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Chapter 5

Chemotaxonomy of Actinobacteria

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Abstract

Actinobacterial classification was originally based largely on morphological observation; it is not adequate in itself to differentiate between many genera, because some are so similar morphologically, but differ from their diagnostic chemical composition. In search of reliable classification methods reflecting phylogenetic relationships, at least to the genus level, it has been demonstrated that the analyses of chemotaxonomic markers fulfill these requirements. Chemotaxonomy of actinobacteria is concerned with the distribution of specific chemicals of the cell envelope such as amino acid, sugar, polar lipids, menaquinones, and fatty acid. For some coryneform genera of actinobacteria, analysis of mycolic acid composition is required specially. In this chapter, we will introduce the methods of chemotaxonomy including the extraction, fractionation, purification, and analysis of the target compounds.

Keywords: Chemotaxonomy, Amino acid, Sugar, Polar lipids, Menaquinones, Fatty acid, Mycolic acid

1. Introduction

Chemotaxonomy is the study of the chemical variation in microbial cell and the use of chemical characteristics in the classification and identification of bacteria including actinobacteria. In search of reliable classification methods reflecting phylogenetic relationships, it has been demonstrated that the analyses of chemotaxonomic markers fulfill these requirements [1]. Therefore, chemotaxonomy is an essential tool in the modern classification of bacteria; it has been recommended in a polyphasic approach to apply to the species, genus, and higher taxa level [2, 3]. Chemotaxonomy of actinobacteria is concerned with the distribution of specific chemicals of the actinobacteria cell envelope such as amino acid, sugar, polar lipids, menaquinones, mycolic acid, and fatty acid (Table 1) by using chemical techniques, including the extraction, fractionation, purification, and resolution of the target compounds [4].

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Categories	Site in cell	Composition
Chemotaxonomic	cell	Sugars
	Cell wall	Amino acid
	Plasma membranes	Polar lipids
	Plasma membranes	Menaquinones
	Plasma membranes	Fatty acids
	Plasma membranes	Mycolic acids

Table 1. Chemotaxonomic markers applied in polyphasic approach of Actinobacteria

2. Amino acid of cell wall

Actinobacteria could be separated into broad groups at the generic level on the basis of morphology and cell wall composition [5]. For such grouping, the compositions of cell wall diaminopimeric acid isomers and whole cell sugars have become widely accepted as the taxonomic markers [6, 7]. Cell wall of actinobacteria consists of a single 20–80 nm thick homogeneous peptidoglycan and frequently represents more than 20% of the cell dry weight. Peptidoglycan constitutes between 40 % and 80 % of the wall weight, while the remainder is made up largely of other macromolecules (lipids, teichoic acids, and acidic polysaccharides and proteins) covalently linked either directly to peptidoglycan or to one another. The structure of peptidoglycan is very stable; it is an enormous polymer composed of many identical subunits; the polymer contains β1–4 linked disaccharides, N-acetylglucosamine and N-acetylmuramic acid, and several different amino acids. The backbone of this polymer is composed of alternating β1–4 linked disaccharides of N-acetylglucosamine and N-acetylmuramic acid. A peptide chain of four alternating D- and L-amino acids is connected to the carboxyl group of N-acetylmuramic acid. Chains of linked peptidoglycan subunits are joined by cross-links between the peptides. Often, the carboxyl group of the terminal D-alanine is connected directly or through a peptide inter-bridge to the amino group of diaminopimelic acid (Figure 1).

The differences in the amino acid sequence of the peptide chains, the mode of cross-links between the chains, and the diaminoacids present give important information for the classification of actinobacteria and have been used for the description of peptidoglycan type [9]. Detection of the presence of diaminoacids at position 3 of the peptide chain is useful for the classification of actinobacteria. *Meso*- and LL-diaminopimelic acid, L-ornithine, L-lysine, and L-diaminobutyric acid are found present at position 3 of the peptide chain [9–11].

A method for analysis of the diaminoacids of peptidoglycan from whole cells has been described by Staneck and Roberts, Lechevalier, Hasegawa et al., Bousfield et al., and Busse et al. [1, 12–15]; this method is rapid, simple, inexpensive equipment and requires only small amount of biomass. The procedure for rapid determination of the diaminoacids present in the

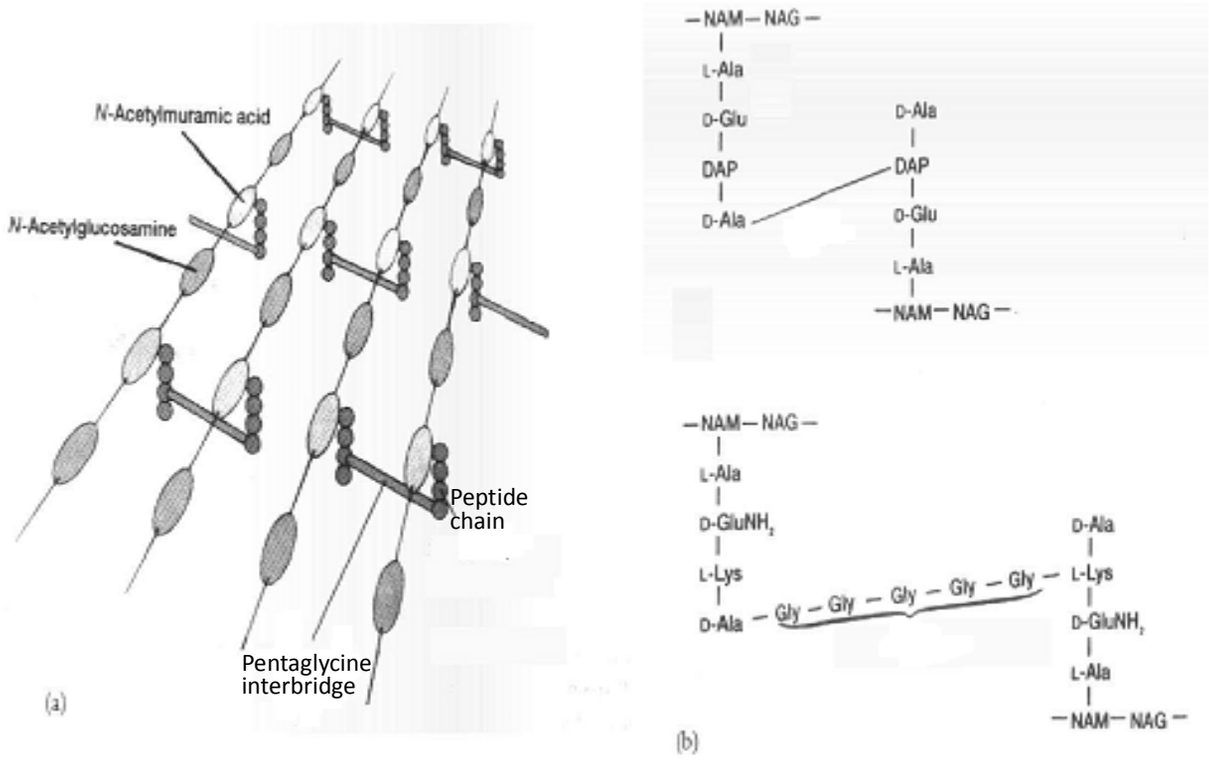


Figure 1. Peptidoglycan structure (a) and peptidoglycan cross-links type (b) (from Prescott et al. [8])

cell, described by Hasegawa et al. [14] with the solvent system of thin-layer chromatography [12], is quite suitable for separation of diaminoacids (Table 2 and Figure 2). However, for analysis of the amino acid in the peptide chains or inter-peptide bridge of the peptidoglycan, the cell wall extraction is required. Detailed cell wall extraction was described by Schleifer and Hancock [16, 17]. Here, we describe a method of cell wall extraction cited from the library of Yunnan Institution of Microbiology, Yunnan University (YIM) (Table 3).

2.1. Extraction of whole cell amino acid

1. A loop of cell mass is added into an ampule, add 0.2 ml of 6 N HCl into the ampule, seal and sand bath to hydrolyze for 16 h at 121°C.

2. Spot 1 μl to the bottom of a 10 × 20 cm of thin-layer plate coated with cellulose.

3. Spot 1 μl of 0.01 M DL-A_2pm containing both LL-and meso-A_2pm on the same plate as a standard.

4. Develop with methanol–water–6 N HCl–pyridine (80:26:4:10, v/v) for 3 h and dry the plates in a fume cupboard.

5. Repeat the fourth step once.

6. Spray the plate very lightly with 0.4% of ninhydrin and heated at 100°C for 2 min to reveal the spots; amino acids are shown as pink spots.

Table 2. Method of extraction and analysis of whole cell amino acids (modified from [12, 14])

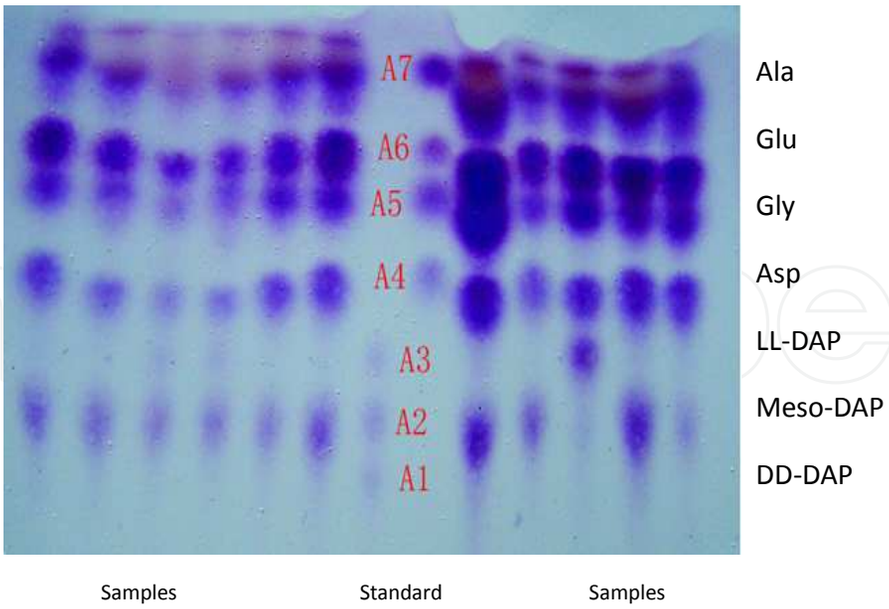


Figure 2. Separation of A_2pm isomers from the hydrolysate of whole cell by thin-layer chromatography

2.2. Preparation of cell wall amino acid

1. Add 1 g of freshly harvested or 0.3 g lyophilized cell mass into 10 ml screw cap test tube; add 1.5 ml of 1 % NaCl (w/v) into the test tube, mix , cap tightly, and stand for 10 min.
2. Add 7 ml of 0.05 mol/l PBS (pH7.6) into the test tube; sonicate for 40 min to lysis the cells (46 w, treatment 5 sec and standing 8 sec, total 40 min).
3. Centrifugate for 15 min at 4,000 rpm; remove the supernatant into a new 10 ml screw cap test tube and discard the precipitate.
4. Centrifugate for 40 min at 12,000 rpm, remove, and discard the supernatant.
5. Add 1 ml of 4 % SDS into the test tube containing precipitate, boiling water bath for 15 min or at room temperature overnight, centrifugate for 30 min at 12,000 rpm, and discard the supernatant.
6. Add 1 ml deionized distilled water, mix and centrifugate for 30 min at 12,000 rpm, and discard supernatant. The addition of deionized distilled water is repeated once.
7. The final insoluble pellet (precipitate) is dried at 65°C; add 200 μl of 6 N HCl into the test tube, mix until the dried pellet dissolved completely, and transfer the solution into the ampule, seal and sand bath overnight at 100°C.
8. Neutralize with 0.2 M NaOH to pH 7.0 and add three volumes sodium borate and mix.

Filter the mixture solution by using 0.45 μm fiber membrane, place the filtered solution into the sample bottle for detecting the amino acid composition by HPLC.

Table 3. Preparation method of cell wall amino acid

2.3. Detection of cell wall amino acid

Amino acids in cell wall hydrolysates were analyzed by precolumn derivatization with o-phthalaldehyde (OPA): ten amino acids standards (10 ml, 0.2 mM) and 10 ml hydrolyzed

purified cell wall were dissolved in 0.1 M (30 ml) borax buffer, and 10 ml OPA was added and allowed to react for 50 sec at room temperature and analyzed by high-performance liquid chromatography (HPLC). The elution time of 10 amino acids standards by HPLC is shown in Figure 3.

High-performance liquid chromatography (HPLC):

Agilent 1100, HPLC system equipped with an Agilent four-unit pump, a 7125 injector, a G1314A UV detector

Columns: ZORBAX Eclipse-AAA (4.6 × 150 mm, 3.5 μm; Agilent)

Columns temperature: 40°C

UV detect wavelength: 338 nm

Mobile phase A: 0.05 mol l^{-1} CH_3COONa and 0.3 % tetrahydrofuran

Mobile phase B: acetonitrile/methanol (1:1, v/v)

Gradient elution: 0–50–50 % buffer B by a linear increase from 0 to 25 to 30

Elution flow rate: 1.0 ml min^{-1}

Injection volume: 20 μl.

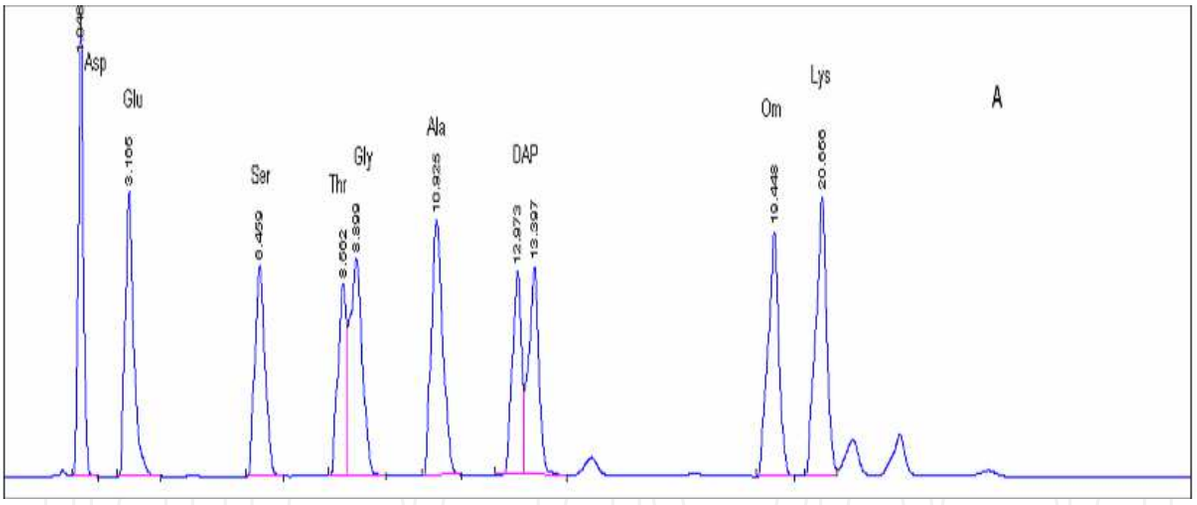


Figure 3. The elution time of 10 amino acids standards by HPLC

The presented method of cell wall amino acids analysis by HPLC will not separate well the LL-, meso-, and dd-A_2pm. So, analysis of the cell wall type of actinobacteria to the genus level requires a combination of HPLC and TLC.

3. Sugar of whole cell hydrolytes

For the classification and identification of actinobacteria, the analysis of sugars from whole cells is needed [10, 11, 19–23]. For discrimination of meso-diaminopimelic acid containing

actinomycetes, five whole cell sugar patterns have been recognized [24], based on the presence of distinct sugars (A: arabinose and galactose; B: madurose; C: no diagnostic sugars; D: arabinose and xylose; E: rhamnose). The combination of the characteristic diaminoacid and some amino acids used cell wall sugars to describe eight wall chemotypes to distinguish actinomycetes [13] (Table 4).

Cell wall chemotype	Characteristic cell wall components
I	L-Diaminopimelic acid, glycine
II	*meso*-Diaminopimelic acid, glycine
III	*meso*-Diaminopimelic acid
IV	*meso*-Diaminopimelic acid, arabinose, galactose
V	Lysine and ornithine
VI	Variable presence of aspartic acid and galactose
VII	Diaminobutyric acid, glycine
VIII	Ornithine
IX	*meso*-Diaminopimelic acid, various amino acids
X	*meso*-Diaminopimelic acid, L-Diaminopimelic acid

Table 4. Chemotypes of cell wall [13]

The analysis of diaminoacids and sugars from whole cell preparations is less time-consuming and often allows an allocation to the correct wall chemotype, but the resulting pattern may be contaminated by non-peptidoglycan-linked saccharides from the cytoplasm, capsules, or slimes. Different methods have been described for whole cell preparations [14, 19, 25], cell wall preparations [9, 26, 27], as well as analysis of sugars [12, 27, 28].

As the methods used to prepare whole cell extracts are similar, the procedure of extraction and analysis of whole cell sugar reported by Hasegawa et al. [14] are briefly described. Although the procedure from Staneck and Roberts [12] for thin-layer chromatography of diagnostic sugar on cellulose plates works reasonably well, it is not able to separate the mannose and arabinose. We described a modified method by changing the developed solvent to separate the mannose and arabinose (Table 5 and Figure 4).

1. Add a loop of cell mass into an ampule, add 0.1 ml of 0.25 N HCl into the ampule, seal and sand bath to hydrolyze for 15 min at 121°C.

2. Spot 2 μl to the bottom of a 10 × 20 cm of thin-layer plate coated with cellulose.

3. Spot 1 μl of standard solution 1 containing rhamnose, xylose, and mannose, and standard solution 2 containing ribose, madurose, arabinose, and glucose on the same plate, respectively.

4. Develop with ethyl acetate–pyridine–acetic acid–water (8:5:1:5, v/v) for 3 h and dry the plates in a fume cupboard.

5. Repeat the fourth step once.

6. Spray the plate very lightly with acid aniline phthalate and heated at 100°C for 4 min to reveal the spots.

Table 5. Extraction and analysis of whole cell sugars (modified from [12, 14])

Besides the procedure of TLC [12, 14], a better procedure to analyze whole cell sugars has been described in our laboratory [18]. It described a method to extract sugars of whole cell and a procedure for preparation of sugar sample for HPLC analysis (Table 6).

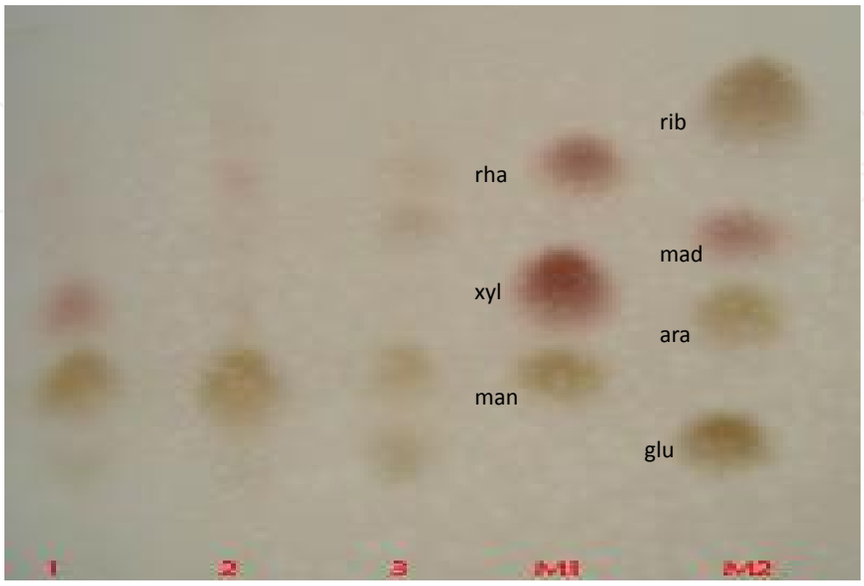


Figure 4. Separation of whole cell sugars from whole cell hydrolysate by thin-layer chromatography (rha = rhamnose, xyl = xylose, man = mannose, rib = ribose, mad = madurose, ara = arabinose, glu = glucose)

3.1. Extraction and preparation of whole cell sugar

1. Add 1 g of freshly harvested or 0.3 g lyophilized cell mass into ampule; add 0.5 ml of 0.5 N HCl into the ampule, seal and sand bath for 2 h.
2. Unseal the ampule, 80 μl hydrolysed whole cell solution and 80 μl 0.25 M methanol solution of 1-phenyl-3-methyl-5-pyrazolone (PMP) and 80 μl 0.2 M NaOH were mixed.
3. Mixture was allowed to react for 30 min at 70°C, cooled to room temperature and neutralized with 80 ml 0.2 M NaOH to pH 7.0, and extracted with isoamyl acetate.
4. After vigorous shaking and centrifugation, the organic phase was carefully discarded to remove the excess reagents.
5. The extraction process was repeated three times, using chloroform instead of isoamyl acetate for the third process; the aqueous layer was then collected and 10 ml was taken for HPLC analysis.

Table 6. Method for extraction and preparation of whole cell sugar [14, 29]

3.2. Analysis of sugar of whole cell hydrolytes

The sugar of whole cell hydrolytes was analyzed by high-performance liquid chromatography (HPLC). The elution time of nine sugar standards by HPLC is shown in Figure 5.

Agilent 1100, HPLC system equipped with an Agilent four-unit pump, a 7125 injector, a G1314A UV detector

Columns: ZORBAX Eclipse XDB-C18 (4.6 × 150 mm, 5 μm; Agilent)

Columns temperature: 40°C

UV detect wavelength: 250 nm

Mobile phase A: acetonitrile

Mobile phase B: 0.05 M sodium acetate (pH 6.9)

Elution: A : B = 17 : 83 (v/v).

Elution flow rate: 1.0 ml min^{-1}

Injection volume: 10 μl

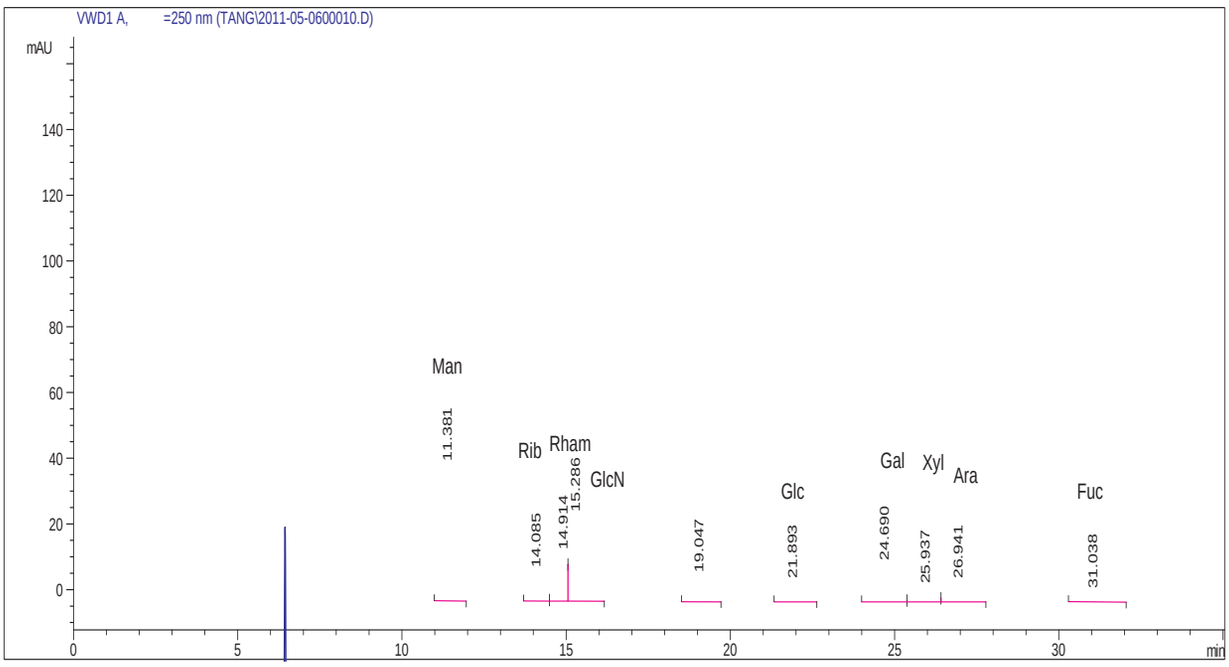


Figure 5. The elution time of nine sugar standards by HPLC (from YIM library) (Man = mannose, Rib = ribose, Rha = rhamnose, GlcN = glucosamine hydrochloride, Glc = glucuronic acid, Gal = galactose, Xyl = xylose, Ara = arabinose, Fuc = fucose)

4. Polar lipids

Polar lipids are important components of bacterial plasma membranes. Bacterial plasma membranes are composed of amphipathic polar lipids associated with specific membrane proteins. Amphipathic polar lipids consist of hydrophilic head groups usually linked to two hydrophobic fatty acid chains. Phospholipids are the most common polar lipids, including phosphatidyglycerol, diphosphatidyglycerol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidyliositol, and other phosphatidylglycolipids. In addi-

tion, glycolipids and acylated ornithine or lysine amides also fall into this category. For the description and differentiation of actinobacteria, five phospholipid types (PI–PV) have been recognized (Table 7) [30, 31].

Phospholipid types	Characteristic phospholipids
P I	No nitrogenous phospholipids
P II	Only one nitrogenous phospholipid phosphatidyl ethanolamine
P III	Phosphatidyl choline and characteristic phospholipid
P IV	Glucosamine-containing phospholipids
P V	Phosphatidylglycerol and glucosamine-containing phospholipids

Table 7. Phospholipid types according to Lechevalier et al. [31]

In taxonomic studies, polar lipids have largely been analyzed by one- or two-dimensional thin-layer chromatography.

4.1. Extraction of polar lipids

The classic method of polar lipid extraction [13] is a time-consuming process taking at least 13 days from start to finish. Subsequently, more rapid procedures have been proposed by Minnikin et al. and Tindall [32, 33]. This utilizes a monophasic methanol for polar lipids extraction; the addition of more chloroform and water forces a phase separation. The lower, mainly chloroform, layer contains the polar lipids, whereas non-lipid components remain in the upper aqueous phase. Minnikin et al. [34] introduced a modified procedure, in which an initial extraction with hexane removes non-polar components such as isoprenoid quinones; in this way menaquinones and polar lipids can be extracted from a single sample of biomass. In this section, a modified procedure for polar lipids extraction is described (Table 8).

1. Place approximately 100~200mg of dried cell mass into a 50 ml tube with Teflon-lined screw cap.
2. Add 2 ml of 0.85% aqueous NaCl, followed by 15 ml methanol.
3. Heat for 10 min at 100°C in a boiling bath and cool to room temperature.
4. Add 10 ml chloroform and 6 ml 0.85% aqueous NaCl , then shake for 10 min.
5. Centrifuge at 8,000 rpm for 10 min, collect the lower layer.
6. The lower layer in a flask is evaporated to dryness under reduced pressure at 40°C on a rotary evaporator.

Table 8. Extraction of polar lipids (modified by Minnikin et al. and Tindall [32, 33])

4.2. Two-dimensional thin-layer chromatography

Separation of the mixture of polar lipids is performed by two-dimensional TLC (Table 9) on silica gel GF254 plate.

1. Dissolve the dried polar lipids in 100 μl of petroleum ether (boiling point: 70-90°C). Spot 10 μl to the bottom of a 10 × 10cm of thin-layer plate coated with silica gel (Merck F_{254}). Develop with chloroform–methanol–water (65:25:4, v/v) in the first dimension and dry the plates overnight in a fume cupboard.
2. Develop with chloroform–acetic acid–methanol–water (80:18:12:5, v/v) in the second dimension. Dry the plates in a fume cupboard.

Table 9. Two-dimensional thin-layer chromatography (modified by Minnikin et al. [35])

4.3. Identification of polar lipid component

Identification of the various lipids is carried out by comparison of their R_f values in the plates and staining behavior (Table 10) with references.

Molybdophosphoric acid for total lipids [36]

1. Dissolve 10% (w/v) molybdophosphoric acid in 95% (v/v) ethanol.
2. Spray the TLC plate and heat at 150°C for at least 10 min. Lipids show as dark spots on a light-green background.

Ninhydrin reagent for lipids containing free amino groups (modified by Consden and Gordon, [37])

1. Dissolve ninhydrin (0.1%, w/v) in acetone.
2. Spray plate and heat at 100°C for 5 min to reveal lipids which contain amino groups as pink spots. Mark the pink spots with a soft pencil to prevent them from fading on storage.
3. The same plate can be used for the detection of lipid phosphorus using molybdenum reagent

α-Naphthol reagent for containing sugar groups [38]

1. Dissolve 15 g α-naphthol in 100 ml 95% (v/v) ethanol.
2. Mix 10.5 ml of this solution with 6.5 ml H_2SO_4, 40.5 ml ethanol, and 4 ml water to make a working solution.
3. Spray the plate lightly and heat a 100°C for 10 min. Glycolipids appear as purple-brown or brown spot.

Dragendorff reagent for lipids containing quaternary nitrogen groups

1. Add bismuth nitrate (1.7 g) to 100 ml of 20 % acetic acid (solution A).
2. Add potassium iodide (40 g) to 100 ml water (solution B).
3. Mix solution A (3.5 ml) and solution B (5 ml) with acetic acid (20 ml) and water (50 ml) to make a working solution.
4. Spray the plate lightly at room temperature; lipids containing quaternary nitrogen shown as orange-red spots. Mark the orange-red spots with a soft pencil to prevent them from fading on storage.
5. The same plate can be used for the detection of lipid phosphorus using molybdenum reagent.

Zinzadze reagent for phosphorus-containing lipids [39]

1. Add molybdenum trioxide (40.11 g) to 1 L of 25 N H_2SO_4 and boil gently in a fume cupboard until all the residue dissolves (solution A).
2. Add powdered molybdenum (1.78 g) to 500 ml of solution A, and boil the mixture gently for 15 min and leave a cool (solution B).
3. Mix equal volumes of solutions A and B and dilute with two volumes of distilled water to make a working solution.
4. Spray the plate very lightly at room temperature, lipids containing phosphorus shown as blue spots.

Table 10. Spray reagents for identification of individual components

5. Menaquinones

Respiratory isoprenoid quinones are constituents of the bacterial cytoplasmic membrane as well as the mitochondrial membrane where they play an important role in the electron transport chain. The potential of analyzing the quinone system for the characterization of bacteria is based on the different types of quinones (e.g., ubiquinones, menaquinones and their derivatives dihydromenaquinone, demethylmenaquinone, and rhodoquinone), the length of isoprenoid side chain, and the number of saturated isoprenoid units. To date, menaquinones are the only type of respiratory isoprenoid quinones found in actinobacteria, and the variations in the number of isoprene units and hydrogenated double bonds make these membrane constituents of considerable chemotaxonomic value [40].

5.1. Extraction and purification of menaquinones

Menaquinones are free lipids that can be readily extracted from freeze-dried cells. Different methods have been described for the extraction of menaquinones [13, 33, 34, 41]. Menaquinones are normally extracted with organic solvents or with their mixture such as acetone, chloroform, and hexane (Table 11). However, they are susceptible to strong acid or alkaline, and photo-oxidation in the presence of oxygen and strong light conditions. But it is not necessary to work in a nitrogen atmosphere or dim light [42]. The menaquinones in these extracts are purified by preparative thin-layer chromatography (TLC), and analysis is then performed by HPLC.

1. Approximately 100 of lyophilized cells are extracted with a volume (40 ml) of chloroform–methanol (2:1 v/v) for approximately 1 h or overnight using a magnetic stirrer.
2. The cell/solvent mixture is passed through filter paper to remove cell debris.
3. The eluate is collected in a flask and evaporated to dryness under reduced pressure at 40°C on a rotary evaporator.

Table 11. Extraction of menaquinones (from Collins et al. [43, 44]).

The menaquinones can be readily purified from extracts by thin-layer chromatographic procedures using silica gel with hexane–diethylether as the developing solvent [43, 44]. Purified menaquinones are revealed by using UV light at 254 nm. In this section, we describe a new developing solvent to purify the menaquinones (Table 12).

1. Dissolve the dried menaquinones in 800 μl of acetone.
2. Apply extract (with 200 μl pipette) as a uniform streak (5 cm long) to a silica gel F254 sheet.
3. Develop the plate in methylbenzene, developing time ~20 min.
4. Allow plate to dry in a fume cupboard (~5 min), view menaquinones by brief irradiation with ultraviolet light at 254 nm. The menaquinones appear as dark-brown/purple bands on a green fluorescent background, R_f ~0.7.
5. Scrape gel containing menaquinones from the plate with spatula, dissolve scraped gel in 500 μl of methanol and elute through syringe and 0.45 μm filter membrane.

Table 12. Purification of menaquinones by thin-layer chromatography

5.2. High-Performance Liquid Chromatography (HPLC) analyzing the menaquinone component

TLC techniques generate only qualitative data. In contrast, high-performance liquid chromatography can be used to generate quantitative data. The resolving power and sensitivity of HPLC are also superior to that of thin-layer chromatographic techniques. The purified menaquinones are rapidly analyzed by reverse-phase high-performance liquid chromatography (rpHPLC) [41, 45].

Menaquinones series is analyzed by HPLC with a UV detector, a C18 column, and an online computer integrator. A large number of different mobile phases have been described [44, 45, 46]. Here, we prefer methanol/isopropanol mixtures (65:35, v/v) as the mobile phase to analyze the menaquinones. The column should be maintained at a constant temperature (40°C).

High-performance liquid chromatography (HPLC):

Agilent 1100, HPLC system equipped with an Agilent four-unit pump, a 7125 injector, a G1314A UV detector

Columns: Zorbax Eclipse XDB-C18 (4.6 × 250 mm, 5 μm; Agilent)

Columns temperature: 40°C

UV detect wavelength: 269 nm

Mobile phase A: methanol

Mobile phase B: isopropanol

Elution: A: B = 65:35 (v/v).

Elution flow rate: 1.0 ml min^{-1}

Injection volume: 20 μl.

6. Mycolic acid

Mycolic acids are high molecular-weight long-chain (up to 90 carbon atoms) 2-alkyl 3-hydroxy fatty acids found in representative of *Corynebacterium*, *Dietzia*, *Gordona*, *Myobacterium*, *Nocardia*, *Rhodococcus*, *Turicella*, and *Tsukumurellu* [47–53]. For the extraction and analysis of mycolic acids, different methods have been described based on TLC, GC, or HPLC [54–59].

6.1. Extraction of mycolic acids from whole cell

In this section, we describe the extraction and analysis of mycolic acids based on TLC [55].

1. Add 50–100 mg freeze-drying cells into a clean, dry test tube.
2. Add 3 ml mixture solvent of methanol, toluene, and conc. sulfuric acid (30:15:1) into the test tube and tightly seal the test tube.

3. Place the test tubes into a water bath at 75°C overnight or 16–18 h.
4. Cool the test tube down to room temperature and add of 2 ml petroleum ether (b.p. 60–80), the mixture is shaken and centrifuged for 10 min at low speed (3,000 rpm), collect the upper solvent phase.
5. Prepare a small column of ammonium hydrogen carbonate and prewash the small column with diethyl ether.
6. Pipette the upper solvent phase into a small column (ca. 1 cm) of ammonium hydrogen carbonate and collect the eluent in a small eppendorf tube (5 ml), then wash the small column again with diethyl ether.
7. Combine the washed eluent and evaporate to dryness under reduced pressure at 40°C on a rotary evaporator.

6.2. Analysis of mycolic acids from whole cell

The mycolic acids of whole cells were analyzed according to the described by Minnikin et al. [55].

1. Dissolve the dried mycolic acids in 200 μl of petroleum ether.
2. Spot 10 μl to the bottom of a 10 × 10cm of thin-layer plate coated with silica gel (Merck F_{254}).
3. Develop with petroleum ether, acetone (95:5, v/v) used for single-dimensional development and dry the plates in a fume cupboard. Develop with petroleum ether acetone (95:5, v/v), followed, in the second direction, by toluene, acetones (97:3) used for two-dimensional development system and dry the plates in a fume cupboard.
4. Staining, spray plate with molybdophosphoric acid and heat at 150°C for 5 min to reveal mycolic acids.

The evaluation of the presence of mycolic acid is only advisable, if other results (e.g., coryneform morphology) allocate the isolate to be identified to the group of coryneform bacteria, but the detection of mycolic acids strongly reduces the number of possible relatives. Further identification to the genus level is often possible by additional application of a few of the other described chemotaxonomic methods (quinones, fatty acids, polar lipids, and/or sugars).

7. Fatty acids

Fatty acid profiles analysis is well introduced for chemotaxonomy of bacteria. Fatty acids most commonly found in the cytoplasmic membrane and lipopolysaccharides of the outer membrane of Gram-negative bacteria as well as lipoteichoic acids in Gram-positive bacteria are relatively simple in structure and possess between 8 and 20 carbon atoms. The variation of carbon chain length, presence of saturated and unsaturated, occurrence of methyl groups fatty acids (iso-, anteiso-, and methylated within the molecule), occurrence of cyclopropane fatty acid (cyclo 17:0, cyclo 19:0), and occurrence of hydroxyl-fatty acid with an OH-group at position 2 or 3 of the molecule all have a taxonomic utility.

Commonly, different bacteria can have different fatty acids. Some fatty acids have a restricted distribution and may be diagnostic for particular groups. Branched fatty acids of the iso and/or anteiso type are important constituents of the *Flavobacterium/Cytophaga/Bacteroides* [60–62]. Cyclohexyl and cycloheptyl fatty acids are characteristic components of some acidothermophilic bacilli [63–65]. Cyclopropane fatty acids are often found in Campylobacter and Lactobacillus [66–68]. 10-Methyloctadecanoic acid and its homologs distribute in many actinomycetes [46, 69].

As the fatty acid composition of bacteria is dependent on the growth phase, temperature, and growth medium, preparing the biomass for analysis of the fatty acids should be taken to ensure that bacteria are grown under standardized conditions. The extraction of fatty acids can be performed with biomass (approximately 40 mg wet weight harvested from agar plates). For most actinomycetes, the reader can select the trypticase soy agar as the growth medium, but for the actinomycetes from extreme environment, the reader should select the optimum growth medium, as well as the possible media should omit material containing fatty acids, such as Tweens and serum.

7.1. Methods for analyzing fatty acids

Different methods have been described involving acid or base [55, 62, 70–72]. In taxonomic studies, it is important to use a consistent method. Here, we introduce a method for preparation of fatty acid methyl esters from whole wet cell material, which is developed by Sasser [73].

7.2. Preparation of reagents

Four reagents are required to liberate, esterify, and extract the fatty acids from living cells.

Reagent 1 Saponification Reagent

Sodium hydroxide (Certified ACS) 45 g

Methanol (reagent Grade) 150 ml

Deionized distilled water 150 ml

Add water and methanol to NaOH pellets in bottle. Stir until NaOH pellets have dissolved.

Reagent 2 Methylation Reagent

12 N hydrochloric acid 195 ml

Methanol (reagent Grade) 275 ml

Deionized distilled water 130 ml

Add acid to water, then to methanol while stirring

Reagent 3 Extraction Solvent

Hexane (HPLC Grade) 200 ml

Methyl-tert-Butyl-ether (HPLC Grade) 200 ml

Add MTBE to hexane and stir

Reagent 4 Base Wash

Sodium hydroxide (Certified ACS) 10.8 g

Deionized distilled water 900 ml

Add water to NaOH pellets in bottle. Stir until NaOH pellets have dissolved.

Warning

Reagent 1 and 2 are caustic, wear safety glasses and gloves.

Methyl-tert-Butyl-ether is extremely flammable. Extinguish all flames and heat sources before use.

Handle in a chemical fume hood.

7.3. Extraction of fatty acids.

Five steps involved in extraction of fatty acids from biomass [73] (see Figure 6):

1. Harvesting: removal of cells from the culture media. Scrap cells (~40 mg for each culture) on growth medium by using inoculation loop; add the scraped cells into a clean, dry 13mm × 100mm Teflon-lined screw cap test tube.

2. Saponification: lysis of the cells to liberate the fatty acid. Add 1 ml of reagent 1 into the test tube, tightly seal with a Teflon-lined screw cap, vortex tube for 5–10 sec, and place each samples tube into a rack. Then, place the rack of the batched samples tubes into a boiling water bath for 5 min, take out the samples tubes, vortex each tube for 5–10 sec, check the tubes for leakage, continue heating the samples tubes in a boiling water bath for 25 min. Remove the rack and cool the tubes at room temperature in a water bath.

3. Methylation: formation of methyl esters of the fatty acid. Add 2 ml of reagent 2 to each tube. Cap each tube tightly and vortex for 5–15 sec. Heat the tubes in an 80 ± 1°C water bath for 10 min. Remove and cool quickly by placing the rack of the batched samples tubes at room temperature in a water bath.

4. Extraction: transfer of the fatty acid methyl esters from the aqueous phase to an organic phase. Uncap each tube in the batch; add 1.25 ml of reagent 3 to each tube. Cap each tube tightly, place bath of tubes in rotator and mix end-over for 10 min. Uncap each tube in the batch, remove and discard the lower (aqueous) phase with a Pasteur pipette.

5. Base wash: aqueous wash of the organic extract prior to chromatographic analysis. Add 3 ml of reagent 4 to each tube, cap each tube tightly and rotate tubes end-over for 5 min. centrifugate for 3 min at 2,000 rpm. Remove the 2/3 of upper solvent phase and place into a GC sample bottle as the sample for detected of fatty acid profiles on the gas chromatograph.

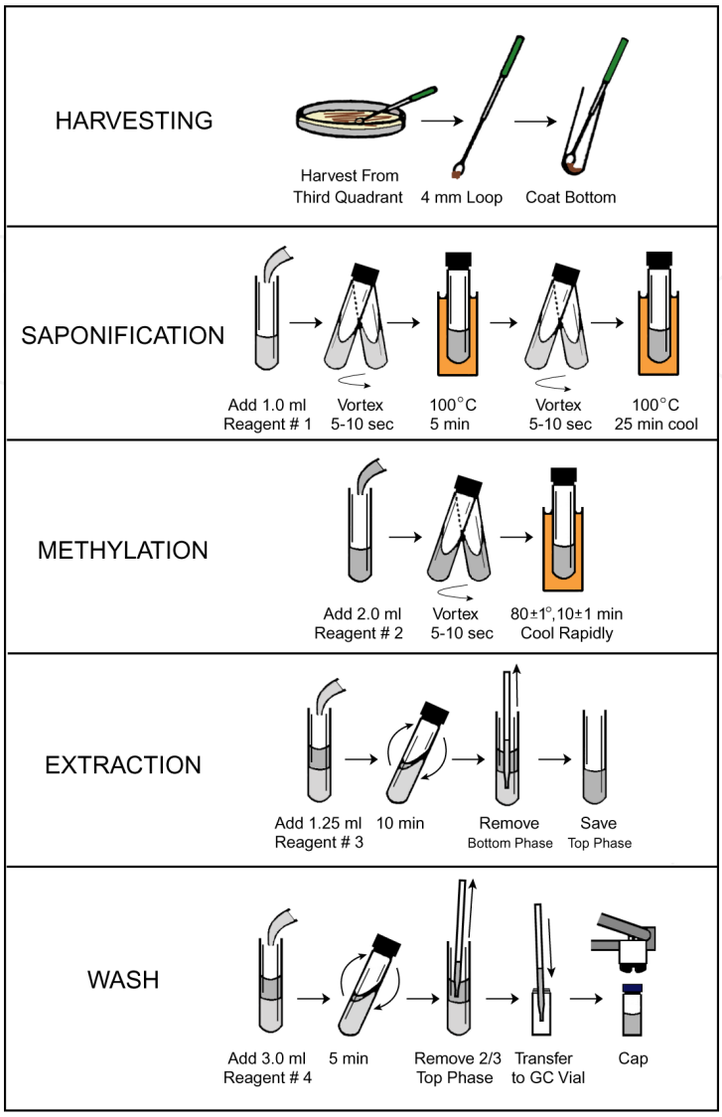


Figure 6. Five steps involved in extraction of fatty acids

Warning:

In the methylation step, excess time or excess temperature of the water bath can degrade some fatty acids.

7.4. Identification of fatty acids

For identification of the fatty acid profiles at the species level, integrated system, including a gas chromatography apparatus with identification software (the Sherlock Microbial Identification System) are required.

Gas chromatographic conditions

Gas chromatographic: Agilent 7890

Agilent 7890, column, flame ionization detector (FID)

Columns:

Agilent 19091B-102, 25 × 200 m × 0.33 μm

Oven temperature 170°C

Injector 250°C

Detector 300°C

Carries gas (hydrogen) 1 ml/min

Inlet pressure 9.000 psi

Electrometer setting 4×10^{12}amps

Splitting 1:20 0.4bar

Hydrogen (for flame) 30 ml/min

Synthetic air (for flame) 400 ml/min

Septum purge 10 ml/min

Auxiliary gas (N2) 30 ml/min

Sample 2 μl

8. Gas chromatography of fatty acids

In general, >5% of fatty acids as "major fatty acid" should be recorded. Fatty acid component of strain YIM 47672 analyzed with gas chromatography, as an example, is showed in Table 13 and Figure 7.

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
1.683	3.664E+8	0.026	----	7.008	SOLVENT PEAK	----	< min rt	
1.838	868	0.019	----	7.305		----	< min rt	
1.877	191	0.018	----	7.379		----	< min rt	
1.912	679	0.023	----	7.446		----	< min rt	
1.956	190	0.020	----	7.530		----	< min rt	
2.045	1201	0.046	----	7.701		----	< min rt	
2.202	1522	0.024	----	8.001		----	< min rt	
2.296	3546	0.031	----	8.181		----	< min rt	
2.628	223	0.021	----	8.817		----	< min rt	
3.028	1020	0.025	----	9.581		----		

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
3.134	837	0.044	----	9.783		----		
3.285	453	0.025	----	10.053		----		
4.676	795	0.032	----	11.754		----		
4.910	221	0.024	1.051	12.001	12:0	0.03	ECL deviates 0.001	Reference -0.002
5.135	567	0.035	----	12.192		----		
6.953	881	0.042	0.994	13.619	14:0 iso	0.13	ECL deviates 0.000	Reference -0.002
7.334	1399	0.044	0.986	13.892	14:1 w5c	0.21	ECL deviates -0.009	
7.479	6652	0.047	0.984	13.996	14:0	0.98	ECL deviates -0.004	Reference -0.005
7.555	4193	0.055	----	14.045		----		
7.674	3021	0.051	----	14.121		----		
7.768	1640	0.038	----	14.181		----		
7.868	1764	0.054	----	14.245		----		
7.976	1064	0.040	----	14.314		----		
8.049	2496	0.059	----	14.361		----		
8.156	2643	0.043	----	14.429		----		
8.231	1716	0.044	0.972	14.477	Sum In Feature 1	0.25	ECL deviates -0.001	13:0 3OH/15:1 i H
8.322	1111	0.044	0.971	14.535	15:1 anteiso A	0.16	ECL deviates 0.008	
8.459	7432	0.037	0.969	14.623	15:0 iso	1.08	ECL deviates 0.000	Reference -0.001
8.600	5278	0.037	0.967	14.713	15:0 anteiso	0.77	ECL deviates 0.000	Reference -0.001
8.821	533	0.039	0.964	14.854	15:1 w6c	0.08	ECL deviates -0.002	
9.049	39473	0.041	0.961	15.000	15:0	----	ECL deviates 0.000	
9.824	3366	0.043	0.952	15.460	16:1 iso H	0.48	ECL deviates -0.001	
10.109	**216646**	**0.041**	**0.949**	**15.629**	**16:0 iso**	**30.90**	ECL deviates 0.002	Reference 0.001

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
10.264	2016	0.042	0.948	15.721	16:0 anteiso	0.29	ECL deviates 0.003	
10.428	14559	0.042	0.946	15.819	Sum In Feature 3	2.07	ECL deviates -0.003	16:1 w7c/16:1 w6c
10.732	**96750**	**0.042**	**0.944**	**16.000**	**16:0**	**13.71**	ECL deviates 0.000	Reference -0.001
11.465	10036	0.043	0.938	16.419	Sum In Feature 9	1.41	ECL deviates 0.003	17:1 iso w9c
11.652	4552	0.045	0.937	16.527	17:1 anteiso w9c	0.64	ECL deviates 0.003	
11.832	19563	0.042	0.936	16.630	17:0 iso	2.75	ECL deviates 0.000	Reference -0.001
11.996	**77103**	**0.043**	**0.935**	**16.724**	**17:0 anteiso**	**10.82**	ECL deviates 0.001	Reference 0.000
12.122	**50353**	**0.045**	**0.934**	**16.796**	**17:1 w8c**	**7.06**	ECL deviates 0.004	
12.286	10094	0.051	0.933	16.890	17:0 cyclo	1.41	ECL deviates 0.002	
12.479	**41837**	**0.043**	**0.932**	**17.001**	**17:0**	**5.85**	ECL deviates 0.001	Reference -0.001
13.202	6556	0.046	0.928	17.408	17:0 10-methyl	0.91	ECL deviates -0.001	
13.307	1495	0.044	0.927	17.468	18:1 iso H	0.21	ECL deviates 0.004	
13.511	1684	0.052	0.927	17.583	18:3 w6c (6,9,12)	0.23	ECL deviates 0.006	
13.598	3730	0.044	0.926	17.632	18:0 iso	0.52	ECL deviates 0.000	Reference -0.002
13.763	**69473**	**0.047**	**0.925**	**17.725**	**Sum In Feature 5**	**9.66**	ECL deviates -0.002	18:0 ante/18:2 w6,9c
13.847	**35208**	**0.048**	**0.925**	**17.772**	**18:1 w9c**	**4.89**	ECL deviates 0.003	
13.937	3878	0.041	0.925	17.823	Sum In Feature 8	0.54	ECL deviates 0.000	18:1 w7c
14.034	14713	0.048	----	17.878		----		
14.141	1445	0.052	----	17.938		----		

RT	Response	Ar/Ht	RFact	ECL	Peak Name	Percent	Comment1	Comment2
14.248	8805	0.046	0.924	17.998	18:0	1.22	ECL deviates -0.002	Reference -0.004
14.601	1763	0.079	----	18.199		----		
14.742	1095	0.043	----	18.279		----		
15.507	2979	0.061	----	18.714		----		
15.583	1055	0.047	0.920	18.757	Sum In Feature 6	0.15	ECL deviates 0.001	19:1 w11c/19:1 w9c
15.784	3100	0.047	0.919	18.871	Sum In Feature 7	0.43	ECL deviates 0.004	19:0 cyclo w10c/19w6
16.010	573	0.038	0.919	19.000	19:0	0.08	ECL deviates 0.000	Reference -0.003
17.223	6351	0.051	----	19.699		----		
17.347	440	0.030	0.916	19.770	20:1 w9c	0.06	ECL deviates 0.000	
----	1716	---	----	----	Summed Feature 1	0.25	15:1 iso H/13:0 3OH	13:0 3OH/15:1 i H
----	14559	---	----	----	Summed Feature 3	2.07	16:1 w7c/16:1 w6c	16:1 w6c/16:1 w7c
----	69473	---	----	----	Summed Feature 5	9.66	18:2 w6,9c/18:0 ante	18:0 ante/18:2 w6,9c
----	1055	---	----	----	Summed Feature 6	0.15	19:1 w11c/19:1 w9c	19:1 w9c/19:1 w11c
----	3100	---	----	----	Summed Feature 7	0.43	un 18.846/19:1 w6c	19:1 w6c/.846/19cy
----	-----	---	----	----		----	19:0 cyclo w10c/ 19w6	
----	3878	---	----	----	Summed Feature 8	0.54	18:1 w7c	18:1 w6c
----	10036	---	----	----	Summed Feature 9	1.41	17:1 iso w9c	16:0 10-methyl

Volume: DATA; File: E068264.61A; Samp Ctr: 5; ID Number: 1107; Type: Samp; Bottle: 3

Method: TSBA6; Created: 5/26/2015 12:39:52 PM; Sample ID: **YIM 47672**

ECL Deviation: 0.003; Reference ECL Shift: 0.002; Number Reference Peaks: 13

Total Response: 756936; Total Named: 708097; Percent Named: 93.55%; Total Amount: 703700; *** No Matches found in TSBA6

Table 13. Fatty acid component of strain YIM 47672 with gas chromatography

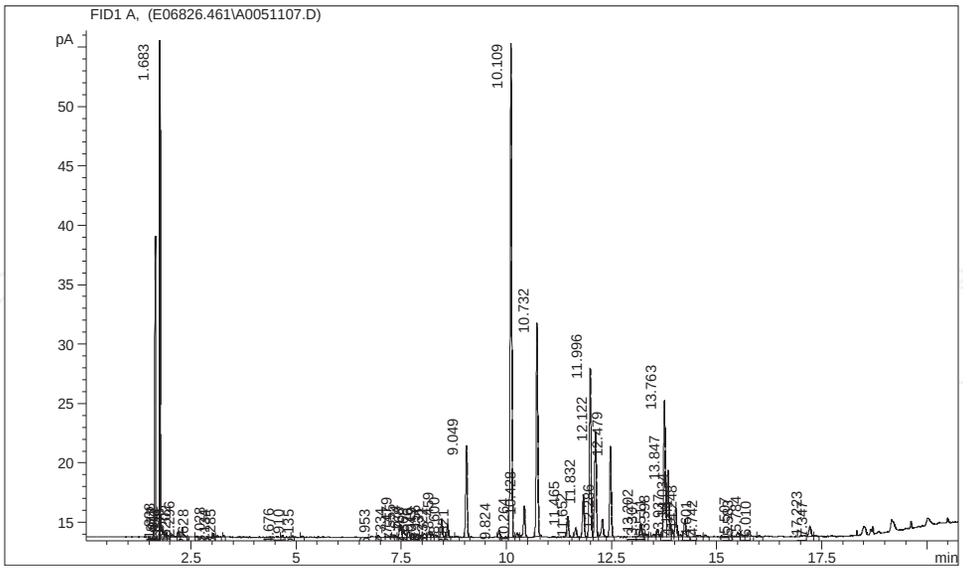


Figure 7. Gas chromatography of fatty acid of strain YIM 47672

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