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Quantitative Analysis of Corynomycolic Acids in Fermentation Broth

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Abstract The mycolic acids and fatty acids of mycolic acid-containing bacteria in various types of fluids were analyzed using capillary gas chromatography and mass spectrometry. As model strains, *Brevibacterium* and *Corynebacterium* species, which have corynomycolic acids in the range of C_{32} C_{36} in the whole cell, were investigated. Optimized solvents extraction procedures for the mycolic acids and fatty acids from the culture fluids were: chloroform/methanol (1:2, v/v) as the first extraction solvents for 4 h; and chloroform/water (1:1, v/v) as the second extraction solvents for 1 h. These conditions gave above 95% recovery yields for mycolic acids from the culture fluids. The mycolic acid profile for the whole cells and the culture fluids were similar for all the media tested. Thus, the procedure described here could be applied for the identification of mycolic acid-containing bacteria in fermentation broth or liquid form of foods.

Key words: *Brevibacterium*, *Corynebacterium*, mycolic acid extraction

The cell wall of *Brevibacterium*, *Corynebacterium*, *Mycobacterium*, *Nocardia*, and *Rhodococcus* species includes three major components: peptidoglycan (a branched and cross-linked glycopeptide), arabinoglycan (a branched polysaccharide), and mycolic acids [2, 5, 14, 15, 18, 23]. Mycolic acids occur in only a few bacterial genera and are the major component of the cell wall structure of these bacteria [1, 2, 7, 22]. The cell wall of mycolic acid-containing bacteria constitutes an efficient permeability barrier and several properties of these bacteria, such as acid fastness, the slow rates of growth, low level of secretion yields for the cellular metabolites (e.g. amino acids), and the natural resistance to a wide range of antibiotics, and these are often thought to be at least partially related to the poor penetration of solutes across the cell wall [12, 17]. Thus, the structure and biosynthesis pathway for mycolic acids of mycolic acid-containing bacteria have been intensively studied [3, 5, 9, 19, 25, 28]. The formulation of mycolic acids varies from species to species. Mycobacterial mycolic acids are the largest in size (containing 60–90 carbon atoms in total size) and the most abundant constituents (40% by weight) of cell walls [20]. The genera *Nocardia* and *Rhodococcus* have mycolic acids with 36–66 carbon atoms which have from zero to four double bonds [1, 21, 26]. The mycolic acids from corynebacteria contain homologous mixtures of saturated and unsaturated components containing 20 to 36 carbon atoms [7, 11]. Based on these differences, it has been suggested that the mycolic acid profile is a form of fingerprint for a single bacterial species [6]. For instance, qualitative differences in mycolic acid composition for three amino acid-producing corynebacteria were reported, where the three species contained: *Corynebacterium glutamicum* NCIB 10025, C_{30}-C_{36}, *Brevibacterium flavum* NCIB 9565, C_{28}-C_{36} and *Brevibacterium lactofermentum* NCIB 9567, C_{32}-C_{36}. The main mycolic acid lipids were $C_{32:0}$ and $C_{34:1}$ in all of the three species tested, while $C_{36:1}$ existed as a minor component [7]. The overall composition of mycolic acids from the three saprophytic species was similar, but this was different to that of pathogenic corynebacteria (including *C. diphtheriae*, *C. ulcerans*, and *C. urealyticum*) found in human, animal, milk, and dairy products [8, 11, 29]. *C. ulcerans* [29] possesses mycolic acids with exceptionally low carbon numbers (C_{20} to C_{32}) while *C. diphtheriae* [8] possesses mycolic acids with C_{20} to C_{35}. *C. urealyticum* exhibited mycolic acids with lipids ranging from C_{26} to C_{30}, and this species is characteristically typified by its content of $C_{30:3}$ and $C_{28:1}$ mycolic acids [11]. However, this type of information was mainly focussed on the cellular mycolic acids rather than extracelluar mycolic acids. In order to detect mycolic acid-producing bacteria in fermentation products, quantitative measurements of mycolic acids in the

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culture fluids are important. In the present work, we optimized the extraction procedure and analyzed fatty acids and mycolic acids from culture fluids using corynebacteria as model strains.

MATERIALS AND METHODS

Bacterial Strains, Media, and Growth Conditions

Corynebacteria strains used were: *B. flavum* [13]; *B. lactofermentum* BL1 [10]; *C. glutamicum* AS019 [30]. Luria broth supplemented with 0.5% glucose (LBG), pH 7.2, contained 10 g of tryptone, 5 g of yeast extract, 5 g of sodium chloride, and 5 g of glucose per liter. All strains of corynebacteria were routinely grown in LBG, at 30°C with agitation (200 rpm) in a shaker incubator for 16 h.

Isolation and Analysis of Mycolic Acids from Whole Cells

Strains were grown to the late exponential phase in shake-flask cultures of LBG, LBG-2% glycine, LBG-4 mg isonicotinic acid hydrazide (INH)/ml, and LBG-2% glycine plus 4 mg INH/ml (LBG-GI) as described above. INH and glycine are reported as the cell wall synthesis inhibitors in *C. glutamium* [10, 16, 30]. The mycolic acid composition was determined by GC and MS for cells following extraction, derivatization, and quantification as described previously [16], with minor modifications. Before derivatization, mycolic acids were separated from fatty acids by TLC. Aliquots of samples (mixture of mycolic acids and fatty acids) were placed on aluminum-baked silica-gel plates (Merck, Germany). The plate was developed in petroleum ether-acetone (95:5, v/v) at room temperature. The mycolic acids were detected by spraying with a 10% (w/v) ethanolic solution of molybdophosphoric acid in ethanol followed by heating in an oven at 120°C for 15 min. Areas corresponding to *Rf* values of mycolic acids were scraped from the plate, extracted by diethyl ether, and then derivatized to trimethylsilyl (TMS) ethers by dissolving in trimethylsilylimidazole (Tri-sil Z, Pierce) at 60°C for 20 min. At the beginning of the sample extraction, 100 µl of 5 mM lignoceric acid methyl ester (LAME) was added as an internal standard to each sample, and all analyses were standardized using the area obtained following extraction and derivatization of the LAME. Mycolic acids were quantified in terms of their peak area and the relative amounts expressed as a percentage of the total area detected for mycolic acids.

Isolation and Analysis of Mycolic Acids from Culture Fluids

To analyze the mycolic acid composition of culture fluids, supernatant fluids were collected at stationary phase by centrifugation (2,000 ×*g*, 4°C, 10 min) and the total volume was filtered (0.22 µm, Millipore) before applying one of two procedures used to extract and concentrate mycolic acids.

(i) Sample concentration by evaporation. Mycolic acids and fatty acids of AS019 were prepared from the culture fluids by heating samples at 80°C for 16 h to concentrate. Cells were grown in the appropriate media (30 ml), harvested, filtered, and then transferred into a 100-ml beaker. The culture broths in the beakers were heated at 80°C for several hours to reduce the volume initially, and then transferred into 20-ml MacCartney bottles for complete dryness at 80°C in a water bath. After 16 h evaporation, the dried samples were subjected to acid methanolysis, extracted by petroleum ether, and then concentrated under a stream of nitrogen. Finally, the mixtures of mycolic acids and fatty acids were silylated.

(ii) Sample preparation by solvent extraction. Mycolic acids of AS019 were also obtained from culture fluids using two different solvent systems: (1) chloroform-methanol and following chloroform-water, and (2) methanol-toluene-H_2SO_4 followed by petroleum ether. These were examined for their efficiencies to extract mycolic acids from culture fluids (30 ml) of LBG-GI.

For the first solvent system, the culture fluids after filtration were incubated with 3.3 volumes of chloroform/methanol (1:2, v/v) for 1 h using a rotary shaker (100 rpm) at 30°C and then held stationary for 4 h. Subsequently, one volume of chloroform and one volume of deionized water were added, the mixture incubated for 1 h using a rotary shaker (100 rpm), and then transferred into a separation funnel, which was kept on the bench for 16 h to allow phase separation. After collecting the top layer, this was transferred into a round-bottomed flask. This procedure was repeated twice. The organic solvent was removed using a rotary evaporator (Eyela) set at 40°C. The extraction that yielded the internal standard of petroleum ether was 60–85% in the first extraction, 90–98% by the second extraction, and over 99% by the third extraction, when comparing the area obtained relative to the total area for all three extractions. After solvents were evaporated to dryness, the extracts were transferred to GC vials by the addition of 2–3 ml of chloroform, and the round bottomed-bottle was washed with chloroform twice and the washes were also collected into the vials. Samples were concentrated by removing chloroform under a nitrogen stream. Mycolic acids and fatty acids were then treated just as samples obtained from whole cells.

For the second solvent system [4], the culture fluids after filtration were shaken at 100 rpm with 100 ml of methanol-toluene-H_2SO_4 (30:15:1, v/v/v) for 1 h in a 500-ml glass bottle, and then samples were held stationary for 4 h. Subsequently, following an addition of 60 ml of petroleum ether, the mixture was shaken for 1 h and transferred into a separation funnel. This was kept on the bench for 16 h to allow phase separation. After collecting the bottom organic layer, the solvent was removed by

rotary evaporation at 40°C, and the residue was derivatized by acid methanolysis and extracted by petroleum ether. Subsequently, the samples were concentrated under nitrogen and silylated, as described previously.

Gas Chromatography on Capillary Column

Trimethylsilyl (TMS) ethers of mycolic acid methyl esters (MAMEs) were analyzed by a gas chromatograph (Varian Star 3400 CX) equipped with flame ionization detector using a 25 m fused silica capillary column BPX5 (0.22 mm i.d.; 0.33 mm o.d., SGE, Scientific Pty, Ltd., Australia). The oven temperature was programmed at 260°C for 1 min, then increased by 10°C/min to 340°C. The injection port and flame ionization detector were kept at 300°C. Nitrogen was the carrier gas (0.9 ml/min).

Gas Chromatography Coupled with Mass Spectrometry (GC/MS)

The identity of the fractionated mycolic acid TMS ethers was determined by gas chromatography/mass spectrometry (GC/MS). The conditions of the gas chromatographic column and the inlet were the same as described above. GC/MS was performed on a Varian Saturn II mass spectrometer combined with electron impact ion source which was held at 300°C, and helium (1.0 ml/min) was the carrier gas.

RESULTS AND DISCUSSION

Evaluation of Evaporation Method at 80°C to Concentrate Mycolic Acids in Culture Fluids

Mycolic acids in culture fluids were concentrated to dryness by heating the samples at 80°C for 16 h. In order to determine whether mycolic acids were stable at this temperature during this procedure, *C. glutamicum* AS019 was first cultivated in LBG. After harvesting, the dried samples from whole cells were divided into four 50 mg lots into four tubes, and 100 µl of the internal standard, LAME (5 mg/ml), was added, respectively. One of these was stored at -20°C without addition of liquid, while the other three were incubated at 80°C after the addition of 3 ml of water or 3 ml of growth media; LBG and LBG-GI. After 16 h incubation, when all liquid from the tubes were evaporated, the four samples were subjected to acid methanolysis, extracted by petroleum ether, then concentrated under a stream of nitrogen, and finally analyzed by GC spectrometry. After injection into the GC, the remaining samples were stored at -20°C for 30 days and then analyzed again using the same GC conditions, in order to check the reproducibility of the method and stability of mycolic acids at -20°C during storage.

The qualitative mycolic acid profiles obtained from four samples were almost identical in terms of the relative percentage of mycolic acids detected, but there were quantitative variations (Table 1). When samples were resuspended in water and evaporated by heating at 80°C, there was little difference in the total peak area detected using fresh material (Day 1 analysis) compared to the control (dry cells, not heated). This indicated that mycolic acids were stable during the heating procedure when cells were in water. However, when cells were resuspended with LBG or LBG-GI and evaporated, the amount of mycolic acids detected by GC analysis decreased by 20% in LBG and 80% in LBG-GI, respectively. This was presumably due to the presence of sugar and amino acid components of the media, such as glucose, yeast extract, and glycine,

Table 1. Recovery of mycolic acids of AS019 from solutions following heating samples at 80°C for 16 h, omitting TLC purification.

Days[a]	Solvent/Temperature[b]	$C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$	Total peak area[c]
1	Control/-20°C	52.7[d]	31.3	8.5	5.3	2.2	493,749
	Water/80°C	53.7	30.8	8.7	4.9	2.0	518,774
	LBG/80°C	49.2	32.9	9.1	6.4	2.5	384,282
	LBG-GI/80°C	57.4	28.2	7.8	5.2	1.5	89,114
20	Control/-20°C	54.3	29.9	8.2	5.3	2.4	214,456
	Water/80°C	55.3	29.3	8.1	5.0	2.4	179,792
	LBG/80°C	55.4	29.4	8.1	5.1	2.0	116,544
	LBG-GI/80°C	58.1	28.0	7.7	4.6	1.6	21,565
30	Control/-20°C	54.5	29.9	8.5	5.1	2.0	161,505
	Water/80°C	55.5	29.6	8.4	4.6	1.9	152,097
	LBG/80°C	55.0	29.6	8.2	5.0	2.0	94,102
	LBG-GI/80°C	56.6	29.1	8.3	4.1	2.0	23,920
Average of MAMEs %±SD		54.8±2.3	29.8±1.3	8.3±0.4	5.1±0.6	2.0±0.3	

[a]Duration of sample (derivatized TMS ethers of MAMEs) storage at -20°C after various treatments.
[b]For controls, no solvent was added and samples stored at -20°C. For other samples, three portions of dried cells were resuspended with water, LBG, or LBG containing 2% glycine and 4 mg/ml INH, and incubated at 80°C for 24 h.
[c]The peak areas for the MAMEs were corrected for variations in the area obtained for the internal standard to account for loss during extraction procedures.
[d]Proportion of each mycolic acid was calculated as a % of the total in terms of peak area detected.

Table 2. Mycolic acid composition of whole cells and culture fluids of three strains of corynebacteria, grown in three different media with 80°C heating to concentrate, with TLC purification.

Strains	Mycolic acids[a]					Total peak area
	$C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$	
Mycolic acids from cells grown in LBG						
BF4	51.3±0.8	31.4±0.2	9.2±0.1	5.5±0.5	2.7±0.2	457198±66890
	(49.1±0.3)	(33.7±2.1)	(11.2±0.7)	(5.0±0.5)	(1.1±0.2)	10250±2790
BL1	50.7±0.6	36.9±0.2	3.8±0.1	7.4±0.4	1.5±0.1	384129±12989
	(50.8±3.6)	(35.3±3.2)	(5.6±0.5)	(6.1±0.5)	(2.3±0.1)	11074±2545
AS019	68.3±1.3	22.5±1.0	6.1±0.3	2.8±0.4	0.9±0.1	435413±48909
	(62.6±0.5)	(26.3±1.2)	(8.2±0.3)	(2.9±0.5)	(ND)	12264±3564
Mycolic acids from cells grown in LBG supplemented with 2% (w/v) glycine						
BF4	49.1±0.3	31.6±1.2	12.8±0.1	5.4±2.1	2.3±0.3	313000±51000
	(51.4±4.3)	(29.9±2.5)	(10.9±0.9)	(6.2±2.5)	(1.5±0.3)	28778±5789
BL1	50.7±0.1	36.3±0.1	3.7±0.1	8.1±0.7	1.3±0.1	281069±21280
	(50.4±2.5)	(36.2±2.5)	(5.7±0.6)	(7.7±0.8)	(ND)	37796±4312
AS019	68.1±0.5	21.2±0.5	7.1±0.1	2.6±0.2	1.1±0.1	386570±32442
	(63.9±1.8)	(21.7±1.8)	(11.0±1.7)	(3.4±1.5)	(ND)	21182±5644
Mycolic acids from cells grown in LBG supplemented with 4 mg INH/ml						
BF4	54.0±0.6	30.9±0.1	7.8±0.6	4.4±0.5	2.1±0.3	414940±35260
	(50.0±3.8)	(35.5±2.9)	(7.3±0.5)	(7.2±0.3)	(1.8±0.3)	19159±6660
BL1	52.5±0.2	34.6±0.4	4.8±0.8	6.6±0.3	1.6±0.3	324500±52342
	(48.3±3.8)	(37.0±3.0)	(7.0±1.1)	(7.6±0.5)	(ND)	22870±3031
AS019	63.3±0.5	25.4±0.4	6.9±0.2	3.1±0.2	1.3±0.1	356788±67769
	(56.0±0.3)	(29.0±1.4)	(10.5±0.3)	(3.5±0.1)	(ND)	18011±1500

[a]Proportion (%) of each mycolic acids was calculated as a % of the total in terms of peak area detected. Numbers in parenthesis i ndicate composition of mycolic acids found in culture fluids. ND, not detected.
Abbreviations: BF4, *B. flavum* BF4; BL1, *B. lactofermentum*; AS019, *C. glutamicum* AS019.

which formed sticky complexes with mycolic acid during the evaporation procedure and decreased extraction yield during the following solvent extractions.

Using these protocols, the mycolic acids were analyzed from both whole cell and culture fluid from three corynebacterial strains (Table 2). To evaluate the composition of mycolic acids in corynebacteria, the strains were grown in a shake-flask culture in LBG and LBG containing either 2% (w/v) glycine or 4 mg INH/ml, and the cells were harvested in the late exponential phase. All strains of corynebacteria had five types of mycolic acids ($C_{32:0}$, $C_{34:0}$, $C_{34:1}$, $C_{36:1}$, and $C_{36:2}$) in whole cells, but the relative proportions of each type varied with the strain and medium composition (Table 2). In terms of mycolic acids composition of whole cell, two *Brevibacterium* strains of BF4 and BL1 looked similar to each other, but different from AS019. Furthermore, the mycolic acid profiles in culture fluids of two *Brevibacterium* strains and a *C. glutamicum* strain, AS019, were almost the same as that from the whole cells. These results show that both mycolic acid profiles in the cell and fermentation broth are similar.

The above results indicated that heating at 80°C to concentrate mycolic acids from culture fluids was probably reliable and can be used to determine the relative proportion of the mycolic acids present, but this method cannot be used reliably for quantitative analysis of mycolic acids from the whole cells with culture fluids. This was presumably due to the presence of sugar and amino acid components of the media, such as glucose, yeast extract, and glycine. Consequently, an alternative approach to quantify mycolic acids in cells with culture fluids was sought based on solvent extraction to remove mycolic acids from culture fluids.

Evaluation of Solvent Extraction Methods for Concentrating Mycolic Acids in Culture Fluids

Two solvent systems were investigated in terms of recovery efficiencies. Table 3 indicated that the first solvent system could be reliably used to extract mycolic acid from culture fluids.

Although the total peak area seen for mycolic acids varied between extractions, corresponding changes in the area of the internal standard could be used to account for these differences, as the ratio between the total area for MAMEs and the area detected for the TMS ether of LAME remained relatively constant. The average for the relative percentage of each mycolic acid in the culture fluid samples showed small standard deviations, indicating that this method could be reliably used to detect the relative proportion of mycolic acids. When samples were prepared from the culture fluids using the first solvent system, there were no or insignificant background peaks detected

Table 3. Recovery of mycolic acids of AS019 from solutions following solvent extractions, omitting TLC purification.

Solvent mixture[a]	N[b]	$C_{32:0}$	$C_{34:1}$	$C_{34:0}$	$C_{36:2}$	$C_{36:1}$	Total peak area[d]	Area of IS[e]	STD[f]
Chloroform/methanol (Chloroform/water)	1	51.7[c]	31.9	9.1	5.0	2.3	57,698	179,120	0.322
	2	49.7	33.8	9.0	5.3	2.3	85,822	253,070	0.339
	3	47.4	33.5	9.0	7.1	3.1	38,913	100,370	0.388
Average of MAMEs %±SD		49.6±2.3	33.1±1.3	9.0±0.4	5.8±0.6	2.6±0.3			
Methanol/toluene/H_2SO_4 (Petroleum ether)	1	N.A[g]	N.A	N.A	N.A	N.A	N.A	19,195	
	2	N.A	N.A	N.A	N.A	N.A	N.A	27,675	

[a]Two combinations of solvents were used for extraction of mycolic acids. Solvent in the parenthesis was used later.
[b]Serial number of experiment for each method.
[c]The proportion of each mycolic acid was calculated as a % of the total.
[d]Sum of peak area of mycolic acid derivatives.
[e]Area of internal standard.
[f]Standardized sum of peak area from mycolic acids, which was obtained from the ratio of d/e.
[g]Because the peaks corresponding to mycolic acids were mixed with strong background, the information was not available (N.A).

above 280°C. In contrast, the second solvent system was found to be not suitable for this purpose, since it failed to extract significant amounts of mycolic acids (Table 3).

Sensitivity of analyses was verified as follows: approximately 50 mg of dried cells were subjected to acid methanolysis, extraction, and purification of MAMEs by TLC as described above. Finally, samples were silyated by using 400 μl of Tri-sil Z, and diluted with dilution factors of 5,10, 50, 100, and 1,000, and then, 1 μl of sample was analyzed by GC. The relationship between the peak area and the amount of TMS ethers of MAMEs applied was linear, with peak area ranging between <2,000 to 65,000. Based on these results, the detection limit of this method is <100 μg of dried cells. In most cases, samples described in the present study contained amounts of mycolic acids within this range of peak area.

TLC Experiments

Nonpolar lipids were extracted by acid methanolysis from the culture fluids and then separated by TLC as shown in Fig. 1. Two major components were observed in all the extracts. The component with the highest chromatographic mobility (R*f*>0.5) corresponded to fatty acid methyl esters (FAMEs), while the ones with lower mobility corresponded to mycolic acid methyl esters (MAMEs). All the extracts produced single spots for the FAMEs and MAMEs. The solvent blank also produced spots on the TLC plates and the absence of mycolic acids in these spots was confirmed by GC/MS analysis of the fractions collected from the TLC plates. The two major spots (FAMEs and MAMEs) were not detected when samples (cells or culture fluids) were replaced by the water. Moreover, it is important to note that mycolic acid profiles obtained from extraction procedures with TLC purification (see Table 2) and omitting TLC purification (see Tables 1 and 3) are totally different. At this stage, it is not clear why the difference occurred. One possible explanation is the differences between each mycolic acid and silica gel interacted in the TLC plate; however, further investigation is warranted.

Mass-Spectrometric Analysis of TLC-Derived MAMEs

TMS derivatives of mycolic acids from *Brevibacterium* and *Corynebacterium* strains, which were prepared from TLC were well separated by GC depending on the number of carbons and double bonds (Fig. 2). This procedure

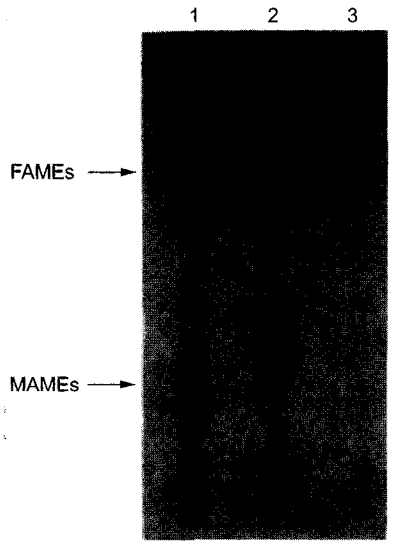

Fig. 1. Thin layer chromatography of whole cell methanolysates of strains of *C. glutamicum* AS019.
Solvent system: single run in petroleum ether (b.p. 60–80°C)-acetone (95:5, v/v). Abbreviations: FAMEs, fatty acid methyl esters, MAMEs, mycolic acids methyl esters. Lanes: 1, *C. glutamicum* AS019 (LBG); 2, mycolic acid standard from *Mycobacterium* species (Sigma, M4537); 3, fatty acid standard (Sigma, 189-6).

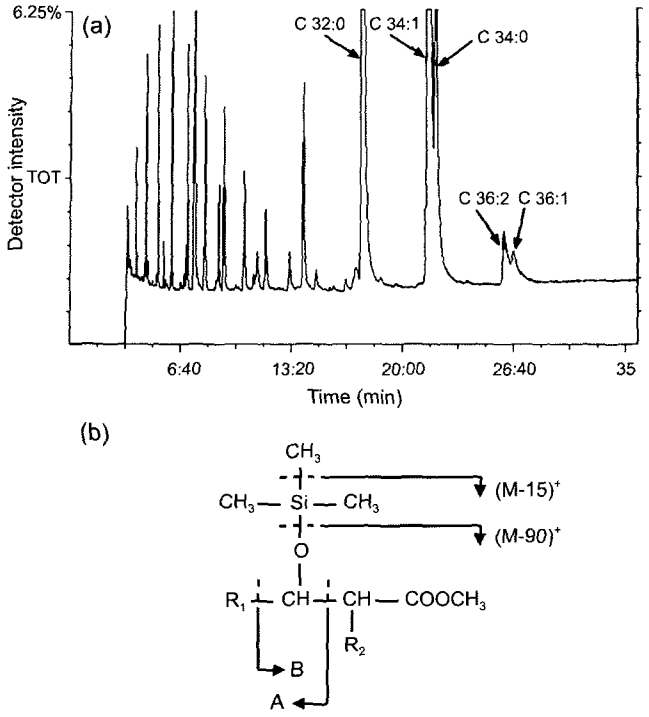

Fig. 2. GC analysis of TMS ethers of MAMEs on a nonpolar BPX5 fused silica capillary column.

Samples were prepared from stationary phase cells of *C. glutamicum* AS019 following growth in LBG-GI. A mass spectrometry detector was used. For each peak, the first number indicates the number of carbon atoms and the second indicates the number of double bonds.

provided 5 separated peaks for mycolic acids from both species. Furthermore, the reproducability of GC profiles was acceptable for fingerprinting of each strain. Further analysis to identify each peak was carried out by combined GC/MS as shown in Fig. 2. The overall structure of the mycolates was determined from the peaks corresponding to the loss of a methyl group (M-15$^+$) and the loss of the trimethylsilyl group (M-90$^+$). The R1 group and the R2 group were determined from the resulting fragments **A** and **B**, respectively. Peak **A** in Fig. 2b was commonly observed corresponding to **B**-29 (from ethyl groups) in Fig. 2, assisting to identify the R2 side chain (Fig. 3). Five major types of mycolic acids were identified ($C_{32:0}$, $C_{34:0}$, $C_{34:1}$, $C_{36:1}$, $C_{36:2}$). The first component eluted from the column was a $C_{32:0}$ mycolate, which showed the characteristic peak at *m/z* 567 (M-15$^+$) (Fig. 3a). Peaks at *m/z* 313, 371, and 342 corresponded to fragments **A**, **B**, and **B**-29, respectively, and showed that the R1 chain was the saturated $C_{15}H_{31}$ and the R2 group was $C_{14}H_{29}$. The second component fractionated was $C_{34:1}$ mycolate, which indicated fragments at *m/z* 593 (M-15$^+$) and 518 (M-90$^+$) (Fig. 3b). This peak consisted of two structural isomers, one form bearing an unsaturated $C_{17}H_{33}R_1$ chain and a saturated $C_{14}H_{29}R_2$ side chian, while the other bearing a saturated $C_{15}H_{31}R_1$ chain and an unsaturated $C_{16}H_{31}R_2$ side chain. The

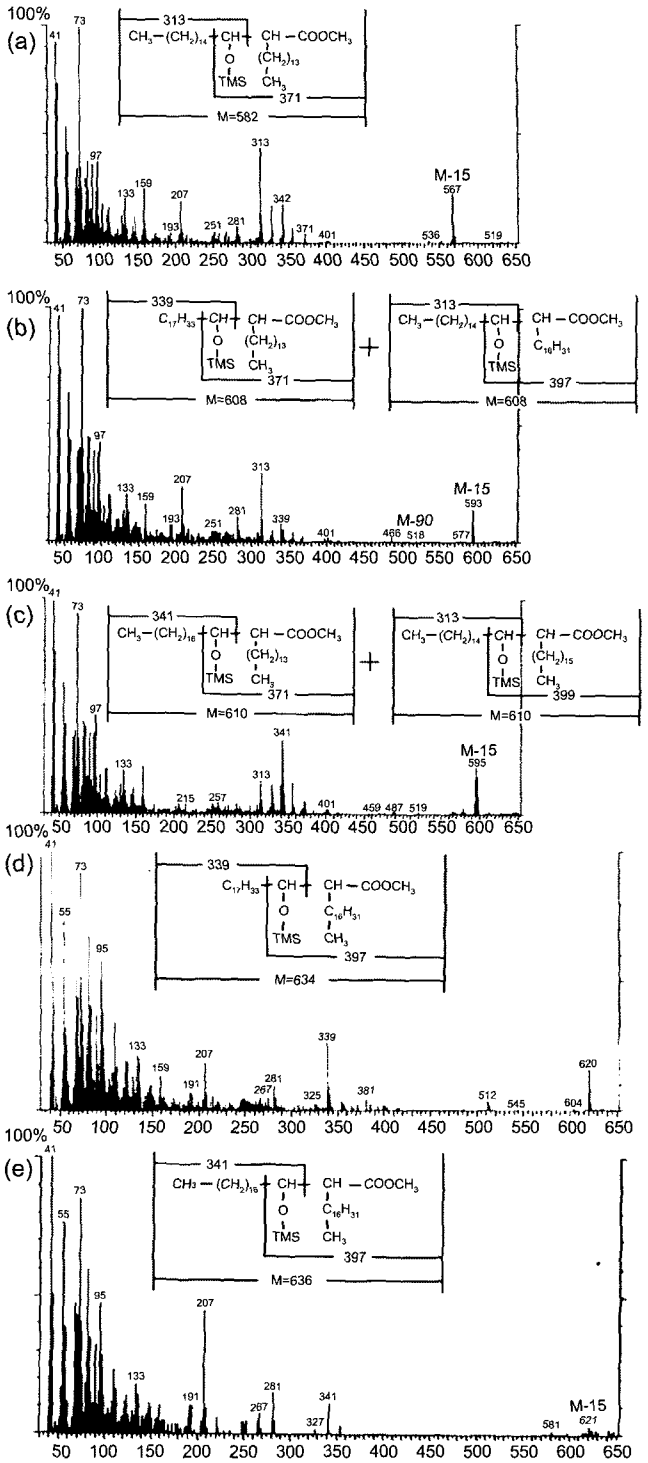

Fig. 3. Mass spectra of TMS ethers of MAMEs of *C. glutamicum* AS019.

The five major peaks detected by GC for the TMS derivatives were identified by GC-MS: the ordinate axis gives the relative fragment abundance (%). a: $C_{32:0}$, b: $C_{34:1}$, c: $C_{34:0}$, d: $C_{36:2}$, e: $C_{36:1}$.

third component fractionated was a $C_{34:0}$ mycolate, which also consisted of two structural isomers (Fig. 3c). One

form had a saturated $C_{17}H_{35}R_1$ chain and a saturated $C_{14}H_{29}R_2$ side chain, the other had a saturated $C_{15}H_{31}R_1$ chain and a saturated $C_{16}H_{33}R_2$ side chain. The fourth component fractionated was a $C_{36:2}$ mycolate (Fig. 3d), which did not yield the expected fragment at *m/z* 619 (*M*-15$^+$). The absence of *m/z* value of 619 may be due to self-ionization in the chamber [27]. The fifth component fractionated was a $C_{36:1}$ mycolate which gave a fragment at *m/z* 621 (*M*-15$^+$) (Fig. 3e).

Based on the present results, we concluded that this approach using solvent extraction was a good method for both qualitative and quantitative analyses of MAMEs from culture fluids, since this procedure yielded above 95% recovery of MAMEs. The successful appilication of this method in other genera might need a similar approach with prior understanding of the gas chromatographic conditions, since resolution of molecular species for mycolic acid on a capillary column became very difficult as the chain-length increased [24].

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