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GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC ANALYSIS OF VOLATILE AMINES PRODUCED BY SEVERAL STRAINS OF *CLOSTRIDIUM*

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SUMMARY

A gas chromatographic—mass spectrometric technique is proposed for the analysis of volatile amines which were isolated from *Clostridium* cultures by vacuum distillation and concentrated as hydrochloride salts. Headspace sampling after alkalinization of the salts under vacuum was the most suitable for subsequent gas chromatographic analysis. With ammonia-loaded helium as carrier gas, methylamines were separated on 4.8% PEG 20M + 0.3% potassium hydroxide on Carbopack B, and other volatile amines on 28% Pennwalt 223 + 4% potassium hydroxide on Gas-Chrom R. Bacterial volatile amines (dimethylamine, trimethylamine, isobutylamine, 3-methylbutylamine, etc.) were detected with a flame-ionization detector and identified by gas chromatography—mass spectrometry in electron-impact and chemical ionization modes.

INTRODUCTION

Volatile amines (VA) have been widely studied in foodstuffs [1—3] and in domestic or animal wastes [4—6], but their gas chromatographic (GC) analysis in bacterial growth media remains little documented.

GC analysis of VA bases is associated with analytical problems due to their high polarity, especially ghosting and severe tailing. These phenomena can be reduced with low reactive column packings such as porous polymers [7, 8] or alkali-coated packings [9—11] eventually pretreated with trimethylchlorosilane [4, 12]. Furthermore the addition of ammonia to the liquid sample [10, 11]

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or to the carrier gas [13] prevents the adsorption of VA and greatly improves their analysis.

When bacterial VA are present at trace levels, the formation of heptafluorobutyryl derivatives and electron-capture detection [14, 15] have been found valuable and avoid the above-mentioned problems. If derivatization is not expected, a vacuum distillation step is interesting to isolate VA from a complex matrix and to concentrate them as hydrochloride salts [16]. VA can be injected as their salts in an alkaline precolumn [17, 18] or as the corresponding bases after realkalinization and extraction [16]. The direct injection of a headspace sample of broth culture after salting out [7] or of an alkalinized culture supernatant fluid [8] has also been described.

A headspace gas chromatographic—mass spectrometric (GC—MS) technique was developed in this work for the analysis of VA produced by some strains of *Clostridium*, with a preliminary reduced pressure distillation step.

EXPERIMENTAL

Bacterial cultures

Nine strains of *Clostridium* were included in this study: *C. ghoni* ATCC 25757, *C. bifermentans* TM (Prévot), *C. sordellii* 82 (Prévot), *C. lituseburense* ATCC 25759, *C. mangenotii* ATCC 25761, *C. histolyticum* Tro 2E (Prévot), *C. perfringens* Lechien (Prévot), *C. difficile* ATCC 9689, and *C. cadaveris* ATCC 25783. A 50-ml volume of meat—liver medium (Infusion Viande-Foie, Institut Pasteur Production, 30 g in 1000 ml of bidistilled water), adjusted to pH 7.2 and contained in an Erlenmeyer flask fitted with two stopcocks (volume ca. 330 ml), was inoculated with 0.5 ml of a 24-h culture. Air was removed from the flask with a vacuum pump and the medium was incubated at 37°C for 120 h. Each strain was studied at least in triplicate along with sterile media processed in the same way.

Isolation of volatile amines

The broth culture was alkalinized with 10 g of sodium carbonate in a round-bottomed flask which was immediately connected to a glass distillation apparatus. Distillation was carried out at 35°C under 15—20 Torr until dryness (about 30 min), VA being trapped in two flasks containing 40 ml and 30 ml of 0.1 *M* hydrochloric acid, respectively. Both acidic solutions were pooled, evaporated and the residue of hydrochloride amine salts dried at 100°C in an air oven for 2 h.

Preparation of headspace sample

Hydrochloride salts, dissolved in 2 ml of bidistilled water, were introduced in a 35-ml flask (Fig. 1). After removing air with a vacuum pump, 1 ml of 6 *M* sodium hydroxide (analytical grade) was added. The flask was held at 50°C for 15 min, filled with helium and a headspace sample, collected in a 5-ml glass syringe through a lateral rubber septum, was injected into the column.

GC analysis

Analyses were performed on two gas chromatographs (Girdel 30 and Girdel

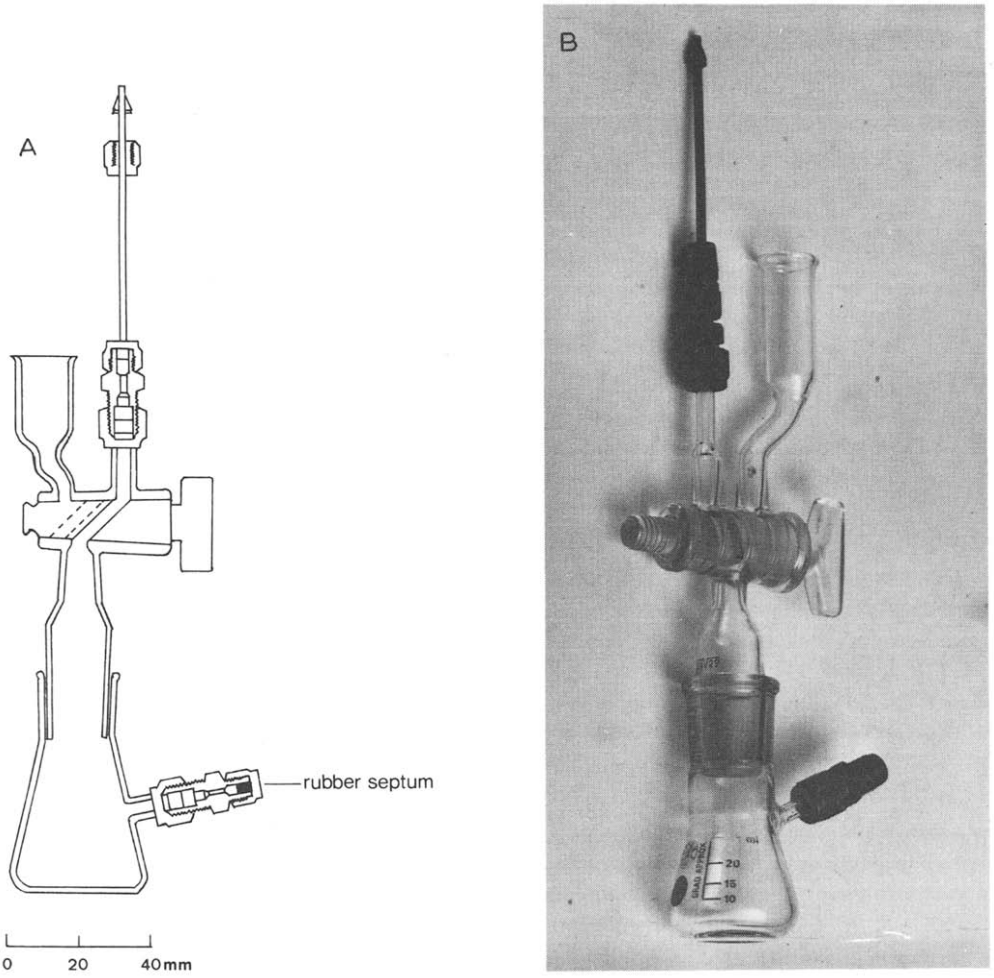

Fig. 1. Flask used for preparation of headspace samples. After alkalinization of volatile amine hydrochloride salts under vacuum, the flask is held at 50°C for 15 min, filled with helium and a headspace sample is collected in a 5-ml glass syringe through the lateral rubber septum.

300) equipped with flame-ionization detectors (air 350 ml min^{-1}, hydrogen 25 ml min^{-1}, sensitivity $5 \cdot 10^{-11}$ A f.s.). Injectors and detectors were heated at 220°C. Ammonia was added to the carrier gas (helium, flow-rate 17.6 ml min^{-1}) with a column conditioner as recommended by Dunn et al. [13] and built with Swagelok® fittings and a 30 mm × 6 mm O.D. glass tube, three-quarters filled with an ammonium hydroxide solution (Carlo Erba 30% RPE). Two borosilicate glass columns (2.10 m long, 6.35 mm O.D., 2 mm I.D.) were used, one packed with 28% Pennwalt 223 + 4% potassium hydroxide on 80—100 mesh Gas-Chrom R (Alltech; packing A) and operated isothermally at 80°C after conditioning overnight at 100°C, the other packed with 4.8% PEG 20M + 0.3% potassium hydroxide on 100—120 mesh Carbopack B (packing B, obtained from Professor Di Corcia, University of Rome) and operated isothermally at 50°C after conditioning overnight at 220°C. Retention times were

determined by co-chromatography with authentic standards of analytical grade: trimethylamine, dimethylamine and ethylamine hydrochlorides (Fluka); isopropylamine, isobutylamine and 2-methylbutylamine (Fluka); methylamine (Prolabo): *n*-butylamine, 3-methylbutylamine and *n*-pentylamine (Poly-Science); piperidine and pyrrolidine (Janssen-Chimica).

GC—MS analysis

A Riber R-10-10 quadrupole mass spectrometer coupled to a Girdel 30S gas chromatograph was operated in both electron-impact ionization mode (ionization potential 70 eV) and chemical ionization mode (ammonia 0.1 kPa, L'Air Liquide, purity ⩾ 99.96%). Chromatographic separation was performed on packing B with a temperature programme from 50°C to 170°C at 10°C min^{-1}. The operating mass range was 20—150 a.m.u. (*m/e* 28 and 32 excluded) and the scan rate was 245 a.m.u. sec^{-1}. Recording and calculation of mass spectra were done with a System Industries—Digital Equipment Corporation PDP/8M calculator. Mass spectra were compared with those of previously listed authentic standards and those of MS registers.

RESULTS AND DISCUSSION

Retention times of VA authentic standards in headspace GC analyses are given in Table I. Two different chromatographic techniques were necessary: Pennwalt 223 Amine Packing separated VA except methylamines as previously

TABLE I

RETENTION TIMES OF VOLATILE AMINE AUTHENTIC STANDARDS IN HEADSPACE GC ANALYSIS

Borosilicate glass columns (2.10 m long, 6.35 mm O.D., 2 mm I.D.). Injector temperature: 220°C. Detector: flame-ionization, temperature 220°C, attenuation $5 \cdot 10^{-11}$ A f.s. Carrier gas: helium (flow-rate 17.6 ml min^{-1}) loaded with ammonia.

	Retention time (min:sec)	
	28% Pennwalt 223 + 4% potassium hydroxide on 80—100 mesh Gas-Chrom R (80°C isothermal)	4.8% PEG 20M + 0.3% potassium hydroxide on 100—120 mesh Carbopack B (50°C isothermal)
Methylamine		0:54
Dimethylamine		2:00
Ethylamine		2:48
Trimethylamine	0:54	3:24
Isopropylamine	1:21	
n-Propylamine	1:54	
Isobutylamine	3:12	
n-Butylamine	4:09	
Pyrrolidine	6:30	
2-Methylbutylamine	6:45	
3-Methylbutylamine	7:00	
n-Pentylamine	8:30	
Piperidine	12:00	

reported by Dunn et al. [13]. For this latter purpose, packing B [9] was chosen. A preliminary study showed that a column packed with 4% Carbowax 20M + 0.8% potassium hydroxide on 60—80 mesh Carbopack B (commercially available from Supelco) could not be proposed for routine analysis of bacterial VA because of important instability of retention times in long-term use as sometimes noted [9, 19]. Furthermore, separation of C_1—C_6 VA on packing B would require temperature programming.

As ammonia prevents adsorption of VA in chromatographic columns, its addition to an amine solution is suitable for injection of liquids [10]. The headspace gas above an aqueous solution of VA and ammonia (1000 ppm) was sampled and injected but ghosting problems were still encountered. Alternative addition of ammonia to the carrier gas was much more efficient; this was attributed to the continuous presence of this base in the column and caused no analytical problem but a small and regular decrease of the baseline which was easily corrected with a second column in dual mode.

Results of analysis of VA produced by nine strains of *Clostridium* are summarized in Tables II and III. Chromatograms obtained with *C. sordellii* 82 are shown in Fig. 2. In previous studies of cultures of *Clostridium* by direct reduced-pressure headspace GC [20, 21], only trimethylamine was found as a VA. Even when replacing the flame-ionization detector by a thermoionic specific detector, no VA other than trimethylamine was detected in this work. This could have been due to the low concentration in the cultures and to the hydrogen-bonding ability of primary and secondary amines in the GC system and the aqueous medium. Problems associated with this phenomenon did not appear with trimethylamine. Thus it was thought advisable to isolate and concentrate VA as hydrochloride salts by vacuum distillation, a suitable method for such volatile compounds [16]. After alkalinization of the corresponding salts, injection of VA bases was tested in three modes: after extraction with a solvent (dodecane, standard for GC, Carlo-Erba), in aqueous solution and by headspace sampling. In the first modality, detection of late eluted dodecane did not interfere with that of low-molecular-weight VA but

TABLE II

VOLATILE AMINES PRODUCED BY NINE STRAINS OF *CLOSTRIDIUM* AND ANALYSED BY HEADSPACE GC ON A PENNWALT 223 COLUMN

Packing: 28% Pennwalt 223 + 4% potassium hydroxide on 80—100 mesh Gas-Chrom R. Other analytical conditions as in Table I. Results are arbitrarily expressed as peak area (mm^2), and are given as mean (n = 3 for each bacterial strain, n = 2 for uninoculated medium) with standard deviation in parentheses. For retention times of amines see Table I.

Bacterial strain	Trimethylamine	Unidentified compound*	Isobutyl-amine	3-Methylbutyl-amine	Unidentified compound**
C. ghoni ATCC 25757	8085 (1534)	1323 (90)	152 (62)	727 (184)	693 (104)
C. bifermentans TM	166,400 (7259)		1507 (496)	1140 (145)	
C. sordellii 82	87,040 (11,809)		237 (95)	24,309 (17,238)	
C. lituseburense ATCC 25759	5056 (832)		201 (44)	705 (359)	
C. mangenotii ATCC 25761	209,333 (15,123)	11,147 (760)	429 (105)	1592 (253)	1591 (362)
C. histolyticum Tro 2E	1495,040 (355,314)		859 (280)	1214 (130)	
C. perfringens Lechien	5679 (1222)		218 (113)	887 (296)	
C. difficile ATCC 9689	5957 (1104)		187 (61)	637 (146)	
C. cadaveris ATCC 25783	425,600 (56,708)		285 (41)	2007 (605)	
Uninoculated medium	5688 (577)		91 (16)	452 (6)	

*Retention time 1 min 48 sec.
**Retention time 10 min 48 sec.

TABLE III

VOLATILE AMINES PRODUCED BY NINE STRAINS OF *CLOSTRIDIUM* AND ANALYSED BY HEADSPACE GC ON A PEG 20M COLUMN

Packing: 4.8% PEG 20M + 0.3% potassium hydroxide on 100—120 mesh Carbopack B. Other analytical conditions as in Table I. Results are arbitrarily expressed as peak area (mm^2), and are given as mean (n = 3 for each bacterial strain, n = 2 for uninoculated medium) with standard deviation in parentheses. For retention times of amines see Table I.

Bacterial strain	Methylamine	Dimethylamine	Ethylamine	Trimethylamine
C. ghoni ATCC 25757		215 (129)	517 (7)*	9755 (2019)
C. bifermentans TM		83 (17)	372 (6)	139,520 (37,989)
C. sordellii 82	6875 (947)	1984 (816)		135,453 (8032)
C. lituseburense ATCC 25759	109 (12)	89 (8)	787 (45)	8176 (1343)
C. mangenotii ATCC 25761	272 (68)*	2500 (399)		144,043 (13,899)
C. histolyticum Tro 2E	88 (14)	107 (10)	1988 (162)	1132,893 (137,116)
C. perfringens Lechien	2107 (158)	102 (16)	254 (30)	8128 (356)
C. difficile ATCC 9689		104 (18)	1053 (724)	6955 (2947)
C. cadaveris ATCC 25783			645 (263)	302,933 (51,177)
Uninoculated medium	35 (7)	61 (27)	2610 (127)	5640 (509)

*The peak area could be determined only from two chromatograms.

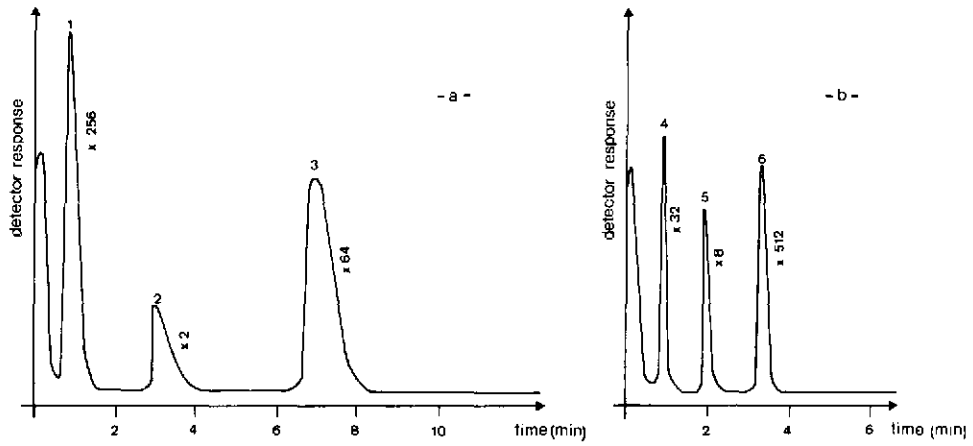

Fig. 2. Chromatograms of volatile amines produced by *C. sordellii* 82. Borosilicate glass columns (2.10 m long, 6.35 mm O.D., 2 mm I.D.) packed with 28% Pennwalt 223 + 4% potassium hydroxide on 80—100 mesh Gas-Chrom R (a) (80°C isothermal) or with 4.8% PEG 20M + 0.3% potassium hydroxide on 100—120 mesh Carbopack B (b) (50°C isothermal). Other analytical conditions as in Table I. Peaks: 1 and 6 = trimethylamine; 2 = isobutylamine; 3 = 3-methylbutylamine; 4 = methylamine; 5 = dimethylamine.

made repetitive analyses impossible. In the second modality, the injected volume was necessarily small, interfering peaks due to water appeared and crystallization of sodium chloride in the head of the column was bothersome. In the third modality, preliminary reduced-pressure headspace GC analyses were performed but adsorption of VA in the stainless-steel gas sampling valve could not be minimized. Therefore the direct injection of helium-diluted headspace gas, sampled with a glass syringe, was preferred.

In addition to co-chromatography, identification of VA was carried out by GC—MS. Electron-impact ionization of primary VA yields a small number of fragment ions (mainly *m/e* 30) and a low intensity molecular peak. Chemical ionization with ammonia as reagent gas was helpful for the determination of

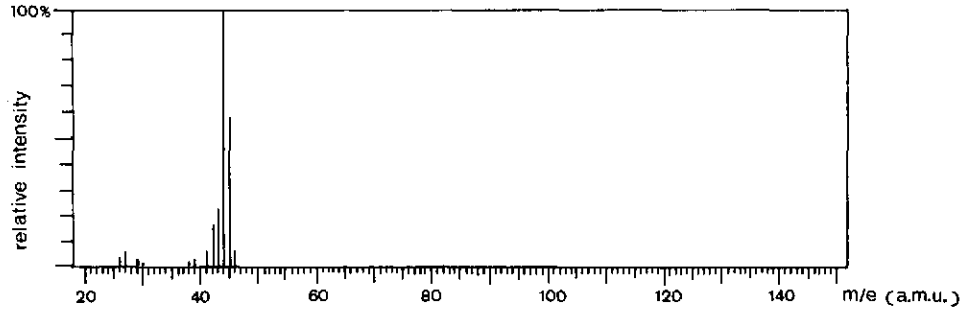

Fig. 3. Electron-impact mass spectrum of dimethylamine (molecular mass M = 45) from a sample of *C. mangenotii* ATCC 25761 (ionization potential 70 eV).

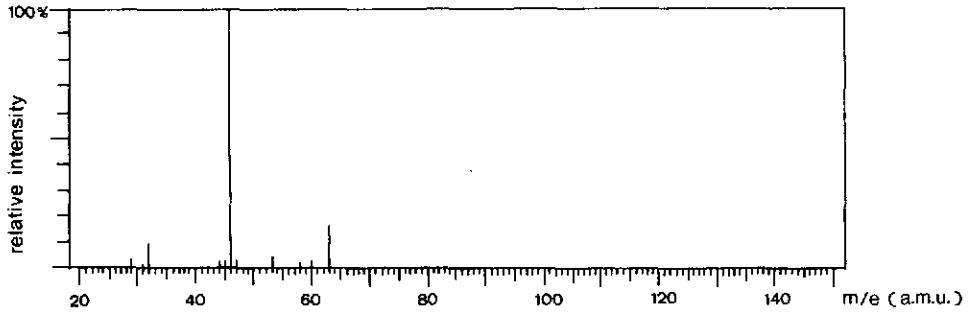

Fig. 4. Chemical ionization mass spectrum of dimethylamine (molecular mass M = 45) from a sample of *C. sordellii* 82, showing characteristic peaks M + 1 = 46 and M + 18 = 63 (ammonia 0.1 kPa).

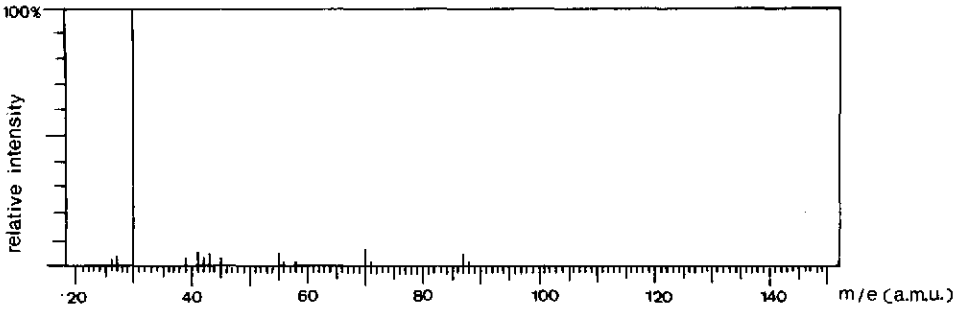

Fig. 5. Electron-impact mass spectrum of 3-methylbutylamine (molecular mass M = 87) from a sample of *C. mangenotii* ATCC 25761 (ionization potential 70 eV).

molecular mass. Mass spectra of dimethylamine (Figs. 3 and 4), trimethylamine, isobutylamine and 3-methylbutylamine (Figs. 5 and 6) were obtained with samples of *C. sordellii* or *C. mangenotii*. The profile of the ion *m/e* 30 showed a characteristic peak at the retention time of methylamine and a small peak of 2-methylbutylamine, eluted just before 3-methylbutylamine, which were difficult to discern on the total-ion current chromatogram. Another compound was detected which could not be identified as diethylamine or methylisopropylamine because of the similarity of their mass spectra.

GC analysis of bacterial amines has been proposed as a tool for identification

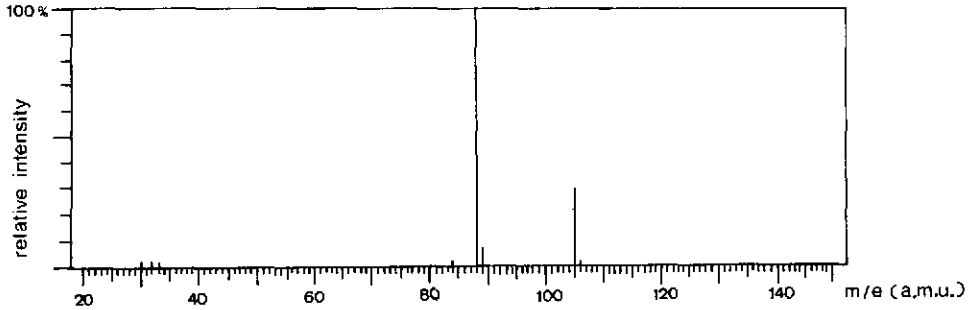

Fig. 6. Chemical ionization mass spectrum of 3-methylbutylamine (molecular mass M = 87) from a sample of *C. sordellii* 82, showing characteristic peaks M + 1 = 88 and M + 18 = 105 (ammonia 0.1 kPa).

of *Proteus* [7, 8, 14, 22], *Clostridium* [7, 23] and other pathogenic bacteria [24]. It was especially developed for the differentiation between *C. sordellii* and *C. bifermentans*: analysis of amine derivatives showed that β-phenylethylamine and tryptamine were produced by *C. bifermentans* [25, 26] whereas 3-methylbutylamine (isopentylamine) was produced by *C. sordellii* [15] as previously reported [27]. In this work, GC—MS analysis of concentrated bacterial VA confirmed 3-methylbutylamine with *C. sordellii* 82. Other decarboxylation amines such as the isomer 2-methylbutylamine and isobutylamine could be detected along with methylamines. Occurrence of methylamine and dimethylamine with *C. sordellii* 82 and of isobutylamine mainly with *C. bifermentans* TM may be mentioned as new distinctive characters of these strains. A preliminary distillation step is required in this work but seems necessary for recovery and concentration of VA traces. The GC technique proposed here presents all the advantages attributed to headspace analysis of amines [12], classical problems of VA analysis being overcome with ammonia conditioning of the column.

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