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Synthesis of 2-hydroxy acid from 2-amino acid by *Clostridium butyricum*

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Abstract: Cultures of *Clostridium butyricum* type strain in synthetic medium supplemented with various L-2-amino acids revealed the presence of the corresponding 2-hydroxy acid. This metabolite is able to produce the polyester poly(2-hydroxyalkanoic acid). The bioconversion is not stereoselective since D-2-amino acids were also converted. Chiral GC analysis demonstrated that only D-enantiomer is formed from L-leucine.

Introduction: Among the main categories of degradable and recyclable materials, the bioresorbables and bioassimilables polymers are the most of interest for the medical applications. Those go from the drug coating (control the release of an active ingredient) to the temporary implant which loses slowly its mechanical properties and thus allows the progressive reconstitution of tissues. Recently, polyhydroxyalkanoates polyesters (figure 1) became of industrial interest as one of the synthetic polymers the most used in these various fields[1].

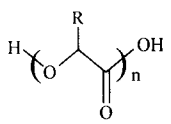

Figure 1: General formula of poly(2-hydroxyalkanoic acid)

The materials most studied until now are derivatives of poly(glycolic acid) (R = H) and poly[(2*S*)-lactic acid] (R = CH_3). These polyesters have attracted considerable interest from academia and industry as they are biodegradable and biocompatible thermoplastics and/or elastomers that are considered for a wide range of technical applications in industry, agriculture, medicine, pharmacology and other areas[2,3]. The degradation will be slow since the aliphatic chain length is high. Few studies were carried out on these polymers which have, because of the bulkier aliphatic groupings, a hydrophobic character and thus a resistance to the hydrolysis higher than polyglycolic and polylactic acids. These poly-α-hydroxyacids have good mechanical properties, they are not toxic for living organisms and their degradation, by hydrolysis, could be controlled.

The biotechnological exploitation of the microbial group of anaerobic bacteria for various industrial purposes is very promising, but, is still far from being accomplished. Some species of the genus *Clostridium* are specialized in the fermentation of individual 2-amino acids whereas others are able to realize the Stickland reaction, in

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which one 2-amino acid is oxidized whereas the other (or the same one) is reduced with 2-hydroxy and 2-enoic acids as intermediates[4-6]. The type species *C. butyricum* has not been reported as an amino-acid fermenting species[6-8], formic, acetic, butyric, and lactic acids are the final products of the carbohydrate fermentation by this glucidolytic, non proteolytic species. Recently, 2-hydroxy-4-methylpentanoic acid was identified by gas chromatography-mass spectrometry (GC-MS) in cultures of *C. butyricum* type strain[9].

The purpose of this paper was to present the ability of *C. butyricum* to convert natural as well as unusual 2-amino acids into the corresponding 2-hydroxy acid in synthetic BMG medium. Acid-extractable metabolites were analyzed by GC-MS as *tert*-butyldimethylsilyl (TBDMS) derivatives and the configuration of the 2-hydroxy acid obtained from L-leucine was determined by chiral GC.

C. butyricum strain used throughout this study is the type strain MGPP 26^T (VPI 3266^T), stored at -80°C in brain-heart medium with 15% (v/v) glycerol. The compositions of the complex medium trypcase-glucose-yeast extract-hemin (TGYH) and the synthetic basal mineral glucose (BMG) medium were as previously reported[9]. The 2-amino acids, supplemented as a 0.22-μm membrane-filtered solution, were presented in table I. The supplemented BMG media were incubated under nitrogen: carbon dioxide (80:20, v/v) at 37°C. No significant variation was observed when the gas phase N_2:CO_2 (80:20, v/v) was changed to H_2:CO_2 (95:5, v/v).

Results and Discussion: After culture of *C. butyricum* type strain in BMG medium in the absence of L-leucine, samples were processed by using a modification of a previously described method[10]. Only metabolites previously reported in the literature, i.e., formic, acetic, butyric, and lactic acids, were detected and 2-hydroxy-4-methylpentanoic acid was not detected [chromatogram (a), figure 2].

The formation of 2-hydroxy-4-methylpentanoic acid from L-leucine in *C. butyricum* cultures has been previously reported[9]. The use of L-(*methyl*-[$^2H_3$])leucine and L-[1-^{13}C]leucine unambiguously demonstrated that L-leucine is a precursor of 2-hydroxy-4-methylpentanoic acid. However, the ability of *C. butyricum* to bioconvert L-2-amino acids other than L-Leu was not known.

After culture in BMG medium supplemented with a given 2-amino acid, GC profiles show the presence of an additional peak(s), identified by GC-MS[11] to the corresponding 2-hydroxy acid. The bioconversion occurred in BMG medium with the hydrophobic L-amino acids L-leucine, L-valine, L-phenylalanine, and L-methionine. Some unnatural 2-amino acids with an hydrophobic chain, i.e., L-2-aminobutanoic, DL-2-amino-4-pentanoic, L-2-amino-4-pentenoic, DL-2-amino-4-hexanoic, L-2-aminooctanoic, and DL-2-amino-4-phenylbutanoic acids, yielded the corresponding 2-hydroxy acid. The two D-2-amino acids included in this study (D-leucine and D-methionine) both yielded the corresponding 2-hydroxy acid. Chromatograms (b) to (e) (figure 2) correspond to cultures supplemented with DL-norvaline (b), DL-norleucine (c), L-methionine (d) or L-phenylalanine (e). However, 2-hydroxy-3-methylpentanoic acid and 1-hydroxycyclopentanecarboxylic acids were not observed in synthetic medium supplemented with L-isoleucine and cycloleucine, respectively. In the case of L-isoleucine, this does not seem to be due to a steric hindrance on the C-3 atom since L-valine is a substrate in the process.

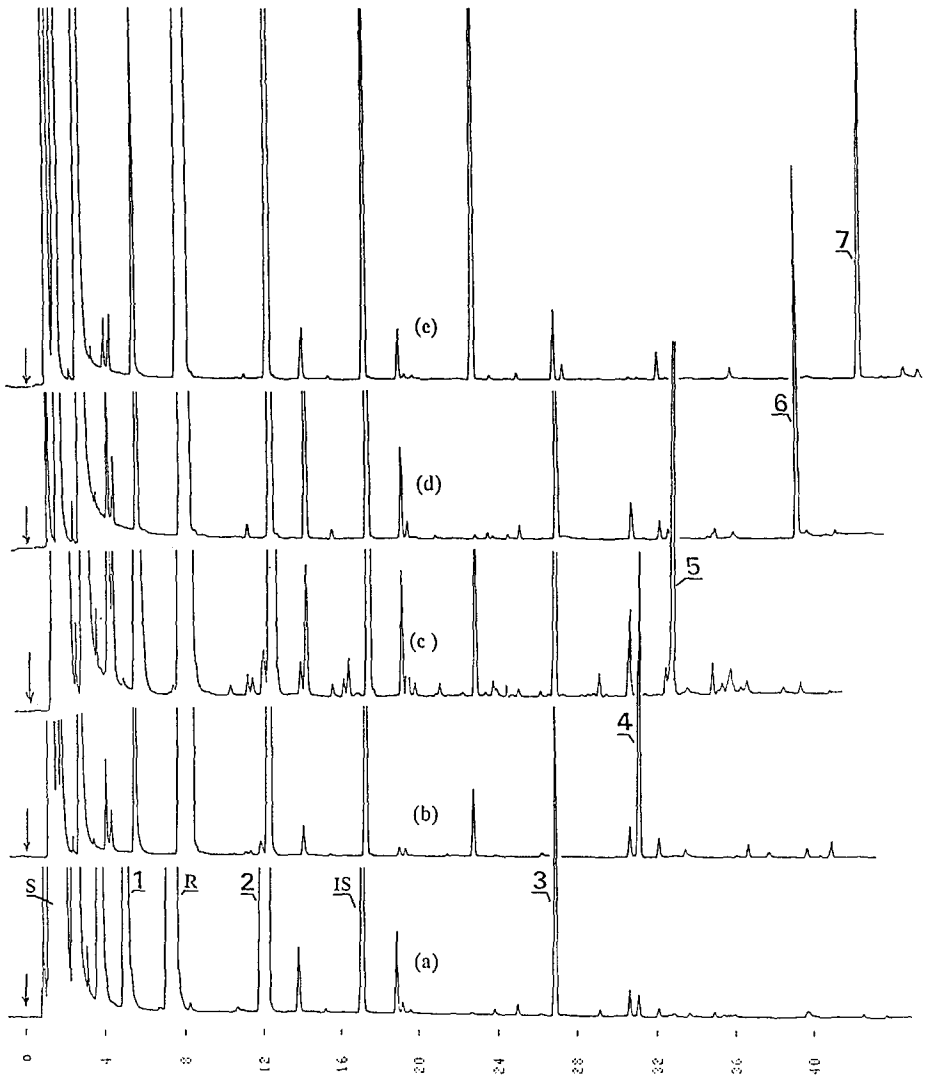

Figure 2: GC profiles obtained for *C. butyricum* cultures grown in BMG medium (a); in BMG medium supplemented with (b) DL-norvaline, (c) DL-norleucine, (d) L-methionine or (e) L-phenylalanine. For identification, S, solvent; R, *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide; IS, internal standard (2-methylpentanoic acid). TBDMS derivatives of: acetic acid (1); butyric acid (2); lactic acid (3); 2-hydroxypentanoic acid (4); 2-hydroxyhexanoic acid (5); 2-hydroxy-4-methylthiobutanoic acid (6) and 2-hydroxy-3-phenylpropanoic acid (7).

On the basis of retention times and comparison of mass spectra with those of authentic standards, the additional peaks were identified to di-TBDMS derivatives of the 2-hydroxypentanoic acid (peak 4), 2-hydroxyhexanoic acid (peak 5), 2-hydroxy-4-methylthiobutanoic acid (peak 6), and 2-hydroxy-3-phenylpropanoic acid (peak 7). The molecular masses observed were compatible and corresponding mass spectra reveal that the highest fragments were at m/z $[M-57]^+$ and $[M-15]^+$ as usually observed with TBDMS derivatives and the presence of

an ion at m/z $[M-57-28]^+$ formed by rearrangement of the fragment $[M-57]^+$ with loss of a carbonyl fragment indicates the presence of a 2-hydroxy carboxylate group, which is exemplified in the mass spectrum of the 2-hydroxy-4-pentenoic acid di-TBDMS derivative (figure 3).

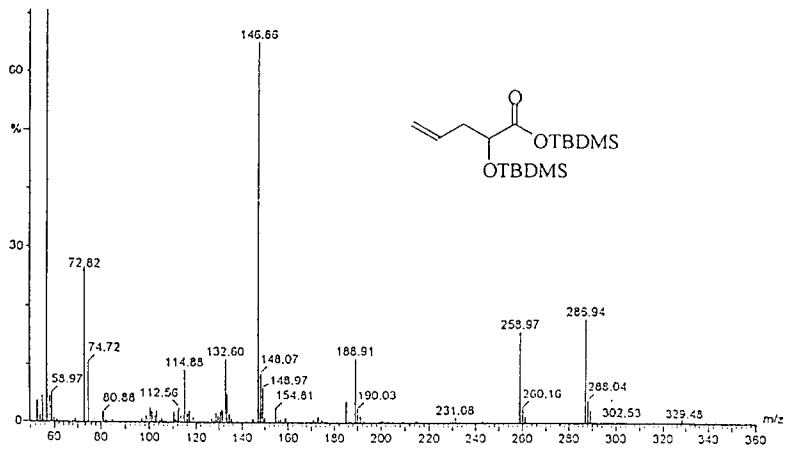

Figure 3: Mass spectra of di-TBDMS derivative of 2-hydroxy-4-pentenoic acid (MW: 344).

For the various 2-amino acids converted by *C. butyricum* in BMG medium after 24-h of incubation, the bioconversion rates were low and similar for D- and L-amino acids (table 1). The maximal rate observed with DL-norvaline being 6.2 %. In our study, there is no need to improve the culture techniques in order to achieve higher concentrations and better productivity for the 2-hydroxyacid produced.

Table 1: Yields of conversion of 2-amino acid into 2-hydroxy acid by *C. butyricum* in synthetic medium.

2-amino acid (substrat)		C*	2-hydroxy acid	yield
Systematic name	usual name			(%)
2-aminobutanoic	-	10	2-hydroxybutanoic	1.3
2-amino-3-methylbutanoic	L-valine	10	2-hydroxy-3-methylbutanoic	3.1
2-aminopentanoic	DL-norvaline	10	2-hydroxypentanoic	6.2
2-amino-4-methylpentanoic	L- or D-leucine	10	2-hydroxy-4-methylpentanoic	2.6
2-aminohexanoic	D,L-norleucine	10	2-hydroxyhexanoic	4.8
2-aminooctanoic	-	5	2-hydroxyoctanoic	1.0
2-amino-4-pentenoic	-	10	2-hydroxy-4-pentenoic	2.3
2-amino-4-methylthiobutanoic	L- or D-methionine	10	2-hydroxy-4-methylthiobutanoic	2.9
2- amino-3-phenylpropanoic	DL-phenylalanine	10	2-hydroxy-3-phenylpropanoic	1.1
2- amino-3-phenylbutanoic	DL-homophenylalanine	10	2-hydroxy-3-phenylbutanoic	0.4

C*: initial concentration of the 2-amino acid in $mmol.l^{-1}$

The absolute configuration of the C-2 atom of the 2-hydroxy acid formed from L-leucine [(*S*)-2-amino-4-methylpentanoic acid] has been determined by chiral GC[12]. The analyses were run after the derivatization of the 2-hydroxy acids into their trifluoroacetyl isopropyl esters[13]. Only the formation of (*R*)-2-hydroxy-4-methylpentanoic acid was observed (figure 4). This observation indicates that a configuration inversion on the C-2 atom is involved and agrees with the results[14] relative to the stereospecificity of 2-oxo acid reductase[15].

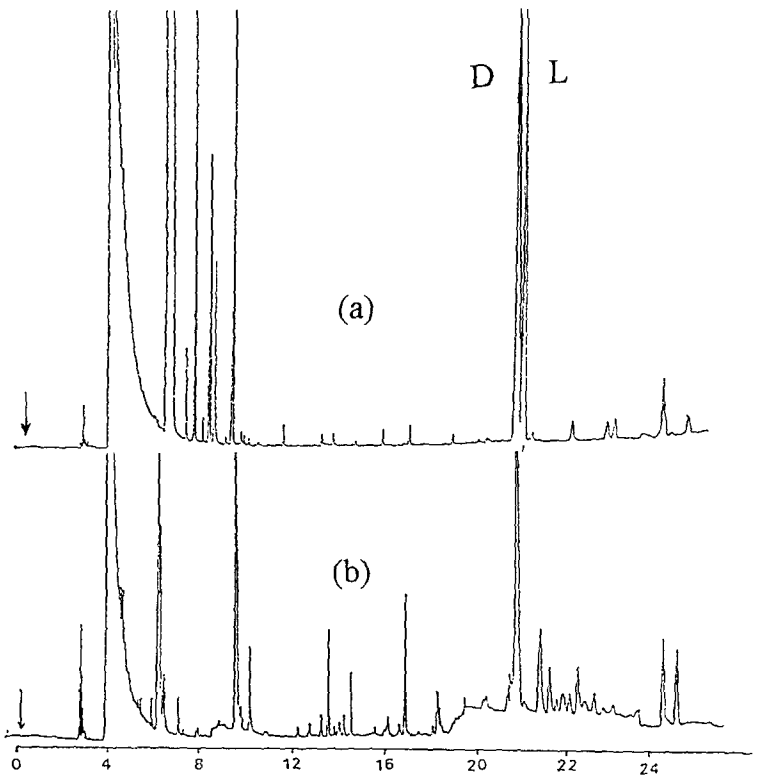

Figure 4: Chiral GC separation of 2-hydroxy-4-methylpentanoic acid: mixture of L-+D-enantiomers (a), and obtained from a 24-h culture of *C. butyricum* in BMG + L-leucine (b).

Results for cultures of *C. butyricum* in the presence of equimolar concentrations of L-leucine and acetate (10 mM each) did not show any significant variation in the 2-hydroxy-4-methylpentanoic acid concentrations. Only a slight increase of butyric acid concentration was observed. Acetate is an electron acceptor necessary for the complete degradation of mannitol by *C. butyricum*[16] and for the oxidation of several 2-amino acids (L-leucine, L-isoleucine, L-valine and L-alanine) by *C. acetireducens*[17,18], yielding butyric acid. In the case of *C. butyricum*, addition of acetate to L-leucine supplemented cultures at the beginning of the incubation was not followed by an increased of both butyric acid formation and L-leucine conversion.

Formation of 2-hydroxy-4-methylpentanoic acid has been reported for several anaerobic bacteria, e.g., for growing cultures in TGYH medium of clostridia (*C. sporogenes*, *C. difficile*, *C. paraputrificum*, *C. perenne*), *Peptostreptococcus anaerobius* and some propionibacteria[19], for resting cells of *Eubacterium brachy*[20] and for different species of *Listeria*[21]. Using the methylation procedure[22], the occurrence of 2-hydroxybutanoic and 2-hydroxypentanoic acids was described in different species of the genus *Fusobacterium* and in *C. innocuum*[19].

Although the 2-oxoacid was only detected after supplementation with L-methionine or L-phenylalanine, a two-step metabolic pathway including the intermediate 2-oxo acid can be postulated: the first reaction is the

oxidative deamination of L-leucine into 4-methyl-2-oxopentanoic acid, catalyzed by leucine dehydrogenase. A reduction by a D-2-hydroxyisocaproate dehydrogenase of the 2-oxo acid into (*R*)-2-hydroxy-4-methylpentanoic acid (first intermediate in the reductive branch of the Stickland reaction[5]) is the second reaction.

This finding opens new areas of research in amino-acid metabolism by *C. butyricum*. For biotechnology's, *C. butyricum* is a candidate for the obtaining of the D-enantiomer of various 2-hydroxy acids as precursors in the synthesis of biodegradable polyesters homologous to poly(glycolic acid) and poly[(2*S*)-lactic acid] or for enantioselective synthesis[23,24].

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10 Method described[9] with modifications as follows: *tert*-butylmethylether for the extraction and the organic layer was drying over anhydrous calcium chloride. TBDMS formation was carried out at room temperature by adding 50 µl of isooctane and 50 µl of silylating agent to the evaporation residue.
11 analyses were run on a Fisons GC800 gas chromatograph coupled to a quadrupole Fisons MD 800 mass spectrometer on a capillary column (methylsiloxane, 5% phenyl; 25 m x 0.25 mm i.d., oven temperature: 100°C to 320°C at 5°C min^{-1}) and electron impact for ionization (70 ev; emission current, 0.2 mA).
12 capillary column Chirasil-L-Val (50 m x 0.32 mm id). Oven temperature: 90°C for 4 min to 220°C at a rate of 4°C min^{-1}. Injector and detector temperatures: 250°C and 275°C, respectively.
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