J. Biosci., Vol. 21, Number 4, June 1996, pp 487–496. © Printed in India.

Biodegradation of commercial linear alkyl benzenes by *Nocardia amarae*

MANJU BHATIA* and H DEVENDRA SINGH†
Department of Biochemistry, Manipur University, Imphal 795003, Manipur, India
*Present address: Department of Chemistry, Oriental College, Imphal 795001, Manipur, India

MS received 18 September 1995; revised 3 April 1996

Abstract. Laboratory degradation studies of two indigeneously produced linear alkyl benzenes by *Nocardia amarae* MB-11 isolated from soil showed an overall degradation of linear alkyl benzenes isomers to the extent of 57-70%. Degradation of 2-phenyl isomers of linear alkyl benzenes was complete and faster than that of other phenyl position (C3–C7) isomers which were degraded to the extent of 40-72% only. Length of alkyl side chains (C_{10}–C_{14}) had little or no impact on the degradation pattern. Major metabolities detected were 2-, 3- and 4-phenyl butyric acids, phenyl acetic acid and *cis, cis*-muconic acid. Minor metabolites were *trans*-cinnamic acid, 4-phenyl 3-butenoic acid and 3-phenyl pentanoic acid along with two unidentified hydroxy acids. On the basis of the formation pattern of these metabolities, three catabolic pathways of linear alkyl benzenes isomers in *Nocardia amarae* MB-11 were postulated. All the phenyl position (C2–C7) isomers of C_{10}, C_{12}, and C_{14} linear alkyl benzenes along with 3-phenyl and 5-phenyl isomers of C_{11} and C_{13} linear alkyl benzenes were degraded via *cis,cis*-muconic acid pathway. Other phenyl position isomers of C_{11} and C_{13} linear alkyl benzenes with phenyl substitution at even number carbon atoms were principally degraded via phenyl acetic acid pathway while *trans*-cinnamic acid formation provided a minor pathway.

Keywords. Linear alkyl benzenes*; Nocardia amarae*; degradation patterns; catabolic pathways.

1. Introduction

In recent years, several reports showed the occurrence of linear alkyl benzenes (LAB) residues in municipal and domestic wastes and in marine and riverine sediments which were considered to arise from detergent bearing wastes (Eganhouse *et al* 1983; Ishiwatari *et al* 1983). These LAB residues are considered deleterious to all forms of life and pose environmental problem. Though there are several reports (Willets 1973; Gledhill 1974; Swisher 1987) on the details of the microbial degradation of alkyl benzene sulphonates (LAS), comparatively little is known on the microbial degradation of commercial LAB which are the main raw materials for many detergents. Utilization of 1-phenyl alkanes by *Nocardia isolates* (Sariaslani *et al* 1974), *Acinetobacter lwoffi* (Amund and Higgins 1985) and *Pseudomonas* species (Reddy *et al* 1983) was reported. It was shown that 1-phenyl alkanes were degraded by a combination of ω-, β- and α-oxidations of the alkyl side chains. It was proposed that 1-phenyl alkanes with odd number carbon chain were degraded to trans-cinnamate whereas compounds with even number carbon chain were catabolized via phenyl acetate and homogentisate (Amund and Higgins 1985). Commercial LAB however do not contain 1-phenyl

†Corresponding author.

alkanes which are not produced in the alkylation of benzene (Gledhill 1974) though they contain all the other possible phenyl position isomers. Characteristics of microbial degradation of complex commercial LAB containing various phenyl position isomers are not well understood.

This paper presents biodegradation of commercial LAB produced in India by a pure bacterial culture *Nocardia amarae* and proposes catabolic pathways for various phenyl position isomers of LAB.

2. Materials and methods

2.1 *Microorganisms*

The bacterial strain *N. amarae* MB-11 was isolated by enrichment culture technique from the soil samples collected from a location where crude oil and petroleum wastes were dumped. Purification of the organism was done by streak plate method using mineral agar medium (Davis and Raymond 1961) containing LAB as the sole carbon source. It was identified following the procedure described in Bergey's Manual of Determinative Bacteriology (1974) and Bergey's Manual of Systematic Bacteriology (1986). The species identification was done on the basis of the fatty acid profile signature using a Microbial Identification System (M/s Microcheck Inc., Northfield, Vermount, USA). The organism was maintained on mineral-agar slants containing LAB (0·5 ml/l).

2.2 *Chemicals*

Commercial LAB samples were obtained from M/s Indian Petrochemical Co. Ltd., Vadodara (LABI) and from M/s Tamilnadu Petrochemicals Ltd., Madras (LAB II).

2.3 *Cultivation*

Cultivation for growth and biodegradation studies was done in a series of 500 ml capacity conical flasks containing 50 ml sterile mineral medium, pH 7·2 (Davis and Raymond 1961) along with 0·5 ml LAB. The inoculum was prepared by cultivation of the organism on mineral-agar slants containing LAB as sole carbon source for 48 h. Active cells from slants were harvested and suspended in sterile mineral medium to serve as inoculum. Each flask was inoculated with 2 ml inoculum containing about 5 mg cells (dry wt) and incubated in a rotary shaker at 35°C for 96-144h.

2.4 *Estimation of biomass and residual LAB concentrations*

For this purpose flasks were withdrawn at regular intervals and the contents were centrifuged at 12,000 *g* for 30 min in a Sorvall Supercentrifuge. The clear aqueous phase between the top oil-cell emulsion layer and bottom cell sediment was removed carefully by suction. The residue containing cells was washed thrice with four volumes of a mixture of acetone and hexane (3:1 v/v) and the solvent washings were pooled and kept for analysis. The washed cells were dried at 60°-70°C for 18 h to constant weight to obtain dry biomass. For the estimation of residual LAB, the pooled solvent washings

were concentrated to a small volume and the concentrate was extracted with equal volumes of hexane twice. The middle aqueous layer obtained after centrifugation of the broth was also extracted twice with hexane. The hexane extracts were pooled and dried over anhydrous Na_2 SO_4 and desolvatized. The residual hydrocarbon was redissolved in a known volume of hexane and analysed by gas liquid chromatography.

2.5 *Isolation of acidic metabolites of LAB*

About 21 of culture broth obtained after cultivation of the organism on LAB for 72 h as described above was collected and centrifuged at 12,000 *g* for 30 min. For solvent extraction of metabolites, the middle aqueous layer free from cells and cell debris was collected and used as suggested by Raymond *et al* (1967) to avoid formation of complicating emulsions and coloured complexes with cell extracts. The aqueous layer was acidified with 6 N HCl to pH 2·0 and extracted thrice with equal volumes of diethylether. The ether extracts were pooled, dried over anhydrous Na_2SO_4 and desolvatized. The dried sample was dissolved in a small amount of diethylether and separation of acidic components was done by TLC on silica gel (Merck) coated plates (5 × 20 cm) according to the procedure of Sariaslani *et al* (1974). The chromatograms were developed in benzene-methanol-acetic acid (90:16:8 v/v) for non-hydroxylated aromatic acids and in petroleum ether (60-80°C)-diethylether-formic acid (45:5:1 v/v) for the hydroxylated acids. Non-hydroxylated acids were detected by spraying the dried plates with 0·4% bromocresol green whereby they appeared as yellow spots against a blue background. Hydroxylated acids were detected as blue spots by spraying with 1% ethanolic Gibb's reagent (2,6-dichloro-*p*-benzoquinone-4-chlorimine) followed by saturated aqueous $NaHCO_3$.

Preparation of individual acidic metabolites was done by preparative TLC (PLC) on 500-1000 μm thick silica gel coated plates (20 × 20 cm) and developed as described above. After detecting the individual spots by comparing with a control run, these were scrapped from the plates. The metabolites were recovered by elution with petroleum ether (60-80°C) and crystallized from petroleum ether (60-80°C)-benzene (100:1 v/v) for spectrometric and other analyses.

Methyl esters of acidic metabolites were prepared with 10% boron trifluoride in methanol (Metcalfe and Schmitz 1961).

2.6 *Instrumental analysis*

Components of LAB samples were analysed using Finnigan Mat INCOS 50 quadruple mass spectrometer interfaced with a Varian 3400 gas chromatograph. Gas chromatography was done using a capillary column (30 m × 0·25 mm) of fused silica coated with DB-5 at programmed temperature of 140°-170°-300°C. Mass spectral analysis was done at an electron beam energy of 70 eV and the mass spectral data were processed by a Finnigan INCOS Data System with a data bank for 42,220 compounds.

The IR absorption spectra were recorded by thin film technique using a Perkin Elmer Model 580-B IR spectrophotometer. NMR spectra were obtained with a Varian T-60 NMR spectrometer using deuterochloroform with trimethylsilane as internal standard. Mass spectral analysis was done by a Finnigan Mat INCOS 50 GC-Mass spectrometer.

3. Results and discussion

3.1 *Growth and utilization of LAB*

N. amarae MB-11 is a Gram positive, coccoidal, capsular, non-motile, acid fast and strictly aerobic bacterium. Its generic identification was confirmed by the fatty acid profile signature but the species identification was tentative as the profile signature had a similarity index of 0·410 which was below the level (0·500-0·990) considered to be an

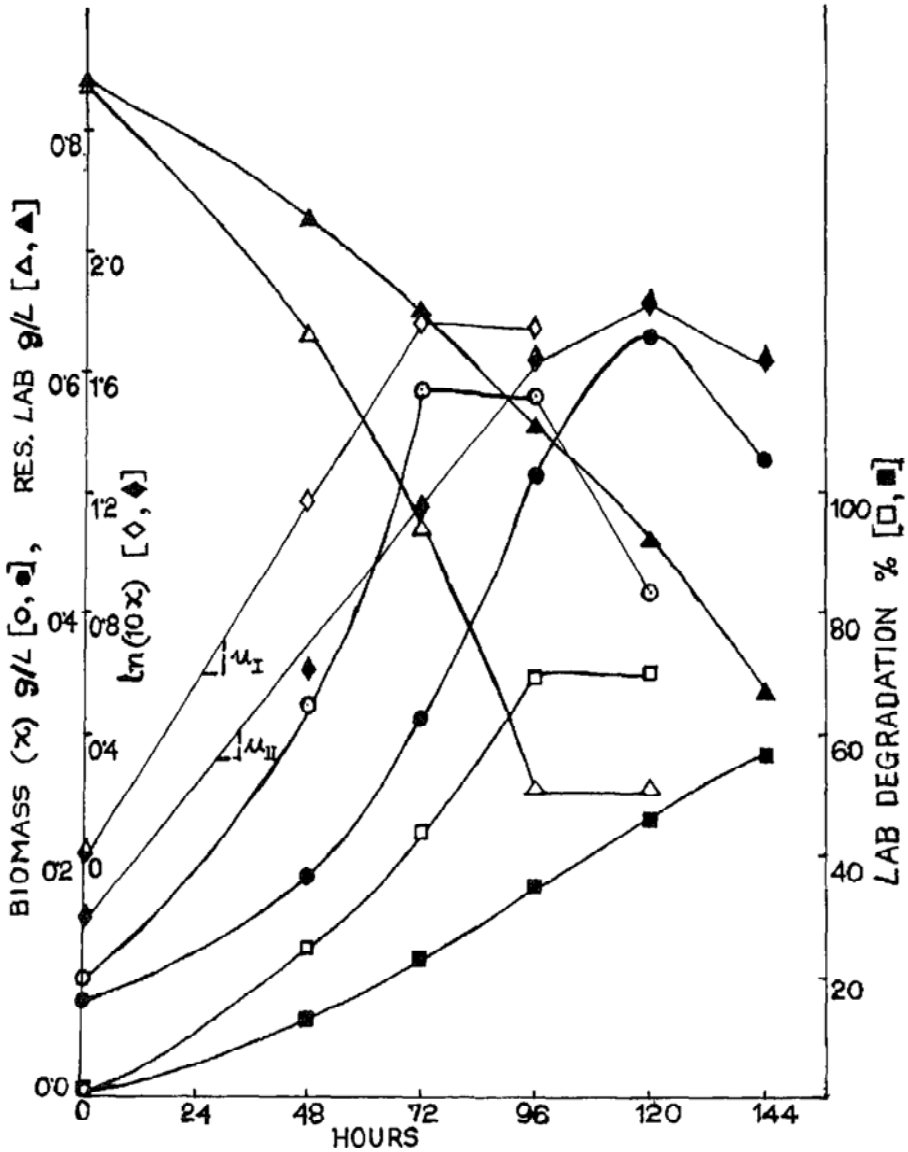

Figure 1. Growth of *N. amarae* MB-11 on LAB I (O,Δ, ◊, □, Al) and LAB II (●,▲,♦,■) (O,) Growth; (◊,♦) Log_e biomass conc.; (Δ , ▲), Residual LAB; (□,■), LAB degradation.

excellent match. The ability of various Nocardia species to degrade and utilize LAB was well documented (Webley *et al* 1956; Davis and Raymond 1961; Sariaslani *et al* 1974).

An exponential growth of *N. amarae* MB-11 on LAB for 72-96 h follwed by a stationary or decline phase was observed (figure 1). The growth was faster on LAB I (μ=0·24 h^{-1}) than on LAB II (μ = 0·018 h^{-1}) probably due to the different compositions of these LAB samples. LAB I contained alkyl side chain with 11 to 14 carbon atoms whereas LAB II had C_{10}–C_{13} alkane side chains (tables 1 and 2). Both LAB samples contained some poorly characterized components constituting less than 1%. Degradation of LAB was concomitant with cellular growth. Overall degradation of LAB I was to the extent of 70% in 96 h and LAB II to 57% in 144 h. With the decline in growth, further degradation was also stopped. Biomass yield was quite low in the range of 0·11-0·13 indicating the possible accumulation of metabolites.

3.2 *Degradation of LAB isomers*

Comparative degradation of various phenyl position isomers of LAB homologues by *N. amarae* MB-11 is shown in tables 1 and 2. Highest rate of degradation was observed with 2-phenyl isomers which were completely degraded during the incubation period. Alkane chain length had generally a negligible effect on the rate of degradation of this isomer. The pattern of degradation of 3-, 4-, 5-, 6- and 7-phenyl isomers of various alkyl chain lengths in each LAB sample was also by and large similar. Maximum degradation attained for these isomers was 62-72% in the case of LAB I and 40-54% in the case of LAB II. The result indicates that LAB degradation by this organism does not follow the "distance principle" postulated by Swisher (1963, 1987) which states that

Table 1. Biodegradation profile of the constituents of LAB I during incubation with *N. amarae* MB-11.

		0 h		Degradation (%)		
GC peak No.	Identification of phenyl (R) alkanes	% of total LAB	g/l	48 h	72 h	96 h
1–6	Unidentified	0·82	0·07	100·00	100·00	100·00
7	5R + 4R–C_{11}	9·74	0·82	13·7	27·2	66·3
8	3R–C_{11}	2·57	0·22	24·7	38·1	72·6
9	2R–C_{11}	2·28	0·19	45·7	100·00	100·00
10	6R + 5R–C_{12}	11·86	1·00	15·4	29·9	61·7
11	4R–C_{12}	4·17	0·35	13·5	30·8	58·8
12	3R–C_{12}	3·77	0·32	18·4	39·5	64·5
13	2R–C_{12}	3·67	0·31	46·0	100·00	100·00
14	6R + 5R–C_{13}	17·88	1·51	25·2	41·2	63·9
15	4R–C_{13}	5·67	0·48	29·2	31·3	62·9
16	3R–C_{13}	4·66	0·39	23·4	35·8	62·0
17	2R–C_{13}	4·06	0·34	46·8	100·00	100·00
18	7R + 6R + 5R–C_{14}	17·22	1·45	23·5	34·7	68·3
19	4R–C_{14}	4·28	0·36	16·4	35·7	67·6
20	3R–C_{14}	3·77	0·32	27·1	38·5	63·9
21	2R–C_{14}	3·53	0·29	64·5	100·00	100·00
Total		99·95	8·42	—	—	—

Table 2. Pattern of biodegradation of LAB II components during incubation with *N. amarae* MB-11.

GC peak No.	Identification of phenyl (R) alkanes	0 h		Degradation (%)		
		% of total LAB	g/l	48 h	72 h	144 h
1 + 2	Unidentified	0·86	0·07	100·00	100·00	100·00
3 + 4	5R + 4R–C_{10}	7·51	0·63	8·5	12·8	52·8
5	3R–C_{10}	5·02	0·42	9·8	15·1	54·5
6	2R–C_{10}	4·85	0·41	34·4	53·6	93·0
7	5R–C_{11}	12·47	1·05	6·5	12·9	47·0
8	4R–C_{11}	7·18	0·61	7·6	14·0	49·1
9	3R–C_{11}	6·87	0·58	8·8	16·2	49·8
10	2R–C_{11}	6·83	0·58	32·5	52·6	93·9
11	6R + 5R–C_{12}	12·42	1·05	6·2	12·2	44·0
12	4R–C_{12}	5·71	0·48	9·6	15·5	45·9
13	3R–C_{12}	5·62	0·47	10·8	17·0	46·3
14	2R–C_{12}	5·49	0·46	35·2	55·8	94·6
15	6R + 5R–C_{13}	9·37	0·79	6·2	11·2	42·4
16	4R–C_{13}	3·82	0·32	9·4	14·3	44·4
17	3R–C_{13}	3·17	0·27	2·3	7·6	40·0
18	2R–C_{13}	2·79	0·24	40·2	62·5	100·0
Total		99·98	8·43	—	—	—

degradation is slower with the decreased distance between the phenyl group and the centre of the alkyl chain and with the decrease in the alkyl chain length. Other investigators (Bayona *et al* 1986) using a *Pseudomonas* sp. however reported that the principle was followed in the degradation of a commercial LAB containing C —C_{14} compounds. It is possible that the microorganism used may be the deciding factor in determining the degradation pattern.

Because of their homologous character and isometric distribution, LAB residues were considered to be useful molecular markers of domestic wastes and even as geochronological tools (Eganhouse 1986). In this respect, the results obtained may have significant implication. The unidentified components in the two LAB samples which were recorded as initial peaks in chromatograms were completely degraded in 48 h of incubation as indicated by the disappearance of the peaks (tables 1 and 2).

3.3 *Metabolites of LAB*

Degradation of LAB is likely to produce a series of acidic metabolites. Identification of these metabolites may throw light on the metabolic pathways of degradation of LAB isomers and homologues. Five major spots and three minor spots of the metabolites from LAB were observed in TLC. Crystalline samples obtained from these spots were designated compound No. I—VIII, and these were subjected to IR, NMR and mass spectrometric analysis for identification. Two spots of hydroxylated acidic metabolites were observed on TLC but sufficient amount could not be recovered for spectrometric analysis.

Compound I. Mass m/e: 163(M^+), 135(2%), 119(20%), 91 (base peak), 77(15%), mol. wt.–164.

Compound II: Mass m/e: 163(M^+), 118(18%), 105(base peak), 77(19%), mol. wt —164.

Compound III. Mass m/e: 163(M^+), 146(12.5%), 117(4.8%), 104(base peak), 91(65·7%), 77(7·9%), mol. wt.-164.

The above mass data are consistent with aromatic compounds with butyric acid side chain. NMR and IR spectra of compounds I, II and III closely correspond with the literature data for 2-phenyl-, 3-phenyl and 4-phenyl butyric acid respectively and these are identified as such.

Compound IV. Mass m/e: 161(M^+), 117(base peak), 115(72.9%), 91(29.7%), 77(4.4%), mol. wt.-162. The data are indicative of an aromatic compound with a C_4-unsaturated side chain having a terminal carboxyl group. Intense peak at m/e 117 suggests a double bond at C_3—C_4 position indicating the compound to be 4-phenyl 3-butenoic acid. NMR and IR spectra of the compound matched well with the known spectra for 4-phenyl 3-butenoic acid and confirmed the identification.

Compounds V, VI and VII. These compounds were identified as phenylacetic acid, *trans*-cinnamic acid and *cis*, *cis*-muconic acid respectively on the basis of close identity of NMR, IR and mass spectra with those of authentic compounds.

Compound VIII. This acidic metabolite was converted to methyl ester and spectrometric analysis was conducted on the methyl ester. Mass m/e: 192(M^+), 160(16%), 118(45%) 105(base peak), 91(74%), 77(21%). Computer matching of the spectra with the library spectra using Finnigan INCOS Data System indicated the compound to be 3-phenyl pentanoic acid methyl ester. IR and NMR data confirmed the identification.

3.4 *Catabolic pathways*

Isolation of 2-, 3- and 4-phenyl butyric acids as major metabolites indicates that initial shortening of alkyl side chain by ω- and β-oxidation takes place without cleavage of the benzene ring in the degradation of these phenyl position isomers of LAB as in the case of 1-phenyl alkane degradation (Webley *et al* 1956; Sariaslani *et al* 1974). Whereas the formation of phenylacetic acid and benzoic acid from 1-phenyl alkanes can be readily understood by ω-oxidation and successive β-oxidative removal of two carbon atoms from the side chain, such a cleavage system alone cannot fully explain the formation of these metabolites from other phenyl position isomers of LAB. On the basis of the pattern of metabolites detected, degradative pathways for these LAB isomers are proposed as shown in figures 2 and 3.

All the phenyl position of isomers of C_{10}, C_{12} and C_{14} LAB probably give rise to a common intermediate, 2-phenyl succinic acid (3) by ω- and β-oxidation (figure 2). This is consistent with the isolation of 2-phenyl (1) and 3-phenyl butyric acids (3) as major metabolites. The same common intermediate is also likely to be formed from

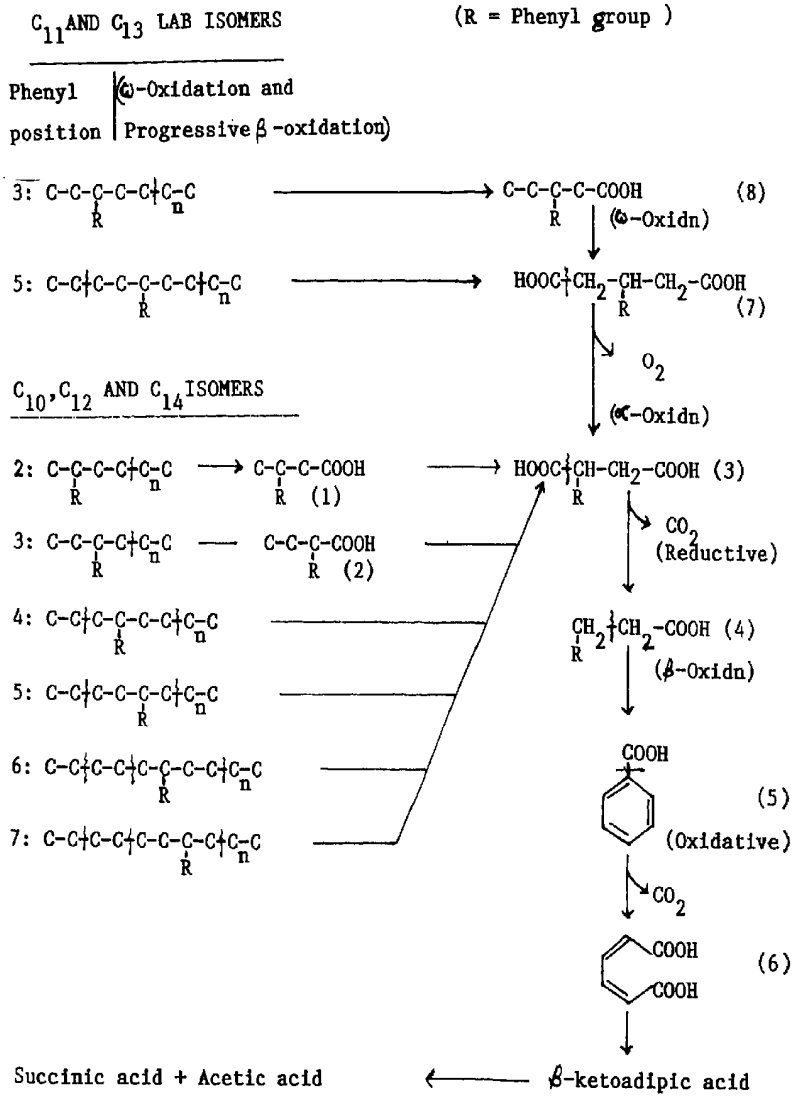

Figure 2. Proposed muconic acid pathway of the catabolism of C_{10}—C_{14} LAB isomers.

3-phenyl and 5-phenyl isomers of C_{11} and C_{13} LAB through the intermediate formation of 3-phenyl glutaric acid (7) and 3-phenyl pentanoic acid (8) which was detected as a minor metabolite. The common intermediate is probably degraded via the formation of 3-phenyl propionate (4) and benzoic acid (5) to *cis*,*cis*-muconic acid (6) which was one of the major metabolites isolated. This conversion necessarily involves the decarboxylation of carboxyl carbon adjacent to the phenyl attachment to form 3-phenyl propionate. Various investigators (Willets 1973; Gledhill 1974) assumed that this is brought about by α-oxidation accompanied by decarboxylation. This is however, unlikely, because such a reaction involves oxidation of the α-carbon atom which, in this case has a phenyl substituent. It is possible that this step is carried out by a special decarboxylation reaction involving the protonation of α-carbon atom and

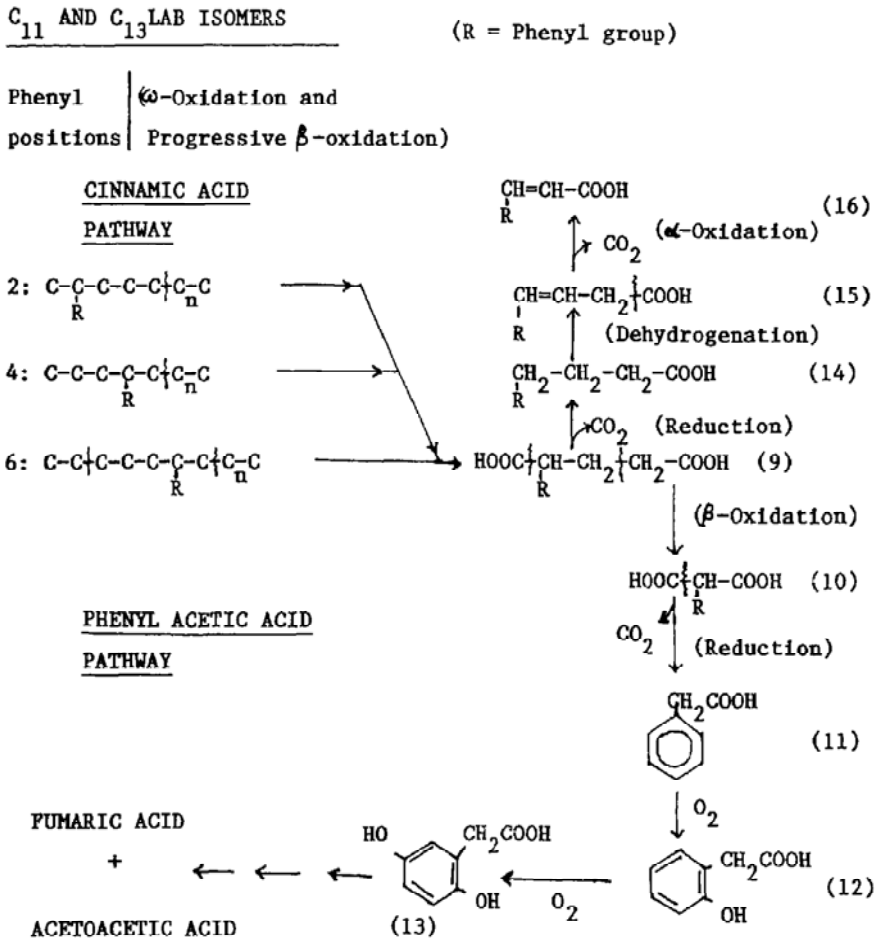

Figure 3. Proposed catabolic pathways of 2-phenyl, 4-phenyl and 6-phenyl isomers of C_{11} and C_{13} LAB.

cleavage of carboxyl carbon as CO_2 (nonoxidative decarboxylation) in a process somewhat similar to the decarboxylation of α-picolinate (Walsh 1978).

By *ω*- and progressive *β*-oxidation, 2-phenyl, 4-phenyl and 6-phenyl isomers of C_{11} and C_{13} LAB may give rise to a common intermediate, 2-phenyl glutaric acid (9) which is subsequently converted to 2-phenyl malonic acid (10) by *β*-oxidative cleavage (figure 3). Phenyl acetic acid (11) which was isolated as a major metabolite may be formed from 2-phenyl malonic acid by nonoxidative (reductive) decarboxylation. Phenyl acetic acid may be further degraded via formation .of α-hydroxy phenyl acetic acid (12) and homogentisic acid (13) to acetoacetic acid and fumaric acid as suggested by Sariaslani *et al* (1974). Detection of two hydroxy aromatic metabolites by reaction with Gibb's reagent (characteristic of a compound hydroxylated at the ortho position of benzene ring) is significant. Phenyl acetic acid pathway is likely to be the major catabolic pathway for these isomers.

A minor pathway may be the formation of *trans*-cinnamic acid (16) from 2-phenyl glutaric acid via the successive formation of 4-phenyl butyric acid (14) and 4-phenyl

3-butenoic acid (15) as shown in figure 3. All these compounds were isolated as major (14) and minor metabolites (15, 16).

References

Amund O O and Higgins I J 1985 The degradation of 1-phenylalkanes by an oil degrading strain of *Acinetobacter lwoffi; Antonie van Leeuwenhoek: J. Microbiol. Serol*. **51** 45-56

Bayona J M, Albaiges J, Solanas A M and Grifoll M 1986 Selective aerobic degradation of LAB by pure microbial cultures; *Chemosphere* **15** 595-598

Davis J B and Raymond R L 1961 Oxidation of alkyl substituted hydrocarbons by a *Nocardia* during growth on n-alkanes; *Appl. Microbial*. **9** 383-388

Eganhouse R P 1986 Long chain alkylbenzenes: their analytical chemistry, environmental occurrence and fate; *Int. J. Environ. Anal. Chem*. **26** 241-263

Eganhouse R P, Blumfield D L and Kaplan I R 1983 Long chain alkylbenzenes as molecular tracers of domestic waste in the marine environment; *Environ. Sci. Technol*. **17** 523-530

Gledhill W E 1974 LAS: Biodegradation and aquatic interactions; *Adv. Appl. Microbial*. **17** 265-293

Ishiwatari R, Takada H, Yun S-J and Matsumoto E 1983 Alkylbenzene pollution in Tokyo Bay Sediments; *Nature* (*London*) **301** 599-600

Metcalfe L D and Schmitz A A 1961 The rapid preparation of fatty acid esters for gas chromatographic analysis; *Anal. Chem*. **33** 363-364

Raymond R L, Jamison V W and Hudson J O 1967 Microbial hydrocarbon co-oxidation I. Oxidation of mono- and dicyclic hydrocarbons by soil isolates of the genus *Nocardia*; *Appl. Microbiol*. **15** 857-865

Reddy P G, Singh H D, Pathak M G, Bhagat S D and Baruah J N 1983 Isolation and functional characterisation of hydrocarbon emulsifying and solubilising factors produced by a *Pseudomonas* species; *Biotechnol. Bioeng*. **25** 387-401

Sariaslani F S, Harper D B and Higgins I J 1974 Microbial biodegradation of hydrocarbons: Catabolism of 1-phenyl alkanes by *Nocardia salmonicolor; Biochemn. J*. **140** 31-45

Swisher R D 1963 Biodegradation of ABS in relation to chemical structure; *J. Water Pollut. Cont. Fed*. **35** 877-892

Swisher R D 1987 *Surfactant Biodegradation* (New York: Marcel Dekker)

Walsh C 1978 *Enzymatic reaction mechanisms* (San Francisco: W H Freeman) p 701

Webley D M, Duff R B and Farmer V C 1956 Evidence for β-oxidation in the metabolism of saturated aliphatic hydrocarbons by soil species of *Nocardia; Nature* (*London*) **178** 1467-1468

Willets A J 1973 Microbial aspects of the biodegradation of synthetic detergents: A review *Int. Biodem. Bull*. **9** 3-10

Corresponding editor: M S S I