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Production of Useful Compounds from Alkane Media in Japan

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A variety of useful compounds, such as amino acids, organic acids, carbohydrates, nucleotides, vitamins and coenzymes, antibiotics and biomass *etc.*, can be produced by microbial processes utilizing alkanes as substrates.

These products are classified into three groups. The Group 1 involves compounds which can be easily produced by conventional processes utilizing carbohydrates. The Group 2 contains products, whose formation is significantly favoured by physiological and metabolic features of alkane-utilizers. The Group 3 consists of products, which have structures closely related to alkane substrates and can be specifically produced in alkane media. At present these compounds are rarely produced from alkanes on industrial scales. However, production of compounds belonging to Group 2 and 3 is not only of academic interest but also of commercial importance even if the price of alkanes will be further elevated.

1 Introduction

Extensive studies have been carried out in an attempt to utilize alkanes as carbon sources for the production of a variety of useful compounds, such as cell mass, proteins, carbohydrates, nucleic acids, lipids, amino acids, organic acids, vitamins and coenzymes, antibiotics and so on[1,2]. These products can be classified into three groups from biochemical standpoints.

The first group (Group 1) involves products common to those of conventional microbial processes using carbohydrate substrates. Cell mass, carbohydrates, amino acids, nucleic acids, antibiotics, several kinds of enzymes, organic acids and vitamins listed in Table 1 can be produced from either alkanes or carbohydrates, although the flow of carbon in the metabolism of these substrates is different in some part and similar in other part (Fig. 1), and strains of microorganisms as well as the cultivation procedures are different. This implies that selection of carbon sources for production of the first group compounds would be made according to their cost performances. However, the production of cell mass or proteins from alkanes, for example, has special importance to relieve food shortage from global viewpoints. Amino acids, such as lysine and glutamic acid, and organic acids, such as citric acid, are also promising products in using alkanes.

The compounds belonging to the second group (Group 2) are also common to carbohydrate processes, but their productivities are enhanced markedly by special features in alkane assimilation (Table 2). Effluent formation of acetyl-CoA by oxidative degradation of alkanes facilitates biosyntheses of isoprenoids, such as carotenoids, xanthophylls, steroids and coenzyme Q. Alkane substrates provide hydrophobic environment which would favour the production of such water-insoluble compounds. Oxidative degradation of alkanes through β-oxidation enhances

Table 1. Products common to carbohydrate substrates (Group 1)

L-*Amino Acids*: Glutamate, Alanine, Lysine, N^{ε}-Acetyl-lysine, Threonine, Valine, Homoserine, Serine, Phenylalanine, Tyrosine, Ornithine, Citrulline, *etc.*
Organic Acids: α-Ketoglutarate, Citrate, Isocitrate, Fumarate, Malate, Succinate, Anglycerate, *etc.*
Carbohydrates: Trehalose, Arabitol, Erythritol, Mannitol, Polysaccharides, *etc.*
Nucleic Acids, Nucleotides, Nucleosides: AMP, GMP, IMP, cAMP, Inosine, Orotic acid, Orotidine, DNA, RNA, *etc.*
Vitamins and Coenzymes: Vitamin B_6 and Pyridoxal 5′-phosphate, Vitamin B_{12}, Porphyrins, *etc.*
Antibiotics: Pyocyanine, Phenazine-1-carboxylic acid, Oxychlororaphine, Cepharosporins, Corynecins, Brevimycin, Cryomycin, Fluopsins, *etc.*
Enzymes: Lipase, Protease, D-Amino acid oxidase, *etc.*
Cell Mass

the cellular level of CoA as well as those of the enzymes and coenzymes relating to the pathway, such as cytochromes and coenzyme Q (Fig. 2). For example, in alkane media, but not in glucose media the production of coenzyme Q by some yeasts can be enhanced markedly by the addition of *p*-hydroxybenzoic acid, a precursor of the quinone moiety of the coenzyme. Much of the coenzyme formed under these conditions was detected in extramitochondrial portion of the cells. The synthesis of a biotin-vitamer (desthiobiotin) by a strain of *Pseudomonas* is stimulated by odd-chain alkanes, from which pimelic acid, a precursor of the biotin-vitamer, is derived *via* diterminal oxidation followed by subsequent β-oxidation. The most important and interesting characteristics of alkane-utilizing yeasts is the conspicuous appearance of specific cytoplasmic organelles — peroxisomes or microbodies. These peroxisomes contain various specific enzymes, especially those participating in degradation and assimilation of alkanes and those producing hydrogen peroxide. The production of several peroxisomal enzymes will be promising.

The third class (Group 3) includes products, which have structures related closely to alkane substrates, and hence can be specifically produced in alkane media (Table 3). Dicarboxylic acids of various chain length are synthesized directly from alkanes through diterminal oxidation. Mutant strains of yeasts, defective in alkane assimilation or dicarboxylic acid degradation, are used for this purpose. Several peroxisomal enzymes specifically induced by alkanes are also included in this group.

In addition, so-called "co-oxidation" techniques are widely used in the conversion of aliphatic, alicyclic and aromatic hydrocarbons[1, 3]. This includes the supply of energy *via* the metabolism of co-substrate or the induction of the substrate-oxidizing

Table 2. Products related to physiological and metabolic features of alkane assimilation (Group 2)

Products Related to Physiological Features
Trehalose lipids, Rhamnolipids, Lipopolysaccharides, *etc.*
Riboflavin, FMN, FAD, Cytochrome c, Coenzyme A, Coenzyme Q, Erogosterol, Carotenoids, *etc.*
Catalase, Uricase, Glycerophosphate dehydrogenase, Lipoproteins, *etc.*
Products Related to Metabolic Features
Biotin-vitamer, Coenzyme Q, Ergosterol, Carotenoids, *etc.*

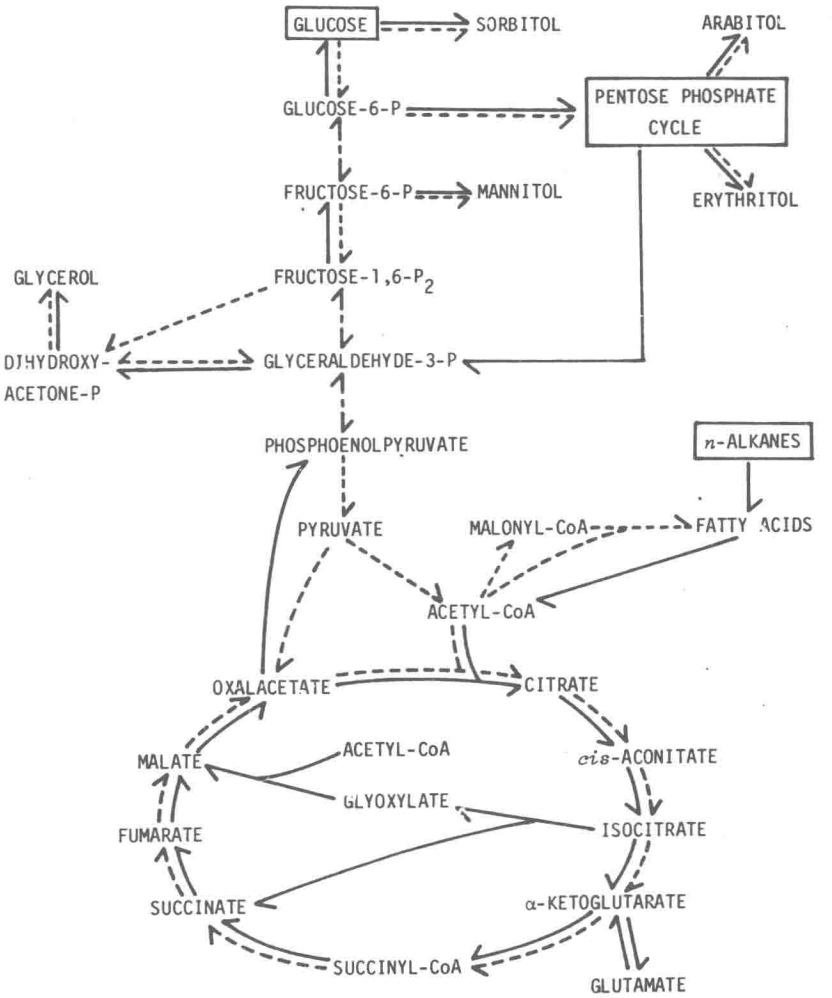

Fig. 1 Metabolic pathways of alkanes and glucose. ———: alkanes; — — —: glucose

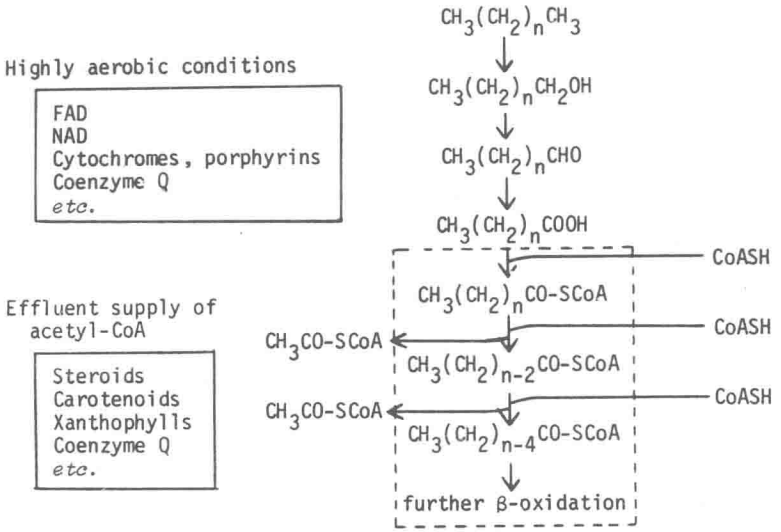

Fig. 2 Relationship of physiological and metabolic features of alkane utilization to microbial products

Table 3. Products specific to alkane substrates (Group 3)

Methylcitrate, Methylisocitrate, *etc.*
Monocarboxylic acids, Dicarboxylic acids, ω-Hydroxy fatty acids, Fatty alcohols, Wax esters, Monoalkenes, *etc.*
Enzymes participating in oxidation and degradation of alkanes

enzymes by co-substrates. A part of the co-oxidation processes will be included in the third group.

This review summarizes the production of useful compounds from alkane substrates by the microbial processes mostly carried out in Japan.

2 Amino Acids

Pioneering work on the amino acid production from alkanes was carried out by Yamada and his coworkers[4, 5, 6]. They reported that bacterial isolates belonging to the various genera could accumulate more or less a considerable variety of amino acids in kerosene media. Thereafter many research groups have improved the techniques by using special cultivation methods and mutant strains. The productivities of amino acids from alkanes are not necessarily superior to those from carbohydrates and petrochemicals.

2.1 L-Glutamic Acid

Yamada et al.[6] described that Strain S1031 of *Corynebacterium hydrocarboclastus* produced 485.5 mg l^{-1} of glutamate in a hydrocarbon medium. This bacterium did not require biotin but did require thiamine, unlike glutamate producers from carbohydrates. At the suboptimal concentration of thiamine (5 μg l^{-1}) the productivity of glutamate was enhanced up to 5 g l^{-1} [7]. They further improved the cultural conditions of the bacterium to produce about 6.3 g l^{-1} of the amino acid[8]. The glutamate formation was accompanied by the accumulation of a large amount of α-ketoglutarate[9]. It was supposed that thiamine deficiency interfered with the tricarboxylic acid cycle at the step of α-ketoglutarate dehydrogenase, leading to the accumulation of α-ketoglutarate and subsequently of glutamate[10].

It is well established that the production of glutamate from carbohydrates is successfully achieved under biotin-deficient conditions by using naturally-occurring biotin auxotrophs. In the case of biotin-containing media, the use of oleate auxotrophs or the addition of penicillin gives high yields. These results are considered to be due to the increased excretion of glutamate by the disturbance of the cell membrane synthesis; the oleate auxotrophs lack the ability to synthesize unsaturated fatty acids and penicillin inhibits the complexing of the cell membrane. The effect of penicillin on the glutamate production from alkanes was also confirmed. *Corynebact. hydrocarboclastus* M-104 [11] accumulated 4.0 g l^{-1} of the amino acid with penicillin, the amount being more than ten-fold higher than that without the antibiotic[12]. Hence, the addition of penicillin has been employed as an excellent technique to produce glutamate from alkanes[13,14,15,16,17]. Accumulation of α-ketoglutarate was found to be reduced by the penicillin addition[16]. Under the improved conditions 82 g l^{-1} [17] or 2.3 g l^{-1} h^{-1} of glutamate[18] was produced by *Arthrobacter paraffineus* KY 4303. Decreases of oxygen absorption and substrate consumption which resulted in the decreased glutamate accumulation were often observed in the presence of penicillin. To avoid this phenomenon, penicillin-resistant mutants of *Corynebact. hydrocarboclastus* were isolated[19]. One of the mutants produced 84 g l^{-1} of the amino acid.

Glutamate-producing bacteria isolated hitherto did not show a biotin requirement in alkane media. In the case of alkane-utilizing bacteria, unsaturated fatty acids are directly derived from alkane substrates. These facts indicate that a novel scheme, substituting for the control of the biotin level or for the use of oleate auxotrophs employed in the carbohydrate processes, might be employed for the production of glutamate from alkanes. As mentioned above, addition of penicillin gave good results on the glutamate production. The effect of penicillin on the glutamate excretion was later found to be correlated to the extracellular accumulation of phospholipids[20,21].

Thus, it would be reasonable to predict that reduction in cellular phospholipids, main components of cell membrane, can be achieved by inhibition of glycerol synthesis, bringing about exocellular glutamate accumulation. A glycerol auxotroph (Strain GL-21) was isolated from *Corynebact. alkanolyticum* Strain No. 314 by Nakao et al.[22,23]. The mutant produced about 40 g l^{-1} of glutamate in the presence of a suboptimal level of glycerol (0.1 mg ml^{-1}) and in the absence of penicillin, while the addition of penicillin was necessary for the glutamate production

by the strain 314 irrespective of the glycerol concentration or by the strain GL-21 in media containing over 0.3 mg ml^{-1} of glycerol. The strain GL-21 was found to be defective in L-glycerol-3-phosphate:NADP oxidoreductase which is indispensable for the glycerol synthesis[24]. From such results, the permeability to glutamate in the mutant was proved to be enhanced, but the activities of the enzymes participating in glutamate synthesis were comparable to those of the parent strain[25]. Under the optimal conditions the strain GL-21 produced 72 g l^{-1} of glutamate[26]. Kikuchi[27] summarized the results on the glutamate permeation under different conditions employed hitherto (Fig. 3). The method to use the glycerol auxotroph is unique in the utilization of alkanes, although the mutant is also useful for glutamate production from different carbon sources.

Typical data on glutamate production from alkanes are summarized in Table 4.

2.2 L-Lysine

Although accumulation of lysine by bacteria in an alkane medium was first demonstrated by Yamada et al.[5,6], the yield was extremely low (24 mg l^{-1}). Ishii et al.[28] tried to produce various amino acids from alkanes by using auxotrophic mutants of *Corynebact. hydrocarboclastus* R-7 and *Alcaligenes marshallii* P-9. Of the auxotrophs obtained, Strain PN-46 (methionine$^-$) of *A. marshallii* accumulated 0.25 g l^{-1} of lysine. High yields of lysine were achieved by a research group of Kyowa Fermen-

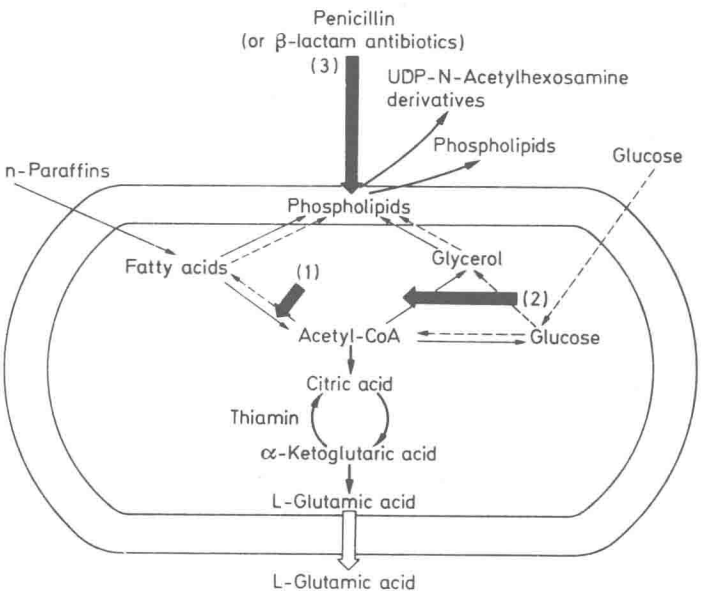

Fig. 3 L-Glutamate excretion through bacterial membrane[27].
————, biosynthesis of phospholipids from alkanes; — — — —, biosynthesis of phospholipids from glucose. (1), biotin auxotroph and oleate auxotroph; (2), glycerol auxotroph; (3) penicillin addition

Table 4. L-Glutamate production from alkanes

Microorganisms	Yield	Ref.
Corynebact. hydrocarboclastus (Thiamine deficiency)	6 g l^{-1}	8)
Arthrobacter paraffineus (Penicillin addition)	82 g l^{-1}	17)
Corynebact. hydrocarboclastus (Penicillin^r mutant)	84 g l^{-1}	19)
Corynebact. alkanolyticum (Glycerol$^-$ mutant)	72 g l^{-1}	26)

tation Industry [see[1,29]]. A homoserine or methionine auxotroph of *Brevibacterium ketoglutamicum* produced 10 g l^{-1} of lysine, and a homoserine auxotroph of *Nocardia sp.* No. 258 accumulated 34 g l^{-1} of the amino acid.

2.3 L-Threonine

An isoleucine leaky auxotroph (Strain KY 7104) of *Arthrobacter paraffineus* accumulated threonine (9 g l^{-1}), valine (9 g l^{-1}), serine (2 g l^{-1}) and leucine (2 g l^{-1}) at a relatively high concentration of thiamine[30]. To increase the yield of threonine and to reduce valine accumulation, an isoleucine plus methionine auxotroph (Strain KY 7137) was derived from Strain KY 7104. A revertant with respect to isoleucine requirement (Strain KY 7135), obtained from Strain KY 7137, showed a high yield of threonine (14.5 g l^{-1}) and a reduced accumulation of valine[31]. The productivity of threonine by the mutant increased up to 18.9 g l^{-1} [see, [1]].

2.4 L-Isoleucine

Micrococcus paraffinolyticus ATCC 15582, *M. paraffineus* KY 4307 and *Corynebact. hydrocarboclastus* ATCC 15592 accumulated 1.55–2.15 g l^{-1} of isoleucine in an alkane medium supplemented with DL-α-aminobutyric acid (α-AB) and fumarate[32]. Relatively high yields of the amino acid were also obtained by adding L-homoserine, α-AB or α-AB plus L-aspartate.

2.5 L-Valine

An isoleucine auxotroph of *Corynebact. hydrocarboclastus* accumulated 2.2 g l^{-1} of valine[28]. The isoleucine leaky auxotroph of *Arthrobacter paraffineus* (Strain KY 7104) produced 9 g l^{-1} of valine accampanied by the production of threonine, as described above[30]. At low concentrations of thiamine the product was converted to glutamate.

2.6 L-Serine

Arthrobacter paraffineus KY 7137 (methionine$^-$, isoleucine$^-$) mentioned above accumulated 1.3 g l^{-1} of serine when 10 mg l^{-1} of isoleucine and 200 mg l^{-1} of

methionine were supplemented to an alkane medium. Addition of glycine (5 g l^{-1}) as a precursor enhanced the serine production up to 3.2 g l^{-1}. An α,ε-diaminopimelic acid plus isoleucine auxotroph of *Arthrobacter paraffineus* was also found to be a serine producer. The accumulation of serine by these mutants was always accompanied by accumulation of threonine[33].

2.7 L-Homoserine

A threonine auxotroph of *Corynebacterium sp.* KY 7142 produced 12 g l^{-1} of homoserine in an alkane medium supplemented with 1 g l^{-1} of threonine. Smaller amounts of isoleucine and valine were also accumulated together with homoserine[34].

2.8 L-Ornithine

An arginine auxotroph (Strain RN-362) of *Corynebact. hydrocarboclastus* R-7 accumulated 3.9 g l^{-1} of ornithine in an alkane medium[28]. By the optimization of the cultural conditions the yield was increased up to 9.3 g l^{-1} [35]. An arginine auxotroph of *Arthrobacter paraffineus* was also utilized for production of ornithine[36]. The maximal yield of ornithine was 8.0 g l^{-1}.

2.9 L-Citrulline

An arginine requiring mutant of *Corynebacterium sp.* KY 4442 accumulated 8 g l^{-1} of citrulline in an alkane medium supplemented with 1 g l^{-1} of arginine[37]. Addition of several amino acids or casamino acid was effective for the production of citrulline. An arginine auxotroph of *Arthrobacter paraffineus* produced 10.2 g l^{-1} of citrulline [see, [1]].

2.10 L-Tyrosine

A phenylalanine auxotroph of *Corynebact. hydrocarboclastus* R-7 was tested for the production of tyrosine from alkanes, but the productivity was rather low (1.0 g l^{-1}) [28]. A phenylalanine auxotroph of *Corynebacterium sp.* KY 4336 accumulated about 19 g l^{-1} of the amino acid when the pH of the medium was shifted from 6.8 to 7.5 at the exponential growth phase[38]. This effect of the pH shift might reflect the activity of transamination of *p*-hydroxyphenylpyruvate to tyrosine, since the accumulation of the acid was high at the acidic pH.

2.11 L-Phenylalanine

A tyrosine auxotroph of *Corynebacterium sp.* KY 4309 produced 10 g l^{-1} of phenylalanine at pH 6.0[39]. When the pH of the medium was kept at 7.0, however, the yield of phenylalanine decreased, and there was accumulation of phenylpyruvate.

2.12 L-Alanine

An arginine auxotroph of *Corynebact. hydrocarboclastus* R-7 accumulated 0.8 g l^{-1} of alanine[28]. A glutamate-producer, *Corynebact. hydrocarboclastus* S10B1 formed 1.5 g l^{-1} of alanine under the thiamine deficient conditions with concomitant

accumulation of large amounts of glutamate and α-ketoglutarate[9]. A strain of *Corynebact. hydrocarboclastus* also produced 4 g l^{-1} of alanine [see, [29]].

2.13 Other Amino Acids

Productions of several amino acids, such as L-tryptophan, L-leucine and L-proline were also examined. Anthranilic acid was found to be effective for the accumulation of tryptophan in alkane media. *Brevibact. ketoglutamicum* (1.5 g l^{-1}) and *Candida tropicalis* (2.0 g l^{-1}) were good producers of tryptophan [see, [1]]. An isoleucine auxotroph of *Corynebact. hydrocarboclastus* R-7 produced 0.7 g l^{-1} of leucine[28]. An arginine auxotroph of the same bacterium accumulated 0.3 g l^{-1} of proline[28].

A large amount of ε-N-acetyl-L-lysine was produced by a lysine-producing mutant (Strain CR-27) of *Corynebact. hydrocarboclastus* KY 8837. The mutant showed a decreased ability to utilize acetyl-lysine as carbon source. Under the optimal conditions Strain CR-27 accumulated 41 g l^{-1} of acetyl-lysine and 9 g l^{-1} of lysine[40].

3 Organic Acids

Accumulation of oxidation products of alkanes has been reported since the early stage of petroleum microbiology [see, [1]]. However, most of these products were not regarded to be useful because of their low yields. In the course of studies on glutamate production from alkanes, concomitant accumulation of α-ketoglutarate was discovered. This finding emphasized possibilities of production of various acids metabolically related to α-ketoglutarate from alkanes. Several acids belonging to the tricarboxylic acid cycle, especially citrate, were found to be efficiently accumulated in alkane media. In addition to these organic acids of the Group 1 (*loc. cit.*), production of long-chain dicarboxylic acids was attempted. These acids belonging to the Group 3, compounds derived directly from alkanes, are promising products.

3.1 α-Ketoglutarate

A glutamate-producing bacterium *Corynebact. hydrocarboclastus* S10B1 accumulated 16 g l^{-1} of α-ketoglutarate under thiamine deficient conditions[9]. Similarly, *Corynebacterium sp.* KY 4439, a glutamate-producer, accumulated the acid with the yield of 85.8% from 8.7% of alkanes[41] at suboptimal concentrations of thiamine. Various strains of *Candida lipolytica* also accumulated α-ketoglutarate in an alkane medium. Among them Strain AJ 5004 produced 65.8 g l^{-1} of the acid[42,43,44]. In these processes the level of thiamine in the medium was kept low.

3.2 Citrate and Isocitrate

Citrate is a promising product from alkanes, because the acid is widely utilized in the food, pharmaceutical and chemical industries.

Tabuchi et al.[45] reported that *C. lipolytica* Strain No. 228, which had been isolated as a potent citrate-producer from glucose, converted 56% of the substrate

n-hexadecane to yield 34 g l^{-1} of citrate. They also found that various strains of *Candida* yeasts have the ability to accumulate isocitrate in addition to citrate in an alkane medium[46]. The ratio of citrate to isocitrate varied depending on cultural conditions. Reduction of iron concentration in a medium resulted in an increase in the citrate production and in the decrease in the isocitrate accumulation. Under these conditions *C. lipolytica* No. 6–20 produced 85–90 g l^{-1} of citrate and about 20 g l^{-1} of isocitrate from 60 g l^{-1} of alkane mixture, whereas an iron sufficient medium gave about 50 g l^{-1} of citrate and 40 g l^{-1} of isocitrate[47]. When $CaCO_3$ was omitted from the medium and the N/C ratio was increased, the productivity of citrate decreased, while the cell yield increased significantly. Thus, it is necessary for obtaining citrate in a high yield to keep the medium at near neutral pH and at a low nitrogen concentration[48]. The effect of ferrous ion deficiency on the citrate accumulation was ascribed to a reduced activity of aconitase, an iron-containing enzyme. Thiamine deficiency was reported to enhance the isocitrate dehydrogenase activity of the yeasts, resulting in the accumulation of α-ketoglutarate[49]. A problem in the citrate production by yeasts is the concomitant accumulation of isocitrate as described above, which reduces the yield of citrate and makes the purification of citrate difficult. *C. lipolytica* showed a relatively high level of aconitase, which could be inhibited by monofluoroacetate (MFA). In fact, the addition of MFA to the medium decreased the accumulation of isocitrate markedly[50].

Akiyama et al.[51,52,53] isolated two mutant strains from citrate-producing *C. lipolytica* IFO 1437. These mutants assimilated citrate only slightly as a sole carbon source, and one of them, Strain S-22, was very sensitive to MFA. Aconitase activity of another strain, K-20, was one-tenth of that of the parent strain, while the activity of the strain S-22 was about one-hundredth. In accordance with the decreased aconitase activity the productivity of citrate increased from 60 g l^{-1} with the parent strain to 110 g l^{-1} with the strain S-22. The ratio of citrate to isocitrate decreased markedly, from 60:40 with the parent to 97:3 with the mutant. The results reported by Akiyama et al. summarized in Fig. 4 together with those of Tabuchi et al.

With *C. zeylanoides* the sum of citrate and isocitrate, produced from 7.75% of *n*-alkanes, reached 150%, but the ratio of these acids was about 50:50[54]. The pH control with ammonia water gave a good result on the citrate production, 99 g l^{-1} of citrate and 11 g l^{-1} of isocitrate[55].

One mutant of *C. lipolytica* produced 170 g l^{-1} of citrate and 4 g l^{-1} of isocitrate[56]. The same research group reported the maximum citrate yield, 183 g l^{-1}

Parent strain	Citrate, 60 g l^{-1}; isocitrate 40 g l^{-1}
Citrate-nonutilizable mutant	Citrate, 90 g l^{-1}; isocitrate 10 g l^{-1}
Monofluoroacetate-sensitive mutant	Citrate, 110 g l^{-1}; isocitrate 4.5 g l^{-1}

Fig. 4 Citrate production from alkanes by *Candida lipolytica*

(isocitrate, 40 g l^{-1}), with *C. hitachinica* [see, [57]]. A mutant of *C. citrica* also produced citrate in a good yield (102 g l^{-1}) with a reduced accumulation of isocitrate (2 g l^{-1})[58]. Kinetic studies on the citrate and isocitrate production by *C. lipolytica* were carried out in an NH_4^+-limited chemostat culture[59].

Tabuchi and Hara[60] tried to produce isocitrate from alkanes by using MFA-resistant mutants of *C. lipolytica*. Although the mutants showed slightly higher aconitase activity than the parent strain, the yield of isocitrate was not enhanced. These mutants accumulated an oxyacid which was identified later as 2-methyl-isocitrate.

3.3 C_7-Acids

As mentioned above, MFA-resistant mutants of *C. lipolytica* did not show an increase in the productivity of isocitrate, but accumulated an oxyacid[60], which was identified as 2-methylisocitrate[61,62]. One of the mutants, No. 2 of *C. lipolytica*, accumulated about 35 g l^{-1} of methylisocitrate from odd-chain alkanes, the amount being comparable to the sum of citrate and isocitrate produced from these substrates. Even-chain alkanes and glucose did not serve as substrates for the methylisocitrate production[63]. Furthermore, Tabuchi et al.[64] demonstrated accumulation of methyl-citrate and 2-methyl-*cis*-aconitate by the mutant grown on alkane mixture. The results suggested that propionyl-CoA derived from odd-chain alkanes by β-oxidation might be the precursor of these C_7-acids. In fact, they identified special enzymes, methylcitrate synthase and methylisocitrate lyase, and proposed so-called "methylcitric acid cycle" (Fig. 5) for the metabolism of propionyl-CoA in yeasts[65]. This cycle seems to be specific to the alkane metabolism, and therefore, these C_7-acids belong to the Group 3 compounds.

3.4 Fumarate, Malate and Succinate

Yamada et al.[66] isolated a strain of *C. hydrocarbofumarica* which produced 39 g l^{-1} of fumarate from alkanes. By optimization of the cultural conditions the productivity was enhanced up to 50 g l^{-1} (Y = 84%)[67]. Fumarate thus accumulated was further converted to a more useful organic acid, *l*-malate, by the associated cultivation of *C. hydrocarbofumarica* with *C. utilis* or *Pichia membranaefaciens* having a high fumarase activity[68]. About 30 g l^{-1} of fumarate (Y = 65%) was also produced by *C. blankii* MT 1025 [69].

As described above, yeasts having a high fumarase activity was used for the bioconversion of fumarate produced by *C. hydrocarbofumarica* in an alkane medium. The yield of malate based on alkane substrate was 71–72% (36–38 g l^{-1})[68]. Direct production of malate was also examined. *C. brumptii* IFO 0731 accumulated 24.5 g l^{-1} of malate (Y = 80%)[70].

Succinate was produced by *C. brumptii* IFO 0731 [71,72,73]. The maximal yield was 23.6 g l^{-1} (Y = 67%).

3.5 Anglyceric Acid

C. tenuis IFO 1303 accumulated about 10 g l^{-1} of anglyceric acid in an alkane medium[74]. Relatively high concentrations of $(NH_4)_2SO_4$ was favourable to produce this acid by reducing the accumulation of citrate.