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CHEMICALS FROM HYDROCARBONS BY THE FERMENTATION PROCESS[1]

by

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SUMMARY

CHEMICALS FROM HYDROCARBONS BY THE FERMENTATION PROCESS [1]

by

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Microbial utilization of petroleum hydrocarbons have been studied in at least four directions in Japan. The first of these is the production of single cell protein, the second is the replacement of the raw materials for fermentation by hydrocarbon or the secondary products derived from petroleum, the third is the production of useful substances characteristic in hydrocarbon fermentation, and the fourth is the chemical modification of petroleum hydrocarbons by means of the oxidative activity of microorganisms.

Recently, some of techniques established initially on the laboratory scale have been developed on the semi-industrial level. For example, with yeast cell protein on a reasonable prospect, there is potential for full industrial operation in near future.

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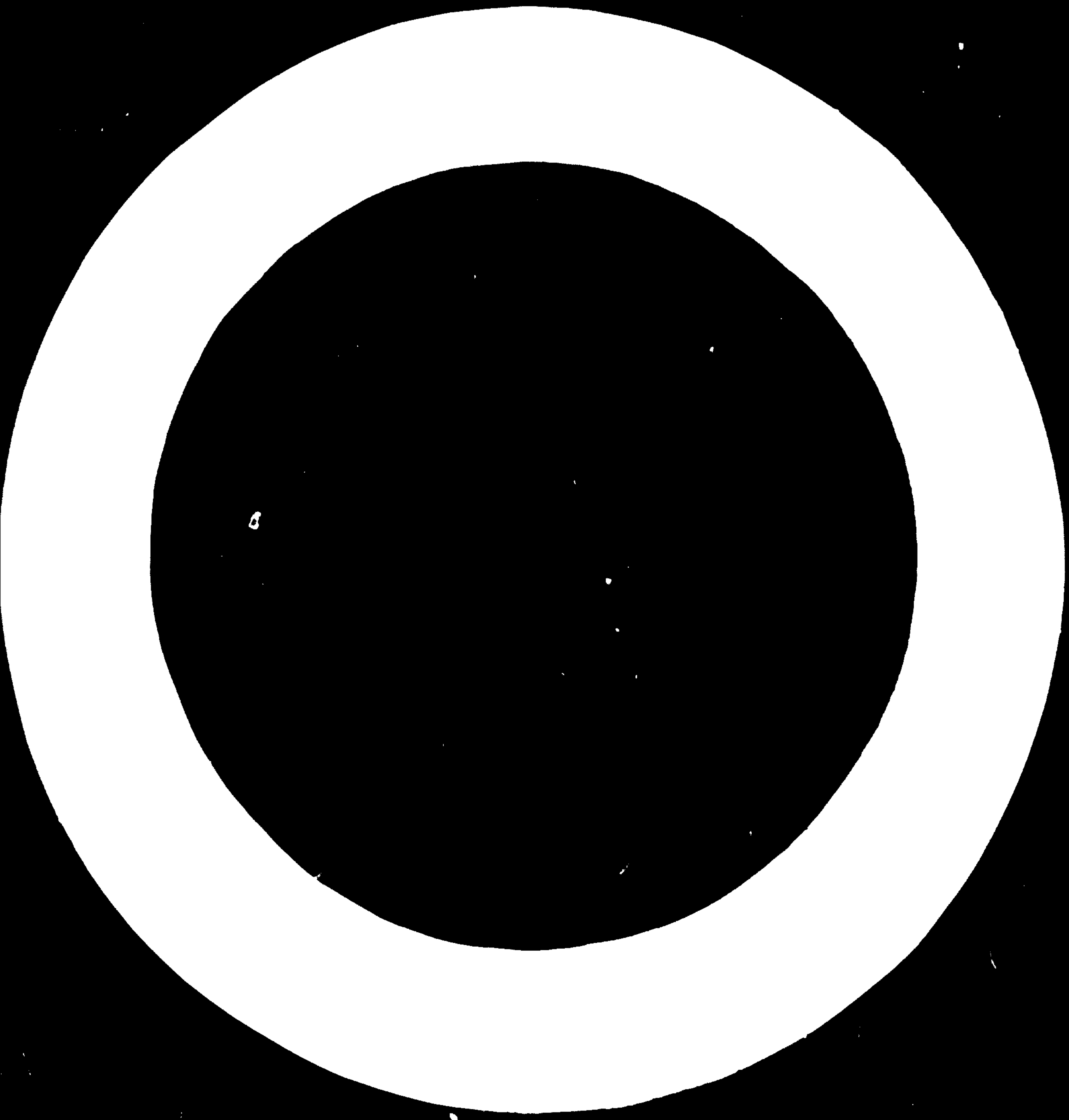
In connection with the fermentation processes of amino acids, particularly glutamic acid which was firstly established in Japan, the possibility is foreseen as the replacement of the raw materials by n-paraffin. Moreover, the production of threonine, homoserine, ornithine, citrulline and other amino acids from hydrocarbon have become possible owing to the success in obtaining a variety of bacterial mutants.

The production of sugars such as glucose, trehalose, mannose and polysaccharides by bacteria and yeast grown on n-paraffin as the sole source of carbon have recently reported. Biochemical studies on the hydrocarbon fermentation have demonstrated the occurrence of considerable amount of glycolipids characteristic in hydrocarbon-utilizing bacteria.

Among the organic acids producible from hydrocarbon by microorganisms, the most promising product may be citric acid. The reported yield is more than 80 g/l from 60 g/l of n-paraffin.

In addition, the production of various chemicals such as vitamins, coenzymes, aromatic compounds and others has been reported in Japan. Many other oxidative products of aromatic hydrocarbon have been demonstrated.



Introduction

With the improvement of the microbe-utilizing techniques, the fermentation process concerning the production of various useful chemicals has been developed to industrial scale, and further a great deal of the raw material has been demanded with it. However, it is now predicted that the supply of more sufficient amount of the raw material which has been so far obtained from agricultural products become more difficult in future because the shortage of the food in the world is foreseen with the rapid increase in the world population. And again, together with the trouble that agricultural product is not supplied constantly by year on account of various natural calamities, an increasing attention has been paid towards the replacement of the raw material of the fermentation by petroleum hydrocarbon which is estimated to be semi-exhaustively contained in the earth.

Considering the possible origin of petroleum on the basis of the scientific evidences, it is likely that organic matter such as proto-petroleum had already existed upon the earth before the living systems were generated. On the other hand, the present evolutionary theory suggests us that the most ancient living organisms might be the microbes capable of growing upon the organic matter which had already been formed on the earth. Therefore, the microbes and petroleum could probably have acted on each other since that period. The discovery that a number of microorganisms capable of utilizing hydrocarbon as the sole source of carbon are distributed widely in the soil is quite reasonable if the above consideration would be acceptable.

The tendancy towards the replacement of the raw material by petroleum hydrocarbon appears to be an adverse current when considering from the evolutional view point of living systems, but rather more extensive possibility could be expected in the attempt for the production of useful material.

In the process of conventional fermentation, it is known that the variation of the metabolic system and the product occurs whenever different substrate is supplied as carbon source. Therefore, more extensive variation in the replacement of the raw material by hydrocarbon is expectable.

Microbial conversion of hydrocarbon to useful products has been investigated in at least four directions in Japan. The first of these is to produce protein more efficiently by hydrocarbon-utilizing yeast. At the international conference on single cell protein in 1967, many developed countries demonstrated that the techniques had been established on the semi-industrial scale. A second direction is to replace the raw material in the fermentation process by petroleum hydrocarbon and to produce the chemicals which have been already manufactured through the conventional processes. Actually, amino acids such as glutamic acid, threonine, homoserine, ornithine, citrulline and others, organic acids such as citric and α-ketoglutaric acid, and vitamins have been obtained by this process. A third direction for the study on hydrocarbon fermentation is on the basis of the assumption that various useful chemicals characteristic in the substrate hydrocarbon might be obtained. The production of fatty acids, alcohols, waxes and other lipids, sugars, and aromatic compounds have been reported. A fourth direction is concerned with the chemical modification of hydrocarbon, in which a part conversion of substrate hydrocarbon by microbial oxidation has been undertaken. Namely, the conversion of n-paraffin into the corresponding dicarboxylic acid by diterminal oxidation, alicyclic hydrocarbon into the corresponding alcohol and ketone, xylene into toluic acid, and cymene into cumic acid have been shown.

Besides the above-mentioned attempts, the materials derived secondarily from petroleum, which will be easily supplied with the recent growth of petrochemical industry, have been applied for fermentation processes as the raw materials. For example, glutamic acid is actually producible from ethanol or acetic acid with good yields.

The purpose of this communication is to review briefly Japanese studies concerning the production of the chemicals from hydrocarbon through the fermentation processes which have been attempted in the above-mentioned directions.

1. Hydrocarbon-utilizing microorganisms

Since there are many excellent publications[1,2] concerning microbial reaction on hydrocarbon, it is not intended here to refer exhaustively to reported findings. The recent data obtained along with the investigation of the production of amino acids, sugars and other chemicals will be described in this paper.

It has been shown that the microbes capable of utilizing hydrocarbon include not only bacteria but also mold , actinomycetes and yeast. Those microbes are found widely in the soil regardless of the presence of hydrocarbon. Therefore it seems not so difficult to select the microbes capable of producing useful products from hydrocarbons. The growth of those microbes is initiated by the supply of aqueous nutrients and vitamins in addition to hydrocarbon as a carbon source under the optimal physical conditions. The most preferable carbon source for their growth is n-paraffins in the range of $C_{11} - C_{20}$ 3-5). The optimum range of carbon number of n-alkane is different according to the [illegible] microbial strains used.5) For instance, some strains of <u>Arthrobacter</u>, <u>Brevibacterium</u> and <u>Micrococcus</u> grow preferably on n-paraffin of C-15 to C-18, while some of <u>Corynebacterium</u> prefer those of C-12 to C-14. A similar tendency is also observed in the productivity of amino acids and organic acids. Aromatic hydrocarbons6) or cyclic compounds2) are generally less utilized by microorganisms.

On the other hand, a number of hydrocarbon-utilizing bacteria is also capable of growing on alcohols4-7), organic acids or saccharides as the sole source of carbon. Particularly, the ability of the growth on alcohols suggests some parallelism between the utilization of alcohols and hydrocarbons. In this case, that of higher alcohol is more preferable carbon source. Similarly, the fact that they can utilize fatty acids which are the intermediate in hydrocarbon oxidation is considered to be natural to them*) As far as it is known, these bacteria are able to grow on carbohydrates as well, for example, on sugar alcohols such as mannitol, sorbitol and glycerol, and ketohexose such as fructose, but glucose and its isomers are not preferable carbon sources8). The characteristics of hydrocarbon-utilizing bacteria in sugar utilization could become a significant clue in an attempt to produce sugars from hydrocarbon.

Another feature of hydrocarbon-utilizing microbes is that the demand of oxygen for their growth is higher than with others9). In an attempt to produce a useful material from hydrocarbon through the fermentation process, the supply of sufficient oxygen should be considered in the growing phase of microbes. Since oxygen-containing products are derived from hydrocarbons which do not contain oxygen, hydrocarbon fermentation could be referred to as oxygen

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*) Acetic acid is also utilized by some of them.

fixing fermentation. The additional effect of metal ions such as iron, copper and others could be explained on the basis of the above findings.

Of the physical conditions for the culture of bacteria, the most important factor is to control pH of culture medium below 0.8, because these bacteria are generally susceptible to death on higher pH and productivity is also depressed under such condition.

Further, the fact that those bacteria can take up both the nutrients of different nature, lipophilic carbon sources and hydrophilic other nutrients, suggests the presence of some special mechanisms characteristic of their cells. It is generally observed that those bacteria display a significant activity of emulsion formation in the mixture of hydrocarbons and aqueous solution of other nutrients. From these observations, we assumed that a surfactant-like substance should be present on the surface of their cells and attempted to characterize it. Consequently, the isolated material was identified as trehalose ester of a hydroxy fatty acid.10/ The glycolipids placed under the same category as the trehalose lipid have also been found in other microbial strains which can utilize n-alkane as the sole source of carbon, and the isolated materials displayed remarkable activity as the surfactant. These findings strengthen the possibility that these lipids play a possible role in the utilization of hydrocarbon by bacteria. In addition, occurrence of sophorose lipid in n-paraffin-utilising yeast was also reported. Comparison of the characteristics of hydrocarbon-utilizing bacteria with those of others is of great interest to either bacteriologist or biochemist.

2. Production of amino acid from n-alkane

Owing to vast progress of the fermentation techniques since the direct process of glutamic acid production was invented in Japan, l-amino acid has been supplied smoothly to various fields, the demand of amino acid has been rapidly increasing not only for food and feed supplement but also for the raw material of synthetic fibres and leathers. On these backgrounds, the replacement of the raw materials for fermentation by cheaper and more constantly supplied materials has been desired, and investigated for about seven years in Japan.

Early in the study, the question was to find the bacteria having both the characters which are capable of growing on n-alkane and also to convert it efficiently to glutamic acid or other amino acids. Fortunately, a number of microbial strains which are producible various amino acids have been discovered. Iizuka et al.[12] first reported the occurrence of microbes in the soil, which were able to produce glutamic acids, alanine, tyrosine and lysine when kerosene was used as the sole source of carbon. And they[13] named them as *Corynebacterium hydrocarboclastus* and *C. oleophilus*. In spite of that these microbes utilized n-alkane actively, the quantity of amino acids produced was not sufficient enough to develop to a further process. Subsequently, Yamada and Takahashi[14] isolated the bacteria belonging to *Corynebacterium*, *Arthrobacter*, and *Bacillus* capable of producing various amino acids. A variety of microorganisms capable of converting kerosene and n-paraffin efficiently into glutamic acid and α-ketoglutaric acid has been isolated from the soil by Tanaka et al.[5] in our laboratory. These were classified to genus *Corynebacterium*, *Arthrobacter*, *Mycobacterium*, *Brevibacterium*, *Micrococcus*, *Bacillus* and *Nocardia*. Iguchi et al.[15] have also shown that the bacterial strain isolated by them produced relatively large amounts of glutamic acid in the culture medium. Almost all of these bacteria have been known to require thiamine essential for the growth. It is of interest to compare this with the action of biotin which is known as the growth factor of the *Corynebacterium glutamicus* and is responsible for glutamic acid production from carbohydrate source.

Glutamic acid

Yamada and his colleagues[16] have demonstrated using Corynebacterium hydrocarboclastus to yield 5 g/l of glutamic acid from n-paraffin under the suboptimal concentration of thiamine for the growth. Iguchi et al.[15] studying on the effect of antibiotics on the production using *Corynebacterium petrophilum* isolated by them, have also shown the accumulation of 13 g/l of glutamic acid from 39 g/l of n-hexadecane by addition of penicillin to the growing culture. Both the techniques, by limitation of the concentration of growth factor[17] and by addition of penicillin[18], have been developed as excellent means for the production of this amino acid by the conventional fermentation process in which carbohydrates were used as the raw material.

During the course of the study on the optimal condition for glutamic acid production, Imada et al.[19) have indicated that the yield of 11 g/l was obtained from 70 g/l of n-pentadecane without penicillin under the concentration of 0.025 % of corn steep liquor. In this work they emphasized the effectiveness of iron ion in the culture medium on the basis that the production of glutamic acid was absolutely dependent upon the concentration of iron higher than [illegible] mg/ml, while in the lower concentration, the alcohol and fatty acid corresponding to the n-paraffin used above was easily utilized, but the oxidation of n-pentadecane was significantly depressed. They speculated from the results that iron ion may participate in oxygenase reaction in the earlier step of n-paraffin metabolism. In addition, it has been reported that the effectiveness of [illegible] on this process was enhanced by the addition of small amounts of oleic acid. [illegible] et al.[20) have found the effectiveness of cysteine as well as iron ion, and also indicated that the optimal concentration of phosphate was at the range of 0.5 to 1.0 % in this process.

In connection with our recent work[21) concerning glutamic acid production from n-paraffin using *Arthrobacter paraffineus* and *Corynebacterium* sp. which also required thiamine for growth, it has been reported that under the sub-optimal condition of thiamine concentration, the yield of glutamic acid was about 30 g/l and that of α-ketoglutaric acid was approximately 50 g/l, from 100 g/l of n-paraffin mixture mainly containing C_{12} to C_{1}[illegible] fractions. On the other hand, the addition of penicillin to the exponentially growing culture of the above strains under the optimal condition of thiamine concentration for their growth stimulated preferably the yield of glutamic acid and resulted in the production of more than 70 g/l, whereas the production of α-ketoglutarate was depressed below 5 g/l. This has been obtained from the experiments of semi-practical scale using the jar-fermenter equipped with some instruments capable of controlling pH of the medium automatically by ammonia solution.

In view of these investigations, it might be possible that the process of glutamic acid production will reach an industrial scale in the near future. Nevertheless, for the further progress of this process, it is necessary to combine systematically the studies from microbiological, biochemical and bioengineering view point.

Lysine and other amino acids

Following [illegible] progress [illegible] glutamic acid production from n-paraffin as shown above, many [illegible] attempts have been made to find the microbes capable of producing [illegible] at the present, the quantities of [illegible] those [illegible]. It has been reported that [illegible] produces methionine and [illegible].[22] [illegible][23] attempting to obtain auxotrophic mutants [illegible], have isolated arginine-requiring [illegible]. [illegible] and reported the production of arginine [illegible] yield was [illegible].4 or 6.2 g/l from [illegible] of the substrate, [illegible] or [illegible] % v/v. Oshima and [illegible] have also reported the extracellular accumulation [illegible] arginine-requiring mutants of [illegible]. [illegible] they have isolated another auxotroph, [illegible] which requires arginine as well and found that it produced [illegible] from n-paraffin mixture containing the fraction of [illegible] g/l from 10 % v/v of [illegible],[25] [illegible]; [illegible] which requires [illegible] by [illegible] et al.[26] in [illegible], (10 to 50 [illegible]), [illegible] value of about 10 g/l [illegible]. On the other hand, [illegible],[27] [illegible], [illegible] and lysine are produced [illegible] patents. In [illegible], [illegible] of hydrocarbon [illegible] prototrophic strain. [illegible] productivity of [illegible] corresponds to those in carbohydrate-utilizing [illegible].

3. Production of [illegible] derivatives

It is [illegible] are one [illegible] principal structural materials in microorganisms. The fact that [illegible] microbes are capable of growing on hydrocarbon [illegible] biosynthetic systems by which hydrocarbons are converted into sugars. Actually, it has been reported that some microorganisms grown with hydrocarbon produced significant amounts of polysaccharide slime which presumably has the function of capsular material. Raymond et al.28) have shown that a polysaccharide was accumulated in the cultures of a *Nocardia* grown with n-octadecane.

Recently, we reported the extracellular accumulation of glucose and trehalose by bacteria grown on n-paraffin as the sole source of carbon.29) Further, the addition of penicillin to the growing culture led to a remarkable increase in the accumulation of trehalose. The yield attained to approximately 8 g/l, corresponding to 60 to 70 % of total amount of sugar accumulated in the culture medium.

Of interest is that these sugars were also produced by other bacteria which can utilize n-paraffin as the sole source of carbon. Moreover, it was explained that trehalose was derived from trehalose lipid which was present on bacterial cells and played a possible role in the utilization of n-paraffin by bacteria. Penicillin significantly suppressed the biosynthesis of this lipid and consequently led the extracellular accumulation of both the precursors, trehalose and fatty acid.40)

We have also found that various polysaccharides were produced from n-paraffin by bacteria.30) For instance, arabomannan was isolated from the culture medium. Arabinose, mannose, galactose, glucose, rhamnose and unknown pentose were identified as constituents of the heteropolysaccharide. Recently, it was found that yeast strains of *Candida* and *Torulopsis* produced considerable amounts of mannose and unknown sugar from n-alkane [illegible] that nonionic surfactant was supplied to the culture medium.31)

The study of sugar production from hydrocarbon is still in the early stage and it can be expected to develop farther.

4. *Production of organic acid*

According to the reported route of microbial oxidation of hydrocarbons 32-34), n-paraffin is firstly oxidized to fatty acids and is further converted to lower acids by the method either of [illegible]- or [illegible]-oxidation. From this viewpoint, it appears to be possible to get a variety of intermediates by this route. Actually, the production of dicarboxylic acid such as pimeric acid and adipic acid have been reported.

On the other hand, it has been published that a considerable amount of citric acid was produced by yeast grown on n-alkanes. Abe and his colleagues,[35,36] who had isolated the yeast strain capable of converting efficiently n-paraffin to citric acid, have shown that [illegible] g/l of citric acid was produced from 50 g/l of n-hexadecane through the fermentation process using *Candida lipolytica*, in which the elimination of iron ions out of the medium was essential for the increase in the rate of citric acid to isocitric acid produced.

We have also performed a study of citric acid production on a semi-industrial scale using mold and yeast strains, *Penicillium janthinellum* KY 1141, *Candida zeylanoides* nov. sp KY 5002[37]. When the latter was grown aerobically on n-paraffin mixture mainly containing C_{12} to C_{15} fractions the yield of citric acid was more than 65 g/l, corresponding to 125 % of n-paraffin used.

The accumulation of α-ketoglutaric acid and pyruvic acid have been also reported using other yeast strain, *Candida lipolytica* AJ 5004, isolated by the research group of Ajinomoto Co. Ltd.[38, 39]. The yields were respectively 60 g/l and 4 g/l from 80 g/l of n-paraffin as the carbon source. In this process, the effectiveness of calcium ion on the production of organic acids was emphasized.

We have also reported the production of α-ketoglutaric acid by *Arthrobacter paraffineus* which was isolated as glutamic acid-producing strain as described above[5,24]. By adjusting pH automatically to 6.5 – 7.0 during the fermentation, more than 70 g/l of keto acid and 30 g/l of glutamic acid have been produced from 100 g/l of n-paraffin mixture under the suboptimal concentration of thiamine in the culture medium.

5. Production of vitamines and co-enzymes

Advantages claimed generally for fermentation processes are that many substances having chemically complex structures are obtainable more easily than by synthetic methods. The fermentation production of vitamines and coenzymes may be placed under this category.

Attempts to produce these chemicals by microbial methods from hydrocarbon has also been actively carried out in Japan[40]. Suzuki et al.[41] have reported the accumulation of B_2 in the culture media of yeast strains belonging to *Pichia*. Associating with the cell growth under the concentration of n-paraffin of 0.5 – 2.0 %, accumulating amounts increased and the maximum yield reached approximately 57 mg/l. Sato et al.[42] have also shown the production of riboflavin from n-hexadecane by *Eremothecium ashbyii* grown on the medium containing ammonium biphosphate, sodium glutamate, liquid paraffin and Tween 80. The yield was approximately 9 mg/l, of which major part extracellularly accumulated.

Vitamin B_6 which is responsible for the oxidative reaction of amino acids or amines is known to be contained widely in microbial cells. When hydrocarbon was applied for the substrate, too, it has been reported[40] that yeast such as *Candida albicans* and some of *Mycobacteria* produced this vitamin in their culture media. The yield was not higher than 400 µg/l, but it is planned to be developed further.

Increasing attention has been paid towards deoxyadenosyl-B_{12} since its biological function was recognized as the factor of antipernicious anemia. Fukui et al.[40] have attempted to produce it by *Corynebacterium simplex* and *Nocardia lutea* and reported the yield of 166 µg/l from n-hexadecane by *Corynebacterium simplex*.

The quantity of these vitamins produced up to now is not enough to develop on a larger scale.

On the other hand, Tsuboi et al.[43] studying the production of biotin, has reported that a strain of *Pseudomonas* isolated from the soil produced about 30 mg/l of biotin-related compound, characterized mostly as desthiobiotin.

The attempts to produce carotenoids and cytochrome b' have been carried out as well. Nishimura et al.[44] have shown that *Mycobacterium smegmatis* grown with kerosene as carbon source accumulated xanthophyll, of which the main components were 4-keto-γ-carotene and its derivatives. Subsequently, Fukui et al.[40] have reported that the yield of xanthophyll was approximately 3 mg/l in a laboratory scale experiment. Besides, cytochrome 'c' has been produced by several yeast strains such as *Candida albicans* and *Torulopsis utilis*. Under the condition that nonionic surfactant was added, about 10 mg/l of cytochrome 'c' has been accumulated.

In addition, the bacterial production of coenzyme Q[45] and coenzyme A[46] have been reported by Fukui et al. and the research group of Takeda Pharmaceutical Company. The production of these compounds probably needs further improvement, because these coenzymes participate in the sequence of oxidative reaction of hydrocarbon, whereby it is presumed to be contained in hydrocarbon-utilizing microorganisms much more than others.

6. Production of other chemicals from n-alkanes

Of the special metabolites derived from n-alkane through the microbial action, the production of phenazine and its derivatives have been shown. It has been reported that Phenazine-1-carboxylic acid was accumulated in the hydrocarbon layer of the culture medium[7]. We have also observed that orange and purple-colored pigments were accumulated in crystalline form in the n-paraffin layer of the culture medium of *Arthrobacter paraffineus* KY 7134 and identified respectively as phenazine-1, 6-hydroxide and phenazinediol-5, 10-N-oxides[48]. These findings are of note for the reason why the ring compounds are producible from straight chain hydrocarbons.

Generally, during the growth of bacteria with hydrocarbon, it is often observed that the lysis of bacterial cells occurs with its growth. It is particularly noted when penicillin, cephalosporin C and its derivatives were added to the growing culture[21]. Applying this technique, various cell components have been reported to be excreted to the culture medium. For example, protein[49], nucleic acids[49], UDPG peptide[50], phospholipid[51], and polysaccharide[29] have been already shown.

7. Production of chemicals from aromatic hydrocarbon

Comparatively, little is known about microorganisms which can utilize aromatic hydrocarbons as compared with aliphatic compounds. By the application of co-oxidation phenomenon, Raymond et al.[52] have reported the microbial oxidation of aromatic compounds.

Following [illegible], [illegible] and [illegible][53] have isolated the bacterium belonging to genus, [illegible], which can grow on aromatic hydrocarbon as the sole source of carbon, and reported the modification of p-cymene or cumene into cumic acid. This oxidation was much dependent upon pH of the culture [illegible]. [illegible][54] [illegible] found the production of 3.[illegible] g/l of [illegible] from 20 g/l of [illegible], Pseudomonas sp.

The [illegible] conversion of [illegible] into salicylic acid has already been known. Recently, [illegible] et al.[55] have [illegible] in [illegible] of 12 g/l of salicylic acid [illegible] over [illegible] percent by use of [illegible]. In this process, [illegible] was used [illegible] strong urease activity. [illegible], [illegible] et al.[56] [illegible] the possibility to improve the production yield through [illegible] in which anion exchange resin [illegible] to remove the salicylic acid produced out of the [illegible].

The [illegible] has been demonstrated by [illegible] et al.[57]. [illegible] isolated by them were able to convert [illegible] (10 g/l) [illegible] acid (1.12 g/l).

Although [illegible] production of aromatic compounds [illegible] to apply them directly to the [illegible] difficulty in microbial [illegible] synthetic activity [illegible] compounds in the microbial systems.

[illegible], studies on the microbial production of various useful chemicals [illegible] from the hydrocarbons. Besides, [illegible] with the utilization of the [illegible] from petroleum. Actually, ethanol[58], acetic acid[59], [illegible][60] and so on, have been applied as the substrate for glutamic acid production.

As it has been described above, it is confident that the techniques of hydrocarbon fermentation will make much progress in Japan. When full industrial operation is accomplished in the near future, we will be able to overcome the difficulties in the conventional process where the supply of the raw materials is obliged to depend on agricultural products, and it will render great contribution to the prosperity of human beings.

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