Tetradecane 1,14-Dicarboxylic Acid Production from *n*-Hexadecane by *Candida cloacae*[†]

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The better condition of cultivation for tetradecane 1,14-dicarboxylic acid (DC-16) production from *n*-hexadecane (*n*-C₁₆) by *Candida cloacae* MR-12 was investigated by using acetic acid as carbon source for the growth. In general, the condition suitable for the growth was also favorable for the production of DC-16. The change of pH during cultivation, the use of NaOH solution as pH controlling agent after pH-change and the addition of antifoam stimulated the production of DC-16.

Under the optimum conditions where the culture medium contained 15% (v/v) *n*-C₁₆, 1.4% (w/v) acetic acid, inorganic salts and growth factors, and pH was changed from 6.5 to 7.75 at 16 hr after the inoculation, the highest level of DC-16 production was attained after about 72 hr cultivation and the amount of the product accumulated was 61.5 g per liter of the medium.

When a mixture of various *n*-alkanes was used as starting material, DCs corresponding to the respective *n*-alkanes were produced as mixture.

In the previous papers,^{1,2)} it has been shown that strain M-1, derived from *n*-alkane-utilizing *Candida cloacae* 310 as a mutant unable to assimilate DC-10, produces a large amount of DCs from *n*-alkanes. Furthermore, it has been also found that a mutant strain MR-12, which was a further derivative of strain M-1 and unable to assimilate *n*-alkane, accumulates markedly DCs from *n*-alkanes.³⁾ On testing, alcohol, diol, aldehyde, fatty acid and methyl or ethylester of fatty acid are also found to be utilized as raw materials for DC production.

The degradation activities of strain M-1 and MR-12 against DC-16 are reduced to 40% and 10% of that of the parent strain, respec-

tively. Thus, it is found that the DC producing activity is closely related to the degradation activity of DC. Resting cells of strain M-1 and MR-12 produce 29.3 g/liter and 42.7 g/liter of DC-16 (tetradecane 1,14-dicarboxylic acid) from *n*-C₁₆, respectively.

On the other hand, production of DC-16 from *n*-C₁₆ in growing culture of strain M-1 is well brought about by using acetic acid as carbon source for the growth.

Present paper deals with the condition of cultivation for DC-16 production from *n*-C₁₆ by strain MR-12 using acetic acid as carbon source for the growth.

MATERIALS AND METHODS

Microorganism. *Candida cloacae* MR-12, derived from strain M-1 as a mutant unable to assimilate *n*-alkane, was used.

Media. Basal medium: 10% (v/v) *n*-alkane, 4.92 g

[†] Microbial Production of Long-chain Dicarboxylic Acids from *n*-Alkanes (Part 4).

The abbreviation used throughout this paper include DC, *n*-alkane ω,ω' -dicarboxylic acid; DC-N, dicarboxylic acid with N carbon atoms; *n*-C_N, *n*-alkane with N carbon atoms; AcH, acetic acid; AcNH₄, CH₃COONH₄; AcNa, CH₃COONa·3H₂O.

AcNH₄, 4.92 g AcNa, 5 g (NH₄)₂SO₄, 2 g KH₂PO₄, 0.3 g MgSO₄·7H₂O, 10 mg FeSO₄·7H₂O, 8 mg ZnSO₄·7H₂O, 8 mg MnSO₄·nH₂O, 100 γ biotin and 900 ml distilled water. pH 6.5. Seed medium: 50 g sucrose, 3.28 g AcNH₄, 3.28 g AcNa, 20 g (NH₄)₂HPO₄, 2 g KH₂PO₄, 0.3 g MgSO₄·7H₂O, 10 mg FeSO₄·7H₂O, 8 mg ZnSO₄·7H₂O, 8 mg MnSO₄·nH₂O, 100 γ biotin and 1000 ml distilled water. pH 6.5.

Cultivation. For the seed culture, a loopful of cells grown overnight on yeast extract-polypeptone medium was inoculated to 25 ml of seed medium in 500 ml flask and cells were cultured on a shaker at 30°C for 24 hr.

In the studies of cultural conditions, all cultivations were carried out by using a 1000 ml-glass jar equipped with a stainless steel sparger, magnetic driven impellers and an automatic pH controlling apparatus. In this case, 15 ml of seed culture broth obtained as mentioned above was inoculated to 300 ml medium in the 1000 ml-glass jar and cells were cultured at 30°C. The aeration and agitation were kept at 1/2 unit* and 1400 rpm, respectively. During cultivation, pH was changed from the optimum for the growth to that for the production of DC-16 as in the case of strain M-1.²⁾ The change of pH was carried out at about the end of growth. The regulation of pH both in the phase of growth and production of DC-16 was carried out by feeding the solution containing acetic acid and by adding alkali such as ammonia gas or NaOH, respectively.

The pH-change was performed partly by the rise of pH caused by the specific consumption of residual acetic acid in the medium and mainly by the addition of alkali. When pH rose more than 7.75, it was adjusted to 7.75 by adding HCl manually.

Determination of DC. DC-16 was determined gas chromatographically using DC-14 as internal standard. The determination of DCs other than DC-16 was carried out as described before.²⁾

Chemicals. All *n*-alkanes used were purchased from Kanto Chemical Co., Inc., and they were all certified as 98% pure.

* One unit of aeration was defined as one liter of air supplied to one liter medium per min.

RESULTS

Growth on acetic acid

Since strain MR-12 was not able to assimilate *n*-alkane, a carbon source which supported the growth of MR-12 was needed for the production of DC-16 from *n*-C₁₆ by growing culture of strain MR-12. Then, acetic acid was selected as a carbon source for the growth, because it was found to be effective for DC-16 production from *n*-C₁₆ by growing culture of strain M-1. It was another reason that acetic acid was obtained as a cheaper carbon source from petrochemical industry.

Since high concentration of acetic acid was found to be inhibitory to the growth of strain M-1,²⁾ acetic acid was fed continuously during cultivation, keeping its concentration very low in the medium. That is, a mixed solution of acetic acid and ammonium acetate or inorganic acids was used for automatic regulation of pH in the growing culture. The cultivation was carried out by using a 1000 ml-glass jar with a automatic pH regulating apparatus.

Table I shows the effect of pH on the growth of strain MR-12 on acetic acid. The favorable pH for the growth on acetic acid was about 6.5.

TABLE I. EFFECT OF pH ON THE GROWTH OF STRAIN MR-12

Medium: Basal medium without *n*-alkane was used.

Culture: Cultivation was carried out at 30°C for 24 hr. pH of the medium was regulated by adding the feed solution containing AcH and AcNH₄ at the ratio of 1 to 0.25 by mole. The concentration of AcH in the feed solution was 60% as free acid.

pH	Growth (O.D. _{562mμ})	
	12 hr	24 hr
5.0		0.015
5.5	0.156	0.220
6.0	0.178	0.580
6.5	0.224	0.600
7.0	0.190	0.530
7.5		0.130

TABLE II. EFFECT OF INITIAL CONCENTRATION OF ACETIC ACID AND NITROGEN, AND OF THE COMPOSITION OF FEED SOLUTION ON THE GROWTH OF STRAIN MR-12

Medium: Basal medium without *n*-alkane was used. pH 6.5.

Culture: Cultivation was carried out at 30°C. pH of the medium was automatically regulated to 6.5 with the feed solution described in the Table.

AcNH ₄ added (%)	AcNa added (%)	(NH ₄) ₂ SO ₄ added (%)	Total acetic acid (% as free acid)	Total nitrogen (% as N)	Feed solution used composition and ratio	Growth (O.D. _{at 564mμ})	
						12 hr	24 hr
0.492	0.492	0.5	0.6	0.142	AcH-AcNH ₄ , 1 : 0.25	0.270	0.725
0.770	0	0	0.6	0.140	AcH-AcNa, 1 : 0.25	0.375	0.860
0.770	0	0	0.6	0.140	AcH-AcNa, 1 : 0.5	0.540	0.800
0.770	0.906	0	1.0	0.140	AcH-AcNa, 1 : 0.25	0.440	0.840
0.770	1.812	0	1.4	0.140	AcH-AcNa, 1 : 0.25	0.400	0.810
1.285	0.227	0	1.0	0.234	AcH-AcNa, 1 : 0.25	0.418	0.780
1.285	1.133	0	1.4	0.234	AcH-AcNa, 1 : 0.25	0.410	0.760

Then, effects of the initial concentration of acetic acid and nitrogen, and of the composition of feed solution on the growth of strain MR-12 were tested. The results are presented in Table II.

The concentrations of acetic acid and nitrogen were suitable at 1.0% and 0.14%, respectively. As to the feed solution, AcH-AcNa mixture at a ratio of 1 to 0.25 was superior to AcH-AcNH₄ and to AcH-AcNa at a ratio of 1 to 0.5.

Furthermore, it was observed that the abundant growth of strain MR-12 occurred even in the absence of (NH₄)₂SO₄ as a nitrogen source.

*DC-16 production from *n*-C₁₆ by growing culture of strain MR-12*

The condition for the production of DC-16 from *n*-C₁₆ by growing culture of strain MR-12 was investigated by adding 10% (v/v) of *n*-C₁₆ to the culture medium. In this case, pH of the medium was changed from pH suitable for the growth (6.5) to that for DC-16 production (7.75)²¹ at the end of growth as described in the method.

However, when feeding of AcH-AcNa solution was stopped in order to change pH from 6.5 to 7.75, pH of the medium rose much over 7.75. The rise of pH was so strik-

ing as not to be regulated even by the addition of 3N HCl manually. Such a striking rise of pH seemed to occur through the consumption of acetic acid which might have remained at a high concentration in the medium at the time of pH-change. Therefore, AcNa in the feed solution was replaced by inorganic acid in order to prevent the needless rise of pH at the change of pH. Namely, it was expected that feeding of the mixture containing acetic acid and HCl in some proportion might cause a gradual decrease of the concentration of acetic acid in the medium and that complete consumption of acetic acid might be brought about at the time of pH-change.

Then, effects of feed solution containing acetic acid and HCl in various proportion and pH after the change of pH on DC-16 production from *n*-C₁₆ were investigated. The results are shown in Table III.

In this experiment, the rise of pH was not so striking as mentioned above. However, when 60% acetic acid solution or mixed solution of 10N AcH and 10N HCl at a proportion of 10 to 1 was used as feed solution, the addition of HCl manually was still necessary to regulate pH in the early phase after the change of pH. In this case, the rise of pH was no longer observed at about 4 to 6 hr

TABLE III. EFFECT OF pH AFTER pH-CHANGE AND FEED SOLUTION ON DC-16 PRODUCTION

Medium: Basal medium containing 7.70 g/liter of AcNH_4 and 9.06 g/liter of AcNa was used. pH before pH-change was 6.5.

Culture: Cultivation was carried out at 30°C for 72 hr. The regulation of pH before and after pH-change was carried out by adding the feed solution shown in the Table and ammonia gas, respectively.

Feed solution ^{a)} AcH : HCl	pH after pH-change	DC-16 produced (g/liter)	
		48 hr	72 hr
1 : 0	7.0	17.2	25.9
1 : 0	7.25	19.1	27.8
1 : 0	7.5	20.0	31.1
1 : 0	7.75	21.0	32.6
1 : 0	8.0	9.8	17.6
6 : 1	7.5	19.8	30.8
8 : 1	7.5	19.4	30.1
10 : 1	7.5	19.4	29.6

^{a)} Each feed solution was made up by mixing 10N AcH and 10N HCl with the volume ratio as shown in the Table.

TABLE IV. EFFECT OF YEAST EXTRACT ON DC-16 PRODUCTION

Medium: Basal medium containing 7.70 g/liter of AcNH_4 and 9.06 g/liter of AcNa was used.

Culture: Cultivation was carried out at 30°C for 72 hr. pH before pH-change was adjusted to 6.5 with the feed solution containing AcH and HCl (6:1). pH after pH-change was adjusted to 7.75 with ammonia gas.

Concentration of yeast extract	DC-16 produced (g/liter)	
	48 hr	72 hr
0 %	20.2	32.4
0.025	23.3	33.5
0.05	25.3	36.2
0.1	26.6	36.5

after pH-change. This was probably due to the occurrence of DC-16 production in addition to the complete consumption of acetic acid. Thereafter, pH of the medium was regulated automatically with ammonia gas. When the feed solution containing AcH and HCl at a proportion of 6 to 1 or 8 to 1 was

used, pH of the medium was apt to fall at about 16 hr after inoculation. In this case, pH-change was carried out by adding ammonia gas.

In any case, a considerable amount of DC-16 was produced from $n\text{-C}_{16}$ by the growing culture of strain MR-12. Since there observed little difference between the amount of DC-16 produced by 60% acetic acid solution and that by AcH-HCl mixture (6:1) as the feed solution at pH 7.5, the latter solution was used as feed solution in the following experiments. After pH-change, pH 7.75 was the most suitable for the production of DC-16 from $n\text{-C}_{16}$.

Since yeast extract enhanced the production of DC-16 from $n\text{-C}_{16}$ by resting cells of strain MR-12,³¹ the effect of the concentration on DC-16 production by growing cells was tested. As shown in Table IV, the amount of DC-16 from $n\text{-C}_{16}$ increased as the increase of the concentration of yeast extract, however, the amounts of DC-16 produced by the addition of 0.05% and 0.1% of yeast extract were practically equal. So that the cultivation for DC-16 production was carried out by adding 0.05% of yeast extract in the following experiments.

Though the data were not presenten, the addition of yeast extract to the medium also promoted the growth of strain MR-12 on acetic acid. Therefore, the effect of initial concentration of acetic acid on DC-16 production was tested under the presence of 0.05% yeast extract. It was also tested whether NaOH solution and AcH- H_2SO_4 mixture were suitable as the pH controlling agent after pH-change and as feed solution instead of ammonia gas and AcH-HCl mixture, respectively. In addition, since a remarkable foaming occurred after pH-change which would be due to the accumulation of DC-16, the effect of anti-foam on DC-16 production was also examined. These results are summarized in Table V. The final volume of the cultured broth was measured and indicated together with the total

amount of DC-16 per jar because of the change of the volume owing to the addition of NaOH and antifoam.

Among the factors tested, the use of NaOH solution as a pH controlling agent after pH-change and the addition of antifoam markedly enhanced the production of DC-16, though the final volume of the cultured broth increased by these additions. The increase of

the initial concentration of acetic acid and the use of the feed solution composed of acetic acid and H₂SO₄ were slightly stimulative for DC-16 production. However, since HCl seems to corrode the metallic jar fermentor more violently than H₂SO₄, the feed solution containing H₂SO₄ was selected and used in the following experiments.

Effects of nitrogen concentration, the ratio

TABLE V. EFFECT OF INITIAL ACETIC ACID CONCENTRATION, FEED SOLUTION, pH CONTROLLING AGENT AFTER pH-CHANGE AND THE ADDITION OF ANTIFOAM ON DC-16 PRODUCTION

Medium: Basal medium containing 7.70 g/liter of AcNH₄ and 9.06 g/liter AcNa (1.0% as free acid) or 7.70 g/liter of AcNH₄ and 18.12 g/liter of AcNa (1.4% as free acid) in addition to 0.5 g/liter of yeast extract was used.

Culture: Cultivation was carried out at 30°C for 72 hr. pH before and after pH-change were adjusted to 6.5 and 7.75, respectively.

Initial AcH concentration (% as free)	Component of feed solution ^{a)}	pH controlling agent after pH-change	Addition of antifoam ^{b)}	DC-16 produced (g/liter)		Final volume ml/jar	Total DC-16 g/jar
				48 hr	72 hr		
1.0	AcH : HCl	ammonia gas	—	23.1	32.6	210	6.84
1.4	AcH : HCl	ammonia gas	—	24.9	34.5	225	7.76
1.4	AcH : H ₂ SO ₄	ammonia gas	—	24.2	34.7	245	8.50
1.4	AcH H ₂ SO ₄	ammonia gas	+	24.6	35.1	290	10.17
1.4	AcH H ₂ SO ₄	3 N NaOH	+	30.2	40.8	335	13.66

^{a)} Each feed solution was made up by mixing 10 N AcH and 10 N HCl or H₂SO₄ at the ratio of 6 to 1.

^{b)} Toshiba silicone TSA 735 purchased from Toshiba Electronic Co., Inc.

TABLE VI. EFFECT OF NITROGEN CONCENTRATION, FEED SOLUTION AND MIEKI ON THE DC-16 PRODUCTION

Medium: Basal medium containing 7.70 g/liter of AcNH₄ and 18.12 g/liter of AcNa (in the case of 0.14% N) or 11.55 g/liter of AcNH₄ and 12.08 g/liter of AcNa (in the case of 0.21% N) in addition to 0.5 g/liter of yeast extract was used. pH 6.5.

Culture: Cultivation was carried out at 30°C for 72 hr by adding 3 N NaOH as pH controlling agent after pH-change and antifoam.

Nitrogen concentration (% as N)	Addition of Miki ^{a)} (ml/liter)	Feed solution ^{b)}		DC-16 produced (g/liter)		Total DC-16 (g/jar)
		AcH	H ₂ SO ₄	48 hr	72 hr	
0.14	0	6	1	33.9	41.5	14.11
0.14	0	8	1	31.6	40.2	13.87
0.21	0	6 : 1		38.6	45.2	14.01
0.21	0	8 : 1		37.9	45.8	14.47
0.21	20	8	1	40.2	51.5	16.48

^{a)} Miki, an acid hydrolyzate of defatted soybean, was added instead of 0.5 g/liter of yeast extract.

^{b)} Each feed solution was made up by mixing 10 N AcH and 10 N H₂SO₄ at the volume ratio shown in the Table.

of the components in the feed solution and the addition of Mieki instead of yeast extract on DC-16 production were investigated.

TABLE VII. DCs PRODUCTION FROM VARIOUS *n*-ALKANES

Medium: Basal medium containing 11.55 g/liter of AcNH_4 , 12.08 g/liter of AcNa and 20 ml/liter of Mieki was used. Each *n*-alkane was added to the content of 10% (v/v).

Culture: Cultivation was carried out as shown in the legend of Table VI.

<i>n</i> -Alkane added	DC produced	Amount of DC (g/liter)		Total DC (g/jar)
		48 hr	72 hr	
<i>n</i> -C ₁₁	DC-11	9.6	13.2	3.23
<i>n</i> -C ₁₂	DC-12	15.2	21.5	5.48
<i>n</i> -C ₁₃	DC-13	21.6	30.7	8.59
<i>n</i> -C ₁₄	DC-14	30.4	44.5	12.68
<i>n</i> -C ₁₅	DC-15	34.1	48.0	15.36
<i>n</i> -C ₁₆	DC-16	38.0	50.2	17.07
<i>n</i> -C ₁₇	DC-17	36.0	45.2	15.59
<i>n</i> -C ₁₈	DC-18	12.6	20.2	6.36

As shown in Table VI, the addition of Mieki, acid hydrolyzate of defatted soybean, brought about the marked increase of DC-16. The increase of nitrogen concentration was also effective. But there was little difference between the mixture of acetic acid and H_2SO_4 at a rate of 6:1 and that at 8:1 in the feed solution in DC-16 production.

Table VII shows the production of DCs from various *n*-alkanes. It was found that various kinds of DC having the same number of carbon atoms as those of the substrates used were produced by growing culture of strain MR-12. Among DCs produced, DC-16 from *n*-C₁₆ was most abundantly accumulated and DC-17 from *n*-C₁₇ was next. Thus, the accumulation of DCs with the carbon number around 16 were relatively high.

The production of DCs from mixture of *n*-alkanes was investigated and compared with that from individual *n*-alkanes. As shown in Table VIII, it was found that DCs corresponding to the respective *n*-alkanes in the mixture were produced as mixture.

TABLE VIII. DC PRODUCTION FROM THE MIXTURE OF *n*-ALKANE

Medium: Same medium as shown in Table VII was employed.

Culture: Cultivation was carried out as shown in the legend of Table VI.

<i>n</i> -Alkane added	<i>n</i> -Alkane content % (v/v)	DC produced	Amount of DC (g/liter)		Total DC (g/jar)	Consumption of <i>n</i> -alkane (%)	Molar yield of DC ^{a)} (%)	Relative yield ^{b)}	
			48 hr	72 hr				A	B
<i>n</i> -C ₁₂	5	DC-12	9.6	15.2	4.18	83.1	33.2	100	
<i>n</i> -C ₁₂	10	DC-12	16.6	20.6	5.98	55.0	35.9		100
<i>n</i> -C ₁₃	5	DC-13	12.6	17.2	4.82	81.5	39.6	100	
<i>n</i> -C ₁₃	10	DC-13	22.0	29.4	9.26	57.6	53.8		100
<i>n</i> -C ₁₆	5	DC-16	24.2	29.2	7.47	94.7	53.6	100	
<i>n</i> -C ₁₆	10	DC-16	41.6	55.2	17.66	97.3	62.1		100
<i>n</i> -C ₁₂	5	DC-12	9.2	16.6	5.15	69.3	49.0	148	137
<i>n</i> -C ₁₆	5	DC-16	6.2	9.6	2.97	56.7	35.6	66	57
<i>n</i> -C ₁₃	5	DC-13	8.6	14.3	4.62	72.0	42.8	108	80
<i>n</i> -C ₁₆	5	DC-16	6.0	10.2	3.22	56.7	36.0	67	58

^{a)} Molar yield of DC was calculated against *n*-alkane consumed.

^{b)} Relative yield was calculated by dividing the molar yield of DC obtained from the mixture of *n*-alkane by that from the individual *n*-alkane.

However, considerable differences were observed between the cultivation with the individual and the mixed *n*-alkanes. One of them was that the consumption rate of *n*-C₁₆ at the cultivation in the mixture of *n*-alkanes was markedly low to compare with that of *n*-C₁₆ alone. On the contrary, the consumption rate of *n*-C₁₂ or *n*-C₁₃ in the mixture of *n*-alkane was somewhat higher than that of 10% of *n*-C₁₂ or *n*-C₁₃ alone, respectively. Secondly, the molar yield of DC-16 from *n*-C₁₆ consumed at the cultivation in the mixture of *n*-C₁₆ and *n*-C₁₂ or *n*-C₁₃ markedly decreased as compared

with that in *n*-C₁₆ alone. On the other hand, the molar yield of DC-12 or DC-13 produced from corresponding *n*-alkane mixed with *n*-C₁₆ increased in almost cases tested. Only exception was the case that the molar yield of DC-13 from *n*-C₁₃ consumed at the cultivation in the mixture of *n*-C₁₃ and *n*-C₁₆ decreased compared with that in 10% *n*-C₁₃ alone.

Table IX shows the effect of *n*-C₁₆ content on DC-16 production. Although the production of DC-16 increased to about twice by increasing the content of *n*-C₁₆ from 5 to 10%, there was no increase of DC-16 production

TABLE IX. EFFECT OF *n*-C₁₆ CONTENT ON DC-16 PRODUCTION

Medium: The same medium as shown in Table VII was used.

Culture: Cultivation was carried out as shown in the legend of Table VI.

<i>n</i> -C ₁₆ content % (v/v)	<i>n</i> -C ₁₆ consumed %	DC-16 produced (g/liter)		Total DC-16 g/jar	Yield of DC-16 ^{a)}	
		48 hr	72 hr		molar %	weight %
5	99.2	22.8	29.6	8.58	58.9	76.2
7.5	98.0	31.7	40.6	12.38	57.3	72.5
10	96.5	40.2	51.2	16.64	58.9	74.7
15	69.3	41.6	54.2	18.16	59.8	75.6
20	53.0	40.6	53.8	18.18	58.5	74.2

^{a)} The yield of DC-16 was calculated against *n*-C₁₆ consumed. The specific gravity of *n*-C₁₆ employed for the calculation was 0.7718 at 25°C.

TABLE X. EFFECT OF SEED CULTURE ON DC-16 PRODUCTION

Medium: Medium containing 15% (v/v) of *n*-C₁₆ was used. Other compositions were the same as described in Table VII.

Seed cell	Seed phase (hr)	Growth of seed (O.D. at 562m μ)	DC-16 produced (g/liter)		Total DC-16 (g/jar)
			48 hr	72 hr	
A-cell ^{a)}	20	0.790	41.4	54.5	17.44
S-cell ^{b)}	18	0.710	41.0	54.7	16.85
S-cell	20	0.800	44.5	60.0	18.90
S-cell	22	0.860	45.9	61.5	19.00
S-cell	24	0.780	42.0	57.4	18.37
S-cell	26	0.650	40.6	54.8	16.99
S-cell	28	0.450	41.6	54.9	17.29
S-cell	30	0.365	38.2	48.1	14.43

^{a)} The cells grown on the same medium as shown in Table VII except *n*-C₁₆ were used.

^{b)} The cells grown on medium F³⁾ were used.

proportional to the increase of n -C₁₆ when the n -C₁₆ content became higher than 10%. However, the molar yield of DC-16 from n -C₁₆ consumed was about 58% in any case. This value was about the same with that obtained by resting cells of strain MR-12 as reported in the previous paper.³⁾

Effect of the seed culture was studied as summarized in Table X. There was little difference between acetate-grown cells and sucrose-grown cells in the production of DC-16. As to the cultural phase of seed tested by using sucrose-grown cells, the maximum production of DC-16, *i.e.*, 61.5 g/liter, was obtained by the inoculation of cells on sucrose for 22 hr. In this case, the consumption rate of n -C₁₆ and the yield of DC-16 from n -C₁₆ added were 65.7% and 54.7% (w/w), respectively. When seed cultures were continued more than 22 hr, marked lysis of cells occurred and, in fact, the cells grown more than 22 hr on sucrose were found to be unsuitable as seed.

Figure 1 shows the time course of fermentation. The cultivation was carried out with

FIG. 1. Time Course of Fermentation.

Medium: Medium containing 15% (v/v) of n -C₁₆ was used. Other components were the same as described in Table VII.

Culture: Cultivation was carried out as shown in the legend of Table VI.

▲—▲ Growth, ×—× AcH consumption, ●—● n -C₁₆ consumption, ○—○ DC-16 production.

15% (v/v) n -C₁₆. As may be seen, the consumption of acetate began rapidly at about 4 hr after inoculation and the complete consumption was observed at about 18 hr after inoculation. The accumulation of DC-16 was enhanced by the change of pH at 16 hr and DC-16 increased according to the consumption of n -C₁₆. The amount of DC-16 and the consumption rate of n -C₁₆ were 56.2 g/liter and 68.6% after 72 hr cultivation respectively.

DISCUSSION

Establishment of a process for the production of long-chain DCs by using microorganism, which was the initial object of this work, was thus accomplished. In the conventional fermentations, a carbon source was used for the growth as well as for the production, for example, the fermentative productions of organic acids, amino acids, nucleosides or nucleotides and so on. On the other hand, in the present fermentation, carbon source for the growth was required in addition to the raw material for the production, because the microorganism employed was not able to assimilate the raw material. Such type of fermentation might be effective for the biochemical performance of chemical unit process. It is likely that the amount of cells which catalyze the reaction may be one of the limiting factors in such a fermentative process. In fact, the condition favorable for the growth was generally favorable for the production of DCs.

Among the various factors tested, the use of NaOH solution as pH controlling agent after pH-change and the addition of antifoam were specifically effective for the production of DC-16. The stimulative effect of antifoam might be due to the dispersion of n -alkane and cells which were apt to be adsorbed on the surface of the foam. The fact that NaOH solution was superior to ammonia gas as pH controlling agent after pH-change would indicate that ammonia gas might be toxic for the cells.

Other factors such as Mieki closely related to the stimulation of both the growth and the production.

Since both the carbon source and the product were organic acid in the present fermentation and considerable difference was observed between the optimum pH for the growth and the production, regulation of pH during fermentation was very important. The regulation of pH both in the phase of growth and production was carried out by adding acetic acid-inorganic acid mixture and NaOH solution, respectively. In this case, the carbon source for the growth was let to the complete consumption at the end of the growth by controlling the ratio of acetic acid to inorganic acid in the feed solution. Then, the change of pH from the optimum for the growth to that for the production was easily carried out by adding NaOH solution. These devices brought about the high yield of DC-16 production. Namely, the maximum yield of DC-16, *i.e.*, 61.5 g/liter, which was corrected to 63.3 g/liter by the volume of cultured broth, was obtained from 15% (v/v) of *n*-C₁₆ after 72 hr cultivation. In this case, the weight yield of DC-16 from *n*-C₁₆ added was about 54.7%. However, 10% (v/v) of *n*-C₁₆ seemed to be more practical than 15% (v/v) because considerable high amount of *n*-C₁₆ was remained in cultured broth when 15% (v/v) of *n*-C₁₆ was used.

The present fermentation had several advantages in addition to the high yield as mentioned above. One of them was that the isolation process of the product was easy, because almost all *n*-C₁₆ added was consumed within relatively short cultivation. The other

was that the product was obtained in considerably pure state, because the by-product other than CO₂ are not accumulated as reported in the previous paper.³⁾

In the production of DCs from *n*-alkane mixture, considerably complicated phenomena was observed. Lower *n*-alkane tended to be preferentially consumed by the cells if it was mixed with higher *n*-alkane. It was presumed that lower *n*-alkane was preferentially adsorbed by enzyme and inhibited the adsorption of higher *n*-alkane. Furthermore, the molar yield of DC produced from lower *n*-alkane in the mixture was somewhat higher than that from lower *n*-alkane alone. Therefore, it was presumed that DC-16 from *n*-C₁₆ was degraded to DC-12 which was added to DC-12 from *n*-C₁₂, but the accumulation of DC-12 from *n*-C₁₆ alone did not occur. On the contrary, the molar yield of DC-16 from *n*-C₁₆ consumed in the mixture was markedly low as compared with that from *n*-C₁₆ alone. It was also presumed that the degradation of DC-16 into such as CO₂ might be stimulated by the lower *n*-alkane.

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