Studies on the Utilization of Hydrocarbons by Microorganisms

Part XI. Effects of Organic Nutrients, Mineral Salts and Other Substances on the Accumulation of Cumic Acid from *p*-Cymene

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The accumulation of cumic acid from *p*-cymene by a newly isolated *Pseudomonas* strain was investigated.

The maximal accumulation of the cumic acid in cultural medium was observed by adjustment of pH value at 6. Among seven organic nutrients tested, only yeast extract and Bacto-tryptone stimulated the acid accumulation. Manganese sulfate and calcium chloride showed a slightly good effect, while ferrous sulfate was appreciably inhibitory. As the maximal yield, 19 g of the acid was obtained by the initial concentration of 50 ml *p*-cymene in 1 liter of the medium.

Although considerable attention has been recently paid, little work has been done from industrial viewpoint on microbial transformation of hydrocarbons besides Davis' work.¹⁾ In this work, the microbial assimilation and transformation of aromatic hydrocarbons were investigated extensively.

In an earlier paper,²⁾ during the course of an investigation on the accumulation of cumic acid by a newly isolated *Pseudomonas* strain, it was shown that addition of calcium carbonate in growth medium and considerable agitation gave much accumulation of cumic acid. And it was shown that this effect of calcium carbonate was ascribed to the change of pH value of the cultural medium. Ishikura *et al.*³⁾ also described an important effect of

pH on microbial formation of salicylic acid. Therefore, it was desired to clarify the effect of pH values on the formation of cumic acid by this strain. Furthermore, it seemed to be interesting, from the viewpoint of industrial application of this process, to determine effects of some organic nutrients, mineral salts and other substances.

In this paper, the prominent results obtained in these studies are described.

MATERIALS AND METHODS

Microorganism. *Pseudomonas* strain S449B6 was used throughout this work, which was newly isolated by the authors and identified as closely resembling *Pseudomonas desmolytica*.

Cells, grown on *p*-cymene-agar-slant for 2 days, were suspended in distilled water. Optical density of the cell suspension was adjusted to about 1.0 at 610 m μ . 1.5 ml of the suspension was inoculated into 50 ml of medium. The medium was incubated in a 500-ml shaking flask at 30°C for 20 or 48 hr on a reciprocal shaker.

Medium. The basal medium is shown in Table I. Unless otherwise stated, all cultivations were carried

1) J. B. Davis and R. L. Raymond, *Appl. Microbiol.*, **9**, 383 (1961).

2) K. Yamada, S. Horiguchi and J. Takahashi, *Agr. Biol. Chem.*, **29**, 943 (1965).

3) T. Ishikura, H. Nishida, K. Tanno, N. Miyachi and A. Ozaki, Annual Meeting of Agr. Chem. Soc. Japan (1965).

TABLE I. BASAL MEDIUM

<i>p</i> -Cymene	1.0	ml
NH ₄ NO ₃	0.4	g
KH ₂ PO ₄	0.15	"
Na ₂ HPO ₄	0.15	"
MgSO ₄ ·7H ₂ O	0.02	"
CaCl ₂ ·2H ₂ O	0.001	"
FeSO ₄ ·7H ₂ O	0.005	"
MnSO ₄ ·4H ₂ O	0.0002	"
Yeast extract	0.05	"
Dist. water	100	ml
pH	7.0	

out in this medium. Urea solution was sterilized separately by addition of a piece of thymol and added to the sterile medium in a flask. When containing vitamins, the medium was sterilized at 100°C for 15 min.

p-Cymene was obtained from Tokyo Chemical Industry Co., Ltd., and was used without further purification.

Organic nutrients used in this experiment were also obtained commercially as follows: yeast extract, malt extract, potato extract, Bacto-tryptone (pancreatic digest of casein USP) and casamino acid (vitamin free) from DIFCO Laboratories, CSL from Nisshoku Co., Polypepton from Daigo Eiyo Kagaku Co. and meat extract from Kyokuto Seiyaku Co.

After incubation, cultural broth was acidified by phosphoric acid and extracted with ether. The ether solution was titrated by 1/10 N NaOH for the estimation of the cumic acid formed.

Details of other methods were given previously.²⁾

RESULTS AND DISCUSSION

Effect of pH control

In the previous paper, it was described that the addition of calcium carbonate was very effective for the production of cumic acid. The experiment showed that pH value was maintained higher by the addition of calcium carbonate.

So, in this work, pH of cultural broth was adjusted at several values by adding sodium hydroxide or ammonia. As shown in Fig. 1, the broth was controlled by 5N sodium hydroxide at pH 6, 7 and 8. In the case of pH 8, the organism did not grow for the first 30 hr of cultivation. After then the broth was incubated without pH control and the

FIG. 1. pH of Cultural Broth Controlled at Each Value with 5N NaOH.

—●—: not controlled.
 × ○
 ↑ ↓ pH was adjusted at each time with 5N NaOH.
 × ○

TABLE II. EFFECT OF pH OF CULTURAL BROTH ON THE ACID PRODUCTION (mg/liter)

pH	Time (hr)		
	24	32	164
Not adjusted	1670	1570	—
6*	2270	3040	—
7*	2160	2420	—
8*	—	—	3160

* pH was adjusted with 5N NaOH as shown in Fig. 1.

FIG. 2. pH of Cultural Broth Controlled at Each Value with 5N NH₄OH.

—●—: not controlled.
 × ○
 ↑ ↓ pH was adjusted at each time with 5N NH₄OH.
 × ○

organism began to grow gradually. Table II shows that pH 6 was favourable value for the acid production. Using ammonia, similar results were obtained as shown in Fig. 2 and Table III.

TABLE III. EFFECT OF pH OF CULTURAL BROTH ON THE ACID PRODUCTION (mg/liter)

pH	Time (hr)	
	34.5	59
Not adjusted	1930	1730
6*	3700	100

* pH was adjusted at 6 with 5N NH_4OH as shown in Fig. 2.

Effect of minerals

Effects of minerals added in the cultural medium are shown in Table IV. The experiments were carried out by omission of the minerals from the basal medium. It is noteworthy that ferrous sulfate showed a clearly inhibitory effect. The amount of the cumic acid accumulated was reduced to a half by the inhibition of ferrous sulfate. Calcium chloride and manganese sulfate were slightly stimulative. Magnesium sulfate seems to be inhibitory to the formation of cumic acid, as shown in Table IV. However, as indicated in Table V, slightly exhibited a stimulative effect when ferrous sulfate was not contained

TABLE IV. EFFECT OF MINERALS ON THE ACID PRODUCTION

Minerals omitted	Acid found (mg/liter)
Control	860
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	1680
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	1010
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	720
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	790
Yeast extract	540

TABLE V. EFFECT OF Fe, Mg AND YEAST EXT. ON THE ACID PRODUCTION

Compound omitted	Growth	Acid found (mg/liter)
Control	++	860
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	++	1820
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	+	1670
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$		
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	+	1190
Yeast extract		
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	$\pm$	500
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$		
Yeast extract

FIG. 3. Effect of Ferrous Sulfate on the Acid Production.

in the cultural medium.

Fig. 3 reveals that the maximum yields were obtained without ferrous sulfate.

Effect of yeast extract and other organic nutrients

Effect of yeast extract on the formation of cumic acid is also shown in Table IV. In this experiments, ferrous sulfate was not omitted from the basal medium. It clearly stimulated the acid formation. Fig. 4 indicates that an optimal concentration of yeast extract was 3000 mg per liter, and that the maximal yield of the acid amounted to 5000 mg per liter from 8600 mg of *p*-cymene.

Effects of six other organic nutrients on the acid production were examined (Table VI). When 3000 mg per liter of each nutrient was added to the cultural medium instead of

FIG. 4. Effect of Yeast Extract on the Acid Production.

* Ferrous sulfate was omitted from basal medium in this experiment.

TABLE VI. EFFECT OF ORGANIC NUTRIENTS ON THE ACID PRODUCTION (mg/liter)

Nutrient added (3000 mg/liter)	Time (hr)	
	25.5	70.5
Control	1420	1500
Yeast extract	5030	4290
CSL	1100	1120
Polypepton	2280	2130
Meat extract	1160	1160
Malt extract	1870	1690
Potato extract	310	350
Bacto-tryptone	4590	4010

yeast extract, only Bacto-tryptone showed similar effect to yeast extract as far as the acid accumulative effect. While potato extract was definitely inhibitory.

Amino acid, for instance casamino acid, was also slightly stimulative (Fig. 5), and then stimulation by vitamin mixture (Table VII) was not prominent as shown in Fig. 6. Both casamino acid and vitamin mixture had less effect than yeast extract. Purine and pyrimidine bases, and ashed yeast extract had

FIG. 5. Effect of Casamino Acid on the Acid Production.

TABLE VII. COMPOSITION OF VITAMIN MIXTURE

	g/liter
Vitamin B ₁ -HCl	1
Vitamin B ₂	1
Vitamin B ₆	1
Ca-pantothenate	1
Nicotinic acid	1
PABA	0.2
Biotin	0.01
Folic acid	0.01

FIG. 6. Effect of Vitamins on the Acid Production.

* Vitamin mixture is shown in Table VII.

also little effect. Mixed system which contained casamino acid, vitamins, bases and ashed yeast extract gave no more than 3000 mg of the acid per liter.

The amount of yeast extract, 3000 mg per liter, seems to be too much as usual stimulative effectors go. It is suggested that yeast extract effected mainly as energy and carbon sources. In this point of view, effect of glucose was tested instead of yeast extract. However, Table VIII shows that it had no

TABLE VIII. EFFECT OF GLUCOSE ON THE ACID PRODUCTION

Nutrient added (mg/liter)	Acid found (mg/liter)
Control	1450
Yeast extract 1000	3610
Glucose* 10	1560
" * 1000	1640

* Glucose was added to basal medium instead of yeast extract.

definite effect. At least, it revealed that glucose did not effect as energy and carbon sources.

Effect of inorganic nitrogen sources

Effect of several inorganic nitrogen sources on the acid accumulation was studied. In this investigation, the medium contained 3 g of yeast extract and other components as shown in Table I, but not ferrous sulfate. As shown in Table IX, ammonium nitrate was rather suitable for the acid production. While even in the absence of inorganic

TABLE IX. EFFECT OF INORGANIC NITROGEN SOURCES ON THE ACID PRODUCTION

Nitrogen source	(g/liter*)	Acid found (mg/liter)
Control	—	3780
NH ₄ NO ₃	4	5470
NH ₄ Cl	5	5170
NH ₄ H ₂ PO ₄	12	4680
Urea	3	4700

* as 1.4 g of nitrogen per liter.

FIG. 7. Time Course of the Acid Production, When Urea was Fed as a Nitrogen Source.

nitrogen sources the acid was produced 70 % as much as in the presence of ammonium nitrate. From this phenomenon yeast extract seems to effect partly as nitrogen source.

Other nitrogen sources such as ammonium sulfate, potassium nitrate and sodium nitrate were also tested. But they had no stimulative effect on the acid production.

When urea was fed as a nitrogen source, pH of the cultural broth was maintained rather high (Fig. 7). But amount of the acid produced did not so much increase.

Effect of initial *p*-cymene concentration

When initial *p*-cymene concentration was

FIG. 8. Effect of Initial *p*-Cymene Concentration.

increased from one to thirty ml per 100 ml of cultural medium, the amount of cumic acid accumulated increased from 5 g per liter to 19 g per liter, giving the maximal yield of the acid at 5 ml per 100 ml (Fig. 8).

From these results, about 60 per cent of conversion ratio was obtained by the addition of good deal of yeast extract. This effect of yeast extract had such decisive superiority that the effects of pH control and agitation shown in the previous paper were overcome. So it was felt that further experiments were of importance for determination of this effect. Especially the phenomenon, that Bactotryptone, Polypepton and casamino acid showed good or slightly stimulative effect, seems to require more detailed investigation about amino acid and peptide.

And also it appears of interest to investigate further the inhibition mechanism of ferrous sulfate for the acid production. Aromatic ring-splitting enzymes are known to be ferrous ion dependent. Ratledge and Winder⁴⁾ showed that *Mycobacterium smegmatis* which had been maintained in their laboratories accumulated a good deal of salicylic acid with iron-deficient medium. In their discussion, it was described that *M. smegmatis* was presumed to require iron for the next steps in the utilization of salicylic acid and a proximate metabolite. However, nothing was known about the iron requirement for the metabolism of these substances.

4) C. Ratledge and F. G. Winder, *Biochem. J.*, **84**, 501 (1962).

Also in the case of cumic acid accumulation, it may be supposed that ferrous ion is required for further degradation of the acid.

In this process, it was noteworthy that no by-product was isolated from the cultural broth. But, from the industrial viewpoint, there are some problems compared with synthetic processes. The problems are, for

instance, concentration of substrate and product in the reaction medium and reaction rate. In this point of the view, it would be interesting to study the fermentation and the engineering of the heterogeneous system. Especially it is an important phenomenon that even in 30 per cent of *p*-cymene concentration the organism could grow and produced the acid.