Production of Nucleic Acid Related Substances by Fermentative Processes

Part VI. Accumulation of 5'-Xanthylic Acid by Guanine-Requiring Mutants of *Micrococcus glutamicus*

II. Studies on Cultural Conditions

By Masanaru MISAWA, Takashi NARA and Shukuo KINOSHITA

Tokyo Research Laboratory, Kyowa Hakko Kogyo Co., Ltd.

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A guanineless mutant of *Micrococcus glutamicus*, KY9978 strain, which was derived from a guanine-adenine doubleless mutant, KY9938 strain, was able to grow on guanine more abundantly than the corresponding nucleoside and nucleotide, but unable to grow on 2,6-diaminopurine. A fermentation medium most suitable for XMP production by KY9978 strain was decided by varying the concentrations and kinds of ingredients in the medium. Of particular importance in media is the concentration of guanine, the excessive addition of which decreased XMP yields remarkably. This might suggest negative feed back mechanisms involved in GMP biosynthesis by this strain. Typical chemical changes in XMP fermentation showed that the maximal amount of the product was approximately 2.75 mg per ml of medium.

INTRODUCTION

It was previously reported that guanineless or guanine-adenine doubleless mutants of *Micrococcus glutamicus* accumulated a large amount of 5'-xanthylic acid (abbreviated as XMP) in the culture fluid.¹⁾

In this report, the results on growth response of the organisms and on experiments to develop a complex medium suitable for XMP production by the organisms are described.

EXPERIMENTAL

Materials and Methods

M. glutamicus KY9978 (a guanine-requiring mutant) was used exclusively in these experiments.

Materials and methods are the same as in the previous report¹⁾ with the exception of the tests for growth response. Procedures for growth response tests are as follows: 1 drop of cell suspension which was prepared by addition

of 10 ml of sterilized water to an agar slant culture of KY9978 strain, was inoculated to 10 ml of the growth medium in a test tube. It was incubated at 30°C with shaking. After 2 days, the cell density was assayed by the same method as described previously.¹⁾ The chemically defined medium comprising casamino acids (vitamin free) as amino acid source, necessary for growth of the organism,²⁾ was used for the test. The composition of the medium is shown in Fig. 1.

RESULTS

Growth Response of KY 9978 Strain.

The growth response of KY9978 strain to guanine, its derivatives and 2,6-diaminopurine was first tested. The results are given in Fig. 1

It was observed that the most abundant growth of the mutant was obtained by guanine supplement, whereas its growth was very poor on a medium to which guanosine or 5'-guanylic acid was added. Addition of approximately

1) M. Misawa, T. Nara, K. Udagawa, S. Abe and S. Kinoshita, This Journal, **28**, 688 (1964).

2) K. Nakayama, Z. Sato and S. Kinoshita, Oral presentation at Meeting of Agr. Chem. Society, Tokyo, Nov. 1963.

FIG. 1. Growth Responses of KY9978 Strain to Guanine Derivatives.

Medium: Glucose 2.0%, $(\text{NH}_4)_2\text{SO}_4$ 0.4%, casamino acids 1.0% K_2HPO_4 0.1%, urea 0.1%, vitamin mixture 1 ml/100 ml and $\text{MgSO}_4 \cdot 7\text{aq}$ 0.03%, $\text{FeSO}_4 \cdot 7\text{aq}$ 0.01%, $\text{CaCl}_2 \cdot 2\text{aq}$ 0.01%.

26 DAP: 2.6 Diaminopurine, DCW.: Dried Cell Weight.

—○— Guanine —△— 5'-GMP
—●— Guanosine —□— 2,6-DAP

FIG. 2. Effect on Growth of KY9938 Strain of Nitrogen Sources in Fermentation Media.

Basal medium: glucose 10.0%, yeast extract 1.0%, $\text{MgSO}_4 \cdot 7\text{aq}$ 0.1%, CaCO_3 2.0%, K_2HPO_4 0.2% and KH_2PO_4 0.2%. Nitrogen sources were added to this basal medium, as indicated in Figure.

* When urea was used as a nitrogen source, CaCO_3 was not present in the medium.

After 6-day incubation.
—●— NH_4 -Acetate —△— NH_4Cl
—×— $(\text{NH}_4)_2\text{HPO}_4$ —●— Urea
—○— $(\text{NH}_4)_2\text{SO}_4$ —□— NH_4NO_3

80 μmoles of guanine to the medium gave the maximal growth. On the other hand, adenine, hypoxanthine, xanthine, the corresponding ribosides and ribotides failed to support the growth of the organism.

FIG. 3. Effect on XMP Accumulation by KY 9938 Strain of Nitrogen Sources in Fermentation Media.

Fermentation medium (Basal): Glucose 10.0%, yeast extract 1.0%, KH_2PO_4 0.2%, K_2HPO_4 0.2%, $\text{MgSO}_4 \cdot 7\text{aq}$ 0.1% and CaCO_3 2%.

Nitrogen sources were added to this basal medium, as indicated in Figure.

* When urea was used as a nitrogen source, CaCO_3 was not contained in the medium.

—○— $(\text{NH}_4)_2\text{SO}_4$ —×— Urea
—●— NH_4 -Acetate —△— NH_4Cl
—□— NH_4NO_3 —▲— $(\text{NH}_4)_2\text{HPO}_4$

Moreover, it could not grow on 2,6-diaminopurine, in contrast with previous findings that the guanine-auxotroph of *Aerobacter aerogenes*,³⁾ or *Ophistoma multianulatum*⁴⁾ is able to grow on 2,6-diaminopurine instead of guanine.

Inorganic Nitrogen Sources.

In attempt to find out media giving better yields of XMP, various inorganic nitrogenous compounds were used as nitrogen sources in the XMP fermentation.

As shown in Fig. 2, all of the nitrogen compounds tested were able to support the growth of the cells and especially ammonium sulfate gave the most abundant growth. The amounts of XMP accumulated on various concentrations of these compounds are shown in Fig. 3. From these results, ammonium sulfate was found to be the best nitrogen source not only for the accumulation of XMP but also for the growth.

Carbon Sources.

The organism could utilize glucose, sucrose, fructose maltose and mannose as carbon sources.

3) B. Magasanik and M.S. Brooke, *J. Biol. Chem.* **206**, 83 (1954).

4) N. Fries, *ibid.*, **200**, 325 (1953).

FIG. 4. Effects of Sterilization Method of Glucose on XMP Fermentation.

Fermentation medium: Glucose 10.0%, Yeast extract 1.0%, KH₂PO₄, 0.2%, K₂HPO₄ 0.2%, MgSO₄·7H₂O 0.1% and CaCO₃ 2.0%. Simultaneously: Glucose was sterilized simultaneously with the other components in the medium.

Separately: Glucose was sterilized separately from the other components in the medium.

Assayed for 6-day culture broth.

—○— Separately.
---●--- Simultaneously.

Although it grew more sufficiently on maltose than on the other sugars, the strain accumulated the highest level of XMP on glucose. It was further observed that the simultaneous sterilization of glucose with the other component in the medium reduced the yield of the product (Fig. 4). Therefore, glucose autoclaved separately was added to the media just prior to inoculation.

Inorganic Phosphate and Magnesium.

The levels of both inorganic phosphate and magnesium sulfate in the fermentation medium affected both the growth of the cells and XMP yields. (Table I). The high levels of monobasic, dibasic potassium phosphates and magnesium sulfate in the medium inhibited the growth of the cells. The most suitable levels of these salts for XMP production were decided to be approximately 0.2% of KH₂PO₄, 0.2% of K₂HPO₄ and 0.4% of MgSO₄·7H₂O.

Natural Nitrogen Sources.

The supplement to fermentation media of natural nitrogen sources such as peptone, casamino acids and NZ-amine stimulated XMP production by KY9938 culture. The results

TABLE I. EFFECT ON XMP FERMENTATION OF INORGANIC PHOSPHATE AND MAGNESIUM IN FERMENTATION MEDIUM

KH ₂ PO ₄ K ₂ HPO ₄ (each)	MgSO ₄ ·7H ₂ O %	4-day incubation		
		pH	DCW mg/ml	XMP mg/ml
0.1%	0.05	5.1	18.1	0.70
	0.1	5.0	18.0	1.17
	0.2	4.9	16.2	1.26
	0.4	5.0	17.6	1.29
	0.6	5.0	17.6	0.93
0.2%	0.05	4.8	15.1	1.41
	0.1	5.0	18.4	1.28
	0.2	4.9	11.0	1.40
	0.4	4.8	11.2	1.71
	0.6	4.8	11.8	1.29
0.4%	0.05	4.6	15.4	1.39
	0.1	4.6	13.9	1.32
	0.2	5.0	13.8	0.75
	0.4	4.8	9.4	0.66
	0.6	4.7	10.1	0.62
0.6%	0.05	4.8	8.3	0.78
	0.1	4.7	7.8	0.80
	0.2	4.8	8.1	0.73
	0.4	4.8	8.2	0.74
	0.6	4.9	7.0	0.59

Basal medium: Glucose 10.0%, (NH₄)₂SO₄ 2.0% yeast extract 1.0%, and CaCO₃ 2%.

KH₂PO₄, K₂HPO₄ and MgSO₄·7H₂O were added to this basal medium, as indicated in Table.

are shown in Fig. 5. Addition of NZ-amine or peptone (up to as much as 1.5%) resulted in more accumulation of XMP, whereas addition of casamino acids more than 0.5% decreased the yields.

Trace Elements.

Effects of trace elements on growth are shown in Fig. 6. Zn⁺⁺, Co⁺⁺, Cu⁺⁺ and Ni⁺⁺ inhibited remarkably growth of KY9938 strain at the level of 0.1%, while no inhibitory effect on growth was noted in media containing Fe⁺⁺, Mn⁺⁺ and Ba⁺⁺ at the same level. Obviously, the growth inhibition by the higher levels of trace elements resulted in lowering of XMP titres.

Effect of Guanine on XMP Accumulation.

It is wellknown that in various fermentations with biochemical auxotrophs, quantities of a

FIG. 5. Effect on XMP Production of Natural Nitrogen Sources.

Fermentation medium (Basal): Glucose 10.0%, yeast extract 1.0%, KH_2PO_4 0.2%, K_2HPO_4 0.2%, $\text{MgSO}_4 \cdot 7\text{aq.}$ 0.4% and CaCO_3 2%.

Natural nitrogen sources were added to this basal medium, as indicated in Figure. Assayed for 6-day culture broth.

—○— NZ-amine
- - -△- - Peptone
- - -●- - Casamino acids

FIG. 6. Effect of Trace Elements on Growth of KY 9938 Strain.

Fermentation medium (Basal): Glucose 10.0%, KH_2PO_4 0.2%, K_2HPO_4 0.2%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.4%, CaCO_3 2.0%, Yeast extract 1.0%, $(\text{NH}_4)_2\text{SO}_4$ 2.0% and NZ-amine 0.75%.

Trace elements were added to this medium, as indicated in Figure. Assayed for 6-day culture broth.

—□— $\text{BaCl}_2 \cdot 2\text{aq.}$ —▲— $\text{NiCl}_2 \cdot 6\text{aq.}$
—○— $\text{FeSO}_4 \cdot 7\text{aq.}$ —△— $\text{ZnSO}_4 \cdot 7\text{aq.}$
—×— $\text{MnSO}_4 \cdot 4\text{aq.}$ —●— $\text{CoCl}_2 \cdot 6\text{aq.}$
—◇— $\text{CuSO}_4 \cdot 5\text{aq.}$

required nutrient added to media exert a marked effect on the amounts of accumulated product. Effects of guanine required by KY9938 strain were examined on XMP fermentation. The results are summarized in Fig. 7 and Fig. 8.

FIG. 7. Effect of Guanine on Growth of KY 9978 Strain in Seed Medium.

Seed medium: Glucose 2.0%, yeast extract 1.0%, peptone 1.0%, and NaCl 0.25%.

Guanine was added to this medium, as indicated in Figure. Assayed for 24-hour culture.

FIG. 8. Effect of Guanine in Media on XMP Accumulation by KY9978 Strain.

Seed medium: Glucose 2.0%, Yeast extract 1.0%, peptone 1.0% and NaCl 0.25%

Fermentation medium: Glucose 10.0%, KH_2PO_4 0.2%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.4%, yeast extract 1.0%, $(\text{NH}_4)_2\text{SO}_4$ 2.0% and CaCO_3 2.0%. Guanine was added to this medium, as indicated in Figure.

Assayed for 6-day culture broth.

Guanine in seed medium

(1) 0 γ/ml
(2) 10 γ/ml
(3) 50 γ/ml
(4) 100 γ/ml

Growth on the seed medium was more stimulated by further increment of guanine concentration up to about 50 γ per ml of medium (Fig.

7). The seed cultures grown on media containing various concentration of guanine was inoculated to fermentation media containing various levels of guanine up to 50 γ per ml.

Excessive addition of guanine to fermentation media suppressed the accumulation of XMP and the presence of guanine in seed media also reduced the yield of the product appreciably.

The growth stimulatory effect of guanine in fermentation media was not clear at the final stage of the fermentation, since the media comprised 1.0% of yeast extract which contained guanine compounds. At an early stage of the fermentation, however, guanine addition evidently stimulated the growth.

The Chemical Changes in XMP Fermentation.

As stated above, the medium most suitable for accumulation of XMP was established with

KY9938 strain. The developed medium consists of 10% of glucose, 2.0% of $(\text{NH}_4)_2\text{SO}_4$, 0.2% of KH_2PO_4 , 0.4% of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0% of yeast extract, 0.5% of peptone and 2.0% of CaCO_3 . Typical chemical changes in XMP fermentation on the medium are given in Fig. 9. The time course shows that XMP began to accumulate after 1-day, and reached its maximal yields around 5-days. Glucose was consumed completely after 5-days and the cells were increased gradually. A small amount of xanthine was accumulated at the earlier stage, but decreased at the later stage. Since pH of the medium was not controlled, it was adjusted to 4.8 at 2 days' incubation, but increased gradually after 5 days. The maximal amount of XMP accumulated was 2.75 mg per ml of medium.

FIG. 9. A Typical Chemical Change in XMP Fermentation.

Seed medium: Glucose 2.0%, yeast extract 1.0%, peptone 1.0% and NaCl 0.25%.

Fermentation medium: Glucose 10.0%, $(\text{NH}_4)_2\text{SO}_4$ 2.0%, yeast extract 1.0%, peptone 0.5%, KH_2PO_4 0.2%, K_2HPO_4 0.2%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.4% and CaCO_3 2.0%.

—○— XMP, —△— D.C.W., —x— pH, —●— Glucose, —○— Xanthine

5) K. Ogata, *Amino Acids and Nucleic Acid*, **8**, 1 (1963).

6) S. Kinoshita, *J. Ferm. Assoc. Japan*, **22**, 101 (1964).

7) K. Nakayama, T. Suzuki, Z. Sato and S. Kinoshita, Oral Presentation at Symposium on Amino Acid and Nucleic Acid Fermentation, Kyoto, Nov., 1963.

DISCUSSION

To the end of producing compounds related to nucleic acid, such as 5'-inosinic, guanylic and

xanthylic acids, many investigations^{5,6)} have recently been focused on microbial accumulation of these compounds, which have been used for seasonings.

However, the production of XMP by fermentation processes has not yet been reported. Nakayama et al.⁷⁾ in our laboratory have recently reported the accumulation of 5'-inosinic acid by adenine-requiring mutants of *Micrococcus glutamicus*.

Under such circumstances, we found that guanine or guanine-adenine requiring mutants of *M. glutamicus* accumulated a large amount of 5'-XMP. Such a marked excretion of XMP might be ascribed to a genetic blocking of XMP aminase, responsible for the conversion of XMP to GMP, as already reported by Magasanik³⁾

The facts that the guanineless mutant of *Aerobacter aerogenes*³⁾ accumulates xanthosine, whereas those of *M. glutamicus* produce XMP are speculated to reflect significant differences of phosphatase activities between both cultures as reported previously.⁸⁾

One of the guanine-requiring mutants of *M. glutamicus*, KY9978 strain, was selected as the best strain for XMP production. Guanine supported the most abundant growth of the strain as compared with the corresponding nucleotide and nucleoside. It is therefore suggested that guanine may be more permeable into the cells than other derivatives or that the activities of

cleavable enzymes of nucleotide or nucleoside, such as phosphatase, nucleosidase and so on are lower in this culture.

It has been demonstrated that the guanineless mutants of *Aerobacter aerogenes*³⁾, *Salmonella typhimurium*⁹⁾ or *Ophiostoma multianulatum*⁴⁾ are able to grow on 2,6-diaminopurine, instead of guanine, whereas the KY 9978 strain cannot grow on the compound. This finding indicates that 2,6-diaminopurine may not be converted to guanine in our strain.

Of particular importance in this fermentation is a fact that an excess addition of guanine to media inhibited the accumulation of XMP. This phenomenon could be accounted for by "negative feed back mechanisms" involved in the biosynthesis of purine nucleotide. In particular, it is of extreme interest regarding XMP biosynthesis that inosinic acid dehydrogenase in *Enterobacteriaceae*¹⁰⁾ involved in the conversion of inosinic acid to XMP is repressed by guanine.

At any rate, possible operation of such control mechanisms in KY 9978 culture is only speculative.

Further enzymatic approaches must be awaited to dissolve the reason why guanine suppresses the XMP accumulation by *M. glutamicus* KY 9978 strain. Experiments along such lines are now in progress.

9) G.P. Kalle and J. Gots, *J. Bact* **81**, 331 (1961).

10) A.P. Levin and B. Magasanik, *J. Biol. Chem.* **236**, 184 (1961).

8) M. Misawa, T. Nara and K. Nakayama, *J. Agr. Chem. Soc. (Japan)*, **38**, 167 (1964).