STUDIES ON INOSINE FERMENTATION — PRODUCTION OF INOSINE BY MUTANTS OF *BACILLUS SUBTILIS*

III. EFFECT OF CULTURAL CONDITIONS

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As reported previously, the authors obtained various inosine-producing mutants from *Bacillus subtilis* No. 2 which was an adenine auxotroph (1). Investigations were further made on the nutrient requirement of these mutants (2), and it was found that the inosine production was markedly accelerated by the addition of dry yeast or yeast extract to the culture medium.

In the present study the effects on inosine productivity of pH and aeration of culture were investigated, and by adopting a condition most favorable for the fermentation the time course of inosine formation in relation to the consumption of carbon source (glucose) was followed in detail.

MATERIALS AND METHODS

Mutant strains. The effects of pH and aeration were investigated using strain B-4 (adenine-histidine double less) as the test organism, for which the composition of the medium favorable for inosine production has been already established (2). The time course of fermentation was studied using strain C-30 (adenine-histidine-tyrosine less), a most potent inosine producer which had been obtained from strain B-4 by X-ray irradiation (1).

Media. The fermentation medium used in the experiment of aeration had the following composition: starch hydrolysate (10 % as glucose), 2 % NH_4Cl , 1 % yeast extract, 3 ml cornsteep liquor/liter, 0.1 % casein hydrolysate, 0.01 % sodium ribonucleate, 0.8 % KH_2PO_4 , 0.04 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 ppm Fe^{++} , 2 ppm Mn^{++} and 2 % CaCO_3 . The pH was adjusted to 7.0. The pH of the medium was varied by the use of various pH-controlling agents. In the experiments in which the time course of fermentation was followed, a medium of the following composition was used; starch hydrolysate (6 % as glucose), 2.6 % NH_4Cl , 1.4 % dry yeast (produced by Tōyō-Bōseki Co.) 2 ml cornsteep liquor/liter, 4 ml *Mi-eki*/liter, 0.1 % KH_2PO_4 , 2 ppm Fe^{++} , 2 ppm Mn^{++} and 2 % CaCO_3 . The pH was adjusted to 7.0. Each medium was sterilized at 120° for 15 min.

Fermentation procedure. The fermentation experiments were carried

out as follows unless otherwise indicated. Precultivated cells of the test strain were inoculated into 20 ml of the medium described above at the concentration of ca. 5×10^7 cells/ml, and cultivation was carried out at 30° using a 500 ml flask on a shaking machine. At intervals aliquots were withdrawn from the culture and subjected to analysis. Glucose was assayed by the method of FEHLING-LEHMAN-SCHOORL, and inosine and hypoxanthine were assayed by the method described in the previous paper (1).

RESULTS

Effect of aeration

When mutant cells of *B. subtilis*, such as strain B-4, are inoculated and cultivated in the fermentation medium, a small amount of inosine was produced unless a large quantity of oxygen was supplied to the culture medium. When cultivation was carried out under static condition without shaking, the cells grew forming a surface mat characteristic of *B. subtilis* and inosine production occurred only negligibly. A large amount of inosine could be accumulated only under a sufficient supply of oxygen such as realized in the shaking culture. To investigate the relationship between the oxygen supply and inosine productivity, the following experiment was designed: 20, 40, 80 and 160 ml of the media which were inoculated at the same concen-

Fig. 1. Effect of aeration on inosine productivity of strain B-4.

Kd value was calculated by the equation, $Kd = (O_2)/p \cdot v \cdot t$, where (O_2) , g-mole of O_2 supplied to the culture medium; p, atmospheric pressure; v, volume (ml) of the culture medium; t, time (min).

tration of cells were poured into 500 ml flasks and fermentation was carried out on a shaking machine. As far as the flasks used have the same size and form and the shaking is done under the same condition, the smaller the volume of the culture medium contained in the flask, the higher the O_2 supply to the culture. After 70 hr of cultivation at 30° , the amount of inosine produced in each flask was estimated. Fig. 1 shows a typical result obtained in such experiment. In the flask containing 20 ml medium, the K_d value (β), that is "oxygen absorption rate" as defined in the legend of Fig. 1, was 7×10^{-6} and inosine was produced in an amount of 6.5 g/liter. In the flask containing 160 ml medium, the K_d value was 3.3×10^{-6} , and the amount of inosine was only 1.6 g/liter. It was thus clearly demonstrated that the sufficient supply of oxygen is a very important factor for the accumulation of inosine in the culture.

Effect of pH

To investigate the effect of pH on the inosine productivity a series of culture were run, whose pH-values were maintained at different levels by feeding appropriate amounts of phosphoric acid or ammonia throughout the whole course of fermentation. In the experiment reproduced in Fig. 2, the pH was maintained at 3, 4, 5, 6, 7, 8 and 9 during 18 hr of cultivation. The variation of pH value in each run was within the range of ± 0.5 . As a control experiment, 2% of calcium carbonate was initially added to the culture medium and cultivation was carried out without any pH-regulation. As may be seen, the optimum pH for the inosine production was in the

Fig. 2. Effect of pH on inosine productivity of strain B-4.

The width of each column indicates the pH range maintained during the cultivation. The column drawn by dotted line indicates the result of experiment in which 2% of $CaCO_3$ was added to the medium initially (see text).

range of 5-7, and there occurred almost no production at pHs lower than 4 and higher than 9. In the control experiment, the initial addition of calcium carbonate maintained the pH in the optimum region (5.0-6.7) throughout the whole course of fermentation, and as the result, the inosine was produced to the maximum degree.

Time course of fermentation

By using strain C-30, which is the most potent inosine producer among the mutants thus far isolated, investigation was made of the relation between the consumption of glucose and the production of inosine during the course of fermentation. (For the arrangement of experiment, see *Materials and Methods*). The initial concentration of glucose (supplied as starch hydrolysate) was fixed at 6%. pH control was done merely by the initial addition of 2% of calcium carbonate, which maintained the pH value in the optimum range, 5-6, during the course of the experiment. The results obtained are summarized in Fig. 3. The formation of inosine began after 10 hr of cultivation, its velocity was the highest at 30-50 hr, and the highest level of accumula-

Fig. 3. Time course of inosine fermentation by strain C-30.

●: inosine, ▲: hypoxanthine,
○: glucose, △: pH.

tion was attained at 70–90 hr of cultivation. During the fermentation, glucose was consumed gradually and decreased to less than one fifth of the initial concentration after 80 hr lapse of time when the maximum accumulation of inosine was observed. The maximum level of inosine production was 7 g/liter of the culture medium and, in terms of molar yield per glucose consumed, it was 9.4%. No detectable amount of hypoxanthine appeared throughout the whole course of fermentation. Further experiments indicated that the increase of glucose concentration from 6% to 15% caused an increase in the absolute yield of inosine in the culture medium, but it apparently caused a decline of the yield against the amount of glucose consumed.

DISCUSSION

The industrial methods for preparing the compounds related to nucleic acids by using microorganisms can be generally classified as follows:

- (a) enzymatic cleavage of nucleic acids (especially RNA or DNA),
- (b) enzymatic (or metabolic) interconversion among the relevant compounds, and
- (c) extracellular accumulation caused by *de novo* synthesis.

As for the category (a), extensive studies have been done, and some of them have already been applied to the industrial production on a large scale (4). In this method, the prerequisite is the preparation of nucleic acids (RNA or DNA) from organisms as the starting material, and the primary products obtained are, in general, mixtures of various nucleotidic compounds. In the case of the category (b), for which several interesting methods have been reported (5), specific compounds must be prepared, in some way or another, as the starting material. In view of such an angle, the procedure of the category (c) seems to be the most promising, provided that the means could be found, by artificial mutation or adoption of specific cultural conditions, to steer the microbial metabolism to the direction of producing useful compound from simple carbon, nitrogen (and phosphate) sources. Trials along this line have been, and being consistently, made by a number of workers (6–7), although their efforts have not yet been met with success on the commercial basis.

Our finding of the mutants producing a large amount of inosine as a sole product indicates a possibility of industrial production of a compound related to nucleic acids by a method based on the category (c), in which inosine is produced using glucose as the main substrate. Since the strains used in the present study were all mutants derived from an adenine auxotroph, the substances accumulated by them must have been the metabolic precursors in the pathway of biosynthesis of 5'-adenylic acid (cf. 8–10). That the accumulation of inosine observed in the present study was due to its *de novo* synthesis occurring in this way was evidenced by our observations

that: (i) in the synthetic medium, the inosine accumulation was suppressed in the presence of excess adenine (2), and (ii) inosine formation occurred in parallel with the consumption of glucose.

SUMMARY

Using two typical inosine-producing mutants of *Bacillus subtilis*, the effect on inosine fermentation of cultural conditions as well as the time course of inosine formation was investigated.

1. Sufficient supply of oxygen was requisite for the production of a large amount of inosine.

2. The optimum pH for inosine production was in the range of 5-7.

3. The inosine formation began at 10th hr of cultivation, and was at the highest rate at 30th-50th hr. At 70th-90th hr, the accumulation attained to the highest level, *i.e.*, 7 g/liter of the culture medium, which contained initially 6 % glucose.

4. The inosine formation proceeded in parallel with the consumption of glucose.

5. Methods for preparing a single compound related to nucleic acids on a large scale was discussed.

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