Microbial Degradation of Nitrosamines—II

Effect of the Conditions of Growth and Enzymic Reaction on the Nitrosamine Breakdown^{*1}

Katsuhiko HARADA^{*2}

(Received January 28, 1980)

The effect of growth conditions of *Rhizopus oryzae* and enzymic reaction conditions in the cell-free extract on the degradation of NDMA and NDPA has been investigated.

Of the growth conditions, the degradation activities in the growing cells were highest when the strains incubated for one day were inoculated into the potato-malt medium of pH 8.0 containing either NDMA or NDPA. Optimal temperature and nitrosamine concentration for the NDMA degradation were different from those for the NDPA degradation, i.e., for both NDMA and NDPA degradation they were 0.03–0.12 and 0.1 mM respectively; while those for the nitrosamine degradation were 20°C and 30°C respectively. No marked difference was found in the activities of the stationary and shaking cultures. The cell-free extract of 3 and 4 days growing-cells which were precultured under the optimal conditions obtained were maximally active in degrading NDPA and NDMA respectively.

Of the enzymic reaction conditions, the degradation activities in the cell-free extract were highest at temperature 30°C and pH 8.0 under anaerobic condition. The nitrosamine degradation was shown to exhibit a concentration dependence different from the relation found in the growth conditions. Namely, the degradation activities were high in the concentration of more than 0.1 mM.

In the previous paper¹⁾, it has been shown by using three microorganisms, i.e., *Rhizopus oryzae*, *Streptococcus cremoris*, and *Saccharomyces rouxii* that NDPA was degraded faster in the suspensions of cells precultured in the nitrosamine-containing medium than in the control cultures, i.e., the suspensions of cells harvested from the nitrosamine-free medium, and the cell-free extract of the precultured cells of *Rhizopus oryzae* was active in degrading NDPA. These findings indicated that an inducible enzyme in *Rhizopus oryzae* might be involved in the breakdown of nitrosamine.

The present study was designed to investigate firstly the effect of the basal culture conditions on the degradation of both NDMA and NDPA in order to increase the production of the enzyme in the moulds, and secondly the effect of the enzymic reaction conditions of the cell-free extract of precultured cells.

(IFO 4705) and by the cell-free extract of precultured cells were performed according to the previous paper¹⁾.

The experiments were carried out successively in various conditions such as stock cultures, stationary or shaking culture, pHs, media, temperatures and nitrosamine concentrations to acquire firstly the basal culture conditions on the nitrosamine breakdown and also anaerobic or aerobic, temperatures, pHs and nitrosamine concentrations to get secondly the enzymic reaction conditions of cell-free extract.

To find the optimal condition of each factor, the optimal condition of factors was chosen one by one according to the above mentioned order, the preceeding factors were kept at the combination of their optimal conditions obtained and the next optimal condition of the factor under consideration was sought.

Experimental

The degradation and assay of both NDMA and NDPA by the growing cells of *Rhizopus oryzae*

Results and Discussion

Effect of Different Culture Conditions on the Degradation of Nitrosamine by Growing Cells

^{*1} Contribution from the Shimonoseki University of Fisheries, No. 870.

^{*2} Lab. Biochem., Dept. Food Sci. Technol., Shimonoseki Univ. Fish., Shimonoseki 759-65 (原田勝彦: 水産大学校).

The following abbreviations are used: NDMA, nitrosodimethylamine; NDPA, nitrosodipropylamine.

The degradation activities in the growing cells were, first of all, examined at 30°C in the potato-malt medium containing 0.1 mM nitrosamine, using one loopful of the three stock cultures, i.e., the strains incubated for one, three and six days which are presumed to be the lag, the log and the stationary phases respectively. The results obtained are shown in the upper left of Fig. 1. It should be noted that both NDMA and NDPA degradation activities were highest in the medium in which the

strains incubated for one day were used. The activities at eight days of incubation, however, were at similar levels regardless of the different stock cultures used. These findings show that young moulds are suitable for the degradation of nitrosamines. The activities of NDMA degradation increased gradually with the incubation days, but those of NDPA reached to maximum at two days of incubation and subsequently decreased apparently. This fact may be due to the cause that

Fig. 1. Effect of different culture conditions on the breakdown of nitrosamine.

The open and closed symbols show NDPA and NDMA, while the circles, triangles and squares indicate the nitrosamine degradation in the case where was used one day-, three days- and six days-stock cultures respectively.

The alphabets, a, b, c, d, e, f and g show potato-malt (2: 1), potato, malt, Sabouraud, candida GE, corn meal and mycobiotic media respectively.

NDPA disappeared more easily than NDMA because of their volatility. All the figures of Fig. 1 supports this fact.

The activities in the stationary and the shaking cultures are shown in the upper middle of Fig. 1. Neither activity of NDMA and NDPA degradation was any significant difference under the two conditions used. From this result, it is conceivable that the nitrosamine degradation activity is free from the quantity of oxygen.

The activities in the seven media are shown in the upper right of Fig. 1. In this figure, the nitrosamine degradation is recognized in five media except corn meal and mycobiotic media. These media are potato-malt, potato, malt, Sabouraud and candida GE. Although the growth extent of moulds in each medium are not presented. The dry weight after two days is highest by 65.4–78.7 mg in candida GE medium, lowest by 0.1–0.4 mg in corn meal and by 1.0–1.4 mg in mycobiotic media, intermediate by 17.2–21.3 mg in potato-malt, by 16.2–18.7 mg in potato, by 17.1–18.3 mg in malt and by 15.9–31.0 mg in Sabouraud media. Among these media which are effective for the nitrosamine degradation, potato-malt medium which showed intermediate growth was of the most activity, while candida GE medium which showed high growth was not always of high activity. This shows the possibility that the degradation correlates to the cofactors which participate in the enzymic breakdown of nitrosamine.

The activities at different temperatures are shown in the lower left of Fig. 1. The activities in degrading both NDMA and NDPA were highest in the growing cells incubated at 20°C and 30°C respectively. The activities at other temperatures, however, varied more or less among the growing cells incubated at each temperature.

The results of the effect of different pHs on nitrosamine degradation are shown in the lower middle of Fig. 1. The activities of both NDMA and NDPA degradation were highest in the growing cells cultured at pH 8.0. ROWLAND *et al.*²⁾ found pH 7.0 to be optimal for the degradation of NDMA and nitrosodiphenylamine in five groups of resting bacteria isolated from the gastrointestinal of rat. It is conceivable from these results that the degradation of nitrosamines by microorganisms may be conducted at near neutral pH.

The activities at different nitrosamine concentrations are presented in the lower right of Fig. 1. NDMA degradation was shown to exhibit a broad concentration dependence, that is, the activities

were high between 0.03 and 0.12 mM of nitrosamine concentration. On the contrary, NDPA degradation was shown to exhibit a narrow concentration dependence, that is, the activities was highest at near 0.1 mM of nitrosamine concentration. These results did not coincide with the result of ROWLAND *et al.*²⁾ that the degradation proportion of NDMA and nitrosodiphenylamine increases at low concentration less than 1 mM. This difference may probably be caused by the strains used. The activity of NDPA degradation was about two times more than that of NDMA regardless of the concentration of the nitrosamine used. This finding may correlate with the relative rates of the formation of both nitrosamines, dipropylamine being nitrosated far more readily than dimethylamine³⁾.

Fig. 2. Effect of incubation days on the activity of enzyme degrading nitrosamine.

Symbols are legend as Fig. 1.

In order to examine how many days of incubation are needed to produce the maximum amount of nitrosamine degrading enzyme under the optimal conditions described above, the activities of nitrosamine degradation were examined in the cell-free extract prepared at each incubation days, 2–6 days. The results are shown in Fig. 2. The activities of NDMA and NDPA degradation were highest in the cell-free extract of the growing cells precultured for 4 and 3 days respectively. These data suggest that the enzyme for nitrosamine degradation will be produced adaptively in the moulds which are precultured with the nitrosamine as described in the previous paper¹⁾.

Effect of Different Enzymic Reaction Conditions on the Degradation of Nitrosamine by Cell-free Extract

The activities of nitrosamine degradation in the cell-free extract were, first of all, examined at 30°C in the Tris buffer of pH 8.0 containing 0.1 mM

Fig. 3. Effect of different enzymic reaction conditions on the breakdown of nitrosamine. Symbols are legend as Fig. 1.

nitrosamine. The results obtained are presented in Fig. 3. Both NDMA and NDPA were degraded anaerobically by the cell-free extract, as opposed aerobically. The activities of both NDMA breakdown in the anaerobic were about two times more than that in the aerobic. This finding shows that the molecule oxygen might not participate in the enzymic degradation of nitrosamine. Optimal temperature and pH in the enzymic degradation were 30°C and 8.0 respectively for both nitrosamines. The activities of enzymic degradation at different concentrations were high in the concen-

tration more than 0.1 mM. This reflects that the enzymic degradation is dependent on nitrosamine concentration.

References

- 1) K. HARADA and K. YAMADA: *Bull. Japan. Soc. Sci. Fish.*, **45**, 925-928 (1979).
- 2) I. R. ROWLAND and P. GRASSO: *Appl. Microbiol.*, **29**, 7-12 (1975).
- 3) F. ENDER and L. CEH: *Z. Lebensmittel-Unters. Forsch.*, **145**, 133-142 (1971).