STEPWISE ENHANCEMENT OF PRODUCTIVITY OF THERMOSTABLE AMYLASE IN *BACILLUS LICHENIFORMIS* BY A SERIES OF MUTATIONS¹

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Methods for increasing the productivity of thermostable amylase of *Bacillus licheniformis* were investigated. Cells of *B. licheniformis* NYK 74 were treated with N-methyl-N'-nitro-N-nitrosoguanidine resulting in mutants which were resistant to D-cycloserine. These were selected first and among them amylase hyper producers were selected. By a series of the same treatments, except that the concentration of D-cycloserine was increased step by step, a strain was developed which produced 2,000 times more amylase than the original one.

In previous paper (1-8) we have reported that in *Bacillus subtilis* 6160, a mutant derived from *B. subtilis* 168, it was possible to increase the α-amylase productivity by about three thousand times using transformations and mutations. D-Cycloserine was found to be effective in developing α-amylase hyper producers (7). Though there are many studies on the thermostable amylase of *B. licheniformis* (9), genetic improvement of its amylase productivity more than several hundred times has seldom been reported. Using D-cycloserine-resistance as a guide mark, we have succeeded in increasing the productivity of thermostable amylase of *B. licheniformis* NYK 74 by about two thousand times by repeated mutations. Details of the processes are reported here.

MATERIALS AND METHODS

*Bacterial strain and media.* The original strain used in this experiment was *Bacillus licheniformis* NYK 74 which is a strain preserved in our laboratory producing only 1 unit of heat stable α-amylase.

Two kinds of media, referred to as BY and ABY, were used. Medium BY

ⁱ Abbreviation used in this text is as follows: NTG, N-methyl-N'-nitro-N-nitrosoguanidine.
contained, per liter: 5 g of meat extract, 10 g of peptone, 2 g of NaCl, and 2 g of yeast extract, while medium ABY had, per liter: 10 g of meat extract, 20 g of peptone, 4 g of NaCl, 4 g of yeast extract, and 100 g of soluble starch. BY was employed for general cultures, and ABY was used in the experiments testing the productivity of α-amylase.

The productivity of heat stable α-amylase was determined as follows: After the cells were cultured in medium ABY for an appropriate time, the amylase activity of the supernatant of the medium treated at 80°C for 10 min was measured. The assay method and amylase unit were as mentioned in previous papers (6, 10). Amylase units found in 1 ml of the medium after 72 hr of culture was taken as the measure of amylase productivity.

Mutagenic experiments were performed originally with B. licheniformis NYK 74 to obtain its derivatives. These are resistant to D-cycloserine, and have higher amylase productivity than the original strain. Bacterial cells were treated with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) in a concentration of 20 µg/ml for 30 min at 37°C. The resulting mutants were tested for their resistance to D-Cycloserine in a concentration of 60 µg/ml. These resistant mutants were tested for

![Graph showing the productivity test for NTG treated B. licheniformis TM4 cells.](image)

Fig. 1. Thermostable amylase productivity test for NTG treated B. licheniformis TM4 cells.

Cells were cultured in BY medium containing 1% soluble starch at 30°C for 72 hr. Amylase activities were measured as mentioned in the text. Symbols: ■, before heat treatment; □, after heat treatment (80°C, 10 min).
their productivity of extracellular heat stable amylase. Mutations were repeated, successively increasing the concentration of D-cycloserine.

RESULTS

In the first treatment of strain NYK 74 with NTG followed by screening, we obtained a mutant (AN 501) which produced 8 U/ml of amylase. After monocell selection, we obtained a stable strain (501TM4) producing 30 U/ml of heat stable amylase. In the next step, we treated strain 501TM4 with NTG and the resulting mutants were selected on plates containing 100 µg/ml of D-cycloserine. Amylase tests on a soluble-starch-agar plate were also made on D-cycloserine resistant mutants, and we obtained 34 strains which seemed to be promising. Small scale amylase productivity tests were made on these strains. The results of the tests are shown in Fig. 1. Among these strains, TM429 was the best, producing about 100 U/ml of heat stable amylase. After monocell selection of the strain TM429, we obtained strain TM4291 which produced about 160 U/ml of heat stable amylase. Then we treated strain TM4291 with NTG and selected the resulting D-cycloserine resistant mutants using plates containing 200 µg/ml of D-cycloserine. The resistant mutants thus obtained were examined for amylase productivity and we found strain (TMN11) producing 200 U/ml of heat stable amylase. Strain TMN11 was further treated with NTG and the resulting mutants
which were resistant to 300 μg/ml of D-cycloserine were selected. Among these resistant mutants, strains which showed amylase hyper productivity on starch-agar plates were subjected to culture test for amylase productivity. In this case, most of the strains produced heat labile amylase. Fortunately, one excellent strain, TMN1147, produced about 500 U/ml of heat stable amylase (Fig. 2).

After strain TMN1147 was exposed to NTG, mutants were selected on plates containing 350 μg/ml of D-cycloserine. Among the resistant mutants, we obtained a strain, MSN5, which produced 1,000 U/ml of amylase. Mono-cell selection of strain MSN5 resulted a stable strain SM5322 which was capable of consistently producing 1,500 U/ml of heat stable amylase.

As the last step, we treated SM5322 with NTG and selected mutants which were resistant to 400 μg/ml of D-cycloserine and were hyper producer of amylase. Among these strains SMN11 showed the highest productivity of heat stable amylase (about 2,000 U/ml). The profile of increase of amylase production in each step of mutation is shown in Fig. 3.

**DISCUSSION**

One of the authors (B.M) has succeeded in enhancing the α-amylase productivity of *B. subtilis* NA64 by a factor of 3,000 using DNA mediated transformation and mutation (1–8). Cells of *B. licheniformis* NYK74, however, do not
become competent to DNA and hence are not transformed by foreign chromosomal DNA. Therefore in this case we tried to apply only mutation to obtain a hyper producer of amylase.

As is seen in Fig. 3, not every step of mutation produced a large increase in amylase synthesis but the accumulation of many small steps resulted in a remarkable increment. This phenomenon suggests that there must be numerous factors which have strong relationships with the productivity of amylase as in the case of B. subtilis.

We selected mutants first by D-cycloserine and then by amylase productivity. This is because we have realized by experience that this method is more efficient than one with no pretreatment, though there seemed to be no direct relationship between these two phenomena. D-Cycloserine is reported to inhibit alanine racemase (E.C. 5.1.1.1) and D-alanine: D-alanine ligase (ADP) (E.C.6.3.2.4) (11). In addition D-cycloserine resistant mutants of Streptococcus had increased levels of alanine racemase (eight times) and D-alanine: D-alanine ligase (five times) (12). MATSUDA et al. reported that methyl viologen treated cells of Escherichia coli had higher levels of superoxide dismutase activity and were more resistant to D-cycloserine than untreated cells. They also found that D-cycloserine had some interaction with DNA (13). Correlation between these phenomena and increase in amylase productivity is now under investigation.

The process of secretion of an enzyme is considered to be very complicated one affected by not only the transcription and translation efficiency of the corresponding gene but also by many kinds of secretion mechanisms such as signal peptide, signal recognition protein (SRP) (14, 15), translocation systems in membrane, and probably by other yet unknown mechanisms. Mutation of any one of these should affect the synthesis, secretion and total accumulation of the enzyme. In the present case, though we do not know which mutation belongs to which mechanism, the fact that the final accumulation of amylase has increased step by step up to two thousand times clearly indicates that each mutation is playing a special role in the processes of amylase synthesis and secretion. Biochemical and genetical analyses of each of these mutations should reveal many new significant processes or mechanisms contributing to the enzyme accumulation.

The final mutant (SMN11) shows not much difference in general appearance from the original strain except that the growth rate of the former is a little slower than the latter.

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REFERENCES