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Continuous Culture Studies on the Biosynthesis of Alkaline Protease, Neutral Protease and α-Amylase by *Bacillus subtilis* NRRL-B3411

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SUMMARY

The amounts of three catabolite repressible enzymes, alkaline protease, neutral protease and α-amylase, produced by *Bacillus subtilis* NRRL-B3411 growing in a chemostat, depended on the growth-limiting substrate. Limiting growth with glucose was advantageous for α-amylase synthesis while nitrogen-limited growth was advantageous for synthesis of the two proteases. Under the conditions used, continuous cultures were unsuitable for large-scale production of the three enzymes since spontaneous mutations to less productive strains occurred in the long term.

INTRODUCTION

Continuous culture studies were initiated by us for two purposes. The first was to develop a better understanding of the regulatory mechanisms involved in the biosynthesis of alkaline protease, neutral protease and α-amylase by *Bacillus subtilis* NRRL-B3411. Such information is fundamental to understanding how to alter these mechanisms in order to provide a desired type of micro-organism. The second was to investigate the possibility of synthesizing these enzymes in a large-scale continuous culture. Decided investment advantages (Ellsworth, Telling & East, 1959) of continuous cultures are discussed in the literature as well as some disadvantages (Butterworth, 1967).

A number of publications have appeared in recent years on the synthesis of exoenzymes, many of which are reviewed by Schaeffer (1969). Coleman (1967) reported that amylase and proteases of *Bacillus subtilis* are synthesized in the stationary phase of growth and appear simultaneously and in parallel fashion. Mandelstam (1967) postulates the concept of a catabolite repressing only the enzymes directly or indirectly producing it. We believe we have data which will help to clarify these concepts even further.

METHODS

Organism. The micro-organism used was *Bacillus subtilis* NRRL-B3411.

Media. These consisted of a basic salt solution plus various carbohydrate and nitrogen sources. The basic salt solution contained (per l of distilled water): $FeSO_4.7H_2O$, 0·01 g; $ZnCl_2$, 0·02 g; $MnCl_2.4H_2O$, 0·05 g; $MgSO_4$, 0·50 g; $CaCl_2.2H_2O$, 0·50 g; K^+, 0·02 M; PO_4^{3-}, 0·02 M; and EDTA, 0·001 M. This salts composition is a slightly modified version of that used by Schaeffer, Millet & Aubert (1965) in their study on the sporulation of *Bacillus megaterium*.

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These modifications resulted from calculations made from the salt composition data for *B. subtilis* given by Murrell (1967). Calcium was added to stabilize the enzymes (Keay & Wildi, 1970; Keay, Moser & Wildi, 1970; Moseley & Keay, 1970). EDTA was added to prevent salt precipitation at the fermentation pH.

All salts were reagent-grade chemicals. Acid-hydrolysed casein and monosodium glutamate were obtained from Sigma Chemical Company, St Louis, Missouri, U.S.A. Vitamin-free casein was obtained from Fisher Scientific Co. (C-203), St Louis, Missouri, U.S.A.

Media for the chemostat were sterilized by filtration through a 0·22 μm Millipore filter. For the experiments with casein, a 10 % (w/v) solution was prepared and neutralized to pH 7·0 with 10 M-KOH before being autoclaved for 30 min at 121 °C. This solution was then diluted to 2 % with filter-sterilized salts solution. All chemostat media were stored in 10 l aspirator bottles. Shake-flask and Petri-dish media were steam-sterilized at 121 °C for 15 min.

Fermentation equipment and conditions. Flasks (500 ml) containing 100 ml of medium were inoculated from lyophilized spore cultures of *Bacillus subtilis* NRRL-B3411 and shaken at 37 °C. Yeast extract (0·2 %) was added to diluted (5:1) chemostat medium to aid spore germination. After full growth was obtained, the shake-flask cultures were added to the chemostat culture vessel (10 % was a typical inoculum).

Shake-flask fermentations were carried out in a New Brunswick Incubator Shaker Model G25 at 37 °C with a gyration speed of 250 rev./min and eccentricity 1 in.

Chemostat fermentations were carried out in a Model MA02F1 2 l continuous culture vessel from Fermentation Design, Allentown, Pennsylvania., U.S.A. The pH value was measured and controlled at 6·3 ± 0·1 by a Fermentation Design pH control unit with an autoclavable Ingold pH electrode; 10 M-H_3PO_4 and 10 M-KOH were used for pH control. Temperature was controlled at 37 ± 0·5 °C. The culture volume varied from 1·0 to 1·2 l, depending on the experiment. Foam was controlled using Mazer DF-21C antifoam.

Turbidity. Turbidity measurements were made at 660 nm using a Beckman DU-2B spectrophotometer. Samples were diluted to give extinction readings in the range from 0·05 to 0·5 in a 1 cm quartz cell.

Bacterial dry weight. Measurements were made in quadruplicate and averaged. Samples were centrifuged for 10 min at 20000*g*, the supernatant fluid decanted and the pellets placed in preweighed pans. The pellets were dried at 105 °C for 24 h to constant weight. The relationship between turbidity and bacterial dry weight was linear. An extinction of 10 corresponded to a bacterial dry weight of 4 mg/ml.

Growth-rate measurements. These were made during batch growth from semilog plots of turbidity versus time. Growth rates under continuous culture conditions were set by the dilution rate of the culture.

Enzyme assays. Whole culture samples for enzymatic assays were frozen in dry ice immediately after sampling and stored at −20 °C. Just before assay, samples were thawed, diluted and stored in an ice bath. Dilutions were carried out with 0·01 % calcium acetate.

Neutral and alkaline protease activities were determined as caseinase activities at pH values of 7·0 and 10·0, respectively. Keay (1971) shows pH profile data which allow the activities of the two enzymes to be distinguished.

Most of the caseinase activity measurements were carried out on a Technicon autoanalyser with reagents as specified for the standard assay (Keay & Wildi, 1970). Extinction was measured at 660 nm after treatment with Folin phenol reagent. Solutions of known protease activity were used to calibrate the autoanalyser at pH of 7 and 10. Blanks were run with each assay by use of dual channels.

α-Amylase assays also were carried out on an autoanalyser. Colour reduction of potassium ferricyanide was measured and compared to values obtained from α-amylase standards. Units were based on the dinitrosalicyclic acid assay method (Moseley & Keay, 1970). Again, blanks were run with each assay.

Glucose assays. A glucose Tes-Tape[R] supplied by Eli Lilly, Indianapolis, Indiana, U.S.A., was used which gave qualitative results specific for glucose. More quantitative results were obtained when desired by use of Glucostat Special[R] supplied by Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.

Ammonia assays. The urea–nitrogen assay was used (Chaney & Marbach, 1962) with reagents from the Hyland Division of Travenol Laboratories, Los Angeles, California, U.S.A. The Hyland procedure was utilized.

Glutamate assays. Deproteinized supernatants of samples were assayed with the ninhydrin method (Greenstein & Winitz, 1961). Deproteinization was carried out by adding 4 ml of 0·4 M-trichloroacetic acid to each 1 ml of sample and centrifuging at 20000*g* for 10 min to remove suspended solids. The results were corrected by subtracting NH_3 values obtained with the urea–nitrogen assay.

Colony characterizations. Standard microbiological tests (Breed, Murray & Smith, 1957; Conn, Jennison & Weeks, 1957) were run on the variants obtained from the chemostat in the long-term experiments. Also, two phages specific for *Bacillus subtilis* NRRL-B3411 were used to check the variants. The standard phage overlay assay technique was used with Fisher J-1096-G phage assay base agar, Fisher J-1097-G phage assay overlay agar and Difco nutrient broth supplemented with 1 % dextrose.

Theoretical equations used to study mutation phenomena. In an attempt to understand the spontaneous mutation phenomena found in this study, a kinetic model was derived based on that of Moser (1958), although his presentation is simplified. It is assumed that the chemostat culture is in a steady-state and contains 100 % parent cells at zero time. Then consider that a small fraction of cells, λ, mutate during each generation. Kubitschek (1970) has recently shown that spontaneous mutations are a function only of the rate of DNA synthesis. Revertant frequencies are not important here as will be shown later. Then,

$$\frac{dS}{dt} = -\frac{1}{Y_1}\frac{\mu_1 S}{K_{m_1}+S}n_1 - \frac{1}{Y_2}\frac{\mu_2 S}{K_{m_2}+S}n_2 + D(S_0 - S) \tag{1}$$

$$\frac{dn_1}{dt} = \frac{\mu_1 S}{K_{m_1}+S}(n_1 - \lambda n_1) - Dn_1, \tag{2}$$

$$\frac{dn_2}{dt} = \frac{\mu_2 S}{K_{m_2}+S}n_2 + \frac{\mu_1 S}{K_{m_1}+S}\lambda n_1 - Dn_2. \tag{3}$$

where at

$$t = 0, \quad S = \frac{D}{\mu_1 - D}K_{m_1}, \quad n_1 = Y_1(S_0 - S), \quad n_2 = 0.$$

D is the dilution rate (h^{-1}); K_m, substrate saturation constant (mg/ml); n, bacterial dry weight (mg/ml); S, substrate concentration (mg/ml); t, time (h); Y, yield coefficient (g/g); λ, mutation rate (mutation frequency per generation); μ, maximal growth rate (h^{-1}); 1 refers to parent micro-organism and 2 refers to mutant micro-organism. The Monod model for growth is assumed to apply.

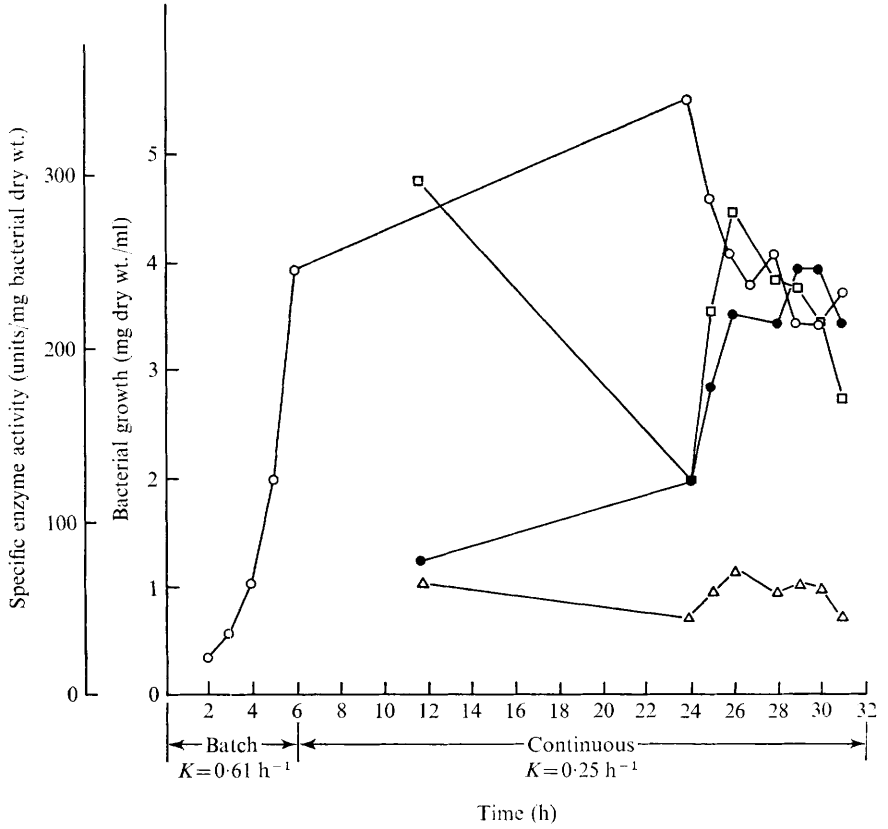


Fig. 1. Enzyme synthesis by *Bacillus subtilis* on glucose (1 %) and NH_4Cl (2 %) with glucose-limiting growth. Runs 1 and 5. ○, Growth; △, alkaline protease; □, neutral protease; ●, α-amylase.

RESULTS

When *Bacillus subtilis* NRRL-B3411 was grown on glucose (1 %) and NH_4Cl (2 %) with the standard salts solution, all three enzymes of interest were repressed during batch growth (Fig. 1). Under chemostatic control with glucose as the limiting nutrient, however, all three enzymes appeared when the growth rate was decreased to 0·25 h^{-1}.

In batch cultures with glucose (1 %) and acid hydrolysed casein (0·5 %), all three enzymes were repressed. Under chemostatic control at a growth rate of 0·3 h^{-1}, the two proteases appeared in good quantity whereas no detectable α-amylase was found (Fig. 2). Here, acid-hydrolysed casein was found to be growth-limiting.

During growth on monosodium glutamate (2 %) in batch cultures all three enzymes appeared during log growth. Under chemostatic control the enzyme levels were the same as in batch growth with glutamate serving as both a carbon and nitrogen source (Fig. 3). Carbon was limiting since ammonia was found in the culture during the first 86 h. At 86 h a switch to media containing glucose (1 %) and glutamate (2 %) was made. This caused a dramatic drop in all three enzyme levels at an equivalent growth rate. The two proteases reached non-detectable levels and the α-amylase was decreased considerably. Assays showed glucose to be limiting in the latter part of the run. The drop in enzyme levels was not a result of a change in population (Fig. 5).

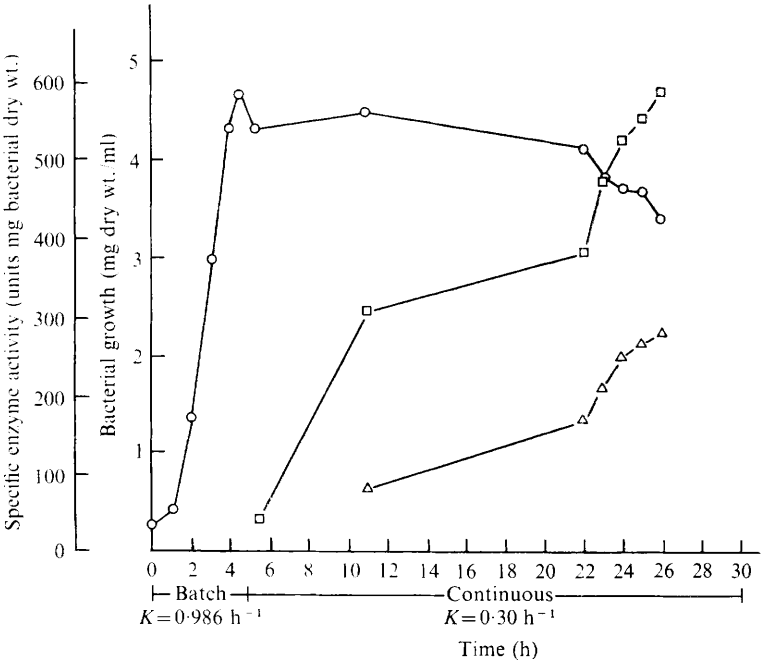


Fig. 2. Enzyme synthesis by *Bacillus subtilis* on glucose (1 %) and Casamino acids (0·5 %) with nitrogen limiting growth. Runs 2 and 6. ○, Growth; △, alkaline protease; □, neutral protease.

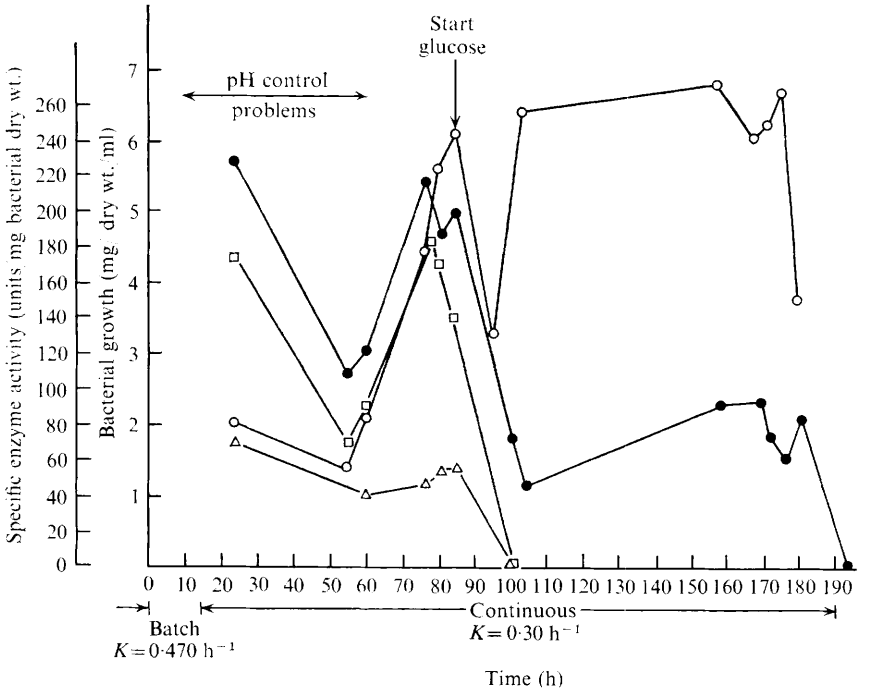


Fig. 3. Enzyme synthesis by *Bacillus subtilis* growing continuously on monosodium glutamate (2 %) and glucose (1 %) (after 86 h). Runs 7 and 7*a*. ○, Growth; △, alkaline protease; □, neutral protease; ●, α-amylase.

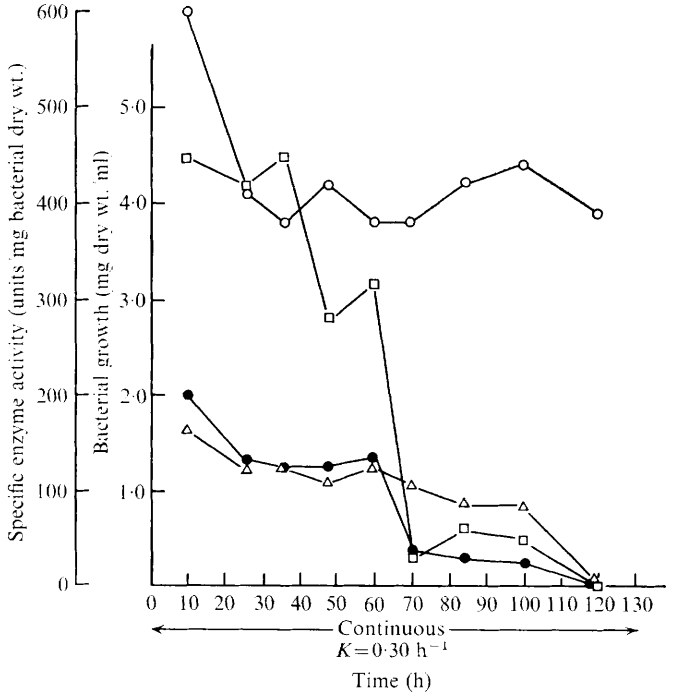


Fig. 4. Enzyme synthesis by *Bacillus subtilis* growing continuously on vitamin-free casein (2 %). Run 8. ○, Growth; △, alkaline protease; □, neutral protease; ●, α-amylase.

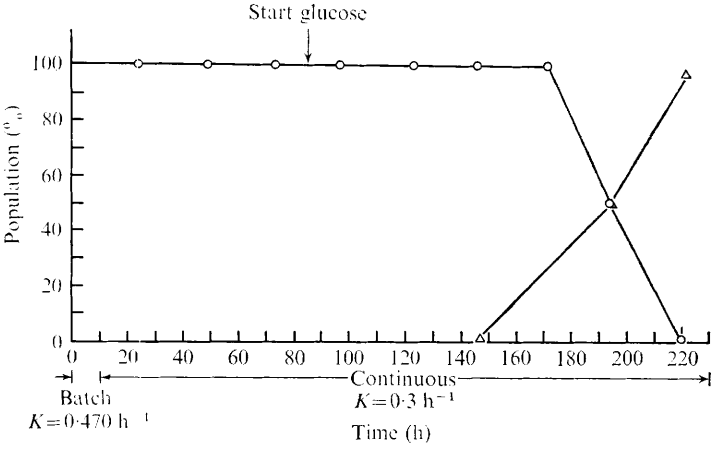


Fig. 5. Typical colony shift of *Bacillus subtilis* growing on monosodium glutamate (2 %) and glucose (1 %). ○, Parent or wild-type micro-organism; △, mutant micro-organism.

During growth on vitamin-free casein (2 %) in batch cultures, the three enzymes were again derepressed during the entire growth curve. In a chemostat with a growth rate of 0.30 h^{-1}, a precipitous drop in enzyme levels occurred at 60 h due to a less productive *Bacillus subtilis* NRRL-B3411 variant taking over the culture (Fig. 4). A takeover of continuous cultures by mutants occurred in other media; for example, after growth of the parent strain on glucose (1 %) and glutamate (2 %, w/v) for about 160 h (Fig. 5).

Theoretical calculations of colony shifts were made with assumed values for some of the

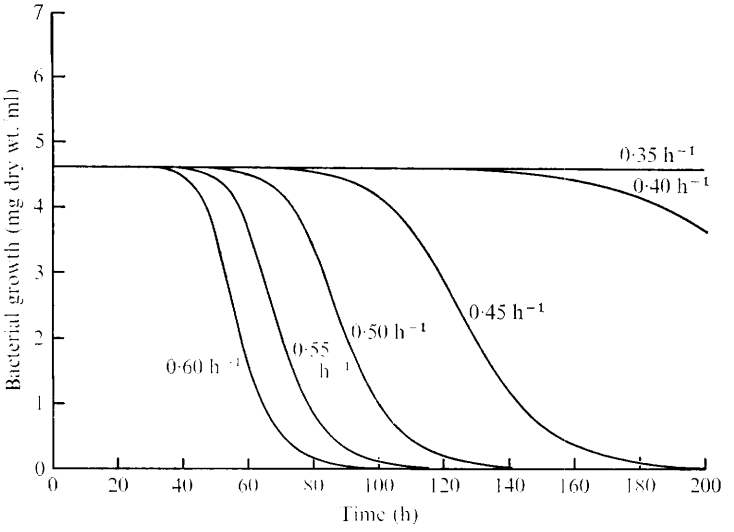


Fig. 6. Theoretical calculations (n_1) for colony shifts of *Bacillus subtilis* at various values of μ_2, the maximal growth rate of the mutant micro-organism.

parameters (Fig. 6). Values derived from our experiments were: Y_1 and Y_2, 0·33; S_0, initial substrate concentration, 20 mg/ml; D, 0.30 h^{-1}; K_{m_1}, 1·0 mg/ml; μ_1, 0.35 h^{-1}; K_{m_2} was assumed equal to K_{m_1}. The calculations were not very sensitive to changes in K_{m_2}. Almost equivalent results were obtained with values for this parameter of 2·0 or 0·5. A relatively high value of 10^{-5} was chosen for λ but values of 10^{-4} or 10^{-6} had little effect on the response curves (Fig. 6). These calculations were not affected by assuming similar values for a revertant mutation frequency. The parameter of greatest sensitivity was μ_2, the maximal growth rate of the mutant micro-organism. Differences of 0·1 to 0·25 h^{-1} in the growth rates between host and mutant micro-organism showed response curves very similar to our data. Such differences are not uncommon (Moser, 1958).

DISCUSSION

The experiments conducted in this study (Table 1) show that the regulation of α-amylase synthesis is different from that of the proteases. The α-amylase is severely repressed whenever glucose is in excess (runs 1, 2 and 6). Glucose repression of amylase synthesis was first demonstrated by Katz (1898).

Although amylase regulation differs from the regulation of the two proteases, the proteinases appear to be regulated co-ordinately and therefore possibly by the same effector molecule. When one protease was completely repressed so was the other (runs 1, 2 and 7*a*). When both were produced the ratio of the specific activities of the neutral to alkaline proteases ranged from 2·2 to 3·6.

Runs 1, 2 and 7 confirm previous observations made on protease syntheses by *Bacillus licheniformis* (Laishley & Bernlohr, 1966, 1968). *Bacillus subtilis* NRRL-B3411 growing at a rate of 0·61 h^{-1} on glucose-NH_4 salts repress protease synthesis completely. This repression is catabolic since it is relieved by growth on glutamate. The slower growth rate on glutamate (0·47 h^{-1}) may contribute to the derepression (Fabian, 1969). However, a more important factor is the nature of the limiting nutrient, as shown in run 7 where a switch to a glucose-limited medium completely repressed the proteases.

Table 1. *Specific enzyme activities from batch and continuous cultures of Bacillus subtilis*

Run	Mode	Growth rate (h^{-1})	Medium	Limiting C or N	Nature of limiting compound	Specific activities of enzymes (units/mg) α-Amylase	NP	AP	Ratio NP/AP
1	Batch	0·61	Glucose-NH_4	Neither	None	0	0	0	—
2		0·99	Glucose-Cas AAs			0	0	0	—
3		0·47	Glutamate (2 %)			200	140	65	2·2
4		0·33	Casein (2 %)			175	450	165	2·7
5	Continuous	0·25	Glucose-NH_4	C	Glucose	225	225	70	3·2
6		0·3	Glucose-Casamino acids	N	Cas-AAs	0	475	225	2·1
7		0·3	Glutamate (2 %)	C	Glutamate	200	180	50	3·6
7*a*		0·3	Glutamate (2 %) and glucose (1 %)	C	Glucose	25	0	0	0
8		0·3	Casein (2 %)	C	—	125	375	125	3·0

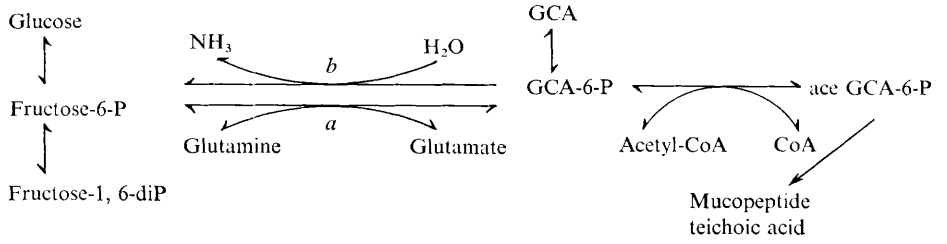
Bacillus protease synthesis is repressed catabolically and by amino acids (Chaloupka, 1969); although both glucose and amino acids each repress megateriopeptidase synthesis, both are required for complete repression and at low amino acid concentrations, glucose has little or no protease-repressing activity. We confirmed his findings (run 6). The best differential rate of synthesis of the two proteases occurred in the presence of glucose-Casamino acids during nitrogen-limited growth.

The dual requirement for a glucose catabolite and a nitrogen source for repression of protease synthesis is identical to the requirements for the repression of *Bacillus subtilis* tricarboxylic acid (TCA) enzymes (Hanson, Blicharska, Arnaud & Szulmajster, 1964). Since the proteases and a functional TCA cycle appear at about the same time during growth and sporulation of *B. subtilis*, all of these enzymes may well be under the control of the same effector molecule. Hanson *et al.* (1964) suggested that this molecule might be 'glutamate or a derivative of glutamate'. The following model suggests that glutamine plays a key role in regulating these enzymes.

While *Bacillus subtilis* NRRL-B3411 is growing on glucose–Casamino acids, it is carrying out the following reactions:

$$\text{glutamate} + NH_4^+ + \text{ATP} \longrightarrow \text{glutamine}.$$

Since this is a highly endothermic reaction, a molecule of ATP is expended for each molecule of glutamine synthesized. The glutamine, in turn, participates in the formation of amino-sugars (wall and membrane precursors; Bates & Pasternak, 1965).



a, Fructose-6-P-aminotransterase; *b*, glucosamine-6-P-deaminase (Freese, Cole, Klofat & Freese, 1970); GCA, D-glucosamine; GCA-6-P, D-glucosamine-6-P; ace GCA-6-P, *N*-acetyl-D-glucosamine-6-P

N-acetyl-D-glucosamine-6-phosphate produces a severe 'permanent catabolite repression' of β-galactosidase synthesis, which, in turn, is reversible by cyclic 3′,5′-adenosine monophosphate (C-AMP) (Goldenbaum, Broman & Dobrogosz, 1970). Our data are consistent with the idea that *N*-acetyl-D-glucosamine-6-phosphate is involved in the repression of the proteases and TCA cycle enzymes. Metabolic conditions favouring its synthesis (the availability of fructose-6-phosphate and glutamine) favour continued growth. Metabolic conditions favouring its degradation (relief of repression of the GCA-6-deaminase by exhaustion of glucose) (depletion of fructose-6-P or glutamine) favour derepression of the proteases, and TCA cycle enzymes; and the initiation of sporulation.

The large scale production of the three enzymes could not be achieved in continuous cultures under the conditions we used. The use of chemostats for this purpose depends on devising conditions under which the selective pressures inherent in a chemostat favour the continued existence of the enzyme producing micro-organism. The potential methods for doing this (e.g. feeding a peptide antibiotic which the wild-type digests but to which the protease deficient mutants are sensitive) are all thwarted by the extracellular nature of the enzymes. Conditions favouring the growth of the wild-type are equally favourable to the enzyme-less mutant, since the product of the enzyme is available to both. Perhaps by employing mutants unable to secrete the various enzymes, one might select hyperproductive strains from heterogeneous populations.

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REFERENCES

BATES, C. J. & PASTERNAK, C. A. (1965). Further studies on the regulation of amino sugar metabolism in *Bacillus subtilis*. *Biochemical Journal* **96**, 147–154.

BREED, R. S., MURRAY, E. G. D. & SMITH, N. R. (1957). *Bergey's Manual of Determinative Bacteriology*, 7th edn. Baltimore: Williams and Wilkins.

BUTTERWORTH, D. (1967). Factors determining the choice of batch or continuous fermentation. In *Biology and the Manufacturing Industries*. Edited by M. Brooks. New York: Academic Press.

CHALOUPKA, J. (1969). Dual control of megateriopeptidase synthesis. *Annales Institute Pasteur* **117**, 631–636.

CHANEY, A. L. & MARBACH, E. P. (1962). Modified reagents for determination of urea and ammonia. *Clinical Chemistry* **8**, 130–132.

COLEMAN, G. (1967). Studies on the regulation of extracellular enzyme formation by *Bacillus subtilis*. *Journal of General Microbiology* **49**, 421–431.

CONN, H. J., JENNISON, M. W. & WEEKS, O. B. (1957). Routine tests for the identification of bacteria. In *Manual of Microbiological Methods*, pp. 140–168. New York: McGraw-Hill.

ELLSWORTH, R., TELLING, R. C. & EAST, D. N. (1959). The investment value of continuous culture. *Journal of Applied Bacteriology* **22**, 138–152.

FABIAN, J. (1969). Protease synthesis of *Bacillus pumilus* grown in continuous culture. In *Continuous Cultivation of Microorganisms*. Edited by I. Malek, K. Beran, Z. Fencl, V. Munk, J. Ricica & H. Smrckova. New York: Academic Press.

FREESE, E. B., COLE, R. M., KLOFAT, W. & FREESE, E. (1970). Growth sporulation and enzyme defects of glucosamine mutants of *Bacillus subtilis*. *Journal of Bacteriology* **101**, 1046–1062.

GOLDENBAUM, P. E., BROMAN, R. L. & DOBROGOSZ, W. J. (1970). Cyclic 3′,5′-adenosine monophosphate and *N*-acetylglucosamine-6-phosphate as regulatory signals in catabolic repression in the lac operon in *Escherichia coli*. *Journal of Bacteriology* **103**, 663–670.

GREENSTEIN, J. P. & WINITZ, M. (1961). *Chemistry of the Amino Acids*, vol. 2, pp. 1299–1317. New York: Wiley.

HANSON, R. S., BLICHARSKA, J., ARNAUD, M. & SZULMAJSTER, J. (1964). Observations on the regulation of the synthesis of the tricarboxylic acid cycle enzymes in *Bacillus subtilis* Marburg. *Biochemical and Biophysical Research Communications* **17**, 690–695.

KATZ, J. (1898). *Jahrbuch für wissenschaftliche Botanik* **31**, 599. Cited by K. Daigen & B. Williams (1970). Catabolite repression and other control mechanisms in carbohydrate utilization. *Advances in Microbial Physiology* **4**, 251–324.

KEAY, L. (1971). Microbial proteases. *Process Biochemistry* **68**, 17–21.

KEAY, L., MOSER, P. W. & WILDI, B. S. (1970). Proteases of the genus *Bacillus*. II. Alkaline proteases. *Biotechnology and Bioengineering* **12**, 213–249.

KEAY, L. & WILDI, B. S. (1970). Proteases of the genus *Bacillus*. I. Neutral proteases. *Biotechnology and Bioengineering* **12**, 179–212.

KUBITSCHEK, H. E. (1970). Nuclear selection in filamentous bacteria: Resolution of the Novick-Szilard Paradox. *10th International Congress of Microbiology*, 1970, Colloquium C11-3.

LAISHLEY, E. J. & BERNLOHR, R. W. (1966). Catabolic repression of several sporulation enzymes during growth of *Bacillus licheniformis Bacteriological Proceedings*, 97.

LAISHLEY, E. J. & BERNLOHR, R. W. (1968). Regulation of arginine and proline catabolism in *Bacillus licheniformis*. *Journal of Bacteriology* **96**, 322–329.

MANDELSTAM, J. (1967). End product repression and the regulation of degradative enzymes. In *Regulation of Nucleic Acid, and Protein Synthesis*, pp. 361–356. Edited by V. V. Koningsberger and L. Bosch. Amsterdam: Elsevier Publishing Co.

MOSELEY, M. H. & KEAY, L. (1970). Purification and characterization of the amylase of *Bacillus subtilis* NRRL-B3411. *Biotechnology and Bioengineering* **12**, 251–271.

MOSER, H. (1958). *The Dynamics of Bacterial Populations Maintained in the Chemostat*. Publication G 14. Washington D.C : Carnegie Institution.

MURRELL, W. G. (1967). The biochemistry of the bacterial endospore. *Advances in Microbial Physiology* **1**, 133–251.

SCHAEFFER, P. (1969). Sporulation and the production of antibiotics, exoenzymes and exotoxins. *Bacteriological Reviews* **33**, 48–71.

SCHAEFFER, P., MILLET, S. & AUBERT, J. P. (1965). Catabolite repression of bacterial sporulation. *Proceedings of the National Academy of Sciences of the United States of America*. **54**, 704–711.