ACTA CHEMICA SCANDINAVICA 26 (1972) 3543–3548

The Effect of Phosphate on α-Amylase Production and Sporulation by *Bacillus subtilis*

PERTTI H. MARKKANEN and TOR-MAGNUS ENARI

Biotechnical Laboratory, The Technical Research Centre of Finland, Box 192, SF-00121 Helsinki 12, Finland

The effect of phosphate on sporulation and α-amylase (E.C. 3.2.1.1.) production was examined using *Bacillus subtilis* NCIB 8646. In fermentor culture the required concentration of phosphate for good growth rate was 0.061 M and with the same phosphate concentration the production rate of α-amylase was also high. Some increase in both growth rate and α-amylase production was noticeable with higher phosphate concentrations. The corresponding values for shake cultures were 0.033 M for good α-amylase production rate and as low as 0.014 M for good growth rate. With increasing phosphate concentrations the degree of sporulation and maximum α-amylase yield both increased, with analogous curves. Intracellular α-amylase activity was very low (less than 1 % of extracellular activity) and there seemed to be a reciprocal correlation between phosphate concentration and the amount of intracellular α-amylase.

α-Amylase production by *Bacillus subtilis* has a higher inorganic phosphate requirement than that for cell growth.[1,2] Sporulation is stimulated by the presence of phosphate, but is not dependent upon phosphate in excess of that required for α-amylase production.[3] Phosphate is one of the main constituents of spores.[4] *Bacillus amyloliquefaciens* has a phosphate requirement of 0.1 M for maximum α-amylase production.[5] As the nutrient media used in α-amylase production in industry are quite complex the effect of different ions upon α-amylase production is not always apparent. In this study the effect of phosphate ion on α-amylase production and bacterial growth was followed, and also the correlation between α-amylase production and sporulation by *Bacillus subtilis* NCIB 8646.

MATERIALS AND METHODS

Microorganism. Bacillus subtilis NCIB 8646 maintained on nutrient agar was used throughout the work.

Medium. The basic medium for the whole work was as follows (% w/v): Bacto peptone (Difco) 0.2; Bacto yeast extract (Difco) 0.3; NH_4Cl (Merck, *p.a.*) 1.6; KCl (Merck, *p.a.*)

0.15; $MnSO_4 . 2H_2O$ (Merck, *p.a.*) 0.0040; $CaCl_2$ (Merck, *p.a.*) 0.0111; Zinc acetate (Merck, *p.a.*) 0.0088; Trisodium citrate hydrate, (Merck, *p.a.*) 0.585; Lintner's soluble starch (Merck) 5.0; KH_2PO_4 (varying concentrations). Before any addition of phosphate, the medium contained 0.0066 % KH_2PO_4.

Culture method. Cells grown on a nutrient agar slant were heated in a water bath for 10 min and inoculated into 50 ml of 2 % yeast extract medium contained in a 250 ml conical flask. After 24 h incubation at 30°C 1 ml of this seed culture was inoculated into bottles containing 50 ml of the growth medium and shake cultured at 30°C on a gyrotory shaker (G 10 New Brunswick Scientific Company). Into the fermentor (FL 103, Biotec) 50 ml of the seed culture were inoculated. In all fermentor experiments the standard conditions were: 2500 ml of growth medium, stirring speed 325 rpm, aeration rate 1 vol./1 vol. medium/min, and temperature 30°C.

Measurement of growth and sporulation. The bacterial growth was followed by the dilution method on nutrient agar in Petri dishes and by turbidity measurements in a colorimeter with a 660 nm filter. Spore counts were made using the dilution method, after heating for 10 min at 100°C in a water bath so that only heat tolerant spores were measured.

Chemical methods

α-Amylase activity. The method of Fisher and Stein [6] was used. This method is based upon the liberation of reducing groups from starch, measured by the dinitrosalicylic acid method. One unit of activity was that amount of enzyme which liberated reducing groups corresponding to 1 mg maltose hydrate. Standard conditions were: 1 % Lintner's soluble starch as substrate, pH 5.9, and reaction time 3 min.

Intracellular α-amylase activity was measured after washing the cells twice and breaking them with a Braun cell homogenizer with glass beads in 0.002 M glycerophosphate buffer pH 5.9. After centrifugation the activity was measured as above.

Dipicolinic acid (DPA). DPA was measured using the method of Jansen *et al.*[7]

Inorganic phosphate. The determination of inorganic phosphate was made using the method of Fiske and Subbarow.[8]

RESULTS AND DISCUSSION

Fermentor experiments

The growth and activity curves with different K_2HPO_4 concentrations are presented in Fig. 1. From the linear parts of the curves the growth rate (μ) was calculated using the following formula:

$$\mu = \frac{2.3 \times \Delta\text{Turbidity}}{\Delta t}$$

where ΔTurbidity is the difference between two readings and Δt the time interval (h) between them. As a measure of the rate of production of α-amylase (μa) the slope of the production curve was used. These values are shown in Fig. 1d. From this it can be seen that good growth rate and α-amylase production rate require the addition of 0.1 % K_2HPO_4. A total phosphate concentration of 0.1066 % (0.061 M) is therefore the minimum requirement (the initial K_2HPO_4 content of the medium was 0.066 %). However, the maximum yield of α-amylase needs in every case more phosphate (Table 1 and Fig. 3). Wallerstein [5] found that for *B. amyloliquefaciens* the required phosphate concentration for α-amylase production was 0.1 M. Similar results are reported also by

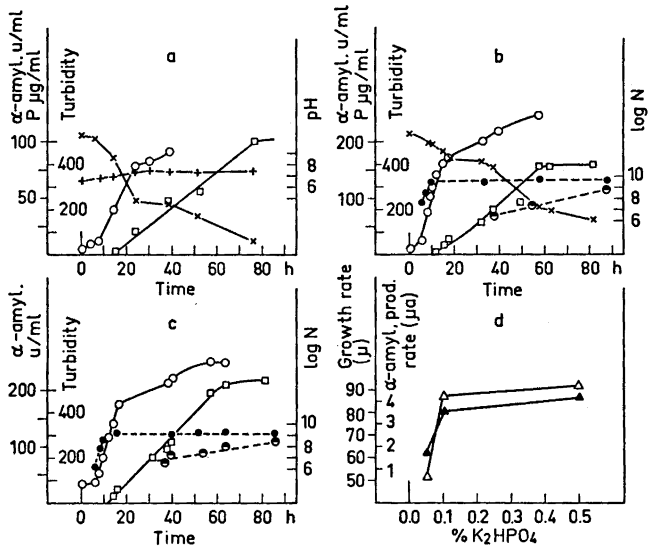

Fig. 1. Effect of phosphate in fermentor cultivation. Phosphate additions: a 0.05 %, b 0.10 %, c 0.50 %. ○ turbidity, □ α-amylase, + pH, × P µg/ml, ● total viable cells ◓ heat stable spores, △ growth rate, ▲ α-amylase production rate.

Fukumoto *et al.*[9] According to Lulla [10] phosphate stimulated α-amylase production more than any other inorganic ion used.

Sporulation is quite strongly repressed in high nutrient content media such as that used in this work. Table 1 shows sporulation as a percentage of viable cells and maximum α-amylase production. Both increase with increasing phosphate concentration. This indicates that the same or the same kind of control mechanism may be involved in both of these events.

Uptake of phosphate. The uptake of phosphate during the course of growth is presented in Figs. 1a and 1b. There is a plateau after the growth reaches its maximum. When sporulation begins some hours later uptake also increases. After α-amylase production has finished phosphate uptake is low as perhaps are all the other activities of the cells.

Table 1. The effect of phosphate concentration on sporulation and α-amylase production in fermentor culture.

Added K_2HPO_4 %	Spores %	Maximal α-amylase activity, µ/ml
0.05	–	99
0.1	0.5	156
0.5	6.0	216

Shake culture experiments

Fig. 2 shows the results of the shake culture experiments. Addition of phosphate had only a small influence on the growth rate (Fig. 2f), but α-amylase production required the addition of 0.05 % K_2HPO_4. Thus for satisfactory growth the requirement of K_2HPO_4 was about 0.0066 % (0.021 M) while for good α-amylase production rate the requirement was 0.0566 % (0.033 M). These values are less than those obtained for the fermentor media. However, the effect of phosphate upon maximum α-amylase production and sporulation of bacteria is of the same nature as in the fermentor (Fig. 3). Increase of phosphate concentration from 0.1 % to 1.0 % had only a small effect upon sporulation percent, DPA concentration, and maximum α-amylase production.

It appears that sporulation and α-amylase secretion are regulated by the same kind of metabolic control mechanism,[11,12] but that α-amylase production

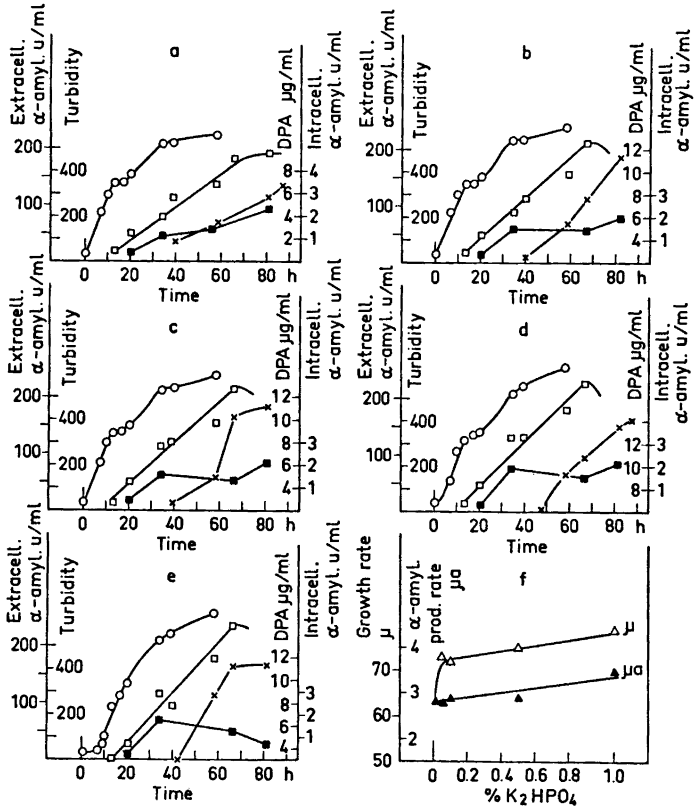

Fig. 2. Effect of phosphate in shake flask experiments. Phosphate additions: a 0.01 %, b 0.05 %, c 0.10 %, d 0.50 %, e 1.0 %. ○ turbidity, □ extracellular α-amylase, ■ intracellular α-amylase, × DPA, △ growth rate, ▲ α-amylase production rate.

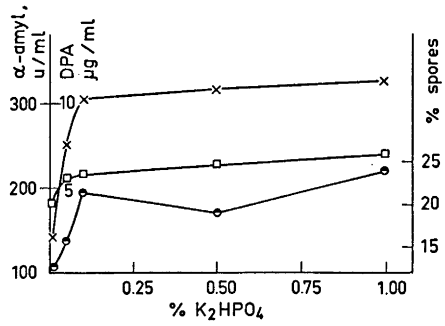

Fig. 3. Effect of phosphate on α-amylase production, DPA-concentration and degree of sporulation. × DPA, □ α-amylase, ◓ % spores of total viable cells, when the α-amylase had reached its maximum value.

is not essential for sporulation. It has been shown [12] that some α-amylase producing mutants do not in fact sporulate. It is therefore thought that the role of α-amylase is only to liberate the large amount of energy required for the sporulation process.

Intracellular α-amylase. In cells actively secreting α-amylase, there is also a small intracellular activity, which is liberated by rupturing the cells. All of that activity remains in the supernatant after centrifugation at 3000 rpm or 18 000 rpm. Table 2 shows the correlation between phosphate concentration and the intracellular α-amylase represented as a percentage of the secreted enzyme when the extracellular activity has reached its maximum value. The reduction of intracellular α-amylase activity with increasing concentration of phosphate is probably explained by the fact that secretion into the medium is more complete at higher phosphate concentrations.

Table 2. The correlation between phosphate concentration and intracellular α-amylase activity as percentage of extracellular activity.

Added K_2HPO_4 %	Intracellular activity, % of extracellular
0.01	0.78
0.05	0.67
0.10	0.61
0.50	0.66
1.0	0.55

Acknowledgements. P.M. is Research Assistant of the Technical Science Research Council in Finland. The authors wish to thank Miss Karita Lindström for her skilful technical assistance.

REFERENCES

1. Amaha, M., Ordal, Z. J. and Tauba, A. *J. Bacteriol.* **72** (1956) 34.
2. Windish, W. W. and Mhatre, N. S. *Advan. Appl. Microbiol.* **7** (1965) 273.
3. Murrel, W. G. In Gould, G. W. and Hurst, A., Eds., *The Bacterial Spore*, Academic, New York 1969, p. 215.
4. Murrel, W. G. *Advan. Microbiol. Physiol.* **1** (1967) 133.
5. Wallerstein, L. *Ind. Eng. Chem.* **31** (1939) 1218.
6. Fisher, E. H. and Stein, E. A. *Biochem. Prep.* **8** (1961) 27.
7. Jansen, F. J., Lund, A. J. and Anderson, L. E. *Science* **127** (1958) 26.
8. Fiske, C. H. and Subbarow, Y. *J. Biol. Chem.* **66** (1925) 375.
9. Fukumoto, J., Yamamoto, T., Tsuru, D. and Ishikawa, K. *Proc. Int. Symp. Enzyme Chem., Maruzen, Tokyo 1957*, (1958), p. 479.
10. Lulla, B. S. *Biochim. Biophys. Acta* **7** (1951) 244.
11. Coleman, G. *J. Gen. Microbiol.* **49** (1967) 421.
12. Schaeffer, P. *Bacteriol. Rev.* **33** (1969) 48.

Received April 10, 1972.