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Characterization of a new *Bacillus stearothermophilus* isolate: a highly thermostable α-amylase-producing strain

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Abstract A novel strain of *Bacillus stearothermophilus* was isolated from samples of a potato-processing industry. Compared to known α-amylases from other *B. stearothermophilus* strains, the isolate was found to produce a highly thermostable α-amylase. The half-time of inactivation of this α-amylase was 5.1 h at 80°C and 2.4 h at 90°C. The temperature optimum for activity of the α-amylase was 70°C; the pH optimum for activity was relatively low, in the range 5.5–6.0. α-Amylase synthesis was regulated by induction and repression mechanisms. An inverse relationship was found between growth rate and α-amylase production. Low starch concentrations and low growth temperatures were favourable for enzyme production by the organism. At the optimal temperature for growth, 65°C, the α-amylase was a growth-associated enzyme. The optimal temperature for α-amylase production, however, was 40°C, with α-amylase increasing from 3.9 units (U)/ml to 143 U/ml when lowering the growth temperature from 65°C to 40°C. Maximal α-amylase production in a batch fermentor run at 65°C was 102 U/ml, which was 26-fold higher than in erlenmeyer flasks at 65°C. The dissolved O_2 concentration was found to be a critical factor in production of the α-amylase.

Introduction

Thermostable α-amylases find a number of applications in commercial processes, such as thinning and liquefaction of starch in the brewing and sugar industries, which take place at temperatures up to 110°C. α-Amylase hydrolyses internal α-1,4-bonds in starch. The result is a rapid decrease in viscosity and formation of dextrins and small amounts of glucose and maltose (Sharp et al. 1989).

The genus *Bacillus* produces a large variety of extracellular enzymes of which amylases and proteases are of significant industrial importance. An extremely thermostable α-amylase is available from the mesophile *B. licheniformis* (Morgan and Priest 1981). This enzyme, which is marketed commercially, has a broad pH range for activity, is highly thermostable in the presence of starch and has a lower requirement for Ca^{2+} than many other bacterial α-amylases.

The thermophilic bacterium *B. stearothermophilus* offers an alternative for the commercial production of thermostable α-amylases. Thermophilic fermentations are considered quite useful for technical and environmental purposes (Kristjansson 1989; Sonnleitner and Fiechter 1983). Advantages are, for instance, a reduction in cooling costs, a better solubility of substrates, a lower viscosity allowing accelerated mixing and pumping and a reduced risk of microbial contamination. However, running α-amylase production processes at higher temperatures will require a new process design and improved knowledge of thermophilic bacteria. Processes using thermophiles still lack the maturity of classical processes with mesophilic bacteria and yeasts. Enzyme yields have remained relatively low since little work has been done on strain selection, growth optimization and enzyme yield optimization (Coolbear et al. 1992).

The α-amylases from *B. stearothermophilus* have been studied extensively (Vihinen and Mäntsälä 1987). Several α-amylases produced by strains of this species have been purified and characterized. The genes encoding for several of these α-amylases have been cloned into mesophilic production strains. We have screened various strains of *B. stearothermophilus*, isolated from samples of a potato-processing industry, for optimum growth

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temperature and α-amylase production. The most promising strain was studied in detail and here we report the effects of growth rates, substrates and cultivation conditions on α-amylase production. In addition, some characteristic properties of the extracellular α-amylase are presented. This strain is particularly interesting because of the production of a highly thermostable α-amylase compared to α-amylases of other *B. stearothermophilus* strains.

Materials and methods

Isolation of the organisms

The organisms were isolated from samples from a potato blancher operating at temperatures up to the boiling point of water. Enrichment cultures in tryptone yeast extract medium containing 0.1% (w/w) glucose at 55–65°C were set up to isolate thermophilic bacteria. Pure cultures were obtained after several transfers on agar. The isolates were all aerobic spore-forming rods and thus classified as strains of the genus *Bacillus*. The selected strains were further identified with the Analytical Profile Index (API) 50 CHB test (API Bio-Mérieux, Den Bosch, The Netherlands). One strain, *Bacillus* MFF4, was studied in more detail because of its high optimal growth temperature, rapid growth and high α-amylase production compared to the other strains.

Media and growth conditions

The growth medium used was minimal medium with starch (MMS) and was a modification of the defined medium employed by Welker and Campbell (1963). MMS contained per litre: 10 g soluble starch (Merck, Amsterdam, The Netherlands), 1.0 g NH_4Cl, 0.5 g CH_3COOK, 1.0 g NaCl, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.025 g $FeSO_4 \cdot 7H_2O$, 0.025 g $MgCl_2 \cdot 2H_2O$, 0.01 g ethylenediaminetetraacetic acid (EDTA), 1.0 g casein hydrolysate, 0.1 g citric acid, 1.0 g $Na_2HPO_4 . 2H_2O$ and 0.1 mg biotin. The buffer components, citric acid and phosphate, were added separately after autoclaving to prevent precipitation of inorganic salts, e.g. calcium phosphates. Mono- and disaccharides were sterilized by membrane filtration (pore size 0.45 µm), whereas all polysaccharides were autoclaved. L-Broth was used as described by Maniatis et al. (1989). *B. stearothermophilus* ATCC 12980 (Vihinen and Mäntsälä 1990) and ATCC 7954 (Hartman et al. 1955), used in control experiments, were grown on MMS at 55°C.

Cells were cultivated in erlenmeyer flasks in a shaking water bath (GFL type 1083, Salm en Kipp, Breukelen, The Netherlands) with a shaking frequency of 110–130 rpm or in a 3-l fermentor with a working volume of 2.0 l (Applikon, Schiedam, The Netherlands). Erlenmeyer flasks of 250 or 300 ml were filled with medium to a volume of 80 ml. The temperature of the fermentor was controlled at 65°C and the pH at 7.0. Dissolved O_2 levels were monitored in the fermentor using an O_2 electrode (Ingold, Frankfurt, Germany). The fermentor was aerated at a flow rate of 0.36 or 0.72 l/min and the stirrer speed was set at 400 or 800 rpm.

All strains were maintained on MMS agar. At intervals of 1 month, fresh cultures were started from frozen stocks (− 80°C).

Assay of α-amylase activity

α-Amylase activity was measured according to the microassay of Sirou et al. (1990) with a few modifications (unless stated otherwise). The substrate, 2-chloro-4-nitrophenol maltotrioside ($CNPG_3$), used in the assay was obtained from the "Enzyline α-amylase direct unitaire" kit (API BioMérieux, Den Bosch, The Netherlands). $CNPG_3$ is hydrolysed by α-amylase and directly produces 2-chloro-4-nitrophenol (CNP).

Per vial, a solution containing 1 ml of 0.1 M CH_3COONa, pH 6.5, and 5 mM $CaCl_2 \cdot 2H_2O$ was added. A 30-µl portion of the substrate (6 mmol/l of $CNPG_3$) was pipetted into individual wells of a microtitre plate. Supernatant (30 µl) was added in the activity range of 0–25 units (U/ml) and diluted with a solution of 0.1 M CH_3COONa, pH 6.5, and 5 mM $CaCl_2$ in the higher activity range. After incubation (1 h, 60°C) the reaction was stopped with 150 µl of 1% TRIS, pH 10.4. Absorbance at 405 nm was read with a microplate reader (Biorad, model 3550, Veenendaal, The Netherlands). One enzyme unit (U) is defined as the amount of enzyme releasing 1 µmol CNP in 60 min under assay conditions. The assay was linear over a 60 min incubation period and proportional to the amount of sample.

Total α-amylase activity was determined by performing the assay with the culture fluid containing whole cells; the absorbance at 405 nm was measured after removal of the cells. Cell-bound α-amylase activity was calculated as the difference between the measured total α-amylase activity and the α-amylase activity present in the supernatant.

The maximum production of α-amylase in cultures was determined by periodically assaying the culture fluid with and without cells. Samples taken from the culture were stored at 4°C until analysis and first centrifuged in the case of determination of α-amylase activity in the supernatant.

Thermostability experiments

Residual α-amylase activity was measured with the modified dinitrosalicylic acid (DNS) method at 60°C (Bernfield 1955). The substrate solution contained 2% boiled soluble starch and 5 mM $CaCl_2$ in 40 mM Na-phosphate buffer, pH 6.0. The reaction at 60°C was stopped after an appropriate incubation period. The activity unit is defined as the amount of enzyme releasing 1 µmol reducing sugars (as maltose) per minute under assay conditions.

Determination of substrates and products

Maltose concentrations were determined by the DNS method (Bernfield 1955). Total sugars in the samples were analysed by the anthrone method as described by Nelson (1952). Cell dry weight was determined by drying a known volume of washed cells overnight at 105°C to constant weight. Alcohols and acids in culture supernatants were analysed by HPLC (Pharmacia LKB, Uppsala, Sweden) equipped with a Skodex KC-811 column. Compounds were detected by a refractive index detector (Waters) or a UV-detector (Pharmacia).

Results

Characterization of strain MFF4

Isolate MFF4 was rod-shaped, cells had a length of 1–2 µm and a diameter of 0.2–0.5 µm. Occasionally chains of two to five bacteria as well as longer filamentous structures were observed. Spores were oval in shape and present in a terminal position in the cells extending the cell diameter. The isolate had a broad temperature range for growth (Table 1). No growth was observed above 72°C or under 39°C. The growth rate was highest at 60–70°C.

Table 1. Growth and α-amylase production in the supernatants of *Bacillus stearothermophilus* MFF4 cultures grown in erlenmeyer flasks at different temperatures

Temperature (°C)	Biomass production (g/l)	Growth rate (h^{-1})	Maximum amylase produced (U/ml)
40	0.38	0.24	143.0
50	0.40	0.33	80.4
60	0.51	0.44	5.5
65	0.54	0.44	3.9
70	0.43	0.44	0.4

Due to its spore-forming ability, its growth at 65°C and the observed starch hydrolysis, the isolate was classified as a strain of the species *B. stearothermophilus* (Claus and Berkeley 1986). This was confirmed with the API 50 CHB test, which tests the ability of a microorganism to utilize a preselected panel of 50 different carbon sources. This "metabolic fingerprint" confirmed classification of the isolate as a strain of the species *B. stearothermophilus*.

A possible stimulation of the maximum biomass production and growth rate of *B. stearothermophilus* MFF4 was examined by adding various supplements to MMS. From the vitamins tested (biotin, nicotinic acid and thiamin, 0.1 mg/l) only biotin was required for growth. Casein hydrolysate addition (0.1%, w/v, in MMS) stimulated growth (0.14 g/l increase in biomass) of strain MFF4. Several amino acids (Ile, Cys, Phe, Asp, Glu, Met) known to stimulate growth of *B. stearothermophilus* strains (Sharp et al. 1989) were tested individually but showed no effect. Stimulatory effects of casein hydrolysate on α-amylase production by strain MFF4 were not observed.

General properties of the enzyme

Temperature and pH optima for activity. The pH optimum of the α-amylase, determined at 60°C, was in the pH range 5.5–6.0 (Fig. 1A). The temperature optimum of the α-amylase was determined at pH 6.5. In all assays 5 mM $CaCl_2$ was included. The incubation time was 20 min to diminish the effects of thermal inactivation. The α-amylase activities were maximal at 70–75°C (Fig. 1B).

Effect of temperature on enzyme stability. The thermostability of the α-amylase was studied by incubating culture supernatants at 80°C and 90°C. The residual amylase activity was measured with an assay based on a direct action on starch. α-Amylase activity was measured with the DNS method at 60°C, which also facilitates comparison with other reports on thermostable α-amylase.

The enzyme was highly stable at all temperatures tested (Fig. 2). The half-time of inactivation was 5.1 h at

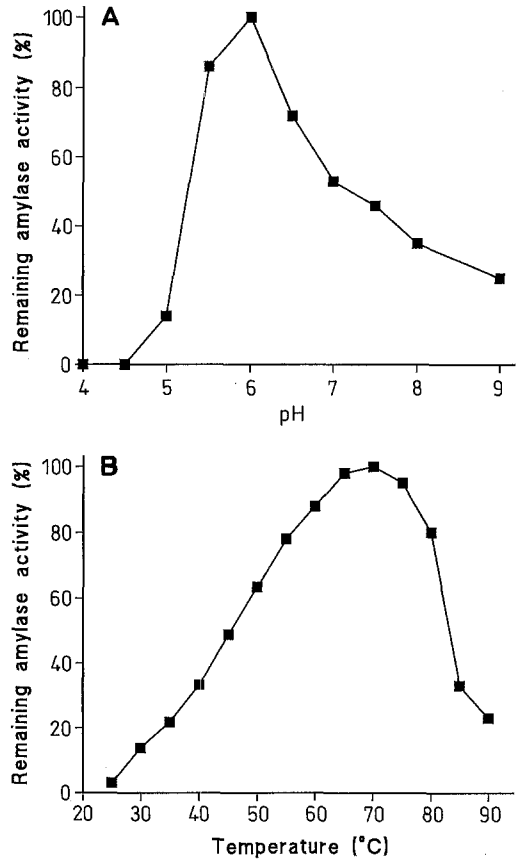

Fig. 1A, B. Effect of pH (**A**) and temperature (**B**) of the extracellular α-amylase of *Bacillus stearothermophilus* MFF4 grown on minimal medium with starch (MMS) in a batch fermentor at 65°C. The 100% activity levels were: 136 units (U/ml) (**A**) and 108 units U/ml (**B**)

80°C and 2.4 h at 90°C. When the thermostability of the α-amylase was tested at 80°C with 0.5 mM $CaCl_2$, the half-time of inactivation was reduced to 1.0 h. Activity at 60°C was not influenced by the concentration of calcium. Consequently Ca^{2+} ions enhance the thermostability of the α-amylase, but not the activity of the α-amylase.

For a comparison, two other strains producing the highest thermostable α-amylases known until now from *B. stearothermophilus* strains, ATCC 12980 (Vihinen and Mäntsälä 1990) and ATCC 7954 (Hartman et al. 1955), were studied. At temperatures of 80°C and 90°C the α-amylase of strain MFF4 was the most thermostable (Fig. 2).

After 1 h heating at 80°C or 90°C, the residual α-amylase activity, measured with the microassay according to Sirou et al. (1990), was 0% in all cases.

Enzyme production in erlenmeyer flasks

α-Amylase production profile. α-Amylase production by strain MFF4 at 65°C was initiated in the early exponential growth phase and became maximal during the

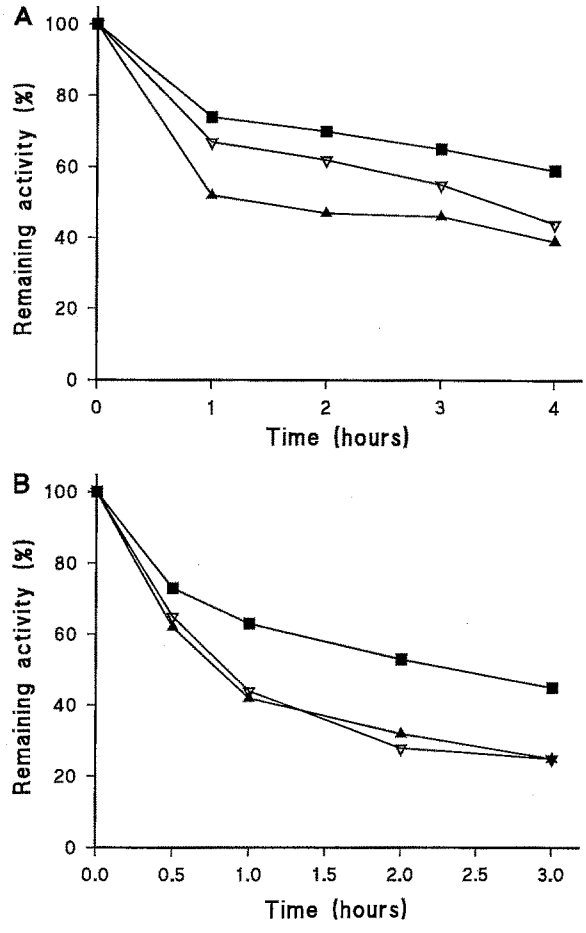

Fig. 2A,B. Thermostability of the α-amylase of *B. stearothermophilus* MFF4 (■), ATCC 12980 (▲) and ATCC 7954 (▽). Cell-free supernatants of cultures grown on MMS at 55°C (t = 24 h) in erlenmeyer flasks were incubated at 80°C (**A**) and 90°C (**B**); $CaCl_2$ (5 mM) was included in all experiments. The 100% activity levels were (**A** and **B**): 2.2 U/ml (MFF4), 2.8 U/ml (ATCC 12980), 3.1 U/ml (ATCC 7954)

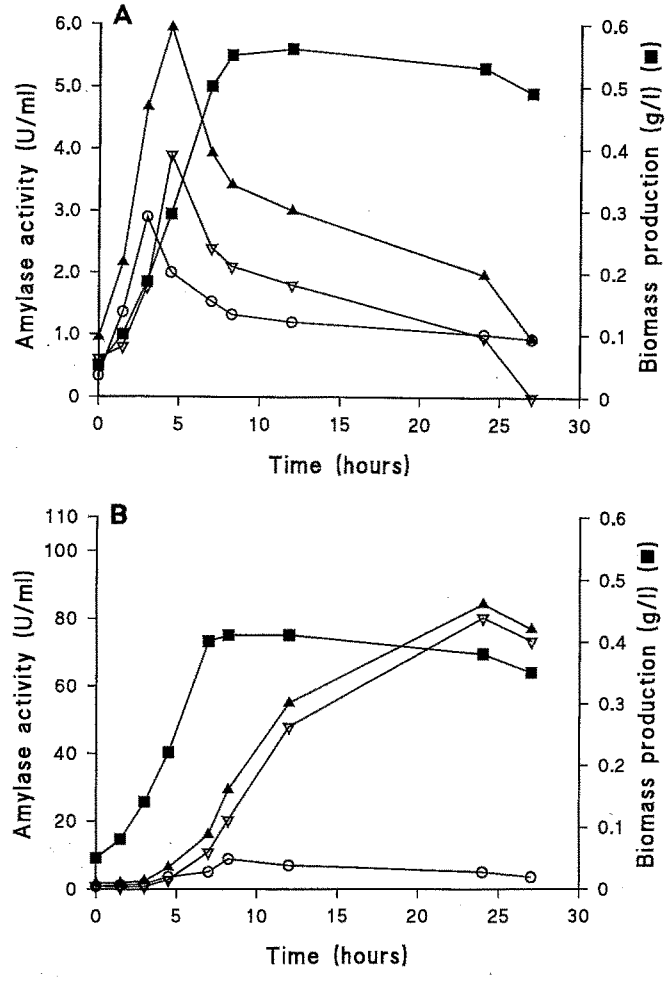

Fig. 3A,B. α-Amylase and biomass production by *B. stearothermophilus* MFF4 in erlenmeyer flasks at 65°C (**A**) and at 50°C (**B**): ▲, total α-amylase activity; ○, cell-bound α-amylase activity; ▽, amylase activity in the supernatant (U/ml); ■, biomass production (g/l)

exponential growth phase (Fig. 3A). A total α-amylase production of 5.9 U/ml was reached within 6 h. At 65°C α-amylase production is growth-associated.

The growth rate of strain MFF4 at 65°C was 0.44 h^{-1}, with a maximal biomass production of 0.54 g/l. Especially in the early exponential growth phase, part of the amylase was produced in a cell-bound form.

Effect of temperature on α-amylase production. α-Amylase production in strain MFF4 grown in erlenmeyer flasks was temperature-dependent (Table 1). The maximum amount of α-amylase present in the supernatant at a growth temperature of 40°C was 143 U/ml, which is a 37-fold increase in α-amylase production when compared to α-amylase production at a growth temperature of 65°C. At 70°C α-amylase production was lowest (0.4 U/ml). Thermostability of the α-amylase produced at lower temperatures was checked; the α-amylases formed at 40°C and 65°C turned out to be equally thermostable.

The α-amylase production profile by cells grown at 50°C was different from that at 65°C. It also started in the exponential growth phase but only reached a maximum late in the stationary growth phase (Fig. 3B). Most of the α-amylase produced at 50°C was present in the supernatant.

Effect of carbon source on α-amylase production. α-Amylase synthesis by cells grown on various substrates was determined at 65°C and 50°C (Table 2). α-Amylase production was induced by starch, amylopectin, maltooligosaccharides (G2–G6) and maltose. Starch and amylopectin were the most effective inducers of α-amylase synthesis. With glucose, fructose or sucrose as the carbon source and L-broth only a low basal level of α-amylase was produced. α-Amylase levels decreased immediately after addition of glucose (Fig. 4). This indicates an immediate cessation of de-novo enzyme synthesis, probably due to glucose catabolite repression of

Table 2. Effect of carbon source on growth and production of α-amylase in the supernatants of *B. stearothermophilus* MFF4 cultures grown at 50°C or 65°C in erlenmeyer flasks

Carbon source (10 g/l)	Biomass production (g/l)		Growth rate (h^{-1})		Max. α-amylase production (U/ml)		Induction (+/−)
	65°C[a]	50°C[b]	65°C[a]	50°C[b]	65°C[a]	50°C[b]	
Starch	0.54	0.40	0.44	0.33	3.9	80.4	+
Amylopectin	0.48	0.26	0.42	0.38	3.9	82.5	+
Maltooligosaccharides[c]	0.64	0.33	0.35	0.29	1.9	32.3	+
Maltose	0.67	0.26	0.44	0.36	1.9	35.6	+
Glucose	0.58	0.37	0.29	0.27	0.0	0.4	−
Sucrose	0.66	0.29	0.53	0.33	0.0	2.1	−
Fructose	0.52	0.25	0.51	0.25	0.0	2.1	−
LB	0.95	0.97	0.48	0.46	0.2	4.1	−

U, Units; LB, Luria broth (Mariatis et al. 1989)
[a] Cells were grown at 65°C
[b] Cells were grown at 50°C
[c] G2–G6

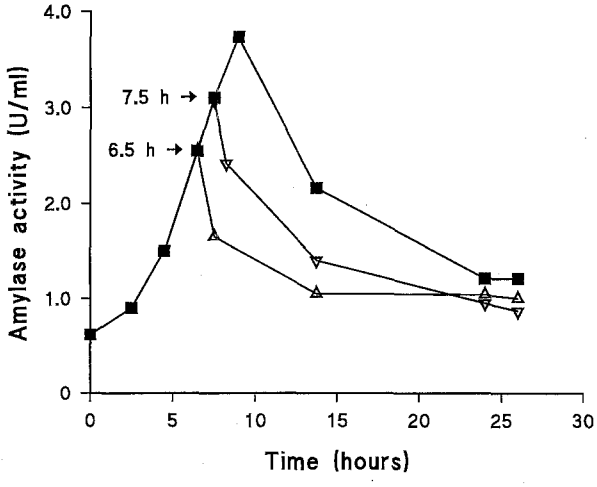

Fig. 4. Repression of α-amylase synthesis by addition of glucose to cells of *B. stearothermophilus* MFF4 on MMS at 65°C in erlenmeyer flasks: ■, no glucose added; △, 5 g glucose/l added after 6.5 h; ▽, 5 g glucose/l added after 7.5 h

enzyme synthesis. α-Amylase synthesis in strain MFF4 was not repressed by sucrose or fructose.

Effect of substrate concentration on α-amylase production. The effect of substrate concentration on α-amylase productivity was studied (Table 3). Biomass production of strain MFF4 increased with increasing starch concentration up to 8 g/l. At higher starch concentrations biomass production reached a plateau; at 22 g/l starch biomass production only decreased slightly. In the case of 0 g/l starch, biomass was formed from the casein hydrolysate present in the medium. The growth rate was more or less constant over the starch concentration range tested. At a concentration of starch of 1–2 g/l, 90% of the starch was used; at higher concentrations, substrate consumption was incomplete, indicating that another component in the medium became growth-limiting. The growth medium used, however, was suitable for studying α-amylase production characteristics, which was the main objective of our study. Ability to produce high levels of α-amylase was sometimes lost by strain MFF4; however, not affecting its growth characteristics in MMS medium due to production of a low basal level of α-amylase.

In the absence of starch in the medium almost no α-amylase was produced. α-Amylase production by strain MFF4 was highest at low substrate concentrations (Table 3): α-amylase production was increased ten-fold when the starch concentration was lowered from 10 g/l to 1 g/l.

Enzyme production in the fermentor

α-Amylase production profile. The growth rate of strain MFF4 in the fermentor was 0.52 h^{-1}, resulting in a biomass concentration of 0.58 g/l cell dry weight within 12 h (Fig. 5A). The α-amylase production profile of strain MFF4 in the fermentor at 65°C (stirrer speed 800 rpm, air flow 0.72 l/min) was the same as that found in erlenmeyer flasks; the α-amylase production was initiated in the early exponential growth phase and became maximal in the later exponential growth phase (Fig. 5A). After 12 h of growth on MMS, total α-amylase production reached a maximum of 108 U/ml and declined thereafter. Most of this α-amylase was freely present in the supernatant. Only in the exponential growth phase was part (40 U/ml) of the α-amylase present in a cell-bound form. α-Amylase production at 65°C in the fermentor was obviously much higher than in erlenmeyer flasks.

Table 3. Growth and substrate consumption by *B. stearothermophilus* strain MFF4 grown on minimal medium with starch in erlenmeyer flasks at 65°C with different concentrations of soluble starch

Concentration starch (g/l)	Biomass production (g/l)	Growth rate (h^{-1})	Starch degradation (g/l)	Starch degradation (%)	Max. α-amylase activity (U/ml)
0.0	0.19	0.41	—	—	0.2
1.0	0.28	0.42	0.9	90	44.9
2.0	0.41	0.41	1.8	90	34.9
4.0	0.47	0.43	2.0	50	19.9
8.0	0.53	0.45	2.3	28	5.0
10.0	0.54	0.45	2.3	23	3.9
22.0	0.51	0.42	2.5	11	0.7

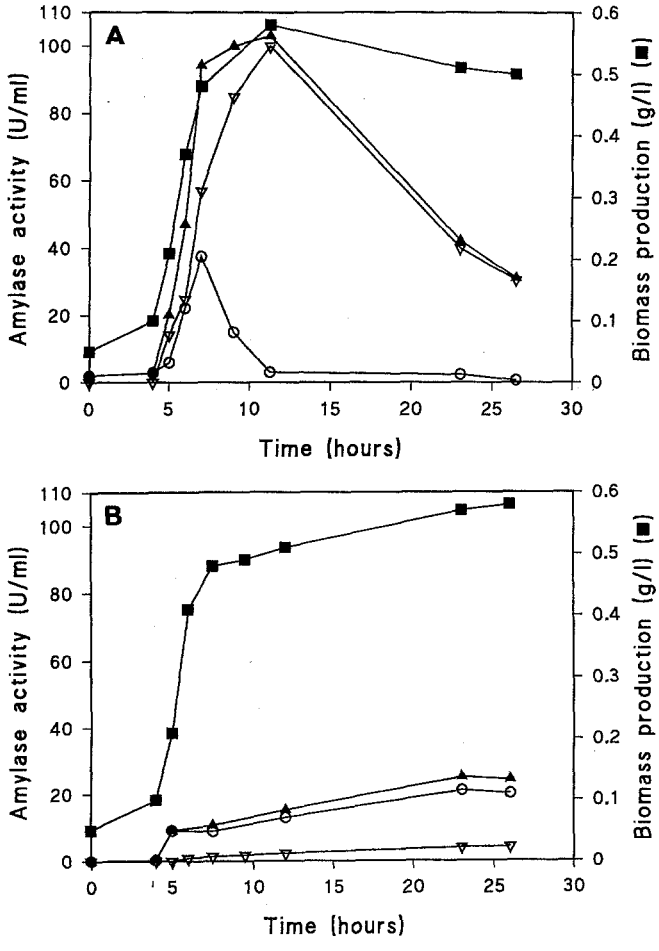

Fig. 5A, B. α-Amylase and biomass production by *B. stearothermophilus* MFF4 growing on MMS at 65°C in a batch fermentor with a high O_2 supply (stirrer speed 800 rpm, air flow 0.72 l/min, **A**) and a low O_2 supply (stirrer speed 400 rpm, air flow 0.36 l/min, **B**) at 65°C: ▲, total α-amylase activity; ○, cell-bound α-amylase activity; ▽, α-amylase activity in the supernatant (U/ml); ■, biomass production (g/l)

Effect of dissolved O_2 concentration. The effect of O_2 concentration on α-amylase production was studied by lowering the air supply (from 0.72 l/min to 0.36 l/min) and stirrer speed (from 800 rpm to 400 rpm) of the fermentor in such a way that growth rate and biomass formation were not affected. The O_2 concentration at normal stirrer speed and air flow was $\geqslant 60\%$ of air saturation; at low stirrer speed and air-flow, O_2 levels decreased for 4 h to 0% during the exponential growth phase. Total α-amylase production was decreased four to five-fold by lowering the O_2 supply (Fig. 5A and B). The maximal total α-amylase production at low O_2 concentration was only 24 U/ml, from which 20 U/ml was cell-bound α-amylase, compared to 108 U/ml at high aeration and stirrer speed.

HPLC analysis of supernatants of cultures ($t = 48$ h) grown in erlenmeyer flasks at 65°C revealed accumulation of lactate (0.27 g/l) and traces of propionate and ethanol, indicating a period of anaerobic growth. No fermentation products accumulated at high O_2 supply rates.

Discussion

Isolate MFF4 was classified as a strain of the species *B. stearothermophilus*. Strain MFF4 differs, however, from other *B. stearothermophilus* strains with an absolute requirement for biotin, nicotinic acid and thiamin (Amartey et al. 1991; Lee et al. 1982; Rowe et al. 1975), since from the vitamins tested only biotin was needed for growth of the organism. Strain MMF4 resembles other near-prototrophic *B. stearothermophilus* strains (Martins and Tempest 1991; Epstein and Grossowicz 1969). Unlike strain MFF4, however, *B. stearothermophilus* var. *non-diastaticus* is not able to hydrolyse starch (Epstein and Grossowicz 1969). Contrary to the situation in *B. stearothermophilus* 1503-4 (Welker and Campbell 1963), stimulatory effects of casein hydrolysate on α-amylase production by strain MFF4 were not observed.

Some general properties of the α-amylase of *B. stearothermophilus* MFF4 were examined in supernatants of cells grown on MMS. The pH optimum of the enzyme was in the range 5.5–6.0 (Fig. 1A). The pH optimal for *B. stearothermophilus* α-amylases vary but generally are in the range 4.5–8.0 (Vihinen and Mäntsälä 1987). The pH optima of the commercially used α-amylases are in the range 6.0–7.0.

The relatively low pH optimum of the α-amylase from *B. stearothermophilus* MFF4, compared to pH optima of commercially used α-amylases, is an advantage when considering a possible use in the starch-liquefaction process (Gacesa and Hubble 1987). The first step in this process, the formation of maltodextrins from starch by the action of α-amylase, takes place at temperatures of 80–110°C and at pH 6.0–7.0. The second step in this process is a saccharification of the maltodextrins formed with glucoamylase at pH 4.0–4.5. Starch liquefaction enzymes active at the saccharifying pH of 4.5 are of interest to the starch industry (Starnes 1990), allowing savings to be made on the amount of acid and base needed for pH adjustment, thereby also reducing the costs of ion-exchange media and chemicals for purification of the syrup.

The temperature optimum of the enzyme was 70–75°C (Fig. 1B), which is high for a *B. stearothermophilus* α-amylase. Temperature optima for activity of *B. stearothermophilus* α-amylases are generally in the range of 50–70°C. The highest *B. stearothermophilus* α-amylase temperature optimum reported so far is 70–80°C (Vihinen and Mäntsälä 1990).

The α-amylase of strain MFF4 was more thermostable than the α-amylases of *B. stearothermophilus* ATCC 7954 and ATCC 12980 (Fig. 2). The residual α-amylase activity in these experiments was measured with the DNS method, which is based on a direct action of the α-amylase on starch. Residual amylase activity measured with the microassay according to Sirou et al. (1990), which is based on action of the α-amylase on $CNPG_3$, was 0% for all strains tested. A possible explanation for this difference in results is a change in the active centre of the α-amylase after heating, after which some substrates, like $CNPG_3$, cannot be hydrolysed. Further research is necessary to examine this.

The production of the α-amylase by strain MFF4 in erlenmeyer flasks was studied in detail. At 65°C the production was found to be growth-associated (Fig. 3A). Production of α-amylase by *B. stearothermophilus* strains generally occurs in the exponential growth phase (Sidler and Zuber 1977; Davis et al. 1980), unlike the situation in *B. subtilis* and *B. licheniformis* where α-amylase production occurs in the late exponential and the stationary phase, prior to the onset of sporulation (Priest 1977).

α-Amylase synthesis in strain MFF4 was regulated by induction and repression mechanisms (Table 2, Fig. 4). These results are in agreement with the general ideas on regulation of exoenzyme synthesis. It is generally accepted that exoenzyme synthesis is induced by low-molecular-mass products that enter the cell and are produced by degradation of their substrates by a low basal level of constitutive exoenzyme (Priest 1977). α-Amylase synthesis in strain MFF4 was repressed by glucose, but not by fructose or sucrose. α-Amylase synthesis in most bacilli is susceptible to catabolite repression by glucose and fructose (Priest 1977).

The α-amylase production in strain MFF4 was temperature-dependent (Table 1). Low growth temperatures were favourable for enzyme production by the organism. To our knowledge the relationship between α-amylase production and temperature, as observed for strain MFF4, has not been reported before in the literature. A clear temperature dependence of α-amylase synthesis in *Bacillus* strains has been demonstrated several times before. In these cases however the temperature optima for α-amylase production and biomass production were the same (Saito and Yamamoto 1975; Babu and Satyanarayana 1993).

The increased α-amylase production by strain MFF4 at lower growth temperature might be due to the decreased growth rates at lower temperatures. An inverse relationship between extracellular enzyme synthesis and growth rate is frequently observed (Priest 1977; Sonnleitner et al. 1984). Another possible explanation could be the increase in O_2 solubility at lower temperatures: the solubility of O_2 in water at 40°C is $10.3 \cdot 10^{-4}$ mol/l; at 70°C, however, it is $8.2 \cdot 10^{-4}$ mol/l.

α-Amylase production in strain MFF4 was dependent on the starch concentration. Low starch concentrations were favourable for enzyme production by the organism (Table 3). These results were not in agreement with a study on optimal culture conditions for the production of a thermostable α-amylase by *B. stearothermophilus* (Srivastava and Baruah 1986), where the optimal starch concentration for α-amylase production was 30 g/l. Higher or lower starch concentrations only resulted in decreased α-amylase production. No other reports on the relationship between starch concentration and amylase production could be found.

A possible explanation for the observed relationship between starch concentration and α-amylase production in strain MFF4 is repression of enzyme synthesis at higher starch concentrations by starch-hydrolysis products such as glucose or a difference in O_2 availability due to a change in viscosity at higher starch concentrations or a combination of both.

The dissolved O_2 concentration was found to be a critical factor in α-amylase production by *B. stearothermophilus* MFF4 (Fig. 5A and B). Total α-amylase production was decreased four to five fold by lowering the O_2 supply. The observed difference between α-amylase production at 65°C in erlenmeyer flasks (Fig. 3A), which reached a maximum total activity of 5.9 U/ml, and production in the fermentor (Fig. 5A), which reached a maximum of 108 U/ml, could be caused by such a difference in O_2 availability.

No reports exists, to our knowledge, on the effect of O_2 concentration on α-amylase production in *B. stearothermophilus* strains in general. Quantitative data on the dependence of specific growth rates and enzyme production rates on the specific O_2 uptake rate

are very scarce for thermophiles. Some reports exist on the relationship between α-amylase production and oxygen supply in mesophilic *Bacillus* strains, but data are often contradictory (Sonnleitner 1983).

At lower starch concentrations (5 g/l) α-amylase production in the fermentor (stirrer speed, 800 rpm; air flow, 0.72 l/min) was not increased (data not shown). For this reason, the observed relationship between starch concentration and α-amylase production in erlenmeyer flasks probably cannot be explained by the repression of enzyme synthesis at higher starch concentrations, but can still be explained by a difference in O_2 availability.

At a high O_2 concentration in the fermentor, most of the α-amylase produced was freely present in the supernatant after 24 h of growth (Fig. 5A), whereas at a low O_2 concentration most of the produced α-amylase was cell-bound (Fig. 5B). Furthermore, an indication was found for a period of anaerobic growth in the case of cells grown at a low O_2 concentration. From these results a parallel can be drawn with anaerobic thermophiles, in which the bulk enzyme activity is associated to the cell surface and cannot be easily released into the culture fluid (Antranikian 1990).

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