Journal of Industrial Microbiology (1996) 17, 96–99

The production of α-amylase (E.C.3.2.1.1.) by *Bacillus amyloliquefaciens*, in a complex and a totally defined synthetic culture medium

CJ Hewitt and GL Solomons

School of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK

The production of α-amylase by *Bacillus amyloliquefaciens* in both complex and synthetic culture media was examined at a laboratory fermenter scale. In a complex medium which supports fast growth rates, enzyme production occurred only when the growth rate declined, principally in the stationary phase. By contrast, in a synthetic culture medium with lactose as the carbon source supporting much lower growth rates, enzyme formation occurred simultaneously with cell growth. The repression of enzyme formation during rapid growth may be due either to catabolite repression or to the low level of mRNA synthesis concerned with the production of exoproteins.

Keywords: α-amylase; complex medium; synthetic medium; *Bacillus amyloliquefaciens*

Introduction

α-Amylase, an amylolytic enzyme, is of considerable commercial significance since its range of hydrolytic products, from high molecular weight dextrins to low molecular weight polymers of glucose, are used in the texile, food, brewing and paper industries [9]. The bacterial source of the enzyme is usually from either *Bacillus amyloliquefaciens* or *Bacillus lichenformis*, the latter now being of greater industrial importance.

In the industrial process, the enzyme is produced by growing the organism on a complex culture medium containing soya bean meal, autolysed yeast, along with other protein and peptide sources [11]. The two salient features of the fermentation are that bacterial growth precedes enzyme formation, which occurs during the stationary phase and that, early on in the fermentation, foaming takes place which is difficult to control.

This work, which continues the studies that have been made on this process in this department [3,10], is concerned with comparing the patterns of enzyme formation in a complex culture medium with that produced in a defined synthetic culture medium, supporting a similar enzyme titre.

Methods and materials

Bacillus amyloliquefaciens B20 was maintained at 4°C on a peptone, casein, yeast extract agar and grown at 37°C on malt extract, yeast extract agar (MEYEA). The composition of the Standard Lactose Medium (SLM) [10] is, in g L^{-1}: lactose 78.2; soya flour (defatted) 19.0; yeast extract 15.8; sodium caseinate 6.6; and $MgSO_4 \cdot 7H_2O$ 0.4; and made up in tap water. The pH was adjusted to 7.0 with 1 M NaOH prior to sterilisation. The composition of the Basal Synthetic Medium (BSM) in g L^{-1} is: lactose 80.0; $(NH_4)_2HPO_4$ 16.0; KH_2PO_4 1.50; $MgSO_4 \cdot 7H_2O$ 0.02; $FeSO_4 \cdot 7H_2O$ 0.02; $Na_2MoO_4 \cdot 2H_2O$ 0.002; $CuSO_4 \cdot 5H_2O$ 0.01; and made up with distilled water. The pH of the medium was not adjusted unless otherwise stated. The $(NH_4)_2HPO_4$ was sterilised separately and added aseptically to the remainder of the medium after it had cooled, in order to prevent browning by the Maillard reaction [7]. Chemicals used were of reagent grade. The silicone-based antifoam used was Assaff III manufactured by Rhone-Poulenc (Manchester, UK). For those BSM fermentations controlled above pH 6.5, a 50% ammonia solution was used as the control agent; in such experiments the concentration of $(NH_4)_2HPO_4$ was reduced to 4 g L^{-1}. Inoculum was prepared by growing the culture on MEYEA plates for 48 h and then transferring cells to the appropriate inoculation medium in shake flasks. For fermentations using either SLM or BSM, the medium was 4% malt extract and 2% yeast extract. The cell biomass was measured turbidimetrically by optical density at 600 nm in a double-beam spectrophotometer, calibrated against cell dry weight at 80°C to constant weight. A 4% inoculum with an optical density of at least 0.25 at a 100-fold dilution was used for all fermentations. Shake flask fermentations were carried out at 37°C with 30 ml per flask in 500-ml baffled Erlenmeyer flasks on a rotary shaker at 320 rpm, with an eccentricity of 5 cm. Laboratory scale fermentations were carried out in a 3-L fermenter vessel, with a working volume of 2.5 L, fitted with two Rushton turbines and equipped with pH, anti-foam, temperature, aeration and impeller speed control, and dissolved oxygen monitoring by an Ingold steam-sterilisable polarographic electrode. Sugars were measured using a Gilson HPLC system with an aminex HPX-87P carbohydrate analysis column (Bio-rad, Hemel Hempstead, UK), with post-column derivitisation using ammoniacal cupric sulphate and measurement with a variable wavelength spectrophotometer (Series 1000 Cecil Instruments, Cambridge, UK). The system, providing a sensitivity of 450 ng per 20 mm^3 of glucose, was calibrated with known concentrations of lactose, glucose and galac-

Correspondence: Dr CJ Hewitt, School of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK
Received 21 February 1995; accepted 12 July 1996

tose. α-Amylase activity was assayed by a modified viscometric method [3], calibrated with a crystalline *Bacillus* sp enzyme preparation of known potency (Sigma, Poole, UK). The concentration of ammonia was determined by micro-Kjeldahl distillation. Amino acids were qualitatively evaluated by single dimension Thin Layer Chromatography (TLC) on type 100 cellulose on polyester plates (Sigma), using a 40:2:10 mixture of 2-propanol, glacial acetic acid and distilled water.

Results

A series of five batch fermentations was carried out in SLM at impeller speeds varying between 600–1000 rpm. Despite severe and intensive foaming, reproducible fermentations were run without the need to lower the impeller speed. The varying patterns of cell growth, lactose utilisation, ammonia concentration, pH, dissolved oxygen concentration (DOC) and enzyme titre at an impeller speed of 1000 rpm are shown in Figure 1. The values of μ_{max} obtained during the early part of the growth cycle were approximately 1 h^{-1} compared to values of 1.3 h^{-1} for growth on glucose [8], and a maximum enzyme titre of 1700 IU ml^{-1} was obtained. Since at the higher impeller speeds lactose was fully utilised by 55 h, a fermentation was run at 1000 rpm where a pulse of 20 g L^{-1} lactose was added at 45 h, just before the initial lactose was exhausted. It was expected that this addition would delay sporulation, since this event not only causes cessation of growth, but also of enzyme production. However, cell growth and enzyme titre remained unchanged from the control.

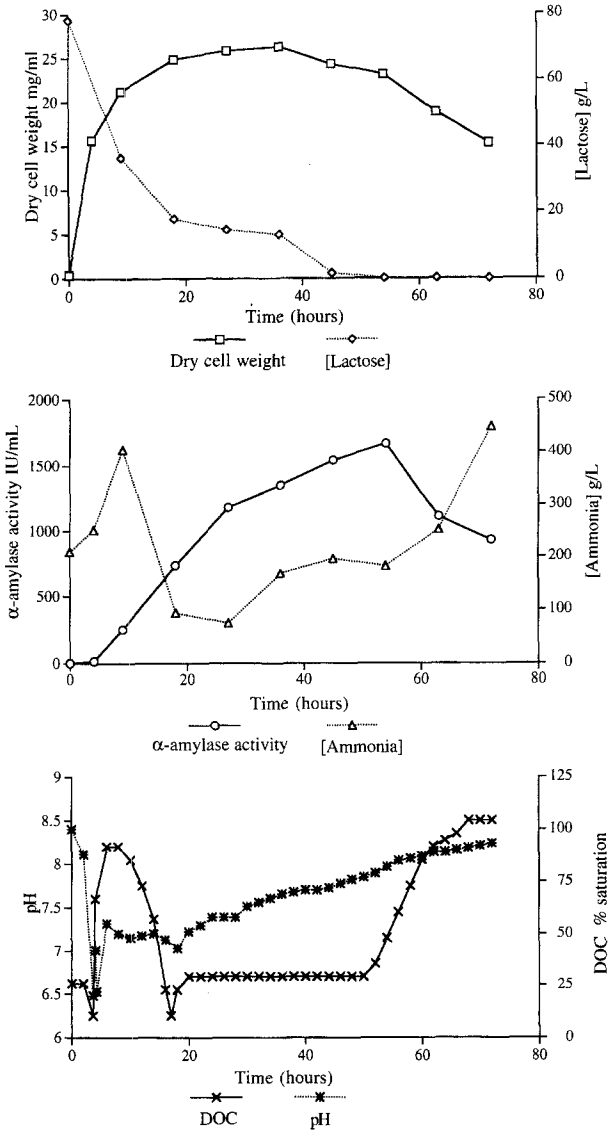

Figure 1 Data for a fermentation carried out at 1000 rpm using SLM.

Having shown that the fermenter system could provide DOC levels to meet the peak oxygen demand of the culture, a development programme was initiated using both shake flask and fermenter experiments to study the effects of a range of nutrient supplementation on growth and enzyme formation in the BSM. In this medium, the organism grew at a reduced level, 7.32 mg ml^{-1} cell dry weight at 36 h, compared to values of 25.19 mg ml^{-1} cell dry weight at 27 h grown on SLM; moreover, no α-amylase was produced in BSM compared to over 1650 IU ml^{-1} in SLM. The amino acids hydroxyproline, proline, glutamine, glutamic acid, arginine and isoleucine, which play an important part in the control of cell turgor pressure associated with growth [13], had a positive effect on growth and enzyme production when added at 0.15 g L^{-1} to BSM. Since hydroxyproline and glutamine could be replaced by proline and glutamic acid respectively, the optimum concentrations of the three amino acids for growth and α-amylase production were found in shaken flask cultures to be 1.0, 1.0 and 0.5 g L^{-1} for glutamic acid, proline and arginine, respectively. Cell dry weight increased to 15.5 mg ml^{-1} and enzyme titre to 545 IU ml^{-1}.

Bacillus sp α-amylase is less stable below pH 6.0, consequently attempts were made to improve the buffering capacity of the BSM. The substitution of mono-basic potassium phosphate by the di-basic salt caused a reduction in growth, but a significant increase in enzyme titre, to values of over 700 IU ml^{-1}, the pH remaining at a level around 6.0. The replacement by the di-basic phosphate caused the precipitation of a calcium salt after sterilisation which could be prevented by addition of 0.5% citric acid to the culture medium, which acts as a chelating agent retaining the calcium in solution. This addition produced a further increase in enzyme levels to 875 IU ml^{-1} with pH values remaining above 6.14. Since the enzyme is known to be a calcium metalloenzyme, it is possible that the increase in calcium solubility and hence availability was the cause of the improvement.

Addition of the bases adenine, thymine, cytosine, guanine and uracil at 10 ppm to BSM increased cell growth and enzyme titre, but when these same additions were made to BSM plus amino acids, cell growth was not affected but enzyme titres fell, except in the case of adenine, when there was no change.

It was thought that the increased growth on complex media was due to the presence of saturated fatty acids, therefore the addition of fatty acid sources to the culture medium was examined. When either corn oil or Tween 80 at 2 ml L^{-1} was added to BSM enzyme production increased; growth was not affected by the corn oil, but

Tween 80 did increase cell dry weight. However, addition of either to the finally developed medium was not useful.

In shake flasks, the addition of inositol at 0.095 g L^{-1} to BSM increased enzyme titres, but had no effect on cell dry weight, whilst *p*-aminobenzoic acid at 1.0×10^{-4} g L^{-1} decreased growth, but increased enzyme levels. The combination of the two vitamins was not however complementary. Unexpectedly, the addition of any other vitamin proved detrimental.

Translation of the results of the shake flask experiments to laboratory scale, commenced with a batch culture using BSM, with an impeller speed of 1000 rpm. The DOC remained above 20% saturation throughout the fermentation. During the first 36 h both growth and lactose uptake rates were low, whilst ammonia uptake was high. Arginine and proline were both exhausted by 36 h, a μ_{max} of only 0.074 h^{-1} was measured at this time with a maximum enzyme titre of 427 IU ml^{-1} found after 45 h, and lactose utilisation remained incomplete even after 72 h. Since pH control was not used, values below 6 were found after 18 h.

Supplementing BSM with the range of vitamins used previously in shake flask experiments resulted in an increased oxygen demand and an increase in μ_{max} to 0.22 h^{-1}. Arginine and proline were depleted within 9 h but the pH fell very rapidly and the cell dry weight fell precipitately after 27 h due to the onset of sporulation. The maximum enzyme titre achieved was less than that reached in the unsupplemented medium, due almost certainly to the more rapid fall in pH.

Provision was therefore made to control pH by the addition, on demand, of 50% v/v ammonia. To maintain an approximate total nitrogen input, the concentration of the di-ammonium hydrogen orthophosphate was reduced to 4 g L^{-1} at which level it was calculated that there was still sufficient phosphate to support the expected biomass. The resulting maximum growth rate was similar to that obtained in the control experiment as were uptake rates of lactose, arginine and proline. The maximum cell density was lower, but the enzyme titre increased to 784 IU ml^{-1}. Since in this experiment the DOC had fallen to 5% saturation, a fermentation using otherwise identical conditions was run with an impeller speed of 1200 rpm. The DOC under these conditions was maintained above 12% saturation and this resulted in an increase in cell dry weight to 22.14 mg ml^{-1}, an enzyme titre of 874 IU ml^{-1} and an increase in the rate of enzyme production, μ_{max} at 0.25 h^{-1} was unchanged. The improvement in enzyme production with increased oxygen transfer led to all further fermentations being carried out at an impeller speed of 1200 rpm.

Under all conditions described above, it was noted that proline and arginine were exhausted from the medium prior to the period where the maximum growth rate was recorded. When the amino acids were added at 22 h, the maximum enzyme titre increased to 1027 IU ml^{-1} and growth continued for a further 9 h, albeit at a low rate (Figure 2). When BSM with vitamins was supplemented with corn oil, μ_{max} was increased to 0.28 h^{-1}, cell dry weight to 24.29 mg ml^{-1} and α-amylase to 949 IU ml^{-1}. The addition of Tween 80 was not tried due to its foaming properties. Even when fermentations were conducted with pH control and vitamins added to BSM, the stationary

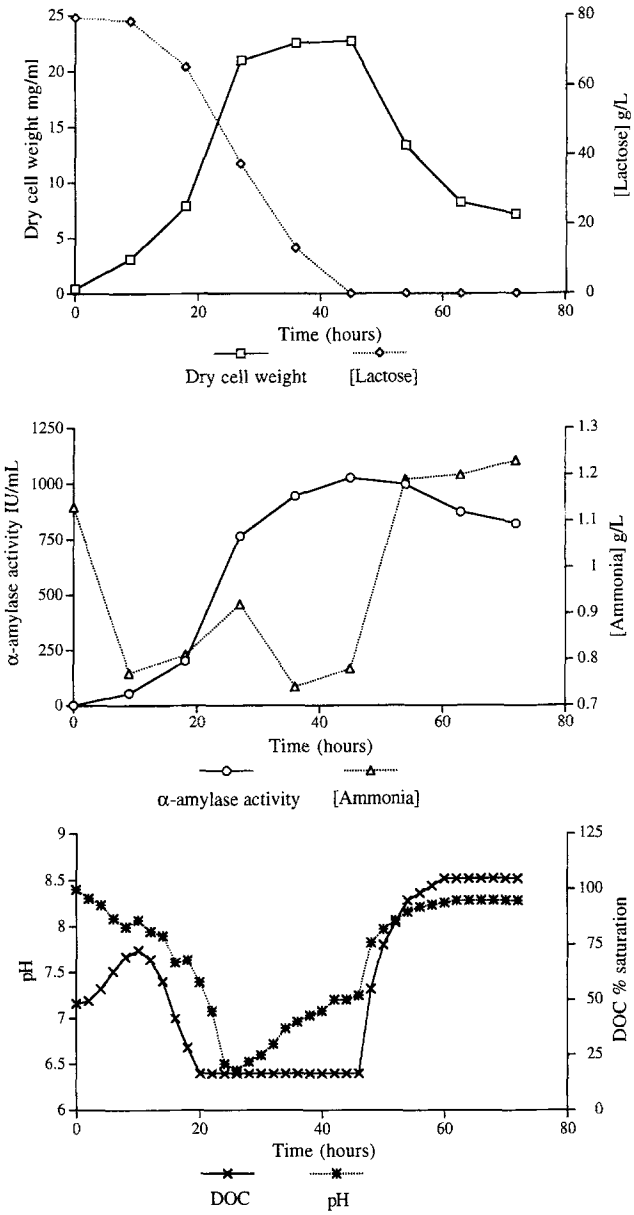

Figure 2 Data for a fermentation carried out at 1200 rpm using BSM, supplemented with vitamins, amino acids with both proline and arginine added after 22 h.

phase occurred before lactose was fully utilised as well as in the presence of excess nitrogen and high levels of DOC. The possibility therefore existed that growth was being limited by other nutrients, perhaps trace elements [1]. To test this assumption, a fermentation was conducted adding trace elements [6]. After 22 h, no effect was noted on any of the parameters measured.

Discussion

The complex SLM provides, not unexpectedly, a culture medium with better growth potential due to its superior buffering capacity and a wider range of nutrients. When either medium was used, the growth phase of the culture terminated when lactose, ammonia and DOC were present in excess, and the addition of known essential growth fac-

tors did not significantly prolong this period of growth. Two possible explanations are either that the cells require a nutrient that is in insufficient supply, or that cytotoxic metabolic products are building up in the cells or more probably in the culture medium. In most cases, the initiation of sporulation is preceded by the depletion of lactose, addition of which prior to full utilisation, can postpone sporulation. Once sporulation is initiated, it is characterised by an immediate rise in culture pH and ammonia concentration.

During fermentations with SLM, the maximum specific growth rate, which is higher than that attained in the synthetic medium, was reached much earlier in the fermentation than that found on BSM. During this period of rapid growth, α-amylase synthesis is at a low level and the rate only increases when growth slows at the onset of the stationary phase. By contrast in BSM, α-amylase production increased with cell density. These findings are in accord with the model for α-amylase regulation [2], in which enzyme synthesis is repressed during rapid growth, either through catabolite repression or due to low levels of mRNA synthesis concerned with the production of exoproteins, increasing 10-fold only at the end of exponential growth. With a slowly metabolised sugar, such as lactose, as the carbon source in a totally synthetic culture medium, the growth rate is never high enough to initiate these constraints. Similar findings on semi-synthetic culture media have been described for *Bacillus subtilis* [5,12], a plasmid-containing bacterium. Another definite advantage of growing cells of *Bacillus* spp on a synthetic medium is the reduction of the extremely severe foaming problems caused by growth on culture media containing high concentrations of amino acids from yeast extracts etc, when the pH is above 7.0. This foaming results from the oxidative deamination of amino acids and the subsequent liberation of oxygen by the activity of catalase [4]. Consequently the quantity of anti-foam used is greatly reduced with the synthetic medium, whilst enzyme production levels are comparable.

Acknowledgements

CJH thanks the University of Birmingham for the award of the Adrian Brown and Bridon Scholarships during the course of this study.

References

1 Brown DE and RN Gaddum. 1988. Linear growth in batch culture caused by conservative trace nutrient limitation. Biotechnol Lett 10: 525–530.
2 Coleman C, S Brown and DA Stormonth. 1975. Accumulation of mRNA for extracellular enzymes as a general phenomenon in *B. amyloliquefaciens*. J Mol Biol 52: 143–148.
3 Dixon K. 1975. Production of alpha-amylase by *Bacillus subtilis*. PhD Thesis, University of Birmingham, UK.
4 Gale EF. 1947. The Chemical Activities of Bacteria. pp. 105–106, University Tutorial Press, London.
5 Liebs P, K Riedel, JP Graba, D Schrapel and U Tischler. 1988. Formation of some extracellular enzymes during the exponential growth of *Bacillus subtilis*. Folia Microbiol 33: 88–95.
6 Martin RS, D Bushel, DJ Leak and BS Hartley. 1992. Development of a synthetic medium for continuous anaerobic growth and ethanol production with a lactate dehydrogenase mutant of *Bacillus stearothermophilus*. J Gen Microbiol 138: 987–996.
7 Mulley EA, CR Stumbo and WM Hunting. 1975. Kinetics of thiamine degradation by heat. J Food Sci 40: 985–986.
8 Pinches A, ME Louwe and TG Watson. 1985. Growth, plasmid stability and alpha-amylase production in batch fermentation using a recombinant *Bacillus subtilis* strain. Biotech Lett 7: 621–626.
9 Sherwood MA. 1969. Enzymes in industry. Process Biochem 4: 279–283.
10 Thayanithy K. 1980. A study of the production of a bacterial alpha-amylase. PhD Thesis. University of Birmingham, UK.
11 Underkofler LA. 1976. Microbial enzymes. In: Industrial Microbiology (Miller BM and W Litsky, eds), pp 128–164, McGraw-Hill, New York.
12 Wei D, SJ Parulekar, BC Stark and WA Weigand. 1989. Plasmid stability and alpha-amylase production in batch and continuous cultures of *Bacillus subtilis* TN106[pAT5]. Biotech Bioeng 33: 1010–1020.
13 Whatmore AM, JA Chudek and RH Reed. 1990. The effects of osmotic upshock on the intracellular solute pools of *Bacillus subtilis*. J Gen Microbiol 136: 2527–2535.