Appl Microbiol Biotechnol (1988) 28:404—408

Applied and Microbiology Biotechnology

Protease production by *Bacillus subtilis* in oxygen-controlled, glucose fed-batch fermentations

Manoj M. Kole, Indira Draper, and Donald F. Gerson*

Alberta Research Council, P. O. Box 8330, Postal Station F, T6H 5X2, Biotechnology Department Edmonton, Alberta, Canada

Summary. An open-loop, on-off control system using the dissolved oxygen level to control a glucose feed was used in a study of growth and production of protease by *Bacillus subtilis* CNIB 8054. With this system, both glucose and oxygen were controlled at low concentrations. In batch fermentations, protease activity in the fermentation broth was maximum when growth had stopped. During oxygen-controlled, glucose fed-batch fermentations, growth and the production of protease activity continued during glucose feeding. Oxygen-controlled, glucose fed-batch fermentations produced more protease activity than batch fermentations, depending upon the set point for dissolved oxygen. These results indicate that control of glucose and oxygen concentrations can result in improvements in protease production.

Introduction

Many bacteria belonging to the genus *Bacillus* excrete large amounts of enzymes into the culture medium. *Bacillus subtilis* is well known for the production of industrially important enzymes such as amylase and protease (Debabov 1982). Bacteria of the genus *Bacillus* are active producers of extracellular proteases, and under most culture conditions, *Bacillus* sp. produce extracellular proteases during the post-exponential and stationary growth phases (Schaeffer 1969; Dawson and Kurz 1969; Kole et al. 1987). Certain species of *Bacillus*, however, produce protease during the exponential growth period (Chaloupka and Kreckova 1966; Chaloupka 1969; Millet and Aubert 1969). The exact mechanisms responsible for the cellular control of protease synthesis are unknown. Production of protease can be inhibited by amino acids, the carbon source or by both nitrogen and carbon sources (Schaeffer 1969; Levisohn and Aronson 1967; May and Elliott 1968).

The majority of the studies of protease production by *Bacillus* have used batch cultivation techniques, where the extracellular environment was constantly changing, with, for example, the concentrations of the carbon source and the nitrogen source constantly decreasing. To maintain optimal conditions, control must be exerted over the concentrations of all relevant substrates. Dissolved oxygen control can be obtained through variation in aeration or agitation rates, carbon source concentration can be maintained by time-dependent or various open-loop controlled feeding strategies; and, ammonium concentration control can be effected by closed-loop, separated sensor strategies (Thompson et al. 1985; Kole et al. 1985; Oguztoreli et al. 1986; Kole et al. 1986). In our experiments, an indirect control of glucose feed rate is presented for the production of protease by *B. subtilis*. The glucose control system is based on the dissolved oxygen levels, which control the on-off activation of a glucose feed pump. The results obtained with this system are compared with a simple fed-batch fermentation. Oxygen controlled, glucose fed-batch fermentations of *B. subtilis* resulted in higher final protease concentrations than were obtained in batch fermentations.

Materials and methods

Microorganism, media and fermentations

The organism used in these experiments was *Bacillus subtilis* NCIB 8054, obtained from the culture collection of the De-

* *Present address:* Connaught Laboratories Ltd., 1755 Steeles Avenue West, Willowdale, Ontario, Canada, M2R 3T4

Offprint requests to: M. M. Kole

partment of Microbiology, University of Alberta. The culture was maintained on a slant on trypticase soya agar at 4° C. The basal medium used for all fermentation was synthetic M9 medium with some modifications. The medium has the following constituents (g/l unless otherwise noted): Na_2HPO_4, 6.0; KH_2PO_4, 3.0; $(NH_4)_2SO_4$, 2.64; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2$, 0.015; glucose, 5.0; thiamine HCl, 1 mg/l; $CuSO_4$, 3 mg/l; $FeCl_3$, 3 mg/l; $MnSO_4$, 3 mg/l; $ZnSO_4$, 3 mg/l. Glucose, $MgSO_4$, and $CaCl_2$ were sterilized separately and added to the cooled medium. The trace metals and thiamine HCl were filter sterilized and added to the fermentor separately. Inoculum (500 ml) was grown to exponential phase at 35° ± 1° C on a gyrorotary shaker.

Fermentations were performed in a standard design fermentor (Chemap A.G., Volketswil, Switzerland) with a 14 l vessel containing 10 l of medium. Temperature was controlled at 35° C. Mixing was with three, 6-bladed flat-blade impellers operating at 500 rpm with an aeration rate of 3 l air/min. The oxygen transfer rate under these conditions was found to be 50 mM/L/h by purging the tank with oxygen-free N_2 and recording the reoxygenation of cell-free medium with an IL530 polaragraphic oxygen electrode system (Instrumentation Laboratories, Andover, Mass. USA). The pH of the culture broth in the fermentor was maintained by an Ingold sterilizable pH electrode attached to a Chemcadet pH/millivolt controller (Cole-Parmer, Chicago, Ill. USA), with set point limits of pH 6.9 and 7.2. The pH was controlled automatically by adding either 4 N HCl or 4 N NaOH by the on-off activation of peristaltic pumps.

Samples were taken every hour throughout each fermentation. Biomass was determined spectrophotometrically ($O.D._{620}$) and converted to grams of cell dry weight (g.c.d.w.) by comparison with a standard conversion curve which was prepared for this strain of *B. subtilis*. Microscopic examinations of the culture were made periodically. Total glucose was determined in cell free medium by the method of Dubois et al. (1956) and with a YSI glucose analyzer, model 27 (Yellow Springs Instrument Co. Inc., Ohio, USA). The ammonium ion concentration of each sample was also determined with an Orion model 95-12 ammonia gas electrode (Orison, Cambridge, Mass., USA), using the manufacturer's recommended sample preparation and calibration techniques. Extracellular protein was determined by the Bio-Rad method.

Protease estimation

Protease activity was assayed using the Azocasein method. In this method, 1 ml of the crude bacterial broth was placed into a test tube and 1 ml of 0.5% (w/v) azocasein (Sigma Chemical Co., St. Louis, Mo., USA) in tris-HCl buffer (200 mM at pH 7.4) was added. This was incubated for 60 min at 30° C. The reaction was stopped by adding 2.0 ml of 10% (w/v) trichloroacetic acid. This was centrifuged at 1500 *g* for 10 min. The supernatant fluid was removed and the O.D. at 380 nm was measured. The enzyme activity was determined from a standard curve which had been made for the enzyme papain (Sigma Chemical Co., USA). The activity of protease in the bacterial supernatants is expressed in terms of units equivalent to the activity of a given weight of papain (1 unit = 1 mg papain). This method assays both neutral and alkaline proteases in the medium and no attempt was made to distinguish between these activities at this stage of our investigations.

Glucose control system

The open-loop, on-off glucose control system is shown diagramatically in Fig. 1. A dissolved oxygen (D.O.) probe in the fermentor was connected to an amplifier and an on-off controller. The amplifier was calibrated to give a reading of 100% saturation when air was sparged through uninoculated medium in the fermentor. After inoculation, the D.O. level slowly decreased, as the demand for oxygen increased during growth. When the glucose was exhausted from the medium, the bacteria became glucose limited and decreased their oxygen demand, resulting in an immediate increase in the D.O. level. At this time, the controller was turned on. When the dissolved oxygen level exceeded the set point, the pump was activated and glucose was supplied to the fermentor until increased oxygen demand brought the D.O. level below the set point, and the pump was automatically turned off. The concentration of the glucose feed solution was 250 g/l. With this system, the D.O. level was controlled at an average value close to the set point by a glucose feed which acted to increase the oxygen demand of the culture. The dissolved oxygen level was monitored with a dual channel recorder.

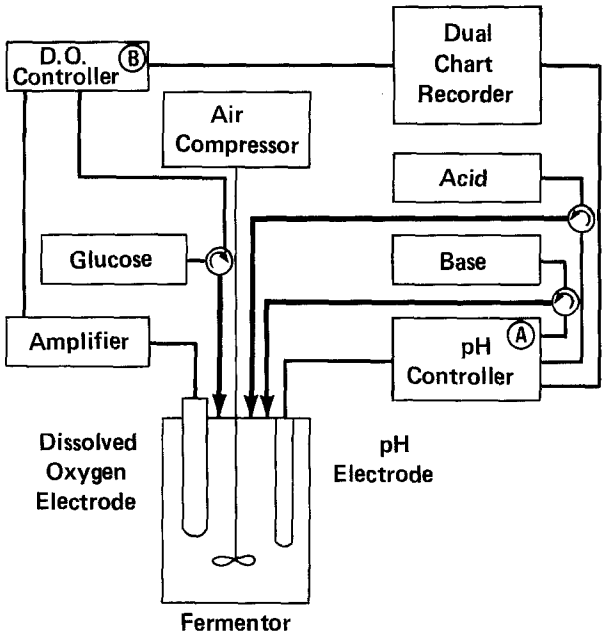

Fig. 1. Oxygen-controlled, glucose fed-batch fermentation system. Controller A maintains the pH in the fermentor. Controller B initiates the glucose feed when the dissolved oxygen concentration in the fermentor exceeds the set point

Results and discussion

a. Batch cultivation

Figure 2 gives the results of a batch cultivation of *B. subtilis* for protease production. Exponential phase terminated at approximately 7 h when glucose was exhausted and was followed by a decline phase, during which most of the protease activity appeared in the medium. The biomass decrease during the decline phase was due to death and lysis of the cells as observed by viable and micro-

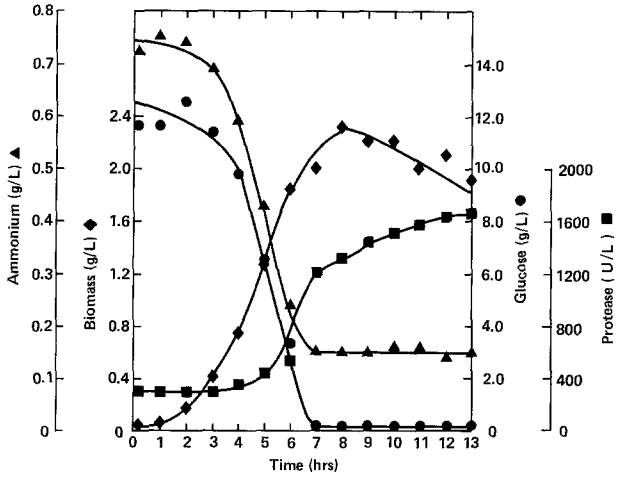

Fig. 2. Batch fermentation of *B. subtilis*. (◆) Apparent biomass determined by optical density (g cell dry wt/l); (▲) ammonium (g/l); (●) glucose (g/l); (■) protease (U/l)

scopic counts. There was no change in the total extracellular protein, but protease production increased in stationary and death phase. There was no spore formation of the *Bacillus* sp. during this study as determined by microscopic observation. Jensen (1972) found enhanced protease production in a two-stage fermentation of *Bacillus*. Both of these results indicate that stationary phase conditions favour protease production in *Bacillus*. Growth parameters for batch cultivation are given in Table 1.

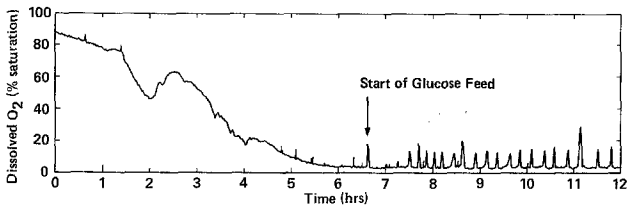

Fig. 3. Trace of dissolved oxygen levels during an oxygen-controlled, glucose fed-batch fermentation. Dissolved oxygen levels of 10%—15% saturation initiated feeding with glucose

b. Oxygen-controlled, glucose fed-batch cultivation

Control System: The glucose control system described diagramatically in Fig. 1 and in "Materials and methods", results in a pattern of dissolved oxygen levels such as the example given in Fig. 3. The sharp spikes result from the depletion of glucose, followed by a sharp rise in D.O. due to decreased oxygen demand, followed by a glucose feed period with subsequently reduced D.O. levels due to increased oxygen demand. This method is very simple, and results in the control of the average glucose concentration in the fermentor at low levels. During periods of rapid glucose utilization, the oxygen level in the fermentor was also quite low, however, the average D.O. concentration was probably close to the set-point.

The results of an oxygen-controlled, glucose fed-batch fermentation are given in Fig. 4. In this fermentation, once the initial supply of glucose

Table 1. Growth parameters and protease production of *Bacillus subtilis* in different fermentation conditions

	Uncontrolled batch fermentation	Oxygen controlled glucose fed-batch fermentations	
		20% saturation	40% saturation
Maximum apparent growth rate μ (h^{-1})	0.91	0.80	0.91
Maximum biomass production (g/l)	2.30	1.85	2.30
Maximum biomass productivity (g/l-h)	0.18	0.14	0.21
Total glucose utilization (g/l)	12.94	12.95	11.00
Average glucose utilization rate (g/l-h)	1.00	1.00	1.00
Total ammonium utilization (g/l)	0.60	0.62	0.62
Average ammonium utilization rate (g/l-h)	0.05	0.05	0.06
Final protease concentration (U/l)	1600	1800	3000
Average protease productivity (U/l-h)	0120	0140	0270
Yp/glucose[a]	120	140	270
Yp/NH_4^+ [b]	2660	2900	4840

[a] Units of protease produced/g of glucose consumed
[b] Units of protease produced/g of NH_4^+ consumed

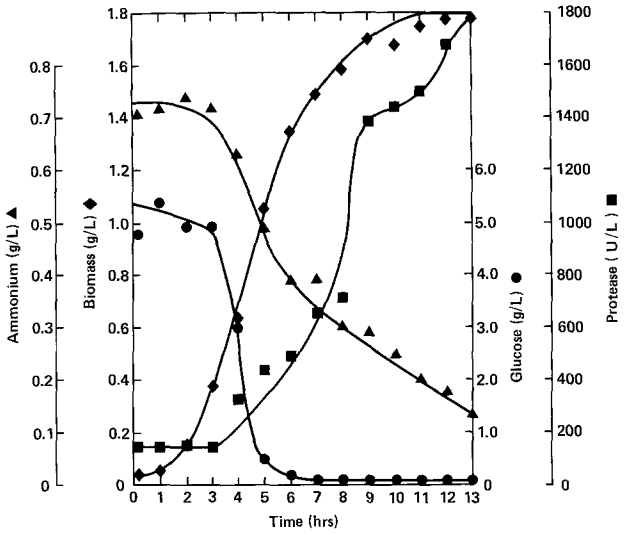

Fig. 4. Oxygen-controlled, glucose fed-batch fermentation of *B. subtilis* with a D.O. set point of approximately 20% saturation. (◆) Apparent biomass determined by optical density (g cell dry wt/l); (▲) ammonium (g/l); (●) glucose (g/l); (■) protease (U/l)

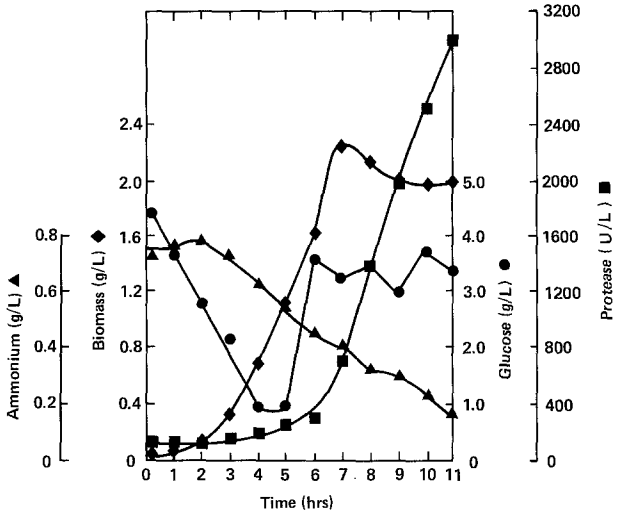

Fig. 5. Oxygen-controlled, glucose fed-batch fermentation of *B. subtilis* with a D.O. set point of approximately 40% saturation (see text). (◆) Apparent biomass determined by optical density (g cell dry wt/l); (▲) ammonium (g/l); (●) glucose (g/l); (■) protease (U/l)

was exhausted and dissolved oxygen concentration increased, a glucose feed was initiated whenever the dissolved oxygen level exceeded 20% saturation. Approximately the same amount of glucose was used under these conditions as in the batch fermentation described above. During the period of glucose feed, there was a steady increase in both biomass and protease concentrations. However, the final biomass was less than that achieved in batch cultivation. The reason for this may be the relatively low oxygen concentration available to the organism during periods when there was ample glucose. Low oxygen concentrations have been shown to inhibit protease production (Fabian 1970). Other fermentation parameters for oxygen-controlled, glucose fed-batch fermentations of this type are given in Table 1.

Automatic control of glucose feed with a high dissolved oxygen concentration set point (>35% saturation) proved difficult with this equipment. In order to maintain the dissolved oxygen concentration at approximately 40%, the agitation rate was increased from 500 to 1000 rpm to increase the oxygen transfer rate. The prime effect of increased agitation rate is to increase oxygen transfer rate and agitation rate control has been commonly used as an open-loop approach to D.O. concentration control. There are, of course, secondary effects of variation in agitation rate on the mass transfer rate of dissolved substrates into the cells, since metabolic rates are dependent on the concentrations of all substrates including oxygen available to the cell. In addition, with high set points the automatic controller tended to deliver excessive glucose to the fermentor. To solve this problem, a manual override was used to more effectively maintain the D.O. levels close to the set point.

Oxygen controlled glucose fed-batch fermentations with a high D.O. set point produced more biomass and protease than similar fermentations with a lower set point (Fig. 5). Final protease concentration in oxygen-controlled glucose fed-batch fermentations with the D.O. set point of 40% was approximately 1.6 times more than cultivation with a D.O. set point of 20%, and 1.9 times more than in batch cultivations. The yields of protease on both glucose and ammonium were highest in oxygen-controlled glucose fed-batch fermentations with a D.O. set point of 40% (Table 1).

These results indicate that fed-batch fermentations which optimize the steady-state concentrations of both glucose and oxygen provide better conditions than batch fermentations for protease production from *Bacillus*.

Acknowledgements. We thank Dr. W. Leps and Dr. B. Thompson for their useful comments during the preparation of this manuscript.

References

Chaloupka J, Kreckova P (1966) Regulating the formation of protease in *Bacillus megaterium*. I. Effect of amino acids in enzyme formation. Folia Microbiol 11:82—88

Chaloupka J (1969) Dual control of megateriopeptidase synthesis. Ann Inst Pasteur 117:631—636

Dawson PSS, Kurz WGW (1969) Continuous phased culture — a technique for growing, analyzing and using microbiol cells. Biotech Bioeng 11:843—851

Debabov VG (1982) The industrial use of Bacilli. In: Dubnau D (ed). The molecular biology of the Bacilli, vol. 1. Academic Press, New York, p 331

Dubois M, Gilles KA, Hamilton JK, Gebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350—361

Fabian J (1970) Synthesis of extracellular protease by *Bacillus pumilus*. Folia Microbiol 15:160—168

Jensen DE (1972) Continuous production of extracellular protease by *Bacillus subtilis* in a two stage fermentor. Biotech Bioeng 14:647—662

Kole MM, Thompson BG, Gerson DF (1985) Ammonium concentration control in fed-batch fermentations of *Saccharomyces cerevisiae*. J Ferment Technol 63:121—125

Kole MM, Ward D, Gerson DF (1986) Simultaneous control of ammonium and glucose concentrations in *Escherichia coli* fermentations. J Ferment Technol 64:233—238

Kole MM, Draper I, Gerson DF (1987) Production of protease by *Bacillus subtilis* using simultaneous control of glucose and ammonium concentrations. J Chem Tech Biotech (in press)

Levisohn S, Aronson AI (1967) Regulation of extracellular protease production in *Bacillus cereus*. J Bacteriol 93:1023—1030

May BK, Elliott WH (1968) Characteristics of extracellular protease formation by *Bacillus subtilis* and its control by amino acid repression. Biochim Biophys Acta 157:607—615

Millet J, Aubert JP (1969) Megateriopeptidase, an extracellular protease of *Bacillus megaterium*. III. Biosynthesis and physiological role. Ann Inst Pasteur 117:461—473

Oguztoreli MN, Ozum B, Gerson DF (1986) Optimal control of substrate concentrations in bioreactors with separated sensors. Biotech Bioeng 28:952—959

Schaeffer P (1969) Sporulation and the production of antibiotics, exoenzymes, and exotoxins. Bacteriol Rev 33:48—71

Thompson BG, Kole MM, Gerson DF (1985) Control of ammonium concentration in *Escherichia coli* fermentations. Biotech Bioeng 27:818—824

Received June 15, 1987/Accepted January 6, 1988