α-Amylase Production in Fed Batch Cultivation of *Bacillus caldolyticus*: An Interpretation of Fermentation Course Using 2-D Gel Electrophoresis

J. Bader, B. Neumann[*], K. Schwab, M. K. Popovic[+], C. Scheler[*], and R. Bajpai[**]
Studiengang Biotechnology, Fachbereich V, TFH-Berlin, Seestraße 64, 13347 Berlin, Germany, popovic@tfh-berlin.de, Fax: 030 4504 3959
[*]Proteome Factory AG, Dorotheenstr. 94, 10117 Berlin, Germany
[**]Department of Chemical Engineering, University of Missouri-Columbia, W2017 EBE, Columbia, MO 65211, USA

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The conditions for increased production of thermostable α-amylase from *Bacillus caldolyticus* DSM 405 were investigated. Preliminary experiments in batch shake flasks led to an optimized initial cultivation medium. Shake flask experiments in extended-batch and in fed-batch mode of operation indicated that the α-amylase production was enhanced by continuous feeding of starch. The activity of the α-amylase with optimized initial medium in batch-operated shake flasks was 5.7 U mL^{-1} compared to 15.4 U mL^{-1} in the extended-batch culture and 21 U mL^{-1} in fed-batch culture. The improvements were achieved by avoiding any excess of starch in medium that led to accumulation of glucose followed by acetate formation. Adding casitone as the second component of the feeding solution in an aerated and agitated fed-batch bioreactor (3-liter working volume) led to an increased α-amylase activity of up to 163.7 U mL.$^{-1}$

All phases of cultivation were analyzed using 2D-gel electrophoresis in combination with nano LC-ESI-MS/MS for identification of altered proteins. Pyruvate kinase, 6-phosphofructokinase, GltC, anti-sigma F factor, glycogen synthase and several important variable proteins were detected. With help of these results, potential improvements of a two-component feeding strategy are discussed.

Key words:
Bacillus caldolyticus, α-amylase, thermophilic microorganism, fermentation, 2D-gel-elektrophoresis

Introduction

α-amylases are endoenzymes that randomly hydrolyze α-1,4-glycosidic bonds. These enzymes are commonly used in liquefaction of starch. Specifically, the enzymes produced by thermophiles are in high demand due to their ability to withstand high process temperatures and longer shelf life.[1] Several thermophilic microorganisms are known to produce α-amylases. These include *Bacillus coagulans*, *Geobacillus stearothermophilus*, *Bacillus brevis*, *Bacillus acidocaldarious*, and *Bacillus thermoamyloliquefaciens*.[2] *Bacillus caldolyticus*, an aerobic caldo-active microorganism, is particularly attractive because it grows optimally at 70 °C[3] and its α-amylase retains considerable hydrolytic activity at temperatures as high as 105 °C.[4] α-Amylase of *Bacillus caldolyticus* requires less calcium for its stability compared to α-amylases of several other thermophiles.[3] However, few publications have dealt with production of amylase by this microorganism.

Emanuilova and *Toda*[5] studied kinetics of growth and product formation by *Bacillus caldolyticus* DSM405 and reported that production of α-amylase by this organism is growth-associated. Starch was found to be an inducer of α-amylase in this strain. Continuous cultivation of the cells under operating conditions where residual concentrations of glucose and starch in broth were undetectable produced significant improvement in α-amylase titers.[5] *Cheng* et al.[6,7] conducted continuous cultivations of *Bacillus caldolyticus* (strain SP) in which maltose induces enzyme production and found that a mutant, *Bacillus caldolyticus* strain M1 with improved enzyme productivity dominated the culture. *Schwab*[8] pursued this reasoning by studying production of α-amylase by *Bacillus caldolyticus* DSM405 under batch, extended-batch, fed-batch, and continuous culture operation. Cultivations with different initial starch concentrations and those with different rates of feeding of starch showed that this strain could produce acetic acid and accumulation of acetic acid in broth associated with low α-amylase activity in broth. These results suggest that a

[+]The author to whom the correspondence should be directed.

desirable mode of reactor operation for high-productivity, high-activity production of α-amylase by *Bacillus caldolyticus* should minimize formation of acetic acid. The production of acetic acid under an excess glucose is also known by the genetically related *Bacillus stearothermophilus*.[9]

Fed-batch cultivations are ideally suited for high-density cultivations. Several authors have investigated strategies for operation of high-density fed-batch cultures targeting maximization of one product while minimizing formation of another product.[10–16] Of these, the method proposed by *Akesson* et al.[11,12] is particularly interesting for production of α-amylase by *Bacillus caldolyticus* because (a) it involves minimization of acetic acid production, (b) it uses response of a dissolved oxygen probe to temporary changes in feed rate to detect production of acetic acid, and (c) it does not require previous knowledge of system parameters.

In this paper, batch, extended batch and fed-batch cultivations of *Bacillus caldolyticus* DSM405 are reported. Metabolic status of the cells during fed-batch operation was determined using the strategy proposed by *Akesson* et al.[12] and feed rates were manipulated accordingly. Changes in cell's proteome under different conditions were monitored by 2-D gel electrophoresis and these are also discussed. Since there is no published information concerning the effect of culture conditions on enzymatic activities of *Bacillus caldolyticus* cells, this information served as an additional window into the nature of limiting nutrients during reactor operation and their impact on cell behaviour.

Materials and methods

Strain and media:

The bacterial strain *Bacillus caldolyticus* DSM 405[17] was used in this study. The media compositions, unless stated otherwise, were: for batch cultivation – 2.0 g L^{-1} casitone, 1.0 g L^{-1} Zulkowsky starch (Merck), 0.05 g L^{-1} KH_2PO_4, 0.25 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.03 g L^{-1} $FeSO_4 \cdot 7H_2O$, 1.57 mg L^{-1} $MnCl_2 \cdot 4H_2O$, and 0.1 g L^{-1} $CaCl_2 \cdot 2H_2O$; for extended batch and fed batch cultivations – same as in batch, except that the concentration of starch was reduced to 0.5 g L^{-1}. Initial pH of the media was 7.0. $MnCl_2 \cdot 4H_2O$ and $CaCl_2 \cdot 2H_2O$ were sterilized together but separately from the rest of the salts. Starch solution was also sterilized separately.

Culture conditions:

The batch- and extended-batch fermentations were conducted in 500 mL Erlenmeyer flasks at 70 °C and n = 150 min^{-1}. In extended-batch fermentations, starch was added to the medium in the Erlenmeyer flasks in the form of 10 g L^{-1} aqueous solution once every hour to increase starch mass concentration in broth by 0.1 g L^{-1} starting with the time when glucose depletion was noticed. In fed-batch fermentations, a 10 g L^{-1} aqueous solution of starch was fed to the bioreactor at a rate of the Γ = 0.1 g L^{-1} h^{-1}. Some fed-batch cultivations were also conducted in Erlenmeyer flasks. Herein, feeding was accomplished by Amersham peristaltic pumps P-1 (GE Healthcare, Wisconsin, U.S.A.).

A 7-liter Biostat E-Fermenter (Sartorius BBI Systems, Melsungen, Germany) with an initial operating volume of 3 L was used for further fed-batch experiments. At the start, the stirring speed was n = 500 min^{-1}, and airflow rate was Q = 0.5 L^{-1} min^{-1}. Stirrer speed and airflow were increased during fermentation to keep p_{O_2} above 50 % of oxygen solubility in water in equilibrium with air at p = 1 bar pressure and 70 °C temperature. This concentration of dissolved oxygen was chosen because of the high oxygen demand of *B. caldolyticus* and in regard to the low solubility of oxygen at 70 °C. The solubility of oxygen was calculated using the following relationship.[18]

$$\gamma_{O_2} = 14.674 - 13.644 \log\left(1 + \frac{T}{12.8}\right)$$

pH was controlled at 7.0 ± 0.1 using 1 mol L^{-1} KOH or 1 mol L^{-1} phosphoric acid solutions. Silicon oil was used to control foam. The feeding rates and the composition of feed solutions have been described in the text. Operating strategy for the Biostat fermenter was to increase the feed rate as long as acetate excretion was negligible. As per *Akesson* et al.[12] acetic acid production in cells is related to respiratory system overload which can be determined by superimposing short (1 min) pulses (up as well as down) on the feed rate. If the dissolved oxygen probe responds downward to pulse-up of feed rate and upward to pulse-down of feed, there is no overload in cells under the operating conditions and no acetic acid production should occur. In this case, the feeding rate was increased linearly.

The respiratory metabolism is supposed to be just saturated (point of 'onset of production of acetic acid') when the probe responds to pulse-down but not to pulse-up of feeding rate. Failure of the probe to respond to either of the pulses shows overloaded respiratory system and acetic acid production by the cells. This strategy was used to determine the state of the system and to control the rate of feeding by an Amersham peristaltic pump P-1. This pump was operated by a PC-interfaced controller and software that permitted ± 20 % modifi-

cations of the feeding rate for the above mentioned duration of pulses.

Analytical procedures:

Cell density was monitored as optical density at a wavelength of λ = 600 nm with Philips PU 8625 UV / VIS spectrophotometer (Philips GmbH, Hamburg, Germany). Optical density OD was converted into cell dry weight by

$$\text{cell dry mass concentration (g L}^{-1}) = \text{OD}_{600\ \text{nm}} \cdot 0.33$$

Starch mass concentration in cell-free broth was analyzed by adding 700-μL water and 15 μL of iodine-solution (w = 4 %) to 300 μL of the sample supernatant and measuring light absorption E (extinction) at 600 nm using a UV-VIS-Photometer. The starch-concentration (γ_{st}, g L^{-1}) was calculated using a calibration curve prepared from solutions of known concentrations of starch:

$$\gamma_{st} = (E - 0.0423)/1.2522$$

Glucose mass concentration was measured with a Glucose-kit [Roche Diagnostik, Mannheim, Germany Kit-No. 0 716 251].

α-amylase activity was measured using a modified *Manning* and *Campbell*[19] method: a mixture of 20 μL culture supernatant, 20 μL starch solution (w = 1 %), and 20 μL 1 mol L^{-1} sodium acetate buffer pH 5.4 was incubated at 70 °C for 10 min. 1 mL water and 15 μL iodine solution (γ = 30 g L^{-1}) were added to the incubated mixture after cooling on ice, and absorbance was measured at 580 nm. As a reference, 20 μL fresh medium was used instead of 20 μL supernatant.

Acetate was assayed with the Acetate-Kit [Roche Diagnostik, Mannheim, Germany Kit-No. 10 148 261 035].

For 2-D gel electrophoresis, cells were separated from medium by centrifugation, washed twice with Tris KCl (0.1 mol L^{-1}, pH 7.3), and stored at –80 °C prior to NEPHGE separation. For NEPHGE, the cells were disrupted by three freeze-thaw cycles, and centrifuged for 30 min at a_c = 150 000 · g. Urea (9 mol L^{-1}), DTT (70 mmol L^{-1}), ampholytes pH 2–11 (ρ = 2 %) and CHAPS (w = 1 %) were added to supernatants. NEPHGE and SDS-PAGE were performed according to Proteome Factory's (Proteomefactory AG, Berlin, Germany) Large Gel Protocol (20 · 30 cm). Spots were excised and applied to tryptic in-gel digest. ESI-MS/MS of tryptic peptides was performed using Bruker's Esquire 3000+ (Bruker BioSpin GmbH, Rheinstetten, Germany) ion trap mass spectrometer coupled to an Agilent 1100 nanoLC (Agilent Technologies, Inc, Palo Alto, USA) instrument.

Results and Discussion

Experiments in shake flasks

Results of the experiments conducted in shake flasks showed that final activity of α-amylase in broth is influenced by the nature of reactor operation. The maximum α-amylase activities obtained in shake flasks under different operations are presented in Fig. 1.

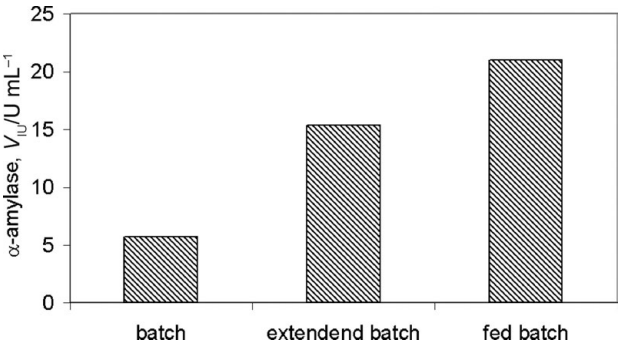


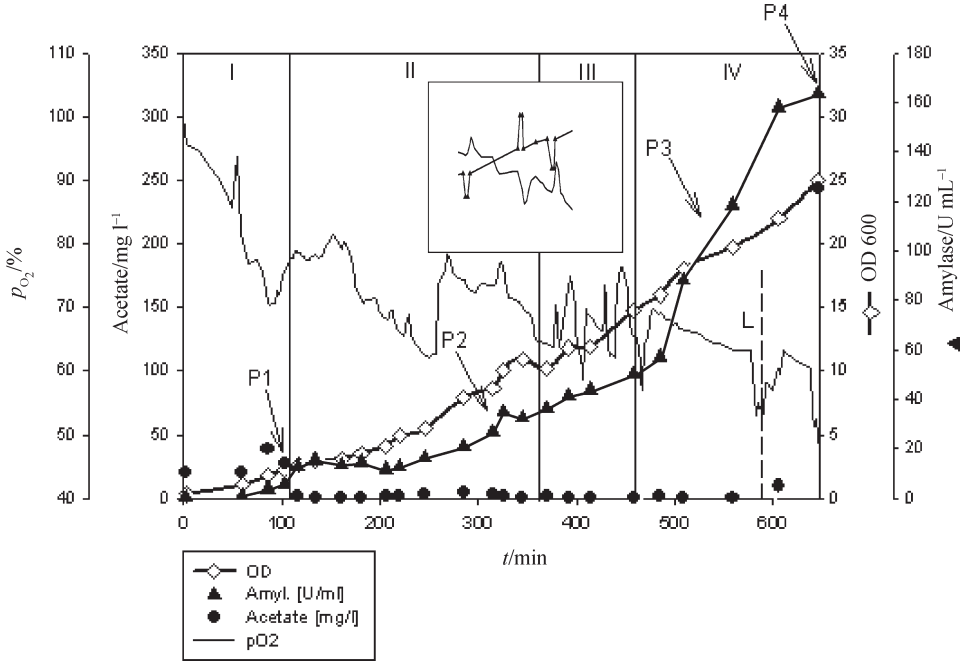
Fig. 1 – *Final amylase activities in shake flasks*

The best activity during batch fermentation was 5.7 U mL^{-1} using medium composition stated under Materials and Methods. In extended- and fed-batch cultivations in shake flasks, initial concentration of starch was reduced to γ_{st} = 0.5 g L^{-1} to reduce accumulation of glucose and acetic acid. 15.4 U mL^{-1} were obtained in extended batch mode with an effective feed rate 0.1 g L^{-1} h^{-1} starch. During fed-batch cultivation in shake flasks, 21 U mL^{-1} amylase activity was obtained with a constant but continuous feed rate of Γ = 0.1 g L^{-1} h^{-1} starch.

Experiments in Biostat E-fermenter

The fed-batch operation in the Biostat E-fermenter with a starch feeding rate of 0.1 g L^{-1} h^{-1} resulted in improved cell growth and α-amylase activity of 70 U mL^{-1} after 780 min of fermentation, ostensibly due to improved oxygen transfer capacity in the fermenter. During the Biostat fermenter operation, a steep increase of p_{O2} was observed in early stage of fed-batch phase. Addition of a pulse of 5 g casitone in the fermenter at this time resulted in a drop in p_{O_2} and increased cell growth rate. This suggested that nitrogen was very likely limiting at this point in the fed-batch culture. As a consequence, a two-component feed solution containing casitone and starch was utilized in later experiments.

Results of a modified fed-batch experiment in the Biostat fermenter (3-L initial operating volume) are shown in Fig. 2. The reactor operation involved a batch phase and three fed-batch phases. During



F i g . 2 – *Production of α-amylase by Bacillus caldolyticus in fed-batch Biostat fermentor with dissolved oxygen and pH control. P1- P4 are samples for proteome analysis. "L" marks onset of casitone limitation in phase IV. The inserted window shows the p_{O_2}-probe reaction (line) to the up and down-pulses of feeding rate (black triangle).*

the fed-batch phases, feeding rate was increased in steps following the strategy of *Akesson* et al.[12]

In phase I, the cells were grown in batch mode starting with a medium containing 0.5 g L^{-1} starch, 2 g L^{-1} casitone and other salts as specified earlier. During this period, dissolved oxygen tension dropped to 70 % saturation and detectable amounts of acetic acid were observed in broth. Towards the end of phase I, acetic acid started to be consumed and dissolved oxygen concentration tended upwards. Amylase activity at the end of phase I was 15 U mL^{-1}.

Feeding of nutrients was initiated starting at 105th min (Fig. 2, phase II). In phase II, the feed solution contained 10 g L^{-1} starch and 40 g L^{-1} casitone. Casitone was included in feed medium due to the suspected nitrogen limitation mentioned above and the ratio of casitone to starch was kept same (ζ = 4:1) as in batch medium. Initial feeding rate was Q = 0.5 mL min^{-1}. This corresponded to feeding rates of Γ = 0.1 g L^{-1} h^{-1} for starch and 0.4 g L^{-1} h^{-1} for casitone. Following the strategy of *Akesson* et al.,[12] feeding rate was raised in steps to 3.6 mL min^{-1} by 370th min. During this phase, acetic acid concentration in broth reduced to non-detect levels and remained so in spite of steadily increased feeding rate. No accumulation of starch was noticed either while amylase activity increased steadily from 15 U mL^{-1} to ~ 40 U mL^{-1}.

During phase II, down-pulses of feeding rate resulted in an upward response of dissolved oxygen probe. However, up-pulses of feeding rate did not elicit any response from p_{O_2} probe. According to *Akesson* et al.,[12] this should have indicated 'onset of acetate production'. However, no accumulation of any starch or acetic acid could be recognized in broth in spite of increasing feed rate of starch and casitone. This indicated a more complex response of our system to feeding of the two-component mixture than suggested by *Akesson* et al.[12]

A response of pH probe to pulse-up of feed rate was noticed. It was suspected that casitone may have been accumulating in broth during this period and it was perhaps also used as a carbon source. Increased consumption of phosphoric acid for pH-correction was an indication of the deamination reactions that precede utilization of amino acids as carbon sources.

To avoid further accumulation of casitone in broth, its concentration in the feed solution was reduced to 20 g L^{-1} in phase III (Fig. 2). Starch concentration was retained at 10 g L^{-1}. The feeding rate at the start of phase III (370th min) was Q = 3.6 mL min^{-1} and it was slowly raised to 7.2 mL min^{-1} by 470th min without invoking any accumulation of acetic acid or starch. Optical density and amylase activity in broth increased steadily during phase III of operation. Both pulse-up and pulse-down experiments invoked the expected response by the dissolved oxygen probe, suggesting that the respiratory metabolism of the cells was not saturated. Dur-

ing this period, amylase activity increased to ~50 U mL^{-1}.

Since starch is an inducer for amylase production in cells, starch concentration in the feed was increased to γ = 15 g L^{-1} in phase IV (Fig. 2). At the same time, the mass concentration of casitone in feed was reduced to 10 g L^{-1} in an attempt to reduce cell growth rate and avoid oxygen transfer limitation. The feeding rate was kept constant at 7.2 mL min^{-1}. Amylase activity increased rapidly from ~50 U mL^{-1} at the start of phase IV (470th min) to 150 U mL^{-1} at 600th min and finally peaked up at 163.7 U mL^{-1} (Fig. 2 – part IV). Obviously the ratio of casitone and starch has a very large influence on α-amylase expression a similar observation was found by *Skolpap* et al.[15] Up to this point of fermentation no acetic acid was observed and pH signal was steady. This indicates that no starch accumulation occurred. At 610th min (Fig. 2 "L"), a rapid drop in p_{O_2} and build-up of acetic acid was observed in the broth. Concomitantly, amylase activity also started to drop. Dissolved oxygen levels could be restored to >50 % saturation by increasing air flow rate from 5 L min^{-1} to 12 L min^{-1} but it could neither stem further increase in concentration of acetic acid nor arrest drop in amylase activity. The experiment was terminated at 670th min.

The fermentation mode according to *Akesson* et al.[12] for *E. coli* fermentation and single glucose feeding is also possible for the two component-feeding during the fermentation of the thermophilic *B. caldolyticus*. In addition to the p_{O_2} probe signals corresponding pH-probe reaction were used for feed control. Congruent response of p_{O_2} and pH probes to the up and down-pulses in feeding rate indicate approaching of optimal nutrition concentrations in broth.[20] In this way the accumulation of starch, glucose and/or the inhibitory metabolite acetate[9] could be avoided.

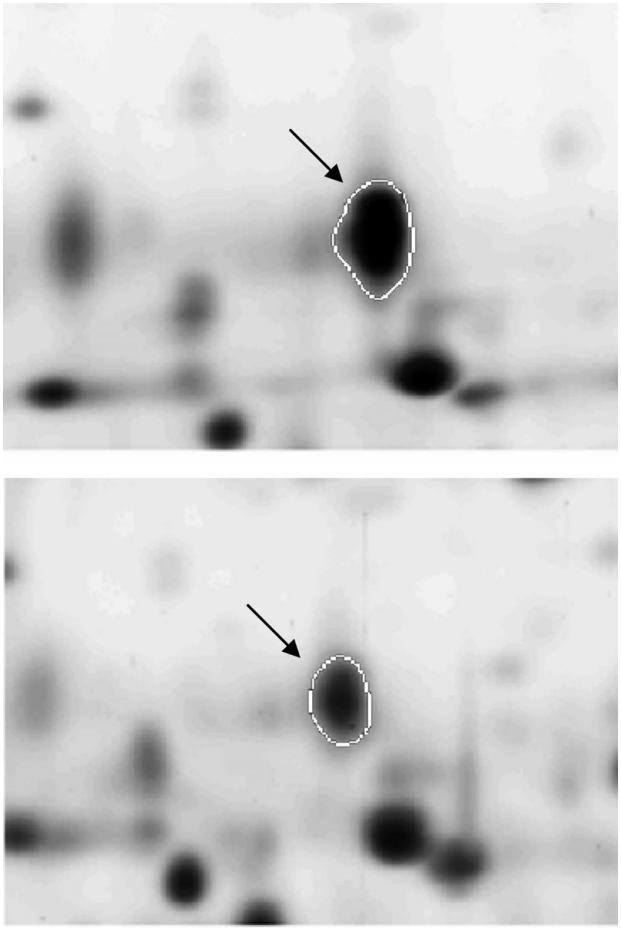
Emanuilova and *Toda*[5] reported a growth-associated production of α-amylase based on their experiments in batch and continuous cultures. In phases I to III, the production of α-amylase appears to increase linearly with increase in cell growth suggesting a growth-associated enzyme formation. However, in phase IV enzyme production rate increased drastically where the cell growth was similar to that in earlier phases. As a result, a more complex dependence between cell growth and product formation may be expected and it needs further exploration.

Metabolic interpretations using 2-D gel electrophoresis

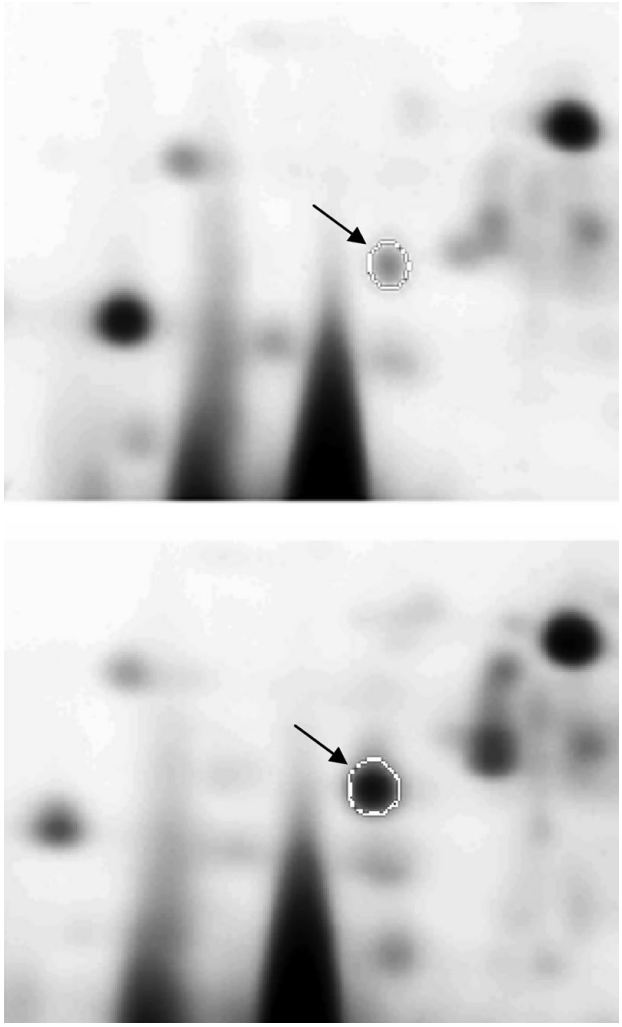
Samples P1 to P4 were collected from different phases of fermentation (Fig. 2) and their proteome was analyzed using 2-dimensional gel electrophoresis. The proteome analysis revealed significant variations in the amount of several proteins that are involved in the energy metabolism and protein synthesis in the cells.

The cells at the end of the batch phase (Fig. 2 – P1) contained very low amounts of pyruvate kinase (Swiss-Prot entry: Q02499), and 6-phosphofructokinase (Swiss-Prot entry: P00512). Both indicate a strong limitation of starch in the medium at the end of batch phase. These cells also show a high intensity of regulatory protein GltC (Swiss-Prot entry: P20668) (see P1 in Fig. 3) which is an indication of limitation of glutamate i.e. casitone in the medium.[21] On the other hand, the intensity of GltC decreased in sample P2 (Fig. 3) suggesting that the shortage of glutamate started to vanish during Phase II.

Intensities of anti-sigma F factor (Swiss-Prot entry: O32727), a stage II sporulation protein AB in samples P1 and P2 are shown in Fig. 4. While this protein is present in very small quantities in P1, it is up-regulated in sample P2. This appears to be a re-



F i g . 3 – *Intensities of the regulatory protein GltC of glutamate biosynthesis in samples P1 (left) and P2 (right).*



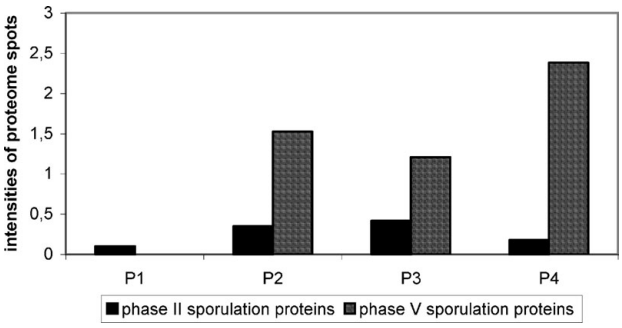
F i g . 4 – *Intensities of the stage II sporulation protein AB in samples P1 (left) and P2 (right).*

sult of starvation of the culture of casitone and carbon source at the end of batch phase, causing the start of sporulation.

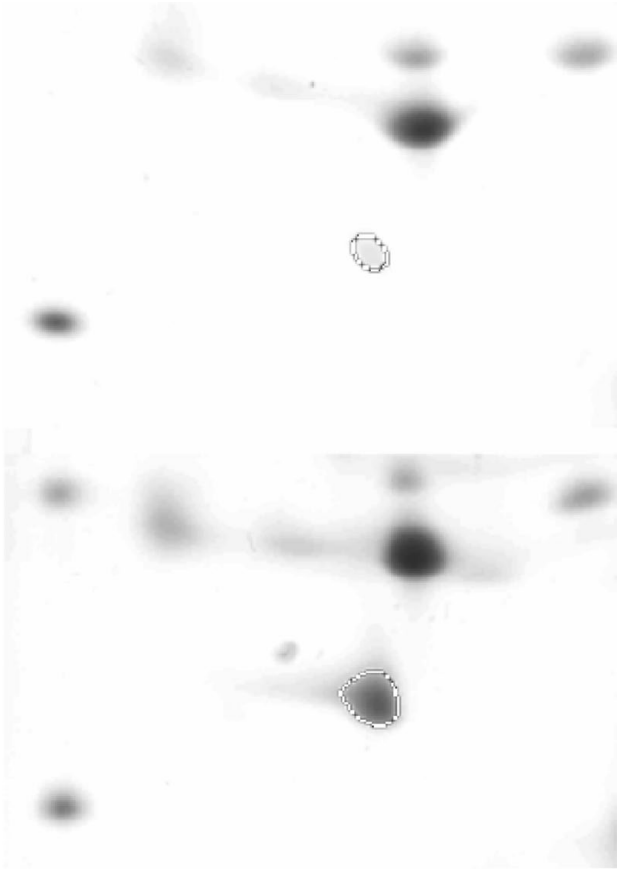
Intensities of sporulation phase II and sporulation phase V (Swiss-Prot entry: P45693) proteins are plotted in Fig. 5 also. The sporulation phase II proteins remained up regulated in samples P2 and P3; their intensity decreased in sample P4. On the other hand, sporulation stage V proteins increased in intensity all along. This suggests that the described starvation at the end of batch operation was the initial event that induced sporulation. This is also supported by the finding of a strong increase of concentration of glycogen synthase (Swiss-Prot: Q816G8) between the end of batch phase (sample P1) and phase II sample P2 in Fig. 2. Glycogen synthase is expressed during the early stages of sporulation.[22]

The observed sporulation of a part of the culture seems to start at the end of batch phase of fermentation. This shows that the length of batch phase is a critical point during fermentation.[15] At this point of time no spores are detectable by microscopy. This start of sporulation can be clearly detected by proteome analysis and offers an opportunity to improve fermentation strategy to avoid the assumed cause of sporulation.

The highest specific growth rate of the culture of $\mu = 0{,}37\ h^{-1}$ was found in Phase II (Fig. 2). The specific growth rate decreased during fermentation to $\mu = 0{,}23\ h^{-1}$ in phase IV. This correlates with observation of strong expression of translational elongation factor Tu (Swiss-Prot entry: O50306) in sample P2.



F i g . 5 – *Intensity of sporulation proteins in different samples.*



F i g . 6 – *Intensity of proteome spots of glycogen synthase in samples P1 (left) and P4 (right)*

Amylase production rate increased at the start of phase IV (470 min) and peaked at 600th min. GltC was up-regulated in P4, indicating glutamate limitation. It was assumed that glutamate limitation together with excess starch in the growth media resulted in the observed acetate formation and, concomitantly, in no further increase in amylase activity. Also glycogen synthase was found up-regulated in sample P4 (Fig. 6). This indicates N-limited growth conditions with excess carbon source in the media,[23] corroborating the above interpretation.

Acetate formation in phase IV was associated with a strong increase of oxygen consumption, possibly due to the "uncoupling effect" caused by acetic acid in cells.[24,25] Acetic acid in cells disturbs the pH gradient between cytoplasm and periplasm, and hence ATP synthesis. One effect of the uncoupling effect is a strong increase of oxygen consumption. A sudden rise of the acetate concentration caused by the so-called overflow metabolism[26] at constant feeding rate of starch and casitone can be seen as an indicator of shortage of nitrogen source under the described fermentation parameters. Drastic changes during fermentation as seen after 610th min can not be explained without proteome analysis.

Conclusions

Fed-batch amylase production by *B. caldolyticus* involving feeding of a solution containing starch and casitone linked to response of dissolved oxygen probe to pulse-up and pulse-down of feeding rate led to high yields of α-amylase with good liquefying abilities. Interpretation of p_{O_2} and pH probe signals in response to the transient changes in feed rates indicated limitations of nitrogen and/or carbon sources in broth at the time of transients. These limitations were confirmed by changes in several important metabolic proteins that were detected by 2-D gel electrophoresis. The results of proteomic analysis led to a better understanding of cell metabolism. Starvation of cells at the end of the batch phase followed by initiation of sporulation was suggested by the proteomic analysis even though subsequent changes in feed composition resulted in continued growth and enzyme production. The highest α-amylase production was observed at a relatively low growth rate. This result was maintained by avoiding any glucose and acetate accumulation during fermentation. Obviously, the ratio of casitone and starch has a high influence on α-amylase expression. The gained information could be used to further optimisation of *Bacillus caldolyticus* fermentation course.

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List of symbols and abbreviation

E – absorption, extinction, nm
n – stirring speed, min^{-1}
OD – optical density
p – presure, %
T – temperature, %
t – time, min
V_{in} – internatiol amylose activity, mL^{-1}
w – mass fraction, %
Γ – feed rate, g L^{-1} h^{-1}
γ – mass concentration, g L^{-1}
ζ – mass ratio
λ – wavelength, nm
μ – specific growth rate, h^{-1}
φ – volume fraction, %

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