Physiological Characterization of a *Bacillus licheniformis* Strain in Chemostat Cultivations

Carla Pinto

*Department of Chemical Engineering, Lund University, Sweden*

June 11, 2012

**Abstract**

The aim of the current work was to collect relevant data on the physiology of *Bacillus licheniformis* in chemostat cultivations. The first experiments have shown some nutrient limitation other than the carbon source in the culture medium. Besides, the growth yields observed were much smaller than expected for bacteria growing in glucose. As a consequence of the abovementioned results, the adaptation of the original culture medium to a suitable one was set as the latest objective of this work. Further experiments were developed in order to test the behavior of the microorganism under lower glucose concentrations and higher trace metal concentration. The results have shown that these adjustments were not sufficient to circumvent the nutrient limitation issue.

**Keywords:** *B. licheniformis*, Chemostat, Culture medium, Nutrient Limitation, Growth Yield

**Introduction**

*Bacillus licheniformis* is a microorganism currently used for the industrial synthesis of products with biotechnological interest. Even though it is closely related to *Bacillus subtilis*, little is known about its physiology, bioenergetic features of growth and exocellular product formation. Data about growth parameters and energy conservation may be of great value for improving the efficiency of production processes.

The initial aim of this work was to get some relevant information on the physiology of *B. licheniformis* in continuous cultivations. However, during its course, the chosen culture medium was shown not to be the most adequate for the desired purposes. Consequently, the direction of the project has changed and finding a suitable medium from the original recipe, by testing different concentrations of carbon source and/or trace metals, has become the new objective.

**Theory**

*Bacillus licheniformis* – the organism used in this project - is a saprophytic bacterium common in soil and other natural environments. The cells are capable of degrading several substrates and growing on a large diversity of nutrient sources due to its capacity of producing and secreting plenty of hydrolytic enzymes. Among others, this ability makes *B. licheniformis* an interesting organism for industrial purposes [1, 2]. Thus, it has been employed in the fermentation industry for a long time, especially for the production of antibiotics and some hydrolytic enzymes [3-5]. Although *B. licheniformis* has remarkable economic importance, the knowledge of its physiology is still limited. It is closely related to *B. subtilis*, which in contrast has been thoroughly analyzed in numerous laboratories all over the world [6].

It has been shown that several features of the general metabolism are similar in *B. licheniformis* and *B. subtilis* although there are also evident dissimilarities. Regulation of
glycolysis pathway and TCA cycle in response to glucose is apparently similar [7, 8]. Some enzymes known to be involved in the overflow metabolism in B. subtilis, e.g. acetate kinase and phosphotransacetylase, are enhanced in B. licheniformis cells grown in excess of glucose [9]. Glucose-limited cells of both species, for instance, induce genes needed for the mobilization of carbon from different sources [10, 11].

A major difference in the energy metabolism of B. licheniformis and B. subtilis consists of the presence of genes homologous to isocitrate lyase and malate synthase within the genome of the former only. The referred enzymes are needed for the metabolism of substrates that enter central metabolism as acetyl-CoA units, e.g. acetate and 2,3-butanediol [12]. In fact, the abovementioned substrates have an additional importance since they are overflow metabolites synthesized during growth on high glucose amounts. Additionally, B. licheniformis may use both acetate and 2,3-butanediol as sole carbon sources [13]. Furthermore, B. licheniformis encodes some proteins that are needed for an anaerobic or facultative anaerobic type of life. In fact, it seems to have the ability of growing anaerobically for a long period of time [11, 14].

α-Amylases (E.C. 3.2.1.1) are starch-hydrolyzing enzymes that catalyze the hydrolysis of internal α-1,4-β-glycosidic bonds in starch polymers [15]. A significant part of the α-amylases are metalloenzymes and so require calcium ions for their enzymatic activity, stability and integrity [16]. Amylases are among the most prominent industrial enzymes and have an extensive variety of applications. In fact, amylases account for approx. 30% of the global enzyme production. The increasing demand for these enzymes in several industries makes of enormous importance to develop enzymes with even better properties and to improve the cost-effectiveness of their production processes [17].

Materials and Methods

Bacterial strain and preparation of the inoculum.

B. licheniformis SJ4628 was obtained from Novozymes A/S Bagsværd, Denmark and kept as frozen stock at -80 °C in 15% (v/v) glycerol. Solid growth medium (SSB agar) used for propagation was prepared from 1% (w/v) soy peptone, 1% (w/v) sucrose, 0.2% (w/v) KH₂PO₄, 0.5% (w/v) Na₂HPO₄, 2.5% (w/v) agar and adjusted to pH 7 using 1 M NaOH. SSB agar was autoclaved at 123 °C for 20 min.

For preparation of the inoculum, B. licheniformis was grown in 250 mL shake flasks for 16 h at 37 °C on 100 mL of culture medium being agitated (180 rpm). The culture medium (original recipe) was prepared from 3% (w/v) tryptone, 0.4% (w/v) MgSO₄·7H₂O, 0.7% (w/v) K₂HPO₄, 0.7% (w/v) Na₂HPO₄·2H₂O, 0.4% (w/v) (NH₄)₂SO₄, 0.5% (w/v) K₂SO₄, 0.078% (w/v) citric acid, 0.00039% (w/v) MnSO₄·H₂O, 0.00157% (w/v) FeSO₄·7H₂O, 0.00016% (w/v) CuSO₄, 0.00033% (w/v) ZnSO₄·7H₂O and 1.5% (w/v) glucose. Culture medium was autoclaved at 123 °C for 20 min in the absence of glucose. Glucose was sterilized in the same conditions and added aseptically.

Bioreactor operation

All the experiments were performed in one of two similar 800 mL fermenters with a working volume of 500 mL. The inoculum volume to the fermenter vessel was 10% (v/v). Also, 0.125×10⁻³% (v/v) antifoam was added aseptically to the fermenter at the beginning of each experiment.

Carbon dioxide production was measured with two gas analyzers. For calibration purposes, two gases were used, the first containing 100% N₂ and the second containing 5% CO₂ and 20% O₂.

Batch cultivation: the pH was controlled at 7.0±0.3 and 1 M NaOH was used for pH control. The temperature was set at 37 °C. The air flow rate was 400 mL/min and the agitation speed was about 900 rpm. Deviations to the referred conditions are conveniently mentioned.

Continuous cultivation: the feeding composition was similar to those mentioned for the culture medium and supplemented with 0.1% (w/v) CaCO₃. Samples were withdrawn from cultures in steady state.

Analytical procedures

The sample amount taken was about 18-22 mL and varied according to the measurements to be performed on each sample.
Dry weight: Triplicate samples (5 mL) were taken from the fermenter vessel and put into pre-weighed test tubes. The test tubes were centrifuged thrice at 3000 rpm for 12 minutes. After the first two centrifugations, the supernatant was poured off and 0.9% NaCl was added to the same initial volume. After the last centrifugation, the supernatant was poured off and the test tubes were dried to constant weight at 105 °C. Dry weight was determined as the difference between the mass of test tube with sample and the mass of the tube. The average of three measurements was considered the actual dry weight.

Turbidity measurements were made at 610 nm. Samples were diluted with 0.9% NaCl to give absorbance measurements up to 0.6. The culture medium (prior to inoculation) was used as blank.

Glucose, acetate and acetoin concentrations: Non proteinaceous compounds, namely glucose, acetate and acetoin, were determined by HPLC analysis.

Additionally, glucose and acetate levels were determined enzymatically with commercial kits (R-Biopharm AG). The measurements were made as described in the instructions, except for the fact that all volumes were halved.

Results and Discussion

First, some preparative batches (results not shown in this article) and further batch and continuous experiments were made considering the initial main purpose of the project (Experiments 1, 2 and 3).

Since the outcome of those first experiments seemed to expose the inadequacy of the selected culture medium for the desired purposes, i.e., continuous cultivation of *B. licheniformis*, the later experiments focused on finding an alternative and suitable recipe by modifying the concentrations of both the carbon source and of trace metals, thus changing the work direction to a new perspective.

**First Series of Experiments**

All the experiments started with a batch cultivation of *B. licheniformis*. First, the cell growth was followed by measuring the culture turbidity and the dry weight (the measurements of the latter are not shown in this article).
high maintenance requirement, i.e. consumption of substrate for purposes other than cell growth, may explain the observed results.

The concentration of acetate and acetoin was also followed during the fermentation process. Figure 3 shows the obtained concentration profiles.

Figure 3. Acetate (top) and acetoin (bottom) concentration profiles for B. licheniformis batch cultivations (values were obtained through HPLC analysis).

Both the concentrations of acetate and acetoin thus increase during the course of the fermentation for the experiments. These compounds may result from the cell metabolism at glucose excess or high glucose fluxes.

Product yields (Y_{acetate/S} and Y_{acetoin/S}) were also determined. Regarding acetate production yield, values of 0.42 g acetate/g glucose, 0.37 g acetate/g glucose and 0.36 g acetate/g glucose were obtained for Experiments 1, 2 and 3, respectively. Besides, the obtained values concerning acetoin production were 0.11 g acetoin/g glucose, 0.13 g acetoin/g glucose and 0.10 g acetoin/g glucose for Experiments 1, 2 and 3, respectively. The obtained results are much higher than expected (it is worth to highlight that approximately 50% of the consumed glucose was used to produce acetate and acetoin, supposedly overflow metabolites). The adequacy of the HPLC method used for their quantification should therefore be validated further.

The feeding has started approximately 6.5 h after the inoculum addition to the reactor-continuous cultivation. Figure 4 shows the results obtained from turbidity measurements for Experiments 1 to 3.

Figure 4. Turbidity as a function of dilution rate for B. licheniformis continuous cultivations.

The biomass profile shown in Figure 4 is fairly in accordance with the expected, since for the steady state operation of a CSTR, the change of cell concentration with time is equal to zero (dX/dt=0).

Evolution of the substrate concentration was also followed and the obtained profile is shown in Figure 5.

Figure 5. Glucose concentration as a function of dilution rate for B. licheniformis continuous cultivations (values were obtained through HPLC analysis).

Glucose concentration decreased with increasing dilution rates from D=0.35 h\(^{-1}\) on. For D=0.30 h\(^{-1}\) the glucose concentration was lower than obtained for D=0.35 h\(^{-1}\), but since no lower dilution rates were tested, the referred trend cannot be confirmed. After the observation of decreasing amounts of biomass for higher dilution rates (within the range of dilution rates tested in this series of
experiments), the concentration of glucose was expected to follow the opposite trend, since lower amounts of cells consume lower concentrations of glucose.

The concentration of acetate and acetoin was also followed during the chemostat cultivation (Figure 6).

![Figure 6. Acetate (top) and acetoin (bottom) concentration profiles for B. licheniformis continuous cultivations (values were obtained through HPLC analysis).](image)

Acetate concentrations are low (about 0.2 g/L) for all the experiments and tested dilutions rates, with the exception of the value found for D=0.50 h⁻¹ in Experiment 1, which was approximately 2.3 g/L. Verifying the formation of overflow metabolites (such as acetate) at dilutions rates close to critical dilution rate was one of the major (and initial) interests of the work and thus the obtained results seem very interesting. Nevertheless, the collected data is not enough to support the referred work hypothesis.

**Second Series of Experiments**

The main purpose of the second series of experiments was to find a plausible explanation for the unexpected results obtained in the first experiments and to plan an adequate strategy for further experiments. In order to do so, two experiments were carried out within this series. The Experiment 4 was pure batch cultivation and the culture medium was the same used for the three previous experiments. The Experiment 5 started with a batch cultivation and then the reactor was fed for 3 days (continuous cultivation). The culture medium was similar to the one used in previous experiments, with exception for the trace metal concentration, which was increased from 0.00025 g/L to 0.025 g/L (it was important to verify whether the highly reduced trace metal concentration could be limiting or not).

Maximum specific growth rates were determined for both experiments and similar values of about 0.51 h⁻¹ were obtained.

The glucose concentration in the culture medium after 72 h of continuous cultivation for Experiment 5 was 14.1 g/L (measurement through HPLC analysis). For a reactor running in continuous mode operation, the dilution rate equals the specific growth rate, in steady state. So, the specific growth rate when the sample was taken should be approximately 0.35 h⁻¹, which is far enough from the maximum specific growth rate (or critical dilution rate). Thus, the expected concentration of glucose was zero or almost zero and the obtained value is a clear sign that the culture medium composition is not adequate for B. licheniformis growth in chemostat cultivation. As the glucose concentration seems not to be limiting, two different alternatives could be put into practice: the concentration of glucose could be decreased to a value that makes it be the limiting nutrient or the concentration of the remaining culture medium compounds could be increased. The former alternative was the chosen procedure for the third series of experiments.

**Third Series of Experiments**

The particular condition for these experiments was the reduced concentration of glucose, 4 g/L.

All the experiments of the present series started with batch cultivation of B. licheniformis. Figure 7 shows the results obtained from turbidity measurements for all the experiments.
The exponential growth phase varied significantly within the developed experiments, since the specific growth rate starts decreasing around 4 h for Experiment 8 and the same phenomenon happened only approximately 6 h after the beginning of the fermentation for Experiment 7. This observation may be partially explained by the variation of the physiological state of the inocula prepared for each experiment. The incubation time of the inoculum used in Experiment 8 was 12 h instead of the “standard” 16 h and cells of a younger inoculum are expected to be in a more active phase regarding multiplication/growth, thus leading to the reduction of lag phase.

Maximum specific growth rates were also determined and similar values of about 0.60 h\(^{-1}\) were obtained for all the experiments.

The feeding started 6.5 h after the inoculum addition to the reactor and its composition was very similar to the one referred for batch cultivation-continuous cultivation. Figure 8 shows the results obtained from turbidity measurements for Experiments 6 to 8 in chemostat cultivation.

For the range of tested dilution rates, the biomass amount seems to decrease with increasing dilution rates, which slightly deviates from what was expected.

Substrate concentration profile was also obtained (Figure 9).

Since the experiments were developed under the same conditions, the obtained results seem not to be reasonable. Considering that the glucose concentration for this series of experiments was lowered in order to guarantee that the carbon source was the limiting substrate, the results obtained for Experiment 7 are according to the expected, i.e all glucose has been consumed.

Acetate concentration profile was also determined (Figure 10).

It is important to highlight that the concentrations of acetate found for the last series of experiments were significantly lower than the ones obtained for the previous series. Even though the HPLC method used for the quantification (first and second series of experiments) may not be adequate, the
knowledge on *B. licheniformis* physiology may also explain this observation. In fact, after the uptake of a substrate, it may be degraded through several metabolic pathways. When oxygen is present in the culture medium, and the microorganism is aerobic or facultative anaerobic, the oxidative metabolism is the most relevant. When there is carbon excess, the excessive production of pyruvate requires the activation of other pathways that do not require oxygen and this is called fermentative metabolism. When the fermentative metabolism happens due to an excess of carbon source, the metabolites formed are also called overflow metabolites. It is still not known which overflow metabolites are produced by *Bacillus licheniformis*, but considering it is closely related to *B. subtilis* it is believed that the overflow metabolites may be the same, i.e., acetate and acetoin. For the first and second series of experiments, the concentration of glucose was 15 g/L and for the third series it was only 4 g/L. As the amount of glucose to metabolize by *B. licheniformis* was considerably lower for the last series of experiments, the oxidative metabolism was certainly preferred to fermentative metabolism and so the concentration of overflow metabolites was lower.

From the results obtained for all the experiments, it is apparent that neither the initial culture medium nor the adjusted versions are adequate for growing *B. licheniformis* in chemostat cultivation. There seems to be an unknown nutrient limitation, the nature of which was not found in the experiments.

Summing up, both finding the nutrient limitation in the original culture medium (the latest objective) and gathering relevant information on the physiology of *B. licheniformis* in chemostat cultivations would require additional experiments and different work strategies.

**Conclusions**

The original culture medium recipe provided by Novozymes was found not to be adequate for growing *B. licheniformis* in chemostat cultivations. Large amounts of precipitates (salts and trace metals) were formed after mixing and/or autoclaving, as a consequence of the extremely high amounts of the referred components.

Nutrient limitation other than the carbon source was found to be present throughout the experiments. The attempts of solving that issue by decreasing the concentration of glucose and increasing the concentration of trace metals were not successful.

Surprisingly high amounts of acetate and acetoin were found in culture media for almost all the experiments. The opposite outcome was obtained for growth yield, which values were significantly lower than the reported for *B. licheniformis* growing in glucose.

Although some interesting data on *B. licheniformis* behavior in batch and continuous cultivations was collected, the strategies implemented on the developed experiments were not sufficient to meet the desired objectives.

**References**


