# The formation of reserve polymers in *Bacillus megaterium*

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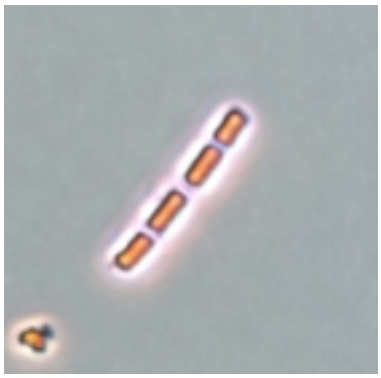

## 1. Abstract

**The formation of reserve polymers in *Bacillus megaterium* under different unbalanced growth conditions was investigated. It was shown, that at culture conditions of 1mM $NH_4^+$ and 33mM glucose polyhydroxybutyrate (PHB) formation reached its maximum – up to 20% per cell dry weight. Polyhydroxyalkanoate (PHA) inclusion bodies were detected either qualitatively after Nile Blue staining or quantitatively by high pressure liquid chromatography (HPLC). After ten hours of cultivation, 50.7 mg pure PHB could be obtained from a culture grown in an airlift bioreactor with a working volume of 2.5 liters.**

## 2. Introduction

**PHA accumulates as energy and carbon reserves in granular inclusion bodies in the cytoplasm of many bacteria and archaea. PHA is a polyester of repeating subunits with the structure [O-$CH(R)(CH_2)_xCO$]. PHB is the most common form where x=1 and R=$CH_3$. PHA accumulation usually occurs in the presence of excess carbon and with a limitation of an essential nutrient such as nitrogen, phosphorus, sulfur, magnesium, potassium or iron. *Bacillus megaterium*, a gram-positive, spore-forming bacterium, known to be able to form PHA up to 50% of its cell dry weight, was used as model organism. Polymer can easily be identified by high pressure liquid chromatography.**

## 3. Questions

**a) How much reserve polymer is formed by *Bacillus megaterium* under different unbalanced growth conditions?**
**b) How can reserve polymer formation be followed in *Bacillus megaterium* by fluorescence microscopy?**
**c) How can PHB be "produced" in an airlift bioreactor?**

## 4. Methods

### a) Determination of PHB-quantity per cell dry weight

**Two liters of sterile nutrient medium (see Appendix) were divided in 16 different solutions with varying C- and N-concentrations (each 1mM, 33mM, 66mM, 100mM) to simulate different unbalanced growth conditions. After inoculation with *B. megaterium* and incubation for 24h at 30°C, samples of 1ml were prepared for HPLC: After centrifugation every pellet was frozen in liquid nitrogen, vacuum dried, and the weight was determined. Cells were lyzed for 30min in boiling sodium hydroxide (2M) and PHB was converted to crotonic acid. After the reaction samples were**

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**cooled on ice and neutralized with 2M hydrochloric acid. Calibration was carried out with pure, commercially available PHB. HPLC was used to determine crotonic acid (absorption at 210nm, with diluted sulphuric acid (5 mM) as eluent.**

**b) Fluorescence microscopy – Staining with Nile Blue**

**2 drops of 0.2% Nile-blue-solution were added to 300µl sample of each culture, vortexed and incubated in a water bath for 10 minutes at 55°C. After 1 min of mild sonication, inclusion bodies were visualized at 1000x magnification under fluorescent light (excitation wavelength 550nm, observation at 580nm) as bright orange intracellular granules.**

**c) Airlift bioreactor – PHB extraction**

**Fig. 1: Airlift bioreactor**
c)

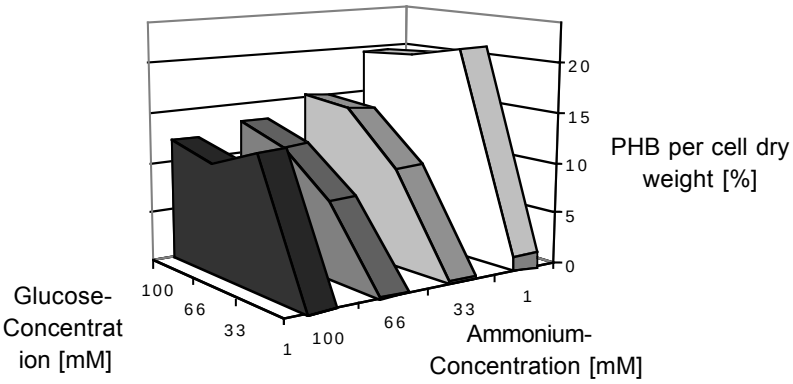
**The airlift bioreactor is a special method to keep the medium stirring without any mechanical help. Only air bubbles rising up in the middle of the reactor cause a current. The size of the bubbles and the amount/minute is regulated. To keep the incubation temperature constant at 30°C, the reactor was equipped with an outer glass column connected to a thermostatted water bath (Fig. 1). In addition to stirring, rising air bubbles supply the medium with enough oxygen. The reactor was autoclaved and filled with 2.5l of sterile medium containing 33mM glucose and 1mM ammonium. An inoculum (30ml) from a stock culture was added. The reactor was maintained for 12 hours. To extract PHB, the whole nutrient solution was centrifuged, the pellet was vacuum dried and dissolved in pure methanol. After 1min of sonication, vortexing, and centrifugation for 10min the methanol was evaporated under an air stream. The pellet was dissolved in chloroform, filtered and air dried.**

## 5. Results

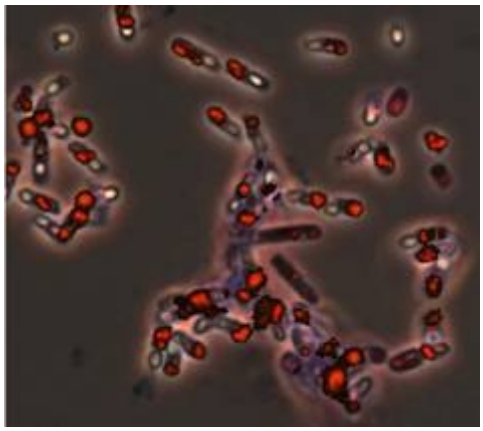
**a) PHB-quantity per cell dry weight**

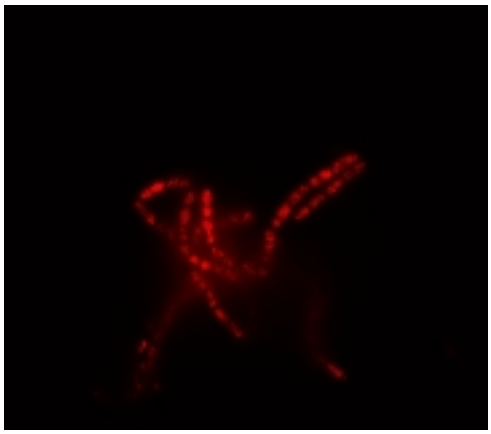
**Figure 2 demonstrates that the more carbon and the less nitrogen is available, the more PHB is formed. PHB formation reached its maximum (more than 20% of cell dry weight) at a concentration of 33mM glucose and 1mM ammonium.**

**Fig. 2: PHB per dry cell weight**



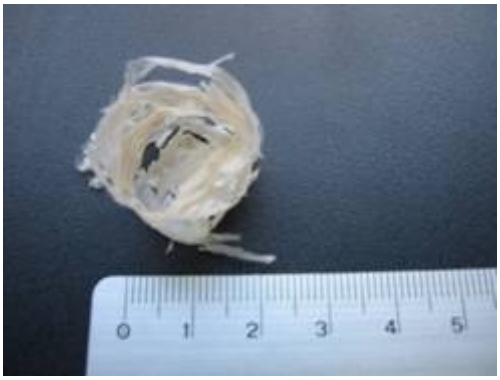
**b) Fluorescence microscopy – Staining with Nile Blue**





**Overlay of a light- and a fluorescence microscopy picture, gives the location of PHB granules in the cytoplasm. The fluorescence microscopy picture of Nile Blue stained bacteria chains, filled with PHB inclusion bodies, shows a bright orange fluorescence.**

**c) Airlift bioreactor – PHB extraction**




**From 2.5l growth medium 418mg cell dry mass was obtained. After PHB extraction and purification 51mg of a plastic material could be produced as shown in the picture beside.**

## 6. Discussion

**a) PHB-quantity per cell dry weight**

**By measuring pH as indicator for bacterial growth it was observed that an ammonium concentration lower than 1mM had a growth inhibiting effect. Concentrations higher than 33 mM (excess nitrogen) and had nearly no effect on bacterial growth. Other components probably became limiting. Figure 1 shows this effect: At ammonium concentrations of ≥33 mM PHB formation does not depend on ammonia- but rather on glucose concentration.**

**c) Air-Lift Bioreactor – PHB extraction**

**Cell dry weight from the 2.5l-airlift reactor was only 418 mg. The optical density in the reactor air-lift was approximately ten times lower than in the shaker flask cultures although there was much more oxygen available and much better growth conditions were expected. The reason may be found in the pH regime: in the bioreactor pH dropped from 7 to 3.73 within ten hours, whereas the shake flasks cultures pH was never <4.7. the low pH might have a negative influence on bacterial growth. As consequence, in further experiments not only ammonia and glucose concentrations need to be regulated, but also other parameters such as pH, in order to increase cell as well as PHB yield.**

**Only 51 mg PHB was recovered from 417 mg dry cells, although the expected value was 84 mg. Insufficient extraction methods as well as losses during purification might be the reason.**

## Appendix

**Nutrient solution**

**Basic medium (1 liter)**

| Compounds | Amount(g) |
|---|---|
| Magnesium sulphate | 0.20 |
| Calcium chloride | 0.07 |
| Iron sulphate | 0.01 |
| EDTA | 0.01 |
| Potassium dihydrogenphosphate | 0.60 |
| Dipotassium hydrogenphosphate | 0.90 |
| Yeast extract | 1.00 |

**Trace elements in 1 liter dist. water**

| | |
|---|---|
| Manganese sulphate | 0.02 |
| Boric acid | 0.01 |
| Copper sulphate | 0.01 |
| Ammonium molybdate | 0.02 |
| Zinc sulphate | 0.01 |

From this solution 1ml is added to the basic medium

**Varied compounds added to 100ml basic medium:**

| | | | | |
|---|---|---|---|---|
| Glucose (mM) | 1 mM | 33mM | 66mM | 100mM |
| Amount (g) added | 0.018g | 0.594g | 1.188g | 1.8g |
| Ammonium (mM) | 1 mM | 33mM | 66mM | 100mM |
| Amount (g) added: | 0.0053g | 0.176g | 0.352g | 0.534g |

**The pH of the medium was adjusted to a value of 7.0.**

## Literature

**McCool G.J., Fernandez T., Li N., Cannon M.C. (1996) Polyhydroxyalkanoate inclusion body growth and proliferation in *Bacillus megaterium*. FEMS Microbiology Letters 138:41-48**

**Ostle A.G., Holt J.G. (1982) Nile Blue A as a fluorescent stain for poly-beta-hydroxybutyrate. Applied & Environmental Microbiology 44:238-241**