Analysing the phosphorus distribution in Corynebacterium glutamicum ΔmcbR using Energy Filtered Transmission Electron Microscopy (EFTEM)

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This work is dedicated to the memory of the late Rudolf Reichelt, who initiated this project and made significant contributions. Regrettably he did not live to see its results.

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Introduction

The Corynebacterium glutamicum is of immense importance in the biotechnological industry. There are different mutants of the wild type, one is the Corynebacterium glutamicum ATCC 13032 ΔmcbR. It was supposed that the removal of the mcbR regulator would increase the biosynthesis of the sulphur containing amino acids cysteine and methionine. Instead an increased amount of Adenosine triphosphate (ATP) was found. X-ray microanalysis showed small granules with a higher phosphorus signal [1].
The Corynebacterium glutamicum is a good specimen to investigate elemental maps of elements with low concentrations. The low phosphorus concentration of 1.4% to 2.2% [2] would lead to a faint phosphorus signal resulting in a low Signal-to-noise ratio (SNR). The small granules contain enough phosphorus to reach a high SNR that would be a proof for the phosphorus signal.
In most of the specimens we found a silicon signal. The silicon is distributed all over the specimen. Because the L-edges of silicon and the L-edges of phosphorus overlap, we developed a method to separate both signals. This work shows that a simple method to subtract the silicon signal from the combined silicon and phosphorus signal provides good results.

Results of Electron Energy Loss Spectroscopy (EELS)

All spectra have been recorded with a Zeiss Libra 200FE at an acceleration voltage of 200 kV. Fig. 1 shows an EEL spectrum of C. glutamicum ΔmcbR. The power law method is used to calculate the background fit on an energy loss interval of 75 eV to 100 eV. The delayed L-edge of phosphorus starts at 132 eV and rises to a maximum at about 155 eV. In Fig. 1 we find already an elemental signal in front of the phosphorus L-edge. This is a silicon L-edge that starts at 99 eV and still shows a high signal even at 150 eV and above.
Fig. 2 shows the extracted elemental signal from Fig. 1 and additionally the extracted elemental signal from an EEL spectrum recorded at the embedding material spurr. The spectra had been normalised before the elemental signal was extracted. Both spectra feature the 3 characteristic peaks of silicon oxide. About 50% of the elemental signal at the energy loss interval of 132 eV to 180 eV originates from the silicon. To prove the presence of silicon we investigated the K-edge of silicon, too. The source of the silicon is still unknown.

The contamination of the specimens with silicon results in two main problems. The energy loss interval to calculate the background fit is limited much more than without silicon. The lower limit is about 65 eV to 70 eV. At lower energy losses the power law method is not suitable. The upper limit is lowered by the silicon signal. Without silicon the phosphor edge at 132 eV is the upper limit. The silicon edge starts at 99 eV. The lower and the upper limit reduce the width of the energy loss interval from about 60 eV to less than 35 eV.

Energy Filtered Transmission Microscopy (EFTEM)

All EFTEM images have been recorded with a Zeiss EM902 at an acceleration voltage of 80 kV. Fig. 3 shows 3 EFTEM images, recorded at different energy losses. The first one is a zero loss image. The dark granules are possibly the once described in [1]. At an energy loss of 155 eV (the maximum of the phosphorus L-edge) these granules are bright. This indicates a high phosphorus signal. If one considers the EFTEM image at 115 eV in Fig. 3, the high signal is present in front of the phosphorus edge, too. To analyse the phosphorus distribution, a background subtraction is necessary.
To create a background corrected elemental map, we use the 4-window method. 3 EFTEM images are recorded in front of the element edge; these are called pre-edge images. 1 EFTEM image is recorded at an energy loss higher than the elemental edge; this is the post-edge image.
The pre-edge images are used to calculate the background signal. One method to do this calculation is the maximum likelihood estimation (MLE). It is used to find the parameters of a function that fits the background best. A function to fit the background is the power law, which is appropriate for a wide energy loss range. An advantage of the 4-window method over the 3-window method is the additional degree of freedom. The 3-window method uses 2 measurements to calculate the 2 parameters of the power law. Using the 4-window method, an additional degree of freedom is available to analyse the agreement of the measurements to the power law fit.
After calculating the power law function, the background signal can be subtracted from the post-edge image. The result is an elemental map. Fig. 4 shows the elemental map calculated by subtracting the background signal from the 155 eV EFTEM image at the Fig. 3.

Corrected elemental map

The embedding material that was used to prepare this specimen does not contain phosphorus. At the line profile (Fig. 5) taken from the elemental map (Fig. 4), however there is an elemental signal present even in the embedding material. This is the silicon signal that was detected using EELS.
To obtain the phosphorus distribution we have to subtract the silicon signal from the elemental map at an energy loss of 155 eV. The silicon L-edge starts in front of the phosphorus L-edge, making it possible to create a silicon map at 115 eV. To estimate the silicon signal at 155 eV a scaling parameter q is introduced. It is defined as:

q = Spurr (155 eV) / Spurr (115 eV),

where Spurr (ΔE) is the mean intensity at an section outside the bacterium at the energy loss ΔE. As we know that spurr does not contain phosphorus, the origin of this elemental signal must be silicon. The parameter q is used to calculate the silicon signal at 155 eV:

[Si_map] (155 eV)=q • [Si_map] (115 eV).

This silicon map is subtracted from the elemental map at 155 eV that contains both, the silicon signal and the phosphorus signal:

[P_map] (155 eV) = [P_Si_map] (155 eV) - [Si_map] (155 eV).

Fig. 6 shows the result of this correction. There is no more element signal at the spurr. Positions with high phosphorus signal are better separated from the surrounding signal. Some high signals at Fig. 4 are no longer visible at Fig. 6.
At Fig. 7 the Signal-to-noise ratio (SNR) is displayed. The display limits are 0 to 3. We used propagation of uncertainty to calculate the SNR of the corrected elemental map. The subtraction leads to a significant raise of the uncertainty and a decrease of the SNR. We cannot be sure that the measured signal is phosphorus.
To achieve a higher SNR we recorded an EFTEM image at 200 eV, calculated the elemental map and performed the correction. The corrected phosphorus map at 200 eV is shown in Fig. 9. The related SNR (Fig. 8) is significantly higher. There is a SNR of more than 5 at positions of high phosphorus signal. At an energy loss of 200 eV the percentage of the silicon signal to the overall element signal is much lower as at an energy loss of 155 eV. As a consequence the uncertainty is of the subtracted silicon signal decreases. This compensates the higher relative uncertainty of the 200 eV elemental map, the SNR is even increased.
The comparison of the corrected elemental map to the uncorrected one underlines the good result of the correction used. The two line profiles (Fig. 10) were created at the same section of the images. This is not the section of the spurr that was used to calculate the scaling parameter q. The corrected phosphorus signal at the spurr is zero, only some noise is visible. This can be observed at the spurr all over the image.

Conclusion

It is possible to create elemental maps of low concentrations of phosphorus when the specimen contains silicon. The subtraction of the silicon signal is a simple method to correct the phosphorus maps. The requirement is a section at the image that shows only the silicon signal and no phosphorus signal. With the corrected elemental map at an energy loss of 200 eV we even reach a SNR larger than 3 to prove the presence of phosphorus.

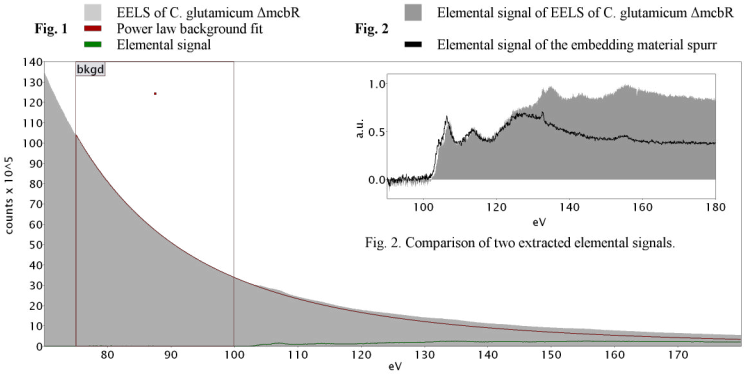

Fig. 2. Comparison of two extracted elemental signals.

Fig. 1. Electron Energy Loss Spectrum of C. glutamicum ΔmcbR.
Gatan Digital Micrograph has been used to calculate the power law background fit.

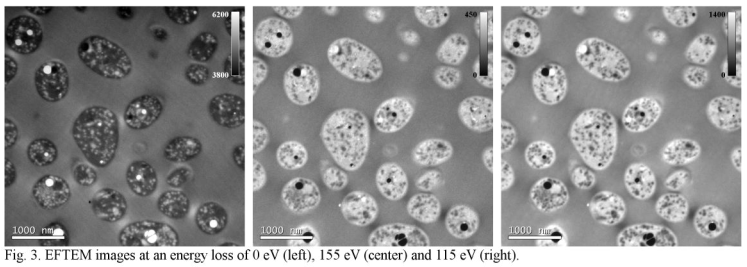

Fig. 3. EFTEM images at an energy loss of 0 eV (left), 155 eV (center) and 115 eV (right).

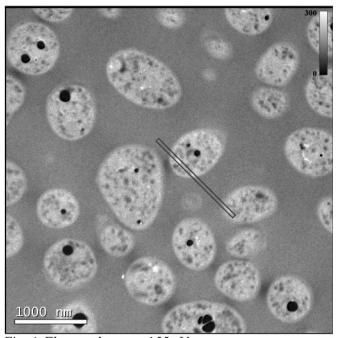

Fig. 4. Elemental map at 155 eV

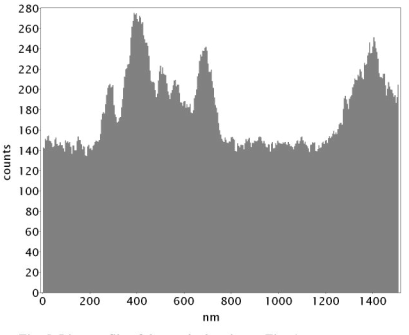

Fig. 5. Line profile of the marked region at Fig. 4.

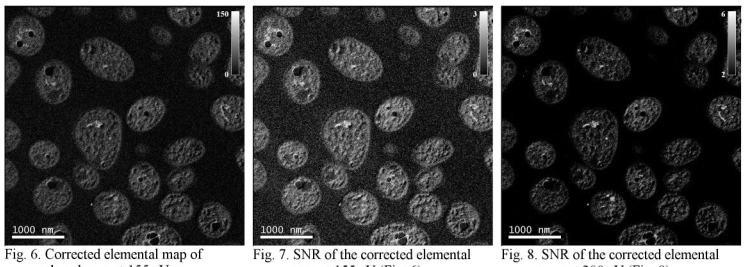

Fig. 6. Corrected elemental map of phosphorus at 155 eV.

Fig. 7. SNR of the corrected elemental map at 155 eV (Fig. 6).

Fig. 8. SNR of the corrected elemental map at 200 eV (Fig. 9).

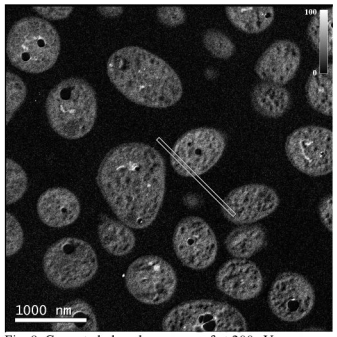

Fig. 9. Corrected phosphorus map of at 200 eV.

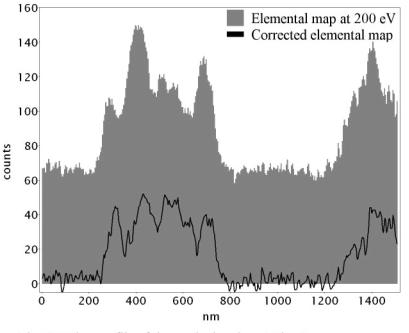

Fig. 10. Line profile of the marked region at Fig. 9.

References & Acknowledgements

1. J. O. Krömer et al., Microbiology 154 (2008), 3917-3930.
2. L. Eggeling, Handbook of Corynebacterium glutamicum (2005)
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