# Shifted-excitation Raman difference spectroscopy for monitoring the algal production of complex polysaccharides

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**Abstract**

The applicability of shifted-excitation Raman difference spectroscopy (SERDS) in combination with signal regression analysis as a non-invasive and alternative approach to monitor the cultivation of phototrophic microorganisms producing complex molecules of pharmaceutical relevance in a bioreactor is demonstrated. As a model system, the cultivation of the red unicellular algae *Porphyridium purpureum* is used focussing on the segregation of polysaccharides that exhibit antiviral activity. The spectroscopic results obtained by linear regression based on partial linear least squares and by nonlinear regression based on support vector machines are discussed against the corresponding results from conventional offline analytics. The SERDS-approach turns out to have strong potential as a non-invasive tool for online-monitoring of biotechnological processes.

## Introduction

The scientific and industrial development of modern biotechnology has created a strong need for methods to monitor and control bioprocesses in order to optimise product formation and output. In addition, acquiring large amounts of data in such processes is important to build models and obtain deeper insights into and a fundamental understanding of the biosystem under investigation. One of the core objectives in bioengineering is to guarantee optimal and homogeneous process and cultivation conditions in order to increase the metabolic performance of a particular microorganism. Non-invasive, in-situ and online measurement of process parameters is the key to achieving this; however suitable technologies are very rare. The state-of-the-art methods for monitoring the substrate and product concentration are offline, at-line or invasive online analytical techniques [1-9]. The latter involves online sensors being immersed into the cultivation broth potentially causing problems with contamination [7-14]. At-line technologies analyse samples directly after being taken and consequently represent a means of analysis almost in real-time [3, 4, 6, 15]. This is why such technologies are greatly increasing in popularity. However, the most common methods are still offline analytics, which are applied on a daily basis or even just at the end of the cultivation.

Those methods are based on sampling and in many cases they are characterized by a very complex and time consuming performance and involve numerous steps of sample preparation, for example enzymatic digestion procedures or dialysis. Thus, spectroscopic methods have great potential to successfully replace offline analytics as they can be employed in situ and deliver data in real time about molecular species facilitating the measurement of concentration and the characterization of molecular interactions[16-21]. Near infrared absorption spectroscopy (NIRS) probes are already used frequently in bioprocess monitoring [1-9, 11, 13-15, 22-27]. However, because they are often based on fibre optical probes that are inserted in the bioreactor, they mean a high risk of contamination associated with financial losses in industrial applications. In principle, online spectroscopy allows instantaneous data acquisition from virtually all components of the matrix (microbial cultivation broth).

Therefore, in this work we present shifted-excitation Raman difference spectroscopy (SERDS) [28-34] in combination with chemometric signal analysis to monitor cultivations in photobioreactors. SERDS is based on the Kasha-Vavilov rule [35, 36] and enables the acquisition of fluorescence-free Raman signals in the presence of fluorescence. This technique is a very promising candidate to replace chemically demanding downstream procedures and, in addition, it has the potential to be directly applied through the transparent walls of the reactor, hence being a truly non-intrusive tool. Photobioreactors are commonly used to grow phototrophic organisms, such as algae, which offer a broad range of applications based on the large variety of algal species. For example, algae serve as production organisms for substances of

biological, medical and physiological relevance [37-39], as feed-stock for renewable chemicals [38, 40] and biodiesel synthesis [41-45]. Additionally they can be used for the absorption of heavy metal ions, e.g. in the field of waste-water treatment [46].

For the present work we chose to investigate the phototropic red microalgae *Porphyridium purpureum* (*P. purpureum*) which can be found in marine systems and fresh water. *P. purpureum* serves as a source of valuable, bioactive compounds [47] including sulphated exopolysaccharides (EPS) [48-58], antioxidative and antiviral compounds[59], polyunsaturated fatty acids [60-62] and phycobilisomes [28-31]. In particular, the sulphated polysaccharides (heteropolymers of large molecular weight) are of great interest because they are antiviral for different types of RNA, DNA and retro viruses, e.g. herpes simplex or human immunodeficiency virus (HIV) [32, 33]. They are able to inhibit the adsorption or penetration of viruses into host cells and with that different reverse transciptases [34, 63, 64]. Thus, it is desirable to optimize the EPS production and maximize its growth dependent segregation to the extra-cellular surrounding where it can be harvested. The segregation predominantly happens between the exponential and stationary growth phases.

In addition to the difficulty of developing an experimental tool, the calibration modelling is a challenging task, in particular concerning the dependency of the mathematical model on the growth phase and thus on the behavior of the cultivation broth. In this context, a temporal segmentation of the calibration model to take the phase dependent behaviour into account seems promising [1, 4-6, 11, 12, 25, 26, 65, 66]. In order to extract information from spectra recorded in a bioreactor, numerous different mathematical models and algorithms have been developed employing multivariate data analysis and chemometrics[26, 67]. A prominent example is the combination of principle component analysis (PCA) and partial regression [68, 69], where PCA is applied initially to identify characteristic spectral signatures, which reflect changes in the bioprocess or the concentration of an analyte. This is often followed by a partial regression analysis. Finally, such models have been internally and externally validated [1-4, 67]. Besides these well-established linear approaches, support vector machines [70](SVM) can be used for nonlinear regression, which is advantageous when the signal-information relation is nonlinear. Generally speaking, nonlinear regression by support vector regression (SVR) [71]is based on the following functional principle. A nonlinear data set, that by definition is not explicable by a linear regression function, is projected into a higher dimensional space by means of Kernel-functions. By application of mathematical optimization techniques, the projection is done in a way that linear regression can be facilitated in the resulting higher dimensional space. The regression function in the higher dimensional space is subsequently back-transformed into the initial data space, and can then be used to explain the nonlinear relationship in the original data set in a mathematically optimal way.

Very recently, the use of SVMs in process monitoring was demonstrated [10, 72-75]. However, the application

of support vector machines for online-concentration measurements based on SERDS signals from a cultivation process has not been reported yet, to the best of our knowledge.

The paper is structured as follows: To examine the potential of product monitoring based on non-invasive SERDS measurements as an alternative to conventional methods, we first compare SERDS with ordinary Raman spectroscopy. Secondly, we validate the concentration predictions from partial linear least squares regression (PLSR) against the reference concentrations determined in a conventional downstream process. Thirdly, we compare the results obtained by the linear PLSR with the nonlinear approach based on support vector machines. Subsequently, we show proof-of-concept results of online concentration determination performed in culture suspensions from the *P. purpureum* cultivation using a bypass flow-loop system evaluated using a specific SVR algorithm, ε-SVR. The last section concludes providing a SWOT (strengths, weaknesses, opportunities and threats) analysis of the presented approach and future prospects.

## Material and Methods

### Measurement system

The optical set-up for the Raman and SERDS measurements consisted of a tunable, continuous wave distributed-feedback diode laser emitting at 785 nm, the beam of which was focused into the cuvette holding the sample. The signal was collected and collimated at 90° with respect to the laser beam using an achromatic lens system. The elastically scattered light was suppressed by longpass filters with 785 and 800 nm cut-off wavelength. For the SERDS measurements the signal was additionally divided into its horizontally and vertically polarized components in a polarizing beam splitter and then spectrally analyzed in two identical fibre coupled spectrometers covering a spectral range from 200 to 4000 $cm^{-1}$. Typical acquisition times in the biological samples were 55 s. The necessary wavelength shift for the SERDS signals was determined by means of binary and ternary ionic liquid mixtures as they have established spectroscopy. A wavelength shift of about 0.65 nm was applied to record the two sets of SERDS spectra. Note that the difference in wavelength was also selected according to the spectral resolution of the spectrometers in such a way that the shifted Raman signals could clearly be distinguished from each other. In addition, the difference was sufficiently small that the difference spectrum can be considered as the differential of the Raman spectrum. This is advantageous when the Raman spectrum needs to be recovered retrospectively.

For each measurement point 10 spectra were averaged and fitted with a suitable Savitzky-Golay polynom. The baseline was corrected by the regression routine *msbackadj* provided in Matlab 2010a before the difference spectrum for each polarization was calculated.

### *Porphyridium purpureum* cultivation

*P. purpureum* was chosen as a model algal system as it is of great interest for biotechnological applications. The microalgae produce a number of valuable compounds [47] including sulphated exopolysaccharides (EPS) [49-57, 76], polyunsaturated fatty acids (PUFAs) [60-62] and phycobilisomes [28-31].
The cultivations were carried out under defined and controlled conditions in 1-l photobioreactor screening modules (PSM) having a reaction volume of 0.9 l(see Figure 1) and a 25-l photobioreactor (airlift loop type reactor) during scale-up. Since the antiviral EPS were chosen as target molecules, the optimization of the cultivation aimed at a maximized EPS segregation. At PSM scale, several cultivations were carried out applying variations in cultivation parameters including the medium composition, photon flux density or gas volume flow rate to find an optimal combination of the cultivation parameters. The optimisation was supported by the Nelder-Mead Simplex algorithm [77]. Cultivation samples were taken daily during the batch cultivations of *P. purpureum.* During the optimisation process and development of the SERDS methodology only cultivation supernatants were investigated and prepared by centrifuging the samples at 26,900 g, 15 °C for 15 minutes by means of the Contifuge Stratos Heraeus centrifuge. For the proof-of-concept experiments cultivation samples were directly investigated without any preparation.

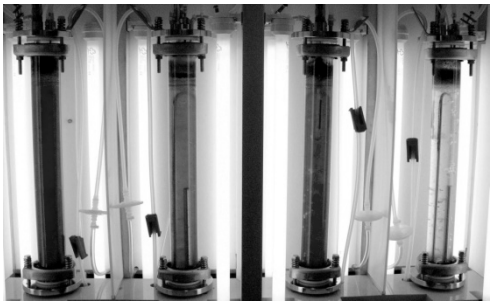

***Fig.1 P****hotobioreactor screening modules for a batch cultivation of Porphyridium purpureum*

### Data evaluation

For the evaluation of the SERDS spectra, principal component analysis (PCA) and partial least squares regression (PLSR) were used. Different algorithms including the non-linear iterative partial least squares (NIPALS) and the singular value decomposition (SVD) algorithm were tested [78]. To accelerate the calibration, we tested additional algorithms based on a Lanczos transformation [79] and an algorithm for PLSR analysis based on the iToolbox of Lars Nørgaard [80]. Eventually, the NIPALS algorithm was found to be the most suitable option. Besides linear regression by means of partial linear least squares, regression by means of support vector machines (SVR) was established and compared to PLSR. For this purpose the *libSVM* toolbox as described by Chang *et al.* [81] was integrated to Matlab2010a. Specifically, we used the ε-SVR algorithm[82], which allows for linear regression in the high dimensional projection

space within a defined margin of error ε. Using the ε-SVR algorithm prevents overfitting of the SVR model to the known data[82]. For the required settings of the ε-SVR algorithm, we used a linear kernel function and an ε-value of 0.08.

The validation of both the PLSR and SVR model was based on a leave-one-out cross-validation, in which the model was first trained on a subset of the data, and then tested on a disjunct subset of the data[83]. The results were averaged over all test runs.

The root mean square error of prediction (RMSEP) value was used as an indicator of the suitability of the calibration model. The RMSEP value is based on the predicted error sum of squares (PRESS) value. In both cases $y_i$ represents the measured value (offline analytics) and $\hat{y}_i$ the concentration predicted by the calibration model using the SERDS-signals as input. The variable $n$ represents the number of samples used.

$$RMSEP = \sqrt{\frac{\sum(y_i - \hat{y}_i)}{n}}$$

$$PRESS = \sum(y_i - \hat{y}_i)^2$$

**Results and Discussion**

In the following, we will first qualitatively compare Raman and SERDS spectra of cultivation supernatants to demonstrate the strong and disturbing fluorescence background. Thereafter, the EPS concentration prediction from SERDS spectra by partial linear least squares (PLSR) and support vector regression (SVR) are compared and discussed. The spectroscopic results are validated against reference concentration data obtained by conventional offline analytics. Eventually, the first proof-of-concept results from online-SERDS measurements in untreated cultivation samples evaluated by SVR are presented.

**Raman spectroscopy**

In principle, conventional Raman spectroscopy can provide all the information required to monitor a *P. purpureum* cultivation process. However, even when using a near-infrared laser source a strong fluorescence background can be observed arising from the excitation of intra- and extra-cellular pigment molecules. To illustrate this, Figure 2 shows a series of Raman spectra recorded during the cultivation process. The data reveal that (1) the fluorescence background overlaps with the Raman spectrum, (2) substantially varies with cultivation duration, and (3) may vary between different cultivation runs as well. Thus data evaluation and interpretation is difficult. One option to overcome this problem is post-experimental data-processing. However recording the experimental data in a way that allows a direct

background correction is advantageous and more robust, especially in cases where the fluorescence background cannot be predicted reliably. Therefore, developing a modified Raman technique appears to be more promising in dealing with the fluorescence signals.

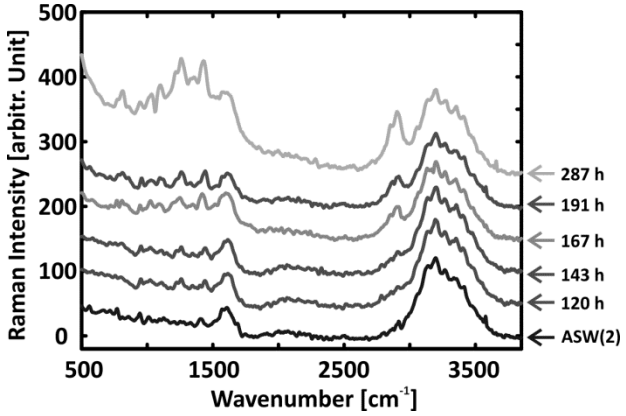


***Fig.2*** *Raman spectra recorded during a batch cultivation of P. purpureum.*

In order to correct for fluorescence interferences we make use of shifted-excitation Raman difference spectroscopy (SERDS) [84-90].

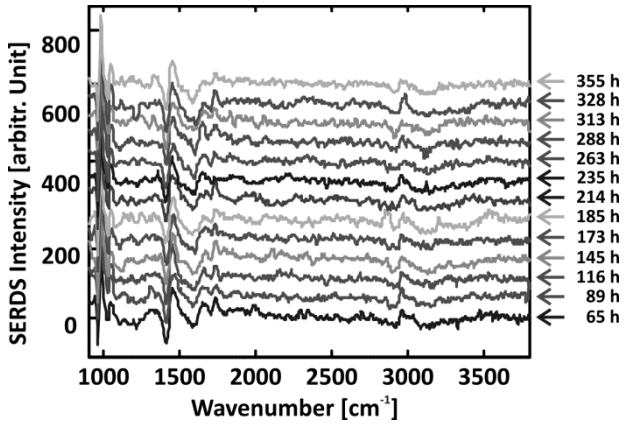


***Fig.3*** *SERDS spectra expressing successively increasing cultivation durations from the bottom to the top for a P. purpureum batch cultivation in a 25-l medusa reactor.*

This approach is based on the Kasha-Vavilov rule, which states that the fluorescence quantum efficiency is independent of the excitation wavelength (within a small spectral range) [35 36]. This empirical rule implies that internal conversion is so fast that other processes, such as fluorescence, intersystem crossing or phosphorescence cannot compete significantly [91]. In other words, when we record two consecutive Raman spectra employing slightly different laser wavelength we will obtain two spectra in which the fluorescence background is the same while the Raman signals are slightly shifted in wavelength. Consequently, the difference of these two spectra is free of fluorescence (see figure 3) and can either be

used to reconstruct a fluorescence-free Raman spectrum or the difference spectrum can directly be evaluated with respect to the EPS concentration.

**Evaluation of the SERDS spectra**

The EPS concentrations derived from the SERDS spectra of the supernatant samples were evaluated against those determined offline by the conventional analytics after a downstream process. For the PLSR calculations the spectral region between 1000 and 1800 $cm^{-1}$ of the spectra shown in Figure 3 provided the basis. Figure 4 shows the EPS concentration over time determined by downstream processing (reference EPS concentration) and SERDS (measured EPS concentration). Figure 5 reveals that the correlation between the SERDS and reference EPS concentrations deviates from ideality (i.e., x = y) only within the measurement accuracy for medium concentrations. Apparently, very low and very high EPS concentrations are less predictable.

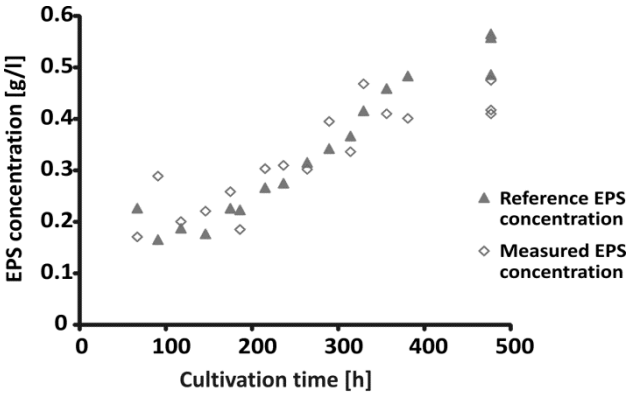


***Fig.4*** *Time dependent segregation of sulphated exopolysaccharide in a P. purpureum cultivation determined by downstream processing as reference (triangle) and SERDS measurement evaluated using linear regression (diamond).*

The RMSEP value for the data plotted in Figure 5 is about 0.07 excluding the higher concentrations and about 0.13 overall.

There are several possible reasons for this behavior, one of which is based on random errors in the downstream processing of the cultivation samples introducing concentration-dependent uncertainties indicated by the horizontal error bars in Figure 5. Constant errors were removed by a post-calibration. For that, the trend line equation of the residual plot of the measured EPS concentrations was used as a projection basis. In addition, the size of the calibration data set may be not sufficient and hence the non-idealities remain dominant.

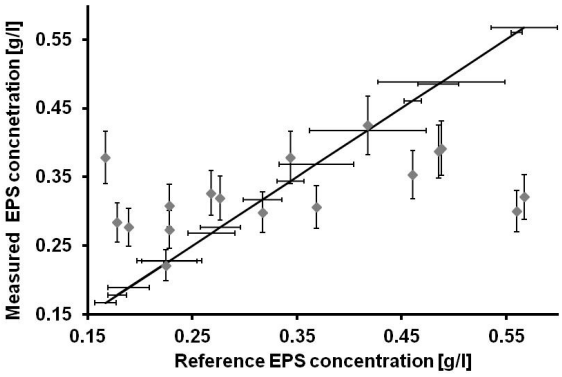


***Fig.5*** *Correlation between the sulphated exopolysaccharide concentrations in supernatants of a P. purpureum cultivation determined by downstream processing (reference data) and SERDS measurements evaluated using linear regression (measured data).*

To test the data set for a nonlinear signal-information relation the residues were analysed and a Durbin-Watson test was performed. Such a statistical test can be used for linear regression models to test for correlation among the residues. If an autocorrelation is indicated, the chosen model can not sufficiently explain the given relationships in the system under investigation. The obtained d-value finally supported the inherent nonlinearity. This finding and the deviations of the prediction from the reference become plausible if three things are considered: Firstly, the EPS molecule changes strongly in size and structure over time and with concentration. Secondly, since the SERDS signal is structural sensitive but the reference method is not, structural changes which influence the SERDS signal unproportional to the correlated concentration change, will insert an inherent nonlinearity between concentration and SERDS signal and with that always deviates from the reference concentration. Finally, for linear models the size of the calibration database is a very important aspect. This means that the larger the database in terms of number and variety, the better a linear model can deal with nonlinearities in the signal-information relationship since their impact will be significantly reduced upon averaging. Therefore, the relatively small calibration dataset provides a third explanation for the deviations. Therefore, we conclude that the PLSR method is not suitable to quantitatively evaluate the nonlinear relationship between the SERDS signals and the corresponding EPS concentrations over the entire concentration range.

To establish a regression model which can deal with the entire EPS concentration range and its nonlinear relationship with the SERDS signal, support vector machines for nonlinear regression (SVR) were used as described in the material and methods section. The results of the concentration prediction by ε-SVR and the conventional PLSR are compared and are shown in Figure 6. The predicted EPS concentrations are based on SERDS signals acquired in batch cultivations of *P.purpureum* in PSM characterized by a variation in cultivation temperature from 15°C to 35°C. As it gets obvious from Figure 5, the SVR

prediction for this cultivation is significantly better than the PLSR prediction. The $PRESS_{PLSR}$ value is about 0.15 and the $PRESS_{SVR}$ is about 0.06, which is less than half the $PRESS_{PLSR}$ value. In the very low concentration regime deviations for both models can still be observed. Anyway, overall, the SVR applied here means a considerable improvement.

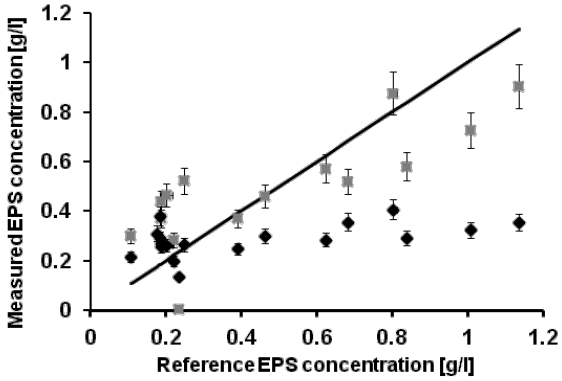


***Fig.6*** *Correlation between the sulphated exopolysaccharide concentrations in supernatants of a P. purpureum cultivation determined by downstream processing (reference data) and SERDS measurements (measured data) evaluated using PLSR (diamond) and SVR (square).*

In order to demonstrate the application of the new analytical tool on untreated cultivation samples containing biomass in an online fashion, a final proof-of-concept experiment was carried out.

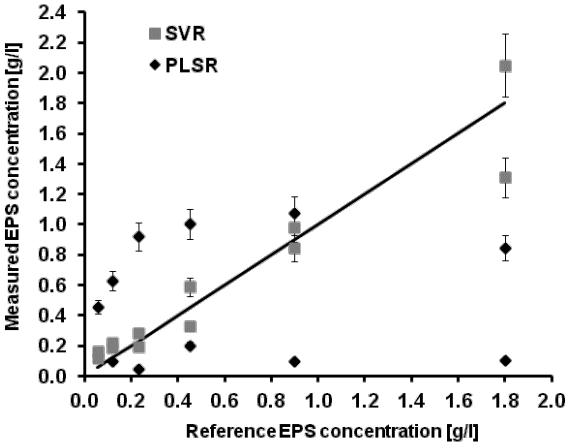


***Fig.7*** *Correlation between the sulphated exopolysaccharide concentrations in whole cultivation samples of a P. purpureum cultivation determined by downstream processing (reference data) and SERDS measurements (measured data) evaluated using PLSR (diamond) and SVR (square).*

Measurements were performed in an optical flow cuvette through which a sample from the cultivation was continuously pumped. Two sets of SERDS spectra were evaluated using ε-SVR and PLSR for

comparison. The results are shown in Figure 6. The PLSR prediction extremely deviates over the entire concentration range from the reference data set leading to an average PRESS value of 0.48 whereas the SVR prediction shows an overall PRESS value of 0.031, which is more than a ten-fold improvement of the EPS concentration prediction. Therefore, we can conclude that the combination of SERDS spectroscopy and ε-SVR has the potential to be applied for non-invasive online monitoring of the *P. purpureum* cultivation. Furthermore, this approach can be easily transferred to other cultivation processes, for example to the cultivation of other algae or even bacteria, e.g. *Escherichia coli*.

**Conclusion**

In this work, we described a Raman spectroscopic approach that allows tracking the segregation of antiviral sulphated exopolysaccharides (EPS) in the course of the cultivation of the microalgae *Porphyridium purpureum*. An analysis of the potentials and limitations of conventional Raman spectroscopy led to the conclusion that its high sensitivity to fluorescence interference makes the data evaluation and thus a reliable performance difficult. To overcome this problem, we developed an innovative approach based on shifted-excitation Raman difference spectroscopy (SERDS), which allows fluorescence-free Raman spectra in the presence of strong fluorescence signals to be acquired.

For quantitative tracking of the bioprocess, the SERDS signals are pre-processed by smoothing (Savitzky-Golay polynom) and baseline correction (regression) as a first step. Thereafter, for data evaluation partial least squares regression (PLSR) based on the NIPALS algorithm to predict the EPS concentration from the SERDS signals was used. The concentrations predicted by PLSR deviates significantly from the reference concentrations determined by conventional offline analytics, especially for very low and very high concentrations. These deviations could be explained by the small calibration data set in the inherently nonlinear signal-concentration relationship. The nonlinearity is based on the concentration and time-dependent structure and size of the EPS molecules, which is recognized by the SERDS signal, but not by the offline analytics which serve as the reference. Furthermore, the surrounding and cultivation status influences the EPS molecule characteristics, too, which can lead to different SERDS spectra for the same EPS concentration. In order to overcome the poor performance of a linear data evaluation by PLSR, an alternative based on nonlinear regression using support vector machines (SVM) was successfully tested. A high prediction accuracy with only small training data sets could be obtained. In the first proof-of-concept experiments with cultivation samples containing biomass the model quality for the EPS concentration prediction was improved by more than an order of magnitude using support vector regression (SVR).

Eventually, to point out the strengths, weaknesses, opportunities and threats of our approach, it is evaluated against the conventional downstream processing. Our spectroscopic method focuses on the

determination of the exopolysaccharide concentration, as this parameter can be directly correlated with the cultivation progress. In this context, the SERDS approach stands out compared to conventional methods due to its potential for fast and direct measurements; hence, the capability of performing real-time process monitoring. The inherently non-invasive nature of the method eliminates the contamination risk of sampling, which is always a big threat to the success of the entire process. Additionally, substantial amounts of time and money can be saved using SERDS, since the downstream processing typically takes at least several hours and involves a large variety of chemicals including acids, alkali, buffer solutions, and enzymes. Even hazardous chemicals such as phenol and sulphuric acid are required to determine the EPS concentration in the conventional way (via phenol-sulphuric-acid-assay). Since these chemicals are very harmful to the human health (e.g., mutagenic) and the environment, they further need a special and expensive waste management. In contrast, SERDS can measure the EPS concentration without any sample preparation or chemical pre-treatment. Therefore, our method provides substantial economic and ecological benefits over the conventional approach. Moreover, the information obtained from the SERDS signal analysis can in principle be directly supplied to a feedback control system to adjust the cultivation parameters, e.g. the temperature, the pH, the acid-base-relation, photon flux, medium composition, etc. This will allow the yield of EPS production to be maximized and, at the same time, the danger of the algal culture die off due to contamination of irregular process conditions to be minimized. Using SERDS, the financial expenses for process analytics can be drastically reduced because costs for personnel, maintenance and consumables are much lower compared with the conventional methods. A weakness of the approach however is the low scattering cross section of the Raman effect which leads to the present detection limit of around 100 mg/l EPS. Utilising resonance enhancement or deep-UV Raman spectroscopy may be a solution to this limitation.

All in all, it can be concluded that the presented SERDS-SVR-approach provides an alternative for conventional offline analytics to determine the EPS concentration and offers the possibility of non-invasive monitoring the cultivation progress of *P.purpureum* online.

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