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On-Line Determination of the Intracellular Poly(β-hydroxybutyric acid) Content in Transformed *Escherichia coli* and Glucose during PHB Production Using Stopped-Flow Attenuated Total Reflection FT-IR Spectrometry

Gunta Jarute,[†,‡] Alexander Kainz,[§] Gerhard Schroll,[§] Josefa R. Baena,[†] and Bernhard Lendl*[,†]

Institute of Chemical Technologies and Analytics, Vienna University of Technology, Getreidemarkt 9/164, A-1060 Vienna, Austria, Department of Physic and Mathematics, University of Latvia, Zellu strasse 2, LV-1002 Riga, Latvia, and Institute for Microbiology and Genetics, Vienna University, Althanstraβe 14, 1090 Vienna, Austria

An automated approach for rapid on-line monitoring of the solid and liquid phases present in bioprocesses based on mid-IR Fourier transform spectrometry is introduced. The principles of this new approach are presented using the example of the quantitative and qualitative analysis of poly(β-hydroxybutyric acid) (PHB) accumulated in living bacterial cells as well as dissolved glucose during two 27-h fermentation processes. FT-IR spectra were recorded on-line using a diamond-attenuated total reflection (ATR) cell connected to the fermentation broth by means of a computer-controlled flow system. For calibration of the FT-IR method, standard reference analysis procedures for PHB (gas chromatography) and glucose (HPLC) were used. While pumping the fermentation broth through the flow cell, the recorded spectra corresponded to the fermentation solution, which allowed the determination of glucose in a range from 3.8 to −10.3 g/L. Upon stopping the flow, the cells settled on the ATR surface, and the thus recorded spectra enabled the determination of the intracellular PHB content of *Escherichia coli* in a range from 0.005 to 0.766 g/L. Errors of cross-validation of 0.264 g/L for glucose and 0.037 g/L for PHB were obtained. Application of one PLS calibration model to another fermentation was possible with prediction errors of 0.493 g/L for glucose and 0.105 g/L for PHB. Furthermore, from the position and shape of the PHB carbonyl band, it could be concluded that the PHB granules inside the *E. coli* are predominantly amorphous.

Using Fourier transform mid-infrared spectrometry, a broad variety of analytical problems can be solved in an elegant way by recording spectra of gaseous, liquid, or solid samples. The molecular specific information contained in the mid-infrared spectral region may be used for a variety of different purposes. It allows rapid identification of pure substances as well as quantitative determination of several analytes also in complex matrixes. The global fingerprint of an infrared spectrum, furthermore, provides access to properties of a sample that cannot be related to the concentration of one or more defined molecules alone. The latter point is of considerable interest in qualitative analysis in which rapid answers in sample qualification are needed. Recent examples for such applications include the identification of scrapie infection of hamsters based on the analysis of mid-infrared spectra recorded from dried blood samples[1] or the identification of red wine varieties from the mid-IR spectra of their phenolic fraction.[2] An appealing feature of infrared spectrometry is, furthermore, the fact that it is a nondestructive technique that requires hardly any sample preparation steps. Therefore, infrared spectrometry leads itself to application in process analytical chemistry where these characteristics are important for obtaining reliable information in short time.[3] So far, the recorded mid-IR spectra have almost exclusively been used for quantitative analysis determining the concentration of selected analytes.[4,5] However, it shall also be noted that qualitative information, such as the physiological status of microorganism, may be deduced from mid-IR spectra in case they can be recorded reliably.[6,7]

If mid-infrared spectrometry is to be used for in- or on-line monitoring of the liquid phase of bioprocesses, then the attenuated total reflection (ATR) technique is the technique of choice for spectrum acquisition because of the strong mid-infrared absorption of water. The ATR technique efficiently ensures a short optical path for measurement without putting restraints on the geometry

* Corresponding author. Phone: +43 1 58801 15140. Fax: +43 1 58801 15199. E-mail: blendl@mail.zserv.tuwien.ac.at.

† Vienna University of Technology.

‡ University of Latvia.

§ Vienna University.

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of the unit hosting the ATR element. As a result, in-line ATR sensors have been developed for process analytical applications. However, when using such in-line ATR sensor systems, care must be exercised that no film is formed on the ATR surface because this would render impossible any reliable measurement. This can be a serious problem in fermentation monitoring in which biofilms may also be easily formed on diamond ATR surfaces, as observed by us in the case of a fed-batch yeast fermentation in which a horizontally mounted ATR was coupled to a bioprocess in a fast loop.[8] The fact that especially proteins rapidly form films on ATR surfaces, including ZnSe and other ATR materials, has also been exploited for the study of these proteins. Nevertheless, recent papers in the literature describe the prolonged use of in-line ATR probes for bioprocess monitoring over several hours.[9,10] However, so far, both in-line and on-line ATR measurements have only been conducted to determine the concentration of a variety of small molecules dissolved in the fermentation broth, such as sugars, alcohols, organic and amino acids, and phosphate.

In this contribution, we introduce a new experimental concept that extends the sampling capabilities of on-line ATR spectroscopy to solid materials present in bioprocesses. The new concept utilizes the stopped flow technique to distinguish between dissolved molecules and microorganisms (solid matter). FT-IR spectra that are recorded while the fermentation broth is pumped through the flow cell predominantly contain information on the chemical composition of the liquid phase. This is because the laminar flow conditions in the flow cell and the short residence time of the microorganism do not come into the reach of the evanescent field. However, upon stopping the flow, the microorganisms settle on the ATR surface where they can be measured. Complete removal of the microorganism from the ATR surface after measurement can be achieved and verified by a sequence of automated rinsing steps involving $NaHCO_3$ and distilled water. Using this concept, dissolved molecules and microorganisms can be reliably analyzed during a bioprocess in a completely automated fashion.

This new concept has been applied to the determination of dissolved glucose and poly-3-D-hydroxybutyric acid (PHB) in different *Escherichia coli* strains during three fermentation runs. PHB is a promising biotechnological product from the class of bacterial polyhydroxyalkanoates (PHAs), a naturally occurring thermoplastic polyester suitable for environmentally friendly packaging materials, especially for biomedical applications. Pure PHB is accumulated in nature by bacterial cells and kept in an amorphous state in the cytoplasma of procaryotic cells, forming granule structures. Several methods have already been used to determine polymer content in bacteria, but they require extensive and complicated sample preparation, such as extraction, purification, and formation of the methyl ester, which is followed by gas chromatography for quantitative analysis of the produced measurand.[11]

Apart from being laborious, the results are available only with a significant time delay, which renders them not useful for rapid decision making. Therefore, in bioprocess monitoring, there is a need for new analytical methods that provide timely information on the chemical composition of the whole fermentation broth, including dissolved molecules and solid matter.

Off-line FT-IR spectrometry has already been applied to the study of PHB in bacterial cells.[12] Recently, a method based on FT-IR spectrometry was developed for quantitative and qualitative analysis of PHAs in lyophilized bacteria. The ester carbonyl band at 1744−1722 cm^{-1}, apart from being important in quantitative analysis, also allows assessment of the degree of crystallinity of the polyesters. This is possible because upon crystallization, this band is shifted toward lower wavenumbers as a result of intensified hydrogen bonding in the crystallized form.

MATERIALS AND METHODS

Bacterial Cultures and Growth Conditions. *E. coli* strain DH5 was originally derived from the German Collection of Microorganisms and Cell Cultures (DSMZ). The strain was transformed freshly with plasmids harboring the three genes of *Ralstonia eutropha* needed for recombinant PHB expression in *E. coli*. Plasmid p4A (amp) harboring the PHA operon was described by Janes et al.[13] The dual plasmid system using pUMS (amp) and pSYN (tet) was set up by Kalousek and Lubitz[14] for enhanced production on glucose-free medium during the active growth phase. For maintenance of the plasmids, the antibiotics amphicillin (100 μg/mL) and tetracycline (10 μg/mL) had to be added to the broth.

The bioprocesses were started in a 2000-mL bioreactor by addition of 100 mL of cell culture to 1500 mL of medium at 28 °C using a thermostatic water bath for constant temperature during the 26-h fermentation experiments. The standard LB (Luria Bertani) medium used contained 1% glucose, 10 g/L peptone from caseine, 5 g/L yeast extract (Difco), and 5 g/L NaCl at pH 7.0. For an additional carbon source, glucose to a concentration of 1% was added. A magnetic stirrer at 150 rpm provided aeration.

Experimental Setup for On-Line FT-IR Measurements. The experimental setup designed for the on-line monitoring of the fermentation process is schematized in Figure 1. A computer-controlled sequential injection analysis (SIA) flow system acted as an interface between the fermentation under study and the ATR cell for FT-IR measurements. The whole system consisted of the bioreactor (volume, 2000 mL), Teflon tubing (i.d., 1 mm), a Cavro (Sunnyvale, CA) XP 3000 syringe pump equipped with a 5-mL syringe, a selection valve (Vici Valco, Schenkon, Switzerland), and a Bruker IFS 88 spectrometer equipped with a three reflections diamond ATR cell connected to the selection valve through a 50-cm-long piece of Teflon tubing. For detection, a liquid nitrogen-cooled mercury−cadmium−telluride (MTC) detector was used. The penetration depth (d_p) for the system diamond−water could be estimated to be in the range of 2 μm. The cumulative penetration depth has been measured by comparison of the absorbance of a 10 g/L glucose solution at 1080 cm^{-1} in a 25-μm flow cell and the absorbance of the same solution in the ATR flow

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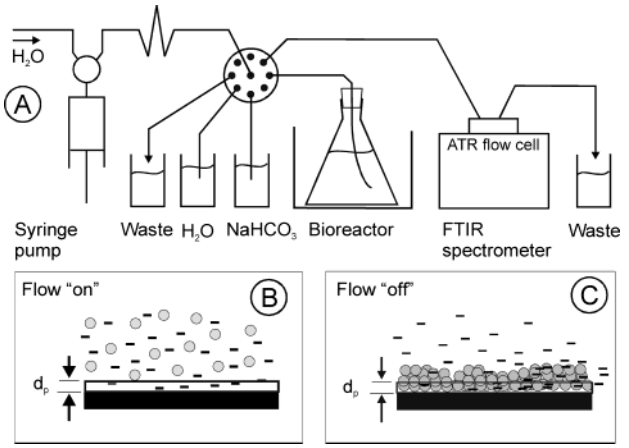

Figure 1. (A) Schematic experimental setup employed for on-line monitoring of intracellular PHB accumulation in *E. coli* during fermentation processes. (B) During flow-on, hardly any cells come into reach of the evanescent field. In the figure, the distance this field reaches into the liquid phase is denoted penetration depth (d_p). (C) As the cells settle, they come into reach of the evanescent field and, thus, contribute to the recorded spectra.

cell used in the experiment and found to be 4.8 μm. The flow system was controlled via an in-house-written MS Visual Basic 6.0-based software Sagittarius V2 (1.02.0003), and FT-IR spectra were recorded using the processing program OPUS (Bruker, Ettlingen, Germany). Each spectrum was obtained at a resolution of 4 cm^{-1} by co-adding 128 scans. The spectral range extended from 4000 to 700 cm^{-1}, the main spectral information was contained within the 1900−800 cm^{-1} region.

Experimental Procedure. For continued monitoring of the bioprocess, the experimental procedure consisting of the following steps was repeatedly applied: background acquisition, system conditioning, sample measurement, and system cleaning. For background acquisition, distilled water was passed onto the ATR cell. Subsequently, two fractions of 3 mL from the fermentation broth were driven through the system to have the sample replace the water in the tubing. For sample measurement, a 3-mL volume of bacterial broth was pumped onto the ATR cell, and the spectrum of the fermentation mixture was recorded, thus providing information on the glucose concentration in solution. Afterwards, the flow was stopped for 15 min, after which the spectrum of the cells settled onto the ATR surface was recorded. From these growth spectra, the amount of PHB produced during the fermentation could be determined. Simultaneously, 30 mL of broth was automatically sampled from the bioreactor for the corresponding reference analysis. The next step implied the cleaning of the flow system, including the ATR flow cell, by flushing with a 5% $NaHCO_3$ solution for 15 min, followed by rinsing with distilled water for 25 min. Every cycle (including background measurement, system conditioning, sample measurement, and cleaning steps) took 1 h to be completed.

Reference Analysis. Samples from the fermentation process were subjected to HPLC analysis to determine the concentration of glucose in the solution and to GC analysis to determine the PHB content in the cells. For this purpose, *E. coli* cells harvested from the bioprocess were centrifuged for 10 min at 13400 rpm to separate the supernatant from the cells. The supernatant was then filtered through a Millipore 0.22-μm filter and stored at 4 °C for HPLC analysis. For PHB determination, *E. coli* cells were washed with saline solution in a 1.5-mL Eppendorf tube and centrifuged for another 10 min at 13400 rpm. The cells were then frozen at −20 °C and lyophilized.

Determination of Biomass. The biomass growth during fermentation was assessed through optical density measurements of the cell mass as determined by standard molecular absorption photometry at 600 nm.

Glucose Determination in the Fermentation Broth. HPLC analysis was performed on an 8% Ca^{2+} column (300 mm × 7.8 mm, Phenomenex, CA) at 75 °C using a Hewlett-Packard series 1100 HPLC analyzer equipped with UV and refraction index detection. The separation was achieved under isocratic conditions (flow rate, 0.6 mL/min) with distilled water as mobile phase. The results were processed using the above-mentioned OPUS software. The total procedure took 20 min for each sample.

PHB Determination in Lyophilized Cells. Lyophilized cells were prepared according to the procedure described by Braunegg et al.[11] The cells were treated with a solution consisting of a 1:1 mixture of chloroform and 3% sulfuric acid in methanol at 103 °C for 210 min to render the constituents into methyl esters. After cooling, the reaction mixture was extracted with distilled water, and the organic phase was dried over anhydrous sodium sulfate and analyzed with GC as described previously.[14] The total procedure (including sample treatment and chromatographic run) took a minimum of 5 h.

Data Analysis. The data of the reference analysis, in connection with the corresponding spectra, were analyzed by partial least squares (PLS) regression, which was performed using the appropriate software package of the available OPUS software. The number of factors to be included in the PLS method was assessed by cross-validation. Calibration models were created to relate the spectra to the known concentrations obtained by reference analysis. These models are characterized by the determination coefficient, R^2, which evaluates the fit of the model to the calibration data, and different errors, such as RMSEC, root mean square error of calibration; RMSECV, root mean square error of cross-validation; and RMSEP, root mean square error of prediction. The correlation coefficient is calculated from a plot of measured versus predicted values, with values closer to 1 indicating a better agreement between the measured and predicted values. The RMSEC is a measure of goodness of the PLS models to the calibration set; the RMSECV indicates the predictive ability of the model within the calibration set and the optimal number of factors to be used, and the RMSEP evaluates the response of an established calibration model versus an independent test sample set.

RESULTS AND DISCUSSION

Bioprocesses for PHB Production under Study. PHB accumulation of two differently transformed *E. coli* strains was monitored for 27 h in separate experiments. Results of the reference analysis are plotted in Figure 2, which indicate that the fermentations had developed as expected. At the start of the process, in the active growth phase, the equilibrium is still in favor of the formation of biomass, so that only minor amounts of PHB are produced. After yielding a certain cell density, the glucose concentration decreased as the storage compound was produced and accumulated in the cells. Recombinant *E. coli* cells producing

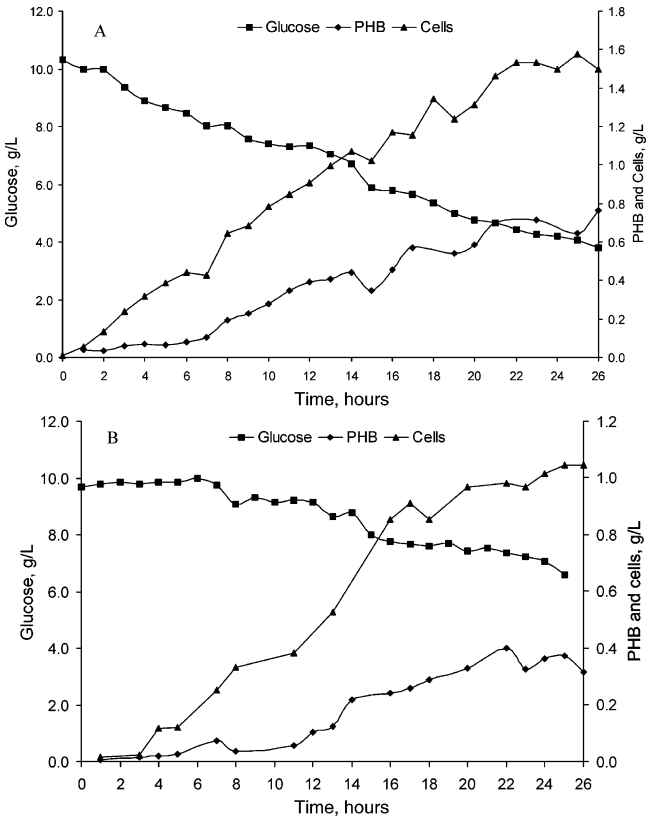

Figure 2. Plot of cell density, expressed as grams per liter; glucose; and PHB concentrations during the fermentation processes of recombinant PHB producing *E. coli* strains: (A) *E. coli* DH5α containing plasmid p4A; (B) *E. coli* DH5α containing plasmids pUMS and pSYN. Reference analysis was used for concentration determination.

PHB were elongated and bloated by the thermoplastic polyester, resulting in oversize cells, as was monitored by light microscopy. Since the bioprocess was not pH-controlled, a shift of the pH toward acidic medium (from pH 6.6 to pH 5.2) was observed in the first hours of the fermentation The pH then remained constant at a value of pH 5 until the end of the experiment. This could also influence the granule size of the storage granules and the chain length of the single polymers that form the granules, although the present study was not focused on the determination of these parameters.

Mid-IR Spectra of PHB and PHB Containing *E. coli*. Reference spectra of crystalline and amorphous PHB recorded with a diamond ATR element are displayed in Figure 3, together with a spectrum of an *E. coli* that contained PHB deposited onto the ATR surface after a settling time of 30 min. One can clearly distinguish between different biomolecule markers, such as the cellular protein bands and others characteristic for PHB. Protein levels could be estimated by the amide I band at 1650 cm^{-1} (amide carbonyl stretching) and the amide II band at 1540 cm^{-1} (out-of-phase of the N−H bend combined with the CN stretching). The most important PHB band is placed at 1738 cm^{-1} (carbonyl stretch); other strong bands are located between 1450 and 1000 cm^{-1} (methyl and methylene deformation and C−O stretch vibrations).[12] The position of the carbonyl band has been used by several authors to evaluate the crystallinity degree of PHB

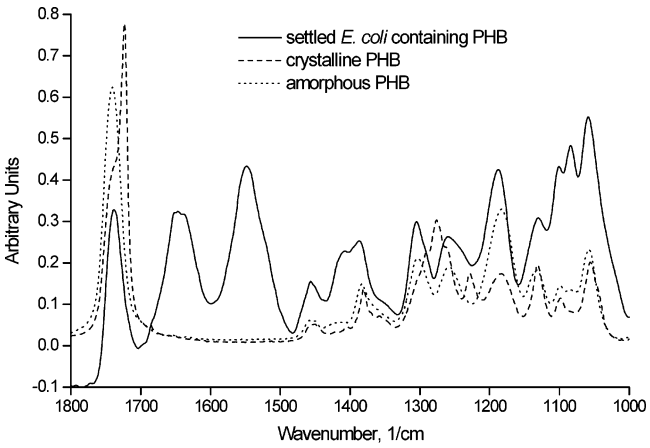

Figure 3. FT-IR reference spectra of amorphous, crystalline PHB compared with a spectrum of *E. coli* DH5α p4A containing PHB that was taken from a stopped-flow experiment during fermentation. The spectra were recorded using a water background spectrum.

films. According to Bloemberger et al.,[15] after 1 min of solvent evaporation, the band was at 1740 cm^{-1}, whereas it was at 1725 cm^{-1} after 17 h of solvent evaporation. The bands at 1740 and 1725 cm^{-1} were attributed to the stretch vibrations of the amorphous and crystalline carbonyl groups, respectively.[16,17] In the crystalline phase, the segments predominantly form 2_1 helices, in which the oxygen atoms of the carbonyl groups are located closer to the hydrogen atoms of the other segments. This gives rise to increased H-bonding in the crystalline phase, which is reflected in the shift of the ν-C═O toward lower frequencies as compared to the amorphous phase.[18] Therefore, the measured band position of the intracellular PHB allows one to conclude that PHB produced in the *E. coli* is amorphous.

On-Line FT-IR Measurements Using the Automated Flow System. The versatility of the proposed bioprocess monitoring system allows for FT-IR measurements to differentiate between dissolved and suspended components of the fermentation broth, depending on the flow rate of the sample. Due to the short penetration depth, which is on the order of 1−2 μm for the diamond−water system in the mid-IR spectral region, only sample in close proximity to the ATR surface contributes to the measured spectra. Therefore, considering the laminar flow inside the flow cell, together with the short residence time of the sample as well as the low diffusion coefficients of the microorganisms, it is clear that these hardly reach the evanescent field upon passing the flow cell. As a consequence, the spectra recorded during flow-on are dominated by the liquid phase of the fermentation broth, which in this application allows the determination of dissolved glucose. On the other hand, when the flow is stopped, the solid bacteria cells settle onto the ATR surface, and the thus recorded spectra provide information on the bacterial cell composition, including its PHB content, which again can be exploited for performing quantitative analysis.

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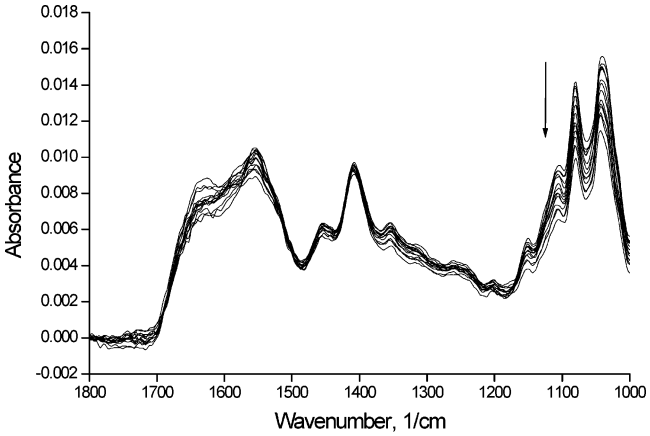

Figure 4. FT-IR spectra of the fermentation broth during PHB production using the *E. coli* DH5α p4A strain. The spectra have been recorded in continuous-flow mode and mainly reflect changes in the composition of the liquid phase. The arrows indicate increasing time.

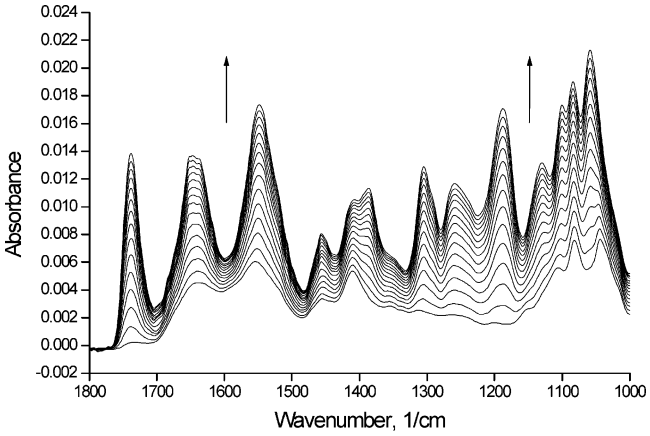

Figure 5. Monitoring of the settling of PHB containing *E. coli* DH5α p4A cells. The FT-IR spectra shown were recorded for 2 min. The arrows indicate increasing time.

As an example, the spectra recorded in the "flow-on" mode during fermentation using the single plasmid system are shown in Figure 4. In these spectra, only the decrease in the bands corresponding to glucose (1150−1000 cm^{-1}) was observed, as was expected from the growing of the bacterial cells. No spectral features corresponding to PHB are noticeable, even when it is present in the reference analyses. This is consistent with the fact that non-water-soluble PHB is accumulated intracellularly and that the *E. coli* cells do not come significantly within reach of the evanescent field.

As discussed previously, by stopping the flow, the solid compounds settle onto the ATR surface, and their IR spectra can be recorded. The change in the recorded spectra during a 30-min settling period is shown in Figure 5. Increasing settling time implies that a greater number of cells are exposed to the evanescent field, which results in an increase in the recorded intensities. In the case of the *E. coli* cells under study, the observed settling was completed after roughly 45 min. However, it must be expected that during the settling process, the composition of the *E. coli* cells continued to change; therefore, the PHB content in the cells measured after a settling period of 45 min will not exactly reflect the one of the sample drawn from the bioprocess. To minimize this time lag, the settling process was fixed to 15 min. After this time, the recorded intensities had reached ∼40% of the possible final values, which was sufficient for reliable quantitative analysis. The change in the cell spectra during fermentation can be seen in Figure 6. The depicted spectra were recorded after a settling time of 15 min in order not to overload the figure, only the spectra recorded every 3 h are shown. Due to the well-separated spectral features of PHB and the proteins contained in the bacterial cells, the increase of the PHB content during the course of fermentation can readily be seen from the recorded spectra. Similar FT-IR spectra have been obtained for the other investigated fermentation using the dual plasmid system.

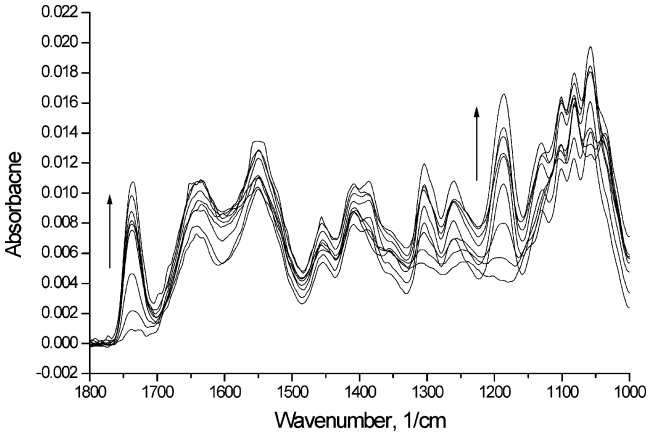

Figure 6. Change in the FT-IR spectra of PHB-containing *E. coli* cells during the 26-h fermentation using the *E. coli* DH5α p4A strain. The time spacing between individual spectra is 3 h. Each spectrum corresponds to a settling time of 15 min. The arrows indicate increasing time.

PLS Analysis. PLS regressions were performed on calibration sets composed initially of 27 samples recorded from each fermentation. The spectra were studied to detect the presence of outliers through the study of the leverage and the spectral residuals. In the case of the spectra recorded during flow-on, no outliers were detected in either fermentation; however, in the case of the spectra recorded after a settling time of 15 min, two and three spectra had to be removed, respectively. Original spectra as well as their second derivatives were subjected to analysis, revealing no significant differences in the obtained results. Therefore, the regression models were finally performed using original spectra. Individual models were developed for each analyte (glucose and PHB) and validated using leave-one-out cross-validation. Table 1 shows the results in terms of correlation coefficients between the predicted and measured concentrations and the errors of calibration, prediction, and estimation in each case, together with the number of samples used in the calibration or test set as well as the number of factors required to obtain the best fit.

Results of Glucose Analysis. PLS regressions for the estimation of glucose concentration were performed on the spectra recorded in the continuous flow mode using the spectral region between 1190 and 960 wavenumbers. For both fermentations, calibration models could be established with good correlation coefficients (R^2 = 99.26 and 96.90). Furthermore, a satisfactory agreement between the predicted values of the leave-one-out cross-validation samples and values obtained from reference analysis (RMSEC and RMSECV between 0.194 and 0.297 g/L) was obtained, which further indicated the accuracy of the models.

Table 1. Results from PLS Regressions for the Fermentation of *E. coli* DH5α P4A[a] and *E. coli* DH5α PUMS PSYN[b]

calibration data, samples	factors	R^2	RMSEC, g/L	RMSECV, g/L	test data, samples	RMSEP, g/L
			Glucose			
E. coli DH5α p4A/27	5	99.26	0.194	0.297		
E. coli DH5α p4A/27	5	99.26	0.194		*E. coli* DH5α pUMS pSYN/27	0.493
E. coli DH5α pUMS pSYN/27	2	95.46	0.252	0.264		
E. coli DH5α pUMS pSYN/27	2	95.46	0.252		*E. coli* DH5α p4A/27	0.469
			PHB			
E. coli DH5α p4A/24	5	98.46	0.029	0.038		
E. coli DH5α p4A/24	5	98.46	0.029		*E. coli* DH5α pUMS pSYN/25	0.108
E. coli DH5α p4A/23	7	99.51	0.017		*E. coli* DH5α pUMS pSYN/24	0.035
E. coli DH5α pUMS pSYN/25	5	96.92	0.026	0.037		
E. coli DH5α pUMS pSYN/25	5	96.92	0.026		*E. coli* DH5α p4A/24	0.105
E. coli DH5α pUMS pSYN/24	8	99.89	0.005		*E. coli* DH5α p4A/23	0.047

[a] 10.3−3.8 g/L Glucose, 0.059−0.661 g/L PHB. [b] 9.98−6.65 g/L Glucose, 0.030−0.364 g/L PHB.

When applying the same calibration model to an independent set of test samples (taken form the other fermentation), the error of prediction (RMSEP) increased, but was still acceptable when taking into account the complexity of the sample matrix.

Results of PHB Analysis. For prediction of the PHB concentration, the spectral information contained in the 1780−930 cm^{-1} region was selected for the PLS regression model. Upon removing outliers, the number of samples was reduced from 27 to 24 in the case of the *E. coli* DH5α p4A fermentation and to 25 in the case of the *E. coli* DH5α pUMS pSYN fermentation. As shown in Table 1, the PLS models yielded good coefficients of determination (R^2 = 98.46 and 96.92), and the errors found in cross-validation were low enough (0.037−0.038 g/L) to demonstrate the suitability of the calibration for the prediction of samples within the same fermentation. However, when the optimum calibration model was applied to the prediction of an independent set of samples, the RMSEP increased to 0.108 or 0.105 g/L, depending on the model applied. This increase in the prediction error may be attributed to the fact that two different strains were used; therefore, including a larger number of factors in the calibration significantly reduces the RMSEP values (down to 0.017 and 0.005 g/L). However, when judging the errors obtained, the performance of the reference method applied needs to be taken into account, as well. Due to the laborious procedure, the precision of the complete reference method is in the range of 8% for a medium concentrated sample (3 g/L), which corresponds to 0.25 g/L PHB. The slightly decreased performance of the on-line method is, however, more than compensated by the fact that it provides information practically and in real time with respect to a delay of 5 h in the case of the reference method. This distinct quality allows for rapid decision making in the case of a badly performing fermentation.

CONCLUSIONS

A new method for the online study of intracellular processes in living bacteria cells by using ATR-FT-IR spectroscopy to obtain molecule-specific information of the target species has been developed. The method provides a rapid and useful tool for on-line monitoring of bioprocesses. The new method was applied for the determination of intracellular thermoplastic polyesters that are accumulated in transformed *E. coli* during fermentation, as well as of glucose in the fermentation broth. Qualitative information about the crystallinity degree of PHB granules as produced in *E. coli* bacteria cells was also obtained. The described ATR-FT-IR monitoring has been demonstrated to enable real time detection of anomalies in the process, thus allowing for fast application of correction actions that would not be feasible using conventional off-line analysis. On the basis of the results obtained, many other useful applications of the developed technology may be expected. Especially, the capability of mid-IR spectrometry to provide information on the physiological status of cells might be of interest in advanced bioprocess monitoring systems.

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