Monitoring bacterial processes by Fourier transform infrared spectroscopy: *Helicobacter pylori* drug inactivation and plasmid bioproduction in recombinant *Escherichia coli* cultures

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***Abstract*—Fourier transform infrared (FTIR) spectroscopy is evaluated as a tool to monitor two bacterial processes: strain discrimination and drug inactivation studies with the gastric pathogen *Helicobacter pylori* and the plasmid production process based on high-density cultures of recombinant *Escherichia coli*. Results show, that after evaluation of different incubation conditions of *H.pylori* with the drug model, the application of principal component analysis to the FTIR spectra assembles the samples into clusters which can be related with the minimal inhibitory concentration. Morever, the same methodology applied to FTIR spectra from 12 different strains can be used to distinguish them. For the *E.coli* cultures it is possible to estimate the concentration of relevant bioprocess monitoring variables, such as plasmid, biomass, and carbon sources (glucose, glycerol, acetate) by using partial least squares (PLS) models based on FTIR spectra.**

***Index Terms*—FTIR spectroscopy; chemometrics; *H.pylori*; *E.coli*; drug inactivation; bioprocess monitoring;**

I. Introduction

Fourier transform infrared (FTIR) spectroscopy is becoming an interesting technique to monitor and analyse biological processes due to recent developments in FTIR spectroscopy instrumentation, which increased wavelength accuracy, spectral quality and reproducibility [1]. It has been applied in many biological areas, as for example in discrimination of the pathogen *Straphylococcus aureus* from other strains of *Straphylococcus* [2], to detect intracellular components, such as granules, capsules and endospores in intact bacteria [3] and to identify the signature of the metabolic changes in prostate cancer cells induced by anticancer drugs [4].

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FTIR spectroscopy usually allows rapid non destructive analysis requiring minimal sample preparation. By applying several preprocessing techniques and chemometric methods, such as principal component analysis and partial least squares regression, it is possible to process FTIR spectra and extract biologically meaningful information. This work studies the applicability of FTIR spectroscopy in two different bacterial processes: strain discrimination (A) and drug inactivation studies (B) with the gastric pathogen *H.pylori* and the plasmid production process based on high-density cultures of recombinant *E.coli* (C).

A. Discrimination of 12 H.pylori strains

H.pylori, a Gram-negative microaerophilic bacterium highly adapted for colonization of the human stomach, infects more than half of the world's human population, contributing to several gastric diseases such as peptic ulcer, MALT lymphoma or gastric carcinoma [5]–[7]. As it presents a high genetic variability [8], [9], patients are often infected with multiple *H.pylori* strains [10]. Eradication therapy generally includes two or more antimicrobials, but resistances of some strains against the most commonly used antibiotics can lead to a failure of the treatment. Moreover, many broad-spectrum antibiotics are expensive and have toxic side effects [11]. For these reasons a rapid identification of the present *H.pylori* strains is desirable in order to provide an effective therapy. This thesis investigates the potential of FTIR spectroscopy to distinguish 12 *H.pylori* strains.

B. The drug inactivation of the gastric pathogen H.pylori

The gastric pathogen *H.pylori* is usually acquired in early childhood [10] and if not treated with antibiotics persists

TABLE I

MAIN CHARACTERISTICS OF FIVE DIFFERENT SETS OF EXPERIMENTS CONDUCTED WITH *H.pylori* STRAINS 26695 AND J99, DIFFERING IN RELATION TO THE QUANTITY OF THE BACTERIA AND THE NUTRIENT BRUCELLA BROTH IN THE FINAL INCUBATION MIXTURE AS WELL AS THE TIME USED FOR INCUBATION OF THE BACTERIA WITH THE DRUG MODEL. THE INCUBATION MIXTURES OF EXPERIMENTS 2 TO 5 WERE WASHED PRIOR TO SPECTRA ACQUISITION IN CONTRAST TO EXPERIMENT 1.

***H.pylori* strain**	**Experiment number**	**OD**	**Brucella broth**	**Tested drug concentration ($\mu g\ ml^{-1}$)**	**Incubation time (h)**	**Analyte volume (μl)**	**Sample type**
26695	1	4	25%	0,2.5,25,50,75,100	1	30	whole incubation mixture
	2	2	5%	0,2.5,5,25,50,75,100,200,400	1	25	washed cells
	3	2	5%	0,2.5,5,25,50,75,100,200,400	3	25	washed cells
	4	2	5%	0,2.5,5,25,50,75,100,200,400	6	25	washed cells
J99	5	4	0	0,2.5,25,50,75,100,200	1	25	washed cells

throughout life. Unfortunately, eradication therapy fails in 10 to 20% of all cases due to growing resistances against the commonly used antibiotics, such as metronidazole and clarithromycin. To overcome these limitations it is crucial to screen for new antibacterial drugs, which in the case of *H.pylori* is difficult due to the high genetic variability of this organism [8], [9]. Therefore a high-throughput method is needed for the drug screening. This thesis evaluates the potential of FTIR spectroscopy as a technique to monitor the effect of a drug model on the bacterium metabolism, which could be used in future high-throughput drug screenings.

C. The plasmid production process based on high-density cultures of recombinant E.coli

In the last decade plasmid vectors have become extremely attractive for use in DNA vaccines and gene therapy, as they offer multiple advantages over viral vectors, including large packaging capacity, stability without integration and reduced toxicity [12]. However, the industrial plasmid production with recombinant *E.coli* cells is a complex process, as many parameters, such as medium composition and cultivation conditions influence the plasmid copy number, stability and the amount of biomass produced [13]. Therefore it is crucial to develop monitoring techniques which allow to increase the understanding of the process, its control and optimisation. Conventional laboratory methods are usually not adequate to measure the significant parameters in a time frame which allows adjustments of the process [14]. Therefore it is relevant to develop rapid monitoring techniques, which give information about the culture state to adjust the characteristics of the culture improving batch to batch reproducibility as well as process efficiency. Being a rapid and sensitive analysis method, FTIR spectroscopy is promising to serve well for this purpose. This thesis evaluates it as a tool to predict the concentration of the relevant variables for control and optimisation of the cultivation bioprocess, which are biomass, plasmid and carbon sources (glucose, glycerol, acetate).

II. GOALS

This thesis aims at monitoring the drug inactivation of *H.pylori* by FTIR spectroscopy to determine if this method can serve as a quick alternative to conventional drug susceptibility assays. Moreover, the potential of FTIR spectroscopy in discriminating 12 *H.pylori* strains is investigated. Furthermore, FTIR spectroscopy is evaluated as a monitoring technique for the plasmid bioproduction in recombinant *E.coli* cultures with the objective of rapidly predicting the most relevant variables for monitoring purposes: the concentration of biomass, plasmid and the carbon sources glucose, glycerol and acetate.

III. MATERIAL AND METHODS

A. Sample preparation for the H.pylori dataset

The minimum inhibitory concentration (MIC) was determined by J.Lopes and M.Oleastro using the agar dilution method as recommended by the *H.pylori* study group [15]. To evaluate if FTIR spectroscopy is capable to estimate the MIC obtained by this conventional method, 5 sets of experiments were conducted representing different incubation conditions of the *H.pylori* strains with the drug model (table I). For this, the bacterial strains J99 and 26695 were grown on *H.pylori* selective media (Biogerm, Portugal) and incubated for 24 to 48 hours at 37°C in microaerophilic conditions (CampyGen, Oxoid, UK). The bacteria were collected from the petri dishes and suspended in NaCl 0.9% (w/v). The optical density (OD) of the resuspended cells was read at 600 nm (using Synergy 2, BioTek Instruments in combination with the GEN5 software, BioTek Instruments, USA) after appropriate dilution and the incubation mixtures as described in table I were prepared.
For investigating the potential of FTIR spectroscopy to discriminate between *H.pylori* strains, samples of the strains 4115, 3485, 4009, 3746, 3861, 26695, 3982, 4101, J99, 3551, 3923 and 4212 with an optical density of ca. 4 were prepared as discribed above. Strains 26695, originally isolated from an English patient suffering from gastritis [16] and strain J99, obtained in the USA from a patient with a duodenal ulcer [17] are reference strains. The remaining strains were isolated in 2008 and 2009 from Portuguese patients and belong to the collection of the National Institute of Health Dr. Ricardo Jorge (Lisbon, Portugal).

B. Sample preparation for the E.coli dataset

Five *E. coli* batch cultures were conducted by Martins (2009) [18] in non selective and complex media using yeast extract and bacto tryptone as nitrogen sources and different mixtures of glucose and glycerol as carbon sources (table II). The concentration of biomass, plasmid, glucose, glycerol and acetate were analysed by Martins (2009) [18] by high-performance liquid chromatography (HPLC) and enzymatic

methods. The cellular *E.coli* pellets obtained from centrifugation of 1 ml of whole broth obtained along the five cultures were resuspended in 5 ml of NaCl 0.9% (w/v) for the subsequent FTIR analysis.

C. Spectra aquisition

For the FTIR analysis 25 or 30 μl of the bacterial cell suspension were placed on IR-transparent Zn-Se-microtiter plates with 96 wells (Bruker optics, Germany) and dehydrated using a vacuum dessicator (ME2, Vaccubrand, Germany). The FTIR spectra were acquired using a HTS-XT associated to a Vertex-70 spectrometer (Bruker optics, Germany) at a resolution of 2 cm^{-1}. Each final spectrum results from the average of 40 spectra acquisitions.

D. Preprocessing and multivariate data analysis

For both datasets several preprocessing strategies (offset correction, spectra normalization, multiplicative scatter correction (MSC), first derivative and using a narrow region of the spectra) have been tested. Manipulation of the spectra and the application of the chemometric methods have been performed with Matlab concerning the *H.pylori* dataset. For the *E.coli* dataset additionally a baseline correction has been performed using the OPUS software provided by Bruker.

IV. Results and discussion

A. Discrimination of H.pylori strains

FTIR spectroscopy provides a "molecular fingerprint" of the analysed cells and thus quantitative information about its total biochemical composition [19], which can be used for identification and discrimination of microorganisms [2]. To evaluate the discriminatory power of FTIR spectroscopy, the spectra of 12 *H.pylori* strains were collected and, thereafter, processed using MSC. Afterwards, PCA was performed on the first derivate of the spectra. In the score plot of the first two principal components (fig. 1) it is possible to distinguish the strains. It is worth to point out, that the two strains on which the inactivation by a drug model was monitored in this thesis, namely the strains J99 and 26695, are located in different areas of the score plot, which indicates big structural differences.

B. Inactivation of H.pylori by a drug model

The minimal inhibitory concentration (MIC) of the drug model, determined by the agar dilution method was 100 $\mu g\ ml^{-1}$ for strains J99 and 26695. With the main goal to evaluate, if the rapid and sensitive technique of FTIR spectroscopy could be used to monitor the *H.pylori* drug inactivation in an alternative way, several experiments were conducted as described in table I.

TABLE II
Medium composition in carbon source of batch cultures A to E. The values were obtained by analyses in the bioreactor immediately after the start of the culture.

Batch	**Glucose** ($g\ l^{-1}$)	**Glycerol** ($g\ l^{-1}$)
A	6.0	0.0
B	0.0	5.1
C	6.8	5.4
D	4.1	5.4
E	8.8	3.9

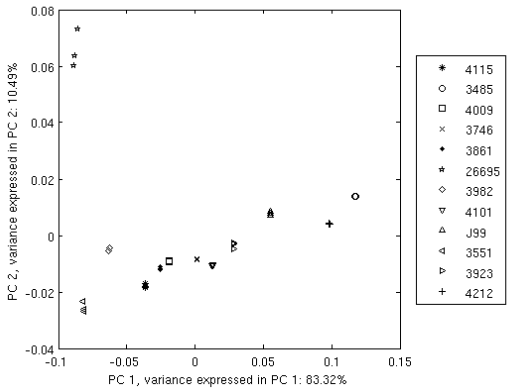


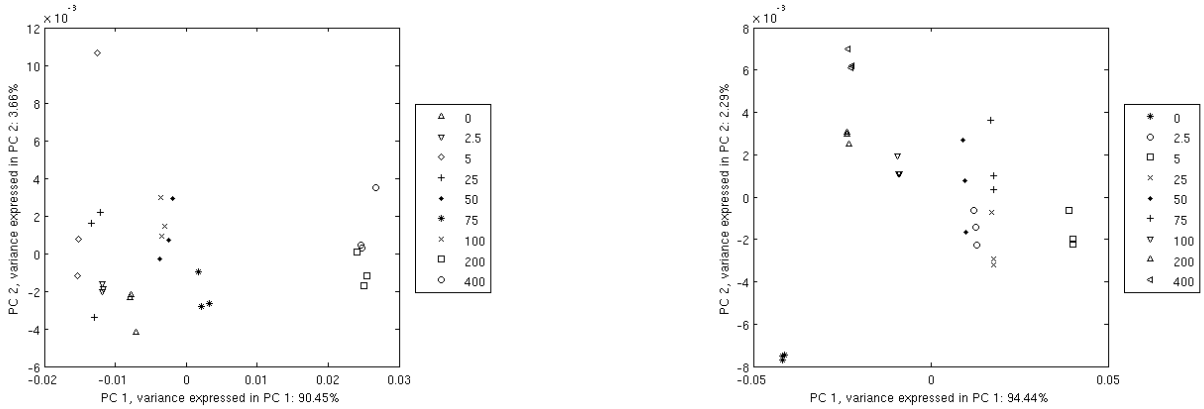
Fig. 1. Score plot of the first two principal components of the spectra of the *H.pylori* strains 4115, 3485, 4009, 3746, 3861, 26695, 3982, 4101, J99, 3551, 3923 and 4212.

After evaluating the above mentioned preprocessing techniques, for the principal component analysis on the *H.pylori* dataset a preprocessing of offset correction and multiplicative scatter correction (MSC) on the sample replicates has been chosen. Moreover, the first derivative was calculated using the Savitzky-Golay filter of window size 15 and polynomial order 4.

The experiments on strain 26695 showed, that cell quantities corresponding to an OD higher than 4 should be used, as lower quantities result in low absorbance values in the spectra, which decrease the signal to noise ratio (SNR). Moreover, brucella broth absorbs in the IR region (data not shown) and thus the cells should be washed before the spectra aquisition.

Principal component analysis showed, that FTIR spectroscopy can be used to monitor the effect of the drug on the *H.pylori* metabolism, even for concentrations far lower than the MIC such as 2.5 $\mu g\ ml^{-1}$ (fig. 2). The results regarding the analysis of the FTIR datasets obtained from *H.pylori* strains 26695 (Experiment 2 and 4) and J99 (Experiment 5) will be presented and discussed.

Fig. 2(a) shows a score plot of the first two principal components (PCs) of the spectra of experiment 2. A clear division into two groups is recognizable: One group contains the scores representing samples incubated with a concentration of the drug of 200 $\mu g\ ml^{-1}$ or higher, the other group contains all the other scores with no evident order related to the drug concentrations used. This clustering indicates a severe metabolic change in the bacteria for concentrations higher than the MIC. Comparison with experiment 4, which differed from experiment 2 only in the incubation period allows an analysis of the drug's effect over time of exposure. Using an incubation time of 6 hours it is possible to detect a structure in the score plot of the first two principal components of the spectra, which is in accordance with the minimal inhibitory concentration (fig. 2(b)). In this plot, three clusters are well



(a) Experiment 2 with an incubation time of 1 hour

(b) Experiment 4 with an incubation time of 6 hours

Fig. 2. Score plots of the first two principal components of experiments 2 and 4 conducted on *H.pylori* strain 26695 using an incubation time of one and 6 hours, respectively.

defined:

- The cluster formed by the scores representing the control sample, which was incubated without the drug.
- The cluster, which contains the scores representing the bacteria incubated with the drug of concentrations from 2.5 up to 75 $\mu g\ ml^{-1}$, which are below the MIC (100 $\mu g\ ml^{-1}$).
- The cluster composed of the scores representing the bacteria incubated with the drug of concentrations of the MIC or higher.

It can be concluded that after an incubation period of 6 hours, FTIR spectroscopy detects metabolic changes induced by the drug. For concentrations at or above 100 $\mu g\ ml^{-1}$ there is a clear change in the cellular metabolism, which is detected in FTIR spectra by the PCA as a distinct cluster. Moreover it is possible to identify the control sample, which shows, that for this incubation time even small concentrations of the drug, such as 2.5 $\mu g\ ml^{-1}$ have an effect on the bactera's metabolism.

For the spectra obtained from this experiment, it was possible to draw conclusions about the apparent translational level of the bacteria. This was achieved by assignment of the the peaks at wavenumbers 1660 and 1240 cm^{-1} to proteins and genomic DNA [11] and estimation of the ratio of produced proteins per genomic DNA. This ratio drops from 5.6 to 3.2 at the threshold of 100 $\mu g\ ml^{-1}$ in accordance with the minimal inhibitory concentration determined for this strain.

In experiment 5, the effect of the drug model has been monitored on the metabolism of *H.pylori* strain J99, which differs strongly from the strain 26695 in its molecular fingerprint (fig. 1). The score plot of the first two PCs of the spectra (fig. 3) shows three clusters: one containing the scores representing the control sample and the sample incubated with a drug concentration of 2.5 $\mu g\ ml^{-1}$, one containing the scores representing the samples incubated with drug concentrations between 25 and 75 $\mu g\ ml^{-1}$ and one containing the scores, which represent the samples treated with drug concentrations equal or higher than the MIC of 100 $\mu g\ ml^{-1}$.

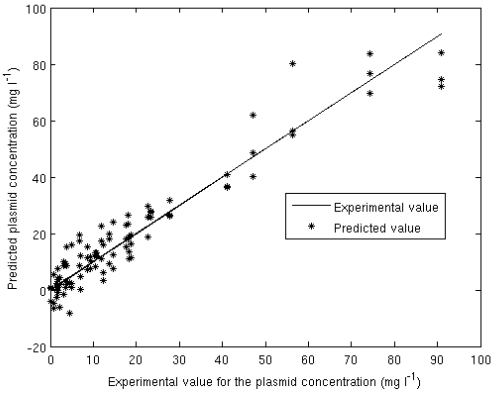
C. Recombinant E.coli batch cultures for plasmid production

PLS regression has been performed on the spectra of the culture samples in order to predict the concentration of the major variables of the batch cultivations, which are plasmids, biomass, glucose, glycerol and acetate. Before this, the spectra have been preprocessed with MSC on the sample replicates and the first derivative, which has been calculated using the Savitzky-Golay filter of window size 15 and order 4. Moreover a smaller region of the spectra was chosen to build the models, which was from wavenumbers 900 to 1900 cm^{-1}.

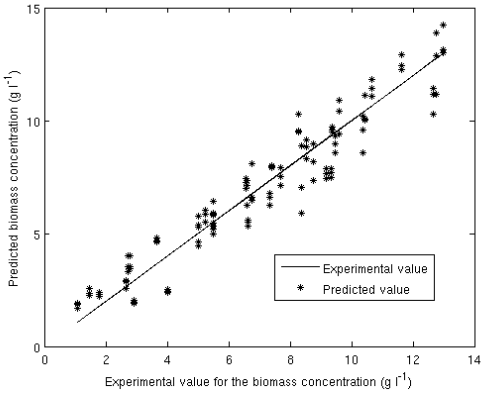
To determine how many latent variables should be used for the PLS model, a leave-one-out (LOO) cross-validation has been performed. The predicted values, which were obtained by the LOO cross-validation have been used to judge the quality of calibration by calculating the correlation coefficient between these values and the experimental ones. As an example, in the following the estimation of the plasmid as well as the biomass concentration will be discussed in detail.

The PLS model of the plasmid concentration, performed on the spectra of all the batches using 17 latent variables gave a correlation coefficient between the predicted and the experimental values of 0.956, indicative of a good prediction performance (fig. 4(a)). The present PLS model to predict the plasmid concentration was developed considering the spectra of all 5 batches. It is worth to mention, that the 5 batches were conducted in different medium compositions resulting in different culture behaviours. To evaluate the capacity of monitoring plasmid production in a defined culture condition, which usually occurs in industrial processes, the PLS model was also developed for batch D. The correlation coefficient, which resulted from this PLS using 9 latent variables, was 0.973, which is higher than the one using the spectra of all cultures.

Economic large-scale plasmid production from *E.coli* requires



(a) Predicted and experimental values of the plasmid concentration



(b) Predicted and experimental values of the biomass concentration

Fig. 4. Predicted and experimental values of the PLS regression on the plasmid and the biomass concentration using 17 and 6 latent variables respectively. The correlation coefficient between the predicted and the experimental values was 0.956 and 0.975 respectively.

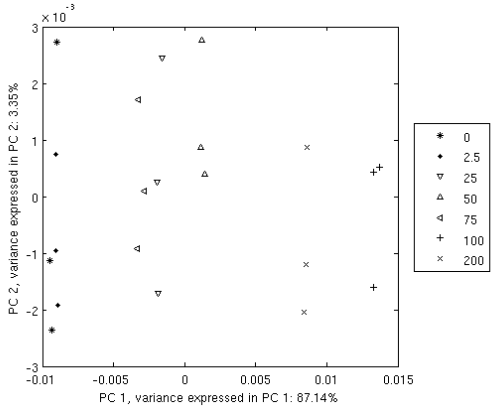


Fig. 3. Score plot of the first two principal components of experiment 5 conducted on *H.pylori* strain J99, using different concentrations of the drug and an incubation time of one hour.

the concomitant optimisation of plasmid copy number (specific yield) and of biomass concentration [13]. Therefore it is necessary to monitor the latter. In this thesis, a PLS regression has been performed on the spectra of all the batches and using the predicted values of the LOO cross-validation a correlation coefficient of 0.975 could be obtained using 6 latent variables (fig. 4(b)). For the other parameters of interest, namely acetate, glucose and glycerol is was also possible to develop PLS models with correlation coefficients higher than 0.95.

V. Conclusions

FTIR spectroscopy was evaluated for two bacterial processes: strain discrimination and drug inactivation studies with the gastric pathogen *H.pylori* and plasmid bioproduction in recombinant *E.coli* cultures.

The application of several preprocessing techniques (offset correction, spectra normalization, multiplicative scatter correction (MSC), first derivative and using a narrow region of the spectra) on the spectra showed, that preprocessing highly influences the output of the subsequent data analysis. Therefore, the preprocessing techniques must be selected carefully.

Principal component analysis of the spectra collected from 12 different *H.pylori* strains showed, that FTIR spectroscopy can be used as a tool for discriminating between different bacterial strains and most probably the bacteria typing.

For the monitoring of the drug inactivation of *H.pylori* several incubation conditions of the bacterium with the drug model were evaluated: the drug concentration, the incubation period, the presence and concentration of the nutrient broth, the bacteria quantity and the bacterial strain. For all the experiments it was possible to monitor the effect of the drug on the bacteria, even for low drug concentrations, such as 2.5 $\mu g\ ml^{-1}$. Moreover, the effect of the drug related to the time of exposure could be studied. In summary, FTIR spectroscopy can be used to estimate the *H.pylori* drug inactivation in a rapid and sensitive way, using low quantities of bacteria and the target drug in comparison to the ones used in the conventional method of agar dilution. The *E.coli* dataset was obtained by collecting the FTIR spectra of the bacteria pellet throughout several batch cultivations using different concentrations of glucose and glycerol as carbon sources. From the FTIR spectra of the bacteria PLS regression models were built to predict the plasmid concentration as well as the concentration of the major metabolites of the culture, namely biomass, glucose, glycerol and acetate. In all PLS models, the leave-one-out cross-validation gave rise to correlation coefficients higher than 0.95 between the predicted and the experimental values, which indicates good prediction performance.

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