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Real-time monitoring of continuous fermentation by Raman spectroscopy

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Abstract

The production of bio-ethanol from lignocellulosic material requires a more efficient process to be feasible and compete with products from fossil fuels. There is a need to rapidly and non-destructively be able to determine key components during fermentation. Raman spectroscopy is a technique, which can be used to monitor the fermentation process in real-time and provide information about key components which can be accessed immediately, thus facilitating process control. A continuous system with membrane cell recycling was set up and fermentations were performed using *Saccharomyces cerevisiae* ATCC 96581. Fermentations were performed to test for optimal dilution rates and operating times, the effect of different sugar concentrations in the media feed, and which position in the system was optimal for Raman data collection. Raman data and aliquot samples for HPLC validation were continuously collected throughout the fermentations. Raman data was analysed with PLS models to obtain component concentrations, for which RMSE was calculated in order to compare to HPLC validation set. Fermentations were performed with synthetic glucose media as well as with poplar hydrolysate. It was shown that the continuous system with membrane cell recycling could achieve a glucose-to-ethanol conversion of between 75-100%. The process could be sufficiently monitored by Raman spectroscopy, and predicted concentrations were within the range of the validation set in most cases. However, the error of prediction varied between the different fermentations.

Keywords

Raman spectroscopy, continuous fermentation, real-time monitoring

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Real-time monitoring of continuous fermentation by Raman spectroscopy

Therese Krieg

Populärvetenskaplig sammanfattning

Sedan Al Gore lämnade Vita Huset och blev miljökämpe med dokumentären *An Inconvenient Truth*, har vi blivit överösta med rapporter om hur vi påverkar vår planet. Allt från bilder på isbjörnar som desperat greppar tag om smältande isflak i Arktis, till reportage om klimatflyktingar. Klimat och miljöfrågor varit ett växande samtalsämne värden över. Oljeberoendet som idag existerar är varken hållbart eller försvarbart inför framtida generationer – det är helt klart en obekväm sanning.

Under en längre tid har flera alternativa bränslen forskats fram. Bio-etanol från lignocellulosa är ett intressant alternativ att ta i beaktande. Lignocellulosa som biomassa innefattar t.ex. restprodukter från skogs och jordbruksindustrin. Råmaterialet är billigt, förnybart och finns i rikliga mängder. Fortsatt utveckling av processerna kring tillverkningen krävs dock fortfarande. Detta projekt har fokuserat på att förbättra processkontrollen vid fermentation. Det behövs ett effektivt sätt att snabbt analysera nyckelprodukter i real-tid utan att påverka processen. Raman spektroskopi är en metod som fångar upp karakteristiska vibrationer i molekylerna och resulterar i ett spektra där olika komponenter representeras kvantitativt. Enskilda koncentrationer för komponenter av intresse tas fram genom att analysera data med kinetiska modeller. Fördelen med real-tids övervakning är resultaten och indikationer på hur processen går är tillhanda direkt, vilket underlättar för processkontroll.

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Abbreviations

ATCC: American type culture collection

APC: Advanced process control

HPLC: High pressure liquid chromatography

MCRB: Membrane cell-recycle bioreactor

PCA: Principal component analysis

PLS: Partial least squares

RMSE: Root-mean-square-error

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1. Introduction

In a world dependent on fossil fuels, with a petrochemistry industry that is exploiting oil reserves, there is an immediate need for an alternative to fossil fuels. The vast global use of petrochemicals has created an increase of greenhouse gases in the atmosphere (van Moris et. al., 2006). Due to the environmental impacts of fossil fuels, eco-consciousness has increased and incentives towards developing alternative fuels are growing stronger (Favaro et. al., 2013). An attractive alternative to fossil fuels is fuel derived from lignocellulosic materials such as wheat straw or poplar. The carbon cycle of fuel derived from plant biomass has a balance between feedstock production and carbon dioxide fixation, thus not impacting the environment in the way fossil fuels do. One of the most promising of alternative fuels is bio-ethanol. In addition to the environmental benefits of alternative fuels they also address the concerns about energy security in the world (van Moris et. al., 2006).

Worldwide the interest in biomass-derived fuel has increased along with the gasoline price spikes. To cope with both the increased cost in products from fossil fuel and the climate changes many countries are investing in alternative sustainably derived fuels, such as bio-ethanol (Limayem et. al., 2012). The primary feedstock for first generation bio-ethanol production is corn, wheat, or sugarcane. This raises a question with concerns addressing impacts on society, whether or not we should be using food crops for fuel (Favaro et. al., 2013). Lignocellulosic derived biofuels is therefore a promising technology. Lignocellulosic materials can be provided in large quantities to supply large-scale production. It is a low-cost and abundant raw material. Even though the material itself is accessible at low costs, it requires high cost and labor intensive processing (Limayem et. al., 2012).

New technologies that are emerging should improve upon current production processes by increasing efficiency and cost effectiveness, while reducing environmental impacts (Limayem et. al., 2012). Lignocellulosic biomass is suitable as an energy source since it is abundant and renewable. As mentioned above it is also inexpensive, which reduces the final cost of the fuel derived from it. The major components of lignocellulose are cellulose, hemicellulose, and lignin. The biomass is converted to sugars through hydrolysis and the hydrolysate can then be fermented by a microorganism. However, a range of inhibitory compounds are formed during the process of hydrolysis, which makes the process of fermentation more complex (Larsson et. al., 2001).

Fermentation can be performed as a batch, fed-batch, or continuous process. The advantage of a continuous fermentation system is that inhibitory products are continuously removed from the bioreactor, leading to less inhibition and better conversion. To increase productivity further it is necessary to obtain and maintain a high cell concentration, this is done through cell recycling. It is not possible to reach cell concentrations at required level by the conventional fermentation processes, mentioned above, even though fed-batch system are frequently used to attain high cell density. However fed-batch is usually more often used when there are no inhibitory products present. Cell recycling can effectively be used to maintain high levels of cell concentration in bioreactors. For a continuous fermentation system immobilization and cell recycle methods are suitable since they can function with high dilution rates, which would otherwise lead to wash-out. To achieve complete cell recycling, membrane based-techniques are preferable. Even though membrane based cell recycling can cause shear damage on the cells and affect the flux, it is advantageous due to the fact that it allows for high cell density and is easily replaced even during operation. (Chang et. al., 1994)

Several analytical methods, such as, ultraviolet-visible spectrophotometry, gas chromatography/mass spectroscopy, capillary electrophoresis, and electrochemical methods can be used to analyse monosaccharides in hydrolysate. However unlike these methods, vibrational spectroscopy methods, like Raman or infrared (IR) spectroscopy, can present information on chemical content of multiple analytes at the same time. In comparison to IR spectroscopy, Raman is advantageous when working with aqueous solutions. This is because water does not produce high amounts of scatter within the region of interest and thus it is a better technique for quantitative analysis in this case (Shin et. al., 2011).

Providing fast and reliable information about reactant and product concentrations during fermentation processes is necessary to facilitate process control. Quantitative measurements of bioconversion processes today mainly rely on off-line technologies, such as high performance liquid chromatography (HPLC). Performing analysis on an off-line instrument delays critical system evaluation, making process control more difficult. Better knowledge of the parameters involved in the process of fermentation combined with a suitable online technology would provide a better understanding of the chemical and physical properties of the systems used for fermentation of bio-ethanol. On-line spectroscopic methods such as Raman, enables fast analysis of the progress of the system. As Raman is able to provide high-resolution information, it has been useful as a validation method in the petrochemical industry (Roberto et. al., 2012).

2. Objective of project

With the aim to make alternative fuel production and process control more efficient, there is a need to rapidly and non-destructively be able to determine the key analytes during bioconversion. Using HPLC as an assessment method requires aliquot samples taken during fermentation and thus the results will be at hand at a time much later than when the sample was taken. Raman spectroscopy could be used to continuously monitor the process and data would be accessible immediately.

The objective of this project was to set up a continuous fermentation with a cell-recycle system and evaluate the possibility of using Raman Spectroscopy as real-time online monitoring for continuous fermentations. Continuous fermentations in a membrane cell-recycle bioreactor (MCRB) were performed using both synthetic glucose media and diluted poplar hydrolysate. Experiments were designed to test different conditions for the continuous system to function at as well as the best way to collect Raman data. Raman data was collected throughout the fermentations and HPLC was used as external validation.

3. Background

3.1 Raman spectroscopy

Raman spectroscopy is a vibrational spectroscopic method that can be used for quantitative modelling. A Raman spectrum provides chemical and physical data that is used for online monitoring. To process the data is more difficult, therefore chemometric tools, such as, principal component analysis (PCA) and partial least square (PLS) are used to analyse the Raman spectral data (Roberto et. al., 2012).

Raman spectroscopy is a technique based on light scattering, or Raman scattering. The laser diode sends out a laser beam, exciting the molecules and causing shifts in energy. The light is scattered and returned, to be detected by the Raman instrument. The characteristic vibrations of the different molecules will be detected and shown as peaks at different Raman shifts. There are certain peaks that are characteristic to each molecule. The intensity and wavelength of these peaks correspond to the quantity of each compound present. The spectra is used to monitor the fermentation process and predicting the concentration of each compound of interest. The main compounds in this project are ethanol and glucose. For ethanol there is a major peak at 883cm^{-1} representing C-C stretching as well as two smaller ones around 1047 and 1084cm^{-1}. For glucose a major peak is located around 1123cm^{-1} (Figure 1).

HPLC is the most regularly utilized off-line instrument. Like most off-line techniques it creates time delays and also limits the number of validation samples that can be taken for a particular time interval (Roberto et. al., 2012). An important aspect of Raman spectroscopy is that it analyses the sample in a non-destructive way. It does not affect the quality or condition of the fermentation process. In addition it can be run without the costly and time-consuming sample preparation and analysis time of HPLC (Ewanick et. al., 2012).

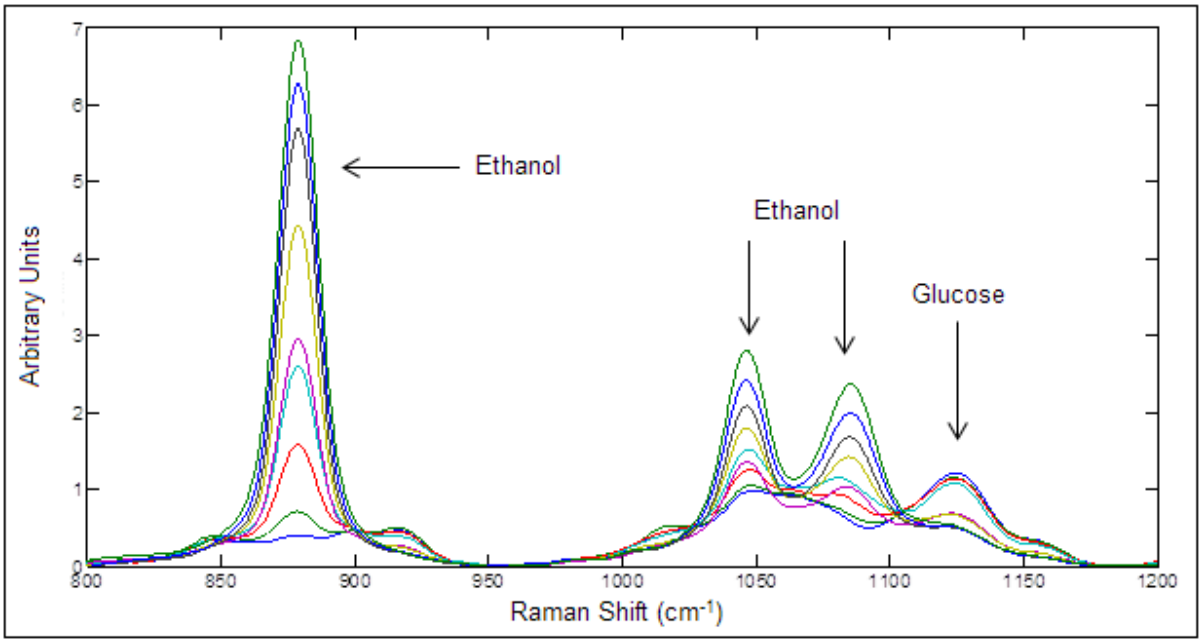

Figure 1: Major peaks for ethanol and glucose in a Raman spectra.

3.2 Saccharomyces cerevisiae ATCC96581

Saccharomyces cerevisiae is a well known yeast and widely used in industry (Snoek and Steensma, 2007). It is commonly used for sugar to ethanol fermentation. Wild type *S. cerevisiae* can ferment hexose sugars such as glucose and mannose. However it is not capable of fermenting pentose sugars such as xylose. In plant biomass hydrolysate, xylose is one of the major compounds, along with glucose (van Maris et. al., 2006). There are many different strains of *S. cerevisiae*, during this project the non-modified strain of *S. cerevisiae* ATCC96581 has been used.

This yeast is more tolerant than other microorganisms (e.g. bacteria) to inhibitory products such as ethanol or glycerol. (Almeida et. al., 2007) It is very robust and can resist high osmotic pressure and is tolerant to low pH levels (>pH 4.0). *S. cerevisiae* ATCC96581 has an optimal growth at a temperature around 30°C (Limayem et. al., 2012) and has in general a high thermotolerance (Favaro et. al., 2013).

3.3 The fermentation process: glucose to ethanol

Glucose is one of the dominant sugars in plant hydrolysate and can be fermented by wild type *Saccharomyces cerevisiae* via the Embden-Meyerhof pathway of glycolysis, producing ethanol. *S. cerevisiae* only requires a concentration gradient across the plasma membrane to take up glucose. After the uptake of glucose it will be dissimilated via the Embden-Meyerhof pathway that oxidizes glucose to two pyruvate molecules, resulting in 2 ATP molecules (van Moris et. al., 2006). The fermentation is always accompanied by the formation of carbon dioxide as a byproduct (Limayem et. al., 2012).
The reaction is a follows:

$$C_6H_{12}O_6 + 2P_i + 2ADP \rightarrow 2CH_3CH_2OH + 2CO_2 + 2ATP$$

4. Methods

4.1 Cell culturing and media

S. cerevisiae ATCC96851 was obtained from agar plate cultures. The cells were grown in a glucose media (10g/l each of peptone, yeast extract, and glucose) for a total of 48 hours before fermentation. Media was inoculated with cell colonies and then grown for 24 hours at 30ºC and 175rpm orbital shaking in a MaxQ4000 incubator (Thermo Fisher Scientific). Cells were then spun down at 4000rpm for 5 minutes using an Allegra 25R Centrifuge (Beckman Coulter) and transferred to fresh media. After another 24 hours cells were harvested by centrifugation, as before, and resuspended in a small volume of 0,9% NaCl. The concentration of cells was determined using absorbance measurements at 600 nm (UV-1700 Pharma Spec, Shimadzu). The cell concentration used for inoculation of fermentation was 5g/l.

Synthetic fermentation media contained 10g/l peptone, 10g/l yeast extract, 2g/l ammonium sulphate, 2g/l sodium nitrate, and 0,2g/l sodium sulphate. Glucose was added to the media to the desired concentration. The poplar hydrolysate was diluted 1:3 with water. The diluted poplar hydrolysate for fermentation was spiked with glucose to a 60g/l concentration. Added to the hydrolysate was also nutrients (2 g/l ammonium sulphate, 2g/l sodium nitrate, and 0,2 g/l sodium sulphate). All media was filtered through a 0,2 µm sterile filter before fermentation.

Prior to fermentation the concentration of sugars, degradation components such as furfural, 5-hydroxymethyl-furfural, and acetic acid, as well as the concentration of total phenolics were measured in the hydrolysate. Table 1 shows the content of these components in the original hydrolysate and the diluted one used for fermentation.

Table 1: Components in poplar hydrolysate. Total phenolics, Acetic Acid, 5-hydroxymethyl-furfural, furfural, and sugars.

Components in Poplar Hydrolysate (g/l)	Phenolics	Acetic Acid	HMF	Furfural	Sugars				
					Arabinose	Galactose	Glucose	Xylose	Mannose
Diluted	2.45	4.60	0.3	2.21	0.18	0.3	4.66	6.53	0.8
Non-diluted	5.45	14.0	1.18	6.08	0.57	0.91	14.21	19.21	2.77

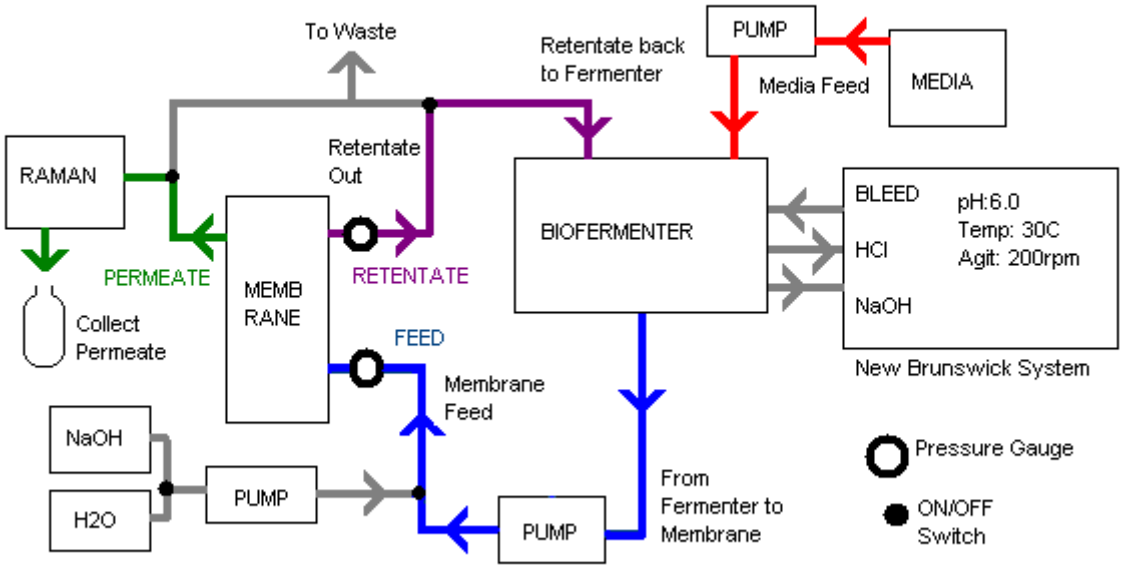

Figure 2: Schematic of the continuous fermentation system set up.

4.2 Continuous fermentation system

The continuous fermentation system consisted of the bioreactor (New Brunswick Scientific BioFlo 115 bioreactor) and a cell recycling membrane (0.1m^2 Pellicon 2 Filter, 0,2 micron) as the main parts. As shown in the schematic of the continuous system in Figure 1 the bioreactor had a feed for recycled cells from the membrane as well as a feed for fresh media, thus continuously adding sugar and nutrient as well as building up the cell concentration. Media and cells are pumped from the fermenter to the feed of the membrane, the components are then separated and cells retained and pumped back into the fermenter. Ethanol and glucose is pumped out and collected through the permeate side.

The continuous fermentations were performed either in a 2*l* or a 1*l* bioreactor. The fermenter was also equipped with a pH probe, temperature probe, level probe, exhaust condenser, and acid/base ports. The constant conditions for fermentation were 30°C, pH 6.0, and 200 rpm continuous agitation. The pH was maintained at 6.0 using 2M NaOH and 2M HCl. A Raman probe was used to collect data of the permeate flow for all the fermentations, as well as in the fermenter for specific experiments.

4.3 Overview of experiments

Four different continuous fermentations were performed using different conditions. Prior to continuous fermentations, batch fermentation using the same glucose concentrations were performed in order to locate the time it takes to reach ethanol peak production. All continuous fermentations were started as a batch process until ethanol peak production was reached. Samples were continuously taken every 2 hours from the fermentation broth and the permeate, for analysis by HPLC for Raman validation. Raman was set to collect data every 15 minutes with a 7 second exposure time, unless otherwise specified below for the specific experiment.

4.3.1 Continuous fermentation 1: testing different dilution rates

Continuous fermentation 1 tested different dilution rates. The fermentation was performed to determine an optimal dilution rate for continuous operation. A total volume of 1*l* synthetic media with 60g/l glucose in a 2*l* bioreactor was used. The continuous system was run for a total of 46 hours. Four different dilution rates (0,05; 0,1; 0,15; and 0,3 h^{-1}) were tested.

4.3.2 Continuous fermentation 2: using raman inside and outside the fermenter

Continuous fermentation 2 studied the difference between collecting Raman data inside the fermenter, in the presence of cells or at the permeate stream, after cell separation. With the hypotheses that collecting Raman data at the permeate flow will give a better prediction. A working volume of 0,5*l* synthetic media with 60g/l glucose in a 1*l* bioreactor was used to determine if there were any changes based on scaling. Two different dilution rates; 0,2 and 0,1 h^{-1}, were utilized, and running for around 24 hours each.

4.3.3 Continuous fermentation 3: using a higher glucose concentration

The difference in Raman prediction using a higher glucose concentration was tested in continuous fermentation 3. Glucose concentration was increased in the synthetic media to 120g/l in a total volume of 1*l* synthetic media in a 2*l* bioreactor for this fermentation. The continuous fermentation was run at dilution rate 0,1h^{-1} for a total of 12 hours. The permeate flow was controlled by a pump to match the dilution rate.

4.3.4 Continuous fermentation 4: using hydrolysate

This fermentation was run using diluted poplar hydrolysate media. It was run with a total volume of 1*l* in a 1*l* bioreactor at a dilution rate of 0,1 h^{-1} for 18 hours. Raman was set to monitor both the permeate stream as well as inside the fermenter. Raman was set to collect data at 7 seconds exposure time initially, but later in the process changed to collect every 3 seconds at the permeate and every 20 seconds inside the fermenter.

4.4 Data acquisition and analysis

HPLC was used to assess the glucose, ethanol, and glycerol concentrations. For hydrolysate HPLC was also used to detect additional sugars present (galactose, arabinose, xylose, glucose and mannose) as well as acetic acid, 5-hydroxymethyl-furfural, and furfural. Total phenolics concentration present in the hydrolysate was measured using Folin Ciocalteau reagent. Analysis of sugars was run on a ICS-3000 Ion Chromatography System (Dionex Corporation), and the remaining components on a Shimadzu Prominence LC. Standards were used to quantify the unknown samples in both cases.

Raman data was collected through the RamanRXN2 system (Kaiser Optical Systems, Ann Arbor, MI) with a probe immersed in the media. Laser wavelength of 785 nm was used with a power around 230mW. Raman spectroscopy was also used to make standard calibration samples using known concentrations of both glucose and ethanol ranging from 1-60g/l. These were used to build a Partial Least Square (PLS) model. The 60g/l PLS model was used for all predictions, and in addition a 120g/l PLS model was also used to predict Raman for Continuous Fermentation 3. The model is used to predict the concentrations of compounds from the Raman data. The processed Raman data is then compared to the HPLC validation set to assess the accuracy of the Raman reading. All data from HPLC was analysed using Excel (Microsoft Works). The data from Raman spectroscopy was analysed using Matlab (MathWorks). Absorbance was measured for the collected samples at 600 nm. The absorbance was then used to calculate the concentration of yeast cells, using a calibration curve determined by *S. cerevisiae* cell dry weight.

5. Results and Discussion

Each continuous fermentation generated validation data from HPLC and the raw Raman data. The raw data was processed with a polynomial fitting to reduce the elevated baseline that is present in the spectra between 300 and 1800 cm^{-1}. The data was normalized based on an internal standard peak at 418cm^{-1}, representing a signature peak from the blue sapphire probe tip. In the processed Raman data (Figure 3, 5, 6, 8, 10, and 11) the major specific peaks of ethanol (883cm^{-1}) and glucose (1123cm^{-1}) can be seen. Only a few specific data points from the collected data are plotted in these figures in order to clearly see the increase and decrease of ethanol and glucose concentrations, respectively. The estimated concentration of components from Raman and HPLC can be seen plotted against each other in the graphs in Figure 4, 7, 9, and 12. Raman data was collected every 15 minutes but is plotted as average over two hours to match the HPLC sampling points. A summary of data including substrate and product concentrations as well as conversion rates can be seen in Table 3. Root-mean-square-errors (RMSE) of predictions are shown in Table 2.

In addition to ethanol, the by-product glycerol was also formed in the fermentation process. However, it was an insignificant amount in comparison to the concentration of ethanol produced. The cell concentration for each fermentation continuously increased throughout the

fermentation. Separating out the ethanol decreases inhibition and cells can continue to grow and ferment. Spikes present in ethanol production and glucose consumption are due to extra media being added to the fermenter in order to maintain volume, thus increasing glucose concentration and reducing ethanol production momentarily.

5.1 Continuous fermentation 1: testing different dilution rates

For the continuous fermentation 1, it is clear that the Raman is able to predict the process of glucose consumption and ethanol production at a satisfactory level. The RMSE for ethanol was 5,5 and 6,6 for glucose (Table 2). As can be seen in Figure 4, the concentrations assessed from fermenter, permeate, and Raman follow a similar trend.

This fermentation was run to test out the different dilution rates. The lower dilution rates (i.e. 0,05; 0,1; and 0,15h^{-1}) appear to work well, showing a steady level of ethanol being produced as well a low level of residual glucose present in the permeate stream, indicating high sugar conversion. On the contrary, for the higher dilution rate of 0,3h^{-1} the cells started to die off and sugar conversion was greatly reduced, which affected ethanol yield.

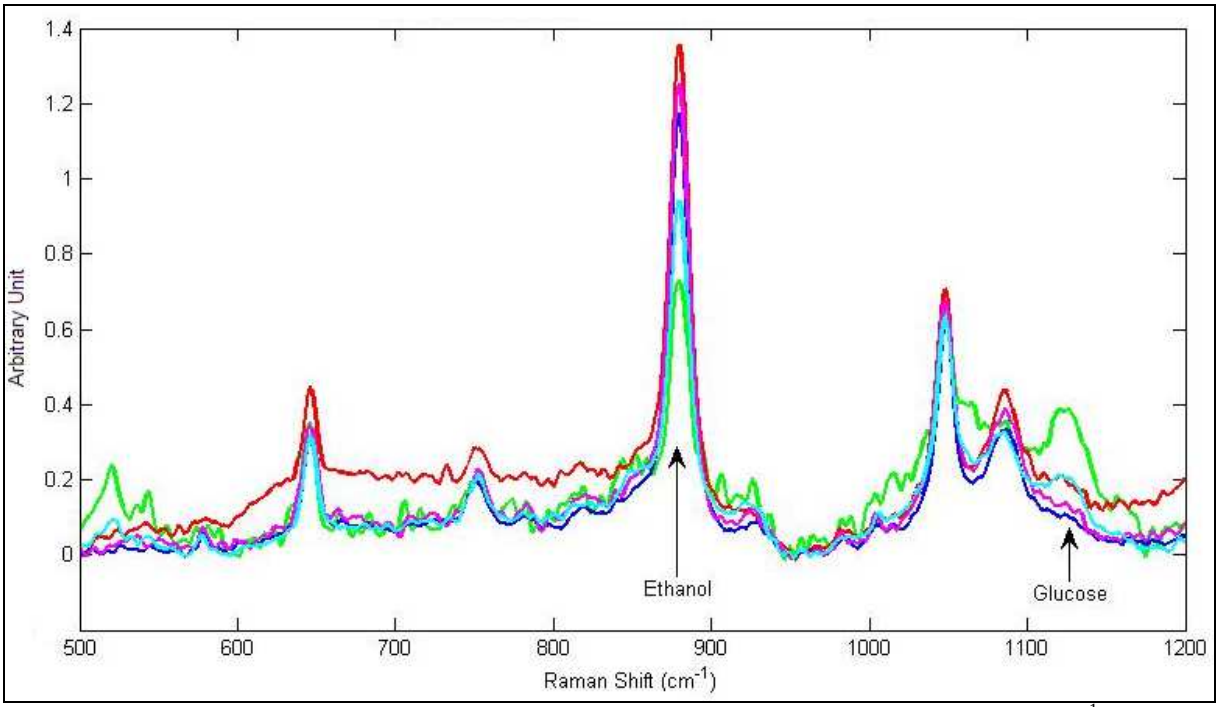

Figure 3: Continuous Fermentation 1: Processed Raman data showing main peaks for ethanol (883cm^{-1}) and glucose (1123cm^{-1}).

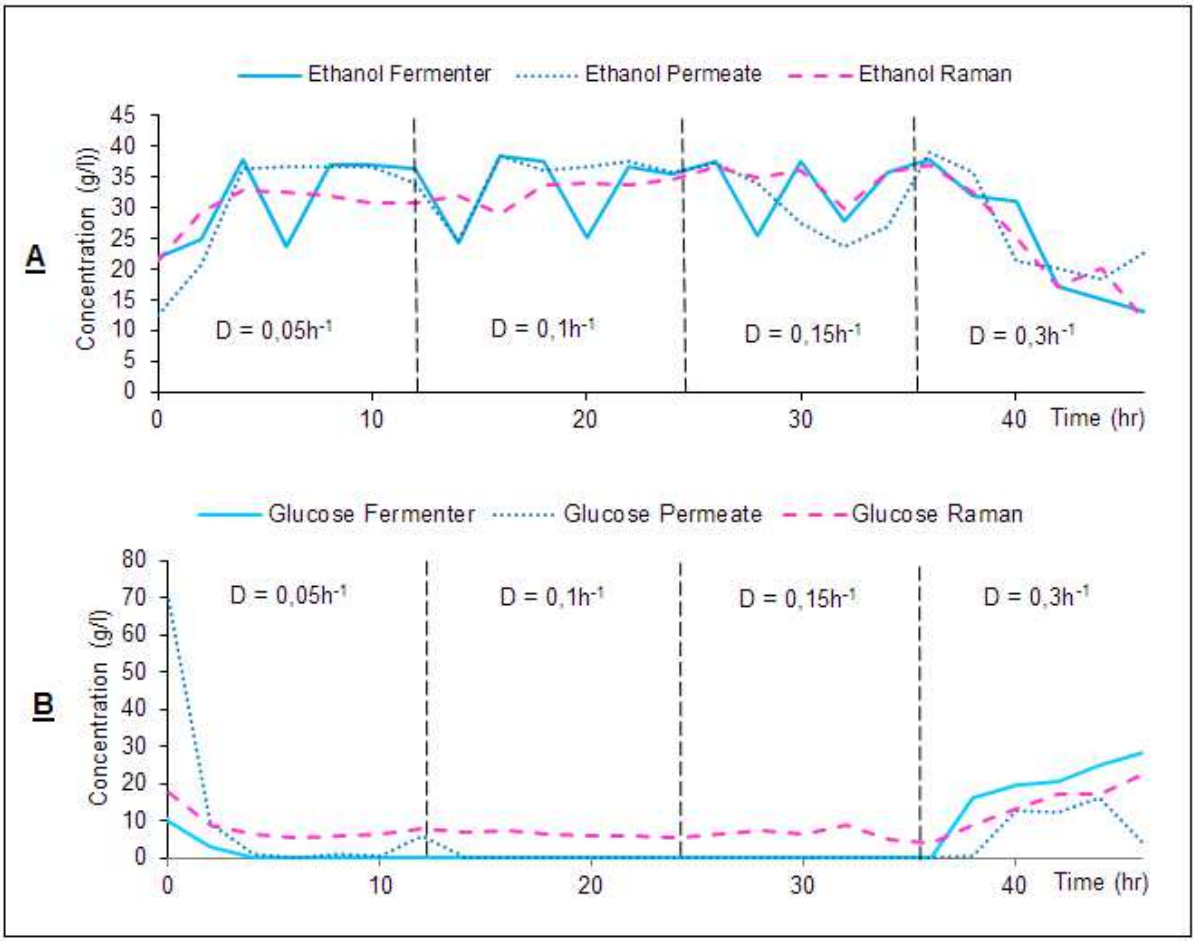

Figure 4: Continuous fermentation 1: Ethanol and glucose concentration. A) Ethanol concentration comparison between measurements inside the fermenter, in the permeate, and predicted by Raman. B) Glucose concentration comparison between measurements inside the fermenter, in the permeate, and predicted by Raman. Vertical dashed lines indicate time point when dilution rate (D) was changed.

5.2 Continuous fermentation 2: using raman inside and outside the fermenter

Continuous fermentation 2 also showed a clean Raman spectra when collecting data at the permeate (Figure 5). Whereas when data was collected inside the fermenter (Figure 6), there is a lot of noise present in the spectra. The additional noise can be correlated to the increasing cell concentration, which reduces the Raman signal-to-noise ratio. The concentration assessed from Raman data inside the fermenter did not give a successful prediction of glucose or ethanol concentrations, as seen (Figure 7) when compared to the results from the HPLC and the Raman collected at the permeate. The concentrations assessed from samples taken at the fermenter, permeate and Raman at the permeate follow a similar trend. Comparing the error in prediction, RMSE, much higher values are obtained when using Raman data collected inside the fermenter than at the permeate (Table 2).

Using a smaller fermentation vessel size with a lower volume than in continuous fermentation 1 proved more difficult to maintain at a constant volume for this system. With the first dilution rate of $0{,}2h^{-1}$ it was more difficult to maintain the volume constant than with the dilution rate of $0{,}1h^{-1}$. The later one gives a better functioning continuous fermentation system where ethanol production is kept at a constant level and sugars completely utilized.

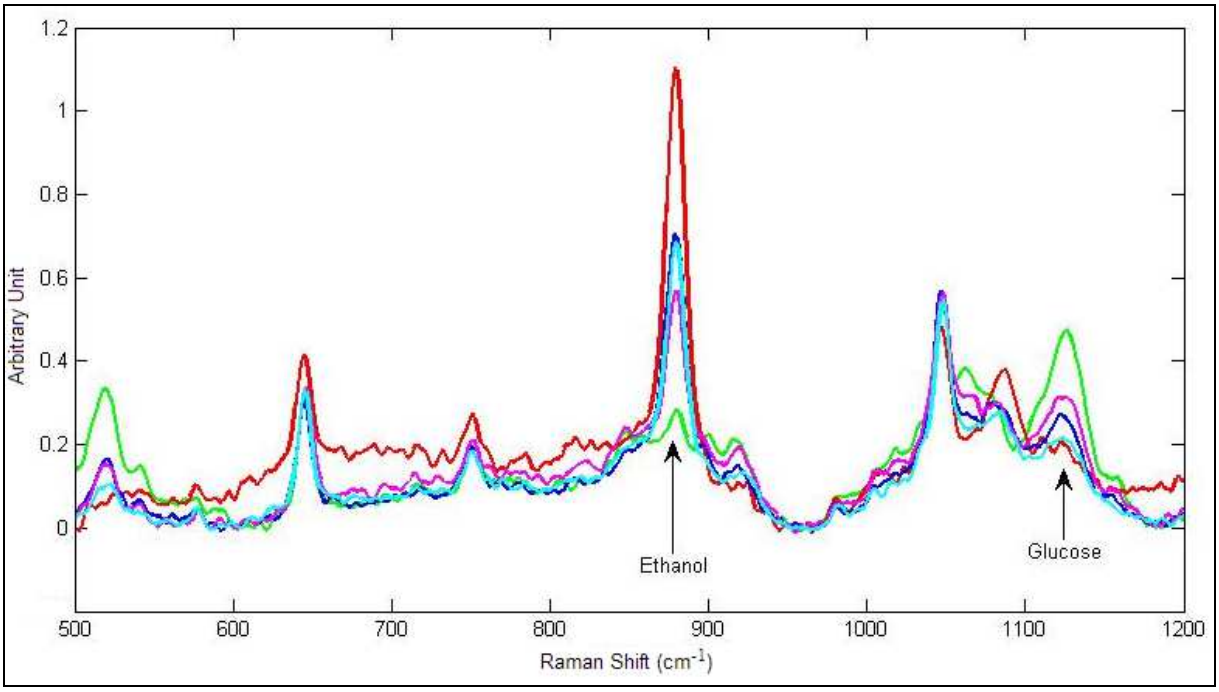

Figure 5: Continuous fermentation 2: Processed Raman data (permeate) showing main peaks for ethanol ($883cm^{-1}$) and glucose ($1123cm^{-1}$).

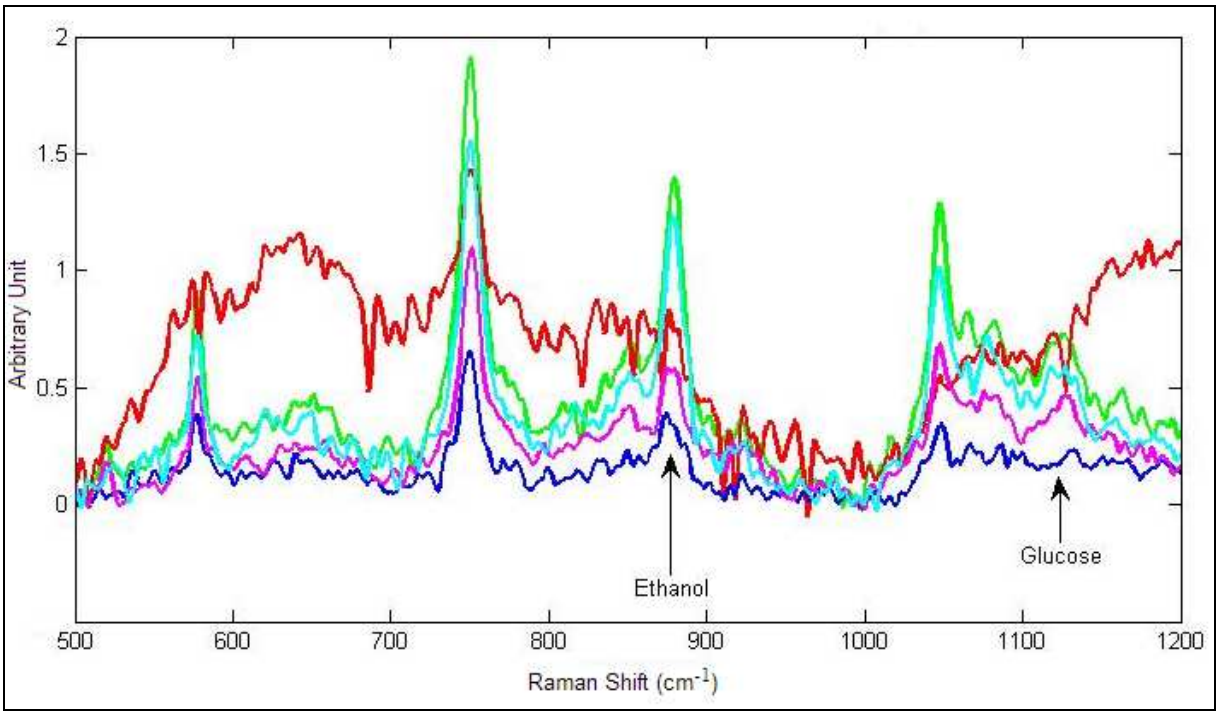

Figure 6: Continuous fermentation 2: Processed Raman data (fermenter) showing main peaks for ethanol ($883cm^{-1}$) and glucose ($1123cm^{-1}$).

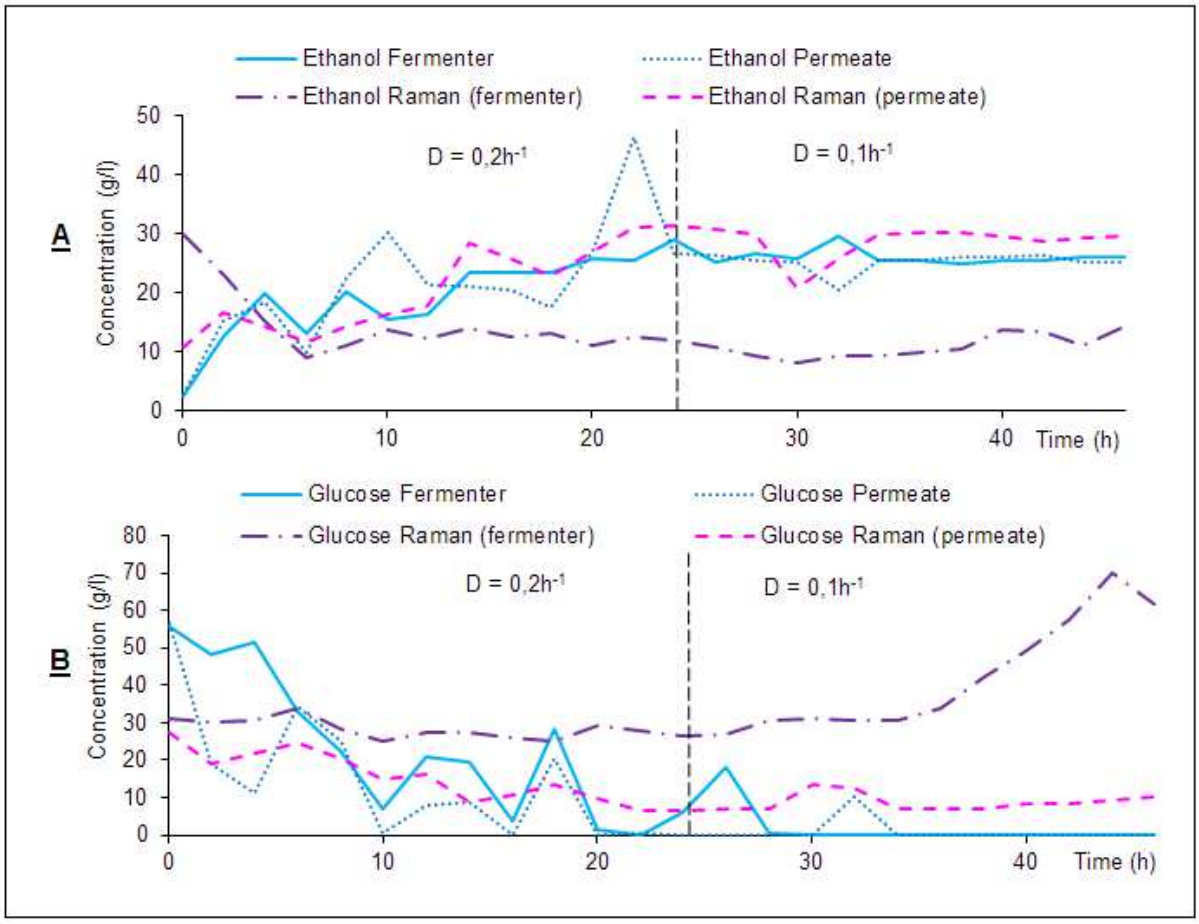

Figure 7: Continuous fermentation 2: Ethanol and glucose concentration. A) Ethanol concentration comparison between measurements inside the fermenter , in the permeate, and predicted by Raman inside the fermenter and in the permeate flow. B) Glucose concentration comparison between measurements inside the fermenter, in the permeate, and predicted by Raman inside the fermenter and at the permeate. Vertical dashed lines indicate the time point when the dilution rate (D) was changed.

5.3 Continuous fermentation 3: using a higher glucose concentration

When testing a higher glucose concentration in the sythetic media with Continuous Fermentation 3 the prediction from the collected Raman data was not as good as for the previous fermentations. However, the Raman spectra was clear and peaks easy to distinguish (Figure 8). During the fermentation issues with maintaining the volume were met and, combined with the shorter time it was run, it was difficullt to get a steady glucose reading.

When using the 60g/l PLS model, Raman for the glucose prediction compared to HPLC results is in a better range than the predicted ethanol concentrations. Where as when using the 120g/l PLS model the ethanol prediction is in close range to the HPLC data and the glucose is not. As can be seen in Figure 9, the concentrations between Raman and HPLC can vary to a difference up to as much as 20-25g/l, where as for previous fermentations the difference was only at a maximum of 5-10g/l. Seen in Table 2, the RMSE values indicate that the 60g/l PLS model was sufficient for predicting the glucose concentration but not the ethanol. On the contrary the 120g/l model could predict the ethanol concentration with a RMSE value of 9,8 compared to the glucose RMSE of 57,4.

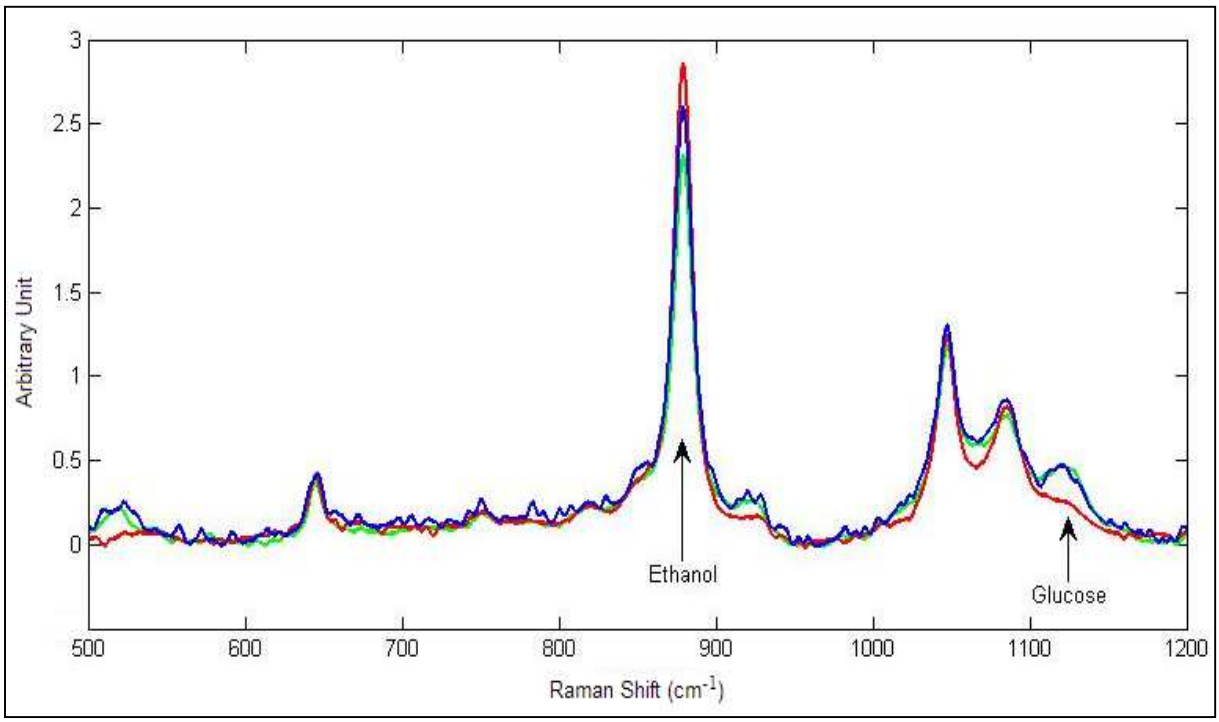

Figure 8**:** Continuous fermentation 3: Processed Raman data showing main peaks for ethanol (883cm^{-1}) and glucose **(**1123cm^{-1}).

Table 2: RMSE for Glucose and ethanol

RMSE	Ethanol	Glucose
Continuous Fermentation 1	5,5	6,6
Continuous Fermentation 2	5,3[*1]	8,4[*1]
	14,1[*2]	33,4[*2]
Continuous Fermentation 3	25,1[*3]	13,0[*3]
	9,8[*4]	57,4[*4]
Continuous Fermentation 4	38,8[*1]	102,9[*1]
	16,4[*2]	81,0[*2]

RMSE=Root mean square error.
[*1] Raman data collected at permeate,[*2] Raman data collected in fermenter,[*3] Raman predicted with 60g/l PLS model,[*4] Raman predicted with 120g/l PLS model.

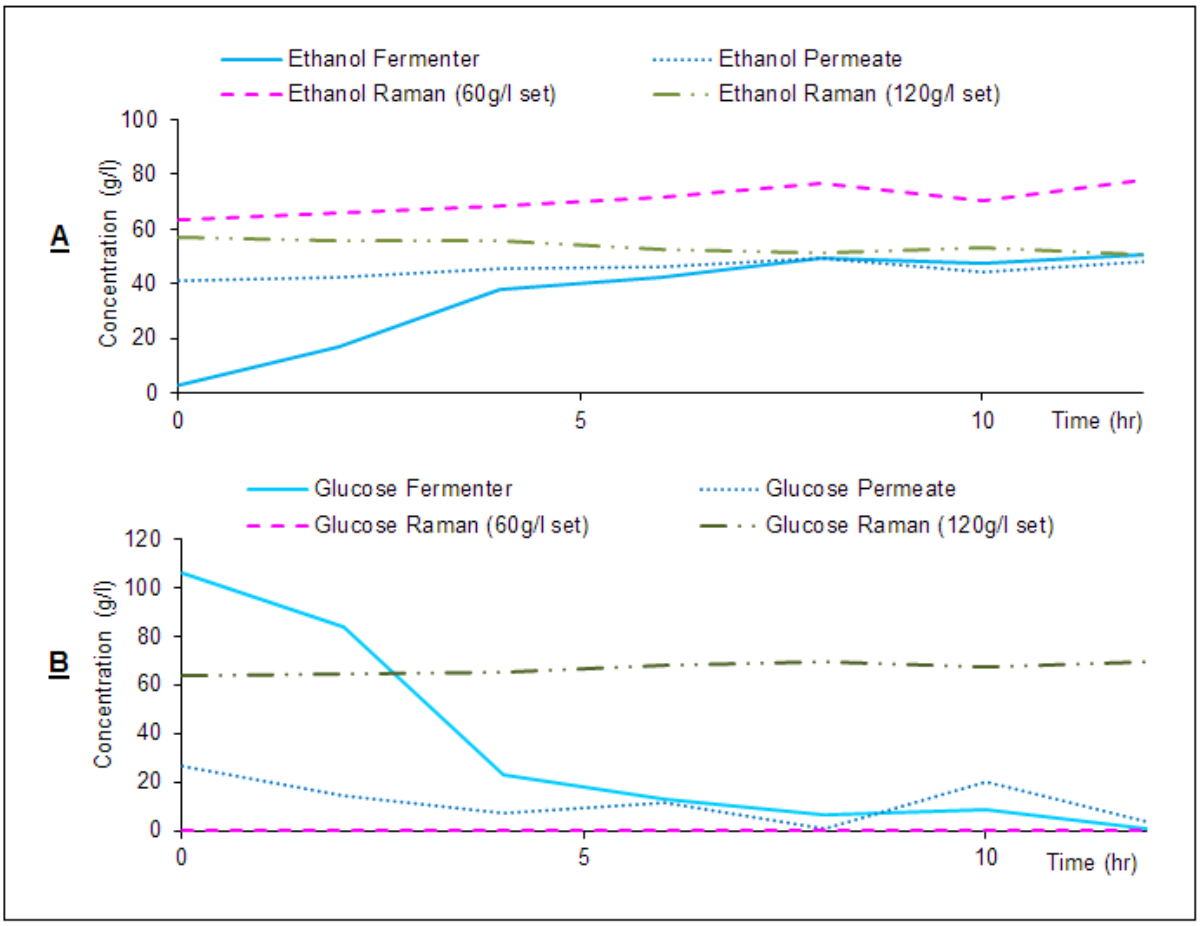

Figure 9: Continuous fermentation 3: Ethanol and glucose concentration. A) Ethanol concentration comparison between measurements inside the fermenter, in the permeate, and predicted by Raman. B) Glucose concentration comparison between measurements inside the fermenter, in the permeate, and predicted by Raman. Raman data has been predicted using both a PLS model with a HPLC validation set with a maximum concentration of 120g/l (120g/l set) and 60g/l (60g/l set).

5.4 Continuous fermentation 4: using hydrolysate

Using hydrolysate for fermentation worked well as far as the fermentation process is concerned. Cells were growing and all glucose and mannose sugars were utilized. Ethanol production was at a constant level throughout the fermentation. Raman data collected inside the fermenter, shown in Figure 10, contained high levels of noise. The permeate Raman data also contained some noise but peaks are detectable, as seen in Figure 11. Glucose concentration levels were not successfully predicted for either of the collected Raman data sets, as seen in Figure 12.

The ethanol concentrations can be seen in Figure 12. The ethanol prediction for the fermenter was close to the HPLC validation set. Varying within a similar range as for the predictions made for 60g/l synthetic glucose media. As for the permeate data, that ethanol prediction was way above the actual levels of concentrations measured by HPLC. Which contradicts the results from continuous fermentation 2, and the hypotheses, where it was shown that the readings at the permeate gave much better predictions, closer to that of the HPLC result, than did the prediction from the Raman collected inside the fermenter. The values for RMSE are all generally high (Table 2), but it can be noted that RMSE, when using Raman data collected inside the fermenter, are lower than the values for RMSE when using Raman data collected

from the permeate flow. It can be noted, in Figure 10 and 11, that the Raman spectra from the fermenter is noisy, although peaks can be detected and the spectra from the permeate is much clearer, indicating that that would make the prediction more accurate.

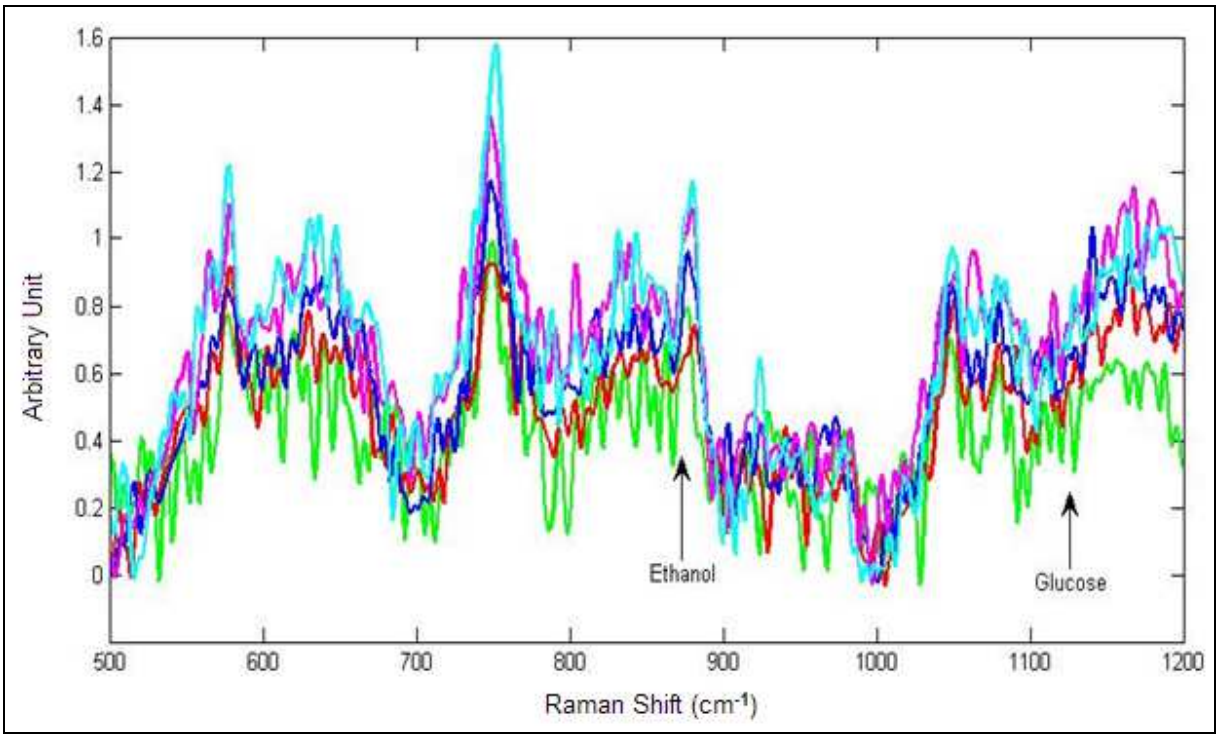

Figure 10: Continuous fermentation 4: Processed Raman data (fermenter) showing main peaks for ethanol (883cm^{-1}) and glucose (1123cm^{-1}).

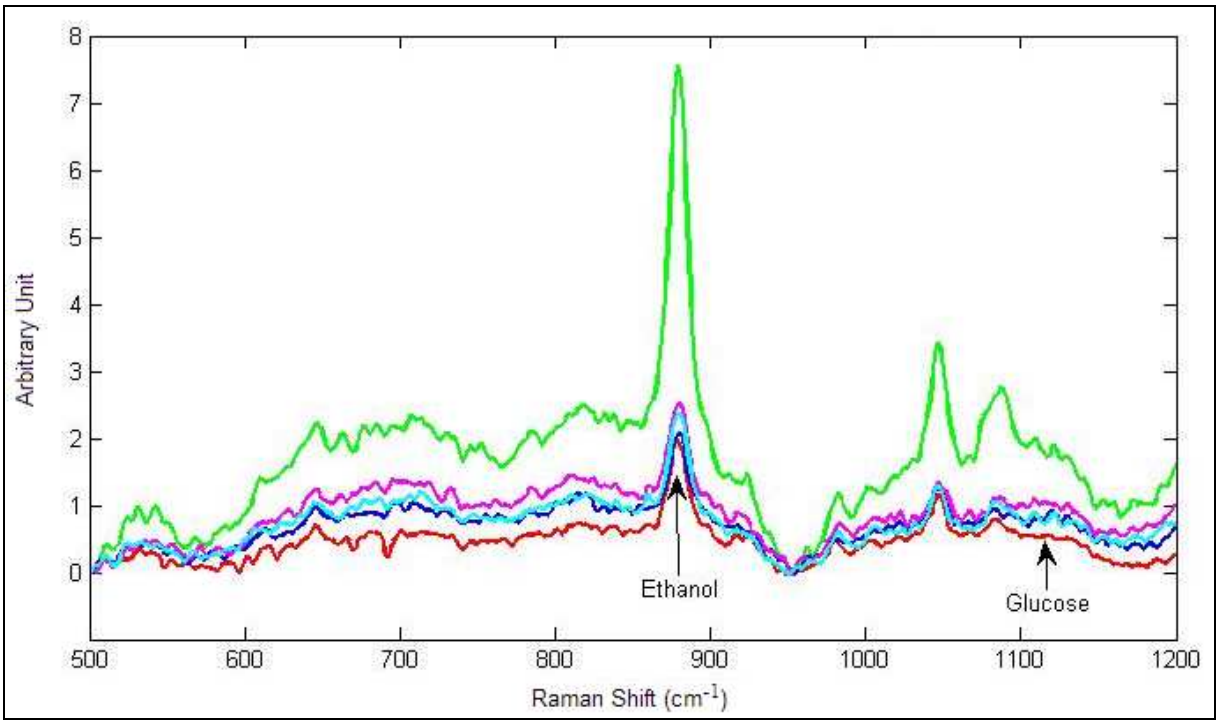

Figure 11: Continuous fermentation 4: Processed Raman data (permeate) showing main peaks for ethanol (883cm^{-1}) and glucose (1123cm^{-1}).

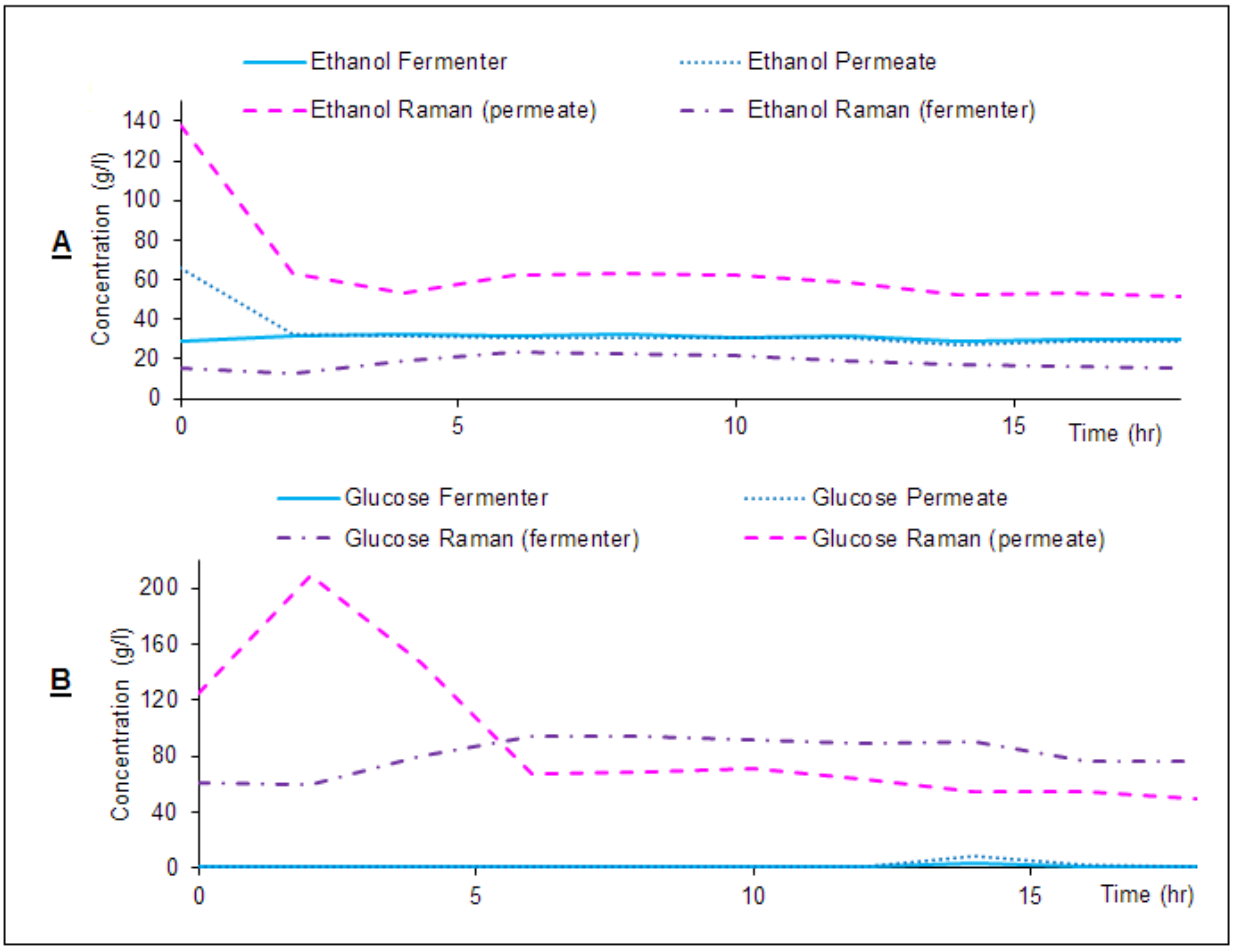

Figure 12: Continuous fermentation 4: Ethanol and glucose concentration. A) Ethanol concentration comparison between measurements inside the fermenter, in the permeate, and predicted by Raman inside the fermenter and in the permeate flow. B) Glucose concentration comparison between measurements inside the fermenter, in the permeate, and predicted by Raman inside the fermenter and at the permeate.

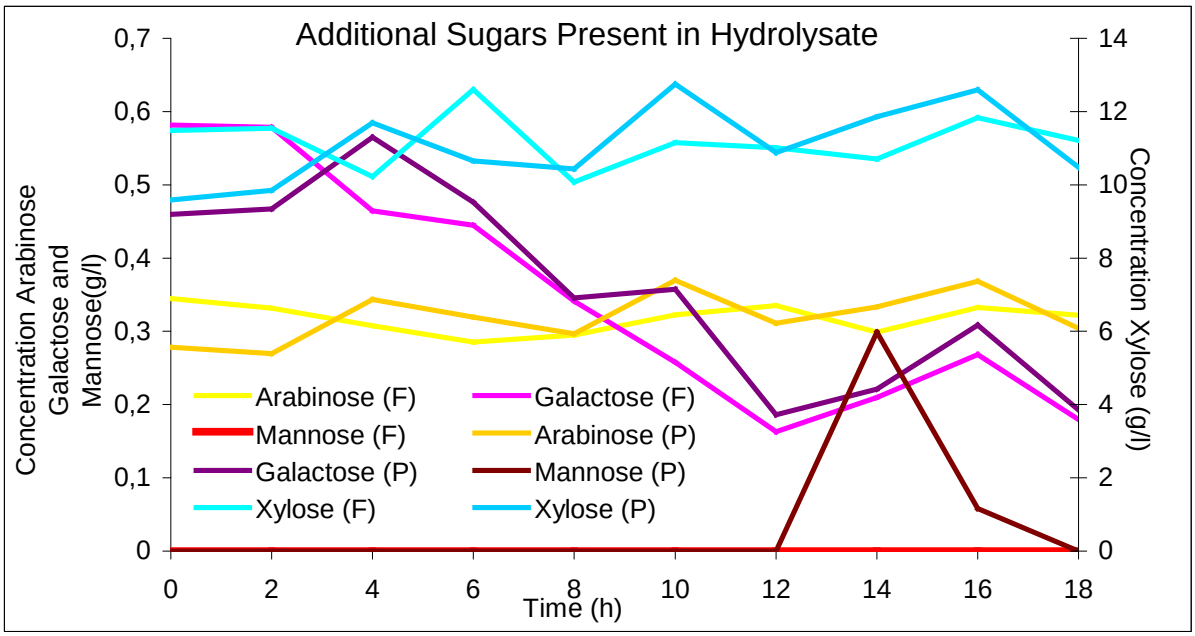

Figure 13: Utilization of additional sugars in hydrolysate.

In Figure 13, the additional sugars present are shown. The pentose sugars, xylose and arabinose, are not utilized by *S. Cerevisiae* as expected. As for the hexose sugars, galactose is partly utilized and mannose is completely utilized along with glucose. Phenolic compound concentration in the poplar hydrolysate was reduced through dilution with water. This reduced the total concentration of phenolics from 5.5g/l to 2.5g/l, as seen previously in Table 1. Neither filtration of the media trough 0,2 µm sterile filter nor going through the membrane reduced the total phenolics or acetic acid concentrations significantly. The concentration of furfural and 5-hydroxymethyl-furfural were reduced to zero after filtration through 0,2 µm sterile filter.

Overall for this specific system set up, a larger volume of 1*l* was optimal for the continuous system to work at and continuously be maintained at a constant volume. Whereas for the smaller volume; higher dilution rates caused problems with holding the volume constant. At a lower dilution rate the smaller volume also worked well. Higher dilution rates are not optimal for this system to function at. A dilution rate of $0{,}1h^{-1}$ seemed to be the most efficient one for this specific system. Although different sizes of fermenters were used in different experiment and thus also different volumes of media it can be noted that a $0{,}1h^{-1}$ dilution rate seemed to be the best to maintain a functioning system. Feeding the media at a 0, $1h^{-1}$ rate, and also controlling the permeate flow to work at the same rate, was ideal for this system.

6. Conclusion

A continuous fermentation system was successfully set up and it was possible to monitor the fermentation process via Raman spectroscopy. Bioreactor size and volume did affect the performance of the system, but a steady state system was achieved with dilution rates below $0{,}2h^{-1}$. Increased sugar concentration in the media fed to the system did not significantly affect sugar conversion. For a synthetic media with 60g/l glucose the Raman data could be accurately processed to give an adequate measurement of the concentrations of substrate and product. For the higher glucose concentration the predictions were not as accurate, most likely due to the PLS model since the Raman spectra looked good. When using the 60g/l PLS model, prediction was better for glucose than ethanol, and vice versa for the 120g/l PLS model. This could be because the glucose concentration during the fermentation will be low, and therefore more accurately predicted by a model based on lower glucose concentrations. The ethanol concentration, during the fermentation, was continuously held at a higher concentration, thus more accurately predicted by a model with more data points in the same range as the observed ethanol production.

It can be concluded that the Raman probe works best when in a loop outside fermenter when collecting data. The Raman spectra obtained from the permeate flow has less noise and more clearly visible peaks. Thus, readings must be taken after cells have been recycled and transferred back into the fermenter in order to get a clean spectra. From readings inside the fermenter it was not possible to retrieve data that gave a clean spectra without noise present. The cell density inside the fermenter is too high and created to much noise for an accurate reading, although peaks are still visible in the spectra.

When looking at fermentation of hydrolysate, there is a large amount of background noise and it is difficult to get a good Raman reading inside the fermenter. The Raman reading at the permeate did give a clearer spectra from which peaks following ethanol and glucose concentrations can be observed. The ethanol prediction is better than the one for glucose,

perhaps because this peak is more specific to the vibrations in ethanol than the 1123cm^{-1} peak is to glucose, which is present in a larger number of peaks. It also makes sense not to have the Raman probe inside the fermenter from an industrial point of view. In a bigger vessel and with a bigger system, that kind of set up would not be realistic. When comparing collecting Raman data inside the fermenter or at the permeate, the fermentation with synthetic media confirms that concentration predictions are more accurate from the data obtained after cells have been removed. However, the fermentation using hydrolysate did not show this. The prediction from the data obtained inside the fermenter gave a more accurate prediction of ethanol concentration. This might be due to the fact that even though the spectra is noisy, the peak at 883 cm^{-1} is clear with no interference of noise. It can also be noted that the cell concentration for fermentation with hydrolysate did not reach a level as high as for synthetic media, thus resulting in less interference from cells in the Raman reading.

Although the fermentations were not run to produce an optimal yield of ethanol, this system was capable of producing high levels of ethanol from synthetic glucose media. The main focus was to monitor and assess the possibility to monitor the fermentation process by Raman spectroscopy. In Table 3, data from all fermentations can be seen, showing concentrations for both glucose and ethanol. With a theoretical yield of 51% for *S. cerevisiae* glucose to ethanol fermentation, conversion rates ranged from 75-100%. The constant concentration level of ethanol through out the fermentations show that the system is capable of running continuously and keeping the production at a constant level. As well as that the yeast is capable of utilizing the sugars completely and managing fermentation.

Future work includes automating the system and improving the process control by applying a system for real-time feedback of Raman measurements, as a tool for advanced process control (APC). A reliable feedback system is necessary for APC to ensure operation of the system as close as possible to optimal production. Further, development of modelling is necessary, to be able to more accurately predict the concentrations of components in hydrolysate fermentation. Combining kinetic models with Raman monitoring and model predictive control, could contribute to a feasible and efficient system for large-scale bio-ethanol production.

Table 3: Summary. Substrate, ethanol, glucose and cell concentrations for each fermentation, as well as conversion, for each specific dilution rate used during operation.

		Continuous Fermentation 1				Continuous Fermentation 2		Continuous Fermentation 3	Continuous Fermentation 4
[Substrate] (g/l)		60				60		120	60
Dilution rate (1/h)		0,05	0,1	0,15	0,3	0,2	0,1	0,1	0,1
[Ethanol] (g/l)	**Raman**	29,7	32,4	35,0	23,8	20,6[*1]	28,8[*1]	70,8[*3]	65,7[*1]
						14,6[*2]	11,0[*2]	53,8[*4]	18,3[*2]
	Permeate	33,5	34,7	32,0	26,2	21,5	25,3	45,4	34,0
	Fermenter	47,9	51,7	52,7	36,1	22,1	26,0	46,2	31,0
[Glucose] (g/l)	**Raman**	8,6	6,7	6,3	13,9	15,4[*1]	8,6[*1]	0[*3]	91,0[*1]
						28,4[*2]	41,1[*2]	67,0[*4]	81,1[*2]
	Permeate	3,0	0,8	0,1	7,7	14,2	0,9	12,0	1,4
	Fermenter	0,5	0	0	18,3	12,3	0	9,0	0,7
[Cell] (g/l)		7,1	9,3	12,0	4,6	12,0	24,8	11,8	10,1
Conversion[*5] (g/g)		98%	100%	100%	74%	75%	89%	79%	100%

[*1] Raman data collected at permeate, [*2] Raman data collected in fermenter, [*3] Raman predicted with 60g/l PLS model, [*4] Raman predicted with 120g/l PLS model, [*5] Calculated with average of HPLC results.

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