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Microalgal process-monitoring based on high-selectivity spectroscopy tools: status and future perspectives

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Abstract

Microalgae are well known for their ability to accumulate lipids into their cells, which can be used for biofuels and mitigate CO₂ emissions; however, due to economic challenges, microalgae bioprocesses have maneuvered towards the simultaneous production of food, feed, fuel, and various high-value chemicals in a biorefinery concept. On-line and in-line monitoring of macromolecules such as lipids, proteins, carbohydrates, and high-value pigments will be more critical to maintain product quality and consistency for downstream processing in a biorefinery to maintain and valorize these markets. The main contribution of this review is to present current and prospective advances of on-line and in-line process analytical technology (PAT), with high-selectivity—the capability of monitoring several analytes simultaneously—in the interest of improving product quality, productivity, and process automation of a microalgal biorefinery. The high-selectivity PAT under consideration are mid-infrared (MIR), near-infrared (NIR), and Raman vibrational spectroscopies. The current review contains critical assessment of these technologies in the context of recent advances in software and hardware to move microalgae productions towards process automation through multivariate process control (MVPC) and software sensors trained on

“big data”. The paper will also include a comprehensive overview of off-line implementations of vibrational spectroscopy in microalgal research as it pertains to spectral interpretation and process automation to aid and motivate development.

Keywords

Near-Infrared Spectroscopy; Mid-infrared Spectroscopy; Raman spectroscopy; Process analytical technology, On-line; *in situ*; software sensor; multivariate process control

1 Introduction

Over the years, high oil producing microalgae have gained much attention as a feedstock for biofuels. Microalgae are most commonly produced because of their high lipid content, which constitutes about 20 to 60% of the microalgal biomass; however, microalgae lipid production for biofuels alone is too costly to justify a full-scale production [1]. Alternatively, multiple products from microalgae can offset production costs in a biorefinery concept [1]. Microalgal lipids, proteins, and carbohydrates can be directed to various markets and potential high-value product (HVP) pharmaceuticals/nutraceuticals such as pigments, fatty acids, and sterols can also be extracted to valorize the production [1–5]. Monitoring the composition of feedstock at the inlet of biorefineries is critical to assess the proper fractionation of the feedstock into valuable components [6] or to optimize downstream conversion processes for bioenergy [7]. However, methods to monitor the content of these various products in highly variable microalgae reactions have received very little attention.

Additionally, there is an overall lack of on-line monitoring tools capable of measuring biological activity for advanced feedback control of microalgal bioreactions [8]. In outdoor reactions, numerous inhomogeneities from low mixing speeds, aphotic zones, high intensity photic zones, diurnal and diel irradiance and temperature, and other anisotropy over large

land areas and photobioreactor settings confound the modelling and scalability of microalgae productions [8–10], which can be further complicated by fluctuating media, in the case of microalgal wastewater treatment. With these dynamic properties in mind, control capabilities for microalgal bioprocesses can benefit greatly from dynamic real-time optimization to correct for these inhomogeneities and inconsistencies as they occur in real-time to improve process controls aimed at maintaining the quality and consistency of sensitive products in the microalgae.

The use of microalgae as food or feed also shift microalgae production considerations towards quality control, in which nutritional content, product quality, culture purity, and contamination are essential to meet standards and regulations. In 2014, a survey of algal experts performed by Enzing *et al.* [11] reported that food safety legislation for standards and regulations was at the top of the list of key non-technical barriers to microalgal productions, beneath capital investments. Meanwhile, on-line/in-line monitoring of bioprocesses in real-time to improve production quality, consistency, homogeneity, and productivity have been widely recommended. In 2004, The United States Food and Drug Administration (USFDA) issued guidance for the bio-pharmaceutical industry under the term “process analytical technology” (PAT) [12]. The goal of PAT is to use timely measurements—high time resolution—of analytes in a process for design, analysis, and, ultimately, control of product quality, in which the product quality becomes built-in to the process. Similar recommendations have been made by the European Medicines Agency under the broad term “Quality by Design” (QbD) [13]. The European Federation of Biotechnology (EFB) Section on Biochemical Engineering Science (ESBES) refers to a similar philosophy of Modelling, Monitoring, Measurement & Control (M3C) [14,15]. The implementation of PAT requires the control of critical process parameters (CPPs), such as mixing/aeration/flowrate, pH, and temperature, while considering critical quality attributes (CQA) [16]. CQAs are defined as

physical, chemical, biological, attributes that should be within a desired limit, range, or distribution in the interest of improving product quality and consistency [16]. Furthermore, it can be argued that PAT should be fundamental to the design of microalgae productions reactor in the interest of producing pharmaceutical grade products and to valorize the complex microalgae processes through a biorefinery strategy.

Meanwhile, large, high time resolution datasets of multiple cellular products can be used to create robust and adaptive models for process automation. Moreover, the need for time-resolute measurements of multiple cellular products in a biorefinery has the potential to be addressed by mid-infrared (MIR), near-infrared (NIR), and Raman vibrational spectroscopies. MIR, NIR, and Raman vibrational spectroscopies can be used to monitor several CQAs in real-time and may even replace some CPPs. Currently, there are few reviews of vibrational spectroscopy as it pertains to the microalgae industry [9,17–19]; however, these reviews do not take into account the technological progress and development of on-line/in-line vibrational spectroscopy implemented in bioprocess, pharmaceutical, and liquid and solid food industries [20–28], which share many of the main implementation challenges with microalgae PAT. Though a microalgae bioprocess infrastructure can vary greatly compared from other industries, the monitoring of intracellular and extracellular macromolecules in bioprocesses share many intrinsic similarities to yeast and bacteria bioprocesses, which have been extensively reviewed together due to these similarities [20,21,29]. Furthermore, databases of single-cell bacteria, yeast, and microalgae spectra can all be used together to improve prediction and classification models of microalgae [30]. Overall, MIR, NIR, and Raman vibrational spectroscopies are promising techniques to monitor multiple molecular analytes in any biological processes on-line/in-line [31].

This paper reviews current applications and prospects of non-invasive, non-destructive, on-line/in-line MIR, NIR, and Raman spectroscopies as PAT, and a comprehensive overview

of off-line implementations of these technologies and their contributions to PAT. The review is critically evaluating these monitoring possibilities, and addresses several challenges for a microalgal biorefinery that these innovative technologies may improve:

- Simultaneous monitoring of carbohydrates, lipids, proteins, and HVPs in a microalgal biorefinery.
- Monitoring culture purity, contamination, or stability of engineered ecosystems.
- Process automation through model predictive control (MPC) of macromolecular profiles of microalgae from real-time measurements.
- Temporary vibrational spectroscopy monitoring to train software sensors: correlating real-time macromolecular data from vibrational spectroscopy to standard critical process parameters (CPPs) for computational automation.

2 Overview of PAT with Vibrational Spectroscopy

In the interest of PAT, this review will address novel tools for measuring critical quality attributes (CQAs) to be used in tandem with critical process parameters (CPPs), with the potential to replace some of them. The CQA monitoring tools under consideration are those capable of real-time and high-selectivity measurements of macromolecules pertinent to the microalgal biorefinery. PAT bioreaction monitoring can be classified into off-line, at-line, on-line, and in-line based on the location of the monitoring system [21,31,32]. A schematic of on-line and in-line measurement is shown in Fig. 1. In general, it is understood that submersed monitoring tools run the risk of contamination of the system.

Of the potential PAT tools, infrared (IR) and Raman vibrational spectroscopies are powerful non-invasive monitoring tools that can be used to monitor multiple molecular analytes on-line/in-line. Unique spectral signatures of molecular bond vibrations are produced by either absorbing (infrared) or scattering (Raman) radiation, which is then

analyzed. The resulting spectral signatures can be used to characterize macromolecules quantitatively and qualitatively.

MIR spectroscopy is used to analyze fundamental vibration absorption spectra of molecular bonds in the 4000-400 cm^{-1} (wavenumber: the inverse of wavelength), in which most organic compounds spectra occur between wavenumbers of 4000-1500 cm^{-1} [33]. Proportions and ratios of these bonds can elucidate information of very specific chemical compounds including lipids, proteins, carbohydrates, and silica (diatoms) and be used to characterize more specific molecules of industrial interest such as fatty acids [34,35], TAG [36], and beta-carotene as a precursor astaxanthin [37]. Additionally, MIR can also be used also differentiate algal species, strain mutants, and phenotypic aberrations within a strain (Fig. 2 & Fig. 3).

NIR spectroscopy is used to analyze vibrational spectra produced from the overtones of fundamental vibrations occurring in the 12 500 cm^{-1} to 4000 cm^{-1} MIR range and from stretch vibrations of hydrogen bonds (3600 – 2400 cm^{-1}); effectively, the range is between 13 000 and 4000 cm^{-1} [20]. NIR research is mainly focused on the qualitative analysis of lipids, fatty acids, proteins, carbohydrates, nucleic acids, and sometimes carotenoids (Fig. 2 & Fig. 3).

Raman spectroscopy is a vibrational spectroscopy that relies on faint differences in scattered light due to molecular vibrations. When detecting Raman scattered light, Rayleigh and Tyndall scattering must first be eliminated, elucidating faint characteristic spectra comprising astaxanthin, chlorophyll a, chlorophyll b, chlorophyll c, lipids, fatty acids, proteins, carbohydrates, nucleic acids, [18,38–44] (Fig. 2 & Fig. 3). Both IR and Raman spectroscopies can elucidate different aspects of a compound even though Raman signals are several orders of magnitude smaller than IR signals. Information related to the principles of vibrational spectroscopy and spectral peaks of interest in biological applications are extensive [20,21,45–47].

To extract relevant information related to macromolecule content from MIR, NIR, and Raman spectra, various chemometric models, statistical, and mathematical treatments are required [40, 46,48]. Chemometrics in MIR are mostly used to characterize and differentiate multiple microalgae in a sample [49] or the relative abundance of the same microalgae with different nutritional histories [50]. On the other hand, on-line chemometric calibration models of NIR spectra of macromolecules in biotechnology can be considered one of the most complex applications of chemometrics in process monitoring [31]. This is because the faint and/or overlapping overtone bands created from fundamental MIR bands, spectral interferences of water [51], and overall dynamic nature of bioreactions. Like NIR, the faint spectral signals from Raman light scattering require advanced chemometrics to interpret Raman spectra (Fig. 2 & Fig. 3). Additionally, advances in high-performance computing and molecular simulations such as density functional theory (DFT) can be used to define additional vibrational modes of chemicals [18, 38,52–54], which can possibly be used in the future in the detection of HVP or contaminants. The various chemometric algorithms are summarized in Fig. 2 and Fig. 3.

3 Mid-Infrared (MIR) spectroscopy of microalgae

Mid-infrared (MIR) spectroscopy is a rapid, high-throughput, non-destructive, qualitative and quantitative tool used to detect the spectra of a number of organic molecules [46, 48,55], and has been applied to microalgae applications in several ways. MIR spectroscopy can be used to elucidate patterns and evolutions of major macromolecules such as lipids, proteins, and carbohydrates, as well as minor components such as pigments in real-time. Most all microalgae MIR research is conducted off-line on dry or lyophilized samples and detected with a Fourier transform infrared (FTIR) spectrometer. These off-line studies reveal the potential versatility of on-line/in-line MIR monitoring [56]. Over the years, off-line

microalgal MIR spectroscopy research has focused largely on characterizing mixed populations of microalgae, macromolecular composition, and phenotypic changes in microalgae from nutrient stress (Fig. 3). Off-line MIR can also be used to characterize mixed populations of microalgae at a strain level due to subtle changes in molecular content between strains [57]. Therefore, MIR may be useful to monitor open and mixed microalgal systems such as high-rate algal ponds or culture purity in closed productions if successfully implemented on-line/in-line. The sensitivity of off-line MIR measurements can also elucidate molecular changes within an organism facilitated by metal sorption [58–60], which may be useful to understand the accumulation of metals of microalgae treating wastewaters. MIR spectroscopy has also been used to monitor intercellular sugars for mixotrophic and heterotrophic microalgae productions [61]. The entire range of microalgal analytes that can be measured using MIR spectroscopy on-line/in-line and off-line can be seen in Fig. 2 and Fig. 3.

While off-line studies of MIR for microalgae analysis continue, little work has been done to advance the use of on-line/in-line MIR spectroscopy in microalgal suspensions most likely because of the spectral interference of water. However, a promising mode of MIR for wet applications is attenuated total reflectance Fourier transform infrared (ATR-FTR), which has had growing applications in pharmaceutical, agro-food, and environmental sectors and can be used on-line/in-line in liquid media (see reviews by [20] and [21]).

Studies exploring MIR PAT of microalgae are scarce; however, the studies available show significant PAT potential, in which ATR-FTIR systems have only recently been introduced. Girard *et al.* [61] studied the ATR-FTIR spectra of intercellular sugar in cultures of mixotrophic *Scenedesmus obliquus*, *in situ* with a fiber optic probe. In the study, the authors successfully predicted lactose, glucose, and galactose in a mixture of Bold's basal media and cheese water permeate wastewater. Using a partial least squares regression (PLSR)

model, the authors established a high correlation ($R^2 > 99.9$) for each sugar with ATR-FTIR spectra as the proportions of these sugars changed over several days of the experiment. Similarly, As recent as 2015, Vogt and White [62] have been developing a Horizontal ATR-FTIR system to monitor chemical composition of *Dunaliella Parva*, which may be applied either on-line/in-line. The work is mainly focused on model development, in which the authors note their future investigations will focus on improving the instrumental and chemometric methodologies for quantitative measurements of *in vivo* cell composition in response to changes in their chemical environment.

Alternatively, flow-through cell microspectroscopy coupled with FTIR (micro-FTIR) is another method to monitor microalgal macromolecules *in vivo*; however, its use is limited to in-line diversion of microalgal suspensions to the micro-FTIR apparatus (Fig. 1). Unlike ATR-FTIR, micro-FTIR is still limited from water interferences; however, microscopically focused radiation and high spatial resolution detectors minimize the interference of water [63]. Heraud *et al.* [64] demonstrated that FTIR spectra for proteins and lipids could be obtained with a micro-FTIR fixed on the large (~200 micron) microalgae *Micrasterias hardyi* observed in a flow-through cell. In the flow-through cell, the microalgae cell was alive and viable, while changes in proteins and lipids were monitored as nutrient amended media flowed by the microalga. Flow-through cells may represent a possible avenue for in-line (*in situ* or *ex situ*) monitoring of microalgal cells with minimal volume loss. These flow-through cells, or microfluidic cells, which have been used off-line, broaden the possibilities of using micro-FTIR in-line, and have thus been overviewed in this work (Fig. 3).

Despite the few applications of MIR in PAT, there are numerous studies addressing MIR spectroscopy for improving microalgal productions; specifically, nutrient stress dynamics (Fig. 3). The contributions of off-line MIR spectra to databases is also important for on-line/in-line MIR development. A study by Laurens and Wolfrum [66] recorded FTIR spectra

of triglyceride and phospholipids of “wet” samples of *Nannochloropsis* sp., *Chlorococcum* sp., *Spirulina* sp. and an unknown diatom without any sample preparation. Triglyceride predictions from FTIR spectra were acceptable ($R^2 > 0.902$), whereas phospholipid predictions were low ($R^2 = 0.588$). Wagner *et al.* [17] showed that relative spectral changes in carbohydrate/protein ratios in *Chlamydomonas reinhardtii* could be differentiated as the microalgae transitioned from an 11 hour dark periods to 13 hours of light demonstrating the significance of monitoring microalgae in outdoor productions in irregular light.

MIR PAT in other bioprocesses, which share many similarities with microalgae bioprocesses, have made numerous advancements in on-line/in-line monitoring. There are numerous cases of *in situ* MIR applications, all of which use ATR-FTIR probes to acquire quantitative MIR spectra of a multitude of media components and bioproducts [20,21]. MIR spectra relevant to the microalgal industry obtained *in situ* in yeast and bacterial bioreaction suspensions in 5 L to 15 L reactors include optical density, ammonium, phosphates, acetate, glucose, fructose, organic acids as well as several bioproducts [20,21]. Overall, the on-going research in other bioprocesses can ultimately be used to improve microalgae applications through improvements in hardware, growing spectral databases of common molecular spectra, or through improved chemometrics used to refine MIR spectra in on-line/in-line applications.

4 Near-Infrared (NIR) spectroscopy of microalgae

Recently, technological advances have rendered NIR spectroscopy increasingly practical because of its simplicity, portability, low energy cost, and improving sensitivity [68]. Unlike other vibrational spectroscopies, NIR has been used extensively as a turbidimetric optical density estimation tool in microalgae productions and has also been used for density control to optimize productions [69]. However, there are many more biological features that NIR can

elucidate if implemented properly. NIR *in vivo* analysis of microalgae has only yet investigated biomass, lipids, and carotenoids (Fig. 2) but the potential use of high-selectivity on-line/in-line NIR in microalgae bioreactions is evidenced by its prolific use in other bioprocesses [20,21]. Furthermore, there is ongoing research of NIR implemented off-line, which largely investigate basic macromolecular composition due to changes in nutrients or environment. Off-line NIR composition measurements comprise of lipids, carbohydrates, proteins, total Kjeldahl nitrogen (TKN), and some cases nucleic acid, chlorophyll, ash content (Fig. 3), and structural differences in microalgae [70] which can benefit on-line/in-line NIR development.

NIR spectroscopy research of *in vivo* microalgae samples has recently emerged in literature in the interest of real-time PAT, with the demonstrated capability of measuring biomass, lipids, and carotenoids in microalgal suspensions. Shao *et al.* [71] used an *in situ* portable visible NIR (Vis-NIR) fiber-optic probe to measure intracellular carotenoid content in *Spirulina* sp. suspensions. In the study, carotenoid content was predicted using three PLSR prediction models with correlation coefficients as high as 0.96. Recently, Challagulla *et al.* [73] demonstrated that biomass dry weight and lipids in *Rhopalosolen saccatus* could be predicted from on-line NIR spectra with a PLSR model. The authors analyzed NIR (transmission mode) of liquid culture through a glass cuvette and compared these results to Fourier transform NIR (FT-NIR: reflectance mode) spectra of wet and dry filter paper containing the same sample. Biomass and lipids were predicted with cross-validation correlation coefficients (R_{cv}) for the on-line liquid suspension and off-line wet and dry filter papers of 0.88, 0.93, and 0.93 for biomass, and 0.72, 0.86, 0.81 for total lipids, respectively.

There are some notable cases in which NIR predictions of microalgal parameters cell suspensions are compared with wet and dry filtered samples. In an earlier study, Challagulla *et al.* [74] monitored biomass and lipids with an on-line visible-short wave NIR (Vis-SW

NIR: interactance mode) spectra through a 2 L culture vessel containing *Chlorella vulgaris*. In this case, on-line biomass prediction with Vis-SW NIR (cross validation correlation coefficient: $R_{cv} = 0.95$) outperformed off-line FT-NIR of wet filtered samples ($R_{cv} = 0.88$), and performed similarly to off-line FT-NIR of off-line dry filtered samples ($R_{cv} = 0.96$). However, total lipid content could not be predicted with much accuracy using the on-line probe. In the same study, Challagulla *et al.* [74] also tested the off-line FT-NIR probe on several other species of microalgae (*Chlorella vulgaris*, *Nitzschia pusilla*, *Navicula* sp. 1, *Navicula* sp. 2, and a mix of all four) observing high, and similar prediction accuracies; however, total lipid predictions were only successful on dry filter papers. Furthermore, for monitoring biomass exclusively, more conventional, and cheaper biomass monitoring tools may be employed. However, advances in chemometrics and continued research of the most viable NIR modes (e.g. transmission, FT-NIR, interactance) and wavelengths have the potential to improve the selectivity of *in vivo* NIR monitoring of microalgae.

While *in vivo* analysis of microalgae using NIR has been largely overlooked, advances of on-line/in-line monitoring of NIR in the food, bio-based, and pharmaceutical industries demonstrate the widespread viability of NIR. There are several reviews of PAT NIR applications in bio-based, agro-food, liquid-food, biofuel, pharmaceutical, and bio-pharmaceutical industries, which encompass PAT of biomass, pH, protein, extracellular protein, pyruvate, viscosity, acetic acid, total sugars, oil, phosphate, astaxanthin, glycerol, nitrogen sources, and casein [20–25]. In the dairy industry, short-wave NIR (SW-NIR; 600–1050 nm) is used for high quality on-line monitoring of milk for fat, protein, lactose, somatic cells (e.g. leukocytes), and milk urea nitrogen [75,76]. Several reviews have comprehensive lists of on-line/in-line and at-line applications of NIR in fermentations and biopharmaceutical aimed at PAT [20,21, 32,77,78]. At scales ranging from lab top to 40 m³ bioreactions in cell

culture bioprocesses, environmental process, and anaerobic digestion applications [21], on-line/in-line NIR development has a very realistic future in microalgae process monitoring.

5 Raman spectroscopy of microalgae

Raman spectroscopy is another high-selectivity spectroscopy tool used to monitor a variety of molecules in microalgae, in which most studies are aimed at the quantitative determination of various lipids and pigments (Fig. 2 and Fig. 3). Unlike MIR and NIR, the vast majority Raman spectroscopy studies focus on *in vivo* or wet microalgal analysis since there is a weak spectral interference of water, making Raman spectroscopy desirable for the analysis of *in vivo* biological samples [79] (Fig. 2 and Fig. 3). Raman spectroscopy is a burgeoning field of spectroscopy, which can be used also in combination with infrared spectroscopy [9, 31,80]. Like FTIR, Raman can also be used to identify microalgae species; however, research related to this is scarce [39, 43, 54,81,82].

Similar to the background interference of water in IR, Raman spectroscopy can be complicated by background fluorescence spectra [21, 42,83], and colored samples with wavelengths similar to the laser light source [84]. Fluorescence can be influenced by a variety of factors such as concentration [85], pH, dissolved oxygen, pressure, and temperature, making it very difficult to correct for [86], especially in microalgal cultures with highly fluorescent pigments. There are a variety of methods to reduce fluorescence interference, but shift excitation Raman difference spectroscopy (SERDS) has gained the most popularity, and has even been produced in a handheld format [86].

The emergence of on-line/in-line Raman spectroscopy research of microalgae shows a very promising outlook for Raman spectroscopy as PAT. Nadadoor *et al.* [88] report using an on-line, real-time, immersion probe to measure glucose, biomass, and oil inside a 2 L bioreactor of heterotrophically grown *Auxenochlorella prototheocoides* in fed-batch mode.

The authors successfully predicted glucose, biomass, and oil with R^2 as high as 0.8867, 0.9822 and 0.8597, respectively, using a support vector regression (SVR). Noack *et al.* [89] used SERDS to monitor extracellular polymeric substances carbohydrates produced by the marine red microalgae *Porphyridium purpureum*. In their proof-of-concept experiment, the authors used SERDS to predict EPS content in optical flow cuvettes receiving a continuous flow of culture. The authors also found that support vector machines (SVM), similar to support vector regression (SVR), had a higher extracellular polymeric substance prediction accuracy (prediction sum of squares: PRESS = 0.031) than a PLSR (PRESS = 0.48). Overall, with hardware capable of minimizing fluorescence and predicting both intracellular and extracellular compounds, Raman spectroscopy has a very probable future in on-line/in-line monitoring of bioreactions of microalgae.

Handheld, at-line, Raman spectrometers using CCDs, which can achieve high spectral resolutions [90] have recently become more common in various industries as well as in microalgal applications. Wood *et al.* [43] used a portable Raman spectrometer with fiber optic probe fixed on an acoustically levitated droplet of microalgal suspension to minimize the side effects of a container. The authors investigated the effects of high light exposure and nitrogen limitation on the spectral proportions of chlorophyll-a and beta-carotene in *Dunaliella tertiolecta*, *Chaetoceros muelleri*, *Phaeodactylum tricornutum*, and *Porphyridium purpureum*. Their work also illustrated that Raman has the capability of species differentiation by observing the differences in spectra.

In-line Raman monitoring of microalgal suspensions has also be realized though microfluidic flow through cells coupled with high resolution Raman micro-spectroscopy, for a number of applications related to microalgae biotechnology. Samek [92] notes that *in vivo* and real-time monitoring of nutrient dynamics, lipid content, and metabolism can be done through microfluidic chips and optical tweezers with Raman spectroscopy. Microfluidic

cytometer “side loops” have already been employed to measure chlorophyll fluorescence induction, in which the microalgae cells can be dark adapted and flows can be decelerated to permit analyses requiring longer measurement times [84,93]. Ren *et al.* [30] used an on-line micro-Raman spectrophotometer with a microfluidic device for Raman activated cell sorting (RACS), similar to fluorescence-activated cell sorting (FACS), which was used for cell phenotyping. The authors compared Raman spectra of the microalgae to a database of 12 011 single-cell Raman spectra containing 14 strains of microalgae, bacteria, and yeast; three of which were microalgae. Using a SVM prediction model trained on the database of Raman spectra, the authors could determine the phenotypic match of the microalgae *Nannochloropsis oceana* in nitrogen deplete/replete conditions with 93.3% accuracy. Similarly, micro-Raman can be used with optical tweezers (i.e. optical trapping and laser trapping), which simulate microfluidic cells by confining a single-cell into a small location using focused lasers. Wu *et al.* [41] used laser trapping Raman spectroscopy (LTRS) to analyze individual cells of *Botryococcus braunii*, *Neochloris oleoabundans*, and *Chlamydomonas reinhardtii*. In their study, a systematic qualitative profiling of Raman spectra of 11 fatty acids (stearic, palmitic, myristic, dodecanoic, decanoic, octanoic, oleic, palmitoleic, linolenic, arachidonic) was used to screen the microalgae and reveal quantitative predictions of lipid unsaturation. The authors note the promise of direct monitoring of microalgal cells *in situ*. Moreover, with microfluidic technologies included, the majority of Raman microscopic advances have PAT potential for microalga suspensions (Fig. 3).

Raman technology is relatively new in biotechnological applications; however, there are numerous studies of Raman PAT. Of those similar to microalgae, Cannizzaro *et al.* [94] used a Raman immersion probe inside a bioreactor to measure carotenoids produced by *Phaffia rhodozyma*. Because of the strong Raman signal of carotenoids [44,95], a simple calibration model was used without the need of complex chemometrics. Lourenço *et al.* [21] reviewed

some of the Raman applications in fermentation, which encompass food, beverage, pharmaceutical and environmental applications. Reviews on Raman spectroscopy PAT in the pharmaceutical industry [25], food agriculture and beverages, [26], and a comprehensive multi-author review of PAT applications [78] may aid in the PAT realization of Raman in microalgal applications. The growing number of on-line/in-line Raman studies as well as the improvements in hardware and software have made Raman a promising candidate for on-line/in-line applications.

6 Vibrational Spectroscopy PAT potential

In general, NIR and Raman are considered the best candidates for on-line/in-line measurements. However, the diversity of microalgae bioreactions –phototrophic, mixotrophic, heterotrophic– and the variety of reactor designs, operational modes, and sought-after products and parameters are all influential when choosing on-line/in-line vibrational spectroscopy. Moreover, there are several key characteristics to consider when selecting a vibrational spectroscopy assembly for on-line/in-line monitoring:

- *High-selectivity, and species and phenotypic cell differentiation* When predicting and identifying unknowns (e.g. contamination) and in terms of the highest-selectivity, MIR (ATR-FTIR) is the best suited for on-line/in-line applications followed by Raman. The availability of MIR spectral libraries, which help with chemometric model building to differentiate species and detect phenotypic changes, give MIR this advantage over Raman, for the time being. However, both Raman and ATR-FTIR have not been used to differentiate microalgal cells in suspensions. Furthermore, apart from measuring intracellular components of microalgae, FTIR has been used to measure or infer a number of critical process parameters (CPPs) and critical quality attributes (CQAs) that are commonly monitored in microalgae reactions. On-line ATR-FTIR spectra of the ratio of

H_2PO_4^- to HPO_4^{2-} can be used for feedback control pH within ± 0.15 pH units of a set point [96]. Alternatively, the ratio of carbohydrate/amide I determined by off-line FTIR can be used to predict photosynthetic efficiency with high accuracy ($R^2 = 0.998$) [27], which is extremely important for understanding microalgae physiology. Other attempts have correlated FTIR spectra to chemical oxygen demand, total organic carbon, and volatile fatty acids, and total and partial alkalinity to some degree [97].

- *Multiple monitoring locations* Another drawback of ATR-FTIR is that fiber optic materials with sufficient MIR transmission are limited and costly [80] and require very short fiber optic cables and may not be suitable for multiplexing [21] to monitor inhomogeneities across large scale photobioreactors. On the other hand, Raman and NIR are more likely to be multiplexed with fiber optic conduits, each which can be up to a few meters long [21].
- *Portability and modularity* NIR and Raman are the best candidates due to their general absence of FT interferometer used in most all MIR applications. Over the years, hardware and chemometric advances have improved the mobility and practicality of NIR devices for industry [98,99], which are also much more simple to construct than MIR and Raman.
- *Culture density* Where low density bioreactions are concerned, MIR is the most suitable followed by Raman and then NIR. The lower detection of NIR, for example, compared with FTIR, makes the latter a desirable option for quantification at sub g/L concentrations [100] often observed in microalgal bioreactions.

7 PAT Automation

A plausible approach to controlling complex microalgal bioprocesses is through spectroscopic on-line/in-line monitoring coupled with chemometric automation. Apart from using chemometrics to interpret vibrational spectra of compounds, real-time critical quality

attributes (CQA) and critical process parameters (CPPs) can be used to enhance model predictive control (MPC), nonlinear model predictive control, or multivariate process control (MVPC) to automate bioreactions. Similarly, off-line analyses of the multitude of media components in fluctuating media (e.g. wastewater) requiring laborious and costly analysis may be circumvented by using spectroscopic techniques measuring real-time nutrient responses of cells inside a reactor [101].

Improvements in chemometrics and computational processing power have made MVPC a promising tool for the automation of bioreactions. Most applications of vibrational spectroscopy PAT require calibration and validation of chemometric models [21, 48,102] with the exception of some analytes, for example carotenoids measured through Raman [94]. However, inhomogeneities in large systems may require the need of non-linear chemometrics (ANNs, SVMs, and Kohonen networks) to be used as a dynamic “fingerprint” of the bioprocess. These non-linearities may arise from a variety of factors, such as the effect of variable temperature on vibrational spectra, changes in culture purity, or unknown cellular responses to dynamic media. ANN MVPCs have already been used to monitor and control the growth of microalgae through monitoring basic CPPs [103,104] and glucose concentrations in heterotrophic microalgal growth [105]. Unsupervised—without *a priori* off-line calibration—pattern recognition and classification of time-course spectra data may also be useful to model relative spectral changes for process control. Moreover, with continued data collection of microalgae spectra into spectral databases, machine learning models (e.g. SVM) can be improved to become more robust [30] and applied to MVPC. Reviews of linear, non-linear, supervised, and unsupervised techniques and their careful consideration in bioprocess are provided by Despagne [106], Faassen and Hitzmann [108], Funes [109], Komives and Parker [110], and Lourenço *et al.* [21]. Nowadays, user-friendly software packages are

available to simplify MVPC, but the extent of their functionality and the available chemometric methods are not within the scope of this paper.

Software sensors or soft sensors, also referred to as inferential sensors [111], virtual on-line analyzers [112], and observer-based sensors [113], can be used to model real-time CPP data (e.g. pH and DO) and infrequent off-line analysis of macromolecules to interpolate artificial real-time values [8,9,114] to monitor a number of bioprocesses. However, the soft sensor building is nontrivial and the robustness is contingent on the frequency of analysis of the desired CQA. However, it is also possible to use real-time vibrational spectroscopy temporarily to train soft sensor models until a robust soft sensor model is created. Temporally segmented modelling can be used to focus efforts on highly controlled growth and production phases in bioprocesses to develop models [115], terminating when a robust soft sensor model has been correlated to a CPP already in use (e.g. pH). An outdoor microalgal model might employ temporal segmentation based on periods of consistent media, temperature, and light to develop primary models. In doing so, future contamination concerns of in-line and *in situ* on-line monitoring are alleviated once a model has been built and the probe is removed. Furthermore, spectroscopic techniques such as fluorescence [107] and NIR spectroscopy [116] have been suggested to refine these soft sensor algorithms.

Though using vibrational spectroscopic techniques ultimately adds to the production cost, the compatibility of these technologies with fiber optics leaves the authors with a broad suggestion: consider sampling ports, optical conduit ports, or transparent surfaces in the design of a reactor to permit permanent or temporary spectroscopy for model building. Despite the appeal of on-line/in-line sensors, the development of at-line/off-line tools may still critical for PAT and model building. Acquiring and sharing spectral information into databases and repositories can aid in model training, which can then be adopted into other microalgal processes. Furthermore, academic data acquisition and model building at the early

stages of microalgal industry development may benefit future productions with models built from on-line spectral databases. Ultimately, facilitating “big data” in the microalgae arena at an early stage may benefit the industry later on from a number of profound advances in data science.

8 Conclusions

The complexity and the potential of microalgae to be used as food, feed, and fuel in various markets in a biorefinery concept underline the need for a very thorough understanding of microalgal systems with an emphasis on quality control. This can be accomplished by high-selectivity and time-resolute vibrational spectroscopic PAT. The purpose of this work was to elucidate some of the aspects of the complex nature of vibrational spectroscopy of microalgae and some insights into the applications and limitations before implementation. Currently, if high-selectivity, mixed cultures, and contamination prediction is of concern MIR instruments are the most promising, and Raman spectroscopy has an outlook of competing with these instruments with growing spectral libraries and technological advancements. NIR instruments remain cheap and reliable for measuring basic constituents in liquid (e.g. lipid, carbohydrates, and proteins), which may improve with advanced chemometrics and modelling of new spectral contributions in the NIR range. Despite the prohibitive cost of Raman spectroscopy, the future of vibrational spectroscopy and the focus of research should consider the continued growth of Raman technology and the ongoing success of IR spectroscopy in *in situ* analysis and their benefits to the microalgal industry. The ultimate return on the investment of early use is academically and industrially lucrative in terms of accelerating an immensely complex, idle, or possibly dwindling industry with profound implications for sustainability and food security.

Declaration of interest

The authors report no declarations of interest.

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Figure captions

Fig. 1. Bioreactor monitoring techniques. On-line measurements acquire light/data directly from the reactor (dotted lines). In-line measurements channel reactor media away from the reactor to acquire data (arrows).

Fig. 2. Comprehensive overview of on-line/in-line, at-line/cell suspension, wet sample/*in vivo* application of various vibrational spectroscopy (NIR, MIR, Raman) modes in microalgal research. The figure is categorized into research focus, major products, and minor products for each investigated microalgae species and strain.

Fig. 3. Comprehensive overview of off-line applications of various vibrational spectroscopy (NIR, MIR, Raman) modes in microalgal research. The figure is categorized into research focus, major products, and minor products for each investigated microalgae species and strain.

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