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Robust near-infra-red spectroscopic probe for dynamic monitoring of critical nutrient ratio in microbial fermentation processes

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ABSTRACT

Near infra-red spectroscopy (NIRS) measurements for bioprocess monitoring and control, are integral to process analytical technology (PAT) initiatives by EMEA and US-FDA. Yet, NIRS is not widely practiced in challenging microbial fermentation processes. We present a practical approach to develop NIRS models for linoleic acid (LA), oleic acid (OA) and ammonia which are critical nutrients in lipstatin fermentation by *Streptomyces toxitricini*. The lipstatin productivity was enhanced and steadied by dynamic monitoring and control of critical nutrient ratio (CNR) of LA to ammonia. The NIRS models were used to develop a novel, soft probe for CNR as an alternative to laborious, hourly, off-line analyses. The calibration was designed with typical data for four industrially useful microbes. The approach enabled direct use of spectra for a generally applicable model with distinct wave number optima of 6250–5555 cm^{-1} (LA), 6666–5882 cm^{-1} (OA), 6800–6300 cm^{-1} (ammonia). The standard errors of calibration and prediction were 1.5×10^{-3} g L^{-1}, 1.6×10^{-3} g L^{-1}, 1.1 ppm, and 8.9×10^{-4} g L^{-1}, 1.8×10^{-2} g L^{-1}, 3.6 ppm, respectively, for the respective nutrients. The robustness of probe is evident from the low mean percentage error of 2.3% for prediction of CNR at low concentration ranges of 0.02–0.24 g L^{-1} and 0.21–0.56 g g^{-1} for LA and CNR, respectively.

1. Introduction

Continuous process tracking, with challenges of limited measurement options [1,2] was significantly helped when European Medicines Agency (EMEA) and U.S. Food and Drug Administration (USFDA) formulated the Process Analytical Technology (PAT) guidance in 2003–2004 (http://www.vt.fi/inf/pdf/workingpapers/2006/W60.pdf, http://www.fda.gov/Cder/OPS/PAT.htm). The developments in near infra-red spectroscopy (NIRS), which is integral to PAT, were motivated by the absorption of near infra-red radiations due to C—H, N—H, and O—H bonds typically found in nutrients fed in biological processes. The use of specific wavenumbers that correspond to the absorbance maxima for the respective bonds in the nutrients, makes the application difficult in culture situations where there is significant interference from the other media components. Thus, a range of wavenumbers is used when NIR monitoring is applied to such complex situations as industrial cultures [3–5]. The range of wavenumbers also helps in generalizing the applicability to this technique to a wide range of industrial microbial processes.

Further, a typical microbial fermentation process has inherent complexities due to complex raw material constituents, vigorous stirring and gas bubbles, ever changing morphology of the growing microbe, varying rheological properties of the broth not only along the cultivation time but also from batch-to-batch and also with the reactor scale [3–7]. Consequently, the use of NIRS for in-process analysis in commercial processes is limited mainly to cell culture [8–10] or insect culture [11,12] where the media matrix and measurement conditions are relatively simpler. Though NIRS measurement of carbon and nitrogen sources has been tried for several microbial processes (Table 1), the observed large mean percentage errors suggest a need to improve the accuracy. For example, the reported NIRS studies have shown relatively higher standard measurement errors of 1.16 g L^{-1} for fructose, 0.22 g L^{-1} for acetate, 2 g L^{-1} for glycerol, 0.24 g L^{-1} for methanol, 20 g L^{-1} for linoleic acid and 4 g L^{-1} for an unknown carbon source when the measurement range for the above carbon sources was 10–35 g L^{-1} [21], 1–6 g L^{-1} [21], 0–62 g L^{-1} [13], 0–2.5 g L^{-1} [13], 440–560 g L^{-1} [22], and 7–36 g L^{-1} [3], respectively. The above applications of NIRS are unsuitable for fermentation processes in which the toxic feed components such as methanol, acetate, linoleic acid have to be maintained in broth at a residual level similar to or lower than the above reported standard errors. For many high cell density industrial fermentations [13,23], key carbon source and/or precursor [24,25], organic acid metabolites [26] need to be maintained at

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Table 1
Use of near infra-red spectroscopy (NIRS) for measurement of typical analytes in microbial fermentation processes.

S. no.	Strain	Analyte	Concentration range	SEP	Mean % error	Reference
1	*Streptomyces clavuligerus*	C-source	7–36 g L^{-1}	4 g L^{-1}	19.0	[3]
		N-source	300–800 ppm	100 ppm	18.0	
2	*Pichia pastoris*	Glycerol	0–62 g L^{-1}	2.01 g L^{-1}	6.4	[13]
		Methanol	0–10 g L^{-1}	0.22 g L^{-1}	44.0	
3	*Saccharomyces cerevisiae*	Glucose	0–5 g L^{-1}	1.386 g L^{-1}	55.0	[14]
4	*Saccharomyces cerevisiae*	Glucose	0–2 g L^{-1}	0.8 g L^{-1}	55.0	[15]
5	*Streptomyces coelicolor*	Glucose	0–43	2 g L^{-1}	8.7	[16]
		Ammonium	39–111	11 mM	15.0	
6	*Penicillium chrysogenum*	Total sugars	5–90 g L^{-1}	2.5 g L^{-1}	5.3	[7,17,18]
		Ammonium	0–2.5 g L^{-1}	0.17 g L^{-1}	13.6	
7	*Anaerobic digestion of manure using mixed culture*	Volatile fatty acids	2.5–35 g L^{-1}	2.4 g L^{-1}	12.9	[19]
8	*Vibrio cholera*	Glucose	0–4.12 g L^{-1}	0.26 g L^{-1}	12.6	[20]
		Acetate	0–3.77 g L^{-1}	0.28 g L^{-1}	14.9	

residual concentration less than 1 g L^{-1}. Similarly, an average absolute error of 2.88 ppm and a corresponding mean percentage error of 27% [27] for a commonly used nitrogen source like ammonia advocate a need to improve the predictive ability, accuracy and robustness of the NIRS model for low concentrations of toxic compounds such as ammonia as well as organic acids [6]. This is a challenge because application of NIRS for accurate at line/online measurement of critical nutrients in microbial fermentation has been limited thus far to concentrations greater than 1% [28].

Effective individual NIRS models for critical nutrients at low concentrations are a prerequisite for developing an integrated probe for accurate measurement of critical nutrient ratio (CNR) of two important nutrients [29] that may be crucial for a microbial fermentation process. This requires a thorough understanding of chemometric techniques and of the dynamics of the fermentation process, and an appropriate integration between the two leading to an improved calibration philosophy [30].

The calibration based on purely synthetic samples made from media mixtures, yields poor results for prediction in broth samples because the likely interactions between components of the spent broth are not given due weightage in model building [9,10]. This is further limited by the fact that a majority of the work with synthetic media has been done for relatively simpler matrix of animal cell culture [8,9] or insect cell culture [10] whereas a majority of industrial microbial fermentations employ high cell densities as well as various complex nutrients like soya flour, oils, vegetable peptones, yeast extracts, and so on. The use of semi-synthetic samples for calibration involved the addition of known amounts of the analytes to be measured, to different aliquots of the spent medium collected from the fermentation process. However, this approach performed poorly in insect cell cultivation samples [4] due to variability inherent to fermentation processes. Another limitation was due to a slight distortion of the analyte distribution toward higher concentrations [11,12,31] of glucose and ammonia in cell culture because the minimum concentration in the calibration samples was higher than the minimum concentration in the applied model. The use of real fermentation samples is necessitated by the inability to synthetically mimic the interactions between the numerous components of the actual fermentation spent media matrix [6,32]. Further, the batch to batch process variations and their impact upon complex interactions and the resulting model question the suitability of this approach itself. Also, the interpretations of spectral information and the process of improving accuracy, particularly at low concentrations of analytes are cumbersome due to large number of variables (wavelengths), presence of components that exhibit overlapping absorbance features, ever-changing micro-organism morphology and solids concentration.

The focus of this work was to develop a robust and distinguishing CNR probe to address some of the above mentioned challenges. The model system used here is a challenging system consisting of structurally similar molecules, linoleic acid (LA) and oleic acid (OA) as carbon sources. The nitrogen source was ammonia, and the ratio of LA to ammonia is the relevant CNR. The study was motivated by pressing requirements of a demanding industrial fermentation in which the key carbon source (LA) needs to be maintained at atypically low concentration of 0–0.2 g L^{-1} due to toxicity considerations, in addition to maintain CNR at an optimum level for maximizing the productivity.

2. Materials and methods

2.1. Fermentation description

Streptomyces toxitricini BICC 6825 (Biocon India culture collection) was grown in a complex media adapted from literature [33]. One mL glycerol vial containing microbial spores was used to inoculate pre-sterilized, 250 mL shake flask containing 50 mL of the seed medium: 10 g L^{-1} soyabean flour (toasted), 10 g L^{-1} glycerol, 5 g L^{-1} yeast extract, 0.1 g L^{-1} silicon antifoam, pH 6.8 + 0.1. The seed shake flask was incubated for 24 h on a rotary shaker at 230 rpm and 28 °C followed by transfer to pre-sterilized, 2 L shake flask containing 300 mL of seed medium. The second stage of seed flask was incubated for 24 h before being transferred to 50 L fermenter containing 30 L of production medium: 30 g L^{-1} soya flour, glycerol 30 g L^{-1}, pH 6.9. The production medium was supplemented with L-leucine, LA, OA, and soyabean flour intermittently. All fermentations were carried out at 28 °C, 600 rpm agitation and 1 vvm aeration. The process pH was kept constant at 7.0 + 0.1 by addition of 10% (w/v) NaOH or 10% (w/v) HNO_3. The DO was controlled above 20% by changing agitation, aeration as well as head pressure in cascade mode. All fermentation studies were done in 50 L stainless steel 316 fermenter (Alfa Laval, India). The media for other microbes (*Pichia pastoris BICC7996*, *Aspergillus niger BICC 5174*, *Escherichia coli BICC 7841*) were adapted from literature [29,34,35].

2.2. Sampling and reference methods

Broth samples were collected from the fermenters at hourly intervals and characterized by the conventional analytical methods. All the broth samples were centrifuged at 10,000 × *g* for 10 min at 4 °C and the biomass concentration was determined as wet pellet weight. Further, the broth samples were also directly extracted with acetone by mixing 10 g of broth with 50 mL of acetone. The supernatant was then used for estimation of lipstatin as product content, LA and OA as carbon sources by analytical HPLC method [33], Ammonia was analyzed by SAN^{++} automated wet chemistry analyzer (Skalar Analytical B.V. Tinstraat, Breda, The Netherlands). These off line values were used as reference for model building.