Viki R. Chopda¹
Mili Pathak¹
Jyoti Batra¹
James Gomes²
Anurag S. Rathore¹

¹Department of Chemical Engineering, IIT Delhi, New Delhi, India

²Kusuma School of Biological Sciences, IIT Delhi, New Delhi, India

Research Article

Enabler for process analytical technology implementation in *Pichia pastoris* fermentation: Fluorescence-based soft sensors for rapid quantitation of product titer

Rapid quantitation of product titer is a critical input for control of any bioprocess. This measurement, however, is marred by the myriad components that are present in the fermentation broth, often requiring extensive sample pretreatment before analysis. Spectroscopy techniques such as fluorescence spectroscopy are widely recognized as potential monitoring tools. Here, we investigate the possibility of using fluorescence of the culture supernatant as a potential at-line monitoring tool to measure the concentration of a recombinant therapeutic protein expressed in a *Pichia pastoris* fed-batch fermentation. We propose an integrated method wherein both the target protein and total protein concentrations are predicted using intrinsic riboflavin fluorescence and extrinsic fluorescence, respectively. The root mean square error for estimating the concentrations of the target protein (using riboflavin fluorescence) and total protein (using extrinsic fluorescence) have been estimated to be <0.1 and <0.2, respectively. The proposed approach has been validated for two different biotherapeutic products, human serum albumin and granulocyte colony stimulating factor, that were expressed using Mut⁺ and Mut^s strains of *P. pastoris*, respectively. The proposed approach is rapid (1 min analysis time, 10 min total with at line sampling) and thus could be a significant enabler for process analytical technology implementation in *Pichia* fermentation.

Keywords: Bioprocess monitoring / Fluorescence spectroscopy / Granulocyte colony stimulating factor / Human serum albumin / *Pichia pastoris*

Additional supporting information may be found in the online version of this article at the publisher's web-site

Received: June 15, 2016; revised: August 11, 2016; accepted: October 13, 2016

DOI: 10.1002/elsc.201600155

1 Introduction

Biotechnology has emerged as a key area of growth in the 21st century with the biotherapeutics dominating the arena of high-value biotech products. These products, as well as the processes used to make them, are known to be complex. Reliable mon-

itoring of these processes is thus very important for achieving high manufacturing yields and consistent product quality [1–3].

The past decade has seen the advent of quality by design and process analytical technology (PAT), which involve use of enhanced understanding of the product and the process so as to create a well-controlled process that is capable of delivering a product with consistent quality [4, 5]. The PAT approach emphasizes on real-time monitoring of process parameters and raw material attributes by application of various in situ and/or at-line analytical tools to ensure that any deviations during processing are identified and immediately dealt with [6, 7]. The complex nature of bioprocesses, however, imposes an inherent challenge in implementing real-time process monitoring and control [8, 9]. Though there has been a deluge of remarkable advancements

Correspondence: Dr. Anurag S. Rathore (asrathore@biotechcmz.com), Department of Chemical Engineering, Indian Institute of Technology Delhi, Hauz Khas, New Delhi, 110 016, India.

Abbreviations: ANS, 8-anilino-1-naphthalene-sulfonic acid; DO, dissolved oxygen; FAD, flavin adenine dinucleotide; GCSF, granulocyte colony stimulating factor; GFP, green fluorescent protein; HPLC, high-performance liquid chromatography; HSA, human serum albumin; PAT, process analytical technology; RMSE, root mean square error

in process analytics both with respect to hardware and approach over the past decade, existing processes are still far from being optimal due to limitations of process-monitoring tools [10–12].

Spectroscopic techniques are particularly desirable for bioprocess monitoring because of their rapid and noninvasive nature, thereby enabling their use as at-line or online tools within the bioreactor [13, 14]. In online mode (in situ/in-line), the probe is immersed in the fermentation medium and data collected continuously. This mode is preferred for process control applications as the objective is prompt execution of control actions [1]. Many a times, due to rigorous conditions in the bioreactor, at-line mode has to be used. In this mode, a sample is taken out of the bioreactor and analyzed in near vicinity such as the Yellow Springs Instruments (YSI) analyzer. Many sensors based on spectroscopic techniques also favor the use of soft sensors in bioprocess monitoring. A brief summary of the commonly used spectroscopic tools is presented in Supporting Information Table S1 [12, 13, 15–17]. Furthermore, fluorescence spectroscopy has emerged as the preferred choice for monitoring and quality control of raw materials and process samples in online as well as off-line mode [18]. Biogenic fluorophores, such as riboflavin, pyridoxine, and amino acids, such as phenylalanine, tryptophan, and tyrosine, can be measured with high sensitivity (at a micromolar level) using this method [15]. Hisiger and Jolicoeur monitored cellular growth by measuring the NADH fluorescence signal [19]. Surribas et al. used tryptophan fluorescence signal for monitoring protein production dynamics and reported that tryptophan signal was not affected by fluctuation in dissolved oxygen (DO) concentration [20]. Advancements such as multiwavelength scanning spectrofluorometry [21], synchronous scanning fluorescence (SSF), and fluorescence excitation–emission matrix (EEM) have enabled data collection over a range of excitation and emission wavelengths [22, 23]. Fluorescence spectroscopy has been successfully applied for monitoring of several critical process variables such as protein concentration [24], cell growth [25], and viability and media component degradation for microbial as well as mammalian cell processes [26, 27].

The methylotrophic yeast *Pichia pastoris* represents one of the predominant workhorse of biotech industry popular not only for its versatility and efficiency in high expression of recombinant proteins but also for its ability to generate high-density cultures. The level of protein expression in *P. pastoris* is significantly influenced by the cultivation parameters, and, therefore, the monitoring of product formation dynamics becomes an imperative need to achieve optimal process development and control [28]. Monitoring of some of the key state variables of *P. pastoris* fermentation such as biomass and substrate concentration has been successfully demonstrated using fluorescence spectroscopy [20, 29]. However, monitoring the concentration of a recombinant protein product is challenging in such high-cell density fermentation. There are few reports that demonstrate fluorescence-based monitoring of protein production in *Pichia* fermentation; however, they require the use of green fluorescent protein (GFP) as a fusion tag [30, 31].

In this paper, we propose the use of an integrated monitoring method based on fluorescence signal for measuring protein concentration in the fermentation broth. Two applications have

been successfully examined for their use in *P. pastoris* fermentation. In the first application, intrinsic fluorescence of riboflavin has been used to predict the concentration of recombinant human serum albumin (rHSA) (66.5 kDa) and recombinant granulocyte colony stimulating factor (rGCSF) (19.6 kDa). In the second, extrinsic fluorescence measurement has been employed to predict the total protein concentration in *Pichia* fermentation. We think that the proposed approach would have wide applicability for monitoring production of therapeutic proteins in *P. pastoris* fermentation and will facilitate implementation of PAT in fermentation.

2 Materials and methods

2.1 Strain

Two strains of *P. pastoris* were employed in this study. *P. pastoris* X-33 Mut⁺ strain was used for the production of rHSA under the regulation of AOX1 promoter. *P. pastoris* Mut⁺ strain was used for the production of the rGCSF under the control of the promoter AOX2 (AOX1[−]).

2.2 Media and cultivation conditions

Both microbial strains were grown overnight in shake flasks at 30°C in a rich standard Buffered Glycerol-Complex Medium (BMGY) medium containing 1% w/v yeast extract, 2% w/v peptone, 100 mM potassium phosphate buffer (pH 6.0), 4×10^{-5} % w/v biotin, and 1% w/v glycerol. This inoculum was used further for bioreactor cultivation. The batch medium used for fermentation is described in ref.32. The batch was operated at 30°C and pH 5. The batch phase took around 22 h, and the fed-batch was started once spike in DO was observed. The fed batch operation was carried out in two different sets based on feeding solution composition. In one set of experiments, methanol was used as the sole carbon source and in the second set of experiments, a mixture of 70% methanol and 30% sorbitol was used for feeding. For fed batch operation, the temperature and pH was decreased to 24°C and 6, respectively, using a prewritten algorithm to maintain uniform conditions for all experiments. The pH was maintained by adding ammonium hydroxide solution (28–30%) and 2N ortho-phosphoric acid. The stirring and airflow rates were varied to ensure that the DO was higher than 10% of saturation. Pure oxygen was supplied as and when required. An integrated graphical user interface program was developed using LabVIEW 10 (National Instruments, Austin, TX) to collect and store the acquired data as well as to give command signals to the respective controller of process variables [33].

2.3 Analysis

2.3.1 Biomass analysis

For measuring biomass concentration, 10 mL of culture liquid was centrifuged at $8000 \times g$ for 5 min in preweighed centrifuge tubes. The sediment was washed twice with distilled water and then dried to constant weight at 105°C.

2.3.2 rHSA and rGCSF estimation

rHSA and rGCSF were analyzed by reversed phase high-performance liquid chromatography (RP-HPLC) using the C4 XBridge® (Waters Corporation, Milford, MA, USA) and Jupiter C4 (Phenomenex, CA, USA) HPLC columns, respectively. Acetonitrile (ACN; HPLC grade, Merck) and Milli-Q water were used for buffer preparation. Organic phases were prepared with mixing of 70% acetonitrile, 30% water, and 0.75% trifluoroacetic acid (TFA) as an ion-pair reagent for rHSA analysis, and 99.9% ACN and 0.1% TFA for rGCSF analysis, respectively.

2.3.3 Total protein determination

Total protein content was measured by Bradford assay using bovine serum albumin as a standard and absorbance of 595 nm [34].

2.3.4 Fluorometry

Off-line fluorescence measurements were performed using 96-well black bottom plate (Greiner) at room temperature using a SpectraMax® M2e (Molecular Devices, CA, USA). The step increment given was 1 nm for all types of spectral scans. For the development of calibration curve, standard HSA of USP grade, 20% solution (Baxter Pvt. Ltd., India) and standard GCSF from a domestic manufacturer were used. For measurement of sample fluorescence, the fluorescence measurement of initial batch medium sample was taken as a blank and subtracted from sample readings. Experiments were carried out under different feeding conditions using different strains to generate raw data which were benchmarked with HPLC data and used further for model development. Furthermore, this model was validated in subsequent experiments to predict target as well as total protein concentration in different strain selected under different feeding conditions.

2.3.4.1 Intrinsic fluorescence measurement. The three aromatic amino acids, namely phenylalanine, tyrosine, and tryptophan, are known to exhibit fluorescence in proteins. Energy absorbed by phenylalanine and tyrosine is often transferred to the tryptophan residues in the same protein, and, therefore, emission scan of proteins is dominated by tryptophan fluorescence [35]. For intrinsic tryptophan fluorescence measurement, samples were excited at 278 nm and emission spectra were scanned in the range from 300 to 400 nm (λ_{max} 340 nm). To measure intrinsic riboflavin fluorescence, culture samples were excited at 450 nm and emission spectra were scanned in the range from 490 to 600 nm (λ_{max} 520 nm).

2.3.4.2 Extrinsic fluorescence measurement. When fluorescent dyes are added externally in the sample that further binds with sample components specifically or nonspecifically to produce fluorescent measurement, the approach is referred to as extrinsic fluorescence [36]. For extrinsic fluorescence, the 8-anilino-naphthalene-1-sulfonic acid (ANS) dye was used, samples were excited at 380 nm, and emission scan was recorded from 400 to 600 nm (λ_{max} 470 nm).

Figure 1. Illustration of at-line monitoring setup for fluorescence measurements.

2.3.5 At-line setup for enabling PAT-based monitoring

Cell-free samples were automatically collected via filtration probes equipped with standard flow.

Cell free samples were withdrawn from the bioreactor at regular intervals using FISP® ceramic membranes (Flownamics®, Inc., USA). The sample was then allowed to go through an in-line mixer. The probe outlet was connected to the inlet of this mixer through one end of a Y-connector made up of polypropylene. The other end of Y-connector was joined to a dye solution reservoir through a peristaltic pump. The mixing and incubation of dye solution with cell-free sample was carried out in the in-line mixer. The outlet of the in-line mixer was connected to the inlet of flow cell through which fluorescence signal was monitored, and the final sample was discarded or collected as per requirement. The setup is shown in Fig. 1.

3 Results and discussion

Commonly used methods for protein estimation are summarized in Supporting Information Table S2. In this paper, we propose an integrated methodology toward monitoring of the concentration of the protein of interest that utilizes both of the above-mentioned approaches so as to not just quantify but also provide an orthogonal confirmation of the results. The analysis time of the proposed approach is less than 1 min, thereby enabling its use for monitoring as well as controlling fermentation processes. Furthermore, the proposed rapid fluorescence method can enable PAT implementation for *P. pastoris* fermentation as it meets all the necessary requirements.

3.1 Case study I: Monitoring rHSA production by *Pichia Mut*⁺ strain

HSA is an important therapeutic protein as it has a number of pharmaceutical applications. The rHSA produced was monitored by measuring extrinsic and intrinsic fluorescence of culture broth. Simultaneous analysis of rHSA concentration was performed by HPLC.

Figure 2. Results obtained from the production of rHSA in *Pichia pastoris*. (A) Plot of maximum tryptophan fluorescence intensity versus protein concentration for HSA standard (0.1–4 mg mL⁻¹). (B) Plot of maximum extrinsic fluorescence intensity versus protein concentration of HSA standard (0.1–4 mg mL⁻¹). (C) Actual versus predicted plot for total protein concentration produced in *Pichia* fermentation fed with methanol. (D) Actual versus predicted plot for total protein concentration produced in *Pichia* fermentation fed with mixed feed. (E) Plot of maximum riboflavin fluorescence intensity versus protein concentration of recombinant HSA (obtained by RP-HPLC) produced in *Pichia* fermentation fed with methanol. (F) Actual versus predicted plot for rHSA production in *Pichia* fermentation fed on mixed feed.

3.1.1 Direct monitoring

3.1.1.1 Reversed phase HPLC (RP-HPLC). Supporting Information Fig. S1A and S1B illustrate the HPLC chromatograms corresponding to the HSA standard along with the calibration curve developed with the HSA standard in the concentration range of 0.1–4 mg mL⁻¹, respectively. Furthermore, Supporting Information Fig. S1C illustrates the change in concentration of rHSA with time for the two different feed compositions with *P. pastoris* Mut⁺ strain fed-batch fermentation, and Supporting Information Fig. S1D illustrates the corresponding optical density profiles (the Supporting Information). rHSA production was induced by feeding methanol at around 22 h, and the process was terminated after rHSA concentration reached a constant value (1.5 g L⁻¹). Though HPLC is a robust, reproducible, and selective analytical tool for protein quantification, its on-line implementation as a PAT tool is not extensively observed for routine bioprocess applications. This is so because fermentation samples consist of cells, unwanted impurities, by-products of cell metabolism, and complex media nutrients. These samples require pretreatment such as centrifugation (approximately 10 min), filtration (approximately 3 min) before HPLC analysis can be performed. In addition, the HPLC analysis time required typically varies from 30 min to several hours [18]. As

a result, HPLC can be used in at-line or off-line monitoring mode.

3.1.1.2 Intrinsic fluorescence. The changes in intrinsic tryptophan fluorescence intensity were utilized to predict protein concentration. Tryptophan intrinsic fluorescence spectrum for standard HSA solution was acquired over a range of concentration (0.1–4 mg mL⁻¹), and it was found that the maximum fluorescence intensity saturates at higher protein concentrations (Supporting Information Fig. S2A) and that a plateau in maximum fluorescence intensity is observed once protein concentration rises above 1 mg mL⁻¹ (Fig. 2A). Hence, it can be concluded that utility of this approach for monitoring of HSA concentration is limited to 1 mg mL⁻¹. Rathore et al. also demonstrated the non-linearity of tryptophan fluorescence intensity with protein concentration, which further necessitated dilution at higher protein concentrations (> 1 mg mL⁻¹) [37]. Thus, intrinsic tryptophan fluorescence spectrum can be employed for protein concentration determination up to a certain concentration (normally 1 mg mL⁻¹).

3.1.1.3 Extrinsic fluorescence. In this study, ANS dye was used for monitoring of protein production in fermentation as

Table 1. Summary of model performance for protein estimation using fluorescence signal in *Pichia* fermentation

Approach	<i>Pichia</i> run	RMSE for total protein estimation	RMSE for target protein (rHSA) estimation
Using extrinsic fluorescence	Fed with only methanol	0.231	0.841
Using extrinsic fluorescence	Fed with mixed feed	0.108	0.356
Using intrinsic riboflavin fluorescence	Fed with mixed feed	0.305	0.093
Integrated approach (extrinsic fluorescence for total protein and intrinsic riboflavin fluorescence for target (rHSA) protein estimation)	Case study	0.102 (Using extrinsic fluorescence)	0.192 (Using riboflavin fluorescence)

fluorescence properties of ANS are strongly dependent on their interaction with protein molecules. Interaction of ANS with protein molecules results in a change of polarity and viscosity of the environment. Hydrophobic interactions and electrostatic interactions play a major role in binding of ANS to proteins. However, complementary interactions such as van der Waals interactions are required to stabilize the ion pairs [38, 39]. An increase in extrinsic fluorescence intensity was observed upon increasing standard HSA concentration (Supporting Information Fig. S2B). A linear relation of maximum extrinsic fluorescence intensity with increasing protein concentration was obtained up to standard HSA concentration of 4 mg mL⁻¹ (Fig. 2B). Since the addition of an external reagent is required, it is evident from the data that extrinsic fluorescence can be used as a monitoring and control tool in at-line mode. When this approach was used to estimate rHSA concentration in *Pichia* fermentation, the root mean square error (RMSE) observed was unacceptably high (RMSE = ±1). However, when this approach was used to measure total protein content and the results were compared to those from the Bradford assay, the correlation was significantly better (RMSE = ± 0.2). These data are presented in Table 1.

This observation may be due to the fact that ANS binds in a nonspecific manner to proteins containing positively charged amino acids. Figures 2C and D illustrate the actual versus predicted empirical correlation plot of estimating total protein concentration for *Pichia* fermentation for both the feed regimes that were examined. It is evident from the results that measurement of extrinsic fluorescence can be used for quantitation of total protein content. The proposed method is found to be as rapid and reproducible as the Bradford assay and henceforth can be used as a potential at-line monitoring tool.

3.1.2 Indirect monitoring using riboflavin fluorescence

In view of the limitations seen in the fluorescence-based measurements discussed above with respect to estimating the target protein concentration, we also examined the possibility of identifying other key cellular metabolites that may play a crucial role in the various protein production pathways. Alcohol oxidase (AOX) is a key enzyme in methanol metabolism and catalyzes the first step in methanol catabolism—oxidation of methanol to formaldehyde with concomitant production of hydrogen peroxide (H₂O₂) [40]. There are two AOX genes in *P. pastoris* that code for the AOX enzyme: The AOX 1 gene (AOX1) is responsible for greater than 90% of the enzyme in the cell, and the

AOX 2 (AOX2) influences the remaining 10% [41]. The mature active form of AOX is an oligomer (600 kDa), reported to consist of eight identical subunits, each of which carries one noncovalently bound flavin adenine dinucleotide (FAD) molecule as the prosthetic group. Flavin synthesis is tightly regulated under AOX promoter. High-cell density cultures of *P. pastoris* grown on methanol tend to develop yellow colored supernatants attributed to the release of free flavins in the culture supernatant. Regulation by FAD is mainly under the control of genes encoding different enzymes involved in the catalytic cascade leading to the generation of riboflavin, Flavin mono nucleotide and FAD, respectively [42]. FAD binding and assembly of the AOX oligomer are necessary steps in AOX biosynthesis and its function [43]. This feature of *P. pastoris* for flavin production, therefore, can be potentially useful in estimation of protein concentration. As the standard human HSA sample does not contain riboflavin, a calibration curve of rHSA using a series of *Pichia* fermentations having methanol as a sole carbon source was generated. The rHSA concentration was estimated using RP-HPLC, and the resulting data plotted against the maximum riboflavin fluorescence intensity of fermentation samples at different time intervals. It is evident from Supporting Information Fig. S2C that intrinsic riboflavin fluorescence intensity also increases with time, and thus a linear calibration curve can be created between the rHSA concentration measured with RP-HPLC and the maximum riboflavin fluorescence intensity (Fig. 2E). This correlation was then applied to subsequent *Pichia* runs to predict protein production. The RMSE for the proposed empirical correlation for estimating rHSA concentration is ±0.1 with respect to the RP-HPLC method (Fig. 2F and Table 1). In addition, fluorescence spectroscopy offers a noninvasive and rapid approach (<1 min) compared to the traditional HPLC analysis (30 min to several hours). Thus, by monitoring the intrinsic riboflavin fluorescence intensity, we are able to successfully estimate rHSA concentration in at-line mode. In addition, after normalization of the riboflavin fluorescence spectra, the three distinct phases of *Pichia* fermentation namely, glycerol batch phase, transition phase, and methanol induction phase, are clearly observed (Fig. 4). To generate this graph, we have used the following normalization formula:

$$x_{\text{new}} = \frac{X_{\text{current}} - X_{\text{min}}}{X_{\text{max}} - X_{\text{min}}} \quad (1)$$

where X is obtained from raw fluorescence measurements. Furthermore, as there are online fluorescence probes available for

bioreactor monitoring, use of the proposed approach as a PAT tool is feasible.

3.1.3 PAT-based implementation of the proposed integrated approach for protein monitoring

Simultaneous monitoring of both the concentration of the product and total protein is of interest for control of a fermentation process. Use of HPLC as an at-line monitoring tool has been successfully demonstrated by several researchers [44, 45]. However, the cost involved in developing such a setup as well as the required maintenance, the need for extensive sample pretreatment, and the use of high-pressure capacity column are potential roadblocks that inhibit routine applications of HPLC in fermentation. In contrast, we are proposing a quick method using single-dimensional fluorescence spectroscopy (extrinsic and intrinsic riboflavin fluorescence signal) for monitoring both total protein as well as target protein in the fermentation broth.

To implement the proposed integrated approach, a key challenge is that of achieving a gentle mixing of the dye and the sample externally followed by incubation for sufficient time. This problem has been overcome by using the setup described in Fig. 1. The setup consists of the sample probe, dye solution, peristaltic pumps, the in-line mixer, and the flow cell with fluorescence detector. Sample and dye were withdrawn from the bioreactor and dye reservoir, respectively, at regular intervals using a pre-programmed pump assembly. Then, both cell-free samples and dye solutions were passed through the in-line mixer such that gentle mixing occurred and a residence time of ~5 min was provided for sample–dye interaction. The sequence of analysis and the time required for mixing are given in Table 2. The total time required for sampling and analysis including both extrinsic and intrinsic fluorescence was around 10 min, of which the mixing and incubation time is ~6 min. First, extrinsic fluorescence was monitored followed by intrinsic fluorescence using the same setup.

The RMSE for predicting the concentration of both the target protein (rHSA) using intrinsic riboflavin fluorescence as well as total protein using extrinsic fluorescence was found to be less than 0.2 (Fig. 3A and 3C and Table 1). Thus, the proposed method can be effective as an online monitoring and control tool for *P. pastoris* fermentation such that production of target protein over unwanted protein can be tracked and appropriate action can be taken. Since the undesired generation of the process and product related impurities adds an extra burden on downstream processing, such kind of process control can result in optimal bioprocesses.

Riboflavin fluorescence was also measured both in glycerol batch (initial 22 h) and methanol induction phase, and a significant correlation was observed with rHSA production. In glycerol batch phase, the change in riboflavin fluorescence intensity was not significant. From initial inoculation in the bioreactor to the end of glycerol batch phase, optical density gradually increased from 0.5 to 66 with a marginal increase in maximum riboflavin fluorescence intensity from 627 to 797 arbitrary fluorescence unit (AFU). On the other hand, riboflavin fluorescence intensity increased significantly from 1000 to 10,000 AFU in the methanol induction phase due to the increase in

flavin release upon AOX activation. Normalization of the riboflavin fluorescence spectra resulted in the identification of the distinct phases of *Pichia* fermentation quite clearly namely, glycerol batch phase, transition phase, and methanol induction phase (Supporting Information Fig. S2D). An additional advantage that flavin-based fluorescence monitoring offers over monitoring based on GFP-based fluorescent proteins is that the fluorescence signal of riboflavin is not sensitive to oxygen fluctuations in the bioreactor. On the other hand, GFP-based fluorescent proteins show weak/no fluorescence emission in low-oxygen environments as reported by Mukherjee et al. [46]. Reischer et al. observed another major limitation of GFP is its poor signal detection with crude fermentation broth [47].

3.2 Case study II: Monitoring GCSF production by *Pichia* Mut^s strain

To demonstrate the applicability of the proposed approach on another *P. pastoris* fermentation-derived product having AOX as a promoter system (which uses methanol as a component for induction), we examined potential application involving the use of a *P. pastoris* Mut^s strain expressing rGCSF. Supporting Information Fig. S3A and S3B illustrate the chromatograms obtained for standard GCSF using RP-HPLC, and the calibration curve developed for standard GCSF in the concentration range of 0.01–0.15 mg mL⁻¹, respectively. Furthermore, while Supporting Information Fig. S3C illustrates the change in product concentration as measured by RP-HPLC as a function of time, Supporting Information Fig. S3D illustrates the corresponding information about the change in biomass concentration measured at OD₆₀₀. It is seen that rGCSF concentration rises to 0.14 mg mL⁻¹ after 120 h of fermentation time. Since the concentration is significantly lower than that for HSA in the first case study, intrinsic tryptophan fluorescence intensity was measured and was found to linearly correlate with rGCSF concentration. Furthermore, a similar linear correlation was observed between the intrinsic riboflavin fluorescence and rGCSF concentration. Finally, extrinsic fluorescence was also found to linearly correlate with total protein concentration. Figures 3D–F show actual versus predicted profiles. Thus, depending on the strain chosen and target protein being produced, the empirical correlation can be developed to measure concentrations of both the product as well as the total protein. Thus, the proposed integrated method is applicable as a potential PAT tool for monitoring protein production in *P. pastoris* culture using AOX as a promoter system.

Culture media used in both case studies does not contain any fluorophore, and hence the media fluorescence was subtracted from the sample signal. Therefore, any variation in fluorescence emission would not face interference from the background emission due to media components and can be attributed solely to the dynamics of protein production [24]. Earlier, use of two-dimensional fluorescence spectroscopy has been demonstrated as a monitoring tool for predicting important state variables such as biomass, DO, and substrate concentration [27, 48], but this requires use of multivariate data analysis for extracting process information from raw fluorescence data [49]. On the other

Table 2. Details of the time events deployed for at-line monitoring of fluorescence measurement

Place	Time (min)	Flow rate (mL/min)	Pump
(From bioreactor and dye reservoir) till Y junction	(a) 0.35	(a) 1.4	(a) A on B on
From Y junction to in-line mixer	(a) 1.8 (b) 6.94	(a) 0.7 (b) 1.4	(a) A on B on (b) A on B off
From in-line mixer to flow cell	Measurement taken at	(a) 1.4 (b) 1.4	(a) A on B off (b) A on B off
	(a) 7.50 (extrinsic fluorescence) (b) 9.30 (intrinsic fluorescence)		

Figure 3. Results from the demonstration of an integrated approach for monitoring protein production in *Pichia* fermentation. (A) The plot of maximum extrinsic fluorescence intensity and maximum intrinsic riboflavin fluorescence intensity at different time intervals for rHSA production from Mut⁺ *Pichia* fermentation. (B) Actual versus predicted plot for rHSA production in *Pichia* fermentation using intrinsic riboflavin fluorescence. (C) Actual versus predicted plot for total protein concentration in Mut⁺ *Pichia* fermentation using extrinsic fluorescence. (D) Actual versus predicted plot for rGCSF production in *Pichia* Mut⁺ fermentation using intrinsic tryptophan fluorescence. (E) Actual versus predicted plot for rGCSF production in *Pichia* Mut⁺ fermentation using intrinsic riboflavin fluorescence. (F) Actual versus predicted plot for total protein concentration in Mut⁺ *Pichia* fermentation using extrinsic fluorescence.

hand, our proposed integrated method is devoid of any such prerequisites and has been demonstrated to accurately predict protein concentration. While riboflavin fluorescence intensity has earlier been correlated with biomass during growth phase in *Pichia* fermentation up to low optical density ($OD_{600} \sim 12$) by Hisiger and Jolicoeur [19]. However, such correlation has not been demonstrated during protein production at higher cell densities [19, 20]. Using tryptophan fluorescence, the *Rhizopus oryzae* lipase production in the *P. pastoris* was successfully

estimated by Surribas et al. [20] with a prediction error of 7% in the exponential growth phase and 20% in the stationary phase. In our case, the prediction error using riboflavin fluorescence signal was less than 10% over the entire process regime of protein production. We have also plotted standard riboflavin concentration versus maximum riboflavin intensity and have obtained a linear correlation between them up to the concentration of 7 μ M and intensity at this point was $\sim 40,000$ AFU (Supporting Information Fig. S4). With respect to this standard graph, the

Figure 4. Normalized riboflavin fluorescence spectra at different time interval samples for rHSA produced in *Pichia* fermentation. A Clear distinction between the different phases of fermentation process viz. batch phase, transition phase and methanol induction phase has been successfully captured.

maximum concentration of riboflavin achieved in the reactor was $\sim 1 \mu\text{M}$.

4 Concluding remarks

Control of a fermentation process requires us to have an ability to real-time or near-real-time monitor concentration of the key metabolites and of course the product of interest. Simultaneous measurement of the various parameters related to cellular growth, metabolism, and product formation allows us to “fingerprint” the process as well as exercise appropriate process control. In this paper, the use of fluorescence spectroscopy as an at-line PAT tool has been explored and successfully demonstrated for monitoring recombinant protein production in *Pichia* fermentation. Supporting Information Table S3 presents a comparison of the performance of the various analytical techniques employed that were examined in this study for monitoring protein production. We have proposed an integrated monitoring method based on intrinsic riboflavin fluorescence and extrinsic fluorescence to monitor both the target proteins (rHSA and rGCSF) as well as total protein content in the *Pichia* fermentation that is quite rapid (1 min analysis time, 10 min total with at line sampling). The proposed approach has been validated under different feeding conditions (methanol and methanol + sorbitol). A significant correlation has been observed between flavin synthesis and target protein production. These findings have been validated using HPLC as an orthogonal approach ($\text{RMSE} < 0.2$). Moreover, monitoring of riboflavin further provides knowledge about different phases of *Pichia* cultivations. Extrinsic fluorescence measurement (using ANS dye) has been proposed for monitoring the total protein production ($\text{RMSE} < 0.1$). Based on the results presented here, we can conclude that the proposed method based on fluorescence signal can act as an enabler for PAT implementation in bioprocess control for monitoring of the protein production dynamics in *Pichia* fermentation.

Practical application

Biological processes are poorly understood due to their intricate nature. This holds true particularly in the case of the recombinant protein production due to the strong interaction between protein expression and cell metabolism. Based on this interaction, we present an integrated fluorescence method which uses intrinsic riboflavin fluorescence and extrinsic fluorescence to monitor both the target as well as total protein content in the *Pichia* fermentation. The method is quite rapid (1 min analysis time, 10 min total with at line sampling). The proposed approach has been validated under different feeding conditions (methanol and methanol + sorbitol) for different target proteins (rHSA and rGCSF). These findings have been validated using HPLC ($\text{RMSE} < 0.2$). Based on the results, we can conclude that the proposed method based on fluorescence signal can act as an enabler for PAT implementation in bioprocess control for monitoring of the protein production dynamics in *Pichia* fermentation.

The research was supported and funded by grant SB/S3/CE/093/2013 from Department of Science and Technology, India, and the High Impact Proposal Award from IIT, Delhi.

The authors do not have any conflict of interest with respect to the work presented here.

5 References

- [1] Lourenço, N. D., Lopes, J. A., Almeida, C. F., Sarraguça, M. C. et al. Bioreactor monitoring with spectroscopy and chemometrics: A review. *Anal. Bioanal. Chem.* 2012, 404, 1211–1237.
- [2] Gomes, J., Chopda, V. R., Rathore, A. S., Integrating systems analysis and control for implementing process analytical technology in bioprocess development. *J. Chem. Technol. Biotechnol.* 2015, 90, 583–589.
- [3] Komives, C., Parker, R. S., Bioreactor state estimation and control. *Curr. Opin. Biotechnol.* 2003, 14, 468–474.
- [4] Rathore, A. S., Winkle, H., Quality by design for biopharmaceuticals. *Nat. Biotechnol.* 2009, 27, 26–34.
- [5] Rathore, A. S., Roadmap for implementation of quality by design (QbD) for biotechnology products. *Trends Biotechnol.* 2009, 27, 546–553.
- [6] Rathore, A. S., Bhambure, R., Ghare, V., Process analytical technology (PAT) for biopharmaceutical products. *Anal. Bioanal. Chem.* 2010, 398, 137–154.
- [7] Read, E. K., Park, J. T., Shah, R. B., Riley, B. S. et al. Process analytical technology (PAT) for biopharmaceutical products: part I. Concepts and applications. *Biotechnol. Bioeng.* 2010, 105, 276–284.
- [8] Persad, A., Chopda, V. R., Rathore, A. S., Gomes, J., Comparative performance of decoupled input–output

- linearizing controller and linear interpolation PID controller: enhancing biomass and ethanol production in *Saccharomyces cerevisiae*. *Appl. Biochem. Biotechnol.* 2013, 169, 1219–1240.
- [9] Chopda, V., Rathore, A., Gomes, J., On-line implementation of decoupled input-output linearizing controller in Baker's yeast fermentation. *IFAC Proc.* 2013, 10, 259–264.
- [10] Vojinović, V., Cabral, J. M. S., Fonseca, L. P., Real-time bioprocess monitoring: Part I: In situ sensors. *Sensors Actuators B: Chem.* 2006, 114, 1083–1091.
- [11] Junker, B. H., Wang, H. Y., Bioprocess monitoring and computer control: key roots of the current PAT initiative. *Biotechnol. Bioeng.* 2006, 95, 226–261.
- [12] Wechselberger, P., Sägmeister, P., Herwig, C., Real-time estimation of biomass and specific growth rate in physiologically variable recombinant fed-batch processes. *Bioprocess Biosyst. Eng.* 2013, 36, 1205–1218.
- [13] Luttmann, R., Bracewell, D. G., Cornelissen, G., Gernaey, K. V. et al. Soft sensors in bioprocessing: a status report and recommendations. *Biotechnol. J.* 2012, 7, 1040–1048.
- [14] Clementschitsch, F., Bayer, K., Improvement of bioprocess monitoring: development of novel concepts. *Microb. Cell Fact.* 2006, 5, 19.
- [15] Pons, M. N., Le Bonté, S., Potier, O., Spectral analysis and fingerprinting for biomedica characterisation. *J. Biotechnol.* 2004, 113, 211–230.
- [16] Abu-Absi, N. R., Martel, R. P., Lanza, A. M., Clements, S. J. et al. Application of spectroscopic methods for monitoring of bioprocesses and the implications for the manufacture of biologics. *Pharm. Bioprocess.* 2014, 2, 267–284.
- [17] Lee, H. W., Carvell, J., Brorson, K., Yoon, S., Dielectric spectroscopy-based estimation of VCD in CHO cell culture. *J. Chem. Technol. Biotechnol.* 2015, 90, 273–282.
- [18] Faassen, S. M., Hitzmann, B., Fluorescence Spectroscopy and chemometric modeling for bioprocess monitoring. *Sensors* 2015, 15, 10271–10291.
- [19] Hisiger, S., Jolicoeur, M., A multiwavelength fluorescence probe: Is one probe capable for on-line monitoring of recombinant protein production and biomass activity? *J. Biotechnol.* 2005, 117, 325–336.
- [20] Surribas, A., Geissler, D., Gierse, A., Scheper, T. et al. State variables monitoring by in situ multi-wavelength fluorescence spectroscopy in heterologous protein production by *Pichia pastoris*. *J. Biotechnol.* 2006, 124, 412–419.
- [21] Jain, G., Jayaraman, G., Kökpınar, Ö., Rinas, U. et al. On-line monitoring of recombinant bacterial cultures using multi-wavelength fluorescence spectroscopy. *Biochem. Eng. J.* 2011, 58, 133–139.
- [22] Ödman, P., Johansen, C. L., Olsson, L., Gernaey, K. V. et al. On-line estimation of biomass, glucose and ethanol in *Saccharomyces cerevisiae* cultivations using in situ multi-wavelength fluorescence and software sensors. *J. Biotechnol.* 2009, 144, 102–112.
- [23] Sikorska, E., Gliszczynska-Świgło, A., Insińska-Rak, M., Khmelinskii, I. et al., Simultaneous analysis of riboflavin and aromatic amino acids in beer using fluorescence and multivariate calibration methods. *Anal. Chim. Acta.* 2008, 613, 207–217.
- [24] Groza, R. C., Calvet, A., Ryder, A. G., A fluorescence anisotropy method for measuring protein concentration in complex cell culture media. *Anal. Chim. Acta.* 2014, 821, 54–61.
- [25] Zimmermann, H. F., Trauthwein, H., Dingerdissen, U., Rieping, M. et al. Monitoring aerobic *Escherichia coli* growth in shaken microplates by measurement of culture fluorescence. *BioTechniques* 2004, 36, 580–585.
- [26] Calvet, A., Li, B., Ryder, A. G., A rapid fluorescence-based method for the quantitative analysis of cell culture media photo-degradation. *Anal. Chim. Acta.* 2014, 807, 111–119.
- [27] Teixeira, A. P., Portugal, C. A., Carinhas, N., Dias, J. M. et al., In situ 2D fluorometry and chemometric monitoring of mammalian cell cultures. *Biotechnol. Bioeng.* 2009, 102, 1098–1106.
- [28] Harms, J., Wang, X., Kim, T., Yang, X. et al. Defining process design space for biotech products: case study of *Pichia pastoris* fermentation. *Biotechnol. Prog.* 2008, 24, 655–662.
- [29] Surribas, A., Montesinos, J. L., Valero, F. F., Biomass estimation using fluorescence measurements in *Pichia pastoris* bioprocess. *J. Chem. Technol. Biotechnol.* 2006, 81, 23–28.
- [30] Sarraemna, V., Talmont, F., de Roch, M. S., Milon, A. et al. Green fluorescent protein as a reporter of human μ -opioid receptor overexpression and localization in the methylotrophic yeast *Pichia pastoris*. *J. Biotechnol.* 2002, 99, 23–39.
- [31] Zhang, Y., Yang, B., In vivo optimizing of intracellular production of heterologous protein in *Pichia pastoris* by fluorescent scanning. *Anal. Biochem.* 2006, 357, 232–239.
- [32] Chopda, V. R., Gomes, J., Rathore, A. S., Bridging the gap between PAT concepts and implementation: An integrated software platform for fermentation. *Biotechnol. J.* 2015, 11, 164–171.
- [33] Chopda, V. R., Rathore, A. S., Gomes, J., Maximizing biomass concentration in baker's yeast process by using a decoupled geometric controller for substrate and dissolved oxygen. *Biore-sour. Technol.* 2015, 196, 160–168.
- [34] Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976, 72, 248–254.
- [35] Lakowicz, J. R. (Ed.), *Principles of Fluorescence Spectroscopy* (third ed.), Springer-Verlag, Norwell, MA, 2006.
- [36] Samanta, A., Paul, B. K., Guchhait, N., Spectroscopic probe analysis for exploring probe–protein interaction: a mapping of native, unfolding and refolding of protein bovine serum albumin by extrinsic fluorescence probe. *Biophys. Chem.* 2011, 156, 128–139.
- [37] Rathore, A. S., Li, X., Bartkowski, W., Sharma, A. et al. Case study and application of process analytical technology (PAT) towards bioprocessing: Use of tryptophan fluorescence as at-line tool for making pooling decisions for process chromatography. *Biotechnol. Prog.* 2009, 25, 1433–1439.
- [38] Gull, N., Sen, P., Khan, R. H., Interaction of bovine (BSA), rabbit (RSA), and porcine (PSA) serum albumins with cationic single-chain/gemini surfactants: A comparative study. *Langmuir* 2009, 25, 11686–11691.
- [39] Hawe, A., Sutter, M., Jiskoot, W., Extrinsic fluorescent dyes as tools for protein characterization. *Pharm. Res.* 2008, 25, 1487–1499.

- [40] Ozimek, P., Veenhuis, M., Klei, I. J., Alcohol oxidase: A complex peroxisomal, oligomeric flavoprotein. *FEMS Yeast Res.* 2005, 5, 975–983.
- [41] Cos, O., Ramón, R., Montesinos, J. L., Valero, F., Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: A review. *Microb. Cell Fact.* 2006, 5, 17.
- [42] Marx, H., Mattanovich, D., Sauer, M., Overexpression of the riboflavin biosynthetic pathway in *Pichia pastoris*. *Microb. Cell Fact.* 2008, 7, 23.
- [43] Zhang, H., Loovers, H. M., Xu, L. Q., Wang, M. et al. Alcohol oxidase (AOX1) from *Pichia pastoris* is a novel inhibitor of prion propagation and a potential ATPase. *Mol. Microbiol.* 2009, 71, 702–716.
- [44] Baker, K. N., Rendall, M. H., Patel, A., Boyd, P. et al. Rapid monitoring of recombinant protein products: A comparison of current technologies. *Trends Biotechnol.* 2002, 20, 149–156.
- [45] Tohmola, N., Ahtinen, J., Pitkänen, J. P., Parviainen, V. et al. On-line high performance liquid chromatography measurements of extracellular metabolites in an aerobic batch yeast (*Saccharomyces cerevisiae*) culture. *Biotechnol. Bioprocess Eng.* 2011, 16, 264–272.
- [46] Mukherjee, A., Walker, J., Weyant, K. B., Schroeder, C. M., Characterization of flavin-based fluorescent proteins: an emerging class of fluorescent reporters. *PLoS One* 2013, 8, e64753.
- [47] Reischer, H., Schotola, I., Striedner, G., Pötschacher, F. et al. Evaluation of the GFP signal and its aptitude for novel on-line monitoring strategies of recombinant fermentation processes. *J. Biotechnol.* 2004, 108, 115–125.
- [48] Rhee, J. I., Kang, T. H., On-line process monitoring and chemometric modeling with 2D fluorescence spectra obtained in recombinant *E. coli* fermentations. *Process Biochem.* 2007, 42, 1124–1134.
- [49] Glassey, J., Measurement, monitoring, modelling and control of bioprocesses, in: Mandenius, C. F., Titchener-Hooker, N. J. (Eds.), *Advances in Biochemical Engineering/Biotechnology*, Springer, Berlin 2013. pp. 167–191.