Process Biochemistry 58 (2017) 51–59

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

On-line monitoring of relevant fluorophores of yeast cultivations due to glucose addition during the diauxic growth

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ARTICLE INFO

Keywords:
On-line monitoring
Saccharomyces cerevisiae
Biogenic fluorophores
2D Fluorescence spectroscopy
Principal component analysis

ABSTRACT

The relevant fluorophores in cells can be a key component to understanding cellular activities, which in turn explains states of cultivation processes. The autofluorescence inside microorganisms can be measured by 2D fluorescence spectroscopy which is an effective and non-invasive device for an on-line monitoring of bioprocesses. We detected the following intrinsic fluorophores which are part of metabolic pathways for yeast growth during fermentation in real-time: tryptophan, pyridoxine, NADH, riboflavin, FAD, and FMN. The changes of these intrinsic fluorophores were observed from the yeast cultivations under three conditions: (i) normal batch, (ii) glucose addition during glucose growth phase, and (iii) glucose addition during ethanol growth phase after a diauxic shift. The glucose addition during ethanol growth phase demonstrated the correlative changes of the fluorophores, which was a key component in the metabolic switch from ethanol to glucose growth phase. Additionally, the quantification of conversion between tryptophan and NADH was shown as a proportional factor. It was calculated from the ratio of the area of NADH and tryptophan fluorescence intensity after the glucose addition until depletion. The proportional factor was independent on various glucose concentrations with the coefficient of determination, R^2 = 0.999.

1. Introduction

An optimum processing is needed in the field of food, pharmaceutical, and biotechnological industry with the ultimate goal of achieving high productivity and high quality products. In order to achieve this goal, there are many different parameters to be realized and controlled, e.g., physical, chemical, and biological aspects of microbial bioprocesses. Microbial cultivations are such a complex process, therefore, reliable and efficient tools are required for an on-line monitoring to receive as much real-time information as possible, so that the processes can be controlled in time. On-line bioprocess monitoring has been studied and developed for many years. In 2002, the US Food and Drug Administration (FDA) launched the Process Analytical Technology (PAT) initiative to be applied in the process monitoring and effectively drove progresses in this field [1–5]. During the past decade, there were many investigations on in-line/on-line monitoring of bioprocesses by using various optical technologies, such as in situ microscopy, near-infrared (NIR), Raman, and fluorescence spectroscopy [6–15].

Fluorescence spectroscopy is one of the potential techniques for an on-line monitoring without any interfering processes, which reduces the risk of contamination in a bioreactor. Besides, it provides real-time information and bypasses the need to sampling data [15–20]. The fluorescence sensor is particularly effective for monitoring the autofluorescence inside cells. Two-dimensional (2D) or multi-wavelength fluorescence spectroscopy was developed to be able to measure biogenic fluorophores in a wider range of excitation and emission wavelengths [17,21,22]. Thus, the non-identified overlapping peaks and the quenching of different fluorophores can be detected by the 2D fluorescence spectroscopy. The sensor is not only applied in monitoring cultivation processes of *Saccharomyces cerevisiae*, but also in many different microorganisms, such as *Claviceps purpurea*, *Streptomyces coelicolor,* and *Escherichia coli* [14,23–26]. The on-line monitoring using 2D fluorescence spectroscopy can enhance the understanding of the diversity in biological systems. For example, Bhatta et al., 2006 applied fluorescence spectroscopy to differentiate species between yeast and bacteria and also between different strains of yeasts [27]. Another study used a fluorescence sensor to monitor growth and stress responses of yeast cells by observing changes of autofluorescence [28]. The fluorescence sensor cannot directly measure glucose and ethanol concentrations because they are not fluorescent. However, it can monitor the fluorescent molecules relating to cellular activities, such as nicotinamide-adenine dinucleotide (NADH), tryptophan, pyridoxine (vitamin B_6), riboflavin (vitamin B_2), flavin-adenine dinucleotide (FAD), and flavin mononucleotide (FMN) [15,16]. Many studies investigated the

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http://dx.doi.org/10.1016/j.procbio.2017.05.007
Received 9 February 2017; Received in revised form 3 April 2017; Accepted 8 May 2017
Available online 10 May 2017
1359-5113/

relation between intrinsic fluorophores and products or substrates. Then they used the fluorophores to estimate concentrations of cell mass, glucose, ethanol, and other products [14–25,29,30].

Cellular metabolism occurring in living microorganisms contains several metabolic pathways, such as glycolysis, the citric acid cycle (TCA cycle), and the electron transport chain. The intracellular fluorophores are key metabolic components for the metabolic pathways [22]. In different fermentation systems or conditions, the behavior of each biogenic fluorophore is different. Therefore, it is necessary to understand and recognize the change of intrinsic fluorophores during each phase of cultivations in order to find a relevant indicator of the metabolic switches. These significant indicators could be used to predict state variables and improve the cultivation process.

The objective of this study was to investigate relevant fluorophores under the three different cultivation conditions. The biogenic fluorophores, such as NADH, tryptophan, pyridoxine, riboflavin, FAD, and FMN, were monitored to observe their behaviors in different operations during the cultivations.

2. Materials and methods

2.1. Yeast strain, culture and fermentation conditions

S. cerevisiae (fresh baker's yeast, Oma's Ur-Hefe) was pre-cultivated before fermentation. 10 g fresh baker's yeast was inoculated into 100 mL Schatzmann medium, which consists of 0.34 g/L $MgSO_4{\cdot}7H_2O$, 0.42 g/L $CaCl_2{\cdot}2H_2O$, 4.5 g/L $(NH_4)_2SO_4$, 1.9 g/L $(NH_4)_2HPO_4$, and 0.9 g/L KCl [31]. The preculture was shaken for 10 min (180 rpm) and then was pumped into a 3-L stainless steel tank bioreactor (Minifors, Inifors HT, Bottmingen, Switzerland) with a working volume of 1.35 L. The medium used for the cultivations was the same as the one for the preculture, but with 10 g/L glucose, 1 mL/L trace elements solution (0.015 g/L $FeCl_3{\cdot}6H_2O$, 9 mg/L $ZnSO_4{\cdot}7H_2O$, 10.5 mg/L $MnSO_4{\cdot}2H_2O$, and 2.4 mg/L $CuSO_4{\cdot}5H_2O$), 1 mL/L vitamin solution (0.06 g/L myoinositol, 0.03 g/L Ca-pantothenate, 6 mg/L thiamine HCl, 1.5 mg/L pyridoxine HCl, and 0.03 mg/L biotin), and 200 µL/L antifoam. The yeast cultivations under three different conditions were run in triplicate. The first cultivation condition was a normal batch (Fig. 1A). The second condition was a cultivation with the glucose addition during the glucose growth phase at ca. 1.5 h. For the last condition, the glucose solution was also added, but during the ethanol growth phase at ca. 6 h. A 10 mL sample was regularly taken. Before the glucose addition at ca. 1.5 h, 4 samplings were taken as shown in Fig. 1B. Then 30 mL glucose feed solution was pumped into the bioreactor with the concentration of 1.35×10^2 g/L. Hence, the glucose concentration in the cultivation after feeding increased around 3.0 g/L. In the case of the glucose addition at ca. 6 h, 12 samplings were taken before adding as demonstrated in Fig. 1C. 30 mL of the glucose feed solution was pumped in as well, but with the different required glucose concentrations, i.e., 1.5, 3.0, 4.5, and 6.0 g/L, in the total medium. Thus, the glucose feed solution was provided with the various concentrations of 63, 1.26×10^2, 1.89×10^2, and 2.52×10^2 g/L, respectively. To realize the biomass and ethanol concentrations, 30 mL increasing from the remaining volume is less than 3%, therefore, the dilution factor was not considered in the theoretical models (in chapter 2.4). All cultivations were operated at a constant temperature, 30 °C and a maintained pH 5. The aeration and agitation rates were kept constant at 3.5 L/min and 430 rpm, respectively. Iris software (Inifors HT, Bottmingen, Switzerland) was applied as a process control system for the bioreactor.

2.2. Off-line analysis

Samples for analyzing concentrations of biomass, glucose, and ethanol were regularly taken from the bioreactor and put into pre-weighed and predried microcentrifuge tubes. Cell dry weight was determined by centrifugation (Universal 16 R, Hettich Zentrifugen GmbH & Co. KG, Tuttlingen, Germany) of a sample with 1.5 mL (2 times)

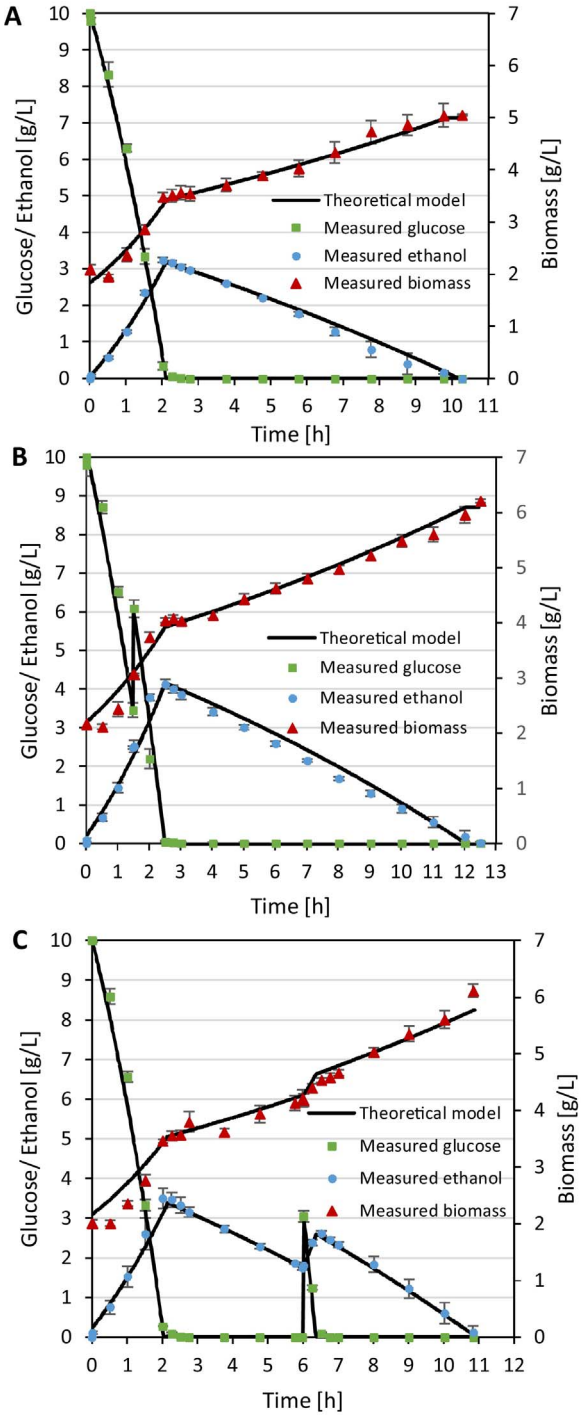

Fig. 1. The characteristics of the yeast cultivation with 10 g/L starting glucose and the theoretical models of the yeast cultivation in different conditions. (A) Normal batch. (B) Cultivation with glucose addition during GP and (C) during EP.

at 14,000 rpm for 10 min at 4 °C. The wet cells were let in a drying oven at 103 °C for 24 h. Subsequently, they were cooled down for 30 min before weighing. The supernatant of the samples after the centrifugation was examined by HPLC (ProStar, Variant, Walnut Creek, CA, USA) to determine the glucose and ethanol concentrations. Firstly, the supernatant was filtrated with pore size filter, 0.45 µm, polypropylene membrane (VWR, Darmstadt, Germany), then 20 µL was injected into a Rezex ROA-organic acid H+ (8%) column (Phenomenex, Aschaffenburg, Germany) and operated at 70 °C with 5 mM H_2SO_4 as an eluent at 0.6 mL/min flow rate. The concentrations of glucose and ethanol were calculated by Galaxie software(Varian, Walnut Creek, CA, USA).