Appl Biochem Biotechnol (2009) 157:237–243
DOI 10.1007/s12010-008-8395-9

On-line Characterization of Physiological State in Poly(β-Hydroxybutyrate) Production by *Wautersia eutropha*

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Received: 13 February 2008 / Accepted: 6 October 2008 /
Published online: 29 October 2008

Abstract Culture fluorescence measurement technique has the potential for *on-line* characterization of metabolic status of fermentation processes. Many fluorophores present inside the living cells such as NADH + H^+, tryptophan, pyridoxine, and riboflavin fluoresce at specific excitation and emission wavelength combinations. Since these key intracellular metabolites are involved in cell growth and metabolism, their concentration change at any time inside the cell could reflect the changes in cell metabolic activity. NADH + H^+ spectrofluorometry was used for on-line characterization of physiological state during batch cultivation of poly-β-hydroxybutyric acid (PHB) production by *Wautersia eutropha*. The culture fluorescence increased with an increase in the biomass concentration with time. A linear correlation between cell mass concentration and net NADH + H^+ fluorescence was established during active growth phase (13 to 38 h) of batch cultivation. The rate of change of culture fluorescence (d*F*/d*t*) exhibited a gradual increase during the predominantly growth phase of batch cultivation (till 20 h). Thereafter, a sudden drop in the d*F*/d*t* rate and its leveling was recorded indicating major changes in culture metabolism status which synchronized with the start-up of accumulation of PHB. After 48 h, yet another decrease in the rate of change of fluorescence (d*F*/d*t*) was observed primarily due to severe substrate limitation in the reactor. On-line NADH + H^+ fluorescence signal and its rate (d*F*/d*t*) could therefore be used to distinguish the growth, product formation, and nutrient depletion stage (the metabolic state marker) during the batch cultivation of *W. eutropha*.

Keywords PHB · *Wautersia eutropha* · NADH + H^+ · Fluorescence · Batch cultivation

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Introduction

Synthetic polymers were initially developed to enhance the quality and comfort of life. These became an essential part of life because of their inherent properties of durability, strength, lightness, permeability, transparency, and cost. However, these desirable qualities of durability and resistance to degradation have made plastic materials a source of environmental hazard and invite waste management problems. Poly-β-hydroxybutyric acid (PHB) is a biodegradable polymer, which is accumulated as an energy reserve material by a large number of bacteria when nutrients such as nitrogen or phosphorous sources are available in limiting concentrations in the presence of excess carbon source [1]. PHB is considered to be a strong candidate as a replacement to conventional plastics because it possesses material properties similar to various synthetic thermoplastics currently in use (polypropylene, synthetic rubber, etc.) and is completely degraded upon disposal by microorganisms in various environments such as soil, sea, lake water, and sewage [2]. The major problem in large-scale production of PHB is the high cost of production. The cost can be reduced by development of high-yielding strains and better bioreactor cultivation approaches. However, the quality and quantity of the PHB can be improved by employing efficient (preferably *on-line*) process control strategies. Culture fluorescence measurement technique is one such technique which has the potential for on-line characterization of metabolic status of fermentation processes. Many fluorophores like reduced pyridine nucleotides (NADH) present inside the living cells fluoresce at specific excitation and emission wavelengths combinations. The reduced pyridine nucleotides are the primary suppliers of reducing power to the catabolic and anabolic pathways in the cells. Since these key intracellular cofactors play an important role in cell growth and metabolism, their concentration change at any time inside the cell could reflect the changes in cell metabolic activity. Among the various fluorophores like NADH, tryptophan, pyridoxine, and riboflavin, NADH is the most important substance because of its central position in metabolic pathways. Intracellular NADH + H^+ can be measured due to its fluorescent properties. NADH + H^+ absorbs light peaking at 340 nm and emit it at a longer wavelength (fluoresce) with a maximum at 460 nm. Fluorometric determination of NADH + H^+ is a reasonably known technique. Application of NADH + H^+ fluorescence measurements for indirect biomass (cell concentration) estimations has been reported with the assumption that under balanced growth (i.e., when there are no environmental changes and the cells grow exponentially), the total NADH + H^+ content in the cells in the bioreactor is proportional to the biomass concentration. The first application of this technique was demonstrated in 1957 [3] when NADH + H^+ fluorescence was measured in the whole cells. Later, this technique was applied to continuous cultures of microorganisms to reflect intracellular NADH + H^+ [4]. Cellular NADH + H^+ level has become a popular *in situ* indicator of cellular mass and/or metabolic state of cells [4–8]. This compound has been conveniently measured through on-line fluorescence for various microbial systems for indirect *in situ* assessment of biomass [9] and establishment of physiological state [10–12]. Thus, fluorescence measurement of cell broth at different excitation wavelengths using non-invasive optical sensing can be used for on-line monitoring of many key physiological parameters of a fermentation process. The optical nature of this fluorescence technique eliminates the time lag in the measurements of the critical intracellular parameters without even taking the sample. Also, “fluorescence” has an additional advantage of monitoring intracellular activities of the cells rather than just the environment around them as is done by normal pH and dissolved oxygen sensors. However, agitation, aeration, pH, dissolved oxygen concentration, and temperature are

important bioprocess operation parameters which are known to affect culture fluorescence signals [13]. Thus, in order to derive meaningful correlations of cell metabolism with culture fluorescence, these environmental parameters should be carefully controlled during the fermentation process. Present study involved the use of NADH + H^+ fluorescence monitoring in the bioreactor during growth and PHB production by *Wautersia eutropha*. The major aim was to develop a correlation between the on-line fluorescence signal and biomass and preferably identify yet another physiological marker for on-line indication of product (PHB) accumulation.

Materials and Methods

Microorganism and Medium

W. eutropha NRRL B14690, procured from Northern Regional Research Laboratory, Peoria, USA, was cultivated in a mineral salt medium as optimized in our earlier studies [1]. The strain was maintained on nutrient agar slants at 5°C and subcultured monthly.

Culture Conditions

Seed culture was prepared in optimized media (urea, 1.0 g/L; corn steep liquor, 0.5 g/L; KH_2PO_4, 1.5 g/L; Na_2HPO_4, 4.0 g/L; $MgSO_4{\cdot}7H_2O$, 0.51 g/L; $CaCl_2$, 0.02 g/L; trace metal solution, 10 mL/L) with 10 g/L fructose. The organism was cultivated under rotational agitation (Adolf Kuhner, Germany) at 150 rpm and 30°C for 24 h in a 1-L Erlenmeyer flask containing 200 mL media. Batch cultivation was carried out at 30°C in a 15-L, Biostat C (B. Braun, Germany) bioreactor containing 5.5 L optimized media with 40 g/L fructose. The reactor was equipped with three conventional turbine-type impellers and four baffles. The fluorescence probe was inserted in one of the standard 25 mm ports of the reactor. The reactor was sterilized *in situ* at 121°C for 20 min, cooled, and then inoculated with 5% inoculum (*v*/*v*). Culture pH was maintained at 7.0 by automatic addition of 2 N NaOH/2 N HCl. Dissolved oxygen concentration was maintained at 20% saturation value by manually adjusting the speed of the agitator and/or flow rate of sterile air. The dissolved oxygen concentration in the fermentor was measured using an *in situ* Ingold (Ingold, Switzerland) dissolved oxygen probe. The dissolved oxygen concentration was measured as % pO_2. Samples were withdrawn at an interval of 3–4 h for the analysis of biomass, PHB, and fructose.

On-line NADH + H^+ fluorescence was measured by NADH + H^+ fluorescence probe in terms of arbitrary fluorescence units (AFU; Varian, VIPL 3120 Hansen Way Palo Alto, California 94304. USA). The experimental setup for the present study is shown in Fig. 1. Sampling was done every 3–4 h from the bioreactor for the estimation of biomass (dry cell weight), PHB produced, and fructose and nitrogen consumed during the fermentation process. A correlation was established between the respective biomass and the corresponding net NADH + H^+ fluorescence (observed NADH + H^+ fluorescence− medium fluorescence) produced for each sampling point during the entire batch cultivation.

Analytical Methods

Optical density (OD) of the suitably diluted cell suspension at 600 nm was measured in a UVIKON 930 Spectrophotometer (Kontron Instruments, USA). Cell mass concentration

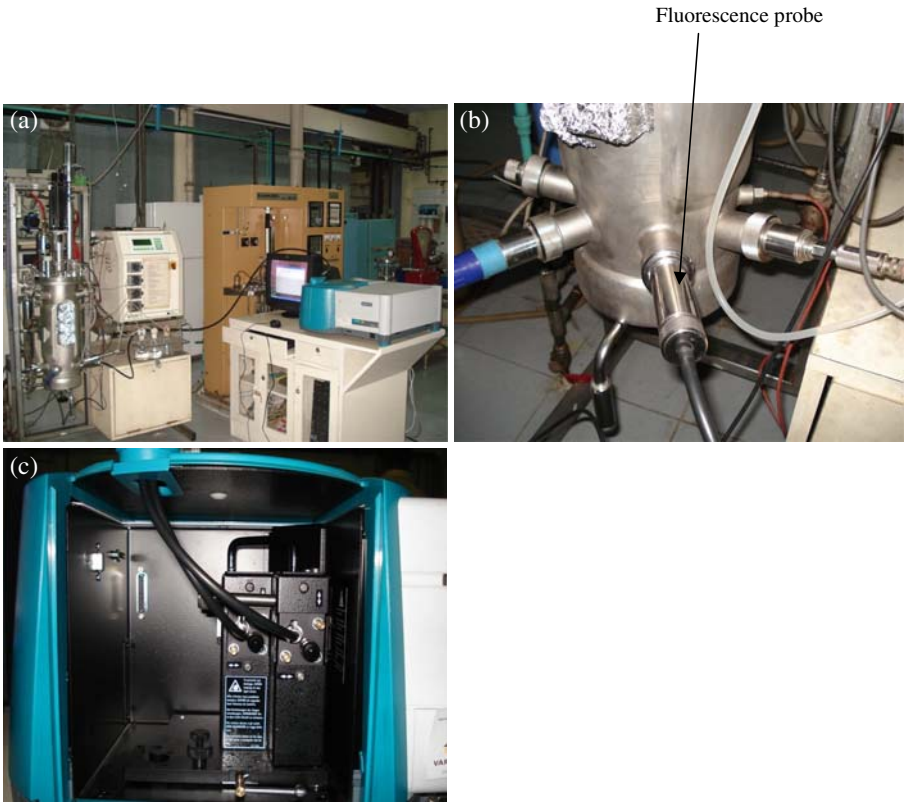

Fig. 1 Batch cultivation of *W. eutropha* in a 15-L bioreactor with fluorescence probe for measuring on-line NADH + H^+ fluorescence. **a** Schematic of reactor with fluorescence probe. **b** Probe (*arrow*) inside the reactor. **c** Fluorescence measuring assembly with probe connections

was determined by standard plot between $OD_{600\ nm}$ and dry cell mass. The supernatant obtained by centrifugation (Sorvall RC5B Centrifuge) of the culture broth at 10,000 rpm (8,944×*g*) for 10 min at 4°C was used for residual substrate analysis. The estimation protocols for PHB, nitrogen and fructose were as reported earlier [14].

Result and Discussion

Batch Cultivation with Fluorescence Probe

Batch cultivation with the NADH + H^+ fluorescence probe was carried out for 60 h. The batch growth kinetics, NADH + H^+ fluorescence, and PHB production profiles with respect to fructose and nitrogen consumption during the batch cultivation of *W. eutropha* cells in the bioreactor can be seen in Fig. 2a. A total biomass of 17.7 g/L was obtained in 60 h of cultivation. The active biomass (total biomass−PHB) leveled off after 29 h (Fig. 2a) due to the limitation of nitrogen. The limitation of nitrogen and presence of fructose resulted in active production of PHB. Maximum PHB production of 8.7 g/L was obtained towards the end of the cultivation (60 h). An increasing trend was observed between biomass production and on-line NADH + H^+ fluorescence till the culture reached the stationary phase (13 to

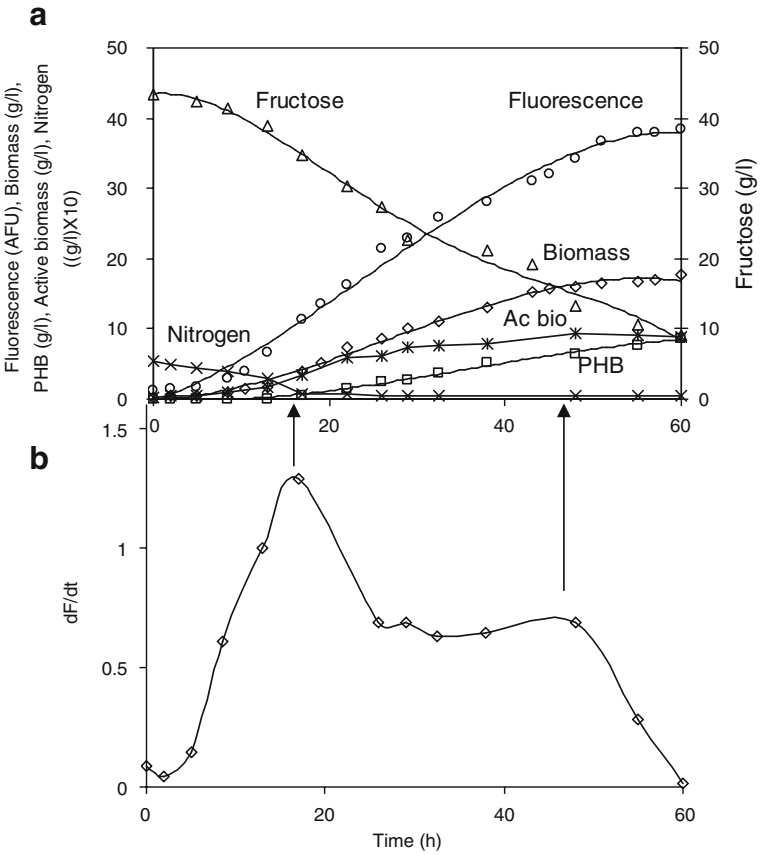

Fig. 2 **a** Batch kinetics of *W. eutropha* in a 15-L bioreactor (*open circles* net NADH + H^+ fluorescence; *open squares* PHB; *open triangles* fructose; *open diamonds* biomass; *X mark* nitrogen; *asterisk* active biomass). **b** Correlation between the rate of change of culture fluorescence (d*F*/d*t*) with time. Decrease in d*F*/d*t* at 20 h indicated a shift in metabolism towards PHB synthesis. Change at 48 h, synchronized with substrate depletion

38 h). A plot between the net NADH + H^+ fluorescence and total biomass during 13 to 38 h cultivation resulted in a linear relationship with a correlation coefficient of 0.98 (Fig. 3).

The synthesis of PHB has been reported to vary directly with the intracellular concentrations of acetyl-CoA and NADH + H^+ [2]. The first two enzymes of the PHB synthetic pathway (acetyl-CoA acyl transferase and acetoacetyl-CoA reductase) are invariably inhibited by high intracellular concentrations of NAD^+ [15]. An enhanced intracellular accumulation of NADH + H^+ is required for the synthesis of PHB. Therefore, the increase in the intracellular NADH + H^+ concentration can possibly be monitored by following the variation in rate of change of cellular fluorescence with time.

An attempt was made to develop a correlation between the rate of change of culture fluorescence (d*F*/d*t*) and the different metabolic activities (growth phase, start of PHB accumulation, etc; Fig. 2b). The rate (d*F*/d*t*) is a parameter which is observable on-line, and therefore, its variation provides an on-line, *in situ* signal to elucidate the metabolic events to some extent. The rate of change of culture fluorescence (d*F*/d*t*) was found to increase

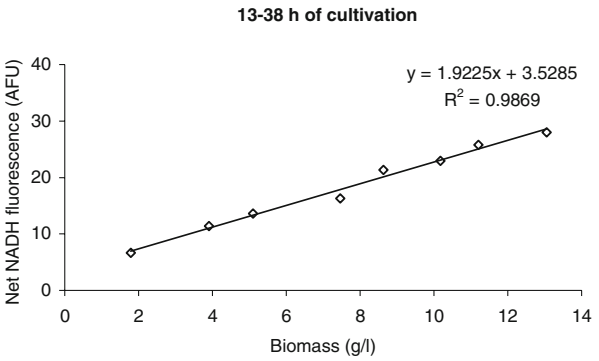

Fig. 3 Correlation between cell concentration (g/L) and on-line NADH + H^+ fluorescence of the culture measured during the batch kinetic study. A linear correlation was obtained between the biomass concentration (g/L) and net NADH + H^+ fluorescence (AFU) values during the log phase (13–38 h) of the batch cultivation (Y=1.92X+3.5; X, biomass (g/L); Y, net NADH + H^+ fluorescence). R=0.98

during the active growth phase of cells till 18 h. The first change was observed at 20 h, which synchronized with accumulation of PHB inside the cell. Physiological state change of cells (start-up of accumulation of PHB) from 20 h was reasonably well reflected by on-line peak of dF/dt and significant decrease later on. This featured reduced growth rate and accumulation of PHB in cells indicating a decrease in dF/dt rate. The relatively constant values of dF/dt (during 25–48 h) reflected no increase in actively growing compound (total biomass−PHB) of the cell (Fig. 3) as also demonstrated by constant values of active biomass values in graph. Another change was reflected at 48 h which somehow indicated the disappearance of the nutrients. As seen in Fig. 2b, the fructose limitation started at around 48 h, after which the rate of change of fluorescence (dF/dt) sharply decreased primarily due to fructose limitation leading to decreased rate of PHB accumulation in the reactor. The dF/dt value of zero at 60 h indicated the leveling of PHB accumulation towards the end of cultivation primarily due to nonavailability of substrate in the reactor.

The on-line NADH + H^+ fluorescence measurement was a function of the biomass concentration during 13 to 28 h (balanced growth phase) of batch cultivation. Therefore, raw fluorescence data can be utilized as a marker for the biomass concentration during the cultivation only in the growth phase for *W. eutropha*. However, dF/dt changes significantly throughout the cultivation due to different metabolic activities (active exponential growth, PHB accumulation, depletion of substrate, etc) of the cells. Therefore, ΔF and d(ΔF)/dt will reflect the biomass formation and/or product accumulation during different times of the cultivation and therefore is an important on-line indicator for process control strategies.

Conclusion

The potential of on-line NADH + H^+ fluorescence monitoring for indirect estimation of biomass and its metabolic activity during PHB production in bioreactors was established. A linear correlation (Y=1.92X+3.5; X, biomass (g/L); Y, net NADH + H^+ fluorescence) between the biomass (g/L) and corresponding culture (NADH + H^+) fluorescence was obtained during the log phase (13–38 h) of the batch kinetic study. The rate of change of culture fluorescence (dF/dt) was found to increase during the active growth phase of cells (0–18 h) and peaked at 20 h, which coincided with the beginning of the product formation (PHB accumulation) phase. dF/dt rate remained constant during 25–48 h when there was not much increase in active biomass formation. The (dF/dt) rate started decreasing sharply from 48 h. This reflected (on-line) that the limitation of fructose had started.

References

1. Khanna, S., & Srivastava, A. K. (2005). *Process Biochemistry*, *40*, 609–619.
2. Lee, S. Y. (1996). *Biotechnology and Bioengineering*, *49*, 1–14. doi:10.1002/(SICI)1097-0290(19960105)49:1<1::AID-BIT1>3.0.CO;2-P.
3. Duysens, L. N. M., & Amesz, J. (1957). *Biochimica et Biophysica Acta*, *24*, 19–26. doi:10.1016/0006-3002(57)90141-5.
4. Harrison, D. E. F., & Chance, B. (1970). *Applied Microbiology*, *19*, 446–450.
5. Groom, C. A., Luong, J. H. T., & Mulchandani, A. (1988). *Journal of Biotechnology*, *8*, 271–278. doi:10.1016/0168-1656(88)90019-3.
6. Kwong, S. C. W., Randers, L., & Rao, G. (1992). *Biotechnology Progress*, *8*, 410–412. doi:10.1021/bp00017a006.
7. Farabegoli, G., Hellinga, C., Heijnen, J. J., & van Loosdrecht, M. C. (2003). *Water Research*, *37*, 2732–2738. doi:10.1016/S0043-1354(03)00064-2.
8. Franz, C., J rgen, K., Florentina, P., & Karl, B. (2005). *Journal of Biotechnology*, *120*, 183–196. doi:10.1016/j.jbiotec.2005.05.030.
9. Scheper, T., Gebauer, A., & Schugerl, K. (1987). *Chemical Engineering Journal*, *34*, 7–12. doi:10.1016/0300-9467(87)85009-8.
10. Srivastava, A. K., & Volesky, B. (1991). *Applied Microbiology and Biotechnology*, *34*, 450–457. doi:10.1007/BF00180569.
11. Srivastava, A. K., & Volesky, B. (1991). *Biotechnology and Bioengineering*, *38*, 191–195. doi:10.1002/bit.260380211.
12. Sriram, G., & Sureshkumar, G. K. (2001). *Process Biochemistry*, *36*, 1167–1173. doi:10.1016/S0032-9592(01)00151-0.
13. Li, J., & Humphrey, A. E. (1990). *Biotechnology and Bioengineering*, *37*, 1043–1049. doi:10.1002/bit.260371109.
14. Khanna, S., & Srivastava, A. K. (2005). *Biotechnology Progress*, *21*, 830–838. doi:10.1021/bp0495769.
15. Zinn, M., Witholt, B., & Egli, T. (2001). *Advanced Drug Delivery Reviews*, *53*, 5–21. doi:10.1016/S0169-409X(01)00218-6.