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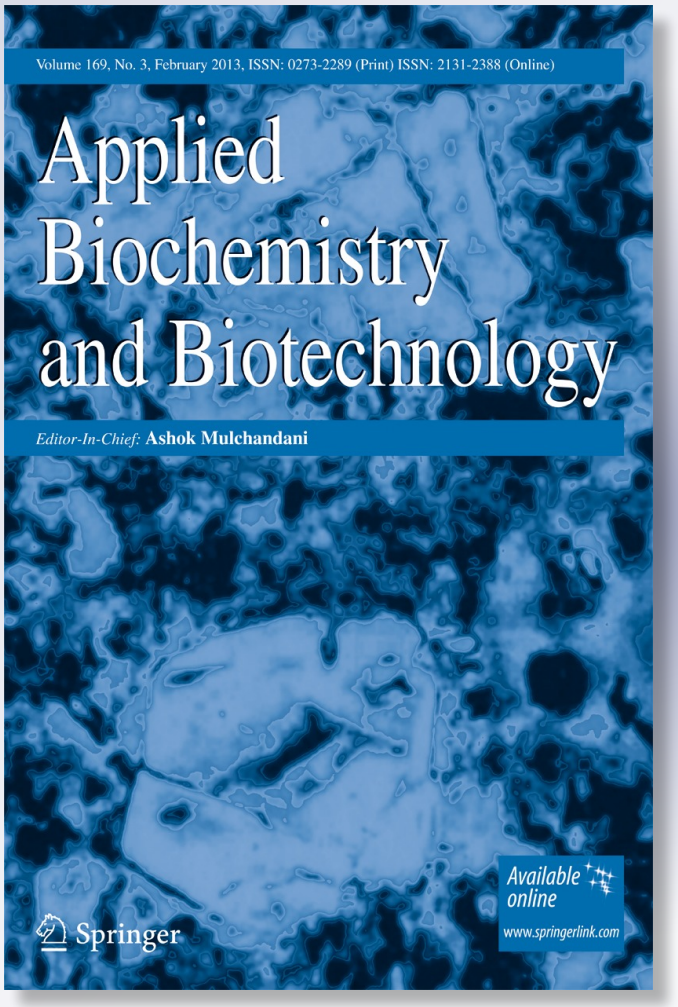

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Abstract Culture fluorescence measurement is an indirect and non-invasive method of biomass estimation to assess the metabolic state of the microorganism in a fermentation process. In the present investigation, NAD(P)H fluorescence has been used for on-line in situ characterization of metabolic changes occurring during different phases of batch cultivation of *Azohydromonas australica* in growth associated poly(3-hydroxybutyrate) or PHB production. A linear correlation between biomass concentration and net NAD(P)H fluorescence was obtained during early log phase (3–12 h) and late log phase (24–39 h) of PHB fermentation. After 12 h (mid log phase) cultivation PHB accumulation shot up and a drop in culture fluorescence was observed which synchronously exhibited continuous utilization of NAD(P)H for the synthesis of biomass and PHB formation simultaneously. A decrease in the observed net fluorescence value was observed again towards the end of fermentation (at 39 h) which corresponded very well with the culture starvation and substrate depletion towards the end of cultivation inside the bioreactor. It was therefore concluded that NAD(P)H fluorescence measurements could be used for indication of the time of fresh nutrient (substrate) feed during substrate limitation to further enhance the PHB production.

Keywords Poly (3-hydroxybutyrate) · *Azohydromonas australica* · NAD(P)H fluorescence · Bioprocess monitoring · Bioreactor · Batch fermentation

Introduction

Poly (3-hydroxybutyrate) or PHB is a natural biodegradable polymer which is produced by various microorganisms intracellularly as an energy and carbon reserve material [1, 2]. This biopolymer can be used as a replacement to conventional plastics because it possesses physico-mechanical properties similar to petro-chemically derived polymers [3]. Apart from biodegradability, PHB also has various interesting properties such as biocompatibility,

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thermoprocessability, piezoelectricity, and nonlinear optical activity with many potential applications particularly in newly emerging areas such as drug delivery, nerve tissue engineering, bone repair, and coating of seeds of plants for pesticides delivery in agriculture [4]. In spite of all these advantages offered by PHB, the main bottleneck in its large-scale production is its high cost of production. The high production cost of PHB can be minimized by using inexpensive substrates, high-yielding strains, and improved process control strategies.

The biosynthesis of PHB is a three-step process involving three different enzymes namely β-ketothiolase (or acetyl-CoA acyl transferase), NAD(P)H-dependent acetoacetyl-CoA reductase and PHB synthase. β-ketothiolase catalyzes the condensation of two molecules of acetyl-CoA to acetoacetyl-CoA which is then reduced to 3-hydroxybutyryl-CoA by the NAD(P)H-dependent acetoacetyl-CoA reductase. Finally, 3-hydroxybutyryl-CoA is polymerized into PHB using PHB synthase [1, 2, 5]. The biosynthesis of PHB has been reported to vary directly with the intracellular concentrations of NADH and acetyl-CoA (key precursor of PHB synthesis) [1]. During nutrient limitation, high intracellular concentrations of NADH inhibit the key regulatory enzymes of tricarboxylic acid cycle (TCA cycle) resulting in slow down of TCA cycle and accumulation of acetyl CoA. Accumulation of acetyl CoA and hence reduction in free Co-A level activates the β-ketothiolase enzyme of the PHB biosynthetic pathway and initiates PHB synthesis [4].

In the present investigation *Azohydromonas australica* was utilized for PHB production because it produces PHB in a growth-associated manner, grows fast, and can accumulate high amounts of PHB (up to 80 % of cell dry weight) during growth phase of cultivation which could improve the PHB yield on the carbon source and efficiency of PHB recovery process [6, 7]. Moreover, *A. australica* is able to utilize inexpensive carbon source, sucrose thereby indicating the possible utilization of various renewable resources rich in sucrose such as beet molasses, cane molasses, and maple sap for PHB production [8–10]. In addition to high PHB accumulation rates (offered by *A. australica*) required for improvement in PHB production research efforts are also needed to minimize the production cost using improved and effective on-line process control and optimization strategies. One of the major limitations in fermentation process control is effective monitoring of the metabolic state of the culture. For the development of improved and efficient on-line monitoring and process control strategies there is a desperate requirement of reliable on-line sensing devices which can describe the metabolic activities of the culture inside the bioreactor [11]. There are many important physiological state markers inside the living cells such as NADH, NADPH (collectively referred as NAD(P)H), DNA, RNA, and tryptophan which are directly involved in cell growth and metabolism, hence their concentration changes at any time inside the cell would reflect different physiological changes occurring in a cell population [12]. Intracellular reduced pyridine nucleotides (NADH and NADPH) are the major suppliers of electrons in catabolic and anabolic processes of the cell and have similar fluorescent properties with a specific absorption and emission maximum wavelength of 340 and 460 nm, respectively. However, oxidized form (NAD^+ and $NADP^+$) of these nucleotides or coenzymes are non-fluorescent. Therefore any physiological changes which affect the ratio of reduced and oxidized pyridine nucleotides inside the cell will be reflected by the on-line NAD(P)H fluorescent signal.

Ever since the discovery of on-line fluorescence measurement technique for the estimation of biomass in suspensions of baker's yeast and alga by Duysens and Amesz [13], there have been various attempts in literature for establishing its potential in characterization of biomass and physiological status of the cell during different fermentation bioprocesses [14–16]. In the present study attempt has been made to investigate the use of NAD(P)H culture

fluorescence for on-line monitoring of biomass and growth-associated PHB production by *A. australica* in which no reports are available so far. The main aim of the study was to establish a correlation between net NAD(P)H fluorescence and biomass concentration during batch cultivation of *A. australica* and on-line, in situ establishment of culture metabolic activities which can be extremely useful for bioprocess control and process optimization particularly in the identification of the appropriate time of nutrient (or substrate) feed during substrate limitation and/or fed-batch cultivation for the enhanced PHB production.

Materials and Methods

Microorganism and Growth Medium

A growth-associated PHB producer, *A. australica* DSM 1124 obtained from German Collection of Microorganisms and Cell Cultures (DSMZ, Germany) was used in the present investigation. The strain was maintained on nutrient agar slants at 4 °C and subcultured monthly.

In the present investigation statistically optimized media recipe (Table 1) was used for cell growth and PHB production [17]. The statistical optimization of media was done by Plackett-Burman and Central Composite Design protocol using Design Expert (version 5.0.9) software (Stat-Ease Corporation, USA). Trace metal solution consisted of: 6 g/L ammonium Fe (III) citrate, 10 g/L $CaCl_2 \cdot 2H_2O$, 0.3 g/L H_3BO_3, 0.2 g/L $CoCl_2 \cdot 6H_2O$, 0.1 g/L $ZnSO_4 \cdot 7H_2O$, 0.03 g/L $MnCl_2 \cdot 4H_2O$, 0.03 g/L $Na_2MoO_4 \cdot 2H_2O$, 0.02 g/L $NiSO_4 \cdot 7H_2O$, and 0.01 g/L $CuSO_4 \cdot 5H_2O$. Phosphate solution was autoclaved separately to prevent precipitation and trace metal solution was sterilized by filtration. All the medium components were mixed aseptically under laminar flow hood before inoculation. Medium pH was adjusted aseptically to 7.0 using 2 N NaOH/HCl.

Inoculum Preparation for Bioreactor

Fifty milliliters sterile medium (described above) was taken in 250-mL conical flask and inoculated aseptically with two loop full of culture grown on nutrient agar slant. The conical flasks were kept in an orbital shaker at 200 rpm and 33 °C (for 48 h) for culture inoculum development. Before the operation of the bioreactor, another inoculum adaptation step was performed by transferring the growing culture (5 % *v*/*v*) into 1-L flasks containing 200 mL media. The flask cultivation was continued for 24 h till log phase was reached and actively growing culture was used to inoculate the bioreactor.

Table 1 Statistically optimized medium recipe used for growth associated PHB production [17]

Components	Optimized concentration (g/L)
Sucrose	25
$(NH_4)_2SO_4$,	2.8
$MgSO_4$	0.20
KH_2PO_4	3.25
Na_2HPO_4	3.25
Trace metal solution	1.5 (mL/L)

NAD(P)H Fluorescence-Based Batch Cultivation

Batch cultivation of *A. australica* was carried out in a custom-designed 8-L stainless-steel stirred tank bioreactor (working volume 5 L) under controlled environmental conditions of pH, temperature, aeration, and agitation rates. Culture fluorescence was measured in situ using NAD(P)H fluorescence probe (Ingold, Switzerland) inserted into the bioreactor through a 25-mm slot for probe (clearly shown in Fig. 1). The Ingold Type Fluorosensor used in the present study serves for the on-line determination of fluorescent substances (NADH and NADPH only) in chemical and biotechnological processes [18]. The absorption and emission wavelength of light (340 and 460 nm, respectively) have been specifically chosen in this probe for the detection and measurements of fluorescence due to nicotinamide adenine dinucleotides in reduced state. Therefore this probe selectively captures fluorescence due to NAD(P)H only. Agitation in the reactor was carried out by a conventional flat blade turbine-type impeller. The temperature was controlled at 33 °C using chilled water circulator unit (Julabo FP50, Germany). The reactor (along with the probe) was sterilized at 121 °C for 40 min, cooled, and then inoculated with 5 % inoculum (*v*/*v*). Medium pH was maintained at 7.0 by automatic addition of 2 N NaOH/HCl. Air was sparged from the bottom of the reactor using perforated stainless steel L-shaped sparger. The aeration rate and agitation speed in the bioreactor were kept constant at 2.0 vvm and 250 rpm, respectively. The dissolved oxygen concentration in the bioreactor was measured by an in situ dissolved oxygen probe (Applisens, The Netherlands). On-line NAD(P)H fluorescence was measured in terms of normalized fluorescence units (NFU) using NAD(P)H fluorescence probe which was calibrated using quinine sulfate which have been reported to have same fluorescent properties as NAD(P)H [19]. Fermentation samples were collected at an interval of 3 h and

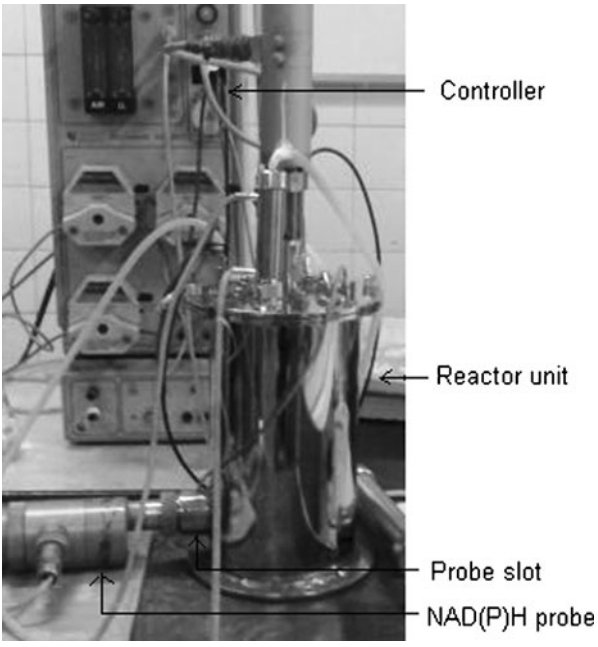

Fig. 1 Experimental set-up of 8-L capacity reactor with NAD(P)H fluorescence probe used for online NAD (P)H fluorescence measurement during batch-cultivation of *A. australica*

analyzed for biomass, PHB, and sucrose. NAD(P)H fluorescence-based batch experiments were done in duplicates to confirm the reproducibility of the fermentation process.

Analytical Methods

The optical density of the samples was monitored spectrophotometrically at 600 nm (OPTIZEN model 3220UV, Mecasys, Korea) after suitable dilution of fermentation broth. Biomass (or cell concentration) was calculated by a standard plot between OD_{600nm} vs. dry cell weight (DCW). Culture samples were centrifuged at 10,000 rpm for 15 min at 4 °C and the supernatant was used to determine residual sucrose concentration. Residual sucrose concentration in an appropriately diluted fermentation sample was measured by the dinitrosalicylic acid method [20]. The cell pellet (obtained after centrifugation) was dried at 90 °C in a hot air oven and DCW was calculated. PHB was analyzed by gas chromatography (GC 2010 Shimadzu Co., Japan) using benzoic acid as an internal standard [21]. Each PHB sample was injected three times into the column and amount of PHB was determined from averaged values in the present study.

Results and Discussion

The batch kinetics of PHB fermentation was independently studied in detail (in a 7-L lab-scale bioreactor) under controlled environmental conditions before carrying out NAD(P)H fluorescence-based cultivation in a custom-made bioreactor [17]. The batch PHB production kinetics study helped in estimation of key kinetic parameters such as specific growth rate, PHB productivity, and PHB yield and content which could then be related to NAD(P)H fluorescence measurements in a more lucid manner. The batch cultivation featured accumulation of 8.71 g/L biomass and 6.24 g/L PHB concentration with a maximum PHB production rate of 0.75 g/h. The maximum specific growth rate (μ_{max}) of the culture was observed to be 0.38 h^{-1} which meant culture grew at faster rates. At the end of fermentation, cells were harvested from the reactor and the metabolic products of PHB fermentation were analyzed by gas chromatography. Upon GC analysis, PHB was found to be the main product of fermentation without any by-products from *A. australica*. Therefore in the absence of any other by-products it was concluded that biomass and PHB were the main or only products of PHB fermentation process and any changes (increase or decreases) in the observed NAD(P)H fluorescence signal could be qualitatively related to biomass (including cellular proteins and enzymes) and PHB concentrations only. To the best of our knowledge from literature survey it was observed that PHB is the main metabolite or product of sucrose fermentation produced by this bacterium [1, 10, 22–24]. Moreover, a significantly high PHB content of 72 % of dry cell weight was accumulated inside the cells at the end of fermentation which was close to the maximum theoretical content (as high as 80 % of cell dry weight when grown on sucrose) of *A. australica* [7, 24] and therefore it further confirmed the formation of PHB only along with the biomass formation.

The growth and product formation kinetics along with corresponding NAD(P)H fluorescence observations were then established in an 8-L batch bioreactor cultivation. Since observed fluorescence is strongly dependent on variations in temperature, pH, and agitation rate, the batch cultivation was carried out under controlled environmental conditions (as mentioned above) in order to eliminate the possible effect of environmental changes on the observed fluorescence. However, the medium do contain certain fluorescent compounds (such as growth factors) which could affect the overall culture fluorescence, therefore the

contribution of medium fluorescence was subtracted from the observed culture fluorescence to calculate the net NAD(P)H fluorescence ($|\Delta F|$). It was observed that the background fluorescence of the culture broth (supernatant) remains more or less constant at each point of time with respect to the changes observed in the total fluorescence. The same fact has already been reported by Reardon et al. [25] and Srivastava and Volesky [26]. Thus the net fluorescence reflects the observed fluorescence minus medium fluorescence, i.e., $|\Delta F|$=observed−medium fluorescence. Figure 2 illustrates typical time course of the batch cultivation of *A. australica* in the bioreactor which could be divided into three phases namely lag phase, 0–3 h; log phase, 3–39 h; and stationary phase or substrate depletion stage, 39–48 h. Experimental data points in Fig. 2 shows the average values of biomass, PHB, and sucrose at different time intervals. From Fig. 2, it becomes clear that after a lag phase of 3 h, the culture starts growing which was observed by the increase in biomass concentration and increase in net NAD(P)H fluorescence. A correlation was established between respective process variables (biomass and PHB) and the corresponding net NAD(P)H fluorescence throughout the batch cultivation and different phases of the cultivation were distinctly described by the fluorescence profile.

As seen in Fig. 2, the entire exponential growth phase could be divided into three regions: region I (3–12 h), region II (12–24 h), and region III (24–39 h). The early log phase of the cultivation (region I) showed an increase in the net NAD(P)H fluorescence. A linear correlation between biomass concentration and net NAD(P)H fluorescence was observed during early log phase (3 to 12 h) of the cultivation. During this period a relatively slower increase in PHB accumulation was observed and biomass concentration (X) and net NAD(P)H fluorescence ($|\Delta F|$) were correlated by the following linear correlation: $|\Delta F|=24.3X+3.45$ ($R^2=0.99$) as shown in Fig. 3a. A dip in net fluorescence was observed just after 12 h which continued till 24 h reflecting change in physiological state of cells arising out of rapid exponential growth along with faster accumulation of PHB (as depicted in Fig. 2). Thus a

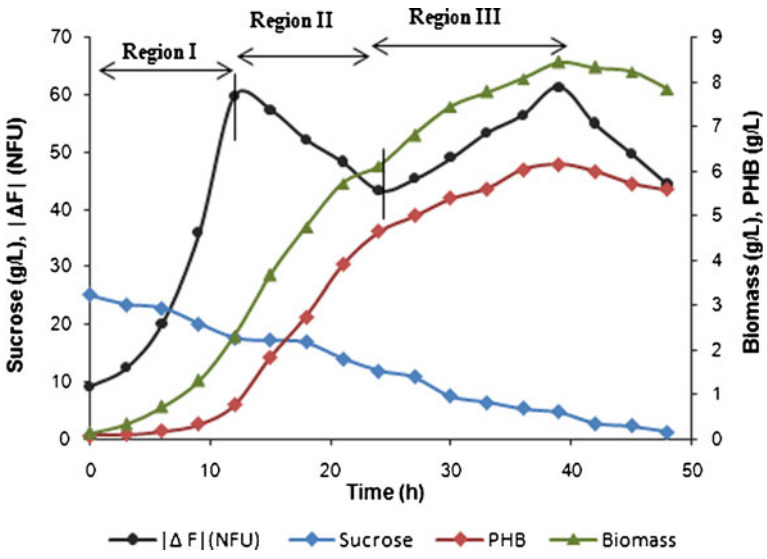

Fig. 2 The batch kinetics profile of *A. australica* in the bioreactor depicting correlation between respective process variables (biomass, product, and residual nutrients concentration) and the corresponding net NAD(P)H fluorescence ($|\Delta F|$, observed—medium fluorescence). *NFU* normalized fluorescence units. Process variables show the average values of experimental data at different time intervals

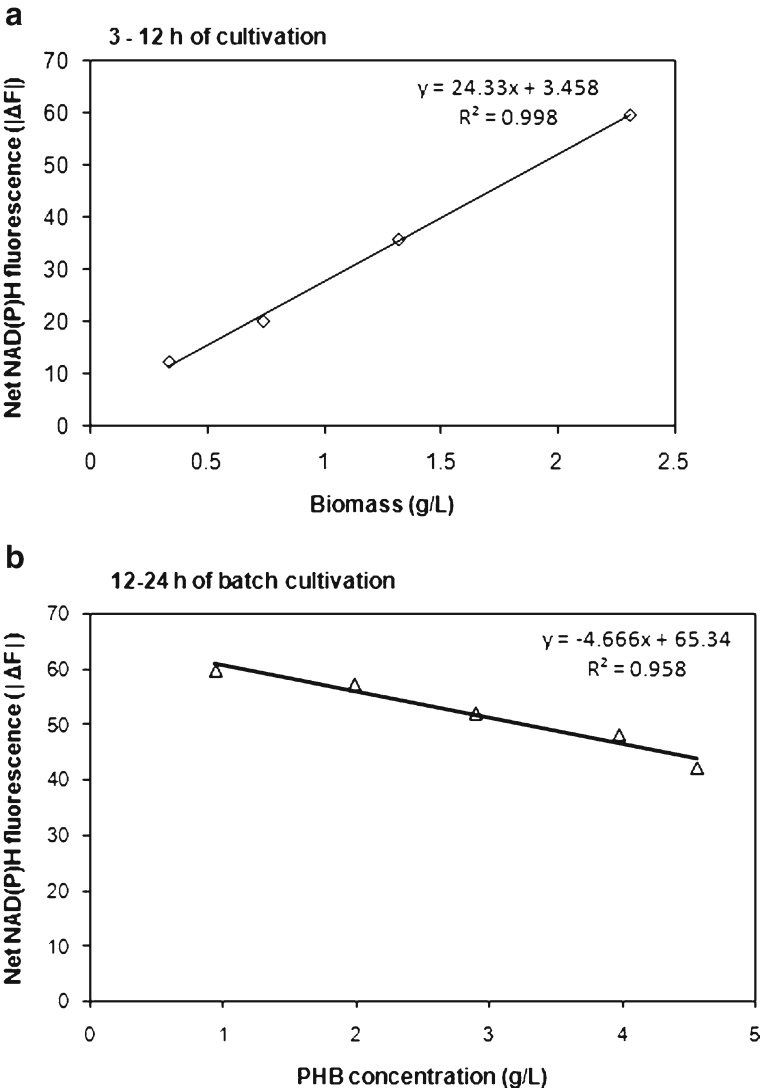

Fig. 3 Correlation between net NAD(P)H fluorescence (|Δ*F*|) and **a** biomass concentration (*X*) of the culture measured during 3 to 12 h of batch cultivation, **b** PHB concentration (*P*) of the culture in region II (12 to 24 h). Data points represent the average values of the samples

linear correlation between net NAD(P)H fluorescence and biomass concentration was not observed throughout the entire exponential phase of batch cultivation. This sharp dip could be attributed to the fact that NAD(P)H was not only used for biomass formation but also channeled into PHB formation pathway simultaneously due to growth-associated PHB production [9, 27]. Figure 3b illustrates the relationship between net NAD(P)H fluorescence and PHB accumulation across the regions II and the slope (negative) of the line in Fig. 3b can be interpreted as the decrease in the fluorescence per unit increase in PHB concentration, which in turn, suggests consumption of reduction equivalents for PHB synthesis and hence decrease in intracellular NAD(P)H concentration. Genser et al. [9] has also reported that *A*.

australica (earlier known as *Alcaligenes latus* DSM1124) has no NADH oxidase activity indicating an impaired respiratory pathway and dependence on PHB synthesis for storing reduction equivalents such as NAD(P)H (generated during TCA cycle) during growth. An impaired NADH oxidase activity results in accumulation of high levels of NAD(P)H which in turn inhibit the isocitrate dehydrogenase and citrate synthase enzymes (key enzymes of TCA cycle). This results in accumulation of high acetyl-CoA levels (main precursor of PHB synthesis), consequently forcing the cells for PHB synthesis. Hence in case of *A. australica*, PHB act as an alternative sink for reduction equivalents during growth and helps in regenerating oxidized nucleotides for TCA cycle [28]. Therefore on-line, in situ NAD(P)H fluorescence measurement will indicate the shift in culture metabolism which promotes the PHB accumulation, i.e., channeling of reduction equivalents towards PHB synthesis and serve as an important on-line signal for bioprocess control purposes. The specific rates of PHB production and PHB productivity were calculated at each point of time of sampling during entire batch cultivation. The specific rates of PHB production (Qp) were plotted across all the regions as shown in Fig. 4. From Fig. 4 it was observed that the specific PHB production rates (accumulation rates) were higher in region II (12–24 h, maximum at 12 h) consequently reinforcing the results of switching of reduction equivalents towards PHB synthesis during NAD(P)H fluorescence-based cultivation.

A linear correlation between net NAD(P)H fluorescence and biomass concentration was again observed in region III (24–39 h) of the cultivation synchronizing with more biomass formation and rather slow increase in PHB concentrations. In this region biomass concentration and $|\Delta F|$ were correlated by the following linear correlation: $|\Delta F|=6.27X+3.51$ (Fig. 5). Therefore culture fluorescence signal could be used as an on-line indicator of biomass formation in two regions (region I, 3–12 h and region III, 24–39 h) except region II which was characterized by vigorous PHB synthesis and its accumulation inside the cells (Fig. 4). NADPH fluorescence cannot be correlated with the PHB concentrations in regions I and III because in these regions very small amount of PHB was accumulated inside the cells. During stationary phase (39–48 h, Fig. 2) a dip in culture fluorescence was again observed because of culture starvation and depletion of sucrose concentration to a very low level

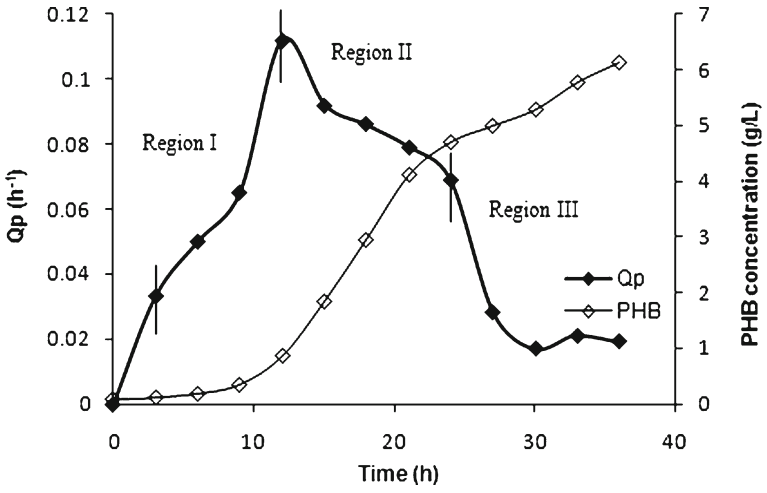

Fig. 4 Specific rates of PHB formation (*Qp*) and PHB concentration profile across all the regions. Region II demonstrates the much faster accumulation of PHB. Experimental data points show the average values of the samples

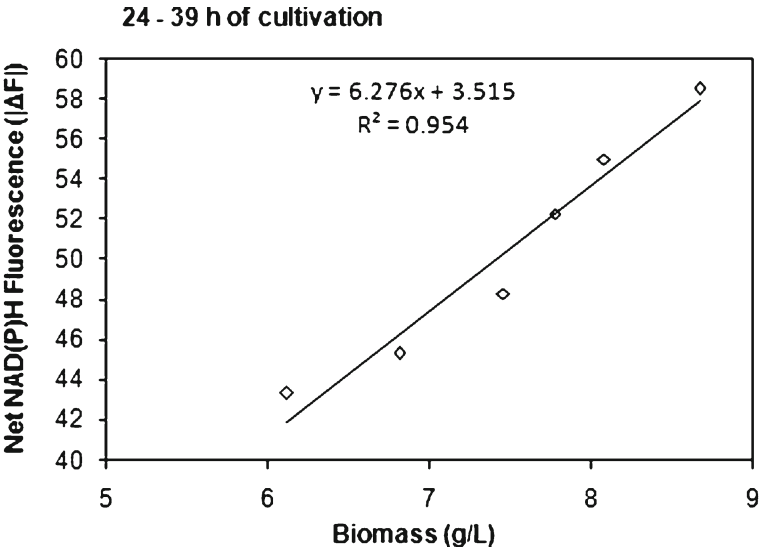

Fig. 5 Correlation between biomass concentration (X) and net NAD(P)H fluorescence ($|\Delta F|$) of the culture measured during 24 to 39 h of batch cultivation. A linear correlation was observed between X and $|\Delta F|$ in region III. Experimental data points reflect the average values of the samples

(1.5 g/L) suggesting excessive limitation of nutrients. This significant drop in the NAD(P)H fluorescence signal (reaching to 43 NFU) reflected the end of batch cultivation due to extreme low availability of substrate in the reactor which helped in deciding the time of harvest of PHB from the reactor before cells enter into stationary phase and undergo death. This dip in the on-line NAD(P)H fluorescence signal at 39 h could be extremely helpful in deciding the time of fresh nutrient (or substrate) feed also to further re-establish the growth of the culture and improve the product formation. The feeding of fresh nutrients could prolong the exponential growth phase of the cells which may eventually lead to increase in the PHB production. Zabriski and Humphrey [29] have also reported that a decrease in culture fluorescence during cell starvation and substrate limitation could serve as an important on-line, in situ signal for fresh substrate (glucose) feeding. A very similar phenomenon of decrease in fluorescence during stationary phase has also been reported by other researchers for various fermentations [13, 15, 30] but in the present study it also served as an important on-line signal for the removal of the cells from the reactor for product recovery to prevent PHB degradation [23]. In addition to monitoring intracellular activities, non-invasive nature of NAD(P)H fluorescence measurement technique provided an opportunity of real time assessment of important biological parameters during batch cultivation without any time lag. The fluorescence measurements are used for gaining an insight of all metabolic events occurring inside the cell without intermittent removal of fermentation broth (sample) for off-line analysis [19].

Conclusions

In the present investigation NAD(P)H fluorescence technique proved to be very useful for on-line monitoring of culture biomass and characterization of metabolic state in the bioreactor for growth-associated PHB production. A linear correlation between biomass

concentration and net NAD(P)H culture fluorescence was observed during the early (3–12 h) and late log phase (24–39 h) not throughout the cultivation which was mainly due to simultaneous rapid utilization of reduction equivalents (NAD(P)H) for the PHB accumulation. This continuous channeling of reducing power for both biomass and PHB accumulation was marked by decrease in culture fluorescence from 12–24 h (exactly at the time of rapid accumulation of PHB concentration). During stationary phase a decrease in NAD(P)H fluorescence was observed primarily because of complete disappearance of sucrose and other nutrients which could thus serve as an important on-line signal for the termination of normal batch cultivation and removal of cells from the reactor to prevent the degradation of PHB and/or addition of fresh substrate during nutrients limitation for enhanced PHB production. Therefore, in the present study use of on-line in situ NAD(P)H fluorescence measurement in *A. australica* adequately reflected key metabolic activities such as beginning of exponential phase, fast accumulation of PHB and depletion of substrates during the batch cultivation. To the best of our knowledge, this is the first report on the use of NAD (P)H fluorescence measurement for on-line monitoring of biomass and growth-associated PHB production by *A. australica* during batch cultivation.

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