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POLY(3-HYDROXYBUTYRATE) PRODUCTION BY *Cupriavidus necator* SUPPLEMENTED WITH MINIEMULSIFIED SOYBEAN OIL

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Abstract - Studies have shown that the supplementation of vegetable oils, in poly(3-hydroxybutyrate) production, provides an increase in the process productivity, besides inducing lipase activity in the medium. The supplementation with miniemulsified oils could potentialize these results. In this work, the influence of supplementation of the medium with soybean oil, without treatment and miniemulsified, on polymerr production was evaluated. The best moment to supplement the medium and its influence on lipase activity were also analyzed. The results showed that the supplementation with miniemulsified soybean oil promoted an increase of 9.7% in polymer content and the productivity increased from 0.58 to 0.63 $g.L^{-1}.h^{-1}$, when compared with the culture without supplementation. The best time to add the supplement is in the beginning of the production phase. The lipase activity was higher for the culture supplemented with miniemulsified soybean oil, promoting the use of the oil.
Keywords: Poly(3-hydroxybutyrate); miniemulsion; soybean oil; supplementation; *Cupriavidus necator*.

INTRODUCTION

Polymers of petrochemical origin have been replacing several materials such as glass and metal. The growing use and incorrect disposal of these materials cause many environmental problems. In face of that, some alternatives have been studied, among which the production of biodegradable polymers (Manangan *et al*., 2010).

Polyhydroxyalkanoates (PHAs) are biodegradable, biocompatible, and non-toxic polymers which stand out as replacements for petroleum-derived polymers. Among PHAs, poly(3-hydroxybutyrate) (P(3HB)) stands out due to its thermoplastic and mechanical characteristics similar to those of polypropylene (Lee *et al*., 2008; Kahar *et al*., 2004). This biopolymer is synthesized through a microbial pathway, where the bacterium *Cupriavidus necator* is one of the most widely-studied microorganisms due to its ability to store up to 80% of its dry mass as polymer and to use different substrates as carbon source (Ramsay, 1994).

Usually, P(3HB) production takes place in two separate phases. In the first (growth phase), all nutrients are available, while in the second (production phase), there is the limitation of one of the essential nutrients other than the carbon source (Lee, 1996). The costs with substrate, operation and control of the bioreactor, and downstream processing make this

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polymer commercially uncompetitive against petroleum-derived polymers. It is expected that lower production costs will help accelerate further commercialization of this polymer (Riedel *et al*., 2012).

An interesting alternative for reducing the production costs is by using supplements or inducers in PHA production. These agents can interfere in the metabolic pathway, causing higher cell production and resulting in greater productivity, but they can also act directly on PHA synthesis (Marangoni *et al*., 2001). The supplementation of the culture medium with oleic and linoleic acids has been assessed in cultures for producing P(3HB) with *Cupriavidus necator*. In these studies, the authors concluded that there was an increase in productivity and higher biopolymer accumulation (Schneider *et al*., 2010).

Since these fatty acids are present in many vegetable oils, some authors studied the supplementation of the culture medium with different oils and noticed that, just like with pure fatty acids, there was an increase in productivity and intracellular polymer accumulation (Lee *et al*., 2008; Ng *et al*., 2010; Dalcanton *et al*., 2010; Pradella *et al*., 2012). The biggest problem in the use these oils in culture media is heterogeneity since the oil, being hydrophobic, tend to remain on the surface even with intense agitation of the medium (Budde *et al*., 2011). Associated with this fact, studies have already shown that vegetable oils are better assimilated when supplied as emulsions (Budde *et al*., 2011). Therefore, supplementation of the culture medium with miniemulsified soybean oil could promote better results in productivity and polymer accumulation.

Vegetable oils must be hydrolyzed into their derived fatty acids, by means of lipases, in order to be metabolized by the bacterium *C. necator*. Some studies (Fukui and Doi, 1998; Kahar *et al*., 2004) confirmed the presence of this enzyme through enzymatic activity analysis, which indicates that the bacterium *C. necator*, when in the presence of vegetable oils, excretes lipase into the culture medium. One of the lipases secreted by *C. necator* H16 plays a critical role in the ability of the strain to create an emulsion with the oil present in the culture medium, which facilitates the hydrolysis and therefore the consumption of substrate (Brigham *et al*., 2010). In the work of Budde *et al*. (2011), the oil was supplied to the culture medium in emulsified form and the results showed that there was a reduction of the adaptation phase of the strain in the medium, indicating that the substrate was easily assimilated.

Miniemulsions are classically defined as an aqueous dispersion with oil particles varying from 50 to 500 nm in size that are relatively stable, prepared by intense agitation of a system containing water, oil, and a surfactant (Landfester *et al*., 1999), while emulsion particles have an average size from 500 to 10,000 nm (Langevin *et al*., 2004). Through the miniemulsification, the contact surface between the water and the oil increases several fold, and the consumption of the substrate by the microorganism is possibly facilitated.

In this context, the goal of the present study was to compare the influence of supplementing pure soybean oil (without treatment) and miniemulsified soybean oil on P(3HB) production in cultures with *C. necator* using a synthetic glucose/fructose medium as the main carbon source. In order to better understand the microorganism behavior in the presence of vegetable oils, the lipase enzymatic activity was also quantified.

MATERIALS AND METHODS

Microorganism and Culture Medium

The strain *Cupriavidus necator* DSM 545 was stored frozen in glycerol at -80 °C until the moment of inoculation. Two pre-cultures were used. The first, nutrient broth (NB), contained 3 $g.L^{-1}$ of meat extract and 5 $g.L^{-1}$ of meat peptone and was used to inoculate the microorganism. The second was a mineral medium (MM) based on Aragão *et al*., (1996), with modifications and no nitrogen limitation. The MM contained (in $g.L^{-1}$): glucose 20; fructose 20; KH_2PO_4 4.39; ammonium and iron citrate III 0.06; $CaCl_2.2H_2O$ 0.01, $MgSO_4.7H_2O$ 0.5; urea 2; nitrilotriacetic acid 0.19; and trace elements 1 $mL.L^{-1}$. The composition of the trace element solution was (in $g.L^{-1}$): H_3BO_3 0.3, $CoCl_2.6H_2O$ 0.2, $ZnSO_4.7H_2O$ 0.1, $MnCl_2.4H_2O$ 0.33, $Na_2MoO_4.2H_2O$ 0.03, $NiCl_2.6H_2O$ 0.02, and $CuSO_4.5H_2O$ 0.01. The composition of the culture medium used in the assays was the same as the MM. During the assays, when the concentration of glucose/fructose was around 15 $g.L^{-1}$, a pulse of a concentrated solution of these nutrients was performed to avoid carbon starvation. The limiting nutrient in the medium, to promote the polymer accumulation during the assays, was nitrogen.

Supplement Addition

The soybean oil used in this work had as the main composition (w/v): 47% of linoleic acid, 24.2% of oleic acid, 17.7% of palmitic acid, 4.86% of linolenic acid and 3.76% of stearic acid. The soybean oil was added to the culture medium, without treatment (pure)

or miniemulsified, at an equivalent oleic acid concentration of 0.3 $g.L^{-1}$. Thus, after the supplementation, the total oleic acid concentration in the culture medium was 0.3 $g.L^{-1}$.

Cultures in Shake Flasks

The pre-cultures and the cultures performed in shake flasks were incubated in an orbital shaker (New Brunswick Sci. Company Inc., New Jersey, USA) at 35 °C and 150 rpm for 24 hours. The cultures were carried out in 1-liter flasks with an initial volume of 0.4 L and the pH was kept at 7.0 with addition of NaOH and HCl solutions.

Seven different cultures were carried out: a culture without supplement (control), three cultures supplemented with pure soybean oil and three cultures supplemented with miniemulsified soybean oil. The difference between the supplemented cultures was the moment that the supplement was added to the culture, which occurred when the nitrogen concentration in the culture medium was 0.5 $g.L^{-1}$ (exponential growth phase), 0.4 $g.L^{-1}$ (time of limiting nitrogen source) and 0.2 $g.L^{-1}$ (the beginning of the stationary phase of growth). The enzymatic activity over time was evaluated for all the cultures.

Cultures in Bioreactor

The pre-cultures were incubated in a shaker (New Brunswick Sci. Company Inc., New Jersey, USA) at 35 °C and 150 rpm for 24 hours.

The bioreactor cultures were carried out in a 5-liter bioreactor (BIOFLO 110, New Brunswick Sci. Company Inc., New Jersey, USA) with initial volume of 4 L. The temperature was kept at 35 °C and the pH at 7.0 by adding NaOH and HCl solutions with concentrations of 100 $g.L^{-1}$ and 270 $mL.L^{-1}$, respectively. The initial conditions of agitation and aeration were 450 rpm and 0.1 vvm, respectively, and gradually increased so that the dissolved oxygen concentration was kept above 30% compared to the atmospheric air saturation.

The supplementation of the vegetable oil (pure and miniemulsified) took place at the best moment defined by the greatest enzymatic activity through cultures performed in shake flasks. One culture with no soybean oil supplementation (control) was also carried out.

Miniemulsion Preparation

To verify the interference of soy lecithin concentration on the particle size of the miniemulsion, the following concentrations of emulsifier were tested: 0.2, 0.4, 0.8, 1.2 and 1.6% (w/v) of soy lecithin. For each case, the lecithin was solubilized in the soybean oil for 20 minutes by mechanical agitation. After that, water was added to the system, at a ratio of 4:1, and the solution remained under mechanical agitation for 1 hour. Finally, this solution underwent the dispersion step in an ultrasonic dismembrator (Ultrasonic Dismembrator 500 (400W) - Fisher Scientific) for two minutes at ultrasound power of 280 W. For each concentration of soy lecithin evaluated, measurements of the particle size were carried out to verify the stability of the miniemulsions. Right after obtaining the miniemulsion, the particle size was measured, then the miniemulsion remained standing for 30 minutes, at room temperature, and a new measurement was performed. This procedure occurred during a period of two hours and, after 24 hours, one last measurement was carried out.

The measurement of the particle size was made by Dynamic Light Scattering (Coulter NP4 Plus or Zetasizer, Nano Series), which determines the average particle diameter from the diffusion rate of particles through the fluid. To perform the measurement, the miniemulsion was diluted in distilled water in a ratio of 1:5.

Analytical Techniques

Samples of the culture medium were taken every two hours, during the assays. For each sampling, 12 mL of culture medium were transferred to 2 ml tubes. Samples of 2 ml were centrifuged at 10,956 xg for 10 min, and the precipitate washed with 1 ml of distilled water. Five samples of supernatant were frozen for later analysis of substrate consumption and one sample was stored at 4 °C (over a period of up to 24 hours) for enzyme activity analysis. Three samples of precipitate were put into a dryer at 100 °C until constant weigh (gravimetric analysis) and three samples were frozen for P(3HB) quantification.

The cell concentration during the culture was determined by turbidimetry at 600 nm and by gravimetric analysis. The concentrations of total reducing sugars were quantified by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959). The residual nitrogen concentration, in the form of ammonium ions, was determined by an enzymatic-colorimetric method (Ureia-ES - Gold Analisa, Belo Horizonte, Minas Gerais, Brazil). The determination of the P(3HB) concentration was made through gas chromatography (GC-90 equipped with a fused-silica capillary column, Supercowax - 10 - 0.53 mm x 30 m, and flame ionization detector) according to the meth-

anolysis method based on Brandl *et al*. (1988), where 1 μL of the sample was injected into the gas chromatographer. Poly(3-hydroxybutyrate) was used as the external standard. The injection, detection, and column temperatures were 190 °C, 230 °C, and 120 °C, respectively. The enzymatic activity (lipase) was assessed by using the Lipase Kit (Gold Analisa, Belo Horizonte, Minas Gerais, Brazil): first of all, the lipases present in the supernatant hydrolyze the thioester 2,3-disulfanylpropan-1-ol (BAL), releasing a thioalcohol and butyric acid. After that, the thioalcohol reacts with 5-(3-carboxy-4-nitrophenyl)disulfanyl-2-nitrobenzoic acid (DTNB) in a buffered medium, forming a yellow anion whose color intensity is proportional to the enzyme concentration and shows maximum absorption at 412 nm.

Data Treatment

The volume variation in the system, during the assays, was negligible, so the data of the cultures were treated in terms of concentration ($g.L^{-1}$). Polynomial adjustments were made to the data of total biomass (Xt) and P(3HB) versus time, through the software Lissage (developed by Simões *et al*., 1995). Residual biomass (Xr) was estimated from the difference between Xt and P(3HB) values obtained from the polynomial fits.

To compare the results of the three assays, the same period of the production phase was considered, and the results were interrupted exactly 13 hours after the beginning of the production phase, i.e., when the nitrogen concentration reached 0.2 $g.L^{-1}$ in the culture medium. To compare the assays at exactly 13 hours of production phase, the final results of Xt, P(3HB), Xr, productivity and final percentage of polymer, were obtained from the polynomial fits to the experimental data.

In order to verify the increase in polymer accumulation between the control and the supplemented cultures, Equation 1 was applied, where $P_{control}$ is the final concentration of accumulated polymer in the control culture and $P_{supplement}$ is the final concentration of accumulated polymer in the culture supplemented with soybean oil, pure or miniemulsified.

$$\text{Increase in accumulation} = \frac{P_{supplement} - P_{control}}{P_{control}} \cdot 100 \quad (1)$$

The global productivity (Equation 2) (Pr), expressed in $g.L^{-1}.h^{-1}$, was calculated by the difference between the P(3HB) concentration at a time t ($P(3HB)_t$) and the initial P(3HB) concentration ($P(3HB)_{t0}$), divided by the corresponding time span, where t_0 is the initial time.

$$P_r = \frac{P(3HB)_t - P(3HB)_{t_0}}{t - t_0} \quad (2)$$

RESULTS AND DISCUSSION

Miniemulsion Stability

The stability of the miniemulsion was studied by testing different concentrations of soy lecithin. This study was important to establish which concentration would promote the formation of a stable miniemulsion. The concentrations of soy lecithin of 0.2 and 0.4% promoted heterogeneous emulsions, with particle sizes greater than 1000 nm (data not shown). These results show that higher concentrations of soy lecithin are necessary.

Table 1 presents the average particle size over time for the concentrations of soy lecithin of 0.8, 1.2 and 1.6%. From these data, the concentration of 0.8% provided an average particle size equal to 272 nm at the first moment, but over time the particles exhibited an increase in their average size. After 24 hours it was noted that the emulsion presented heterogeneous particles.

Table 1: Particle average size over time for miniemultions with different concentrations of soy lecithin, with two minutes of sonication at an ultrasound power of 280 W.

% (w/v) soy lecithin	Particle average size (nm)					
	0 min	30 min	60 min	90 Min	120 min	24 hours
0.8	272	289	245	291	277	862
1.2	247	242	250	255	252	259
1.6	226	238	242	252	250	258

The concentration of 1.2% of soy lecithin was more stable than the concentration of 0.8%, since after 24 hours the average size of the particles remained the same. The increase of the soy lecithin concentration from 1.2 to 1.6% did not promote the reduction of the particle size. Based on these results, the soybean oil miniemulsion was made up of a dispersed phase (soybean oil - 19.7%), a continuous phase (water - 79.1%) and a surfactant (soy lecithin – 1.2%).

Study of the Best Moment for the Soybean Oil Supplementation

Figure 1 presents the results of enzymatic activity for the seven cultures performed in shake flasks. It is

noteworthy that all culture conditions were the same, the only difference being the nitrogen concentration at the moment of adding the supplement.

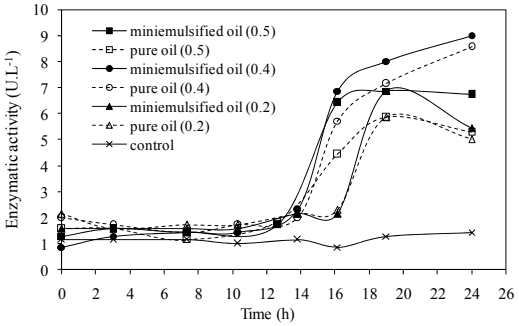

Figure 1: Profile of the enzymatic activity over time in cultures performed in shake flasks. Control culture (solid line with asterisk), supplemented with pure soybean oil (dashed line with open square) and with miniemulsified soybean oil (solid line with closed square), when the nitrogen concentration was 0.5 $g.L^{-1}$; supplemented with pure soybean oil (dashed line with open circle) and with miniemulsified soybean oil (solid line with closed circle), when the nitrogen concentration was 0.4 $g.L^{-1}$; supplemented with pure soybean oil (dashed line with open triangle) and with miniemulsified soybean oil (solid line with closed triangle), when the nitrogen concentration was 0.2 $g.L^{-1}$.

Initially, all cultures showed a basal production of lipase. Only after adding the supplement was there the induction and excretion of the enzyme into the culture medium, for both conditions of soybean oil, pure and miniemulsified. In all cases, the lipase activity was higher when the supplement was added as a miniemulsion, suggesting that the miniemulsion promotes greater enzyme excretion by the microorganism *C. necator*.

Evaluating the different moments when the supplement was added, the nitrogen concentration that promoted the highest lipase activity was 0.4 $g.L^{-1}$, considered to be the limiting concentration of this substrate in the medium, which promotes the beginning of the synthesis of P(3HB) (Ienczak *et al*., 2011), for both ways in which the oil was supplemented. From these results, to achieve better enzymatic activities of lipases, the moment at which the supplement should be added to the cultures is when the nitrogen concentration in the culture medium is 0.4 $g.L^{-1}$.

Influence of Supplementation with Miniemulsified Soybean Oil on P(3HB) Production

Three cultures were performed in the bioreactor: a control culture and two supplemented with soybean oil, pure and miniemulsified. The final concentration results of total biomass (Xt), P(3HB), polymer percentages (% P(3HB)) and global productivity (Pr), after 13 h of production phase can be observed in Table 2. Figure 2 presents the evolution of Xt, P(3HB) and Xr along the time.

Table 2: Final values of total biomass (Xt), P(3HB), polymer percentages (% P(3HB)) and global productivity (Pr), after 13 hours of production phase, for the control cultures and those supplemented with pure and miniemulsified soybean oil.

Culture	Xt ($g.L^{-1}$)	P(3HB) ($g.L^{-1}$)	Pr ($g.L^{-1}.h^{-1}$)	P(3HB) (%)
Control	21.5	15.5	0.58	72.0
Supplemented with pure soybean oil	23.0	16.0	0.60	69.6
Supplemented with miniemulsified soybean oil	23.0	17.0	0.63	73.9

The increase in polymer accumulation, in terms of percentage of P(3HB) in the cells was 3.2% for the one supplemented with pure soybean oil and 9.7% for the one supplemented with the miniemulsified soybean oil. These values are in agreement with the results found by Dalcanton *et al*. (2010), who obtained an increase of 22% in the P(3HB) accumulation when hydrolyzed rice starch was used as the main substrate supplemented with pure soybean oil, at an equivalent oleic acid concentration of 0.3 $g.L^{-1}$ in *C. necator* cultures.

The culture supplemented with pure soybean oil had a productivity similar to the control culture. In the culture supplemented with soybean oil miniemulsion, there was an increase in productivity when compared to the control culture and to the culture supplemented with pure soybean oil. This difference between the results of the culture supplemented with pure and miniemusified soybean oil may have occurred because, in the miniemulsion, the contact area of the oil droplets is greater than in pure oil, which may have facilitated its absorption.

This increase in accumulation and, consequently, in productivity is due to the fact that, as suggested by Lee *et al*. (2008), substrates containing higher carbon content, like fatty acids present in vegetable oils, promote higher yields in PHAs when compared to simple sugars like glucose and fructose. Theoretically, a molecule of glucose or fructose, which contains six carbon atoms, is metabolized in the Entner–Doudoroff pathway to form two acetyl-CoA and two molecules of carbon dioxide, thus leading to the formation of one 3HB monomer unit. On the other

hand, a molecule of linoleic acid, which contains eighteen carbon atoms, is oxidized via the β-oxidation pathway to form nine acetyl-CoA, without CO_2 release, which probably contributes to the production of more 3HB monomer units (Akiyama, *et al*., 2003).

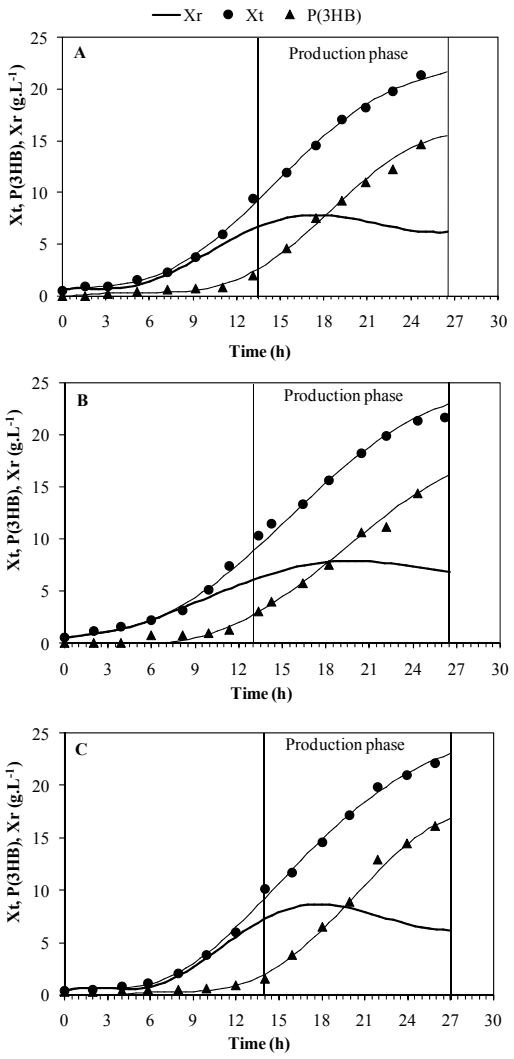

Figure 2: Evolution of the concentration of Xt (solid circle), P(3HB) (solid triangle) and Xr (solid line) over time for the control culture (A), culture supplemented with pure soybean oil (B), and culture supplemented with miniemulsified soybean oil (C).

Figure 3 shows the enzymatic activity during the cultures. Firstly, a basal production of the lipase can be seen at the beginning of the three cultures. However, in the supplemented cultures there was an increase in the enzymatic activity after the addition of the supplement. This increase took place because the soybean oil serves as an inductor for the microorganism to begin excreting the enzyme into the culture medium, as has been reported in the literature (Kahar *et al*., 2004). When the oil was supplemented in the miniemulsion form, the enzymatic activity reached higher values, as occurred in the cultures in shake flasks. After achieving a maximum value, the enzymatic activity presents a decrease with time. This can be due to the fact that the small amount of soybean oil added to the culture medium is quickly assimilated by the bacterium, which means that there is no more substrate for the enzyme.

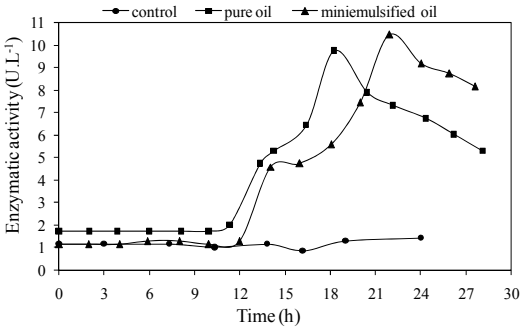

Figure 3: Profile of the enzymatic activity over time in cultures performed in the bioreactor. Control culture (closed circle), cultures supplemented with pure soybean oil (closed square), and with miniemulsified soybean oil (closed triangle).

In conclusion, this study shows that supplementing the culture medium with either pure or miniemulsified soybean oil led to an increase in P(3HB) production: 3.2% and 9.7%, respectively, when compared to the control culture. The best moment to add the soybean oil to the culture medium was at the beginning of the production phase (0.4 $g.L^{-1}$ of nitrogen in the medium). The lipase activity and the polymer productivity were higher for the culture supplemented with miniemulsified soybean oil than in the others. This behavior strongly suggests that the supplementation of the culture medium with miniemulsified soybean oil acts directly on the P(3HB) production, and the moment to add the oil is crucial to reach these results, since if the supplementation occurs in the growth phase, the oil will be used to produce biomass instead of polymer. These results demonstrate that miniemulsified soybean oil is a good alternative for increasing P(3HB) productivity.

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NOMENCLATURE

Latin Letters

DNS	Dinitrosalicylic acid
MM	Mineral medium
NB	Nutrient broth
PHA	Polyhydroxyalcanoate
P(3HB)	Poly(3-hydroxybutyrate) ($g.L^{-1}$)
$P(3HB)_t$	P(3HB) at a time t ($g.L^{-1}$)
$P(3HB)_{t0}$	P(3HB) at initial time ($g.L^{-1}$)
$P_{control}$	Final concentration of P(3HB) in control culture ($g.L^{-1}$)
Pr	Global productivity ($g.L^{-1}.h^{-1}$)
$P_{supplemented}$	Final concentration of P(3HB) in supplemented culture ($g.L^{-1}$)
t	time (h)
t_0	Initial time (h)
Xr	Residual biomass ($g.L^{-1}$)
Xt	Total biomass ($g.L^{-1}$)

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