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Mathematical Modelling and Scale-up of Batch Fermentation with *Burkholderia cepacia* B27 Using Vegetal Oil as Carbon Source to Produce Polyhydroxyalkanoates

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A mathematical model that predicts biomass growth and polyhidroxyalkanoates (PHAs) accumulation in *Burkholderia cepacia* B27 and its validation at pilot plant scale is presented in this work. During the fermentation process, the mutated strain was capable to produce 15 g/L of PHA and accumulate 86 % of bacteria weight as polymer, with corn oil as carbon source. Bioreactor assays were performed with a 5 L working volume, pH 7.0 and 32 °C. The model development steps were the following: 1) kinetic behavior analysis; 2) formulation of differential equations to predict kinetic behavior; 3) fitting of model parameters with Matlab® solvers, and 4) model validation with statistical and experimental tests. The experimental validation was developed under batch conditions with a work volume of 60 L, showing a good prediction of the system behavior. This model will be useful for the optimization of the feed-batch process that is the next step in the research.

1. Introduction

Pollution problems and regulations for a safe and clean environment have served as driving force to stimulate increased research for potential solutions like bio-based and biodegradable polymers as well as other more sustainable materials to replace synthetic plastics (Kettl et al. 2012). Polyhydroxyalkanoates (PHAs) are a group of natural biodegradable polymers which are usually synthesized by various microorganisms, as energy reserve material, under limitation of essential nutrients and excess of carbon source (Amache et al. 2013). PHAs have many applications in several fields, such as several fields, as packaging, food services, bio-medical, and agriculture industries has increased due to their biodegradability, compatibility and piezoelectricity (Nicolò et al. 2014; Rai et al. 2011). However, wider use of PHAs is limited mainly by their high production cost which depends on the substrate cost, the PHA/substrate yield factor and the quality of the product required by the downstream process (Yang et al. 2006). Currently, various approaches have been used to tackle this problem, one of them is the optimization strategy (Gahlawat and Srivastava 2013; Riascos 2004). A bioprocess engineering approach to address process optimization could be developed through mathematical modelling, which not only helps in the understanding of the system but also predicts the system dynamic to facilitate the optimization of the process. Process optimization based on models saves much of the time and cost of performing experiments (Gahlawat and Srivastava 2013). In the present research Burkholderia cepacia was utilized for PHA production due to its capacity to produce PHA in a growth-associated manner, accumulating high amounts of PHA (up to 86 % of cell dry weight) during growth phase of cultivation, which could improve the PHA yield on the carbon source (Akiyama et al. 2003). Moreover B. cepacia is able to utilize inexpensive carbon sources, like hydrolyzed wood residues, vegetal oil and glycerol (Chee et al. 2010; Wang and Liu 2014). This feature makes possible to use various renewable resources for PHA production. The aim of the present study was to propose a kinetic mathematical model that adequately describes the observed batch kinetics of cultivation with B. cepacia. In addition, this model elucidates substrate limitation and inhibition under different scenarios of nutrient feed conditions. A mathematical model seems to be a valuable, fast and reliable tool for optimizing the nutrients feeding for fed-batch cultivation aiming at maximizing the PHA productivity. This way, the model provides reliable information to reduce the limitation of substrate and also overcome the inhibition problem caused by the substrate (Kaur et al. 2012; Gahlawat and Srivastava 2013).

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2. Materials and methods

2.1 Culture Conditions and Fermentation Process

Burkholderia cepacia B27 was obtained from genetically modified process in the Biotechnology Institute of the Universidad Nacional de Colombia (IBUN) (Florez et al. 2014). The strain was selected due to its capabilities to use oils as carbon source and to accumulate intracellular PHAs. It was maintained on cryovials with L.B. broth and 40 % glycerol. 5 % of *B. cepacia* B27 stock inoculum was introduced into 250 mL conical flask with 50 mL of L.B. medium and incubated in shaking (SK-333-PRO SCILOGEX, United States) with temperature room at 32 °C and 200 rpm during 24 hours for culture inoculum development, another inoculum adaptation step was performed by transferring the inoculum into 2000 mL conical flask containing 450 mL of culture media under the same conditions mentioned before; finally, the inoculum was seeded into BioFlo CelliGen® 115 (Eppendorf, United States) with 5000 mL culture medium. Culture media was prepared with (g/L) KH_2PO_4 2.65, Na_2HPO_4 3.39, $(NH_4)_2SO_4$ 2.8, $MgSO_4$ 0.3, vegetal oil 20, tween 80 at 22.5 % (v/v oil) and antifoam at 5.5 % (v/v tween 80). Trace elements containing (g/L) $FeSO_4$ 2, $CaCl_2$ 2, $CoCl_2 \cdot 6H_2O$ 0.2, $CuCl_2 \cdot 2H_2O$ 0.01, $NiCl_3 \cdot 6H_2O$ 0.2, $MnCl_2 \cdot 4H_2O$ 0.03, $ZnSO_4 \cdot 7H_2O$ 0.1, H_3BO_3 0.3, $NaMoO_4.2H_2O$ 0.03, dissolved in 1N HCl were added at 0.2 % (v/v) to the culture medium and then sterilized prior inoculation. All the culture medium was fitted at pH 7 (± 0.1) with 3 N NaOH solution.

2.2 Preliminary substrate inhibition and limitation studies

To study the effects of different concentrations of nutrients (vegetal oil and nitrogen) on the growth rate of *B. cepacia* B27, preliminary inhibition studies were carried out in 500 mL shake flasks containing 150 mL medium. The effect of increasing vegetal oil concentration on the growth of *B. cepacia* B27 was studied by monitoring the maximum specific growth rate in the medium varying vegetal oil concentration (ranging from 0.5 to 170 g/L) while the concentration of the medium components was keeping constant at their optimized values. The effects of varying the nitrogen concentration (0.2–10 g/L) on the culture growth were also studied in a similar way. Shake flasks containing 150 mL medium were inoculated with 5 % inoculum and kept in a rotary shaker at 32 °C and 200 rpm for 24 h. Samples were withdrawn at regular interval of 6 h and analyzed for biomass content.

2.3 Batch-cultivation in bioreactor

Batch kinetics was studied in a 7 L stirred tank reactor (STR) (BioFlo CelliGen 115) containing 5 L optimized medium. The reactor was sterilized at 121 °C for 30 min, cooled and then inoculated with 5 % (v/v) inoculum. Agitation in the reactor was carried out by using a conventional flat blade turbine-type impeller. The temperature was controlled at 32 °C. Medium pH was maintained at 7.0 with addition of 2 N NaOH/HCl. Air was sparged from the bottom of the reactor using perforated stainless steel ring sparger. Inlet air was maintained at 2 vvm. Fermentation samples were collected at intervals of 6-12 h and analyzed for biomass, PHA, vegetal oil and nitrogen concentrations. Batch experiments were done in triplicate for verifying the reproducibility of the results. Furthermore the model obtained was validated under batch conditions with a bioreactor of 100 L with 60 L of work medium and similar conditions of 7 L bioreactor.

2.4 Modeling and simulation procedure

The batch kinetics data and preliminary flash data were used for developing the mathematical model of the PHA fermentation process. Model parameters were estimated by minimizing the difference between experimental observations and model simulations. The optimization program for kinetic parameters estimation was developed in Matlab®. For the estimation of model parameters a system of differential equations was solved using a numerical integration program based on the Runge-Kutta Method of 4th order. The optimization program for the direct search of the minimum of the multivariable function (or objective function) was based on the algorithm followed by (Patwardhan and Srivastava 2008; Kaur et al. 2012; c). The minimization criteria is the Residual Sum of Squares (Eq 1) where y_i is the experimental data, $f(x_i)$ is the model prediction and n is the number of experimental data.

$$RSS = \sum_{i=1}^{n} (y_i - f(x_i))^2 \quad (1)$$

2.5 Analytical procedures

2.5.1 Biomass, residual ammonium, PHA and vegetal oil quantification

Five mL samples of the fermentation medium were taken at set intervals, and centrifuged at 5000 rpm for 10 min. The precipitate was washed twice with 5 mL distilled water, collected, dried at 80–90 °C until it reached a stable weight (Yang et al. 2006). Residual ammonium was quantified using phenol hypochlorite method as described by Gumel et al. (2012). Cell free supernatant (2 mL) was diluted with distilled water (dilution factor

depends on the amount of ammonium sulfate, which maximum readable concentration is 4 mg /L. Resulting light blue coloration was measured using a spectrophotometer Genesys 10s UV-Vis (Thermo Fisher scientific, USA) at 640 nm against blank distilled water. Residual ammonium concentration was then quantified in reference to standard calibration curve. PHA quantification was developed with five mL samples of the fermentation medium, centrifuged at 5000 rpm for 10 min. The precipitate was washed by vortexed and centrifuged with 5 mL distilled water twice, then 0.55 mL of sodium dodecyl sulfate (SDS) per biomass at 20 % concentration was added for digestion at 80 °C during one hour, the mixture was washed by vortexed and centrifuged with 5 mL distilled water twice, collected, dried at 80–90 °C until stable weight. Residual vegetal oil was quantified using the method described by Kahar et al. (2004): 2 ml of the culture broth was adopted to a screw tube and then mixed with 5 mL hexane. After vigorous shaken for 1 min, 1 mL of hexane layer was transferred to pre-weighted tube, and then rotaevaporated at 78 °C, until the hexane phase evaporated. Then the screw tube was weighted. The vegetal oil concentration was estimated from the extracted amount, according to the predetermined calibration curve.

3. Results and discussion

3.1. Mathematical model development

The developing of batch mathematical model for PHA production was based on the following assumptions:

1. Vegetal oil and nitrogen are the only limiting substrates affecting growth and PHA production.
2. No process limitation by phosphorous nor by any other medium components takes place and these are present in excessive amount in the fermentation medium.
3. Vegetal oil is the carbon source for biomass growth, product formation and maintenance of the cell.
4. Temperature (32 °C) and pH (7.0) of culture remain constant throughout the fermentation.

From preliminary flasks studies, kinetic behavior was found to be limited by the concentration of vegetal oil (Sc) and nitrogen (Sn), which were expressed by Monod kinetics in both cases Figure 1A and 1B. On the other hand, from inhibition studies (Figure 1C and 1D), it was observed a reduction in specific growth rate at concentration of 100 g/L and a complete inhibition at 166 g/L of vegetal oil; likewise for nitrogen, inhibitory concentration was close to 10 g/L.

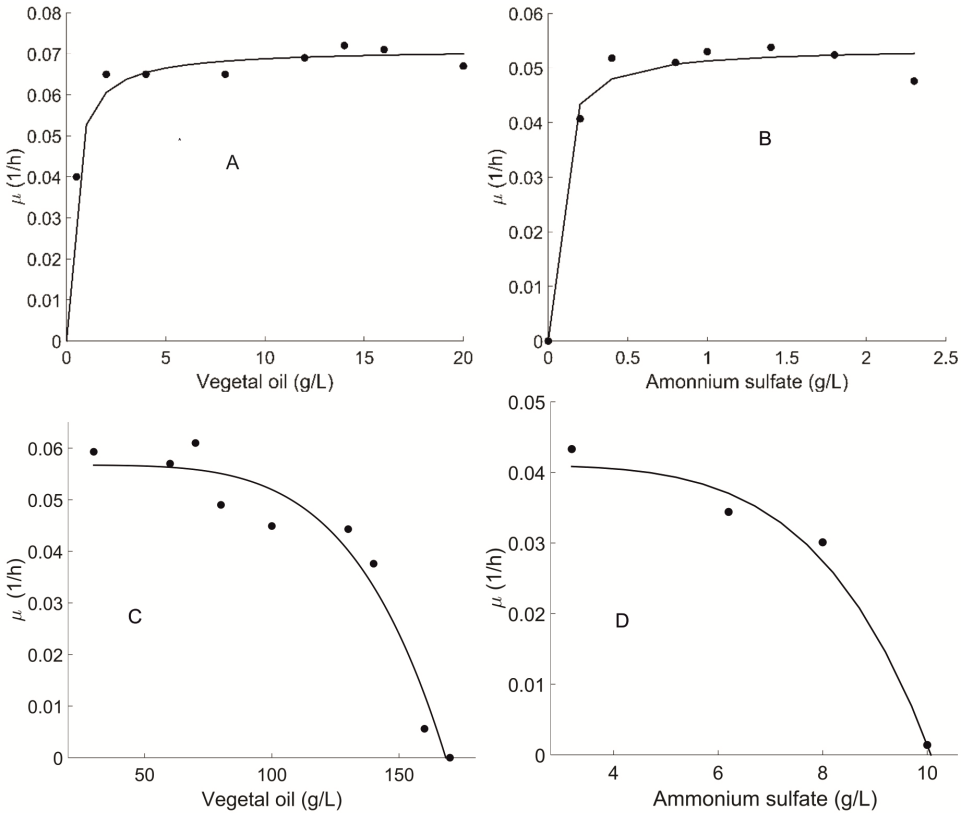

Figure 1. Behavior of specific growth rate (μ) as a function of concentration of limiting substrates. A) vegetal oil and B) nitrogen. And inhibiting substrates concentrations. C) vegetal oil and D) nitrogen. Smooth lines represent the fitted values (–), experimental data (•).

The empirical model for inhibition behavior for both substrates was based on Luong model (Luong 1985) Eq(2), this model was appropriate taking into account experimental data obtained.

$$\mu = \mu\max \cdot \left(1 - \left(\frac{Sc}{Scm}\right)^{a1}\right)\left(1 - \left(\frac{Sn}{Snm}\right)^{a2}\right) \quad (2)$$

Where $\mu\max$ is the maximum specific growth rate, Scm, Snm the inhibitory substrate concentrations, at which the specific growth rate is zero, Sc, Sn the substrate concentrations and $a1, a2$ describe the type of relationship between μ and substrate. Approximate values for the empirical model parameters were calculated from the plots of μ vs S through nonlinear regression, minimizing the RSS.

The mathematical model consists of a system of differential mass balance equations, which can describe the kinetic behavior of *B. cepacia* B27. The functions for limitation and inhibition together with the parameter values obtained previously were considered. In that way, biomass growth rate in batch fermentation is modeled as follows:

$$\frac{dX}{dt} = \left[\mu\max \cdot \left(\frac{Sc}{Ksc + Sc}\right) \cdot \left(\frac{Sn}{Knc + Sn}\right) \cdot \left(1 - \left(\frac{Sc}{Scm}\right)^{a1}\right) \cdot \left(1 - \left(\frac{Sn}{Snm}\right)^{a2}\right)\right] \cdot X \quad (3)$$

Where Ksc and Ksn are vegetal oil and nitrogen affinity constant, $a1$ and $a2$ are kinetic parameters and X is the biomass concentration. Considering growth associated production, the differential equation of vegetal oil consumption is as follows:

$$\frac{dSc}{dT} = -\left[\left(\frac{1}{Y_{\frac{x}{Sc}}} * \mu\right) + m_c\right] * X \quad (4)$$

Where $Y_{\frac{x}{Sc}}$ is the total biomass (active biomass plus intracellular polymer) yield factor and m_c is the maintenance coefficient for vegetal oil. Nitrogen was assumed to be utilized for the growth of biomass and maintenance only, as follows.

$$\frac{dSn}{dt} = -\left[\left(\frac{1}{Y_{\frac{x}{Sn}}} * \mu\right) + m_n\right] * X \quad (5)$$

Where $Y_{\frac{x}{Sn}}$ is the biomass/nitrogen yield factor, m_n is the maintenance coefficient for nitrogen. Finally, product formation was observed as proportional to biomass formation. Therefore it was adequately described by growth associated component only as follows:

$$\frac{dP}{dT} = [\propto * \mu] * X \quad (6)$$

Where $\propto$ is the constant associated to product formation.

3.1.1 Model parameters estimation

For the estimation of optimal values for model parameters (equations 3, 4, 5 and 6) the optimization program starts with the initial values estimated from the empirical models of inhibition and limitation flasks experiments. Experimental data from three different 5 L batch fermentations and the RSS function were employed for the fitting. Fermentation for parameter fitting considered 2.8 g/L ammonium nitrate, and 40, 20 and 5 g/L vegetal oil. Optimal values for parameters values that minimizing the RSS are presented in Table 1. The model fitting is presented in Figure 2. Figure 2A shows the results for 20 g/L vegetal oil in 5 L fermentation and 3B depicts the results for 40 g/L and 60 L. The main observations in Figure 2 are the satisfactory fitting and the good prediction of the overall fermentation behavior.

Table 1: Optimal values for model parameters

parameter	*value*	*parameter*	*value*	*parameter*	*value*
$\mu\max$	0.07 h^{-1}	Ksc	1.25 g/L	Ksn	0.650 g/L
$\propto$	0.983 g/g	Snm	14.52 g/L	Scm	199 g/L
		$a1$	1.079	$a2$	4.79
		$Y_{\frac{x}{Sc}}$	0.88 g_{cel}/g_{oil}	$Y_{\frac{x}{Sn}}$	0.98 $g_{cel}/g_{nitrogen}$
		m_c	0.0016 $g_{oil}/g_{cel}.h$	m_n	0.0035 $g_{nitrogen}/g_{cel}.h$

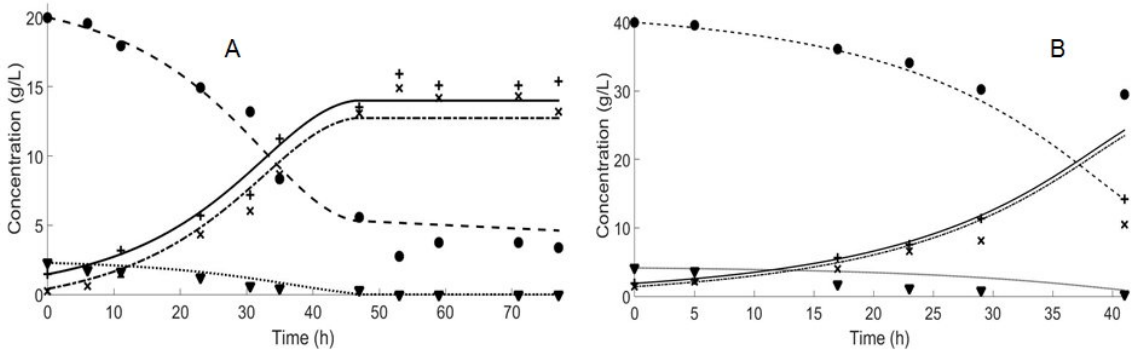

Figure 2. Results in batch bioreactor. A) 5 L fermentation with 20 g/L vegetal oil and B) 60 L with 40 g/L vegetal oil. Experimental data: (▾) ammonium sulfate, (●) vegetal oil, (×) product PHA, (+) biomass and model prediction: (——) Biomass, (– – –) vegetal oil, (.....) ammonium sulfate, (– . – .) PHA.

3.2.2 Model validation

The model was validated using the degree of reliability applying a statistical method suggested by different authors (Patwardhan and Srivastava 2008; Kaur et al. 2012; Gahlawat and Srivastava 2013) on the 5 L batch fermentation results. The statistical evaluation value (λ-value) for reliability of the model was 11.25 which was less than the $F_{(m,n-m)}$ value (observed from F table 11.35) for 99 % confidence level of the model, where n is the number of experimental data points and m the number of process variables $F_{(4,9)}$. Considering the process scale-up, the model was validated under batch conditions with a bioreactor of 100 L and 60 L of culture medium with 40 g/L of vegetal oil and 4.2 g/L of ammonium sulfate, under the same operational conditions of the experiment with the 7 L stirred tank reactor, for 41 hours. Figure 2B shows the data predicted by the model which had a good fitting with the experimental data. The fitting factors (R-square) were 0.95, 0.95, 0.95 and 0.81 for biomass, PHA, vegetal oil and ammonium respectively.

3.2 Batch cultivation in bioreactor

As additional result, experimental analysis allowed to determine optimal conditions for batch fermentation. These conditions consider initial concentrations of 20 g/L vegetal oil and 2.8 g/L ammonium sulfate. Figure 2 shows the batch kinetics of *B. cepacia* B27 under optimal condition, where the maximum concentration of biomass (15.4 g/L) and PHA (14.0 g/L) was reached at 72 h, with a PHA content as high as 86 % of DCW. Thus, Figure 2 allows to observe a strong correlation between production of PHA and biomass, becoming in a growth-associated manner of PHA production since the beginning of the kinetic. In the literature (Steinbüchel et al., 2003) it was observed that PHA medium chain length is the main product obtained from different vegetal oil produced by various bacteria. This statement agrees with our results. During the fermentation process 18.5 g/L vegetal oil concentration was metabolized from its initial concentration of 20 g/L and only 3.3 g/L vegetal oil was left by *B. cepacia* B27 in culture broth. The maximum PHA production rate and PHA yield was 0.84 g/h and 0.79 g PHA /g vegetal oil respectively, which is higher than reported for another authors (Gahlawat and Srivastava 2013; Khanna and Srivastava 2006; López-Cuellar et al., 2011)

4. Conclusions

Batch kinetics of PHA by *Burkholderia cepacia* B27 under optimal culture medium was established. A batch mathematical model was proposed from flash fermentation with inhibition and limitation substrate concentration of nitrogen and vegetal oil. These experiments generated feasible and reliable initial parameters of the kinetic batch model. The statistical 'F' test confirmed the validity of the proposed model. Moreover the evaluation of the model under scale-up conditions with different substrate concentrations showed a good prediction and reliability which validated the parameters obtained. Furthermore this model would be useful for optimization process under fed-batch conditions. Obtained values for productivity (0.27 g/L.h) and yield (0.88 g_{cel}/g_{oil}) confirm that vegetal oil is an interesting option for PHA production and *B. cepacia B 27* is an efficient strain for this fermentation process.

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References

Akiyama M., Tsuge T, Doi Y., 2003, Environmental life cycle comparison of polyhydroxyalkanoates produced from renewable carbon resources by bacterial fermentation, Polymer Degrad. and Stabil., 80, 183–194.

Amache R., Sukan A., Safari M., Roy I. Keshavarz T., 2013, Advances in PHAs production, Chemical Engineering Transactions, 32, 931–936.

Chee J-Y., Tan Y., Samian M-R., Sudesh K., 2010, Isolation and Characterization of a Burkholderia sp. USM (JCM15050) Capable of Producing Polyhydroxyalkanoate (PHA) from Triglycerides, Fatty Acids and Glycerols, Journal of Polymers and the Environment, 18, 584–592, DOI:10.1007/s10924-010-0204-1.

Florez D. V., Suarez Z. R., Merchan N. A., Gutierrez L. C., Olmos L., Moreno N. C., 2014, Mutagenesis studies for enhancement of PHA production by Burkholderia cepacia 2G57, isolated from colombian soils, International Symposium on Biopolymers Santos-Brazil, 92.

Gahlawat G., Srivastava A.K., 2013, Development of a mathematical model for the growth associated Polyhydroxybutyrate fermentation by Azohydromonas australica and its use for the design of fed-batch cultivation strategies, Bioresource Technology, 137, Elsevier Ltd, 98–105.

Gumel A.M., Annuar M.S.M., Heidelberg T., 2012, Biosynthesis and Characterization of Polyhydroxyalkanoates Copolymers Produced by P. putida Bet001 Isolated from Palm Oil Mill Effluent, PLoS ONE, 7.

Kahar P., Tsuge T., Taguchi K., Doi Y., 2004, High yield production of polyhydroxyalkanoates from soybean oil by Ralstonia eutropha and its recombinant strain, Polymer Degradation and Stability, 83, 79–86.

Kaur G., Srivastava A.K., Chand S., 2012, Mathematical modelling approach for concentration and productivity enhancement of 1,3-propanediol using Clostridium diolis, Biochemical Engineering Journal, 68, 34-41, DOI: 10.1016/j.bej.2012.07.004.

Kettl KH., Shahzad K., Eder M., Narodoslawsky M., 2012, Ecological footprint comparison of biobased PHA production from animal residues, Chemical Engineering Transactions, 29, 439–444.

Khanna S., Srivastava A.K., 2006, Optimization of nutrient feed concentration and addition time for production of poly(β-hydroxybutyrate), Enzyme and Microbial Technology, 39, 1145–1151.

López-Cuellar M.R., Alba-Flores J., Rodríguez J.N.G. and Pérez-Guevara, F., 2011, Production of polyhydroxyalkanoates (PHAs) with canola oil as carbon source, International Journal of Biological Macromolecules, 48, 74–80.

Luong J.H.T., 1985, Kinetics of ethanol inhibition in alcohol fermentation, Biotechnology and Bioengineering, 27, Wiley Subscription Services, Inc., A Wiley Company, 280–285, DOI:10.1002/bit.260270311.

Nicolò M.S., Franco D., Camarda V., Gullace R., Rizzo M.G., Fragalà M., Licciardello G., Catara A.F., Guglielmino S.P.P., 2014, Integrated microbial process for bioconversion of crude glycerol from biodiesel into biosurfactants and phas, Chemical Engineering Transactions, 38, 187–192.

Patwardhan P., Srivastava A.K., 2008, Fed-batch cultivation of Wautersia eutropha, Bioresource Technology, 99, 1787–1792.

Rai R., Keshavarz T., Roether J.A., Boccaccini A.R., Roy I., 2011, Medium chain length polyhydroxyalkanoates, promising new biomedical materials for the future, Materials Science and Engineering: R: Reports, 72, Elsevier B.V., 29–47.

Riascos C., 2004, Optimal control of bioreactors: a simultaneous approach for complex systems, Chemical Engineering Journal, 99, 23–34.

Steinbüchel A., Lütke-Eversloh T., 2003, Metabolic engineering and pathway construction for biotechnological production of relevant polyhydroxyalkanoates in microorganisms, Biochem. Eng. Journal, 16, 81–96.

Wang Y., Liu S., 2014, Production of (R)-3-hydroxybutyric acid by Burkholderia cepacia from wood extract hydrolysates, AMB Express, 4, Springer Berlin Heidelberg, 1–10, DOI: 10.1186/s13568-014-0028-9>.

Yang J., Huang J., Ni J., 2006, Mathematical modeling of the batch fermentation of Zoogloea sp. GY3 used for synthesizing polyhydroxyalkanoates, Journal of Chemical Technology and Biotechnology, 81, 5, 789–793.