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Growth of *Burkholderia sacchari* LFM 101 cultivated in glucose, sucrose and glycerol at different temperatures

Valkirea Matos Nascimento[1], Luiziana Ferreira Silva[2], José Gregório Cabrera Gomez[2], Gustavo Graciano Fonseca[1]*

[1]Federal University of Grande Dourados/FCBA – Lab. of Bioengineering, Rod. Dourados/Itahum, km 12 – 79804-970 – Dourados, MS – Brazil.
[2]University of São Paulo/Institute of Biomedical Sciences – Dept. of Microbiology, Av. Prof. Lineu Prestes, 1374 – 05508-900 – São Paulo, SP – Brazil.
*Corresponding author <ggf@ufgd.edu.br>

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ABSTRACT: Polyhydroxyalkanoates (PHAs) have attracted major industrial interest as alternatives to conventional plastics. They are produced by several bacteria as cytoplasmic inclusions when nutrients are in limited supply. Among the many factors influencing bacterial growth, the effect of temperature on both specific growth rates and growth yields in terms of carbon source intake is of considerable interest. This study aimed to evaluate the influence of the bacterium *Burkholderia sacchari* LFM 101 on growth and PHA production, using glucose, sucrose or glycerol as a carbon source, at 30 and 35 °C. The results showed that *B. sacchari* cultured with glucose at 35 °C presented both higher productivity and polymer yield in dried cell mass. There were no differences in growth rates (μ_{max}) in sucrose and glucose. The growth conditions studied were not favorable to glycerol consumption due to limitations in the energy supply from glycerol.

Keywords: polyhydroxyalkanoates (PHAs), metabolism, kinetics

Introduction

Polyhydroxyalkanoates (PHAs) are biopolymers that can be accumulated in the form of cytoplasmic inclusions by several bacteria. Potential applications of the PHAs vary due to the diversity of possible monomer combinations (Mendonça et al., 2013; Rodríguez-Contreras et al., 2015). Polyhydroxybutyrate (P3HB) is one of the most studied representatives of the PHA family. It has attracted attention because of its biodegradability, sustainability, durability and plasticity. It has properties similar to polypropylene (Mousavioun et al., 2013).

Burkholderia sacchari was reported as a Gram-negative bacterium with significant potential for industrial production of bioplastics (Silva et al., 2000; Squio and Aragão, 2004). The strain *B. sacchari* LFM 101 was isolated from Brazilian sugarcane plantation soil (Brämer et al., 2001). Bacterial PHAs synthesis normally occurs more expressively when there is carbon source in excess and, simultaneously, at least one limiting nutrient, e.g. nitrogen, phosphorus or oxygen, essential for growth (Singh and Parmar, 2011). Once the polymer is synthesized as energy reserve, bacteria can later consume it when other available carbon sources have been exhausted. Thus, it is extremely important to understand the physiological behavior of bacteria.

The PHA-production cost is highly dependent on the price of the substrate (Lopes et al., 2014). As a consequence, PHAs are substantially more expensive than synthetic plastics. Sugarcane sucrose is a low cost alternative substrate. The integration of PHA production into sugarcane and ethanol mills can constitute a green cycle for bioplastic production (Nonato et al., 2001). Likewise, the use of residual glycerol from biodiesel production might foment low-cost PHA production (Squio and Aragão, 2004; Zhu et al., 2010).

There are studies in the literature on *B. sacchari* and PHA production from different substrates, but, for the mostpart, cells were grown at 30 °C (Silva et al., 2000; Mendonça et al., 2013; Lopes et al., 2014). Temperature is an important condition for bioprocesses. Changes in temperature may result in different microbial responses (Fonseca et al., 2013). Thus, this study aimed to evaluate the influence of *Burkholderia sacchari* LFM 101 on growth and PHA production, using glucose, sucrose or glycerol as carbon sources, at 30 or 35 °C.

Materials and Methods

Microorganism and preservation

Burkholderia sacchari LFM 101 were obtained lyophilized. Cells were reactivated by cultivation in 125 mL-Erlenmeyer flasks shaken with 50 mL of nutrient broth (3 g L^{-1} of meat extract; 5 g L^{-1} of peptone) (24 h, 30 °C, 2.2 × g) and cryopreserved by the addition of 20 % glycerol solution in the same proportion as the medium. The final solution (glycerol, 10 % v v^{-1}) containing the cells was distributed into 2 mL sterile microtubes, which were kept in a domestic freezer (-18 °C, 40 min) before being stored at -80 °C in an ultrafreezer.

Pre-cultures were prepared by transferring a loop from the frozen stock vial to a Petri plate containing nutrient broth agar (48 h, 30 °C). A loop from the plate was transferred to a 500 mL-Erlenmeyer flask containing 250 mL of mineral medium (as described below) added from each carbon source. After 16-18 h of growth in an orbital shaker (3.5 × g) at 30 or 35 °C (depending on the temperature of the subsequent cultivation, which was the same), an aliquot (calculated OD_{600} = 0.1) was utilized as inoculum for the main cultivation in the same synthetic medium, as described below.

Culture medium

Cultivations were performed in a defined mineral medium, containing (per liter of distilled H_2O): KH_2PO_4, 6.67 g; $(NH_4)_2HPO_4$, 4 g; $MgSO_4.7H_2O$, 0.8 g; $C_6H_8O_7.H_2O$, 0.8 g; thiamine, 0.01 g and trace element solution, 0.5 mL. The composition of the trace element solution was (per liter of 5 M HCl): $FeSO_4.7H_2O$, 10 g; $CaCl_2$, 2 g; $ZnSO_4.7H_2O$, 2.2 g; $MnSO_4.4H_2O$, 0.5 g; $CuSO_4.5H_2O$, 1 g; $(NH_4)_6Mo.7O_24.4H_2O$, 0.1 g; $Na_2B_4O_7.10H_2O$, 0.02 g (Lee and Choi, 2001). All medium components, except the carbon source, and thiamine were sterilized by autoclaving at 121 °C for 15 min. The solution containing the carbon source (glucose, sucrose or glycerol, 10 g L^{-1}) was autoclaved separately and thiamine sterilized by filtration. All components were mixed after reaching room temperature.

Cultivations

Cultivations were performed in triplicate in 500 mL Erlenmeyer flasks with 250 mL working volume and started by adding a certain volume of the pre-culture, so that the initial cell concentration in the flask was 0.1 absorbance units at 600 nm (Abs_{600}). Cultivation conditions were 30 or 35 °C, initial pH of 6.0 in an orbital shaker at 3.5 × g.

Samples (maximum 4 mL every 1 h) were collected regularly from the flasks in centrifuge tubes in an ice-water bath; 2 mL were used for $OD_{600\ nm}$ measurement, after appropriate dilution (when necessary). The remaining 2 mL was centrifuged (17,609.0 × g, 5 min, 5 °C). The supernatant was frozen at -80 °C and later used for determining the concentration of extracellular metabolites. The pellet was utilized to determine the biomass concentration, by mass difference, in terms of grams of dry biomass weight per culture. The pH was obtained by potentiometric measurements (Hanna).

Biomass determination

The biomass pellet obtained after sample centrifugation was dried in an oven (105 °C) until constant weight. The dried cell mass (g L^{-1}) was obtained by the quotient of the difference in weight by the volume of centrifuged medium. Biomass concentration (X) was also indirectly determined via optical density (OD) measurements taken with a spectrophotometer at 600 nm. For this purpose, the absorbance values measured were converted into mass values using a linear relationship (OD units per gram dry cell mass) determined for each experiment.

Determination of kinetic parameters

The exponential growth phase (EGP) was identified as the linear region on an ln (X) vs. time plot for batch cultivation data. The maximum specific growth rate (μ_{max}) was determined as the slope of this linear region. The biomass yield from substrate ($Y_{X/S}$) was determined as the slope of the line on an X vs. S plot, exclusively including points belonging to the EGP. The specific rate of substrate consumption (μ_S) was calculated by the quotient of μ_{max} by $Y_{X/S}$. The maximum biomass concentration (X_{max}) was indicated by the maximum dried cell mass concentration or $OD_{600\ nm}$ observed in each experiment. The remaining biomass X (R) was identifed by the difference between total biomass and accumulated polymer.

Polymer extraction

On reaching the stationary phase, experiments were stopped, centrifuged (202.9 × g, 40 min), and the biomass pellet was dried in an oven (40 °C, 24 h). Extraction was performed by using a Soxhlet extractor with chloroform as solvent, during a 12 h flow. Then the solution was concentrated and the polymer precipitated with a 95 % ethanol solution.

Determination of extracellular metabolite concentration

Glucose, glycerol, ethanol and organic acids were separated as described elsewhere (Fonseca et al., 2007), with some modification. They were determined by high performance liquid chromatography (HPLC) equipped with an Aminex HPX-87H ion-exclusion column (300 × 7.8 mm). The column was eluted at 55 °C using water acidified with trifluoroacetic acid (TFA) at 0.005 M as mobile phase, at a flow rate of 0.6 mL min^{-1}. These compounds were detected by a UV-absorbance detector at 254 nm connected in series with a differential refractometer detector (RID) coupled to a data acquisition module.

PHA composition

PHA composition was determined by gas chromatography of propyl esters, as described elsewhere (Mendonça et al., 2013). A volume of 1 mL of organic phase was analyzed after fractionation of the sample (split 1:25) in a gas chromatograph equipped with an HP-5 column (5 % phenyl methyl siloxane, 30 m in length, 0.25mm in diameter, and 0:25 µm in film thickness). Analysis was conducted under the following conditions: carrier gas helium (0.8 mL min^{-1}); injector temperature of 250 °C; detector temperature of 300 °C; detection system by flame ionization detector (FID), with the oven set at 100 °C for 1 min, with a temperature increase up to 185 °C at 8 °C min^{-1}, thereafter maintained at 185 °C for 15 min. Benzoic acid was used as the internal standard.

Statistical analysis

The Statistica 8.0 software (Stat Soft, USA) was used to calculate the analysis of variance (ANOVA). The Tukey test was used to determine differences between the variables' temperature and substrate, with a 95 % confidence interval.

Results and Discussion

Table 1 shows the kinetic parameters obtained from the experiments with glucose, sucrose and glycerol as unique carbon sources, at 30 and 35 °C (glycerol only at 30 °C).

Table 1 – Kinetic parameters of the cultivations with *Burkholderia sacchari* LFM 101 from glucose, sucrose and glycerol at 30 and 35 °C.

S_o	T	CF	μ_{max}	X_{max}	DT	P_X	P_{PHA}	μ_S	$Y_{X/S}$	PHA	3HB	3HB	X (R)
	°C		h^{-1}	g L^{-1}	h	g L^{-1} h^{-1}	g L^{-1} h^{-1}	h^{-1}	gDW g^{-1}	% DCW	mol %	g L^{-1}	g L^{-1}
GLU	30	0.2	$0.526^a \pm 0.007$	$1.874^b \pm 0.004$	$1.397^b \pm 0.129$	$0.103^b \pm 0.005$	$0.041^b \pm 0.002$	$2.701^a \pm 0.089$	$0.194^c \pm 0.003$	$39.58^a \pm 1.266$	100	0.742	1.132
	35	0.2	$0.539^a \pm 0.036$	$2.878^a \pm 0.001$	$1.288^b \pm 0.086$	$0.130^a \pm 0.005$	$0.054^a \pm 0.002$	$1.780^b \pm 0.019$	$0.303^a \pm 0.013$	$41.20^a \pm 0.898$	100	1.186	1.692
SUC	30	0.2	$0.544^a \pm 0.005$	$1.892^b \pm 0.116$	$1.274^b \pm 0.012$	$0.104^b \pm 0.006$	$0.027^c \pm 0.001$	$2.614^a \pm 0.131$	$0.208^{bc} \pm 0.008$	$25.93^b \pm 2.496$	100	0.490	1.401
	35	0.2	$0.546^a \pm 0.003$	$2.654^a \pm 0.076$	$1.269^b \pm 0.007$	$0.131^a \pm 0.004$	$0.044^b \pm 0.001$	$1.957^b \pm 0.197$	$0.281^{ab} \pm 0.037$	$33.12^{ab} \pm 5.989$	100	0.879	1.775
GLY	30	0.5	$0.408^b \pm 0.010$	$0.457^c \pm 0.026$	$1.698^a \pm 0.041$	$0.041^c \pm 0.003$	ND	ND	ND	ND	ND	ND	ND

GLU: glucose; SUC: sucrose; GLY: glycerol; T: temperature; S_0: initial substrate; DT: doubling time; μ_{max}: maximum specific growth rate; X_{max}: maximum biomass concentration; PHA: polyhydroxyalkanoate; DCW: dry cell weight; μ_S: specific rate of substrate consumption; $Y_{X/S}$: specific rate of substrate consumption; 3HB: 3-hydroxybutyrate; CF: conversion factor of optical density (600 nm) into biomass; R: remaining; ND: not determined. Same letters in the same column were not significantly different ($p \geq 0.05$).

When the maximum specific growth rate (μ_{max}) values obtained with glucose and sucrose were compared, no difference ($p > 0.05$) between them was observed (an average of 0.539 h^{-1}). A differences was seen in the case of glycerol only, whose μ_{max} was 0.408 h^{-1}. The μ_{max} values were higher (except for glycerol) than that previously reported for *Burkholderia sacchari* IPT 101 with glucose as substrate (μ_{max} = 0.45 h^{-1}) (Gomez et al., 1996).

However, the highest PHA productivity (0.054 g L^{-1} h^{-1}) was seen with glucose at 35 °C. Experiments with both glucose and sucrose attained the stationary phase after approximately 19.5 h, but with glycerol it was reached after only 6 h.

The literature shows that *Burkholderia* sp. was able to grow between 25 and 37 °C with 28 to 30 °C as the optimal range (Lopes et al., 2014). In our study, 35 °C was found to be the most favorable temperature for maximal X_{max} and P_x parameters for the *Burkholderia sacchari* strain.

The highest polymer yield was obtained with glucose at 35 °C (41 %), a value greater than the results obtained by Mendonça et al. (2013) with the same strain after 24 hours of cultivation (32 % PHA in its cell dry weight), using glucose (10 g L^{-1}) as the carbon source. It was also observed that, up to 24 hours of culture, the bacteria used the carbon and nitrogen sources for growth, and that after 24 hours, once the nitrogen had been exhausted, the excess of carbon present was used for PHA accumulation.

In this study, experiments were interrupted at the beginning of the stationary phase of growth because this coincided with the depletion of the carbon source, so there was no excess carbon for polymer accumulation (Figure 1).

It is observed that PHA productivity on sucrose at 35 °C is equivalent to that obtained on glucose at 30 °C (Table 1). As reported by Squio and Aragão (2004), *Burkholderia sacchari* has great potential for industrial production, mainly because of its ability to consume sucrose. This potential was confirmed by Gomez (2006) in his study where *Burkholderia sacchari* IPT 101 (= LFM 101) was able to accumulate 75 % of its dry weight in

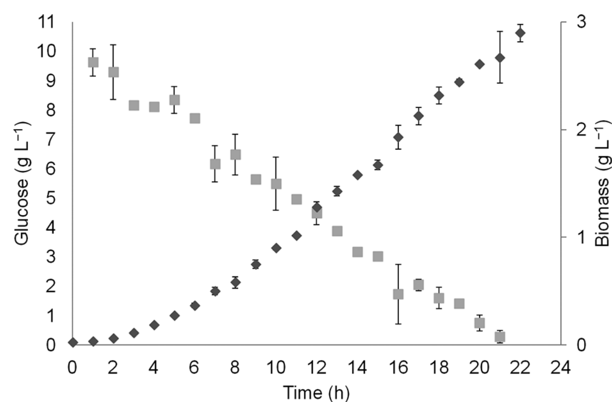

Figure 1 – Growth kinetics of Burkholderia sacchari LFM 101 with glucose as carbon source at 35 °C.

P3HB from glucose plus fructose and 69 % from sucrose, with an efficiency higher than 80 % of the maximum theoretical yield in 48 hour cultivations. Thus, it may be considered that, although a slightly smaller yield was observed on sucrose than on glucose, sucrose is still a very attractive carbon source for polymer production.

The type of polymer synthesized by the bacteria depends on the substrate used, as it determines which pathway will be utilized on it. In this study, for the substrates sucrose and glucose, P3HB was the sole PHA monomer accumulated by *Burkholderia sacchari* LFM 101. There was no expressive production (> 0.1g L^{-1}) in any of the evaluated metabolites.

The parameters related to substrate consumption were not calculated for the glycerol substrate. It was not considered feasible to continue with the analysis, since there was neither a significant glycerol consumption nor biomass formation. X_{max} obtained was extremely low (0.46 g L^{-1}) (Table 1). In a previous study using synthetic medium with glycerol as the sole carbon source, cell growth was also found in yeast cultures, even when working with strains capable of consuming glycerol. However, growth was observed when yeast extract was added to the culture medium, which was related to trace compounds present

in the yeast extract that would be essential for an effective assimilation of glycerol by yeasts (Rivaldi et al., 2012).

When glycerol is utilized as the sole carbon source, it is initially metabolized to dihydroxyacetone phosphate (Figure 2). For this, there are two possible pathways: one is dehydrogenation and the other phosphorylation. The first one is NAD^+ (oxidized nicotinamide adenine dinucleotide) and ATP (adenosine triphosphate) dependent, the second one is ATP and FAD (flavin adenine dinucleotide) dependent (Rivaldi et al., 2012). Then, dihydroxyacetone phosphate is directed at energy production and/or PHA biosynthesis.

The glycerol assimilation is dependent on external electron receptors. The carbon source must ensure the overall redox balance within the cell. Therefore, the choice of the carbon source becomes a determinant factor if satisfactory product yields are to be obtained (Clomburg and Gonzalez, 2013).

Thus, in the experiment conducted with *Burkholderia sacchari* LFM 101 with glycerol as the sole carbon source, the poor growth could be attributed to no significant growth being observed due to the lack of energy (NAD^+; FAD; ATP) for breaking the glycerol molecule. The small biomass production is probably due to the consumption of the other nutrients present in the mineral medium, e.g. citric acid.

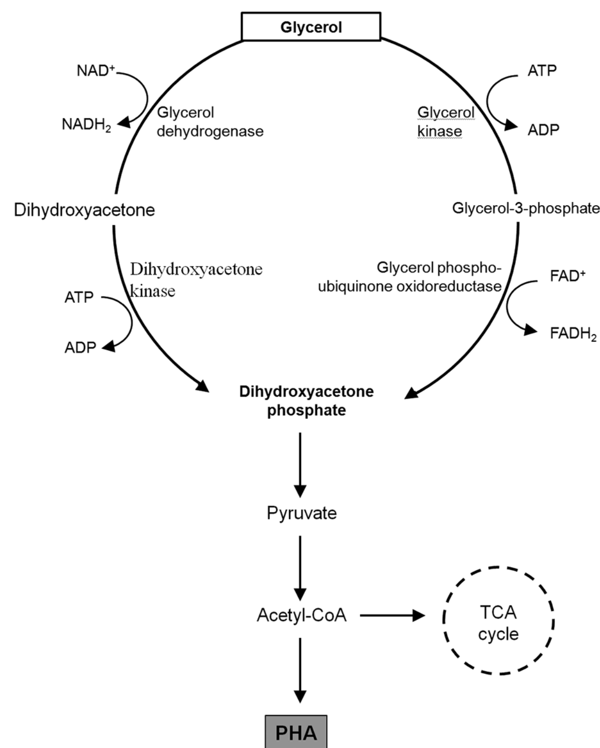

Figure 2 – Metabolic pathways for glycerol assimilation by microorganisms and polyhydroxyalkanoate (PHA) formation (adapted from Rivaldi et al., 2012).

A PHA yield of 10 % for *Burkholderia sacchari* on glycerol (Rodríguez-Contreras et al., 2015) has been published elsewhere. However, glucose was utilized in the initial stages of cultivation, and glycerol as the sole carbon source only in the phase of PHA accumulation. Thus, it can be concluded that it is possible to produce polymers and cells from glycerol if cultivation is supplemented with another substrate that has the necessary energy for breaking glycerol molecules.

Conclusion

It was found that the *Burkholderia sacchari* LFM 101 presented the best results in cultures containing glucose at 35 °C. Although higher productivity was observed with glucose, sucrose revealed great potential for polymer accumulation by the bacterium under higher temperatures. The temperature did not influence the maximum specific growth rates (μ_{max}), which remained around 0.54 h^{-1} for both the glucose and sucrose substrates. However, *B. sacchari* showed greater accumulation of polymer at 35 °C, indicating that the temperature adjustment is important for optimizing the production of biopolymer from *B. sacchari*. The growth conditions analyzed were not favorable to glycerol consumption probably due to limitations in the energy supply from glycerol.

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