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INFLUENCE OF GLYCEROL ON POLY(3-HYDROXYBUTYRATE) PRODUCTION BY *CUPRIAVIDUS NECATOR* AND *BURKHOLDERIA SACCHARI*

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Abstract

Glycerol is a co-product of many industrial processes and is generated in large quantities from different origins. In this study, glycerol is used as a cheap carbon source for the production of poly(3-hydroxybutyrate) (PHB) with two different collection strains, *Cupriavidus necator* and *Burkholderia sacchari*, in order to provide an alternative outlet for glycerol and produce value-added bioproducts. The objective of this work was to study the influence of this carbon source on their growth kinetics, on their polymer production, and on the molecular mass of the produced biopolymer. Therefore, fermentations in bioreactors were carried out with these strains. Different results for both strains were obtained showing, for the first time, a high cell dry mass and growth rate, when glycerol was used together with glucose in the fermentation with *C. necator*. In the first fermentation with *B. sacchari* using glycerol as a sole carbon source, the strain properly developed synthesising PHB. The biopolymers obtained from both fermentations with glycerol showed low molecular masses about 300 kDa with a polydispersity of 4.72 with *C. necator*, and 200 kDa with polydispersity of 2.50 with *B. sacchari*.

Keywords: *Cupriavidus necator, Burkholderia sacchari,* poly(3-hydroxybutyrate), glycerol, bioreactor, molecular mass

1. Introduction

Poly(3-hydroxybutyrate) (PHB) is the most widely studied member of the polyhydroxyalkanoates (PHAs). These are biopolyesters synthesized by numerous microorganisms as energy reserve materials when an essential nutrient is limited [1]. They possess properties similar to various synthetic thermoplastics like polypropylene, and can also be reutilized by proper microorganisms as internal carbon substrate when the supply of the growth-limiting nutrient is provided again [2]. Microbial production of PHB is predominantly investigated in the bacterium *Cupriavidus necator,* which can store PHA up to 96% of its cell dry mass (CDM) under conditions of nitrogen or phosphate limitation and excess of carbon source. It is used as a PHA producer on an industrial scale [3]. On the other hand, *Burkholderia sacchari* (DMS 17165) is a less studied strain in this field, but some studies already show that the strain is a good polymer producer [4, 5, 6, 7]. It is a Gram-negative bacterium isolated from the soil of a sugar-cane plantation in Brazil and found to accumulate up to 68% of the CDM as PHB with sucrose as the sole carbon source [4].

Industrial PHB production by microorganisms has been widely attempted but there is still a need to overcome the problem of its high production cost, so that it can be economically competitive with conventional plastics [4]. The main factors that increase these biopolymer production costs are reported by Choi and Lee [8]. Among the current approaches to decrease these costs, the search of new strains capable of synthesizing biopolymer improving the productivity of the process [9, 10] or the replacement of the carbon source for cheaper ones are the main strategies studied [11]. It was pointed out that the raw materials claim the major part of the production cost for biopolymers [12]. Therefore, the substitution of pure sugars as substrates such as glucose or sucrose by applying cheaper carbon sources as basis feedstock is of importance to enhance the cost efficiency of the production process. Among these substrates, molasses [12], starch [13], whey from the dairy industry [14], the waste water from olive oil production [15], xylose [5], and a broad range of plant oils [16] are available. Glycerol is a co-product of many industrial processes and is generated in large quantities. The large scale production of biodiesel, as an alternative and renewable energy source, already results in a surplus of glycerol [17]. This side carbon compound could be the ideal source for industrial production of PHAs. Taking this into account, it was interesting to examine the availability of this industrial by-product with respect to the microbial PHB production with *C. necator* DSM 545 and *B. sacchari* DSM 17165.

There are already some studies showing that some bacterial and archaeal strains are able to produce low molecular mass PHB using glycerol as a carbon source [18, 19]. On the one hand, when glucose is used as carbon source, it is metabolized to pyruvate *via* the Entner-Doudoroff pathway (2-keto-3-deoxy-6-phosphogluconate pathway). Pyruvate can be converted by a dehydrogenase to acetyl-CoA, the central intermediate of the cellular metabolism, and the starting compound for the PHB synthesis. On the other hand, glycerol can be metabolized to pyruvate as well, but *via* the intermediate compound glyceraldehyde-3-phosphate [12, 20].

In this study, two fermentations in bioreactors were carried out with two different strains from the German strain collection, *C. necator* (DMS 545) and *B. sacchari* (DMS 17165). In both cases, glycerol was used as an inexpensive carbon source during the phase of predominant PHA accumulation. The objective was to study glycerol's influence on the growth kinetics of the above mentioned strains, on their polymer production performance, and on the molecular mass of the produced biopolymer in order to assess an alternative outlet for glycerol and generate value-added of biopolymers.

2. Materials and methods

2.1. Microorganisms - *Cupriavidus necator* DSM 545 (formerly known as *Wautersia eutropha*, *Ralstonia eutropha*, *Alcaligenes eutrophus* and *Hydrogenomonas eutropha*) and *Burkholderia sacchari* DSM 17165 were obtained from *Deutsche Sammlung von Mikroorganismen und Zellkulturen* GmbH, Germany.

2.2. Materials - All simple salts and chemical products were at least technical grade and obtained from Sigma-Aldrich Chemical Company. ROTIPURAN® (≥86 % glycerol from Carl Roch, Germany) was used as glycerol (density of 1.26 g/cm^3).

2.3. Culture media - The strains were cultivated in a mineral medium (M), containing (g/L): $Na_2HPO_4{\cdot}2H_2O$, 4.5; KH_2PO_4, 1.5; $MgSO_4{\cdot}7H_2O$, 0.8; NaCl, 0.9; $(NH_4)_2SO_4$, 2; $CaCl_2{\cdot}2H_2O$, 0.02; NH_4Fe(III) citrate, 0.05; agar, 10; trace element solution SL6, 1 mL; glucose, 15; adjusted to a pH value of 7,0. SL6 was composed as follows (mg/L): $ZnSO_4{\cdot}7H_2O$, 100; H_3BO_3, 300; $CaCl_2{\cdot}6H_2O$, 200; $CuSO_4$, 6; $NiCl_2{\cdot}6H_2O$, 20; $Na_2MoO_4{\cdot}2H_2O$, 30; $MnCl_2{\cdot}2H_2O$, 25. The batch growth medium for the bioreactors consisted of (g/L):

KH_2PO_4, 4.3; NaCl, 0.8; $(NH_4)_2SO_4$, 3; $MgSO_4{\cdot}7H_2O$, 0.8; $CaCl_2{\cdot}2H_2O$, 0.02; NH_4Fe(III) citrate, 0.05;

glucose, 20; and trace element solution SL6, 3 mL. All media were adjusted to a pH value of 7.0. The components susceptible to precipitation at sterilization conditions were sterilized separately (21 min-120 ºC). All media used for *B. sacchari* DSM 17165 required no NaCl [1, 21].

2.4. Precultures and inoculum - Precultures of the strains were first inoculated from solid medium grown for 24 hours. The first seed cultures were incubated overnight in 300 mL shake flasks containing 100 mL of the medium at 30 ºC for *C. necator* DSM 545 and at 37 ºC for *B. sacchari* DSM 17165, and with a pH value of 7.0 in a rotary shaker at 120 rpm. Glycerol (15 g/L) was used in the precultures with *B. sacchari* DSM 17165 to pre-adapt it to the carbon source. The inoculums for the bioreactors were prepared from 5 mL of these precultures, which were then transferred to 1000 mL shake flasks containing 250 mL of the medium and incubated under the same conditions as the precultures. One and half liters of inoculum cultures were used to inoculate the bioreactors with *C. necator* DSM 545 and *B. sacchari* DSM 17165, respectively.

2.5. Bioreactors operating – The two strains were cultivated separately using a medium with a common carbon source, glucose, but combining it with a cheaper one, glycerol, in order to provide it an alternative

outlet and generate value-added PHB. Different fermentation strategies were used depending on the strain requirements. The fermentations with *C. necator* DSM 545 was adapted by combining glycerol and glucose for the re-feeding in the growth phase, while the re-feeding on the fed-batch fermentations with *B. sacchari* DSM 17165 was carried out only with glycerol. The fermentation with *C. necator* DSM 545 was carried out in Labfors 3 bioreactors (Infors AG, Bottmingen, Switzerland). It had a total volume of 7.5 L with a working volume of 5 L and 1 L of inoculum. The fermentation with *B. sacchari* DSM 17165 was carried out in a smaller stirred tank reactor of Labfors 3 (Infors AG, Bottmingen, Switzerland). It had a total volume of 3.6 L with a working volume of 2.4 L. For this experiment a total volume of 1.5 L was utilized with 0.5 L of inoculum. Temperatures of the cultures were 30 (DSM 545) and 37 ºC (DSM 17165), and the pH-values were maintained at 6.8 and 7.0, respectively. All relevant fermentation parameters (temperature, pH-value and consumption of pH correction solutions, flow rate, dissolved oxygen concentration, stirrer speed, and antifoam activity) were monitored and recorded by an IRIS software program. The water temperature and pH were constantly controlled by a sensor (pt-100 Labfors, Infors AG, Bottmingen, Switzerland) and pH-electrode (Hamilton, Switzerland). The pH-value was maintained at the required pH-value by automatic addition of 25% ammonia hydroxide (at the same time alkaline pH correction solution and nitrogen source for microbial growth), 10% sodium hydroxide (alkali for the reactors when the nitrogen source was limited) or 10% sulfuric acid solution. The concentration of the dissolved oxygen was achieved by automatic adjustment of the stirrer speed and the air flow rate. These were used to monitor the cell activity and were initially adjusted to 5 L/min and 500 rpm, respectively. Dissolved oxygen concentration was maintained at 40% of air saturation in water and was monitored by the oxygen partial pressure (pO_2) (Ingold sensor). Oxygen was supplied at 150 L/h through an absolute filter (Sartorius, Midisart 2000). The concentration of the dissolved oxygen was followed by an oxygen electrode (Hamilton, Switzerland) and was calibrated inside the bioreactor using N_2 for the 0 value. Foam formation was controlled by an electrode and the antifoam agent Struktol J700 was added automatically in order to avoid heavy foam formation. Glucose was added from a concentrated solution of 50 % (w/v) during both fermentations to avoid the carbon source limitation and to maintain the activity of the cells according to the fermentation requirements. In the accumulation phase, glycerol was also added from a 100% sterile solution.

2.6. Determination of the cell dry mass (CDM), residual biomass (RB) and PHB content - Samples of 10 mL of culture broth were taken throughout the fermentations and centrifuged in pre-weighed glass screw-cap tubes for 15 min at 4000 rpm (Megafuge 1.0R Heraeus Sepatech). The pellet was frozen, lyophilised and weighed to determine the CDM by weight difference. The PHA in lyophilized biomass samples was transesterificated by acidic methanolysis following Braunegg's method [22]. Analyses were carried out with Agilent Technologies 6850 gas chromatograph (30-m HP5 column, Hewlett-Packard, USA; Agilent 6850 Series Autosampler). The methyl esters of PHA constituents were detected by a flame ionization detector with helium as a carrier gas (split- ratio of 1:10). P(3HB-co-19.1%-3HV) (Biopol; Imperial Chemical Industries) was used for 3HB and 3HV calibration and hexanoic acid was used as an internal standard. The PHB content

(wt %) was defined as the percentage of the ratio of 3HB concentration to CDM. The RB was then calculated as the difference between cell concentrations and the PHB content.

2.8. Carbohydrate determination - Carbohydrate concentration from supernatant was monitored by means of HPLC equipment composed of a thermostated Aminex HPX 87H column (thermostated at 75 ºC, Biorad, Hercules, USA), a LC-20AD pump, a SIC-20 AC autosampler, a RID-10A refractive index detector and a CTO-20 AC column oven. Also the LC solution software for registration and evaluation of the data obtained was used. One and half mL of liquid media was sterile filtrated and transferred into vials. Water was used as an eluent at a flow rate of 0.6 mL/min. Standards of different concentrations of glucose or glycerol, respectively, were prepared.

2.9. Determination of nitrogen source – Two mL of supernatant was mixed with 50 µL alkaline ISAB solution containing 5 M sodium hydroxide, 10 % methanol, 0.05 M Na_2-EDTA and a colour indicator. The mixture was immediately analysed with an Orion ion selective electrode; the signal was monitored by a voltmeter. The standard curve was calculated measuring different ammonium sulphate standards solutions of defined concentrations.

2.10. PHB isolation - At the end of the fermentations, the biomass was pasteurized increasing the temperature of the bioreactor up to 70 ºC for 30 min. The culture broths were centrifuged for 30 min at 4°C and 6000 rpm in a Sorvall® RC-5B Refrigerated Superspeed centrifuge (DuPont Instruments); the obtained cell pellets were frozen and lyophilized. The dry biomasses were stirred with a tenfold quantity (mass) of ethanol for 24 hours at room temperature for removing the lipid components. Degreased cell masses were separated from the ethanol solution by vacuum filtration. The remaining cell masses were air-dried and stirred with a thirtyfold quantity of chloroform for 24 hours. The solutions of PHA in chloroform were separated from the residual cell masses *via* vacuum-assisted filtration. Afterwards, the major part of the chloroform was removed by rotary evaporator until viscous solutions were obtained (concentration of biopolymer approximately 40 g/L). The PHA was precipitated by adding a tenfold amount of ice-cooled ethanol, separated by vacuum filtration and left at room temperature for the remaining solvent to evaporate [21].

2.11. Polymer characterization - The chemical structure was characterised with a Perkin Elmer Fourier Transform Infrared (FTIR) using optical Perkin Elmer software. The line-scan spectra were based on 32 scans and a resolution of 4 cm^{-1}. ^1H NMR spectra were recorded at 25 °C on a Bruker AM300 spectrometer. The polymer samples were dissolved in chloroform and a drop of TMS (tetra methyl silane used as internal standard for calibrating chemical shift for ^1H) was added as reference. Ten mg of the sample dissolved in 1 mL of deuterated solvent was used. Proton spectra were recorded at 300.1 MHz with a spectrum of 32 K data points. A total of 64 scans were utilized with a relaxation delay of 1 second. The GPC measurements were performed utilising chloroform as an eluent at a flow rate of 0.80 mL/min with a stabilisation pressure of 35 bars and a sample concentration of 1.5 mg/mL. A Waters Styragel HT column for mid-range molecular-mass distributions was used and samples of polystyrene with different molecular masses were used as standard. DSC experiments were performed on a Perkin-Elmer Pyris 1 instrument with a dry nitrogen gas flow of 50

mL/min. The apparatus was calibrated using Indium of high purity. Approximately, 5 mg of the sample was sealed in an aluminium planched and analyzed. The melting temperature (T_m), melting enthalpy (ΔH_m) and the glass transition temperature (T_g) were determined by the second heating run of DSC endothermic peaks. The crystallinity degree (X_c) of P(3HB) was calculated assuming that the ΔH_m value of 100% crystalline P(3HB) is 146 J/g [23]. Scans started at -30 ºC and were ramped at 10 ºC/min to 230 ºC.

3. Results and Discussion

3.1. Fermentation with *Cupriavidus necator* DSM 545 – *C. necator* DSM 545 was cultivated in fed-batch fermentation in a 7L-bioreactor to study the glycerol influence on the growth phase. Glucose and glycerol were co-supplemented in the PHA production phase for the biosynthesis of PHB. The evolutions of the CDM, RB and PHB content are shown in Figure 1a. The CDM concentration increased throughout the fermentation, initially, due to the strain growth phase, and later, in the stationary phase, because of the biopolymer accumulation. The strain required 8.5 hours of adaptation, and then it grew until around 19 hours with a specific maximum growth rate (μ_{max}) of 1.21 h^{-1}. The polymer production increased until the end of the fermentation, achieving a volumetric productivity of 0.76 g/L·h and a maximal PHB content of 64.55% in CDM (Table 1). The biopolymer accumulation was induced by the interruption of the nitrogen source supply after 18.5 hours and it got limited after 25.5 hours of cultivation (Figure 1b). Its limitation acts as an initiator for PHA production due to the formation of proteins (RB) stops and the carbon is transferred to PHB synthesis [14]. Figure 1b shows the time courses of the carbon sources, glucose and glycerol. A common concentration of 20g/L of glucose was initially used for *C. necator* due to the fact that this concentration is generally known as the optimum concentration for this strain at the beginning of the cultivation process. The amount of glucose (black dots in Figure 1b) was varied along the fermentation depending on the requirements of the strain to grow ("Fed-batch feeding strategy"). At the point when glucose was first limited (after 30.5 hours of cultivation) the cells activity decreased, requiring its addition, thus causing the reactivation of the cells. It was consumed together with glycerol in the stationary phase. Later, after 4 more re-feedings, glucose was limited again after 50 hours of fermentation. However, the cell activity remained constant until the end of the cultivation. This could be explained because at this point the strain was already adapted to the use of glycerol as a sole carbon source. Glycerol was added only one time (48 g/L) and it was constantly consumed. Its consumption by *C. necator* DSM 545 was very slow compared to the consumption of glucose. Results of this fermentation can be compared to previous studies. Initially, Bormann et al. [24] used *C. necator* to produce PHA in a 2.5 L fermenter with glycerol and casein, obtaining 0.17 g/g of polymer conversion. In later studies, glycerol and different concentrations of salts were used together with yeast extract as a carbon source for fermentation in a 2 L bioreactor, obtaining a polymer conversion of 0.30 g/g from crude glycerol [17]. In the studies carried out by Cavalheiro et al. [25], different types of glycerol were used together with glucose as carbon sources in a 2 L bioreactor. Polymer conversions of 0.34 and 0.37 were obtained. In all reported cases, the carbon substrate was substituted by glycerol, and the conversion results considered only for glycerol. In this study, however, both glucose and glycerol were used together throughout the fermentation process, according to the requirements of the strain, reaching a PHB yield of 0.34 g/g from the combination of carbon

sources, glucose and glycerol. Nevertheless, if the conversion results of this fermentation are separately analysed, the PHB yields from glucose and from glycerol were 0.26 g/g (between 0 and 19 hours of fermentation) and 0.75 g/g (between 50 and 57.5 hours of fermentation), respectively. This last result is much higher compared to the previous mentioned studies for glycerol as sole carbon source. Regarding PHB production, *C. necator* JMP 134 was used to synthesize PHB in Mothes et al. [17] studies, attaining around 70% of PHB in CDM from pure glycerol. However, only 0.13 h^{-1} of maximum specific growth rate was reached. In the experiments of Cavalheiro et al. [25] with *C. necator* DSM 545 and commercial glycerol, a specific growth rate of 0.11 h^{-1}were attained with maximum PHB content of 62%. When waste glycerol was used instead, PHB maximal accumulation was 38% in CDM and a specific growth rate of 0.15 h^{-1}. The results attained in this fermentation are higher from those reported (Table 1). It is important to point out that a high growth rate of 1.21 h^{-1} was obtained in this fermentation by the initial use of glucose as a carbon source. Together with the results of the volumetric productivity, maximal PHB content and PHB conversion, it could be concluded that the combination of both carbon substrates could be a great strategy for PHB production since higher growth rates and the use of cheaper feedstock are advantageous with respect to the reduction of production costs.

3.2. Fermentation with *Burkholderia sacchari* DSM 17165 - Fed batch fermentation in a 3L-bioreactor was carried out in this work with the strain *B. sacchari* DSM 17165, utilizing glycerol as an only carbon source in the accumulation phase. The evolutions of the CDM, PHB content and RB curves along this fermentation are shown in Figure 2a. The curves show a constant growth of the strain during the entire fermentation. It synthesised PHB almost constantly during the fermentation, although the nitrogen source was not totally limited until 40 hours of fermentation. PHB was accumulated in 10% of the CDM at the end of the fermentation, reaching the maximal value and the volumetric productivity 0.08 g/L·h.The nitrogen source limitation was attained at 39.75 hours of fermentation, and at this point a slight increase in the PHB accumulation can be observed. Evolutions of the nitrogen and carbon sources (glycerol and glucose) as well as the time of their additions are shown in Figure 2b. In the *B. sacchari* precultures, 15 g/L glycerol was used in order to adapt the strain to the carbon source according to pre-experiments on shaking flask scale that indicated a slightly inhibiting effect of glycerol already at concentrations around 20 g/L. In these pre-experiments, 15 g/L of glycerol turned out to be suitable for this organism. Glucose was the initial carbon substrate and was first consumed as a sole carbon source until around 10 hours of fermentation. It was added twice along the cultivation to support both cell growth and polymer accumulation, and was totally limited after 35.5 hours. Thus, both carbon substrates were consumed by the strain between 10 and 35.5 hours of fermentation, the exponential growth phase. For this period of time, the strain shows a specific growth rate of 0.42 h^{-1}. Hereafter, glycerol was the only remaining carbon source in the fermentation, which was added until it was finished, and it was consumed at a constant rate. Its consumption increased in the last part of the fermentation, since it was the only carbon source utilized by the strain from 35.5 hours until the end (0.66 g/L·h). The biopolymer accumulated throughout the last 22 hours of fermentation by *B. sacchari* DSM 17165 was synthesised only from the glycerol source. The total consumption of carbon source was 1.63 g/L·h,

shared between glucose (1.02 g/L·h) and glycerol (0.62 g/L·h). The conversion to CDM and to PHB from 35.5 to 57.25 hours was 0.63 and 0.094 g/g respectively (Table 1).

There are only a few studies where the bacterial strain *B. sacchari* is used for the production of PHAs [4, 5, 7]. One of the first fermentations with this strain was carried out by Silva et al. [5]. It used xylose plus glucose under phosphate limitation, reaching a PHB-content of 62%, and conversion yield of 0.22 g/g. Regarding PHB accumulation, this strain showed better results with phosphate limitation if compared to nitrogen limited conditions. Recently, Pradella et al. [7] carried out cultivation with sucrose as a carbon source for PHB production by *B. sacchari* IPT 189 *via* nitrogen limitation to induce the polymer accumulation. Up to 42% of polymer content, a biomass concentration of around 150 g/L·h, a productivity of 1.7 g/L·h, and a conversion yield from sucrose of 0.22 g/g were achieved. Since this is the first attempt for cultivating this strain on glycerol for PHA production, it is not possible to find similar studies to compare it to. The only similar work was carried out with *B. cepacia* and glycerol as a carbon substrate by Zhu et al. [26], obtaining a 5.8 g/L of CDM at the end of the shake flask cultivation and 81.9% PHB of CDM using 3% of biodiesel-glycerol as feedstock over 96 hours of growth. When the glycerol percentage was increased the CDM decreased. Although the polymer content reached by *B. cepacia* is higher than the result obtained in the present work with *B. sacchari*, the fermentation lasted double the time thus negatively impacting the productivity, and the strategy for glycerol addition were different.

3.3. Biopolymer characterization - The biopolymers extracted from each fermentation were characterized by FTIR and ^{1}H NMR to assess their chemical structure, while the determination of the molecular masses and thermal analysis was carried out by GPC and DSC, respectively. FTIR and ^{1}H NMR analyses showed typical bands and signals of PHB for both PHA extracted from *C. necator* DSM 545 and *B. sacchari* DSM 17165 fermentations. FTIR transmission spectra of the PHAs extracted from both bacteria (Figure 3a) showed an intensive band corresponding to the stretching of the carbonyl group at 1726 cm^{-1}, two intense bands of the methyl and methylene groups at 2960-2850 cm^{-1}, a vibration of symmetric torsion of the methyl group at 1390-1370 cm^{-1}, a double band corresponding to the tension of anti-symmetric vibration of the ester group at 1230-1050 cm^{-1}. The characterization by ^{1}H NMR confirmed the structure of PHB homopolymer (Figure 3b). These spectra showed the presence of three groups of signals: a doublet at 1.29 ppm attributed to the methyl group coupled to one proton, a doublet of quadruplet at 2.57 ppm attributed to the ethylene group adjacent to an asymmetric carbon atom bearing a single proton and a multiplet at 5.27 ppm characteristic of the methylene group. The signal observed at 7.25 ppm corresponds to the residual chloroform [27]. It can therefore be concluded that the strains produced PHAs exclusively in the form of PHB. Regarding the GPC results (Figure 4), the PHB extracted from *C. necator* DSM 545 showed a mass-average molecular mass (M_w) of 302,500±10,500 Da with a PDI of 4.72±0.12, and from *B. sacchari* DSM 17165 it was 200,000±980 with a PDI of 2.50±0.43. In general, the molecular masses of PHAs produced from bacteria have a relatively high molecular mass around 200,000 to 3,000,000 Da, depending on the microorganism and the culture conditions [28]. Considering the common high molecular mass of the biosynthesised PHA, the PHB produced from glycerol by both bacteria in this work showed low molecular mass. Such results have been previously

reported when glycerol was used as a carbon source. Initially, the results reported by Madden et al. [29] showed that the molecular mass of the PHB produced by *Ralstonia eutropha* NCIMB 40529 from glycerol was substantially lower than for polymer produced from glucose. Other strains also showed similar results on the molecular masses of the synthesized PHBs when glycerol was used as feedstock. Solaiman and Ashby [30] reported the decrease in the molecular masses of PHA from *Pseudomonas oleovorans* when glycerol was used as a carbon source. In direct comparison, Koller et al. [18] reported that *Haloferax mediterranei* produced polyester with a mass average molecular mass of 250,000 Da from GLP (which contains 70% glycerol). Madden et al. [29] and Taidi et al. [31] concluded that glycerol acts as a chain transfer agent in the chain termination step of the polymerization and this effect is more pronounced with a high concentration of glycerol in the medium (more than 10 g/L). Moreover, the molecular mass of PHA is dependent on the producing bacteria [31]. Already Madden et al. [29] concluded that when glycerol or glycols are present in the medium, these substances cause termination of chain propagation by covalent linking at the carboxyl terminus of the polyester ("end capping"). For certain special fields of application, low molecular mass PHA might be desired, for utilization as softeners or for the design of special polymers such as amphiphilic block copolymers [14, 10]. Zhu et al. [26] used *B. cepacia* in a study where the glycerol concentration was varied. The molecular mass of the polymer decreased when the glycerol amount was increased (3% to 9% of glycerol, Mw decreased gradually from 304,000 Da to 162,000 Da, respectively). In the present work, glycerol was maintained in excess (10 g/L maximal glycerol concentration in the medium) and the molecular mass obtained is found within this range for the PHB extracted from *B. sacchari*. Through the DSC analysis (Figure 4), the values for T_m and X_c of the extracted PHBs were obtained. *Cupriavidus necator* DSM 545 shows a T_m of 173.09 ºC and X_c of 62.53%, while *B. sacchari* DSM 17165 shows lower T_m of 163.32 ºC and higher X_c of 72.82%. These thermal properties are similar to the common bacterial PHBs producers [28, 32]. However, the differences between the thermal parameters of the two PHBs here synthesised can be explained by the different molecular masses, thus different degrees of polymerization. Normally, the reorganization of smaller chains into more compact structures contributes to the increase of the X_c [33]. The biopolymer synthesized by *C. necator* DSM 545 showed a crystallinity degree lower than the PHB from *B. sacchari* DSM 17165 which shows a lower molecular mass and lower chain lengths. On the other hand, the T_m for the PHB produced by *Burkholderia sacchari* DSM 17165 is higher than the T_m of the PHB produced by *C. necator* DSM 545. This is due to the fact that different melting point values are related to polymer fractions (different chain lengths). It is well established in polymer chemistry that an end group acts as an impurity and lowers polymer melting points [27].

3.4. Economical study

The study of the cost comparing both strains and fermentations is very complex since there are many factors to consider. The major cost factor, namely the carbon source amounts to about 50% to the entire PHA production cost. However, glycerol is a cheaper carbon source compared to glucose. Therefore, it is demonstrated in this study that better and cheaper carbon source can be used for both strains to decrease the raw material costs in the PHA production. On one hand, the PHA production costs of the fermentation with *C.*

necator decrease using the combination of glucose with glycerol compared to a conventional fermentation where only glucose is used. On the other hand, the fermentation with *B. sacchari* used only glycerol which decreased the production costs, but producing less amount of PHA. However, the objective of this study was both utilize a low-costly raw material while obtaining PHB of low molecular mass. So, it was demonstrated that this strain is able to produce LMW PHB from a cheap raw material as glycerol is. A rough estimation can be made considering a price of about 0,2-0,4 US-$ per kg of glucose (depending on the source) and the assumption that glycerol constitutes a surplus material available at no cost (similar assumptions for PHA production on surplus whey as C-source [34]. As the second cost factor, the downstream processing for PHA recovery from surrounding biomass is reported. In our present case, the method of downstreaming will be the same for both processes because of the similar cell composition of the two investigated strains, and because of the similar final mass fractions of PHA in cell mass that can be obtained. The only factor that is decisive for the different economic performance of the two processes (of course beside the C-source, see before) is the volumetric productivity. As a consequence, the difference in economic performance can be estimated by the ratio of the volumetric productivities, and a factor describing the cost for the applied glucose per kg of polymer.

4. Conclusions

Glycerol differently influenced the growth kinetics, the polymer production and the polymer molecular mass produced by both bacteria. The PHB obtained from *C. necator* DSM 545, combining glucose and glycerol, resulted in a LMW-PHB. According to the literature, this is the first time that a high cell dry mass was achieved with a high growth rate when glycerol is used in a fermentation with *C. necator*. It seems that the combination of these two carbon substrates is potentially a great strategy to improve the growth and polymer production conditions of the strain. On the other hand, this work reports for the first time PHA biosynthesis with *B. sacchari* DSM 17165 using glycerol as a sole carbon source in the accumulation phase. Although no high polymer content was achieved if compared with *C. necator* DSM 545, the strain develops perfectly with only the addition of glycerol as a carbon source and a lower LMW-PHB than *C. necator* DSM 545 was obtained.

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Figure Captions:

Figure 1. Evolution of different parameters in *C. necator* DSM 545 fermentation. a) Time courses of the CDM, RB and PHB content concentrations. b) Evolution of the carbon sources (glucose is defined by black dots and grey rhombus while glycerol with grey squares and triangles) and nitrogen concentration in the media during the fermentation. Black dots: re-feeding time with glucose (50% w/v). Dark grey triangle: re-feeding time with glycerol. Grey rhombus: stop of the nitrogen source supply

Figure 2. Evolution of different parameters in *B. sacchari* DSM 17165 fermentation. a) Time courses of the CDM, RB and PHB content concentrations. b) Evolution of the carbon source (glucose is defined by black dots and grey rhombus while glycerol with grey squares and triangles) and nitrogen concentration in the media during the fermentation. Black dots: re-feeding time with glucose (50% w/v). Dark grey triangle: re-feeding time with glycerol. Grey rhombus: stop of the nitrogen source supply

Figure 3. a) FTIR spectra of the PHB produced by *C. necator* DSM 545 and *B. sacchari* DSM 17165. b) ^{1}H NMR spectrum from PHB extracted from *C. necator* DSM 545

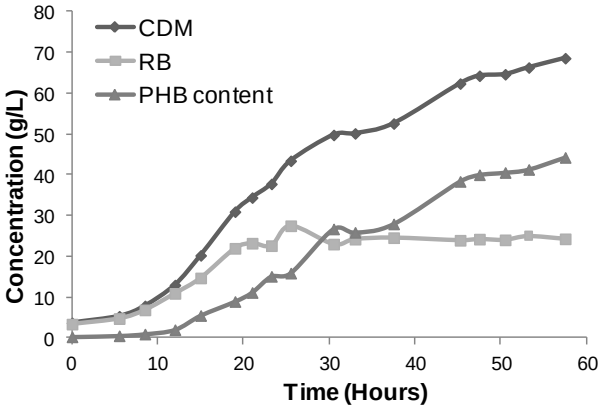
Figure 4. GPC (a) and DSC (b) spectra of the PHB produced by *C. necator* DSM 545 and *B. sacchari* DSM 17165.

Table 1. Main parameters of the fed-batch fermentation with *C. necator* DSM 545 and *B. sacchari* DSM 17165

Parameter		*C. necator* DSM 545	*B. sacchari* DSM 17165
Final CDM [g/L]		68.56	43.79
Final PHB [g/L]		44.25	4.48
Maximal content of PHB in biomass [%]		64.55	10.22
Volumetric productivity PHB [g/L·h]		0.76	0.08
Specific maximum growth rate, μ_{max} [h^{-1}]		1.21	0.42
Total consumption of carbon source [g/L·h]		2.29	1.63
Consumption of glucose [g/L·h]		1.91	1.02
Consumption of glycerol [g/L·h]	from 19 to 57.5 h	0.57	-
	from 35.5 to 57.25 h	-	0.66
Yield $_{(CDM/glucose)}$ [g/g]	from 0 to 19 h	0.82	-
Yield $_{(CDM/glycerol)}$ [g/g]	from 50 to 57.5 h	0.80	-
	from 35.5 to 57.25 h	-	0.63
Yield $_{(PHB/glucose)}$ [g/g]	from 0 to 19 h	0.26	-
Yield $_{(PHB/glycerol)}$ [g/g]	from 50 to 57.5 h	0.75	-
	from 35.5 to 57.25 h	-	0.094
Yield $_{(PHB/carbon\ source)}$ [g/g]		0.34	0.41

Highlights:

- LMW-PHB production by two collection strains using glycerol
- Glycerol differently influences growth kinetics, PHB production and its Mw
- Combining glycerol & glucose improve growth and PHB production by *C. Necator*
- Using glycerol as a sole carbon source by *B. sacchari* produces LMW-PHB



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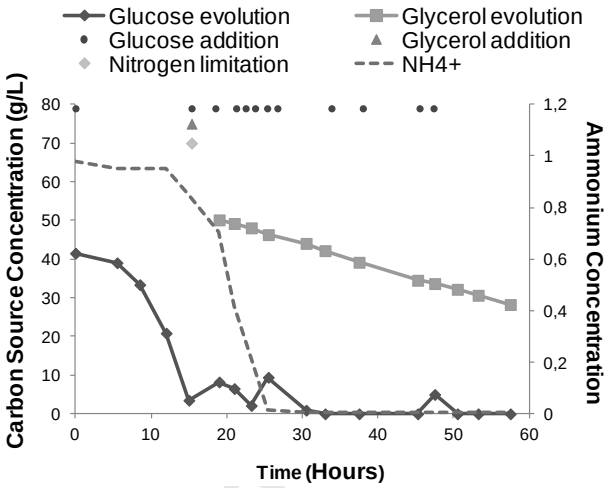

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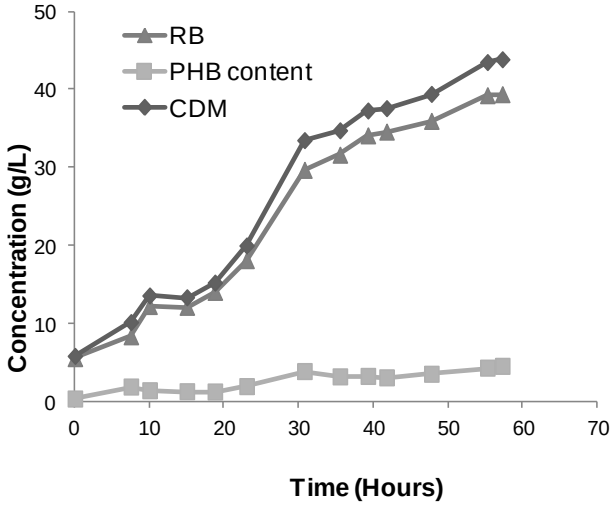
Fig. 1



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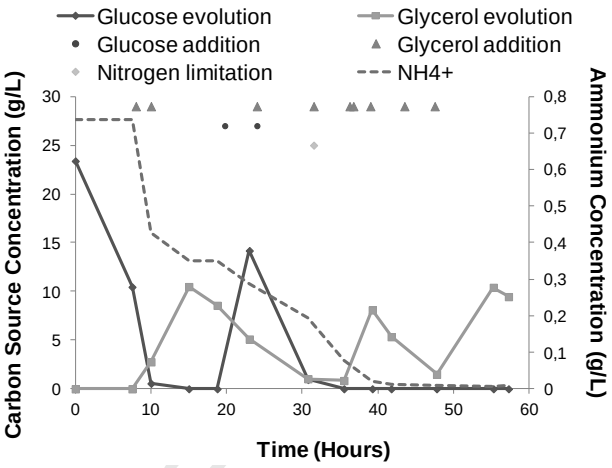

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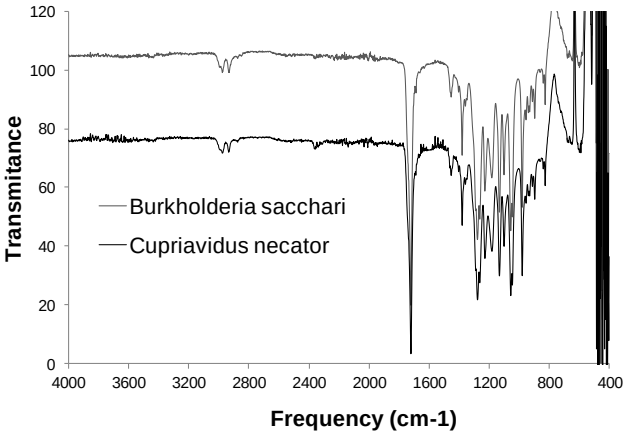
Fig. 2



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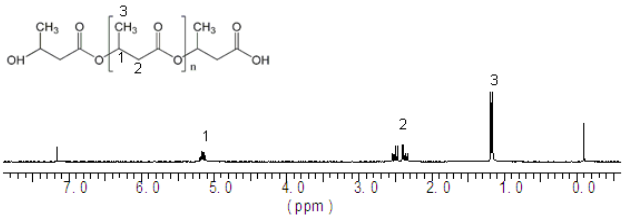

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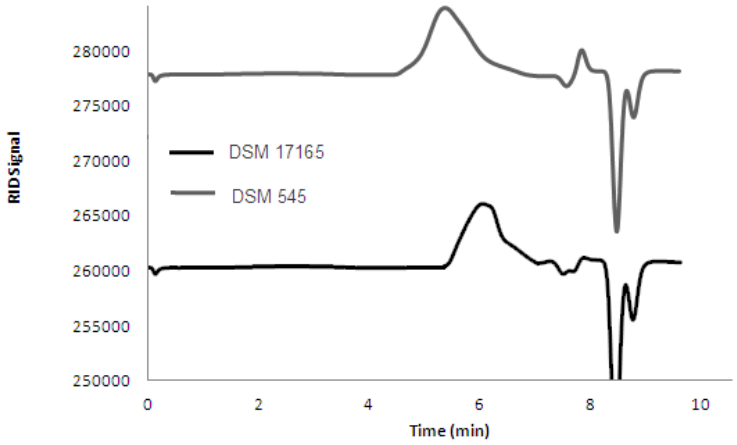
Fig. 3



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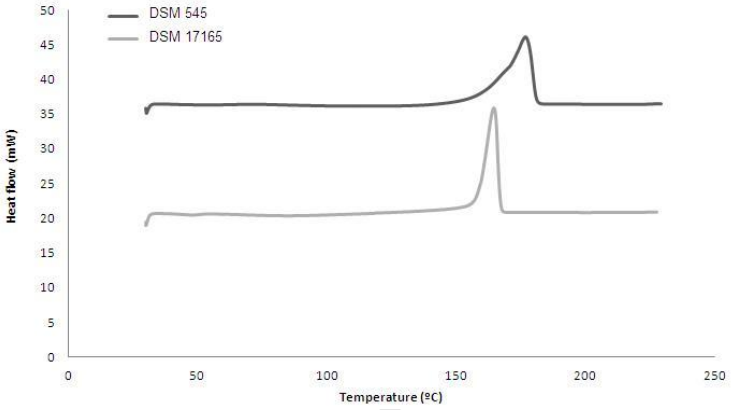


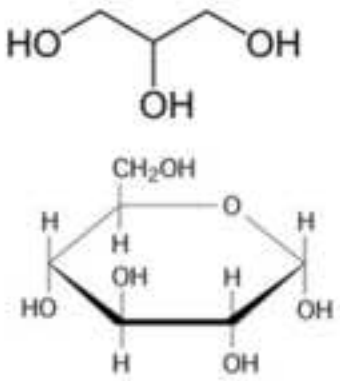
b)

Fig. 4

Glycerol is a co-product of many industrial processes → it is here used as a raw material for biopolymer synthesis

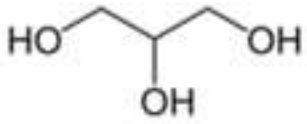
2 STRAINS 2 FERMENTATION STRATEGIES:

GLYCEROL + GLUCOSE



Cupriavidus necator

GLYCEROL



Burkholderia sacchari

Low Molecular Mass PHB