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Article

PHB Production in Biofermentors Assisted through Biosensor Applications

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Abstract: Polyhydroxyalcanoates (PHAs) are biodegradable polymers synthesized in cytoplasmic granules in bacteria, such as *Cupriavidus necator* (*Ralstonia eutropha*), *Alcaligenes latus*, *Pseudomonas* spp., *Comamonas* spp. and other species. PHAs accumulation occurs in response to stress conditions, i.e. under high carbon and low nitrogen (24:1 ratio). PHA can be synthesized using recombinant microorganisms (provided with the operon *phbA/phbB/phbC),* escaping the constrains of nutrient request, except addition of high amount of sugar (glucose, lactose, fructose). In this study; *E. coli* was genetically modified for PHB production in biofermentors. The production of PHA at industrial scale requires a continuous supplementation of fermentable sugars to support the availability of nutrients and to assess the level of the exponential growth phase; since sugars are required either for bacterial growth either for PHA synthesis and energy storage. The biofermentors need to be run in automated system. Sensors are used at many points in fermentators; in the evaluation of parameters: consumption of sugars; cell density; quantification of PHB synthesis. The need of operational control during the fermentation has prompted us to application of three measurements; one unit linked to a Nanodrop to evaluate OD; one linked to a reaction chamber to measure sugars consumed by enzyme based fluorescence detection; and one for bacteria Nile Blue staining and fluorescence intensity reads. The growth of bacteria on three different plant by-products was monitored and PHB production in four days using a banana by-product feed was optimised. These detectors will make possible to exploit the full potential of bioreactors optimizing the time of use and maximizing the number of bacteria synthesizing PHA.

Keywords: *Cupriavidus necator*; *E. coli*; bofermentors; biosensors; growth phase; biosynthetic pathways; P(3HB); PHB: polyhydroxybutyrate,; spectrofluorometry; SERS: Surface Enhanced Raman Spectroscopy; turbidity as optical density (OD)

1. Introduction

Many bacterial species accumulate during the stationary phase and store in intracellular organelles polymers of hydroxypropionate, hydroxybutirate and hydroxyvalerate (poly hydroxyalcanoates, PHAs). These polymers are biodegradable and biocompatible and used in production of bioplastics. PHAs based bioplastics possess good mechanical properties and are components of bottles, medical devices and are easily molded. PHB is produced by *Pseudomonas* spp. and by *Ralstonia eutropha* (*Cupriavidus necator*) [1]. The bacteria in presence of nitrogen limitation [2] convert the carbon sources into energy storing polymers. In the production of PHB in fermentors, one-stage batch culture [3], two-stage batch culture [4,5] and fed-batch cultures [6,7] has shown potential to be used as production methods.

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PHB was also produced in *E. coli* using genetic engineering [8,9]. The genetically modified E. coli system overcomes the nitrogen limitation problem [10], and the need to change the composition of the growth medium. Carbohydrate stocks are injected in continuous into the fermentors to provide the substrates for PHB production [11-14].

Since in the industrial production of PHA 50% of the costs are due to the carbohydrate substrates, new methods to derive bacterial feed from plant byproducts have been developed. Significant research was performed on agro–industrial even agro–waste streams as feedstock for fermentation. Researchers realized a high-productivity fermentation of PHB [15], and PHB fermentation process using chicory roots [16], whey, glycerol, vegetable oils and lignocellulosic feedstock [17-26].

In the project TRANSBIO of the EU commission [27] aimed to the production of PHB and organic acids from microorganisms, we tested the effectiveness of use of plant byproducts in the reduction of costs of industrial fermentation and PHB recovery.

Recombinant *E. coli* producing PHB grow rapidly, accumulate PHB for about 50% of dry weight [28-32], and are able to exploit various carbohydrates [33,34]. Several factors (i.e., type of feed, aeration conditions) influence the biomass growth rate and PHB production and molecular weight size. Several authors showed that PHB production using *E. coli* recombinant systems could be optimised by increasing the oxygen dissolved into the medium, for instance using high rate sparging and aeration [35-37].

PHAs costs may also vary depending on the type of application, since materials for drug delivery and medical device components have high value [38,39]. The main problem is the high cost of the feedstock, about 1 dollars/kg of PHB, in addition to the operational costs, the extraction solvents and purification costs. There is need to reduce bioreactor costs by optimisation of fermentation conditions, and to optimize the PHB yield.

Several companies are active in the field of PHA production, such as Tepha, Metabolix, Telles, Mitsubishi, TianAn, Kaneka, Cheil Jidang, Meredian, Sirim and PHB Industries (some examples in Table 1).

The application of sensors in the biofermentors is mainly envisaged to monitor bacterial growth, the level of nutrients, and to PHA production. A typical bioreactor (i.e. BIOSTAT Q Bioreactor System) is provided with three basic sensors for the monitoring of physico-chemical parameters: a temperature probe, a sensor of pH, and a probe for oxygen tension. Biosensors are based on (chemical, physicochemical, or biological) sensing components and transducing elements; in addition the system requires miniaturised reaction chambers, to deliver small volumes, servo drives, high-precision mechanical components, syringe pumps, pump modules and pumping systems for fluid stream control.

Recently a metabolic/polymerization/macroscopic modelling system was developed, to assess process variables, and to control process operating variables (i.e., nutritional and aeration conditions) in order to optimize biomass production rate, PHB accumulation and molecular weight of PHB [41]. Various types of sensors can be used to determine bacterial concentration, able to quantify bacteria at high density. Biosensors for whole-cell bacterial detection have been recently described [42-45]. Detection systems for bacteria quantification are various, ranging from spectrophotometric detection, to Surface Enhanced Raman Spectroscopy (SERS) [46], to electrochemical biosensors, such as Alternate Current (AC) susceptometry methods to assess

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concentration of bacterial biomass, to methods based on electrochemical impedance spectroscopy (EIS) [44,46].

Several methods are available for determination of dissolved carbohydrates, based on glucose oxidase activity, and exploiting viscosimetric [48] or amperometric detection. Nanoencapsulation of enzymes for glucose concentration has been recently achieved [49].

PHB production is a critical point in industrial fermentation, since keeping the process for the shortest time possible is economically advantageous. PHB screening methods for PHB determination in bacteria are based on Nile Blue staining [47]. Nile Blue dye stains PHB as well as neutral lipids in bacterial membranes. Determination of PHB based on Nile Blue involves several steps, fixing with alcohol or acetone, for dye permeability through the membrane, requiring few hours and operator manuality. Recently a highly sensitive and quantitative method was developed based on bacteria spotted on a microarray slide and fluorescence quantification using laser scanner (unpublished results) [27].

In industrial fermentation for production of PHB, three new sensors are here described: a) a sensor for bacterial biomass determination, b) a sensor to quantify carbohydrates feedstock, and c) a sensor to quantify the level of produced PHB during time. The combination of these three sensors could make possible the exploitation of bioreactors to optimize the time of use (bacterial biomass) and maximize synthesis of PHB in shortest fermentors operating time.

Public scientists, devoid of constrains due to patents from private companies, may support development of sensors in fermentors for the production of bio-based products for the green chemistry.

The growth of bacteria on three different plant by-products was monitored, i.e. potato enzymatic hydrolysate, corn enzymatic hydrolysate, and banana juice, a by-product of the infant food industry. The PHB production in fermentors was optimised in four days using the banana by-product feed. We monitored bacteria growth in biofermentors for media acidification, sugar consumption, bacterial cell density, and level of PHB synthesis: this parameter, together with the type of feed used, influences the economy of the process for the correlation between costs of instrumentation and running time of operations.

2. Experimental Section

2.1 Microorganisms and Plasmid Preparation for E. coli Transformation

A series of *Ralstonia eutropha* environmental isolates were selected for their ability to produce high amounts of PHB, and used as controls and comparison to establish the maximum yield of product under bioreactor conditions.

Cupriavidus necator ATCC 17699 (LGC Standards), deposited as *Ralstonia eutropha*, was used to amplify *phaCAB* operon. *Escherichia coli TOP10* chemically competent [F⁻*mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG λ*⁻] and *Escherichia coli BL21(DE3)* chemically competent [F⁻*ompT hsdIS*$_B$(*r*$_B$⁻*m*$_B$⁻) *gal dcm*(DE3)] (Invitrogen, Life Technologies) were used. Linear cloning pUC19 vector (Invitrogen, Life Technologies) was used for cloning of the genes through homologous recombination. Expression vector pET24b characterized by strong hybrid T7/LacO promoter (Novagen, Inc.) was used for inducible *phaCAB* genes expression. DNA was extracted from an overnight cell culture of *Cupriavidus necator* ATCC 17699 using DNA Purification Kit (Wizard®, Promega).

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After PCR amplification of *phaCAB* operon devoid of native promoter, the PCR product was purified using PureLink PCR Purification Kit (Invitrogen, Life Technologies) and used for the homologous recombination by GENEART Seamless Cloning and Assembly Kit (Invitrogen, Life Technologies). The 4100bp amplified *phaCAB* operon devoid of its promoter was cloned in pUC19 vector by homologous recombination, as these primers shared terminal ends of linearized vector. Chemically competent *E.coli TOP10* cells were transformed and plated in LB plates containing 100 μg/mL Ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) for blue/white screening. The recombinant pUC19/*phaCAB* vector (6700 bp) was extracted by PureLink Hipure Plasmid Miniprep kit (Invitrogen, Life Technologies), digested by *Eco*RI and *Hind*III restriction enzymes (37 °C for 2 hours) and purified fragment ligated in the pET24b vector (5310 bp), previously linearized by same restriction enzymes. *E.coli BL21(DE3)* cells were transformed and plated in LB plates containing 50 μg/mL kanamycin. Colonies of *E. coli* expressing the recombinant vector were selected by detection of *phaCAB* gene by PCR after plasmid linearization (Figure 1).

2.2 Culture Media and Conditions

Recombinant *E. coli BL21(DE3)* harbouring heterologous *phaCAB* operon from *Cupriavidus necator* ATCC 17699 was cultivated at 30 °C and 150 rpm in fed-batch conditions, both in 500 mL shake flasks with a starting volume of 150 mL and in 20 L bioreactor (BIOSTAT®, Sartorius) with a starting volume of 15 L. Optical density (OD) was used to monitor the bacteria biomass, achieved by maintaining a 40% oxygen saturation with a constant flow of compressed air (2 vvm) and with a cascade control speed. pH was monitored automatically at 6.9 ± 0.1 through addition of a stock 15% v/v H_2SO_4 (Sigma, Ireland) and NH_4OH (20% v/v). The induction phase was conducted adding galactose 10 mM or lactose 30 mM at 25 °C, pH 6.9 and air flow 3 L/min after 24h, when the bacteria reached their stationary phase. Feeding solution was added at 4 mL/min for 48 h.

The fed-batch hydrolysate media containing Sweet Corn Enzymatic Hydrolysate 10%, Banana juice 5% or Potato Skin Enzymatic hydrolysate 25% were supplied from TRANSBIO Consortium [27]. The sugar content in Sweet Corn Enzymatic Hydrolysate, Potato Skin waste and Banana juice hydrolysate is shown in Table 2. A buffer solution, based on Na_2HPO_4/Na_2PO_4 x H_2O 10% (v/v) (stock 1M, pH 7) was used. To maintain plasmid stability, kanamycin (50μg/mL stock solution) was added 1:100 to the medium. A Trace Element Solution (TES) stock was prepared as follows (g/L): 10 $FeSO_4x7H_2O$, 2 $CaCl_2x2H_2O$, 2,2 $ZnSO_4x7H_2O$, 0,5 $MnSO_4x4H_2O$, 1 $CuSO_4x5H_2O$, 0,02 $Na_2B_4O_7x10H_2O$ and 1% of TES solution was added to hydrolysate medium. The feeding solution consisted of only hydrolysate medium and 10% phosphate buffer; without additional sugar added.

2.3 Analytical Procedures during Biofermentor Use

2.3.1 Quantification of Reducing Sugars

The carbohydrates content in the bacterial medium was daily measured by commercially available kit (Sucrose, D-Fructose and D-Glucose kit, Megazyme, Ireland), through which NADH is quantified by its absorbance. This was made using microwell plates and spectrofluorometer reads at 340 nm in a TECAN Infinite 200Pro instrument. Glucose and fructose have been quantified every 12 h. The enzymatic reactions allowed to determine the levels of consumed sugars. Depending on sugar availability, the bacteria stopped producing PHB, therefore additional sugar stock solution was injected into the biofermentor. Based on the volumes to be added as sugar stock solution to

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sustain bacterial growth for 5 days, it was estimated that the optical density of cells before inducing them to synthesise PHB is 25 or higher.

2.3.2. Cell Dry Weight (CDW)

The cell growth in shaking flasks and 20 L bioreactor was monitored by measuring the DO_{600nm} of washed aliquots, using a spectrophotometer (SHIMADZU Corporation).

The Cell Dry Weight (CDW) of a known volume of culture broth (5mL) was estimated by centrifugation at 8000 rpm for 5 minutes, and washed, followed by bacteria lyophilisation. The weight of the dry pellet was expressed in g/L.

2.3.3. Monitoring the Production of PHB by Nile Blue Staining

Gene expression was induced after 24 h of bacterial growth by the addition of lactose 30 mM or galactose 10 mM instead of IPTG; polymers production was monitored daily by Nile blue staining.

We used a rapid method to monitor PHB production, as follows. An aliquot of bacterial culture was washed twice with water and 5 µL were pipetted onto clean glass slide, air-dried, heat fixed and stained with a Nile blue solution for 10 minutes at 55 °C. Then, the slide was washed and treated with 8% acetic acid for 30 seconds to remove the stain excess. The glass slide was washed, air-dried and analyzed by Affymetrix 428 array Scanner, at excitation 460 nm/ 550 nm emission.

When the signal reached maximal fluorescence (white signal, Fig. 2d), the fermentation was stopped, the suspension centrifuged and the pellet dried for PHB extraction.

2.3.4. PHB/PHA Quantification and Extraction

To measure intracellular polymers, direct cellular digestion in sulphuric acid was used. In particular, 50 µL of medium were added in 500 µL 96% sulphuric acid into a water bath at 95-98 °C for 30 min as PHB was converted to crotonic acid by heating in concentrated sulphuric acid. Spectrophotometric assay at 235 nm was conducted by spectrophotometer (SHIMADZU Scientific Instruments Inc., Columbia). Pure PHB (Sigma-Aldrich) was used for calibration curve. The PHB/PHA concentration was defined as gram of polymer per litre of culture broth.

To extract PHB/PHA polymers, cellular lysis was obtained by enzymatic digestion with 50 µL lysozyme (50 mg/mL stock, added 1:100) for 1 h and 30 min and proteinase K (1 mg/ml stock added 1:100) for 3 hours. Then, the digested cellular material was transferred in a Corex glass tube. Hot chloroform was added and samples were kept in a boiling water bath for 2 h (vortexing every 10 min) (Fig 4a).For each mg of pellet, 12,5 µl of chloroform and 12,5 µl of 6% Na hypoclorite solution are added to the pellet and incubated at 37 °C for 15 min. A centrifugation at 3000 g for 25 minutes was conducted to remove non-PHB cell material and to recover the chloroform lower phase, containing PHB polymers. Finally, solid PHB was achieved by adding methanol (7:3 v/v of methanol and water) (5 volumes in respect to chloroform) and filtration (Fig. 3b).

3. Results and Discussion

Recombinant *E. coli BL21(DE3)* cells, harbouring a heterologous *phaCAB* operon, were grown in scaling up experiments before reaching the biofermentor scale, using media containing plant carbohydrates, such as Sweet Corn Enzymatic Hydrolysate 10%, Banana juice 5% and Potato Skin Enzymatic hydrolysate 25%. The composition of these three concentrates is shown in Table 2, while in Table 3 is shown the method to produce the hydrolysates and the energy requirements. No

glucose was added, and lactose was used as inducer of expression of PHB synthesis operon. The aim was to determine PHB yields that can be obtained using plant waste as ingredients for cell growth media. After 24 h, when bacteria reached a high density, *phaCAB* operon was induced and expressed, under the strong hybrid T7/LacO promoter of pET system, by addiction of lactose or galactose (Fig. 2). The pET vector is a powerful system for expressing recombinant proteins as it possesses a strong and inducible hybrid T7/LacO promoter, but the costs of the chemical inducing agent, IPTG, are very high. Therefore, several authors used lactose as inducer. In fact, when lactose is not present, the *lac* repressor (LacI) binds to the operator sequence, blocking access of T7 RNA polymerase to the promoter site; conversely, when lactose binds to LacI, T7 RNA polymerase can bind to the promoter site and genes are expressed. The bacterial β-galactosidase (*lacZ*) hydrolyzes lactose to galactose and glucose; glucose is used as carbon source, while galactose is used only as inducer as *E. coli BL21* is a gal(-) strain and it is not able to metabolize it. Moreover, to lower the costs of medium, we used lactose derived from whey source. The advantage of using an pET induction system rather than a constitutive expression system is linked to the possibility of allowing the recombinant bacteria to reach the maximum growth rate, influencing from the outside the timing of PHB induction after cells have reached the stationary phase, thus optimizing energy consumption in the system.

In this work, *pET/CAB E.coli* strains engineered with the *phaC* operon were aerobically grown using three agro-industry hydrolysates supplemented with buffer and trace elements solution, in 20 L bioreactors. The experiments were replicated several times. Empty *E. coli BL21(DE3)*, without pET/CAB vector, was used to compare bacterial growth in the same hydrolysate medium, verifying the possible toxicity of phaCAB proteins. Nile blue staining was used to confirm heterologous PHA/PHB production. Moreover, we tested the same hydrolysate media using a natural strain, *Ralstonia taiwanensis*. As shown in Figure 2, we observed a very limited basal expression in the first hours for the recombinant *pET/CAB E.coli* strains (green signal, Fig. 2b) and the induction of the operon with PHB synthesis after addition of galactose or lactose (white signal, Fig. 2d). No variations in bacterial growth were observed after *phaCAB* operon expression, indicating that the heterologous proteins are not toxic to *E. coli BL21*. To evaluate the polymer synthesis, 50 μL of medium were directly digested in 500 μL 96% sulphuric acid into hot water bath for 30 minutes; poly-β-hydroxybutyric acid was converted to crotonic acid by heating and spectrophotometric assay was conducted at 235 nm. However, although direct acid digestion is a rapid method to measure polymers inside the cells, there is an overestimation since all degradation products contribute to the absorbance. To extract PHB polymers, cell lysis was performed by enzymatic digestion with Lysozyme (50 mg/mL) and Proteinase K. After digestion, lysed cells were transferred in a 30 ml Corex glass tube (Fig. 4a), hot chloroform was added and finally, after centrifugation to remove non-PHB cell material, PHB polymers were recovered after filtration and methanol precipitation (Fig. 4b).

Considering the three different agro-industrial wastes tested, the best result in both bacterial growth and PHB production was observed with banana juice hydrolysate and the recombinant strain (Table 4). The natural strain *Ralstonia taiwanensis*, usually good PHB producer when tested in two-stage batch production (nutrient medium followed by limiting medium), when grown exclusively in agro-industrial wastes showed very low yield (Table 4). Although in the literature natural strains are reported as the best PHB producers, the results depend on the medium

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composition. Whilst recombinant strain does not require carbon/nitrogen imbalance in the bacterial medium, PHB synthesis in natural strains depends on a precise C:N ratio; in addition, the depolymerase may degrade PHB when carbon is unavailable in the medium. *E. coli pET/CAB*, conversely, is able to grow and produce PHB polymers using plant by-product hydrolysates, without the necessity of carbon/nitrogen imbalance. Banana juice, supplied from TRANSBIO Consortium, used to 5% (v/v) of the final medium, particularly rich in glucose and fructose (89.80 ± 0.5 g/L and 82.24 ± 0.4g/L, respectively) and nitrogen (6 g/L); was shown to be an excellent medium for recombinant *E. coli* grown to produce PHB (Table 4). Conversely, a natural strain needs two different media: a nutrient medium to increase biomass and a limiting medium, with carbon/nitrogen imbalance, to produce PHB; the composition of concentrated banana juice is disadvantageous for natural strains, as it contains excessive nitrogen concentration that decrease the C/N ratio expected for a limiting medium. To overcome this drawback, glucose syrup or molasses can be used as carbon feeding, with an increase of the production costs. An additional advantage in the use of banana juice is related to its citric acid content. In fact, the utilisation of the available sugars in the medium is divided into two steps. Acetyl-CoA, intermediate of the central carbon metabolism, is required for bacterial growth, but is used to produce PHB when the biomass has reached the maximum density. PHB is produced starting from acetyl-CoA, with phbA dependent conversion of two acetyl-CoA molecules into acetoacetyl-CoA; the presence of citrate inhibits the tricarboxylic acids (TCA) cycle, causing a shift of most of the acetyl-CoA generated in glycolytic pathway towards PHB synthesis.

The pH was maintained stable in the range of 6.9 ± 0.1 by the addition of NH_4OH (20% v/v) as bacterial growth leads to an acidification of the medium; ammonium hydroxide represents a nitrogen source and also in this case it is not suitable for a natural strain, causing the need to seek an alternative buffer system. Another parameter that needs to be optimised for bacterial growth is the amount of oxygen in the medium. In small scale experiments, we used Erlenmeyer flasks with baffles that facilitate the oxygenation of bacterial cultures under stirring, observing improvements in terms of biomass. This strategy is in agreement with Chen and Page, which used high aeration rate to promote cell growth in the first fermentation step, and lower aeration rate in the second stage, to promote PHB production [4]. However, various strategies were proposed to increase biomass and to produce PHB: one-stage or two-stage batch culture, or high-cell-density fed-batch cultures [3]. Based on fed-batch culture, drainage system in the bioreactor would be a good strategy as it removes the supernatant depleted of nutrient and it would concentrate the bacterial culture, reducing an excessive increase of the volume for a longer and more efficient feeding.

The advantages of using recombinant *E. coli* are fast growth and high cell density cultures; fast growth rate and good level of PHB production, These factors are dependent on medium composition: a nutrient medium rich in carbon sources impacts PHA yield as well as production costs. Today, costs of production of PHAs is high, around US$ 4-6/ kg, including also costs for polymer extraction and recovery [28, 50]. PHB granules being cytosolic, mechanical or chemical methods are required for cell disruption.

The possibility to use plant byproducts such as sugar cane molasses as growth media made the use of naturally producing strains economically convenient [51, 52]. Several authors used agroindustrial wastes for PHB production by adding them as supplementary carbon source in synthetic media such as LB or PCA, together with additional fructose, glucose or ammonium sulphate.

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The growth of bacteria on three different plant by-products was monitored, i.e. potato enzymatic hydrolysate, corn enzymatic hydrolysate, and banana juice, a by-product of the infant food industry. The PHB production in fermentors was optimised in four days (120 hr) using the banana by-product feed (Figure 4). We monitored bacteria growth in biofermentors for sugar availability and level of PHB synthesis. The rapid consumption of sugars in the medium prompted us to accelerate the addition of increasing amounts of banana juice feed during three days, until the staining of the slides showed the saturation of PHB signal (Figure 3).

To further reduce the production costs and avoid the use of antibiotics for selection plasmid containing *E. coli*, it is envisaged in the future to integrate the recombinant system into *E. coli* chromosome. T7 promoter allows to use lactose, a by-product from whey and dairy productions, as inducing agent for expression in recombinant E. coli [53].

Li *et al*. integrated the *phaCAB* operon with a 5CPtacs promoter cluster into *E. coli* chromosome, to create a system of repetitive promoters for high and stable overexpression; the resulting engineered bacteria accumulated 23.7% PHB of the cell dry weight in batch fermentation [54]. Lee *et al*. produced pure (*R*)-3-hydroxybutyric acid (R3HB) from glucose, with a yield of 49.5% (85.6% of the maximum theoretical yield), by integration of the PHA biosynthesis genes into the chromosome of *E. coli* [55].

Interestingly, Yin *et al*. produced poly(hydroxybutyrate-co-hydroxyvalerate) (PHBV) inserting one copy of *methylmalonyl-CoA mutase* and *methylmalonyl-CoA decarboxylase* genes into the porin site in *Halomonas* TD08 *2-methylcitrate synthase* (*prpC*$^{\Delta}$) deletion mutant. They observed that active transcription start site and gene copy number made *Halomonas* TD08 better suited for chromosome engineering, compared with *E. coli*, for PHA production [56].

Recently, amperometric electrochemical sensor was set up for in-line measurement of acetate, using a microbial electrode based om *Geobacter* spp. for the determination of acetate [57]. The bacteria were shown to oxidize acetate making the electrode a terminal electron acceptor. When acetate is oxidized by the bacteria, a current signal is produced. The microbial electroactive films as sensing elements are promising sensor components. The organic acids that can be measured are propionate, butyrate, and other volatile fatty acids (VFA). Thus, in different settings and conditions, real-time inline monitoring of VFA concentration has been shown to be feasible [57-59].

Currently, VFA are measured using off-line, gas or liquid chromatography methods. In-line measurements may be achieved by integration of a sensor directly into the process. Electrochemical methods in general are a promising tool for real-time measurements, as the current or the measured potential give instantaneous signals. However, a sensor element is needed, selective for VFA. In fermentors, the determination of short chain fatty acids, for instance isovalerate, may be useful in the quantification of supplemented VFA for the synthesis of mixed-type PHA. In addition, advances in nutrient requirement, expressed as complex carbohydrates from plant by-products, of recombinant *E. coli* cells producing poly-lactate-poly-butyrate in bioreactors, have been described [60].

The substrates for PHB production are mainly derived from food-based carbon source. Therefore new opportunities for non-food carbon sources are the subject of present studies [51]. Plant by-prodcuts, second generation bioethanol industry, hemicelluloses and lignin hydrolysates have been the subject of the new trend of studies for PHA production using inexpensive carbon sources. The fed-batch hydrolysate media here studied were: Sweet Corn Enzymatic (SCE) hydrolysate 10%, Banana juice (BJ) by-product 5% and Potato Skin Enzymatic (PSE) hydrolysate 25%. Banana juice, differently from other hydrolysate sources, possesses a discrete amount of citric acid (3,50 g/L) and this can help shifting toward P(3HB) pathway. The data of experiments comparing *Ralstonia* spp.

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and *E. coli* grown on three hydrolysate feeds are shown in Table 4. The highest PHB production was obtained with recombinant *E. coli pET/CAB* grown in fed-batch banana juice medium. The highest PHB amount obtained in this work was 0.252 g/L, corresponding to 50% of bacteria dry weight (Table 5). PHB polymers were extracted by enzymatic digestion and hot chloroform in Corex glass tubes (Figure 4); after non-solvent precipitation and filtration, granules were lyophilized and collected.

In this preliminary work, we showed the feasibility to use plant byproducts as feeds. Among three types of by-products tested, banana juice showed to be the most performing in PHB synthesis, supplying the carbohydrates needed by bacteria. The real time monitoring of the culture conditions allowed to optimize the growth of bacteria and the operation time for PHB synthesis, exploiting the full potential of bioreactors and minimising the operation costs. Recently, another publication reported on the feasibility to use banana by-products for PHB fermentation [61] and for other processes such as biogas production, two thematic areas in the bio-economy era. Therefore, these efforts may lead to more affordable and less costly methods of PHB production based on suitable plant by-products and efficiently engineered bacteria able to produce the required bioplastic types.

Figure 1. Detection of recombinant plasmid *pET24/phaCAB* by PCR and DNA digestion. Nested PCR of *phbC* gene (550bp) to confirm insert in presumed positive colonies (1a) and digestion of positive pET24/phaCAB plasmid with *Bgl*II restriction enzyme to linearize it (9400bp) (1b).

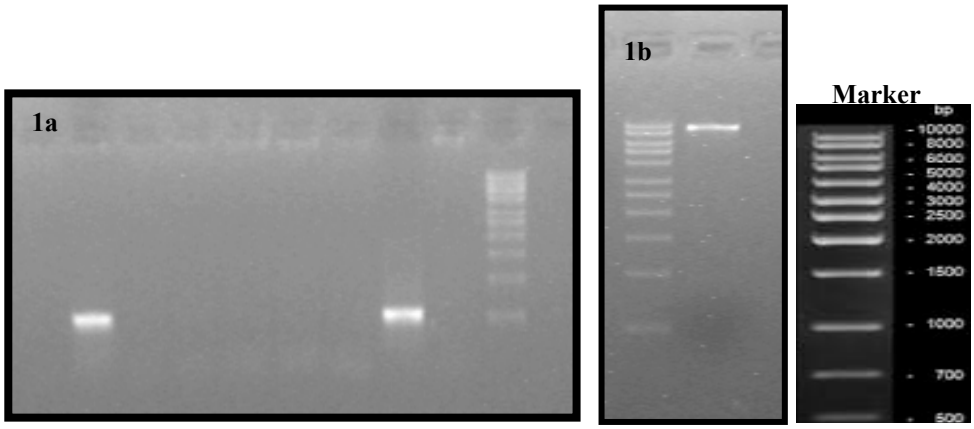

Figure 2: **Nile blue staining of cells spotted onto glass slides.** Sampling of cultured cells from the small scale growth in flask to slide preparation. Every spot contained 5 μL of bacterial cells from cell cultures at three different dilutions (from bottom to top: 1:1; 1:2; 1:3), The slides were heat fixed and Nile blue stained; and fluorescence analyzed in an Array Scanner at 460 nm excitation (2a). Before induction there is only basal expression (blue signal, 2b); after 24 h from lactose addition, the signal increased (green and orange spots, 2c) with maximal production at 72 h (white spots, 2d).

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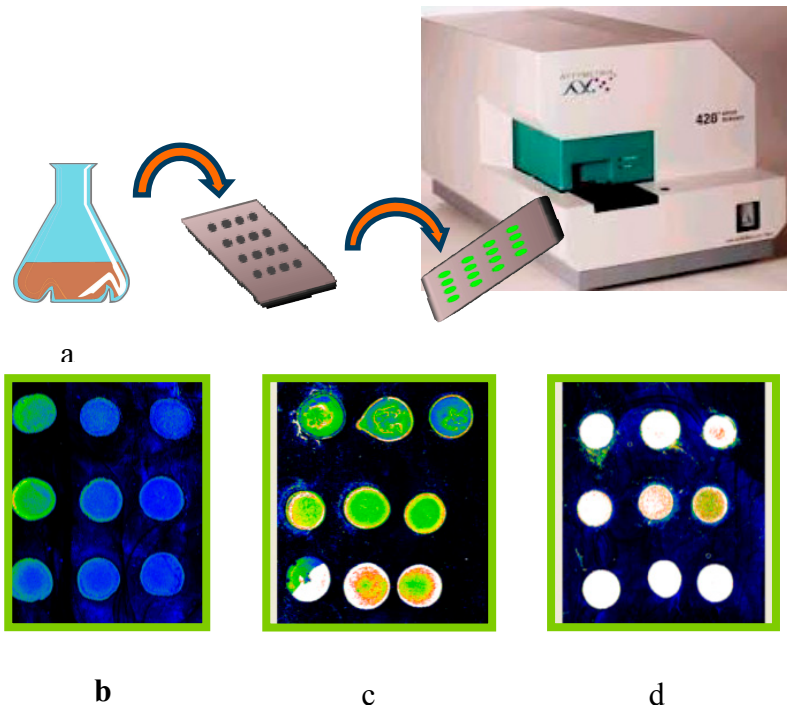

Figure 3. PHB production over 120 hr incubation: monitoring sugars consumed and addition of new feed

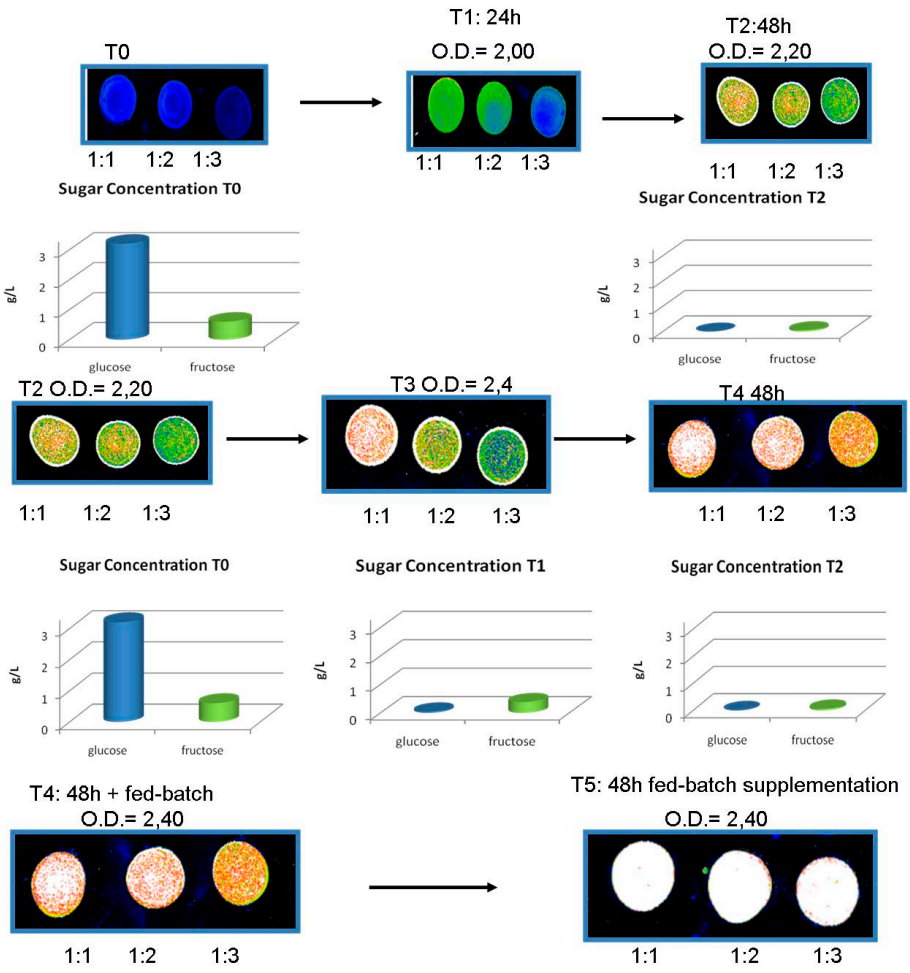

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Figure 4: **PHB extraction and lyophilisation.** PHB polymers were extracted by enzymatic digestion and hot chloroform in Corex glass tubes (4a); after non-solvent precipitation and filtration, granules were lyophilized and collected (4b).

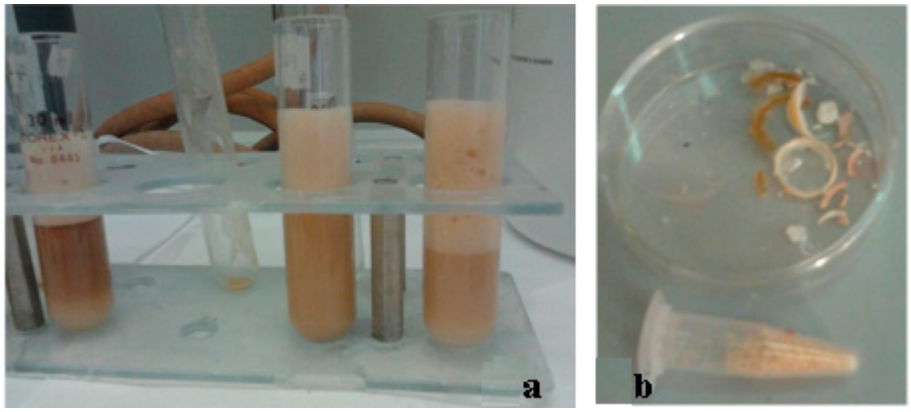

Table 1. Currently used carbohydrate carbon sources in industrial fermentation

CARBOHYDRATE	PRODUCT	COMPANY
Dextrose, molasses	PHBV	TianAn Biologic Materials, China www.tianan-enmat.com
Sugar cane, molasses	PHB	PHB Industrial S.A., Brazil www.biocycle.com.br
Methanol	PHB, mixed type	Mitsubishi Gas Chemicals, Japan
vegetable oil	PHB, mixed type	Kaneka, Japan; Meredian, USA

Table 2. Composition of three enzymatic hydrolysates used in this study (values provided by TRANSBIO Consortium partners; nd: not determined).

Composition	Sweet Corn (g/L)	Potato skin (g/L)	Banana juice (g/L)
Glucose	104	45	90
Fructose	10	≤5	82
Maltose	≤5	10	nd
Sucrose	≤5	≤5	nd
Nitrogen	3,6	4	6
Citric acid	nd	nd	3,5
Magnesium	nd	nd	0,3
Phosphate	nd	nd	0,25

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Table 3. Enzymatic hydrolysis protocols (centr. = centrifugation; red. sug. = reducing sugars; chars. = characteristics)

	Inputs					**Output chars.**	
Pretreated byproduct	**byproduct mass (g)**	**CH_3COONa 0.05 M (ml)**	**enzymes (ml)**	**incubation**	**centr.**	**Red. sug. (g/L)**	**Glucose (g/L)**
sweet-corn	125	375	5.3	24h 50 °C 600 rpm	x	129-148	52.4-61.5
potato	105	300	0.3	100 °C + 48h 70 °C 600 rpm	x	194-198	163.2-182.4
Banana	100			-	x	172	90

Table 4: *Ralstonia* spp. and *E. coli* PHB accumulation in three fed-batch hydrolysate media, after 72h**.** The reported values were means of triplicate experiments.

Fed-Batch hydrolysate	CDW(g l^{-1})	PHB (g l^{-1})
Sweet Corn Enzymatic hydrolysate 10%		
Recombinant *E. coli BL21(DE3)*	8,2	1,7
Empty *E. coli BL21(DE3)*	7,9	/
Ralstonia taiwanensis	4,3	/
Potato Skin Enzymatic hydrolysate 25%		
Recombinant *E. coli BL21(DE3)*	15,3	2,9
Empty *E. coli BL21(DE3)*	14,9	/
Ralstonia taiwanensis	9,5	0,4
Banana juice 5% hydrolysate		
Recombinant *E. coli BL21(DE3)*	20,6	3,9
E. coli BL21(DE3 without plasmid	20,2	/
Ralstonia taiwanensis	15,8	0,2

Table 5. Highest amount of PHB obtained in fermentors using *E. coli* fed with banana juice stock

PHB production in fermentor		
	(g/L)	% DW
Total Biomass	3,15	
PHB	**0,252**	50%

4. Conclusions

For a greater commercialisation and industrial sustainability, the use of bacterial PHB-producers and cheap agro-industrial residues have attracted research and commercial interest worldwide. We

showed the feasibility and convenience of setting up and optimization of the parameters for bacteria growth and PHB synthesis in bioreactors, through the monitoring of sugars consumed, evaluation of cell density, and quantification of PHB. The use of recombinant *E. coli* cells growing on plant by-products may lower the costs of the fermentation and the instrumentation, together with the operational costs. The development of biosensors and automatable devices measuring these parameters could make more affordable the industrial production of bioplastics.

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Author Contributions

V.M. and O.F.D. performed the microbiology studies and P.P. provided the biofermentor and biochemical analyses, and the writing the manuscript.

Conflict of Interest

"The authors declare no conflict of interest".

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