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PHB Production in Biofermentors Assisted through Biosensor Applications †

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† Presented at the 3rd International Electronic Conference on Sensors and Applications, 15–30 November 2016; Available online: https://sciforum.net/conference/ecsa-3.

Published: 29 November 2016

Abstract: Poly-hydroxy-alcanoates (PHAs) are biodegradable and biocompatible polymers synthesized and accumulated in intracellular compartments in several bacterial species. Polyhydroxyalcanoates (PHAs) are synthesized by numerous prokaryotes, such as *Cupriavidus necator* (*Ralstonia eutropha*), *Pseudomonas* spp., *Comamonas* spp., 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). Recombinant *E. coli* systems were studied to produce PHB using metabolic engineering. In biofermentors, the critical points are the excess of fermentable sugars and the ratio of nutrients versus cell optical density. In order to allow production in biofermentors in automated system, sensors are envisaged to evaluate critical parameters such as sugar consumption, bacteria concentration and level of synthesis of PHA. The need of fermentors and operation control has compelled for application of three biosensing units, one linked to a Nanodrop to evaluate OD, one linked to an enzymatic reaction chamber to measure sugars consumed by enzyme linked sugar biosensing tools, and one for sampling the bacteria, Nile Blue staining, and fluorescence intensity reads. 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: *Ralstonia eutropha*; *E. coli*; Biofermentor; biosensing; exponential growth; biosynthesis

1. Introduction

Polyhydroxyalcanoates (PHAs) are biodegradable and biocompatible polymers synthesized by bacteria and accumulated in intracellular compartments. Poly(3-hydroxybutyrate) (PHB) has been considered to be good candidate for completely biodegradable polymers due to the mechanical properties similar to petroleum-derived polymers and complete biodegradability. PHB, the most common PHA, is synthesized by numerous prokaryotes as *Cupriavidus necator* (*Ralstonia eutropha*) [1], in response to limitation of nitrogen [2] in presence of high carbon sources. Several strategies are used to produce P(3HB), one-stage batch [3], two-stage batch [4,5] or high-cell-density fed-batch cultures [6,7].

PHA can be synthesised and produced using recombinant microorganisms [8,9]. The use of recombinant bacteria enables to escape the need to limit nitrogen sources [10], while sugars (glucose, lactose, fructose) are continuously added to the medium to sustain exponential growth and PHB synthesis [11,12], as well as for shunt of PHB substrates by modification of glycolysis and other metabolic pathways [13,14].

In the composition of medium used to feed the bacteria, attempts have been made to reduce the costs by extracting the nutrients from wastes and byproducts. Significant research was

performed on agro–industrial even agro–waste streams as feedstock for fermentation. Researchers realized a high-productivity fermentation of P(3HB) [15], and implemented successfully a P(3HB) fermentation process using chicory roots (*Cichorium intybus*) [16] after hydroponic cultivation as a carbon source.

Complex organic by-products are a good opportunity, both from an environmental and an economic perspective, to produce PHB and organic acids; this was also the topic of a recently concluded EU project [17].

The advantages of using recombinant *E. coli* for the production of PHB include rapid growth, accumulation of PHB greater than 50% of cell weight [18–22], and the ability to utilize inexpensive carbon sources [23,24]. Key process operating variables (i.e., nutritional and aeration conditions) affect the biomass production rate and the PHB accumulation in the cells and its associated molecular weight distribution. Previous studies have demonstrated that PHB production using recombinant systems such as *E. coli* have been hindered upon scaling up in part due to the use of large amounts of oxygen required for high bacterial growth and PHB generation [25,26].

Bubble gas microaeration and sparging has been shown useful to increase the oxygenation of the medium and the synthesis of PHB [27]. The process optimization may lead to high intracellular PHB accumulation (up to 95% of PHB/g of dry cell weight). Applications of PHA are coatings, packaging films and in bottling, medicine, drug delivery and bioplastic components [28,29]. The principal bottleneck is the cost of production, being higher than 2 dollars/kg, due to costs of running the fermentors, and for extraction and purification. Therefore, it is highly desirable to optimise bioreactor conditions to improve the yield, and to scale up the processing capacity of fermentors.

Various companies produce PHB and PHB-V heteropolymers. TephaElast (by Tepha), Biopol (by Metabolix, Cambridge, MA, USA), Mirel (by Telles, Clinton, IA, USA, this joint venture has been closed), Biogreen (by Mitsubishi Gas Chemical, Tokyo, Japan), Enmat (by Tinan, Tianjin, China), Nodex (by Kaneka, Osaka, Japan), Biocycle (by PHB Ind., Serrana, Sao Paulo, Brazil). Of these, Telles has the highest production capacity, projected to reach 50.000 tonn/year in 2020, and SIRIM, Selangor, Malaysia, has a fully automated fermentor system [30]. In 2016, Metabolix announced the intention to sell the patents and assets for PHA to Cheil Jedang (Seoul, Korea), making this company one of the strongest in this field for the next years.

Bioreactors are provided with sensors to monitor physico-chemical parameters, such as temperature probe and pH sensor (linked to pumps to add NaOH or HCl), stirring speed, air flux regulation or micro-bubble dispersion by sparging (BIOSTAT Q Multi-Fermentor Bioreactor System with dissolved oxygen probe), to provide dissolved oxygen, needed for aerobic growth. In order to make the process sustainable and economically convenient, two factors need to be optimised: high optical density (OD) of cell suspension, and continuous presence of 5%–10% sugars. Turbidity (OD 600) and glucose consumption need to be measured, at 0, 4, 8, 12, 24, 48, 72 and 96 h. PHA production needs to be evaluated too, since after PHA accumulation bacterial cells are collected for PHA extraction. The bacteria need to reach an OD close to 50, to obtain an optimum ratio of cells/volume, exploiting the maximum volume capacity of bioreactors, without diluting the sugars.

Recently researchers have described a combined metabolic/polymerization/macroscopic modelling approach, relating the process performance with the process variables, controlling the key process operating variables (i.e., nutritional and aeration conditions) affecting biomass production rate and PHB accumulation in the cells and PHB molecular weight distribution [31].

The potential of application of sensors and biosensors in the bioreactors applied to monitoring the exponential phase growth, the level of nutrients, and the synthesis of PHA is envisaged for a feasible and sustainable increase in production of bioplastics at industrial level. There is a need to control the availability of sugar substrate, to monitor the synthesis of PHB, an to check the viability of bacteria after exponential growth and at growth curve saturation.

Sensors based on an enzymatic reactions can measure sugars concentration. This is made at laboratory scale using microwell plates and spectrofluorometer reads, based on enzymatic

reactions. A sensor must be provided with an autosampler, a microfluidic pump, and a reaction chamber where enzyme produced NADH is quantified by its absorbance.

Microfluidic systems allow the controlled flow of operations like solvent and solute transport, valving, mixing, separation, concentration and detection with a dedicated biosensor (chemical, physicochemical, or biological method). All the components, from micro-reaction chambers, delivering small volumes through servo drives, high-precision mechanical components, and pumping systems with pulsation free fluid streams, syringe pumps, pump modules may be assembled in a Lab-on-Chip (LoC) system, under automated control, reducing operation times and operator errors.

Other types of sensors can be used to determine bacterial concentration, alternative to optical density measures, unsuitable when working with such high density of bacteria. Biosensors for whole-cell bacterial detection have been recently reviewed [32–35]. Various detection systems have been applied in bacteria quantification, from spectrophotometric detection, as Fourier Transformation-IR spectrometry (FTIR) and Reflectometric Interference Spectroscopy (RIfS), to Surface Enhanced Raman Spectroscopy (SERS) [36], to electrochemical biosensors, such as Alternate Current (AC) susceptometry measuring the magnetic field, suitable for bacteria concentration evaluation, to impedance-based systems, as electrochemical impedance spectroscopy (EIS) [34,36].

Finally, a sensor needs to be dedicated to the detection and evaluation of PHB production. This is a critical point in industrial fermentation, since keeping the process for the shortest time possible is economically convenient, and bacteria that do not synthesise still consume sugars and keep the fermentors busy. Traditional PHB screening methods for PHB quantification in whole cells have exploited the Nile Blue staining and fluorescence of PHB containing bacteria [37]. Nile Blue dye stains PHB and other neutral lipids in bacteria. Quantitative assessments of PHB based on Nile Blue fluorescence involve various fixing steps, executed with an alcohol or acetone treatment, that facilitate the permeation of the dye through the membrane. The time required is between one and two hours and some manual passages. New methods have been proven less time consuming than standard Nile Blue colorimetric staining of PHB, and may be run in automatic, providing measures even over-night. Recently a more sensitive measurement has been obtained detecting bacteria fluorescence on a laser scanner (unpublished results) [17]. In this way, a colour scale was obtained, from blue, green, red, to white as the highest saturation signal. The chip slides loaded with serial dilutions of bacteria, are slightly dried for fixing the pellets to the glass, and incubated with the solutions for staining, centrifuged and loaded onto the scanner.

To the aim of automated detection of PHB in cells still in the fermentors, it is envisaged that SERS methods [36] could be efficiently applied not only to quantification of PHB, but also to discrimination of the types of polyhydroxyalkanoates produced. This may support the technologists and substitute the standard HPLC analyses for quality and quantity of PHA products.

The combination of these three sensors could make possible the exploitation of the full potential of bioreactors in optimization of the time of use (bacterial growth cycles) and maximization of bacterial synthesis of PHB in the shortest time possible.

Among the measuring methods or biosensors that can be applied to determine bacterial concentration, since the achievement is the determination of the maximum density, the methods most favoured for industrial applications are those cost effective and with few equipment maintenance problems. Therefore EIS, SPR and other applications requiring calibration curves are less favoured, while optical measurements requiring minimal liquid handling, such as few diluting steps and sample reproduction are most probable candidates.

Concerning the measurement of sugars to be quantified, there are already several methods on stick or test strip, exploiting viscosimetric [38] or amperometric detection of glucose oxidase activity. It has to be kept in mind that fermentors may need to work with higher concentration of sugars, proximal to 10% of the volume used. Nanoencapsulation of enzymes to read glucose concentration has been achieved [39].

Lab-on-a-chip (LOC) devices have a strong potential to be used in the field since they can be miniaturized and automated; being also potentially fast and very sensitive. There are still several

issues to be solved before application in field applications, including the pre-treatment of a sample, such as dilution of bacteria within the linear range of calibration curve, proper storage of reagents, full integration into a battery-powered or energy-failure proof system, range of linearity measurements and comparability of each method, and easiness of operations, to allow the operator to overcome troubleshoots of the system. Each biosensor technique has its own advantages and disadvantages in terms of equipment required, sensitivity, simplicity and cost effectiveness, being optical sensors affordable and with maintenance procedures easy to be performed, and enzyme based sensors highly reliable and based on well established protocols in medical applications.

In industrial fermentation for production of PHB at cost-effective scale, in addition to sensors controlling the standard parameters temperature, pH, oxygen, three additional sensors are proposed, a sensor to evaluate bacteria concentration, and a sensor for sugar concentration, and a sensor to monitor PHB synthesis over the time and the amount of PHB produced. The perspectives and future vision for using biosensors for industrial synthesis of PHA and related processes based on industrial fermentors (bio based biochemicals industry) are sound, but not disclosed and discussed due to protection of intellectual property rights and economic interests. Therefore, it is requested from academia to disclose the applications of sensor components with a broader view, such as the production in fermentors of recombinant proteins or organic acids as intermediate products for the green chemistry.

In the following sections we discuss the monitoring of bacteria growth in biofermentors according to media acidification, sugar consumption, cell density, and PHB synthesis, this latter being the parameter influencing the economy of the process for the correlation between costs of instrumentation and running time of operations.

2. Materials and Methods

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 cells and *Escherichia coli* BL21(DE3) chemically competent cells (Invitrogen, ThermoFisher, Waltham, MA, USA) were used for cloning and gene expression, respectively. Linear cloning pUC19 vector (Invitrogen, ThermoFisher, Waltham, MA, USA) was used for genes cloning by homologous recombination. Expression vector pET24b characterized by strong hybrid T7/LacO promoter (Novagen, Merck Millipore, Darmstadt, Germany) was used for inducible phaCAB genes expression.

2.2. Culture Media and Conditions

Recombinant *E. coliBL21(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. DO was maintained at 40% oxygen saturation with a constant flow of compressed air (2 vvm) and with a cascade control speed. The pH was controlled automatically at 6.9 ± 0.1 by the addition of 15% *v*/*v* H_2SO_4 (Sigma Aldrich, Merck Millipore, Darmstadt, Germany) 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 [17]. The sugar content in Sweet Corn Enzymatic Hydrolysate, Potato Skin waste and Banana juice hydrolysate was reported in Table 1. Na_2HPO_4/Na_2PO_4 x H_2O buffer 10% (*v*/*v*) (stock 1M, pH 7) was used; to maintain plasmid stability, kanamycin antibiotic (50 μg/mL) was added to the medium.

A Trace Element Solution (TES) stock was prepared as follows, (g/L): 10 $FeSO_4 \times 7H_2O$, 2 $CaCl_2 \times 2H_2O$, 2.2 $ZnSO_4 \times 7H_2O$, 0.5 $MnSO_4 \times 4H_2O$, 1 $CuSO_4 \times 5H_2O$, 0.02 $Na_2B_4O_7 \times 10H_2O$ and 1% of TES solution was added to hydrolysate medium. The feeding solution consisted of only hydrolysate medium and 10% phosphate buffer; no additional sugar was added.

Table 1. Composition of Enzymatic hydrolysates used in this study. The values were supplied 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

2.3. Amplification and Cloning of phaCAB Genes in E. coli BL21(DE3)

Genomic DNA was extracted from an overnight cell culture of *Cupriavidus necator* ATCC-17699 using Wizard® Genomic DNA Purification Kit (Promega, Fitchburg, WI, USA), according to the manufacturer's instructions.

Amplification of phaCAB operon without native promoter. The PCR product was purified using PureLink PCR Purification Kit (Invitrogen, ThermoFisher, Waltham, MA, USA) and used for the homologous recombination by GENEART Seamless Cloning and Assembly Kit (Invitrogen, ThermoFisher, Waltham, MA, USA). The 4100 bp 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.coliTOP10* 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, ThermoFisher, Waltham, MA, US), digested by *Eco*RI and *Hin*dIII restriction enzymes (37 °C for 2 h) and the purified fragment was ligated to pET24b vector (5310 bp), previously linearized by same restriction enzymes. BL21(DE3*) E.coli* cells were transformed and plated in LB plates containing 50 μg/mL kanamycin. Transformed cells and positive colonies were identified by PCR on picked colonies and on purified plasmids (Figure 1a). Nested PCR of *phbC* gene (550 bp) was followed by linearization with *Bgl*II restriction enzyme to cut the pET24/phaCAB plasmid (9400 bp) (Figure 1b).

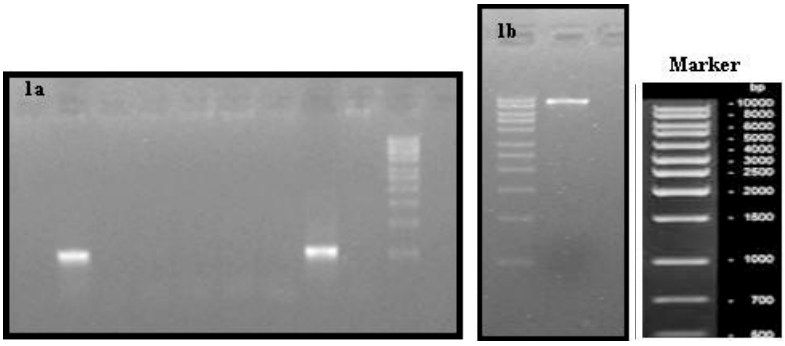

Figure 1. Molecular detection of recombinant plasmid pET24/phaCAB by Nested PCR and Digestion. Nested PCR of *phbC* gene (550bp) to confirm insert in presumed positive colonies (**a**) and digestion of positive pET24/phaCAB plasmid with *Bgl*II restriction enzyme to linearize it (9400 bp) (**b**).

2.4. Analytical Procedures during Biofermentor Use

2.4.1. 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, Wicklow, Ireland). Glucose and fructose have been quantified every 12 h. The enzymatic reactions based on NADH production allowed to determine the levels of consumed sugars, using microwell plates and spectrofluorometer reads at 340 nm in a TECAN Infinite 200Pro instrument. Depending on sugar availability, the bacteria will stop growing and producing PHB, therefore additional sugar stock solution needs to be injected into the biofermentor. Based on the volumes to be added as sugar stock solution to sustain bacterial growth for 5 days, it was estimated that the best optical density of cells at the start of fermentation in this volume is 25 or higher. This density will allow the cells to focus on PHB production instead of continuing the exponential phase growth.

2.4.2. 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, Kyoto, Japan).

The Cell Dry Weight (CDW) of a known volume of culture broth (5 mL) was estimated by centrifugation at 8000 rpm for 5 min, washed with distilled water and lyophilized. The weight of the lyophilized cells was expressed in g/L.

2.4.3. 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 10mM instead of IPTG; polymers production was monitored daily by Nile blue staining.

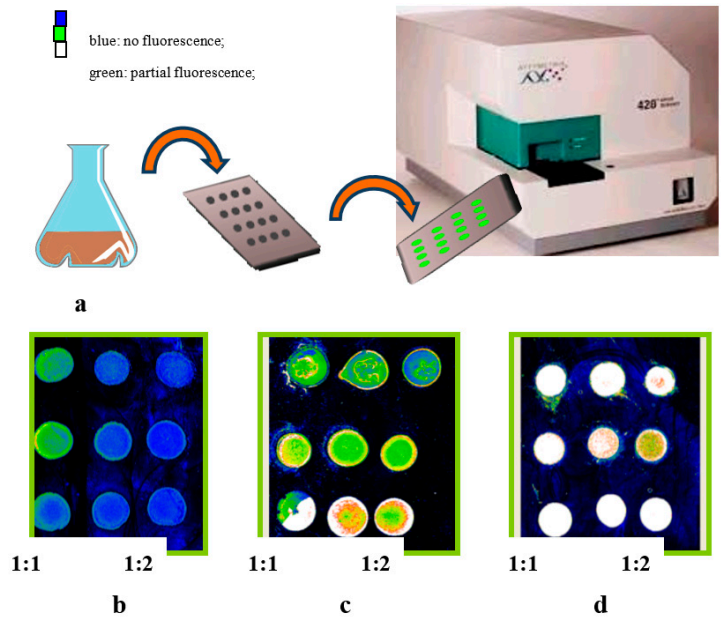

Figure 2. Nile blue staining of cells spotted onto glass slides. Sampling of cultured cells from the biofermentors and slide preparation. Every spot contained 5 µL of bacterial cells from cell cultures at three different dilutions (1:1; 1:2; 1:3), The slides were heat fixed and Nile blue stained; and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

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 min at 55 °C. Then, the slide was washed and treated with 8% acetic acid for 30 s to remove the stain excess. The glass slide was washed, air-dried and analyzed by Affymetrix 428 array Scanner, at excitation 460 nm/emiss. 550 nm.

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

2.4.4. 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., Kyoto, Japan). Pure PHB (Sigma-Aldrich, Merck Millipore, Darmstadt, Germany) 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 h. 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) (Figure 3a). A centrifugation at 3000 g for 25 min was conducted to remove non-PHB cell material and to recover lower chloroform phase, containing PHB polymers. Finally, solid PHB/PHA was obtained by non-solvent precipitation (five times the volume of chloroform) and filtration (Figure 3b). The non-solvent used was a mixture of methanol and water (7:3, vol/vol).

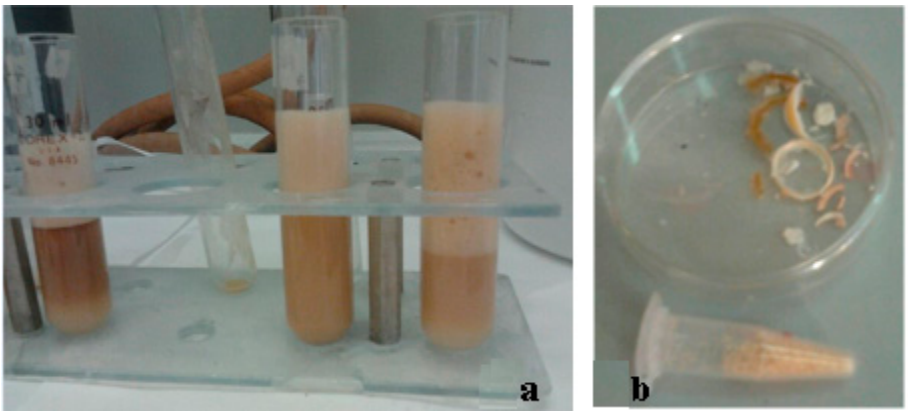

Figure 3. PHB extraction and lyophilisation. PHB polymers were extracted by enzymatic digestion and hot chloroform in Corex glass tubes (**a**); after non-solvent precipitation and filtration, granules were lyophilized and collected (**b**).

3. Results and Discussion

Recombinant BL21(DE3*) E. coli* cells, harbouring a heterologous *phaCAB* operon, were used for bacterial growth and PHB production in biofermentors using media containing plant carbohydrates, such as Sweet Corn Enzymatic Hydrolysate 10%, Banana juice 5% or Potato Skin Enzymatic hydrolysate 25%. 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 (Figure 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, in the absence of lactose, 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 study, recombinant pET/CAB *E. coli* strains were aerobically grown using agroindustrial hydrolysates supplemented with buffer and trace elements solution, in 20 L bioreactors. The experiments were replicated several times. Empty BL21(DE3*) E. coli,* 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, Figure 2b) and the induction of the operon with PHB synthesis after addition of galactose or lactose (white signal, Figure 2d). No variations in bacterial growth were observed after *phaCAB* operon expression, indicating that the heterologous proteins are not toxic to *E. coliBL21*. To evaluate the polymer synthesis, 50 μL of medium were directly digested in 500 μL 96% sulphuric acid into hot water bath for 30 min; 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 obtained by enzymatic digestion with Lysozyme (50 mg/mL) and Proteinase K. After the digested cellular material was transferred in a Corex glass tube (Figure 3a), hot chloroform was added and finally, after centrifugation to remove non-PHB cell material, PHB polymers were recovered by filtration and methanol precipitation (Figure 3b).

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 2). 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 2). In fact, even if in the literature natural strains are reported as the best PHB producers, the results depend on the medium 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, whilst the depolymerase may degrade PHB when carbon is unavailable in the medium pET/CAB *E. coli*, conversely, is able to grow and produce PHB polymers in hydrolysate waste, without the necessity of carbon/nitrogen imbalance. Banana by-product supplied from TRANSBIO Consortium, used to 5% (*v*/*v*) of the final medium, is particularly rich in glucose and fructose (89.80 ± 0.5 g/L and 82.24 ± 0.4 g/L, respectively) and nitrogen (6 g/L); was shown to be an excellent medium for recombinant *E. coli* grown to produce P(3HB). Conversely, a natural strain needs two different media: a nutrient medium to increase biomass and a limiting medium, with carbon/nitrogen imbalance, to produce PHA/PHB; the particular composition of concentrated banana juice results in a disadvantage for natural strain manipulation, as it contains excessive nitrogen concentration amount that decrease the C/N ratio expected in the limiting medium. To overcome this drawback, a glucose syrup can be used as carbon feeding, increasing the costs of production. An additional advantage to use banana juice waste 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 cell synthesis during the exponential phase but is diverted to produce PHB polymers in the stationary phase. PHAs are produced from acetyl-CoA as phbA enzyme condenses two

acetyl-CoA molecules into acetoacetyl-CoA, while the addition of citrate blocks the TCA cycle, causing a shift of most of the acetyl-CoA generated in glycolysis pathway towards PHA synthesis. Banana juice, conversely to other hydrolysates, possesses a discrete amount of citric acid (3.50 g/L) and it can help shifting toward P(3HB) pathway. Another important aspect is that recombinant strains can not degrade the PHB polymers inside the cell, due to absence of depolymerises.

Table 2. PHB accumulation in different fed-batch hydrolysate media after 72 h. The highest PHB production was obtained with recombinant pET/CAB *E. coli* grown in fed-batch banana juice medium. 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 BL21(DE3*) E.coli*	8.2	1.7
Empty BL21(DE3) *E.coli*	7.9	/
Ralstonia taiwanensis	4.3	/
Potato Skin Enzymatic hydrolysate 25%:		
Recombinant BL21(DE3)*E. coli*	15.3	2.9
Empty BL21(DE3) *E. coli*	14.9	/
Ralstonia taiwanensis	9.5	0.4
Banana juice 5% hydrolysate:		
Recombinant BL21(DE3) *E. coli*	20.6	3.9
Empty BL21(DE3) *E. coli*	20.2	/
Ralstonia taiwanensis	15.8	0.2

The pH was controlled automatically at 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 not to be neglected for a better bacterial growth of both, recombinant and natural strains, 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, who used high aeration rate to promote cell growth in the first stage and lower aeration rate in the second stage to promote PHB production [4]. However, many strategies are proposed to increase biomass and to produce P(3HB): one-stage batch, two-stage batch 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* as the host organism are well known, as fast growth and high cell density cultures are easily achieved; high bacterial growth and PHB production are reported in literature but these factors are dependent on medium composition: a nutrient medium, rich in pure carbon sources, will increase the final PHB/PHA yield but, at the same time, the production costs. Unfortunately, the cost of producing PHAs is still high, approximately US$ 4–6/ kg, as there are included also polymer extraction and recovery costs [18,40]. In fact, PHB granules are cytosolic and 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 [41,8]. These authors used agroindustrial wastes for PHB production but adding them as supplementary carbon source in synthetic media such as LB or PCA, together with additional fructose, glucose or ammonium sulphate.

Moreover, to further reduce the production costs, it is possible 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* [42].

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 accumulate 23.7% PHB of the cell dry weight in batch cultivation [43]. Lee et al. produced enantiomerically 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 and depolymerase genes into the chromosome of *E. coli* [44]. Interestingly, Yin et al. produced poly(hydroxybutyrate-*co*-hydroxyvalerate) (PHBV) inserting one copy of genes methylmalonyl-CoA mutase and methylmalonyl-CoA decarboxylase into the strongest expression site porin in 2-methylcitrate synthase (*prpC*) deleted mutant *Halomonas* TD08. They observed that active transcriptional site and gene copy number made *Halomonas* TD08 more suitable for chromosome engineering, compared with *E. coli*, for PHA production [45].

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 that it is possible to set up the parameters for optimum cell growth and PHB synthesis by monitoring the sugars consumption, the cell density, and the synthesis of PHB during fermentation in bioreactors. 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 operator costs. The development of biosensors and automatable devices measuring these parameters could make more affordable the industrial production of bioplastics.

Acknowledgments: We acknowledge the European Community project TRANSBIO for economic support (FP7/2007-2013) under grant agreement n. 289603, Prof. Carmela Velasquez from Centro Nacional de Ciencia y Tecnología de Alimentos, University of Costa Rica for providing the banana juice concentrate, and Thomas Dietrich and the Tecnalia staff for providing the enzymatic hydrolysate of various vegetables, and for project coordination.

Author Contributions: V.M. performed the molecular studies and engineering of *E. coli*, O.F.D. the Ralstonia and natural producing bacteria analyses, and P.P. supervised the biofermentor application.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

PHA	poylhydroxyalanoate
PHB	polyhydroxybutyrate
PHB-V	mixed type PHA, containing hydroxyvalerate
EIS	electrochemical impedance spectroscopy
FTIR	Fourier Transformation-IR Spectrometry
SERS	Surface Enhanced Rama Spectroscopy

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