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Optimization of Polyhydroxyalkanoate Production by Recombinant *E. coli* Supplemented with Different Plant By-Products

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Abstract

Polyhydroxyalkanoates (PHAs) are biodegradable polymers synthesized in cytoplasmic granules in bacteria, such as *Cupriavidus necator* (*Ralstonia eutropha*), and several other bacteria. PHAs accumulation occurs in response to stress conditions, i.e. under high carbon and low nitrogen (24:1 ratio). In this study, *E. coli* was genetically modified for PHA production in biofermentors. PHA was synthesized in bacteria transformed with the operon *phbA/phbB/phbC*. The bacteria were fed using a basal medium supplemented with three different plant by-products, potato tuber skin hydrolysate, corn hydrolysate, and banana juice supplement. The growth in biofermentor was monitored through the evaluation of consumption of sugars and quantification of PHA synthesis. A microarray scanner was used to read fluorescence intensity of Nile Blue stained bacteria. PHA production by *E. coli* fed on a banana juice supplement outperformed all the other fermentation media, with highest amount of PHA per dry cell weight.

Keywords:* *Biodegradable polymers; Bacteria; E. coli; Hydrolysate

Introduction

Many bacterial species, such as the chemolytotrophs *Cupriavidus necator* (*Ralstonia eutropha*), *Cupriavidus metallidurans* and *Alcaligenes latus* [1-9], *Pseudomonas putida, P. aeruginosa* [10-14], *P. pseudoflava* [15] and other *Pseudomonas* spp [16], *Azotobacter vinelandii* [17], *Halomonas campisalis* [18], *Enterobacter* spp. [19], *Thermus thermophilus* [20]*; Bacillus subtilis* [21,22] and *Bacillus cereus* [23] among others, can synthesise and accumulate and store polyhydroxyalkanoates (PHAs) in intracellular organelles [24], co-polymers composed of hydroxypropionate, hydroxybutyrate and hydroxyvalerate, depending on the availability of precursors (such as octanoic acid) [11,25-27] and intermediate compounds regulating the metabolic flux toward their synthesis, such as citrate. These polymers are biodegradable, biocompatible and useful for production of bioplastics. PHAs based bioplastics are easily moulded, possess good mechanical properties and are components of bottles and medical devices. PHAs are produced by bacteria in presence of nitrogen limitation [28], converting the carbon sources into energy storing polymers. In the production of PHA in biofermentors, one-stage culture

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[29,30], two-stage batch culture [9,17,29], fed-batch [1,16,31-33] high-cell density cultures [1,34-37] and mixed cultures [38-40], as well as submerged and solid state fermentation processes [41] have shown potential to be exploited as production methods [42].

Carbohydrate stocks may be injected in continuous into the fermentors or supplied at determined time points (fed-batch), to provide the substrates for PHA production [43-46]. Bacterial cells respond to environmental stress (pressure, N or P limitation) by increasing the synthesis of PHAs [10,47].

PHA has been also produced in *E. coli* [48], using genetic engineering [44,45,47,49-57] and through bacterial cell factories [58]. The genetically modified *E. coli* system overcomes the nitrogen limitation problem [38,46,59,60] and the need to change the composition of the growth medium. Other bacterial species have been used for biotechnology application and gene engineering, such as *Aeromonas hydrophila* [61] *Halomonas* spp. [62], mutants of *Pseudomonas putida* [63], *P. aeruginosa* [13], and *Bacillus* spp. [64], or using other biotechnological systems [65].

Recombinant *E. coli* producing PHA grow rapidly, accumulate PHA for about 50% of dry weight [38,66-69] and are able to exploit various carbohydrates and intermediate compounds [53,57,70,71]. Several factors (i.e., type of feed, aeration conditions) influence the biomass growth rate and PHA production and molecular weight size. Several authors showed that PHA 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 [72].

PHAs costs may also vary depending on the type of application, since materials for drug delivery and medical device components have high value [73,74]. The main problem is the high cost of the feedstock, about 1 dollars/kg of PHA, in addition to the operational costs, the extraction solvents and purification costs. In the industrial production of PHAs, more than 50% of the costs are due to the carbohydrate substrates. Therefore, new methods to feed bacteria using plant by-products have been developed. Significant research was performed on agro–industrial even agro–waste streams as feedstock for fermentation. Researchers realized a high-productivity of PHA [75], and fermentation process using C1 carbon sources [76,77], sugarcane molasses [9,19,21,40,44,55,78,79], soy molasses [80], fruit pomace and agroindustrial byproducts [52,81], chicory roots [7, 50], whey, spent sulfite liquor [82], glycerol [53,54,57], grass press juice [36], animal fats and vegetable oils [14,83,3,16,8,13] microalgae [71] and lignocellulosic feedstock [26,34,35,38,53, 58,71,79 84,85].

There is need to reduce bioreactor costs by optimisation of fermentation conditions, and to optimize the PHA yield. The application of sensors in the biofermentors is mainly envisaged to monitor bacterial growth, the level of nutrients, and the effectiveness of 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.

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, PHA accumulation and molecular weight of PHA [86]. 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 [69,87,88].

PHA production is a critical point in industrial fermentation, since keeping the process for the shortest time possible is economically advantageous. PHA screening methods for PHA determination in bacteria are based on Nile Red (λ excitation: 543 nm, λ emission: 560-710) [89] or Nile Blue staining [82,90,91]. Recently, highly sensitive and quantitative methods to read quantitatively the Nile Blue stained bacteria have been developed, based on fluorometry combined with a flow cell [2], and based on laser scanner (λ exc 460 nm/λ emiss 550 nm,) for fluorescence quantification of stained bacteria spotted on microarray slides [92].

In the project TRANSBIO of the EU commission [92] aimed to the production of PHA and organic acids from microorganisms, we tested the effectiveness of use of plant by-products in the reduction of costs of industrial fermentation and PHA recovery.

In industrial fermentation for production of PHA, three devices are envisaged for monitoring the following parameters: a) bacterial biomass determination, b) quantification of carbohydrates feedstock, c) monitoring of the level of PHA produced during time. A combination of these three parameters has been set up to optimize the use of bioreactors for: time of use (bacterial biomass), and maximum synthesis of PHA in fermentors at short operating time.

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 PHA production in fermentors was optimised in three days (after reaching the desired biomass) using the banana by-product feed. We monitored bacteria growth in biofermentors for media acidification, sugar consumption, bacterial cell density, and the timing and the amount of PHA synthesised: 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.

Material and Methods

All reagents were of high grade purity (Sigma-Aldrich, Merck Millipore, Darmstadt, Germany).

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 PHA, and used as controls and comparison to establish the maximum yield of product under bioreactor conditions.

Cupriavidus necator (*Ralstonia eutropha)* was used to amplify *phaCAB* operon. *Escherichia coli TOP10* chemically competent and *Escherichia coli BL21 (DE3)* chemically competent (Invitrogen) were used. Linear cloning pUC19 vector (Invitrogen, ThermoFisher, Waltham, MA, US) was used for cloning of the genes through homologous recombination. Expression vector pET24b characterized by strong hybrid T7/LacO promoter (Novagen, Merck Millipore, Darmstadt, Germany) was used for inducible *phaCAB* genes expression. DNA was extracted from an overnight cell culture of *Cupriavidus necator* ATCC 17699 using DNA Purification Kit (Promega, Fitchburg, WI, US).

After PCR amplification of *phaCAB* operon devoid of native promoter, the PCR product was purified using PureLink PCR Purification Kit (Invitrogen) and used for the homologous recombination by GENEART Seamless Cloning and Assembly Kit (Invitrogen). 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. 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), 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. Several methods of transformation of *E. coli* have been described previously, but is is difficult to reproduce the same results obtained or the same yield of PHA produced.

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 Biostat bioreactor (Sartorius, Goettingen, Germany) 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 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 24 h, 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 1. A buffer $Na_2HPO_4/Na_2PO_4 \times H_2O$ (pH 7) was used in the initial growth culture. 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 $FeSO_4 \times 7H_2O$, 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; without additional sugar added.

Quantification of Reducing Sugars

The carbohydrates content in the bacterial medium was daily measured by Sucrose, D-Fructose and D-Glucose kit (Megazyme, Wicklow, Ireland), through which NADH is quantified by its absorbance. This was made using microwell plates and spectrofluorometer reads at 340 nm in an Infinite 200 Pro instrument (Tecan, Männedorf, Switzerland). 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 PHA, therefore additional sugar stock solution was injected

TABLE 1. **Composition of three enzymatic hydrolysates used in this study (values by TRANSBIO Consortium partners).**

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	-	-	3,5
Magnesium	-	-	0,3
Phosphate	-	-	0,25

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 optical density of cells before inducing them to synthesise PHA is 25 or higher.

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

Monitoring the Production of PHA by Nile Blue Staining and Fluorescence Quantification

Determination of PHA based on Nile Blue involves several steps, fixing with alcohol or acetone, for dye permeability through the membrane, requiring few hours and transfer of bacteria from aliquot sample to eppendorf tubes. After induction of PHA synthase after 24 h of bacterial growth by the addition of lactose 30 mM or galactose 10 mM; production of polymers was monitored daily by Nile blue staining. An aliquot of bacterial culture was washed twice with MilliR H_2O 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. Three aliquots were done for each sampling, with two serial dilutions of the sample (1:2, 1:3). 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 analysed by Affymetrix 428 array Scanner, at excitation 460 nm/550 nm emission (FIG. 1). When the signal reached maximal fluorescence (white signal, FIG. 2), the fermentation was stopped, the suspension centrifuged and the pellet dried for PHA extraction.

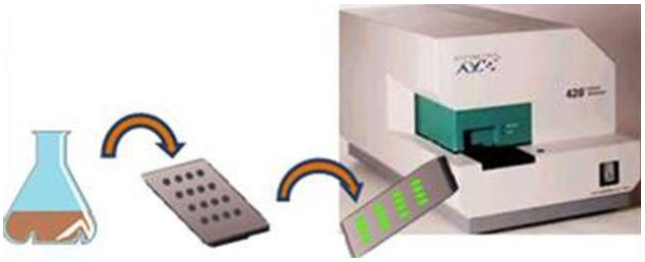

FIG. 1. **Nile blue staining of cells spotted onto glass slides. Samples (5 µL) of cultured cells were spotted onto a glass slide at three dilutions in H_2O (1:1; 1:2; 1:3, vol/vol). The fluorescence analysed in an Array Scanner at 460 nm excitation.**

PHA 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°C to 98°C for 30 min as PHA was converted to crotonic acid by heating in concentrated sulphuric acid. Spectrophotometric assay at 235 nm was conducted by spectrophotometer (Shimadzu). Pure polyhydroxybutyrate (PHB) (Sigma-Aldrich, Merck Millipore, Darmstadt, Germany) was used for calibration curve. The PHA concentration was defined as gram of polymer per litre of culture broth.

To extract PHA polymers, cellular lysis was performed using enzymatic digestion. 50 µL lysozyme (50 mg/mL stock, added 1:100) were added and incubated for 1 h and 30 min followed by proteinase K (1 mg/ml stock addition (1:100), left for 3 hours. Then, the digested cellular material was transferred in a 30 ml Corex glass tube. Hot chloroform was added and samples were kept in a boiling water bath for 2 h (vortexing every 10 min) (FIG. 3a). For each mg of pellet, 12,5 µl of chloroform and 12,5 µl of 6% Na hypochlorite 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-PHA cell material and to recover the chloroform lower phase, containing PHA polymers. Finally, solid PHA was achieved by adding methanol (7:3 v/v of methanol and water) (5 volumes in respect to chloroform) and filtration (FIG. 3b).

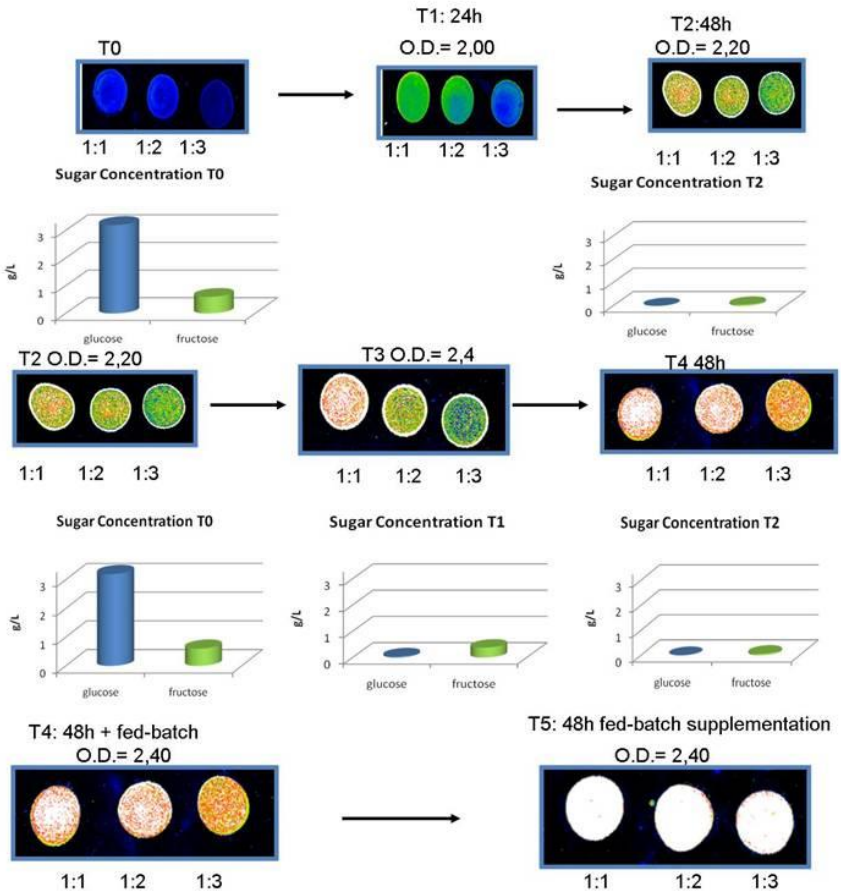

FIG. 2. **PHA production over 120 hr incubation: monitoring sugars consumed and addition of new feed. The glass slides show the spots of three serial dilutions of bacteria (1:1, 1:2, 1:3) to avoid saturation of fluorescence signals.**

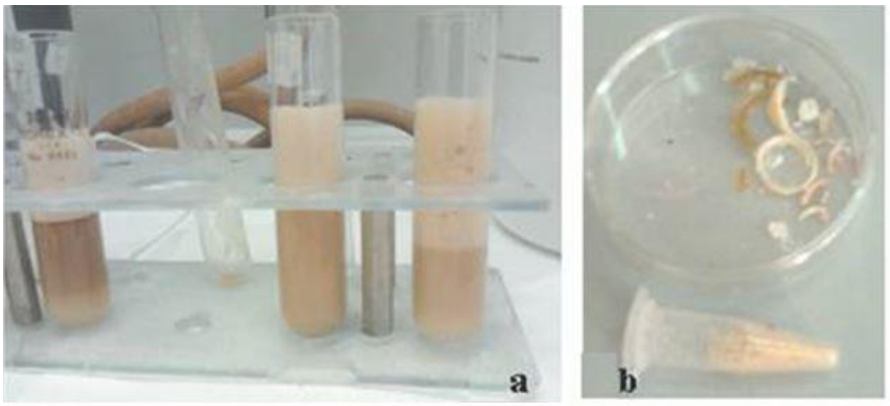

FIG. 3. **PHA extraction in Corex glass tubes (3a); and lyophilisation (3b).**

Cell Dry Weight (CDW)

The Cell Dry Weight (CDW) of a sampled volume of culture broth (5 mL) or the entire fermentation was obtained 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.

Results

Biomass growth for PHA production

Recombinant *E. coli BL21(DE3)* cells, harbouring a heterologous *phaCAB* operon, were grown in scaling up experiments before reaching the biofermentor scale in 20 L bioreactors, using media containing plant carbohydrates, such as Sweet corn enzymatic hydrolysate 10%, Banana juice 5% and Potato skin enzymatic hydrolysate 25%. No glucose was added, and lactose was used as inducer of expression of PHB synthesis operon. 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). Previously several authors used lactose as inducer.

Moreover, we tested the same hydrolysate media using a natural strain, *Ralstonia taiwanensis*. A limited basal expression in the first hours for the recombinant *pET/CAB E.coli* strains was present (green signal, FIG. 2b). After addition of lactose, the PHA synthesis was detected (white signal, FIG. 2d). 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. After centrifugation to remove non-PHA cell material, PHA polymers were recovered by filtration and methanol precipitation (FIG. 3b).

When testing the three different agro-industrial wastes as feed, the best result in bacterial growth and PHA production was observed with banana juice hydrolysate and the recombinant strain (TABLES 2 and 3). The natural strain *Ralstonia taiwanensis*, usually good PHA 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).

TABLE 2. ***Ralstonia* spp. and *E. coli* PHA accumulation in three fed-batch hydrolysate media, after 72h. The reported values were means of triplicate experiments. Cell dry weight (CDW).**

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

TABLE 3. **PHA synthesised by *E. coli* fed with banana juice stock in biofermentors.**

PHA production in biofermentor		
	(g/L)	**% DW**
Total Biomass	3,15	-
PHA	0,252	50%

Although in the literature natural strains are reported as the best PHA producers, the results depend on the medium composition. While recombinant strains do not require carbon/nitrogen imbalance in the bacterial medium, PHA synthesis in natural strains depends on a precise C:N ratio. *E. coli pET/CAB* is able to produce PHA polymers using plant by-product hydrolysate. Banana juice, 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* for PHA production using the optimised protocol and fermentors incubation for three days (TABLE 3).

To overcome the C:N imbalance, glucose syrup or molasses are used as carbon feed, with an increase of the production costs. Therefore new strains, mutants or engineered for PHA production, have been studied worldwide. The recent application of agricultural by-products, lignocellulosic biomass, oils and C1 carbons have widened the potential to produce PHAs at lower costs. In this study, three different by-products were tested to supplement the bacteria with carbohydrates. However, as shown in TABLE 4, the first two hydrolizates required higher energy costs, and enzymes for the breakdown of

polysaccharides. Potato hydrolysate showed to contain the highest content of glucose, while fructose is probably required in the high synthesis of PHAs. Therfore, banana juice by-product affected the growth of *E. coli* due to a well-balanced content of reducing sugars (TABLE 4). 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 PHA when the biomass has reached the maximum density. PHA 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 PHA synthesis.

TABLE 4. **Requirement of energy and output of available reducing sugars for banana juice stock compared to other plant by-products. Enzymes mixture hydrolysis protocols and incubation times are unique for each reaction.**

	Inputs					**Outputs**	
Pretreated byproduct	**byproduct mass (g)**	**CH_3COONa 0.05 M (ml)**	**enzymes (ml)**	**Incubation time**	**Energy (kWh) cutting, drying, milling**	**Reducing sugar (g/L)**	**Glucose (g/L)**
sweet-corn	125	375	5.3	24h 50°C 600 rpm	2.45	129-148	52.4-61.5
endive	105	330	4.4	48h 50°C 600 rpm	7.3	107-119	32.1-47.6
potato	105	300	0.3	100°C+48h 70°C 600 rpm	12.7	194-198	163.2-182.4
Banana	100	-	-	-	-	172	90

The pH was maintained stable in the range of 6.9 ± 0.1 by the addition of NH_4OH (20% v/v). Another parameter that needs to be optimised for bacterial growth is the amount of oxygen in the medium. Previously, researchers used high aeration rate to promote cell growth in the first fermentation step, and lower aeration rate in the second stage, to promote PHA production [17]. However, various strategies were proposed to increase biomass and to produce PHA [29]. 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; high levels of PHA 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 PHA are around US$ 4-6/kg, including costs for polymer extraction and recovery [5,38]. PHA granules being intraorganellar, mechanical or chemical methods are required for cell disruption. W developed an extraction process based on enzymatic lysis of bacteria followed by repeated extractions, as described in material and methods.

The possibility to use plant byproducts such as sugar cane molasses as growth media made the use of naturally producing strains economically convenient [19,52]. Several authors used agroindustrial wastes for PHA production by adding them as supplementary carbon source in synthetic media such as LB or PCA, together with additional fructose, glucose or ammonium sulphate. In this work, we showed that optimization of cell biomass growth and PHA production may be achieved by

implementing the sensing devices integrated with a biofermentor, using three sensing units able to quantify the sugar availability, the bacterial biomass, and the PHA production level. This allowed to individuate the critical sugar needs able to sustain bacterial PHA production, especially favoured by the presence of banana juice sugars.

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 PHA production in fermentors was optimised in three days of PHA synthesis induction (72 hr) using the banana by-product feed (FIG. 2). We monitored bacteria growth in biofermentors for sugar availability and level of PHA synthesis. The rapid consumption of sugars in the medium (either during exponential phase growth as well as during PHA synthesis induction) led to the need to fed-batch addition of increasing amounts of banana juice during the three day fermentation, until the staining of the slides showed the saturation of PHA signal (FIG. 2).

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 using lactose, a by-product from whey and dairy productions, as inducing agent for expression in recombinant *E. coli* [93].

Researchers previously 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% PHA of the cell dry weight in batch fermentation [61]. Others 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* [94].

Interestingly, researchers produced poly (hydroxybutyrate-co-hydroxyvalerate) (PHBV) [95] 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.

Recently, sensor detection was set up for in-line measurement of bio-based chemicals, using a *Geobacter* spp. microbial electrode for the determination of acetate [96]. 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 [97,98].

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 of

complex carbohydrates from plant by-products in recombinant *E. coli* cells producing poly-lactate-poly-butyrate in bioreactors, have been described [30].

Discussion

In this work, we showed the differential requirement of three plant byproducts by recombinant *E. coli* in biofermentors, as feed for producion of PHAs, as highly needed biobased biochemicals. The data of experiments comparing *Ralstonia* spp. and *E. coli* grown on three hydrolysate feeds, shown in TABLE 4, indicate that banana juice based feed was the best performing feed in PHA production by *E. coli*, and was also the best feed for *Ralstonia* spp. Among the by-products tested, banana juice showed to contain the nutrient required for PHA synthesis, supplying the carbohydrates and the intermediate carbons. The real time monitoring of the culture conditions allowed to optimize the growth of bacteria and the operation time for PHA 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 PHA production [99] and for other processes such as biogas production, two thematic topics in the bio-economy era. Therefore, these efforts may lead to more affordable and less costly methods of PHA production based on suitable plant by-products and efficiently engineered bacteria [100-103].

The use of bacterial PHA producers and cheap agro-industrial residues have attracted commercial interest worldwide aiming to industrial sustainability, especially using cheap carbohydrates as feed. This study showed the feasibility of recombinant *E. coli* culturing in bioreactors for PHA synthesis, through the monitoring of sugars consumed, determination of PHA synthesis and quantification of PHA. The use of recombinant *E. coli* cells growing on banana juice by-products at the site of banana juice transformation, such as in Costa Rica, may lower the costs of fermentation for PHA production, and decrease the operational costs of the banana juice industry.

Compliance and Ethics

The authors declare there is no conflict of interest.

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