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Bioconversion of cassava starch by-product into *Bacillus* and related bacteria polyhydroxyalkanoates

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Abstract

Background: Unlike petroleum-based synthetic plastics, biodegradable biopolymer generation from industrial residue is a key strategy to reduce costs in the production process, as well as in the waste management, since efficient industrial wastewater treatment could be costly. In this context, the present work describes the prospection and use of bacterial strains capable to bioconvert cassava starch by-product into biodegradable polyhydroxyalkanoates (PHAs). **Results:** The first step of this study was the bacterial competence screening which was conducted with 72 strains covering 21 *Bacillus* and related species. The microorganism growth in a medium with a starch substrate was measured by an innovative MTT assay, while the ability of the bacteria to secrete amylase and produce PHA was evaluated by the Nile Red Dye method. Based on growth and potential for PHA production, four isolates were selected and identified as *Bacillus megaterium* by 16S rRNA sequencing. When cultivated in hydrolyzed cassava starch by-product, maximum production reached 4.97 g dry biomass/L with 29.7% of Poly-(3-hydroxybutyrate) (characterized by FTIR). **Conclusions:** MTT assay proved to be a reliable methodology for monitoring bacterial growth in insoluble media. Selected amylolytic strains could be used as an alternative industrial process for biodegradable plastics production from starchy residues, reducing costs for biodegradable biopolymer production and wastewater treatment operations.

Keywords: *Bacillus megaterium*, biodegradable plastics, biopolymer, MTT assay, starch substrates

INTRODUCTION

Polymeric materials are important for society due to their characteristics of versatility, durability and resistance to degradation. However, there are many environmental problems resulting from the use of petrochemical derived polymers making necessary to find alternatives to replace these biorecalcitrant materials. One solution to this problem is the use of biodegradable polymers, which are polymers with the desirable properties of conventional plastics but also with rapid biodegradation in the environment after discarding (Sankhla et al. 2010). One such biodegradable type of polymer are polyhydroxyalkanoates (PHAs), which are polyesters containing hydroxyalkanoic acid monomers which

can be biosynthesized from renewable (waste) resources (Mengmeng et al. 2009). PHAs are accumulated intracellularly by bacteria as carbon or energy reserves, mainly when there is a limitation of an essential nutrient, such as nitrogen, phosphorus, oxygen or sulphur (Verlinden et al. 2007).

Due to their chemical and physical characteristics, PHAs have a wide range of applications including conventional plastic materials, utensils, and cosmetic containers, as well as in medicine (as bone plates, surgical sutures and blood vessel replacements), in the pharmaceutical and food industries, and as biodegradable carriers for the long-term dosage of drugs, medicines, hormones, insecticides and herbicides (Reddy et al. 2003). The most studied PHA is the poly-(3-hydroxybutirate), P(3HB), which exhibits high crystallinity and higher resistance to UV than polypropylene.

The commercial use of PHAs is, however, limited mainly due to its relatively high production cost. Therefore, the use of low cost substrates is an important factor, along with the need to obtain strains for efficient substrate to polymer conversion (Khardenavis et al. 2007). Low cost substrates include starchy substrates, which are important compounds in food industry by-products. Also, agro-based industrial wastewaters, activated sludge, paper mill wastewater, crude glycerol waste stream, and waste cooking oil can be a suitable alternative as low cost substrate and biomass respectively for PHA production (Beun et al. 2000; Bengtsson et al. 2008; Costa et al. 2009; Chakravarty et al. 2010; Dobroth et al. 2011). Cassava is widely cultivated in the world, which implies that a considerable amount of by-products is produced, and an appropriate destination for these by-products must be found to minimize environmental impacts. According to Boonnop et al. (2009), typical cassava starch composition is 68.9% of dry matter (essentially carbohydrates) which can be detailed as follow: 95.9% of organic matter, 2.8% of protein, and 2.3% of fat matter. In relation to the limitations of cassava starch by-product as a substrate for PHA producing microorganisms, the most important parameter is the water solubility. Thus, PHA yield can be limited by amylase hydrolytic activity from microorganisms. Alternatively, chemical hydrolysis (alkaline or acid), high pressure or temperature could increase carbohydrate release from cellulosic material and increase PHA production.

One group of environmental microorganisms with great biodegradation potential is the *Bacillus* which is genetically and biochemically well understood and offers several advantages, such as, short generation time, attractive host for gene expression, it can easily grow to very high cell density using inexpensive carbon and nitrogen sources, and capability of secreting large amounts of enzymes (Law et al. 2003). With respect to the bacterial growth determination in turbid medium, most available methodologies are laborious and time-consuming. In this respect, available methodologies for bacterial growth determination considered as laborious and time-consuming for insoluble substrates are the microscopic counts or culture and colony counts that represent only the viable cells. In relation to soluble substrates, these methodologies can be resumed as assessing cell density by spectrophotometry or determination of cellular mass. In the first case a calibration curve is necessary to establish the correspondence between the cellular density and the number of cells, while in the second case, the invasive character of the sampling method must be considered. In other words, acceptable quantification implies a reasonable mass sampled, which may hinder the removal of aliquots from a single vial. An alternative method to measure bacterial growth is the use of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is widely used for *in vitro* measurement of metabolic viability of cell cultures subjected to different culture conditions (Sieuwerts et al. 1995). This assay is based on the ability of viable cells to produce formazan, which can be measured by visible spectrophotometry. Thus, the objectives of this study were to select a bacterial strain capable of growing and producing PHA from a cassava starch by-product as the carbon source, to identify selected microorganisms by molecular methods, and to assess the applicability of the MTT assay for the bacterial growth determination.

MATERIALS AND METHODS

Bacterial strains and culture media

The collection of 72 strains evaluated was obtained in the Applied Microbiology Laboratory (UNIVALI, SC, Brazil) (Table 1). The strains were isolated from environmental samples and classified by morphological, growth and biochemical characteristics, according to a published methodology (Logan and De Vos, 2009). For the culture maintenance, the microorganisms were grown in M9 media, which contained (per liter of distilled water): 6.78 g $Na_2HPO_4 \cdot 7H_2O$; 3.0 g KH_2PO_4; 0.5 g NaCl; 1.0 g NH_4Cl,

enriched with pure starch or cassava starch by-product (5 g/L), to evaluate the growth of the strains. In the assaying step, two pre-cultures were used: one in Luria-Bertani (LB) medium (per liter of distilled water: 10 g tryptone; 5 g yeast extract; 10 g NaCl), and the other in the medium described by Ramsay et al. (1990) without nutrient limitation (per liter of distilled water: 6.7 g $Na_2HPO_4 \cdot 7H_2O$; 1.5 g KH_2PO_4; 1.0 g $(NH_4)_2SO_4$; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.01 g $CaCl_2$; 0.06 g ferrous ammonium citrate; 1 mL trace-element solution. Each liter of trace-element solution in distilled water contained 0.3 g H_3BO_3; 0.2 g $CoCl_2 \cdot 6\ H_2O$; 0.1 g $ZnSO_4 \cdot 7H_2O$; 0.03 g $MnCl_2 \cdot 4H_2O$; 0.03 g $NaMoO_4 \cdot 2H_2O$; 0.02 g $NiCl_2 \cdot 6H_2O$; 0.01 g $CuSO_4 \cdot 5H_2O$), enriched with cassava starch by-product (20 g/L). For PHA production assays, the medium described by Ramsay et al. (1990) with nutrient limitation (the same as that described above, except with 2.5 g/L $Na_2HPO_4 \cdot 7H_2O$ and 0.83 g/L KH_2PO_4) and cassava starch by-product (20 g/L) were used.

Table 1. **Code and species of the evaluated bacteria from LAMA (Applied Microbiology Laboratory-Universidade do Vale do Itajaí, Brazil).**

Code	Species	Code	Species
002	*Bacillus cereus*	090	*Bacillus simplex*
032	*Bacillus cereus*	156	*Bacillus smithii*
055	*Bacillus cereus*	023	*Bacillus subtilis*
100	*Bacillus cereus*	146	*Bacillus subtilis*
102	*Bacillus cereus*	148	*Bacillus thuringiensis*
113	*Bacillus cereus*	098	*Geobacillus kaustophilus*
135	*Bacillus cereus*	099	*Geobacillus kaustophilus*
142	*Bacillus cereus*	139	*Paenibacillus apiarius*
159	*Bacillus cereus*	149	*Paenibacillus apiarius*
167	*Bacillus cereus*	151	*Paenibacillus apiarius*
169	*Bacillus cereus*	181	*Paenibacillus borealis*
174	*Bacillus cereus*	186	*Paenibacillus borealis*
176	*Bacillus cereus*	272	*Paenibacillus dendritiformis*
180	*Bacillus cereus*	273	*Paenibacillus dendritiformis*
258	*Bacillus cereus*	274	*Paenibacillus dendritiformis*
260	*Bacillus cereus*	032	*Paenibacillus dendritiformis*
091	*Bacillus ehimensis*	094	*Paenibacillus koreensis*
190	*Bacillus ehimensis*	097	*Paenibacillus koreensis*
031	*Bacillus firmus*	010	*Paenibacillus macquarensis*
018	*Bacillus licheniformis*	261	*Paenibacillus macquarensis*
020	*Bacillus licheniformis*	263	*Paenibacillus macquarensis*
062	*Bacillus licheniformis*	264	*Paenibacillus macquarensis*
084	*Bacillus licheniformis*	266	*Paenibacillus macquarensis*
259	*Bacillus licheniformis*	268	*Paenibacillus macquarensis*
073	*Bacillus megaterium*	051	*Paenibacillus pallidus*
095	*Bacillus megaterium*	015	*Paenibacillus polymyxa*
252	*Bacillus megaterium*	050	*Paenibacillus polymyxa*
262	*Bacillus megaterium*	012	*Paenibacillus polymyxa*
265	*Bacillus megaterium*	138	*Paenibacillus polymyxa*
075	*Bacillus mycoides*	184	*Paenibacillus polymyxa*
076	*Bacillus mycoides*	133	*Paenibacillus thiaminolyticus*
157	*Bacillus mycoides*	136	*Paenibacillus thiaminolyticus*
054	*Bacillus racemilacticus*	140	*Paenibacillus thiaminolyticus*
083	*Bacillus racemilacticus*	254	*Paenibacillus thiaminolyticus*
086	*Bacillus racemilacticus*	255	*Paenibacillus thiaminolyticus*
092	*Bacillus racemilacticus*	256	*Paenibacillus thiaminolyticus*

Selection of amylolytic microorganisms

The different measured parameters for the selection of microorganisms for the PHA production were based in their ability to grow in medium containing the residue as a source of carbon (diameter of the colony and MTT absorbance assays) and their ability to produce amylase to degrade the substrate (diameter of the halo and enzymatic index). Thus, the 72 strains were inoculated in solid M9 media with 5 g/L of commercial starch and were cultivated for 48 hrs (37ºC). The strain growth was evaluated by visual comparison and classified into: i) without, ii) poor or iii) vigorous growth. The strains with vigorous growth were selected and inoculated in solid M9 media with 5 g/L of cassava starch by-product at four points on each plate, and cultivated for 24 hrs (37ºC). These plates were stained with iodine solution (1% in ethanol), and the colony diameter and starch degradation halo were measured. The enzymatic index was calculated from the ratio between the average halo and colony diameters, to evaluate the starch degradation capacity through sugar consumption. The differences among treatments were analyzed using ANOVA ($P \leq 0.05$) and the Tukey's test ($P \leq 0.05$).

Determination of bacterial growth by MTT assay

The microorganisms were cultivated in two pre-cultures: 5 mL of LB medium for 24 hrs (37ºC, 150 rpm), followed by transfer to 50 mL of the medium described by Ramsay et al. (1990) without limitation, and 20 g/L of cassava starch by-product for 24 hrs. A 10% solution of pre-culture was transferred to 300 mL of limited medium with 20 g/L of cassava starch by-product and cultivated for 48 hrs. Bacterial growth was determined by MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Freshney, 2005). The culture was diluted (1:6) in medium without limitation of starch by-product, with six replicates, and 10 µl of MTT (5 mg/mL diluted in PBS buffer) were added followed by incubation for 1 hr (37ºC). Subsequently, 100 µL of 10% SDS in 0.01 M HCl were added and the mixture was incubated for 18 hrs at 25ºC. Spectrophotometry measurements were carried out at 540 nm. To compare the viable bacteria cells with the MTT assay response, the microorganisms from the medium containing cassava by-product were inoculated at different times in a Petri dish (LB medium). Aliquots were sampled after different time periods for unity forming colony (UFC) count and absorbance measurement.

PHA production and characterization

For qualitative detection of PHA production, cells were cultivated with Nile Red Dye (0.5 µg/mL diluted in DMSO), which stains PHA granules, as described for Jendrossek et al. (2007), and observed under an epifluorescence microscope (Olympus BX40). For PHA production and quantification strains were cultivated (48 hrs, 150 rpm at 37ºC) in hydrolyzed starch cassava by-product. Although the selected microorganisms showed ability to grow in the medium of raw starch cassava by-product, the final assays were performed with hydrolyzed products for two reasons: i) it would be difficult to determine the bacteria ability to produce PHA using crude insoluble substrate, since the unmetabolized substrate fraction can interfere with the polymer quantification, and ii) in previous tests was observed under microscope that two of isolates showed no precipitate of Nile Red Dye inside the cells (*i.e.*, no PHA accumulation) under the tested conditions. For this purpose appropriated amount of by-product were hydrolyzed at 121ºC for 30 min with 1% HCl solution (Law et al. 2003; Dalcanton et al. 2010). Hydrolysis of cassava starch by-product using this methodology released about 5% (m/m) in sugar (glucose-quantified by standard DNS procedure). After cells growth, they were recovered by centrifugation (1,800 g; 10 min; 20ºC; Eppendorf 5810R centrifuge), freezed (-20ºC) and lyophilized for 5 hrs in a Jouan LP3 lyophilizer. Samples were then stored in a freezer (-20ºC) for further analysis. The accumulated PHA was determined by gas chromatography, as described by Chia et al. (2010). PHA was extracted from lyophilized cells by the method described by Shishatskaya and Volova (2004). The characterization of PHA was carried out by Fourier transform infrared spectroscopy (FTIR) (Perkin Elmer).

The 16S ribosomal RNA gene amplification and analysis

Genomic DNA from Bacillus isolates was obtained (DNeasy Blood & Tissue Kit, Qiagen - MD) from overnight culture cells grown in LB medium (37ºC, 150 rpm) and used as a DNA template for the PCR reactions (Sambrook and Russell, 2001). The 16S rRNA gene fragment (1.5 Kb) was amplified by PCR using primers (F-5'-AGAGTTTGATCCTGGCTCAG-3'; R-5'-AAGGAGGTGATCCAGCCGCA-3') as described by Hiraishi (1992). Amplification reaction mixtures (20 µL) consisted of 0.2 mM of each

dNTP, 0.5 µM of each primer, 1 U of Platinum Taq DNA polymerase (Invitrogen, Brazil), 1X Taq polymerase buffer, 5 mM of MgCl2, 5% DMSO and 50 ng of DNA. The thermal cycling conditions consisted of one cycle at 94ºC for 5 min followed by 35 cycles of 30 sec at 94ºC, 30 sec at 58.5ºC, and 1.75 min at 72ºC, and a final extension step of 4 min at 72ºC. 16S rRNA amplicons were sequenced at Macrogen (Seoul, Korea) using standard protocols with dye terminator and capillary sequencer (ABI3730XL, Applied Biosystems, CA). For this purpose, each PCR product was cloned in pTZ57R/T using the InsTAclone™ PCR Cloning Kit (Fermentas, MD) and the recombinant plasmid introduced into chemically competent *Escherichia coli* DH5α cells (Sambrook and Russell, 2001). Cells were spread onto solid LB supplemented with ampicillin (100 µg/mL) and X-gal (0.02 mg/mL 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside (Invitrogen, CA). White transformant clones were selected and vector isolated (PureLink™ Quick Plasmid Miniprep Kit, Invitrogen, CA). Insert was sequenced using universal M13 primers, as well as a 16S rRNA internal primer (5'-GGATGACAGTACCTGAAGAATAAGCAC-3'). Sequences were deposited in the GenBank at NCBI. The homologies of the determined sequences of 16S rRNA gene were compared with full length sequences in the GenBank database by the BLAST method (Camacho et al. 2008).

RESULTS AND DISCUSSION

Selection of amylolytic microorganisms and determination of enzymatic index

From the 72 strains inoculated in pure starch as the sole source of carbon, 55 were able to grow in this substrate with varied growth rates classified as poor or vigorous. When these 55 selected strains were inoculated in pure starch, 16 bacterial strains exhibited a higher growth rate after 24 hrs of culture. These 16 bacterial strains were then selected and their amylolytic potential for the use of cassava starch by-products was evaluated. From these 16 strains, 14 visually showed vigorous colony formation (data not shown) and were selected for the next selection step. The enzymatic index (ratio between average halo and colony diameters) was determined to verify the capacity of the enzyme secretion to hydrolyze the starch of the culture media (Table 2). Statistical analysis (Tukey test) of the enzymatic index showed significant variation among the different indexes.

Table 2. **Bacterial growth on cassava starch residue and their hydrolytic activity.**

Bacterial strain	Colony diameter[a] (mm)	Halo diameter[a] (mm)	Enzymatic index[b]
LAMA23	1.067 ± 0.253^{C}	$2.925 \pm 0.218^{C,D}$	$2,997 \pm 0.399^{A}$
LAMA73	0.521 ± 0.031^{A}	1.317 ± 0.383^{A}	$2,592 \pm 0.869^{A}$
LAMA95	0.488 ± 0.022^{A}	1.333 ± 0.052^{A}	$2,748 \pm 0.220^{A}$
LAMA98	$0.733 \pm 0.113^{A,B}$	$2.958 \pm 0.253^{C,D}$	$4,193 \pm 0.360^{B}$
LAMA133	0.442 ± 0.007^{C}	2.267 ± 0.270^{B}	$5,221 \pm 0.704^{B}$
LAMA190	0.550 ± 0.033^{A}	$2.608 \pm 0.076^{B,C}$	$4,869 \pm 0.284^{B}$
LAMA261	0.475 ± 0.033^{A}	1.317 ± 0.014^{A}	$2,789 \pm 0.212^{A}$
LAMA262	0.454 ± 0.019^{A}	1.233 ± 0.038^{A}	$2,765 \pm 0.168^{A}$
LAMA263	0.470 ± 0.026^{A}	1.200 ± 0.043^{A}	$2,562 \pm 0.216^{A}$
LAMA265	0.500 ± 0.057^{A}	1.325 ± 0.115^{A}	$2,718 \pm 0.195^{A}$
LAMA268	$0.525 \pm 0.012^{A,B}$	1.291 ± 0.062^{A}	$2,470 \pm 0.182^{A}$
LAMA272	0.483 ± 0.040^{A}	1.258 ± 0.080^{A}	2.620 ± 0.197^{A}
LAMA409	$0.512 \pm 0.012^{A,B}$	1.275 ± 0.050^{A}	$2,504 \pm 0.097^{A}$
LAMA410	0.483 ± 0.019^{A}	1.283 ± 0.080^{A}	$1,283 \pm 0.117^{A}$

[a] Mean in mm ± SD for three replicates. Values in the same column with different superscript letters are significantly different from each other ($P < 0.05$, Tukey test). Separate analysis was carried out for each column.
[b] Ratio between starch degradation halo and bacterial colony.

The data in Table 2 show that the colony diameter of strain LAMA23 showed the highest value, whereas for the halo diameter strains LAMA23 and LAMA98 showed the highest values. Also, it can be observed that *B. ehimensis* (LAMA190), *G. kaustophilus* (LAMA098) and *P. thiaminolyticus* (LAMA133) had small colonies but high enzyme secretion levels, showing no direct relation between colony and halo diameters. Additionally, the results of Table 2 revealed that all microorganisms tested are able to secrete amylolytic enzymes at varied concentrations.

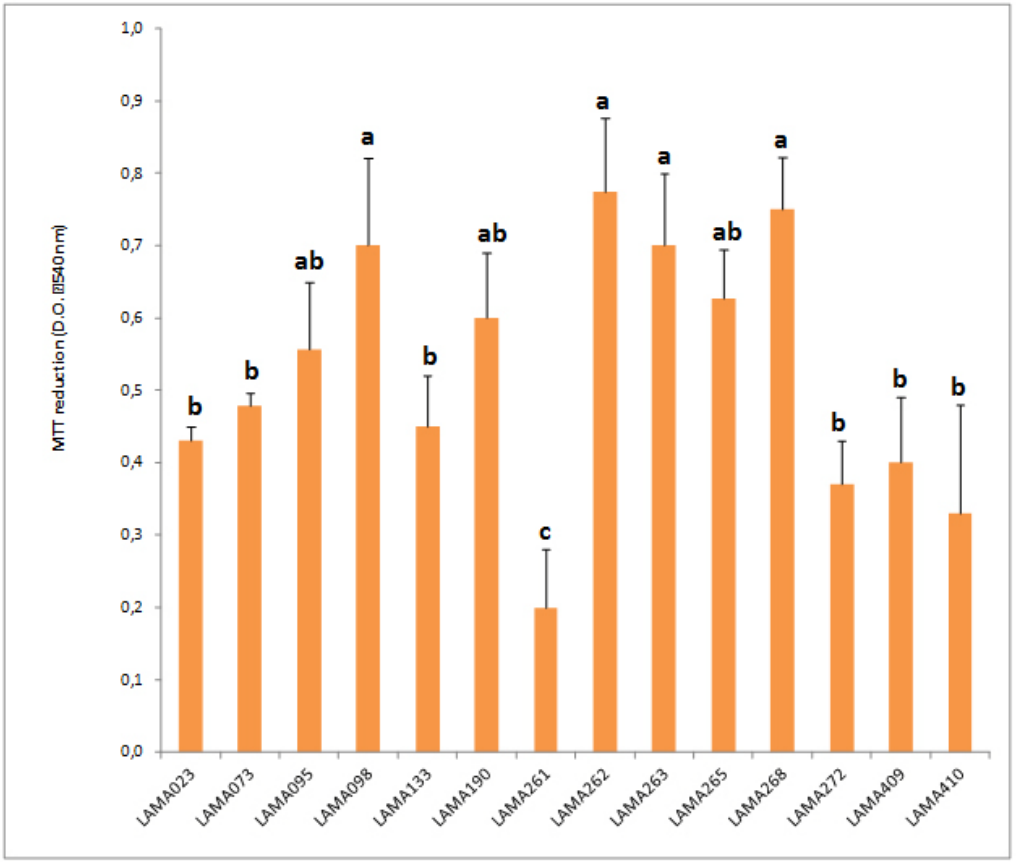

Fig. 1 Results from MTT assay to measure cellular growth of strains in M9 medium, with 5 g/L of cassava starch by-product (λ = 540 nm). The vertical bars in the columns are the standard deviation of the sample averages. The strains indicated by the same letter are not different at the 5% level of significance (Tukey test).

Thus, these results showed that the cassava starch by-product substrate enabled the microorganisms to grow in a minimal composition medium, and to secrete the enzymes responsible for PHA biosynthesis. This is a key feature for application in the industrial recycling processes, providing a rational way to reduce the environmental discharge of this by-product (Gupta et al. 2003). According to Reddy et al. (2003), three biochemical reactions are implicated in the PHA synthesis. In the first reaction, there is a condensation of two molecules of acetyl-coenzyme A (acetyl-CoA) to acetoacetyl-CoA (β-ketothiolase enzyme), followed by reduction of acetoacetyl-CoA to hydroxybutyryl-CoA (enzyme dependent of NADPH-acetoacetyl-CoA reductase), and finally, hydroxybutyryl-CoA monomers are polymerized to PHA (PHA-polymerase enzyme).

Determination of bacterial growth by the MTT assay

Bacterial growth of viable cells in this experimental context was determined by MTT assay. To verify the feasibility of this assay, a previous experiment was carried out with the strain LAMA 262 (selected randomly) grown in both raw and hydrolyzed cassava by-product.

The MTT assay results indicated no difference at the 5% level of significance between the microorganisms grown in the raw and hydrolyzed cassava by-product. Besides demonstrating the MTT assay feasibility, this finding confirms the versatility of the microorganisms to secrete all of the enzymes necessary for growth in a medium comprising this industrial residue. Therefore, the following tests were carried out with raw cassava by-product, considering minimization of the cost of PHA production on an industrial scale. The 14 selected bacterial strains were cultured in a medium containing raw cassava by-product and the results of the MTT assay for cellular growth are shown in Figure 1. It is possible to verify that some selected strains exhibited a vigorous growth in the limited medium composed by cassava starch by-product according to the MTT assay, except LAMA 261, which showed a lower growth potential than the other strains.

As MTT assay is proposed here as a new method for bacterial growth determination in insoluble media. Figure 2 show the relationship between formazan production and the number of viable cells of four strains cultivated in M9 media supplemented with glucose or cassava residue. A correlation of 0.97 between MTT reads and units forming colony average points was determined. This result clearly indicates the applicability of MTT for growth monitoring purposes.

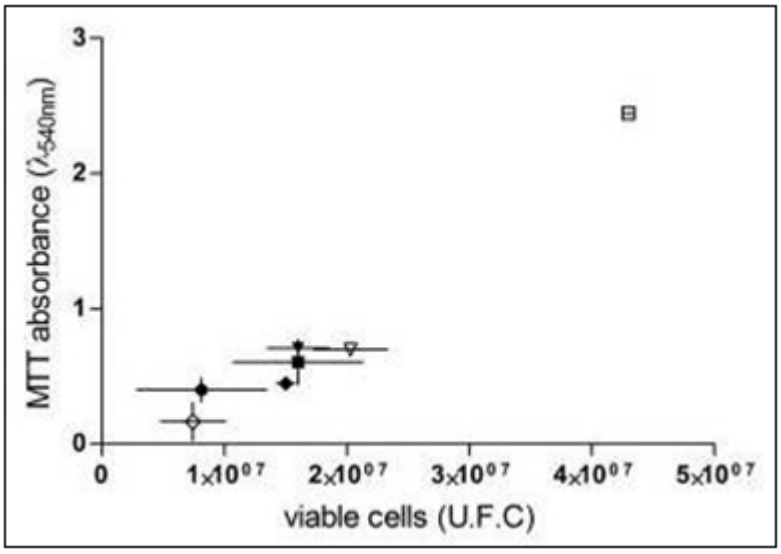

Fig. 2 Relationship between MTT reads (λ = 540 nm) and number of viable cells (U.F.C- unity forming colony). Strains were cultivated in M9 medium supplemented with 5 g/L of glucose (filled symbols) or cassava starch by-product (open symbols). Strains: LAMA 265 (diamonds), LAMA 073 (circle), LAMA 09 (square) and LAMA (triangle). The horizontal/vertical bars are the standard deviation of the sample averages (three replicates).

In this study, the determination of bacterial growth in a turbid medium could be performed only by means of culture analysis and microscopy microorganism counts, which are laborious and time-consuming. The MTT assay is rapid and easy to perform, although it can be significantly affected by a number of conditions (Sieuwerts et al. 1995). In this respect, MTT assay can be affected directly by: i) the sample dilution, since compound incubated with cells must be reduced by succinate dehydrogenase prior to spectrophotometric measurement; ii) exposure time of MTT to the cells, which could be variable according to the cell physiology. Furthermore, MTT solution conservation is important to preserve reaction effectiveness. Thus, this compound must be stored in a freezer, as well as the solution prepared in PBS buffer (5 mg/mL). In our experiments the MTT assay was found to be a reliable methodology to measure bacterial growth rates, without interference due to the culture medium or the industrial residue composition.

Production, purification and characterization of PHA

To evaluate the PHA production capacity, two microorganisms LAMA262 and LAMA268 (*Bacillus megaterium* and *Paenibacillus macquariensis*) were selected from MTT assay and were inoculated in

limited medium containing 20 g/L of pure glucose or 20 g/L of raw cassava starch by-product, both with the addition of Nile Red Dye. As a positive control, a recombinant strain of *E. coli* (DH10B) transformed with plasmid pRLC2, which contains the genes for PHA production (gene phaC of *Chromobacterium violaceum* and genes phbA and phbB of *Cupriavidus necator*, all regulated by Lac promoter of the plasmid) was used. After culture, observation under an epifluorescence microscope indicated that the strains grew in both carbon sources; however, they did not produce PHA from the cassava by-products media (data not shown), perhaps the few starch hydrolysis products were used to the cellular multiplication, only.

In order to identify which among the 14 pre-selected amylolytic strains are the most PHA producers, they were cultivated in rich glucose (20 g/L) complex media (10 g/L tryptone; 5 g/L yeast extract; 10 g/L NaCl; 2.5 g/L $Na_2HPO_4 \cdot 7H_2O$ and 0.83 g/L KH_2PO_4) and hydrolyzed cassava by-product (20% of hydrolyzed cassava (v/v), equivalent to 2 g of glucose/L media). After 48 hrs of incubation, the cultures were observed under an epifluorescence microscope and the results showed that LAMA073, LAMA262 and LAMA265 produced a higher degree of granule fluorescence than the other bacterial strains (Figure 3).

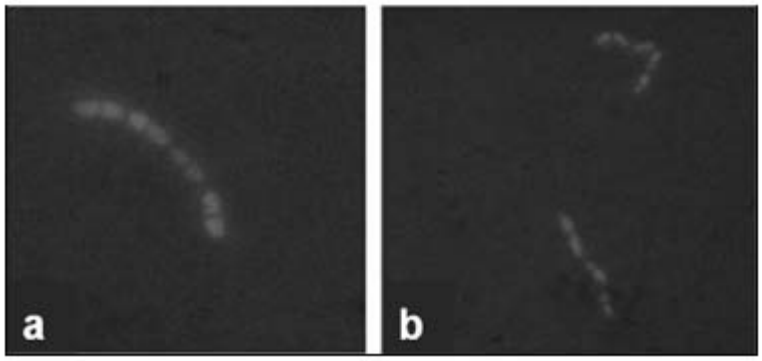

Fig. 3 PHA accumulation observed under epifluorescence microscope. a) LAMA262 cultivated in cassava by-product medium; b) LAMA 262 cultivated in glucose medium. Magnification 1000X.

The four selected strains (LAMA095, LAMA073, LAMA262 and LAMA265) were then cultivated in pure glucose and cassava starch by-product in order to quantify the enzyme biomass and polyhydroxybutyrate [P(3HB)] polymer production. Through methanolysis and using benzoic acid as the internal standard, samples were analyzed by gas chromatography (Table 3).

According to Table 3, the LAMA095 strain produced the highest biomass amount when cultivated in the cassava starch by-product. However, LAMA073 produced a higher amount of P(3HB) when cultivated in glucose, where the polymer corresponds to approximately 41.95 wt.% of its biomass. Interestingly, LAMA265 showed a yield of 25% (w/w dry cell) in the production of P(3HB) when cultivated with 20% of hydrolyzed cassava starch by-product (v/v), which increased to 31.6% when cultivation was carried out in pure glucose. Nevertheless, the LAMA095 strain should be highlighted because it showed a higher biomass production when cultivated with cassava starch by-product than when cultivated with pure glucose. This strain produced the highest amount of P(3HB), while the polymer recuperation percentage was similar for the two substrates (24.1% of P(3HB) for glucose culture, and 23.9% for hydrolyzed cassava starch by-product).

To improve the P(3HB) production yield, the proportion of hydrolyzed cassava starch by-product in the culture medium was increased to 40% (v/v), equivalent to 4 g/L of sugar. As expected, an increase in the strain biomass and in P(3HB) production were observed when the carbon source was increased in the medium, most notably for the strain LAMA095, which reached 1.48 g P(3HB)/L. The highest P(3HB) yield was achieved by the LAMA073 strain, 30.4% (w/w dry cell), which is 57% higher than the amount produced by the strain cultivated in the medium with 20% hydrolyzed cassava starch by-product.

Table 3. Production of biomass (dry weight) and P(3HB) as well as polymer recuperation percentage of strains cultivated in limited media with 20% and 40% of hydrolyzed cassava starch by-product (v/v) and 20 g/L of pure glucose incubated for 48 hrs at 37ºC.

Strain	Substrate	Biomass (g/L)[a]	Tukey test (biomass)[b]				P(3HB) (g/L)	P(3HB) (%)[c]
			all	glu.	cas. 20%	cas. 40%		
073	Glucose	1.74 ± 0.4	A	A			0.73	41.95
	Cassava 20% (v/v)	2.07 ± 0.9	B		B		0.27	13.04
	Cassava 40% (v/v)	3.35 ± 0.7	C			B	1.02	30.45
095	Glucose	1.37 ± 0.5	D	C			0.33	24.09
	Cassava 20% (v/v)	4.48 ± 0.5	E		A		1.07	23.88
	Cassava 40% (v/v)	4.97 ± 0.9	E			A	1.48	29.78
262	Glucose	1.58 ± 0.6	F	B			0.34	21.51
	Cassava 20% (v/v)	1.69 ± 0.3	F		C		0.10	5.92
	Cassava 40% (v/v)	3.36 ± 0.8	G			B	0.19	5.65
265	Glucose	1.55 ± 0.7	H	B			0.49	31.61
	Cassava 20% (v/v)	1.40 ± 0.7	H		D		0.35	25.00
	Cassava 40% (v/v)	2.92 ± 0.5	I			C	0.52	17.81

[a] Biomass (dry weight) was determined using three replicates for each microorganism, and lyophilized cells were mixed to carry out gas chromatography analysis. [b] Tukey test ($P < 0.05$) - strains indicated by the same letter are not significantly different from each other. all - Tukey test (TT) all culture conditions; glu.- TT for treatments cultivated in glucose; cas. 20% - TT for treatments cultivated in cassava 20% and cas. 40% - TT for treatments cultivated in cassava 40%. [c] % P(3HB) = (weight of P(3HB)/weight of biomass) x 100.

The percentage of P(3HB) observed in this study (around 30% based on w/w dry cells) can be compared with results of other studies available in the literature. Dalcanton et al. (2010) obtained 35% of PHB (w/w dry cell) when *Ralstonia eutropha* (recently renamed *Cupriavidus necator*) was cultivated in hydrolyzed rice processing by-product. Recombinant *E. coli* showed 34.8% yield of PHB (w/w dry cell) when this bacterium was cultivated in hydrolyzed corn starch (Fonseca et al. 2008), which is the same magnitude found in the present work. Better results were obtained by Zhang et al. (1994) who cultivated recombinant *E. coli* and *Klebsiella aerogenes* in 6% of cane molasses. These authors showed that the bacteria accumulated 45% of P(3HB) (w/w dry cell).

With regard to *Bacillus* studies, Gouda et al. (2001) cultivated *B. megaterium* in cane molasses, obtaining a yield of 46.2% of P(3HB) (w/w dry cell), similar to the yield obtained by Omar et al. (2001) (around 50%) using beet molasses and the same bacterial species. In another study, Law et al. (2003) carried out assays with recombinant *B. subtilis* cultivated in hydrolyzed malt waste and obtained 15.3% (w/w dry cell) of yield, while *B. megaterium* CQPBA036-07 DMP01 cultivated in 50 g/L of sucrose yielded 75% of PHB (w/w dry cell) (Faccin et al. 2009). More recently, Kulpreecha et al. (2009) achieved a yield of 61.6% of PHB (w/w dry cell) cultivating *B. megaterium* BA-019 for 12 hrs. The production of polyhydroxybutyrate (PHB) using agro-industrial residues as the carbon source has also been studied using seven substrates, viz., wheat bran, potato starch, sesame oil cake, groundnut oil cake, cassava powder, jackfruit seed powder and corn flour (Ramadas et al. 2009). These residues were hydrolyzed using commercial enzymes and the hydrolysates assessed to select the best substrate for PHB production. Jackfruit seed powder allowed the maximum production of PHB under submerged fermentation using *Bacillus sphaericus* (19%) at an initial pH of 7.5 (Ramadas et al. 2009). These literature data show that the P(3HB) production yield presented in this study is acceptable, despite the poor medium content and low cost substrates used in our experiments. In this regard, it is important to note that the quantity of hydrolyzed cassava starch by-product used in these experiments was lower than the quantity of glucose, and thus it may be possible to generate more significant results using equivalent quantities of substrate. Also, the P(3HB) yield can be increased in purified/hydrolyzed substrate raw material.

Table 4 shows the molecular sequencing of four selected bacterial strains based on the 16S ribosomal RNA gene amplification and a comparison of the results with NCBI GenBank data. The segments

isolated from the strains LAMA073, LAMA095, LAMA262 and LAMA265 were compared to nucleotides accessed in GenBank. After comparison, the bacterial strains were identified as *B. megaterium* and registered in the Genbank (NCBI) under the following access numbers: LAMA 073 - HM104230, LAMA 095 - HM104231, LAMA 262 - HM104232 and LAMA 265 - HM104233. Although we obtained the same identification for the four strains, it is clear that this species presents great diversity and plasticity concerning its metabolism, which can be exploited in several industrial processes including, more recently, the recycling waste industry.

Table 4. 16S rDNA sequence for molecular identification: comparison of four selected bacterial strains with data from GenBank (NCBI).

Bacterial strain	Specie	Most similar GenBank-NCBI Access	Identity[a]	NCBI access registered
LAMA73	*B. megaterium*	AF142677.4	99%	HM104230
LAMA95	*B. megaterium*	AJ717381.1	99%	HM104231
LAMA262	*B. megaterium*	DQ660362.1	99%	HM104232
LAMA265	*B. megaterium*	DQ267829.1	99%	HM104233

[a] percentage based in number of identical nucleotides in relation to the total number of compared nucleotides.

Figure 4 shows the FTIR spectra for the P(3HB) produced by LAMA095 (Figure 4a) and LAMA265 (Figure 4b). Both spectra exhibited the main characteristic bands of PHAs, which confirmed the presence of the polymer: an intense band at 1,726 cm^{-1}, related to carbonyl stretching, which is typical of PHA (Hong et al. 1999). Several other bands with varied intensity, between 1,450 and 1,000 cm^{-1}, are also present, which are attributed to deformation of methyl and methylene groups and due to C-O-C stretching. It is important to point out that the exact wave number of the peaks varies with the crystallinity and with the chain length of the PHA (Hong et al. 1999; Kansiz et al. 2000). These results are in agreement with the P(3HB) characterization data available in the literature (Kansiz et al. 2000).

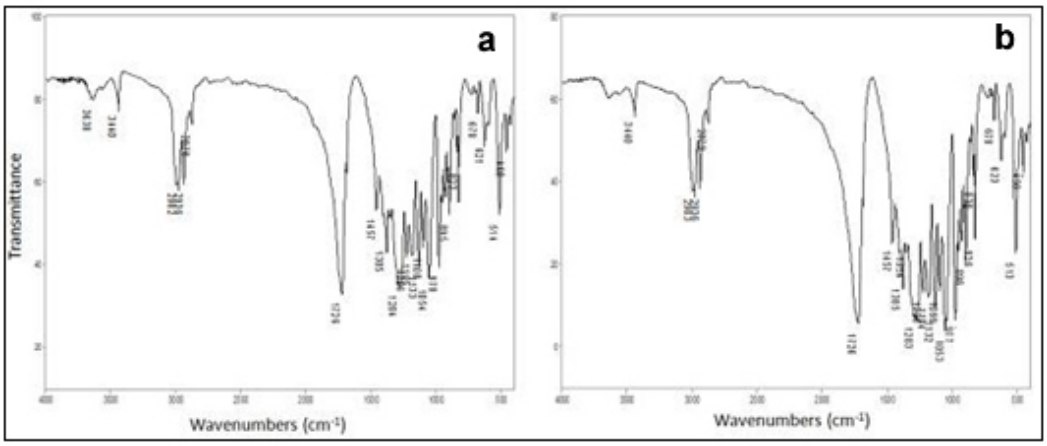

Fig. 4 FTIR spectrum for P(3HB) produced by the strains LAMA095 (a) and LAMA265 (b) of *B. megaterium.*

The cassava processing by-product possesses high levels of starch and reducing sugar when hydrolyzed, which will generate an environmental imbalance if discarded to the environment without prior treatment (FAO, 2001). In this perspective, production of PHA from cassava starch residues meets with two goals: i) recycling of cassava processing waste to give an appropriate destination for this industrial by-product, and ii) development of high value polymeric products for use in areas such as medicine, agriculture, tissue engineering, nanocomposites, polymer blends and chiral synthesis (Philip et al. 2007). The results of this study revealed that PHA production by microorganisms is dependent on

the culture conditions and substrate concentration, as well as the addition of supplements. Most microorganisms, including *Bacillus*, require nutrient limitation and carbon excess in the medium in which they are grown, allowing them to conserve energy by means of metabolism regulation and PHA accumulation. This processes starts with carbon absorption, followed by PHA synthesis and accumulation (Reddy et al. 2003, Philip et al. 2007).

From the different species tested in this study, *B*. *amyloliquefaciens*, *B. stearothermophilus*, *B. subtilis, B. megaterium* and *B. licheniformis* were effective in P(3HB) biosynthesis. Furthermore, four *B. megaterium* strains were selected as natural PHAs producers when cultivated in media containing cassava starch by-product as the sole carbon source, which is consistent with other studies on PHA production by this species (Gouda et al. 2001; Faccin et al. 2009; Kulpreecha et al. 2009). PHA was first discovered in a *B. megaterium* culture, but this species was subsequently neglected by researchers, probably because it utilizes the polymer in the presence of carbon and nutrient sources as energy for the sporulation process, while other bacteria consume the polymer only when there are no carbon and nutrient sources (Wu et al. 2001). Microorganisms that undergo the sporulation process are easily preserved and replicated, and are more stress-tolerant. Thus, *B. megaterium* is found in abundance in soil and water, and has important industrial advantages, *e.g*., it is non-pathogenic, it can grow in different carbon sources and it produces PHAs from industrial by-products (Gouda et al. 2001; Law et al. 2003).

CONCLUDING REMARKS

From a total of 72 strains, four isolates of *B. megaterium* were selected based on their capacity to bioconvert hydrolyzed cassava starchy by-product into P(3HB). One of them reached 4.97 g dry biomass/L with 29.7% of P(3HB), demonstrating its efficiency to produce biodegradable polymers from residues. The bacterial growth was monitored thought the MTT assay, which proved to be a reliable methodology for this purpose. Finally, the alternative treatment proposed for cassava by-products will minimizes environmental impacts, ensuring a sustainable life-cycle for polymeric products.

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