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Full Length Research Paper

Bioprospecting and characterization of poly-β-hydroxyalkanoate (PHAs) producing bacteria isolated from Colombian sugarcane producing areas

Moreno Nubia*, Gutiérrez Ivonne, Malagón Dionisio, Grosso Victoria, Revelo Dolly, Suárez Diego, González Juan, Aristizábal Fabio, Espinosa Armando and Montoya Dolly

Universidad Nacional de Colombia, Instituto de Biotecnología, Ciudad Universitaria, Edificio Manuel Ancizar, Bogotá, Colombia, AA 14490.

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Bioprospecting for poly-β-hydroxyalkanoate (PHA)-accumulating micro-organisms was carried out in sugarcane growing areas of Colombia. They were isolated in unbalanced culture medium (high carbon/nitrogen ratio) with sucrose, fructose and glucose used as carbon source. PHAs producing bacteria were identified by staining with Sudan black and solubilising cellular components in sodium hypochlorite. A arbitrary scale was established (ranging from 1 to 4) for selecting the best strains, acording to growth, staining with Sudan black and solubilising cellular material. 108 isolates rated higher than 2 were obtained by using this scale; 44 of these were selected for evaluating them in a balloon flask for their ability to grow in sucrose. Scheffe's test grouped the isolates evaluated in the balloon flask for polymer production and productivity. The 6 best isolates were evaluated in a fermenter to determine their kinetic growth profiles, substrate consumption and polymer accumulation. Differential scanning calorimeter (DSC) was used on the recovered polymer for determining fusion temperature and the conclusion was reached that 2 strains accumulated poly-β-hydroxybutyrate (PHB) and another 4 accumulated hydroxy-butyrate copolymers and other monomer units. These 6 strains were molecularly characterised by partially sequencing the 16s rRNA ribosomal gene, localizing them in 4 clusters on the taxonomic tree.

Key words: Biopolymer, PHA, native microorganism, fermentation, microbiology biodiversity.

INTRODUCTION

Biological polymers (due to their physical characteristics and ability to become rapidly degraded) are candidates for replacing those having a petrochemical origin. Biopolymers are synthesised by microorganisms from agricultural substrates and are able to become degraded to carbon dioxide and water in aerobic conditions or to methanol in anaerobic conditions in such diverse habitats as soil, sea, stagnant water or sewage (Lee, 1996; Reusch, 2002). Poly-β-hydroxyalkanoates (PHAs) are inclusion bodies accumulated by some bacterial genera as reserve material, when culture medium is unbalanced due to limited oxygen, nitrogen, phosphorous, sulphur or magnesium and an excess of carbon source (Kim et al., 1994; Lee, 1996; Madison and Huisman, 1999). More than 90 PHA polymers are currently known; poly-β-hydroxybutyrate (PHB) has been the most widely studied, being a homopolymer whose monomer units have a D (-) configuration due to the stereospecificity of the enzymes involved in the synthesis (Lee, 1996).

Production and marketing of PHAs have been limited in two ways. The first deals with the bacteria's ability to accumulate the polymer, since PHA accumulation has been detected in about 300 bacterial species but accumulation levels in many of them is very low. Nevertheless, species such as *Ralstonia eutropha* (previously known as *Alcaligenes eutrophus*), *Alcaligenes latus*, *Pseudomonas oleovorans*, *Pseudomonas putida*, recombinant *Escherichia coli* and *Azotobacter vinelandii* have

*Correspondig author. E-mail: ncmorenos@unal.edu.co. Tel: 0571-3165000 Ext 16972. Fax: 0571-3165415.

been widely investigated. The other aspect which has limited PHA production and marketing is related to the high costs of biological materials *cf* those of petrochemical origin. It has been shown that the cost of substrate (mainly carbon source) affects final PHA cost the most (Khanna and Srivastava, 2005).

New strains, using economic substrates and having a high accumulation percentage, must thus be isolated to resolve these problems. Interest has been centred on gram negative bacteria from soil where sugarcane is being cultivated due to the microbial richness present and the selective pressure caused by the imbalance of the carbon/nitrogen ratio, by the decomposition of residues in the soil leading to an excess of carbon and nitrogen becoming limited (Gómez et al., 1996; Rodríguez et al., 1995; De Lima et al., 1999; Wang, 1998). Certain parameters must be born in mind when selecting a microorganism as a candidate for the industrial production of PHAs, such as the cell's ability to use cost-effective carbon sources, speed of growth, ability to synthesise the polymer and the percentage of polymer accumulation (Lee, 1996; Khanna and Srivastava, 2005). Agricultural and food waste has been used as substrate in an attempt to reduce costs (Lee and Gilmore, 2005).

New applications have been sought when trying to find a market niche where cost will not be a limiting factor for commercialising PHAs, such as package for foodstuffs (Bucci et al., 2005), preparation of stereospecific isomers (Lee et al., 2000) and biomedical applications (Gogolewiski et al., 1993; Cheng and Wu, 2005; Wang et al., 2005).

This work presents the methodology employed for isolating strains from sugarcane producing areas which were able to accumulate PHAs, selecting the best isolates, their molecular characterisation by partially sequencing the ribosomal 16s RNA gene, the best isolates evaluation in both balloon flask and fermenter and characterising the biopolymer so obtained by differential scanning calorimeter.

MATERIALS AND METHODS

Sample soil collection

Isolates were obtained from soil and rhizosphere samples taken from sugarcane growing farms located in the Colombian departments of Nariño, Valle del Cauca, Santander and Cundinamarca. The samples so collected were identified and kept in plastic bags. They were then transferred to the Microbiology laboratory at the Universidad Nacional de Colombia's Instituto de Biotecnología and remained protected from the light in a refrigerated room at 4°C. Each sample was homogenised by sieving (2.0 mm pores); dry weight equivalents were established by treating 3 samples having 20 g of fresh soil, incubating them at 105°C for 12 h (Alef and Nannipieri, 1995).

Isolating Gram negative microorganisms PHA-producing bacteria

The colonias were isolated by the methods described by Gómez et al. (1996) and Ramsay et al. (1990) in a minimum mineral salt medium (MSM), having inhibitors for Gram positive bacteria and fungi and a mixed carbon source (glucose, fructose and sucrose) for obtaining just Gram negative microorganisms. The equivalent of 5 g of dry soil was taken and cultured in 100 ml of MSM medium; following 12 h incubation, seried dilutions were carried out from enriching culture using isotonic saline solution until a 10^{-8} dilution was reached. 100 µl of the last three dilutions were spread on Petri dish with unbalanced MSM solid medium or unbalanced C/N ratio, containing the three separate carbon sources. The sowings were incubated at 30°C to isolate bacterial colonies (Alef and Nannipieri, 1995).

The isolated colonies were streaked on nutritive agar until obtaining pure cultures for identifying each separate morphotype from the colonies. Gram staining was used to determine the form, size, type of aggregation and the presence of characteristic structures, such as reserve granules, endospores or cysts.

Two techniques were used for detecting the presence of polymer: staining with Sudan black (Burdon, 1946) and dissolving cellular components (except for PHA) with sodium hypochlorite (Law and Slepecky, 1960; García et al., 2001). Micro-organisms being able to accumulate PHAs were selected in MSM and grown in duplicate in 3 fresh Petri dishes of MSM agar, each dish containing a different carbon source (glucose, fructose or sucrose) and incubated at 30°C for 5 days. An arbitrary scale running from 1 to 4 was established as criteria for rejecting or accepting an isolate; this took into account growth in unbalanced MSM agar (1: scarce; 2: moderate; 3: abundant; 4: very abundant), Sudan black (1: less than 20% of cells presenting polymer; 2: 20% to 50% of cells presenting polymer granules; 3: 51 to 80% of cells presenting polymer granules; 4: more than 80% of cells presenting granules) and hypochlorite test (1: scarce; 2: moderate; 3: abundant; 4: very abundant). A relative weight was also established for each of the criteria evaluated as follows: 20% growth, 30% Sudan black and 50% hypochlorite, in such a way that isolates rated equal to or greater than 50% were considered to be promising or had good quality and were thereby criopreserved.

Molecular characterisation of isolates

The six best native PHA-accumulator isolates were first characterised by partially sequencing the ribosomal 16s RNA gene. Template DNA used for the amplification was obtained from a bacterial lysate (50 - 200 ng DNA). Conditions for amplifying the fragments were: one 2-min-cycle at 94°C; 25 cycles of 15 s at 94°C, 30 s at 62°C and 50 s at 72°C and an extension cycle of 15 s at 94°C, 30 s at 62°C and 7 min at 72°C. Each 25 µl reaction contained: 1X buffer (10 mM Tris-HCl, pH 9.0 at 25°C, 50 mM KCl and 0.1% Triton X-100) (Promega), 0.18 mM dNTPs and 0.3 µM of each primer A, E (direct primers) and J (reverse primer) (Keis et al., 1995) (A and J were modified at their 3'-OH extreme according to Montoya et al. (1999), 25 mM $MgCl_2$ and 0.1 U *Taq* polymerase (Promega). All PCR reactions were carried out using a Hybaid Omn-E thermocycler. The IBUN 22A (*Clostridium* sp.) strain from IBUN's Microbiology laboratory was used as positive control. The expected size for the IBUN 22A *Clostridium* sp. fragment amplified with primers A and J was 711 bp (Montoya et al., 1999) and was around 1,400 bp with primers E and J. the PCR product was analysed on 1% agarose gels-1X TBE and run at 3V/cm for 2 h.

REVSEQ, ClustalW, Clustalx 1.81 and GeneDoc software was used for analysing data re partial sequences from sequenced PCR fragments (MACROGEN-Korea). Similarity was sought by aligning final partial sequences against GenBank/EMBL/DDBJ databases using BLASTN software (version 2.8.8). Taxonomic relationship was determined by aligning partial sequences from the 16 S ARNr gene from isolate sequences from this study, with reference sequences obtained from GenBank/EMBL/DDBJ databases using Clustalx 1.81 software. The tree was constructed using MEGA 2.0

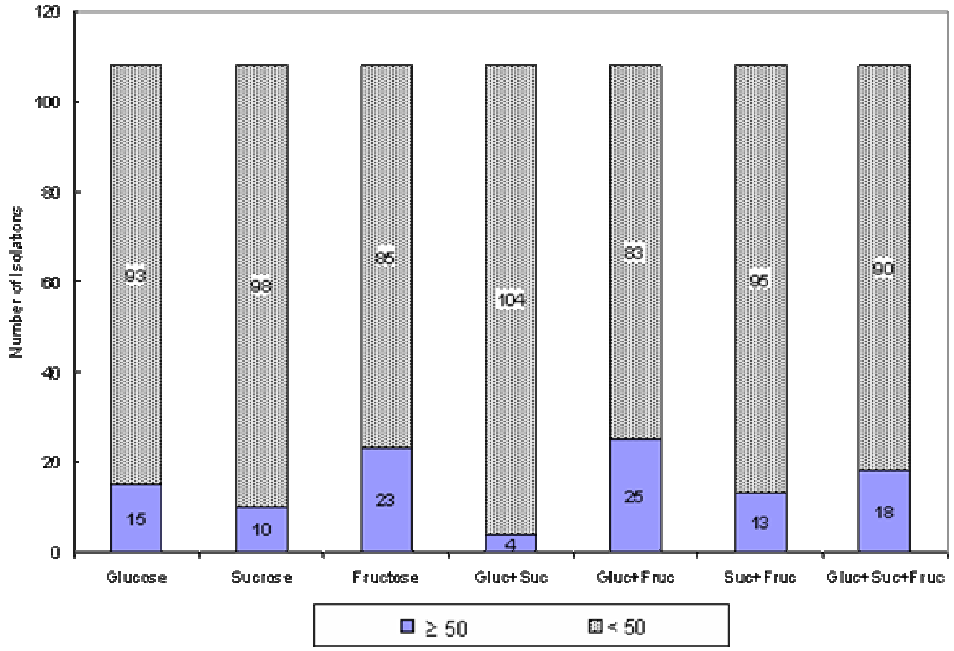

Figure 1. Number of promising isolates according to carbon source in which PHAs accumulate. n = 108.

software, employing the Neighbour-Joining algorithm grouping strategy (Saitou and Nei, 1987) and using a *p* distance matrix. Around 1,400 to 1,500 basepairs were used. The dendogram so obtained was subjected to Felsentein's Bootstrap test.

Determining kinetic parameters

Accumulated biomass was quantified by spectrophotometry using a standard curve relating biomass absorbance to dry weight; target was saline solution at 600 nm wavelength on VARIAN DSM 100 equipment. Sucrose was determined by quantifying reducing sugars with DNS, following hydrolysis with commercial invertase at 60°C for 30 min (Miller, 1959). Microscopy (using Gram staining) was performed to confirm culture purity; polymer accumulation was observed by quantifying using the turbidimetric test using sodium hypochlorite (Law and Slepecky, 1960) and staining with Sudan black (Burdon, 1946).

Determining profiles for PHA accumulation in balloon flasks

Promising isolates able to grow in sucrose were evaluated in 250 mL flask. The isolate being evaluated was sown in 10 ml nutritive broth, incubated at 30°C, with shaking at 100 rpm for 12 h. At the end of this time, it was put in a 250 ml balloon flask containing 100 ml unbalanced MSM liquid medium, using sucrose as sole carbon source. It was incubated at 30°C, with shaking at 100 rpm. 2 ml samples were taken each 4 h during the first 12 h and then each 6 h for determining biomass and substrate concentration as well as polymer accumulation. Fermentation was halted when the carbon source was completely exhausted. All assays were done in duplicate.

PHA accumulation in 7-litre fermenter

Strains selected during balloon flask evaluation as being excellent PHA formers were then evaluated in a 7-litre BIOFLO III fermenter (5-litre operational volume) equipped with temperature control, pH control, shaking control and dissolved oxygen sensor. An inoculum (10% of fermentor volume) was prepared with the strain to be evaluated and incubated at 30°C under rotational agitation at 200 rpm for 24 h. The fermenter was inoculated at the end of this time. The culture was kept at 30°C, with shaking at 300 rpm and pH was maintained at 7.0 by adding sterile soda. The medium used is a modification of the medium used by Barbosa et al. (2005) for *R. eutropha* where fructose was replaced by sucrose. Fermentation was carried out by two-stage fed lot; biomass was left to grow without limitation during the first stage. Once the carbon source became exhausted, a pulse of sucrose was added to the fermenter restabilising initial concentration. Fermentation was halted when sucrose became totally consumed or its concentration did not change over time.

RESULTS AND DISCUSSION

Isolating Gram negative PHA-producing bacteria

440 colony forming units (UFC) were isolated having different cellular morphology and types of colony. 291 out of the 440 isolates were able to accumulate polymer in any of the 3 evaluated sugars; when they were stained with Sudan black, 108 obtained a rating equal to or greater than 2 on the defined semi-quantitative scale. 15 (14%) of these isolates accumulated in glucose as sole carbon source, 10 (9%) in sucrose and 23 (21%) in fructose. On the other hand, 25 isolates (23%) accumulated in glucose and fructose as carbon source, 4 (4%) in glucose and sucrose, 13 (12%) in sucrose and fructose and 18 isolates (17%) were able to accumulate in any of the 3 sugars used (Figure 1). These results are in agree-

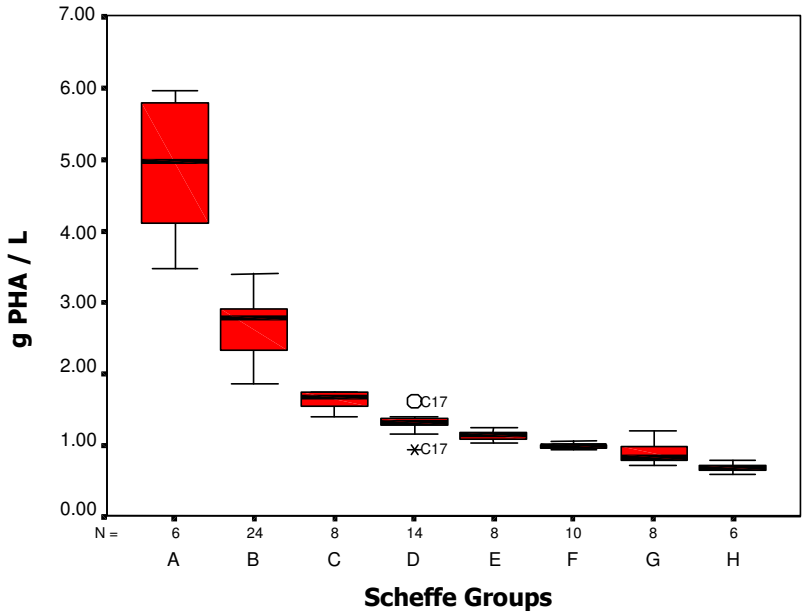

Figure 2. Scheffe groups for PHA production.

ment with has been reported in the literature regarding finding a great quantity of micro-organisms in sugarcane crops able to accumulate PHAs due to selective pressure caused by high carbon: nitrogen ratio (Gómez et al., 1996; Rodrigues et al., 1995; De Lima et al., 1999; Wang and Bakken, 1998).

44 of the 108 isolates considered to be promising accumulated polymer in sucrose; this makes them good candidates for industrial production of PHAs due to the possibility of using complex and economic substrates such as molasses which has 36% of this sugar in its composition. This is a great advantage, since the cost of the substrate become reduced by 22.4% when the microorganism uses sucrose as carbon source via the Entner-Doudoroff route as reported by Yamane et al. (1995).

The 44 isolates presenting PHA accumulation in sucrose were evaluated at flask level using this substrate as carbon source. Variance analysis determined whether a strain had a significant effect on response variables considered here: production (g PHA/l) and productivity (gPHA/l.h). Scheffe's test was used for determining which levels of factor work considered (44 strains) showed significant differences. The groups of strains formed by Scheffe's test indicated that there were no significant differences between each of them; however, a 5% significant difference was presented between groups. SAS software version 6.12 for Windows was used for analysing statistical data.

Figure 2 shows the 8 groups (A to H) formed by Scheffe's statistical test for the variable PHA production. Group A was formed by 3 strains (2G-57, B69 and C14) which presented the highest production values (5.55, 5.28 and 3.79 g/L, respectively). Group B was formed by 12 strains (C16, N2004, N2109, N3510, S0305, S1306, S1308, S1402, S1407, S1602, S1804 and S1901) having production values ranging from 3.21 to 1.99 g/L. The other strains were located in the remaining groups, having production values ranging from 1.71 to 0.63 g/L, these being extremely low for being considered as strains having potential for industrialization.

Scheffe's test for productivity (gPHA/l.h) grouped the strains into 6 groups (A to F) (Figure 3). Group A was formed by 2 strains (C14 and S1407) which presented the highest productivity values (0.0795 and 0.070 g/L.h, respectively). Group B was formed by 16 strains (2G-57, B69, C16, C22, C29, C3-26, C52, C54, N2004, N2109, N3510, S0305, S1306, S1308, S1402, S 1602 and S1804) having productivity ranging from 0.066 to 0.0266 g/L.h. The other strains were located amongst the remaining groups, having productivity values ranging from 0.026 to 0.009 g/L.

It can be seen that the groups forming Scheffe for the production and productivity variables were not equal; this was due to when productivity was being evaluated, time was involved as a factor. The 3 isolates presenting the highest production levels (2G-57, B69 and C14) were located in group B regarding productivity; this was due to the time being employed for reaching such production being prolonged (75, 98 and 54 h respectively). The opposite happened with isolate S1407 which did not present the highest production levels (Group B) but did have a high level of productivity since it reached such production within 36 h. 20 (Sheffe groups A and B) of the 44 isolates evaluated therefore presented interesting characteristics regarding production and productivity, making them promising candidates for industrial PHA production.

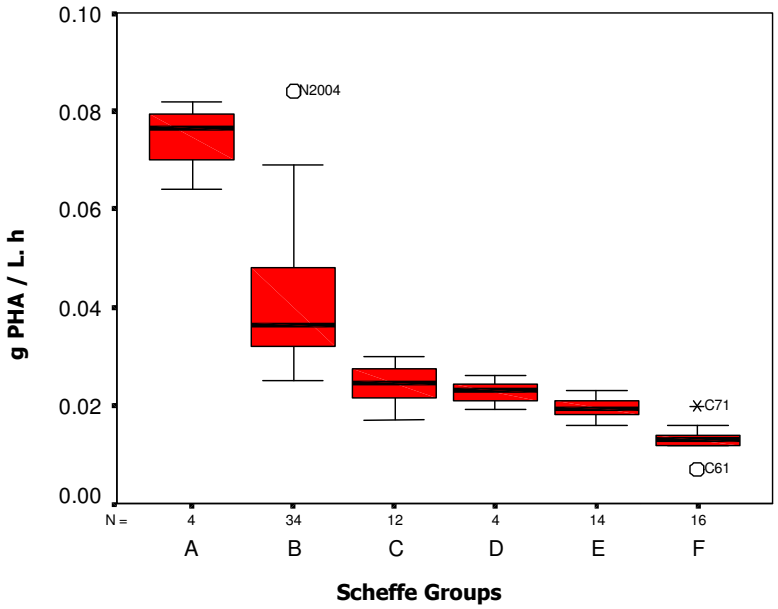

Figure 3. Scheffe groups for biopolymer productivity (gPHA/l.h).

The isolates having the greatest capacity for producing PHAs were selected (i.e. 2G-57, B69, C14, C16 and N2004), plus isolate S1407 for its rapid accumulation, for chosen for further analysis.

Molecular characterisation

The results of analysing the partial sequence of the 16s rRNA ribosomal gene are showed in the Figure 4. Four well-separated clusters are defined from the external group (*Bacillus megaterium*). Isolate B69 was located in the first cluster, closely related to species from the *Rhizobium* genera 2G-57 was located in the second cluster, closely related to *Bulkholderia cepacia.* The isolates C14, C16 and S1407, and C16 and S1407 isolates were located in the third cluster, closely related to "strict group" of *Pseudomonas* sp to belong to the 16S rRNA homology I group (Anzai et al., 2000). The reference enterobacteria were grouped in the fourth cluster considered for this affiliation; isolate N2004 was located here. The clusters so formed corresponded to that reported in the literature (Holmes et al., 1987; Younf and Haukka, 1996; Berkum et al., 1996; Anzai et al., 1997; Anzai et al., 2000; Coenye et al., 2001 a and b).

Determining PHA accumulation profiles in 7-litre fermenter

Accumulation of PHAs for the 6 isolates grown in 7 L fermentor was compared to the *R. eutropha* pattern strain (Table 1). Three groups (high, medium and low) were formed for all parameters considered to facilitate analysis, taking results obtained with the reference strain as standard.

Specific rate of growth (μ_x) revealed that isolates from the low group were S1407 and C14 (speeds of 0.1055 and 0.1134 h^{-1}, respectively), C16 and 2G-57 from the medium group (speeds of 0.1741 and 0.2881 h^{-1}, respectively) and isolates N2004 and B69 and *R. eutropha* from the high group (0.4170, 0.4800 and 0.4676 h^{-1}, respectively). All strains evaluated, except for C16, reduced their rate of growth around 12 h of fermentation, coinciding with the fall in dissolved oxygen.

Isolates N2004, C16 and S1407 were classified in the low accumulation percentage group (4, 8 and 15.32%, respectively), 2G-57 and *R. eutropha* were in the medium group (32.59 and 44.53%, respectively) and B69 and C14 in the high group (61.19 and 74.44%, respectively). Isolate C14 presented an accumulation percentage which is not usual for *Pseudomonas* spp. strains which are generally between 30 - 50% as reported by Ramsay et al. (1989); He et al. (1998); Lee et al. (1996) and Braunegg et al. (1995). This makes it an unusual strain, having high potential for producing medium chain PHAs. The B69 strain, from the *Rhizobium* sp. genus, presented a high accumulation percentage (61.19%), this being very close to that reported by Kshama and Shamala (2003) for some *Rhizobium* sp. strains (27 to 57%). Accumulated percentage for isolate 2G-57 (32.59%), belonging to the *Burkholderia* sp. genera, was close to that reported by Silva et al. (2000) for the *B. straincia* IPT 101 strain (37.58%) which is a strain used for producing industrial biopolymer, even though in different conditions. It is worth stressing that this strain was found at the same level as

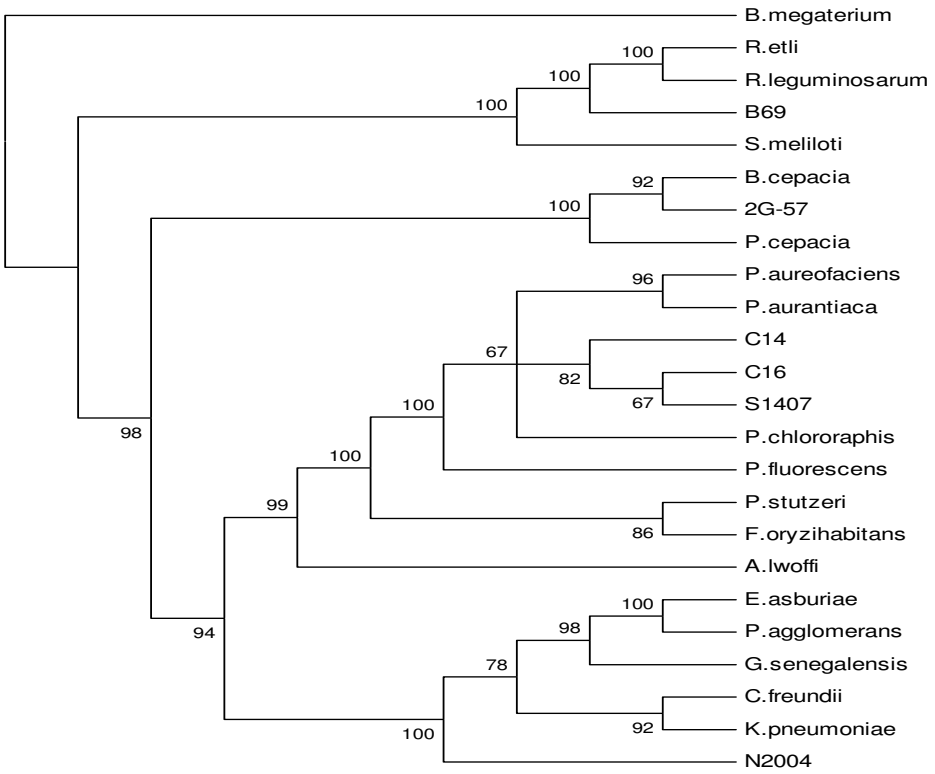

Figure 4. Phylogentic tree of native PHA-producing strains. The tree was constructed by Neighbour-Joining on a p distance matrix.

Table 1. Comparison of different kinetic parameters for isolated strains and *R. eutropha.*

Parameter	*R. eutropha*	C16	S1407	C14	2G-57	B69	N2004
Speed of growth, h^{-1}	0.4676	0.1741	0.1055	0.1134	0.2881	0.4800	0.4170
Percentage of accumulation	44.53	8.00	15.32	74.40	32.59	61.19	4.00
Productivity, g PHA/l h	0.058	0.0168	0.0210	0.0671	0.059	0.039	0.0102
Fusion temperature, °C	170.53	146.76	128.26	122.90	172.68	155.48	129.54

the *R. eutropha* pattern strain. As it has 2 isolates outperforming the industrially used pattern strain, then it can be concluded that the isolation strategy was successful, as well as the selection criteria for the best strains. In the future, directed mediums will be employed for these strains, according to the genus to which the isolates belonged, to increase accumulation percentage. The strains grouped in the low group should be studied in other culture mediums and with another essential element being limited for increasing accumulation percentage.

Regarding productivity, the low group was formed by N2004 and C16 (0.0102 and 0.0168 g polymer/l h, respectively), the medium group by S1407 and B69 (0.0210 and 0.0390 g polymer/l h, respectively) and the high group by *R. eutropha*, 2G-57 and C14 (0.058, 0.059 and 0.0671 g polymer/l h). Isolate C14 is a very important strain since its productivity was in the same order as that for *R. eutropha* and international reports have shown that *Pseudomonas* sp. productivity is less than that for *R. eutropha* (Lee et al., 1999): 1.59 and 1.79 g/L h for *Pseudomonas* and 4.94 g/L h for *R. eutropha*. Isolate 2G-57, also located in the high productivity group, is a strain whose genera has been used industrially for producing PHB, as reported by Silva et al. (2000). This led the group to conclude that this strain has possibilities for producing a technological process in the future; unfortunately, no productivity values have been reported for this strain. The medium and low productivity groups presented productivity values which were much lower than those for *R. eutropha*; they must therefore be evaluated in new culture medium conditions and it must be guaranteed that there is no limitation in terms of dissolved oxygen, since this is one of the causes of low productivity. Even though being far from internationally reported productivities, the new isolates are becoming promising strains and can be improved on by studying and optimising fermentation

conditions.

We characterized polymer by DSC to detect fusion temperature; the values were compared with those reported internationally for different types of PHAs. Polymer melting temperature obtained when using *R. eutropha* strains and 2G-57 were very similar (170.53°C and 172.68°C, respectively), suggesting that the polymer synthesized by isolate 2G-57 is poly-β-hydroxybutyrate (PHB). Polymer synthesised by isolate B69 from the *Rhizobium* genera is also PHB, having a lower fusion temperature than that synthesised by *R. eutropha*, showing that PHB properties depend on the strain and the conditions used during fermentation. This was corroborated later on by gas chromatography (data not shown). Polymer from the strain N2004 presented a very similar fusion temperature to that of *Pseudomonas* but it has not been found to have been reported in the literature that the *Enterobacteria* family are PHA accumulators; they could not thus be compared. Polymers produced by the other isolates (*Pseudomonas* genera) would be expected to be hydroxybutyrate copolymers (3HB) and others monomers, this being a significant advance in PHA production because these strains are able to accumulate these biopolymers from sucrose and thus facilitate the search for more economic substrates such as molasses or sugarcane industry residues and thereby reduce production costs. The new isolates are able to produce copolymers from just sucrose. Internationally, copolymers have been sought by various research groups as they have better mechanical properties than PHB and it is difficult to find strains synthesising them from a single substrate. Generally two substrates are required for obtaining the copolymer, making the process costly (Khanna and Srivastava, 2005). In the future, the materials will be better characterised by determining their molecular weight and composition.

The techniques developed for isolating and evaluating strains are appropriate. It is hoped to standardise a molecular technique in the future which will allow selecting strains, bearing in mind the presence of genes involved in the PHA synthesis, and thus carry out more rapid, safer screening. It is also hoped to optimise the mediums and fermentation conditions, bearing in mind each strain's nutritional requirements according to the genera to which they belong. The new materials obtained should be applied to producing supports for fibroblast growth as part of this project and thereby evaluate their application in the field of biomedical engineering where biopolymers have very high added value.This work also achieved integrating microbiological and molecular techniques with fermentation techniques at shake-flask and fermenter levels and has shown the experience of a group which has carried out a process through from the basic part to the applied or industrial part.

Conclusions

This work has contributed the discovering and evaluate on of Colombia's biodiversity by obtaining 108 isolates which is able to accumulate PHAs from carbohydrates (glucose, fructose and sucrose); 44 of them are able to grow in sucrose were evaluated in shake-flasks. Two of the isolated strains (2G57 and C14) presented very similar productivity to that of *R. eutropha* (0.059 and 0.0671 g PHA/l h for 2G57 and C14, respectively; 0.058 g PHA/l h for *R. eutropha*) at fermenter level, without optimising either the medium or the fermentation conditions, making them candidates for an industrial process. Characterising the materials obtained showed that two strains produced PHB and four produced hydroxybutyrate copolymers and other monomer units, this being a great advance in knowledge regarding PHAs internationally. Molecular techniques were used for determining the taxonomic distance between promising isolates, forming 4 defined clusters.

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