Development of a cost-effective technology for the production of the bioplastic poly-(3-hydroxyalkanoate) from sugarcane harvest residues

G Umrit, S Ganeshan, K Mulleegadoo and A Dookun-Saumtally
Mauritius Sugarcane Industry Research Institute, Mauritius Cane Industry Authority, Reduit, Mauritius; gunshiam.umrit@msiri.mu

Abstract Volatility in sugar prices is leading sugarcane industries worldwide to broaden their revenue base by moving from a single commodity manufacturer to one of renewable biomass for production of a broad range of value-added products. Of the numerous potential pathways for producing biofuels and biochemicals based on the sugarcane crop, the bioplastic poly-(3-hydroxyalkanoate) (PHA) has attracted great interest due to its intrinsic biodegradability and biocompatibility. Although industrial scale production of PHAs is beginning in some countries, they still currently remain niche materials within certain high value markets, due mainly to the high cost of production, particularly the high cost of feedstock that constitutes 50% of the total production cost. The objective of this research was to use low cost biomass such as harvest residues that have zero value as feedstock for PHA biosynthesis with a view to reduce production cost. Sugarcane trash was evaluated as a feedstock for the production of PHA by bacterial isolates collected and purified from different niches, as well as reference isolates known to produce PHAs (e.g. Ralstonia eutropha, Azospirillum brasilense, Rhizobium sp., Bacillus sp.). Dilute acid pretreatment followed by enzymatic hydrolysis yielded 51–59 g fermentable sugars per 100 g trash. Different fermentation conditions, both in shake flask culture as well as a 7-L biofermentor, were evaluated so as to optimize cellular biomass production and PHA synthesis. Ralstonia eutropha showed the highest PHA accumulation, yielding up to 50% by weight of dry cell mass. Fourier transform-infra-red spectroscopy (FT-IR) and nuclear magnetic resonance (NMR) analysis showed that poly-(3-hydroxybutyrate) (PHB) was the predominant biopolymer synthesized from the trash hydrolysates. Fermentable sugars from sugarcane trash hydrolysis without the addition of nutrient supplements can be used for PHB production. Projections for further optimization of the process for PHB production from sugarcane trash hydrolysate and further reduction of production cost are discussed.

Key words Sugarcane, trash, hydrolysates, polyhydroxyalkanoates, biopolymers

INTRODUCTION

The world sugar price has been especially volatile over the past decades increasing from USD 360 per ton in 2005 to reach a peak of USD 626 per ton in 2010, the highest in 30 years (Fig. 1; OECD/FAO 2015). This provided incentives to growers around the world to grow more sugarcane and beet resulting in a sharp increase in global sugar production. As a result of these global production surpluses, international sugar prices have been on a declining trend since 2011 to reach USD 306 per ton in 2014. It is estimated that world raw sugar prices will remain low for much of the next few years (OECD/FAO 2015). This decline in the price of sugar, coupled with an increase in the costs of production, has put pressure on the sugar industry to diversify its revenue base in order to remain economically sustainable.

![Fig. 1. Raw and white sugar prices and projections until 2024 (OECD/FAO 2015).](image-url)
The current revenue base for the sugar industry is a narrow range of products including raw and refined sugar, ethanol from molasses and cogeneration using bagasse (Twine 2005). In the face of declining world sugar prices, this narrow product base represents a significant challenge to the industry. For the industry to remain profitable and competitive there is a need to develop new technologies for creating novel bio-based products that are not only substitutes for existing petroleum-based products but may offer unique product properties unattainable with fossil-based alternatives (e.g. biodegradability and biocompatibility). A multitude of products, including polyols, polymer precursors, oligosaccharides, enzymes, aromatic compounds, therapeutic proteins, levulinic acids, succinic acids, furanics and their derivatives and bioplastics, have been identified as potential bio-based products from sugarcane feedstock (Twine 2005; O’Hara et al. 2013).

Of the numerous potential pathways for producing biochemicals based on the sugarcane crop, the bioplastic poly-(3-hydroxyalkanoate) (PHA) has attracted increased interest due to its intrinsic biodegradability and biocompatibility. PHAs are a family of biopolymers with diverse structures and are the only bioplastics completely synthesized by microorganisms. PHAs are degraded upon exposure to soil, compost or marine sediment under both aerobic and anaerobic environments, without forming any toxic products (Tokiwa et al. 2009). Apart from their biodegradability, the major advantages of PHAs include their bio-based nature; they do not contribute to GHG emission and are biocompatible especially for medical applications (Koller et al. 2010).

Polyhydroxybutyrate (PHB) and polyhydroxyvalerate (PHBV) are common types of PHAs seen in nature. PHB is the most widespread and best characterized member of the PHA family, is similar in its mechanical, physical and thermal properties to many different plastics, and is currently mainly used in the medical industry for internal suture as it is non-toxic, compatible and naturally absorbed and so does not need to be surgically removed. Other potential uses include automotive, design, high-tech electronics, and as controlled drug release carriers and packaging (Chen 2010; E4Tech 2015).

Global bioplastics production capacity is set to increase from around 1.7 Mt in 2014 to approximately 7.8 Mt in 2019 (European Bioplastics 2015). The key players in PHA production via fermentation of sugar are Metabolix in USA, Biomer in Germany, Bio-On in Italy and TianAn Biopolymer Co in China. Nonetheless, PHAs still currently remain niche materials within certain high value markets because the cost of production is still high. In that context, the integration of PHA production into a combined sugar and ethanol factory has the potential for reducing production cost and making large-scale PHA production economically profitable (Koller et al. 2009). However, when using pure substrates such as sucrose for production of PHA, the production cost will still remain high, since the cost for the carbon substrate used accounts for 50% of the total process costs. Consequently, the major thrust in PHA research is a reduction in production costs by switching to cheaper feedstocks. A number of investigations on utilization of sugarcane molasses as a substrate for PHA production have been reported (Purushotaman et al. 2001; Albuquerque et al. 2007; Bengtsson et al. 2010). Similarly, hydrolysis of the hemicellulose and cellulose fractions of bagasse to convertible sugars and their utilization by suitable bacterial strains for PHA biosynthesis has been successfully demonstrated (Silva et al. 2004; Yu and Stahl 2008). However, most sugar factories burn bagasse to cogenerate steam and electricity for running the factory and sell any surplus electricity to the local or national grid. Other large-scale use of the bagasse includes the production of second-generation biofuels.

Sugarcane trash, the agricultural residue left after harvesting, is another important biomass resource in the sugarcane industry that is under-used or improperly utilized. On average, some 12-15 t of trash are produced annually on 1 ha of land under sugarcane production. While sugarcane trash has been extensively studied, the main focus has been on its use in the energy industry. To our knowledge, there is no report on the use of sugarcane trash as a feedstock for PHA production.

The purpose of this study was to develop a technology for the production of PHA from sugarcane trash, laying emphasis on the utilization and valorization of this hitherto under-utilized waste product of the industry. This could help further reduce the cost of PHA production.

MATERIALS AND METHODS

Sugarcane trash pretreatment and hydrolysis

We used sugarcane trash that was collected from fields soon after mechanical harvest when all crop residues had dried out. The trash was milled to pass through a 1 mm sieve and pre-treated with either dilute phosphoric acid or dilute sulphuric acid. The treatment was performed at 121°C for 1 hour in a solution containing either 3.5% H₃PO₄ or 0.5% H₂SO₄ at a solution: solid ratio of 10:1.
The lignocellulosic fraction was separated by filtration, and delignified using 1.5% sodium hydroxide solution in an autoclave at 100°C for 1 hour. The delignified residue was hydrolysed using the enzyme Accellerase® 1500 after adjustment of the substrate pH to 4.8 using sodium citrate buffer. The enzyme-substrate mixture was incubated in an incubator-shaker in a 1-L flask at 50°C for 48 hours. The enzyme hydrolysate was analysed for total reducing sugars and used as C source for PHA synthesis by Ralstonia eutropha H16 G+ (Westfälische Wilhelms-Universität, Germany).

Fermentation conditions

Ralstonia eutropha was grown for 2 days on nutrient agar medium, cultured overnight in 10 mL Terrific Broth at 30°C and 200 rpm. We transferred 1 mL of the culture to 50 mL of either: (i) M10 medium (composition: (g/L) (NH₄)₂SO₄ 0.2, KH₂PO₄ 13.3, MgSO₄ 1.2, citric Acid 1.7, trace element solution 10 mL/L, (g/L, FeSO₄.7H₂O 10, ZnSO₄.7H₂O 2.25, CuSO₄.5H₂O 1, MnSO₄.5H₂O 0.5, CaCl₂.2H₂O 2.0, Na₂B₄O₇.10H₂O 0.23, (NH₄)₆Mo₇O₂₄ O.1, 35% HCl 10 mL); or (ii) trash hydrolysate contained in 250 mL flasks. The cultures were shaken at 30°C and 200 rpm for 72 h. After cultivation the cells were harvested by centrifugation at 4000 rpm for 10 minutes.

To screen different bacterial strains for PHA production ability, we grew them on M10 medium as well as on hydrolysate medium. The presence of PHA as intracellular granules was examined by staining the bacterial smear with Sudan black-B.

PHA production from enzymatic hydrolysate of sugarcane trash using a 7-L biofermentor

With a view to scale up the PHA production, fermentation was also carried out in a 7-L biofermentor at 30°C for 72 h at 500 rpm and 40% dissolved oxygen.

Extraction of PHA

We used a detergent-based extraction method where the bacterial pellet was treated successively with sodium dodecyl sulphate (SDS) and sodium hypochlorite, followed by washing with water. PHA was extracted in hot chloroform under reflux, filtered to remove cell debris and precipitated with cold methanol. PHA content was calculated as percentage of cell dry weight.

Analytical procedures

Structural carbohydrates and lignin in sugarcane trash were determined using the Standard Biomass Analytical Procedures, National Renewable Energy Laboratory, USA. Total reducing sugars in trash hydrolysate was determined using the Lane-Eynon Method (Chen 1985). Characterization of the PHA recovered was carried out using ¹H NMR analysis and FT-IR Spectroscopy.

RESULTS AND DISCUSSION

Screening of bacterial strains for ability to synthesize PHA

Our approach was to isolate and screen local naturally occurring non-pathogenic strains for their ability to accumulate PHA. The evaluation of some 50 bacterial isolates collected and purified from different niches (vinasse, sugarcane trash, soil, refinery mud) including Azospirillum brasilense, Rhizobium sp. and Bacillus sp. as well as the reference bacterial strain Ralstonia eutropha H16 G+ for their ability to produce PHA led to the conclusion that none of the locally isolated strains was better than Ralstonia eutropha H16 G+. The latter was, therefore, selected for subsequent work on PHA biosynthesis. Ralstonia eutropha (also known as Cupriavidus necator) is the bacterium that has been most extensively studied and claimed to be among the most effective for PHA production.
Trash hydrolysates

The composition of sugarcane trash is shown in Table 1. The measured amounts of polymeric carbohydrate (cellulose + hemicellulose) and lignin were within the range of 58-71% dry weight (DW) and 14% DW respectively, reported elsewhere for sugarcane trash (Patureau 1989; Rossel 2006; Gonçalves et al. 2005). Canilha et al. (2013), on the other hand, reported higher lignin contents of Brazilian sugarcane trash, varying between 26 and 41%. It is relevant to note that the sugarcane trash we used has contents of cellulose and hemicellulose that are not far from reported values of 45-80% DW for sugarcane bagasse.

Table 1. Composition of sugarcane trash.

<table>
<thead>
<tr>
<th>Component</th>
<th>Range</th>
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</thead>
<tbody>
<tr>
<td>Dry matter</td>
<td>91.8 - 94.8%</td>
</tr>
<tr>
<td>Mineral matter</td>
<td>3.0 - 7.0% DW</td>
</tr>
<tr>
<td>Carbon</td>
<td>42 - 44% DW</td>
</tr>
<tr>
<td>Acid-soluble lignin</td>
<td>0.44 - 0.45% DW</td>
</tr>
<tr>
<td>Acid-insoluble lignin</td>
<td>23.0 - 25.5% DW</td>
</tr>
<tr>
<td>Polymeric carbohydrates (cellulose and hemicellulose)</td>
<td>68 - 71% DW</td>
</tr>
</tbody>
</table>

The yields of simple sugars after acid pre-treatment and enzymatic hydrolysis are shown in Table 2. At a solid load rate of 10%, i.e. 50 g dry trash in 500 mL acid solution, nearly 30% of the of the trash dry weight could be decomposed and dissolved in the dilute acid solution during pre-treatment and a further 22-30% of the trash could be dissolved during enzymatic hydrolysis. Overall, 58-59% of the dry weight of trash could be converted to simple sugars, representing 83-87% of the total polymeric carbohydrates. Based on data available in the literature, the efficiency of conversion of cellulosic solids to simple sugars ranges from 80 to 99%, with the highest sugar yield being approximately 60 g/100 g dry biomass (Benjamin et al. 2011; Silva et al. 2010).

Table 2. Yield of total reducing sugars from sugarcane trash hydrolysis.

<table>
<thead>
<tr>
<th>Pre-treatment step</th>
<th>Enzymatic hydrolysis step</th>
<th>Reducing sugars yield (g/100 g trash)</th>
</tr>
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<tbody>
<tr>
<td>3.5% phosphoric acid</td>
<td>Accellerase® 1500: 1 mL/g biomass, 48 h incubation @ 50°C</td>
<td>59</td>
</tr>
<tr>
<td>@ 121°C, 1 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5% sulphuric acid</td>
<td>Accellerase® 1500: 1 mL/g biomass, 48 h incubation @ 50°C</td>
<td>58</td>
</tr>
<tr>
<td>@ 121°C, 1 h</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Biosynthesis of PHA

Fermentations of Ralstonia eutropha in hydrolysate fraction obtained after dilute acid pre-treatment of trash did not show any cell multiplication or PHA accumulation in the bacterial cells after 48 h of incubation. This was to be expected since it is known that along with the release of monomers, dilute acid pre-treatment also generates a number of hydrolysis products that are inhibitory to microorganisms, including PHA-producing bacteria, even at very low concentrations (Dietrich et al. 2013; Jönsson and Martin 2016). Whilst we made no attempt to remove the inhibitory effects, a number of techniques to circumvent the problem of inhibitors are known and have been used elsewhere (Pan et al. 2012; Jönsson and Martin 2016).

Highest cellular biomass and biopolymer yields were obtained at 72 h of growth on the enzyme hydrolysate fraction diluted to contain 20 g/L reducing sugars, under shake-flask conditions (Table 3). Compared to shake-flask conditions, about 28% lower biopolymer yields were obtained with the 7-L biofermentor. Compared to the M10 mineral medium, sugarcane trash hydrolysate yielded 2-3 times more biopolymer, indicating that trash hydrolysate could be effectively used as a carbon source for PHA production. However, the measured maximum biomass yield of 3.5 g DCM/L was much lower than reported values of 19.2 g/L of lignocellulose hydrolysate medium (Sandhya et al. 2013) or of 9.6-10.2 g/L nutrient medium (El Sayed et al. 2009). Similarly, the measured maximum biopolymer yield of 50% DCM was much lower than values reported in the literature. Thus, Aznury et al. (2012) reported PHA content of 74% DCM in Ralstonia eutropha grown on a medium containing 40 g/L glucose enriched with volatile fatty acids. Similarly, Kim et al. (2016), using a recombinant strain of
Ralstonia eutropha reported PHA content of 72.5% wt from lignocellulosic biomass hydrolysate. This suggests that there is potential for further optimization of the fermentation process using sugarcane trash as C source. The strategies for enhanced PHA production include the use of chemical detoxification of fermentation inhibitors (Jönsson and Martin 2016), use of adapted tolerant strain of Ralstonia eutropha (Yu and Stahl 2008) or recombinant strains capable of utilizing the xylose as well as glucose derived from the hemicellulosic fraction of sugarcane trash (Kim et al. 2016), and the use of fed-batch cultivation for more efficient PHA biosynthesis (Pan et al. 2014).

**Table 3.** Total dry cell mass (DCM) and biopolymer accumulation by Ralstonia eutropha grown on M10 medium and sugarcane trash hydrolysate for 72 h.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Biomass yield (g dry cell mass per L)</th>
<th>Biopolymer yield (% of dry cell mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M10 medium (containing 20 g/L glucose)</td>
<td>3.5</td>
<td>17</td>
</tr>
<tr>
<td>Sugarcane trash hydrolysate (diluted to contain 20 g/L glucose)</td>
<td>3.5</td>
<td>1.6 (50)</td>
</tr>
</tbody>
</table>

![Fig. 2.](image)

1H NMR (A) and FT-IR (B) spectra obtained from biopolymer produced from sugarcane trash hydrolysate by *Ralstonia eutropha.*
Biopolymer characterization

The 1H NMR spectra of the biopolymer from trash hydrolysates (Fig. 2A) showed chemical shifts at 1.25, 2.55 and 5.25 typical of PHB. The absence of other characteristic peaks indicate that pure PHB was the only biopolymer produced by Ralstonia eutropha grown on trash hydrolysate. FT-IR analysis (Fig. 2B) showed the presence of characteristic absorption bands at 1718.57 cm⁻¹ and 1274.67 cm⁻¹, confirming the presence of PHB.

CONCLUSION

The major cost factor in the commercial production of PHA bioplastics remains the cost of raw materials. Our study has shown that it is possible to produce PHA bioplastics from hydrolysates of sugarcane trash, converting an unutilized waste of the sugarcane industry into a value-added product. In view of the lower PHA productivity compared to that reported for other feedstocks, further optimization of the process is required to make it cost-effective.

REFERENCES


Developpement d'une technologie rentable pour la production du bioplastique poly-(3-hydroxyalcanoate) à partir des paillasses de canne à sucre

Résumé. L'instabilité des prix du sucre a amené des industries de la canne à sucre dans le monde entier à passer de fabricant de produits de base unique à l'un de la biomasse renouvelable pour la production d'une large gamme de produits à valeur ajoutée afin d'élargir leur base de revenus. Parmi les nombreuses possibilités pour la production de produits biochimiques à base de la canne à sucre, le bioplastique Poly-(3-hydroxyalcanoate) (PHA) a suscité un grand intérêt en raison de sa biodégradabilité et de sa biocompatibilité. Bien que la production à l'échelle industrielle du PHA a commencé dans certains pays, ils demeurent toujours des matériaux de choix au sein des certains marchés à forte valeur ajoutée, principalement en raison du coût élevé de production, en particulier le coût élevé des matières premières qui constituent 50 % du coût total de production. L’objectif de cette recherche était d’utiliser une biomasse peu coûteuse telle que la paille de la canne à sucre comme matière première pour la biosynthèse de PHA en vue de réduire les coûts de production. Le paillasses de canne a été évalué comme matière première pour la production de PHA par des souches bactériennes recueillis et purifiés à partir de différentes sources ainsi que des souches de référence connues pour produire des PHA (par exemple Raistonia eutropha, Azospirillum brasilense, Rhizobium sp., Bacillus sp.). Un prértraitement à l’acide dilué suivi d’une hydrolyse enzymatique a donné entre 51-59 g de sucres fermentables pour 100 g de paillasses. Des conditions de fermentations différentes, à la fois dans des flacons de culture ainsi qu’un bio-férentisseur d’une contenance de 7 litres, ont été évalués de manière à optimiser la production de biomasse cellulaire et la biosynthèse de PHA. Raistonia eutropha a démontré la plus grande accumulation de PHA, contenant jusqu’à 50 % en poids sec de cellules. L’analyse par spectroscopie infrarouge (FT-IR) et par résonance magnétique nucléaire (RMN) a montré que la poly-(3-hydroxybutyrate) (PHB) est le polymère prédominant synthétisé à partir de ces hydrolysats de paillasses. Les sucres fermentables obtenus à partir de l’hydrolyse du paillasses de canne à sucre sans apport de suppléments nutritifs peuvent être utilisés pour la production de PHB. Les projections pour une meilleure optimisation du processus de production de PHB à partir des hydrolysats de paillasses de canne à sucre en vue de réduire les coûts de production sont discutées.

Mots-clés: Canne à sucre, la paille, les hydrolysats, les polyhydroxyalcanoates, les biopolymères

Desarrollo de una tecnología económicamente efectiva para la producción del bioplástico poli-(3 hidroxialcanoato) a partir residuos de la cosecha cañera

Resumen. La volatilidad del precio del azúcar induce mundialmente a ampliar su base de ganancias moviéndose desde una monocultura a una de biomasa renovable que sostiene la producción de un amplio rango de productos de valor agregado. Entre los numerosos caminos de producción para producir bio combustibles y bioquímicos basados en la caña de azúcar, el bioplástico poli-(3 hidroxialcanoato) (PHA) ha atraído gran interés debido a su bio degradabilidad intrínseca y su biocompatibilidad. A pesar de que la producción de PHAs ha comenzado en algunos países su nicho como un producto dentro de ciertos mercados de alto valor, debido principalmente al alto costo de producción, específica de las materias primas de alto costo que resultan el 50% del costo total de producción. El objetivo de esta investigación fue el utilizar biomasa, como los residuos de la cosecha que tienen valor cero, para la biosíntesis de PHA, en el objetivo de reducir los costos de producción. Los residuos de la caña se evaluaron como materias primas para la producción de PHA, empleando bacterias recolectadas, aisladas y purificadas, obtenidas de varios nichos, así como microorganismos conocidos como productores de PHA (Raistonia eutropha, Azospirillum brasilense, Rhizobium sp., Bacillus sp.). El tratamiento con ácido diluido, seguido de un hidrolisis enzimática rinde 51-59 g de azúcares fermentables por 100 g de residuos. Se evaluaron diferentes condiciones de fermentación: en zarandas con frascos de vidrio así como en fermentadores de 7 l, para optimizar la bio producción celular y la síntesis de PHA. La Raistonia eutropha mostró la mayor acumulación de PHA, rindiendo hasta el 50% en peso de materia seca celular. Los análisis de espectroscopia infrarroja Fourier de transformación (FT-IR) y la resonancia magnética nuclear (NMR) mostraron que el poli-(3-hidroxibutirato) (PHB) resultó el biopolímero sintetizado predominantemente de los hidrolizados de residuos. Es posible utilizar azúcares fermentables de los hidrolizados de residuos cañeros, sin adición de nutrientes suplementarios, para la producción de PHB. Se discute una proyección para la optimización del proceso de producción de PHB a partir residuos cañeros y la reducción posterior de los costos de producción.

Palabras clave: Caña de azúcar, residuos, hidrolizados, polihidroxialcanoatos, biopolímeros