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Use of a mannitol rich ensiled grass press juice (EGPJ) as a sole carbon source for polyhydroxyalkanoates (PHAs) production through high cell density cultivation

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Abstract

This study demonstrates the use of a mannitol rich ensiled grass press juice (EGPJ) as a renewable carbon substrate for polyhydroxyalkanoates (PHA) production in shaking flask experiments and fed-batch stirred tank reactor cultivations. Fed-batch cultivations of *B. sacchari* IPT101 using EGPJ as sole carbon source produced 44.5 g/L CDW containing 33% polyhydroxybutyrate (PHB) in 36 h, while *P. chlororaphis* IMD555 produced a CDW of 37 g/L containing 10% of medium chain length (mcl-PHA) in 34 h. PHB and mcl-PHA extracted from *B. sacchari* IPT101 and *P. chlororaphis* IMD555, grown on EGPJ, had a molecular weight of 548 kg/mol and 115.4 kg/mol respectively. While mcl-PHA can be produced from EGPJ, PHB production is more interesting as there is a 4 fold higher volumetric productivity compared to mcl-PHA.

Keywords

Biorefining, ensiled grass press juice, polyhydroxyalkanoates, mannitol, pulse feed

1. Introduction

Various bio-based materials such as polyhydroxyalkanoate (PHA), polylactic acid (PLA), and polybutylene succinate (PBS) have been recommended as alternative solutions to petrochemical plastics (Chen and Patel, 2012). Among them, PHA is the only polymer which can be completely synthesized and degraded by bacteria (Lageveen et al., 1988) and has similar properties to conventional plastics (Chen and Patel, 2012). PHA is accumulated within the cytoplasm of bacteria as a carbon and energy storage granule, in the presence of excess carbon (Lageveen et al., 1988). In order to be competitive with petrochemical plastics market, PHA production has to rely on cheap or zero cost resources. Finding cheap ways to take advantage of most of the fermentable carbohydrates that are present in a renewable biomass is one of the challenges of a biorefining approach (McEniry and O'Kiely, 2014). Grass is a worldwide resource that roughly covers 30 million Km^2 of the earth (Panunzi et al., 2008), and grassland pasture is extensively available in Europe primarily for grazing of ruminant and equine. Several countries are advancing in the green biorefinery concept of utilizing the whole biomass to generate various valuable products or precursors for value added products. In a green biorefinery setting the preservation of grass as silage and its subsequent fractionation into press-juice and press-cake fractions represent the two major process steps in the utilization of grass biomass (Wachendorf et al., 2009). Grass has water soluble carbohydrates (WSC) in solution and insoluble carbohydrates in the form of cellulose and hemicellulose. Depending on the climate and environmental stress different carbohydrate proportions can be present in the grass (Stoop et al., 1996). Press juice from ensiled grass contains most of the WSC and carbohydrates released from

the hemicellulose fibers (due to the acidic fermentation during the ensiling) and is an available substrate (Mandl, 2010) for bacterial fermentation. The press cake portion can be used as a feedstock for ruminants (McEniry and O'Kiely, 2014), as an insulating material in the building sector (McEniry and O'Kiely, 2014) or it can be further treated (Cerrone et al., 2014a) and digested to yield fermentable sugars for biorefining (Davis et al., 2013). Press juice has been demonstrated as a good substrate for biogas and bioethanol production (Wachendorf et al., 2009), as a source of lactic acid (McEniry and O'Kiely, 2014) or as a nutrient supplement for microbial growth (Koller et al., 2005). However, there are no reports on the use of press juice from ensiled grass as a sole carbon source for production of PHA.

Press juice from the ensiled grass can have different carbohydrate composition depending on the bacterial and yeast fermentation taking place during the ensilation process. This study focused on mannitol rich ensiled grass press juice (EGPJ) as a growth and PHA production substrate. Mannitol has been isolated from perennial ryegrass (Harwood, 1954) and is produced by the plants to prevent the stress effect of drought (Stoop et al., 1996) or the oxidative effect of free radicals (Rasmussen et al., 2008), and it can also be produced as a result of the free glucose and fructose conversion by hetero fermentative facultative anaerobic lactic acid bacteria (LAB) (Saha and Racine, 2011) and yeast (Onishi and Suzuki, 1968) during the ensiling process.

This study developed a bioprocess for fed-batch cultivation of selected bacterial strains using EGPJ as sole carbon and energy source with the aim of achieving both high cell density and PHB/PHA volumetric productivities.

2. Materials and Methods

2.1 Bacterial Strains

A total of 17 bacterial strains [five different strains of *P. putida* (ATCC 47054, NCIMB 41538, NCIMB 14927, NCIMB 41162, NC010501), four different strains of *P. fluorescens* (IMD472, IMD512, IMD9035, IMD332) one strain of *P. jesseni* (IMD491), one strain of *P. chlororaphis* (IMD555), two *Pseudomonas* sp. (IMD495 and IMD498), three *Cupriavidus necator* (NRRL 14690, NRRL 4385, ATCC 17699) and *Burkholderia sacchari* IPT101 (DSM17165)] were used in this study. These strains were maintained in Luria Bertani broth (LB) with 50% glycerol at -80 °C. In preparation for the PHA production experiments the strains were streaked on to minimal salt medium (MSM) agar with glucose and incubated at 30 °C for 24 h except that 0.5 g/L of yeast extract was used as a supplement for *B. sacchari* IPT101.

2.2. Ensiled grass press juice (EGPJ) production

Perennial ryegrass, grown at the Teagasc Grange Animal & Grassland Research and Innovation Centre (Dunsany, Co. Meath, Ireland), was cut with a precision-chop harvester (Pottinger, Mex VI) with a target chop-length of 19 mm. Approximately 1.5 tonnes of fresh grass were ensiled for a total of 7 weeks. Batches of ensiled ryegrass were subjected to primary processing at AFBI (Plant Health & Environmental Protection Branch, Newforge Lane, Belfast, Northern Ireland) using a pilot-scale Model PP-7RL-S screw press (Ponndorf Maschinenfabrik, Germany), as described by Sharma et al. (2012). The un-pressed, ensiled grass had high moisture content (~79-80%; i.e. a dry matter content of

20.5%) as the ensiled grass was not pre-wilted prior to ensiling. Due to the relatively high moisture content of the ensiled grass, the screw press was adjusted during pressing to give a target dry matter content of approximately 30%. The extruded pressed cake and juice fractions were separated and collected in plastic bags and the press juice and washings were combined to give the final press juice sample and stored at -20 °C in 200 L plastic drums until use. Centrifugation (9000 RPM for 20 min) was carried out to remove any suspended solids, followed by rotary evaporation (with a Heidolph ™ Laborota 20 model) to concentrate the press juice supernatant (up to 300 g/L of total sugar). EGPJ was steam sterilised and pH was adjusted to neutral when necessary.

2.3 Characterization of EGPJ.

The press juice from the ensiled grass was analyzed for its total carbohydrate content by phenol sulphuric acid (PSA) method (Dubois et al., 1956) and sugar (monosaccharides and mannitol) composition by HPLC (Davis et al., 2013). Total protein was estimated by Bicinchoninic acid method and available phosphorus was estimated by ammonium molybdate method (USEPA 1983). The concentration of ammonia was measured using the method previously described by Cerrone et al. (2014a). (D)-Lactic and (L)-lactic acid were detected by Megazyme© specific kit for lactic acid detection by colorimetric method with a SpectraMax 340 Molecular Devices© microplate reader. Three different analyses were performed for each parameter.

2.4 PHA/PHB biosynthesis using analytical grade sugars as carbon source

Shaking flasks experiments were conducted using 17 strains of bacteria in 250 mL shaking flasks containing 50 mL of nitrogen limited (0.25 g/L NH_4Cl) MSM with 7.2 g/L of different analytical grade sugars (glucose, xylose, arabinose, galactose, mannose, and mannitol) at 30 °C and 200 RPM for 48 h. The bacterial strains which had the ability to utilize more than 2 individual sugars (both for growth and PHA production) were further tested for their ability to utilize a mixture of simple sugars (final concentration of 7.2 g/L) equivalent to that of EGPJ. Three independent experiments were carried out using each sugar and each bacterial strain.

2.5 PHA/PHB biosynthesis using EGPJ as sole carbon and energy source

Shaking flasks experiments were conducted using three selected strains of bacteria (*B. sacchari* IPT101, *P. putida* W619 and *P. chlororaphis* IMD555) using buffered EGPJ (pH-7) as sole carbon and energy source (7.2 g/L final sugar concentration). These experiments were conducted in 250 mL flasks containing 50 mL of MSM without any added nitrogen. Flasks were inoculated with 1% overnight inoculum and incubated at 30 °C and 200 RPM for 24-48 h. Decanoic acid (2 mM) was added as a mcl-PHA precursor at 24 h to improve mcl-PHA production when needed. Three independent experiments were carried out using each bacterial strain.

2.6 Conditions for fed-batch cultivation of bacteria using EGPJ as carbon source.

A Sartorius® 5L stirred tank reactor was used for fed-batch cultivation of bacteria with a starting volume of 3L. The fermentor was equipped with an oxyferm FDA 325

oxygen sensor (Hamilton, Switzerland), a ring sparger, and a 6-blade disk (Rushton turbine) impeller. The media used for the fed-batch cultivation contained the following components per liter: 9g $Na_2HPO_4 \cdot 12H_2O$, 1.5g of KH_2PO_4, 0.8g $MgSO_4$ and trace elements as defined by Sun et al. (2007). Initial sugar and $(NH_4)_2SO_4$ concentrations were 20.0 and 4.0 g/L, respectively. DO was maintained at 40% oxygen saturation with a constant flow of compressed air (2 vvm) and with increasing the agitation speed as a cascade control (initial agitation was set at 500 RPM). The pH was controlled automatically at 6.9 +/- 0.1 by the addition of 15% v/v H_2SO_4 (Sigma, Ireland) and NH_4OH (20% v/v) which was also used to provide nitrogen source. Whenever nitrogen limitation was needed the pH was controlled by the addition of 6M NaOH. Foaming was controlled by manual addition of antifoam (polypropylene glycol P2000, Sigma) when required. The temperature was maintained at 30 °C using a thermostat controlled double-jacketed system. The data were recorded into BioPAT® MFCS SCADA fermentation software (Sartorius AG, Germany). An inoculum of 400 mL of overnight culture was used for a 3 L initial working volume. Pulse feeds of EGPJ or simulated EGPJ (total sugars of 20 g/L) were added manually when needed, based on the increase in DO value.

2.7 HPLC analysis

Monosaccharides and sugar alcohol composition of the EGPJ and culture broth were determined by anion exchange chromatography using an ICS-300 system (Dionex) equipped with an Amperometric detector and a gold electrode. The column was a CarboPac SA10 (4 x 250 mm) equipped with a security guard cartridge. The samples were eluted with 1 mM KOH under isocratic condition at 1 mL/min and 25 °C. Quantification

was achieved by reference to appropriate monosaccharide (glucose, xylose, arabinose, fucose, galactose, and mannose) and sugar alcohol (mannitol) standards. Appropriate dilutions of the substrates or culture supernatant were prepared and a 0.5 mL of each sample was filtered using Whatman Mini- UniPrep^{TM} syringeless filters (0.45 µM Whatman Inc. NJ, USA) and a sample volume of 10 µL was used for all injections.

2.8 Cell dry weight and polymer analyses.

The bacterial cells were harvested from a known volume (50 mL from shaking flasks and 4 mL from fermentor) of the culture broth by centrifugation and the pellet was washed with deionized water and lyophilized. The weight of the lyophilized cells was determined and the CDW was expressed in g/L. The polymer content and the monomer composition of the polymer were determined by Gas chromatography (GC) and confirmed by GC-Mass Spectroscopy (GC-MS). Approximately 5-10 mg of lyophilized cells were subjected to acidic methanolysis, as described by Lageveen et al. (1988) and the resultant 3-hydroxyalkanoic acid methyl esters were assayed using an Agilent 6890N chromatography equipped with a HP-INNOWAX capillary column (30 m by 0.25 mm with 0.5 mm of film thickness) and a flame-ionization detector (FID). A temperature program of 110 °C for 5 min; temperature ramp of 3 °C per min up to 130 °C followed by 5 °C per min, up to 250 °C was applied for mcl-PHA. The temperature program for PHB was set as follows: 120 °C for 4 min; a first temperature ramp of 10 °C per min up to 170 °C, a second temperature ramp of 40 °C per min up to 230 °C. PHA was extracted from 5-8 g of dried cells by soxhlet extraction using chloroform as the solvent and the extracted PHA was concentrated by rotary evaporator (Rotavapour® Buchi) and precipitated with 10 times volume of ice

cold ethanol (Koller et al., 2005). Molecular weight distribution and thermal properties such as melting temperature (Tm), glass transition temperature (Tg), thermal degradation onset point (TGA), and enthalpy of fusion (ΔH) of the extracted mcl-PHA and PHB were carried out using the methods (Differential Scanning Calorimetry and Gel Permeation Chromatography) described by Cerrone et al. (2014b). Nuclear Magnetic Resonance (NMR) (in particular: ^{1}H-NMR and ^{13}C-NMR) technique was utilized to identify the polymers (PHAs) synthesized by the strains. A Bruker Avance AM-400 Ultrashield^{TM} with 4 nucleus (Inova model) spectrometer in the pulse-Fourier transform mode at a frequency of 250 MHz using glass tubes with $CDCl_3$ solution was employed.

3. Results and Discussion.

3.1 Composition of ensiled grass press juice (EGPJ)

Compositional analysis indicated that press juice obtained from ensiled perennial ryegrass contained 54 g/L of total sugars (based on PSA method), 20 g/L of proteins, 15 g/L of (L)-lactic acid, 5.4 g/L of (D)-lactic acid, 0.5 g/L soluble phosphorous and 0.2 g/L of NH_4. Based on HPLC analysis, the major sugars present were mannitol (42%), glucose (35%), xylose (11%), arabinose (6%) and traces of other sugars such as mannose, rhamnose and galactose. The HPLC method was specifically for monosaccharides and sugar alcohol but there is a possibility that oligosaccharides contribute to the total sugars content. Koller et al. (2005) and Sieker et al. (2011) reported high content of fructose in press juice obtained from ensiled grass in contrast to negligible amount of fructose in EGPJ used in the present study. The lactic acid content of EGPJ was 60% lower than that reported by Sieker

et al. (2011). The monomeric sugars present in the EGPJ might be released from hemicellulose and cellulose fractions due to the activity of plant hemicellulases: these can be active, particularly in the earlier stages of ensilage (Dewar et al., 1963). Pentoses (arabinose and xylose) are the main products of hydrolysis and the ratio of the products differ from the ratio in the grass (i.e. they may preferentially split some pentoses from the xylan molecular chains). Another possible reason for the monomeric sugar release is the acidic hydrolysis of grass fibers during the ensiling; the reduction in herbage pH from 6.0 to 4.0 results from the accumulation of fermentation acids (particularly lactic acid) (Dewar et al., 1963). Presence of high levels of mannitol in EGPJ is most likely due to the reduction of fructose to mannitol by hetero fermentative lactic acid bacteria and yeast during the ensiling process (Saha and Racine, 2011; Onishi and Suzuki, 1968). Grass contains a large indigenous epiphytic microflora. Typically grass in Ireland contains 10^4 to 10^6 lactic acid bacteria and 10^1 to 10^3 yeasts per 1 g of grass (McEniry et al., 2010). In other countries, due to the UV-sterilising effect of sunlight (intensity or daily duration) or the desiccation effect of lower relative humidity in the grass crop they have much lower counts of epiphytic microorganisms.

3.2 Growth and PHA production using analytical grade sugars in shaking flask experiment

In order to achieve complete utilization of all the sugars present in the EGPJ, 17 bacteria were screened for their ability to grow and produce PHA from mannitol, glucose, xylose, arabinose, and mannose. Three strains (*B. sacchari* IPT101, *P. putida* W619, and *P. chlororaphis* IMD555) which produced highest amount of PHA from mannitol, glucose

and xylose were selected for further experiments using EGPJ. *B. sacchari* IPT101 and *P. putida* W619 were known for their ability to use mannitol for growth (Bramer et al., 2001; Taghavi et al., 2009) however, there are no reports on PHA production by these strains from mannitol. Growth and PHA accumulation by *P. chlororaphis* IMD555 with mannitol is reported for the first time in this study. There is limited literature available on PHB production using commercial mannitol as carbon source by other strains of bacteria, such as *Rhizobium elti* and *Pseudomonas stutzeri* (Belal et al., 2013).

Using a mixture of analytical grade sugars (total of 7.2 g/L) mimicking the sugar composition of EGPJ (simulated EGPJ), *B. sacchari* IPT101 produced 3.1 g/L of CDW with 34.5% of PHB (Table 1) under nitrogen limiting conditions. Monosaccharides and sugar alcohol analysis from the culture media at different time points by HPLC indicated that the sugars were consumed sequentially; glucose was consumed first, followed by xylose and finally mannitol. Whilst glucose usually diffuse at high concentration into the cytoplasmic space with a porin (OprB)/ABC uptake mediated system, regulated by the osmolarity of the environment (del Castillo et al., 2007); it has been reported that uptake of mannitol works through a fructose homologous mannitol-specific PTS transporter, with a multiple interconnected genic regulation in many gram negatives bacteria (Tan et al., 2009). *P. putida* W619 and *P. chlororaphis* IMD555 achieved a CDW of 1.2 and 1.1 g/L, respectively using 7.2 g/L of simulated EGPJ. *P. putida* W619 accumulated 31% of the CDW as PHA under N-limiting growth conditions in 48 h whereas *P. chlororaphis* IMD555 accumulated 38% of CDW as PHA. The PHA accumulated by both *Pseudomonas* strains had the same 3-hydroxyalkanoic acid monomers, in a slightly different proportion

(Table 1). *Pseudomonas* strains showed similar trend of sugar utilization as that of *B. sacchari* IPT101.

3.3. Growth and PHA production using EGPJ as sole carbon source in shaking flask experiments

All the three tested strains were able to grow and produce PHA utilizing EGPJ as the sole carbon (7.2 g/L of total sugars) and energy source (Table 1). *B. sacchari* IPT101 achieved CDW of 2.3 g/L and accumulated 23% of the CDW as PHB. CDW and PHB content on EGPJ were lower than that compared to analytical grade sugars. The reduction in CDW is probably due to the presence of some inhibitory compounds present in the grass press juice which include organic acids and phenolics such as p-coumaric acid and ferulic acid and these compounds are known to inhibit microbial growth and accumulation of bioproducts (Sieker et al., 2011). Similar effects have been earlier reported by Keenan et al. (2006) where *Burkholderia* grew better on commercial grade xylose and levulinic acid compared to real hydrolyzate. Keenan et al. (2006) have shown that the use of liming and activated charcoal as a suitable pretreatment for the removal of phenolics, similar approach towards EGPJ could increase the volumetric productivities of CDW and PHB. *Burkholderia* sp. is known to utilize different sugars and accumulate PHB from different types of hydrolyzate derived from biomass (Bramer et al., 2001; Cesario et al., 2014). Even though, *Burkholderia* can accumulate high levels of PHB under inorganic nutrient limitation (Cesario et al., 2014), PHB production data (Table 1) indicated that strict limitation of inorganic nutrients is not a prerequisite for PHB accumulation by this strain, making it interesting for a biotechnological bioprocess using a complex substrate such as

EGPJ. *P. putida* W619 and *P. chlororaphis* IMD555 achieved a CDW of 0.5 and 0.7 g/L, respectively, using EGPJ which is lower compared to the CDW achieved using simulated EGPJ; a similar trend shown by *B. sacchari* IPT101, probably due to inhibitors present in the EGPJ. *P. putida* W619 and *P. chlororaphis* IMD555 accumulated 6 and 7% of CDW as mcl-PHA, respectively using EGPJ as the sole carbon and energy source. Generally *Pseudomonas* produces mcl-PHA from sugars under strict nutrient limitation (such as nitrogen or phosphorous) (Lageveen et al., 1988). EGPJ naturally contains nitrogen and phosphorous, hence imposing a strict nitrogen or phosphorous limitation was not possible. This would possibly be a reason for reduced mcl-PHA accumulation by *Pseudomonas* strains using EGPJ compared to simulated EGPJ (where strict nitrogen limitation was imposed). To improve the mcl-PHA accumulation in *Pseudomonas* strains, low concentrations of fatty acid precursor (2mM of decanoic acid) were added to the culture medium. This resulted in a two fold increase (17-19% of CDW) of PHA content in *P. putida* W619 and *P. chlororaphis* IMD555. A two stage strategy (composed by growth phase on a less expensive substrate and PHA accumulation phase on fatty acids precursors) has already been shown to decrease the cost of PHA production and increase PHA productivity (Sun et al., 2007; Cerrone et al., 2014b; Davis et al., 2015).

Monosaccharide and sugar alcohol analysis from the culture supernatant at different time intervals indicated that the tested strains showed a similar pattern of sugar utilization as that of simulated EGPJ. In a previous study, green grass juice and silage juice were used as nutrient supplement for PHA producing bacteria using analytical grade sugars as carbon source (Koller et al., 2005). This is the first report on EGPJ as a sole carbon and energy

substrate for PHA accumulation. EGPJ is an interesting substrate because of its ease of production, without the need for chemical pre-treatment, hydrolysis and purification.

3.4. Fed-batch cultivation of *B. sacchari* IPT101 using pulse feed of EGPJ.

If mixed sugars are present in a substrate, accumulation of a less preferred sugar in the fermentation medium is expected due to carbon catabolite repression (CCR) (Del Castillo et al., 2007). This accumulation can be prevented by pulse feed cultivation, as reported by Cesario et al. (2014). A pulse feed cultivation was carried out in order to achieve high cell density of *B. sacchari* IPT101 using both EGPJ as carbon source. A maximum CDW of 44.5 g/L after 36 h of growth, with a PHB content of 35% of CDW (Fig. 1) was obtained when EGPJ was used as carbon substrate. The PHB content remained constant (~30% of CDW) from 20 h, though the cell dry weight linearly increased throughout the cultivation. PHB accumulation plateaued from hour 20 of the cultivation probably due to the presence of organic nitrogen in every pulse of EGPJ. The final PHB content was 13.8 g/L and the CDW yield was 0.47 g/g of sugar. The PHB and CDW volumetric productivity were 0.38 g/L/h and 1.23 g/L/h, respectively. Complete exhaustion of the sugars was ensured after each pulse to avoid the accumulation of a less preferred sugar due to CCR. While pulse feeding decreases the volumetric productivity compared to a continuous feed, this is necessary in order to balance the preferential utilization of sugars with the need to ensure all sugars are completely consumed (Cesario et al., 2014). In order to investigate if there were any inhibition on both bacterial growth and PHB accumulation, due to the use of EGPJ, a similar pulse feed strategy was carried out using simulated EGPJ. A maximum of 64 g/L CDW was achieved after 48 h of cultivation (total 160 g of

sugars/L) using simulated EGPJ (Fig. 2). PHB content was 19.8 g/L and the final CDW yield was 0.4 g/g of substrate. PHB contributed to 10% of the CDW of *B. sacchari* IPT101 prior to the onset of nitrogen limitation. PHB increased to 31% of CDW at the end of the nitrogen limited phase (Fig. 2). Pulse feed strategy using simulated EGPJ resulted in a PHB and CDW volumetric productivity of 0.41 g/L/h and 1.33 g/L/h, respectively which were comparable to the productivity values achieved with EGPJ. Maximum specific growth rates (μ_{max}) for *B. sacchari* IPT101 using either EGPJ or simulated EGPJ were same (0.03 h^{-1}).

There are few reports on the use of other renewable sugar rich substrates such as wheat straw hydrolyzate, rice straw hydrolyzate, and wood hydrolyzate (Koller et al., 2010) for PHB production. However, these substrates require physico-chemical pretreatment and hydrolysis to release the sugar from the biomass. On the other hand EGPJ does not require any chemical pretreatment or enzyme hydrolysis. While ensiling is a slow biological process it is a useful storage/preparation method for grass so that a biorefinery can produce a product that can be used throughout the year. Ensiling involves microbial activities which lead to acidification of the grass biomass and this causes the WSC to be released from the free fraction and from the hemicellulosic fibers. EGPJ only requires minimal processing such as rotary evaporation to avoid dilution effect in the pulse feed process. There is limited literature available on the use of untreated or minimally treated substrates for PHB production. Those include oil palm trunk sap (Lokesh et al., 2012) oil palm frond juice (Zahari et al., 2012) and Mahua flower extract (Anil Kumar et al., 2007) which are rich in sucrose (60% or more). CDW and PHB yields of *B. sacchari* IPT101 grown on EGPJ were similar to *Bacillus megaterium* grown on oil palm trunk sap (Lokesh et al.,

2012), and both yields were slightly lower than *Cupriavidus necator* CCUG 52238 grown on oil palm frond juice (0.44 vs 0.53 g CDW/g of sugars and 0.12 vs 0.17 g PHB/g of sugars) (Zahari et al., 2012), CDW yield for *B. sacchari* IPT101 was two-fold higher than that reported for *Bacillus* sp. on Mahua flower extract, while PHB yield was similar (Anil Kumar et al., 2007). None of these studies conducted fed-batch fermentations to achieve high cell densities. Higher volumetric productivities of biomass and PHB are necessary for an industrial bioprocess outcome. This study achieved high volumetric productivities for both CDW and PHB using a chemically or enzymatically untreated substrate as a sole carbon source.

EGPJ is a by-product of the ensiling process and it has been previously used for bioethanol (Sieker et al., 2011) and biogas production (Wachendorf et al., 2009), however there is no report on the use of EGPJ as sole carbon source for PHA production. Koller et al. (2005) used green grass juice and silage juice as nutrient supplements to enhance PHB production by 6% using *Wautersia eutropha* (presently known as *C. necator*). PHB production using EGPJ as a carbon substrate avoids the competition with food as grass is a non-food renewable resource and can also be integrated into the animal feed sector (McEniry and O'Kiely, 2014). Approximately, 50% of PHA production cost accounts to the cost of carbon substrate. Under current grassland management and production practices there is an estimated average of 33% excess grass available in addition to the livestock requirements (Koller et al., 2005; McEniry and O'Kiely, 2014). Considering that one hectare yields 12-14 tonne of silage/year; each tonne of silage will yield 400 L of EGPJ, therefore one hectare would produce 5-6 tonne of EGPJ/year. EGPJ, a zero cost carbon resource which can yield 0.164 g of PHB/g of sugars that results in 8.8 kg of PHB/tonne of

EGPJ. Based on the current market price of minimal media components one can produce PHB at US$ 6.13/kg. Increasing the yield of sugar conversion into PHB will ultimately lead to a cost-effective fermentation bioprocess. Compared to the industrial PHB production using commercial sugars the yield of the current study is 4 fold lower but the price is in the same range (US$ 4/kg). Furthermore the conversion of an effluent into value added bioproducts has additional benefits through integration with animal food production (silage), and reduction in the cost of cellulosic pretreatment by avoiding the use of purified crude enzyme preparations.

3.5. Fed-batch cultivation of *P. chlororaphis* IMD555 using EGPJ and simulated EGPJ as carbon source.

Pulse feed strategy of EGPJ combined with nitrogen limitation resulted in a maximum CDW of 37.5 g/L and accumulation of 10% mcl-PHA by *P. chlororaphis* IMD555 at 34 h (Fig. 3). This corresponds to a maximum mcl-PHA content of 3.75 g/L. Biomass and mcl-PHA volumetric productivities were 1.1 g/L/h and 0.1 g/L/h, respectively. While pulse feed of EGPJ to *P. chlororaphis* IMD555 resulted in an increase in CDW, mcl-PHA accumulation reached a plateau of 10% at 24 h. Pulse feed strategy of simulated EGPJ resulted in a maximum CDW of 57.5 g/L and accumulation of 2.2% mcl-PHA by *P. chlororaphis* IMD555 at 31 h (Fig. 4). This corresponds to a maximum mcl-PHA content of 1.26 g/L. CDW and mcl-PHA volumetric productivities were 2.07 g/L/h and 0.04 g/L/h, respectively. Onset of nitrogen limitation (from 29 h) resulted in the accumulation of mannitol along with xylose, caused foaming and microbial cell lysis, this resulted in limited accumulation of mcl-PHA. (Fig. 4). Pulse-feed strategy is especially

useful when mixed sugars are used in order to prevent substrate accumulation. As *P. chlororaphis* IMD555 cannot use xylose, it is expected that, with high cell densities fermentations, build-up of xylose will occur at the end of the pulse-feeding strategy. Another possible reason for low mcl-PHA accumulation on simulated EGPJ can be due to a higher μ_{max} compared to EGPJ (0.13 h^{-1} vs 0.08 h^{-1}). Koller et al. (2010) reported that there is a strict connection between reduced mcl-PHA accumulation and higher growth rate. EGPJ addition is able to guarantee a reduced μ_{max}, while simulated EGPJ increase the growth rate of *P. chlororaphis* IMD555 by 61%, therefore EGPJ guarantees better conditions for mcl-PHA accumulation rather than simulated EGPJ

This is the first report of using a minimally treated (mechanically) grass derived renewable substrate for mcl-PHA production; though a previous study (Davis et al., 2013) used chemically and enzymatically treated grass hydrolyzate for the growth and mcl-PHA accumulation by *Pseudomonas* strains, in flasks experiments. Though *Pseudomonas* species grown on unrelated substrates (sugars, glycerol and VFAs) can achieve high cell densities they require strict inorganic nutrient limitation or addition of fatty acids precursors for mcl-PHA production (Cerrone et al., 2014b; Davis et al., 2015). Follonier et al. (2014) also achieved low levels of mcl-PHA using an enzymatically treated sugar rich substrate (apple pomace) for *P. putida* during the growth phase. Therefore a two-phase system where lignocellulose derived mixed sugars (or unrelated substrates) used for bacterial biomass production and fatty acids precursors used during the mcl-PHA accumulation phase should considerably reduce the overall cost of PHA production. Using a biphasic system (fruit pomace and waste frying oil) Follonier et al. (2014) achieved a CDW of 10.2 g/L and mcl-PHA of 1.4 g/L, in 45 h (CDW and mcl-PHA volumetric productivity of 0.24

g/L/h and 0.05 g/L/h, respectively). The mcl-PHA and biomass productivity from EGPJ is three and five fold higher respectively, compared to fruit pomace (Follonier et al., 2014). The ensiling of grass can reduce costs as one can avoid the addition of enzymes for pre-treatment of grass and the pressed cake byproduct arising from pressing can be used for other applications (McEniry and O'Kiely, 2014).

3.6. Physico-chemical properties of PHB and mcl-PHA.

NMR and GC data indicated that PHB was composed of 100% (R)-3-hydroxybutyrate, while mcl-PHA was composed of 49 (± 4) % (*R*)-3- hydroxydecanoate (3-HD), 39 (± 4) % (*R*)-3-hydroxydodecanoate (3-HDD) and 10 (± 1) % (*R*)-3-hydroxyoctanoate (3-HO). PHB extracted from *B. sacchari* grown on EGPJ had a polydispersity of 2.3, molecular weight (Mw) of 548 kg/mol and molecular number (Mn) of 238 kg/mol, respectively. DSC showed that Tg and Tm of PHB were 2.44 and 172.6 °C, respectively and these parameters were comparable to the PHB produced with simulated EGPJ. TGA (288 °C) and ΔH (-16 J/g) of PHB produced using EGPJ were slightly lower than that produced using simulated EGPJ (ΔH:-12 J/g and TGA: 303 °C). Polymer extracted from *P. chlororaphis* IMD555 grown on EGPJ has a PDI of 2.3, Mw and Mn of 115.4 and 48.8 kg/mol, respectively. Tg, Tm, and TGA of the extracted mcl-PHA were -42.1, 38.2 and 284 (°C), respectively. Molecular weight distributions and thermal properties of mcl-PHA produced by *P. chlororaphis* IMD555 on EGPJ were very similar to mcl-PHA produced by commercially available sugars (Ashby et al., 2001; Davis et al., 2013).

4. Conclusions

A process for biopolymer production using an effluent from grass silage process (EGPJ) has been demonstrated. The mannitol rich ensiled grass press juice (EGPJ), a cheap and renewable substrate, without any chemical or enzymatic pre-treatments was useful for achieving high CDW for both mcl-PHA and PHB accumulating bacterial strain but high PHB productivity (13.4 g PHB/L) compared to mcl-PHA (3.75 g mcl-PHA/L).

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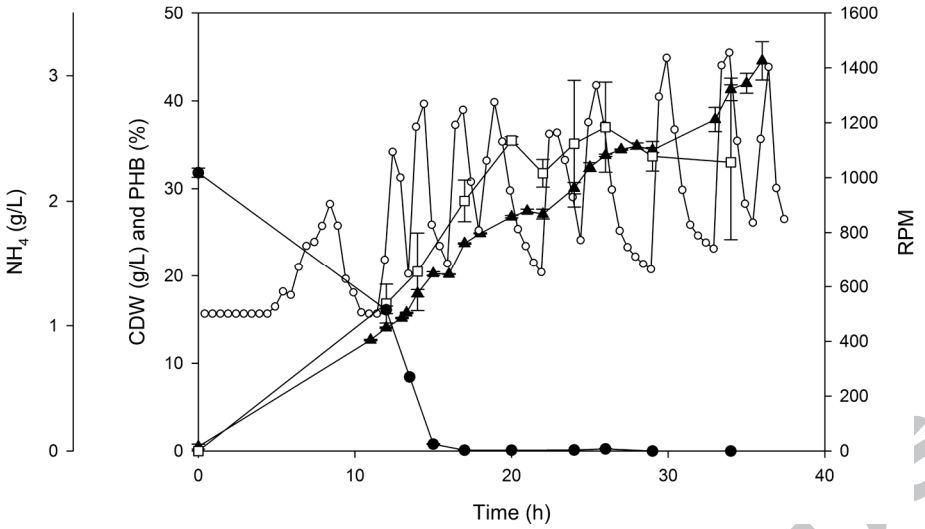
Figure captions

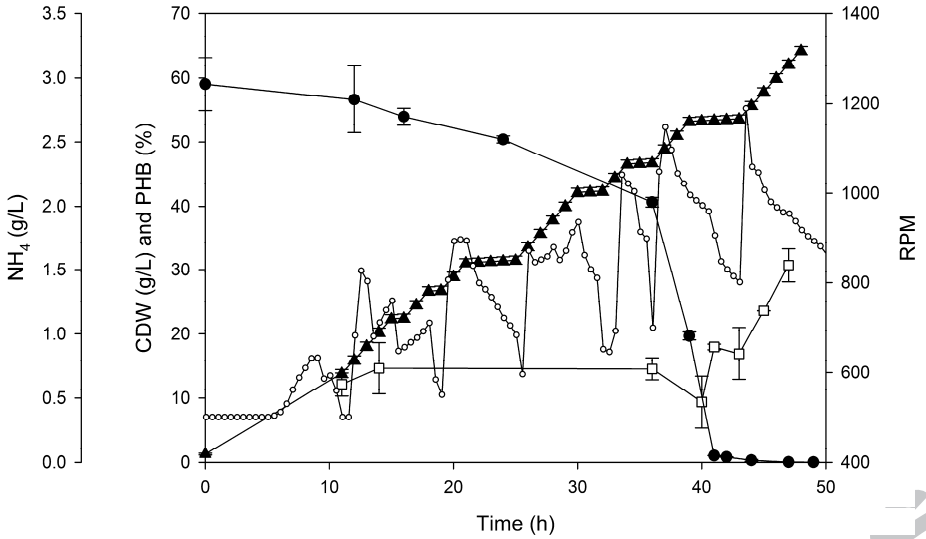
Figure 1 Fed-batch cultivation of *B. sacchari* IPT101 with pulse feed of ensiled grass press juice as carbon source. (▲) cell dry weight (g/L), (○) impeller speed (RPM), (●) NH_4 (g/L) and (□) PHB (%) were tracked over time.

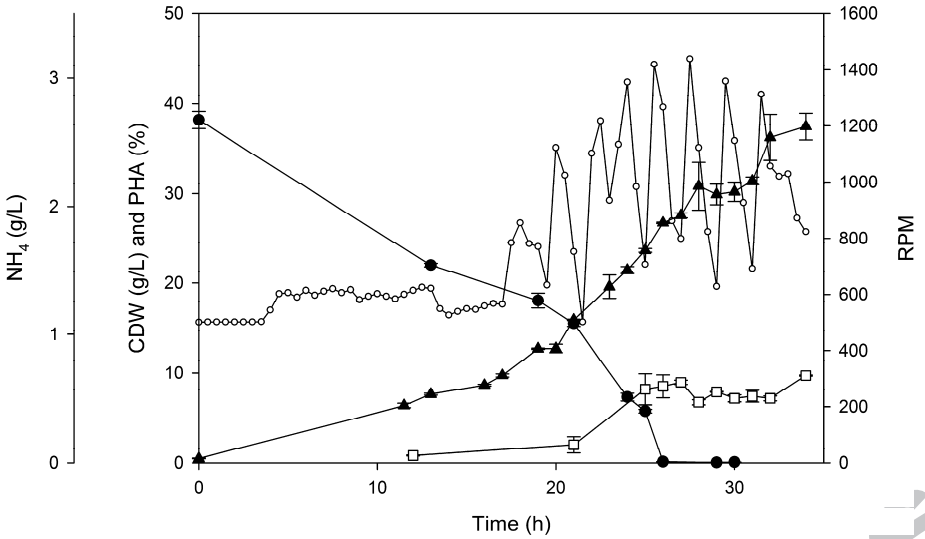
Figure 2 Fed-batch cultivation of *B. sacchari* IPT101 with pulse feed of simulated ensiled grass press juice as carbon source. (▲) cell dry weight (g/L), (○) impeller speed (RPM), (●) NH_4 (g/L) and (□) PHB (%) were tracked over time.

Figure 3 Fed-batch cultivation of *P. chlororaphis* IMD555 with pulse feed of ensiled grass press juice as carbon source. (▲) cell dry weight (g/L), (○) impeller speed (RPM), (●) NH_4 (g/L) and (□) mcl-PHA (%) were tracked over time.

Figure 4 Fed-batch cultivation of *P. chlororaphis* IMD555 with pulse feed of simulated ensiled grass press juice as carbon source. (▲) cell dry weight (g/L), (○) impeller speed (RPM), (●) NH_4 (g/L), (□) mcl-PHA (%), (Δ) reducing sugars (g/L) and (X) mannitol (g/L) were tracked over time.







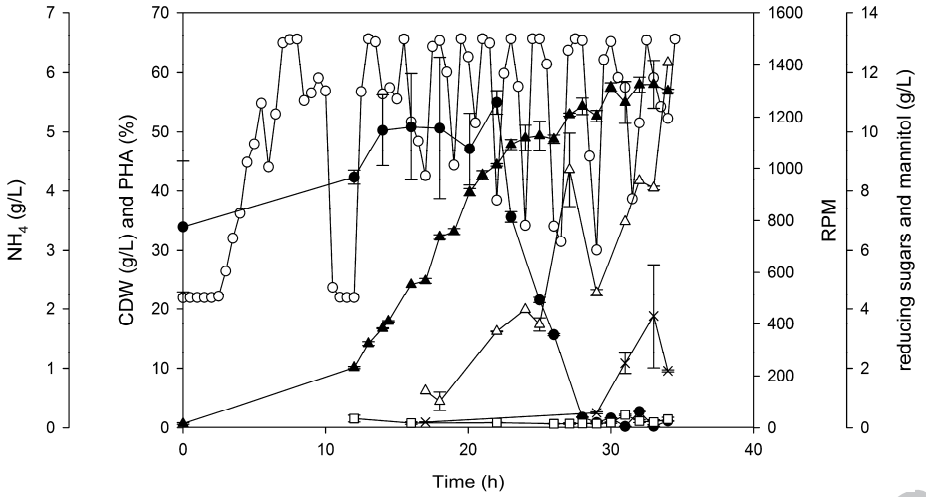


Table 1 Biosynthesis of polyhydroxyalkanoates (PHA) in shaking flask experiments using EGPJ and simulated EGPJ.

Strains used	Substrates	CDW (g/L)	PHA (%)	Monomer composition (mol %)				
				3-HB	3-HH	3-HO	3-HD	3-HDD
P. putida W619	EGPJ	0.5	6.8	0	6	19	68	5
P. chlororaphis IMD555		0.7	9	0	0	15	56	28
B. sacchari IPT101		2.3	23.3	100	0	0	0	0
P. putida W619	Simulated	1.04	31	0	1	14	63	21
P. chlororaphis IMD555	EGPJ	1.05	38	0	5	10	54	32
B. sacchari IPT101		4.3	34.5	100	0	0	0	0

Experiments were conducted in 250 mL shaking flasks with MSM containing 7.2 g/L (total sugars) of EGPJ and simulated EGPJ (mimicking sugars composition of EGPJ) as carbon source. Flasks were inoculated with 1% of overnight inoculum and incubated for 48 h at 30 °C and 200 RPM (shaking table incubator).

EGPJ: Ensiled grass press juice

CDW: Cell dry weight was estimated gravimetrically after freeze-drying of washed cell pellet from 45 mL of culture (g dry biomass/volume mL)*1000

PHA: polyhydroxyalkanoates content (% w/w) was estimated by GC with FID detector after methanolysis of the freeze dried cells.

Monomer composition: % distribution of each (R)-3-hydroxyalkanoic acid on the total polyhydroxyalkanoate content after methanolysis [3-HH: (R)-3-hydroxyhexanoic acid, 3-HO: (R)-3-hydroxyoctanoic acid, 3-HD: (R)-3-hydroxydecanoic acid, 3-HDD: (R)-3-hydroxydodecanoic acid]

Highlights

1. First report on the use of a mannitol rich renewable carbon source for PHA production
2. Achieved PHB and mcl-PHA production using EGPJ as sole carbon source.
3. EGPJ is a suitable substrate to achieve high cell density and high PHB productivity.
4. PHB productivity on EGPJ was comparable to that on commercial sugars.

Graphical abstract