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Supervisor: Prof. Dra. Luiziana Ferreira da Silva

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CERTIFICADO DE ISENÇÃO

Certificamos que o Protocolo CEP-ICB Nº **564/12** referente ao projeto intitulado: "*Estudo do metabolismo de xilose em Burkholderia sacchari para gerar polihidroxibutirato a partir de hidrolisados lignocelulósicos mediante desenvolvimento de novas linhagens*" sob a responsabilidade de **Linda Priscila Guamán Bautista,** foi analisado na presente data pela CEUA - COMISSÃO DE ÉTICA NO USO DE ANIMAIS e pela CEPSH- COMISSÃO DE ÉTICA EM PESQUISA COM SERES HUMANOS, tendo sido deliberado que o referido projeto não utilizará animais que estejam sob a égide da lei 11.794 de 8 de outubro de 2008, nem envolverá procedimentos regulados pela Resolução CONEP nº196 de 1996.

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PROF. DR. WOTHAN TAVARES DE LIMA
Coordenador da CEUA - ICB/USP

PROF. DR. PAOLO M.A ZANOTTO
Coordenador da CEPsh - ICB/USP

A mis padres Gustavo y Rosa.

Por haberme dado el mejor título de mi vida, ser su hija.

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“*Well-behaved women seldom make history*”

Laurel Thatcher Ulrich.

RESUMO

Guamán LP. Clonagem e superexpressão dos genes do metabolismo da xilose em *Burkholderia sacchari* e avaliação do efeito na repressao catabólica e no acúmulo de Polihidroxibutirato usando açúcares lignocelulósicos. Tese (Doutorado em Microbiologia). São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2016.

Apesar das muitas vantagens que os Polihidroxialcanoatos apresentam, uma das principais razões que limitam seu uso a grande escala, é o alto custo da fonte de carbono usada como substrato, por tanto, o uso de fontes de carbono mais baratas obtidas de resíduos lignocelulósicos são uma alternativa para reduzir os custos de produção, além de serem completamente renováveis. No Brasil, a quantidade de bagaço de cana de açúcar disponível é de 20.8×10^6 por ano, o que representa, 10×10^6 toneladas de xilose disponíveis, que são geralmente queimadas ou descartadas. A pesar de ser o segundo açúcar mais abundante da natureza, o uso de xilose ainda representa uma barreira técnica, devido ao fenômeno de repressão catabólica quando a glicose está presente, e também devido ao ineficiente consumo de xilose em várias linhagens. Usamos como modelo de estudo Burkholderia sacchari, uma bactéria isolada no Brasil, que consome xilose, e acumula ate um 80% de P(3HB) como massa seca, com o objetivo de melhorar a velocidade especifica máxima de crescimento (0.14 h^{-1}) e o teor de acumulo de P(3HB), a traves da expressão de genes metabólicos, reguladores e de transporte de xilose. Primeiro, nós descrevemos a organização dos genes responsáveis da assimilação da xilose, e avaliamos a superexpressão dos dois primeiros genes metabólicos xylAB. Nós demonstramos que a superexpressão destes genes melhorou a velocidade específica máxima de crescimento assim como o acúmulo de P(3HB), atingindo o mais alto fator de conversão de xilose a P(3HB) (0.35 g/g), com um incremento na velocidade a 0.203 h^{-1}. Depois, a foram realizados ensaios em misturas de açúcares para avaliar a repressão catabólica (CCR) na presença de xilose, glicose e arabinose. Foi possível identificar uma forte CCR de glicose sob xilose, e uma CCR mais relaxada na mistura de glicose sob arabinose. A super expressão dos genes xylE-xylAB permitiu abolir a repressão catabólica melhorando assim o acúmulo de P(3HB) ate 66%. Finalmente, e devido a que uma das maiores limitações para a aplicação de biologia sintética e engenharia metabólica em B. sacchari é a falta de ferramentas moleculares apropriadas, nos decidimos construir um set de plasmídeos adaptando o sistema BglBrick usando origens de replicação compatíveis com B. sacchari. Foram avaliados também, dois diferentes promotores e se identificaram os melhores níveis de cada um dos indutores. Usando este set de plasmídeos, nos reportamos que a superexpressão de XylR, o fator de regulação transcricional do operon de xilose, nos permitiu atingir a maior velocidade máxima específica de crescimento para B. sacchari, $0.25h^{-1}$, quando usada xylose como fonte de carbono, o fator de conversão de xilose a P(3HB) e o teor de acúmulo foram também incrementadas. Em resumo, nos reportamos os diferentes níveis de repressão catabólica em B. sacchari em misturas de xilose, glicose e arabinose, e também, concluímos que a superexpressão dos genes xylAB e xylR melhoram a velocidade específica de crescimento, o fator de conversão e o teor de acumulo de P(3HB) em B. sacchari usando xilose como fonte de carbono

Palavras chave - Xilose. *Burkholderia sacchari*. Poli-3-Hidroxibutirato. Lignocellulose.

ABSTRACT

Guamán LP. Cloning and overexpression of xylose catabolism genes of Burkholderia sacchari and evaluation of the impact on catabolic repression and Polyhydroxybutyrate production using hemicellulosic sugars. [Ph.D. thesis (Microbiology). Microbiology] São Paulo University, São: Paulo Instituto de Ciências Biomédicas, Universidade de São Paulo; 2016

Despite the many advantages of polyhydroxyalkanoates, their higher production costs when compared with petroleum-based polymers still represent a barrier for make them competitive. One of the major reasons is the high cost associated at the carbon source used as substrate, therefore, the use of low-cost carbon sources obtained from lignocellulose residues, is an alternative to reduce production costs, besides of being completely renewable. In Brazil, the amount of sugarcane bagasse available is around 20.8 × 106 tonnes year−1 which means 10 × 106 tonnes year−1 of xylose available, mostly burned or discarded. Despite of being the second most abundant sugar in nature, xylose utilization still represents a technical barrier, because of carbon catabolite repression when in presence of glucose, and due to inefficient xylose uptake in several strains. In this research we study Burkholderia sacchari, a bacterium isolated in Brazil, which consumes xylose and accumulate up to 80% of the cell mass as P(3HB), with the aim of improving its specific growth rate (0.14 h-1) and P(3HB) yield, through overexpression of xylose catabolic, transport and regulator genes. First, we described the organization of the genes responsible for xylose assimilation, and we tested the first two metabolic genes (xylAB) demonstrating through its overexpression, that it is possible to improve B. sacchari ability to growth and use xylose as a sole carbon source and production of P(3HB). The highest conversion rate of xylose to P(3HB) (0.35 g/g) was achieved and also, the highest titer (11.3 g L-1) at a specific growth rate of 0.203 h-1. Then a series of sugar mixtures assays were performed to assess carbon catabolite repression (CCR) in mixtures of xylose, glucose, and arabinose. We identified strong CCR over xylose when glucose is supplied, and a relaxed CCR in glucose arabinose mixtures. xylE-xylAB genes were overexpressed to abolish CCR allowing us also improve P(3HB) accumulation up to 62%. Finally, and because one of the major limitations for applying metabolic engineering or synthetic biology approaches in B. sacchari is the lack of appropriate molecular tools, we decided to construct a set of inducible vectors adapting the existing Bgll Brick system, using compatible replication origins to B. sacchari, two different promoters were assessed, and the best induction levels were described. Using this set of plasmids we reported that XylR (transcriptional xylose regulator) overexpression allowed us to achieve the highest growth rate reported to B. sacchari when cultivated in xylose as the sole carbon source (0.25h-1), improving also P(3HB) conversion factor and yield. In summary, we report B. sacchari has different levels of CCR in mixtures of xylose, glucose and arabinose; and also, we conclude that overexpression of xylAB and xylR genes improve growth rate, conversion factor and yield when P(3HB) is produced using xylose as carbon source in B. sacchari.

Keywords - Xylose. *Burkholderia sacchari*. Polyhydroxybutyrate. Lignocellulose.

LIST OF ABBREVIATIONS

PHA - polyhydroxyalkanoates
P(3HB) - poli-3-hydroxybutyrate
P(3HB) - polyhydroxybutyrate
CDW - cell dry weight (g/l or g)
$Y_{P(3HB)/XIL}$ - Conversion rate of xylose in polyhydroxybutyrate
GC-MS - Gas chromatography - Mass spectrometry.
G6P - glucose-6-phosphate
F6P - fructose-6-phosphate
G3P - Glyceraldehyde 3-phosphate
E4P - erythrose 4-phosphate
S7P - sedoheptulose 7-phosphate
Rbl5P - ribulose-5-phosphate
Rb5P - ribose-5-phosphate
AcCoA - acetyl-CoA
xylA - xylose isomerase
xylB - xylulokinase
xylF - xylose-binding protein
xylG - ATP-binding protein
xylH - membrane transporter ABC-type
xylR - transcriptional regulator of the the AraC family of regulators
μ_{max} - maximum specific growth rate
OD - Optical Density
MM - Minimal Media
LB - Luria Bertani
Km - Kanamycin
IPTG - Isopropyl β-D-1-thiogalactopyranoside
LFM - Laboratorio de Fisiología de Microorganismos
CCR - Carbon Catabolite repression
PTS - Phophotransferase system

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1 INTRODUCTION

Polyhydroxyalkanoates (PHA's), are intracellular granules of polyester, accumulated as carbon and energy storage materials, synthesized by many microbial strains under unbalanced growth conditions such as the presence of excess carbon source and limitation of at least one essential nutrient. Its biodegradability, biocompatibility and similar physical properties to synthetic polymers make them an environmentally friendly alternative to petrochemical based plastics (1).

Since PHA's first description in 1926 by Lemoigne (2), several advances towards PHA's large scale production have been conducted, however, currently, the high production costs make PHA's more expensive than conventional plastics. One of the main reasons, is the high cost associated to carbon source used. According to Nonato and coworkers, it can account for up to 29% of its overall production cost, even when integrated to sugarcane mills (3). Several attempts including use of inexpensive lignocellulosic biomass have been conducted in recent years (4). In Brazil sugarcane production doubled the last decade, increasing at the same level bagasse availability by around 208 million tonnes (5). It is estimated around 30% (on a dry weight basis) of the harvested sugarcane corresponds to lignocellulosic byproducts (6).

The amount of sugarcane bagasse estimated to be used only for hydrolysis process is around 7.0×10^6 tonnes year (5) which means 35×10^3 tons per year of xylose available, mostly burned or discarded (7). *Burkholderia sacchari* LFM 101 (8,9), is able to accumulate up to 75% of cell dry weight (CDW) as PHA (10), this bacteria can utilize a variety of carbon sources including xylose, and even hemicellulosic hydrolysates from sugarcane bagasse (11–13) to produce not only PHA's but also other high-value chemicals like xylitol and xylonic acid (9,14). Despite of its capability to use xylose as carbon source, its slow growth rate ($0.16\ h^{-1}$) associated with xylose consumption, represent a barrier for using this bacteria as a chassis to produce PHA's or other high value chemicals on industrial scale. Therefore, it is highly relevant to understand the particular features of xylose metabolism in *B. sacchari* and use this information to overcome metabolic roadblocks and exploit its potential for renewable bioproduction.

Within this context, the aim of this research was to improve xylose uptake in *B. sacchari,* through (1) Genome analysis and organization of operon(s) involved in xylose metabolism in *B. sacchari*. (2) Overexpression of key genes related to xylose metabolism, transport, or regulation, (3) Evaluate gene overexpression impact in growth rate and P(3HB) production. (4) Evaluate carbon catabolite repression in glucose, arabinose and xylose mixtures. (5) Finally, we also wanted to contribute to future synthetic biology approaches constructing a set of plasmids with replications origins compatible with *B. sacchari,* testing also two different promoters and appropriate induction levels.

2 CHAPTER 1: Literature Review

2.1.1 Petroleum based plastics

Plastics have been present in our daily lives since 1920 and their use has increased exponentially (15). The annual production of petroleum based plastics exceeded 300 million tons in 2015 (16). In every facet of our lives, industry, in homes, food packaging, plastics are involved, and the demand keeps growing. Millions of tons of plastic wastes are generated worldwide, disposing 93% of them in landfills and oceans (17). Even in some european countries highly engaged in recovery plastic wastes, such as Germany, Netherlands, Sweden, Denmark and Austria, recycling reached only 28% on average (18).

In developing countries including China, Indonesia, Philippines, Sri Lanka and Vietnam were reported to generate more than 50% of global plastic pollution in marine environment (19). Although the technologies for recovering the plastics wastes have been improved, an increase in the world population to about 9 billion in 2050 requires a higher demand for plastic production and consequently, an increase in the amount of plastic wastes (18).

Final disposal for plastics are landfilling, incineration and recycling. First two are known to produce negative environmental and health effects. Many plastics can be recycled, and some of the materials used to make plastics can be recovered. However, this method is not fully utilized, due to difficulties with the collection and sorting of plastic waste (20). Finally, certain polymers are designed to biodegrade, thereby preventing long-term environmental damage from pollution. However, many biodegradable plastics may not biodegrade rapidly enough under ambient environmental conditions to avoid accumulation from continuous inputs (21).

In a recent work, published in Science journal in 2015, researchers linked worldwide data on solid waste, population density, and economic status, and estimated the mass of land-based plastic waste entering the ocean. They calculated around 12.7 million metric tons entering the ocean (21) (Figure 1).

Figure 1 - Estimative of plastic waste deposited into the ocean. Source (Jambeck et al., 2016)

Based on tonnage, China was judged the biggest plastic polluter among 192 coastal countries surveyed. Researchers estimate that the annual rate of plastic disposal in the ocean could grow to 155 million metric tons by 2025, they also note that if the world pursued a strategy of total waste management and a cap on waste levels, the flow of plastic into the oceans could be reduced by more than 75 percent by 2025.

Is not difficult to conclude that one of the biggest challenges as society in the 21st century, is to meet the growing demand for energy for transportation, heating and industrial processes, besides, to ensure raw material for the industry in a sustainable manner (22). Petroleum is still the most widely source used for fuels and plastics production, however, not only its reserves are finite, but also, it contributes to global warming among others negative effects for the environment as mentioned before (23). Thus, several studies have sought oil substitution for lignocellulosic residues, not only to produce ethanol or plastics, but also a wide variety of high value chemicals using renewable raw materials, low cost, and available

in large quantities (9,24,25). One of the most broadly studied biopolymers are the Polyhydroxyalkanoates (PHA's), and several researchers around the world have been working the last decade aiming to replace in the future petrochemical plastics for PHA's.

2.1.2 Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHA's) are linear polyesters (Figure 2), naturally produced by a wide variety of bacteria, that synthesize these polymers as a carbon and energy storage (26). They are accumulated under certain conditions, in carbon excess and limiting concentration of essential growth nutrients, such as nitrogen or phosphate (27). When the carbon source is depleted, the collected biopolymers are depolymerised, and their degradation products can be used as a source of carbon and energy materials (28).

These biopolymers possess diverse and easily variable physical properties that allow them, to directly compete with synthetic plastics (29). The first reported of PHA's occurred in 1923 at the Pasteur Institute, where microbiologist Maurice Lemoigne characterized for the first time polyhydroxybutyrate P(3HB) composition (2) (Figure 2). PHA's were classified according to carbon chains length of their monomeric units. Short chain length PHA's (PHA'S_{CL}) which carbon chain is constituted of 3 to 5 monomers, medium chain length (PHA_{mcl}) consisting between 6 to 14 monomers, and the long chain monomers with more than 15 monomers (PHA_{LCL}) (1).

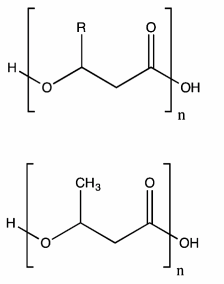

Figure 2 - Chemical structure of PHA's. Top: General structure of PHA's. Bottom: Structure of P(3HB). Source: (Steinbuchel et al., 1995)

More than 250 PHA's producing bateria have been described, among them, very few are suitable for produce PHA's achieving high concentration and productivities. Some of

these bacteria includes *Ralstonia eutropha*, *Azotobacter vinelandii*, recombinant *Escherichia coli*, some *Pseudomonas* species, among others (31).

The high production cost when compared with its counterpats petroleum based is the main barrier for its commercialization. Consequently, much effort has been devoted to reducing PHA's cost, including development of better bacterial strains, more efficient bioreactor cultivation conditions and recovery processes (30,32).

2.1.2.1 P(3HB)

For many years, P(3HB) was the only polymer known. In 1964, Davis identified the 3-hydroxy-2-butenoic acid as a constituent PHA produced by *Nocardia* (33). By 1974, Wallen and Rohwedder identified different monomers others than 3HB in activated sludge samples, they described 3-hydroxyvalerate (3HV) and 3 hydroxyhexanoate (3HHx) (34). Years later, Findlay and White (1983) demonstrated the presence of 3HB units, 3-hydroxyheptanoate (3HHp) and 3-hydroxyoctanoate (3HO) extracted from *Bacillus megaterium* polymer (35). In the 80's, a growing number of different constituents of PHA's were identified (36–38).

P(3HB) raised industrial interest early in the 60's, because of its thermoplastic properties similar to polypropylene. In the late 70's and early 80's, with the oil crisis, a new development was carried out in order to develop new technology for the production of these polymers as plastics substitutes of petrochemical origin (39,40).

In Brazil, a process for producing P(3HB) using sucrose as a main carbon source integrated in sugar and ethanol mill was developed (3). Also, in the early 90's, the project "Biodegradable plastics production from sugarcane through biotechnological processes" began, which was developed through a cooperation with the Biotechnology Laboratory of the Institute Technological Research of the State of São Paulo (IPT), the Copersucar Technology Center and the Institute of Biomedical Sciences from São Paulo University (41).

The main reason to raise such a big interest is because of its features. P(3HB), is a highly crystalline, hard and brittle thermoplastic, with similar characteristics to polystyrene or poly vinyl chloride (PVC). Its physical properties are often compared to those of polypropylene, for having similar melting point, crystallinity, and glass transition temperature (42). Several approaches have been conducted to reduce this material fragility. Some of these

studies rely on obtaining copolymers with hydroxyvalerate units that have lower crystallinity and better mechanical properties than the P(3HB) (14).

2.1.2.2 PHA's properties and applications

The increased interest in producing heteropolymers with improved physical and mechanical qualities, is due to P(3HB) melting temperature (177 °C), which is within 2 degrees of its thermal decomposition, making very difficult to melt it without polymer breakdown. Copolymers of (3HB-co-3HV) displays several interesting features such as being water resistant, impermeable to oxygen, less brittle, and have melting temperatures that are 30 °C lower than the homopolymer P(3HB) (13). By adding substrates such as propionic acid, valeric acid (39) and levulinic acid to nitrogen-limited cultures of many bacteria (14), copolymers of P(3HB-co-3HV) can be produced.

The structure of the side groups in the polymer chain determines PHA properties, as well as the distance between ester groups in the molecule (43) . The physical properties of the P(3HB) polymer (Glass transition temperature (Tg) = 2 °C, melting temperatures (Tm) = 177 °C, and 70% crystallinity) closely resemble those of polypropylene (44). In order to reduce down the cristalinity degree to 40-60%, P(3HV) monomer should be incorporated into the P(3HB-co-3HV) copolymer making the polymer more flexible, with great extension to break percentages, and Tm values ranging from 60 °C to 177 °C depending on Mol % 3HV content (45) (Table 1). Thermal characteristics, Tm and decomposition temperature (Td), of the polyhydroxyalkanoate copolymers are lower than those for polyhydroxybutyrate, whose melting temperature and thermal decomposition are 168-170 and 260-265 °C, respectively. Both parameters decrease to 150-160 and 200-220 °C, respectively, when the hydroxyvalerate mole fraction is raised (46).

Table 1 - Physical Properties of PHA's and Polypropylene. Source: (Harper, 2005)

Parameter	P(3HB)[b]	P(3HB-3HV)	P(3HB-4HB)	P(3HO-3HH)	PP
Tm (^{o}C)[c]	177	145	150	61	176
Tg (^{o}C)[d]	2	-1	-7	-36	-10
Crystallinity (%)	70	56	45	30	60
Extension to break (%)	5	50	444	300	400

[b]P(3HB) poly(3-hydroxybutyrate), P(3HB-3HV) poly(3-hydroxybutyrate-*co*-3 hydroxyvalerate) containing 20% 3HV, P(3HB-4HB) poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) containing 16% 4HB, P(3HO-3HH) poly(3-hydroxyoctanoate-*co*-3-hydroxyhexanoate) containing 11% 3HH, and PP polypropylene. [c]Tm is melting temperature. [d]Tg is glass transition temperature.

As mentioned before, depending on monomeric composition, PHA's can have different applications and useful properties such as: biodegradability, thermoplasticity, biocompatibility, non-toxicity, etc. In recent years companies have been interested in the use of PHA's in packaging, biomedical, agricultural applications (Figure 3). It is well known that PHA's were initially used for manufacturing cosmetic containers such as shampoo bottles (48), moisture barriers in sanitary products (49) or pure chemicals as raw materials for the production oflatex paints (50) . Also, they can be used as carriers for long term release of herbicides or insecticides (51) . P (3HB) homopolymer and copolyester of P(3HB-co-3HV) are the most studied PHA's for medical applications. In recent years they are considered as materials in the fabrication of cardiovascular products (heart valves, stents, vascular grafts) (29) , in drug delivery system (tablets, microcarriers for anticancer therapy), in wound management (sutures, nerve cuffs, swabs, straples), in orthopaedy (bone plates, spinal cages) (29).

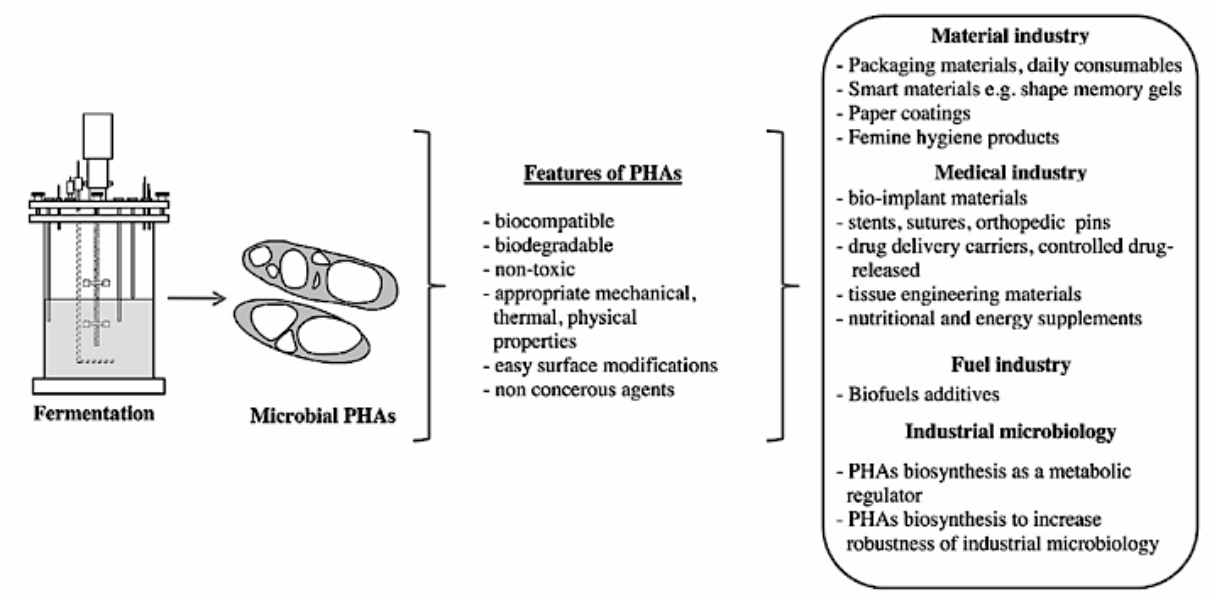

Figure 3 - Different applications of PHA's. Source (Możejko et al., 2016)

2.1.2.3 PHA's biodegradability

PHA's are fully biodegradable into final components of carbon dioxide and water (1,52). Various bacteria and fungi degrade PHA's through extracellular PHA depolymerase enzymes activity (52) (Figure 4).

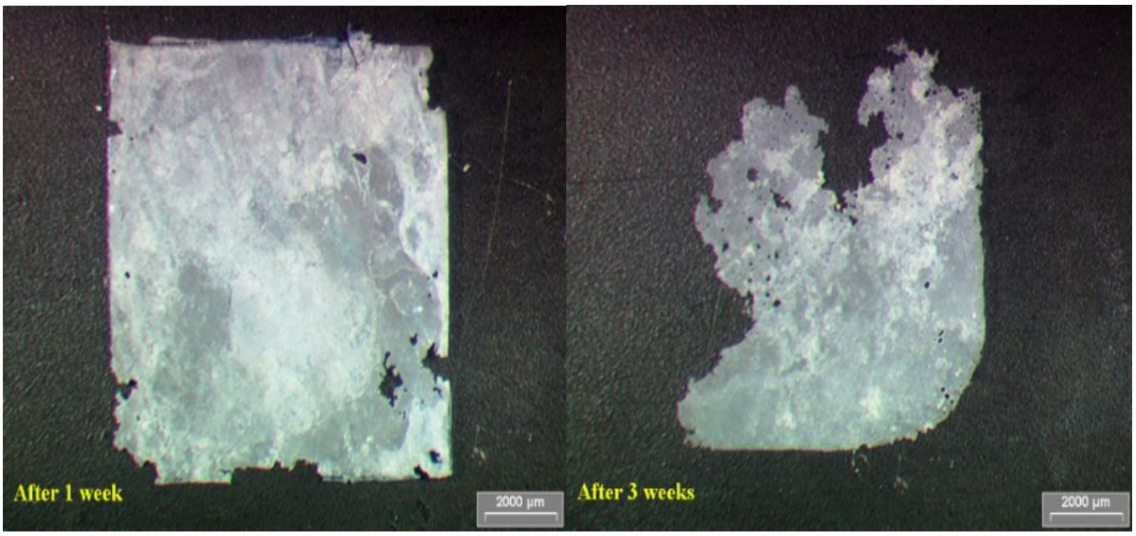

Figure 4 - Pictures showing P(3HB) film degrading to almost 50% of its original size in a 3 week time frame. Source: (Thompson et al., 2004)

These low-molecular-weight degradation products are then transported into the cell and subsequently metabolized as carbon and energy sources (54) (Figure 5).

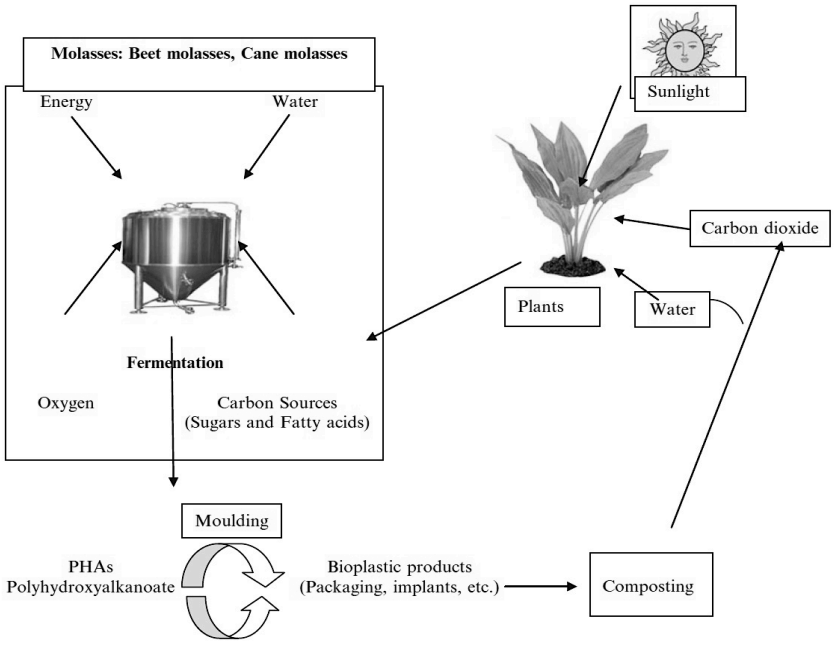

Figure 5 - Biodegradation cycle for PHA's. Source (Tokiwa et al., 2009)

Chowdhury and coworkers, reported for the first time P(3HB)-degrading microorganisms from *Bacillus*, *Pseudomonas* and *Streptomyces* species. From then on, several aerobic and anaerobic P(3HB)-degrading microorganisms have been isolated from soil (*Pseudomonas lemoigne*, *Comamonas* sp. *Acidovorax faecalis*), and anaerobic sludge

(*Alcaligenes faecalis, Pseudomonas, Illyobacter delafieldi*), seawater and lakewater (*Comamonas testosterone, Pseudomonas stutzeri*) (55). It has been estimated that between 0.5–9.6% of total microorganisms are P(3HB) degraters (56).

2.1.2.4 PHA's biosynthesis

PHA biosynthesis has been extensively studied in many bacteria, and it depends on three main factors: the carbon source provided, metabolic pathways in producing bacteria and the bacterial PHA synthase (57). The simplest short chain length PHA, involves three enzymatic reactions; first, condensation of two molecules of acetyl CoA, mainly from the tricarboxylic acid (TCA) cycle, into acetoacetyl-CoA by the β -ketothiolase (*phaA*). Then, acetoacetyl-CoA is reduced to 3-hydroxybutyryl-CoA (3HB-CoA) thanks to acetoacetyl-CoA reductase (*phaB*). Finally, the PHA synthase (*phaC*) polymerizes the 3-hydroxybutyryl-CoA monomers to P(3HB), with the subsequent liberation of CoA (58,59) as shown in Figure 6.

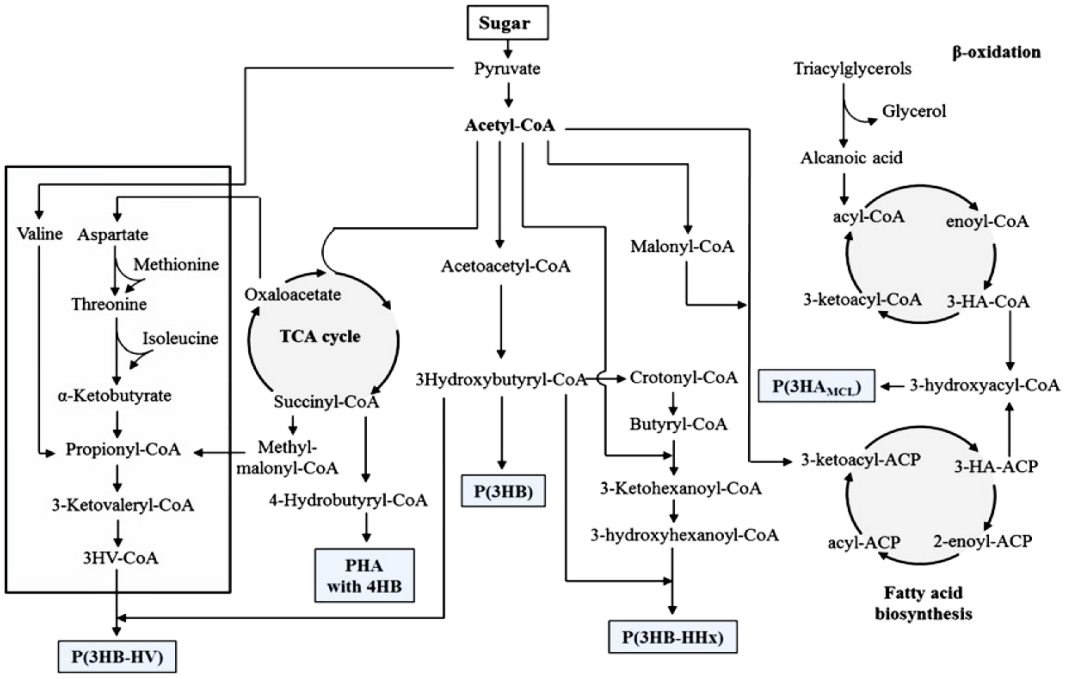

Figure 6 - Pathways involved in the biosynthesis of polyhydroxyalkanoates. Amino acid metabolic pathways, the tricarboxylic acids cycle, butyrate metabolism, fatty acid biosynthesis, and β-oxidation pathways are shown. Abbreviations: ACP acyl-carrier protein, 3HB 3-hydroxybutyric acid, 3HA 3-hydroxyalkanoic acid, HV hydroxyvaleric acid, 4HB 4-hydroxybutyric acid, HHx hydroxyhexanoic acid, MCL medium chain length. Source: (Pena et al., 2014)

Precursors for PHA's synthesis may be derived from fatty acid de novo biosynthesis when the microorganism is grown on unrelated carbon sources, such as glucose, gluconate or acetate and oxidation when the microorganism is grown on related carbon sources, such as fatty acids (60). In the first case, the resulting PHA composition depends on the carbon source, whereas in the second case, no relationship between the carbohydrates used as carbon sources and the resulting PHA composition exists (Figure 6).

The capability of incorporating different hydroxyacyl-CoA units will be dependent on the PHA synthase (*phaC*). Based on the constituent subunits, amino acid sequence and substrate specificity, PHA synthases can be divided into four classes. It is known that class I, III and IV polymerize short-chain-length (scl) monomers (C3–C5), whereas class II PHA synthase utilizes medium-chain-length (mcl) monomers (C6–C14). Class I (e.g. in *Ralstonia eutropha*) and class II (e.g. in *Pseudomonas putida*) PHA synthases consist of one subunit (PhaC) with molecular masses between 61 kDa and 73 kDa (61). Whereas, class III (e.g. *Allochromatium vinosum*) and class IV (e.g. *Bacillus megaterium*) require two types of subunits for their activity, PhaC (40.3 kDa) and PhaE (20 or 40 kDa), PhaC (41.5 kDa) and PhaR (22 kDa), respectively.

2.1.3 P(3HB) production using xylose and related hemicellulosic sugars

Several factors influence bioplastic production economics. The most significant factor is the cost of the carbon source, which can account for up to 50% of the overall production cost (62). Plant biomass available worldwide is around 200 trillion kg with only 3% of that total currently utilized (52). The hemicellulose fraction of that biomass consists of various monosaccharide components including pentose sugars such as D-xylose and *L*-arabinose and hexose sugars such as D-glucose, D-galactose and D-mannose (63). Researchers have speculated that costs for hemicellulose hydrolysate would be as low as 5 cents per kilogram (4), or up to 14 cents per kilogram (64).

In order to utilize sugars from hemicellulose, they must be extracted from the biomass first. Currently, there are several extraction methods, such as acid and/or solvent, enzymatic, or physical extraction. Acid extraction consists in the use acid at low concentrations (2-6%) of H2SO4 or HCL with high temperatures of 170 °C, and pressure of 10 atm (65). A greener extraction process is through steam explosion. Under this process high temperatures and

pressures are applied with a sudden pressure drop, resulting in disruption of the lignocellulosic structure (66). Enzymes are used also to improve amounts of sugar available in the hydrolysate. For example, xylanases, which hydrolyze glycosidic bonds in xylan and xylose oligomers, is used even after chemical or thermal procedures to increase hydrolysis efficiency (67).

As mentioned before, xylose is the second most abundant carbohydrate in nature, surpassed in quantity only by the glucose present in the cellulose. The main natural source of xylose is the lignocellulosic material of different agricultural residues. Hemicellulosic fraction composed of 70 - 80% of xylose (68) represents between 20 to 35% of dry weight of lignocellulosic material depending on plant species, growing conditions and localization (69). In Brazil, the amount of sugarcane bagasse estimated to be used only for hydrolysis process is around 7.0 × 106 tons per year (5) which means 35 × 103 tons of xylose available per year, mostly burned or discarded (7). The utilization of xylose for P(3HB) production was evaluated for the first time by Bertrand and coworkers in *Pseudomonas pseudoflava*, although, when concentrations of hydrolyzated above 30% were supplied, cellular inhibition was observed, maybe due inhibitory compounds (70).

Silva and coworkers, selected bacteria for P(3HB) production using sugarcane bagasse hydrolyzates, (glucose and xylose mixtures) they reached high cell concentrations (~ 60 g / L) and higher P(3HB) accumulation yields (~ 60% of cell dry weight) both in *Burkholderia cepacia* IPT048 and *Burkholderia sacchari* IPT101 (13). Lopes et al. (2009) isolated bacteria from different environments, and assessed their potential production of P(3HB) from xylose. The isolate with the best performance was a *Bacillus* sp. strain, which reached about 65% of P(3HB) accumulation, with a conversion factor of 0.25 g/g and a volumetric productivity of 0.06 g L^{-1}.h. These values were similar to those observed for *B. sacchari* IPT101 that produced P(3HB) from xylose with a conversion factor of 0.26 g/g and a volumetric productivity of 0.07 g L^{-1}.h (12). The higher xylose conversion factor from xylose to P(3HB) achieved so far is 0.25 g/g and of 0.28 g L^{-1}. h. the higher volumetric productivity (9,71) (Table 2).

Table 2 - Summary of P(3HB) producers strains using xylose as carbon.

Strain	μmáx (h^{-1})	DCW ($g\ L^{-1}$)	P(3HB) (%)	YP(3HB)/xyl ($g\ g^{-1}$)	Prod. vol ($g\ L^{-1}\ h$)	References
Burkholderia cepacia ATCC 17759	0.34	7.5	49	0.11	[a]	Young et al. (1994)
Burkholderia cepacia IPT 048	[a]	4.1	54	0.20	[a]	Silva et al. (2004)
Burkholderia sacchari IPT 101	[a]	5.5	58	0.26	0.07	Lopes et al. (2009)
Burkholderia sacchari LMF828 (mutant PTS glu−+)	0.35	5.3	50	0.17	0.07	Lopes et al. (2011)
Bacillus cereus CFR06	[a]	1.1	35	[a]	0.06	Halami (2008)
Bacillus sp. MA3.3 (*Bacillus megaterium*)	[a]	5.5	64	0.24		Lopes et al. (2009)
Escherichia coli TG1(pSYL107)	[a]	4.8	36	[a]	0.028	Lee (1998)
Pseudomonas pseudoflava ATCC 33668	0.13	4	22	0.04		Bertrand et al. (1990)
Isolated bacterium strain QN271	[a]	4.3	29	[a]	0.04	Doan and Nguyen (2012)

DCW: Dry cell wall. Prod vol: Volumetric productivity. YP(3HB)/xyl: Yield xylose to P3HB. PTS: Phosphotransferase.

2.1.4 Sugar cane bagasse

One of the most studied biomass residues in Brazil is the sugarcane bagasse. Every ton of sugarcane results in about 0.3 ton of bagasse, which is burned for electricity production to supply all energy needed in the mills. The prognosis for 2013/2014 season indicated that the total volume of sugarcane to be processed by the sugar-ethanol sector reached a total of 602 million tons, which represents an increase of 5.1% over the last season resulting in an addition of 31 million tons of processed sugarcane (72). As sugarcane production increases, bagasse production also increases at the same proportion. The estimative of bagasse availability for 2013/2014 season was 169 million tons only in the sugar-ethanol sector, with an addition of 39 million tons of bagasse from sugarcane used in other sectors (5).

Sugarcane bagasse is currently used in sugar mills as a heat source in boilers for steam generation, heating and plant operation (73). However, as mentioned before a high percentage can be used for other purposes, especially for production of high value added chemicals.

Second generation ethanol production is already being applied in several countries, but is only considered the cellulosic fraction for fermentation, because, efficient xylose fermenting yeasts are still in development (25).

The main composition of the lignocellulosic residues is cellulose, hemicellulose and lignin (74), which in case of bagasse sugarcane, after hydrolysis process catalyzed by acids or cellulolytic enzymes, result in simple carbohydrates around of 75.7 g L^{-1} of xylose, 13.5 g L^{-1} of arabinose, and 13.2 g L^{-1} of glucose, letting it available for bacteria transformation into bioproducts (75,76).

In Brazil, bioethanol industry was established in 1970, via extraction sugars and its fermentation to obtain the final product by distillation (74); this solution, however, has been strongly criticized due to needed of large land extensions and the "food vs. fuel debate" which claims the use of different carbon sources for fuel and no for food (77), within this context, second-generation biofuels, which use lignocellulosic residues as substrate to produce different bioproducts, represent an alternative to traditional carbon sources used so far. The first significant step, towards oil replacement for a bio renewable source, was given with the "Proálcool" program, created by the Brazilian government to promote several initiatives to make ethanol broadly accepted and economically viable (78). Mills, before essentially sugar producers, became major agrobusiness enterprises by adding distilleries to produce ethanol on a large scale (79). Nowadays, those mills, are facing a new challenge, adopt the Biorefinery concept in order to use biomass efficiently and produce different compounds in a sustainable manner.

2.1.5 Burkholderia sacchari

B. sacchari was isolated from a sugarcane plantation soil in Brazil (8,10) . Cells are Gram negative, oxidase and catalase positive, rod shaped and motile presenting polar flagella. Optimum growth temperature is 30 °C. A transmission electronic microscope (TEM) image of *B. sacchari* cells with PHA granules is presented in Figure 7.

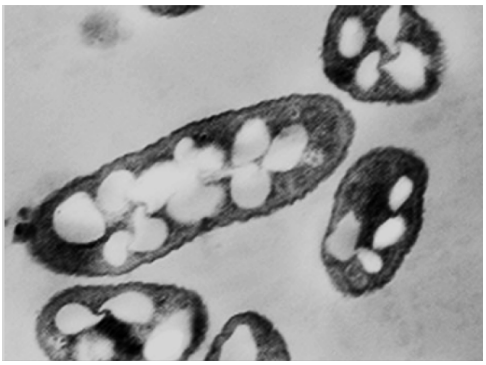

Figure 7 - TEM image of *B. sacchari* containing 70% dry weight of P(3HB). Source: (Birgit, 2015)

When cultivated on excess of sucrose, glucose, or xylose, and under limitation of an essential nutrient such as nitrogen, phosphorous, oxygen, etc, *B. sacchari* accumulates the homopolymer P(3HB), and if a co-substrate is offered bacteria accumulates a copolymer of poly-3-hydroxybutyrate-co-3-hydroxyvalerate (P(3HB)-co-3HV) (81). *B. sacchari* was improved to incorporate 3HV units from propionate, as well as its ability to use xylose and hydrolyzate bagasse sugarcane (82). A schematic representation of glucose and xylose metabolism for PHA production in *B. sacchari* is presented in Figure 8.

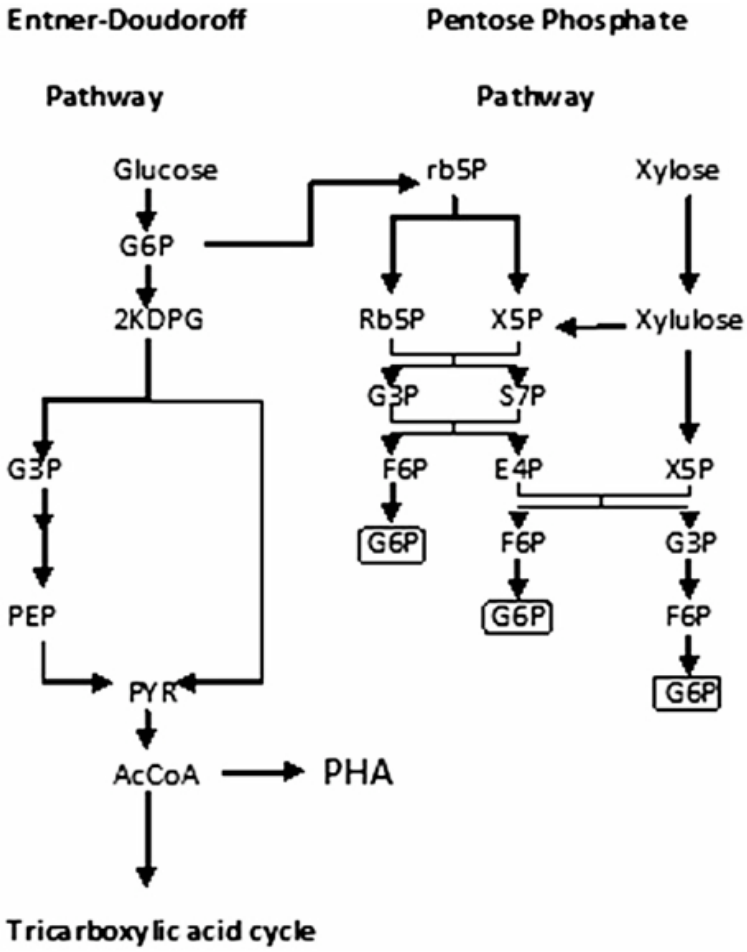

Figure 8 - Representation of the sugar to PHA metabolism in *B. sacchari*. Adapted from (83) (G6P glucose 6-phosphate, 2KDPG 2-keto-3-deoxy-6- phosphogluconate, G3P D-glyceraldehyde 3-phosphate, PEP phosphoenolpyruvate, PYR pyruvate, rb5P ribulose-5-phosphate, Rb5P ribose-5-phosphate, X5P xylulose 5-phosphate, S7P sedoheptulose 7-phosphate, F6P fructose 6-phosphate, E4P erythrose 4-phosphate, AcCoA acetyl-coenzyme A). Source (Kamm, 2014)

As shown in Figure 8, *B. sacchari*, is one of the few strains able to metabolize pentoses from hemicellulose-rich lignocellulosic hydrolyzates and produce PHA's (8,10,13). This strain has been shown to accumulate about 60% of P(3HB) from lignocellulosic hydrolyzates (71) and synthesize P (3HB-co-3HHx) from glucose and hexanoic acid (14). *B. sacchari* was recently proposed as a cell factory because of its capability to produce different high value chemical compounds as xylitol and xylonic acid (9,14).

2.1.6 Xylose catabolism

The major metabolic pathways for xylose fermentation are similar in bacteria, yeast, and fungi, with the notable exceptions of significant differences in transport, regulation, cofactor requirements, and the products of pyruvate fermentation. After transport into the cell by different mechanisms depending on the strain, and considering xylose isomerase as the pathway, xylose must be converted to xylulose and then phosphorylated to xylulose-5-phosphate before entering the pentose phosphate pathway (PPP). Within the PPP cycle, xylulose- 5-phosphate is metabolized to glyceraldehyde-3-phosphate and fructose-6-phosphate, then these compounds are converted to pyruvate in the EMP pathway (84).

It has been proposed that sugar transport across the cell membrane, and the regulation of the various sugar transport systems, play a key role in determining how productively microorganisms ferment xylose. The general importance of sugar transport is evidenced by the fact that transport and phosphorylation of glucose have previously been proposed as the rate-limiting steps in glycolysis (85,86). It is, therefore, likely that sugar transport may also limit the rate of bioproducts from xylose in xylose-fermenting yeasts and bacteria, althouh, other researches have described xylose isomerase as responsable for the defficient xylose uptake.

The characteristics of *D*-xylose transport systems in different microorganisms has been extensively reviewed (87,88). Microorganisms can utilize facilitated diffusion and/or active (energy requiring) mechanisms for sugar uptake. Active transport systems, generally high specific, in contrast to facilitated diffusion systems (more promiscous), require metabolic energy and can uptake sugars against a concentration gradient. Xylose can be transported by a chemiosmotic system symport of protons, low affinity (XylE or XylT) (89), and other high-affinity transport system, XylFGH (90), in which XylF represents a periplasmic protein that binds with high affinity to xylose, XylH is a permease and XylG an ATPase that provides the energy for xylose translocation process. In Figure 9 Xylose uptake in *E. coli* through both transport systems is ilustrated. XylE is a *D*-xylose proton symporter which uses the proton gradient as a source of energy. It possesses a relatively low affinity with high Km values between 63 and 169 μM. XylFGH, the other *D*-xylose transport system, belongs to the ATP binding cassette (“ABC”) family of transporters and requires one ATP per D-xylose

transported. XylFGH exhibits a high affinity with an apparent Km between 0.2 and 4 μM (91).

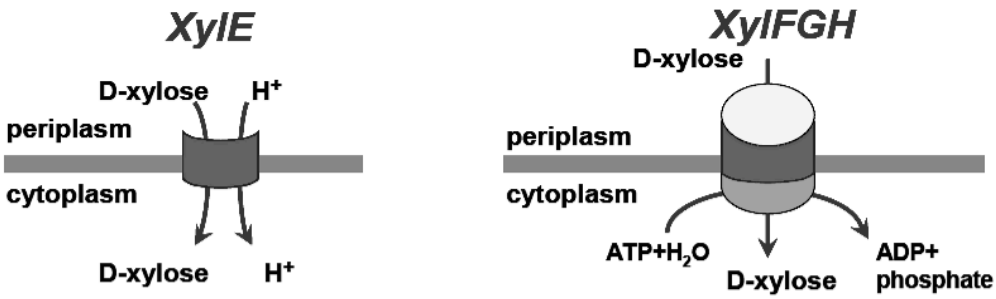

Figure 9 - Xylose transport systems in *E. coli* . XylE is a D-xylose proton symporter wich uses the proton gradient as a source of energy. XylFGH belongs to the ATP binding cassette "ABC" family of trasnporters and requires one ATP per D-xylose transported. Source: (Sumiya et al., 1995)

The metabolic pathways for xylose catabolism described in literature are: via oxidoreductase via isomerase, and via Weimberg Via Dahms as shown in Figure 10.

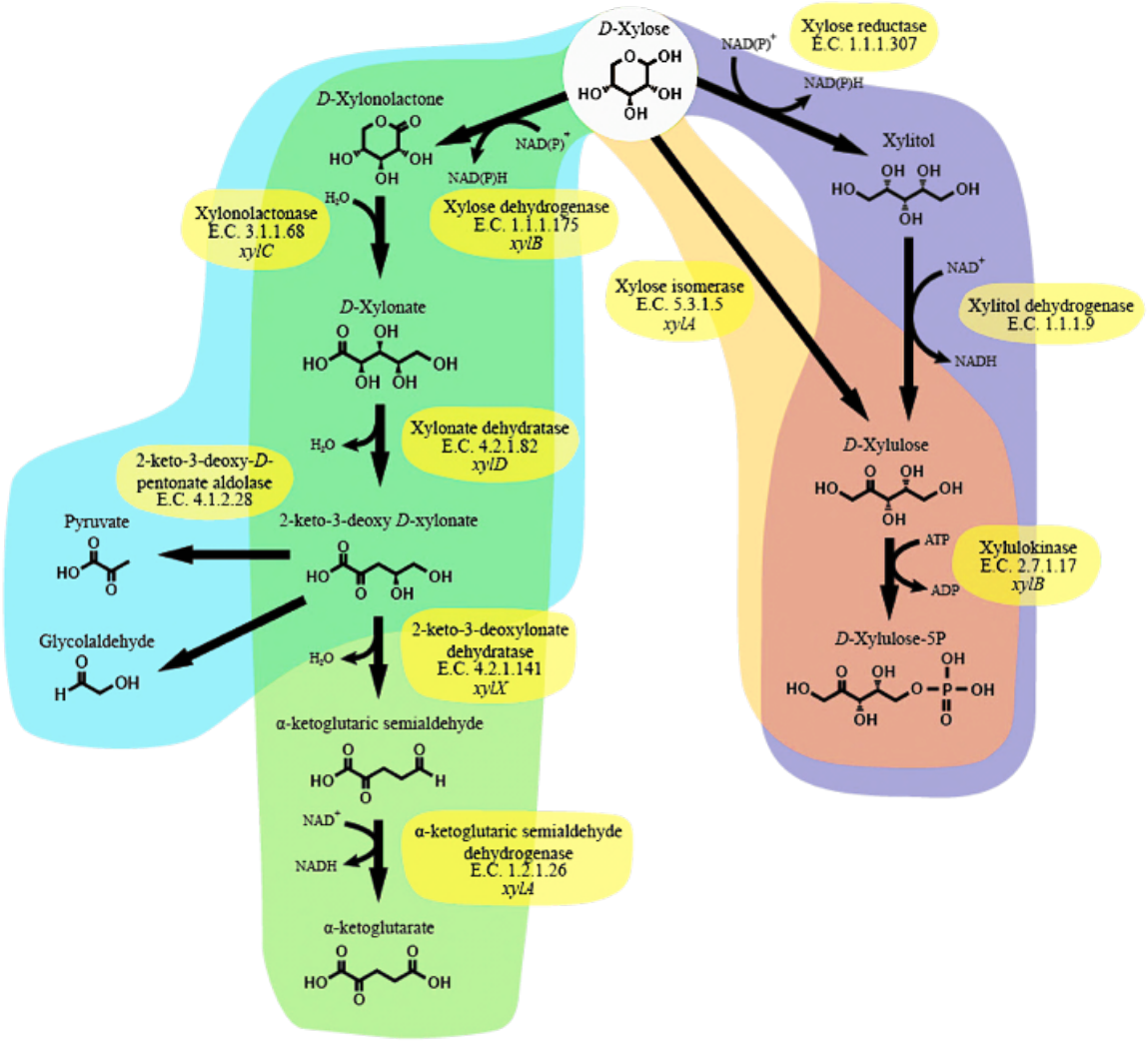

Figure 10 - Xylose metabolic pathways in bacteria. Source: (Roncallo, 2016)

Song and Park characterized the functions and regulation of xylose operon in *E. coli* , which utilizes xylose through xylose isomerase pathway, they concluded that transcriptional regulation of the *XylA* (xylose isomerase), *XylB* (xylulokinase) and *XylFGH* transporter is mediated by *XylR* (transcriptional regulator), and the cylic-AMP receptor protein CRP In *E. coli* , as well as in most micro-organisms xylose is metabolized by the PPP (Figure 10). Regardless of the pathway, xylose uptake and activation (phosphorylation) require energy derived from the hydrolysis of 2 ATP molecules. In contrast, glucose transport uses a single ATP equivalent (PEP) for both transport and activation (96).

Intracellular xylulose-5-phosphate is metabolized by the pentose-phosphate pathway. Through a series of reactions catalyzed by transketolase and 10 transaldolase, xylose is

converted into intermediates of glycolysis (fructose-6-phosphate and glyceraldehyde-3-phosphate). For every 6 xyloses consumed (30 carbon atoms), 4 fructose-6-phosphates and 2 glyceraldehyde-3-phosphates are produced. These molecules are further metabolized by glycolysis to ultimately yield 10 molecules of pyruvate. Thus, all 30 carbon atoms which began in xylose are converted into pyruvate (96).

Since xylose is a pentose and requires separate energy for transport and activation, growth on xylose results in a relatively low ATP yield. The transport and activation of 6 xylose molecules (30 carbons) requires 12 ATPs, assuming 1 ATP is required for transport regardless of the pathway. In the conversion of this xylulose-5-phosphate to 10 molecules of glyceraldehyde-3-phosphate, an additional 4 ATPs are consumed. The conversion of these 10 molecules of glyceraldehyde-3-phosphate to pyruvate yields 20 ATPs (83). The net gain of energy in the conversion of 6 molecules of xylose to 10 pyruvate is 4 ATPs. An equal amount of glucose on the basis of moles of carbon (5 molecules; 30 carbon atoms) produces a net of 10 ATPs during conversion to 10 molecules of pyruvate. The net energy gain for glucose catabolism is 1 ATP per pyruvate, 2.5-fold more ATP than from xylose catabolism (96)

2.1.7 Carbon catabolic repression (CCR) in bacteria

By definition CCR is the preference of a primary carbon source (generally glucose), over a less preferred sugar (99). Most bacteria use various compounds as sources of carbon. These carbon sources can either be co-metabolized or the bacteria can preferentially use the carbon sources that are most easily accesible, allow fastest growth and provide more energy (ATP). In 1942, Monod observed this phenomena with the glucose–lactose diauxie *E. coli* (98). Subsequent research with bacteria and higher organisms has revealed that selective carbon-source utilization is common, and that glucose is the preferred carbon source in many of the model organisms that have been studied.

CCR is one of the most important regulatory systems in bacteria, around 5–10% of all bacterial genes are subject to CCR (100). Succes over other competitors can be determined by CCR, since this mechanism allows cell invest energy in the preferred carbon source which will determine a faster growth rate, and more energy provided (101).

When hydrolysates or mixtures of sugars are used as carbon source, bacteria activate the mechanism of catabolic repression. A highly sophisticated signal transduction networks receive and incorporate information on the physiological state of the cell and the availability of carbon sources, finely regulating the expression of catabolic enzymes (99). In Gram-negative bacteria, the phosphotransferase system (PTS) uses phosphoenolpyruvate as a donor of phosphoryl groups for concomitant transport and phosphorylation of sugars into the cell (Figure 11). A phosphorylation relay proceeds sequentially from phosphoenolpyruvate (PEP) as a phosphoryl donor for Enzyme I (encoded by ptsI), HPr, Enzyme IIA, Enzyme IIB and finally, the incoming sugar is transported through the membrane via the integral membrane protein Enzyme IIC (encoded by ptsG) (97).

When glucose is absent, phosphorylated IIA enzyme stimulates adenylate cyclase for cyclic AMP synthesis (cAMP). A complex consisting of cAMP and cAMP receptor protein (CRP or CAP), called CRP-cAMP, stimulates the transcription of cAMP-dependent genes related to the consumption of non-PTS sugars. In contrast, during glucose transport, dephosphorylated EIIG1 and no longer stimulates adenylate cyclase (99).

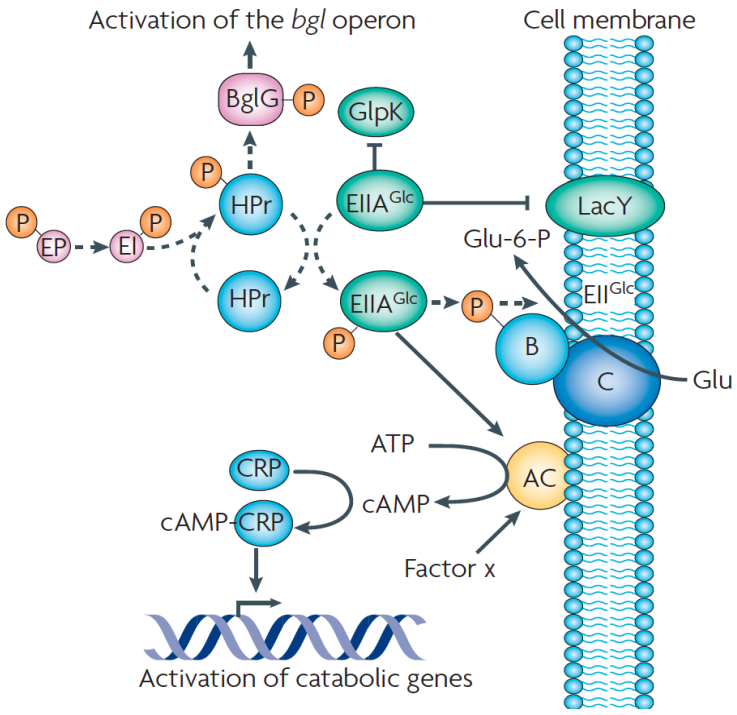

Figure 11 - Carbon catabolite repression mechanism in *E. coli*. Source: (Gorke and Stulke, 2008). The EIIA domain of the glucosemtransporter (EIIAGlc) is the central processing unit in CCR in E. coli. When phosphorylated, EIIAGlc binds and activatesmadenylate cyclase (AC), which leads to cyclic AMP (cAMP) synthesis. An unknown 'factor x' is also required for the activation of AC. High cAMP concentrations trigger the formation of cAMP–CRP complexes, which bind and activate the promoters of catabolic genes.

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3 CHAPTER 2: Improving growth rate and poly-3-hydroxybutyrate P(3HB) production from xylose by *Burkholderia sacchari*, through *xylA* and *xylB* overexpression

3.2.1 Introduction

Polyhydroxyalkanoates (PHA's) are intracellular polyester granules, accumulated as carbon and energy storage materials (1), synthesized by many bacterial strains under unbalanced growth conditions such as the presence of excess carbon source and limitation of at least one essential nutrient. Its biodegradability, biocompatibility and similar physical properties to synthetic polymers make them an environmentally friendly alternative to petrochemical based plastics (2).

Since the first description of PHA's in 1926 by Lemoigne (3), several advances towards large scale PHA production have been achieved. However, high production costs make PHA's more expensive than conventional plastics. One of the main factors for this phenomenon is the high cost of carbon sources, which can account for up to 29% of overall production cost, even when production is combined with onsite sugarcane milling (4). Several attempts, including use of inexpensive hemicellulosic biomass have been conducted in recent years (5,6). In Brazil, sugarcane production has doubled over the last decade, corresponding to increased bagasse availability by around 208 million tonnes. (7,8). It is estimated that around 30% (by dry weight basis) of the harvested sugarcane is comprised of hemicellulosic byproducts (9) composed of 70-80% of xylose (10) of which at least 50% could be recovered after hydrolysis, then, around 10^6 tons of xylose may be available each season (5), which currently is mostly burned to generate energy in thermoelectrics or discarded (11).

Burkholderia sacchari LFM 101 (12,13) is able to accumulate up to 75% of its cell dry weight (CDW) as PHA (14), utilizing a variety of carbon sources including xylose, and even hemicellulosic hydrolysates from sugarcane bagasse (15–17) to produce not only PHAs but also other high-value chemicals like xylitol and xylonic acid (13,18). Despite its capability to use xylose as carbon source, the slow growth rate (0.16 h^{-1}) under xylose utilization represents a barrier to use this bacterium as a chassis to produce PHA's and/or other high value chemicals on industrial scale. Therefore, it is imperative to understand the dynamics of xylose catabolism in *B. sacchari* in order to overcome metabolic roadblocks and exploit its potential for renewable bioproduction.

In this work, the organization of the genes responsible for xylose assimilation in *B. sacchari* is described. We also develop a strain overexpressing XylA and XylB, that demonstrated improved growth on and utilization of xylose as a sole carbon source for production of poly-3-hydroxybutyrate P(3HB) with the highest yield reported to date and reaching around 95% of theoretical yield.

3.2.2 Materials and Methods

3.2.2.1 Strains and Plasmids

B. sacchari LFM101, recently reclassified as *Paraburkholderia sacchari* (19), hereafter LFM101 (12) was used in this study as a final host for plasmids. *B. sacchari* LFM1402 strain was created transforming pBBR1MCS-2 xylAB into LFM101. *E. coli* DH10B (F– endA1 deoR+ recA1 galE15 galK16 nupG rpsL Δ(lac)X74 φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) StrR λ–), was used for plasmid construction and propagation. The plasmid pBBR1MCS-2 (20) was used as a cloning and expressing vector.

3.2.2.2 DNA manipulations

Plasmid DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, and DNA ligation were performed by standard procedures (21). Genes *xylA* and *xylB* were amplified from LMF101 genome using the primers xylA_F1 (ATAGCACCGCGGCTTCCCAGGTAGCGGGAAGC) and xylB_R1 (GTGACTCTCGAGCGCGCTACCAGGTAACGCGCC). The fragment was amplified using Phusion® High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, Massachusetts, USA), in a Mastercycler nexus gradient thermal cycler (Eppendorf AG, Hamburg, Germany). Cycling conditions were as follows: 98 ºC 1', 30x (98 ºC 30", 63 ºC 30", 72 ºC, 2') 72 ºC 5').

The purified 2.94-kb PCR product was digested and ligated into *Sac*I and *Xho*I sites of pBBR1MCS-2 to create pBBR1MCS-2-*xylAB*. Colonies were screened using blue/white selection and digestion with SacI and XhoI to confirm correct construction of pBBR1MCS-2-*xylAB*. Finally, plasmids from 3 colonies with expected sizes were isolated and sequenced using M13 primers (M13fwd, GTAAAACGACGGCCAGT, M13rev GAGCGGATAACAATTTCACACAGG). Electrocompetent cells of *B. sacchari* LFM101 were prepared according to previously established protocols. Briefly, 1 mL of mid-exponential PHA'se cell culture was inoculated into 50 mL of LB (250 mL flask) and

incubated at 30 ºC, 170 rpm for 6 h until cells reached OD_{600}= 0.6. The flask was incubated for 15 min on ice, cells were collected by centrifugation (15 min, 4500 rpm), washed twice with 50 mL of sterile nanopure water and once in sterile 10% glycerol. Finally, cell pellet was resuspended and aliquoted (50 µL each) in sterile 20% glycerol. Plasmids were transformed by electroporation using Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Inc. Hercules, California, USA). 100 ng of DNA was added to 50 µL of electrocompetent cells and transferred to a 0.2 cm electroporation cuvette. Electroporation parameters were: 25 µF, 200 Ohm, 2500 V.

3.2.2.3 Culture Media and Growth Conditions

Luria-Bertani (LB) (NaCl, 5 $g.L^{-1}$; tryptone, 10 $g.L^{-1}$; and yeast extract, 5 $g.L^{-1}$) was used to construct and propagate plasmids in *E. coli* DH10ẟ. When necessary, kanamycin (50 µg.mL−1), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, 40 µg/mL), and Isopropyl β-D-1-thiogalactopyranoside (IPTG, 200 µM) were added to the medium. For growth curve and P3HB production assays, 10 mL of cells from an LB culture in mid-exponential PHA'se were inoculated into 100 mL of mineral salts medium (MM) (22). Xylose was autoclaved separately and aseptically added to the medium; the final concentration used is indicated in each case. For bioreactor assays, LMF101 and LMF1402 cells were pre-incubated 24 h at 30 °C and agitation of 150 rpm in 1 L flasks with 200 mL MM (22) containing (NH4)2SO4 3 $g.L^{-1}$ and xylose 6 $g.L^{-1}$. A set of fed-batch experiments was performed in two identical bioreactors (Applikon Biotechnology Inc. Delft, Netherlands) using a working volume of 2 L at 30 °C for 60 h. The pH was set at 7.0 and controlled by automatic addition of NaOH (1M) or H2SO4 (1M). Dissolved oxygen (DO) was maintained above 40% of saturation by varying the agitation speed. Composition of MM was adapted (as follows) to limit nitrogen availability (in $g.L^{-1}$): KH2PO4 (0,39); (NH4)2SO4 (2,91); MgSO4·7H2O (0.31); CaCl2·2H2O (0.010); $(NH_4)_5Fe(C_6H_4O_7)_2$ (0.06); NaCl (1); Xylose (40); trace elements solution (2 $mL.L^{-1}$), which was prepared with H3BO3 (0.30 $g.L^{-1}$); CoCl2·6H2O (0.20 $g.L^{-1}$); ZnSO4·7H2O (0.10 $g.L^{-1}$); MnCl2·4H2O (0.03 $g.L^{-1}$); NaMoO4·2H2O (0.03 $g.L^{-1}$); NiCl2·6H2O (0.02 $g.L^{-1}$); CuSO4·5H2O (0.01 $g.L^{-1}$). Cell dry weight (CDW), xylose and P3HB content were measured at the indicated times.

3.2.2.4 Gene annotation

B. sacchari xylose isomerase gene (*xylA*) sequence previously reported (23) was used to locate the *xyl* operon in the LFM101 genome. To annotate genes and to determine xylose

operon structure, promoters, and terminators, a combination of Artemis Comparison Tool (24), Fgenes B and BLAST (25) was used. In addition, synteny analysis across *Burkholderia* species was performed using Absynte (26)

3.2.2.5 Xylose isomerase activity measurements.

Xylose isomerase activity (27) was measured in a buffer containing 50 mM Tris-HCl buffer (pH 7.5), 0.15 mM NADH, 1mM TEA, 10 mM $MgCl_2$, 10 U·mL and 50 mM of xylose. Cells in early exponential growth PHA'se grown in MMX medium (18h, 30 ºC, 150 rpm), were collected by centrifugation, washed twice with cold washing buffer (10% glycerol, 40 mM pH8,0 Tris, 10 mM $MgCl_2$, 5 mM NaCl and 10 mM β-mercaptoethanol) and resuspended in 1 mL of the same buffer. Cells pellets were disrupted by sonication and 35 µL of each cell extract was used for the xylose isomerase activity assay. Total cell protein concentration of extracts was determined by Bradford assay (28). Absorbance at 340 nm for each strain was measured in biological triplicates in 96-well format, using Biotek Synergy H1 (BioTek, Winooski, Vermont, United States) at 25 °C and expressed as average ± SD.

3.2.2.6 Analytical methods

3.2.2.6.1 Dry biomass concentration

10 mL of culture was harvested by centrifugation at 10,600 *g* and lyophilized in microtubes. Dry biomass was weighed using an analytical balance (Ohaus Adventurer, Parsippany, New Jersey, USA) and expressed in grams per liter as cell dry weight (CDW).

3.2.2.6.2 Xylose determination

Xylose concentration was determined by high performance liquid chromatography (HPLC). 10 µl of each sample was injected into a Dionex HPLC (Ultimate 3000, Thermo Fisher Scientific Inc, Waltham, MA, USA) equipped with a sugar separation column (Aminex-HPX-87H). For detection, a differential refractometer (Shodex IR-101) was used. Separation occurred at 45 °C with H_2SO_4 solution (5 mM) at a flow rate of 0.6 $mL.min^{-1}$. The standard curve was constructed using xylose solutions containing up to 3 $g.L^{-1}$.

3.2.2.6.3 Polyhydroxybutyrate P(3HB) content and composition

12 mg of freeze-dried cells P(3HB) were subjected to propanolysis (29). P3HB content was determined as described previously by Gomez, 1996 (14) with an Agilent 7890A GC System (Agilent Technologies, Santa Clara, California, USA) equipped with a HP5 capillary column after sample split (1:25). Helium (0.8 $mL·min^{-1}$) was used as carrier gas . Injector and

FID temperature were 250 ºC and 300 ºC, respectively. The oven was programmed to maintain temperature at 100 ºC for 1 min, then increase temperature at a rate of 8 ºC min^{-1} up to 210 ºC, which was maintained for 15 min. Benzoic acid was used as the internal standard (Sigma-Aldrich, Saint Louis, Missouri, USA).

3.2.3 Results and Discussion

3.2.3.1 Xylose metabolism gene organization

Although the ability to use xylose and other pentoses as carbon sources has been described for different bacteria, including some *E. coli* and *Burkholderia* strains, the metabolic pathway responsible for xylose utilization in *B. sacchari* LFM101 is poorly understood. In *E. coli ,* xylose isomerase pathway enzymes are encoded by two catabolic genes (*xylA* and *xylB*), two transporters (ABC-type transporters encoded by *xylFGH* genes and symporter *xylE*), and a transcriptional regulator encoded by *xyl*R. Sequences of other *xyl* genes are available for different *Burkholderia* species (30), but for *B. sacchari* LFM101, only xylose isomerase (*xylA*) has been annotated (23) and the organization of *xyl* genes in *B. sacchari* LFM101 was predicted based on a comparison of known sequences of *xyl* genes with other *Burkholderia* and *Pseudomonas* species. According to the same study, in *B. sacchari, xylB,* encoding for xylulokinase, is distal to the *xyl* operon (>500 000 bp) and may be encoded on a different chromosome. To clarify the organization of *xyl* operon and further understand some of the particular features of xylose catabolism in this bacterium, we identified and annotated the sequences corresponding to *xylB, xylFGH, xylE* and *xylR* using Artemis Comparison Tool (24), FgenesB (31) and BLAST (25).

B. sacchari LFM101 catabolic, transport, and regulator genes identified in the present study (Figure 12), belong to the xylose isomerase pathway also present in other bacteria. In this pathway, xylose enters the cell either through a low-affinity symporter (XylE) or a high-affinity, ATP-driven (ABC) transport system (XylFGH). Once inside the cell, xylose isomerase (XylA) converts it to *D*-xylulose and afterwards a xylulokinase (XylB) converts it to *D*-xylulose 5-phosphate, an intermediate of the pentose phosphate pathway. Promoter analysis of the complete *xyl* locus (8542 bp) using BPROM (32) (Softberry, 2016) revealed the presence of two promoters located in opposite directions, in the intergenic region of xylR-A (Figure 12). No promoter was identified in the intergenic region located between *xylB* and *xylF* (254 bp) using the same algorithm. Surprisingly, the only promoter able to activate transcription of *xylF* is located inside *xylB* coding sequence, suggesting that, in *B. sacchari*

LFM101, *xylF* transcription can potentially be initiated from this internal promoter in a similar way to the one described for *xylFGH* operon of *Thermoanaerobacter ethanolicus* 39E (33).

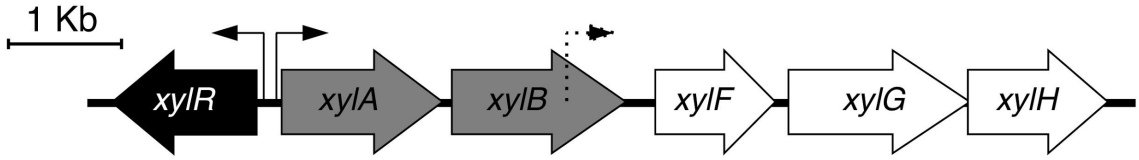

Figure 12 - Organization of *B. sacchari* LFM101 *xyl* locus. *xylAB* (gray) encoding xylose isomerase and xylulokinase respectively; *xylF* encodes a xylose-binding protein, *xylG* encodes an ATP-binding protein, and *xylH* encodes a membrane transporter ABC-type xylose transport (white). *xylR* (black), encodes a transcriptional regulator of the the AraC family of regulators. Arrows represent the promoters identified using BPROM algorithm, a bidirectional region with two promoters in the intergenic region of xylR-A (solid arrows) and a promoter identified inside the coding sequence of *xylB* (dashed arrow).

3.2.3.2 Construction of a B. sacchari strain overexpressing xylAB genes

The overexpression of genes involved in xylose catabolism, especially *xylA,* has been assessed in various microorganisms as a strategy to increase growth rate using xylose, but with variable success (34–37). Lopes and coworkers, reported that the overexpression of XylA did not improve either growth rate or biomass yield in *B. sacchari* (23). In a separate study, overexpression of both XylA and XylB from *Piromyces furiosus* improved the production of ethanol in *S. cerevisiae* by 8-fold. (34). Further, overexpression of *E. coli* XylA and XylB genes improved production of 1-3 propanediol by allowing simultaneous utilization of xylose and glycerol in *Klebsiella pneumoniae* (38). We therefore constructed a strain overexpressing the *B. sacchari* XylAB in attempt to improve the growth rate of *B. sacchari* on xylose and to potentially enhance the production of P(3HB) from xylose as a sole carbon source. Improved P(3HB) production from xylose is a key step towards the development of strains capable of producing bioplastics from renewable and inexpensive carbon sources.

Based on the currently organization of the xylose operon in *B. sacchari* LFM101, we constructed a strain overexpressing *B. sacchari xylAB* to test its effect not only on growth rate ($\mu_{max}.h^{-1}$), but also to potentially enhance production of P(3HB) using xylose as a sole carbon source, as a step forward for the development of strains capable of efficiently produce bioplastics from renewable carbon sources. To do so, a 2.94-Kb fragment including the native promoter located 81 bp upstream *xylA* CDS, the native RBS, and the coding sequences of both *xylA* and *xylB* were amplified by PCR with the primers xylA_F1 and xylB_R1. The product was digested and ligated into the broad host range cloning vector pBBR1MCS-2 (20). The confirmed pBBR1MCS2-*xylAB* plasmid was transformed in *B. sacchari* LFM101 to construct *B. sacchari* LFM1402. Due to the lack of knowledge regarding molecular biology in *B. sacchari* LFM101, native promoter and RBS sequences were maintained to guarantee transcription and translation of *xylAB.*

3.2.3.3 XylAB overexpression leads to a substantial increase in xylose isomerase activity.

Xylose isomerase is involved in the first step for assimilation of this pentose in bacteria. To evaluate the activity of this enzyme, *B. sacchari* LFM1402 and LFM101 transformed with pBBR1MCS-2 as a control (LFM 1403), were grown in MM with xylose, total protein was extracted, and xylose isomerase activities were measured using a coupled enzymatic assay modified for 96-well plate format. As shown in Table 3, after 18 h the strain LFM1402, transformed with pBBR1MCS2-*xylAB* plasmid showed a substantial improvement (63%) in xylose isomerase specific activity in comparison to both the wild type LMF101 (0.0282 μmol.min^{-1}.mg protein^{-1}) and the strain LFM1403 harboring pBBR1MCS-2 without *xylAB* (0.0304 μmol.min^{-1}.mg protein^{-1}). These data showed that, in *B. sacchari*, the multicopy effect resulted in overexpression of XylA in LFM1402 strain. Although the activity of XylB was not tested, XylB is expressed from the same operon, therefore allowing for similar XylB and XylA overexpression in the LFM1402 strain.

Table 3 - Xylose isomerase activities using a coupled enzymatic assay in *B. sacchari*

Strain	Xylose isomerase specific activity (μmol·min^{-1}·mg protein^{-1})
B. sacchari LFM101	0.0282 ± 0.006
B. sacchari pBBR1MCS-2	0.0304 ± 0.002
B. sacchari LFM1402	0.0845 ± 0.003

3.2.3.4 Overexpression of XylAB in LFM1402, significantly improved growth rate and P(3HB) production using xylose as a sole carbon source

Given the great potential of *B. sacchari* as a microbial biofactory (13,18) and its natural ability to accumulate P(3HB), we next sought to determine whether XylAB overexpression may improve the specific growth rate and production of P(3HB) in LFM1402 from xylose. We tested the production of P(3HB) in LFM101 (wild type) and LFM1402 (recombinant) strains by developing fed-batch assays (2 L working volume) to control multiple relevant parameters (pH, availability of oxygen, and carbon source), increase cell density, and evaluate the performance of the recombinant strain on a bigger scale. Two fed-batch experiments were performed in parallel to compare both strains. The results are summarized in Table 4.

Table 4 - Data from P(3HB) production and growth rate in minimal media supplemented with xylose in Fed Batch assay.

Strain	CDM $g\ L^{-1}$	P(3HB) %	P(3HB) $g\ L^{-1}$	Time h	μ_{max} h^{-1}	$Y_{P(3HB)/xyl}$ g/g
B. sacchari LFM101	12.78	58.07	8.400	54	0.154	0.26
B. sacchari LFM1402	16.89	67	11.294	54	0.203	0.35

Cell dry mass (CDM), polymer content of CDM (%P(3HB)), P(3HB) yield from xylose (YP(3HB)/xyl)

In both assays, cell growth continued until nitrogen was exhausted and polymers began to accumulate within the cells (at a CDW of approximately 11 $g\ L^{-1}$). All experiments were performed using mineral media (MM) with xylose as sole carbon source. Gas chromatography analysis showed the presence of only four carbon monomers, characterizing the P(3HB) homopolymer, as previously described for *B. sacchari* when xylose was provided as carbon source (17).

The accumulation PHA's initiated at 15h of cultivation, biomass improved by 32% from 12.78 to 16.89 g L-1 by the overexpression of xylAB (Figure 13), indicating significantly improved xylose catabolism and cell growth. The enhanced utilization of xylose most strikingly improved the production of P(3HB) to 11.29 g L-1, which represents a 34% improvement compared to the parental strain (8.4 g L-1). Xylose consumption was significantly increased, achieving the highest yield of xylose to P(3HB) reported to date (0.35 g/g), representing a 35% improvement. LFM1402 accumulated 67% P3HB of cell dry weight

using xylose. The specific growth rate reached a μmax of 0.20 h-1 and P(3HB) productivity increased 25,5% when compared to wild type (LFM 101).

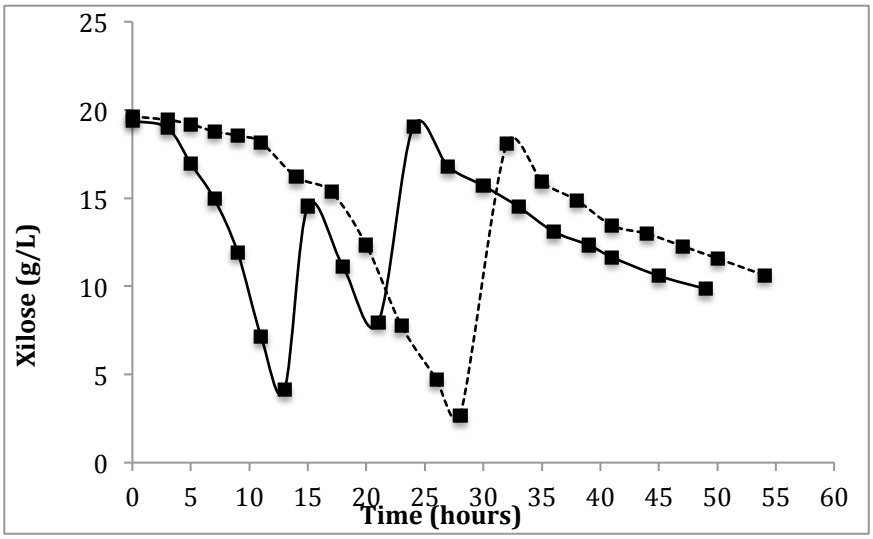

Figure 13A. Xylose feeding profiles of fed batch culture. *B. sacchari* LFM1402 (solid line), *B. sacchari* LFM101 (dotted line).

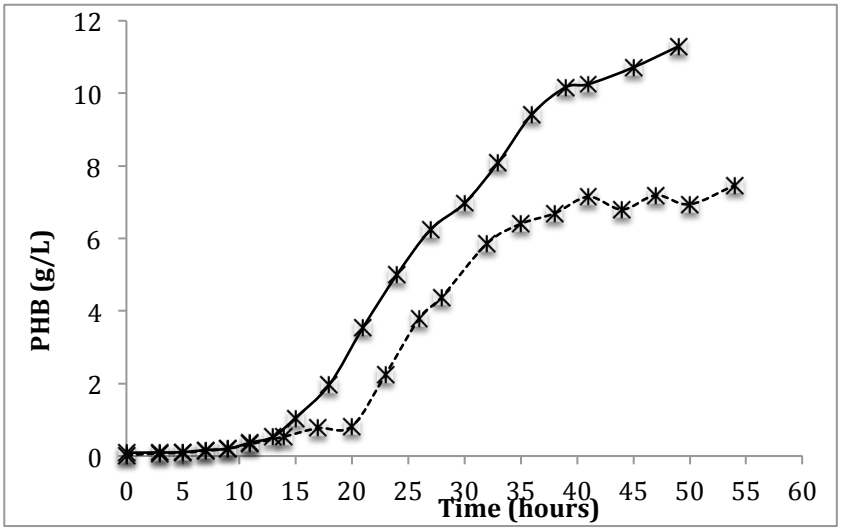

Figure 13B. Curves showing accumulation of P3(HB) during fed batch assay using xylose. B. sacchari LFM1402 (solid line), B. sacchari LFM101 (dotted line).

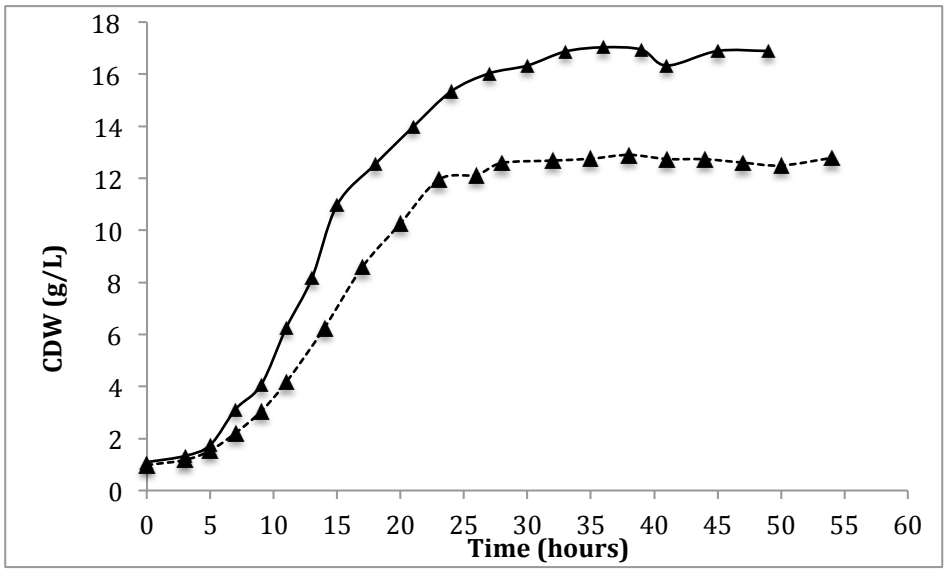

Figure 13C Cell dry weight (CDW) profiles. B. sacchari LFM1402 (solid line), B. sacchari LFM101 (dotted line).

In 2012, a metabolic flux análisis was conducted by Lopes and coworkers, considering that *B. sacchari* accumulates PHB without formation of residual biomass in a pseudo-stationary state using Metatool software (15). The analysis allowed to determine the possible intracelular flux balance using stoichiometric models: Pentose phosphate pathway (PPP), Entner-Doudoroff (ED), tricarboxylic acid pathway (TCA) and PHA production pathway.

Figure 14 shows two of the metabolic flux possibilities that reach the lowest and highest yields represented by flows 2 and 10.

One of the hypotheses based on elementary, suggests that in order to achieve yield improvements there must be a difference in the flux between Glucose-6-Phosphate (G6P) and 2-keto-desoxi-6-phosphogliconate (2KDPG) absent in flow 2 and present in flow 10. Figure 14. In the first situation there is a strong competition in the ED pathway for the Glucose-3-Phosphate (G3P) formed in the pentose phosphate patway (PPP). Thus, the flux of G3P is higher toward PEP of the ED pathway than in the G6P of the PPP. In the second situation, in flux 10, an increase in the lower part of the PPP is observed, stimulating G6P formation. Subsequently G6P is metabolized at the top of the ED pathway. In this flux distribution, higher production of NADPH produced during the conversion of G6P to PG6, which is a coenzyme necessary for the production of PHB, is verified. Thus, this model proposes that increased flow at the bottom of PPP may result in higher yields (0.4 g/g vs 0.25 g/g)). Thus, it is likely that the overexpression of the *xylAB* genes allows to increase the availability of NADPH with the consequent increase in yield, as demonstrated by Lopes.

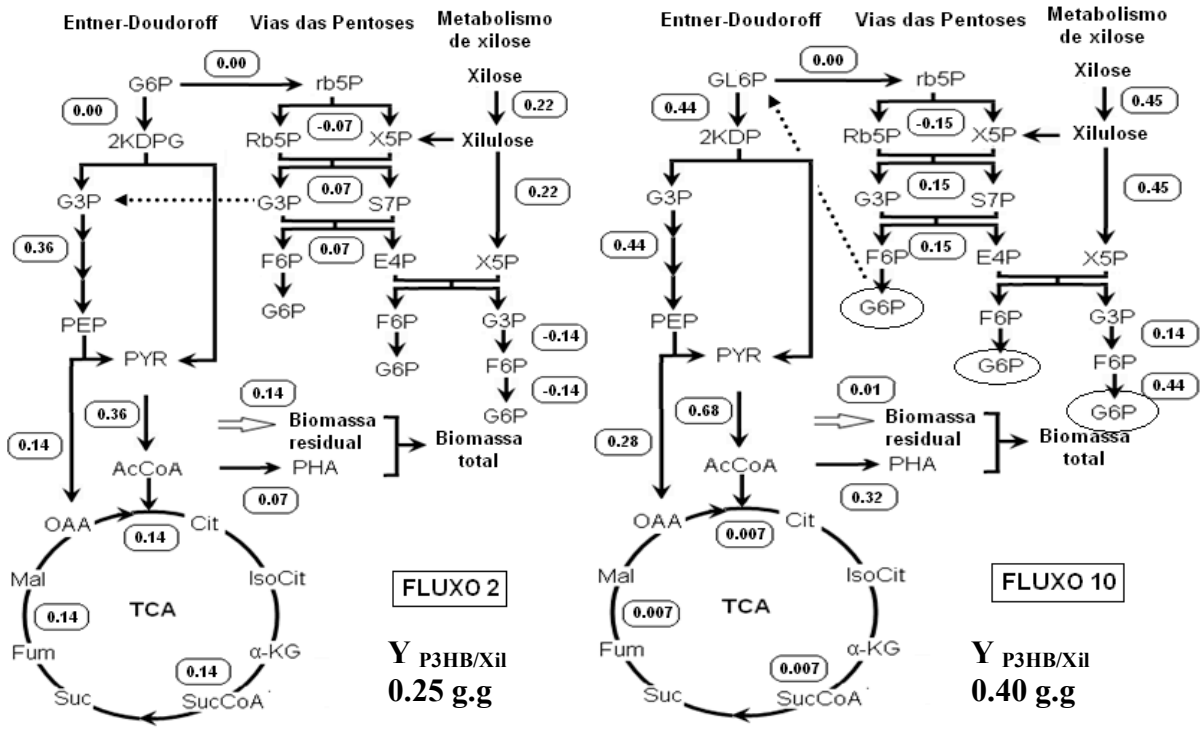

Figure 14. In silico flux analisis of xylose metabolism in *B. sacchari* using Metatool software. Arrows show reaction direction and negative values show reaction opposite.

Several groups have worked over a decade to develop strains suitable for efficient pentose fermentation. Most of this groups are focused on using metabolic engineering for the development of novel, efficient pentose-fermenting strains of *S. cerevisiae* with varying levels of success, (39–41). Conversely, bacteria have been much less studied as targets for pentose fermentation. Only a few bacterial strains have been demonstrated to catabolize pentoses and to accumulate PHAs. Within the *Burkholderia* genus, *B. cepacia* has been reported to accumulate P(3HB-*co*-3HV) copolymers using xylose and levulinic acid as carbon source (42). Pan and coworkers achieved a production around 52% of P(3HB) of CDW using the same strain; however, low productivity (0.09 g L^{-1} h) was reported due to the formation of inhibitory compounds after sugar maple hydrolysis to obtain xylose (43).

Other approaches using *B. sacchari* have been conducted in order to improve xylose and other hemicellulosic sugars uptake and to produce P(3HB) and other bioproducts. Lopes et al. conducted a search for new xylose-utilizing strains in various environmental niches, and also searched for strains able to grow with toxic byproducts of hemicellulose hydrolisis, etc. It has

been previously described a *B. sacchari* LFM828 mutant (PTS - glu +) growing at 0.35 h^{-1} in xylose (44), however this resultat data has never been possible to be reproduced in our laboratory after several attempts. (45,46).

Using wheat straw hydrolysates rich in xylose and glucose, Cesareo and coworkers studied growth and P(3HB) accumulation in *B. sacchari* LFM101, a 65% of P(3HB) was achieved with 0.22 g/g yield (47). In a recent work of the same group, the maximum specific growth rate for *B. sacchari* growing in MM using xylose as carbon source was 0.18 h^{-1}, although it is important to note this medium was supplemented with yeast extract 1 g L^{-1}. The authors were able to produce not only P(3HB) but also xylitol and xylonic acid (13) making *B. sacchari* even more interesting for industry because of its diverse metabolism and versatility to use a broad range of carbon sources as previously indicated. (18)

This research significantly improved the capacity of P(3HB) production, an advance which significantly enhances the cost effectiveness of P(3HB) production. Other researchers have demonstrated that the generation of improved reduction potential achieved through pentose catabolism may impact the overall biosynthetic capacity of strains that produce P(3HB) and other products. The optimization in P(3HB) accumulation (reaching 11.29 g L^{-1} with a yield of 0.35 g/g, without direct engineering of the P(3HB) pathway under engineered improved xylose utilization conditions suggests that the reduction potential (NADPH) in xylose catabolism contributed to the improvement in P(3HB) yield. This result indicates that beyond improving the cost of carbon source utilization, our system to improve xylose catabolism further serves to generally improve P(3HB) production via improved reduction potential. Additional efforts to improve reduction potential may therefore serve as key targets for the improvement of P(3HB) production in *B. sacchari.*

3.2.3.5 Concluding remarks

Further studies should consider genetic modifications downstream of the xylose utilization pathway genes or combine this with other strategies to improve P3HB accumulation. For example, in addition to engineering the xylose utilization pathway, a transaldolase (*talA*) which catalizes a conversion of sedo-7-heptulose (S7P) and glyceraldehyde-3-phosphate (G3P) in fructose-6-phosphate (F6P) and erythrose-4-

phosphate (E4P) can be overexpressed to improve NADPH needed for P3HB biosynthesis as described by Song and coworkers (48)

The results presented here are of great industrial interest since this work improved the maximum specific growth rate in xylose by 31% as well as P3HB titer, by overexpression of *xylA* and *xylB* in *B. sacchari*, achieving the highest yield from xylose to P3HB (0.35 g/g) reported to date with approximately 95% of the maximum theoretical yield (11).

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4 CHAPTER 3: Engineering Xylose Metabolism for production of Polyhydroxybutyrate in the non-model bacterium *Burkholderia sacchari.*

4.3.1 Introduction

Lignocellulose consisting of cellulose, hemicellulose, and lignin is the most abundant renewable resource worldwide. Utilization of xylose, the second most abundant sugar in lignocellulosic materials, is essential for the efficient conversion of biomass to high-value chemicals (1). A few bacteria are known to directly transform xylose to P(3HB), however, the rates and yields need to be improved to make production economically viable (1). Strategies to improve production parameters often includes overexpression of xylose transporters or catabolic proteins.

In the recent years, the application of synthetic biology principles (abstraction, modularity, and standardization) in *E. coli, S. cerevisiae* and other model organisms opened new possibilities for pathway engineering and optimization (2,3). The BglBrick vectors, one of the most widely used expression platforms in synthetic biology, has been used successfully to introduce synthetic pathways and engineer native ones to allow the production of biofuels, bioplastics precursors, pharmaceuticals and other high-value chemicals. (4,5) Despite the exciting progress in fields such as systems and synthetic biology or metabolic engineering, often the potential for scaling-up is limited by the use of expensive precursors or the conversion of non-renewable feedstocks.

B. sacchari, a Gram-negative bacterium isolated in Brazil (6), has an enormous potential for industrial scale production because among other interesting features, can produce high-value compounds such as xylonic acid, xylitol and P(3HB) using renewable carbon sources, like xylose and arabinose (7,8). Besides, is able to accumulate up to 80% cell dry weight as P(3HB) (3) and produce copolymers of polyhydroxyalkanoates (incorporating hydroxyalkanoate monomers other than 3HB) (9). Despite its great potential, the lack of proper molecular tools and the inherently slow growth rate and P(3HB) accumulation must be improved to allow the use of this bacterium for industrial scale production (10). In the present study, a set of BglBrick plasmids was successfully adapted to control protein expression in the non-model bacterium *B. sacchari.* Arabinose (pBAD promoter) and IPTG (lacUV5 promoter) inducible expression systems were functional showing a maximum induction of 2.5 and 65-fold. The constructed plasmids were used to individually overexpress all transporters

(*xylE* and *xylFGH*) and catabolic (*xylAB*) proteins involved in xylose utilization and evaluate its individual effects on growth rate and production of poly-3-hydroxybutyrate (P(3HB)). *xylAB* overexpression resulted in improvements of 46.8% in growth rate, P(3HB) production and yield respectively.

Finally, expression of appropriate levels of the transcriptional regulator (*xylR*) further enhance growth rate (67%), P(3HB) production (34%), and yield (80%). According to our knowledge, this paper represents the first approach of improving growth rate and PH3B production using of synthetic biology in *B. sacchari.* This work emphasizes the relevance of having tools allowing precise and tunable control of expression in non-model organisms.

4.3.2 Experimental Procedures

4.3.2.1 Chemicals and media

Unless otherwise specified, all chemicals were obtained from Sigma-Aldrich ® (Sigma-Aldrich, Saint Louis, Missouri, USA) . Luria-Bertani (LB) medium (10 $g.L^{-1}$ tryptone, 10 $g.L^{-1}$ NaCl, and 5 $g.L^{-1}$ yeast extract, pH 7.4) was used for cloning purposes and maintaining the plasmids. Minimal medium (MM) used for growth and P(3HB) accumulation assays was modified from (11) and contains in $g \cdot L^{-1}$: KH_2PO_4 (0,39); $(NH_4)_2SO_4$ (2,91); $MgSO_4 \cdot 7H_2O$ (0.31); $CaCl_2 \cdot 2H_2O$ (0.010); $(NH_4)_5Fe(C_6H_4O_7)_2$ (0.06); NaCl (1); trace elements solution (2 $mL \cdot L^{-1}$), which was prepared with H_3BO_3 (0.30 $g \cdot L^{-1}$); $CoCl_2 \cdot 6H_2O$ (0.20 $g \cdot L^{-1}$); $ZnSO_4 \cdot 7H_2O$ (0.10 $g \cdot L^{-1}$); $MnCl_2 \cdot 4H_2O$ (0.03 $g \cdot L^{-1}$); $NaMoO_4 \cdot 2H_2O$ (0.03 $g \cdot L^{-1}$); $NiCl_2 \cdot 6H_2O$ (0.02 $g \cdot L^{-1}$); $CuSO_4 \cdot 5H_2O$ (0.01 $g \cdot L^{-1}$).Use of xylose, glucose or arabinose as carbon sources for MM is indicated as MMX, MMG or MMA respectively. Sterile filtered solutions of arabinose or Isopropyl β-D-1-thiogalactopyranoside (IPTG) were used as inducers at the indicated concentrations.

4.3.2.2 Strains and growth conditions

E. coli DH10B (F– endA1 deoR+ recA1 galE15 galK16 nupG rpsL Δ(lac)X74 φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) StrR λ–) was used as a host for plasmid construction and propagation. *E. coli* cultures were grown in LB broth (200 rpm) or agar at 37 ºC. When needed, media was supplemented with kanamycin (kan, 50 μg/mL). *B. sacchari* LMF101 was used as the final host for pBuB8k-RFP and pBuB5k-RFP plasmids. It was grown at 30 ºC, 150 rpm in MM with $5g.L^{-1}$ for 24 h for growth assays, and $15g.L^{-1}$ for 48 h for P(3HB) accumulation.

4.3.2.3 Bioinformatic analysis and primer design

The genes in the present study, were selected using data from a recent analysis of *B. sacchari* genome from our group. Minimum Tm of 60 ºC and 18 bp hybridization to target was used as a standard for primer design. Primers were designed to amplify only the complete coding sequence (CDS, from ATG to STOP codon) of the genes of interest (GI) and add the desired restriction site for cloning in the compatible BglBrick plasmid (Table 5). Synthetic ribosome binding site (RBS) described elsewhere (4) was added in each forward primer to guarantee the efficient translation of the cloned sequences. When needed, a stop codon was added at the end of the corresponding CDS.

Table 5 - Primers sequences used in this study. RBS are shown in Italic. Restriction sites are underlined.

Name	Sequence	Restriction site
xylA_F	AAAAAAGAATTC*TTTAAGAAGGAGATATACAT*ATGGGAGCCGAGCGCAAA	*Eco*RI
xylB_R	AAAAAAGGATCCTCAGGCGCCGGATGCGAATA	*Bam*HI
xylF_F	AAAAAACATATGAAGTTCGCCAAACGCCGTTCG	*Nde*I
xylH_R	AAAAAACTCGAGTCAGCGGCGGTTCGACCCC	*Xho*I
xylE_F	AAAAAAAGATCT*TTTAAGAAGGAGATATACAT*ATGACCGCGCTGGAAAAGAAAG	*Bgl*II
xylE_R	AAAAAACTCGAGTCAGTGTGAAAACCCGGAGGCCG	*Xho*I
xylR_F	AAAAAAAGATCT*TTTAAGAAGGAGATATACAT*ATGACCCGCGCCACCGCTTCCC	*Bgl*II
xylR_R	AAAAAACTCGAGTCAGGAGTTTGATGTGCATGCGGCGCGC	*Xho*I
EcoRIBglII_pBuB8K_F	AAAAAAGAATTCAAAAGATCT*TTTAAGAAGGAGATATACAT*ATGGCGAG	*Eco*RI, *Bgl*II
Prom_Xba_pBuB8K_R	AAAAAATCTAGAGGAGAAACAGTAGAGAGTTGCGATAAAA	*Xba*I
XbaI_RBS_xylE_F	AAAAAATCTAGA*TTTAAGAAGGAGATATACAT*ATGACCGCGCTGGAAAAGAAAG	*Xba*I
xylESTOP_BglII_R	AAAAAAAGATCTTCAGTGTGAAAACCCGGAGGCCG	*Bgl*II
XbaI_RBS_xylA_F	AAAAAATCTAGA*TTTAAGAAGGAGATATACAT*ATGGGAGCCGAGCGCAAA	*Xba*I
xylBSTOP_EcoRI_R	AAAAAAGAATTCTCAGGCGCCGGATGCGAATA	*Eco*RI
XbaI_RBS_xylE_F	AAAAAATCTAGA*TTTAAGAAGGAGATATACAT*ATGACCGCGCTGGAAAAGAAAG	*Xba*I
xylESTOP_BglII_R	AAAAAAAGATCTTCAGTGTGAAAACCCGGAGGCCG	*Bgl*II
XbaI_RBS_xylA_F	AAAAAATCTAGA*TTTAAGAAGGAGATATACAT*ATGGGAGCCGAGCGCAAA	*Xba*I
xylBSTOP_EcoRI_R	AAAAAAGAATTCTCAGGCGCCGGATGCGAATA	*Eco*RI

4.3.2.4 Gene amplification

Genes were amplified from *B. sacchari* genome, using Q5® High-Fidelity DNA Polymerase (New England Biolabs, Inc, Ipswich, Massachusetts, United States) in a Mastercycler nexus gradient thermal cycler (Eppendorf AG, Hamburg, Germany) following the protocol recommended by the manufacturer. Cycling conditions were as follow: 98 ºC 2', 35X (98 ºC 20", 60 ºC 20", 72 ºC (25" per Kb)) 72 ºC 5'.

4.3.2.5 Plasmid construction

Plasmids used in this study are listed in Table 6. pBuB backbones were constructed amplifying pBBR1 ori from pBBR1MCS-2 (12) with the primers pBBR1_fullori_F and pBBR1_fullori_R. Purified PCR product was digested and ligated into pB8k-RFP and pB5k-RFP plasmids using *Bcu*I and *Avr*II restriction sites. For construction of pBuB plasmids with xylose metabolism and trasnport genes, PCR products amplified with the indicated primers (Table 5) were isolated from gel using GeneJET Gel Extraction and DNA Cleanup Micro Kit (Thermo Fisher Scientific Inc.) according to manufacturer's instructions and cloned into pBuB8k-RFP and/or pBuB5k-RFP using the corresponding restriction enzyme (Table 6).

4.3.2.6 Plasmid stability.

Plasmid stability in *B. sacchari* harboring plasmids recently constructed, was assessed as described by (10) Briefly, cultures of *B. sacchari,* harboring either pBuB8k-RFP and pBuB5K-RFP, were grown to the mid-logarithmic phase and used to inoculate LB broth tubes without kanamycin at an initial (OD600) of 0.02. At an OD_{600} of 0.32, aliquots were obtained (four generations approximately) and plated onto LB plates with and without kanamycin. A third aliquot was transferred to fresh LB broth and incubated until a set of 20 generations was completed. Plates were incubated at 30 °C for 24 h, and colonies were counted. To calculate the percent plasmid stability (in 20 generations) the number of colonies in the LB plates supplemented with kanamycin was divided by the number of colonies in the LB plates without antibiotic.

Table 6 - Plasmids used in this study.

Name	ORI	Promoter	Resistance	Gene	Plasmid source
pBbB8k-RFP	BBR1*	pBAD	Kan	RFP	(Lee et al., 2011)
pBbB5k-RFP	BBR1*	placUV5	Kan	RFP	(Lee et al., 2011)
pBBR1MCS-2	BBR1	plac	Kan	lacZ	(Kovach et al., 1994)
pBuB8k-RFP	BBR1	pBAD	Kan	RFP	Present study
pBuB5k-RFP	BBR1	placUV5	Kan	RFP	Present study
pBuB1k-GFP	BBR1	pTrc	Kan	GFP	Present study
pBuB6k-RFP	BBR1	pLlacO1	Kan	RFP	Present study
pBuB8k-xylE	BBR1	pBAD	Kan	xylE	Present study
pBuB8k-xylFGH	BBR1	pBAD	Kan	xylFGH	Present study
pBuB8k-xylAB	BBR1	pBAD	Kan	xylAB	Present study
pBuB8k-xylR	BBR1	pBAD	Kan	xylR	Present study
pBuB8k-xylE_RFP	BBR1	pBAD	Kan	xylE, RFP	Present study
pBuB8k-xylFGH_RFP	BBR1	pBAD	Kan	xylFGH, RFP	Present study
pBuB8k-xylAB_RFP	BBR1	pBAD	Kan	xylAB, RFP	Present study
pBuB5k-xylR	BBR1	placUV5	Kan	xylR	Present study

* Modified BBR1 as described in (Lee et al., 2011). Kan: Kanamycin.

4.3.2.7 B. sacchari transformation

Electrocompetent cells of *B. sacchari* were prepared according to previously established protocols. Briefly, 1 mL cells from a culture in mid-exponential phase was inoculated into 50 mL of LB (250 mL flask) and incubated at 30 ºC, 170 rpm until cells reached OD_{600} 0.6. Flask was incubated 15 min on ice, collected by centrifugation (15 min, 4500 rpm), washed twice with 50 mL of sterile water. Cell pellet was finally resuspended and aliquoted (50 µL each) in sterile 20% glycerol. Plasmids were transformed by electroporation using Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Inc. Hercules, California). 100 ng of DNA were added to 50 µL electrocompetent cells, transferred to a 0.2 cm electroporation cuvette. Electroporation parameters: 25 µF, 200 ohm, 2500 V.

4.3.2.8 Fluorescence and growth assays

Strains were inoculated in 120 µl of MMX and transferred to 96-well plate as pre inoculum. Plate was incubated 24 h at 30 ºC and 170 rpm. Absorbance at 600 nm was measured in the Synergy H1 96-well plate reader (BioTek® Instruments, Inc, Winooski, Vermont, United States) and adjusted to start with an OD_{600} 0.05 for each strain. 96-well plates were incubated at 30 ºC with continuous orbital agitation (548 cpm) in the Synergy H1

96-well plate reader. Growth (OD_{600}) and fluorescence (RFP, exc: 535, emi: 620, gain 80) were monitored during 24 h (15 min intervals). Both parameters (fluorescence and growth) were measured in triplicate and data is expressed in arbitrary units, adjusted with the growth in each case.

4.3.2.9 P(3HB) accumulation assay

MM (1g.L^{-1} Ammonium Sulphate; Xylose 15g.L^{-1}) was used to promote P(3HB) accumulation. 125 mL erlenmeyers containing 50 mL of MM were used. Initial optical density (OD_{600}) was adjusted to 0.1 for all cultures. For the production analysis, two samples (10 mL each) were taken one at the beginning of the experiment and one after 72 h of culture (30 °C, 200 rpm) . These samples were centrifuged (8,000 rpm, 4 °C, 10 min). The supernatant was frozen in a freezer at -20 °C for dosing sugars by liquid phase chromatography (HPLC), while the precipitate was frozen and lyophilized to measure dry cell mass (g / L), and to determine composition of PHA by gas phase chromatography of propyl esters (13)**.**

4.3.2.10 Analytical Methods

4.3.2.10.1 Biomass concentration

10 mL of culture were harvested by centrifugation at 10,600 *g* and lyophilized in microtubes. Dry biomass was weighed using analytical balance (Ohaus Adventurer, Parsippany, New Jersey, USA) and expressed in grams per liter as cell dry weight (CDW).

4.3.2.10.2 Xylose determination

Xylose concentration was determined by liquid chromatography (HPLC). 10 µl of each sample was injected into a Dionex HPLC (Ultimate 3000, Thermo Fisher Scientific Inc, Waltham, MA, USA) equipped with a sugar separation column (Aminex-HPX-87H). For detection, a differential refractometer (Shodex IR-101) was used. Separation occurred at 45 °C with H_2SO_4 solution (5 mM) at a flow rate of 0.6 mL·min $^{-1}$. The standard curve was constructed using xylose solutions containing up to 3 g·L^{-1}.

4.3.2.10.3 Poly-3-hydroxybutyate (P3HB) content and composition measurements

12 mg of freeze-dried cells were subjected to propanolysis. (13) P(3HB) content was determined as described previously by Gomez, 1996 (14) with an Agilent 7890A GC System (Agilent Technologies, Santa Clara, California, USA) equipped with a HP5 capillary column

after sample split (1:25). Helium (0.8 mL·min^{-1}) was used as carrier gas .Benzoic acid was used as the internal standard. (Sigma-Aldrich, Saint Louis, Missouri, USA).

4.3.3 Results and Discussion

4.3.3.1 Adapting Bglbrick vectors for using in Burkholderia sacchari

The most efficient and widely used prokaryotic host for recombinant protein production and metabolic engineering at industrial scale is *E. coli* (15). However, since 1990's *B. sacchari* and other Gram-negative bacteria have demonstrated enormous potential for industrial scale bioproduction. *B. sacchari* has been proposed as a cell factory due to its natural ability use renewable carbon sources and to efficiently produce a wide range of chemicals, including P(3HB) in high yield with xylose as a sole carbon source and other high value compounds such as xylitol, xylonic acid (7–9). Despite its remarkable potential, one of the major limitations for applying metabolic engineering or synthetic biology approaches in *B. sacchari* is the lack of appropriate molecular tools. Previously constructed broad-host-range plasmid vectors derived from IncP and IncW incompatibility groups have shown to be able to replicate in some species of *Burkholderia* (10). Nevertheless, most of research in *Burkholderia* species, is mainly focused on studying multiresistant clinical isolates, then, appropriate reporters systems, inducible promoters and other essential tools for metabolic engineering and synthetic biology are not available for *B. sacchari.*

Aiming to engineer *B. sacchari* as a chassis for bioproduction, one of the most widely described expression platforms available, the BglBrick vectors (4) was tested in *B. sacchari*. Since plasmids with pBBR1 replication origin have been successfully transformed into *B. sacchari* (16), first BglBrick plasmids containing the broad host range replication origin pBBR1 (pBbB plasmids in BglBrick nomenclature) were tested. Surprisingly, as opposed to the observed with the control plasmid pBBR1MCS-2 which produced around 2X10^3 colonies·µg^{-1}, transformation with different pBbB plasmids resulted in no colonies. Trying first to understand this phenomenon, and finally, to have a functional plasmid, sequences of both pBBR1 replication origins were analyzed.

pBBR1 plasmid originally isolated from *Bordetella bronchiseptica* encodes an essential replication protein (Rep) that shares sequence homology with replication proteins in Gram-negative bacteria (17). In addition, contains a series of sequences (direct repeats, putative IHF binding, and AT-rich regions) described as important for plasmid replication.

The sequence alignment and comparison revealed differences that may explain the observed phenomenon. In addition to one point mutation changing serine 100 to leucine in Rep protein, described to improve copy number in *E coli*, a region of 198 bp present in the original pBBR1MCS (12), is missing in the pBBR1 origin used in the BglBrick plasmid. Although there is no detailed description of the mechanism responsible of pBBR1 replication or the potential role of the missing region, the similarity in the arrangement of direct repeats, putative IHF binding site and AT-rich regions with other plasmids present in Gram-negative bacteria, suggest the possibility that direct repeats absent in pBBR1 from BgblBrick, are important for interaction with DnaA or other proteins essential for efficient replication of the plasmid in *B. sacchari.* Understanding in detail the mechanism responsible for this difference goes beyond the scope of this work, however supporting the proposed hypothesis, evidence from previous studies, suggested that differences in position, spacing, and arrangement of DnaA binding sequences, can significantly affect plasmid replication even in close related Gram-negative bacteria(18). In order to overcome this issue and have a functional plasmid, a set of vectors where the BBR1 replication origin was replaced with the pBBR1 origin from pBBR1MCS-2 previously used in *B. sacchari* were created. The new plasmids preserve almost the same nomenclature than BglBrick vectors, changing only the prefix pBb (Bglbrick) to pBu (Burkhobrick) (Figure 14). Following this nomenclature, pBuB8k-RFP includes replication origin from pBBR1MCS-2, promoter pBAD indicated by number 8 and letter k representing kanamycin resistance. Correct colonies were confirmed by digestion after plasmid isolation from DH10ß. Transformation of *B. sacchari* with pBuB8k-RFP or pBuB5K-RFP, produced a close number of colonies than pBBR1MCS-2 (data not shown) confirming that the modified version of the pBBR1, present in the pBbB8k-RFP was not able to efficiently replicate in *B. sacchari.*

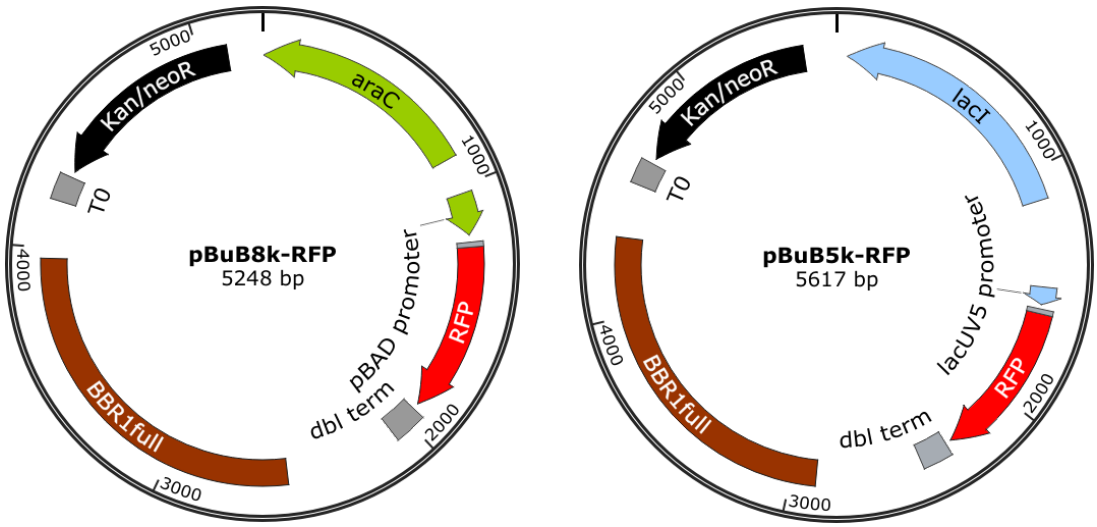

Figure 13 - Burkhobrick vectors, a set of Bglbrick-based expression vectors for *Burkholderia sacchari***.** pBuB8k-RFP (5284 bp) and pBuB5k-RFP (5617 bp) vectors were constructed changing the replication origin of BglBrick pBbB8k-RFP and pBbB5k-RFP plasmids respectively. Except for the change of prefix (Bb in BglBrick to Bu for Burkhobrick vectors), nomenclature of this plasmids is identical to Bglbrick vectors (details in text). The figure depicts the origin of replication from pBBR1MCS-2 (brown), kanamycin resistance (black), red fluorescent protein, RFP (red) pBAD expression system including BAD promoter and *E. coli araC* (light blue), placUV5 expression system including lacUV5 promoter and *E. coli lacI* (light green).

4.3.3.2 Stability of pBuB8k-RFP and pBuB5k-RFP in B. sacchari

Segregational plasmid stability, frequently referred as plasmid instability, is crucial in industrial processes and bioproduction for a simple reason: one plasmid-free cell, means overall less product. Several factors including plasmid copy number, replication mechanism, culturing conditions (19,20) and host cell affect segregational stability. Given the potential of *B. sacchari* for industrial scale production the stability of BurkhoBrick vectors were tested. Bacteria carrying pBuB8k-RFP and pBuB5k-RFP were grown continuously in the absence of kanamycin for 20 generations. Samples were withdrawn at the indicated points, diluted and spread on LB plates with and without kanamycin (Figure 15). Plasmid stability was expressed as a percent value from the number of colonies obtained with antibiotic selection relative to the number of colonies obtained in the absence of selection. Figure 15 shows that after 20 generations in the absence of antibiotic selection, the stability in *B. sacchari* of pBuB8k-RFP was (81%) and pBuB5k-RFP (77%).

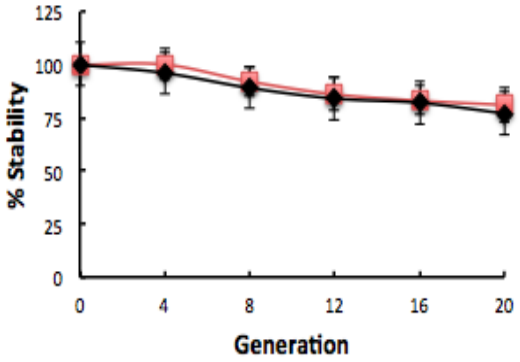

Figure 14 - Segregational stability of pBuB8k-RFP (red squares) and pBuB5k-RFP (black diamonds) in *B. sacchari*. Cells were grown in LB broth without kanamycin for 20 generations and spread on plates without and with kanamycin. The stability of plasmids was determined by dividing the number of colonies obtained on kanamycin plates by the number of colonies obtained on LB plates without antibiotic. The data points represent the mean and standard deviations from the experiments completed in triplicate.

4.3.3.3 Inducible expression of RFP in B. sacchari

Protein expression level is a fundamental parameter for metabolic engineering that often can be tuned using different concentrations of inducer. Although pBAD and placUV5 promoters were used previously in non-enteric bacteria (21,22) and other *Burkholderia* species (10), no information describing its use in *B. sacchari* is available. To explore the effectiveness of both promoters, RBS sequences, and terminators from BglBrick vectors for metabolic engineering applications in this bacterium, RFP expression in cells transformed with pBuB8k-RFP and pBuB5k-RFP was evaluated.

pBAD expression system used in pBuB8k-RFP, is based on the dual activity (repression/activation), exerted by the regulatory protein AraC. In absence of arabinose, AraC efficiently represses transcription and translation by binding to DNA and forming a loop. In the presence of L-arabinose, AraC becomes activated and promotes transcription from the pBAD promoter. (23–25). Previous studies described that pBAD is functional in *Burkholderia cepacia* (10), but no information for *B. sacchari* has been reported so far. On the other hand, lacUV5 promoter used in pBuB5k-RFP, is a variant of the wild type *E. coli* lac core promoter (26) with improved ability to recruit RNA Polymerase due to a 2 base pair mutation in the -10 hexamer region. Expression from placUV5 is controlled by the LacI repressor and can be induced with IPTG. Previous attempts to use pLac in *B. cepacia* were unsuccessful (10). To monitor expression with different inducer concentrations, Red fluorescent protein (RFP) was used as reporter. As shown in Figure 16, both promoters were able to drive expression of RFP in *B. sacchari* demonstrating also that synthetic RBS and terminator sequences present in the BglBrick vectors are functional in *B. sacchari.*

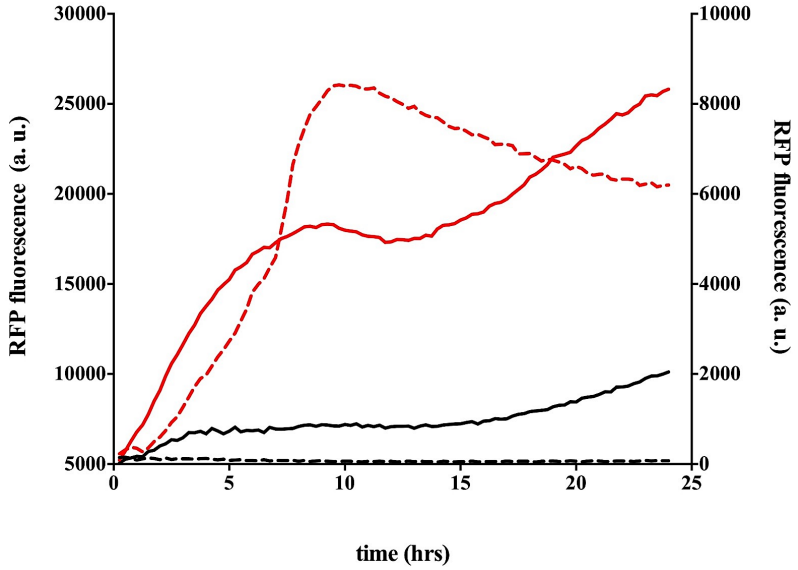

Figure 15 - Time-dependent expression of RFP using plasmids pBuB8k-RFP and pBuB5k-RFP in *B. sacchari*. Late-exponential cells were diluted 100 times in MMX (with and without inducer) and transferred to 96-well plates. RFP fluorescence and optical density (OD600) were measured in triplicate every 15 min during 24 h. IPTG 0.5 mM (solid red line) or arabinose 5 mM (dashed red line) were used as inducers. Fluorescence was normalized with optical density and is expressed in arbitrary units. No induced cells are shown in black. Expression profile of pBuB5k-RFP is plotted on the left Y axis and pBuB8k-RFP on the right Y axis.

As shown in Figure 16, and 17A, RFP expression from pBuB8k-RFP was strongly dependent of arabinose addition reaching a maximum of 65-fold induction (7.5 mM arabinose) compared to the uninduced plasmid. Induction with less than 0.1 mM arabinose, resulted in RFP expression indistinguishable from uninduced control (Figure 17A). An important issue to use arabinose as inducer is that *B. sacchari*, efficiently uses this pentose as a carbon source (6,27). Considering that concentrations of xylose, glucose or other carbon sources used in different minimum media, often vary between 0.3 and 0.5%, and that concentrations used to induce pBAD in *B. sacchari* (2 to 20 mM), corresponding to 0.03 to 0.3% respectively, then, these concentrations are high enough to cause significant effects in growth. Besides, differently from other expression systems were synthetic inducers like IPTG or aTC are not metabolized, arabinose concentration diminishes over time affecting also induction kinetics.

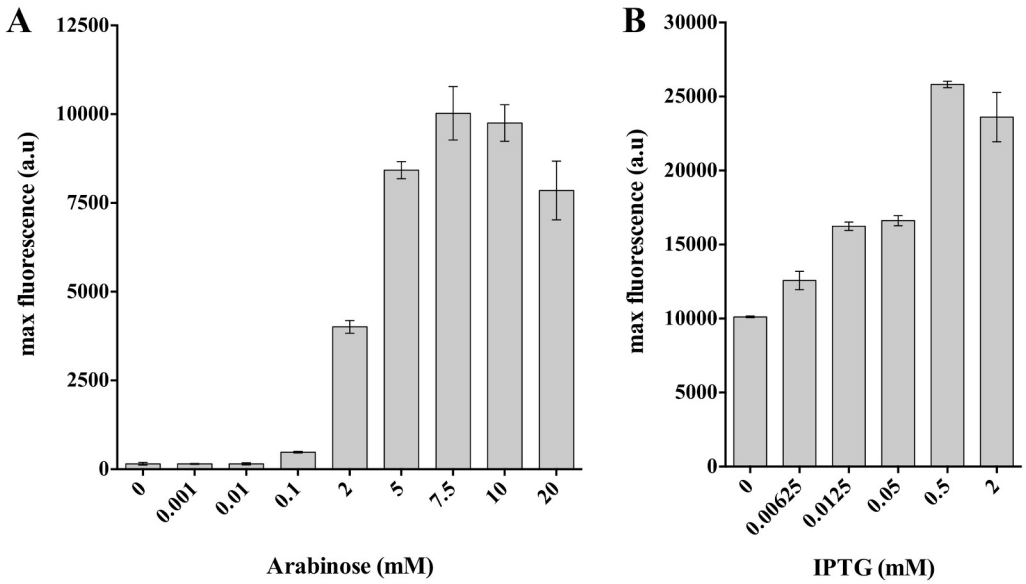

Figure 18 - Inducer-dependent expression of pBuB8k-RFP and pBuB5k-RFP in B. sacchari. Late-exponential pBuB8k-RFP (A) and pBuB5k-RFP (B) cells were diluted 100 times in MMX with increasing concentrations of inducer and transferred to 96-well plates. RFP fluorescence and optical density (OD600) were measured in triplicate every 15 min during 24 h. For each inducer concentration, max normalized fluorescence ± SD is shown.

RFP expression was also effectively activated in pBuB5k-RFP (Figure 16), but in contrast to the strong inducer-dependence of pBuB8k-RFP, high levels of fluorescence were observed in the strain transformed with pBuB5k-RFP under low induction levels (3.125 to 12.5 mM) and even in uninduced conditions (Figure 17A). Maximum induction (2.5-fold) was reached adding 0.5 mM IPTG. This result shows that in *B. sacchari* as in *E. coli*, lacUV5 promoter drives high basal expression possibly due to its intrinsically higher ability to recruit RNA polymerase.

5.4.2.8 xylAB overexpression improved growth rate and P(3HB) production of B. sacchari.

Once developed the tools for controlling expression, and pursuing to identify the best strategy to improve growth rate and P(3HB) production of *B. sacchari* using xylose as a sole carbon source, genes involved in xylose transport and catabolism were overexpressed. Xylose transporters *xylE* (low-affinity xylose symporter) and *xylFGH* (ABC-type transporters ATP-dependent) genes and catabolic genes *xylA* (xylose isomerase) and *xylB* (xylulokinase) from *B. sacchari* were cloned in pBuB8k using the restriction sites specified in Table 5. pBuB8k was selected to clone the xylose genes to minimize the basal expression and evaluate the role of proteins only when overexpressed.

Confirmed plasmids carrying transporter or catabolic genes were transformed into *B. sacchari* and specific growth rate (μ_{max}, h^{-1}) for each strain was determined using Growth Rates software (28) from growth curves obtained in 96-well Synergy H1 plate reader. *B. sacchari* pBuB8k-RFP served as the control strain . As shown in Figure 18, overexpression of *xylAB* resulted in significant growth rate improvement (46.8%), compared to the control strain expressing RFP. *xylFGH* overexpression slightly improved growth rate, compared to RFP-expressing strain. All strains showed comparable growth rate when uninduced (data not shown).

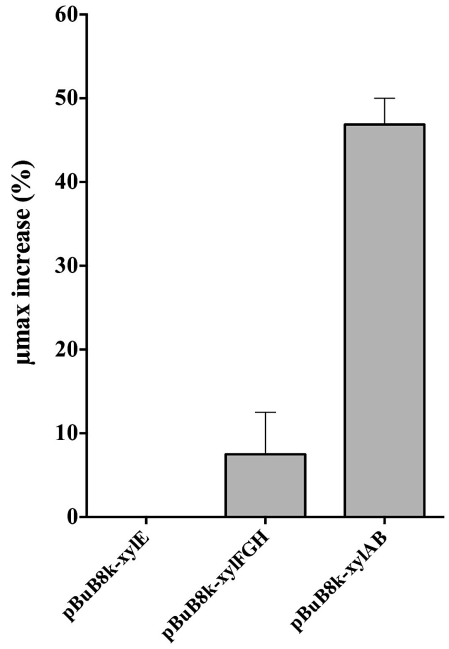

Figure 16 - Expression of xylAB and xylFGH improve xylose utilization in *B. sacchari.* Late-exponential cells grown in MMG were diluted 1:100 in MMX with arabinose (5mM) and transferred to 96-well plates. Optical density (OD_{600}) was measured in triplicate every 15 min during 24 h Improvement was determined comparing growth rate of each strain with pBuB8k-RFP

4.3.3.5 Adequate levels of transcriptional regulator xylR, further improved growth rate and P3HB production.

Xylose utilization pathway recently described in *B. sacchari*, is composed by two transporters (*xylE*, *xylFGH*) two catabolic genes (*xylA*, *xylB*) and one regulator (*xylR*). Overexpression of transporters or catabolic proteins is a common strategy used to improve xylose utilization in different microorganisms. (3,29,30). Using a different approach, *xylR* transcriptional regulator, was overexpressed in *E. coli* leading to the coordinated increment of *xylA*, *xylB*, *xylF* and *xylG*. In addition, cells were able to co-utilize arabinose and xylose and produce ethanol more efficiently (31). Seeking to coordinately increase the levels of proteins

responsible for xylose utilization and further improve production of P(3HB) in *B. sacchari*, *xylR* was amplified and cloned in the pBuB8k plasmid to asses the effect of its overexpression on the growth and P(3HB) production. pBuB5k-xylR was confirmed by restriction and transformed into *B. sacchari.* As shown in Fig. 19, the growth rate of pBuB8k-*xylR* strain was improved 19.1% compared to strain expressing RFP with the same concentration of arabinose when 0.1 mM arabinose was added to induce xylR expression. Surprisingly, the addition of , 0.5 mM or higher arabinose concentrations, resulted in drastic impairment of cell growth in MMX (Figure 19). This results suggest that although increased expression of XylR enhanced xylose utilization, when both xylose and arabinose are present at significant concentrations, the strain expressing only RFP can clearly co-utilize this sugars. On the contrary, in the xylR overexpressing strain, the augmented levels of XylR can inhibit arabinose utilization (31) by a mechanism called reciprocal regulation (32). In this mechanism described in *E. coli*, XylR antagonizes AraC binding to target promoters, inhibiting expression of arabinose utilization genes. Although there is no description of XylR role in *B. sacchari* or the potential mechanism responsible for this effect, the similar phenotype with *E. coli* and the conserved regulation of central carbon among several Gram-negative bacteria, suggest that this mechanism could be also present in this bacterium. This result emphasizes the critical role of having tools to fine tune protein expression for biological engineering purposes.

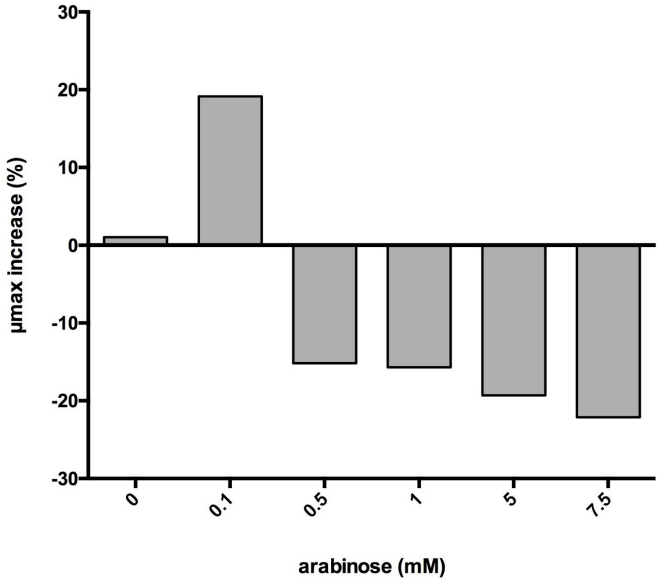

Figure 20 - Adequate levels of xylR improved growth rate of *B. sacchari*. Late-exponential cells grown in MMG were diluted 1:100 in MMX with increasing concentrations of arabinose (0 to 7.5 mM) and transferred to 96-well plates. Optical density (OD_{600}) was measured in triplicate every 15 min during 24 h. The improvement was

determined by comparing the growth rate of each strain with the control strain expressing RFP induced with the same concentration of arabinose.

4.3.3.6 XylR overexpression under control of LacUV5 promoter

To further understand the effect of *xylR* overexpression in xylose utilization, we took advantage of the set of plasmids created in this work to create pBuB5k-*xylR*, a plasmid where *xylR* expression is controlled by placUV5, and confirming the positive effect on growth caused by xylR overexpression, induction with low level (6.25 μM IPTG) or no induction (due to high basal expression), increased growth rate significantly up to 0.25 h^{-1} the highest growth rate achieved in xylose to date, Figure 20. According to this results, medium expression of *xylR* allowed the cells to adapt faster to the shift of carbon source (MMG was used as carbon source in the pre inoculum). Surprisingly, addition of higher concentration of IPTG (50 or 500 μM) caused delayed growth, even when xylose was used as a sole carbon source. The reason of this phenomenon is unclear but is in agreement with previous data showing that constitutively high levels of xylR have deleterious effects for *E. coli* cells (31). (Figure 20). A protein interaction prediction analysis using STRING (33) showed that besides xylose catabolism, *E coli* XylR also is predicted to interact with different proteins involved in sugar metabolism (lacZ, ptsN), nitrogen-limitation response (rpoN), and phage response (pspN). Maybe this predicted interactions or XylR participation in other physiological process is altered by the substantial increase in XylR expression and is responsible for the observed phenotype caused by XylR overexpression in *E. coli* and *B. sacchari.* Figure 21. No data is available about xylR interactions in *B. sacchari*, but it may also interact with different proteins other than xylose related , which may explains why its expression needs to be finely modulated to avoid detrimental effects.

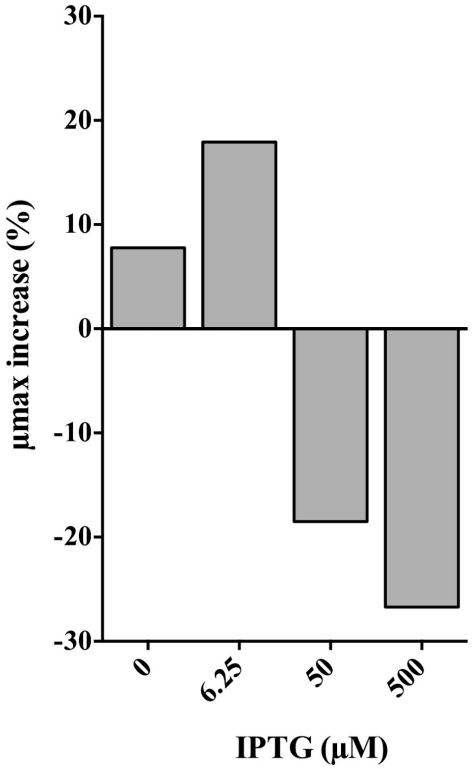

Figure 21 - Adequate levels of xylR improved growth rate of *B. sacchari*. Late-exponential cells grown in MMG were diluted 1:100 in MMX with increasing concentrations of IPTG (0 to 500 µM) and transferred to 96-well plates. Optical density (OD_{600}) was measured in triplicate every 15 min during 24 h. The improvement was determined by comparing the growth rate of each strain with the control strain expressing RFP induced with the same concentration of IPTG.

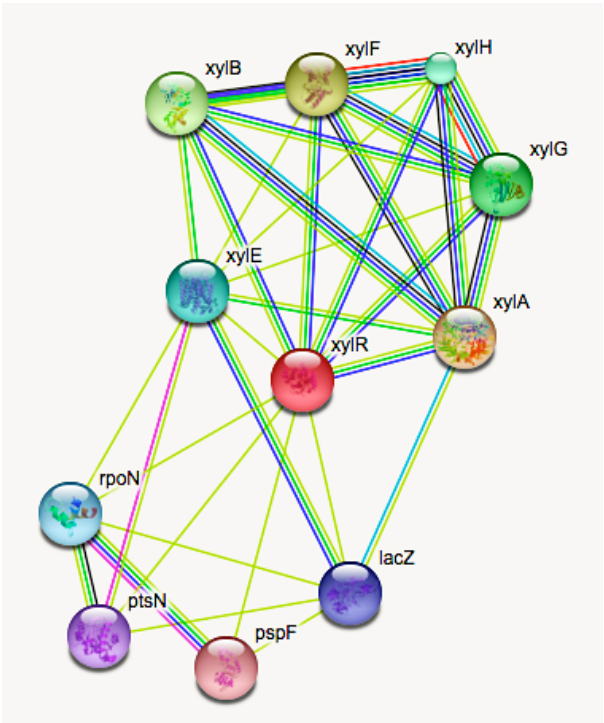

Figure 22 – Protein interaction Network using String software. XylR sequence from E. coli MG1655 was used for the analysis.

4.3.3.7 Overexpression of xylR further improved P3HB accumulation

Once demonstrated that xylR overexpression is an effective strategy to improve xylose utilization, its impact for P(3HB) production was evaluated after 48 hrs in batch cultures. As shown in Table 7, strains expressing xylR improved growth rate, produced significantly higher amounts of P(3HB) (34% increase compared to control strain) and resulted in the highest polymer yield reported for *B. sacchari* using xylose as a sole carbon source of 0.396 $g \cdot g^{-1}$, which represents an 80% increment compared to control strain.

In a recent research where *B. sacchari* wild-type strain was used to produce P(3HB) the maximum growth rate achieved was 0.18 h^{-1} with a polymer yield of 0.2 g/g. Must be mentioned that this result was only obtained when using a ratio of glucose to xylose of 2:1 (8). Previously, a study included in a PhD dissertation (1), estimated the value of the theoretical maximum conversion efficiency of xylose in P (3HB), a metabolic model was constructed for *B. sacchari*, in which carbohydrate central metabolism, cellular biomass biosynthesis and formation P(3HB) were considered. To constructed and test the model, Metatool software (34) was used. The *in silico* analysis of elementary modes was performed with the aim of identifying modifications in *B. sacchari* metabolism that would allow to improve the xylose yield ($Y_{PHB/Xyl}$). According to this analysis the most efficient way to improve yield is to increase the flux in the non-oxidative branch of the pentose phosphate pathway, increasing also NADPH availability, required for P3HB biosynthesis (1). The maximum yield obtained from this analysis was 0.40 $g \cdot g^{-1}$ when xylose is being used with 100% maximum theoretical to produce P(3HB). Considering that the yield obtained in the present work for xylR (0.396 $g \cdot g^{-1}$) match with the previously modeled data for the best yield, is plausible to suggest that in agreement with the modeling data, XylR overexpression improved the accumulation of P(3HB) by increasing flux of non-oxidative branch of the pentose phosphate pathway with the consequent increment in NADPH pool, achieving the highest yield reported to date for *B. sacchari*.

Table 7. Summary of the results of P3HB accumulation assay in *B. sacchari*

Strain	**CDW**	**%PHB**	**PHB**	μ_{max}	$Y_{P3HB/xyl}$
	g l^{-1}		**g l^{-1}**	**h^{-1}**	**g/g**
B. sacchari 8k xylR	7.15 ± 0,58	65 ±1.98	4.65	0,20 ± 0,003	0.348±0.007
B. sacchari 8k RFP	5.12 ±0,72	51.95 ±3.01	2.66	0,156 ±0,099	0.237±0.005
B. sacchari 5k xylR	8.02 ± 0,44	71.07 ±2.46	5.7	0,252 ± 0,11	0.396±0.009
B. sacchari 5k RFP	5.34 ± 0,66	54 ±2.88	2.89	0,162 ± 0,16	0.225±0.006

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5 CHAPTER 4: Carbon Catabolite repression in *B. sacchari*

5.4.1 Introduction

Plastics have been present in our daily lives since 1920's and their use has increased exponentially (1). It was estimated the annual production of petroleum based plastics exceeded 300 million tons in 2015 (2) generating an environmental problem because of its resistance to degradation, accumulating in the environment. Since bioplastics materials are expected to gradually replace petrochemical materials in the next decades, multidisciplinary research groups have been working toward make bioplastics commercially competitive, trying to reduce costs and expanding its applications (2). Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible thermoplastics, naturally produced by a wide variety of bacteria, which synthesize these polymers as a carbon and energy storage (3) under unbalanced growth conditions such as limitation of an essential nutrient (phosphorous, nitrogen, sulphur, magnesium or oxygen) and excess of carbon source. (4). A major obstacle preventing the large scale production of polyhydroxyalkanoates (PHAs) has been the high production cost, since most of carbon sources used for PHA production are from pure carbohydrates such as glucose, sucrose, fatty acids, among others (5,6). As an alternative, inexpensive feedstocks including hemicellulosic biomass from sugarcane, wood, and agricultural residues have been tested for production of different compounds.

One of the most studied biomass residues in Brazil is the sugarcane bagasse. The estimative of bagasse availability for 2016/2017 season is around 208 million of tons (7,8), after hydrolysis process catalyzed by acids or cellulolytic enzymes, result in simple carbohydrates around of 75.7 $g.L^{-1}$ of xylose, 13.5 $g.L^{-1}$ of arabinose, and 13.2 $g.L^{-1}$ of glucose, which are available for bacteria transformation into bioproducts (9,10). Most of industrial interest microorganisms do not utilize all these sugars efficiently. Even model organisms present somo challenges such as lack of consume of pentoses in *S. cerevisiae*, and carbon catabolite repression (CCR) in *E. coli*, which consumes xilose, glucose and arabinose, but glucose repression prevents pentose compsuntion in this microorganism.

In order to utilize bagasse, acid hydrolysis is usually performed to release sugars. However, this treatment generates a number of toxic compounds, such as acetic acid, furfural and hydroxymethylfurfural (HMF) (11). Several procedure have been successfully applied to

eliminate these toxic compounds and to permit the utilization of sugars by microorganisms (9), within this context, *B. sacchari* have shown resistance to toxic compounds and achieves higher polymer contents and yields from the carbon source were observed with bagasse hydrolysate, compared with the use of analytical grade carbon sources. (12)

Several efforts have been conducted to develop a strain able to utilize simultaneously multiple lignocellulose sugars. In *Saccharomyces cerevisiae,* xylose utilization and transport genes have been expressed with different levels of success to avoid carbon catabolite repression, most of the cases, even when xylose and glucose are consumed simultaneously, most of xylose remains at the time when glucose was already depleted. Moreover, pentoses accumulation in a continuous fermentation process may lead to a strain inhibition by the osmotic imbalance (13).

Another approach has been studied in *E. coli*, disrupting the phosphotransferase system (PTS) of glucose uptake by ptsG knockout which encodes the enzyme IICBGlc to promote simultaneous sugar compsuntion, although, most of xylose always remains (14). Also, in a different research conducted in *B. sacchari* in a UV mutant for the PTS system, a simultaneous composition was reported, however, PHB biosynthesis was affected, being necessary yeast extract supplementation to maintain higher values of growth rate and sugar consumption rate, the authors conclude, PTS plays an important role in cell physiology and the elimination of its components has a significant impact on carbon flux distribution, and PHB biosynthesis (15). *B. sacchari*, has an great potential as a cell factory because of its capacity to produce diverse high value compounds (16,17), then, overcoming CCR would be useful in order to reduce costs associated to carbon source. The purpose of this study was first, characterize if there is a hierarchy between xilose, arabinose and glucose when supplied sugar mixtures since these are the main sugar in lignocellulosic hydrolysate from sugarcane bagasse, and to abolish CCR in xilose, arabinose and glucose mixtures by overexpressing two synthetic xylose operon comprised by *xylE* and *xylAB* genes (transport and catabolic genes from *B. sacchari*) and *gatC xylAB* (galactitol permease from *E. coli*) previously reported as an alternative transported for xylose (18) , and evaluating its impact in growth rate and P(3HB) accumulation.

5.4.2 Materials and Methods

5.4.2.1 Strains and vector

Escherichia coli DH10B (F– endA1 deoR+ recA1 galE15 galK16 nupG rpsL Δ(lac)X74 φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) StrR λ–)

was used as a host for plasmid construction. *E. coli* cultures were grown in LB broth (150 rpm) or agar 1.5% at 37 ºC. When needed, media was supplemented with kanamycin (kan, 50 µg/mL). *B. sacchari* LFM101 (recently reclassified as *Paraburkholderia sacchari* (19), hereafter LFM101 (20) was used in this study as a final host for plasmids. *B. sacchari* LFM1403 strain was created transforming pBBR1MCS-2 *xylE xylAB* into LFM101. As a cloning and expression vector, plasmid pBBR1MCS2 (21) was used.

5.4.2.2 Growth conditions

Luria-Bertani (LB) medium (10 g/L tryptone, 10 g/L NaCl, and 5 g/L yeast extract, pH 7.4) was used for cloning purposes. Minimal medium (MM) used for growth and P3HB accumulation assays was modified from (22) and containing in $g \cdot L^{-1}$: KH_2PO_4 (0,39); $(NH_4)_2SO_4$ (2,91); $MgSO_4 \cdot 7H_2O$ (0.31); $CaCl_2 \cdot 2H_2O$ (0.010); $(NH_4)_5Fe(C_5H_4O_7)_2$ (0.06); NaCl (1); ; trace elements solution (2 $mL \cdot L^{-1}$), which was prepared with H_3BO_3 (0.30 $g \cdot L^{-1}$); $CoCl_2 \cdot 6H_2O$ (0.20 $g \cdot L^{-1}$); $ZnSO_4 \cdot 7H_2O$ (0.10 $g \cdot L^{-1}$); $MnCl_2 \cdot 4H_2O$ (0.03 $g \cdot L^{-1}$); $NaMoO_4 \cdot 2H_2O$ (0.03 $g \cdot L^{-1}$); $NiCl_2 \cdot 6H_2O$ (0.02 $g \cdot L^{-1}$); $CuSO_4 \cdot 5H_2O$ (0.01 $g \cdot L^{-1}$). Use of xylose, glucose or arabinose as carbon sources for MM is indicated as MMX, MMG or MMA respectively. For growth assays to confirm CCR in *B. sacchari*, initial OD_{600} was 0.1 for all conditions.

5.4.2.3 P3HB accumulation assay

For P(3HB) accumulation assays, 5g/L of each sugar (15g/L total) was provided. 125 mL erlenmeyers containing 50 mL of MMX were used. Experiments were done in triplicate, two samples (10 mL each) were taken one at the beginning of the experiment and and one after 72 h of culture (30 °C, 200 rpm) .Samples were centrifuged (8,000 rpm, 4 °C, 10 min). The supernatant was frozen in a freezer at -20 ° C for sugar quantification by liquid phase chromatography (HPLC). Pellet cell was frozen and lyophilized to measure dry cell mass (g / L), and to determine composition of PHA by gas phase chromatography of propyl esters (23).

5.4.2.4 Primer design

Gene sequences were selected from *B. sacchari* genome annotation, sequence identity was determined using the online Basic Local Alignment Search Tool (BLAST) (24). Primers were designed to amplify the native RBS and the complete coding sequence and add the desired restriction site for cloning. Plasmid DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, and DNA ligation were performed by standard procedures (25).

Primers are listed in Table 1. Genes were under *E. coli* Lac promoter control; pBBR1MCS2 plasmid doesn't harbor lacI repressor, a desirable feature to avoid CCR.

Table 8 - Primers used in this study

Primer Name	Sequence	Restriction site
xylE_ApaI_F1	ACGTCAGGGCCCGCTCTGATGCATGCCGCAA	*Apa*I
xylE_HindIII_R1	GACTAGAAGCTTATTCCCGTCTGCGTCAA	*Hind*III
xylAB_HindIII_F1	TCCAGTAAGCTTCTCGCTGTGCATCGACCAA	HindIII
xylAB_XbaI_R1	ACGTAATCTAGATGCGAATACGGGTTCGAGTG	*Xba*I
M13fwd	GTAAAACGACGGCCAGT	NA
M13rev	GAGCGGATAACAATTTCACACAGG	NA
gatC_ApaI_F1	CTTGAGGGGCCCTGAATTAATCCAGTGTCGGG	*Apa*I
gatC_HindIII_R1	ATCCAGAAGCTTGATTGTTAAGGGGGATAACC	*Hind*III

5.4.2.5 Gene amplifications

The fragments were amplified using Phusion® High-Fidelity DNA Polymerase (Thermo Scientific, Waltham, Massachusetts, USA), in a Mastercycler nexus gradient thermal cycler (Eppendorf AG, Hamburg). Cycling conditions were for *xylE*: 98 ºC 1', 30X (98 ºC 30", 55 ºC 30", 72 ºC, 1') 72 ºC 5'), *xylAB:* 98 ºC 1', 30X (98 ºC 30", 58 ºC 30", 72 ºC, 2') 72 ºC 5') *gatC:* 98 ºC 1', 30X (98 ºC 30", 61 ºC 30", 72 ºC, 2') 72 ºC 5')

5.4.2.6 Plasmids construction

The purified 1.48 kb *xylE* and 1.54 kb *gatC* PCR products were digested and ligated into *Apa*I and *Hind*III sites of pBBR1MCS-2 to create pBBR1MCS-2-*xylE* and pBBR1MCS-2-*gatC* respectively, then the 2.92-kb *xylAB* PCR product was digested and ligated into *Hind*III and *Xba*I sites of pBBR1MCS-2-*xylE* to create pBBR1MCS-2-*xylE xylAB* and pBBR1MCS-2-*gatC* creating pBBR1MCS-2-*gatC xylAB*. Colonies were screened using blue/white selection and digestion with *Apa*I and *Xba*I to confirm correct construction. Finally, M13 primers (Table 1) were used to to confirm constructions by sequencing.

5.4.2.7 B. sacchari transformation

Electrocompetent cells of *B. sacchari* LFM101 were prepared according to previously established protocols. Briefly, 1 mL cells from a culture in mid-exponential phase was inoculated into 50 mL of LB (250 mL flask) and incubated at 30 ºC, 150 rpm during 6 h until cells reached OD_{600}= 0.6. Flask was incubated 15 min on ice, collected by centrifugation (15 min, 4500 rpm), washed twice with 50 mL of sterile nanopure water and once in sterile 20% glycerol .Finally, cell pellet was resuspended and aliquoted (50 µL each) in sterile 20% glycerol. Plasmids were electroporated using Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Inc. Hercules, California, USA). 100 *n*g of DNA were added to 50 µL electrocompetent cells, transferred to a 0.2 cm electroporation cuvette. Electroporation parameters were: 25 µF, 200 Ohm, 2500 V.

5.4.2.8 Analytical methods

5.4.2.8.1 Biomass concentration

Biomass was expressed in grams per liter as cell dry weight (CDW). First 10 mL of culture were harvested by centrifugation at 10,600 g and lyophilized. Dry biomass was weighed using analytical balance (Ohaus Adventurer, Parsippany, New Jersey, USA) and expressed in grams per liter as cell dry weight (CDW).

5.4.2.8.2 Xylose, glucose and arabinose determination

Sugars concentrations were quantified through liquid chromatography (HPLC). 10 µl of each sample was injected into a Dionex HPLC (Ultimate 3000, Thermo Fisher Scientific Inc, Waltham, MA, USA) equipped with a sugar separation column (Aminex-HPX-87H). For detection, a differential refractometer (Shodex IR-101) was used. Separation occurred at 45 °C with H_2SO_4 solution (5 mM) at a flow rate of 0.6 $mL \cdot min^{-1}$. Standard curves were constructed using xylose, glucose and arabinose solutions containing up to 3 $g \cdot L^{-1}$ each.

5.4.2.8.3 Growth rate

The OD measured at 600 nm (DU-650 spectrophotometer, Beckman Instruments, San Jose, CA) was used to monitor cell growth.

5.4.2.8.4 Poly-3-hydroxybutyate (P3HB) content and composition measurements

15 mg of lyophilized cells P(3HB) were subjected to propanolysis, (23) according to Gomez, 1996. (26) with an Agilent 7890A GC System (Agilent Technologies, Santa Clara,

California, USA) equipped with a HP5 capillary column after sample split (1:25). Helium (0.8 $mL \cdot min^{-1}$) was used as carrier gas. Injector and FID temperature were 250ºC and 300ºC, respectively. Benzoic acid was used as the internal standard. (Sigma-Aldrich, Saint Louis, Missouri, USA).

5.4.3 Results and Discussion

5.4.3.1 Different levels of CCR in B. sacchari

Microorganisms must survive in constantly fluctuating environments, where, nutrient absorption provides optimal fitness which is key to be competitive. Owning CCR mechanism, is a benefit for microorganisms survival during the changing nutritional conditions of the environment (27).

The basic principle underlying CCR is universal for all microbes; the preferred substrate, is the one that provides more energy. This is normally achieved by inhibiting expression of genes involved in the catabolism of secondary carbon sources, which are not preferred (28). The low expression level of genes responsible for sugars other than glucose, which is generally the preferred sugar, leads to diauxic growth profile (13). This occurs because initially cells are grown using glucose which is consumed more rapidly and provides a higher growth rate. Then, when only the second sugar is consumed, we have a drastic reduction in specific growth rate due to slower metabolism of this, thus reducing productivity when the process is applied on an industrial scale.

In *E. coli* for example, the ranking of the hierarchy based on promoter activity and growth rate is glucose >lactose> arabinose > xylose > sorbitol (29) , whereas in some *Lactobacillus species*, which are naturally able to simultaneous utilization of numerous carbon sources it seem this hierarchy does not exist, for instance, in *L. brevis*, co-utilization of xylose and glucose is a common trait for this species. (30). Earlier research in our laboratory with *B. sacchari* suggested xylose utilization rate was increased when glucose was present in the media, which is the opposite to CCR (31), on the other hand, Lopes and coworkers reported a UV mutant for PTS system in *B. sacchari* which was able to consume xylose, glucose and arabinose, although sugars hierarchy was not described and PHB production was strongly affected in the mutant strain (15). Aiming to understand sugar compsuntion preference in *B. sacchari*, shaker flasks assays containing xilose-glucose, and xilose-arabinose

mixtures were performed and the profile growth rate and sugar utilization is shown in Figure 20,

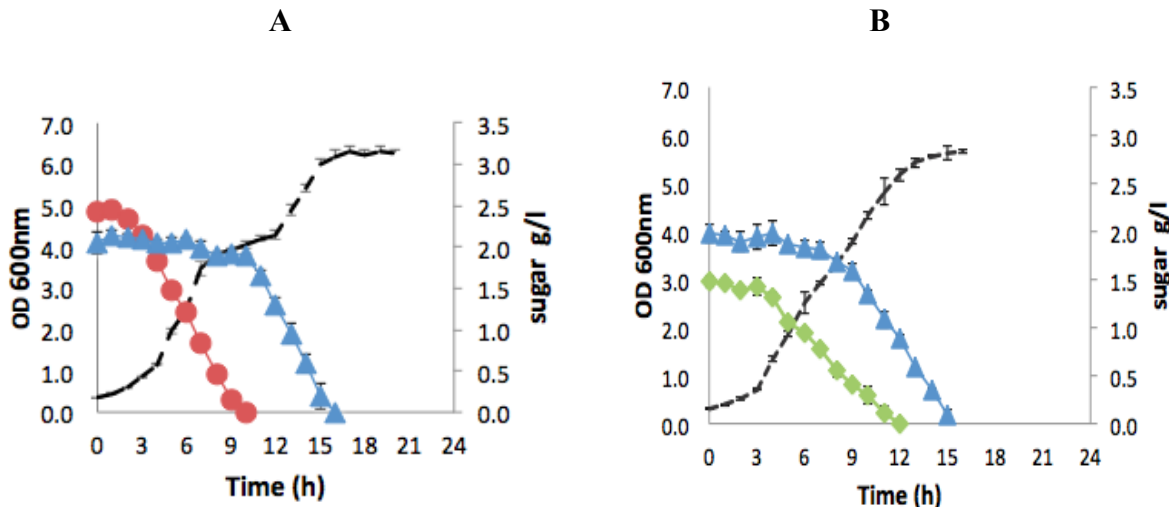

Figure 23 - Growth and sugar compsuntion profiles. (A) Xilose-Glucose Mixture. (B) Arabinose-Xilose mixture. Red circles: Glucose, Blue triangles: Xylose, Green diamonds: Arabinose. Dotted line: Optical density 600nm

As shown in Figure 20 A, glucose is first metabolized while xylose was inhibited, glucose is completely depleted at hour 10, then xylose is consumed been exhausted at hour 16. It is also possible to see diauxic growth with a lag phase between 9-14h, time when glucose was being depleted and cells need to adjust its cellular machinery to utilize xylose; growth rate was about 0.35 h^{-1}. In arabinose-xylose mixture, Figure 20 B, *B. sacchari* consumed arabinose preferentially, however by hour 12 when arabinose was depleted 50% of xilose was also consumed, therefore, both pentoses were consumed simultaneously although arabinose was depleted first. Unlike glucose-xylose mixture, growth profile in arabinose-xilose does not present strong diauxic growth, leading to growth rate of 0.44 h^{-1}. In both mixtures, sugars were totally depleted after 16 h.

In *E. coli* has been described that arabinose is strongly repressed by glucose, and that, a second hierarchy exists, in which, *E. coli* will consume xylose once arabinose is completely exhausted (32). In a different research involving growth studies showed that *Lactobacillus brevis* simultaneously consumes numerous carbon sources and appears to lack normal hierarchical control of carbohydrate utilization. (30)

Despite of being reported preferential consumption of glucose over xilose in *B. sacchari* (15,16) less is known about xilose-arabinose utilization. As shown in Figure 20 B, simultaneous compsuntion of pentoses unlike previously reported for *E. coli* was observed. This feature is interesting since, 80% of sugar in lignocellulose are pentoses (10).

5.4.3.2 Overexpression of xylE xylAB and gatC xylAB in B. sacchari

Several approaches have been conducted to abolish CCR in *E. coli* which is used for the production of a diverse number of high-value metabolites. Inactivation of the PTS components have been reported to cause growth reduction as a consequence of diminished glucose uptake capability, so different approaches including modifying intracellular cAMP levels as a strategy to change the role of the cAMP–CRP complex (33), adaptive selection in chemostat culture system of PTS− mutants, replacement in PTS− strains of the native GalP promoter by strong promoters or the substitution of this permease by recombinant glucose transport system (15).

In order to overcome strong repression of glucose over xylose and arabinose, and knowing xylose and arabinose can be simultaneously consumed, we proposed to over express both xylose catabolic genes in *B. sacchari*, (*xylAB*) in two different constructions, one with xylose *xylE* and one with *gatC,* both described as xylose transporters, which have been described to improve xylose uptake when overexpressed (18,34).
Overexpression results are shown in Figures 21 B-C.

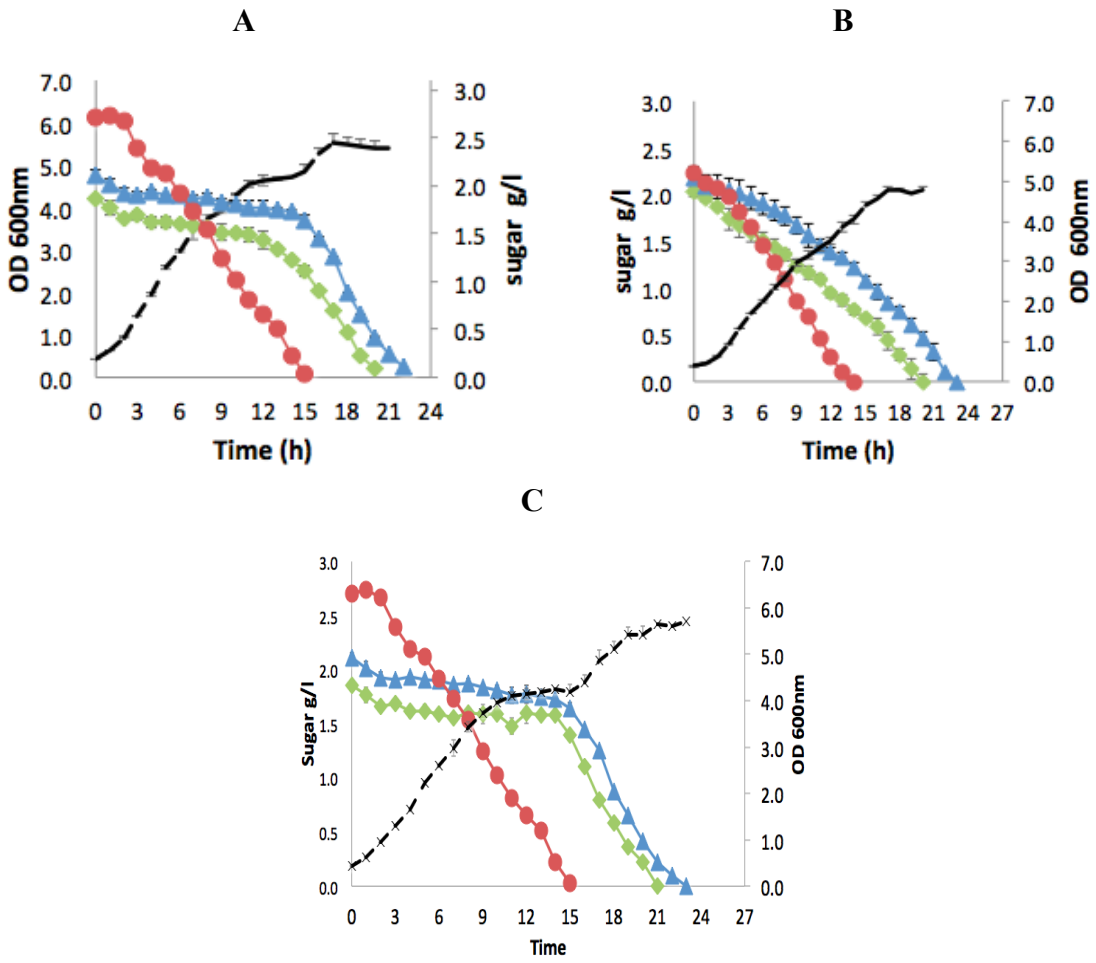

Figure 24 - Growth profiles in xylose arabinose and glucose mixtures. Red circles: Glucose, Blue triangles: Xylose, Green diamonds: Arabinose. Dotted line: Optical density 600nm. **A**. *B. sacchari* control. **B**. *B. sacchari* xylE xylAB. **C**. *B. sacchari* gatC xylAB

As shown in Figure 21 B, *xylE xylAB* overexpression succesfully overcomed glucose repression over arabinose and xylose. All three sugars were consumed simultaneously, although glucose is always the preferred carbon source, being depleted at hour 14, when 63% of arabinose and 45 % of xylose was already consumed. By hour 23 all sugars were consumed with a growth rate of 0.35 h^{-1}, whereas, *gatC xylAB* overexpression didnt not resulted in any improvement showing a similar behaviour than control strain Figure 21C.

Sugar cane bagasse is mainly composed of xylose, glucose and arabinose, and as discussed by Kim 2010, the main problem associated to glucose repression over pentoses in a fed batch fermentation, is that pentoses are accumulated while glucose is totally consumed. In a continuous process inhibitory products from fermentation such as ethanol, or acid lactic can be increased with pentoses utilization rate being slower. Furthermore, if a small amount of glucose is left in the media, pentoses will not be utilized and remain unfermented delaying the process and decreasing yield and productivity. (35) Besides of that, increasing amounts of pentoses left in the media can inhibit cellular growth after several cycles of feeding. So it is interesting to have a strain able to metabolize three sugars simultaneously. Elimination of diauxic growth and simultaneous sugar compsuntion in *B. sacchari* represents a great improvement because that implies a increase in productivity reducing operating time (36).

5.4.3.3 P(3HB) accumulation in B. sacchari pBBR1MCS2-xylE xylAB

After verifying that *xylE xylAB* gene expression allowed simultaneous consumption of xylose, glucose and arabinose, a P(3HB) accumulation assay was performed as described in materials and methods. Results are summarized in Table 9. The recombinant strain presented an increase of 18% in P(3HB) accumulation when compared to control strain, likewise, it is possible to verify that yield improved by 45.8% which represents, *B. sacchari* is converting xylose in P3HB with around 95% of theoretical maximum accumulating 66% of PHB as CDW.

Table 9 - P(3HB) accumulation assay

Strain	CDW g l^{-1}	%P(3HB)	P(3HB) g l^{-1}	$Y_{P(3HB)/Xil}$ g/g
B. sacchari pBBR1MCS-2::xylE xylAB	7,03 ±0,33	66 ±2,89	4,64 ±0,31	0,35 ±0,03
B. sacchari pBBR1MCS-2	5,34 ±0,28	56,03 ±3,11	2,99 ±0,19	0,24 ±0,02

This is the first time that simultaneous and complete consumption of lignocellulose sugars glucose, xylose and arabinose is demonstrated in shaken flasks assays of *B. sacchari*. The glucose-mediated inhibition of xylose uptake was removed. We consider *B. sacchari* a useful platform for the development of PHB production processes using lignocellulosic sugars. Since low-cost substrates will be preferred in second-generation biorefineries, *B. sacchari's* ability to utilize all sugars is a great advantage that should be harnessed.

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6 CHAPTER 5

In this chapter different results, relevant for future research with *B. sacchari* are summarized even though this results won't be submitted for publication.

A major challenge that must be addressed to allow the broad use of *B. sacchari* as a model organism for large scale production of different high-value compounds, is the lack of proper molecular tools for applying metabolic engineering and synthetic biology approaches. Since *B. sacchari* was first isolated 16 years ago, (1), no plasmid other than pBBR1MCS has been used. Several strategies aiming to improve xylose utilization, avoid CCR, and produce different PHB monomer composition have been conducted in *B. sacchari,* always using this plasmid with different levels of success (2–4).

As detailed in the original article, (5) and shown in Figure 22, pBBR1MCS-2 is a plasmid mainly designed for cloning purposes and includes no features to control protein expression. The main feature incorporated in pBBR1MCS-2 is the multiple cloning site (MCS) within lacZα gene to facilitate blue/white selection by α-peptide complementation (6). pBBR1MCS-2 includes pBBR1 replication origin, kanamycin resistance and pLac promoter. However must be mentioned that no repressor (lacI) is present in the plasmid and therefore control of expression relies only on the lacI encoded in the chromosome of the host cell. Furthermore, due to the fact that MCS is included into lacZ CDS, proteins cloned in the MCS will include two partial sequences of lacZ in the 5' and 3' ending of the cloned protein which may be detrimental for protein function or expression. In addition, pBBR1MCS-2 is not compatible with the most common and widely used biopart standards (BioBrick and BglBricks), essential for working in synthetic biology.

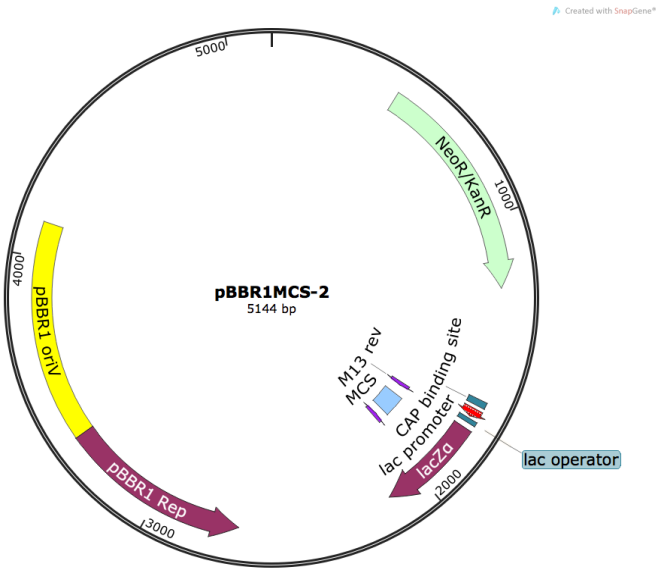


Figure 25 - pBBR1MCS2 map. LacZ promoter is shown in red, MCS flanked by M13 primers are shown in light blue.

Intending to expand the tools available and to further develop *B. sacchari* as a microbial platform for engineering and bioproduction, different replication origins and promoters were tested using fluorescent proteins.

6.5.1 Replication origins

One of the main challenges to use different plasmids in *B. sacchari*, is the lack of knowledge about the origins able to efficiently replicate in this bacterium. Replication origins of BglBrick vectors (Figure 23) and other from different compatibility groups were evaluated.

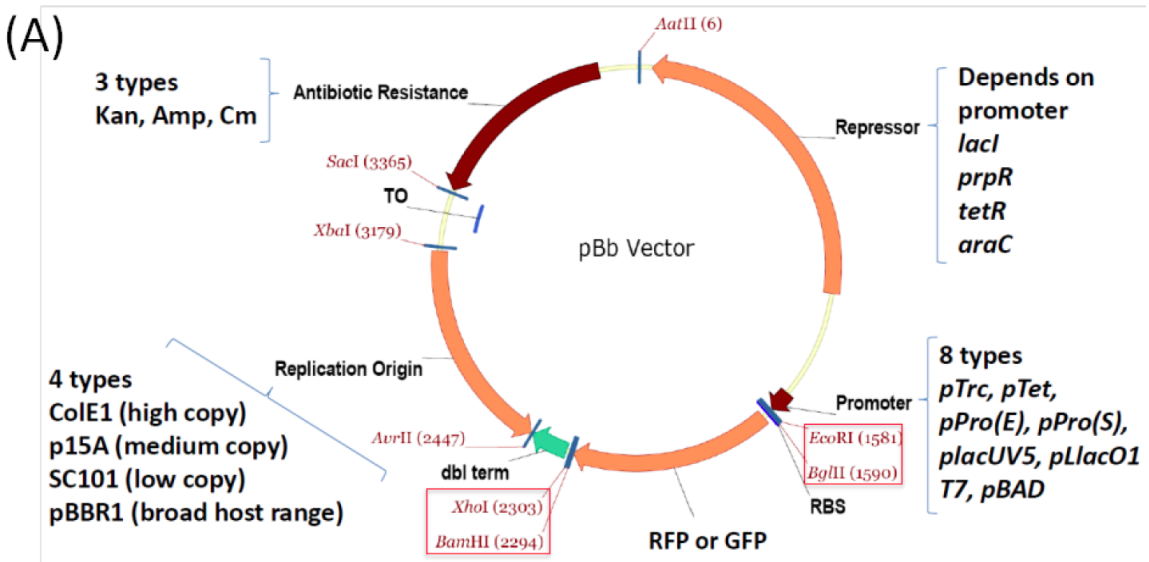


Figure 26 - BglBrick plasmids structure and principal features. Plasmids are composed of three modules antibiotic resistance gene module, replication origin module, and expression module, which includes the repressor, promoter, gene of interest (rfp or gfp) and terminator. BglBrick sites are in red boxes.

Plasmids were transformed by electroporation as described in section 4.3.2.7. Qualitative results summarized in table 10. Cells were considered as positives when 20 or more colonies were observed on plate 30 hrs after electroporation. To discard contamination, catalase (9) and oxidase (10) tests were performed for three colonies per plate.

Table 10 - Different replication origins tested in *B. sacchari.*

Plasmid Name	Ori	Growth
p**A**8a-CFP	p15A	+
p**B**6a-GFP	BBR1	+
p**E**6a-RFP	colE1	+
p**S**2a-RFP	SC101	+
pCV0021-YFP	RSF1010 oriV	-
pEXT21	IncW pSF6	-
pEXT22	IncFII R100	-

All BglBrick replication origins showed colonies after electroporation, strengthen the idea to use this platform for controlling expression in *B. sacchari.* Plasmids with replication origin from other groups of bacteria were also tested to increase our chances to find compatible replication origins. As shown in Table 10, no growth was detected for origins RSF1010 oriV and for origins from incompatibility groups IncW and IncFII. All transformations were performed including pBBR1-MCS2 plasmid as positive control. It should be noted that in this work, only the ability to generate colonies after transformation was tested and therefore no data for stability is available and should be performed to guarantee reproducibility.

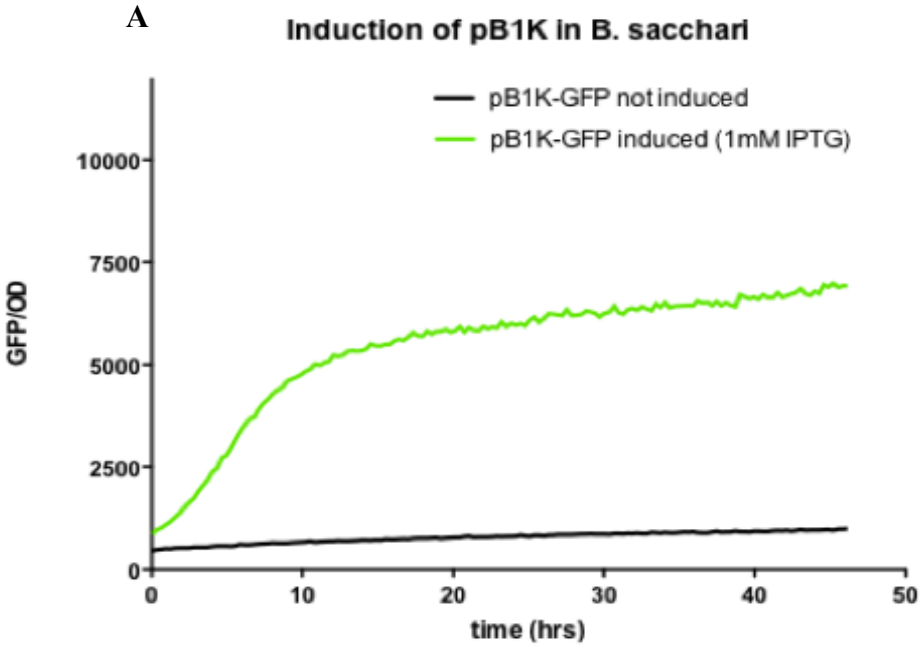
6.5.2 Alternative promoters in *B. sacchari*

Besides pLacUV5 and pBAD promoters detailed in Chapter 3, ability to drive expression of fluorescent proteins was evaluated for three more promoters using 96-well plate assay following the protocol in 4.3.2.8 section. Results shown in Table 11 and Figures 24 and 25, indicate that pTrc and pLlacO-1 were able to activate expression of GFP and showed induction of 18.1 and 2.5-fold respectively compared to the uninduced strain. pTet promoter

was unable to activate expression of GFP showing that maybe minimal promoter included in the plasmid (54 bp) is not recognized by transcription machinery of *B. sacchari.*

Table 11. Alternative promoters tested in *B. sacchari.*

Name	Promoter	Inducer	Induction
pB**1**k-GFP	Trc (*E. coli*)	IPTG	+++
pB**2**a-GFP	Tet (*E. coli*)	aTc	-
pB**5**k-RFP	lacUV5 (*E. coli*)	IPTG	+++
pB**6**a-GFP	LlacO-1 (*E. coli*)	IPTG	+
pB**8**k-RFP	BAD (*E. coli*)	arabinose	+++++



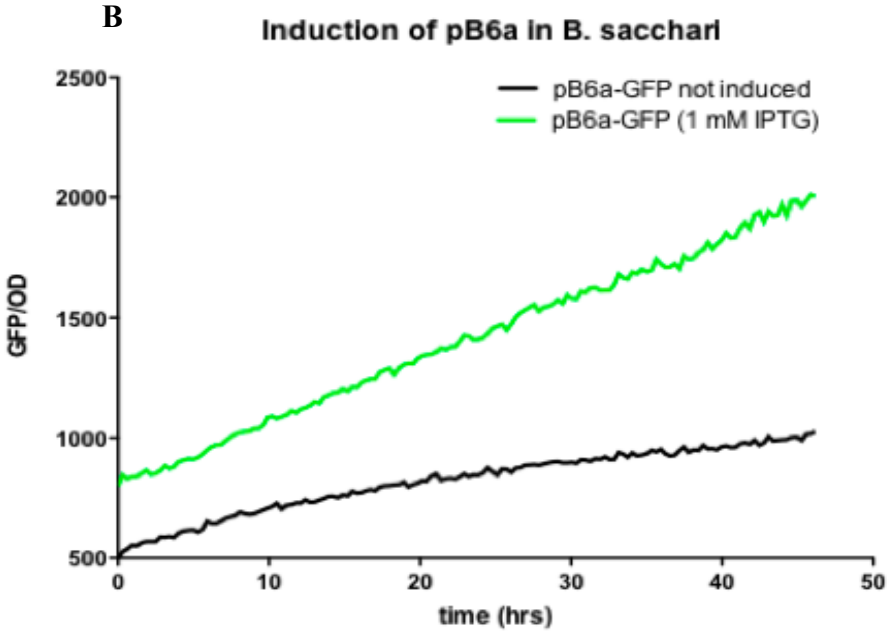


Figure 27 - Time-dependent expression of GFP activated by pTrc and pLlacO-1 in *B. sacchari*. Late-exponential cells of pB1k-GFP (A) and pB6a-GFP (B) were diluted 100 times in MMX (with and without inducer) and transferred to 96-well plates. GFP fluorescence and optical density (OD_{600}) were measured in triplicate every 15 min during 48 h. IPTG 1 mM (green line) was used as inducers. Fluorescence was normalized with optical density and is expressed in arbitrary units. No induced cells are shown in black for each case.

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Appendixes

A - Papers Published

Bentley GJ, Jiang W, **Guamán LP**, Xiao Y, Zhang F. Engineering Escherichia coli to produce branched-chain fatty acids in high percentages. Metab Eng. 2016 Jul 12;38:148–58.

Alexandrino, P.M.R., Mendonça, T.T., **Guamán Bautista, L.P**., Cherix, J., Lozano-Sakalauskas, G.C., Fujita, A., Ramos Filho, E., Long, P., Padilla, G., Taciro, M.K., Gomez, J.G.C. and Silva, L.F. 2015. Draft Genome Sequence of the Polyhydroxyalkanoate-Producing Bacterium Burkholderia sacchari LMG 19450 Isolated from Brazilian Sugarcane Plantation Soil. *Genome Announc* 3(3).

B - Manuscripts in progression

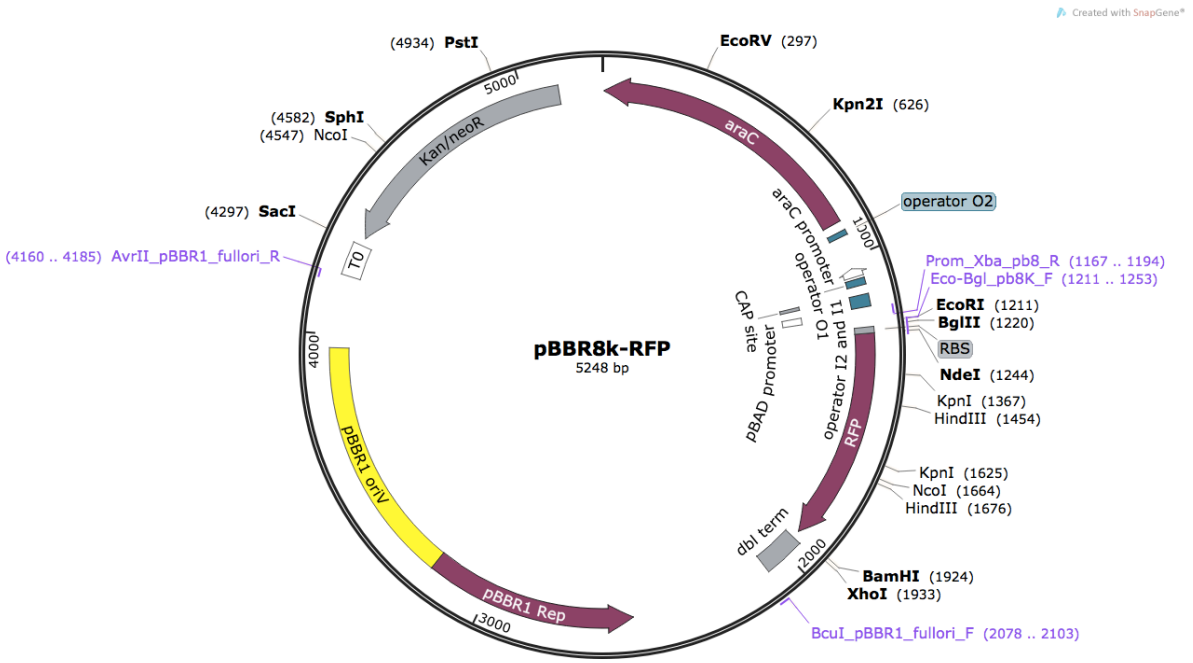
Guamán LP, Ramos de Oliveira E, Barba-Ostria C.A,Taciro, M.K., Gomez, J.G.C. and Silva, L.F. Improving growth rate and P(3HB) production in xylose, through xylA and xylB overexpression in Burkholderia sacchari.

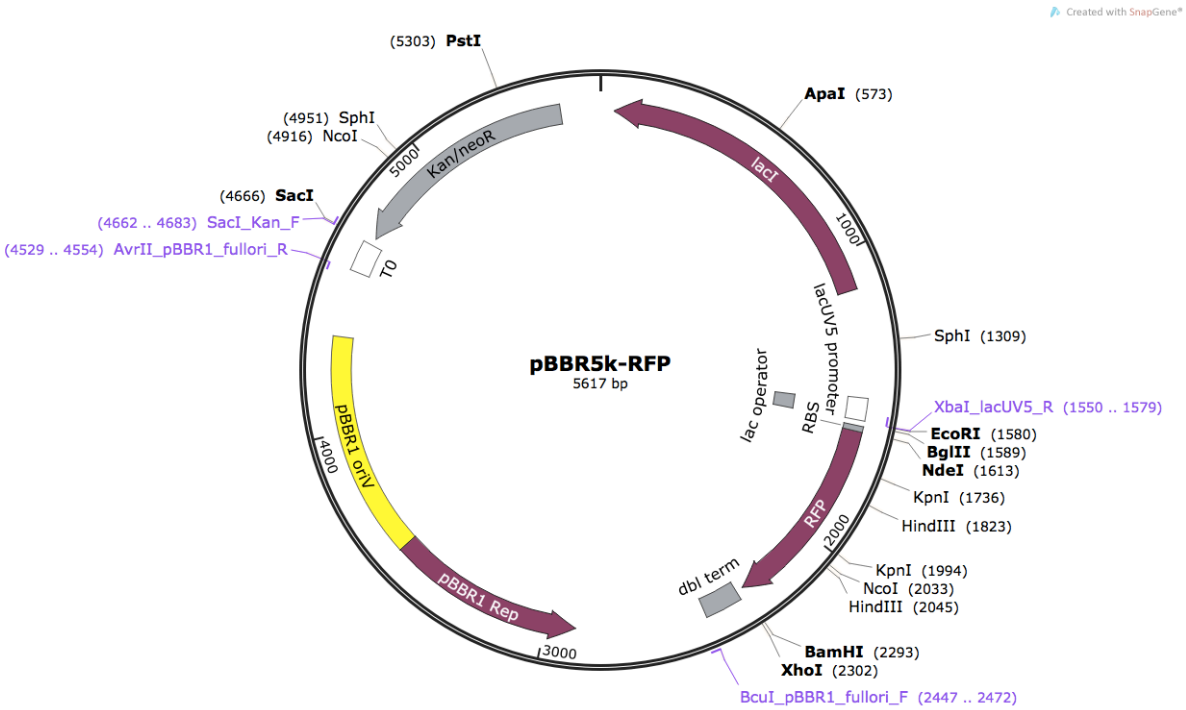
Guamán LP, Barba-Ostria C.A, Zhang F, Taciro, M.K., Gomez, J.G.C. and Silva, L.F. Engineering Xylose Metabolism for renewable production of Polyhydroxybutyrate in the non-model bacterium Burkholderia sacchari.

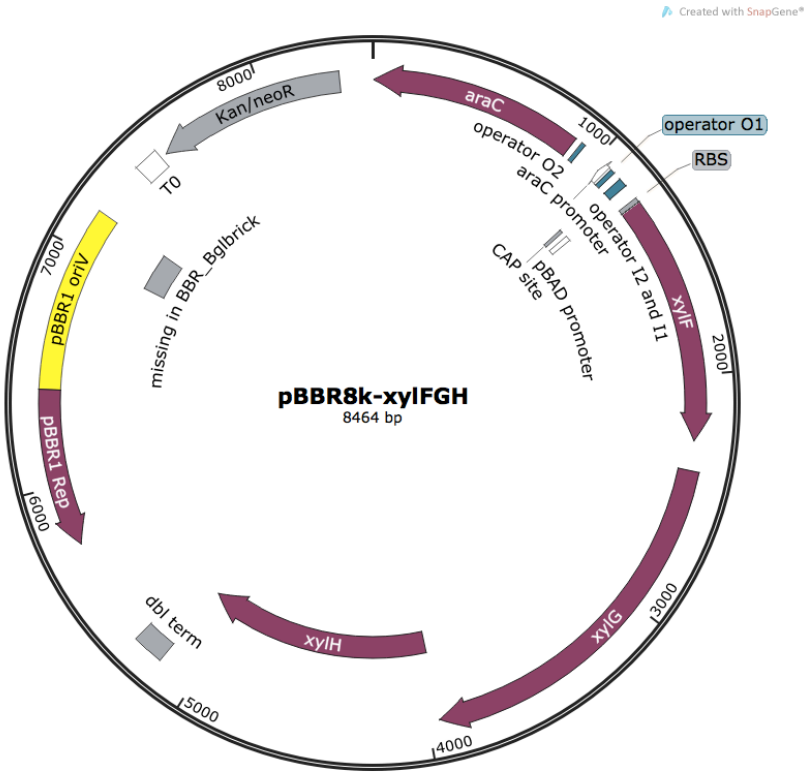
Guamán LP, Barba-Ostria C.A, Gomez, J.G.C. and Silva, L.F. Simultaneous compsuntion of xylose, arabinose and glucose for production of Polyhydroxybutyrate in *B. sacchari*

Ramos de Oliveira E, **Guamán LP**, Mendonça, T.T, Taciro, M.K., Gomez, J.G.C. and Silva, L.F. Genomic insertions of different PHA synthase genes using two transposon systems and its effects on P(3HB-co-3HA MCL) production by Pseudomonas sp. LFM046 and LFM461.

C - Plasmid maps



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8464 bp
araC
operator O2
araC promoter
operator O1
RBS
operator I2 and I1
xylF
xylG
xylH
pBAD promoter
CAP site
dbl term
missing in BBR_Bglbrick
T0
Kan/neoR
pBBR1 oriV
pBBR1 Rep
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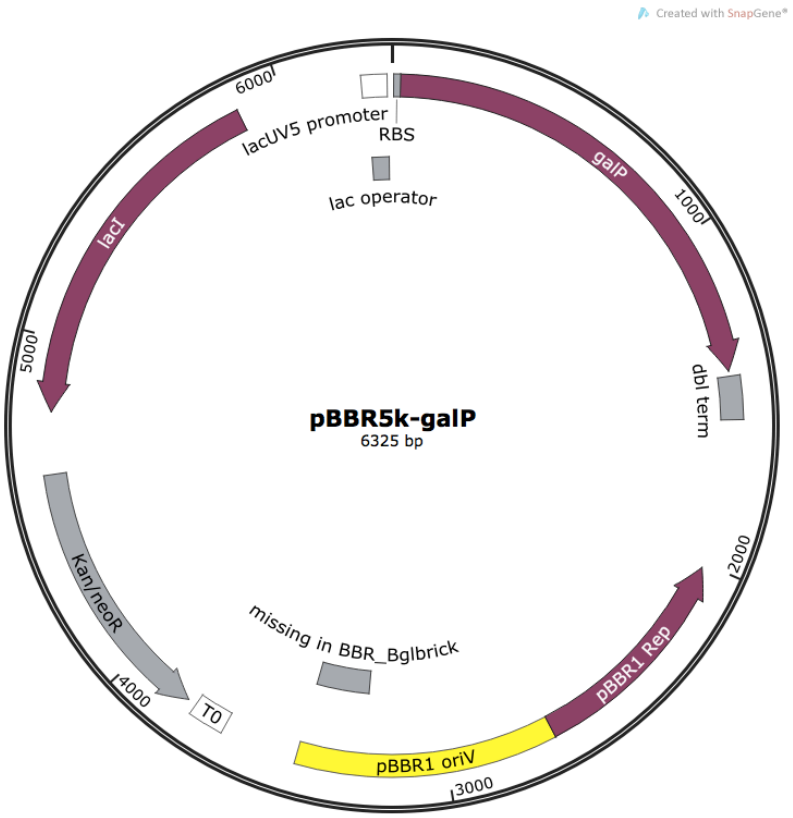
2000
3000
4000
5000
6000
7000
8000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lac operator
galP
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3000
4000
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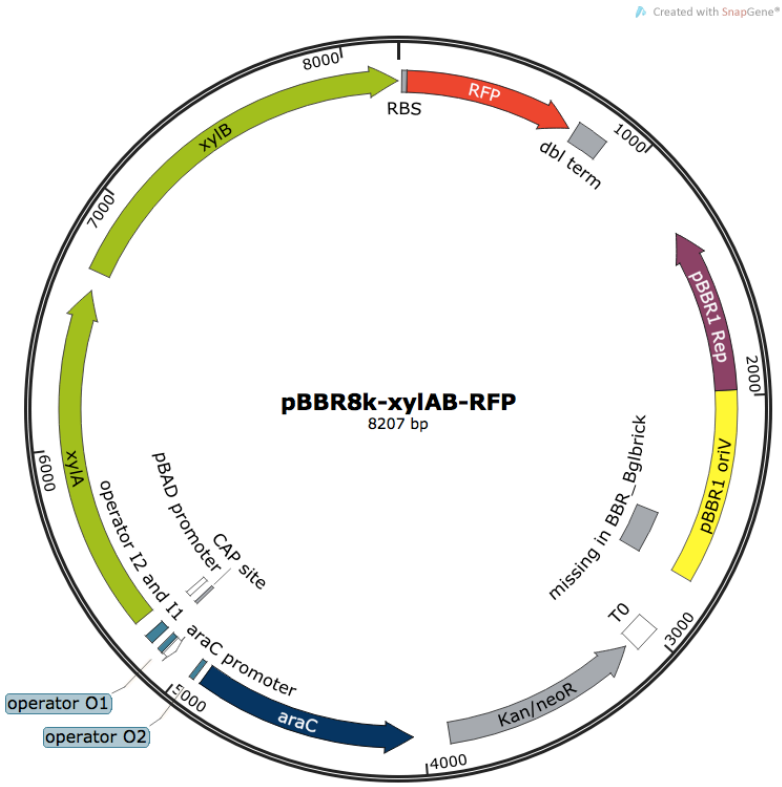
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RBS
RFP
dbl term
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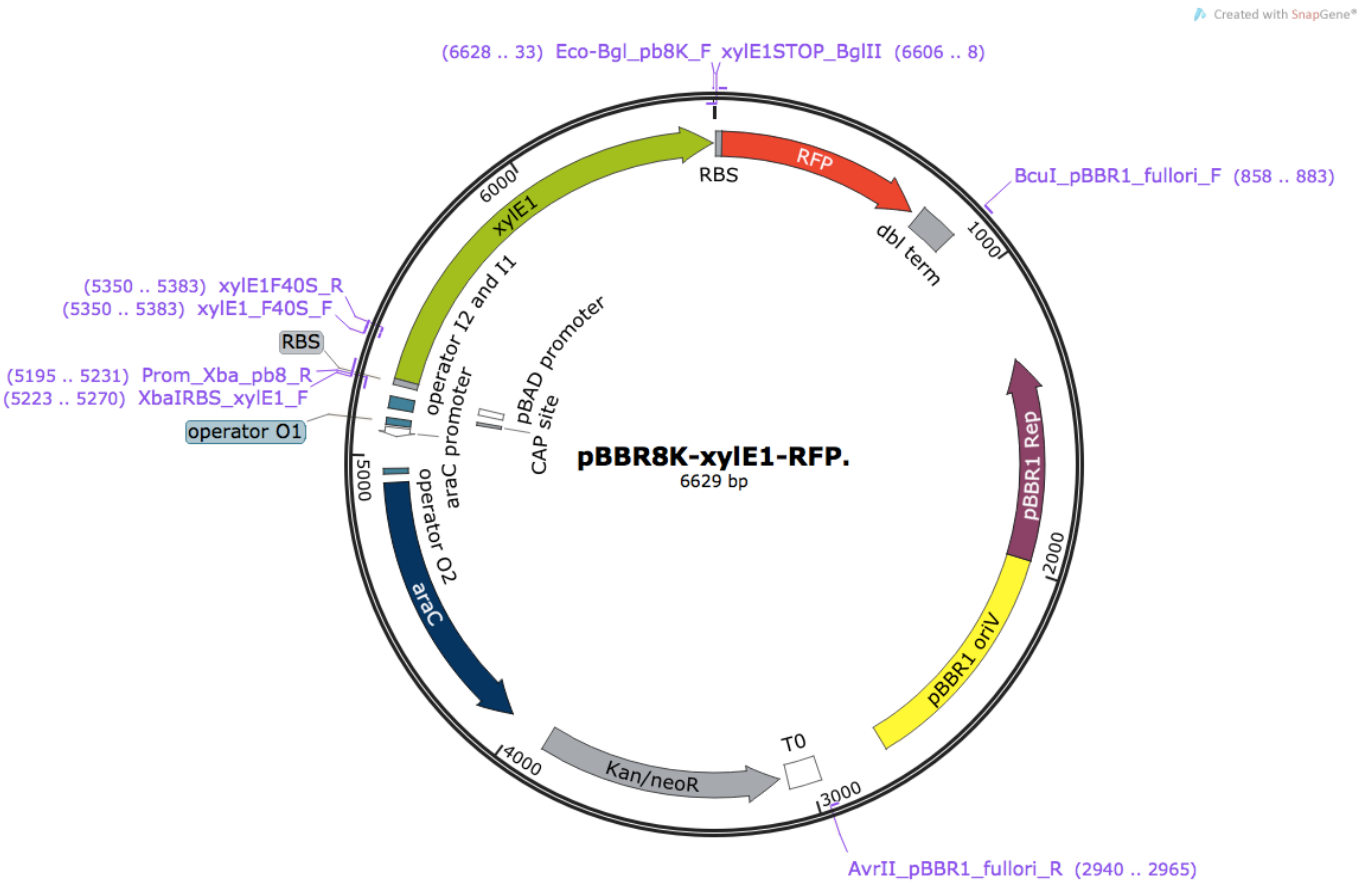
pBBR1 oriV
missing in BBR_BglBrick
T0
Kan/neoR
araC
araC promoter
operator O1
operator O2
operator I2 and I1
pBAD promoter
CAP site
xylA
xylB
1000
2000
3000
4000
5000
6000
7000
8000


Created with SnapGene®
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(5350 .. 5383) xylE1_F40S_F
RBS
(5195 .. 5231) Prom_Xba_pb8_R
(5223 .. 5270) XbaIRBS_xylE1_F
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pBBR8K-xylE1-RFP.
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RBS
RFP
dbl term
pBBR1 Rep
pBBR1 oriV
T0
Kan/neoR
araC
araC promoter
operator O2
operator I2 and I1
pBAD promoter
CAP site
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2000
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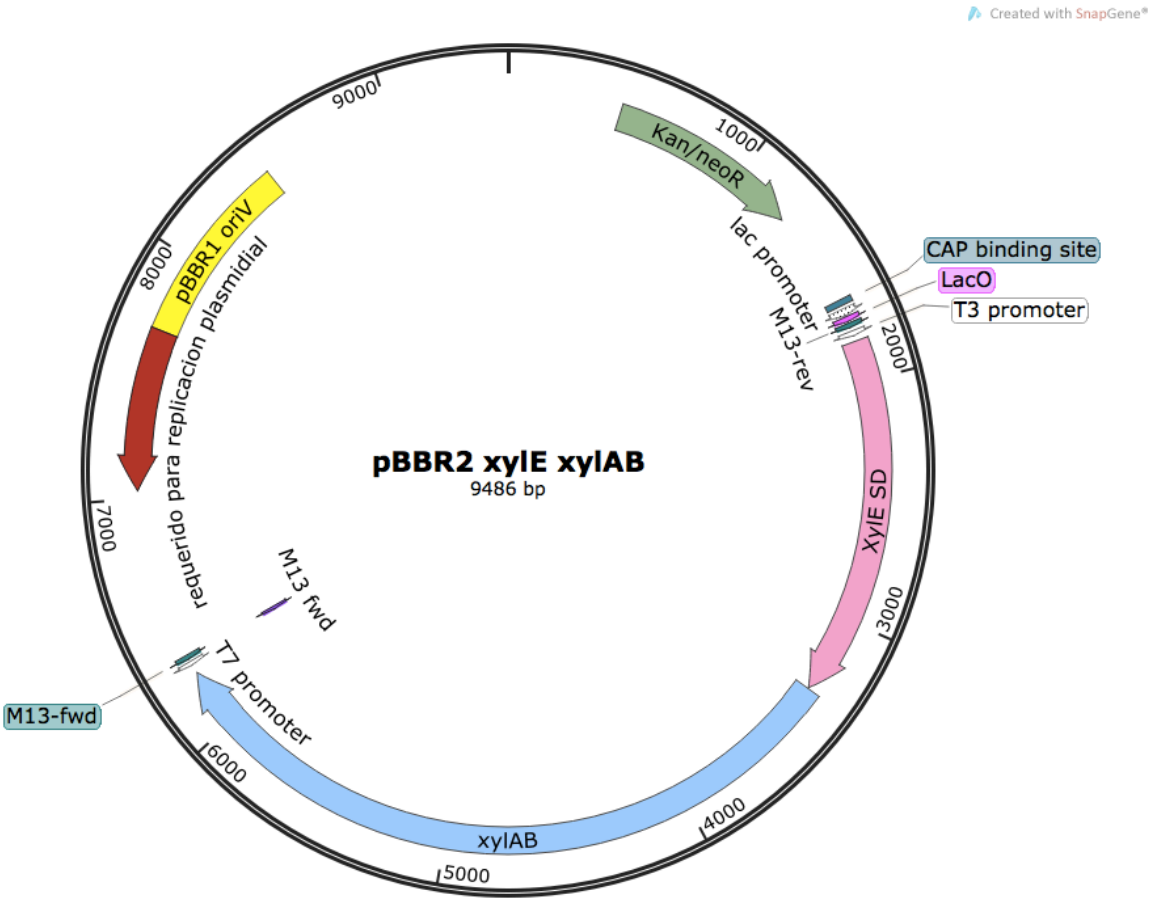
4000
5000
6000
AvrII_pBBR1_fullori_R (2940 .. 2965)


Created with SnapGene®
pBBR2 xylE xylAB
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5000
6000
7000
8000
9000
Kan/neoR
lac promoter
M13-rev
CAP binding site
LacO
T3 promoter
XylE SD
xylAB
T7 promoter
M13 fwd
M13-fwd
requerido para replicacion plasmidial
pBBR1 oriV