Production of polyhydroxybutyrate from milk whey fermentation by *Bacillus megaterium* TRQ8

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Producción de polihidroxibutirato a partir de la fermentación de suero de leche por Bacillus megaterium *TRQ8.*

Abstract

Plastics derived from oil represent a major environmental problem, due to their long period of degradation, high emissions of CO_2 and consumption of non-renewable resources. Thus, it is necessary to generate sustainable alternatives to produce natural polymers, such as polyhydroxybutyrate (PHB), a biopolymer produced from the fermentation of agro-industrial wastes by some microorganisms.
The aim of this work was to analyze the optimum culture medium composition and culture conditions to improve the yield of PHB production in *Bacillus megaterium* TRQ8. Milk whey was used as the carbon source and the fermentation was performed inside a bioreactor under controlled conditions. In order to achieve this purpose an initial conditioning pretreatment to remove the nutrients from the whey was implemented, afterwards, the fermentation and extraction of PHB were carried out; furthermore, different culture media containing ethanol and sodium acetate at different concentrations were tested to enhance the synthesis of PHB. As a result of the tests performed it was concluded that the medium with ethanol 1% v/v was the one that increased significantly the PHB production.

Key words: Bacillus megaterium, milk whey, fermentation, polyhydroxybutyrate (PHB), biofilms.

Resumen

Los plásticos que son producidos a partir del petróleo representan un problema ambiental importante, debido a su largo periodo de degradación, altas emisiones de CO_2 y por el consumo de recursos no renovables. Por lo tanto, es necesario generar alternativas sostenibles para producir polímeros naturales, como el polihidroxibutirato (PHB), que es un biopolímero producido a partir de la fermentación de residuos agroindustriales llevada a cabo por algunos microorganismos.
El objetivo de este trabajo fue analizar la composición óptima del medio de cultivo y las condiciones de cultivo para mejorar el rendimiento de la síntesis de PHB en *Bacillus megaterium* TRQ8. La fermentación se realizó dentro de un biorreactor con condiciones controladas y se utilizó suero de leche como fuente de carbono. Inicialmente se hizo un pretratamiento con la finalidad de eliminar los nutrientes del suero y posteriormente se llevó a cabo la fermentación y extracción del PHB; además, diferentes medios de cultivo que contenían etanol y acetato de sodio a distintas concentraciones fueron analizados con el objetivo de incrementar la producción de polihidroxibutirato. Con base en los resultados obtenidos se concluyó que el medio con etanol al 1% v / v fue el que aumentó significativamente la producción de PHB.

Palabras claves: Bacillus megaterium, Suero de leche, fermentación, Polihidroxibutirato (PHB), biopelículas.

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Introduction

Synthetic polymers include a wide range of materials synthetized by the polymerization of monomers derived from oil or gas, such as: polypropylene, polyethylene, polyvinyl chloride, polystyrene and polyethylene terephthalate (Andrady and Neal, 2009). Likewise, plastics are usually made from these polymers using different chemical additives. These materials are cost-effective, durable, lightweight and corrosion-resistant materials with high thermal and electrical insulation properties that can be extruded, molded and casted (Thompson *et al*, 2009b). They are used in the manufacture of everyday items that generate social benefits and comfort; many aspects of daily life involve the use of plastics in many different areas like transportation, telecommunications, clothing, footwear and especially for packaging (Thompson *et al*, 2009b).

The great variety of today´s polymers and the versatility of their properties provide an ideal raw material for the manufacture of many different plastic products that facilitate the transportation of food, beverages and other goods (Thompson *et al*, 2009b). Packaging is the most used application for these polymers and accounts for about 40.1% of the overall consumption (Lambert, 2013), however, most of them are non-biodegradable, and their increasing accumulation in the environment is generating several environmental problems (Tokiwa *et al*, 2009). Each year, an estimated 500 billion plastic bags are consumed worldwide (Myint *et al*, 2012), in addition, the global demand for these polymers has increased, which negatively impacts the economy around these products, because the crude oil is limited (North and Halden, 2013) and it has been estimated that 260 million tons of plastic are used per year, accounting for approximately 8% of world oil production (Thompson *et al*, 2009).

Among the most significant problems are that plastic pollution and accumulation in the environment (natural habitats, shores, oceans, etc.) affects wildlife due to entanglement and ingestion of plastic residues by animals, causing serious injuries, restriction of movement, breathing, lacerations, ulcers, reproductive problems and death (Webb *et al*, 2013). Also, many of the chemicals that are used in the fabrication of plastics are known to be toxic (Thompson *et al*, 2009). It has been proven that the chemicals used in the manufacture of plastics are present in human population through ingestion, inhalation or dermal contact (Talsness *et al*, 2009), this toxic substances, like the bisphenol A, polybrominated diphenyl ether (PBDE) and tetrabromobisphenol A (TBBPA), affect the reproductive system, disrupt thyroid hormone homeostasis, generate an anti-androgen action and cardiovascular diseases (Stahlhut *et al*, 2009). However the most concerning problem is the current unsustainable production and high consumption of natural resources like oil to satisfy the immense demand of plastic items (North and Halden, 2013).

Thus, the generation or production of other kind of sustainable and biodegradable materials using renewable sources to mitigate the high fossil energy consumption (Gautam, 2009) and CO_2 emissions is required (Naranjo, 2010). Natural polymers, such as polyhydroxybutyrate (PHB), represent a feasible and sustainable alternative for the fabrication of different biodegradable plastics (Getachew and Woldesenbet, 2016). One of the most important characteristics of this kind of polymers is their production using renewable carbon sources like milk whey, which is generated as an agro-industrial waste (González *et al*, 2013).

PHB is a polyester belonging to the polyhydroxyalkanoates family (Figure 1) that can be produced by several bacterial strains growing under stress culture conditions like nutrient depletion and an excess carbon source (Campuzano and Vasquez, 2013). Under these conditions, bacterial cells store PHB intracellularly as a reserve of carbon and energy in the form of granules (Babruwad *et al*, 2015).

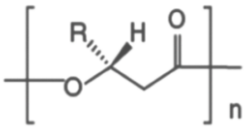

Figure 1. Chemical structure of Polyhydroxyalkanoates

PHB is considered as one of the main alternatives to replace traditional plastics because it has favorable physicochemical characteristics such as being a completely biodegradable, strong and highly hydrophobic material (Wang *et al*, 2007). The

chemical structure and physical properties of PHB are similar to that of polypropylene; however, its high melting temperature of 177°C makes the processing of this polymer difficult (Table 1) (Seoane *et al*, 2013).

The polymerization of the hydroxyalkanoic acids occurs by the action of intracellular enzymes forming an ester bond between the carboxyl group of one monomer with the hydroxyl group of the following (Figure 1) (Lemos and Mina, 2015). There are two ways for the synthesis of polyhydroxyalkanoates, the first is the in vitro production performed through a cell-free system from monomers just as lactones, hydroxyalkanoic acids or thioesters, which are not naturally synthesized, the second form, the in vivo production, is given by the genetic modification of plants or by the fermentation of substrates through microorganisms in order to induce the metabolic pathways of production of polyhydroxyalkanoates (Martínez, 2013).

The monomeric composition of these biopolymers depends on the metabolic pathways by which they are synthesized and the external carbon source used as raw material (Peña *et al*, 2014). The three metabolic pathways by which PHAs are synthesized to obtain their derivatives is through the degradation of fatty acids, fatty acid biosynthesis and the degradation of sugars to obtain Acetyl-CoA (Serrano, 2010). PHB is a derivative of PHAs and its metabolic pathway has been generally studied, as shown in figure 2. This explains how keto-thiolase enzyme catalyzes the reversible addition of an acetyl group to an acetyl-CoA molecule, the Acetoacetyl-CoA reductase reduces the Acetoacetyl-CoA molecules into 3 hydroxybutyryl-CoA and finally, the PHB synthetase catalyzes the polymerization reaction between the hydroxybutyrate molecules (Rivera, 2009).

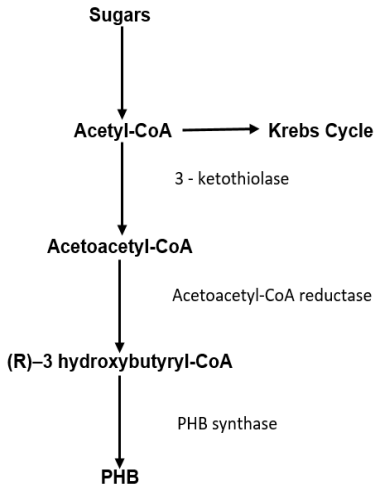

Figure 2. Metabolic pathway for the PHB synthesis (Rivera, 2009).

PHB is synthesized by more than 300 microorganisms, but not all of them accumulate enough to be used in large-scale production, the best producing bacteria that are capable of accumulating large amounts are: *C. necator*, *Azohydromonas*, *Pseudomonas oleovorans*, *Pseudomonas putida*, *Aeromonas hydrophila*, *Paracoccus denitrificans*, *Methylobacterium extorquens*, *Bacillus* spp., *Azotobacter vinelandii* and recombinant *E. coli* (Peña *et al*, 2014).

The required microbial characteristics for selecting a promissory strain is its short duplication time, high yield of PHB synthesis, consumption of a cheap carbon source, and the quality of the PHB produced (Lemos and Mina, 2015). Thus, the species *Bacillus megaterium*, a Gram-positive bacilli, aerobic and sporulated bacteria presents all the characteristics previously mentioned (Vary *et al*, 2007). The macroscopic characteristics of this species are large and convex colonies with uniform borders, they are also moist and non-hemolytic (Forbes, 2009). In addition, the PHB is produced by this bacterial species by the fermentation of raw materials like agro-industrial wastes, as it is the case

Table 1. Comparison between polyhydroxybutyrate and polypropylene properties (Singh, 2015).

Property	Polyhydroxybutyrate	Polypropylene
Melting temperature (°C)	177	176
Glass transition temperature (°C)	2	-10
Crystallinity (%)	60	50-70
Tensile Strength (MPa)	43	38
Extension to break (%)	5	400

of the milk whey, which is the liquid obtained during cheese production by coagulating and separating casein proteins from milk (Naranjo, 2010). Whey contains a large amount of nutrients such as proteins, lipids and minerals, since it retains 55% of the total milk nutrients it also has a high lactose content close to 5% or 94 g l^{-1} (Bovo, 2014). It is estimated that 1 or 2 kg of cheese can be produced from every 10 liters of cow's milk, and an approximate from 8 to 9 kg of milk whey is produced in this industrial process. Thus, this product is the most viable alternative to be used as substrate for the production of PHB (Naranjo, 2010). The milk whey composition is characterized by the origin of the milk, generally 70% of crude proteins (beta-lactoglobulin, alpha-lactoglobulin, and immunoglobulin), protease-peptones, and native enzymes remains in the serum (Valencia, 2009).

Therefore, the synthesis of PHB by the bacterial fermentation of milk whey is a promissory alternative to replace the plastics derived from petroleum, such as propylene and polypropylene (Naranjo, 2010). In addition, the use of milk whey as a carbon source in this process mitigates the environmental problems caused when it is discarded to the environment without any treatment (Valencia, 2009). PHB can also be used in 3D printing, tissue repair and polymer-based depots for controlled drug release or implants (Luef *et al*, 2015).

The objective of this work is to present the PHB as a new alternative polymer for the production of biofilms and to determine the optimum culture conditions to enhance its synthesis in *B. megaterium* TRQ8.

Materials and methods

Bacterial strain

The strain *Bacillus megaterium* TRQ8 used in this work was isolated from wheat rhizosphere in the Yaqui Valley, Sonora, México. This strain belongs to Colección de Microorganismos Edáficos y Endófitos Nativos del Instituto Tecnológico de Sonora (www.itson.mx/COLMENA) (de los Santos-Villalobos *et al*., 2017).

Pre-treatment of the Milk Whey

To be able to use the milk whey as a substrate for the production of PHB by B. megaterium TRQ8 fermentation, it was necessary to sterilize and to remove the proteins present in the serum. The milk whey was sterilized at 115 °C for 15 minutes, causing the precipitation of a fraction of proteins (Bell *et al*, 1983). The supernatant obtained from the sterilization was filtered using a cellulose-acetate fiber membrane, then the pH of the supernatant was adjusted to 7.0 using a 12N NaOH solution. Finally the solution was centrifuged at 4,400 rpm for 15 minutes and the supernatant obtained in this step was used to prepare the culture medium. (Campuzano *et al*., 2013).

Growth Conditions for Fermentation

For the fermentation of B. megaterium TRQ8 were used 1.0 x 103 Unit Forming Colonies ml-1, which was carried out in a bioreactor with a capacity of 5 l during 48 hours at 32 °C and 200 rpm. The total volume of whey and culture medium were 2 l, with 10% v/v of the culture medium composed by KH2PO4, 1.5 g l^{-1}; Na_2 PO_4, 9 g l^{-1}; $MgSO_4$ $7H_4O$, 0.2 g l^{-1}; and 1 ml l^{-1} of a solution of trace elements composed of: $FeSO_4$ $7H_2O$, 10 g l^{-1}; $ZnSO_4$ $7H_2O$, 2.25 g l^{-1}; $CuSO_4$ $5H_2O$, 1 g l^{-1}; $MnSO_4$ $4H_2O$, 0.5 g l^{-1}; $CaCl_2$ $2H_2O$, 2 g l^{-1}; H_3BO_4, 0.23 g l^{-1}; $(NH4)Mo7O_{24}$, 0.2 g l^{-1}; and 35% HCl, 10 ml. (Heinrich *et al*, 2012; Campuzano *et al*., 2013).

PHB Extraction, Quantification and Production of the Biofilms

After 48 hours of fermentation, the culture was centrifuged at 4,400 rpm during 30 minutes, the supernatant was discarded and the dry weight of the cells in g/ml was measured from the obtained pellet. Then, it was digested with a 4% sodium hypochlorite solution at a temperature of 37 °C for 20 minutes, the solution was centrifuged again at 4,400 rpm for 30 minutes. The pellet was then washed with acetone, water and ethanol 70%. Once the PHB was obtained, its weight was measured and the percentage of accumulation, in g/ml, was calculated using the following formula (Mikkili *et al*, 2014):

$$\%\ of\ PHB\ production = \frac{Dry\ weight\ of\ PHB}{Dry\ weight\ of\ cells} * 100$$

Finally, 100 ml of chloroform were added at 60 °C during 15 minutes for each 3 g of PHB obtained, the mixture was placed on a cellulose paper inside a Petri dish and the solvent was evaporated at room temperature in a vacuum for 24 hours (Cyras *et al*, 2007).

Optimization of PHB Production

The experiment was carried out with the same inoculation, culture medium, and conditions previously mentioned, however, 3 modifications were evaluated with the aim of improving the yield of the PHB, such as the addition of: i) 1% v/v of ethanol, ii) 3% v/v of ethanol, iii) 1% v/v of sodium acetate, and iv) 3% v/v of sodium acetate, 27 hours after incubation, when the bacteria was in the stationary phase. In addition, the amount of oxygen available in a flask was increased to quantify its effect on the PHB synthesis (Table 2) All the samples were done by duplicate. After 48 hours of fermentation, the amount of PHB was quantified, according to the protocol mentioned before.

Statistics analysis

Data were analyzed by ANOVA analysis of variance, using Fisher LSD test (P= 0.5), using the Statgraphics Plus 5.1 software.

Results and discussion

All the treatments, evaluated by duplicate, allowed the synthesis of PHB from the milk whey fermentation by B. megaterium TRQ8, since all of them had the required conditions aforementioned.

The application of external stress has been reported as an efficient strategy to improve the production of PHB (Obruca *et al*, 2011). Thus the culture medium containing 1% of ethanol increased the production of polyhydroxybutyrate to 46% (control treatment: 28%) (Figure 3).

In this case, the strain TRQ8 oxidized the ethanol present in the medium and transformed it to Acetyl-CoA, which is the main substrate of the PHB pathway (Obruca *et al*, 2011). As it is shown in figure 4, also during these oxidative reactions, reduced coenzymes NAD(P)H are produced, stimulating the flux of acetyl-CoA towards the PHB biosynthetic pathway (Obruca *et al*, 2010c).

Table 2. Composition of the culture medium inside the flasks.

	Milk Whey (ml)	Buffer (ml)	Solution of trace elements (µl)	Ethanol (ml)	Acetate (g)
Control	189	21	210	0	0
Ethanol 1%	189	21	210	2.19	0
Ethanol 3%	189	21	210	6.57	0
Acetate 1%	189	21	210	0	3.076
Acetate 3%	189	21	210	0	9.23
Oxygen	54	6	60	0	0

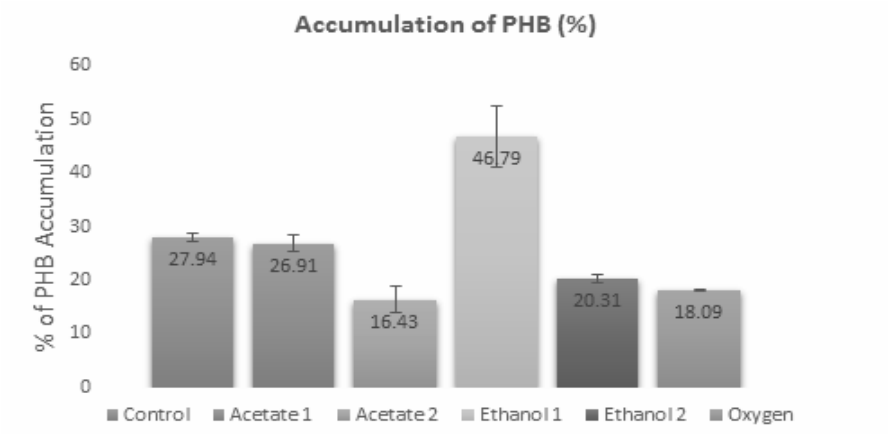

Figure 3. Percentage of PHB accumulation in Bacillus megaterium TRQ8, growing under different compositions of culture media.

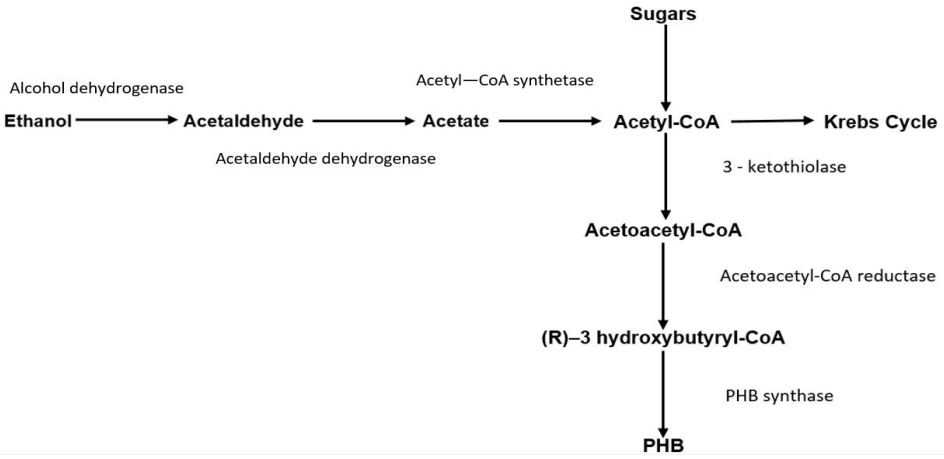

Figure 4. Metabolic pathway of ethanol oxidation and PHB fermentation.

Reduced coenzymes suppress the TCA cycle, furthermore, it has been proven that the accumulation of reduced coenzymes during ethanol oxidation stimulates the activity of the 3-ketothiolase and acetoacetyl-CoA reductase enzymes from the PHB pathway resulting in an overproduction of the polymer (Figure 4) (Oeding and Schlegen, 1973). Hence, less free CoA, which inhibits PHB biosynthesis, is formed as a result of TCA cycle inhibition and instead it is used to build acetyl-CoA (Obruca *et al*, 2010b). For this reason the concentration of NAD(P)H is considered to be the major regulatory factor determining the flux of acetyl-CoA to either TCA cycle or PHB Pathway (Obruca *et al*, 2010c).

However, the production of PHB diminished with 3% of ethanol in the culture medium, which suggests the toxic effect of high ethanol concentration for the strain, affecting its growth, viability, and metabolism, due to ethanol-induced leakage of the plasma membrane (Ingram, 1990). Ethanol causes an increase in membrane fluidity and can alter its integrity because it interacts with membrane proteins, causing conformational changes and thereby influencing their function (Tóth *et al*, 2014). For this reason, it is possible that a concentration of 3% of ethanol in the medium killed a large number of bacteria, and therefore caused a decrease in the synthesis of PHB.

On the other hand, the culture media containing sodium acetate did not show higher production. It is known that a large number of bacteria use acetate for the synthesis of lipids and other compounds (Guifang *et al*, 2002), therefore, there is a high probability that B. megaterium TRQ8 has used the sodium acetate available in the culture medium to produce lipids instead of PHB (Fei *et al*, 2016), since the percentage of accumulation at both concentrations were lower in comparison with the control (Figure 3).

The production of PHB was lower in the flask with a higher amount of oxygen because the carbon flux was bigger towards the Krebs cycle than the fermentation pathway (Nath *et al*, 2008). A limited amount of dissolved oxygen concentration increases the fluxes of acetyl CoA towards PHB. The shortage of oxygen prevents the electron transport chain to work due to the absence of an electron acceptor, consequently, an alternative pathway involving the production of an organic compound, in this case, PHB is promoted to regenerate the electron carrier NAD+ (Baron, 1996). Therefore, a limited dissolved amount of oxygen increases the flux of acetyl-CoA, the common precursor of both polyhydroxybutyrate and TCA pathways, towards PHB accumulation instead of TCA cycle (Nath *et al*, 2008).

Conclusions

Bacillus megaterium TRQ8 is able to produce high quantities of PHB under exogenous stress conditions, such as a culture medium containing 1% of ethanol. Thus, it is possible to increase the yield of PHB synthesis by adding a volume of 1% of ethanol to the culture medium for this strain. The

other media did not have the expected results due to various factors like alternative metabolic pathways or inhibition of the development and death of the microbial population. Also the amount of available oxygen is another important factor to take into account, since the PHB production is stimulated by oxygen limitation.

However the high cost of production is the main drawback of this kind of plastics and has prevented the PHB from being used in different items, thus the reduction of the manufacturing costs is the main aspect to be improved.

The application of an external stress, like ethanol, is a promising strategy to enhance the production of PHB from cheese whey using B. megaterium. Nevertheless many other strategies can be applied in order to obtain better results. For instance, the utilization of a recombinant bacterium with better growth characteristics will require less time to produce the polymer. Moreover, the deletion of other metabolic pathways (fermentation) and the overexpression of the 3-ketothiolase enzyme can redirect the carbon flux towards the desired pathway and hence increase the yield of synthesis thereby reducing the costs of production to obtain similar values to those of synthetic polymers.

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