STUDY FOR POLY[(R)-3-HYDROXYBUTYRATE] PRODUCTION BY BACTERIAL STRAINS AND ITS MODIFICATION FOR DRUG DELIVERY SYSTEMS

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I have become my own version of an optimist. If I can't make it through one door, I'll go through another door - or I'll make a door. Something terrific will come no matter how dark the present

Tinc la meva pròpia versió de l'optimisme. Si no puc creuar una porta, creuaré una altra o faré una altra porta. Una cosa meravellosa vindrà, no importa lo fosc que estigui el present

Tengo mi propia versión del optimismo. Si no puedo cruzar una puerta, cruzaré otra o hare otra puerta. Algo maravilloso vendrá, no importa lo oscuro que esté el presente

Rabindranath Tagore

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The international concern of finding a solution to the need of replacing the petroleum-based polymers that are creating significant environmental problems due to the produced wastes is well-known. Biopolymers appear to be a great sustainable alternative since they are biodegradable and are produced from renewable sources. Biopolyesters, such as polyhydroxyalkanoates (PHAs), which are synthesized by microorganism, are of special interest lately. The most common and probably the most abundant of this general class of microbial polyesters is the biocompatible and biodegradable polyhydroxybutyrate (PHB). Nowadays, these biopolymers are not yet competitive with the traditional polymers, mainly due to their high production costs. However, a decrease in these costs has been achieved by the latest researches in this field. It is therefore important to continue investigating new bacteria capable of accumulating and producing greater amounts of biopolymer than bacteria already used in the industry, such as *Cupriavidus necator*, with the aim of reducing the production cost even more. All this knowledge was motivation enough to start a new line of research based on this type of biopolymers in order to investigate the possible solution to the environmental problematic issues.

Knowing that some halophilic bacteria accumulate significant amounts of PHB, the opportunity to travel to Bolivia was taken to obtain some samples from different salty lakes in order to find PHB producing bacteria. Therefore, the thesis was mainly focused on finding the greatest bacterium capable of accumulating biopolymer, and on the study of its biosynthesis, together with the material transformation and its application as biocompatible material for drug delivery particles. Taking all this into consideration, it can be said that this is a highly interdisciplinary thesis for which, not only a thorough knowledge of bio-/chemistry, microbiology, cell biology, physiology, and enzymologist have been indispensable but also biotechnological and engineering techniques. For this reason, and in order to collaborate with specialist researchers in these sciences/disciplines, the thesis has been carried out in different departments of the *Universitat Politècnica de Catalunya-Barcelona Tech* (UPC-Spain), and in collaboration with the *Institut für Biotechnologie und Bioprozesstechnik* from the *Technische Universität Graz* (TU Graz-Austria).

This thesis manuscript has been structured into different parts. It starts with an **introduction** where the problematic situation related to the plastic residues is described. It also includes a summarized review of the previous research (state-of-the-art) as well as the explanation of the basic concepts about PHAs, and specifically, about the biosynthesis and the industrial production of PHB. Likewise, the possible applications of these biopolyesters are explained, especially as a drug delivery system. The main **objectives** of the thesis are separately compiled.

In order to clarify the content of the main body of the thesis and taking into account the chronology of the study, this manuscript has been divided into three main **chapters**. Each chapter features two or more sections. Some of the sections have been submitted as manuscripts to specific scientific journals and several have been published.

CHAPTER I. Biopolymer production. It includes the bacteria isolation from environmental samples, the detection of the biopolymer-producing bacterial strains, the selection of the greatest biopolymer-

producing bacteria, the determination of the biopolymer production by the selected strain, and the entire characterization of the biopolymer produced by the selected bacterium. Also, two fermentations for PHB production from different collection bacterial strains are included here. The initial work was fulfilled at the *Departament d'Òptica i Optometria* from the UPC-Campus Terrassa, in the *Laboratori de Microbiologia Sanitària i Mediambiental* (MSMLab). It was carried out in collaboration with the TU Graz and was performed within the installations of *Institut für Biotechnologie und Bioprozesstechnik*. As a result of this study, four articles have been written and sent to scientific journals. The first and the second sections of this chapter have been accepted for publication as an original scientific paper in *Food Technology and Biotechnology* and in *Journal of Applied Microbiology*, respectively. The third and the fourth sections are being considered for publication.

CHAPTER II. Biopolymer transformation. Once the first part was concluded, the thesis was mainly focused on the transformation of the biomaterial. Thus, the decrease in the molecular mass of the biopolyester by enzymatic reaction was the main study in this second part. In both sections of this second chapter, the enzymatic degradation of a PHA copolymer, and the application of such degradation to the studied PHB are included. All these tasks were carried out at the *Departament d'Ènginyeria Química* from the UPC-Campus Terrassa, exactly in the *Laboratori de Catàlisi Enzimàtica* (*Enginyeria i Biotecnologia* group). As a result of these studies, two articles have been published in the journal *Polymer Degradation and Stability*.

CHAPTER III. Biopolymer application. The study of the material application was focused on the field of drug delivery systems. Specifically, the preparation of biopolyester micro- and nano-particles loaded with an antibiotic for the treatment of destructive periodontal diseases. The study of their antibacterial properties was also carried out. This third chapter is divided into two sections and the study was achieved by the collaboration of two departments from the UPC: the *Departament d'Òptica i Optometria* and the *Departament de Ciència de Materials I Enginyeria Metal·lúrgica* (Biomaterials, Biomechanics and Tissue Engineering group). A manuscript containing all of the work described in this chapter has been submitted for publication as original scientific paper.

All **conclusions** resulting from the different studies that constitute the thesis are compiled after the main chapters. It was also important to include the summary of the **scientific production** which is the fruit of the labour of the thesis, and a list of the **future and further studies** related to the thesis.

The thesis was mainly economically supported by the participant organisms and by the *Comisión Interministerial de Ciencia y Tecnología*: MAT2006-05979 (Synthesis, study and application of novel polymeric materials with controlled structure and fluorescence sensors- study of the environmental degradation) and MAT2009-13547 (Biomaterials with antimicrobial and bone regeneration properties for oral and maxillofacial surgery).

ABSTRACT/RESUMEN

Nowadays biopolymers are a good alternative to petrochemical polymers which are one of the most frequently used materials in our society, and due to their non-biodegradability have created serious ambient problems. One of these biopolymers is Poly[(R)-3-Hydroxybutyrate] (PHB) which is produced from renewable resources by various microorganisms such as the bacterium *Cupriavidus necator* and, after its use it can be composted. It has different applications as bulk material in packing films, containers or paper coatings. Due to its excellent biocompatibility and biodegradability, PHB is a potential material for applications in drug delivery. It is therefore important to continue investigating the different strategies to ensure the competitiveness between these biopolymers and petroleum-based polymers.

The **first part** of the thesis is mainly focused on finding a new PHB-producing bacterium capable of developing and producing a significant concentration of biopolymer using one of the conventional medium for PHB industrial production. Therefore, four water and mud samples were taken from four Bolivian salty lakes: Laguna Colorada, Laguna Hedionda, Laguna de Chiguana, and Uyuni Salt Lake. The microorganisms were isolated in order to detect the best PHB-producing bacterial strain. A bacterium identified as a new strain of the genus *Bacillus* was selected for polymer production studies. The strain was deposited in the Spanish Type Culture Collection (CECT), as *Bacillus megaterium* uyuni S29. This microorganism was tested under fed-batch fermentations using an industrial media for biopolymer production, with glucose as a sole carbon source, and nitrogen limitation was the main strategy to induce PHB accumulation. The results of the first fermentation under controlled conditions showed a high specific growth rate, and a final percentage of 30% PHB in cell dry mass (CDM), with no limitation in nitrogen source. In a second fed-batch experiment, the limitation of the nitrogen source was achieved and the polymer content reached its maximal value of around 70% of the CDM. The strain not only grew properly in the industrial condition proposed, but it also produced and accumulated a larger content of PHB than ever reached before for its genus. Also, fermentations in shaking flasks with different NaCl concentrations showed that the bacterium is capable of producing PHB in the presence of high salt concentrations. These results make the strain appealing not just to utilize it in the biotechnological production of PHB, but for other applications such as the treatment of salty wastewater.

On the other hand, the polymer extraction was carried out with a solvent/anti-solvent method using chloroform and ethanol. An alternative polymer extraction method was done via acetone in order to study the possibility of extracting different types of biopolyesters. The characterization of the resultant biopolymers concluded that *Bacillus megaterium* uyuni S29 synthesized PHB homopolymer with two main molecular masses around 600 and 125 kDa with polydispersity index (PDI) of 1.2 and 1.5 respectively. Moreover, the thermal analyses of the biomaterial showed different thermal properties compared to the ones of common PHB, enlarging the application possibilities to this biopolyester.

Furthermore, two fermentations in a bioreactor were carried out from two collection strains, *Cupriavidus necator* (DMS 545) and *Burkholderia sacchari* (DMS 17165), and using a cheap carbon

substrate, glycerol. The purpose of this work was to study the influence of this carbon source on the growth kinetics of the above mentioned strains, on their polymer productions, and on the molecular mass of the produced biopolymer. The PHBs produced in these fermentations were used in the thesis for studies of polymer transformation and application, together with the PHB produced by *Bacillus megaterium* uyuni S29.

After obtaining the polymer from the bacteria, the **second part** was focused on its transformation. The molecular masses of PHA produced from bacteria usually have a relatively high molecular mass (M_w) around 200 and 3,000 kDa. They are unsuitable for molecular design of special polymers such as amphiphilic block copolymers. For such applications, more manageable molecular masses are required. Therefore, a sustainable degradation process set out for an easy industrial scale application was proposed in the second part of this thesis. First, a copolymer from PHA family, poly[3-hydroxybutyrate-co-4-hydroxybutyrate] (P(3HB-co-4HB), was used to carry out an enzymatic degradation of the material. Two commercially available lipases were used to decrease the polymer molecular mass to oligomers between 5 and 1 kDa. It was the first time that these triglyceride lipases were used for this type of application and due to the positive results one of them was used for degradation of PHB. The results confirmed the enzymatic reaction of the used lipase with PHB and showed a controlled decrease of the molecular mass from 300 kDa to 4 kDa.

In the **third part** of the study, the application of the PHB as a drug delivery system was analyzed. There are very few papers reporting on the incorporation and subsequent release of therapeutic agents with PHB systems and none of them relate to doxycycline (DOXY). DOXY is a well known broad-spectrum antibiotic, and it has been frequently used in treating destructive periodontal diseases such as juvenile periodontitis or acute periodontal abscesses. However, there are some concerns over possible side effects such as gastro-intestinal disturbance. In order to avoid the side effects and reach the infection with an effective drug concentration, protection and controlled delivery of DOXY are desirable. Thus, different drug-entrapment and emulsification methods were studied in order to obtain optimal DOXY-loaded PHB micro- and nano-particles. The results showed that the combination of ultrasounds with high speed stirring in the preparation of double emulsion is highly effective in obtaining DOXY-loaded PHB particles with high drug loading and entrapment efficiency, and with great method efficiency. Furthermore, the qualitative antibacterial activity found in different formulations show that these particles are good candidates for drug delivery systems. Due to these great results, the best methods found for PHB particles formation were used with the PHAs produced throughout the thesis in order to confirm their suitability for different PHAs and to study the possibility of improving the results of drug entrapment and method efficiency.

Actualmente, los biopolímeros son una buena alternativa a los polímeros derivados del petróleo, que son uno de los materiales más utilizados en nuestra sociedad, y junto con su escasa o nula biodegradabilidad crean graves problemas ambientales. Uno de estos biopolímeros es el poli[(R)-3-hidroxibutirato] (PHB) producido a partir de recursos renovables por diversos microorganismos tales como la bacteria *Cupriavidus necator* y que después de su uso se puede compostar. Tiene diferentes aplicaciones como material a granel para films de embalaje, recipientes o revestimientos de papel. Debido a su excelente biocompatibilidad y biodegradabilidad, el PHB es un material con gran potencial para ser aplicado como vehículo para administración de fármacos. Por tanto, es importante investigar las diferentes estrategias para garantizar la competitividad de estos biopolímeros enfrente de los polímeros procedentes del petróleo.

La **primera parte** de la tesis se centra principalmente en la búsqueda de una nueva bacteria productora de PHB capaz de desarrollar y producir una concentración significativa de biopolímero usando un medio convencional para la producción de PHB de forma industrial. Se partió de cuatro muestras de agua y barro tomadas de cuatro lagos salados de Bolivia: Laguna Colorada, Laguna Hedionda, Laguna de Chiguana y Salar de Uyuni. Los microorganismos se aislaron con el fin de detectar las mejores cepas bacterianas productoras de PHB. Para los estudios de producción de polímeros, se seleccionó una nueva cepa del género *Bacillus*, identificada como *Bacillus megaterium* uyuni S29. Esta cepa fue depositada en la Colección Española de Cultivos Tipo (CECT). Para la producción de biopolímero se utilizó este microorganismo en diferentes fermentaciones fed-batch con un medio industrial con glucosa como única fuente de carbono y limitación de nitrógeno para inducir su acumulación. Los resultados de la primera fermentación en birreactor mostraron una alta tasa de crecimiento específico y un porcentaje final de PHB de 30% en masa celular (CDM), sin llegar a limitar la fuente de nitrógeno. En un segundo experimento se limitó la fuente de nitrógeno y el contenido de polímero alcanzó su valor máximo (70% del CDM). La cepa no sólo creció adecuadamente en las condiciones industriales propuestas, sino que también produjo y acumuló un gran contenido de PHB que no se había logrado antes con este género. Además, las fermentaciones con diferentes concentraciones de NaCl que se llevaron a cabo en un nuevo experimento mostraron que la bacteria es capaz de producir PHB en presencia de altas concentraciones de sal. Estos resultados determinan que la cepa sea atractiva no sólo para utilizarla en la producción biotecnológica de PHB, sino también para otras aplicaciones como por ejemplo en el tratamiento de aguas residuales saladas.

La extracción de polímero se llevó a cabo con un método de solvente/anti-solvente utilizando cloroformo y etanol. Se utilizó un método alternativo de extracción de polímero mediante acetona con el fin de estudiar la posibilidad de extraer diferentes tipos de biopoliésteres. La caracterización de los biopolímeros resultantes concluyó que *Bacillus megaterium* uyuni S29 sintetiza PHB homopolímero con dos masas moleculares principales de alrededor de 600 y 125 kDa con índices de polidispersidad (PDI) de 1,2 y 1,5 respectivamente. Además, los análisis térmicos mostraron

propiedades térmicas diferentes a las del PHB común, ampliando las posibilidades de aplicación de este biopoliéster.

Asimismo, se llevaron a cabo dos fermentaciones en biorreactores a partir de dos cepas de colección, *Cupriavidus necator* (DMS 545) y *Burkholderia sacchari* (DMS 17165), usando un sustrato de carbono barato, glicerol. El propósito de este estudio fue analizar la influencia de la fuente de carbono sobre: la cinética de crecimiento estas cepas, la producción de polímero, y la masa molecular del biopolímero producido. El PHB obtenido en estas fermentaciones se utilizó en la tesis para los estudios posteriores de transformación y aplicación del polímero, junto con el PHB producido por *Bacillus megaterium* uyuni S29.

Después de obtener el polímero, la **segunda parte** de la tesis se centró en el estudio de su transformación. Normalmente, las masas moleculares de los PHAs producidos a partir de bacterias son relativamente altas entre 200 y 3.000 kDa, por lo que no son adecuadas para el diseño de polímeros especiales tales como copolímeros anfifílicos en bloque. Para tales aplicaciones se requieren masas moleculares más manejables. Por lo tanto, en esta segunda parte se estudió una propuesta de proceso sostenible de degradación de PHAs pensada para una posterior aplicación a escala industrial. Inicialmente, se utilizó un copolímero de la familia de los PHAs, el poli(3-hidroxibutirato-co-4-hidroxibutirato) (P(3HB-co-4HB)), para llevar a cabo su degradación enzimática. Se emplearon dos lipasas comerciales para disminuir la masa molecular del polímero y obtener oligómeros con masa molecular entre 5 y 1 kDa. Fue la primera vez que dichas lipasas de triglicéridos han sido utilizadas con este polímero y, debido a los buenos resultados obtenidos, se empleó una de ellas para la degradación de PHB. Se confirmó que la reacción enzimática de esta lipasa con el PHB produjo una disminución controlada de la masa molecular de 300 kDa a 4 kDa.

En la **tercera parte** de la tesis se analizó la aplicación del PHB como sistema de liberación de fármaco. Existen pocas publicaciones que informan sobre la liberación y la posterior incorporación de agentes terapéuticos con sistemas de PHB y ninguno de ellos hace referencia a la doxiciclina (DOXY). DOXY es un conocido antibiótico de amplio espectro y que se utilizada frecuentemente en el tratamiento de enfermedades periodontales destructivas, tales como periodontitis juvenil o dolores periodontales agudos. Sin embargo, puede producir algunos posibles efectos secundarios como trastornos gastrointestinales. Con el fin de evitar estos efectos y llegar a la infección con una concentración de fármaco eficaz, se requiere una protección y administración controlada de DOXY. Por lo tanto, se estudiaron diferentes métodos de atrapamiento molecular y de preparación de emulsificaciones con el fin de obtener micro- y nano-partículas de PHB cargadas con DOXY. Los resultados mostraron que la combinación de ultrasonidos con agitación a alta velocidad en la preparación de emulsión doble es muy eficaz para la obtención de estas partículas. La carga de fármaco, la eficacia de atrapamiento y la eficiencia del método así obtenidos fueron elevados en comparación con los otros métodos estudiados. Además, los resultados de actividad antibacteriana llevados a cabo con estas partículas mostraron que son buenas candidatas para la liberación DOXI.

Los mejores métodos encontrados para la formación de partículas de PHB se utilizaron con los PHAs producidos a lo largo de la tesis para confirmar su idoneidad con otros PHAs, y e ser así, estudiar la posibilidad de mejorar los resultados de la captura del fármaco y la eficiencia del método.

INTRODUCTION

1. Plastic environmental problems and bioplastics

Generally, plastics are synthetic polymeric materials of macromolecular constitution, possessing great versatile properties. They are relatively cheap and durable materials which can be easily transformed by the application of heat or pressure, and moulded into almost any desired shape, including fibres and thin films. They serve as raw material for the production of a large variety of commodities and are utilized in almost every manufacturing industry ranging from automotive and packaging to medicine. Therefore, plastics are at the present time one of the most significant materials used in our society.

The total global capacity of commodity plastic production dramatically increased in the last decades. This polymer production rose from 1.5 million tons in 1950 to 245 million tons in 2008, an annual growth rate of 9% (**Chanprateep 2010)**. In 2009, global plastics production fell to around 230 million tons as a result of the economic crisis (**Mudgal 2011**). Global plastics production has grown noticeably faster than European production, presumably due to the growth of plastics production in Asia, China being the greatest producer with 15% of world plastic production. In the European Union (EU), the total 2009 plastic production was 55 million tons, a decrease of 8.3% over 2008[1]. Germany manufactures the largest amount of any European country, and is also the major plastic-consumer followed by Italy. Spain produces about the 1.5%, and after France it is the fourth highest plastic consuming country in the EU (**Mudgal 2011**). Four sectors represent the plastic demand in the EU: packaging (38%), building & construction (21%), automotive (7%) and electrical & electronic equipment (6%). At world level, polyethylene (PE) has the highest share of the total production of any polymer type (30%), followed by polyethyleneterepthalate (PET) (20%), polypropylene (PP) (18%), polyvinyl chloride (PVC) and polystyrene/expanded polystyrene (PS/EPS).

On one hand, polymers are made from the by-products of refining crude oil. Therefore, the prices of this non-removable raw material influence the petroleum-based plastics industry. With an ever increasing demand for fuel, the price of crude oil has risen dramatically in recent years and the price of petroleum-based plastics could increase by 50- 80% in 2012, based on the increasing price of oil (**Baker 2009, Chanprateep 2010**). As a result it has become more economically appealing to consider other possibilities for petroplastics replacement (**Baker 2009**).

On the other hand, the high production of plastic generates large amounts of waste, creating a risk to human health and to the environment. In 2008, 40% of the produced plastic commodities were short-life products, and the rest were long-life ones. Consequently, the total generation of post-consumer plastic waste in the EU was 24.9 million tons. Packaging is by far the largest contributor to plastic waste at 63% (**Mudgal 2011**). The total post-consumer waste in 2009 was about 24.3 million tons from the total plastic production (55 million tons), which represents a decrease of 2.6% over 2008[1]. Average generation of plastic packaging waste in the EU per-capita was around 30.6 kg. The reason why plastics generate such a large amount of wastes, it is because they are usually non-

[1] Plastic*Europe*, Association of plastic manofacturers. Plastics – the Facts 2010, an analysis of European plastics production, demand and recovery for 2009. www.plasticseurope.org.

biodegradable, and can therefore remain in the environment for a very long time. Polymers are characterized by their long molecular chains. This excessive molecular size seems to be mainly responsible for the resistance of these macromolecules to biodegradation. Natural mechanisms cannot deal with novel pollutants since these are unfamiliar to it (**Reddy 2003**).

The search for solutions to these economical and environmental problems has constituted a great challenge for the scientific community. For waste solution, several disposal options are already available to deal with plastic remainders. They include composting, recycling, energy recovery (including anaerobic digestion, incineration, gasification and pyrolysis), and landfill. For instance, the 24.3 million tons of waste produced in 2009 by the EU were managed in three different ways: 5.5 million tons were recycled, 7.6 million tons were converted into energy and the rest went to landfills for their disposal[1]. However, these strategies for waste management are not optimal, and remain the subject of much controversy and discussion among both scientists and the public. Composting predominantly consists of aerated static piles that, if not properly aerated, can release methane into the atmosphere. At the moment, the majority of composting companies are unwilling to take materials that are not guaranteed to be non-degradable petroplastic-free (**Baker 2009**). Recycling presents two major disadvantages: the difficulty of sorting the wide variety of plastics and the changes in the material properties that limits the application range (**Reddy 2003**). Incineration is a suitable means of plastic disposal for the production of heat and electricity, and incinerating plastic has been one option in dealing with non-biodegradable plastics. Nevertheless, it is the most expensive option for waste disposal and it is also dangerous: harmful chemicals like hydrogen chloride and hydrogen cyanide are released during incineration (**Reddy 2003, Baker 2009**). The European directive that controls waste incineration is the Directive 2000/76/EC[2]. With regard to landfill, it is considered to be the worst option both economically and environmentally. The waste materials to be disposed of in landfills require previous sorting or treatement. This is dictated by the European Directive 1999/31/EC Landfill Directive[2]. It prevents or reduces possible negative effects on the environment by introducing severe technical requirements for waste and landfills. For instance, there are several wastes which may not be accepted in a landfill such as liquid waste; flammable, explosive or oxidising waste; hospital and other clinical waste which is infectious; and used tires, with certain exceptions. These requirements are a crucial but costly stage in the process of plastic waste management.

Because these proposed disposal solutions in waste management still present some weaknesses, and plus the current problematic situation in the oil industry that increases the prices of its derived products, it is important to find and study other options. In the early 1990s, an intensive activity started to develop novel plastics with properties comparable to that of conventional polymers, but also susceptible to microbial degradation. The use of these materials would reduce waste deposit volume while undergoing degradation in a landfill, or alternatively they could be treated in composting plants, without pollutant production. The combination of biodegradable plastic and landfill and composting

[2] Official Journal of the European Communities. http://ec.europa.eu/environment/waste/landfill_index.htm

technologies attempt to offer a new approach to the waste management system. In addition, the use of renewable resources for biopolymer production results in the independence of the petroleum industry (**Müller 2005**). Consequently, it has become more economically appealing to consider bioplastics as a potential replacement for petroleum-based plastics (**Baker 2009**).

Bioplastics are not a single class of polymers but rather a family of products. They can be separated into either or both of the following categories: bio-based plastics and biodegradable or compostable plastics. Bio-based plastics are polymers derived from renewable resources such as starch, sugar, vegetable oil or wood pulp. They can be either biodegradable or non-biodegradable. For example, PE derived from bio-ethanol would be bio-based but not biodegradable (**Mudgal 2011**). On the other hand, biodegradables are polymers capable of undergoing decomposition into carbon dioxide (CO_2), methane, water, inorganic compounds, or biomass in which the predominant mechanism is the enzymatic action of micro-organisms, reflecting available disposal conditions. It can be measured by standard tests, over a specific period of time[3]. Compostable plastic is a polymer that undergoes degradation by biological processes during composting to yield CO_2, water, inorganic compounds and biomass, and leaves no toxic residue. Biodegradable or compostable plastics are often bio-based or petroleum-based polymers such as polycaprolactone (PCL) that can be completely broken down by microorganisms in the environment into non-toxic compounds. Some biodegradable plastics even contain a mixture of petroleum-based polymers and biopolymers. **Figure 1** shows a possible classification for biodegradable polymers depending on the evolution of the synthesis process.

Two of the most promising biopolymers are polylactic acid (PLA) and polyhydroxyalkanoats (PHAs). PLA has received much attention as a potential alternative to existing materials while PHA is challenging and price-competitive with petroleum-based polymers (**Chanprateep 2010**). In relation to both biodegradable and economic polymers, the PHAs appear to be a good alternative because as well as possessing thermoplastic properties, they come from natural and renewed sources. These are polyesters produced by a wide range of microorganisms as intracellular carbon and energy reserve materials. They are recognized as completely biosynthetic and biodegradable with zero toxic waste, and are completely recyclable into organic waste. Due to these properties, PHAs have found immense applications, especially in the biomedical field (**Chanprateep 2010**). Poly(3-hydroxybutyrate) (PHB) is the most abundant natural aliphatic biopolyester belonging to the PHA family.

[3] Plastics Standards: ASTM D-6400-99. www.astm.org/index.shtml. ASTM D-6400-99

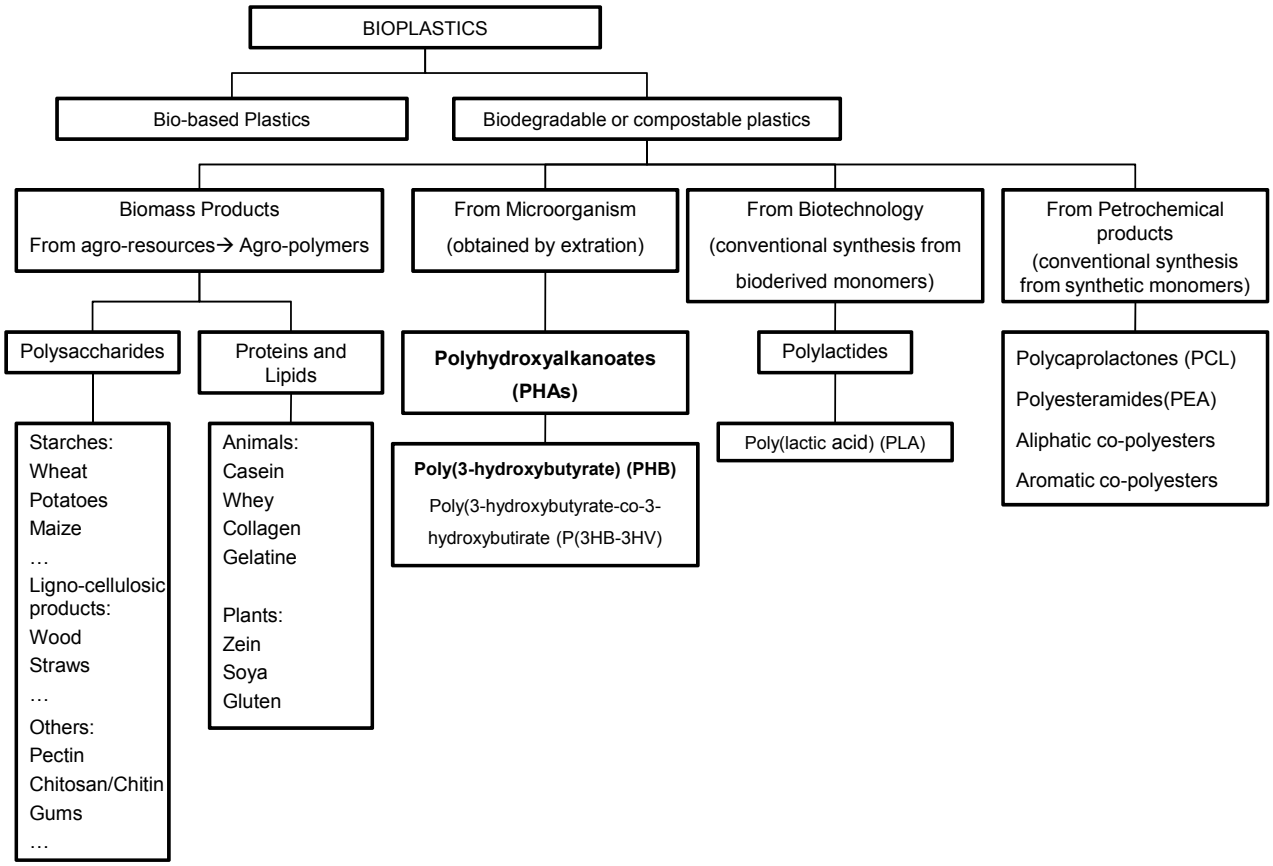


Figure 1. Biodegradable polymer classification (**Bergeret 2011**)

2. Historical review

The discovery of PHAs dates back to 1923 when Maurice Lemoigne, a chemist and microbiologist at the Lille branch of the Pasteur Institute, observed that some granular bacterial component from *Bacillus megaterium* were not soluble. In 1927, Lemoigne identified as PHB the chemical composition of these inclusions (**Lemoigne 1927**). Later, he recognized its structure and chemical formula (**Lemoigne 1946**). By the end of the 1950s, enough evidence was already collected from studies on the genus Bacillus to suggest that PHB functions as an intracellular reserve for carbon and energy in several bacteria (**Macrae 1958, Doudoroff 1959**). Throughout the 1960's, the discovery of the thermoplastic properties of the PHAs increased the interest in their research, and in their industrial production (**Lundgren 1964**). The first time that PHB was commercialized was in 1959 by W. R. Grace and Company in the United States (W.R. Grace & Co., New York, US) (**Baptist 1965**). However, the company shut down the process due to low production efficiency. At this time, the 3-hydroxybutyrate (3HB) unit was thought to be the only hydroxyalkanoate (HA) constituent that formed these material reserve polymers. During this decade, there were different contributions covering enzymology, PHB morphology, and the establishment of its chemical and macromolecular structure. In the 1970's, hydroxyalkanoates (HA) other than 3HB were identified (**Wallen 1974**). 3-

hydroxyvalerate (3HV) and 3-hydroxyhexanoate (3HHx) were the major and the minor constituents, respectively. In 1970, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-3HV) was commercialized by Imperial Chemical Industries Ltd. (ICI/Zeneca BioProducts, Bellingham, UK) under the trade name of Biopol™. Based on *Cupriavidus necator* (also known as *Ralstonia eutropha*), P(3HB-3HV) had different properties than PHB and could be employed in a wide range of applications. Later in the 1980's, the medium-chain-length (MCL)-PHAs were also discovered as the product of *Pseudomonas oleovorans* (**De Smet 1983**). At this time, PHB applications were focused on fiber and packaging applications due to its similar properties to the PP (**Holmes 1985**). In the middle of the 1980's, the successful large scale production of PHB was achieved by Chemie Linz GmbH, a new Austrian PHA producing company. They attempted industrial production of PHB using a newly isolated strain of *Azohydromonas lata* (former *Alcaligenes latus*), and published several patents (EP0144017 A1 and B1) (**Lafferty 1985, 1988, Hrabak 1992**). Research on bacterial PHAs cloning and characterization of genes involving their biosynthesis were developed by the end of the 1980s. The genes coding the enzymes responsible for PHA biosynthesis were already cloned from *Cupriavidus necator*. These genes were also shown to be functionally active in *Escherichia coli* (**Slater 1988, Peoples 1989, Liu 2007**). By this time, it was already clear that these storage polymers were synthesized not only in Gram-negative bacteria but also in a wide range of Gram-positive bacteria, aerobic (cyanobacteria) and anaerobic (non-sulfur and sulfur purple bacteria) photosynthetic bacteria, as well as in some archaebacteria.

The development of several international companies interested in the PHAs business from the 1990's until nowadays, helped to increase the knowledge of these biopolymers, their applications and their processing, and to improve the economical efficiency of their production processes. In China, many companies have recently been developed in the area of PHAs. Kaneka Corporation (a result of the collaboration of Tsinga University in China and the Riken Institute in Japan) has developed a wide range of applications for PHB and has commercialized a wide range of PHAs copolymers (Nodax™) as fibers, nonwoven materials, aqueous dispersions, and disposable products (**Rudnik 2008**). Recently, Kaneka Corporation has announced its plan to start the production of a plant-derived polymer called Kaneka P(3HB-3HHx), with a high production capacity[4]. TianAn Biologic[5] has focused on the production and application development of P(3HB-3HV) since its establishment in 2000. In 2004, it became the first company in the world to achieve commercial production of P(3HB-3HV) using a water-based extraction technology. Tianjin GreenBio Materials Co. (Green Bio™) was found in 1925 and is dedicated to the development, production and sale of bio-based polymer materials. Later PHAs were included[6]. In Japan, Mitsubishi Gas Chemical has made progress in the production of PHB from methanol fermentation (BioGreen™) (**Kosior 2006**).

[4] www.kaneka.co.jp/kaneka-e/news/pdf/090310.pdf
[5] www.tianan-enmat.com
[6] www.tjgreenbio.com/en/index.aspx

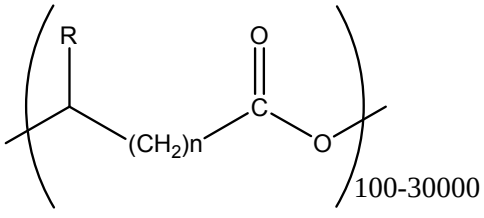
In South-America, PHB Industrial S.A. uses sugar cane to manufacture PHB (Biocycle™) taking advantage of the fact that Brazil is one of the largest sugar-exporting countries. Since 1992, the company has been running a pilot plant at 50 tons per year and plans to increase its production capacity (**Pessoa-Jr 2005**). In North-America, Nodax is a registered trademark introduced by the Procter & Gamble Company (P&G, Cincinnati, USA). (**Noda 2004**). It is poly(3-hydroxybutyrate-co-hexanoate) (P(3HB-HHx)) made from corn, sugar beet and/or vegetable oils[7]. Biomatera Inc.[8] in Canada specializes in the manufacture of PHA by fermentation of agricultural residues. The biopolymers are used in the manufacture of creams and gels that are used as slow-release agents in drug manufacturing, and as cosmetic agents and tissue matrix regeneration (**Archambault 2004**)[9]. In Europe, the German company Biomer Inc. began production on a commercial scale of PHB for special applications in 1993 (**Schmack 2000**), and registered the trade name Biomer™ in 1995. Nowadays, Biomer Inc. is exploring new markets for its PHB and other polyesters such as PLA. They develop microorganisms that ferment sugar or starch syrup through a toll manufacturing arrangement. The polyester extract is then compounded with LMW- and HMW-plasticizers, nucleators, and processing aids to process them *via* injection molding. The applications include medical "Immunostick" diagnostic tools, fireworks casings, and practice artillery shells for the military. PHB is also extruded into multi-filaments for woven surgical patches. The company is also developing PHB grades with higher melt strength for blown film. The company is experiencing strong growth particularly in the US. Capacity is several tons per month and PHB prices range from $5 to $10/lb. Lately, PHB is being applied in food packaging such as yogurt cups and beverage bottles. However, a big obstacle is obtaining food-contact approvals due to the many substances present in the residual biomass. Food-approval testing therefore becomes prohibitively expensive[10]. Parallel to the development of Biomer Inc., the first and only PHA production company, Imperial Chemical Industries Ltd., sold its Biopol™ business to Monsanto in 1996, and later to Metabolix Inc. Metabolix Inc. was formed in 1992; however they have not acquired Monsanto's patent related to bio-based plastics, which included the Biopol assets until 2001. They have developed an integrated manufacturing process using transgenic strains for fermentation and a proprietary recovery process. In 2006, they formed commercial alliance with ADM Polymer (Archer Daniels Midland Company), one of the largest agricultural processors in the world, and together they established a joint venture company, Telles, a manufacturing facility capable of producing 110 million pounds of PHA biopolymers annually. In 2008, Metabolix Inc. announced the combined production of PHA bio-based polymers and biomass energy with a target to obtain PHA from switchgrass (**Somleva 2008**). On January 9, 2012, ADM announced the termination of the

[7] www.pgchemicals.com
[8] www.biomatera.com
[9] Recommended reading about the current and potential large volume manufacturers of PHAs: Chanprateep 2010
[10] www.ptonline.com

commercial alliance with Metabolix Inc., effective as of February 8, 2012. Nowadays, Metabolic Inc. is restructuring its business plan[11].

3. Polyhydroxyalkanoates-PHAs

PHAs are biologically synthesized polymers. Many bacteria synthesize and accumulate them as carbon and energy storage materials under specific conditions of nutrient depletion in the presence of a surplus of carbon source. Moreover, when the supply of the limiting nutrient is restored, the PHA can be degraded by intracellular depolymerases and subsequently metabolized as a carbon and energy source. To a minor extent, their function is related to certain enzymatic mechanisms in the cells (**Lee 1996a, Chen 2010**). PHAs differ widely in their structure and properties depending on the producing microorganism, the conditions of biosynthesis, and the type of carbon source used in the production process (**Volova 2004, Philip 2007, Valappil 2007a, Chen 2009**). They are accumulated within the microorganism in the shape of granules. Proteins and lipids form a membrane coat surrounding the core region and stabilize the transition between the hydrophobic core region and its hydrophilic surroundings. Every granule consists of at least a thousand polymer chains forming right-handed 2-helices with a twofold screw axis and a fibre repeat of 0.596 nm, stabilized by hydrogen bonds and van der Waals intramolecular forces (**Koller 2010**). Initially, it was thought that the biopolymer inside the granules had a crystalline character, thus explaining the brittleness of some PHAs, especially PHB. However, later nuclear magnetic resonance spectroscopic studies demonstrated that it is a mobile amorphous bulk, which crystallizes immediately in the extraction process (**Koller 2010**). Due to their low solubility and their high molecular weight, PHAs do not cause an increase in osmotic pressure. They are therefore ideal storage compounds.



n	R	Polymer name
	Hydrogen	Poly(3-hydroxypropionate)
	Methyl	Poly(3-hydroxybutyrate)
1	Ethyl	Poly(3-hydroxyvalerate)
	Propyl	Poly(3-hydroxyhexanoate)
	Pentyl	Poly(3-hydroxyoctanate)
2	Hydrogen	Poly(4-hydroxybutyrate)
3	Hydrogen	Poly(5-hydroxyvalerate)

Figure 2. PHAs structure

[11] Metabolix annual Report: www.mirelplastics.com

PHAs are linear biopolymers composed of hydroxyalkanoate units (HA) as the basic structure in **figure 2** shows. Chemically, they are polyesters which the hydroxyl and the carboxyl groups of the hydroxyalkanoic acids linked together *via* oxoester bonds.

In addition to their plastic-like properties, PHAs also features optical activity, antioxidant properties, piezoelectricity, and, most importantly, biodegradability and biocompatibility (**Lee 1996a, Akaraonye 2010**).They can be divided into three main groups, depending on the number of carbon atoms in the monomer units: short-chain-length (SCL) PHAs in which carbon numbers range from C3 to C5, MCL-PHAs which consist of 6-14 carbon numbers, and long-chain-length (LCL) PHAs which shows more than 14 carbon atoms (**figure 3**). The type of polyester to be synthesised is dependent on the strain. *Cupriavidus necator* or *Azohydromonas lata*, for example, can polymerize only 3HAs consisting of 3-5 carbon atoms (SCL-PHAs producers), while *Pseudomonas putida* only accepts 3HAs of 6-14 carbon atoms. This is mainly due to the substrate specificity of the PHA syntheses that can only accept 3HAs of a certain range of carbon length (**Koller 2010**). The vast majority of microorganisms synthesize either SCL-PHAs containing primarily 3HB units or MCL-PHAs containing 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) as the major monomers (**Lee 1996a, Reddy 2003**). Physical and mechanical properties of SCL and MCL-PHAs are distinct. SCL-PHAs are thermoplastics like PP with a high degree of crystallinity, forming stiff crystalline materials that are brittle and cannot be extended without breakage, whereas MCL-PHAs are biodegradable elastomers with a low crystallinity and a low glass transition temperature (**Shang 2008, Chen 2010, Koller 2010**). SCL-MCL PHA copolymers can have properties between the two states, dependent on the ratio of SCL and MCL monomers in the copolymer (**Nomura 2004**).

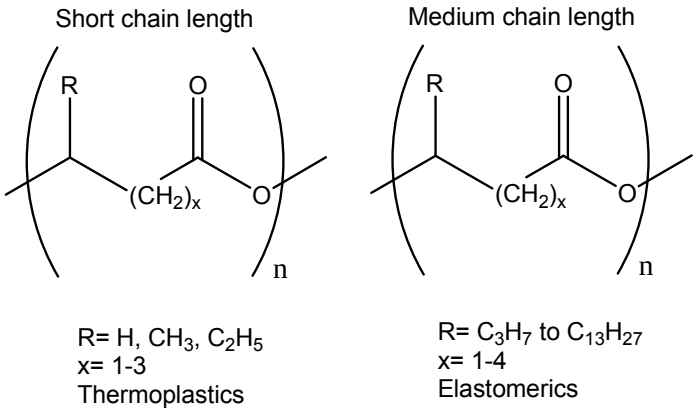
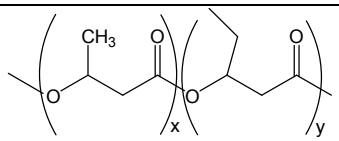
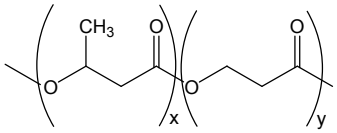
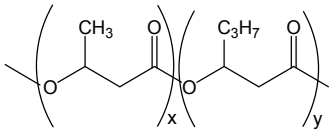
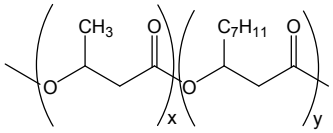
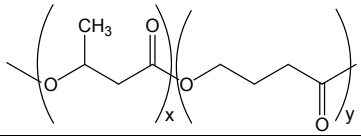


Figure 3. Schema of SCL-PHAs and MCL-PHAs.

The production of PHAs copolymers *via* fermentation is also possible. Several copolymers can be produced by feeding the microorganism with different substrates (precursors). P(3HB-3HV) and poly(3-hydroxybutyrate-co-4-hydroxybutyrate) (P(3HB-4HB)), consisting of 3HB combined with 3HV or 4-hydroxybutyrate (4HB) units respectively are the main copolymers produced directly in the bacterial cells. **Table 1** shows some PHA copolymers synthesized by different bacterial strains depending on the precursor used in the fermentation process (**Sudesh 2000**).

Table 1. Microbial synthesis of PHA copolymers (**Sudesh 2000, Koller 2010)**

Bacterial strain	Carbon substrate	Copolymer	Formula Copolymer
Cupriavidus necator *Alcaligenes latus*	propanoic acid pentanoic acid	P(3HB-3HV)	
Cupriavidus necator	Pentanoic acid 3-hydroxypropionic acid	P(3HB-3HP)	
Alcaligenes latus *Aeromonas cavie*	1,5-Pentanediol Plant oils	P(3HB-3HHX)	
Pseudomonas sp. *Cupriavidus necator*	Sugar 4-Hydroxybutyric acid	P(3HB-3HD)	
Alcaligenes latus *Comamonas acidovorans* *Delftia acidovorans*	g-Butyrolactone 1,4-Butanediol 1,6-Hexanediol On mixtures of acetic acid and g-buty rolactone	P(3HB-4HB)	

Currently, the production of PHAs copolymerized with mercaptanoes is being investigated. The biosynthesis of poly(3-hydroxybutyrate-co-3-mercaptopropionate) (P(3HB-co-3MP)) have been biosynthetised by *Cupriavidus necator* during cultivation with gluconate in the presence of 3,3′-thiodipropionic acid) or 3,3′-dithiodipropionic acid (**Yu 2007**, **Peplinski 2010**).

The biosynthetic production of copolymers generates many advantages such as the extension of the polymer properties. However, the incorporation of different building blocks into the polyester chains normally requires expensive co-substrates and two-stages for the fermentations. It therefore constitutes an added cost factor. These precursors do not only contribute to the production cost, but, in addition, are often toxic for the microbial strain (**Koller 2010**). A possible solution would be the use of microorganisms that can produce copolymers from simple sugars. Copolymers consisting of 3HB and 3-hydroxy-4-pentenoic acid (3H4PE) building blocks are reported to be produced by *Burkholderia cepacia* from sucrose or gluconate (**Rodrigues 1995**). Therefore, the large-scale production of complex PHAs is still complicate.

It is important to take into account that the importance of PHAs is not only due to their biodegradability, but due to their thermoplastic and mechanical properties as well. They have sufficiently high molecular mass to own polymer properties similar to the conventional petroplastics. For instance, the molecular mass of PHAs is found in the range of 200-3,000 kDa which can vary with the microorganism and the substrate used in the biosynthesis (**Sudesh 2000, Ravenelle 2002**). **Table 2** and **table 3** summarize and compare various thermal and mechanical properties of PHB to

some of the most common PHAs copolymers[12], and to a petroleum-based polymer such as PP (**Madison 1999, Sudesh 2000**, **Koller 2010, Akaraonye 2010**).

Table 2. Properties of some PHAs

Parameter	Values for					
	PHB	P(3HB-3% 3HV)	P(3HB-20%3HV)	P4HB	P(3HB-16%4HB)	P(3HO-3HHx)
Melting temperature (T_m) (°C)	177	170	145	60	152	61
Glass transition temperature (T_g) (°C)	4	-	-1	-50	-8	-36
Tensile strength (MPa)	40	38	32	104	26	9
Young´s modulus (GPa)	3.5	2.9	1.2	149	-	0.008
Elongation at break (%)	6	-	50	1000	444	380

Table 3. Main thermo and mechanical properties of PHB, P(3HB-3HV) and PP

Properties	PHB	P(3HB-3HV) for 4 – 95% (mol/mol) 3HV	PP
Molecular mass (10^5 g/mol)	1 – 8	3	2.2 – 7
Density (kg/m^3)	1.25	1.20	0.905
T_m (ºC)	171 – 182	75 – 172	176
Crystallinity degree (X_c) (%)	80	55 – 70	70
T_g (ºC)	4 – 10	-13 – +8	-10
O_2 Permeability [(cm^3/(m^2atm)]	45	n. d.	1700
UV Resistance	Good	Good	Bad
Resistance to solvents	Bad	Bad	Good
Tensile strength (Mpa)	40	25 – 30	38
Elongation to break (%)	6	8 – 1200	400
Young's modulus (GPa)	3.5	2.9 (3% 3HV); 0.7 (25% 3HV)	1.7
Biodegradability	Yes	Yes	No

[12] Recommended reading about PHAs copolymers: Hazer B, Steinbüchel A. Increased diversification of polyhydroxyalkanoates by modification reactions for industrial and medical applications. Appl Microbiol Biotechnol 74(2007)1–12

The major advantageous characteristics of PHAs are underlined along these lines:

Biodegradability - It is a typical quality from a product biologically synthesized. Due to their biodegradability, PHAs can be composted after their use (**Chen 2002, Reddy 2003, Cheng 2006, Bonartsev 2007**). Consequently, PHAs do not contribute to increase the residues accumulated by the human action. In contrast to conventional plastics, PHAs fit perfectly in the ecosystem due to its complete biodegradation.

Carbon balance - The carbon cycle is closed for PHAs synthesis, exploitation and degradation. PHAs can be used in the making of plastics, which upon disposal, will be degraded and converted to carbon dioxide and water by the actions of microorganisms commonly present in the natural environment, land or sea. The biomass of the microorganisms can be further utilized as soil fertilizers for the plants; CO_2 will be recycled to the aerosphere undergoing photosynthesis, producing carbohydrates, alcohols, and organic acids which will be re-used by the microorganisms, forming a system of recycling (closed circle). Thus, these biodegradable plastics are called “green polymer” (**Ariffin 2010**).

Independence from oil industry - Due to the fact that PHAs are produced from renewable resources, they are not directly dependent on the availability of fossil feedstocks. This is therefore a great advantage to the petroleum-based polymers because of the actual crisis regarding the oil reserves and the derived economical problems (**Araffin 2010**, **Koller 2010**).

Biocompatibility - It is the property of being biologically compatible by not producing a toxic, injurious, or immunological response in living tissue[13]. Complex sanitary-chemical, toxicological, and medico-physiological studies of PHA, performed in cell cultures of various origin, as well as in acute and chronic experiments with laboratory animals are indicative of biocompatibility of PHAs at the level of cells, tissues, and the whole body (**Zinn 2001, Valappil 2006, Wu 2009**). Biocompatibility of PHAs *in vitro* has been demonstrated on different cell cultures (fibroblasts, mesenchymal stem cells, osteoblasts, bone marrow cells, articular cartilage chondrocytes, endothelial and glial cells or smooth muscle cells) (**Chen 2005, Xiao 2007**). Their biocompatibility has also been demonstrated in vivo under subcutaneous implantation (**Bonartsev 2007, Sun 2007**). The low tissue reaction to implanted PHAs films indicates their high biocompatibility in vivo that was observed by many investigators (**Chen 2005**, **Qu 2006, Bonartsev 2007**). Therefore, in special fields of applications, PHAs are superior to conventional plastics.

4. Poly[(R)-3-hydroxyburyrate]-PHB

PHB was the first PHA to be discovered by Maurice Lemoigne in 1923. It is homo-polymer synthesized and, as a member of the PHAs family, is accumulated as a membrane enclosed inclusion by several microorganisms as a carbon and energy reservoir, usually when grown under a limitation

[13] Definition by the medical dictionary: www.medical-dictionary.thefreedictionary.com

of a nutrient such as oxygen, phosphor, nitrogen, sulfur, or trace elements like magnesium, calcium, iron and in the presence of an excess of carbon source (**Lee 1996a, Khanna 2005**).

Although PHB is the most widely studied and best characterized PHA, it is still very promising due to its ease of production **(Lee 1996, Peoples 1989**) and due to the wide number of advantages it shows. On one hand, PHB is easily produced compared to other PHAs (**Lee 1996**). On the other hand, what makes PHB so special is first, its biodegradability and, second, its material properties. PHB is clearly biocompatible, since it is composed of R-3-hydroxybutyric acid (3HB units). The hydrolytic degradation of PHB proceeds to obtain this acid which is a normal constituent of blood (concentrations between 0.3 and 1.3 mM). It is also found in the cell envelope of eukaryotes (**Zinn 2001, Nair 2007, Bonartsev 2007**). PHB has a rather low degradation rate in the body compared to synthetic polyesters presumably due to its high crystallinity (**Nair 2007**).

This natural biopolymer combines three exceptional features: thermoplastic properties, biodegradability and biocompatibility. Besides, PHB shows material properties comparable to those of the petrochemical derived plastics (see **table 3**). It exhibits good barrier properties like PVC and PET (**Sharma 1995**). Furthermore, it has some additional properties which favour interest in this material. Firstly, the 3HB acid monomer is a chiral molecule and the natural polymer consists purely of D(-) monomers. Therefore, this monomer can be used for the chemical production of complex chiral pharmaceutical or agrochemical agents (**Hrabak 1992**).

PHB as well as P(3HB-3HV) are brittle materials outside the prokaryotic cell. Brittleness is caused by high crystallinity, and there are three main reasons for the high crystallinity of PHB. First, at ambient temperature the secondary crystallization of the amorphous phase takes place during storage at room temperature. Second, its T_g is usually close to room temperature (4-10 ºC). Because PHB has a low nucleation density, large spherulites are formed inside the biopolymer bulk, exhibiting inter-spherulitic cracks. This brittleness can be overcome by the addition of a nucleating agents that increases the number of small spherulites or plasticizers which enhance the molecular motion. Thus, the glass transition decreases producing a more ductile material (**El-Hadi 2002**).

PHB also attracts special ecological interests since it can undergo rapid degradation and biodegradation under various environmental conditions. It can be used in the packaging industry as a biodegradable plastic for solving environmental pollution problems. Other advantages of PHB, when compared to chemically produced polymers such as polyglycolic acid (PGA), PLA, and PLGA, which are mostly well known as biologically degradable drug carriers with good retarding characteristics, is that it has an excellent biocompatibility, and a propensity to biodegradation under different environmental conditions (**Chen 2002, Bonartsev 2007, Errico 2009**).

The properties described in this section confirm that PHB is still a very promising biopolymer which shows great potential in many different fields. This is the reason why the thesis was focused on the study of this biopolyester.

5. Economic competitiveness of PHAs

As previously stated, the oil price has doubled in recent years and at the same time world production and consumption of polymeric materials has quickly grown and continuous to do so. As a consequence, big international chemical companies such as BASF or CIBA are strongly interested in the academic research work on sustainable production of non-petroleum-based chemicals, with the objective of replacing the classical technologies by "White Biotechnology". The reason for their interest is the need to stay competitive in the market. Besides production of bio-fuels such as bioethanol or biodiesel, the biotechnological synthesis of biopolymers as PHAs is at the centre of interest (**Koller 2010b**).

PHAs are undoubtedly one of the potential candidates for replacing petroleum-based plastics. However, a drawback for the broad utilization of PHAs is the fact that in most cases their production costs are still higher than costs for conventional petroleum-based plastics. In order to overcome this problem and to be competitive face to face with conventional plastics, PHAs must have a large volume of production with low cost. The high cost of the PHAs production is mainly due to the high raw material costs, small production volumes, and high processing costs, particularly for polymer purification and isolation.

The cost of the carbon source contributes significantly to the overall production cost of PHAs, being about 40-50% of the total production cost (**Solaiman 2006a**). The tendency is to use cheaper feedstock which does not interfere with the needs of food or feed production, for biopolymer manufacture (**Braunegg 2008, Povolo 2010, Koller 2010, Koller 2010b**). **Table 4** summarizes the cost of some feedstock already used for PHA production.

Table 4. Effect of the substrate cost on the production cost of PHB (**Chanprateep 2010**)

Substrate	Approximate price (€/kg)	PHB yield (g PHB/ g substrate)
Glucose	0.41	0.38
Sucrose	0.35	0.40
Ethanol	0.31	0.50
Methanol	0.28	0.43
Acetate	0.59	0.38
Cassava starch	0.19	0.20
Cheese whey	0.07	0.33
Cane molasses	0.10	0.42
Palm oil	0.79	0.65
Soya oil	0.92	0.70

Based on this, utilization of sugars like sucrose has to be excluded in many countries, even in countries that have a surplus of sucrose such as Brazil (**Braunegg 2008**). In addition, some of these carbohydrates as sugar and starch from crops are unavoidably used for biofuels production, since the prices of fossil fuels keep on increasing. Also, inexpensive carbon sources such as agricultural wastes and industrial by-products may include additional costs due to pre-treatment steps, extended cultivation times, and purification. Therefore, the selection of carbon sources should not be focused only on market prices but also on availability and global price consistency. Recent attention has been paid to agricultural wastes and industrial by-products. The utilization of waste materials for PHA biosynthesis constitutes a viable strategy for the improvement of cost-efficient biopolymer production, and helps industries to overcome disposal problems. It also gives added value to the PHAs formed products. Among these substrates which occur in large quantities, molasses (**Zhang 1994**), starch (**Chen 2006**), whey from the dairy industry (**Koller 2006**), waste water from olive oil production (**Gonzalez-Lopez 1996**), xylose (**Silva 2004**), a broad range of plant oils (**Kahar 2004**), glycerol liquid phase (GLP) from the biodiesel production (**Koller 2010b**) and, lately, waste frying oil (**Verlinden 2011**) are available[14]. Recently, waste glycerol, a by-product of the biodiesel industry, has received much interest from many researchers as an inexpensive organic source for PHA production. As reported by **Cavalheiro (2009),** *Cupriavidus necator* was used to produce PHB using waste glycerol. Taking this into account, it was interesting to examine in the thesis the availability of this industrial by-product with respect to the microbial PHB production (**CHAPTER I, section 4**).

Other important factors affecting the economics of PHAs production by bacterial fermentation have already been reported by **Choi** (**1999**). The costs of complex nitrogen sources when the strategy for PHAs production is the limitation of a phosphate source have to be considered. One solution is trying to work in conditions of nitrogen limitation and using inexpensive nitrogen sources as silage juice hydrolyzed, instead of complex nitrogen sources such as fish peptone or meat extract. In order to avoid costs from a nitrogen source, the strategy employed in the fermentation processes carried out in the thesis work was the limitation of this nutrient for the inducement of the polymer biosynthesis. On the other hand, when PHA is produced in culture with a high density of cells, dissolved oxygen often acts as a limiting factor. Consequently, it is necessary to keep the dissolved oxygen concentration constant in aerobic fermentation. The requirement for avoiding oxygen limitation like a pressurized vessel, high mixing energy, high gas-flow rates and oxygen-enriched air feeds is a costly factor. Recovery of PHA should also be considered because it significantly affects the overall economics, especially on a large scale. After biosynthesis and separation of the bacterial biomass, a solid-liquid extraction procedure is generally required. In this case it could be interesting to use recyclable solvents to decrease the economical costs (**Lee 1996a, 1998**). The microorganism and its productivity is another important factor affecting the production cost. The selection of a microorganism for the production of PHA should be based on several factors including the ability of the cells to utilize

[14] Recommended reading about the selection of carbon source for PHA production: **Chanprateep 2010**

an inexpensive carbon source, growth rate, polymer synthesis rate, and the maximum extent of polymer accumulation. By considering these factors, only some bacteria as previously described are suitable for the production of PHAs. Nevertheless, there is still the possibility of discovering and studying new strains capable of synthesizing biopolymer to improve the productivity of the entire process. There are two ways of acquiring new bacterial species to meet the above demands: to isolate them from the natural environment and to design genetic recombination strains (**Tian 2009**). For instance, Tian (**2009**) recently reported a list of new bacterial species, either isolated or re-constructed for producing PHAs.

The costs of these factors have been studied and improved throughout the last years. Consequently, the prices of PHAs have been reduced. For example, the cost of P(3HB-3HV) Biopol in 1998 was 4-8 $/kg compared to 0.6-0.9 $/kg for conventional synthetic plastics (**Liu 1998**). The latest market price of Mirel™ is quoted at about 1.50 €/kg (**Chanprateep 2010**). PHAs combine biodegradability with premium pricing relative to most petroleum-based plastics (**Chanprateep 2010**). The summary of market prices for PHAs in 2010 is given in **table 5**.

However it is important to keep on investigating and improving the factors affecting PHAs competitiveness. In this thesis, two important factors affecting the biopolymer production cost have been studied. The discovery of novel PHB-producing bacteria from environmental samples which could be a possible replacement for the bacterial strains currently used was one of the factors (**CHAPTER I, sections 1-3**). To a minor extent, the use of glycerol as cheap feedstock has been studied in the development of two known strains (**CHAPTER I, section 4**).

Table 5. Current and potential large volume manufacturers of PHAs

Polymer	Trade names	Manufacturers	Price (€/kg) in 2010
PHB	Biogreen®	Mitsubishi Gas Chemical Company Inc. (Japan)	2.5–3.0
PHB	Mirel™	Metabolix Inc.	1.50
PHB	Biocycle®	PHB Industrial Company (Brazil)	n/a
P(3HB-3HV) and PHB	Biomer®	Biomer Inc. (Germany)	3.0–5.0
P(3HB-3HV) P(3HB-3HV) +Ecoflex blend	Enmat®	Tianan Biologic (China)	3.26
P(3HB-3HHx)	Nodax™	P&G (US)	2.50
P(3HB-3HHx)	Nodax™	Lianyi Biotech (China)	3.70
P(3HB-3HHx)	Kaneka PHBH	Kaneka Corporation (Japan)	n/a
P(3HB-4HB)	Green Bio	Tianjin Gree Bio-Science Co/DSM	n/a

6. PHA-producing microorganisms

The microorganism of choice for the industrial production of PHA varies depending on factors that include the cell's ability to utilize an inexpensive carbon source, the growth rate, the polymer synthesis rate, the quality and quantity of PHAs, and the cost of downstream processes (**Lee 2001**). Although, there are more than 300 different microorganisms synthesizing PHAs, only a few of these cultures are used in the industry for polymer production. *Cupriavidus necator* (formerly known as *Ralstonia eutropha* or *Alcaligenes eutrophus*), *Alcaligenes latus*, genus Pseudomonas, *Paracoccus denitrificans*, *Protomonas extorquens*, and recombinant *Escherichia coli* are able to produce sufficient PHA for large-scale production (**Lee 1996a, 1996b**). Also, PHAs can be produced by insect cells (**William 1996**), and can be efficiently produced by transgenic plants (**Snell 2009**). Some examples of patented PHA-producing bacterial strains currently used in industrial-scale production are summarized by **Chanprateep** (**2010**).

At present, bacterial fermentation with *Cupriavidus necator* is used widely in industrial processes toward PHAs. It is the most extensively studied strain for the production of PHA due to its ability to accumulate large amounts of high-molecular-mass (HMW) PHB from simple carbon sources such as fructose and acetic acid. *Cupriavidus necator* grows properly in minimal medium at 30 °C on a multitude of carbon sources, but not on glucose. Accumulation of PHB by *Cupriavidus necator* proceeds preferentially under nitrogen- or phosphate-limiting conditions. A glucose-utilizing mutant was first selected by Imperial Chemical Industries Ltd. in 1979 for the development of commercial production of PHB from glucose on a large scale, and P(3HB-3HV) from a mixture of glucose and propionic acid by fed-batch culture of *Cupriavidus necator* (**Vandamme 2004, Khanna 2005b**). *Alcaligenes latus,* a mutant strain of *Azotobacter vinelandii*, has also attracted much attention because of its ability to accumulate PHB following the growth-associated pattern (PHB-synthesis during exponential growth phase), and because it is also able to grow on the less expensive carbon sources, such as sucrose, cane sugar and beet sugar, a part from growing on glucose and fructose (**Braunegg 1995**). Chemie Linz GmbH produced PHB from sucrose at up to 1,000 kg per week by using *Alcaligenes latus.* ***Azotobacter sp.*** is an aerobic bacterium that accumulates PHB when cultivated on several carbon sources, including sucrose. It was the first chosen bacteria for the industrial synthesis of PHB since it was microbiologically well understood and was recognized as a putative production organism. However, the strain was unstable and secreted polysaccharides. The formation of any by-product is undesirable and should be kept to a minimum due to the direct impact in the yield of product. Thus, the fact of producing polysaccharides parallel to the production of PHB makes the process difficult to control, since it is the choice of the bacteria discarded (**Madison 1999, Pettinari 2003**). Recombinant *Escherichia coli* is capable of accumulating great amounts of polymer, more than 80% of the cell dry mass (CDM). It grows fast even at high temperatures and is easy to lyse. Fast growth will enable it to accumulate a large amount of polymer and the easy lyses of the cells decreases the cost of the purification of the PHA granules. This bacterium can also use diverse carbon sources as lactose, sucrose and xilose which allow the use of a cheap raw material as

substrate (molasses, hydrolysed hemicelluloses and milk serum among others). Another advantage is the fact that it does not possess intracellular depolymerases that could degrade the polymer while it is accumulating (**Reddy 2003, Akaraonye 2010**). The bacteria of the genus Pseudomonas can produce MCL- and LCL-PHAs from substrate that contains alkanes or alkanoic acids. Several studies showed that *Pseudomonas putida* and *Pseudomonas aeruginosa* strains are able to convert acetyl coenzyme A (acetyl CoA) to MCL monomers for PHA synthesis (**Madison 1999, Khanna 2005b**). Genetically changed *Pseudomonas* can produce a poly(3-hydroxybutyrate-co-3-hydroxyacyl (P(3HB-co-3HA)) copolymer with a high number of 3HB units from glucose. The production of MCL-PHAs by fed-batch and continuous cultures of *Pseudomonas oleovorans* has been investigated for reaching higher concentrations of PHA with a great productivity. Furthermore, a great feature of many *Pseudomonas* species as PHA-producing organisms is their capability of synthesizing PHAs bearing various functional groups such as halogens, branched alkyls, phenyl, phenoxy, olefin and esters when they are grown on substrates containing the corresponding chemical structures (**Kim 2000**). Methylotrophs are a diverse group of microorganisms that can use reduced carbon compounds, such as methanol or methane, as the carbon source for their growth. The use of these microorganisms was considered by Imperial Chemical Industries Ltd. for the production of PHB from methanol, which is one of the cheapest carbon sources available. However, accumulation of less than satisfactory amounts of PHA made the recovery process difficult, and the company abandoned the process. Nevertheless, the low price of methanol has stimulated the researchers to develop processes for the production of PHA from these kinds of substrates (**Lee 1996a**, **Madison 1999, Khanna 2005b**). There is continuing effort to find new possibilities to produce PHAs on a large scale and at more competitive cost. For these reasons, some plants have been genetically modified introducing bacterial genes to be able to produce PHAs. The first demonstration of PHB production by plants was in 1992 with *Arabidopsis thaliana* (a small flowering plant found in Europe, Asia and Africa) (**Poirier 1992**), through the creation of novel metabolic pathways either in the cytoplasm, plastid or peroxisome **(Khanna 2005b)** (**figure 4**). Later, PHAs were synthesized by several other kinds of plants like tobacco, cotton or corn. The production of PHAs directly in plant crops provide low-cost with high-volume for the manufacture of renewable resource-based polymers and create new value-added markets for agriculture. However, the huge diversity of PHAs structures that are possible to be found through microbial fermentation will not be possible with this plant system, which will be limited to fewer types (**Snell 2009**).

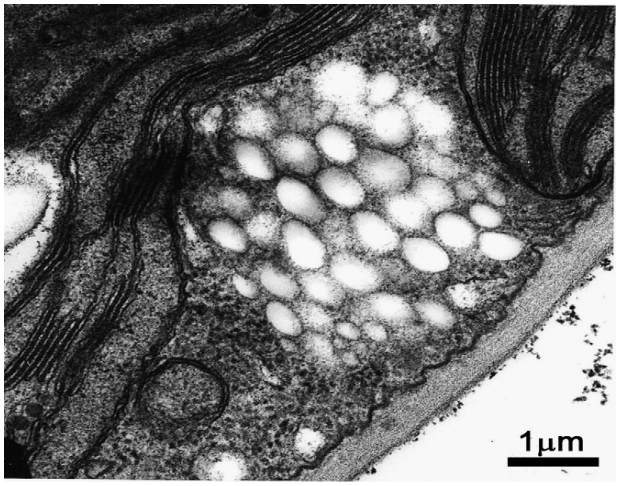


Figure 4. Electron micrograph of PHA granules in the cytoplasm of transgenic *Arabidopsis thaliana* cells (**Nawrath 2008**)

It is important to take into account that among the microorganisms able to produce PHA it is possible to find Gram-positive or negative bacteria, and archaea. Some Gram-negative bacteria contain lipopolysaccharides (LPS) in the outer membrane, which are endotoxins (pyrogens) and these can be produced together with the PHAs in the fermentation process. The presence of LPS induces a strong immunogenic reaction and is therefore undesirable for the biomedical application of the PHAs (**Chen 2005**). Gram-positive bacteria lack LPS and are hence potentially better PHA producers to be used for biomedical applications (**Valappil 2007b**).

Among the strains considered to be good candidates for PHB industrial production, *Cupriavidus necator* and *Alcaligenes latus*, and some strains form the genera *Azotobacter*, *Pseudomonas* and recombinant *Escherichia coli* have attained the highest polyester accumulation (around 60–80% PHB) and concentrations when cultured in batch and fed-batch fermentation systems (**Chen 2009**). At present, bacterial fermentation of *Cupriavidus necator* seems to be the most cost-effective process and even if production switches to other bacteria or agricultural crops, these processes are likely to use *Cupriavidus necator* genes (**Verlinden 2007**). A few important other strains that were recently studied include: *Bacillus spp*., *Alcaligenes spp*., *Pseudomonas spp.*, *Aeromonas hydrophil*a, *Rhodopseudomonas palustris*, *Escherichia coli*, *Burkholderia sacchari* and *Halomonas boliviensis.* As it was previously commented on, there is still the possibility to find new species that could replace the already used.

Halophiles, which constitute a wide group of organisms including protozoa, algae and bacteria, are characterized by their requirement for a high concentration of inorganic salts, mostly sodium chloride, to grow and survive. They inhabit in saline environments with extreme conditions of osmolarity. This forces those organisms to develop special physiological mechanisms to survive in this environment (**Quillaguaman 2005**). Most authors recognize three kinds of halophilic bacteria (**Rodríguez-Valera 1981, Ramires 2004, Quillaguaman 2010**): halotolerant which are able to live in presence of salt (0-15% w/v), extreme halotolerant that are able to live in a salt concentration of approximately 15 %

(w/v) NaCl (2.5 M), moderate halophiles which grow better in media with salt and somehow there is the possibility that some of them required salt to survive in a salinity range of 3-15% (w/v) NaCl (0.5-2.5 M), and extreme halophiles that show optimal growth in media containing 25% (w/v) NaCl (2.5-5.2 M). Some halophiles have shown a great potential for biotechnological production of PHAs. For example, the halophilic archaeon *Haloferax mediterranei* which grows optimally with 25% (w/v) salts in the medium can accumulate PHB up to 60-65 wt% of its CDM under phosphorous limitation conditions and a surplus of glucose or starch as carbon source. It also accumulates P(3HB-3HV) in large amounts using glucose, starch, and hydrolyzed whey as carbon sources. Several bacterial species of the family Halomonadaceae have also been detected that accumulate PHAs. *Halomonas boliviensis* reached PHA yields and volumetric productivities close to the highest reported so far (**Quillaguaman 2004, 2005**).

In light of these previous works, the initial studies of this thesis were developed using environmental samples of water and mud from different hypersaline Bolivian lagoons (Laguna Colorada, Laguna Hedionda, Laguna de Chiguana and Uyuni salt lake) with the purpose of finding new bacterial strains either halophilic or halotolerant capable of producing PHA, specifically PHB (**CHAPTER I, sections 1-3**).

7. PHA biosynthesis. The route from substrate to PHA

The PHA biosynthesis is divided into three general phases by **Steinbüchel** (**1995**) (**figure 6**). In phase I, a suitable carbon source enters in the cell *via* simple diffusion or *via* specific transport systems located in the cytoplasmatic membrane. In phase II, the compound is intracellularly metabolized into a hydroxyacyl CoA, and this will be the substrate of the PHA synthase. In phase III, PHA synthase forms oxoester bonds by releasing CoA (**Sudesh 2000**).

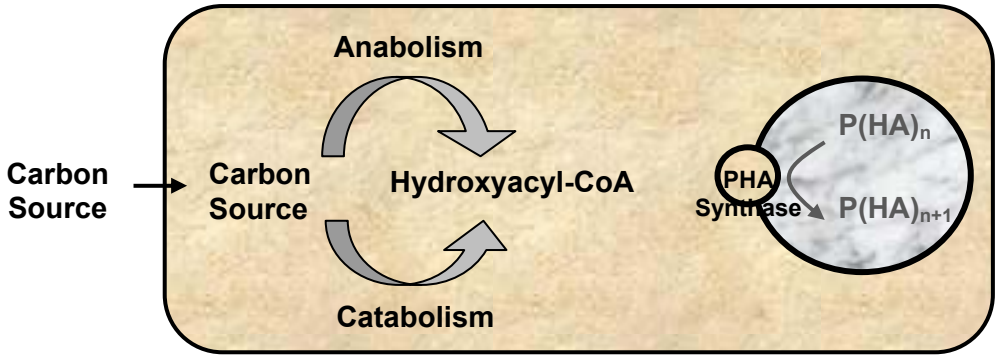


Figure 5. Phases in the biosynthesis of PHA (**Sudesh 2000**)

There are four different pathways reported in detail for PHAs biosynthesis (**Stainbücheln 1991, Khana 2005)**.

Pathway I - In *Cupriavidus necator*, β-ketothiolase carries out the condensation of two molecules of acetyl CoA to acetoacetyl CoA. Then a NADPH-dependent acetoacetyl CoA reductase carries out its

conversion to R-3-hydroxybutyryl CoA. The third and final step is the polymerisation reaction catalysed by PHA synthase (**Anderson 1990**).

Pathway II - In *Rhodopseudomonas rubrum*, the pathway differs after the second step where the acetoacetyl CoA formed by β-ketothiolase is reduced by a NADH-dependent reductase to L-(+)-3-hydroxybutyrylbCoA which is then converted to D-(-)-3-hydroxybutyryl CoA by two enoyl CoA hydratases.

Pathway III - A third type of PHA biosynthetic pathway is found in most *Pseudomonas* species belonging to rRNA homology group I. *Pseudomonas oleovorans* and other *Pseudomonas* species accumulate PHA consisting of 3-hydroxyalkanoic acid of MCL if cells are cultivated with alkanes, alkanols or alkanoic acids (**Lagaveen 1988, De Smet 1983, Zinn 2001**).

Pathway IV - The fourth type of PHA biosynthetic pathway is present in almost all *Pseudomonas* species belonging to rRNA homology group II. This pathway involves the synthesis of copolyesters consisting of MCL 3HAs from acetyl CoA. This pathway has not been studied in detail.

7.1. PHB Biosynthesis: *Cupriavidus necator* Pathway I

PHB biosynthesis is investigated in detail with *Cupriavidus necator* (**Madison 1999, Rehm 2009**). Sugar substrate is transported into the cell and transformed into acetyl CoA which is the central compound in PHB metabolism. Three enzymes are implicated in the biosynthesis of PHB from acetyl CoA. Ketothiolase (EC: 2.3.1.16, encoded by PhbA gene) which dimerizes acetyl CoA by condensing two acetyl CoA molecules to form acetoacetyl CoA. Acetoacetyl CoA dehydrogenase (EC:1.1.1.36, encoded by PhbB gene) which reduces acetoacetyl CoA to R-3-hydroxybutyryl CoA. R-3-hydroxybutiryl CoA constitutes the real substrate for the PHA synthase. PHA synthase (EC: 2.3.1.-, encoded by PhbC) polymerizes the R-3-hydroxybutiryl CoA to monomers. The growing polymer chain is elongated by the incorporation of additional monomers. 3-, 4-, 5-hydroxyalkanoates from the isomers of 4 or 5 carbons hydroxyacetyl CoAs are accepted by *Cupriavidus necator* PHA synthase (**Madison 1999**).

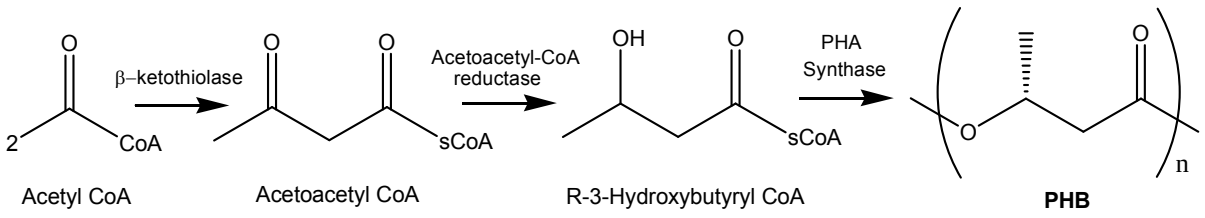


Figure 6. Biosynthetic pathway I for PHB biosynthesis by *Cupriavidus necator*

7.2. Regulation of PHA metabolism

PHA biosynthesis is regulated by the activity of β-ketothiolase and acetoacetyl CoA reductase, while the PHA biodegradation is dependent on the activity of 3-hydroxybutyrate dehydrogenase. The control of the activation and the inhibition of these enzymes are regulated by various compounds that are important factors responsible for their behavior: acetyl CoA, free CoA, and, to a lower extent, $NADP^+$ or NADPH, ATP, pyruvate and oxalacetate. In general, high yields of PHA are achieved as a consequence of an excess of carbon and energy source together with a decrease of sulfur, phosphorus, nitrogen or oxygen (**Anderson 1990, Braunegg 1998**). Under oxygen limiting conditions, PHA synthesis provides the cell with a possibility to regenerate reducing power, but if sufficient oxygen is available, CoA increases because of entry of acetyl CoA in the carboxylic acid cycle (TCA-also known as the citric acid cycle, Krebs cycle, or more rarely, the Szent-Györgyi-Krebs cycle). On the other hand, if sulfur is limited, it stops the protein synthesis due to the inability of production of sulfur-containing aminoacids, as methionin or cystein, and phosphate limitation stops the nucleic acid biosynthesis. In these two cases, there is an accumulation of NADPH and acetyl CoA. This energy increase produces inhibition of the TCA circle enzymes. Consequently, acetyl CoA is accumulated and it enters in the PHA circle by the conversion of acetyl CoA in acetoacetyl CoA *via* β-ketothiolase as can be observe in **figures 7 and 8**.

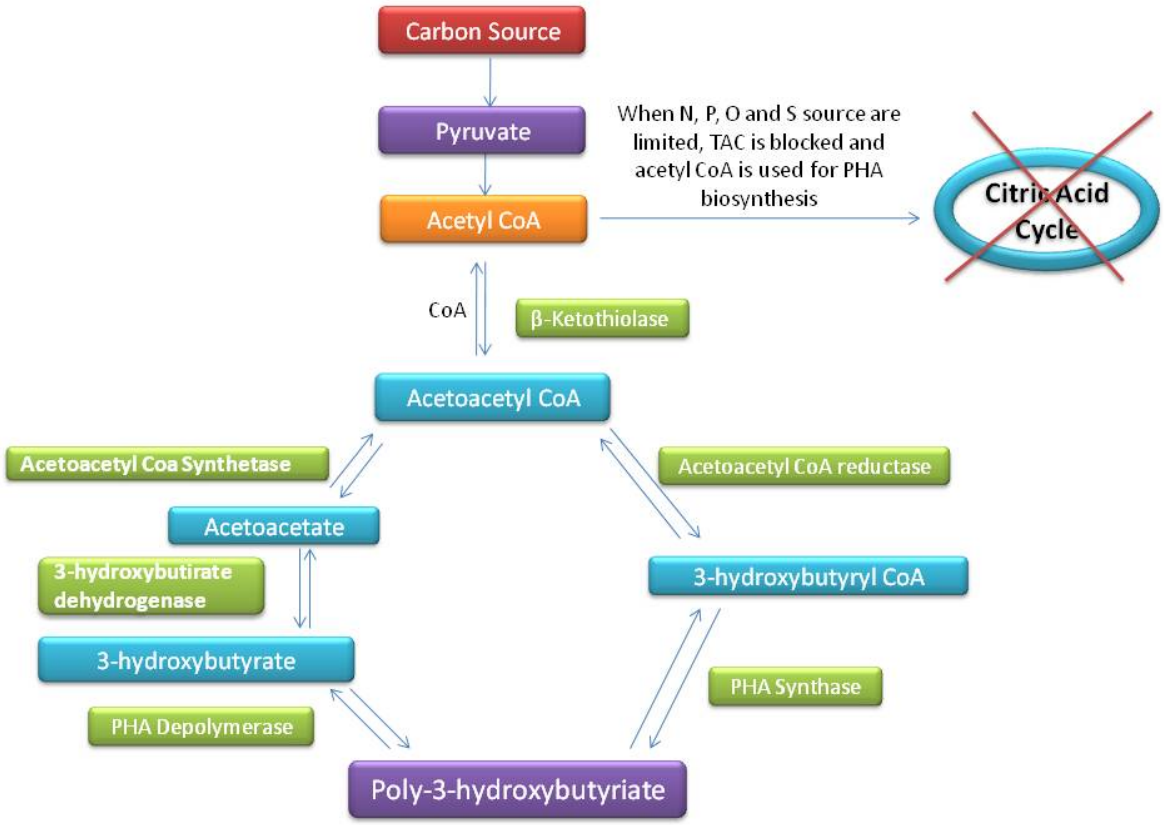


Figure 7. Diagram of the metabolic pathway I for PHB biosynthesis.
(**Lee 1996a, Madison 1999, Sudesh 2000, Zinn 2001, Braunegg 2004**)

7.3. Glucose metabolism

PHAs are produced from a wide variety of substrates such as renewable resources (sucrose, starch, cellulose, triacylglycerols), fossil resources (methane, mineral oil, lignite, hard coal), by-products (molasses, whey, glycerol), chemicals (propionic acid, 4-hydroxybutyric acid) and CO_2. The major part of the PHAs is produced by microorganisms that catabolize glucose *via* the Enter-Doudoroff pathway (KDPG) which is only found among prokaryotic organisms (**Solaiman 2006b**).

The final product of this pathway is pyruvate which can be converted by the pyruvate dehydrogenase enzymatic system to acetyl CoA, the key compound towards TCA circle and PHA biosynthesis.

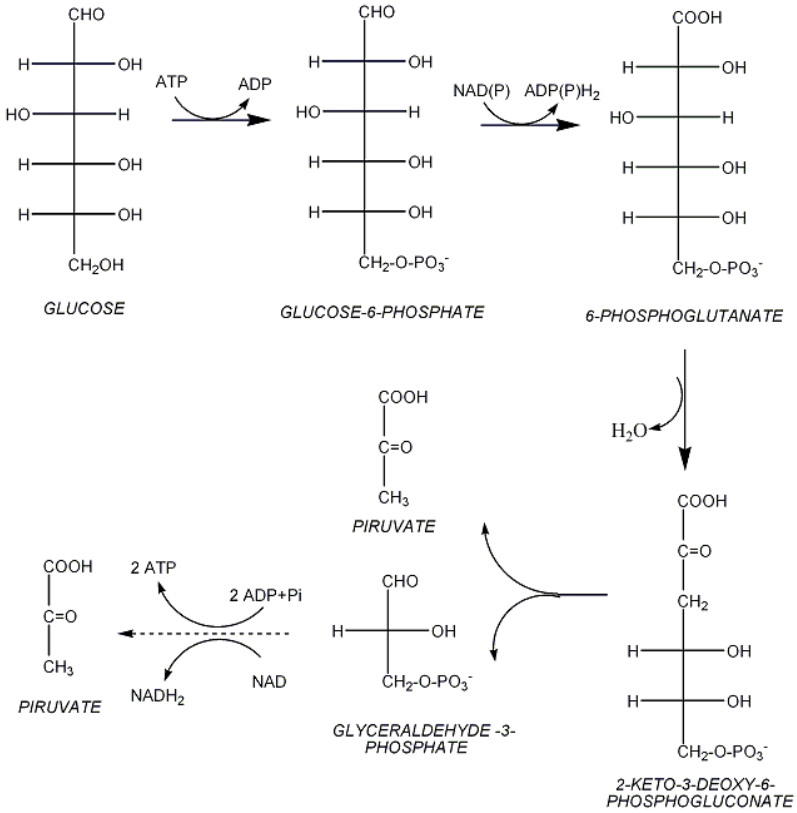


Figure 8. Glucose metabolism *via* Enter-Doudoroff pathway

7.4. Biodegradation of PHAs

PHAs can be degraded by two different depolymerases: intracellular and extracellular PHA depolymerases (i-PHA, e-PHA). I-PHAs of the accumulating bacteria act in order to consume PHA in the absence of the suitable exogenous carbon source (**Jendrossek 1996**, **Tokiwa 2004**). As with other polymers synthesized by living systems, PHB can be degraded at a later stage by the microorganisms producing it. The diagram in **figure 8** shows not only the pathway I for PHB biosynthesis but also its degradation by i-PHA. When the supply of the limiting nutrient is restored the

TCA activity is regenerated, and the increased CoA levels lead to inhibition of the β-kethothiolase. As a consequence, the cells start to break down the polymer and its carbon flows into the TCA circle.

E-PHAs are secreted from various microorganisms for the utilization of the possible PHA left in the environment after accumulating bacteria have died **(Hiraishi 2009, knoll 2009**). PHB depolymerases have been isolated and purified from various microorganisms belonging to the *Alcaligenes*, *Comamonas* and *Pseudomonas* species (**Tokiwa 2004**). The ability to degrade PHA is not restricted to bacteria, and many PHA-degrading fungi have also been identified (**Kim 2003**). The activities of these enzymes depend on the composition of the polymer, stereo regularity of the polymer (only PHAs with R- configuration are degraded by the depolymerases), crystallinity (degradability decreases as the overall crystallinity increases), molecular mass (LMW polymers are generally biodegraded more rapidly), and the environmental conditions (temperature, moisture level, pH, and nutrient supply). The degradation rate of a piece of PHB typically ranges from a few months in aerobic sewage to years in seawater (**Jendrossek 1996, Madison 1999**) (**figure 9**). PHAs are degraded by specific e-PHAs, which hydrolyze the polymer by surface erosion to water soluble monomers and/or oligomers. PHA-degrading bacteria differ with respect to the type of polyester they can degrade. Most of the characterized bacteria are specific to PHB, but there are also some bacteria that can utilize a large variety of PHAs (S or MCL, and copolymers). Also, copolymers containing 4HB monomers units are degraded faster than PHB or 3HB-3HV copolymers (**Jendrossek 1996, Madison 1999, Sudesh 2000**).

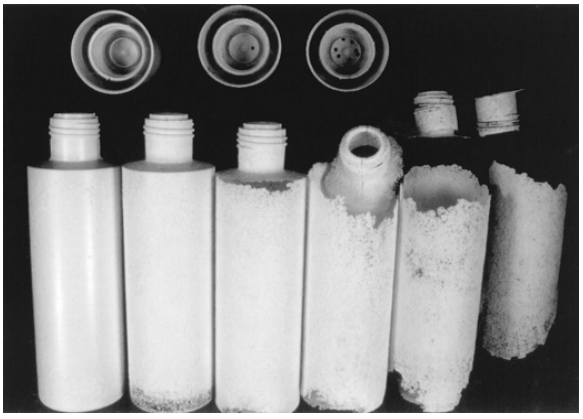


Figure 9. Degradation of P(3HB-3HV) in aerobic sewage sludge. Bottles were incubated during the summer (average temperature, 20 °C) in aerobic conditions. The progress of degradation is demonstrated with bottles that have been subjected to this treatment for 0, 2, 4, 6, 8, and 10 weeks (from left to right) (**Madison 1999**)

PHAs enzymatic degradation studies have been basically focused on utilizing depolymerases. In this thesis, studies of polymer degradation have been carried out. Since lipases (triacylglycerol hydrolases E.C. 3.1.1.3) present some advantages compared to other enzymes, they have been used in this thesis to degrade P(3HB-4HB) and PHB in a sustainable process set out for an easy industrial scale application (**CHAPTER II, sections 1 and 2**).

8. Industrial production of PHA

PHAs production is commonly carried out by fermentation using an aqueous medium, and with bacterial strains such as the ones commented on in section 6. Other production possibilities have been tried out, as the use of transgenic plants or solid state fermentation, to propose alternatives for the PHAs manufacture with lower production costs, but with lower outcomes (**Oliveira 2007**). PHAs production by fermentation can be carried out by different operating modes: batch, fed-batch and continuous fermentations. In the industry the fermentation for polymer production is achieved by means of bioreactors in continuous processes.

Batch - It is a closed system culture in which cells are grown in a fixed volume of nutrient culture medium under specific environmental conditions (temperature, pressure, aeration, nutrient type, etc). The substrates are added at the beginning and products are removed at the end of the process. This fermentation process is acceptable for growth studies and screenings for potential PHA accumulation organism. In batch fermentation, the medium is prepared to be growth-limited in one essential nutrient as nitrogen or phosphate, while other nutrients, especially the carbon source, are in excess. Depending on the microorganism used, the substrate and the fermentation volume, the experiment can be performed in 24 to 48 hours. During this time, the microorganism goes through the main phases: lag, growth, stationary and finally death phase. The PHA produced in the fermentation can be consumed by the same microorganism, therefore this method rarely gives an indication of the maximum capacity of the cells to accumulate PHA (**Zinn 2001**).

Fed-batch - Fed-batch fermentation is basically a batch culture that is continuously supplemented with selected nutrients after entering the late exponential phase (**Zinn 2001**). It is probably the most common operation in industrial biotechnology processes. Here, selected nutrients, mostly the carbon source, are supplemented after their depletion or are maintained at certain constant levels. This produces a high density of cells, reducing the costs of PHA production significantly (**Lee 1998**). Classically, PHA production is accomplished under fed-batch feeding conditions, where substrates are supplied to the fermentation broth when required. In a bioreactor system, this addition can be automated to a high degree by coupling the substrate addition to process parameters like the dissolved oxygen tension, pH-value or the CO_2 concentration, or by automatic feeding under a well-known kinetic control of the process (**Koller 2010**). For instance, **figure 10** shows fermentation carried out in a stirred tank reactor of Labfors3 (Infors, Switzerland). It had a total volume of 3 L, with a working volume of 1.5 L (0.5 L of inoculum). All relevant fermentation parameters (pH value, flow rate, dissolved oxygen concentration, stirrer speed, consumption of pH correction solutions, and activity of antifoam probe) were monitored and recorded by IRIS software program.

Continuous culture - It is an open system which allows matter to flow in and out of the process. Continuous production is achieved when the added medium displaces an equal volume of the fermentation broth. If medium is added continuously, a steady state is achieved eventually, which means that the formation of new biomass by the culture is balanced by the loss of cells from the flow

out. In this steady state the main fermentation parameters such as temperature, pH, volume, pressure, and mass do not vary with the time. The most important advantage of continuous cultures fermentation is that it allows the production of polymers with higher constant quality and higher productivities compared with discontinuous processes (**Akaraonye 2010**).

Figure 10. Photography of a 3L-Bioreactor while producing PHB by a novel strain

For the PHAs production, it is also important to take into account that there are two groups of PHA-producing bacteria that have been classified based on the culture conditions required for efficient PHA biosynthesis (**Lee 1996a, Khanna 2005b**). Therefore, it is important to develop a suitable fermentation strategy for each type in order to enhance the production of PHAs. The first group of bacteria (nutrient limitation) requires the limitation of an essential nutrient, such as nitrogen, phosphorous, magnesium or sulphur, and an excess of carbon. The bacteria included in this group are *Cupriavidus necator*, *Protomonas extorquens*, and *Protomonas oleovorans*. Strategy for biopolymer production consists of a two-step fed-batch fermentation. Cells are first grown to a desired concentration without nutrient limitation, after which an essential nutrient is limited to induce the PHA biosynthesis. When the limitation is applied, the cells no longer grow, and they use the carbon source provided in excess to produce and accumulate polymer. The cell concentration increases only as a result of the intracellular accumulation of polymer (**Choi 1999**). The second group of bacteria (growth-associated PHA producer) which include *Alcaligenes latus* and recombinant *Escherichia coli* do not require nutrient limitation for PHA biosynthesis and can accumulate polymer during growth. For this type, an optimal nutrient feeding strategy, which supports both cell growth and PHA accumulation, needs to be applied (**Choi 1999**).

Batch and fed-batch fermentation with nutrient limitation were the main methodologies used in this thesis to work with the used strains (*Cupriavidus necator, Burkholderia sacchari* and the isolated *Bacillus megaterium* uyuni S29) (entire **CHAPTER I**).

8.1. Bacterial growth curve

The increase in the cell size and cell mass during the development of an organism is termed as growth, affected by both physical (pH, temperature, osmotic pressure, hydrostatic pressure, etc) and nutritional factors (amount of carbon, nitrogen, other trace elements provided in the growth medium). The organisms require these basic parameters for their energy generation and cellular biosynthesis.

To study the growth of a bacterial population, the sterile broth should be inoculated with the viable cells of the bacterium and incubated under optimal growth conditions. The bacterium starts utilizing the components of the media and it will increase its size and cellular mass. Bacteria are unicellular organisms which divide by binary fission when they reach a certain size. The bacterium is then known to be in an active growth phase. **Figure 11** shows a diagram of the typical bacteria growth curve with the different growth phases[15].

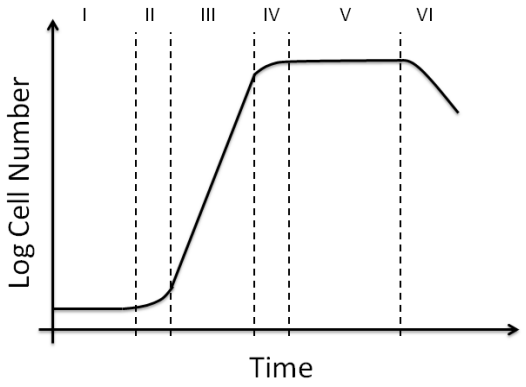


Figure 11. Sigmoid curve of growth of population

I. **Lag Phase** - When a microorganism is introduced into a fresh medium, it takes some time to adapt to the new environment. This phase is termed as the Lag Phase and is a period of no net growth after inoculation (no increase in cell numbers). It is unproductive in terms of biomass/product accumulation and should be as short as possible. Therefore, it is very important to decrease the lag period to increase the productivity, by inoculating with exponential phase cells, pre-acclimating inoculums in growth media, using large cell inoculums size (5-10% by volume). The length of this phase varies with each organism and with the previous cultivation conditions.

[15] http://amrita.vlab.co.in/?sub=3&brch=73&sim=1105&cnt=1

II. **Acceleration Phase -** once the cells have adapted to the new environment, cell division occurs at increasing frequency until the maximum growth rate is reached.

I. **Exponential or Logarithmic Growth Phase** - Cell mass and cell number increase exponentially with time. Cell population doubles at regular intervals of time. The microorganism metabolic activity increases and the organism begins the DNA replication by binary fission at a constant rate. The growth medium is exploited at the maximal rate, the culture reaches the maximum growth rate, and the number of bacteria increases logarithmically (exponentially). This will result in a balanced growth. The time taken by the bacteria to double in number during a specified time period is known as the generation time. The generation time tends to vary with different organisms. For instance, *Escherichia coli* divides every 20 min, hence its generation time is 20 minutes, and for *Staphylococcus aureus* it is 30 min.

II. **Deceleration Phase** - In this phase, an essential nutrient in culture begins to run out. This nutrient is often referred to as the growth limiting substrate. Media are often designed in a way that this nutrient is generally the carbon source.

III. **Stationary Phase** - As the bacterial population continues to grow, all the nutrients in the growth medium are used up by the microorganism for their rapid multiplication. This results in the accumulation of waste materials, toxic metabolites and inhibitory compounds such as antibiotics in the medium. This shifts the conditions of the medium such as pH and temperature, thereby creating an unfavorable environment for the bacterial growth. The reproduction rate will slow down, the cells undergoing division is equal to the number of death cells, and finally bacterium stops its division completely. The cell number is not increased and thus the growth rate is stabilized.

IV. **Death/Decay/Decline Phase**- The depletion of nutrients and the subsequent accumulation of metabolic waste products and other toxic materials in the media will facilitate the bacterium to move on to the death phase. During this phase, the bacterium completely loses its ability to reproduce. Individual bacteria begin to die due to the unfavorable conditions. The death is rapid and at a uniform rate. The number of dead cells exceeds the number of living cells. Some organisms which can resist this condition can survive in the environment by producing endospores.

8.2. Kinetics in batch culture

Quantitative description of cellular processes is an indispensable tool to characterize fermentation processes. Thus, the most important quantitative parameters, specific and maximum growth rate, yield and productivity, are quantitative measures that specify how the cells grow and convert the substrates to the product (**Ratlege 2006**). Using mass balances, those process characteristic

parameters among others like final substrate, product and biomass concentrations can be determined for the different reactor operating modes. For a general reactor system, rates of change of component masses in the system can be related to the rate of reaction using the following general equation:

$$\frac{dm}{dt} = M_i - M_0 + R_G - R_C \quad \textbf{(1)}$$

Where m is a mass of component A (g) in the vessel, t is time (h), M_i is the mass flow of A entering the reactor (g/h), M_o is the mass flow rate of A leaving the reactor, R_G is the mass rate of generation of A by reaction, and R_C is the mass rate of consumption of A by reaction (**Doran 1995**).

In order to describe the exponential phase, a mass balance is performed on cells in a batch fermentor using the **equation 1**. Cells do not flow into or out of the vessel, so that $M_i=M_0=O$. Mass of cells in the reactor, m, is equal to the cell concentration X (g/L) multiplied by the liquid volume V (L). The mass rate of cell growth R_G is equal to r_xV where r_x is the volumetric rate of biomass formation (g/Lh). The growth rate, r_x is defined as the increment of biomass per time ($\Delta X/\Delta t$) (g/Lh). Since the growth rate is proportional to the biomass concentration, it can also be defined as $r_x=\mu X$, where μ is the specific growth rate (h^{-1}). If cell death takes place in the reactor beside growth, then $R_C=r_dV$ where r_d is the volumetric rate of cell death. r_d can be expressed using the first order equation $r_d=k_dX$ where k_d is the specific death constant. Therefore, **equation 1** for cells in a batch reactor is:

$$\frac{d(XV)}{dt} = \mu XV - k_d XV \quad \textbf{(2)}$$

Specific growth rate (μ):

The exponential growth phase is the most important step on the cellular growth cycle when the product to be produced is either the biomass itself or a growth associated product. Quantification of the exponential growth rate is the first fundamental step in the quantification of culture kinetics. The establishment of exponential growth is dependent on a number of factors: viable inoculums, a suitable energy source, the presence of excess nutrients and growth factors, the absence of inhibitors, a suitable environment (i.e. temperature, dissolved oxygen). The specific growth rate, μ, defines the exponential growth as follows[16].

For constant volume V in the bioreactor and if the rate of cell death is negligible in the exponential phase, **equation 2** is reduced to (**Doran 1995**):

$$\frac{dX}{dt} = \mu X \quad \textbf{(3)}$$

Because μ in batch culture remains approximately constant during the exponential phase, it can be directly integrated to find the relationship between batch time and cell concentration. When t = 0 at

[16] Dr. Clem Kuek:ZIP/Lect+Prac/IndusMicrobiol/Lectures/GrowthKinetics.doc

the beginning of the exponential growth phase, then $X = X_0$ (X_0 is the biomass concentration at the start of the fermentation. Thus integrating **equation 3**,

$$\int_{X_0}^{X} \frac{dX}{X} = \int_{0}^{t} \mu dt \Rightarrow Ln\frac{X}{X_0} = \mu t$$

$$X = X_0 e^{\mu t} \quad \textbf{(4)}$$

Where X_0 is the original biomass, X is the biomass after a certain time, and e is the base of the natural log. Thus, the natural log in **equation 4** becomes: $\ln X = \ln X_0 + \mu t$. As a result, the plot of ln X versus t gives a straight line in the exponential phase and the slope corresponds to the specific growth rate (h^{-1}). Therefore, the determination of the specific growth rate from data would be calculated by numerical definition as the difference between the values on either side of the data point.

$$\mu = \frac{X_2 - X_1}{t_2 - t_1}$$

Maximum specific growth rate (μ_{max}) and saturation constant (K_s):

Throughtout the entire fermentation process in batch culture, μ does not remain constant (**figure 12**) and can be estimated by using the values for growth determined as described previously. Therefore, specific growth rate may be estimated by the relationship: $\mu = r_x/X$.

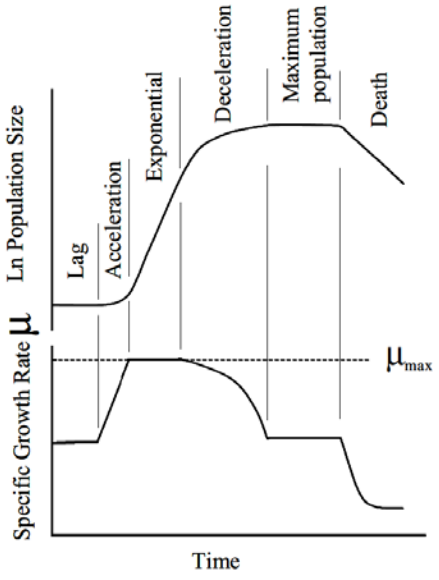


Figure 12. Phases of growth in batch culture and evolution of the specific growth rate along a fermentation process[16]

There is a moment in the fermentation process when the μ achieves its maximal value (μ_{max}). For processes where maximal growth rates are desirable, attainment of μ_{max} in culture is important because μ_{max} is determined by the genetics of the microorganism and the conditions of culture. As substrate concentration is the major affecting factor, μ_{max} can be calculated by their relationship. **Figure 13** shows the correlation between μ and the substrate concentration.

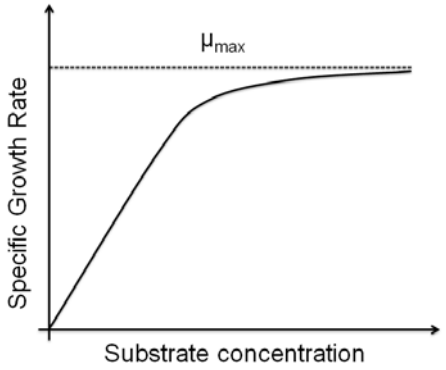


Figure 13. Relationship between μ and the substrate concentration in a batch culture

Monod's growth model (1941) was proposed as an empirical model to describe microbial growth (**equation 5**). It differs from classical growth models, such as those proposed by Gompertz (1825), Verhulst (1845, 1847) or Richards (1959), because it introduces the concept of a limiting nutrient (**Lobry 1992**). The model defines the correlation between the growth rate and the concentration of the limiting nutrient. A nutrient is said to be limiting when there is a causal relationship between its exhaustion and the end of growth. It is applied only where a single substrate (S) is limiting and the accumulation of growth associated toxins can be ignored (which is generally the case in dilute microbial suspensions).

$$\mu = \mu_{max} \cdot \frac{S}{K_S + S} \Rightarrow \frac{dX}{dt} = \frac{\mu_{max} S X}{K_S + S} \quad \textbf{(5)}$$

μ_{max} is the maximum specific growth rate (h^{-1}) and is an indication of how fast the organism grows in conditions where all limiting substrates are in excess. K_S is the saturation constant (g/L of substrate). It is numerically equal to the substrate concentration at which $r_x = \mu_{max}/2$. It is an indication of how quickly μ migrates from μ_{max} to 0 as the concentration of limiting substrate (maybe glucose) moves towards 0. These two parameters are constants for a given organism for a specified substrate. Both can be estimated using the Lineweaver-Burke equation:

$$\frac{1}{\mu} = \frac{K_S}{\mu_{max}} \frac{1}{S} + \frac{1}{\mu_{max}}$$

The equation now has the form of a straight line with an interception. By plotting1/μ as a function of 1/S, the slope of the obtained straight line is K_S/μ_{max} and the y-axis intercept is $1/\mu_{max}$.

Yield coefficients:

The yield (Y) specifies the amount of product or cell quantity obtained from the substrate or raw material. It indicates the degree of efficiency of the conversion of substrates into desired products. Attainment of efficient Y translates directly into economic efficiency, and thus productivity. It is determined by the genetics of the microorganism, the conditions of the culture, and the nature of the input (substrate). For instance, the Yield of cell mass per unit mass substrate utilized ($Y_{x/s}$) or the Yield of product mass per unit mass of substrate utilized ($Y_{p/n}$) can be calculated:

$$Y_{X/S} = \frac{\Delta X}{\Delta S} = \frac{\text{mass of cells formed}}{\text{mass of substrate consumed}}$$

$$\Delta S = \Delta S_{\text{assimilate d into biomass}} + \Delta S_{\text{assimilate d into extracelul ar products}} + \Delta S_{\text{growth energy}} + \Delta S_{\text{maintenanc e energy}}$$

Because ΔS changes with growth conditions, $Y_{X/S}$ is not a constant.

$$Y_{P/S} = \frac{\Delta P}{\Delta S} = \frac{\text{mass of product formed}}{\text{mass of substrate consumed}}$$

Volumetric productivity (Qp):

The productivity specifies the rate of the product formation. Overall Yield of cell mass per unit mass substrate utilized ($Y_{x/s}$) or the Yield of product mass per unit mass of substrate utilized ($Y_{p/n}$) (g/Lh) is the average production capacity per unit volume and time of the bioreactor. In order to maximize Q_p, it is best to operate the bioreactor as close as possible to the physical restrictions, which are the result of mass and heat transfer during the process. These limitations are stoichiometrically related to product formation (**Van't Riet 1991**). In the case of suspended cells producing PHAs, physical restrictions are the available room for the cell growth in the reactor and the intracellular space available for the PHA accumulation. Therefore, the variable costs of a bioprocess are related to the volumetric capacity of the fermentation unit, and the process time required to produce a defined amount of the product (**Maurer 2006**). Thus the volumetric productivity Q_P is the most plausible target for optimization cost and is defined as:

$$Q_P = \frac{P}{V \cdot t}$$

where P is product concentration (g/L) and V is total volume (L). In the case of batch culture V is constant. Consequently the productivity in batch culture (Q_p) may be described by the equation

$$Q_P = \frac{X_{max} - X_0}{t_1 - t_2}$$

where Q_p is the output of the culture in terms of biomass concentration per hour, X_{max} is the maximum cell concentration achieved at the stationary phase, X_0 is the initial cell concentration at inoculation, t_1,

is the time during which the organism grows at μ_{max} and t_2 is the time during which the organism is not growing at μ_{max} and includes the lag phase, the deceleration phase, and the periods of batching, sterilizing and harvesting (**Stambury 1995**).

Specific production rate (q_p):

Irrespective of the PHA production type and in analogy to the derivation of the relations for the biomass from **equation 1**, the volumetric rate of product formation r_p in cell culture can be expressed as a function of residual biomass:

$$\frac{dP}{dt} = r_P = q_P X_R$$

where P is product concentration (g/L), X_R is residual biomass concentration (g/L) calculated as the difference between total biomass and product concentration (X_R=X-P), and q_P is the specific rate of product formation with units (g/gh). q_P can be evaluated at any time during fermentation as the ratio of production rate and residual biomass concentration.

8.3. Kinetics in fed-batch culture

In fed-batch fermentation, selected nutrients, mostly the carbon source, are supplemented after their depletion or are maintained at certain levels. The advantage of fed-batch cultures in general is the high cell densities that can be obtained which reduce the costs of PHA product significantly. A disadvantage of the process is that the cells grow at a decreasing rate when the feed rate and the feed concentration are kept constant. The reason for this is that the added nutrients are consumed by an ever-increasing cell concentration during identical time units.

The increase in working volume during fed-batch fermentation is described by the fee flow rate F.

$$F = \frac{dV}{dT}$$

where F is the flow rate (L/h), V is the volume (L) and T is the time (h).

When **equation 1** is applied for mass balance on biomass, product and substrate, expressions for the rates of biomass formation, product formation and substrate consumption can be derived and, after simplifications, the next equations are obtained (**Doran 1995**):

$$\frac{dX}{dt} = X(\mu - D)$$

$$\frac{dP}{dt} = (r_P + \mu \cdot Y_{PX})X_t - PD$$

$$\frac{dS}{dt} = D(S_1 - S) - \frac{\mu X}{Y_{XS}}$$

where D is the dilution rate which is constant in a fed-batch fermentation (in a continuous operation mode, it would not be a constant), and Y_{XS} is the biomass yield from substrate,

9. Applications of PHAs

9.1. Drug Delivery Systems

The systems for drug delivery provide significant advantages compared to conventional strategies (**Shi 2010**). For example, they could improve the therapeutic activity of drugs by enhancing their bioavailability and their effectual concentration, improving solubility of hydrophobic drugs and prolonging half-life. They could also reduce the toxic side effects by releasing drugs in a sustained or stimuli-triggered manner. In addition, nano-particles could be passively accumulated in specific tissues such as tumor or inflammatory tissues through the enhanced permeability and retention effect (**Langer 1998**). To be suitable as a drug carrier the material has to be biocompatible, biodegradable in certain applications, and nontoxic. Many different materials including liposome, polymers, and some self-assembled macromolecules have been developed as drug delivery carriers. Among these, biodegradable polymers are of interest because of their versatility and biocompatibility properties (**Mundargi 2008, Xiong 2010**). Due to the biopolymer characteristics, it becomes possible to engineer multiple functionalities required for efficient drug delivery, simultaneously maintaining biocompatibility, facile manufacturing and stable formulation (**Xiong 2010**). PHAs particularly have been studied for implant biomedical and controlled drug-release applications because of their biocompatibility and biodegradability (**Sun 2007, Xiao 2007, Wu 2009, Xiong 2010**). In the past, some success was reported with the use of PHB, P(3HB-3HV), P(3HB-4HB), and P(3HB- 3HHx) studying the possibility of using these biopolymers as controlled drug release matrices: PHB as a drug carrier (**Koosha 1989**), cellular response to different PHAs particles **Saad 1996**, P(3HB-3HV) and P(3HB-4HB) rods as drug carriers (**Gürsel 2001**), P(3HB-3HV) controlled release systems (**Rossi 2004**), P(3HB- 3HHx) nano- and micro-particles for drug release (**Lu 2010**), PHB and P(3HB-3HHx) nano-particles (**Xiong 2010**). There are very few papers reporting on the incorporation and subsequent release of therapeutic agents with PHB systems.

In this thesis, the different methods for the PHB-loaded nano- and micro-particles preparation were studied for the optimal particle-entrapment of an active principle (entire **CHAPTER III**).

9.2. Other applications

Different applications require different material properties of the biopolyester. As previously commented on, PHAs properties can be generated by the fine-tuning of their composition during biosynthesis. Also, their specific properties of biodegradability, biocompatibility and thermo-plasticity, common to all of them, make PHAs suitable for a wide range of applications. Therefore, the use of

PHAs is not limited to simple packaging materials, but covers commodity items, materials for agro-industrial purposes and for pharmaceutical and medical applications (**Chen 2005, Chen 2009**).

Packaging industry – PHAs can be utilized as raw material for all packaging products that are used for a short period of time, including food utensils, films, daily consumables or electronic appliances. In this case, biodegradability is a very important property because these products are used for a short period of time, often discarded soon after the first use, and a very big amount of waste is rejected. Therefore, the possibility of biodegradation is an advantage of PHAs over the conventional plastics (**Chen 2009, Koller 2010**). As domestic & industrial products, PHAs can be found among others in cosmetics packages, cups, cutlery, packages for milk and juice through the lamination with cardboard, and also engine oil bottles and shampoo (for example Wella®) (**Sudesh 2000**).

Bulk chemicals - Heat sensitive adhesives[17], latex[18], and smart gels (**Hoffman 2001**).

Block copolymerization - LMW-PHA can be changed into PHA diols for block copolymerization with other polymers (**Ravenelle 2002, Chen 2006**).

Plastic processing - PHAs can be used as a processing aids for plastic processing (improve heat resistance).

Textile industry - Like nylons, PHAs can be processed into fibers[19] (**Schmack 2000**, **Li 2008**).

Agriculture - PHAs are used in products which have controlled release of growth regulators of plants or pesticides and can also be applied in mulching film material or seeding pots (**Sudesh 2000, Philip 2007**).

Healthy food additives - PHAs oligomers can be used as food supplements for obtaining ketone bodies[20].

Industrial microbiology - The PHAs synthesis operon can be used as a metabolic regulator or resistance enhancer to improve the performance of industrial microbial strains.

Biofuels or fuel additives - PHAs can be hydrolyzed to form hydroxyalkanoate methyl esters that are combustible (**Gao 2011**).

Protein purification - PHAs granules binding proteins phasin or PhaP are used to purify recombinant proteins (**Wang 2008**).

Fine chemical industry - PHA monomers are all chiral R-forms. Therefore they have many potential applications as intermediates for the synthesis of antibiotics, vitamins, aromatics, pheromones and other fine chemicals. Recent studies revealed that 3HB could stimulate growth of several mammalian cells (**Chen 2005**, **Liu 2007, Philip 2007**).

[17] Rutherford DR, Hammar WJ, Babu GN. Patent US5614576 (1997)
[18] George N,Hammond T, Liddell JM, Satgurunathan R, Turner PD. Patent US5977250 (1999)
[19] Martin DP, Rizk s, Ahuja A, Williams SF. Patent US 20040234576 A1 (2004)
[20] Martin DP, Peoples OP, Williams SF, Luhua Z.Patent US 6380244 B2 (1999)

Medical applications - Due to their biodegradability and biocompatibility, PHAs could easily be developed into medical materials. PHB was the first PHA polymer used in biomedical applications[21]. The first PHAs used in this area were developed for composed surgical sutures that are degraded in the proper body forming products that are absorbed by the organism. PHAs are used in a wide variety of medical fields such as cardiovascular, wound healing, orthopedic, and tissue engineering. Some current examples are the manufacturing of scaffold for bone and cartilage regeneration (**Puppi 2010),** PHB scaffolds for tissue engineering (**Misra 2010**), PHA nano-fiber scaffolds (**Xu 2010**), and skeletal tissue engineering (**Khan 2010**). They are also used as osteosynthetic materials in the stimulation of bone growth owing to their piezoelectric properties, in bone plates, surgical sutures and blood vessel replacements (**Khanna 2005b**). Moreover, PHA monomers, especially R-3HB, have therapeutic effects on Alzheimer's and Parkinson's diseases, osteoporosis and even memory improvement (**Zou 2009**). In the case of the PHA particles, the drugs are encapsulated inside of a PHA matrix allowing its release in the ideal concentration and in the place desired for the period of time intended (**Zinn 2001, Chen 2005, Misra 2006**).

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OBJECTIVES

According to the introduction, the main goal of the thesis is to obtain and transform PHB from different environmental sources in order to develop drug delivery systems. To attain this objective, the thesis has been structured into three chapters with specific goals in each, compiled as follows:

CHAPTER I. PHB PRODUCTION

In order to replace the well-known industrial production bacterial strains with new ones and to solve the most costly factors in its production, the objectives are:

- To isolate a novel potential PHB-producing bacterium from Bolivian hypersaline lake water and mud samples capable of growing in one of the conventional media for industrial PHB production without excessive salt concentrations, to taxonomically identify the selected microorganism and to characterize the biopolymer produced.
- To study the maximal PHB production potential of the novel strain in the conventional media used for industrial biopolymer production, the possibility that the strain accumulates different types of PHAs, their characterization, and the cellular morphology during the biosynthesis process.
- To analyze the influence of different concentrations of salt on the cell growth and on the PHB production by the novel *Bacillus megaterium* uyuni S29.
- To explore the influence of glycerol as a cheap carbon source on the growth kinetics of two collection strains, *Cupriavidus necator* (DMS 545) and *Burkholderia sacchari* (DMS 17165), on their polymer productions, and on the molecular mass of the produced biopolymer.

CHAPTER II. PHB TRANSFORMATION

In order to obtain more manageable molecular masses of PHB to design anphiphilic block copolymers, the objectives are:

- To study the enzymatic degradation of P(3HB-co-4HB) by means of two different commercial lipases, in order to reduce the molecular mass of the biopolymer.
- To analyze the enzymatic degradation of the PHB by means of a commercial lipase in order to obtain low molecular mass PHB.

CHAPTER III. APPLICATION

In order to know the capacity of PHB to obtain micro- and nano-particles the objectives are:

- To determine the optimal doxycycline-loaded PHB micro- and nano-particles by comparing different physico-mechanical methodologies and varying different processing conditions in order to determine their influence on morphology, size, and drug entrapment capabilities.

- To evaluate the suitability of the most promising methods for the preparation of doxycycline - loaded micro- and nano-spheres previously tested with different PHAs and to attempt to improve the results of drug entrapment capabilities and method efficiency.

CHAPTER I. PHB PRODUCTION

Section 1: *Novel Poly[(R)-3-hydroxybutyrate]-producing bacterium isolated from a Bolivian hypersaline lake* [1]

1. Summary

Poly[(*R*)-3-hydroxybutyrate] (PHB) constitutes a biopolymer synthesized from renewable resources by various microorganisms. This work focuses on finding a new PHB-producing bacterium capable of growing in conventional media used for industrial biopolymer production, its taxonomical identification, and characterization of its biopolymer. Thus, a bacterial isolation process was carried out from environmental samples of water and mud. Among the isolates, strain S-29 was selected and used in a fed-batch fermentation to generate a biopolymer. This biopolymer was recovered and identified as PHB homopolymer. Surprisingly, it featured several fractions of different molecular masses, and thermal properties unusual for PHB. Hence, the microorganism S-29, genetically identified as a new strain of *Bacillus megaterium*, turned out to be interesting not only due to its growth and PHB accumulation kinetics under the investigated cultivation conditions, but also due to the thermal properties of the produced PHB.

2. Introduction

The rapid increase in human population during the 20th century has raised the global consumption of goods, thus increasing the volume of non-biodegradable residues, especially plastics. These growing piles of resistant waste constitute a severe environmental problem of soaring impact. Therefore, there is a need to study and develop new biodegradable polymers with plastic-like properties (*1*). Polyhydroxyalkanoates (PHAs) are biodegradable polyesters synthesized by microorganisms as carbon and energy storage materials under the conditions of limiting nutrients such as nitrogen, phosphate or oxygen together with an excess of carbon source (*2*). Under these conditions, several microorganisms are able to divert the usual carbon flux (conversion of acetyl-CoA in the tricarboxylic acid cycle for creation of energy and metabolites for biomass formation) towards synthesis of PHA. Such microorganisms can also reutilize the produced PHA as internal carbon substrate when the supply of the growth-limiting nutrient is provided again (*1*,*3*). The most extensively studied strain for PHA production on an industrial scale is *Cupriavidus necator*; this is due to its versatility in the accumulation of poly[(*R*)-3-hydroxybutyrate] (PHB) and its copolymers (*4,5*). To a lesser extent, *Azahydromonas lata* (formerly known as *Alcaligenes latus*) (*4,6*), *Azotobacter* sp. and recombinant *Escherichia coli* are also used for polymer production (*4,6,7*). PHAs are biobased, biodegradable and biocompatible biopolyesters which possess thermal properties similar to some petroleum-based polymers such as polypropylene (*4*). PHB is the most frequently occurring PHA and constitutes a

[1] *It corresponds to the article accepted for publication in Food Technology and Biotechnology: Novel Poly[(R)-3-hydroxybutyrate]-producing bacterium isolated from a Bolivian hypersaline lake by Rodríguez-Contreras Alejandra, Koller Martin, Miranda-de Sousa Dias Miguel, Calafell Margarita, Braunegg Gehart, Marqués-Calvo María Soledad.*

linear, unbranched homopolymer consisting of (*R*)-3-hydroxybutyric acid (3HB) units. It is considered very promising as a biodegradable plastic mainly for packaging industries to solve environmental pollution problems (*7*). In addition, it exhibits potential applications in medicine, veterinary practice and agriculture due to its biocompatibility (*7*).

So far, PHAs are not competitive with petroleum-based polymers in economic terms due to their high production costs (*6*). Therefore, efforts are focused on improving the production steps that generate the major part of the costs. Recent studies attempt to solve the most costly factors (feedstock, polymer extraction and microorganism efficiency) by investigating the use of cheaper carbon sources (*8*), novel polymer isolation methods, different fermentation strategies (*9*), and by discovering new microorganisms (*10*). The importance of investigating novel strains lies in the possibility of replacing well-known industrial production strains with new ones, aspiring to a more productive and efficient polymer production process. Current studies report the isolation of new PHA-producing species from extreme environments, and some of them might replace well-established, industrially implemented microorganisms in the near future (*11*,*12*).

3. Objectives

The main goal of this work was to isolate a novel potential PHB-producing bacterium from Bolivian hypersaline lake water and mud samples, capable of growing in one of the conventional media for industrial PHB production without excessive salt concentrations. Taxonomical identification of the selected microorganism and the characterization of the produced biopolymer were further objectives of the study.

4. Materials and Methods

Samples and media

Four water and mud samples were collected, three from three different lagoons and one from a salt lake in Bolivia (Potosí Department): Laguna Colorada, Laguna Hedionda, Laguna de Chiguana and Salar de Uyuni (Fig. 1 13). The samples were taken from the surface of the lagoon shore.

For the isolation of the bacteria, HM was used, composed of (in g/L): NaCl 44.5, $MgSO_4 \cdot 7H_2O$ 0.25, $CaCl_2 \cdot 2H_2O$ 0.09, KCl 0.5, NaBr 0.06, peptone 5, yeast extract 10, glucose 1 and granulated agar 20 adjusted to the pH value of 7.0 (*14*).

The conventional medium used for PHB production was the minimal mineral medium according to Küng (*15*) which contained (in g/L): $Na_2HPO_4 \cdot 2H_2O$ 4.5, KH_2PO_4 1.5, $MgSO_4 \cdot 7H_2O$ 0.2, NaCl 1, $(NH_4)_2SO_4$ 2, $CaCl_2 \cdot 2H_2O$ 0.02, NH_4Fe(III) citrate 0.05, agar 15, trace element solution SL6 1 mL, glucose 10 g and adjusted to the pH value of 7.0. SL6 was composed of (in mg/L): $ZnSO_4 \cdot 7H_2O$ 100,

H_3BO_3 300, $CoCl_2 \cdot 6H_2O$ 200, $CuSO_4$ 6, $NiCl_2 \cdot 6H_2O$ 20, $Na_2MoO_4 \cdot 2H_2O$ 30 and $MnCl_2 \cdot 2H_2O$ 25. The components susceptible to precipitation were sterilized separately (for 21 min at 120 ºC).

Fig. 1. Bolivian map with the location where the samples were taken from. Inside the black circle: Laguna Colorada (*1*), Laguna Hedionda (*2*), Laguna Chiguana (*3*) and Salar de Uyuni (*4*). The environmental conditions and chemical-physical characteristics of these lakes are described by Hurlbert and Chang (13)

Bacterial isolation

A procedure for viable counting using serial dilutions of the samples was carried out to obtain the appropriate colony number. The liquid used for the dilutions was sterile saline solution with a dilution factor of 10^5. Aliquots of 0.1 mL of each diluted sample were spread over the surface of agar plates containing HM medium using a sterile Drigalski glass spreader. The cultures were incubated at 35 ºC for 72 hours (*16*). Once pure cultures were obtained by propagating single colonies on new HM agar plates, the strains were differentiated by their macro- and microscopic characteristics (shape and colour of the colonies, opaque character, motility and shape of the cells, sporulation, and inclusions). For storage, all strains were frozen at -80 ºC. The observation of endospores produced after 7 days on solid medium (HM) was made by means of phase contrast microscopy (Labophot Microscope, Nikon Instruments, New York, NY, USA).

Bacterial selection

For the detection of polymer-producing bacteria, Nile Blue A (Sigma-Aldrich, St. Louis, MO, USA) solution in dimethylsulfoxide (DMSO, 0.25 mg/mL) was added to the sterile HM medium. Each isolated strain was incubated at 35 ºC for 48 h. The agar plates were exposed to UV light (312 nm) after cultivation to detect PHB accumulation in the grown colonies. The cells were observed under fluorescent microscope (Labophot Microscope, Nikon Instruments) (*17*). Afterwards, the detected polymer-producing bacteria were grown in conventional liquid medium. A scale up of liquid batch cultures was performed by inoculating selected pure colonies from solid medium to 300-mL baffled shake flasks containing 100 mL of conventional medium. All strains were incubated at 35 ºC for 48 h under continuous shaking at 120 rpm. The bacterial strain, which grow significantly faster under these conditions, will be selected.

Bacterial identification

Bacterial genomic DNA was extracted by the lysozyme-proteinase K-sodium dodecyl sulphate method (modified by increasing the reagent concentration to 2.4 mg/mL of lysozyme, 0.5 mg/mL of proteinase K and of 0.8 % of sodium dodecyl sulphate). The DNA product was amplified by PCR (Bio-Rad iCycler, Madrid, Spain) reaction according to the following procedure: 30 µL of the reaction mixture contained 6 µL of the 5x Taq & Go^{TM} (MP Biomedicals, Carlsbad, CA, USA), 1.5 µL of primer pair mix EubI-forward (5'-GAGTTTGATCCTGGCTCAG-3') and 907 r-reverse (5'-CCGTCATTTC(AC)TTT(AG)AGTTT-3') both at a concentration of 10 pmol/µL and 20-30 ng of template (*19*). The PCR products were purified with GeneClean Turbo Kit (MP Biomedicals) as recommended by the manufacturer. The fragments were sequenced in Molecular Biology-ZMF (Centre for Medical Research in Graz, Austria) using the reverse primer 907r. The fragments were taxonomically identified according to partial 16S rDNA and compared to sequences available in the GenBank databases using BLAST. The nucleotide sequence data reported in this paper have been deposited at GenBank (NCBI, Bethesda, MD, USA) (http://blast.ncbi.nlm.nih.gov/Blast.cgi), with an accession number of JF508445.

Polymer production

Fed-batch fermentation in shake flasks was performed with the selected strain. Seed culture of the same strain was used to prepare the inoculum cultures. Two parallel setups in 300-mL shake flasks with 100 mL of conventional medium were inoculated from solid medium and incubated for 24 h at 35 ºC and 120 rpm. A volume of 5 mL of selected precultures was then used to inoculate 300-mL fermentation flasks containing 150 mL of conventional medium for 72 h at 35 ºC and 120 rpm. Glucose was added as a solution of 50 % (by mass per volume) during the fed-batch fermentation to avoid the carbon source limitation. Samples of 5 mL were taken at regular time intervals to trace the polymer production.

In order to determine the Cell dry mass (CDM), samples of 5 mL of culture broth were taken during the fed-batch fermentation and centrifuged at 12000x for 20 min (Heraeus Megafuge 1.0 R refrigerated centrifuge, DJB Labcared, Newport Pagnell, UK). The pellet was frozen, lyophilised and weighed to determine the CDM. For PHA determination, the PHA in lyophilized biomass samples was transesterified by acidic methanolysis according to Braunegg's method (*18*). Analyses were carried out with an Agilent Technologies 6850 gas chromatograph (GC) equipped with 30-m HP5 column (Hewlett-Packard, USA) and a 6850 Series Autosampler (Agilent Technologies, Santa Clara, USA). The methyl esters of the PHA constituents were detected by a flame ionization detector with helium as carrier gas (split ratio of 1:10). Pure Poly[3-hydroxybutyrate-co-3-hydroxyvalerate] (P(3HB-co-19.1-%-3HV)) (Biopol; Imperial Chemical Industries London, UK) was used for 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) calibration by external calibration; hexanoic acid was applied as internal standard. The PHA content (in % by mass) in cells was defined as the percentage of the ratio 3HB and CDM concentration. Residual biomass (RB in g/L) was calculated as the difference between CDM (g/L) and PHB (g/L) (*5,8*).

For glucose determination, carbohydrate (glucose) concentration in the sample supernatant was monitored by means of high-performance liquid chromatography (HPLC) equipment consisting of a thermostated Aminex HPX 87H column (thermostated at 75 ºC, BioRad, Hercules, CA, USA), a LC-20AD pump, a SIC-20 AC auto-sampler, a RID-10A refractive index detector and a CTO-20 AC column oven. Likewise, the LC solution software for registration and evaluation of the data was used. Water was used as eluent at a flow rate of 0.6 mL/min. The standards were prepared using different glucose concentrations (*5*).

Nitrogen source was determined as follows: 2 mL of supernatant were mixed with 50 µL of alkaline ISAB (ionic strength adjustment buffer) solution containing 5 M sodium hydroxide, 10 % methanol, 0.05 M Na_2-EDTA and colour indicator. The mixture was analysed with an Orion ion selective electrode; the signal was monitored by a voltmeter. The standard curve was calculated measuring ammonium sulphate standard solutions of defined concentrations (*5*).

Polymer extraction, identification and characterization

The cells cultivated in the shake flask experiments were pasteurized *in situ* for 30 min at 70 ºC. The pasteurized biomass was then centrifuged at 12 000x for 20 min, frozen and lyophilized for 24 h. The pellet was degreased by overnight stirring with ethanol (12.5 mL of ethanol per g of biomass). Then, PHA was recovered from the dried degreased biomass by overnight Soxhlet extraction with chloroform. After the removal of the major part of chloroform on a rotary evaporator (Büchi Rotavapor RE111, Flawil, Switzerland), the polymer was precipitated by adding cold ethanol and separated from the liquid by filtration. The purity of the extracted material and the completeness of extraction were determined by GC (*5,8*).

The extracted polymer was characterised by means of Fourier transform infrared (FTIR) spectroscopy and nuclear magnetic resonance (NMR). FTIR spectra of the biopolymer were collected using a

PerkinElmer Paragon 1000 FT-IR spectrometer (PerkinElmer, Waltham, MA, USA). The line-scan spectra were based on 32 scans and a resolution of 4 cm^{-1}. ^1H NMR spectra were recorded at 25 °C on a Bruker AM300 spectrometer (Bruker Optik, Ettlingen, Germany). The polymer samples were dissolved in chloroform; a drop of tetramethylsilane (TMS), internal standard for calibration, was added as reference. A mass of 10 mg of sample dissolved in 1 mL of deutered solvent was used. Proton spectra were recorded at 300.1 MHz with a spectrum of 32 kB data points. A total of 64 scans were utilized with a relaxation delay of 1 s. The gel performance chromatography (GPC) measurements were performed using chloroform as an eluent at a flow rate of 0.80 mL/min with a stabilisation pressure of 35 bars and a sample concentration of 1.5 mg/mL. A Waters Styragel HT column (Waters Corporation, Milford, PA, USA) for mid-range molecular-mass distributions was used; samples of monodisperse polystyrene with different molar masses were applied as standard. Differential scanning calorimetry (DSC) analyses were performed on a Perkin-Elmer Pyris 1 instrument (PerkinElmer) with dry nitrogen gas flow of 50 mL/min. The apparatus was calibrated using indium of high purity. Approximately 5 mg of sample were sealed in an aluminium pan and analyzed. The melting temperature (t_m), melting enthalpy (ΔH_m) and the glass transition temperature (t_g) were determined from DSC endothermic peaks of the second heating scan. The degree of crystallinity (X_c) of PHB was calculated assuming a ΔH_m value of 100 % crystalline PHB of 146 J/g. Scans started at -30 ºC and were ramped at 10 ºC/min to 230 ºC (*3*,*5*).

Electron microscopy

Transmission electron microscopy (TEM) observations were achieved with a JEOL 1200 EX-II electron microscope (Jeol Ltd., Tokyo, Japan) operating at 90 kV. The cells and their intracellular PHB granules were observed as thin sections prepared as follows: after 4 h of fermentation in shake flasks, samples were fixed with a freshly prepared mixture of 2 % (by volume) glutaraldehyde, 3 % (by mass per volume) of freshly prepared paraformaldehyde, 5 % (w/v) sucrose, and 0.1 M sodium cacodylate buffer, pH 7.4. Afterwards, bacterial cells were dehydrated using ethanol solutions with increasing concentrations, and finally embedded in a low-viscosity embedding resin polymerizing at 60 ºC overnight. Resin-embedded bacteria were sectioned using ultramicrotomy (thickness of 70 nm) (20).

5. Results

Bacterial isolation and selection

HM medium was used to isolate the bacteria from the water and mud samples taken from Laguna Colorada, Laguna Hedionda, Laguna de Chiguana and Salar de Uyuni. Several distinguishable colonies were obtained after spreading the four diluted samples on solid HM medium. When the pure cultures were separated by transferring each colony to a new solid HM medium, a detailed macro-

and microscopic observation was done in order to differentiate the microorganisms. Forty-nine different bacteria were considered for this study, as the sum of clearly distinct microorganisms.

Twenty of these 49 bacteria produced spores within 7 days in HM solid medium. These species were not used for further studies of polymer production. The remaining bacteria were observed by fluorescence microscopy after being stained with Nile Blue A to distinguish the PHB-producers. After 48 h of incubation, the viable colonies that showed bright orange colour under UV light were selected for further studies of polymer production. Altogether 12 bacteria were found that showed polymer inclusions.

A scale-up to liquid fed-batch cultures of the 12 selected bacterial strains using conventional medium was carried out. The strain from Salar de Uyuni, labelled S-29, grew significantly faster under these conditions compared to the other selected strains. Its absorbance (A_{420nm}) increased from 0.2 to 10.1 after 48 h of incubation (growth rate 0.21 h^{-1}). Consequently, strain S-29 was chosen for studies of polymer production and, therefore, the morphological and taxonomical identification of the strain was carried out.

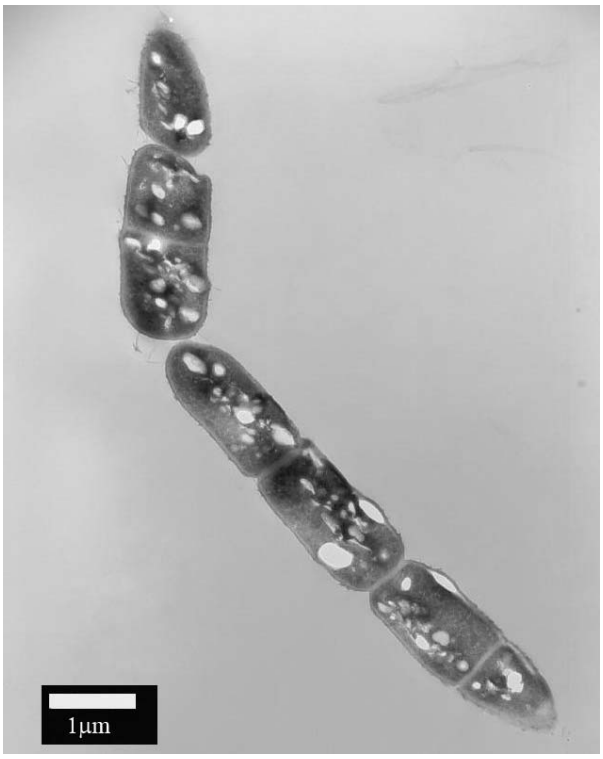


Fig. 2. TEM micrograph of *B. megaterium* uyuni S29 after 4 h of fermentation

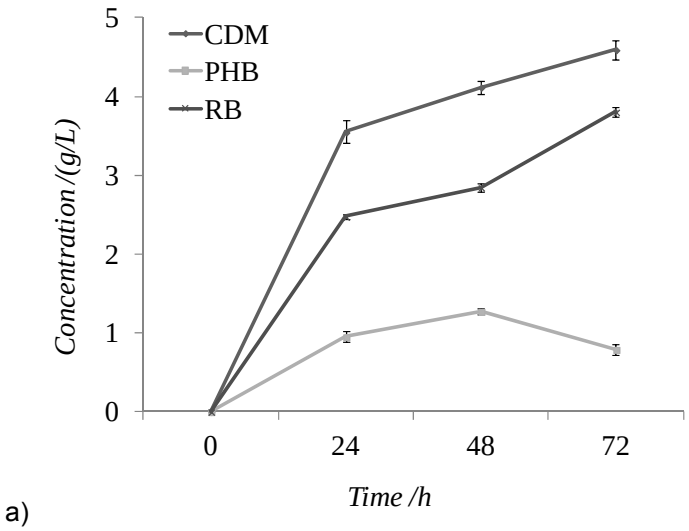
The strain labeled S-29 has been identified as a new strain of *Bacillus megaterium.* It was deposited in the Spanish Type Culture Collection (CECT) with the number 7922 and given the name of *Bacillus megaterium* uyuni S29.

Bacterial identification

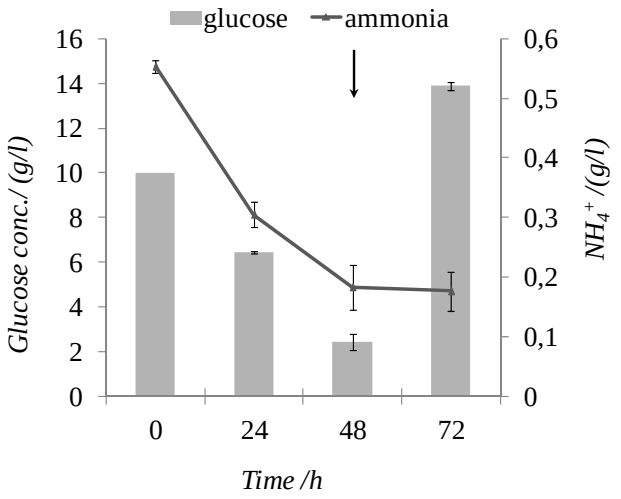
Macroscopic identification of the selected strain showed white, viscous colonies with regular edges and flat elevation. Microscopic observation of the cells showed rod-shaped Gram-positive bacteria. The cells are often found in chains where the walls are joined or slightly separated. TEM micrographs are shown in figure 2. Here, it is possible to observe the cells as round-ended rods (1.0 x2.0 µm) occurring in pairs and in short chains (7.0 µm). Some polymer inclusions are already visible as clear grey granules after 4 h of fermentation.

Polymer production, identification and characterization

As a preliminary study of the polymer production capacity of *Bacillus megaterium* uyuni S29, fed-batch fermentation in shake flasks was carried out in conventional medium. The purpose was the identification and characterization of the accumulated biopolymer. Limitation of nitrogen source (ammonium sulphate) in the presence of a surplus of carbon source was used to provoke the strain to accumulate biopolymer. These conditions were realized by providing a conventional medium nitrogen source at the beginning of the cultivation, and the repeated addition of glucose (carbon source) during the fermentation. Spore formation was always controlled by bacterial observation under microscope. Fig. 3a shows the fermentation pattern by indicating the time curves of CDM, RB, and PHB concentrations. The concentration of CDM increased to (4.59±0.12) g/L after 72 h of fermentation. The maximal concentration of PHB (1.30±0.04) g/L was reached after 48 h with a polymer content of (31±0.20) % in CDM. After this time, the polymer concentration decreased again. The time courses for substrates (glucose and inorganic nitrogen) are shown in Fig. 3b. The nitrogen source (ammonium sulphate) was almost depleted after approx. 48 h. This coincides with the time when the maximal polymer concentration was obtained. Regarding the sugar consumption, the bacteria were consuming it throughout the fermentation to grow and to synthesise biopolymer. The total consumption of sugar after 48 h was (7.55±0.36) g/L, thus the polymer and CDM yields at this point were (17±5) % and (55±3) %, respectively. The glucose concentration at the end of the fermentation was higher than 10 g/L (initial concentration) because 12 g/L of glucose were added after 48 h (black arrow in fig. 3b). Table 1 summarizes the most significant results of the cultivation of *Bacillus megaterium* uyuni S29.



a)



b)

Fig. 3. Fermentation time curves during 72 h with *B. megaterium* uyuni S29: a) cell dry mass (CDM), polymer (PHB) and residual biomass (RB) concentrations; b) Time curves of substrates (carbon and nitrogen source) of the fermentation with *B. megaterium* uyuni S29. The arrow indicates the time of refeeding with glucose (50 % by mass). The error bars indicate the deviation between two parallel set-ups

Table 1. Average and standard deviation (s.d.) values of the main parameters resulting from the fermentation with *B. megaterium* uyuni S29

Parameter	Average±s.d.
Final cell dry mass/(g/L)	4.59±0.12
Maximum PHA/(g/L)	1.30±0.04
Maximum content of PHA in biomass at 48 h/%	31.00±0.20
Maximum specific growth rate μ_{max} /h^{-1}	0.10±0.04
Volumetric productivity of PHA/(g/L·h)	0.03±0.03
Total consumption of glucose from 0 to 48 h/(g/L)	7.55±0.36
$Yield_{(PHA/sugars)}$ from 0 to 48 h	0.17±0.05
$Yield_{(CDM/sugars)}$ from 0 to 48 h	0.55±0.03

FTIR and NMR results showed typical bands and signals of PHB. FTIR transmission spectrum of the
60 PHA extracted from the fermentation with *Bacillus megaterium* uyuni S29 showed the main bands at 1726, 2960-2850, 1390-1370 and 1230-1050 cm^{-1} corresponding to the carbonyl group, methyl and methylene groups, the methyl group, and the ester group, respectively. ^1H NMR results showed a spectrum with the presence of three groups of signals characteristic of the PHB homopolymer: at 1.29 ppm attributed to the methyl group, at 2.57 ppm for the ethylene group adjacent to an asymmetric carbon atom and at 5.27 ppm characteristic of the methylene group. The signal observed at 7.25 ppm corresponds to the residual chloroform. For polymer characterization, GPC and DSC analyses were carried out in order to determine the molar mass distribution and the thermal properties of the biopolymer. Table 2 shows the results of the GPC analyses. The measurements of the refraction index show three main peaks, corresponding to different molar masses. Their proportions are also indicated in table 2. The thermal analyses of the PHB extracted from *Bacillus megaterium* uyuni S29 indicate an X_c and t_g of (36.7±1.2) % and (-17.10±0.81) ºC, respectively, and three different t_m peaks at (96.50±3.50), (118.50±3.50) and (134.34±2.51) ºC. These results are shown in the DSC curve of figure 4.

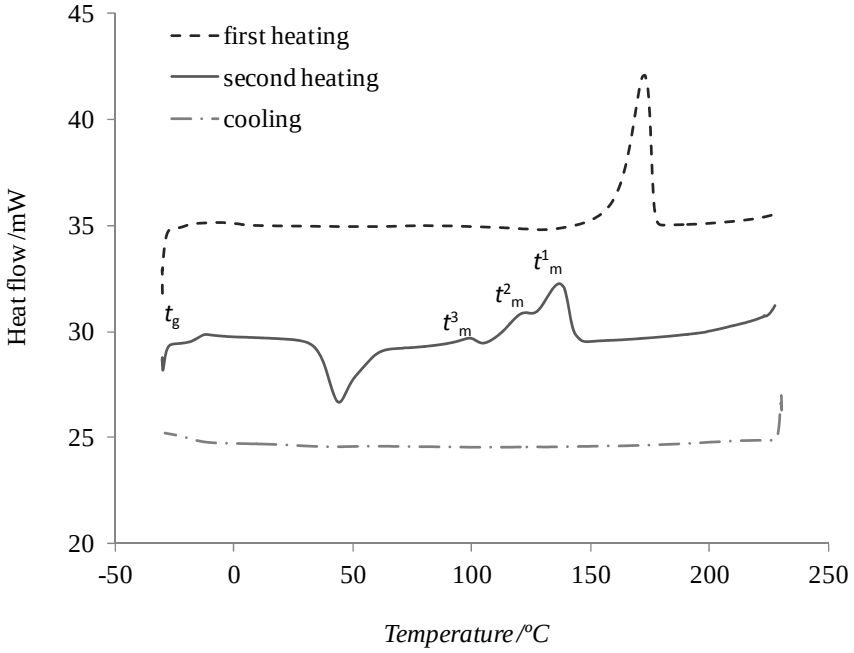


Fig. 4. DSC curve of PHB extracted from *B. megaterium* uyuni S29. t^1_m= (134.34±2.51) ºC, t^2_m= (118.50±3.50) ºC, t^1_m= (96.50±3.50) ºC, t_g= (-17.10±0.81) ºC, X_c= (36.70±1.20) %

Table 2. Molecular mass distribution of polymer extracted from *B. megaterium* uyuni S29 and the proportion of each different molecular mass

Peak	Fraction/%	M_n /kDa	M_w /kDa	PDI (M_w / M_n)	M_p /kDa
1st	76	705	795	1.12	740
2nd	13	135	190	1.40	174
3rd	11	27.0	39.6	1.47	31.3

M_n is the average number of the molecular mass, M_w is the average mass of the molecular mass, PDI is the polydispersity index and M_p is the maximum of the molecular mass

6. Discussion

The importance of this work was the discovery of a strain from environmental samples available for cultivation in a conventional medium with low salt content which is already used in the industrial production of PHAs. The samples were taken from Bolivian hypersaline lakes based on previous studies (*13,21*) reporting the isolation of important PHA producers from regions with similar extreme conditions such as *Halomonas boliviensis* (*11*), or *Haloferax mediterranei* (*12*). The advantages of the use of the industrial conditions for polymer production, especially moderate amounts of salt in the medium, are twofold. Firstly, if the novel strains do not require any additional change of the culture conditions, there is no additional cost for its adaptation to the production process. Secondly, high

salinity levels make the fermentation medium quite corrosive and might incur higher investment cost for the bioreactor equipment used in large-scale. Salt in the medium must be concentrated and recycled in order to reduce the overall process cost as well as to minimize ecological drawbacks implicit in the disposal of post-fermentation residues (*8,11*). Therefore, avoiding the use of high salt concentrations during the fermentation improves economically and ecologically the production process.

HM medium was used for the isolation of microorganisms in similar studies (*11*,*14*). Regarding the work at hand, a high diversity of bacterial species was found in the Bolivian samples. However, some of them formed endospores under conditions of nutrient depletion, coinciding with the requirement to induce biopolymer production. As this constitutes an undesired competition for the external carbon source between both metabolic processes (*22*), endospore-producing strains were eliminated from the study.

Bacteria without visible spore formation that showed PHB inclusions after staining with Nile Blue A were grown in conventional liquid medium, which was selected for polymer production because it is industrially used medium for the controlled accumulation of PHAs. Its composition, especially the nitrogen content, is optimized in order to induce the polymer accumulation already present after a few hours of fermentation (*5,8*,*15*). *B. megaterium* uyuni S29 was selected among the PHA-positive bacteria, because it showed fastest growth in conventional liquid medium. Taking into account its origin and its promising growth kinetics in conventional medium at low salt content, *B. megaterium* uyuni S29 can be classified as a halotolerant bacterium, characterized by a tolerance of high NaCl concentrations (*16*).

The fed-batch fermentation study was carried out with *B. megaterium* uyuni S29 in order to produce PHA biopolymer for further characterization. The strain was able to accumulate 31 % of PHB in CDM in this first experiment. This value can be considered promising, if compared to other novel bacteria described in recent works (*10*). The maximal polymer production took place when the nitrogen was limited. Nitrogen limitation acts as the initiator of PHA production because the formation of proteins (residual biomass) stops and the flux of carbon is directed to polymer synthesis (*8*). However, the values presented in Fig. 3 imply that the polymer is already accumulated during the exponential phase of bacterial growth. This can also be concluded from Fig. 2, which shows both cell division and polymer granules at the same time. These results are commonly observed when the depletion of other element(s) in the medium, besides nitrogen, restricts cell multiplication. Most likely, the agitation speed (120 rpm) was insufficient for optimum cultivation of strain S-29 in shake flasks, inducing oxygen limitation. At the end of fermentation, the RB increased after 48 h without any addition of nitrogen source. This indicates that the cell autolysis could have occurred (*23*). Also during this time, the PHB concentration decreases, indicating that the strain is degrading and consuming the biopolymer again, as a result of the decrease of the carbon source concentration. Consequently, further studies are required in order to optimise the biopolymer production process and to determine

the potential of the strain as PHB-producer. However, the results achieved by this preliminary study of polymer production by the bacterial *B. megaterium* uyuni S29 (Table 1) are not far from other published results (*8*,*12*). For instance, Koller *et al.* (*8*) used the same conventional medium to compare the potential of three different wild-type bacteria, among them the halophilic archaeon *Haloferax mediterranei*, as industrial scale PHA producers. The maximum specific growth rate and the volumetric productivity were obtained in the range from 0.03 to 0.05 and 0.08 to 0.29, respectively. *B. megaterium* uyuni S29 showed a higher growth rate growing in conventional medium (0.1±0.14) h^{-1}. Quillaguamán *et al.* (*11*) reached a high PHB content and volumetric productivity, 88 % of CDM and 1.1 g/(L·h) respectively, using a complex strategy for PHB production by *H. boliviensis*. Therefore, it could be possible to capitalize on the maximal PHB production potential of *B. megaterium* uyuni S29 through the control and fine-tuning of the fermentation conditions (pH value, dissolved oxygen, temperature, providing sub-optimal concentrations of nitrogen during PHA accumulation phase) by means of a laboratory bioreactor. In addition, the application of a bioreactor enables the investigation of different strategies for PHB production, such as PHA copolymer production for tailoring the polyester properties or continuous production mode for enhanced productivity.

This first fermentation with *B. megaterium* uyuni S29 enabled the identification and characterization of the produced biopolymer. The main bands and peaks of the FTIR and ^{1}H NMR spectra correspond to PHB, according to those found in the literature (*9*). These results together with the GC analysis show that *B. megaterium* uyuni S29 exclusively accumulates PHB homopolymer using conventional medium with glucose as a substrate. Wild type bacteria typically produce PHB molecular masses between 10-3000 kDa, with a polydispersity index (PDI) of about 2 (*4*,*24*). The results of the GPC analysis show that the values for PHB produced by *B. megaterium* uyuni S29 are within this range. However, three different fractions could be identified, with the highest proportion corresponding to the highest molecular mass. Regarding the thermal analyses, X_c is generally reported in a range between 60-80 %, t_g about 4 ºC and t_m about 160-180 ºC (*25*). In contrast, PHB extracted from *B. megaterium* uyuni S29 shows significantly lower values of these thermal parameters (Table 3; 5,8,21,26-28). The thermal properties that are closer to the PHA copolymers than to PHB might be a consequence of a formation of a blend of the three different PHB fractions, as determined by GPC analyses. This can be explained by the fact that the thermal behaviour of some polymers is influenced to a high extent by their chain length (*9*). The polymer fractions with a low degree of polymerization show a relatively large quantity of chain ends; here, the end groups act as ‘impurities’, lowering the polymer melting points (*25*). Therefore, there is evidence that the synthesised biopolymer features a blend of different PHB fractions with different molar masses (different degrees of polymerisation).

Table 3. Thermal properties of different PHAs

Polymer	Species	M_n-PDI /kDa	t_m /ºC	t_g /ºC	X_c /%	Ref.
PHB from glucose	*B. megaterium* uyuni S29	705-1.12	134.34	-17.10	36.7	Present paper
PHB from glucose	*C. necator* DSM 545	665-2.6	178	2.9	68	(*5*)
PHB from molasses	*C. necator*	220	173	-	55	(*26*)
PHB from sugars	*C. necator*	230	150	-	60	(*26*)
PHB from glucose	*B. cereus* SPV	1100-1.75	169.71	2.04	57.66	(*27*)
PHB from glucose	*B. cereus* SPV	882-2.6	160.83	-2.45	54.42	(*27*)
PHB from glucose	*B. cereus* SPV	885-3.1	171.71	2.72	64.08	(*27*)
P3HV	*H. pseudoflava*	-	110-112-118	-15	56	(*28*)
P(3HB-co-6 %3HV) from whey sugar	*H. mediterranei*	1057-1.5	150.80-158.90	6	-	(*8*)
P(3HB-co-12 %3HV) from molasses	*C. necator*	245	165	-	45	(*26*)
P(3HB-co-20 %3HV)	-	-	145	-1	-	(*28*)
P4HB	*H. pseudoflava*	-	53	-40	-	(*28*)
P4HB	-	-	56	-48	55	(*21*)
P(3HB-co-18 %4HB)	-	-	165	-4	30	(*21*)
P(3HB-co-69 %4HB)	-	-	50	-36	-	(*21*)

The phylogenic analyses of the 16S rDNA from the isolate showed that a new strain, *B. megaterium* uyuni S29, has been found. Thus, its macro- and microscopic characteristics coincide with those found for other strains of *B. megaterium* in literature (*29*): an aerobic, Gram-positive, rod-shaped bacterium with flat and viscous colonies. This is the first time that a wild-type strain of *B. megaterium* has been isolated from an extreme environment with high salinity as is the case with the Salar de Uyuni. The compilation of the chemical analyses of brine samples from Salar de Uyuni are given by Retting (*30*). A previous classification of *B. megaterium* indicates that it is halotolerant (*31*), thus coinciding with the initial conclusion of S-29. Although *B. megaterium* uyuni S29 is a member of the Bacillaceae family which are characterised by their endospore formation in unfavourable environments (*29*), no endospores were detected throughout the study. The used culture conditions probably induced the production of the biopolymer but were not favourable for the spore formation. Recently, some studies have shown many strains from this family that can be great polymer producers with high industrial potential (*33*) such as *B. cereus,* which can accumulate 48 % PHB in CDM with starch as a carbon source (*34*), or *Bacillus* sp. IPCB-403, which can accumulate 70 % PHB in CDM at optimum culture conditions (*35*). Thus, it is possible to avoid sporulation during polymer synthesis if the required conditions are found.

7. Conclusion

This study describes for the first time the isolation of a wild-type strain of *B. megaterium* (uyuni S29) from extreme saline environments. The novel strain grew well in a conventional medium with low salt content as typically used for industrial production of PHAs. The data presented in this preliminary study about PHB production by strain S-29 indicate a high potential of this bacterial isolate as PHB-producer. The biosynthesised PHB features thermal properties differing from conventional PHB (lower crystallinity, glass transition temperature and melting point) probably due to polymer fractions of different molecular masses. Therefore, further studies are needed in order to optimise the biopolymer production process and to assess the potential of the material for different applications.

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Section 2: *High production of poly(3-hydroxybutyrate) from a wild Bacillus megaterium Bolivian strain* [1]

1. Abstract

A novel strain of *Bacillus megaterium* was isolated from Uyuni salt lake (Bolivia) in a preliminary work. In this new work, the strain was used for the study of poly(3-hydroxybutyrate) (PHB) biosynthesis. The objectives were to determine the maximal PHB production potential of *B. megaterium* strain uyuni S-29 in an industrial conventional media, the possibility that the strain accumulates different types of PHA, the cellular morphology during the biosynthesis process, and the characterization of the produced biopolymers. The microorganism was first tested in a 3L-bioreactor obtaining a high specific growth rate of 1.64 h^{-1}. A second fed-batch experiment was carried out in shaking flasks, reaching up to 70% PHB of cell dry mass. The synthesised polymers were characterized and the thermal analyses showed properties differing from conventional PHB. According to the literature, the wild *B. megaterium* strain uyuni S29 showed in this work the highest polymer accumulation for the genus *Bacillus.*

2. Introduction

Nowadays plastics are one of the most used materials because of their versatility and material properties, especially as packaging materials due to their durability and resistance to degradation. However, these properties have a very high environmental impact. Consequently, biopolymers such as polyhydroxyalkanoates (PHAs) which are produced by several microorganisms are an attractive alternative to conventional plastics, not just because they are 100% biodegradable, but because they can be produced from renewable resources. This allows their production to be independent of the oil industry. PHAs are also biocompatible with a wide range of applications in medicine, pharmacy, veterinary and food packaging. Poly-3-hydroxybutyrate (PHB) is the simplest and most commonly produced PHA. Because of its competing thermoplastic and mechanical properties, which are similar to plastics or elastomers derived from petroleum, it is gaining interest as a substitute to conventional plastics [1, 2]. However, the major commercial drawback of the bacterial PHB is its high production cost. It makes it substantially more expensive than synthetic plastics. The main factors that increase the polymer production cost are reported by Choi and Lee [3]. Researchers have been focusing on different strategies to overcome this production cost [2]. With the aim of commercializing PHA, a great deal of effort has been devoted to reducing the production cost by the development of better bacterial strains [2- 4].

[1] *It corresponds to the article accepted for publication in Journal of Applied Microbiology: High production of poly(3-hydroxybutyrate) from a wild Bacillus megaterium Bolivian strain by Rodríguez-Contreras Alejandra, Koller Martin, Miranda-de Sousa Dias Miguel, Calafell Margarita, Braunegg Gehart, Marqués-Calvo María Soledad.*

The genus *Bacillus* was identified as one of the first Gram-positive bacteria capable of producing PHB [5]. This genus has been widely used for a long time in industry and academia, due to the stability of its replication and maintenance of plasmids [6]. Up to now, many species of PHA-producing bacilli have been isolated from various environments [7]. Among them, great polymer producers have been reported [7, 8], such as *B. cereus* which can accumulate 48% PHB of CDM in a starch containing medium [9], or *Bacillus sp.* IPCB-403 which is able to accumulate PHB in 70% of the CDM in optimum culture conditions [10]. However, there is a drawback to working with *Bacillus* species for large-scale production of biopolymer: sporulation is the reason for low PHB productivity. It is obvious, considering the fact that spore formation and PHA accumulation are provoked by similar nutritional stress conditions [11]. Nevertheless, there are studies on a large-scale PHB production as reported by Valappil et al. [12] with *Bacillus cereus* where the acidic pH used in the medium avoids the spore formation. Hence, it is promising to explore strategies that prevent sporulation by *Bacillus* species in order to increase the production of PHAs and its efficiency yield from the applied carbon source.

This genus seems to be a potential candidate for production of PHB due to its better polymer yields and less severe fermentation conditions [13]. It is still being widely studied not only because new species with new properties are still appearing [9, 14], but also because *Bacillus* is used for the production of a variety of different PHAs by utilizing different carbon sources [13]. Among the new PHAs-producing *Bacillus*, a wild-type strain *B. megaterium* uyuni S29 was recently isolated from extreme saline environments [14]. This novel strain grew well in a conventional medium with low salt content as typically used for industrial production of PHAs.

3. Objectives

The main objectives of this new work were to study the maximal PHB production potential of *B. megaterium* strain uyuni S29 in the conventional media used for industrial biopolymer production and the possibility that the strain accumulates different types of PHAs. The characterization of the produced biopolymers and the examination of the cellular morphology during the biosynthesis process were further objectives of this study.

4. Materials and Methods

Microorganism

B. megaterium uyuni S29 was isolated from water and mud samples from the Uyuni hypersaline lake (Bolivia). The new strain was deposited in the Spanish Type Culture Collection (CECT) with the number 7922 [14].

Culture medium

The strain was maintained at 4 ºC on solid minimal mineral medium (M) according to Küng (1982) containing (per litre): $Na_2HPO_4{\cdot}2H_2O$, 4.5 g; KH_2PO_4, 1.5 g; $MgSO_4{\cdot}7H_2O$, 0.2 g; NaCl, 0.9; $(NH_4)_2SO_4$, 2 g; $CaCl_2{\cdot}2H_2O$, 0.02 g; NH_4Fe(III) Citrate, 0.05 g; agar, 15; trace element solution SL6[1], 1 mL; glucose, 10 g. M medium was used to grow the strain in liquid medium for the pre-cultures of the experiments. A modified M medium was used in the fermentation in the 3L-bioreactor containing: KH_2PO_4, 5 g; $MgSO_4{\cdot}7H_2O$, 0.40 g; NaCl, 0.9; $(NH_4)_2SO_4$, 2 g; $CaCl_2{\cdot}2H_2O$, 0.02 g; NH_4Fe(III) Citrate, 0.05 g; trace element solution SL6[1], 2 mL; glucose, 10 g [14]. The same M medium was used for fermentation in the shaking flasks experiment, but citric acid 1 M was added. Sugars and mineral salt solutions were autoclaved separately at 121 ºC for 20 min [15].

Fermentation strategy for bioreactor

Precultures of the strain were first inoculated from solid M medium grown for 24 hours. They were incubated overnight in 300 mL shaking flasks containing 100 mL of M medium at 35 ºC and a pH value of 7.0 in a rotary shaker at 120 rpm. The strain was cultivated until the cell density reached an OD_{420} of 14. The inoculum was prepared with 10 mL of these precultures in 1 L erlenmeyer flasks containing 250 mL of modified M medium. 0.5 L of these cultures with OD_{420} 10.7 and 11.5 were used to inoculate 1 L of modified M medium in the bioreactor. The fermentation was carried out in a stirred tank reactor of Labfors3 (Infors, Switzerland). It had a total volume of 3 L, with a working volume of 1.5 L (0.5 L of inoculum). All relevant fermentation parameters (pH value, flow rate, dissolved oxygen concentration, stirrer speed, consumption of pH correction solutions, and activity of antifoam probe) were monitored and recorded by IRIS software program. The temperature of the system was maintained by water flow at 35 ºC. The pH-value (Hamilton sensor) was maintained at 7.0 by using 10 % solution of H_2SO_4/NaOH-NH_4OH. NH_4OH was used in the growing phase, and when the ammonium source was limited, NaOH was used instead. The air inflow rate and agitation speed were used to monitor the cell activity and they were initially adjusted to 5 L/min and 500 rpm respectively. The oxygen partial pressure (pO_2) (Ingold sensor) was kept at about 40% of the saturation concentration of oxygen in water and oxygen was supplied at 150 L/h through an absolute filter (Sartorius, Midisart 2000). Glucose was added from a concentrated solution of 50 % (w/v) during fermentation to avoid the carbon source limitation.

Shaking flasks experiment

Precultures were prepared from solid medium and incubated for 24 hours at 35 ºC and 120 rpm. When the precultures reached an OD_{420} of 13.2, pH value 6.7, 10 mL were used to inoculate two parallel set-ups with 250 mL of modified M medium in 1 liter flask. Glucose was added as a concentrated solution (50% w/v) during fermentation to avoid the carbon source limitation. Growth was monitored *via* optical density at λ of 420 nm.

Analytical procedure

Determination of the CDM, residual biomass (RB) and PHB content

Samples of 10 mL of culture broth were taken along the fermentations and centrifuged at 4000 rpm for 15 min (Megafuge 1.0R Heraeus Sepatech). The pellet was frozen, lyophilised and weighed to determine the CDM by weight difference. The PHA in lyophilized biomass samples was transesterificated by acidic methanolysis following Braunegg's method [16]. Analyses were carried out with Agilant Technologies 6850 gas chromatograph (GC) (30-m HP5 column, Hewlett-Packard, USA; Agilant 6850 Series Autosampler). The methyl esters of PHA constituents were detected by a flame ionization detector with helium as carrier gas (split-ratio of 1:10). P(3HB-co-19.1%-3HV) (Biopol; Imperial Chemical Industries) was used for 3HB and 3HV calibration and hexanoic acid was used as internal standard. The PHB content (wt %) was defined as the percentage of the ratio of 3HB concentration to CDM. The RB was then calculated as the difference between cell concentrations (CDM) and the PHB content.

Determination of glucose

Carbohydrate concentration from supernatant was monitored by means of HPLC equipment composed of a thermostated Aminex HPX 87H column (thermostated at 75 ºC, Biorad, Hercules, USA), a LC-20AD pump, a SIC-20 AC autosampler, a RID-10A refractive index detector and a CTO-20 AC column oven. Also the LC solution software for registration and evaluation of the data obtained was used. 1.5 mL of liquid media were sterile filtrated and transferred into vials. Water was used as eluent at a flow rate of 0.6 mL/min. The standards were prepared with different concentrations of glucose.

Determination of nitrogen source

2 mL of supernatant was mixed with 50 µl alkaline ISAB solution containing 5 M sodium hydroxide, 10 % methanol, 0.05 M Na_2-EDTA and a colour indicator. The mixture was immediately analysed with an Orion ion selective electrode; the signal was monitored by a voltmeter. The standard curve was calculated measuring different ammonium sulphate standards solutions of defined concentrations.

Polymer extraction

The cells cultivated were *in situ* pasteurized and the culture broth was then centrifuged at 6000 rpm for 20 min (Inula Sorvall RC-5B Refrigerated Superspeed centrifuge), frozen and lyophilized for 24 h in all cases. After degreasing the biomass by shaking overnight with ethanol, the polymer was extracted from the cells *via* chloroform, by stirring 1 g of freeze-dried cell in 100 mL chloroform for 48 h and purified by re-precipitation with 10 volumes of ice-cold methanol [17]. The alternative polymer extraction method was done *via* Soxhlet with acetone under reflux at 56 ºC for 5h. Polymer precipitation was carried out by cooling the solution [18]. The purity of the extracted material was determined by GC.

Polymer characterization

The chemical structure was characterised with a Perkin Elmer Fourier Transform Infrared microscopy using optical Perkin Elmer software. The line-scan spectra were based on 32 scans and a resolution of 4 cm^{-1}. ^{1}H NMR spectra were recorded at 25 °C on a Bruker AM300 spectrometer. The polymer samples were dissolved in chloroform and a drop of TMS (tetra methyl silane used as internal standard for calibrating chemical shift for ^{1}H) was added as reference. 10 mg of the sample dissolved in 1 mL of deutered solvent was used. Proton spectra were recorded at 300.1 MHz with a spectrum of 32 K data points. A total of 64 scans were utilized with a relaxation delay of 1 second. The GPC measurements were performed utilising chloroform as an eluent at a flow rate of 0.80 mL/min with a stabilisation pressure of 35 bars and a sample concentration of 1.5 mg/mL. A Waters Styragel HT column for mid-range molecular-mass distributions was used and samples of polystyrene with different molecular masses were used as standard. DSC experiments were performed on a Perkin-Elmer Pyris 1 instrument with a dry nitrogen gas flow of 50 mL/min. The apparatus was calibrated using Indium of high purity. Approximately, 5 mg of the sample was sealed in an aluminium planched and analyzed. The melting temperature (T_m), melting enthalpy (ΔH_m) and the glass transition temperature (T_g) were determined by the second heating run of DSC endothermic peaks. The crystallinity degree (X_c) of PHB was calculated assuming that the ΔH_m value of 100% crystalline PHB is 146 J/g [19]. Scans started at -30 ºC and were ramped at 10 ºC/min to 230 ºC.

Electron microscopy

Transmission electron microscopy (TEM) observations were achieved with a JEOL 1200 EX-II electron microscope operating at 90 kV and the PHB granules contained were observed as thin sections prepared following the method in Tian et al. [8]. Samples from the fermentation in shaking flasks were taken at 3 different times (4, 12 and 21 hours) and were fixed with a freshly prepared mixture of 2% (v/v) glutaraldehyde, 3% (w/v) paraformaldehyde made fresh, 5% (w/v) sucrose, and 0.1 M sodium cacodylate buffer, pH 7.4. Afterwards, bacterial cells were dehydrated using ethanol solutions then embedded in low-viscosity embedding resin, which polymerized at 60 ºC overnight. Resin-embedded bacteria were sectioned using ultramicrotome with a thickness of around 70 nm.

5. Results

Polymer production

Two different fed-batch fermentations were carry out in order to study the possibilities of the strain *B. megaterium* uyuni S29 of producing and accumulating PHB. Table 1 summarises the main results obtained from both fed-batch fermentations. Comparing the results of both assays, higher specific growth rate (μ_{max}) and final CDM were obtained in the growth curve of the bioreactor experiment, while the sugar conversion was higher for the shaking flasks assay as a result of the higher polymer accumulation.

Fermentation in the bioreactor

Fed-batch fermentation in a 3L-bioreactor was first carried out, showing the data a μ_{max} of 1.64±0.01 with a volumetric productivity of 0.25±0.03 g/Lh PHB (table 1). The strain started to accumulate biopolymer after 15 hours of fermentation and it stayed constant until it was ended. The maximal polymer content reached 29.70±0.28 % (w/w) of CDM from glucose as a carbon source. This first fermentation lasted 18 hours and the CDM reached a final value of 28.59±0.09 g/L with 8.50±0.65 g/L of biopolymer. Figure 1a shows the results of the bioreactor fermentation in terms of CDM, PHB content and RB concentrations.

Table 1. Values of the main parameters of the fermentations: bioreactor and shaking flasks

Parameter	Bioreactor	Shaking flasks
Final Cell Dry Mass [g/L]	28.59±0.09	7.14±0.08
Final PHB [g/L]	8.50±0.65	2.35±0.05
Maximal Content of PHA in biomass [%]	29.70±0.28	69.20±4.12
Volumetric Productivity PHA [g/L·h]	0.25±0.03	0.45 ±0.01[a]
μ_{max} [1/h]	1.64±0.01	0.58±0.01
Total consumption of sugars [g/L·h]	1.81	0.51
Yield $_{(PHA/Sugars)}$	0.14±0.02	0.18±0.01
Yield $_{(CDM/Sugars)}$	0.47±0.00	0.56±0.01

[a]At 12 hours of fermentation

The ammonium source was added during the growth phase as ammonium hydroxide (25 % w/w) together with the adjustment of the pH-value. After 15 hours of fermentation, this addition was terminated in order to induce polymer accumulation as indicated in figure 1b by the grey arrow. The consumption of ammonium started, however it was never limited during the entire fermentation. Regarding the carbon source evolution, the consumption and addition of glucose is also shown in figure 1b as a result of HPLC analysis. The initial glucose concentration was 10 g/L and it was totally consumed by the cells after 8.3 hours, then it was added from a concentrated glucose solution (50 % w/v) constantly maintained in excess.

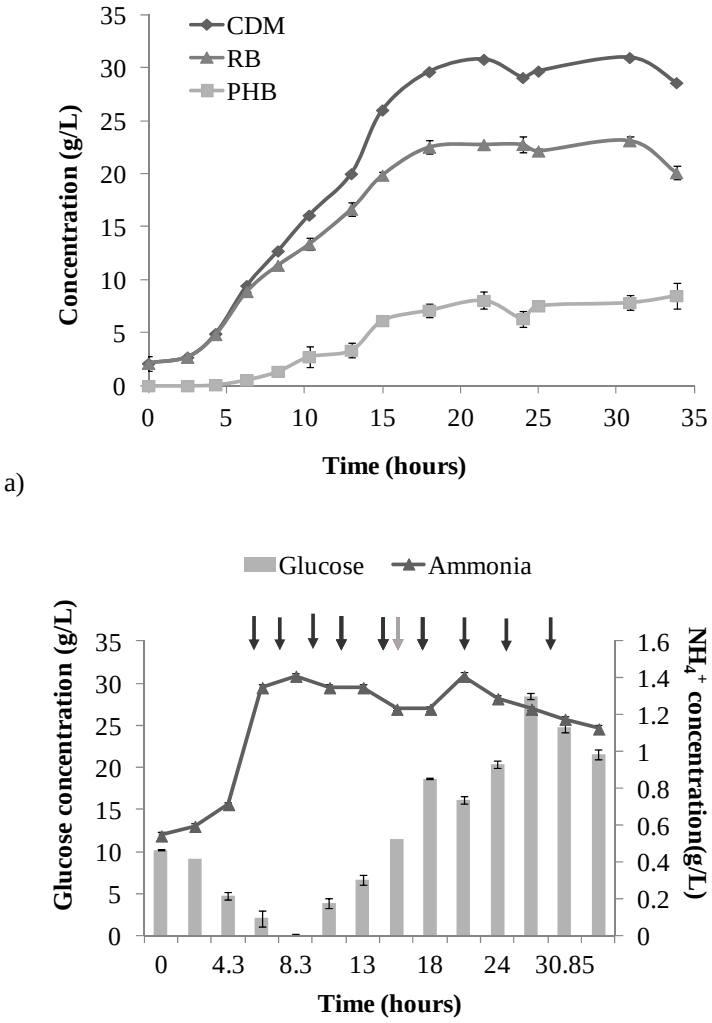
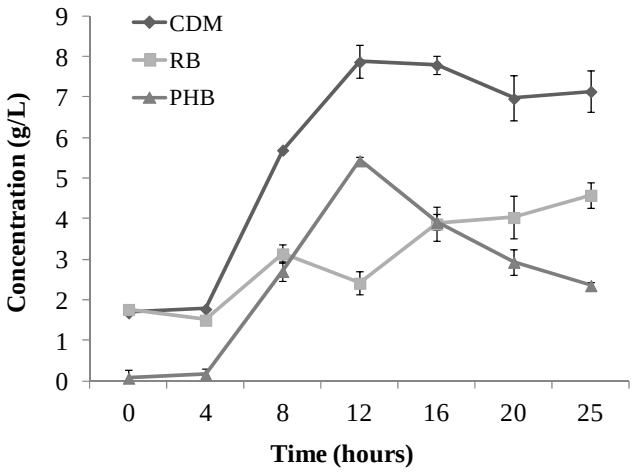


Figure 1. Growth curves of *B. megaterium* strain uyuni S29 under controlled conditions in the bioreactor fermentation: cell dry mass (CDM), polymer content (PHB) and residual biomass (RB) (a). Substrates evolution during fermentation: glucose and ammonium concentration. The grey arrow shows the cut in the addition of nitrogen source (ammonium hydroxide) and the black arrows indicate the addition of glucose (b). The error bars refer to deviations between three samples taken

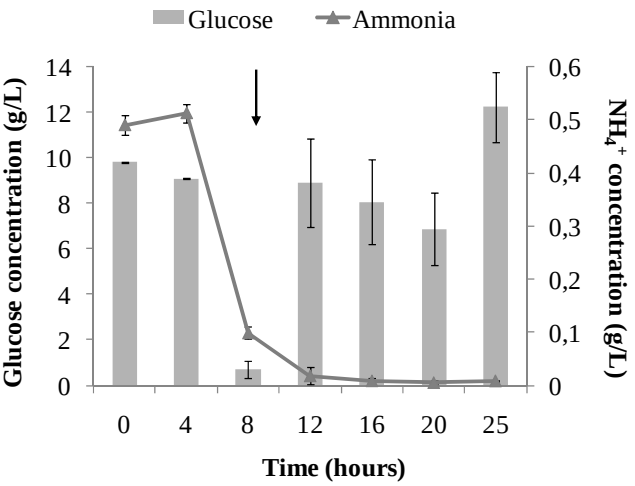
Fermentation in shaking flasks

A total limitation of the nitrogen source was achieved after 12 hours of fermentation. Exactly at this moment, the maximal PHB content was reached with a 70% (69.20 ± 4.10 % of 3HB from the gas chromatography after transesterification) of CDM (figure 2) obtaining a volumetric productivity of 0.45±0.01 g/Lh PHB. In this fermentation, the RB decreased between 8 and 12 hours of fermentation coinciding with a decrease in the nitrogen source. After 12 hours of fermentation the RB increased without any addition of nitrogen. After the maximal polymer concentration was reached, the RB increased and the polymer content decreased. Hence, the polymer and the cell concentrations

coincide in the growth curve after 16 hours of fermentation. The decrease of PHB concentration in the medium implied the re-utilization of biopolymer by the cells.



a)



b)

Figure 2. Growth curves of *B. megaterium* strain uyuni S29 in the shaking flasks experiment: cell dry mass (CDM), polymer content (PHB) and residual biomass (RB) (a). Substrates evolution during fermentation: glucose and ammonium concentration. The black arrows indicate the glucose addition (b). The error bars refer to deviations between two parallel experimental set ups

Polymer characterization

After polymer extraction with chloroform for both experiments and also with acetone for the bioreactor experiment, three polymers were obtained and characterized by means of FTIR, ^{1}H NMR, GPC and DSC.

FTIR results show no differences between the spectra of the three extracted polymers. Carbonyl and ester groups at 1726 and 1330-1050 cm^{-1}, respectively and methyl group at 1390 $cm^{-1}$are shown in the three spectra corresponding to the main bands of PHB. ^{1}H NMR spectra show the presence of three groups of signals, characteristics of the homopolymer PHB (figure 3): a doublet at 1.29 ppm

which is attributed to the methyl group coupled with one proton (signal 3, figure 3), a doublet of quadruplet at 2.57 ppm which is attributed to a methylene group adjacent to an asymmetric carbon atom bearing a single proton (signal 2, figure 3) and a multiplet at 5.27 ppm characteristic of the methylene group (signals 1, figure 3). Two other signals are also observed, one at 3.70 ppm which is due to the methylene group at the end of the chain (signals 4, figure 3) and another one at 7.25 ppm which is attributed to chloroform (signals $CHCl_3$, figure 3) [20].

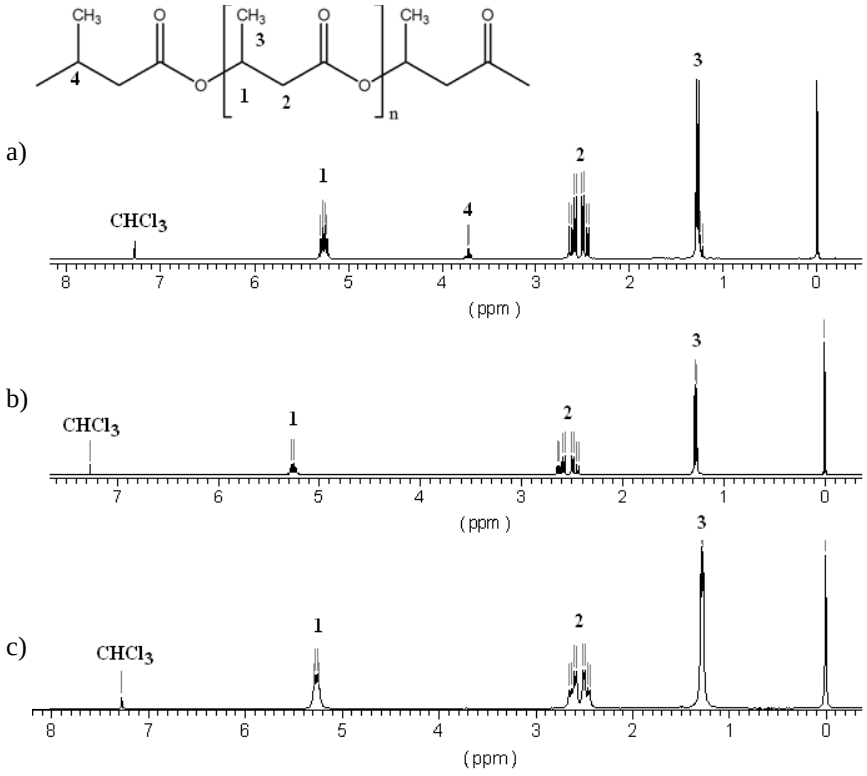


Figure 3. ^{1}H NMR spectra of polymer extracted from fermentation in the bioreactor with acetone (a), with chloroform (b) and from shaking flasks experiments (c). The chemical shift is expressed in parts per million (ppm). Signal 1 is characteristic of the methylene group, signal 2 corresponds to the methylene group adjacent to an asymmetric carbon atom bearing a single proton, signal 3 corresponds to the methyl group coupled to one proton, and signal 4 corresponds to the methylene group at the end of the chain

GPC and DSC analyses were carried out in order to determine the molar mass distribution and the thermal properties of the biopolymer, respectively. The molar masses from GPC analyses did not vary considerably in the polymers from both experiments and with both extraction methods. The results presented two main peaks in all cases that correspond to two different molar masses: one peak represents a molecular mass of 600 kDa while the other represents a molecular mass of 125 kDa with a polydispersity index of 1.2 and 1.5, respectively. From the DSC thermal analyses, differences in the thermal properties of the extracted PHBs were observed. Table 2 shows different T_m, T_g and X_c for the three PHB samples characterised.

Table 2. Main thermal parameters of DSC analyses for the bioreactor and shaking flasks experiments

		Bioreactor		Shaking Flasks
	2nd Heating	acetone	chloroform	chloroform
T_m (ºC)	1st peak	93	100	-
	2nd peak	115	122	151
	3rd peak	131	136.8	161
T_g (ºC)		-17	-16	-11
X_c (%)		37	35	65.95

Cellular morphology

Figure 4 shows TEM micrographs taken at different fermentation times. The evolution of the cells and their polymer inclusions can be observed. Micrographs of samples after 4 hours of fermentation show long, growing cells, while growth 12 hour-cells are already synthesizing polymer. Micrographs taken from samples of 21 hours of fermentation show shorter and wider cells deformed due to the pressure of the big internal inclusions. In this last case, several difficulties were encountered in cutting off the samples with the microtome due to the high amount, and hence, resistance of the intracellular inclusions. Consequently, the cellular walls of several cells were fractured because of the jump of some internal inclusions. This can be observed in the micrographs of the samples at 12 and 21 hours of fermentation (black arrow in figure 4).

6. Discussion

PHB production

In a previous study [14], *B. megaterium* uyuni S29 showed the formation of intracellular PHA granules. The granules were processed for analysis and the proton displacements and chemical shifts observed in the ^{1}H NMR spectrum confirmed the chemical structure of PHB homopolymer. The polymer biosynthesis was carried out utilizing a conventional medium with low salt content as typically used for industrial production of PHAs [15, 21]. Considering the involved advantages of using conditions used industrially [14], the same medium was used in both fed-batch experiments.

The results of both fed-batch fermentations show that better growth conditions were achieved in the bioreactor experiment, while higher polymer content was achieved in fed-batch experiments in shaking flasks (table 1). This is possible because the bioreactor allows the total control of fermentation parameters, while the shaking flasks experiment indicates the tendency of the behaviour [22].

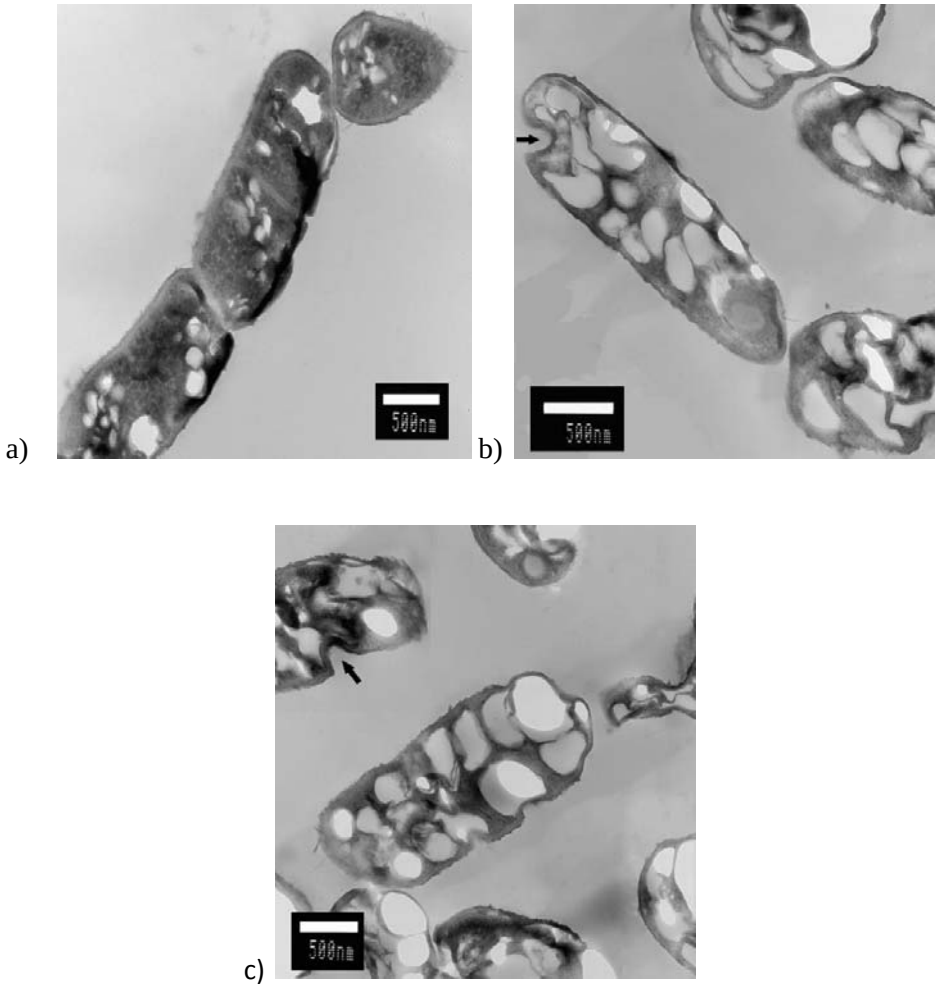


Figure 4. Micrographs of *B. megaterium* strain uyuni S29 after 4 hours (a), 12 hours (b) and 21 hours (c) of fermentation. Black arrows indicate the jump of a polymer inclusion.

In the first fed-batch experiment carried out in a 3L-bioreactor, no limitation of nitrogen was achieved in the entire fermentation. Because all other nutrients were under control, the system was likely to be in suboptimal conditions for biopolymer accumulation and no high amount of polymer content could be attained. Thus, there was a margin for further improvement on the PHB production. Consequently, a second fed-batch experiment was carried out in order to totally restrict the nitrogen source. This time, nitrogen limitation was achieved after 12 h of fermentation, attaining the maximal polymer content at this point (69.20 ± 4.10 % PHB). In this fermentation, the RB increased without any addition of nitrogen after 12 hours, indicating that cell autolysis could occur [23].

In the literature, studies with *B. megaterium* for PHB production were initially reported to show a maximal polymer content of 40% of CDM in a glucose medium containing acetate [24]. Later, a polymer yield of 48.13% with *B. megaterium* Y6 was reached [25], and 42% in a fed-batch experiment using sugar cane molasses and urea as cheap carbon sources under nitrogen limiting conditions [26]. Most recently, it was found that *B. megaterium* strain OU303A yielded a maximum of 62.43% DCM

polymer in the medium containing glycerol as a carbon source [27]. As mentioned before, sporulation is the cause of low PHB productivity in *Bacillus* genus. *B. megaterium* produces spores under similar conditions to PHA formation, mainly as a consequence of the depletion of essential nutrients, leading to a decrease of the accumulated PHB [28]. This could be the reason why such a low PHB content was reached by this genus before. To our knowledge and compared with the percentages found in the literature, the 70% of PHB (69.20 ± 4.10 % PHB) reached by *B. megaterium* uyuni S29 is up to now, the highest polymer content obtained by a *Bacillus* genus. Moreover, the results from the experiments carried out in this study with *B. megaterium* uyuni S29, indicate that PHB production and accumulation takes place when the nitrogen source is limited, reaching a high polymer concentration with no visible spore formation.

In addition and considering an already industrial scaled strain such as *A. lata* which reaches its high PHB content (88% of CDM) with ammonium limitation [29], *B. megaterium* uyuni S29 is not far from this result. It has to be considered that *A. lata* has been studied for a long time and its optimal fermentation conditions are being constantly improved. In comparison, the percentage reached with strain uyuni S29 (70% of CDM) was obtained from a shaking flasks experiment with feed-batch fermentation, where the conditions were not completely optimal. Therefore, it is very important to continue the research with bacterial strain *B. megaterium* uyuni S29 to further improve its PHB content.

Furthermore, it is possible to consider *B. megaterium* strain uyuni S29 for polymer production on an industrial scale. First, because the 70% of PHB obtained in this fermentation is higher compared to the 60% of polymer that is necessary for considering a strain suitable to be used in an industrial process [24]. Second, because industrial fermentation conditions (the used conventional medium and moderate salt content) were already taken into account for the bacterium selection in the previous study [14] and those conditions were maintain in this work. Thus, no additional changes on the culture conditions are needed to adapt *B. megaterium* strain uyuni S29 to the required industrial conditions for biopolymer synthesis.

Polymer extraction and characterization

Two extraction methods have been carried out in this work in order to study the possibility of the strain to produce other qualities of PHA besides PHB. Extraction with acetone by Soxhlet was used in previous works to extract medium chain length PHA [18]. These are PHA biopolymers that contain 6 to 14 carbons in their subunits and possess attractive properties such as low melting points, high elasticity, and biodegradability [18]. However, the main bands and peaks of the FTIR and ^{1}H NMR spectra from the polymers extracted using both extraction procedures (*via* chloroform and *via* acetone) correspond to the characteristic ones of PHB homopolymer, a short chain length (scl) PHA, according to the literature [30]. These results together with the GC analysis indicate that *B. megaterium* uyuni S29 produces and accumulates not other PHA but PHB homopolymer from

glucose as sole carbon substrate, matching with the results from the preliminary study of polymer characterization with strain *B. megaterium* strain uyuni S29 [14].

The molecular masses of PHB produced from wild type bacteria are usually in the range of 10 and 3,000 kDa with a polydispersity around 2 [31]. The results of the GPC analysis showed that the values for the biopolymer production by *B. megaterium* strain uyuni S29 are within this range. The values of the two molar masses concur with the results obtained in previous works with this bacterium [14].

Usually, scl-PHAs, especially PHB, constitute highly crystalline materials, although they are amorphous within the bacterial cell. The crystallization rapidly occurs after disruption of cells when the polymer is extracted. A common X_c is typically found between 60-80%, the T_g about 4 ºC and the T_m about 160-180 ºC [1, 12, 31]. In this study, the three PHB samples extracted from *B. megaterium* uyuni S29 showed very different thermal properties compared to the usual PHAs. The biopolyesters extracted from both fed-batch experiments (bioreactor and shaking flasks) showed lower thermal parameters compared to the common ones from PHBs. These results coincide with the thermal properties of the biopolymer extracted in initial studies with this strain [14]. Particularly, the extracts from the bioreactor experiment showed even lower X_c and T_g (table 2). The obtained thermal values confirmed that different fermentation processes and extraction techniques can influence the thermal properties, corresponding to the findings reported by Valappil et al. [12]. Low thermal properties shown by the chloroform extraction technique could be explained by the presence of possible impurities. The method may allow lipids, fatty acids and other hydrophobic cellular materials to be extracted along with PHB. However, the fact that the polymer extracted *via* Soxhlet-acetone also showed low thermal properties confirms that this is a property of the biopolymer synthesized by *B. megaterium* uyuni S29.

On the other hand, these uncommon thermal properties could be the result of the blend of the different PHBs fractions, as the two different molar masses resulted from the GPC analyses show. Therefore, the results obtained from DSC and GPC for these extracted PHBs could be connected to each other. The thermal behavior of some polymers is influenced by the chain length and molecular mass of the polymer used [32]. As already reported by Lundgren et al. [33], lower molar masses tend to melt at lower temperatures, since the end groups act as impurities. The different melting point values are related to polymer fractions which have undergone different degrees of degradation, yielding polyester with a relatively large fraction of chain ends (low degree of polymerization). The different peaks of the melting points from DSC results could be a consequence of these different chain lengths. Thus, there is evidence that the synthesised PHB features a blend of different PHB fractions with different molar masses (different degrees of polymerization). This matches with the DSC results obtained in previous studies with this strain [14].

The crystallinity of a polymer is known to play a major role in the degradation of a polymer: the amorphous regions in polymers degrade at a much faster rate compared to crystalline regions [34].

The relatively lower crystallinity of the PHB isolated from *B. megaterium* uyuni S29 can be an advantage, since it will depend on the application needs. Otherwise, the thermal properties of the polymers influence their mechanical properties [35]. Thus, the relatively low thermal properties of the PHBs isolated from *B. megaterium* uyuni S29 can be reflected on a decrease of the strength and on an increase of the extensibility of the material, enlarging its possible applications.

7. Conclusions

Strain uyuni S29 is a wild type of *B. megaterium* never tested before for PHB or any other PHA biosynthesis. In this work, the strain not only grew properly in the industrial conditions proposed without spore formation, but it also produced and accumulated a large content of PHB never reached before for its genus (70% w/w of CDM). Consequently, *B. megaterium* uyuni S29 could be considered for polymer bioproduction on an industrial scale. Additionally, the different thermal properties of the PHB produced by the strain could make the use of the material in a wider range of applications possible.

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Section 3: *PHB production by a novel strain of Bacillus megaterium in different salt conditions*[1]

1. Abstract

Bacillus megaterium uyuni S29 was isolated from Bolivian salt lake, Uyuni. A previous study shows the capability of the strain to produce and accumulate poly(3-hydroxybutyrate) (PHB) without spore formation, when it is grown in an industrial conventional media with low salt content. In order to analyze the influence of salt content in the medium on the cell growth and PHB production, fermentations in shaking flasks with different sodium chloride concentrations were carried out. The strain showed a high adaptability to the different media, obtaining the best growth and PHB production results with a salt concentration of 45 g/L, with a PHB accumulation of 40% in cell dry mass (CDM). The strain is also able to develop in a salt concentration of 100 g/L, producing PHB in 22.5% of the CDM. It can survive in a 250 g/L concentration of salt without spore formation but its PHB accumulation is very low. The tolerance to high salt concentrations together with the production of biopolymer makes this strain appealing not just for utilization in the biotechnological production of PHAs, but for other applications such as the treatment of salty wastewater.

2. Introduction

Halophiles constitute a wide group of microorganisms characterized by their requirement for high concentrations of inorganic salts, mostly sodium chloride. The adaptation to life in high salt concentrations can be accomplished in different ways. The most common strategy involves the accumulation of organic osmotic solutions without the need for specialized adaptation of intracellular proteins to the high quantity of salt. The second strategy is the intracellular accumulation of high concentrations of K^+. This approach, unlike the use of organic solutions, requires extensive adaptation of the intracellular enzymatic machinery to be functional in the presence of high ionic concentrations. The great diversity of strategies used by halophiles to deal with high salinity in their environment, associated with the fact that the halofilia takes place throughout the tree of life, suggests that adaptation to life at high salt concentrations is easy to develop, and probably emerged several times during the course of the life. The only feature halophiles have in common is their ability to live in hypersaline environments (**Ramírez 2006**).

This group of microorganisms has shown a great potential for biotechnological production of PHAs. Most authors recognize three kinds of halophilic bacteria: halotolerant (tolerate 0-15% NaCl), moderate halophiles (require 1-15% NaCl) and extreme halophiles (require 15-30%NaCl) (**Rodríguez-Valera 1981, Quillamana 2006**). For example, the halophilic strain *Halomonas*

[1] *It corresponds to the article send for publication in Journal of Applied Microbiology: PHB production by a novel strain of Bacillus megaterium in different salt conditions by Rodríguez-Contreras Alejandra, Koller Martin, Miranda-de Sousa Dias Miguel, Calafell Margarita, Braunegg Gehart, Marqués-Calvo María Soledad.*

boliviensis which grows with 4.5% (w/v) salts in medium, can accumulate PHB around 54 wt% from the CDM (**Quillamanan 2010**). The extremely halophilic archaea *Haloferax mediterranei* grows optimally with 25% (w/v) of salt accumulating biopolymer up to 60–65 wt% of its CDM (**Quillamanan 2006**).

Bacillus species are aerobic, Gram-positive, rod-shaped bacteria and seem to be a potential candidate for production of PHB (**Thirumala 2010**). Many *Bacillus* species have been classified as halotolerant (**Smith 2009**). The application of this microorganism is being studied for high-salinity wastewater treatment. In general, halophilic (moderate or extreme) Archaea are able to carry out denitrification, thus can provide excellent models to explore large-scale bioremediation processes to remove nitrogen compounds from brines (**Nájera-Fernandez 2012**). A biological solution of environmental problems associated with considerable salt concentrations in wastewater, cannot be achieved by the action of microorganisms without the dilution to lower salinity concentrations. However, this is not feasible from the point of view of the engineer or the economist. Another approach is to enrich the media with competent microorganisms that tolerate higher salt concentrations found in the polluted environment (**Hinteregger 1997, Sivaprakasam 2008**). For example, **Kargi** (**2002**) reported some studies using salt-tolerant bacteria such as *Halobacter halobium* for biological treatment of saline wastewater. Sivaprakasam (**2008**) also used some halotolerant bacteria to treat wastewater from the leather industry with a high salt concentration (1-10% NaCl by wt).

3. Objectives

Bacillus megaterium strain uyuni S29 was recently isolated from Bolivian saline water and mud samples from Uyuni salt lake and was studied for PHB production in an industrial conventional media (**Rodríguez-Contreras, in press**). As indicated above, halophiles show a great potential for biotechnological production of PHAs. Therefore, the main goal of this study was to analyze the influence of the salt concentration on the cell growth and on the PHB production by the novel *Bacillus megaterium* uyuni S29.

4. Materials and methods

Microorganism and culture medium

The wild type of *Bacillus megaterium*, the strain uyuni S29 isolated from the hypersaline Uyuni Lake (Bolivia), was used in this study (**Rodríguez-Contreras, in press**). The strain was maintained at 4 ºC on solid minimal mineral medium (M) according to Küng (**1982**) (**Atlić 2011**).

Fermentation conditions

The pre-cultures were inoculated from solid M medium and grown for 24 hours at 35 ºC in 100 mL of M medium. The pre-culture with an OD_{420} of 10.9 and pH value of 7.0, was selected to inoculate the

four set-ups, each one supplemented with different salt concentration: 5, 45, 100 and 250 g/L NaCl. The fermentations consisted of two set-ups prepared in 1 L flask containing 250 mL of M medium with its corresponding salt content. The incubation was carried out at 35 ºC and 130 rpm. Glucose was added through a concentrated solution of monohydrated glucose (50% w/v) to avoid carbon source limitation. Five samples were taken during the incubation from every flask, and afterwards CDM, PHB content, residual biomass (RB), carbon, and nitrogen source content were analysed to study the evolution of the fermentation.

Analytical methods

Samples of 5 mL of culture medium were taken throughout the fermentations, centrifuged twice at 6000 rpm for 20 min in order to eliminate the salt, which influences the analytical results. The pellet was frozen, lyophilised and weighed to determine the CDM. The PHB in lyophilized biomass samples was transesterificated by acidic methanolysis following Braunegg's method (**1978**). Analyses were carried out with an Agilant Technologies 6850 gas chromatograph (30-m HP5 column, Hewlett-Packard, USA; Agilant 6850 Series Autosampler). The methyl esters of the PHB constituents were detected by a flame ionization detector with helium as a carrier gas (split- ratio of 1:10). P(3HB-co-19.1%-3HV) (Biopol; Imperial Chemical Industries) was used for 3HB and 3HV calibration and hexanoic acid was used as an internal standard. The PHB content (wt %) was defined as the percentage of the ratio of 3HB concentration to CDM. The RB was then calculated as the difference between CDM and PHB concentrations.

Determination of substrates

Glucose and salt content were determinated by means of high performance liquid chromatography (HPLC) equipment, consisting of a thermostated Aminex HPX 87H column (thermostated at 75 ºC, Biorad, Hercules, USA), a LC-20AD pump, a SIC-20 AC autosampler, a RID-10A refractive index detector and a CTO-20 AC column oven. LC solution software was used for registration and evaluation of the data. Samples of 1.5 mL of the medium were transferred into vials and water was used as an eluent at a flow rate of 0.6 mL/min. The standards were prepared with different concentrations of glucose and NaCl.

For the determination of the nitrogen source, 2 mL of supernatant was mixed with 50 µL alkaline ISAB (ionic strength adjustment buffer)) solution containing 5 M sodium hydroxide, 10 % methanol, 0.05 M Na_2-EDTA and a colour indicator. The mixture was immediately analysed with an Orion ion selective electrode; the signal was monitored by a voltmeter. The standard curve was calculated measuring different ammonium sulphate standards solutions of defined concentrations.

5. Results

Figure 1 shows the growth curves of *B. megaterium* uyuni S29 according to the media used with different NaCl concentrations. The results show two different groups of curves in relation to the salt

concentrations. One of them is associated with the lower concentrations of NaCl (5 and 45 g/L) and the other one, corresponds with the highest (100 and 250 g/L). In both cases, the optimal salt concentration for the strain growth is 45 g/L NaCl, although it also grew at 5 and at 100 g/L NaCl. The cultures with 250 g/L NaCl show a very slight increase of the OD until 11 hours of fermentation, and then decrease to practically zero. Therefore, the results of these salt concentration cultures are not further considered. None of the *B. megaterium* uyuni S29 culture showed spores throughout the fermentation in M medium, when they were observed by light microscopy.

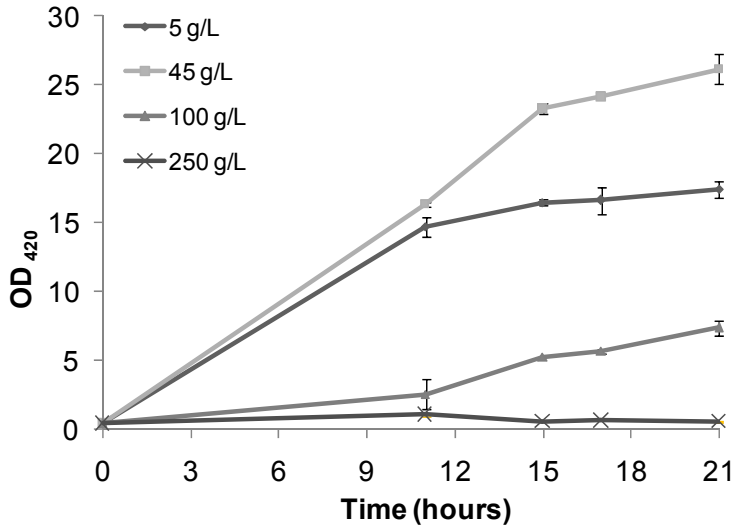
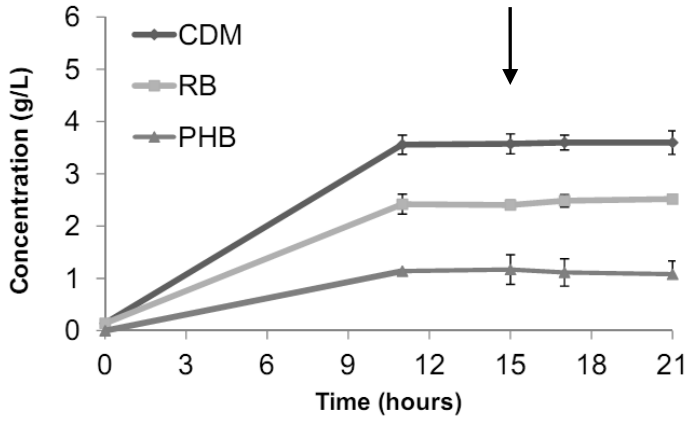


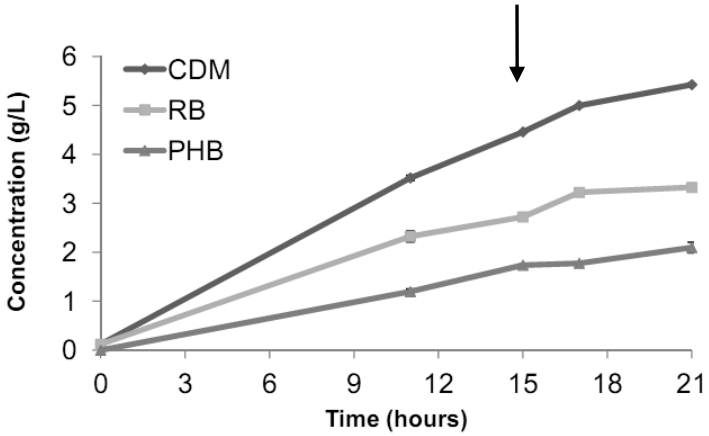
Figure 1. OD_{420} variation during the fermentation with *B. megaterium* uyuni S29 in four different salt concentrations. The error bars refer to deviations between two parallel experimental set ups.

The main results of the fermentations are shown in table 1 and figures 1 and 2. The growth curves of the fermentations by means of CDM, RB and PHB content (3HB from the gas chromatography after transesterification) with the different salt concentrations are shown in figure 2. Figure 2a shows the growth curve of the cultures with 5 g/L of salt in the medium. It shows a stationary phase reached after 11 hours of fermentation with a specific growth rate (μ_{max}) of 0.22±0.08 h^{-1} in the growth phase. The ammonia source was limited at 17 hours, but any changes were observed in the PHB production, since the maximal PHB accumulation was achieved before this time (1.17±0.28 g/L at 15h of fermentation). The growth curves of the cultures with 45 g/L salt are shown in figure 2b. The strain grew during the entire fermentation and the μ_{max} was 0.21±0.05 h^{-1} until 11 hours. Nitrogen was limited at 17h of fermentation and an increase in the PHB accumulation can be observed, obtaining the maximal PHB content (2.22±0.11g/L). Figure 2c shows the growth curves of the cultures with 100 g/l salt. The strain shows a long lag phase of around 11 hours and a growth phase of 6 hours (from 11 hours till 17 hours), with a μ_{max} of 0.30±0.06 h^{-1} which is higher compared to the other fermentations. An increase in the PHB production after 17 hours of fermentation can also be observed, when the ammonia source was limited (0.72±0.06 g/L PHB). Figure 3 shows the highest CDM and PHB content for each fermentation. For instance, the maximum CDM achieved in the

fermentation with 45 g/L salt was 5.42±0.04 g/L after 21 hours. At the same time, the maximum polymer concentration was obtained, corresponding to 40 % PHB in CDM.



(a)



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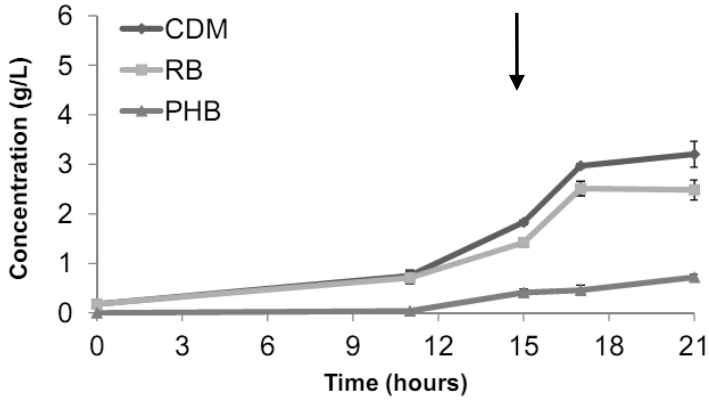

(c)

Figure 2. CDM, RB and PHB content during the fermentation with *B. megaterium* uyuni S29 in four different salt concentrations in medium: 5 g/L NaCl (a), 45 g/L NaCl (b) and 100 g/L NaCl (c). The error bars refer to deviations between two parallel experimental set ups. Black arrow indicates the limitation of the nitrogen source

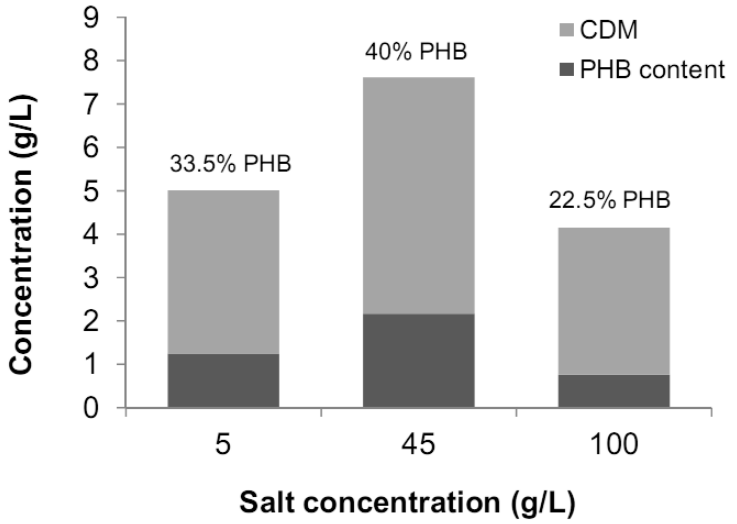

Figure 3. Maximum CDM and PHB values for *B. megaterium* uyuni S29 after 21 hours of fermentation with 5, 45 and 100 g/L NaCl

Table 1. Values of the main parameters of the fermentations: bioreactor and shaking flasks

Salt % in each medium	5 g/L	45 g/L	100 g/L
Final CDM (g/L)	2.60±0.23	5.42±0.04	3.20±0.26
Maximum PHB (g/L)	1.17±0.28	2.22±0.11	0.72±0.06
Final Content of PHB in biomass (g/L)	1.07±0.25	2.09±0.11	0.72±0.06
Maximum PHB content (%)	32.55±6.16	40.97±0.64	22.49±0.02
Maximum specific growth rate $\mu_{max\ (1/h)}$	0.22±0.08*	0.21±0.05*	0.30±0.06**
Volumetric Productivity PHB (g/L·h)	0.06±0.01	0.10±0.03	0.03±0.00
Total consumption of glucose from t = 0 to 21 hours/ (g/L)	9.67	15.44	3.32
Yield $_{(PHB/Sugars)}$ from t = 0 to 21 hours	0.11±0.03	0.13±0.04	0.22±0.01
Yield $_{(CDM/Sugars)}$ from t = 0 to 21 hours	0.27±0.02	0.35±0.00	0.96±0.07

[1] Calculated from the beginning of the fermentation until 11h
[1*] Calculated from 11h until 17h

By means of HPLC, the real glucose and salt concentration in every sample taken was analysed. Glucose consumption was constant in all cultures and it was maintained in excess from a concentrated solution. The salt concentration evolution during the fermentation process is shown in figure 4. As shown, the salt concentration is constant at the beginning and then slightly decreases in almost all set-ups. After 15 hours, the decrease of NaCl concentration is higher for set-ups with higher salt concentration: for 250 and 100 g/L the decrease is 6%. The cultures with 45 g/L of salt show a

decrease of the salt in the medium at the end of the fermentation of 5.8% and the NaCl concentration is maintained constant in cultures with 5 g/L.

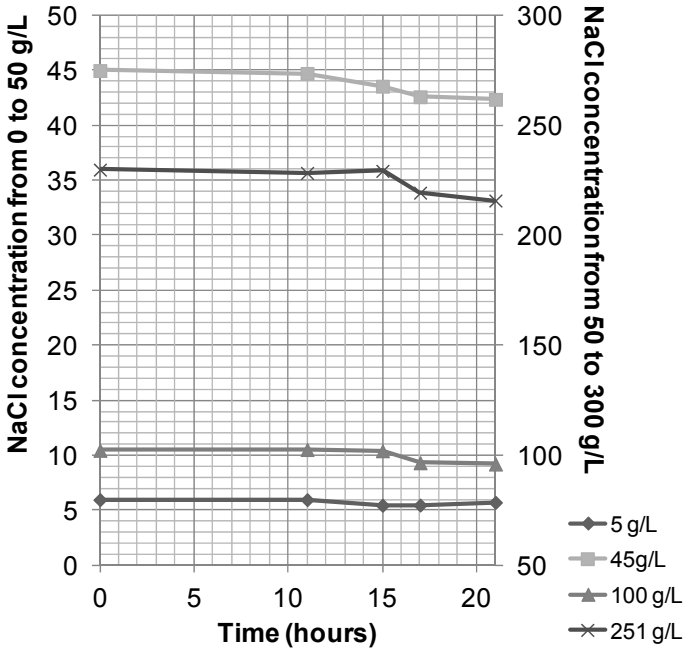

Figure 4. Salt concentration along the fermentations

6. Discussion

Bacterial growth and salt concentration

There is not much information about the influence of salt concentration on the growth and biopolymer production in *B. megaterium*. Thus, this preliminary study was focused on the analysis of the influence of NaCl concentrations on the growth-cell and PHB production by the novel *Bacillus megaterium* uyuni S29.

The results of the study show that the strain behaves differently depending on the quantity of salt in the medium. Curves of fermentation with salt concentrations of 5 and 45 g/L (0.5 and 4.5 % NaCl) are very similar, showing no long lag phase and similar μ_{max} values, around 0.2 h^{-1}. However, when the salt concentration was increased to 100 g/L NaCl, the lag phase also increased. **Nekolny** (**2002**) reported that an elevated concentration of NaCl in liquid medium causes a concentration-dependent growth delay and a decrease in the growth rate of *B. megaterium*. It seems obvious that the bacterium faces a salt stress situation that causes a physiological reaction to overcome an unusual situation. The adaptation to salt stress is accompanied by a longer lag phase and a reduction in the μ_{max}, since the strain needs more time to adapt to the stressful medium. Taking this into account, the results found in the fermentations with *B. megaterium* uyuni S29 with the different NaCl concentrations indicate that the salt content in which the strain lives in its natural environment could

be in the region of 5 and 45 g/L. These salt concentrations do not involve a stress situation for the bacterium and consequently it did not show a long adaptation phase. However, salt concentrations of around 100 g/L cause a stress situation to the bacterium, requiring a longer time for adaptation. The bacterium requires extensive adaptation of the intracellular enzymatic machinery to be functional in the presence of high ionic concentrations. On the other hand, the μ_{max} increases for these set-ups (0.30 h^{-1}) when comparing them with the μ_{max} of cultures with less salt concentration. This result differs from the ones reported by **Nekolny** (**2002**). This could be because *B megaterium* uyuni S29 has the ability to adapt to stressful media. Taking into account that it was originally isolated from Uyuny salt lake, which salt concentration varies during the year and can be extremely high in the dry season, it could have developed special skills to survive (**Retting 1980, Hurlbert 1988**).

B. megaterium uyuni S29 did not develop in cultures with 250 g/L NaCl during the 21 hours that the fermentation lasted. This high concentration of salt inhibits its growth, but it does not provoke its death. The strain uses NaCl as a strategy to compensate for high salt concentration in the medium and keeps it alive. This NaCl concentration can be the maximum salt content tolerated by the strain to survive. As **Obruka** (**2010**) reports for *C. necator*, when the stress factor, in this case high salt concentration, is applied at the beginning of cultivation, culture growth is inhibited. Consequently, it is probable that 250 g/L NaCl in the medium inhibits the cell growth of *B. megaterium* uyuni S29 and, consequently, its polymer production.

PHB production and salt concentration

Studies with *Cupriavidus necator* show that cultures exposed to a stress factor (0.5% NaCl) resulted in unaffected biomass formation and enhanced PHB accumulation (**2010 Obruka**). *B megaterium* uyuni S29 also developed pretty well in a culture with 0.5% NaCl. However, the CDM and the PHB accumulated in a salt concentration of 0.5% (w/v) were not better than in a culture with 4.5% (w/v) NaCl. This higher salt concentration (45 g/L NaCl) enhanced not just the growth cell, but the polymer production as well. Something similar was found for *Rhizobium* DDSS-69 (**Natarajan 1995**) indicating that NaCl stress results in an improvement of the PHB production. Therefore, 4.5% NaCl might be around the optimal salt concentration for *B megaterium* uyuni S29 to grow and to synthesize biopolymer.

The decrease in PHB content in cells exposed to high NaCl concentrations can be explained by inhibition of PHB biosynthetic pathway and activation of other metabolic processes related to osmotic stress response (**Obruka 2010**). This could be the case of *B. megaterium* uyuni S29 when grow in 100 g/L NaCl because the results show no higher growth and PHB production in relation to the fermentation with 45 g/L NaCl.

Role of the salt concentration in the cell

Excluding the salt concentration of 250 g/L, it was observed in this study that the salt concentration decreased for all set-ups around 6% after 21 hours of fermentation. This indicates that the strain

somehow uses the NaCl in its metabolism. Halophilic and halotolerant bacteria are characterised by their ability to balance the osmotic pressure. One way to adjust the salt concentration of the medium is by accumulating small molecules to counteract the osmotic pressure. The osmolytes are necessary for halophilic microorganisms because they increase the stability of some proteins in the stressful media (**Roberts 2005**), acting like charge stabilizers (**Lanky 1974**). Osmolytes do not inhibit the cell functions or modulate some enzyme activity, and their accumulation helps to maintain turgor pressure, cell volume, and concentration of electrolytes, all important elements for cell proliferation (**Roberts 2005**). Otherwise, there is the "salt-in-cytoplasm"-strategy, which means that inorganic ions, mainly K^+ and Cl^-, accumulate in the cytoplasm until the internal salt concentration is similar to the extracellular one. This strategy is found in extremely halophilic Halobacteria (Archaea) and halophilic, anaerobic Haloanaerobiales (Bacteria) (**Averhoff 2010**). In the case of *B. megaterium* uyuni S29 it is possible that it introduces NaCl to use both ions as osmolytes. Roberts (**2005**) reports that solutes like K^+ and Na^+ also contribute to osmotic balance in cells and, together with Cl^- ion, are the major solutes shown in halophilic aerobic Archaea. This could explain the decrease in the salt concentration in the experiments reported here.

On the other hand, some halophytic microorganisms synthesise their own osmolytes. **Robers (2005**) reported some examples where PHB could act as a conventional osmolyte: the halotolerant *Photobacterium profundum* SS9 and the halophilics *Methylarcula marina* and *M. terricola. Photobacterium profundum* SS9 (**Martin 2002**) accumulates a high concentration of PHB at high hydrostatic pressure and NaCl concentration, indicating that the monomer, and possibly the polymer, function as conventional osmolytes. Therefore, PHB could be for *B. megaterium* uyuni S29 not just a carbon and energy storage, but an osmolyte as well, since it is more evident at higher salt concentrations.

In the present study, regarding the fermentation with high salt content (250 g/L NaCl), *B. megaterium* uyuni S29 incorporates NaCl (decrease in salt concentration in a medium of about 6%) but it did not grow. This could be because many enzymes (65, 124), including those of extremely halophilic bacteria (80, 83, 84), are denatured by high concentrations of salting-intype salts, suggesting the involvement of hydrophobic forces in their structure (**Langy 1974, Ramírez 2006**), not allowing the proper growth of the microorganism. When the salt concentration was around 100 g/L NaCl, the strain integrated salt inside the cells and also grew, producing PHB. It showed both mechanisms of osmolyte synthesis: by taking Na^+ cations from the environment and *via* PHB accumulation. It seemed that 45g/L NaCl in the medium was the optimal salt concentration for both, the cell growth and PHB accumulation. Also, there was a lower decrease in the salt content during the fermentation, showing both mechanisms of osmolyte synthesis in this lower salt concentration. When the concentration of salt in the media was around 5 g/L NaCl, this salt content in media also decreased during the fermentation. Most likely the strain used salt as well as PHB to equilibrate the osmotic pressure in low concentrations of salt.

Halophilic classification of *B. megaterium* uyuni S29

According to Vreeland (**1987**) who thoroughly studied the mechanisms of halotolerance in microorganisms and their halophilic classification, *B. megaterium* uyuni S29 can be classified as moderately halotolerant, since it can develop at salt concentrations between 5 and 100 g/L (corresponding to 0.08 and 1.7 M). However, a recent criterion according to Oren (**2008**) indicates that halophiles grow optimally at NaCl concentrations of 5% (w/v) (50 g/L) or higher, and tolerate at least 10% (w/v) (100 g/L) salt. *B. megaterium* uyuni S29 fulfills this condition as well and therefore, can be classified as a halophile.

Application

The use of moderately halophilic bacteria, in various biotechnological applications, has been suggested due to their versatility in the choice of a wide range of substrates and their simple cultivation requirements (**Ventosa 1998**). Haloarchaea present some advantages for the overall economical efficiency of PHA production. For example, the sterilization cost is reduced, since at such a high concentration of salt, the growth of non-halophilic microorganisms is prevented, allowing a process without strict sterile conditions (**Koller 2007b, Quillamana 2010**). The fact that *B. megaterium* uyuni S29 is classified as moderately halotolerant according to **Vreeland** (**1987**) or as halophilic according to Oren (**2008**) makes possible to apply the advantages of haloarcheae in the PHB production with the strain.

Several Bacillus species have been used in the treatment of wastewater as denitrifiers (**Bernhard 2012**), such as *B. cereus* (**Zhao 2009**) or B. Cereus (**Yang 2011**), and as phosphate removers such as *Bacillus sp* RS-1 (**Krishnaswamy 2011**). As halotolerant bacteria, some of them have been used as a biological solution for environmental problems associated with considerable salt concentrations in wastewater (**Kargi 2002, Sivaprakasam 2008**). *B. megaterium* uyuni S29 has shown that it tolerates a wide range of salt content (range of 0.1-10 % NaCl) and it is able to synthesize biopolymer in all cases. Because of these characteristics, *B. megaterium* strain uyuni S29 could be a competent bacterium to be applied in treatments for wastewater that contains high concentrations of salt. It might be possible to utilize it to eliminate the excess of phosphates, nitrogen and xenobiotics present in the water and at the same time produce biopolymer with added value. Moreover, the fact that *B. megaterium* uyuni S29 can develop in a salt concentration very similar to the concentration of sea water (around 3.5% w/v salts and 98% of these is NaCl) makes the strain very appealing because of the potential to use this cheap source as a culture medium (**Grosa 2008, Pandian 2010**).

7. Conclusion

The salt concentration influences the cell growth and on PHB production by the novel *Bacillus megaterium* uyuni S29. The strain behaves differently depending on the quantity of salt in the medium, requiring adaptation of the intracellular enzymatic machinery to be functional and equilibrate

the osmotic pressure in the presence of high salt concentrations. The optimal salt concentration improves the cell growth as well as the PHB production. The fact that the strain can properly develop and synthesise PHB in the presence and absence of salt, suggests that the strain is a great candidate for biotechnological applications in different areas.

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Section 4: *Influence of glycerol on PHB production by Cupriavidus necator and Burkholderia sacchari* [1]

1. Abstract

Glycerol is a co-product of many industrial processes and is generated in large quantities. In this study, glycerol is used as a cheap carbon source for the production of PHB with two different strains from the German strain collection, *Cupriavidus necator* (DMS 545) and *Burkholderia sacchari* (DMS 17165) in order to provide an alternative outlet for glycerol and produce value-added products. The objective of this work was to study the influence of this carbon source on their growth kinetics, on their polymer productions, and on the molecular mass of the produced biopolymer. Therefore, two fermentations in 7L- and 3L-bioreactors were carried out with each strain. The results show, for the first time, a high cell dry mass and growth rate, when glycerol is used in the fermentation with *Cupriavidus necator* DSM 545. The molecular mass obtained was relatively low, about 300 kDa with a polydispersity 4.72. In the first fermentation carried out with *Burkholderia sacchari* DSM 17165 with glycerol as a sole carbon source, low molecular mass PHB was produced, 200 kDa and polydispersity of 2.50.

2. Introduction

Poly(3-hydroxybutyrate) (PHB) is the most widely studied member of the Polyhydroxyalkanoates (PHAs). These are biopolyesters of various hydroxyalkanoates which are synthesized by numerous microorganisms as energy reserve materials when an essential nutrient is limited (**Khanna and Srivastava 2005a**). They possess properties similar to various synthetic thermoplastics like polypropylene and hence can be used in their place. They are also completely degraded to water and carbon dioxide under aerobic conditions and to methane under anaerobic conditions by microorganisms in soil, sea, lake water and sewage (**Lee 1996**).

Among the microorganisms able to produce PHAs, it is possible to find Gram-positive or -negative bacteria, and archea. In addition, the production of PHAs in organelles of genetically modified eucaryontic organisms like plants (**Poirier 2010, Brumbley 2010**), yeasts (**Breuer 2002**) or even insects (**Williams 1996**) is reported in literature. Microbial production of PHB is predominantly investigated in the bacterium *Cupriavidus necator* (also known as *Ralstonia eutropha* and *Alcaligenes eutrophus*). *Cupriavidus necator*, which can store PHA up to 96% of its cell dry mass (CDM), is known to produce PHB under conditions of nitrogen or phosphate limitation and excess of carbon source. Furthermore, it is the only PHA producing organism with an entirely reported genome and is used as a PHA producer on an industrial scale (**Madison 1999**). On the other hand, *Burkholderia*

[1] *It corresponds to the article send for publication in Journal of Applied Microbiology: Effect of glycerol in the production of PHB by Burkholderia sacchari by Rodríguez-Contreras Alejandra, Koller Martin, Miranda-de Sousa Dias Miguel, Calafell Margarita, Braunegg Gehart, Marqués-Calvo María Soledad.*

sacchari (DMS 17165) is a less studied strain in this field, but some studies already show that the strain is a good polymer producer (**Brämer 2001, Silva 2004, Rocha 2008, Pradella 2010**). It is a Gram-negative bacterium isolated from the soil of a sugar-cane plantation in Brazil and found to accumulate up to 68% of the CDM as PHB with sucrose as the sole carbon source and up to 65–69% of the CDM as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) P(3HB-co-3HV)] with glucose and propionic acid as precursor substrates (**Brämer 2001**). These two bacterial strains were used in this work, C*upriavidus necator* because it is the one that has been most extensively studied and industrially used (**Verlinden 2007**), and *Burkholderia sacchari* because it is a relatively novel strain with high potential for the PHA production.

Industrial PHB production by microorganisms has been widely attempted but there is still a need to address the problem of improvement of PHB yield and productivity so that it can economically compete with the production cost of conventional plastics (**Khanna and Srivastava 2005b**). The main factors that increase these biopolymer production costs are those reported by **Choi (1999)**. The raw materials, chemicals, equipment and energy used in the process increase the price of the product making the biopolyesters uncompetitive with the conventional plastics. The researchers have been focusing on different strategies to decrease this cost. Among them, the search and study of new strains capable of synthesizing biopolymer improving the productivity of the process (**Oliveira 2007, Rodriguez-Contreras 2012**) or the replacement of the carbon source for the cheapest ones to decrease the cost of the raw materials (**Braunegg 2008, Povolo 2010, Koller 2010a, Koller 2010b**). It was pointed out that the raw materials claim the major part of the production cost for biopolymers; they can contribute up to 50% of the entire process expenses (**Solaiman 2006a**). The substitution of pure sugars substrates such as glucose or sucrose by applying cheaper carbon sources as basis feedstock is of importance to further enhance the cost efficiency of the production process. Among these substrates which occur in large quantities, molasses (**Zhang 1994**), starch (**Chen 2006**), whey from the dairy industry (**Koller 2006**), the waste water from olive oil production (**Gonzalez-Lopez 1996**), xylose (**Silva 2004**), a broad range of plant oils (**Kahar 2004**) and glycerol liquid phase (GLP) from the biodiesel production (**Koller 2005a, Koller 2010b**) are available.

Glycerol is a co-product of many industrial processes and is generated in large quantities. It is also a by-product of the biodiesel production. The large scale production of biodiesel, as an alternative and renewable energy source, will result in a surplus of glycerol- the transesterification of the plant oil produces around 200000 t of glycerol (**Mothes 2007, Koller 2010b**). This side product could be the ideal source for industrial fermentation. Besides, PHA produced from renewable resources becomes more and more competitive with common plastics due to the rising oil prices (**Ashby 2005**, **Mothes 2007**). Taking this into account, it was interesting to examine the availability of this industrial by-product with respect to the microbial PHB production with *Cupriavidus necator* DMS 545 and *Burkholderia sacchari* DMS 17165.

There are already some studies showing that some strains are able to produce low molecular mass PHB using glycerol as a carbon source (**Koller 2005a**, **Ashby 2005**). When glucose is used as carbon source, it is metabolized to pyruvate *via* the Enbner-Doudoroff pathway (2-keto-3-deoxy-6-phosphogluconate pathway). Pyruvate can be converted by a dehydrogenase to Acetyl-CoA, the central intermediate of the cellular metabolism, and the starting compound for the PHB synthesis. On the other hand, glycerol can be metabolized to pyruvate as well, but via glyceraldehyde-3-phosphate (**Solaiman 2006b, Khanna 2011**)

3. Objectives

In this study, two fermentations in 7L- and 3L-bioreactors were carried out with two different strains from the German strain collection, *Cupriavidus necator* (DMS 545) and *Burkholderia sacchari* (DMS 17165) respectively. In both cases, glycerol was used as a cheap carbon source in the accumulation phase. The main purpose of this work was to provide an alternative outlet for glycerol and generate value-added biopolymers. Therefore, the objective was to study its influence on the growth kinetics of the above mentioned strains, on their polymer productions, and on the molecular mass of the produced biopolymer.

4. Materials and methods

Microorganisms

Cupriavidus necator DSM 545 (formerly known as *Wautersia eutropha*, *Ralstonia eutropha*, *Alcaligenes eutrophus* and *Hydrogenomonas eutropha*) and *Burkholderia sacchari* DSM 17165 were obtained from *Deutsche Sammlung von Mikroorganismen und Zellkulturen* GmbH, Germany.

Materials

All simple salts and chemical products were at least technical grade and obtained from Sigma-Aldrich Chemical Company. ROTIPURAN® (≥86 % glycerol and 1,2,3-propantriol from Carl Roch, Germany) was used as glycerol (density of 1.26 g/cm^3).

Culture media

The strains were cultivated in a mineral medium (M) according to Küng **(1982) (Koller 2005b, Atlić 2011),** containing (g/L): $Na_2HPO_4{\cdot}2H_2O$, 4.5; KH_2PO_4, 1.5; $MgSO_4{\cdot}7H_2O$, 0.8; NaCl, 0.9; $(NH_4)_2SO_4$, 2; $CaCl_2{\cdot}2H_2O$, 0.02; NH_4Fe(III) Citrate, 0.05; agar, 10; trace element solution SL6, 1 mL; glucose, 15; adjusted to a pH value of 7,0. SL6 was composed by (mg/L): $ZnSO_4{\cdot}7H_2O$, 100; H_3BO_3, 300; $CoCl_2{\cdot}6H_2O$, 200; $CuSO_4$, 6; $NiCl_2{\cdot}6H_2O$, 20; $Na_2MoO_4{\cdot}2H_2O$, 30; $MnCl_2{\cdot}2H_2O$, 25. The batch growth medium for the bioreactors consisted of (g/L): KH_2PO_4, 4.3; NaCl, 0.8; $(NH_4)_2SO_4$, 3; $MgSO_4{\cdot}7H_2O$, 0.8; $CaCl_2{\cdot}2H_2O$, 0.02; NH_4Fe(III)Citrate, 0.05; glucose, 20; and trace element solution SL6, 3 mL. All

media were adjusted to a pH value of 7. The components susceptible to precipitate were sterilized separately (21 min-120 ºC). All media used for *Burkholderia sacchari* DSM 17165 required no NaCl.

Precultures and Inoculum

Precultures of the strains were first inoculated from solid medium grown for 24 hours. The first seed cultures were incubated overnight in 300 mL shake flasks containing 100 mL of the medium at 30 ºC for *Cupriavidus necator* DSM 545 and at 37 ºC for *Burkholderia sacchari* DSM 17165, and with a pH value of 7.0 in a rotary shaker at 120 rpm. Glycerol (15 g/L) was used in the precultures with *Burkholderia sacchari* DSM 17165 to pre-adapt it to the carbon source. The inoculums for the bioreactors were prepared from 5 mL of these precultures, which were then transferred to 1000-mL shake flasks containing 250 mL of the medium and incubated under the same conditions as the precultures. One and half liters of inoculum cultures were used to inoculate the bioreactors with *Cupriavidus necator* DSM 545 and *Burkholderia sacchari* DSM 17165, respectively.

Bioreactors operating

The fermentation with *Cupriavidus necator* DSM 545 was carried out in Labfors 3 bioreactors (Infors AG, Bottmingen, Switzerland). It had a total volume of 7.5 L with a working volume of 5 L (1 L of inoculum). The fermentation with *Burkholderia sacchari* DSM 17165 was carried out in a smaller stirred tank reactor of Labfors 3 (Infors AG, Bottmingen, Switzerland). It had a total volume of 3.6 L with a working volume of 2.4 L. For this experiment a total volume of 1.5 L was utilized with 0.5 L of inoculum. The temperatures of the cultures were 30 and 37 ºC, and the pH-values were maintained at 6.8 and 7, respectively.

The sterilization of all the bioreactor equipment was done inside an autoclave at 121 ºC for 1 hour. All relevant fermentation parameters (bath temperature, pH-value and consumption of pH correction solutions, flow rate, dissolved oxygen concentration, stirrer speed, and antifoam activity) were monitored and recorded by an IRIS software program. The bioreactor vessel is constituted of two layers of glass, into which the water flow is used to control the temperature of the culture bath. The bioreactor controlled the water temperature by cooling and heating until it adjusted the bath temperature, which was constantly controlled by a sensor (pt-100 Labfors, Infors AG, Bottmingen, Switzerland). The regulation of pH was automatically controlled by a pH-electrode (Hamilton, Switzerland). The electrode was polarized for 3-5 hours and, after calibration and sterilization, was introduced into the bioreactor. The pH-value was maintained at the required pH-value by automatic addition of 25% ammonia hydroxide (alkali and nitrogen source for microbial growth), 10% sodium hydroxide (alkali for the reactors when the nitrogen source was limited) or 10% sulfuric acid solution. The concentration of the dissolved oxygen was achieved by automatic adjustment of the stirrer speed and the air flow rate. These were used to monitor the cell activity and were initially adjusted to 5 L/min and 500 rpm respectively. Dissolved oxygen concentration was maintained at 40% of air saturation in water and was monitored by the oxygen partial pressure (pO_2) (Ingold sensor). Oxygen was supplied at 150 L/h through an absolute filter (Sartorius, Midisart 2000). The concentration of the dissolved

oxygen was followed by an oxygen electrode (Hamilton, Switzerland) and was calibrated inside the bioreactor using N_2 for the 0 value. Foam formation was controlled by an electrode and the antifoam agent Struktol J700 was added automatically in order to avoid heavy foam formation. Glucose was added from a concentrated solution of 50 % (w/v) during both fermentations to avoid the carbon source limitation and to maintain the activity of the cells according to the fermentation requirements. In the accumulation phase, glycerol was also added from a 100% sterile solution (table 1).

Table 1. The addition times and amount of glucose for the fermentation with *Cupriavidus necator* DSM 545 and with *Burkholderia sacchari* DSM 17165, respectively

Cupriavidus necator DSM 545			*Burkholderia sacchari* DSM 17165		
Time (h)	Glycerol (g/L)	Glucose (g/L)	Time (hours)	Glycerol (g/L)	Glucose (g/L)
15.35		8.26	8	2	
18.50	48.22	8.26	10	10	
21.25		4.13	19.75		20
22.50		4.13	24	5	15
23.75		4.13	31.5	5	
25.35		8.26	36.25	5	
26.70		8.26	36.75	5	
33.85		4.13	39	1	
38.00		10.33	43.5	2	
45.50		4.13	47.5	10	
47.35		4.13			

Optical density

Optical density (OD) was measured for rapid assessment of the cell growth. The measurements were carried out at a wavelength of 420 nm using Spectronic® Genesys™ 2PC spectrophotometer.

Determination of the CDM, RB and PHB content

A gravimetrical method was used to determine the biomass concentration expressed as CDM in fermentation samples. Samples of 10 mL of culture broth were taken throughout the fermentations and centrifuged in pre-weighed glass screw-cap tubes for 15 min at 4000 rpm (Megafuge 1.0R Heraeus Sepatech). The pellet was frozen, lyophilised and weighed to determine the CDM by weight difference. The PHA in lyophilized biomass samples was transesterificated by acidic methanolysis following Braunegg's method (**Braunegg 1978**). Analyses were carried out with Agilant Technologies 6850 gas chromatograph (30-m HP5 column, Hewlett-Packard, USA; Agilant 6850 Series Autosampler). The methyl esters of PHA constituents were detected by a flame ionization detector

with helium as a carrier gas (split- ratio of 1:10). P(3HB-co-19.1%-3HV) (Biopol; Imperial Chemical Industries) was used for 3HB and 3HV calibration and hexanoic acid was used as an internal standard. The PHB content (wt %) was defined as the percentage of the ratio of 3HB concentration to CDM. The RB was then calculated as the difference between cell concentrations and the PHB content.

Carbohydrate determination

Carbohydrate concentration from supernatant was monitored by means of HPLC equipment composed of a thermostated Aminex HPX 87H column (thermostated at 75 ºC, Biorad, Hercules, USA), a LC-20AD pump, a SIC-20 AC autosampler, a RID-10A refractive index detector and a CTO-20 AC column oven. Also the LC solution software for registration and evaluation of the data obtained was used. 1.5 mL of liquid media were sterile filtrated and transferred into vials. Water was used as an eluent at a flow rate of 0.6 mL/min. The standards were prepared with different concentrations of glucose and glycerol.

Determination of nitrogen source

2 mL of supernatant was mixed with 50 µl alkaline ISAB solution containing 5 M sodium hydroxide, 10 % methanol, 0.05 M Na_2-EDTA and a colour indicator. The mixture was immediately analysed with an Orion ion selective electrode; the signal was monitored by a voltmeter. The standard curve was calculated measuring different ammonium sulphate standards solutions of defined concentrations.

PHB isolation

At the end of the fermentations, the temperature of the bioreactor was set up to 70 ºC for 30 min in order to pasteurize the biomass. The fermentation broths were centrifuged for 30 min at 4°C and 6,000 rpm in a Sorvall® RC-5B Refrigerated Superspeed centrifuge (DuPont Instruments); the obtained cell pellets were frozen and lyophilized. The dry biomasses were stirred with a tenfold quantity (mass) of ethanol for 24 hours at room temperature in order to remove the lipid components. Decreased cell masses were separated from the ethanol solution of the extracted lipids by vacuum filtration. The remaining cell masses were air-dried. To extract PHB, the cell masses were stirred with a thirtyfold quantity of chloroform for 24 hours under light protection. The solutions of PHA in chloroform were separated from the residual cell masses *via* vacuum-assisted filtration. Afterwards, the major part of the chloroform was removed *via* vacuum-assisted distillation using a rotary evaporator until viscous solutions were obtained (concentration of biopolymer approximately 40 g/L). The PHB was precipitated by adding a tenfold amount of ice-cooled ethanol to the chloroform solutions, separated by vacuum filtration and left at room temperature for the remaining solvent to evaporate.

Polymer characterization

The chemical structure was characterised with a Perkin Elmer Fourier Transform Infrared microscopy using optical Perkin Elmer software. The line-scan spectra were based on 32 scans and a resolution

of 4 cm^{-1}. ^1H NMR spectra were recorded at 25 °C on a Bruker AM300 spectrometer. The polymer samples were dissolved in chloroform and a drop of TMS (tetra methyl silane used as internal standard for calibrating chemical shift for ^1H) was added as reference. 10 mg of the sample dissolved in 1 mL of deutered solvent was used. Proton spectra were recorded at 300.1 MHz with a spectrum of 32 K data points. A total of 64 scans were utilized with a relaxation delay of 1 second. The GPC measurements were performed utilising chloroform as an eluent at a flow rate of 0.80 mL/min with a stabilisation pressure of 35 bars and a sample concentration of 1.5 mg/mL. A Waters Styragel HT column for mid-range molecular-mass distributions was used and samples of polystyrene with different molecular masses were used as standard. DSC experiments were performed on a Perkin-Elmer Pyris 1 instrument with a dry nitrogen gas flow of 50 mL/min. The apparatus was calibrated using Indium of high purity. Approximately, 5 mg of the sample was sealed in an aluminium planched and analyzed. The melting temperature (T_m), melting enthalpy (ΔH_m) and the glass transition temperature (T_g) were determined by the second heating run of DSC endothermic peaks. The crystallinity degree (X_c) of P(3HB) was calculated assuming that the ΔH_m value of 100% crystalline P(3HB) is 146 J/g (**Barham 1984**). Scans started at -30 ºC and were ramped at 10 ºC/min to 230 ºC.

5. Results and Discussion

The fed-batch fermentations with the bacterial strains lasted 57.5 and 57.25 hours with *Cupriavidus necator* DSM 545 and *Burkholderia sacchari* DSM 17165 respectively. During the fermentation processes, the cell activity was controlled in real time by means of the base and the oxygen evolutions. These two parameters are given by the pO_2 and base pump sensors. Normally, the consumption of base and oxygen increase while the cells are growing, decreasing the pO_2. When the pO_2 increased it is a consequence of a decrease in the cell activity. Also the OD (420 nm) is another parameter that provides information about the bacterial activity. After analysing the samples taken, it was confirmed that the kinetics of the OD and the CDM coincided (figure 1).

When comparing the OD and the CDM from both fermentations, higher values were reached on the *Cupriavidus necator* DSM 545 culture compared to the ones obtained from *Burkholderia sacchari* DSM 17165. This is due to the different development capability that each strain showed in the medium. A common element in both fermentations was that glycerol was used as cheap carbon source. However, they are different from each other in several aspects and therefore fermentation values are not comparable. First, the bioreactor used for cultivating *Cupriavidus necator* DSM 545 had a higher working volume than the bioreactor used with *Burkholderia sacchari* DSM 17165. The bioreactor's total volumes were 7.5 and 3.6 L with a real working volume of 3.6 and 1.5 L, respectively. The reason why a higher volume was used for the cultivation with *Cupriavidus necator* DSM 545 is that a higher amount of biopolymer was expected to be synthesized with this strain, so that it could be used for further investigations of polymer transformation and application. Second, the fermentation strategy was different for each fermentation, adjusting it to the bacterial requirements.

For instance, fermentation with *Cupriavidus necator* DSM 545 was carried out with two carbon sources whereas the one with *Burkholderia sacchari* DSM 17165 was carried out with just glycerol. Third, the inoculums amounts were also different for both fermentations due to technical requirements and its influences on the bacterial growth.

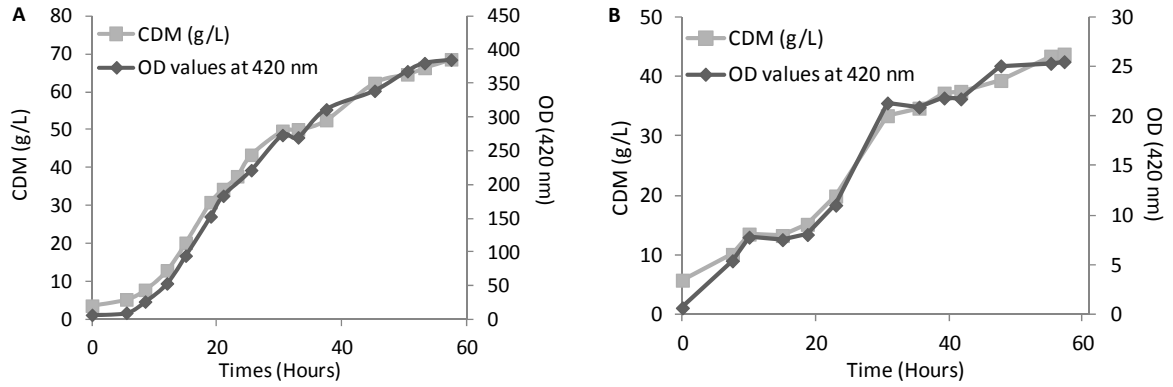

Figure 1. Comparison of the OD and CDM kinetics during the fermentations with *Cupriavidus necator* DSM 545 (A) and *Burkholderia sacchari* DSM 17165 (B)

Fermentation with *Cupriavidus necator* DSM 545

Cupriavidus necator DSM 545 was cultivated in fed-batch fermentation in a 7L-bioreactor in order to not only study the glycerol influence, but also to produce a high amount of PHB. Glucose and glycerol were co-supplemented in the PHA production phase for the biosynthesis of PHB. The evolution of the CDM, that is the addition of the RB and the biopolymer content, is shown in figure 2. The CDM concentration increased throughout the fermentation, initially, due to the strain growth phase, and later, in the stationary phase, because of the biopolymer accumulation. The evolutions of both RB and PHB content are also shown in this figure. The RB shows how the strain developed during the cultivation. In this case, the strain required 8.5 hours of adaptation (lag phase), and then it grew until around 19 hours with a specific maximum growth rate (μ_{max}) of 1.21 h^{-1}. The polymer production increased until the end of the fermentation, achieving a volumetric productivity of 0.76 g/L·h and a maximal PHB content of 64.55% in CDM.

Nitrogen limitation acts as an initiator for PHA production because the formation of proteins (RB) stops and then the carbon flux is used for polymer synthesis (**Koller 2007, Sudesh 2000**). Therefore, the biopolymer accumulation was induced by the interruption of the nitrogen source supply. The additions of a nitrogen source were initially made by using NH_4OH as an alkali source. After 18.5 hours of fermentation, the limitation of the nitrogen source (PHB accumulation conditions) was initiated by switching the ammonia solution to NaOH for pH correction. Grey rhombus in figure 3 indicates the time when the nitrogen source was no longer supplied. The nitrogen limitation occurred after 25.5 hours of cultivation. At this time, the production of biomass stopped, beginning the stationary phase and the polymer production was enhanced.

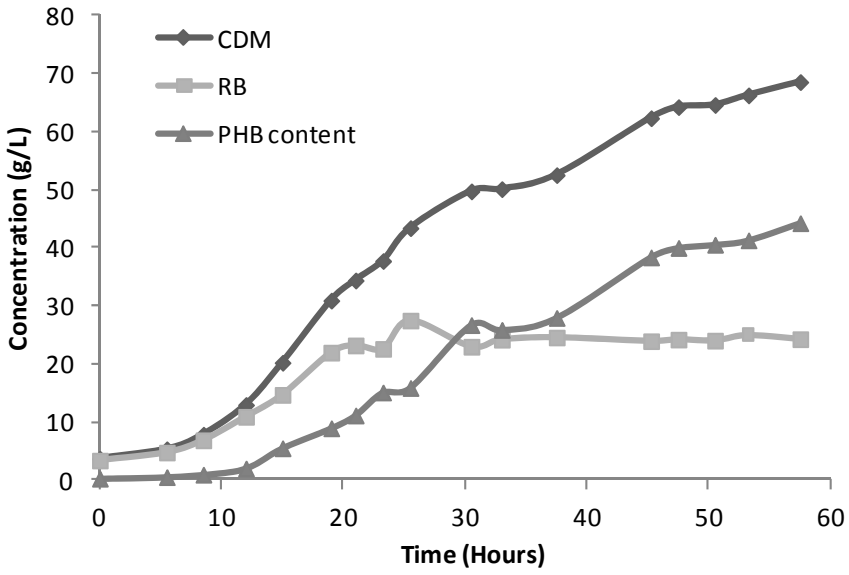

Figure 2. Time courses of the CDM, RB and PHB content concentrations

Figure 3 not only shows the evolution of the nitrogen source and the time when it was limited, but also the time courses of the carbon sources used in the fermentation, glucose and glycerol. Glucose was the initial carbon substrate and was being first consumed as a sole carbon source until 19 hours of fermentation. Between 19 and 50.5 hours of fermentation both carbon substrates were consumed by the strain as figure 3 shows by the decrease on the glucose and glycerol curves. After 50.5 hours, the only carbon substrate consumed by the strain was glycerol. Glucose was being added during the strain cultivation due to fermentation requirements – the black dots in figure 3 show its additions. It was first limited after 30.5 hours of cultivation having been totally consumed by the strain. At this point, the cells activity decreased, and glucose was therefore added at 33.5 hours, thus causing the reactivation of the cells. Later, after 4 more re-feedings, glucose was limited again after 50 hours of fermentation. However, the cell activity remained constant until the end of the cultivation. This could be because at this point the strain was already adapted to the use of glycerol as a sole carbon source. Glycerol was added only one time (48 g/L) and it was constantly consumed. Its addition is indicated by the dark grey triangle in figure 3.

The consumption of glycerol by *Cupriavidus necator* DSM 545 was very slow compared to the consumption of glucose. However, the conversion yield of PHB from glycerol was higher than from glucose (table 2). The yield of PHB from the total carbon source during the entire fermentation was 0.34 g/g from the combination of both glucose and glycerol. This result can be compared to previous studies with *Cupriavidus necator* and glycerol as carbon substrate. Initially, Bormann (**1999**) used this strain to produce PHA *via* 2.5 L fermenter with glycerol and casein, obtaining 0.17 g/g of polymer conversion. In later studies, glycerol and different concentrations of salts were used together with yeast extract as a carbon source for fermentation in a 2 L bioreactor (**Mothes 2007**). Here, a polymer conversion of 0.33 g/g could be obtained from crude glycerol**.** In the studies carried out by Cavalheiro

(**2009**), different types of glycerol (commercial/pure and waste glycerol from biodiesel manufacturers) were used together with glucose as carbon sources in a 2 L bioreactor. Polymer conversions of 0.34 and 0.37 were obtained. In all mentioned and reported cases, the carbon substrate was substituted by glycerol in the fermentation process, and the conversion results considered only for glycerol. However, in this study, both glucose and glycerol were used together throughout the fermentation process. The polymer yield reached from the total carbon source was 0.4 g/g. Nevertheless, if the conversion results of this fermentation are separately analysed, the PHB yields from glucose and from glycerol were 0.26 g/g (between 0 and 19 hours of fermentation) and 0.75 g/g (between 50 and 57.5 hours of fermentation) respectively. This last result is much higher compared to the previous mentioned studies for just glycerol.

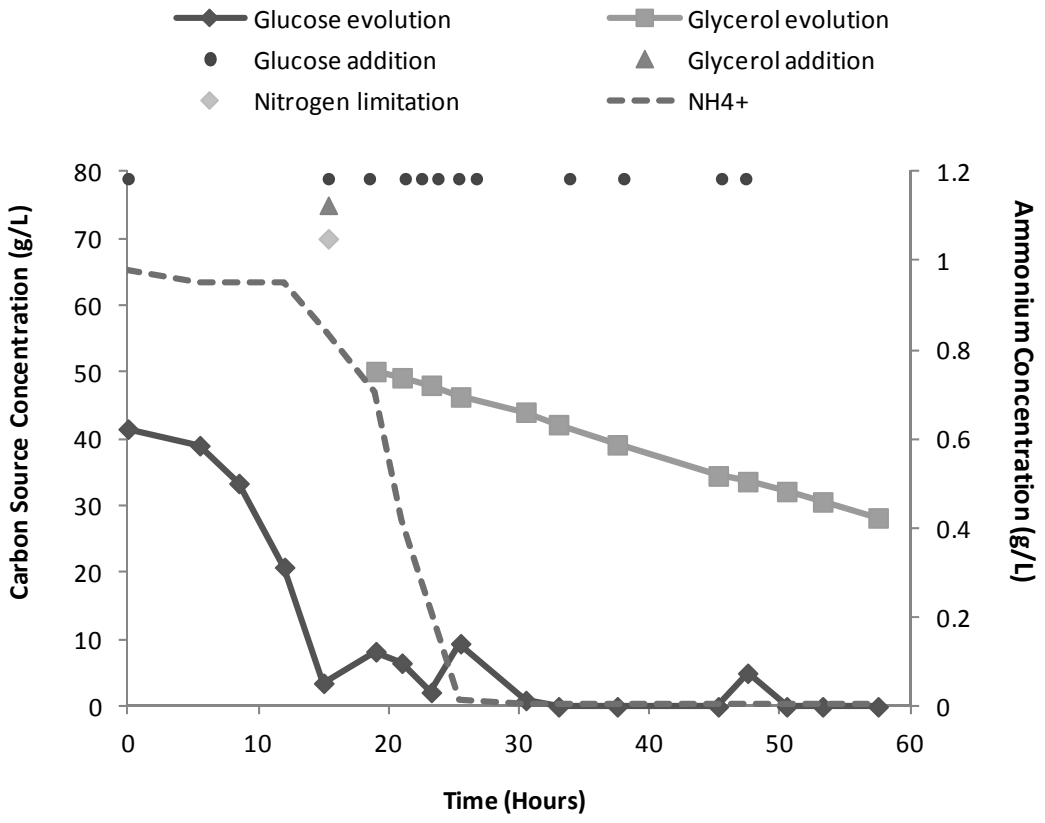

Figure 3. Evolution of the carbon sources (glucose and glycerol) and nitrogen concentration in the media during the fermentation. Black dots: re-feeding time with glucose (50% w/v). Dark grey triangle: re-feeding time with glycerol. Grey rhombus: stop of the nitrogen source supply

Table 2. Main parameters of the fed-batch fermentation with *Cupriavidus necator* DSM 545

Parameter	Value
Final CDM [g/L]	68.56
Final PHB [g/L]	44.25
Maximal content of PHB in biomass [%]	64.55
Volumetric productivity PHB [g/L·h]	0.76
Specific maximum growth rate, μ_{max} [h^{-1}]	1.21
Total consumption of carbon source [g/L·h]	2.29
Consumption of glucose [g/L·h]	1.91
Consumption of glycerol from 19 to 57.5 [g/L·h]	0.57
Yield $_{(CDM/glucose)}$ from 0 to 19h [g/g]	0.82
Yield $_{(CDM/glycerol)}$ from 50 to 57.5h [g/g]	0.80
Yield $_{(PHB/glucose)}$ from 0 to 19h [g/g]	0.26
Yield $_{(PHB/glycerol)}$ from 50 to 57.5h [g/g]	0.75
Yield $_{(PHB/carbon\ sourcel)}$ [g/g]	0.34

Cupriavidus necator JMP 134 was used to synthesize PHB, attaining around 70 % of PHB in CDM from pure glycerol and 0.13 h^{-1} of maximum specific growth rate (**Mothes 2007**). *Cupriavidus necator* DSM 545 was tested with two types of glycerol by Cavalheiro (**2009**). On a commercial glycerol, productivities of around 0.6 g/L·h and 1.5 g/L·h and a specific growth rate of 0.11 h^{-1}were attained. The maximum CDM and the PHB content were 82.5 g/L and 62% respectively. When waste glycerol was used instead, a productivity of 0.84 g/L·h and a specific growth rate of 0.15 h^{-1} were achieved. The PHB maximal accumulation was 38% in CDM. The results attained in this fermentation (table 2) are not far from those reported by Cavalheiro (**2009**).

It is important to point out that a high growth rate of 1.21 h^{-1} was obtained in this fermentation by the initial use of glucose as a carbon source. Together with the results of the volumetric productivity, maximal PHB content and PHB conversion, it could be concluded that the combination of both carbon substrates could be a great strategy for PHB production since higher growth rates and the use of cheaper feedstock are advantageous with respect to the reduction of production costs.

Fermentation with *Burkholderia sacchari* DSM 17165

Fed batch fermentation in a 3L-bioreactor was carried out in this work with the strain *Burkholderia sacchari* DSM 17165. By utilizing glycerol as a carbon source in the accumulation phase, the production of LMW-PHB directly from the bacterial cells was attempted. The evolutions of the CDM,

PHB content and RB curves along this fermentation are shown in figure 4. The curves show a constant growth of the strain during the entire fermentation. However it is possible to separate the exponential growth phase between 10 and 30.5 hours. For this period of time, the strain shows a specific growth rate of 0.42 h^{-1}. It synthesised PHB almost constantly during the fermentation, although the nitrogen source was not totally limited until 40 hours of fermentation. PHB was accumulated in 10% of the CDM at the end of the fermentation, reaching the maximal value and the volumetric productivity 0.08 g/L·h.

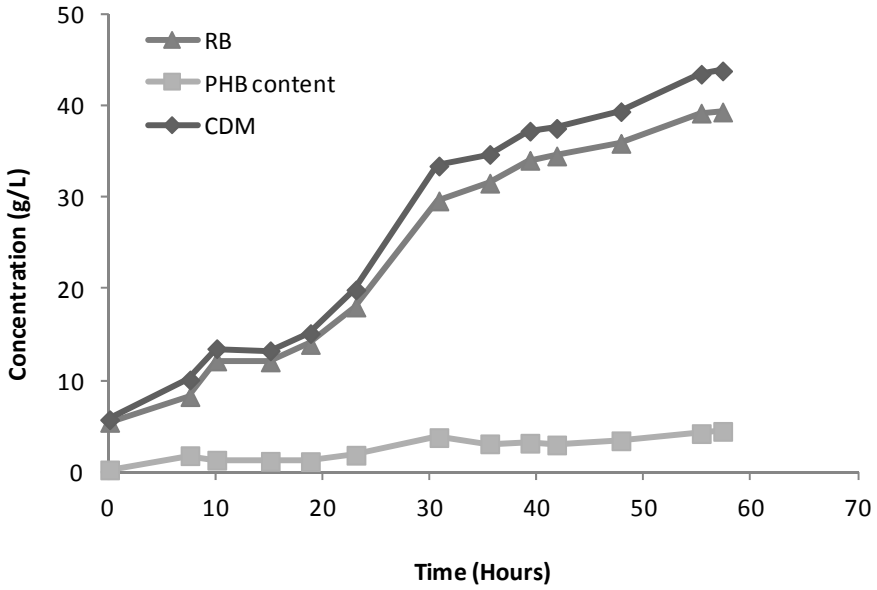

Figure 4. Time courses of the CDM, RB and PHB content concentrations

A nitrogen source was added at the beginning of the fermentation as ammonium sulphate and during the fermentation as ammonia hydroxide solution. It was consumed by the strain until the stationary phase was achieved (30.75 h). At this point, the nitrogen source was no longer supplied, and its limitation was attained at 39.75 hours of fermentation. A slight increase in the PHB accumulation can be observed when the nitrogen source was limited. Evolutions of the nitrogen and carbon (glycerol & glucose) sources as well as the time of their additions are shown in figure 5.

The time courses of the carbon sources used in the fermentation, glucose and glycerol, are also shown in figure 5. Glucose was the initial carbon substrate and was being first consumed as a sole carbon source until around 10 hours of fermentation. As the black dots show in figure 5, glucose was added twice along the cultivation to support both cell growth and polymer accumulation, and was totally limited after 35.5 hours. Thus, both carbon substrates were being consumed by the strain between 10 and 35.5 hours of fermentation. Hereafter, glycerol was the only remaining carbon source in the fermentation, which was added until it was finished. Its additions are indicted with the dark grey triangle in figure 5. The biopolymer accumulated throughout the last 22 hours of fermentation by *Burkholderia sacchari* DSM 17165 was synthesised only from the glycerol source.

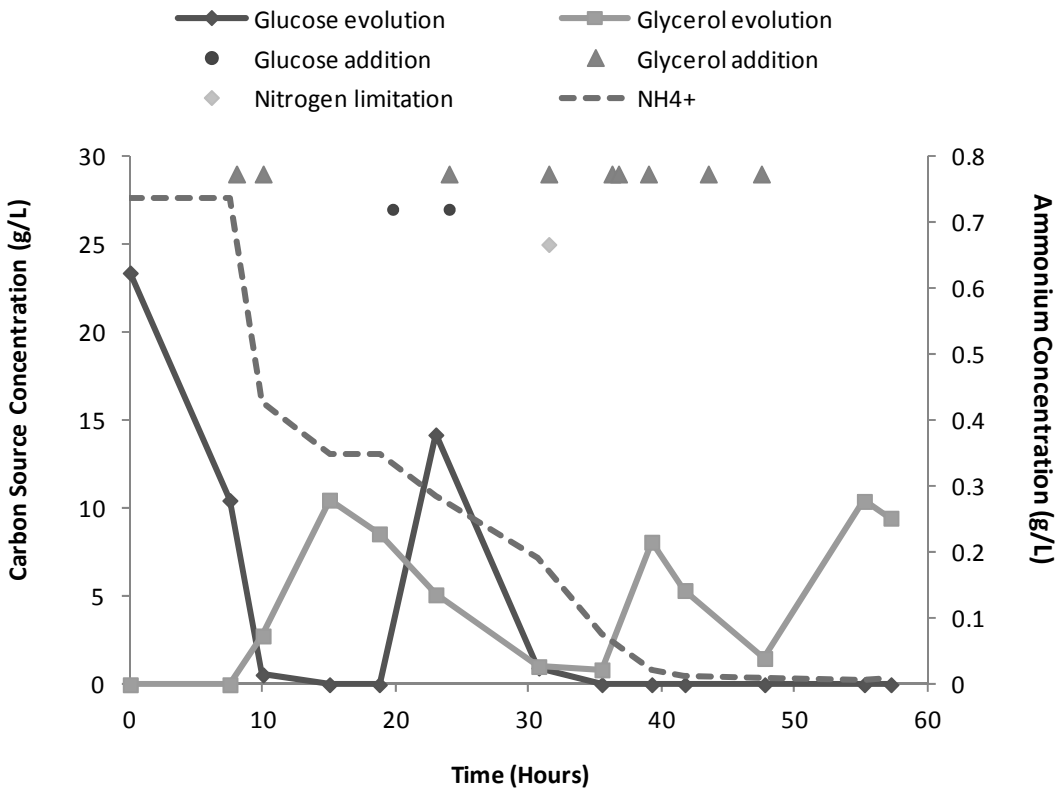

Figure 5. Evolution of the carbon source (glucose and glycerol) and nitrogen concentration in the media during the fermentation. Black dots: re-feeding time with glucose (50% w/v). Dark grey triangle: re-feeding time with glycerol. Grey rhombus: stop of the nitrogen source supply

Taking into account the amount of substrates consumed by the strain in the cultivation, the total consumption of carbon source was 1.63 g/L·h. This value is shared between glucose (1.02 g/L·h) and glycerol (0.62 g/L·h). The consumption of glycerol by *Burkholderia sacchari* DSM 17165 was constant from the first addition. It increased in the last part of the fermentation, since it was the only carbon source utilized by the strain from 35.5 hours until the end (0.66 g/L·h). The conversion to CDM and to PHB from 35.5 to 57.25 hours was 0.63 and 0.094 g/g respectively (table 3).

There are only a few studies where the bacterial strain *Burkholderia sacchari* is used for the production of PHAs (**Brämer 2001, Silva 2004, Rocha 2008, Pradella 2010**). One of the first fermentations with this strain was carried out by Silva (**2004**). It used xylose plus glucose under phosphate limitation, reaching a PHB-content of 62%, and conversion yield of 0.22 g/g. This strain showed better results with phosphate limitation. Recently, Pradella (**2010**) carried out cultivation with sucrose as a carbon source for PHB production by *Burkholderia sacchari* IPT 189 *via* nitrogen limitation to induce the polymer accumulation. Up to 42% of polymer content, a biomass concentration of around 150 g/L·h, a productivity of 1.7 g/L·h, and a conversion yield from sucrose of 0.22 g/g were achieved.

Table 3. Main parameters of the fed-batch fermentation with *Burkholderia sacchari* DSM 17165

Parameter	Value
Final CDM [g/L]	43.79
Final PHB [g/L]	4.48
Maximal content of PHB in biomass [%]	10.22
Volumetric productivity PHB [g/L·h]	0.08
Specific maximum growth rate, μmax [h-1]	0.42
Total consumption of carbon source [g/L·h]	1.63
Consumption of glucose [g/L·h]	1.02
Consumption of glycerol [g/L·h]	0.62
Consumption of glycerol from 35,5 to 57.25 hours [g/L·h]	0.66
Yield $_{(PHB/glycerol)}$ from 35,5 to 57.25 hours [g/g]	0.094
Yield $_{(PHB/carbon\ source)}$ [g/g]	0.045
Yield $_{(CDM/glycerol)}$ from 35,5 to 57.25 hours [g/g]	0.63
Yield $_{(CDM/carbon\ source)}$ [g/g]	0.41

Since this is the first attempt at cultivating this strain with glycerol for the production of PHA, it is not possible to find similar studies to compare it to. The only similar work was carried out with *Burkholderia cepacia* and glycerol as a carbon substrate by Zhu (**2010**), obtaining a 5.8 g/L of CDM at the end of the shake flask cultivation and 81.9% PHB of CDM using 3% of biodiesel-glycerol as feedstock over 96 hours of growth. When the glycerol percentage was increased the CDM decreased. Although the polymer content reached by *Burkholderia cepacia* is higher than the result obtained in the present work with *Burkholderia sacchari*, the fermentation lasted double the time and the strategy for glycerol addition was different (in the present work glycerol was being added at different fermentation times as shown in table 1).

The results obtained from each fermentation show that *Cupriavidus necator* DSM 545 has problems properly developng with glycerol as the only carbon substrate; therefore it was necessary to add glucose throughout the fermentation. On the other hand, *Burkholderia sacchari* DSM 17165 grow properly with glycerol as a sole carbon source. However, the entire polymer produced by *Cupriavidus necator* DSM 545 was converted from the combination of both glucose & glycerol, and by the polymer produced by *Burkholderia sacchari* DSM 17165 was accumulated from just glycerol.

Biopolymer Characterization

The biopolymers extracted from the lyophilized biomasses at the end of each incubation were used for the assessment of chemical structure by FTIR and ^{1}H NMR, determination of molecular weight by GPC and thermal analysis by DSC.

FTIR and ^{1}H NMR analyses showed typical bands and signals of PHB for both PHA extracted from *Cupriavidus necator* DSM 545 and *Burkholderia sacchari* DSM 17165 fermentations. FTIR transmission spectra of the PHAs extracted showed an intensive band corresponding to the stretching of the carbonyl group at 1726 cm^{-1}, two intense bands of the methyl and methylene groups at 2960-2850 cm^{-1}, a vibration of symmetric torsion of the methyl group at 1390-1370 cm^{-1}, a double band corresponding to the tension of anti-symmetric vibration of the ester group at 1230-1050 cm^{-1} (figure 6).

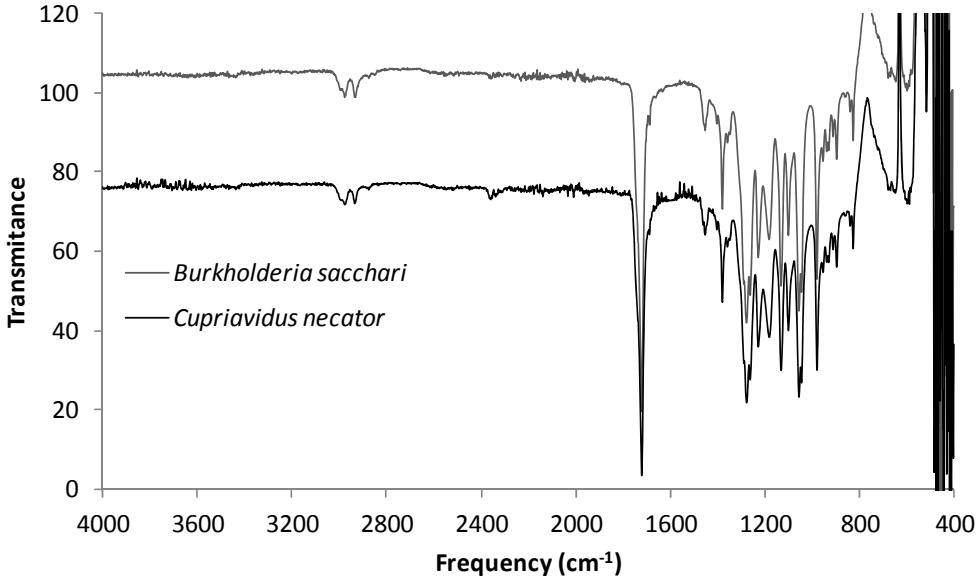

Figure 6. FTIR spectra of the PHB extracted from both bacteria

The characterization of the PHA samples by ^{1}H NMR clearly indicated the PHA structure as attributable to the homopolymer PHB (figure 7). ^{1}H NMR results showed spectra with the presence of three groups of signals characteristic of the PHB homopolymer. A doublet at 1.29 ppm which is attributed to the methyl group coupled to one proton, a doublet of quadruplet at 2.57 ppm which is attributed to the ethylene group adjacent to an asymmetric carbon atom bearing a single proton and a multiplet at 5.27 ppm characteristic of the methylene group. Another signal is also observed at 7.25 ppm which is due to the residual chloroform (**Lundgren 1965**, **Jan 1996**). Figure 7 shows the spectrum of the PHB extracted from *Cupriavidus necator* DSM 545 as an example. From the results, it can be concluded that the strains produced PHAs exclusively in the form of PHB.

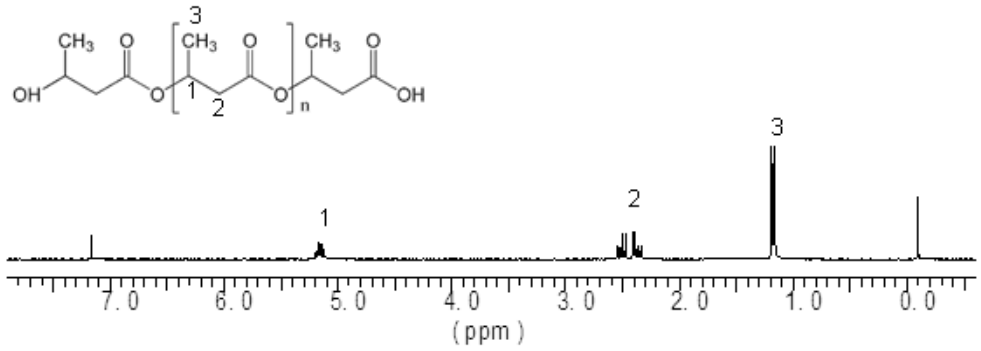

Figure 7. ^{1}H NMR spectrum from PHB extracted from *Cupriavidus necator* DSM 545

The GPC results indicated that the mass-average molecular mass (M_w) of the biopolyester extracted from *Cupriavidus necator* DSM 545 was 302,500±10,500 Da with a PDI of 4.72±0.12, and from *Burkholderia sacchari* DSM 17165 was 200,000±980 with a PDI of 2.50±0.43. In general, the molecular masses of PHAs produced from bacteria have a relatively high molecular mass around 20,000 to 3,000,000 Da, depending on the microorganism and the culture conditions (**Sudesh 2000**). In this work, the PHB produced by both bacteria in a medium containing glycerol showed a relatively low molecular mass. Such results have been previously reported when glycerol was used as a carbon source. Initially, the results reported by Madden (**1999**) showed that the molecular mass of the PHB produced by *R*alstonia *eutropha* NCIMB 40529 from glycerol was substantially lower than for polymer produced from glucose. Other strains also showed similar results on the molecular masses of the synthesized PHBs when glycerol was used as feedstock. Solaiman (**2005**) reported the decrease in the molecular masses of PHA from *Pseudomonas oleovorans* when glycerol was used as a carbon source. In direct comparison, Koller (**2005a**) reported that *Haloferax mediterranei* produced polyester with a mass average molecular mass of 250,000 Da from GLP (which contains 70% glycerol). Madden (**1999**) and Taidi (**1994**) concluded that glycerol acts as a chain transfer agent in the chain termination step of the polymerization and this effect is more pronounced with a high concentration of glycerol in the medium (more than 10 g/L). Moreover, the molecular mass of PHA is dependent on the producing bacteria (**Taidi 1994**). Also Koller (**2007**) concluded that when glycerol or glycols are present in the medium, these substances cause termination of chain propagation by covalent linking at the carboxyl terminus of the polyester ("end capping"). If GLP as well as pure glycerol is applied as raw material for PHA biosynthesis, it has to be considered whether the molecular masses are high enough for the application required. For certain special fields of application, low molecular mass PHA might be desired, for utilization as softeners or for the design of special polymers such as amphiphilic block copolymers (**Koller 2007**).

Zhu (**2010**) used *Burkholderia cepacia* in a study where the glycerol concentration was varied. The molecular mass of the polymer decreased when the glycerol amount was increased (3% to 9% of glycerol, Mw decreased gradually from 304,000 Da to 162,000 Da, respectively). In the present work,

glycerol was maintained in excess (10 g/L maxim glycerol concentration in the medium) and the molecular mass obtained is found within this range for *Burkholderia sacchari.*

Through the analysis of the second heating curve from DSC, the values of the T_m and X_c of the extracted PHBs were obtained and are shown in table 4. These thermal properties are similar to the common bacterial PHBs (**Sudesh 2000, Valappil 2007**). A regular X_c is found between 60-80%, and the T_m about 160-180ºC for PHB.

Normally, the reorganization of smaller chains into more compact structures, contributes to the increase of the X_c (**Luzuriaga 2006**). The biopolymer synthesized by *Cupriavidus necator* DSM 545 showed a crystallinity degree lower than the PHB from *Burkholderia sacchari* DSM 17165 which shows a lower molecular mass and lower chain lengths. On the other hand, the T_m for the PHB produced by *Burkholderia sacchari* DSM 17165 is higher than the T_m of the PHB produced by *Cupriavidus necator* DSM 545. This is due to the fact that different melting point values are related to polymer fractions (different chain lengths). It is well established in polymer chemistry that an end group acts as an impurity and lowers polymer melting points (**Lundgren 1965**).

Table 4. Thermal properties of the PHBs produced by *Cupriavidus necator* DSM 545 and *Burkholderia sacchari* DSM 17165

	T_m ºC	X_c%
Cupriavidus necator DSM 545	173.09	62.53
Burkholderia sacchari DSM 17165	163.32	72.82

6. Conclusions

Glycerol differently influenced the growth kinetics, the polymer production and the polymer molecular mass produced by both bacteria. The PHB obtained from *Cupriavidus necator* DSM 545, combining glucose and glycerol, resulted in a LMW-PHB. Although this result is inside the range of the molecular masses of common PHAs, this is the first time that a high cell dry mass was achieved with a high growth rate when glycerol is used in the fermentation. If the results are compared to previous studies using only glycerol, it seems that the combination of these two carbon substrates is potentially a great strategy to improve the growth and polymer production conditions of the strain.

This was the first fermentation carried out with *Burkholderia sacchari* DSM 17165, using glycerol as a sole carbon source in the accumulation phase for PHB production. Although no high polymer content was achieved, the strain develops perfectly with only the addition of glycerol as a carbon source. Moreover, it produced a lower molecular mass PHB compared to the polymer produced by *Cupriavidus necator* DSM 545.

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CHAPTER II. PHB TRANSFORMATION

Section 1: *Enzymatic degradation of P(3HB-co-4HB) by commercial Lipases*[1]

1. Abstract

Polyhydroxyalkanoates (PHAs) are biocompatible and biodegradable polymers utilized in a wide range of applications. Among these applications, low-molecular-mass (LMW) PHAs are currently being applied in different studies such as amphiphilic block copolymers synthesis. The main goal of this study is to obtain poly[3-hydroxybutyrate-co-4-hydroxybutyrate] (P(3HB-co-4HB)) oligomers - between 5000 to 1000 Da. A sustainable process is proposed for controlled depolymerisation by means of enzymatic degradation using commercial triglyceride lipases. Therefore, two different lipases were used for the degradation of commercial PHB composed of 8.4% 4HB fraction. Depolymerisation was carried out changing the exposure time and it was monitored together with the analyses of the products by means of gel performance chromatography (GPC), differential scanning calorimetry (DSC), Fourier transform infra red (FTIR), and nuclear magnetic resonance (NMR). It is suggested that the enzymatic degradation with lipases is a feasible process of controlled depolymerisation, which allows obtaining specific LMW-PHB oligomers.

2. Introduction

Polyhydroxyalkanoates (PHAs) are biopolyesters synthesized and accumulated by a large number of microorganisms as carbon and energy reserve. PHAs have been widely studied because they are a possible alternative to conventional polymers (oil-dependent polymers). They can be produced from renewable resources, which make their production independent of the oil industry. PHAs are biodegradable and biocompatible biopolyesters, with mechanical and thermal properties similar to some conventional polymers such as polypropylene [1, 2, 3]. Poly-3-hydroxybutyrate (PHB) is the simplest and most commonly known PHA. It constitutes a linear-unbranched homopolymer consisting of (R)-3-hydroxybutyric acid (3HB) units. Depending on the fermentation strategy and the producer microorganisms, different copolymers can be synthesised. Poly(3-hydroxybutyrate-co-4-hydroxybutyrate) (P(3HB-co-4HB)) copolymer is one of the most well known members of the PHAs family. PHB with high 4-hydroxybutyric acid (4HB) content is thermoplastic, elastomeric, and the elongation at break is much longer than some similar common plastics [4].

The molecular masses of PHA produced from bacteria have a relatively high molecular mass (Mw) around 200 to 3000 kDa [5, 6]. These high molecular masses are unsuitable for molecular design of special polymers such as amphiphilic block copolymers [7]. For such applications, more manageable molecular masses, around 1 to 5 kDa are required [6]. Methods such as acid [8] and base [9] hydrolysis as well as pyrolysis [10] are frequently used to depolymerize the natural PHA. The

[1] *It corresponds to the published article: Rodríguez-Contreras Alejandra, Calafell-Monfort Margarita, Marqués-Calvo María Soledad. Enzymatic degradation of P(3HB-co-4HB) by commercial Lipases. Polymer Degradation and Stability 97 (2012) 597-604.*

hydrolysis by base is known as saponification and Lemoigne was the pioneer [11] who degraded the obtained bacterial polymer to prove the PHB structure. The chemical hydrolysis with PHB in acidic and basic media conditions has been reviewed by Yu [12]. Also, there are many studies on the thermal degradation of bacterial PHB. It is already accepted that PHB and its copolymers degrade at high temperatures from 180 to 200 ºC. It is also known that random chain scission occurs when the temperatures are moderate (around the melting point). Yu [13] published a process to produce LMW-PHA by means of thermal degradation (patent US 7,361,725 B2). However, the major drawbacks associated with these degradation processes are ecological and economic. In addition if a degradation proceeds randomly, as in these cases, the polydispersity index (PDI) is relatively high [14].

PHAs enzymatic degradation studies have been basically focused on utilizing depolymerases. PHA is degraded by intracellular (i-PHA) and extracellular (e-PHA) depolymerases secreted from microorganisms. In PHA recycling systems, the discarded PHA materials are enzymatically degraded to oligomers or monomers by e-PHA depolymerases, and the resulting decomposed compounds are then utilized by the microorganisms [15]. The main applications of these enzymes have been focused on the degradation of PHA leading recyclable systems**.** Although a number of PHA-depolymerases from microorganisms have been purified and characterized [16], these enzymes are not commercially available yet.

Lipases (triacylglycerol hydrolases E.C. 3.1.1.3) are enzymes that catalyze the hydrolysis of glycerol fatty acid esters. They are water-soluble enzymes that are able to react with insoluble substrates, and to form enzyme-substrate complex (E-S) in the emulsion interface. This is an advantage in the case of water insoluble substrates but soluble in other organic immiscible in water solvents [17, 18]. Lipases are characterized by specificity and are classified by: regioselectivity, substrate or enantioselectivity. The regioselective lipases are positional specific and are subdivided into two groups [19]: nonspecific lipase, responsible for the random hydrolysis of all glyceride bonds formed between fatty acid and glyceride, and specific 1,3 lipases with positional specificity towards ester bonds in position 1,3 of the tryacylglycerol. Steric hindrance avoids the fatty acid in position 2 to enter in the active site. Lipases have also been studied for polyesters degradation [20, 21, 22]. However, no studies have been found using lipases for degradation of bacterial PHB or P(3HB-co-4HB) .

3. Objectives

In this study, the degradation of P(3HB-co-4HB) by means of lipases is proposed with the aim of obtaining LMW-PHB in a range of 5000-3000 Da. The proposed depolymerization is a sustainable process set out for an easy industrial scale application. Therefore, commercial PHB composed of both 3HB and 4HB fractions, was degraded with two different commercial regiospecific lipases, in order to study the possible enzymatic hydrolysis. The polymeric degradation was analyzed using DSC and GPC, while the mechanism of the enzymatic hydrolysis was analyzed with NMR and FTIR.

4. Material and methods

PHB and Lipases

Bacterial P(3HB-co-8.4%4HB) copolymer with molecular mass of 400 kDa was used in the study. Two triglyceride lipases were used for the biopolymer degradation. Lipase AK (LA) supplied by Amano (Amano Pharmaceutical, Tokyo, Japan), is extracted from a selected strain of *Pseudomonas fluorescens.* It has a lipolytic activity of around 20,000 U/g, a molecular mass of 64 kDa [23], and it is a non-regiospecific lipase**.** Lipopan Conc BG (LB), a concentrated formulation of Lipopan 50 BG produced by Novozymes (Denmark) from a genetic modified strain of *Aspergilus niger,* being the initial source of *Thermomices lanuginosus*, was also used. It is 1,3 regiospecific with a molecular mass of 39 kDa [24]. It has no standardized activity, although Lipopan 50 BG activity is 50,000 U/ [25].

Activity method

The hydrolytic activity of the enzyme was measured by titration of the released olive oil fatty acids substrate. The method was proposed by Amano: FIP (Fungi-Lipase International method) method for Lipase, pH value 7. The method is based on the hydrolysis of glycerol fatty acids esters by the enzyme, monitoring the NaOH consumption to neutralize the acid produced at pH 7 and 30 ºC. Gum Arabic was used as an emulsifier. The results were processed by applying a pseudo Michaelis-Menten [26] equation: the oleic acid concentration released after 10 min was related to the different enzyme concentrations.

Emulsion preparation

The preparation of the water suspension of PHB was carried out with a modified method from Horowitz [27]. A chloroform solution of PHB was emulsified with sodium deoxycholate (10 mM) using Ultra-Turrax (T 25 digital) at 15 000 rpm for 10-15 min. The organic solvent was removed from the emulsion by heating at 75ºC for 90 min.

Dynamic light scattering (DLS)

The polymer particle size and size distribution were determined by (DLS) using a nano-particle size analyzer DL-135 (Cordouan Technologies, France). Each analysis lasted for 200 s and was performed at 25 °C with an angle detection of 900.

Lipase-catalyzed degradation of P(3HB-co-4HB)

The polymer suspension was used in the heterogeneous enzymatic degradation, since the only solvent present in the solution was water. Each sample was placed in a vial filled with 6 ml of polymer suspension (17 mg P(3HB-co-4HB)//mL) with phosphate buffer (0.05 M), pH 7.4, containing between 25-35 mg of enzyme. Then they were incubated in a thermostatic oven at 37 ºC. Samples were removed from incubation at different periods of time (6, 24, 48, 72 hours), and the enzymes were denaturalized by heating at 100 ºC for 5-10 min. The samples were then centrifuged (Nahita 2615) for

10 min at 4000 rpm. The precipitate was washed with distilled water, re-suspended and centrifuged again. The polymer free of enzyme was then frozen and lyophilized. The pellet was re-suspended in chloroform and centrifuged. The chloroform was finally removed with the rotary evaporator and the dried polymer, free of chloroform and water soluble impurities, was analyzed. The blank was done following the same procedure but without enzyme.

Polymer degradation studies

Directly after enzymatic degradation, the P(3HB-co-4HB) was analyzed under GPC, which provided the relative molecular mass and the molecular mass distribution of the samples. GPC measurements were performed at room temperature using the Agilent 1200 system and Agilent ChemStation software (Agilent technologies, USA) to keep and reproduce the results. An Agilent 1200 refractive index detector was used at working temperature of 35 ºC, with a temperature controller and wolfram lamp. Chloroform was used as eluent at a flow rate of 0.80 mL/min with a stabilisation pressure of 35 bars and a sample concentration of 1.5 mg/mL. A Waters Styragel HT column (Waters Corporation, Milford, USA) was used and different molecular masses polystyrene as standard.

Thermal analyses were carried out with DSC analyses. Experiments were performed on a Perkin-Elmer Pyris 1 DSC (Perkin-Elmer, USA) with a dry nitrogen gas flow of 50 mL/min. The apparatus was calibrated using Indium of high purity. Approximately 5 mg of the sample was sealed in an aluminium planchet and analyzed. The melting temperature (T_m), melting enthalpy (ΔH_m) and the glass transition temperature (T_g) were determined from the second heating run of DSC endothermic peaks. The crystallinity degree (X_c) of P(3HB-co-4HB) was calculated assuming that the ΔH_m value of 100% crystalline PHB is 146 J/g [28]. Scans started at 30 ºC and were ramped at 10 ºC/min to 230 ºC.

The chemical structure was characterised with Perkin Elmer Fourier transform infrared (FTIR) (Perkin-Elmer, USA) using optical Perkin Elmer software. The line-scan spectra were based on 32 scans and a resolution of 4 cm^{-1}.

^{1}H and ^{13}C NMR spectra were recorded at 25 °C on a Bruker AM300 spectrometer (Bruker, Germany). The polymer samples were dissolved in chloroform and a drop of tetra methyl silane was added as reference. 10 mg of sample dissolved in 1 mL of deutered solvent were used. Proton spectra were recorded at 300.1 MHz with a spectrum of 32 K data points. A total of 64 scans were utilized with a relaxation delay of 1 second.

5. Results

Enzymatic activity

One unit of enzyme activity is defined as that amount of lipase in mg which liberates 1 µmol of fatty acid from olive oil per minute under the described assay conditions. Lipase-catalyzed hydrolysis of

olive oil followed pseudo Michaelis-Menten kinetics. Results of the enzymes activity are shown in table 1. The results found for both lipases agree with the activities reported by the suppliers, being LB more active than LA.

Table1. Enzyme activity: the parameters of the Michaelis-Menten kinetic equation were determined by the reciprocal plot of the reaction rate vs. enzyme concentration [37]

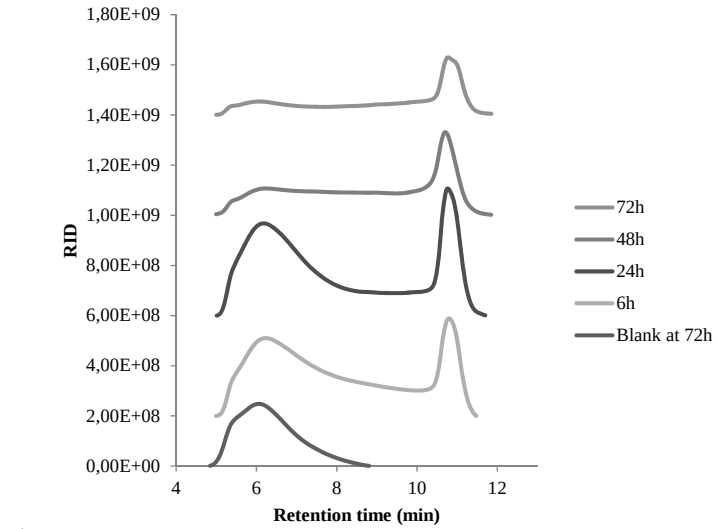
Enzyme	Michaelis-Menten kinetic parameters		U/mg	V_{max}/K_m (min^{-1})
	V_{max} (mM min^{-1})	K_m (mM)		
LA	2.29	0.033	30.60	70
LB	5.23	0.0031	50.97	1700

Dynamic light scattering

The DLS measurements show polymer particles around 0.2 μm with a polydispersity of 0.95.

Polymer degradation studies

The GPC analyses revealed a correlation between the enzymatic reaction time and the molecular mass of degraded P(3HB-co-4HB). The GPC results for LA are visible in figure 1B. The initial polymer sample shows a peak at a retention time of 6 min, corresponding to a molecular mass of 300 kDa with a PDI_{6min} of 1.80. After 6 hours of incubation, a small peak appeared at a retention time of 11 min. It represents a molecular mass of 5000 Da and it has a PDI_{11min} of 1.45. The initial peak at 6 min is maintained in all samples, except in the last one, where the peak at the higher retention time predominates. Figure 1A shows the evolution of P(3HB-co-4HB) degradation with LB. The decrease in the molecular mass follows a similar behavior, comparing with that for LA. However, the high-retention-time peak (11 min) for the 6-hour-sample is higher with LB. Also, this peak shows less polydispersity (PDI_{11min} of 1.15). The initial peak at a retention time of 6 min, is present in all samples except in the P(3HB-co-4HB) degraded during 72 hours. Moreover, this sample shows a shoulder on the peak at a retention time of 11 min, which corresponds to a lower molecular mass polymer.



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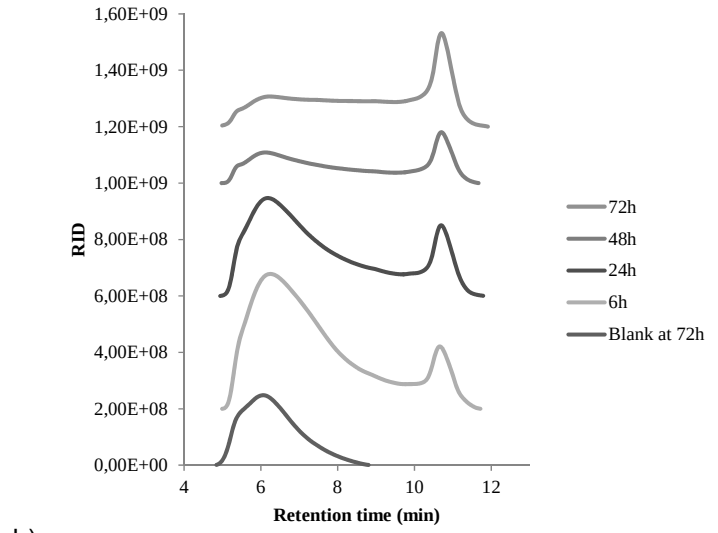
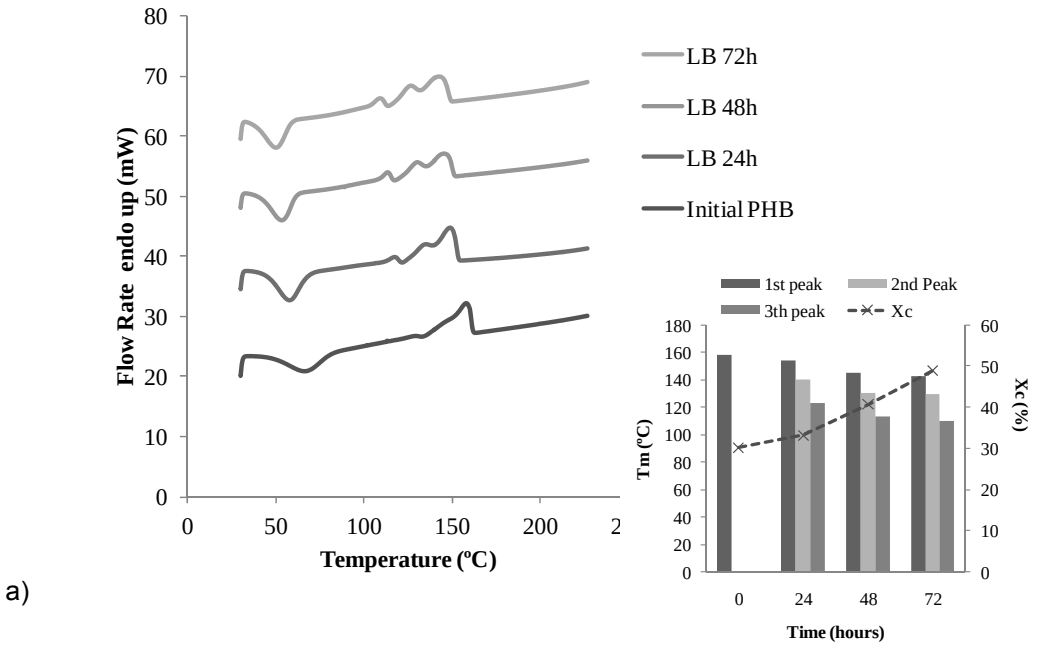


b)

Figure 1. GPC results of LB (a) and LA (b)

The results of DSC analyses are shown in figure 2. There is a relationship between the polymer degradation and the T_m and X_c. The initial T_m is 158 ºC and this value decreases with hydrolysis time with both lipases. The X_c increases 38% and 40% for LA and LB respectively.



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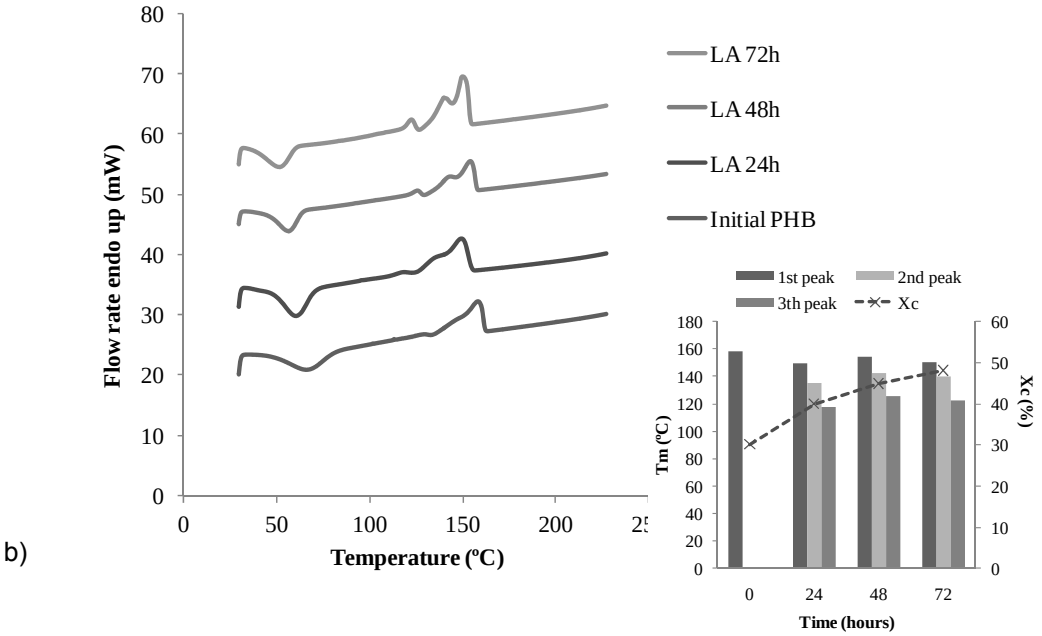
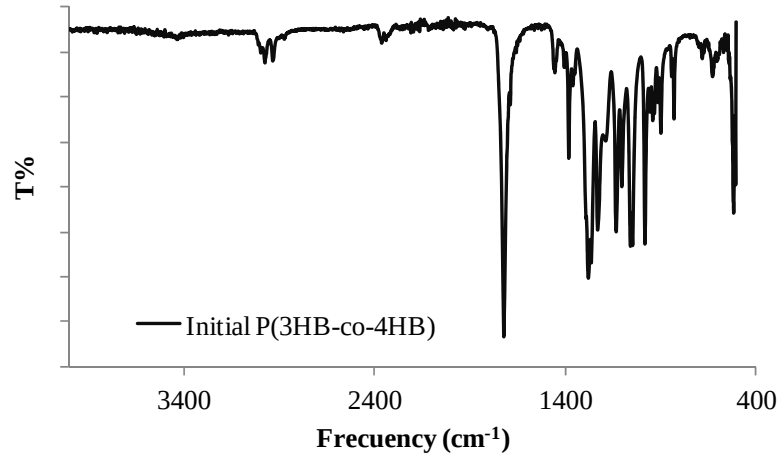
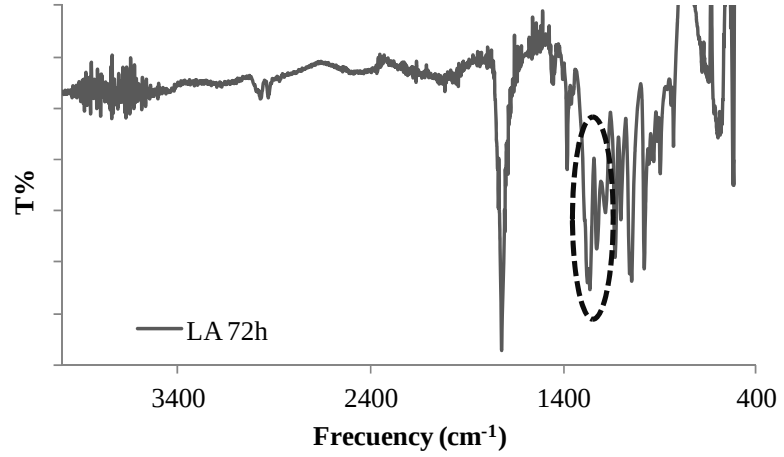


b)

Figure 2. DSC thermograms (second heating), melting temperature (T_m), and crystallinity degree (X_c) of P(3HB-co-4HB) samples degraded with LB (a) and LA (b) at different periods of time. The 0 sample correspond to Blank at 72h



a)



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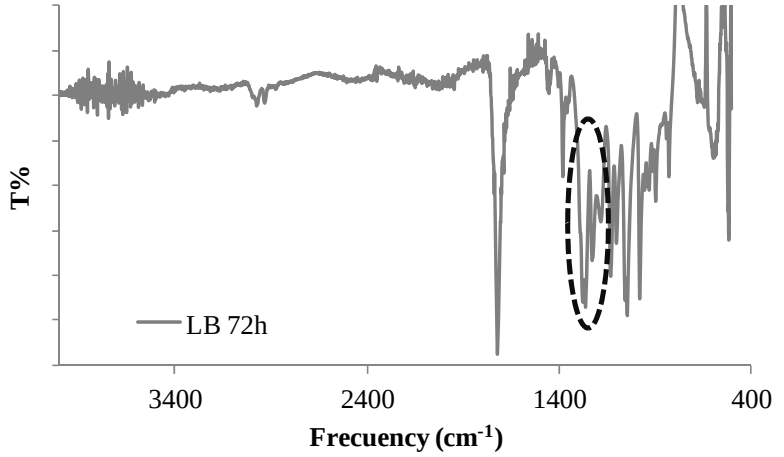
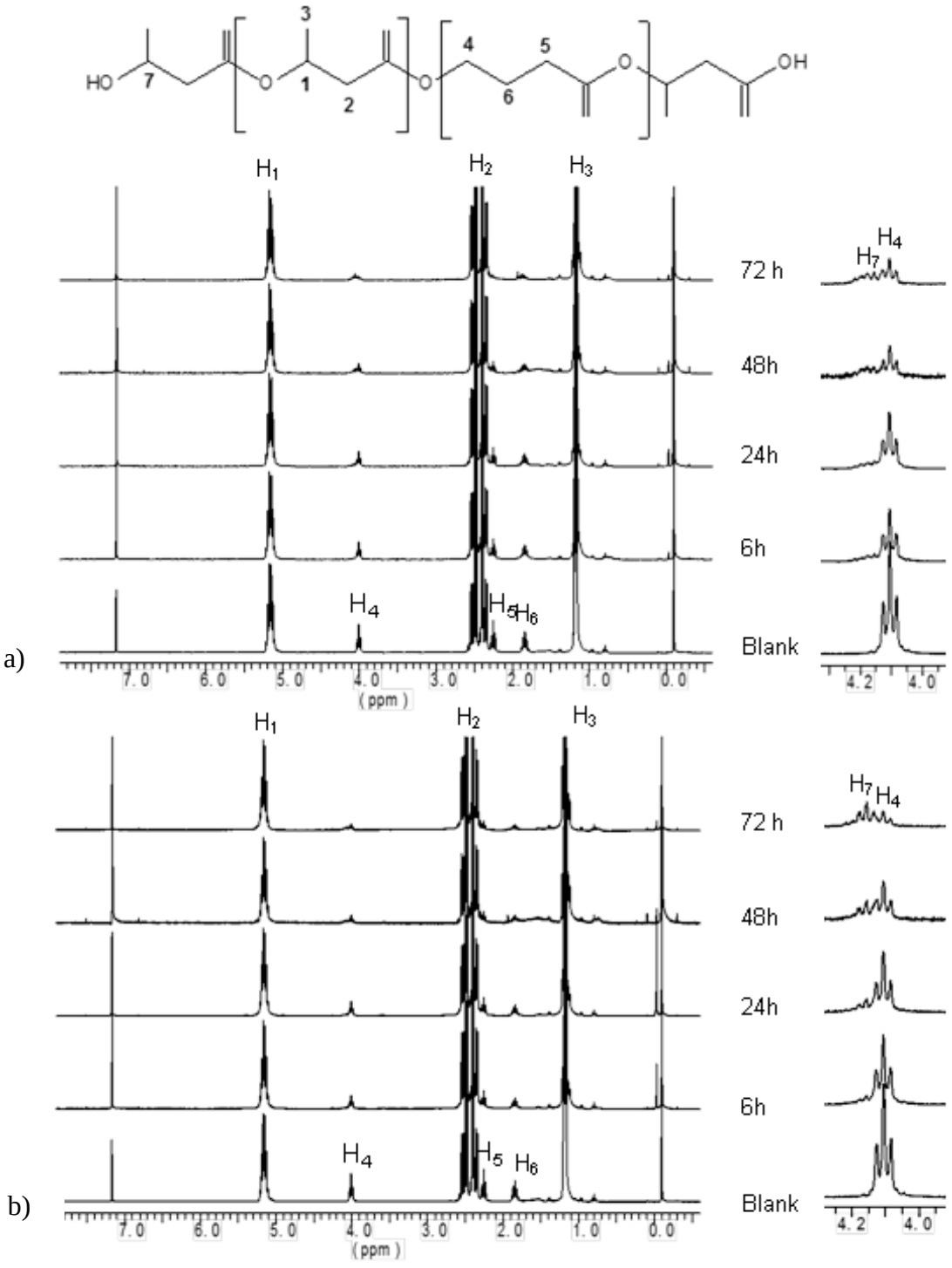


c)

Figure 3. FTIR spectra of P(3HB-co-4HB) (a) after 72 hours of enzymatic reaction with LB (b) and LA (c). The dotted circle shows the variation in the bands at 1230, 1278, 1186 cm^{-1}, as a result of the enzymatic hydrolysis

FTIR spectra does not show significant differences between the P(3HB-co-4HB) degraded samples with in both enzymes. All samples show the main PHB chemical groups corresponding peaks: bands around 1726 cm^{-1} belong to carbonyl group, near 2925 cm^{-1} represent methylene C-H vibrations and bands in between 1150 cm^{-1} and 1270 cm^{-1} stand for ester C=O vibrations. However, samples taken at longer time of enzymatic hydrolysis show an intensity variation of the bands at 1230, 1278, 1186 cm^{-1}. The band intensity increases at 1278 cm^{-1} CH_2 wagging of the crystalline phase respectively. The bands at 1230 and 1186 cm^{-1} are assigned to the chain conformation and the stretching vibration of C-O-C respectively. Figure 3 shows the polymer spectra after 72 hours of enzymatic hydrolysis with both lipases comparing with the initial P(3HB-co-4HB).

The ^{1}H and ^{13}C NMR spectra for the initial material are shown in the figure 4. The resonances of corresponding carbons mainly presented the chemical shifts for PHB at 19.7 ppm for the methyl carbon (figure 4 signal C_8), 40.7 ppm for the methylene carbon (figure 4 signal C_6), 67.6 ppm for the methyne carbon (figure 4 signal C_7), and 169.2 ppm for the carbonyl carbon (figure 4 signal C_5). It also presented the lower peaks corresponding to the 4HB fraction at 172.75 ppm for the carbonyl group (figure 4 signal C_1), 64 ppm (figure 4 signal C_4), 24 (figure 4 signal C_3), and 31 ppm (figure 4 signal C_2) for the methylene carbons. Similar results are shown in ^{1}H NMR spectra. All samples presented the three characteristics groups of signals for PHB: a doublet at 1.29 ppm (figure 4 signal H_3), a doublet of quadruplet at 2.57 ppm (figure 4 signal H_2), and a multiplet at 5.27 ppm (figure 4 signal H_1). All spectra also show signals of lower intensity corresponding to 4HB fraction: a triplet at 4.10 ppm (figure 4 signal H_4), and two multiplets at 2.30 and 2.00 ppm (figure 4 signals H_5 and H_6). The monomer 4HB represents an 8.4 % of the polymer, calculated in both fractions through their representative peaks areas: for 3HB signal at 5.27 ppm and for 4HB signal at 4.10 ppm. Regarding the degradation process, a decrease of the three signals corresponding to 4HB fraction with the reaction time of both enzymes is observed.


HO
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a)
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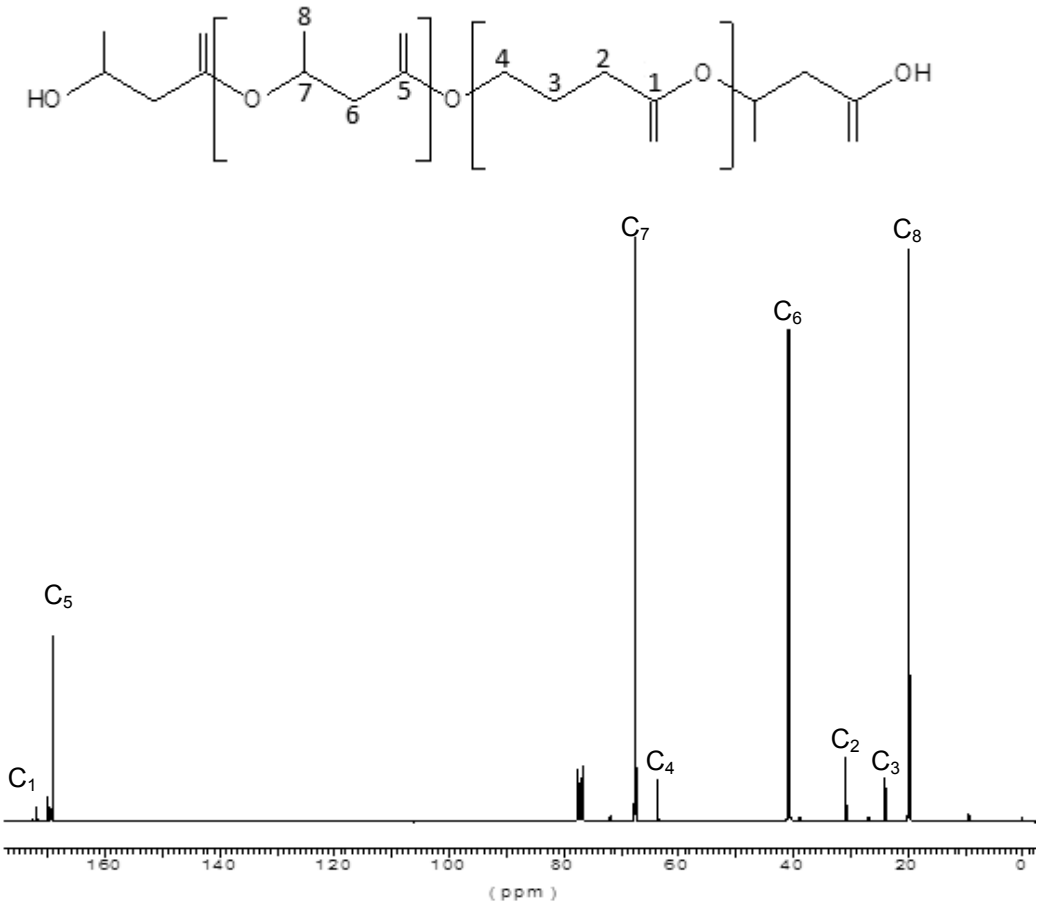

c)

Figure 4. P(3HB-co-4HB) chemical structure. ^{1}H NMR spectra of P(3HB-co-4HB) samples degraded with LA (a) and LB (b). ^{13}C NMR spectra of initial P(3HB-co-4HB) (c)

6. Discussion

Lipase kinetics restricted to an interface cannot be described by the classic Michaelis-Menten model, in which the enzyme and substrate are considered to be soluble in the same solvent. Therefore a simple model is applied here which describes lipase activity at an interface. Consisting of two successive equilibriums, the first one describes the physical adsorption of the enzyme at the interface, and the second one describes the formation of the E-S complex. The latter equilibrium may be described by a pseudo Michaelis–Menten mechanism occurring on an interface, rather than in a bulk level [26**,** 17]. The main parameters that describe the enzymatic reaction are: turnover number (k_{cat}) (specific rate of the enzymatic reaction) and Michaelis-Menten constant (K_m) (inverse of the affinity of the enzyme for the substrate). The studied enzymatic reactions are heterogeneous, consequently the k_{cat} cannot be determined by $V_{max}= k_{cat}\ [E]_0$ (the initial enzyme concentration is not the concentration of the E-S complex). Therefore, the specificity of the enzyme is defined in this work by V_{max}/K_m [29]. The kinetic results (table 1) showed that LB has more specificity for the olive oil than LA. However, this specificity can be just for guidance in the case of P(3HB-co-4HB), since the

substrate is not a triacylglyceride, but an unbranched polyester. Although the P(3HB-co-4HB) specificity of the enzymes is not known, it is possible to anticipate that LB would be more specific for P(3HB-co-4HB) than LA, taking into account the degradation results of this study (GPC and DSC).

The lipases enzymatic degradation produced a decrease in the molecular mass of the bacterial P(3HB-co-4HB) as a result of the enzymatic hydrolysis. The GPC analyses of the P(3HB-co-4HB) particles degraded by both lipases show two main peaks (figure 1). It is possible that the enzyme first hydrolyzes the polymer molecules of the particle surface. Therefore the peak at longer retention time corresponds to LMW-PHB which was hydrolyzed with the enzymes on the particle surface forming the E-S complex. The remaining polymer, unattainable for the enzyme, was not hydrolyzed. This HMW-PHB is shown in the GPC analyses with the peak at less retention time. Also, it can be observed for the enzymatic hydrolysis of P(3HB-co-4HB) with LB, that the peak related to the initial molecular mass almost totally disappears after 72 hours of enzymatic hydrolysis, while the same sample with LA still shows this peak. Moreover, the shoulder observed in the 72-hours-degraded sample with LB shows that the enzyme is starting to further degrade the lower molecular mass polymer (or oligomers). Regarding the PDI, the P(3HB-co-4HB) degraded by means of LB show less disperse mass distribution than the polymer samples degraded with LA. This is an indication of the specificity of LB (1,3 regiospecific). The enzyme hydrolyzes the polymer in specific regions of the molecule. The molecular masses after enzymatic hydrolysis are more similar and therefore a finer GPC peak is obtained at longer retention time with LB. All this explains the possible higher specificity that LB shows for P(3HB-co-4HB) than LA.

Through DSC analyses the P(3HB-co-4HB) degradation is observed as a decrease on the T_m and an increase of the X_c (Figure 2). The significant T_m decrement is due to lower P(3HB-co-4HB) molecular mass, as a direct consequence of the chain scission reactions occurring in the enzymatic hydrolysis. The different melting point peaks from DSC results can be a consequence of the different chain lengths. Thus, there is an increase of the lower T_m peaks with the degradation time due to the increase of the lower molecular masses. Moreover the reorganization of smaller chains, resulting from PHB scission, into more compact structures, contributes to the increase in the X_c [30]. If both enzymes are compared, the main difference in the DSC results can be observed for the Xc. In the case of LB the Xc increase with time as a result of the increase of the LMW-PHB. Especially the polymer sample hydrolyzed for 72 hours with LB shows the higher crystallinity. Also the GPC results show the higher decrease of the molecular mass from this sample. This matches the higher enzyme activity of LB compared with LA.

All infrared spectra show the main peaks of the P(3HB) [28, 31] since this is the main fraction of the copolymer (91.6% 3HB). The FTIR analyses show a variation on the shape and intensity of the bands at 1186, 1230 and 1278 cm^{-1}. It is known that these changes are often associated to the variation of the polymer crystallinity degree [28, 32]. The increment with the reaction time of the intensity at 1278 (CH_2 wagging) of the crystalline phase [32] concurs with the DSC analyses previously discussed.

The NMR results give information about the changes on the polymer molecular structure, as a consequence of the P(3HB-co-4HB) hydrolysis by both used lipases The spectra of the degraded samples show the main peaks of the PHB [33], matching with FTIR results. They also show a decrease in the intensity of the 4HB-unit peaks. It was found in the literature some studies reported that lipases hydrolyze the ester bonds of polyesters but cannot hydrolyze PHB [34]. However, the results of the present study demonstrate that both lipases used hydrolysed the polymer through the 3HB fraction, as well. The increase with the reaction time on the intensity of the peak H_7 (figure 4), which corresponds to the methylene group of the 3HB fraction at the end of the chain, indicates that both lipases hydrolyze P(3HB-co-4HB) through 3HB fraction. LB which is a 1,3 regiospecific Lipase, mainly hydrolyzes primary alcohols as the 4HB fraction is. Therefore, it can be observed in the ^{1}H NMR results that the 4HB peak (figure 4B, H_4) after 72 h reacting with LB is lower than the same peak with LA, which is non-regiospecific. Non-regiospecific lipase can also hydrolyse the secondary alcohol and therefore LA hydrolyzes preferably the ester bond of the 3HB fraction. On the other hand, the triplet corresponding to H_7 (figure 4) shows a higher increase after 72 hours with LB than with LA. This can be explained because of the higher activity and regiospecificity of LB in front of LA. These results correlated with GPC and DSC because P(3HB-co-4HB) degradation with LB was more effective than with LA.

Altogether lipases present some advantages to be used in the degradation of PHAs, among the other processes commented on before. On one hand, the use of enzymes instead of chemicals as in acid/basic degradation, or high temperatures in thermal degradation, is a more sustainable process [35]. On the other hand, lipases can be *a priori* a suitable alternative for PHA degradation instead of depolymerases. First of all, because lipases have an analogous aminoacid structure in the catalytic domain as PHA-depolymerase (lipase box) [16, 36] and, second, because lipases are enzymes that are extensively studied and have an extensive commercial availability**.** Also when compared with PHA-depolymerases, another advantage is that lipases are active in their monomeric form. They do not need a quaternary structure as depolymerase does. Thus, lipase can easily surround the polymer particle to saturate the substrate in the catalytic action interface [37]**.** In the light of these facts together with the results from the work at hand, the sustainable P(3HB-co-4HB) degradation is proposed in order to obtain LMW polymer as a replacement for conventional methods.

7. Conclusion

In this study it was possible to degrade HMW-PHB composed of 3HB and 4HB fractions into LMW-PHB by means of two lipolytic enzymes. LB showed higher activity than LA and better specificity for P(3HB-co-4HB). Even though the enzymes have different regiospecificity, both lipases hydrolyzed the backbone chain without distinguishing the ester bonds from primary (4HB) or secondary (3HB) alcohols. The main factor in the polymer degradation is the enzyme activity.

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Section 2: *Enzymatic degradation of poly(3-hydroxybutyrate) by a commercial lipase* [1]

1. Abstract

Polyesters such as poly(3-hydroxybutyrate) (PHB) have attracted commercial and academic interest as new biotechnological materials. In previous studies it was confirmed that two commercial lipases hydrolyzed the ester bonds from the 3HB fractions of P(3HB-co-4HB) copolymer. In this study, one of the previously used commercial lipases has been used for the enzymatic degradation of PHB homopolymer obtained *via* fermentation with *Cupriavidus necator* in order to obtain low molecular mass polymer. The results confirmed the enzymatic reaction of the used lipase with this PHB and show a controlled decrease of the molecular mass from 300 kDa to 4000 Da.

2. Introduction

A wide range of microorganism like bacteria can produce intra- and extra-cellular biopolymers, which fulfill diverse functions and ecosystem services [1]. It is well established that Polyhydroxyalkanoates (PHA) are synthesized by bacteria and some archaea as intracellular storage compounds to serve as carbon and energy sources under unfavorable conditions. Poly[(R)-3-hydroxybutyrate] (PHB) is the most frequently occurring and commonly known PHA. It constitutes a linear, unbranched homopolymer consisting of (R)-3-hydroxybutyric acid (HB) units. It is very promising as a biodegradable plastic. It has many potential applications in medicine, veterinary practice and agriculture due to its biocompatibility and biodegradability [2, 3]. The most extensively studied strain for PHA production on an industrial scale is *Cupriavidus necator*, because of its versatility in the accumulation of P(3HB) and its copolymers [4].

Relatively low-molecular mass (LMW) PHB has been used for the preparation of amphiphilic block copolymers because high molecular masses are unsuitable for molecular design of special polymers. Amphiphilic block copolymers have attracted special attention in both fundamental and applied research because of their unique chain architecture and physical properties [5]. For instance, the preparation of biodegradable nanoparticles based on amphiphilic triblock copolymer combining LMW-PHB with poly(ethylene glycol) or poly(oxyethylene) [6, 7] or grafting PHB oligomers onto chitosan for the preparation of the amphiphilic block copolymers [7] have been reported using PHB oligomers. Since the molecular masses (Mw) of PHA produced from bacteria are relatively high -around 200000 - 3000000 Da [8, 9] - it is important to have methods to depolymerase this natural PHA in order to obtain LMW chains. Methods such as acid [10] and alkaline [11] hydrolysis as well as pyrolysis [12] are frequently used. However a more sustainable process is desired. Enzymatic degradation of poly(3-hydroxybutirate-co-4-hydroxybutirate) (P(3HB-co-4HB)) by commercial lipases was previously

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studied [13] and the results showed that the used lipases hydrolyzed the backbone chain without distinguishing between the ester bonds from 3HB and 4HB fractions.

3. Objectives

The degradation of PHB by means of a commercial lipase is proposed in this study, with the aim of obtaining LMW-PHB in a range of 4000-3000 Da.

4. Material and methods

PHB

The PHB used in this study was extracted from a bacterial fermentation with *Cupriavidus necator* strain DSM 545 cultivated on glycerol as the sole carbon source during the cultivation process. The results of the biopolymer characterization are shown together here with the characterization of the samples of the enzymatic degradation study.

Lipase

Triglyceride lipase was used for PHB degradation. Lipopan Conc BG, a concentrated formulation of Lipopan 50 BG commercialized by Novozymes (Denmark) from a genetic modified strain of *Aspergilus niger,* being the initial source of *Thermomices lanuginosus*, was used. It is 1,3 regiospecific with a molecular mass of 39000 Da [14]. It has no standardized activity, although Lipopan 50 BG activity is 50000 U/g [15].

Lipase-catalyzed degradation of PHB

Initially, a polymer suspension was prepared following a modified method from Horowitz [13, 16]. The suspended spherical polymer particles showed a diameter average of 0.2 μm with a polydispersity of 0.95 [13]. This polymer suspension was used in the heterogeneous enzymatic degradation, water being the only solvent present in the solution. Each sample was placed in a vial filled with 6 ml of polymer suspension (17 mg PHB/mL) with a phosphate buffer (0.05 M), pH 7.4, and containing 35 mg of enzyme. The vials were then incubated in a thermostatic oven at 37 ºC. Samples were removed from incubation at different periods of time (6, 24, 48, 72 h). The enzymatic reaction was stopped by denaturalizing the enzyme, heating the vials at 100 ºC between 5 and 10 min. The samples were then centrifuged (Nahita 2615) for 10 min at 4000 rpm. The precipitate was washed with distilled water, re-suspended and centrifuged again. The polymer free of enzyme was then frozen and lyophilized. The pellet was washed with chloroform and centrifuged. Finally, the chloroform was removed with the rotary evaporator and the dried polymer, free of chloroform and water soluble impurities, was analyzed. The blank was done following the same procedure but without enzyme.

Characterization

Directly after enzymatic degradation, the PHB was analyzed under GPC, which provided the relative molecular mass and the molecular mass distribution of the samples. GPC measurements were performed at room temperature using the Agilent 1200 system and Agilent ChemStation software (Agilent Technologies, USA) to keep and reproduce the results. An Agilent 1200 refractor index detector was used at a working temperature of 35 ºC, with a temperature controller and a wolfram lamp. Chloroform was used as an eluent at a flow rate of 0.80 mL/min with a stabilization pressure of 35 bars and a sample concentration of 1.5 mg/mL. A Waters Styragel HT column (Waters Corporation, Milford, USA) was used together with different molecular masses of polystyrene as standard.

Thermal analyses were carried out with DSC analyses. Experiments were performed on a Perkin-Elmer Pyris 1 DSC (Perkin-Elmer, USA) with a dry nitrogen gas flow of 50 mL/min. The apparatus was calibrated using Indium of high purity. Approximately 5 mg of the sample was sealed in an aluminum planchet and analyzed. The melting temperature (T_m), melting enthalpy (ΔH_m) and the glass transition temperature (T_g) were determined by the second heating run of DSC endothermic peaks. The crystallinity degree (X_c) of PHB was calculated assuming that the ΔH_m value of 100% crystalline PHB is 146 J/g [17]. Scans started at 30 ºC and were ramped at 10 ºC/min to 230 ºC.

^{1}H NMR spectra were recorded at 25 °C on a Bruker AM300 spectrometer (Bruker, Germany). The polymer samples were dissolved in chloroform and a drop of tetra methyl silane was added as reference. 10 mg of sample dissolved in 1 mL of deutered chloroform were used. Proton spectra were recorded at 300.1 MHz with a spectrum of 32 K data points. A total of 64 scans were utilized with a relaxation delay of 1 s.

5. Results

The PHB used in this study shows a molecular mass of 313000 Da with a polydispersity index (PDI) of 4.15. The analyses of the blank sample of the enzymatic degradation show very similar results from GPC analyses (Mw of 292000 with PDI of 4.73), indicating that the enzymatic degradation only took place when the enzyme was present in the reaction. The GPC analyses of the degraded samples show a decrease in the molecular mass with the enzymatic reaction time. Figure 1 shows the evolution of the PHB degradation with the enzyme. The initial polymer sample shows a wide peak at a retention time of around 6 min, corresponding to a molecular mass of around 300000 Da. After 6 h of incubation, a small peak appeared at a retention time of 11 min. It corresponds to a molecular mass of around 4000 Da. Although the initial peak at 6 min is present in all samples, its intensity decreases with the enzymatic reaction time. The peak at a higher retention time (11 min) increases with the reaction time and shows less polydispersity than the initial peak. The PHB degraded for the

duration of 72 h (Mw of 3700 with PDI of 1.52) shows a small shoulder on the 11-min-retention-time peak which corresponds to lower molecular mass.

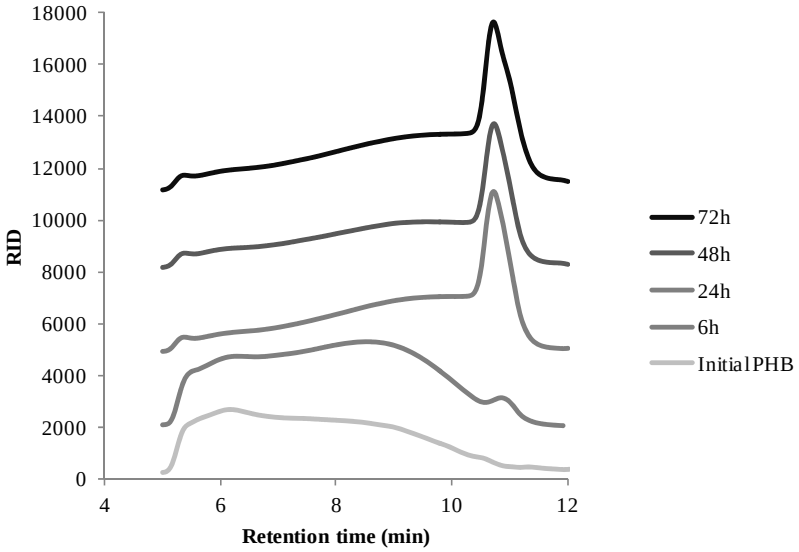

Figure 1. GPC results of PHB degraded with Lipopan at different periods of time

The results of DSC analyses are shown in Table 1. There is a decrease in the T_m of the samples degraded with the enzyme that increases with the reaction time. The X_c values are also shown in the table. Although these values were expected to increase with the reaction time, the polymer samples degraded for 48 and 72 h show a decrease.

Table 1. DSC values (second heating). Melting temperature (T_m) and crystallinity degree (X_c) of PHB samples degraded with Lipopan at different periods of time

Sample	X_c (%)	T_m (ºC)
Initial PHB	62.53	173.093
PHB after 6 h with Lipase	64.21	169.790
PHB after 24 h with Lipase	62.79	162.792
PHB after 48 h with Lipase	59.95	160.295
PHB after 72 h with Lipase	56.51	160.296

Figure 2 shows the ^{1}H NMR spectra of the biopolyester degraded by the enzyme at different periods of time. The results show that all samples presented the three main groups of signals for PHB: a doublet at 1.29 ppm (signal H_3), a doublet of quadruplet at 2.57 ppm (signal H_2), and a multiplet at 5.27 ppm (signal H_1). Regarding the degradation process, an increase of the signal corresponding to

the proton at the end of the chain (signal H_4) is observed. The number-average molecular mass (M_n) of the PHB samples from the enzymatic degradation was also calculated by means of ^1H NMR spectra. The peak corresponding to the end-group protons (methylene group) together with the peaks of the monomer protons, were accurately integrated. Once the ratio of protons on the end-groups to protons on the polymer chain is determined, the monomer molecular mass (86 g/mol) was applied to generate the Mn value. The molecular masses of the sample calculated in this way showed a clear decrease with the degradation time. The PHB sample that was exposed longer to the enzyme (72h) showed a Mn of 3998 Da.

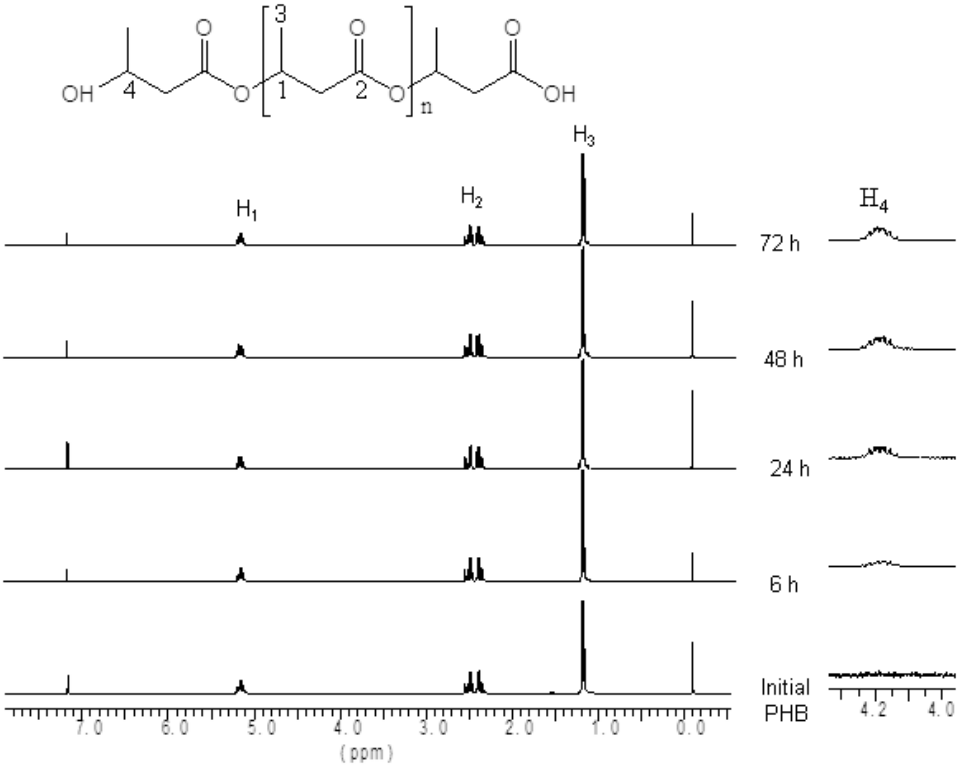

Figure 2. PHB chemical structure. ^1H NMR spectra of PHB samples degraded with Lipopan

H_2

6. Discussion

In the literature some studies reported that lipases hydrolyze the ester bonds of polyesters but cannot hydrolyze PHB [18]. However, in our previous studies it was confirmed that some commercial lipases can react with the 3HB fraction of the copolymer P(3HB-co-4HB) [13]. Lipopan was chosen because it is highly active (50000 U/g) and it showed more specificity for PHB.

Wild type bacteria typically produce PHB with molecular masses between 10000-8000000 Da, with a PDI about 2 [8]. The PHB used in this study and produced by *C. necator* shows a molecular mass inside this typical range; however the polydispersity is a little higher. This value can still be considered

common taking into account the strain and the type of fermentation [19]. As expected, the biopolyester suffered a decrease in the molecular mass as a result of the chain scission when it was degraded *via* lipase enzymatic reaction. The two main peaks showed by GPC analyses (figure 1) can be related with the particle spherical shape. It is possible that the enzyme hydrolyzed the PHB molecules of the surface of the polymer particles first. Therefore the peak at longer retention time corresponds to LMW-PHB which was hydrolyzed with the enzymes on the particle surface. The remaining polymer was unattainable for the enzyme and consequently not hydrolyzed. In the GPC analyses, this non-hydrolyzed PHB is shown by means of the peak at less retention time (6 min). Between 6 and 24 h of enzymatic reaction, the GPC analyses of these samples show the main peak of the initial molecular mass. The peak corresponding to the lower molecular mass considerably increases for the polymer samples degraded more than 24h. This slow degradation during the first 24 hours can indicate a resistance of the particle surface to react with the enzyme. Once the surface is degraded, the enzyme could easily reach the remaining inner polymer and degrade it almost immediately. On the other hand, the shoulder observed in the 72-hours-degraded sample shows that the enzyme was starting to further degrade the lower molecular mass polymer (or oligomers). It is also important to note that the low PDI of the GPC peak of the degraded PHB indicates the specificity of the enzyme for the biopolymer.

The used PHB showed thermal properties similar to the common bacterial PHAs. A common X_c is found between 60-80 %, and the T_m about 160-180 ºC [8, 20]. Through DSC analyses the PHB degradation is observed as a decrease on the T_m (Table 1). The significant T_m decrease indicated a lower PHB molecular mass that confirms the efficiency chain scission reactions occurring in the enzymatic hydrolysis. On the other hand, the reorganization of smaller chains, resulting from chain scission, into more compact structures, contributes to the increase in the X_c [21]. However the crystallinity did not show an important increase in the polymer samples with lower molecular masses in this case. This can be explained because some crystallization difficulties can be encountered by crystallizing LMW-oligomers.

The ^{1}H NMR results give information about the changes on the polymer molecular structure, as a consequence of the PHB hydrolysis by the enzyme. The spectra of the degraded samples show the main peaks of the PHB [22]. The increase with the reaction time on the intensity of the peak, which corresponds to the proton of the methylene group at the end of the chain, indicates that the polymer was hydrolysed by the enzyme, reinforcing the GPC and DSC results commented on before. The Mn values of the PHB samples calculated according to Yu [23] decreased during the assay, matching with the results from GPC experiments. Moreover, the value of the Mn of 72-hours-degraded sample coincides with Mn from the GPC analyses (4000 Da).

All these results with PHB homopolymer obtained *via* fermentation with *C. necator* match with the results of our previous studies, where the enzymatic degradation with Lipopan was carried out with a PHB copolymer [13].

7. Conclusion

The results of the present study together with previous one [16] demonstrate that the triglyceride commercial lipase, Lipopan Conc. BG with hydrolysis activity in primary ester bonds of triglycerides is able to decrease the homopolymer PHB molecular mass from *Cupriavidus necator*. The specificity that the enzyme presents for the biopolymer was also demonstrated in this study.

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CHAPTER III. APPLICATION

Section 1: *Methods for the preparation of doxycycline-loaded PHB micro- and nano-spheres* [1]

1. Abstract

Natural and synthetic biodegradable polymers have been investigated for controlled drug release. Poly(3-hydroxybutyrate) can be produced by bacteria and is remarkable for this application due to its excellent biocompatibility and biodegradability. The objectives of this work were to study and compare different drug-entrapment and emulsification methods for obtaining doxycycline-loaded PHB micro- and nano-spheres, and to select the more effective process. The micro-/nano-particles were prepared by polymer precipitation *via* dialysis, simple emulsion (O/W) or multiple emulsion (W1/O/W2) applying solvent evaporation in the last two cases. This was carried out either by ultrasonication, dripping and/or high speed stirring. Different processing conditions were varied in order to evaluate their influence on morphology, size, and drug entrapment capabilities. The highest drug load was obtained by single emulsion with high speed stirring. In the case of multiple emulsion, the combination of ultrasound with high speed stirring resulted in the most elevated process yield and drug loading capability.

2. Introduction

Poly(3-hydroxybutyrate) (PHB) is a biopolyester from the family of polyhydroxyalkanoates which is produced by microorganisms from removable resources (**Xiong 2010**). Among natural and synthetic biodegradable polymers, PHB is found to be remarkable for its applications in drug delivery due to its excellent biocompatibility and degradability (**Tian 2008**). The biopolymer production *via* microbial fermentation lowers the presence of toxic products from the synthetic polymerization process (**Korsatko 1984**), while the hydrolytic degradation of PHB leads to the obtainment of the monomer D-3-hydroxybutyric acid. This acid is a normal constituent of blood and is one of the three ketone bodies which are produced endogenously by the process known as ketogenesis (**Pouton 1996**). Other advantages of PHB, when compared with chemically produced polymers such as polyglycolic acid (PGA), polylactic acid (PLA), and poly(lactide-co-glycolide) (PLGA), which are mostly well known as biologically degradable drug carriers with good retarding characteristics, are their excellent biocompatibility, and their propensity to biodegradation under different environmental conditions (**Chen 2002, Errico 2009**). In addition, the controllable retarding properties of drug delivery systems based on PHB can be modulated by variations in processing and molecular weight of the polymer and copolymer composition (**Kim 2000, Errico 2009**).

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[1] *It corresponds to the article send for publication in the European Polymer Journal: Methods for the obtaining of doxycycline-loaded PHB micro- and nano-spheres by A. Rodríguez-Contreras, C. Canal, M. Calafell-Monfort, G. Julio-Moran, M. Pau Ginebra-Molins, M.S. Marqués-Calvo.*

There are very few papers reporting on the incorporation and subsequent release of therapeutic agents with PHB systems. **Mora-Huertas** (**2010**) reported a summary of the methods for the formation of polymeric capsules formation for drug delivery, and PHB is restricted to a few of them. The use of PHB in drug delivery systems (DDS) has been reported in recent years, and emulsion followed by solvent evaporation has been the most frequently used microencapsulation technique (**Mora-Huertas 2010, Tiwari 2011**). Although some studies report low entrapment efficiency (EE) (**Baran 2002**), double emulsion (water-in-oil-in-water, $W_1/O/W_2$) with solvent evaporation is an advantageous technique because it provides higher protection, allowing for the substance to be encapsulated. Nevertheless, it is possible to increase the encapsulation capability of the system by modifying the double emulsion preparation conditions (**Freytag 2000, Kalaji 2009, Ayoub 2011, Ahmed 2012**). Also, **Errico** (**2009**) recently reported a new technique for PHB drug-loaded capsules *via* dialysis. It is the aim of this work to evaluate the suitability and compare the efficiency of these three techniques in order to produce antibiotic-loaded PHB capsules.

Doxycycline (DOXY) is a well-known broad-spectrum antibiotic, which is effective against both Gram-positive and Gram-negative bacteria, protozoa, and various anaerobes. As a tetracycline analogue, it can work as a bacteriostatic which is capable of inhibiting the bacterial protein synthesis at the ribosomal sites. DOXY presents a long half-life, high lipid solubility, and very good oral absorption. After being introduced into clinical practice in 1967, DOXY has been frequently used in treating destructive periodontal diseases such as juvenile periodontitis and acute periodontal abscesses. It has been used against periodontal infection and for enhancing bone regeneration after periodontal diseases. Additionally, it has also been used to prevent bacterial infection related to septic arthritis. However, there are some concerns over possible side effects such as gastro-intestinal disturbance, esophageal erosion, and photosensitivity when administrated orally. In order to reach the infection deep inside affected tissues with an effective drug concentration and to circumvent the systemic side effects, controlled local delivery of DOXY is desirable (**Feng 2010**). A possible route to achieve it is by encapsulating or entrapping DOXY within a biodegradable matrix such as PHB and obtaining appropriate particle sizes or drug loads.

3. Objectives

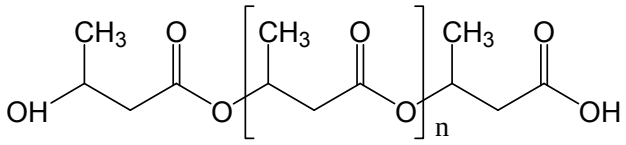
In this study, different drug-entrapment and emulsification methods were studied in order to obtain optimal DOXY-loaded PHB micro- and nano-spheres. The methods compared were: a) Polymer precipitation *via* dialysis; b) simple emulsion (O/W); or c) multiple emulsion ($W_1/O/W_2$), applying solvent evaporation in the last two cases. This was carried out either by ultrasonication, dripping and/or high speed stirring, and their combination. Different processing conditions were also varied in order to evaluate their influence on morphology, size, and drug entrapment capabilities (drug loading, encapsulation efficiency and method efficiency).

4. Material and methods

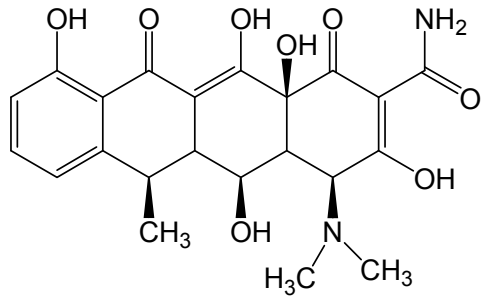
PHA and reagents

PHB from microbial fermentation with *Cupriavidus necator* and glycerol as a carbon source (Mw 302,500±10,500 Da with a polydisersity index of 4.72±0.12) was purchased from the Institute of Biotechnology and Biochemical Engineering (Graz University of Technology) (**CHAPTER I, section 4**). The antibiotic used was Doxycycline hyclate (DOXY) (Sigma-Aldrich) (Doxycycline hydrochloride hemiethanolate hemihydrate, $C_{22}H_{24}N_2O_8 \cdot HCl \cdot 0.5H_2O \cdot 0.5C_2H_6O$, M_W 1025.89 Da), which is freely soluble in water (1:1-10 w/w) (**Parfitt 1999**). Figure 1 shows the chemical structure of both, biopolymer (figure 1a) and antibiotic (figure 1b). Chemical products such as dimethyl sulfoxide (DMSO), trifluoroethanol 99% (TFE) and dichloromethane (DCM) were purchased from Sigma Chemical Co. and used without further purification. The gelling agent was bovine gelatin (Gel) and the surfactants used were Pluronic-F127, poly(vinyl alcohol) (PVA) and sodium taurocholate (TAU) (Sigma Chemical Co.). The dialysis bags used were porous cellulose membranes with a molecular weight cut off of 12,000.

a)



b)



* HCl
* 1/2 H_2O
* 1/2 CH_3CH_2OH

Figure 1. Chemical structure of PHB and DOXY.

PHB-based particles

Different methods already reported were applied in this study with variations to prepare DOXY-containing micro- and nano-particles: a) precipitation *via* dialysis; b) O/W or c) $W_1/O/W_2$ emulsions with solvent evaporation. Three different routes were used to obtain the emulsions, namely ultrasound, dripping, and high speed stirring. Data relevant to individual experiments are summarized in table 1, whereas the general procedures are described in detail in the following paragraphs.

PHB-particle production by precipitation *via* dialysis

The method followed was previously reported by **Errico** (**2009**). For unloaded particles, 10 mg of PHB dissolved in 9 mL of TFE and 1 mL of DMSO was introduced in the dialysis membrane. To prepare DOXY-loaded particles, 10 mg of PHB dissolved in 9.6 mL of TFE with different amounts of Pluronic-F127 solution in TFE (10 mg/mL) and DOXY dissolved in different amounts of DMSO were used. The resulting solutions were stirred at room temperature for 20 minutes, then dialyzed against 1 L of distilled water for 3 hours and then the distilled water was replaced at intervals of 3-4 hours during 24 hours.

PHB-particle production by single and double emulsions

Dripping method

The dripping method was used to produce simple and double stable emulsions. The unloaded PHB particles were produced by simple emulsion by dripping a PHB solution in a 1% PVA aqueous solution (150 mL). The resultant emulsion was stirred at 900 rpm for 8 h at room temperature until complete solvent evaporation (**Chen 2002**).

Simple emulsion technique (O/W): the method used was based on that reported by **Chen and Davis** (**2002**) and was similar to the method used by **Mundargy (2007**). The simple emulsion was carried out by dissolving PHB in DCM (10 mL) (organic phase). DOXY was dispersed into the polymer solution. This dispersion was added drop-wise into an aqueous phase (150 mL) containing the surfactant. The resulting emulsion was stirred at 900 rpm for 8 h at room temperature until complete solvent evaporation.

Double emulsion technique ($W_1/O/W_2$): A primary emulsion (W_1/O) was prepared by mixing an aqueous solution of DOXY with the PHB solution in DCM by ultrasonic agitation for 2 min (Bandelin Sonopuls with microtip MS 73). This emulsion was dripped onto 150 mL of the second aqueous phase (W_2) which contained the surfactant, either PVA or TAU, and NaCl (4%). The resultant emulsion was stirred at 900 rpm for 8 h at room temperature until complete solvent evaporation (Chen and Davis, 2002).

Ultrasounds method

Unloaded particles of PHB were formed by means of one-step ultrasonication (Bandelin Sonopuls with microtip MS 73). PHB was dissolved in 1 mL DCM and was then emulsified into 10 mL of distilled water with 2% PVA and NaCl (4% w/w) under an ultrasonic homogenizer at 5W (3 min at 60%). The emulsion obtained was magnetically stirred at 700 rpm for 5 h until complete solvent evaporation. DOXY-containing PHB spheres were produced by double emulsion ($W_1/O/W_2$), adapting a sonication method reported by **Feng** (**2010**). The amount of DOXY was dissolved in 200 µL of distilled water and was equivalent to 30% (w/w) of the dry weight of PHB. This aqueous solution was emulsified with the organic phase which consisted of PHB dissolved in 1 mL DCM, by means of a probe ultrasonic homogenizer at 2W (2 min at 50%). This primary W_1/O emulsion was then emulsified into 10 mL of

distilled water with either PVA or TAU and NaCl (4% w/w) under ultrasonic homogenizer at 5W (3 min at 60%) to form the $W_1/O/W_2$ emulsion. The resulting secondary emulsion was magnetically stirred at 700 rpm for 5 h until complete solvent evaporation.

High speed stirring

Simple emulsion technique (O/W): Some modifications from **Mundargi** (**2007**) were adopted to formulate the DOXY-loaded PHB microspheres. In this method, DOXY equivalent to 30% (w/w) dry weight of the polymer was dispersed into 10 mL of the PHB solution in DCM. Then, the dispersion was slowly added into 100 mL of an aqueous solution containing either PVA or TAU, and 4% NaCl and emulsified using an Ultra-Turrax (IKA –Werke GmbH ¬Co. KG, Germany). Mechanical stirring was maintained at 12,000 rpm for 15 min to form the O/W emulsion. Further, solvent removal and hardening of the microspheres was achieved by continued stirring at 900 rpm for up to 2 h. A similar protocol was followed to produce the unloaded PHB particles.

Double emulsion technique ($W_1/O/W_2$): The combination of two methods was applied to produce the double emulsion. First, ultrasounds were applied for the initial emulsion (W_1/O), followed by high speed stirring to reach the second emulsion ($W_1/O/W_2$). The method reported by **Mundargi** (**2007**) was followed. DOXY equivalent to 30% (w/w) dry weight of PHB was dissolved in 2 mL of distilled water to form a DOXY aqueous solution. PHB was dissolved in 10 mL of DCM (oil phase). Both aqueous and organic solutions were emulsified using the probe ultrasonicator previously described. Different sonication times were tested (2, 3, and 5 min) to prepare different W_1/O emulsions. Then, this first emulsion was slowly added into 100 mL of an aqueous solution containing the surfactant and NaCl (4% w/v). Different concentrations of PVA and TAU were tested. The double emulsion was achieved by mechanical stirring using Ultra-turrax (IKA –Werke GmbH ¬Co. KG, Germany) at 12,000 rpm for 15 min. The solvent removal and hardening of the microspheres was attained by continued stirring at 900 rpm for up to 2 h.

In order to improve the drug loading capabilities of the micro-/nano-spheres, different variations and tests were carried out in the method. Table 1 shows all modified parameters: variation on the W_1 volume, addition of gelatine in either W_1 or W_2 or different initial DOXY contents.

Particle recovery

The micro- and nano-spheres obtained by the different processes were collected by centrifugation at 4,000 rpm for 15 min (Medifriger centrifuge, JP Selecta, Barcelona, Spain), separated and washed with distilled water. The resultant suspension was frozen and freeze-dried overnight (laboratory freeze-dryer Teslar Cryodos) to obtain a white powder. The product was stored, desiccated, and kept in the dark at 20°C until required (**Mora-Huertas 2010**). Method yield or efficiency (ME) was calculated according to the following equation: Method efficiency (%) = [(weight initial PHB- weight polymer debris)/weight initial PHB] x100. The suspended particles were sieved (100 µm) to separate

particles from the polymer debris, which did not have spherical shape. These were dried and accurately weighed to be considered for the equation.

Determination of the encapsulation efficiency (EE) and drug loading

The obtained particles (10 mg) were dissolved in 1 mL chloroform, and the DOXY within the micro-particles was extracted by adding 5 mL physiological phosphate buffer saline (PBS) solution (pH 7.4, 0.1 M). The solvent was removed by bubbling nitrogen gas. After adding 2 mL of PBS solution and vigorously mixing by vortexing, the polymeric debris was removed by filtration through a 0.45 mm filter (Millipore, USA). The resultant DOXY solution was analyzed with a UV/Vis spectrophotometer (Cary 100 Bio, with Cary Win UV software) at the λ_{max} of 275 nm. This extraction process was applied to a minimum of two sets of particles, with at least three batches of formulations being analyzed. The % of EE and the % of drug loading of the microspheres were calculated as follows (**Mundargi 2007, Sendil 1999**): Encapsulation Efficiency (%)= (weight of drug loaded/weight of drug initially added)x100 and Drug Loading (%) =(weight of drug in particles/weight of particles)x100.

Scanning electron microscope (SEM) and particle size analysis

Dried micro- and nano-particles were gold coated (coating thickness between 10-15 nm) under vacuum by means of a BAL-TEC (SCD 005, Germany) cool sputter coater and their scanning electron micrographs were obtained using a Jeol (JSM-5610, Japan) scanning electron microscope, operating at magnificatins from 500 to 20.000X. The median particle size was obtained after the evaluation of the microscopic images with a AnalySIS software tool (Soft Imaging System GmbH, Münster, Germany).

***In vitro* antibacterial assay**

A commonly used procedure for studying the antibacterial action of the particles was followed (**Madigan 2000, Nonsee 2011**). The assay was performed with both Gram-positive and Gram-negative bacteria (*Staphylococcus aureus* and *Escherichia coli*, respectively). The overnight incubated bacteria solutions were diluted with a sterile PBS solution until it reached an absorbance value of 0.2 at 600 nm (equivalent to 10^8 bacteria). The solutions were used to inoculate (100 µL) Petri plates with Chromocult® (Merck) media for *E.coli* and Triptoy Soy Agar (TSA) media for *S. aureus*. The micro- and nano-particles (6 mg) were placed on the surface of the inoculated medium by means of a mould to obtain the same contact area for all tested samples. The plates were incubated at 37 ºC for 24 hours, when the diameter of inhibition was measured. The controls were carried out with drug-free particles.

5. Results

In general, and for all tested processes, the results are quite different when unloaded particles are produced in comparison with the results of the drug loaded ones. In this case, a new component is added to the system and this is noticeable in the topography of the particles surface, their shape and size, as well as in the process yield. Table 1 and figures 2-4 show the influence of DOXY and of the different processes on the PHB-based particles obtained.

PHB-particle production by precipitation via dialysis

As shown in figure 2a, unloaded spheres between 400-200 nm were obtained with the dialysis method. When the active principle was introduced in the system, the spheres obtained showed higher diameter (around 1-2 µm) (figure 2b and 2c). The DMSO and Pluronic-F127amounts were varied to optimize the sphere formation. The highest loading, EE and ME were obtained for sample D3 (2.94, 5.90 and 30% respectively), which was carried out without surfactant and 300 µL DMSO. Sample D6 also shows an improvement on the ME (figure 2c), due to the presence of Pluronic-F127 but lower drug loading.

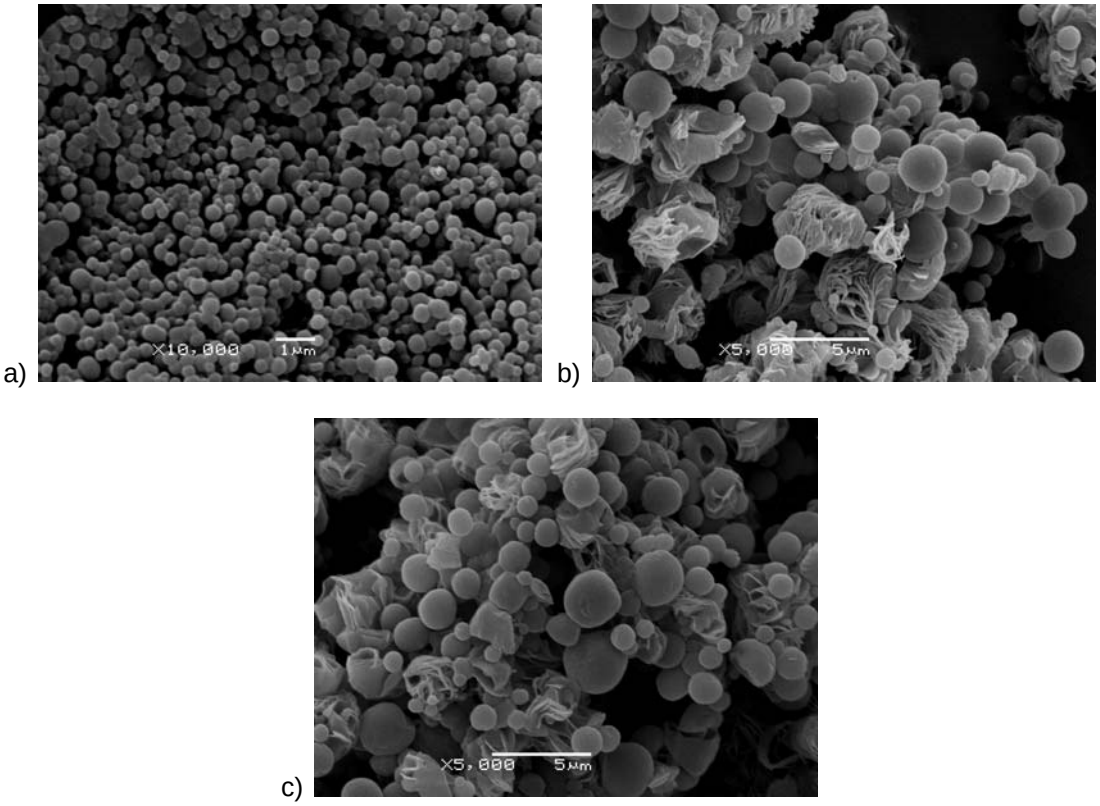


Figure 2. SEM micrographs of the samples obtained from the dialysis method: a) unloaded PHB particles (D1 sample), b) DOXY-loaded PHB particles prepared with 300 µL DMSO (Sample D3), c) DOXY-loaded PHB particles carried out with 200 µL Pluronic-F127 and 300 µL DMSO (Sample D6).

Table 1. PHB-based micro- and nano-particles formulations prepared by different methods. Drug loading, entrapment efficiency (EE), method efficiency (ME), and PHB-based particle sizes.

Method	Combined methods	Technique	Sample	PHB (mg)	DOXY (mg)	Other compounds	% Loading (w/w)	EE %	ME %	Diameter size (μm)
Dialysis	Dialysis	Control	D1	10	-	1000 μL DMSO	-	-	45	0.34±0.10
		Precipitation	D2	10	3	NO DMSO	1.12	1.29	20	1.32±0.68
			D3	10	3	300 μL DMSO	2.94	5.90	30	1.47±0.63
			D4	10	5	1000 μL DMSO (5mg DOXY/mL)	0.23	0.50	25	1.42±0.30
			D5	10	3	500 μL Pluronic-F127	0.31	1.28	23	1.55±0.63
			D6	10	3	200 μL Pluronic-F127 - 300 μL DMSO	0.85	3.12	31	2.12±0.87
			D7	10	3	300 μL Pluronic-F127 - 300 μL DMSO	0.75	2.52	26	1.54±0.68
Dripping	Dripping	Control	DR1	200	-	1% PVA	-	-	68	50.48±24.60
		O/W	DR2	200	20	1% PVA	0.08	0.42	32	64.08±36.14
			DR3	200	40	1% PVA	0.06	0.64	33	33.51±24.48
	Ultrasound+Dripping	$W_1/O/W_2$	US/DR4	300	90	1% PVA - 4% NaCl	1.34	4.00	65	33.62±23.49
			US/DR5	300	90	0.5% TAU - 4% NaCl	2.61	10.15	76	5.35±5.89
Ultrasound	Ultrasound	Control	US1	30	-	2% PVA - 4% NaCl	-	-	85	0.14±0.04
	Double Ultrasounds	$W_1/O/W_2$	US/US2	30	9	2% PVA - 4% NaCl	3.48	13.64	44	0.60±0.27
			US/US3	30	9	0.5% TAU - 4% NaCl	1.35	4.29	47	0.31±0.17
High speed stirring	High speed stirring	Control	TU1	300	-	0.5% PVA - 4% NaCl - 2 min US	-	-	57	32.82±9.52
		O/W	TU2	200	60	2% PVA - 4% NaCl	4.29	14.37	54	29.68±7.63
			TU3	200	60	0.5% TAU - 4% NaCl	4.83	16.18	47	0.43±0.18
	Ultrasound + High speed stirring	$W_1/O/W_2$	US/TU4	300	90	0.5% PVA - 4% NaCl - 2 min US	2.44	8.11	72	19.17±9.43
			US/TU5	300	90	0.5% PVA - 4% NaCl - 3 min US	2.24	7.38	77	19.17±9.43
			US/TU6	300	90	0.5% PVA - 4% NaCl - 5 min US	2.17	4.75	73	19.17±9.43
			US/TU7	300	90	1% PVA - 4% NaCl - 2 min US	2.13	7.05	73	24.03±7.38
			US/TU8	300	90	2% PVA - 4% NaCl - 2 min US	2.58	8.58	78	22.60±8.82
			US/TU9	300	90	3% PVA - 4% NaCl - 2 min US	2.41	8.00	69	22.84±9.43
			US/TU10	300	90	0.5% TAU - 4% NaCl - 2 min US	2.12	7.51	81	0.30±0.13
			US/TU11	300	90	1% TAU - 4% NaCl - 2 min US	1.17	3.92	72	0.35±0.17
			US/TU12	300	90	2% TAU - 4% NaCl - 2 min US	0.60	2.09	76	0.33±0.13
			US/TU13	300	90	2% PVA - 4% NaCl - W_1= 1mL H_2O	1.93	11.56	69	17.43±6.01
			US/TU14	300	90	0.5% TAU - 4% NaCl - W_1= 1mL H_2O	2.90	14.84	73	0.32±0.16
			US/TU15	300	90	2% PVA - 4% NaCl - 1% Gel* in W_2 - W_1= 1mL H_2O	2.23	7.54	87	8.65±2.34
			US/TU16	300	90	0,5% TAU - 4% NaCl - 1% Gel* in W_2 - W_1= 1mL H_2O	1.42	4.72	76	0.79±0.36
			US/TU17	300	90	2%PVA - 4% NaCl - 2% Gel* in W_1 - W_1= 1mL H_2O	3.32	11.36	89	10.95±3.65
			US/TU18	300	90	0,5%TAU- 4% NaCl - 2% Gel* in W_1 - W_1= 1mL H_2O	3.66	12.55	78	0.38±0.22
			US/TU19	300	45	2% PVA - 4% NaCl - 1% Gel* in W_2 - W_1= 0.5 mL H_2O	1.72	11.72	81	4.93±1.92
			US/TU20	300	45	0,5% TAU - 4% NaCl - 1% Gel*in W_2 - W_1= 0.5mL H_2O	1.84	11.54	84	0.43±0.24

*Gel: Gelatine

PHB-particle production by emulsion

Dripping method

The drug loading and EE obtained from PHB microspheres produced from a simple emulsion (O/W) with drug dispersion were very low. However, the drug load, EE and ME were improved with the double emulsion technique ($W_1/O/W_2$), particularly when a TAU emulsifier is used. It was observed by SEM that the particle size was smaller when this surfactant was used. Figure 3 shows the micrographs of the PHB-particles obtained by droplet addition method. When the particle surface was observed by SEM, it was confirmed that the surface of the control (figure 3a) and the simple emulsion samples (figure 3b), which do not have the inner aqueous phase, show less roughness on the surface than the particles obtained from double emulsion (figure 3c and 3d).

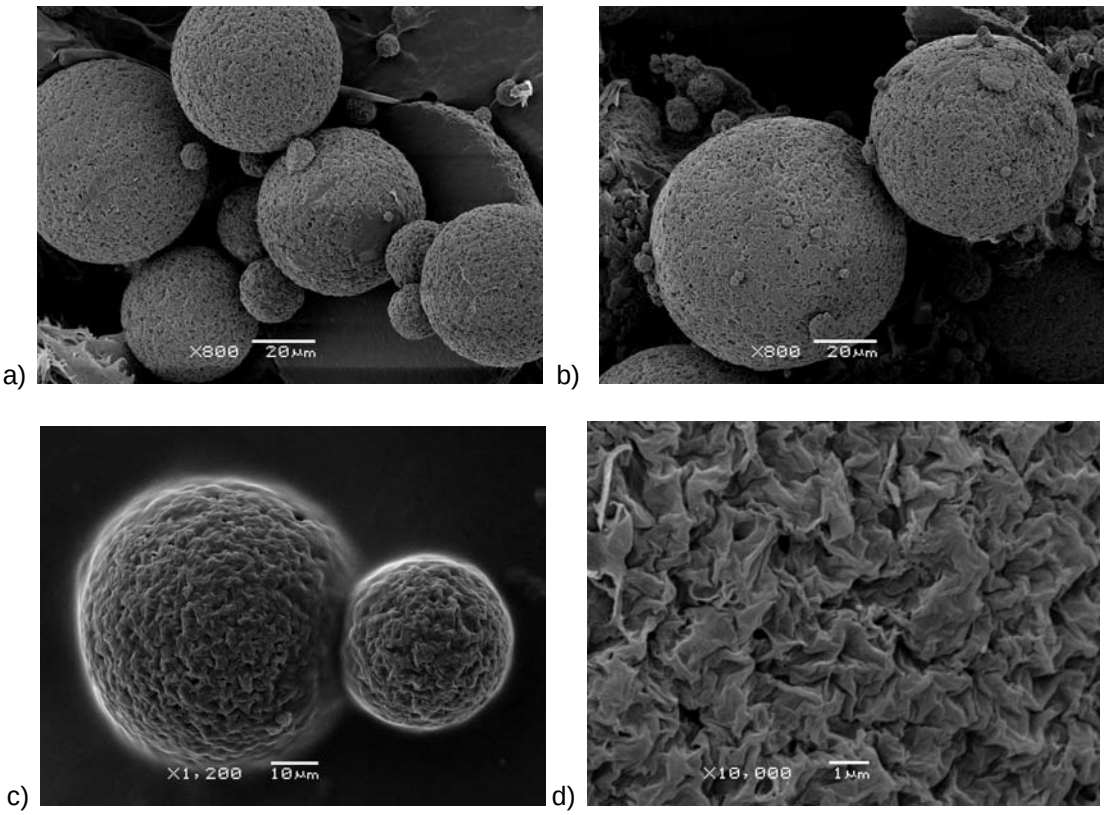
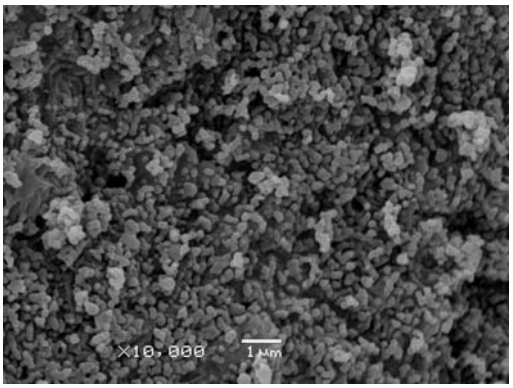


Figure 3. SEM micrographs of the samples obtained by using the dripping method: a) unloaded PHB particles (DR1sample), b) DOXY-loaded PHB particles from O/W carried out with 1% PVA (DR2 sample), c) DOXY-loaded PHB particles from $W_1/O/W_2$ carried out with 1% PVA (sample US/DR4), and d) US/DR4 Particle surface from

3.2.2. Ultrasound method

The particles obtained by double emulsion employing ultrasounds were in the nano-scale range (figure 4). In parallel with the previously described methods, spheres with no DOXY showed smaller diameters than those containing the active principle. The EE and loading obtained from drug encapsulation using PVA as surfactant in the W_2 are of 13.64% and 3.48% respectively. When this

emulsifier was used, particle size (around 600 nm) and entrapment capabilities were higher compared to those using TAU (particle size around 300 nm).

a) 

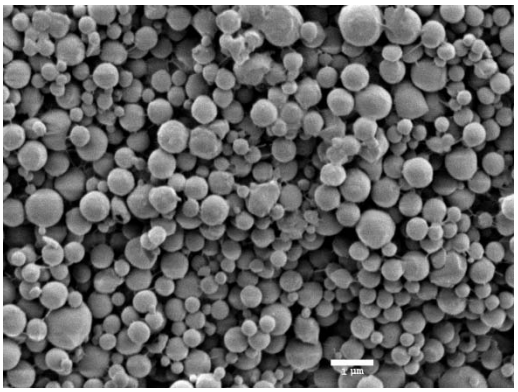
b) 

Figure 4. SEM micrographs of the samples obtained from the ultrasounds method: a) unloaded PHB particles (US1 sample), b) DOXY-loaded PHB particles carried out with PVA (sample US/US2).

High speed stirring

When DOXY was dispersed in the PHB solution to carry out the O/W emulsion *via* high speed stirring with an ultra-turrax, high drug loading and EE were obtained. However, the system precipitated, forming non-spherical particles when the solvent evaporated. Therefore the ME was low (54 and 47 % with PVA and TAU respectively). On the other hand, the double emulsion obtained by combining ultrasounds for the first emulsion (W_1/O) with high speed stirring by the ultra-turrax for the second emulsion ($W_1/O/W_2$), showed the best ME compared to the other methods tested in this work (between 70-80% in all formulations studied). The results showed that the optimal ultrasounds time to obtain a stable W_1 emulsion was 2 min. Best results of drug loading, EE and ME were obtained when the concentrations of PVA and TAU were 2 and 0.5%, respectively. During the study these initial results were improved when other parameters were varied. The volume of the first aqueous phase (W_1) was decreased (from 2 mL to 1 mL) and consequently the drug loading and the EE improved. Scanning electron micrographs of particle surfaces showed a more porous matrix structure for micro-particles with higher volume in the inner aqueous phase (figure 5a) than the ones with lower volume (figure 5c). The surface of nano-particles could not be observed under SEM because of the poor resolution of the micrographs. The EE was improved from 8.11 to 11.56% with PVA and from 7.51% to 14.84% with TAU, also increasing the loading, when the W_1 volume was decreased. The addition of gelatin to the system, in either W_1 or W_2 also improved the results in both cases, with the best ones obtained for gelatin present in W_1.

The particle size was in the nano-scale when TAU was used as an emulsifier. The particle size increased to the micro-scale with PVA. Figures 5a and 5b show the micro- and nano-particles respectively obtained with PVA and TAU as emulsifiers. Figures 5c and 5d show the particles obtained with 50% less volume in the aqueous phase (W_1). In this last case, slightly smoother particle

surface was observed, when comparing the morphology of particles in micrographs on figures 5a and 5c.

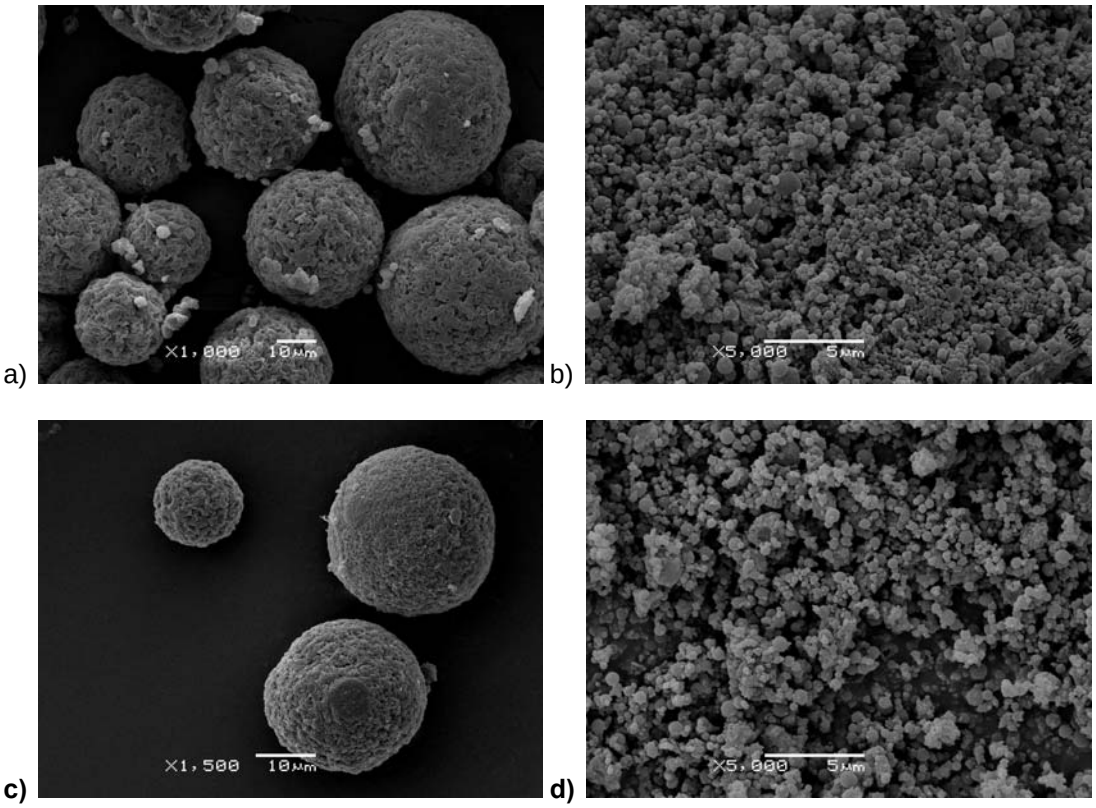


Figure 5. SEM micrographs of the samples obtained from high speed stirring method: a) DOXY-loaded PHB particles carried out with 2% PVA (US/TU8 sample), b) US/TU10 sample carried out with 0.5% TAU, c) US/TU13 samples carried out with 2% PVA and 50% less volume in W_1 (1 mL), d) US/TU14 sample carried out with 0.5% TAU and 50% less volume in W_1 (1 mL).

Table 2. Inhibition zone diameter of the DOXY-loaded PHB micro- and nano-particles.

Sample	Diameter of the inhibition zone (mm) for *Staphylococcus aureus*	Diameter of the inhibition zone (mm) for *Escherichia coli*
Control	0	0
TU2	21.00±2.8	19.00±2.8
TU3	21.50±2.1	19.50±2.1
USTU13	15.50±0.7	13.50±0.7
USTU14	14.75±0.3	13.75±0.3
USTU17	20.50±2.1	18.50±0.7
USTU18	21.50±2.1	20.50±2.1

In vitro antibacterial assay

Testing of the diffusive qualitative antibacterial activity (table 2) of the DOXY-loaded particles versus Gram-positive and Gram negative bacteria (*S. Aureus* and *E. coli*) revealed proper diffusion. Particles that showed higher drug load in the DOXY extraction test, showed slightly higher inhibition zone diameter. This was observed in all cases that the particles in contact with *S. aureus* showed higher diameter than the particles in contact with *E. coli*. Particles free of the drug showed no inhibition zone.

6. Discussion

To obtain DOXY-loaded micro- and nano-particles of PHB, different methodologies were studied and compared in this work. Polymer precipitation *via* dialysis, simple emulsion (O/W) and multiple emulsions ($W_1/O/W_2$) were the three applied techniques which lead to PHB micro- or nano-particle formation in all cases. In order to obtain stable emulsions, ultrasonication, dripping and high speed stirring by an ultra-turrax were used. Depending on the method used, the particle size, surface morphology, drug loading, EE, and ME could be adjusted.

PHB-particle production by precipitation *via* dialysis

The dialysis process has some requirements for the production of spherical particles: the solvent is limited to water-miscible ones, and both the polymer and the drug must be soluble in the same solvent (**Errico 2009**). A binary solvent mixture of TFE and DMSO (in different ratios) was chosen because it was able to dissolve both PHB and DOXY fulfilling the requirements of the technique**.** DMSO/TFE formed a more stable binary system than the mono-solvent system of TFE (**Errico 2009**), resulting in an improved drug entrapment. The DMSO amount was varied to improve drug entrapment, and consequently, the drug loading and the EE. In order to decrease the formation of flakes and precipitates, Pluronic-F127 was added at different concentrations, helping to stabilize the system and improving the ME. It is a nonionic surfactant that has been used in different applications to facilitate the solubilization of different substances and as adisperser.

Despite the improvements obtained through the variations of the method reported by **Errico (2009)** to adapt it to the DOXY-PHB system, dialysis was not an effective method, since the lowest entrapment capabilities and ME were thus obtained compared to the results from the rest of the techniques tested in this study. This is probably because DOXY interferes somehow in the process. When the unloaded particles were produced *via* dialysis, polymeric spheres could readily be obtained. However, when the loaded spheres were being produced, part of the polymer was not able to transform into spherical shape. Consequently, the ME was higher when DOXY was not present in the system. Also, when the drug was within the system, the particle size increased (figure 1) differing with **Errico** (**2009**) results. This difficulty of sphere formation and augment of the particle diameter when DOXY was in the system can be a consequence of the influence of the molecular interactions between the drug and the biopolymer, since **Errico** (**2009**) used a different active principle (retinoic acid), with smaller molecular

weight and less polar groups. Therefore, it can be assumed that, in the case of DOXY-PHB system, the interactions between the drug and the biopolymer are stronger. Also the higher molecular mass of DOXY with respect to the drug used in the previous work referred to may account for the bigger particle size.

Single and double emulsion techniques

Simple oil-in-water (O/W) emulsion with solvent evaporation was studied in this work in order to compare it to multiple water-in-oil-in-water ($W_1/O/W_2$) emulsion technique. When preparing microspheres by O/W emulsion, after the organic phase is added into the aqueous phase, the solvent dissolves into the aqueous phase and evaporates at the air-liquid interface (**Maia 2004**). In this case, the polymer and the drug should be soluble in the same solvent, which should be immiscible or partially miscible in water (**Chen 2002**). However, and due to the limited number of common solvents between DOXY and PHB, a dispersion method following **Freytag** (**2000**) and **Mundargy** (**2007**) was carried out to obtain a single emulsion. This solid-oil-water technique (S/O/W) was recommended before for producing microspheres with high drug loadings (**Spenlehauer 1986**). Thus, DOXY was dispersed in the PHB solution with DCM (organic phase), and then it was added to the aqueous phase, avoiding the step of dissolving the drug into the internal aqueous phase. In addition, higher theoretical drug loading can be achieved because the internal drug phase consists only of solid particles and not of a drug solution (**Freytag 2000, Feng 2010**).

The double emulsion technique is considered to be the most promising because it permits the encapsulation of hydrophilic and hydrophobic drug in both oily and aqueous phases (**Ahmed 2012**). This technique can be easily used to entrap hydrophilic drugs taking into account an important prerequisite for high encapsulation efficiencies: the drug must be insoluble in the organic polymer solution. Therefore, it was the main method of choice to study the encapsulation of the water-soluble DOXY. As it is difficult to entrap very small hydrophilic molecules such as DOXY into spheres with acceptable EE (more than 5%), since the high solubility of the drug in the external phase and its small size enables its diffusion to the external phase during the emulsion and solvent evaporation process (**Feng 2010**), different strategies were used in this work to improve the encapsulation capability.

For both simple and double emulsion, it is also of high importance to control the particle hardening step. Solvent evaporation depends mostly on temperature and the concentration of solvent in the air. As **Maia** (**2004**) reports, the solvent elimination procedure may influence properties of the final spheres and that is one of the effects of processing conditions that should be known during spherical particles production, before the design of controlled DDS. Therefore, time and temperature have to be precisely controlled during the evaporation process (**Ayoub 2011**).

PHB-particle production by dripping method

In the simple emulsion, DOXY dispersion in the PHB solution was added drop-wise into the aqueous phase following the process proposed by **Chen** (**2002**). **Mora-Huertas** (**2010**) reported that the

addition of the organic phase into the aqueous phase causes the diffusion of the solvent towards the external phase, resulting in the formation of solid spheres. In this case, only very low amounts of DOXY were loaded. When the dispersion was added to the aqueous solution, DOXY probably diffused through the organic polymer solution towards the external aqueous phase due to solubility gradients. PHB precipitated with spherical shape but with no active principle (insignificant drug loading and EE). However, when W_1/O emulsion is used instead of the drug dispersion in the multiple emulsion method (W_1/O/W_2), the results improved noticeably (DR2 with respect to US/DR4 in table 1). The drug is retained in the inner water phase, facilitated by the presence of NaCl solution in the external water phase. NaCl was added to the external aqueous phase (W_2) of the double emulsion in order to decrease the drug solubility (**Freytag 2000, Mundargy 2007**). Thus the hydrophilic drug does not tend to diffuse to the external phase, enhancing the EE and loading. Therefore, NaCl was added in all subsequent experiments to the external aqueous phase.

The type of surfactant influenced the entrapment capability of the spheres because of their different nature. PVA is a non charged polymer and has been frequently used as a stabilizer in biodegradable microsphere fabrication due to the colloidal stability that it provides (**Kalaji 2009**). When TAU, an ionic surfactant, was used in the W_2 instead of PVA, almost double drug loading and EE was obtained. This higher entrapment capability could be explained because TAU presents a higher number of hydroxyl groups in its molecular structure compared to PVA, linking with DOXY through hydrogen bonds. This probably helps to retain the antibiotic in the polymer matrix.

On the other hand, the sphere size and the surface morphology are in agreement with the results reported by **Chen** (**2002**). SEM micrographs show that the spheres produced *via* simple emulsion are more defined and the surface texture is finer. According to **Freytag** (**2000**), sphere porosity increased with the volume of the W_1 phase. This is because no water from the inner phase evaporates during the hardening of the polymeric particles.

PHB-particle production by ultrasounds

Ultrasounds were used in order to obtain DOXY-loaded nano-particles of PHB following recent studies with poly(L-lactic acid) (**Feng 2010**). Although in the process reported by **Feng** (**2010**) different materials were used for a water-in-oil-in-oil (W/O/O) emulsion preparation, ultrasounds were used in this work to attempt the formation of water-in-oil-in-water emulsion (W_1/O/W_2). The results showed that the DOXY-loaded PHB particles were obtained successfully and all of them were in the nano-sized scale. These results concur with **Mora-Huertas** (**2010**) who reported that spheres in the order of nano-scale are obtained when ultrasounds were used for nano-particle production. A different size of nano-spheres was obtained among the control (140±0.4 nm) and the drug-loaded samples with either of the surfactants - (600±270 nm) with PVA and (310±170 nm) with TAU. On a small scale, any different component in the system can produce variations in the process. Therefore, the presence of DOXY could aid in the increase of the particle size. Accordingly, **Mohanraj** (**2006**)

reported that the particle size is influenced by the type and concentration of stabilizer, homogenizer speed and polymer concentration.

PHB-particle production by high speed stirring

Ultrasound was chosen to be used as the method to emulsify the first emulsion in all double emulsion experiments because it has been shown to be a highly effective method (**Horowitz 1994, Sendil 1999, Freytag 2000, Baran 2002, Mundargi 2007, Mora-Huertas 2010**).

No previous studies were found in the literature reporting DOXY-loading on PHB spheres. The most comparable work was made by **Sendil** (**1999**), in which the tetracycline encapsulation in poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) was studied. In our system, the polymer and the antibiotic are chemically very similar to those previously reported. Therefore the results obtained with similar methodology (ultrasonication combined with high speed stirring for multiple emulsion) have been found to be very similar as well.

The drug loading and EE of the spheres produced by drug-dispersion (O/W) using ultra-turrax and PVA as surfactant (control and TU2) were slightly higher compared to the $W_1/O/W_2$ technique. This was probably due to a less porous matrix produced by the absence of the inner water phase (**Freytag 2000, Feng 2010)**. Nevertheless, part of the biopolymer hardened during solvent evaporation lost its spherical shape and the ME was consequently very low. Taking this into account, different parameters affecting the sphere formation and the encapsulation capability of the system have been studied with the double emulsion by ultrasounds combined with ultra-turrax such as the variation of the type of stabilizer and their concentrations, the different volume of the inner phase, the use of gelatin in either aqueous phase, and the emulsification time (for the 1^{st} and 2^{nd} emulsions).

Regarding the stabilizers, the optimal surfactant concentration for the stabilization of the system and for the improvement of the drug loaded was important to determine. Below a critical concentration, the amount of emulsifier in the medium is not enough to stabilize all the microcapsules. Thus, part of the drug is dissolved in the aqueous phase and is lost before the evaporation of the solvent (and hardening of the particles). On the contrary, when the emulsifier used is above the critical level it might interact with the available drug during spheres formation resulting in its leakage towards the aqueous phase (**Freytag 2000**). Therefore, different concentrations of the two emulsifying agents, PVA and TAU, were tested. As a result, 2 % of PVA and 0.5% of TAU were the optimal stabilizer concentration obtained to stabilize the system, confirmed through the best results of drug loading, EE and ME obtained for these concentrations. The use of different amounts of surfactant had a significant influence on particle size coinciding with that reported by other authors (**Mohanraj 2006, Tian 2008**). When TAU was used as an emulsifier agent, nano-particles were more easily obtained. Particles on the micro-scale were obtained with PVA. As previously commented on, TAU and PVA have different dissociation in water. It is possible that the ionic interaction between TAU with the biopolymer and/or the drug enhanced the formation of small particles.

The volume of the inner aqueous phase is primarily determined by the required dose and the water solubility of the drug, and it influenced the drug loading and particles surface (porosity). As was also observed in previous studies (**Freytag 2000**), the EE decreased by increasing the amount of the internal aqueous phase.

Some studies using gelatin in the drug encapsulation procedure (**Chen 2002, Sendil 1999**) reported that the release rate decreases with the increase of the gelatin. In this work, the encapsulation capabilities improved when gelatin was in either W_1 or W_2. However, best results were obtained when gelatin was present in the inner phase. That can be explained by the decrease of the water activity produced by the presence of the gelatin. When gelatin is present in the W_1, DOXY migrates to the polymeric solution phase. The same occurs when gelatin is in the external phase.

***In vitro* antibacterial assay**

In the antibacterial assay carried out, if the active principle is capable of diffusing from the material to the media, a region of bacterial growth inhibition is thus created (in this case observed for *E. coli* and *S. aureus* with all particles tested). The diameter of this zone was proportional to the amount of antibacterial agent added to the medium, the solubility of the agent, the diffusion coefficient, and its overall effectiveness (**Madigan 2000**). The registered inhibition zones effectively show that the particles containing higher drug loading have higher antibacterial properties. Thus, it has been shown, that the Doxy loaded in the particles may diffuse from the particles to the surrounding media. From that point of view, it can be considered that the DOXY-PHB micro- and nano-particles prepared may be good candidates as drug delivery systems, and will therefore be selected for further studies.

7. Conclusion

The doxycycline-loaded PHB micro- and nano-spheres have been obtained by the different methods studied. By comparing the different drug-entrapment and emulsification methods tested, the more effective process was achieved by the combination of ultrasounds with high speed stirring, although other methods like ultrasounds for multiple emulsion or drug dispersion by high speed stirring for simple emulsion also show high drug loading and EE. In order to select the optimal process to obtain micro- or nano-particles, it is necessary to reach an agreement between the drug loading and EE, with the ME. These results, and the qualitative antibacterial activity found for different formulations points out their candidacy for doxycycline delivery systems.

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Section 2: *Preparation of doxycycline-loaded micro- and nano-spheres with different PHAs*

1. Abstract

In a previous work, different drug-entrapment and emulsification methods for the preparation of doxycycline-loaded micro- and nano-spheres were studied with a bacterial PHB from *Cupriavidus necator* DSM 545. The most promising methods from this previous work have been used in the present study in order to confirm that they are suitable for the preparation of micro- and nano-spheres with other PHAs and, in this case, to study the possibility if improving the results of drug entrapment and method efficiency. Therefore, an attempt was made to prepare spherical particles with bacterial PHB samples of different origins and a copolymer by means of different physico-mechanical methods of solvent evaporation (ultrasounds, dripping and/or high speed stirring, and their combination). The results showed that it was possible to form doxycycline-loaded micro- and nano-spheres by the same methodology used in the previous work, obtaining similar results with all PHAs used. Regarding the copolymer, P(3HB-co-4HB) did not show good entrapment capabilities with doxycycline, compared with the other PHAs.

2. Introduction

Microencapsulation is described as a process of enclosing micron-sized particles that can be in an inert shell which isolates and protects the kept substance from the external environment. The particles can be either solid or droplets (**Ghosh 2006**). When the diameter is between 3-800 μm, the products thus obtained are called micro-particles, micro-capsules and micro-spheres which differentiate in morphology and internal structure. When the particle size is below 1 μm they are known as nano-particles, nano-capsules, and nano-spheres respectively (**Remuñán 1997**). Particles larger than 1000 μm are known as macro-particles (**Thies 1996**).

Microencapsulation is carried out in order (i) to protect the sensitive substances from the external environment, (ii) to mask the organoleptic properties like color, taste, and odor of the substance, (iii) to obtain controlled release of the drug substance, (iv) to safely handle toxic materials, (v) to get targeted release of the drug and (vi) to avoid adverse effects like gastric irritation (**Jyothi 2010**).

There are different ways to achieve the production of micro- or nano-particles: chemical, physico-chemical and physico-mechanical methods (**Jyothi 2010**). Previously, different physico-mechanical methods of solvent evaporation have been studied to produce micro-/ and nano-particles of PHB loaded with doxycicline (DOXY) (**Chapter III, section 1**). DOXY-loaded PHB micro-/nano-particles were obtained by polymer precipitation *via* dialysis, simple emulsion (O/W) and multiple emulsion ($W_1/O/W_2$) applying the solvent evaporation method in the last two cases. The emulsions were mechanically prepared by ultrasounds, dripping and/or high speed stirring, and by their combination. This study was carried out with a single PHB from a microbial fermentation with *Cupriavidus necator*

DSM 545 and the combination of glucose and glycerol as carbon sources (**Chapter I, section 4**). The method that was highly effective in obtaining DOXY-loaded PHB particles with high method efficiency (ME) was the combination of ultrasounds with high speed stirring to obtain a double emulsion. Also, the use of ultrasounds for the preparation of double emulsion or drug dispersion by high speed stirring for simple emulsion show high entrapment capabilities. On the other hand, reported studies indicate that polymer properties such as the molecular mass influence the method and encapsulation efficiency (ME, EE) (**Jyothi 2010**). Therefore, an attempt was made to use these three physico-mechanical techniques in this study to produce DOXY-loaded micro- and nano-spheres, using three PHAs with different properties.

3. Objectives

The most promising methods for the preparation of DOXY-loaded micro- and nano-spheres tested in a previous work were used in this study in order to confirm their suitability for the preparation of micro- and nano-particles with other PHAs (a PHA copolymer and PHB samples of different origins and with different polymer properties). The work also studies the possibility of improving the results of drug entrapment capabilities and method efficiency of results obtained from the PHB micro- and nano-spheres obtained in the previous study. Hence, the particle size, their drug loading, EE, and ME has been evaluated by comparing the results obtained to the ones from the PHB produced by *C. necator* DSM 545.

4. Material and methods

PHAs

Three different PHAs were used in this study: PHB from microbial fermentation with the environmental novel *Bacillus megaterium* strain uyuni S-29 using glucose as substrate, PHB from microbial fermentation with *Burkholderia sacchari* DSM 17165 and glycerol as carbon source, and a bacterial P(3HB-co-8.4% 4HB) copolymer. The main properties of the biopolymers are summarized in **table 1** and are compared to the PHB from *C. necator* used in the previous work (**Chapter III, Section 1**).

The antibiotic used was doxycycline hyclate (DOXY) (Sigma-Aldrich) (doxycycline hydrochloride hemiethanolate hemihydrate, $C_{22}H_{24}N_2O_8 \cdot HCl \cdot 0.5H_2O \cdot 0.5C_2H_6O$, M_W 1025.89 Da), which is freely soluble in water (1:1–10 w/w) (**Parfitt 1999**). Chemical products such as Pluronic-F127, poly(vinyl alcohol) (PVA) and sodium taurocholate (TAU) were purchased by Sigma Chemical Co. The gelling agent was bovine gelatin (Gel).

PHA-based particles

Three different methods were applied in this study to prepare DOXY-containing micro- and nano-particles. Ultrasounds and high speed stirring were used to obtain the emulsions by means of which the PHB-loaded particles were obtained. Ultrasound was used twice to prepare multiple emulsion ($W_1/O/W_2$) with solvent evaporation to remove the polymer solvent, and high speed stirring was used to prepare both single (O/W) and multiple emulsion ($W_1/O/W_2$) with solvent evaporation as well. Data relevant to the individual experiments is summarized in table 2, whereas the general procedures are described in detail in the following paragraphs.

Table 1. Summary of the main values of the PHAs characterization

	Polymer	PHB	PHB	P(3HB-4HB)	PHB
	Origin	*B. megaterium* uyuni S-29	*B. sacchari* DSM 17165	Commercial	*C. necator* DSM 545
Thermal properties (DSC analyses)	Tm (ºC)	134.34	163.32	158	172.09
	Xc (%)	36.7	72.82	30	62.53
Molecular mass distribution (GPC analyses)	Mw (KDa)	705	200	300	302
	PDI	1.12	2.50	1.80	4.72

Table 2. Formulations and methods used to prepare the PHA-based micro- and nano-particles.

Method	Combined methods	Technique	Sample	PHB (mg)	DOXY (mg)	Other compounds
High speed stirring	Dispersion	O/W	TU1	200	60	2% PVA - 4% NaCl
	Dispersion	O/W	TU2	200	60	0.5% TAU - 4% NaCl
	Ultrasound + High speed stirring	$W_1/O/W_2$	US/TU1	300	90	2% PVA - 4% NaCl - 2% Gel in W_1 - W_1= 1mL H_2O
	Ultrasound + High speed stirring	$W_1/O/W_2$	US/TU2	300	90	0,5% TAU- 4% NaCl - 2% Gel in W_1 - W_1= 1mL H_2O
Ultrasound	Double Ultrasounds	$W_1/O/W_2$	US/US1	30	9	2% PVA - 4% NaCl
	Double Ultrasounds	$W_1/O/W_2$	US/US2	30	9	0.5% TAU - 4% NaCl

PHB-particle production by emulsion

High speed stirring

Some modifications from **Mundargi** (**2007**) were adopted to formulate the DOXY-loaded PHB microspheres *via* a simple emulsion technique (O/W). In this method, DOXY equivalent to 30% (w/w)

dry weight of the polymer was dispersed into 10 mL of PHB solution in DCM. Then, the dispersion was slowly added into 100 mL of an aqueous solution containing either PVA or TAU, and NaCl (4% w/v), and emulsified using an ultra-turrax (IKA-Werke GmbH Co. KG, Germany). Mechanical stirring was maintained at 12000 rpm for 15 min to form the O/W emulsion. Further, solvent removal and hardening of the microspheres was achieved by continued stirring at 900 rpm for up to 2 h. A similar protocol was followed to produce the unloaded PHB particles.

The method reported by **Mundargi (2007)** was followed to carry out the double emulsion technique ($W_1/O/W_2$). First, ultrasounds were applied for the initial emulsion (W_1/O), followed by high speed stirring to reach the second emulsion ($W_1/O/W_2$). DOXY equivalent to 30% (w/w) dry weight of PHB was dissolved in 2 mL of distilled water to form a DOXY aqueous solution. PHB was dissolved in 10 mL of DCM (oil phase). Both aqueous and organic solutions were emulsified using the probe ultrasonicator previously described (Bandelin Sonopuls with microtip MS 73). Then, this first emulsion was slowly added into 100 mL of an aqueous solution containing either PVA or TAU, and NaCl (4% w/v). Different concentrations of PVA and TAU were tested. The double emulsion was achieved by mechanical stirring using the ultra-turrax at 12000 rpm for 15 min. The solvent removal and hardening of the microspheres was attained by continued stirring at 900 rpm for up to 2 h.

In order to improve the drug loading capabilities of the micro-/nano-spheres, different variations and tests were carried out in each method. Table 1 shows all modified parameters: variation on the W_1 volume, addition of gelatine in either W_1 or W_2 or different initial DOXY contents.

Ultrasounds method

DOXY-containing PHB spheres were produced by double emulsion ($W_1/O/W_2$), adapting a sonication method reported by **Feng (2010)**. An amount of DOXY equivalent to 30% (w/w) of the dry mass of PHB was dissolved in 200 µL of distilled water. This aqueous solution was emulsified with the organic phase which consisted of PHB dissolved in 1 mL DCM, by means of a probe ultrasonic homogenizer at 2W (2 min at 50%). This primary W_1/O emulsion was then emulsified into 10 ml of distilled water with either PVA or TAU and NaCl (4% w/w) under ultrasonic homogenizer at 5W (3 min at 60%) to form the $W_1/O/W_2$ emulsion. The resulting secondary emulsion was magnetically stirred at 700 rpm for 5 h until complete solvent evaporation.

Particle recovery and ME

The micro- and nano-spheres obtained by the three different processes were collected by centrifugation at 4000 rpm for 15 min (Medifriger centrifuge, JP Selecta, Barcelona, Spain), separated and washed with distilled water. The resultant suspension was frozen and freeze-dried overnight (laboratory freeze-dryer Teslar Cryodos) to obtain a white powder. The product was stored, desiccated, and kept in the dark at 20 °C until required (**Mora-Huertas 2010**). Method yield or efficiency (ME) was calculated according to the following equation: Method efficiency (%) = [(weight initial PHB- weight polymer debris)/weight initial PHB] x100. The particle suspension was sieved (100

μm) to separate particles from the polymer debris, which did not have a spherical shape. These were dried and weighed.

Determination of the EE and drug loading

The particles obtained (10 mg) were dissolved in 1 mL of chloroform, and the DOXY within the microparticles was extracted by adding 5 mL of a physiological phosphate buffer (PBS) solution (pH 7.4, 0.1 M). The solvent was removed by bubbling nitrogen gas. After adding 2 ml of the PBS solution and vigorously mixing by vortexing, the polymeric debris were removed by filtration through a 0.45 mm filter (Millipore, USA). The resultant DOXY solution was analyzed with a UV/Vis spectrophotometer (Cary 100 Bio, with Cary Win UV software) at the λ_{max} of 275nm. This extraction process was applied to at least two sets of particles. The % of EE and the % of drug loading of the microspheres were calculated as follows (**Sendil 1999, Mundargi 2007**): Encapsulation Efficiency (%)= (weight of drug loaded/weight of drug initially added)x100 and Drug Loading (%) =(weight of drug in particles/weight of particles)x100.

Scanning electron microscope (SEM) and particle size analysis

Dried micro- and nano-particles were gold coated (coating thickness between 10-15 nm) under vacuum by means of a BAL-TEC (SCD 005, Germany) cool sputter coater and their scanning electron micrographs were obtained using a Jeol (JSM-5610, Japan) scanning electron microscope, operating at magnitudes from 500 to 20.000X. The median particle size was obtained after the evaluation of the microscopic images with AnalySIS software tool (Soft Imaging System GmbH, Münster, Germany).

5. Results and discussion

The results obtained in this study are compared to the results attained in previous studies with the biopolymer produced by *Cupriavidus necator* DSM 545 **(Chapter III, section 1).** All these results are shown in **table 3**.

Theoretically, the enhancement of the EE and drug loading is achieved by: low solubility of the polymer in organic solvent, high solubility of the organic solvent in water, high polymer concentration, low ratio of disperse phase to continuous phase and fast solvent removal rate. All these factors produce a fast solidification or hardening of particles leading to high encapsulation efficiency (**Jyothi 2010**). This is mainly because the drug diffusion into the continuous phase, W_1 or W_2 depending on if it is a simple or multiple emulsion, respectively, takes place during the first 10 min of emulsification. In order to obtain high entrapment capabilities of the particles, it is also important to consider the interactions between the drug and the polymer. On the other hand, such interaction can influence the drug release from the particle.

The methods used in this study for the formation of DOXY-loaded PHA micro- and nano-particles were previously optimized. Therefore, many of these factors such as the polymer concentration, the disperse phase to continuous phase ratio, and the solvent removal rate have been fixed. Other parameters like the drug and emulsified concentrations, and the amount of agents such as NaCl and gelatin, which were found to improve the entrapment capabilities of the formed particles, were also unchanged. In this study, PHBs with different properties compared to the properties from the PHB used in the previous work, and a PHA co-polymer were used for the formation of DOXY-loaded micro- and nano-particles. Following, the possible factors affecting the entrapment capabilities of the particles formed from these different PHAs are compared.

Table 3. Summary of the results: micro- and nano- particles produced from different PHAs

Sample		TU1	TU2	US/TU1	US/TU2	US/US1	US/US2
PHB from *B. megaterium* uyuni S29	Size (µm)	1.34±0.42	0.26±0.75	2.26±0.75	21.34±9.27 0.42±0.19	0.34±0.14	0.48±0.38
	L %	2.40	3.72	2.04	9.08	1.33	3.14
	EE %	7.38	10.48	6.76	15.56	4.87	10.82
	MM %	62	57	86	81	46	45
PHB from *B. sacchari* DSM 17165	Size (µm)	2.67±0.81	0.19±0.08	27.55±19.47	0.16±0.05	0.28±0.12	0.21±0.10
	L %	1.17	3.28	0.98	3.83	3.57	3.07
	EE %	3.74	10.72	3.39	12.87	9.92	9.19
	MM %	69	54	56	63	68	49
P(3HB-co-4HB)	Size (µm)	37.70±11.39	0.28±0.13	11.08±9.32	0.81±1.11	0.29±0.09	0.36±0.11
	L %	2.86	1.86	0.94	0.67	0.78	0.91
	EE %	9.43	6.43	3.16	2.22	2.57	2.70
	MM %	78	73	87	86	52	56
PHB from *C. necator* DSM 545	Size (µm)	29.68±7.63	0.43±0.18	10.95±3.65	0.38±0.22	0.60±0.27	0.31±0.17
	L %	4.83	4.29	3.32	3.66	1.35	3.48
	EE %	16.18	14.37	11.36	12.55	4.29	13.64
	MM %	54	47	89	78	44	47

PHB from *Bacillus megaterium* uyuni S29

The PHB produced by *B. megaterium* uyuni S29 is mainly characterized by its higher molecular mass and its lower thermal properties (melting point and crystallinity degree) compared to the ones of the PHB produced by *C. necator* DSM 545.

In general, the particles achieved with this PHB are smaller compared to the ones obtained from PHB of *C. necator* DSM 545. Agreeing with previous results with PHB from *C. necator* DSM 545, the particles obtained using PVA as an emulsifier are higher compared to the ones obtained using TAU. Also, the particles are in a nano-scale when ultrasound is used twice for the double emulsion method (**Mora-Huertas 2010**). Figure 1 shows the SEM micrographs taken of the particles from the different methods used.

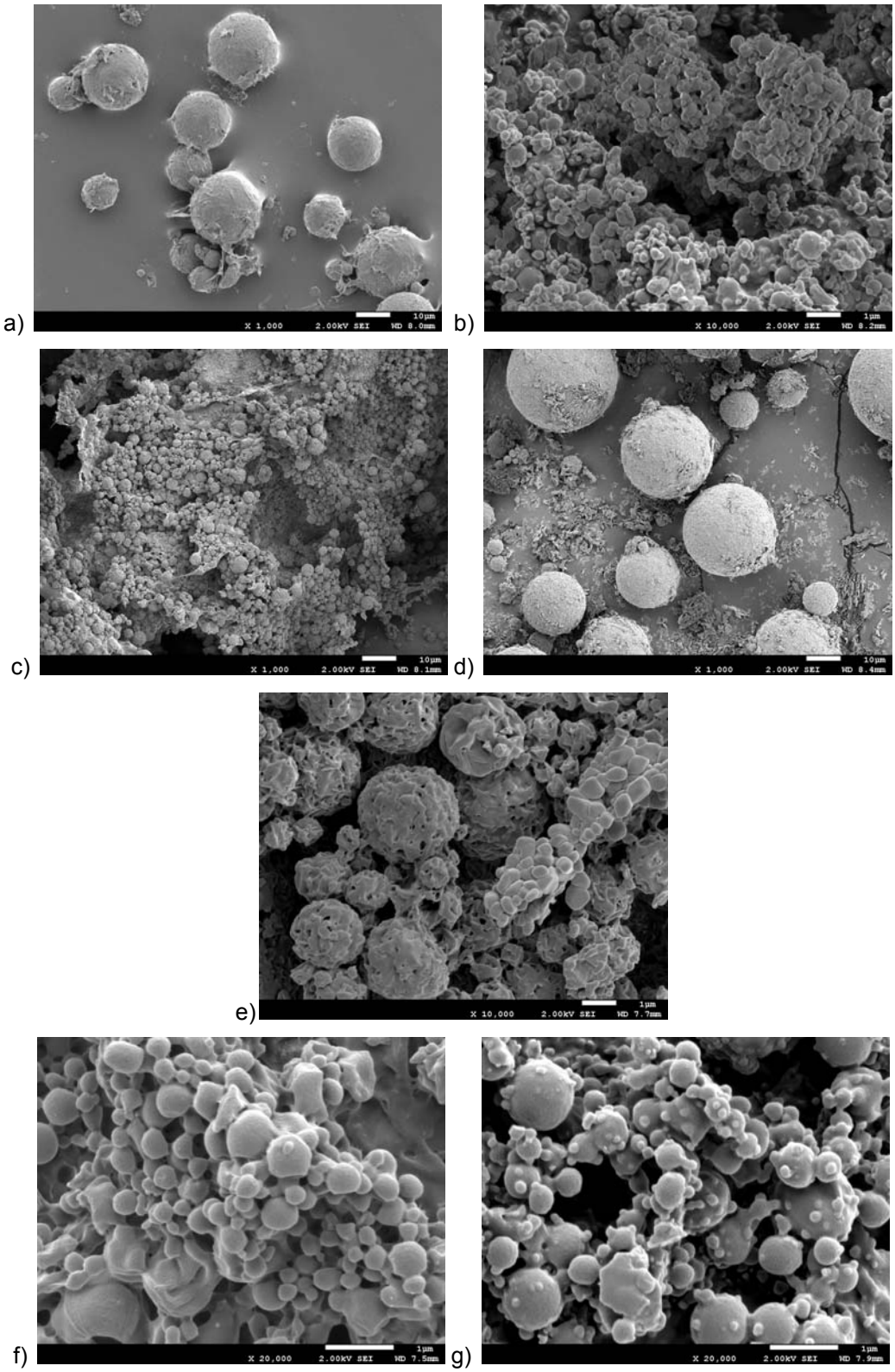

Figure 1. Particles formed by dispersion with (a) PVA and (b) TAU, combination of ultrasound and high speed stirring with (c) PVA and (d, e) TAU, and ultrasound with (f) PVA, and (g) TAU from PHB homopolymer produced by *B. megaterium* uyuni S29

The results of the particle formation with ultrasound and high speed stirring using TAU as an emulsifier (US/TU2) show that both, micro- and nano-size spheres, were formed. Figure 1 (d) shows the micro-particles (21.34±9.27) µm and figure 1 (e) shows the nano-size particles (0.42±0.19) µm. In the previous work, the results of the particle size obtained using TAU are in the nano-scale. However, there has been an aggregation process, forming bigger spheres. This might be because the stirring speed was not high enough to homogenize the system.

The entrapment capabilities of the particles produced by the different methods with PHB homopolymer from *B. megaterium* uyuni S29 are all a little bit lower compared to the ones of the PHB produced by *C. necator* DSM 545. Since both biopolymers were chemically the same, similar results in drug entrapment capabilities and method efficiency were expected to be obtained. Also, a small decrease in the EE and drug loading was expected due to the different material properties. The factors affecting the EE are the solubility in the organic solvent and the drug/polymer interactions. Solubility is influenced by the molecular mass of the polymer (**Jyothi 2010**), and the drug/polymer interactions are enhanced by hydrogen bonding and polar interactions, also related to the polymer molecular mass (**Johansen 1998**). Because of its higher molecular mass, the material has less ending groups, hydroxyl and carboxylic groups. Consequently, less hydrogen bonding and polar interactions are produced between the polymer and the antibiotic, giving the drug less affinity for the biopolymer. On the other hand, PHB with higher molecular mass is more hydrophobic and less soluble in the organic solvent (DCM) (**Jyothi 2010**), worsening the entrapment capabilities of the system and, thus, coinciding with the results obtained.

These statements concur with all results, except for one. The PHB particles produced by the combination of ultrasound and high speed stirring (US/TU2) using TAU as an emulsifier show the high entrapment results. This result can be explained by means of the particle size obtained with this method. It is possible that this aggregation process could enhance the drug entrapment by the polymer net.

Compared to the results obtained with PHB from *C. necator* DSM 545, the ME is higher in most cases, perhaps due to the lower crystallinity degree that this PHB shows. In a previous study with this bacterium, it was concluded that it produces a PHB with lower thermal properties than the common PHBs reported in the literature (**Chapter I, section 1 and 2**). The less crystalline PHB produced by *B. megaterium* uyuni S29 could be an advantage for the particles preparation.

Comparing all methods used, it seems that the combination of ultrasound with high speed stirring (US/TU) using both surfactants is the best suitable method for particle formation with the high-molecular-mass PHB from *B. megaterium* uyuni S-29, especially when TAU is used as an emulsifier. In this case, high ME was also achieved. The other two methods show no better results compared to the PHB from *C. necator* DSM 545.

PHB from *Burkholderia sacchari* DSM 17165

As discussed in previous studies (**Chapter I, section 4**), the PHB produced by *B. sacchari* DSM 17165 with glycerol as a single carbon source is characterized by its relatively low molecular mass (200 kDa with polydispersity of 2.50).

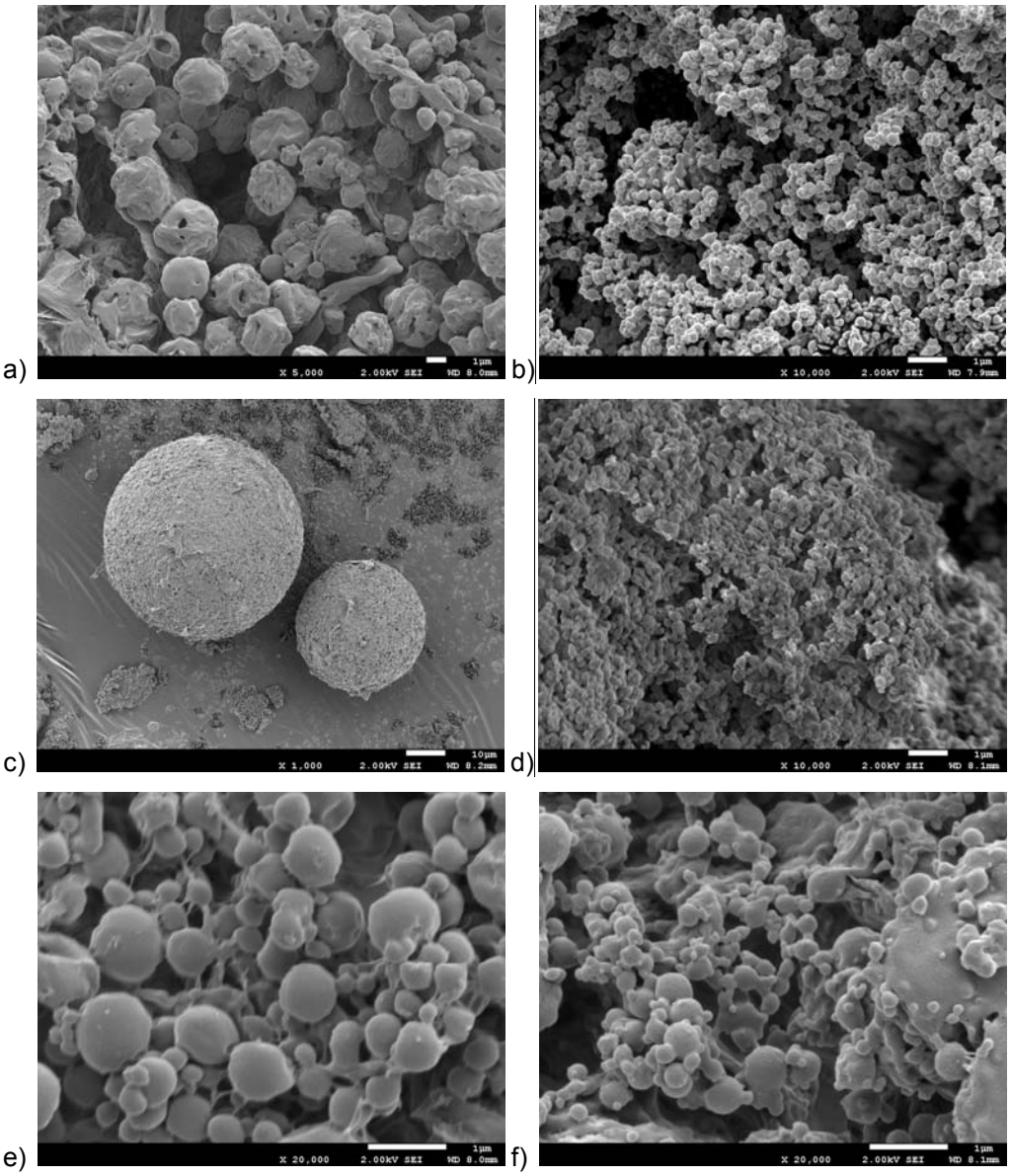

Figure 2. Particles formed by dispersion with (a) PVA and (b) TAU, combination of ultrasound and high speed stirring with (c) PVA and (d) TAU, and ultrasound with (e) PVA and (f) TAU from PHB homopolymer produced by *Burkholderia sacchari* DSM 17165

Figure 2 shows the DOXY-loaded spheres obtained with PHB from *B. sacchari* DSM 17165. The particle sizes obtained using this PHB are in general smaller compared to the particles from *C. necator* DSM 545 and *B. megaterum* uyuni S29. This is probably due to its thermal properties and molecular mass, since it is reported that the particle diameters of some polyesters depend on the crystallinity degree of the material (**Sosnowski 2001**). As with the results obtained for PHB from *C.*

necator DSM 545 and *B. megaterum* uyuni S29, nano-size spheres were obtained with TAU as an emulsifier and when ultrasound is used in the emulsification process (**Mora-Huertas 2010**). On the other hand, the results of the particle formation with ultrasound and high speed stirring using PVA as an emulsifier (US/TU1) show a high particle size. However, the high standard deviation (±19.47 µm) indicates that this method produces a wide range of particles with different sizes- high particle size dispersion.

Regarding the entrapment capabilities, the results obtained with PHB from *B. sacchari* DSM 17165 are not better than the results of the other PHB samples from *C. necator* DSM 545 and *B. megaterum* uyuni S29. Specifically, the entrapment capability results of the particles obtained by means of ultrasounds and high speed stirring using TAU as an emulsifier (US/TU2) are very similar compared to the particles produced the same way with PHB from *C. necator* DSM 545. Also, the use of ultrasounds for the double emulsion method formed particles with similar EE and loading using PVA as an emulsifier (US/US1). The PHB from *B. sacchari* DSM 17165 was expected to have a higher affinity to the drug than a higher molecular mass PHB such as the one produced by *B. megaterium* uyuni S29 or *C. necator* DSM 545, because of its low molecular mass. However, the results show, in most of the cases, a lower entrapment capability. This can be explained by the increment on the solubility of the biopolymer in the organic phase due to its lower molecular mass. This higher solubility produces a longer particle-hardener time, resulting in low encapsulation efficiencies (**Mehta 1996**).

On the other hand, the ME is lower for most of the cases. The explanation can be found in the different polymer properties that this PHB presents compared to the PHB from *C. necator* DSM 545 and *B. megaterium* uyuni S29. It is highly crystalline and has a lower molecular mass. These polymer properties influence the sphere formation (**Sosnowski 2001**).

Regarding the methodology, the use of ultrasounds for the preparation of double emulsion (US/US) was the method which showed better results of encapsulation efficiency and ME with low-molecular-mass PHB from *B. sacchari* DSM 7165. The ultrasound with high speed stirring method (US/TU) using TAU as an emulsifier also showed good results.

P(3HB-co-8.4% 4HB) copolymer

P(3HB-co-4HB) is a very common member of the PHA family and is characterized by the presence of the 4HB fraction which changes the mechanical and thermal properties of the PHB (**Sudesh 2000, Vigneswari 2010, Jing 2012**). Therefore, the main difference between the bacterial P(3HB-co-4HB) copolymer and the rest of the PHB samples used in this study is the introduction of 8.4% of the 4HB fraction. The molecular structure of the copolymer is shown in figure 3.

The sphere formation was successfully carried out by the same processes as the ones used for the rest of the PHBs. Figure 4 shows the micrographs of the resulting P(3HB-co-4HB)-spheres. The particle sizes in most of the cases are higher for the copolymer. On the other hand, and agreeing with the other results, the use of TAU as an emulsifier for the preparation of the DOXY-loaded spheres

produced particles on the nano-scale. Also, the use of PVA as an emulsifier produced bigger particles depending on the method used. The results of the double ultrasound method with P(3HB-co-4HB) copolymer show the production of nano-spheres with the use of ultrasound in the process (**Mora-Huertas 2010**).

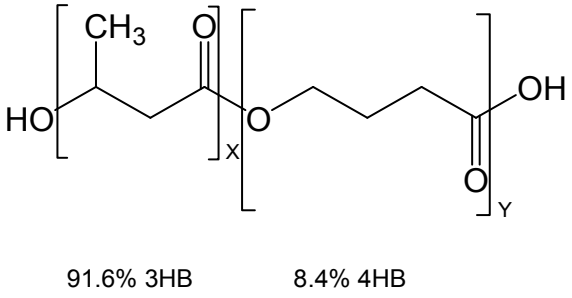

Figure 3. P(3HB-co-4HB) structure

The entrapment capabilities of the spheres obtained with P(3HB-co-4HB) did not improve compared to the results of the other PHAs. The fact that the 4HB fraction is a simple linear monomer which does not attach any chemical group capable of linking to the drug molecule could be the explanation. Therefore the drug/polymer interactions do not improve using this PHA copolymer (**Jyothi 2010**). This can be confirmed by comparing the entrapment capability results from *C. necator* DSM 545 to the ones of the copolymer because both PHAs chemically differ from each other in the 4HB fraction that the copolymer shows, having similar molecular mass (300 kDa). Therefore, and taking into account the obtained entrapment capabilities results, P(3HB-co-4HB) copolymer is not adequate for the production of DOXY-loaded PHB spheres.

The values of the ME obtained with the copolymer are in general higher compared to the results with PHB from *C. necator* DSM 545. It is possible that the low crystallinity degree of the copolymer enhanced the sphere-formation processes. Consequently, it is possible to conclude that these physico-mechanical methods are also good strategies for preparing spheres on the micro- and nano-scale with P(3HB-co-4HB), especially the drug dispersion by high speed stirring method (TU) for simple emulsion, which shows the higher ME.

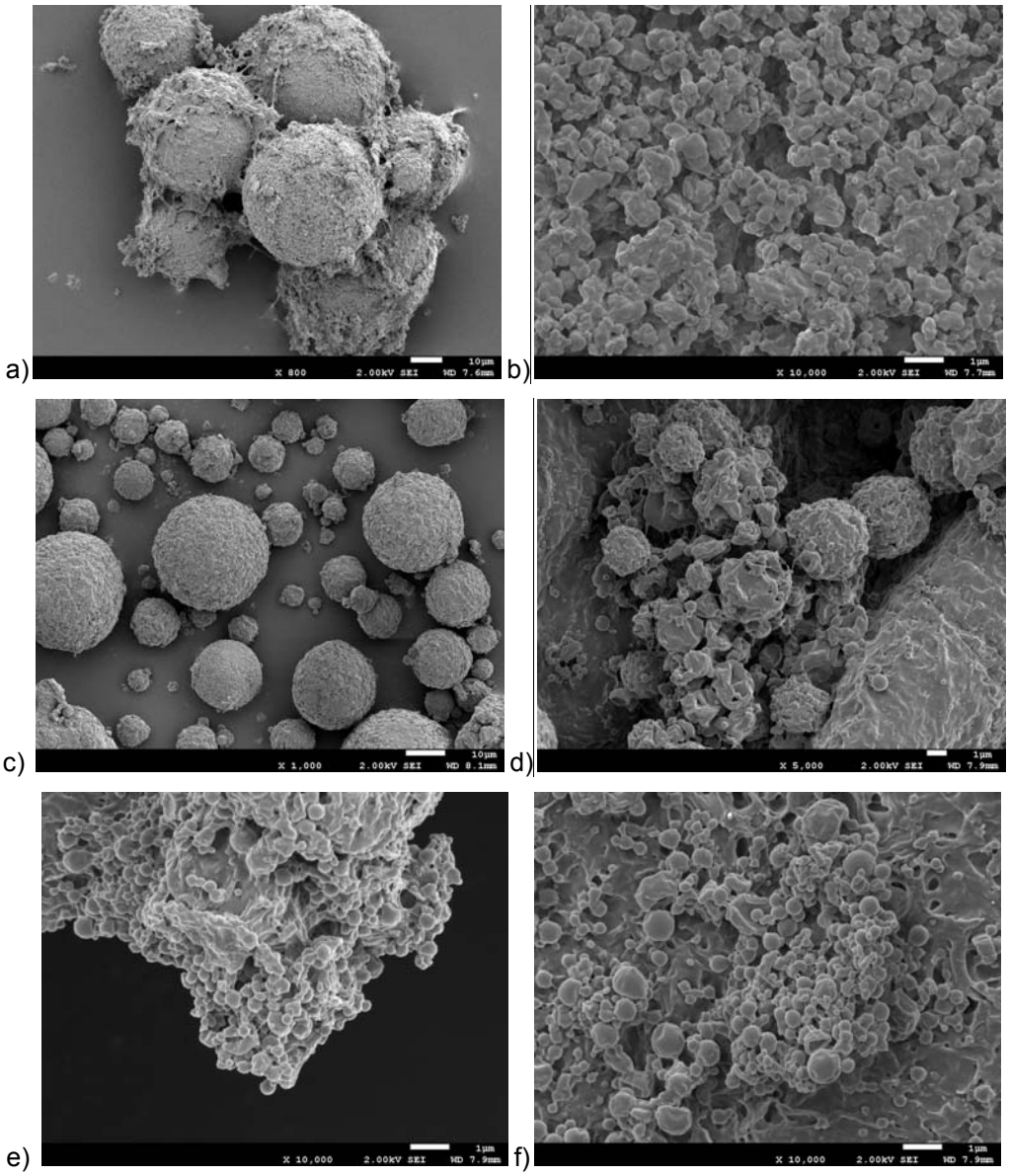

Figure 4. Particles formed by dispersion with (a) PVA and (b) TAU, combination of ultrasound and high speed stirring with (c) PVA and (d) TAU, and ultrasound with (e) PVA and (f) TAU from P(3HB-co-4HB) copolymer

6. Conclusions

This study confirms that the methods previously used for the preparation of DOXY-loaded PHB micro- and nano-spheres successfully work using PHB from different origins and properties. In general, very similar entrapment capabilities and method efficiency results were achieved with all PHBs used compared to the previous studies carried out with PHB from *C. necator* DSM 545.

Although low entrapment capabilities were achieved with P(3HB-co-4HB) copolymer compared to the rest of the PHAs tested, the methods used in this study are also suitable for the formation of micro- and nano-spheres of P(3HB-co-4HB).

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CONCLUSIONS

The main conclusions of each chapter are gathered together here:

CHAPTER I. PHB PRODUCTION

1. The isolation of a wild-type PHB-producer strain of *Bacillus megaterium* (uyuni S29) from extreme saline environments is described for the first time. Thus, the strain is currently deposited in the Spanish Type Culture Collection (CECT) with the number 7922.
2. The novel strain properly develops in a conventional medium with low salt content as typically used for industrial production of PHAs. The strain produces and accumulates a larger content of PHB than ever reached before for the genus *Bacillus* in the industrial conditions proposed without spore formation. Consequently, *Bacillus megaterium* uyuni S29 shows a high potential for PHB production and could be considered for its bioproduction on an industrial scale.
3. From the biopolymer extraction and analyses experiments, it can be concluded that *Bacillus megaterium* uyuni S29 does not synthesise any other PHA but PHB homopolymer from glucose as a carbon substrate.
4. The biosynthesised PHB features thermal properties differing from conventional PHB (lower crystallinity, glass transition temperature and melting point) probably due to the different polymer fractions of molecular masses it is composed of. Consequently, PHB produced by the strain could be considered for use in a wider range of applications.
5. The salt concentration influences the cell growth and the PHB production by the novel *Bacillus megaterium* uyuni S29. The strain behaves differently depending on the quantity of salt in the medium so that the optimal salt concentration improves the cell growth as well as the PHB production.
6. The fact that the strain can properly develop and synthesise PHB in the presence and absence of salt suggests that the strain is a great candidate for biotechnological applications in different areas.
7. A high cell dry mass and a high growth rate are achieved for the first time when the combination of glycerol and glucose is used in the fermentation with *Cupriavidus necator* DSM 545 for the production of PHB. This carbon source combination is potentially a great strategy to improve the growth and polymer production in the presence of glycerol.
8. *Burkholderia sacchari* DSM 17165 properly develops in the presence of glycerol as a sole carbon source producing low molecular mass PHB thus providing an alternative outlet for glycerol and producing a value-added products PHB

CHAPTER II. PHB TRANSFORMATION

9. For the first time, it is possible to degrade high molecular mass PHB composed of 3HB and 4HB fractions into low molecular mass PHB by means of two commercial lipases, Lipopan Conc BG and Lipase AK. Therefore, the proposed depolymerization can be considered for a sustainable process on a larger scale.

10. The main factor in the polymer degradation is the enzyme activity. Lipopan Conc BG shows higher activity than Lipase AK and better specificity for P(3HB-co-4HB). Therefore it is used in the degradation of pure PHB.
11. Even though the enzymes have different regiospecificity, both lipases hydrolyzed the backbone chain without distinguishing the ester bonds from primary (4HB) or secondary (3HB) alcohols. Particularly, the specificity that Lipopan Conc BG lipase presents for PHB is confirmed. This commercial enzyme with hydrolytic activity in primary ester bonds of triglycerides is able to decrease the homopolymer PHB molecular mass.

CHAPTER III. APPLICATION

12. It is necessary to find a balance between the entrapment capabilities (drug loading and entrapment efficiency) with the method efficiency when doxycycline-loaded micro- or nano-spherical particles are prepared by precipitation *via* dialysis, single emulsion, and multiple emulsion utilizing emulsification methods such as dropping, ultrasounds, high speed stirring and their combinations.
13. The combination of ultrasounds with high speed stirring in the preparation of double emulsion is highly effective in obtaining doxycycline-loaded particles of PHBs from different origins with high method efficiency. Furthermore, other methods like ultrasounds for multiple emulsion or drug dispersion by high speed stirring for simple emulsion, show high drug loading and entrapment efficiency.
14. Although low entrapment capabilities were achieved with P(3HB-co-4HB) copolymer compared to the rest of the PHAs tested, the methods used are also suitable for the formation of micro- and nano-spheres of the copolymer.
15. The qualitative antibacterial activity found in particles obtained *via* double emulsion with the combination of ultrasounds with and without high speed stirring, and *via* simple emulsion with drug dispersion by high speed stirring, points out their potential candidacy for drug delivery systems.

FUTURE AND FURTHER STUDIES

Production and application of PHA needs an interdisciplinary approach. This is already provoking a high potential impact in sectors like agriculture, fermentation, materials, energy and medicine. The future of the PHA industry follows two main paths: reducing production costs and developing high added value applications. In the first case, genetic engineering related to metabolic pathway alteration and synthetic biology is one of the main fields of tendency, aiming the generation of genetically modified strains capable of producing more or bigger PHAs granules. Also, the use of other organisms in PHA production, such as genetically modified plants, is still a possible alternative. Some other approaches to attaining a less expensive PHA production process include improvements in the fermentation conditions by the use of cheaper carbon sources such as dairy or fuel wastes, the use of new strains, genetically modified or not, with which less restrictive culture techniques are possible, and the application of new continuous fermentation technologies.

High-value-added PHAs applications should be developed simultaneously to decrease their production cost. Such applications should include their use as materials for bio-implants and the improvement of biopolymer degradation control, tissue engineering, and monomers and oligomers for drugs.

Other areas to explore can be found in the production of PHAs with special properties. This can be based on the use of modified PHA syntheses which will allow PHAs with chemically modifiable functional groups. Such modification would include producing mixed copolymers with controllable properties. On the other hand, a novel process for the improvement of the different polymer properties can be studied by the addition of inorganic nanotubes in order to prepare advanced polymer nanocomposites. The most simple, cost-effective and ecologically friendly way, the melt-processing route, is currently being applied to PHAs to fine-tune their properties.

Further studies from the thesis

- Application of the optimal salt concentration found in this thesis in a bioreactor fermentation with the novel strain *Bacillus megaterium* uyuni S29 in the industrial medium and the confirmation of its optimality in a culture under controlled conditions in order to improve the PHB production.
- Reduction of the PHB production cost by the study of *Bacillus megaterium* uyuni S29 growth and its polymer production utilizing glycerol as a cheap carbon source, and the analysis of its ability to convert it into PHB or any other PHA.
- Study of the utilization of the novel strain *Bacillus megaterium* uyuni S29 for salty wastewater treatment, and the consequent study of its biopolymer production in this environment. This is based on the fact that several *Bacillus* species such as *Bacillus cereus* **(Krishnaswamy 2011**) have been used in the treatment of wastewater as denitrifiers and as phosphate removers. As halotolerant bacteria, some of them have been used in the treatment of salty wastewater **(Sivaprakasam 2008**).

- Improvement of the entrapment capabilities of doxycycline and other drugs in a PHB system and the application of the low molecular weight PHB oligomers obtained in this thesis by the preparation of specialty graft and block copolymers as amphiphilic macromolecules. Di/triblock copolymers of PHB and, for example, polyethilenglycole could be applied in the encapsulation of drugs and act as amphiphilic drug carriers in the form of micro- or nano-particles or as frozen micelles (**Chen 2006**).
- Determination of the drug delivery profile of the doxycycline-loaded micro- and nano-particles of the different PHAs produced and utilized in this thesis in order to determine the retarding properties of these drug delivery systems, since they can be controllable by variations in processing and molecular mass of the polymer and copolymer composition (**Errico 2009**).

SCIENTIFIC PRODUCTION

Articles

M. Koller, A. Salerno, E. Ingolic, M. Dias, A. Rodríguez-Contreras, R. Raposo, A. Reiterer, G. Braunegg (**2009**). Contexts between the bio-mediated production of polymers, chemicals, and fuels from waste streams. Biologi italiani 8, 8-11.

A. Rodríguez-Contreras, M. Calafell-Monfort, M. S. Marqués-Calvo (**2012**). Enzymatic degradation of P(3HB-co-4HB) by commercial lipases. Polymer Degradation and Stability 97(4) 597-604.

A. Rodríguez-Contreras, M. Calafell-Monfort, M. S. Marqués-Calvo (**2012**). Enzymatic degradation of poly(3-hydroxybutirate) by a commercial lipase. Polymer Degradation and Stability 97: 2473-2476.

A. Rodríguez-Contreras, M. Koller, M. Miranda-de Sousa Dias, M. Calafell, G. Braunegg, M. S. Marqués-Calvo (**2013**). Novel Poly[(R)-3-hydroxybutyrate]-producing bacterium isolated from a Bolivian hypersaline lake. Food Technology and Biotechnology 51. **Accepted**.

A. Rodríguez-Contreras, M. Koller, M. Miranda-de Sousa Dias, M. Calafell, G. Braunegg, M. S. Marqués-Calvo (**2013**). High production of poly(3-hydroxybutyrate) from a wild *Bacillus megaterium* Bolivian strain. Journal of Applied Microbiology. **Accepted**.

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Conferences

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> A. Rodríguez, M. Calafell, M.S. Marqués, F. Roman and J. Bou. *Study of chemical and enzymatic degradation of bacterial poly(3-hydroxybutirate).*

Congress of Industrial Microbiology and Microbial Biotechnology (CMIBM2008): Barcelona (Spain), from12th to 14th November, 2008. Poster and abstract:

> A. Rodríguez, M. Koller, M. Calafell, M.S. Marqués-Calvo. *Study for poly [(R)-3-hydroxybutyrate] production by microorganisms from natural water samples.*

12th European Congress (EPF'09): Graz (Austria) from 12th to 17th of July, 2009. Poster and abstract:

A. Rodríguez-Contreras, G. García-Grillasca, J. Bou, M. Calafell, M.S Marqués-Calvo. *Study of enzymatic degradation of bacterial poly(3-hydroxybutirate).*

14th European Congress on Biotechnology (ECB14): Barcelona (Spain) from13th to 16th September, 2009. Posters and abstracts:

A. Rodríguez-Contreras, G. García-Grillasca, M.Calafell, J.J. Bou, M.P. Almajano, M.S Marqués-Calvo. *Study of enzymatic degradation of bacterial poly(3-hydroxybutyrate).*

A. Rodríguez Contreras, M. Koller, M. Dias, G. Braunegg, M. Calafell, M. S. Marqués-Calvo. *Study for poly[(R)-3-hydroxybutyrate] by new microorganism production from natural water samples.*

M. Miranda de Sousa Dias, M. Koller, A. Rodríguez-Contreras, A. Reiterer, M. Calafell, J. Bou, G. Braunegg. *Study of the influence of saponificated sunflower oil in the growth of Pseudomonas oleoverans ATCC 29347 aiming the production of medium length chain PHAs - appliance of a novel method utilizing emulsifier.*

I want to take this opportunity to express gratitude to

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My family

My closest friends

Everybody who somehow have participated for the conception of this PhD thesis

Abreviations

ADM	Archer daniels midland
Acetyl-CoA	Acetyl coenzyme A
ATP	Trifosfato de adenosina
Mw	Average mass of the molar mass
Mn	Average number of the molar mass
TCA	Carboxylic acid cycle circle, citric acid cycle, Krebs cycle, Szent-Györgyi-Krebs cycle
CDM	Cell dry mass
CO_2	Carbon dioxide
CoA	Coenzime A
DSC	Differential scanning calorimeter
DSM	*Deutsche Sammlung von Mikroorganismen*
DDS	Drug delivery systems
EC	Enzyme Commission number
KDPG	Enter-Doudoroff pathway
EE	Entrapment efficiency
e-PHA	Extracellular PHA depolymerases
EPS	Expanded polystyrene
EU	European union
CDM	Cell dry mass
DOXY	Doxycycline
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatograph
GLP	Glycerol liquid phase
GmbH	Gesellschaft mit beschränkter Haftung/ company with limited liability
GPC	Gel performance chromatography
hydroxyacyl-CoA	Hydroxylacyl coenzime a
HPLC	High performance liquid chromatography
HMW	High-molecular-mass
HA	Hydroxyalkanoate
3HB	3-hydroxybutyrate
3HV	3-hydroxyvalerate
3HHx	3-hydroxyhexanoate
3HD	3-hydroxydecanoate
3HO	3-hydroxyoctanoate
4HB	4-hydroxybutyrate

3H4PE	3-hydroxy-4-pentenoic acid
ICI	Imperial chemical industries
i-PHA	Intracellular PHA depolymerases
ISAB	Ionic strength adjustment buffer
Ltd	Limited company
LCL	Long-chain-length
LPS	Lipopolysaccharides
LMW	Low molecular mass
MCL	Medium-chain-length
ΔH_m	Melting enthalpy
ME	Method efficiency
NADP($^+$/H)	Nicotinamida adenina dinucleótido fosfato
NADH	Nicotinamida adenín dinucleótido
NMR	Nuclear magnetic resonance
Mp	Peak-maximum of the molar mass
OD	Optical density
pO_2	Oxygen partial pressure
P&G	Procter & gamble company
PDI	Polydispersity index
PE	Polyethylene
PET	Polyethyleneterepthalate
PCL	Polycaprolactone
PGA	Polyglycolic acid
PHA	Polyhydroxyalcanoates
P(3HB-HHx)	Poly(3-hydroxybutyrate-co-hexanoate)
P(3HB-co-3HA)	Poly(3-hydroxybutirate-co-3-hydroxyacyl
P(3HB-4HB)	Poly(3-hydroxybutyrate-co-4-hydroxybutyrate)
P(3HB-3HD)	Poly(3-hydroxybutyrate-co-3-hydroxydecanoate)
P(3HB-3HP)	Poly(3-hydroxybutyrate-co-3- hydroxypropionate)
P(3HB-3HV)	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
P(3HB-co-3MP)	Poly(3-hydroxybutyrate-co-3-mercaptopropionate)
PLGA	Poly(lactate-*co*-glycolate)
PLA	Polylactic acid
PP	Polypropylene
PHB	Poly[(r)-3-hydroxybutyrate]
PS	Polystyrene
PVC	Polyvinyl chloride
RB	Residual biomass

RID	Refractive index
rRNA	Ribosomal ribonucleic acid
SCL	Short-chain-length
CECT	Spanish type culture collection
TEM	Transmission electron microscopy
TCA	Citric acid cycle, Krebs cycle, the Szent-Györgyi-Krebs cycle
UK	United kingdom
USA	United States of America
UV	Ultra violet

Abbreviations and symbols for the equations

X	Cell concentration
X_c	Crystallinity degree
X	Dry biomass
F	Flow rate
T_g	Glass transition temperature
r_x	Growth rate
X_0	Initial cell concentration at inoculation
m	Mass
$M_¡$	Mass flow of A entering the reactor
M_i	Mass flow rate entering the reactor
M_o	Mass flow rate leaving the reactor
R_G	Mass flow rate of generation by reaction
R_C	Mass rate of consumption
X_{max}	Maximum cell concentration
μ_{max}	Maximum specific growth rate
M_w	Molecular mass
T_m	Melting temperature
P	Product concentration
P_{batch}	Productivity in batch culture
X_R	Residual biomass concentration
K_s	Saturation constant
k_d	Specific death constant
μ	Specific growth rate
q_p	Specific production rate
S	Substrate mass
t	Time
V	Volume

Q_p	Volumetric productivity
r_d	Volumetric rate of cell death
r_x	Volumetric rate of biomass formation
v/v	Volume/ volume
w/v	Weight/volume
Y	Yield
$Y_{x/s}$	Yield of cell mass per unit mass substrate utilized
$Y_{p/n}$	Yield of product mass per unit mass of substrate utilized
λ	Wave length

***Bacillus megaterium* strain uyuni S29, 16S ribosomal RNA gene, partial sequence**
GenBank: JF508445.1[1]

LOCUS JF508445 881 bp DNA linear BCT 07-MAY-2011
DEFINITION Bacillus megaterium strain uyuni S29 16S ribosomal RNA gene, partial sequence.
ACCESSION JF508445
VERSION JF508445.1 GI:331687455
SOURCE Bacillus megaterium
ORGANISM Bacillus megaterium
Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus.
REFERENCE 1 (bases 1 to 881)
AUTHORS Rodriguez-Contreras,A., Koller,M., Miranda-de Sousa Dias,M., Calafell,M., Braunegg,G. and Marques-Calvo,M.S.
TITLE Poly[(R)-3-hydroxybutyrate] with new thermal properties produced by a novel bacteria isolated from hypersaline lakes
FEATURES Location/Qualifiers
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[1] http://www.cect.org/bacterias.php

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