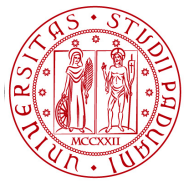

UNIVERSITÀ
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DI PADOVA

Department of Agronomy, Food, Natural Resources, Animal and Environment

PhD COURSE OF CROP SCIENCE

CYCLE: XXVI

Biopolymer production from agro-industrial wastes: bacterial strains selection and genetic improvement.

Head of Course: Ch.mo Prof. Antonio Berti

Supervisors: Ch.mo Prof. Sergio Casella

Ch.ma Prof.ssa Marina Basaglia

Co-supervisor: Dr.ssa Silvana Povolo

PhD student: Maria Giovanna Romanelli

Declaration

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma of the university or other institute of higher learning, except where due acknowledgment has been made in the text.

31 January 2014 Maria Giovanna Romanelli

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I pensieri sono perle false finché non si trasformano in azioni.

Sii il cambiamento che vuoi vedere avvenire nel mondo.

(Mahatma Gandhi)

To Patrizia Stefani

Table of Contents

Abstract

This work is included in a wider research program carried out at the Dipartimento di Agronomia Animali Alimenti Risorse Naturali e Ambiente (DAFNAE) within the EU project ANIMPOL (*Biotechnological conversion of carbon containing wastes for eco-efficient production of high added value products*). The aim of the entire project was to produce high-added-value products using inexpensive agricultural by-products as carbon source. The fatty by-products from slaughterhouses have been adopted for its conversion into biopolymers such as polyhydroxyalkanoates (PHA) by properly selected and/or developed microbes.

In more detail the objective of this research was to look for, select and characterise bacterial strains able to utilise, as carbon source, low cost industrial lipid wastes such as triacylglycerols (TAGs) from animal fats, with the final goal to produce PHAs. For this reason lipolytic bacteria were isolated from a variety of different environments, such as soil or waste water of a slaughterhouse. Several bacterial strains were found to possess remarkable lipolytic activities but not efficient PHAs production capabilities.

As a consequence, a molecular biology program started in order to obtain a microbial strain capable of both hydrolysing lipids and producing high levels of PHAs.

Delftia acidovorans DSM39, a well-known producer of PHAs with high molar fractions of 4-hydroxybutyrate (4HB), although unable to metabolize lipids, was selected as host strain. On the other hand, *Pseudomonas stutzeri* BT3, the most efficient lipase producer among the obtained isolates, was designated as potential donor of lipolytic genes.

The *lipC* and *lipH* sequences of *P. stutzeri* BT3 were successfully co-expressed into *D. acidovorans* DSM39 and the resulting recombinant strain displayed high extracellular enzymatic activity on corn oil. The PHAs production from corn oil achieved high levels (26% of cell dry weight, with about 7% of 4HB). Surprisingly, the recombinant strain produced greater values directly from slaughterhouse residues such as udder and lard (43 and 39%, respectively, with almost 7% of 4HB).

Moreover, this work proved the ability of the recombinant *D. acidovorans* DSM39 strain to produce PHAs with significant percentage of 4HB, without the supplementation of any precursor in the liquid broth. This research paves the way to the efficient one-step

conversion of fatty residues into PHAs having valuable properties exploitable in several medical and industrial applications.

Riassunto

Questo lavoro è inserito in un ampio programma di ricerca svolto presso il Dipartimento di Agronomia Animali Alimenti Risorse Naturali e Ambiente (DAFNAE), nell'ambito del progetto europeo ANIMPOL (*Biotechnological conversion of carbon containing wastes for eco-efficient production of high added value products*). Lo scopo dell'intero progetto era di ottenere prodotti ad alto valore aggiunto utilizzando sottoprodotti agricoli a basso costoso, come fonte di carbonio.

I grassi, sottoprodotti dell'industria della lavorazione della carne, sono stati utilizzati per la conversione in biopolimeri, come poilidrossialcanoati (PHA), da parte di microbi adeguatamente selezionati e /o ricombinanti.

In particolare, l'obiettivo di questa ricerca è stato quello di cercare, selezionare e caratterizzare ceppi batterici in grado di utilizzare, come fonte di carbonio a basso costo, trigliceridi derivati da grassi animali, con l'obiettivo finale di produrre PHA. Per questo motivo, batteri lipolitici sono stati isolati da differenti nicchie ecologiche, come suolo o acque reflue di un macello. Diversi ceppi batterici hanno dimostrato notevoli attività lipolitiche ma non efficienti capacità di produrre PHA.

Di conseguenza è stato avviato un programma di biologia molecolare per ottenere un ceppo microbico capace di idrolizzare i trigliceridi e di usarli per produrre elevati livelli di PHA. *Delftia acidovorans* DSM39, noto produttore di PHA con consistenti frazioni molari di 4-idrossibutirrato (4HB), è stato selezionato come ceppo ospite, anche se incapace di metabolizzare i lipidi. *Pseudomonas stutzeri* BT3, selezionato per la sua elevata attività lipolitica tra gli isolati microbici ottenuti, è stato designato come potenziale donatore di geni lipolitici.

Le sequenze codificanti per i geni della lipasi *lipC* e *lipH* di *P. stutzeri* BT3 sono state co-espresse con successo in *D. acidovorans* DSM39. Il risultante ceppo ricombinante ha dimostrato promettenti attività enzimatiche extracellulari a partire da olio di mais. La produzione di PHA da olio di mais ha raggiunto livelli elevati (26 % del peso secco cellulare, con circa il 7% di 4HB). Sorprendentemente, il ceppo ricombinante ha prodotto valori nettamente superiori utilizzando direttamente, come fonte di carbonio, scarti di macello quali grasso di mammella bovina e lardo (43 e 39 %, rispettivamente, con quasi il 7 % di 4HB).

Inoltre, questo lavoro ha dimostrato la capacità del ceppo ricombinante *D. acidovorans* DSM39 di produrre PHA con percentuale significativa di 4HB, senza l'impiego, nel liquido colturale, di specifici precursori.

In base ai risultati preliminari finora conseguiti, questo studio rappresenta un primo passo verso lo sviluppo di microrganismi idonei alla conversione one-step di sottoprodotti lipidici in PHA con promettenti proprietà chimico-fisiche sfruttabili in numerose applicazioni mediche e industriali.

1. Introduction

1.1. Plastics and the environment

1.1.1. Plastic waste

Most plastics are made from petroleum that is a non-renewable resource and a relevant amount of additional energy is also required for its extraction and processing. The utilization of constrained fossil resources for the production of polymers significantly contributes to current problems like “greenhouse effect” and “global warming”. This is caused by the fact that these materials are utilized only during a relatively short time span. After that, they are often incinerated, elevating the atmospheric CO_2 concentration. The main problem arising from incineration of plastics is the same as for energy recovery from fossil feedstocks: carbon that was fixed during millions of years, and within this time was not part of the natural carbon cycle, is converted to CO_2. Afterwards the CO_2 can accumulate in the atmosphere, contributing to the mentioned climatic effects. In addition, incineration of plastics often generates toxic compounds (Braunegg *et al.,* 2004). Besides, more and more waste of highly resistant plastics that are not incinerated is piled up every year because this material is almost completely not biodegradable. Recycling systems demand a certain degree of purity and a high sorting accuracy. The collection costs are fairly high, and recycling has a negative impact on the quality of the materials, such as an increase in brittleness (Braunegg *et al*., 2004). In addition to these ecological considerations, the price of crude oil is unpredictably fluctuating, not least due to miscellaneous developments in the global political situation. This constitutes a factor of immense uncertainty especially for the highly petrol-dependent polymer industry.

Currently, the production plastic materials demands about 5-7% of the entire delivered amounts of fossil feedstocks. This percentage is forecasted to rise significantly during the next decades as a consequence of the increase of economy and life level of emerging countries and countries in transition. Furthermore data for the remaining amounts of fossil oil in the earth's interior are changing quickly due to advanced methods for tracing and discharging of mineral oils. Finally, one day fossil feedstock will be completely depleted.

1.1.2. Biodegradable plastics

The safe and efficient distribution of goods worldwide requires a rapidly increasing production of polymeric compounds acting as packaging materials. Furthermore, polymers have a growing importance as niche products for special applications as in the medical field.
Therefore, taking into account the current environmental problems, the involved industrial branches are much more aware of the necessity to promote novel biopolymers production techniques based on renewable resources.
In fact, sustainable production of polymers, fine chemicals, bulk chemicals and fuels could be achieved by the so called 'white biotechnology', which includes the action of living organisms, or their microbial metabolites (such as enzymes) or the use of renewable resources as starting material.
In these contexts, the social benefits obtained by shifting from fossil-based to bio-based plastics could be enormous, especially if these polymers are biodegradable or, even more, if they are also compostable. Indeed, bio-based materials have the potential to produce fewer greenhouse gases, require less energy, and produce less toxic pollutants over their lifecycle when compared to products made from fossil fuels.
The recalcitrance of common plastic to be degraded by microorganisms derives from their chemical-physical characteristics, such as the high molecular weight, high degree of branching, the insolubility in water, the presence of additives (harmful for microorganisms themselves) or of functional groups that prevent the attack of microbial enzymes (Titz *et a*l., 2012).
A product hazard for the environment is assessed by estimating the degree of its degradation and depends on the time needed for its complete mineralization, i.e. its oxidation to CO_2 and H_2O (biodegradation).
According to the International Standard Organization (ISO) a polymer is classifiable as biodegradable if its chemical structure can be modified when subjected to the action of organisms (such as bacteria, fungi, algae ...) giving rise to the loss of some typical properties of the starting material. The biodegradability shall be demonstrated for each of its significant organic constituent present in greater than 1% with respect to the dry mass. Basically, ISO allowed organic constituents whose non-biodegradable component appears in a quantity not exceeding 1%.

Biodegradable polymers are certified according to any of the following legally binding international standards:

- ISO 17088:2012,
- EN 13432:2002, EN 14995:2006
- ASTM D6400-12

According to the UNI EN 13432:2002 features, a compostable material should have the following characteristics:

- **Biodegradability** determined by measuring the actual metabolic conversion of the compostable material into carbon dioxide. This property is quantitatively assessed with the standard test method UNI EN14046 (also published as "ISO 14855: biodegradability under controlled composting conditions"). The level of acceptance is equal to 90% to be reached in less than six months.
- **Disintegrability** assessed by the fragmentation and loss of visibility in the final compost (absence of visual contamination). It is measured by a composting test pilot scale (UNI EN14045), where the test material is biodegraded together with organic waste for three months and in the end the compost is sieved with a sieve of two millimeters. The residues of the test material larger than two millimeters are considered not disintegrated and this fraction must be less than 10% of the initial mass. No adverse effects on the composting process: requirement occurred with a composting test pilot scale.

Low levels of heavy metals must be below the default values and should not have any adverse effects on the quality of the compost (e.g. the reduction of the agronomic value and presence of eco-toxicological effects on plant growth). A test of plant growth (OECD test 208 modified) is carried out on samples of compost where degradation of the test material has taken place. There should be no difference from control compost.

Other physico-chemical parameters which must not differ from the control compost after the degradation are: pH, volatile solids, nitrogen, phosphorus, magnesium, potassium and salt content.

The UNI EN 13432:2002 is a harmonized standard, which was reported in the "Official Journal of the European Union". Therefore, it must be transposed at national level in

European countries and when actuated it would provide a presumption of conformity with the European Directive 94/62 EC on packaging and packaging waste. For assessing the compostability of the plastic material the UNI EN 14995:2007 “Plastics Scheme of testing and specification” specifies the requirements and the procedures for the determination of the anaerobic treatment of plastics with reference to the biodegradability and the disintegrability during the biological treatment (effect on the quality of the resulting compound).

Unfortunately, there are some significant and valid concerns about biopolymers. For example, the current use of genetically modified corn as a feedstock for PLA (PolyLactic Acid) is a major concern as much as the environmental impacts of producing corn. Furthermore, the inclusion of potentially harmful materials in manufacturing raises other alarms. In addition, the recycling and disposal of these products are potential problems, especially for the impact on the current recycling and disposal infrastructure. For example, bottles made with PLA can contaminate the recycling of polyethylene terephthalate (PET) bottles. Since most recycling technologies are unable to distinguish between the two types of plastic, many recyclers oppose the use of PLA until the recycling technology is capable of weeding out products made with PLA. In order to maximize the overall benefits of biopolymers, these issues need to be addressed without impeding their commercial viability. This will likely require a combination of policy incentives and regulations, private-public engagement and support, and market development supporting economic, environmental and social objectives.

Current and future research developments in biodegradable polymers relate mainly to the scaling-up of production and improvement of product properties. Large scale production will definitively increase the supply of bioplastic thus reducing their prices.

1.2. Polymers, bioproducts, bioplastics

Polymers are natural or synthetic compounds structured as chains of repeating basic molecular units (monomers). They are classified as macromolecules, due to their high molecular weight. Schematically, the polymeric macromolecules can be divided into:

- Natural polymers, such as proteins composed of amino acids
- Synthetic polymers, traditional plastics such as polyethylene and polyvinyl chloride

- Biopolymers, such as, PHA.

Cellulose, starch and chitin, proteins and peptides, DNA and RNA are all examples of natural polymers produced by living organisms. The term “biopolymer” (or "bioplastic") is actually used as synonym of bioplastic but two different criteria underline its definition: the source of the raw materials and the biodegradability of the polymer.

According to these criteria it is possible to differentiate between three types of biopolymers:

(1) Biopolymers that are made from renewable raw materials (bio-based) and are biodegradable.

(2) Biopolymers that are made from renewable raw materials (bio-based) and are not biodegradable.

(3) Biopolymers that are made from fossil fuels and are biodegradable.

Furthermore, the bioproducts composed by biopolymers can be divided into two broad groups, namely biodegradable and non-biodegradable, or alternatively, into bio-based and non-bio-based bio-products.

Bio-products can be also classified on the ways in which they respond to heat as thermoplastics, thermosets (Raquez *et al*., 2010) or elastomers. Finally, it is noteworthy that bioproducts blends are mixtures of polymers from different origins, while biocomposites are biopolymers or synthetic polymers reinforced with natural fibers and/or fillers and additives.

According to the European Bioplastics association, a bioplastic is either bio-based, biodegradable, or features both properties. For example biopolyethylene derive from sugarcane is classified as bioplastic because it is bio-based but it is also considered bioplastic even if not biodegradable (European Bioplastics Association, 2008).

1.2.1. The bio-based polymers

"Bio-based" is a term focused on raw materials, and is applied to polymers derived from renewable resources. Raw materials are defined as renewable if they are replenished by natural procedures at rates comparable or faster than their rate of consumption (Ravenstijn *et al.,* 2010).

Bio-based polymers may be divided into three main categories according to their origin and

production:

(1) Polymers directly extracted/removed from biomass. Examples are polysaccharides such as starch and cellulose, and proteins like casein and gluten.

(2) Polymers produced by classical chemical synthesis using renewable bio-based monomers. A good example is PLA, a biopolyester polymerized from lactic acid monomers. The monomers themselves may be produced via fermentation of carbohydrate feedstock.

(3) Polymers produced by microorganisms or genetically modified bacteria. To date, this group of bio-based polymers consists mainly of polyhydroxyalkanoates, but developments with bacterial cellulose are in progress.

Currently, there are no standards quoting what can be called as "bio-based product". However, there are objective ways to quantify the bio-based content of a product. ASTM (American National Standards Institute) and ISO have developed standards for measuring the bio-based content of materials via carbon isotope analysis. Relevant standards include: AST D6866-12 and ASTM D7026-04.

The most widely used biopolymers are:

- Starch thermoplastic, constituting about 50% of the bioplastic market, which represents the most widely used bioplastic. Flexibiliser and plasticizer are added so the starch can also be processed thermo-plastically. By varying the amounts of these additives, the properties of the material can be tailored to specific needs (also called "thermo-plastical starch"). Industrially, starch based bioplastic are often blended with biodegradable polyesters. These blends are mainly starch/polycaprolactone or starch/Ecoflex (polybutylene adipate-co-terephthalate produced by BASF) (BASF, Germany; PHBISA, Brazil). These blends remain compostables. Other producers, such as Roquette, have developed another strategy based on starch/polyolefine blends. These blends are no longer biodegradables, but display a lower carbon footprint compared to the corresponding petroleum based plastics (Bergeret, 2011).
- Cellulose bioplastic are mainly the cellulose esters and their derivatives.
- PLA is a transparent plastic produced from corn. Corn starch is hydrolysed to glucose, which is converted into lactic acid and then subjected to polymerization.
- Polyhydroxyalkanoates (PHAs) are aliphatic polyesters produced directly by microorganisms through fermentation of the carbon substrate from natural

substances or from wastes of industrial processes. PHAs can be produced as homopolymers (P(3HB)) or as copolymers (for example, P(3HB-*co*-3HV). The final properties obviously depend on the content of co-monomers.

These last polymers (PHAs) attracted the attention of both the scientific community and the industries because of their promising properties, and also for the possibility to be produced from a wide range of different cheap by-products. For this reason the research and investigation on specific microbial species able to produce PHAs represent the subject of the present study.

1.3. Polyhydroxyalkanoates

1.3.1. General information

PHAs are polyesters produced by numerous prokaryotic strains starting from renewable sources like carbohydrates, lipids, alcohols or organic acids classically under unfavourable growth conditions due to imbalanced nutrient supply (Koller *et al*., 2010).

PHAs are accumulated as carbon/energy storage or reducing power materials by over 30% of soil-inhabiting bacteria (Wu *et al*., 2000) usually under the condition of limiting nutritional elements such as N, P, S, O, or Mg and in the presence of excess carbon source (Lee *et al*., 1996). Many bacteria in activated sludge, in open ocean, and in extreme environments are also capable of producing PHA as reported in Table 1.1.

Table 1.1.

PHA-accumulating microbial genera (Koller *et al.,* 2010)

Acidovorax	*Erwinia*	*Oscillatoria*[a]
Acinetobacter	*Escherichia (wil tipe)*[d]	*Physarum*[e]
Actinobacillus	*Ferrobacillus*	*Paucispirillum*
Actinomycetes	*Gamphospheria*	*Pedomicrobium*
Aeromonas	*Gloeocapsa*[a]	*Photobacterium*
Alcaligenes [a,b]	*Gloeothece*[a]	*Protomonas*
Allochromatium	*Haemophilus*	*Pseudomonas*[a,b]
Anabaena[b]	*Halobacterium* [a,c]	*Ralstonia* [a,b]
Aphanothece[a]	*Haloarcula* [a,b,c]	*Rhizobium*[a,b]
Aquaspirillum	*Haloferax*[a,b,c]	*Rhodobacter*
Asticcaulus	*Halomonas*[a]	*Rhodococcus*[b]
Azomonas	*Haloquadratum*[c]	*Rhodopseudomonas*
Azospirillum	*Haloterrigena*[c]	*Rhodospirillum*[b]
Azotobacter[a,b]	*Hydrogenophaga*[a,b]	*Rubrivivax*
Bacillus[a,b]	*Hyphomicrobium*	*Saccharophagus*
Beggiato[a]	*Klebsiella (recombinant)*	*Shinorhizobium*
Beijerinckia[b]	*Lamprocystis*	*Sphaerotilus*[a]
Beneckea	*Lampropedia*	*Spirillum*
Brachymonas	*Leptothrix*	*Spirulina*[a]
Bradyrhizobium	*Methanomonas*	*Staphylococcus*
Burkholderia[a]	*Methylobacterium*[b]	*Stella*
Caryophanon	*Methylosinus*	*Streptomyces*
Caulobacter	*Methylocystis*	*Synechococcus*[a]
Chloroflexus	*Methylomonas*	*Syntrophomonas*
Chlorogloea[a]	*Methylovibrio*	*Thiobacillus*
Chromatium	*Micrococcus*	*Thiococcus*
Chromobacterium	*Microcoleus*	*Thiocystis*
Clostridium	*Microcystis*	*Thiodictyon*
Comamonas[a,b]	*Microlunatus*[b]	*Thiopedia*
Corynebacterium[b]	*Moraxella*	*Thiosphaera*
Cupriavidus[a,b]	*Mycoplana*[a]	*Variovorax*[a,b]
Cyanobacterium[b]	*Nitrobacter*	*Vibrio*
Defluviicoccus[b]	*Nitrococcus*	*Wautersia*[a,b] *(today Cupriavidus)*
Derxia[b]	*Nocardia*[a,b]	*Xanthobacter*
Delftia[a,b]	*Nostoc*	*Zoogloea*[a]
Ectothiorhodospira	*Oceanospirillum*	
Erwinia	*Paracoccous*	
Escherichia (recombinant)[a]	*Paucispirillum*	

a detailed knowledge about growth and production kinetics available
b accumulation of copolyesters known
c archaea
d PHA found in cell membranes
e eukaryotic genera with poly-b-malic acid (PMA) production known

PHAs are deposited as intracellular water-insoluble inclusions and become refractive granules that are clearly evident in PHA-rich cells using a light-optical microscope. These granules have a typical diameter of 0.2-0.7 µm and consist of 97.7 % PHA, 1.8 % protein and 0.5 % lipids. Proteins and lipids form a coat membrane around the core region. When available carbon is limiting, microorganisms completely degrade the PHAs internal granules producing water and CO_2, thus providing the cell with an advantage for surviving during starvation periods.

1.3.2. Structure of PHAs

PHAs are molecules with a simple structure, synthetized by a wide range of different bacteria: Gram-positive and Gram-negative, aerobic and anaerobic. They are accumulated in an amount ranging from a few granules up to 90% of the dry weight of the cell.

PHAs have different interesting properties in relation to the structures they assume. Depending on the bacterial species and growth conditions, homopolymers, random copolymers or block copolymers of PHA can be produced. In fact, PHAs compose a large group of polymers extremely various for the structure. Over 150 different PHA monomers are being reported (He *et al.* 1999). This variability depends from the number of CH_2 groups in the principal chain and from the alkyl group in the (R) position (Fig. 1.1).

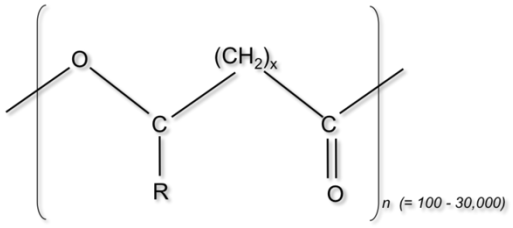


Figure 1.1. Chemical structure of PHA

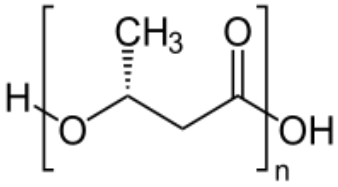
According to the number of carbon atoms of their building blocks, PHAs are divided into three different groups:

- short-chain-length (*scl*) PHAs: 3–5 carbon atoms
- medium-chain-length (*mcl*) PHAs: 6–14 carbon atoms

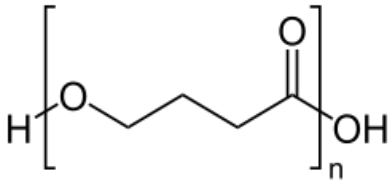
- long-chain-length (*lcl*) PHAs: more than 15 carbon atoms (until today, only *in vitro* production has been described; *lcl*-PHA building blocks have not been detected yet in naturally occurring PHAs).

Examples of monomers of PHAs considered in this work thesis are:

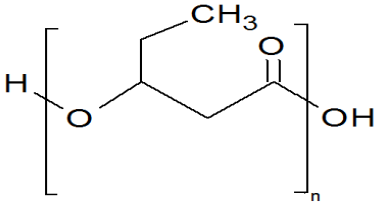
- 3 hydroxybutyrate (3HB) is the most frequent microbial monomer. 3HB contains one methyl group in the lateral chain and three carbon atoms in the principal chain.



- 4-hydroxybutyrate (4HB) forms a copolymer with 3HB. This polymer is particularly important for its mechanical and physical properties. The principal chain of 4HB has four carbon atoms and no side chain is present.



- 3-hydroxyvalerate (3HV) forms particular copolymer with 3HB. 3HV have one ethyl group in lateral chain and the principal chain is composed of three carbons.



The type and the number of repetition (n) of the monomers (Fig. 1.1) ranging from 100 to 30000 is variable and determine the identity of the polymer. The monomer carboxyl group form an ester bond with the hydroxyl group of the consecutive monomer, thereby creating the polymer.

1.4. PHAs synthesis

1.4.1. PHAs metabolisms

PHAs can be synthesized from fatty acids or other carbon sources and their final aliphatic composition depends on the growth substrate. Acetyl-CoA is the key molecule for their synthesis, as it provides the monomers of 3-hydroxyalkanoyl-CoA of different length at the PHA synthetase. In addition, 3-hydroxyalkanoyl-CoA can also be supplied from β-oxidation of fatty acids of different chain lengths. A number of genes, encoding various enzymes, are directly or indirectly involved in PHA synthesis. So far, eight different synthetic pathways have been identified for different groups of microorganisms. The first pathway involves the three key enzymes beta-ketothiolase, NADPH-dependent acetoacetyl-CoA reductase and PHA synthase, encoded by genes *phaA*, *phaB*, and *phaC*, respectively.

The second PHA synthesis pathway is connected to the absorption of fatty acids by the organism. After the beta-oxidation of the acyl chains, acyl-CoA enters in the process of synthesis of PHA monomers. The enzymes required for this pathway include 3-ketoacyl-CoA reductase, epimerase, R-enoyl-CoA hydratase, acyl-CoA oxidase (putative), enoyl-CoA hydratase I (putative). Cases of PHAs production from this pathways are: mcl-PHA, or/and P(3HB-*co*-HHx) P(3HB) copolymers (Chen *et al*., 2010). Concerning the remaining six metabolic pathways, all enzymes involved are summarized below in Fig. 1.2 and Tab. 1.2.

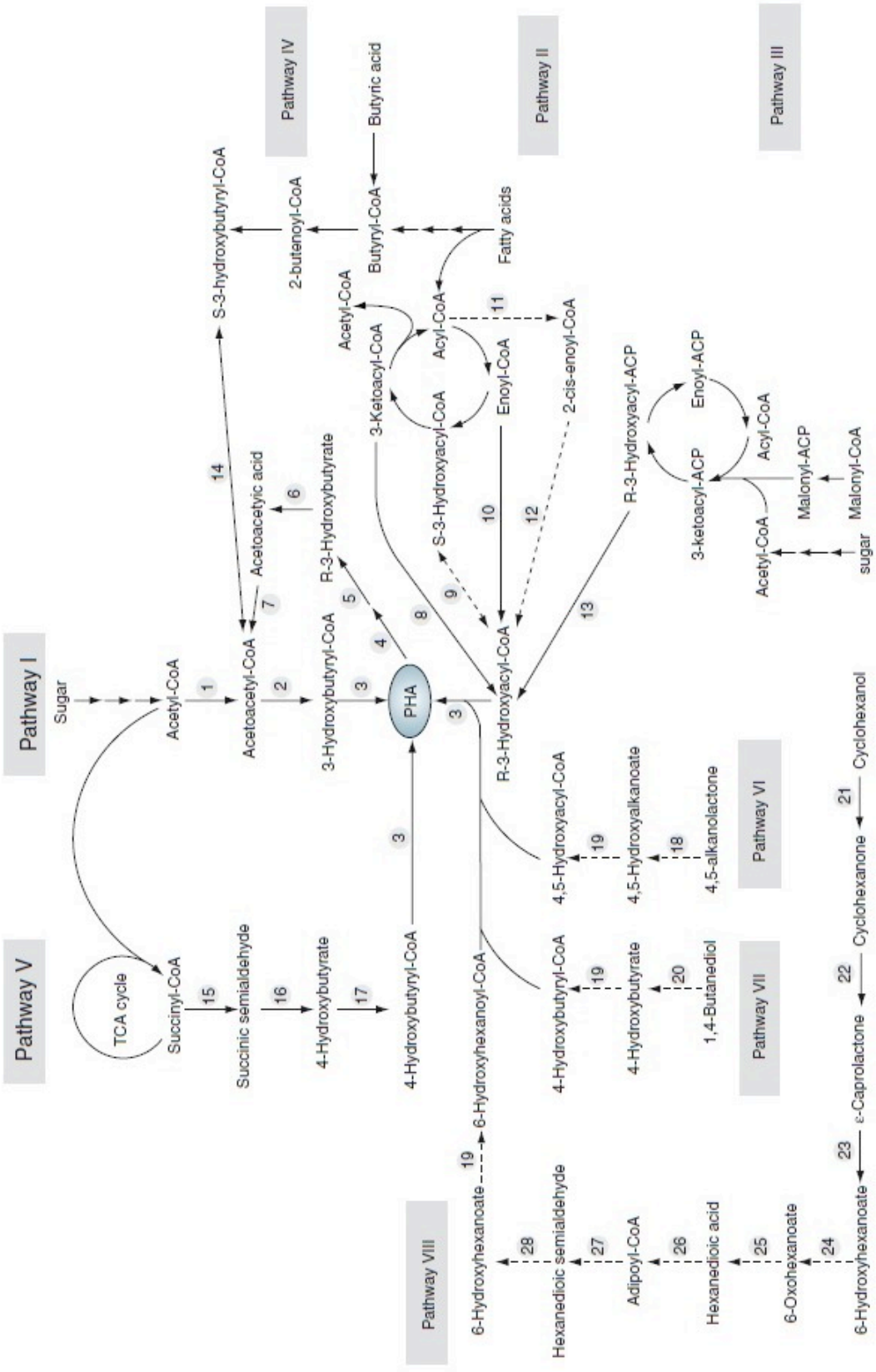


Figure 1.2.

Scheme of the eight metabolic pathways of PHA synthesis (Chen *et al*., 2010).

Table 1.2.

Genes and enzymes associated with the production pathways of PHA in bacteria (Chen *et al.,* 2010).

Pathway	Abbreviation	Enzime
I	PhaA	β-Ketothiolase
	PhaB	NADPH dependent acetoacetyl-CoA reductase
	PhaC	PHA synthase
Associated	PhaZ	PHA depolymerase
way		Dimer hydrolase
		(R)3-Hydroxybutyrate dehydrogenase
		Acetoacetyl-CoA synthetase
II	FabG	3-Ketoacyl-CoA synthetase
		Epimerase
	PhaJ	Enoyl-CoA Hydratase/enoyl-CoA Hydratase I
		Acyl-CoA oxidase, putative
		Enoyl-CoA hydratase I, putative
III	PhaG	3-Hydroxyacyl-ACP-CoA transferaseMalonyl-
	FabD	CoA-ACP transacylase
IV		NADH-dependent acetoacetyl-CoA reductase
	SucD	Succinic semialdehyde dehydrogenase
V	4hbD	4-Hydroxybutyrate dehydrogenase
	OrfZ	4-Hydroxybutyrate-CoA:CoA transferase
VI		Lactonase, putative
		Hydroxyacyl-CoA synthase, putative
VII		Alcohol dehydrogenase, putative
VIII	ChnA	Cyclohexanol dehydrogenase
	ChnB	Cycloexanone monooxygenases
	ChnC	Caprolactone hydrolase
	ChcD	6-Hydroxyhexanoate dehydrogenase
	ChnE	6-Oxohexanoate dehydrogenase
		Semialdehyde dehydrogenase, putative
		6-Hydroxyhexanoate dehydrogenase, putative
		Hydroxyacyl-CoA synthase, putative

In most of cases, bacteria are able to produce PHB, preferentially short-chain-length (scl) PHA copolymers consisting of C3-C5 chains, including P[(R)-3-hydroxybutyrate-*co*-4-hydroxybutyrate] P(3HB-*co*-4HB) (Saito *et al*., 1996) and poly[(3- hydroxybutyrate-co-*3*-hydroxyvalerate] P(3HB-*co*-3HV) (Alderete *et al.,* 1993).

For instance, *Cupriavidus necator* and *Azohydromonas lata* can polymerize only 3-hydroxyalkanoates (3HAs) consisting of 3–5 carbon atoms, while *Pseudomonas putida* only produces 3HAs of 6–14 carbon atoms. This different behaviour is due to the specificity of the PHA synthase, responsible for polymerization of hydroxyalkanoic acid thioesters. Due to this high enzyme specificity, it was believed that only one strain could not produce both scl-PHAs and mcl-PHAs. On the contrary, some exceptions have been found, as for example the production of a P(3-hydroxybutyrate-co-3-hydroxyhexanoate) copolyester by strains of *Aeromonas* sp., when grown on fatty acids of more than 12 carbon atoms (Shimamura *et al*., 1994; McCool *et al.,* 2001), or the production of a random copolyester of different 3HAs consisting of 4–12 carbon atoms from gluconate by a *Pseudomonas* strain (Abe *et al*., 1994).

1.4.2. Biodegradation of PHAs

As stated before, granules of P(3HB) may be hydrolysed when the bacteria require carbon source. In fact, the energy and 3-hydroxybutyric acid released during the granules degradation allow bacteria to grow and survive when external carbon becomes limiting, thus sustaining at least one or two cell divisions, even in the absence of exogenous carbon sources.

All research carried out on the biodegradation of P(3HB) led to a clear distinction between extracellular and intracellular degradation. Extracellular degradation involves the use, even by microorganisms not necessarily producers, of a source of exogenous carbon represented by P(3HB) released into the environment by producing bacteria that are no longer viable. The ability to degrade this polymer is widely distributed among bacteria and several fungi, and depends on the secretion of specific enzymes such as poly-3HB-depolymerases. The extracellular depolymerases are carboxyiesterases that hydrolyse water-insoluble polymers into monomers and soluble oligomers, which are absorbed into the cells as nutrients and then metabolised (Jendrossek *et al*., 2002).

On the other hand, intracellular degradation consists in the active mobilization of endogenous carbon (Jendrossek *et al.,* 2001) by an intracellular depolymerase, encoded by *phaZ* gene, as reported by Saegusa and coworkers (2001), that showed no similarity with known extracellular depolymerases (Fig. 1.3).

It has been reported that this protein, designated as PhaZ1, is actively expressed and its properties were examined in raw extracts. PhaZ1 is a protein of 47 kDa and its activity involves the conversion of the poly-3HB in oligomers and in (R)-3-hydroxybutyrate monomers.

In addition to PhaZ1, another esterase has been characterized, PhaZ2, and it has been proven its ability to hydrolyse linear and cyclic oligomers of 3HB, but not crystalline P(3HB) (York *et al.,* 2003).

Furthermore, Saegusa and colleagues have observed that, during the stationary growth phase, depolymerases remain inactive if the culture medium is rich in carbonaceous nutrients (Saegusa *et al*., 2001).

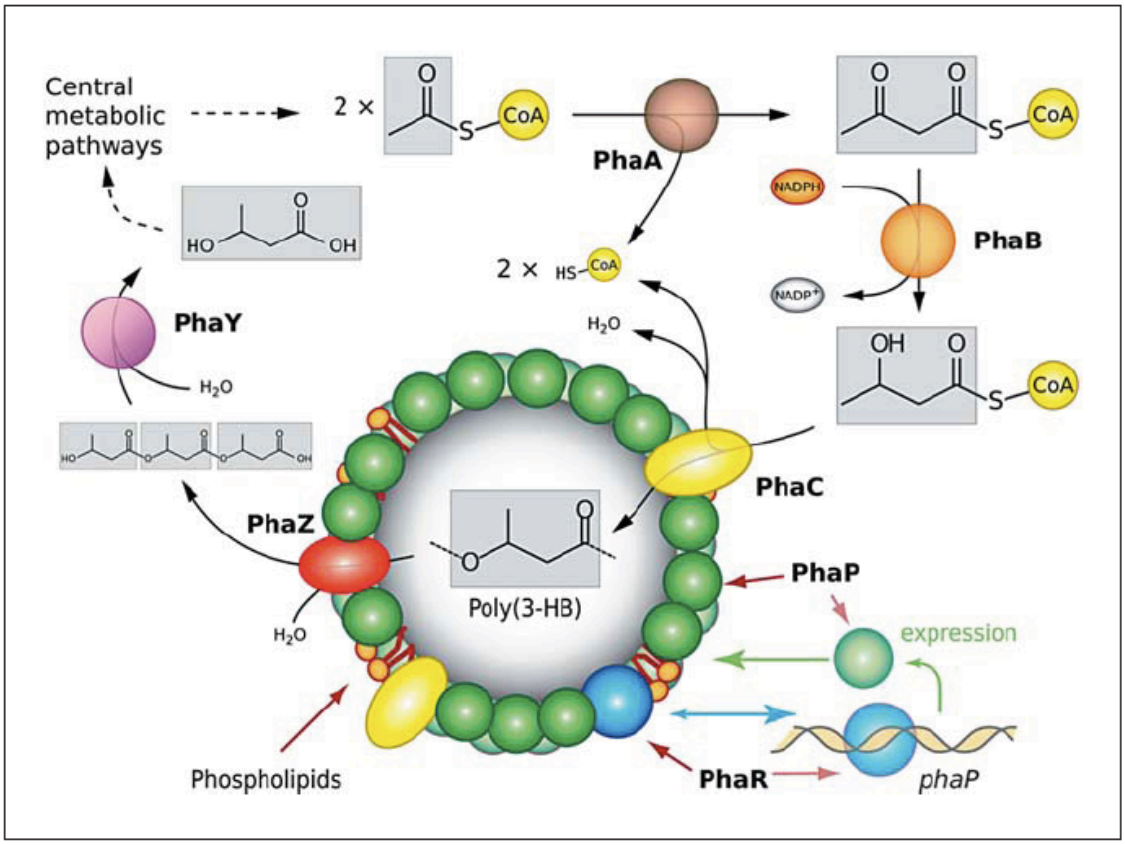


Figure 1.3.

Schematic representation of all the known elements involved in the synthesis of P(3HB), in its granular structure and in its degradation (Steinbüchel *et al.,* 2009).

It is known that the *in vivo* P(3HB) degradation is accomplished in several steps: the PhaZ1 and PhaZ2 cut the chains of the molecules of the P(3HB) and amorphous products, such as oligomers of 3HB of medium size, remain attached to the granules due to their hydrophobicity, with the free ends protruding from the granules (Fig 1.3). A small amount of short chain oligomers and monomers diffuse into the cytosol. Subsequently, the PhaZ2 degrades the oligomers of 3HB remained on the granules and the free ends of the amorphous P(3HB). Finally, the PhaZ2 localized in the cytosol hydrolyses the diffused 3HB oligomers (Kobayashi *et al*., 2003).

In conclusion, considering that the microorganisms capable of degrading P(3HB) are present in all natural environments, it is possible to consider the P(3HB) a biodegradable polymer.

1.5. Industrial application of PHAs

1.5.1. Price and strategy for PHA production

Once accepted the attracting nature and the enormous potential of PHAs as a possible solution to the accumulation of oil-derived plastic in the environment, significant efforts have been devoted to develop different strategies to obtain the industrial production of this biopolymer as a commercial "bioplastic". However, the production cost is not completely competitive, yet. Several factors influence the cost of PHAs, especially the nature and availability of the substrate. In view of a sustainable production of PHAs, one of the most interesting strategies is the use of wastes as carbon source. A promising approach could be the utilization of diverse wastes that cause severe disposal problems, like waste from dairy industry, slaughterhouse or waste waters from other food processing and food service industries. These materials contain large amounts of carbon compounds that could be used as building blocks for PHAs.

Other substrates containing considerable amounts of fat, oil and grease (FOG) have been found to be possible substrates in the production of PHAs, such as plant oils (Alias *at al.,* 2005) or cheaper waste streams from oil mills or used oils. Moreover, a potential added value is the need to treat these residues for their disposal that this strategy would solve.

1.6. PHA production from waste

1.6.1. Production of PHAs from agro-food residues

One of the most expensive item in PHA production is the substrate for culturing bacterial cells, which alone constitutes more than 50% of the costs (Chen *et al*., 2010). Therefore, agro-food and industrial wastes can be used as cheap carbon source for growing the bacteria of interest. These materials are low cost and some of these residues, such as serum and oil, may constitute a serious environmental problem if their disposal is not correctly addressed.

Among the waste materials candidates to serve as substrate for the production of PHAs there are:

- Serum from the dairy industry (studied in the European project WHEYPOL)
- Glycerol from the production of biodiesel
- Grass clippings, silage for animal feed
- Scraps of the sugar: molasses, starch, alcohol,
- Residual lignin-cellulosic materials from farms, maintenance of green areas, timber industry and paper and many other enterprises.
- Lipids such as waste oils, food waste, waste water from the production of oil
- Rendering: animal fats, meat meal and bone meal (studied in the European project ANIMPOL).

Since the characteristics of a production process depends largely on the culture substrate of the bacteria, in order to adapt the available technologies or build new ones it is necessary to perform extensive targeted studies for each of these materials.

To make this possible, it is necessary to bring together in a single group working skills required to assess all aspects of a production line, such as microbiology, molecular biology, chemistry, process engineering and management, marketing. Some of these research projects are supported by the European Union, such as ANIMPOL which studies the biotechnological conversion of carbon containing wastes (such as slaughter waste) for eco-efficient production of high added value products, as PHA polymers.

1.7. PHA production by microorganisms

The polymers synthesized by microorganisms were first investigated by Lemigne, who isolated the reserve polymers in *Bacillus megaterium* (1925) and identified the polymer 3HB (Chee et al., 2010).

A wide variety of microorganisms are capable of accumulating PHAs when various factors like the type of microbial production strain, the feeding regime for nutrient supply, and the process parameters during the biosynthesis are appropriate. The condition for PHAs production by microorganisms need, into appropriate culture media, low nitrogen (or other basic nutrients) and high carbon source.

In the seventies, Imperial Chemical Industry was the first production company to use methylotrophs bacteria, as they use methanol that it is a cheap substrate. Unfortunately, this type of bacteria achieved little production of polymers with low molecular weight. It was then decided to focus on *Azotobacter*, but the instability of the *Azotobacter* sp. strains and the excessive production of polysaccharides were the cause of the early abandon of this path. Afterwards, the attention focused on *Ralstonia eutropha,* a bacterium able to accumulate P(3HB) with high molecular weight. This strain, which grows preferably on fructose in the absence of nitrogen or phosphorus, was chosen for the production of the copolymer poly (3HB-*co*-3HV). It has been demonstrated that the bacterium *Protomonas extorquens*, growing on methanol, can also produce substantial amounts of P(3HB). Even the Gram-positive microorganisms were taken in consideration in different studies (Valappil *et al*., 2007). The genus *Bacillus* would be the best candidate for the production of P(3HB). However, the drawback of spore-forming microorganisms as *Bacillus* is the use of PHAs to obtain the energy required for sporulation. Thus, the identification of sporulated microorganisms with excellent productions of P(3HB) could be useful, but only for the search of new genes of the biosynthesis of the polymer that can be used in a not spore-forming host .

Although the ability to synthesize PHAs is widespread among microorganisms, it is equally true that not all of these bacteria are suitable for an industrial production (Chen *et al.,* 2010). Moreover it is very rare that a microorganism found in nature is both excellent producer of PHA and also able to metabolize a substrate of any waste origin (Povolo *et al.,* 2012; Titz *et al.,* 2012).

1.8. Delftia acidovorans

1.8.1. *Delftia acidovorans* for PHAs production

Delftia acidovorans, a Gram-negative not spore-forming microbe, is the best known wild type bacterium that can efficiently accumulate PHAs containing high molar fractions of 4-hydroxybutyrate (4HB) (Saito et al., 1996). *D. acidovorans* belongs to the Comamonadaceae family.

Scientific name	*Delftia acidovorans*
Synonym	*Comamonas acidovorans*

Based on 16S gene sequence analysis *Comamonas acidovorans* was found phylogenetically distant from type species of *Comamonas*. Therefore, it was removed from genus *Comamonas* and was renamed as *Delftia acidovorans*. This name refers to the city of Delft, the site of isolation of the type strain (1926).

Microscopically, *D. acidovorans* is a straight to slightly curved gram negative bacillus, which occur singly or in pairs. It is motile by means of polar or bipolar tufts of one to five flagella. The organism is strictly aerobic and its optimal growth occurs at 30 °C.

D. acidovorans has the most suitable metabolic pathway for the controlled biosynthesis of P(4HB) homopolymers as well as poly(3-hydroxybutirate-*co*-4 hydroxybutyrate) [P(3HB-*co*-4HB)] that is potentially one of the most useful PHAs for biomaterial applications (Ch'ng *et al*., 2012). When the 4HB molar fraction in the copolymer is increased, the property of the polymer changes from one with high crystallinity to another with strong elastomer (Siew *et al*., 2008). In *D. acidovorans*, the 4HB monomer content could be easily controlled by supplying substrate mixtures of 4HB and other carbon sources. This peculiarity results in the ability to biosynthesize PHAs with wide ranges of elasticity and tensile strength and, most importantly, controlled rates of biodegradation.

This microorganism is capable of producing PHA copolymer with a high molar fraction of 4HB monomer content and it can arrive to 90% of the total copolymer. Until now, 4HB was found to be incorporated into PHA only when related carbon sources (such as 4HB, γ-butyrolactone and 1,4-butanediol) are provided in the culture media.

1.8.2. P(4HB) homopolyester production and utilization

Several bacteria possess a scl-PHA synthase enzyme capable to incorporate 4-hydroxybutyric acid (4HB) into PHAs. However, the incorporation of 4HB strongly depends on the use of precursor substrates as carbon sources. This is also true for all other PHAs that contain non-3HA constituents, which are normally synthesized by bacteria grown on substrates with precursors inside. Since no 4HB has been synthesized so far from wild-type strain without precursors like carbon sources in the media, recent resarches have been focused on the engineering of microbes able to produce 4HB from simple carbon sources, without the expensive supplementation of precursors. Intensive efforts are going on nowadays to achieve a biotechnological production of P(4HB) from cheap carbon sources because of its interesting properties (Saito *et al*., 1994). 4HB have attracted much attention as environmentally degradable thermoplastics for a wide range of agricultural, marine and medical applications.

Finally, it is important to note that U.S. Food and Drugs Administration has approved a PHA containing 4-hydroxybutyrate monomer units produced by recombinant technology for application as absorbable suture material. Therefore, these polyesters have great potential for both biomedical and pharmaceutical applications (Williams *et al.,* 2002).

1.9. Hystory of lipases

Lipases are water-solution enzymes that hydrolyse ester bonds of water insoluble substrates such as triglycerides. Lipids constitute a large part of the earth's biomass, and lipolytic enzymes play an important role in the turnover of these compounds. In 1856 Claude Bernard firstly discovered a lipase in pancreatic juice as an enzyme that hydrolysed insoluble oil droplests obtaining soluble products (Sangeetha *et al.,* 2011).

Animal pancreatic extracts were traditionally used as the source of lipase for commercial applications. Initial interest in microbial lipases was generated because of a shortage of pancreas and difficulties in collecting available material. Bacterial lipases were first observed in the year 1901 in *Serratia marescens* and *Pseudomonas aeruginosa* (Hasan *at al*., 2006). Microbial sources of lipase were deeply explored when the industrial potential of lipases

enhanced and when demand for lipases could not be met by the supply from animal sources.

Microbial lipases are often more convenient for enzyme production than enzymes derived from plant and animals, and this can be attributed to the easy way they can be mass cultured and genetically manipulated (Hasen *et al.,* 2006).

Commercial microbial lipases are produced from bacteria, fungi and actinomicetes. Until now lipases isolated from fungi are the most studied among all microbial lipases but bacterial strains are being constantly screened and improved for lipase production.

The high level production of microbial lipases requires not only the efficient overexpression of the lipase genes but also a detailed understanding of the molecular mechanisms governing their folding and secretion. For this reason, lipase production of many different bacterial species has been extensively studied and reported, particularly for *Pseudomonas* sp., *Bacillus sp*. and *P. aerugionosa* (Madan *et al*., 2010).

1.10. Cellular localizzation of lipase

1.10.1. Secretory system of lipase

Bacterial lipase may be intracellular, membrane-bound or extracellular. The most interesting are the extracellular lipase because the producers of intracellular lipase can only grow on glycerol and simple lipids but not on long chain triglycerides.

Bacteria secrete lipase to the external medium through different types of secretory systems. The type I Secretory System is an energy driven exporter complex made up of three protein subunits. The type II secretion system (or GSP, General Secretory Pathway) comprises two steps for the process. In the first step, the protein is translocated across the inner membrane of the Gram-negative bacterium from a path-dependent Sec or Tat-dependent Sec (Angkawidjaja *et al.,* 2006).

Anchored to the inner membrane of the Gram-negative bacterium there is the protein Lif. This protein, associating closely with lipases determines their correct folding. Subsequently to the process of folding and degradation of N-terminus, the lipase is secreted outside of the cell by a protein complex aspecific (XCP machinery) (Rosenau *et al.,* 2004).

1.10.2. Lif proteins

Experimental evidence has shown that the natural conformation of single proteins or protein complex is not only determined by the amino acid sequence.

It is known that specific accessory proteins, called molecular chaperones, are required to mediate the formation of the correct tertiary structure of some proteins, or protein complex, even if they are not themselves components of the final functional structure.

About a decade ago, it was found that extracellular lipase gene of family I was encoded in an operon together with a second gene, shown to be necessary to the lipase activity. In fact, this second gene assisted the correct folding of its associated lipase. Therefore, they were named Lifs to indicate that they constitute a unique class of *li*pase-specific *f*oldases. (Rosenau *et al*., 2004)

Lipase subfamilies I.1 and I.2 fold into an enzymaticly active conformation in periplasm and are subsequently transported through the bacterial outer membrane by means of a complex machinery consisting of up to 14 different proteins. To achieve a secretion-competent conformation, lipases require specific intermolecular folding catalysts, the Lif proteins.

Lifs represent a unique family of proteins without any significant homology to other classes of proteins and have been shown to specifically activate only their cognate lipases. A lipase gene and its foldase gene usually form an operon suggesting a 1:1 ratio for both lipase and foldase expression. In vitro experiments revealed a 1:1 stoichiometry for lipase-lif complexes during the action process suggesting that Lifs function as single-turnover catalysts (Fig. 1.4).

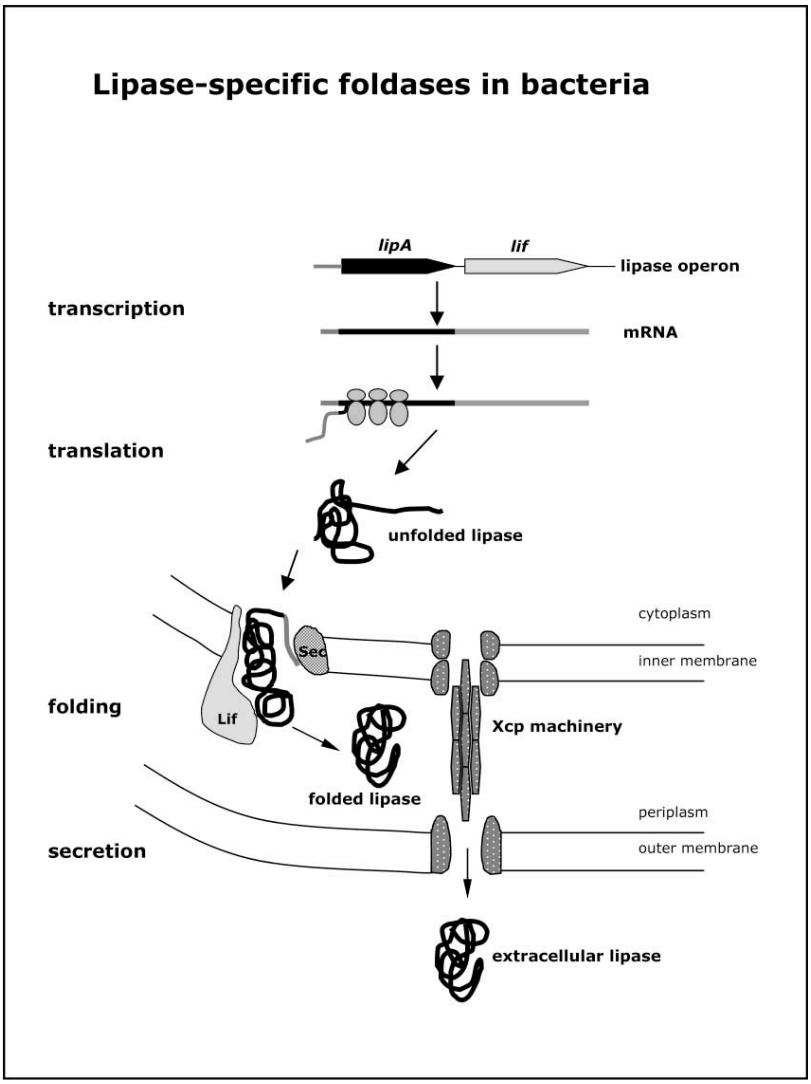


Figure 1.4. Scheme of lipase secretion. (Rosenau *et al*., 2004)

1.11. Features of the family of enzymatic lipase.

1.11.1. Triacylglycerol hydrolase

Lipase (triacylglycerol hydrolase, EC.3.1.1.3) are an important class of enzymes with many uses in biotechnology and biomedical applications and represent one of the most commercially important class of enzymes (Angkawidjaja *et al*., 2006). Lipase natural substrates are long-chain triglycerides that are insoluble in water and separate from the aqueous phase as emulsions and micelles (Jaeger *et a*l., 1998). Lipase hydrolyses the carboxyl ester bonds in mono-, di- and tri-glycerides to liberate fatty acids and alcohols in aqueous solutions (Angkawidjaja *et al*., 2006).

The fatty acids are then transported into the cell where they are catabolized via the beta-

oxidation cycle. Some bacteria also synthesize surfactants that may increase the surface area and bioavailability of hydrophobic carbon sources, allowing for more efficient growth on these compounds (Rosenberg *et al*., 1999).

All lipases belong to the superfamily of "α / β hydrolase", one of the largest groups of structurally related enzymes, although with different functionality (Angkawidjaja *et al*., 2006). The lipase activity is related to a catalytic triad of amino acids with a nucleophilic residue, a triad in which lipase is generally represented by Ser, His and Asp. The serine residue is usually placed in a penta-peptidic GxSxG pattern, located near the center of the enzyme (Angkawidjaja *et al*., 2006).

The lipase assume an active conformation in the moment in which they are in contact with a substrate insoluble in water in concentrations close to the solubility limit of the substrate itself. This phenomenon is described as "interfacial activation" (Angkawidjaja *et al.,* 2006). Virtually all of the lipase used for purposes of research or commercial are extracellular enzymes of microbial origine, then extracted by bacteria or fungi. The bacterial lipolytic enzymes have been classified into eight families based on sequence homologies and properties (Arpigny *et al*., 1999). Among these, the family I is a larger group and it has been classified into seven subfamilies.

Lipase subfamily I.1, I.2 and I.3 are produced by gram-negative bacteria. The subfamilies I.1 and I.2 have a relatively high sequence similarity (30-40%) and are secreted by the type II secretion system (T2SS), which is described previously.

1.12. Recombinant bacterial lipases

Recombinant DNA technology allows to choose the right lipases for an appropriate host and permit the overexpression of the lipases in the host to fulfil commercial demands.

Many bacterial lipases have been cloned, sequenced and expressed in homologous or heterologous hosts (Sangeetha *et al*., 2011).

Several studies have demonstrated the specificity of lif-protein to its associated lipase (Shibata *et al.,* 1998; El Khattabi *et al.,* 1999). These results show that it is necessary to clone together lipase gene and the relative lif gene to achieve an efficient heterologous expression of a lipase.

It was also reported that the transposition of these two genes (lipase and chaperon sequences) between phylogenetically close related organisms is sufficient to obtain a proficient production of lipase (Jorgensen *et al*., 1991). Another study indicates that in the secretion of lipase 30 other cellular proteins are involved (Rosenau *et al*. 2004), and therefore, they are all present in the majority of organisms phylogenetically close to the donor microorganisms possessing the lipase genes.

1.13. Project outline

This work is included in a wider research program carried out at the Dipartimento di Agronomia Animali Alimenti Risorse Naturali e Ambiente (DAFNAE) within the EU project ANIMPOL (*Biotechnological conversion of carbon containing wastes for eco-efficient production of high added value products*). The aim of the entire project was to produce PHAs using as carbon source the fatty wastes from slaughterhouses.

In more detail the objective of this research was to look for, select and characterise bacterial strains able to utilise, as carbon source, low cost industrial lipid wastes such as triacylglycerols (TAGs) from animal fats, with the final goal to produce PHAs. For this reason lipolytic bacteria were isolated from a variety of different environments, such as soil or waste water of a slaughterhouse. Several bacterial strains were found to possess remarkable lipolytic activities but not efficient PHAs production capabilities.

As a result, a molecular biology program started in order to obtain a microbial strain capable of both hydrolysing lipids and producing high levels of PHAs. *Delftia acidovorans* DSM39*,* well known for its high PHAs production but unable to metabolize lipids, was selected as host strain. On the other hand, *Pseudomonas stutzeri* BT3, the most efficient lipase producing isolates, was designated as potential donor of lipolytic genes which were identified, sequenced and co-expressed into *D. acidovorans* DSM39.

The resulting engineered strain, *D. acidovorans*-pBBR1MCS-5-*lipH-lipC*, was studied for its ability to produce PHAs from fatty substrates. It was successfully grown in TAG-containing medium, showed high lypolytic activities and was able to grow on several fatty substrates. Moreover, the PHAs production content from corn oil achieved high levels. Surprisingly, the recombinant strain produce greater values from slaughterhouse residues such as udder and lard.

2. Materials and Methods

2.1 Strains, plasmids and media

The sources of bacterial strains used in this work are summarized in Table 2.1

Table 2.1 Bacterial strains and plasmid used in this study.

Strains or plasmids	**Genotype**	**Source or reference**
strains		
Pseudomans stutzeri BT3	Wild type	This work
Pseudomans sp. AA4	Wild type	This work
Aeromonas sp .PU7	Wild type	This work
Escherichia coli DH10B	F^- endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ-	Durfee *et al.,* 2008
Escherichia coli pDrive-*lipC*	Amp^+ (pDrive-*lipC*)	This work
Escherichia coli pDrive-*lipH*	Amp^+ (pDrive-*lipH*)	This work
Escherichia coli pBBR1MCS-5-*lipC*	$Gent^+$ (pBBR1MCS-5-*lipC*)	This work
Escherichia coli pBBR1MCS-5-*lipH-lipC*	$Gent^+$ (pBBR1MCS-5-*lipH-lipC*)	This work
Delftia acidovorans DSM 39	Wild tipe	This work
Delftia acidovorans pBBR1MCS-5-*lipH-lipC*	Gent+ (pBBR1MCS-5-*lipH-lipC*)	This work
Plasmid		
pDrive	Amp^+	Quiagen
pBBR1MCS-5	$Gent^+$	CBS-KNAW Fungal Biodiversity Centre of Royal Netherland Academy of Arts and Sciences)
pDrive-*lipH*	Amp^+ *lipH* gene	This work
pDrive-*lipC*	Amp^+ *lipC* gene	This work
pBBR1MCS-5-*lipC*	$Gent^+$ *lipC* gene	This work
pBBR1MCS-5-*lipH-lipC*	Gent+ *lipC* and *lipH* genes	This work

Culture media used in this work are reported in Table 2.2. All media were autoclaved at 120°C for 20 minutes before utilization. When necessary, liquid media were solidified adding 1.8% agar-agar. All chemicals, media components and supplements were of analytical grade standard.

Table 2.2 Culture media used in this study.

Medium	Reference or supplier
Luria-Bertani (LB)	DIFCO (United States)
Nutrient Broth (NB)	OXOID (England)
DSMZ81	DSMZ (Germany)
Mininimal medium for *D. acidovorans*	Saito *et a*l., 1994
Minimal Salt Medium (MSM)	Ramsay *et al*., 1990

2.2 Isolation of microorganisms

2.2.1 Isolation of lipolytic bacteria from soil and slaughterhouse wastes

Indigenous bacteria from soil of the Experimental Station of the University of Padova and from waste waters of a slaughterhouse (Fratelli Tosetto, Commercio e Lavorazione Carni S.A.S. Via Provinciale, 1235010 Campo San Martino-PD) were isolated by plate dilution standard methods. In short, 20 g soil or waste water were suspended in autoclave sterilized NaCl 0.9% and maintained on a rotary shaker for 45 min at the maximum speed. Serial dilutions (1:10) were prepared and aliquots inoculated onto Agar plates of Minimal Salts Medium (MSM) (Ramsay at all, 1990) containing commercial corn oil (2.5 % w/v) or commercial lard (2.5 % w/v) as only carbon source and 1.0 % (w/v) arabic gum as emulsifier and rhodamine B (Sigma) (0.001 % w/v) (Kouker *et al.,* 1987). Plates were then incubated at 37°C for 3-5 days till appearance of colonies.

Isolated colonies were then streak-plated onto both solidified MSM and Nutrient medium containing corn oil and rhodamine B and plates incubated at 37 °C. Lipolytic activity associated with bacterial colonies was visualised using a hand-held UV transilluminator (Model UVGL55; UVP Inc., CA, USA) at a wavelength between 350 and 365 nm. The lipolytic colonies show orange-fluorescence haloes when plates are irradiated with UV light at 350-

365 nm. Lipolytic colonies were isolated and stored in glycerol at -20° for molecular and physiological analyses.

2.2.2 Genetic characterization of newly isolated strains: DNA amplification and 16S rDNA sequencing

Genomic DNA was extracted and purified from newly isolated strains as described by (Sambroock *et al.,* 1989), and its purity was assessed using the A260/A280 and A260/A230 ratios. Universal primers R1n (5'-GCTCAGATTGAACGCTGGCG-3') e U2 (5'-ACATTTC ACAACACGAGCTG-30) were used to amplify 1-kb 16S rDNA fragment, corresponding to positions 22–1,066 in *Escherichia coli* 16S rDNA (Weidner *et al.*, 2000). PCR products were purified using QIAquick PCR Purification kit (Quiagen) and then resuspended in 30 µl deionised water. DNA sequencing on both strands was performed by the dideoxy chain termination method with an ABI Prism 3100 DNA Analyzer, using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Biosystems) according to the protocol provided by the manufacturer. GenBank and RDP databases were used to search for 16S rDNA sequence similarities (Maidak *et al.,* 2000).

2.2.3 Culture media and growth conditions of environmental isolates

Inocula of the environmental isolates were grown under shaking at 150 RPM, in aerobic condition at 37° C in 15 mL Falcon containing 3 mL of NB medium (pH 7.0).

With the aim to evaluate if fatty waste could be metabolized by the isolated bacteria to produce PHAs, a two-step cultivation procedure was used: the first step consisted in bio-mass production in 200 mL minimal medium DSMZ81 containing both high carbon sources (glucose 4% and 1% corn oil) and nitrogen source, for 2 days. Each isolate was grown in shaken flasks with aeration at 37 °C. Once produced, the biomass was collected by centrifugation at 4000 *g* at 4°C for 15 min, and transferred to 200 mL minimal medium DSMZ81 with low nitrogen content (1/3 of the normal content) and high amount of fatty acids (2%) as only carbon source (accumulation condition). The incubation in this second

step was under aerobic condition for 3 days. As emulsifier, Arabic gum or DMSO (Dimethyl sulfoxide) were added. All liquid cultures were grown at 37 °C in shaking flasks at 150 rpm.

2.3 Collection strain: growth conditions and PHA production

2.3.1 PHA synthesis by *D. acidovorans* DSM39 and derivatives

PHA synthesis by *D. acidovorans* DSM39 and derivatives was carried out by two-step cultivation. In the case of derivatives all cultures were performed with addition of antibiotic Gentamicin (20 µg/mL).

In the first step aimed to produce biomass, the microorganism was grown under shaking at 150 RPM, in aerobic condition at 30° C in 1L flasks containing 200 mL of NB medium (pH 7.0). An overnight culture ($OD_{600\ nm}$=1.5) at 0.25% (vol/vol) was used as preinoculum. After 24 h, the cells were harvested by centrifugation at 4000 g at 4°C for 15 min. In the second step, to promote PHA synthesis, the centrifuged cells were transferred into a 200 mL nitrogen-free medium (pH 7.0) for 2-3 days in shaking flasks at 150 rpm; the medium contained 2.65 g/L K_2HPO_4, 7.16 gL^{-1} $Na_2HPO_4 \cdot 12H_2O$, 0.33 gL^{-1} $MgSO_4 \cdot 7H_2O$, 0.033 gL^{-1} $(NH_4)_2SO_4$, and was supplemented with 2% (wt/vol) corn oil, udder, lard, tallow (see chapter2.6), meristic acid, stearic acid, palmitic acid or oleic acid and 10 mL L^{-1} microelements solution. The microelements solution contained 119 mg $CoCl_2$, 9.7 g $FeCl_3$, 7.8 g $CaCl_2$, 118 mg $NiCl_2 \cdot 6H_2O$, 62.2 mg $CrCl_2$ and 156.4 mg $CuSO_4 \cdot 5H_2O$ (per litre of 0.1 M HCl) (Saito *et al.*, 1994).

2.3.2 Polyhydroxyalkanoates (PHA) analyses

PHA concentration was determined by the method of Braunegg *et al.* (1978); 3-hydroxyalkyl esters were quantified by gas chromatography with a silica fused capillary column AT-WAX (Alltech Italia s.r.l., Milan, Italy) and a flame ionization detector. The gas carrier was helium, the injection port temperature was 250 °C, the detector temperature 270 °C and the oven temperature 150 °C. The GC-temperature programme was as follows: initial oven temperature 90 °C (maintained for 1 min), with increases of 5 °C/min to a final temperature of 150 °C (maintained for 6 min). The internal standard was benzoic acid, and the external

standards were: 3-hydroxybutyric acid (Sigma-Aldrich, Italy), P(3HB-co-3HV) copolymer (Biopol TM; Imperial Chemical Industries, Great Britain), P(3HB-co-11.2 mol%4HB) copolymer (SoGreenTM 00A; Tianjin, China), mcl-copolyester samples consisting of (R)-3-hydroxyhexanoate (3Hx), (R)-3-hydroxyoctanoate (3HO), (R)-3-hydroxydecanoate (3HD), and (R)-3-hydroxydodecanoate (3HDD) (Metabolix Bio-Industrial Evolution, USA). Results were expressed as percentage of PHAs on the bacterial biomass measured as described in paragraph 2.3.3.

For microscopy examination of PHA granules, dry smears of formalin-treated cells were stained with Nile Red (25 mM Nile Red in dimethyl sulfoxide, diluted 1/500 in sterile water) for 30 min, rinsed with water and examined by epifluorescence microscopy. Smears were observed using an Olympus 24 fluorescent microscope BX60 equipped with a blue 420-nm exciter filter. Nile Red stained PHA granules and produced a strong green-yellow fluorescence inside bacteria.

2.3.3 Biomass measurements

10 mL culture broth were centrifuged, washed twice in 10 mL of distilled water and the pellet dried to constant mass at 80 °C.

2.3.4 Antibiotic resistance evaluation

D. acidovorans DSM 39 was cultured in liquid NB media containing one of the following antibiotics: Gentamicin 20 µg/mL, Tetracycline 45 µg/mL, Kanamycin 45 µg/mL, Chloramphenicol 30 µg/mL. A 0.25% (vol/vol) inoculum ($OD_{600\ nm}$=1.5) was used in a 15 mL Falcon containing 3 mL of NB medium (pH 7.0) and each antibiotic; test tubes were incubated aerobically at 30° C in shaking flasks at 150 rpm. After 3 days antibiotic resistance/sensitivity was recorded measuring $OD_{600\ nm}$.

2.3.5 Growth evaluation

To obtain growth curves, a 500 µL inoculum ($OD_{600\ nm}$=1.5) was used in 1L flasks containing 300 mL of sterile NB medium amended with 2% of corn oil. Cell growth was evaluated every 12 hours as described in paragraph 2.3.3.

2.4 Lipase production

2.4.1 Growth of bacteria for lipase analyses

To evaluate lipase production microbial cells were cultured at 30°C (*Delftia acidovorans* and *Acinetobacter venezianus*) or 37°C (all the newly isolated strains), for 24-48 h in 1000-mL flasks containing 300 mL of NB with 2% of corn oil. *D. acidovorans* pBBR1MCS-5-*lipH-lipC* was cultured at 30°C for 24-48 h in 1000-mL flasks containing 300 mL of NB.

Cells were centrifuged and the supernatant was used for the titrimetric method (the protocol is described in paragraph 2.4.2). The lipase activity was also confirmed in solid media using the rhodamine B assay as described in paragraph 2.2.1 (Kouker *et al.,* 1986).

2.4.2 Lipase assay in liquid medium

Lipase activity for newly isolated strains was measured in liquid medium by titrimetric assay (Pinsirodom *et al.*, 2001) adding 5 mL of the supernatant of the bacterial cultures to 50 mL of 5 % (v/v) corn oil emulsion in 50 mM Tris–HCl buffer (pH 8), containing 5 % (w/v) of arabic gum. The mixture was incubated at 37 °C for 3 h, and every 30 min, to stop the reaction, 5 mL were sampled and added to 10 mL ethanol. With the aim to optimize the assay conditions for *P. stutzeri* BT3, the lipase activity was measured at both 30° and 37°C and at pH 6.5, 7.5 and 9.5. The activity of *D. acidovorans* pBBR1MCS-5-*lipH-lipC* was determined at 37°C and pH 7.5

The released fatty acids were titrated with 0.05 M NaOH using phenolphthalein as an indicator. The difference in titer values between samples and blank was used to calculate the amount of released fatty acid. One unit of lipase was defined as the amount of enzyme that released 1 µmol of fatty acid per minute under assay conditions.

2.5 GMM construction

In any metabolic engineering programme, the choice of host strain and heterologous gene(s) of interest to be expressed is crucial to fulfil the final goal. In this work, the non lipolytic *D. acidovorans* DSM 39 was selected as recipient because it can accumulate PHAs with high molar fractions of 4-hydroxybutyrate (4HB) (Saito *et al*., 1994). Starting from the newly isolate *P. stutzeri* BT3 used as lipase gene donor, the plasmid pBBR1MCS-5-*lipH-lipC* was obtained and introduced in *D. acidovorans* DSM39 as described in the following paragraphs.

2.5.1 Ability of *D. acidovorans* to grow in free fatty acid and glycerol.

Before starting with the genetic engineering program, the ability of *D. acidovorans* to use free fatty acid and glycerol as carbon source was evaluated. With this purpose *D. acidovorans* was inoculated onto Agar plates of appropriate minimal medium (described in paragraph 2.3.1) containing as only carbon source palmitic acid, stearic acid, meristic acid, oleic acid or glycerol (2.5 % w/v). Plates were then incubated at 30°C and growth recorded after 3-5 days.

2.5.2 Primers construction

With the aim of identifying and isolating a *lip* gene from the new strain *P. stutzeri* BT3 for the production of the recombinant plasmid, PCR primers for the amplification of *lip* genes were set up. PCR primers construction is an essential aspect for the success of amplification reaction. The primers used in this work (Table 2.3) were designed using the software Primer 3 (http://frodo.wi.mit.edu/primer3/). PCR primers for *lipC* and *lipH* genes were designed on the basis of DNA sequence of *Pseudomonas stutzeri* A1501 present in GeneBank (accession n. NC_009434.1). Once the sequences were selected, they were synthetized by EUROfins MGW Operons (Ebersberg, Germany) and lyophilized; when necessary they were re-dissolved in water at a concentration 100 µM and stored at -20°C.

The primer LIP-GMO was designed complementary on the sequence upstream to MCS of the plasmid vector pBBR1MCS-5 and it was used with LIPC-R and LIPH-R to verify the presence of the genes in the plasmid pBBR1MCS-5 (provided by CBS-KNAW Fungal Biodiversity Centre of Royal Netherlands Academy of Arts and Sciences).

Table 2.3. Sequences of primer used for PCR amplification.

Primer	sequence
LIPC-F	ATGAACAAGAACAAAACCTTGCT
LIPC-R	GTCAGAGCCCCGCGTTCTTCAATC
LIPH-F	ATGAGCAGATCCATCCTTTT
LIPH-R	TCAGCGAGTCCGATCCTCC
LIP-GMO	GTTTTCCCAGTCACGACGTT

2.5.3 DNA amplification

Bacterial genomic DNA was extracted and purified as described by Sambroock *et al.* (1989).

A master mix was prepared containing template DNA (approximately 100 ng), 25 pmol of each primer, 0.25 mM dNTPs, and 19 µL buffer amended with 1.25 mM $MgCl_2$ and 0.5U of Taq DNA-polymerase (Euroclone S.p.A., Milano, Italy).

The reaction conditions for amplification of *lipC* were: 1 cycle (95 °C for 4 min), 40 cycles (95°C for 1 min, 55.3 °C 1 min, 72 °C, 1 min) and a final cycle (72°C for 5 min).

Reaction conditions for amplification of *lipH* were: 1 cycle (95 °C for 4 min), 40 cycles (95°C for 1 min, 53.2 °C 1 min, 72 °C, 1 min) and a final cycle (72°C for 5 min).

The cycles program for LIP-GMO LIPC-R and LIP-GMO and LIPH-R were respectively: 1 cycle (94 °C for 60 sec), 25 cycles (94°C for 1 min, 55.3 °C 1 min, 72 °C, 30 sec), a final cycle (72°C for 2 min) and 1 cycle (94 °C for 60 sec), 25 cycles (94°C for 1 min , 55.2 °C 1 min, 72 °C, 30 sec), a final cycle (72°C for 2 min).

To verify the correct insertion of the *lipC* and *lipH* genes in the vector, a PCR with the primer pairs LIPH-F/LIPC-R LIPC-F/LIPH-R was also carried out, using the following amplification program: 1 cycle (94 °C for 90 sec), 25 cycles (95°C for 90 sec, 57.3 °C 90 sec, 72 °C, 90 sec) and a final cycle (72°C for 5 min)

Amplified DNA fragments were separated by electrophoresis on agarose gel and stained by EuroSafe (EuroClone S.p.A., Milano, Italy).

2.5.4 Electrophoresis parameters

A 100 mL gel 0.8% agarose for Pulsed field (SIGMA) was prepared with 1X TAE buffer, also used as the corresponding running buffer. The bands were visualized after acid stain EuroSafe (EuroClone S.p.A., Milano, Italy). Digital images were acquired with an EDAS290 image capturing system (Kodak, Rochester, NY).

2.5.5. Cloning of PCR fragments.

PCR products were ligated into the pDrive commercial plasmid. These procedures were carried out following the protocol "Qiagen PCR cloning handbook" (Qiagen). The sequences of interest were then excised with specific restriction enzymes reported in paragraph 2.5.5.1

2.5.5.1 Plasmid construction.

The pBBR1MCS-5-*LipH-lipC* plasmids were constructed as follows. The PCR DNA fragment encoding for *lipC* and *lipH* gene were inserted into the pDrive cloning vector (QIAGEN PCR Cloning Kit) and selected with α- complementation and ampicillin resistance. The plates for the selection contained solid LB amended with ampicillin (100 µg/mL), IPTG (50 mM) and X -gal (80 µg/mL). The correct insertion was verified by PCR amplification of specific genes. Protocol is reported in paragraph 2.5.3.

lipC gene was cut with *EcoR*I (Thermo Scientific) and inserted by ligation in pBBR1MCS-5, obtaining pBBR1MCS-5-*lipC*. The used ligase was T4 ligase (Biolabs). The ligation mix contained: 120 ng of pBBR1MCS-5, 250 ng of insert suspended in 1 µL of DNA ligase, 2 µL of buffer and MilliQ water to 20 µL (the ratio between pBBR1MCS-5 and the insert was 1/10).

The mixture was incubated at 4°C overnight. Plasmid vector pBBR1MCS-5 was cut with EcoRI and treated for 60 min at 37°C with alkaline Fosfatase (Roche) to prevent recircularization. The enzymatic reaction was performed in water mixing 1/10 of the total volume of buffer 10X and 1U of alkaline phosphatase per pmol of plasmid pBBR1MCS-5.

The *lipH* fragment was subcloned with T4 ligase into the corresponding sites ClaI-ApaI of pBBR1MCS-5-*lipC*, generating pBBR1MCS-5-*lipH-lipC*. Each restriction enzyme provided by Thermo Scientific (EcoRI, ClaI, ApaI, MluI, NaeI) was mixed with 16 µL of MilliQ water, 2 µL

of appropriate buffer 10X, 1 µL of DNA substrate. After stirring, the samples were incubated overnight at the temperature indicated for each enzyme. The LB plates used for the selection of recombinants contained gentamicin (20 µg/mL), IPTG (50 mM) and X -gal (80 µg/mL).

Plasmid screening by minipreparation was performed as described by Sambrook *et al.* (1989). Large-scale plasmid preparations were obtained by using a Plasmid Midi Kit (Qiagen, Valencia, Calif., USA). DNA restriction fragments were analyzed by agarose gel electrophoresis in TAE buffer system (0.04 M TRIS-acetate, 0.001 M EDTA, 57.1 mL/L glacial acetic acid).

Finally, pBBR1MCS-5-*lipH-lipC* was transformed in *D. acidovorans* DSM39 as described in 2.5.6.

To verify the functional lipase expression in both recombinant *E. coli* and *D. acidovorans* the rhodamine B agar method described in paragraph 2.2.1 was used.

2.5.6 Preparation of *E. coli* and *D. acidovorans* competent cells and transformation.

To obtain competent cells of *E. coli* DH10B e *D. acidovorans* and for the trasformation using $CaCl_2$ technique, the protocol of Ausubel *et al.* (2003) was used.

2.5.7 Verification of T3 promoter funtionality in *E. coli* pBBR1MCS-5-*lipH-lipC*.

In order to confirm the functionality of the T3 promoter, RNA was extracted, cDNA from transcribed inserted gene (under the control of T3 promoter) was obtained and amplified as described in the following paragraphs.

2.5.7.1 RNA extraction

RNA was extracted from *E. coli* pBBR1MCS-5-*lipH-lipC* using the commercial kit, "explains RNAspin Mini Kit" (GE Healthcare).

2.5.7.2 (RT) Reverse Transcription PCR and PCR amplification of lipC gene

The extracted RNA was quantified by Nanodrop (Thermo Scientific Instrument Inc.).

cDNA was obtained from isolated RNA using reverse transcriptase (Quiagen) as follows.

The reaction mix contained:

RNA	1 µL
Primer (LIPC-R o LIPH-R)	1 µL
dNTP's mix (10mM)	1 µL
H_2O MilliQ	17 µL

A negative control without the enzyme was prepared to exclude possible contamination by genomic DNA.

The mix was incubated for 5 min at 65 °C, transferred to ice and 4 µL of "first strand buffer" 5X and 2 µL DTT (0.1 M) were added. After incubation for 2 'at 42 ° C, 1 µL of Superscript II RT (200U) was added only to positive reaction.

The samples thus obtained were amplified using the following PCR program:

- 10' a 22°C
- 50' a 42°C
- 15' a 70°C

Once cDNA was obtained, PCR amplification of *lipC* gene was performed using the protocol described in paragraph 2.5.3.

2.5.8 Screening of lipase-secreting transformants of *E. coli* and *D. acidovorans.*

The rhodamine B agar method (Kouker *et al.*, 1986) was used to detect the colonies of trasformants having extracellular lipase activities. With this purpose, bacterial cells transformed with pBBR1MCS-5-*lipH-lipC* plasmid were plated on rhodamine agar plates and incubated at 37° or 30 °C for 24 h (*E. coli* at 37°C and *D. acidovorans* 30°C) as described in paragraph 2.4.1.

2.5.9 Bioinformatics tools

In this work the following bioinformatics tools were used for genes identification and to define the best protocol for cloning.

• RDP

The Ribosomal Database Project (RDP) is a curated database that offers ribosome-related data, services analysis and associated computer programs including phylogenetically ordered alignments of ribosomal DNA (rDNA) sequences, derived phylogenetic trees, rRNA secondary structure diagrams and various software for handling, analyzing and displaying alignments and trees.

This tool was used in the phase of identification of the new isolated strains.

• BLAST

This tool enables alignment and comparison of DNA sequences. BLAST was used to identify the *lip* and *lif* genes in *P. stutzeri*. The sequence of lip and lif of *Pseudomans cepacia* were used to saerch the genes with high similarity in the genome of *P. stutzeri,* deposited in Genbank. Blast was used to compare the sequence similarity of the *lipH and lipC* genes from *P. stutzeri BT3* to the sequences of *lipH and lipC* in *P. stutzeri* ATCC A1501 (accession no. NC_009434.1) deposited in Genbank.

• GenBank

This tool is the NIH genetic sequence database, an annotated collection of all public available DNA sequences (Nucleic Acids Research, 2013 Jan; 41(D1):D36-42). GenBank is part of the International Nucleotide Sequence Database Collaboration, which comprises the DNA DataBank of Japan (DDBJ), the European Molecular Biology Laboratory (EMBL), and GenBank at NCBI. These three organizations exchange data on a daily basis.

• Pfam

The Pfam database is a large collection of protein families, each represented by *multiple sequence alignments* and *hidden Markov models* (HMMs)

Lipase and lipase chaperone aminoacidic sequences were inserted in Pfam for the identification of domains. This tool was used to verify the proteins function.

• Primer3

This is an online bioinformatics tools for primers designing .

• PRED – TAT

This tool Identifies specific peptide sequences within functional motifs that reveal a secretion mechanism of type Sec or Tat (Bagos *et al*., 2010).

It was used for the verification of the pathway of secretion of *lipC*.

2.6 Fatty carbon sources used in this work.

The fatty carbon sources used in this work and their origin, are reported in Tab. 2.4.

Table 2.4. Origin of fatty carbon sources used in this work

FAT	Origin
Corn oil	Commercial product
Lard	Commercial product
Udder	Prof. Mittelbach (Karl-Franz University of Graz-Austria)
Tallow	Prof. Mittelbach (Karl-Franz University of Graz-Austria)

2.6.1 Chemical composition of tallow and udder.

The chemical composition of udder and tallow (reported in Tab 2.5 and Tab. 2.6) used in this work was kindly provided by Prof. Mittelbach (Karl-Franz University of Graz-Austria), one of the European partners within EC-ANIMPOL project .

Table 2.5. Chemical composition of udder samples.

Parameter	Unit	Test method	Result
Free fatty acids	%(m/m)	DGF-C-V-2 (06)	2.35
Unsaponifiable	%(m/m)	DGF-C-III 1 a (77)	3.10
Total fatty acids	%(m/m)	DGF-C-III 2 (97)	92.17
Sulfur content	mg/kg	EN ISO 20846	28
Nitrogen content	mg/kg	ISO/TR 11905-2	172
Water content	mg/kg	EN ISO 12937	89.5
Phosphorus content	mg/kg	EN 14107	206
Sodium content	mg/kg	EN 14538	12.3
Potassium content	mg/kg	EN 14538	47.5
Calcium content	mg/kg	EN 14538	64.3
Magnesium content	mg/kg	EN 14538	4.7
Fatty acid composition:		A.O.C.S. Ce 1-62	
Capric acid (C10:0)	%(m/m)		0.06
Lauric acid (C12:0)	%(m/m)		0.09
Myristic acid (C14:0)	%(m/m)		1.54
Pentadecanoic acid (C15:0)	%(m/m)		0.09
Palmitic acid (C16:0)	%(m/m)		26.15
Palmitoleic acid (C16:1)	%(m/m)		2.21
Margaric acid (C17:0)	%(m/m)		0.34
Heptadecenoic acid (C17:1)	%(m/m)		0.16
Stearic acid (C18:0)	%(m/m)		16.29
Oleic acid (C18:1)	%(m/m)		36.31
Linoleic acid (C18:2)	%(m/m)		12.53
Linolenic acid (C18:3)	%(m/m)		0.77
Arachidic acid (C20:0)	%(m/m)		0.36
Gadoleic acid (C20:1)	%(m/m)		1.06
Lignoceric acid (C24:0)	%(m/m)		0.49
Not identified	%(m/m)		1.55
Saturated fatty acids	%(m/m)		45.41
Unsaturated fatty acids	%(m/m)		53.04
Fat content	%(m/m)		57.63
Iodine value	g I_2/100 g	EN 14111	57.60

Table 2.6. Chemical composition of the tallow samples.

Parameter	Unit	Test method	Result
Free fatty acids	%(m/m)	DGF-C-V-2 (06)	8.46
Unsaponifiable	%(m/m)	DGF-C-III 1 a (77)	1.86
Total fatty acids	%(m/m)	DGF-C-III 2 (97)	92.9
Polymer content	%(m/m)	ISO 16931	< 0.1
Polyethylene content	mg/kg	ISO 6656	5
Insoluble impurities	%(m/m)	Internal method	0.24
Sulfur content	mg/kg	EN ISO 20846	46
Nitrogen content	mg/kg	ISO/TR 11905-2	367
Water content	mg/kg	EN ISO 12937	1150
Phosphorus content	mg/kg	EN 14107	167
Sodium content	mg/kg	EN 14538	99.0
Potassium content	mg/kg	EN 14538	40.7
Calcium content	mg/kg	EN 14538	129
Magnesium content	mg/kg	EN 14538	22.0
Fatty acid composition:		A.O.C.S. Ce 1-62	
Capric acid (C10:0)	%(m/m)		0.10
Lauric acid (C12:0)	%(m/m)		0.12
Myristic acid (C14:0)	%(m/m)		2.61
Myristoleic acid (C14:1)	%(m/m)		0.44
Pentadecanoic acid (C15:0)	%(m/m)		0.51
Pentadecenoic acid (C15:1)	%(m/m)		0.18
Palmitic acid (C16:0)	%(m/m)		24.14
Palmitoleic acid (C16:1)	%(m/m)		2.24
Margaric acid (C17:0)	%(m/m)		1.21
Heptadecenoic acid (C17:1)	%(m/m)		0.73
Stearic acid (C18:0)	%(m/m)		20.60
Oleic acid (C18:1)	%(m/m)		38.93
Linoleic acid (C18:2)	%(m/m)		2.98
Linolenic acid (C18:3)	%(m/m)		0.62
Arachidic acid (C20:0)	%(m/m)		0.21
Gadoleic acid (C20:1)	%(m/m)		0.28
Not identified	%(m/m)		4.10
Saturated fatty acids	%(m/m)		49.5
Unsaturated fatty acids	%(m/m)		46.4
Iodine value	g I_2/100 g	EN 14111	42.88

3. Results and Discussion

This work is included in a wider research program carried out at the DAFNAE Department within the EU project ANIMPOL (*Biotechnological conversion of carbon containing wastes for eco-efficient production of high added value products*).

The aim of the entire project was to produce polyhydroxyalkanoates (PHAs) using fatty wastes as carbon source.

The experimental work of my PhD was divided into the following phases:

(1) Isolation of bacteria able to produce extracellular lipases: new strains were isolated on the basis of their high lypolitic activities, then they were genetically characterized and their PHA production evaluated.

(2) Lipase genes (*lipC* and *lipH* genes) were identified in one of the isolates with high lipolytic activity and cloned in a suitable host (*Delfia acidovorans)*, appropriately selected among a number of known bacterial species able to efficiently synthesize PHA, and in particular P(4HB) homopolymers as well as co-polymers such as P(3HB-*co*-4HB).

(3) As a final validation both the lipolytic activity of the obtained recombinant *D. acidovorans* pBBR1MCS-5-*lipH-lipC* and its PHB production was evaluated by directly using fatty wastes as carbon source.

3.1 Isolation of lipolytic bacteria from slaughterhouse and soil

A number of microorganisms of different habitat are known to have the ability to produce extracellular lipases (Ko *et al*., 2004). Therefore, a program of isolation of new bacterial strains was opened starting from soil, generally considered as a wide source of unknown microorganisms, and from the waste streams of a slaughterhouse, where the probability to find lipolytic bacteria was assumed to be high. Indigenuos soil bacteria were searched and isolated from the experimental station of the University of Padova, while slaughterhouse Fratelli Tosetto (Campo San Martino, Padova) kindly made available their plant.

The first stage was based on a selection of bacteria potentially able to degrade oils or animal fats. The isolates showing lipolytic activity were kept for further studies. In a second step,

the above selected bacteria were directly grown on corn oil, and PHA accumulation was analysed.

Serial dilutions of soil and slaughterhouse waste samples were plated, as described in ‘‘Materials and methods’’, on a solid medium containing rhodamine B, suitable for a preliminary selection of lipolytic bacteria. Among all the isolates obtained, 26 showed a significant halo around the colonies when examined under both UV and white light (Fig. 3.1).

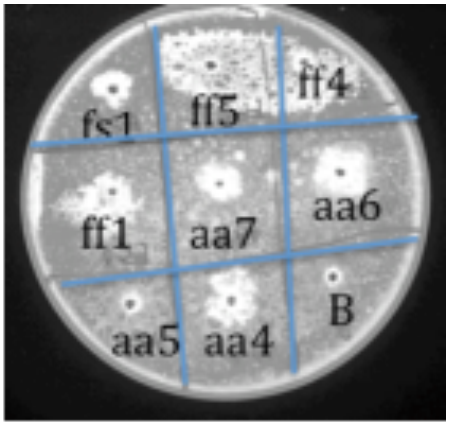


Figure 3.1.

Identification of lipase positive colonies on rhodamine B agar plates. Plates were subjected to UV irradiation (350 nm) and positive bacteria formed orange fluorescent halos around the colonies, indicating production of lipase. All the strains in tihs immage were positive.

B: negative control, without cells.

For all these strains 16S ribosomal DNA (16S rDNA) was amplified and sequenced as described in “Material and method” and their identification obtained by a comparison with available data in Genbank. The possible occurrance of sure pathogen bacteria was accurately checked and the most suspected isolates were definitely discharged. A list of the remaining strains, kept for the subsequent studies, is reported in Table 3.1.

In the same Table 3.1 the ability to accumulate PHA by the selected isolates is reported, as tested by a preliminary plate Nile Red staining (see ‘‘Materials and methods’’ section), where the accumulation of PHA was indicated by the presence of bright green-yellow fluorescent granules within the cells.

Table 3.1. Identification of the isolates at species level and qualitative evaluation of both lipolytic activity (rhodamine B test) and PHA production (Nile Red test) on solid medium.

Isolate	Origin	Identification (16S rDNA)	% identity	Accession number	Lipolytic activity (rhodamine B)	PHA production (Nile Red)
BT1	s	*Acinetobacter sp.*	97	HM584298.1	+	+
BT2	s	*Pseudomonas stutzeri*	99	AB682251.1	+	+
BT3	s	*Pseudomonas stutzeri*	99	AB682251.1	+	+
BT4	s	*Acinetobacter calcoaceticus*	98	FJ357635.1	+	+
BT5	s	*Sinorhizobium sp.*	92	DQ898296.1	+	+
BT6	s	*Chryseobacterium sp.*	98	EF208922.1	+	+
AA4	sw	*Pseudomonas sp.*	83	GQ284542.1	+	+
AA5	sw	*Acinetobacter sp*	67	EF204262.1	+	+
AA6	sw	*Acinetobacter baumannii*	99	HQ632003.1	+	-
AA7	sw	*Acinetobacter sp.*	100	EF204262.1	+	+
LU2	sw	*Acinetobacter johnsonii ì*	99	HM854248.1	+	-
FF1	sw	*Bacillus altitudinis*	99	FJ174623.1	+	+
FF4	sw	*Stenotrophomonas maltophila*	100	AB680524.1	+	+
FF5	sw	*Bacillus pumilus*	88	GU084145.1	+	+
FS1	sw	*Bacillus pumilus*	98	AY647298.1	+	+
FS2	sw	*Acinetobacter sp.*	99	EF204262.1	+	-
PE1	sw	*Acidovorax temperans*	99	JF496561.1	+	+
PE6	sw	*E.coli*	87	JN180963.1	+	-
PE8	sw	*Acinetobacter sp.*	99	JN849072.1	+	+
PE10	sw	*Acinetobacter sp.*	99	EF204262.1	+	-
PE14	sw	*Aeromonas media*	100	JF928564.1	+	+
PU3	sw	*Acinetobacter sp.*	100	JN228325.1	+	-
PU6	sw	*Acinetobacter sp.*	98	GQ246681.1	+	+
PU7	sw	*Aeromonas sp.*	100	GU356308.1	+	-

Isolates from soil (s) and slaughterhouse waste water (sw)

In order to obtain further confirmation of the lipolytic ability of the isolates, lipase activity was quantitatively determined in liquid minimal medium containing corn oil, by using the supernatant of the growth suspensions. The results, shown in Table 3.2, indicated a range from 0.07 to 0.7 µmol/(min × mL supernatant). These values, with *Pseudomonas sp*. isolate AA4, *Aeromonas sp*. isolate PU7 and *P. stutzeri* isolate BT3 showing the highest activities, are in the range of previously reported extra-cellular bacterial lipases (Sangeetha *et al*., 2011).

In order to quantify the ability to produce PHAs directly from fats, the isolates were then subjected to specific growth conditions, known to support the PHA metabolism. In step one, in order to produce a thick biomass, the bacteria were grown aerobically at 37° C for 48 h in a liquid minimal medium containing both high carbon and nitrogen sources. In the following stage, the obtained biomass was transferred to a secondary culture medium containing high

carbon, but low nitrogen sources (accumulation conditions). Corn oil (2.0 % v/v) was used as the only carbon source and Arabic gum (0.7 % w/v) was added as emulsifier because long-chain triacylglycerols (such as oil) have very low solubility in water. The cultures were incubated for 72 h and then analysed for intracellular PHA accumulation.

Table 3.2. Lipolytic activity and PHAs accumulation by the new isolates**.**

Isolate	Identification	Lipolytic activity	
		µmol/(min × mL surnatant)	PHA% (CDM)
BT1	*Acinetobacter sp.*	0.20	11.22
BT2	*Pseudomonas stutzeri*	0.10	0.01
BT3	*Pseudomonas stutzeri*	0.40	0.01
BT4	*Acinetobacter calcoaceticus*	0.20	0.05
BT5	*Sinorhizobium sp.*	0.10	0.07
BT6	*Chryseobacterium sp.*	0.05	0.01
FF1	*Bacillus altitudinis*	0.20	0.02
AA4	*Pseudomonas sp.*	0.70	0.06
AA5	*Acinetobacter sp.*	0.05	1.09
AA7	*Acinetobacter sp.*	0.10	0.25
FS1	*Bacillus pumilus*	0.30	0.47
FS2	*Acinetobacter sp.*	0.05	-
LU2	*Acinetobacter johnsonii*	0.05	-
PE6	*Escherichia coli*	0.10	-
PE8	*Acinetobacter sp.*	-	0.05
PE10	*Acinetobacter sp.*	0.30	-
PU3	*Acinetobacter sp.*	0.06	-
PU6	*Acinetobacter sp.*	0.07	0.11
AA6	*Acinetobacter baumannii*	0.20	-
FF4	*Stenotrophomonas maltophilia*	0.07	0.71
PE14	*Aeromonas media*	0.20	3.12
PE1	*Acidovorax temperans*	0.20	71.99
FF5	*Bacillus pumilus*	0.20	0.01
PU7	*Aeromonas sp.*	0.70	-

As reported above and in Tab. 3.2, although most of the isolates showed lipase activity, the production of PHAs is low in the majority of cases. Particularly, two strains demonstrated a significant PHAs accumulation in terms of PHA% on CDM, but the biomass production was very low. Indeed, isolate BT1, identified as *Acinetobacter sp*., and strain PE1, identified as *Acidovorax temperans*, grew only to reach 0.4 and 0.2 g/L of dry weight, respectively. Although these two isolates could be improved for biomass production within an *ad-hoc* process-study, their use was not suitable for the present research because the objective was not the optimization of lipase production, but the finding of suitable source of genes encoding for such an activity. With this aim, the three most proficient strains, in terms of

lipase activity, were selected as possible candidates (*Pseudomonas stutzeri* BT3, *Pseudomonas sp.* AA4 and *Aeromonas sp*. PU7).

3.2.1 The Construction of a recombinant strain.

The results reported above confirm, unfortunately, that it is very rare that a microorganism found in nature is both excellent producer of PHA and also suitable for an industrial process requiring the ability to metabolize a substrate from waste materials. The response of the scientific community to this problem could be the design of recombinant microorganisms, which simultaneously possess the enzymatic equipment to synthesize PHAs and the ability to metabolize particular substrates.

In the case of a PHAs production from waste oils or fats, which is the objective of the present study, bacteria should be excellent PHAs producers and equipped with enzymatic activities allowing to hydrolyse triglycerides and use the acyl chains obtained for their metabolism and finally for PHA production.

Gram-negative bacteria produce a number of lipolytic enzymes; among them, extracellular lipases, encoded by *lipA* or *lipC* genes, seems to be among the most common. However, other genes such as *xcp* cluster genes are involved in the mechanism of lipase secretion and experimental evidence has shown that the natural conformation of lipase is not always only determined by the amino acid sequence (Rosenau *et al.,* 2004).

About a decade ago, it was found that extracellular lipase gene of family I was encoded by an operon together with a second gene, necessary to lipase activity. This second gene (*lipH*) assisted the correct folding of its associated lipase. Therefore, these proteins were named Lifs to indicate that they constitute a unique class of *lipase-specific foldases* (Jaeger *at al.*, 2000).

The three isolates selected above that gave higher lipase activity were *Pseudomonas stutzeri* BT3, *Pseudomonas* sp AA4, *Aeromonas* sp, PU7 respectively (see Tab. 3.2). These results confirm literature data reporting that the genera *Pseudomonas* and *Aeromonas* have high lipase activity (Fauré *et al*, 2011) and the levels of lipase here detected for the above new isolates are comparable to those recently reported (Hasanuzzaman *et al.,* 2004). Therefore,

a decision was taken to search lipase genes directly into these isolates in order to possibly use these bacterial strains as a source of genes.

With the aim to detect the presence of *lipC* (lipase gene) and *lipH* (lipase-specific foldase) into these isolates, a first approach has been to design appropriate primers through a preliminar in silico analysis of complete DNA genome of collection strains *Pseudomonas stutzeri* ATCC 17588 (accession no. CP_002881.1) and ATCC A1501 (accession no. NC_009434.1), available in NCBI database. Once *lipH* and *lipC* genes were effectively identified in the two collection strains of *Pseudomans stutzeri*, it has been possible to design the most appropriate primers to be utilised on the new isolates. These primers resulted as follows:

Primers for *lipC* amplification:

LIPC-F:5'-ATGAACAAGAACAAAACCTTGCT-3';

LIPC-R:5'-GTCAGAGCCCCGCGTTCTTCAATC-3';

Primers for *lipH* amplification:

LIPH-F,5'-ATGAGCAGATCCATCCTTTT-3';

LIPH-R, 5'-TCAGCGAGTCCGATCCTCC-3'.

PCR annealing temperature and number of cycle are reported above in the ‘‘Materials and methods’’ section.

The products of PCR amplification to detect into the three selected isolates the presence of *lipC* gene, known to encode for extracellular lipases in pseudomonads are shown in Fig. 3.2. As evident, only newly isolated strain BT3 gave positive results. Those PCR products were then sequenced and alignement performed using BLAST (Basic Local Alignment Search Tool). The alignment evaluation indicated that *lipC* gene of this isolate has similar sequences of *lipC* gene of *Pseudomonas stutzeri* collection strains, suggesting that *lipC* are conserved within the same species. This is also confirmed by positive PCR amplification of strain *Pseudomonas stutzeri* BT2 (data not shown), but already discharged because of lower lipase activity.

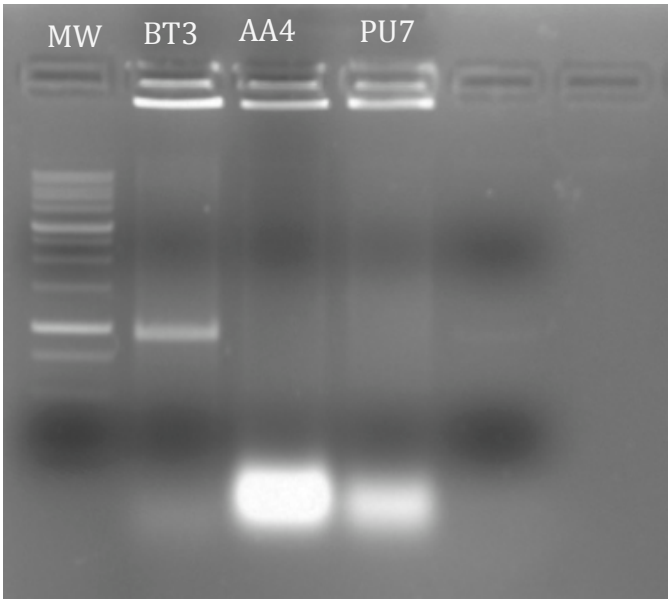


Figure 3.2. Amplification of *lipC* gene from DNA extracts of the new isolates *Pseudomonas stutzeri* BT3, *Pseudomonas sp.* AA4 and *Aeromonas sp*. PU7

This result helped to take a decision on the most suitable strain to adopt as a lipase gene donor. Therefore, the final selection relapsed on *Pseudomonas stutzeri* BT3.

To obtain a preliminar-optimization of the production of lipases from *P. stutzeri* BT3, growth curves were assessed at 37°C in nutrient broth, previously found as optimal conditions for lipase secretion. *Acinetobacter venezianus* DSM 23050 was used as positive control for lipase activity. This bacterium was isolated from an oil-degrading consortium in the Venice lagoon (Italy) and it has been demonstrated as capable of metabolizing fuel oil (Di Cello *et al*., 1997).

Aliquots of cultures were sampled to evaluate the growth. The samples were centrifuged, the pellet was dried and used for the construction of growth curves and the supernatant was utilized for lipase analysis. Lipase activity was measured by titrimetric assay (Pinsirodom *et al*., 2001) at various pHs and temperatures to determine optimal enzymatic activities.

The growth kinetics showed that, as also previously reported (Sangeetha *et al.,* 2011), major production of lipase occurs at late stationary phase (data not shown). Interestingly, *P. stutzeri* BT3 reached stationary phase after 48 hour growth, while the reference strain *A. venezianus* only after 72 hour.

The optimum pH and temperature for *P. stutzeri* BT3 were pH 7.5 and 37°C, respectively, resulting in a lipase activity of 0.5 U/(mL x min), comparable with that of known lipolytic bacteria. This obserevation reinforced the choice of *P. stutzeri BT3* as the genes donor.

3.3. Selection of a suitable lipase genes recipient capable to produce P(4HB) from fatty waste

Poly-4-hydroxybutyrate P(4HB) has been identified as a new absorbable material for chirurgical medical applications such as suture thread and offers a specific set of proprieties that are not currently available, such as absorption without problems of rejection.

P(4HB) have attracted much attention as environmentally degradable thermoplastics for a wide range of agricultural, marine and medical applications. The adsorbable biomaterial is strong yet flexible, and degrades *in vivo* at least in part by a surface erosion process.

It is important to note that U.S. Food and Drugs Administration has approved a PHA containing 4-hydroxybutyrate monomer units produced by recombinant technology for application as absorbable suture material. Therefore, these polyesters are considered for biomedical and also pharmaceutical applications (Williams *et al.,* 2002).

For this reasons, in our experimental project, we searched for a bacterial strain capable to produce a PHA containing 4-hydroxybutyrate monomer units. After an evaluation of bibliographic information the choice relapsed on *Delftia acidovorans*, a Gram-negative non spore forming species belonging to the Comamonadaceae family, well known because it can efficiently accumulate PHAs containing high molar fractions of 4-hydroxybutyrate (4HB) such as the co-polymer P(3HB-*co*-4HB) (Saito *et al*., 1996).

Once obtained from “Leibniz-Institute DSMz-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH”, the collection strain *Delftia acidovorans* DSM 39 was tested for its ability to metabolize fats, but, as expected, this strain resulted unable to use triglycerides. A subsequent test was performed in order to evaluate the ability of the strain to use, as the only carbon source, the free fatty acids and the glycerol released by lipase action. This point represents a key aspect to be taken into consideration in view of the possible genetic modification of this strain by inserting lipase genes. Indeed, in order to give a sense to the

present work, once the lipases are expressed, the products of their activity must represent a suitable carbon source for the recombinant strain.

Therefore, a preliminary test was performed onto solid substrate to verify the possible utilization of some representing free fatty acids and glycerol by *D. acidovorans* DSM 39. As reported in Table 3.3 the strain appeared able to grow well on oleic acid and glycerol, less better on palmitic and myristic acid and did not form any colony on stearic acid.

Table 3.3. Utilization of free fatty acids by the collection strain *D. acidovorans* DSM 39 in plate.

Strain		Palmitic acid	Stearic acid	Myristic acid	Oleic acid	Glycerol
Delftia DSM39	acidovorans	+	-	+	+++	+++

+= growth; - = no growth

However, the growth in Petri dishes could not offer optimal conditions for bacterial growth and accumulation of polymer and a liquid culture cultivation was needed to also observe this second phase. As Table 3.4 reports, experiment in liquid cultures,also assessed to verify the possible production of PHAs by starting from the single fatty acids, shows a different behaviour of the strain, even if stearic acid was confirmed unsuitable. Identification by GC analysis of the different monomers accumulated by the strain is also reported in Table 3.4. The maximum values, in terms of percentage of PHA on the bacterial biomass produced and the molar % composition of the co-polymers P(3HB), P(4HB) and P(3HV), were obtained always after 48 h growth.

Table 3.4.

Production of PHAs from free fatty acids as carbon source by *D. acidovorans* DSM39.

	Time	PHA (% of CDM)[a]	3HB (mol%)	4HB (mol%)	3HV (mol%)
MYRISTIC ACID	48	42.03 ± 9.06	90.84	8.35	0.16
OLEIC ACID	48	22.26 ± 6.85	29.46	-	70.55
STEARIC ACID	48	0.26 ± 0.02	-	-	-
PALMITIC ACID	48	32.22 ± 5.26	90.69	8.07	0.25

[a] : CDM (cell dry mass) in g/L

The most interesting results were related to myristic acid that enabled PHA accumulation up to more than 40% of the total biomass produced. Palmitic and oleic acids were also suitable as a substrate allowing the bacteria to store 32.22 and 22.26 % PHA, respectively. . Interestingly, 4HB monomer was produced in relevant percentages by starting from myristic and palmitic acids (more than 8 mol%).

These results clearly indicated that, once *D. acidovorans* will be able to cut triglycerides through the expression of lipases, it will be suitable to produce different kinds of PHAs containing high molar fractions of P(4HB).

3.4. Recombinant strain construction

At this stage both the donor (*Pseudomonas stutzeri* BT3) and the recipient (*Delftia acidovorans* DSM 39) strains were available, accurately selected from a new isolation program and from an international collection, respectively.

By using LIPC-R, LIPC-F, LIPH-R and LIPH-F primers, already described above and employed to verify the presence of lipase genes in the newly isolated strains, a PCR reaction was performed utilising the genomic DNA extracted from *P. stutzeri* BT3 as template. The size of the amplicons, evaluated by gel electrophoresis (Fig. 3.3), resulted clearly compatible with the genes of interest (*lipH* and *lipC:* 1007 bp and 935 bp, respectively). PCR products were sequenced (by BMR Genomics) and their sequence identity were compared with that of *lipH* and *lipC* genes present in Genbank obtaining 97% and 98% of identity, respectively.

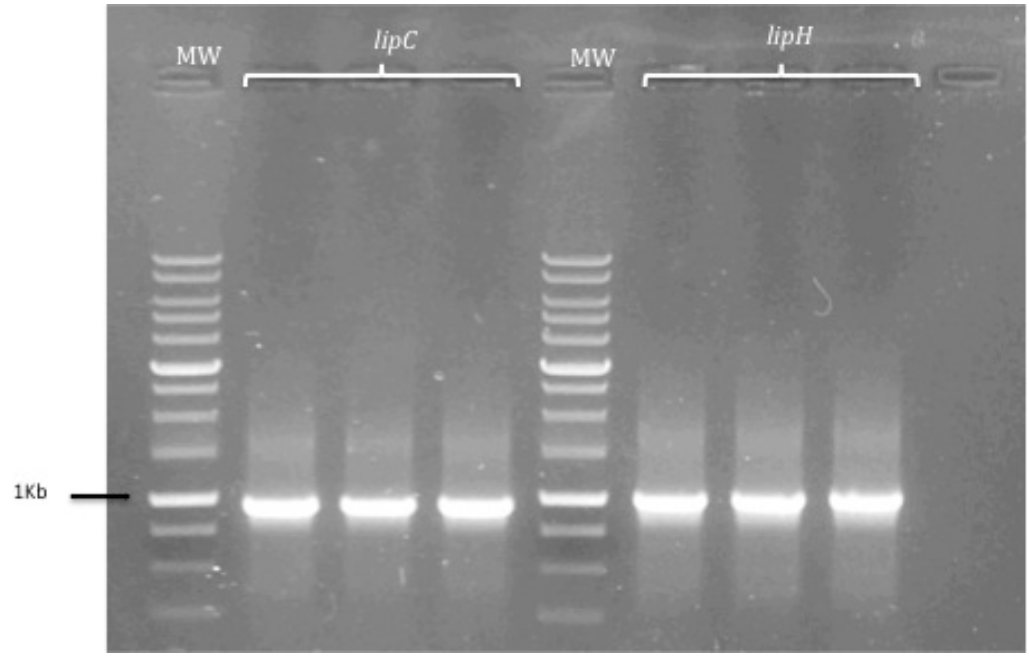


Figure 3.3. Electrophoresis of PCR products of *P. stutzeri* BT3 after amplification with the specific couples of primers LIPC-R/LIPC-F and LIPH-R/LIPH-F.

M: markers.

3.4.1 Construct design

The transfer of *lif* and *lip* genes (in our case *lipH* and *lipC,* respectively) between microorganisms phylogenetically related proved functional in obtaining lipase production (Jorgensen *et al*., 1991), although 30 other cellular proteins are involved in the production and secretion of this enzyme (Rosenau *et al*., 2004). This is possible because such proteins are not specific for the lipase, but are involved in the transport routes of many similar enzymes, such as proteases.

It was therefore necessary to ensure that these transport routes are present in the microorganism selected for the gene's acceptance. The main pathway of secretion of the gram negative is called GSP (Generic Secretion Pathway) and it is responsible for the translocation of many proteins in the periplasm. Secretion proteins to be recognized by the

proteins targeted for secretion all possess N-terminal, particular amino acid sequences that act as a signal peptide (Pugsley, 1993).

The identification of these peptides also allows for recognition of the type of secretion pathway. In particular it is possible to distinguish the Sec-dependent and Tat-dependent pathways. In the first pathway proteins are translocated in the periplasm in an unfolded state, while the latter are secreted already correctly folded.

The presence and the type of Sec-type signal peptide was verified in *lipH* and *lipC* genes of *P. stutzeri* BT3 using the bioinformatic tool PRED-TAT (Bagos *et al.,* 2010). The first 16 amino acid residues of LipC were found to be Sec-type signal with a reliability score value of 0.93/1, while in LipH the first 26 amino acids presumably act as Sec-type signal having a score of 0.97/1. These values are comparable with those calculated for *lipH* and *lipC* genes of *P. stutzeri* ATCC 17588.

These findings demonstrate that the proteins *lipC* and *lipH* are secreted by the bacterium using the GSP path through the Sec-dependent pathway. GSP proteins are generic for all protein products destined for the periplasm and the secretion of lipase in recombinant organisms for this pathway should work fine.

The positioning in *P. stutzeri*, bacterium of origin of the genes, of the sequences *lipH* and *lipC* in the same operon suggest a 1:1 ratio between the two protein products; these are placed under the control of the same promoter.

Many studies have sought to elucidate the genetic regulation of the operon in microorganisms, particularly to investigate if the 1:1 ratio is maintained in transcription, translation and transport of the protein products or if during these processes other regulation mechanisms happen.

By heterologous expression of *lif* and *lip* in *Escherichia coli*, it was determined that, in such conditions, *lif* interacts with *lip* only once, thus confirming the *in vitro* studies which assign to *lif* a catalytic function in single-turnover (Rosenau *et al.,* 2004).

These considerations suggest the maintenance of expression of the two genes 1:1, which allows for them to be placed under the same promoter, as occurs in the organism of origin.

Unlike the original bacteria, the expression of the two genes will be made constitutive, thus allowing the constant production of lipase, a feature conducive to the growth of the bacterium in media rich in lipids.

In order to select the appropriate plasmid vector to be used for genes cloning, a preliminar antibiotic resistance test was assessed in liquid media for *D. acidovorans* DSM 39. As shown in Tab. 3.5, the strain resulted as sensitive to both Gentamicin and Tetracycline at the concentrations generally used for genetic engeenering purposes.

Table 3.5. Antibiotic resistance test in liquid Nutrient Broth (NB). += growth; - = absence of growth

Hours	Gentamicin 20 µg/mL	Tetracycline 45 µg/mL	Kanamycin 45 µg/mL	Chloramphenicol 30 µg/mL
24	-	-	-	-
48	-	-	+	+
72	-	-	+	+

The antibiotic resistance test suggested to adopt pBBR1MCS-5 as plasmid vector. This vector contains a sequence coding for the replication origin (pBBR1 Rep), the gene for gentamicin resistance (GMR), the *lacZ* gene and a region MCS (Multiple Cloning Site). Promoters T7, T3 and *lac* are also present and opportunely positioned in relation to MCS (Fig. 3.4).

In particular, the sequence pBBR1 Rep allows plasmid replication in gram-negative bacteria such as *Delftia acidovorans*, while the position of MCS within the *lacZ* gene enables the α-complementation.

Within this plasmid vector, genes had to be inserted under the control of the constitutive promoter T3, thus allowing constitutive expression of genes. Upstream there is the *lac* promoter of *lacZ* gene that can be induced to transcription by IPTG. Figure 3.5 schematically illustrates the final construct to be obtained.

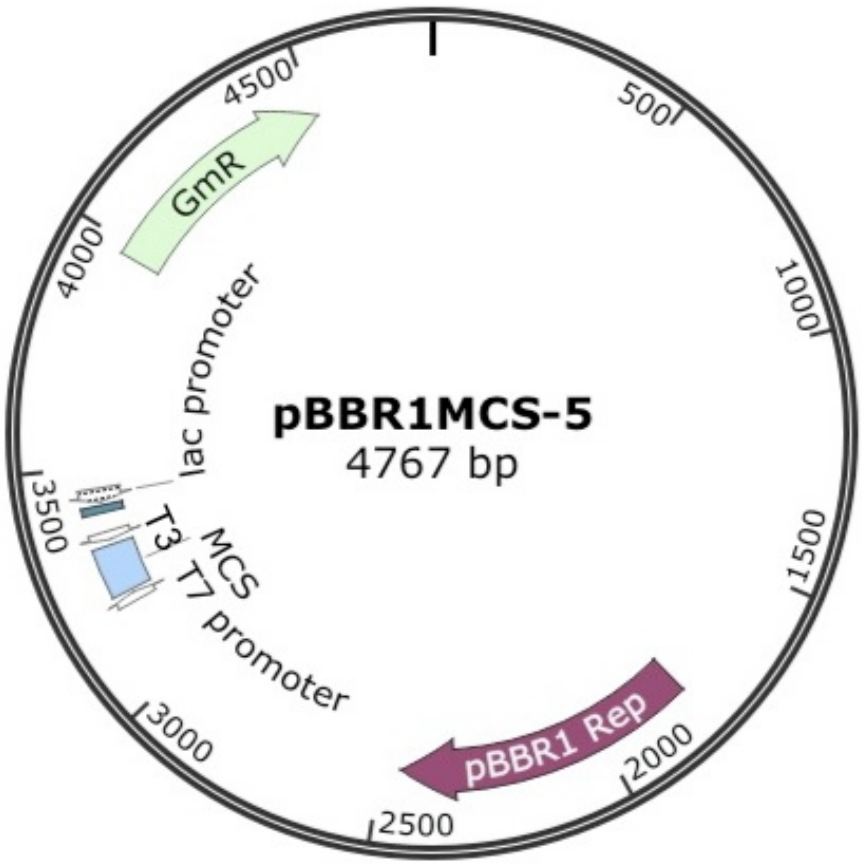


Figure 3.4. Plasmid map of pBBR1MCS-5 vector.

The gentamicin resistance genes (GMR), the *lacZ* gene, the origin of replication (pBBR1 Rep), the area MCS and the T3, T7 and *lac* promoters are highlighted.

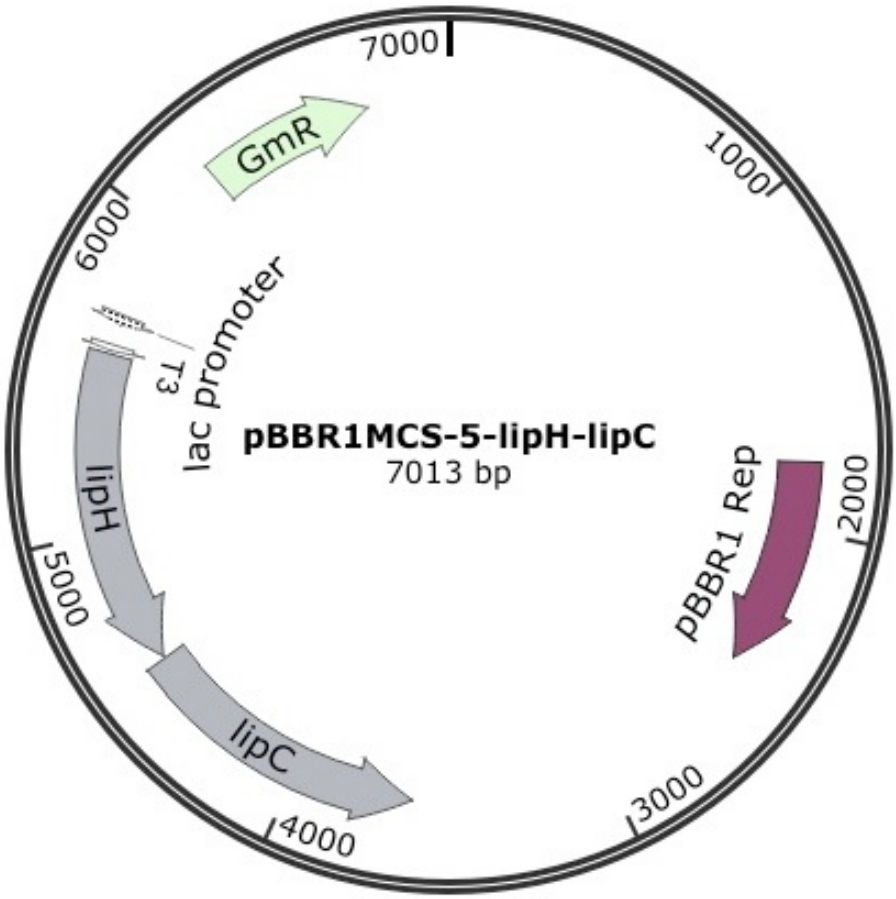


Figure 3.5. Diagram of the final plasmid vector pBBR1MCS-5, with *lipH* and *lipC* genes inserted under the control of the promoter T3.

The constructed plasmid vector shown in Fig. 3.5 was attained by multiple steps.

Initially, *lipC* and *lipH* genes, from *P. stutzeri* BT3, were inserted, by separate processes, within the commercial plasmid pDrive, designed to bond PCR products by uracil overhang, so obtaining pDrive-*lipC* and pDrive-*lipH* (Fig. 3.6). Subsequently, the gene *lipC* was excised from pDrive-*lipC* using the restriction enzyme EcoRI and inserted into the vector pBBR1MCS-5. *LipH* gene was similarly excised from pDrive-*lipH* using the enzymes ApaI and MluI and inserted into pBBR1MCS-5 that already contained *lipC*. These specific restriction enzymes were used to obtain the correct insertion of the fragment on the control of the T3 promoter. The final vector pBBR1MCS-5-*lipH-lipC* was initially cloned into *E. coli* DH10B.

3.4.2 Genes insertion in pDRIVE

lipC and *lipH* amplified fragments were inserted in pDrive at a specific site (317 bp) within MCS region (Fig. 3.6). This will enable the genes to be excised due to the presence of specific restriction sequences.

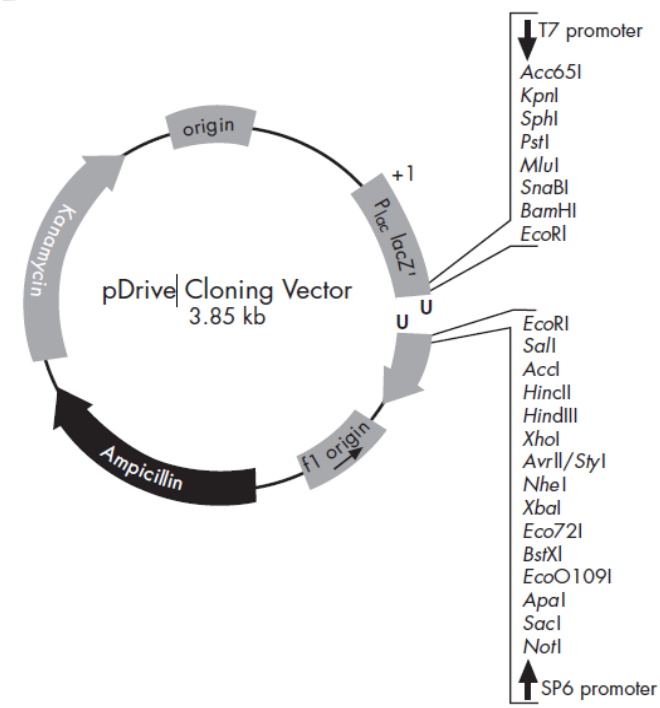


Figure 3.6. pDrive plasmid vector with the restriction sites located in the MCS region, inserted in the *lacZ* gene necessary for α-complementation.
The two uracil nucleotides shown (U) represent the site where PCR amplification products will be inserted.

Following the protocol provided by the manufacturer, the insertions by transformation of the pDrive vectors, containing *lipC* or *lipH*, was performed in two distinct colonies of *E. coli* DH10B. The selection of the transformed colonies were carried out through the acquired antibiotic resistance and by α-complementation.

A further verification of the presence of the desired gene was carried out by PCR for the transformed *E. coli* containing pDrive-*lipC*, using the primers LIPC-F and LIPC-R. The test gave positive results for all the colonies (Fig. 3.7).

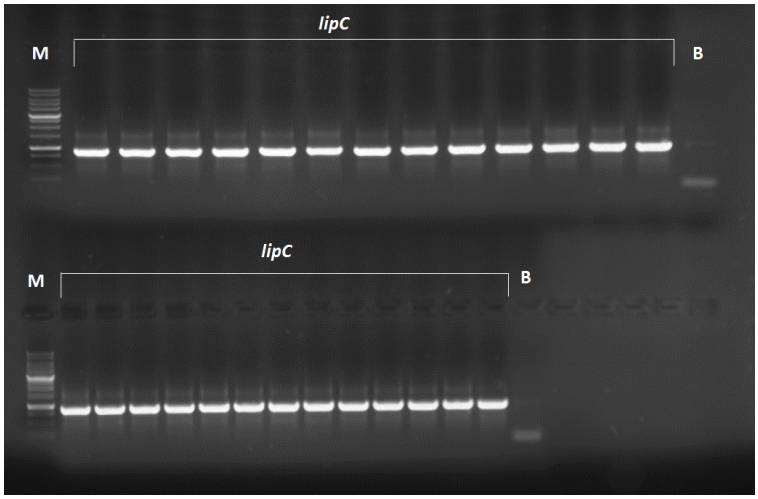


Figure 3.7. Electrophoresis of the *lipC* gene amplification from the plasmid vector pDrive-*lipC*. M: marker; B: negative control.

The *lipC* gene could now be excised by EcoRI digestion.

On the other hand, since the excision of *lipH* from pDrive for the subsequent insertion in pBBR1MCS-5 foresees different restriction sites at the two ends (MluI and ApaI), it was necessary to verify the correct orientation of the gene in pDrive. This was provided by an enzymatic digestion with NaeI. Indeed, by comparing the site restriction maps present in the genes and in the plasmid, it results evident that there is only one NaeI restriction site within *lipH* at base pair number 243, and another single site within pDrive at bp 919. As a consequence, if the gene is inserted correctly, the digestion with NaeI will produce a fragment of 842 bp and one of 4018 bp (Fig. 3.9a). Otherwise, if it was inserted in the wrong orientation, a fragment of 1364 bp and one of 3496 bp will be produced (Fig. 3.9b).

Gel electrophoresis of the NaeI digestion products allowed the selection of those colonies containing the gene properly inserted.

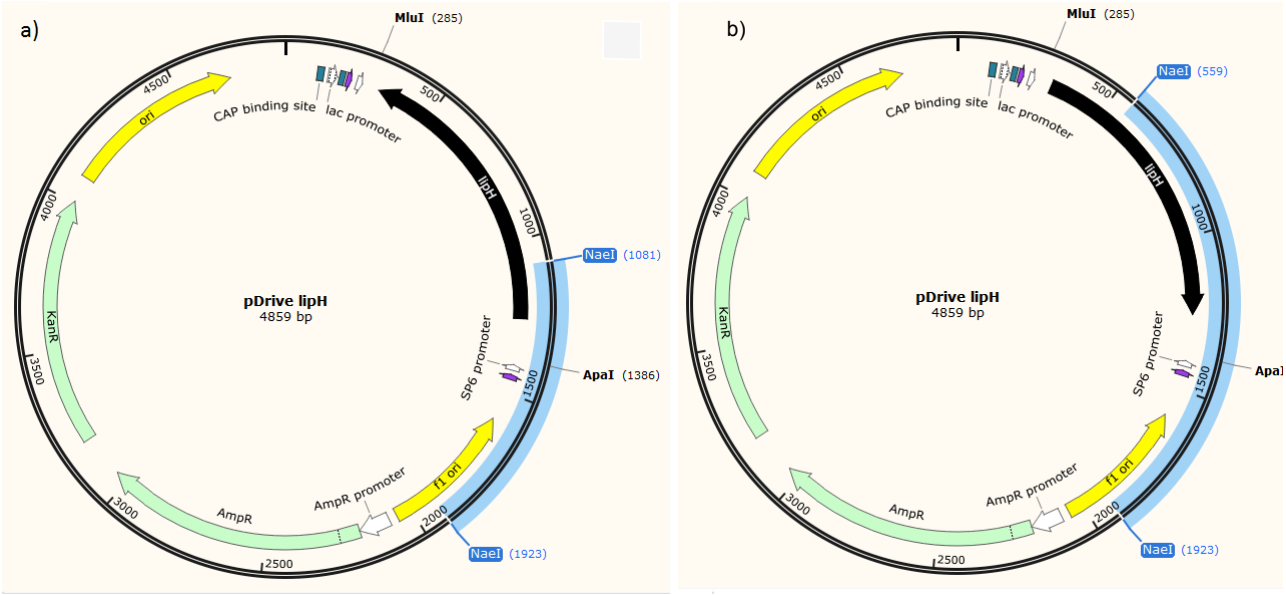


Figure 3.9. pDrive schematic representation of the two possibilities obtainable by the insertion of *lipH* gene. The MluI, ApaI and NaeI restriction sites are also indicated.

a) *lipH* gene is correctly inserted: the segment resulting from NaeI digestion (highlighted in blue) will be of 842 bp.

b) *lipH* gene is inserted in the opposite direction: the segment resulting from NaeI digestion (highlighted in blue) will be of 1364 bp.

3.4.3. Insertion of *lipC* gene in plasmid pBBR1MCS-5

Once the two pDrive constructs (pDrive-*lipC* and pDrive-*lipH*) became available, it was possible to excise the two genes for the subsequent cloning within plasmid pBBR1MCS-5. Therefore, a preparation of this latter plasmid was digested with EcoRI, treated with alkaline phosphatase to avoid recircularization, and a subsequent ligation was achieved first with *lipC* gene that was previously excised from pDrive plamsid.

After ligation the plasmid obtained was trasformed into *E. coli* DH10B.

The trasformed *E. coli* was plated on solid medium with gentamicin, IPTG and X-gal in order to allow the selection of the recombinant colonies by antibiotic resistance and α-complementation.

From the colonies that have successfully acquired the gene (withe colonies), once cultured, plasmidic DNA was extracted.

The presence of the gene within the plasmid was also confirmed by PCR using the primers LIPC-R and LIPC-F, as Fig. 3.10 shows for 2 of the 6 positive colonies obtained from the transformation.

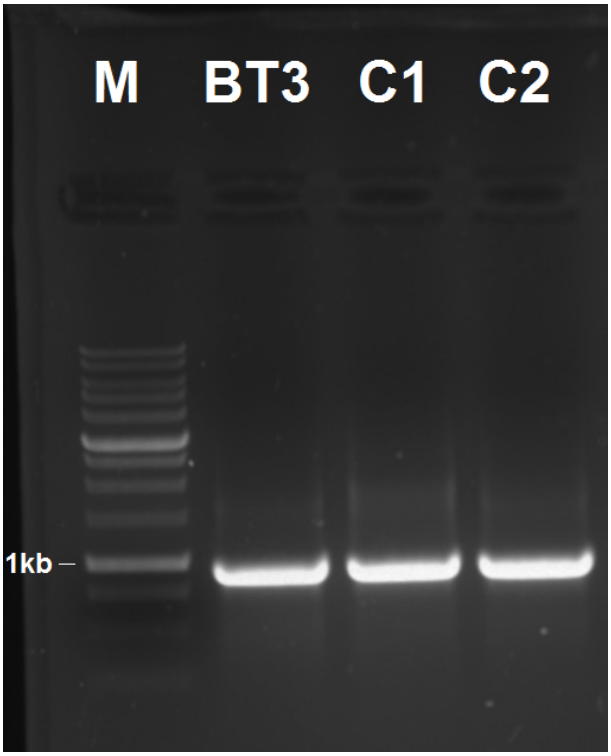
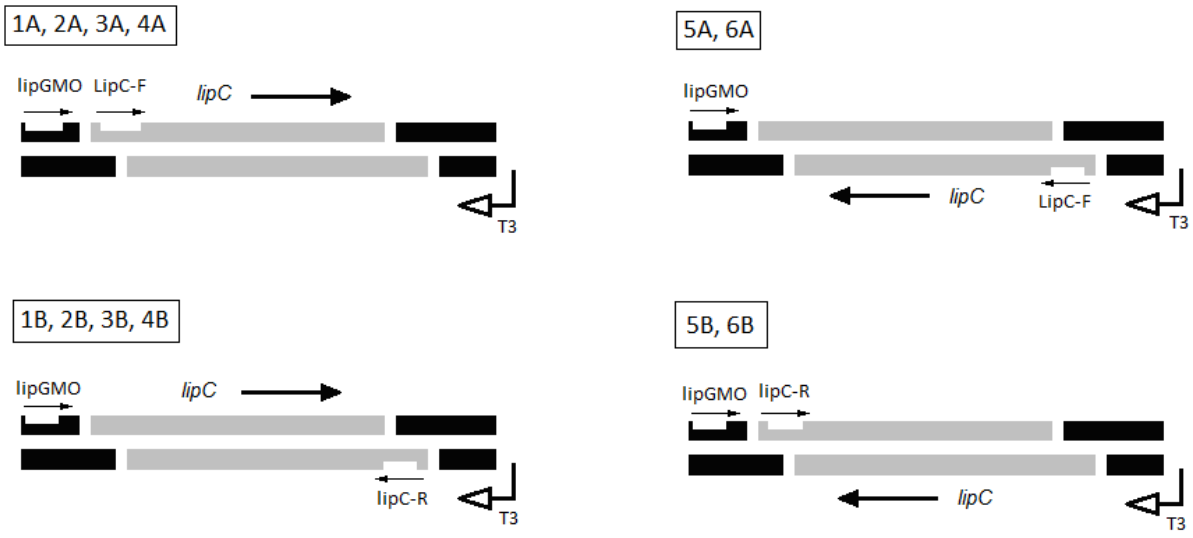


Figure 3.10. Electrophoresis of PCR products amplified with primers LIPC-F and LIPC-R
C1 and C2: plasmidic DNA extracted from two of the 6 transformed colonies
BT3: genomic DNA of *P. stutzeri* BT3
M: markers

Once the presence of *lipC* gene was confirmed, it was of key importance to verify that its insertion correctly occurred downstream and on the same strand of T3 promoter.
With this aim the use of LIP-GMO primer was adopted in combination with LIPC-F or LIPC-R. LIP-GMO is a primer designed on the pBBR1MCS-5 sequence as reported in section 2.5.1. By using plasmidic DNA extracted from the 6 positive colonies obtained above, the four different possibilities described in Fig. 3.11a can occur. Particularly, the amplification by LIP-GMO and LIPC-F would not produce any PCR product if *lipC* gene was inserted in the strand opposite to T3 promoter, while the PCR reaction will be successful if *lipC* gene is located downstream of the promoter. On the other hand, the couple of primers LIP-GMO/LIPC-R will give rise to an amplification product only if *lipC* gene does not lie downstream the T3

promoter. By looking at Fig. 3.11b it is clear that only the plasmids from colonies 5 and 6 acquired *lipC* gene in the correct position (A5, A6, B5 and B6 columns).

a)



b)

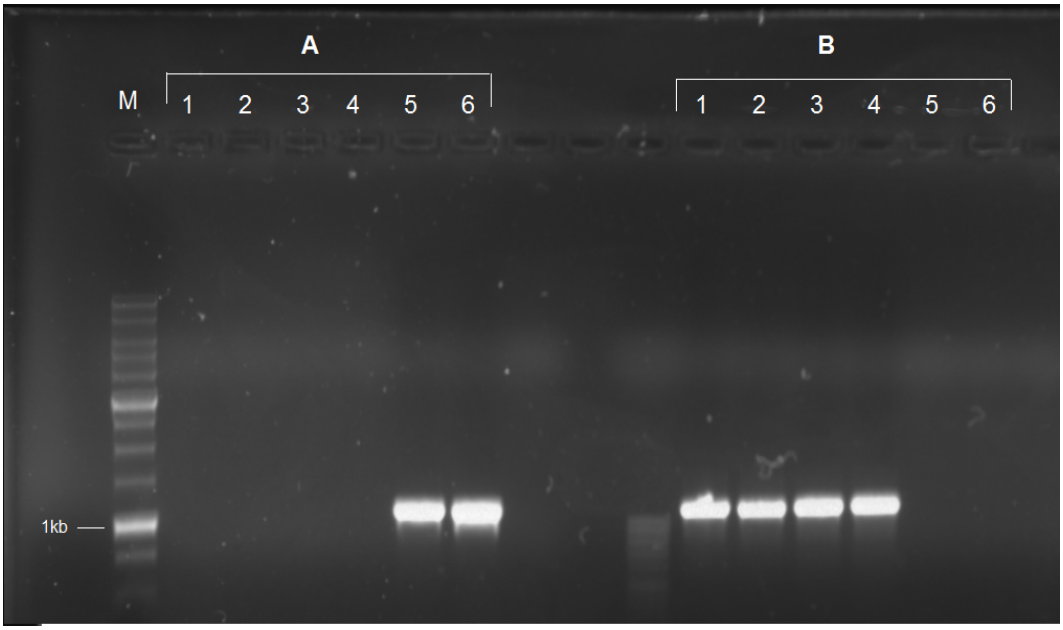


Figure 3.11.

a) Schematic representation of the possible *lipC* gene insertion in pBBR1MCS-5, and the subsequent results of amplification with a mix of primer described in b).

b) Electrophoresis of PCR products amplified with primers:

A: primers LIP-GMO and LIPC-F

B: primers LIP-GMO and LIPC-R

M: markers

3.4.4. Insertion of *lipH* gene in plasmid pBBR1MCS-5-*lipC*

Since the gene was excised from pDrive-lipH using two different restriction enzymes (*lipH* gene was cutted from pDrive with ApaI and MluI), for the correct insertion of the gene into pBBR1MCS-5-*lipC* it was necessary to choose the sites that meet the desired direction. In the MCS of pBBR1MCs-5-*lipC* MluI was absent, therefore ApaI and ClaI enzymes were chosen because the sticky end derived from MluI and ClaI digestion are known to be compatible.

An extraction of plasmidic DNA was performed from the *E. coli* colonies transformed with plasmid pBBR1MCS-5-*lipC* harbouring *lipC* in the right position. After digestion with ApaI and ClaI endonucleases and treatment with alkaline phosphatase, the ligation of *lipH* excised from pDrive-*lipH* was performed to obtain plasmid pBBR1MCS-5-*lipH*-*lipC*. This latter vector was transformed in *E. coli* DH10B to obtain the recombinant strain *E. coli* DH10B-pBBR1MCS-5-*lipH*-*lipC.*

Plating the recombinants on medium contining gentamicin enabled to select those colonies that acquired the plasmid. However, since the *lacZ* gene remains as interrupted, irrespective of the insertion of *lipH* because of the presence of *lipC*, a specific PCR was needed to verify the presence of *lipH* in the recombinant colonies. This examination was also designed to obtain information on the direction assumed by *lipH* in the final vector. In other words, once cultured, the plasmidic DNA was extracted from the colonies and subjected to two different PCR by using the couple of primers LIPH-F/LIPC-R and LIPH-F/LIPC-F, respectively. As shown in Fig. 3.12, if *lipH* is present and oriented in the right direction, a fragment of about 2000 bp will be produced with primers LIPH-F/LIPC-R, while no amplification would occur with the second couple of primers.

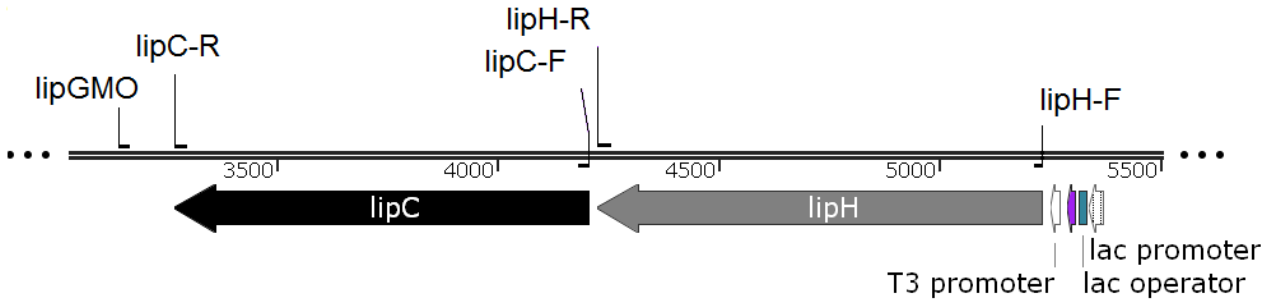


Figure 3.12. Schematic representation of a plasmid portion of pBBR1MCS-5-*lipC-lipH* with the genes properly inserted. Primer T3 and *lac* promoters are also indicated**.**

The gel electrophoresis of PCR products indicated that, together with *lipC*, *lipH* gene is present and correctly oriented, at least in 30% of the recombinant colonies that acquired the plasmid (Fig. 3.13).

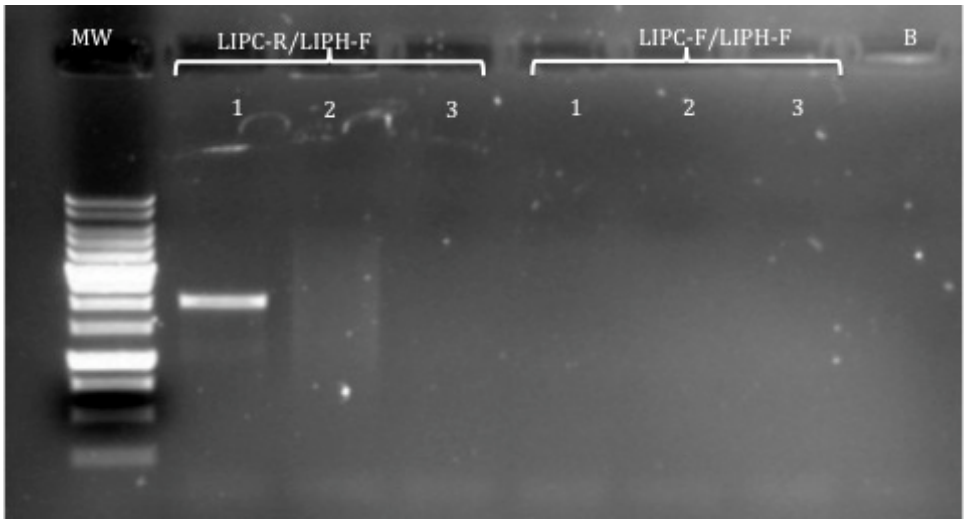


Figure 3.13. Electrophoresis of PCR products amplified with primers LIPH-F/LIPC-R and with primers LIPH-F/LIPC-F of three colonies (1, 2, 3).

M: marker

B: no cells

3.4.5. Verification of T3 promoter

After the insertion of the *lipH* gene the functionality of the T3 promoter in pBBR1MCS-5-*lipH-lipC* was tested. RNA extraction was performed from one of the colonies with the

correct insertion of the *lipC and lipH* genes and cDNA obtained by reverse transcription was used as a template for *lipC* gene amplification.

The results reported in Fig 3.14 demonstrate the presence of mRNA syntetized from *lipC*. Since T3 promoter controls both *lipH* and *lipC*, the transcription of *lipC* indicates that both genes have been transcribed.

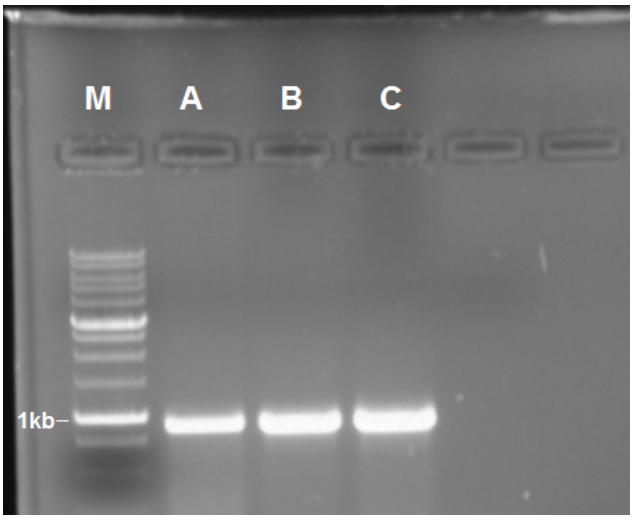


Figure 3.14. PCR of cDNA obtained from cells of *E. coli* pBBR1MCS-5-*lipH-lipC* using primers LIPC-R and LIPC-F.
A, B, C: replicates.
M: markers

3.5. Verification of the acquired lipolytic ability in E. coli pBBR1MCS-5-lipH-lipC

Following the verification that plasmid pBBR1MCS-5-*lipH-lipC* effectively contains the two cloned genes properly inserted and placed under the constitutive promoter T3, the lipolytic activity of the transformed *E. coli* DH10B-pBBR1MCS-5-*lipH-lipC* strain needed to be tested. This validation was performed on plates containing rhodamine B, as described above.

Fig. 3.15 allows the comparison between the transformants and the wild type *E. coli* DH10B. The fluorescence shown by the recombinant strains (a and b), absent in the wild type (c),

testify for a clear lipolytic activity, thus demonstrating the expression of the genes and the correct folding of the produced protein.

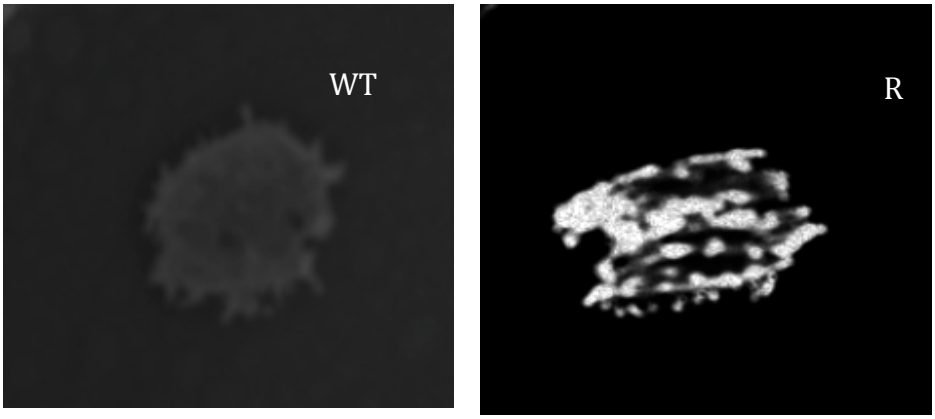


Figure 3.15. Growth on LB medium containing rhodamine B.

WT) *Escherichia coli* DH10B wild-type.

R) Recombinant *Escherichia coli* DH10B-pBBR1MCS-5-*lipH-lipC*.

3.6. Trasformation of plasmid pBBR1MCS-5-lipH-lipC in Delftia acidovorans DSM 39

Once proven effective in *E. coli*, plasmid pBBR1MCS-5-*lipH-lipC* was moved into the final recipient *D. acidovorans* DSM 39 (now becoming *Delftia acidovorans* DSM 39-pBBR1MCS-5-*lipH-lipC*) , chosen on the basis of its ability to produce PHAs containing high molar ratios of 4HB and on its ability to use free fatty acids, as described above. The transformants, obtained by $CaCl_2$ technique described in ‘‘Materials and methods’’, were selected through their gentamicin resistance and used for a *lipC* and *lipH* PCR. Fig. 3.16, that shows the results of PCR electrophoresis from one of the transformants as compared to the wild type strain, confirmed the presence of plasmid pBBR1MCS-5-*lipH-lipC* in the recipient strain.

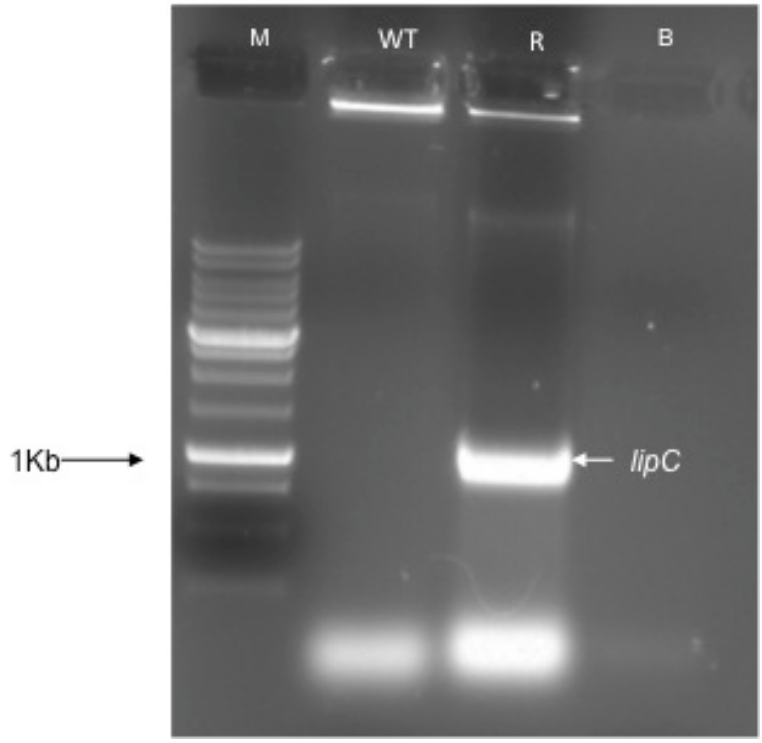


Figure 3.16. Gel electrophoresis of PCR products by using LIPC primers.

WT: *Delftia* acidovorans WT

R: recombinant *Delftia acidovorans* pBBR1MCS-5-*lipH-lipC*

M: markers

B: no cells

An additional verification on the obtained recombinant *Delftia acidovorans* DSM 39-pBBR1MCS-5-*lipH-lipC* was obtained by rhodamine B plate assay that, on the basis of the fluorescence intensity, confirmed a considerable lipase activity (Fig. 3.17).

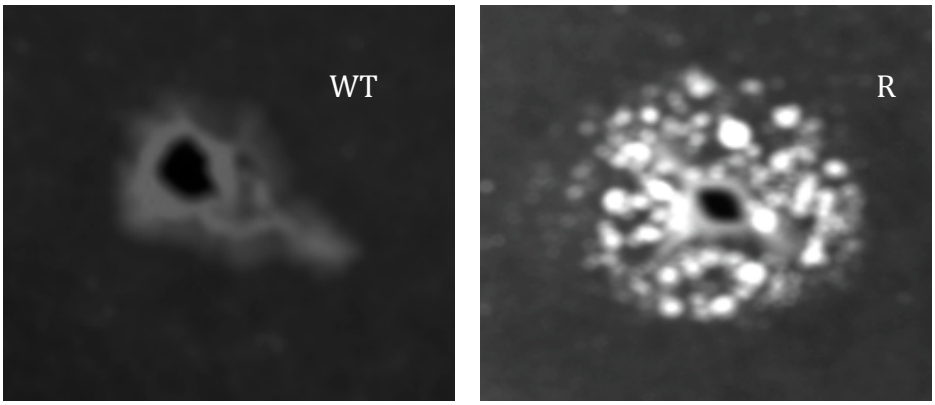


Figure 3.17. Growth of the wild type (WT) and the recombinant (R) *D. acidovorans* on NB medium containing rhodamine B.

However, a titrimetric assay was necessary to determine the content of extracellular lipase units and the ability to constitutively express the cloned lipase genes. Therefore, wild type and recombinant strains were grown for 48h in rich medium (NB) not containing any lipidic carbon source, and lipase activity was checked at 24 and 48h in the cultures supernatant.
Tab. 3.8 shows that a suitable activity, comparable with literature data (Hasanuzzaman *et al*., 2004), was present in the supernatant of the recombinant *D. acidovorans*, while it was completely absent for the wild type strain.

Table 3.8. Lipase activity measured with titrimetric method at 37°C and pH7.5 in the supernatant of *D. acidovorans* pBBR1MCS-5-*lipH-lipC* and WT suspensions.

strain	U/mL 24h	U/mL 48h	µmol/mL*min 24h	µmol/mL*min 48h
D. acidovorans pBBR1MCS-5-lipH-lipC	0.28	0.33	0.64	0.80
D. acidovorans WT	0.00	0.00	0.00	0.00

*3.7. Ability of Delftia acidovorans DSM 39-pBBR1MCS-5-*lipH-lipC *to produce PHAs from fatty wastes.*

The next and crucial step was to check the ability of *Delftia acidovorans* DSM 39-pBBR1MCS-5-*lipH-lipC* to produce PHA using fatty waste as the only carbon source.
In all the following tests the evaluation of the production of PHAs from fatty wastes was performed using a two-step cultivation procedure: the first step consisted in biomass production in rich medium (NB) for 1 day. Aerobic growth was done in shaken flasks at 30 °C, then biomass was collected by centrifugation and transferred into a minimal medium (Saito Y. *et al.,* 1994) with low nitrogen content and high amount of fats as the only carbon source. Growth in this second step was maintained for 2 days. Polyhydroxyalkanoates content was then quantified in biomass using the method of methanolysis (Braunegg *et al.,* 1978), analysing 3-hydroxyalkyl methyl esters by gas chromatography.
Before directly using animal fats as carbon source (udder, lard and tallow), corn oil was

adopted for a preliminary test, due to its emulsion easiness. The related results are reported in Table 3.9.

Tab. 3.9. Production of PHA by wild type and recombinant *D. acidovorans* from corn oil as carbon sources.

Strain	Growth time (h)	PHA (%CDM)	3HB (mol%)	4HB (mol%)	3HV (mol%)
D.acidovorans DSM 39WT 24h	24	0.12 ± 0.06	98.93	0.00	1.06
D.acidovorans DSM 39WT 48h	48	0.15 ± 0.05	100.00	0.00	0.00
D.acidovorans DSM 39-pBBR1MCS-5-*lipH-lipC*	24	12.07 ± 5.49	92.91	6.97	0.12
D.acidovorans DSM 39-pBBR1MCS-5-*lipH-lipC*	48	26.72 ± 6.66	92.71	7.18	0.11

After 48h, recombinant *D. acidovorans* DSM 39-pBBR1MCS-5-*lipH-lipC* accumulated PHA more than 26% of cell dry weight, with about 7% of 4HB.

Taking into consideration that the wild type strain accumulated only 0.15% of PHA on dry biomass, these data confirm that *D. acidovorans* DSM 39 acquired the property to utilize corn oil as a carbon source to produce PHAs.

Moreover, the production of 4HB without precursor is a very interesting event. Indeed, it has been reported that 4HB occurred only when 4HB or appropriate precursors were present in the growth media, such as 1,4-butanediol and γ-butyrolactone, and that the choice of a precursor depends on the type of microorganisms employed (Lee *et al.,* 2004).

Considering the good result obtained with corn oil, next step was to directly use animal fatty wastes as carbon source to obtain PHA.

Udder, the fat extracted from the mammary glands of cow, was the fatty wastes used in this further experiment and the related results are reported in Tab. 3.10.

Tab. 3.10. Production of PHA by wild type and recombinant *D. acidovorans* from udder as carbon source.

Strain	Accumulation time (h)	PHA (%CDM)	3HB (mol%)	4HB (mol%)	3HV (mol%)
D. acidovorans DSM 39 WT	24	3.30 ± 2.14	93.24	6.39	0.37
D. acidovorans DSM 39 WT	48	0.87 ± 0.62	94.41	5.22	0.37
D. acidovorans DSM 39-pBBR1MCS-5-*lipH-lipC*	24	35.90 ± 4.94	91.32	8.46	0.22
D. acidovorans DSM 39-pBBR1MCS-5-*lipH-lipC*	48	42.79 ± 1.86	93.02	6.71	0.28

As compared to the use of corn oil, udder gave even superior results, producing 35-42% of PHA on cellular dry weight, with 6-8% of 4HB within 48 hours (Tab 3.10).

These results unambiguously proved the ability of the recombinant *D. acidovorans* strain to

produce PHA, directly from animal fatty waste, with a significant percentage of 4HB without any precursor in the liquid media.

In the wake of such interesting results, other fatty wastes originating from food industry were tested as a carbon source for the constructed PHA producing strain. Specifically, other two slaughterhouse residues were used: lard and tallow.

Lard is obtained in high amounts from any part of pig with high concentration of fatty tissue and, beside its limited culinary use, it can be also used to produce biofuels and soap. However, in order to prevent spoilage and rancidity, lard must be often treated with bleaching and deodorizing agents, emulsifiers and antioxidants, or it must be refrigerated or frozen.

Tallow is a rendered form of beef or mutton fat. It is solid at room temperature and, if stored in appropriate container to prevent oxidation, it can be maintained for extended periods without the need for refrigeration to prevent decomposition. As well as vegetable oil, this animal residue can be used for the production of biodiesel and, since it is derived from animal by-products, which have little to no value, the food vs. fuel debate does not apply.

As reported in Table 3.11, the recombinant *D. acidovorans* pBBR1MCS-5-*lipH-lipC* resulted able also to use lard, producing remarkable amounts of polymer (up to almost 39% of the cell dry weight), while the wild type strain did not.

Therefore, the genetic modification of the strain under study was effective in delivering to these bacteria the right enzymatic equipment for hydrolyzing the fatty substrate to produce PHAs. Even in this case, without the presence of any precursor, appreciable amounts of 4HB have been produced, reaching about 6% of the total PHA. In the present case, the maximum values of PHA accumulation was achieved at 24h, indicating that such a substrate is easier to be hydrolyzed.

However, additional 24h incubation resulted in the rapid depolymerization of PHA, especially at the expense of P(3HB).

Tab. 3.11. Production of PHA by wild type and recombinant *D. acidovorans* from lard as carbon sources**.**

Strain	Accumulation time (h)	PHA (%CDM)	3HB (mol%)	4HB (mol%)	3HV (mol%)
D.acidovorans DSM 39 WT	24	1.43 ± 0.10	95.98	0.00	4.02
D.acidovorans DSM 39 WT	48	0.55 ± 0.10	94.75	0.00	5.25
D.acidovorans DSM 39-pBBR1MCS-5-*lipH-lipC*	24	39.33 ± 1.04	82.02	6.15	11.83
D.acidovorans DSM 39-pBBR1MCS-5-*lipH-lipC*	48	14.50 ± 5.63	46.24	33.01	20.73

The other fatty waste utilised in this work as a carbon substrate was tallow. The related results, reported in Table 3.12, could be surprising because both the strains, wild type and recombinant *D. acidovorans*, produced appreciable amounts of PHAs. However, a rapid analysis on the emulsified substrate showed that the rendering process of tallow could result in a considerable release of residual free fatty acids that may easily be used by the wild type strain, too. On the other hand, by considering the not significant difference between the amounts and composition of the polymers produced by the two strains, it can be deduced that the use of tallow as a direct substrate for the recombinant *D. acidovorans* will probably require additional investigations of technological nature.

Tab. 3.12. Production of PHA by wild type and recombinant *D. acidovorans* from tallow as carbon sources.

Strain	Accumulation time (h)	PHA (%CDM)	3HB (mol%)	4HB (mol%)	3HV (mol%)
D.acidovorans DSM 39 WT	24	10.33 ± 4.51	88.36	9.12	0.50
D.acidovorans DSM 39 WT	48	7.33 ± 4.04	89.31	9.24	0.28
D.acidovorans DSM 39-pBBR1MCS-5-*lipH-lipC*	24	13.67 ± 1.53	88.31	8.60	0.61
D.acidovorans DSM 39-pBBR1MCS-5-*lipH-lipC*	48	15.33 ± 6.11	89.48	7.54	0.28

4. Concluding remarks

A possible utilization of fatty waste deriving from slaughterhouse and rendering industry attracts much interest because (i) these residues are available in huge amounts, more then 500,000 ton/year in Europe (Titz *et al*., 2012) (ii) there is no concern about food vs. fuel debate because this material is derived from animal by-products, which have little to no value, (iii) their non-petroleum dependence.

In the present study, the possibility to convert such a waste into added value products, such as biodegradable and biocompatible biopolymers, has been attained in a single step process by genetically modifying a non-lipolytic bacterial strain (*D. acidovorans* DSM 39). This bacterial species is also known because it can efficiently accumulate PHAs containing high molar fractions of 4-hydroxybutyrate (4HB) such as the co-polymer P(3HB-*co*-4HB) that shows very attractive chemical-physical-mechanical properties and a wide range of potential applications.

As a source of the genes, instead of using a known collection strain, an attempt to find new lipolitic bacteria in soil or directly from a slaughterhouse was faced. From the results obtained it can be deduced that there are still many opportunities to isolate and select very interesting bacterial strains, possessing attracting properties. The new isolate *Pseudomonas stutzeri* strain BT3 represent a good example of this hypothesis, resulting as an optimal *lip* genes donor. However, as expected, the chance to directly find in nature new bacterial strains specifically tailored for human requirements, has been confirmed as very unlikely.

The transfer of *lipC* and *lipH* genes from *P. stutzeri* BT3 to *D. acidovorans* DSM 39 was assumed to be functional in obtaining lipase production because the two microorganisms are phylogenetically related. Although many other cellular proteins are involved in the production and secretion of this enzyme (Rosenau *et al.,* 2004), they are not specific for lipase, but are assigned to the transport routes of many similar enzymes. The presence and the type of Sec-type signal peptide (Rosenau *et al.,* 2004) was then confirmed in *lipH* and *lipC* genes of *P. stutzeri* BT3 using the bioinformatic tool PRED-TAT (Bagos et al., 2010).

In *P. stutzeri*, the donor of the genes, *lipH* and *lipC* are located in the same operon, suggesting a 1:1 ratio between the two protein products. Therefore, for their heterologous expression, the two genes have been placed under the control of the same promoter. Moreover, unlike the original bacteria, the expression of the two genes was made

constitutive, thus allowing the constant production of lipase, a feature conducive to the continuous utilization of lipids when cultured in appropriate media.

Interestingly, the synthesis of co-polymers containing appreciable amounts of P(4HB) was obtained by the newly constructed strain even in the absence of any specific precursor. Indeed, the high cost of 4HB and, as a consequence, of PHA containing this monomer, is generally due to the necessary use of 4HB, 1,4-butanediol and γ-butyrolactone as precursors. Particularly, 4HB is produced by chemical synthesis from fossil oil-based resources (Zhang *et al.,* 2008).

Finally, the variable results obtained by testing different fatty substrates (i.e. tallow) indicated that the optimization of a hypothetic process should be needed case by case, taking into account both the nature of the fatty waste under use and the treatments it usually withstand.

5. REFERENCES

H. Abe, I. Matsubara, Y. Doi, Y. Hori, A. Yamaguchi, Physical properties and enzymatic degradability of poly- (3-hydroxybutyrate) stereoisomers with different stereoregularities, Macromolecules 27 (1994) 6018–6025.

J.E. Alderete, D.W. Karl, C.H. Park, Production of poly(hydroxybutyrate) homopolymer and copolymer from ethanol and propanol in a fed-batch culture, Biotechnol. Progr. 9 (1993) 520–525.

C. Angkawidjaja and S.Kanaya, Family I.3 lipase: bacterial lipases secreted by the type I secretion system, Cell. Mol. Life Sci. 63 (2006) 2804–2817.

J. Arpigny, K. Jaeger, Bacterial lipolytic enzymes: classification and properties, J. Biochem. 343 (1999) 177-183.

F. M. Ausubel, R. Brent, R. E.Kingston, D. D. Moore, J.G. Seidman, J. A. Smith, K. Struhl, Current Protocols in Molecular Biology, John Wiley & Sons, Inc. Unit 1.8, 2003.

P.G. Bagos, E.P. Nikolaou, D.L. Theodore, K.D. Tsirigos, Combined prediction of Tat and Sec signal peptides with hidden Markov models, Bioinformatics, 26 (2010) 2811-2817.

A. Bergeret, Environmental-Friendly Biodegradable Polymers and Composites, Integrated Waste Management, Mr. Sunil Kumar (Ed.), Volume I, 2011.

G. Braunegg, R. Bona, M. Koller, Sustainable polymer production, Polym. Plast. Technol. Eng. 43 (2004) 1779–1793.

G. Braunegg, B. Sonnleitner, R.M. LaVerty, A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass, Eur. J. Appl. Microbiol. Biotechnol. 6 (1978) 29–37.

D.H.E. Ch'ng, W.H Lee, K. Sudesh, Biosynthesis and lipase-catalysed hydrolysis of 4-hydroxybutyrate-containing polyhydroxyalkanoates from *Delftia acidovorans,* Mal. J. Microbiol. 8 (2012) 156-163.

J.Y. Chee, S.S. Yoga, N.S. Lau, S.C. Ling, MMR Abe, K. Sudesh, Bacterially Produced Polyhydroxyalkanoate (PHA): Converting Renewable Resources into Bioplastics, Cur. Res. Tech. Ed. Top. App. Microbiol. Microb. Biotech. 2 (2010) 1395-1404.

G.Q. Chen, Plastics completely synthesized by bacteria: polyhydroxyalkanoates, Microb. Mon. 14 (2010) 17-37

F. Di Cello, M. Pepi, F. Baldi, R. Fani, Molecular characterization of an n-alkane-degrading bacterial community and identification of a new species, *Acinetobacter venetianus*, Res. Microbiol. 148 (1997) 237–249.

T. Durfee, R. Nelson, S. Baldwin, G. Plunkett III, V. Burland, B. Mau, J. F. Petrosino, X. Qin, D. M. Muzny, M. Ayele, R. A. Gibbs, B. Csörgő, G. Pósfai, G. M. Weinstock, F. R. Blattner, The Complete Genome Sequence of *Escherichia coli* DH10B: Insights into the Biology of a Laboratory Workhorse, J. Bacteriol. 190 (2008) 2597-2606.

N. Fauré, A. Illanes, Immobilization of *Pseudomonas stutzeri* lipase for the transesterification of wood sterols with fatty acid esters, Appl. Biochem. Biotech. 65 (2011) 1332 – 1341.

H. G. Fritz, W. Aichholzer, T. Seidenstücker, B. Widmann, Abbaudare polymerwerkstoffe auf der basis nachwachsender rohstoffe – Möglichkeiten und grenzen. Starch/Stärke 47 (1995) 475-491.

M. Hasanuzzaman, K.M. Umadhay-Briones, S. M. Zsiros , N. Morita, Y. Nodasaka, I. Yumoto, H. Okuyama, Isolation, identification, and characterization of a novel, oil-degrading bacterium, *Pseudomonas aeruginosa* T1, Curr. Microbiol. 49 (2004) 108–114.

W.N. He, Z.M. Zhang, P. Hu, G.Q. Chen, Microbial synthesis and characterization of polyhydroxyalkanoates by strain DG17 from glucose, Acta Polym. Sin. 6 (1999) 709–714.

F. Hasan, A. A. Shah, A. Hameed, Industrial applications of microbial lipases, Enzym. Microb. Technol. 39 (2006) 235–251.

K.E. Jaeger, M.T. Reetz, Microbial lipases form versatile tools for biotechnology, Trends Biotechnol. 16 (1998) 96–403.

D. Jendrossek, Microbial degradation of polyesters, in: W. Babel and A. Steinbüchel (Eds.), Advances in biochemical engineering/biotechnology: biopolyesters, vol. 71. Springer-Verlag Berlin, Heidelberg, Germany, 2001, pp 293-325.

D. Jendrossek, Extracellular polyhydroxyalkanoate depolymerases: the key enzymes of PHA degradation, in: Y. Doi, A. Steinbüchel (Eds) Wiley-VCH, Weinheim Biopolymers, Vol 3b (2002) 41–84.

S. Jørgensen, K.W. Skow, B. Diderichsen, Cloning, sequence and expression of a lipase gene from *Pseudomonas cepacia*: lipase production in heterologous hosts requires two *Pseudomonas* genes, J. Bacteriol. 173 (1991) 559-567

M. E. Khattabi, C. Ockhuijsen, W. Bitter, K. E. Jaeger, J. Tommassen, Specificity of the lipase-specific foldases of gram-negative bacteria and the role of the membrane anchor, Mol. Gen. Genet. 261, 7 (1999) 770 -776.

W.H. Ko, I.T. Wang, P.J. Ann, A simple method for detection of lipolytic microorganism in soils, Soil Biol. Biochem. 37 (2004) 597-599.

T. Kobayashi, M. Shiraki, T. Abe, A. Sugiyama, T. Saito, Purification and properties of an intracellular 3-hydroxybutyrate oligomer hydrolase (PhaZ2) in *Ralstonia eutropha* H16 and its identification as a novel intracellular poly(3-hydroxybutyrate) depolymerase, J. Bacteriol. 185 (2003) 3485-3490.

M. Koller, A. Atlic, M. Dias, A. Reiterer, G. Braunegg, Microbial PHA Production from Waste Raw Materials, in: G. Q. Chen (ed.), Microbiology Monographs: Plastics from Bacteria: Natural Functions and Application, s Springer-Verlag Berlin Heidelberg, 14 (2010) 85-119.

G. Kouker, K.E. Jaeger, Specific and sensitive plate assay for bacteria lipases, Appl. Environ. Microbiol. 53 (1987) 211-213

S.Y. Lee, Plastic bacteria? Progress and prospects for poly-hydroxyalkanoate production in bacteria, Trends Biotechnol. 14 (1996) 431-438.

S.Y. Lee, Y. Lee, Metabolic engineering of *Escherichia coli* for production of enantiomerically pure (R)-hydroxycarboxylic acids, Appl. Environ. Microbiol. 69 (2003) 3421–3426.

B. Madan, P. Mishra, Co-expression of the lipase and foldase of *Pseudomonas aeruginosa* to a functional lipase in *Escherichia coli*, Appl. Microbiol. Biotechnol. 85 (2010) 597-604.

B.L. Maidak, J.R. Cole, T.G. Lilburn, C.T. Parker Jr, P.R. Saxman, J.M. Stredwick, G.M. Garrity, B. Li, G.J. Olsen, S. Pramanik, T.M. Schmidt, J.M. Tiedje, The RDP (Ribosomal Database Project) continues, Nucleic Acids Res. 28 (2000) 173–174.

G.J. McCool, M.C. Cannon, PhaC and PhaR are required for polyhydroxyalkanoic acid synthase activity in *Bacillus megaterium,* J. Bacteriol. 183 (2001) 4235–4243.

K. Mukai, Y. Doi, Y. Sema, K. Tomita, Substrate specificities in hydrolysis of polyhydroxyalkanoates by microbial esterases, Biotech. Lett. 15 (1993) 601–604.

Pinsirodom P., Parkin K.L., Current protocols in food analytical chemistry. Wiley, London, C3: 1.1–1.13, 2001.

S. Povolo, M. G. Romanelli, D. Di Nunno, M. Basaglia, S. Casella, Production of polyhydroxyalkanoates from fatty wastes, J. Polym. Environ. 20 (2012) 944–949

A.P. Pugsley, The complete general secretory pathway in gram-negative bacteria, Microbiol. Rev. 57 (1993) 50-108.

B.A. Ramsay, K. Lomaliza, C. Chavarie, B. Dubè, P. Bataille, J.A. Ramsay, Production of poly (β-hydroxybutirric-co-βhydroxyvaleric) acids, Appl. Environ. Microbiol. 56 (1990) 2093–2098.

J.M. Raquez, M. Deléglise, M.F. Lacrampe, P. Krawczak, Thermosetting (bio)materials derived from renewable resources: A critical review, Prog. Polym. Sci. 35 (2010) 487-509.

J. Ravenstijn, Bioplastics in consumer electronics, Ind. Biotechnol. 6 (2010) 252-263.

F. Rosenau, J. Tomassen, K.E. Jaeger, Lipase-specific foldases, ChemBioChem. 5 (2004) 152-161.

E. Rosenberg, E.Z. Ron, High- and low-molecular-mass microbial surfactants, Applied Microbiol. Biotech. 52 (1999) 154–162.

Y. Saito, Y. Doi, Microbial synthesis and properties of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) in *Comamonas acidovorans*, Int. J. Biol. Macromol. 16 (1994) 99–104.

Y. Saito, S. Nakamura, M. Hiramitsu and Y. Doi, Microbial synthesis and properties of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate), Polym. Int. 39 (1996) 169-174**.**

J. Sambroock, E.F. Fritsch, T. Maniatis, Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbour Laboratory, Cold Spring Harbour, 1989.

H. Saegusa, M. Shiraki, C. Kanai, T. Saito, Cloning of an intracellular poly[D-3-hydroxybutyrate] depolymerase gene from *Ralstonia eutropha* H16 and characterization of the gene product, J. Bacteriol. 183 (2001) 94-100.

R. Sangeetha, I. Arulpandi, A. Geetha, Bacterial lipases as potential industrial biocatalysts: An overview, Res. J. Microbiol. 6 (2011) 1-24.

H. Shibata, H. Kato, J. Oda, Molecular properties and activity of amino-terminal truncated forms of lipase activator protein, Biosci. Biotechnol. Biochem. 62 (1998) 354-357.

E. Shimamura, M. Scandola, Y. Doi, Microbial synthesis and characterization of poly(3-hydroxybutyrateco-3-hydroxypropionate), Macromolecules 27 (1994) 4429–4435

E. L. Siew, N. F. Rajab, A. Bin Osman, K. Sudesh, S. H. Inayat-Hussain, Mutagenic and clastogenic characterization of post-sterilized poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) copolymer biosynthesized by *Delftia acidovorans,* J. Biomed. Mater. Res. 91A (2009) 786- 794.

A. Steinbüchel, F. Reinecke, *Ralstonia eutropha* Strain H16 as model organism for PHA metabolism and for biotechnological production of technically interesting biopolymers, J. Mol. Microbiol. Biotechnol. 6 (2009) 91-108.

M. Titz, K.H. Kettl, K. Shahzad, M. Koller, H. Schnitzer, M. Narodoslawsky, Process optimization for efficient biomediated PHA production from animal-based waste streams. Clean Technol. Environ. Pol. 14 (2012) 495–503.

S. Valappil, D. Peiris, G. Langley, J. Herniman, A. Boccaccini, C. Bucke, I. Roy, Polyhydroxyalkanoate (PHA) biosynthesis from structurally unrelated carbon sources by a newly characterized *Bacillus* spp., J. Biotechnol. 127 (2007) 475-480.

S. Weidner, W. Arnold, E. Stackbrandt, A. Pühler, Phylogenetic Analysis of Bacterial Communities Associated with Leaves of the Seagrass *Halophila stipulacea* by a Culture-Independent Small-Subunit rRNA Gene Approach. Microb. Ecol. 39 (2000) 22–31.

S.F. Williams, D.P. Martin, Applications of PHAs in medicine and pharmacy, in: A. Steinbüchel R. H. Marchessault (Eds), Biopolymers for Medical and Pharmaceutical Applications: Humic Substances, Polyisoprenoids, Polyesters, and Polysaccharides, Wiley-Blackwell, 2005, 91–127.

Q. Wu, S.Q. Sun, P.H.F. Yu, A.X.Z. Chen, G.Q. Chen, Environmental dependence of microbial synthesis of polyhydroxyalkanoates, Acta Polym. Sin. 6 (2000) 751–756.

G.M. York, J. Lupberger, J. Tian, A.G. Lawrence, J. Stubbe, A.J. Sinskey, Ralstonia eutropha H16 encodes two and possibly three intracellular poly [d-(−)-3-hydroxybutyrate] depolymerase genes, J. Bacteriol. 185 (2003) 3788-3794.

Y.H.P. Zhang, Reviving the carbohydrate economy via multi-product lignocellulose biorefineries, J. Indust.l Microbiol. Biotechnol. 35 (2008): 367-375.

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Annexes