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CYCLE: XXXI

Polyhydroxyalkanoates (PHAs) production from lipids containing agri-food wastes.

Coordinatore: Ch.mo Prof. Sergio Casella

Supervisore: Ch.mo Prof. Sergio Casella

Ch.mo Prof. Marina Basaglia

Co-Supervisore: Dr. Lorenzo Favaro

Doctoral Student: Jesús Enrique Rodríguez Gamero

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.

30th October 2018 Jesús Enrique Rodríguez Gamero

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Nuestra recompensa se encuentra en el esfuerzo y no en el resultado. Un esfuerzo total es una victoia absoluta.
Puesto que yo soy imperfecto y necesito la tolerancia y la bondad de los demás, también he de tolerar los defectos del mundo hasta que pueda encontrar el secreto que me permita ponerles remedio
(Mahatma Gandhi)

To Michela, Naily , Yara, Magaly and Juan

ABSTRACT.

In the last decades, economic and environmental concerns about oil shortage and fossil-based economy stimulated the need to shift from conventional plastics to bio-based options like polyhydroxyalkanoates (PHAs). PHAs are stored in many bacteria as intracellular carbon and energy source under limiting environmental conditions. PHAs have a great promise because of their material properties comparable to petrol-based plastomers. Although PHAs could have many applications, their replacement over the oil-based plastics is limited by their expensive production. Therefore, the selection of suitable resources as carbon feedstock for PHAs synthesis and extraction/recovery methods of PHAs are the main factors in the entire PHAs production chain, contributing up to 80% of the operating cost.

Cheap and abundant biomass waste streams have been considered as renewable substrates for the production of polymers, fuels, enzymes and bulk chemicals. The use of industrial or agricultural by-products can be a strategy to decrease also PHAs price. It has been calculated that in the European Union (EU), the slaughterhouses produce around 500,000 tons/year of fatty discards, which could be used efficiently for the production of PHAs, unfortunately, no bacteria with high lipolytic capacity and at the same time ability to accumulate high amounts of PHAs have been found.

Another bottleneck in the PHAs purification steps, is determined by the release of large amounts of chromosomal DNA that causes a dramatic viscosity increase and hampers the following filtration, centrifugation and PHAs recovery steps. High pressure homogenization (HPH) is one of the most widely known methods for large scale cell disruption. HPH is considered environmentally friendly since it does not need solvents to mediate an efficient microbial cells disintegration. After the HPH application, decrease of viscosity is generally achieved by the supplementation of hypochlorite, commercially available nucleases, or heat treatment. Although these methods may be applicable in small-scale fermentation systems, they are not environmentally and economically suitable for industrial PHAs manufacturing.

Looking for a cost-effective solution to the lipolytic activity issue, lipolytic genes (*lipH-lipC*) from *Pseudomonas stutzeri* BT3 have been integrated into *Cupriavidus necator* DSM 545, a well-known PHAs producer. The lipolytic enzymes have been proficiently expressed in the recombinant strain, greatly increasing the PHAs production from the slaughterhouses fatty wastes, indicating that the engineered strain can contribute to increase the economic efficiency of future PHAs **upstream** processing.

On the others hands, looking for a cost-effective solution to the viscosity issue, the staphylococcal nuclease gene *nuc* from *Staphylococcus aureus* has been integrated into the chromosomes of two efficient PHAs-producing bacteria, namely *C. necator* DSM 545 and *Delftia acidovorans* DSM 39. The viscosity of the lysates of *C. necator* recombinant cells was greatly reduced without affecting PHAs production, indicating that the engineered strain is expected to increase the economic efficiency of future PHAs **downstream** processing.

RIASSUNTO.

Negli ultimi decenni, le preoccupazioni economiche e ambientali in merito alla carenza di petrolio e all'economia basata sui fossili, hanno stimolato la necessità di passare dalla plastica convenzionale a opzioni convenzionali fondate su biomassa come i poliidrossialcanoati (PHAs). I PHAs sono sintetizzati ed immagazzinati intracellularmente per alcuni batteri come fonti di carbonio ed energia in condizioni ambientali limitanti. I PHAs, per le loro proprietà materiali paragonabili ai plastomeri a base di petrolio, sono promettenti per sostituire le plastiche sintetiche. Sebbene i PHAs possano avere molte applicazioni, la loro sostituzione sulla plastica a base di petrolio è limitata dalla loro costosa produzione. Pertanto, la selezione di risorse idonee come materia prima di carbonio per la sintesi e il metodi di estrazione/recupero dei PHAs, sono i principali fattori dell'intera catena di produzione che contribuiscono con l'80% ai costi operativi.

Flussi di rifiuti di biomassa sono stati considerati come substrati rinnovabili per la produzione di polimeri, combustibili, enzimi e prodotti chimici. L'uso di sottoprodotti industriali o agricoli può essere una strategia per ridurre anche il prezzo di PHAs. È stato calcolato che nell'Unione europea (UE), i macelli producono circa 500.000 tonnellate/anno di scarti di materia grassa, che potrebbero essere utilizzati in modo efficiente per la produzione di PHAs, sfortunatamente, nessun batterio con elevate capacità lipolitiche e allo stesso tempo capacità per accumulare quantità elevate di PHAs è stato trovato.

Un altro ostacolo nelle fasi di estrazione del PHAs è determinato dal rilascio di grandi quantità di DNA cromosomico che provoca un notevole aumento della viscosità e difficolta le seguenti fasi di recupero di filtrazione e centrifugazione. L'omogeneizzazione ad alta pressione (HPH) è uno dei metodi più noti per l'interruzione cellulare su larga scala. HPH è considerato rispettoso dell'ambiente in quanto non ha bisogno di solventi per mediare un'efficiente disintegrazione delle cellule microbiche. Dopo l'applicazione di HPH, la diminuzione della viscosità viene generalmente ottenuta mediante l'integrazione di ipoclorito, nucleasi commerciale o trattamento termico. Sebbene questi metodi possano essere applicabili nei sistemi di fermentazione su piccola scala, non sono idonei dal punto di vista ambientale ed economico per la produzione industriale di PHAs.

Alla ricerca di una soluzione economicamente vantaggiosa al problema dell'attività lipolitica, i geni lipolitici (*lipH-lipC*) di *Pseudomonas stutzeri* BT3 sono stati integrati in *Cupriavidus necator* DSM 545, un noto produttore di PHAs. Gli enzimi lipolitici sono stati notevolmente espressi nel ceppo ricombinante, aumentando la produzione di PHAs dagli scarti

grassi dei macelli, indicando che il ceppo ingegnerizzato può contribuire ad aumentare l'efficienza economica della futura elaborazione dei PHAs.

Ad ogni modo, alla ricerca di una soluzione economica al problema della viscosità, il gene della nucleasi (*nuc*) dello *Staphylococcus aureus* è stato integrato nei cromosomi di due batteri efficaci produttori di PHAs (*C. necator* DSM 545 e *Delftia acidovorans* DSM 39). La viscosità dei lisati delle cellule ricombinanti di *C. necator* DSM 545 è stata notevolmente ridotta senza influenzare la produzione di PHAs, indicando che il ceppo ingegnerizzato potrebbe aumentare l'efficienza economica dei futuri processi di estrazione di PHAs.

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1. INTRODUCTION.

1.1. Polymers, synthetic plastics and the environment.

Polymers are molecules of large size (macromolecules) and large molecular mass, composed by many simpler units (monomers) linked together by covalent bonds also called hydrogen bonds. When a polymer is composed by the same monomers, it is called homo-polymer, while the presence of different monomers makes it a co-polymer, resulting in different physical-chemical and mechanical characteristics (molecular morphologies, stereo-chemical structures, crystallinity, molecular weight, and others) (Cowie and Arrighi, 2007).

Polymers can be of natural origin, namely those produced by living organisms, such as proteins, DNA, RNA, cellulose, or synthetic origin, such as polyethylene, polyvinyl chloride, and others (Azapagic et al., 2003).

1.1.1. Synthetic plastics.

Synthetic plastics are high molecular weight polymers that contain carbon and hydrogen as the main elements, created by a polymerization process of a variable number of monomers.

In the manufacturing process, plastics are often mixed with additives as dyes, plasticizers, stabilizers, fillers and reinforcements resulting in different chemical, physical and mechanical properties. Although valuable, this can increase the production costs and produce polluting substances at the time of disposal (Gourmelon, 2015).

The majority of these polymers are solid in their final state but, at some stage of manufacturing, they are soft enough to be melted by means of heat and/or pressure.

Currently, the plastics industry is one of the most important and prosperous in the world, increasing production every year (Worm et al., 2017). Worldwide, factories produce approximately 400 million tons per year of plastic, more than a billion Kg per day. Thousands of machines can, collectively, produce plastic soft-drink and water bottles at a rate of nearly 20.000 units/second. Economic engines are so powerful that global plastic production tonnage has been doubled in less than two decades. Due to the great demand, the world's corporations and governments encourage further and continuous increase of plastics production in the future (Fig. 1), much more than other materials such as metals (Ryan, 2015).

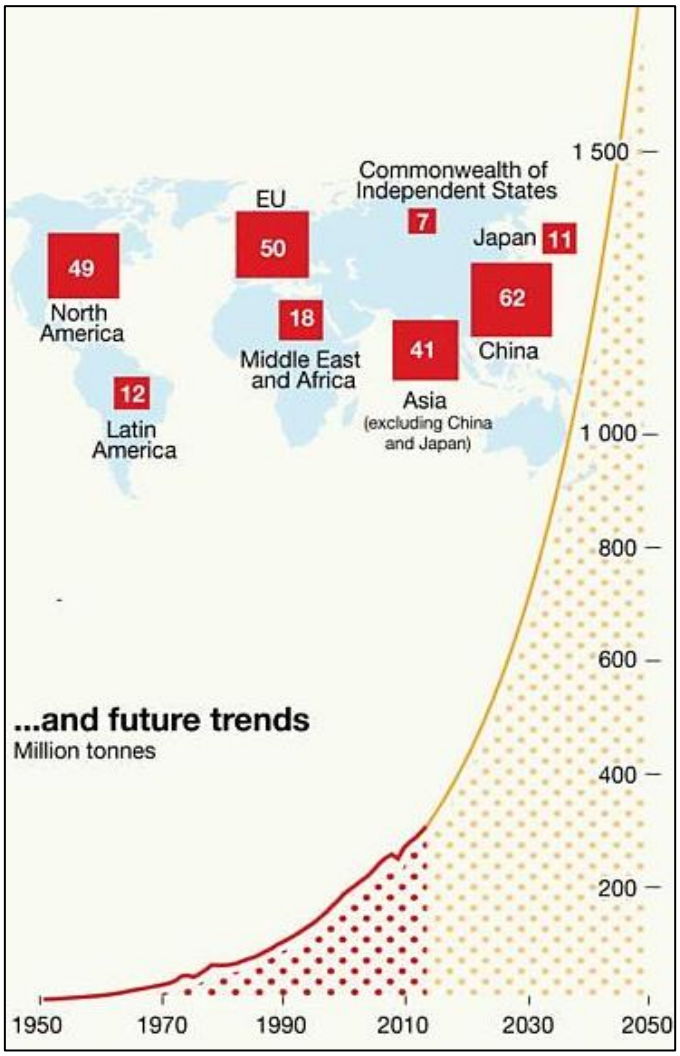


Fig. 1. Increase in the world production of plastics from 1950 to 2010 and future projections up to 2050 (Ryan, 2015).

The increase of synthetic plastics demand is consequence of their commercial versatility and characteristics (Selke and Culter, 2016), such as:

a. lightness: low weight, with a low density (between the ranges of 10 as foams and 3500 Kg/m^3 as reinforced plastics);

b. elasticity and resistance: support great mechanical stress without breaking and recovers its original shape once the force is removed;

c. low coefficient of friction: under friction do not heat up very much, even without lubrication;

d. thermal insulation: low thermal conductivity;

e. corrosion resistance: support weak acids and salty solutions;

f. low cost: taking into account the volume, the raw material of plastic is cheaper than metal;

g. low electric coefficient: can be used as electrical insulators;

h. easy to manufacture;

i. vibration and sound absorbents.

Currently, with the new technologies, plastics can be so resistant that they can be used as a shield, heat and UV ray resistant.

According to their resistance to heat, synthetic plastics can be divided in:

a. thermoplastics: polymers that become flexible above a specific temperature and solidifies upon cooling;

b. thermosets or thermostables: polymers that do not undergo deformation in the presence of heat;

c. elastomers: polymers that allow elongation capacity up to 30 times their normal size, these elastomers can be thermoplastic elastomers or thermoset elastomers.

According to their mechanical properties, synthetic plastics can be:

d. rigid: polymers that break immediately in the presence of a bending force;

e. semi-rigid: polymers that support certain bending force;

f. flexible: polymers that bear the dubbing, these plastics after a certain time can return to its original form.

According to their optical properties, synthetic plastics can be:

g. transparent: polymers that let light through, it can be seen through these materials;

h. translucent: polymers that let a small amount of light pass through, only shadows can be seen when looking through;

i. opaque: these polymers do not let light through.

According to their molecular structure, they can be amorphous, semi-crystalline, crystalline, commodities or engineering polymers.

The most used synthetic plastics are reported in Table 1:

Abbreviation	Full name
ABS	Acrylonitrile butadiene styrene
EPS	Expanded polystyrene
E-PVC	Polymerized expanded PVC
EVA	Ethylene-vinyl acetate copolymer
HD-PE	High density polyethylene = low pressure polyethylene
LD-PE	Low density polyethylene = high pressure polyethylene
MD-PE	Medium density polyethylene
MF	Resins or castings melamine-formaldehyde
M-PVC	PVC polymerized in mass
PA	Polyamide
PB	Polybutene
PC	Polycarbonate
PE	Polyethylene
PEPT	Polyethylene glycol terephthalene
PF	Resins or castings melamine-formaldehyde
PMMA	Polymethyl methacrylate
PMP	Polymethyl-pentene
POM	Polyacetal
PP	Polypropylene
PS	Polystyrene
PSAN	Styrene-acrylonitrile copolymers (= SAN)
PTFE	Poloetrafluoroethylene
PVAC	Polyvinyl acetate
PVC	Polyvinyl chloride
PVCAC	Copolymers chloride-vinyl acetate
PVDC	Polyvinylidene chloride
PUR	Polyurethanes
SAN	Styrene-acrylonitrile copolymers
SB	Copolymers styrene-butadiene-polystyrene anti-shock
S-PVC	Polymerized PVC in suspension
UF	Resins or molding compounds of urea-formaldehyde
PET	Polyethylene terephthalate

Table 1. Most used synthetic plastics and their acronyms (Lozano Olmedo, 2018).

1.1.2. Synthetic plastics waste.

Synthetics plastics are mainly derivatives of petroleum, a non-renewable resource usually requiring much energy to obtain some polymers (W. C. Li et al., 2016). Generally, the intensive utilization of fossil resources contributes to environmental problems such as global warming or greenhouse effects. Therefore, plastics are part of the problem. Indeed, plastics are used only during a short time span and after that they are often incinerated, discarded and accumulated, thus increasing the atmospheric CO_2 concentration or rising the presence of not-degradable material in the environment (Braunegg et al., 2004; Jambeck et al., 2015). The accumulation of plastics has been increasing: in the USA (5% of the world's population) 500 million of plastics water bottles are weekly discarded, almost three million plastic bottles thrown per hour, enough to cover eight football fields. The figures for the European Union (EU) are even higher.

In 2012, 280 million tons of plastic were produced worldwide, with a prevision of an increase of 33 million tons by 2030 (http://elplasticomata.com/crisis-global/). In 2010 it was appraised that in 192 coastal countries, 275 million metric tons (MT) of plastic waste were produced, with around 4.8 to 12.7 million MT entering the ocean. Population volume and the efficiency of waste management systems mostly define which countries provide the greatest quantities of marine waste plastic. The amount of plastic waste potentially entering the ocean will increase by an order of magnitude by 2025 (Romanelli et al., 2014).

Recycling procedures are not effective and request a high degree of purity and sorting accuracy. The costs are high, and recycling has a negative influence on the characteristics of the materials, such as an increment in brittleness (Braunegg et al., 2004). In addition to these ecological reflections, the value of oil is unpredictably unstable, due to miscellaneous developments in the global political situation. This constitutes an aspect of immense uncertainty particularly for the vastly petrol-dependent polymer industry. Currently, the manufacture plastic demands around 5-7% of the fossil feedstock. This fraction is estimated to increase considerably through the next years because of the growth of economy, the increase of the population and the improvement of the quality of life. Furthermore, the remaining amounts of fossil oil in the earth are changing quickly due to innovative systems for tracing and discharging of mineral oils. (Romanelli et al., 2014).

Taking into account these reasons, scientists have been searching for materials that could substitute synthetic plastics, of renewable origin but with similar physical, chemical and mechanical characteristics although not causing negative environmental impact (eco-friendly).

1.1.3. Biodegradable plastics.

Worldwide the safe and efficient distribution of food and other products is in continuous growth. Moreover, the industry of packaging products and plastic materials is increasingly important, in the food, medical and many other areas. Therefore, taking into account the current environmental problems previously mentioned, the industrial sectors are more aware to promote novel biodegradable plastics production techniques based on renewable resources. In fact, sustainable production of polymers could be achieved applying the 'white biotechnology', which involves the use of living organisms, or their metabolites and the use of renewable resources as carbon source. In this case, the social benefits from common petrol-based polymers to bio-based plastics could be enormous, particularly if these are biodegradable and compostable. Certainly, bio-based materials create fewer greenhouse gases, need lower energy and in their lifecycle produce less toxic pollutants compared to fossil products.

The international standards that certify biodegradable polymers are:

a. International Standard Organization (ISO) 17088:2012
b. EN 13432:2002, EN 14995:2006 and EN 14995:2007
c. ASTM D6400-12

ISO indicates that a polymer enters in the biodegradable classification when its chemical structure can be modified by the microorganisms action (bacteria, fungi, algae) losing some typical properties. Among all its components, less than 1% of non-biodegradable material should be present with respect to the dry mass.

UNI EN 13432:2002 is the European standard on packaging and packaging waste. This rule shows how to assess the plastics material compost-ability, while UNI EN 14995:2007 rule shows the "schemes of testing and plastic specification", for the biodegradability estimation of material during biological treatment and effect on the resulting compound quality.

UNI EN 13432:2002 says that a compostable material should have the following properties:

a. biodegradability: the values must be greater than or equal to 90% in not more than 6 months. It is measured by evaluating the metabolic digestion of the compostable material into CO_2. This characteristic is assessed with the standard test method UNI EN 14046 (also published as "ISO 14855: biodegradability under controlled composting conditions").
b. disintegration: measured by the total disintegration of the polymer in the final compost (absence of particles). It uses a composting test pilot scale (UNI EN 14045) that consist of incubating the material together with biodegraded organic waste, for three months, and passing the compost through a sieve of two millimeters. The particles with greater size are

considered as not disintegrated material and this fraction must be less than 10% of the initial mass.

c. presence of metals and/or other pollutants: presence of heavy metal is very important for the use of biodegradable material like compost. The heavy metal level must be very low and should not have any adverse effects on the plant growth, quality of the cultivated land, the reduction of the agronomic value, pH, volatile solids, nitrogen, phosphorus, magnesium, potassium, salt content and others. Plants growth has to be tested by using normal compost as positive control (OECD test 208 modified), and compost made with biopolymers low in metals. (Müller, 2005).

According to the previously mentioned principles, three biopolymers types can be considered:

a. made from renewable raw materials (bio-based) and biodegradable.
b. made from renewable raw materials (bio-based) but not biodegradable.
c. made from fossil fuels and biodegradable.

Although the three categories are considered biopolymers (European Bioplastics Association, 2008), to find solutions for most of the problems mentioned in the paragraph 1.1.2., the most suitable strategy is to make polymers from renewable raw materials (bio-based) and biodegradables (Fig. 2).

The bio-based and biodegradables polymers can also be divided into three main types according to their origin and production (Fig. 3):

a) From protein biomass: Polymers directly extracted/recovered from biomass (proteins such as casein and gluten, lipids like triglycerides and polysaccharides such as starch and cellulose). Thermo-plastic starch constitutes more than 50% of the bioplastic market, being the most used. In the manufacturing process, in order to facilitate the thermo-plastic process, flexibiliser and plasticizer can be added in varying amounts so that the properties of the material can be tailored to specific needs. Industrially, starch based bioplastic are often blended with biodegradable polyesters, mainly starch/polycaprolactone or starch/Ecoflex (polybutylene adipate-co-terephthalate produced by BASF) (BASF, Germany; PHBISA, Brazil); these blends remain compostable. Other producers, such as Roquette, have developed another strategy based on starch/polyolefin blends no longer biodegradables, but displaying a lower carbon footprint compared to the corresponding petroleum based plastics (Anne, 2011).

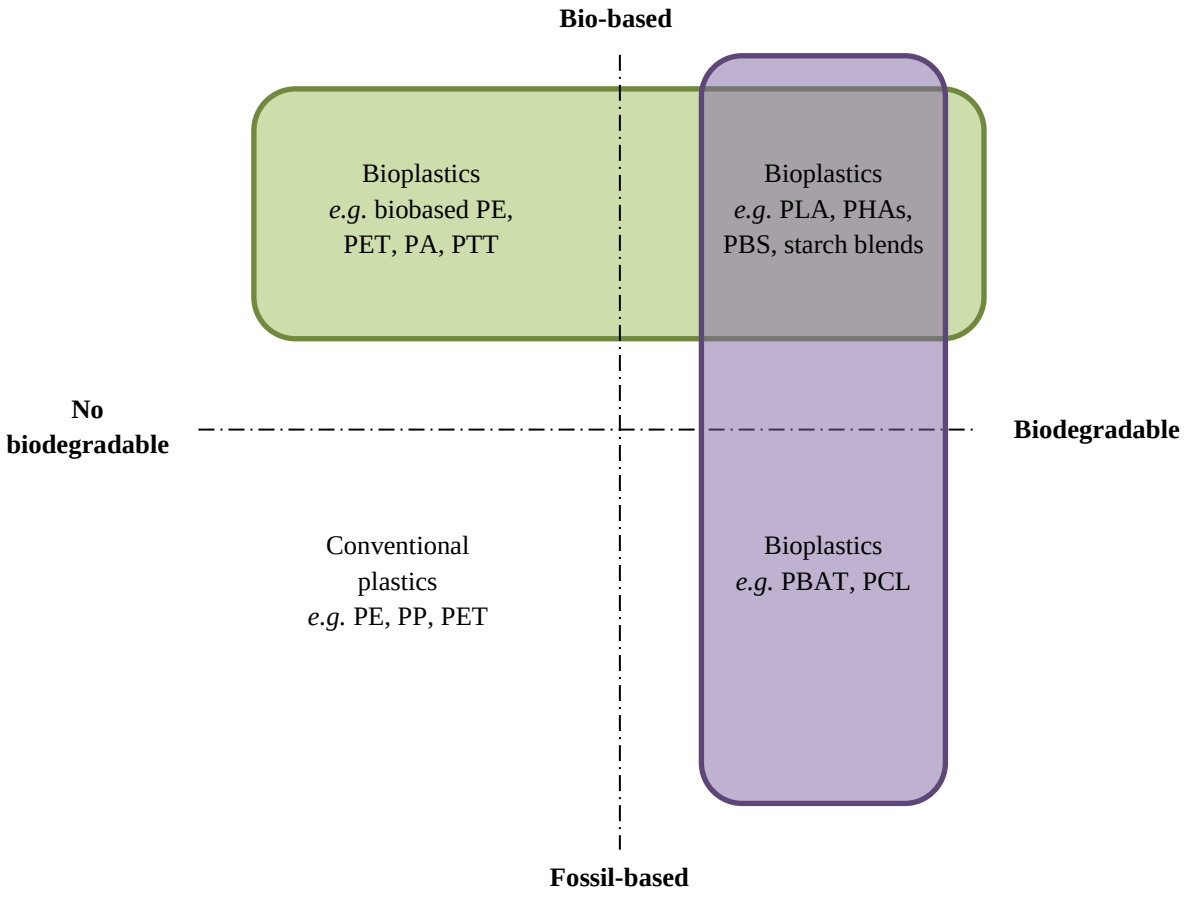


Fig. 2. Bioplastics classification (European Bioplastics Association, 2008).

b) from biotechnology: polymers made by classical chemical synthesis using renewable bio-based monomers. A good example is PolyLactic acid (PLA), a bio-polyester polymerized from lactic acid monomers. The monomers themselves may be produced via fermentation of carbohydrate feedstock. PLA is a transparent plastic usually made from corn. Corn starch is hydrolysed to glucose, which is transformed into lactic acid and then exposed to polymerization (Castro-Aguirre et al., 2016).
c) from microorganism: bio-based polymers produced by microorganisms consists mainly of polyhydroxyalkanoates (PHAs); PHAs are aliphatic polyesters formed directly by microorganisms through fermentation of a variety of carbon sources (eg glucose, fatty acids and others).

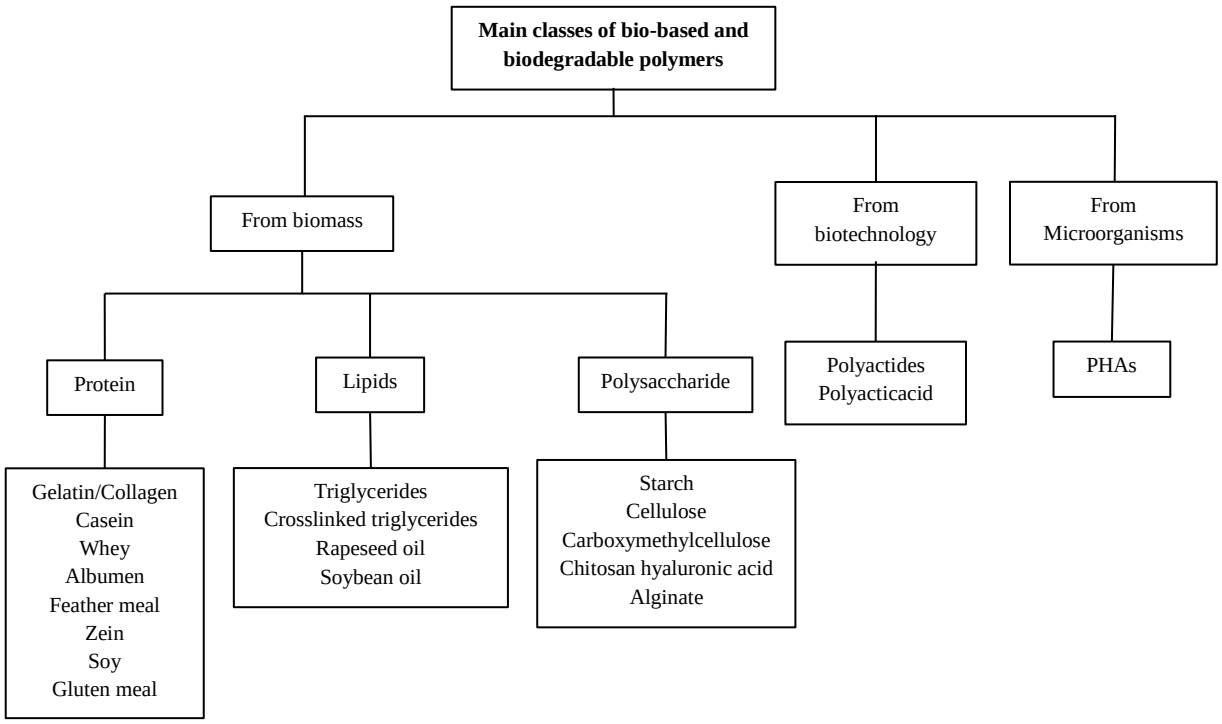


Fig. 3. Different classes of polymers: are bio-based and biodegradable (therefore not including biodegradable plastics from petrochemical resources and non-biodegradable partly or fully bio-sourced plastics) (Bugnicourt et al., 2014).

Some oppositions arose in the public opinion on the PLA biopolymers production, mainly about the use of genetically modified corn as feedstock, the inclusion of potentially harmful compounds and the recycling problems, since PLA can contaminate the recycling of Polyethylene terephthalate (PET), because these materials are very similar and difficult to differentiate. Some biopolymer are very expensive, limiting large-scale industrial production. To maximize the global benefits of biopolymers, these problems need to be treated without obstructing their commercial practicability. This will probably need a mixture of policy motivations and regulations, private and public engagement and support, market advance supporting economic, environmental and social aims.

Present investigations in biodegradable polymers relate principally to the production scaling-up, the improvement of product properties and the production costs reduction.

PHAs are attracting the interest of the scientific community and the industries because of their promising chemical, physical and mechanical properties, they are bio-based polymers, 100% biodegradable, compostable, and they can be also produced from a wide range of different cheap by-products.

1.2. Polyhydroxyalkanoates (PHAs).

PHAs are polyoxoesters of hydroxyalkanoic acids. Their existence in bacteria was first reported decades ago (Lemoigne, 1923). Nowadays, it is well established that PHAs, synthesized by some bacteria and archaea as intracellular storage compounds under unfavourable conditions (see Fig. 4), serve as carbon and energy reserve (Penczek, 2018). Usually, the natural condition that promote the synthesis of PHAs is when an essential nutrient (nitrogen, phosphorus, oxygen, magnesium, among others) is limited, and there are excess of carbon sources (carbohydrates, lipids, alcohols or organic acids) (Lee, 1996).

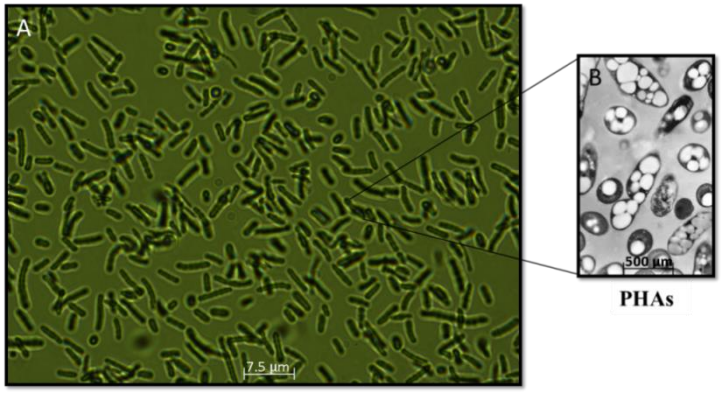


Fig. 4. *Cupriavidus necator* DSM 545. A) Cells observed by optical microscopy with objective 100X. B) Granules of PHAs as spherical cytoplasmic inclusions of variable size.

There are microorganisms able to accumulate PHAs as much as more than 90% of their cell dry weight, thus taking advantage of this storage material especially in environments with fluctuating availability and limitation of nutrients. PHAs-producing bacteria belong to a number of different genera (Table 2) and have been found in both aquatic and terrestrial environments, as well as activated sludge or other artificial environments.

PHAs-accumulating microbial genera		
Acidovorax	*Erwinia*	*Oscillatoria*[a]
Acinetobacter	*Escherichia (wil tipe)*[d]	*Physarume*[e]
Actinobacillus	*Ferrobacillus*	*Paucispirillum*
Actinomycetes	*Gamphospheria*	*Pedomicrobium*
Aeromonas	*Gloeocapsa*[a]	*Photobacterium*
Alcaligenes[a,b]	*Gloeothecea*[a]	*Protomonas*
Allochromatium	*Haemophilus*	*Pseudomonas*[a,b]
Anabaenab[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] PHAs found in cell membranes; [e] Eukaryotic genera with poly-b-malic acid (PMA) production known.

Table 2: PHAs-accumulating microbial genera (Koller et al., 2010).

PHAs particles are deposited as intracellular water-insoluble inclusions and become refractive granules visible to a light-optical microscope (Rehm, 2007). These granules have a typical diameter of 0.2-0.7 μm and consist of 97.7% PHAs, 1.8% protein (Granule Associated Proteins or GAPs) among which are the fasins, intracellular depolymerase, polymerases and 0.5% phospholipids (in grey in the diagram) (Bresan et al., 2016). Proteins and lipids form a coated membrane around the core region (Fig. 5).

When in the culture medium the carbon is limited, PHAs producer bacteria activate the acyl-CoA synthetase, enzyme responsible for depolymerization of PHAs grains. Depolymerized PHAs particles, then are oxidized by enzymes of β-oxidation for finally converting them into water, CO2 and energy, thus providing the cell with an advantage for surviving during starvation periods.

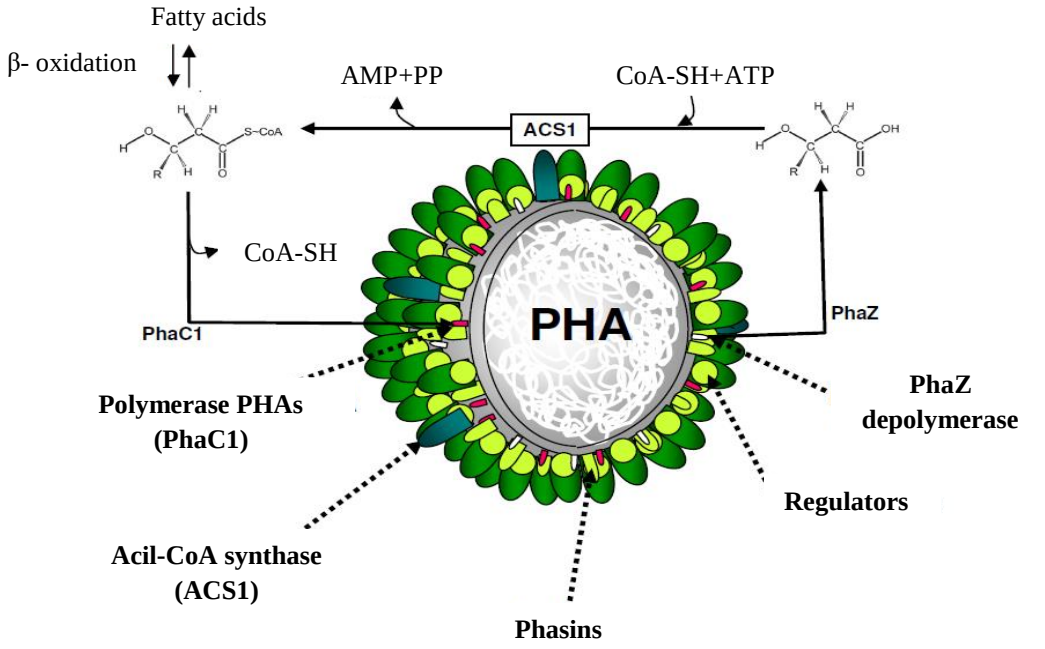


Fig. 5. Schematic representation of a of PHAs granule (Martínez et al., 2009).

1.2.1. Structure of PHAs.

PHAs are molecules with a very complex structure (Fig. 6). They can be omo-polymers of different dimensions or co-polymers of various monomers. To date 150 different PHAs have been identified (He et al., 1999; Wang et al., 2014). This variety is due to the number of CH_2 groups present in the chain and the alkyl group in the R position.

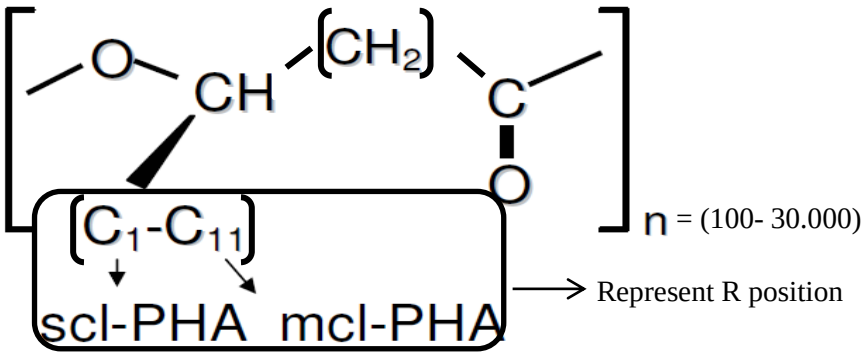


Fig. 6. Chemical structure of the PHAs and variability of the side chain.

From PHAs principal structure, the alkyl group present in this position varies from methyl (CH_3) to tridecyl ($C_{13}H_{27}$) and can be saturated, methylester, cyanophenoxy, cyano, aromatic, unsaturated, phenyl, nitrophenoxy, phenoxy, hydroxyl, halogenated, epoxidized or branched (Steinbüchel et al., 1992). This aspect determines the different polyesters that are naturally obtained; the mechanical and physical-chemical properties such as stiffness, brittleness, melting point, glass transition temperature and resistance to organic solvents depend on the monomeric composition of the polymer.

PHAs are classified into two main types according to their monomeric structure (Fig. 7): short-chain PHAs (scl-PHAs) obtained from 3 to 5 carbon atoms and medium chain (mcl-PHAs) with 6 to 14 carbon atoms.

Some authors speculate about long-chain PHAs (lcl-PHAs) with more than 14 carbon atoms, but there is not enough theoretical and research material to develop this topic.

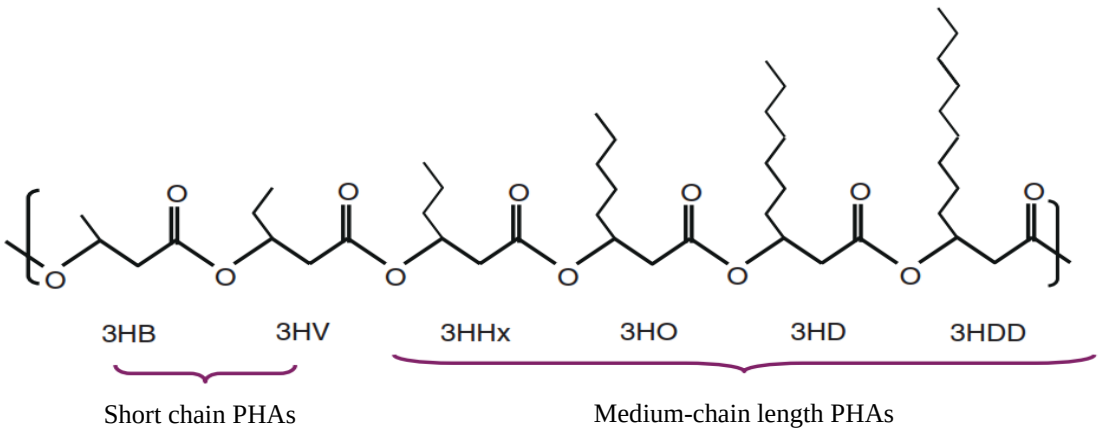


Fig. 7. Common PHAs monomer structures. Short-chain-length monomers: 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV). Medium-chain-length monomers: 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), 3-hydroxydodecanoate (3HDD) (Chen, 2010).

Type and monomer structures of PHAs are normally very dependent on the strain, the fermentation conditions and the substrates used for cell growth (Anderson and Dawes, 1990; Chen, 2010; Madison and Huisman, 1999).

Few microorganisms have the ability to produce different PHAs at the same time. In some cases, specific chemical substances, also called precursors, are used as substrates or supplements to produce different monomers from a single strain. For example butyrolactone and valeric acid are used to produce 3-hydroxybutyarate (3HB), 4-hydroxybutyarate (4HB) and 3-hydroxyvalerate (3HV). Using mix of the two precursors, poly(3-hydroxybutyarate-*co*-4-hydroxybutyarate-*co*-3-hydroxyvalerate) (3HB-*co*-3HV-*co*-4HB) was obtained using *Cupriavidus* sp. USMAA2-4 strain (Aziz et al., 2012).

However, the physical-chemical and mechanical properties very similar or equal to synthetic plastics, depend in turn on the monomers of the biopolymer. For example, PHAs integrated with 3HB plus 3HV and 4HB or the combination between them, have better physical-chemical and mechanical properties than single monomers (El-Hadi et al., 2002; Modi, 2010).

Among the 150 known PHAs monomers, the most commercially important are mentioned in Table 3.

x	Type	Side chain (R)	PHAs Name	Nomenclature
x=1	scl-PHAs	H	Poly(3-hydroxypropionate)	3HP
		CH_3	Poly(3-hydroxybutyarate)	3HB
		C_2H_5	Poly(3-hydroxyvalerate)	3HV
x=2	scl-PHAs	H	Poly(4-hydroxybutyrate)	4HB
		CH_3	Poly(4-hydroxyvalerate)	4HV
x=3		H	Poly(5-hydroxyvalerate)	5HV
x=4		H	Poly(6-hydroxyhexnoate)	6HHx

Table 3: Most important PHAs monomers.

1. 3-hydroxybutyrate (3HB): it is the more frequent monomer. It contains methyl group in the lateral chain and three atoms of carbons in the principal chain (Fig. 8).

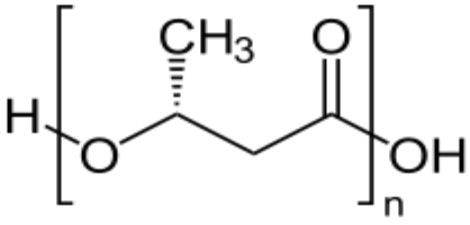


Fig. 8. 3-hydroxybutyrate (3HB).

2. 4-hydroxybutyrate (4HB): the principal chain has four carbon atoms but does not have a lateral chain (Fig. 9); when forming a copolymer with the 3HB and/or 3HV, this PHAs acquire specific mechanical and physical properties.

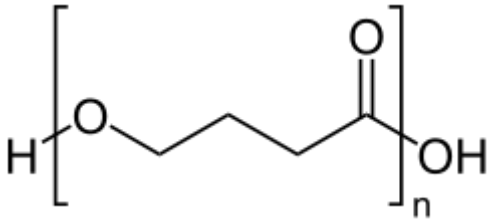


Fig. 9. 4-hydroxybutyrate (4HB).

3. 3-hydroxvalerate (3HV): it presents an ethyl group in the lateral chain, while the principal chain is formed with three carbon atoms (Fig. 10). This monomer also forms a copolymer with 3HB and/or 4HB.

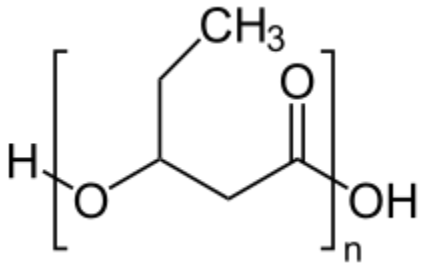


Fig. 10. 3-hydroxvalerate (3HV).

In the present PhD work, the 3HB and 4HB monomers have been taken into consideration.

1.2.2. PHAs synthesis.

There are eight known metabolic pathways adopted by microorganisms to synthesize PHAs monomers (Fig. 11). All these pathways pass through the cycle of tricarboxylic acids (TCA), beta oxidation and fatty acid biosynthesis (Aldor and Keasling, 2003).

Acetyl-CoA is the key molecule for PHAs biosynthesis but (Oeding and Schlegel, 1973).

When the microorganism is in a nitrogen limiting condition, the NADPH/NADP ratio increases and as a result, the citrate synthase and isocitrate dehydrogenase enzymes in the tricarboxylic acid (TCA) cycle are inhibited. Consequently, the flow of acetyl-CoA towards the

TCA is sharply reduced and therefore becomes available for B-ketotiolase and PHAs-synthesis (**Pathway I**) enzymes that convert acetyl-CoA into PHAs (Table 4). This explains why, when nitrogen is in sufficient quantities for the microorganism, the synthesis of PHAs does not take place: the acetyl-CoA enters the TCA cycle and therefore it is not available for the enzymes that are used for the conversion into PHAs (Lee et al., 1995).

The second pathway (**Pathway II**) is linked to the absorption of fatty acids by the microorganism. Following the β-oxidation of the acyl chains, acetyl-CoA can enter the PHAs synthesis pathway. The enzymes involved in this pathway are different: 3-ketoacyl-CoA reductase, epimerase, (R)-enoyl-CoA hydratase/enoyl-CoA hydratase I, acyl-CoA oxidase (putative), and enoyl-CoA hydratase I (putative). When this route is followed, mcl-PHAs or copolymers of R-3PHB and PHBHHx are produced (Chen, 2010).

Pathway III involves 3-hydroxyacyl-ACP-CoA transferase (PhaG) and malonylCoA-ACP transacylase (FabD), which help providing 3-hydroxyacyl-ACP to form monomer 3-hydroxyacyl-CoA, leading to PHAs production under the reaction of PHAs synthase (Sudesh et al., 2000; Taguchi et al., 1999; Zheng et al., 2005) .

Pathway IV employs NADH-dependent acetoacetyl-CoA reductase to oxidize (S)-(+)-3-hydroxybutyryl-CoA. A high portion of NADPH to NADP+ could enhance the delivery of the reductant to nitrogenase in *Rhizobium* (*Cicer*) sp. strain CC 1192 (Chohan and Copeland, 1998). This can also help the reduction of acetoacetyl-CoA for poly[(R)-3 hydroxybutyrate] (PHB) synthesis.

Pathway V uses succinic semialdehyde dehydrogenase (SucD), 4-hydroxybutyrate dehydrogenase (4hbD), and 4-hydroxybutyrate-CoA:CoA transferase (OrfZ) to synthesize 4-hydroxybutyryl-CoA to form 4-hydroxybutyrate-containing PHAs. Pathway V was reported in *Clostridium kluyveri* (Valentin and Dennis, 1997).

The pathway VI uses the lactonase with hydroxyacyl-CoA synthase to convert 4,5-alkanolactone into 4,5-hydroxyacyl-CoA for PHAs synthesis (Valentin and Steinbüchel, 1995).

Pathway VII is based on the putative alcohol dehydrogenase in *Aeromonas hydrophila* 4AK4. In the pathway VII, 1,4-butanediol is oxidized to 4-hydroxybutyrate, then to 4-hydroxybutyryl-CoA for 4-hydroxybutyrate containing PHAs synthesis (Xie and Chen, 2008).

Pathway VIII turns 6-hydroxyhexanoate into 6-hydroxyhexanoate-containing PHAs under the actions of eight enzymes (Table 4).

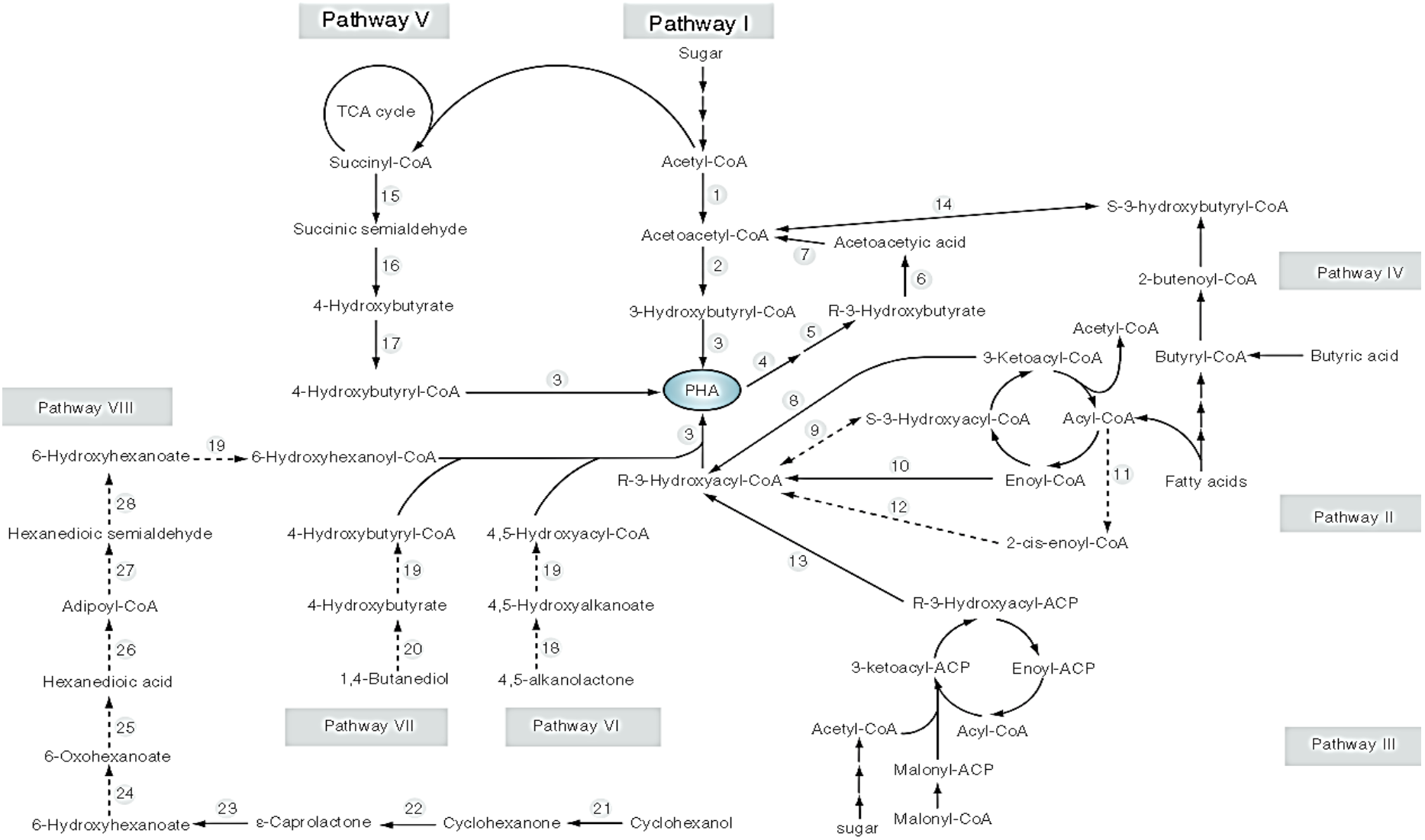


Fig. 11. PHAs biosynthesis pathways (Chen, 2010).

N°	Pathway	Abbreviation	Enzyme	Species	References
1		PhaA	b-Ketothiolase		
2	I	PhaB	NADPH dependent acetoacetyl-CoA reductase	*C. necator*	(Sudesh et al., 2000)
3		PhaC	PHAs synthase		
4		PhaZ	PHAs depolymerase	*A. hydrophila* 4AK4	(Sudesh et al., 2000)
5			Dimer hydrolase	*Pseudomonas stutzeri* 1317	
6	Associated way		(*R*)-3-Hydroxybutyrate dehydrogenase	*R. eutropha*	
7			Acetoacetyl-CoA synthetase	*Pseudomonas oleovorans*	
8		FabG	3-Ketoacyl-CoA reductase	*Pseudomonas putida* KT2442,	(Sudesh et al., 2000)
9			Epimerase	*A. hydrophila 4AK4*,	(Mittendorf et al., 1998)
10		PhaJ	*(R)*-Enoyl-CoA hydratase/enoyl-CoA hydratase I	*Pseudomonas aeruginosa*	
11	II		Acyl-CoA oxidase, putative		
12			Enoyl-CoA hydratase I, putative		
13	III	PhaG FabD	3-Hydroxyacyl-ACP-CoA transferaseMalonyl-CoA-ACP transacylase	*Pseudomonas mendocina*, recombinant *Escherichia coli*	(Sudesh et al., 2000; Taguchi et al., 1999; Zheng et al., 2005)
14	IV		NADH-dependent acetoacetyl-CoA reductase	*Rhizobium* (Cicer) sp. CC 1192	(Chohan and Copeland, 1998)
15		SucD	Succinic semialdehyde dehydrogenase	*Clostridium kluyveri*	(Valentin and Dennis, 1997)
16	V	4hbD	4-Hydroxybutyrate dehydrogenase		
17		OrfZ	4-Hydroxybutyrate-CoA:CoA transferase		
18	VI		Lactonase, putative	Mutans and recombinant of	(Valentin and Steinbüchel, 1995)
19			Hydroxyacyl-CoA synthase, putative	*Alcaligenes eutrophus*	
20	VII		Alcohol dehydrogenase, putative	*A. hydrophila 4AK4*	(Xie and Chen, 2008)
21		ChnA	Cyclohexanol dehydrogenase	*Acinetobacter* sp. SE19,	(Brzostowicz et al., 2002)
22		ChnB	Cyclohexanone monooxygenases	*Brevibacterium epidermidis* HCU	
23		ChnC	Caprolactone hydrolase		
24		ChcD	6-Hydroxyhexanoate dehydrogenase		
25		ChnE	6-Oxohexanoate dehydrogenase		
26	VIII		Semialdehyde dehydrogenase, putative		
27			6-Hydroxyhexanoate dehydrogenase, putative		
28			Hydroxyacyl-CoA synthase, putative		

Table 4: Enzymes involved in PHAs biosynthesis (Chen, 2010).

1.2.3. PHAs properties.

As mentioned above, PHAs fulfil the promising physical-chemical and mechanical functions for the replacement of conventional plastics: PHAs are thermoplastics, biodegradable, biocompatible, piezoelectric, brittle to elastic, have a Mw between 20,000 to 30 million D and can be hydrophobic, permeable or no permeable (to gas or water).

3HB and 4HB are the monomers of interest in this research work, since their unions form polymers with great interest in the medical, pharmaceutical and packaging industry (Amirul et al., 2008; Mitomo et al., 2001; Norhafini et al., 2017; Valentin and Dennis, 1997).

High melting temperature and relatively high tensile strength of PHAs are comparable to petroleum-based polymers. However, pure PHAs have had only limited use mainly because of its intrinsic brittleness (influenced by its slow crystallinity). In fact, the break elongation is very different between 3HB and polypropylene (5% and 400%, respectively) mainly affected by their structures and molecular weights, giving consequently slow crystallization points (El-Hadi et al., 2002). Nevertheless, blending PHAs and in particular 3HB with other polymers or plasticizers, can offer a chance to improve the process by reducing the processing temperature and lowering the fragility of PHAs based plastics (Bugnicourt et al., 2014).

Recent studies have shown that the combination of two or more monomers of PHAs exceptionally improve their characteristics. For example the copolymer, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (3HB-*co*-3HV), exhibits increased temperature of crystallization as compared to the homopolymer 3HB. The molecular increase of the 3HV in the copolymer 3HB-*co*-3HV, firstly produced a decrease in the melting temperature from 175.4 to 168.5°C, with 3HV concentration of 20 mol%. Increase 3HV part shows a typical isodimorphic relationship (Modi, 2010). poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) (3HB-*co*-4HB) (Che et al., 2018; Ye et al., 2018) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxybutyrate) (3HB-*co*-3HV-*co*-4HB) (Aziz et al., 2017; Huong et al., 2015; Kaur and Roy, 2015) present also great improvements, obtaining generally the physical-chemical properties shown in Table 5.

Property* (units)	Values
T_g (°C)	2
T_m (°C)	160-175
X_{cr} (%)	40-60
E (GPa)	1-2
σ (MPa)	15-40
ε (%)	1-15
WVTR ($g \cdot mm/m_2 \cdot day$)	2.36
OTR ($cc \cdot mm/m_2 \cdot day$)	55.12

Table 5: Typical properties of PHAs; *T_g: glass transition temperature, T_m: melting temperature, X_{cr}: crystallinity degree, E: Young's modulus, σ: tensile strength, ε: elongation at break, WVTR: water vapour transmission rate; OTR: oxygen transmission rate (Bugnicourt et al., 2014).

1.2.4. Biodegradation of PHAs.

Stored PHAs provide carbon and energy for the organism when it cannot be obtained externally. PHAs granules can be hydrolysed producing energy , that allows the microorganism to carry metabolic reactions, including cell division (Sudesh and Abe, 2010). In the first investigations about the PHAs biodegradation, scientists observed that *C. necator* H16 (Hippe, 1967; Hippe and Schlegel, 1967), *Rhizobium* sp., *Spirillum* sp. and *Pseudomonas* sp. (Hayward et al., 1959), reached a maximum accumulation of PHAs after the stationary growth phase followed by a gradual decrease of PHAs. Later studies, showed that *Legionella pneumophila* (James et al., 1999), *C. necator* H16 (Handrick et al., 2000) and *Rhizobium tropici* (Povolo and Casella, 2004) continued to grow for a long time after having exhausted all the exogenous carbon, using the 3HB accumulated inside the cell as endogenous carbon source.

Currently, many studies have been carried out to determine the PHAs synthesis and biodegradation (Bugnicourt et al., 2014; Urtuvia et al., 2014). It has been shown that this hydrolysis can be both extracellular in bacteria and fungi (by extracellular depolymerases) and intracellular in bacteria (by intracellular depolymerases) (Sudesh and Abe, 2010).

Extracellular hydrolysis indicates that some microorganisms (not necessarily PHAs producers) can use, as exogenous carbon source, the polymers released in the medium by PHAs producer microorganisms that have already finished their life cycle. The ability of extracellular hydrolysis is distributed among several species of bacteria and fungi, and depends on the secretion of specific enzymes. Poly-3HB-depolymerase hydrolyses the polymers in monomers and soluble

oligomers that are used by the cells as nutrients and metabolized in CO_2 and water (Jendrossek, 2005; Mergaert et al., 1994, 1993; Quinteros et al., 1999). This is a direct demonstration of the biodegradability of these bacterial polymers and, consequently, goods made up by PHAs.

Intracellular hydrolysis consists in the endogenous carbon metabolism (Jendrossek, 2005) by the action of an intracellular depolymerase (PhaZ1), encoded by *phaZ* gene (Saegusa et al., 2001), which does not have common characteristics in extracellular depolymerases. The PhaZ1 protein is a 47 kDa protein and is actively expressed. Its activity determines the conversions of poly-3HB in oligomers and in 3HB (monomer) (Povolo et al., 2015).

In addition, PhaZ2 esterase has also been studied; this enzyme is able to hydrolyse 3HB linear and cyclic oligomers, but unlike PhaZ1, it is not capable of degrading crystallized poly-3HB. However, it has been found that because of the modifications that poly-3HB undergoes during industrial production processes, PhaZ2 is able to degrade it with the same intensity and efficiency as PhaZ1 (Povolo et al., 2015; Saegusa et al., 2001). It is noteworthy, that, in the stationary growth phase, with the presence of exogenous carbon, nutrients and adequate growth conditions in the medium, these polymerases remain inactive (Saegusa et al., 2001).

A PHAs degradation scheme is reported in Fig. 12. Enzymes PhaZ1 and PhaZ2 hydrolyze the chain of the amorphous poly-3HB molecule, producing oligomers of 3HB of medium size. Due to their hydrophobicity, they remain attached to the granules, then PhaZ2 degrades the free ends of the oligomers bound to the grains resulting the total hydrolysis and releasing H_2O, CO_2 and energy (Kobayashi et al., 2003).

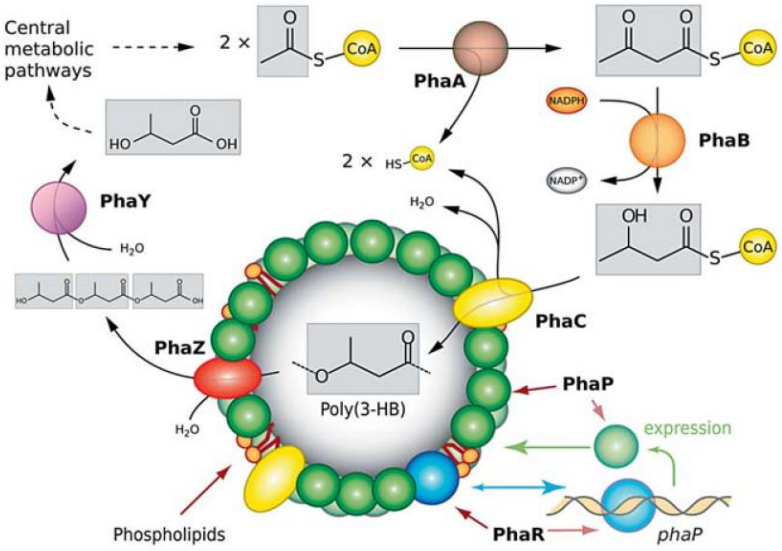


Fig. 12. Poly-3HB intracellular hydrolysis by PHAs producer bacteria (Reinecke and Steinbüchel, 2009).

1.3. Industrial production of PHAs.

Many years of research have led to the improvement of PHA production on a large scale, reaching the manufacture of homopolymers of 3HB, copolymers of 3HB and 3HV, 3HB and 4HB, as well as copolymers of 3HB and 3HHx and small quantities of PHAs medium-chain-length. Some companies are known to be engaged in PHAs production, among them Kaneka in Japan, P&G Chemical, BP and Metabolix in USA, Monsanto and others. Monsanto, in its Zeneca plant , has a production capacity of 660000 Lb/year of PHBV that is expected to increase in the coming years. However, to rapidly increase the global production of PHAs , it is necessary to reduce the manufacturing cost. Therefore, it is very urgent to develop low-cost PHAs production technology (Reddy et al., 2013).

1.3.1. Methods and strategies of PHAs production.

There are several industrial methods of PHAs producing of one, two, three and up to four step as reviewed in (Burk et al., 2011), but in this work we will only focus on two methods:

"The discontinued (batch) and continuous (fed-batch) cultivation method". They are very commonly used processes; the choice depends on the microorganism used in the process. Microorganisms can be in fact divided into two groups, based on the culture conditions required for PHAs synthesis: the first group of bacteria requires the limitation of an essential nutrient such as nitrogen, phosphorus, magnesium, potassium, oxygen or sulfur, and an excess carbon source (for example *C. necator, Pseudomonas oleovorans* among others). The second group of bacteria does not require nutrient limitation for PHAs synthesis (*Alcaligenes latus*, a mutant strain *Azotobacter vinelandii* and recombinant *E. coli* harboring the *A. eutrophus* PHAs biosynthesis operon) and can accumulate polymer during growth (Philip et al., 2007; Quillaguamán et al., 2010; Reddy et al., 2003).

During these production processes, pure cultures are usually used in a sterile medium to avoid competition for the substrate and the growth of non-PHAs producing bacteria.

Both processes generally consist of three phases: biomass production, PHAs accumulation and finally the extraction/recovery of PHAs.

For the bacteria of the first group, the two-step method is normally used; the procedure can be carried out in one or two different bioreactors. In the **step-one** (biomass production) microorganisms are grown in a cultural medium with optimal conditions (nutrients, pH, oxygen, carbon, temperature and others) in order to obtain the highest cells number to be used later on PHAs

production. Then, the cells undergo the **step-two** (PHAs accumulation) where the cells are transferred into a cultural medium (same or different from step-one) with limited essential nutrients (not optimal) but with high amount of carbon (Fig. 13a). In step-two PHAs accumulation is promoted, there is not cell growth, but the weight and cell density increases due to the PHAs cell content (Bugnicourt et al., 2014; Lee, 1996; Philip et al., 2007; Reddy et al., 2003). Both step can be realized in the same bioreactor but there must be optimal relationships among carbon source, other nutrients and fermentation time,: for example a premature limitation of nutrients will result in both low final cell number and PHAs quantity or if application of nutrient limitation is delayed too long, at the end of the process, cells will not contain high amounts of polymer, even though high cell concentrations is achieved (Anjum et al., 2016; Atlić et al., 2011; Kim et al., 1994).

C. necator, the bacterium currently most employed for the commercial production of 3HB and P(3HB-*co*-3HV), accumulates a large amount of polymer (up to 80% of cell dry mass) when nitrogen or phosphorus is completely depleted (Atlić et al., 2011). However, many other bacteria belonging to the first group, such as *P. extorquens* and *P. oleovorans*, produce PHAs more efficiently when a nutrient is limited but not completely absent (Preusting et al., 1993; Suzuki et al., 1986; Villano et al., 2014; Wang et al., 2012).

In the bacteria belonging to the second group, the PHAs production is elaborated in a single fermentation (Fig. 13b). The nutrient feeding strategy is crucial to the success of PHAs production. Cell growth and PHAs accumulation need to be balanced to avoid incomplete accumulation of PHAs or premature termination of fermentation at low cell concentration. There is an interesting relationship between the residual cell concentration and PHAs content. Since PHAs are accumulated in the cytoplasm, the residual cell concentration will determine how much PHAs can potentially be produced. A high PHAs content with a low residual cell concentration will result in a low final PHAs concentration and productivity. A high final cell concentration with a low PHAs content will also reduce the final PHAs concentration, productivity and yield. A high residual cell concentration with a high PHAs content will give the best results (Lee, 1996; Preusting et al., 1993; Suzuki et al., 1986).

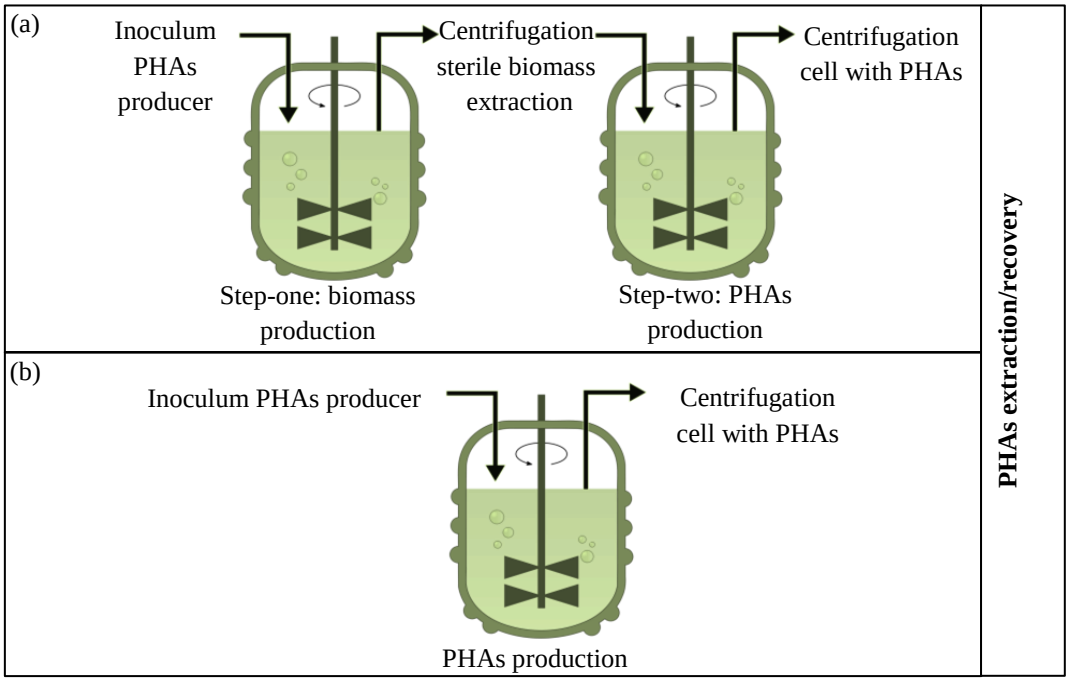


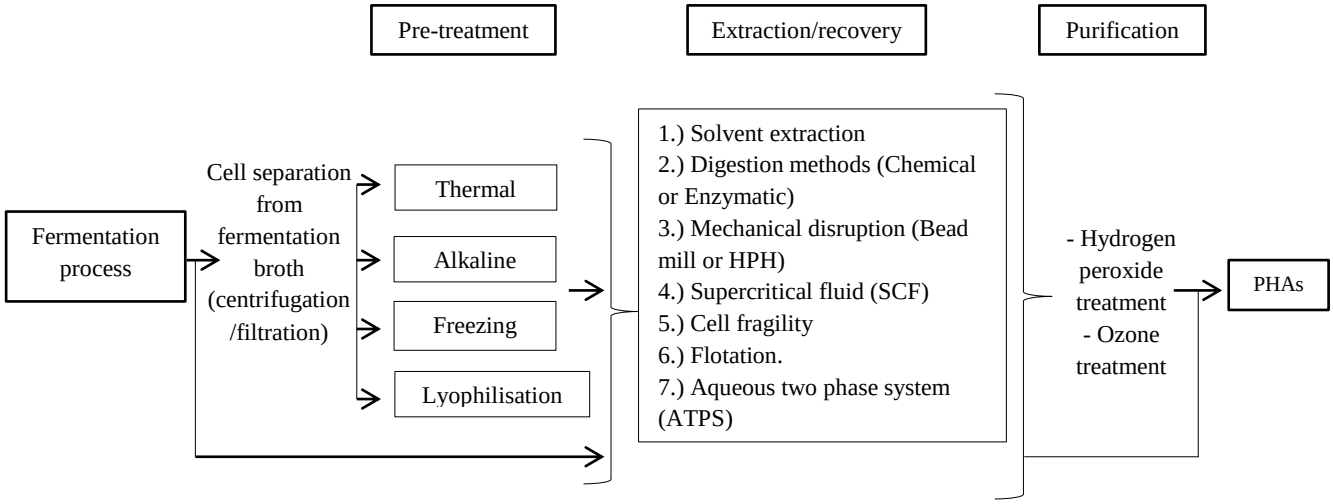
Fig. 13. PHAs production methods by: a) Bacteria of the first group (for example *C. necator*), using a two-steps PHAs production method with two bioreactors and b) bacteria of the second group (for example *Alcaligenes latus*), using a single bioreactor.

1.3.2. PHAs extraction/recovery.

After PHAs production, biopolymers must be extracted and recovered. Since PHAs is an intracellular product, cell pre-treatment and extraction/recovery methods are required. The extraction/recovery method could account for up to 40% of the overall manufacturing cost. Therefore, the downstream process of PHAs extraction/recovery is one of the key steps to ensure profitability of the system. Moreover, it must be eco-friendly and efficiently preserve material quality (Anis et al., 2013; Koller et al., 2013).

There are some factors influencing the choice of PHAs extraction/recovery method: type of microorganism (varying cell membrane fragility), type of PHAs (short, medium or long-chain-length-PHAs), PHAs load (content) in the biomass, purity required in accordance with the polymer application, availability and disposability of chemicals used and impact on the PHAs molecular weight (Koller et al., 2013). PHAs extraction is the major operation during recovery; therefore pre-treatment and purification steps can be added to improve the cell disruption process or to obtain PHAs of higher purity.

PHAs recovery includes three steps, i.e., pre-treatment, extraction/recovery and purification (Fig. 14) (Abdullah, 2015).



HPH: high pressure homogenization.

Fig. 14. General method of PHAs recovery (modified by Abdullah, 2015).

In the scientific literature several PHAs extraction/recovery methods are reported (see Table 6); the most important are:

1. Solvent extraction.

Usually for the extraction, halogenated solvents such as chloroform, dichloromethane and polychlorinated ethane are used. These solvents weaken the cell membrane and subsequently dissolve the PHAs (Harrison et al., 1992; Ramsay et al., 1994). In the laboratory, the PHAs-containing solvents are concentrated by evaporation using Soxhlet apparatus and then precipitated using low molecular weight alcohols, such as methanol and ethanol, at lower temperatures (Hrabak, 1992; Kessler and Witholt, 1998).

2. Chemical digestion methods.

The chemical approach is based on the solubilisation of non-PHAs cellular mass (NPCM) (Furrer et al., 2007). Instead of dissolving PHAs in a solvent, the digestion method acts by removing the surrounding NPCM which results in releasing free-PHAs grains. This is realized using chemicals such as surfactants and sodium hypochlorite (NaClO) (Koller et al., 2013).

3. Enzymatic digestion methods.

The enzymatic digestion uses proteolytic and hydrolytic enzymes such as lysozymes, phospholipases and nucleases to digest the biomass. This digestion method doesn't alter the PHAs granules in fact, unlike solvent extraction, the natural morphology of PHAs granules are

maintained, which is especially useful when the synthesis of strong fibres is needed (Koller et al., 2013; Martino et al., 2014).

4. Mechanical disruption method of bacterial cells by using bead mill.

Bead mill is an equipment with a cylindrical grinding chamber and a rotor inside the concentric cylinder to supply agitation. The bead mill is supplemented with cooling water which circulates outside the grinding chamber to counteract the high temperatures generated during the operation. Bead mill performance is not affected by biomass concentration; therefore, it is highly reproducible and feasible when applied on an industrial scale (Martino et al., 2014; I M Tamer et al., 1998b).

5. Mechanical disruption method of bacterial cells by high pressures homogenization (HPH).

The high pressure homogenizer is essentially a positive-displacement pump that forces cell suspension through a valve, before impacting the stream at high velocity on an impact ring. Operating pressures range up to 1500 bar. This method shows its effectiveness on a large scale as the extra pre-treatment is not required. However, it works well over a small range of biomass concentrations (Jacquel et al., 2008; Nonato et al., 2001).

6. Supercritical fluid (SCF).

SCF is widely used and it mainly utilize supercritical CO_2, due to its high solubility with other compounds, low toxicity, low reactivity and the unique properties of high densities and low viscosities. The effectiveness of this extraction method is highly dependent on the optimization of the operating parameters, such as temperature, pressure, specificity of the solvent, as well as the exposure times (Khosravi-Darani et al., 2004).

7. Cell fragility.

This method relies on the fact that the accumulation of PHAs could increase the osmotic fragility and weakens the cell wall. Therefore, is possible to manipulate cell fragility by changing the composition of the medium to break cell walls and extract PHAs. This was demonstrated for the first time, using fish peptone into the medium, which consequently led to fragile cell walls before being subjected to extraction with aqueous ammonia (Koller et al., 2013; Page and Cornish, 1993; Quillaguamán et al., 2010).

8. Flotation.

The flotation method consists of PHAs recovery by self-flotation of cell debris. It was tested after the extraction of various organic solvents, to obtain of 3HB from *Zobellella denitrificans* MW1. The cells were mixed with chloroform at 30°C for 72 h and later subjected to self-flotation of cell debris overnight at room temperature, obtaining a recovery of 85% (w/w) and purity of 98% of 3HB (Ibrahim and Steinbüchel, 2009). The use of green solvents together with flotation technique would add more advantages for the downstream processing of PHAs. Previously, selective dissolved-air flotation was also applied to extract mcl-PHAs from the cell debris of *P. putida* (van Hee et al., 2006). The main limitation of dissolved-air flotation is linked to the need of several consecutive flotation steps.

9. Aqueous two phase system (ATPS).

ATPS is formed by mixing two polymers or one polymer and an inorganic salt at low concentrations with mix incompatibility. As soon as two immiscible phases coexist, the PHAs released in the medium is solubilized in one of the liquids and can be separated (Yang et al., 2008). In a recent investigation, *B. flexus* cells were subjected to enzymatic hydrolysis, then cells were filtrated and re-suspended in a salt solution of Polyethylene glycol 8000/phosphate, pH 8.0 and 28°C, to separate the PHAs of non-PHAs biomass; in this experiment, a PHAs with 97% purity was obtained. In addition, the used hydrolytic enzyme was recovered (Divyashree et al., 2009). Nevertheless, some elements have to be considered in order to choose a good ATPS recovery system, as polymer molecular weight, concentration of polymer-salt, pH, molecular mass, charge etc. This method is considered attractive because of the short processing time, low material cost and energy consumption, good resolution, high yield and relatively high capacity (Rito-Palomares, 2004; Yang et al., 2008). However, ATPS is not yet used at industrial scale due to problems such as robustness, reproducibility, absence of commercial kits to evaluate ATPS at bench scale as well as poor understanding of the mechanism (Rito-Palomares, 2004).

10. Gamma irradiation.

Gamma irradiation is used on wet cells to support cell disruption and release of PHAs. Previous investigation with *B. flexus* irradiated with gamma rays (10 kGy) and subjected to chloroform extraction at room temperature in a short period, resulted in a 54% recovery. It was reported that Gamma irradiation offers advantages such as generate optimal rupture of cells at low dosage of irradiation, easier recovery of PHAs and low degree of cross-linking (Divyashree and

Shamala, 2009). Furthermore, radiation induced cell disruption is independent from chemicals, resulting in a relatively contamination free process. The major disadvantages are the length of irradiation time and the initial investment costs that hinders industrial scale applications (Bhattacharya, 2000). Overall, chemicals (alkaline or acidic solutions, surfactants and organic solvents) have been extensively proved and used in most of the recovery methods under different operating conditions although enzymatic digestion seems to be more eco-friendly; however, pure enzymes are expensive. In order to decrease the cost, some researchers have used microbial cultures as the source of the enzymes. For example, the use of *Microbispora* sp. culture instead of pure enzymes, to hydrolyse S. meliloti cells was found to be effective. Some methods like bead mill, HDH and supercritical fluid disruption are also eco-friendly since no chemicals are involved in the PHAs recovery process but special and costly equipments are often required. The use of gamma irradiation has to be fully studied to demonstrate its efficiency (Kunasundari and Sudesh, 2011).

Extraction method	Details	Strain	Results	References
Solvent extraction	Chloroform ($CHCl_3$)	*Bacillus cereus* SPV	Purity: 92%; Yield: 31%	(Valappil et al., 2007)
		C. necator DSM 545	Purity: 95%; Yield: 96%	(Fiorese et al., 2009)
	1,2-Propylene carbonate	*C. necator* DSM 545	Purity: 84%; Yield: 95%	(Fiorese et al., 2009)
	Acetone-water process		Yield: 80-85%	(Narasimhan et al., 2008)
	Methyl *tert*-butyl ether	*Pseudomonas putida* KT2440	Yield: 15-17.5%	(Wampfler et al., 2010)
	Methylene chloride	*C. necator*	Purity: 98%	(Zinn et al., 2003)
	Non halogenated solvents	*C. necator*		(Mantelatto and Durao, 2013)
	Acetone, room temperature	*P. putida* GPo1	Yield: 94%	(Elbahloul and Steinbüchel, 2009)
Digestion method				
Surfactant	SDS	Recombinant *E. coli*	Purity: 99%; Yield: 89%	(Choi and Lee, 1999)
	Palmitoyl carnitine	*C. necator, A. latus*	Purity: 56-78%	(Lee et al., 1993)
Sodium hypochlorite (NaClO)		*C. necator*, recombinant *E. coli*	Purity: 86%; Yield: 93%	(Hahn et al., 1995)
		C. necator DSM 545	Purity: 98%	(Berger et al., 1989)
Surfactant- NaClO	SDS- NaClO	*Azotobacter chroococcum* G-3	Purity: 98%; Yield: 87%	(Dong and Sun, 2000)
	Triton X-100- NaClO	*C. necator* DSM 545	Purity: 98%	(Ramsay et al., 1990)
Surfactant-chelate	Triton X-100-EDTA	*Sinorhizobium meliloti*	Purity: 68%	(Lakshman and Shamala, 2006)

	Betaine-EDTA-disodium salt	*C. necator* DSM 545	Purity:≥96%; Yield: 90%	(Chen et al., 2001)
Dispersion of $CHCl_3$ and NaClO	$CHCl_3$ - NaClO	*B. cereus* SPV	Purity: 95%; Yield: 30%	(Valappil et al., 2007)
		C. necator recombinant *E. coli*	Purity:≥98%	(Hahn et al., 1995)
Selective dissolution by protons	Sulfuric acid	*C. necator*	Purity:≥97%; Yield:≥95%	(Yu and Chen, 2006)
Enzymatic digestion	*Microbispora* sp culture- $CHCl_3$	*S. meliloti*	Purity: 94	(Lakshman and Shamala, 2006)
	Enzyme combined with SDS-EDTA	*P. putida*	Purity: 93%	(Kathiraser et al., 2007)
	Bromelain; pancreatin	*C. necator*	Purity: 89%; Yield: 90%	(Kapritchkoff et al., 2006)
Mechanical disruption	Bead mill	*A. latus*		(I M Tamer et al., 1998b)
	High pressure homogenization	*A. latus*		
	SDS-high pressure homogenization	*Metylobacterium* sp V49	Purity: 95%; Yield: 98%	(Ghatnekar et al., 2002)
	Sonification	*B. flexus*	Purity: 92%; Yield: 20%	(Divyashree et al., 2009)
Supercritical fluid	$SC\text{-}CO_2$	*C. necator*	Yield: 89%	(Hejazi et al., 2003)
Cell fragility	$CHCl_3$	*B. flexus*	Yield: 43%	(Divyashree and Shamala, 2010)
	Sodium hydrolysis	*B. flexus*	Yield: 50%	
	Alkaline hydrolysis	*B. flexus*	Yield: 50%	
Self-flotation of cell debris	$CHCl_3$	*Z. dinitrificans* MW1	Purity: 98%; Yield: 85%	(Ibrahim and Steinbüchel, 2009)
Dissolved air flotation	Enzymatic hydrolysis, sonification, flotation	*P. putida*	Purity: 86%	(van Hee et al., 2006)
Aquerous two phase system	*Microbispora* sp culture- ATPS	B. *flexus*	Purity: 95%; Yield: 50%	(Divyashree et al., 2009)
Gamma irradiation	Radiation- chloroform	B. *flexus*	Yield: 45-54%	(Divyashree and Shamala, 2009)
Air classification		*E. coli*	Purity: 97%; Yield: 90%	(Noda, 1998)
		C. necator	Purity: 95%; Yield: 85%	
Spontaneous liberation list of		*E. coli*	Purity: 80%	(Jung et al., 2005)

Table 6. PHAs recovery methods, modified from (Kunasundari and Sudesh, 2011).

1.4. BOTTLENECKS IN THE PHAs PRODUCTION CHAIN.

As already reported above, although PHAs are the most promising biopolymers for the substitution of the conventional plastics, their large-scale production is limited by several economic and manufacturing issues.

Costs in biopolymer production are mainly determined by the price of required raw materials and also by the extraction/recovery technologies: both can approximately account for up to 90% the expenses of the entire process.

1.4.1. High substrate costs.

Plastics like PE and PP are produced at a price of less than US $1.3-1.9/Kg. On the other hand, PHAs cost about 15-17 times more. In 1995 Monsanto produced and sold the PHAs at around US $17/Kg (Fornasiero and Graziani, 2011). Metabolic engineering, improved fermentation condition, and higher production capacities were able to reduce the cost to around US $4.9-6.1/Kg in 2009, which was still three times higher than the price for PP (DiGregorio, 2009). Therefore, PHAs still have a limited market, despite their potential to substitute 33% of commercial polymers (Castilho et al., 2009).

Numerous obstacles hinder cheaper PHAs production: the low petroleum prices due to shale gas exploitation. the increased price of glucose from corn starch and glycerol and fatty acids as oleic and palmitic, as feedstocks.

In general, fermentation processes have higher process costs related to lower yields when compared to processes in chemical reactors (Dietrich et al., 2017). The type of substrate greatly affects both cost and yield of PHAs (Table 7) taking into consideration that the substrate could account for up to 50% of the overall manufacturing cost. Therefore, in order to contain the cost, the selection of cheap raw material becomes one of the key factors in the PHAs production chain.

Substrate	Substrate price (US $ per Kg)	3HB yield (Kg 3HB/Kg substrate)	PHAs price per Kg of substrate [(US $ per Kg)/ (Kg 3HB/Kg substrate)]
Glucose	0.493[a] (0.220[c])	0.38[b]	1.30 (0.58)
Sucrose	0.290[d]	0.40[b]	0.72
Methanol	0.180[e]	0.43[b]	0.42
Acetic acid	0.595[e]	0.38[b]	1.56
Ethanol	0.502[a]	0.50[b]	1.00
Cane molasses	0.220[a]	0.42[a]	0.52
Cheese whey	0.071[a]	0.33[a]	0.22
Hemicellulose hydrolysate	0.069[a]	0.20[a]	0.34

[a] Data taken from Hocking and Marchessault (Griffin, 1994); [b] Calculated by multiplying the theoretical yield by 0.8 (assuming 80% of polymer accumulation) (Yamane, 1993); [c] Estimate of the value of hydrolysed corn starch; [d] International market price of raw sugar; [e] International market price from Chem. J. (Korea).

Table 7. Effect of substrate cost and 3HB yield on the production cost of 3HB, modified from (Dietrich et al., 2017).

1.4.2. Downstream processing costs and hazards.

After PHAs biosynthesis, microbial mass is separated from the cultural medium by well-established techniques like sedimentation, filtration, centrifugation or, less frequently, flocculation. PHAs extraction/recovery from non-PHAs cell mass (NPCM) also known as “residual biomass”, mainly comprising polypeptides, (phospho)lipids, DNA, RNA, and peptidoglycans (Braunegg et al., 1998) implies substantial and often underestimated cost aspect, particularly for large scale and (semi)industrial biopolymer fabrication (Choi and Lee, 1999). Industrial high usage of dangerous solvents and excessive requirement for energy input, are still common features in PHAs extraction/recovery, strongly antagonizing sustainability and economic feasibility. Hence, product extraction and recovery (downstream processing) displays a decisive process step of PHAs manufacturing.

Extraction using chemicals solvents (chloroform, methylene chloride, propylene carbonate, dichloromethane, amounts other) can give very good results (Ramsay et al., 1994) although the removal of cell debris in the solution is difficult. Moreover this technique needs large quantities of toxic and volatile solvent, which not only increases the total production cost but has a significant environmental impact (Choi and Lee, 1999). To decrease the costs and hazardousness non-halogenated solvents such as lactic acid esters, amides, ketones, acetic acid, acetic acid anhydride, tetrahydrofuran (Kurdikar et al., 2000; Nonato et al., 2001) has been also utilized together with cyclic carbonates such as ethylene carbonate and propylene carbonate (Baptist, 1962; Koller et al., 2013; Lafferty and Heinzle, 1979), but unfortunately with unsatisfactory yields.

The direct digestion using the hypochlorite solution (Berger et al., 1989), the pre-treatment with surfactants followed by the hypochlorite digestion (Ramsay et al., 1990), the enzymatic digestion process developed by Zeneca (Holmes and Lim, 1990) or the extraction with chloroform and sodium hypochlorite solution (Hahn et al., 1993) seem to be promising alternative. However, the large reagents quantities and high costs discourage their uses.

Mechanical methods (Divyashree and Shamala, 2009; Ghatnekar et al., 2002; I M Tamer et al., 1998a) and supercritical fluids (Hejazi et al., 2003) have less environmental impact and high performances, although they require a high initial investment.

Other factors affecting fermentation costs have to be taken into account although less significative: the oxygen supply, mainly when PHAs is produced in high-cell-density culture and dissolved oxygen often acts as a limiting factor, the quality of water and the energy needed for sterilization and incubation.

Therefore, the selection of economical feedstocks and cheap eco-friendly extraction/recovery methods, become the key factors in the PHAs production chain to reduce costs, increase production making PHAs competitive with oil-derived plastics .

1.5. POSSIBLE SOLUTIONS FOR THE BOTTLENECKS IN THE PHAs PRODUCTION CHAIN.

In the last 24 years, and especially from 2012 until now, there has been a huge increase of studies aimed at introducing bioplastics into the market at competitive prices in comparison to fossil fuel-based plastics (Rodriguez-Perez et al., 2018).

During this period, scientists mostly focused on the reduction of PHAs production costs. Toward such an objective, different strategies have been adopted, such as genetic studies (genetic improvement or genetic transformation of strains), optimization of the purification extraction step to obtain PHAs with higher economic value and/or promising new applications, implementation of continuous processes to diversify the feed streams, re-use of the bacterial biomass generated from PHAs production, reduction of the energy consumption during the process, integration into a bio-refinery system to improve the yield and obtain PHAs from waste streams (Rodriguez-Perez et al., 2018).

1.5.1. PHAs production from wastes as alternative low cost substrates.

The use of alternative substrates as organic wastes for PHAs production, which is also one the topics of this research work, has been taken into account as a promising alternative (Koller, 2016; Povolo et al., 2012). Moreover , the use of industrial by-products takes the advantage of decreasing disposal costs of wastes, obtaining at the same time value-added products (Casella et al., 2016). A broad range of wastes and by-product streams associated with agricultural, urban and industrial sectors can be identified as suitable feedstock for the PHAs biotechnological production (Casella et al., 2016; Du et al., 2012; Koller et al., 2010). Table 8 reports low cost substrates successfully used as carbon substrates for PHAs production. A number of interesting results are already available starting from these cheap substrates. For example, the use of vegetal residues, such as rice bran, pea-shells, chicory roots, potato peels, apple pomace, onion peels, grape pomace, animal farm wastes, poultry litter. In addition, the use of industrial wastes was also approached, including water streams as wastewater from olive oil extraction process, leguminous processing wastewater and fruit processing wastewater. Additionally, low and medium added value by-products such as cheese whey and olive oil distillate, respectively, have been studied for PHAs production (Casella et al., 2016; Koller, 2016; Rodriguez-Perez et al., 2018). Food wastes comprise solid wastes, such as spent coffee grounds and food waste composite including boiled rice, cooked vegetables, un-cooked vegetables, cooking oil, vegetable peelings, cooked meat, boiled spices and a liquid waste such as used cooked oil. Other substrates of non-agro-industrial origin were also evaluated, like crude glycerol, oil cake hydrolysate and biodiesel fatty acid by-product from glycerol purification. In addition, carboxylic acids contained in glycerol anaerobic digestion effluent from 1,3-propanediol production were successfully studied as carbon source (Rodriguez-Perez et al., 2018).

Reference	Culture	Employed substrate	Operation mode	Yield	PHAs %
(Gómez Cardozo et al., 2016)	*Bacillus megaterium*, *Bacillus* sp., and *Lactococcuslactis*	Cheese whey, cooking oil Crude glycerol	1Phase: Growth and PHAs production[+]	0.2	87
(Cruz et al., 2016)	*C. necator*	Olive oil distillate, cooking oil biodiesel fatty acids-by-product	1Phase: PHAs production*	0.9	62
(Alsafadi and Al-Mashaqbeh, 2017)	*H mediterranei*	Olive mill wastewater	3Phases: Pre-treatment; Growth and PHAs production*	0.009[b]	43
(Kourmentza et al., 2015)	Mixed culture	Olive mill wastewater	3Phases: Pretreatment; Enrichment under stress conditions and PHAs production*	0.18/0.68	64.4/18.2
(Campanari et al., 2014)	Mixed culture: activated sludge	olive mil wastewater	3Phases: Pre-treatment; Enrichment and PHAs production*	0.86	30
(Valentino et al., 2015)	Mixed culture	Cheese whey	3Phases: Pre-treatment; feast-famine regime and PHAs production*; 3 Phases: Pre-treatment; Growth and PHAs production*	0.28/0.3; 0.46	58/75; No data
(Koller et al., 2012)	*C. necator* mRePT	Cheese whey	2Phases: Growth and PHAs production	0.21-2.4[h]	21
(Pais et al., 2016)	*H mediterranei*	Cheese whey	2Phases: Pre-treatment and PHAs production[+]	0.78	53
(Colombo et al., 2016)	Mixed culture: Activated sludge	Cheese whey	3Phases: Pre-treatment, Enrichment (feast/famine) and PHAs production*	0.9[c]	39
(Cruz et al., 2015)	*Pseudomonas*, *C. necator* DSM 428	used cooking oil	2Phases: Growth and PHAs production[+]	0.41[d]	63
(Bera et al., 2015)	*Halomonas hydrothermalis*	crude glycerol + oil cake hydrolysate	3Phases: Pretreatment, Growth and PHAs production*	0.75	73.3
(Ray et al., 2016)	*Pannonibacter phragmitetus ERC8*	Crude glycerol	3Phases: Pretreatment, Isolation and enrichment and, PHAs production*	0.16	64.34
(de Paula et al., 2017)	Pandoraea sp.	Crude glycerol	2Phases: Isolation and Growth and, PHAs production*	0.16/0.22/0.05/0.05/0.04	37/49/12/12/10
(Ntaikou et al., 2014)	Mixed culture//Pure culture: *Pseudomonas* sp.	olive-mill wastewater	3Phases: Pre-treatment, enrichment (acclimation) and PHAs production[&]	7.58 ± 0.06[h]	25
(Chandrasekhar et al., 2015)	Mixed culture: activated sludge	boiled rice, cooked vegetables, un-cooked vegetables (spoiled), cooking oil, vegetable peelings, cooked meat, boiled spices	3Phases: Pre-treatment, enrichment (feast/famine) and PHAs production[&]	0.17	24
(Romanelli et al., 2014)	*D. acidovorans* DSM 39 recombinant + *liph-lipC* gene	slaughterhouse waste	2Phases: Culture grow and PHAs production*	No data	28/15/15
(Riedel et al., 2015)	Pure culture : *C. necator* H16	Waste plant oil and animal fats	2Phases: Culture grow and PHAs production*	No data	79-82

Not specified chemical; [b] g PHAs/g VFA; [c] Cmmol PHAs/Cmmol substrate; [d] g substrate (VFA)/g PHAs; [e] Cmol HB/Cmol VFA 0.142 Cmol HV/Cmol VFA; [f] Cmol PHAs/Cmol VFA; [g] maximum modelled biomass specific PHAs production rate (Cmmol/Cmmol/h); [h] g PHAs per L of initial OMW 0.00758 g/g; Yield: g PHAs/g substrate; * Batch operation; [+] Sequential operation; [&] Continuous operation.

Table 8. Different wastes streams reported as carbon source for PHAs production, modified from (Rodriguez-Perez et al., 2018).

In 2010 an EU research project called EC-ANIMPOL started. The consortium was composed by several European Countries, including Italy (Università di Padova) and coordinated by Graz University of Technology (Austria). The main objective of the whole project was to study the biotechnological conversion of fats-containing agro-industrial wastes for the eco efficient production of high added value products, such as PHAs polymers. This project contemplates the use of slaughterhouse wastes as carbon source, taking advantage of their high fat content at the almost zero costs.

In the EU, the amounts of animal lipids from the slaughtering animal process can be quantified with more than 500,000 ton/year, of which 450,000 ton are destined to biodiesel production and 50,000 ton to crude glycerol production. ANIMPOL project proposed the use of these animal fats or the waste from these processes as carbon sources for PHAs production (Fig. 15) (Koller and Braunegg, 2015; Titz et al., 2012).

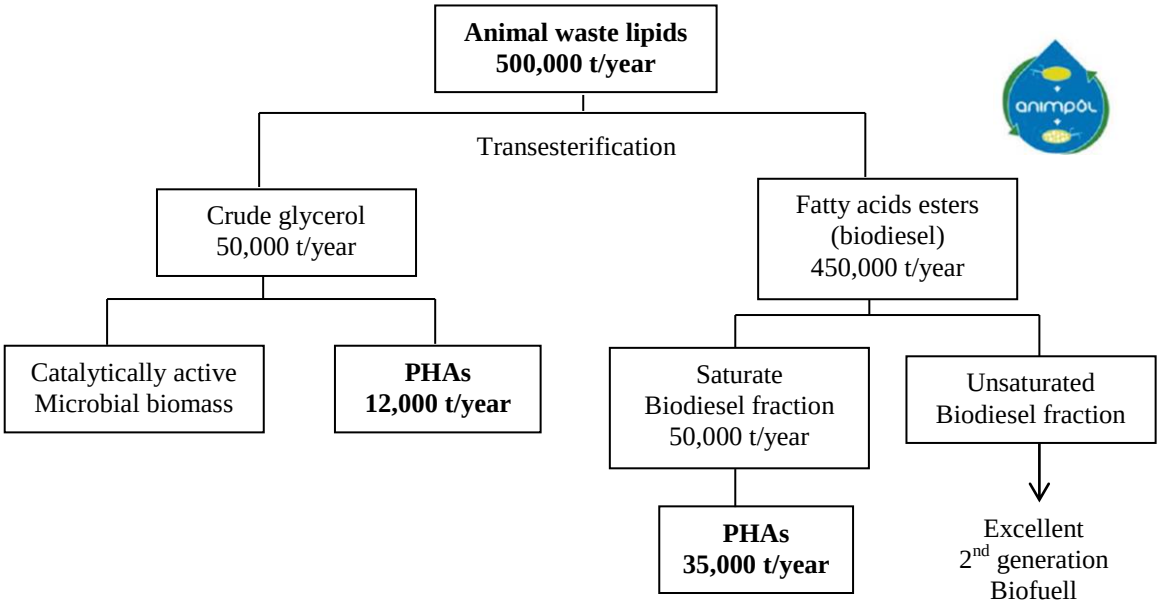


Fig. 15. The ANIMPOL Project: available quantities of waste lipids from the animal processing industry, and theoretical attainable quantities of PHAs (Koller and Braunegg, 2015).

Unfortunately, few PHAs-producing strains have the ability to hydrolyse complex fats, moreover the microorganisms showing this property accumulate low amounts of PHA from these wastes (Romanelli et al., 2014). Hence, the possibility of integrating genes encoding for lipase enzymes into the genomes of active PHAs-producing bacteria, has been investigated in the Project.

1.5.2. Downstream processing, alternative methods of PHAs extraction/recovery.

Low cost PHAs extraction steps, with high performance and low environmental impact is an important guideline to be followed in future research. The current PHAs extraction steps are indeed very energy and solvent intensive and cause losses in PHAs.

Highly pure PHAs can be produced by processes that break the cell and solubilize cellular material other than PHAs. Therefore, a simple digestion method obtained by inexpensive chemicals or mechanically, could lower purification costs in an environmentally friendly way.

Mechanical methods are apparently those with the lower environmental impact and economic needs (only an initial investment is in fact requested) among all the conventional methods that have been studied.

HPH (high pressures homogenization) is one of the most widely known methods for large-scale cell disruption. HPH is considered environmentally friendly since it does not need solvents to obtain an efficient microbial cells disintegration (Koller et al., 2013). However, cell lysis causes the release of large amounts of chromosomal DNA which results in a dramatic increase in viscosity, hampering following filtration and centrifugation steps (Ling et al., 1998; I M Tamer et al., 1998b; Van Wegen et al., 1998). Since the efficiency of both filtration and centrifugation is inversely related to the viscosity, quick removal of the DNA is crucial (Atkinson and Mavituna, 1991). Drop in viscosity is generally achieved by the supplementation of hypochlorite, commercially available nucleases, or heat treatment. Although these methods may be applicable in small-scale fermentation systems, they are not environmentally and economically suitable for industrial PHAs manufacturing (Koller et al., 2013). As a solution to this problem, Boynton et al., 1999, integrated a nuclease-encoding gene from *Staphylococcus aureus* into the genome of *P. putida* . Staphylococcal nuclease is readily expressed extracellularly in *P. putida* strains without affecting PHAs production or strain stability. During downstream processing, the viscosity of the lysate from nuclease-integrated *P. putida* strain was reduced to a level similar to that observed for the wild type strain after treatment with commercial nuclease (Boynton et al., 1999).

Recent studies describes the green and sustainable partial recovery and purification of PHAs using insects that use the bacteria as feed and excrete PHAs granules in the form of fecal pellets since their digestive system is able to assimilate the bacterial cells but not the PHAs (Ong et al., 2018). Other groups recently proposed the use of 1-Ethyl-3-methylimidazolium Diethyl Phosphate (Dubey et al., 2017), Dimethyl carbonate (DMC) and switchable anionic surfactants (Samorì et al., 2015), obtaining yields of 60%, 85% and 90%, respectively. These studies indicate the possibility to find new , cheaper, easy, fast and eco-friendly ways for PHAs extraction/recovery.

1.6. Relevant PHAs producing microorganisms.

In 1923 Lemoigne identified 3-hydoxybutyric acid from *B. megaterium*; nowadays, we know that this is a products of PHB hydrolysis (Volova, 2004).

Nowadays, more than 150 PHAs types have been identified and more than 300 microorganisms producing PHAs. However, not all PHA-producing strains have high yields. In the seventies, Imperial Chemical Industry was the first company to apply *methylotroph* microorganisms, using methanol as a cheap substrate. Unfortunately, this type of microorganisms produced little polymers amounts with a low molecular weight. The company decided to focus on *Azotobacter* sp., but the instability of the *Azotobacter* sp. strains and the excessive production of polysaccharides were the reason of the early abandon of this path.

Nowadays, *Cupriavidus necator* and *Delftia acidovorans* are among the most important PHAs producers.

C. necator is able to accumulate up to more than 80% of PHAs/dry weight and currently it is one of the most used industrial species for the production of 3HB (Berezina, 2013). *D. acidovorans* is a well-known wild type bacterium that can efficiently accumulate PHAs containing high molar fractions of 4HB (Saito et al., 1996), a very important copolymer that, once blended with 3HV and/or 3HB, provides important properties to PHAs material (Romanelli et al., 2014).

1.6.1. PHAs production by *Cupriavidus necator*.

C. necator has gone through a series of name changes along its history. It was originally named *Hydrogenomonas eutrophus*, then *A. eutrophus*, *Ralstonia eutropha*, *Wautersia eutropha* and finally, after a deep characterization in terms of cell morphology, metabolism, GC content, phenotyping, lipid composition and 16S rRNA analysis, it was named *C. necator*.

It is a non-pathogenic, rod shaped, Gram-negative bacterium, non-spore forming, with optimal growth temperature of 30°C. It is a facultative aerobe which can live in both aerobic and anaerobic environments, and it has motility due to two flagella. There are differences among strains of the same *C. necator* specie; for instance *C. necator* JMP 134 has multiple habitats, while *C. necator* H16 has a specialized habitat; however, both strains requires non-salty (not halophilic) environments (Slonczewski and Foster, 2013).

C. necator is nowadays one of the most used in the industrial PHAs production and serves as model organism for genetics and control of autotrophic carbon dioxide and hydrogen fixation. It is able to accumulate up to 80% of high molecular weight PHAs, to degrade a large list of

chloroaromatic compounds, chemically related pollutants, carbohydrates and oils. *C. necator* is able to grow only with hydrogen and CO_2 as its only energy and carbon source. When O_2 is not present, it can use different metabolic pathways to growth, such as denitrification (Slonczewski and Foster, 2013).

This microorganism can use different pathways to produce PHAs, depending on the carbon sources. It can also produce 4HB and 3HV, but in this case the incorporation of precursors such as γ- butyrolactone, 1,4-butanediol, valeric acid, 1-pentanol, among others, is needed (Aziz et al., 2012).

As stated earlier, it is able to grow using fatty acids as a substrate, but differently from *D. acidovarans*, *C. necator* is able to produce only low amounts of lipase enzyme (Koller and Braunegg, 2015; Riedel et al., 2015; Verlinden et al., 2011).

1.6.2. PHAs production by *Delftia acidovorans*.

Initially belonged to the Comamonadaceae family, *Comamonas acidovorans* was found genetically different from *Comamonas* species, based on 16SrDNA analysis. Therefore, it was re-assigned to *Delftia* genus as *D. acidovorans*. This name refers to the city of Delft, the location where the strain was first isolated in 1926.

It is a Gram-negative, not spore-forming bacterium, a straight to slightly curved bacillus, which occurs singly or in pairs. It has mobility by means of polar or bipolar flagella; the optimal growth temperature is 30°C and is strictly aerobic. The metabolic pathway for PHAs production is number II (see paragraph 1.2.2.), linked to the absorption of fatty acids for the biosynthesis mainly of 3HB and 4HB, the most important PHAs at industrial level for biomaterial production (Ch'ng et al., 2012).

D. acidovorans can accumulate up to 20% of high molecular weight PHAs with high quantities of 4HB. So far, 4HB was found to be incorporated into PHAs of *D. acidovorans* only when precursors such γ- butyrolactone and 1,4-butanediol are provided in the culture medium (Mothes and Ackermann, 2005) or when slaughterhouse fatty wastes were used as carbon sources (Romanelli et al., 2014).

Although it is a microorganism that uses fatty acids as carbon sources, it does not have the capacity to produce the lipases. Therefore, it cannot directly utilize complex fats to produce PHAs. Recent studies have used genetic tools to introduce lipase encoding genes into *D. acidovorans* DSM 39, thus making possible the hydrolysis of complex fats (triglycerides) by a genetically modified micro-organism (Romanelli et al., 2014).

1.7. Lipases.

1.7.1. History of lipases.

Pancreatic juice, extracted for the first time in 1856 by Claude Bernard, was found able to hydrolyze insoluble oil droplets, thus obtaining soluble products (Sangeetha et al., 2011).

From there, mainly animal pancreatic lipolytic extracts were used as the source of lipase for commercial applications. Once the demand for lipases could not be met by the supply from animal sources, because of pancreas shortage and difficulties in collecting available material, scientists began to explore new sources of lipases.

Bacterial lipases were first observed in 1901 in *Serratia marcescens* and *Pseudomonas aeruginosa* (Hasan et al., 2006). After their discovery within prokaryotes, microorganisms were used as the main source of lipolytic enzymes due to their easy growth and manipulation (Hasan et al., 2006). The microbial lipases are often more convenient than enzymes derived from plant and animals. Other than from bacteria and actinomycetes, lipases can also be produced by fungi and for this reason the current market for microbial lipases became very wide.

Until now, lipases obtained from fungi are the most used, even if bacteria are constantly studied and improved. The manufacturing of microbial lipases needs not only the effective overexpression of the lipase genes, but also a detailed molecular mechanisms governing their folding and secretion. For this reason, lipase synthesis by several bacterial species has been extensively studied and reported, principally in *Pseudomonas* sp., *Bacillus* sp. and *P. aeruginosa* (Madan and Mishra, 2010).

1.7.2. Mechanisms of production and secretion of lipases.

Lipids represent a substantial fraction of the earth's biomass and lipolytic enzymes play an important role in their hydrolysis and, therefore, in natural organic matter re-cycling. Lipases (triacylglycerol hydrolase, EC.3.1.1.3) are water-soluble enzymes that hydrolyse ester bonds in mono-, di- and triacylglycerol in water-soluble fat acid and alcohols. The fatty acids are transported into the cytosol and 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 using these compounds (Rosenberg and Ron, 1999).

Lipases are part of the superfamily of "α/β hydrolases", one of the largest groups of enzymes structurally bound together (Angkawidjaja and Kanaya, 2006). The lipolytic activity is associated with the catalytic activity of three amino acids, a catalytic triad with a nucleophilic

residue, a histidine and an acid. These triads in the lipases are represented by serine, histidine and aspartate.

The active conformation is taken by the lipase when it comes into contact with a substrate insoluble in water (for example oil) at a concentration close to the solubility limit of the substrate. This phenomenon is called “interfacial activation” (Angkawidjaja and Kanaya, 2006).

Lipolytic enzymes derived from bacteria are classified into eight families based on their sequence homology: family I is a large group and it has been classified into seven subfamilies of which subfamilies I.1, I.2 and I.3 are lipases produced by gram-negative bacteria. The subfamilies I.1 and I.2 have a comparatively high sequence similarity (around 30-40%) and are secreted by the type II secretion system (T2SS), as mentioned below (Arpigny and Jaeger, 1999).

The bacterial lipases can be intracellular, membrane-bound or extracellular. The most interesting for our work are the extracellular lipases because they are the only one that can be produced in the presence of long chain triglycerides, while the other two do not.

Bacteria secrete lipase into the medium through different systems:

- Type I secretory system: is an energy driven exporter complex made up of three protein subunits.
- Type II secretion system (GSP or General Secretory Pathway): comprises two steps. In the first step, the protein is translocated across the inner membrane of the Gram-negative bacteria from a path-dependent Sec (Angkawidjaja and Kanaya, 2006); the protein Lif. anchored to the inner membrane determines correct coding of lipases. After the process of folding and the degradation of N-terminus, the lipase is secreted outside the cell by a specific protein complex (XCP machinery) (Rosenau et al., 2004).

In general the natural conformation of lipase is determined not only by the amino acid sequence, but also by the presence of chaperones (specific accessory proteins or molecular chaperones). These chaperones facilitate the formation of the tertiary structure of some proteins, or protein complex, although they are not components of the final structure.

Extracellular lipase gene of family I is located in an operon together with a second gene, that is necessary to the lipase activity. In fact, the proteins coded by this second gene assists the correct folding of the associated lipase. For this reason, they were named Lifs to indicate that they constitute a unique class of *lip*ase-specific foldases (Rosenau et al., 2004); lipase subfamilies I.1 and I.2 fold into an enzymatically dynamic conformation in periplasm and then they are transported through the bacterial outer membrane by means of a complex process consisting of up to 14 different proteins. To complete a secretion-competent conformation, lipases need specific

intermolecular folding catalysts, the Lif proteins. Lifs are a unique family of proteins without any significant homology to other classes of proteins that 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 (Fig. 16).

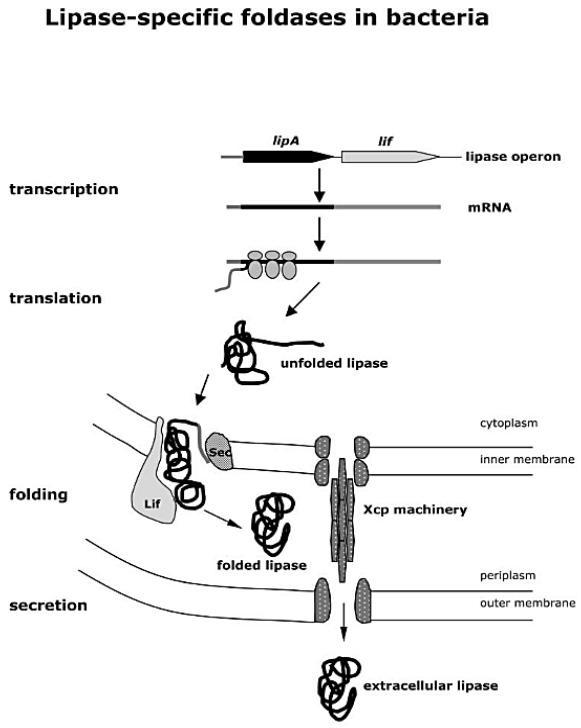


Fig. 16. Lipase secretion in Gram negative bacteria (Rosenau et al., 2004).

1.7.3. Recombinant bacterial lipases.

Genetic developments and new technologies allowed to sequence the structure of a large number of lipases. Thus, it has been possible to understand how they are produced and work. As a consequences of this knowledge, genetic technology enables to choose the right lipolytic genes and improve their performance or insert them into other non-lypolitic bacteria, allowing lipases overexpression in the host with the final objective to fulfil commercial demands.

Many bacterial lipases have been in fact cloned, sequenced and expressed in homologous or heterologous hosts (Sangeetha et al., 2011). However, to obtain the competent heterologous expression of a lipase, it is required to clone together lipase gene and the relative *lif* gene. Many

investigations have indicated the specificity of *lif*-protein to its corresponding lipase (El Khattabi et al., 1999; Shibata et al., 1998). These results demonstrated that lipase transposition and chaperon sequences, between phylogenetically close related organisms, is adequate to obtain a successful production of lipase (Jørgensen et al., 1991). Another study shows that, in the production of lipase, other 30 cellular proteins are involved (Rosenau et al., 2004). Fortunately, they are all present in most of the organisms phylogenetically close to the donor microorganisms.

1.8. Nucleases.

1.8.1. History of nuclease.

The first nuclease was described in the late 1960s by Stuart Linn and Werner Arber, that isolated an enzyme responsible for phage growth restriction in *E. coli*. In this work authors noticed that one of these enzymes added a methyl group (CH_3) to the DNA, generating methylated DNA (methylase enzyme), while another cleaved un-methylated DNA in a wide variety of locations along the length of the molecule (restriction nuclease enzyme) (Arber and Linn, 1969; Linn and Arber, 1968). Nevertheless, these enzymes had the capacity to break the DNA and RNA chain in non-specific places or in a random way; with the need of a tool to cut and paste the DNA in specific places, the scientists were encouraged to search and develop new restriction enzymes with specific places of action.

In 1970 Smith and Welcox, separated and characterized the first restriction nuclease acting on a specific DNA nucleotide sequence. The enzyme, extracted from *Haemophilus influenzae* (Smith and Welcox, 1970) and called *HindII*, always cuts directly in the middle of this sequence (5'-GTYRAC-3', 3'-CARYTG-5'). Nowadays, many restriction enzymes are known, nominated on the list of REBASE (http://rebase.neb.com),

The nucleases are classified into two types, depending on the place of action: the exonucleases that digest nucleic acids from the ends and the endonucleases that act on the central region of target molecules. These are subdivided into deoxy-ribonucleases that acts on DNA and ribonucleases that acts on RNA (Rittié and Perbal, 2008).

Owing to the current technological development, the restriction enzymes have a great use in the molecular biology industry, day by day are improvements and developed new enzymes with different and greater restriction specificity (Rasala and Mayfield, 2015).

1.8.2. *Staphylococcus aureus* nuclease.

Staphylococcus aureus is one of the types of bacteria most responsible for food poisoning and it has been identified as the causative agent in many food poisoning outbreaks The disease is due to the action of the heat-stable enterotoxins produced by the bacterium and that cannot be removed from the finished product by heat treatments that kills the vegetative bacterial cells but doesn't inactivate the enterotoxins (Schaumburg et al., 2014). For this reason and due to the fact that the analyses of enterotoxins are costly and time consuming , the presence of the nuclease (that is thermostable too) in food is usually analysed as indicator of the possible presence of enterotoxins (F. Li et al., 2016).

The staphylococcal nuclease is a relatively non-specific endo-exonuclease, Ca2+-activated extracellular phosphodiesterase which degrades both DNA and RNA to 3'-nucleotides. The enzyme has a molecular weight of 16,807 Daltons, 149 amino acid residues with no disulphide bonds or free sulfhydryl groups, and is strongly inhibited by deoxythymidine-3',5'-diphosphate (pdTp) (Rosman et al., 2018). *S. aureus* nuclease production is a character associated to the pathogenicity of *S. aureus* and was associated with delayed bacterial clearance in the lung and increased mortality after intranasal infection and promotes resistance against NET-mediated antimicrobial activity of neutrophils and contributes to disease pathogenesis in vivo (Wolter et al., 2018).

Nowadays *Staphylococcus aureus* nuclease is industrially produced by a recombinant *E. coli* strain and it is able to digest double-stranded, single-stranded, circular and linear nucleic acids (Hu et al., 2013). The enzyme is active in the pH range of 7.0 - 10.0, with optimal activity at pH 9.2 for both RNA and DNA substrates.

It is suitable for the degradation and removing of nucleic acids present in protein preparation and to reduce viscosity of cell lysates during cell lysis preparation. Unfortunately the high costs limit the industrial utilization (Gamero et al., 2018).

To reduce the cost, *nuc* gene from *S. aureus* was cloned into the PHAs producer *P. putida* strain, in order to avoid the use of commercial nuclease in the final polymer extraction process (Boynton et al., 1999; Chesneau and El Solh, 1992).

1.9. PROJECT OUTLINE.

As already stated, PHAs are polymers that meet a series of promising physical-chemical, mechanical and biological characteristics allowing them to replace plastics from crude oil, in addition they are "biobased", "biodegradable" and "biocompatible" (Krzan et al., 2006; Miertus and Ren, 2002). According to a recent report, published in 2017, the global PHAs market is expected to increase from US$ 73.6 million within 2016 to US$ 93.5 million by 2021, characterized by a compound annual growth rate (CAGR) of 4.88% (Kourmentza et al., 2017), but to achieve this increase successfully, it is necessary to make the production processes of PHAs more efficient and economical.

As an option to reduce PHAs production cost, the use of organic industrial wastes as carbon sources and alternative methods of PHAs recovery were evaluated.

On the basis of previous results obtained during the EU project ANIMPOL (Biotechnological conversion of carbon containing wastes for eco-efficient production of high added value products), the use of fatty wastes from slaughterhouses could be a strategy to decrease PHAs price, unfortunately strains with high PHAs production level hydrolyse complex fats (triglycerides) with low efficiency.

In principle, these problems could be solved mainly by two strategies: 1) the isolation, selection and characterization of new natural microbial strains capable of efficiently converting the slaughterhouses fatty wastes into PHAs; 2) the engineering of microorganisms that exhibit high PHAs productions yields, so that can convert the slaughterhouses fatty wastes into PHAs.

Although the first approach is fascinating, no wild type strain has been so far reported to achieve these objectives, meanwhile the other option though challenging, is considered to be the most suitable (Casella et al., 2016).

In this research work, a molecular biology program started in order to obtain a microbial strain capable of both hydrolysing lipids and producing high levels of PHAs. *Cupriavidus necator* DSM 545, a bacterium producing high PHAs amounts but poorly able to metabolize lipids, was selected as host strain of lipolytic genes (*lipH-lipC*) from the efficient lipase producer *Pseudomonas stutzeri* BT3.

On the other hand, to reduce the cost of downstream process of PHAs extraction, *C. necator* DSM 545 and *D. acidovorans* DSM 39 were selected as recipients of the staphylococcal nuclease gene *nuc* from *Staphylococcus aureus*, with the aim of avoiding the use of commercial nuclease during the PHAs extraction/recovery phase.

2. MATERIALS AND METHODS.

2.1. Media, strains and plasmids.

The media used in this work are reported in Table 9. All chemicals, media components and supplements were of analytical grade (Sigma).

The strains were grown routinely in Nutrient Broth, DSMZ 81 medium (Povolo et al., 2010) and Nitrogen reduced medium (Romanelli et al., 2014).

The Nitrogen reduced medium sometime was modified with 1 g/L $(NH_4)_2SO_4$.

Medium	Composition	Reference or supplier
DSMZ 81 medium	NH_4Cl 1 g/L; $MgSO_4 \cdot 7H_2O$ 0.5 g/L; $NaHCO_3$ 0.5 g/L; KH_2PO_4 2.3 g/L; $Na_2HPO_4 \cdot 2H_2O$ 2.9 g/L; $CaCl_2 \cdot 2H_2O$ 0.01 g/L; Ferric ammonium citrate 0.05 g/L; Standard vitamin solution: Riboflavin 0.0005 mg; Thiamine-$HCl \cdot 2H_2O$ 0.0025 mg; Nicotinic acid 0.0025 mg; Pyridoxine-HCl 0.0025 mg; Ca-pantothenate 0.0025 mg; Biotin 0.000005 mg; Folic acid 0.00001 mg; Vitamin B12 0.00005 mg	DSMZ
Nutrient broth (NB)	Glucose 1 g/L; peptone 15 g/L; NaCl 6 g/L; yeast extract 3 g/L	DIFCO
Super optimal broth medium (SOC medium)	Tryptone 20 g/L; Yeast Extract 5 g/L; $MgSO_4$ 4.8 g/L; dextrose 3.6 g/L; NaCl 0.5 g/L; KCl 0.2 g/L	(Aneja et al., 2009)
DNAse agar	Tryptose 20 g/L; Deoxyribonucleic acid 2 g/L; NaCl 5 g/L; Agar 12 g/L	(Favaro et al., 2014)
Nitrogen reduced medium	K_2HPO_4 2.7 g/L; $Na_2HPO_4 \cdot 12H_2O$ 7.2 g/L; $MgSO_4 \cdot 7H_2O$ 0.3 g/L; $(NH_4)_2SO_4$ 0.03 g/L; microelements of 0.1 M HCl containing per liter: $COCl_2$ 119 mg/L; $FeCl_3$ 9.7 g/L; $CaCl_2$ 7.8 g/L; $NiCl_2 \cdot 6H_2O$ 118 mg/L; $CrCl_2$ 62.2 mg/L and $CuSO_4.5H_2O$ 156.4 mg/L	(Romanelli et al., 2014)
Nitrogen rich medium	Peptone from meat 10 g/L; NB 10 g/L; Yeast extract 10 g/L; $(NH_4)_2SO_4$ 5 g/L	(Povolo et al., 2013; Tsuge et al., 2004)

DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen).

Table 9. Media used in this study.

Media for recombinant strains were supplemented with kanamycin or gentamicin when requested. All media were autoclaved at 121°C for 20 min before utilization. When necessary, liquid broths were solidified adding 20 g/L agar-agar.

The bacterial strains and plasmids used this work are listed in Table 10.

Strain and plasmid	Genotype	Reference or source
E. 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)
C. necator DSM 545	Wild type	DSMZ
D. acidovorans DSM 39	Wild type	DSMZ
Pseudomonas stutzeri BT3	Wild type	(Romanelli et al., 2014)
E. coli pBBR1MCS 5-*lipH-lipC*	$Gent^+$ (pBBR1MCS-5-*lipH-lipC*)	(Romanelli et al., 2014)
C. necator DSM 545 pBBR1MCS-5-*lipH-lipC*	$Gent^+$ (pBBR1MCS-5-*lipH-lipC*)	This work
E. coli pHM2-*nuc*	Km^+ (pHM2-*nuc*)	This work
C. necator DSM 545 pHM2-*nuc*	Km^+ (pHM2-*nuc*)	This work
D. acidovorans DSM 39 pHM2-*nuc*	Km^+ (pHM2-*nuc*)	This work
pDrive	Amp^+	Quiagen
pBBR1MCS-5	$Gent^+$	CBS-KNAW Fungal Biodiversity Centre of Royal Netherland Academy of Arts and Sciences)
pDrive-*lipH*	Amp^+ *lipH* gene	(Romanelli et al., 2014)
pDrive-*lipC*	Amp^+ *lipC* gene	(Romanelli et al., 2014)
pBBR1MCS-5-*lipC*	$Gent^+$ *lipC* gene	(Romanelli et al., 2014)
pBBR1MCS-5-*lipH-lipC*	$Gent^+$ *lipC* and *lipH* gene	(Romanelli et al., 2014)
pGEM-T	Km^+, Amp^+	Promega
pHM2	Km^+	Addgene
p*nuc*	*nuc* from *S. aureus*	Technische Universität München
pGEM-T-*nuc*	Km^+, Amp^+	This work
pHM2-*nuc*	Km^+	This work

Table 10. Strains and plasmids used in this study.

2.2. SCREENING FOR PHAs PRODUCTION AND RECOVERY FROM C. NECATOR DSM 545 AND D. ACIDOVORANS DSM 39 STRAINS.

2.2.1. Evaluation of substrates and growth media.

To select the optimal medium for growth and PHAs production of *C. necator* DSM 545 and *D. acidovorans* DSM 39, several broths and different carbon sources were tested (Table 11). Seed cultures of *C. necator* DSM 545 and *D. acidovorans* DSM 39 were obtained overnight in 5 mL of NB at 30°C under shaking at 150 rpm and were used to inoculate 100 mL of each medium previously sterilized, to an initial $OD_{600\ nm}$: 0.3 in 300 mL flasks and incubated in the same conditions. The optical density $OD_{600\ nm}$ (using a Pharmacia Biotech Ultrospec 2000 UV/VIS Spectrophotometers) and colony forming units (CFU/mL) (obtained plating 100 µL of serially diluted samples on Nutrient Agar) were measured twice a day. All experiments were performed in triplicate and standard deviation reported.

Medium	Substrate	Strain
NB	-	*C. necator* DSM 545
DSMZ 81	Glucose (30 g/L)	
DSMZ 81	Glycerol (30 g/L)	
NB	-	*D. acidovorans* DSM 39
NB with glucose	Glucose (25 g/L)	
NB with glucose	Glucose (50 g/L)	
Nitrogen rich medium	-	
Nitrogen reduced medium with 1 g/L $(NH_4)_2SO_4$	Glucose (30 g/L)	
Nitrogen reduced medium with 1 g/L $(NH_4)_2SO_4$	Glycerol (30 g/L)	

Table 11. Media and substrates used for optimizing grow and PHAs production from the strain (- medium without addition of other carbon source).

2.2.2. Optimization of carbon/nitrogen ratio in the biomass production.

Once the media and substrate were optimized for the growth of *C. necator* DSM 545, further studies were conducted on different carbon/nitrogen ratio, using NH_4Cl as nitrogen source.

Pre-inoculum of *C. necator* DSM 545 was obtained as indicated in the paragraph 2.2.1., but using DSMZ 81 medium and glucose 30 g/L as carbon sources. Seed cultures of *C. necator* DSM 545 were inoculate in 100 mL of DSMZ 81 medium ($OD_{600\ nm}$= 0.3) with increasing concentrations

of NH_4Cl (0.5, 1, 2.5 and 5 g/L) and glucose (10, 20 and 30 g/L) in 300 mL flasks and incubated 72 h at 30°C. The optical density $OD_{600\ nm}$ (measured using a Pharmacia Biotech Ultrospec 2000 UV/VIS Spectrophotometers) and colony forming units (CFU/mL) (obtained plating 100 µL of serially diluted samples on Nutrient Agar) were measured twice a day. All experiments were performed in triplicate and standard deviation reported.

2.2.3. PHAs synthesis by *C. necator* DSM 545, *D. acidovorans* DSM 39 and recombinant strains.

To evaluate the PHAs production, two different procedures were adopted: the one-step process was used when fats were used as carbon source for biomass production and PHAs accumulation, while the two-steps procedures were carried out when fats or glucose were used as carbon source for biomass production and glucose as carbon source for PHAs accumulation.

In short, in the one-step process, strains were aerobically grown for 96 h at 30 or 37°C under shaking at 150 rpm in 1 L flasks containing 300 mL of DSMZ 81 medium for *C. necator* DSM 545 and recombinants or Nitrogen reduced medium with 1 g/L $(NH_4)_2SO_4$ for *D. acidovorans* DSM 39 and recombinants; both media contained 30 g/L of carbon substrate. After incubation, cells were recovered and PHAs extracted as described in paragraphs 2.2.4. and 2.2.5.

When the two-steps procedure was adopted, after biomass production phase (according to the step-one described above), cells were aseptically recovered by centrifugation at 4°C (4000 x g for 10 min), washed and re-suspended in the same media used above, containing 30 g/L glucose (for *C. necator* DSM 545) or glycerol (for *D. acidovorans* DSM 39) but with one-third of initial nitrogen content. Then flasks were incubated at 30 or 37°C (150 rpm) and after 72 or 96 h cells were recovered and PHAs extracted as described in paragraphs 2.2.4. and 2.2.5. All experiments were performed in triplicate and standard deviation reported.

2.2.4. Biomass measurements.

After PHAs accumulation process, the cells were harvested by centrifugation at 4°C (4000 x g for 10 min), washed twice with sterile water, frozen at -80°C for 24 h and then lyophilized (Freeze Dryer Modulyo, Edwards) until complete water removal. After freeze-drying, samples were weighed to assess cell dry biomass (CDM).

When incubation was performed in media containing fatty wastes as a carbon source, the pellets were washed with 5 mL a cold mixture of water-n-hexane (5:2) to remove residual fats before freezing (Riedel et al., 2015). The possible loss of PHAs and biomass due to washing with water-n-hexane was also evaluated as described below. All experiments were performed in triplicate and standard deviation reported.

2.2.4.1 Evaluation of biomass and PHAs loss due to washing with water-n-hexane.

To evaluate the possible loss of biomass and PHAs content due to washing with organic solvents, after PHAs accumulation process using fatty wastes as carbon source, cells were recovered by centrifugation at 4°C (4000 x g for 10 min) and the pellets washed with 5 mL a cold mixture of water-n-hexane (5:2) or only with water to remove residual fats (Riedel et al., 2015); cells were them frozen at -80°C for 24 h and finally lyophilized (Freeze Dryer Modulyo, Edwards) until total water removal. After freeze-drying, samples were weighed and PHAs content was assessed. All experiments were performed in triplicate and standard deviation reported.

2.2.5. PHAs analyses.

PHAs concentration was determined in cells according to (Braunegg et al., 1978; Torri et al., 2014). In short, samples of freeze-dried cells (10 mg) were hydrolyzed in a mixture of dichloroethane-propanol-HCl (5:4:1) for 4 h at 100°C. The propyl esters of hydroxylalkanoic acids were analyzed by gas chromatography using a Thermo Finnigan Trace GC, equipped with FID detector and AT-WAX column (30m x 0.25mm x 0.25 µm). The gas carrier was helium at flow rate 1.2 mL/min and the split/splitless injector with a split ratio 1:30 was set at 250°C, the FID temperature was 270°C and the oven was set at 150°C. Benzoic acid served as internal standard; the external standards, 3-hydroxybutyric acid (3HB), Poly(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid P(3HB-*co*-12 mol% 3HV) and Poly(3-hydroxybutyric acid-*co*-4-hydroxybytyric acid) P(3HB-*co*-11.2 mol% 4HB) were purchased from Sigma-Aldrich (Italy). Results were expressed as the percentage of PHAs on cell dry biomass (CDM) or as g of PHAs for a liter of culture. All experiments were performed in triplicate and standard deviation reported.

2.2.6. Evaluation of antibiotic resistance of *C. necator* DSM 545 and *D. acidovorans* DSM 39.

Pre-inocula of *C. necator* DSM 545 and *D. acidovorans* DSM 39, were cultivated overnight at 30°C in NB medium as indicated in the paragraph 2.2.1. 100 µL of culture of each strain were plated in Petrie dishes containing Nutrient Agar supplemented with increasing amounts of gentamicin or kanamycin (from 5 up to 500 µg/mL). The plates were incubated for 72 h at 30°C and inhibition of growth recorded when present.

2.3. STRAINS CONSTRUCTION FOR THE IMPROVED PRODUCTION OF PHAs FROM SLAUGHTERHOUSE FATTY WASTES.

In order to reduce production costs, bovine and swine slaughterhouse fatty wastes were evaluated as substrates for the microbial growth and the production of PHAs.

2.3.1. Fatty carbon sources used in this work.

The fatty carbon sources used in this work were obtained from local slaughterhouses and are reported in Table 12 together with their rough analyses carried out in the Chemistry Laboratory (LaChi) of DAFNAE; Padua University, following official analysis described in (Nollet, 2004).

Origin	Source	Humidity	Ashes	Protein	Lipid	pH
Bovine	Udder	69.7	0.9	14.8	14.9	6.3
	Kidney fat	10.6	0.1	3.0	86.5	6.4
	Visceral fat	30.9	0.2	5.5	62.3	6.7
Swine	Lard	4.1	0.04	1.5	93.1	6.4
	Jowl fat	20.9	0.3	7.7	69.6	6.0
	Membrane caul fat	10.7	0.2	1.5	87.2	5.6

Table 12. Chemical composition (% of dry matter) of slaughterhouse by-products used in this study.

2.3.2. Fatty acid composition of slaughterhouses wastes.

The fatty acid analysis was performed in the Chemistry Laboratory (LaChi) of DAFNAE; Padua University, following official analysis Folch method (Folch et al., 1957).

The fatty acid composition of carbon sources used in this work is reported in Table 13.

Fatty acid	Udder	Kidney fat	Visceral fat	Lard	Jowl fat	Membrane caul fat
SFA	7.3	52.7	26.2	33.9	23.6	38.9
UFA	6.9	30	33.4	55.1	42.9	44.5
MUFA	6.4	26.4	30.9	34.1	34.2	32.2
PUFA	0.5	3.6	2.4	21	8.7	12.3
OMEGA3	0.05	0.2	0.1	1.5	0.6	0.9
OMEGA6	0.4	3.1	1.6	18.7	7.4	10.7
SFA/UFA	0.1	1.5	0.5	0.5	0.4	0.7
MUFA/PUFA	0.02	0.06	0.04	1.4	2.6	2.2
OMEGA3/OMEGA6	0.02	0.06	0.04	0.07	0.05	0.07

SFA: Saturated fatty acids; UFA: Unsaturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids.

Table 13. Fatty acid composition of slaughterhouse by-products (% of dry matter).

2.3.3. Transformation of *C. necator* DSM 545 strain with *lipH-lipC* genes.

Ad reported above, *C. necator* DSM 545 strain was selected as recipient for genetic modifications because of its high PHAs accumulation ability (up to 80% w/w of its CDM), moreover, the stored PHAs contains high molar fractions of 3HB (Abbondanzi et al., 2017; Marudkla et al., 2018).

C. necator DSM 545 was electroporated with pBBR1MCS-5-*lipH-lipC* (obtained from (Romanelli et al., 2014)) according to a procedure slightly modified from that described by Aneja and colleagues (Aneja et al., 2009). Seed cultures of *C. necator* DSM 545 were grown overnight in 5 mL of NB (30°C, 150 rpm shaking). Two mL of pre-inoculum were used to inoculate 125 mL Erlenmeyer flask containing 48 mL NB. The cultures were grown for 1 h at 30°C on a rotary shaker set at 150 rpm. Aliquots were centrifuged at 7000 x g at 4°C for 35 min. The harvested cells, washed in a pre-chilled and sterile sucrose solution (0.3 M), were re-suspended in the same solution to an optical density at 600 nm ($OD_{600\ nm}$) of 5. 100 µL of the concentrated cell suspensions were mixed with 2-5 µg of pBBR1MCS-5-*lipH-lipC* in an electroporation cuvette (0.1-cm gap width). After 5 min incubation in ice, electroporation was performed at 2.5 kV, 25 µF, and 200 Ω in a Gene Pulser II electroporator equipped with a Pulse Controller Plus module (Bio-Rad Gene Pulser). Immediately after electroporation, 0.9 mL of SOC medium were added to cell suspension in a 2 mL polypropylene Eppendorf. Cells were kept for 2 h at 30°C (150 rpm), centrifuged at 7000 x g for 10 min, and re-suspended in 300 µL of NB. Gentamicin-resistant clones were selected after spreading

150 µL of the cell suspension on Nutrient agar supplemented with gentamicin (500 µg/mL) and incubate them for 48 h at 30°C.

2.3.3.1. Detection of *lipH-lipC* genes in recombinant strains.

After *C. necator* DSM 545 transformation with pBBR1MCS-5-*lipH-lipC* plasmid, gentamicin-resistant strains were selected for the *lipH-lipC* genes presence by amplification using a thermocycler (Bio-Rad iCycler PCR Thermal Cycler). Master mix was prepared to amplify lipase genes with approximately 100 ng of DNA, 25 pmol of each primer (see Table 14), 0.25 mM dNTPs, and 19 µL buffer amended with 1.25 mM $MgCl_2$ and 0.5 U of Taq DNA-polymerase (Euroclone S.p.A., Milano, Italy).

The reaction conditions to amplify *lipH* and *lipG* genes were: 1 cycle (95°C for 4 min), 40 cycles (95°C for 1 min, 55.3°C for 1 min, 72°C for 1 min) and a final cycle (72°C for 5 min).

Amplified DNA from *lipH-lipC* genes was separated by electrophoresis on agarose gel. A 100 mL gel 10 g/L agarose (Sigma-Aldrich) was prepared with 1× TAE buffer, as well as the running buffer. After running, bands were visualized using acid stain EuroSafe (EuroClone S.p.A., Milano, Italy). Digital images were acquired with a GENi Gel Documentation System (Syngene).

Liquid enzymatic assays were also performed by following the amount of *p*NP released from *p*NPP as described in the section 2.3.3.2.3.

Plasmid	Sequences	Reference
LIPC-F	ATGAACAAGAACAAAACCTTGCT	(Romanelli et al., 2014)
LIPC-R	GTCAGAGCCCCGCGTTCTTCAATC	
LIPH-F	ATGAGCAGATCCATCCTTTT	
LIPH-R	TCAGCGAGTCCGATCCTCC	

Table 14. Sequences of primer used for PCR amplification.

2.3.3.2. Lipolytic activity evaluation.

With the aim to find the best conditions to evaluate the lipolytic activity of recombinant *C. necator* DSM 545 pBBR1MCS-5-*lipH-lipC*, different growth media and fatty substrates were firstly screened for their ability to stimulate the production of lipolytic enzyme in the wild types strains *C. necator* DSM 545 and *P. stutzeri* BT3.

With this aim, pre-inocula of the *C. necator* DSM 545 and *P. stutzeri* BT3 strains were prepared as described in paragraph 2.2.1. and used to inoculate 100 mL of NB medium or DSMZ 81 medium supplemented with 30 g/L olive or corn oil as carbon source (initial $OD_{600\ nm}$: 0.3) in 300 mL flasks. Flasks were incubated at 30 and 37°C under shaking at 150 rpm for 96 h. At 24, 48, 72 and 96 h samples were collected and centrifuged (7000 x g per 10 min); the supernatants were used to measure extracellular lipase activity.

Once identified the fatty substrate with the best ability to stimulate the production of lipase of wild type strains, the identification of lipolytic recombinant strains was conducted The samples were prepared as described above and tested with and without gentamicin at 500 µg/mL in the selected growth medium.

As positive control, a lipase from *Candida rugosa* (Sigma-Aldrich L8525; ≥40.000 units/mg) was used at a dilution of 1:100000. All experiments were performed in triplicate and standard deviation reported.

2.3.3.2.1. Lipolytic activity evaluation by plates assays.

Lipase activity was evaluated using plates assays following the method of (Lomolino et al., 2012) with slight modifications. Plates were filled with 20 g/L agarose gel (Sigma-Aldrich). Agarose gel was prepared with Tris-buffered saline at pH 7 by dissolving in microwave oven. The solution was cooled to 70°C and different lipase substrates (Table 15) at 50 mM and 0.2% v/v Triton X-100 (BDH Chemicals, Poole, UK) were added. The gel solution was transferred into the plates and cooled at room temperature. The thickness of the resulting gel was approx. 3 mm. Wells were obtained using a 200 µL pipette tip and were loaded with 10 µL supernatant of the bacterial cultures. Plates were then incubated at 30 and 37°C; the fluorescence of positive samples was observed using a GENi Gel Documentation System (Syngene) after 10 min.

Rhodamine B plates were also tested, using the method described by (Clausen and Dabelsteen, 1969). All experiments were performed in triplicate and standard deviation reported.

Lipase substrates (Sigma-Aldrich)	Reference
Rhodamine b (Sigma-Aldrich 83689)	(Clausen and Dabelsteen, 1969)
3(2-benzoxazolyl) umbelliferyl acetate (Sigma-Aldrich 12832)	(Ting et al., 2016)
Ethyl 7-acetoxycoumarin-3-carboxylate (Sigma-Aldrich 00838)	(Velasco-Lozano et al., 2012)
Fluorescein butyrate (Sigma-Aldrich 46942)	(Lomolino et al., 2012)
Fluorescein sodium (Sigma-Aldrich F6377)	(Lomolino et al., 2012)
Resorufin acetate (Sigma-Aldrich 83636)	(Rodrigues et al., 2012)
Resorufin butyrate (Sigma-Aldrich 83637)	(Glogauer et al., 2011)

Table 15. Substrates of lipases used in the experiments.

2.3.3.2.2. Lipolytic activity measurements by titrimetric method.

Lipase activity was also measured in liquid medium by a titrimetric assay (Pinsirodom and Parkin, 2001); in short 5 mL of the supernatant of the bacterial cultures were added to 50 mL of 50 g/L corn oil emulsion in 50 mM Tris-HCl buffer (pH 8), containing 50 g/L 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 10 mL ethanol added. With the aim to optimize the assay conditions, the lipase activity was measured at both 30 and 37°C and at pH 6.5, 7.5 and 9.5. 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 quantity 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 the assay conditions. All experiments were performed in triplicate and standard deviation reported.

2.3.3.2.3. Lipolytic activity measurements by spectrophotometric assay.

Enzymatic activity was also determined as described by (Glogauer et al., 2011). Briefly, every 24 h, the amount of *p*-nitrophenol (*p*NP) released from *p*-nitrophenyl palmitate (*p*NPP) was monitored at 410 nm for at least 30 min at 37°C using the TECAN Spark 20M microplate spectrophotometer (TECAN, Salzburg, Austria). The substrate solution was made by mixing a stock solution of 20 mM of *p*NPP in acetonitrile/isopropanol (1/4 v/v) with an assay buffer containing Tris-HCl pH 7.5, $CaCl_2$ and Triton X-100, under agitation in a water bath at 60°C until the solution became transparent. 180 μL of the substrate solution were pipetted into a 96-well microtiter plate and the reaction was initiated by addition of 20 μL of the supernatant from samples. The final

volume of the reaction mixture was 200 µL (Tris-HCl 50 mM, pH 7.5; 1 mM $CaCl_2$, 0.3% v/v Triton X-100, 1 mM *p*NPP, 4% v/v isopropanol, 1% v/v acetonitrile). A commercial lipase from *Candida rugosa* (Sigma-Aldrich L8525; ≥40.000 units/mg) was used as positive control at a dilution of 1:100000 in the mix.

Lipolytic activity was determined under each reaction condition and non-enzymatic hydrolysis of substrates was also taken into consideration. One unit of lipase activity was defined as 1 µmol of *p*NP produced per minute (mU/ (min·mL)). All experiments were performed in triplicate and standard deviation reported.

2.3.4. Slaughterhouses fatty waste pre-treatment.

Slaughterhouse fatty wastes were finally crushed using a meat grinder and stored separately in 0.5 Kg bags under vacuum at -20°C.

For the fermentation process, the fatty samples were processed as reported in (Riedel et al., 2015): each fatty waste was melted in a water bath at 60°C and filtered through a standard cellulose coffee filter to separate solid impurities (e.g., fibers, tissues and others); the blood was also removed. For each 5 kg samples, 3.5 kg of fat were recovered.

2.3.5. Optimization of growth conditions using slaughterhouse wastes as carbon source.

To optimize growth conditions using slaughterhouse fatty wastes as carbon source, the following parameters have been considered: incubation temperature, the use of Tween-80 as an emulsifier to improve the dispersion of fats, NH_4Cl concentration (needed to support both microbial growth and PHAs production). Moreover, the most appropriate sterilization procedure of media was defined.

2.3.5.1. Temperature selection to PHAs production using slaughterhouse fatty wastes as carbon source.

DSMZ 81 medium, formulated as indicated in the Table 9, was supplemented with each of the six fatty carbon sources reported in Table 12 at 30 g/L and incubated for 6 h at 30 and 37°C (150 rpm) to evaluate the melting levels of the added fatty wastes.

2.3.5.2. Evaluation of Tween-80 as fats emulsifier.

DSMZ 81 growth medium was supplemented with 30 g/L of each of the six slaughterhouse fatty wastes and increasing concentrations of Tween-80 (0, 2.5, 5, 7.5 and 10 g/L). Each flask was incubated for 6 h at 37°C (150 rpm) and the fat emulsification in the culture medium was evaluated observing the homogeneity state.

2.3.5.3. Sterilization method.

After the above described experiments, five, among the six slaughterhouse fatty wastes, were selected on the basis of their melting at the temperature of incubation (30°C) and the low Tween-80 requirement for emulsification.

The fatty wastes, DSMZ 81 medium and Tween-80 were mixed before autoclaving or sterilized separately and mixed at the required concentrations after autoclaving. Media supplemented only with Tween-80 or only with fatty wastes were also evaluated. All media were inoculated at $OD_{600\,nm}$ of 0.3 with pre-inoculum of *C. necator* DSM 545 and incubated at 37°C for 96 h (150 rpm). Non inoculated samples were also prepared as negative control.

Cells growth was monitored twice a day by determining CFU/mL on Nutrient Agar. At the end of incubation time, cells were recovered and final PHAs concentrations were also determined as described in paragraphs 2.2.4. and 2.2.5. All experiments were performed in triplicate and standard deviation reported.

2.3.5.4. Nitrogen source (NH_4Cl) optimization for biomass and PHAs production from slaughterhouse fatty wastes.

Membrane caul fat was selected as carbon source to perform additional studies focused on the optimization of nitrogen concentration during growth and PHAs production.

Seed culture of *C. necator* DSM 545 was obtained overnight in 5 mL of DSMZ 81 medium supplemented with 30 g/L corn oil at 30°C under shaking at 150 rpm and used to inoculated 100 mL of DSMZ 81 medium in 300 mL flask, formulated as indicated in the Table 9 but with different NH_4Cl concentrations (0.5, 1, 2.5, g/L) and supplemented with 30 g/L of membrane caul fat; flask were incubated at 37°C for 96 h.

Cells growth was monitored twice a day by determining CFU/mL on Nutrient Agar. Lipolytic enzymatic activity was evaluated every 24 h while PHAs concentrations and fat content of the spent fermentation broth were determined after 96 h. All experiments were performed in triplicate and standard deviation reported.

2.3.6. PHAs production.

Two methods for PHAs production from slaughterhouses fatty wastes were used, the one-step and the two-steps as described below.

2.3.6.1. One-step PHAs production by *C. necator* DSM 545 and *C. necator* DSM 545 pBBR1MCS-5-*lipH-lipC* using membrane caul fat as carbon source.

According to the results obtained in the previous experiments, the best growth conditions were found to be: DSMZ 81 medium amended before sterilization with 30 g/L membrane caul fat, 1 g/L NH_4Cl and without Tween-80.

Pre-inocula of *C. necator* DSM 545 and *C. necator* DSM 545 pBBR1MCS-5-*lipH-lipC* obtained overnight in DSMZ 81 medium with 30 g/L corn oil as carbon source were used to inoculate 300 mL flasks containing 100 mL of medium at 0.3 initial $OD_{600\ nm}$. After 96 h of incubation at 37°C under shaking (150 rpm), the cells were harvested by centrifugation at 4°C (4000 x g for 10 min), washed with 5 mL water-n-hexane cold mixture (5:2) to remove residual fats (Riedel et al., 2015) and then frozen at -80°C. CDM and PHAs content were evaluated as described in paragraph 2.2.4. and 2.2.5.

Fats content of the spent fermentation broth was also determined after 96 h, using the official Folch method of analysis (Folch et al., 1957).

Recombinant strain was evaluated with or without gentamicin (500 μg/mL) addition. All experiments were performed in triplicate and standard deviation reported.

2.3.6.2. Two-steps PHAs production by *C. necator* DSM 545 pBBR1MCS-5-*lipH-lipC* using membrane caul fat and glucose as carbon sources.

In order to increase the PHAs accumulation in the recombinant strains, a two-steps PHAs production procedure was developed. Membrane caul fat was used as carbon source in step-one, as described above, to obtain biomass.

In step-two the biomass undergoes PHAs accumulation using glucose as carbon source.

Glucose and nitrogen (NH_4Cl) concentrations were firstly optimized to support the highest PHAs accumulation during the second step using the lower glucose amounts.

C. necator DSM 545 parental strain (with limited extracellular lipase activity) was not used in this experiment, because of the low biomass archived using membrane caul fat as carbon source in the first step.

The biomass of *C. necator* DSM 545 pBBR1MCS-5-*lipH-lipC* was obtained using the same conditions described in the paragraph 2.3.6.1. using membrane caul fat as carbon sources. Cells were recovered by centrifugation at 4°C (4000 x g for 10 min), washed with cold water and then re-suspended in 50 or 100 mL of the same media used above but with different glucose concentrations (7.5, 15 and 30 g/L) and different NH_4Cl concentrations (0, 0.075, 0.15 and 0.3 g/L). The incubation was performed at 37°C (150 rpm) for 72 h; every 24 h residual glucose concentrations were determined.

Once the accumulation phase was completed, cells were then harvested by centrifugation at 4°C (4000 x g for 10 min), washed with 5 mL water-n-hexane cold mixture (5:2) to remove fats residual from phase one (Riedel et al., 2015), frozen at -80°C per 24 h and lyophilized (Freeze Dryer Modulyo, Edwards) until total water removal.

CDM and final PHAs concentration were them evaluated as described in paragraph 2.2.4. and 2.2.5. 3HB yield was determined as gram of 3HB produced during the second step per gram of consumed glucose.

Gentamicin (500 μg/mL) was used to amend only the pre-inoculum medium of *C. necator* DSM 545 pBBR1MCS-5-*lipH-lipC*.

2.4. STRAINS CONSTRUCTION FOR THE NUCLEASE EXPRESSION.

Industrial manufacturing of PHAs requires its purification from high-cell-density cultures. Cells are broken by homogenization and PHAs granules are cleansed and treated to obtain PHAs latexes. However, cell lysis releases large amounts of DNA, which results in an increasing viscosity of the medium, hampering the following downstream steps. Drop in viscosity is generally achieved by "**costly procedures**", among them, the use of commercially available nucleases.

Searching for a cost-effective solution to this issue, a nuclease gene from *Staphylococcus aureus* has been integrated into *C. necator* DSM 545 and *D. acidovorans* DSM 39 strains. These bacteria are currently the most used for the bioplastics production, because have high yielding of PHAs production (80% w/w of its cell dry mass) with high molar fractions of 3HB and/or 4HB (Abbondanzi et al., 2017; Marudkla et al., 2018; Romanelli et al., 2014).

Strategies, activities and methods applied for transformation of *C. necator* DSM 545 and *D. acidovorans* DSM 39 with *nuc* gene from *S. aureus* are described below.

2.4.1. Integrative *nuc* plasmid construction.

The restriction enzyme digestion, electrophoresis, DNA ligation, *E. coli* DNA isolation and transformation were performed using standard methods according to Sambrook, 2001. The *nuc* gene, expressing the nuclease from *S. aureus*, was obtained as an amplified fragment of 700 bp from plasmid p*nuc* kindly provided by Prof. Schleifer (Technische Universität München, Germany). Primers *nucA* (5'-TTCTCTAGAATTCAGGAGGTTTTTATGGCTATCAGTAATGTTTCG-3') and *nucB* (5'-GCCGGTACCTTATTGACCTGAATCAGCGTTG-3') were used for PCR amplification using a thermocycler (Bio-Rad iCycler PCR Thermal Cycler). The thermal protocol was designed as follows: initial denaturation 94°C for 2 min, followed by 50 cycles of denaturation at 95°C (30 s), annealing at 55°C (45 s), and extension at 72°C (45 s). PCR products were purified and cloned into pGEM, thus yielding the vector pGEM-*nuc*. pGEM-*nuc* was then digested with *Nae*I and *Sac*I and *nuc* gene was introduced in the broad host range plasmid pHM2, thus resulting in the final vector named pHM2-*nuc*. Plasmid pHM2-*nuc* was transformed into *E. coli* DH10B.

2.4.2. Transformation of *C. necator* DSM 545 and *D. acidovorans* DSM 39.

C. necator DSM 545 and *D. acidovorans* DSM 39 were electroporated with pHM2-*nuc* according to a procedure slightly different from that described by Aneja and colleagues (Aneja et al., 2009). Seed cultures of *C. necator* DSM 545 and *D. acidovorans* DSM 39 were grown overnight in 5 mL of NB (30°C, 200 rpm shaking). Two mL of pre-inoculum were used to inoculate 125 mL Erlenmeyer flask containing 48 mL NB. The cultures were grown for 1 h at 30°C on a rotary shaker set at 250 rpm. Aliquots were centrifuged at 7000 x g at 4°C for 35 min. The harvested cells, washed in a pre-chilled and sterile sucrose solution (0.3 M), were re-suspended in the same solution to an optical density at 600 nm ($OD_{600\ nm}$) of 5. 100 µL of the concentrated cell suspensions were mixed with 2-5 µg of pHM2-*nuc* in an electroporation cuvette (0.1-cm gap width). After 5 min incubation in ice, electroporation was performed at 2.5 kV, 25 µF, and 200 Ω in a Gene Pulser II electroporator equipped with a Pulse Controller Plus module (Bio-Rad Gene Pulser). Immediately after electroporation, 0.9 mL of SOC (Super Optimal broth with Catabolite repression) medium was added to cell suspension in a 2 mL polypropylene Eppendorf. Cells were kept for 2 h at 30°C (250 rpm), centrifuged at 7000 x g for 10 min, and re-suspended in 300 µL of NB. Kanamycin-resistant integrants were selected after spreading 150 µL of the cell suspension on NB agar supplemented with kanamycin (100 µg/mL), and incubation overnight at 30°C. The successful transformation of the wild type strains was confirmed by PCR using primers *nucA* and *nucB* as described in section 2.4.3.

2.4.3. Detection of *nuc* gene in recombinant strains.

After *C. necator* DSM 545 and *D. acidovorans* DSM 39 transformation with pHM2-*nuc* plasmid, kanamycin-resistant strains were selected for the *nuc* genes presence by amplification using a thermocycler (Bio-Rad iCycler PCR Thermal Cycler).

Master mix was prepared to amplify nuclease genes with approximately 50 ng of DNA, 1 µL of each *nucA* and *nucB* primer (10 mM) (see paragraph 2.4.1.), 0.5 µL dNTPs (10 mM), 5 µL (5x) buffer and 0.12 µL Taq DNA-polymerase (5 U/µL) (Euroclone S.p.A., Milano, Italy).

The reaction conditions to amplify *nuc* gene were: initial denaturation 94°C for 2 min, followed by 50 cycles of denaturation at 94°C (30 s), annealing at 55°C (45 s), and extension at 72°C (45 s min).

Amplified DNA (*nuc* gene) fragments were separated by electrophoresis on agarose gel. 100 mL gel 1.2% w/v agarose (Sigma-Aldrich) was prepared with 1× TAE buffer, as well as the running buffer. After running, bands were visualized using acid stain EuroSafe (EuroClone S.p.A., Milano, Italy). Digital images were acquired with a GENi Gel Documentation System (Syngene).

2.4.4. Characterization of nuclease activity in the engineered strains.

Nuclease activity was usually examined by a plate assay, observing the appearance of clearing zones around colonies grown on DNAse agar (Favaro et al., 2014). The engineered strains with the largest DNA hydrolysis halos were selected for further studies. To appropriately estimate nuclease activity, agarose gel electrophoresis with high-molecular-weight DNA (phage λ DNA, Sigma-Aldrich) was also used (Boynton et al., 1999).

Engineered and parental strains were grown for 72 h at 30°C in NB medium (200 rpm). Then, 500 µL of cultures were centrifuged. In parallel, samples of broth were treated with 100 µL of chloroform to release periplasmic nuclease and then centrifuged (7000 x g for 10 min). After centrifugation, supernatants (17 µL) were mixed with λ DNA (1 µL) and 2 µL $CaCl_2$ 1 mM. The blends were incubated at 37°C for 30, 60 and 120 min. DNA was run in an agarose gel electrophoresis (120 V for 90 min) and nuclease activity was evaluated by determining the reduction in the molecular weight of phage λ DNA.

Liquid enzymatic assays were also performed; in this case nuclease activity was based on the increase in absorbance at 260 nm which usually accompanies hydrolysis of nucleic acids (Cuatrecasas et al., 1967). *C. necator* DSM 545, *D. acidovorans* DSM 39 and recombinant strains, grown overnight at 30°C in 5 mL of NB, were used to inoculate 50 mL NB to an initial optical density at 600 nm ($OD_{600\ nm}$) of 0.2 and incubated at 30°C on a rotary shaker. After 72 h, 2 mL of cultures were periodically sampled and centrifuged (7000 x g per 10 min). The supernatants were used to assess nuclease activity. The assay mixture, consisting of a total volume of 300 µL of 0.025 M Tris-HCl buffer (pH 8.8) with 15 µL of λ DNA, 15 µL of 0.2 M CaCl and 30 µL of supernatant, was placed at 30°C in a polymethylmethacrylate cuvette; the increase in absorbance at 260 nm was measured by a Spectrophotometer (Pharmacia Biotech Ultrospec 2000) until constant readings was achieved. Commercial nuclease from *Serratia marcescens* (Benzonase® Nuclease ≥250 units/µL, Sigma-Aldrich) at a final concentration of 0.025 units/µL was used as positive control for all experiment. The experiment was carried out in triplicate.

2.4.5. PHAs synthesis by *C. necator* DSM 545, *C. necator* DSM 545 pHM2-*nuc*, *D. acidovorans* DSM 39 and *D. acidovorans* DSM 39 pHM2-*nuc*.

For PHAs production, a two-steps cultivation procedure was carried out. In the first step, targeted to produce biomass, *C. necator* DSM 545 and its *nuc*-recombinant were aerobically grown at 30°C under shaking at 150 rpm in 1L flasks containing 300 mL of DSMZ 81 medium with 30 g/L glucose. *D. acidovorans* DSM 39 and its *nuc*-recombinant were grown in Nitrogen reduced medium containing 1 g/L $(NH_4)_2SO_4$ and 30 g/L glycerol. After 72 h, cells were aseptically recovered by centrifugation at 4°C (4000 x g for 10 min) and washed with sterile water. In the second step, to promote polyester accumulation, cells were moved to the same media used for the biomass production phase, but supplemented with only one third of the nitrogen content. The incubation was performed at 30°C (200 rpm) for 72 h. Cells were then harvested, centrifuged (4000 x g for 10 min), washed twice with sterile water and frozen at -80°C, before being lyophilized (Freeze Dryer Modulyo , Edwards). After freeze-drying, samples were weighed to assess cell dry biomass and PHAs analyses as indicated the paragraphs 2.2.4. and 2.2.5. All experiments were performed in triplicate and standard deviation reported.

2.4.6. Reduction of cell lysates viscosity by recombinant *C. necator* DSM 545 pHM2-*nuc*.

C. necator DSM 545 and *C. necator* DSM 545 pHM2-*nuc* strains were grown for PHAs accumulation as described in the section 2.4.5. Once the accumulation phase was completed, 1 mM $CaCl_2$ was added and the cells disrupted using the Constant Systems Cell Disrupter One Shot at five different pressures (0.25, 0.5, 0.75, 1.5 and 2.25 kbar). To estimate the efficiency of cell lysis, the protein content was analyzed in the supernatants of intact or disrupted cells by the Bradford protein assay method (Boynton et al., 1999) and the cells morphology was observed at 100X objective with an optical microscope (Leica DM2000 Led, equipped with Leica DFC450C). The disrupted cultures were then incubated at 37°C for 1 h, and the viscosity of the lysates measured at room temperature using a Rotational Rheometer (Malvern Kinexus Pro and geometry CP2/60: PL 61 ST). Positive controls were obtained adding aliquots of Benzonase® to the lysates. All experiments were performed in triplicate and standard deviation reported.

3. RESULTS AND DISCUSSION.

3.1. Evaluation of substrates and growth media.

With the aim of optimizing growth and PHAs production by *C. necator* DSM 545 and *D. acidovorans* DSM 39, different microbial growth media reported in materials and methods (paragraph 2.2.1.) were tested.

C. necator DSM 545 showed good growth with all media tested (Table 16), obtaining the lowest growth with DSMZ 81 medium supplemented with 30 g/L of glycerol as carbon sources (absorbance: 12.5 and CFU/mL: $5.3x10^8$). However, growing *C. necator* DSM 545 for 72 h at 30°C in DSMZ 81 medium supplemented with glucose at 30 g/L as carbon source supported the highest growth, with a value of 19.3 $OD_{600\ nm}$ and $4.1x10^9$ CFU/mL.

On the other hand, *D. acidovorans* DSM 39 exhibited only a slight growth in broths such as NB, NB supplemented with glucose (25 and 50 g/L) and Nitrogen reduced medium supplemented with 1 g/L $(NH_4)_2SO_4$ and glucose (30 g/L) (Table 16) obtaining absorbance values between 0.2 and 2.6 ($OD_{600\ nm}$) and CFU/mL of $5.6x10^7$-$6.1x10^7$. Nitrogen rich medium did support better growth ($OD_{600\ nm}$ = 3.8 and $7.1x10^7$ CFU/mL). This could related to the high concentration of yeast extract and peptone available in the broth (Table 9). Nevertheless, once grown for 96 h at 30°C in Nitrogen reduced medium supplemented with 1 g/L $(NH_4)_2SO_4$ and 30 g/L glycerol as carbon sources, achieved the absorbance ($OD_{600\ nm}$) and CFU/mL with highest values (4.4 and $2.3x10^8$, respectively). As such, glycerol and not glucose, seems to be the most suitable carbon source for *D. acidovorans* DSM 39, in agreement with recently reported papers (Cavalheiro et al., 2009; Posada et al., 2011; Romanelli et al., 2014).

	Medium	*C. necator* DSM 545	*D. acidovorans* DSM 39
Absorbance ($OD_{600\ nm}$)	NB	18.6± 0.3	1.0± 0.1
	NB with glucose (25 g/L)	nt	2.6± 0.5
	NB with glucose (50 g/L)	nt	2.3± 0.2
	Nitrogen rich medium	nt	3.8± 0.1
	Nitrogen reduced medium with 1 g/L $(NH_4)_2SO_4$ and 30 g/L glucose	nt	0.2± 0.1
	Nitrogen reduced medium with 1 g/L $(NH_4)_2SO_4$ and 30 g/L glycerol	nt	4.4± 0.2
	DSMZ 81 with glucose (30 g/L)	19.3± 0.2	nt
	DSMZ 81 with glycerol (30 g/L)	12.5± 1.0	nt
CFU/mL	NB	$8.2x10^8$± $8.3x10^7$	$4.6x10^7$± $2.1x10^6$
	NB with glucose (25 g/L)	nt	$6.1x10^7$± $1.0x10^6$
	NB with glucose (50 g/L)	nt	$5.2x10^7$± $5.3x10^6$
	Nitrogen rich medium	nt	$7.1x10^7$± $1.6x10^6$
	Nitrogen reduced medium with 1 g/L $(NH_4)_2SO_4$ and 30 g/L glucose	nt	$5.6x10^7$± $3.0x10^6$
	Nitrogen reduced medium with 1 g/L $(NH_4)_2SO_4$ and 30 g/L glycerol	nt	$2.3x10^8$± $3.5x10^7$
	DSMZ 81 with glucose (30 g/L)	$4.1x10^9$± $3.6x10^8$	nt
	DSMZ 81 with glycerol (30 g/L)	$5.3x10^8$± $6.x10^6$	nt

Table 16. Growth values of *C. necator* DSM 545 and *D. acidovorans* DSM 39 strains once grown for 72 h in different media (nt: no tested). Values represent the mean of three replicates and standard deviation is reported.

Growth kinetics of *C. necator* DSM 545 and *D. acidovorans* DSM 39 grown in DSMZ 81 and Nitrogen reduced medium with 1 g/L $(NH_4)_2SO_4$ and 30 g/L glucose or glycerol, respectively, are reported in Fig. 17. *C. necator* DSM 545 completed the growth on glucose within 72 h (Fig. 17a) whereas *D. acidovorans* DSM 39 exhibited slower growth rates (Fig. 17b), reaching the stationary phase later.

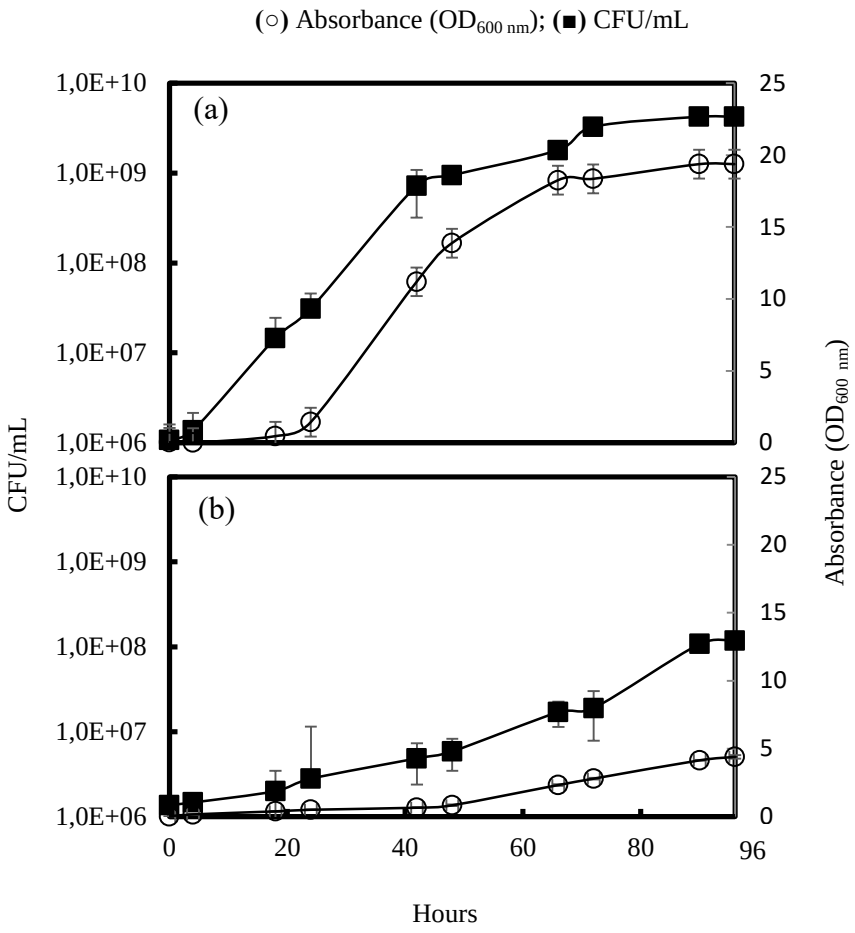


Fig. 17. Growth curves of (a): *C. necator* DSM 545 and (b) *D. acidovorans* DSM 39, incubated for 96 h at 30°C in DSMZ 81 and Nitrogen reduced medium with 1 g/L $(NH_4)_2SO_4$ and 30 g/L glucose or glycerol, respectively. Absorbance ($OD_{600\ nm}$) and CFU/mL values are indicated on the main and secondary y-axis, respectively. The values represent the mean of three replicates and standard deviation is reported.

According to the data reported, both in Table 16 and in Fig. 17, DSMZ 81 medium supplemented with 30 g/L glucose (*C. necator* DSM 545) and Nitrogen reduced medium with 1 g/L $(NH_4)_2SO_4$ and 30 g/L glycerol (*D. acidovorans* DSM 39) were selected as optimal media for further research activities.

3.1.1. Optimization of carbon/nitrogen ratio in the biomass production.

C. necator DSM 545 was selected for further studies tailored to the optimization of the carbon/nitrogen ratio, using different glucose concentrations (10, 20 and 30 g/L) and NH_4Cl concentrations (0.5, 1, 2.5 and 5 g/L), as described in the paragraph 2.2.2.

Fig. 18. shows the growth kinetics monitored for all the tested conditions. When 1 g/L of NH_4Cl with different glucose concentrations (10, 20 and 30 g/L) was used (Fig. 18a-c, respectively), the values of CFU/mL oscillated between $3.5x10^9$and $7.2x10^9$, with the highest values achieved in the presence of NH_4Cl 1 g/L and glucose 30 g/L (Fig. 18c). In the presence of glucose concentrations 20 and 30 g/L (Fig. 18b, c), cell growth monitored using CFU/mL seems to be completed within 48 h meanwhile the absorbance values continue to increase up to 72 h. This finding could be related to the on-going PHAs accumulation consequent to both the lack of nitrogen and the availability of the glucose as confirmed by HPLC analysis along with cell growth (data not shown).

In the presence of NH_4Cl 2.5 g/L (Fig. 18d-f), CFU/mL values ranged between $3.5x10^9$ to $5.2x10^9$ for all tested glucose combinations, achieving the highest value in the broth supplemented with 2.5 g/L of NH_4Cl and 30 g/L of glucose (Fig. 18f).

When 5 g/L of NH_4Cl (Fig. 18g-i) was supplemented together with 10, 20 and 30 g/L of glucose, *C. necator* DSM 545 did not exhibit significant differences in terms of both CFU/mL and $OD_{600\ nm}$ data. The maximum growth was achieved within 40 h. Then, cells viability quickly dropped. This could be due to a metabolic stress related to the high concentrations of NH_4Cl (Ayub et al., 2004; Obruca et al., 2017). However, further studies and analyses are required to verify such hypothesis.

Optical density ($OD_{600\ nm}$) in the last two cases (when 2.5 and 5 g/L of NH_4Cl were used) were directly proportional to CFU/mL trend, i.e. in the exponential phase the absorbance ($OD_{600\ nm}$) increases with cell growth, but once exponential phase is completed, the absorbance becomes consistent along with the stationary phase. Considering these results, it could be said that cells do not accumulate PHAs once the exponential phase is completed consequent to the presence of high nitrogen concentration in the medium.

(□) Absorbance $OD_{600\ nm}$; (◊) CFU/mL

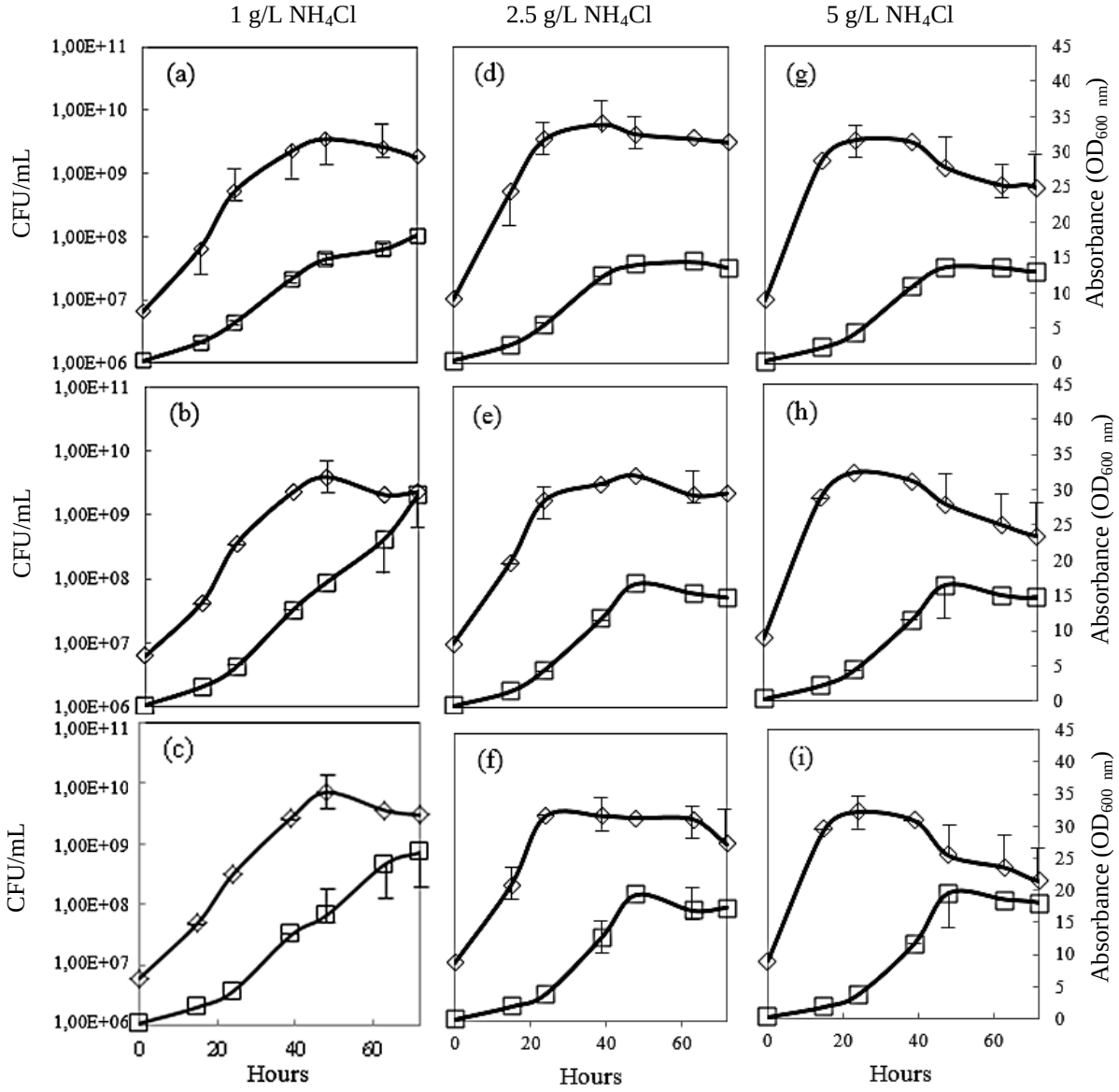


Fig. 18. *C. necator* DSM 545 growth, incubated at 30°C for 72 h at different concentrations of carbon and nitrogen (NH_4Cl) sources in DSMZ 81 medium containing: (a), (d) and (g): 10 g/L glucose; (b), (e) and (h): 20 g/L glucose; (c), (f) and (i): 30 g/L glucose. Values represent the mean of three replicates and standard deviation is reported.

As described in Fig. 18, the concentrations of nitrogen and glucose appearing the most suitable to support the bacteria growth was 1 g/L of NH_4Cl and 30 g/L of glucose (Fig. 18c), where the largest amount of CFU/mL ($7.2x10^9$) was achieved in a short time (48 h approximately) and NH_4Cl level used is the lowest, thus helping to reduce the costs in a future production process.

3.2. PHAs synthesis by *C. necator* DSM 545 and *D. acidovorans* DSM 39.

As initial experiments for PHAs production, the wild type strains *C. necator* DSM 545 and *D. acidovorans* DSM 39 were processed by using a two-steps cultivation procedure in their respective optimal cultural media and carbon sources. In the first step, the broths were supplemented with 30 g/L carbon and 1 g/L NH_4Cl, whereas in the step two 30 g/L carbon and 1/3 nitrogen content were used. These latter conditions are known to support PHAs accumulation. For each step, the incubation was under shaking at 30°C for 72 h.

C. necator DSM 545 and *D. acidovorans* DSM 39 showed good growth and high PHAs production (Table 17) with results in agreement with those described in recently published manuscripts (Abbondanzi et al., 2017; Gamero et al., 2018; Marudkla et al., 2018; Romanelli et al., 2014). *C. necator* DSM 545 obtained 11.6 g/L of cell dry mass (CDM) with 84.6% w/w PHAs content, whereas *D. acidovorans* DSM 39 produced 2.9 g/L of CDM with 15.4% w/w PHAs .

Strain	CDM (g/L)	3HB (% CDM)
C. necator DSM 545	11.6 ± 0.3	84.6 ± 3.2
D. acidovorans DSM 39	2.9 ± 0.1	15.4 ± 1.6

CDM= cell dry mass.

Table 17. PHAs production by *C. necator* DSM 545 and *D. acidovorans* DSM 39. Values represent the mean of three replicates and standard deviation is reported.

Both strains were selected for genetic improvements in view of PHAs production at industrial levels, with the objective to reduce the cost and improve biopolymer extraction/recovery efficiency.

3.3. Evaluation of antibiotic resistance of *C. necator* DSM 545 and *D. acidovorans* DSM 39.

Wild type bacteria could only be transformed with vectors containing dominant selection markers such as gentamicin (e.g. pBBR1MCS) or kanamycin (e.g. pHM2). The resistance to these antibiotics were determined for *C. necator* DSM 545 and *D. acidovorans* DSM 39 (Table 18).

The concentration of 500 and 20 μg/mL of gentamicin was chosen for the selection of the recombinants of *C. necator* DSM 545 and *D. acidovorans* DSM 39, respectively. Regarding

kanamycin, 10 µg/mL was enough to inhibit both the growth of *C. necator* DSM 545 and *D. acidovorans* DSM 39 strains (Table 18).

Antibiotic	*C. necator* DSM 545	*D. acidovorans* DSM 39
Gentamicin (µg/ mL)		
0	++++	++++
10	++++	++
20	++++	ng
50	++++	ng
100	++++	ng
200	++++	ng
300	+++	ng
400	++	ng
500	ng	ng
Kanamycin (µg/mL)		
0	++++	++++
5	++	++
10	ng	ng

Table 18. Dominant selection marker resistance of *C. necator* DSM 545 and *D. acidovorans* DSM 39 strains, grown on NB plate supplemented with increasing concentration of gentamicin and kanamycin. (++++: consistent growth; ng: no growth).

3.4. STRAINS CONSTRUCTION FOR THE IMPROVED PRODUCTION OF PHAs FROM SLAUGHTERHOUSE FATTY WASTES.

Slaughterhouse fatty wastes were selected as carbon sources for PHAs production, since they are highly available as by-products in EU (about 500,000 ton/year) and very cheap (Koller et al., 2017; Casella et al., 2016; Titz et al., 2012).

In order to effectively utilize these substrates, *C. necator* DSM 545, although with a basal lipolytic activity (Cruz et al., 2016; Obruca et al., 2014), has been selected as host strain for the expression of efficient lipase genes. As such, the recombinant strains of *C. necator* DSM 545 engineered for addition lipase activities would result in novel and proficient lipid-to-PHAs converters.

3.4.1. Transformation of *C. necator* DSM 545 strain with *lipH-lipC* genes.

In a previous work carried out in the microbiology laboratory of DAFNAE (University of Padova), twenty-six strains capable of producing lipase have been isolated from fatty wastes samples of the slaughterhouses (Povolo et al., 2012; Romanelli et al., 2014). The most promising was found to be a strain of *Pseudomonas stutzeri* (BT3), showing a high extracellular lipolytic activity. *P. stutzeri*, indeed, is described in the literature for such a reason (Fauré and Illanes, 2011; Lalucat et al., 2006; Sangeetha et al., 2011). Therefore, *P. stutzeri* BT3 strain was chosen as possible donor of the genes encoding for efficient extracellular lipases. As previously reported (Fauré and Illanes, 2011; Lalucat et al., 2006; Sangeetha et al., 2011) the best known and efficient lipase genes are called *lipH* and *lipC*. Once verified the presence of both genes into BT3 strain, they were cloned into an appropriate plasmid vector (pBBR1MCS-5) containing the pBBR1 Rep sequence, which enables the replication of plasmid in Gram negative bacteria such as *D. acidovorans* DSM 39 (Romanelli et al., 2014). Once plasmid pBBR1MCS-5-*lipH-lipC* was obtained, it was cloned in *E. coli* DH10B and the lipolytic activity of the recombinant strain *E. coli* pBBR1MCS-5-*lipH-lipC* verified on plates containing Rhodamine B. (Romanelli et al., 2014).

From *E. coli* pBBR1MCS-5-*lipH-lipC*, plasmid pBBR1MCS-5-*lipH-lipC* was extracted using the standard methods according to Sambrook, 2001, and used for the transformation of *C. necator* DSM 545. Recombinants of *C. necator* DSM 545 expressing lipase were then obtained through chromosomal integration of plasmid pBBR1MCS-5-*lipH-lipC*, carrying the *lipH-lipC* genes from *P. stutzeri* BT3. About 800 gentamicin-resistant clones, possible plasmid transporters pBBR1MCS-5-*lipH-lipC* were selected and the presence of *lipH-lipC* genes in the recombinant strains were confirmed with PCR using gene-specific primers: LIPC-F (5'-ATGAACAAGAACAAAACCTTGCT-3'); LIPC-R (5'-GTCAGAGCCCCGCGTTCTTCAATC-3'); LIPH-F (5'-ATGAGCAGATCCATCCTTTT-3'); LIPH-R (5'-TCAGCGAGTCCGATCCTCC-3'). As described in Fig. 19, all the newly engineered bacteria gave positive results with a PCR product of 1.004 and 0.935 Kb, for *lipH* and *lipC* genes, respectively. These size are consistent with those of the native genes of *P. stutzeri* BT3 (Romanelli et al., 2014).

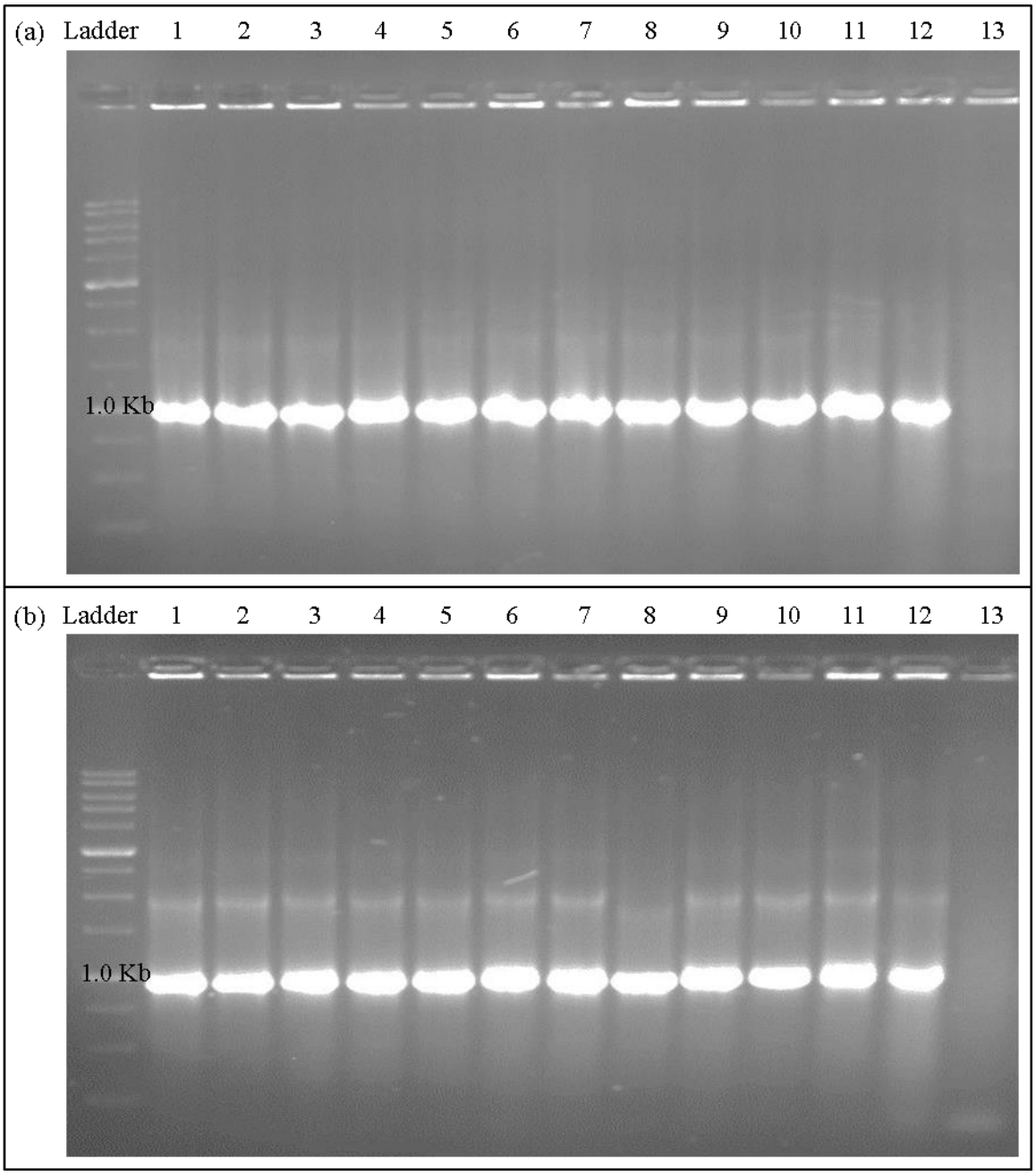


Fig. 19. Gel electrophoresis of PCR amplification products obtained with primers LIPC-F, LIPC-R, LIPH-F and LIPH-R in *P. stutzeri* BT3 (lane 1a and 1b), *C. necator* DSM 545 pBBR1MCS-5-*lipH-lipC* (from lane 2a to 12a and from 2b to 12b) and *C. necator* DSM 545 (lane 13a and 13b) strains. 1 Kb molecular weight marker (Euroclone) was used.

3.4.2. Substrate identification for lipolytic activity evaluation.

Before characterizing the lipolytic activity in the recombinant *C. necator* DSM 545 strains engineered for extracellular lipase production expression, different growth media and fatty

substrates were tested to identify the most suitable ones to stimulate the greatest production of lipolytic enzyme by the parental *C. necator* DSM 545 already reported for a basal lipolytic activity (Cruz et al., 2016; Obruca et al., 2014). *P. stutzeri* BT3 has been included as reference lipolytic strain (Fendri et al., 2010; Li et al., 2014; Romanelli et al., 2014)

Plate assays, titrimetric and spectrophotometry methods were applied to determine lipolytic activity. The results obtained from plate assays and titrimetric method were found to be not reliable for inconsistency in the values obtained, whereas spectrophotometric assay showed excellent results, which are described below.

Spectrophotometric assay was applied using a TECAN spark 20M microplate spectrophotometer (TECAN, Salzburg, Austria) to detect the amount of *p*-nitrophenol (*p*NP) released from *p*-Nitrophenyl palmitate (*p*NPP) at 410 nm.

The two wild type strains, *C. necator* DSM 545 and *P. stutzeri* BT3, were grown at 30°C in three different media (NB and DSMZ 81 supplemented with 30 g/L of olive or corn oil) and the lipolytic activity of the supernatant monitored every 24 h in Tris-HCl buffers at pH 7.5 for 30 min, using two incubation temperatures, 30 and 37°C. The enzymatic values showed no significant differences between 30 and 37°C. Highest values, reported in Table 19, were obtained after 72 hour incubation.

When both *C. necator* DSM 545 and *P. stutzeri* BT3 strains were grown in NB medium, an extremely low lipolytic activity was detected: 12 and 53 mU/(min·mL), respectively (Table 19). These findings could be considered expected since NB does not have any lipids useful to induce the lipase production by both tested strains. However, when DSMZ 81 medium was supplemented with olive oil, *C. necator* DSM 545 and *P. stutzeri* BT3 showed higher activity: 58 and 84 mU/(min·mL), respectively. On the other hand, DSMZ 81 medium supplemented with corn oil, obtained the highest values after 72 h of growth for both strains: *C. necator* DSM 545 with 67 mU/(min·mL) and *P. stutzeri* BT3 with 167 mU/(min·mL).

Strain	NB	DSMZ 81 medium with olive oil	DSMZ 81 medium with corn oil
P. stutzeri BT3	53± 0.5	84± 1.3	167± 1.6
C. necator DSM 545	12± 1	58± 0.9	67± 1.2

Table 19. Lipolytic activity (mU/(min·mL)) of *C. necator* DSM 545 and *P. stutzeri* BT3 grown for 72 h in NB and DSMZ 81 medium with olive or corn oil. The enzymatic activity was measured on cell-free supernatants after 72 h incubation. The assays were performed at 30°C in buffer Tris-HCl at pH 7.5. Values represent the mean of three replicates and standard deviation is reported.

3.4.3. Characterization of lipolytic activity in the engineered strain.

Once the integration of both *lipH* and *lipC* genes into the recombinant strains of *C. necator* DSM 545 was successfully confirmed, a representative number of clones were screened for lipolytic activity using a TECAN spark 20M microplate spectrophotometer (TECAN, Salzburg, Austria), as previously described. Determining the enzymatic activities at 30 and 37°C for 30 min was useful to select a cluster of 86 recombinants with great promise. Furthermore, four integrants with high lipolytic activities were creamed off for future studies and named DSM 545 *lip3*, DSM 545 *lip4*, DSM 545 *lip8* and DSM 545 *lip11*.

As reported in Table 20, recombinant strains exhibited the highest lipolytic activity after 72 h of growth, DSM 545 *lip3*, DSM 545 *lip4* and DSM 545 *lip8* had lipolytic activities ranging from 68 to 98 mU/(min·mL), values slightly higher than those reported for their parental strain (67 mU/(min·mL)).

Strain	24 h	48 h	72 h
C. necator DSM 545 *lip3*	13± 0.2	61± 0.8	69± 2
C. necator DSM 545 *lip4*	19± 1	34± 2.3	68± 1.6
C. necator DSM 545 *lip8*	12± 1	74± 1.2	98± 0.5
C. necator DSM 545 *lip11*	31± 0.4	39± 1.8	121± 0.3

Table 20. Lipolytic activity of *C. necator* DSM 545 pBBR1MCS-5-*lipH-lipC* clones after 24, 48 and 72 h grown in DSMZ 81 broth supplemented with 30 g/L corn oil. The assays were performed at 30°C in buffer Tris-HCl at pH 7.5. All values are expressed in mU/(min·mL). Values represent the mean of three replicates and standard deviation is reported.

On the other hand, *C. necator* DSM 545 *lip11* showed superior enzymatic activity (121 mU/(min·mL) after 72 h of growth, similar to that of the *P. stutzeri* BT3, donor strain of the lipase genes: 167 mU/(min·mL).

The kinetics of the enzymatic activities at 24, 48 and 72 h is shown in Fig. 20 for, *P. stutzeri* BT3, *C. necator* DSM 545 and *C. necator* DSM 545 *lip11* (recombinant strain with greater lipolytic activity), grown in DMSZ 81 broth supplemented with 30 g/L corn oil and incubated at 30°C.

In the first 48 h of growth, *P. stutzeri* BT3 showed low lipolytic enzymes production. However, the enzymatic production increased up to 4 times within 72 h of incubation (from 39 to 167 mU/(min·mL)). *C. necator* DSM 545, on the other hand, took approximately 48 h to produce

the lipolytic enzyme, and then the production stopped, until it reached 67 mU/(min·mL). Regarding *C. necator* DSM 545 *lip11*, its behavior was very similar to the donor strain of the genes, but after the first 48 h the enzymatic activity increased a little less, until (121 mU/(min·mL)).

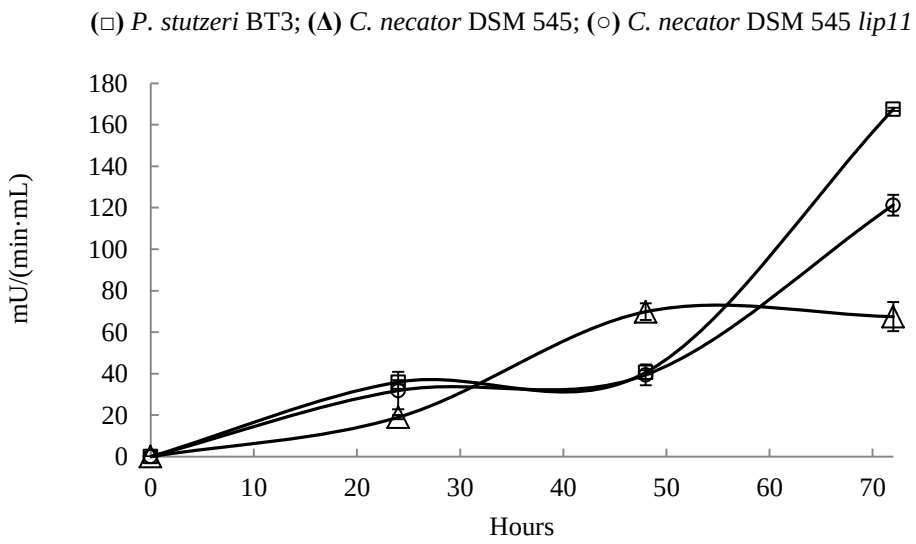


Fig. 20. Kinetic of lipolytic activity of *P. stutzeri* BT3, *C. necator* DSM 545 and *C. necator* DSM 545 *lip11* grown in DMSZ 81 broth supplemented with 30 g/L corn oil. The assays were performed at 30°C in buffer Tris-HCl at pH 7.5. All values are expressed in mU/(min·mL) and represent the mean of three replicates with standard deviation reported.

Considering the high enzymatic activities exhibited on *p*NPP (Table 20 and Fig. 20), *C. necator* DSM 545 *lip11* was selected for further studies on PHAs production from slaughterhouse fatty wastes as carbon source.

3.4.4. Optimization of growth conditions using slaughterhouse fatty wastes as carbon source.

To optimize growth conditions of both parental and recombinant strains of *C. necator* DSM 545 using slaughterhouse fatty wastes as carbon source, several parameters have been considered. Specifically, incubation temperature, the use of Tween-80 as an emulsifier to improve the availability of fats, NH_4Cl concentration needed to support reasonable microbial growth levels and production of PHAs have been considered. The most appropriate sterilization procedure for fats and growth media has been also defined.

3.4.4.1. Temperature selection to PHAs production using slaughterhouse fatty wastes as carbon source.

Melting conditions of slaughterhouse fatty wastes have been tested in DSMZ 81 medium at 30 g/L of each samples and incubated for 6 h at 30 and 37°C (Fig. 21). All assayed by-products were completely solid at 30° C. On the contrary, at 37°C good melting levels have been detected, except for the kidney fatty waste (Fig. 21).

This means that incubating the feedstocks at higher temperature could be a reasonable option since *C. necator* DSM 545 and *P. stutzeri* BT3 were found to grow well also at 37°C.

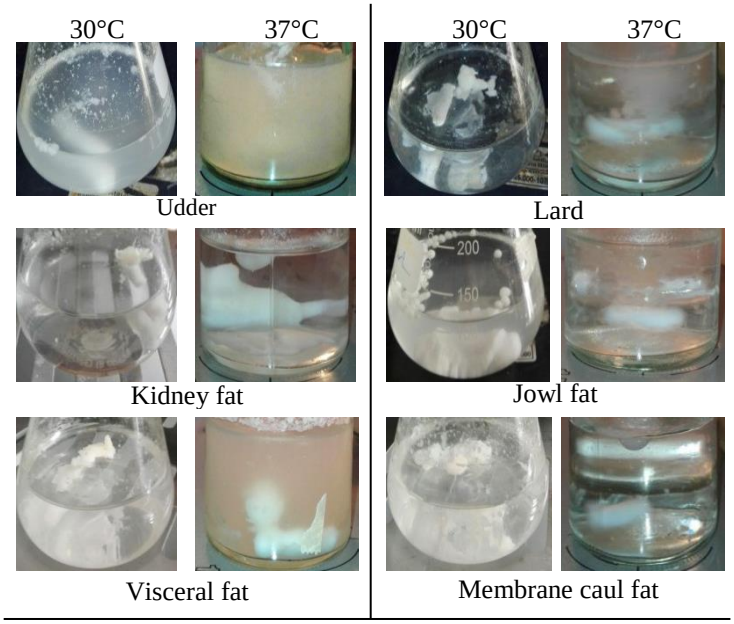


Fig. 21. Effect of temperature incubation on melting state of 30 g/L slaughterhouses fatty wastes in DSMZ 81 medium.

3.4.4.2. Evaluation of Tween-80 as fats emulsifier.

Different concentrations of Tween-80 (0, 2.5, 5, 7.5 and 10 g/L) were added to DSMZ 81 medium with 30 g/L of each fatty waste. They were incubated at 37°C and were evaluated to assess the minimum quantity of Tween-80 needed to obtain a proper emulsion. Tween-80 in fact, has been already reported to be a good emulsifier for several lipid-rich materials such as coco nut, palm Oil and others (Muktar et al., 2017; Thinagaran and Sudesh, 2017). It has been used both at industrial and laboratory levels as stabilizer and antifoam with loadings of up to 10 g/L. For this reason, the maximum concentration used in this study was set to 10 g/L.

Fig. 22 shows that the use of Tween-80 effectively improves the emulsification of all the tested wastes in DSMZ 81 medium. The higher concentration of Tween-80, the more homogeneous the emulsions becomes (Fig. 22).

Lard, membrane caul fat and jowl fat in the presence of 5 g/L Tween-80 presented a good homogeneity. While visceral fat and kidney fat required 7.5 and 10 g/L of Tween-80, respectively. On the other hand, as reported in Fig. 22, the udder required no Tween-80 to dilute in the medium; this may be due to its low lipids percentage in its composition (Table 13).

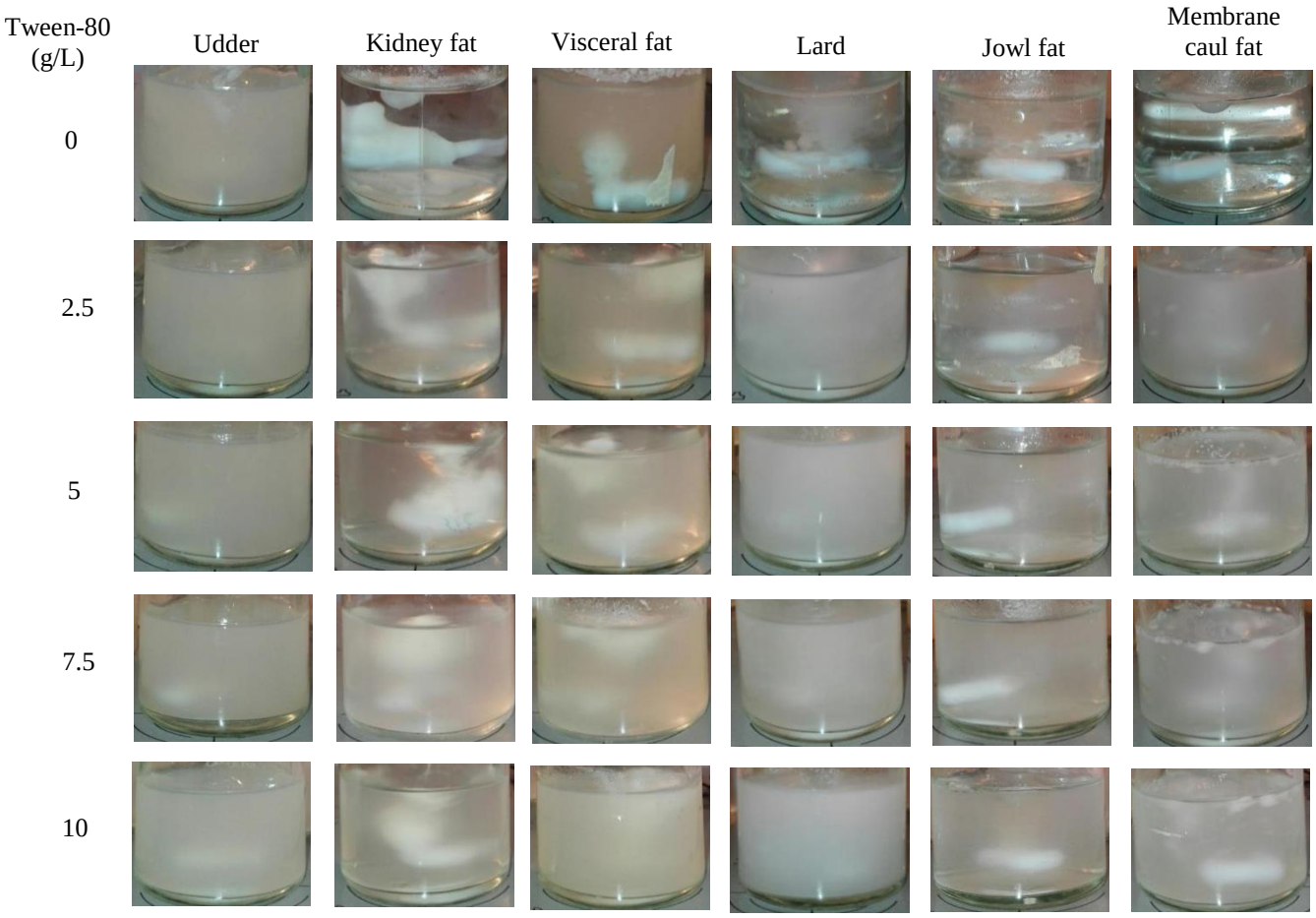


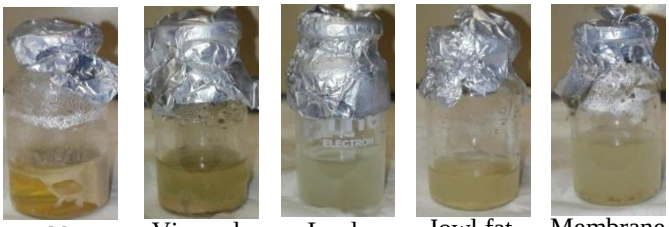
Fig. 22. Effect of increasing concentrations of Tween-80 on the homogeneous state at 37°C of several slaughterhouses fatty wastes (30 g/L) in DSMZ 81 medium.

For future studies, the following Tween-80 concentrations have been selected: kidney fat 10 g/L, visceral fat and lard 7.5 g/L, jowl and membrane caul fat 5 g/L. No addition of Tween-80 has been designed for udder.

3.4.4.3. Sterilization method.

With the objective to optimize the sterilization method of fatty by-products, has been selected the udder, visceral fat, lard, jowl fat and membrane caul fat by their suitable melting levels at 37°C and their low Tween-80 requirements.

Fatty wastes, DSMZ 81 medium and Tween-80 were mixed before sterilization or were sterilized separately and then mixed together. The sterilization of each substrate separately resulted in the formation of solid particles of tissue, probably deriving from the blood components solidified as a consequence of the high sterilization temperatures (Fig. 23). As such, the separation of the sterilized fats from the solid particles was efficient, which improved the quality of the fats added into the DSM81 broth.



Udder | Visceral fat | Lard | Jowl fat | Membrane caul fat

Fig. 23. Slaughterhouses fatty wastes (30 g/L) sterilized at 121°C per 20 min.

Once prepared, the media were incubated at 37°C under shaking and, after 6 h, evaluated for their melting levels (Fig. 24). The media sterilized together with the fatty waste and Tween-80 showed a degree of homogeneity greater than those formulated adding fats and Tween-80 sterilized separately to the Mineral medium (Fig. 24). This could indicate that the high sterilization temperatures supported the fats homogenization. As such, the sterilization of the mixture of DSMZ 81 broth, fatty waste and Tween-80 was selected as procedure for future studies on PHAs production. Nevertheless, it is necessary to perform further investigations on the high temperatures effects at molecular levels on fats and the possible reactions with the DSMZ 81 medium components.

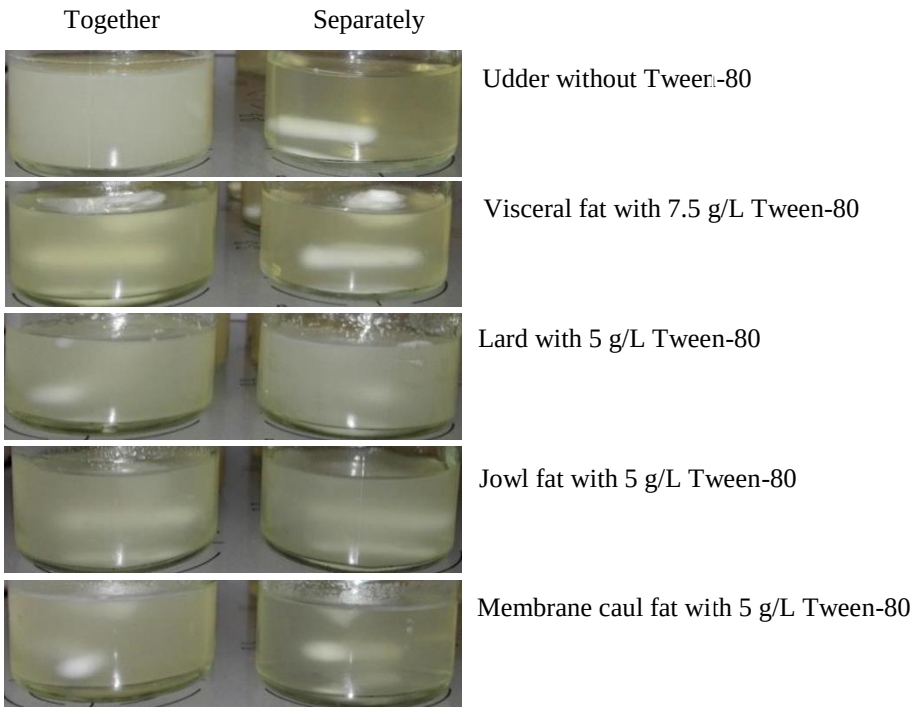


Fig. 24. Effect of sterilizing fatty wastes, DSMZ 81 medium and Tween-80 together or separately and then mixed. Sterilization has been performed at 121°C per 20 min.

C. necator DSM 545 was then used to process all the selected fatty by-products into PHAs in a one-step configuration setting at 37°C. Tween-80 supplementation were provided according to the fatty waste considered (see 3.4.4.2.). Moreover, benchmark broths supplemented only with Tween-80 or fatty substrates have been included in the experimental design. After 72 h of growth, CDM and PHAs content were determined (Table 21).

The presence in the broth of only Tween-80 was sufficient to support both growth and PHAs production. However, CDM and 3HB concentration were quite low, indicating that Tween-80 could be an unusual carbon source for *C. necator* DSM 545.

Using udder as carbon source, both CDM (1.7 g/L) and PHAs production (0.02 g/L) were higher, confirming its good dissolubility in the medium without Tween-80 addition. Nonetheless, when 5 g/L Tween-80 was supplemented to lard, jowl fatty and membrane caul fatty, *C. necator* DSM 545 produced CDM and PHAs levels lower than those detected without the emulsifier addition.

On the other hand, the addition of Tween-80 (7.5 g/L) to visceral fat was beneficial for CDM and PHAs production. 3HB content was almost 3-times higher in the presence of Tween-80,

whose presence seemed to support the ability of *C. necator* DSM 545 to produce PHAs out from this fatty residue (Table 21).

Overall, the effect of Tween-80 supplementation on PHAs production from slaughterhouse waste streams seems to be substrate-specific and needs further and deeper investigations.

Considering now the final values of CFU/mL, it seems that the addition of Tween-80 to lard, jowl fat and membrane caul fat did not result in higher cell growth whereas the emulsifier did increase the CFU/mL in the presence of visceral fat.

Carbon source	CDM (g/L)	3HB (% CDM)	CFU/mL
Tween-80 (5 g/L)	0.9± 0.1	1.0± 0.3	$2.1x10^7$± $1.4x10^6$
Tween-80 (7.5 g/L)	1.1± 0.3	1.0± 0.1	$3.8x10^8$± $6.3x10^7$
Udder	1.7± 0.5	1.2± 0.1	$5.3x10^7$± $5.3x10^5$
Visceral fat	1.2± 0.3	0.6± 0.1	$9.1x10^7$± $7.1x10^6$
Visceral fat with Tween-80 (7.5 g/L)	1.7± 0.1	1.7± 0. 1	$8.0x10^8$± $3.5x10^6$
Lard	0.8± 0.02	2.2± 0.7	$8.2x10^7$± $7.8x10^6$
Lard with Tween-80 (5 g/L)	0.3± 0.01	1.7± 0.7	$7.8x10^7$± $7.1x10^6$
Jowl fat	2.1± 0.4	3.1± 0.2	$2.0x10^9$± $1.4x10^7$
Jowl fat with Tween-80 (5 g/L)	1.0± 0.03	2.5± 0.2	$9.3x10^8$± $2.8x10^7$
Membrane caul fat	2.4± 0.2	4.0± 0.8	$7.4x10^8$± $1.1x10^7$
Membrane caul fat with Tween-80 (5 g/L)	1.0± 0.4	2.7± 0.3	$3.1x10^8$± $7.7x10^6$

CDM= Cell dry mass.

Table 21. Growth and PHAs accumulation by *C. necator* DSM 545 using slaughterhouse fatty wastes as carbon sources with or without Tween-80 supplementation. Values represent the mean of three replicates and standard deviation is reported.

3.4.4.4. Nitrogen source (NH_4Cl) optimization for biomass and PHAs production from slaughterhouse fatty wastes.

Membrane caul fat waste was selected among the other fatty wastes as the most promising substrate for PHAs accumulation, since *C. necator* DSM 545 produced the highest 3HB levels (Table 21) without Tween-80 addition. Moreover, such substrate is quite rich in lipid (Table 13) and has achieved a good melting level once incubated at 37°C in the presence of 5 g/L Tween-80 (Fig. 22).

C. necator DSM 545 cell growth and PHAs production were evaluated in DSMZ 81 medium, supplemented with three different concentrations of NH_4Cl (0.5, 1 and 2.5 g/L) and 30 g/L membrane caul fat (Fig. 25 and Table 22).

The strain was able to grow in all three tested conditions. In the presence of 0.5 g/L NH_4Cl lower CFU/mL levels were achieved after 48 h (up to $1.3x10^9$), whereas the other two nitrogen concentrations gave similar growth kinetics after 48 h of growth, with nearly $6.0x10^9$ CFU/mL (Fig. 25).

The lower values of biomass detected in the medium with the lowest NH_4Cl content could be due to a shortage of nitrogen, which was limited the *C. necator* DSM 545 develop (Ayub et al., 2004; Obruca et al., 2017).

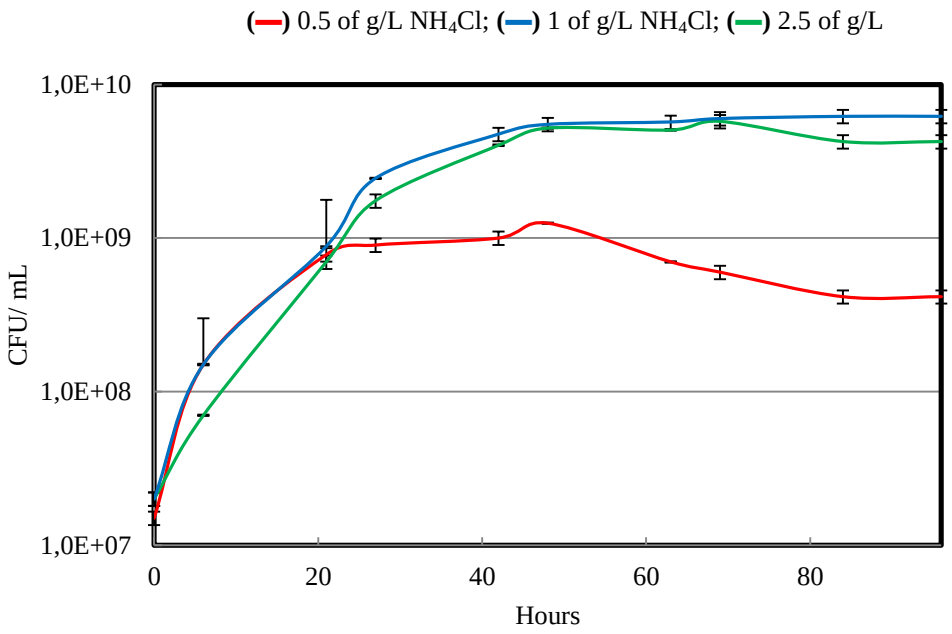


Fig. 25. Growth kinetics of *C. necator* DSM 545 in DSMZ 81 supplemented with different NH_4Cl concentrations and 30 g/L of membrane caul fat as carbon source. Values represent the mean of three replicates and standard deviation is reported.

Strain	Carbon source	NH_4Cl (g/L)	CDM (g/L)	3HB (% CDM)	3HB (g/L)	Lipolytic activity (mU/ (min·mL))	% fat consumption
C. necator DSM 545	Membrane caul fat	0.5	1.3± 0.4	12.4±0.1	0.2	30.2± 1.8	13.0± 0.3
		1	1.6± 0.1	9.8±0.2	0.2	69.4± 3.1	17.2± 1.1
		2.5	1.4± 0.2	2.2±0.1	0.03	60.1± 2.3	15.1± 0.8

CDM= Cell dry mass.

Table 22. PHAs production of *C. necator* DSM 545 in DSMZ 81 medium supplemented with different NH_4Cl concentrations (0.5, 1 and 2.5 g/L) and 30 g/L membrane caul fat as carbon source at 37°C for 96 h of incubation. Values represent the mean of three replicates and standard deviation is reported.

CDM and PHAs content were also affected by NH_4Cl availability in the broth (Table 22). The use of 0.5 g/L NH_4Cl was suboptimal in terms of CDM (1.3 g/L) but had a beneficial PHAs accumulation (12.4% CDM). This is a consequence of the carbon/nitrogen ratio imbalance, since *C. necator* DSM 545 when being in the presence of carbon excess and low nitrogen concentration, activates the metabolism of PHAs accumulation in advance (Wen et al., 2010). On the other hand, supplementing the broth with 2.5 g/L of NH_4Cl resulted in a CDM similar to that of 1 g/L of NH_4Cl, but with lower PHAs levels.

Considering now the lipolytic activity detected in the supernatant of the broths after 96 h of incubation, the medium supplemented with 0.5 g/L of NH_4Cl supported significantly lower enzymatic capabilities. This finding could be related to the fast depletion of nitrogen, which reduces the expression of lipolytic enzymes (Fickers et al., 2004; Gupta et al., 2004). Such limited lipase activity could also explain the lower consumption of membrane caul fat by *C. necator* DSM 545 once grown in the presence of the lowest NH_4Cl concentrations. At higher nitrogen availability, both lipolytic activities and fat consumption were more evident (Table 22).

Overall, the results reported in Fig. 25 and Table 22 seem to indicate that the best concentrations for both biomass production and PHAs accumulation process using slaughterhouse fatty waste were 1 g/L of NH_4Cl and 30 g/L of carbon source.

3.4.5. PHAs production from slaughterhouse residues

The optimization of nitrogen and carbon source concentration was crucial for the setting of experiments focused to process fatty by-products into PHAs by using both the parental *C. necator* DSM 545 and the most efficient recombinant *C. necator* DSM 545 *lip11*. One-step and two-steps procedures have been applied as described below.

3.4.5.1. One-step PHAs production by *C. necator* DSM 545 and *C. necator* DSM 545 *lip11* using membrane caul fat as carbon source.

Parental and recombinant strains were grown for 96 h in a one-step cultivation procedure as described in 2.3.6.1. DSMZ 81 medium was formulated with 1 g/L NH_4Cl and 30 g/L of membrane caul fat (Table 23). Noteworthy, the recombinant strain *C. necator* DSM 545 *lip11* produced high levels of CDM (2.8 g/L) and PHAs content (0.8 g/L). On the other hand, the parental strain did confirm the ability to process membrane caul fat into biomass and 3HB, although at a much lower

rate. 3HB concentration (g/L) in the presence of the recombinant microbe was found to be more than 5-fold that detected in the broth inoculated with the parental *C. necator* DSM 545.

The production of lipolytic enzyme by *C. necator* DSM 545 *lip11* recombinant strain was almost 1.6-fold that of the wild type strain. The higher availability of lipase resulted in higher biomass production ($3.1x10^{10}$ instead of $5.3x10^{9}$ CFU/mL) and greater consumption of membrane caul fat, 26.7 instead of 16.8% measured in the spent broth of the parental *C. necator* DSM 545.

Strain	CDM (g/L)	3HB (% CDM)	3HB (g/L)	CFU/mL	Lipolytic activity (mU/(min·mL))	% fat consumption
C. necator DSM 545	1.5± 0.1	9.8± 0.02	0.15	$5.3x10^{9}$± $2.8x10^{8}$	69.1± 3.1	16.8± 1.1
C. necator DSM 545 *lip11*	2.8±0.3	28.5±0.03	0.80	$3.1x10^{10}$± $3.4x10^{9}$	117.0± 2.8	26.7± 0.04

CDM= Cell dry mass

Table 23. Growth and PHAs production of *C. necator* DSM 545 and *C. necator* DSM 545 *lip11* in DSMZ 81 medium supplemented with 1 g/L NH_4Cl and 30 g/L membrane caul fat as carbon sources after 96 h of incubation at 37°C. Values represent the mean of three replicates and standard deviation is reported.

3.4.5.2. Two-steps PHAs production by *C. necator* DSM 545 *lip11* using membrane caul fat and glucose as carbon sources.

In order to both increase the productivity of PHAs and lower their cost, a novel two-steps PHAs production procedure has been defined using membrane caul fat and glucose, both at 30 g/L, as carbon source in the first and second phase, respectively. Since the parental strain did not produce significant levels of PHAs and biomass from the membrane caul fat (Table 23), only the engineered microbe *C. necator* DSM 545 *lip11* has been considered in this experiment.

To further optimize the glucose-to-3HB yield of the second step, bacterial cells grown in the first phase into 100 mL of DSMZ 81 with membrane caul fat have been transferred into 100 or 50 mL of DSMZ 81 broth supplemented with increasing concentrations of glucose (7.5, 15 and 30 g/L) and NH_4Cl, 0, 0.075, 0.15 and 0.3 g/L (Table 24). The incubation was performed at 37°C (150 rpm) for 72 h, and at 24 h intervals glucose concentrations were determined.

The rational supporting such experimental plan is threefold. Firstly, the use of a cheap carbon source to produce cell biomass in the first phase would result in lower production cost. Secondly, the use of different glucose concentration and volumes of broth in the second phase is

focused on optimizing the yield of PHAs from consumed glucose. Finally, nitrogen availability was considered as variable to assess the ability of the strain to differentially consume glucose and produce PHAs.

Nitrogen occurrence in all the tested conditions seems to have promoted the glucose consumption. The higher starting glucose concentrations the more sugar depletion has been detected in the presence of NH_4Cl. (Fig. 26). Overall, a general trend of glucose utilization can be highlighted: after approximately 48 h of growth, the glucose consumption stopped. Moreover, the results indicated that the use of high glucose concentration (30 g/L) could be non-beneficial for PHAs production as the majority of the sugar remained unused over a long incubation period.

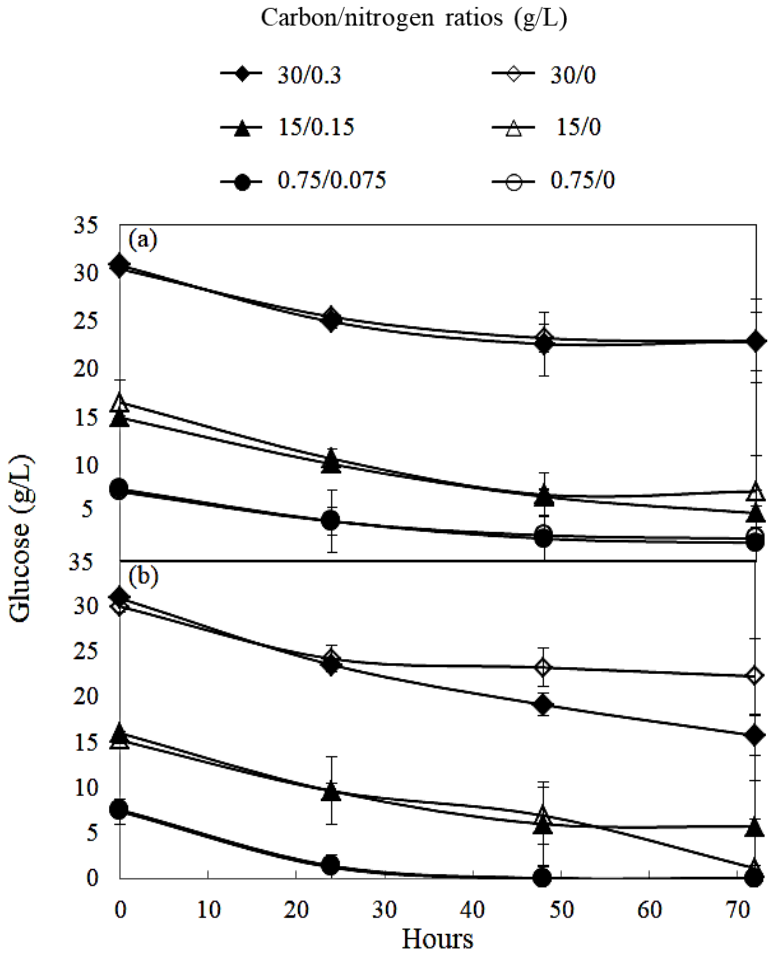


Fig. 26. Kinetics of glucose consumption by *C. necator* DSM 545 *lip11* in a) 100 mL or b) 50 mL of DSMZ 81 medium supplemented with different carbon/nitrogen ratios in step two and incubated at 37°C. Values represent the mean of three replicates and standard deviation is reported.

As reported in Table 24, nitrogen did support also higher cell biomass in all the kinetics accomplished with the exception of those performed in 50 mL volumes and 15 or 7.5 g/L glucose, which showed similar amount of cell biomass. PHAs production, both in terms of 3HB (g/L) and % CDM, was generally higher in the presence of nitrogen, too. Only, the experiment conducted with 50 mL broth having 7.5 g/L glucose reached low PHAs accumulation values.

					Glucose (g/L)			
Step-two	Carbon/Nitrogen ratios (g/L)	CDM (g/L)	3HB (% CDM)	3HB (g/L)	Initial	Final	Consumed	3HB Yield
100 mL	30/0.3	5.5± 0.2	77.2± 6.4	4.0	30	18.2± 4.3	11.8	0.34
	30/0	3.3± 0.1	53.3± 4.7	1.5		22.9± 3.0	7.1	0.21
	15/0.15	4.3± 0.1	59.9± 2.8	2.4	15	5.0± 0.6	10.0	0.24
	15/0	2.5± 0.2	52.8± 1.4	1.1		7.3± 3.6	7.7	0.14
	7.5/0.075	3.2± 0.6	63.5± 2.5	1.8	7.5	1.9± 0.5	5.6	0.32
	7.5/0	2.7± 0.1	52.9± 1.7	1.2		2.4± 4.1	5.1	0.24
50 mL	30/0.3	6.6± 0.6	67.5± 4.1	4.1	30	15.7± 2.2	14.3	0.28
	30/0	4.3± 0.7	38.3± 6.7	1.2		22.2± 4.1	7.8	0.16
	15/0.15	6.4± 0.6	75.5± 2.4	4.4	15	5.7± 1.3	9.3	0.47
	15/0	6.6± 0.9	57.0± 3.5	3.3		1.1± 0.2	13.9	0.24
	7.5/0.075	5.1± 0.8	40.9± 0.3	1.6	7.5	0	7.5	0.22
	7.5/0	4.9± 0.4	55.8± 2.7	2.3		0	7.5	0.30

CDM= Cell dry mass.

Table 24. Two-steps PHAs production by *C. necator* DSM 545 *lip11* in DSMZ 81 medium supplemented with different carbon/nitrogen concentrations (g/L glucose/ g/L NH_4Cl) and different volumes of step-two (100 and 50 mL). 3HB yield was determined as gram of 3HB produced during the second step per gram of consumed glucose. Values represent the mean of three replicates and standard deviation is reported.

In order to better compare the PHAs performances contents and yields achieved by *C. necator* DSM 545 *lip11* in this two-steps method, 3HB concentrations and yields have been plotted in Fig. 27.

The highest biopolymer accumulation value has been detected in the 50 mL broth supplemented with 15 g/L glucose and 0.15 g/L NH_4Cl (4.4 g/L PHAs). Slightly lower concentrations were reached by the same recombinant strain once grown in the presence of 30 g/L glucose with 0.3 g/L NH_4Cl in both volumes, 100 and 50 mL, of DSMZ81 medium. At lower glucose levels available at the beginning of the second step (7.5 g/L), 3HB productions and yields decreased suggesting that such sugar levels were too low in both tested volumes.

The highest 3HB yield (0.47 g of 3HB per g of consumed glucose) was obtained in 50 mL supplementing the strain with 15 g/L glucose and 0.15 g/L NH_4Cl. This indicates that *C. necator* DSM 545 *lip11* was able, in a medium with high cellular concentration, to produce a high quantity of PHAs using a lower amount of glucose.

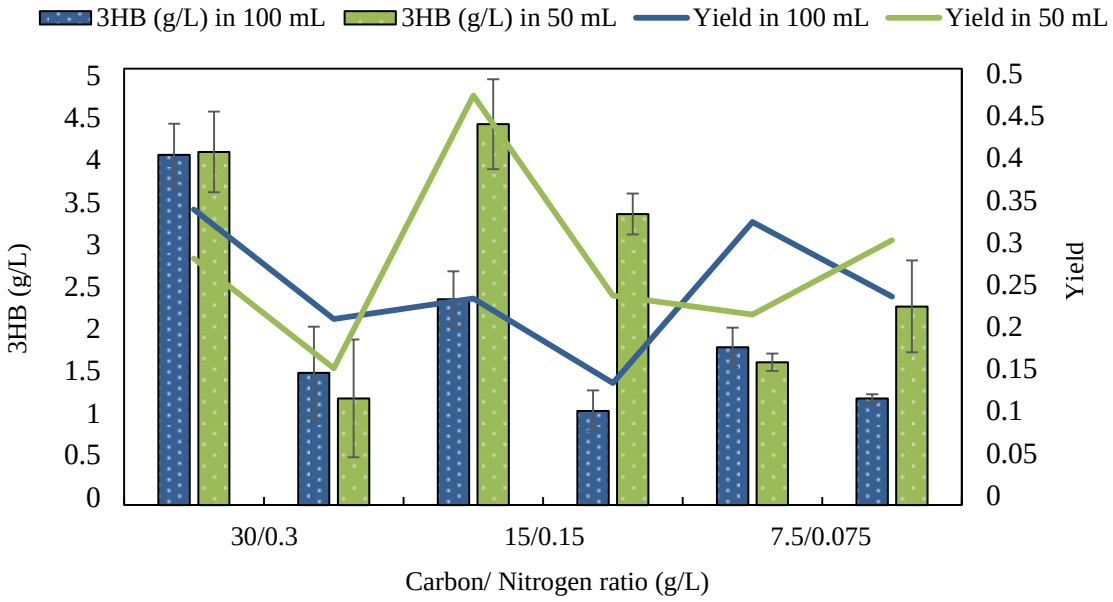


Fig. 27. 3HB g/L production by *C. necator* DSM 545 *lip11* in 100 mL or 50 mL of DSMZ 81 medium supplemented with different carbon/nitrogen concentrations (g/L glucose/ g/L NH_4Cl) after 72 h of incubation at 37°C. 3HB yield was determined as gram of 3HB produced during the second step per gram of consumed glucose. Values represent the mean of three replicates and standard deviation is reported.

3.5. STRAINS CONSTRUCTION FOR THE NUCLEASE EXPRESSION.

As mentioned before, the high pressure homogenization (HPH) is one of the most widely known method for large scale cell disruption, it is considered environmentally friendly and does not need solvents to mediate an efficient microbial cells disintegration (Koller et al., 2013). However, cell lysis causes the release of large amounts of chromosomal DNA which results in a dramatic increase in viscosity, hampering following filtration and centrifugation steps (Atkinson and Mavituna, 1991; Ling et al., 1998; I M Tamer et al., 1998a; I M Tamer et al., 1998b; Van Wegen et al., 1998). Drop in viscosity is generally achieved by the supplementation of commercially nucleases, which increasing the production costs in the downstream step (Koller et al., 2013).

In order look a cost-effective solution to the viscosity issue, two selected strains (*C. necator* DSM 545 and *D. acidovorans* DSM 39) have been genetically modified and tested as following.

3.5.1. Integration of nuclease into *C. necator* DSM 545 and *D. acidovorans* DSM 39.

C. necator DSM 545 and *D. acidovorans* DSM 39 expressing a staphylococcal nuclease were constructed through random integration of plasmid pMH2-*nuc*, carrying the *nuc* gene from *S. aureus*. About 1000 kanamycin-resistant clones were screened for nuclease activity by plating on DNase agar and a cluster of engineered strains with the highest levels of nuclease expression was selected, obtaining 181 and 186 clone of *C. necator* DSM 545 pHM2-*nuc* and *D. acidovorans* DSM 39 pHM2-*nuc*, respectively. Furthermore, two integrands with the largest hydrolysis halos were creamed off and named *C. necator* DSM 545 pHM2-*nuc* and *D. acidovorans* DSM 39 pHM2-*nuc*. Both whole cell culture and cell-free supernatant clearly demonstrated the ability to cleave DNA in the recombinant strains (Fig. 28 a-d). On the contrary, the two wild type bacteria displayed no nuclease activity (Fig. 28 e, f).

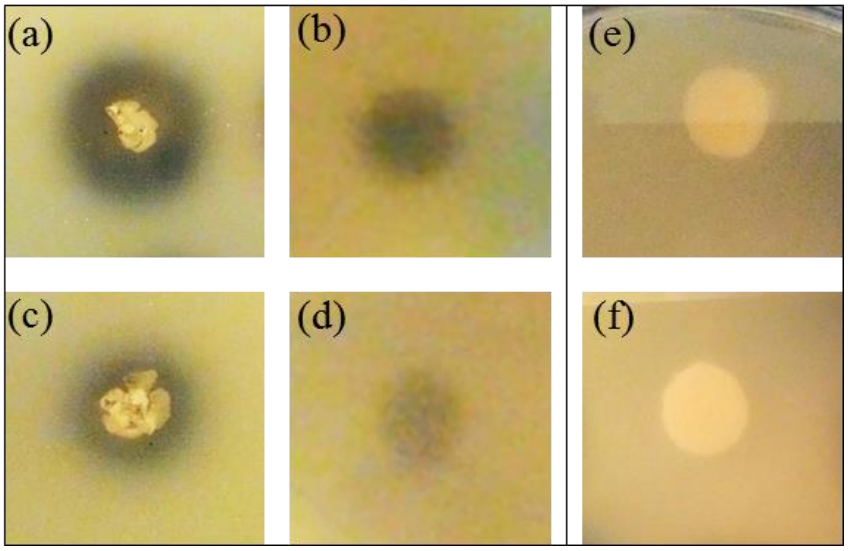


Fig. 28. The DNase activities of wild type and recombinant strains were examined on DNase agar. The plates were incubated at 30°C for 72 h. Halos around colonies indicate DNA digestion of cell broths (a, c, e, f) or cell-free supernatants (b,d) of microbial cultures. The following strains were tested: *C. necator* DSM 545 pMH2-*nuc* (a, b), *D. acidovorans* DSM 39 pMH2-*nuc* (c,d), *C. necator* DSM 545 (e) and *D. acidovorans* DSM 39 (f).

The presence of *nuc* gene in the recombinant strains was confirmed with PCR using gene specific primers. As described in Fig. 29, only the newly engineered bacteria gave positive results producing a fragment of 0.7 Kb, consistent with that of *nuc* gene of *S. aureus* (Brakstad et al., 1992; Kim et al., 2001).

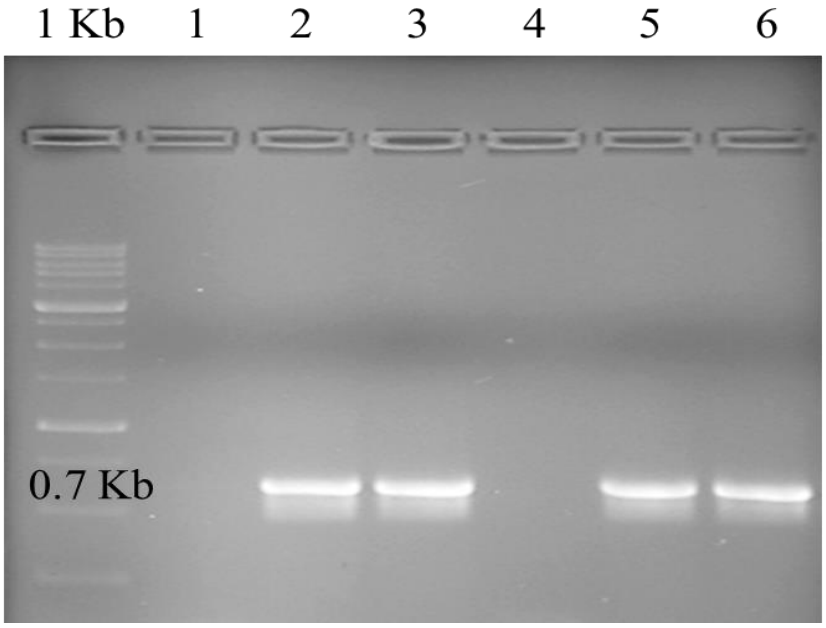


Fig. 29. Gel electrophoresis of primers *nucA* and *nucB* PCR amplification product in *C. necator* DSM 545 (lane 1), *C. necator* DSM 545 pHM2-*nuc* (lanes 2 and 3), *D. acidovorans* DSM 39 (lane 4) and *D. acidovorans* DSM 39 pHM2-*nuc* (lanes 5 and 6) strains. 1 Kb molecular weight marker (Euroclone) was used.

3.5.2. Characterization of nuclease activity in the engineered strains.

The nuclease activity was assessed in both the extracellular broth (using the cell free supernatants) and in the periplasm (using $CHCl_3$ as permeabilization agent) of parental and recombinant cells. Bacterial strains were grown in NB for 72 h and cell free supernatant or whole cell culture treated with $CHCl_3$ were used to assess hydrolytic activity on phage λ DNA increasing residence times. The highest extent of DNA hydrolysis by both engineered strains was obtained after 1 h incubation whereas, as expected, parental bacteria did not show any nuclease ability (Fig. 30). Cell free supernatant samples of both recombinants readily hydrolysed DNA (lanes 3, 7, 11, 15) and cell fractions permeabilized by chloroform, representing the periplasm (lanes 4, 8, 12, 16), did not demonstrate higher enzymatic activity as no further reduction in molecular weight of λ

DNA occurred (Fig. 30). As a result, the nuclease seems to be primarily secreted into the medium through the periplasm by both recombinant strains. This finding is consistent with Boynton and colleagues reporting that recombinant nuclease of *S. aureus* was mainly secreted by an engineered *Pseudomonas putida* strain (Boynton et al., 1999).

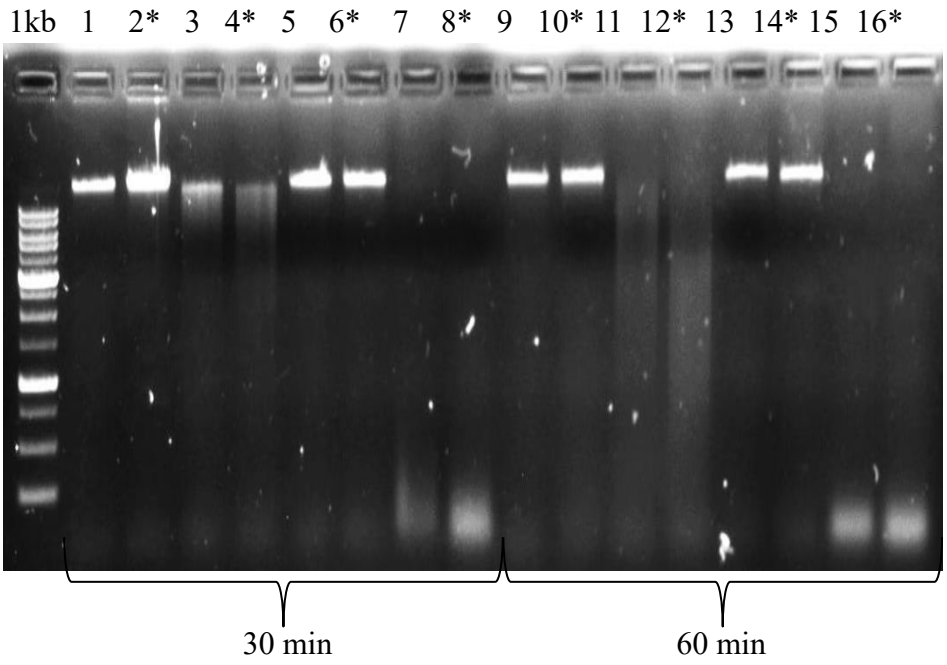


Fig. 30. Nuclease activity in *D. acidovorans* DSM 39, *C. necator* DSM 545 and relative recombinant strains after incubation at 30°C for 30 and 60 min. Growth medium and chloroform-permeabilized cell fractions (indicated by *) were incubated with phage λ DNA in the case of *D. acidovorans* DSM 39 (lanes 1, 2, 9 and 10), *D. acidovorans* DSM 39 pHM2-*nuc* (lanes 3, 4, 11 and 12), *C. necator* DSM 545 (lanes 5, 6, 13 and 14) and *C. necator* DSM 545 pHM2-*nuc* (lanes 7, 8, 15 and 16).

Nuclease activity of both recombinants was confirmed in liquid assays. Bacterial strains were grown in NB for 96 h and, at regular intervals, the supernatant was used to determine phage λ DNA hydrolysis by spectrophotometrically, measuring the absorbance of acid soluble polynucleotides at 260 nm. Both strains exhibited the highest nuclease activity after 72 h of growth, accounting for 67 and 17 mU/(min·mL) for *C. necator* DSM 545 pHM2-*nuc* and *D. acidovorans* DSM 39 pHM2-*nuc*, respectively. This outcome is in agreement with the greater extent of DNA hydrolysis for *C. necator* DSM 545 pHM2-*nuc* (Fig. 30).

3.5.3. PHAs synthesis by *C. necator* DSM 545, *C. necator* DSM 545 pHM2-*nuc*, *D. acidovorans* DSM 39 and *D. acidovorans* DSM 39 pHM2-*nuc*.

In order to successfully replace parental strains as PHAs producers, the recombinant microbes should maintain the same strain stability and PHAs productivity as the wild type bacteria. Parental and recombinant strains were grown, using a two-steps cultivation procedure. Their respective media were formulated with high carbon and low nitrogen content, conditions known to support PHAs accumulation. Noteworthy, the nuclease-expressing microbes produced cell biomass similar to that of their parental strains, with *C. necator* DSM 545 strains achieving the highest levels (Table 25). Moreover, PHAs granules, composed exclusively of 3HB monomers, accumulated in the recombinant cells at concentrations comparable to those of the relative wild type strain. Both findings indicated that genetic transformation and nuclease production do not cause any evident metabolic burden to the recombinants, which could be efficiently used as PHAs producers.

Strain	CDM (g/L)	3HB (% CDM)
C. necator DSM 545	11.6 ± 0.3	84.6 ± 3.2
C. necator DSM 545-pHM2-*nuc*	11.9 ± 0.3	83.6 ± 4.3
D. acidovorans DSM 39	2.9 ± 0.1	15.4 ± 1.6
D. acidovorans DSM 39-pHM2-*nuc*	2.7 ± 0.1	16.7 ± 1.2

CDM= Cell dry mass.

Table 25. PHAs production by *C. necator* DSM 545, *D. acidovorans* DSM 39 and their recombinant strains expressing *nuc* of *S. aureus.* Values represent the mean of three replicates and standard deviation is reported.

3.5.4. Reduction of cell lysates viscosity by recombinant *C. necator* DSM 545 pHM2-*nuc.*

C. necator DSM 545 pHM2-*nuc* exhibiting relatively high levels of nuclease activity and confirming the innate aptitude to accumulate extraordinary levels of PHAs (Table 25) was selected as promising microbe for further studies. In particular, both recombinant and parental strains were then grown in batch experiments using the above described two-steps method. After PHAs accumulation phase, the cells were homogenized at 0.25, 0.5, 0.75, 1.5 and 2.5 kbar, to release the PHAs. For the wild type strain, homogenization took place with or without Benzonase® addition. The viscosities of the lysates are reported in Fig. 31a, as a function of the pressure applied during

homogenization. Protein content of lysate samples was also measured using the Bradford protein assay method, in order to relate the efficacy of cell lysis to each homogenization pressure (Fig. 31b).

(○) *C. necator* DSM 545; (■) *C. necator* DSM 545 pHM2-*nuc*; (▲): *C. necator* DSM 545 with added Benzonase.

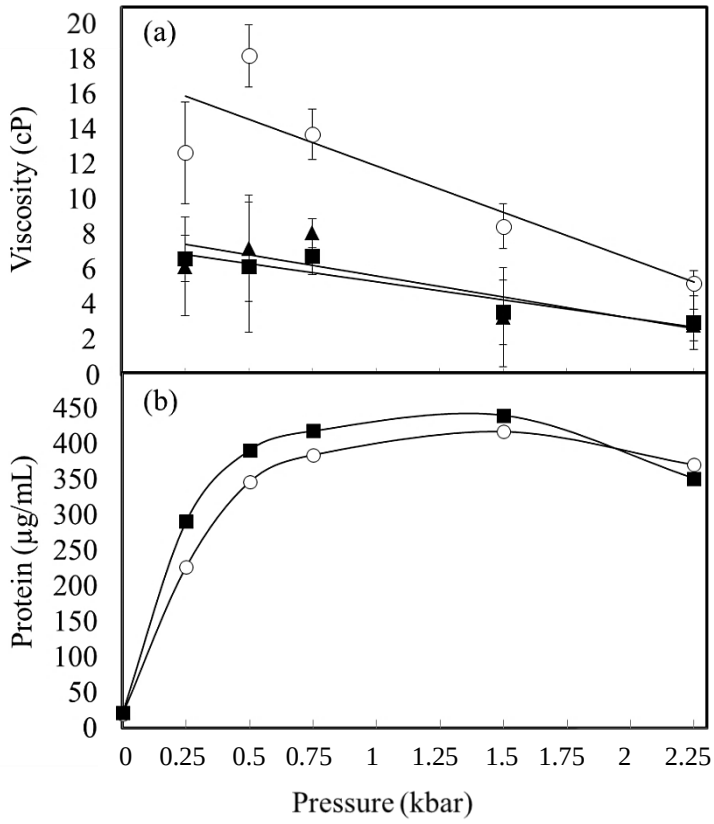


Fig. 31. Viscosities of cell lysates (a) and protein content for cell lysates (b) of *C. necator* DSM 545 and *C. necator* DSM 545 pHM2-*nuc*. Values are the mean of three replicates and error bars represent the standard deviation.

Cell disruption was assessed microscopically for both strains, confirming that the higher pressure the more cell debris was evident (Fig. 32).

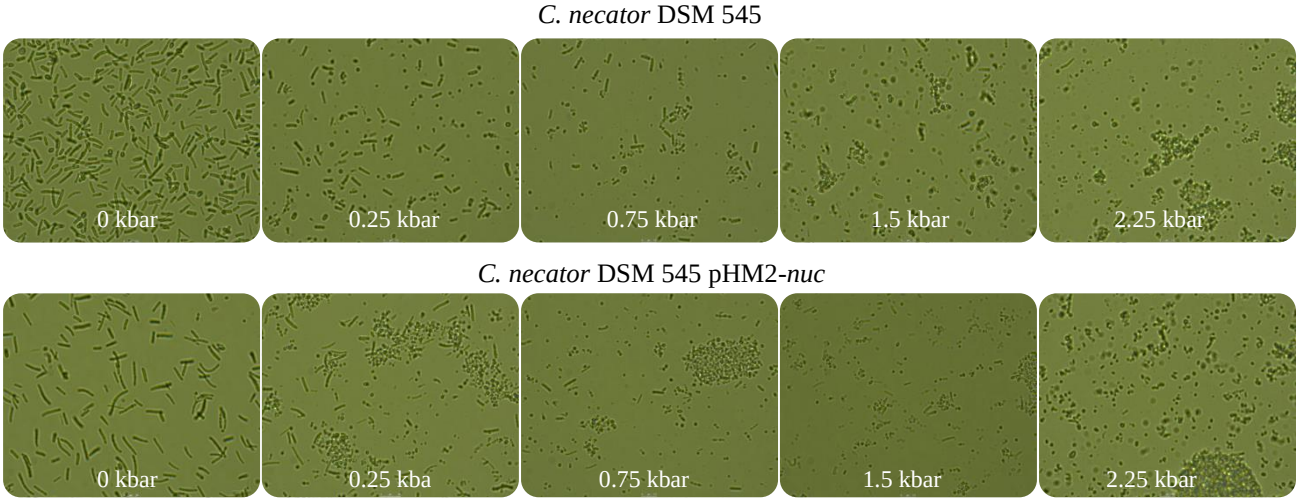


Fig. 32. *C. necator* DSM 545 and *C. necator* DSM 545 pHM2-*nuc* cells exposed to increasing pressures, under optical-microscope at 100X objective.

Protein concentrations increases with pressure until 1.5 kbar, reaching a maximum of 417.4 µg/mL. However, at 2.25 kbar the protein content decreased, presumably because stronger pressures may begin to denature measurable proteins.

In terms of viscosity, already at the lowest homogenization pressure (0.25 kbar), the recombinant strain *C. necator* DSM 545 pHM2-*nuc* was able to reduce viscosity levels of the lysate at similar values to *C. necator* DSM 545 with Benzonase® (positive control), maintaining the same tendency to all homogenization pressures.

This result indicates that recombinant nuclease was functionally produced in high-cell-density fermentations and that the use of such microbe eliminates the need for expensive commercial nuclease to reduce the viscosity of the cell lysate.

4. CONCLUSIONS.

The main obstacle of the PHAs production on a large scale is its high production cost, mainly influenced by both the expensive raw material and extraction/recovery technologies. Therefore, the use of agricultural and industrial by-products as PHAs feedstocks and the development of simple methods for the extraction of PHAs could represent a low cost sustainable alternative.

Wastes from slaughterhouses could represent an important carbon source for the PHAs production, because they are very cheap and largely available in EU. Unfortunately, these wastes are rich in fats and the scientific community has not found yet microorganisms with both high lipolytic activity and the ability to produce high PHAs quantities.

Furthermore, in this research study, the high pressure homogenization (HPH) was selected for the PHAs extraction, because is an economic method and is considered environmentally friendly. However, cell lysis causes the release of large amounts of chromosomal DNA which results in a dramatic increase in viscosity, hampering the following filtration and centrifugation steps. Drop in viscosity is generally achieved by the supplementation of commercial nucleases, being this extremely expensive.

Looking for a solution to these problems, the strategy here followed focused on two pillars. First, the genetic engineering of microorganisms with high PHAs production potential to obtain recombinants with high lipolytic activities. *C. necator* DSM 545 has been selected as host strain, because of being one of the most popular strains used in the PHAs production industry. The lipolytic genes *lipH-lipC* from *P. stutzeri* BT3 were used to develop the plasmid pBBR1MCS-5-*lipH-lipC* which then has been integrated into *C. necator* DSM 545 by the electroporation method. One promising recombinant with the highest lipolytic activity, namely *C. necator* DSM 545 *lip11*, was selected and efficiently used to process slaughterhouse waste streams into PHAs.

The recombinant strain showed interesting results, accumulating 3HB up to 29% CDM. With the aim of further reducing the production cost, a two-steps procedure for PHAs production has been developed, using membrane caul fat and glucose in the first and second phase, respectively. In the second step, glucose and nitrogen concentrations have been optimized and two different volumes tested. The results so far obtained seem to indicate that the recombinant strain *C. necator* DSM 545 *lip11* could have great potential as PHAs producer from fatty waste materials.

In a second research pillar, the staphylococcal nuclease gene *nuc* from *S. aureus* has been used to develop the plasmid pHM2-*nuc* which then has been integrated into two well-known PHAs producing strains *C. necator* DSM 545 and *D. acidovorans* DSM 39. The *C. necator* DSM 545

pHM2-*nuc* recombinant with the highest nuclease activity has been selected for future experiments. Finally, the recombinant strain was used for the viscosity reduction test, homogenizing the cells by HPH after the PHAs accumulation step. The viscosities of the lysates of *C. necator* recombinant cells were greatly reduced without the need of expensive commercial nucleases. As such, this is an interesting novelty on the reduction of viscosity of cell lysates by an engineered *C. necator* strain producing high 3HB levels.

Overall, this research work showed important preliminary results that could be improved in the future by using more up-to-date fermentation equipments. Furthermore, it has been demonstrated that downstream and upstream processes of PHA production can be further improved and optimized in order to increase the production of PHAs at lower costs, to finally facilitate their introduction in the market and replace the synthetic polymers.

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6. REFERENCES.

Abbondanzi, F., Biscaro, G., Carvalho, G., Favaro, L., Lemos, P., Paglione, M., Samorì, C., Torri, C., 2017. Fast method for the determination of short-chain-length polyhydroxyalkanoates (scl-PHAs) in bacterial samples by In Vial-Thermolysis (IVT). New biotechnology 39, 29–35.

Abdullah, A.A.-A., 2015. Microbial-based polyhydroxyalkanoates. Smithers Rapra.

Aldor, I.S., Keasling, J.D., 2003. Process design for microbial plastic factories: metabolic engineering of polyhydroxyalkanoates. Current opinion in biotechnology 14, 475–483.

Alsafadi, D., Al-Mashaqbeh, O., 2017. A one-stage cultivation process for the production of poly-3-(hydroxybutyrate-*co*-hydroxyvalerate) from olive mill wastewater by *Haloferax mediterranei*. New biotechnology 34, 47–53.

Amirul, A.A., Yahya, A.R.M., Sudesh, K., Azizan, M.N.M., Majid, M.I.A., 2008. Biosynthesis of poly (3-hydroxybutyrate-*co*-4-hydroxybutyrate) copolymer by *Cupriavidus* sp. USMAA1020 isolated from Lake Kulim, Malaysia. Bioresource technology 99, 4903–4909.

Anderson, A.J., Dawes, E.A., 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiological reviews 54, 450–472.

Aneja, K.K., Ashby, R.D., Solaiman, D.K.Y., 2009. Altered composition of *Ralstonia eutropha* poly (hydroxyalkanoate) through expression of PHA synthase from *Allochromatium vinosum* ATCC 35206. Biotechnology letters 31, 1601–1612.

Angkawidjaja, C., Kanaya, S., 2006. Family I. 3 lipase: bacterial lipases secreted by the type I secretion system. Cellular and molecular life sciences CMLS 63, 2804–2817.

Anis, S.N.S., Iqbal, N.M., Kumar, S., Amirul, A.-A., 2013. Effect of different recovery strategies of P (3HB-*co*-3HHx) copolymer from *Cupriavidus necator* recombinant harboring the PHA synthase of *Chromobacterium* sp. USM2. Separation and purification technology 102, 111–117.

Anjum, A., Zuber, M., Zia, K.M., Noreen, A., Anjum, M.N., Tabasum, S., 2016. Microbial production of polyhydroxyalkanoates (PHAs) and its copolymers: a review of recent advancements. International journal of biological macromolecules 89, 161–174.

Anne, B., 2011. Environmental-friendly biodegradable polymers and composites, in: integrated waste management-volume I. InTech.

Arber, W., Linn, S., 1969. DNA modification and restriction. Annual review of biochemistry 38, 467–500.

Arpigny, J.L., Jaeger, K.-E., 1999. Bacterial lipolytic enzymes: classification and properties. Biochemical journal 343, 177.

Atkinson, B., Mavituna, F., 1991. Biochemical engineering and biotechnology handbook. Stockton.

Atlić, A., Koller, M., Scherzer, D., Kutschera, C., Grillo-Fernandes, E., Horvat, P., Chiellini, E., Braunegg, G., 2011. Continuous production of poly ([R]-3-hydroxybutyrate) by *Cupriavidus necator* in a multistage bioreactor cascade. Applied microbiology and biotechnology 91, 295–304.

Ayub, N.D., Pettinari, M.J., Ruiz, J.A., López, N.I., 2004. A polyhydroxybutyrate-producing *Pseudomonas* sp. isolated from antarctic environments with high stress resistance. Current microbiology 49, 170–174.

Azapagic, A., Emsley, A., Hamerton, I., 2003. Polymers: the environment and sustainable development. John Wiley & Sons.

Aziz, N.A., Huong, K.-H., Sipaut, C.S., Amirul, A.A., 2017. A fed-batch strategy to produce high poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxybutyrate) terpolymer yield with enhanced mechanical properties in bioreactor. Bioprocess and biosystems engineering 40, 1643–1656.

Aziz, N.A., Sipaut, C.S., Abdullah, A.A., 2012. Improvement of the production of poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-4-hydroxybutyrate) terpolyester by manipulating the culture condition. Journal of chemical technology and biotechnology 87, 1607–1614.

Baptist, J.N., 1962. assignor to WR Grace & Co. US Patents 3, 1962.

Bera, A., Dubey, S., Bhayani, K., Mondal, D., Mishra, S., Ghosh, P.K., 2015. Microbial synthesis of polyhydroxyalkanoate using seaweed-derived crude levulinic acid as co-nutrient. International journal of biological macromolecules 72, 487–494.

Berezina, N., 2013. Novel approach for productivity enhancement of polyhydroxyalkanoates (PHA) production by *Cupriavidus necator* DSM 545. New biotechnology 30, 192–195.

Berger, E., Ramsay, B.A., Ramsay, J.A., Chavarie, C., Braunegg, G., 1989. PHB recovery by hypochlorite digestion of non-PHB biomass. Biotechnology techniques 3, 227–232.

Bhattacharya, A., 2000. Radiation and industrial polymers. Progress in polymer science 25, 371–401.

Boynton, Z.L., Koon, J.J., Brennan, E.M., Clouart, J.D., Horowitz, D.M., Gerngross, T.U., Huisman, G.W., 1999. Reduction of cell lysate viscosity during processing of poly(3-hydroxyalkanoates) by chromosomal integration of the staphylococcal nuclease gene in *Pseudomonas putida*. Applied and environmental microbiology 65, 1524–1529.

Brakstad, O.G., Aasbakk, K., Maeland, J.A., 1992. Detection of *Staphylococcus aureus* by polymerase chain reaction amplification of the *nuc* gene. Journal of clinical microbiology 30,

1654–1660.

Braunegg, G., Bona, R., Koller, M., 2004. Sustainable polymer production. Polymer-plastics technology and engineering 43, 1779–1793.

Braunegg, G., Lefebvre, G., Genser, K.F., 1998. Polyhydroxyalkanoates, biopolyesters from renewable resources: physiological and engineering aspects. Journal of biotechnology 65, 127–161.

Braunegg, G., Sonnleitner, B.Y., Lafferty, R.M., 1978. A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. Applied microbiology and biotechnology 6, 29–37.

Bresan, S., Sznajder, A., Hauf, W., Forchhammer, K., Pfeiffer, D., Jendrossek, D., 2016. Polyhydroxyalkanoate (PHA) granules have no phospholipids. Scientific reports 6, 26612.

Brzostowicz, P., Blasko, M., Rouvière, P., 2002. Identification of two gene clusters involved in cyclohexanone oxidation in *Brevibacterium epidermidis* strain HCU. Applied microbiology and biotechnology 58, 781–789.

Bugnicourt, E., Cinelli, P., Lazzeri, A., Alvarez, V.A., 2014. Polyhydroxyalkanoate (PHA): Review of synthesis, characteristics, processing and potential applications in packaging.

Burk, M.J., Van Dien, S.J., Burgard, A.P., Niu, W., 2011. Compositions and methods for the biosynthesis of 1, 4-butanediol and its precursors.

Casella, S., Favaro, L., Basaglia, M., 2016. Bacterial genetic modifications for improving polyhydroxyalkanoates production from inexpensive carbon sources, in: Koller, M. (Ed.), Recent advances in biotechnology volume 1: Microbial biopolyester production, performance and processing: microbiology, feedstocks, and metabolism. Bentham eBooks, pp. 380–435.

Campanari, S., e Silva, F.A., Bertin, L., Villano, M., Majone, M., 2014. Effect of the organic loading rate on the production of polyhydroxyalkanoates in a multi-stage process aimed at the valorization of olive oil mill wastewater. International journal of biological macromolecules 71, 34–41.

Castilho, L.R., Mitchell, D.A., Freire, D.M.G., 2009. Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation. Bioresource technology 100, 5996–6009.

Castro-Aguirre, E., Iñiguez-Franco, F., Samsudin, H., Fang, X., Auras, R., 2016. Poly (lactic acid)—Mass production, processing, industrial applications, and end of life. Advanced drug delivery reviews 107, 333–366.

Cavalheiro, J.M.B.T., de Almeida, M.C.M.D., Grandfils, C., Da Fonseca, M.M.R., 2009. Poly (3-

hydroxybutyrate) production by *Cupriavidus necator* using waste glycerol. Process biochemistry 44, 509–515.

Ch'ng, D.H.-E., Lee, W.-H., Sudesh, K., 2012. Biosynthesis and lipase-catalysed hydrolysis of 4-hydroxybutyrate-containing polyhydroxyalkanoates from *Delftia acidovorans*. Mal J Microbiol 9, 33–42.

Chandrasekhar, K., Amulya, K., Mohan, S.V., 2015. Solid phase bio-electrofermentation of food waste to harvest value-added products associated with waste remediation. Waste management 45, 57–65.

Che, X.-M., Ye, H.-M., Chen, G.-Q., 2018. Effects of uracil on crystallization and rheological property of poly (R-3-hydroxybutyrate-*co*-4-hydroxybutyrate). Composites Part A: Applied science and manufacturing 109, 141–150.

Chen, G.-Q., 2010. Plastics completely synthesized by bacteria: polyhydroxyalkanoates, in: Plastics from Bacteria. Springer, pp. 17–37.

Chen, Y., Yang, H., Zhou, Q., Chen, J., Gu, G., 2001. Cleaner recovery of poly (3-hydroxybutyric acid) synthesized in *Alcaligenes eutrophus*. Process biochemistry 36, 501–506.

Chesneau, O., El Solh, N., 1992. Nucleotide sequence of a *nuc* gene encoding the thermonuclease of *Staphylococcus intermedius*. Nucleic acids research 20, 5232.

Chohan, S.N., Copeland, L., 1998. Acetoacetyl coenzyme A reductase and polyhydroxybutyrate synthesis in rhizobium (Cicer) sp. strain CC 1192. Applied and environmental microbiology 64, 2859–2863.

Choi, J., Lee, S.Y., 1999. Efficient and economical recovery of poly (3-hydroxybutyrate) from recombinant *Escherichia coli* by simple digestion with chemicals. Biotechnology and bioengineering 62, 546–553.

Clausen, F.P., Dabelsteen, E., 1969. Increase in sensitivity of the rhodamine B method for keratinization by the use of fluorescent light. Acta pathologica microbiologica scandinavica 77, 169–171.

Colombo, B., Sciarria, T.P., Reis, M., Scaglia, B., Adani, F., 2016. Polyhydroxyalkanoates (PHAs) production from fermented cheese whey by using a mixed microbial culture. Bioresource technology 218, 692–699.

Cowie, J.M.G., Arrighi, V., 2007. Polymers: chemistry and physics of modern materials. CRC press.

Cruz, M. V, Freitas, F., Paiva, A., Mano, F., Dionísio, M., Ramos, A.M., Reis, M.A.M., 2016. Valorization of fatty acids-containing wastes and byproducts into short-and medium-chain

length polyhydroxyalkanoates. New biotechnology 33, 206–215.

Cruz, M. V, Sarraguça, M.C., Freitas, F., Lopes, J.A., Reis, M.A.M., 2015. Online monitoring of P (3HB) produced from used cooking oil with near-infrared spectroscopy. Journal of biotechnology 194, 1–9.

Cuatrecasas, P., Fuchs, S., Anfinsen, C.B., 1967. Catalytic properties and specificity of the extracellular nuclease of *Staphylococcus aureus*. The journal of biological chemistry 242, 1541–1547.

de Paula, F.C., Kakazu, S., de Paula, C.B.C., Gomez, J.G.C., Contiero, J., 2017. Polyhydroxyalkanoate production from crude glycerol by newly isolated *Pandoraea* sp. Journal of King Saud University-Science 29, 166–173.

Dietrich, K., Dumont, M.-J., Del Rio, L.F., Orsat, V., 2017. Producing PHAs in the bioeconomy—towards a sustainable bioplastic. Sustainable production and consumption 9, 58–70.

DiGregorio, B.E., 2009. Biobased performance bioplastic: Mirel. Chemistry & biology 16, 1–2.

Divyashree, M.S., Shamala, T.R., 2010. Extractability of polyhydroxyalkanoate synthesized by *Bacillus flexus* cultivated in organic and inorganic nutrient media. Indian journal of microbiology 50, 63–69.

Divyashree, M.S., Shamala, T.R., 2009. Effect of gamma irradiation on cell lysis and polyhydroxyalkanoate produced by *Bacillus flexus*. Radiation physics and chemistry 78, 147–152.

Divyashree, M.S., Shamala, T.R., Rastogi, N.K., 2009. Isolation of polyhydroxyalkanoate from hydrolyzed cells of *Bacillus flexus* using aqueous two-phase system containing polyethylene glycol and phosphate. Biotechnology and bioprocess engineering 14, 482–489.

Dong, Z., Sun, X., 2000. A new method of recovering polyhydroxyalkanoate from *Azotobacter chroococcum*. Chinese science bulletin 45, 252–256.

Du, C., Sabirova, J., Soetaert, W., Ki Carol Lin, S., 2012. Polyhydroxyalkanoates production from low-cost sustainable raw materials. Current chemical biology 6, 14–25.

Dubey, S., Bharmoria, P., Gehlot, P.S., Agrawal, V., Kumar, A., Mishra, S., 2017. 1-Ethyl-3-methylimidazolium diethylphosphate based extraction of bioplastic “polyhydroxyalkanoates” from bacteria: green and sustainable approach. ACS sustainable chemistry & engineering 6, 766–773.

Durfee, T., Nelson, R., Baldwin, S., Plunkett 3rd, G., Burland, V., Mau, B., Petrosino, J.F., Qin, X., Muzny, D.M., Ayele, M., Gibbs, R.A., Csorgo, B., Posfai, G., Weinstock, G.M., Blattner, F.R., 2008. The complete genome sequence of *Escherichia coli* DH10B: insights into the biology of

a laboratory workhorse. Journal of bacteriology 190, 2597–2606. https://doi.org/10.1128/JB.01695-07 [doi]

El-Hadi, A., Schnabel, R., Straube, E., Müller, G., Henning, S., 2002. Correlation between degree of crystallinity, morphology, glass temperature, mechanical properties and biodegradation of poly (3-hydroxyalkanoate) PHAs and their blends. Polymer testing 21, 665–674.

El Khattabi, M., Ockhuijsen, C., Bitter, W., Jaeger, K.-E., Tommassen, J., 1999. Specificity of the lipase-specific foldases of gram-negative bacteria and the role of the membrane anchor. Molecular and general genetics MGG 261, 770–776.

Elbahloul, Y., Steinbüchel, A., 2009. Large-scale production of poly (3-hydroxyoctanoic acid) by *Pseudomonas putida* GPo1 and a simplified downstream process. Applied and environmental microbiology 75, 643–651.

Fauré, N., Illanes, A., 2011. Immobilization of *Pseudomonas stutzeri* lipase for the transesterification of wood sterols with fatty acid esters. Applied biochemistry and biotechnology 165, 1332–1341.

Favaro, L., Basaglia, M., Casella, S., Hue, I., Dousset, X., de Melo Franco, B.D.G., Todorov, S.D., 2014. Bacteriocinogenic potential and safety evaluation of non-starter *Enterococcus faecium* strains isolated from home made white brine cheese. Food microbiology 38, 228–239.

Fendri, I., Chaari, A., Dhouib, A., Jlassi, B., Abousalham, A., Carrière, F., Sayadi, S., Abdelkafi, S., 2010. Isolation, identification and characterization of a new lipolytic *Pseudomonas* sp., strain AHD-1, from Tunisian soil. Environmental technology 31, 87–95.

Fickers, P., Nicaud, J.M., Gaillardin, C., Destain, J., Thonart, P., 2004. Carbon and nitrogen sources modulate lipase production in the yeast *Yarrowia lipolytica*. Journal of applied microbiology 96, 742–749.

Fiorese, M.L., Freitas, F., Pais, J., Ramos, A.M., de Aragão, G.M.F., Reis, M.A.M., 2009. Recovery of polyhydroxybutyrate (PHB) from *Cupriavidus necator* biomass by solvent extraction with 1, 2-propylene carbonate. Engineering in life sciences 9, 454–461.

Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J biol Chem 226, 497–509.

Fornasiero, P., Graziani, M., 2011. Renewable resources and renewable energy: a global challenge. CRC press.

Furrer, P., Panke, S., Zinn, M., 2007. Efficient recovery of low endotoxin medium-chain-length poly ([R]-3-hydroxyalkanoate) from bacterial biomass. Journal of microbiological methods 69, 206–213.

Gamero, J.E.R., Favaro, L., Pizzocchero, V., Lomolino, G., Basaglia, M., Casella, S., 2018. Nuclease expression in efficient polyhydroxyalkanoates-producing bacteria could yield cost reduction during downstream processing. Bioresource technology 261, 176–181.

Ghatnekar, M.S., Pai, J.S., Ganesh, M., 2002. Production and recovery of poly-3-hydroxybutyrate from *Methylobacterium* sp V49. Journal of chemical technology and biotechnology 77, 444–448.

Glogauer, A., Martini, V.P., Faoro, H., Couto, G.H., Müller-Santos, M., Monteiro, R.A., Mitchell, D.A., de Souza, E.M., Pedrosa, F.O., Krieger, N., 2011. Identification and characterization of a new true lipase isolated through metagenomic approach. Microbial cell factories 10, 54.

Gómez Cardozo, J.R., Mora Martínez, A.L., Yepes Pérez, M., Correa Londoño, G.A., 2016. Production and characterization of polyhydroxyalkanoates and native microorganisms synthesized from fatty waste. International journal of polymer science 2016.

Gourmelon, G., 2015. Global plastic production rises, recycling lags. New worldwatch institute analysis explores trends in global plastic consumption and recycling. Recovery from http://www. worldwatch. org.

Griffin, G.J.L., 1994. Chemistry and technology of biodegradable polymers. Blackie academic and professional.

Gupta, R., Gupta, N., Rathi, P., 2004. Bacterial lipases: an overview of production, purification and biochemical properties. Applied microbiology and biotechnology 64, 763–781.

Hahn, S.K., Chang, Y.K., Kim, B.S., Lee, K.M., Chang, H.N., 1993. The recovery of poly (3-hydroxybutyrate) by using dispersions of sodium hypochlorite solution and chloroform. Biotechnology techniques 7, 209–212.

Hahn, S.K., Chang, Y.K., Lee, S.Y., 1995. Recovery and characterization of poly (3-hydroxybutyric acid) synthesized in *Alcaligenes eutrophus* and recombinant *Escherichia coli*. Applied and environmental microbiology 61, 34–39.

Handrick, R., Reinhardt, S., Jendrossek, D., 2000. Mobilization of poly(3-hydroxybutyrate) in *Ralstonia eutropha*. Journal of bacteriology 182, 5916–5918.

Harrison, S.T.L., Chase, H.A., Amor, S.R., Bonthrone, K.M., Sanders, J.K.M., 1992. Plasticization of poly (hydroxybutyrate) in vivo. International journal of biological macromolecules 14, 50–56.

Hasan, F., Shah, A.A., Hameed, A., 2006. Industrial applications of microbial lipases. Enzyme and microbial technology 39, 235–251.

Hayward, A.C., Forsyth, W.G.C., Roberts, J.B., 1959. Synthesis and breakdown of poly-β-

hydroxybutyric acid by bacteria. Microbiology 20, 510–518.

He, W.N., Zhang, Z.M., Hu, P., Chen, C.Q., 1999. Microbial synthesis and characterization of polyhydroxyalkanoates by DG17 from glucose. Acta polymerica Sinica 6, 10.

Hejazi, P., Vasheghani-Farahani, E., Yamini, Y., 2003. Supercritical fluid disruption of *Ralstonia eutropha* for poly (β-hydroxybutyrate) recovery. Biotechnology progress 19, 1519–1523.

Hippe, H., 1967. Abbau und wiederverwertung von poly-β-hydroxybuttersäure durch *Hydrogenomonas* H 16. Archiv für mikrobiologie 56, 248–277.

Hippe, H., Schlegel, H.G., 1967. Hydrolyse von PHBS durch intracelluläre depolymerase von *Hydrogenomonas* H16. Archiv für mikrobiologie 56, 278–299.

Holmes, P.A., Lim, G.B., 1990. Separation process.

Hrabak, O., 1992. Industrial production of poly-β-hydroxybutyrate. FEMS microbiology letters 103, 251–255.

Hu, Y., Meng, J., Shi, C., Hervin, K., Fratamico, P.M., Shi, X., 2013. Characterization and comparative analysis of a second thermonuclease from *Staphylococcus aureus*. Microbiological research 168, 174–182.

Huong, K.-H., Kannusamy, S., Lim, S.Y.H., Amirul, A.A., 2015. Biosynthetic enhancement of single-stage poly (3-hydroxybutyrate-*co*-4-hydroxybutyrate) production by manipulating the substrate mixtures. Journal of industrial microbiology & biotechnology 42, 1291–1297.

Ibrahim, M.H.A., Steinbüchel, A., 2009. Poly (3-hydroxybutyrate) production from glycerol by *Zobellella denitrificans* MW1 via high-cell-density fed-batch fermentation and simplified solvent extraction. Applied and environmental microbiology 75, 6222–6231.

Jacquel, N., Lo, C.-W., Wei, Y.-H., Wu, H.-S., Wang, S.S., 2008. Isolation and purification of bacterial poly (3-hydroxyalkanoates). Biochemical engineering journal 39, 15–27.

Jambeck, J.R., Geyer, R., Wilcox, C., Siegler, T.R., Perryman, M., Andrady, A., Narayan, R., Law, K.L., 2015. Plastic waste inputs from land into the ocean. Science 347, 768–771.

James, B.W., Mauchline, W.S., Dennis, P.J., Keevil, C.W., Wait, R., 1999. Poly-3-hydroxybutyrate in *Legionella pneumophila*, an energy source for survival in low-nutrient environments. Applied and environmental microbiology 65, 822–827.

Jendrossek, D., 2005. Extracellular polyhydroxyalkanoate (PHA) depolymerases: the key enzymes of PHA degradation. Biopolymers online.

Jørgensen, S., Skov, K.W., Diderichsen, B., 1991. Cloning, sequence, and expression of a lipase gene from *Pseudomonas cepacia*: lipase production in heterologous hosts requires two Pseudomonas genes. Journal of bacteriology 173, 559–567.

Jung, I.L., Phyo, K.H., Kim, K.C., Park, H.K., Kim, I.G., 2005. Spontaneous liberation of intracellular polyhydroxybutyrate granules in *Escherichia coli*. Research in microbiology 156, 865–873.

Kapritchkoff, F.M., Viotti, A.P., Alli, R.C.P., Zuccolo, M., Pradella, J.G.C., Maiorano, A.E., Miranda, E.A., Bonomi, A., 2006. Enzymatic recovery and purification of polyhydroxybutyrate produced by *Ralstonia eutropha*. Journal of biotechnology 122, 453–462.

Kathiraser, Y., Aroua, M.K., Ramachandran, K.B., Tan, I.K.P., 2007. Chemical characterization of medium-chain-length polyhydroxyalkanoates (PHAs) recovered by enzymatic treatment and ultrafiltration. Journal of chemical technology and biotechnology 82, 847–855.

Kaur, G., Roy, I., 2015. Strategies for large-scale production of polyhydroxyalkanoates. Chemical and biochemical engineering quarterly 29, 157–172.

Kessler, B., Witholt, B., 1998. Synthesis, recovery and possible application of medium-chain-length polyhydroxyalkanoates: a short overview, in: macromolecular symposia. Wiley online library, pp. 245–260.

Khosravi-Darani, K., Vasheghani-Farahani, E., Shojaosadati, S.A., Yamini, Y., 2004. Effect of process variables on supercritical fluid disruption of *Ralstonia eutropha* cells for poly (R-hydroxybutyrate) recovery. Biotechnology progress 20, 1757–1765.

Kim, B.S., Lee, S.C., Lee, S.Y., Chang, H.N., Chang, Y.K., Woo, S.I., 1994. Production of poly (3-hydroxybutyric acid) by fed-batch culture of *Alcaligenes eutrophu*s with glucose concentration control. Biotechnology and bioengineering 43, 892–898.

Kim, C.-H., Khan, M., Morin, D.E., Hurley, W.L., Tripathy, D.N., Kehrli Jr, M., Oluoch, A.O., Kakoma, I., 2001. Optimization of the PCR for detection of *Staphylococcus aureus nuc* gene in bovine milk. Journal of dairy science 84, 74–83.

Kobayashi, T., Shiraki, M., Abe, T., Sugiyama, A., Saito, T., 2003. 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. Journal of bacteriology 185, 3485–3490.

Koller, M., 2016. Recent advances in biotechnology volume, 1: Microbial biopolyester production, performance and processing microbiology, feedstocks, and metabolism. Bentham science publishers.

Koller, M., Atlić, A., Dias, M., Reiterer, A., Braunegg, G., 2010. Microbial PHA production from waste raw materials, in: Plastics from bacteria. Springer, pp. 85–119.

Koller, M., Braunegg, G., 2015. Biomediated production of structurally diverse poly

(hydroxyalkanoates) from surplus streams of the animal processing industry. Polimery 60.

Koller, M., Maršálek, L., de Sousa Dias, M.M., Braunegg, G., 2017. Producing microbial polyhydroxyalkanoate (PHA) biopolyesters in a sustainable manner. New biotechnology 37, 24–38.

Koller, M., Niebelschütz, H., Braunegg, G., 2013. Strategies for recovery and purification of poly [(R)-3-hydroxyalkanoates] (PHA) biopolyesters from surrounding biomass. Engineering in life sciences 13, 549–562.

Koller, M., Salerno, A., Muhr, A., Reiterer, A., Chiellini, E., Casella, S., Horvat, P., Braunegg, G., 2012. Whey lactose as a raw material for microbial production of biodegradable polyesters, in: polyester. InTech.

Kourmentza, C., Ntaikou, I., Lyberatos, G., Kornaros, M., 2015. Polyhydroxyalkanoates from *Pseudomonas* sp. using synthetic and olive mill wastewater under limiting conditions. International journal of biological macromolecules 74, 202–210.

Kourmentza, C., Plácido, J., Venetsaneas, N., Burniol-Figols, A., Varrone, C., Gavala, H.N., Reis, M.A.M., 2017. Recent advances and challenges towards sustainable polyhydroxyalkanoate (PHA) production. Bioengineering 4, 55.

Krzan, A., Hemjinda, S., Miertus, S., Corti, A., Chiellini, E., 2006. Standardization and certification in the area of environmentally degradable plastics. Polymer degradation and stability 91, 2819–2833.

Kunasundari, B., Sudesh, K., 2011. Isolation and recovery of microbial polyhydroxyalkanoates. Express polymer letters 5.

Kurdikar, D.L., Strauser, F.E., Solodar, A.J., Paster, M.D., Asrar, J., 2000. Methods of PHA extraction and recovery using non-halogenated solvents.

Lafferty, R.M., Heinzle, E., 1979. Use of cyclic carbonic acid esters as solvents for poly-(β-hydroxybutyric acid).

Lakshman, K., Shamala, T.R., 2006. Extraction of polyhydroxyalkanoate from *Sinorhizobium meliloti* cells using *Microbispora* sp. culture and its enzymes. Enzyme and microbial technology 39, 1471–1475.

Lalucat, J., Bennasar, A., Bosch, R., García-Valdés, E., Palleroni, N.J., 2006. Biology of *Pseudomonas stutzeri*. Microbiology and molecular biology reviews 70, 510–547.

Lee, E.Y., Kang, S.H., Choi, C.Y., 1995. Biosynthesis of poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) by newly isolated *Agrobacterium* sp. SH-1 and GW-014 from structurally unrelated single carbon substrates. Journal of fermentation and bioengineering 79, 328–334.

Lee, K.M., Chang, H.N., Chang, Y.K., Kim, B.S., Hahn, S.K., 1993. The lysis of gram-negative *Alcaligenes eutrophus* and *Alcaligenes latus* by palmitoyl carnitine. Biotechnology techniques 7, 295–300.

Lee, S.Y., 1996. Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria. Trends in biotechnology 14, 431–438.

Lemoigne, M., 1923. Production d'acide β-oxybutyrique par certaines bact'eries du groupe du *Bacillus subtilis*. CR.Hebd.Seances Acad.Sci 176, 1761.

Li, F., Xie, G., Zhou, B., Yu, P., Yu, S., Aguilar, Z.P., Wei, H., Xu, H., 2016. Rapid and simultaneous detection of viable *Cronobacter sakazakii*, *Staphylococcus aureus*, and *Bacillus cereu*s in infant food products by PMA-mPCR assay with internal amplification control. LWT-Food science and technology 74, 176–182.

Li, W.C., Tse, H.F., Fok, L., 2016. Plastic waste in the marine environment: A review of sources, occurrence and effects. Science of the total environment 566, 333–349.

Li, X.-L., Zhang, W.-H., Wang, Y.-D., Dai, Y.-J., Zhang, H.-T., Wang, Y., Wang, H.-K., Lu, F.-P., 2014. A high-detergent-performance, cold-adapted lipase from *Pseudomonas stutzeri* PS59 suitable for detergent formulation. Journal of molecular catalysis B: enzymatic 102, 16–24.

Ling, Y., Wong, H.H., Thomas, C.J., Williams, D.R.G., Middelberg, A.P.J., 1998. Pilot-scale extraction of PHB from recombinant *E. coli* by homogenization and centrifugation. Bioseparation 7, 9–15.

Linn, S., Arber, W., 1968. Host specificity of DNA produced by *Escherichia coli*, X. In vitro restriction of phage fd replicative form. Proceedings of the national academy of sciences 59, 1300–1306.

Lomolino, G., Di Pierro, G., Lante, A., 2012. A quantitative fluorescence-based lipase assay. Food technology and biotechnology 50, 479.

Lozano Olmedo, C.E., 2018. Comparación del grado de inserción en polipropileno (PP), entre anhidrido acético y anhidrido maleico para su utilización en mezclas de plástico.

Madan, B., Mishra, P., 2010. Co-expression of the lipase and foldase of *Pseudomonas aeruginosa* to a functional lipase in *Escherichia coli*. Applied microbiology and biotechnology 85, 597–604.

Madison, L.L., Huisman, G.W., 1999. Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. Microbiology and molecular biology reviews : MMBR 63, 21–53.

Mantelatto, P.E., Durao, N.A.S., 2013. Process for extracting and recovering polyhydroxyalkanoates (PHAs) from cellular biomass.

Martínez, L.I.D.E., PRIETO, M.A., GARCÍA, P., 2009. Estudio bioquímico, genético y fisiológico de la degradación intracelular de polihidroxialcanoatos en *Pseudomonas putida*: aplicaciones biotecnológicas.

Martino, L., Cruz, M. V, Scoma, A., Freitas, F., Bertin, L., Scandola, M., Reis, M.A.M., 2014. Recovery of amorphous polyhydroxybutyrate granules from *Cupriavidus necator* cells grown on used cooking oil. International journal of biological macromolecules 71, 117–123.

Marudkla, J., Lee, W.-C., Wannawilai, S., Chisti, Y., Sirisansaneeyakul, S., 2018. Model of acetic acid-affected growth and poly (3-hydroxybutyrate) production by *Cupriavidus necator* DSM 545. Journal of biotechnology.

Mergaert, J., Anderson, C., Wouters, A., Swings, J., 1994. Microbial degradation of poly (3-hydroxybutyrate) and poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) in compost. Journal of environmental polymer degradation 2, 177–183.

Mergaert, J., Webb, A., Anderson, C., Wouters, A., Swings, J., 1993. Microbial degradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) in soils. Applied and environmental microbiology 59, 3233–3238.

Miertus, S., Ren, X., 2002. Environmentally degradable plastics and waste management. POLIMERY-WARSAW- 47, 545–550.

Mitomo, H., Hsieh, W.-C., Nishiwaki, K., Kasuya, K., Doi, Y., 2001. Poly (3-hydroxybutyrate-*co*-4-hydroxybutyrate) produced by *Comamonas acidovorans*. Polymer 42, 3455–3461.

Mittendorf, V., Robertson, E.J., Leech, R.M., Kruger, N., Steinbuchel, A., Poirier, Y., 1998. Synthesis of medium-chain-length polyhydroxyalkanoates in arabidopsis thaliana using intermediates of peroxisomal fatty acid beta-oxidation. Proceedings of the national academy of sciences of the United States of America 95, 13397–13402.

Modi, S.J., 2010. Assessing the feasibility of poly-(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) and poly-(lactic acid) for potential food packaging applications. The Ohio State University.

Mothes, G., Ackermann, J., 2005. Synthesis of poly (3-hydroxybutyrate-*co*-4-hydrobutyrate) with a target mole fraction of 4-Hydroxybutyric acid units by two-stage continuous cultivation of *Delftia acidovorans* P4a. Engineering in life sciences 5, 58–62.

Muhammadi, Shabina, Afzal, M., Hameed, S., 2015. Bacterial polyhydroxyalkanoates-eco-friendly next generation plastic: production, biocompatibility, biodegradation, physical properties and applications. Green chemistry letters and reviews 8, 56–77.

Muktar, M.Z., Rose, L. bt C., Amin, K.A.M., 2017. Formulation and optimization of virgin coconut

oil with Tween-80 incorporated in gellan gum hydrogel, in: AIP conference proceedings. AIP publishing, p. 20044.

Müller, R., 2005. Biodegradability of polymers: regulations and methods for testing. Biopolymers online.

Narasimhan, K., Cearley, A.C., Gibson, M.S., Welling, S.J., 2008. Process for the solvent-based extraction of polyhydroxyalkanoates from biomass.

Noda, I., 1998. Process for recovering polyhydroxyalkanotes using air classification.

Nollet, L., 2004. Handbook of food analysis: physical characterization and nutrient analysis. Vol. 1. CRC PressI Llc.

Nonato, R., Mantelatto, P., Rossell, C., 2001. Integrated production of biodegradable plastic, sugar and ethanol. Applied microbiology and biotechnology 57, 1–5.

Norhafini, H., Thinagaran, L., Shantini, K., Huong, K.-H., Syafiq, I.M., Bhubalan, K., Amirul, A.A., 2017. Synthesis of poly (3-hydroxybutyrate-*co*-4-hydroxybutyrate) with high 4HB composition and PHA content using 1, 4-butanediol and 1, 6-hexanediol for medical application. Journal of polymer research 24, 189.

Ntaikou, I., Peroni, C.V., Kourmentza, C., Ilieva, V.I., Morelli, A., Chiellini, E., Lyberatos, G., 2014. Microbial bio-based plastics from olive-mill wastewater: generation and properties of polyhydroxyalkanoates from mixed cultures in a two-stage pilot scale system. Journal of biotechnology 188, 138–147.

Obruca, S., Petrik, S., Benesova, P., Svoboda, Z., Eremka, L., Marova, I., 2014. Utilization of oil extracted from spent coffee grounds for sustainable production of polyhydroxyalkanoates. Applied microbiology and biotechnology 98, 5883–5890.

Obruca, S., Sedlacek, P., Koller, M., Kucera, D., Pernicova, I., 2017. Involvement of polyhydroxyalkanoates in stress resistance of microbial cells: Biotechnological consequences and applications. Biotechnology advances.

Oeding, V., Schlegel, H.G., 1973. Beta-ketothiolase from *Hydrogenomonas eutropha* H16 and its significance in the regulation of poly-beta-hydroxybutyrate metabolism. The Biochemical journal 134, 239–248.

Ong, S.Y., Zainab-L, I., Pyary, S., Sudesh, K., 2018. A novel biological recovery approach for PHA employing selective digestion of bacterial biomass in animals. Applied microbiology and biotechnology 1–11.

Page, W.J., Cornish, A., 1993. Growth of Azotobacter vinelandii UWD in fish peptone medium and simplified extraction of poly-β-hydroxybutyrate. Applied and environmental microbiology 59,

4236–4244.

Pais, J., Serafim, L.S., Freitas, F., Reis, M.A.M., 2016. Conversion of cheese whey into poly (3-hydroxybutyrate-*co*-3-hydroxyvalerate) by *Haloferax mediterranei*. New biotechnology 33, 224–230.

Penczek, S., 2018. Models of biopolymers by ring-opening polymerization: 0. CRC press.

Philip, S., Keshavarz, T., Roy, I., 2007. Polyhydroxyalkanoates: biodegradable polymers with a range of applications. Journal of chemical technology & biotechnology: International research in process, Environmental & clean technology 82, 233–247.

Pinsirodom, P., Parkin, K.L., 2001. Lipase assays. Current protocols in food analytical chemistry.

Posada, J.A., Naranjo, J.M., López, J.A., Higuita, J.C., Cardona, C.A., 2011. Design and analysis of poly-3-hydroxybutyrate production processes from crude glycerol. Process biochemistry 46, 310–317.

Povolo, S., Basaglia, M., Fontana, F., Morelli, A., Casella, S., 2015. Poly (hydroxyalkanoate) production by *Cupriavidus necator* from fatty waste can be enhanced by phaZ1 inactivation. Chemical and biochemical engineering Quarterly 29, 67–74.

Povolo, S., Casella, S., 2004. Poly-3-hydroxybutyrate has an important role for the survival of *Rhizobium tropici* under starvation. Annals of microbiology 54, 307–316.

Povolo, S., Romanelli, M.G., Basaglia, M., Ilieva, V.I., Corti, A., Morelli, A., Chiellini, E., Casella, S., 2013. Polyhydroxyalkanoate biosynthesis by *Hydrogenophaga pseudoflava* DSM1034 from structurally unrelated carbon sources. New biotechnology 30, 629–634.

Povolo, S., Romanelli, M.G., Fontana, F., Basaglia, M., Casella, S., 2012. Production of polyhydroxyalkanoates from fatty wastes. Journal of polymers and the environment 20, 944–949.

Povolo, S., Toffano, P., Basaglia, M., Casella, S., 2010. Polyhydroxyalkanoates production by engineered *Cupriavidus necator* from waste material containing lactose. Bioresource technology 101, 7902–7907.

Preusting, H., Kingma, J., Huisman, G., Steinbüchel, A., Witholt, B., 1993. Formation of polyester blends by a recombinant strain of *Pseudomonas oleovorans*: Different poly(3-hydroxyalkanoates) are stored in separate granules. Journal of environmental polymer degradation 1, 11–21. https://doi.org/10.1007/BF01457649

Quillaguamán, J., Guzmán, H., Van-Thuoc, D., Hatti-Kaul, R., 2010. Synthesis and production of polyhydroxyalkanoates by halophiles: current potential and future prospects. Applied microbiology and biotechnology 85, 1687–1696.

Quinteros, R., Goodwin, S., Lenz, R.W., Park, W.H., 1999. Extracellular degradation of medium chain length poly (β-hydroxyalkanoates) by *Comamonas* sp. International journal of biological macromolecules 25, 135–143.

Ramsay, J.A., Berger, E., Ramsay, B.A., Chavarie, C., 1990. Recovery of poly-3-hydroxyalkanoic acid granules by a surfactant-hypochlorite treatment. Biotechnology techniques 4, 221–226.

Ramsay, J.A., Berger, E., Voyer, R., Chavarie, C., Ramsay, B.A., 1994. Extraction of poly-3-hydroxybutyrate using chlorinated solvents. Biotechnology techniques 8, 589–594.

Rasala, B.A., Mayfield, S.P., 2015. Photosynthetic biomanufacturing in green algae; production of recombinant proteins for industrial, nutritional, and medical uses. Photosynthesis research 123, 227–239.

Ray, S., Prajapati, V., Patel, K., Trivedi, U., 2016. Optimization and characterization of PHA from isolate *Pannonibacter phragmitetus* ERC8 using glycerol waste. International journal of biological macromolecules 86, 741–749.

Reddy, C.S.K., Ghai, R., Kalia, V., 2003. Polyhydroxyalkanoates: an overview. Bioresource technology 87, 137–146.

Reddy, M.M., Vivekanandhan, S., Misra, M., Bhatia, S.K., Mohanty, A.K., 2013. Biobased plastics and bionanocomposites: Current status and future opportunities. Progress in polymer science 38, 1653–1689.

Rehm, B.H.A., 2007. Biogenesis of microbial polyhydroxyalkanoate granules: a platform technology for the production of tailor-made bioparticles. Current issues in molecular biology 9, 41.

Reinecke, F., Steinbüchel, A., 2009. *Ralstonia eutropha* strain H16 as model organism for PHA metabolism and for biotechnological production of technically interesting biopolymers. Journal of molecular microbiology and biotechnology 16, 91–108.

Riedel, S.L., Jahns, S., Koenig, S., Bock, M.C.E., Brigham, C.J., Bader, J., Stahl, U., 2015. Polyhydroxyalkanoates production with *Ralstonia eutropha* from low quality waste animal fats. Journal of biotechnology 214, 119–127.

Rito-Palomares, M., 2004. Practical application of aqueous two-phase partition to process development for the recovery of biological products. Journal of Chromatography B 807, 3–11.

Rittié, L., Perbal, B., 2008. Enzymes used in molecular biology: a useful guide. Journal of cell communication and signaling 2, 25–45.

Rodrigues, D., Rocha-Santos, T.A.P., Gomes, A.M., Goodfellow, B.J., Freitas, A.C., 2012. Lipolysis in probiotic and synbiotic cheese: The influence of probiotic bacteria, prebiotic

compounds and ripening time on free fatty acid profiles. Food chemistry 131, 1414–1421.

Rodriguez-Perez, S., Serrano, A., Pantión, A.A., Alonso-Fariñas, B., 2018. Challenges of scaling-up PHA production from waste streams. A review. Journal of environmental management 205, 215–230.

Romanelli, M.G., Povolo, S., Favaro, L., Fontana, F., Basaglia, M., Casella, S., 2014. Engineering *Delftia acidovorans* DSM39 to produce polyhydroxyalkanoates from slaughterhouse waste. International journal of biological macromolecules 71, 21–27.

Rosenau, F., Tommassen, J., Jaeger, K., 2004. Lipase-specific foldases. Chembiochem 5, 152–161.

Rosenberg, E., Ron, E.Z., 1999. High-and low-molecular-mass microbial surfactants. Applied microbiology and biotechnology 52, 154–162.

Rosman, C.W.K., Pastrana, F.R., Buist, G., Heuker, M., van Oosten, M., McNamara, J.O., van Dam, G.M., van Dijl, J.M., 2018. Ex vivo tracer efficacy in optical imaging of *Staphylococcus Aureus* nuclease activity. Scientific reports 8, 1305.

Ryan, P.G., 2015. A brief history of marine litter research, in: Marine Anthropogenic Litter. Springer, pp. 1–25.

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

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

Sambrook, J., 2001. Preparation and analysis of eukaryotic genomic DNA. Molecular cloning 6.1-6.5.

Samorì, C., Basaglia, M., Casella, S., Favaro, L., Galletti, P., Giorgini, L., Marchi, D., Mazzocchetti, L., Torri, C., Tagliavini, E., 2015. Dimethyl carbonate and switchable anionic surfactants: two effective tools for the extraction of polyhydroxyalkanoates from microbial biomass. Green chemistry 17, 1047–1056.

Sangeetha, R., Arulpandi, I., Geetha, A., 2011. Bacterial lipases as potential industrial biocatalysts: an overview. Research journal of microbiology 6, 1.

Schaumburg, F., Pauly, M., Schubert, G., Shittu, A., Tong, S., Leendertz, F., Peters, G., Becker, K., 2014. Characterization of a novel thermostable nuclease homolog (NucM) in a highly divergent *Staphylococcus aureus* clade. Journal of clinical microbiology 52, 4036–4038.

Selke, S.E.M., Culter, J.D., 2016. Plastics packaging: properties, processing, applications, and regulations. Carl Hanser Verlag GmbH Co KG.

Shibata, H., Kato, H., ODA, J., 1998. Molecular properties and activity of amino-terminal truncated forms of lipase activator protein. Bioscience, biotechnology, and biochemistry 62, 354–357.

Slonczewski, J.L., Foster, J.W., 2013. Microbiology: An Evolving Science: Third international student edition. WW Norton & Company.

Smith, H.O., Welcox, K.W., 1970. A restriction enzyme from *Hemophilus influenzae*: I. Purification and general properties. Journal of molecular biology 51, 379–391.

Steinbüchel, A., Hustede, E., Liebergesell, M., Pieper, U., Timm, A., Valentin, H., 1992. Molecular basis for biosynthesis and accumulation of polyhydroxyalkanoic acids in bacteria. FEMS microbiology letters 103, 217–230.

Sudesh, K., Abe, H., 2010. Practical guide to microbial polyhydroxyalkanoates. ISmithers.

Sudesh, K., Abe, H., Doi, Y., 2000. Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. Progress in polymer science 25, 1503–1555.

Suzuki, T., Yamane, T., Shimizu, S., 1986. Mass production of poly-β-hydroxybutyric acid by fed-batch culture with controlled carbon/nitrogen feeding. Applied microbiology and biotechnology 24, 370–374.

Taguchi, K., Aoyagi, Y., Matsusaki, H., Fukui, T., Doi, Y., 1999. Over-expression of 3-ketoacyl-ACP synthase III or malonyl-CoA-ACP transacylase gene induces monomer supply for polyhydroxybutyrate production in *Escherichia coli* HB101. Biotechnology letters 21, 579–584.

Tamer, I.M., Moo-Young, M., Chisti, Y., 1998a. Optimization of poly (β-hydroxybutyric acid) recovery from *Alcaligenes latus*: combined mechanical and chemical treatments. Bioprocess engineering 19, 459–468.

Tamer, I.M., Moo-Young, M., Chisti, Y., 1998b. Disruption of *Alcaligenes latus* for recovery of poly (β-hydroxybutyric acid): comparison of high-pressure homogenization, bead milling, and chemically induced lysis. Industrial & engineering chemistry research 37, 1807–1814.

Thinagaran, L., Sudesh, K., 2017. Evaluation of sludge palm oil as feedstock and development of efficient method for its utilization to produce polyhydroxyalkanoate. Waste and biomass valorization 1–12.

Ting, A.Y., Baker, D., Richter, F., Nivon, L., Liu, D.S.-C., 2016. Methods and compositions for protein labeling using lipoic acid ligases.

Titz, M., Kettl, K.-H., Shahzad, K., Koller, M., Schnitzer, H., Narodoslawsky, M., 2012. Process optimization for efficient biomediated PHA production from animal-based waste streams. Clean technologies and environmental policy 14, 495–503.

Torri, C., Cordiani, H., Samorì, C., Favaro, L., Fabbri, D., 2014. Fast procedure for the analysis of poly (hydroxyalkanoates) in bacterial cells by off-line pyrolysis/gas-chromatography with flame ionization detector. Journal of chromatography A 1359, 230–236.

Tsuge, T., Imazu, S., Takase, K., Taguchi, S., Doi, Y., 2004. An extra large insertion in the polyhydroxyalkanoate synthase from *Delftia acidovorans* DS-17: its deletion effects and relation to cellular proteolysis. FEMS microbiology letters 231, 77–83.

Urtuvia, V., Villegas, P., González, M., Seeger, M., 2014. Bacterial production of the biodegradable plastics polyhydroxyalkanoates. International journal of biological macromolecules 70, 208–213.

Valappil, S.P., Misra, S.K., Boccaccini, A.R., Keshavarz, T., Bucke, C., Roy, I., 2007. Large-scale production and efficient recovery of PHB with desirable material properties, from the newly characterised *Bacillus cereus* SPV. Journal of biotechnology 132, 251–258.

Valentin, H.E., Dennis, D., 1997. Production of poly (3-hydroxybutyrate-*co*-4-hydroxybutyrate) in recombinant *Escherichia coli* grown on glucose. Journal of biotechnology 58, 33–38.

Valentin, H.E., Steinbüchel, A., 1995. Accumulation of poly (3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid-*co*-4-hydroxyvaleric acid) by mutants and recombinant strains of *Alcaligenes eutrophus*. Journal of environmental polymer degradation 3, 169–175.

Valentino, F., Riccardi, C., Campanari, S., Pomata, D., Majone, M., 2015. Fate of *β*-hexachlorocyclohexane in the mixed microbial cultures (MMCs) three-stage polyhydroxyalkanoates (PHA) production process from cheese whey. Bioresource technology 192, 304–311.

Van Hee, P., Elumbaring, A.C.M.R., van der Lans, R.G.J.M., Van der Wielen, L.A.M., 2006. Selective recovery of polyhydroxyalkanoate inclusion bodies from fermentation broth by dissolved-air flotation. Journal of colloid and interface science 297, 595–606.

Van Wegen, R.J., Ling, Y., Middelberg, A.P.J., 1998. Industrial production of polyhydroxyalkanoates using *Escherichia coli*: an economic analysis. Chemical engineering research and design 76, 417–426.

Velasco-Lozano, S., Volke-Sepulveda, T., Favela-Torres, E., 2012. Lipases production by solid-state fermentation: the case of *Rhizopus homothallicus* in perlite, in: lipases and phospholipases. Springer, pp. 227–237.

Verlinden, R.A.J., Hill, D.J., Kenward, M.A., Williams, C.D., Piotrowska-Seget, Z., Radecka, I.K., 2011. Production of polyhydroxyalkanoates from waste frying oil by *Cupriavidus necator*. AMB express 1, 11.

Villano, M., Valentino, F., Barbetta, A., Martino, L., Scandola, M., Majone, M., 2014. Polyhydroxyalkanoates production with mixed microbial cultures: from culture selection to polymer recovery in a high-rate continuous process. New biotechnology 31, 289–296.

Volova, T.G., 2004. Polyhydroxyalkanoates-plastic materials of the 21st century: production, properties, applications. Nova publishers.

Wampfler, B., Ramsauer, T., Rezzonico, S., Hischier, R., Kohling, R., Thony-Meyer, L., Zinn, M., 2010. Isolation and purification of medium chain length poly (3-hydroxyalkanoates)(mcl-PHA) for medical applications using nonchlorinated solvents. Biomacromolecules 11, 2716–2723.

Wang, B., Sharma-Shivappa, R.R., Olson, J.W., Khan, S.A., 2012. Upstream process optimization of polyhydroxybutyrate (PHB) by *Alcaligenes latus* using two-stage batch and fed-batch fermentation strategies. Bioprocess and biosystems engineering 35, 1591–1602.

Wang, Y., Yin, J., Chen, G.-Q., 2014. Polyhydroxyalkanoates, challenges and opportunities. Current opinion in biotechnology 30, 59–65.

Wen, Q., Chen, Z., Tian, T., Chen, W., 2010. Effects of phosphorus and nitrogen limitation on PHA production in activated sludge. Journal of environmental sciences 22, 1602–1607.

Wolter, F., Klemm, J., Puchta, H., 2018. Efficient in planta gene targeting in *Arabidopsis* using egg cell-specific expression of the Cas9 nuclease of *Staphylococcus aureus*. The plant journal 94, 735–746.

Worm, B., Lotze, H.K., Jubinville, I., Wilcox, C., Jambeck, J., 2017. Plastic as a persistent marine pollutant. Annual review of environment and resources 42, 1–26.

Xie, W.P., Chen, G.-Q., 2008. Production and characterization of terpolyester poly (3-hydroxybutyrate-*co*-4-hydroxybutyrate-*co*-3-hydroxyhexanoate) by recombinant *Aeromonas hydrophila* 4AK4 harboring genes phaPCJ. Biochemical engineering journal 38, 384–389.

Yamane, T., 1993. Yield of poly-D (-)-3-hydroxybutyrate from various carbon sources: A theoretical study. Biotechnology and bioengineering 41, 165–170.

Yang, S., Huang, Z., Jiang, Z., Li, L., 2008. Partition and purification of a thermostable xylanase produced by *Paecilomyces thermophila* in solid-state fermentation using aqueous two-phase systems. Process biochemistry 43, 56–61.

Ye, J., Hu, D., Che, X., Jiang, X., Li, T., Chen, J., Zhang, H.M., Chen, G.-Q., 2018. Engineering of *Halomonas bluephagenesis* for low cost production of poly (3-hydroxybutyrate-*co*-4-hydroxybutyrate) from glucose. Metabolic engineering 47, 143–152.

Yu, J., Chen, L.X.L., 2006. Cost-effective recovery and purification of polyhydroxyalkanoates by

selective dissolution of cell mass. Biotechnology progress 22, 547–553.

Zheng, L.Z., Li, Z., Tian, H.-L., Li, M., Chen, G.-Q., 2005. Molecular cloning and functional analysis of (R)-3-hydroxyacyl-acyl carrier protein: coenzyme A transacylase from *Pseudomonas mendocina* LZ. FEMS microbiology letters 252, 299–307.

Zinn, M., Weilenmann, H., Hany, R., Schmid, M., Egli, T.H., 2003. Tailored synthesis of poly ([R]-3-hydroxybutyrate-*co*-3-hydroxyvalerate)(PHB/HV) in *Ralstonia eutropha* DSM 428. Engineering in life sciences 23, 309–316.