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# Study of *Halomonas* sp. SF2003 biotechnological potential: Application to PolyHydroxyAlkanoates (PHA) production

Tatiana Thomas

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# THESE DE DOCTORAT DE

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Par **Tatiana THOMAS**

**Étude du potentiel biotechnologique de *Halomonas* sp. SF2003 :  
Application à la production de PolyHydroxyAlcanoates (PHA).**

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*« Failure is only the opportunity to begin again more intelligently. »*

*Henry Ford*

*« I dettagli fanno la perfezione e la perfezione non è un dettaglio. »*

*Leonardo Da Vinci*



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---

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# Préface

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De nos jours, l'exploitation des matériaux plastiques (PP, PE, PVC, ...) s'est imposée dans tous les secteurs industriels et de la vie courante. Leur faible coût associé à leurs propriétés physico-chimiques (légèreté, durabilité, transparence, ...) leur confèrent de nombreux avantages en comparaison des autres types de matériaux (verre, bois, métal,...) et les ont rendu indispensables. La production mondiale n'a, en conséquence, pas cessé de progresser ces dernières décennies et a atteint le niveau record de 359 millions de tonnes en 2018 soit l'équivalent de 11,38 tonnes produites par seconde. À l'heure où la préservation de l'Environnement est une problématique mondiale prioritaire, la production et la fin de vie de ces matériaux soulèvent de nombreuses controverses, leur persistance dans le milieu naturel s'étendant sur des dizaines d'années. Bien que des moyens de préventions et de traitements aient été développés pour limiter la pollution liée à l'utilisation de plastiques (à usage unique), leur accumulation dans la nature, y compris dans les océans, est alarmante. De plus, la majeure partie des plastiques conventionnels étant issus de l'industrie pétrochimique, l'amenuisement des réserves pétrolières pose la question de la pérennité de la production.

La recherche de solutions alternatives, durables et respectueuses de l'Environnement s'est donc intensifiée ces dernières années conduisant au développement des biopolymères et notamment de ceux étant à la fois biosourcés (issus de ressources naturelles renouvelables) et biodégradables tels que les PolyHydroxyAlcanoates (PHA).

Dans le même temps, l'intérêt porté au milieu marin, a orienté naturellement les recherches vers cet environnement, véritable gisement de molécules et/ou de substances aux propriétés spécifiques : lipides, antioxydants ou encore biopolymères dont les exopolysaccharides (EPS) ou les polyhydroxyalcanoates (PHA), produits notamment par des (micro)organismes halophiles.

Fort de ces constats, l'Institut de Recherche Dupuy de Lôme (IRDLD) et le Laboratoire de Biotechnologies Marines (LBCM) de l'Université Bretagne Sud, ont développé conjointement des compétences permettant l'exploitation et la valorisation de ressources naturelles, y compris issues du

milieu marin, pour répondre à la problématique du remplacement des plastiques conventionnels par des matériaux biosourcés.

Les travaux présentés dans ce manuscrit se situent à l'interface de ces deux domaines et s'intègrent totalement dans la thématique de recherche de l'équipe « Ingénierie des biopolymères » de l'IRDL dont l'activité se concentre, en particulier, sur la production à façon de PHA. À travers la mise en œuvre de différentes techniques d'étude, allant de la biologie moléculaire jusqu'aux procédés d'extraction, les polymères produits répondent à des critères précis. L'étude menée, ici, vise à caractériser la bactérie marine, *Halomonas* sp. SF2003, isolée de mollusques pêchés sur les côtes bretonnes, en vue d'exploiter son potentiel biotechnologique, et notamment, sa capacité de production de PHA. Des précédents travaux développés au Laboratoire dans le cadre de projets collaboratifs (BIOCOMBA, PHAPACK et BLUECOPHA) axés sur une montée en échelle de la production de PHA, avaient laissé présager du caractère prometteur de cette souche bactérienne.

Le premier chapitre de cette étude dresse un état des lieux de la diversité des (micro-)organismes marins et de leur potentiel pour le secteur des biotechnologies au regard de leurs capacités d'adaptation et de production de composés d'intérêts. Dans un second temps, l'accent est mis sur le potentiel d'une famille de composés, les polyhydroxyalcanoates, qui s'imposent comme une alternative aux matériaux plastiques conventionnels.

Le second chapitre introduit la partie expérimentale en décrivant l'ensemble des techniques mises en œuvre pour la réalisation de cette étude.

La section « Résultats » est présentée en trois chapitres. Le chapitre III relate la caractérisation génétique et métabolique de la souche *Halomonas* sp. SF2003, le séquençage du génome et son annotation nous ayant permis de classer la souche et de cibler certains phénotypes. Cette partie sert de base à la caractérisation de la souche et de son métabolisme. Le chapitre IV se focalise sur l'étude de la versatilité de la souche vis-à-vis des sources de carbone afin d'identifier les substrats les plus adaptés à sa croissance et à la production de PHA. La compréhension de la biosynthèse des PHA chez *Halomonas* sp. SF2003 est également approfondie par l'étude des enzymes clés des voies métaboliques empruntées : les PHA synthases PhaC1 et PhaC2. Finalement, le chapitre V étudie l'impact de différents facteurs



(sources de carbone et salinité) sur la production de PHA, et plus spécialement sur la productivité de la souche. Cette partie a également permis d'initier la mise au point d'une méthode de suivi, en temps réel, de la production de PHA.

À termes, l'ensemble des résultats obtenus permettront d'optimiser la production de PHA par *Halomonas* sp. SF2003 afin d'exploiter en totalité le potentiel biotechnologique de cette souche.

Une partie des travaux ayant été réalisée en collaboration avec l'équipe du Pr. Kumar SUDESH, de l'EcoBiomaterial Research Lab de l'Université Sains Malaysia (School of Biological Sciences) et un des chapîtres ayant déjà été publié, l'intégralité de la partie résultat de ce manuscrit est présentée sous le format « article », en anglais.

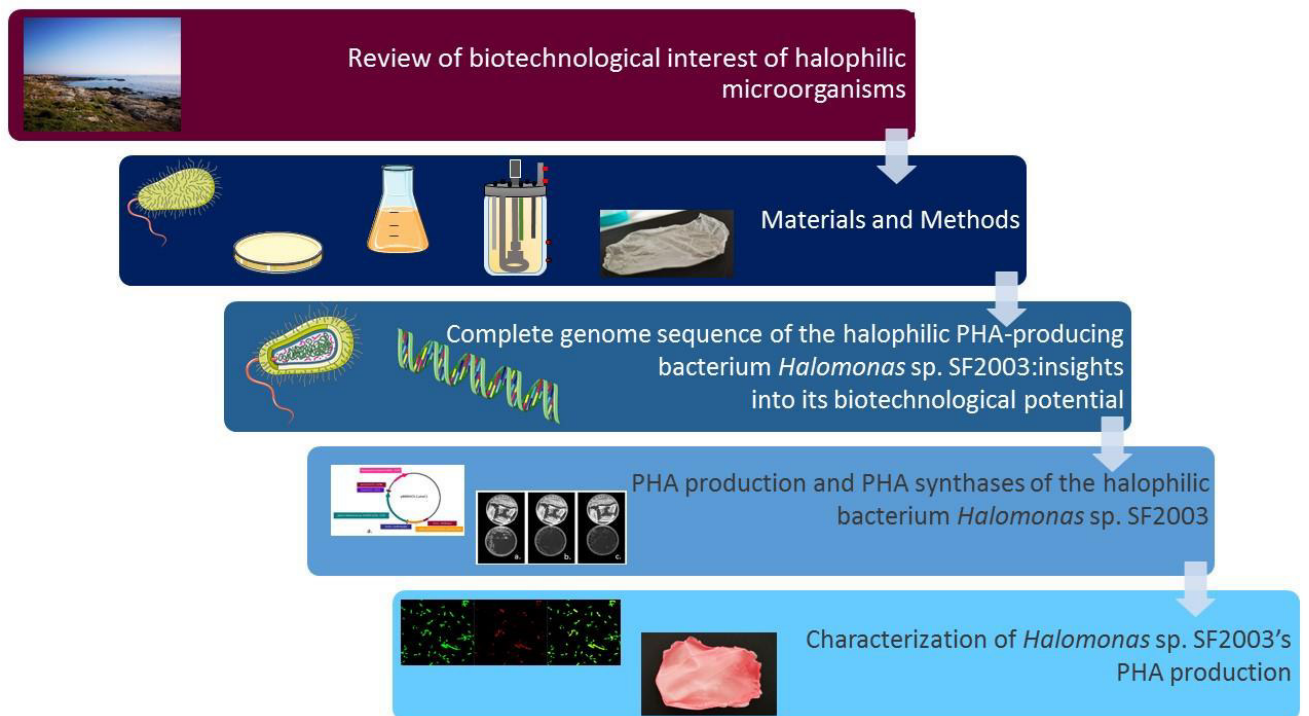


Figure 1: Représentation schématique du contenu de la thèse.



# **Chapter 1 State of the art**

## **Halotolerant/Halophilic microorganisms and their biotechnological potential**

---

## Introduction

Halophiles and halotolerants are organisms living in saline or hypersaline environment, sometimes considered as extreme environments, and are encountered in the three domain of life: *Archaea*, *Bacteria* and *Eukarya*. Because they produce a diversity of valuable compounds and expose a strong adaptability in front of harsh environmental conditions, they are ardently studied for their use in various fields. This report discusses the interest of using halophilic and halotolerant microorganisms in biotechnological process. In a first part, microbial communities and their natural environment will be presented, then some examples of biotechnological applications using halophiles or halotolerant will be exposed. Finally, and based on data described in the previous part, a particular interest will be given to PolyHydroxyAlkanoates (PHA). Usually, several studies are still carried out in order to demonstrate that the use of halophile/halotolerant bacteria could be an interesting and promising way for a sustainable production of these eco-friendly bioplastics. It will be demonstrated that an important effort is given to find new strains, exploit different by-products, optimize systems and increase yield of production in order to reduce cost of production and facilitate PHA extraction and purification steps.

### I. Microbial communities

#### I.1 Habitats and characteristics

Hypersaline environments are characterized by a concentration of salts higher than 10-12%, caused by dissolution of minerals. These environments are widely distributed and include saline soils, inland salterns and aquatic habitats like saline lakes, salty marshy places, seas or marine salterns <sup>1,2</sup>. They are considered as representative of extreme environments because normal cell physiology cannot withstand the strong salt concentrations leading to considerable (micro-)organisms leaving inside, also, as representative of the primitive (microbial) population <sup>3,4</sup>. Indeed saline environments represent a source of (micro-)organisms, named halophiles or halotolerants, that can belong to the three domain of life: *Archaea*, *Bacterial/Prokarya* and *Eukarya* and expose interesting properties <sup>5</sup>. Organisms living in hypersaline environments are qualified of extremophilic because of their adaptability in front of constraining living conditions like high/low temperature, low oxygen availability, alkaline pH, or presence of heavy metals/toxic compounds <sup>4,6-8</sup>. Therefore, populations found in these environments are of great interest and are strongly studied for biotechnological applications. In this review, we will only discuss only about halotolerant and halophilic microorganisms belonging to *Archaea* and *Bacteria* domains.

Isolation and identification of microorganisms from these environments require special enrichment techniques, physiological and biochemical characterization but also special genotyping<sup>4</sup>. Numerous studies have been conducted to understand metabolic adaptation of halophilic microorganisms in front of these environments and, consequently, different classifications have been proposed. The most widely used is based on the optimal growth of microorganisms according to salt concentration<sup>2,4,6,7</sup>.

Accordingly, four categories of microorganisms have been defined depending on their salt concentration tolerance:

- “Non-halophiles bacteria”, which have the best growth in media with salt concentration lower than, or equal to, 0.2 M (1% (w/v)) of NaCl. Several bacteria of this category can also tolerate higher salt concentrations and are named halotolerant,
- “Slight halophiles”, for marine bacteria, which have best growth with salt concentration between 0.2 and 0.5 M (1% to 3% (w/v)) of NaCl,
- “Moderate halophiles” with optimal growing rate for salt concentrations from 0.5 to 2.5 M of NaCl (3% to 15% (w/v)),
- “Extreme halophiles” showing best growth for salt concentration from 2.5 to 5.2 M of NaCl (15%-30%(w/v))<sup>6,9</sup>.

Maintenance of the osmotic pressure on either side of the cell is function of two principal mechanisms depending on the category of the microorganism. Aerobic and halophilic *Archaea* and anaerobic halophilic bacteria accumulate inorganic ions, like KCl, to obtain a high salt concentration in order to balance the osmotic pressure. This mechanism is also known as « salt-in strategy ». This strategy requires an adaptation of the intracellular chemistry and proteins, including enzymes, to preserve their functionalities<sup>4,10,11</sup>. In comparison, the « salt-out strategy » is used by halophilic or halotolerant *Eubacteria*, *Eukarya* and by some methanogenic *Archaea*. This second mechanism consists in increasing the intracellular concentration of soluble organic solutes (or osmolytes) like amino acids and/or their derivatives, nitrogen-containing compounds, polyols and sugars and/or to expulse salts out of the cell. Organic solutes are non-ionic and show a high water solubility. Consequently, they do not disturb the cell metabolism. Accumulation of these compounds results from an uptake from the environment or *de novo* synthesis. The accumulated osmolytes also act for cell adaptation to cold, freezing conditions, heat or dessication and help for stabilization of biomolecules like DNA, enzymes or membranes. These molecules have low molecular weight and are classified into zwitterionic, non charged or anionic solutes. Zwitterionic solutes include amino acids and their derivatives (e.g. glycine betaine, ectoine or hydroxyectoine) whereas non charged solutes involve amino acids or carbohydrates such as N-acetylglutaminylglutamine amide, trehalose or sucrose. Finally, anionic solutes include L- $\alpha$ -glutamate, hydroxybutyrate (HB) and its polymerized form: poly- $\beta$ -hydroxybutyrate (PHB) which is the main representative of the polyhydroxyalkanoates (PHA) family<sup>4,10,11</sup>. Interestingly, a number of these

compounds are used in biotechnological fields for various applications. Examples of such applications, and particularly production of PHA, will be discussed later in this manuscript.

For a long time, only two groups have been differentiated: the moderately halophilic Bacteria and the extremely aerobic halophilic Archaea but recent studies allow isolation and identification of new species, genera or higher taxa <sup>7</sup>.

## I.2 Taxonomy

### I.2.1 Haloarchaea

At the present time and based on data available in the second edition of the Bergey's Manual of Systematic Bacteriology and on LPSN database <sup>12</sup>, Archaea includes a total of 5 phyla, 12 classes and 22 orders. Only the Euryarchaeota phylum includes halophilic Archaea grouped within different orders (**Table 1**) <sup>4,6,7,13,14</sup>.

Halophilic Archaea, also named haloarchaea, are included within the Halobacteria class (formely designated Halomebacteria). They belong to the *Halobacteriaceae* family within the *Halobacteriales* order. Identification and classification of haloarchaea is based on the description of typical features obtained by the study of phylogenetic characteristics (comparison of 16S rRNA gene sequences), genotypic and phenotypic characteristics (growth using different media and growth conditions) and/or polar lipid analysis. Indeed, haloarchaea produce ether-linked lipids which are typically used as key characteristics for differentiation of the members, especially at the genus level. The most relevant characteristics of halophilic Archaea are their requirement for high salt concentrations (up to 20-25% of NaCl) and their inability to grow in freshwater media, which result in cells lysis <sup>7</sup>. They are considered as the best adapted prokaryotes to hypersaline environments <sup>6</sup> as they can easily grow aerobically in media containing 20 to 25 % of NaCl. Additionally, some of them can optimally grow in alkaline medium and are consequently named haloalkaliphilic. Moreover, recent studies of hypersaline environments allow identification of halophilic methanogenic species.

Even if a majority of haloarchaea can grow in presence of oxygen, some of them are strictly anaerobic and obtained their energy by the formation of methane by dismutation of methyl compounds (methyl amines, methyl sulfides or methanol). This group of microorganisms, named methanogenic haloarchaea, is essential in hypersaline environment but only few species have been isolated, or are well described. For the moment, methanogenic *Archaea* are classified within the class *Methanomicrobia* of the Euryarchaeota phylum. They have been described in seven orders (**Table 1**), but only *Methanosarcinales* and *Methanomicrobiales* orders include halophilic species. Four genera included methanogenic haloarchaea: *Methanohalobium*, *Methanohalophilus*, *Methanosalsum* and *Methanocalculus*.

Table 1: Haloarchaea and Methanogenic Haloarchaea classification.  
Based on data of de la Haba *et al.*, 2011 and completed with data from other studies.

Phylum	Class	Orders	Family	Genus/Species
Euryarchaeota	Halobacteria (or Halomebacteria)	Halobacteriales	<i>Haloarculaceae</i>	<i>Haloarcula</i> <i>Halomicrobium</i> <i>Halorhabdus</i> <i>Halosimplex</i>
			<i>Halobacteriaceae</i>	<i>Haladaptatus</i> <i>Halalkalicoccus</i> <i>Halarchaeum</i> <i>Halobacterium</i> <i>Halorussus</i> <i>Natronoarchaeum</i> <i>Natronomonas</i> <i>Salarchaeum</i>
			<i>Halococcaceae</i>	<i>Halococcus</i>
	Haloferrales	<i>Haloferaceae</i>	<i>Haloferax</i> <i>Halogeometricum</i> <i>Halosarcina</i> <i>Haloplanus</i> <i>Halopelagius</i> <i>Haloquadratum</i> <i>Halobaculum</i> <i>Halogramum</i> <i>Halonotius</i> <i>Halorubrum</i>	
		<i>Halorubraceae</i>		
	Natrialbales	<i>Natrialbaceae</i>	<i>Halobiforma</i> <i>Halopiger</i> <i>Halostagnicola</i> <i>Haloterrigena</i> <i>Halovivax</i> <i>Natrialba</i> <i>Natrinema</i> <i>Natronobacterium</i> <i>Natronococcus</i> <i>Natronolimnobi</i> <i>Natronorubrum</i>	
Methanomicrobia	Methanosarcinales	Methanosarcinaceae	<i>Methanohalobium</i>	
			<i>Methanohalophylus</i>	
			<i>Methanosalsum</i>	
Methanomicrobiales	Unassigned	<i>Methanocalculus</i>		

### 1.2.2 Halophilic and halotolerant bacteria

Until now, domain of Bacteria groups 34 phyla which include 77 classes, but only the following phyla include halophilic bacteria: Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Proteobacteria, Spirochaetes, Tenericutes and Thermotogae. These phyla regroup moderately or extremely halophilic bacteria in contrast to halophilic *Archaea* which are mainly extremely halophilic species.

Halophilic Bacteria include a large number of heterogeneous species including Gram positive and Gram negative cocci or rods which are aerobic or anaerobic with a heterotrophic or phototrophic metabolism. The height phyla (**Table 2, 3, 4 and 5**) not only include halophilic genera/species but also halotolerant genera/species.

Actinobacteria phylum is divided in 6 classes and 48 families for a total of 218 species representing one of a major phylum of the domain. Among the different classes, only *Actinomycetales* order, from *Actinobacteria* class, contains halophilic species inside the following suborders: *Actinopolysporinae*, *Corynebacterineae*, *Glycomycineae*, *Micrococcineae*, *Pseudonocardineae* and *Streptoporangineae*.

Comparatively, three classes inside the Bacteroidetes phylum contain halophilic species: *Bacteroidia*, *Flavobacteriia* and *Sphingobacteriia*. For all these classes, only one family containing halophilic species is described: *Marinilabiaceae* (*Bacteroidia* class), *Flavobacteriaceae* family (*Flavobacteriia* class) and *Rhodothermaceae* (*Sphingobacteriia* class).

Cyanobacteria phylum (also named blue-green algae or blue-green bacteria or *Cyanophyta*) has a taxonomy not clearly defined yet, depending on the followed rules (bacteriological or botanical). Species inside this phylum expose interesting properties and are essential for photosynthesis of their natural environment of origin. Slightly halophilic bacterial species are only described in the *Oscillatoriales* order.

Traditionally three classes are described in the Firmicutes phylum: *Bacilli*, *Clostridia* and *Mollicutes* but recently the last one, *Mollicutes* class, was moved to the *Tenericutes* phylum (described below). Two new classes were added to the phylum: *Erysipelotrichi* and *Thermolithobacteria*. This phylum groups heterogenous microorganisms in morphologic and physiologic point of view.



Table 2: Taxonomy of Halobacteria (Part1).

Phylum	Class	Order	Family	Genus or Species
Actinobacteria	Actinobacteria	Actinomycetales	<i>Actinopolysporaceae</i>	<i>Actinopolyspora</i>
			<i>Corynebacterineae</i>	<i>Corynebacterium</i>
			<i>Glycomycetaceae</i>	<i>Haloglycomyces</i>
			<i>Jiangellaceae</i>	<i>Haloactinopolyspora</i>
			<i>Nocardiopsaceae</i>	<i>Haloactinospora</i> <i>Nocardiopsis</i> <i>Streptomonospora</i>
			<i>Pseudonocardiaceae</i>	<i>Amycolatopsis</i> <i>Haloechinothrix</i>
			<i>Bogoriellaceae</i>	<i>Georgenia</i>
			<i>Micrococcaceae</i>	<i>Nesterenkonkia</i> <i>Isoptericola</i> <i>Prauserella</i>
			<i>Promicromonosporaceae</i>	<i>Saccharomonospora</i> <i>Saccharopolyspora</i>
			<i>Ruaniaceae</i>	<i>Haloactinobacterium</i>
Bacteroidetes	<i>Bacteroidia</i>	<i>Bacteroidales</i>	<i>Marinilabiliaceae</i>	<i>Anaerophaga</i>
	<i>Flavobacteriia</i>	<i>Flavobacteriales</i>	<i>Flavobacteriaceae</i>	<i>Gramella</i> <i>Psychroflexus</i>
	<i>Sphingobacteriia</i>	<i>Rhodothermales (or Sphingobacteriales)</i>	<i>Rhodothermaceae (or Salinibacteraceae)</i>	<i>Salinibacter</i> <i>Salisaeta</i>
Cyanobacteria		<i>Chroococcales</i>	Unassigned	<i>Rubidibacter</i>
	Unassigned	<i>Oscillatoriales</i>	« <i>Prochlorococcaceae</i> »	<i>Halospirulina</i>
		<i>Prochlorales</i>	Unassigned	<i>Prochlorococcus</i>
Firmicutes	<i>Bacilli</i>	<i>Bacillales</i>	<i>Bacillaceae</i>	<i>Alkalibacillus</i>
				<i>Aquisalibacillus</i>
				<i>Bacillus</i>
				<i>Filobacillus</i>
				<i>Gracilibacillus</i>
				<i>Halalkalibacillus</i>
				<i>Halolactibacillus</i>
				<i>Halobacillus</i>
				<i>Lentibacillus</i>
				<i>Oceanobacillus</i>
				<i>Ornithinibacillus</i>
				<i>Paraliobacillus</i>
				<i>Pontibacillus</i>
				<i>Salimicrobium</i>
				<i>Salinibacillus</i>
<i>Salirhabdus</i>				
<i>Salsuginibacillus</i>				
<i>Sediminibacillus</i>				
<i>Tenuibacillus</i>				
<i>Thalassobacillus</i>				
<i>Virgibacillus</i>				
<i>Jeotgalibacillus</i>				
<i>Planococcaceae</i>				
<i>Staphylococcaceae</i>	<i>Salinicoccus</i>			
<i>Clostridia</i>	<i>Halanaerobiales</i>	<i>Halanaerobiaceae</i>	<i>Halanaerobium</i> <i>Halocella</i> <i>Halothermothrix</i>	

Table 3: Taxonomy of Halobacteria (Part2).

Phylum	Class	Order	Family	Genus or Species	
Firmicutes	Clostridia	Halanaerobiales	Halanaerobiaceae	<i>Halanaerobium</i> <i>Halocella</i> <i>Halothermothrix</i>	
			Halobacteroidaceae	<i>Acetohalobium</i> <i>Halanaerobacter</i> <i>Halobacteroides</i> <i>Natronella</i> <i>Orenia</i> <i>Selenihalanaerobacter</i> <i>Sporohalobacter</i>	
Proteobacteria	Alphaproteobacteria	Caulobacterales	Hyphomonadaceae	<i>Hyphomonas</i> <i>Maribaculum</i> <i>Maricaulis</i> <i>Woodsholea</i>	
		Rhizobiales	Hyphomicrobiaceae Rhodobiaceae	<i>Dichotomicrobium</i> <i>Rhodobium</i> <i>Antarctobacter</i> <i>Citreimonas</i> <i>Hwanghaeicola</i> <i>Jannaschia</i> <i>Maribius</i> <i>Marivita</i> <i>Methylarcula</i> <i>Oceanibulbus</i> <i>Oceanicola</i> <i>Palleronia</i> <i>Paracoccus</i> <i>Ponticoccus</i>	
		Rhodobacterales	Rhodobacteraceae	<i>Rhodothalassium</i> <i>Rhodovulum</i> <i>Roseicitreum</i> <i>Roseinatronobacter</i> <i>Roseisalinus</i> <i>Roseovarius</i> <i>Salinihabitans</i> <i>Salipiger</i> <i>Sediminimonas</i> <i>Shimia</i> <i>Sulfitobacter</i> <i>Tropicibacter</i> <i>Yangia</i>	
		Rhodospirillales	Rhodospirillaceae	<i>Fodinicurvata</i> <i>Marispirillum</i> <i>Rhodovibrio</i> <i>Roseospira</i>	
		Deltaproteobacteria	Desulfobacterales	Desulfobacteraceae Desulfobulbaceae	<i>Desulfocella</i> <i>Desulfurivibrio</i>
			Desulfovibrionales	Desulfohalobiaceae Desulfovibrionaceae	<i>Desulfohalobium</i> <i>Desulfovibrio</i> <i>Desulfonatronospira</i> <i>Desulfovermiculus</i>
		Epsilonproteobacteria	Campylobacterales	Campylobacteraceae Helicobacteraceae	<i>Arcobacter halophilus</i> <i>Sulfurovum lithotrophicum</i> <i>Sulfurimonas autotrophica</i>

Table 4: Taxonomy of Halobacteria (Part3).

Phylum	Class	Order	Family	Genus or Species		
Proteobacteria	Gammaproteobacteria	<i>Aeromonadales</i>	<i>Aeromonadaceae</i>	<i>Aeromonas</i>		
		<i>Alteromonadales</i>	<i>Alteromonadaceae</i>	<i>Alteromonas</i> <i>Aestuariibacter</i> <i>Glaciecola</i> <i>Marinobacter</i> <i>Marinobacterium</i> <i>Melitea</i>		
				<i>Idiomarina</i> <i>Pseudidiomarina</i>		
				<i>Pseudoalteromonadaceae</i> <i>Pseudoalteromonas</i>		
				<i>Psychromonadaceae</i> <i>Psychromonas</i>		
				<i>Cellvibrionaceae</i> <i>Gilvimarinus</i>		
		<i>Cellvibrionales</i>	<i>Halieaceae</i> <i>Haliea</i>			
			<i>Microbulbiferaceae</i> <i>Microbulbifer</i>			
			<i>Chromatiales</i>	<i>Chromatiaceae</i>	<i>Halochromatium</i> <i>Marichromatium</i> <i>Thiohalocapsa</i> <i>Alkalilimnicola</i> <i>Aquisalimonas</i> <i>Arhodomonas</i> <i>Ectothiorhodospira</i> <i>Ectothiorhodosinus</i> <i>Halorhodospira</i> <i>Thioalkalivibrio</i> <i>Thiohalospira</i>	
		<i>Hahellaceae</i> <i>Halospina</i>				
		<i>Halothiobacillaceae</i> <i>Halothiobacillus</i>				
		<i>Thioalkalibacteriaceae</i> <i>Thioalkalibacter</i>				
		<i>Thioalkalispiraceae</i> <i>Thiohalophilus</i>				
		<i>Oceanospirillales</i>			<i>Alcanivoraceae</i>	<i>Alcanivorax</i> <i>Aidingimonas</i> <i>Carnimonas</i> <i>Chromohalobacter</i> <i>Cobetia</i> <i>Halomonas</i> <i>Kushmeria</i> <i>Modicisalibacter</i> <i>Salinicola</i> <i>Nitrincola</i> <i>Oleispira</i>
						<i>Halomonadaceae</i>
						<i>Oceanospirillaceae</i>
« <i>Saccharospirillaceae</i> » <i>Saccharospirillum</i>						
<i>Unassigned</i> <i>Salicola</i>						
« <i>Nevskiales</i> »						
« <i>Salinisphaeraceae</i> » <i>Salinisphaera</i>						
<i>Pseudomonadales</i>	<i>Pseudomonadaceae</i>	<i>Pseudomonas</i>				
<i>Vibrionales</i>	<i>Vibrionaceae</i>	<i>Salinivibrio</i>				
<i>Thiotrichales</i>	<i>Piscirickettsiaceae</i>	<i>Thiomicrospira</i>				
Unassigned	Unassigned	<i>Methylohalomonas</i> <i>Thiohalobacter</i> <i>Thiohalomonas</i> <i>Thiohalorhabdus</i>				

Table 5: Taxonomy of Halobacteria (Part4).

Phylum	Class	Order	Family	Genus or Species
<b>Spirochaetes</b>	<i>Spirochaetes</i>	<i>Spirochaetales</i>	<i>Spirochaetaceae</i>	<i>Spirochaeta</i>
<b>Tenericutes</b>	<i>Mollicutes</i>	<i>Haloplasmales</i>	<i>Haloplasmataceae</i>	<i>Haloplasma contractile</i>
<b>Thermotogae</b>	<i>Thermotogae</i>	<i>Kosmotogales</i>	<i>Kosmotogaceae</i>	<i>Kosmotoga</i>
		<i>Petrotogales</i>	<i>Petrotogaceae</i>	<i>Marinitoga</i> <i>Petrotoga</i>
		<i>Thermotogales</i>	<i>Thermotogaceae</i>	<i>Thermosipho</i> <i>Thermotoga</i>

Inside the *Bacilli* class (or *Bacillales* order) various halophilic species have been described in *Bacillaceae*, *Planococcaceae* and *Staphylococcaceae* families but the *Bacillaceae* family contains the highest number of halophilic species.

*Proteobacteria* represents the most important and phenotypically diverse phylum of the Bacteria domain and contains an important number of halophilic or halotolerant genera or species. This phylum is strictly composed of Gram-negative species and is divided in 5 classes (*Zeta-*, *Alpha-*, *Beta-*, *Delta-*, *Gamma-* and *Epsilonproteobacteria*) which are ubiquitous and physiological heterogenous. All the classes include halophilic species except *Betaproteobacteria*.

*Rhodobacterales*, *Rhizobiales* and *Rhodospirillales* are composed of moderately halophilic species in the *Alphaproteobacteria* class. Halophilic bacteria of *Deltaproteobacteria* (also named *Deltabacteria*) are described as strictly anaerobic chemoorganotrophic, chemolithoautotrophic or chemolithoheterotrophic sulfate-reducing organisms with respiratory metabolism and are classed in different families. Comparatively, inside the *Epsilonproteobacteria* class only three moderately halophilic species, isolated from hydrothermal environments, have been described: *Arcobacter halophilus*, *Sulfurovum lithotrophicum* and *Sulfurimonas autotrophica*.

Finally, *Gammaproteobacteria* phylum contains the largest number of genera, approximately 250, including 51 moderately halophilic species<sup>6,7</sup>.

The *Oceanospirillales* order contains the family with the most important number of halophilic species described until now: *Halomonadaceae*<sup>15,16</sup>. This family is composed of species exposing different phenotypic characteristic making classification quite difficult in some case. Moreover, the nine genera composing this family are phylogenetically close except the *Halomonas* genus which is polyphyletic. Hence *Halomonadaceae* family exposes interesting features, and numerous research have been conducted for its study as well as its members leading to a reconsideration and a reorganization of the family in the 1990's<sup>17,18</sup>.

In comparison with previous phyla, *Spirochaetes* and *Tenericutes* phyla contain only few halophilic species: *Spirochaeta halophila*, *S. africana*, *S. alkalica* and *S. asiatica* and *Haloplasma contractile*.

Finally, several moderate, slight or marine species, have been described in Thermotogae phylum. All belonging to *Kosmotogaceae*, *Petrotogaceae* or *Thermotogaceae* families. But actually, *Petrotoga halophila* is the only moderately halophilic species clearly identified <sup>6,12</sup>.

This part depicted taxonomy of halophilic archaea and bacteria and demonstrated their important representation in different phyla and at the same time represented as an important variety of (micro-)organisms which can be studied. Molecular mechanisms of their adaptation to hypersaline conditions have only recently been studied. Moreover, identification of genes conferring salt resistance, along with the presence in halophiles of novel and stable biomolecules, explain the growing interest of both scientists and industry to study more deeply and to exploit these species. The next section will expose the specific characteristics and properties of these microorganisms and how they provide significant opportunities for biotechnology.

## II. Biotechnological applications

The use of biotechnologies in our way of life is inescapable. They are employed for production of added value compounds by bioprocessing from sustainable (co-)products provided by agriculture, environment or industries, used in various domains <sup>19</sup>. Even if the most famous known applications are the production of biopolymers and biofuels, biotechnologies are also exploited for applications including production of detergents, food and drink. Research and exploitation of halophiles already started during last century. Regarding physiological features, adaptability and characteristics of halophiles in front of extreme environments (alkalic/acidic, hot/cold or extremely salty) their employment in biotechnology industry seems to be an advantageous solution to overcome important energy and water consumptions that are required by some biotechnological processes <sup>4,9</sup>.

### II.1 Pigments

Among the important variety of compounds produced by halophiles, including biosurfactants/detergents, biopolymers, enzymes, compatible solutes, some are more studied and yet produced at a large industrial scale.

Since the end of the 19<sup>th</sup> century and the beginning of the 20<sup>th</sup> century, haloarchaea have been identified as responsible for the pink to red coloration of saline lakes and/or highly salt concentrated ponds of salterns <sup>6</sup>. This coloration, observed in animals, plants, algae or microorganisms, is due to production of yellow-red or orange-red liposoluble pigments called carotenoids. There is more than 750 carotenoids, some of natural origin and others obtained by chemical synthesis, including cathaxanthin, phytoene, phytofluene, lycopene, lycopersene,  $\beta$ -carotene, C<sub>50</sub>-bacterioruberin and its derivatives

(isopentenyldehydrorhodopin, anhydrobacterioruberin, bisanhydrobacterioruberin or monoanhydrobacterioruberin)<sup>20,21</sup>. Bacterioruberin is the main carotenoids produced by haloarchaea. It is composed of 50 atoms of carbon and can be found as *trans* or *cis* isomer as its derivatives<sup>13</sup> (**Figure 1**). Bacterioruberin have different biological roles in haloarchaea. It acts for protection of DNA and consequently cells, against ionizing by UV-radiation or hydrogen peroxide (antioxidant activity) demonstrated in *Halococcus morrhuae* and *Halobacterium salinarium*'s extracts. Bacterioruberin acts also for cellular membrane reinforcement, by limiting water permeability and increasing rigidity<sup>21,22</sup>. Different species of haloarchaea have been identified as natural producers of carotenoids like *Haloferax alexandrinus* that produces canthaxanthin or the green algae *Dunaliella salina* and *D. bardawil* which are already exploited for production of  $\beta$ -carotene<sup>9,23</sup>. But usually, only few are cultivated for viable commercialisation of carotenoids due to the high production costs.

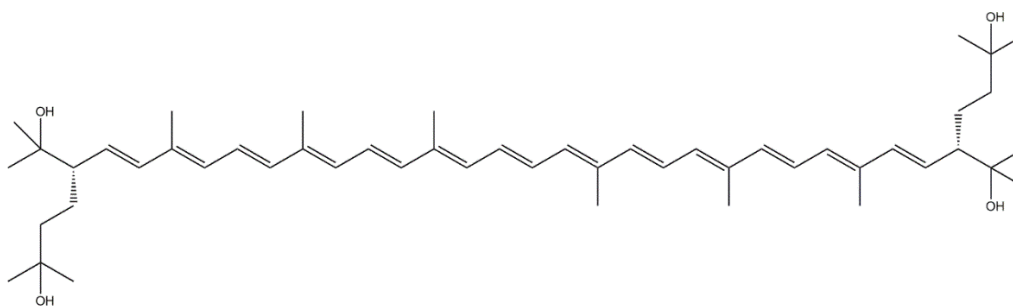


Figure 1: Bacterioruberin.

Another pigment of interest produced by halophiles is the membrane-bound retinal pigment named bacteriorhodopsin<sup>4</sup>. This compound is a light-driven proton pump which is involved in a photocycle, initiated by light excitation, leading to proton release outside the cell. This release creates a proton gradient usable for adenosine triphosphate (ATP) generation<sup>4</sup>. Bacteriorhodopsin was discovered in the 1970s in *Halobacterium salinarium*'s proteome and is qualified as an unusual protein due to its characteristics. Indeed this protein, of approximately 25kDa, tolerates a large range of temperature and does not need high salt concentration, in contrast to other proteins of *H. salinarium*, to keep its structural stability and activity. Yet bacteriorhodopsin is sold as purple membrane patches but there is no true commercial applications even if use of bacteriorhodopsin seems to be adapted to many different applications like spatial light modulators, artificial retinas<sup>23</sup> or for desalination of seawater<sup>24</sup>. The pigment could be used for conversion of light energy into chemical energy (and also electricity)<sup>4</sup> or for back conversion of ADP to ATP, allowing production of ATP which is highly necessary for some biotechnological process<sup>24</sup>. But one of the most interesting and promising applications is the use of bacteriorhodopsin as erasable and photosensitive material for optical information recording and processing in holography especially<sup>11</sup>.

Carotenoids are produced by numerous halophiles : haloarchaea or halobacteria and count a large variety of pigments exposing interesting properties (strong immune boosting and antioxidant properties

but also protective action against premature ageing)<sup>25</sup>. They can be used as additives in baking process or emulsions, or in cosmetic industry<sup>22,23</sup>. Even if the yield of production and downstream process are very interesting they must be improved to optimize production costs at their maximum. Solutions can be to increase production yield, use of by-product such as nutritive source or transgenic systems (as well as production of  $\beta$ -carotene by *Halomonas elongata* expressing production genes of *Pantoea agglomerans*)<sup>24</sup>. Usually the use of these pigments have been poorly studied however in view of the high demand of carotenoids in cosmetic, food or pharmaceutical industries new studies should be conducted.

## II.2 Enzymes and Compatible solutes

Among the important variety of compounds produced by halophiles, a special attention is given to enzymes and compatible solutes. Indeed, enzymes are referred as polyextremophilic and unusual stable proteins due to their intrinsically stability and activity under harsh conditions<sup>25-27</sup>. Compatible solutes, then, could be used for stabilization of (bio-)molecules.

Halophilic microorganisms secrete halophilic hydrolases (proteases, lipases, amylases, esterases or nucleases) able to catalyze reactions under high salt concentrations<sup>28</sup>. Currently, glycosyl hydrolase, protease and lipase represent 70% of sold enzyme. Proteases alone are the most used enzymes, especially in detergents formulations, pharmaceuticals, food processing or waste management<sup>10</sup>. Different halophilic species have been identified as protease producer such as *Halobacillus karajensis* strain MA-2, *Pseudoalteromonas* sp., *Halobacillus* sp. or *Chromohalobacter* sp.<sup>10,25</sup>. Lipase represents also an important industrial enzyme exploited in detergent formulation or organic synthesis and has been reported to be produced by the moderately halophilic species *Salinivibrio* sp.<sup>24</sup>. Finally, halophilic amylases, which are commonly cyclomaltodextrinases, are produced by various halobacteria (*Halomonas meridiana*, *Halobacillus* spp., *Halotheotrix orenii*, *Haloarchula* sp.S-1 or *Micrococcus halobius*) and can also be used in detergent formulation or for waste water treatment<sup>10</sup>. But even if a large variety of halophilic enzymes are produced by different halobacteria there is only few which have found industrial application due to limited requirement for salt-tolerant enzymes. Currently, nuclease H of *Micrococcus varians* subsp. *halophilus* is used for production of flavoring agent is one of the few enzymes with industrial applications<sup>24</sup>.

In comparison, demand for compatible solutes is quiet more developed. The term compatible solutes refers to zwitterionic, non charged or anionic, low molecular mass compounds allowing adaptation, maintenance and survival of organisms under harsh conditions by providing an osmotic balance<sup>25</sup>. These compounds avoid or limit detrimental action of high salt concentration, high temperature, freezing or dessication on (bio-)molecules (DNA, proteins, ...). Additionnaly, some of them expose ability to counteract UV-A effects.

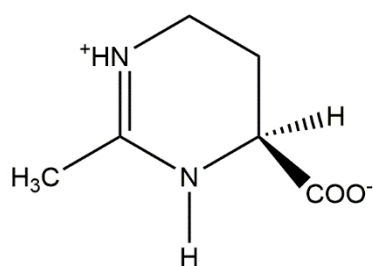


Figure 2: Ectoine.

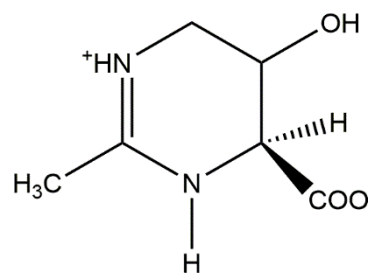


Figure 3: Hydroxyectoine.

This mechanism is considered as one of the main defense of halophiles <sup>10</sup>. This class of molecules includes amino acids (alanine, glycine, proline), sugars (trehalose and sucrose), polyols (glycerol, mannitol, sorbitol), betaine, ectoine and its derivative hydroxyectoine (**Figures 2 and 3**). Both last ones are the most produced by halophiles in front of high salt concentrations. Degree of halotolerance seems to be linked to type of compatible solutes accumulated by cells: low salt-tolerant strains, frequently, accumulated sucrose and/or trehalose, moderately salt-tolerant glucosylglycerol while ectoine and quaternary ammonium compounds are found in highly salt-tolerant species. Ectoine was first observed in *Ectothiorhodospira halochloris* (a haloalkaliphilic photosynthetic sulfur bacterium) before being found, as its derivative hydroxyectoine, in a large variety of species such as *Halomonas elongata*, *H. salina*, *H. boliviensis*, *H. ventosa*, *Chromohalobacter salexigens*, *Virgibacillus halodenitrificans* or *Marinococcus* M52. Industrial productions of ectoine and hydroxyectoine are developed using *Halomonas elongata* and *Marinococcus* M52, respectively. One biotechnological process, called « bacterial milking », was developed for production of ectoine with *H. elongata* and consists to induce excretion of ectoine, to maintain osmotic balance, by transferring bacterial biomass alternatively into low and high osmolarity medium <sup>5,29</sup>. Studies have demonstrated that optimization of medium composition, significantly impacts yield of production <sup>30</sup>. Employment of halophilic strain, engineered or not, could allow production of compatible solutes and their use as proteins stabilizers, dessication protector in medical applications or for skin care protection in cosmetic formulations <sup>4,9,25</sup>.

### II.3 Biopolymers

Microorganisms, including halophiles, are responsible for production of various biopolymers like polyamides, polyanhydrides, polyesters or polysaccharides, whose majority are extracellular products. They are mainly synthesized to protect cells in front of stressful conditions <sup>31</sup>. In natural environment, biopolymers are completely degraded by depolymerases and hydrolase action making them good candidates for replacement of recalcitrant oil-based polymers. Consequently, bacterial polymers are exploitable in diverse application fields (food industry, pharmaceuticals technology, packaging, bioremediation,...) <sup>32</sup>. In this section we will present and describe some of them in order to underlign their biotechnological potential.



### II.3.1 Polysaccharides

There are already many studies looking for discovery and exploitation of marine polysaccharides such as agar, agarose, carrageenans and alginates, that are extracted from seaweeds (macroalgae) <sup>33</sup>. But marine bacteria can also produce an important variety of polysaccharides: structural, intracellular or extracellular polysaccharides also named exopolysaccharides (EPS). This last type of polysaccharides represents another important source of marine polymers with promising or actual applications. Numerous bacterial species have been reported to produce EPS in large amounts <sup>24</sup>, nevertheless only few of these polysaccharides have been yet fully studied <sup>33</sup>. They are water soluble molecules, which may be ionic or non-ionic, literally extracellularly produced by halophilic microorganisms. EPS are composed of very regular units of repeat (branched or not) <sup>4</sup> that can be sugars including amino sugars (D-Glucosamine and D-Galactosamine), pentoses (D-Arabinose, D-Ribose, D-Xylose), hexoses (D-Allose, L-Fucose, D-Galactose, D-Glucose, D-Mannose, L-Rhamnose) or uronic acids (D-Glucuronic acids, D-Galacturonic acids). They can also contain various organic or inorganic compounds (acetic, phosphoric, pyruvic, sulfuric or succinic acids, phosphate or sulfate) <sup>33,34</sup>. EPS can be considered as protective compounds against adverse conditions and many halophilic species have been described to possess an EPS capsule surrounding cell to protect it. But they are also involved in different processes in marine environment such as bacterial attachment on surfaces, biochemical interaction between cells and absorption of dissolved organic materials <sup>33,35</sup>. EPS production has been described in various halophilic microorganisms like Halobacteria and especially *Halomonas* species (*H. alkaliantartica*, *H. anticariensis* strain FP36, *H. eurihalina*, *H. maura*, *Halomonas* sp. AAD6, *H. ventosae* sp. nov.,...) <sup>5,11,24,36,37</sup> or in *Cyanobacteria* species (*Aphanothece halophytica* or *Cyanospira capsulate*) <sup>24,38</sup> but also in haloarchaea (*Haloferax mediterranei*, *Haloferax gibbonsii*, *Haloarcula* strain T5). Their production can be induced by various environmental factors: salinity, pH, temperature, light intensity, competition for nutrients or for adaptation in front of extreme habitats. Consequently their biological functions depend on microorganisms' environment <sup>39</sup>.

EPS produced by *Halomonas* species are polyanionic with an elevated sulphate content and glucuronic acid <sup>4,38</sup> giving them good gellifying properties. Comparatively, EPS from the haloarchaea, *Haloferax mediterranei*, are anionic sulfated acidic heteropolysaccharides exposing good rheological properties, high viscosity at low concentration and resistance against harsh conditions (temperature and pH) <sup>9,11,24</sup>. EPS also expose immunomodulation properties (sulfated EPS can limit virus penetration in host cells like EPS of *A. halophytica* which inhibit pneumonia caused by influenza virus H1N1 <sup>24</sup> or those produced by *H. mauran*<sup>4</sup>), anticancer activity (EPS from *Pseudomonas* sp. exposing cytotoxic effects on cancer cell lines MT-4) or bone-healing properties (EPS produced by *Vibrio diabolicus*) <sup>5,31,33,38</sup>.

These properties allow marine EPS to have applications such as surfacting, emulsifying, gellifying, thickening agent in various industries. They can be used in food or pharmaceutical applications but also for oil recovery and bioremediation of heavy metals.

EPS expose interesting properties, as well as those produced by *Halomonas almeriensis* (emulsification of hydrophobic compounds ; bio-detoxifier, emulsifier or biological agent) <sup>40</sup>. Consequently, studies for optimization of their production and recovery are conducting. Actually, production and recovery process are key points. Indeed, in some cases production yields are low and production of EPS using fermentation process leads to the obtention of a final growth medium revealing a high viscosity. Consequently, recovery of biopolymer is difficult and can be too expensive for the final quantity of EPS. Different approaches are studied, i.e. the use of genetically modified strains or optimization of production parameters. Several studies have been conducted to determine impact of temperature on EPS production by marine strains (members of *Colwellia*, *Hallela* and *Pseudoalteromonas* genera) and demonstrated its key function for increasing production yields <sup>39</sup>. Influence of media composition on biopolymer production was also investigated for different bacterial species (belonging to *Pseudoalteromonas*, *Pantoea*, *Halomonas* genera) and highlights the importance of this parameter and the necessity to adapt it to microorganism's metabolisms.

### II.3.2 Polyesters/Polyhydroxyalkanoates

Among biopolymers, microbial polyesters named polyhydroxyalkanoates (PHA) (**Figure 4**), probably represent the heterogeneous biopolymers of greatest interest.

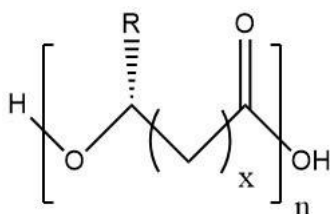


Figure 4: PolyHydroxyAlkanoates general structure.

PHA are biodegradable and biocompatible storage polymers naturally accumulated by different organisms: Prokaryotes, Bacteria and Archaea. PHA are stored as insoluble inclusions of 0.1-0.5  $\mu\text{m}$  in diameter in cytoplasm <sup>32</sup>. PHA granules are carbon and energy reserves, but their composition suggests a multifunctional role and leads to another way to call them: “carbonosomes” <sup>41</sup>.

An important structural diversity has been described for these biopolymers. Indeed, more than 150 different monomers have been reported (**Table 6**) conferring to PHA a wide range of properties <sup>10</sup>. Because they are biodegradable, thermoplastic and elastomeric materials, they are considered as good alternatives for replacement of conventional plastics <sup>11,42-44</sup>.

Table 6: Common monomer units of PHA.

Class	Pendant R group		Name	Abbreviations
scl-PHA	CH <sub>3</sub>	Methyl	Poly(3-Hydroxybutyrate)	P-3HB
	CH <sub>2</sub> CH <sub>3</sub>	Ethyl	Poly(3-Hydroxyvalerate)	P-3HV
mcl-PHA	CH <sub>2</sub> CH <sub>2</sub> CH <sub>3</sub>	Propyl	Poly(3-Hydroxyhexanoate)	P-3HHx
	(CH <sub>2</sub> ) <sub>3</sub> -CH <sub>3</sub>	Butyl	Poly(3-Hydroxyheptanoate)	P-3HHp
	(CH <sub>2</sub> ) <sub>4</sub> -CH <sub>3</sub>	Pentyl	Poly(3-Hydroxyoctanoate)	P-3HO
	(CH <sub>2</sub> ) <sub>8</sub> -CH <sub>3</sub>	Nonyl	Poly(3-Hydroxydodecanoate)	P-3HDD

Due to these interesting characteristics, their use can be considered in various field such as plastics processing industry (extruded or moulded products and packing or mulching films) for food packaging, consumer goods <sup>45</sup> or for medical applications (cardiovascular products, surgical sutures, wound dressing, tissue scaffolds, artifacts/implants, bone replacement, ...) <sup>5,32,46,47</sup>. They can also be used as biodegradable carriers of valuable compounds (hormones, drugs or biocides) <sup>48,49</sup>. Exploitation of PHA in agricultural/environmental fields, as *in situ* bioremediation of contaminated sites or treatment of municipal wastewater, can also be considered <sup>50,51</sup>. Finally, PHA-producing bacteria have been recently used for the enhancement of crop productivity by improvement of nitrogen fixation <sup>52</sup>.

Currently, only few companies worldwide can furnish large amount of PHA such as Biomer (Germany) <sup>53,54</sup>, Bio-on (Italy) <sup>55</sup>, Imperial Chemical Industry (U.K), Metabolix, Inc. (U.S.A) <sup>56</sup> or Telles LLC (U.S.A) <sup>57</sup>. The main restriction for development of their marketing is their final cost. Indeed, production of PHA still requires high-priced carbon substrates as well as expensive downstream processing leading to an overall production cost 5 to 10 times more expensive (7 to 10 €/kg) than those of conventional plastics <sup>13,32</sup>.

One of the tracks studied in order to reduce final cost of PHA is the use of halophilic/halotolerant strains since <sup>10</sup>:

- Seawater can be exploited as producing medium, limiting freshwater consumption,
- High salt concentration limits external contamination,
- Simple carbon substrates can be used,
- Limited downstream processes are required as osmotic shock may be sufficient to lyse bacteria, depending on the species,
- PHA biosynthesis can be coupled with other valuable compounds production (such as EPS)

All these characteristics allow reduction of production costs but still demonstrate some limitations. For example, high salt concentrations can be harmful for equipment (leading to corrosion) or coupled productions can complicate downstream process.

First description of PHA-producing Haloarchaea was reported in 1972 and 1986, in *Haloferax mediterranei* and *Haloarcula marismortui*, before being reported in different Haloarchaeal and Halobacterial species such as *Haloquadratum walsbyi*, *Halogeometricum borinquense*, *Natrinema altinense*<sup>46</sup>, *Cobetia marina*, *Halomonas boliviensis* LC1, *H. elongata* DSM 2581, *H. salina*, *Halomonas* sp. TD01<sup>58-61</sup>.

Production by *Haloarcula* and *Haloferax* species are the most studied and efficient haloarchaea to produce various PHA<sup>13</sup>. Indeed, *Haloferax mediterranei* has demonstrated ability to produce both poly-3-hydroxybutyrate (homopolymer, P-3HB) and poly-3-hydroxybutyrate-co-3-hydroxyvalerate (P-3HB-co-3HV) and production of terpolymer poly-3-hydroxybutyrate-co-3-hydroxyvalerate-co-4-hydroxybutyrate (P-3HB-co-3HV-co-4HB) has also been reported<sup>62</sup>.

Additionally to haloarchaea species, halobacteria are also deeply studied for PHA production and especially *Halomonadaceae*. In this family, numerous PHA-producers were identified as well as *Halomas aquamarina* ATCC 14400, *H. boliviensis* DSM 15516, *H. cupida* CECT 5001, *H. elongata* CECT 4279, *H. eurihalina* ATCC 49336, *H. meridiana* DSM 4225, *H. pantelleriensis* DSM 9661, *H. variabilis* DSM 3051 or *H. ventosae* DSM15911. Like haloarchaea, halobacteria are able to produce different type of PHA (P-3HB, P-3HB-co-3HV,...).

### III. Generalities about Polyhydroxyalkanoates

Polyhydroxyalkanoates are biopolyesters detected in various eukaryotic (plants, animals,...) and prokaryotic organisms (bacteria and archaea) which show thermal, mechanical and physicochemical properties similar to those of conventional petroleum-based plastics<sup>43,44,63</sup>. This part will only deal with generalities about bacterial PHA (production, characteristics, properties and applications).

First observation was reported in 1926 by Lemoigne in *Bacillus megaterium*, a Gram positive bacterium, before being observed in various Gram positive (like *Bacillus* sp., *Clostridium* sp., *Actinomyces* sp., *Corynebacterium* sp., *Staphylococcus* sp. or *Streptomyces* sp.) and Gram negative (*Acinetobacter* sp., *Alcaligenes* sp., *Chromobacterium* sp., *Haemophilus* sp., *Nitrococcus* sp., *Pseudomonas* sp.,...) species including halobacteria and haloarchaea species (*Haloferax mediterranei*, *Halomonas boliviensis*, *Halomonas elongata*,...). To date more than 300 species are able to produce these biopolymers<sup>56</sup>.

PHA are water insoluble compounds accumulated into the bacterial cytoplasm as one to ten granules (**Figure 5**)<sup>64,65</sup>. PHA production occurs during bacterial growth between end of exponential growth phase and beginning of stationary phase under nutritional imbalance (excess of carbon and a limitation of another essential nutrients like nitrogen, oxygen or phosphorus)<sup>65,66</sup> or in response to an environmental stress (harsh physical conditions)<sup>32,67-69</sup>.

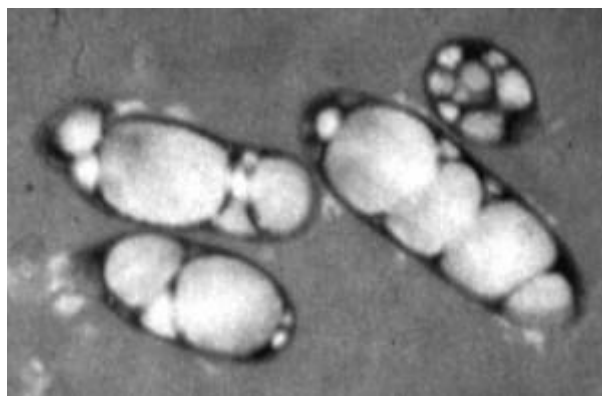


Figure 5: Transmission Electron Micrograph of P-3HB-co-3-HV granules in *Cupriavidus necator* H16. (<http://www.ecobiomaterial.com>)

PHA granules serve mainly as carbon and energy reserve but others biological functions have been described<sup>41</sup>. For instance, it has been reported that PHA can limit alteration of cellular components as protein or RNA and can enhance resistance against desiccation, thermal or osmotic shock, UV irradiation...<sup>41</sup>. Others studies have demonstrated horizontal transfert of PHA metabolism related genes by bacterial species in order to resist to stressful environment<sup>70</sup>. In *Bacillus cereus*, *Clostridium botulinum* or *Azotobacter vinelandii*<sup>41,49</sup>, PHA production has been reported to be linked to various cellular mechanisms like spore or cyst formation, synthetic nitrogen fixation or to prevent production of acidic compounds of cellular metabolisms<sup>49,64,71</sup>. In *Methylophilus marina*, *M. terricola* and *Photobacterium profundum*,  $\beta$ -hydroxybutyrate accumulations have been reported when extracellular concentration of NaCl increases, suggesting a potential osmolytic role of  $\beta$ -hydroxybutyrate or its polymer<sup>72</sup>. PHA in the form of oligomer, meaning 100 to 200 units of monomer, and more precisely as oligo-PHB, have been detected complexed with polyphosphate and calcium ions (PHB-Ca-polyphosphate complexes) in cellular membrane of different PHA-producing species or not as *Bacillus subtilis* or *Escherichia coli*. Additional research have demonstrated that PHB-Ca-polyphosphate complexes are linked to competence of bacterial cells<sup>64</sup>. Actually, it is clear that biological functions of PHA can not be limited to simple storage compounds and need to be deeply studied to demonstrate their importance in various species. Indeed, considering PHA granules's composition, it is suggested that they can have other different biological functions<sup>49,64</sup> and consequently PHA granules are also named« carbonosomes » to illustrate the multifunctionnal functions of these structures.

## III.1 Structure and properties of PHA

### III.1.1 Structure

Polyhydroxyalkanoates are linear polyesters composed of R-hydroxy fatty acids monomer (-HA) with a side chain wearing alkyl, branched alkyl or substituted alkyl groups which are saturated or not. Monomers are connected together by an ester bond which appears when a carboxylic group of a monomer is connected to hydroxyl group of the adjacent monomer<sup>65,73</sup>. Due to the stereospecificity of the key enzyme involved in the biosynthesis: PHA synthase (PhaC), all monomer units of R group are in R(-) configuration<sup>56,74</sup>. Nature of pendent group is directly linked to the carbon substrates assimilated for biosynthesis. A wide variety of carbon substrates such as sugars (glucose, fructose, sucrose, cellulose,...), triacylglycerols, hydrocarbons, fatty acids or acids (4-hydroxybutyric acid, propionic acid,...) are frequently employed for PHA biosynthesis. In addition to conventionnal sugars or complex mix used for production, there is an important variety of atypical carbon sources, as industrial co-products or wastes, which have been tested or are still exploited<sup>75,76</sup>. This important variety of potential substrates, in conjunction with the diversity of producing species, leads to a wide structural range of PHA. To date, there are more than 150 structures referenced in the literature<sup>13</sup>, some of them are illustrated in **Figure 6**. Bacterial PHA expose a molecular weight ( $M_w$ ) generally ranging from  $5.0 \times 10^5$  to  $3.0 \times 10^6$  g/mol with a polydispersity ranging from 1.6 to 2.2<sup>69,77,78</sup>. Bacterial strain and composition of pendent group R influence value of n which is generally ranging from 100 to 30000.

General structure of PolyHydroxyAlkanoates

	R group		PHA
sol-PHA	Methyl	CH <sub>3</sub>	Poly-3-Hydroxybutyrate
	Ethyl	CH <sub>2</sub> -CH <sub>3</sub>	Poly-3-Hydroxyvalerate
mel-PHA	Propyl	(CH <sub>2</sub> ) <sub>2</sub> -CH <sub>3</sub>	Poly-3-Hydroxyhexanoate
	Butyl	(CH <sub>2</sub> ) <sub>3</sub> -CH <sub>3</sub>	Poly-3-Hydroxyheptanoate
	Pentyl	(CH <sub>2</sub> ) <sub>4</sub> -CH <sub>3</sub>	Poly-3-Hydroxyoctanoate
	Hexyl	(CH <sub>2</sub> ) <sub>5</sub> -CH <sub>3</sub>	Poly-3-Hydroxynonanoate
	Heptyl	(CH <sub>2</sub> ) <sub>6</sub> -CH <sub>3</sub>	Poly-3-Hydroxydecanoate
	Octyl	(CH <sub>2</sub> ) <sub>7</sub> -CH <sub>3</sub>	Poly-3-Hydroxyundecanoate
	Nonyl	(CH <sub>2</sub> ) <sub>8</sub> -CH <sub>3</sub>	Poly-3-Hydroxydodecanoate
	Decyl	(CH <sub>2</sub> ) <sub>9</sub> -CH <sub>3</sub>	Poly-3-Hydroxytridecanoate
	Undecyl	(CH <sub>2</sub> ) <sub>10</sub> -CH <sub>3</sub>	Poly-3-Hydroxytetradecanoate
	Dodecyl	(CH <sub>2</sub> ) <sub>11</sub> -CH <sub>3</sub>	Poly-3-Hydroxypentadecanoate
	Tridecyl	(CH <sub>2</sub> ) <sub>12</sub> -CH <sub>3</sub>	Poly-3-Hydroxyhexadecanoate

Figure 6: General nomenclature of main PHA.

A classification based on the number of carbon atoms in the alkyl chain gives two groups of PHA: short-chain length PHA (scl-PHA), with monomers composed of 3 to 5 carbon atoms, and medium-chain length PHA (mcl-PHA) composed by monomers with 6 to 14 carbon atoms (**Figures 6 and 7**). Based on polymer composition, homo- and heteropolymers (such as P-3HB-co-P-4HB or P-3HB-co-3HV) can be distinguished.

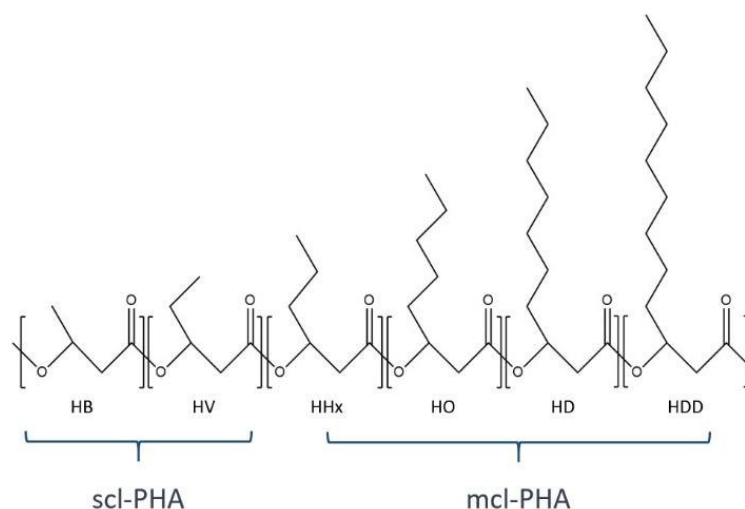


Figure 7: Common monomers of PHA.

Production of scl- or mcl-PHA is dependent on PHA synthase (PhaC) specificity, i.e. its class (described below), and substrates used. Furthermore, monomers with functionalized monomers such as cyano, halogen and hydroxy carboxyl groups, have already been described in mcl-PHA <sup>67</sup>.

Beside homopolymers, heteropolymers expose a great interest. Their production occurs when carbon substrates mix are used during production step or if there is an alternation of carbon sources <sup>65</sup>. For example, production medium composed of glucose and valerate leads to the biosynthesis of P-3HB-co-3-HV. Proportions of each monomer composing the heteropolymers are dependent on conditions, including bacterial strains and carbon substrates used. The employment of carbon substrates mix does not allow to perfectly control PHA composition and produces random heteropolymers. Heteropolymers consisting of copolymers of scl- and mcl-PHA expose better mechanical properties than scl-PHA (see below) and hence focused main of the research <sup>73,79</sup>.

In addition, diverse chemical modifications have been envisaged to obtain new functionalized polymers after their biosynthesis. For example, Bassas-Galià *et al.*, have used epoxidation of PHA in order to obtain biomaterials allowing conjugation of biological molecules <sup>80</sup>. Others chemical reactions which can be applied to functionalize PHA are chlorination, hydroxylation, carboxylation, thiolation or esterification to obtain grafted polymers like cellulose-g-P-3HB-co-3HV or P-3HB-co-3HV-g-chitin <sup>81,82</sup>.

### III.1.2 Properties

PHA are biopolymers exhibiting numerous advantageous properties. They are entirely biodegradable, non toxic, biocompatible, and expose a wide range of physicochemical, thermal and mechanical properties. Their thermomechanical properties are closely dependent of their own composition. General data in literature gives an overview of the range of these different characteristics like cristalinities ranging from 30% to 70% and melting temperatures ranging from 50°C to 180°C <sup>77,83</sup>.

Table 7: Mechanical and thermal properties of PHA.

Based on data of Taguchi *et al.*,2012 and Rudnik, 2008 and completed with data from other studies.

PHA	Copolymer content	Molecular weight (g/mol)	Melting temperature (°C)	Glass transition temperature (°C)	Tensile strenght (MPa)	Elongation (%)
PP	-	n.i	170	-10	35	400
P-3HB	-	0.6x10 <sup>6</sup>	177	4	43	5
P-4HB	-	n.i	60	-50		1000
P-3HB-co-4HB	3mol% 4HB	n.i	159	n.i	24	242
P-3HB-co-4HB	16mol% 4HB	n.i	150	-7	26	444
P-3HB-co-3HV	8mol% HV	1.0x10 <sup>6</sup>	165	1	19	35
P-3HB-co-3HHx	5mol% HHx	0.8x10 <sup>6</sup>	160	-2	32	260
P-3HO	n.i	n.i	61	n.a	6-10	300-450
P-3HHx-co-3HO	8mol% HHx and 92 mol% HO	n.i	61	33	10	300

Scl-PHA are generally stereoregular polymers with a degree of cristallinity ranging from 55 to 80%. They have glass transition temperatures around 5-10°C and melting temperatures ranging from 170°C to 180°C (**Table 7**). These characteristics make them stiff and brittle materials. However, scl-PHA with a high molecular weight, that can be produced by recombinant strains, expose improved physical properties <sup>84</sup>. An exception can be notified in scl-PHA as P-4HB is a highly flexible polymer due to incorporation of a comonomer into backbone of the polymer. Compared to scl-PHA, mcl-PHA expose a lower degree of cristallinity, around 25%. They also have glass transition temperatures and melting temperatures lower than those of scl-PHA, which are ranging from -40 to -25°C and 39°C to 61°C, respectively (**Table 7**) <sup>67,85</sup>. Mcl-PHA are consequently more elastic and flexible. This difference of properties determines final use of each class of PHA.

### III.2 Metabolism of PHA

Currently, numerous studies are available or are still conducting, for a better understanding and/or modification of metabolic pathways of PHA biosynthesis. Final objectives remain to tailor-make these biopolymers and also to increase yields of production according to an optimal exploitation of different metabolisms <sup>45,86</sup>.



### III.2.1 Molecular organisation

There are numerous molecular data concerning PHA metabolism available in literature but the most studied organization is *Cupriavidus necator* (formerly *Ralstonia eutropha*). The three main genes coding for key enzymes of PHA biosynthesis: *phaC*, *phaA* and *phaB*, coding for PHA synthase,  $\beta$ -ketothiolase and acetoacetyl-Coa reductase respectively, are usually organized in one operon: *phaCAB*, such as in *C. necator*<sup>69</sup> or *Pseudomonas* sp.<sup>66,87</sup>. However, different organizations have been evidenced<sup>56</sup> in other bacterial species. Indeed, there is a high number of genes coding for enzymes directly or indirectly involved in PHA biosynthesis. For example, Meng *et al.* have identified about 44 enzymes involving in one of the different pathways (discussed below) for PHA biosynthesis, suggesting the high number and the diversity of genes involved in these mechanisms<sup>88</sup>. Organization of the biosynthesis operons depends on bacterial species and also on the PHA synthase class (**Figure 8**)<sup>89</sup>.

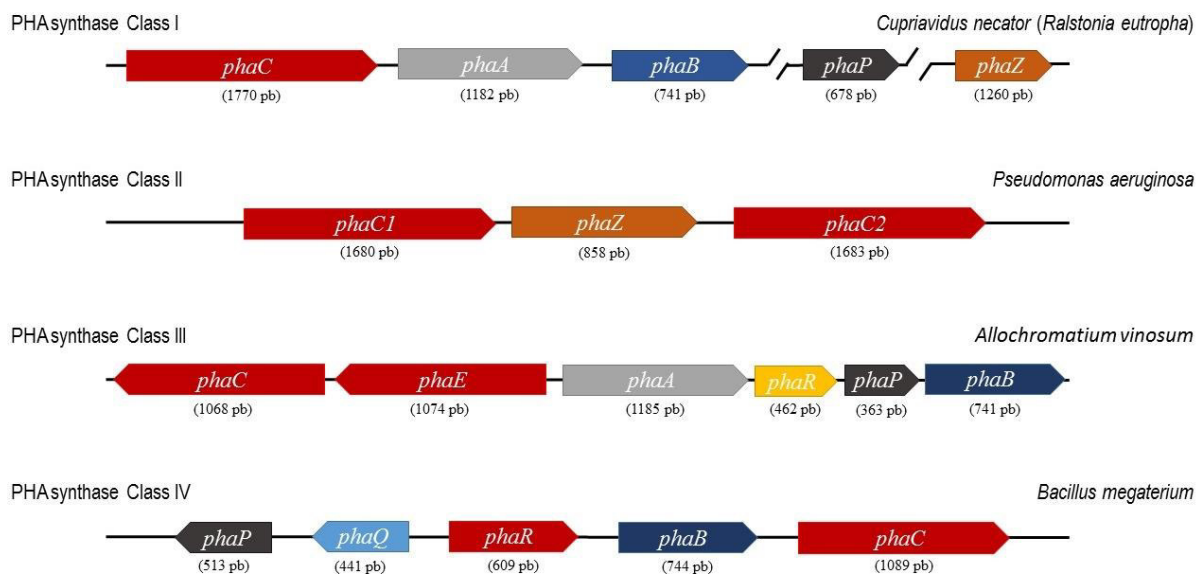


Figure 8: PHA biosynthesis operon organisation in representative bacterial species.

Indeed, there are four classes of PHA synthases which have their own features. Synthases of class I and class II are only composed by one *phaC* gene while those of classes III and IV are constituted by two genes. Both *phaC* and *phaE* genes are required for PHA synthesis by class III PHA synthases while for the class IV enzymes, *phaC* and *phaR* genes are necessary (**Figure 8**). Consequently, biosynthesis operon organization is dependent of synthases classes.

### III.2.2 Metabolic pathways

Three main metabolic pathways (I, II and III) are generally used to describe PHA biosynthesis but a total of 14, natural or engineered, pathways have been yet described<sup>88</sup>. Pathways use by microorganisms depend on both PHA synthase (PhaC) specificity and nature of the carbon source, the latter including a wide variety of compounds ranging from usual carbohydrates to complex mix or co-

products. Metabolism of PHA is regulated at enzymatic and transcriptional level. Indeed it has been shown that intracellular concentrations of free Coenzyme A, acetyl-CoA, of NADPH and ratio of NADPH/NADP can act to regulate PHA biosynthesis <sup>41</sup>.

The three main and natural pathways (**Figure 9**) include acetyl-CoA or acyl-CoA as intermediate. Pathway I leading to the production of P-3HB, it starts with condensation of two acetyl-CoA, obtained after glycolysis of sugars (i.e. glucose), by acetyl-CoA acetyltransferase (PhaA), to produce acetoacetyl-CoA. Acetoacetyl-CoA reductase (PhaB) reduced acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA which is finally converted to P-3HB by polyhydroxyalkanoate synthase (PhaC).

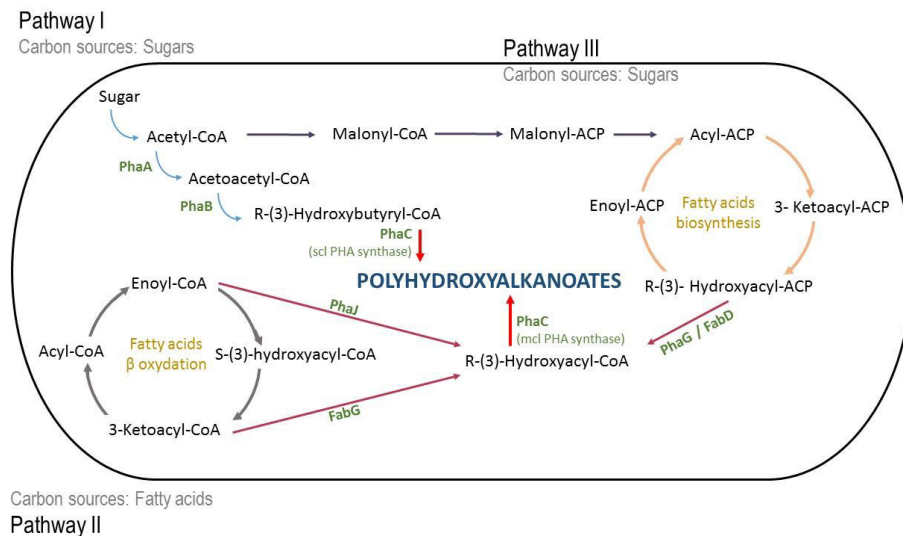


Figure 9: Metabolic pathways for PHA biosynthesis. Adapted from Nomoto and Yano, 2009; Sudesh *et al.*, 2000 and Verlinden *et al.*, 2007.

Pathway II is a derivative of  $\beta$ -oxydation of fatty acids and involves production of 3-hydroxyacyl-CoA from 2,3-dehydroacyl-CoA, 3-hydroxyacyl-CoA or 3-ketoacyl-CoA, by (R)-enoyl-CoA hydratase, 3-hydroxyacyl-CoA epimerase or ketoacyl-CoA reductase, respectively. Then 3-hydroxyacyl-CoA is used by PhaC for PHA synthesis (**Figure 9**).

Finally, pathway III, involves the conversion of 3-hydroxyacyl-Acyl carrier protein (ACP) derived from the fatty acid synthesis from sugars, to 3-hydroxyacyl by 3-hydroxyacyl-ACP-CoA transferase followed by a synthesis of PHA by PhaC <sup>69,74,90</sup>. Complementarily to these pathways, a fourth one is more and more described, pathway IV, and involves conversion of butyric acids to S-hydroxyacyl-CoA and then in acetoacetyl-CoA, without entering in  $\beta$ -oxydation cycle <sup>88</sup> (**Figure 9**).

The need to control biopolymer structure has led to further studies using genetic engineering and deep knowledges of these main metabolic pathways. These research have allowed the development of ten additional pathways that can be exprimed simultaneously to “natural” pathways in PHA-producing strains or in non PHA-producing strains such as *E. coli* <sup>88</sup>. But all metabolic pathways include enzyme PhaC, demonstrating its key function in PHA synthesis.

### III.2.3 Granules of PHA

Granules of PHA, recently renamed carbonosomes because of their composition, are spherical inclusions with a size ranging from 0.1 to 0.5 $\mu\text{m}$ <sup>91</sup>. These structures naturally refract light allowing their observation using phase contrast microscope but also with staining techniques using Sudan Black or fluorescent stains such as Nile Blue or Nile Red<sup>69,92</sup>. Granules characteristics are functions of the bacterial strain and mode of production/culture conditions<sup>63,64</sup>. Composition and formation of PHA granules are studied for a long time and different models have been proposed. In 1968 Griebel *et. al* described chemical composition of PHA granules in *B. megaterium* and highlighted influence of harvest, preparation and observation technics on composition<sup>93</sup>. Consequently, terms of native PHA granules and denaturated PHA granules were proposed, the first one being closer to *in vivo* PHA granules and wearing PHA synthase enzyme, and can partially explain the difference of point of view. In this study chemical composition was determined such as 98% of PHA (and especially P-3HB), 2% of proteins and trace of lipids leading to the proposal that, *in vivo*, PHA granules are surrounding by lipidic (mono-)layer with anchored proteins<sup>93</sup>. Since this work, numerous research have been conducted and are still ongoing to elucidate biogenesis and PHA granule composition. To date, and based on data obtained with *C. necator*, three models of PHA granules formations have been proposed: micelle model, scaffolds model and budding model (**Figure 10**).

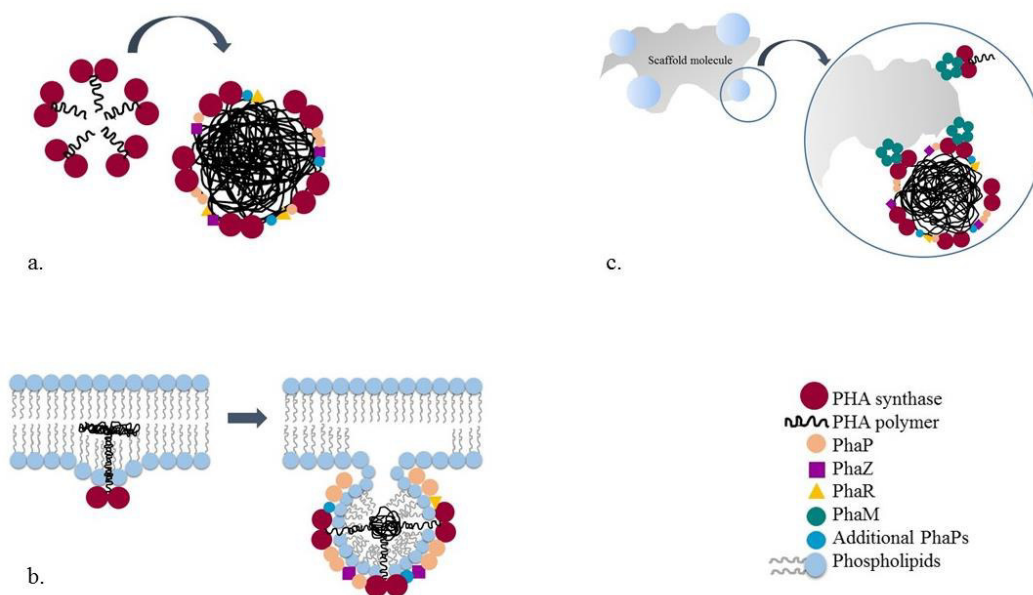


Figure 10: Schematic representation of PHA granules formation, a. Micelle model, b. Budding model and c. Scaffold model. Adapted from Jendrossek and Pfeiffer, 2014 and Williams and Patricia, 2018<sup>64,73</sup>.

The micelle model suggests that PHA synthase (PhaC), key enzyme of PHA metabolism (described below), is free in bacterial cytoplasm with a random localisation. Once a sufficient quantity of substrate is detected, polymerisation is engaged and the neo-formed polymer chains start to be grouped together and form a micelle-like structure, due to the insolubility of PHA. PhaC, which is

partially hydrophile, stays on the micelle-like structure surface. Later, other proteins in relation with PHA metabolism (called PHA granule-associated proteins (PGAP)), will attach to the micelle-like structure (**Figure 10**). This model can explain that PHA granules are randomly distributed in bacterial cells <sup>56,64,91,94</sup>.

In budding model, in contrary to the previous model, PHA synthase is supposed to be attached to the inner face of plasma membrane possibly in response to detection of appropriate substrates (such as 3HB oligomer or a part of a long chain fatty acids). Synthesis of polymer is initiated in the membrane bilayer conducting to bud a vesicle when PHA chains reach a specific size. Finally, PHA granules formation started, the structures (which could be surrounded by a (mono-)layer of lipids) are released in cytoplasm before to be complete by PGAP and phasin Figure 10) <sup>56,64,91</sup>. Location of PHA synthase, PhaC, near to plasma membrane is unusual, consequently a third model was proposed.

The scaffold model proposes the creation of a link between PHA synthase and a scaffold molecule within the cell (still not identified/unknown at the time of writing) during formation of PHA granules. With this model, location of PHA granules is necessarily dependent of the scaffold molecule location. Some studies conducted with observations performed by transmission electron microscopy (TEM) or cryotomography advance the hypothesis that the scaffold molecule could be a mediation element localized in nucleoid region <sup>95,96</sup>. Usually there is no clear differentiation between micelle and scaffold models but replacement of the first one by the second one is frequent <sup>56,64</sup>.

Presence of phospholipids at surface of PHA granules is still hardly discussed. All proposed models since the 1960's include a monolayer of phospholipids at the surface of the granules based on data obtained by TEM analysis which determined a membrane size of 4nm (half of cytoplasmic membrane composed of a phospholipidic bilayer). But recently, Jendrossek *et al.* conducted a study on different bacterial species with expression of fusion proteins which have led to exclude (phospho-)lipids of PHA granules composition. It was proposed that detection of (phospho-)lipids is linked to isolation technic of PHA granules <sup>56,64,97</sup>. Even if conclusions of this study lead to exclude the presence of (phospho-)lipids at PHA granules surface, newest research (including this one within the scope of this PhD) are still using Nile Red or Nile Blue, which are fluorescent lipophilic stains. These technics allow to obtain results, leaving in suspension question of PHA granules composition <sup>98-100</sup>.

In contrast to phospholipid, the presence of proteins surrounding PHA granules has long been accepted and are commonly called PHA granule-associated proteins (PGAP). Studies for identification and analysis of PHA granules surface, and protein composition, are performed using different technics such as atomic force microscopy (AFM), transmission electron microscopy (TEM), fluorescent microscopy, liquid chromatography coupled with mass spectrometry (LC-MS), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or trypsin digestion. Diversity of PGAP have been highlighted at the PHA granules surface, for example Jendrossek *et al.*, 2009, have identified a high

number of polypeptides by proteomic analysis in *C. necator*, some of which are not essential for PHA metabolism<sup>94</sup>. Usually PGAP, which can be detected in different microorganisms, are classified in four groups : PHA synthases, PHA depolymerases, regulatory proteins and phasins<sup>91</sup>.

PHA synthases are key enzymes of PHA biosynthesis and are responsible for conversion of (R)-3-hydroxyacyl CoA in PHA simultaneously to liberation of Coenzyme A. Similarly to other proteins of PHA metabolism, first studies and description were performed with *C. necator* (formerly *Ralstonia eutropha*) and now approximately 90 PHA synthases have been identified and characterized. PHA synthases are classified in four classes, depending on their structures and their substrates specificity<sup>56</sup>. PHA synthases of class I are constituted of one PhaC sub-unit, encoded by *phaC* genes, and have a molecular weight ranging from 60 to 70 kiloDalton (kDa). They use substrates with 3 to 5 carbon atoms for synthesis of scl-PHA. PHA synthases belonging to class I have been described in numerous bacterial species including *C. necator*. Class II regroups PHA synthases consisting of one PhaC sub-unit, similarly to PHA synthases of class I, with a molecular weight also ranging from 60 to 70 kDa. But this class of synthases are responsible of synthesis of mcl-PHA from substrates constituted of 6 to 14 carbon atoms. They are mainly described in *Pseudomonas* species. Members of class III PHA synthases, synthesize also scl-PHA from substrates with 3 to 5 carbon atoms but also from substrates with 6 to 8 carbon atoms, such as PHA synthase of *Thiocapsa pfenningii*. They have been identified in *Allochromatium vinosum* or Archaea and are composed of two sub-units: PhaC and PhaE with both a molecular weight of approximately 40 kDa. PHA synthases composing class IV are also formed by two sub-units: PhaC and PhaR with a molecular weight of approximately 40 kDa and 20 kDa, respectively. This class of synthases, which are mainly responsible for scl-PHA synthesis, are for now exclusively found in *Bacillus* genus (*B. megaterium*, *B. cereus*,...) <sup>58,69,87,101</sup>.

PHA depolymerases, named PhaZ, are responsible for PHA depolymerisation (« degradation ») and were the first time identified in *P. putida* (*P. oleovorans*)<sup>94,102</sup>. Two type of depolymerases can be reported: intracellular depolymerases (i-PHA depolymerases or i-PhaZ) and extracellular depolymerases (e-PHA depolymerases or e-PhaZ). PhaZ have been identified in PHA producing strains themselves, algae and fungi<sup>41</sup>. Additionnally to these two main classes, a third class has been described in *Rhodospirillum rubrum* and corresponds to PHA depolymerases with a periplasmic location (p-PHA depolymerases or P-PhaZ). PHA depolymerases expose a « specificity to substrates » in the sense that there are specific PHA depolymerases for scl-PHA or for mcl-PHA<sup>103</sup>. Usually PHA depolymerases are considering as hydrolases and/or esterases which are potentially not only involved in PHA metabolism. They start their activity when bacteria are stressed, under a carbon limitation, and hydrolyze polymers in 3-hydroxyalkanoic acid, the monomeric unit of PHA. Then a dehydrogenase oxidizes 3-hydroxyalkanoic acid to form acetoacetyl-CoA which will be convert in acetyl-CoA by  $\beta$ -ketothiolase<sup>104</sup>.

Phasins (commonly called PhaP) are members of an important group of PGAP. They were identified for the first time in 1994 in *Rhodococcus ruber*, before being detected in other numerous PHA-producing bacteria (*C. necator*, *B. megaterium*, *Pseudomonas putida*,...) and also some Archaea (*Haloferax mediterranei*)<sup>56,64,105</sup>. They constitute the most important and widespread group of PGAP and are classified in four families, depending on a characteristic domain, and bacterial species where they have been identified<sup>105</sup>. Phasins PhaP1 of *C. necator*, PhaF and PhaI of *P. putida* are the most well studied. Phasins are amphiphilic oligomers, mainly found in a tetramer conformation in solution, with a molecular weight ranging from 11 to 25 kDa and play different role linked to PHA metabolism and characteristic of PHA granules. Indeed, they are suggested to be essential for PHA-granule formation and to bring them a structural role. It seems that PhaP regulate surface, size and number of granules, determine the surface-to-volume ratio and help the stabilization of these structures<sup>56</sup>. Additionally, some phasins seems to have a role in the level expression and activity of PHA synthase and in activation of PHA depolymerase. Expression of PhaP, and consequently their activities, are regulated by the regulatory protein PhaR, which is found at the surface of PHA-granules but also bound to DNA (region upstream to *phaP* and *phaR* genes)<sup>94,105</sup>. Another phasin, PhaF, has been identified in *P. putida* KT2440 and described to play the role of stabilizers of PHA granules<sup>73</sup>.

The transcriptional regulator PhaR of *C. necator* is a repressor of PhaP phasin. Based on data obtained from studies conducted with *C. necator*, different hypothesis have been proposed concerning the mode of action of PhaR. PhaR binds PHA-granules as soon as polymerisation is started by PHA synthase, leading to a decrease of the free-PhaR cytoplasmic concentration and consequently stopping repression of phasin PhaP by PhaR. Synthesis of PhaP is possible and the phasin can attach the PHA granules leading to a decrease number of available site for PhaR. Cytoplasmic concentration of PhaR therefore increases and the protein can bind to DNA on promoter region of *phaP* gene (coding for PhaP protein) inhibiting its transcription, but also its own transcription (autoregulation of PhaR). Inhibition of PhaP also occurs when there are no optimal conditions for PHA accumulation/production. Due to its capacity to bind irreversibly PHA-granules and reversibly DNA, regulatory protein PhaR exposes a bifonctionnal character<sup>56,91,94</sup>.

Some multifunctional proteins have also been described to have a multifunctional role. For example, PhaM protein of *C. necator* H16 is able to bind PHA granules and DNA (*in vitro*) and nucleoid region (*in vivo*). Moreover this proteins seems to expose phasin-like properties and capacity to assist distribution of PHA-granules<sup>73</sup>.

### III.3 Production of PolyHydroxyAlkanoates

PHA production involves several steps ranging from optimization of bacterial culture to extraction and purification of the polymer (**Figure 11**). Most of the steps can be performed using different methods and the choice of these methods must result in an optimal balance between quality of PHA and final cost. Next section will expose some of methods which can be used in the different steps.

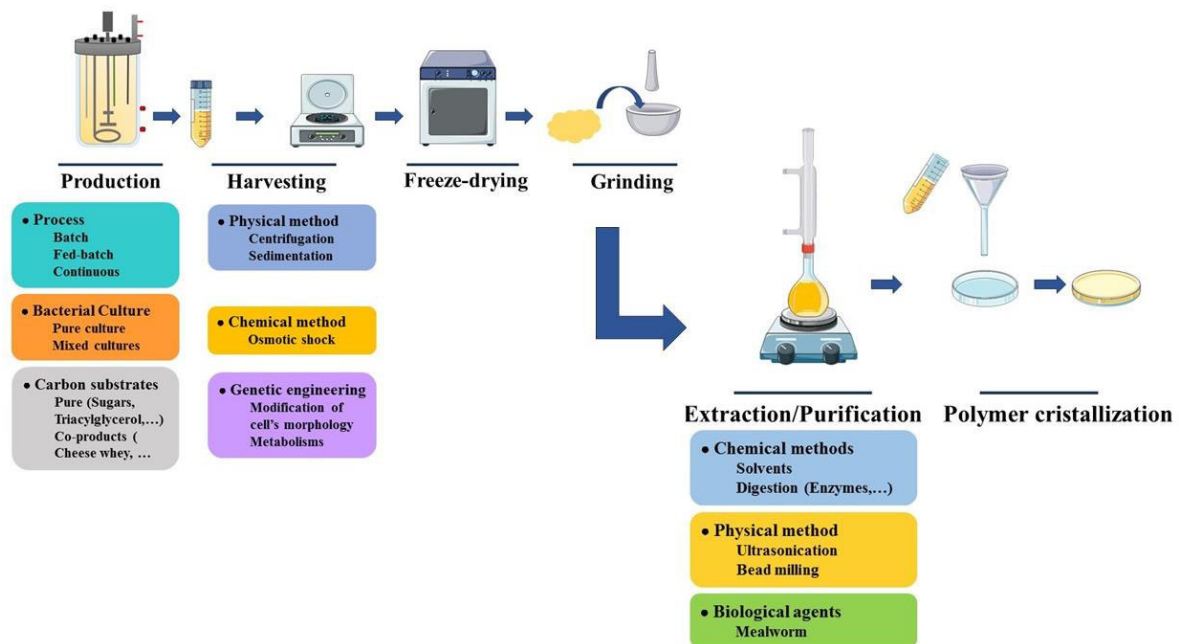


Figure 11: Schematic representation of general mode of production of PHA.

#### III.3.1 Operational modes of Production

Usually three modes of PHA production are used: batch, fed-batch and continuous modes (**Figures 11 and 12**). Pure or mixed cultures can be used, the second solution allowing exploitation of open-unsterile processes. All modes are still deeply investigated in order to optimize PHA yield and productivity, indeed production of PHA can be non-growth associated to some species. For efficient large-scale production, PHA-producing organisms have to be cultivated under stable physical and chemical parameters (pH-value, temperature, oxygen supply, substrate concentration) and under aseptic conditions to exclude microbial competition phenomena.

As exhaustively described in literature, PHA production encompasses two easily distinguishable steps for majority of PHA producing strains. First one aims to produce a maximal active cell density in a nutritionally balanced growth medium and the second one leads to PHA accumulation by producing a nutrient stress inducing deviation of the carbon flux from biomass production towards PHA synthesis. Even if there is a separation between two phases, a PHA production can be observed during biomass accumulation step, but is negligible as compared to that occurring during bacterial growth<sup>63,106</sup>.

Although even efforts for solid-state PHA production are reported, these processes result in modest productivity. The two-step process characteristic for PHA production can only be efficiently performed in planktonic cultures non-immobilized cells.

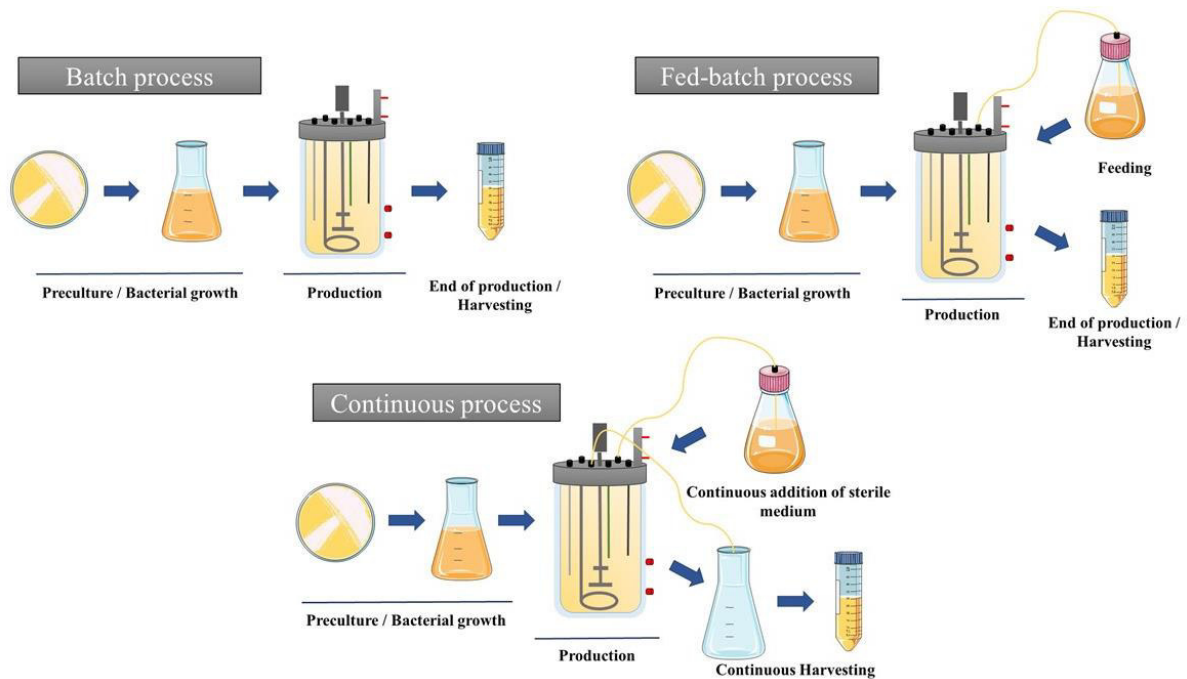


Figure 12: Three main modes of PHA production.

Batch cultures are frequently used to study PHA production. With this process bacteria are growing in a medium exposing an excess of carbon and a limitation of essential nutrient (oxygen, nitrogen or phosphorus). During this process, bacteria cells are subjected to a gradual change of nutrient concentration in medium. Optimally, bacterial growth is pushed to its maximum (end of exponential phase/beginning of stationary phase), but it is necessary to stop the fermentation at the right time to prevent the use of neo-synthesized PHA (depolymerization) and death phase. Usually, batch production is running for 24 to 72h. Cell harvest occurs only at the end of the cultivation batch (**Figure 12**). This process has already been employed with different bacterial species/strains like *Cupriavidus* sp., *Bacillus* sp. or *Chelatococcus* sp.<sup>107</sup>.

Discontinuous fed-batch strategies are most widely used at pilot- and industrial-scale such as with *Methylobacterium* sp. ZP24 or *Bacillus megaterium*<sup>63,106–108</sup>. Here, the culture is supplemented with selected nutrients until the working volume of the reactor is reached (**Figure 12**). This mode allows obtention of higher cell density than during batch cultivation and is generally well reliable and reproducible. Nonetheless, addition of some substrates, especially those used for mcl-PHA biosynthesis (i.e. fatty acids), can lead to a growth-inhibiting effect of the bacterial cells and consequently reduce productivity levels. Moreover, product quality (i.e. molecular weight and polydispersity) can fluctuate between different batches. Even if fed-batch culture exposes some disadvantages, it is the most common



used technic. **Table 8** exposes comparative data between batch and fed-batch process and illustrates difference of results between both using same strains and carbon substrates.

Table 8: Comparison of PHA production using different process.

Strain	Carbon sources	Process	PHA	PHA/CDW (wt%)	Production (g/L)	Ref.
<i>Cupriavidus necator</i> H16	Residual oil	Batch	P-3HB	20	1.24	109
		Fed-Batch		41	6.36	
<i>Pseudomonas oleovorans</i>	Residual oil	Batch	P-3HHx-co-3HO-co-3HD-co-3HDD	17	0.87	109
		Fed-Batch		39	6.38	
<i>Bacillus megaterium</i> BA-019	Sugarcane molasses	Batch	P-3HB	27	8.75	108
		Fed-Batch		46	41.58	
Bacterial consortium	Sugarcan molasses	Continuous, Feast and Famine Batch	P-3HB-co-3HV	~17	~0.43	110

Continuous culture, chemostat, is also employed for PHA biosynthesis. This method consists in replacing culture broth with sterile medium in order to extend production period and maintain specific growth rate with adjustment of dilution rate. This technic allows to obtain the highest productivities with a constant product quality. However, longer production time with this technic increases risk of contamination and consequently sterility must be better controlled during production period. In comparison to batch and fed-batch cultures, harvesting occurs continuously in continuous process (**Figure 12**)<sup>63,106,107</sup>. This method has already been used with different species such as *C. necator* DSM 545, *Haloferax mediterannei* DSM 1411, *Pseudomonas putida* (also known as *P. oleovorans*) GPO1 or *P. putida* KT2440<sup>63,106,107</sup>.

### III.3.2 Bacterial cultures

Additionally to the mode of production, different bacterial cultures can be used: pure cultures, mixed cultures or recombinant strains cultures. For a long time, pure cultures were only used and allowed commercialisation of heteropolymers (by Monsanto, Imperial Chemical Industries Ltd.). Pure cultures are still used for production of fonctionalized PHA but also for exploitation of genetically engineered strains. With this technic, PHA composition is dependent of carbon substrates used and usually lead to production of one type of polymer. In contrast, mixed cultures/consortium of bacteria has been developed in order to reduce production cost. Indeed, this mode of production does not require sterilization of reactor and allows the use of atypical « cheap » carbon substrates (fermented cheese whey, municipal solid waste, sugar cane molasses,...) due to the high diversity of metabolisms presents<sup>111-113</sup>. This mode of production is based on ecological selection pressure during feast and famine cycles leading to select high capacity PHA-producing strains. PHA production with mixed cultures is also deeply investigated in case of halophilic PHA-producing strains. Indeed halophilic strains expose a high and similar capacity to uptake different substrates<sup>58</sup>. Moreover, use of halophilic strains eases

exploitation of open and unsterile systems which can highly participate to decrease the production cost<sup>114</sup>. Even if mixed culture production presents interesting advantages, rate of production and structural homogeneity of polymer are still limiting and require to be deeply investigated.

In addition to wild type strain which can be found in common pure or mixed cultures, recombinant strains are also widely used.

The oldest strategy is the use of genetic engineered non-producing strain, such as *Escherichia coli*, which frequently expose a faster growth rate and have low substrates specificity<sup>107</sup>. Other option is to promote PHA metabolisms by weakening competitive pathways: TCA cycle for improve P-3HB production or methyl citrate cycle (MCC) for improve P-3HB-co-3HV production<sup>61,115</sup>. Modifications of bacterial cell sizes can also be considered. Indeed, different studies using genetically engineered *E. coli* (CRISPRi), have demonstrated that larger cells allow a higher PHA accumulation. Futhermore, higher bacterial cell size can influence conversion of substrates into PHA and facilitate biomass harvesting by the use of gravity<sup>116</sup>. Many studies have already been conducted during the last decades for production of scl- and mcl-PHA using genes of various PHA-producing strains such as *C. necator* or *Pseudomonas* species for example<sup>73,107,117,118</sup>.

Modification of PHA genes expression levels, designing of ribosome binding site (RBS) or modification of promoters are other strategies which already gave good results<sup>116</sup>. However, expression of additional copies of PHA genes is sometimes not sufficient to improve polymer production<sup>73</sup> and consequently other strategies must be employed.

Genetic engineering of a wide variety of PHA-producing bacteria can be performed using well-known and applied molecular biology technics. However, they expose a low success rate and are time-consuming. Futhermore, it seems very difficult to apply these technics to large gene clusters like those involved in PHA production. Consequently, additional technics have been developped like CRISPR/Cas9, CRISPRi, single-strand overlapping annealing (SSOA) or Nickling Endonucleases for DNA ligation-independant cloning (NE-LIC). SSOA consists of a digestion of gene cluster and to anneal and covalently attach single-strand DNA (ssDNA) to a circular and linear vector and NE-LIC to produce ssDNA overlaps with 3'-end and 5'-end<sup>73</sup>. These technics allow to express more easily PHA-production pathway by non-producing species, as well as *E. coli*<sup>119</sup>, or to increase co-production of hydrogen and P-3HB simultaneously<sup>120</sup>.

### III.3.3 Carbon substrates

A major parameter of PHA production is the selection of appropriate carbon substrates. In addition to their impact on polymer structures, they can also negatively affect final cost of materials.

Carbon substrates used for PHA production can be divided into three groups: simple carbohydrates (sugars), triacylglycerol and hydrocarbons. The first one being the most widely and easily assimilated

by bacteria. In comparison, exploitation and conversion of triacylglycerol and hydrocarbons is only observed in some species <sup>76,121</sup>.

Monosaccharides and oligosaccharides can directly be incorporated and use by bacteria (i.e. glucose, fructose, sucrose, lactose,...) while polysaccharides, (i.e. cellulose, hemicellulose, starch...) must be hydrolyzed before their assimilation.

Triacylglycerol can be used as carbon substrates in PHA production systems exploiting adapted bacteria, meaning lipase-producing and secreting strains: *Pseudomonas* sp., *Aeromonas caviae*, *Chromobacterium* sp.,... For example, Sharma *et. al* have shown that the use of canola oil with *P. chlororaphis* PA23 can lead to PHA production <sup>76,122</sup>. Moreover, production of mcl-PHA with *Pseudomonas putida* strains CA-3 has also been performed using mixture of volatile fatty acids (VFA) composed of acetic acid, propionic acid, butyric acid and valeric acid <sup>123</sup>.

Among the wide variety of bacterial species with hydrocarbons related metabolism only some of them are able to use them for PHA production. For example, *Sphingobium scionense* sp. WP01<sup>T</sup> <sup>124</sup>, *Bacillus* sp. CYR1 <sup>125</sup> or *C. necator* <sup>126</sup> have already demonstrated their ability to synthesize PHA using different hydrocarbons. *Sphingobium scionense* sp. WP01<sup>T</sup> is able to convert biphenyl and naphthalene into P-3HB <sup>124</sup>, while *Bacillus* sp. CYR1 can use phenolic compounds or naphthalene for P-3HB synthesis <sup>125</sup>. In the case of *C. necator*, benzaldehyde allows PHA productions and presence of other different toxic compounds, such as benzene dimethanol, represent an efficient stress to improve PHA production <sup>126</sup>.

Unfortunately even if the use of carbohydrates, triacylglycerol or hydrocarbons is easy, these substrates can be expensive and/or can require costly and time-consuming pre-treatment. Moreover, they are derived of food crops creating a competition with food provisions. Consequently, numerous studies are conducting for valorisation of inexpensive carbon substrates as found in agroindustrial, municipal wastes <sup>51,121,127</sup> or petrochemical plastic waste <sup>128</sup>.

### III.3.4 Downstream processing

Final steps of PHA production consist of harvesting biomass, polymers extraction and purification (**Figure 11**). All these steps can significantly impact the PHA yield, the PHA structure and the overall production cost. Moreover, these steps can have a negative environmental impact. Numerous researches are conducted for the replacement of conventional methods by eco-friendly or bio-based techniques accordingly to the principles of green-chemistry.

Biomass harvesting can be performed by different easy/common chemical or physical methods such as (ultra-) centrifugation, gravity sedimentation, (micro-)filtration or flocculation and has the objective of separating biomass from culture broth and potential extracellular compounds <sup>42,129</sup>. Depending on final volume of production, these traditional harvesting methods can be tedious and

constraining. Consequently, various strategies have been developed to modify cell morphology using genetic engineering on genes/proteins related to cell division processus (FtsZ) or cytoskeletal protein expression <sup>129</sup>. These modifications lead to higher PHA accumulation, formation of filamentous cells and aggregates and easier/faster gravimetric separations.

Extraction step commonly requires the use of high quantity of chemicals, solvents, water and energy which negatively impact environment <sup>130</sup>. Several methods have been tested and extractions can be performed by the use of solvents (halogen-free or halogenated), by digestion (biological agents, enzymes, chemicals or surfactants) or by supercritical fluids. Additionally to chemicals technics, mechanical methods such as high pressure, ultrasonication or bead milling can also be applied for PHA recovery. Sometimes, pre-treatment steps (i.e. drying, grinding, (bio-)chemical treatments,..) can be applied and allow a better extraction efficiency. Among extraction technics, a particular interest is given to biological agents (i.e. laboratory rats or mealworms) during the last few years according to results of several research teams which have demonstrated viability of coupling breeding worms and PHA recovery. It has been shown that dried bacterial biomass of PHA-producing strains (*C. necator*) can be used as an effective nutrient source for mealworm. They will then digest biomass without use of PHA which are recovered from fecal pellets <sup>131</sup>. This method allows the recovery of PHA without the alteration of PHA granules and consequently represents a promising solution, which could be integrated to a circular economy concept and contribute to reduction of production costs <sup>132</sup>. Each method exposes advantages and disadvantages and selected method must expose best balance between all parameters and must consider final use of PHA (for example biomedical applications require obtention of PHA exposing a high purity). For a long time, solvents have been the most used but cost and environmental impact led to increase the research for alternative solutions that are more eco-responsible/eco-friendly and less expensive <sup>42</sup>.

### III.4 PHA production employing halophilic microorganisms

Previously, capacity of halophilic microorganisms to produce various compounds of interest has been exposed. Among the examples, production of PHA by halophilic microorganisms exposes several advantages, as well as the use of high salt concentrations or simplified extraction processes which allow the reduction of production cost.

#### III.4.1 Haloarchaea

PHA production by haloarchaea can be achieved using different modes of production can be used exploiting typical (sugars or starch <sup>46,62</sup>) or atypical carbon substrates (by-product of ethanol industry <sup>133</sup>, olive mill wastewater <sup>134</sup>, cheese whey <sup>135</sup> or macroalgal co-products <sup>136</sup>). For example, P-3HB-co-3HV production has been reported with *Hfx. mediterranei* when by-products are added in production medium which was also the case without the addition of precursors <sup>62</sup>. Similarly, P-3HB-co-

3HV-co-4HB production has been performed with a mix of  $\gamma$ -butyrolactone, valerate and whey (Table 9)<sup>46,62</sup>.

Table 9: Comparative data of PHA production in haloarchaea species.

Bacterial strain	Carbon substrates	PHA	PHA/CDW (wt%)	Production (g/L)	Ref.
<i>Haloferax mediterranei</i>	Whey sugars	P-3HB-co-3HV (6mol% 3HV)	73	12.2	62
	Pretreated vinasse	P-3HB-co-3HV (12.4mol% 3HV)	70	19.7	133
	<i>Ulva</i> sp. derivated carbohydrates	P-3HB-co-3HV (8.0mol% 3HV)	58	2.2	136
	Olive mill wastewater	P-3HB-co-3HV (6.5mol% 3HV)	43	0.2	134
	Cheese whey	P-3HB-co-3HV (1.5mol% 3HV)	53	7.9	135
	$\gamma$ -butyrolactone, valerate and whey	P-3HB-co-3HV-co-4HB (21.8mol% 3HV and 5.8mol% 4HB)	88	14.7	46,62
<i>Haloferax gibbonsii</i>	Glucose	P-3HB	1	n.i	137,138
<i>Haloferax volcanii</i>	Glucose	P-3HB	7	n.i	137,138
<i>Haloarcula</i> sp. IRU1	Glucose	P-3HB	63	n.i	139
<i>Haloarcula hispanica</i>	Glucose	P-3HB	2	n.i	13,138
<i>Haloarcula marismortui</i>	Glucose	P-3HB	21	n.i	13,140
<i>Halogeometricum borinquense</i> strain E3	Glucose	P-3HB-co-3HV (21.5mol% 3HV)	74	~4.0	137
<i>Natrinema altunense</i> RM-G10	Glucose	P-3HB-co-3HV (13.9mol% 3HV)	61	14.8	141

In addition to *Hfx. mediterranei*, other Haloarchaea can be exploited such as *Hfx. volcanii*<sup>137,138</sup> or *Haloarcula* sp. IRU1<sup>139</sup> for P-3HB production and *Halogeometricum borinquense* strain E3<sup>137</sup> or *Natrinema altunense* strain RM-G10<sup>141</sup> for P-3HB-co-3HV production (Table 9).

Haloarchaea strains have already shown their ability to produce homo- or copolymer of PHA (Table 9), which can be less expensive than using recombinant strains<sup>142</sup>. Furthermore, the use of these strains allows easy downstream processing as osmotic shock can be employed<sup>24,46</sup>.

### III.4.2 Halobacteria

Among Halobacteria, *Halomonas boliviensis* LC1 has been used in different studies with various carbonaceous substrates as typical sugars (glucose, sucrose or xylose) and also agricultural by-products or hydrolysates of starch. For example, production of P-3HB was achieved using sucrose under oxygen limitation and led to 54% of PHA in cdw (7.7 g/L of PHA)<sup>143,144</sup>. Production of copolymer such as P-3HB-co-3-HV can also be achieved with halobacteria as well as *Halomonas campisalis* MCM B-1027 (Table 10)<sup>46,145,146</sup>.

Table 10: Comparative data of PHA production in Halobacteria species.

Bacterial strain	Carbon substrates	PHA	PHA/CDW (wt%)	Production (g/L)	Ref.
<i>Halomonas boliviensis</i> LC1	Sucrose	P-3HB	54	7.7	143,144
<i>Halomonas campisalis</i> MCM B-1027	Maltose	P-3HB-co-3HV (36.8mol% 3HV)	81	4.5	145,146
<i>Vibrio harveyi</i> MCCB 284	Glycerol	P-3HB	72	2.3	148
<i>Bacillus</i> sp. KSN5	Molasses	P-3HB	72	10.8	149
<i>Yangia</i> sp. CCB MM3	Glycerol	P-3HB-co-3HV (7.0mol% 3HV)	n.i	n.i	151
<i>Yangia</i> sp. ND 199	Fructose	P-3HB	62	2.7	150
	Fructose and sodium heptanoate	P-3HB-co-3HV (4.9mol% 3HV)	56	1.5	
	Fructose and 1,4 butanediol	P-3HB-co-4HB (5.7mol% 4HB)	55	1.3	
<i>Aeromonas halophila</i> 4AK4	Lauric acid	P-3HB-co-3HHx (23.6mol% 3-HHx)	20	1.5	152,153
	Oleic acid	P-3HB-co-3HHx (25.1mol% 3HHx)	6	0.3	

In addition to *Halomonas* species, different other halophilic bacteria have been reported to be efficient PHA-producing <sup>147</sup>. For example, *Vibrio* species have already been identified as potential PHA-producing bacteria since the 1990's and new studies continue to identify new producing strains. Recently *Vibrio harveyi* MCCB 284 <sup>148</sup> and *Bacillus* sp. KSN5, a bacterium from marine soil <sup>77</sup>, have demonstrated their ability to produce P-3HB from different carbon substrates (**Table 10**). In the same way, two species of *Yangia* genus have also been identified as PHA producing strains: *Yangia* sp. CCB MM3, isolated from Malaysian Mangrove, and *Yangia* sp. ND199, isolated from Vietnamese mangrove. Interestingly, both of them are able to produce copolymers using various pure or mix of substrates (**Table 10**) <sup>150,151</sup>.

Halophilic species produce mainly short chain length PHA (scl-PHA), but some members of *Aeromonadales* order have been described as efficient producers of medium chain length PHA (mcl-PHA), which are elastomeric materials. Indeed, *Aeromonas halophila* 4AK4 have been used for P-3HB-co-3HHx production from lauric and oleic acid in shake flasks and 20,000-L fermentor (**Table 10**) <sup>152,153</sup>.

### III.4.3 Genetically engineered halophilic bacteria

As mentioned before, complementary to the study of halophilic wild type strains, various engineered strains have been developed and studied in order to increase production yields, by managing different metabolisms, with the final objective of reducing production costs or obtaining new types of PHA <sup>154</sup>. One of the most studied is the halophilic strain *Halomonas bluephagenesis* TD01 (also named *Halomonas* TD01), which was initially isolated from a Chinese lake <sup>155</sup>. The strain was exploited for P-3HB production on glucose with a fed-batch process productions reach 80% of PHA in cdw (64.0 g/L of PHA) (**Table 11**).

Table 11: Comparative data of PHA production in genetically engineered *Halomonas* species. *Halomonas bluephagenesis* TD01<sup>a</sup>: Production performed with a fed-batch process, *Halomonas bluephagenesis* TD01<sup>b</sup>: Production performed with an open unsterile and continuous process, *Halomonas bluephagenesis* TD01<sup>c</sup>: Strain engineered with CRISPR/Cas9 technic and *Halomonas bluephagenesis* TD01<sup>d</sup>: Strain engineered with CRISPRi technic.

Bacterial strain	Carbon substrates	PHA	PHA/CDW (wt%)	Production (g/L)	Ref.
<i>Halomonas bluephagenesis</i> TD01 <sup>a</sup>	Glucose	P-3HB	80	64.0	155
<i>Halomonas bluephagenesis</i> TD01 <sup>b</sup>	Glucose	P-3HB	60	24.0	
<i>Halomonas bluephagenesis</i> TD08	Glucose	P-3HB-co-3HV (4.1mol% 3HV)	61	3.5	156
	Glycerol	P-3HB-co-3HV (6.1mol% 3HV)	67	4.5	
<i>Halomonas bluephagenesis</i> TD01 <sup>c</sup>	Glucose	P-3HB-co-4HB (16.1mol% 4HB)	61	50.1	157
<i>Halomonas bluephagenesis</i> TD01 <sup>d</sup>	Glucose and propionic acid	P-3HB-co-3HV (11.8mol% 3HV)	80	7.2	115
<i>Halomonas bluephagenesis</i> TD01 <sup>d</sup>	Glucose	P-3HB	72	9.7	61

Comparatively, an open unsterile and continuous process with same species and carbon substrates allows the production of 60% of PHA in cdw (24.0 g/L of PHA) <sup>155</sup>. In order to tailor-make PHA or to manage production, several genetic modifications have been studied. The recombinant strain *Halomonas bluephagenesis* TD08, with a 2-methyl citrate synthase gene and three depolymerase genes deleted, was able to produce P-3HB-co-3HV <sup>156</sup>. Production of P-3HB-co-4HB was also achieved using a *Halomonas bluephagenesis* TD01 (Table 11) with a controlled expression of the 4HB-CoA transferase (responsible of conversion of 4-HB to 4HB-CoA) <sup>157</sup>. Traditionnal molecular technics; such as plasmid transformation, gene deletion or plasmid expression systems; allowed to obtain interesting results. However, they are time consuming with low yield of success. Consequently, studies have been conducted to apply new technics of genome editing on PHA-producing strains, on both halophilic and non-halophilic bacteria <sup>19,154</sup>. Indeed, systems such as Clustered Regularly Interspaced Short Palindromic Repeats associated protein 9 (CRISPR/Cas9) or Clustered Regularly Interspaced Short Palindromic Repeats Interference (CRISPRi), initially developed in other bacterial species, have been tested and effectively exploited with halophilic species. CRISPR/Cas9 was used with *Halomonas bluephagenesis* TD01 that can naturally produce P-3HB-co-3HV but with a low of 3-HV units content (<0.73mol%). Repression of *prpC* gene, responsible for expression of 2-methyl citrate synthase, was performed and leads to blocking the methyl citrate cycle (MCC). Consequently, the use of glucose and propionic acid mix in addition to inhibition of MCC allow to increase molar ration of 3-HV up to 11.8mol% (Table 11) <sup>115</sup>. CRISPRi system was also exploited with *Halomonas bluephagenesis* TD01 for repression *gltA*, coding for citrate synthase allowing to use more acetyl-CoA (initially provided for tricarboxylic acid cycle) for P-3HB synthesis. Genetically enginnered strain obtained, *Halomonas*

*bluephagenesis* TD-gltA2, exposes a stronger P-3HB production up to 9.7 g/L of PHA (71.8 % of PHA in cdw) while the wild type strain, *Halomonas bluephagenesis* TD01 produces 7.4 g/L of PHA (77.7% of PHA) (Table 11)<sup>61</sup>. In addition to experiments on (un-)related PHA-metabolisms, other studies were conducted to manage the size of PHA granules by working on *ftsZ*, *minC* and *minD* genes, which are coding for bacterial fission ring protein (Z rings) involved in cell division. Experiments allow to manage bacterial cell size, in order to increase PHA granule size by deleting *phaP* genes which code for phasins and are involved in granules formation<sup>61,158</sup>.

To sum up, there is a huge numbers of possibilities to genetically engineered halophilic strains. Different metabolic pathways can be exploited to increase production yield and productivity or to reduce costs of production but difficulties with genetic engineering of halophilic strains are often encountered making management of bioprocess essential.

## Conclusion

This report gives an overview of the wide variety of halophilic and halotolerant (micro-)organisms existing in the three domains of life and demonstrates their importance. Halophilic/halotolerant (micro-)organisms have very interesting and promising properties making them unavoidable candidates for biotechnological applications in a near future. Indeed, they can be used for productions of different valuable compounds usable in various fields ranging from agri-food industries to biomedical applications. In this report we first described, in the most efficient way, the taxonomic diversity of halophile and halotolerant (micro-)organisms. In a second part the potential of halophile/halotolerant as biotechnological tools for industry and the need to continue research for the optimization of their use have been discussed. Finally, we focused on PolyHydroxyAlkanoates (PHA) for their high potential as new bio-based and biodegradable materials. In this last section structure, molecular basis, metabolisms and production related to the biopolymers have been presented, non-exhaustively in some cases, due to the importance of this research field.

Accordingly, there is a wide variety of PHA available or which can be produced. Numerous studies are still running to elaborate the best way to produce and obtain tailor made PHA. Indeed, bacterial strain used, monomeric composition, length of the side chain, range of molecular weight and potential physical and/or chemical modification of PHA have a crucial impact on material properties and determine its final potential application. Consequently, it seems obvious that production of PHA using halophile or halotolerant (micro-)organisms could be a promising solution in a near future to produce tailor-made/valuable biopolymers, reduce cost of production and finally enhance use of PHA in everyday life.



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## Chapter 2

### Matériels et Méthodes

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# I Souches bactériennes et conditions de culture

## I.1 Souches et milieux étudiés

### I.1.1 Souches bactériennes

L'ensemble des espèces bactériennes utilisées dans cette étude est listé dans le **Tableau 1**. Pour chaque souche, une cryobanque a été réalisée en utilisant 25% de glycérol puis les tubes ont été stockés à -80°C jusqu'à leur utilisation.

Tableau 1: Souches bactériennes utilisées dans cette étude.

Souche	Caractéristiques	Origine
<i>Halomonas</i> sp. SF2003 ID CNCM – I-4786	Souche sauvage productrice de P-3HB et de P-3HB-co-3HV	Mer d'Iroise, Collection IRDL, UBS
<i>Cupriavidus necator</i> H16 (DSM 428)	Souche sauvage productrice de PHA	Collection DSMZ
<i>C. necator</i> H16 PHB <sup>-4</sup> (DSM 541)	Souche mutée non productrice de PHA	Collection DSMZ
<i>C. necator</i> H16 PHB <sup>-4</sup> pBBRMCS-2 <i>phaC1</i>	Souche mutée/recombinante avec le plasmide pBBRMCS2 portant le gène <i>phaC1</i> de <i>Halomonas</i> sp. SF2003, identifié par annotation automatique. Étude du rôle du gène <i>phaC1</i> . Km <sup>R</sup>	Cette étude USM, Penang, Malaisie
<i>C. necator</i> H16 PHB <sup>-4</sup> pBBRMCS-2 <i>phaC2</i>	Souche mutée/recombinante avec le plasmide pBBRMCS2 portant le gène <i>phaC2</i> de <i>Halomonas</i> sp. SF2003, identifié par annotation automatique. Étude du rôle du gène <i>phaC2</i> . Km <sup>R</sup>	Cette étude, USM, Penang, Malaisie
<i>E. cloni</i> ® 10G cells	Cellules (électro/chimio) compétentes	USM, Penang, Malaisie
<i>Escherichia coli</i> S17-1	Cellules (électro/chimio) compétentes	USM, Penang, Malaisie

Km<sup>R</sup> : résistance à la Kanamycine. DSMZ : Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures.

### I.1.2 Milieux

Différents milieux, adaptés à la culture de chaque espèce ou la production de PHA, ont été préparés à partir de réactifs sous forme déshydratés.

#### I.1.2.1 Croissance

- *Halomonas* sp. SF2003

La souche est cultivée en milieu Zobell (en g/L : Bacto Tryptone (Difco, BD) 4, extraits de levures (Fisher BioReagents) 1, sea salts (Instant Ocean, Aquarium systems) 30, pH 7,5). La culture est réalisée à 30°C, sous agitation (200rpm). Le milieu est complété de glucose (Labogros), à une concentration finale de 10 g/L, pour les pré-cultures dédiées aux productions.

- *Cupriavidus necator*

Les souches de *Cupriavidus necator* H16, *C. necator* H16 PHB<sup>-4</sup>, *C. necator* H16 PHB<sup>-4</sup> pBBRMCS-2 *phaC1* et *C. necator* H16 PHB<sup>-4</sup> pBBRMCS-2 *phaC2* sont cultivées sur milieu riche en nutriment (Nutrient rich medium, NR medium) (en g/L : extraits de viande (Sigma-Aldrich) 10, extraits

de levures (Fisher BioReagents) 2, peptone de gélatine, digestion enzymatique 10). Les cultures sont réalisées à 30°C et sous agitation (200 rpm). Le pH du milieu est ajusté à 7.

Les milieux utilisés pour les souches transformantes sont supplémentés en kanamycine (Km) à une concentration finale de 50 µg/mL à partir d'une solution mère à 100 mg/mL. L'antibiotique est ajouté après stérilisation du milieu.

#### I.1.2.2 Tests de croissance en conditions de stress toxique

Pour l'étude de la croissance de *Halomonas* sp. SF2003 en présence de composés toxiques un milieu T (en g/L : Glucose 10 ; Bacto Tryptone (BD, Difco) 0,5 ; extraits de levures (Fisher BioReagents) 0,25 et sea salts (Instant Ocean, Aquarium systems 1, pH 7,5) est utilisé. Les pré-cultures sont réalisées dans le milieu classique et la composition est modifiée pour les tests de croissance en remplaçant le glucose par les hydrocarbures aromatiques polycycliques sélectionnés à des concentrations finales de 5, 50 or 250 ppm.

#### I.1.2.3 Tests de production

Pour la production de PHA, deux milieux sont utilisés : le milieu Référence 1, favorisant la production de biomasse avec un rapport C/N de 24,6, et le milieu Référence 2, présentant un rapport C/N de 187,2 favorable à l'accumulation de PHA.

Milieu Référence 1, en g/L : sucres/carbohydrates/substrats carbonés 10 ; Bacto Tryptone (Difco, BD) 1 ; extraits de levures (Fisher BioReagents) 0,5 ; sea salts (Instant Ocean, Aquarium systems) 11, pH 7,5.

Milieu Référence 2, en g/L: sucres/carbohydrates/substrats carbonés 20 ; extraits de levures (Fisher BioReagents) 0,4 ; sea salts (Instant Ocean, Aquarium systems) 11, pH 7.

Le milieu Référence 2 est également utilisé pour les tests de criblage des sources de carbone. Pour ces tests du Nile Red est ajouté, à une concentration finale de 0,5%, après autoclavage. Pour cela une solution mère de Nile Red à 25% en DMSO (DiMethylSulfOxide) est préparée et stérilisée sur filtre de 0,2 µm de porosité. Les milieux Référence 1 et Référence 2 sont supplémentés de kanamycine à une concentration finale de 50 µg/mL pour les souches transformants/transformées *C. necator* H16 PHB<sup>-4</sup> pBBRMCS-2 *phaC1* et *C. necator* H16 PHB<sup>-4</sup> pBBRMCS-2 *phaC2*.

Tous les milieux sont stérilisés 20 minutes à 121°C et à une pression de 1,2 Bar.

## I.2 Cinétique de croissance des souches bactériennes

Les cinétiques de croissance des souches utilisées dans cette étude ont été établies en milieu liquide dans différentes conditions de culture (**Tableau 2**). Pour l'étude de la croissance le milieu Référence 1 a été utilisé pour l'ensemble des souches (*Halomonas* sp. SF2003, *C. necator* H16, *C. necator* H16 PHB<sup>-4</sup>, *C. necator* H16 PHB<sup>-4</sup> pBBRMCS-2 *phaC1* et *C. necator* H16 PHB<sup>-4</sup> pBBRMCS-2 *phaC2*). La composition du milieu est modifiée selon les conditions et les substrats carbonés testés.

Tableau 2: Conditions étudiées pour les cinétiques de croissance dans cette étude.

	Conditions étudiées		
	Température (°C)	Salinité (% m/v)	Substrats
<i>Halomonas</i> sp. SF2003	6, 20, 25, 30 et 37	0,5 ; 3 ; 5 ; 10 et 20	Aldoses, Cétoses et diholosides Mélanges oses/acides
<i>C. necator</i> H16	/	/	Aldoses et Cétoses
<i>C. necator</i> H16 PHB <sup>-4</sup>	/	/	Aldoses et Cétoses
<i>C. necator</i> H16 PHB <sup>-4</sup> pBBRMCS-2 <i>phaC1</i>	/	/	Aldoses et Cétoses
<i>C. necator</i> H16 PHB <sup>-4</sup> pBBRMCS-2 <i>phaC2</i>	/	/	Aldoses et Cétoses

Les milieux sontensemencés à partir de pré-cultures d'une nuit (15h-16h) à 30°C en milieu Zobell pour *Halomonas* sp. SF2003, en milieu NR pour *C. necator* H16, *C. necator* H16 PHB-4 et en milieu NR supplémenté de kanamycine pour *C. necator* H16 PHB<sup>-4</sup> pBBRMCS-2 *phaC1* et *C. necator* H16 PHB<sup>-4</sup> pBBRMCS-2 *phaC2* ; puis incubés sous agitation (200 rpm pendant 24h à 30h à 30°C).

La croissance est suivie par la mesure de la densité optique à 600nm à l'aide d'un spectrophotomètre de paillasse (Cell Density Meter Model 40, FisherScientific). Les mesures sont réalisées toutes les demi-heures pendant les 7 premières heures puis toutes les heures par la suite.

## II Caractérisation bio-informatique de *Halomonas* sp. SF2003

### II.1 Séquençage et annotation du génome de *Halomonas* sp. SF2003

L'ADN génomique d'*Halomonas* sp. SF2003 a été extrait et séquençé par l'entreprise GATC Biotech (Cologne, Allemagne). Le séquençage a été réalisé à l'aide d'un séquenceur RS II d'une plateforme de séquençage Pacific Biosciences (Pacbio). Pour l'assemblage des données, le logiciel Hierarchical Genome Assembly Process (HGAP), de Pacific Biosciences, a été utilisé avec une librairie de séquences unique.

L'annotation du génome a été réalisée à l'aide des plateformes de données MicroScope Microbial Genome Annotation & Analysis Platform (MaGe, Genoscope, Évry, France) et National Center for Biotechnology Information (NCBI). L'étude des voies métaboliques a été réalisée à l'aide des bases de données MaGe et Kyoto Encyclopedia of Genes and Genomes (KeGG).

La séquence complète du génome d'*Halomonas* sp. SF2003 a été déposée sur GenBank sous le numéro d'accèsion CP028367.

## II.2 Étude phylogénétique d'*Halomonas* sp. SF 20003

L'identification de la souche a été réalisée par étude et comparaison des gènes codant l'ARNr 16S. La construction de l'arbre et le remplacement de *Halomonas* sp. SF2003 ont été réalisés avec le logiciel MEGA7 en utilisant la méthode NJ (Neighbor Joining). Les séquences d'ARNr 16S de trente-trois souches, représentatives ou non de la famille des *Halomonadaceae*, ont été utilisées pour la réalisation de cette étude. Toutes les séquences utilisées pour l'étude sont en accès libre sur NCBI.

Les distances d'évolution ont été calculées avec la méthode Maximum Composite Likelihood et sont exprimées dans l'unité du nombre de substitutions de bases par sites. Toutes les positions avec un écart ou des données manquantes ont été éliminées permettant une analyse sur un total de 821 positions.

## II.3 Étude bio-informatique des PHA synthases

L'analyse bio-informatique des enzymes PhaC1 et PhaC2 a été réalisée par comparaison des séquences en acides aminés des PHA synthases. L'alignement et la détection des domaines similaires aux lipases (lipase box-like sequence), aussi appelés séquence consensus des PhaC, ont été réalisés avec le logiciel BioEdit. L'ensemble des séquences analysées sont en libre accès sur NCBI.

## II.4 Étude phénotypique d'*Halomonas* sp. SF2003

### II.4.1 Croissance de *Halomonas* sp. SF2003 en conditions de stress osmotique et toxique

#### II.4.1.1 Salinité

Des précultures d'une nuit (15h à 16h) en milieux Zobell à 25°C sous agitation sont préparées. La réponse de *Halomonas* sp. SF2003 face au stress salin a été étudiée en milieu Luria Bertani (en g/L : Bacto Tryptone (Difco, BD) 10 ; extraits de levures (Fisher BioReagents) 5). Les milieux sont complétés de NaCl (LaboGros) pour des concentrations finales (m/v) de 0,5% ; 3% ; 5% ; 10% et 20%. Les milieux sontensemencés à partir des pré-cultures pour une concentration finale de 5% (v/v) et sont incubés pendant 24h à 27°C sous agitation. Chaque condition est testée en triplicat.

#### II.4.1.2 Composés toxiques : Hydrocarbures Aromatiques Polycycliques

Cinq Hydrocarbures Aromatiques Polycycliques (HAP) ont été testés individuellement : naphthalène 99 + % (Janssen Chemica), benzène 99.5 % (Janssen Chemica), toluène (Carlo Erba), phénanthrène 98 + % (Janssen Chemica) et le pyrène (Merck). Pour tester la réponse de la souche face à différentes concentrations en HAP, des solutions mères en éthanol ou méthanol absolu, ont été préparées.

Des pré-cultures d'une nuit (15h à 16h) à 25°C (200 rpm) sont réalisées en milieu T puis transférées dans des milieux T modifiés, en remplaçant le glucose par un des HAP à des concentrations finales de 5, 50 or 250 ppm. Les erlenmeyers sont scellés à l'aide de coton cardé, d'aluminium et de parafilm pour limiter l'évaporation des HAP puis incubés 24h à 30°C sous agitation. Des contrôles

positifs et négatifs sont réalisés avec ou sans glucose (10g/L), d'éthanol ou de méthanol. Chaque condition est testée en triplicat.

#### II.4.1.3 Tests d'antibiorésistance

L'antibiorésistance de *Halomonas* sp. SF2003 est étudiée par la technique de l'antibiogramme (diffusion sur disque en milieu gélosé).

Des géloses Zobell sontensemencées par inclusion à partir de pré-cultures d'une nuit de *Halomonas* sp. SF2003. Des disques de papiers stériles sont déposés à l'aide d'une pince sur les géloses puis 10 µL de solution d'antibiotique sont déposées dessus, un témoin négatif avec de l'eau ultra-pure stérile est réalisé en parallèle. Les géloses sont incubées entre 24h et 48h à 30°C puis la présence de halos d'inhibition est évaluée et s'ils sont détectés leurs diamètres est mesuré. La sensibilité de *Halomonas* sp. SF2003 est testée pour des concentrations de 1 et 5 mg/mL d'ampicilline, de gentamycine, de kanamycine, de pénicilline et la streptomycine.

### III Techniques de biologie moléculaire

#### III.1 Extraction d'ADN

L'ADN génomique d'*Halomonas* sp. SF2003 est extrait, à partir d'une culture d'une nuit de 10 mL, à l'aide du kit QIAamp DNA Mini Kit (Qiagen) selon les instructions du fournisseur. Pour la réalisation des PCR, l'ADN chromosomique de la souche peut également être extrait à partir d'une colonie isolée sur gélose.

Les extractions d'ADN plasmidiques sont réalisées en utilisant le kit GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific), selon les instructions du fournisseur. Pour obtenir des concentrations en ADN plasmidique suffisantes, les extractions sont réalisées sur un volume de pré-culture de 10 mL. Les deux plasmides construits lors de cette étude sont répertoriés dans le **Tableau 3**.

Tableau 3: Plasmides utilisés dans cette étude.

Plasmide	Caractéristiques	Provenance
pBBRMCS-2	Plasmide d'expression chez les bactéries à Gram négatif. Km <sup>R</sup>	EcoBioMaterial Lab, UMT, Malaisie
pBBRMCS-2 <i>phaC1</i>	Plasmide d'expression portant le gène <i>phaC1</i> de <i>Halomonas</i> sp. SF2003	Cette étude
pBBRMCS-2 <i>phaC2</i>	Plasmide d'expression portant le gène <i>phaC2</i> de <i>Halomonas</i> sp. SF2003	Cette étude

Km<sup>R</sup> : Résistance à la kanamycine

#### III.2 Réaction de polymérisation en chaîne (PCR)

Les amorces utilisées pour l'amplification des fragments d'intérêts sont désignés à l'aide des logiciels A Plasmid Editor (ApE), BioEdit ou SerialCloner et les T<sub>m</sub> sont déterminés avec les outils « NEB T<sub>m</sub> Calculator » ([www.tmcaculator.neb.com/#!/main](http://www.tmcaculator.neb.com/#!/main)) ou « Thermo Scientific T<sub>m</sub> Calculator » ([www.thermoscientificbio.com/webtools/tmc/](http://www.thermoscientificbio.com/webtools/tmc/)) ou « IDT Oligo Analyzer »

(<https://eu.idtdna.com/pages/tools/oligoanalyzer>). L'ensemble des amorces utilisées dans cette étude est listé dans le **Tableau 4**.

Tableau 4: Amorces oligonucléotidiques utilisées dans cette étude.

Amorces	Séquences (5' – 3')	Cible
PhaC1-F PhaC1-R	AGTAAGCTTAGGAGGAGGCGCATGCAGTCGCCAGCCCA AGTAGCATTAAATTCAGGTTTGCTTCACGTAGGTG	<i>phaC1</i>
PhaC2-F PhaC2-R	AGTAAGCTTAGGAGGAGGCGCATGGACTCAGCCCAGCA AGTAGCATTAAATTCAACTCTTGTCGCTATCCTTG	<i>phaC2</i>
F <i>phaC1</i> A	AATGGTGGCAACAGGCGGTG	<i>phaC1</i> (1/2)
F <i>phaC1</i> B	TGCCATCCAGGCAATCACAG	<i>phaC1</i> (2/2)
F <i>phaC2</i> A	TTCCGCCGAAGGCATCAATGTC	<i>phaC2</i> (1/3)
F <i>phaC2</i> B	GCAGGACGGCTATCTGGATG	<i>phaC2</i> (2/3)
F <i>phaC2</i> C	AGAGCGTGCCGGAAGAGATC	<i>phaC2</i> (3/3)

Les PCR de routines pour les gènes *phaC1* et *phaC2* sont réalisées dans un volume réactionnel final de 50 µL contenant du mélange 2X KAPA HiFi HotStart ReadyMix (KAPABIOSYSTEMS, Wilmington, MA, États-Unis), 10 µM de chaque amorce (IDT Integrated DNA technologies, Singapour, République de Singapour) et environ 200 ng d'ADN matrice. Le mélange KAPA HiFi HotStart ReadyMix contient déjà l'ADN polymérase KAPA HiFi HotStart, le tampon, le MgCl<sub>2</sub> et les dNTPs nécessaires aux réactions d'amplification.

Les PCR de contrôles des clonages et des transconjugaisons sont réalisées dans un volume réactionnel de 10 µL contenant du mélange 2X EconoTaq Master Mix (Lucigen, Middleton, WI, États-Unis) et 1 µM de chaque amorce (IDT Integrated DNA technologies, Singapour, République de Singapour). Le mélange EconoTaq Master Mix contient déjà l'ADN polymérase EconoTaq, le tampon, le MgCl<sub>2</sub> et les dNTPs nécessaires aux réactions d'amplification. Une colonie, prélevée de façon stérile, est incorporée et diluée dans le mélange réactionnel.

Les réactions de PCR sont réalisées dans un MJ Mini™ Personal Thermal Cycler (Biorad, Hercules, CA, États-Unis). Les cycles de dénaturation primaire, de dénaturation, d'appariement, d'élongation et d'élongation finale (température et durée) utilisés lors de cette étude sont décrits dans le **Tableau 5**. Pour l'ensemble des PCR les cycles d'amplification ; comprenant les étapes de dénaturation, d'appariement et d'élongation ; sont répétés 30 fois.

Tableau 5: Cycles de PCR utilisés dans cette étude.

Étape	PCR de routine		PCR de contrôle	
	Température (°C)	Durée	Température (°C)	Durée
Dénaturation primaire	95	3 minutes	94	10 minutes
Dénaturation	98	0,33 minute	94	0,5 minute
Appariement	53-55	0,25 minute	55-56	0,5 minute
Élongation	72	0,67 minute	72	2,5 minutes
Extension finale	72	2 minutes	72	10 minutes

L'amplification des fragments d'ADN est contrôlée par électrophorèse en gel d'agarose 1% (Invitrogen) dans un tampon TAE 1X (Tris HCl 40 mM, acétate de sodium 20 mM, EDTA 10 mM pH 7,9) (Biosolve). La migration des échantillons est réalisée dans un système d'électrophorèse RunOne (Embi Tec) pendant 35 minutes à 100V. Les échantillons sont observés après fixation de l'ADN par du RedSafe™ (iNtRON) et les gels sont passés sous lumières UV à l'aide d'un transilluminateur Gel Doc™ XR + System (Bio-Rad, Hercules, CA, États-Unis). Le marqueur de taille moléculaire (1 kb Plus DNA Ladder, ThermoFisher Scientific) permet de déterminer la taille des fragments de 0,1 à 20 kb.

Les fragments séparés par électrophorèse peuvent être purifiés à partir du gel à l'aide du kit « QIAquick Gel Extraction » (QIAGEN). La concentration et la pureté des échantillons sont contrôlés par calcul du rapport  $DO_{260nm}/DO_{280nm}$ .

### III.3 Double digestion

Les doubles digestions, préparant le clonage, sont réalisées dans un volume réactionnel de 30 µL contenant du tampon de digestion FastDigest (ThermoFisher Scientific, Waltham, MA, États-Unis), les enzymes de digestion SmaI et HindIII (ThermoFisher Scientific, Waltham, MA, États-Unis), 0,2 µg d'ADN et de l'eau ultra-pure stérile. Le mélange réactionnel est incubé pendant 50 minutes à 37°C avant d'être inactivé par choc thermique de 15 minutes à 80°C. La pureté des fragments d'ADN digérés est contrôlée par électrophorèse d'après la technique décrite dans la section III.2 Réaction de polymérisation en chaîne (PCR).

### III.4 Clonage/ligation

Les produits de digestion sont récupérés sur le gel et purifiés à l'aide du kit MYgengel and PCR purification system (Gene Xpress, Selangor, Malaysia), d'après les instructions du fournisseur. Le milieu réactionnel contient le plasmide pBBRMCS-2 et les produits PCR digérés et purifiés ainsi que de la solution de ligation 1 (TaKaRa Bio Inc., Kusatsu, Shiga, Japon).



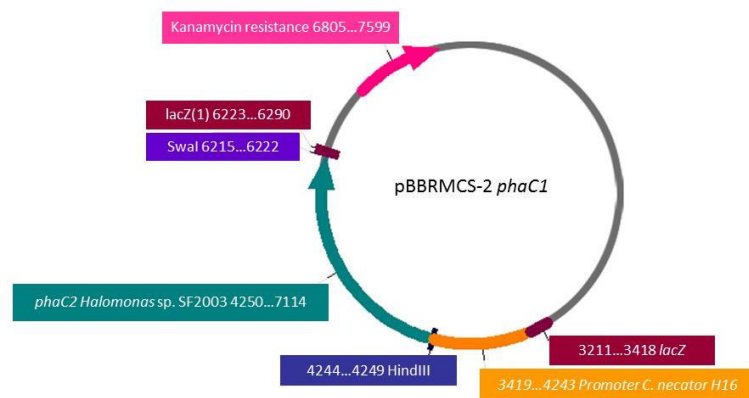


Figure 1: Carte du plasmide pBBRMCS-2 *phaC1*.

Les ligations sont réalisées en utilisant une concentration en vecteur de 40 ng et un ratio molaire insert : vecteur de 3 :1. Une fois complété le milieu est incubé pendant 1h à 16°C avant d’être utilisé pour la transformation des cellules compétentes. Les plasmides, pBBRMCS-2 *phaC1* (**Figure 1**) et pBBRMCS-2 *phaC2* (**Figure 2**) sont obtenus par cette technique.

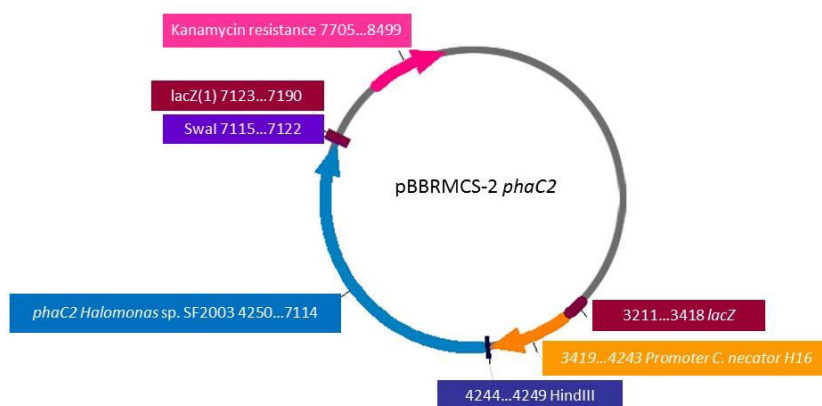


Figure 2 : Carte du plasmide pBBRMCS-2 *phaC2*.

### III.5 Conjugaison/transformation

#### III.5.1 Transformation des cellules *E. cloni*® et *Escherichia coli* S17-1

La transformation des cellules compétentes *E. cloni*® (Lucigen, Parmenter St, Middleton, WI, États-Unis) et *Escherichia coli* S17-1 est réalisée par choc thermique.

Dans un premier temps, les cellules *E. cloni*® sont placées 10 minutes sur de la glace avant d’être mise en contact avec 4 µL du produit de clonage/ligation. L’ensemble est homogénéisé avant d’être placé de nouveau 30 minutes sur glace. Un choc thermique de 45 secondes à 42°C est appliqué puis les cellules sont de nouveau placées sur de la glace pendant, au moins, 2 minutes. Les cellules sont reprises avec 960 µL de Recovery medium et la suspension est mise à incuber pendant 1h à 37°C. Les cellules sont étalées sur gélose LB supplémentée de kanamycine (50 µg/mL) avant d’être incubées à 37°C

pendant 24h pour sélectionner les transformées. Les colonies observées après 24h sont prélevées et des PCR de contrôle sont réalisées, pour vérifier le clonage et le sens d'insertion du fragment d'ADN, directement à partir des colonies par introduction et homogénéisation de ces dernières dans le milieu réactionnel. Les paramètres d'amplification et le contrôle sur gel d'électrophorèse sont réalisés avec les techniques décrites précédemment (III.2 Réaction de polymérisation en chaîne (PCR)).

Les colonies de *E. coli*® ayant correctement intégré les plasmides sont cultivées une nuit en milieu LB supplémenté de kanamycine (50 µg/mL) pour réaliser des extractions plasmidiques à l'aide du kit GeneJet Plasmid MiniPrep (ThermoFisher Scientific, Waltham, MA, États-Unis) selon les instructions du fabricant. Les plasmides extraits et purifiés sont utilisés pour réaliser la transformation des cellules compétentes *E. coli* S17-1 en utilisant le même protocole que pour les cellules *E. coli*, décrit précédemment. Après transformation les cellules sont sélectionnées sur des géloses LB supplémentées de kanamycine (50 µg/mL) et des PCR de contrôle sont réalisées selon le même protocole que pour les cellules *E. coli*®.

### III.5.2 Transconjugaison *E. coli* S17-1/*Cupriavidus necator* H16 PHB<sup>-4</sup>

Pour réaliser la transconjugaison, la souche *Cupriavidus necator* H16 PHB<sup>-4</sup> est mise en contact avec des cellules *E. coli* S17-1 portant les plasmides d'intérêt. Des précultures d'une nuit sont réalisées pour les deux souches, en milieu LB supplémenté de kanamycine (50 µg/mL) à 37°C pour les cellules *E. coli* S17-1 et en milieu NR à 30°C pour la souche *C. necator* H16 PHB<sup>-4</sup>. Les deux précultures sont centrifugées à 14000 rpm pendant 2 minutes puis les culots de *E. coli* S17-1 et *C. necator* H16 PHB<sup>-4</sup> sont repris respectivement avec 300 µL de milieu NR et de milieu LB (sans addition de kanamycine). Cent microlitres de chaque suspension cellulaire sont prélevés avant d'être mis en contact dans un tube stérile. Après homogénéisation, 100 µL sont prélevés et déposés au centre d'une gélose NR qui est mise à incuber à 30°C pendant 8h. À la fin de la période d'incubation, la culture est prélevée stérilement et déposée dans 5 mL de milieu NR avant d'être homogénéisée et étalée sur des géloses citrate de Simmons. Deux géloses sont ensemencées avec 100 et 20 µL de suspension bactérienne avant d'être incubées à 30°C pendant 48h. Après incubation, des PCR de contrôle sont réalisées sur les colonies bleues en utilisant les mêmes paramètres décrits précédemment (III.2 Réaction de polymérisation en chaîne (PCR)).

Des extractions plasmidiques sont réalisées à partir de précultures des colonies de transconjugants positives à l'aide du kit GeneJet Plasmid MiniPrep (ThermoFisher Scientific, Waltham, MA, États-Unis). Les plasmides sont dosés avant d'être préparés, avec les amorces correspondantes, pour le séquençage.

Le séquençage des produits clonés est réalisé par la société 1st BASE Sdn. Bhd. (Malaysia). L'analyse des séquences est effectuée par comparaison avec les séquences des bases de données MaGe

et NCBI à l'aide des logiciels SerialCloner (©Franck Perez [Serial Basics]) et BioEdit (©Tom Hall [Ibis Therapeutics]).

## IV Production de PHA

### IV.1 Criblage de sources de carbone assimilables

#### IV.1.1 Source de carbone

Différents glucides et mélanges de glucides/acides organiques ont été sélectionnés selon qu'ils étaient retrouvés à l'état naturel dans la biomasse végétale ou dans des gisements de co-produits ou d'effluents industriels. Les mélanges glucides/acides organiques sont réalisés en proportion 95/5 mol/mol % (**Tableau 6**).

Tableau 6: Substrats carbonés testés dans cette étude.

	Substrats carbonés	Fournisseur
Glucide	Fructose	Sigma
	Galactose	Acros Organic
	Glucose	Labogros
	Mannose	Acros Organic
	Maltose	Acros Organic
	Melibiose	Acros Organic
	Rhamnose	Acros Organic
	Saccharose	Acros Organic
	Substrats carbonés	Fournisseur
Mélange Glucide/Acide organique	Acide trans-2-pentanoïque	Acros Organic
	Acide lévulinique	Acros Organic
	Acide malique	Acros Organic
	Acide heptanoïque	Acros Organic
	Acide hexanoïque	Acros Organic
	Acide dodécanoïque	Alfa aesar
	Acide palmitique	Alfa aesar

#### IV.1.2 Marquage des granules de PHA au Nile Red

##### IV.1.2.1 Géloses au Nile Red

Le Nile Red (NR) est un marqueur de fluorescence à la phenoxazine ( $\lambda_{\text{excitation}} = 530\text{nm}$  et  $\lambda_{\text{émission}} = 635\text{nm}$ ) (**Figure 3**). Il est utilisé pour la détection des lipides intracellulaires et des domaines

hydrophobes protéiques. Son utilisation pour la détection des granules de PHA est largement répandue depuis la mise au point d'une technique par Spiekermann *et al.*, en 1999 <sup>1</sup> permettant d'observer les granules de PHA.

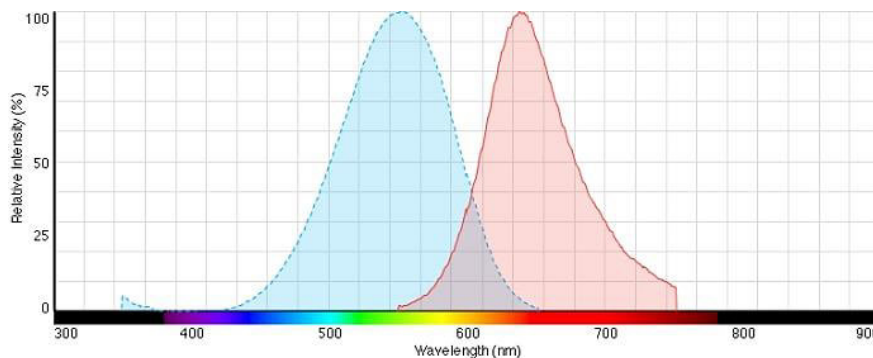


Figure 3: Spectre d'absorption et d'émission de fluorescence du Nile Red.

L'accumulation de PHA dans les cellules bactériennes est visualisée par ajout d'une solution de Nile Red (Sigma) à 0,5% (m/v) dans du DMSO dans les milieux gélosés (Référence 2). Les géloses sontensemencées, à partir d'une pré-culture d'une nuit et incubées 3 jours à 30°C. La fluorescence des colonies est observée sous lampe UV au transilluminateur.

Les souches *C. necator* H16 (DSM 428) et *C. necator* H16 PHB<sup>-4</sup> (DSM 541) sont utilisées respectivement comme témoin positif (PHA<sup>+</sup>) et négatif (PHA<sup>-</sup>).

#### IV.1.2.2 Microscopie confocale à balayage laser

Le screening des substrats de biosynthèse en milieu gélosé est complété par des observations sur des cultures liquides par microscopie confocale à balayage laser (MCBL, Zeiss LSM 710). Le marquage au Nile Red est complété par un marquage au Syto9 qui est un marqueur de fluorescence des acides nucléiques (ARN et ADN) permettant d'observer les cellules procaryotes ( $\lambda_{excitation}= 488\text{nm}$  et  $\lambda_{émission}= 493\text{-}543\text{nm}$ ) (Figure 4).

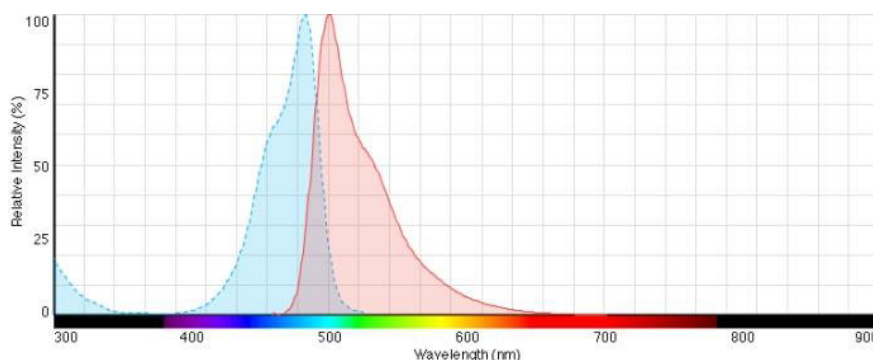


Figure 4: Spectre d'absorption et d'émission de fluorescence du Syto9.

Pour ces observations, le Nile Red est incorporé dans le milieu Référence 2 au début de la phase de biosynthèse de PHA à une concentration finale de 0,4% (v/v). Des prélèvements de 2 mL sont réalisés

à intervalles de temps réguliers durant 72h. Les échantillons prélevés sont lavés à deux reprises avec du tampon phosphate salin (PBS) à 1X à pH 7,4 (en g/L : NaCl 8 ; KCl 0,2 ; Na<sub>2</sub>HPO<sub>4</sub> 1,44 et KH<sub>2</sub>PO<sub>4</sub> 0,24) puis centrifugés 10 minutes à 7500 rpm à température ambiante. Dix microlitres de suspension cellulaire sont prélevés et déposés sur lame puis 8 µL de Syto9 à 5 µM sont ajoutés. Les états-frais sont incubés 15-20 minutes à température ambiante et à l'obscurité avant observation.

#### IV.1.3 Taux de recouvrement

Les observations qualitatives effectuées au microscope confocale à balayage laser sont analysées à l'aide du logiciel ImageJ. Le taux de recouvrement des clichés des lames est déterminé après traitement des images en noir et blanc.

### IV.2 Production

#### IV.2.1 Production en erlenmeyers

Des productions en erlenmeyers sont réalisées, notamment pour le screening de l'utilisation et l'assimilation de sources de carbone pour la production de PHA. Les productions sont réalisées selon un protocole en 2 phases.

Dans la première phase, l'obtention d'une quantité importante de biomasse est recherchée. Le milieu Référence 1 est inoculé à 10% (v/v) partir de pré-culture de 7h, en milieu Zobell additionné de glucose pour *Halomonas* sp. SF2003, Nutrient Rich (NR) pour *C. necator* H16 (DSM 428) ou NR + kanamycine (50 µg/mL) pour *C. necator* H16 PHB<sup>-4</sup> *phaC1* et *C. necator* H16 PHB<sup>-4</sup> *phaC2*.

La quantité de biomasse est suivie par mesure de la DO à 600 nm pendant 17h en incubateur orbital (30°C, 200 rpm). Lorsque la quantité de biomasse atteinte est maximale, les cellules sont culotées par centrifugation à 7500 rpm pendant 10 minutes à 4°C. Le culot cellulaire est lavé à deux reprises avec de l'eau saline (en g/L : Seasalts 11) suivi d'une centrifugation de 7500 rpm pendant 10 minutes à 4°C. À la suite des lavages, le culot est repris dans un volume minimum d'eau saline.

La seconde phase est initiée en transférant le culot de l'étape précédente dans le milieu référence 2, pauvre en azote pour induire la production de biopolymère. La culture est poursuivie pendant 72h à 30°C et 200 rpm. À la fin de l'étape de production, la culture bactérienne est culotée par centrifugation puis le culot cellulaire est lavé avec de l'eau distillée stérile avant d'être surgelé à -80°C puis lyophilisé.

Les productions sont réalisées de façon à n'occuper qu'un cinquième ou un quart du volume maximal du contenant, de cette manière une surface de contact avec l'air de proportion convenable est conservée, permettant une oxygénation.

#### IV.2.2 Production en bio-réacteurs

Les productions sont réalisées dans un fermenteur de paillasse (modèle BioStatB+, Sartorius, France) équipé de 2 cuves agitées (version twin) en verre borosilicaté d'un volume utile de 3L.

Les productions sont réalisées en suivant un protocole en 2 étapes. Une pré-culture de 7h est réalisée dans 300 mL de milieu Zobell additionné de glucose pour *Halomonas* sp. SF2003, Nutrient Rich (NR) pour *C. necator* H16 (DSM 428) ou NR + kanamycine (50 µg/mL) pour les souches *C. necator* H16 PHB<sup>-4</sup> *phaC1* et *C. necator* PHB<sup>-4</sup> *phaC2*.

L'intégralité des pré-cultures est transférée stérilement dans les cuves du fermenteur contenant 2,7 L de milieu Référence 1. Pendant la phase d'accumulation de biomasse, la température est maintenue à 30°C avec une agitation de 400 rpm et une concentration en oxygène dissous de 50%. Le pH des milieux est ajusté à 7,5 à l'aide du pilote et de solutions de soude (NaOH) et d'acide chlorhydrique (HCl) à 1M. Après 17h, la culture est stoppée et centrifugée à 7500 rpm pendant 10 minutes à 4°C. Le culot cellulaire est lavé à deux reprises avec de l'eau saline (en g/L : Seasalts 11) suivi d'une centrifugation de 7500 rpm pendant 10 minutes à 4°C. À la suite des lavages, le culot est repris dans un volume minimum d'eau saline.

La phase de production de PHA est initiée en transférant le culot dans les cuves du réacteur contenant 2L de milieu référence 2, pauvre en azote. La culture est poursuivie pendant 72h à 30°C et 400 rpm avec une concentration en oxygène dissous de 50%. Le pH est maintenu à 7,0 à l'aide du pilote et de solutions de soude (NaOH) et d'acide chlorhydrique (HCl) à 1M.

À la fin de l'étape de production, la culture bactérienne est culotée par centrifugation puis le culot cellulaire est lavé avec de l'eau distillée stérile avant d'être surgelé à -80°C puis lyophilisé.

### IV.3 Extraction et purification des PHA

Les PHA sont extraits dans le chloroforme à partir du culot cellulaire lyophilisé et broyé. À 1 g de masse cellulaire sèche (MCS), 25 mL de chloroforme sont ajoutés dans un ballon puis chauffés une nuit à 60°C sous agitation. La suspension est lavée avec ajout d'eau distillée puis centrifugée pendant 7 minutes à 5000 rpm.

La phase organique est récupérée puis filtrée à l'aide de filtres d'acétate de cellulose 0,2 µm (Clear Line). Le filtrat est distribué dans des boîtes de Pétri en verre jusqu'à évaporation totale du chloroforme à température ambiante.

## Références

1. Spiekermann, P., Rehm, B. H. A., Kalscheuer, R., Baumeister, D. & Steinbüchel, A. A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. *Archives of Microbiology* **171**, 73–80 (1999).





## **Chapter 3**

**Complete genome sequence of the halophilic PHA-producing bacterium *Halomonas* sp. SF2003: insights into its biotechnological potential.**

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## Contexte

Le travail réalisé dans cette partie se concentre sur l'étude du génome et du métabolisme de la bactérie marine *Halomonas* sp. SF2003. Cette souche marine est naturellement productrice de PolyHydroxyAlcanoates (PHA) et présente une grande capacité d'adaptation face à des conditions variées. L'étude de son génome vise à identifier les gènes directement reliés, ou non, au métabolisme des PHA ainsi que ceux liés à son adaptation. L'annotation du génome, couplée à des études phénotypiques, sert de base à ce travail afin de définir les meilleurs paramètres de croissance de la souche mais également de production dans le but de mieux contrôler et d'améliorer la production de PHA par *Halomonas* sp. SF2003.

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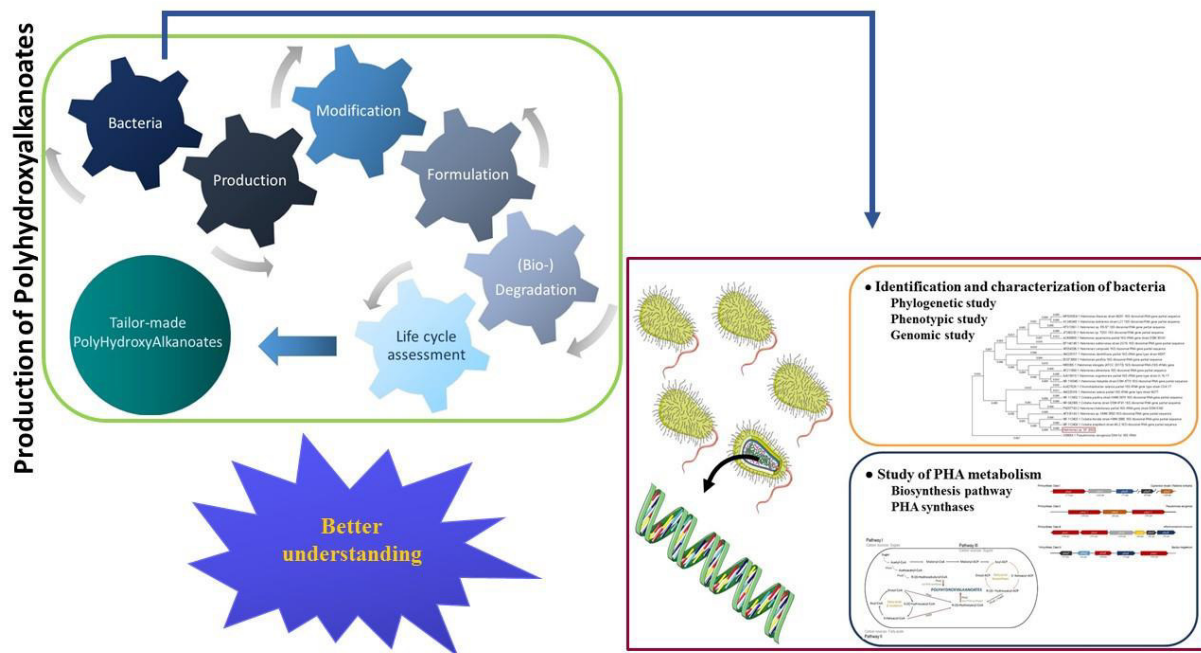
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**Keywords:** *Halomonas* sp. SF2003, Halophilic bacteria, Genome sequencing, Metabolism, Polyhydroxyalkanoates (PHA), Stress-related proteins.

## Highlights:

- *Halomonas* sp. SF2003 exposes interesting versatility in front of growth conditions
- The strain possess carbohydrates and fatty acids metabolisms linked to PHA metabolism
- PHA biosynthesis pathway exhibits an atypical organization with involvement of two PhaC

## Graphical abstract



## Abstract

A halophilic Gram-negative bacterium was isolated from the Iroise Sea and identified as an efficient producer of polyhydroxyalkanoates (PHA). The strain, designated SF2003, was found to belong to the *Halomonas* genus on the basis of 16S rRNA gene sequence similarity. Previous biochemical tests indicated that the *Halomonas* sp. strain SF2003 is capable of supporting various culture conditions which sometimes can be constraining for marine strains. This versatility could be of great interest for biotechnological applications. Therefore, a complete bacterial genome sequencing and *de novo* assembly were performed using a PacBio RSII sequencer and Hierarchical Genome Assembly Process (HGAP) software in order to predict *Halomonas* sp. SF2003 metabolisms, and to identify genes involved in PHA production and stress tolerance.

This study demonstrates the complete genome sequence of *Halomonas* sp. SF2003 which contains a circular 4,36 Mbp chromosome, and replaces the strain in a phylogenetic tree. Genes related to PHA metabolism, carbohydrate metabolism, fatty acid metabolism and stress tolerance were identified and a comparison was made with metabolisms of relative species. Genes annotation highlighted the presence of typical genes involved in PHA biosynthesis such as *phaA*, *phaB* and *phaC* and enabled a preliminary analysis of their organization and characteristics. Several genes of carbohydrates and fatty acid metabolisms were also identified which provided helpful insights into both a better knowledge of the intricacies of PHA biosynthetic pathways and of production purposes. Results show the strong versatility

of *Halomonas* sp. SF2003 to adapt to various temperatures and salinity which can subsequently be exploited for industrial applications such as PHA production.

## Introduction

*Halomonas* sp. SF2003 is a non-pathogenic Gram-negative bacterium isolated from the Iroise Sea (Brittany, France), deposited with the National Collection of Microorganisms Cultures (CNCM) of the Pasteur Institute (Paris, France). Based on a 16S rRNA sequence analysis, this bacterium belongs to the *Halomonadaceae* family which regroups a total of 90 species, mainly halophilic, divided into 10 genera. The most important genus of this bacterial family is the *Halomonas* genus<sup>1,2</sup>. An important taxonomic reorganization among these species was started in the 1990's and was responsible for a review of *Halomonadaceae* phylogeny and phenotypic features. To-date the classification of bacteria within the *Halomonas* genus is still not totally clarified and some bacteria are currently being reclassified, such as *Cobetia* or *Halomonas*<sup>3</sup>. Aside from their biological importance, marine bacteria belonging to the *Halomonadaceae* family have promising applications in biotechnology<sup>4,5</sup> as a source of compatible solutes stabilizers of biomolecules and cells, salt-tolerant enzymes, biosurfactants, biopolymers (exopolysaccharides (EPS) and PHA) and also for bioremediation applications<sup>6-9</sup>. To understand and exploit the features of these bacteria, different genomic projects have been carried out or are in progress<sup>10-12</sup>. In a previous work, we have reported the potential of *Halomonas* sp. SF2003 to produce short chain length PHA (scl-PHA) up to 78% of the cell dry weight<sup>13,14</sup>.

PHA are bacterial polyesters naturally synthesized by numerous bacterial species, belonging to more than 90 genera, including Gram-negative and Gram-positive organisms such as *Bacillus* spp., *Alcaligenes* spp., *Pseudomonas* spp., and *Azotobacter* spp. For these bacteria, PHA serves as carbon and energy reserves that are accumulated as inclusions of 0.2-0.5  $\mu$ m in diameter in response to unbalanced nutrient conditions such as a depletion of phosphorous, oxygen or nitrogen, whilst carbon is plentiful<sup>15-17</sup>. Owing to the diversity of monomers from different carbon sources that can be incorporated into PHA, and in turn change the polymer properties, a wide range of new polymeric materials are potentially available. Besides their renewable nature, PHA are readily biodegradable in the natural ecosystem<sup>17-19</sup>, and have shown mechanical, physicochemical and thermal properties close to those of conventional plastics<sup>20</sup> making them great sustainable alternatives for end-user applications such as food packaging, consumer goods, adhesives or 3D-printing for the plastics processing industry<sup>21</sup>. Due to their biocompatibility and ability to support cell growth and adhesion, PHA have been also utilized in the biomedical field for the development of different medical materials (cardiovascular products, suture, wound dressing, tissue scaffolds)<sup>22</sup>. In addition, PHA can be also very beneficial for biotechnological processes and increasing potential applications are emerging. For instance, PHA-producers and members of the genera *Cupriavidus* and *Pseudomonas* have been found to be efficient for *in situ* bioremediation of contaminated sites<sup>23</sup>. Recently, Bengtsson et al., 2017 have demonstrated the

performance of PHA-enriching mixed microbial consortia for the treatment of municipal wastewater. Other applications include the controlled release of herbicides in PHA-carriers or the enhancement of crop productivity using a PHA-rich bacterial inoculant to improve nitrogen fixation <sup>25,26</sup>.

Currently, numerous studies are being conducted in order to understand, exploit and/or to modify metabolic pathways and structures of these biopolymers and also to increase yields of production <sup>21,27</sup>.

Results of previous works on *Halomonas* sp. SF2003 PHA production <sup>13,14,28</sup> prompts us to continue its study to understand and to control its production. Consequently, sequencing of its genome should enable the identification of genes involved in the biopolymer metabolism. In the literature, numerous bacterial strains belonging to the *Halomonas* or *Cobetia* genera such as *C. marina*, *H. boliviensis* LC1, *H. elongata* DSM 2581, *H. salina*, *Halomonas* sp.TD01 <sup>29-32</sup> or to other genus, have already been described as PHA producers. PHA metabolisms have also been studied enabling us to compare them with *Halomonas* sp. SF2003 PHA metabolism.

Until now, three different metabolic pathways of PHA biosynthesis, involving acetyl-CoA or acyl-CoA as intermediate, have been described. The pathway depends on both the nature of the carbon source and the PHA synthase (PhaC) specificity of the microorganism.

Pathway I, for PHB production, starts with condensation of two acetyl-CoA, obtained after glycolysis of sugars (i.e. glucose), by acetyl-CoA acetyltransferase (PhaA), also named 3-ketoacyl-CoA thiolase or  $\beta$ -ketothiolase to produce acetoacetyl-CoA. Acetoacetyl-CoA reductase (PhaB) reduced acetoacetyl-CoA to (R)-3-hydroxybutyryl-CoA which is converted to PHA, and especially to PHB, by polyhydroxyalkanoate synthase (PhaC). Pathway II, derivative of  $\beta$ -oxydation of fatty acids, included production of 3-hydroxyacyl-CoA from 2,3-dehydroacyl-CoA, 3-hydroxyacyl-CoA or 3-ketoacyl-CoA by (R)-enoyl-CoA hydratase, 3-hydroxyacyl-CoA epimerase or ketoacyl-CoA reductase, respectively. Then 3-hydroxyacyl-CoA is used by PHA synthase for PHA synthesis. Finally, pathway III, involved the conversion of 3-hydroxyacyl-Acyl carrier protein (ACP), derived from the fatty acid synthesis from sugars (i.e. glucose), to 3-hydroxyacyl by 3-hydroxyacyl-ACP-CoA transferase followed by a synthesis of PHA by PHA synthase <sup>17,33,34</sup>. These three pathways are represented in **Figure 1**.

The objectives of this study were to sequence the *Halomonas* sp. SF2003 genome and to identify genes involved in PHA, carbohydrates, fatty acids and stress metabolisms for a more thorough understanding and utilization of the strain. Results will enable a better understanding of the strain itself and its potential industrial uses.

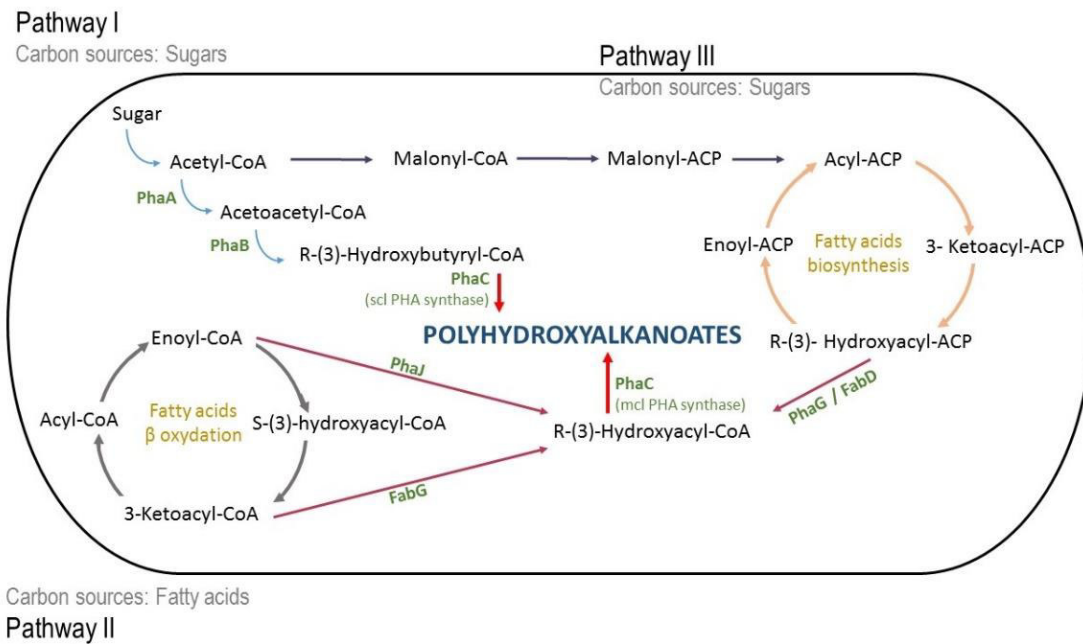


Figure 1: Schematic representation of the three metabolic pathways of PHA production identified in representative bacterial species. Adapted from Nomoto and Yano, 2009; Sudesh et al., 2000 and Verlinden et al., 2007.

## I. Results

### I.1. Genome features

Sequencing of *Halomonas* sp. SF2003 whole-genome produced 73,337 raw reads. It is composed of only one 4,358,421-bp circular chromosome, with a high GC content of 62.5%. A total of 4013 genes, including 3,408 protein-coding genes, 21rRNA, 72 tRNA and 21 ncRNA were identified. All the information is summarized in **Figure 2**, **Table 1** and **Table 2**.

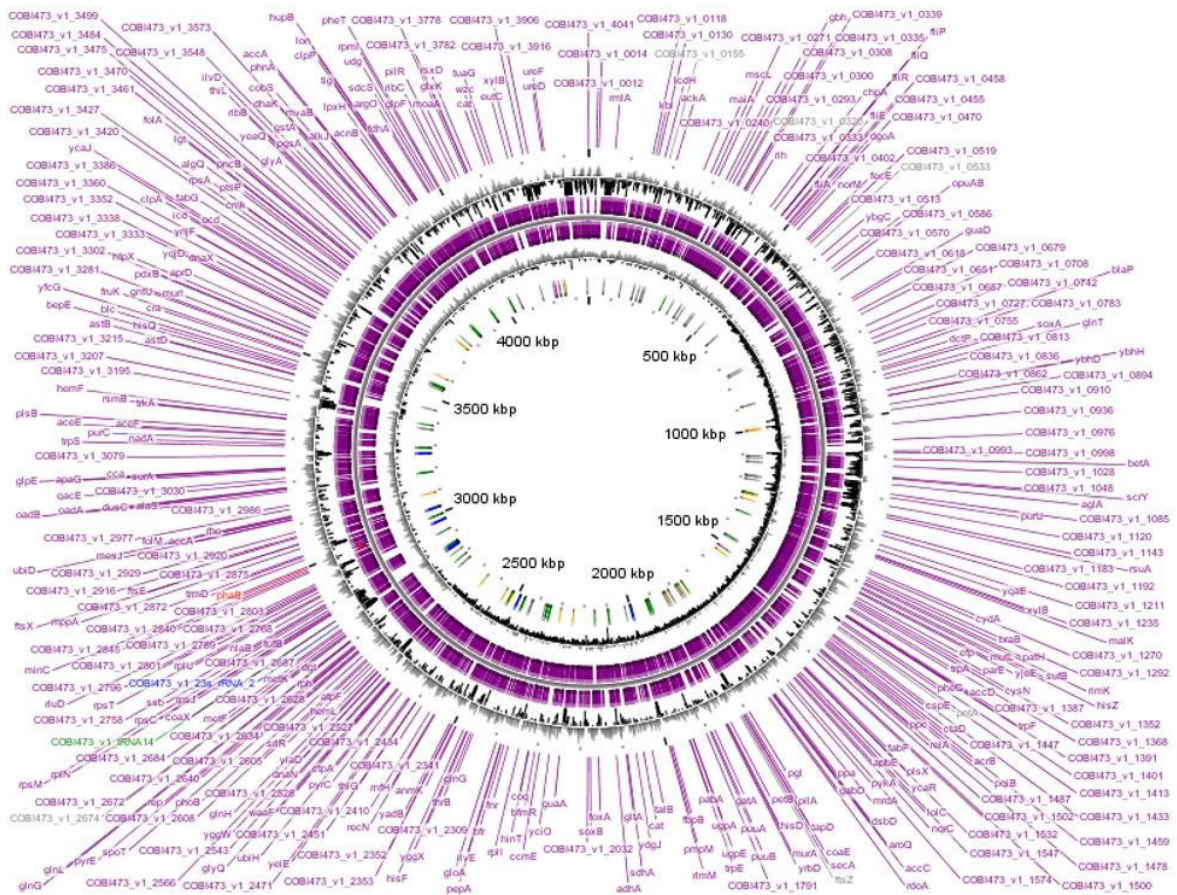


Figure 2: Circular map of *Halomonas* sp. SF2003 genome.  
 Legend: tRNA (green), rRNA (blue), misc\_RNA (orange), Transposable elements (pink) and pseudogenes (grey).

Comparisons were made with closely related species. Data concerning their genome size and GC% are summarized in **Table 3**<sup>11,12,35-41</sup> and are also available on NCBI. These comparisons show the genome size in the average size of other genomes but the number of genes and the GC% is higher than their respective averages. Some of the *Halomonas* species used for comparison also possess a plasmid unlike *Halomonas* sp. SF2003. A comparison of the percentage of associated genes shows that there are a higher number of genes involved in cell cycle control, cell division, chromosome partitioning, amino acid transport and metabolism in *Halomonas* sp. SF2003 than in the other species. Regarding these results *Halomonas* sp. SF2003 has similar genomic characteristics to other *Halomonas* species.

Table 1: Genome features of *Halomonas* sp. SF2003.

Attribute	Values
Genome size (bp)	4,358,421
DNA G+C (%)	62.49
Plasmid	0
DNA scaffolds	1
rRNA genes	21
tRNA genes	72
Other ncRNA genes	4
Protein	3,408
Predicted genes	3,598
Pseudo genes	93

Table 2: Number of genes associated with general COG functional categories.

Code	Value	%age	Description
J	185	4.6	Translation, ribosomal structure and biogenesis
A	1	0	RNA processing and modification
K	278	6.9	Transcription
L	137	3.4	Replication, recombination and repair
B	2	0	Chromatin structure and dynamics
D	42	1.1	Cell cycle control, Cell division, chromosome partitioning
V	39	1	Defense mechanisms
T	157	3.9	Signal transduction mechanisms
M	232	5.8	Cell wall/membrane biogenesis
N	74	1.8	Cell motility
U	89	2.2	Intracellular trafficking and secretion
O	146	3.6	Posttranslational modification, protein turnover, chaperones
C	256	6.4	Energy production and conversion
G	251	6.3	Carbohydrate transport and metabolism
E	438	10.9	Amino acid transport and metabolism
F	85	2.1	Nucleotide transport and metabolism
H	148	3.7	Coenzyme transport and metabolism
I	126	3.1	Lipid transport and metabolism
P	223	5.6	Inorganic ion transport and metabolism
Q	108	2.7	Secondary metabolites biosynthesis, transport and catabolism
R	473	11.8	General function prediction only
S	284	7.1	Function unknown
-	862	21.5	Not in COGs



Table 3: Comparison of genome statistics of different *Halomonas* species. Based on the data of Gao *et al.*, 2015 and completed with data from other studies.

Species	Genome size (Mb)	GC content (%)	Gene count
<i>H. anticarriensis</i> FP35	5.07	58.5	4,817
<i>H. aquamarina</i> 558	3.50	56.7	3,470
<i>H. boliviensis</i> LC1	4.14	54.5	3,915
<i>H. campaniensis</i> LS21	4.07	52.7	3,665
<i>H. elongata</i> DSM 2581	4.06	63.6	3,556
<i>H. elongata</i> K4	3.47	63.4	4,726
<i>H. halocynthiae</i> DSM 14573	2.88	53.8	2,773
<i>H. halodenitrificans</i> DSM 735	3.47	64.0	3,256
<i>H. hydrothermalis</i> MTCC 5445	3.93	60.2	3,637
<i>H. jeotgali</i> Hwa	2.85	62.9	2,636
<i>H. lutea</i> DSM 23508	4.53	59.1	4,368
<i>H. meridiana</i> R1t3	3.51	56.6	3,526
<i>H. salina</i> CIFRI1	4.26	64.2	3,882
<i>H. smyrnensis</i> AAD6	3.56	67.9	3,326
<i>H. stevensii</i> S18214	3.69	60.3	3,523
<i>H. sulfidaeris</i>	4.44	54.0	4,143
<i>H. titanicae</i> BH1	5.34	54.6	2,908
<i>H. zhanjiangensis</i> DSM 21076	4.06	54.5	3,739
<i>H. zincidurans</i> B6	3.55	66.4	3,392
<i>Halomonas</i> sp. R5-57	5.03	55.6	4,677
<i>Halomonas</i> sp. KM-1	4.99	63.9	4,220

## I.2. Identification and phylogenetic study for the classification of *Halomonas* sp.

### SF2003

Genome of *Halomonas* sp. SF2003 contains seven copies of the conserved 23S rRNA genes and 16S rRNA genes. Studies of the seven copies of each, revealed the existence of nine differences between the seven copies of the 23S rRNA genes and the existence of only one difference between the seven copies of 16S rRNA (a cytosine in 2 copies at the position 454 and a thymine in the other 5 copies). It was decided to use only 16S rRNA genes for the phylogenetic classification of *Halomonas* sp. SF2003. In order to limit disparate results, a consensus sequence was generated using 16S rRNA. **Figure 3** illustrates the optimal phylogenetic tree of strain obtained using a Neighbour-joining method (MEGA7), with a sum of branch length equal to 0.33835159. The analysis involved 23 nucleotide sequences. All positions containing gaps and missing data were disregarded. There were a total of 821 positions in the final dataset.

This analysis shows that *Halomonas* sp. SF2003 is closely related to *Cobetia amphilecti* strain 46-2, *Cobetia litoralis* strain KMM 3880, *Cobetia* sp. MACL02 and *Halomonas* sp. KMM 3550 species without a precise differentiation between *Cobetia* or *Halomonas*. These results are in accordance with other data in the literature and confirm that classification of *Halomonadaceae* members is still difficult to clarify<sup>1,3,42</sup>. In fact, de La Haba *et al.*, 2010, highlighted that the *Halomonas* genus is the only one atypical genus within the family *Halomonadaceae*. This genus is described as polyphyletic and is divided in two clusters. Some species are clustered with other species like *Chromohalobacter salarius*, as represented in **Figure 3**, or are still not classified into one of the two main clusters but cannot form another cluster within the genus.

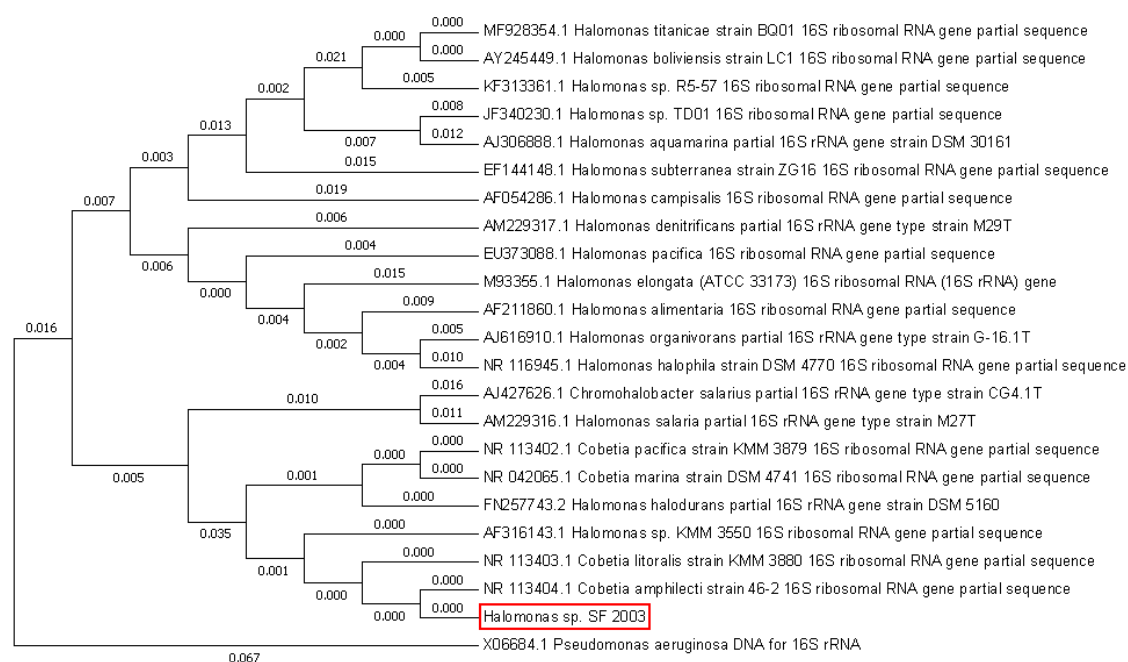


Figure 3: Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequence comparison, showing the evolutionary distance of *Halomonas* sp. SF2003 (framed in red) to a selection of *Halomonadaceae* family members and types strains. *Halomonas alimentaria* (AF211860), *Halomonas* sp. TD01 (JF340230), *Halomonas* sp. R5-57 (KF313361), *Halomonas* sp. KMM 3550 (AF316143), *Halomonas* sp. SF 2003, *H. organivorans* (G-16) (AJ616910), *H. halophila* (DSM4770) (NR 042064), *H. elongata* (ATCC 33173) (M93355), *H. pacifica* (EU373088), *H. denitrificans* (M29T) (AM229317), *H. campisalis* (AF054286), *H. salaria* (M27T) (AM229316), *H. subterranea* (ZG16) (EF144148), *H. titanicae* (BQ01) (MF928354), *H. boliviensis* (LC1) (AY245449), *H. aquamarina* (DSM30161) (AJ306888), *H. halodurans* (DSM5160) (FN257743), *Chromohalobacter salarius* (CG4) (AJ427626), *Cobetia pacifica* (KMM3879) (NR113402), *Cobetia marina* (DSM4741) (NR042065), *Cobetia litoralis* (KMM3880) (NR113403), *Cobetia amphilecti* (46-22) (NR113404), *Cobetia* sp. MACL02 (EF198244) and *Pseudomonas aeruginosa* (X06684). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to collate the phylogenetic tree. The Phylogenetic tree was obtained using a Neighbour-joining method from MEGA7 software.

## I.3. Metabolism

### I.3.1. Carbohydrates metabolism

Analysis of the carbohydrate metabolism of *Halomonas* sp. SF2003 revealed a total of 91 genes coding for biosynthesis or degradation pathways. The strain has genes responsible for fructose, lactose (III), melibiose and sucrose (IV) assimilation and some of the genes involved in galactose (I and II), glucose, glucose-1-phosphate, glycogen (I and II), L-arabinose (III), L-rhamnose (II), lactose (II), ribose and xylose (I and IV) degradation. The presence of fructose, lactose and melibiose degradation pathways is unexpected in a marine bacterium<sup>43-45</sup>, yet it has been reported in different *Halomonas* species like *Halomonas* sp. strain C2SS100<sup>46</sup>, *Halomonas halmophila*, *H. aquamarina*, *H. variabilis*, *H. marina*, *H. magadiensis* or *H. halodurans*<sup>47</sup>, demonstrating the flexibility and adaptation of *Halomonas* strains.

These results are in accordance with other reports on *Halomonas* or *Cobetia* genus phenotypic features<sup>3,46-50</sup>. They also confirm experimental findings that have demonstrated PHA production from agro-industrial processing waters, rich in vegetable proteins and reducing sugars, including mainly fructose<sup>14</sup>. Such capacity to use different carbon sources found in various environment suggested a potential variability of PHA products and a great biotechnological tool. We have for now tested eight carbohydrates and the subsequent PHA production is promising in term of quantity and variability (personal data).

### I.3.2. Polyhydroxyalkanoates production

Genes coding for key enzymes of PHA biosynthesis (PhaA, PhaB and PhaC) are frequently organized in one operon, such as *Cupriavidus necator* (formerly *Ralstonia eutropha*)<sup>17</sup> or *Pseudomonas* sp. (Luengo et al., 2003; Babel and Ackerman, 2001). Organization of the biosynthesis operons depends on bacterial species and also on the PHA synthase class<sup>52</sup> as represented in **Figure 4**. Four classes of PHA synthases are described: Synthase class I and class II are both composed of one *phaC* gene but class I synthases specifically use substrates composed of 3 to 5 carbon atoms and class II substrates with 6 to 14 carbon atoms. Class I synthases are responsible for the production of scl-PHA and can be found in species like *C. necator* while class II synthases produce medium chain length PHA (mcl-PHA) and are described in *Pseudomonas* species. Class III PHA synthases is composed of two genes, *phaC* and *phaE*, and possess specificity for substrates with 3 to 5 carbon atoms like class I but can also synthesize PHA with 6 to 8 carbon atoms. Finally, class IV PHA synthases are also composed of two genes: *phaC* and *phaR* but synthesized mainly scl-PHA and are found in bacteria of the *Bacillus* genus<sup>17,29,51,53</sup>.

Genome annotation, using the platforms MaGe and NCBI, enabled the identification of different genes involved in PHA metabolism such as *phaA*, *phaB*, *phaC1/phaC2* and *phaR* coding for acetyl-CoA acetyltransferase (PhaA), acetoacetyl-CoA reductase (PhaB), poly-beta-hydroxybutyrate polymerase

(PhaC1 and PhaC2) and polyhydroxyalkanoate synthesis repressor (PhaR) respectively. All the data are shown in **Table 4**.

These results orientate classification of the PHA synthase of *Halomonas* sp. SF2003 to class I. The strain belongs to the *Halomonas* genus, produces scl-PHA and does not have gene *phaE*, so the PHA synthase clearly does not belong to class II and III. Results also show that genes coding for the three key enzymes of the PHA synthesis pathway are not clustered in one operon but are clearly distant from each other. To our knowledge, this kind of organization has never been reported elsewhere. Classically, these genes are organized in one operon, or not, with a different order or orientation depending on the bacterial species genes related to PHA biosynthesis, as represented in **Figure 4**<sup>54</sup>.

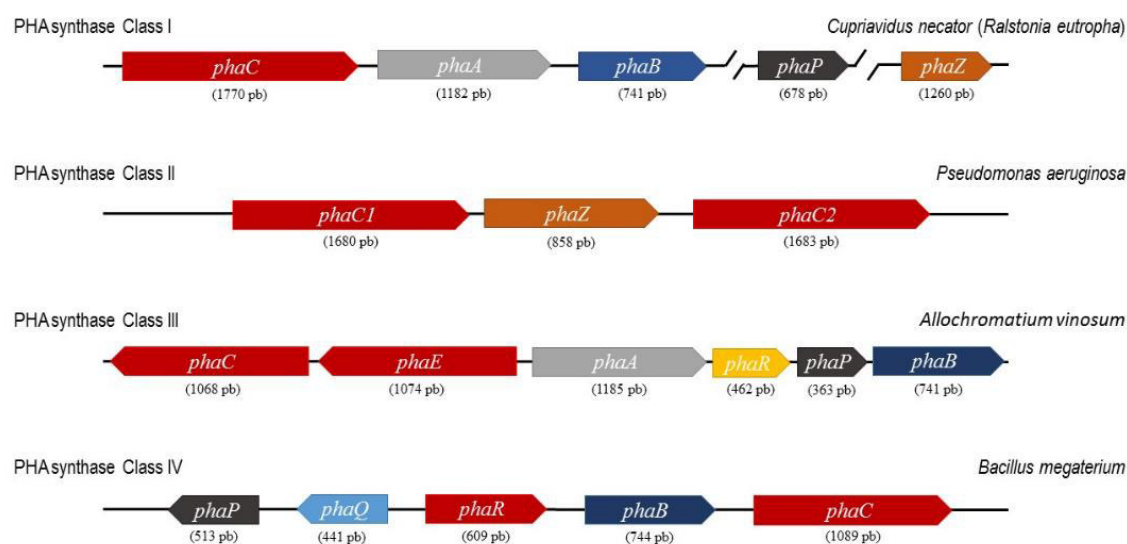


Figure 4: Schematic representation of a PHA cluster for different representative bacterial species. Based on data from previous papers<sup>51-53,55</sup>.

Studies of PhaC1 and PhaC2 proteins were carried out, using server Rapid Annotation and Subsystem Technology (RAST). MaGe, NCBI and UniProt databases provided additional information. Protein PhaC1, being the size of 71.5 kDa, is involved in butanoate and PHA metabolisms. Sequence alignments showed the presence of functional domains of PHA synthase and identity, of about 60-70%, with PHA synthases of other bacterial species like *Cobetia marina*, *Halomonas boliviensis* LC1, *Halomonas* sp. R57-5, *Sulfitobacter guttiformis* KCTC 32187, *Puniceibacterium sediminis* or *Halocynthiibacter arcticus*. These data are in accordance with first results of the bioinformatics analysis and production tests. Like PhaC1, the study of PhaC2 revealed its implication in PHA metabolism and the presence of a functional domain of PHA synthase (in N-term) with an identity, ranging from 65% to 96%, with other bacterial species such as *Cobetia amphilecti*, *Cobetia marina*, *Halomonas anticariensis*

DSM 16096, *Halomonas denitrificans* or *Halomonas boliviensis* LC1. Results of sequence alignments, exposing conserved domains of proteins PhaC1 and PhaC2, are available in **Figure 5**. However, bioinformatics analysis also revealed atypical characteristics of protein PhaC2: the protein being the size of 101.7 kDa and is potentially involved in the metabolism of aromatic compounds. Usually, PhaC proteins have a molecular mass of between 40 to 70 kDa, depending on PHA synthase classes<sup>29,53</sup>. The implication of PHA synthase in aromatic compounds metabolism has, to our knowledge, never been described. This characteristic of protein PhaC2 can be linked to the adaptability of *Halomonas* sp. SF 2003 to polycyclic aromatic compounds, (see below in the stress-related proteins section).

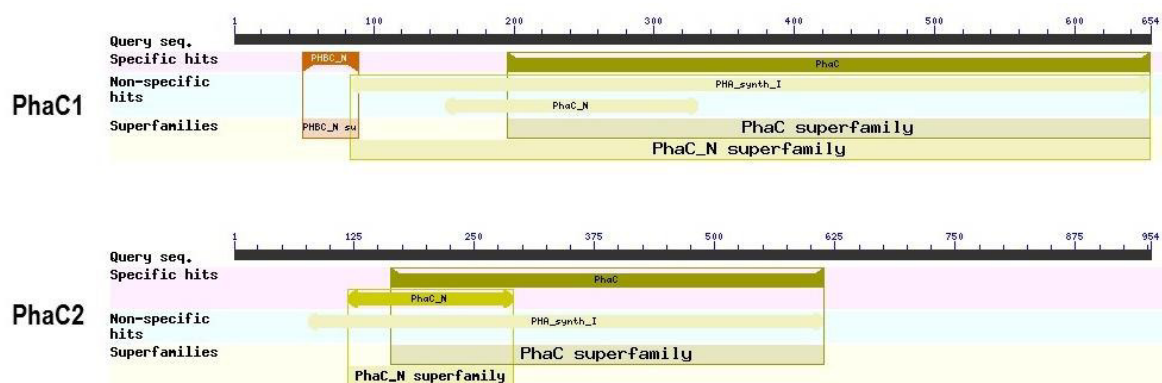


Figure 5: Conserved domains of PHA synthases PhaC1 and PhaC2 of *Halomonas* sp. SF2003 using NCBI database.

First results of the automatic annotation of the *Halomonas* sp. SF2003 genome also included *fabG* in the pathway of PHA production (MaGe Database). This gene codes as for a 3-oxoacyl-ACP reductase involved in fatty acid metabolism and especially in their biosynthesis, as described in Figure 1<sup>56,57</sup>. These results suggested that PHA and fatty acid metabolism are closely related. More relevant research was thus conducted to identify potential genes involved in fatty acid metabolism to determine if *Halomonas* sp. SF2003 can deviate the  $\beta$ -oxidation cycle for PHA production. Genome analysis revealed the presence of characteristic genes encoding enzymes of fatty acid metabolisms: *accB* (acetyl-CoA carboxylase), *fabI* (enoyl-ACP reductase), *yngl/fadD* (acyl-CoA synthetase), *acdA/fadE* (acyl-CoA dehydrogenase). A selection of identified genes is summarized in **Table 4**. These data are in accordance with results of preliminary experiments of PHA production using fatty acids, oleic and undecenoic acids which enabled the production of 0.082 and 0.152g/L of PHA, respectively (unpublished data).

Table 4: Genes from *Halomonas* sp. SF2003 predicted to be involved in PHA and fatty acid biosynthesis and in fatty acid biodegradation.

Metabolic pathway	Protein (MaGe)	Protein (NCBI)	Function (MaGe and/or NCBI)	Locus tag (NCBI)	MW (kDa)
<b>PHA biosynthesis</b>	<b>PhaA</b>	/	Acetyl-CoA C-acyltransferase	C8233_08335	42.4
	<b>PhaA</b>	/	Acetyl-CoA C-acyltransferase	C8233_10360	40.6
	<b>PhaB</b>	/	Beta-ketoacyl-ACP reductase	C8233_12530	27.0
	<b>PhaC1</b>	/	Poly-beta-hydroxybutyrate polymerase	C8233_00780	71.5
	<b>PhaC2</b>	/	Poly-beta-hydroxybutyrate polymerase (modular protein)	C8233_06040	101.8
	<b>PhaR</b>	<b>PhaR</b>	Polyhydroxyalkanoate synthesis repressor	C8233_09450	19.9
<b>Fatty acid biosynthesis</b>	<b>YngI</b>		Putative acyl-CoA synthetase	COBI473_v1_3625 (MaGe)	64.0
	<b>AccB</b>	/	Acetyl-coa carboxylase carboxyl transferase subunit alpha	C8233_13005	34.9
		/	Acetyl-coa carboxylase carboxyl transferase Subunit beta	C8233_05995	37.2
	<b>FabD</b>	<b>FabD</b>	[Acyl-carrier-protein] S-malonyltransferase	C8233_06630	34.2
	<b>AccC</b>	<b>AccC</b>	Acetyl-coa carboxylase biotin carboxylase subunit	C8233_07040	48.6
	<b>FabG</b>	<b>FabG</b>	3-oxoacyl-ACP reductase	C8233_15110	27.5
		<b>FabG</b>	3-oxoacyl-ACP reductase	C8233_06625	25.9
	<b>FabI</b>	/	Enoyl-[acyl-carrier-protein] reductase	C8233_00775	29.1
<b>Fatty acid degradation (<math>\beta</math>-oxidation)</b>	<b>FadA</b>	<b>FadA</b>	Acetyl-coa C-acyltransferase	C8233_08120	41.1
	<b>FadB</b>	<b>FadB</b>	Fatty acid oxidation complex subunit alpha	C8233_08125	77.4
	<b>FadD</b>	/	Long-chain fatty acid--coa ligase	C8233_02555	61.4
			Long-chain fatty acid--coa ligase	C8233_08395	64.3
<b><math>\beta</math>-oxidation deviation/ PHA biosynthesis</b>	<b>FadB</b>		Fatty acid oxidation complex subunit alpha	C8233_08125	77.4
	<b>CaiD</b>	/	Enoyl-CoA hydratase	C8233_00770	52.1
		/	Enoyl-CoA hydratase	C8233_16045	30.3
		/	Enoyl-CoA hydratase	C8233_16135	29.1
		/	Enoyl-CoA hydratase	C8233_16140	43.4
		/	Crotonase/enoyl-coa hydratase family protein	C8233_13655	29.2
		/	Enoyl-coa hydratase/isomerase family protein	C8233_03930	31.3

## I.4. Stress regulation-related proteins

As mentioned above, *Halomonas* sp. SF2003 can grow under high salt concentrations and temperatures, as well as in the presence of toxic compounds like polycyclic aromatic hydrocarbons (PAH) <sup>58</sup>. Results of the pre-tests are illustrated in **Tables 5** and **6** and described below. Adaptability of the strain can be the consequence of the expression of numerous stress-related proteins. The presence of genes involved in such a response was thus researched. Main results are illustrated in **Table 7**.

Like other halotolerant species such as *Halomonas* sp. R5-57 or *H. elongata* DSM 2581, *Halomonas* sp. SF2003 produces the compatible solute 1,4,5,6-tetrahydro-2-methyl-4-pyrimidinecarboxylic acid, also named ectoine, as an osmoprotectant <sup>12,41</sup>. This cyclic amino acid is synthesized by three enzymes i.e. EctA, EctB and EctC, identified in the genome of *Halomonas* sp. SF2003 and respectively encoded by *ectA*, *ectB* and *ectC*. These genes are localized in a same gene cluster as in many others species <sup>41</sup>. Furthermore, as described by Schwibbert in 2011, ectoine hydroxylase encoded by *ectD*, is responsible for ectoine derivative synthesis, hydroxyectoine was also identified in another gene cluster. This protein is found in metabolisms of microorganisms as an adaption tool for resistance to extreme salinity <sup>41,59</sup>. Presence of these enzymes can explain part of the adaptation of *Halomonas* sp. SF2003 to high salinity in the order of 10% to 20% (w/v), data are shown in **Tables 5** and **7**.

Table 5: OD<sub>max</sub> (600nm) of growing tests of *Halomonas* sp. SF2003 under stressful conditions (salinity and polycyclic aromatic compounds).

\*: OD value significantly different from OD of other percentage of salinity (Kruskal-Wallis, significance level of 0.05), <sup>1</sup>: OD value significantly different from OD of control media (Kruskal-Wallis, significance level of 0.05) and <sup>2</sup>: OD value significantly different from OD value of control media + glucose (Kruskal-Wallis, significance level of 0.05).

Stressful conditions		OD <sub>max</sub> (600nm)
Salinity	0.5% (w/v)	2.00 ± 0.16*
	3% (w/v)	3.87 ± 0.04*
	5% (w/v)	7.02 ± 0.21*
	10% (w/v)	8.83 ± 0.32*
	15% (w/v)	5.97 ± 0.42*
	20% (w/v)	0.53 ± 0.08*
Polycyclic Aromatic Hydrocarbons	Benzene (250 ppm)	0.53 ± 0.08 <sup>1,2</sup>
	Naphthalene (50 ppm)	0.64 ± 0.07 <sup>2</sup>
	Phenanthrene (50 ppm)	0.54 ± 0.07 <sup>1,2</sup>
	Pyrene (50 ppm)	0.54 ± 0.02 <sup>1,2</sup>
	Toluene (125 ppm)	0.73 ± 0.08 <sup>2</sup>
	Control media	0.70 ± 0.09
	Control media + glucose	1.75 ± 0.05

*Halomonas* sp. SF2003 is able to grow at 20°C, 25°C, 30°C and 37°C with OD<sub>max(600nm)</sub> of 1.24, 1.90, 1.57 and 1.62 respectively, suggesting the existence of general stress proteins (GSP) enabling such adaptability. Exploitation of genome data revealed the existence of different heat-shock and cold-shock proteins like GroEL, GroES or CspC. These proteins were already identified in other bacterial species as necessary systems for resistance to temperature variations<sup>60-62</sup>. Considering the results of bioinformatics analysis and data from other studies, growing tests at 6°C were conducted, OD<sub>max (600nm)</sub>: 1.56, and confirmed the adaptability of *Halomonas* sp. SF 2003 to temperatures below 10 °C and could suggest the presence of genes coding for cold shock proteins (CSP)<sup>63</sup>.

*Halomonas* sp. SF2003 is able to grow in the presence of a PAH such as; benzene, naphthalene, pyrene, phenanthrene and toluene when used as the main carbon source at different concentrations ranging from 5 to 250 ppm. The tested concentrations depend on PAH solubility and the medium used. The maximal OD<sub>max(600nm)</sub>, of 0.73, was observed with toluene (125 ppm) and is significantly lower when compared to a medium containing glucose, such as a carbon source, suggesting the capacity of *Halomonas* sp. SF2003 to tolerate, in part, the presence of PAH in its environment. Results are illustrated in **Table 5** and show that growth of the strain changes depending on the PAH. These results suggest the presence of gene encoding PAH tolerance enzymes.

Antibiotic susceptibility did not allow to highlight a resistance of *Halomonas* sp. SF2003 against tested antibiotics, results are exposed in **Table 6**. Previous studies have already investigated antibiotic susceptibility of various *Halomonas* strains and have demonstrated the important variability inside the genera. Indeed, even if some species expose resistance against tested antibiotics data in literature expose also susceptibility of different species to various antibiotics.

Table 6: Results of antibiotic susceptibility of *Halomonas* sp. SF2003.

Antibiotic	Inhibition diameter (cm)	
Ampicillin	1 µg/µL	2,0
	5 µg/µL	2,6
Gentamycin	1 µg/µL	1,6
	5 µg/µL	2,4
Kanamycin	1 µg/µL	1,0
	5 µg/µL	1,8
Penicillin	1 µg/µL	1,8
	5 µg/µL	2,0
Streptomycin	1µg/µL	1,0
	5 µg/µL	1,8

For example, susceptibility to antibiotics of beta-lactam family, as ampicillin and penicillin, has been observed for *Halomonas elongata*, *H. eurihalina*, *H. hamiltonii* sp. nov. (DSM 21196<sup>T</sup>), *H. saccharevitans* sp. nov., *H. salina*, *H. stevensii* sp. nov. (DSM 21198<sup>T</sup>) or *H. subglaciescola*<sup>47,49,64,65</sup>.



Same results have been reported for antibiotics belonging to aminoglycosides as susceptibility of *Halomonas alkaliphila* sp. nov. (DSM 16354<sup>T</sup>), *H. campaniensis* sp. nov. (Strain 5AGT), *H. maura* and *H. salina* to gentamycin or susceptibility of *Halomonas hydrothermalis* (DSM 15725), *H. meridiana* (DSM 5425) and *H. stevensii* sp. nov. (DSM 21198<sup>T</sup>) to kanamycin<sup>47,64-67</sup>. The last one, *H. meridiana* (DSM 5425), exposes also a susceptibility to streptomycin similarly to *H. aquamarina* (DSM 30161) or *H. salina*. At the time of writing (August 2018), *Halomonas* sp. SF2003 does not expose a resistance against tested antibiotics probably due to absence gene coding for antibiotics resistance mechanisms<sup>68</sup>. Additional tests, with different antibiotics and different concentrations, are required to complete data but usually tested antibiotics could be used for production of recombinant strains is currently feasible.

Tolerance and the degradation of toxic compounds have already been described in (marine) bacteria like *Pseudomonas*<sup>69</sup>, *Halomonas*<sup>70</sup> or *Neptunomonas*<sup>71</sup>. Results of automatic annotation on MaGe and NCBI databases show the presence of proteins annotated differently on both databases but possibly involved in a tolerance to toxic compounds: YrbD, Ttg2B and Ttg2A (MaGe) and MlaD, MlaF and an unnamed protein (NCBI). Results are illustrated in **Table 7**.

PAH-catabolic pathways usually contain a multicomponent dioxygenase enzyme system, responsible for the dioxygenation of an aromatic nucleus. These pathways are described thoroughly for naphthalene, phenanthrene and anthracene metabolism in the *Pseudomonas* species. Genes coding for these enzymes are found in the same group and are named *nah*-like genes. This group contains classical genes of these pathways like *ndo* (naphthalene dioxygenase), *nah* (naphthalene degradation), *dox* (dibenzothiophene oxidation), *pah* (polycyclic aromatic hydrocarbon (phenanthrene) degradation). New studies, conducted on different Gram negative and positive bacteria, have also revealed the existence of other genes coding for PAH catabolism enzymes ; including *nag* (naphthalene dioxygenase genes), *phd* (phenanthrene degradation), *nid* (dioxygenase systems), *nar*, *phn*, *dbf/pln*, *tod*, *ipb*, *bed*, *tcb*, *bph* ; with the same functions as *nah*-like genes but showing a low homology to those genes<sup>72-74</sup>. Not one of these genes is present in the *Halomonas* sp. SF2003 genome. However, a toluene tolerance pathway involving 3 genes (*yrbD*, *ttg2b* and *ttg2A* encoded for a toluene transporter subunit and 2 toluene tolerance efflux transporters, respectively) involved in PAH tolerance is present according to bioinformatics data available on the MaGe database. These genes encode toluene efflux pumps responsible for the expulsion of the toxic compounds. Tolerance to solvents by Gram-negative bacteria was already described in the literature as being the result of an expulsion of toxic compounds using the efflux system belonging to the RND (resistance, nodulation, cell division) family<sup>75-77</sup>.

Table 7: Stress-related proteins of *Halomonas* sp. SF2003.

Role	Protein (MaGe)	Protein (NCBI)	Function	Locus tag (NCBI)	MW (kDa)
Osmoprotectant	<b>EctA</b>	<b>EctA</b>	Diaminobutyrate acetyltransferase	C8233_02170	21.3
	<b>EctB</b>	<b>EctB</b>	Diaminobutyrate--2-oxoglutarate transaminase	C8233_02175	46.3
	<b>EctC</b>	/	L-ectoine synthase	C8233_02180	14.8
	<b>EctD</b>	<b>Thpd</b>	Ectoine hydroxylase	C8233_10080	40.5
	<b>HtpX</b>	<b>HslU</b>	ATP-dependent protease ATPase subunit HslU	C8233_13125	49.2
	<b>HslV</b>	/	ATP-dependent protease subunit HslV	C8233_13130	18.6
	<b>HslO</b>	/	Hsp33 family molecular chaperone HslO	C8233_15780	33.8
Temperature	<b>GroEL</b>	<b>GroL</b>	Chaperonin GroEL	C8233_15260	57.7
	<b>GroES</b>	<b>GroES</b>	Co-chaperone GroES	C8233_15265	10.5
	<b>GrpE</b>	/	Nucleotide exchange factor	C8233_10485	22.5
	/	/	Cold-shock protein	C8233_12885	16.8
	<b>CspC</b>	/	Cold-shock protein	C8233_09345	7.2
	<b>CspE</b>	/	Cold-shock protein	C8233_05185	7.4
	<b>CspE</b>	/	Cold-shock protein	C8233_06225	7.2
Toxic compounds	<b>YrbD</b>	/	Toluene transporter subunit: membrane component of ABC superfamily	COBI473_v1_1746 (MaGe)	16.6
	<b>Ttg2B</b>	/	Toluene tolerance efflux transporter (ABC superfamily, membrane)	COBI473_v1_1747 (MaGe)	27.9
	<b>Ttg2A</b>	/	Toluene tolerance efflux transporter (ABC superfamily, atp_bind)	COBI473_v1_1748 (MaGe)	33.1
	/	<b>MlaD</b>	Outer membrane lipid asymmetry maintenance Protein	C8233_07605	16.6
	/	/	ABC transporter permease	C8233_07610	27.9
	/	<b>MlaF</b>	Phospholipid ABC transporter ATP-binding protein	C8233_07615	37.5

Growth of *Halomonas* sp. SF2003 in the presence of PAH can then be explained by the presence of this efflux pump rather than the presence of a degradation system, including a multicomponent dioxygenase enzyme system. Comparison of these results with data available on NCBI, identified other proteins at the same position as these identified by the MaGe database. In the NCBI we identified 3 proteins: MlaD, MlaF and an unnamed protein, whereas they were respectively named YrbD, Ttg2A and Ttg2B in MaGe. The function of these proteins was studied using NCBI and UniProt BLAST tools. Protein MlaD, found in place of YrbD, of *Halomonas* sp. SF2003 showed an identity with MlaD proteins of other *Halomonas* species ranging from 65% to 94%. This protein also contains a domain with an identity ranging from 71.7% to 74% with the organic solvent ABC transporter substrate-binding proteins of *Halomonas* sp. KHS3, *Halomonas meridiana*, *Halomonas* sp. SUBG004 and *Halomonas*

*campaniensis*. The protein also presents an identity, around 70%, with a toluene transporter subunit: membrane component of ABC superfamily of *Halomonas* sp. R57-5 and *Halomonas* sp. A3H3. These data suggest, in part, the implication of these proteins in tolerance to PAH. The unnamed protein, identified instead of Ttg2B, demonstrated an identity ranging from 70.0% to 100% with ABC transporter permease MlaE of different bacterial species like *Cobetia* sp. UCD-24C, *Halomonas* sp. 1513 or *Chromohalobacter japonicas*. Protein MlaE is involved in the maintenance of the asymmetry of the outer membrane<sup>78</sup>. Finally, protein MlaF, found in place of Ttg2A, shows an identity, ranging from 72.0% to 99.6%, with proteins MlaF of different bacterial species such as *Halomonas salina* or *Cobetia amphilecti*. This protein participates, like MlaE, in the maintenance of the asymmetry of the outer membrane. Regarding the results of both databases, even if identification differs from one to another, both predict a potential implication of proteins encoded by this part of the genome in a transport/efflux pump system for organic solvents (or especially toluene) which can explain the growth of *Halomonas* sp. SF2003 in the presence of PAH.

## Discussion

The marine bacterium *Halomonas* sp. SF2003, showed promising capabilities for the production of PHA in addition to a high adaptability faced with atypical growth conditions. This report enlarges the knowledge of the genomic characteristics of such a PHA-producer and the central role of PHA synthesis in cellular metabolism. Likewise, the results of the genomic analysis emphasize the relationship between the capability of accumulating PHA, and the enhancement of the stress resistance of producing microorganisms as recently pointed out in some studies<sup>23,26,79</sup>. Resistance to starvation or non-optimal environmental conditions can be the result of the expression of various stress-related proteins. Indeed, genes might be responsible for the resistance of *Halomonas* sp. SF2003 against salinity, temperature or polycyclic aromatic hydrocarbons which were identified in the strain's genome. Nevertheless, the phasins associated to PHA granules as well as some hydroxyl acyl monomers have been reported to exhibit a chaperone-like activity, and might also be involved in the protective mechanism<sup>80</sup>. Conversely, Obruca *et al.* (2018) also stated that the application of certain amounts of a stress factor (osmotic pressure, heavy metals, hydrogen peroxide) supports PHA accumulation in *Cupriavidus necator* and can thus be used as an effective and innovative strategy to improve PHA production. In the particular case of *Halomonas* sp. SF2003 it can thus be hypothesized that PHA production may be boosted in a fermentation medium supplemented with appropriate amounts of naphthalene or phenanthrene as it possesses unusual genes resistant to these compounds.

Biotechnological PHA production processes can be further enhanced by an appropriate feeding strategy deduced from the knowledge of PHA biosynthesis pathways of the producing microorganism. Hence, analysis of the carbohydrate metabolism of *Halomonas* sp. SF2003 revealed the presence of numerous genes involved in carbohydrate biosynthesis or degradation metabolism suggesting that the

strain has the capability to convert various carbonaceous sources into PHA. Owing to its robustness, many industrial waste streams can be considered as potential cheap substrates to support PHA biosynthesis. Indeed, scaling-up the PHA production with inexpensive and readily available feedstocks is the main current challenge for the development of a sustainable and economically competitive production of these biopolymers.

Fatty acids, including those that originate from waste lipids, may also be supplied as substrate for PHA production from *Halomonas* sp. SF2003, as in the *in silico* study conducted here, which revealed the existence of a close relation between the PHA and fatty acid metabolisms. Production of oleic acid and undecenoic acids has already been found to be effective however the quantity of PHA produced by *Halomonas* sp. SF2003 is limited (unpublished data). These results open up the path for new tests to enhance production, testing other fatty acids like hexanoic acid to study expression rates of the different genes involved, as well as the associated enzymatic activities. Utilization of exotic fatty acids with, for instance, terminal carbon rings, side chains or rings as side chains has also to be considered as a potential way to produce PHA with specific characteristics as reported elsewhere <sup>81,82</sup>.

The most adopted approach to overproduce PHA is genetic metabolism modification of producer strains. Not only specific production (g PHA/g biomass) but also volumetric production can be increased up to several fold in comparison with wild type strains. Unusual PHA with attractive properties from the end-user application point of view can be synthesized, such as co-polymers composed of both short chain length (SCL) and medium chain length (MCL) monomers as recently reported in the literature <sup>83</sup>.

Deletions or weakening of PHA unrelated pathways, mainly deletions of genes *fadA* and *fadB*, has been largely well-tried. The deletion of these enzymes in the  $\beta$ -oxidation pathway of the *Pseudomonas* species, enables most fatty acids to channel to PHA synthesis, thus significantly improving substrates to product PHA ratios <sup>84,85</sup>.

Regarding the atypical results of PHA biosynthesis operon and PHA synthases (PhaC1 and PhaC2), the implication of each of these enzymes together or separately on PHA biosynthesis has to be investigated. New arrangements of the PHA biosynthesis operon of *Halomonas* SP2003 can probably provide a better rate of production or cell content as previously reported by Hiroe *et al.*(2012).

Consequently, the strain genome sequencing and analysis opens the door to future developments that will enable a better use of *Halomonas* sp. SF2003 in biotechnological applications.

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## Chapter 4

# PHA production and PHA synthases of the halophilic bacterium *Halomonas* sp. SF2003.

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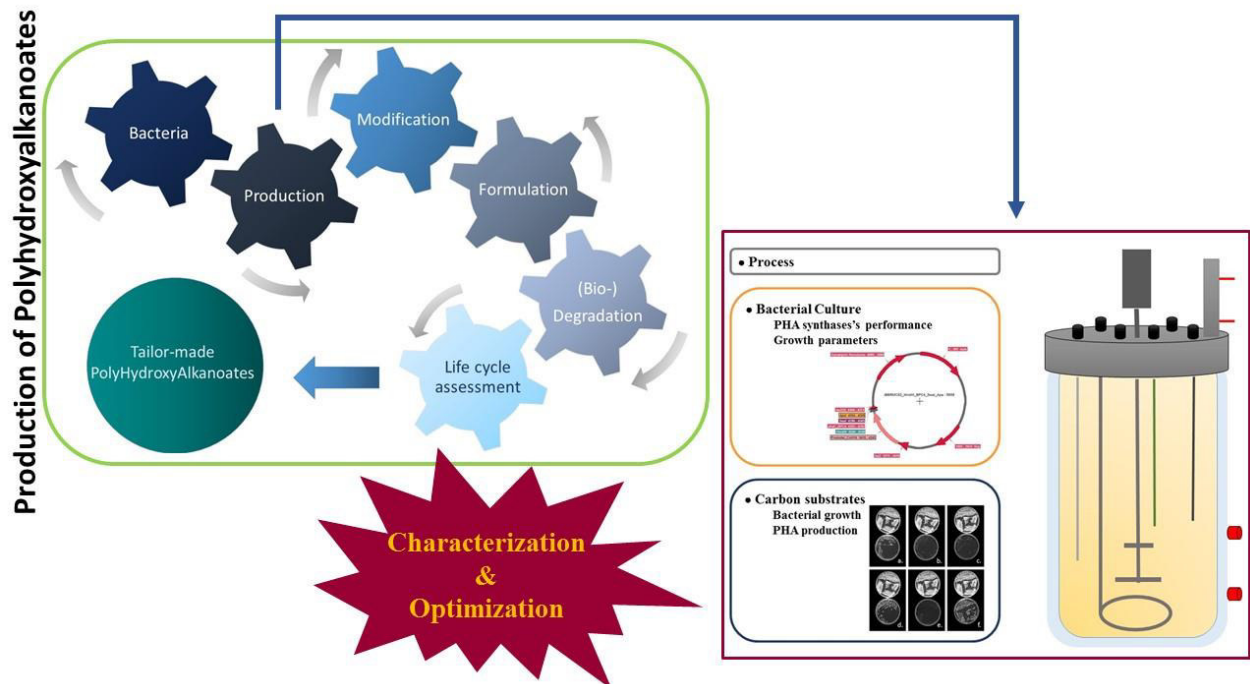
**Keywords:** *Halomonas* sp. SF2003, Halophilic bacteria, PolyHydroxyAlkanoates (PHA), PHA synthases.

## Highlights:

- *Halomonas* sp. SF2003 can employed various carbohydrates and mix for PHA synthesis
- PHA synthases PhaC1 and PhaC2 show differences in structure and synthesis activity

## Graphical abstract

Among the different tools which can be studied and managed to tailor-make PolyHydroxyAlkanoates and enhance their production, bacterial strain and carbon substrates are essential. Assimilation of carbon sources is dependant of strain's metabolism and consequently cannot be dissociated. Both must wisely be studied and well selected to ensure the highest production yield.



## Introduction

PolyHydroxyAlkanoates (PHA) are valuable bio-based and biodegradable polymers produced by numerous bacterial species <sup>1,2</sup>. Because their properties are close to those of conventional petroleum-based plastics, in addition to their biocompatibility, they are considered as materials with high potential <sup>3</sup>. Actually they can be used in various fields ranging from packaging <sup>2</sup> to biomedical applications <sup>1,4</sup>, but one of the main locks to their commercialization and exploitation is the overall cost of production. Currently, several tools can be managed to reduce final cost of PHA production, including characterization of selected microorganisms coupled with optimal selection of carbon substrates <sup>5,6</sup>. Indeed, better understanding of strain metabolisms and response in front of different growth and/or production conditions participate to tailor-make PHA and enhance production yield. To date, there is an important diversity of carbon sources (monomers) which can be exploited for PHA synthesis and as a result, a wide range of (co-)polymer which can be synthesized <sup>6</sup>. PHA properties are closely linked to their bacterial producer strain, carbon substrates and production mode <sup>6,7</sup>, meaning that an accurate study of each parameter is required. On the other hand, the PHA production cost is still limiting their more widespread use. Over the last decade, research have notably focused on the use of low-value

substrates like industrial co-products (from agri-food, waste treatment,...) <sup>6,8,9</sup> as they can represent up to 50% of the production cost. But with these carbon substrates, a perfect control of PHA's structure, molecular weight and properties could be difficult. Therefore, complete studies of carbon substrates utilization and PHA synthesis are required to soundly select the most adapted carbon sources, whether it is pure carbohydrates or co-products. Another way to reduce production costs is to study the strain genome using bioinformatic and genetic engineering. These tools are also exploiting for expression of PHA synthesis operon in non-producing strains exposing less restrictive growth and production conditions <sup>5,10</sup>.

*Halomonas* sp. SF2003 is a halophilic bacterium identified as a PHA-producing strain <sup>11</sup>. Previous studies have shown its capacity to produce polymer up to 78% of cell dry weight (CDW), using conventional carbon sources but also carbonaceous by-products from food wastes <sup>12</sup>. Bioinformatic and phenotypic studies of *Halomonas* sp. SF2003 have demonstrated its versatility under various atypical growth conditions making it an adaptable bacterium. Additionally, genomic annotation also allows to identify various metabolic pathways directly involved, or not, in synthesis of PHA which can be studied for a stronger understanding of *Halomonas* sp. SF2003 PHA metabolism. Our previous study highlighted atypical characteristics and organisation of PHA biosynthesis genes (*phaA*, *phaB*, *phaC1*, *phaC2* and *phaR*) <sup>13</sup>. Regarding its original properties, *Halomonas* sp. SF2003 is an excellent candidate for innovative development of biotechnological production of PHA.

The objectives of this work are to go further into the unravelling/understanding of PHA biosynthesis capability and metabolism of *Halomonas* sp. SF2003 and to identify potential carbon substrates, and in later stage potential industrial co-products, which can be exploited for PHA production. Our work will also contribute to better understand the activity of both PHA synthases of *Halomonas* sp. SF2003 in order to later optimize its PHA production.

## I. Results

### I.1. *In silico* study of PHA synthases PhaC1 and PhaC2 of *Halomonas* sp. SF2003

In a previous work, the whole genome of *Halomonas* sp. SF2003 was sequenced and annotated leading to the identification of two genes potentially encoding two distinct PHA synthase proteins PhaC1 and PhaC2; belonging to class I (based on gene organization and biosynthesized PHA) <sup>13</sup>. To further characterize this first analysis, the consensus lipase box-like sequence of both PHA synthase has been studied.

Lipase box-like sequences are highly conserved patterns present in PHA synthase sequences and are considered as active sites of the enzymes <sup>14-16</sup>. These domains expose similarities with those of lipase but difference is in the replacement of the essential active site of lipase, a serine, by a cysteine in

lipase box-like domain of PHA synthase<sup>17</sup>, leading to rename these sequences as PhaC box consensus sequences<sup>18</sup>. In this pattern, similarly to lipase, Cysteine (Cys or C) represents the catalytic amino acid and is involved in a catalytic triad (C-H-D) participating, supposedly, in elongation step of PHA polymer<sup>18</sup>. The most common described pattern is Glycine-X-Cysteine-X-Glycine (G-X-C-X-G), including Glycine-Tyrosine-Cysteine-Methionine-Glycine sequence (G-Y-C-M-G) detected in *Bacillus cereus* (ATCC 14579) or *Haloferax mediterranei* (ATCC 33500), Glycine-Tyrosine-Cysteine-Leucine-Glycine sequence (G-Y-C-L-G) found in *Cupriavidus metallidurans* strain CH34 or *Halomonas boliviensis* LC1 (DSM15516), or Glycine-Alanine-Cysteine-Serine-Glycine sequence (G-A-C-S-G) in *Cupriavidus necator* strain N-1 or *Pseudomonas fulva* strain 12-X<sup>14,17-19</sup>. However, variations in amino acids composition have also been described in various bacterial species like with *Halomonas elongata* (DSM 2581) or *Halomonas* sp. KM-1 for which sequences have been described (**Figure 1**)<sup>14</sup>.

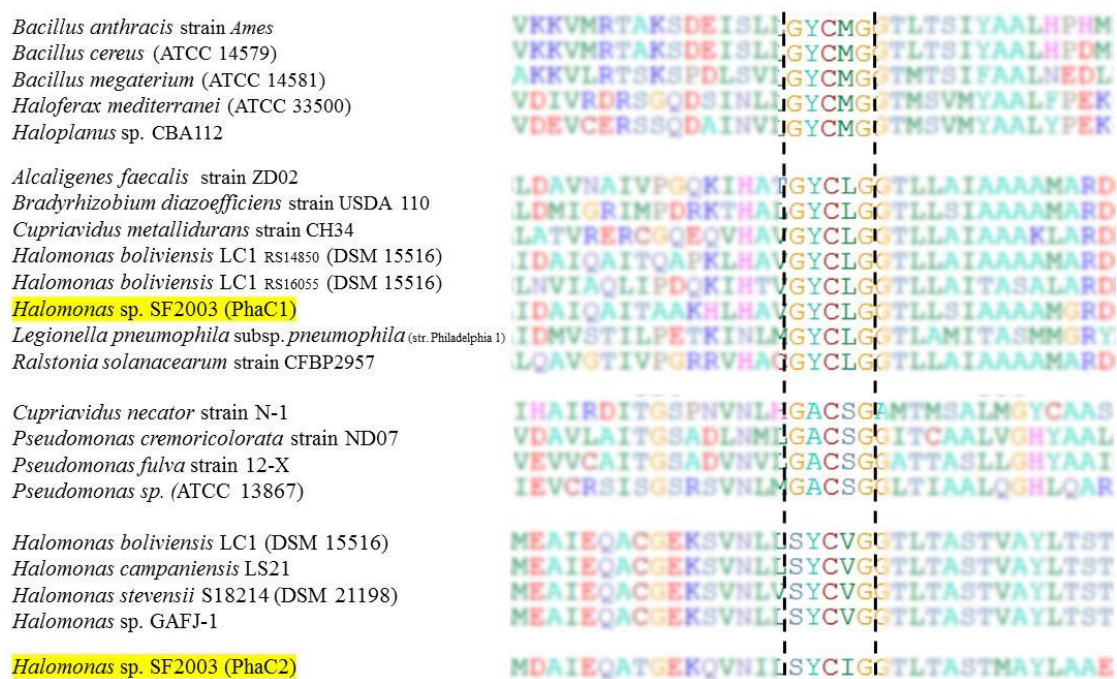


Figure 1: Multiple alignment of partial amino acids sequences of PHA synthase exposing lipase box-like patterns from different bacterial species.

All the sequences are available on NCBI database. Highlighted sequences correspond to PHA synthases PhaC1 and PhaC2 of *Halomonas* sp. SF2003.

Amino acids sequences of PHA synthases PhaC1 and PhaC2 of *Halomonas* sp. SF2003 have been analyzed and allowed the identification of two distinct lipase box-like patterns in both enzymes, beginning at position 384 for PhaC1 and position 343 for PhaC2. In PhaC1 sequence pattern is composed of Glycine-Tyrosine-Cysteine-Leucine-Glycine (G-Y-C-L-G) and pattern in PhaC2 is Serine-Tyrosine-Cysteine-Isoleucine-Glycine (S-Y-C-I-G) (**Figure 1**). Results obtained for PHA synthases of *Halomonas* sp. SF2003 still demonstrated distinction of both enzymes, additionally to their size and location in genome<sup>13</sup>. Indeed, two different patterns have been reported: G-Y-C-L-G for PhaC1 and S-Y-C-I-G for PhaC2. The G-Y-C-L-G pattern detected in PhaC1 amino acids sequence has already been

reported in different halotolerant/halophiles (or not) PHA/PHB-producing strains as *C. metallidurans* strain CH34<sup>20</sup> or *Halomonas boliviensis* LC1 (DSM 15516)<sup>21</sup>. The second pattern, S-Y-C-I-G, founds in PhaC2 amino acids sequence has also been reported in sequence of other halotolerant/halophiles PHA/PHB-producing strains as *Chromohalobacter salexigens* (DSM 3043) and *Halomonas* sp. KM-1<sup>14,22,23</sup>. Additionally, to this difference of pattern in PhaC box consensus sequences, analysis of amino acids sequences framing these active sites suggests a difference in final structure of proteins. The existence of different PHA synthase enzymes in a same bacterial strain has already been observed, as well as several lipase box-like sequences, like for *Halomonas boliviensis* LC1 (DSM 15516). This strain has several PHA synthases in which different lipase box-like pattern have been detected, as illustrated in **Figure 1**.

Differences between both PHA synthases of *Halomonas* sp. SF2003 could generate a difference in catalytic activity and potentially, at the end, impact yield of polymer production. Further research must be performed to elucidate impact of each pattern on enzymes's substrates specificity and selectivity and also to validate identification of catalytic core of both. Indeed, studies on domain of PhaC box consensus sequences of *Halomonas* sp. O-1 have been conducted and demonstrated that modifications of sequence have an impact on synthesis of PHA<sup>14</sup>. Same kind of experiments would be one of the prospects to deeply characterize PHA synthases, PhaC1 and PhaC2, of *Halomonas* sp. SF2003.

## I.2. Screening of carbon substrates for PHA production by *Halomonas* sp. SF2003

To investigate the ability of *Halomonas* sp. SF2003 to produce different PHA, various carbon substrates and mix have been screened for growth and biopolymer accumulation (See section IV.I “Criblage de sources de carbone assimilables” in Chapter 2 Matériels et Méthodes). Bacterial growth and PHA accumulation were first checked using visual examination of Nile Red agar plates (shake flasks or bioreactor). Nile Red is a fluorescent stain of intracellular lipids and hydrophobic domain frequently used to detect PHA<sup>24</sup>. Indeed, Nile Red represents an easy and fast detection tool for PHA biosynthesis using various technic as agar plates or epifluorescence microscopy<sup>11,25-27</sup>. Work has been mainly focused on “pure” carbon substrates, as carbohydrates, for a better understanding of PHA synthase activity and specificity. Height pure carbohydrates, including 5 monosaccharides (glucose, fructose, galactose, rhamnose, mannose) and 3 disaccharides (maltose, melibiose, sucrose) found in food or natural (co-) products including fruits, vegetables, milk or red algae, have been tested based on data available in literature and results of previous studies on *Halomonas* sp. SF2003<sup>12-14,28,29</sup>.

Visual examinations of Nile Red agar plates allow to detect colonies and bacterial growth and PHA production has been screened by detection of Nile Red florescence under UV-lights. *Halomonas* sp. SF2003 was able to use all the tested carbohydrates as substrates for both bacterial growth and PHA accumulation: (D)-Glucose (**Figure 2a**), (D)-Fructose (**Figure 2b**), (D)-Galactose (**Figure 2c**), (D)-Mannose (**Figure 2d**), (D)-Maltose (**Figure 2e**) and (D)-Sucrose (**Figure 2h**), only (L)-Rhamnose and (D)-

Melibiose were not used (**Figure 2f, Figure 2g** and **Table 1**). On Nile Red agar plates, number of UFC as well as the size of the colonies vary from one carbohydrate to another. Qualitatively, growth of *Halomonas* sp. SF2003 seems to be more important on (D)-Glucose, (D)-Mannose and (D)-Maltose but, comparatively, PHA production looks to be more efficient on (D)-Glucose, (D)-Galactose and (D)-Maltose, based on fluorescence intensity.

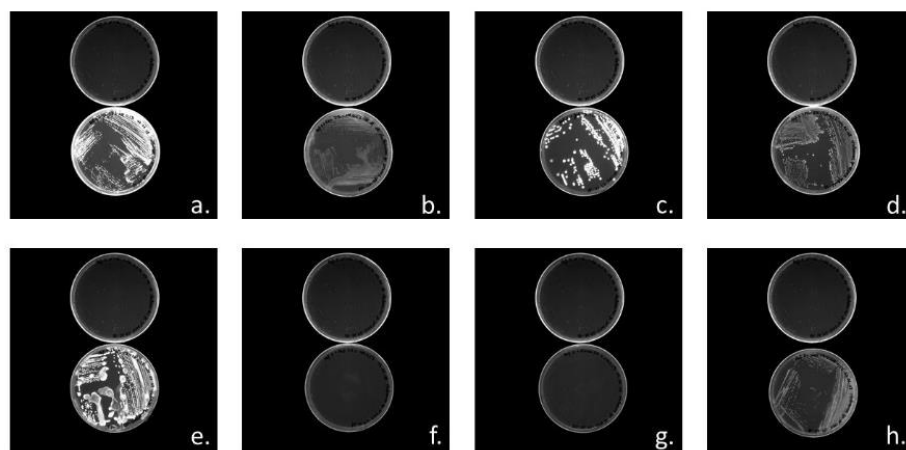


Figure 2: Nile Red agar plates screening with *Halomonas* sp. SF2003 using 2% (w/v) of different carbon substrates. The positive control (medium without addition of carbon substrates) is the upper plate on Figure 2a to Figure 2h. a. (D)-Glucose, b. (D)-Fructose, c. (D)-Galactose, d. (D)-Mannose, e. (D)-Maltose, f. (D)-Melibiose, g. (L)-Rhamnose and h. (D)-Sucrose. Observations under UV-lights performed with transillumination.

Only one carbohydrate in a (L) configuration has been tested: (L)-Rhamnose, and it does not allow both bacterial growth and PHA accumulation by *Halomonas* sp. SF2003. At this time and to our knowledge, there is not yet reports of PHA production with (L)-rhamnose using *Halomonas* species. In contrast, ability to use this sugar as carbon source and substrate for PHA production is variable since *Halomonas* species as *H. cupida*, *H. elongata*, or *H. maura*<sup>30,31</sup> or other species as *C. necator* or *Pseudomonas oleovorans*<sup>32</sup> are capable of doing so while *Halomonas* species as *H. aquamarina*, *H. hamiltonii* (DSM 21196<sup>T</sup>) or *H. subterranea* (JCM 14608T) are not, in accordance to results obtained with *Halomonas* sp. SF2003.

Table 1: Growth and PHA accumulation in *Halomonas* sp. SF2003 using different carbon sources.

Carbon source	Growth	PHA accumulation
(D)-Glucose	+	+
(D)-Fructose	+	+
(D)-Galactose	+	+
(D)-Mannose	+	+
(D)-Maltose	+	+
(D)-Melibiose	-	-
(L)-Rhamnose	-	-
(D)-Sucrose	+	+

Results of screening tests suggest that the inability of use (L)-Rhamnose for bacterial growth or PHA production by *Halomonas* sp. SF2003 is a result of a lack in part or in totality, of required metabolic tools. This hypothesis is in accordance with results of a previous study, which showed the presence of only few genes responsible for rhamnose (II) degradation in the *Halomonas* sp. SF2003 genome (**Table 2**)<sup>13</sup>.

Table 2: Listing of common carbohydrates used for PHA production.

\*: Tested in this study for PHA accumulation, n.i: not identified in *Halomonas* sp. SF2003 genome yet.

Carbohydrates	Origin	Identification of pathway for assimilation
Fructose*	Fruits, Honey	Total
Galactose*	Milk, Honey, Red algae	Partial
Glucose*	Food, Metabolims of living organisms	Partial
Lactose	Dairy products	Total
Maltose*	Starch degradation (barley)	n.i
Mannose*	Fruits, Plants, Mannitol	n.i
Melibiose*	Plants, Fruits	Total
Ribose	RNA	Partial
Rhamnose*	Plants	Partial
Sucrose*	Plants	Total
Xylose	Plants	Partial

Within all tested pure carbohydrate, (D)-Melibiose is the second one which does not allow bacterial growth. Results of screening tests performed suggest that *Halomonas* sp. SF2003 does not or only possesses a part of enzymes require for (D)-Melibiose degradation contrary to results suggested by our previous *in silico* study<sup>13</sup>. A limited number of studies deal with use of (D)-Melibiose for bacterial growth and only sometimes for PHA production as *Burkholderia sacchari* sp. nov.<sup>33</sup>, *Halomonas cupida* is also able to use (D)-Melibiose for its growth<sup>31</sup> like *Bacillus* sp. (Strain SKM11)<sup>34</sup>, *Bacillus subtilis* (Strain PHA 012), *Aeromonas* sp. (Strain PHA 046) or *Alcaligenes* sp. (Strain PHA 047)<sup>35</sup>. Comparatively, and similarly to *Halomonas* sp. SF2003, numerous PHA-producing species have also been reported for their disability to exploit (D)-Melibiose for growth as *Pandora* sp. (Strain MA 03)<sup>36</sup> or *Bacillus cereus* (Strain FC11)<sup>37</sup>.

As described previously and illustrated in **Table 1**, *Halomonas* sp. SF2003 is able to grow in medium with (D)-Glucose, (D)-Fructose, (D)-Galactose, (D)-Mannose, (D)-Maltose and (D)-Sucrose. The strain seems also able to use these carbohydrates for PHA production in accordance with genomic analysis<sup>13</sup>. Indeed, study of *Halomonas* sp. SF2003 genome highlighted the presence a various genes coding for enzymes responsible for carbohydrates assimilation as fructose or sucrose. However, some of the tested carbohydrates have also been used by *Halomonas* sp. SF2003 even if preliminary study of its genome only identified a part of genes required for their total assimilation (**Table 2**). These results

suggest the interest of performing a re-examination and annotation of *Halomonas* sp. SF2003 genome but also open the door for new studies/productions using these pure carbohydrates which can easily be found in various food or agri-food (co-)products.

Use of these carbohydrates for PHA production has already been reported in different bacterial species including, or not, *Halomonas* species. In literature, numerous studies have been conducted to evaluate the use of various carbon sources, including pure carbohydrates, for PHA production. For example, glucose has been used with *Bacillus cereus* UW85 (2.9 g/L of P-3HB)<sup>38</sup>, *Pseudomonas* species<sup>33</sup> but also with some *Halomonas* species such as *Halomonas* sp. TD01 (64.0 g/L of P-3HB)<sup>39</sup> or *Halomonas profundus* (0.3 g/L of P-3HB)<sup>40</sup>. Use of glucose has also been evaluated with *C. necator*, the most studied PHA producing strains, as well as employment of fructose or sucrose<sup>33</sup>. Fructose is also exploited by *Bacillus aryabhatai* PHB10 (2.18 g/L of P-3HB)<sup>41</sup> or halophiles strains such as recombinant strain *Halomonas* sp. TD08 (2.02 g/L of P-3HB-co-3HV)<sup>42</sup>, *H. halophila* (2.31 g/L of P-3HB), *H. organivorans* (2.57 g/L of P-3HB) and *H. salina* (0.17 g/L of P-3HB)<sup>43</sup>. PHA production using sucrose has been successfully achieved with *Azotobacter vinelandii* (0.70 g/L of P-3HB)<sup>6,44</sup> or *Burkholderia sacchari* DSM 17165 (36.50 g/L of P-3HB)<sup>45</sup>. Similarly to fructose, PHA productions have been observed when sucrose is used with halophile strains: *Natrinema* sp. 5TL6 (0.11 g/L of P-3HB-co-3HV)<sup>46</sup>, *H. halophila* (4.85 g/L of P-3HB), *H. organivorans* (2.57 g/L of P-3HB) and *H. salina* (0.14 g/L of P-3HB)<sup>43</sup>. Similarly to *Halomonas* sp. SF2003, various *Halomonas* species such as *H. halophila* (3.41 g/L of P-3HB) or *H. salina* (0.12 g/L of P-3HB) have successfully employed galactose PHA production<sup>43</sup>.

Even if numerous *Halomonas* species such as *H. elongata*, *H. pacifica* or *H. halodurans*<sup>33</sup> can use mannose as growth substrate, only some studies report a possible conversion into PHA. Indeed, Pernicova *et al.*, and Kucera *et al.*, have demonstrated capacity of *H. halophila*, *H. organivorans* and *H. salina* to produce PHA from mannose, up to 0.96, 3.87 and 0.13 g/L of P-3HB, respectively<sup>43,47</sup>. Finally, among already cited strains some of them are also able to produce PHA from maltose as well as *B. aryabhatai* PHB10 (1.47 g/L of P-3HB)<sup>41</sup> or *Geobacillus* sp. AY946034 (0.15 g/L of P-3HB)<sup>48</sup>. In addition to these strains, PHA production from maltose has also been investigated with additional species such as *Halomonas* sp. TD08 (1.56 g/L of P-3HB-co-3HV)<sup>42</sup>, *H. boliviensis* LC1 (0.55 g/L of P-3HB)<sup>33,49</sup> or *H. campisalis* MCM B-365<sup>50</sup> (**Table 3**).



Table 3: Listing (not exhaustive) of various bacterial strains using the different tested carbohydrates for PHA production. Based on data of Verlinden *et al.*, 2007 and completed with data from other studies. Strains in bold expose highest PHA concentrations.

Carbohydrates	Bacterial strains/species
Glucose	<i>Bacillus cereus</i> UW85, <i>Halomonas profundus</i> , <b><i>Halomonas sp. TD01</i></b> , <i>Halomonas sp.</i> SF2003
Fructose	<i>Bacillus aryabhatai</i> PHB10, <i>C. necator</i> , <i>Halomonas</i> TD08, <i>Halomonas sp.</i> SF2003, <i>H. halophila</i> , <b><i>H. organivorans</i></b> , <i>H. salina</i>
Galactose	<b><i>Halomonas halophila</i></b> , <i>H. salina</i> , <i>Halomonas sp.</i> SF2003
Mannose	<i>Halomonas halophila</i> , <b><i>H. organivorans</i></b> , <i>H. salina</i>
Maltose	<i>B. aryabhatai</i> PHB10, <b><i>Halomonas sp. TD08</i></b> , <i>H. boliviensis</i> LC1 and <i>H. campisalis</i>
Melibiose	<i>Burkholderia sacchari</i> sp. nov.
Rhamnose	<i>C. necator</i> , <i>P. oleovorans</i>
Sucrose	<i>Azotobacter vinelandii</i> , <b><i>Burkholderia sacchari</i> DSM 17165</b> , <i>C. necator</i> , <i>Natrinema sp.</i> 5TL6

Results of production show that the employed bioprocess (meaning strain, carbon sources and production systems) significantly impacts production yields and composition of the polymer. Because there is a plenty of systems which can be used, it is difficult to designate which one is the most effective. However, data described previously and in **Table 3** show importance of a deep study and judicious choose of employed bioprocess. Data also demonstrate capacity of *Halomonas* species to use a wide variety of carbohydrates for PHA production, in accordance to results obtained with *Halomonas sp.* SF2003, and are sometimes more efficient than non-halophilic strains. To complete data about *Halomonas sp.* SF2003 carbohydrates metabolisms, additional tests have been conducted on one simple sugars: fructose, galactose and glucose or mixed with one fatty or organic acids, in the proportion 95:5% (mol/mol). Such acids have already been reported as precursor for biosynthesis of copolymers when simple sugars were used as the main substrate. The following acids, which are components of plants, fruits or different industrial effluents (agri-food, chemical, cosmetic, pharmaceutical) were tested: dodecanoic, heptanoic, hexanoic, levulinic, malic, palmitic and trans-2-pentenoic. In the same way than for screening tests with pure carbohydrates, bacterial growth has been evaluated by visual examination and PHA production by detection of fluorescence under UV-lights.

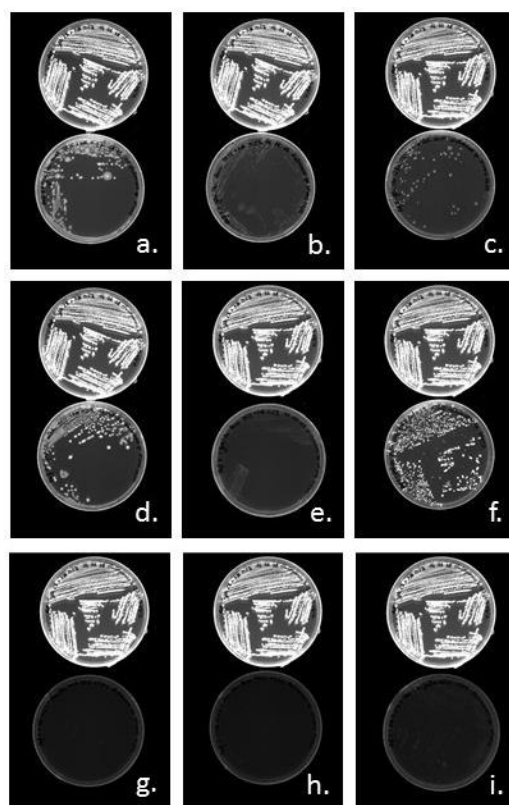


Figure 3: Nile Red agar plates screening with *Halomonas* sp. SF2003 using carbohydrates/acid mix with molar ratio 95/5%, final concentration 2% (w/v).

The positive control (Glucose only) is the upper plate on Figure 3a to Figure 3i. a. Mix Glucose-Malic acid, b. Mix Glucose-Levulinic acid, c. Mix Glucose-Palmitic acid, d. Mix Galactose-Malic acid, e. Mix Galactose-Levulinic acid, f. Mix Galactose-Palmitic acid, g. Mix Fructose-Malic acid, h. Mix Fructose-Levulinic acid and i. Mix Fructose-Palmitic. Observations under UV-lights performed with transillumination.

*Halomonas* sp. SF2003 can grow on majority mix composed of glucose or galactose and organic acids except the following: glucose-dodecanoic acid and galactose-dodecanoic/heptanoic/hexanoic acids. Mix of fructose and acids cannot be used for bacterial growth nor PHA production whatever the acid (**Table 4**). This finding suggests an inhibitory effect of acids depending on the sugar used as co-substrate. Among the mix allowing growth, only five produce fluorescence under UV-lights, suggesting PHA production: glucose-malic acid (**Figure 3a**), glucose-levulinic acid (**Figure 3b**), glucose-palmitic acid (**Figure 3c**), galactose-malic acid (**Figure 3d**) and galactose-palmitic acid (**Figure 3f** and **Table 4**).

Table 4: Growth and PHA accumulation in *Halomonas* sp. SF2003 using different mix of carbohydrates and acids.

	Carbon source	Growth	PHA accumulation
Glucose	Dodecanoic acid	-	-
	Heptanoic acid	+	-
	Hexanoic acid	+	-
	Levulinic acid	+	±
	Malic acid	+	+
	Palmitic acid	+	-
	Trans-2-pentenoic acid	+	-
Galactose	Dodecanoic acid	-	-
	Heptanoic acid	-	-
	Hexanoic acid	-	-
	Levulinic acid	+	-
	Malic acid	+	+
	Palmitic acid	+	±
	Trans-2-pentenoic acid	+	-
Fructose	Dodecanoic acid	±	±
	Heptanoic acid	-	-
	Hexanoic acid	-	-
	Levulinic acid	-	-
	Malic acid	-	-
	Palmitic acid	-	-
	Trans-2-pentenoic acid	-	-

Levulinic, malic and palmitic acids can easily be found in plant co-products and have already been tested in mix with different carbon substrates for PHA production by different bacterial strains. Levulinic acid has been employed in a mix with xylose to perform PHA production with *Burkholderia cepacia*<sup>51</sup> or combined to glucose/fructose with *C. necator*<sup>52</sup>. Quantities of acid employed vary to those tested here and lead to production of P-3HB-co-3HV up to 2.40 g/L with *B. cepacia*<sup>51</sup> and P-3HB synthesis up to 2.41 g/L for *C. necator*<sup>52</sup>. Alongside, previous studies for assimilation of levulinic acid has also been evaluated with *Halomonas hydrothermalis* using seaweed-derived crude levulinic acid and lead to accumulation of P-3HB-co-3HV up to 1.07 g/L<sup>53</sup>. The second acid, malic acid, has been used as co-substrates for PHA production with different bacterial species such as *Burkholderia sacchari* which accumulates P-3HB up to 2.80 g/L from mix of glucose and malic acid<sup>54</sup>. By-products composed of malic acid from fruit pomace have successfully been exploited by *Pseudomonas resinovorans* for P-3-HydroxyHexanoates-co-3-Hydroxyoctanoate-co-3-HydroxyDecanoate-co-3-HydroxyDodecanoate-co-3-HydroxyTetradecenoate (P-3HHx-co-3HO-co-3HD-co-3HDD-co-3HTD) production reaching 1.27 g/L<sup>55</sup>. Other papers reported that addition of malic acid in production medium of *Methylobacterium trichosporium* can promote production of P-3HB up to 1.94 g/L<sup>56</sup>. Finally, palmitic acid is also frequently exploited for PHA production and with various bacterial species. Cruz *et al.*, have tested several by-products and wastes as carbon substrates for PHA production including olive oil, cooking oil

or biodiesel fatty acids by-products. All these products contain more or less important quantities of various fatty acids including palmitic acid. PHA production has been estimated with different species as *Pseudomonas citronellolis*, *P. oleovorans*, *P. resinovorans*, *Cupriavidus necator* H16 and *C. necator* NRRL B-4383 and demonstrated viability of use waste and by-products<sup>57</sup>. Another study exploited oil of spent coffee ground, which contains palmitic acid, for P-3HB production with *C. necator* H16 and led to productions reaching up 10.0 g/L<sup>58</sup>. Additionally, to *Pseudomonas* and *Cupriavidus* species, tests have been conducted on *Burkholderia* sp. USM (JCM15050) to evaluate the exploitation of representative quantities of palmitic acid, alone or in different by-products. Results of this study demonstrated a higher production of P-3HB, up to 1.25 g/L, using palm oil product rather than pure palmitic acid (0.14 g/L of P-3HB)<sup>59</sup>.

According to data reported here and in the literature, it appears that numerous bacterial species, including *Halomonas* sp. SF2003, can use several pure carbohydrates for growth but also for PHA production.

In the case of *Halomonas* sp. SF2003, and based on results of screening tests, it makes more senses to use (D)-Glucose, (D)-Galactose and (D)-Maltose, which shows to qualitatively allow a stronger PHA production. To complete these results, it is necessary to test a mix of these sugars with different ratio of each of them in order to evaluate if PHA production is stronger when exploiting them alone or combined. Moreover, evaluation of PHA production with a mix of carbohydrates will allow to identify potential co-products usable with *Halomonas* sp. SF2003. Indeed, bacterial growth and PHA production of various strains using pure carbohydrates is frequently tested and well reported<sup>33,43</sup>. However, because of their high cost, their use at the industrial scale cannot be reasonably considered and exploitation of by-products is privileged<sup>6</sup>. A majority of pure carbohydrates tested for bacterial growth and PHA production in this study can be found in industrial or natural products (**Table 2**) allowing to test assimilation/exploitation of different (co-)products by *Halomonas* sp. SF2003.

This ability to exploit various carbon substrates, in addition to its capacity to grow in front of atypical/stressful conditions make *Halomonas* sp. SF2003 a versatile strain with a high potential for biotechnological application/use<sup>13,29</sup>. Results of this study identify several potential carbon substrates allowing PHA production and open the door for future tests studying exploitation of each one.

### I.3. Study of PHA synthases

PHA biosynthesis activity of *Halomonas* sp. SF2003 is due to the presence of genes coding for enzymes linked to PHA metabolism (i.e *phaA*, *phaB*, *phaC1*, *phaC2* and *phaR*). Interestingly, genes coding for Acetyl-CoA C-acyltransferase (*phaA*), Beta-ketoacyl-ACP reductase (*phaB*) and PHA synthases (*phaC1* and *phaC2*) are not organized in one operon but are distant from each other on *Halomonas* sp. SF2003 genome sequence. Moreover, *phaC1* and *phaC2* genes expose atypical sizes (1965 pb and 2865 pb, respectively) and conserved domain which led to further study both genes.

### I.3.1. Cloning of PHA synthases *phaC1* and *phaC2* of *Halomonas* sp. SF2003

To better understand the role of each PHA synthase (PhaC1 and PhaC2), their respective genes were cloned in pBBRMCS-2 plasmid before expression in mutant strain *C. necator* H16 PHB<sup>-4</sup>, a non PHA-producing bacterium. Gene *phaC1* has been amplified using PhaC1-F and PhaC1-R primers and *phaC2* gene using PhaC2-F and PhaC2-R (See section III “Techniques de biologie moléculaire” in Chapter 2 Matériels et Méthodes). PCR allowed amplicons production of approximately 2000 and 3000 pb, respectively, corresponding to *phaC1* and *phaC2* size (1965 pb and 2865 pb, respectively). Each amplicon has been digested using SmaI and HindIII enzymes before to be cloned in pBBRMCS-2 plasmid allowing production of pBBRMCS-2 *phaC1* and pBBRMCS-2 *phaC2* plasmids. Both plasmids have been used for transformation of *E. cloni*® and *E. coli* S17-1 cells before transconjugation with mutant strain *C. necator* H16 PHB<sup>-4</sup>, a non PHA-producing strain. Results of control PCR and sequencing confirm correct insertion and total identity between gene sequences of wild type *Halomonas* sp. SF2003 and recombinant *C. necator* H16 PHB<sup>-4</sup> *phaC1* or *phaC2*.

### I.3.2. Characterization of PHA production by transformant strains *C. necator* H16 PHB<sup>-4</sup> *phaC1* and *C. necator* H16 PHB<sup>-4</sup> *phaC2*

To evaluate activity of PHA synthases PhaC1 and PhaC2 of *Halomonas* sp. SF2003, screening for bacterial growth and PHA production have been performed (See section IV.I “Criblage de sources de carbon assimilables” in Matériels et Méthodes section). Likewise, with wild type *Halomonas* sp. SF2003, a total of eight carbohydrates and twenty-one mix have been tested. Bacterial growth and PHA production were qualitatively checked using Nile Red agar plates technic with white light and UV-light evaluation (**Figure 4a to 4h** and **5a to 5h**). Objectives of this part were to evaluate synthesis activity and to start characterization of PHA synthases PhaC1 and PhaC2. This part also allowed to compare production of wild type and transformant strains.

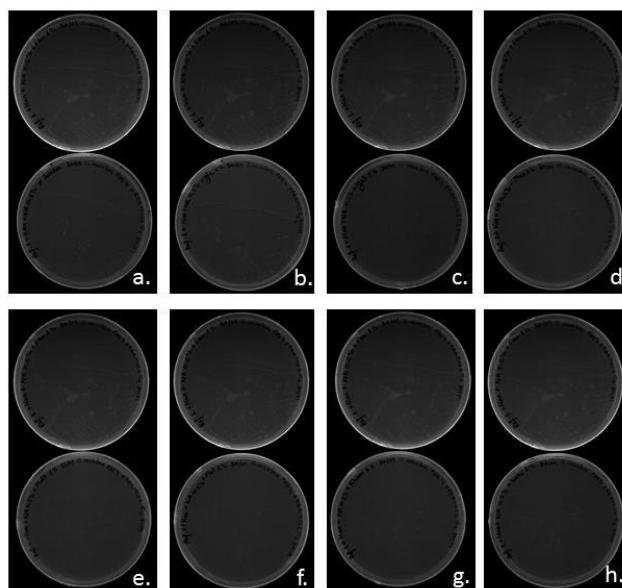


Figure 4: Nile Red agar plates screening with *C. necator* H16 PHB<sup>-4</sup> *phaC1* using 2% (w/v) of different carbon substrates. The positive control (Fructose only) is the upper plate on Figure 4a to Figure 4h. a. Control negative, b. Glucose, c. Galactose, d. Mannose, e. Maltose, f. Melibiose, g. Rhamnose and h. Sucrose. Observations performed under UV-lights with transillumination.

*C. necator* H16 PHB<sup>-4</sup> *phaC1* is able to exploit all pure carbohydrates and a majority of mix carbohydrates/acids tested for bacterial growth (Table 5) excepted following mix: glucose – heptanoic/hexanoic/trans-2-pentenoic acids, galactose – heptanoic/hexanoic/palmitic/trans-2-pentenoic (data not shown). In comparison, results obtained with *C. necator* H16 PHB<sup>-4</sup> *phaC2* are similar, for pure carbohydrates and mix, except for mix galactose-palmitic acid for which a growth is recorded. Some results do not appear clearly positive and have been noted as “±” making interpretation of substrates use and PHA production difficult.

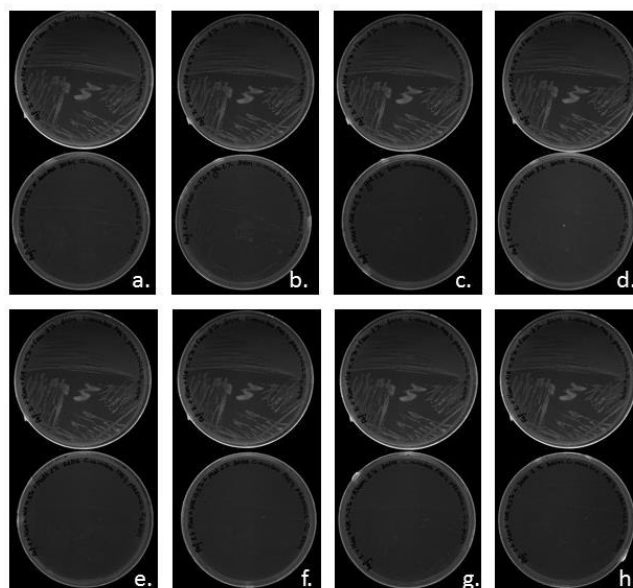


Figure 5: Nile Red agar plates screening with *C. necator* H16 PHB<sup>-4</sup> *phaC2* using 2% (w/v) of different carbon substrates. The positive control (Fructose only) is the upper plate on Figure 5a to Figure 5h. a. Control negative, b. Glucose, c. Galactose, d. Mannose, e. Maltose, f. Melibiose, g. Rhamnose and h. Sucrose. Observations performed under UV-lights with transillumination.

PHA production has been detected with both transformant strains demonstrating the success of cloning experiments and functionalities of both PHA synthase genes, *phaC1* and *phaC2*. Screening tests have allowed the confirmation of correct annotation of *phaC1* and *phaC2* genes and attest to existence of difference between both PHA synthases of *Halomonas* sp. SF2003. Indeed, qualitative analysis of PHA accumulation, by detection of fluorescence under UV-light highlighted several differences between both transformant strains. Indeed, among all carbon substrates tested only three seem to allow PHA accumulation in *C. necator* H16 PHB<sup>-4</sup> *phaC1* (Fructose, Glucose-dodecanoic/palmitic acid) and nine for *C. necator* H16 PHB<sup>-4</sup> *phaC2* (Fructose, Mannose, Sucrose, Glucose-dodecanoic/palmitic acids, Glucose-levulinic/malic acids, Galactose-levulic/malic acids).

Table 5: Growth and PHA accumulation in transformant strains *C. necator* H16 PHB<sup>-4</sup> *phaC1* and *C. necator* H16 PHB<sup>-4</sup> *phaC2* using different carbon sources.

Legend for growth +/-: Positive/Negative, PHA accumulation P/N: Positive/Negative.

Carbon source	<i>C. necator</i> H16 PHB <sup>-4</sup> <i>phaC1</i>		<i>C. necator</i> H16 PHB <sup>-4</sup> <i>phaC2</i>	
	Growth	PHA accumulation	Growth	PHA accumulation
(D)-Fructose	+	±	+	+
(D)-Galactose	+	-	+	-
(D)-Glucose	+	-	+	-
(D)-Maltose	+	-	+	-
(D)-Mannose	+	-	+	±
(D)-Melibiose	+	-	+	-
(L)-Rhamnose	+	-	+	-
(D)-Sucrose	+	-	+	±
Glucose +	Dodecanoic acid	+	±	+
	Heptanoic acid	-	-	-
	Hexanoic acid	-	-	-
	Levulinic acid	+	-	+
	Malic acid	+	-	+
	Palmitic acid	+	±	+
	Trans-2-pentenoic acid	-	-	-
Galactose +	Dodecanoic acid	+	-	+
	Heptanoic acid	-	-	-
	Hexanoic acid	-	-	-
	Levulinic acid	±	-	+
	Malic acid	+	-	+
	Palmitic acid	-	-	+
	Trans-2-pentenoic acid	-	-	-
Fructose +	Dodecanoic acid	-	-	-
	Heptanoic acid	-	-	-
	Hexanoic acid	-	-	-
	Levulinic acid	-	-	-
	Malic acid	-	-	-
	Palmitic acid	-	-	-
	Trans-2-pentenoic acid	-	-	-

These results suggest that synthase PhaC1 is less active or more selective than PhaC2. Indeed, qualitatively, there are more carbon substrates (pure or in mix) that generate a fluorescence under UV-light when *C. necator* H16 PHB<sup>-4</sup> *phaC2* is used than *C. necator* H16 PHB<sup>-4</sup> *phaC1*. Previous study of *Halomonas* sp. SF2003 genome and metabolisms demonstrated several difference between both synthases<sup>13</sup>. Actually PhaC1 and PhaC2 expose an identity of 60-70% and 65-96%, respectively, with different PHA synthases<sup>13</sup>. Results of both studies are in accordance. Even if PhaC2 exposes some atypical characteristics (size and structure of conserved domains), it seems to be the main PHA synthase responsible of HA polymerization.



To confirm this result, production tests must be conducted and new genetic constructions could be tested. These tests will allow to definitively evaluate the activity of PHA synthases. Moreover, in this study a pBBRMCS-2 plasmid with *C. necator* H16 PHA biosynthesis operon has been used. This construction, could be responsible, in part, of weak activity of PhaC1. Indeed, it could be possible that PhaC1 specifically requires proteins (PhaC2, PhaA, PhaB, ...) or promoter of *Halomonas* sp. SF2003 metabolisms to ensure polymer synthesis despite that it has been identified to belong to class I of synthase (meaning that PhaC are constituted of only one subunit and does not require any additional protein to be active). To confirm this hypothesis, several different constructions might be designed, using *Halomonas* sp. SF2003 promoter and PHA biosynthesis genes simultaneously, and evaluated for PHA production<sup>17,60-62</sup>. Among all the different constructions which could be tested, plasmid harboring both *phaC1* and *phaC2* genes, together, must be design like it has been done with PHA synthases of *Halomonas* sp. O-1<sup>14</sup>. This construction will allow to control the influence of each one on the other and to check if PHA synthase PhaC1 requires PhaC2 to be active. It could also be necessary to test activity of each PHA synthase by designing plasmid harboring also *phaA* and *phaB* genes of *Halomonas* sp. SF2003, coding for 3-ketoacyl-CoA thiolase (or  $\beta$ -ketothiolase) and Acetoacetyl-CoA reductase, respectively. Testing different constructions will allow a better understanding of genes activity and to identify best combination to optimize production.

### I.3.3. Polyhydroxyalkanoates production in shake flasks

Results of screening tests demonstrated that several carbohydrates can be used for PHA production. To evaluate and compare synthesis activity in function of strains and sugars exploited production tests in flask have been performed (See section IV.2.1 “Production en erlenmeyers (non-contrôlées)” in Chapter 2 Matériels et Méthodes). In accordance to results of screening tests, and focusing on PHA synthase activity, only three pure carbohydrates have been tested: glucose, fructose and galactose, with *Halomonas* sp. SF2003, *C. necator* H16, *C. necator* H16 PHB<sup>-4</sup> *phaC1* and *C. necator* H16 PHB<sup>-4</sup> *phaC2*.

For *Halomonas* sp. SF2003, glucose was the favorite carbohydrate to produce PHA production (2.25 g/L) followed by galactose (1.23 g/L) and then fructose (1.02 g/L) (**Table 6**). Comparatively *C. necator* H16 produces more PHA when fructose is used as main carbon source in medium (2.25 g/L) rather than glucose (2.05 g/L). Production using galactose cannot be estimated due to low cell dry weight obtained.

Table 6: Comparative PHA productions in flasks with glucose, fructose or galactose.  
N.D : Not determined.

Strain	Carbon source	Dry cell weight (g/L)	PHA (g/L)	PHA content (%)
<i>Halomonas</i> sp. SF2003	Glucose	2.63	2.25	86
<i>C. necator</i> H16		2.89	2.05	71
<i>C. necator</i> H16 PHB <sup>-4</sup> <i>phaC1</i>		1.05	0.32	30
<i>C. necator</i> H16 PHB <sup>-4</sup> <i>phaC2</i>		2.63	1.38	52
<i>Halomonas</i> sp. SF2003		2.63	1.02	39
<i>C. necator</i> H16	Fructose	3.16	2.25	71
<i>C. necator</i> H16 PHB <sup>-4</sup> <i>phaC1</i>		0.79	0.26	33
<i>C. necator</i> H16 PHB <sup>-4</sup> <i>phaC2</i>		3.42	1.83	54
<i>Halomonas</i> sp. SF2003		3.16	1.23	39
<i>C. necator</i> H16	Galactose	0.79	N.D	N.D
<i>C. necator</i> H16 PHB <sup>-4</sup> <i>phaC1</i>		1.06	N.D	N.D
<i>C. necator</i> H16 PHB <sup>-4</sup> <i>phaC2</i>		0.79	N.D	N.D
<i>C. necator</i> H16 PHB <sup>-4</sup> <i>phaC2</i>		0.79	N.D	N.D

Expression of pBBRMCS-2 *phaC1* and pBBRMCS-2 *phaC2* plasmids allow PHA accumulation in mutant strain *C. necator* H16 PHB<sup>-4</sup> (a non PHA-producing strain), confirming the synthesis activity of PhaC1 and PhaC2. Similarly to *C. necator* H16, *C. necator* H16 PHB<sup>-4</sup> *phaC2* uses more efficiently glucose for PHA production (1.38 g/L) than fructose (1.83 g/L). Interestingly, inverse results are observed with *C. necator* H16 PHB<sup>-4</sup> *phaC1* which exposed a production of 0.32 g/L with glucose and 0.26 g/L with fructose. Likewise with *C. necator* H16, PHA production tests using galactose did not allow to determine production yield for both *C. necator* H16 PHB<sup>-4</sup> *phaC1* and *C. necator* H16 PHB<sup>-4</sup> *phaC2*.

It was also highlighted that galactose is more adapted for growth of *Halomonas* sp. SF2003 than for PHA synthesis since only 39% of PHA content was estimated whereas 86% when glucose was used (Table 6). With *C. necator* H16, fructose seems to be more exploited for bacterial growth than for PHA production, PHA content was the same as in glucose condition (71%). *C. necator* H16 PHB<sup>-4</sup> *phaC1* showed a stronger growth with galactose, very close to those obtained with glucose, rather than with fructose. However, PHA content was quite similar using fructose or glucose: 33% and 30%, respectively (Table 6). Finally, *C. necator* H16 PHB<sup>-4</sup> *phaC2* uses more efficiency fructose for bacterial growth and PHA production than glucose even if PHA contents are again quite similar (54% for fructose and 52% for glucose). The results also confirmed that transformant *C. necator* H16 PHB<sup>-4</sup> *phaC2* is a better PHA-producer than *C. necator* H16 PHB<sup>-4</sup> *phaC1* whatever the used carbonaceous substrate. However, expression of both *phaC1* or *phaC2* genes using pBBRMCS-2 system did not allow to obtain more efficient PHA-producers than the wild type strain *C. necator* H16, suggesting to test different construction to induce an overproduction. Composition of production medium and production parameters used for these tests can be responsible in part of the low production yields. Indeed, same medium and parameters have been used for all the strains. However, *Halomonas* sp. SF2003 and *C.*

*necator*'s wild and transformant strains do not exhibit same origin and metabolisms. Consequently, new production medium and different production parameters must be tested.

## Discussion

Lipase box-like sequences are highly conserved domain which have been identified to play a crucial role in elongation of the polymer<sup>18</sup> and are identified in several PHA-producing species. *In silico* study of PHA synthases allowed to identify two different lipase box-like sequences: Glycine-Tyrosine-Cysteine-Leucine-Glycine (G-Y-C-L-G) for PhaC1 and Serine-Tyrosine-Cysteine-Isoleucine-Glycine (S-Y-C-I-G) for PhaC2. Both lipase box-like sequences of *Halomonas* sp. SF2003 PHA synthases have a tyrosine, a cysteine and a glycine (Y-S-G) suggesting that these residues can potentially have a crucial role in catalytic activity of the enzymes. To complete data, structural study of enzymes exploiting X-ray crystallography and/or molecular biology could be performed to confirm, or not, that the identified lipase box-like sequences play a key role in synthesis of PHA by *Halomonas* sp. SF2003. Alterations/modifications of PHA synthase sequences will lead to change proteins tertiary structures and potentially synthesis activity. Other studies have already been performed to elucidate tertiary structure of different PHA synthases and to identify active sites. For example, Ilham *et al.*, (2014) have studied PHA synthases of *Halomonas* sp. O-1 by performing site-directed mutagenesis on different residues and studying production of the strain. They determined that appropriated changes can, positively or negatively, affect synthesis activity, bacterial growth or molecular weight of polymers. Indeed, substitution of alanine for Cys329 or Cys331 in *Halomonas* sp. O-1 or *H. elongata* DSM 2581 PHA synthase sequence leads to a total inhibition of PHA synthesis while substituting glycine for serine impacts polymer molecular weight. These results allowed identification of catalytic sites in enzymes and to imagine modifications in strain genes to enhance production<sup>14</sup>. Studies exploiting X-ray crystallography will allow to apprehend structure of catalytic site and to confirm the role of each residues<sup>17</sup>. Similar studies have already been performed and reported with different species such as *Chromobacterium* sp. USM2<sup>63</sup>, *C. necator*<sup>64</sup> or *Pseudomonas* sp. 61-3<sup>65</sup>.

Results obtained with *Halomonas* sp. SF2003 confirmed substrate versatility of this species for both growth and PHA production. Among tested carbohydrates, positive results have been recorded with glucose, fructose, galactose, mannose, maltose and sucrose. Interestingly, the higher content of polymer was achieved using glucose rather than fructose or galactose even if the last one seems to allow a stronger bacterial growth. Indeed, production cost on pure carbohydrates is still a major lock to a larger commercialization of PHA and exploitation of co-products are deeply studying. For example, production of PHA is rarely tested with “pure” galactose but rather using products constituted by itself such as lactose sources (lactose or cheese whey and milk) or in its polymeric form such as agar in red algae in order to promote use of various co-products. Indeed, PHA productions have successfully been performed with *Haloferax mediterranei* and *Pseudomonas hydrogenovora* on whey lactose<sup>66-68</sup> or with

*Bacillus megaterium*<sup>69</sup> using acid-treated red algae. Actually, there is an important number of studies using this group of carbon substrates. However, without more precise analyzes it is difficult to know which carbohydrates is preferentially used for PHA accumulation. Moreover, the use of these products in their original forms by bacterial strains is difficult and consequently some pre-treatments (hydrolysis) are required, leading sometimes to an increase of the cost and time of production<sup>5</sup>. Similar experiments of PHA production have also been performed using (pre-treated) co-products composed of mannose such as spent coffee ground<sup>70</sup>, sugar maple hemicellulosic hydrolysate<sup>71</sup> or ensiled grass press juice<sup>72</sup>. Based on results of screening tests performed with *Halomonas* sp. SF2003, and using mix of carbohydrates and acids, production tests might be achieved. In fact, mix exposing positive results are composed by carbohydrates and acids which are easily found in various natural products or by-products. As described, levulinic acid can be found in seaweed<sup>53</sup> or paper industry co-products<sup>73</sup>; malic acid, as a component of fruits and vegetables, is easily found in natural products or agri-food co-products<sup>56</sup>. Finally palmitic acid, due to its important representation in vegetal and animal kingdom as well as component of cooking waste, also represents a co-substrate with high potential<sup>59</sup>. To evaluate viability of exploiting these co-products, screening tests must be performed. Furthermore, previous tests could also be completed using more or less important different carbohydrates/acids ratio, as performed in different studies. However, Nile red agar plates's tests are only used as screening tool and must be completed with production tests to evaluate the impact of each mix or pure carbon substrates on production yield and polymer composition.

Consequently, additional tests exploiting different by-products derivated of dairy, waste treatment or agri-food industries might be performed in order to optimize PHA production by *Halomonas* sp. SF2003. This kind of production has already been done with different bacterial species, including *Halomonas* species, as well as other ones, as described previously. Indeed, Pernicova *et al.*, 2019, have studied viability of several *Halomonas* strains to produce PHA from waste cooking oil. They demonstrated that *Halomonas hydrothermalis* exposes the highest production yield (0.38 g/L) but also influence of NaCl concentration on production<sup>74</sup>. These results demonstrate that production medium must be wisely studied and elaborated.

The efficiency of two transformed strains harboring *phaC1* and *phaC2* genes was estimated and compared to those of the wild bacteria through lab scale production. Data of this study confirm their polymerase activity and existence of differences between them including their size, sequences, location on genome and synthesis activity. Indeed, a stronger synthesis activity has been observed for PHA synthase PhaC2 with a polymer content reaching up to 54% as compared to 33% for transformant strain expressing PhaC1. This is in accordance with previous results demonstrating a higher percent of identity of PhaC2 with PHA synthases of other bacterial species than PhaC1<sup>13</sup>. However, both synthase enzymes cannot induce an increase in PHA production as compared to wild type strain *C. necator* H16. For a better understanding of respective activities, a monitoring of bacterial growth and production kinetics

for each wild type and transformant strains might be performed. Following production of each strains in different conditions will allow to more precisely understand activity of each strain/PHA synthase and to adjust more precisely the production step.

Finally, a better characterization of polymerase activity of PhaC1 and PhaC2 can participate to confirm higher synthesis activity of PhaC2. In addition, these results will potentially allow to design new constructions, harbouring *phaC1* and/or *phaC2* genes, to increase PHA production by *Halomonas* sp. SF2003.

## Conclusion

This study has demonstrated the functionality of both PHA synthases, PhaC1 and PhaC2, confirming annotation of *Halomonas* sp. SF2003 genome performed in our *in silico* study. Results also highlighted stronger PHA biosynthesis activity of PHA synthase PhaC2 as compared to PhaC1. Performed screening tests allowed identification of several carbon substrates, pure carbohydrates or mix of sugars and acids, potentially usable for PHA production by *Halomonas* sp. SF2003. Substrate versatility of this bacterium opens the door for new tests in order to optimize production and also confirm its high biotechnological potential. Preliminary biosynthesis tests expose a better PHA production using glucose with *Halomonas* sp. SF2003 while *C. necator* wild type and transformant strains preferably exploit fructose. Additional research, such as kinetics of bacterial growth and PHA production, should optimize production step.

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## **Chapter 5**

# **Characterization of the *Halomonas* sp. SF2003 PHA production**

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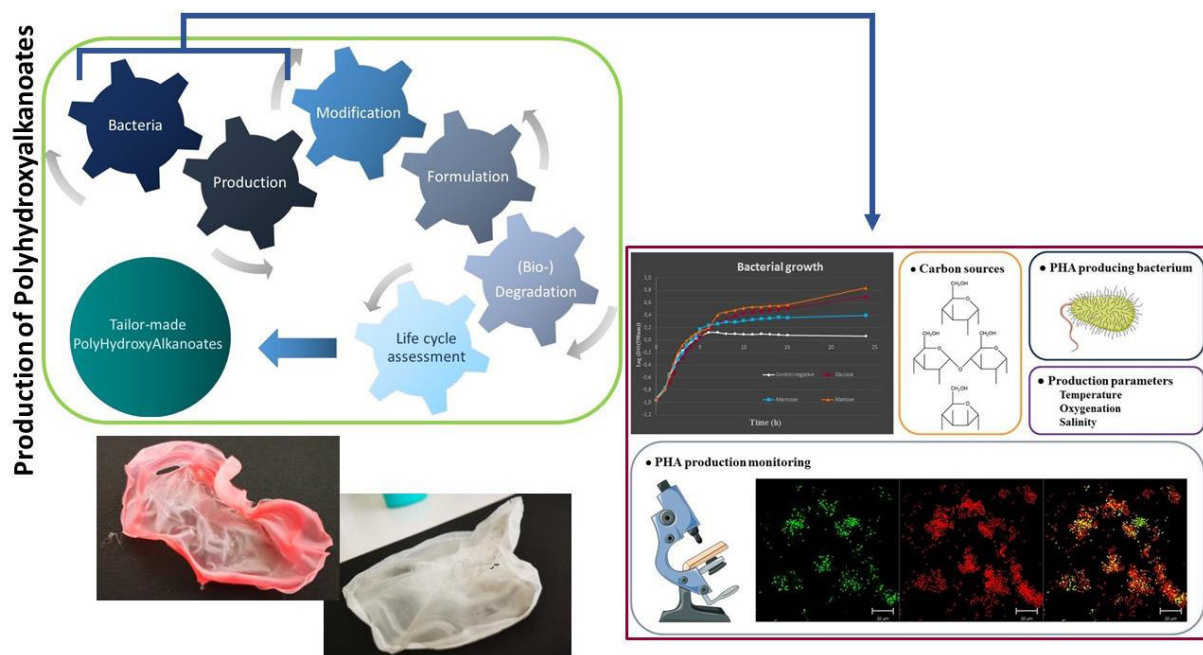
**Keywords:** *Halomonas* sp. SF2003, Halophilic bacteria, PolyHydroxyAlkanoates (PHA), PHA production kinetics, Fluorescence.

## Highlights:

- *Halomonas* sp. SF2003 uses similarly fructose, galactose, glucose, maltose, mannose and sucrose for its growth
- The strain exposes a highest PHA production using glucose rather than galactose or fructose
- Salinity impacts *Halomonas* sp. SF2003 PHA production
- A fluorescence based method allows monitoring and represents a fast and easy tool for optimization of PHA production

## Graphical abstract

Several parameters influencing bacterial PHA production must be considered such as carbon sources, oxygenation, temperature or salinity.... The influence of each one must be studied to optimize bacterial growth simultaneously to PHA production. Among different available analytical tools to characterize production depending on selected parameters combination, fluorescence microscopy monitoring can represent an easy and fast method of real time study.



## Introduction

Polyhydroxyalkanoates are biobased and biodegradable polymers<sup>1-4</sup> produced by numerous microorganisms from various carbon substrates ranging from pure carbohydrates to different by-products<sup>5</sup>. However, even if they can easily be produced and exposed similar properties to those of conventional plastics, their production cost is still too high to reasonably consider replacement of usual plastic materials<sup>6</sup>. Numerous research are conducted to reduce production cost by considering all production modes and parameters. In fact, the production cost can be impacted at all production steps ranging from bacterial strains selected to the process. Because PHA features are closely linked to the used bacterial strain and carbon sources, their selection is under particular attention to tailor-make polymer<sup>7</sup>. Depending on the systems exploited, the production cost can become an insurmountable brake. A perfect knowledge and management of production parameters are required to optimize bioprocess and PHA productions. PHA production can be quantified by performing extraction followed by analysis of production parameters (dry cell weight, PHA concentration and content). But PHA extraction can sometimes require several days before the acquisition of exploitable results. Quantitative technics coupled with qualitative technics (i.e microscopy and/or staining) can hence be employed.



*Halomonas* sp. SF2003 is a marine bacterium which has already demonstrated its biotechnological potential<sup>8</sup>. Indeed, the strain can grow in front of various conditions/environments and is able to synthesize polyhydroxyalkanoates (PHA) using various carbon sources<sup>8,9</sup>. Studies of its genome and its metabolisms allowed identification of genes related to different metabolisms including PHA biosynthesis<sup>8</sup>. Among them, two PHA synthases (PhaC1 and PhaC2) exposing atypical size and genomic environment have been identified. Results of screening tests for carbon substrates and PHA batch productions demonstrated activity of both synthases and also highlighted difference between them in term of PHA yields.

The objectives of this work were to design production bioprocess for each combination of *Halomonas* sp. SF2003 and carbon substrates and to confirm the biosynthetic activity of PHA synthases PhaC1 and PhaC2 in these conditions. Results will enable adaptation and optimization of production process in order to limit time consumption and production cost. This work will also contribute to the development of PHA production monitoring method, based on an image analysis method exploiting confocal laser scanning microscopy (CLSM). This method can be applied for faster monitoring and characterization of PHA production.

## I. Results

### I.1. Optimization of PHA production process

#### I.1.1. Response of *Halomonas* sp. SF2003 growth in function of carbohydrates

Genome analysis of *Halomonas* sp. SF2003 revealed the presence of two PHA synthases and many genes for metabolisms of several carbohydrates, both potentially involved in the ability of the bacterium to exploit them for growth and PHA production<sup>8</sup>. Indeed, among the different tested sugars, fructose, galactose, glucose, maltose, mannose and sucrose led to qualitatively positive results for PHA production (See Chapter IV). As previously described, these carbohydrates can be found in different ratios in by-products which suggests potential feedstocks for PHA production using these products. To design the most efficient PHA production process and to complete our previous study<sup>8</sup>, *Halomonas* sp. SF2003 growth kinetic using biomass accumulation medium (Reference 1) complemented with these different carbohydrates has been performed (See section I.2 “Cinétique de croissance des souches bactériennes” in Chapter 2 Matériels et Méthodes).

For all production media, including negative control (meaning a production media without addition of any supplementary carbon sources), a growth was recorded, confirming the ability of the bacterium to use them as sole carbon sources (**Figure 1**). Exponential phases began after 1h and ended after 5h of growth for all conditions. Then, a slow-down of growth led to the stationary growth phase which started at around 7-8h for all carbohydrates.

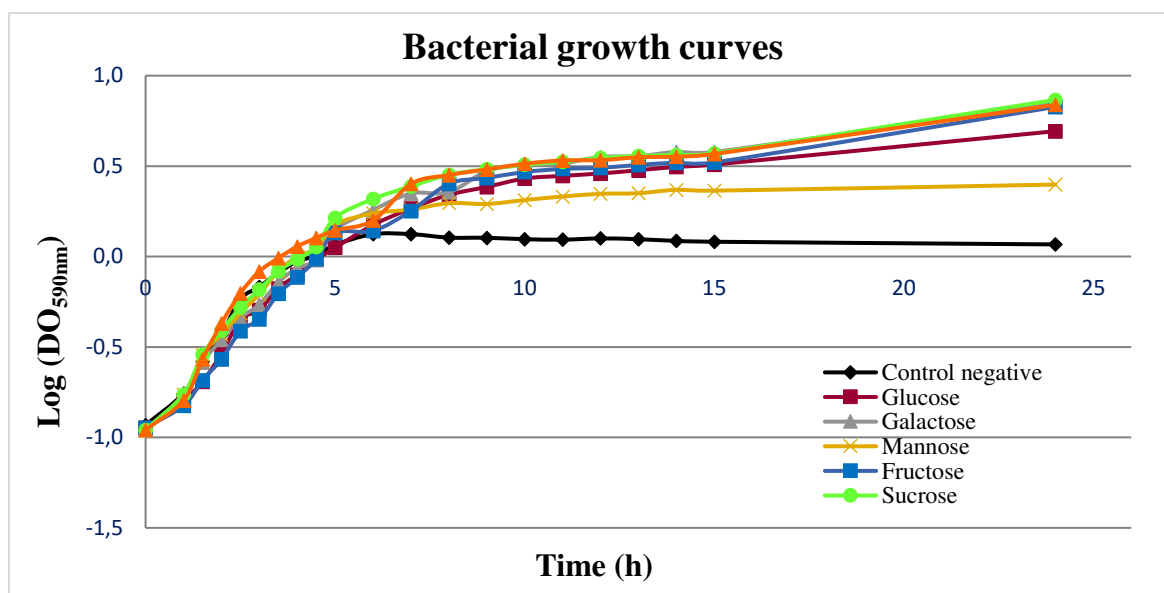


Figure 1: *Halomonas* sp. SF2003 growth kinetics as a function of carbohydrates source.

Considering specific growth rates ( $\mu$ ) and generation time (G) obtained for each tested sugars (Table 1), *Halomonas* sp. SF2003 seems to have a similar use of all of them. Highest specific growth rate was obtained with maltose ( $0.591 \text{ h}^{-1}$ ), followed by sucrose ( $0.536 \text{ h}^{-1}$ ), mannose ( $0.533 \text{ h}^{-1}$ ) and fructose ( $0.532 \text{ h}^{-1}$ ). Main differences were observed regarding on the  $\text{OD}_{590\text{nm}}$  max. Indeed, an  $\text{OD}_{590\text{nm}}$  value of 2.50 was recorded for mannose while sucrose allowed to reach 7.35 (Figure 1).

These results also showed that no addition of any supplementary carbon sources (i.e. carbohydrates, acids, ...) is sufficient to obtain bacterial growth. However, this growth is limited and complementation of one of the tested carbohydrate extends the growth. Indeed, maximal  $\text{OD}_{590\text{nm}}$  reached 1.17 in the negative control whereas it reached a maximum of 7.35 when sucrose was added. Regarding results, maltose and sucrose allowed a faster growth of *Halomonas* sp. SF2003. However, except for mannose, results obtained for all carbohydrates are very close to each other and demonstrated versatility of *Halomonas* sp. SF2003 towards various carbon sources.

Table 1: Growth parameter of *Halomonas* sp. SF2003 using different carbohydrates.

Carbohydrates	Specific growth rate ( $\mu$ ) ( $\text{h}^{-1}$ )	Generation time (G) (h)
Fructose	0.532	1.5
Galactose	0.503	1.5
Glucose	0.512	2.0
Maltose	0.591	1.5
Mannose	0.533	1.5
Sucrose	0.536	1.5
Negative control	0.502	2.5

Recorded data give a better framing of *Halomonas* sp. SF2003 growth phases during biomass accumulation step and allow to adapt production process for each carbohydrate. Actually, batch process used for PHA production consists in two main phases: biomass accumulation and PHA biosynthesis.

The objective of biomass accumulation phase is to obtain the highest cell concentration in order to have an important quantity of viable cells, in optimal physiologic and metabolic state, producing PHA<sup>10</sup>. Bacteria growth must consequently be studied and controlled to stop biomass accumulation phase at the most suitable time. The growth kinetic curves obtained for *Halomonas* sp. SF2003 with the different tested carbohydrates suggest to stop biomass accumulation between 5h and 10h. However, bacterial growth monitoring has been performed using shake-flask systems without any control of pH or oxygenation. Hence, different results may potentially be observed at the bioreactor scale meaning that some readjustements could be required. To complete these data, PHA production kinetics depending on the used carbohydrates and growth phase duration could be performed for better process optimization and biopolymer yields.

The study of *Halomonas* sp. SF2003 growth has been conducted to complete screening tests. Recorded results allow a better characterization of bacterial strain growth conditions and optimization production systems for each carbohydrate. To complete OD<sub>590nm</sub> measures, counting of viable cells could enable to more precise monitoring for systems applied at a superior scale (i.e. 5L-bioreactor).

### I.1.2. Impact of carbon source and salinity on PHA production by *Halomonas* sp.

#### SF2003

*Halomonas* sp. SF2003 exhibits an interesting versatility in front of carbon substrates as well as salinity. Consequently, the influence of both parameters on PHA production has been evaluated by confronting, first, different carbohydrates (Galactose, Fructose and Glucose) as sole carbon substrate and then different medium salt concentrations (11, 30 and 50 g/L).

#### I.1.2.1. Carbon sources

We previously showed, using Nile Red agar plates tests, the versatility of *Halomonas* sp. SF2003 to use different carbohydrates (fructose, galactose, glucose, mannose, maltose) for its growth and PHA production (See Chapter IV « PHA production and PHA synthases of halophile bacterium *Halomonas* sp. SF2003. »). Nile red agar plate is an experimental set-up designed to quickly screen conditions allowing bacterial PHA production. However, this technic does not lead to any quantitative results. Therefore, we quantified PHA production using fructose, galactose and glucose (with a fixed salinity of 11 g/L) to evaluate the impact of each one on both growth and polymer synthesis.

Table 2: Comparison of *Halomonas* sp. SF2003 PHA production depending on carbon sources and/or salinity, n: 3.

Carbon source	Salinity (g/L)	Dry cell weight (g/L)	PHA (g/L)	PHA content (%)
Galactose	11	1.80 ± 0.00	1.02 ± 0.05	57 ± 2.7
Fructose	11	2.24 ± 0.56	0.86 ± 0.22	39 ± 0.1
Glucose	11	3.00 ± 0.91	1.70 ± 0.56	59 ± 19.8
	30	4.10	2.31	56
	50	1.60 ± 0.08	0.66 ± 0.00	41 ± 2.3

PHA production tests using fructose, galactose or glucose demonstrate that the highest dry cell weight (DCW) (3.00 g/L), PHA concentration (1.70 g/L) and content (59%) were obtained with glucose as the main carbon source (**Table 2**). In comparison, the use of galactose and fructose allows obtaining a DCW of only 1.80 and 2.24 g/L, PHA concentrations of 1.02 and 0.86 g/L and PHA contents of 57% and 39%, respectively (**Table 2**).

Coupling results of bacterial growth kinetics and PHA production, under tested parameters, show that glucose allows a highest PHA production (1.70 g/L) even if *Halomonas* sp. SF2003 does not expose its better specific growth rate (0.512 h<sup>-1</sup>) and generation time (2.0 h) (**Table 1**). Production results with galactose and fructose show a highest PHA content and production using galactose (1.02 g/L and 57%, respectively) rather than fructose (0.86 g/L and 39%, respectively) (**Table 2**). The results suggest a better employment of galactose even if bacterial growth kinetics suggest a faster assimilation of fructose with a specific growth rate of 0.532 h<sup>-1</sup> against 0.503 h<sup>-1</sup> with galactose (**Figure 1** and **Table 1**).

Similar results have already been described with other *Halomonas* species. Indeed, PHA production of *H. halophila* and *H. salina* was better with glucose rather than fructose or galactose. In the same way, *Halomonas* sp. SF2003, *H. halophila* and *H. organivorans* preferentially use galactose rather than fructose for polymer synthesis while *H. salina* exposes opposite tendency, with very low PHA concentration (0.17 g/L for fructose and 0.12 g/L for galactose)<sup>11</sup>.

#### 1.1.2.2. Salinity

Previous work on *Halomonas* sp. SF2003 genome and metabolism has demonstrated the capacity of the strain to grow at various NaCl concentrations, ranging from 0.5% to 20% (w/v)<sup>8</sup>. Also, PHA production using various carbohydrates demonstrated better PHA concentration and content using glucose. Hence, in order to evaluate if modification of salinity alters PHA biosynthesis, comparative productions have been conducted using different salt concentrations: 1.1%, 3.0% and 5.0% (w/v), i.e. 11, 30 and 50 g/L respectively, with the same main carbon source: glucose.

As already observed, salinity seems to impact bacterial growth<sup>8</sup>. Salt concentration of 30 g/L allows to obtain the highest DCW (4.10 g/L) simultaneously to the highest PHA concentration (2.31 g/L) (**Table 2**). Comparatively, salt concentration of 11 g/L allows reaching the highest PHA content

(59%) but exposes lower DCW and PHA concentration values: 3.00 g/L and 1.70 g/L, respectively. Finally, salt concentration of 50 g/L exposes lowest productivity values (PHA concentration and content: 0.66 g/L and 41%, respectively) demonstrating that using a too high salinity with *Halomonas* sp. SF2003 can inhibit PHA production (**Table 2**).

High salt concentrations can be considered as a stressful condition potentially responsible for a stimulation or an inhibition of the PHA biosynthesis. Consequently, influence of salinity on PHA production is frequently studied with various bacterial species including halophiles. For example, three salt concentrations have been tested with *Bacillus megaterium* uyuni S29 and showed that 45 g/L of NaCl allows reaching a PHA content higher than 5 or 100 g/L<sup>12</sup>. In *Halomonas hydrothermalis* and *H. neptunia*, the highest PHA contents were reached with a salt concentration of 40 g/L and 60 g/L, respectively<sup>13</sup>. Finally, similar study has been conducted with the haloarchaea *Haloferax mediterranei* and showed that salt concentration of 250 g/L enhanced PHA content, reaching the highest value, even if it the salt concentration was not the optimal for growth<sup>14</sup>. Results obtained with *Halomonas* sp. SF2003 expose the same tendency and are in accordance with these data demonstrating the crucial role of salinity on PHA biosynthesis.

## I.2. Development of a fluorescence based monitoring method

Nile Red agar plates and extraction methods allow to characterize and study PHA production depending on employed strain and carbon substrate. The first one is easy and fast to perform but can only be used as a qualitative tool while extraction gives quantitative data but requires time and could be arduous to execute. Therefore, a fluorescence based monitoring method has been developed using Syto 9 and Nile Red dyes which stains bacterial cells and PHA granules, respectively (See sections “Cinétique de croissance des souches bactériennes” and “Microscopie confocale à balayage laser” in Matériels et Méthodes chapter). This simple method can be quickly achieved and provides quantitative data. Indeed, by determination of the coverage percent of both Nile Red and Syto 9 fluorescence, a Nile Red/Syto9 ratio can be calculated providing informations about the production and the PHA content. For a better understanding of PHA biosynthesis systems of each wild type and transformant strains, PHA production monitorings have been performed exploiting this fluorescence method. Simultaneously, bacterial growth kinetics have been followed. Tests were conducted using shake flask production systems with production medium complemented by glucose or fructose since positive results were recorded with both of them for Nile Red agar plates screening tests and based on data available in literature<sup>9,15,16</sup>.

### I.2.1. PHA production kinetics monitoring using fluorescence staining

Nile Red is a fluorescent stain used for screening of PHA production and PHA granules observation<sup>15,17-19</sup>. We have used Nile Red in a coupled staining method with Syto 9, which allows bacterial cell observations. Syto 9 stains in green bacterial cells (**Figures 2a.1, 2a.3, 2b, 2c and 2d**),

Nile Red stains in red PHA granules and/or cells (**Figures 2a.2, 2a.3, 2b, 2c and 2d**), bacterial cells stained by both markers appear in yellow (**Figures 2a.3, 2b, 2c and 2d**). During production step, samplings have been performed every twelve hours and bacterial cells were spread on slides and observed using confocal laser scanning microscopy (CLSM). CLSM pictures were analysed and coverage percent of fluorescence emitted from Syto9 (green) and Nile Red (red) on slides were determined using image processing software ImageJ.

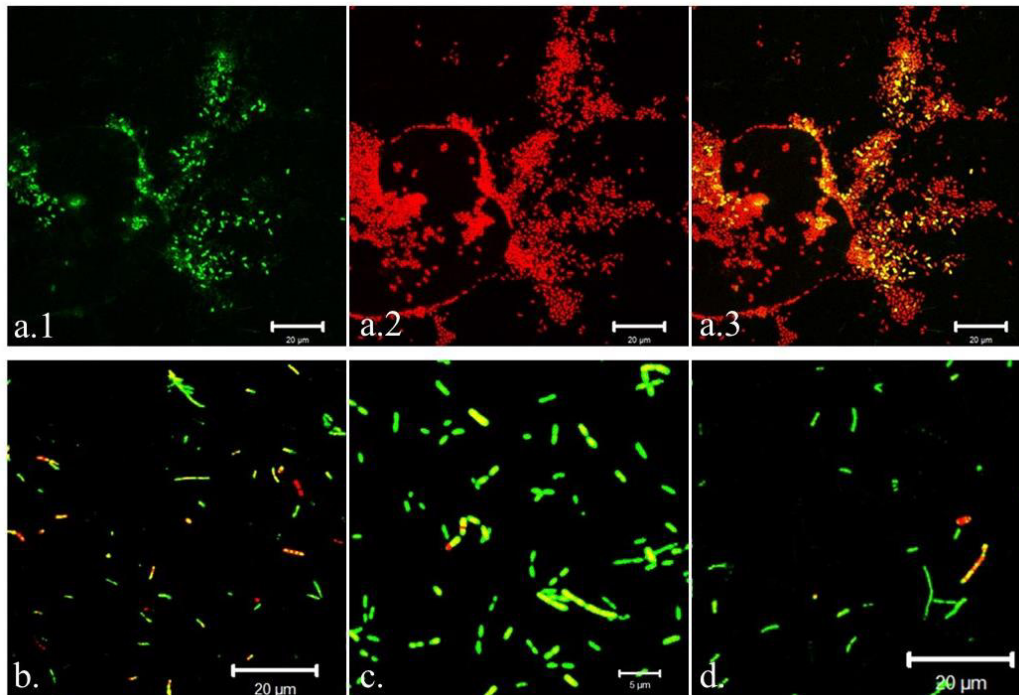


Figure 2: Confocal Laser Scanning Microscopy observations of cells stained by Nile Red and Syto 9.  
Legend: a.: *Halomonas* sp. SF2003, a.1 Detection of Syto9 staining, a.2 Detection of Nile Red staining, a.3 Simultaneous detection of Syto9 and Nile Red stainings, b. *C. necator* H16 PHB<sup>-4</sup> *phaC2* simultaneous detection of Syto9 and Nile Red stainings, c. *C. necator* H16 PHB<sup>-4</sup> *phaC1* simultaneous detection of Syto9 and Nile Red stainings and d. *C. necator* H16 PHB<sup>-4</sup> *phaC2* simultaneous detection of Syto9 and Nile Red stainings.

#### I.2.1.1. PHA Production using fructose

For *Halomonas* sp. SF2003, red fluorescence increases from 0h to 72h (**Figures 3a to 3f**) with a maximal intensity at 72h (**Figure 3f**). Red fluorescence (Nile Red) appears between 24h and 36h (**Figures 3c and 3d**), suggesting a beginning of PHA production and accumulation step around these times. Coverage percent of the slides are in accordance with these observations and show a maximal Nile Red/Syto9 ratio reached at 72h (4.90) (**Table 3**). Data of PHA production kinetics are in accordance with bacterial growth monitoring which demonstrates an increase of Log(OD<sub>590nm</sub>) values until 12h and a stabilization around 0.80 even if fluctuations are recorded from 12h to the end of the kinetic (**Table 3**). These results show a PHA production step starting at around 12h with a maximum reached at 72h. CLSM observations show cell cluster organization (**Figure 3d to 3f**). Similar observations were observed during sample preparation step, even after several resuspension steps. It must be notified that this cell organization could partially disturb image processing and distort fluorescence values.

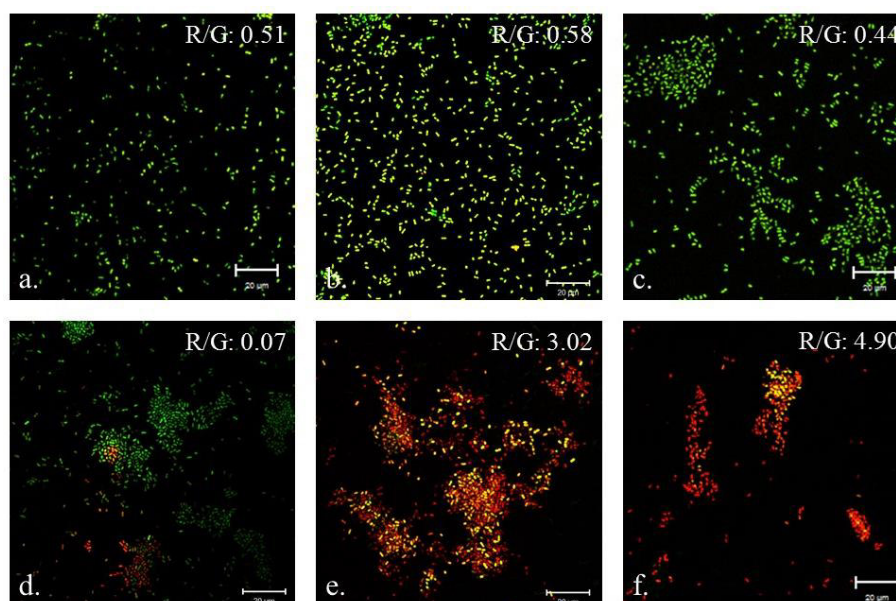


Figure 3: Confocal Laser Scanning Microscope observations of PHA production kinetic from *Halomonas* sp. SF2003 using fructose as the main carbon source.

Legend: a. 0h, b. 12h, c. 24h, d. 36h, e. 60h and f. 72h.

Table 3: Coverage percent of slides and Nile Red/Syto9 ratio of PHA production kinetic with *Halomonas* sp. SF2003 using fructose as the main carbon source.

\*: Highest Nile Red/Syto9 ratio value.

Time (h)	Log(OD <sub>590nm</sub> )	Cells (Syto 9) (%)	PHA (Nile Red) (%)	PHA/Cell
0	0.25	2.31	1.17	0.51
12	1.06	6.59	3.85	0.58
24	0.81	1.62	0.72	0.44
36	0.81	0.59	0.04	0.07
48	0.78	0.81	0.76	0.93
60	0.85	2.89	8.74	3.02
72	0.78	0.51	2.52	<b>4.90*</b>

Same monitorings have been conducted with *C. necator* H16, *C. necator* H16 PHB<sup>-4</sup> *phaC1* and *C. necator* H16 PHB<sup>-4</sup> *phaC2*. Results expose different trends with important variations. For *C. necator* H16, fluorescences from both staining appear from 24h and are detected until the end, 72h (**Figures 4a to 4f**). The maximal Nile Red/Syto9 ratio value (0.36) is recorded after 24h, before a stabilization around 0.25-0.30 until 72h (**Table 4**). These results suggest a beginning and maximum of PHA production starting from 24h. Results of PHA production kinetics are in accordance with bacterial growth kinetics which exposes an increase of Log(OD<sub>590nm</sub>) values from 0h to 24h, before stabilization around 1.20-1.30 until 72h (**Table 4**).

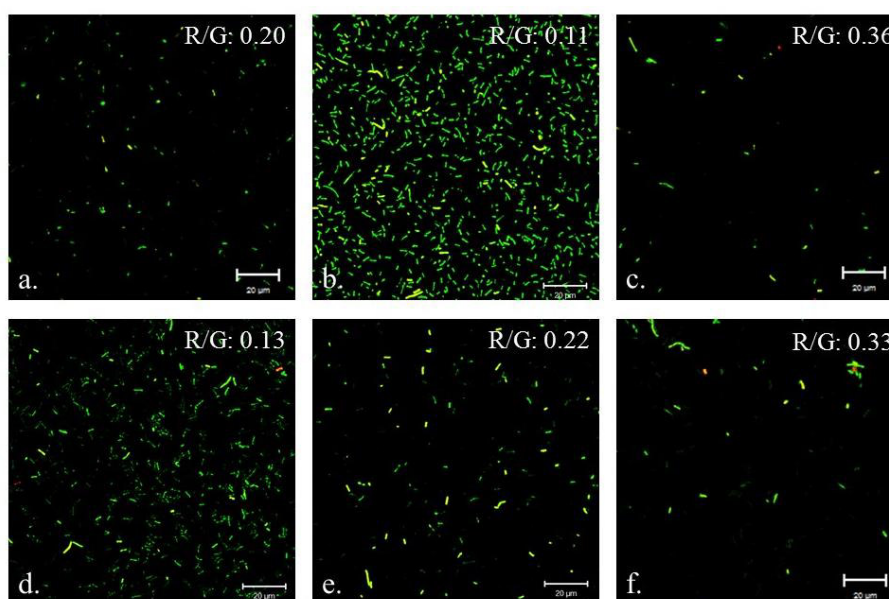


Figure 4: Confocal Laser Scanning Microscope observations of kinetics tests with *C. necator* H16 using fructose as the main carbon source.

Legend: a. 0h, b. 12h, c. 24h, d. 36h, e. 60h and f. 72h.

Table 4: Coverage percent of slides and Nile Red/Syto9 ratio of PHA production kinetic with *C. necator* H16 using fructose as the main carbon source.

\*: Highest Nile Red/Syto9 ratio value.

Time (h)	Log(OD <sub>590nm</sub> )	Cells (Syto 9) (%)	PHA (Nile Red) (%)	PHA/Cell
0	0.29	0.54	0.11	0.20
12	0.99	8.04	0.87	0.11
24	1.08	0.32	0.12	<b>0.36*</b>
36	1.18	1.16	0.15	0.13
48	1.16	0.14	0.04	0.27
60	1.27	1.09	0.24	0.22
72	1.22	0.30	0.10	0.33

Results of *C. necator* H16 PHB<sup>-</sup> *phaC1* are quite similar to those of *C. necator* H16 with detection of yellow fluorescence (Syto9 and Nile Red fluorescences) starting from from 24h (**Figures 5a to 5f**). Then, red fluorescence (Nile Red) is more intensively observed starting from 60h (**Figure 5e**) suggesting a beginning of PHA production at around 24h. Similarly, Nile Red/Syto9 ratio reaches its maximum (0.41) at 60h (**Table 5**), demonstrating a higher PHA production at this time. Bacterial growth kinetic confirms beginning of PHA production at around 24h. Indeed, Log(OD<sub>590nm</sub>) values are stabilized, around 0.30, from 24h to the end (**Table 5**) demonstrating that the strain reaches stationary growth phase from this time.



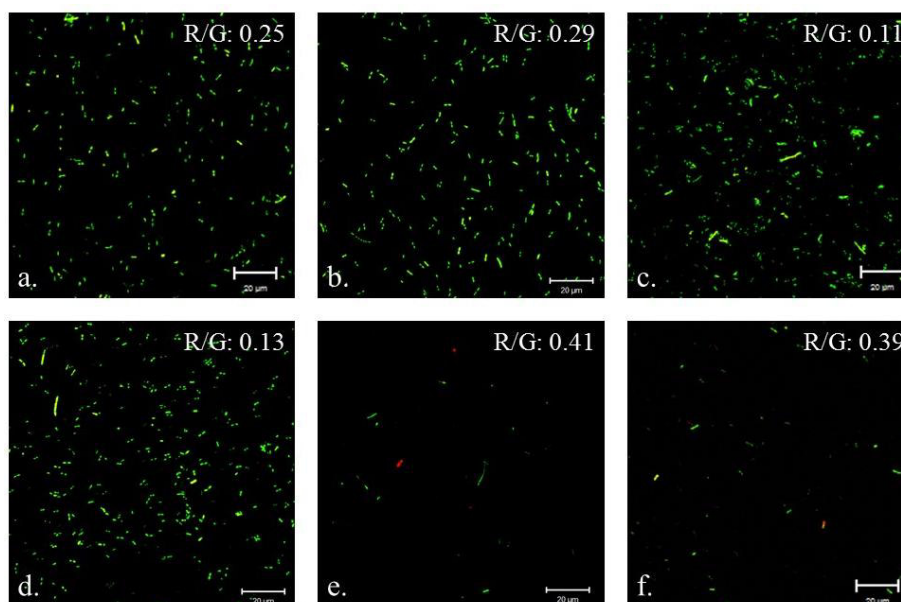


Figure 5: Confocal Laser Scanning Microscope observations of kinetics tests with *C. necator* H16 PHB<sup>-4</sup> *phaC1* using fructose as the main carbon source.

Legend: a. 0h, b. 12h, c. 24h, d. 36h, e. 60h and f. 72h.

Table 5: Coverage percent of slides and Nile Red/Syto9 ratio of PHA production kinetic with *C. necator* H16 PHB<sup>-4</sup> *phaC1* using fructose as the main carbon source.

\*: Highest Nile Red/Syto9 ratio value.

Time (h)	Log(OD <sub>590nm</sub> )	Cells (Syto 9) (%)	PHA (Nile Red) (%)	PHA/Cell
0	0.00	1.45	0.36	0.25
12	0.00	1.83	0.53	0.29
24	0.30	1.62	0.18	0.11
36	0.18	1.72	0.23	0.13
48	0.00	4.65	1.17	0.25
60	0.30	0.10	0.04	<b>0.41*</b>
72	0.30	0.25	0.10	0.39

CLSM observations obtained with *C. necator* H16 PHB<sup>-4</sup> *phaC2* using fructose show a yellow fluorescence already at the beginning of the monitoring until the end (72h) (**Figures 6a to 6f**). Then, starting from 24h, Nile Red fluorescence appears more intense (**Figure 6c**) until the end of the monitoring. At the same time, Nile Red/Syto9 ratio values expose a maximum of 1.08 and Log(OD<sub>590nm</sub>) values show a stabilization of bacterial growth at 36h. These data suggest a beginning of PHA production from 0h with a maximum reached at 36h (**Table 6**).

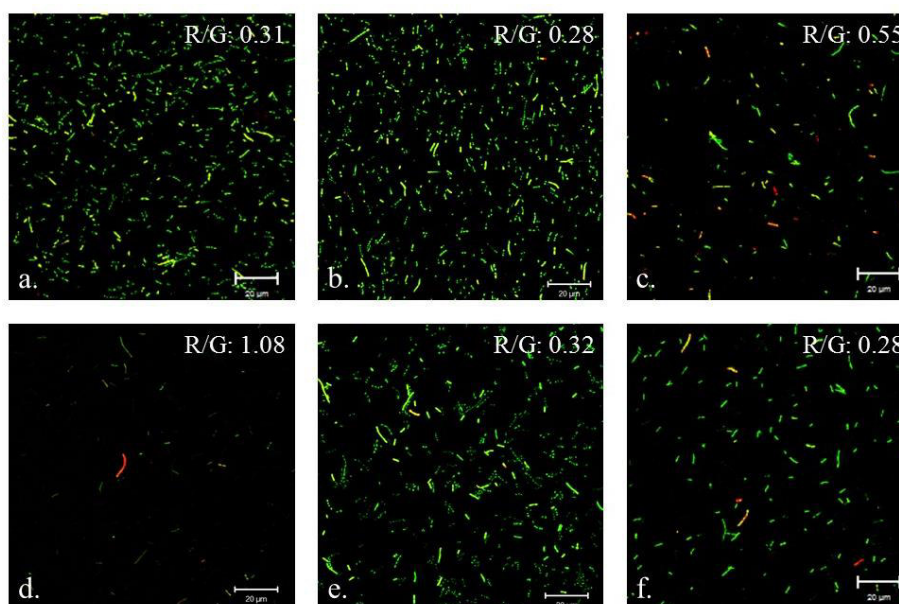


Figure 6: Confocal Laser Scanning Microscope observations of kinetics tests with *C. necator* H16 PHB<sup>-4</sup> phaC2 using fructose as the main carbon source.

Legend: a. 0h, b. 12h, c. 24h, d. 36h, e. 60h and f. 72h.

Table 6: Coverage percent of slides and Nile Red/Syto9 ratio of PHA production kinetic with *C. necator* H16 PHB<sup>-4</sup> phaC2 using fructose as the main carbon source.

\*: Highest Nile Red/Syto9 ratio value.

Time (h)	Log(OD <sub>590nm</sub> )	Cells (Syto 9) (%)	PHA (Nile Red) (%)	PHA/Cell
0	0.28	3.98	1.23	0.31
12	0.58	3.16	0.89	0.28
24	0.95	1.04	0.56	0.55
36	1.11	0.14	0.15	<b>1.08*</b>
48	1.11	0.27	0.13	0.47
60	1.23	1.72	0.55	0.32
72	1.20	1.66	0.47	0.28

Differently to CLSM observations of *Halomonas* sp. SF2003, cell cluster organizations are not detected. However, *C.necator* wild type and transformant strain cells look like to change their morphology by increasing their length (**Figures 6c to 6f, 7c to 7e and 8a to 8f**).

#### I.2.1.2. Production using glucose

Similar monitorings have been conducted using production medium complemented with glucose. For *Halomonas* sp. SF2003 yellow (Syto9 and Nile Red fluorescences) and red (Nile Red) fluorescences are already detected on CLSM observations from 12h (**Figures 7a to 7f**). Then, a steady increase of Nile Red fluorescence is observed from 24h to 60h before a decrease at 72h (**Figures 7c to 7f**). Same observations can be done exploiting Nile Red/Syto9 ratio values. Indeed, an improvement is observed from 0h to a maximum of 4.85 at 36h (**Table 7**) in accordance with bacterial growth results which show an increase of Log(OD<sub>590nm</sub>) values from 0h to 24h followed by a stabilization (**Table 7**).

These data suggest a beginning of PHA production by the strain from 0h with a reinforcement from 24h and a maximum reached at around 36h, once the stationary growth phase is reached. Then, the ratio decreases and at 72h yellow-orange fluorescences are again observed (**Figure 7f**).

Similarly to monitoring performed with fructose, cell clusters are observed from 24h to 72h (**Figure 7b** to **7f**). This cells organization could also be responsible of an image processing disturbed/distorted resulting in an artefactual rise of fluorescences at 72h. Comparatively to production tests using fructose, *Halomonas* sp. SF2003 begins its PHA biosynthesis at around 12h with glucose, based on CLSM observations (**Figure 7b**).

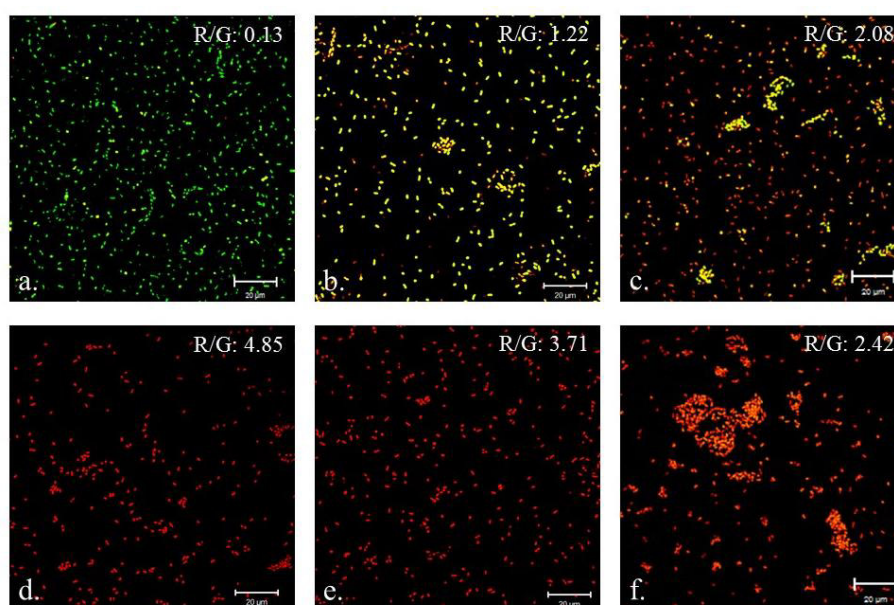


Figure 7: Confocal Laser Scanning Microscope observations of kinetics tests with *Halomonas* sp. SF2003 using glucose as the main carbon source.

Legend: a. 0h, b. 12h, c. 24h, d. 36h, e. 60h and f. 72h.

Table 7: Coverage percent of slides and Nile Red/Syto9 ratio of PHA production kinetic with *Halomonas* sp. SF2003 using glucose as the main carbon source.

\*: Highest Nile Red/Syto9 ratio value.

Time (h)	Log(OD <sub>590nm</sub> )	Cells (Syto 9) (%)	PHA (Nile Red) (%)	PHA/Cell
0	0.30	3.83	0.48	0.13
12	0.81	3.11	3.79	1.22
24	1.10	2.33	4.86	2.08
36	1.10	0.51	2.45	<b>4.85*</b>
48	1.06	1.77	3.36	1.89
60	1.10	0.81	3.01	3.71
72	1.06	2.58	6.25	2.42

With *C. necator* H16, red fluorescence (Nile Red) is clearly detected from 60h (**Figures 8a** to **8f**). Maximal Nile Red/Syto9 ratio, 0.28, is recorded at 72h, but all ratios are very similar during the monitoring. These results show a beginning of PHA production between 24h and 36h (based on CLSM

observations **Figures 8c and 8d**), at the same time as stationary growth phase of the strain (**Table 8**), and highest PHA production around 60h.

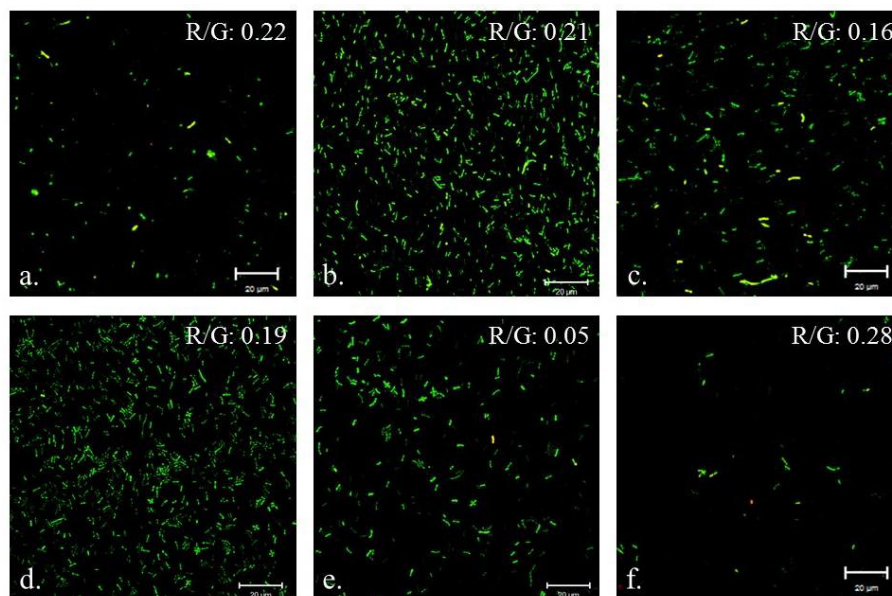


Figure 8: Confocal Laser Scanning Microscope observations of kinetics tests with *C. necator* H16 using glucose as the main carbon source.

Legend: a. 0h, b. 12h, c. 24h, d. 36h, e. 60h and f. 72h.

Table 8: Coverage percent of slides and Nile Red/Syto9 ratio of PHA production kinetic with *C. necator* H16 using glucose as the main carbon source.

\*: Highest Nile Red/Syto9 ratio value.

Time (h)	Log(OD <sub>590nm</sub> )	Cells (Syto 9) (%)	PHA (Nile Red) (%)	PHA/Cell
0	0.30	0.77	0.17	0.22
12	0.93	4.12	0.87	0.21
24	1.09	1.89	0.29	0.16
36	1.16	3.19	0.60	0.19
48	1.19	0.13	0.03	0.26
60	1.22	1.98	0.11	0.05
72	1.18	0.28	0.08	<b>0.28*</b>

Yellow fluorescence (Syto9 and Nile Red fluorescences) is detected from 0h for *C. necator* H16 PHB<sup>-4</sup> *phaC1*, but red fluorescence is only clearly observed at 60h (**Figures 9a to 9f**). Nile red/Syto9 ratio values expose the same trends with maximal values, 0.30 and 0.38, recorded at 60h and 72h, respectively (**Table 9**). Similarly to fructose, *C. necator* H16 PHB<sup>-4</sup> *phaC1* growth using glucose exposes the smallest values with a maximal Log(OD<sub>590nm</sub>) of 0.48 at 72h.

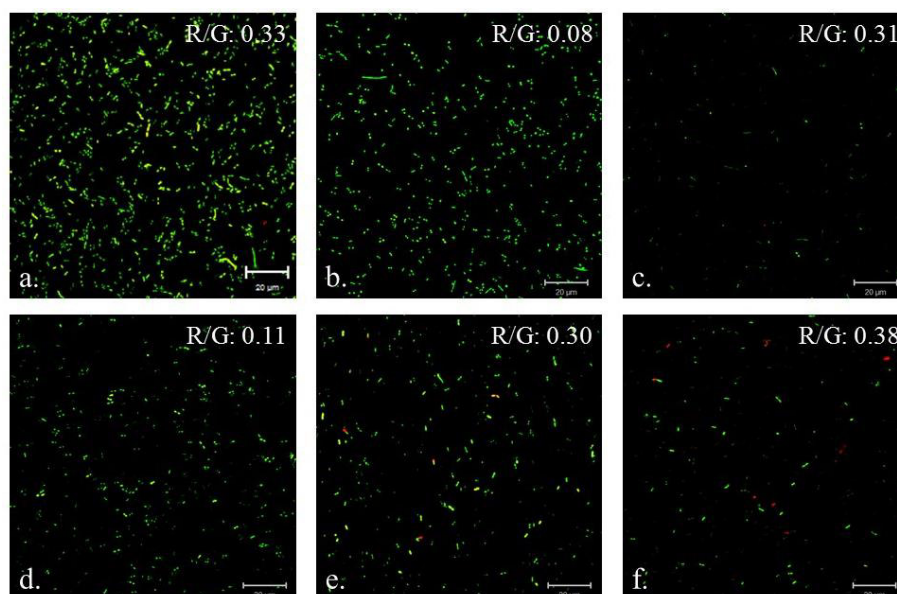


Figure 9: Confocal Laser Scanning Microscope observations of kinetics tests with *C. necator* H16 PHB<sup>-4</sup> *phaC1* using glucose as the main carbon source.

Legend: a. 0h, b. 12h, c. 24h, d. 36h, e. 60h and f. 72h.

Table 9: Coverage percent of slides and Nile Red/Syto9 ratio of PHA production kinetic with *C. necator* H16 PHB<sup>-4</sup> *phaC1* using glucose as the main carbon source.

\*: Highest Nile Red/Syto9 ratio value.

Time (h)	Log(OD <sub>590nm</sub> )	Cells (Syto 9) (%)	PHA (Nile Red) (%)	PHA/Cell
0	0.08	5.25	1.74	0.33
12	0.18	2.41	0.19	0.08
24	0.18	0.19	0.06	0.31
36	0.18	1.04	0.11	0.11
48	0.18	4.08	0.24	0.06
60	0.48	1.05	0.31	0.30
72	0.48	0.42	0.16	<b>0.38*</b>

Finally, data recorded with *C. necator* H16 PHB<sup>-4</sup> *phaC2* show a yellow fluorescence (Syto9 and Nile Red fluorescences) already from 0h and a red fluorescence from 12h. Then, both fluorescences increase with a reinforcement of Nile Red fluorescence from 24h (**Figures 10a to 10f**). Maximal Nile Red/Syto9 ratio value (0.81) is recorded at 60h but an increase is already recorded from 36h (**Table 10**). Similarly, bacterial growth is stabilized in stationary phase from 36h with Log(OD<sub>590nm</sub>) values around 1.00 (**Table 10**) demonstrating beginning of PHA production around 36h.

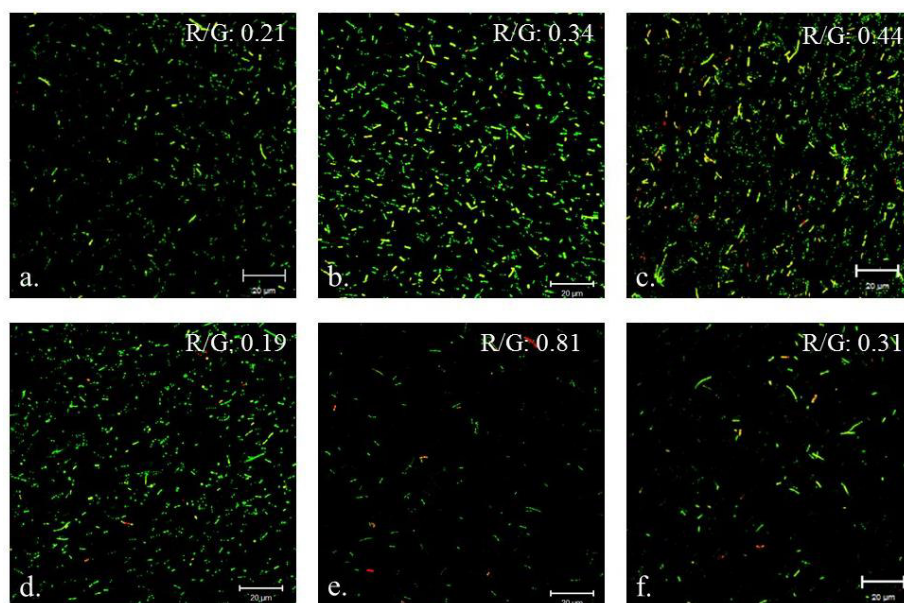


Figure 10: Confocal Laser Scanning Microscope observations of kinetics tests with *C. necator* H16 PHB<sup>-4</sup> *phaC2* using glucose as the main carbon source.

Legend: a. 0h, b. 12h, c. 24h, d. 36h, e. 60h and f. 72h.

Table 10: Coverage percent of slides and Nile Red/Syto9 ratio of PHA production kinetic with *C. necator* H16 PHB<sup>-4</sup> *phaC2* using fructose as the main carbon source.

\*: Highest Nile Red/Syto9 ratio value.

Time (h)	Log(OD <sub>590nm</sub> )	Cells (Syto 9) (%)	PHA (Nile Red) (%)	PHA/Cell
0	0.28	2.27	0.47	0.21
12	0.78	6.47	2.19	0.34
24	0.78	8.21	3.63	0.44
36	0.98	4.87	0.91	0.19
48	1.00	0.50	0.22	0.44
60	1.06	0.10	0.08	<b>0.81*</b>
72	1.04	0.90	0.28	0.31

Observations performed with CLSM with PHA production kinetics using glucose also show a change of bacteria cell length for *C. necator* wild type and transformant strains (**Figure 8a to 8f, 9a to 9f and 10a to 10f**).

### I.2.2.PHA production

To complete monitoring tests, PHA production were estimated at the end of PHA production kinetics, after 72h of production. Using fructose, the highest DCW and PHA concentration were recorded for *C. necator* H16 PHB<sup>-4</sup> *phaC2* and *C. necator* H16 (DCW: 3.16 g/L and 3.42 g/L and PHA concentrations: 2.25 g/L and 1.83 g/L). PHA content observed suggests that productivity of *C. necator* H16 PHB<sup>-4</sup> *phaC2* is limited. Indeed, even if the strain exposes the second highest PHA concentration, its PHA content (34%) is quite similar to those of *Halomonas* sp. SF2003 and *C. necator* PHB<sup>-4</sup> *phaC1* with 39% and 33%, respectively (**Table 11**). Finally, despite its capacity to produce PHA, *C. necator*

H16 PHB<sup>-4</sup> *phaC1* exposes the lowest results for DCW, PHA concentration and content 0.79 g/L of DCW, 0.26 g/L of PHA and 33% of PHA content.

Table 11: Comparative PHA productions of kinetic monitoring using fructose and glucose.

Strain	Carbon source	Dry cell weight (g/L)	PHA (g/L)	PHA content (%)
<i>Halomonas</i> sp. SF2003	Fructose	2.63	1.02	39
<i>C. necator</i> H16		3.16	2.25	71
<i>C. necator</i> H16 PHB <sup>-4</sup> <i>phaC1</i>		0.79	0.26	33
<i>C. necator</i> H16 PHB <sup>-4</sup> <i>phaC2</i>		3.42	1.83	34
<i>Halomonas</i> sp. SF2003		2.63	2.25	86
<i>C. necator</i> H16	Glucose	2.89	2.05	71
<i>C. necator</i> H16 PHB <sup>-4</sup> <i>phaC1</i>		1.05	0.32	30
<i>C. necator</i> H16 PHB <sup>-4</sup> <i>phaC2</i>		2.63	1.38	52

Comparatively, for PHA production tests with glucose highest values were recorded with *Halomonas* sp. SF2003 and *C. necator* H16. For both strains dry cell weight of 2.63 and 2.89 g/L, PHA concentrations of 2.25 and 2.05 g/L and PHA contents of 86 and 71% are recorded, respectively (Table 11). Production with *C. necator* H16 PHB<sup>-4</sup> *phaC2* allows reaching similar DCW (2.63 g/L) than those of *Halomonas* sp. SF2003. However, PHA concentration (1.38 g/L) and content (52%) are lower (Table 11). These results demonstrate the ability of transformant strain *C. necator* H16 PHB<sup>-4</sup> *phaC2* to produce PHA and activity of PHA synthase PhaC2. Similarly to the production tests performed with fructose, here, *C. necator* H16 PHB<sup>-4</sup> *phaC1* still exposes lowest results for DCW (1.05 g/L), PHA concentration (0.38 g/L) and content (30%).

For *Halomonas* sp. SF2003 and *C. necator* H16, results of PHA production tests using both fructose and glucose are in accordance with Nile Red/Syto9 ratio obtained with the fluorescence based monitoring method. Indeed, results expose highest Nile Red/Syto9 values and PHA production/content values for *C. necator* H16 using fructose while for *Halomonas* sp. SF2003 highest values are recorded when glucose is employed. For transformant strains, results of both extraction and fluorescence monitoring do not expose same trends. However, based on Nile Red/Syto9 ratio values and CLSM observations, it appears that the quantities of PHA decreased at 72h, potentially caused by the polymer use or degradation, meaning that extractions performed at this time does not exactly represent the “true” productivity of the strains. These results demonstrate that PHA metabolism of each strain is different and suggest to perform extraction at different time, depending on the strain, in order to avoid PHA degradation and to obtain the highest quantities of polymer. Like previously described with screening tests (See Chapter IV), transformant strains *C. necator* H16 PHB<sup>-4</sup> *phaC1* and *C. necator* H16 PHB<sup>-4</sup> *phaC2* can produce PHA but less effectively than wild type strains *Halomonas* sp. SF2003 or *C. necator* H16.

## Discussion

The impact of medium composition on *Halomonas* sp. SF2003 growth and PHA production has been investigated. Study of sugar use by *Halomonas* sp. SF2003 allowed to frame optimal growing and stationary phases depending on each used carbohydrates. Recorded data demonstrated a quite similar assimilation of fructose, galactose, glucose, maltose, mannose and sucrose by the strain. However, *Halomonas* sp. SF2003 seems to assimilate maltose, fructose and mannose faster than the other carbohydrates. Lowest generation times (1.5 h) are also recorded with these sugars and highest value is obtained with glucose (2.0 h). At the same time, highest OD<sub>590nm</sub> values are not observed with maltose and fructose but with sucrose (7.35) and galactose (7.00) suggesting a higher cell, cellular debris or bacterial compounds concentration in culture supernatant. An *in silico* study could be run to predict potential production of extracellular compounds of interest, and supernatant composition could also be determined to confirm synthesis of additional compounds of interest by *Halomonas* sp. SF2003. Bacterial growth kinetics showed that stationary phase is reached for all tested carbohydrates at around 7-8h, meaning that biomass accumulation step must be stopped before this time. Indeed, in order to preserve the highest viable/active cells concentration and for an optimal beginning of PHA production step, biomass accumulation phase must be stopped before stationary phase. Exploiting these results, designing and testing of production system could be performed in order to optimize *Halomonas* sp. SF2003 PHA production. Previous studies have also reported PHA productions employing fructose, galactose, glucose, mannose and sucrose with *Halomonas* species. Indeed, Pernicova *et al.*, have compared the use of various carbohydrates with *H. halophila*, *H. organivorans* and *H. salina*. Results highlighted a stronger productivity of *H. halophila* (4.85 g/L of PHA) and *H. organivorans* (3.87 g/L) using sucrose and mannose, respectively <sup>11</sup>. Results of this work coupled with data of the literature demonstrated that an accurate study of bacteria and carbon sources combination participates to optimally manage and improve PHA production.

At the same time, influence of salinity on *Halomonas* sp. SF2003 PHA production was investigated. Indeed, salinity is an external parameter which can stimulate PHA production and consequently different studies are conducting in order to evaluate the impact of salt concentration on PHA synthesis <sup>20,21</sup>. Production tests were conducted with *Halomonas* sp. SF2003 testing different salinities. Data from previous work coupled to results obtained in this work here demonstrated that high salt concentration in medium does not significantly influence bacterial growth but seems to impact PHA production. Indeed, *Halomonas* sp. SF2003 can grow at various salt concentrations ranging from 5g/L to 200 g/L <sup>8</sup>. However, comparison of PHA production yields demonstrates a highest productivity for salinity of 30 g/L. These results are in accordance with data of other studies performed with different PHA producing strains such as *Bacillus megaterium* <sup>12</sup>, *Halomonas hydrothermalis*, *H. neptunia* <sup>13</sup>, *H. organivorans* <sup>11</sup> or *Haloferax mediterranei* <sup>14</sup>. Actually, results of these different studies demonstrate that salinity differently influences strain growth and PHA production. Results report that a too important



salt concentration can inhibit PHA production <sup>11,21</sup>. To deeply study and understand the impact of salinity on *Halomonas* sp. SF2003 metabolisms, additional experiments like production tests or transcriptomic analysis could be run using different salt concentrations. Coupling all data will allow a better characterization and management of *Halomonas* sp. SF2003 PHA production.

A coupling method, based OD<sub>590nm</sub> monitoring and CLSM observations, was developed for monitoring and studying PHA production kinetics in real time. Bacteria cells have been stained by Syto9 and PHA granules using Nile Red. The use of fluorescent stains to observe and study PHA granules with different species is frequently reported in literature. Among the different usable markers, Nile Red allows to obtain good observations of PHA granules and consistent results <sup>15,22,23</sup>. Usually these techniques are mostly exploited to study PHA granules location in cell cytoplasm even if today, they are also used to elucidate granules formation mechanisms or production kinetics. Recently, Mravec *et al.*, have developed a confocal fluorescence microscopy analysis with *C. necator* H16, using DiD® fluorescent probe and Nile Red <sup>21</sup>.

Results obtained for production tests using both fructose or glucose as main carbon source expose different trends and important fluctuations depending on bacterial strains tested. However, recorded data allow the identification of the beginning of PHA production as well as stabilization and/or fall of cellular density (**Table 12**). Consequently, to increase production yield and limit decrease of viable cells concentration, PHA production process could be designed more wisely. Moreover, adjustment of used production method could allow to improve quantities of produced PHA. Indeed, it could be conceived to start harvesting as soon as PHA production begins or to change batch process to fed-batch in order to extend production phase and maintain synthesis activity of cells.

Table 12: Summary of highest Nile Red / Syto9 ratio values and PHA production obtained from fluorescence based monitoring method.

\*: PHA production after 72h of production step

Strain	Fructose		Glucose	
	Highest Nile Red /Syto9 ratio	PHA (g/L)*	Highest Nile Red /Syto9 ratio	PHA (g/L)*
<i>Halomonas</i> sp. SF2003	4.90 (72h)	1.02	4.85 (36h)	2.25
<i>C. necator</i> H16	0.36 (24h)	2.25	0.28 (72h)	2.05
<i>C. necator</i> H16 PHB <sup>-4</sup> <i>phaC1</i>	0.41 (60h)	0.26	0.38 (72h)	0.32
<i>C. necator</i> H16 PHB <sup>-4</sup> <i>phaC2</i>	1.08 (36h)	1.83	0.81 (60h)	1.38

To complete results and for a better management of PHA synthesis mechanism by each strain, polymer extraction and production analysis could be performed simultaneously to kinetics monitoring. By coupling all data, the method developed in this work could allow a better understanding and control of production process for each bacterial strain, carbon substrates and process combination selected.

Additionally, important variations in results have been recorded with *C. necator* wild type and transformant strains at the same time as changes of cell morphology. During monitoring Syto 9 fluorescence intensity results does not follow the same tendency as compared to results of OD<sub>590nm</sub>. Some results expose stabilization or small decrease of cellular density/OD<sub>590nm</sub> while at the same time Syto 9 fluorescence seems to increase or to be stabilized. Simultaneously, the morphology of *C. necator*'s cells changes with an increase of their length. Previous study using confocal laser scanning microscope has highlighted an increase of cell length at the same time as volume fraction of PHA granules under nutrient limitation<sup>18</sup>. Modifications of cell morphology and/or organization have also been reported and described during PHA biosynthesis in other studies<sup>18,19,24,25</sup>. Morphology changes detected on CLSM observations are in accordance with these data and could, partially, be responsible for important variations recorded with wild type and transformant strains of *C. necator*. In comparison, results recorded for *Halomonas* sp. SF2003 show less variations at the same time as preservation of cells morphology. However, cell cluster organization is detected. Based on literature and CLSM observations, it is possible that the increase of cell length interferes with cell counting and fluorescence measurements meaning that fluorescence recorded does not really reflects evolution of cellular density and PHA production. Finally, use of Nile Red stain leads to pink PHA films production. To limit this repercussion extraction and careful purification process must be developed.

Results obtained with this monitoring method suggest that improvements of data processing software are required in order to take in consideration potential cell's morphology evolutions. However, this method represents an interesting additional/alternative quantitative tool allowing a faster monitoring and understanding of PHA production in real time.

## Conclusion

This work has demonstrated the influence of various parameters, such as carbon sources or salinity, on *Halomonas* sp. SF2003 growth and PHA production. Results of PHA production (depending on used carbon sources demonstrate a higher production employing glucose. At the same time, study of salinity has highlighted an increase in production using salt concentration of 30 g/L rather than 11 or 50 g/L, confirming the crucial role of both these parameters on halophile strain's metabolisms. Results obtained in this part allow designing of different biosynthesis systems and open the door to new assays to compare strain's productivity depending on used carbohydrates used. PHA production monitoring method, based on CLSM observation and bacterial growth, began to be developed and tested. Even if improvements are required, it represents an interesting and rapid tool for studying and monitoring the PHA production.

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# General conclusion and perspectives

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The work presented in this study investigates PHA synthesis by the marine bacterium *Halomonas* sp. SF2003. The bacterial strain characterization, using biotechnological tools, participates to understand, manage and optimize PHA production.

Sequencing and exploitation of *Halomonas* sp. SF2003 genome allowed the identification of genes and metabolisms related to strain versatility in front of various growth conditions. Bioinformatic analysis demonstrates the presence of genes coding for various stress-related proteins that are probably involved in strain resistance against temperature, salinity or toxic compounds. Analysis of carbohydrates metabolism highlights presence of numerous genes suggesting the strain capability to employ and convert various carbon substrates into PHA. In addition, *in silico* analysis of PHA metabolisms identifies several key genes of PHA biosynthesis exposing atypical organization and characteristics. At the same time, a close connection between *Halomonas* sp. SF2003 PHA and fatty acid metabolisms has been highlighted, demonstrating all the complexity of strain metabolism. Bioinformatic study, coupled with phenotypic tests, allows framing of production conditions to test in order to optimize PHA synthesis.

PHA production capacity of the strain depending on used carbon substrates has been evaluated. Screening tests confirmed results of bioinformatic analysis and demonstrated capacity of *Halomonas* sp. SF2003 to use various pure or mix carbohydrates for PHA synthesis. Production of transformant strains *C. necator* H16 PHB<sup>4</sup> *phaC1* and *C. necator* H16 PHB<sup>4</sup> *phaC2*, combined with production tests, proved the synthesis activity of both PHA synthase PhaC1 and PhaC2, and confirmed results of bioinformatic analysis. Results also showed a preferential employment of glucose by *Halomonas* sp. SF2003 for PHA production. However, polymer has also been obtained with fructose and galactose. In comparison *C. necator* wild type and transformant strains prefer fructose, rather than glucose. At the same time, *in silico* study attests of difference between both synthases demonstrating the necessity to go further into genetic work of *Halomonas* sp. SF2003 PHA metabolism.

Adaptative character of the strain was assessed confronting influence of various growth and production conditions. Even if the strain exposes a versatility of great interest for future industrial exploitation, results also highlight the importance of a perfect knowledge of growth and PHA production used with this bacterium in order to optimize at its maximum its biotechnological potential. Production kinetics have been investigated by the development of a PHA production fluorescence based monitoring method in real time. Study of kinetics shows difference of PHA biosynthesis depending on tested carbon substrates and allows the optimization of the bioprocess. First assays of PHA production demonstrate easy and fast implementation of the designed monitoring method, giving interesting and favorable results, encouraging us to persist the technic development in order to improve it.

All results obtained in this study confirm the biotechnological potential of *Halomonas* sp. SF2003 and can also be used for designing new production bioprocess with this strains. Combined data obtained from genome analysis and production tests open the door to a variety of perspectives in order to optimize *Halomonas* sp. SF2003 PHA production.

Because of its atypical organization in addition to an incomplete identification of genes related to *Halomonas* sp. SF2003 PHA metabolism, investigations must continue. Re-examination of strain's genome could allow (i) to identify new genes directly, or not, linked to PHA metabolism and (ii) to deeply elucidate its organization/functioning. Moreover, because all genes already identified are far from each other on genome sequence, their transcription should be dependent from different promoters which are activated by different stimuli. The identification of each promoter could participate to find activation signals and use them to promote or inhibit the promoter activity. They will also help to identify factors activating PHA biosynthesis. The production and testing of new different transformant strains, harbouring different constructions of PHA biosynthesis operon, might also participate to elucidate influence of each identified genes (*phaA*, *phaB*, *phaC1*, *phaC2*, *phaR*, ...) and operon organization on PHA production. In the long term, production of new transformant strains could allow obtention of PHA overproduction by *Halomonas* sp. SF2003 or by other bacterial host. Actually, analysis of *Halomonas* sp. SF2003 genome highlights the presence of genes related to various metabolism and in agreement with versatility of the strain. Indeed, adaptability of *Halomonas* sp. SF2003 in front of carbon sources and/or stressfull conditions is, clearly, an attractive feature for its biotechnological exploitation. Capacity to grow at different temperature or salinity, and in the presence of toxic compounds, using various carbohydrates enable to imagine its use in harsh environment (i.e., cold/hot, hypersaline or polluted). Results of genome analysis, screening and PHA production tests identify different carbon substrates wich can be encountered in various (by-)products. From these knowledges, and starting from observation that carbon substrates represent up to 50-60% of overall production cost, suggest to evaluate viability of the employment of such "cheap" and sustainable carbon feedstocks for PHA production. Among tested carbon substrates exposing positive results for PHA production, several are found in various (by-)products such as lactose sources (lactose or cheese whey and milk), red algae, spent coffee ground, sugar maple hemicellulosic hydrolysate, ensiled grass press juice, paper industry co-products or cooking waste, opening the field to a large number of new productions possibilities. Actually, owing to the strain's robustness, many industrial products can be considered as potential carbon substrates. Some previous experiments, conducted in other projects (data not shown), using *Halomonas* sp. SF2003 demonstrate the possibility to convert wastewaters from agro-industries, as well as oleic and undecenoic acids, into PHA confirming the existence of a close relation between PHA and fatty acid metabolisms. In addition to a potential cost reduction, use of atypical carbon sources could allow to obtain different homopolymers or heteropolymers (exposing different mol% of monomer). Moreover, the use of unusual



carbon substrates with terminal carbon rings, side chains, or rings as side chains must also be considered as a potential way to produce PHA with specific characteristics or to increase production yields.

Impact of salinity on PHA production has been investigated. Results show a highest productivity of the strain using a salt concentration of 30 g/L. To complete the study and for a better characterization of *Halomonas* sp. SF2003, RNA sequencing analysis are engaged to elucidate impact of salinity on proteins expression level.

In the case of this study, productions have only been performed by a two-step batch process. Based on results obtained from screening, production and monitoring tests ajustement of production bioprocess could be considered. Duration of biomass accumulation and PHA production stages might be adapted to reach a maximal biomass quantity and to stop the fermentation course before depolymerization of the PHA stock by the bacteria themselves for carbon reuse. Extension of PHA production stage could also be tested in a fed-batch bioprocess. The fluorescence method developed to monitor PHA production would be a helpful tool for this purpose.

Finally, recovery method is another part of future prospects which could be investigated in order to improve extraction yield and reduce the ecological footprint of the PHA value chain. Currently, PHA extractions are performed using organic solvents, mainly hot chloroform (as in this work). Different other extraction methods might be tested as ultra high pressure or osmotic shock as pre-treatment, once determination of the critical salinity done.

Actually, there are plenty of perspectives, ranging from molecular work to extraction process, which can be suggested to wholly develop biotechnological potential of *Halomonas* sp. SF2003. This wide variety of perspectives demonstrate all the complexity and all the interest to characterize PHA production depending on the employed bioprocess.



# Contributions scientifiques

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## Articles publiés

**Tatiana Thomas**, Alexis Bazire, Anne Elain, Stéphane Bruzard, Complete genome sequence of the halophilic PHA-producing bacterium *Halomonas* sp. SF2003: insights into its biotechnological potential. World J Microbiol Biotechnol (2019) 35: 50.

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## Communication internationale

**Tatiana Thomas**, Alexis Bazire, Anne Elain, Stéphane Bruzard, Development of genetic tools for an optimized production of polyhydroxyalkanoates (PHAs), 9<sup>th</sup> European Symposium on Biopolymers, Toulouse, 2016

**Tatiana Thomas**, Alexis Bazire, Anne Elain, Stéphane Bruzard, Characterization and exploitation of the marine bacterium *Halomonas* sp. SF2003 for optimization of PHA production, 10<sup>th</sup> European Symposium on Biopolymers, Straubing (Allemagne), 2019

## Communication nationale

**Tatiana Thomas**, Alexis Bazire, Anne Elain, Stéphane Bruzard, Characterization and exploitation of the marine bacterium *Halomonas* sp. SF2003 for optimization of PHA production. Poymerix, Rennes (France), 2019

## Development of genetic tools for an optimized production of polyhydroxyalkanoates (PHAs)

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*Halomonas* SF 2003, a marine bacterium isolated from sea of Iroise, is a polyhydroxyalkanoates (PHAs) producer. Productions can reach 1.6 g/L depending in the culture conditions <sup>1,2</sup>. Because of their properties these biopolymers present environmentally and economically interests in order to replace conventional plastics. The aim of our study is to enhance and to control production of biopolymers using genetic tools and especially genetic materials of *Halomonas* SF 2003.

Sequencing of *Halomonas* SF 2003 allowed study and characterization of its genome. The strain has a genome of approximatively 4,3kpb with a GC content of 62,5%. Analysis revealed the presence of resistance genes against bacteriocin, antibiotics (beta-lactams and fluoroquinolons) and toxic compounds (arsenic, mercury and zinc). Genome annotation enabled also the identification of genes involved in PHAs production including *phaA*, *phaB*, *phaZ*, etc. Two polyhydroxyalkanoates synthases were identified and named *phaC1* and *phaC2* with a size of approximatively 2000 pb and 3000 pb, respectively. Previous studies already reported presence of different polyhydroxyalkanoates synthases in different bacterial strains <sup>3,4</sup>. The second polyhydroxyalkanoates synthase identified in *Halomonas* SF 2003 and named *phaC2* presents particularities including its size and its involvement in biochemical pathways of aromatic compounds degradation. Using these data, it is possible to study PHAs production of *Halomonas* SF 2003 and to use its genes in order to enhance production.

To obtain an overproduction of PHAs we have used two strategies: the first one consisted to overproduce PHAs in *Escherichia coli* and the second one to enhance production of PHAs directly in *Halomonas* SF 2003.

For the first part plasmid pGEM<sup>®</sup>AB(*phaC<sub>s</sub>*), furnished by Pr. Kumar SUDESH's team (Universiti of Sains Malaysia, School of Biological Sciences), was used. This plasmid is a high copy number plasmid composed by *phaA* and *phaB* genes from *Cupriavidus necator* and *phaC* gene of *Chromobacterium* sp. USM2 <sup>5</sup>.

In order to enhance production, the plasmid was introduced in *Escherichia coli* and manipulated to replace the *phaC* gene from *Chromobacterium* by *phaC1* and/or *phaC2* genes from *Halomonas* SF 2003.

In order to modify production of PHAs by *Halomonas* SF 2003, pBBRMCS5 plasmid was used. The *phaA*, *phaB* and *phaC* genes were cloned separately or together into pBBRMCS5 and *Halomonas* SF 2003 was transformed. These manipulations allowed us to study influence of each gene for PHAs production by *Halomonas* SF 2003 but also to control its production of PHAs.

1. Elain, A. *et al.* Valorisation of local agro-industrial processing waters as growth media for polyhydroxyalkanoates (PHA) production. *Ind. Crops Prod.* **80**, 1–5 (2016).
2. Elain, A. *et al.* Rapid and qualitative fluorescence-based method for the assessment of PHA production in marine bacteria during batch culture. *World J. Microbiol. Biotechnol.* **31**, 1555–1563 (2015).
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## **Characterization and exploitation of the marine bacterium *Halomonas* sp. SF2003 for optimization of PHA production**

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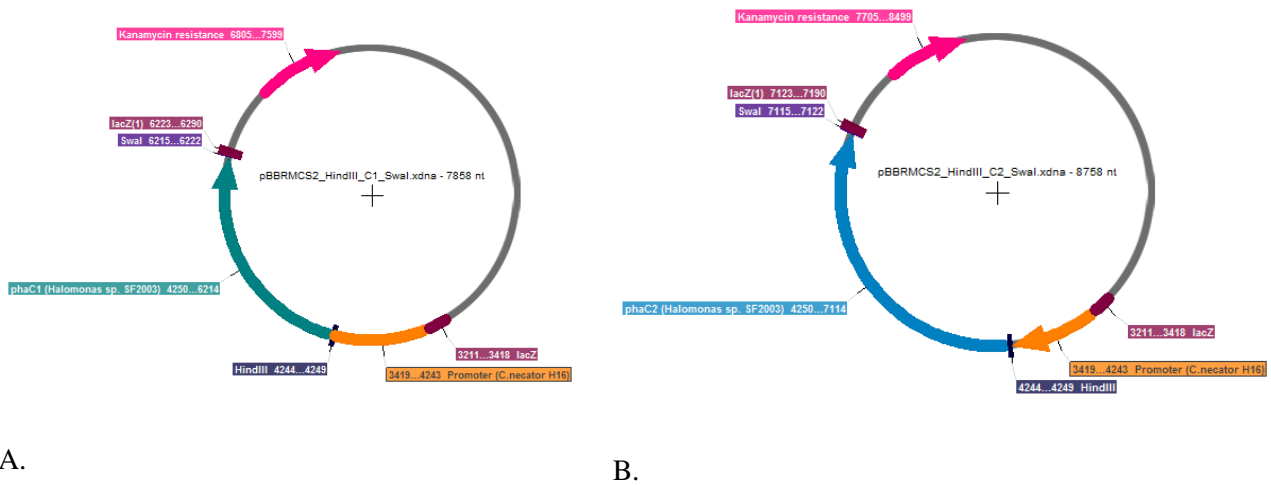
*Halomonas* sp. SF2003 is a halophilic Gram-negative eubacterium isolated from sea of iroise (Brittany, France) which already demonstrated its capacity to produce and accumulate polyhydroxyalkanoates (PHA) up to 78% of the cell dry weight <sup>1,2</sup>. PHA are biocompatible and completely biodegradable bacterial polyesters exposing thermal, mechanical and physicochemical properties close to petroleum based plastics <sup>3,4</sup>. Because of their environmental and economic interests, numerous studies are conducting to identify and exploit new bacteria capable of accumulating these biopolymers <sup>5,6</sup>.

Interestingly, *Halomonas* sp. SF2003 can naturally produce P3-HB, the most studied PHA, and the co-polymer P3HB-3HV, using different carbon substrates even those originate from industrial wastes. This strain also exposes a strong versatility to various temperature and salinity conditions making it a good candidate for a production of PHA at an industrial.

The aim of our work is to study and manage *Halomonas* sp. SF2003 metabolism, using genetic, microbiology and bioprocess tools, in order to enhance and to manage production of PHA.

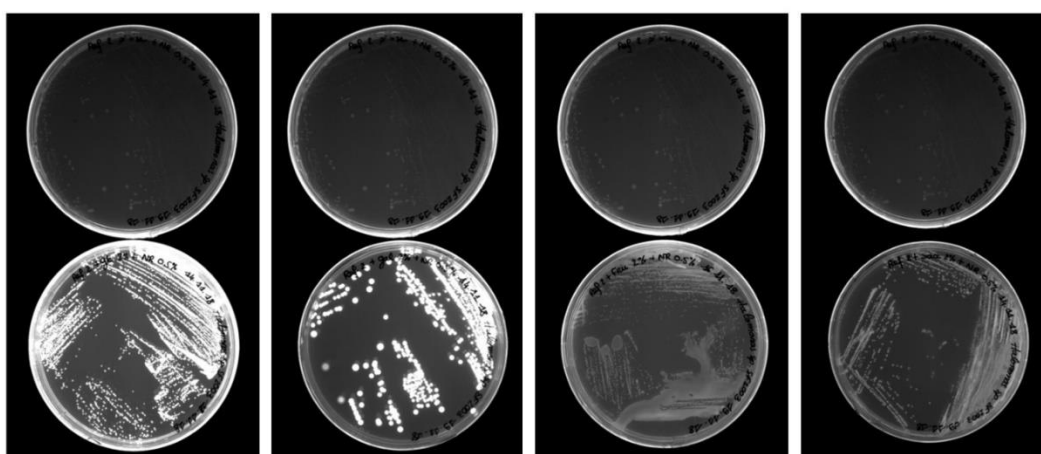
First part of our work consisted to identify and start characterization of the key enzymes involved in the PHA biosynthesis: PHA synthases, and genes belonging to PHA metabolism of *Halomonas* sp. SF2003 using bioinformatics tools and MaGe and NCBI databases. Sequencing and annotation of *Halomonas* sp. SF2003 genome allowed identification of different genes including two potential PHA synthases named *phaC1* and *phaC2* with a size of 1965bp and 2865bp, respectively and distant from each other in the sequence. Because of the atypical organization and size of genes presumably involved in PHA biosynthesis, experiments were conducted to isolate and study them. Thus, *phaC1* and *phaC2* genes were individually cloned in a plasmid pBBRMCS2. Figure 1A exposes new recombinant plasmid pBBRMCS2 HindIII *phaC1* SwaI and Figure 1B new recombinant plasmid pBBRMCS2 HindIII *phaC2* SwaI. Recombinant plasmids were then express in *Cupriavidus necator* H16 PHB<sup>-4</sup> (DSM541), a non-

producing PHA strain. These manipulations allowed us to study the influence of each gene required for PHA production by *Halomonas* sp. SF2003 but also to manage the bioproduction.



**Figure 1: Plasmid constructions for recombinant strains of *C. necator* H16 PHB<sup>-4</sup>, A. pBBRMCS2 HindIII *phaC1* SwaI and B. pBBRMCS2 HindIII *phaC2* SwaI.**

Second part of our work was focused on screening of optimal carbon substrates to optimize bacterial growth and PHA production at same time. A selection of several carbohydrates that can be found in agri-food or marine products was test for PHA production. Screening was conducted using Nile Red agar plates method <sup>1,7</sup>. Figure 2 exposes some typical results. Assimilation of the carbon sources, bacterial growth and batch PHA production were recorded to compare the performances of native and recombinant strains using gas chromatography GCFID and confocal Laser Scanning Microscopy (CLSM) allowed to determine the structural characteristics of the biopolymers that were synthesized in each condition.



**Figure 2: Nile red agar plates for PHA production screening tests with *Halomonas* sp. SF2003, A. Control negative/Glucose 2% (w/v), B. Control negative/Galactose 2% (w/v), C. Control negative/Fructose 2% (w/v) and D. Control negative/Saccharose 2% (w/v). Observations performed with transillumination.**

1. Elain, A. *et al.* Rapid and qualitative fluorescence-based method for the assessment of PHA production in marine bacteria during batch culture. *World J. Microbiol. Biotechnol.* **31**, 1555–1563 (2015).
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3. Deroiné, M. *et al.* Accelerated ageing and lifetime prediction of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in distilled water. *Polym. Test.* **39**, 70–78 (2014).
4. Deroiné, M. *et al.* Seawater accelerated ageing of poly(3-hydroxybutyrate-co-3-hydroxyvalerate). *Polym. Degrad. Stab.* **105**, 237–247 (2014).
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6. Sudesh, K., Abe, H. & Doi, Y. Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Prog. Polym. Sci.* **25**, 1503–1555 (2000).
7. Spiekermann, P., Rehm, B. H. A., Kalscheuer, R., Baumeister, D. & Steinbüchel, A. A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. *Arch. Microbiol.* **171**, 73–80 (1999).



## **Characterization and exploitation of the marine bacterium *Halomonas* sp. SF2003 for optimization of PHA production**

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*Halomonas* sp. SF2003 is a halophilic Gram-negative eubacterium isolated from the Iroise sea (Brittany, France) which already demonstrated its capacity to produce and accumulate polyhydroxyalkanoates (PHA) up to 78% of the cell dry weight <sup>1,2</sup>. Because of their properties, these biopolymers present environmentally and economically interests in order to replace conventional plastics <sup>3,4</sup>. Consequently numerous studies are conducting to identify and exploit new bacteria capable of accumulating these biopolymers <sup>5,6</sup>.

Interestingly, *Halomonas* sp. SF2003 can naturally produce P3-HB and the co-polymer P3HB-3HV, using different carbon substrates even those originate from industrial wastes. This strain also exposes a strong versatility to various temperature and salinity conditions making it a good candidate for biotechnological applications.

The aim of our work is to study and manage *Halomonas* sp. SF2003 metabolism, using genetic, microbiology and bioprocess tools, in order to enhance and to manage its production of PHA.

First part of our work consisted to identify and start characterization of the key enzymes involved in the PHA metabolism of *Halomonas* sp. SF2003 using bioinformatics tools. Sequencing and annotation of *Halomonas* sp. SF2003 genome allowed identification of different genes including two potential PHA synthases named *phaC1* and *phaC2* with a size of 1965bp and 2865bp, respectively and distant from each other in the sequence <sup>7</sup>. Because of the atypical organization and size of genes presumably involved in PHA biosynthesis, experiments were conducted to isolate and study them. Thus, *phaC1* and *phaC2* genes were individually cloned in a plasmid pBBRMCS2. Recombinant plasmids were express in *Cupriavidus necator* H16 PHB<sup>-4</sup> (DSM541), a non-producing PHA strain to study the influence of each gene on PHA production by *Halomonas* sp. SF2003.

Second part of our work was focused on screening of carbon substrates to optimize bacterial growth and PHA production at same time. A selection of several carbohydrates and mix of carbohydrates/acids was test for PHA production. Screening was conducted using Nile Red agar plates method <sup>1,8</sup>. Assimilation

of the carbon sources, bacterial growth and batch PHA production were recorded to compare the performances of wild type and recombinant strains using confocal Laser Scanning Microscopy (CLSM) and gas chromatography GCFID allowed to study and determine characteristics of each condition/production.

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5. Kourmentza, C. *et al.* Recent Advances and Challenges towards Sustainable Polyhydroxyalkanoate (PHA) Production. *Bioengineering* **4**, 55 (2017).
6. Sudesh, K., Abe, H. & Doi, Y. Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Prog. Polym. Sci.* **25**, 1503–1555 (2000).
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**Titre :** Étude du potentiel biotechnologique de *Halomonas* sp. SF2003 : Application à la production de PolyHydroxyAlcanoates (PHA).

**Mots clés :** Biopolymères, Polyhydroxyalcanoates (PHA), Bactéries halophiles, *Halomonas* sp. SF2003, PHA synthase.

**Résumé :** L'amenuisement des ressources pétrochimiques couplé à la pollution engendrée par l'exploitation des plastiques posent de nombreuses questions et conduisent à un besoin urgent de solutions alternatives. Les polyhydroxyalcanoates (PHA) sont des polymères qui ont su se démarquer et naturellement s'imposer comme matériaux de remplacement, étant donné leurs caractères à la fois biosourcé et biodégradable. Leur synthèse par un grand nombre d'organismes procaryotes et eucaryotes, à partir d'une large gamme de substrats carbonés, rend leur production quasi illimitée et conduit à l'obtention de polymères aux propriétés allant de celles d'un thermoplastique à un élastomère. Malgré cela, les coûts de production représentent toujours le principal verrou au développement de leur utilisation. Parmi les solutions envisagées, l'exploitation de ressources marines, telles que

les bactéries halophiles, suscite un fort intérêt tant les capacités d'adaptation de ces souches sont étendues et avantageuses. *Halomonas* sp. SF2003 est une souche marine naturellement productrice de PHA possédant une grande capacité d'adaptation face à de nombreux substrats et conditions environnementales. L'étude et l'optimisation de sa production de PHA font l'objet de ce travail. Le séquençage de son génome a permis l'étude de différents gènes et voies métaboliques confirmant son caractère adaptatif. Dans le même temps, l'influence de différents paramètres sur la production de PHA a été étudiée. Ces travaux, faisant appel à des techniques de biologie moléculaire et des bioprocédés, contribueront au développement du potentiel biotechnologique de la souche *Halomonas* sp. SF2003.

**Title :** Study of *Halomonas* sp. SF2003 biotechnological potential: Application to PolyHydroxyAlcanoates (PHA) production.

**Keywords :** Biopolymers, Polyhydroxyalkanoates (PHA), Halophilic bacteria, *Halomonas* sp. SF2003, PHA synthases.

**Abstract :** Depletion of oil resources coupled to pollution caused by over-exploitation of plastics generate a plentiful of issues and lead to an urgent need for alternatives. PolyHydroxyAlcanoates (PHA) are biopolymers which have distinguished and naturally imposed themselves due to their biosourcing and biodegradability features. Their synthesis by a wide variety of eukaryotic and prokaryotic organisms, from various carbon substrates, makes their production almost unlimited and allows obtaining polymers exposing thermoplastic to elastomeric properties properties. Despite that, production cost is still the main lock to development of their employment. Among possibilities studied, exploitation of marine resources, like halophilic bacteria, arouse a strong interest since adaptability of these strains

is extensive and attractive. *Halomonas* sp. SF2003 is a PHA producing marine bacterium which naturally exposes a considerable versatility in front of carbon substrates and environmental conditions. Characterization and optimization of its PHA production are the main subjects of the presented study. Genome sequencing and annotation, in addition to phenotypic tests, allowed characterization of various genes and metabolic pathways attesting of the adaptative strain character. In the same time, impact of various parameters on *Halomonas* sp. SF2003 PHA production has been investigated. This work, employing molecular biology and bioprocess tools, will contribute to future development of biotechnological potential of *Halomonas* sp. SF2003.