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Screening of polyhydroxyalkanoates producing bacteria isolated from marine ecosystems

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"If people feel they understand the world around them, or, probably, even if they have the conviction that they could understand it if they wanted to, then and only then are they also able to feel that they can make a difference through their decisions and activities".

Frank Oppenheimer

To my greatest treasure,

My parents…

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RESUMO

Os Polihidroxialcanoatos (PHAs) são biopoliésteres biodegradáveis e termoplásticos ecológicos, que são acumulados sob a forma de materiais de armazenamento de energia em várias bactérias em condições de crescimento limitado e fonte de carbono em excesso.

Neste estudo, as bactérias foram isoladas a partir de amostras recolhidas a partir de vários ecossistemas marinhos no arquipélago da Madeira, no Oceano Atlântico e seleccionadas pela sua capacidade de acumular poli-hidroxialcanoatos. Estas amostras foram extraídas do fundo do mar a uma profundidade de 30 e 1,700 metros para se obter uma maior diversidade de microorganismos e, por conseguinte, numa tentativa de obter novas estruturas de PHAs. As estirpes foram directamente isoladas de 612 placas mãe onde as amostras marinhas tinham sido previamente espalhadas. Na totalidade foram isoladas 724 estirpes a partir das placas-mãe, das quais 174 demonstraram resultado positivo durante o screening com o corante Vermelho do Nilo. Todas as estirpes com resultado positivo sintetizaram inclusões intracelulares durante o crescimento em amido como fonte de carbono. Vinte e cinco isolados bacterianos foram testados em cultura de 25 mL com resultados promissores quanto à produção de PHA com armazenamento de 17,71 % para a estirpe MD12-107 e 9,30 % para a estirpe MD12-581. As inclusões foram analisadas através da técnica de cromatografia gasosa como sendo predominantemente poli-ß-hidroxibutirato (PHB). A produção a partir das estirpes MD12-107 e MD12-581 foi testada em 100 ml e em bioreactor. Os melhores resultados foram alcançados pela estirpe MD12-581 com um armazenamento de PHA de 15,40% em menos de 6,5 horas, com 5,5 g/L de concentração de biomassa e uma taxa específica de crescimento de 0,24 h^{-1} quando cultivada num meio contendo 40 g/L de amido, 8 g/L de extracto de levedura e 4 g/L de peptona durante a produção em bioreactor.

Palavras-chave

Polihidroxialcanoatos, biodegradável, bactérias marinhas, vermelho do Nilo, azul do Nilo, cultura pura.

RESUMO ALARGADO

A descoberta e o isolamento de novos compostos a partir de bactérias marinhas sempre foram estimulados pela indústria farmacêutica em pesquisa de novas entidades químicas. Os microrganismos isolados são cultivados a fim de extrair os metabólitos secundários, que geralmente apresentam interesse terapêutico. Com os métodos de extracção normalmente usados, os compostos de elevado peso molecular, tais como proteínas, polissacáridos ou poliésteres produzidos pelo microorganismo são normalmente descartados. No entanto, os biopolímeros, tais como polihidroxialcanoatos (PHAs), produzido por estes microorganismos apresentam um potencial enorme em termos de aplicações industriais. Com efeito, o PHA é um poliéster biodegradável e trata-se de um polímero de armazenamento de energia produzida pelas células em condições de excesso e limitação de nutrientes e que podem ser usados como materiais termoplásticos e elastómeros. A longo prazo, eles podem substituir os plásticos comuns, produzidos a partir de derivados de combustíveis fósseis.

O objectivo deste estudo visa um isolamento de bactérias a partir de uma colecção de 612 placas mãe e posterior screening para identificar novas estirpes que apresentam capacidade para produzir PHA.
A fase inicial deste estudo passou pelo isolamento de bactérias em meio sólido a partir das placas mãe onde as amostras marinhas tinham sido inicialmente semeadas. O meio utilizado no isolamento designou-se meio A1 e foi constituído por 10 g/L de amido, 2 g/L de peptona, 4 g/L de extracto de levedura. Foram obtidos 724 isolados que posteriormente foram sujeitos ao screening com o corante vermelho do Nilo utilizando um meio com condições de excesso de fonte de carbono e limitação de fonte de azoto, condições ideais para a produção de PHA pelas bactérias. O Meio utilizado nesta fase designou-se de Meio A2 e foi constituído por 20 g/L de amido, 1 g/L de peptona e 2 g/L de extracto de levedura. Após o meio ser autoclavado, foi adicionado o corante lipofílico vermelho do Nilo com uma concentração final de 0.5 µg/mL. Após 72 horas de crescimento, as placas foram visualizadas através de uma lâmpada azul (Safe Imager™, Invitrogen) que possibilitou distinguir claramente as bactérias produtoras de PHA das bactérias não produtoras.
Após o screening, foram escolhidas 25 bactérias que apresentaram sinal de fluorescência mais significativo. Como o corante vermelho do Nilo não é específico para o PHA, permitindo também a coloração de lípidos intracelulares, era necessário conferir que a acumulação de PHA era responsável pelo sinal de fluorescência, e não a acumulação de lípidos.

Foi feito um crescimento em 25 mL em meio líquido e a produção de PHA foi posteriormente analisada por cromatografia gasosa. Nesta fase do estudo, foram identificadas duas estirpes: a Gram-positiva MD12-107 e Gram-negativa MD12-581 como sendo produtoras de maior percentagem de PHA, com 17,71 % e 9,30 % respectivamente.

Para determinar o máximo de capacidade de acumulação de PHA pelas estirpes MD12-107 e MD12-581, foi feito um crescimento em 100 mL de meio líquido com a mesma proporção de fonte de carbono e azoto do passo anterior. Durante o crescimento em 25 mL em meio líquido, a estirpe MD12-107 revelou-se melhor produtora que a estirpe MD12-581, todavia, durante o crescimento em 100 mL obteve apenas 2,3 % de PHA após 16 horas de crescimento, relativamente à estirpe MD12-581 que produziu 11,1 % de PHA em após 26 horas de crescimento. Nesta fase foram também testados dois meios sintéticos: o meio Marinho Mineral e meio E suplementado de 40 g/L de glucose. No primeiro caso as bactérias não apresentaram crescimento significativo e no segundo caso, a inoculação nem sequer foi possível devido à precipitação de fosfatos quando se adicionou glucose ao meio E.

Numa produção em bioreactor, a estirpe MD12-581 foi testada em meio A2 obtendo 10,23 % de PHA ao fim de 8 horas de crescimento, com uma taxa específica de crescimento de 0.30 h^{-1}. Para aumentar a produção de PHA, a proporção de fonte de carbono e azoto foi alterada. Foram realizados duplicados para a produção das duas estirpes em meio A3 constituído por 40 g/L de amido, 4 g/L de peptona e 8 g/L de extracto de levedura. No caso da produção de PHA pela estirpe MD12-107, os valores obtidos foram inferiores a 1 % com uma taxa específica de crescimento de 0,18 h^{-1} e 0,16 h^{-1}. No caso da produção pela estirpe MD12-581 foi obtido um valor máximo de 15,40 % de PHA após 6,5 horas de crescimento, com uma taxa específica de crescimento de 0,24 h^{-1}; e 5,37 % após 7 horas de crescimento, com uma taxa específica de crescimento de 0,34 h^{-1}.

ABSTRACT

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters and environmentally friendly thermoplastics, which are accumulated as carbon and energy storage materials in various bacteria in limited growth conditions with excess carbon sources.

In this study, bacteria were isolated from samples taken from various marine ecosystems in the Archipelago of Madeira in the Atlantic Ocean, and screened for their ability to accumulate polyhydroxyalkanoates. These samples were taken from the seabed at depths of 30 and 1,700 meters to obtain a larger diversity of microorganisms and therefore, in an attempt to obtain new structures of PHAs. Strains were directly isolated from 612 mother plates where marine samples had been initially plated. A total of 724 isolates from mother plates were obtained, of which 174 were found PHA-positive using Nile red viable-colony screening. All synthesized intracellular inclusions during growth on starch carbon source. Twenty-five bacterial isolates in 25 mL-scale cultivation were proven promising for PHA production with PHA storage maximum 17.71 % for MD12-107 and 9.30 % for MD12-581 strain. The inclusions were predominantly identified as poly-ß-hydroxybutyrate (PHB) using gas chromatography. Strains MD12-107 and MD12-581 were tested in 100 mL-scale and bioreactor cultivation. The best results were achieved with strain MD12-581 accumulated PHA storage 15.40 % in less than 6.5 hours with 5.5 g/L of cell dry weight and a specific growth rate was 0.24 h^{-1} when grown in medium containing 40 g/L of starch, 8 g/L of yeast extract and 4 g/L of peptone during batch cultivation.

Keywords

Polyhydroxyalkanoates, biodegradable, marine bacteria, Nile red, Nile blue, pure culture.

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Nomenclature

%PHA – PHA content (% w/w)

CDW – Dry Weight Cell, cell concentration (g/L)

DMSO - dimethylsulfoxide

DOC – Dissolved Oxygen Concentration (pO2 (%))

FTIR - Fourier Transmission Infra Red spectroscopy

GC – gas chromatography

HA – hydroxyalkanoate

HB – hydroxybutyrate

HPLC - High Performance Liquid Chromatography

LDPE - low density polyethylene

MCL – medium-chain length

MMC - microbial mixed cultures

NMR - Nuclear Magnetic Resonance spectroscopy

OD600 – Optical Density at 600 nm

[Organic Carbon]- Organic Carbon concentration (ppm)

PHAs – polyhydroxyalkanoates

PHB – polyhydroxybutyrate

PHH - polyhydroxyhexanoate

P(3HB) – poly(3-hydroxybutyrate)

rpm – rotation per minute

SCL – short-chain length

TOC – total organic carbon

UV – ultraviolet

μ- Specific growth rate (h^{-1})

Chapter I - Introduction

1.1. Plastic and Daily Life

Plastics have infiltrated every part of daily life and have basically become indispensable. Because of their versatility and durability, petroleum based plastics have had a variety of both small and large scale uses for over seventy years now (Keshavarz *et al.,* 2010). While they are extremely pliable and can be formed into almost any shape including fibers and thin films, they remain chemically resistant and maintain their durability and strength under a wide range of temperatures (Andrady, 2009; Reddy *et al.,* 2003).

Despite these valuable characteristics, the drawbacks of plastics have become increasingly apparent during the last century. First, plastics are derived from crude oil, a non-renewable energy source that will eventually be depleted. During production, the petroleum-based plastics emit gases considered to be non-environmentally friendly, negatively affecting the global climate (Andrady, 2003). Second, the non-biodegradable nature of plastic wastes has become a cause for environmental concern. These wastes accumulate in landfills and the marine environment if not incinerated. When incinerated, they emit toxic compounds (Suriyamongkol *et al.,* 2007; Reddy *et al*, 2003). For these reasons, there is special interest in producing plastics from materials able to be eliminated in an "environmentally friendly" way (Gross and Karla, 2002).

1.2. Bioplastics

Thanks to chemical engineering and biotechnology companies, biodegradable plastics are being manufactured using renewable resources and their availability is increasing significantly. For example, starch-based plastics have become an important variety of bioplastics used in the production of biodegradable products and an increase in plastics biodegradability would certainly help to alleviate waste problem of our current society (Kim, 2000).

Bioplastics are materials that contain biopolymers in varying percentages and are moldable when applied with heat and pressure. Therefore, they are a potential alternative option to the above mentioned conventional petroleum based plastics (Queiroz *et al*., 2009). Both polymers produced from renewable biological sources and polymers which are biodegradable can be classified as bioplastics. Figure 1 illustrates Bio-based to biodegradable plastics life cycle (Albuquerque, 2009). While some conventional plastics today, such as polyethylene

(PE), polyvinyl chloride (PVC) and polyethylene terephthalate (PET) are able to be manufactured using renewable resources, they remain non-biodegradable, which causes confusion in the plastic market (Razza *et al.*, 2012; Schut, 2008).

Biopolymers can be classified into three groups as follows: polymers extracted directly from biomass, having been modified or not, such as starch or cellulose-based polymers; polymers produced directly by microorganisms in their natural or genetically modified state, exemplified by polyhydroxyalkanoates (PHAs); and polymers obtained with the utilization of bio-intermediaries, produced with renewable raw materials. Examples include: polylactic acid (PLA); bio-polyethylene (BPE), from the polymerization of ethylene produced from bio-ethanol; bio-nylons via diacids from biomass and bio-polyurethanes, incorporating polyols of vegetal origin (Albuquerque, 2009; Queiroz *et al.*, 2009; Reddy *et al*, 2003)

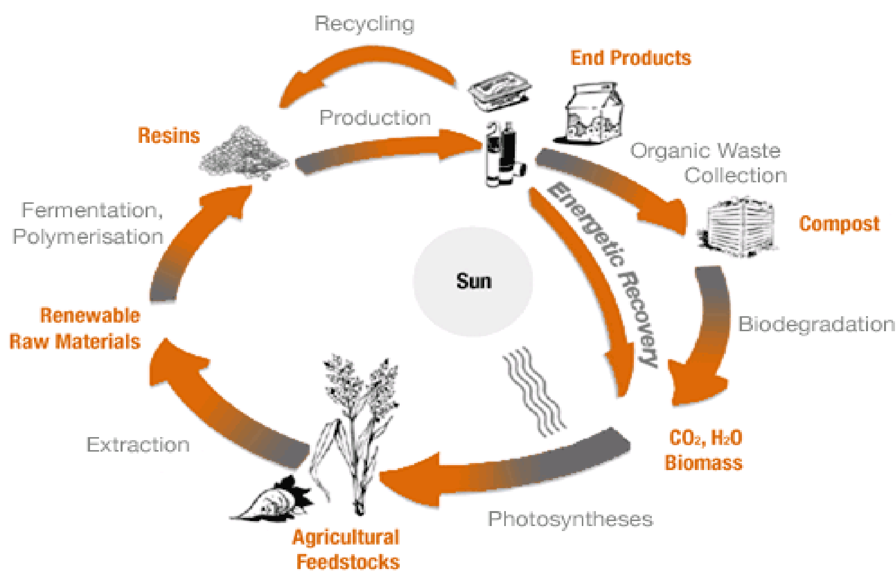

Figure 1- Bio-based to biodegradable plastics life cycle (copyright: EBA, 2009).

1.3. Polyhydroxyalkanoates (PHA): characteristics and advantages

Biopolymers can be produced by a variety of microorganisms. As mentioned before, bacterial polyhydroxyalkanoates (PHAs) represent a unique family of polymers acting as a carbon/energy store for more than 300 species of Gram-positive and Gram-negative bacteria as well as for a wide range of *Archaea* (Chansatein *et al.*, 2012; Laycock *et al.*, 2013).

PHAs are biopolyesters that generally consist of 3-, 4-, 5-, and 6-hydroxycarboxylic acids. In the presence of a limited quantity of essential nutrients such as oxygen, phosphorous or nitrogen, they are synthesized intracellularly as insoluble cytoplasmic inclusions in the presence of excess carbon. Since they do not substantially alter its osmotic state, these polymeric materials are able to be stored at high concentrations within the cell (Chansatein *et al.*, 2012; Laycock *et al.*, 2013; Park *et al.*, 2012). As intracellular granules, these polymers can accumulate to levels as high as 90 % of the cell dry weight (Reddy *et al.*, 2003). PHA-producing bacteria have been reported to be found in various environments such as wastewater, industrial waste, municipal waste, soil, compost, hot spring water, fresh water and marine water (Chansatein *et al.*, 2012). Utilization of these economically appealing substrates allows for a sustainable closed cycle process for PHA production and use (Figure 1) (Satoh *et al.*, 1998; Braunegg *et al.*, 1998; Reis *et al.*, 2006). Additionally, PHAs are non-toxic, biocompatible, biodegradable thermoplastics, with a high degree of polymerization, highly crystalline, optically active and isotactic, piezoelectric and insoluble in water (Reddy *et al.*, 2003).

The first PHA to be identified was poly-3-hydroxybutyrate P(3HB), which showed that could be cast into a transparent film by forming intracellular granules in Gram-positive bacterium *Bacillus megaterium* (Lemoigne, 1926; Laycock *et al*, 2013; Castilho *et al*, 2009). This homopolymer is highly crystalline, between 55–80 %, rendering it brittle and giving it little strength to withstand impact. These characteristics limit its uses. The glass transition temperature of P(3HB) is approximately 5ºC and its melting point is approximately 175 ºC (Reis *et al.*, 2003).

Although over 150 different types of hydroxyalkanoate (HA) monomers synthesized by over three hundred different bacterial have been identified since the discovery of P(3HB), it remains the most commonly observed and well-studied PHA (Albuquerque, 2009; Laycock *et al.*, 2013; Steinbuchel and Valentin, 1995). PHAs are essentially composed of monomers of R-(-)-3-hydroxyalkanoic acid. The different types of PHAs are distinguishable by chain size, type of functional group and unsaturated bonds degree (Braunegg *et al.*, 1998; Alves, 2009). Figure 2 represents the general polyhydroxyalkanoate chemical structure.

PHAs are classified into two main groups by their carbon numbers. The two groups with different material properties are short-chain-length (SCL) and medium-chain-length (MCL)-PHAs. SCL-PHAs are composed of monomers having 3 to 5 carbon atoms. They are often crystalline, stiff and brittle in texture and display thermoplastic material properties similar to polypropylene. MCL-PHAs are composed of monomers having 6 to 14 carbon atoms. They present lower crystallinity (20-40 %) and have elastic material properties similar to rubber and elastomer. Recently, PHAs with low density polyethylene (LDPE)-like material properties have been synthesized by incorporating a small amount of MCL-monomers into the poly-3-

hydroxybutyrate backbone (Farinha, 2009; Keshavarz *et al*. 2010; Lee *et al*, 1995; Park *et al*, 2012; Suriyamongkol *et al*, 2007). The average number of repeating units in each polymer chain can range between 100 and 30,000, resulting in polymer molar masses ranging from 10,000 to $3x10^6$ Da (Albuquerque, 2009; Cranc and Pattel, 2005; Lee, 1996).

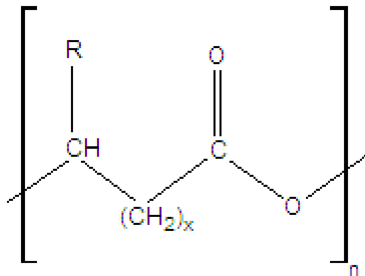

Figure 2- General structure of polyhydroxyalkanoates. R is alkyl groups (Lee, 1996).

Biosynthetic polymers can be produced through microbial or plant route. Currently, microbes are considered to be the major source for production of PHAs (Steinbüchel *et al.*, 2003), although they can also be produced in plants (Keshavarz *et al.*, 2010). Production of bioplastics via microbial route requires technical viability and feasibility for a challenging production (Keshavarz *et al.*, 2010).

Due to their varied monomer content, PHAs differ in their physical and chemical characteristics. Types of organisms, media ingredients, fermentation conditions, modes of fermentation (batch, fed-batch, continuous), and recovery are all factors that affect PHA monomer content (Keshavarz *et al.*, 2010).

Exposure to soil, compost, or marine sediment can degrade PHAs. Biodegradation depends on a variety of factors including microbial activity of the environment, the amount of exposed surface area, temperature, pH, polymer composition, as well as crystallinity (Farinha, 2009; Reddy *et al.*, 2003). Biodegradation of PHAs under aerobic conditions results in carbon dioxide and water, while carbon dioxide and methane are produced during degradation in anaerobic conditions. According to studies, 85 % of PHAs can be degraded in seven weeks (Farinha, 2009; Reddy *et al.*, 2003).

1.4. PHA Extraction, Industrial Production and Applications

PHA recovery process contributes significantly to the overall production cost, therefore there is a need to pursuit a simple, low-cost and highly efficient extraction method, environmentally friendly (Dias *et al.*, 2006). The methods used for cell disruption can be divided into chemical, enzymatic, and mechanical processes (Dias *et al.*, 2006).

There are two commonly employed methods of PHA extraction from bacteria. Using solvents such as chloroform, methylene chloride, propylene carbonate and dichloroethane, is the most common method of extraction (Dias *et al.*, 2006). The conventional method is based on the solubility of PHA in chloroform and insolubility in methanol. Unfortunately, this method is neither environmentally friendly nor suitable for mass production. The chloroform extraction method is widely used at the laboratory scale because it allows for high purity without polymer fractionation of the bioplastic (Dias *et al.*, 2006; Suriyamongkol *et al.*, 2007). However, it requires a large amount of solvent which makes this method too costly for large scale processes (Dias *et al.*, 2006). The second method purposely avoids the use of organic solvents. In this method, bacterial cells are treated with a cocktail of enzymes such as proteases, nucleases and lysozymes, and detergents to remove proteins, nucleic acids, and cell walls, while leaving the PHA intact (Suriyamongkol *et al.*, 2007).

Recently, two new methods were discovered during the search for an environmentally friendly and more cost-efficient extraction. They consist in supercritical CO_2 extraction and non-PHA-selective cell mass dissolution by protons, with PHA crystallization (Dias *et al.*, 2006). Supercritical CO_2 extraction results in higher final purities and a maximum reported value of 89 % efficiency in polymer recovery due to the removal of lipid impurities, its main advantage (Dias *et al.*, 2006). The non-PHA-selective cell mass dissolution by protons seems more cost-effective than conventional chemical treatment methods, with recovery efficiency and purity of 95 and 97 %, respectively (Dias *et al.*, 2006).

Production of PHAs at an industrial scale has been achieved using pure culture, either of natural strains such as *Alcaligenes latus* or *Burkholderia sacchari*, or recombinant strains of *Escherichia coli* and *Cupriavidus necator*. These strains are currently being used to produce four commercial brands of PHA: Biomer®, Nodax®, Biocycle® and Mirel® (for review see Albuquerque, 2009; Lemos *et al.*, 2006).

PHAs have a large variety of uses including packaging, cosmetic containers, shampoo bottles, cardboards and papers, milk cartons and films, moisture, barriers in diapers and sanitary napkins and other personal hygiene materials, pens, combs, bullets, flavor delivery agents in foods, dairy cream substitutes, and bulk chemical production using depolymerised PHA (Keshavarz *et al.*, 2010). Recently, medical uses for PHAs have captured a reasonable amount of attention. These include cardiovascular products, prodrugs, efficacy in nerve and soft tissue repair, dental and maxillofacial treatment, drug delivery, nutrition, orthopedic and urology procedures and wound management (Chen, 2010; Keshavarz *et al.*, 2010).

1.5. Strains for PHA production

1.5.1. PHA production by pure cultures

Since the 1980's, a considerable amount of research has been devoted to the development of pure microbial culture strategies for the production of PHA by microorganisms at an industrial scale. Approximately 14 companies are currently involved in PHA production with either wild type PHA producers or genetically modified organisms (Chen, 2009). Although many types of PHA have been discovered, only a few of these have been able to be produced at large scale including the homopolymer PHB, copolymer PHB and PHV, and the copolymer of PHB and PHH (polyhydroxyhexanoate). Generally, PHA production by pure microbial culture is performed in batch or fed-batch mode, taking anywhere from 38 to 72 hours for fermentation. In order to attain a high cell density, the microorganisms are pre-cultivated in growth medium. The accumulation of PHA during this period is typically minimal. Once the growth medium is depleted, the growth limiting medium is fed to the microorganisms in order to induce and maximize the PHA content of the biomass (Chen, 2009; Albuquerque, 2009). In other words, growth limiting conditions are imposed in order to induce PHA storage. Subsequently, the microbial cells accumulate PHA until saturation is reached and the fermenter is then discharged. In following, extraction of the intracellular polymer takes place by recovering and disrupting the cells (Albuquerque, 2009).

In recent years, a considerable effort has been made to develop pure culture fermentation strategies for more cost-efficient viable PHA production. Research goals were set, attempting to increase process productivity including maximizing PHA cell content. This in turn increases fermentation productivity and improves PHA recovery yield while maximizing cell growth and polymer production rates (Albuquerque, 2009).

PHA production using pure cultures involves a two-stage batch production process. In the first stage (growth), an inoculum of bacteria is introduced into a sterile solution of trace metal nutrients and a suitable carbon source (Laycock *et al.*, 2013). In the second stage, an essential nutrient (such as N, P or O_2) is purposely limited so that PHA accumulation can take place. The properties of the final polymer depend on at least three factors including the mix of carbon feedstocks fed during accumulation, the metabolic pathways that the bacteria use for the following conversion into precursors, and the substrate specificities of the enzymes involved (Laycock *et al.*, 2013).

The most commonly used wild type strain for production of SCL PHA is *R. eutropha*. In order to generate PHB or a copolymer of PHB and PHV, this strain is usually fed with glucose or a mixture of glucose and propionate. The maximum PHA content (% w/w) of *R. eutropha* can

reach 75 % to 80 % of cell dry weight in 48 hours to 60 hours (Chen, 2009). Besides *R. eutropha*, *Alcaligenes latus* is also used for SCL PHA production (Biomer, Germany). *A. latus* can accumulate up to 50 % PHB on glucose or sucrose in 18 hours of growth (Yamane *et al.*, 1996). MCL PHA production by wild type strain on a commercial scale is rare. In addition, *Amerononas hydrophila* and *Pseudomonas oleovorans* have also been used for PHB and PHH copolymer production. The maximum PHA contents of these two strains were reported as 50 % and 63 %, respectively (Chen, 2001; Jung, 2001).

Cost analysis of PHA production identified two main factors contributing to high PHA price: high fermentation operating costs and high downstream processing costs (Castilho *et al.*, 2009; Choi and Lee, 1997; Povolo and Romanelli, 2012). The major contributor to fermentation operating costs is the cost of substrate. In almost all instances, substrates used in current industrial processes are refined sugar feedstocks such as glucose, sucrose, and corn which have a high market price (Albuquerque, 2009; Lee, 1996; Madison and Huisman, 1999; Reddy *et al.*, 2003). Compared with the prices of conventional polymers, it is evident that even the most efficient processes will not allow PHAs to compete with petroleum-based polymers on the basis of price so long as expensive substrates such as glucose are used, as can be seen in Table 1 (Castilho *et al*, 2009). Therefore, it became necessary for genetic/metabolic engineering strategies for PHA production by bacterial fermentation to include the ability to use several inexpensive substrates. These have been attempted mostly at laboratory scale, often with promising results (Albuquerque, 2009; Reddy *et al*, 2003). Some of the best results obtained using pure microbial cultures fed with low cost feedstocks (starch, sugar molasses, tapioca, and whey) are displayed in Table 2 (Albuquerque, 2009).

Table 1- Market prices of bio-based polymers and conventional, petroleum-based polymers

Polymer Market	**Price**	**Reference**
P(3HB) from Biomer (Germany)	€12/kg	Hänggi (2004)
P(3HB-co-3HV) from Metabolix (US)	€10–12/kg	Crank *et al.* (2004)
Modified starch polymers from Novamont (Italy)	€2.5–3.0/kg	Crank *et al.* (2004)
Polypropylene (PP)	€0.74/kg	CMAI Global (2009)
High-density polyethylene (HDPE)	€0.78/kg	CMAI Global (2009)
Low-density polyethylene (LDPE)	€0.74/kg	CMAI Global (2009)
Polyvinyl chloride (PVC)	€0.72/kg	CMAI Global (2009)
Polystyrene (PS)	€0.70/kg	CMAI Global (2009)
Polyethyleneterephtalate (PET)	€0.81/kg	CMAI Global (2008)

Source: adapted from Castilho et al., 2009

Table 2- PHA production using pure microbial cultures grown on refined sugars as substrates or low-cost carbon feedstocks

	Feedstock	Microorganism	Fermentation strategy	CDW (g/L)	PHA content (%)	PHA composition	Ref
Refined Substrates	Glucose	*Cupriavidus necator*	Fed-batch	281	82	PHB	Lee *et al.*(1999)
	Sucrose	*Alcaligenes latus*	Fed-batch	112	88	PHB	Lee *et al.*(1999)
	Glucose	r Escherichia *coli*	Fed-batch	204	77	PHB	Lee *et al.*(1999)
Low cost feedstocks	Starch	*Haloferax mediterranei*	Batch	10	60	PHB	Lillo *et al.*(1990)
	Starch	*Azotobacter chroococcum*	Fed-batch	54	46	PHB	Kim(2000)
	Molasses	*Azotobacter vinelandii* UWD	Fed-batch	33	66	PHB	Page *et al.*(1993)
	Tapioca	*Cupriavidus Necator*	Fed-batch	106	58	PHB	Kim *et al.*(1995)
	Whey	r *Escherichia coli*	Fed-batch	31	80	PHB	Kim(2000)
	Whey	r *Escherichia coli*	Fed-batch	55	57	PHB	Kim(2000)
	Whey	r *Escherichia coli*	Fed-batch	87	80	PHB	Lee *et al.*(1999)
	whey	r *Escherichia coli*	Fed-batch	83-150	67-80	PHB	Lee et al.(2000)
	Different vegetable oils (5g/L)	*Cupriavidus necator*	Shake flask	4.4-5.6	67-80	PHB	Lee *et al.*(2008)
	Different vegetable oils (5g/L) + Propionic Acid	*Cupriavidus necator*	Shake flask	3.6-7,5	74-90	P(HB/HV)	Lee *et al.*(2008)

Source: adapted from Albuquerque, 2009.

1.5.2. PHA production in recombinant *Escherichia coli*

In recent years, both genetic engineering and molecular microbiology techniques have contributed to the enhancement of PHA production in microorganisms. In order to develop optimal recombinant host strains, several mutants with phenotypes in PHA synthesis were characterized. However, little difference in polymer accumulation was achieved due to over-expression of PHA genes in the natural PHA producer. Unfortunately, growth time during fermentation is lengthy for most natural producers, and extraction of polymers from their cells is difficult, making these PHA producers unsuitable for biopolymer production on an industrial scale (Suriymongkol *et al*, 2007).

Though *E. coli* does not naturally produce PHA, it is considered to be an appropriate host for generating higher yields of the biopolymer for several reasons including its fast growth rate, high productivity, easy lysis, easily manipulated genome, easy scale up, and well understood genetics and biochemistry. In addition, a wide range of substrates can be used, its PHA production is not tied to natural regulation, and PHA polymerase is absent (Lee, 1997; Li *et al*., 2006; Sudesh *et al*, 2000; Suriymongkol *et al*, 2007).

In 1988, PHA genes were introduced into *E. coli* for the first time and PHB granules were formed in recombinant *E. coli* host cells (Slater *et al*., 1988; Suriymongkol *et al*., 2007). In order to enhance PHA synthesis, and produce novel PHA, metabolic engineering is being intensely explored. By inserting the genes involved in PHA synthesis from different bacteria into *E. coli,* it gained the capacity for producing both SCL and MCL PHA. Furthermore, the PHA degradation genes were not transferred into *E. coli* (Reddy *et* al., 2003). *E. coli* ability to utilize various carbon sources, including glucose, sucrose, lactose and xylose, further reducing production cost of PHA by using cheap substrates such as molasses, whey and hemicellulose hydrolysate (Lee and Chang, 1995; Reddy *et al*., 2003). This strategy can be extended to virtually any bacterium if it possesses metabolic advantages over those currently in use (Reddy *et al*., 2003). The maximum PHA content from the recombinant *E. coli* harboring *R. eutropha* PHA synthesis gene reached up to 90 % of the cell dry weight (Lee and Choi, 1998).

1.5.3. PHA synthesis in transgenic plants

In comparison with bacteria or yeast, crop plants are capable of producing large amounts of a variety of useful chemicals at low cost. In comparison, PHA production in bacteria or yeasts has a costly fermentation process because it requires growth under sterile condition with an external energy source such as electricity (Reddy *et al.*, 2003; Snell and Peoples, 2002). In contrast, PHA production in plant systems is considerably less expensive because the system only relies on water, soil nutrients, atmospheric CO_2 and sunlight. The cost of producing starch and sugar in plants is less than the cost of commodity plastics, so it may be possible to produce PHA at a similar low cost. Unlike the bacterial cell, the plant cell has different subcellular compartments in which PHA synthesis can be metabolically localized (Hanley *et al*, 2000; Moire *et al*, 2003). Plants use photosynthetically fixed CO_2 and water to generate the bioplastic, which after disposal is degraded back to CO_2 and water, which makes a plant production system much more environmentally friendly. Synthesis of PHAs in crops is also an excellent way of increasing the value of the crops (Reddy *et al.*, 2003; Somerville and Bonetta, 2001). However, the production of PHA in transgenic plants presents barriers associated with the expression of transgenes and metabolic load on plant growth and the constitutive expression of PHA synthesis genes is still a crucial obstacle (Suriyamongkol et al., 2007).

1.5.4. PHA production by mixed cultures

One proposed way of lowering production costs is PHA production by microbial mixed cultures (MMC) (Dias *et al*, 2006; Serafim *et al*, 2008). Costs are reduced by using low value substrates, such as waste or surplus feedstocks, and by eliminating the necessity of reactor sterilization since the culture is able to adapt to various complex waste feedstocks, which reduces fermentation equipment costs (Albuquerque, 2009; Dias *et al*, 2006; Kim, 2000; Laycock *et al*, 2013; Reddy *et al.*, 2003; Serafim *et al*, 2008). Another advantage of MMC processes is based on the fact that these cultures can use a wide variety of complex substrates, even substrates which are rich in nutrients, since PHA storage by mixed culture is not induced by nutrient limitation (Albuquerque, 2009). Mixed cultures are composed of microbial populations of unknown composition, selected by the operational conditions imposed on the open biological system (Albuquerque, 2009).

PHA storage in mixed microbial cultures takes place in systems where the substrate is not continuously available to the microorganisms or in systems where electron donor and acceptor availability are interrupted (Reis *et al.*, 2003). In the case where the substrate is not continuously available to the microorganism, the culture is subjected to a transient carbon supply. Unbalanced growth occurs under these conditions as the sludge is subjected to

consecutive periods of external substrate accessibility (feast) and unavailability (famine). During the famine phase, the carbon uptake is mainly driven to PHA storage and, to a lesser extent, to biomass growth. Following substrate exhaustion, the stored polymer becomes an accessible source of energy and carbon. The ability to internally store carbon gives these microorganisms a competitive advantage over those that do not have this capacity. Due to this quality, they have become predominant in the system. This enrichment strategy is known as aerobic dynamic feeding (ADF) or feast and famine (Reis *et al.*, 2003; Serafim *et al.*, 2004).

1.6. Bioproduction Process

Currently, there are four methods of producing PHAs: *in vitro*, via PHA-polymerase catalyzed polymerization; and *in vivo* with batch, fed-batch and continuous cultures (Zinn *et al.*, 2001).

The synthesis of PHA *in vivo* has been and is still being investigated using batch cultures because batch cultures are easy to handle, and they are suited for growth studies and screenings for potential PHA accumulating organisms. Typically, the medium is designed in such a way that one nutrient limits growth of biomass whiles other nutrients, including the carbon source, are in excess. The experiments are performed within 1-2 days, during which time the cells undergo a progression of growth stages (Zinn *et al*, 2001). Simultaneously, the cells perceive continuous changes of their environment due to constant change in nutrient concentration caused by the cell metabolism. This method seldom gives an accurate indication of the maximum capacity of the cells to accumulate PHA since cells starved for carbon degrade PHA again (Zinn *et al.*, 2001).

1.7. Methods for quantification and/or detection of PHA and compositional analysis.

Several techniques are appropriate to detect organisms that are able to synthesize PHA and determine PHA inside intact cells. The lipophilic dyes Sudan black, Nile blue and Nile red are used to stain colonies and to distinguish between PHA-accumulating and non-accumulating strains (Amara, 2008; Spiekermann *et al.*, 1999).
Using Nile blue and Nile red allows for a more specific and sensitive visualization of PHA granules (Serafim *et al.*, 2002). The Nile blue and Nile red are able to penetrate the membrane and stain biopolymer granules in live cells, allowing for the identification of PHA producing bacteria using fluorescence. However, they cannot identify the monomer composition of PHA produced (Spiekermann *et al.*, 1999). The Nile blue is soluble in water

and ethanol while Nile Red has low solubility in water, though it dissolves in most organic solvents (Ostle and Holt, 1982; Greenspan *et al.*, 1985).
Of these screening techniques for isolating potential PHA-producing strains from environmental samples, Nile red fluorescence offers an easy, rapid screening option (Berlanga *et al.*, 2006). The Nile red is introduced directly in the agar medium, resulting in fluorescent colonies of PHA-positive strains that can be observed by UV illumination omitting the microscopic observation step (Tanamool *et al.*, 2011).
Neither the growth of the bacteria nor PHA production was affected by the presence of the solvents and Nile red or Nile blue dyes (Spiekermann *et al.*, 1999).

Gas Chromatographic (GC) analysis of PHA offers measurable information about the total number and concentration of monomers present in the PHA. When used in addition with Mass Spectroscopy, information about the mass and identity of the monomer involved is also obtained (Lee *et al.*, 1997). This method involves simultaneous extraction and methanolysis of PHA, in mild acid or alkaline conditions, to form hydroxyalkanoate methyl esters which are then analyzed by GC (Reetha, 2008). This method can handle small quantities of samples and can be described as rapid, sensitive, and reproducible (Reetha, 2008).
Other methods of analyzing PHA include High Performance Liquid Chromatography (HPLC), Nuclear Magnetic Ressonance spectroscopy (NMR) and Fourier Transmission Infra Red spectroscopy (FTIR) (Reetha, 2008).

Chapter II- Motivation

Synthetic plastics have become one of the most utilized materials over many years because of their versatility and outstanding technical properties including high chemical resistance and elasticity as well as their relatively low production cost, however there has been an increasing concern for the environment over the harmful effects of petrochemical-derived plastic materials, especially because they are not biologically degraded (Zinn *et al.*, 2001; Reddy *et al.*, 2003).

Polyhydroxyalkanoates (PHAs) are polyesters synthesized by a number of microorganisms as an energy reserve material, particularly in the presence of excess carbon and an essential nutrient such as nitrogen or phosphorous is limited (Laycock *et al.*, 2013). PHAs possess properties similar to currently used synthetic thermoplastics and elastomers making them strong candidates for biodegradable polymer material. Upon disposal, they are completely degraded to water and carbon by microorganisms in various environments such as soil, sea and lake water (Lee, 1996).

The objective of this study was to screen for strains with capability of producing innovative PHAs. Although many bacteria can produce PHA when supplied with the suitable growth conditions and carbon substrates, not all bacteria can yield a high production level of PHA. The selection of the best producers was made and optimization of PHA production by the selected microorganisms was performed.

Chapter III- Materials and methods

3.1. Isolation of microorganisms

In this study, bacteria were isolated from samples taken from various marine ecosystems in the Archipelago of Madeira in the Atlantic Ocean, obtained through the *Ocean Treasures* project. Strains were directly isolated from 1,918 mother plates where marine samples had been initially plated.

The composition of the agar medium used for isolation of the microorganisms was named A1 and contained the following compounds: 10 g/L of starch, 4 g/L of yeast extract, 2 g/L of peptone, 18 g/L of Agar, 750 ml of filtered sea water and 250 ml of Milli Q water. To ensure sterility and total dissolution, the medium was autoclaved at 121ºC for 20 minutes before using. The plates were prepared in a laminar flow chamber to ensure total sterility and prevent contamination of the plates.

3.2. Primary screening of bacterial isolates for PHA production on solid medium

In order to evaluate the PHA content in cells, the lipophilic dye Nile Red was added directly to the agar medium to a final concentration of 0.5 µg/mL was used. A solution of 5 mg Nile red per 10 mL dimethylsulfoxide (DMSO) was prepared previously.

Medium A2 used for screening of the PHA-producing microorganisms contained the following compounds: 20 g/L of starch, 2 g/L of yeast extract, 1 g/L of peptone, 18 g/L of Agar, 750 ml of filtered sea water and 250 mL of Milli Q water. The medium was autoclaved at 121 ºC for 20 minutes after 500 µL of Nile red solution was added separately to the sterilized medium.

Previously isolated colonies were spread on the agar plates. After growth during 72 hours at room temperature, plates were exposed to Blue Light Transilluminator (Safe Imager^{TM}, Invitrogen) and PHA positive isolates were seen as orange/yellow fluorescent colonies.

3.3. Secondary screening in 25 mL liquid cultivation

To confirm the production of PHA, the 25 bacteria presenting a fluorescence signal with the highest intensity were selected and grown in liquid medium. The 25 PHA positive bacteria isolates were grown in 250 mL Erlenmeyer flasks containing 25 mL of the Medium A2 (without

agar). One isolated colony from agar plates was used to inoculate. The inocula were incubated at 28 ºC and 200 rpm. After 120 hours, the bacterial cells were harvested. The biomass was analyzed for PHA content.

3.4. 100 mL-scale cultivation for growth and PHA determination

The 2 strains with the highest PHA content obtained during 25 mL liquid culture were grown in 100 mL liquid culture to determine better growth parameters. Three media were tested: Medium A2, medium E (adapted from Brandl *et al.*, 1988) supplemented with glucose and Mineral Marine Medium (Brauman *et al*, 1971). Solutions 1 and 2 were autoclaved separately at 121 ºC for 20 minutes.

Table 3- Constitution of Mineral Marine (Brauman *et al*, 1971)

	Mineral Marine medium	*g/L*
Solution 1	NaCl,	11.7
	$MgSO_4.7H_2O$;	12.3
	KCl;	0.75
	$CaCl_2.2H_2O$;	1.47
	$C_4H_{11}NO_3$;	6.05
	NH_4Cl	6.65
	$K_2HPO_4.3H_2O$	0.062
	$FeSo_4.7H_2O$.	0.026
Solution 2	Starch	20

Table 4- Constitution E medium (Adapted from Brandl et al., 1988)

	Mineral Marine medium	*g/L*
Solution 1	$(NH_4)2HPO_4$	3.3
	K_2HPO_4	5.8
	KH_2PO_4;	3.7
	$MgSO_4$	10 ml
	Micronutrient	1 ml
Solution 2	Glucose	20

Inocula were prepared in 25 mL of respective medium using one isolated colony from agar plates and incubated for 17 hours at 28 ºC and 200 rpm. 25 mL of inocula in exponential phase (with 17 hours of incubation time) were added to 100 mL of fresh medium. Cultures were performed in 250 mL shake flasks without pH control at 28 ºC and 200 rpm. The assays were run for 46 hours. 2 mL samples were taken periodically. Optical density was monitored immediately and 1 mL samples were frozen at -20 ºC for further PHA quantification.

3.5 Bioreactor cultivation

3.5.1. Medium preparation

The medium used for inocula preparation in the batch reactor was named A3 and contained the following compounds: 40 g/L of starch, 8 g/L of yeast extract, 4 g/L of peptone, 750 mL of filtered sea water and 250 mL of Milli Q water. The medium was autoclaved at 121 ºC for 30 minutes. The Medium A2 was also tested.

3.5.2. Inocula preparation

One isolated colony from agar plate was used to inoculate 100 mL of medium in a 1 L shake flask. The inocula were incubated 24 hours at 28 ºC and 200 rpm.

3.5.3. Operating conditions

This assay was performed in a 2 L reactor (BioStat® B-Plus, Sartorius) with 2 L of working volume where sterile conditions were maintained (Figure 3). The reactor was operated at 28 ºC. pH was maintained at 7.0±0.05 by automatic addition of 2M HCl and 2M NaOH. The dissolved oxygen inside the bioreactor was maintained above 30 % by manually increasing the agitation speed from 400 rpm to 900 rpm. Air flow rate was maintained at 1 vvm. Foam formation was suppressed by addition of an Antifoam solution (BDH Prolabo – VWR).
100 mL of inoculum in the exponential phase were used to inoculate the bioreactor. 10 mL samples were taken periodically during approximately 12 hours. OD at 600 nm was monitored immediately after sampling. Samples were frozen for further analysis of biomass production, PHB content and total organic carbon quantification.

Figure 3- Batch reactor (BioStat® B-Plus, Sartorius)

3.2. Analytical Methods

3.2.1. Cell growth

Cell growth was monitored by measuring the optical density of 1 mL of sample, at 600 nm (OD600) with a spectrophotometer (Elios α, ThermoSpectronic).
For cell dry weight determination, biomass was collected from 10 or 25 ml of culture broth by centrifugation at 10,000 rpm for 10 minutes. The supernatant was collected for further analysis (see 2.2.3). The pellet was resuspended in 10 or 25 mL of NaCl 0.9 % (w/v) and centrifuged again (10,000 rpm for 10 minutes). After centrifugation, the supernatant was discarded and the pellets were frozen in liquid nitrogen before being lyophilized (Telstar, Cryodos) for 20 hours, at approximately -50 ºC and 0.07 mbar. Cell dry weight (CDW) was defined by the dry weight of cells per L of culture broth.
(Equation 1):

$$CDW(g/L) = \frac{m}{V} \qquad (1)$$

Where m is the mass of the biomass lyophilized (g) and V is the volume of culture (L).

3.2.2. PHA quantification

PHAs were determined by gas chromatography (GC) using a method adapted from Serafim et al., 2004. After lyophilization, 2-4 mg of biomass was resuspended in 1 mL of a solution of methanol with 20 % sulfuric acid and 1 mL of chloroform containing 0.88 mg/ml of heptadecane (internal standard) to dissolve walls and extract PHA granules. Samples were digested in a thermoblock (AccuBlock™, Labnet) for 3.5 hours at 100 ºC. After cooling, 1 mL of distilled water was added. Samples were shaken for 1 minute in vortex. Chloroform phase contains the dissolved PHA was extracted and transferred to a 2 mL vial (0.3nm, Merck) with molecular sieves to remove traces of water. 2 µL of sample were injected in a gas chromatographer equipped with a flame ionization detector (Bruker 430-GC) and a BR-SWax column (60 m, 0.53 mm internal diameter, 1 µm film thickness, Bruker, USA), using helium as carrier gas at 1.0 mL/min. Samples were analyzed under a temperature regime starting at 40 ºC, increasing to 100 ºC at a rate of 20 ºC/min, to 175 ºC at a rate of 3 ºC/min and reaching a final temperature of 220 ºC at a rate of 20 ºC / min for ensuring cleaning of the column after each injection. Injector and detector temperatures were 280 ºC and 230 ºC, respectively. Hydroxybutyrate (HB) and hydroxyvalerate (HV) concentrations were determined through the use of two calibration curves, one for HB and other for HV, using standards (0.1-10 g L^{-1}) of a commercial co-polymer P(HB-HV) (88 %/12 %) (Sigma), and corrected using heptadecane as internal standard (concentration of approximately 1 g L^{-1}).Standards were processed in the same way as the samples.

3.2.3. Total organic carbon (TOC)

Total Organic Carbon was determined using a Shimadzu TOC automatic analyzer. Calibration curves in the range of 1 to 500 ppm were obtained with potassium hydrogen phthalate standards for total carbon and with sodium hydrogen carbonate standards and sodium carbonate standard for inorganic carbon. For the analysis, liquid samples were unfrozen, and 5 ml of sample was diluted to 5 mL with milliQ water.

3.2.4. Film preparation

Extraction of PHB from the biomass with chloroform is described in Fiorence et al., 2009 with modifications. 4 g of lyophilized biomass was heated in 200 mL of chloroform to a temperature of 65 ºC, under constant stirring, for 2 hours. Then, the cell debris was separated by centrifugation at 8,000 rpm for 20 min at room temperature. The polymer was recovered by solvent evaporation.

3.2.5. Microscopical analysis

3.2.5.1. Routine visualization

For microscopic visualization of fresh samples, a few drops of culture were put in a slide and observed under a microscope in phase contrast (Olympus BX51).

3.2.5.2. PHA content visualization

For visualization of the samples, 1 ml of culture broth was centrifuged at 8,000 rpm for 10 minutes and the supernatant was discarded. Then the pellet was resuspended in 1 mL of sea water and centrifuged again (8,000 rpm for 10 minutes). After centrifugation, the supernatant was discarded and it was added 50 µL of Nile blue solution and 500 µL of sea water was added to the pellet. This pellet was incubated at 40 ºC for 15 minutes for the purpose of cell immobilization. 20 µL were put in a slide and were observed in the same microscope using fluorescent lighting.

3.2.5.3. Gram staining preparations

To know if the samples are gram-positive or gram-negative, a thin smear of the culture was prepared on a glass slide and heat fixed. Primary crystal violet was added for 1 minute. Excess stain was washed off with distilled water. Lugol solution was added for 1 minute. The smear was then rinsed with water and ethanol. The secondary stain, Safranin, was added for 30 seconds. The slide was then washed with water and observed under a light microscope (Olympus BX51).

3.2.6. Calculations

The proportion of PHA monomers was calculated as a percentage of the total polymer produced on a Cmmol.
Assuming that the isolated PHA is a P(HB-co-HV) polymer, the PHA content (% PHA) was determined by Equation 1:

$$\%(PHA) = \%(HB) + \%(HV) \qquad (1)$$

The PHA content (%PHA) was determined by the Equation 2

$$\%PHA = \frac{m_{PHA}}{m_{cells}} \times 100\% \qquad (2)$$

Where m_{PHA} is the amount of PHA (mg) and m_{cells} (mg) is the amount of liophylized biomass in sample.

The specific growth rate (μ) was determined by the equation 3:

$$\mu = \frac{dX}{Xdt} \qquad (3)$$

Where X represents the concentration in biomass during time.

Chapter IV- Results and Discussion

4.1. Marine bacterial isolates

It is evident from various studies (Shrivastav *et al.*, Quillaguamán *et al.*, 2010) that marine environments can be an excellent source of PHA producing bacteria. However, despite the remarkable potential of the marine ecosystems to provide new and interesting strains for the production of polymers, there are only a limited number of marine bacteria with such characteristics (Quillaguamán et al. 2010).

In the present study, bacteria were isolated from samples taken from various marine ecosystems in the Archipelago of Madeira in the Atlantic Ocean. These samples were taken from the seabed at depths of 30 and 1,700 meters to obtain a larger diversity of microorganisms in an attempt to obtain new microorganisms and possibly new structures of PHAs.
A total of 724 strains were isolated from 612 mother plates where marine samples had been initially plated. All of the strains were successfully grown and pure colonies show different characteristics in morphologies and sizes (see Appendix I). Figure 4 illustrates an example of a mother plate with various colonies and an example with bacteria isolate with one single strain.

Figure 4- Agar plates. Left Mother plate; Right Bacteria isolate.

4.2. Screening of bacterial isolates for PHA production on solid medium

The production of PHA by microorganisms depends on the cultivation conditions including substrates and supplements provided. For regulation of their metabolism and for starting the process of carbon uptake and carbon accumulation in the form of granules as an energy reserve, the limitation of an essential nutrient as well as an excess of carbon in the environment in which they develop is necessary (Luengo *et al.*, 2003).
Nile Red was used during this work to develop a simple and highly sensitive staining method to detect PHAs directly in growing bacterial colonies (Spiekermann *et al.*, 1999). It is known that the Nile Red stain emits strong positive orange/yellow fluorescence signals only when it is linked to hydrophobic compounds like PHAs and lipids (Desouky *et al.*, 2007).
During growth, the Nile red diffuses into the cytoplasm and subsequently into the PHA inclusions, colonies of PHA-accumulating strains should fluorescence when the cells were cultivated under conditions permitting PHA accumulation, so, the direct addition of this dye to the medium provides a tool to discriminate between PHA-negative and PHA-positive strains without killing the cells (Spiekermann *et al.*, 1999).

The medium used at this stage contained 20 g/L of starch, 2 g/L of yeast extract and 1 g/L of peptone. The lipophilic dye Nile Red was added directly to the agar medium to a final concentration of 0.5 µg/mL (Shrivatav *et al.*, 2010; Spiekermann *et al.*, 1999).
The 724 isolated strains previously obtained were grown on agar plates containing Nile Red and incubated for 72 hours, time long enough to permit PHA accumulation but avoid PHA reutilization by the cells.

A total of 174 isolate strains from the 724 tested were found to emit a strong orange/yellow fluorescence under Blue Light after growth of 72 hours, suggesting the accumulation of polymer through the lipophilic dye Nile red (see Appendix II). Almost 75% of the isolated strains did not present any fluorescence.

Fluorescence signal was evaluated macroscopically as followed:
(+ + +) intense orange fluorescence;
(+ +) bright yellow fluorescence;
(+) yellow fluorescence and
(-) no fluorescence.

Table 5- Level of fluorescence intensity of 25 selected strains

Strain	Intensity level	Strain	Intensity level
MD12-071	++	MD12-581	+
MD12-103	++	MD12-582	+
MD12-107	++	MD12-017	+
MD12-125	++	MD12-018	+
MD12-222	++	MD12-020	+
MD12-226	+++	MD12-025	+
MD12-398	++	MD12-028	+
MD12-511	++	MD12-030	+
MD12-115	+	MD12-033	+
MD12-117	+	MD12-034	+
MD12-575	++	MD12-037	+
MD12-337	++	MD12-038	+
MD12-580	+	MD12-006	-

Bacteria with the most intense fluorescence signal were selected for further studies. Table 5 shows the fluorescence intensity of the 25 strains presenting the strongest fluorescence signal. Figure 5 shows the fluorescence signal emitted by four selected strains.

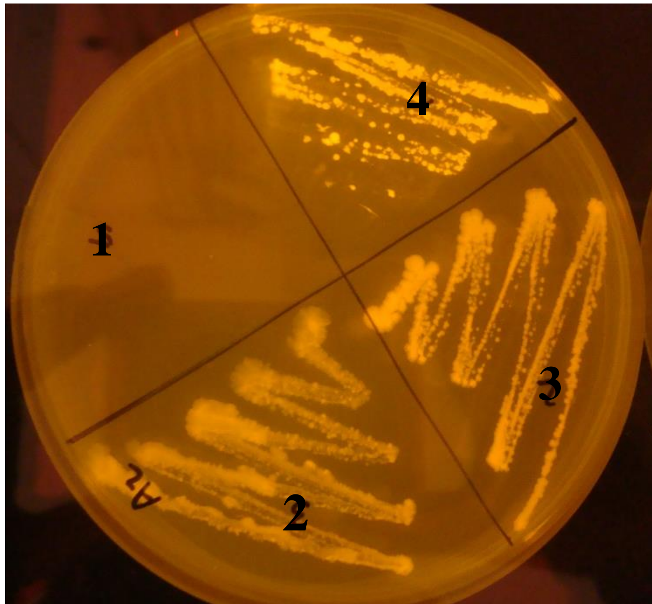

Figure 5-Fluorescent Nile red staining on agar plates of isolates 1: MD12-006; 2: MD12-581; 3: MD12-226; 4: MD12-107.

Colonies of PHA-positive strains of MD12-581(+), MD12-107(+ +), MD12-226(+ + +) exhibited a strong fluorescence, whereas the MD12-006 corresponded to a PHA-negative strain without any fluorescence. Isolated colonies of PHA-positive and PHA-negative strains located on the same agar plate were this way clearly distinguished.

As Nile red staining is not specific to PHA but could also bind to hydrophobic polymers such as lipids, and therefore to confirm the content of PHA, these 25 strains were grown in liquid medium for further analysis of PHA content via GC analysis.

4.3. Secondary screening in 25 mL liquid cultivation

The 25 strains (Table 5) presenting the highest signal on Nile Red plate were grown in 25 mL liquid Medium A2 to assess precisely the production of PHA for further selection of the best PHA producer strain. The medium contained 20 g/L of starch, 2 g/L of yeast extract and 1 g/L of peptone. The inocula were incubated at 28 ºC and 200 rpm. PHA, hydroxybutyrate (HB) and hydroxyvalerate (HV) storage contents and cell dry weight (CDW) for the 25 strains are presented in Table 6.

Table 6- PHA storage content, %HB, %HV, cell dry weight and level of fluorescence on plates with Nile Red

Strain	PHA	HB g(HB)/g(PHA)	HV g(HV)/g(HA)	CDW	Intense level
	(%)	(%)		(g/L)	
MD12-071	2.53	90.12	9.88	0.07	++
MD12-103	0.42	100.00	0.00	0.17	++
MD12-107	17.71	100.00	0.00	0.16	++
MD12-125	2.18	60.08	39.92	0.16	++
MD12-222	0.43	100.00	0.00	0.17	++
MD12-226	0.64	25.18	74.82	0.16	+++
MD12-398	3.58	87.43	12.57	0.03	++
MD12-511	3.94	4.06	95.94	0.17	++
MD12-115	0.68	100.00	0.00	0.11	+
MD12-117	1.95	87.69	12.31	0.06	+
MD12-575	1.83	93.58	6.42	0.16	++
MD12-337	1.28	87.75	12.25	0.12	++
MD12-580	0.98	87.51	12.49	0.15	+
MD12-581	9.30	97.76	2.24	0.16	+
MD12-582	1.53	89.59	10.41	0.15	+
MD12-017	3.41	91.89	8.11	0.24	+
MD12-018	3.22	89.24	10.76	0.18	+
MD12-020	2.47	89.40	10.60	0.24	+
MD12-025	4.28	90.44	9.56	0.15	+
MD12-028	2.64	89.24	10.76	0.22	+
MD12-030	3.19	89.24	10.76	0.18	+
MD12-033	3.44	89.24	10.76	0.17	+
MD12-034	3.74	68.37	31.63	0.21	+
MD12-037	2.43	89.24	10.76	0.24	+
MD12-038	4.02	89.30	10.70	0.15	+
MD12-006	0.00	90.12	9.88	0.00	-

(+++) Orange fluorescence; (++) Bright yellow fluorescence; (+) Yellow fluorescence; (-) No fluorescence

The results obtained showed that all 25 strains selected, were able to produce PHA. MD12-107 strain was considered the best PHB producer, because it accumulated up to 17.71 % PHA content, in agreement with its bright yellow fluorescence (see Figure 5) signal, indicating PHA accumulation during growth on plates. However, biomass production was quite low, below 0.24 g/L.

Although it presented a weak positive fluorescence signal, the MD12-581 strain still produced 9.3% PHA (Figure 5). Similarly, although strain MD12-103 presented a bright yellow fluorescent colonies with Nile red (+ + +), it had the lowest PHA content with only 0.42 %, Besides, the strain MD12- 226 showed high positive fluorescence signal (Figure 5), but was a weak producer PHA with only 0.64 % PHA content.
In conclusion, Nile red screening cannot be directly correlated to PHA content as a strong fluorescence signal does not correspond necessarily to a high percentage of PHA within the bacteria. It estimates only the level of hydrophobic compounds whether PHA or other types of lipids (Doan and Obbard, 2011).
To confirm that the MD12-006 strain was PHA-negative, the strain was included in the 25 mL liquid culture screening. As expected, this strain did not accumulate PHA (proving to be a true negative).

The 2 best PHA-producers strains were further tested with Nile blue. Nile blue is another lipophilic dye used to stain colonies and to distinguish between PHA-accumulating and non-accumulating strains (Spiekermann et al., 1999). This way, it is possible to observe the PHA accumulation by fluorescence microscopy (Ostle and Holt, 1982). 1 ml of culture broth with 50 µL of Nile blue solution was used. Figure 6 illustrates the florescence obtained after staining with Nile blue with MD12-107 and MD12- 581 strains, showing clearly accumulation of PHA granules.

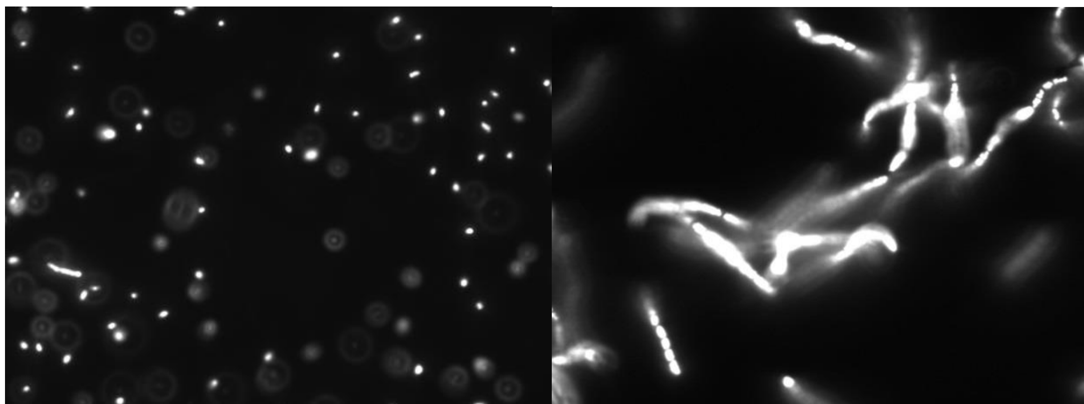

Figure 6- Fresh samples of MD12-107 and MD12-581 strains with Nile blue solution after 72 hours of incubation (1000x); Left: MD12-107; Right: MD12-581.

Based on these results, the strains selected for further optimization were strains MD12-107 and MD12-581. However, one cannot despise the remaining 149 strains that were not tested because, although they showed no intense fluorescence signal, they might still be able produce high percentage of PHA.

4.4. 100 mL-scale cultivation for growth and product determination

In order to determine better the maximum PHA storage capacity of strains MD-107 and MD-581, cells were grown still in medium A2 but in a 100 mL-scale cultivation. Inocula were prepared in 25 mL of 20 g/L of starch, 2 g/L of yeast extract and 1 g/L of peptone and incubated for 17 hours at 28 °C and 200 rpm.

The medium used in the present work is composed of sea water (75 % per Liter) with a salinity of about 3.5 % (Stanley and Morita, 1965). However, variation in sea water composition could interfere with reproducibility of experiments. In order to insure reproducibility, two media of known composition were tested: Marine Mineral medium (Baumam *et al.*, 1971) and the E medium (Adapted from Brandl *et al.*, 1988) (with 75 % seawater) supplemented with glucose.

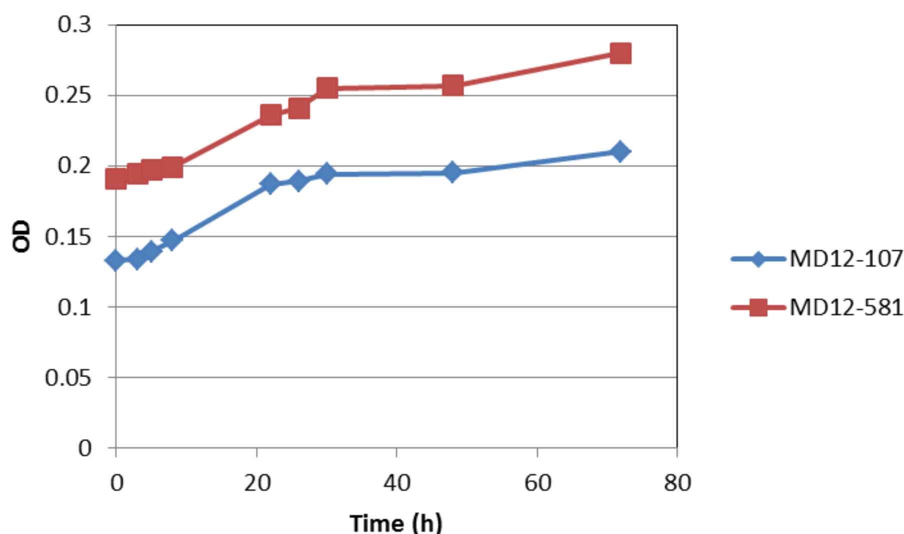

Figure 7- Growth of strains MD12-107 and MD12-581 in Marine Mineral Medium**.**

The synthetic Marine Mineral medium has a defined composition substituting seawater. This medium was constituted with 11.7 g/L of NaCl, 12.3 g/L of $MgSO_4.7H_2O$; 0.75 g/L of KCl; 1.47 g/L of $CaCl_2.2H_2O$; 6.05 g/L of $C_4H_{11}NO_3$; 6.65 g/L of NH_4Cl; 0.062 g/L of $K_2HPO_4.3H_2O$ and 0.026 g/L of $FeSo_4.7H_2O$. Figure 7 illustrates the growth of MD12-107 and MD12-581 on Mineral Marine medium. Bacteria showed very slow growth with very low biomass production with a maximum OD of 0.210 after 72 hours.

As previously mentioned, the environment where strain growth has been performed was a starch carbon source. Starch is a low cost carbon source available in large quantities (Kim, 2000). It has become of interest to use less expensive substrates as an alternative to glucose in PHA production (Song *et al.*, 2012) but a traditional and commonly used substrate for bacterial production of PHAs is glucose (Lee, 1996). A medium with 20 g/L of glucose; 3.3 g of $(NH_4)2HPO_4$; 5.8 g of K_2HPO_4; 3.7 of g KH_2PO_4; 10 mL of $MgSO_4$ and 1 mL of micronutrient was tested. However, when glucose was added to the medium E, phosphates precipitated due to the high salt concentration of seawater, turning the cultivation impossible.

Biomass production is depicted in Figures 8 and 9 for strains MD12-107 and MD12-581 respectively. pH was followed during the cultivation to evaluate if there was any kind of acid production during growth and verify that the culture would not stop because of a too low pH.

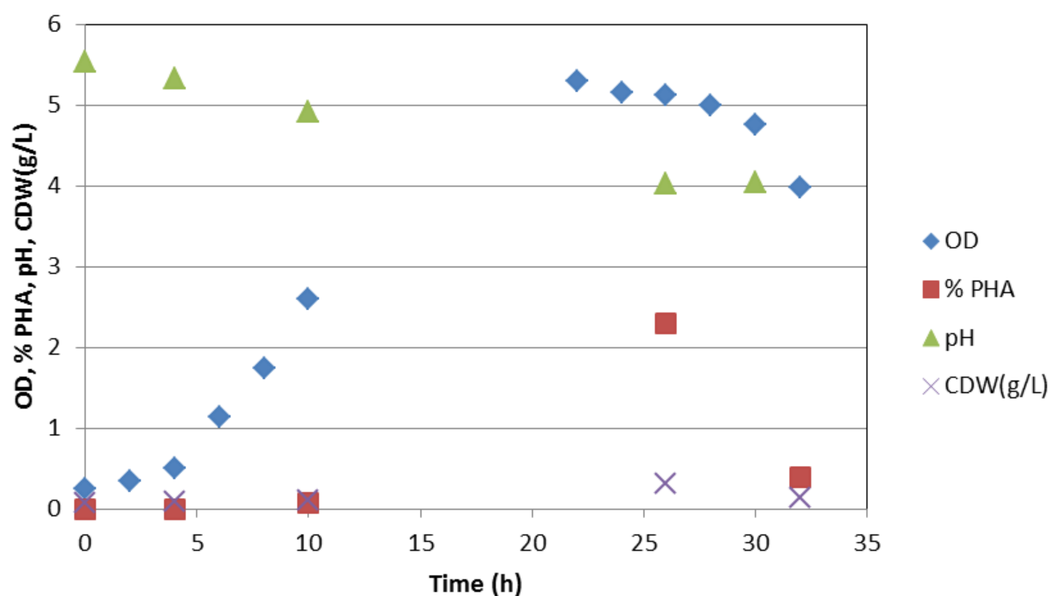

Figure 8- Growth of MD-107 on Medium A2 in 100ml-scale cultivation.

From the results obtained during cultivation in 25 mL liquid, the strain MD12-107 showed to be the best in terms of biomass production and PHA storage. However, during cultivation in 100 mL flaks, this strain only showed a negligible biomass production and PHA content, reaching only of 2.3 % PHA content after 26 hours of growth with OD 5.12, whereas it reached 17.71 % in 25 mL culture. pH was 4.02 after 26 hours, corresponding the highest percentage of PHA. During PHA content accumulation stage, pH was decreasing continuously from 5.5 down to 4.02. During the growth, the strain eventually produces any acid substance which causes pH decrease. When growth terminates, the strain stops producing PHA and pH keeps constant. Biomass concentration only reached 0.306 g/L.

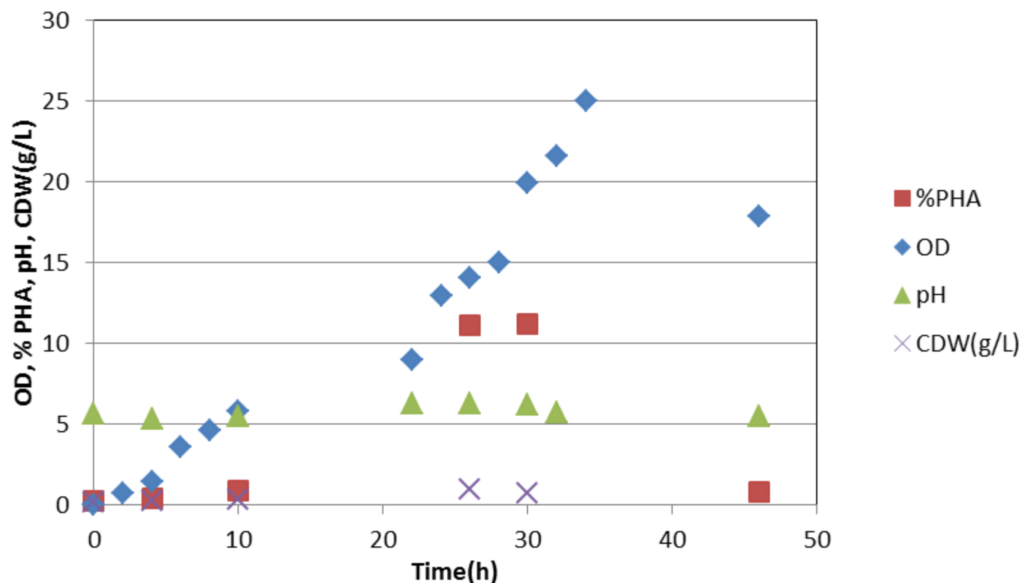

Figure 9- Growth of MD-581 on Medium A2 in 100ml-scale cultivation.

MD12-581 showed the most promising results, with a higher percentage of PHA content in 100 mL than in 25 mL (9.30 %) as well as having a significant biomass production. This strain accumulated a maximum content of 11.2 % of PHA in 30 hours (Figure 9). The highest biomass concentration was observed after 26 hours at 0.966 g/L.

These results show that the strain MD12-581 performed better than the strain MD12-117, producing a higher amount of PHA.

4.5. Bioreactor cultivations

4.5.1. Bioreactor cultivation with Medium A2

To evaluate the performance of the strain MD-581 and determine better growth parameters, the strain was grown in controlled batch reactor. Bioreactor cultivation offers many advantages for production, provided that contamination is avoided and the stability of the strain is guaranteed. The advantages include simplicity of culture control, homogeneity of the production, and constancy of culture conditions (Lillo *et al.*, 1990).

The medium used contained 20 g/L of starch, 2 g/L of yeast extract, 1 g/L of peptone. The results obtained regarding PHA content, % pO_2 and CDW are shown in Figures 10 and 11.

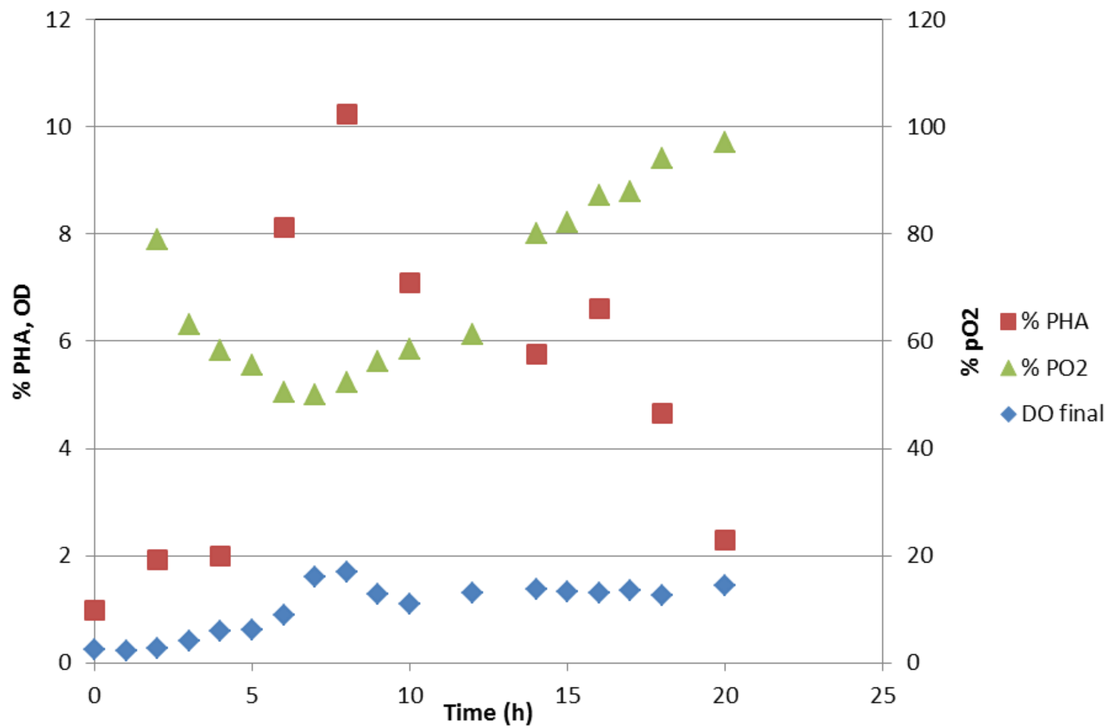

Figure 10- Growth of the Gram-negative MD12-581 with Medium A2 in bioreactor, % PHA, OD and % pO_2.

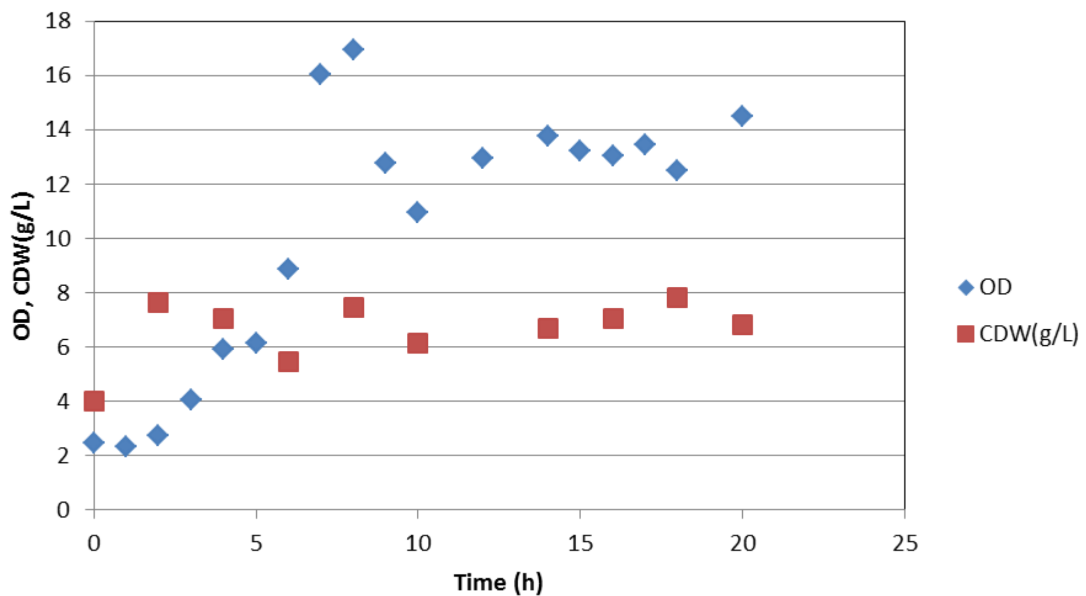

Figure 11- Growth of Gram-negative MD12-581 with Medium A2 in Bioreactor.

The higher supply of air contributed for enhancing cells growth, going from 0.966 g/L in 100 mL cultivation to 7.45 g/L in bioreactor (Figure 11). Exponential phase started just 2 hours after inoculation. After 8 hours, cells reached already the decline phase. Specific growth rate was 0.30 h^{-1}. The PHA production began after 2 hours and within 8 hours reached 10.23 %. However, after 10 hours of cultivations, PHA concentration started to decrease as the strain switches to PHA storages reserve.

4.5.1 Bioreactor cultivation with medium A3

PHA content using Medium A2 (20 g/L of starch; 2 of yeast extract and 1 g/L of peptone) reaches approximately 10 %. This is a promising value but still very low to be of practical relevance. In order to further increase PHA content, the medium was modified with both higher content in carbon source and nitrogen source. Nitrogen limitation was maintained to promote PHA storage.

The medium A3 contained 40 g/L of starch; 8 of yeast extract and 4 g/L of peptone. Both strains MD-107 and MD-581 were grown in duplicates.

4.5.1.1. Strain MD12-107:

The results obtained with the Gram-positive MD12-107 strain in bioreactor cultivation regarding PHA content, pH, % pO_2, CDW and Organic Carbon concentration are illustrated in Figures 12 and 13.

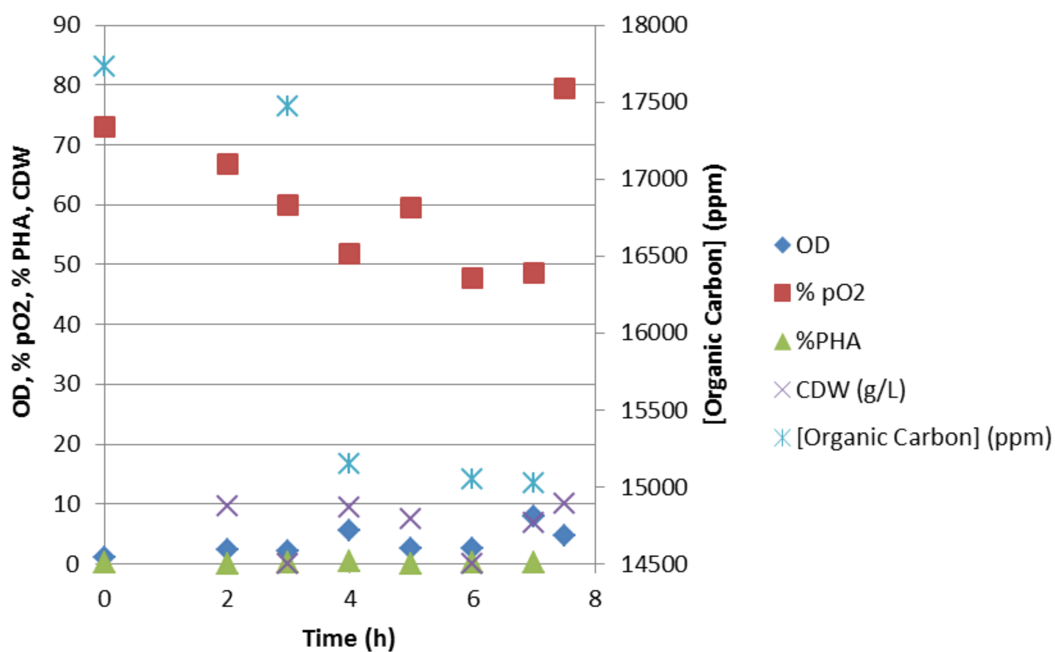

Figure 12- Growth of Gram-positive MD12-107 strain in Bioreactor with Medium A3. OD, % pO_2, % PHA, CDW (g/L) and [Organic Carbon] (ppm). First bioreactor cultivation.

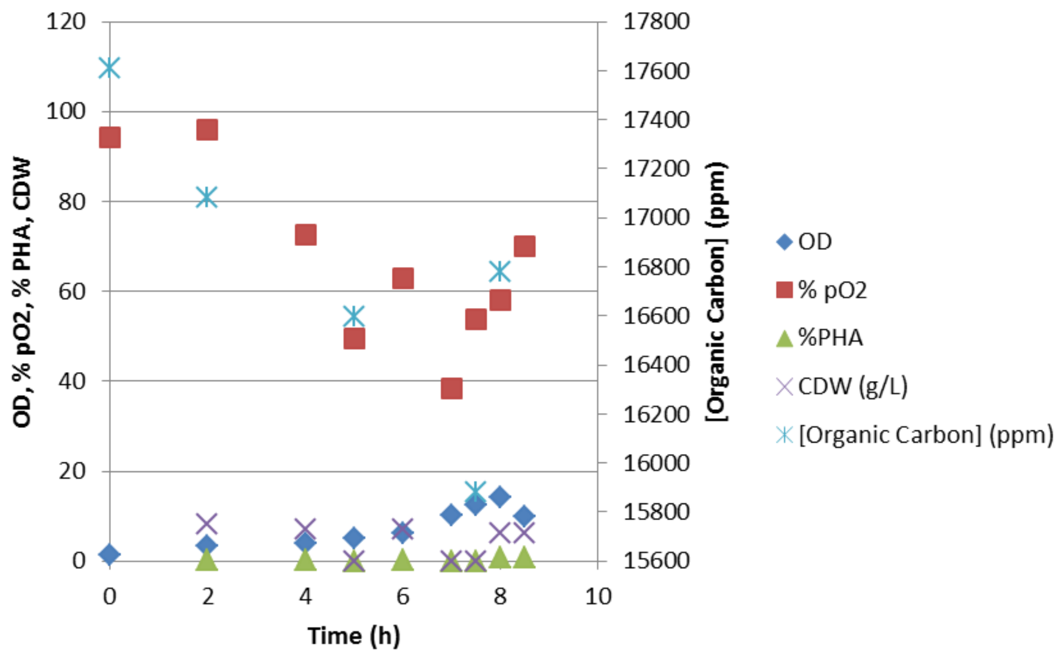

Figure 13- Growth of Gram-positive MD12-107 strain in Bioreactor with Medium A3. OD, % pO2, % PHA, CDW (g/L) and [Organic Carbon] (ppm). Second bioreactor cultivation.

The results obtained with the strain MD12-107 showed that PHA content storage in bioreactor was much lower than in 100 ml cultivation (10.23 %) (Figures 12 and 13). PHA percentage in both experiments was less than 1 % whereas biomass reached 8-10 g/L.

In both experiments, the dissolved oxygen showed a decrease of throughout time but organic carbon consumption was insignificant (less than 3000 ppm).Organic carbon estimation was used to get an estimation of starch consumption, as its complex polymeric structure does not allow a precise measurement. Specific growth rate was 0.18 h^{-1} (Figure 12) and 0.16 h^{-1} (Figure 13). pH was maintained at 7.0±0.05 by automatic addition of 2 M HCl and 2 M NaOH for efficient production. A possible explanation for no PHA accumulation may be this pH value. In 100 mL cultivation, the pH value was around 4-5 which may be an optimal pH for PHA accumulation in the marine bacterium MD12-107. Another explanation could actually be excess of nitrogen source: in flasks, the ratio starch:YE:peptone (20:2:1) was used whereas in the bioreactor a ratio of 40:8:4; in these conditions, the limitation in nitrogen might not be strong enough for PHA accumulation .

4.5.1.2. Strain MD12-581

The results obtained in bioreactor cultivation for the Gram-negative MD12-581 strain regarding PHA content, pH, % pO_2, CDW and Organic Carbon concentration are illustrated in Figures 14 and 15.

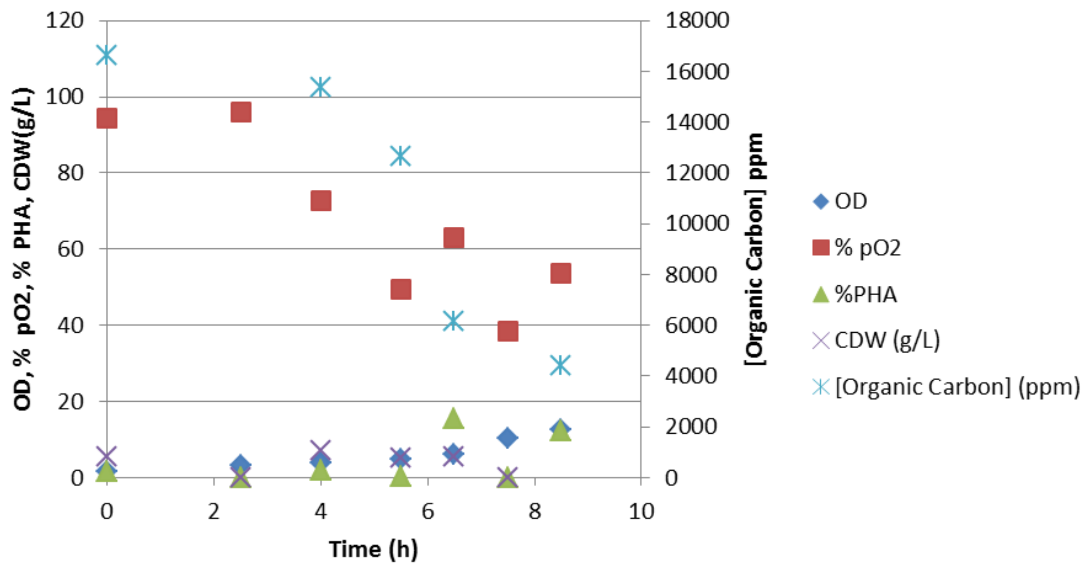

Figure 14- Growth of Gram-negative MD12-581 strain in Bioreactor with Medium A3. OD, % pO_2, % PHA, CDW (g/L) and [Organic Carbon] (ppm). First bioreactor cultivation.

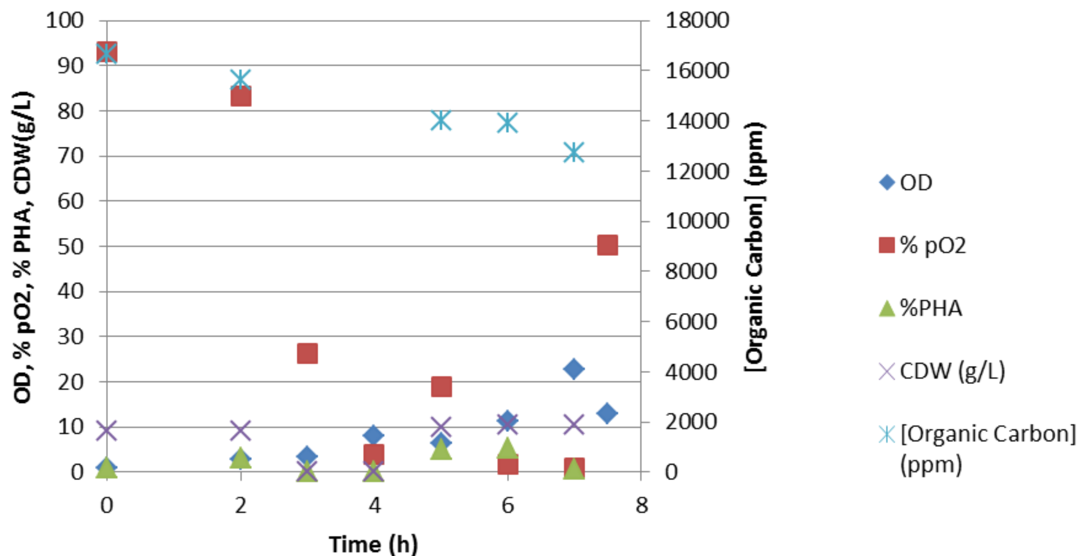

Figure 15- Growth of Gram-negative MD12-107 strain in Bioreactor with Medium A3. OD, % pO_2, % PHA, CDW (g/L) and [Organic Carbon] (ppm). Second bioreactor cultivation.

In first bioreactor cultivation (Figure 14), exponential phase started just 2.5 hours after inoculation. After 8.5 hours, cells reached already the decline phase. Specific growth rate was 0.24 h^{-1}. The PHA was produced after 2 hours and maximum PHA production was obtained at 15.40 % within 6.5 hours of batch fermentation. The highest CDW in microbial cell was 7.12 g/L when OD_{600nm} was 3.96. A significant consumption of organic carbon 12200 ppm (corresponding to around 12.2 g organic carbon/L) was verified. The pH was maintained at 7.0±0.05 by automatic addition of 2 M HCl and 2 M NaOH.
The PHA content obtained is promising, but still low compared to others studies. The maximum PHA content of *Ralstonia eutropha* can reach 75 % to 80 % of cell dry weight in 48 hours to 60 hours (Chen, 2009). *Alcaligenes latus* can accumulate up to 50 % PHB on glucose or sucrose in 18 hours of growth (Yamane et al., 1996). Maximum PHA contents for *Amerononas hydrophila* and *Pseudomonas oleovorans* were reported as 50 % and 63 %, respectively (Chen, 2001; Jung, 2001). Azotobacter chroococum in fed-batch cultivation with carbon source hydrolyzed starch and oxygen limitation had PHA content 46 % (Dalcanton *et al.*, 2010).

It seems that during the second cultivation, PHA production was actually compromised by low levels of air in the reactor (Figure 15 compared to Figure 14). The main difference between the two experiments was the level of dissolved oxygen during growth. In the first cultivation, pO_2 did not decrease below 38 % whereas in the second cultivation the dissolved oxygen presented very low values, reaching minimum value 0.9 % after 7 hours. Cell concentration and PHA productivity usually increased with increasing maximum agitation speed (Kim et al., 2000). Exponential phase started just 3 hours after inoculation. After 7 hours, cells reached the decline phase. In this second bioreactor, specific growth rate was slightly higher at 0.34 h^{-1}. The PHA was produced after 3 hours and maximum PHA production was obtained at 5.37 % within 7 hours of batch fermentation. The biomass production reached 10.54 g/L, much higher than during the 1st batch where it reached 7.5 g/L. only 3920 ppm of organic carbon (corresponding to around 3.9 g/L) was consumed, suggesting that limitation in aeration actually compromised carbon consumption and therefore PHA accumulation.

4.7. PHA film

The main objective of PHA production by microorganisms is the substitution of common fossil-fuel based plastics by biodegradable ones. However, to compete with these already established plastics, PHAs have to fulfill several mechanical properties. In particular, the produced polymer has to be able to form films.
In order to assess the film formation capacity of the PHA produced in this work, PHA was extracted from 4 g of lyophilized biomass in 200 mL chloroform at 65 ºC, under constant stirring, for 2 hours. The polymer was recovered by solvent evaporation.

MD12-581 strain produced a weak and breakable film with 0.12 g from 50 ml of culture broth. Figure 15 illustrates the PHA film obtained. The film still contains remaining's of biomass. On the other hand, PHA extracted from the MD12-107 strain did not produce any film certainly because of the low percentage of PHA produced during the bioreactor cultivations (Figures 12 and 13).

Figure 16- PHA film produced from MD12-581 biomass.

Chapter V- Conclusion and future work

The efficiency of PHA production by bacteria depends on the species and how the nutrient is given to the bacteria. Plastics produced from bacteria have become a possible solution to dumping waste because these plastics can break down easily when compared with conventional plastics produced from petroleum-based compounds.

The carbon sources contribute significantly to the high production costs of PHA, generally representing about 50 % of the cost (Posada *et al.*, 2011). Therefore, the selection of microorganisms capable of producing PHA efficiently using inexpensive substrates is of utmost importance (Kim, 2000). In order to analyze the polymer's ability to synthesize from renewable sources at a low cost, starch was used as a carbon source. According to Kim, 2000 and Sheu et al. 2009, since starch is a renewable carbon source available in large quantities, several studies have been conducted to assess the production of the polymer from this substrate.

The marine ecosystem is an important resource for searching for PHA producing bacteria. In this study, 25 % of the organisms showed high fluorescence through the lipophilic dye Nile red under the growth conditions studied, indicative of accumulation of the polymer and 25 strains were grown to confirm PHA content by gas chromatography. MD12-107 and MD12-581 strains presented a high PHA production capacity (17.71 % and 9.30 % respectively) relatively other 23 strains studied. PHA granules were identified in microscopic samples of fresh cultures of strains MD12-107 and MD12-581 stained with Nile blue.

The MD12-581 strain proved to be efficient in PHA production with a maximum production of 15.4 %, during bioreactor cultivation on 40 g/L starch demonstrating its potential for bioconversion in PHA. This strain produced a weak and breakable PHA film.

The MD12-107 strain was able to produce 17.71 % in 25 ml culture, but showed no significant results when grown in 100 mL or in a bioreactor, presenting a PHA maximum of 2.3 % and 0.69 % respectively. This strain might be less tolerant in the reactor at pH 7 and the culture conditions might have had carbon and nitrogen source in excessive amounts.

Biomass production showed low values for production in 25 mL and 100 mL cultures. However, in the bioreactor that showed higher percentage of PHA content, the CDW maximum obtained was 10.54 g/L and 10.01 g/L for strain MD12-107 and MD12-581 respectively.

The values for specific growth route (0.18 h^{-1} - 0.16 h^{-1} for MD12-107 and 0.24 h^{-1}-0.34 h^{-1}) showed that strains MD12-107 and MD12-581 had a very rapid growth in a bioreactor.

The objective of this work was to screen and isolate promising PHA-producing bacteria from a marine environment. Selection of the best producers was made and preliminary optimization of PHA production by the selected microorganisms was performed. However, the results regarding the production of PHA content obtained are still low compared to what can be found in the literature (Dalcanton *et al.*, 2010; Chen, 2009; Chen, 2001; Jung, 2001;; Yamane *et al.*, 1996).
For this reason, the following strategies could be implemented:

- Better optimization and control of the PHA production system by using several other carbon sources and environmental factors such as pH, temperature and the availability of oxygen. These factors can be manipulated to check whether the production can be enhanced and whether or not high productivity can be accumulated;

- Optimization of culture conditions for strains MD12-107 and MD12-58 to increase production;

- Evaluation of the polymer's physical properties obtained and film purity;

- Testing of the other positive PHA-accumulators to find more efficient bacteria;

- 16S rRNA sequencation to characterize better the producing bacteria.

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Chapter VI- Annexes

Anexx 1- Colony picking of 612 mother plates.

07/09/12	Sample Number	Mother Plating date	Medium/Method	Colony ID	Color	Shape 1	Shape 2
MD12	30	7-06-12	S1	A	white	atomic bomb shape	dry
MD12	30	7-06-12	S1	B	white	atomic bomb shape	
MD12	30	7-06-12	S1	C	clear brwon	dry	
MD12	530	14-06-12	A1 S2	A	white pink	wet	
MD12	234	11-061-2	A1	A	white pink	translucide	semi-wet
MD12	234	11-06-12	A1	B	small transparente		
MD12	234	11-06-12	A1	C	yellow opaque	semi-wet	
MD12	560	15-06-12	112 S2	A	yellow	wet	
MD12	560	15-06-12	112 S2	B	clear brown	semi-wet	
MD12	560	15-06-12	112 S2	C	white brownish	semi-wet	
MD12	517	14-06-12	SWA S2	A	orange		
MD12	517	14-06-12	SWA S2	B	translucide creamy		
MD12	517	14-06-12	SWA S2	C	yellow creamy		
MD12	540	14-06-12	112 S2	A	pink white	wet	
MD12	540	14-06-12	112 S2	B	white yellow	dry	
MD12	540	14-06-12	112 S2	C	orange wrinkles		
MD12	540	14-06-12	112 S2	D	red pink		
MD12	540	14-06-12	112 S2	E	white creamy		
MD12	523	14-06-12	SWA S2	A	yellow		
MD12	523	14-06-12	SWA S2	B	pink creamy		
MD12	523	14-06-12	SWA S2	C	yellow spread		

MD12	523	14-06-12	SWA S2	D	white		
MD12	85	7-06-12	SWA S1	A	orange		
MD12	85	7-06-12	SWA S1	B	translucide creamy		
MD12	85	7-06-12	SWA S1	C	white creamy		
MD12	40	7-06-12	112 S1	A	beige creamy		
MD12	40	7-06-12	112 S1	B	white creamy		
MD12	518	21-07-12	A1 P1	A	brownish beige	translucide	
MD12	518	21-07-12	A1 P1	B	yellow	wet	
MD12	518	21-07-12	A1 P1	C	beige	translucide	
MD12	518	21-07-12	A1 P1	D	tan		
MD12	521	21-07-12	A1 P1	A	white slight grey	semi-wet	
MD12	521	21-07-12	A1 P1	B	dark pink	wet	
MD12	521	21-07-12	A1 P1	C	yellow translucide	wet	
MD12	521	21-07-12	A1 P1	D	yellow grey translucide	wet	
MD12	521	21-07-12	A1 P1	E	pink grey	dry	
MD12	558	15-06-12	M2 S2	A	huge white	wet	
MD12	130	8-06-12	A1 S2	A	white	shining	
MD12	130	8-06-12	A1 S2	B	brown on dry spread		
MD12	130	8-06-12	A1 S2	C	very dry wrinkles	light brwon	
MD12	130	8-06-12	A1 S2	D	pinkish	dry	
MD12	130	8-06-12	A1 S2	E	dry flat	pink	
MD12	528	14-06-12	M2 S2	A	yellow	translucide	wet
MD12	528	14-06-12	M2 S2	B	completely translucide	wet	
MD12	528	14-06-12	M2 S2	C	translucide	pink	
MD12	528	14-06-12	M2 S2	D	brown with white ring		
MD12	528	14-06-12	M2 S2	E	blach with yelloe	wet	
MD12	528	14-06-12	M2 S2	F	whitish	translucide	

MD12	515	21-07-12	A1 P1	A	orange	dry	
MD12	515	21-07-12	A1 P1	B	spread transparent	translucide	
MD12	515	21-07-12	A1 P1	C	light yellow		
MD12	515	21-07-12	A1 P1	D	grey brownigh		
MD12	555	15-06-12	M2 S2	A	transparent	white pink	
MD12	555	15-06-12	M2 S2	B	red pink	dry	
MD12	555	15-06-12	M2 S2	C	white brownish	semi-wet	
MD12	365	11-06-12	112 S2	A	yellow white	opaque	big
MD12	365	11-06-12	112 S2	B	white	translucide	
MD12	365	11-06-12	112 S2	C	greyish	semi-wet	
MD12	523	14-06-12	A1 S2	A	yellow grey	translucide	wet
MD12	523	14-06-12	A1 S2	B	yellow white	opaque	wet
MD12	523	14-06-12	A1 S2	C	white pink	wet	
MD12	523	14-06-12	A1 S2	D	pink brown	translucide	semi-wet
MD12	523	14-06-12	A1 S2	E	red	dry	
MD12	512	14-06-12	112 S2	A	pink orange	slimmy	
MD12	512	14-06-12	112 S2	B	grey white	slimmy	
MD12	512	14-06-12	112 S2	C	transparent		
MD12	512	14-06-12	112 S2	D	light brown	dry	
MD12	115	8-06-12	SWA S2	A	red orange	dry	
MD12	115	8-06-12	SWA S2	B	light orange	wet	
MD12	115	8-06-12	SWA S2	C	white		
MD12	115	8-06-12	SWA S2	D	white		
MD12	3	7-06-12	SWA S1	A	orange	dry	
MD12	3	7-06-12	SWA S1	B	pink	wet	
MD12	3	7-06-12	SWA S1	C	clear brwon	dry	
MD12	506	14-06-12	SWA S2	A	orange	wet	

MD12	506	14-06-12	SWA S2	B	greyish	small	dry
MD12	506	14-06-12	SWA S2	C	light orange		dry
MD12	506	14-06-12	SWA S2	D	white translucide	wet	
MD12	369	11-06-12	A1 S2	A	white	wet	
MD12	369	11-06-12	A1 S2	B	white	dry and flat	

12/09/13	Sample Number	Mother Plating date	Medium/Method	Colony ID	Color	Shape 1	Shape 2
MD12	579	15-06-12	SWA S2	A	roasted yellow		
MD12				B	white	dry	
MD12				C	dark red		
MD12	517	14-06-12	1/2 S2	A	grey	dry	
MD12				B	dirty white	wet	
MD12				C	roasted yellow		
MD12	99	7-06-12	S1	A	white yellow	semi-wet	
MD12	137	6-08-12	SWA S1	A	green		
MD12				B	black		
MD12	513	14-06-12	1/2 S2	A	pink	wet	
MD12					yellow	wet	
MD12	299	11-06-12	S2	A	salmon	wet	
MD12				B	orange	umber	semi-wet
MD12				C	bright yellow	wet	
MD12	396	1-106-12	1/2 S2	A	white yellow	semi-wet	rugous
MD12	528	14-06-12	A1	A	white yellow	wet	
MD12				B	translucide creamy	wet	
MD12				C	brownish beige	wet	
MD12	175	8-06-12	1/2 S2	A	transparent	rugous	big
MD12	100	8-06-12	A1	A	black center	dry	

MD12				B	grey		
MD12	512	14-06-12	SWA S2	A	green center		
MD12				B	yellow	wet	small
MD12	294	11-06-12	S2	A	yellow translucide	flowery	
MD12	35	7-06-12	1/2 S1	A	beige creamy	shining	
MD12	252	11-06-12	S2	A	brown		
MD12	361	11-06-12	1/2 S2	A	beige	rugous	
MD12				B	white		
MD12				C	yellow	wet	
MD12	183	30-17-12	1/2 P1	A	white slight grey	dry	
MD12				B	brownish beige	relief	
MD12	138	30-07-12	SWA P1	A	light pink	semi-wet	
MD12				B	transparent	wet	
MD12	174	30-07-12	A1 P1	A	orange	semi-wet	
MD12	178	30-07-12	A1P1	A	old rose	pale	shining
MD12				B	greenish brown	dry	
MD12	107	30-07-12	A1P1	A	light brown	spleen	
MD12				B	beige	pale	
MD12	438	28-07-12	A1P1	A	orange	shining	wet

13/09/12	Sample Number	Mother Plating date	Medium/Method	Colony ID	Color	Shape 1	Shape 2
MD12	487	13-06-12	S2	A	orange	shining	
MD12	157	8-06-12	A1	A	light beige	shining	wet
MD12				B	dark beige	dry	spleen
MD12	555	13-06-12	A1	A	yellow	shining	wet
MD12	123	8-06-12	A1	A	beige transparent	semi-wet	

MD12				B	transparent	shining	
	545	15-06-12	SWAS2	A	white translucide		
MD12				B	yellow translucide	wet	
MD12	213	23-07-12	SWAP1	A	dark beige	shining	
MD12				B	beige	spleen	
MD12	576	23-07-12	A1	A	salmon		
MD12				B	yellow		
MD12				C	dark yellow		
MD12	514	11-07-12	A1	A	dark yellow	wet	shining
MD12	56	7-06-13	SWAS1	A	light beige		
MD12	348	23-07-13	SWAP1	A	white	shining	

14/09/12	Sample Number	Mother Plating date	Medium/Method	Colony ID	Color	Shape 1	Shape 2
MD12	311	11-06-12	A1S2	A	dark orange	shining	
MD12	340	11-06-12	1/2S2	A	light yellow		
MD12	331	11-06-12	SWAS2	A	white	small	
MD12	59	7-06-12	S1	A	orange	shining	
MD12	52	7-06-12	A1s1	A	dark orange	small	shining
MD12				B	white	dry	pale
MD12	400	13-06-12	1/2S2	A	dark green	dry	
MD12	352	11-06-12	S2	A	light yellow	spleen	
MD12				B	dark orange	dry	
MD12				C	dark yellow	aqueous	wet
MD12				D	light yellow		
MD12	454	13-06-12	1/2S2	A	grey	shining	wet
MD12	384	11-06-12	1/2S2	A	dark orange	shining	wet
MD12				B	dark beige	wet	shining

MD12				C	white yellow	wet	
MD12	9	7-06-12	1/2S1	A	dark yellow	dry	
MD12				B	dark yellow	wet	
MD12	225	11-06-12	1/2S2	A	orange		
MD12				B	light pink	dry	
MD12				C	light yellow	spleen	
MD12	319	28-07-12	A1	A	red		
MD12				B	dark yellow	dry	

1 8/09/12	Sample Number	Mother Plating date	Medium/Method	Colony ID	Color	Shape 1	Shape 2
MD12	442	13-06-12	SWA	A	orange/salmon	dry	
MD12	382	23-07-12	A1	A	dark yellow	wet	
MD12	553	20-07-12	P1	A	light pink		
MD12	531	21-07-12	P1	A	beige	wet	
MD12				B	dark pink	wet	
MD12				C	black	dry	
MD12	655	21-07-12	P1	A	beige creamy		
MD12				B	dark yellow		
MD12	554	25-07-12	A1	A	dark beige	wet	
MD12	535	21-07-12	A1	A	yellow	shining	
MD12				B	light yellow	shining	
MD12	657	21-07-12	SWAP1	A	pink		
MD12				B	green		
MD12	657	21-07-12	A1	A	dark yellow	dry	
MD12	582	21-07-12	A1	A	dark pink	dry	
MD12				B	beige	wet	
MD12	3?5	23-07-12	P1	A	dark yellow		

MD12	224	21-07-12	A1	A	pink		
MD12	224	21-07-12	SWA	A	pink		
MD12	257	23-07-12	SWAP1	A	beige translucide	dry	
MD12	351	23-07-12	P1	A	beige translucide	dry	
MD12	203	23-07-12	P1	A	dark yellow	spleen	
MD12	257	23-07-12	P1	A	beige translucide		
MD12				B	beige	spleen	
MD12	208	23-07-12	P1	A	dark yellow	verry shining	
MD12				B	salmon creamy		
MD12	21?	21-07-12	P1	A	orange	dry	
MD12				B	brownish beige	shining	
MD12	652	21-07-12	SWAP1	A	light pink		
MD12	544	21-07-12	P1	A	light yellow	semi-wet	
MD12	642	21-07-12	P1	A	dark yellow	shining	
MD12				B	light yellow	shining	
MD12	632	21-07-12	A1	A	orange		
MD12				B	dark orange	creamy	
MD12	276	23-07-12	A1 P1	A	dark pink		
MD12				B	dark yellow		

19/09/12	Sample Number	Mother Plating date	Medium/Method	Colony ID	Color	Shape 1	Shape 2
MD12	384	11-06-12	1/2S1	A	dark yellow	shining	
MD12				B	yellow creamy	atomic bomb shape	wet
MD12				C	light yellow creamy	wet	
MD12	333	23-07-12	A1 P1	A	beige	wet	
MD12	201	23-07-12	SWAP1	A	pink	translucide	semi-wet
MD12	72	7-061-2	SWAS1	A	light orange		

MD12				B	orange wrinkles	semi-wet	
MD12	228	21-07-12	SWAP1	A	white		
MD12	569	15-06-12	A1P1	A	orange creamy	wet	
MD12				B	beige	wet	
MD12				C	light orange		
MD12				D	dark beige		
MD12	590	21-07-12	A1P1	A	brown	branched	
MD12	237	21-07-12	SWAP1	A	light orange		
MD12	287	23-07-12	A1P1	A	grey	dry	
MD12	251	21-07-12	A1P1	A	dark yellow		
MD12				B	black		
MD12	231	21-07-12	A1P1	A	dark brown		
MD12	231	21-07-12	SWAP1	A	transparent		
MD12	569	23-07-12	SWAP1	A	pink creamy		
MD12	274	23-07-12	A1P1	A	beige creamy	wet	
MD12				B	white		
MD12				C	light brown	wet	
MD12	271	23-07-12	A1P1	A	light salmon	dry	
MD12				B	brown	creamy	
MD12	271	23-07-12	SWAP1	A	ligth green		
MD12				B	dark green		
MD12	598	21-07-12	SWAP1	A	white	translucide	
MD12	346	23-07-12	A1 P1	A	yellow	wet	translucide
MD12	347	23-07-12	A1 P1	A	brown	rugous	
MD12	135	8-06-12	A1S2	A	dark beige		
MD12				B	white slight grey	dry	
MD12				C	beige	dry	

MD12				D	transparent	small	
MD12	45	23-07-12	SWAP1	A	light orange	wet	
MD12				B	tan	dry	
MD12	355	23-07-12	A1P1	A	dark red	dry	
MD12	420	23-07-12	SWAP1	A	orange	shining	
MD12	343	23-07-12	SWAP1	b	white	dry	
MD12	267	23-07-12	SWAP1	A	very dry wrinkles	light green	
MD12	367	13-07-12	SWAP1	A	white	dry	
MD12	425	23-07-12	A1P1	A	dry flat	light brown	
MD12				B	beige	creamy	wet
MD12	338	23-07-12	A1P1	A	orange	wet	small
MD12	255	23-07-12	A1P1	A	light salmon	semi-wet	
MD12	367	23-07-12	230712	A	brown with white ring	shining	
MD12	254	23-07-12	A1P1	A	yellow	wet	gelatinous
MD12	281	23-07-12	A1P1	A	beige	creamy	
MD12	255	23-07-12	SWAA1	A	light yellow	creamy	wet
MD12				B	green	semi-wet	small
MD12	420	23-07-12	A1P1	A	light orange	semi-wet	small
MD12	341	23-07-12	SWAP1	A	orange	small	semi-wet
MD12	423	23-07-12	A1P1	A	light beige	wet	big
MD12				B	beige	dry	big
MD12	282	32-07-12	A1P1	A	white brownish	wet	gelatinous
MD12	272	23-07-12	A1P1	A	dark beige	opaque	semi-wet
MD12				B	transparent	semi-wet	small
MD12	404	23-07-12	A1P1	A	light green	pale	spleen
MD12	288	23-07-12	A1P1	A	brown		
MD12				B	yellow brown	opaque	wet

MD12	340	23-07-12	A1 P1	A	orange	dry	
MD12	341	23-07-12	A1 P1	A	dirty transparent	translucide	semi-wet
MD12				B	beige		
MD12	278	23-07-12	A1P1	A	dark green	small	semy-wet

20/09/12	Sample Number	Mother Plating date	Medium/Method	Colony ID	Color	Shape 1	Shape 2
MD12	290	23-0712	SWAP1	A	white	opaque	
MD12	269	23-07-12	SWAP1	A	dark salmon	wet	small
MD12	289	23-07-12	SWAP1	A	white	aqueous	
MD12	387	23-07-12	A1P1	A	dark orange	wet	small
MD12	490	13-06-12	A1S2	A	beige	dry	
MD12				B	white creamy	big	
MD12				C	beige	big	
MD12	411	23-07-12	A1P1	A	white brownish	dry	
MD12				B	beige	rough	
MD12	553	21-07-12	A1P1	A	light brown	shining	
MD12				B	orange	pale	
MD12	374	23-07-12	A1	A	brown mud		
MD12				B	dark yellow	small	semi-wet
MD12	216	21-07-12	A1	A	dark beige	wet	
MD12	630	21-07-12	A1	A	beige	dry	
MD12				B	caramel	wet	big
MD12	652	21-07-12	A1P1	A	beige	rough	
MD12				B	light green		
MD12	339	23-07-12	A1	A	dark beige	wet	big
MD12				B	light caramel	dry	
MD12	342	23-07-12	SWAP1	A	yellow		

MD12	348	23-07-12	A1P1	A	light yellow	translucide	
MD12				B	beige translucide	creamy	
MD12	261	23-07-12	A1P1	A	dark beige	gelatinous	
MD12				B	yellow	verry shining	translucide
MD12	378	23-07-12	A1P1	A	white		
MD12	275	23-07-12	SWAP1	A	dark beige		
MD12				B	brownish beige	shining	rugous
MD12				C	dark beige	opaque	
MD12	114	8-06-12	1/2S2	A	salmon	semi-wet	
MD12				B	dark beige	shining	
MD12				C	light beige	shining	
MD12	49	7-06-12	A1S1	A	dark beige	shining	
MD12				B	beige	dry	small

21/09/12	Sample Number	Mother Plating date	Medium/Method	Colony ID	Color	Shape 1	Shape 2
MD12	111	8-06-12	A1S2	A	orange		
MD12				B	tan	shining	wet
MD12				C	beige	creamy	
MD12				D	white		
MD12	118	8-06-12	SWAS2	A	orange	semi-wet	
MD12	22	7-06-12	A1S1	A	beige	translucide	
				B	beige greyish		
MD12	45	7-06-12	A1S1	A	beige creamy	wet	
MD12				B	yellow	pale	
MD12					white	spleen	
MD12					beige creamy	semi-wet	
MD12	523	14-06-12	1/2S2	A	dirty white	dry	

MD12	245	21-07-12	A1	A	dark yellow	wet	
MD12					dark beige	creamy	shining

24/09/12	Sample Number	Mother Plating date	Medium/Method	Colony ID	Color	Shape 1	Shape 2
MD12	626	28-07-12	SWAP1	A	dark salmon		
MD12	613	28-07-12	A1P1	A	dark yellow	dry	small
MD12	245	21-07-12	SWA	A	white		
MD12				B	salmon		
MD12	10	7-06-12	SWAS1	A	black star	dry	
MD12				B	light orange		
MD12				C	white orange		
MD12				D	beige creamy		
MD12				F	caramel	wet	creamy
MD12	159	30-07-12	P1	A	beige	dry	
MD12				B	beige	wet	
MD12	75	7-06-12	SWAP1	A	white creamy		
MD12				B	white	umber	dry
MD12				C	light salmon	dry	
MD12	143	8-06-12	SWAS2	A	dark caramel	semi-wet	rugous
MD12	200	23-07-13	A1	A	light beige	rugous	
MD12				B	translucide creamy	star	
MD12	258	23-07-12	P1	A	brownish beige	gelatinous	
MD12				B	light brown	dry	
MD12	291	23-07-12	P1	A	dark beige center	dry	
MD12				B	light beige center	dry	
MD12	269	24-07-12	P1	A	light brown	gelatinous	
MD12	277	23-07-12	P1	A	grey	rugous	big

MD12	277	23-07-12	SWAP1	A	white	flowery	dry
MD12	292	23-07-12	P1	A	beige creamy	flowery	big
MD12				B	brown	creamy	
MD12	267	23-07-12	P1	A	caramel	dry	
MD12				B	caramel	gelatinous	
MD12	343	23-07-12	P1	A	beige creamy	dry	
MD12				B	brown	gelatinous	
MD12	57	23-07-12	SWAP1	A	brownish yellow	relief	
MD12	201	23-07-12	P1	A	caramel	semi-wet	
MD12				B	brown	wet	
MD12	370	23-07-12	P1	A	beige	relief	gelatinous
MD12				B	beige	pale	rugous
MD12	577	23-07-12	SWAP1	A	greenish beige	creamy	
MD12	273	23-07-12	SWA	A	orange	spleen	
MD12	373	23-07-12	P1	A	old orange	pale	
MD12	438	28-07-12	A1P1	A	orange	shining	wet
MD12	386	23-07-12	P1	A	beige	atomic bomb shape	gelatinous
MD12				B	beige	translucide	
MD12				C	light yellow	creamy	
MD12	337	23-07-12	SWAP1	A	white	atomic bomb shape	
MD12	337	23-07-12	P1	A	brown	translucide	semi-wet
MD12				B	beige	creamy	wet
MD12	604	23-07-12	A1P1	A	beige opaque	semi-wet	
MD12				B	caramel	wet	
MD12	586	210712	P1	A	beige		
MD12	594	210712	p1	A	yellow		
MD12				B	caramel	wet	

MD12	594	210712	SWA	A	yellow creamy	very wet	big
MD12	592	210712	P1	A	dark beige	gelatinous	
MD12				B	light salmon creamy	wet	
MD12	584	210712	112 S2	A	light orange	dry	
MD12				B	dark yellow		
MD12	645	210712	P1A1	A	dark beige creamy		
MD12				B	light yellow		
MD12				C	beige	translucide	wet
MD12				D	white		
MD12	651	210712	SWA	A	dark salmon		
MD12				B	light salmon		
MD12	651	210712	P1	A	orange and black		
MD12				B	beige	gelatinous	
MD12				C	dark beige		
MD12				D	white creamy		
MD12	219	210712	A1	A	dark beige creamy		
MD12				B	caramel	translucide	
MD12				C	white brownish	wet	
MD12				D	beige	spore	
MD12	219	210712	SWAP1	A	white		
MD12	27	70612	1/2S2	A	light yellow	semi-wet	
MD12				B	dark yellow	wet	
MD12				C	beige creamy	wet	
MD12	593	210712	P1	A	white	shining	
MD12				B	caramel		
MD12	597	210712	P1	A	dark red	dry	small
MD12				B	light salmon	shining	

MD12				C	dark beige		

28/09/12	Sample Number	Mother Plating date	Medium/Method	Colony ID	Color	Shape 1	Shape 2
MD12	654	210712	A1P1	a	dark orange	small	shining
MD12				B	dark yellow	gelatinous	
MD12				C	white brownish	small	wet
MD12	661	210712	SWAP1	A	dark yellow	wet	
MD12				B	dark orange	small	semi-wet
MD12	653	210712	P1	A	dark yellow	big	wet
MD12				B	beige/white	creamy	
MD12	658	210712	P1	A	white	translucide	small
MD12	575	230712	A1P1	A	white brownish	semi-wet	small
MD12				B	grey	dry	small
MD12	366	230712	A1P1	A	light pink	dry	small
MD12				B	dark yellow	small	semi-wet
MD12				C	dark beige	big	dry
MD12	366	230712	SWAP1	A	light yellow	small	dry
MD12				B	dark yellow	small	semi-wet
MD12				C	dark beige	big	dry
MD12	366	230712	SWAP1	A	light yellow	small	dry
MD12	356	230712	SWAP1	A	transparent	small	
MD12	375	230712	SWAP1	A	beige	semi-wet	small
MD12				B	light beige	big	dry
MD12	389	230712	SWAP1	A	orange	translucide	small
MD12	407	230712	A1P1	A	dark yellow	small	semi-wet
MD12				B	light brown	dry	
MD12	352	230712	A1P1	A	light yellow	gelatinous	wet

MD12				B	light brown	dry	
MD12				C	beige creamy	semi-wet	
MD12				D	castanho creamy	semi-wet	
MD12	375	230712	SWA	A	light orange	small	dry
MD12	389	230712	A1 P1	A	light beige	dry	
MD12				B	dark beige	rugous	
MD12	552	210712	SWAP1	A	dark salmon	small	semi-wet
MD12				B	dark orange	small	semi-wet
MD12	656	210712	SWAP1	A	white	small	dry
MD12				B	dark yellow	small	semi-wet
MD12				C	white creamy	small	
MD12	658	210712	A1 P1	A	light beige	translucide and gelatinous	wet
MD12				B	caramel creamy	wet	
MD12				C	light beige	wet	translucide
MD12	356	230712	A1P1	A	light brown	dry	
MD12				B	light beige	gelatinous	wet
MD12				C	white	dry	
MD12	206	230712	A1P1	A	brown	semi-wet	
MD12				B	beige	semi-wet	creamy
MD12				C	dark yellow	gelatinous	wet
MD12	266	230712	SWAP1	A	dark pink	small	semi-wet
MD12				B	light pink	small	wet
MD12	275	230712	A1P1	A	yellow translucide	wet	small
MD12				B	light salmon creamy	small	semi-wet
MD12				C	light pink	semi-wet	small
MD12				D	beige creamy	small	wet
MD12	285	230712	A1P1	A	light salmon creamy	dry	

MD12				B	dark yellow	dry	
MD12				C	white brownish	semi-wet	
MD12				D	white	opaque	dry
MD12				E	beige	dry	
MD12				F	salmon	small	semi-wet
MD12	273	230712	A1P1	A	dark beige	gelatinous	wet
MD12				B	dark beige	semi-wet	
MD12	388	230712	A1P1	A	brown	small	wet
MD12				B	beige	wet	small
MD12				C	white brownish	creamy	small
MD12				D	dark yellow	small	dry
MD12	388	230712	SWAP1	A	yellow	small	semi-wet
MD12	336	230712	A1P1	A	caramel	small	semi-wet
MD12				B	caramel	dry	
MD12	374	230712	A1P1	A	light salmon	semi-wet	small
MD12				B	green earth	dry	
MD12	372	230712	A1P1	A	beige creamy	semi-wet	
MD12				B	dark beige	gelatinous	wet
MD12	376	230712	A1P1	A	dark yellow	small	dry
MD12				B	dark beige	semi-wet	creamy
MD12				C	light beige	semi-wet	creamy
MD12	380	230712	SWAP1	A	light orange	small	wet
MD12	354	230712	A1P1	A	brown	semi-wet	
MD12				B	light brown	semi-wet	
MD12				C	beige translucide	wet	big
MD12				D	beige creamy	wet	
MD12	259	230712	A1P1	A	light pink	dry	

MD12				B	dark beige	dry	

01/10/12	Sample Number	Mother Plating date	Medium/Method	Colony ID	Color	Shape 1	Shape 2
MD12	466	23-07-12	A1P1	A	light yellow		
MD12				B	yellow	small	semi-wet
MD12	415	23-07-12	A1P1	A	dark beige	wet	small
MD12				B	light brown	wet	small
MD12	264	23-07-12	A1P1	A	caramel	dry	
MD12				B	beige creamy	wet	
MD12				C	brick color	small	wet
MD12	406	23-07-12	A1P1	A	light salmon	dry	small
MD12				B	white brownish	semi-wet	small
MD12				B	dark beige	dry	
MD12	390	23-07-12	A1P1	A	beige creamy	wet	
MD12				B	light beige	dry	
MD12	380	23-07-12	A1P1	A	beige	dry	
MD12				B	white brownish	semi-wet	big
MD12	285	23-07-12	A1P1	A	white	wet	
MD12	268	23-07-12	A1P1	A	white	semi-wet	
MD12	567	23-07-12	A1P1	A	light salmon	small	dry
MD12	242	21-07-12	A1P1	A	orange	wet	
MD12	521	21-07-12	SWAP1	A	transparent	dry	small
MD12	279	23-07-12	SWAP1	A	light pink aqueous	wet	small
MD12				B	dark salmon	small	semi-wet
MD12	292	23-07-12	SWAP1	A	white creamy	wet	small
MD12				B	orange	wet	small
MD12				C	white	dry	

MD12	371	23-07-12	A1P1	A	dark yellow	dry	
MD12				B	caramel	dry	
MD12	340	21-07-12	SWAP1	A	transparent	small	dry
MD12	535	21-07-12	SWAP1	A	yellow creamy	wet	small
MD12				B	salmon	wet	
MD12				C	light salmon	wet	
MD12				D	white	semi-wet	
MD12	655	21-07-12	SWAP1	A	light pink translucide	small	wet
MD12				B	dark yellow	small	semi-wet
MD12				C	dark yellow translucide	small	wet
MD12	345	21-07-12	A1P1	A	white	dry	
MD12	650	21-07-12	A1 P1	A	brick color	wet	
MD12				B	dark beige	gelatinous	wet
MD12				C	beige	wet	
MD12	226	21-07-12	A1P1	A	beige	dry	
MD12				B	orange	dry	
MD12				C	brown	semi-wet	
MD12	629	21-07-12	A1P1	A	beige creamy	wet	
MD12				B	beige	semi-wet	
MD12	234	21-07-12	SWAP1	A	orange	small	semi-wet
MD12	234	21-07-12	A1P1	A	beige	gelatinous	wet
MD12				B	dark beige	semi-wet	
MD12	381	23-07-12	SWAP1	A	yellow	semi-wet	small
MD12				B	light yellow	semi-wet	
MD12	402	23-07-12	A1P1	A	beige	semi-wet	
MD12				B	beige creamy	wet	

02/10/12	Sample Number	Mother Plating date	Medium/Method	Colony ID	Color	Shape 1	Shape 2
MD12	404	23-07-12	SWAP1	A	white	wet	
MD12				B	orange	small	semi-wet
MD12	648	21-07-12	A1	A	white	dry	small
MD12	381	21-07-12	A1P1	A	orange	wet	
MD12	662	21-07-12	A1P1	A	light pink	dry	
MD12				B	beige creamy	semi-dry	
MD12	540	21-07-12	A1P1	A	dark beige creamy	small	wet
MD12	643	21-07-12	A1P1	A	caramel	translucide	dry
MD12	306	21-07-12	A1P1	A	light salmon	semi-wet	small
MD12	301	21-07-12	A1P1	A	orange	dry	
MD12	unknown	unknown	A1P1	A	dark orange	dry	
MD12				B	dark salmon	dry	
MD12	549	21-07-12	SWAP1	A	light pink	wet	small
MD12	446	21-07-12	SWAP1	A	pink	semi-wet	small
MD12	530	21-07-12	A1	A	salmon	semi-wet	
MD12	546	21-07-12	A1P1	A	yellow	semi-wet	
MD12	412	23-07-12	SWAP1	A	light yellow translucide	small	wet
MD12	372	11-06-12	A1S2	A	yellow fluorescent	wet	
MD12	85	7-06-12	1/2S1	A	dark yellow	wet	small
MD12	296	11-06-12	A1	A	salmon	semi-wet	
MD12	150	8-06-12	1/2S2	A	dark yellow	wet	small
MD12	72	7-06-12	1/2S1	A	light yellow	wet	
MD12				B	dark yellow	wet	
MD12	373	11-06-12	1/2S2	A	light yellow	rugous	dry
MD12	32	7-06-12	A1P1	A	brown	rugous	semi-wet
MD12				B	light beige with halo	dry	

MD12				C	light brown creamy	wet	
MD12	170	8-06-12	SWAS2	A	transparent	dry	
MD12				B	salmon	small	wet
MD12	653	21-07-12	SWAP1	A	dark brown	small	dry
MD12				B	green earth	small	dry
MD12	545	21-07-12	SWAP1	A	transparent	gelatinous	wet
MD12				B	pink translucide	dry	
MD12				C	yellow	small	semi-wet
MD12	298	21-07-12	SWAP1	A	yellow brownish	small	semi-wet
MD12	645	21-07-12	SWAP1	A	light yellow	dry	
MD12	641	21-07-12	SWAP1	A	white translucide	wet	small
MD12				B	light pink	small	semi-wet
MD12				C	dark salmon	dry	
MD12	407	13-06-12	1/2S2	A	light beige	semi-wet	granulated
MD12	264	11-06-12	1/2S2	A	dark yellow	small	semi-wet
MD12				B	black with ring white	semi-wet	
MD12	427	13-06-12	A1S1	A	beige creamy	wet	
MD12	83	7-06-12	A1S1	A	brown creamy	granulated	semi-wet
MD12	83	7-06-12	1/2S1	A	light brown	small	wet
MD12				B	dark beige	dry	
MD12	509	21-07-12	SWAP1	A	light pink	semi-wet	small
MD12				B	white	opaque	dry
MD12	638	21-07-12	A1P1	A	light brown	wet	
MD12				B	beige/yellow	granulated	dry
MD12	242	21-07-12	SWAP1	A	dark beige	dry	small
MD12	214	21-07-12	SWAP1	A	white	translucide	dry
MD12	527	21-07-12	SWAP1	A	dark pink	wet	small

MD12	527	21-07-12	A1P1	A	dark pink	wet	small
MD12	217	21-07-12	A1P1	A	brown point with dark beige ring	wet	small
MD12	522	21-0712	SWAP1	A	dark yellow	dry	small

31/10/12	Sample Number	Mother Plating date	Medium/Method	Colony ID	Color	Shape 1	Shape 2
MD12	232	21-07-12	A1P1	A	orange	small	gelatinous
MD12	157	8-06-12	SWAS2	A	beige point whith white ring	semi-wet	
MD12				B	beige and shining	semi-wet	small
MD12	145	8-06-12	SWAS2	A	white flour	dry	small
MD12				B	brick color	small	dry
MD12				C	black whith grey ring	small	
MD12	145	8-06-12	A1S2	A	light orange with beige extension	dry	
MD12	127	8-06-12	1/2S2	A	lemon yellow	semi-wet	
MD12	unknown	unknown	A1	A	beige	granulated	semi-wet
MD12				B	dirty beige	dry	
MD12	554	15-06-12	SWAS2	A	brown earth	shining and wet	small
MD12	105	8-06-12	1/2S2	A	dark yellow	small	semi-wet
MD12				B	beige creamy	opaque	wet
MD12	321	11-06-12	A1S2	A	old orange	rugous	semi-wet
MD12				B	dark beige	gelatinous	wet
MD12	27	30-07-12	SWAP1	A	dirty orange	dry	small
MD12				B	light yellow translucide	small	semi-wet
MD12	46	30-07-12	SWAP1	A	orange translucide	small	wet
MD12	36	30-07-12	SWAP1	A	yellow ocher		
MD12	337	11-06-12	SWAS2	A	beige translucide	small	wet
MD12	321	11-06-12	SWAS2	A	beige creamy	wet	small
MD12	220	21-07-12	SWAP1	A	light pink translucide	small	semi-wet

MD12	519	21-07-12	SWA	A	dark yellow	dry	small
MD12	252	21-07-12	A1P1	A	dark beige creamy	big	semi-wet
MD12	516	21-07-12	A1P1	A	yellow shining	wet	small
MD12				B	beige creamy translucide	big	wet
MD12	516	21-07-12	SWAP1	A	white	opaque	small and dry
MD12	375	23-07-12	SWA	A	light orange	small	dry
MD12	511	21-07-12	SWAP1	A	light pink translucide	small	wet
MD12	511	21-07-12	A1P1	A	darl salmon	wet	small
MD12	552	21-07-12	SWAP1	A	dark salmon	small	semi-wet
MD12				B	yellow shining	small	wet
MD12				C	light brown	small	wet
MD12				D	dark yellow creamy	semi-wet	
MD12				F	dark brown	small	wet
MD12	109	8-06-12	1/2S2	A	amarelo/orange	small	semi-wet
MD12				B	beige creamy	wet	

04/10/12	Sample Number	Mother Plating date	Medium/Method	Colony ID	Color	Shape 1	Shape 2
MD12	487	13-06-12	1/2S2	A	white star	semi-wet	
+MD12	479	13-06-12	1/2S2	A	beige	atomic bomb shape	granulated
MD12	238	21-07-12	A1P1	A	dirty white translucide	wet	gelatinous
MD12	414	13-06-12	1/2S2	A	beige	opaque	semi-wet
MD12	445	13-06-12	A1S2	A	green earth	rugous	semi-wet
MD12	445	13-0612	1/2S2	A	dark beige	small	wet
MD12				B	light beige	semi-wet	small
MD12				C	transparent	dry	small
MD12	445	13-06-12	SWAS2	A	beige creamy	small	wet
MD12				B	light orange	small	semi-wet

MD12				C	white	creamy	
MD12				D	beige	semi-wet	
MD12	443	13-06-12	A1S2	A	dark beige	dry	
MD12	170	8-06-12	A1S2	A	light orange	semi-wet	
MD12	70	7-06-12	A1S1	A	brown	rugous	dry and small
MD12	188	8-06-12	1/2S2	A	red	small	wet
MD12				B	dark yellow	dry	
MD12				C	yellow	small	semi-wet
MD12				D	white	dry	
MD12	381	11-06-12	A1S2	A	dark beige	rugous	semi-wet
MD12				B	beige	granulated	semi-wet
MD12	359	11-06-12	SWAS2	A	white star	small	semi-wet
MD12	375	11-06-12	SWAS2	A	dark beige translucide	small	semi-wet
MD12	385	11-06-12	SWAS2	A	white translucide	semi-wet	
MD12	381	11-06-12	SWAS2	A	white shining	semi-wet	
MD12	308	11-06-12	SWAS2	A	dark beige	small	creamy
MD12				B	white	dry	
MD12	275	11-06-12	A1S2	A	brown	gelatinous	wet
MD12	285	11-06-12	A1S2	A	old orange	gelatinous	wet
MD12	337	11-06-12	172S2	A	bright yellow	semi-wet	
MD12	183	8-06-12	A1S2	A	old orange	dry	
MD12	600	21-07-12	SWAP1	A	yellow translucide	small	wet
MD12	538	14-06-12	1/2S2	A	dark green	wet	
MD12	538	14-06-12	A1S2	A	yellow	wet	
MD12				B	light brown	wet	
MD12	568	23-07-12	A1P1	A	yellow	semi-wet	
MD12				B	orange	semi-wet	

MD12				C	dark orange	dry	
MD12	588	23-07-12	A1P1	A	light pink	dry	
MD12				B	light yellow	gelatinous	wet
MD12				C	yellow/orange	wet	
MD12	575	23-07-12	A1P1	A	orange	wet	
MD12				B	brown	wet	
MD12	506	21-07-12	A1P1	A	dark salmon	small	semi-wet
MD12				B	dark oprange	small	semi-wet
MD12				C	light pink translucide	gelatinous	wet
MD12	443	13-06-12	1/2S2	A	light salmon	wet	
MD12	350	11-06-12	1/2S2	A	dark orange with black spores	dry	
MD12	543	21-07-12	A1P1	A	brown/yellow	semi-wet	
MD12	303	21-07-12	A1 P1	A	light salmon	semi-wet	
MD12	514	21-0712	A1 P1	A	dark red with grey		
MD12				B	brown/orange	dry	
MD12	433	13-06-12	A1S2	A	dark red	dry	
MD12	525	21-07-12	A1P1	A	light salmon	wet	
MD12	519	21-07-12	A1P1	A	dark salmon	creamy	wet
MD12				B	salmon	semi-wet	
MD12				C	light salmon	semi-wet	
MD12	548	21-07-12	SWAP1	A	pink translucide	semi-wet	
MD12	561	23-07-12	A1P1	A	pink translucide	gelatinous	wet
MD12	25	7-06-12	1/2S1	A	brown with light brown ring	wet	
MD12	32	7-06-12	SWAS1	A	baby pink	dry	
MD12				B	light yellow	granulated	wet
MD12	512	21-07-12	A1P1	A	old orange	dry	
MD12				B	light yellow	granulated	wet

MD12	550	14-06-12	A1S2	A	light yellow creamy	semi-wet	
MD12				B	yellow creamy	semi-wet	
MD12	622	28-07-12	A1P1	A	old pink creamy	semi-wet	
MD12	236	21-07-12	SWAA1	A	white translucide	ice	wet
MD12	220	21-07-12	A1P1	A	violet	semi-wet	
MD12	240	21-07-12	A1P1	A	old pink	semi-wet	
MD12				B	caramel	ice	wet
MD12	535	14-06-12	A1S2	A	orange	dry	small
MD12				B	yellow	semi-wet	big
MD12				C	beige granulated	semi-wet	big
MD12	463	13-06-12	A1S2	A	yellow shining	granulated	semi-wet
MD12	364	11-06-12	A1S2	A	yellow shining	semi-wet	big
MD12	321	11-06-12	1/2S2	A	light orange	semi-wet	big
MD12	235	21-07-12	A1P1	A	old pink	wet	creamy
MD12	535	14-06-12	1/2S2	A	dark green	dry	
MD12	340	11-06-12	A1 S2	A	white shining	granulated	semi-wet
MD12	325	11-06-12	A1S2	A	caramel/orange	semi-wet	
MD12	619	28-07-12	A1S2	A	violet	wet	small
MD12				B	orange	gelatinous	small and wet
MD12	624	28-07-12	SWAP1	A	white translucide	gelatinous	big
MD12	457	13-06-12	1/2S2	A	light orange	dry	big
MD12	9	7-06-12	A1S1	A	beige	granulated	dry and pale
MD12				B	dark beige	wet	
MD12	123	8-06-12	A1S2	A	beige with white ring	dry	
MD12				B	white translucide	granulated	big and dry
MD12				C	grey	granulated	big and dry
MD12				D	light brown	semi-wet	

MD12	391	13-06-12	1/2S2	A	beige with a white points	dry	big
MD12	27	7-06-12	A1S1	A	dark red	rugous	dry
MD12	480	28-07-12	A1P1	A	caramel	ice	wet
MD12				B	orange	semi-wet	
MD12				C	black	semi-wet	semi-wet
MD12	547	14-06-12	1/2S2	A	green earth	wet	

Annex 2- Level intensity with Nile red of strains.

	A2 plate	Mother plate	Date	Colony ID	Intensity level		A2 plate	Mother plate	Date	Colony ID	Intensity signal
MD12	1	244	18-09-12	A	-	MD12	311	527	02-10-12	A	+
MD12	2	224	18-09-12	A	-	MD12	312	214	02-10-12	A	+
MD12	3	642	18-09-12	A	+	MD12	313	249	02-10-12	A	+
MD12	4	212	18-09-12	B	+	MD12	314	638	02-10-12	A	-
MD12	5	279	18-09-12	A	+	MD12	315	109	02-10-12	B	-
MD12	6	652	18-09-12	A	-	MD12	316	511	02-10-12	F	+
MD12	7	582	18-09-12	B	+	MD12	317	511	02-10-12	C	-
MD12	8	341	19-09-12	B	-	MD12	318	511	02-10-12	D	-
MD12	9	367	19-09-12	A	-	MD12	319	511	02-10-12	B	+
MD12	10	255	19-09-12	A	+	MD12	320	511	02-10-12	A	+
MD12	11	338	19-09-12	A	-	MD12	321	511	02-10-12	A	+
MD12	12	655	18-09-12	B	+	MD12	322	516	02-10-12	A	-
MD12	13	531	18-09-12	A	-	MD12	323	516	02-10-12	B	-
MD12	14	531	18-09-12	B	-	MD12	324	516	02-10-12	A	-
MD12	15	531	18-09-12	C	-	MD12	325	252	02-10-12	A	-
MD12	16	657	18-09-12	A	-	MD12	326	519	02-10-12	A	+
MD12	17	535	18-09-12	B	+	MD12	327	321	02-10-12	A	+
MD12	18	535	18-09-12	A	+	MD12	328	337	02-10-12	A	-
MD12	19	276	18-09-12	B	-	MD12	329	427	02-10-12	A	-
MD12	20	553	18-09-12	A	+	MD12	330	264	02-10-12	B	-
MD12	21	382	18-09-12	A	-	MD12	331	407	02-10-12	A	-
MD12	22	279	18-09-12	A	+	MD12	332	545	02-10-12	C	-
MD12	23	208	18-09-12	A	-	MD12	333	545	02-10-12	B	-
MD12	24	208	18-09-12	B	-	MD12	334	545	02-10-12	A	-

MD12	25	203	18-09-12	A	+	MD12	335	567	01-10-12	A	-
MD12	26	311	18-09-12	B	-	MD12	336	242	01-10-12	A	+
MD12	27	9	18-09-12	B	-	MD12	337	286	01-10-12	A	+
MD12	28	255	19-09-12	A	+	MD12	338	268	01-10-12	A	-
MD12	29	331	14-09-12	A	-	MD12	339	285	01-10-12	A	-
MD12	30	340	19-09-12	A	+	MD12	340	380	01-10-12	B	-
MD12	31	287	19-09-12	A	-	MD12	341	380	01-10-12	A	-
MD12	32	52	14-09-12	A	-	MD12	342	390	01-10-12	B	-
MD12	33	255	14-09-12	A	+	MD12	343	390	01-10-12	A	-
MD12	34	257	18-09-12	A	+	MD12	344	406	01-10-12	B	-
MD12	35	642	18-09-12	A	-	MD12	345	406	01-10-12	A	-
MD12	36	9	14-09-12	A	-	MD12	346	264	01-10-12	C	-
MD12	37	299	12-09-12	A	+	MD12	347	264	01-10-12	B	
MD12	38	384	14-09-12	A	+	MD12	348	264	01-10-12	A	-
MD12	39	352	14-09-12	B	-	MD12	349	415	01-10-12	B	-
MD12	40	5	12-10-12	C	+	MD12	350	415	01-10-12	A	-
MD12	41	175	12-10-12	A	+	MD12	351	466	01-10-12	B	+
MD12	42	528	12-10-12	B	+	MD12	352	240	01-10-12	B	-
MD12	43	528	12-10-12	A	-	MD12	353	240	01-10-12	A	+
MD12	44	396	12-10-12	A	-	MD12	354	220	01-10-12	A	+
MD12	45	545	12-10-12	B	+	MD12	355	236	01-10-12	A	-
MD12	46	213	12-10-12	B	+	MD12	356	622	01-10-12	A	-
MD12	47	213	12-10-12	A	+	MD12	357	512	01-10-12	B	-
MD12	48	576	12-10-12	C	+	MD12	358	512	01-10-12	A	-
MD12	49	214	12-10-12	B	-	MD12	359	23	01-10-12	B	-
MD12	50	157	12-10-12	A	+	MD12	360	32	01-10-12	A	-
MD12	51	157	12-10-12	B	+	MD12	361	25	01-10-12	A	-

MD12	52	555	12-10-12	A	-	MD12	362	561	01-10-12	A	-
MD12	53	123	12-10-12	A	+	MD12	363	525	01-10-12	A	-
MD12	54	123	12-10-12	B	-	MD12	364	433	01-10-12	A	-
MD12	55	487	12-10-12	A	-	MD12	365	514	01-10-12	B	-
MD12	56	187	12-10-12	A	+	MD12	366	514	01-10-12	A	-
MD12	57	119	12-09-12	A	-	MD12	367	27	01-10-12	A	-
MD12	58	361	12-10-12	A	-	MD12	368	341	01-10-12	A	-
MD12	59	512	12-10-12	A	-	MD12	369	303	01-10-12	A	-
MD12	60	599	19-09-12	B	-	MD12	370	543	01-10-12	A	-
MD12	61	333	19-09-12	A	+	MD12	371	350	01-10-12	A	-
MD12	62	288	19-09-12	A	+	MD12	372	443	01-10-12	A	+
MD12	63	315	18-09-12	A	-	MD12	373	506	01-10-12	C	-
MD12	64	274	19-09-12	C	+	MD12	374	506	01-10-12	B	-
MD12	65	271	19-09-12	B	+	MD12	375	506	01-10-12	A	-
MD12	66	271	19-09-12	A	-	MD12	376	558	01-10-12	B	-
MD12	67	271	19-09-12	B	-	MD12	377	558	01-10-12	A	+
MD12	68	346	19-09-12	A	-	MD12	378	560	01-10-12	A	-
MD12	69	425	19-09-12	B	-	MD12	379	568	01-10-12	B	-
MD12	70	423	19-09-12	B	-	MD12	380	568	01-10-12	A	+
MD12	71	72	19-09-12	B	++	MD12	381	123	01-10-12	C	-
MD12	72	257	18-09-12	B	-	MD12	382	123	01-10-12	B	-
MD12	73	274	19-09-12	A	-	MD12	383	123	01-10-12	A	-
MD12	74	282	19-09-12	A	-	MD12	384	9	01-10-12	B	-
MD12	75	423	19-09-12	A	-	MD12	385	9	01-10-12	A	-
MD12	76	420	19-09-12	A	-	MD12	386	457	01-10-12	A	-
MD12	77	425	19-09-12	A	+	MD12	387	624	01-10-12	A	-
MD12	78	420	19-09-12	A	+	MD12	388	624	01-10-12	B	-

MD12	79	355	19-09-12	A	-	MD12	389	619	01-10-12	B	-
MD12	80	45	19-09-12	A	-	MD12	390	325	01-10-12	A	-
MD12	81	45	19-09-12	B	-	MD12	391	340	01-10-12	A	-
MD12	82	135	19-09-12	D	+	MD12	392	535	01-10-12	A	-
MD12	83	456	12-09-12	A	-	MD12	393	235	01-10-12	A	+
MD12	84	513	12-09-12	A	-	MD12	394	445	01-10-12	D	-
MD12	85	183	12-09-12	B	+	MD12	395	445	01-10-12	C	-
MD12	86	183	12-09-12	A	-	MD12	396	445	01-10-12	A	-
MD12	87	384	12-09-12	C	-	MD12	397	414	01-10-12	A	-
MD12	88	352	12-09-12	A	+	MD12	398	414	01-10-12	A	++
MD12	89	352	12-09-12	D	-	MD12	399	238	01-10-12	A	-
MD12	90	137	12-09-12	B	+	MD12	400	479	01-10-12	A	-
MD12	91	5	12-09-12	A	-	MD12	401	487	01-10-12	B	+
MD12	92	517	12-09-12	C	+	MD12	402	538	01-10-12	A	-
MD12	93	294	12-09-12	A	-	MD12	403	463	01-10-12	C	+
MD12	94	99	12-09-12	A	-	MD12	404	535	01-10-12	B	-
MD12	95	454	12-09-12	A	-	MD12	405	535	01-10-12	A	-
MD12	96	5	12-09-12	B	-	MD12	406	547	01-10-12	C	-
MD12	97	183	12-09-12	B	-	MD12	407	480	01-10-12	A	-
MD12	98	183	12-09-12	A	-	MD12	408	660	01-10-12	B	-
MD12	99		12-09-12	A	+	MD12	409	285	01-10-12	A	-
MD12	100	517	12-09-12	B	+	MD12	410	188	01-10-12	A	-
MD12	101	361	12-09-12	B	-	MD12	411	70	01-10-12	A	+
MD12	102	187	12-09-12	B	-	MD12	412	170	01-10-12	A	+
MD12	103	517	12-09-12	A	++	MD12	413	232	03-10-12	A	+
MD12	104	35	12-09-12	A	-	MD12	414	337	04-10-12	A	-
MD12	105	107	12-09-12	A	-	MD12	415	183	04-10-12	A	-

MD12	106	170	12-09-12	A	-	MD12	416	275	04-10-12	A	-
MD12	107	352	14-09-12	C	+	MD12	417	308	04-10-12	B	-
MD12	108	400	14-09-12	A	-	MD12	418	308	04-10-12	A	+
MD12	109	338	19-09-12	A	-	MD12	419	375	04-10-12	A	-
MD12	110	278	19-09-12	A	-	MD12	420	359	04-10-12	A	-
MD12	111	135	19-09-12	C	-	MD12	421	381	04-10-12	B	-
MD12	112	135	19-09-12	B	-	MD12	422	381	04-10-12	A	-
MD12	113	135	19-09-12	A	-	MD12	423	165	04-10-12	D	-
MD12	114	114	20-09-12	C	-	MD12	424	165	04-10-12	C	-
MD12	115	333	19-09-12	A	+	MD12	425	165	04-10-12	B	-
MD12	116	341	19-09-12	A	-	MD12	426	188	04-10-12	B	-
MD12	117	590	19-09-12	A	+	MD12	427	36	03-10-12	A	-
MD12	118	275	20-09-12	B	-	MD12	428	27	03-10-12	A	-
MD12	119	114	20-09-12	B	-	MD12	429	Unknown	03-10-12	B	-
MD12	120	378	20-09-12	A	-	MD12	430	145	03-10-12	A	-
MD12	121	261	20-09-12	B	+	MD12	431	145	03-10-12	C	-
MD12	122	261	20-09-12	A	-	MD12	432	145	03-10-12	B	-
MD12	123	347	20-09-12	A	-	MD12	433	145	03-10-12	A	-
MD12	124	347	20-09-12	A	+	MD12	434	157	03-10-12	B	+
MD12	125	594	24-09-12	A	++	MD12	435	157	03-10-12	A	+
MD12	126	594	24-09-12	B	+	MD12	436	255	11-10-12	2	-
MD12	127	594	24-09-12	A	+	MD12	437	255	11-10-12	1B	-
MD12	128	290	20-09-12	A	-	MD12	438	311	11-10-12	2A	+
MD12	129	269	20-09-12	A	+	MD12	439	311	11-10-12	1A	-
MD12	130	553	20-09-12	A	+	MD12	440	288	11-10-12	2A	-
MD12	131	490	20-09-12	A	-	MD12	441	576	11-10-12	2A	-
MD12	132	490	20-09-12	B	-	MD12	442	576	11-10-12	1A	-

MD12	133	133	20-09-12	C	+	MD12	443	251	11-10-12	2A	-
MD12	134	411	20-09-12	A	+	MD12	444	251	11-10-12	1A	-
MD12	135	411	20-09-12	B	-	MD12	445	59	11-10-12	2A	-
MD12	136	387	20-09-12	A	-	MD12	446	59	11-10-12	1A	-
MD12	137	118	21-09-12	A	-	MD12	447	251	11-10-12	1B	-
MD12	138	22	21-09-12	A	+	MD12	448	231	11-10-12	1A	+
MD12	139	111	21-09-12	D	+	MD12	449	231	11-10-12	2	+
MD12	140	111	21-09-12	B	+	MD12	450	274	11-10-12	2	+
MD12	141	111	21-09-12	A	-	MD12	451	404	11-10-12	1A	-
MD12	142	348	29-09-12	B	+	MD12	452	598	11-10-12	1A	-
MD12	143	348	29-09-12	A	-	MD12	453	288	11-10-12	1B	-
MD12	144	342	29-09-12	A	-	MD12	454	274	11-10-12	1B	+
MD12	145	245	21-09-12	B	+	MD12	455	231	11-10-12	2A	-
MD12	146	245	21-09-12	A	+	MD12	456	231	11-10-12	1A	+
MD12	147	523	21-09-12	A	-	MD12	457	251	11-10-12	3B	-
MD12	148	45	21-09-12	D	-	MD12	458	251	11-10-12	B	-
MD12	149	45	21-09-12	C	-	MD12	459	107	11-10-12	1B	-
MD12	150	45	21-09-12	B	-	MD12	460	404	11-10-12	2A	-
MD12	151	45	21-09-12	A	-	MD12	461	438	11-10-12	A	-
MD12	152	22	21-09-12	B	-	MD12	462	569	11-10-12	A	-
MD12	153	339	20-09-12	B	+	MD12	463	569	11-10-12	A	-
MD12	154	339	20-09-12	A	+	MD12	464	554	11-10-12	2A	-
MD12	155	652	20-09-12	B	-	MD12	465	554	11-10-12	1A	-
MD12	156	630	21-09-12	B	-	MD12	466	665	11-10-12	A	-
MD12	157	630	21-09-12	A	-	MD12	467	276	11-10-12	1A	-
MD12	158	216	21-09-12	A	+	MD12	468	632	11-10-12	D	-
MD12	159	374	21-09-12	B	-	MD12	469	513	11-10-12	1B	-

MD12	160	625	21-09-12	A	-	MD12	470	282	11-10-12	2A	+
MD12	161	269	24-09-12	A	-	MD12	471	282	11-10-12	1A	+
MD12	162	291	24-09-12	B	-	MD12	472	569	11-10-12	2	+
MD12	163	291	24-09-12	A	-	MD12	473	569	11-10-12	1D	-
MD12	164	258	24-09-12	A	+	MD12	474	114	11-10-12	2A	-
MD12	165	200	24-09-12	B	-	MD12	475	114	11-10-12	1A	-
MD12	166	75	24-09-12	B	-	MD12	476	282	11-10-12	3A	+
MD12	167	75	24-09-12	A	+	MD12	477	254	11-10-12	3A	+
MD12	168	159	24-09-12	B	+	MD12	478	254	11-10-12	1A	-
MD12	169	159	24-09-12	A	-	MD12	479	254	11-10-12	A2	-
MD12	170	10	24-09-12	D	-	MD12	480	340	11-10-12	2B	-
MD12	171	10	24-09-12	A	-	MD12	481	340	11-10-12	1B	-
MD12	172	246	24-09-12	B	+	MD12	482	255	11-10-12	2A	-
MD12	173	245	24-09-12	A	+	MD12	483	255	11-10-12	1A	-
MD12	174	219	24-09-12	C	+	MD12	484	582	11-10-12	2A	+
MD12	175	219	24-09-12	B	-	MD12	485	544	11-10-12	1A	-
MD12	176	219	24-09-12	A	-	MD12	486	582	11-10-12	1A	-
MD12	177	651	24-09-12	D	+	MD12	487	544	11-10-12	2A	-
MD12	178	651	24-09-12	C	+	MD12	488	57	11-10-12	1A	-
MD12	179	651	24-09-12	B	-	MD12	489	57	16-10-12	2A	+
MD12	180	651	24-09-12	B	-	MD12	490	319	11-10-12	2A	-
MD12	181	651	24-09-12	A	-	MD12	491	319	11-10-12	2A	-
MD12	182	277	24-09-12	A	-	MD12	492	201	16-10-12	3A	-
MD12	183	292	24-09-12	B	-	MD12	493	201	16-10-12	1A	+
MD12	184	292	24-09-12	A	+	MD12	494	201	16-10-12	4B	-
MD12	185	597	24-09-12	B	+	MD12	495	201	16-10-12	3	-
MD12	186	597	24-09-12	A	+	MD12	496	201	16-10-12	1B	-

MD12	187	593	24-09-12	B	-	MD12	497	642	16-10-12	3B	+
MD12	188	27	24-09-12	C	-	MD12	498	201	16-10-12	1A	+
MD12	189	27	24-09-12	B	+	MD12	499	642	16-10-12	2B	+
MD12	190	27	24-09-12	A	+	MD12	500	201	16-10-12	1B	++
MD12	191	219	24-09-12	D	-	MD12	501	373	16-10-12	2A	-
MD12	192	593	24-09-12	A	+	MD12	502	373	16-10-12	1A	-
MD12	193	584	24-09-12	B	-	MD12	503	27	16-10-12	2D	-
MD12	194	370	24-09-12	B	-	MD12	504	27	16-10-12	1D	+
MD12	195	370	24-09-12	A	+	MD12	505	277	16-10-12	2A	-
MD12	196	343	24-09-12	B	-	MD12	506	277	16-10-12	1A	-
MD12	197	343	24-09-12	A	-	MD12	507	553	16-10-12	2B	-
MD12	198	373	24-09-12	A	-	MD12	508	553	16-10-12	1B	-
MD12	199	604	24-09-12	B	+	MD12	509	513	15-10-12	1B	-
MD12	200	577	24-09-12	A	+	MD12	510	72	15-10-12	2A	-
MD12	201	604	24-09-12	A	-	MD12	511	72	15-10-12	1A	++
MD12	202	337	24-09-12	A	-	MD12	512	281	15-10-12	2A	-
MD12	203	386	24-09-12	C	+	MD12	513	281	15-10-12	1A	-
MD12	204	386	24-09-12	B	+	MD12	514	237	15-10-12	3A	-
MD12	205	386	24-09-12	A	+	MD12	515	237	15-10-12	2A	-
MD12	206	645	24-09-12	D	-	MD12	516	597	16-10-12	1B	+
MD12	207	645	24-09-12	C	-	MD12	517	597	16-10-12	2B	-
MD12	208	645	24-09-12	B	-	MD12	518	292	16-10-12	1A	++
MD12	209	404	02-10-12	B	+	MD12	519	292	16-10-12	2A	+
MD12	210	404	02-10-12	A	-	MD12	520	10	16-10-12	1B	-
MD12	211	648	02-10-12	A	-	MD12	521	10	16-10-12	1C	-
MD12	212	306	02-10-12	A	-	MD12	522	626	16-10-12	2A	+
MD12	213	634	02-10-12	A	+	MD12	523	626	16-10-12	1A	-

MD12	214	662	02-10-12	B	+	MD12	524	558	16-10-12	3B	-
MD12	215	656	02-10-12	B	-	MD12	525	258	16-10-12	2B	-
MD12	216	656	28-09-12	A	-	MD12	526	258	16-10-12	1B	-
MD12	217	389	28-09-12	B	-	MD12	527	10	16-10-12	2E	-
MD12	218	389	28-09-12	A	-	MD12	528	10	16-10-12	1E	-
MD12	219	375	28-09-12	A	-	MD12	529	296	25-10-12	4A	-
MD12	220	357	28-09-12	D	+	MD12	530	234	25-10-12	1A	-
MD12	221	352	28-09-12	C	-	MD12	531	292	25-10-12	1C	+
MD12	222	352	28-09-12	B	++	MD12	532	301	02-10-12	A	-
MD12	223	352	28-09-12	A	+	MD12	533	10	16-10-12	C	-
MD12	224	549	02-10-12	A	-	MD12	534	10	16-10-12	B	-
MD12	225	546	02-10-12	A	-	MD12	535	237	15-10-12	A	-
MD12	226	562	02-10-12	A	+++	MD12	536	292	25-10-12	2C	-
MD12	227	259	28-09-12	B	-	MD12	537	83	25-10-12	1C	-
MD12	228	530	02-10-12	A	-	MD12	538	509	25-10-12	1B	+
MD12	229	356	28-09-12	C	-	MD12	539	509	25-10-12	3B	-
MD12	230	356	28-09-12	B	+	MD12	540	638	25-10-12	2B	-
MD12	231	354	28-09-12	A	-	MD12	541	638	25-10-12	1B	-
MD12	232	259	28-09-12	A	-	MD12	542	296	25-10-12	1A	-
MD12	233	373	28-09-12	A	-	MD12	543	296	25-10-12	2A	-
MD12	234	366	28-09-12	C	-	MD12	544	296	25-10-12	3A	-
MD12	235	356	28-09-12	A	-	MD12	545	535	25-10-12	1B	-
MD12	236	658	28-09-12	B	-	MD12	546	345	25-10-12	1A	-
MD12	237	658	28-09-12	A	-	MD12	547	234	25-10-12	A	-
MD12	238	258	28-09-12	E	-	MD12	548	535	25-10-12	2B	-
MD12	239	380	28-09-12	A	-	MD12	549	656	25-10-12	2B	-
MD12	240	376	28-09-12	C	-	MD12	550	656	25-10-12	1B	+

MD12	241	385	28-09-12	F	-	MD12	551	301	25-10-12	2A	-
MD12	242	285	28-09-12	D	+	MD12	552	301	25-10-12	1A	-
MD12	243	285	28-09-12	C	-	MD12	553	661	25-10-12	2A	-
MD12	244	285	28-09-12	B	+	MD12	554	661	25-10-12	1A	-
MD12	245	285	28-09-12	A	+	MD12	555	388	25-10-12	1A	+
MD12	246	273	28-09-12	D	-	MD12	556	538	02-10-12	1A	-
MD12	247	273	28-09-12	C	+	MD12	557	538	02-10-12	2A	-
MD12	248	273	28-09-12	B	-	MD12	558	538	02-10-12	1A	-
MD12	249	266	28-09-12	B	+	MD12	559	538	02-10-12	2A	-
MD12	250	266	28-09-12	A	+	MD12	560	550	02-10-12	1A	-
MD12	251	266	28-09-12	B	-	MD12	561	550	02-10-12	2A	+
MD12	252	266	28-09-12	C	+	MD12	562	568	02-10-12	1B	-
MD12	253	376	28-09-12	B	-	MD12	563	568	02-10-12	B	-
MD12	254	376	28-09-12	A	-	MD12	564	548	02-04-12	1A	+
MD12	255	372	28-09-12	B	+	MD12	565	548	02-04-12	2A	+
MD12	256	372	28-09-12	A	+	MD12	566	641	02-04-12	1B	+
MD12	257	374	28-09-12	B	-	MD12	567	641	02-04-12	2B	-
MD12	258	374	28-09-12	A	+	MD12	568	298	02-04-12	1A	-
MD12	259	388	28-09-12	E	-	MD12	569	298	02-04-12	2A	-
MD12	260	388	28-09-12	D	-	MD12	570	105	02-04-12	1B	-
MD12	261	388	28-09-12	C	-	MD12	571	105	02-04-12	2B	-
MD12	262	388	28-09-12	B	-	MD12	572	46	02-04-12	1A	-
MD12	263	292	01-10-12	A	+	MD12	573	46	02-04-12	2A	-
MD12	264	373	28-09-12	B	-	MD12	574	321	02-04-12	1A	-
MD12	265	273	28-09-12	A	-	MD12	575	321	02-04-12	2A	++
MD12	266	366	28-09-12	B	-	MD12	576	641	02-04-12	2C	-
MD12	267	575	28-09-12	A	-	MD12	577	641	02-04-12	1C	-

MD12	268	653	28-09-12	B	-	MD12	578	407	02-04-12	2B	+
MD12	269	653	28-09-12	A	-	MD12	579	407	02-04-12	1B	+
MD12	270	661	28-09-12	B	-	MD12	580	266	02-04-12	2A	+
MD12	271	654	28-09-12	C	-	MD12	581	266	02-04-12	1A	+
MD12	272	354	28-09-12	D	+	MD12	582	407	02-04-12	2A	+
MD12	273	354	28-09-12	C	-	MD12	583	407	02-04-12	1A	-
MD12	274	354	28-09-12	B	+	MD12	584	662	02-04-12	A	-
MD12	275	366	28-09-12	A	-	MD12	585	662	02-04-12	1A	-
MD12	276	656	28-09-12	A	-	MD12	586	552	02-04-12	2B	-
MD12	277	535	01-10-12	D	-	MD12	587	552	02-04-12	1B	-
MD12	278	371	01-10-12	A	-	MD12	588	552	02-04-12	1A	-
MD12	279	535	01-10-12	C	-	MD12	589	552	02-04-12	2A	-
MD12	280	650	01-10-12	B	-	MD12	590	DESC	02-04-12	2B	+
MD12	281	650	01-10-12	A	-	MD12	591	Unknown	02-04-12	1B	+
MD12	282	629	01-10-12	A	-	MD12	592	Unknown	02-04-12	2A	-
MD12	283	340	01-10-12	A	+	MD12	593	Unknown	02-04-12	1A	-
MD12	284	371	01-10-12	B	-	MD12	594	658	02-04-12	2C	+
MD12	285	292	01-10-12	B	+	MD12	595	558	02-04-12	1C	-
MD12	286	226	01-10-12	C	-	MD12	596	165	03-10-12	A	-
MD12	287	226	01-10-12	B	-	MD12	597	443	03-10-12	A	-
MD12	288	226	01-10-12	D	-	MD12	598	285	03-10-12	A	-
MD12	289	226	01-10-12	A	+	MD12	599	321	03-10-12	A	-
MD12	290	283	01-10-12	A	-	MD12	600	554	03-10-12	A	+
MD12	291	283	01-10-12	B	+	MD12	601	Unknown	03-10-12	A	+
MD12	292	279	01-10-12	B	+	MD12	602	111	03-10-12	C	-
MD12	293	279	01-10-12	A	+	MD12	603	267	03-10-12	A	+
MD12	294	234	01-10-12	A	-	MD12	604	337	03-10-12	A	++

MD12	295	234	01-10-12	B	-	MD12	605	576	03-10-12	A	-
MD12	296	381	01-10-12	A	-	MD12	606	364	03-10-12	A	-
MD12	297	402	01-10-12	A	+	MD12	607	381	03-10-12	A	+
MD12	298	402	01-10-12	B	-	MD12	608	385	03-10-12	A	-
MD12	299	170	02-10-12	B	-	MD12	609	632	03-10-12	B	-
MD12	300	85	02-10-12	A	-	MD12	610	10	03-10-12	F	-
MD12	301	372	02-10-12	A	-	MD12	611	651	03-10-12	A	-
MD12	302	170	02-10-12	A	+	MD12	612	592	03-10-12	A	-
MD12	303	32	02-10-12	C	-	MD12	613	267	03-10-12	B	-
MD12	304	32	02-10-12	A	-	MD12	614	519	03-10-12	A	-
MD12	305	373	02-10-12	A	-	MD12	615	480	03-10-12	A	-
MD12	306	72	02-10-12	B	-	MD12	616	375	03-10-12	B	-
MD12	307	72	02-10-12	A	+	MD12	617	109	03-10-12	A	+
MD12	308	150	02-10-12	A	-	MD12	618	366	03-10-12	A	-
MD12	309	546	02-10-12	A	+	MD12	619	654	03-10-12	B	+