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COSM&BUGS COLLECTION

High Throughput Screen for molecules and metabolites with potential cosmetic application

Vera Junqueira Santos

Mestrado em Microbiologia Aplicada

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Abstract

The cosmetic world has a big gap in the range of available natural origin products with long-lasting efficiency for the resolution of skin ageing problems. This gap challenged the cosmetic industry to focus on the research of this kind of products.

This project offered a new line of investigation using BIOALVO's GPS D^2 Platform and know-how to search for natural compounds with future potential application in cosmetics. The research was based on a unique extremophile prokaryotes collection, SEAVENTbugs, coming from 4 hydrothermal vents located nearby Azores islands. The first step was to select 34 bacterial strains, which formed the COSM&BUG collection, derived from the pre-existing one. From data obtained in previous research using phylogenetic analysis and assays on enzymatic activities, a new dendrogram was established for COSM&BUG collection. Its analysis concluded that the new data did not alter the previous established phylogenetic relations. An aqueous extracts library of the COSM&BUG collection was produced to develop the high throughput screening (HTS) assays. After an extensive analysis, collagen type I and HA were selected as the cosmetic targets to integrate HTS assays: P4HA1 (type I isoform of Prolyl Hydroxylase α subunit) and MMP-1 (Matrix Metalloproteinase 1) for collagen type I, regarding synthesis and degradation pathways respectively; HAS2 (HA Synthase 2) and HYAL2 (Hyaluronidase 2) for HA, regarding synthesis and degradation pathways respectively. Using these targets two types of applications were designed for GPS D^2 Platform: *SyEn Application* allowing to search for P4HA1 and HAS2 enhancers, and *DeIn Application* for MMP-1 and HYAL2 inhibitors. Of these, *SyEn Application* for P4HA1 was developed and in the established conditions the COSM&BUG aqueous extracts showed no effect on target. In parallel, an assay was developed to test protective capabilities against UV radiation of the COSM&BUG aqueous extracts, of which 9 revealed to have that potential.

Key-words: extremophile; cosmetic; anti-ageing; collagen type I; HA – hyaluronic acid; UV radiation

Resumo

As fontes hidrotermais são uma ocorrência característica dos meios marinhos, resultantes da infiltração de água do mar em fendas do fundo oceânico por contracção das rochas, provocada pelo arrefecimento brusco do magma ascendente em contacto com a água fria. Por serem organismos com enorme capacidade de adaptação a condições inóspitas, os microrganismos são comummente encontrados nestes locais. As gamas de variação acentuada de temperatura, a salinidade, o pH, a pressão, as concentrações elevadas de metais, os níveis de actividade de água, entre outras, criam uma pressão selectiva para o tipo de sobreviventes que os compõem. Aos microrganismos capazes de resistir a estas condições dá-se o nome de extremófilos (Gomes and Steiner, 2004). A variedade de microrganismos existente nestes ecossistemas constitui uma vasta fonte de recursos naturais para exploração comercial diversificada.

A indústria cosmética procura cada vez mais compostos/princípios activos de origem natural para integração nos seus produtos (www.bitop.de), de forma a satisfazer os requisitos preferenciais tanto por parte dos consumidores, como das entidades reguladoras do transporte e comercialização de produtos químicos, que têm demonstrado uma preferência crescente pelos produtos amigos do ambiente. Os produtos de beleza, *skin care produtcs*, com maior procura no mercados são os relacionados com o tratamento do envelhecimento precoce da pele e os anti-rugas, *anti-ageing*. Esta gama de produtos tornou-se um negócio de excelência a nível mundial.

Presentemente, muitas empresas da área de *drug discovery* procuram criar alternativas para combater a baixa especificidade da maioria dos ensaios experimentais utilizados, tentando aliar a sua capacidade de alto débito, *high throughput*, testes rápidos e de metodologia simples, à especificidade oferecida pelos ensaios *in vivo*. A BIOALVO S. A., empresa especializada na utilização deste tipo de ensaio, detém a sua própria tecnologia à qual atribuiu o nome de GPS D^2 (Global Platform Screening for Drug Discovery), actualmente aplicada em diversos segmentos terapêuticos do mercado farmacêutico, focando-se nas doenças neurodegenerativas. No entanto, e apesar das grandes dimensões do mercado farmacêutico, a indústria cosmética é por si só um negócio lucrativo em larga expansão, e a aposta a nível biotecnológico nesta indústria tem vindo a ser uma constante à escala global. Nos produtos *anti-ageing* existe uma grande lacuna, tanto na existência de um composto/princípio activo com verdadeira capacidade duradoura para o retardamento do desenvolvimento das rugas, como nos problemas imunológicos causados pela utilização de produtos de preenchimento, *fillers*.

Este projecto desenvolveu uma nova linha de investigação através da utilização da plataforma e do *knowhow* da BIOALVO S.A., na pesquisa de novas moléculas com potencial para futura integração em produtos cosméticos como os *anti-ageing*, contribuindo também com uma via

alternativa para a resolução daqueles problemas. Com este objectivo, o trabalho principal consistiu na pesquisa de compostos/princípios activos numa colecção única de procariótas extremófilos, oriundos de quatro fontes hidrotermais localizadas ao longo da Crista Média Atlântica (MAR), ao lado do arquipélago dos Açores, SEAVENTbugs. O seu desenvolvimento iniciou-se com a selecção das estirpes bacterianas que integraram a colecção em estudo, à qual foi atribuído o nome de COSM&BUG Collection. Esta derivou da colecção pré-existente, a SEAVENTbugs, pertencente ao ICAT, FCUL. Das 243 estirpes que hoje constituem a colecção SEAVENTbugs foram seleccionadas 34 para integrarem a COSM&BUG. Tenreiro T. desenvolveu em 2005 um estudo para avaliar a diversidade procariota da mesma, criando uma base de dados que engloba toda a informação recolhida. Posteriormente, um grupo de investigação do ICAT, FCUL, desenvolveu um estudo enzimático sobre a mesma colecção. Neste projecto, estabeleceu-se um novo dendrograma para a colecção COSM&BUG, incluindo toda a informação disponível referente às 34 estirpes, baseado na comparação de perfis densitométricos, utilizando o coeficiente de correlação de Pearson e o método de agrupamento UPGMA. A análise do novo dendrograma permitiu concluir que as relações entre as 34 estirpes COSM&BUG são sensivelmente as mesmas, em comparação com as exibidas no dendrograma inicial de Tenreiro T. Seguidamente produziu-se uma biblioteca de extractos aquosos da mesma colecção, armazenada a -80^{o}C, para integrar os ensaios de *high throughput screening* (HTS). Para estes ensaios, o primeiro passo constituiu na selecção dos alvos cosméticos genéticos a estudar. Depois de uma profunda análise, o colagéneo do tipo I e o ácido hialurónico foram os dois constituintes considerados relevantes no processo de envelhecimento da pele e seleccionados como alvos. Sendo o objectivo específico, pesquisar na colecção COSM&BUG compostos/princípios activos com capacidade para aumentar os níveis de colagéneo do tipo I e o ácido hialurónico, ou manter os níveis existentes numa pele jovem, seguiram-se dois caminhos possíveis. Tanto os *enhancers* das vias de síntese como os inibidores das vias de degradação dos dois alvos podem conduzir ao resultado desejado. Assim, foram determinados quatro alvos específicos: P4HA1 (type I isoform of Prolyl Hydroxylase α subunit) e HAS2 (Hyaluronic Acid Synthase 2) referentes às vias de síntese, e MMP-1 (Matrix Metalloproteinase 1) e HYAL2 (Hyaluronidase 2) referentes às vias de degradação, ambos os casos reportando-se respectivamente ao colagéneo do tipo I e ao ácido hialurónico. Seguiu-se o desenho de duas aplicações diferentes para a plataforma GPS D^2. A *SyEn Application (Synthesis Enhancer Application)* para os alvos das vias de síntese, e a *DeIn Application (Degradation Inhibitor Application)* para os alvos das vias de degradação. A *SyEn Application* visou pesquisar o aumento dos níveis de expressão dos alvos correspondentes – *enhancers* de P4HA1 e HAS2 –*,* e a *DeIn Application* a diminuição dos níveis de expressão dos alvos respectivos – inibidores de MMP-1 e HYAL2. Para tal, foram utilizadas zonas dos genes dos respectivos alvos implicadas na transcrição dos mesmos e construídos clones que permitiram a detecção das alterações dos seus níveis de activação/inibição.

Neste âmbito a *SyEn Application* para o alvo P4HA1 foi desenvolvida e concluída com êxito. Após o ensaio concluiu-se que, nas condições estabelecidas, nenhum dos extractos aquosos COSM&BUG revelou capacidade para aumentar os níveis de expressão da zona do gene de P4HA1. Paralelamente, foi desenvolvida um outro ensaio para a pesquisa de capacidade protectora contra radiação UV na biblioteca de extractos aquosos da colecção COSM&BUG. Sendo a protecção contra a radiação ultra violeta (UV) um factor de extrema importância quer para a saúde geral, quer para o retardamento do envelhecimento precoce da pele e o desenvolvimento de rugas, optou-se por incluir também este ensaio na análise realizada. Este ensaio permitiu concluir que 9 dos 34 extractos aquosos COSM&BUG apresentaram competências protectoras contra a radiação de 254nm de comprimento de onda.

Numa perspectiva de trabalho futuro, novos extractos aquosos da colecção COSM&BUG sob condições totalmente estéreis deverão ser produzidos. Também se deverá produzir uma biblioteca de extractos orgânicos da colecção, por forma a alargar o estudo de possíveis compostos/princípios activos de interesse para a indústria cosmética.

O ensaio da *SyEn Application* para o alvo P4HA1 deverá ser repetido para confimar os resultados obtidos. As restantes plataformas que foram desenhadas deverão ainda ser implementadas para se obter um painel mais completo de ensaios. Tendo em vista a optimização do ensaio de sensibilidade aos raios UV, os novos extractos aquosos COSM&BUG, produzidos sob condições totalmente estéreis, deverão incorporar uma repetição deste ensaio. Para além do comprimento de onda utilizado, poderão testar-se comprimentos de onda superiores, entre os 280 e 400 nm, de maneira a estudar intensidades mais próximas das radiações UVB e UVA.

Este projecto pode ser considerado como a primeira abordagem ao estudo da colecção COSM&BUG relativamente às suas influências sobre a expressão de constituintes primordiais da pele, como o colagéneo e o ácido hialurónico, na prevenção do desenvolvimento de sinais de envelhecimento. As aplicações *SyEn Application* e *DeIn Application,* desenhadas para a plataforma GPS D^2 da BIOALVO, detêm uma aplicabilidade futura de enorme valor comercial na contribuição para o desenvolvimento da tecnologia cosmética na área dos produtos anti-ageing.

Palavras-chave: extremófilos; cosmética; "*anti-ageing*"; colagéneo do tipo I; ácido hialurónico; radiação UV

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Abbreviations

aa	Amino acids
AP-1	Factor Activator Protein-1
bp	Base pair
CAT	Catalase
CoA	Coenzyme A
CuZnSOD	Copper-Zinc Superoxide Dismutase
DeIn	Degradation Inhibitors
DMSO	Dimethyl Sulfoxide
dNTP	Deoxyribonucleotide triphosphate
E. coli	*Escherichia coli*
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
FCUL	Faculdade de Ciências de Lisboa
GAGs	Glycosaminoglycans
GFP	Green Fluorescence Protein
GLU	Glucose
Gly	Glycine
GPS D^2	Global Platform Screening for Drug Discovery
HA	Hyaluronic Acid
HAS	Hyaluronic Acid Synthase
HTS	High Throughput Screening
HYAL	Hyaluronidase
ICAT	Instituto de Ciência Aplicada e Tecnologia
LB	Luria Bertani broth
MAR	Mid Atlantic Ridge
MMP	Matrix Metalloproteinase
MnSOD	Copper-zinc manganese
nt	Nucleotide
OD	Optical Density
OH	Hydroxyl radical
ON	Over Night
PCR	Polymerase Chain Reaction

PDI	Disulphide isomerase chaperone
Pro	Proline
P4HA1	Type I isoform of prolyl hydroxylase α subunit
RAFF	Raffinose
R&D	Research & Development
rpm	Revolutions per minute
rt	Room temperature
ROS	Reactive Oxygen Species
S. cerevisiae	*Saccharomyces cerevisiae*
SAP	Stabilized Ascorbyl Pentapeptide
SEAHMA	Seafloor and Sub-seafloor Hydrothermal Modelling in the Azores Sea
SyEn	Synthesis Enhancers
SN	Supernatant
ssDNA	Salmon Sterm DNA
TAE buffer	Buffer containing Tris, Acetate and EDTA
URA3	Orotidine-5'-phosphate (OMP) decarboxylase
UV	Ultraviolet radiation
UVA	Ultraviolet radiation A
UVB	Ultraviolet radiation B
WT	Wild type
yEGFP3	Yeast-enhanced GFP
YNB	Yeast Nitrogen Base
YPD	Yeast Peptone Dextrose
5-FOA	5-Fluoroorotic Acid Monohydrate
5-FU	5-Fluorouracil
5-FUMP	5-Fluorouridylic acid

I. Introduction

1. The added value of the deep oceans to human wellbeing – Extremophiles

Marine ecosystems are very rich and unique, where an endless number of species can be found. Innumerous researches were developed looking for the benefits of such diversity. This diversity represents an increasingly growing number of specimens available for study and, therefore, the number of possible commercial applications becomes exponential.

One of the marine environments characteristic is the occurrence of hydrothermal vents. These are geological phenomenon resulting from the ocean waters infiltration in cracks on the seafloor. These cracks result from the rocks contraction effect caused by the rapid cooling of ascending magma in contact with cold water. The large seamounts where columns of molten magma of mantle ascend to the surface forming new crust are called Mid-ocean ridges. Microorganisms are commonly found in these locations since they have an enormous adaptation capability to inhospitable conditions. The particularities of these environments, such as variation ranges of sharp temperature, salinity, pH, pressure, high metals concentrations, levels of water activity, among others, create a selective pressure in the kind of survivors therein found. The microorganisms able to resist to these variations are called extremophiles. (Gomes and Steiner, 2004). As proposed by Mac Elroy in 1974, the extremophile term arises as the definition for any given microorganism that requires extreme environmental conditions in order to survive. The concept of extreme habitat is itself relative, as the condition beyond the limit for a species can be the optimum required for another. Extremophile microorganisms prosper where most others would not survive. This is why they have kept the term extremophile until today. (Madigan and Marrs, 1997; Gomes and Steiner, 2004). This group of microorganisms presents a set of biological characteristics, resulting from adaptations at a molecular level, which grant them protection and, consequently, allowing survival in those circumstances.

Academic research groups and companies Research & Development (R&D) departments focus on this kind of environments to discover compounds or active ingredients differing from those currently known, that can be applied in different areas from pharmaceutical to cosmetics.

In pharma industry, Yondelis is an example of extreme success as it is a marine origin antitumor agent discovered in the colonial tunicate *Ecteinascidia turbinata*. Yondelis is PharmaMar's most advanced compound. In September 2007, it received marketing authorisation from the

European Commission for the treatment of advanced or metastatic soft tissue sarcoma. In November 2009 it has received its second marketing authorisation for the treatment of relapsed platinum-sensitive ovarian cancer (www.pharmamar.com) In cosmetic industry, an example of a successful product based on marine extremophile microorganisms is the line of products called *Photo-aging Defense Line* containing the active ingredient ectoin, a natural anti-stress, low weight molecule, property of Bitop, a German biotechnology company (www.bitop.de).

2. Skin problems - Precocious ageing

As time goes by, the skin suffers degradation and alterations of its components, altering the external appearance. This biological process is named skin ageing. Skin serves as a protective barrier between the body internal organs and environment. Being a complex organ, skin consists of different cell types and structures. It is divided into three main regions, epidermis (cell-rich layer mainly composed by differentiating keratinocytes, which are the most numerous cell type of the skin), dermis (separated from the epidermis by the basement membrane composed by extracellular matrix (ECM) proteins produced in fibroblasts) and hypodermis (lobules of adipose, fat tissue, which support the connective tissue framework). (Callaghan and Wilhelm, 2008; Dermatologia – Ficheiro Clínico e Terapêutico, 2010).

Skin ageing is influenced by several factors including genetic background, environmental exposure, hormonal changes and metabolic processes. All together these factors cause the accumulation of changes in the skin structure, function and appearance (Verdier-Sevrain *et al.*, 2006; Callaghan and Wilhelm, 2008). That process is caused in part due to the reduction of the ECM constituents as fibronectin, collagen, elastin, hyaluronic acid and laminin, among others. The most affected part of the ageing process are collagen, elastin and glycosaminoglycans which are the major components of the dermis. Wrinkles as ageing apparent signs are the result of the weakening connection between the dermis and epidermis due to the decrease of linking fibers – anchorage fibers. This is also the result of many other vital functions reduction of the skin that diminish in efficiency as it ages, being an important example the decrease of components as the collagens type I and III and VII (Craven *et al*., 1997; Callaghan and Wilhelm, 2008).

There are two main processes of skin ageing, one intrinsic and one extrinsic. The genetic background of each individual is responsible for the appearance, through time, of all the ageing signs, in skin and in all other organs. Theoretically, this is inevitable. Concerning the first process, the telomeres, specific structures found in every chromosomes end, are referred as having a fundamental role, in the skin ageing process, in a cellular level. Their main function is to keep the

chromosomes functional stability, which looses its effectiveness through time with the shortening of their structure. On an extrinsic level, ageing is caused by external factors as agents like sun exposure, bad eating habits, smoking and alcohol (Tsoureli-Nikita *et al.*, 2006; Geserick *et al*., 2006; Baumann, 2007; Papanagiotou, 2009).

The influence of the environment, mainly the solar ultraviolet radiation (UV), is of utmost importance for the human skin ageing. It causes deleterious effects such as burnings, immune suppression, erythema, desquamation, solar elastosis (the photo-ageing most prominent histological feature, characterized by collagen degradation and elastine abnormal accumulation in the dermis surface) cancer and premature skin ageing (Sander *et al*., 2002; Kennedy *et al*., 2003). More than one million new cases of skin cancer are diagnosed every single year, representing 40% of all diagnosed types of cancer (Bickers and Athar, 2006; Ming and He, 2009). In the year 2008 alone 1.000.000 new cases were estimated in the United States of America (American Cancer Society, 2008). The loss of tonicity and elasticity of the dermis, as well as the weakness of the skin, are directly related to ageing due to excess sun exposure. The alterations of dermal collagen caused by UV radiation occur primarily through two pathways: (i) by the stimulation of collagen breakdown, resulting in fragmented and disorganized collagen and (ii) by the inhibition of collagen precursor biosynthesis (Quan *et al.*, 2009). 80% of facial ageing is due to this factor alone (Fisher *et al*., 1997; Uitto *et al*., 1997; Baumann, 2007). This phenomenon is clinically characterized by dryness and roughness of the skin texture, uneven pigmentation and fine and deep wrinkles (Berneburg *et al.*, 2000; Ichihashi *et al*., 2009).

Ageing is also associated with the consequences of free radical damages caused by various endogenous reactive oxygen species (ROS). These are short-lived species and are continuously generated in low levels during the course of normal aerobic metabolism. ROS can be classified into two categories, free radicals, such as superoxide anion (O^{2-}) and hydroxyl radical (OH), and non-radicals, like singlet oxygen (O_2) and hydrogen peroxide, being the mitochondria the major local of their production (Xu and Fisher, 2005; Harman, 2006, quoted *in* Callaghan and Wilhelm, 2008; Bickers and Athar, 2006; Callaghan and Wilhelm, 2008; Papanagiotou, 2009). The oxidative stress is appointed as a key role in the development of the cellular events that happens upon UV radiation exposure. Both UVA and UVB radiation can cause the appearance of human skin damage by oxidation of proteins through the formation of H_2O_2, among others ROS species (Sander *et al*., 2002). UVA light, longer wavelengths (320-400 nm), enters deeper into the skin reaching the dermis and being absorbed by fibroblasts. This radiation also interacts with epidermal keratinocytes. UVB light, shorter wavelengths (280-320 nm), is mostly absorbed in the epidermis affecting mainly keratinocytes and induces DNA damages (Berneburg *et al.*, 2000).

Besides the ROS increasing, as hydrogen peroxide, UV radiation decreases anti-oxidant enzymes, such as copper-zinc superoxide dismutase (CuZnSOD), manganese SOD (MnSOD) and catalase (CAT) (which interact with ROS or with its by-products to eliminate them or to decrease their deleterious effects). This characteristic is also observed in the cellular natural ageing process. In both cases, the increasing of ROS production changes the structure and function of genes and proteins causing skin damages. These events cause variations in the intra and extracellular homeostasis leading to modifications both in cell behavior and cell-matrix interaction. Such kind of evidences suggests that UV radiation accelerates many key factors of human natural skin ageing process (Winter *et al*., 2001; Sander *et al*., 2002; Martindale and Holbrook, 2002; Matsumura and Ananthawamy, 2002; Bickers and Athar, 2006; Rattan, 2006; Callaghan and Wilhelm, 2008). Even though there are many unexplored consequences from extreme solar exposure it has been proven that skin cancer and photoageing result, in first hand, from damages accumulation caused by repeated solar radiation exposures, particularly UVA between 320 and 400nm (Marrot *et al*., 2005; Bickers and Athar, 2006; American Cancer Society, 2008). Wrinkles and pigmentation alterations are the most evident skin modifications associated with photo-damage.

3. Cosmetic Industry

Nowadays, more than ever, the grey area between cosmetics and medicine is expanding. This difference has become less and less obvious regarding quite a considerable amount of products. This happened because the cosmetic industry has brought to everyday life beauty products responding to pathological levels of care. Most of these items do not even need a prescription, making them easy to commercialize and obtain by all individuals. Pharmas are mirroring this, especially those that sell mostly beauty products. This is so evident that one can witness the growing empire of stores like *Boots*, for example, as well as their presence in our everyday life. Competing marketing wars among cosmetic giants result on an ever-growing market of products with great variety and variable price.

3.1. Current focus and market needs

Investment on personal care, time and money spent on health and beauty products are progressively growing in modern societies. Environmental harmless product consumption is exponentially growing. *Beauty and health care* is a growing industry, possibly reaching a leading role in world economy. Worldwide, the Cosmetic Industry represents 63.5 billion Euros, being the European market identical to the United States and Japanese combined (Global Insight, 2007). This is worth over 170 billion Euros per year. In Europe it has reached 35 billion Euros per year and regarding natural products it can generate around 3.9 billion Euros per year (euromonitor). From 2006 to 2016, a general growth at a rate of 4,4% is estimated (Global Insight, 2007). However, regarding natural compounds only, the rate is at a much higher level of 9% per year (euromonitor). The skin care market, the leader of the cosmetics industry, is becoming a worldwide power. Until 2006 the sun care and anti-ageing products shared the highest percentages of the total skin care segment (Global Insight, 2007).

On a global scale, there is an ever-growing tendency for the replacement of chemical compounds in cosmetic lines and an investment on natural instead. Many political actions are being developed with this purpose. In Europe there is the REACH program, dealing with Registration, Evaluation, Authorization and Restriction of Chemicals. Active since June 2007, this program imposes a thorough identification of the intrinsic properties of every single chemical product that circulates in Europe (www.apambiente.pt). Therefore, there is a centralization of all available information on any commercialized substance promoting its safer usage. This leads to a progressive replacement of any dangerous or instable compounds by safer ones available on the market. Measures like this show the growing concern for the environment, focusing in protective alternatives as well as investing on

human health. This way, it also enhances the adaptation of various industries, as the Cosmetic. The substitution of chemical compounds by natural ones (from animal, vegetal and microbial origin) has revealed itself an alternative of general success.

There are innumerous products on the market to diminish all the signs of the precocious skin ageing process and to improve the skin appearance and health in general. CosmDerm and CosmoPlast are the only collagen filler materials available derived from human collagen, from a single cell line of human fibroblast. They were developed due to the hypersensitivity effects the former products caused to the consumers, Zyderm and Zyplast with bovine derived collagen. There are many products available on the market as HA fillers. Hylaform, marketed by Allergan, is derived from avian source. Restylane, marketed by Medicis, Inc. is a non-animal stabilized HA, produced from the fermentation of equine *streptococci*. Based on non-animal origin, this product averted the potential immunological problems associated with those based on avian sources. Juvederm, marketed by Allergan, Inc., is also produced from the bacterial fermentation of equine *streptococci*. HA filler, known as Belotero, is being studied, also a non-animal product manufactured by Merz Pharmaceuticals. (Gold, 2007; Beauty and Personal Care in Portugal, Euromonitor, 2010). Most of these products are of topic use even when their main goal is the resurfacing of the epidermis, since it is the only way to decrease those signals. The main active targets for these products are collagen, elastin, and hyaluronic acid. For example, the rejuvenative medicine commonly uses HA as a filler for facial wrinkles. HA is either used alone or with other compounds and its popularity has dramatically increased since 2004. By the following years this had increased almost 10-fold. (Prince, *et al*., 2007; Beauty and Personal Care in Portugal, Euromonitor, 2010). Nivea, one of the leading brands within the cosmetic market, has recently presented one of the most successful products concerning reducing face treatments – Anti-Wrinkle Care System – with active co-enzyme Q10, responsible for activating the energy metabolism on the most inner layers of the skin, providing energy for cellular renovation. Increases in the natural Q10 level, fights wrinkles from within and visibly smoothes the skin, with increased UVA protection for better prevention results. (www3.nivea.com). L'Oreal is still a market leader due to its sales strategy. It offers a large range of different products within beauty and personal care areas. Its positioning reflects the brands innovation and diversification result, as for example Garnier increase. Another good example in the cosmetic area is Protector & Gamble, owner of brands such as Gillette, Pantene and Boost, among many others (Beauty and Personal Care in Portugal, Euromonitor, 2010).

The Portuguese consumers are more and more aware of the damages caused by UV radiation. In Portugal, in 2006, sun care products had the highest market share of the total skin care

segment. An increased use of anti-ageing products was also noticed, showing the consumers care with aesthetic appearance (Global Insight, 2007).

4. Where pharma industry meet cosmetic industry

4.1. HTS technologies applied to cosmetic

High Throughput Screening (HTS) consists of a modern scientific technique using a robotic system. Such methodology enables to quickly conduct innumerous test, such as genetic, biochemical, pharmacological, among others – the highest added value of this kind of scientific intervention. The automated station tests the chosen compounds, analysis the results and selects the hits (positives) for further assays. These research protocols are based on predefined computer programs. Companies and researchers benefits with the reduction of material, human resources and time.

BIOALVO S.A. named GPS D^2 (Global Platform Screening for Drug Discovery) to its HTS technology, and is presently applied in several therapeutic segments of the pharmaceutical market, focusing on degenerative diseases. The drugs discovery platform developed by BIOALVO is based on *in vivo* assays performed in humanized yeasts (yeast strains modified to express the human therapeutic target in analysis and sensible to the presence of the same target, through the incorporation of a DNA sensor functionally connected to a reporter gene). The yeast constitutes a representative and simple life cellular model of the physiological conditions where the therapeutic target is found in human beings, and is easily adaptable to high throughput circumstances. As they are organisms of easy, rapid and economic growth, their usage in BIOALVO's platforms enables to fight the *in vitro* assays nonspecificity, enabling to obtain information from the studied molecules, not only regarding the specific result as to the researched characteristic(s) but also regarding their behavior in a cellular environment – precocious cytotoxicity detection. Through the robotics provided by HTS, the high throughput necessities and the low cost of classic drug discovery programs are granted. The overall association of this microorganism utilization with an HTS assay enables a high profitability of resources, as well as result achievement, which otherwise would take much longer. Following a screening platform work out, the performance of a second screening, in human cellular lines expressing the therapeutical targets in analysis, enables the confirmation of hits molecule potency and toxicity levels (BIOALVO's Annual Report 2009).

In spite of the strong and growing pharmaceutical market, the cosmetic industry is expanding and the biotechnological approach by this industry has been increasing, making it one of the most interesting markets worldwide. To reduce costs and potentiate the researches performed to get new

molecules a continuous growth of screening platforms are being made available lately. This technology and therefore the know-how owned by BIOALVO allows it to extend its business relationship with other potential markets. Through the GPS D^2 Platform it is possible to research new molecules to be integrated in cosmetic products, offering a good reply to the different areas needs of this industry. For example, there is a big gap in the anti-ageing products, regarding an available compound/active principle with real lasting capability in the reduction of wrinkles development. These could be screened using typical biopharmaceutical HTS approaches.

Plants natural extracts, like silymarin, soy isoflavones and green tea polyphenols, among others, have been the most used raw material in cosmetic industry (Papanagiotu, 2009). Nevertheless new approaches are being explored, such as microorganisms and marine macroorganisms. Bearing in mind the compounds diversity produced by these organisms, there is a high probability of getting new compounds or active principles with potential application to the cosmetic industry. Natural extracts libraries (aqueous and/or organic) coming from marine microorganisms represent a huge variability and are potentially interesting for that purpose. Using such kind of libraries and using BIOALVO's know-how and its GPS D^2 Platform, the focus in the research of new molecules for cosmetic application is a worthy and promising future possibility.

5. Aims of this research

The main objective of this project was the research, in a unique collection of extremophile prokaryotes, coming from hydrothermal sources, of compounds/active principles with application in the cosmetic area. A first approach intended to define the phylogenetic relations between these strains taking into account new data available. Next, natural bacteria extracts from that collection were obtained. Additionally, four new HTS assays were designed specifically for cosmetic targets and tried to be implemented. On one of such assays, a HTS was performed using the previous described natural extracts. Finally, it was established an assay to study the protective effect, conferred by this natural bacterial extracts, under UV radiation.

The specific targets of this project were:

1) To select the bacterial strains to integrate the collection to be studied, COSM&BUG, and obtain its aqueous extracts library.

2) To establish a new dendrogram for the selected stains including all the data obtained previously by Tenreiro T., 2005 and Tenreiro A., Conduto A.,2008

3) To select the cosmetic genetic targets to be studied

4) To develop high throughput screening platforms to evaluate the potential utilization of the COSM&BUG aqueous extracts in cosmetics.

5) To develop an assay to investigate the protective capability of the COSM&BUG aqueous extracts to UV radiation.

II. Scientific rational for the identification of aqueous extracts with cosmetic application

1. COSM&BUG Collection

1.1. Strains selection

To meet the goals of this project, a selection of bacterial strains was made. This group of selected strains was called COSM&BUG collection. COSM&BUG, presented in Appendix A, is derived from another collection, the SEAVENTbugs, owned by ICAT, FCUL.

SEAVENTbugs collection was isolated from 25 samples collected in four hydrothermal vents located along Mid Atlantic Ridge (MAR), next to Azores islands. The Portuguese SEAHMA-1 mission was created to study these vents, under the project SEAHMA (Seafloor and Sub-seafloor Hydrothermal Modelling in the Azores Sea). During this mission several samples of water, small animals, sediments, rocks and chimneys were collected. The first three hydrothermal places – Menez Gwen, Lucky Strike and Rainbow – show intense activity and are characterized by the presence of chimneys from which super-heated waters (approximately 300^{o}C) and several other compounds are expelled. The Monte Saldanha is a still developing hydrothermal field, showing small holes scattered along the seafloor with a temperature 3-4^{o}C higher than the normal sea temperature (Tenreiro T., 2005).

SEAVENTbugs collection includes 246 prokaryote isolates. Around 49% of the isolates were obtained from samples collected in Menez Gwen, 27% from Rainbow, 21% from Lucky Strike and only 3% from Monte Saldanha. Regarding the sample type, around 48% of prokaryotes were successfully isolated from small animals, 29% from sediment samples, 14% from chimneys samples and 9% from processed water samples (Tenreiro T., 2005).

This collection comprises 223 psycrotolerant[1] isolates (82 aerobic and 141 anaerobic) and 23 thermophile isolates (anaerobic). This classification regards only the established isolation conditions and consequently it is important to refer that under the designation of psycrotolerant all isolated at 22^{o}C are included, while the isolated at 45^{o}C (11), 65^{o}C (7) and 85^{o}C (5) are considered as thermophile.

T. Tenreiro recovered the 82 aerobic isolates from previous stocks conserved in 20% glycerol at -80^{o}C and only 144 from the 164 original anaerobic isolates from the previous stocks in 5% Dimethyl Sulfoxide (DMSO) In the optimization tests it was possible to re-isolate the 57 anaerobic

isolates in aerobic conditions. These isolates were called as their original plus O2, such as MS SD 260O2 which was isolated from MS SD 260. Four operational phenotypic groups were defined, according to their need of oxygen and temperatures of grow: Group I – aerobes or facultative anaerobes, psycrotoletant; Group II – strict or facultative anaerobes, psycrotolerant; Group III – strict anaerobes, mesophiles[3] Group IV – strict anaerobes, thermophiles. (Tenreiro T., 2005).

The 34 COSM&BUG strains belong to Group I (set of isolates which includes the aerobic and facultative anaerobic, psycrotolerant) (strains list in Appendix A). They were selected based on availability and respiratory characteristics; due to lab practical reasons anaerobic strains were not used. 28 of them (from the 1st to the 29th) are aerobic and the remaining 6 are facultative anaerobes.

1.2. Strains classification

To evaluate the prokaryotic diversity of the 243 strains from the SEAVENT collection, Tenreiro T., 2005, has created a database using the software BioNumerics (Applied Maths), inserting all data obtained in the assays made with selected techniques.

Among the several techniques with different discriminative potential, primers csM13, PH (directed to rDNA 16S) and 1281 (random sequence) for PCR fingerprinting were used, total cellular protein profiles analyzed and subsequently the dimension polymorphisms conservation of the ITS regions evaluated. Through the analysis of dendrograms, based on the comparison of densitometric profiles and using the Pearson correlation coefficient and the UPGMA clustering method, it was possible to get a perspective of the relationship between the studied prokaryotic diversity. Figure 1 shows the dendogram corresponding to the Group I isolates PCR fingerprinting profiles evaluation, obtained with the primers csM13, PH and 1281, and total cellular protein profiles. The 34 strains of COSM&BUG collection are highlighted in green. The figure shows that the selected strains are distributed almost all along the formed clusters (from A to J) in Group I, with exception of F, I and J, mirroring the diversity of the mother collection.

1 – Psycrotolerant microorganisms are the ones which optimum growth temperature is between 15-30^{o}C but can also survive at 0^{o}C (Gomes and Steiner, 2004).

2 – Thermophiles microorganism are the ones which optimum growth temperature is superior to 45^{o}C (Gomes and Steiner, 2004).

3 – Mesophiles microorganisms are the ones which optimum growth temperature is between 30-40^{o}C tolerating temperatures up to a maximum of 45^{o}C (Gomes and Steiner, 2004).

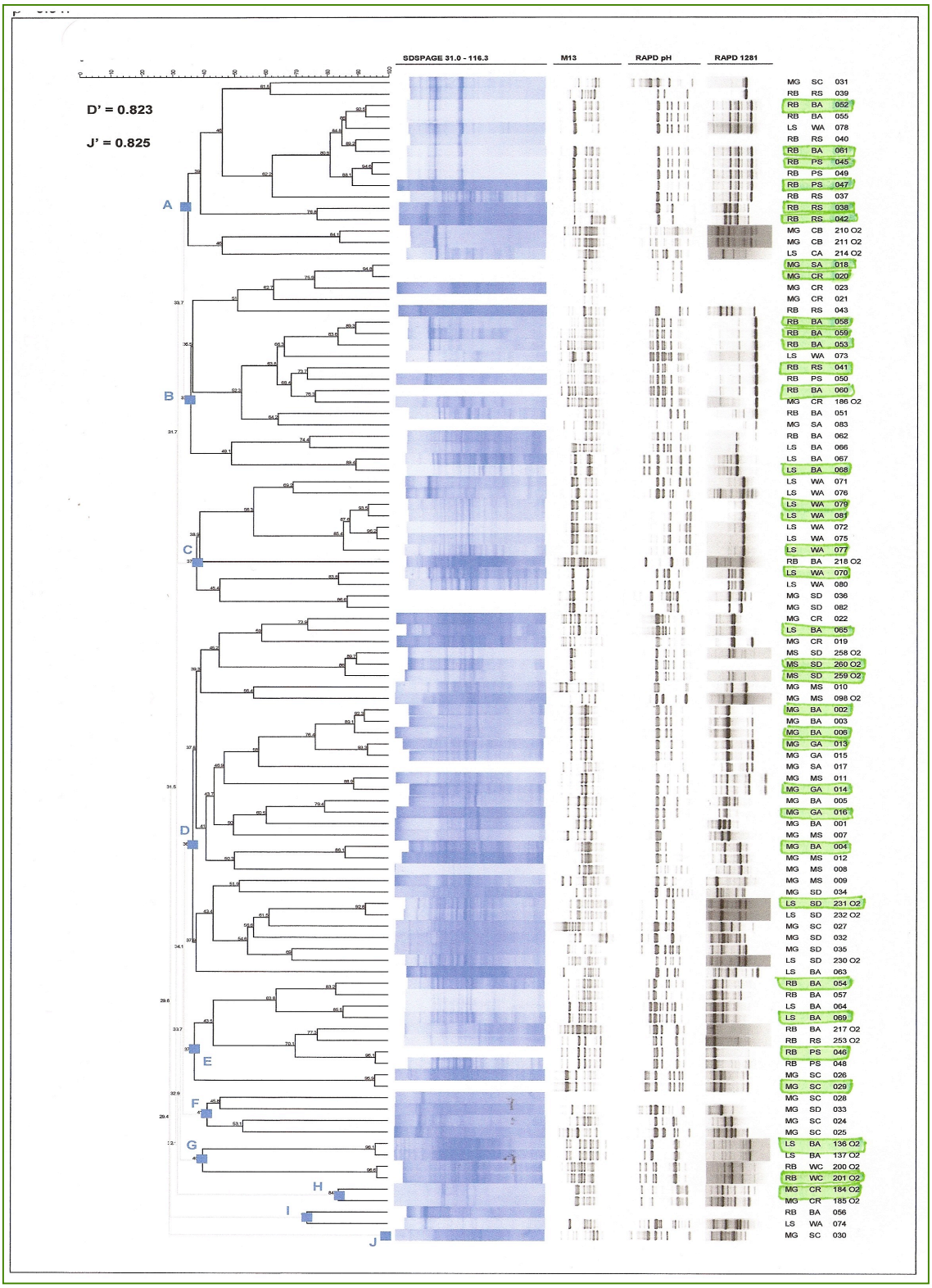

Figure 1 – Dendrogram corresponding to the evaluation of the PCR fingerprinting profiles obtained with primers csM13, PH and 1281 and the total cellular protein profiles of the isolates from Group 1 (Tenreiro T., 2005). The 34 strains of COSM&BUG collection are highlighted in green.

2. Cosmetic targets definition

As mentioned above, cosmetic is becoming a worldwide industry power through its wide range of products available on the market. The exponential cosmetic products demand ensuring more and better results has combined the innovating products availability with competitive prices. This consumer demand focus the cosmetic companies to create new product lines and/or innovate the top brand products. One of the XXI century tendencies is the use of natural origin compounds/active principles in all kinds of products, from basic ones of personal hygiene up to the esthetic beauty care range. One of the most incessant concerns of modern societies in front of the inevitable skin ageing process is the search for a rejuvenated and magnificent skin appearance. Anti-ageing products are one of the most demanded products type within the beauty care range.

This project studied and evaluated skin constituents, which could be considered relevant in this ageing process. Many skin constituents suffer a level decrease as time goes by and ageing develops. Upon this research the selected constituents were named targets.

Of all the constituents of the dermis the selected targets were collagen and hyaluronic acid (HA).

Several approaches to this project could have been done. Simple *in vitro* tests or *in vivo* tests, for example in human cells, could have been performed. Any of these assays could have contributed to obtain answers about the bacterial aqueous extracts interference in the skin constituents selected as targets. However, a different investigation approach was adopted. Over the selected targets a testing assay to screen the COSM&BUG aqueous extracts was performed which could help keeping the collagen and HA levels as in a healthy and young skin. Using the expertise and technology property of BIOALVO S.A., GPS D^2 Platform Applications were designed for the selected targets in order to allow the research of COSM&BUG aqueous extracts, helping in the preservation of the selected skin constituent levels.

The ideal situation would be to study several elements involved in the skin aging process, in order to obtain a more effective response of which would be the best target to focus on; nevertheless, due to practical reasons only two were selected.

Collagen is one of the main dermis components and of extreme relevance in its functions. Is also one of the skin constituents which deteriorates with time, leading to the appearance of aging signs (Gniadecka *et al*., 1998). The collagen synthesis, mainly produced by fibroblasts, undergoes a characteristic decrease in aging skin either *in vivo* or *in vitro*. The changes suffered by collagen have a major role in the skin aging process. An aged dermis is characterized by alterations in the production

of this compound developing fiber fragments of it. The fragmented collagen fibrils accumulation changes the skin structure and function with time. The UV radiation is an external effect increasing the occurrence of disorganized collagen fibers. (Fisher *et al.,* 1997, Quan *et al.*, 2009; Ichihashi *et al*., 2009). These facts reveal the collagen importance for a young and healthy skin. Among the several known collagen types, type I and III are the main components of the dermis. They represent 70% of the dry skin mass, being therefore the most affected in the skin ageing process (Gniadecka *et al*., 1998).

HA is one of the essential components of the skin and a key component of the extracellular matrix (ECM) (Baumann, 2007). It is involved in a wide variety of cellular functions, such as the hydric balance of the skin, preponderant for its hydration and good external appearance. It has also an important role in the structural integrity of the collagen matrix in the dermis (Baumann, 2007; Edwards and Fantasia, 2007).

Two approaches can be considered to achieve the intended purpose – the selected target levels existing in a young and healthy skin. The two targets concentrations can either be obtained through the synthesis or the degradation pathways. The desired concentrations maintenance can be reached either through the synthesized level increase or the degraded level decrease. Both synthesis pathway enhancers and degradation pathway inhibitors could contribute to maintain the skin metabolite levels. Thus, to decrease the collagen and HA degradation values on elderly skins, the choice of the specific targets regarding these components followed.

2.1. Collagen Type I

As there are several types of collagen in the human skin, it was primarily necessary to select the one which is more suitable to this study. Twenty different types of collagen are known which are the ECM predominant structural fibers, together with the elastin fibers. The maintenance of this matrix is done through these components synthesis and degradation balance. Collagen types form a multigenes family of at least 39 members, which are dispersed in 15 chromosomes. The collagens superfamily can be divided into 9 different families according to their polymeric structures, among other characteristics. Families forming fibers, for example, are of Type I, II, III, IV, V and XI. (Trojanowska *et al*., 1998; Chen *et al.,* 2006; Callaghan and Wilhelm, 2008; Fratzl, 2008).

Collagen Type I is the most abundant and operates on the structural integrity, the adherence and cell migration, the tissue remodelling and healing processes (Trojanowska *et al.,* 1998; Fratzl, 2008). It is flagrant the change in the ratio of the various types of collagen with age; in young skins

type I is around 80%, but type III represents 15% of the skin total collagen. In aged skin type I suffers a significant decrease, leading the ratio type III collagen to type I much superior. (Oikarinen, 1990, quoted *in* Bauman, 2007). Per year it is noticed a decrease of approximately 1% of the total collagen existing per unit surface area of skin (Shuster *et al.,* 1975, quoted *in* Bauman, 2007). As it is well known, the exposure to UV radiation changes the levels of the skin constituents and the collagen type I decreases around 59% in aged skins (Fisher *et al.,* 1997).

All the evident facts described have led many scientists to focus on the importance of collagen type I in the process of skin aging (Fisher *et al.,* 1997; Gniadecka *et al*., 1998; Callaghan and Wilhelm, 2008).

Due to all the particularities mentioned above concerning the decrease of collagen type I over the years in human skin, it was selected as one of the targets to study.

2.1.1. P4HA1

The biosynthesis of collagen component is initiated by the production of its precursor – procollagen. It production is followed by a set of post-translational multiple modifications leading to the constitution of collagen functional molecules, which are later secreted to the ECM. The modifications, which occur during this metabolite synthesis, represent a unique characteristic of collagen production pathway. Some of those modifications occur before procollagen secretion into the ECM and some others after the secretion. Intracellular steps need the activity of 5 specific enzymes, 3 collagen hydroxilases (Myllyla *et al*., 1978; Myllyharju and Kivirikko, 1999; Fratzl, 2008) and 2 glycosyltransferases. Two of the first, and main reactions consist of hydroxylation of prolyl and lysyl residues of procollagen, with the formation of hydroxyproline and hydroxylysine, respectively. These two hydroxylations are catalyzed by distinct enzymes, prolyl 4-hydroxylase (P4H) and lysyl hydroxylases (LH) (Fratzl, 2008).

P4H, an intracellular enzyme located at the Endoplasmic Reticulum (RE), plays a vital role in the metabolism of all types of collagen, including collagen type I. Its activity is directly related with collagen production rate. The activated form consists of a tetramer complex, with about 240 kDa molecular weight, composed of two α subunits and two β subunits, with approximately 64 kDa and 60 kDa molecular weight, respectively. The β subunit, identical to enzyme disulphide isomerase chaperone (PDI), facilitates the folding of the enzyme in the correct conformation. It is produced in excess compared with the α subunit, which contains the largest portion of the enzyme catalytic sites and the excess of which restricts the enzyme activity (Kivirikko *et al.,* 1989; Fratzl, 2008). The α subunit is however regarded as the limiting and regulatory part in the synthesis of P4H, meaning that

determines the formation rate of this enzyme (Berg *et al*., 1980). P4H catalyzes the formation of 4-hydroxyproline through the hydroxylation of proline residues located in the triple sequences X-Pro-Gly in procollagen, in peptide linkages during the post-translational processing. The hydroxylation reaction catalyzed by the enzyme P4H includes the incorporation, mediated by iron, of a hydroxyl group at the conserved proline residue, with the consumption of one dioxygen molecule and the release of carbon dioxide, while 2-oxoglutarate is converted into succinate. The reaction is 100% dependent on oxygen, and during the reaction an oxygen atom is incorporated into the newly formed hydroxyl group, while the other atom is incorporated into succinate. (Kivirikko *et al.,* 1989; Myllyharju and Kivirikko, 1997; Siddiq *et al.,* 2007). The formation of hydroxyproline has a crucial effect on collagen subsistence and it is considered the essential process to the occurrence of the correct folding of procollagen polypeptidic chains into stable triple-helical molecules (Kivirikko and Myllyharju, 1997; Fratzl, 2008).

Being the modifications in the production of collagen related to the expression and activity of the prolyl hydroxylase, this enzyme won the recognition of being a crucial step in the production of that component. The inhibition of P4H generates unstable collagen, which due to the fact of not being functional, is degraded within the cell. Not being secreted most of the time, its permanence inside the cell creates an enhancer activity to the reduced of collagen production (Rocnik *et al*., 1998; Han *et al*., 1999; Fratzl, 2008).

Relatively to the prolyl hydroxylase α subunit three isoforms are known, I, II and III (Myllyhardju *et al*., 2003). The type I isoform is the most abundant in most cell types including fibroblasts, the most responsible for the ECM constituents production (Nissi *et al*., 2001).

Because P4H was the enzyme responsible for the most crucial step in the survival of collagen and the inhibition of its expression and activity having a direct impact on its production, leading to the formation of unstable molecules, P4H was the target enzyme choice for the collagen synthesis pathway. The type I isoform of P4H - P4HA1 - being the most abundant in fibroblasts, was the selected target to include in the project.

2.1.2. MMP-1

Mammals fibril collagen hidrolysis is done through a set of unique endopeptidases enzymes named collagenases. Degradation initiates at a single site within its central triple helix (Quan *et al.*, 2009). The cleavage sites are all at Y-Gly connections, in the collagen repetitive sequences Gly-X-Y. Once cleaved, collagen is degraded by other enzymes such as gelatinases and stromelysins. Collagenases belong to a family which includes innumerous enzymes called Matrix Metalloproteinases (MMPs). All MMPs are zinc dependent and are synthesized in a latent form, a single polypeptide. Then, they are secreted as proenzymes needing to be cleaved by extracellular proteins, so that the activation peptide is removed and the proteolytic activity acquired (Pardo and Selman, 2004; Nagase and Murphy, 2004, Fratzl, 2008). They comprise a major unglycosylated form with approximately 57 kDa, and a minor glycosylated form with 61 kDa (Pardo and Selman, 2004). The MMPs expression is generally low in normal cells, showing higher levels in older ones, causing visible skin aging, such as wrinkles surfacing (Brinckerhoff and Matrisian, 2002, quoted *in* Pardo and Selman, 2004; Pardo and Selman, 2004 ; Song *et al*., 2007). With collagenese activity there are only 4 MMPs – MMP-1, 8, 13 e 18. MMP-1 is considered a powerful enzyme in the ECM degradation, being the major protease capable of initiating the degradation of native fibrillar type I and type III collagen, as well as elastin fibrils. This enzyme is considered an essential element in the human skin ageing by exposure to solar radiation. (Seiki, 2002; Quan *et al*., 2009).

Human skin exposure to UV radiation leads to some collagenases induction, among others MMPs (Quan *et al*., 2009). In particularly, UVB radiation induces MMP-1, -3 and -9 in the epidermis, and UVA radiation increases the expression of MMP-1, -2 and -3 in fibroblasts (Ichihashi *et al.,* 2009; Scharffetter *et al.,* 1991, quoted *in* Berneburg, 2000). MMPs expression remains high after several exposures to UV radiations. It can be conclude that this enzymes group constitutes the primary mediators of connective tissue (dermis) degradation in radiation exposed skins and thus resulting in precocious ageing (Fisher *et al*., 1997; Xu and Fisher, 2005). Repetitive inducing of these enzymes through solar radiation exposure during years, or even decades, is responsible for collagen fragmentation and its presence in the skin leads to its synthesis inhibition (Xu and Fisher, 2005; Varani *et al.*, 2006). Among all colagenases, MMP-1 is the most expressed, showing greater susceptibility to UV radiation and is therefore considered an essential element in human skin ageing pathogenicity due to sun exposure (Honda, 2007).

Skins aged naturally and skins degraded through solar radiation can be distinguished but these two skin types share important characteristics including changes in signal transduction pathways, which promote MMPs expression (Callaghan and Wilhelm, 2008). The alterations cause

GTP-binding proteins activation, which are key upstream regulators of MAP kinases (Mitogen-Activated Protein kinase). Certain GTP-binding proteins activation results in the increase of superoxide oxygen reactive specie formation. This and other oxygen reactive species play a key role in the MAP kinases multiple pathways (Callaghan and Wilhelm, 2008; Pantano *et al.,* 2006, quote in Callaghan and Wilhelm, 2008). MAP kinases activation causes the transcription factor Activator Protein-1 induction, commonly known as AP-1, which regulates the expression of many genes involved in growth regulation and cell differentiation. This transcription factor has a very important regulatory role in many MMPs transcription, by up-regulating MMP-1, MMP-3 and MMP-9. AP-1 is also induced by exposure to UV radiation, especially UVB rays, which indirectly lead to collagenases induction, resulting in ECM destruction. The increase in ROS occuring either during the natural aging process or after sun exposure periods, also leads to AP-1 induction (Sato *et al.,* 1993; Fisher *et al.*, 1997**;** Brenneisen *et al*., 2002, quoted *in* Callaghan and Wilhelm, 2008; Aho, *et al.,* 1997). Induced by UV radiation AP-1 partially influences the down-regulation of collagen type I, by inhibiting the transcription of the genes encoding type I procollagen (Bickers and Athar, 2006; Callaghan and Wilhelm, 2008).

Tobacco is appointed as another factor with a great impact in skin ageing, setting the grounds for potential elastosis development. Skin damage by smoking becomes grey (Demierre *et al*, 1999; Kennedy*,* 2003). This effect is related to the increase of collagenases enzymes production. MMP-1 presents high levels in skins of smoking individuals when compared with non-smoking ones, thus suggesting that MMP-1 has a key role in the effect caused by tobacco in the skin ageing process. **(**Yin *et al.*, 2000, Lahmann *et al.*, 2001, Yin *et al.*, 2001, and Knuutinen *et al.*, 2002 quoted *in* Callaghan and Wilhelm, 2008). Smokers aged 40 have as many face wrinkles as the 60 years old non-smoking ones (Callaghan and Wilhelm, 2008). The more an individual smokes, higher is the risk of skin precocious ageing and wrinkles appearance, being these irreversible damages (Kennedy *et al.*, 2003; Freiman *et al.*, 2004; Koh, 2002 and Kadunce *et al.*, 1991, quoted *in* Callaghan and Wilhelm, 2008).

MMP-1 is the major protease capable of initiating the native fibrillar type I collagen degradation by collagenase, being the most sensitive to UV radiation, both in the range of UVA and UVB rays, as well as presenting the highest levels in smoking individuals. For these reasons, the enzyme MMP-1 was selected as the specific target regarding the collagen degradation pathway.

2.2. HA

Besides collagen, other dermis main constituents involved in photodamage are elastine and glycosaminoglycans (GAGs) (Lavker, 1995, quoted *in* Baumann, 2007, Baumann, 2007). GAGs, non-fibrous components, are responsible for the skin external appearance, acting as a protective and stabilizing function of the same. HA, or hyaluran or hyaluronate, initially discovered in the eye humor vitreous in 1934 by Meyer and Palmer, is one of the most important members of GAGs family, as well as dermatansulphate, chondroitin-4-sulphate, chondroitin-6-sulfate, heparan sulfate, keratan sulfate, heparin (Weigel *et al.*, 1997; Baumann, 2007; Edwards and Fantasia, 2007). It can be found either in the dermis or in the epidermis intracellular spaces (Baumann, 2007). It consists of a basic unit of two sugars, glucuronic acid and β1,4-N-acetyl-glucosamine. They are polymerized in macromolecules that could exceed 30.000 repeating units, being one of the ECM biggest components and the only one not connected to a nucleus protein. HA polymers reach sizes from 4 kDa up to 20.000 kDa *in vivo* (Weigel *et al.*, 1997; Baumann, 2007; Price *et al.,* 2007; Edwards and Fantasia, 2007). The fact that HA half-life is less then 1 day in the epidermis and about a third of the total HA present in the human body being removed and replaced every day, demonstrates the enormous need for the production of this metabolite to keep its cellular levels (Tammi *et al*., 1991, quoted *in* Weigel *et al.,* 1997; Tammi *et al.,* 2002).

Influent in the epidermis activation and wound healing, HA is an important contributor in the reepithelization, being used in several therapies such as surgical wound and acne scars. Its capacity of connecting 100 times its weight in water molecules has also a very important role. As it is hydrosoluble, it produces a gel which behaves as a lubricant and water absorber originating hygroscopic and homeostatic properties which preserve the skin hydric balance. (Lupo, 2006; Price *et al*., 2007; Edwards and Fantasia, 2007; Allemann and Baumann, 2008; Tammi *et al.,* 2009). A further HA major function is its capacity to protect the cells from damages caused by free radicals which, as already mentioned in skin issues chapter, play an important role in skin ageing (Wiest and Kerscher, 2007; Presti and Scott, 1994). Another relevant characteristic of this ECM component is its ability to promote the collagen synthesis through fibroblasts proliferation and migration stimulation (Wang *et al*., 2007; Edwards and Fantasia, 2007).

HA synthesis intensity and its concentration in the skin are associated to the keratinocytes proliferation and differentiation in the epidermis basal layer, local where this component exists in significant quantities (Baumann, 2007). In young skins it can be found in the periphery of collagen and elastine fibers and in the places where they interact keeping them fixed; in more aged skins, these connections lose their strength, leading to gradual extinction, or even ceasing to exist. Another feature observed with aging, which contributes to the loss of skin hydration and elasticity, is the

reduced ability of HA to bind water molecules (Ghersetich, 1994, quoted *in* Baumann, 2007; Bauman, 2007; Edwards and Fantasia, 2007; Allemann and Baumann, 2008). The radiation exposure intensify the decreases of metabolites like collagen an elastin during the natural skin ageing process. The GAGs are not an exception, HA being the GAG that undergoes the most significant decrease (Ghersetich, 1994 and Bernstein *et al*., 1996, both quoted *in* Baumann, 2007). In women aged between 19 and 47 HA keeps around 0.03%, at ages above 60 years it is at 0,015% and at ages above 70 years it decreases to 0.007% (Longas, 1987).

Due to all the particularities mentioned above concerning the decrease in human skin over the years, HA was selected as the second target to study in the present project.

2.2.1. HAS2

In ECM, HA is synthesized in long chains through successive addition of sugar residues, by a cytoplasmatic protein complex present in the membrane, called hyaluronidase synthases, being afterwards secreted to the extracellular space. The synthesis of this metabolite occurs in the cell surface and this reaction is developed both in the dermis and the epidermis intracellular spaces, mostly in fibroblasts and keratinocytes (Edwards and Fantasia, 2007; Baumann, 2007).

Only three enzymes are known with the capacity to synthesize HA, HAS1 and HAS2, located in dermis and epidermis, either in fibroblasts and keratinocytes, and HAS3, located in the dermis basal layer and fibroblasts (Weigel, *et al.,* 1997). The vertebrate HAS isoenzymes are named in order of their discovery (Weigel, *et al.,* 2007). The HA is catalyzed by the three HA synthases. Nevertheless, HAS2 is the one showing more synthesis capacity producing long HA chains with approximately $2x10^6$ Da. Found in the plasmatic membrane as a multipass transmembrane protein, it catalyzes the UDP-esterified residues addition to form glucuronic acid and N-acetylglucosamine motifs existing in HA molecules. This enzyme is also responsible for the HA deposition in the ECM (Camenisch *et al.,* 2000). Also relevant in this process, is the fact that the major part of the dermis cells express more significant HAS2 mRNA levels then HAS1 mRNA. Besides this fact, HAS2 seems to be expressed in all fibroblasts, i.e., it is constitutively expressed. Thus, this enzyme is synthesized in higher quantities, being the major responsible for HA synthesis (Sugiyama *et al.*, 1998). The fact that HAS2 gene deletion causes embryonic lethality, while HAS1 and HAS3 genes knockout do not show any kind of phenotype, reflects the importance of HAS2 to HA synthesis (Camenisch *et al*., 2000). It has been demonstrated by a study performed in mice, by Dai G. and his colleagues, that down-regulation affected by the synthetases mentioned above, after repeated UVB radiation is an important inductor of dermis HA loss, also tested *in vitro* verifying that the HAS2 is, in fact, the most negatively regulated

enzyme by this exposure. The same research group was demonstrated that by this same group. HA produced by HAS2 in physiological concentrations is, indeed, necessary for the proliferation and migration of skin fibroblast. The facts presented reveal this enzyme leading role and the importance to keep its expression levels. HA levels maintenance prevents skin ageing signs development (Papanagiotou, 2009; Dai *et al.,* 2007).

Due to the fact that HAS2 is present both in the dermis and epidermis; as it is constitutively expressed in fibroblasts and the one with more HA synthesis capacity; as it is the enzyme which synthesizes higher HA chains; as it presents a superior transcription level than HAS1 enzyme in dermis; as it is of utmost importance to skin fibroblast development; as it is more susceptible to UVB radiation; HAS2 was selected as the specific target to be studied regarding HA synthesis pathway.

2.2.2. HYAL2

HA degradation typically occurs by its internalization to a membrane compartment through a mechanism involving the CD44 cell surface receptor, HA predominant receptor. Subsequently HA fragments are transported to lysosomes suffering a complete enzymatic degradation (Tammi, *et al*., 2001). HA can also be externally degradated by oxygen reactive species and also through hyaluronidases – enzymes with HA degradation capacity – secreted by bacteria (Stern *et al*., 2007, quoted *in* Lauer *et al*., 2008). In the skin HA is degraded by the enzymatic activity of hyaluronidases, and it can also happen due to the presence of free radical in the cells (Allemann and Baumann, 2008). The enzymatic degradation cleaves the HA macromolecules in small polymers of variable lengths which are later degraded in smaller units (Price *et al*., 2007; Edwards and Fantasia, 2007).

In the Hyaluronidase category there are 6 enzymes classified but only 5 of them present HA degradation capacity, HYAL1, 2, 3, 4 and PH20. HYAL1 is the hyaluronidase prevailing in plasma existing also in the majority of tissues and organs in the human body. It degrades the HA polymers of any length in oligomers (Frost *et al*., 1997). HYAL2 presents, unlike HYAL1, an apparent specificity for HA molecules of high molecular weight, >20 kDa, degrading the long HA polymers into products of intermediate lenth, with approximately 20 kDa (Lepperdinger *et al*., 1998). This characteristic makes HYAL2 the one that most likely destroys HA active and functional molecules, which is the first step of HA degradation (Edwards, and Fantasia, 2007). It does not imprint continuity to the degradation of the previously formed fragments into monomers, activity developed by HYAL1. Regarding HYAL3, its major transcript is enzymatically inactive and seems to play nothing but a supporting role to HYAL1 activity. The knockout of this gene do not causes HA accumulation, showing that this enzyme is not preponderant in its degradation (Atmuri *et al.*, 2008, quoted *in* Nykopp *et al.*, 2009). Not much is known about HYAL4 enzymatic activity, only that it is extremely limited. PH20 hyaluronidase

expression in humans is almost exclusively detected in testicles and sperm and shows activity only at high pH (Gmachl *et al.,* 1993; Nykopp *et al.*, 2009).

HYAL1 and HYAL2 hyaluronidases are those with a higher expression rate and enzymatic activity in the process of HA degradation. Nevertheless, in terms of optimal pH for its functioning, HYAL1 shows maximum activity at acid pH and HYAL2 is active either at acid pH and neutral pH, although its optimal pH in humans is 4. This occurrence is due to HYAL2 presence in the membrane surface, which can be found not only in the lysosomes, as it happens with HYAL1. After HYAL2 expression detection in different cells, it was verified that it also exists as cellular surface protein linked to the plasma membrane via a GPI anchor. The HYAL2 membrane form interacts with a Na^+-H^+ exchanger creating a more acidic microenvironment both inside and outside the cell. Thus, this enzyme initiates the HA breakdown outside the cell and the fragments generated are transported into cellular compartments, where the degradation proceeds by other enzymes, including the acid-active HYAL1. This characteristic turns the hyaluronidase HYAL2 in the one with higher versatility in HA degradation, having not just lysosomal activity as primarily thought (Frost *et al*., 1997; Lepperdinger *et al*., 1998; Lepperdinger *et al.,* 2001).

As HYAL2 only degrades high molecular weight HA polymers corresponding to active form, and HYAL1 degrades both entire HA molecules and their fragments; and as it presents enzymatic activity both inside lysosomes and at cellular surface, i.e., it has a membrane form; HYAL2 hyaluronidase was selected the specific target to be included in the study regarding HA degradation pathway.

3. Cosmetic screening assays development (Confidential)

These parts can be found in the confidential annex.

3.1. GPS D^2 Platform Applications

3.1.1. Synthesis Enhancers Application – SyEn Application

3.1.2. Degradation Inhibitors Application – DeIn Application

III. Materials and Methods (Confidential)

These parts can be found in the confidential annex.

1. SyEn and DeIn Applications construction

1.1. PCR reactions

1.2. Cloning

1.2.1. Digestion for cloning

1.2.2. Ligation of DNA to plasmid vector

1.2.3. Transformation into competent *E. coli* cells

1.2.4. Restriction analysis

1.2.5. Sequencing

1.3. Yeast transformation

2. Yeast EGFP3 signal confirmation

3. Production of yeast spheroplasts – P4HA1 promoter induction by Coenzyme A (CoA)

4. COSM&BUG aqueous extracts

5. HTS assay

6. UV sensitivity assay

6.1. Determination of BY4741 inactivation dose to UV light with 254 nm wavelength

6.2. The assay

IV. Results and Discussion (Confidential)

These parts can be found in the confidential annex.

1. COSM&BUG Collection – Strains classification

2. SyEn and DeIn Applications construction

2.1. PCR reactions, Cloning strategies and Bacteria transformation

2.2. Control clone for SyEn Application

2.3. Yeast transformation

3. SyEn Application validation - Yeast EGFP3 signal confirmation

4. P4HA1 promoter induction by Coenzyme A (CoA)

5. COSM&BUG aqueous extracts

6 HTS assay

6.1. Primary Screening Assay for P4HA1 promoter

7. UV sensitivity assay

7.1. Determination of BY4741 inactivation dose to UV light with 254 nm wavelength

7.2. The assay

8. Global conclusions

V. Future prospects (Confidential)

This part can be found in the confidential annex.

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Appendixes (Confidential)

This part can be found in the confidential annex.

Appendix A – COSM&BUG strains list

Appendix B – P4HA1 promoter induction by Coenzyme A (CoA)

Appendix C – COSM&BUG aqueous extracts dry

Appendix D – HTS assay: Primary Screening Assay for P4HA1 promoter

Appendix E – UV sensitivity assay