Student: Ana Isabel Novo de Figueiredo

Supervisor: Paula M. L. Castro

Co-Supervisors: Irina S. Moreira e Isabel Vasconcelos

Thesis Tittle: Search of new enzymes for the biodegradation of sulfur compounds.

Abstract

In recent years there has been an attempt to substitute Bisphenol A (BPA:4,4'-(propane-2,2-diyl) diphenol) in the production of polycarbonates and epoxy resins, particularly in products proposed for infants, labelling them as "BPA-free". The concern is related to the possible effects of this compound on human health mainly as an endocrine disruptor (EDC). The problem is that some of the common substituents are also bisphenols (BPs), such as the sulfur analogue Bisphenol S (BPS: 4,4'-sulfonyldiphenol), which can have similar physiological effects on organisms [1].

The intensive and global use of these phenolic EDCs results in spread environmental contamination with these compounds Industrial and municipal wastewaters are common sources of BPs contamination to aquatic ecosystems. Of the different techniques for removing BPs from wastewaters, biodegradation seems to be the most effective [2].

The aim of this study was to study the biodegradation of BPA and/or BPS by bacterial strain *Labrys portucalensis* F11, previously isolated from an industrially contaminated area and which has been shown to degrade a variety of pharmaceuticals and aromatic compounds [3].

The biodegradation of BPA and BPS was evaluated using *L. portucalensis* strain F11, first in mineral medium supplemented with 5.9 mM of acetate and then in a real wastewater matrix. BPA and BPS concentration was evaluated by HPLC.

Complete degradation of both compounds supplied at 2.0 mg. L^{-1} was reached in independent assays in mineral medium, via co-metabolism, over a 15 days period. Biodegradation reactions of these compounds by F11 follow a first-order kinetic with a biodegradation rate and half-time life of 0.190 d^{-1} and 2.4 days for BPA and 0.106 d^{-1} and 2.9 days for BPS. At higher BPs concentration, 5 mg.L^{-1}, and the same previous conditions, strain F11 was able to remove 85% of BPA and 91% of BPS at the end of the assay (28 days).

BPA and BPS biodegradation by strain F11 was also evaluated in a real matrix of municipal wastewater and preliminary results showed a complete degradation of the supplied amount of BPs. BPA supplied at 2 mg L^{-1} was fully removed in experiments containing F11 cells in 13 days, 17 days before the control samples without the strain F11. BPS (6 mg.L^{-1}) was fully removed in 8 days in samples with and without F11 strain.

Toxicity assays were carried out on samples of biodegradation experiments using S-YES kit (New Diagnostics, Germany) according to manufacturer instructions. BPS shows no significant estrogenicity

over *Saccharomyces cerevisiae* BJ3505 yeast cells and the products of BPA biodegradation by F11 strain have approx. 90% less estrogenic activity than the parent compound.

Labrys portucalensis F11 evidence the ability to fully degrade BPA and BPS. Identification of degradation metabolites is ongoing to better understand the metabolic pathway. To the best of our knowledge, this is the first report of BPS complete degradation by a single bacterial strain isolated from the environment.

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Student: Ana Luiza Rodrigues Fontes

Supervisor(s): Prof.ª Doutora Ana Gomes; Doutor Luis Rodríguez-Alcalá; Prof.ª Doutora Maria do Rosário Domingues

Thesis Tittle: Development of a new functional dairy product enriched in microbial bioactive conjugated fatty acids through an industrial-based approach

Abstract (max. 3000 characters, including spaces)

Obesity is among the major risk factors in the development of certain non-communicable diseases such as cardiovascular diseases and some types of cancer. Nevertheless, during the last decade several food-derived lipids with potential bioactive properties have been identified. These include conjugated linoleic acid (CLA) and conjugated linolenic acid (CLNA) isomers, which have promising anti-obesity and anti-cancer properties, among others. Due to limitations in terms of concentration and availability in their natural sources (e.g. ruminants' milk and meat or vegetable oils), the *in situ* microbial production may reveal itself to be a good strategy to increment CLA/CLNA daily intake. Several lactobacilli and bifidobacteria strains have been reported to produce CLA/CLNA isomers using linoleic (LA) and alpha-linolenic (α-LNA) acids as precursor substrates, respectively. However, few studies have explored the potential to use such bacteria in functional products, therefore, the aims of this research study are i) the identification of CLA/CLNA-producing strains among a wide range of bacterial collections from food and human origins, ii) characterization and understanding of the transformation pathways involved in the CLA/CLNA microbial production, iii) development of a dairy product enriched in microbial conjugated fatty acids (CFA), iv) assessment of their stability throughout storage and v) evaluation of their anti-carcinogenic activity.

With the first objective concluded, the next step was to search for new compounds within the 4 strains previously identified as CLA/CLNA producers and certain non-producing strains, grown in the presence of LA or LNA. Fatty and hydroxy fatty acids analyses enabled the detection of two unknown compounds among two strains. Further mass spectrometry analyses are to be performed to identify those, and possibly other, undetected compounds and, therefore, get new insights into LA/LNA isomerization pathways.

Due to economic and safety concerns, the possibility of using previously hydrolyzed edible vegetable oils rich in LA/LNA instead of pure substrates was investigated. Three lipases (*Candida rugosa*, *Pseudomonas fluorescens* and Pancreatic porcine lipases) were tested for the hydrolysis of each of three commercial oils (flaxseed, hemp seed and soybean oils) to determine which combination yielded higher

free LA/LNA amounts. *Candida rugosa* lipase revealed hydrolysis rates above 90% for all oils, and was thus selected for the treatment of soybean and flaxseed oils, which are rich in LA and LNA, respectively.

According to the CLA/CLNA yields previously observed, the best producing strain was selected to proceed with the milk CLA/CLNA-enrichment. Hydrolyzed soybean and flaxseed oils were tested individually or in combination to provide different proportions of LA and LNA. Highest CFA enrichment was achieved with flaxseed oil alone; this was selected for the following assays. Final optimization assays are being performed to conclude the elaboration of CFA-enriched milk for the development of an enriched dairy product.

Student: Cláudia Maciel

Supervisor(s): Prof. Paula Teixeira, Dr. Vânia Ferreira

Thesis Tittle: Contributions to fill knowledge gaps in the interactions between probiotic bacteria and *Listeria monocytogenes* in the host gastrointestinal tract

Abstract (max. 3000 characters, including spaces)

In the past decades, hundreds of novel bacteriocins have been discovered and, amongst those with potential to counteract the widespread emergence of hypervirulent, drug resistant pathogens, bacteriocins secreted by lactic acid bacteria (LAB) constitute the most extensively investigated. LAB present fastidious growth requirements, generally provided by complex culture media, which formulation encompasses protein hydrolysates, a prominent source of low molecular weight peptides, whilst a scarce bacteriocin production is documented. Moreover, such peptides detain physicochemical features similar to those of bacteriocins; hence intricate purification procedures comprising a multitude of laborious, time consuming steps are pivotal for removal of those contaminants. This fact prompted us to develop a peptide-devoid chemically defined medium (CDM) eliciting a simplified, interferent-free purification procedure towards obtainment of high-purity bacteriocins.

The rationale for the development and optimization of the novel CDM comprised a sequential experimental design, encompassing a one variable at a time (OVAT) approach, a Resolution III two-level fractional factorial design (FFD) and a face-centered central composite design (FCCCD). A protocol composed by ion exchange chromatography (IEX) *per se* - CDM - or in combination with hydrophobic ionic chromatography (HIC) - MRS - was implemented to purify pediocin PA-1 (*Pediococcus acidilactici* and *Lactobacillus plantarum*) and enterocin B (*Enterococcus faecium*).

On the basis of *in silico* genome prediction, the bacterial strains appeared to be endowed with a prominent repertoire of enzymes and transport systems involved in a plethora of metabolic pathways, namely the bacteriocin biosynthetic network.

Initially, OVAT allowed establishing clusters and grouping 45 components into 25 factors, considering the importance pattern, along with the chemical similarity referring to the biological function, of the distinct chemical species. Afterwards, FFD analysis of variance demonstrated that glucose, phosphate buffer and pH impacted bacterial growth and bacteriocin production, whereas purines and pyrimidines only influenced cellular density and surfactant Tween 80 solely impacted peptide concentration. Pertaining bacteriocin production, FCCCD unraveled statistical significance regarding phosphate buffer and pH linear interaction. The novel CDM elicited a promising 4-fold higher peptide synthesis comparatively to the conventional medium (MRS).

Concerning purification, a high resolution IEX was employed and the peptide was eluted as a single symmetrical peak detaining a high degree of purity. MALDI-TOF MS analysis disclosed that the most abundant tryptic fragments corresponded to pediocin PA-1 and enterocin B. The output of the peptides'

purification was also portrayed concerning yield and specific activity and it emerged that in comparison with MRS medium, CDM elicited noteworthy increases of 2.4 and 2.8-fold, respectively.

Student: Helena Alexandra Gonçalves Ferreira

Supervisor(s): Elisabete Pinto, Marta W. Vasconcelos, Ana M. Gil

Thesis Tittle: IMPULSE - IMpact of a PULSE-based partial replacement diet on metabolome and health

Abstract

The IMPULSE project generally intends to assess human health adaptations of daily replacing one typical omnivorous lunch meal with a vegetarian meal using protein-rich grain legumes, such as beans, chickpeas, lentils or peas, also known as pulses, as animal-based foods, like meat, alternatives. For this purpose, we will make use of a combination of food and nutritional research classical tools with cutting edge "omics approaches", namely, Metabolomics through body fluid analysis by Nuclear Magnetic Resonance (NMR) spectroscopy. The first year of this PhD was dedicated to the planification of the quasi-experimental dietary intervention and data collection protocols. From the end of the first year and through the second and third years, three 2-month dietary interventions have been carried out: from March to May 2018 (n=19), from October to December 2018 (n=8) and from March to May 2020 (n=18), respectively. However, due to the COVID-19 world pandemic, the last intervention had to be reduced to a smaller 6-week food trial. As so, my third PhD year was dedicated to the execution of the third dietary intervention together with data collection, as well as to the progression of NMR biologic sample analysis. Until present date, we have gathered data from 45 volunteers, and all has been assembled in databases. About 40% of all biologic samples available were acquired through NMR technique, namely, 53% of urine (193 out of 363) and 26% of plasma samples (30 out of 117). Still, the need to perform a 3rd trial, together with the interruption of laboratory work due to COVID-19 situation has put this task on hold and has not allowed the start of the analysis of the faecal samples yet. Likewise, no NMR spectrums processing has been fully performed yet, hence no results regarding metabolomics can be presented at this seminar. Nevertheless, preliminary analysis from other data sources like food preference tests, as well as, physical measures and blood biochemistry assessments, was performed. In short, results point out to general good acceptance by participants to the proposed diet, to an overall maintenance of anthropometric parameters and to a maintenance (e.g. iron) or slight improvement in few health indicators (e.g. blood lipid profile). Scientific papers are being planned in order to publish relevant results. Yet, in this past year, a first-author systematic review has been published on the journal *Critical Reviews in Food Science and Nutrition (*Q1). Another first-author manuscript has been accepted for revision by the *International Journal of Sociology for Agriculture and Food*. Also, I was co-author of a paper accepted for publication in the *World Review of Nutrition and Dietetics*. All in all, most efforts until now have been dedicated to optimizing data collection in order to produce better quality results. As future work we intend to resume NMR metabolomics analysis of all biological samples as quick as possible, as well as advance all data analysis.

Student: Marta Sofia de Almeida Mendes

Supervisor(s): Prof. Paula Castro (Supervisor), Prof. Manuela Pintado, Prof. Patrícia Moreira (Co-Supervisors)

Thesis Tittle: *Dye decolourisation by yeasts: insights on pathways towards an innovative solution for textile effluents*

Abstract

Environmental pollution is one of the most important problems associated with industrialization. Industrial processes lead to the release of environmental pollutants, which contaminate air, water and soil and may have harmful effects on the ecosystems and also, directly or indirectly, on the human health. Textile industry is one of the most important industry worldwide producing high volumes of dyed effluents due to the large quantities of water and chemicals used in fabric processing. These dyes contribute to environmental pollution as they are resistant to biodegradation and cause toxicity to the aquatic life. Traditional wastewater treatment methods for dye degradation are not very effective and are also expensive and can generate toxic by-products, and biotechnological approaches usually represent more environmentally friendly technologies while allowing for the mineralization of organic pollutants.

The purpose of this PhD work is to develop a yeast-based solution for the degradation of dyes present in textile wastewaters in order to remove colour and decrease toxicity of the dyed effluents.

Three yeast strains isolated from wastewater treatment plants were tested for the ability to decolourise some common groups of commercial textile synthetic dyes (reactive, disperse, direct, acid and basic) and simulated textile effluents, as single strains and as consortia. Extra and intra-cellular enzymes possibly involved in the decolourisation process were searched for, through enzymatic assays, by the isolated strains. Toxicity of the decolourisation products from some selected simulated effluents were evaluated using different tests, covering organisms from different trophic levels. Identification of resultant metabolites are under analysis. Freeze and spray drying of the most relevant strains were carried out as strategies to obtain stable starter cultures able to maintain viability under different conditions of storage. Future work will envisage the incorporation of strains in alginate and/or alginate/chitosan capsules and test for its efficiency as an immobilization agent. Optimized developed prototype solutions will be tested at a pilot scale and introduced in an aerobic granular sludge of bioreactors operating at ESB for wastewater treatment using current procedures. The resulting bioaugmented granules will be tested for their capacity to remove the dyes in the simulated effluents under operating conditions mimicking those of real effluents and the microbial dynamics of the granules in such conditions will be investigated.

Student: Nádia Suati Caetano da Silva

Supervisor(s): Patrícia Moreira da Costa

Manuela Pintado

Raquel Madureira

Thesis Tittle: Exploring new sustainable solutions based on chitosan and nanocellulose towards preventive conservation of Cultural Heritage

Abstract

Artworks and cultural objects made of stone, such as outdoor sculptures, represent a relevant part of the world's cultural heritage and, consequently, its preservation and consolidation are of great importance. However, stone art objects exposed to outdoor conditions are subject to decay, as a consequence of several deteriorating agents of physical, chemical and biological nature. This process, over time, favours the destruction of cultural heritage and the loss of its economic, cultural and historical value. In particular, the colonization and growth of microorganisms are problematic, since the development of intricate and dynamic ecosystems in stone artwork accelerates its degradation resulting in a significant damaging impact on its structural and aesthetical characteristics. Therefore, the design of solutions to delay biodeterioration issues of stone artwork is necessary, namely the development of technological-based preventive measures using sustainable and non-toxic products. In this regard, this study proposes the development of coatings based on chitosan and nanocellulose to apply in outdoor stone sculptures.

Thus, the initial task of the project includes the evaluation of the epilithic microbiome of outdoor sculptures to understand the level of biocontamination to which these artworks are exposed and to identify the main bacterial and fungal taxonomic groups that colonize their surfaces. Five outdoor stone and mortar sculptures located in the Metropolitan Area of Porto were selected for the study. An initial test was performed on one of the selected sculptures to evaluate the efficacy of two noninvasive methodologies to sample stone surfaces: the use of swabs was compared to the use of a poly(HEMA) cryogel, which was tested as an alternative to swabbing, while using flow cytometry to calculate microbial cell density and cell viability. Better recovery of microorganisms was achieved from swabs, although the separation of viable and nonviable populations was clearer in samples obtained from cryogels. Sample collection was therefore carried out with swabs in other sculptures and the microbial quantification by flow cytometry was complemented by ATP bioluminescence assays, colorimetry and SEM observations in selected parts of the sculptures. High-throughput sequencing of the V3-V4 and ITS2 variable regions of the 16S rRNA gene and ITS region revealed a diverse and rich microbial ecosystem thriving on the sculptures' surface, with many of the identified taxonomic groups being commonly associated with the deterioration of stone and mortar cultural heritage.

Student: Ricardo Gómez García

Supervisor(s): Dr. Cristóbal N. Aguilar, Dra. Ana Raquel Madureria and Dra. Manuela Pintado

Thesis Tittle: Functional ingredients from valorization of melon (*Cucumis melo L.)* by-products: production, bioactivity and potential application

Abstract (max. 3000 characters, including spaces)

Inodorus melon peels are part of the enormous overgeneration of fruit by-products by the fruit processing industries, which are mismanaged and rejected to the landfills, causing economic and environmental issues. However such peels can be valorised because they still keep considerable amounts of value-added compounds such as proteins with biological activity (enzymes) and can be recovered by green and cost-effective process. In this study Cucumisin enzyme (CUC) from industrial melon by-products (peel) was separated for the first time through biological precipitation using carrageenan (CRG); such technique could represent a cost-effective process to the industries, avoiding the use of expensive equipment and toxic salts or solvents. In this study, different methods were applied for protein extraction namely precipitation using carrageenan and with ammonium sulphate precipitation and identification was achieved by SDS-PAGE gel and FPLC. The molecular weight of the isolated cucumisin was estimated at 68 KDa and shown highly stable proteolytic (PA) and milk-clotting (MCA) activities in a wide range of $CaCl_2$ concentrations (20 to 60 mM), pH (5 to 7) and temperatures (30 to 85 ºC). Melon peels juice demonstrated to possess significant PA (4.24 U/mg protein) and MCA (191.50 MCU/mg protein), but such values were improved when coupled to biological precipitation (17.65 and 2.11-folds, respectively), showing the capability to be an effective strategy to isolate and purify CUC, keeping its biological properties with a yield of 0.17 g CUC/100 g of by-products. The use of melon by-products as new feedstock for proteins recovery by biological precipitation could decrease the environmental impact as well as minimize the costs associated to the traditional extractive processes. This research demonstrated that melon peels possess relevant proteolytic (4.24 U/mg protein) and milk-clotting (6300 MCU/mg protein) activities with an MCA/PA ratio of 1485 at 85 ºC. Besides, biological precipitation allows to maintain and improve the biological activity of the proteins recovered from melon peels with the best conditions of precipitation at pH value 3 and low concentration of CRG (0.003% w/v). Furthermore, these findings support the importance to valorise melon by-products to avoid economic and environmental issues through their reincorporation into the industrial chains as rich sources of value-added proteins.