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Photolyase production and nanoencapsulation for topical applications

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Photolyase production and nanoencapsulation for topical applications

BY

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**“Equipped with his five senses, human explores
the universe around him and calls
the adventure SCIENCE”**

- Edwin Powell Hubble -

Dedico esta tesis a mi familia
que siempre ha creído en mí más que yo misma

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RESUMO

TORRES-OBREQUE, K.M. **Produção e nanoencapsulação da fotoliase para aplicações tópicas**, 2024. 126f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2024.

A radiação ultravioleta (UV) da luz solar pode induzir danos ao DNA, levando à formação de fotoprodutos que podem, eventualmente, resultar em câncer de pele. A aplicação tópica de enzimas reparadoras de DNA, como a fotoliase, representa uma estratégia inovadora para fotoproteção ativa. Nesse sentido, a nanoencapsulação da fotoliase oferece alternativa promissora para facilitar o acesso da enzima às camadas mais profundas da pele. Este projeto teve como objetivo produzir uma fotoliase recombinante, caracterizá-la e nanoencapsular a enzima em polimerossomas (PL), lipossomas (LP) e nanopartículas poliméricas (PNP). A fotoliase foi produzida por *E. coli* recombinante em processos conduzidos em agitador metabólico e biorreatores descontínuos e alimentados. Após extração e purificação por cromatografia de afinidade, a fotoliase exibiu massa molecular de 47 kDa com um rendimento de 480 mg de proteína pura por litro de cultura em batelada alimentada. Concentrações muito baixas de fotoliase, como 15 µg/mL, apresentaram 90% de reparo de CPD em amostras de DNA *in vitro*. Posteriormente, nanoestruturas foram desenvolvidas usando estratégias de planejamento de experimentos (DoE) para otimizar o diâmetro hidrodinâmico (Dh) e a eficiência de encapsulamento (EE). Valores nanométricos de Dh foram obtidos para todas as nanoestruturas. EE de 23% foram alcançados para PL e LP, e >90% para PNP. Os PL exibiram a melhor liberação da enzima ao longo do tempo (até 35% após 24h), sendo que as PNP apresentaram a menor taxa de liberação atingindo somente ≈ 15% após 10 dias. Baseados em estudos de citotoxicidade *in vitro*, os PL, LP e PNP foram classificados como materiais não irritantes. Finalmente, tanto a fotoliase livre quanto a encapsulada em PL e LP foi capaz de proteger as células da radiação UV, resultando em viabilidades celulares de 37% para a enzima livre e 50-60% na forma nanoencapsulada, comparado a 28% de viabilidade sem o tratamento com a enzima. No geral, nosso estudo demonstra a produção eficiente da fotoliase de *T. thermophilus* e sua capacidade em reparar danos de CPD, principalmente quando encapsulada em PL, oferecendo uma alternativa promissora e inovadora como ingrediente ativo para incorporação em produtos dermatológicos.

Palavras-chave: fotoliase, biorreator de batelada alimentada, polimerossoma, lipossoma, nanopartículas poliméricas.

ABSTRACT

TORRES-OBREQUE, K.M. **Photolyase production and nanoencapsulation for topical applications**, 2024. 126p. Thesis (PhD) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2024.

Ultraviolet (UV) radiation from sunlight can induce DNA damage, leading to the formation of photoproducts that can eventually result in skin cancer. Topical application of DNA repair enzymes, such as photolyase, represents an innovative strategy for active photoprotection. In this sense, photolyase nanoencapsulation offers a promising route to facilitate the access of enzymes to the deeper layers of the skin. This work aimed to produce the recombinant photolyase, characterize it, and investigate nanostructures for the enzyme encapsulation, namely polymersomes (PL), liposomes (LP) and polymeric nanoparticles (PNP). Photolyase production by recombinant *E. coli* was investigated in metabolic shaker and batch and fed-batch bioreactors. After extraction and purification by affinity chromatography, the photolyase exhibited a molecular weight of 47 kDa with a yield of 480 mg of pure protein per liter of fed-batch culture. Very low photolyase concentrations, such as 15 µg/mL, already presented 90% of CPD repair in DNA samples *in vitro*. Subsequently, nanostructures were developed based on design of experiments (DoE) to optimize the hydrodynamic diameter (Dh) and encapsulation efficiency (EE). Nanometric values of Dh were obtained for all nanostructures. EE of 23% was achieved for both PL and LP, and >90% for PNP. PL exhibited the best enzyme release profile to the external medium over time (up to 35% after 24h), while PNP presented the lowest release reaching only ≈ 15% after 10 days. Based on *in vitro* cytotoxicity studies, PL, LP and PNP were classified as non-irritating material. Finally, both free and photolyase-loaded PL and LP were able to protect cells from UV radiation, resulting in cell viability recoveries of 37% for the free enzyme and 50-60% when nanoencapsulated, compared to 28% without the enzyme. Overall, our study demonstrates efficient production of *T. thermophilus* photolyase and the enzyme ability to repair CPD damage, particularly when encapsulated in PL, offering a promising and innovative alternative as an active ingredient for incorporation into dermatological products.

Keywords: photolyase, fed-batch bioreactor, polymersome, liposome, polymeric nanoparticle.

RIASSUNTO

TORRES-OBREQUE, KM. **Produzione e nanoincapsulamento di fotoliasi per applicazioni topiche**, 2024. 126f. Tesi (PhD) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, San Paolo, 2024.

Le radiazioni ultraviolette (UV) provenienti dalla luce solare possono indurre danni al DNA, portando alla formazione di fotoprodotti che possono eventualmente provocare il cancro della pelle. L'applicazione topica degli enzimi riparatori del DNA, come la fotoliasi, rappresenta una strategia innovativa per la fotoprotezione attiva. In questo senso, la nanoincapsulazione della fotoliasi offre un'alternativa promettente per facilitare l'accesso dell'enzima agli strati più profondi della pelle. Questo progetto ha oggettivato produrre una fotoliasi ricombinante, caratterizzarla e nanoincapsulare l'enzima in polimerisomi (PL), liposomi (LP) e nanoparticelle polimeriche (PNP). La fotoliasi è stata prodotta da *E. coli* ricombinante in processi condotti in agitatori metabolici e bioreattori batch e fed-batch. Dopo l'estrazione e la purificazione mediante cromatografia di affinità, la fotoliasi presentava una massa molecolare di 47 kDa con una resa di 480 mg di proteina pura per litro di coltura fed-batch. Concentrazioni molto basse di fotoliasi, come 15 µg/mL, hanno mostrato una riparazione CPD del 90% nei campioni di DNA *in vitro*. Successivamente, sono state sviluppate nanostrutture utilizzando strategie di progettazione degli esperimenti (DoE) per ottimizzare il diametro idrodinamico (Dh) e l'efficienza di incapsulamento (EE). Per tutte le nanostrutture sono stati ottenuti valori nanometrici di Dh. Un EE del 23% è stato raggiunto per PL e LP e >90% per PNP. PL ha mostrato il miglior rilascio dell'enzima nel tempo (fino al 35% dopo 24 ore), mentre PNP ha mostrato il tasso di rilascio più basso, raggiungendo solo ≈ 15% dopo 10 giorni. Sulla base degli studi di citotossicità *in vitro*, PL, LP e PNP sono stati classificati come materiali non irritanti. Infine, sia la fotoliasi libera che quella incapsulata con PL e LP sono state in grado di proteggere le cellule dalle radiazioni UV, determinando una vitalità cellulare del 37% per l'enzima libero e del 50-60% nella forma nanoincapsulata, rispetto al 28% di vitalità senza trattamento enzimatico. Nel complesso, il nostro studio dimostra l'efficiente produzione della fotoliasi di *T. thermophilus* e la sua capacità di riparare il danno CPD, soprattutto se incapsulato in PL, offrendo un'alternativa promettente e innovativa come ingrediente attivo da incorporare nei prodotti dermatologici.

Parole chiave: fotoliasi, bioreattore fed-batch, polimerisoma, liposoma, nanoparticella polimerica

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INTRODUCTION

Life on Earth evolved under the influence of solar radiation. The human body senses the incidence of radiation through several mechanisms. Infrared radiation (IR) is detected in the form of heat, visible radiation is detected through the different colors by the optical system, and ultraviolet (UV) radiation is detected as photochemical reactions (Saewan & Jimtaisong, 2013; Watson et al., 2016).

Generally, UV radiation is absorbed and diffused by oxygen and ozone present in the atmosphere. Unfortunately, the total amount of ozone in the atmosphere, known as the ozone layer, is decreasing and more solar radiation is reaching Earth's surface. Overexposure to UV radiation is associated with an increased incidence of skin cancer, in particular non-melanoma skin cancer, and actinic keratosis, a benign neoplasm with the potential to transform into skin cancer (WHO, 2016).

According to the World Health Organization (WHO), one in every three cancer diagnostics refers to skin cancer worldwide. Specifically, between two and three million cases of non-melanoma skin cancer occur globally each year and it is estimated that a 10% reduction in ozone levels will result in an additional 300,000 cases of non-melanoma skin cancer. Non-melanoma skin cancers represent more than 90% of all skin cancers (Leccia et al., 2019). In Brazil, according to the National Cancer Institute (INCA), non-melanoma skin cancer (basal cell carcinoma and squamous cell carcinoma) accounts for 30% of all malignant neoplasms registered in the country. It is estimated that 220.490 people were diagnosed with skin cancer in Brazil in 2022 (INCA, 2022). However, early diagnosis results in more than a 90% chance of cure.

The skin is the largest organ of the human body, it covers the entire surface of the body and is continuously exposed to the harmful external environment. Nonetheless, it presents several endogenous protection mechanisms against the effects of UV radiation, such as the physical barrier provided by the body hair. Sweat also plays a role by absorbing and reflecting incident radiation and flowing to the hair follicle. Additionally, melanin and the complex system of anti-radical and DNA repair agents

constitute an intrinsic skin defense mechanism. However, individual characteristics such as skin phototype, duration of radiation exposure and geographic position are imperative for the need to supplementary protection (Cestari et al., 2012; Sambandan & Ratner, 2011).

Since photoaging is partly related to DNA damage induced by UV radiation, the topical application of DNA repair enzymes, such as photolyase, emerges as a preventive strategy. They enhance the endogenous capacity for DNA repair, thereby increasing protection against UV radiation and reducing photocarcinogenesis (Gilaberte & González, 2010). However, due to its protein nature, photolyase presents poor stability, being easily degraded under extreme pH and temperature conditions. Furthermore, its high molecular mass (approximately 60 kDa) may pose a potential challenge to cutaneous penetration. In this regard, the use of nanostructures that enable the payload protection and penetration into the skin presents itself as an alternative for the topical use of photolyase. Nanostructures, in general, can enhance the cutaneous penetration profile of certain actives, for example, by forming an occlusive layer on the skin and increasing superficial hydration (Apolinário et al., 2020).

This study proposes the production and nanoencapsulation of the photolyase enzyme from *Thermus thermophilus*, aiming to establish an innovative and functional alternative for active photoprotection by topical application. The approach involves the production of the enzyme using recombinant *E. coli* BL21, purification and its DNA repair enzymatic activity evaluation. Subsequently, we investigated different types of nanostructures, namely polymersomes, liposomes and polymeric nanoparticles to encapsulate the enzyme, as well as the *in vitro* cytotoxicity and UV radiation protection of both free and encapsulated photolyase.

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CHAPTER I

Theoretical Framework

CHAPTER I

Theoretical Framework

1. Skin

Skin is the largest organ in terms of surface area and performs several functions such as sensorial, protection of the body from the external environment, body temperature control, and vitamin D synthesis. In adults, the skin covers an area of about 2 m², its average thickness is about 2.5 mm, and represents up to 6% of total body weight (Čižmarová et al., 2023). The skin is composed by three main layers: the epidermis (most external and avascular), the dermis (rich in blood vessels, lymphatic vessels, nerve endings, fibroblasts, macrophages and extracellular matrix), and the subcutaneous tissue composed of fat cells (lipocytes). (Nascimento et al., 2024). As we can see in Figure 1, the first layer is formed mostly by epithelial tissue and the second mostly by connective tissue (Soutor & Hordinsky, 2015).

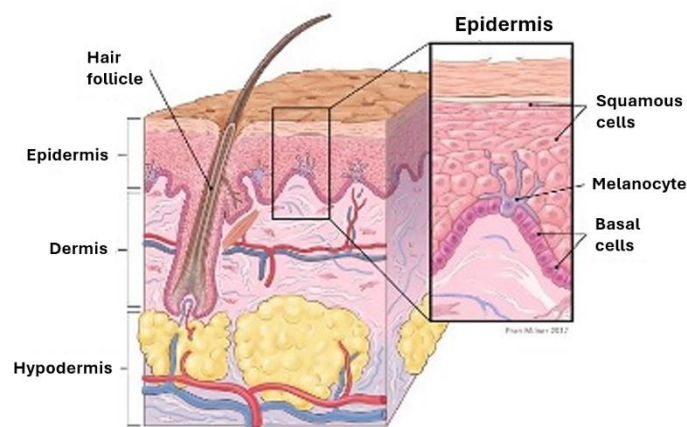


Figure 1. Illustrative cross-section of the skin with the respective names of layers and skin components (Soutor & Hordinsky, 2015).

More specifically, the epidermis is the superficial layer, whose primary function is to serve as a physical and chemical barrier between the interior of the body and the external environment. The epidermis is 0.05 to 1 mm thick, depending on the region of the body, and is mainly made up of three types of cells: keratocytes, melanocytes and

Langherhans cells (Čižmárová et al., 2023; Nafisi & Maibach, 2018). The most superficial layer of the epidermis, called stratum corneum, is made up of 10 to 30 thin layers of dead, anuclear and organelle-free keranocytes, known as corneocytes, which cover the epidermis, giving the skin almost complete impermeability. As the outermost cells age and wear down, they are replaced by new layers of strong, long-wearing cells. Complete cell turnover occurs every 28 to 30 days in young adults, while the same process takes 45 to 50 days in elderly adults (Nafisi & Maibach, 2018; NIH, 2024). Each corneocyte, in addition to being mainly composed of ceramides, cholesterol and fatty acids, is surrounded by protein envelopes containing keratin, with water retention characteristics. Corneodesmosomes provide the connections among corneocytes. This structure is responsible for protection against dehydration and external microorganisms. In addition, gaps known as 'pores' emerge within the stratum corneum (Nafisi & Maibach, 2018).

Melanocytes are phenotypically relevant, responsible for hair and skin pigmentation, contributing not only to the appearance of the skin, but to protection against ultraviolet (UV) radiation. This is due to its greater resistance to stress and genotoxicity caused by UV radiation. Melanocytes can suffer the effects of radiation resulting in melanoma, the most aggressive type of skin cancer (Tang et al., 2020; Yousef et al., 2021).

As the skin is the largest organ in the human body and is completely visible, its changes directly affect self-esteem, with social and psychological impacts. In short, the complexity of skin functioning encompasses much more than just physical and thermoregulatory protection. This list becomes long as it includes immunological, endocrine, metabolic, psycho-emotional, neuro-psycho-immunological functions, and also incorporates essential support systems such as blood, innervation, muscle, endocrine and even sensitization to UV radiation (Čižmárová et al., 2023; Tobin, 2017).

2. Photodamage caused by UV radiation

The energy of solar radiation increases with decreasing wavelength (λ); therefore, low-wavelength UV radiation is more energetic. The spectrum of UV radiation is classified into: UVC (100-290 nm), UVB (290-320 nm) and UVA (320-400 nm). Furthermore, UVA radiation is subdivided into: UVA I (340-400nm) and UVA II (320-340nm). UVB and UVA are the spectral components that reach the planet's surface. UVC radiation, on the other hand, is absorbed by oxygen and ozone present in the atmosphere (He et al., 2022; Lopes et al., 2023; Watson et al., 2016).

Exposure to UV radiation has beneficial and adverse effects on human health. UVB radiation on the skin is important for the biosynthesis of vitamin D, which plays an essential role in our metabolism, specifically in maintaining calcium homeostasis. In contrast, skin exposure to UV rays can cause photoallergic reactions, erythema (redness), burns, spots such as freckles and moles, premature wrinkles and photoaging. UV radiation can also damage DNA, proteins and lipids, which can result in harmful consequences such as cell death, mutagenesis and skin cancer, including malignant cutaneous melanoma and the non-melanoma skin cancers basal cell carcinoma and squamous cell carcinoma (Schuch et al., 2017).

Non-melanoma skin cancer is more common in adults over 40 years old, but constant exposure to solar radiation can lower the average age of affected individuals. Skin cancer is considered a public health problem, considering that 40% of cancers in the United States are skin cancers (LAN, 2019). According to the World Health Organization (WHO), one in every three types of cancer diagnosed worldwide is skin cancer.

UVA radiation is absorbed deep into the dermis, as shown in Figure 2, mainly affecting fibroblasts (Yarovaya et al., 2021). It comprises around 95% of all UV radiation on Earth surfaces and promotes higher oxidation in cells than UVB radiation. It is absorbed by chromophores such as melanin and riboflavin, which will promote the protection of skin constituents. However, they generate reactive species, promoting oxidative stress, through the damage of lipids, proteins and DNA – including mitochondrial genetic material, which, when repeatedly exposed to this type of light, presents around 40% more deletion in its genetic material than normal (He et al., 2022; McDaniel et al., 2018).

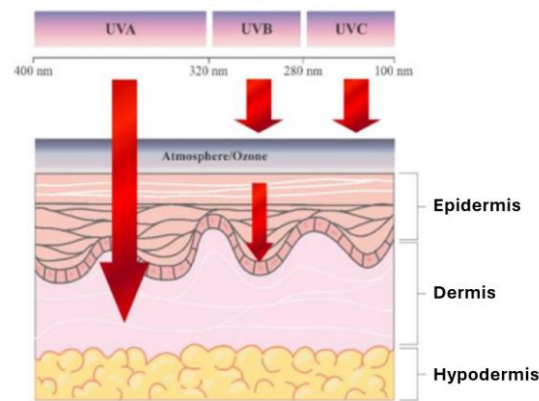


Figure 2. Ultraviolet (UV) radiation reach in the skin layers (Pérez-Sánchez et al., 2018).

In addition to direct effects, sun exposure can also reduce the body defenses that normally limit the progressive development of skin tumors (WHO, 2016). The complexity of the cascade of events extends to the production of reactive oxygen species (ROS), intrinsic to the inflammatory process. ROS are induced by UV radiation and in high levels can disrupt homeostasis, acting as cytotoxic and pro-tumorigenic molecules (Nascimento et al., 2024) and cause oxidative damage to DNA, such as 8-oxo-7,8-dihydroguanine (8-oxoGua) (Huang et al., 2012). Furthermore, UV radiation induces the expression of cutaneous matrix metalloproteinases (MMPs) that degrade collagen - a complex of structural proteins in the skin - and cause photoaging (Lawrence et al., 2018). UV radiation can also be directly absorbed by DNA inducing dimeric photoproducts between adjacent pyrimidine bases (cytosine and thymine). These photolesions are called cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone (6-4PP) photoproducts (Figure 3) (Leccia et al., 2019). Specifically, CPDs occur when adjacent pyrimidine bases absorb energy from UV radiation, forming a cyclobutane ring through the covalent bond between C5 and C6 carbon atoms of both nitrogenous bases. The 6-4PPs can also be present as a covalent bond between pyrimidines of the same strand, however, this is not cyclic, involving the C6 and C4 carbons of the 5' and 3' pyrimidines, respectively. CPD-type damage can lead to the interruption of correct cellular replication and the DNA transcription process, that is, mutagenesis can increase the risk of carcinogenesis, ultimately leading to non-melanoma skin cancer (Banaś et al., 2020; Berardesca et al., 2012; A. Sancar, 2008).

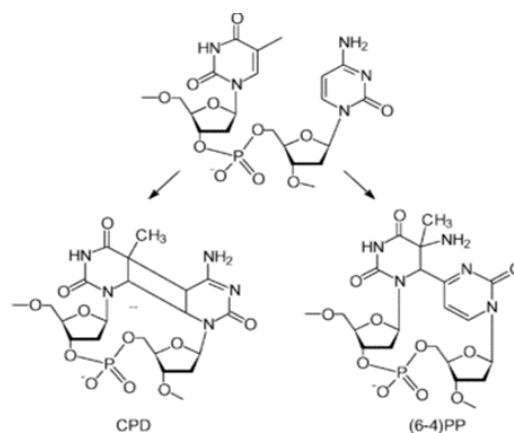


Figure 3. Main lesions caused by UV radiation in DNA: pyrimidine cyclobutanes (CPDs) and photoproducts (6-4)-pyrimidine-pyrimidone (6-4PP).

In general, damage to DNA by UV radiation is 80-90% in the form of CPD and 10-20% of (6-4) PP (Kavakli et al., 2019; A. Sancar, 2003). Furthermore, DNA photolesions may be involved with different molecular responses such as cytokine release, apoptosis and pigmentation (Marrot & Meunier, 2008). Therefore, strategies to reduce the damage caused by UV radiation on the skin are needed.

3. Photolyase

Generally, topical photoprotection involves molecules that absorb or reflect UV radiation, such as organic and inorganic UV filters. However, this passive strategy does not prevent the effects of continuous exposure to UV radiation. In this sense, the topical application of enzymes consists of an "active photoprotection" strategy (Megna et al., 2017). Specifically, the topical use of the enzyme photolyase is of increasing interest in the last decade.

Photolyases are flavoproteins capable of repairing crosslinks in DNA formed by exposure to UV radiation. They repair lesions such as CPD or (6-4) PP, reversing the damage to form intact pyrimidine bases using the energy of blue light (photons emitted between 350-500 nm) (Hosokawa et al., 2019). Thus, two types of photolyase are described in the literature, with different substrate specificity: CPD repair photolyase and (6-4)PP repair photolyase (Hosokawa et al., 2019; A. Sancar, 2008; Santos, 2010). Figure 4 shows the tertiary structure of the *E. coli* CPD-photolyase enzyme.

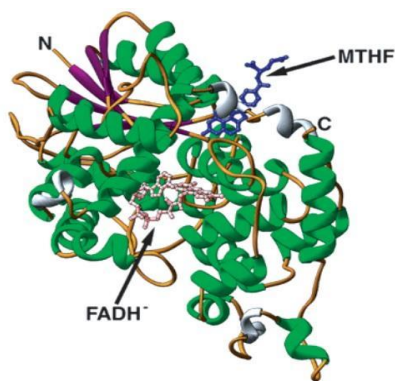


Figure 4. Schematic representation of the tertiary structure of *E. coli* CPD-photolyase showing the *N*-terminal and *C*-terminal domain and the positions of the two cofactors (A. Sancar, 2003).

Photolyases are monomeric enzymes of 53-66 kDa, 450-550 amino acids, active at pH 7.0 and presenting two non-covalently linked cofactors or chromophores. One of the cofactors is always flavin adenine dinucleotide (FAD) and the second can be methenyltetrahydrofolate (MTHF) or 8-hydroxy-7,8-didemethyl-5-deseazariboflavin (8-HDF). The noncovalent active cofactor, FAD, is essential for specific binding to damaged DNA and catalysis. The second cofactor is not necessary for catalysis, however, it functions as a “photoantenna” that absorbs photons from the blue light spectrum and transfers the energy to the FAD increasing the repair rate by 10 to 100 times (Kavakli et al., 2019; Liu et al., 2015).

In 2015, Aziz Sancar was recognized with the Nobel Prize in Chemistry for his work elucidating the mechanisms of DNA repair by photolyase. Enzymatic activity in photolesion repair is described as a two-step mechanism: (i) the enzyme binds to the dimer with high affinity and specificity, which can occur in the dark, and (ii) photoreduction or reversal of the dimer, which occurs only in the presence of light, specifically blue light (Sancar et al., 1987). The key point of the reaction is the fully reduced FAD cofactor (FADH^-) after blue light activation, which is capable of transferring an electron to damaged DNA leading to DNA repair (Figure 5) (A. Sancar, 2003; Zhou et al., 2019).

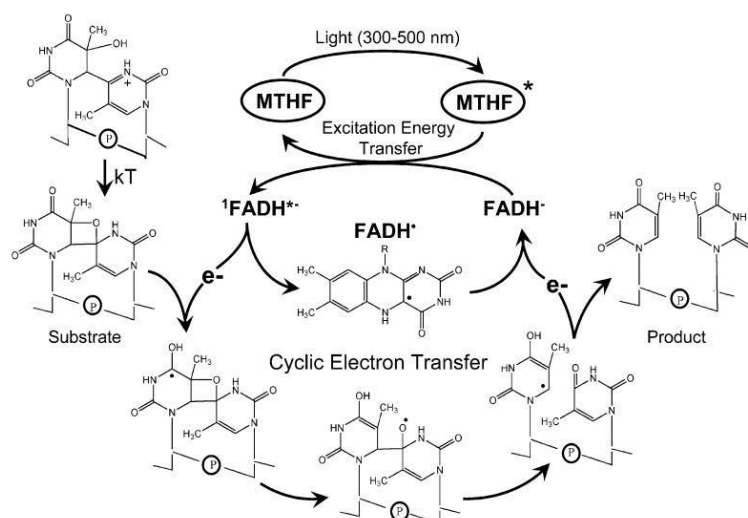


Figure 5. Photolyase reaction mechanism, showing the photochemical steps that include energy and electron transfer, followed by electron transfer back to restore the catalytic cofactor, FADH- (Sancar, 2003).

These enzymes are found in plants, reptiles, amphibians, and marsupials. In general, plant and animal photolyases show limited degree of homology compared to microbial enzymes, generally at the FAD binding site. Photolyase is not innately present in humans, who have other DNA repair mechanisms such as the nucleotide excision repair (NER) pathway, reported to repair UV-induced DNA damage. NER is, however, more effective in recognizing and repairing pyrimidine-primidone lesions and relatively inefficient in repairing CPDs (Leccia et al., 2019). The possibility of incorporating photolyase into a sunscreen - and with it the ability to repair UV-induced DNA damage - adds a new dimension to the strategic approach against solar radiation damage. In 2000, Stege et al. concluded their publication with the words “The exogenous application of photolyase differs from conventional photoprotection in its ability to remove damage that has already occurred. This enzyme therapy approach could therefore be ideally combined as an after-sun strategy with conventional sunscreens to provide photoprotection and repair at the same time”.

There are currently products on the market that combine plankton extract (source of photolyase) encapsulated in liposomes with UV filters, such as Eryfotona® AK-NMSC (Isdin, Barcelona, Spain), Ateia® (Kwizda Pharma GmbH, Vienna, Austria) and Neova®

(Pharma Cosmetics, Oradell, New Jersey, USA) (Ramírez-Gamboa et al., 2022). Recent studies present reviews of the beneficial effects of Eryfotona® in the treatment of actinic keratosis, a benign skin neoplasm induced mainly by UV radiation and with the potential to transform into carcinoma (Leccia et al., 2019; Puig et al., 2019). In Puig et al. (2019), the authors cite 11 clinical studies totaling 228 volunteers and demonstrated the beneficial effect of the formulation in the treatment of mild to moderate actinic keratosis. It is worth noting that all clinical trials were open and the number of patients was reduced. Furthermore, the reviews have among their authors members of the company ISDIN, which raises awareness of a possible conflict of interest. Moscarella et al. (2017) conducted a double-blinded randomized parallel clinical trial pilot study using Eryfotona® and a commercially available SPF 50 sunscreen as a comparator. The total of 36 patients were investigated and after six months both groups showed significant improvement in actinic keratosis lesions, however the effect was more pronounced in the group treated with Eryfotona®.

Yarosh et al. (2019) evaluated 125 scientific publications from the last 20 years with the aim of answering the most prominent questions about the effectiveness of DNA repair enzymes for active photoprotection: Do DNA repair enzymes really work? Do they penetrate the skin and repair DNA? How much cellular damage can they repair? Do they prevent skin cancer? How do they affect skin photoaging? The authors concluded that the topical use of DNA repair enzymes effectively promotes the removal of DNA damage, reduces the appearance of new manifestations of actinic keratosis and promotes regression of existing lesions. Additionally, according to the authors, literature data supports the action of enzymes in preventing photoaging and skin cancer, but additional studies could either strengthen the theory or refute it. We must consider that Eryfotona® contains a plankton extract in its composition without effective control of the enzyme concentration, what can difficult proof of effectiveness. In this sense, studies with formulations containing the pure enzyme might contribute to elucidate the potential of photolyase.

4. Recombinant production of photolyase

The production of photolyase in recombinant *E. coli* cultures has already demonstrated positive results. Marizcurrena et al. (2019) present an efficient and low-cost production of recombinant photolyase, with 35 mg of pure enzyme per L of culture and good results in repairing damage induced by UV radiation.

The production of recombinant proteins using microorganisms, especially bacteria and yeast, has many advantages such as: growth in the form of easily cultivable individual cells, which use low-cost media and are easy to genetically manipulate, giving rise to improved processes for the production of biopharmaceutical products and industrial enzymes (Bahreini et al., 2014; Gupta & Shukla, 2016). The bacterium *E. coli*, in particular, is considered one of the most efficient hosts/producers, as it has well-known physiology and metabolism and presents a high growth rate in simple media. Furthermore, its genome is widely known, facilitating genetic manipulation, and it presents several well-defined cloning vectors for the production of recombinant proteins. (Rosano et al., 2019; Tripathi & Shrivastava, 2019). Currently, 30% of recombinant proteins for therapeutic use are expressed in *E. coli* (L. Li et al., 2022).

To optimize yield in the production of recombinant enzymes, some factors must be considered, such as the culture medium, with several options available on the market. In relation to the composition, culture media can be differentiated into (i) complex media, which do not have a chemical characterization of their components and contain water-soluble extracts of biological origin, rich in nutrients, and (ii) defined media, which are constituted of molecularly homogeneous materials or a mixture of characterized and quantified ingredients, of known chemical composition (Jagschies et al., 2018; Sigma-Aldrich, 2021). Luria-Bertani (LB) for example is a complex medium widely used because it is easy to formulate, promotes rapid bacterial growth and results in high yields in terms of cell mass (Sezonov et al., 2007). However, the use of chemically defined media can be advantageous to produce recombinant proteins, it contains carbon sources, metals and salts at specific proportions needed to produce the molecule of interest. Defined media also allow for better industrial scaling and batch-to-batch quality control, achieving high cellular concentrations and generally facilitating purification processes

(Riesenberg et al., 1991). Li et al. (2017) investigated the global response of the *E. coli* BL21 (DE3) proteome to the production of a recombinant protein (hFGF-2) in complex and defined culture media. Growth and production in complex medium were faster than in defined medium, however, growth inhibition was more pronounced in complex medium, resulting in defined cultures that reached higher total biomass values and higher volumetric concentrations of the recombinant protein.

Specifically, the BL21 (DE3) strain of *E. coli* is widely used in the production of recombinant proteins. It is a modified form of *E. coli* that does not produce significant concentrations of acetate and is also deficient in the two main proteases responsible for recombinant protein degradation, encoded by the genes *lon* (cytoplasmic protease) and *ompT* (periplasmic protease) (Rosano et al., 2014).

5. Nanobiotechnology

Photolyase needs to reach the viable epidermis to exert its effect, but penetration through the upper layer, the stratum corneum, is the main barrier to the passage of topically applied molecules and drugs, representing a major challenge. In this sense, nanotechnology can be an alternative for topical application of photolyase.

According to the National Nanotechnology Institute (nano.gov), nanotechnology is the science, engineering and technology conducted at the nanoscale of 1 – 100 nanometers (nm), or at least containing one dimension at this scale, often being extended to the range of up to 500 nm (Nafisi & Maibach, 2018). Specifically, nanobiotechnology is a field of nanotechnology applied to life sciences, which combines the ideas, tools and materials of nanotechnology and biotechnology.

For the topical application of nanostructures, different routes of penetration into the skin are possible: (I) Intercellular route: corneocytes are not ordered in parallel, allowing particles to make their way between cells, a common path for the transport of amphiphilic molecules; (II) Transcellular Route: unlike the previous route, in this one the nanoparticles cross the cells, passing through their hydrophilic and hydrophobic regions;

(III) Follicular Route: through the follicular system, using hair follicles, sebaceous glands and sweat ducts (Figure 6) (Carter et al., 2019; Yu et al., 2021).

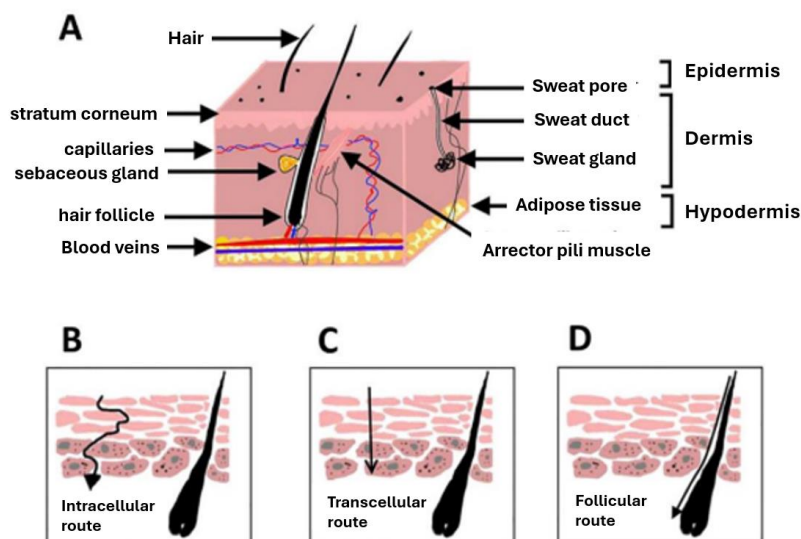


Figure 6. Schematic of nanoparticle penetration pathways into human skin (Carter et al., 2019).

Laboratory tests are normally carried out using pig and human skin and present results comparable to those observed *in vivo*, but the distribution of substances in an *in vivo* model presents relative differences due to the different thickness of the epidermis and dermis layers (Nohynek et al., 2007). Furthermore, research shows that for nanostructures to penetrate the layers of the skin, crossing the stratum corneum, the formation of an occlusion layer, which hydrates the skin, promotes entry into the layers of the epidermis, where the nanostructures can release the bioactive to deeper layers of the epidermis and dermis (Jankowski et al., 2017; Netto MPharm & Jose, 2018; Osorio-Blanco et al., 2020).

Among the main nanocarriers investigated for the delivery of biomolecules, we can highlight: liposomes (LP), polymersomes (PL) and polymeric nanoparticles (PNP) (Alcalá-Alcalá et al., 2015; Apolinário et al., 2017; Cheng & Lee, 2016).

5.1. Liposomes

Liposomes (LP) are spherical vesicles with an aqueous interior and composed of a lipid membrane formed by the self-aggregation of phospholipids (Figure 7). Therefore, LP are biodegradable, biocompatible, non-toxic, and non-immunogenic. substances They can transport lipid-soluble (in the lipid bilayer) and water-soluble (inside the vesicle) compounds (Atanasio et al., 2020; Bozzuto & Molinari, 2015; Pachioni-Vasconcelos et al., 2016), improving solubility and tissue penetration of both lipophilic and hydrophilic drugs. In addition, LP demonstrate remarkable attributes including enhanced site-specific targeting and controlled drug release, shielding drugs from degradation and clearance, and reducing toxic side effects. Liposome sizes vary from 30 nm to the micrometer scale, with the phospholipid bilayer typically measuring 4–5 nm in thickness. (Giordani et al., 2023).

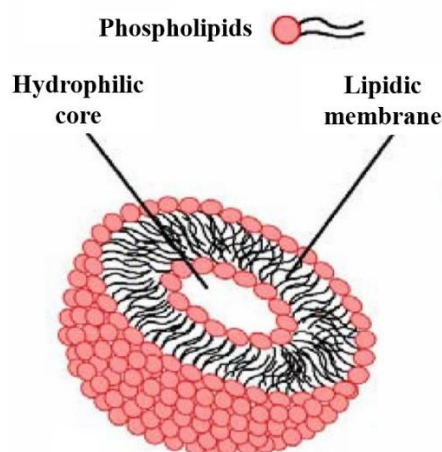


Figure 7. Schematic illustration of a lipid vesicle or liposome.

Thin-film hydration is one of the most prevalent techniques for LP preparation, involving the dissolution of lipid components, with or without a drug, in an organic solvent. Subsequently, the solvent is evaporated via rotary evaporation, followed by rehydration of the film in an aqueous solvent. Other methods include reverse-phase evaporation, freeze-drying, and ethanol injection. Techniques such as membrane extrusion, sonication, homogenization, and/or freeze-thawing are also employed to control the size and size distribution of LP. (Bulbake et al., 2017).

Liposomes can be formulated and processed to vary in size, composition, charge, and lamellarity. Moreover, modifying the constituents of traditional LP can yield structures with unique characteristics. Utilizing alternative lipid types can create pH-sensitive or thermosensitive vesicles, extensively utilized in cancer therapy to prevent lysosomal degradation and induce hyperthermia, respectively (Tavares Luiz et al., 2021).

In 1961, Alec Bangham first described liposomes and in 1995, the American Food and Drug Administration (FDA) approved the first liposomal formulation, Doxil®, for the treatment of Kaposi's sarcoma (Bulbake et al., 2017). Currently, approximately 14 types of liposomal products are authorized to the market by EMA and FDA and many others have been approved by other national agencies, to be used in diverse applications from cosmetic products to anticancer drugs (Giordani et al., 2023).

Among the liposomal formulations approved and available on the market, stands out: Onivyde™, used to treat metastatic adenocarcinoma of the pancreas; Marqibo®, used in the treatment of adult patients with Philadelphia chromosome-negative (Ph-) acute lymphoblastic leukemia (ALL); AmBisome®, indicated to treat severe fungal infections and visceral leishmaniasis (FOOD AND DRUG ADMINISTRATION, 2008, 2015, 2020) and the recent success of the mRNA vaccines developed by Moderna and BioNTech/Pfizer against COVID-19. In this last case, structure is more complex and considered as lipid nanoparticles (LNPs) (Pilkington et al., 2021).

Despite being the most common nanocarriers for pharmaceutical applications, liposomes have some disadvantages, such as the physicochemical instability caused by their lipidic nature. When used topically, liposomes tend to break down into their constituent ingredients, being retained in the stratum corneum (Daraee et al., 2016; Yu et al., 2021). Liposomes application for topical drug delivery date to the research of Mezei and Gulasekharam, who studied the penetration of complete multi-lamellar vesicles into the epidermis and dermis, resulting in enhanced deposition of triamcinolone acetonide compared to conventional topical formulations (such as creams, gels, or solutions) (Mezei & Gulasekharam, 1980; Schlich et al., 2021).

Several articles refer to liposomes as suitable nanostructures for the administration of plankton extract how photolyase source (Berardesca et al., 2012; Gilaberte & González, 2010;

Stege et al., 2000). Stege et al. (2000) observed that nanoencapsulation of photolyase in liposomes resulted in 50% reduction in the production of CPDs and apoptosis in ICAM-1 keratinocytes. Qu et al. (2022) studied the CPD photolyase from *Antarctic Chlamydomonas sp.* nanoencapsulated in liposomes, evaluating the protective mechanisms against UVB-induced damage in mice, and observed DNA repair, antioxidant, anti-inflammatory, and anti-wrinkle properties as well as collagen protection.

5.2. Polymersomes

Polymeric vesicles or polymersomes (PL) are nanostructures formed from the self-aggregation of amphiphilic copolymers in contact with an aqueous medium; therefore, similar to liposomes, they have a hydrophobic bilayer that can incorporate hydrophobic proteins or drugs and an aqueous core capable of encapsulating hydrophilic proteins or drugs (Figure 8) (Pachioni-Vasconcelos et al., 2016). PL were developed by Discher and collaborators in 1999 and have attracted attention due to their colloidal stability and ability to encapsulate various types of molecules, in addition to being usually more stable and versatile than liposomes (Apolinário et al., 2017; C. A. Oliveira et al., 2020). The size, structure and physicochemical properties of PL can be precisely tuned by changing the combination of polymers and their molecular mass, allowing different membrane thickness and permeability. It is possible to design PL in the range of 10 nm to 5 μ m, with controlled surface topology (irregular and/or smooth) and even with responsive behavior to environmental stimuli, such as pH, temperature, light/UV, oxidation and presence of glucose (Pachioni-Vasconcelos et al., 2016; Rodríguez-García et al., 2011). Compared to LP, PL have a membrane thickness of approximately 5 – 50 nm (liposomes $\sim$ 3 nm), which provides better mechanical resistance (Rideau et al., 2018). Despite all the advantages, there are still no pharmaceutical products based on PL on the market.

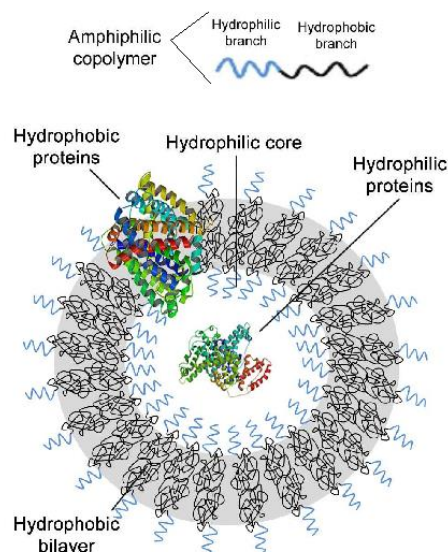


Figure 8. Schematic representation of a polymeric vesicle or polymersome (Pachioni-Vasconcelos et al., 2016).

The forming unit of PL are amphiphilic copolymers, structures that contain two or more homopolymer sequences with different hydrophilic character joined by covalent bond. When in contact with solvents, they self-organize into different morphologies, which will depend on parameters such as polymer concentration, temperature, chemical structure of the amphiphilic copolymer, proportion and molecular mass of each homopolymer block, solvent properties, agitation and pH (Apolinário et al., 2017; Chang et al., 2014). The homopolymer sequences that form the amphiphilic copolymer are called blocks and according to the number of blocks present in the structure, they are considered diblock (AB), triblock (ABA) or even multiblock. This versatility allows the formation of nanostructures with different characteristics with the ability to incorporate drugs with different properties. The polymers used must present a set of ideal specific characteristics: they must be water-soluble, non-toxic, non-immunogenic and degraded or eliminated by the body (Mvango et al., 2018; Wagner et al., 2018). Some examples of amphiphilic copolymers that have been used are: poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide), PEO-PPO-PEO; poly(ethylene oxide)-poly(lactic acid), PEO-PLA and poly(ethylene oxide)-polycaprolactone (PEO-PCL) (Apolinário et al., 2017; Pachioni-Vasconcelos et al., 2016).

Two strategies can explain the formation of PL, the first based on the formation of lamellar aggregates (bilayers) that come together to form the final vesicle and the second on the effect of solvent diffusion into preformed micelles, which gives rise to vesicles. Also, different methods are used to produce PL such as nanoprecipitation, emulsification and film hydration (Apolinário et al., 2017; Matoori & Leroux, 2020; Pachioni-Vasconcelos et al., 2016). The choice of method directly affects the final characteristics of the nanostructures such as size and polydispersity index (PDI). It is important to mention that, depending on the characteristics of the polymers, for example the glass transition temperature, other methods can also be used to prepare PL, such as direct dissolution of the polymer in an aqueous medium.

An example of an amphiphilic copolymer for the preparation of polymersomes refers to the class of Pluronics® or poloxamers. Poloxamers are nonionic amphiphilic copolymers formed by combining poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO). They are arranged in a three-block structure: PEO-PPO-PEO, where the central PPO chain forms a hydrophobic region, while the PEO side chains form the hydrophilic blocks. Due to biocompatibility, several poloxamers are approved by the American and British pharmacopoeias for human use and widely used in a variety of pharmaceutical formulations (Batrakova & Kabanov, 2008; Pitto-Barry & Barry, 2014).

For topical application, the ability to deform and compress, and thus be able to penetrate the narrow pores of the skin, is highly desirable. Pegoraro et al. (2014) demonstrated in membrane models the translocation of malleable polymersomes through narrow pores. According to the authors, flexible vesicles can translocate without disruption through pores with diameters an order of magnitude smaller. Furthermore, the authors state that larger PL (400 nm) may be more flexible than smaller ones (100-200 nm), as they can deform more easily through the pores (50 nm). In particular, PL formed by Pluronic® L-121 or poloxamer 401 (PEO₅PPO₆₈PEO₅) present ideal flexibility to incorporate large biomolecules and potential ability to cross small gaps in the skin (C. Oliveira et al., 2021; Pitto-Barry & Barry, 2014). In our research group, Apolinário et al. (2019) and Oliveira et al. (2020) studied the encapsulation of L-asparaginase and catalase, respectively, in poloxamer 401 polymeric vesicles, presenting

stable structures with a hydrodynamic diameter of approximately 100-200 nm and reaching 5% encapsulation efficiency.

5.3. Polymeric nanoparticle

Polymeric nanoparticles (PNP), more specifically polymeric nanospheres, correspond to continuous polymeric networks between 1 and 1000 nm in which drugs can be incorporated dissolved, entrapped, or attached to the nanoparticle matrix (Figure 9) (Tavares Luiz et al., 2021; Zielinska et al., 2020).

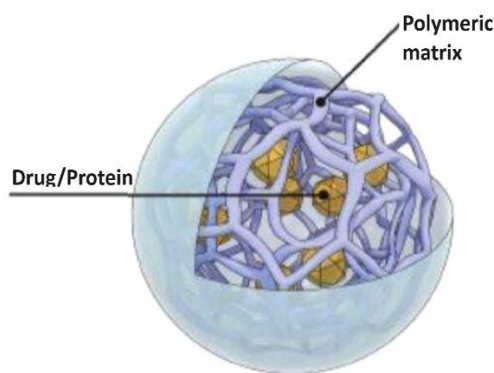


Figure 9. Schematic representation of a polymeric nanoparticle (Bhardwaj & Jangde, 2023).

Factors like the amount of polymer, oil, and surfactants; drug concentration; drug/polymer ratio; aqueous and organic phase volumes; stirring rate and time; homogenization rate; centrifugation time and rate; and sonication time and frequency should be better understood when preparing PNP with properties that are suited to clinical purposes (Tavares Luiz et al., 2021). For example, a great variety of natural and synthetic polymers have been exploited for the preparation of nanoparticles. Among them, polycaprolactone (PCL), poly (lactic acid) (PLA), poly (glycolic acid) (PGA), and their copolymer, poly (lactic-co-glycolic acid) (PLGA) have been extensively investigated (Atanasio et al., 2020). Particularly, polycaprolactone (PCL) is a biodegradable biopolymer with excellent properties such as biocompatibility, non-toxicity, and thermal stability. These qualities have led to its extensive adoption in the biomedical sector, including its approval by the Food and Drug Administration (FDA) (Drago et al., 2021). Alvarez-Román et al. (2001) detailed the synthesis of PCL nanoparticles for epidermal

delivery of the sunscreen agent octyl methoxycinnamate and observed improved sun protection and partial mitigation of erythema.

Several methods are employed for the preparation of polymeric nanoparticles (PNPs), including nanoprecipitation, spray-drying, emulsion solvent evaporation and microfluidic systems. However, none of these methods is universally preferred due to potential variations in the properties of the resultant nanoparticles. Discrepancies may arise in terms of size, stability over time and the efficacy of drug incorporation, depending on the chosen preparation technique. Furthermore, the production method can influence the kinetics of drug release, which is another crucial factor to consider when working with nanoparticles. Among the techniques proposed for PNP production, the emulsion evaporation method is most commonly used. (Atanasio et al., 2020). It involves emulsifying polymers and organic solvents within an aqueous phase. Specifically, the double emulsion water-in-oil-in-water (W/O/W) is commonly employed. In this technique, the drug or active ingredient is first dissolved or dispersed in the internal water phase. This water phase is then emulsified in an organic solvent containing the polymer, forming the primary water-in-oil emulsion. Finally, this primary emulsion is dispersed within an external aqueous phase containing stabilizers, leading to the formation of nanodroplets. Subsequently, nanoparticles are formed by removing the organic solvent. This method allows for better control over particle size distribution, reduces solvent residue, and yields high entrapment efficiency (Bhardwaj & Jangde, 2023).

Tomoda et al. (2011) fabricated spherical PLGA nanoparticles encapsulating indomethacin via an emulsion solvent evaporation method for transdermal delivery. An *in vitro* permeation study conducted using rat skin demonstrated improved transdermal delivery of indomethacin when the drug was loaded into PLGA nanoparticles. In another work, PLGA and chitosan were used to fabricate surface-modified bilayered nanoparticles incorporating two anti-inflammatory compounds, spantide II and ketoprofen. *In vitro* permeation studies on human skin demonstrated a significant increase in the penetration of the drugs into the epidermis and dermis layers compared to control groups (Shah et al., 2012).

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CHAPTER II

Recombinant production of a highly efficient photolyase from *Thermus thermophilus*

This chapter demonstrated the possibility to obtain a recombinant and pure photolyase, fully active, and presents for the first time the enzyme kinetics. This study was published in the Biotechnology Journal (<https://doi.org/10.1002/biot.202300325>).

CHAPTER II

Recombinant production of a highly efficient photolyase from *Thermus thermophilus*

ABSTRACT

Ultraviolet (UV) radiation from sunlight can damage DNA, inducing mutagenesis and eventually leading to skin cancer. Topical sunscreens are used to avoid the effect of UV irradiation, but the topical application of DNA repair enzymes, such as photolyase, can provide active photoprotection by DNA recovery. Here we produced a recombinant *Thermus thermophilus* photolyase expressed in *E. coli*, evaluated the kinetic parameters of bacterial growth and the kinetics and stability of the enzyme. The maximum biomass (X_{max}) of 2.0 g/L was reached after 5 h of cultivation, corresponding to $P_x = 0.4$ g/L.h. The μ_{max} corresponded to 1.0 h^{-1} . Photolyase was purified by affinity chromatography and high amounts of pure enzyme were obtained (3.25 mg/L of cultivation). Two different methods demonstrated the enzyme activity on DNA samples and very low enzyme concentrations, such as 15 $\mu\text{g/mL}$, already resulted in 90% of CPD photodamage removal. We also determined photolyase k_M of 9.5 nM, confirming the potential of the enzyme at very low concentrations, and demonstrated conservation of enzyme activity after freezing (-20°C) and lyophilization. Therefore, we demonstrate *T. thermophilus* photolyase capacity of CPD damage repair and its potential as an active ingredient to be incorporated in dermatological products.

KEYWORDS: Photolyase, active photoprotection, CDP-DNA, photodamage DNA, *Thermus thermophilus*.

1. INTRODUCTION

Life on Earth evolved under the influence of solar radiation. Unfortunately, the total amount of ozone in the Earth's atmosphere (i.e., the ozone layer) is decreasing and more solar radiation is reaching the Earth's surface (Barnes et al., 2019). UV radiation can damage DNA inducing the formation of photoproducts such as cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone (6-4PP). (Leccia et al., 2019) Overexposure to UV radiation is associated with an increased incidence of skin cancer, in particular non-melanoma skin cancer and actinic keratosis, a benign neoplasm with the potential to transform into skin cancer (Barnes et al., 2019; Berardesca et al., 2012).

Human skin has several endogenous mechanisms against UV radiation, including hair as a physical barrier, sweating that reflects and absorbs radiation, melanin, and the complex system of anti-radical and DNA repair enzymes. Nonetheless, additional protection must be required depending on exposure time, geographic location and individual characteristics such as skin phototype. (Cestari et al., 2012; Sambandan & Ratner, 2011) Topical photoprotection usually involves molecules that absorb or reflect UV radiation, such as organic and inorganic filters. However, this is a passive and unspecific strategy to prevent the effects of continuous exposure to UV radiation. In this sense, increased interest on the topical application of DNA repair enzymes, such as photolyases, has been witnessed in the last decade; it can be seen as an "active photoprotection". (Megna et al., 2017) The possibility of incorporating photolyase - and with it the ability to repair UV-induced DNA damage - in a sunscreen adds a new dimension to the strategies against solar radiation damage. (Stege et al., 2000)

Photolyase (PHL) is a flavoprotein capable of repairing lesions such as CPD or (6-4) PP employing the energy of blue light (photons emitted between 350-500 nm), therefore reversing the damage to form intact pyrimidine bases. (Hosokawa et al., 2019) They are divided into class I CPD photolyases, class II CPD photolyases, class III CPD photolyases, (6-4) photolyases, and single-stranded DNA-specific photolyases (CRY-DASH). (Dikbas et al., 2019) This monomeric enzyme presents two non-covalent linked cofactors or chromophores. One of the cofactors is always flavin adenine dinucleotide (FAD) and the second can be 5,10-methenyltetrahydrofolate (MTHF), 8-hydroxy-7,8-didemethyl-5-deeazariboflavin (8-HDF), flavin mononucleotide (FMN), or 6,7-dimethyl 8-ribityl-

lumazin (DMRL) depending on the type of photolyase.(Dikbas et al., 2019) The non-covalent FAD cofactor is essential for catalysis. The second cofactor is not required for catalysis, nonetheless it functions as a “photoantenna” that absorbs photons from the blue light spectrum and transfers the energy to the FAD, increasing the repair rate by 10 to 100 times. (Kavakli et al., 2019; Liu et al., 2015) The amino acid residues which interact with FAD in *Escherichia coli* photolyase are mostly conserved in other bacterial photolyases. On the other hand, the residues interacting with MTHF are conserved only in folate-type and not in deazaflavin-type photolyases. (Kato et al., 1997)

Kato et al. (1997) expressed the *Thermus thermophilus* photolyase in *E. coli* and found it has FAD as a chromophore and the amino acid sequence is consistent with a folate-type, consequently, the second chromophore binding site was not conserved. The authors also proved the enzyme stability when subjected to heat and acidic pH, however enzyme activity was only indirectly demonstrated. In addition, the production of the enzyme was not deeply investigated and it would be important to evaluate process parameters. (Kato et al., 1997)

Here, we investigated the bioprocess parameters to produce the photolyase from *T. thermophilus* by recombinant expression in *E. coli* BL21 (DE3) and demonstrated for the first time the actual CPD damage reversion by the enzyme, providing the k_M value. Both aspects bring a real commercial perspective for this photolyase enzyme as an innovative and functional alternative of active photoprotection upon topical application.

2. MATERIALS AND METHODS

2.1. Photolyase production

The enzyme was produced by a recombinant *E. coli* BL21 (DE3) strain containing the pET15b vector (histidine-tagged N-terminal fusion protein) with the *Thermus thermophilus* photolyase gene with codon optimization to *E. coli* expression (GenScript - Piscataway NJ, USA). The transformed strain of *E. coli* BL21 (DE3) was stored in glycerol (8%) at -80 °C.

The pre-inoculum was prepared from the strain stored in glycerol in 50 mL of sterile culture Luria-Bertani (LB) medium supplemented with carbenicillin (50 µg/mL) and grown at 37°C, 200 rpm for 16 h. The cultures were inoculated in fresh LB medium supplemented with carbenicillin (50 µg/mL), with an initial optical density (OD_{600nm}) of 0.1 and the culture was incubated in a metabolic shaker at 37°C, 200 rpm. Initially, a bacterial growth curve was constructed without induction over time and the cell concentration at half-exponential phase was determined. This concentration was defined as the induction time and the IPTG inducer (isopropyl β d-1-thiogalactoside) was added at a final concentration of 0.1 mM. The culture was kept under incubation in a metabolic shaker at 37°C, 200 rpm for 3 h for the expression of photolyase. Cultivations were carried out in triplicate. A correlation curve between optical density (OD_{600nm}) and dry biomass (g/L) was generated and used to calculate cell concentration (biomass) in all bacterial growth curves ($y = 2.44x$).

The total protein concentration was determined from the same samples used to estimate cell concentration at different times (pellet of centrifugation) using the BCA Protein Assay Kit according to the manufacturer instructions (Sigma Aldrich, St Luis, USA), immediately after complete cell disruption using BugBuster® reagent (Merck Millipore, Darmstadt, Germany).

2.2. Determination of growth kinetic parameters

The maximum cell concentration (X_{max} , expressed in g/L) was based on the correlation curve between optical density (OD_{600nm}) and dry biomass (g/L). The specific growth rate (h^{-1}) was calculated by the following equations that describes the typical growth of a bacterial population: (Vitolo et al., 2015)

$$\mu_X = \frac{1}{X} \frac{dX}{dt} \quad (1)$$

$$\ln(X - X_0) = \mu_X(t - t_0) \quad (2)$$

in which X is the biomass at a specific time (g/L), X_0 is the initial biomass (g/L), $t - t_0$ is the time interval (h) and μ_X is the specific growth rate. By means of a logarithmic growth

curve ($\ln(X)$) as a function of time (h), we calculated the slope of the exponential phase line that provides the maximum specific growth rate (μ_{max}).

P_X corresponds to the volumetric productivity in cells per unit of time and was calculated according to equation 3.

$$P_X = \frac{X - X_0}{t} \quad (3)$$

2.3. Photolyase purification

At the end of the culture, the cells were collected by centrifugation at 4,000 x *g* for 30 min at 4°C, resuspended in 20 mM Tris-HCl pH 7.4, 300 mM NaCl, 50 mM imidazole, 1 mM EDTA (ethylenediamine tetraacetic acid) and 1 mM PMSF (phenylmethylsulfonyl fluoride), and finally lysed by ultrasound probe at 30% of amplitude, 18 pulses of 10 seconds with 30 seconds intervals between pulses, keeping the cells in an ice bath. The protein extract obtained was centrifuged at 16,000 x *g* for 30 min and the supernatant filtered through a 0.45 µm membrane (Merck Millipore, Darmstadt, Germany). Purification was performed by affinity chromatography, using a 5 mL Hi-Trap nickel affinity column (Ge Healthcare, Chicago, USA), and elution with two steps (20 and 70%) of 500 mM imidazole, in an AKTA Fast Protein Liquid Chromatographer (Ge Healthcare, Chicago, USA). The presence of protein in the collected fractions was verified by the BCA method and confirmed by gel electrophoresis – SDS PAGE 12%. For the SDS-PAGE, samples were treated with a reducing agent (β-mercaptoethanol) and heated to 95°C for 5 min.

2.4. Determination of photolyase molecular mass by size exclusion chromatography (SEC)

A Superdex 200 Increase 10/300 GL molecular exclusion column (GE Life Sciences) was used to determine the photolyase molecular weight, based on a calibration curve correlating distribution constant (K_{av}) with molecular weight. Elution was carried out isocratically with phosphate buffer saline (PBS) pH 7.4 in an AKTA FPLC equipment (Ge Healthcare, Chicago, USA). The following proteins (Sigma Aldrich, St Luis, USA) were use

as molecular weight standards: catalase (240 kDa), L-asparaginase (136 kDa), bovine serum albumin (BSA) (66.5 kDa) and lysozyme (14.5 kDa). The distribution constant (K_{av}) of each standard was calculated by the following equation:

$$K_{av} = \frac{v_e - v_0}{v_c - v_0} \quad (5)$$

in which v_c is the geometric volume of the column (24 mL), v_0 is the dead volume (8.08 mL) and v_e correspond to elution volume.

2.5. Photolyase wavelength scan

Wavelength scans (350 to 700 nm) of both folded and denatured photolyase were performed on a Spectramax plus 384 spectrophotometer (Molecular Devices, California, USA) to confirm the presence of the chromophore. The enzyme was denatured by the addition of 50 mM phosphoric acid at pH 2.0 and incubated at 25°C for 2 h.

2.6. Determination of photolyase activity: indirect method by T4-endonuclease V repair activity

2.6.1. Production and extraction of the enzyme T4-endonuclease V

The T4-endonuclease V was recombinantly overexpressed in *E. coli* BL21 with pET15b vector in LB culture medium supplemented with 50 µg/mL of carbenicillin. Initially, an overnight pre-inoculum was carried out at 37°C and 250 rpm and following 50 mL of fresh medium was inoculated with the pre-inoculum to reach a OD_{600nm} of 0.1. The cultivation was carried out in a 250 mL Erlenmeyer at 37°C and 250 rpm. After 3.5 h, the culture was induced with 1mM IPTG and continued under agitation for another 2.5 h. The culture was centrifuged at 4,000 xg for 20 min at 4°C and the cells resuspended in NET buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl pH 8.0 and 100 mM PMSF) and subjected to cell disruption by ultrasound probe using the same protocol described in section 2.4. After lysis, the suspension was centrifuged at 10,000 xg for 1 h and the supernatant (protein extract) stored at 4°C for further use.

2.6.2. Purification and irradiation of pCMUT plasmid

Construction of the plasmid used as substrate, pCMUT, is described elsewhere (Schuch et al., 2009). The plasmid was amplified in cultures of *E. coli* DH10b, purified using the commercial Qiagen Maxi Kit (Qiagen, Germantown, USA) and then stored in TE buffer (10 mM Tris-HCl pH 0.8; 1 mM EDTA). The pCMUT concentration and purity was determined in a NanoDrop micro-volume spectrophotometer (Thermo-Fischer Scientific, Massachusetts, USA). Aliquots of 100 ng/μL were diluted and stored to be used in enzyme activity assays. Before each assay, 20 μL of pCMUT (100 ng/μL) were added in a 30 mm diameter petri dish and irradiated with 300 J/m² of UVC (Philips UVC lamp TUV 15 W/G15 T8, t = 9.5 min, irradiation dose determined with a UV VLX 3W radiometer (Vilber Lourmat, Germany)).

2.6.3. Photoactivation and enzymatic reaction

Different concentrations of photolyase (40, 20 and 10 μg/mL) were added to 100 ng/μL of irradiated plasmid DNA. Droplets of this mixture (20 – 30 μL) were transferred to a petri dish and exposed to a white light transilluminator for 60 min. Samples left in the dark were used as control. After photoactivation, 4 μL of the mixture (Photolyase + irradiated DNA) were mixed with 2 μL of T4 enzyme extract and the volume was completed to 20 μL with NET buffer. The samples were incubated at 37°C for 60 min and submitted to electrophoresis for 1.5 h at 100 V in an agarose gel (0.7% w/v) diluted in TBE buffer (89 Mm Tris-H₃BO₃, 2 mM EDTA, pH 8.1), with the addition of 1.5 μL of ethidium bromide (0.5 μg/mL) and to the samples was added 3 μL of bromophenol blue.

The CPD-type lesions (T4 sensitive sites) were determined based on densitometry analysis of the DNA bands in the electrophoresis gel using image generating equipment (Image Quant 300, GE healthcare, Chicago, USA) and the ImageJ® software. The number of breaks induced by the T4 enzyme per kilo base pair (kbp) corresponds to a Poisson distribution, therefore the number of lesions can be estimated based on equation (6):

$$Nb = -\ln \left(\frac{1.4 * FI}{1.4 * FI + FII} \right) / 1.8 \quad (6)$$

where Nb is the number of breaks in the plasmid DNA molecule per kbp, FI represents the fluorescence intensity of the supercoiled plasmid DNA band (form I), FII represents the fluorescence intensity of the circular DNA band (form II), 1.8 is the size of the plasmid pCMUT (kbp) and 1.4 was a factor applied to correct the excess of fluorescence that occurs in circular DNA due to its higher affinity with ethidium bromide. (Schuch et al., 2009)

One-way analysis of variance (ANOVA) was used to compare treated groups with the positive control, as well as the effect among different photolyase concentrations; p value < 0.05 was considered statistically significant. Statistical analysis was conducted using GraphPad Prism® Software.

2.7. Determination of photolyase activity: CPD detection by immunochemistry

Photolyase activity was also evaluated by immunochemistry with the ab242297 CPD Elisa Kit (Abcam, Cambridge, United Kingdom). Initially, the CPD-DNA and the DNA provided in the kit were converted to single-stranded DNA (denatured) by incubating at 95°C for 10 min and rapidly chilling on ice for 10 min. Solutions of denatured CPD-DNA or DNA in cold phosphate buffer (100 mM, pH 7.4) were freshly prepared (4 µg/mL) and serial dilutions of CPD-DNA in denatured reduced DNA were further prepared for the standard curve. To perform the assay, 50 µL of denatured CPD-DNA (100 ng/mL) was incubated with 50 µL of different photolyase concentrations (150, 75, 30, 15, 10, 5 and 1 µg/mL) on visible light at room temperature for 60 min. Replicates were wrapped in aluminium foil and left in the dark for the same time. Following, 50 µL of the systems as well as of the denatured CPD-DNA standards were added to the wells of a DNA High-Binding plate and the test was performed according to the manufacturer instructions.

Additionally, the kinetics of the enzyme reaction was investigated. Specifically, 50 µL of denatured CPD-DNA (substrate) at different concentrations (1.56 to 100 ng/ml) was incubated with 50 µL of 10 µg/mL photolyase on visible light at room temperature for 60 min. Immediately, the immunochemistry test was carried out as described above and

the Michaelis-Menten constant (k_M) calculated with the help of the GraphPad Prism 10 software (La Jolla, California/USA).

2.8. Photolyase stability under different temperature and storage conditions

The effect of temperature on photolyase stability was investigated by wavelength scans (215 to 700 nm) on a Spectramax plus 384 spectrophotometer (Molecular Devices, California, USA) after the enzyme incubation for 10 minutes at different temperatures (30, 40, 50, 60 and 70 °C). All the experiments were performed with 1 mL samples of photolyase at 1 mg/mL in phosphate buffer 0.1 mM pH 7.4. For spectrophotometric measurements, samples were diluted to 0.2 mg/mL. Three different conditions of storage were also evaluated: (i) 4°C for 14 days, (ii) one cycle of freezing at -20°C and (iii) lyophilization (freezing at -80°C and, sequentially, dry at low pressure). For these experiments, 50 mM sucrose was added to promote enzyme stability and the enzymatic activity was evaluated by immunochemistry, as described in section 2.7.

3. RESULTS AND DISCUSSION

3.1. Photolyase production

Recombinant *E. coli* BL21 (DE3) was cultivated in LB medium and a classic bacterial growth profile was observed (Figure 1). The maximum biomass (X_{max}) of 2.0 g/L was reached after 5 h of cultivation, corresponding to $P_x = 0.4$ g/L.h. The μ_{max} corresponded to 1.0 h^{-1} , in agreement with previous values reported in the literature (Carmignotto & Azzoni, 2019; Li et al., 2017; Torres-Obreque et al., 2019).

Based on the cell curve, induction time was defined at 3.5 h of cultivation, corresponding to the middle of the exponential phase. The induction was realized by IPTG, a non-metabolizable inducer of the “*lac* operon” and, consequently, after its addition the photolyase expression is uninterrupted. (Xu et al., 2023) Figure 1 presents the growth curves of the induced cultivations and one can observe a decrease in the specific growth rate after induction owing to the stress related to the recombinant protein

overexpression. Decreased levels of specific sugar transport systems have been reported, as well as of enzymes involved in central carbon metabolism and amino acid biosynthesis, which may point to a decrease in the ability of cells to produce sugars, precursors needed for protein synthesis and cell growth. (Li et al., 2017)

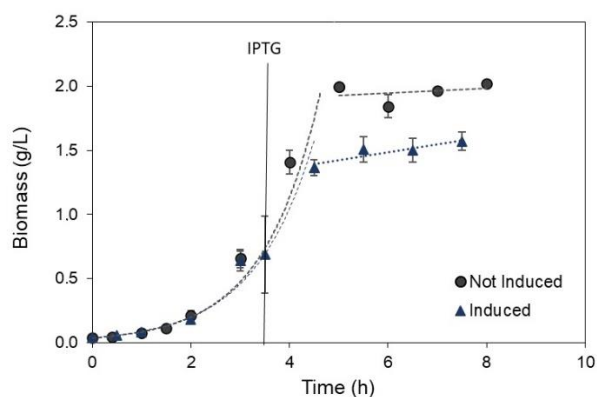


Figure 1. Growth curve of recombinant *E. coli* BL21 (DE3) expressing the *T. thermophilus* photolyase in Luria-Bertani medium with and without IPTG induction. The error bars correspond to the standard deviation.

Total protein concentration was determined after cell disruption and, as can be seen, induced cultivation resulted in approximately 3 times more protein than non-induced cultivation, which was attributed to the photolyase overexpression (Figure 2A). To confirm this assumption, the protein extracts were analysed by reducing SDS-PAGE (Figure 2B) and an intense band was observed after induction, corresponding to the expected photolyase size of approximately 45-50 kDa (red arrow).

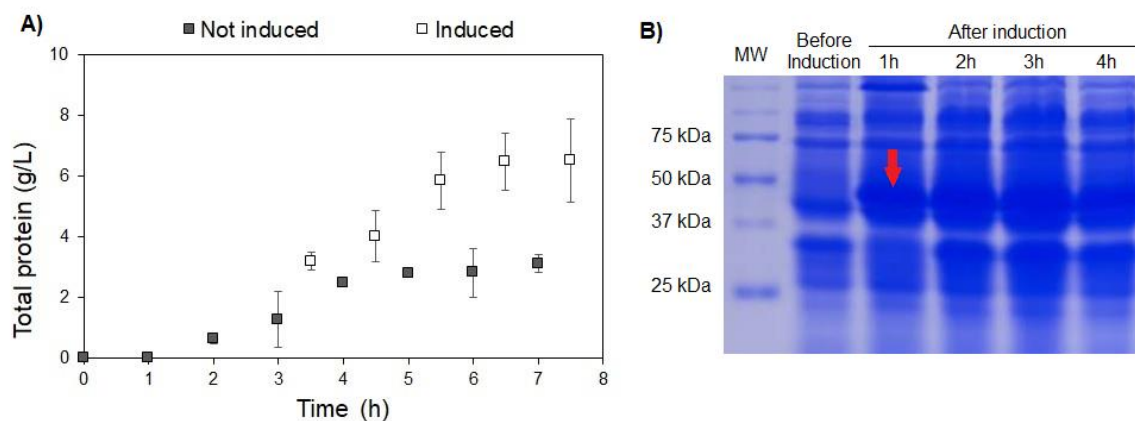


Figure 2. A) Total protein concentration of induced and non-induced recombinant *E. coli* BL21 (DE3) cultivation in Luria-Bertani medium. B) Electrophoresis (SDS-PAGE 12%) gel of the protein extract from induced cultivation at different times. The red arrow indicates the band corresponding to photolyase. The error bars correspond to the standard deviation.

3.2. Photolyase purification and physical characterization

The *E. coli* biomass was collected by centrifugation and submitted to cell disruption and protein purification (Figure 3). The second peak on the chromatogram (Figure 3A) corresponded to yellow fractions owing to the presence of photolyase with the FAD cofactor. Figure 3B presents the SDS-PAGE analysis of the crude extract and affinity purification fractions. A 47 kDa band can be seen after purification, corresponding to the molecular mass of *T. thermophilus* photolyase. Based on protein and cell concentration determinations, we obtained 3.25 mg of protein per L of cultivation.

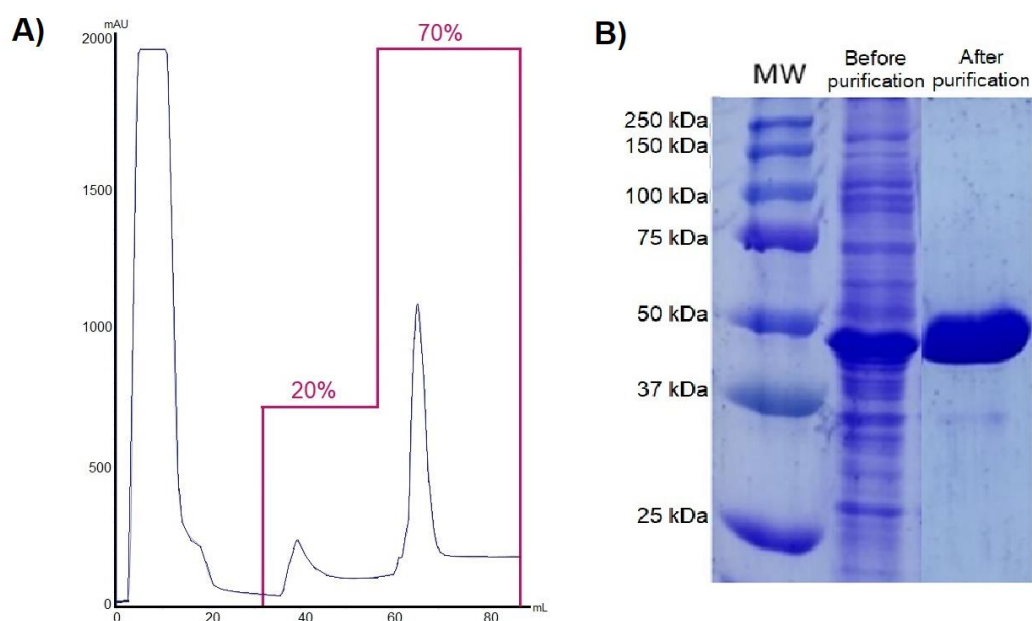


Figure 3. Photolyase purification. A) Chromatogram of photolyase purification by IMAC. Blue line: absorbance at 280nm, purple line: 500 mM imidazole steps. B) Gel electrophoresis (SDS-PAGE, 12%) of the crude extract (Before purification) and the purified fraction eluted at 70% step (After purification). The first lane corresponds to the molecular weight (MW) standards (Precision Plus Protein™ Standards, Biorad).

The hydrodynamic diameter of the photolyase produced was confirmed by molecular size exclusion chromatography (SEC). Commercial proteins, with known molecular masses, were used to generate a curve correlating molecular size (distribution constant, K_{av}) and elution volume (according to item 2.5). The chromatogram of standard proteins is shown in Figure 4A and resulted in the correlation curve described by the equation of $K_{av} = -0.4227 \log MW + 1.2151$ (Figure 4B). Based on the correlation equation, photolyase molecular mass (MM) was estimated to be approximately 47 kDa, in agreement with the electrophoresis result and with the MM estimated from the amino acid sequence (approximately 47.9 kDa). These results indicated that *T. thermophilus* photolyase was produced in an expected monomer form and globular size.

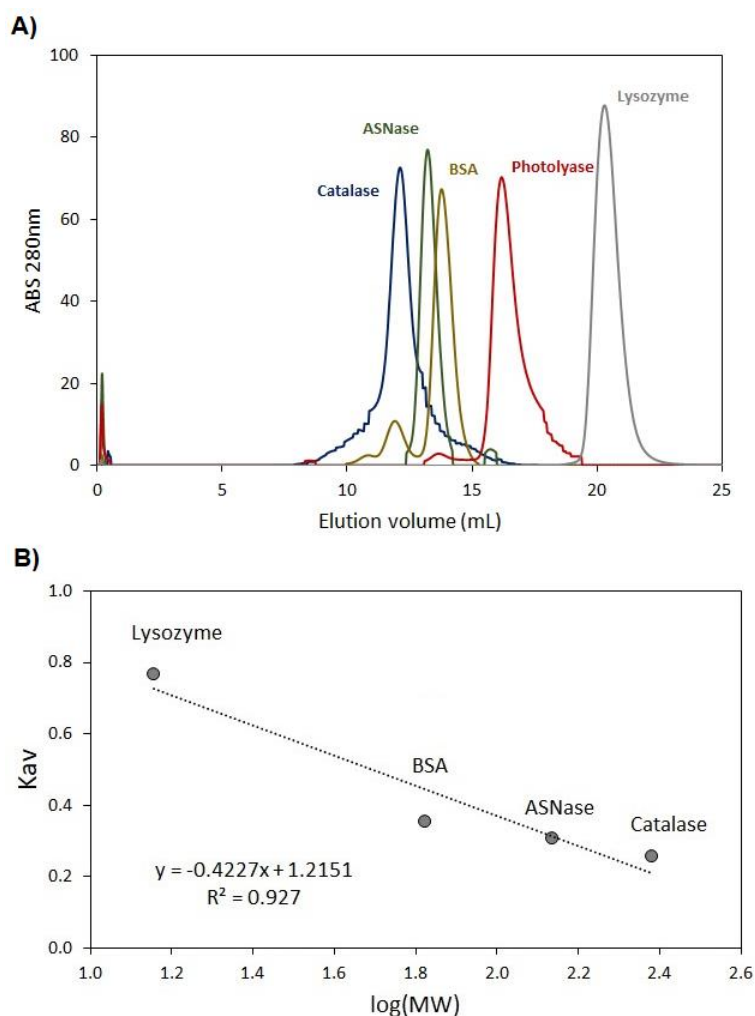


Figure 4. A) Chromatogram of molar mass standards: catalase (240 kDa, ve 12.20 mL), ASNase (L-asparaginase, 136 kDa, ve 13.02 mL), BSA (66.5 kDa, ve 13.77 mL) and lysozyme (14.5 kDa, ve 20.29 mL), carried out on a Superdex 200 Increase 10/300 GL molecular exclusion column, along with photolyase enzyme elution (ve 16.17 mL) and B) Correlation curve between molecular weight and distribution constant (K_{av}).

The absorption spectrum of the purified protein was measured and highest absorption peaks were confirmed at around 350, 440, and 600 nm (Figure 5). The resulting spectrum was identical to that reported by Kato et al. (1997) for the *T. thermophilus* photolyase and represents a typical FAD spectrum. Similar profile was reported to *Potorous tridactylis* photolyase, which also does not have a second chromophore. (Yasui et al., 1994)

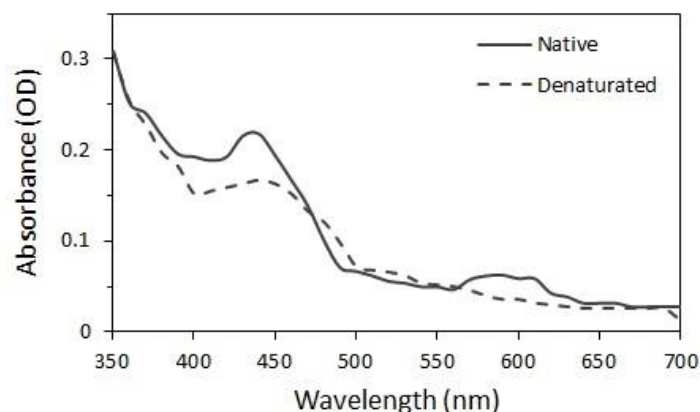


Figure 5. Absorption spectra of native (solid line) and acid-denatured (dotted line) photolyase measured between 350 and 700 nm.

3.3. Determination of photolyase activity and k_M

Plasmid *ex vivo* assay allows to distinguish cleaved from undamaged DNA, including the one that has been repaired. In pCMUT electrophoresis, two forms are most prevalent, namely Form I, the supercoiled DNA, and Form II, a relaxed circular form generated by the phosphodiester bond cleavage in one of the strands. The different topologies of the DNA confer different mobility on an electrophoresis gel and the mixture of Form I and II can be separated and quantified. (Higgins & Vologodskii, 2015)

We confirmed the presence of CPD lesions in the DNA after exposure to UVC radiation and T4 enzyme activity. The T4 extract, non-irradiated (DNA) and irradiated (DNA+UVC) plasmid were used as controls. As can be seen in Figure 6A, both intact (non-irradiated) and damaged (UV irradiated) DNA preserved the supercoiled Form I. However, in the presence of T4 the irradiated DNA was cleaved and the band corresponding to Form I disappeared and only Form II is present corresponding to the highest number of breaks per kbp. A small amount of Form II is also present in the non-irradiated DNA sample, what could indicate non-specific effects of T4 or unintentional damage to the DNA.

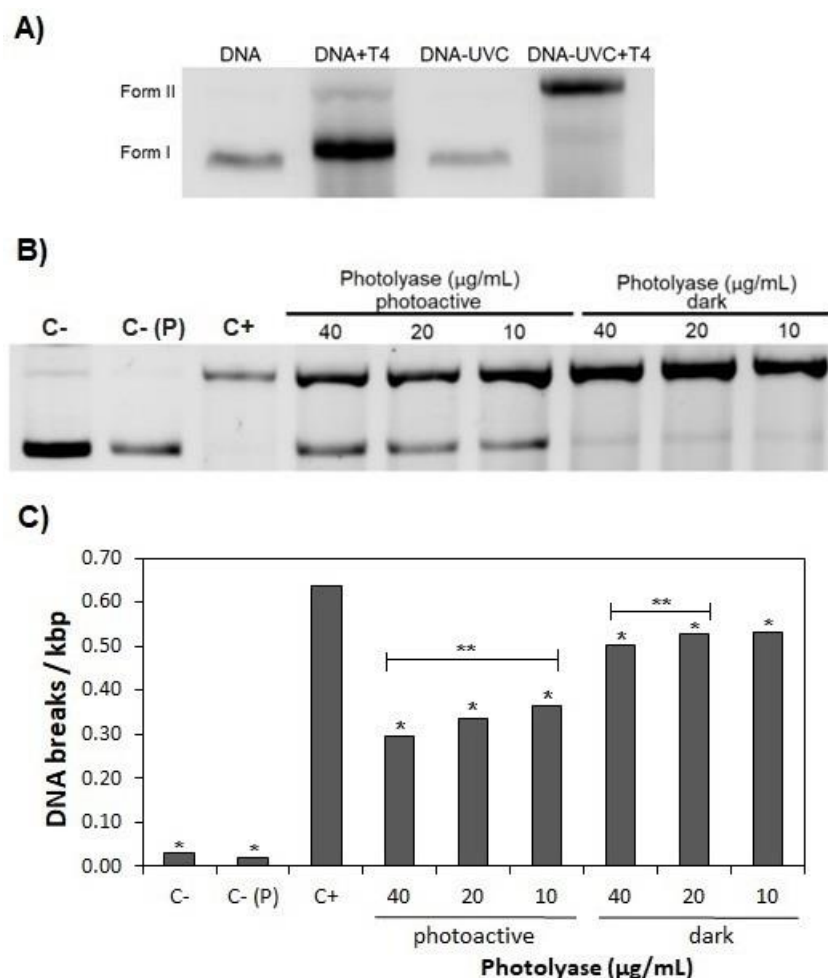


Figure 6. Photolyase activity measured by indirect assay. A) Agarose gel (0.7%) of the pCMUT plasmid non-irradiated (DNA) and irradiated (DNA-UVC), in the presence or not of T4-endonuclease V. B) Agarose gel (0.7%) of the pCMUT plasmid in the presence of different concentrations of photolyase with exposition (photoactive) or not (dark) to white light. C) Densitometry quantification of the agarose gel bands. C- : Negative control (without T4). C- (P): Negative control with addition of photolyase. C+: Positive control (with T4 action). Statistics refers to the positive control (*), and the effect among different photolyase concentrations (**); p value < 0.05.

Following, photolyase activity was tested and a dose dependent reduction on the number of DNA breaks was observed after photoactivation for 60 min, indicating a repairing activity (Figure 6B-C). At 40 $\mu\text{g/mL}$ of photolyase, the number of DNA breaks/kbp reached a value of 0.30, which represents 54% DNA repair. The same concentration of photolyase resulted in 21% of DNA recovery in the dark. Statistics also indicate a dose-response effect (Figure 6C).

The enzymatic activity was also evaluated by immunochemistry (ELISA), which means direct quantification of CPD dimers (Figure 7A). This highly sensitive method showed that the photolyase produced here can remove up to 98% of CPD damage. Furthermore, Figure 7B shows that recovery was similar with or without previous enzyme photoactivation, demonstrating that this step is not needed to test enzyme activity. Concentrations such as 15 $\mu\text{g/mL}$ already resulted in 90% of recovery. Our results are similar with previous one from the literature; Marizcurrena et al., (2019) showed that *Hymenobacter sp.* UV11 photolyase can repair UV DNA damage at concentrations in the micromolar range. (Marizcurrena et al., 2019)

The higher values obtained with the enzyme immunoassay are explained by higher sensitivity and specificity of this test. Moreover, immunoassay reduce interference, leading to more accurate results and better control of the DNA concentration. Nonetheless, the cost of the test is significantly higher, and the indirect quantification can be an alternative to first proof photolyase enzymes activity. Literature shows efforts to quantify photolyase activity. Recently, the use of a synthetic substrate and quantification by liquid chromatography tandem mass spectrometry (UHPLC–MS). was proposed.(Vallejos-Almirall et al., 2020) More specifically, the method was based on the enzyme conversion of a 15mer oligonucleotide containing a center cyclobutane thymidine dimer, to the restored 15mer oligonucleotide, with detection of both species by UHPLC–MS.(Vallejos-Almirall et al., 2020) The oligonucleotides recovery from reaction mixture was $\sim 94\%$ and the limit of quantification was 13.4 nM for 15mer, confirming the method in this work as an ease and routine alternative to quantify photolyase activity *in vitro*. In addition, a k_M of 291.10 ± 52.97 nM was calculated with this artificial substrate. Nonetheless, one should keep in mind it does not apply to photolyase activity determination in biological samples, such as DNA extracted from cell and tissue culture, what could effectively prove the enzyme effective capacity to repair DNA damage *in vivo*.

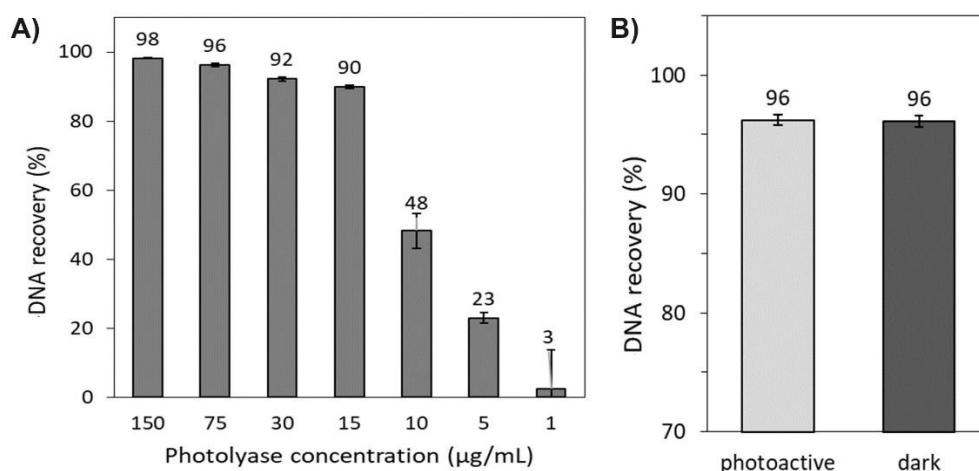


Figure 7. Photolyase activity measured by direct assay using standard denatured CPD-DNA (100 ng/mL) as substrate and detected by immunochemistry assay. Error bars correspond to standard deviation. A) CPD-DNA recovery (%) at different photolyase concentration (150 – 15 µg/mL) and B) CPD-DNA recovery (%) at 75 µg/mL photolyase with exposition (photoactive) or not (dark) to the white light. The error bars correspond to the standard deviation.

Here, the kinetics of the photolyase catalysed reaction was studied (Figure 8) and the enzyme was found to be Michaelian ($R^2 = 0.982$), with a $k_M = 9.7 \pm 0.9$ nM. Sancar et al. (1987)(Sancar et al., 1987) showed for the first time the catalytic parameters from *E. coli* photolyase with an association constant (k_A) of $4.7 \times 10^{-7} \text{ M}^{-1}\text{s}^{-1}$, what, in addition to the catalytic parameter $k_3 = 5.8 \times 10^{-3} \text{ s}^{-1}$, represents a k_M of 25 nM. Also, Nakayama et al. (2004) (Nakayama et al., 2004) reported a k_M of 100 nM for *E. coli* photolyase when cis-syn CPD was used as a substrate. To the best of our knowledge, this is the first report of k_M the *T. thermophilus* photolyase.

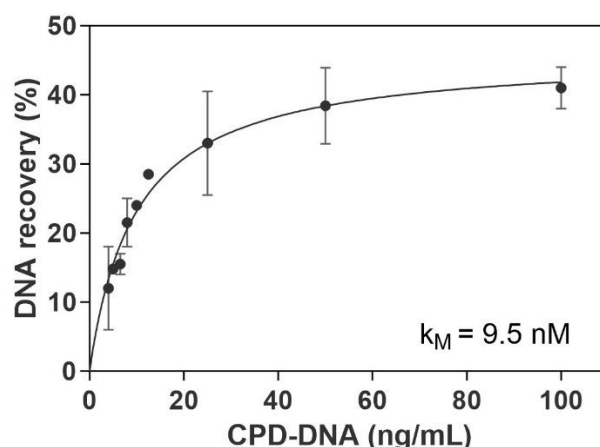


Figure 8. Saturation curve for photolyase activity: CPD-DNA recovery as a function of CPD-DNA concentration. The reaction was incubated at room temperature during 1 h at different concentration of CPD-DNA concentrations. The black line corresponds to the adjusted Michaelis-Menten equation ($R^2 = 0.982$ and $Sy.x = 1.498$). The error bars correspond to the standard deviation ($n = 3$).

3.5. Photolyase stability under different temperatures and storage conditions

Enzymes as proteins are associated to inherent molecular instability, at some extension (Cerdobbel et al., 2010). Consequently, storage conditions are important due to the potential loss of structural integrity and/or activity caused by time and temperature, which may result in denaturation and aggregation. Here we show that photolyase stored at 4°C for 2 weeks preserved only 46% of its activity compared to the freshly purified enzyme (Figure 9A). On the other hand, preserved activity was observed after a freezing cycle at -20°C, demonstrating this can be a strategy for enzyme storage. Lyophilization was also investigated, and the enzyme kept 81% of its activity after this process. Lyophilization offers the advantage of reducing both weight and volume of the sample. Furthermore, lyophilized samples usually are stable at room temperature, facilitating long-term storage and alleviating the challenges of cost-efficiency during storage and transportation. From an industrial perspective, lyophilization may present itself as the optimal choice, despite the minor loss of enzymatic activity.

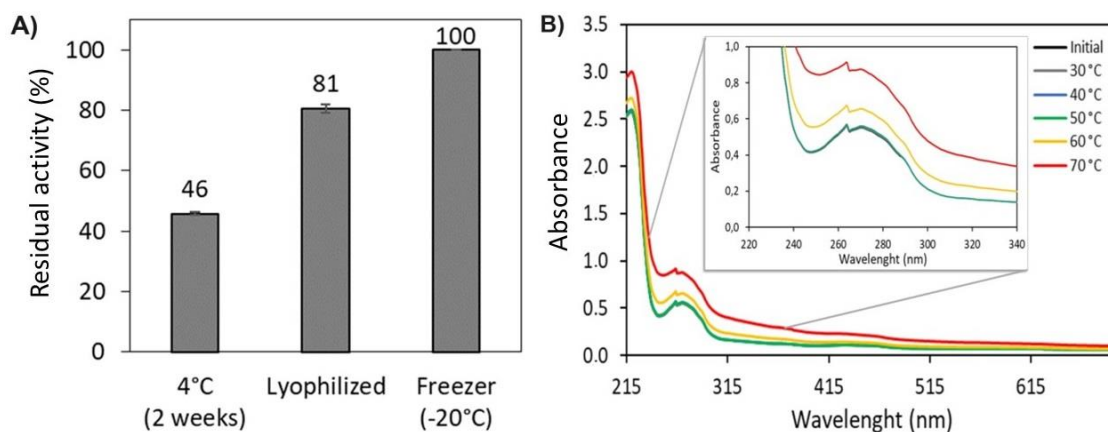


Figure 9. Photolyase stability: A) Residual activity of photolyase after different conditions of storage. The error bars correspond to the standard deviation. B) Wavelength scan of photolyase after 10 min of exposition to different temperatures. The black line corresponds to the initial wavelength scan prior to exposition to the different temperatures.

Finally, Figure 9B shows the photolyase wavelength scans after the enzyme incubation for 10 minutes at different temperatures. As can be seen, similar spectra were obtained for the enzyme not exposed to temperature (initial) and after expositions to 30, 40 and 50°C, demonstrating enzyme stability at these temperatures. On the other, upon temperature increase the spectra moved to higher absorbance values at 280nm, what can correlate to the exposition of aromatic residues as a result of protein unfolding. Our results are in agreement with the literature since *T. thermophilus* is classified as an extremely thermophilic bacteria, with an optimal growth temperature of about 65 °C.(Beffa et al., 1996) This results, reinforces the investigated photolyase as a promising active component for topical formulations, offering potent DNA repair activity and resistance to thermal stress.

3. CONCLUSIONS

In this work, we efficiently produced the *Thermus thermophilus* photolyase by recombinant expression in *E. coli* BL21 (DE3) and demonstrated its highly efficient enzymatic activity. Enzyme production and purification altogether resulted in a yield of 3.25 mg of photolyase per L of cultivation. The photolyase produced presented the expected size and absorbance spectrum and was found to be highly active in repairing CPD lesions to DNA exposed to UV radiation with 90% of repair at 15µg/mL. The Michaelis-Menten constant was determined to be $k_M = 9.7$ nM and confirms the enzyme affinity for the substrate. Finally, we demonstrate the enzyme resists to temperatures up to 50 °C, as well as freezing and lyophilization. In conclusion, *T. thermophilus* photolyase presents great potential as a novel alternative for UV photodamage repair to be incorporated in dermatological products.

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CHAPTER III

Fed-batch bioreactor process scale-up for *Thermus thermophilus* photolyase production

This chapter presents an optimization of photolyase production using different processes and scale up to bioreactors.

CHAPTER III

Fed-batch bioreactor process scale-up for *Thermus thermophilus* photolyase production

ABSTRACT

The production of recombinant proteins by microorganisms, particularly *Escherichia coli*, offers numerous advantages including ease of cultivation, genetic manipulation, and high growth rates. Culture media composition, whether complex or defined, significantly impacts protein expression, with defined media often yielding higher biomass and recombinant protein production. Moreover, batch and fed-batch bioreactors are pivotal in bioprocess engineering, offering distinct advantages and catering to different production requirements. The choice between these bioreactor types and overall process optimization, are crucial roles in biotechnological applications. Different fermentation processes, including metabolic shaker, batch and fed-batch processes by recombinant *E. coli* BL21, were studied and compared for the effective *T. thermophilus* photolyase production. The main kinetic fermentation parameters in all the processes were compared, namely, maximum cell concentration (X_{max}), specific growth rate (μ_{max}), volumetric productivity (P_x), yield of biomass production per consumed substrate ($Y_{x/s}$) and product volumetric productivity (P_{prod}). The results demonstrated that the batch bioreactor doubles the maximum biomass compared to the metabolic shaker and that the fed-batch process reaches X_{max} of 20.2 g/L, doubling it again compared to the batch process. This is reflected in obtaining 480 mg/L of pure enzyme culture at the end of the process for fed-batch, which is 2.8 times higher. These values symbolize a volumetric productivity of pure enzyme of 20.9 mg/L.h for the fed-batch process compared to 8.1 mg/L.h for the batch process. This new strategy for large-scale production of photolyase enzyme opens a new opportunity to incorporate a high effective DNA repair enzyme to dermatological and pharmaceutical products.

Keywords: photolyase, bioreactor, fed-batch bioreactor, recombinant protein, bioprocess.

1. INTRODUCTION

The production of recombinant proteins by microorganisms such as bacteria and yeasts has many advantages, highlighting ease of cultivation and genetically manipulation (Bahreini et al., 2014; Gupta & Shukla, 2016). *Escherichia coli* is considered the first-line system for heterologous production of proteins and one of the most efficient producers. *E. coli* has well-known physiology and metabolism and a high growth rate in simple media. It is also amenable to various cultivation techniques, achieving high cell density under favorable growth conditions (Dhaver et al., 2023; Zare et al., 2019). Furthermore, its genome is widely known facilitating genetic manipulation. Several cloning and expression vectors are available to produce recombinant proteins from *E. coli* (Rosano et al., 2019; Tripathi & Shrivastava, 2019) and currently 30% of the recombinant proteins for therapeutic use are expressed in this host (L. Li et al., 2022; Rosano et al., 2014). In particular, the BL21 (DE3) strain is widely used to produce recombinant proteins. It is insensitive to glucose concentration, therefore growing faster and producing less acetate than wild type *E. coli*. This strain is also deficient in two proteases encoded by the genes lon (cytoplasmic protease) and ompT (periplasmic protease), the main proteases that participate in the degradation of recombinant proteins (Rosano et al., 2014). In addition to host selection, culture media plays an important role in protein expression. In relation to composition, they can be differentiated into (i) complex media, which are composed by water-soluble biological extracts rich in nutrients and do not present chemically characterized components, and (ii) defined media presenting a well-known chemical composition (Jagschies et al., 2018; Sigma-Aldrich, 2021). Luria-Bertani (LB) for example is a complex medium easy to formulate that promotes rapid bacterial growth resulting in high biomass yields. For this reason, it is widely used in laboratory and industrial environments (Sezonov, Joseleau-Petit, & D'Ari, 2007; Wang et al., 2023). Nonetheless, chemically defined media can be advantageous to produce recombinant proteins by adjusting the concentrations of carbon sources, metals, and salts. In addition, chemically defined media is usually more reproducible, allowing- batch-to-batch quality control, and generally facilitates purification processes (Riesenberg et al., 1991). Li et al., (2017) studied the global response of *E. coli* BL21 (DE3) proteome to the production of the recombinant protein hFGF-2 (Human Growth Factor-2) in complex and

defined culture media. Growth rate was higher in complex medium; however growth inhibition was more pronounced. Therefore, defined medium resulted in higher total biomass and volumetric production of the recombinant protein.

The type of bioprocess can also influence recombinant protein production. In batch processes, all nutrients are available at the beginning of the process, making it easy to implement with low probability of contamination. However, when the substrate has a toxic or inhibitory effect, fed batch processes can be a better alternative. In this modality, one or more nutrients are fed throughout the process at defined flow rates, resulting in better metabolic control (Behl et al., 2023).

Specifically, the production of photolyase is of increasing interest in the last decade. Photolyase is an enzyme that repairs ultraviolet light radiation damage to DNA by photoreactivation (Ramírez-Gamboa et al., 2022). Therefore, it provides an active strategy against solar radiation damage, including photoaging and cancer (Nascimento et al., 2024; Megna et al., 2017). Berardesca et al., (2012) studied the only photolyase commercial source currently on the market, the extract of the cyanobacterium *Anacystis nidulans*, and confirmed the potential of photolyase in human patients at a concentration of 1% in the product. In the same way, Marizcurrena et al., (2019) presented the first report of photolyase recombinant production from the bacterial *Hymenobacter* sp. They worked with in the *E. coli* host, LB medium and included the N-terminal 6-His fusion, resulting in a yield of 210 mg of protein per litre of culture and reported repair activity with 1 µg/mL of photolyase. More recently, our group reported the high enzymatic activity of *T. thermophilus* photolyase, with 90% of DNA repair at 15 µg/mL of enzyme (Torres-Obreque et al., 2024).

In this work, we optimized the production of photolyase from *Thermus thermophilus* by the recombinant *E. coli* BL21 strain. Different culture media and cultivation processes were studied, including batch and fed-batch bioreactor. This is an important step to future industrial production of photolyase, an enzyme that shows promising results as an active ingredient for cosmetic and pharmaceutical formulations (Torres-Obreque et al., 2024; Marizcurrena et al., 2019).

2. MATERIALS AND METHODS

2.1. Cloning and transformation

The photolyase was produced by a recombinant *E. coli* BL21 (DE3) strain with a pET15b vector (histidine-tagged N-terminal fusion protein) encoding the *Thermus thermophilus* photolyase gene (GenBank: AB001637.1). The vector with codon optimization to *E. coli* expression was synthesized by GenScript (Piscataway NJ, USA). Two restriction enzymes, NcoI and BamHI, were used to corroborate the integrity of the transformed vector due the temperature stress over time. The experiment included positive and negative controls (Table 1).

Table 1. Experimental parameters for assessment of transformed vector integrity of pET15b with *T. thermophilus* photolyase gene.

	Volume (μL)			
	Sample 1	Sample 2	Control +	Control -
Transformed plasmid	1.5	1.5		1.5
Empty plasmid	-	-	1.5	-
NcoI enzyme	2	-	2	-
BamHI enzyme	0.5	0.5	0.5	-
Buffer	-	2	-	-
dH ₂ O	16	16	16	18.5

Following, the vector was used to transform the *E. coli* BL21 (DE3) bacteria. Briefly, 50 ng of plasmid were inserted into 50 μL of electrocompetent *E. coli* by electroporation at 2.5 kV in the Gene Pulser(R) II equipment (Bio-Rad laboratories, Hercules, CA, USA). The electroporated cells were resuspended in 1 mL of Luria-Bertani (LB) medium and incubated for 1 h at 37 °C under shaking at 180 rpm. After incubation, 100 μL of the cells were inoculated in LB solid medium supplemented with carbenicillin (50 μL/mL) and the plate was incubated at 37°C overnight. The colonies selected by antibiotic resistance were placed in liquid LB medium and incubated under shaking (37°C, 180 rpm) overnight. Finally, the transformed strain was stored in glycerol (10%) at -80 °C.

2.2. Photolyase production in shaker incubator

Two media were used for the growth of *E. coli*: the complex Luria-Bertani (LB) medium and the chemically characterized medium proposed by Riesenberger et al. (1991) (Glucose 10 g/L; KH_2PO_4 13.3 g/L; $(\text{NH}_4)_2\text{HPO}_4$ 4 g/L; citric acid 1.7 g/L; MgSO_4 1.2 g/L; Iron citrate (III) 0.06 g/L; MnCl_2 1.5×10^{-2} g/L; $\text{Zn}(\text{CH}_3\text{COO})_2$ 0.8×10^{-2} g/L; H_3BO_3 0.3×10^{-2} g/L; Na_2MoO_4 0.25×10^{-2} g/L; CoCl_2 0.25×10^{-2} g/L; CuCl_2 0.15×10^{-2} g/L; $\text{EDTA} \cdot \text{Na}_2$ 0.84×10^{-2} g/L; thiamine HCl 0.45×10^{-2} g/L).

The pre-inoculum was prepared from the strain stored in glycerol in 50 mL of sterile LB culture medium supplemented with carbenicillin (50 $\mu\text{g/mL}$) and grown at 37°C, 200 rpm for 16 h. The cultures were inoculated in the corresponding fresh medium (LB or defined) supplemented with carbenicillin (50 $\mu\text{g/mL}$), with an initial optical density ($\text{OD}_{600\text{nm}}$) of 0.1. The culture was incubated in a metabolic shaker at 37°C, 200 rpm. Initially, a bacterial growth curve was constructed without induction over time and the cell concentration at half-exponential phase was determined based on a pre-obtained analytical curve correlating $\text{OD}_{600\text{nm}}$ and dry biomass (g/L) ($y = 2.44x$). This concentration was defined as the induction time and the IPTG inducer (isopropyl β -D-thiogalactoside) was added at a concentration of 0.1 mmol per g of cell. The culture was kept under incubation in a metabolic shaker at 37°C, 200 rpm for 3 h for the expression of photolyase. Cultivations were carried out in triplicate.

2.3. Photolyase production in bioreactor

2.3.1. Batch process.

The BIOSTAT B(R) 2 L bioreactor (Sartorius, Germany) was operated with 1 L of modified Riesenberger defined medium with 27.5 g/L of glucose and supplemented with carbenicillin (50 $\mu\text{L/mL}$). The cultivation process parameters were temperature of 37 °C, pH at 6.8 controlled by the addition of NH_4OH (50% v/v) or H_3PO_4 (50% v/v), total gas flow of 1 vvm (liter of air per liter of medium per minute) and dissolved oxygen (DO) above 20% of air saturation, with cascade control of agitation between 300 and 1500 rpm. Silicon (Y30, Sigma-Aldrich, St Luis, USA) was added as antifoam when necessary. Pre-inoculum was prepared as described in Section 2.3 and inoculation corresponded to

an OD_{600nm} of 0.1. Samples of 1-2 mL were collected every hour to construct a bacterial growth curve. Then, the induction was carried out at the end of the exponential growth phase with a single pulse of IPTG (0.1 mmol per g of cell). The culture was kept at the same condition overnight for the expression of photolyase.

2.3.2. Fed-batch process

Fed-Batch cultivation began with a batch cultivation in a BIOSTAT B(R) 2 L bioreactor (Sartorius(r), Germany) at the same conditions described above, until reaching total glucose consumption at 9 h, represented by a rapid increase in the concentration of dissolved oxygen (DO) indicating the end of the exponential growth phase. At this exact time, the feeding was initiated with the strategy of keeping static dissolved oxygen concentration (DO-stat) constant. The DO-stat control was performed in a closed-loop, when the DO sensor detected oxygen concentration above 20% of air saturation, the feeding solution (glucose 596 g/L; MgSO₄ 20 g/L; EDTA 0.813 g/L; citrate 0.04 g/L; CoCl₂ 0.004 g/L; MnCl₂ 0.0235 g/L; CuCl₂ 0.0023 g/L; H₃Bo₃ 0.0047 g/L; Na₂MoO₄ 0.004 g/L; Zn(CH₃CooH)₂ 0.016 g/L) was activated, and the reactor fed until the oxygen concentration dropped below 20%. When the culture reached the stationary phase, feeding stopped and IPTG was added at 0.1 mmol per g of cell. Culture was kept overnight under batch conditions for the expression of photolyase.

2.4. Determination of growth kinetic parameters

The maximum cell concentration (X_{max} , expressed in g/L) was based on the correlation curve between optical density (OD_{600nm}) and dry biomass (g/L). The specific growth rate (h^{-1}) was calculated with the help of the following equations that describes the typical growth of a bacterial population:

$$\mu_X = \frac{1}{X} \frac{dX}{dt} \quad (1)$$

$$\ln (X - X_0) = \mu_X (t - t_0) \quad (2)$$

in which X is the biomass at a specific time (g/L), X_0 is the initial biomass (g/L), $t - t_0$ is the time interval (h) and μ_x is the specific growth rate. By means of a logarithmic growth curve ($\ln(X)$) as a function of time (h), we calculated the slope of the line corresponding to the exponential phase, what corresponds to the maximum specific growth rate (μ_{max}).

P_X corresponds to the biomass volumetric productivity (g/L) per unit of time and was calculated according to equation 3.

$$P_X = \frac{X_{max} - X_0}{t - t_0} \quad (3)$$

$Y_{X/S}$ is the yield of biomass production per consumed substrate and was calculated based on equation 4, in which S_0 and S_F correspond to initial and final substrate concentrations, respectively.

$$Y_{X/S} = \frac{X_{max} - X_0}{S_0 - S_F} \quad (4)$$

P_{prod} corresponds to the product volumetric productivity in mg of pure enzyme per liter of culture and per unit of time and was calculated according to equation 5.

$$P_{prod} = \frac{E}{V * (t - t_0)} \quad (5)$$

in which E is the mass of pure enzyme obtained after purification (mg) and V is the volume of culture from which the enzyme was purified.

2.5. Determination of reducing sugars

The concentration of total reducing sugars as substrate in the culture samples was determined by the DNS method (Gusakov et al., 2011). Samples were collected at different times during cell growth with defined medium, centrifuged at 4,000 x g for 20 min at 4°C and the supernatant was collected. Then, 175 µL of 3,5-dinitrosalicylic acid (5 g/L) were mixed with 75 µL of the supernatant and reacted at 90 °C for 10 min. The mixture was immediately placed in the freezer for 5 min. Finally, 1 mL of ultrapure water was added and absorbance measured at 515 nm. The analytical curve was constructed using glucose at different concentrations from 0.1 to 1.0 mg/mL.

2.6. Photolyase purification

At the end of the culture, the cells were collected by centrifugation at 4,000 x *g* for 30 min at 4°C, resuspended in 20 mM Tris-HCl pH 7.4, 300 mM NaCl, 50 mM imidazole, 1 mM EDTA (ethylenediamine tetraacetic acid) and 1 mM PMSF (phenylmethylsulfonyl fluoride), and finally lysed by ultrasound probe at 30% of amplitude, 18 pulses of 10 seconds with 30 seconds intervals between pulses, keeping the cells in an ice bath. The protein extract obtained was centrifuged at 16,000 x *g* for 30 min and the supernatant filtered through a 0.45 µm pore diameter membrane (Merck Millipore, Darmstadt, Germany). Purification was performed by affinity chromatography, using a 5 mL Hi-Trap nickel affinity column (Ge Healthcare, Chicago, USA) and elution in two steps (20 and 70% of 500 mM imidazole), in an AKTA Fast Protein Liquid Chromatographer (Ge Healthcare, Chicago, USA). The presence of protein in the collected fractions was confirmed by gel electrophoresis – SDS PAGE 12% (Laemmli, 1970). For the SDS-PAGE, samples were treated with the reducing agent β-mercaptoethanol and heated to 95°C for 5 min. The total quantity of pure enzyme was measured by the BCA Protein Assay Kit according to the manufacturer instructions (Sigma Aldrich, St Luis, USA).

3. RESULTS AND DISCUSSION

3.1. Cloning and transformation

The BL21 (DE3) strain was transformed with the pET15b vector with the integrated gene of *T. thermophilus* photolyase. Initially, the integrity of the transformed plasmid was confirmed by using two restriction enzymes, NcoI and BamHI. The empty vector was used as a positive control and the transformed vector without treatment with the restriction enzymes as a negative control. Figure 1 shows the results of the agarose gel, in which Sample 1 (with the digestion by both enzymes) presents two bands (~6000 and ~1700 bp approximately). The first is equivalent to the positive control (the empty plasmid with 5708 bp) and the second has a mass equivalent to that reported for the *T. thermophilus* photolyase gene (1677 bp). Sample 2 presents a single band of 7500-8000 bp, corresponding to the linearized vector, equivalent to the sum of the empty vector plus the gene. Therefore, the desired gene was correctly inserted into the vector and no

losses were observed due to thermal stress over the time. Following, the transformation of an electrocompetent *E. coli* bacterium was carried out successfully and the recombinant strain stored with 10% glycerol under -80°C .

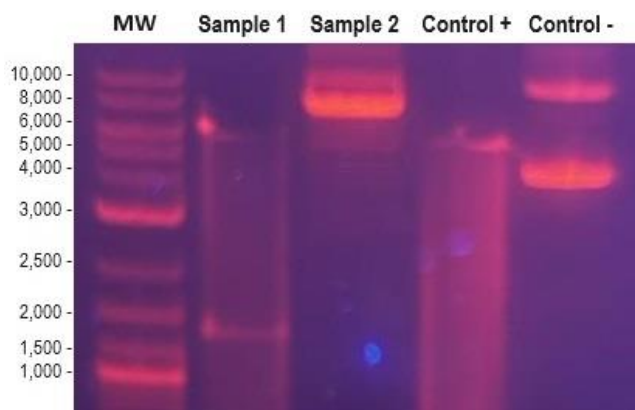


Figure 1. Agarose electrophoresis gel analysis of transformed vector integrity of pET15b with *T. thermophilus* photolyase gene. **MW:** molecular weight (pb). **Sample 1:** transformed vector digested by NcoI and BamHI. **Sample 2:** transformed vector digested only by BamHI. **Control +:** empty vector. **Control -:** transformed vector without treatment with both restriction enzymes.

3.2. Photolyase production in shaker incubator

The recombinant *E. coli* was cultivated in LB complex medium with a total volume of 500 mL. A classic bacterial growth profile was observed, with the maximum biomass (X_{max}) of 2.0 g/L reached after 5 h of cultivation (Figure 2A). Biomass productivity was $P_X = 0.4$ g/L.h, corresponding to μ_{max} of 1.0 h^{-1} , in agreement with previous values reported in the literature. Torres-Obreque et al., (2019) reported μ_{max} of 0.66 h^{-1} and Li et al., (2017) obtained a value of 0.92 h^{-1} for *E. coli* BL21 (DE3) in LB medium, expressing different proteins. Berney et al., (2006) studied the *E. coli* specific growth rate sensitivity and describe a fast-growing culture in LB medium with a $\mu = 1.0 \text{ h}^{-1}$.

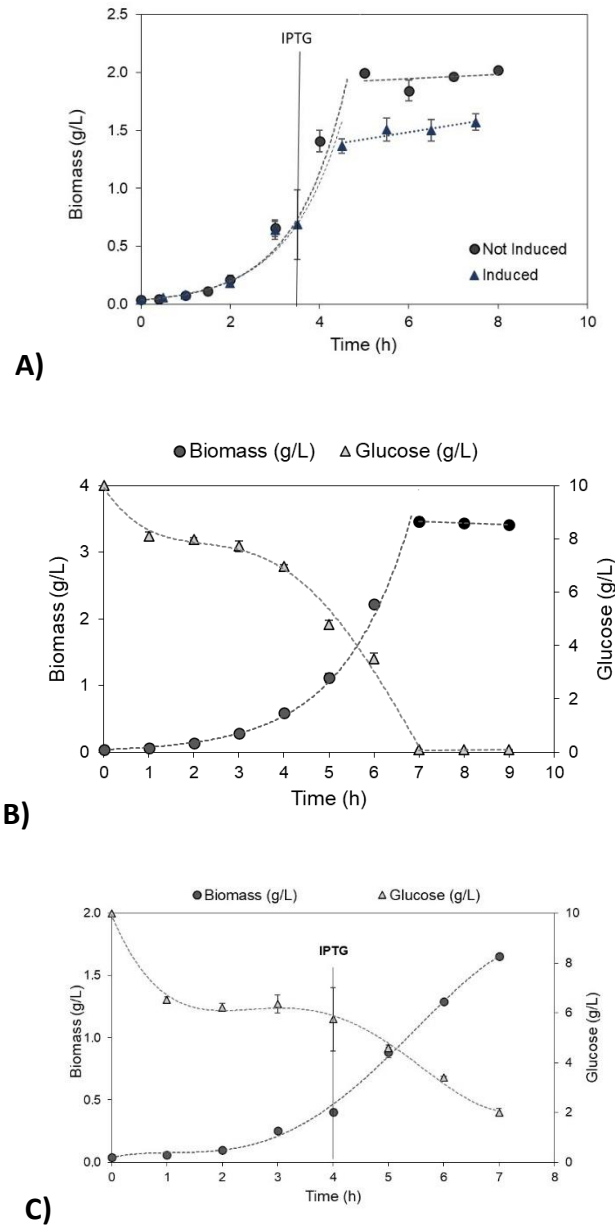


Figure 2. A) Growth curves of recombinant *E. coli* BL21 (DE3) expressing the *T. thermophilus* photolyase: (A) Luria-Bertani medium with and without IPTG induction; (B) chemically defined medium without induction, altogether with substrate consumption (glucose); (C) chemically defined medium with IPTG induction, altogether with substrate consumption. The error bars correspond to the standard deviation.

It is worth noting that the variation observed among the results is partly due to the low reproducibility in the complex medium. The LB medium does not have its chemical components accurately characterized and quantified and its composition can vary from batch to batch. In addition, LB medium is composed mainly of yeast extract and for this reason the concentration of fermentable sugars is very low, therefore quantification of $Y_{x/s}$ was not possible (Sezonov, Joseleau-Petit, & D'Ari, 2007).

Based on the growth curve, induction with IPTG was carried out at 3.5 h of cultivation. As IPTG is a non-metabolizable inducer of the "lac operon", the transcription and consequent expression of photolyase is uninterrupted (Nelson & Cox, 2013). The specific growth rate (μ_x) after IPTG addition was calculated to study the effects of induction on cell growth and a $\mu_x = 0.74 \text{ h}^{-1}$ was obtained, confirming that IPTG addition shifts the metabolic flow to uninterrupted production of the recombinant protein, resulting in a metabolic imbalance associated with the drop in μ_x value (Rozkov et al., 2004).

The recombinant *E. coli* was also cultivated in a defined culture medium (Figure 2B), what could be more appropriate to industrial production due to batch standardization, facilitating downstream operations and compliance with regulatory criteria (de Almeida Parizotto et al., 2021). The $X_{max} = 3.6 \text{ g/L}$ was reached at 7h of cultivation after complete glucose consumption. The μ_{max} corresponded to 0.66 h^{-1} , $P_x = 0.51 \text{ g/L.h}$ and $Y_{x/s} = 0.36 \text{ g cells/g substrate}$. All the kinetic parameters are summarized in Table 2. The μ_{max} was higher in LB complex medium (1.0 h^{-1}) compared to the chemically defined medium (0.66 h^{-1}), however this is not necessarily advantageous for recombinant protein production since μ_{max} is linearly related to acetate production that can in turn inhibit cellular growth at high concentrations (Lulit & Strohl, 1990; Ponce, 1999). In fact, chemically defined medium resulted in twice higher X_{max} than complex medium and this is mostly related to the composition of media. LB has yeast extract, peptone and sodium chloride in its composition; therefore, the concentration of fermentable sugars is low and the bacterial cells are forced to metabolize amino acids as the main carbon source. Consequently, high biomass concentrations cannot be reached (Sezonov, Joseleau-Petit, & D'ari, 2007). The higher P_x value obtained with the defined medium also represents an advantage in time and economic resources.

Table 2. Comparison of kinetic growth of recombinant *E. coli* BL21 (DE3) cultures in Luria-Bertani (LB) and defined medium: $X_{\max}$ - maximum biomass, P_x - biomass productivity, $\mu_{\max}$ - specific growth rate; $Y_{x/s}$ - substrate to biomass conversion factor.

Kinetic parameters	Shaker incubator		Bioreactor (Defined medium)	
	LB medium	Defined medium	Batch	Fed-Batch
$X_{\max}$ (g/L)	1.99	3.61	10.5	20.2
P_x (g/L.h)	0.39	0.51	1.16	1.84
$\mu_{\max}$ (h ⁻¹)	1.0	0.66	0.72	0.49

The *E. coli* biomass produced in LB or defined culture medium was collected by centrifugation and submitted to photolyase purification process according to section 2.7. The SDS-PAGE analysis of the purified enzyme (data not shown) showed a unique band of 47 kDa, corresponding to the molecular mass of *T. thermophilus* photolyase (Kato et al., 1997; Torres-Obreque et al., 2024). Cultivation with LB medium resulted in 3.25 mg of protein per L of culture. On the other hand, 15.6 mg of protein per L of culture were obtained when chemically defined medium was used. Therefore, the defined medium was considered superior and selected to proceed with the scale-up of photolyase production.

3.3. Photolyase production in bioreactor – Batch process

Bioreactors allow better control of the cultivation parameters, leading to better efficiency in substrate consumption to avoid growth inhibition. For this reason, the defined medium was supplemented with higher concentrations of glucose in comparison to the metabolic shaker. To avoid the oxygen limitation, the DO in the bioreactor was controlled by agitation and air injection, maintained at a minimum of 20% approx., as can be seen in Figure 3. When the cultivation reached stationary phase, a sharp increase in the DO was observed indicating less oxygen consumption, as expected. Batch cultivation in the bioreactor resulted in $X_{\max} = 10.5$ g/L at 9h, with a $\mu_{\max} = 0.72$ h⁻¹. The higher oxygen availability, in comparison to metabolic shaker, the pH

control and high initial substrate allowed the exponential growth phase to be extended. The growth rate was similar to that observed in shaker, but $X_{\max}$ was almost 3 times higher. As a result, biomass volumetric productivity ($P_x = 1.16$ g/L.h) increased 127% in comparison to the culture in shaker.

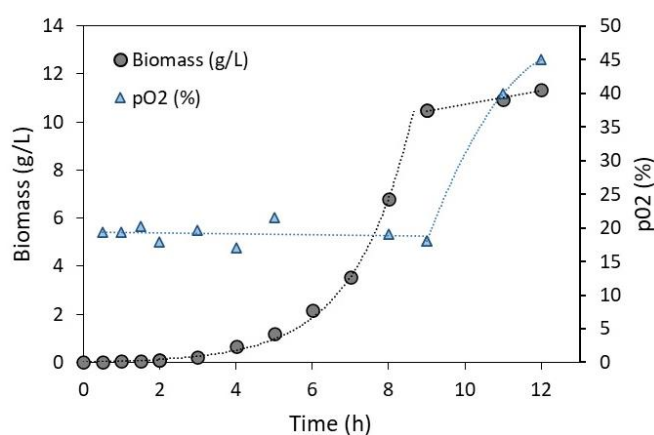


Figure 3. Growth curve (Biomass, g/L) and dissolved oxygen curve (pO₂, %) of recombinant *E. coli* BL21 (DE3) expressing the *T. thermophilus* photolyase cultivation in chemically defined medium in a batch bioreactor.

The IPTG induction was carried out at the end of the exponential growth phase in one single pulse and, 12h after induction, the biomass was obtained by centrifugation for further purification. In the end of the process, 170 mg of pure enzyme was produced per L of cultivation, 10-fold higher than the quantity produced in the shaker incubator and representing a product volumetric productivity (P_{prod}) of 8.1 mg/L.h (Table 3).

Similar results are reported in literature for other complex biomolecules. For example, Carmignotto & Azzoni (2019) discussed the expression of recombinant Cas9 protein in *E. coli* BL21 (DE3). The maximum cell concentration obtained was 5.6 g/L, and the maximum specific growth rate (μ_{max}) was 0.53 h⁻¹. The Cas 9 volumetric productivity after 8 h of induction was 11.9 mg/L.h.

Table 3. Summary of enzyme concentration and product volumetric productivity (P_{prod}) after photolyase purification for different cultivation processes.

Parameters	Shaker incubator		Bioreactor (Defined medium)	
	LB medium	Defined medium	Batch	Fed-Batch
Concentration of pure enzyme (mg/L)	3.25	15.6	170	480
P_{prod} (mg/L.h)	0.25	0.36	8.1	20.9

3.4. Photolyase production in bioreactor – Fed-batch process

The fed-batch strategy is typically used in industrial processes to reach high cell density in bioreactors, usually employing highly concentrated feed solutions to avoid dilution (Behl et al., 2023). Production of heterologous proteins by fed-batch cultures of recombinant microorganisms have been extensively studied. The controlled addition of the nutrient directly affects the growth rate of the culture and helps to avoid overflow metabolism (formation of side metabolites, such as acetate for *E. coli*) by oxygen limitation (anaerobiosis) (Z. Li et al., 2017). Several feeding strategies can be used to cultivation processes, for instance, constant feeding rate, constant substrate concentration, constant specific growth rate, and constant oxygen level. We adopted the strategy of DO-stat (constant dissolved oxygen level), since a minimum oxygen level is required for the glucose metabolism and cell growth (de Almeida Parizotto et al., 2021).

The process (Figure 4) started with an initial glucose concentration of 27 g/L and the batch phase finished at 9h when glucose was totally consumed. At this point, the feeding started and continued by 2 h with a μ_{max} of 0.49 h⁻¹. A X_{max} = 20.2 g/L was observed at the end of feeding phase, with 11h of cultivation. As can be seen, cell concentration duplicated by extending the exponential phase for two hours. More important, employing a DO-stat strategy resulted in a marked enhancement of biomass volumetric productivity, reaching 1.84 g/L.h, an increment of approximately 60% compared to the conventional batch culture.

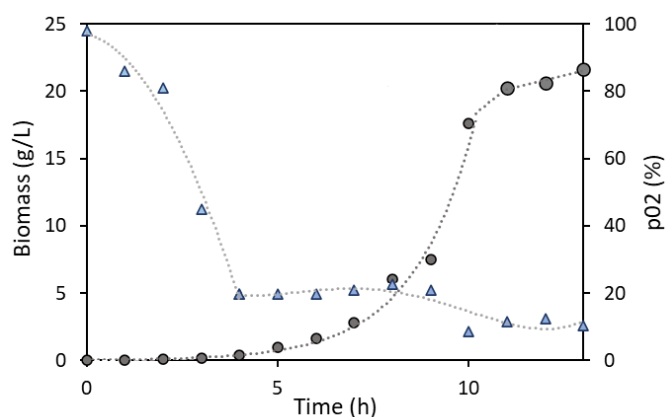


Figure 4. Growth curve (Biomass, g/L) and dissolved oxygen curve (pO₂, %) of recombinant *E. coli* BL21 (DE3) expressing the *T. thermophilus* photolyase cultivation in chemically defined medium in a fed-batch bioreactor.

For the fed-batch bioreactor we obtained 480 mg of pure enzyme per L of culture (Table 3). This value is 2.8 times higher than the one obtained in the batch bioreactor and 30.8 times higher than the one obtained in shaker incubator. Moreover, this value represents an increase of 160% in P_{prod} (20.9) compared to the batch condition.

Miret et al. (2020) used fed-batch process for the production of the enzyme complex phosphite dehydrogenase - cyclohexanone monooxygenase (PTDH–CHMO) in an *E. coli* NEB10 β strain and a 9.1-fold increase in total protein concentration was observed, compared to the batch process (460 mg/L). According to the authors, 1990 mg/L of enzyme were produced 6 hours after induction, which is in the same order of magnitude of increase we observed for the photolyase production from batch (170 mg/L) to fed-batch (480 mg/L). Also, in terms of volumetric productivity, they observed 3 to 4-fold increase in PTDH–CHMO production from the batch (19 mg/L.h) to the fed-batch (71 mg/L.h) process, a slightly higher increment than the one we observed in the volumetric productivity from the batch (8.1 mg/L.h) to the fed-batch (20.9 mg/L.h) process of photolyase production.

4. CONCLUSION

Here we presented the scale up of *T. thermophilus* photolyase production by *E. coli* from shaker conditions to fed-batch bioreactor. Batch bioreactor already exhibited a three-fold increase in maximum biomass (10.5 mg/L) compared to the metabolic shaker (3.6 g/L), however the fed-batch process resulted in $X_{\max} = 20.2$ g/L, the double of the biomass produced in batch system. This enhancement translated into an yield of 480 mg/L of pure enzyme by fed-batch process, representing a 2.8-fold increase. Consequently, the volumetric productivity of pure enzyme was 20.9 mg/L.h for the fed-batch process, significantly superior to the 8.1 mg/L.h observed in the batch process. The demonstrated superiority of the fed-batch process in producing *T. thermophilus* photolyase, with significantly higher biomass and volumetric productivity compared to batch culture, presents a promising avenue for large-scale production of this enzyme with potential applications in dermatological and pharmaceutical products.

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CHAPTER IV

Comparative study of nanostructures for the encapsulation of photolyase

This chapter focus on the nanoencapsulation of photolyase to promote the penetration into skin for future topical applications, with the study of three different nanostructures. In addition, *in vitro* cytotoxicity and photorepair activity in HaCaT cells were evaluated for free and nanoencapsulated photolyase.

CHAPTER IV

Comparative Study of nanostructures for the encapsulation of photolyase

ABSTRACT

The topical application of enzymes, such as photolyase, for the repair of UV-induced DNA photodamage represents an interesting strategy of "active photoprotection". However, a large and hydrophilic molecule like photolyase, needs to penetrate the skin to exert its biological activity and, in this sense, nanocarriers can provide an aid for barrier crossing. In this work, we investigated different nanostructured systems to encapsulate photolyase, namely polymersomes (PL), liposomes (LP), and polymeric nanoparticles (PNP). Both, PL and LP, presented favorable characteristics, such as nanometric size (100-180 nm), similar encapsulation efficiency values (23%) and adequate release to the external aqueous medium (up to 35% for the PL after 24h). On the other hand, PNPs presented the best EE performances, with an adequate size and an easy-scaling methodology. Unfortunately, the slow release of the enzyme towards the external phase ($\approx 15\%$ after 10 days) and cytotoxicity in HaCaT cells represent disadvantage of this system. We also achieved remarkable improvements in cell viability after UV irradiation with photolyase-loaded PL and LP, with cell viability recoveries of 37% for the free enzyme and 50-60% when nanoencapsulated, compared to 28% without the enzyme. This work proposes for the first time an innovative and efficient encapsulation technology for the enzyme photolyase as an alternative for its incorporation in skin care, sun care and skin cancer prevention products.

Keywords: photolyase, polymersome, liposome, polymeric nanostructures, DNA-repair.

1. INTRODUCTION

The exposure to ultraviolet (UV) radiation has beneficial and adverse effects on human health. UVB radiation on the skin is responsible for the synthesis of vitamin D, which plays an essential role in our body metabolism, specifically in maintaining calcium homeostasis (Schuch et al., 2017). In contrast, UV radiation can be directly absorbed by DNA and induce dimeric photoproducts between adjacent pyrimidine bases (cytosine and thymine) resulting in harmful consequences such as cell death, mutagenesis, and skin cancer (Berardesca et al., 2012; Schuch et al., 2017). The photolesions caused by UV radiation are called cyclobutane pyrimidine dimers (CPDs) and photoproducts of pyrimidine (6-4) pyrimidone ((6-4)PP) (Leccia et al., 2019; Sancar, 2008). In general, damage to DNA is 80-90% in the form of CPD and 10-20% in the form of (6-4)PP (Kavakli et al., 2019; Sancar, 2003). Sunscreens have been important allies to avoid the effects of UV radiation, however this passive strategy does not reverse photolesions. Therefore, new active strategies to reduce the damage caused by UV radiation on the DNA are desired. Specifically, the topical use of DNA repair enzymes, such as photolyase, is of increasing interest in the last decade (Torres-Obreque et al., 2024).

Photolyases are flavoproteins capable of repairing crosslinks in DNA formed by exposure to UV radiation. They repair injuries such as CPD or (6-4) PP, reversing the damage to form the bases of intact pyrimidines, using the energy of blue light (photons emitted between 350-500 nm) (Hosokawa et al., 2019). Photolyase is not innately present in humans, which have other DNA repair mechanisms, but not so much effective for CPD damage repair. To exert biological activity, photolyase needs to reach the viable epidermis, but skin penetration of proteins is a challenge owing to the stratum corneum, the main barrier to the passage of molecules and drugs applied topically. In this sense, nanostructures can be an alternative for topical application of photolyase.

Here, we investigated three different nanostructures as alternatives for photolyase nanoformulation, namely liposomes, polymersomes and polymeric nanoparticles. Liposomes (LP) are the most used drug delivery systems. They are spherical vesicles comprised of an aqueous core surrounded by a lipid membrane obtained by the self-aggregation of phospholipids (Bozzuto & Molinari, 2015). Liposomes made their successful entry into the market in 1995 with Doxil®. Since then, about 20 liposomal

formulations are available in the market for clinical use and more than 28 are enrolled in clinical trials, from cosmetic ingredients to anticancer drugs (Bulbake et al., 2017; Giordani et al., 2023). Similar to liposomes, polymersomes (PL) are nanostructures formed by the self-aggregation of amphiphilic block copolymers in aqueous medium (Pachioni-Vasconcelos et al., 2016). PL have attracted attention due to their stability and versatility compared to liposomes (Apolinário et al., 2017; C. A. Oliveira et al., 2020a). The size, structure, and physicochemical properties of polymersomes can be adjusted depending on block copolymer composition and Mw, allowing different membrane thickness and permeability. Nonetheless, there are still no pharmaceutical products based on polymersomes on the market. Finally, polymeric nanoparticles (PNP) correspond to continuous polymeric networks in which drugs can be incorporated (Zielinska et al., 2020). Over the years, a great variety of natural and synthetic polymers have been explored to produce PNP, such as polycaprolactone (PCL), an FDA approved biopolymer of fossil origin but biodegradable and biocompatible (Drago et al., 2021).

Currently, some photolyase-based nanoproducts are available on the market. Nonetheless, they consist of a plankton extract as a source of photolyase encapsulated in liposomes and associated with UV filters. There are no reports on the encapsulation of pure photolyase in nanostructures.

2. MATERIALS AND METHODS

2.1. Production and purification of photolyase

The enzyme was produced by a recombinant *E. coli* BL21 (DE3) strain containing the pET15b vector (histidine-tagged N-terminal fusion protein) with the *Thermus thermophilus* photolyase gene. The pre-inoculum was prepared in 50 mL of sterile culture Luria-Bertani (LB) medium supplemented with carbenicillin (50 µg/mL) and grown at 37°C, 200 rpm for 16 h. The cultures were inoculated in fresh LB medium supplemented with carbenicillin (50 µg/mL), with an initial optical density (OD_{600nm}) of 0.1 and incubated in a metabolic shaker at 37°C, 200 rpm. At half-exponential phase (OD_{600nm} = 0.7-0.8) 0.1 mM of IPTG (isopropyl β d-1-thiogalactoside) was added to induce photolyase expression. The culture was kept under incubation in a metabolic shaker at

the same condition for 3 h. Following, cells were collected by centrifugation at 4,000 x g for 30 min at 4°C, resuspended in 20 mM Tris-HCl pH 7.4, 300 mM NaCl, 50 mM imidazole, 1 mM EDTA (ethylenediamine tetraacetic acid) and 1 mM PMSF (phenylmethylsulfonyl fluoride), and finally lysed by ultrasound probe at 30% of amplitude, 18 pulses of 10 seconds with 30 seconds intervals between pulses, keeping the cells in an ice bath. The protein extract obtained was centrifuged at 16,000 xg for 30 min. Purification was performed by affinity chromatography, using a 5 mL Hi-Trap nickel affinity column (Ge Healthcare, Chicago, USA), and elution with two steps (20 and 70%) of 500 mM imidazole, in an AKTA Fast Protein Liquid Chromatographer (Ge Healthcare, Chicago, USA). More details on the photolyase production can be find in a previous work (Torres-Obreque et al., 2024).

2.2. Production of polymersomes

A design of experiments (DoE) of 2 factors and 3 levels (3^2) was used to optimize photolyase encapsulation in polymersomes (Table 1). The independent variables (factors) were the photolyase concentration and sucrose concentration, an osmolyte to prevent protein aggregation. The response variables were the hydrodynamic diameter (Dh) and the encapsulation efficiency (%EE). The polymersomes (PL) were obtained by direct dissolution of 5 mg/mL of poloxamer 401 (Pluronic L-121®) in 100 mM potassium phosphate buffer (pH 7.4) containing the photolyase and sucrose according to the factorial design. The systems were stirred at 4 °C and 400 rpm for 1 h and subsequently kept under stirring (800 rpm) for 12 h at room temperature to allow the polymer self-aggregation. Any remaining bulk polymer was removed by centrifugation at 1000 x g for 5 min.

Table 1. Design of experiments (DoE) 3^2 for the photolyase encapsulation in poloxamer 401 polymersomes.

Factors	Levels		
	-1	0	1
Photolyase (mg/mL)	1	3	5
Sucrose (mM)	0	50	100

2.3. Production of liposomes

Liposomes (LP) were produced using the film hydration method. Phosphatidylcholine (PC) was dissolved in 50 mL of ethanol and the solvent was removed using a rotary evaporator (Laborota 4000 model, Heidolph, Schwabach, Germany) to form a thin film. Different enzyme concentrations were dissolved in 50 mM phosphate buffer (pH 7.0) supplemented with 50 mM of sucrose and 10 mL of each solution was used to hydrate the thin film obtained. The solution was left under magnetic stirring for 2.5 h at room temperature. Finally, extrusion with 0.45 μm membrane was carried out (10 cycles). A DoE 2^2 was used, to optimize the hydrodynamic diameter and the encapsulation efficiency (%EE) (Table 2).

Table 2. Design of experiments (DoE) 2^2 for the photolyase encapsulation in phosphatidylcholine liposomes.

Factors	Levels	
	-1	1
Photolyase (mg/mL)	1.5	3.0
Phospholipid (mg/mL)	5	20

2.4. Production of polymeric nanoparticles

The production of polymeric nanoparticles (PNP) for photolyase encapsulation was carried out using a DoE 2³. Concentrations of enzyme and polymer, as well as different emulsification conditions were investigated (Table 3). Polymeric nanoparticles (PNP) were produced using the solvent evaporation method of water/oil/water (W/O/W) double emulsions. First, the internal aqueous phase was prepared by dissolving the enzyme at different concentrations in 50 mM phosphate buffer (pH 7.0) with 50 mM of sucrose and 0.5% PVA (polyvinyl alcohol). The oil phase was prepared by dissolving polycaprolactone (PCL), in 19 g of ethyl acetate until its complete dissolution. 1 mL of aqueous internal phase containing photolyase was added to the oil phase. The primary emulsion was obtained by ultrasonic process using a Vibra-Cell™ ultrasonic liquid processor (Sonics & Materials, Inc., Newtown, CT, USA) with a 20 kHz ultrasonic generator probe at 60% amplitude in pulsed mode (30 sec on and 30 sec off) for 2 min. The external aqueous phase (80 mL) was saturated with ethyl acetate and PVA (1%, w/v) and added to the primary emulsion. Secondary emulsion was conducted using a rotor stator emulsifier (Silverson, Massachusetts, USA) under different conditions (according to the Table 3). The emulsion produced was kept for 4 h under magnetic stirring at 35 °C to allow complete evaporation of the organic solvent.

Table 3. Design of experiments (DoE) 2³ for the photolyase encapsulation in polycaprolactone (PCL) polymeric nanoparticles.

Factors	Levels	
	-1	1
Photolyase (mg/mL)	1.5	3.0
PCL (mg)	100	1000
Emulsion condition (rpm/min)	3000 / 2	7000 / 6

2.5. Determination of particle size

The size of the nanostructures was estimated based on Dynamic Light Scattering (DLS) measurements using a Malvern Zetasizer Nano ZS instrument operating with a 633 nm He-Ne laser and a detector adjusted at an angle of 173°. For analysis, 100 µL aliquots of each system were diluted in 1.0 mL of 100mM potassium phosphate buffer (pH 7.4). The analysis was carried out using a polystyrene cuvette with an optical path of 1.0 cm. Measurements were performed in triplicate and the results presented as the average $\pm$ standard deviation.

2.6. Determination of encapsulation efficiency

The different nanostructure systems were centrifuged at 12,000 xg for 30 min (room temperature for PL and 4 °C for LP and PNP) and the total protein concentration of the supernatant was determined by the BCA method following the manufacturer instructions (Sigma Aldrich, St Luis, USA). The absorbance of the samples was read at 562 nm using a microplate reader Spectra Max M2 ((Molecular Devices, California, USA). Encapsulation efficiency percentage (%EE) was calculated based on Eq.1:

$$EE \% = \frac{Initial\ protein - Free\ protein}{Initial\ protein} \times 100 \quad (Eq.1)$$

in which *Initial protein* correspond to the initial photolyase concentration in the system and *Free protein* is the concentration of photolyase in the supernatant after the centrifugation step, i.e., non-encapsulated. Experiments were performed in triplicate and the results presented as the average $\pm$ standard deviation.

2.7. *In vitro* release of the encapsulated photolyase

Samples of the nanostructures were centrifuged at 12,000 xg for 30 min (room temperature for PL and 4 °C for LP and PNP) and the pellets resuspended in the same volume of 100 mM potassium phosphate buffer, pH 7.4, and stored at room temperature under constant stirring. Samples were collected periodically, centrifuged at 12,000 xg, and protein concentration quantified in the supernatant by BCA

assay. Results were expressed as percentage of photolyase released over the time. Release studies were performed in triplicate and results presented as average $\pm$ standard deviation.

2.8. Cytotoxicity assay

Cytotoxicity of photolyase and nanostructures (PL, LP and PNP) was evaluated in HaCaT cells (immortalized human keratinocytes) by the MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). The cells were grown in DMEM supplemented with 10% FBS, in an incubator at 37 °C, 95% humidity and 5% CO₂ atmosphere. The cells were plated in a 96-well plate at a concentration of 5 x 10⁴ cells/well and 100 mL of DMEM. After incubation for 24 h, the cells were exposed to 100 mL of fresh media containing photolyase (10 to 300 µg/mL) or PL (3 to 1000 µg/mL) or LP (3 to 20000 µg/mL) or PNP (3 to 2500 µg/mL) and incubated for more 24 hours (37 °C, 5% CO₂). Following, the samples (100 mL) were replaced by 100 µL of fresh DMEM and 20 µL MTS and incubated at the same condition for 2 h. The absorbance of the dye was assessed at 490 nm on a spectrophotometer UV Vis (LabSystemsTitertek Multiskan MCC/340 Plate Reader; Thermo Labsystems, USA). The positive control corresponded to the absorbance of cells exposed only to DMEM and sodium dodecyl sulfate (SDS) 100 mM was used as the negative control. The effect on cell viability was estimated in comparison to control cells.

2.9. In vitro effect of UV radiation

Before evaluating the effect of nanoencapsulated photolyase on UV-exposed HaCaT cells, enzyme activity was confirmed by immunochemistry with the ab242297 CPD Elisa Kit (Abcam, Cambridge, United Kingdom), as described in Torres-Obreque et al. (2024). The test was performed before and after each formulation process, *i.e.*, for PL, LP and PNP. For the *in vitro* effect of UV radiation, cells were exposed to the UV lamp in a laminar flow cabinet. The lamp emission intensity was determined by VLX3W radiometer (Vilber Lourmat, Torcy, Ile de France, France) in 0.56 J/m²/s. Different times

of exposure were used to achieve UV doses from 33 to 1000 J/m². In a 96-well plate, immediately before UVA exposure, the culture medium of HaCat cells was replaced by phosphate buffer solution (PBS) or the sample containing free or nanoencapsulated photolyase. Concentration of 5 and 50 µg/mL of photolyase and 40, 20 and 10 µg/mL of photolyase-loaded PL or LP were evaluated. The samples of photolyase-loaded nanostructure were incubated 1h before irradiation to allow the enzyme release and or nanostructures penetration. After irradiation, the plates were washed and fresh DMEM was added. Finally, the plates were incubated overnight, and MTS test carried out as explained in the previous item. The positive control corresponded to cells not exposed to UV radiation and the negative control to cells exposed to 100 mM SDS solution.

2.10. Statistics

Statistical analyses were performed in Minitab (version 16) software. Data were treated using one-way ANOVA (analysis of variance) followed by the Tukey test for multiple comparisons. P-value < 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Production of polymersomes

Aiming to improve the permeability of photolyase through the skin in topical applications, different nanostructures were studied. Initially, polymeric vesicles or polymersomes (PL) were produced based on the self-aggregation of poloxamer 401. The preparation of the PL was carried out by changing the temperature, initially stirring at 4°C to dissolve the polymer in the buffer, and subsequently stirring at room temperature to form the nanostructures by self-aggregation and to encapsulate the proteins. Previous data from our group demonstrated that poloxamer 401 aggregates into vesicles at the concentrations and conditions used here (Apolinário et al., 2019; C. A. Oliveira et al., 2020a).

The formation of nanostructures was initially confirmed by the turbidity of the systems visible to the naked eye after a few hours under stirring (Tyndal effect). Moreover, according to the results of DLS curve (Figure 1), nanostructures of about 150 nm were obtained and a second peak at 450 nm was observed corresponding to some protein

precipitation, *i.e.*, photolyase aggregates. In fact, an analysis performed on the ProtPram software indicates an instability index of 47.73 for photolyase, which classifies it as an unstable protein (values above 40) (Gasteiger et al., 2005).

To avoid this aggregation and improve the encapsulation, the osmolyte sucrose was added in all the formulations. Osmolytes are small organic molecules that favor the balance of macromolecules to their native conformation. Therefore, they are employed as excipients in pharmaceutical protein formulations. Włodarczyk et al., (2018), studied different osmolytes for protein stability in aqueous solution and sucrose provided the best results with low protein aggregation.

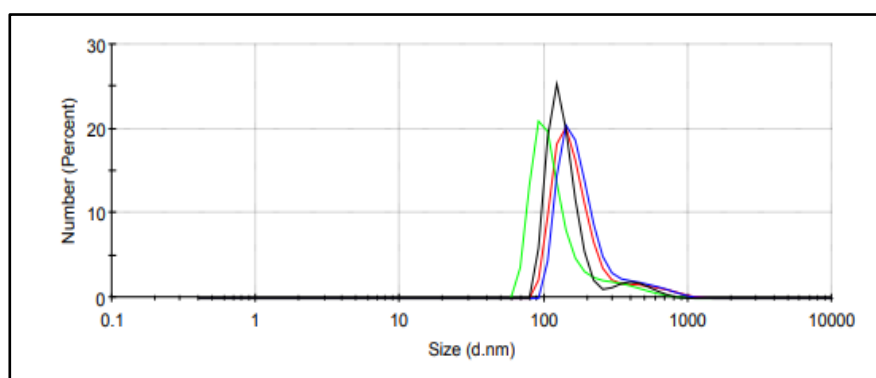


Figure 1. Dynamic light scattering – size distribution by number - of photolyase-loaded polymersomes (5 mg/mL of poloxamer 401 and 1 mg/mL of photolyase).

Following, a 3^2 -factorial design was carried out to optimize the polymersomes production and the results are presented in Table 4 and Figure 2.

According to the literature, poloxamer 401 forms small unilamellar vesicles, measuring approximately 100-200 nm, like those obtained in this work (APOLINÁRIO et al., 2019). Regarding the PDI, values between 0.299 and 0.573 were observed (data no shown). For both dependent variables, Dh and %EE, the pareto diagram of standardized effects (Figure 2C-E) indicated that no single factor was significant, even the combined factors, and the sucrose concentration represents the main effect. More specifically, increasing sucrose concentration results in a decrease in particle size (Figure 2A-B). in addition,

sucrose concentration had a positive effect on %EE (Figure 2D-E); it resulted in an increase from 2% to 23%. However, for the systems with 5 mg/mL of photolyase (with or without sucrose) DLS readings showed a second peak at approximately 580 nm, corresponding to 10% of the number of particles in the sample. We attributed this second peak to photolyase aggregates that end up increasing the polydispersity of the system and affecting the encapsulation efficiency, hence, this concentration of photolyase was not considered adequate for nanostructures production. It is worth noting that literature reports that self-assembled systems such as PL and LP present some degree of polydispersity, what is not an impediment to topical formulations.

Table 4. Results of the experimental design 3² applied to the preparation of photolyase-loaded polymersomes Dh: hydrodynamic diameter; EE: encapsulation efficiency. The error values correspond to the standard deviation.

Photolyase (mg/mL)	Sucrose (mM)	Dh (nm)	EE (%)
1	0	162 ± 9	2 ± 0.2
1	50	150 ± 20	13 ± 0.3
1	100	115 ± 11	16 ± 2
3	0	165 ± 18	2 ± 0.6
3	50	181 ± 18	21 ± 2
3	100	126 ± 12	23 ± 11
5	0	136 ± 20	3 ± 2
5	50	143 ± 15	6 ± 4
5	100	126 ± 11	6 ± 2

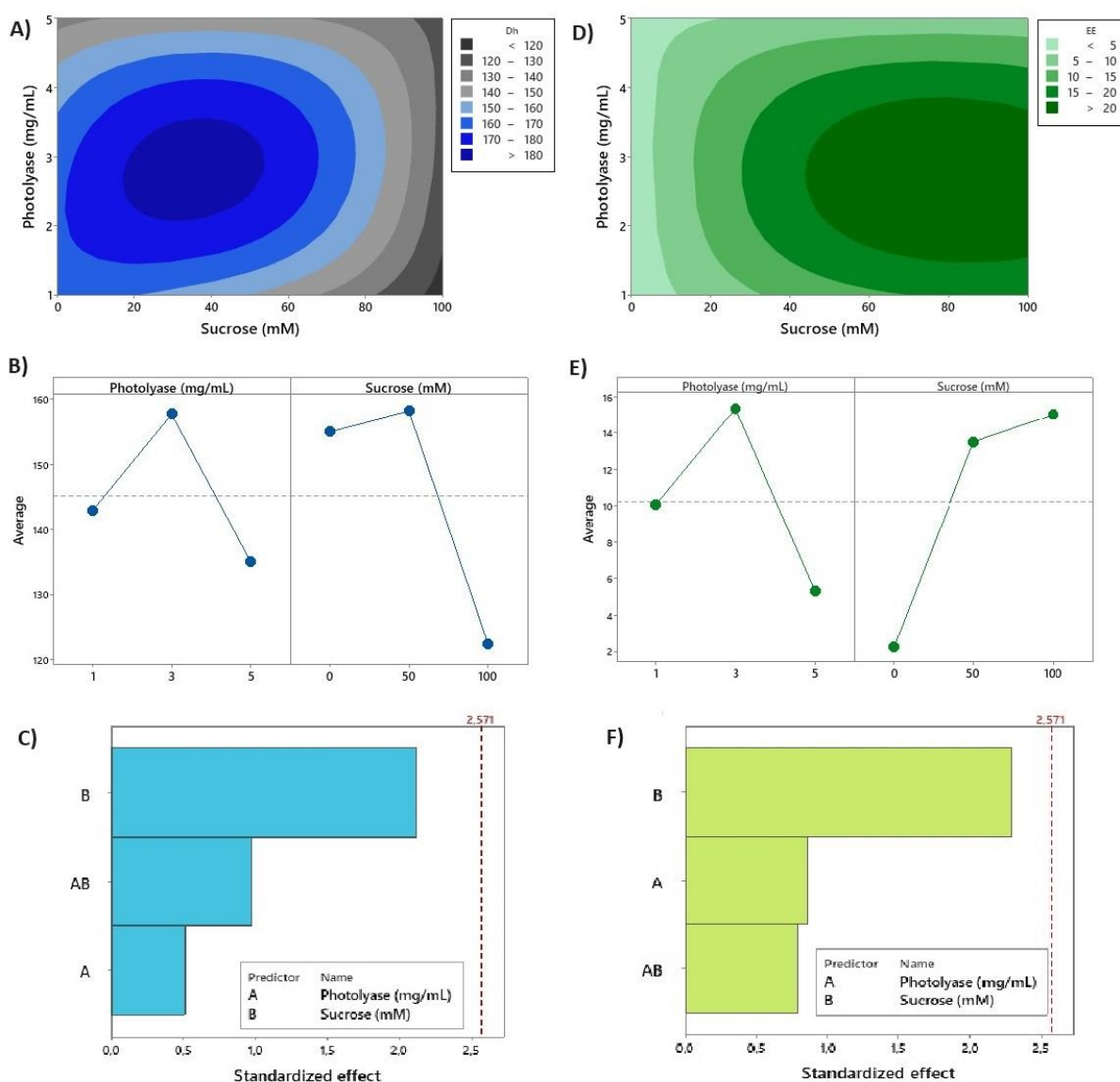


Figure 2. Contour plot, graph of the main effects and pareto chart for the (A-C) hydrodynamic diameter (Dh) and (D-F) encapsulation efficiency (EE) based on the experimental design 3^2 applied to the preparation of photolyase-loaded polymersomes.

The literature reports no clear correlation between protein size and encapsulation efficiency, even so, similar results were found in the literature for different protein encapsulation in polymeric vesicles of poloxamer 401. Oliveira et al., (2020) reported about 5% for catalase encapsulation and Muso-Cachumba et al., (2023) showed values up to 6% and 17% for BSA and L-asparaginase, respectively.

Furthermore, the minimum sucrose concentration investigated in this study effectively prevented protein aggregation and enhanced the percentage of encapsulation efficiency (%EE). Consequently, when utilizing 50 or 100 mM osmolyte, there were not significantly difference in the encapsulation rate with a p-value > 0.05 in the Tukey test. Hence, our results show that 50 mM sucrose adequately ensures enzyme stability and enhances %EE.

Based on the DoE experiments performed, the best conditions to produce photolyase-loaded PL are 3 mg/mL of enzyme and 50 mM sucrose. Also, based on the results obtained with sucrose, this osmolyte was used in photolyase solutions to prepare LP and PNP.

3.2. Production of Liposomes

LP were produced using the thin-film hydration method combined with extrusion. In this approach, a lipid film was formed and subsequently hydrated using an aqueous solution of photolyase. The favorable interactions between water and phospholipids lead to the spontaneous formation of LP. The results of photolyase encapsulation in LP based on a DoE 2² are presented in Table 5 and Figure 3.

The photolyase concentration represents the main factor influencing Dh (Figure 3C); increasing the enzyme concentration resulted in decreasing the LP size, with average values of 94 ± 7 nm and 63 ± 4 nm for 1.5 and 3.0 mg/mL of photolyase, respectively. In general, LP presented a smaller average size compared to PL, what is not necessarily superior for topical application. Literature shows that PL of poloxamer 401 are flexible enough to squeeze across the tight gaps of the skin, owing to the larger size and more loosely packed bilayer (Oliveira et al., 2021; Pachioni-Vasconcelos et al., 2016; Pitto-Barry & Barry, 2014).

Table 5. Results of the experimental design 2² applied to the preparation of photolyase-loaded liposomes. Dh: hydrodynamic diameter; EE: encapsulation efficiency. The error values correspond to the standard deviation.

Photolyase (mg/mL)	Phosphatidylcholine (mg/mL)	Dh (nm)	EE (%)
1.5	5	99 ± 30	23 ± 8
1.5	20	89 ± 6	10 ± 3
3.0	5	66 ± 11	23 ± 8
3.0	20	60 ± 14	9 ± 3

The photolyase concentration did not influence EE results, with a p-value of 0.9 in the Tukey test. It should be noted that all LP systems were supplemented with sucrose based on previous model results, enhancing enzyme solubility. In contrast, the phosphatidylcholine concentration represented the main effect on EE (Figure 3F), more specifically the lowest phosphatidylcholine concentration corresponded to a significantly higher EE (p-value < 0.05). The highest EE value of 23% was obtained with 5 mg/mL of phosphatidylcholine, and is similar to the highest %EE obtained for the PL. In this point, it is important to highlight that LP and PL are vesicles that share structural similarities since both are composed of a bilayer membrane surrounding a water compartment. In this sense, the EE is strongly related to the volume and concentration of the protein aqueous solution effectively entrapped.

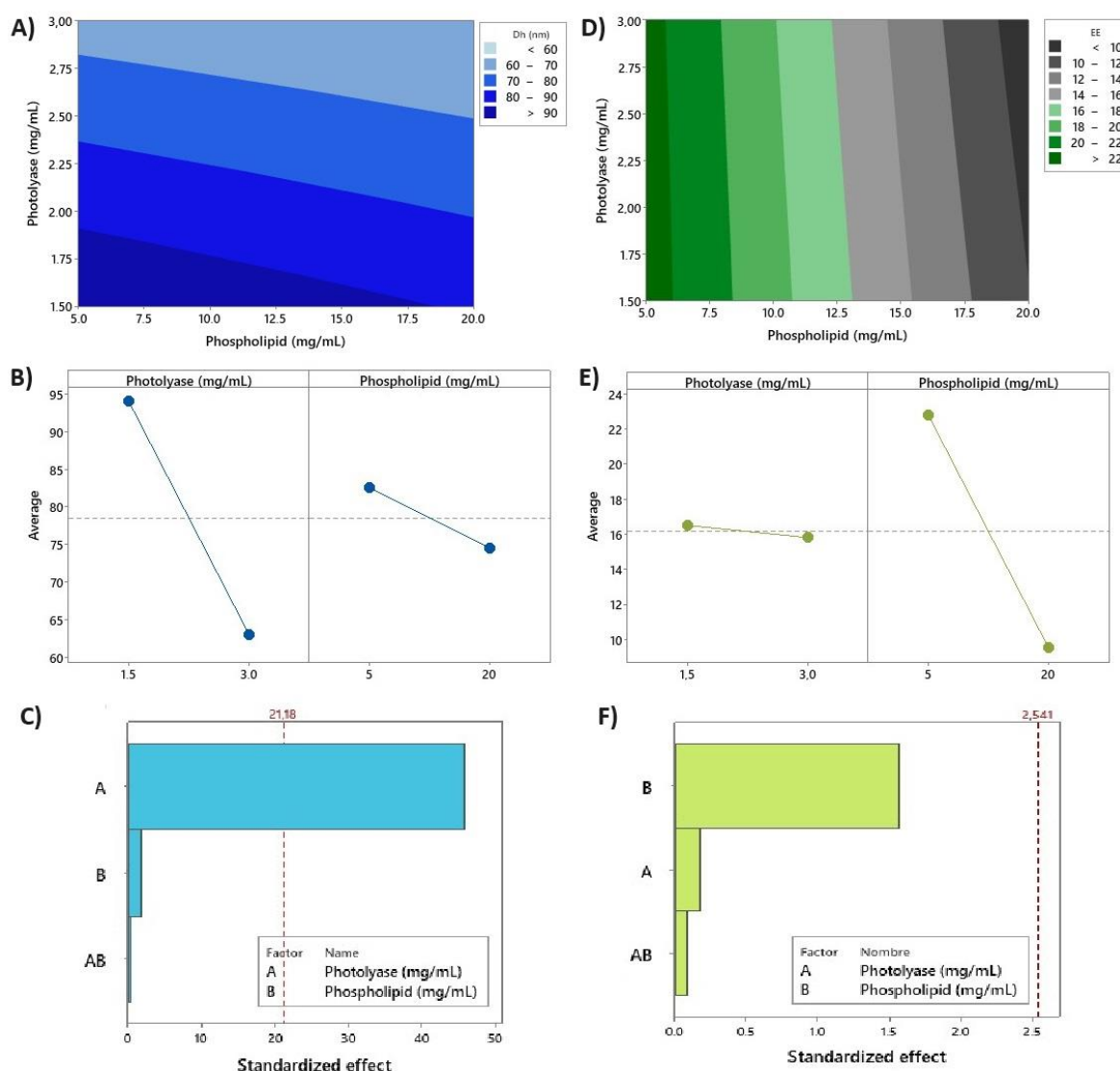


Figure 3. Contour plot, graph of the main effects and pareto chart for the (A-C) hydrodynamic diameter (Dh) and (D-F) encapsulation efficiency (EE) based on the experimental design 2^2 applied to the preparation of photolyase-loaded liposomes.

3.3. Production of polymeric nanoparticles

The production of photolyase-loaded polymeric nanoparticles (NPs) was carried out using a DoE 3^2 as presented in item 2.5. In addition to evaluating the concentration of enzyme and polymer, agitation during emulsification was also tested. The results are presented in Table 6 and Figure 4.

The Pareto graph of standardized effects (Figure 4C) indicated that only the emulsion conditions were a significant factor for. Effectively, we observe that less agitation (3000 rpm) resulted in a significant increase in Dh, with structures even outside the nanoscale range. Higher stirring speed (7000 rpm), on the other hand, resulted in smaller structures with Dh between 86 and 184 nm, depending on the polymer concentration.

Table 6. Results of the experimental design 2³ applied to the preparation of photolyase-loaded polymeric nanoparticle. Dh: hydrodynamic diameter; EE: encapsulation efficiency. The error values correspond to the standard deviation.

Photolyase (mg/mL)	Polycaprolactone (mg)	Emulsion condition (rpm/min)	Dh (nm)	EE (%)
1.5	100	3000/2	678 ± 25	94 ± 0.1
1.5	100	7000/6	86 ± 4	88 ± 1.6
1.5	1000	3000/2	1230 ± 26	89 ± 0.8
1.5	1000	7000/6	184 ± 8	85 ± 1.2
3.0	100	3000/2	642 ± 29	96 ± 0.02
3.0	100	7000/6	99 ± 3	93 ± 0.04
3.0	1000	3000/2	1310 ± 28	96 ± 0.4
3.0	1000	7000/6	179 ± 7	89 ± 0.04

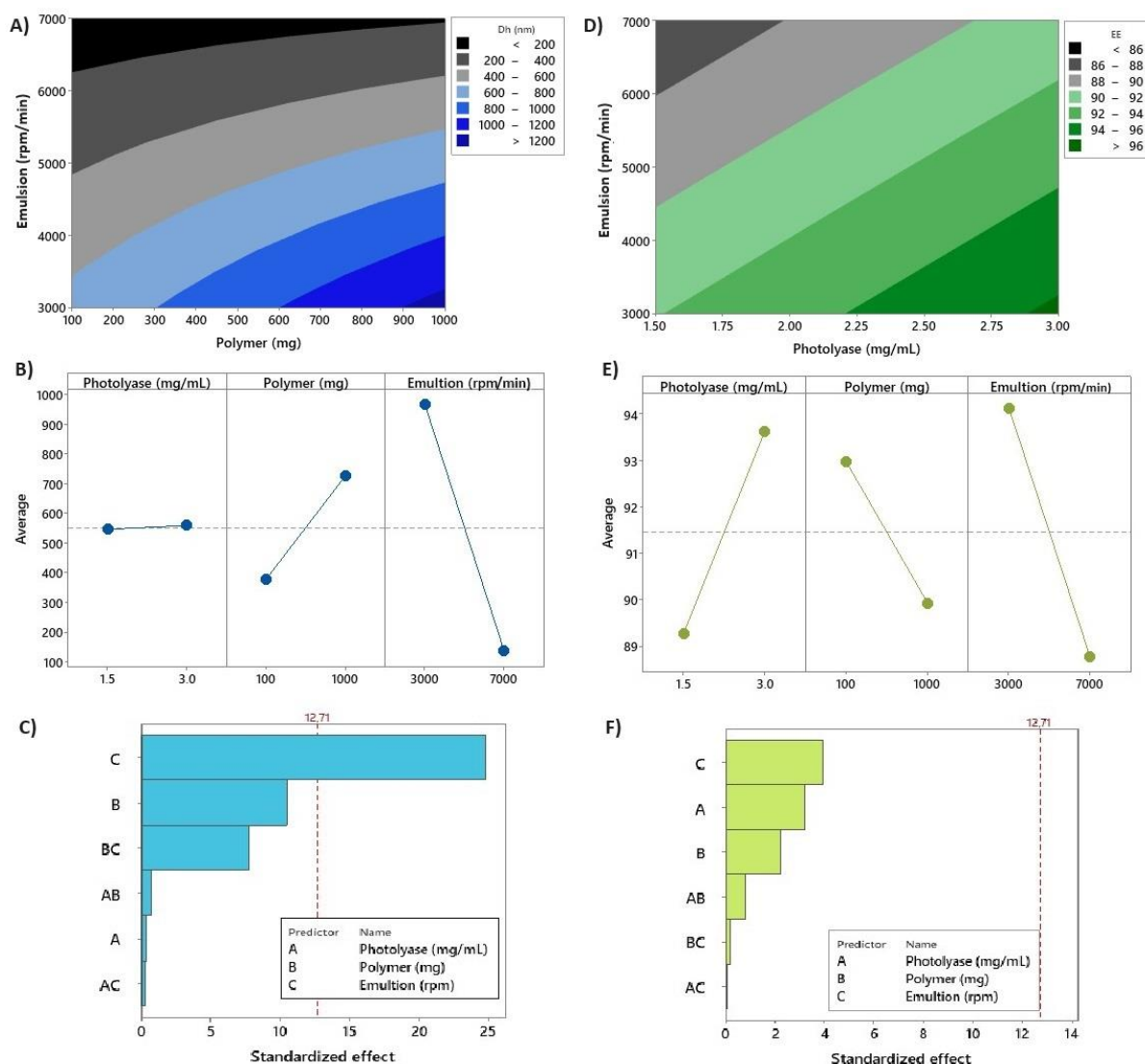


Figure 4. Contour plot, graph of the main effects and pareto chart for the (A-C) hydrodynamic diameter (Dh) and (D-F) encapsulation efficiency (EE) based on the experimental design 2^3 applied to the preparation of photolyase-loaded polymeric nanoparticle.

Encapsulation efficiency values ranged from 85% to 96%, with no significant differences according to Tuckey test. In fact, high EE values were expected for the PNP since the total volume of the initial aqueous phase containing the enzyme is incorporated inside the resulting nanoparticles. Similarly, the photolyase concentration did not influence Dh and EE. Thus, the best condition for PNP productions correspond to 7000 rpm/6 min, 3.0 mg/mL of photolyase and 100 mg of polymer.

3.4. *In vitro* release of the encapsulated photolyase

Photolyase release over time was evaluated for PL, LP and PNP produced at the optimal condition of each nanostructure. The results revealed an initial burst release of photolyase from all nanostructures followed by a slow and linear release (Figure 5). We can probably attribute it to a certain amount of enzyme adsorbed at the surface of the nanostructures. In fact, the burst release is faster and more evident for the polymeric nanostructures. In the case of the PL, the photolyase might be entangled in the PEG net of the polymersomes corona, what justifies the fast release (Musso-Cachumba et al., 2023). For the PNP, we believe the enzyme ins partially accommodate in the polymeric net and can easily diffuse from the nanostructures. Nonetheless, considering a topical application, a delay in release would be detrimental to the effect of the enzyme on the skin.

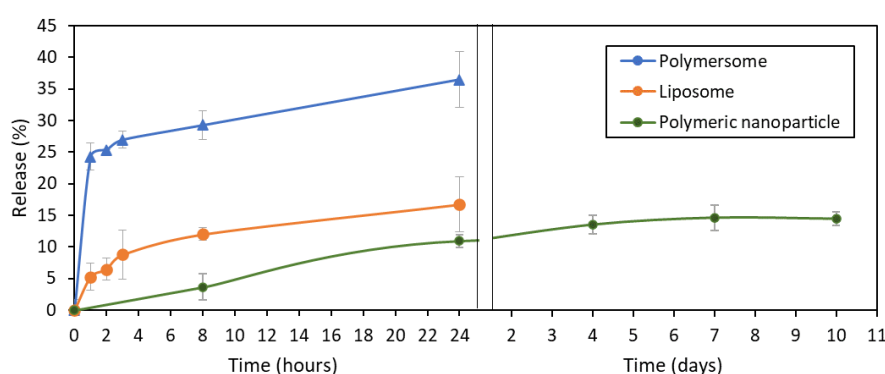


Figure 5. Results of photolyase release to the external aqueous phase over time, for the photolyase encapsulated in polymersomes (PL), liposomes (LP) and polymeric polycaprolactone nanoparticles (PNP). Error bars represent standard deviation.

On the other hand, we observe that after 24 hours PL resulted in twice the percentage of release (37%) than LP (17%) and three times more compared to the PNP (11%), demonstration that poloxamer 401 PS are more permeable to release photolyase to external phase and probably more adequate for topical enzyme application.

3.5. *In vitro* cytotoxicity of photolyase and nanostructures

Biocompatibility stands as a paramount parameter in nanotechnological applications, alongside encapsulation efficiency (EE) and optimal particle size. It indicates the capacity of a material to produce an appropriate host response under defined conditions. In this study, biocompatibility assessment involved *in vitro* evaluation of cytotoxicity in human keratinocyte cells (HaCaT), quantified through cell viability by MTS assay.

Initially, we evaluated the cytotoxicity of the pure photolyase and 100% cell viability was observed for concentrations up to 50 $\mu\text{g/mL}$ and about 75% for 100 $\mu\text{g/mL}$ (Figure 6A). Our previous work (Torres-Obreque et al., 2024) demonstrated the enzyme activity at very low concentrations, with values as low as 10 $\mu\text{g/mL}$ yielding 90% CPD damage recovery. Additionally, qualitative data from other studies indicate DNA repair activity at concentrations as low as 1 $\mu\text{g/mL}$ (Marizcurrena et al., 2019). Based on these insights, it is evident that the enzyme is biocompatible at necessary concentrations for its biological function.

Regarding the nanostructures, diverse outcomes were observed (Figure 6B-D), consistent with expectations from their fabrication using different materials. PL at 25 $\mu\text{g/mL}$ demonstrated a cell viability of at least 86%. Furthermore, PL formulations exhibited no cytotoxicity at concentrations below 12.5 $\mu\text{g/mL}$. As expected, LP formulations were found to be very biocompatible with 100% cell viability at concentrations as high as 5 mg/mL. In contrast, PNP formulations exhibited some cytotoxic effect across all concentrations studied, with maximum cell viability values reaching approximately 90% for concentrations below 12.5 $\mu\text{g/mL}$. According to the IC_{50} value, materials are classified as strongly irritant ($< 7 \mu\text{g/mL}$), slightly irritant (≥ 7 and $\leq 175 \mu\text{g/mL}$) and non-irritant ($> 175 \mu\text{g/mL}$) (Bracher et al., 1987). Based on this classification, PL, LP and PNP were considered non-irritant to HaCaT cells.

The literature report no statistically significant differences when comparing the control with concentrations of 500 $\mu\text{g/mL}$ of LP and also PNP on human endothelial cells (EA.hy926) over time (Atanasio et al., 2020). Moreover, Oliveira et al., (2021) determined IC_{50} values of 83 and 56 $\mu\text{g/mL}$ for poloxamer 401 in NCTC 929 and SIRC cell lines, respectively, measure by the neutral red assay. This difference in IC_{50} can be

explained by the differences in cell lineages and by the dye used in the cytotoxicity assay (MTT or neutral red).

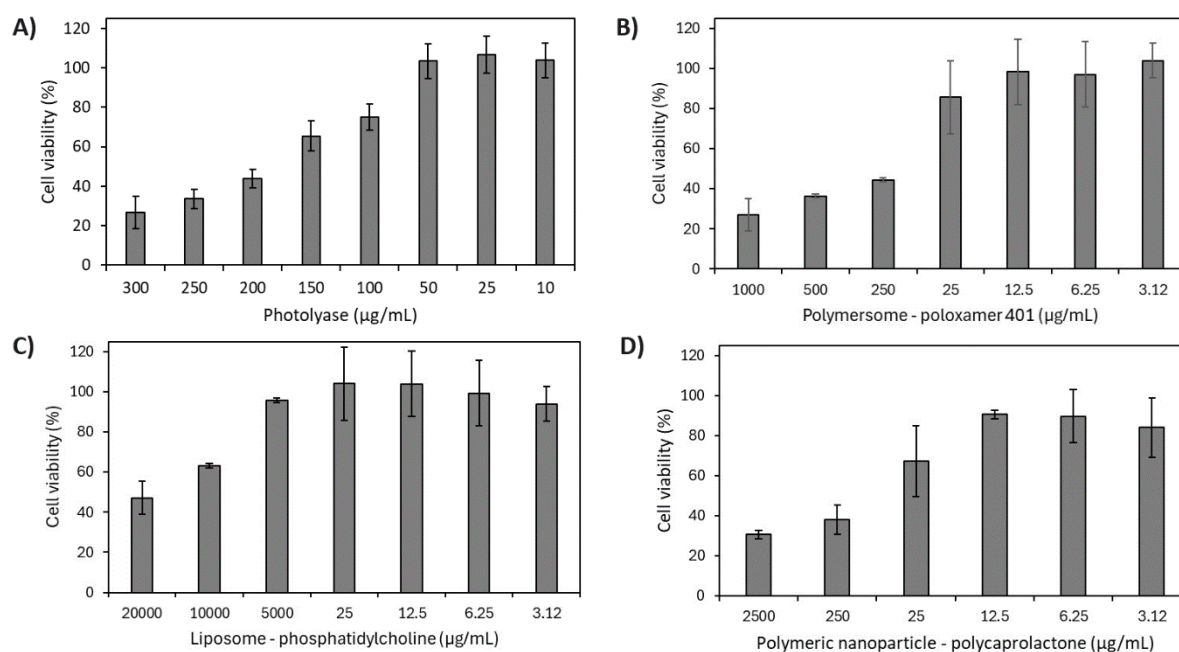


Figure 6. Cellular viability of HaCaT cells (4×10^4 cells/well) after 24h exposition to (A) free photolyase and (B) photolyase-loaded polymersome, (C) photolyase-loaded liposome and (D) photolyase-loaded polymeric nanoparticle. Error bars correspond to standard deviation ($n = 3$).

3.6. *In Vitro* evaluation of nanoencapsulated photolyase in UV exposed HaCaT cells

Prior to the evaluation of photoprotection, we confirmed by Elisa assay that enzyme preserved activity when nanoencapsulated. As can be seen in Figure 7, no significant differences in enzymatic activity were observed before and after each process, suggesting that the established protocols maintain the activity of the enzyme.

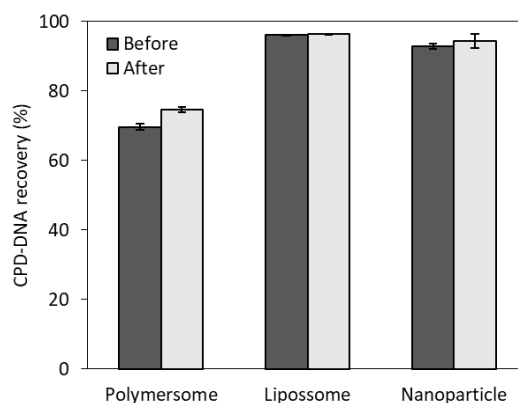


Figure 7. Photolyase activity (%CPD-DNA recovery) by Elisa before and after enzyme nanoencapsulation into polymersomes, liposomes and nanoparticles. Error bars correspond to standard deviation.

Subsequently, the impact of UV radiation and the protective effect of free and encapsulated photolyase in PL and LP formulations were evaluated. The Total Solar Irradiance (TSI) value is equal to 1361 W/m^2 but varies when it reaches the Earth surface since UV radiation is diffused and absorbed by the atmosphere. Therefore TSI can range from approximately 200 to 1000 W/m^2 depending on geographic location, time of day, weather conditions and other factors (Almorox et al., 2021). Here, we do not intend to simulate direct solar radiation at the Earth surface, but rather to elucidate the impact of photolyase activity on cell viability, what could reflect the enzyme capacity of DNA repair.

Our results reveal that HaCaT cell viability was only 28% after exposition to 33 J/m^2 of UV radiation (Figure 8A). In contrast, photolyase at concentrations of 5 and $50 \mu\text{g/mL}$ increased cell survival up to 37% and 47%, respectively. This trend persisted across all radiation intensities examined, nonetheless it was less intense for higher and more damaging UV doses (more than 333 J/m^2). Consequently, it can be inferred that photolyase protects cells from UV-radiation damage.

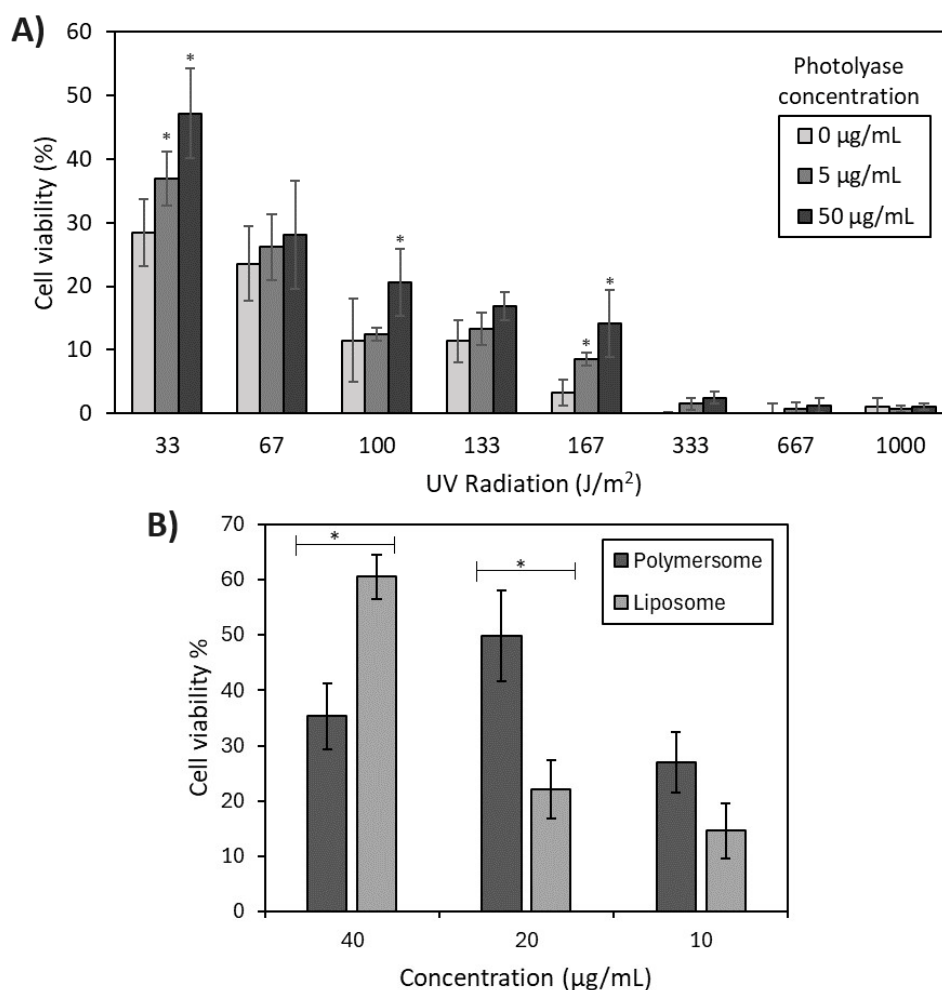


Figure 8. Cell viability of HaCaT cells (4×10^4 cells/well) after exposition to UV irradiation **(A)** at different intensities in the presence of different concentrations of pure photolyase and **(B)** at 33 J/m² and in the presence of photolyase-loaded polymersomes and liposomes at different concentrations. Error bars correspond to standard deviations ($n = 3$). *Significance differences ($p > 0.05$).

Finally, HaCaT cells were incubated with photolyase-loaded PL and LP at different dilutions and UV irradiated with 33 J/m² (Figure 8B). The concentration of 40 µg/mL corresponds approximately to 5 µg/mL of encapsulated photolyase, considering the initial concentration of 3 mg/mL and an approximate EE of 22%. As can be seen, under these condition PL resulted in cell viability (35%) similar to that obtained in the presence of pure photolyase, while LP resulted in approximately 60% cell viability. Therefore, nanostructures preserved or even increased the enzyme effect on UV irradiated cells. At

higher dilutions, PL appears superior, presenting a maximum of 50% cell viability versus 22% for LP when concentration of 20 $\mu\text{g/mL}$ were used. In this context, the photolyase-loaded PL demonstrates superior efficacy in UV radiation protection compared to LP, also outperforming the protective capacity of the pure enzyme. The reduced efficacy observed with higher PL concentrations can be attributed to the cytotoxic effects observed in concentrations exceeding 25 $\mu\text{g/mL}$, as presented above. These cytotoxic effects may counteract the protective capacity of photolyase on cell viability.

Furthermore, it is important to highlight that skin penetration studies were previously carried out by our group. Oliveira et al., (2021) performed cutaneous permeation assays associated with laser scanning confocal fluorescence microscopy and demonstrated that PL increased ten times the percentage of skin penetration. In addition, compared to LP, encapsulation into PL usually offers superior thermal and physicochemical stability and higher resistance to mechanical stresses (Pegoraro et al., 2014).

4. CONCLUSION

We investigated different nanostructures, namely polymersomes, liposomes and polymeric nanoparticles, as strategies for photolyase delivery in the skin. Nanostructures were developed using design of experiments (DoE) approach. Nanometric Dh values and EE values exceeding 23% were obtained for both PL and LP, whereas for PNP EE was higher than 90%. However, enzyme release was lower with PNP, while PL exhibited the best enzyme release profile into the external medium over time, reaching 37% after 24 hours. Concentrations below 50 $\mu\text{g/mL}$ of photolyase, 25 $\mu\text{g/mL}$ of PL, and 5 mg/mL of LP showed no cytotoxicity in HaCaT cells. Finally, both free and encapsulated photolyase in PL and LP were able to protect cells from UV radiation, but PL was shown to be superior. In conclusion, our study demonstrates the potential of photolyase, particularly when encapsulated into PL, to protect the cells of the UV photodamage induced by UV radiation. This new strategy offers a promising and innovative alternative for photolyase incorporation into dermatological products.

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FINAL CONSIDERATION

Our investigation underscores the significant potential of photolyase in mitigating UV-induced DNA damage, a key factor in skin cancer development. The recombinant production and purification of photolyase represented crucial steps to obtain a highly pure photolyase, with a molecular weight of 47 kDa. Moreover, our methodology yielded a greater final productivity of 480 mg of pure photolyase per L of fed-batch culture, highlighting the efficiency of our production process. Our findings reveal that even at low concentrations, photolyase exhibits elevated effectiveness in repairing cyclobutane pyrimidine dimers (CPDs) in DNA. We showed for the first time the Michaelis-Menten constant (k_M) for *T. thermophilus* photolyase, with value similar to reported by the Nobel Prize Sancar (1987) for *E. coli* photolyase. Subsequently, the encapsulation in different nanostructures, including polymersomes, liposomes and polymeric nanoparticles, have resulted in optimizing both the hydrodynamic diameter and encapsulation efficiency of photolyase. Specifically, the presence of sucrose has significantly increased the encapsulation efficiency. Furthermore, our cytotoxicity analysis in HaCaT cells indicate the biocompatibility of all encapsulated formulations, affirming their potential for dermatological applications. Notably, polymersome-encapsulated photolyase has emerged as the most promising candidate, showcasing superior cell protection against UV radiation. Taken together, the results presented in this thesis, demonstrate the extraordinary ability of photolyase, particularly when encapsulated in polymersomes, how and innovative approach to serve as an effective active ingredient in photoprotective skincare formulations.

APPENDIX A: Academic Transcripts

9135 - 8730260 / 2 - Karin Mariana Torres Obreque

Email: ktorres@usp.br
Data de Nascimento: 23/12/1990
Cédula de RNE - V935072-S - DF
Local de Chile
Nacionalidade: Chilena
Graduação: Licenciado en Ciencias de la Ingenieria - Universidad de la Frontera - Chile - 2014
Graduação: Ingeniero Civil en Biotecnologia - Universidad de La Frontera - Chile - 2015
Mestrado: Mestra em Ciências - Área: Tecnologia Químico-Farmacêutica - Faculdade de Ciências Farmacêuticas - Universidade de São Paulo - São Paulo - Brasil - 2017

Curso: Doutorado
Programa: Tecnologia Bioquímico-Farmacêutica
Modalidade: Acadêmica
Área: Tecnologia Químico-Farmacêutica
Data de Matrícula: 22/07/2019
Início da Contagem de Prazo: 22/07/2019
Data Limite para o Depósito: 16/09/2024
Orientador na USP: Prof(a). Dr(a). Carlota de Oliveira Rangel Yagui - 22/07/2019 até o presente Email: corangel@usp.br
Aluno USP em convênio de dupla titulação com instituição estrangeira
Instituição Conveniada: Università degli Studi di Genova, Itália
Orientador na Instituição Conveniada: Patrizia Perego

Proficiência em Línguas:

Inglês, Aprovado em 22/07/2019

Trancamento(s): 60 dias

Período de 08/02/2023 até 08/04/2023

Data de Aprovação no

Exame de Qualificação: Aprovado em 04/03/2022

Data do Depósito do
Trabalho:

Título do Trabalho:

Data Máxima para
Aprovação da Banca:

Data de Aprovação da
Banca:

Data Máxima para
Defesa:

Data da Defesa:

Resultado da Defesa:

Histórico de

Ocorrências:

Primeira Matrícula em 22/07/2019

Trancado em 08/02/2023

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 7493 em vigor a partir de 29/03/2018).

Última ocorrência:

Matrícula de Acompanhamento em 29/01/2024

Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação	Pres.
BTC5700-9/4	Economia de Projetos Biotecnológicos (Curso Interunidades: Biotecnologia - Universidade de São Paulo)	02/09/2019	10/11/2019	90	6	75	A	N	Concluída	S
PGF5357-1/1	Técnicas de Caracterização de Sistemas de Relevância Biológica (Instituto de Física - Universidade de São Paulo)	11/10/2019	17/10/2019	15	1	100	A	N	Concluída	S
BMF5881-1/2	Medicina Personalizada: Contribuições da Farmacogenômica e da Nanotecnologia	04/11/2019	15/12/2019	90	0	-	-	N	Matrícula cancelada	S

Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação	Pres.
	(Instituto de Ciências Biomédicas - Universidade de São Paulo)									
FBT5700-4/1	Preparo de Artigos Científicos na Área de Tecnologia Bioquímico-Farmacêutica	03/02/2020	05/04/2020	90	6	90	A	N	Concluída	S
BTC5721-5/4	Tópicos Avançados em Química de Proteínas e Peptídeos para Aplicação Biotecnológica (Curso Interunidades: Biotecnologia - Universidade de São Paulo)	31/03/2020	11/05/2020	90	0	-	-	N	Turma cancelada	S
FBT5728-1/3	Inglês Científico - Pronúncia e Apresentação Oral TOX036	13/04/2020	26/04/2020	30	0	-	-	N	Turma cancelada	S
Crédito Externo	Enzimologia básica e aplicações (1)	11/05/2020	22/05/2020	-	4	90	T	-	-	S
BTC5721-5/5	Tópicos Avançados em Química de Proteínas e Peptídeos para Aplicação Biotecnológica (Curso Interunidades: Biotecnologia - Universidade de São Paulo)	12/05/2020	22/06/2020	90	6	100	A	N	Concluída	S
FBT5749-1/1	Quality-by-Design: aplicação na produção de biofármacos	15/06/2020	28/06/2020	30	2	100	A	N	Concluída	S
QBQ5904-1/1	Proteômica Baseada em Espectrometria de Massas (Instituto de Química - Universidade de São Paulo)	22/04/2021	02/06/2021	30	2	100	A	N	Concluída	S
FBT5773-9/3	Tópicos Especiais em Tecnologia Bioquímico-Farmacêutica I	02/08/2021	10/10/2021	30	2	100	A	N	Concluída	N

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito de tese (Totais/Presenciais)	Totais/Presenciais
Disciplinas:	0	20 / 12	29 / 29*
Estágios:			
Total:	0	20 / 12	29 / 29*

Créditos Atribuídos à Tese: 172

*Créditos obtidos anteriormente a 01/01/2024 serão computados como presenciais de acordo com a Resolução CoPGr 8546, de

Observações:

1) Disciplinas(s) cursada(s) na(o) Instituto Butantan. Atribuição de créditos aprovada pelo(a) Comissão de Pós-Graduação em Sessão de 03/08/2020

Conceito a partir de 02/01/1997:

A - Excelente, com direito a crédito; B - Bom, com direito a crédito; C - Regular, com direito a crédito; R - Reprovado; T - Transferência.

Um(1) crédito equivale a 15 horas de atividade programada.



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APPENDIX B: Scientific Publications

1. **Torres-Obreque, KM**; Gonçalves, F; Ferraro, R; Fuentes-León, F; Menck, CF; Costa-Silva, TA; Monteiro, G; Perego, P; Rangel-Yagui, CO. Recombinant production of a highly efficient photolyase from *Thermus thermophilus*. *Biotechnology Journal* (2024). <https://doi.org/10.1002/biot.202300325>
2. Santos do Nascimento, N; **Torres-Obreque, KM**; Oliveira, C; Rabelo, J; Baby, A; Long, PF.; Young, A; Rangel-Yagui, CO. Enzymes for dermatological use. *Experimental Dermatology* (2024). <https://doi.org/10.1111/exd.15008>
3. **Torres-Obreque, KM**; Kleingesinds, EK; Santos, JHPM; Carretero, G. et al. PEGylation versus glycosylation: effect on the thermodynamics and thermostability of crisantaspase. *Preparative Biochemistry & Biotechnology* (2023) <https://doi.org/10.1080/10826068.2023.2249100>
4. **Torres-Obreque, KM**; Meneguetti, GP; Muso-Cachumba, JJ. et al. Building better biobetters: From fundamentals to industrial application. *Drug Discovery Today* 27 (1) (2022). <https://doi.org/10.1016/j.drudis.2021.08.009>
5. de Souza Guimarães, M; Cachumba, JJM; Bueno, CZ; **Torres-Obreque, KM**. et al. Peg-grafted liposomes for L-asparaginase encapsulation. *Pharmaceutics* 14 (9), (2022). <https://doi.org/10.3390/pharmaceutics14091819>
6. Sotomayor-Gerding D, Troncoso JM, Díaz-Riquelme K, **Torres-Obreque KM**, Cumilaf J, Yañez AJ, Rubilar M. Microencapsulation of *Piscirickettsia salmonis* Antigens for Fish Oral Immunization: Optimization and Stability Studies. *Polymers* (2022). <https://doi.org/10.3390/polym14235115>
7. Lima, GM; Atrazhev, A; Sarkar, S; Sojitra, M; Reddy, R; **Torres-Obreque, KM**. et al. DNA-Encoded Multivalent Display of Chemically Modified Protein Tetramers on Phage: Synthesis and in Vivo Applications. *ACS Chemical Biology* 17 (11), (2021). <https://doi.org/10.1021/acscchembio.1c00835>