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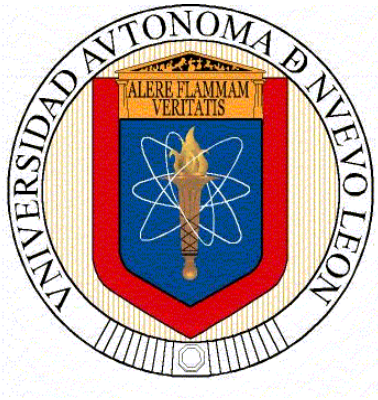

TESIS

CHARACTERIZATION OF A NOVEL ENZYME ISOLATED FROM *Bacillus cereus* 6P RESPONSIBLE FOR GLYPHOSATE BIODEGRADATION BY AN ALTERNATIVE PATHWAY TO C-P LYASE

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ELENA GUADALUPE VERDUZCO SUÁREZ

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CHARACTERIZATION OF A NOVEL ENZYME ISOLATED FROM *Bacillus cereus* 6P RESPONSIBLE FOR GLYPHOSATE BIODEGRADATION BY AN ALTERNATIVE PATHWAY TO C-P LYASE

by

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Characterization of a novel enzyme isolated from *Bacillus cereus* 6P responsible for glyphosate biodegradation by an alternative pathway to C-P lyase.

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SUMMARY

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Characterization of a novel enzyme isolated from *Bacillus cereus* 6P responsible for glyphosate biodegradation by an alternative pathway to C-P lyase.

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Candidate for the degree of Master of Science Major in Applied Microbiology

Study area: Applied microbiology.

Aim and Method of study: To demonstrate that an intracellular enzyme isolated from *Bacillus cereus* 6P can biodegrade glyphosate, cleaving the C-P bond by an alternative pathway to C-P lyase independent of the phosphate level in the environment.

Conclusions: A semi-purified cytoplasmatic enzyme from *B. cereus* 6P could biodegrade glyphosate by an alternative pathway to C-P lyase, independent of phosphate levels in the milieu.

_____

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To my beloved Aracely, for giving me all her faith, even when I was lost.

DEDICATION

To my fiancé, Aracely, for my first (and probably last) thesis.

I love you.

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SYMBOLS AND ABBREVIATIONS

AMPA	Aminomethylphosphonic acid
BSA	Bovine Serum Albumin
FAD	Flavin Adenine Dinucleotide
GOX	Glyphosate Oxidoreductase
GP	Glyphosate
LB	Luria Bertani
MMM	Minimal Mineral Medium
MPn	Methylphosphonate
MWCO	Molecular Weight Cut-Off
OD	Optical Density
rpm	Revolutions per minute
t	Tonnes

CHAPTER 1

INTRODUCTION AND JUSTIFICATION

1.1 Introduction

Agriculture has been one of the most essential activities of humankind throughout history. The current agriculture model relies on the intensive use of fertilizers, insecticides, fungicides, and herbicides (Sosa et al., 2019). Since the middle of the twentieth century, the use of herbicides for weed control has played an important role in improving crop productivity (Clapp, 2021). In this context, during the 1970s, Monsanto introduced the use of glyphosate (GP), an organophosphorus molecule (Székács & Darvas, 2012), which has become the most intensively used herbicide around the globe since the introduction of GP-tolerant crops in the nineties (Benbrook, 2016; Gillezeau et al., 2019; Székács & Darvas, 2012). GP belongs to a class of compounds called phosphonates, characterized by a carbon-to-phosphorus (C-P) bond that provides the molecule with relatively high degradation resistance (Schowanek & Verstraete, 1990).

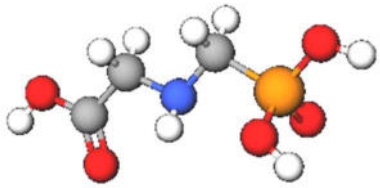

Figure 1. *Chemical structure of glyphosate (in grey: carbon, white: hydrogen, red: oxygen, blue: nitrogen, orange: phosphorous).*

After the expiration of Monsanto´s patent in 2000, several companies began to develop generic products at a lower cost, with China as the biggest producer and exporter of GP. China alone can produce enough GP to satisfy the global demand even if all other manufacturers cease production (Bravo & Naranjo, 2016; Székács & Darvas, 2012).

Initially, GP was presented as a benevolent product that will not persist in soil and will only affect plants, as it inhibits the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzyme found in the shikimic acid pathway that governs essential metabolic processes in plants, fungi, and some bacteria (Myers et al., 2016; Werner et al., 2021). GP was considered harmless to humans because of the absence of this pathway in mammals (Werner et al., 2021). However, recent studies have shown the possible long-term low-level effects of GP in humans as a carcinogenic, endocrine disruptor, or neurotoxic substance (Peillex & Pelletier, 2020; Richmond, 2018). In this sense, the International Agency for Research on Cancer (IARC) from the World Health Organization (WHO) has recognized GP as part of Group 2A, substances considered “probably carcinogenic to humans” after the revision of several publicly available studies (Bravo & Naranjo, 2016; IARC, 2017). After the resolution from IARC, the opinions have been divided, with agribusiness declaring opposition to IARC’s conclusions (Gillezeau et al., 2019; Richmond, 2018). IARC also informed that all decisions respecting legislation or regulation about GP are the responsibility of each agency and government (Richmond, 2018). Thus, governments around the world have

taken restrictive actions to reduce GP consumption to protect their populations (Bravo & Naranjo, 2016).

The Mexican government adopted a total ban on GP to control these adverse effects. An official decree was initially published on December 31, 2020, declaring that Mexico will gradually avoid all use, distribution, and importation of GP, achieving a total transition from GP to ecological alternatives by 2024; simultaneously, the use of genetically modified (GM) maize was prohibited (Alcántara-de la Cruz et al., 2021; DOF, 2020). After several economic and political issues between the US and Mexican governments, a new decree was published in February 2023, where the ban on GP was maintained. Still, GM maize was regulated for industrial use and prohibited for human consumption (DOF, 2023). The Consejo Nacional de Humanidades, Ciencia y Tecnología (CONAHCyT), published every year, after the decree, the recommendation for the importation of GP, having for 2022: a maximum of 8 263.1 t of formulated GP and a maximum of 628.6 t of technical GP, a 50% reduction compared with 2021 (CONAHCyT, 2022).

Despite governments' efforts, GP's presence is widespread in different ecosystems. This extensive distribution indistinctly affects humans, animals, non-target plants, and microorganisms. It has been reported that GP reduces the microbial richness of the soil, affecting plants also by increasing phytopathogen populations. Other examples are the alteration of trophic chains due to behavioral and reproductive changes in organisms like earthworms and honeybees, the surge of resistant weeds, and the toxic effects of GP in marine organisms and

ecosystems (Richmond, 2018). GP has been detected in diverse matrixes such as soil, foods, surface, and groundwater, even in human fluids such as blood, urine, and breast milk. As a result, it has portrayed a warning about the exposition that the planet has suffered because of the intensive use of this herbicide and urges to evaluate levels of GP in the population and ecosystems regularly (Benbrook, 2016; Gillezeau et al., 2019; Lozano-Kasten et al., 2021; Rendón-Von Osten & Dzul-Caamal, 2017). Urinary levels of GP in humans show values of 0.26–73.5 μg/L in exposed workers and 0.16–7.6 μg/L in the general population, reinforcing the evidence of the ubiquitous presence of GP. Nevertheless, standardized studies are still required to confirm this data (Gillezeau et al., 2019).

To date, there is no standardized process to remediate the environmental effects of GP. It is, therefore, necessary to take advantage of the biological resources available for their elimination. In this sense, it is known that in bacteria, there are two major pathways for the biodegradation of GP: (1) C-P lyase (E.C. 4.7.1.1) complex, and (2) GP oxidoreductase (GOX, E.C. 1.5.3.23) pathway. C-P lyase is rarely active because it requires a state of inorganic phosphate (Pi) starvation, and the GOX pathway, even when high Pi levels do not hinder it, produces aminomethylphosphonic acid (AMPA) provoking secondary contamination (Singh et al., 2020). Recent studies from our laboratory revealed the existence of a new enzyme independent of Pi concentration that does not produce AMPA as a contaminant by-product. Therefore, it has shown a real potential to be used in the bioremediation of GP.

1.2 Project Justification

Considering the pollution caused by the intensive use of GP in the past decades and the absence of any process for GP elimination, the urgent need to offer options for environmental remediation to reduce the presence of this herbicide in ecosystems is undoubtedly reinforced.

Although there is research on the biodegradation of GP using microorganisms, it is notorious that its application *in situ* is still complex due to the factors involved in microbial control, mainly due to high levels of phosphate in the environment. This project will allow us to isolate a new enzyme of bacterial origin that can biodegrade GP by an alternative pathway to C-P lyase, independent of phosphate levels, which could offer a bioremediation tool with more practical application.

CHAPTER 2

LITERATURE REVIEW

2.1 Phosphonates

Phosphoric acids or phosphonates are organophosphorus compounds characterized by the presence of the C-P bond, which gives the molecule stability and resistance against hydrolysis, whilst the phosphonyl moiety mimics several chemical states of enzymatic by-products (Engel, 1977; Horsman & Zechel, 2017). The phosphonate portion also provides bioactivity; therefore, the use of phosphonates is extended in the industry as antibiotics (White & Metcalf, 2007), lubricants, flame extinguishers (Sviridov et al., 2011), herbicides or chelates (Nowack, 2003). The same moiety serves as a source of Pi for several microorganisms that have developed the molecular machinery to cleavage the C-P bond (Horsman & Zechel, 2017). Phosphonates have natural and anthropogenic origins, with GP as an example of a synthetic phosphonate (Nowack, 2003).

2.2 Glyphosate in Mexico

Agriculture has been closely related to the development of Mexico since before the Spanish colonization, and nowadays, it represents a significant input to the country's economic sector (SADER, 2023). With more than 43 thousand T

of pesticide use reported for the FAOSTAT, Mexico has the highest pesticide use in Central America, with herbicides as the second most used after fungicides and bactericides (FAO, 2023). However, there is no information about the active ingredients used per category, which complicates the analysis of the actual impacts of such usage.

Even without exact numbers on the use of GP, it is known that the majority corresponds to unjustified use in crops that are not GP-resistant and the illegal distribution of GP-resistant crops in the country (Alcántara-de la Cruz et al., 2021). Since 2020, the Mexican government applied actions to achieve a total ban on GP use and commercialization by March 2024 (DOF, 2023). Since then, several agencies implied in the federal administration have applied efforts to find agroecological alternatives to the use of GP (González-Moscoso et al., 2023). However, various concerns are presented yet to control the use and distribution of herbicides and pesticides in the national territory, primarily due to long-standing issues such as the decentralization of the legal framework for pesticide regulation (Albert, 2019) and smuggling (OECD, 2021). Also, the general disinformation of the population and underestimation of the risks play an essential role in the routine use of these substances.

Despite the conscious actions taken by the Mexican government to decrease the exposure of the population to GP, the system indeed lacks certainty to assert that the objective is being achieved in its entirety; also, the effects of GP will not disappear as swiftly as desired when the total ban on the substance comes into force in 2024. Considering the extensive distribution of GP reported in diverse

matrixes, including food for human consumption, it is vital to ensure actions from every sector (academic, scientific, medical, governmental) oriented to mitigate or eradicate the long-term affectations of GP as the one proposed in this study.

2.3 Microbial Glyphosate Biodegradation

The pure compound GP presents low toxicity both orally and dermally, but commercial GP formulations make the mechanisms of toxicity complex (Bradberry et al., 2007). As mentioned before, the intensive and neglected use of GP has rendered a ubiquitous presence of the herbicide in the environment. Hence, it is necessary to intervene to solve the adverse effects of GP and its metabolites; a viable option is using microbial enzymes to biodegrade GP.

In the case of bacteria, the most extended active pathway for GP biodegradation is the GOX pathway, where GP cleaves at the C-N bond, producing glyoxylic acid and AMPA (Figure 2). GOX is an enzyme usually independent of the Pi levels but dependent on FAD as a cofactor (Habte & Beyene, 2020). Most of the time, AMPA is not mineralized for the bacteria and is excreted into the environment. When AMPA is further metabolized in bacteria, the enzyme responsible for the transformation is C-P lyase (Singh et al., 2020; Sviridov et al., 2015); AMPA exhibits a higher toxicity and half-life time in soil than GP itself [up to 958 days and up to 197 days, respectively (Ojelade et al., 2022; Tresnakova et al., 2021)]. Also, it has been demonstrated that GP and AMPA can be bioaccumulated in biofilms in concentrations 2 to 4 orders of magnitude higher

than the surrounding water in systems like wetlands, promoting the availability of these pollutants to the trophic chains (Beecraft & Rooney, 2021).

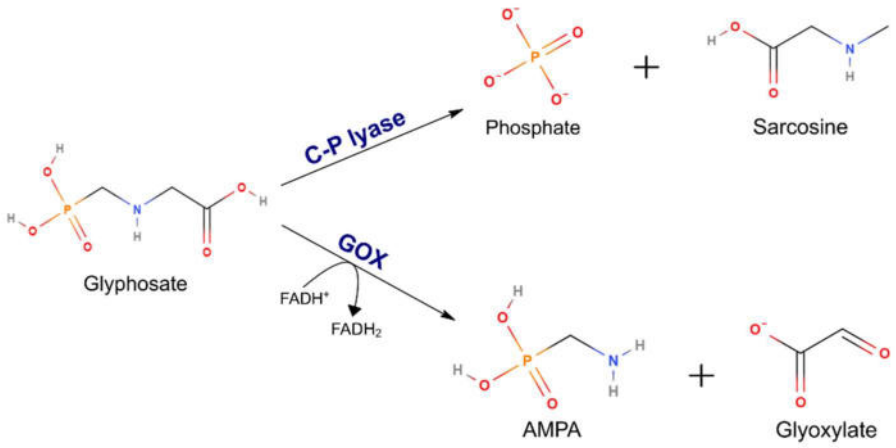

Figure 2. *Bacterial pathways for glyphosate biodegradation.*

The second major pathway is the C-P lyase complex, in which the C-P bond is cleaved, generating sarcosine and Pi (Figure 2). However, this pathway is active only when Pi concentration is <4 μM, a rare condition to be found in nature; also, its activity has been reported to be associated with the cell membrane and is not highly specific for GP (Singh et al., 2020).

2.3.1 C-P Lyase Isolation

Evidence about the mechanism for the C-P cleavage in a variety of phosphonates (including the positive biodegradation of GP) was provided by Wackett et al. (1987), using the *Agrobacterium radiobacter* strain as a model. Subsequent studies showed that the enzymatic activity of C-P lyase was lost upon

the destruction of the cell membrane, so it was deduced that it is a membrane-associated enzyme (Stosiek et al., 2019).

Since then, multiple studies have demonstrated the ability of C-P lyase to metabolize natural and synthetic phosphonates, including GP. The most studied C-P lyase comes from *E. coli,* known to be the product of 14 operon genes [*phnCDEFGHIJKLMNOP* (Singh et al., 2020)]. In 1990, it was demonstrated that the expression of this complex was independent of earlier exposure to phosphonates and confirmed the correlation between the presence of C-P lyase activity and low Pi levels available for the microorganisms (Schowanek & Verstraete, 1990). Among the species that could consume GP were *Arthrobacter* sp. GLP-1, *Pseudomonas* sp. G2982 and *Alcaligenes eutrophus*.

Selvapandiyan and Bhatnagar (1994) reported partially purifying a C-P lyase enzyme from a *Pseudomonas* sp. GLC11, cultured in a GP concentration of up to 125 mM. The molecular mass of the enzyme was estimated at *ca.* 200 kDa and presented activity for GP and AMPA. The enzyme presented an unstable activity, and stabilizing it was impossible. This portrayed an example of the activity loss due to the complex C-P lyase forming part of the cell membrane.

In 2013, Kamat *et al*. elucidated for the first time the crucial role of PhnJ as the catalytic center of a C-P lyase from E. coli using methyl phosphonate as a substrate (Kamat et al., 2013). Later, Selvi and Manonmani (2015) published the purification and characterization of an enzyme from *Pseudomonas putida* T5 supplied with 10 ppm of GP for 72 h. The biodegradation of GP was confirmed through sarcosine production using the purified enzyme. Conditions for the

enzymatic activity used 50 mM of phosphate buffer (pH 7.0) and 60 μM of GP. The molecular mass of the purified enzyme was a monomer of 70 kDa, and its optimal pH and temperature were around 7.0 and 30 °C, respectively. The loss in enzyme activity was presented with an increase in temperature. This would represent the first report of a pure and active C-P lyase against GP.

2.3.2 GOX Reports

Despite all the studies on microorganisms demonstrating C-P lyase activity on GP, no biotechnological process has been developed. This may be due to the negative effect of phosphate on the enzymatic activity. Nevertheless, some microorganisms possess unique alternative metabolic pathways for GP metabolization.

Since the 1980s, several studies have deepened their knowledge of the GOX pathway, referring to microorganisms capable of biodegrading GP while producing AMPA. For example, Sviridov et al., (2012) studied the phosphonate catabolism in *Ochrobactrum anthropi* GPK 3 and reported the ability of this strain to degrade GP via C-P lyase and a novel enzyme called GOX. This phenomenon was also present in *Bacillus cereus* CB4 (Fan et al., 2012). On the other hand, some authors have reported the use of the GOX pathway for GP degradation in several strains, i.e., *Providencia rettgeri,* which can tolerate high concentrations of GP (>120,000 mg/L), showing a 71.4% degradation in 24 h at 10,000 mg/L GP (Xu et al., 2019); and *Ochrobactrum* sp. GDOS can degrade GP in the presence

of phosphate but releases AMPA to the supernatant (Hadi et al., 2013). Another study reported that *Bacillus aryabhattai* FACU3 could grow in concentrations of GP up to 250 mg/mL and expressed a FAD-dependent GOX-like protein (Elarabi et al., 2020). All these studies show the potential of several strains to be applied for the bioremediation of highly GP-contaminated matrixes but with the unfortunate release of AMPA.

2.3.3 Enzyme Prospects

New studies from our research group have demonstrated the existence of a novel enzyme that can degrade GP without releasing AMPA. Acosta-Cortés et al. (2019) published the capacity of the *Bacillus cereus* 6P strain native to Nuevo León to degrade GP by cleavage of the C-P bond and use the Pi as a source for the accumulation of intracellular polyphosphate (polyP) and even release Pi into the medium reaching concentrations higher than 30 µM. The later evidence opposes the typical mechanism of C-P lyase. A BLAST analysis did not find C-P lyase homology, suggesting the existence of another enzyme able to catabolize GP by C-P scission independently of Pi levels. The authors also propose developing a biotechnological process with economic and environmental connotations that allow the association of bioremediation of ecosystems with the production of polyP that could serve as a renewable source of phosphorus.

Additionally, Martínez Ledezma (2019) reported the ability of the same strain to biodegrade GP by an alternative pathway to C-P lyase independent of Pi levels. He probed the absence of C-P lyase's genetic catalytic component (*phnJ*)

by molecular analysis, which would theoretically make it impossible for this strain to degrade the herbicide by C-P cleavage. The study also confirmed the capacity of the strain to accumulate polyP when using GP as the sole phosphorus source, reaffirming the enzyme's ability to attack the C-P bond of GP. Acute toxicity bioassays in *E. putida* exposed to the exhausted culture medium showed no affectations to the worms and increased offspring compared to the control, as a correlation to the production of non-harmful by-products from the enzymatic activity on GP contrary to the AMPA production of GOX. Finally, the enzyme was reported to be in the cell's cytoplasm, which also differs from the typical C-P lyase complex attached to the membrane, suggesting that further isolation and characterization of the enzyme can be addressed.

Every pathway for GP biodegradation has significant ecological and biotechnological implications. Research in these areas continues to advance our understanding of phosphorus cycling and the metabolism of organophosphorus compounds like GP, with potential applications in industry, agriculture, bioremediation, and environmental science.

CHAPTER 3

HYPOTHESIS AND OBJECTIVES

3.1 Hypothesis

A cytoplasmic enzyme isolated from *Bacillus cereus* 6P can biodegrade glyphosate.

3.2 General Objective

This study aims to identify and characterize an enzyme from *Bacillus cereus* 6P responsible for glyphosate's biodegradation by an alternative pathway to C-P lyase, independent of phosphate levels in the environment.

3.3 Specific Objectives

1. To reactivate the strain *Bacillus cereus* 6P.
2. To produce and semi-purify the enzyme responsible for glyphosate biodegradation.
3. To evaluate the enzymatic reaction conditions (such as temperature and pH) for glyphosate biodegradation by the cell-free extracts.

CHAPTER 4

METHODOLOGY

This project was executed in the Laboratorio de Procesos Microbiológicos in the Centro de Investigación en Biotecnología y Nanotecnología (CIBYN). The laboratory provided all the necessary materials, reagents, and equipment.

4.1 Strain Reactivation and Preservation

Bacillus cereus 6P strain was obtained from the Laboratorio de Procesos Microbiológicos in CIBYN. Its reactivation was performed by culture in non-selective nutrient media, such as LB broth or nutrient broth. These were incubated in aerobic conditions for 24 h at 28°C with constant agitation of 150 rpm. For long-term preservation, 400 µL of the *B. cereus* 6P culture was added to a polypropylene microtube, and 600 µL of sterile 50% glycerol solution was added immediately; the solution was homogenized by hand and stored at -20°C until further use.

4.2 Production of the Enzyme

4.2.1 Growth Kinetics of the Strain

Knowing the capacity of *B. cereus* 6P to biodegrade GP, the behaviour of the strain at standard and stressful nutrient conditions was determined. Growth kinetic in LB broth and MMM (1 mM GP as the sole P source) were assayed. An initial 0.05 OD inoculum (620 nm) was incubated for 72 h for LB broth and 120 h for MMM at 28 °C and constant agitation of 150 rpm. Samples of 1 mL of culture were taken, and its OD at 620 nm was registered. For LB medium, samples were taken each 2 h for the first 18 h and approximately each 8 h afterwards. For MMM, samples were taken each 2 h for the first 12 h and then each 8-16 h. Values of time vs OD were graphed and compared. Assays were run in triplicate.

4.2.1.1 Nile Red Staining to Determine the Production of PHA.

To confirm the presence of nutritional stress in the bacterial cells, detection tests for polyhydroxyalkanoates (PHA) were performed. This was done by Nile red staining of 1 mL of sample, which was centrifuged for 5 minutes at 13 000 rpm. The supernatant was discarded, and 30 µL of 1% Nile red solution was added. The mixture was incubated for 10 min in a 55°C water bath. After this time, the pellet was washed to remove excess dye and resuspended in saline for smearing. This was observed under a DM-3000 fluorescence microscope (Leica Microsystems) using a Texas red filter (excitation λ= 553 nm, and emission λ=636 nm).

4.2.2 Culture Conditions to Produce the Enzyme

Modified growth conditions proposed by Martínez Ledezma (2019) were used. The initial inoculum consisted of MMM-washed cells from an initial 100 mL LB broth culture in the *log* phase. Cells were added in MMM containing 10 mM sodium acetate ($C_2H_3NaO_2$) as a carbon source, 4 mM ammonium chloride (NH_4Cl) as a nitrogen source, and 1 mM GP as the sole phosphorus source. The culture was incubated at 28 °C and 150 rpm for at least 120 h.

4.2.3 Obtention of the Enzyme

Cell-free extract. Cells were separated from the MMM broth by centrifugation at 7830 rpm, at 4 °C for 10 min. The supernatant was discarded, and cells were washed twice in 50 mM Tris-HCl (pH 7). Cells were resuspended in 50 mM Tris-HCl (pH 7) and sonicated in a Fisher Scientific FB120 sonicator at 80 % amplitude for 15 min (30 seconds pulse, 60 seconds break), always maintaining an ice bath. The homogenate was centrifuged for 10 min at 7830 rpm, at 4 °C; the supernatant was recovered and designated as crude extract (CE).

Solvent precipitation. CE was precipitated using a modified protocol from von der Haar (2019) to eliminate the contaminating free Pi in the samples. A known volume of protein solution was added to a polypropylene tube. Then, 4 volumes of ethanol were added and vortexed, and 1 volume of chloroform was added and vortexed. Immediately, 3 volumes of water were added to the tube, vortexed, and centrifuged at 7197 ×g, at 4°C for 10 min. After the centrifugation,

without disturbing the interface, the upper aqueous phase was carefully discarded, and 3 volumes of ethanol were added, vortexed, and centrifuged at 7197 ×g, at 4°C for 15 min. Finally, all liquid was discarded, and the pellet dried at room temperature for several minutes. Once dried, proteins were stored at -20 °C until further use.

4.2.4 Protein Quantification

Protein concentration was measured using Bradford's Method with BSA as a standard protein. Samples of 100 µL of protein solution were added to 1 mL of Bradford's reagent, incubated 10-20 min at room temperature, and absorbance at 595 nm was registered in a Cary 50 spectrophotometer.

For highly diluted samples, a microplate Bradford assay was used: 50 µL of the sample was mixed with 250 µL of Bradford's reagent, incubated at room temperature for 10 min, and absorbance at 620 nm was registered in a microplate absorbance reader Accuris Smartreader 96, using BSA as the standard protein for the measurement of the calibration curve.

4.2.5 Protein Visualization by Polyacrylamide Gel Electrophoresis (PAGE)

The proteins obtained from each step of the semi-purification process were subjected to SDS-PAGE to determine their homogeneity and estimate their molecular mass. SDS-PAGE was performed on a 15% polyacrylamide gel.

Proteins were stained with an EZBlue staining reagent, and SigmaMarker™ wide range (Sigma-Aldrich) was used as the molecular weight marker.

4.3 Characterization of Enzymatic Reaction

4.3.1 Enzymatic Assay

Two modifications of the Method from Sviridov et al. (2012) were followed to evaluate the enzymatic activity (final volume of 80 µL). The first method used a reaction mixture containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 µM FAD, 35 µM GP, and protein extract. In the second method, FAD was excluded. Samples were incubated for 1 h at 30 °C.

The decrease in the concentration of GP was determined indirectly by quantification of equimolar Pi release from the GP molecule after the cleavage of the C-P bond. According to the provider's instructions, this was achieved using the Green Malachite Assay (Sigma Aldrich). One unit (U) of activity was defined as the release of 1 µM of $Pi \cdot min^{-1}$. The preparation of a phosphate calibration curve ranging from 0 to 40 µM was held; 80 µL of reaction mixture was transferred into separate wells of a multiwell plate, and 20 µL of Working Reagent (WR) was added to each well (stopping the reaction). The microplate was incubated for 30 minutes in the dark at room temperature for colour development. Absorbance at 620 nm was measured in a microplate absorbance reader Accuris Smartreader 96.

Effects of pH. Enzymatic activity as a function of pH was determined using 50 mM Tris-HCl buffer with pH values of 7, 8, and 9 incubating for 1 h at 30 °C.

Effects of temperature. Enzymatic activity as a function of temperature was determined by incubating for 1 h at ideal pH at temperatures ranging from 30 °C to 45 °C.

4.4 DISPOSAL OF GENERATED WASTE

All waste generated was classified according to the hazardous waste classification procedures document PR-CLB-SRR/000 of the Facultad de Ciencias Químicas established by the institution's Waste Management and Control Department.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 ENZYME PRODUCTION

5.1.1 Growth Kinetics of the Strain

B. cereus 6P was reactivated using a non-selective media to obtain fresh high OD cultures. Then, the growth kinetics of the strain were determined in two mediums (LB broth and MMM) to compare the development of the bacteria in a nutrient-rich medium and a challenging medium with the herbicide as the sole source of phosphorus (P), a well-known growth-limiting nutrient (White & Metcalf, 2007). This xenobiotic promotes stressful conditions for the microorganism, forcing it to express genes to produce a GP-degrading enzyme capable of cleaving the C-P bond in the herbicide molecule.

Figure 3a shows the enormous difference between the growth reached in each media. In the case of LB broth, the lag phase was almost nonexistent as the inoculum had already been adapted to this medium. In less than 24 h, the OD suffered a drastic decrease due to culture death. Still, in the following hours, the culture increased the OD anew, where the remaining cells were adapting to harsh conditions and nutrient depletion in the medium.

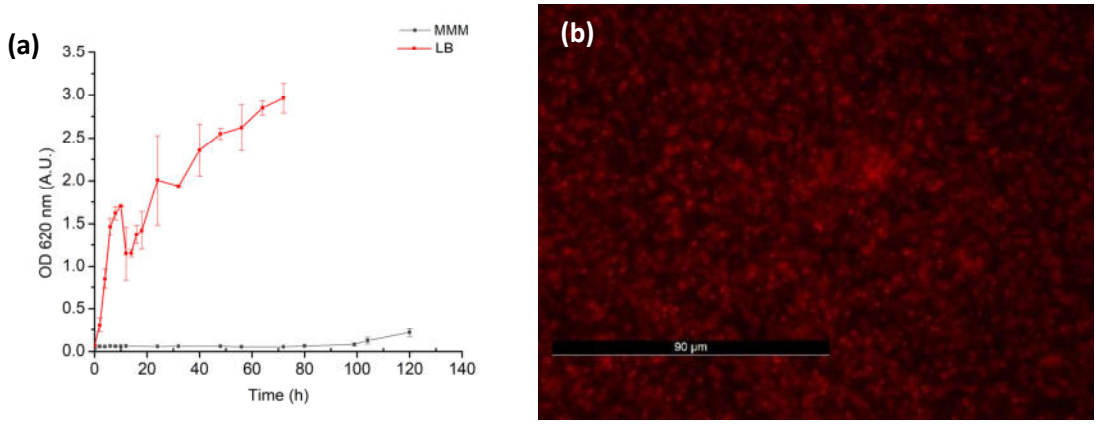

Figure 3. *(a) Growth kinetics of B. cereus 6P in MMM and LB broth (n=3). Samples with absorbance higher than 1.0 were calculated using serial dilutions; (b) PHA granules detected in B. cereus 6P using Nile red staining after 72 h incubated in LB broth. Objective 100x.*

Samples of the LB culture at 72 h showed the presence of PHA granules after the Nile Red staining (Figure 3b), indicative of the adaptative metabolism to nutrient stress (depletion of essential nutrients in the face of excess carbon) reached after the high consumption in the log phase of the strain. Similar growth behaviour was reported by Yasin and Al-Mayaly (2021) during the fermentation of glucose by *B. cereus* ARY73 to produce PHA. The PHA production by *B. cereus* has been extensively reported in diverse mediums varying the carbon source and ratio of essential nutrients (Halami, 2008; Maheshwari et al., 2018; Mizuno et al., 2010; Tsuge et al., 2015).

For the MMM culture, at approximately 100 h, a slight increase in OD was observed (Figure 3a); this coincided with the results of Martínez Ledezma (2019), indicating the adaptation of the bacterium to the adverse conditions and the expression of the metabolic machinery necessary to metabolize the herbicide to obtain the essential P. Similar results were reported for GP pre-conditioned *Lysinibacillus sphaericus* cultures (González-Valenzuela & Dussán, 2018), where

the principal difference is that their lag time was shorter than the one reported in studies from our laboratory, as a consequence of the pre-condition at the herbicide. The reduction in OD in MMM compared to LB broth is greater than 90%, but the strain can survive and grow in such conditions.

B. cereus 6P has been demonstrated to metabolise GP, taking advantage of the Pi produced to accumulate polyP while not producing polyhydroxyalkanoates (PHA) under such conditions (Acosta-Cortés et al., 2019). The above denotes that, despite the stress, the bacterium is not compromising its development, known that PHA production is regularly activated as a survival measure under adverse conditions (Zuriari et al., 2013).

Previous studies by Martínez Ledezma (2019) and Acosta-Cortés et al. (2019) with 6P strain showed that it does not have the genes for the expression of the catalytic part of C-P lyase, supporting the notion that another enzyme is responsible for cleaving the C-P bond. Furthermore, the production of Pi, which is indicative of such cleavage, supports the fact that a GOX enzyme, which produces AMPA instead, is also not responsible for the degradation of the pesticide.

5.1.2 Culture Conditions to Produce the Enzyme

Figure 4 shows the regular rod-shaped morphology of *B. cereus* 6P. This gram-positive bacterium was 1-1.5 μm in diameter and 3-5 μm long when cultured in LB medium.

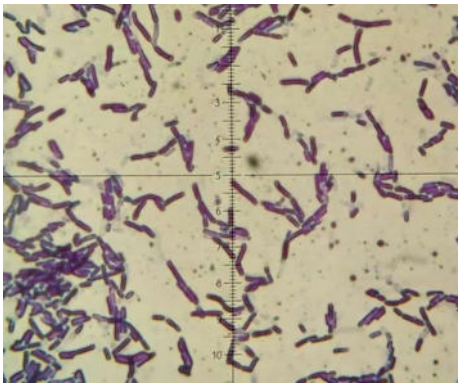

Figure 4. *Gram staining of B. cereus 6P (24 h in LB broth). Objective 100x.*

During the culture of *B. cereus* 6P in MMM, morphological changes attributed to the nutritional stress to which the bacteria are subjected were observed. Pi is a necessary component for the metabolic processes of all living cells, growth being one of the most notorious (Vera et al., 2008; White & Metcalf, 2007). Therefore, limiting its environmental availability is expected to decrease cell growth.

In Figure 5b, the same bacteria cultured for several days in MMM containing GP showed changes in its dimensions and capacity to retain staining reagents, maybe because the rinse of reagents was severe enough to sweep almost all the outer membrane of the significantly smaller cells. The rod shape was shortened several times until the cell looked almost round. The decrease in size was observed to be more noticeable as the days of culture progressed (Figure 5a-b).

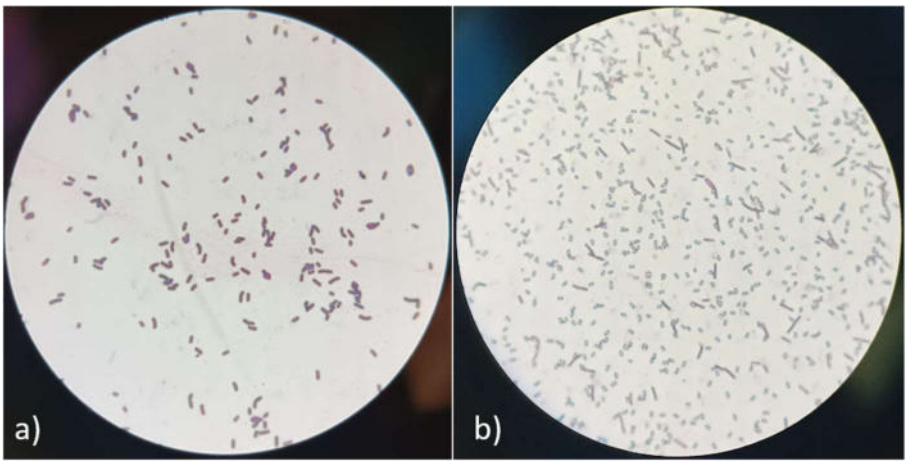

Figure 5. *Gram staining of B. cereus 6P. Comparison between (a) 120 h in MMM and (b) 210 h in MMM. Objective 100x (both).*

Additionally, the production of polyP in *B. cereus* 6P cultured in the presence of 1 mM GP (Figure 6) can be contrasted with the results of *Acidithiobacillus ferrooxidans* cultured in the presence of 1 mM of methyl phosphonate (M-Pn) as sole source of P, in both cases, the bacteria were able to degrade the Pn to use it as P source, but in the case of *A. ferrooxidans* the pool of polyP was consumed during the incubation process to promote growth, whilst *B. cereus'* pool of polyP is increased (Acosta-Cortés et al., 2019; Vera et al., 2008).

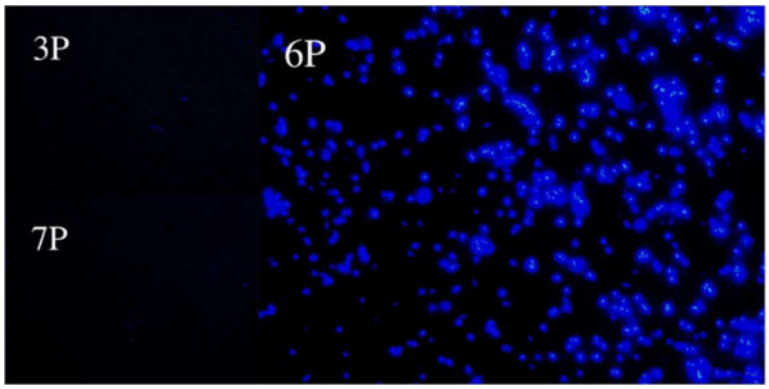

Figure 6. *DAPI staining for polyP evaluation after 120 h of incubation on MMM* (Acosta-Cortés et al., 2019)*. PolyP is observed as yellow-green granules after the staining with DAPI (4',6-diamidino-2-phenylindole). Isolates 3P and 7P were used as negative controls.*

These results are comparable to recent studies with a *P. putida* strain grown on MS1 minimal medium supplemented with GP (500 mg/L) as a P source, where the authors observed similar morphological changes in the rod shape of the bacteria. Other study results showed that P. putida Ch2 accumulated PHA granules under these culture conditions (Esikova et al., 2023), contrasting with the behaviour of *B. cereus* 6P.

All the results mentioned above are a great example of this strain's metabolic plasticity (Martínez-Herrera et al., 2023), allowing it to adapt to several conditions, including a high-stress environment and produce metabolites of human interest.

5.1.3 Protein and Phosphate Quantification

Total protein (TP) measurement was performed using Bradford's method to determine the protein concentration in the cell-free extracts. Two main calibration curves were performed. The first was achieved using a standard working range of 0.05 to 0.5 mg/mL of BSA (Figure 7). For this method, the absorbance was registered at 595 nm.

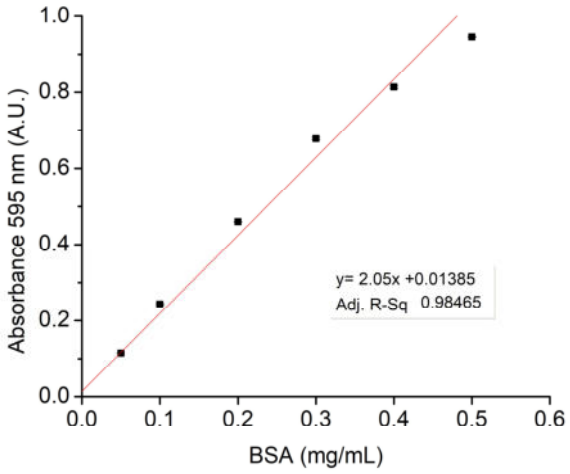

Figure 7. *BSA calibration curve for protein quantification (n=3).*

A second curve was performed to quantify significantly diluted samples, with a calibration curve ranging from 0.01 to 1.0 µg de BSA in a 50 µL sample. For this calibration curve, absorbance was registered at 620 nm (Figure 8).

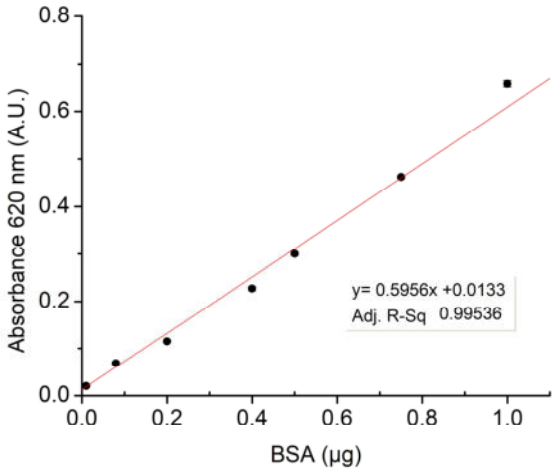

Figure 8. *BSA calibration curve for protein quantification for diluted samples (n=3).*

To proceed with the Pi quantification in the extracts, the colourimetric Malachite Green Phosphate Assay Kit (MAK307, Sigma-Aldrich) was used, where

a green complex is formed between Malachite Green, molybdate, and free Pi. A calibration curve for phosphate concentration was performed in a working range of 4 to 40 µM according to the provider's instructions (Figure 9). All materials and reagents were assayed to verify that no contaminating Pi was present.

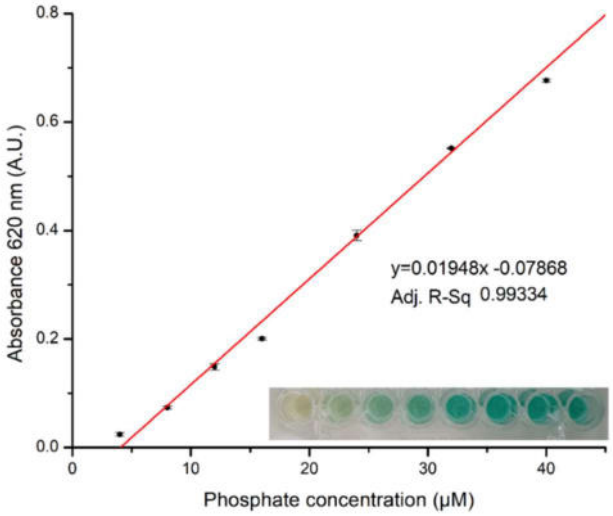

Figure 9. *Calibration curve for phosphate concentration (n=3).*

5.1.4 Obtention of the Enzyme and Elimination of Contaminating Pi

After analyzing the morphology of 6P cells on GP-rich MMM, the cell lysis of 210 h cultures was performed to obtain the intracellular content. Martínez Ledezma (2019) reports the potential localization of the enzyme. The pelleted cells from the MMM culture were sonicated in 50 mM Tris-HCl (pH 7). The homogenate was centrifuged for 10 min at 7830 rpm, at 4 °C to eliminate cell debris; the clear supernatant was recovered and designated as crude extract (CE).

Studies report the optimal intracellular Pi concentration for procaryotic cells such as *E. coli* to be between 1 and 10 mM (McCleary, 2017). The Pi concentration of CE from *B. cereus* 6P (173.13 µM) was several times lower than those optimal concentrations; this could be explained by the capability of 6P to rapidly store free Pi as polyP, which makes this resource less available, as observed in these results.

To have another contrast, the normalized free Pi concentration of CE samples was 20,084.7 nM Pi/µg of protein. Results can be compared to a study on *Salmonella enterica* where authors reported a stable intracellular Pi concentration of 1250 nM/µg of protein in a wild-type strain (Pontes & Groisman, 2018). Pi concentration values from the 6P strain are folds-higher than the *S. enterica* strain, depicting the *B. cereus* 6P's capability of continuously uptake Pi from the cleaved GP independent of the environmental Pi concentration and, perhaps, also showed an unstable state of Pi consumption due to active growth at harvest time.

To semi-purify the enzyme, solvent precipitation of CE was performed to eliminate the contaminating Pi present in the extract. During precipitation, the formation of a whitish disc in the interphase of solvents mentioned as a precipitated protein was observed by von der Haar (2019). After the elimination of solvents, such precipitate was distributed in the bottom of the polypropylene tube as fine granules that were stored at -20 °C until further use. Total dryness of the granules was avoided to facilitate later resuspension of the protein (Scopes, 1982).

All samples assayed after sonication (CE) presented less than 1.0 mg/mL of TP. After protein precipitation, the resuspension of the protein pellet in 50 mM Tris-HCl pH 7 remained partial even after the addition of several drops of buffer, avoiding high dilution of the proteins. Also, the loss of protein was significant, 88% of initial TP. This result can be compared with reports of Nickerson and Doucette (2020), where a rapid solvent precipitation resulted in recovery of 30% of TP, where results are enhanced after prolonged exposition at temperatures well below 0 °C. Even with the severe loss of protein suffered, this method was preferred over other desalting methods (UF centrifugal devices, liquid chromatography SEC) because the removal of residual Pi showed superior results (Table 1), and the enzymatic assay could be performed.

Table 1. Phosphate removal from cell-free extracts using different desalting methods.

Method	***Initial PO_4^{3-} concentration***	***Pi removal***	***Observations***
UF centrifugal devices 30K MWCO	173.13 µM	<20%	Even though some Pi was washed into the filtrate, most was in the retentate. There was a high protein retention in the membrane.
SEC	173.13 µM	100%	Total elimination of Pi, but the dilution of samples was too high.
Solvent precipitation	173.13 µM	>90%	High loss in TP was obtained, but samples were almost free of residual Pi.

The results after the protein precipitation are expected because the ionic strength of the protein mix was drastically changed by the removal of Pi and other salts naturally included in the intracellular content. This suggests that deeper evaluation of the resuspension's conditions can be addressed to enhance the TP

quantification, resulting in a complete resuspended pellet; also, a change in solvents could be evaluated to produce less denaturation of proteins, which is unavoidable in this type of separation method (Scopes, 1982).

To assess the protein profile of the samples (CE and precipitate), 10 µL of each sample was run in an SDS-PAGE using a 15% polyacrylamide (PA) gel for the protein separation. Results are shown in Figure 10. Lane 1 corresponds to SigmaMarker™ wide range with 12 standard proteins ranging from 6.5 to 200 kDa; lane 2 corresponds to CE, and lane 3 contains the precipitated fraction. It can be observed that all visible bands from CE were conserved and intensified in the protein precipitation. During this study, it was impossible to attribute the enzymatic activity to only a single band or a reduced group of proteins.

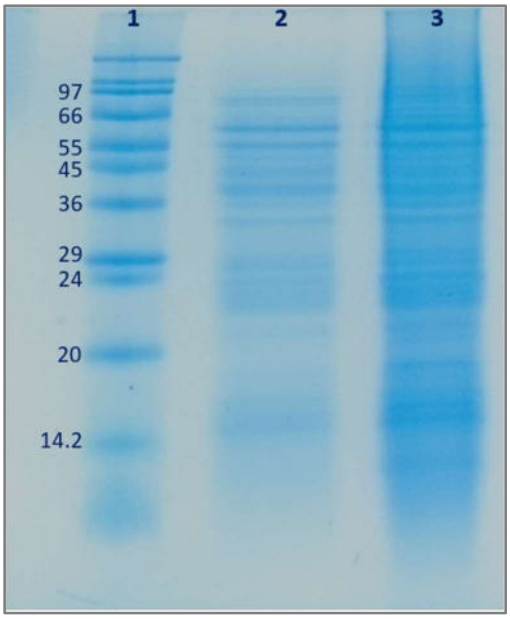

Figure 10. *SDS-PAGE of cell-free extract. Separation achieved in 15% PA gel. In lane 1: molecular weight marker with myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20 kDa), α-lactalbumin (14.2 kDa), aprotinin (6.5 kDa) as standards; lane 2: crude extract after sonication; lane 3: solvent precipitation fraction.*

5.2 CHARACTERIZATION OF ENZYMATIC REACTION

5.2.1 Enzymatic Assay

The cleavage of the C-P bond in GP releases Pi to the reaction mix in an equimolar ratio (Figure 11). Therefore, the concentration of Pi released was an indirect measure of the GP biodegradation.

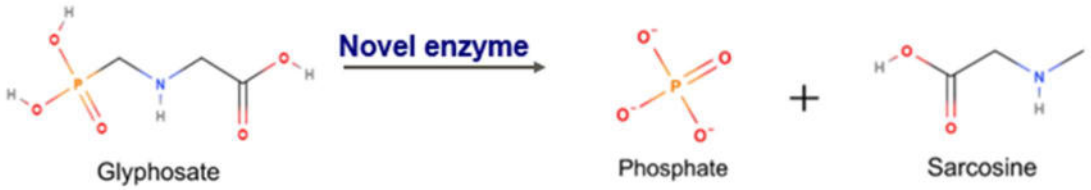

Figure 11. *Proposed activity of the novel enzyme.*

Samples of CE and precipitate were assayed to determine the GP-degrading enzymatic activity. Two reaction mixes were used for enzymatic assays, one containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 35 µM GP, 10 µM FAD and protein, and the second where FAD was excluded. The presence of FAD in the reaction mix was to evaluate the possible FAD dependency of the unknown enzyme. Samples were incubated for 1 h at different temperatures and pH values to assess the best conditions to perform the rest of the assays. Proper controls were considered: 1) reaction mix containing every component but protein (negative control), and 2) reaction mix containing every component but GP (negative control).

Samples of CE were assayed to evaluate the GP-degrading reaction in both reaction mixes (FAD and No FAD). The final volume of the response was 80 µL. Reaction mixes at different pH values were incubated for 1 hour at 30 °C. The highest values for specific activity were obtained at pH 8, with values of 27.01 U/mg and 24.98 U/mg for FAD-containing and No FAD media, respectively (Figure 12). These values corresponded to approximately 39.92% and 36.91% of GP degradation for FAD and No FAD media, respectively (Table 2). For both pH 7 and 9, activity values were halved.

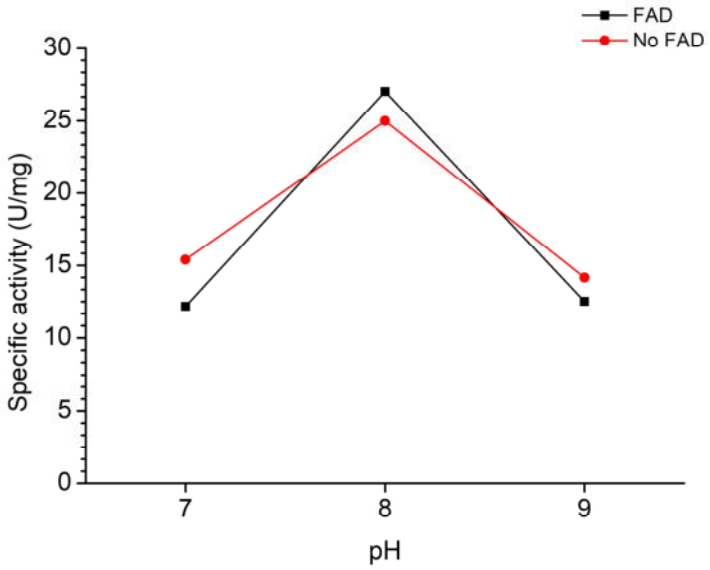

Figure 12. *The specific activity of CE incubated at 30°C at different pH values; results are the mean of two independent assays.*

Table 2. Percentage of GP degradation by CE after 1 h incubation at 30 °C at different pH values (n=2).

		% GP degradation		
FAD presence	***Temperature (°C)***	***pH***		
		7	***8***	***9***
+	30	17.99	39.92	18.51
-	30	22.76	36.91	20.93

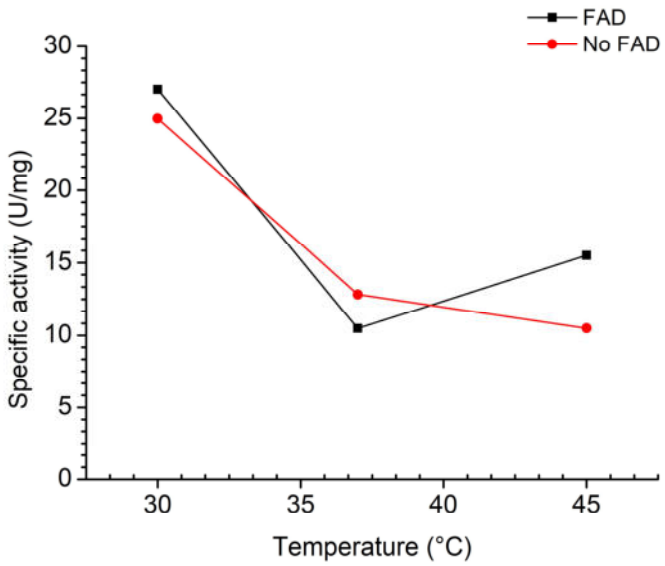

Figure 13. *Specific activity of cell-free extract incubated at pH 8.0 at different temperatures, results are the mean of two independent assays.*

Table 3. Percentage of GP degradation by CE after 1 h incubation at pH 8 at different temperature values (n=2).

		% GP degradation		
		Temperature (°C)		
FAD presence	***pH***	***30***	***37***	***45***
+	8	39.92	15.50	22.91
-	8	36.91	18.95	15.50

The reaction was also performed in both media (FAD and No FAD) at pH 8, 1 h incubation at different temperatures: 30, 37, and 45 °C. Results are shown in Figure 13 and Table 3. It is notorious that the specific activity of the CE decayed abruptly at higher temperatures. In the case of the No FAD media, the activity decreased gradually, showing at 45 °C a value of 10.49 U/mg, whilst, in the FAD-containing media, the specific activity at 45 °C showed a slight increase compared with the value at 37 °C, 15.50 U/mg, and 10.48 U/mg, respectively. This could be

attributed to the enhanced thermal stability of the enzymatic activity when FAD acts as a cofactor (Gouda et al., 2003; Pey et al., 2014). However, further analysis of specific activity against the presence of FAD needs to be performed.

These results showed that the activity of the GP-degrading enzyme from *B. cereus* 6P is independent of Pi levels, as the consumption of GP in the MMM culture was active during the harvest of cells. No activity inhibition was detected during the CE assays with high Pi levels. Also, the presence of FAD does not seem necessary for the reaction occurrence because an almost identical pattern of the specific activity was observed in both reaction mixes. This information led me to think that the enzyme of interest is not a GOX-like enzyme, but further molecular determination of the presence of GOX genes would be of much interest to confirm with certainty the total absence of both GP-degrading pathways in *B. cereus* 6P.

A two-factor ANOVA was performed to evaluate whether there were significant statistical differences between the factors pH and the presence of FAD on enzyme activity. The results are presented in Tables 4 and 5. pH value is statistically significant on the enzymatic activity ($p=0.034$). When comparing the influence of FAD on the enzymatic activity, no significant differences were detected between the assay with FAD and No FAD ($p=0.607$). There was no statistical significance of temperature and FAD presence on the enzymatic activity (ANOVA not shown).

Table 4. Summary of factors to evaluate the effects of pH and FAD on the enzymatic activity.

Summary	*Count*	*Sum*	*Average*	*Variance*
pH 7	2	40.75	20.375	11.376
pH 8	2	76.83	38.415	4.530
pH 9	2	39.44	19.72	2.928
FAD	3	76.42	25.473	156.597
No FAD	3	80.60	26.866	76.488

Table 5. ANOVA to evaluate the effects of pH and FAD on the enzymatic activity.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
pH	450.249	2	225.125	28.277	0.034	19
FAD/No FAD	2.912	1	2.912	0.366	0.607	18.513
Error	15.922	2	7.961			
Total	469.084	5				

After determining the best-tested temperature and pH conditions to evaluate the enzymatic reaction, the residue was assayed at 30 °C and pH 8 in the reaction mix containing FAD, as shown in Table 6.

Table 6 summarizes the steps in semi-purifying a glyphosate-degrading enzyme from *B. cereus* 6P. The experiments were assayed in a reaction mix containing FAD.

Step	*Total activity (U)*	*Total protein (mg)*	*Specific activity (U/mg) **	*Purification (fold)*
Crude extract	51.62	1.293	27.02	1.0
Solvent precipitation	4.39	0.155	28.32	1.05

* Results of specific activity are the mean of two independent assays.

Although to date there are reports of microorganisms capable of expressing the proteins of the C-P lyase complex, GOX, and even some others

with Pn consumption as Pi source without a specific degradation pathway (Hove-Jensen et al., 2014), this is the first time that a strain of the genus *Bacillus* is reported as capable of degrading GP by direct cleavage of the C-P bond, with no presence of genes for the catalytic fraction of C-P lyase (*phnJ*), and independent of phosphate levels. All evidence contrasts with both C-P lyase and GOX pathways.

Studies mentioning specific activity against GP as a consequence of C-P lyase or GOX action were considered to compare these results with other authors. Selvi and Manonmani (2015) reported the purification of an intracellular C-P lyase from *P. putida* T5 with a specific activity of 0.28 U/mg (same unit as in this study) and 3.775 U/mg for the CE and solvent precipitation, respectively, both values comparably lower than the specific activity from our cell-free extracts. Their reaction mix contained 50 mM phosphate buffer pH 7.0, 60 µM GP, and enzyme. HPLC followed GP degradation after derivatization. The optimal pH and temperature were 7.0 and 30 °C, respectively.

Moreover, their purification steps reported a minimal loss of TP and a purification fold of 627 for the pure enzyme (175.60 U/mg). These authors also stated that the enzyme was a 70 kDa monomer. This is the only report of a purified intracellular C-P lyase enzyme highly active against GP.

A study from Selvapandiyan and Bhatnagar (1994) reported C-P lyase activity for periplasm extracts from two Pseudomonas strains, GLC11 and PG2982. The specific activity for GP degradation in a mix with 50 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, and 50 mM was 262 and 280 nmol Pi released·$hour^{-1}$·mg^{-1} for

GLC11 and PG2982, respectively. Nonetheless, the activity was unstable. In the present study, the highest specific activity value, obtained with FAD, pH 8.0, at 30 °C was 259 nmol Pi released·hour^{-1}·mg^{-1}, a close value compared to the C-P lyase from *Pseudomonas* strains.

Studies of enzymatic activity of the GOX are available with values ranging from <3.0 nmol AMPA·min^{-1}·mg^{-1} to 93 nmol AMPA·min^{-1}·mg^{-1} in GM GOX (Barry & Kishore, 1998), but would not be further compared with our results as technical differences could interfere, for example, how the oxygen ratio utilization of GOX can vary depending of the GOX origin, and the use of advanced technics such as radiometry.

CHAPTER 6

CONCLUSIONS

After culture in stressful conditions (phosphorous starvation, GP presence), the strain *B. cereus* 6P suffered morphological changes, most likely due to the limitation of several metabolic pathways regulated by phosphate concentration.

The production of a cytoplasmic GP-degrading enzyme independent of Pi levels was successfully achieved, maintaining GP as the sole source of phosphorous in the medium.

The TP concentration in CE samples was less than 1.0 mg/mL, suggesting that optimizing growth conditions and a lysis process is necessary to utilize the best-limiting reagents, such as GP and TP.

Quantification of Pi released from the GP molecule can be accurately followed by the Malachite Green Assay, supposing a cheaper method than the typical analysis using derivatization and HPLC.

The specific activity of cell-free extracts (CE and precipitate) presented higher values at pH 8.0 and 30 °C. The specific activity in the reaction mix containing FAD at pH 8.0 was slightly higher than its counterpart without the dinucleotide. Still, both reaction types had almost the same behavior as a function of pH. An ANOVA showed no significant difference between the effect of FAD and no FAD reaction mixes on the enzymatic activity. Still, significant statistical

differences were found in the effect of pH values on the enzymatic activity. This implies that the presence of FAD is not a determinant of the enzyme's activity, as in the case of GOX.

The reaction mix containing FAD seems to minimize the activity loss at higher temperatures (45 °C), while the reaction mix without FAD presents activity loss as temperature rises. The highest GP degradation percentage was 39.92% after 1 h of CE incubation in the presence of FAD at pH 8.0 and 30°C; degradation values for almost all other conditions were much lower.

In the present study, it was not possible to further purify the enzyme, remaining unknown the molecular weight.

6.1 Suggestions and Propositions

It would be significant to optimize the lysis method to enhance the TP obtained per batch of cells. Another suggestion is to secure the supply of the most vital reagent for subsequent projects, as in this case was GP. If GP is guaranteed, several tests can be followed (such as increasing the culture batch volume) to obtain better performance and confirm the reproducibility of every result that needs to be addressed. Also, it is prudent to use another method for TP quantification with higher sensibility and fewer interferences, as can be BCA.

CHAPTER 7

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