Mechanism of biotransformation of phosphogypsum by urease and carbonic anhydrase produced by *Lysinibacillus sphaericus*

A Dissertation report for

Course code and Course Title: GBO 381 Dissertation

Credits: 8

Submitted in partial fulfilment for the degree of

MASTERS OF SCIENCE IN BIOTECHNOLOGY

by

SELCEA SAVIA D'COSTA

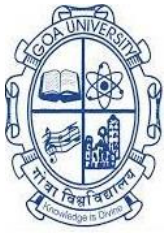
21P047017

Under the Supervision of

Dr. MEGHANATH PRABHU

School of Biological Sciences and Biotechnology

Biotechnology Discipline



Goa University

APRIL 2023

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "**Mechanism of biotransformation of phosphogypsum by urease and carbonic anhydrase produced by *L. sphaericus***" is based on the results of investigations carried out by me in the Department of Biotechnology at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Dr. Meghanath Prabhu and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will be not be responsible for the correctness of observations / experimental or other findings given the dissertation.

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Selcea Savia D'Costa
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Date: 12/5/23.

Place: Goa University

Examined by: 12/5/23.

Seal of the School

COMPLETION CERTIFICATE

This is to certify that the dissertation report on **"Mechanism of biotransformation of phosphogypsum by the urease and carbonic anhydrase enzymes produced by *L. sphaericus*"** is a bonafide work carried out by **Ms. Selcea Savia D'Costa** under my supervision/mentorship in partial fulfillment of the requirements for the award of the degree of Masters of Science in Biotechnology in the Discipline Masters of Science in Biotechnology at the School of Biological Sciences and Biotechnology, Goa University.

12/5/23

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sanitakerkar 12/5/23

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Place: Goa University

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LIST OF ABBREVIATIONS AND SYMBOLS:

PG: Phosphogypsum

CA: Carbonic anhydrase

SDS-PAGE: sodium dodecyl sulphate- polyacrylamide gel electrophoresis

TAE: Tris acetate buffer

µl: microliter

h: hour

g: grams

°C: degree Celsius

mM: millimolar

SRB: Sulphate reducing bacteria

TSS: Total Suspended Solids

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1. ABSTRACT

1. ABSTRACT

In the fertilizer industry, the waste by-product of phosphoric acid synthesis is phosphogypsum. A huge amount of phosphogypsum is produced which is highly toxic to the environment. This study focussed on the biotransformation of phosphogypsum using *L. sphaericus*. The pH of the culture in different parameters was checked to find out which the growth behaviour of the culture. The pH of the PG test media over the period of 7 days was found to be 8.4. The pH of the media control was 8 and the pH of the culture control was 9.25, indicating that the culture prefers an alkaline environment.

The ability of the *L. sphaericus* to grow in the environment containing phosphogypsum and to transform it was studied by plotting a growth curve to observe if there was an increase in the spectrophotometric readings with or without phosphogypsum. The biotransformation mechanism of *L. sphaericus* was determined by using a different calcium source for the biotransformation to compare the differences in the growth pattern. An uneven growth pattern was observed in the samples as the growth curve was increasing and decreasing during the incubation period.

The protein determination in the media was done by Folin-Lowry's method which helped to determine if the culture was able to function in the particular environment. The standard protein for Folin Lowry's method was Bovine serum albumin. The protein concentration in the $CaCl_2$ test samples was the highest with an increase of 1.06 mg/ml after 7 days. The protein concentration of the PG test samples had an increase of 0.66 mg/ml during the incubation period.

SDS-PAGE was run for the comparative analysis of the presence of the enzymes produced by the culture to detect the presence of carbonic anhydrase and urease in the biotransformation mechanism. The quantitative tests to detect the presence of urease and carbonic anhydrase enzyme was conducted and both were found to be positive.

2. INTRODUCTION AND REVIEW OF LITERATURE

2.1. INTRODUCTION

Hazardous waste, particularly radioactive waste, is a distinct type of industrial wastes. Chemical industries (organic and inorganic); oil refining; and thermal processes are the major sources of hazardous waste (Ines Hammas-Nasri et al., 2016).

The primary by-product of the production of phosphoric acid is the acidic digestion of phosphate rock, which results in the formation of phosphogypsum. It has a number of impurities, including radioactive and rare earth elements, fluorine compounds, residual acid (P_2O_5), and trace elements. During the process of making phosphoric acid, the rare earths are concentrated in phosphogypsum. Phosphate gypsum contains between 70% and 85% of the phosphate rock's original contents. The remaining portion remains dissolved in the phosphoric acid-containing leaching solution. Phosphate gypsum is an excellent source of rare earths due to the fact that rare earths are merely a by-product of the production of phosphoric acid and the enormous quantities of phosphate rock that are processed annually. When its large volumes are taken into consideration, phosphogypsum, which has low concentrations of rare earths, may therefore provide significant quantities of these elements (Ines Hammas-Nasri et al., 2016).

Phosphorus is a mineral found in phosphate rock that is used in some fertilisers to aid in the development of robust roots in plants. Small levels of naturally occurring radionuclides, primarily uranium and radium, can be found in phosphate rock. By dissolving the rock in an acidic solution, the phosphorous is extracted from phosphate rock while processing it to generate fertiliser. Phosphogypsum is the name given to the waste that is produced. This waste is where the majority of the naturally occurring uranium, thorium, and radium contained in phosphate rock ends up. Radium, a radioactive gas, is produced when the elements uranium and thorium decay into it. Phosphogypsum is more radioactive than the natural phosphate rock because of the concentrated wastes. In huge piles known as stacks, the waste generated during fertiliser manufacture is kept. Some stacks are hundreds of feet high and span hundreds of acres.

When phosphogypsum is first placed on the stack, it is quite watery. On the stack, a crust develops as the phosphogypsum dries up. As the crust gets thicker, less radon can escape and the waste is less likely to be carried away by the wind. A portion of the water could seep out the bottom and contaminate the nearby groundwater (*Radioactive Material From Fertilizer Production*, 2021).

The four promising resource utilization directions of phosphogypsum are prefabricated building materials, eco-friendly materials and soil materials, and new green functional materials and chemical fillers (Wu et al., 2022).

To maintain the ideal concentration of metals inside their cells, microorganisms have evolved mechanisms of tolerance and resistance. A microorganism can withstand metal toxicity by exploiting intrinsic features and metabolism-independent strategies including adsorption by membranes, cell walls, or surface layers (S-Layer) (Shaw &Dussan, 2018).

Prokaryotes have a wide range of resistance mechanisms, but the primary energy-dependent homeostasis mechanism for regulating intracellular metal concentrations is the extrusion of cations by efflux pumps (Shaw &Dussan, 2018).

The metal resistance efflux pumps are widely studied in gram negative bacteria. The mechanisms help in the transport of the metal to the periplasm from the cytoplasm, or it is sent to the outer membrane of the cell. This is led by the proton motive force of P-type ATPases and the cation diffusion facilitator proteins. The ATPases which are present in the transmembrane control the movement of ions and molecules across the membrane which help to maintain the intracellular concentrations of ions (Shaw &Dussan, 2018).

In SDS-PAGE, the sodium dodecyl sulphate (SDS) is an anionic detergent which is used to linearize the proteins which provides them a negative charge. In the majority of proteins, SDS binding to the polypeptide chain imparts an equitable distribution of charge per unit mass, leading to an approximate size fractionation during electrophoresis. The greater unpredictability in the ratio of bound SDS makes it inherently more difficult to appropriately treat proteins using this approach, such as numerous membrane proteins and those that interact with surfactants in their natural environment (Suvra Roy & Vikash Kumar, 2014).

2.2. Review of literature

2.2.1. Phosphogypsum:

The main by-product which is produced in the phosphoric acid production sites is phosphogypsum (Yassine Ennaciri& Mohammed Bettach, 2021). Phosphogypsum is produced in the processing of phosphate rock which leads to the production of phosphoric acid. The chemical composition of phosphogypsum is 96% $CaSO_4.2H_2O$. The impurities present in it are P_2O_5, SiO_2, Al_2O_3, Fe_2O_3, MgO, Na_2O and K_2O (Yassine Ennaciri& Mohammed Bettach, 2021).

The majority of manufactured PG has a particle size distribution that primarily falls within the 10-1000 m range and is classified as fine powder. Because of the PG's 5%–28% moisture level, fine particles agglomerate and less dust is produced. The majority of the impurities are adsorbed on the surface of PG, which has a micromorphology that is mostly flake irregular, rhombic, and other crystal morphologies (Wu et al., 2022).

Radioactivity is also present in phosphogypsum due to the presence of Uranium-238 and Thorium-232. PG has been identified by the USEPA as a "Technologically Enhanced Naturally Occurring Radioactive Material" (TENORM). Phosphate ore is treated using either dry thermal or wet acid techniques to create phosphoric acid. Phosphorus is made via the dry thermal process in an electric arc furnace. The wet chemical phosphoric acid treatment process, or ‘‘wet process’’, is widely used to produce phosphoric acid and calcium sulfate – mainly in dihydrate form. The raw phosphate (fluorapatite) decomposes with concentrated sulphuric acid at a temperature of 75–80°C, and phosphoric acid and calcium sulfate dihydrate – phosphogypsum are produced. (Yassine Ennaciri& Mohammed Bettach, 2021).

Production of 1 ton of phosphoric acid is known to result in the formation of about 5-ton phosphogypsum waste (Baena et al., 1998). The management of phosphogypsum is become a difficult task due to its highly toxic substances; only 15% of the phosphogypsum is recycled. Most of the countries have opted to store phosphogypsum in open air in special landfills or to dump it into the seawaters or estuaries. The dumping of phosphogypsum causes contamination of the surface water, ground water, soil and the atmosphere due to the leaching or the erosion of the hazardous elements. In Poland PG dumps comprise as much as 35 million tons.

The phosphate industry discharges toxic contaminants into air, water and soil. Air pollutants from phosphate fertilizer plants are fluorides and particulate matter, SO_2 and SO_3. The effluents are TSS (Total Suspended Solids), Phosphorous, Fluoride and Cadmium.

In an article, by Ortega-García et al. (2017), suggest a possible association between proximity to phosphogypsum industries and increased risk of paediatric cancer, specifically neuroectodermic tumours (Ortega-García et al., 2017).

Industrial fluoride, one of the dangerous components of PG, exists in both gaseous and particulate forms in industrial zones, causing serious health problems after prolonged inhalation or ingestion. Thus, increasing sulphate may have negative effects on aquatic organisms: in Microcystis aeruginosa, it inhibits photosynthesis, causes oxidative stress, increases toxin production, and affects expression of the related genes (Guo, Tingzong et al., 2001).

2.2.2. Characteristics of phosphogypsum:

The characteristics of phosphogypsum varies as per the ore origin and the attack process which determines the crystalline morphology of the phosphogysum. The phosphogysum is observed in the form of very fine sand after filtration. The porous PG is regarded as an acidic by-product since it still contains phosphoric, sulfuric, and hydrofluoric acids (pH less than 3). PG is soluble in saltwater (approx. 4.1 g/l). This may be because its solubility is pH dependent (Guo, Tingzong et al., 2001).

Phosphogypsum is a grey, damp, fine grained powder, silt or silty-sand material with a maximum size range between 0.5 and 1.0 mm, and 50–75% of particles finer than 0.075 mm. The specific gravity of phosphogypsum ranges from 2.3 to 2.6. The moisture content is usually in the range 8–30%. The main compounds of PG are: SO_4, CaO, SiO_2, Na_2O and P_2O_5 (Yassine Ennaciri& Mohammed Bettach, 2021).

2.2.3. Applications of Phosphogypsum in:

2.2.3.1. Agriculture:

The well-known agricultural uses of PG are reclamation of land, remediation of saline and sodic soils, amendment of soil to prevent crusting and to enhance water retention, and fertilization of soil for growing crops and pasture.

The addition of PG and lime in soil improves its plasticity and swell-shrink characteristics, and results in the formation of a dense compact mass of stabilized soil. (EzzeddineSaadaoui et al., 2017)

Phosphogypsum is used as a fertilizer in agriculture because it contains large amounts of calcium, phosphorus, and sulphur. PG application is known to improve seed growth and yield for several species. Liu et al. (2010) put three different amounts (15, 30 and 45 tonnes per hectare) to rice fields in saline-sodic soils of North-East China and found a substantial increase mostly for 30 tonnes per hectare (Liu, M. et al., 2010).

In an article, Al-Hwaiti and Al-Khashman (2017) indicated a relative absence of health risks associated with the consumption of vegetables/fruits grown in PG-amended soils (Nascimento, R. et al., 2017). In another article, Mahmoud and Abd El-Kader (2015) used PG alone or mixed with compost (mix ratio of 1:1) at 10 or 20 g/kg dry soil to mobilize heavy metals in contaminated soil, and showed that this practice improves canola growth and immobilizes heavy metals mainly for PG alone (Mahmoud, E. & Abd El-Kader, N., 2015).

2.2.3.2. Plaster manufacturing:

Phosphogypsum can be used instead of gypsum for the manufacturing of plaster. But the impurities present in phosphogypsum needs to be removed as even a small amount of these impurities affects the settling time and strength of the plaster (Yassine Ennaciri& Mohammed Bettach, 2021).

2.2.3.3. Cement sector:

Building materials are in higher demand as a result of the construction industry's rapid growth and ongoing improvements in people's living conditions. The majority of building materials' raw materials come from natural sources and harm the environment. PG can be utilized in place of raw materials for construction because of its enormous output and storage capacity as well as its unique features. Building materials frequently employ the by-product PG. The following primarily introduces a few key ways that phosphogypsum is used in the building materials industry (Wu et al., 2022).

Research and application of the technology for producing sulfuric acid and cement using PG as the primary raw material have advanced. In the production of sulfuric acid and cement, the apparent solid-solid reaction between Calcium sulphide and phosphogypsum has been extensively utilized. It was found that PG could be microencapsulated in a sulfur polymer matrix to make a new kind of PG-sulfur polymer cement that could be used to make building materials. Wu Sichuan et al. gypsum serves as the sole source of calcium oxide, allowing it to be incorporated into the mineral phases of the prepared iron-rich calcium sulfoaluminate cement. Even though research into this technology is fairly mature, it still has a number of drawbacks, like the high cost of preparation, high energy use, and gas pollution. As a result, most of its applications have been stopped, and a relatively inexpensive and safe treatment technology needs to be developed (Wu et al., 2022). Phosphogypsum is used in the cement industry to regulate its hydraulic setting. The addition of phosphogypsum affects the resistance, consistency and the setting time of the cement processed (Yassine Ennaciri& Mohammed Bettach, 2021).

2.2.4. Biotransformation of phosphogypsum:

2.2.4.1. Using anaerobic bacteria:

Biotransformation of phosphogypsum isolated from crude petroleum refining wastewaters by sulfate reducing bacteria.

SRB uses organic compounds as an electron donor and sulfates as electron acceptor. Sulfates are known to comprise about 50% of the mass of phosphogypsum and can be used by the sulfur reducing bacteria as a source of final electron acceptor.

Samples of the studied wastewaters (crude petroleum-refining wastewaters or purified by the activated sludge method) were placed in transparent 100 mL containers and 5 g/L of phosphogypsum was added. The containers were tightly closed and set aside for 6 weeks to allow the selection of anaerobic, sulfidogenic consortia capable of carrying out the biotransformation of phosphogypsum. Incubation was at 30 or 55°C (Dorota Wolicka et al., 2005).

2.2.4.2. Using Sulphate-reducing bacteria:

50% of the PG mass comprises sulphates that can be used in the process of dissimilative reduction of sulphates by sulphate reducing bacteria (SRB) as the final electron acceptor (Wolicka and Kowalski, 2005, 2006).

SRB are used in anaerobic conditions in all types of sewage treatment bioreactors. They are known to compete for the available organic compounds with various groups of bacteria at all decomposition levels except hydrolysis, because of the absence of hydrolytic enzymes in SRB.

The SRBs which are extensively studied for the biotransformation of phsophogypsum to calcite are *Desulfomonas, Desulfococcus, Desulfobacter, Desulfosarcina, Desulfotomaculum* and *Desulfovibrio* (Dorota Wolicka et al., 2005).

2.2.4.3. Using aerobic bacteria:

2.2.4.3.1. Microbially induced calcite precipitation (MICP):

The MICP formation of calcium carbonate occurs due to the presence of microbial cells and biochemical activities from supersaturated solution. There are four parameters which lead to the formation of biomineral in MICP: pH, dissolve inorganic carbon (DIC), calcium concentration, nucleation site. These parameters help in the formation of a stable calcium carbonate.

Bacteria have negatively charged cell surface calcium ions which binds to negatively charged cell surfaces of the bacteria. Cation calcium ions reacts with the anion carbonate to form calcium carbonate. The bacteria cell exopolymers, biofilms, inactive spores provides a nucleation site for precipitation of calcium carbonate.

The precipitation of calcium carbonate results in the formation of different calcium carbonate crystals, i.e., calcite, aragonite, and valerite. Calcite is formed in the monohydrate calcite phase. Calcite and valerite are formed by the MICP process (Kamble& Prabhu, 2020).

The ability of urease-mediated ureolysis and carbonic anhydrase to dehydrate CO_2 and precipitate carbonate minerals is well recognised, but many studies of the environmental microbial population rely on marker gene or metagenomic techniques that cannot detect in situ activity (Medina Ferrer et al., 2020).

The environmental process known as microbially induced calcium carbonate precipitation (MICP) has been investigated as an alternative approach to address a number of engineering and environmental problems, including the remediation of concrete structures, soil consolidation, pollutant bioremediation, and CO_2 sequestration. MICP can be challenging to analyze and detect in a given setting, despite its environmental relevance and numerous applications. Medina Ferrer et al., (2020) developed a method for the field detection of the two enzymes which are associated with MICP, that is, the urease and carbonic anhydrase activity. These were used to reveal the microbial communities that potentially promote carbonate precipitation in situ.

Metabolisms that enhance carbonate saturation and encourage MICP locally include photosynthesis, methane oxidation, nitrate reduction, bicarbonate transport, and ureolysis. Microorganisms can utilise urea as a source of carbon and nitrogen through the process of ureolysis, which is catalysed by urease. Through the carbon dioxide-bicarbonate interconversion, carbonic anhydrase allows quick carbon transfer into the cell. As a result of their action, both enzymes produce carbonate anions and raise pH levels. When urea is broken down by urease, ammonia and carbon dioxide are produced. Ammonia hydrolysis and CO_2 hydration, a reversible reaction catalysed by carbonic anhydrase, combine to produce one mol of hydroxide and one mol of bicarbonate for every mol of urea (Medina Ferrer et al., 2020). Hydroxide and bicarbonate production increase saturation with respect to calcium carbonate to promote precipitation.

The MICP process using carbonic anhydrase has four stages: (a) the CA bacteria multiplied and produced the CA; (b) the CA bacteria promoted the carbon dioxide hydration to produce HCO_3, which was then converted to carbonate ion in alkaline solution; (c) carbonate ions were formatted and positive charged Ca^{2+} aggregated on the surface of CA bacteria; and (d) used CA bacteria as nucleation sites to form a significant number of mineralized products in the solution (Zheng & Qian, 2020).

2.2.4.3.2. Urease producing bacteria:

The urease producing microorganism produces urease enzyme which converts urea to ammonia and carbon dioxide. The carbon dioxide reacts with the calcium ions of phosphogypsum to yield calcium carbonate and ammonia and sulphate reacts with each other to yield ammonium sulphate.

One mole of urea is hydrolyzed to one mole of ammonia and one mole of carbonate. Carbonate produces one mole of NH_3 and carbonic acid by spontaneous hydrolysis. NH_3 and carbonic acid are balanced in the solution to form bicarbonate. Two moles of each ammonia and hydroxide are formed. The balance of bicarbonate shifts carbonate due to the increase in pH.

There is a high influx of calcium ions and excessive efflux of protons and to survive and compensate for the loss of the protons, the cells on its outside export calcium. The dissolved inorganic carbon and calcium ions in the environment causes the extracellular precipitation of calcium carbonate. (Pandey & Prabhu, 2019)

2.2.4.3.3. Carbonic anhydrase producing bacteria:

It was found out in the article of biosequestration of carbon dioxide using carbonic anhydrase from novel *Streptomyces kunmingensis* by Sangeetha et al., 2022, that at pH 7 and 28 °C, the carbonic anhydrase enzyme had its highest level of optimum activity. The main variables that affect sequestration rate and efficiency are pH and temperature. It was discovered that the pH range of 8.5 and the temperature range of 30°C were ideal for microbial metabolic activity (Sangeetha et al., 2022).

There are currently six genetic subgroups of carbonic anhydrases which are found out. Carbonic anhydrase is a superfamily of metalloenzymes that catalyse the reaction between CO_2 and bicarbonate. The Bacteria, Archaea and Eukarya kingdoms require carbon dioxide, bicarbonate and protons for their daily physiological processes. Due to this a high concentration of carbonic anhydrase enzyme is present in the cellular compartments of these organisms. The physiological processes which take place with carbonic anhydrase enzyme are respiration, the conversion of carbon dioxide and bicarbonate and its transport, homeostasis of carbon dioxide, secretion of electrolytes in tissues, calcification processes, various biosynthetic reactions and various other processes (Supuran, 2016). The bacteria are negatively charged, when external calcium sources are present, positive charge Ca^{2+} gets adsorbed on their surfaces. This provides the nucleation sites for the deposition of mineralized products, which ultimately became calcite crystalline $CaCO_3$ (Zheng & Qian, 2020).

Prokaryotic carbonic anhydrase can range in molecular weight from 45 to 60 kDa. There are three structurally significant types of CA found in prokaryotes, namely, **α**, **β**, and **γ**. Of these, **β**, **γ** are found in bacteria, whereas, **α** is only occasionally seen in bacteria (Sangeetha et al., 2022).

Carbonate deposition is mostly promoted by carbonic anhydrase which is produced by microorganisms. It has been found out that the hydration of carbon dioxide and hydrogen bicarbonate can be accelerated by carbonic anhydrase. The carbon dioxide and hydrogen bicarbonate is known to react with the hydroxide and calcium ions which lead to the formation of calcium carbonate. The calcium carbonate precipitation is highly observed in the alkaline environment and the presence of calcium sources (Zheng & Qian, 2020).

Zinc ions are present in carbonic anhydrase enzyme, due to which it is classified as a metal enzyme. It has a catalytic role as it helps in the reaction reversibility in the conversion of carbon dioxide and hydrogen carbonate. In the research article, 'Influencing factors and formation mechanism of $CaCO_3$ precipitation induced by microbial carbonic anhydrase' by Tianwen Zheng and Chunxiang Qian, it was stated that the bovine red blood cells, dunaliella and bacteria produced carbonic anhydrase which was involved in the crystallization of calcium carbonate. This was known as microbial induced calcium carbonate precipitation (MICP).

The reactions which take place during the formation of calcium carbonate induce by carbonic anhydrase are as follows:

1) hydrated aqueous form of carbon dioxide is produced from gaseous carbon dioxide. It is a reversible reaction:

CO_2 (g) ↔ CO_2 (aq)

2) carbonic acid is produced when carbon dioxide reacts with water:

CO_2 (aq) + H_2O ↔ H_2CO_3

3) hydrogen ions and bicarbonate are produced by the ionization of carbonic acid in water:

H_2CO_3 ↔ $H^+ + CO_3^-$

4) carbonate ion and water is produced from bicarbonate ions in alkaline conditions:

$HCO_3^- + OH^-$ ↔ $CO_3^{2-} + H_2O$

5) Calcium carbonate precipitates in the presence of calcium ions and carbonate ions:

$Ca^{2+} + CO_3^{2-}$ ↔ $CaCO_3$

(Zheng & Qian, 2020)

2.2.5. Esterase enzyme of carbonic anhydrase:

To detect the presence of carbonic anhydrase, some amount of inoculum is added to the reaction solution and observed for colour change. The appearance of yellow colour indicates the presence of carbonic anhydrase. In this reaction, p-nitrophenyl acetate acts as a substrate of carbonic anhydrase enzyme. A change in yellow colour is seen due to the hydrolysis of p-nitrophenyl acetate. This indicates that carbonic anhydrase enzyme forms bicarbonate which reacts with the calcium ions of phosphogypsum in alkaline conditions and forms calcium carbonate.

The *Lysinibacillus sphaericus* is known to produce carbonic anhydrase which regulates bicarbonate metabolism. This metabolism is responsible for the incorporation of Ni metalloenzyme into the active site of the urease enzyme. It leads to the activation of the urease enzyme which further hydrolyses urea into ammonia and carbon dioxide. The bicarbonate

formed in this process forms carbonate ions in alkaline conditions. The carbonate ions react with calcium ions of calcium sulphate (Kamble& Prabhu, 2020).

2.2.6. Test sensitivity for the detection of urease and carbonic anhydrase

In a microcentrifuge tube cap, pH-sensitive dye encapsulated in cellulose can detect ammonia or carbon dioxide, which is followed by pH increase induced by urease activity or pH decrease induced by carbonic anhydrase. The CA assay yields only subtle color changes compared to negative controls containing acetazolamide. This is due to the rapid and spontaneous dehydration of bicarbonate even in the absence of enzymes, which turns the meta-cresol violet indicator yellow within 30 minutes. However, detection of CA hydratase activity by any method is limited by its speed. Since it is a non-enzymatic reaction, it should always be compared with a negative control for a given reaction time.

Various factors affect the quality of the CA field detection method, such as, incubation period on ice, close comparison to negative controls at specific time points, lower sensitivity as compared to other assays. Low-temperature incubation limits non-enzymatic CO_2 hydration more than CA-mediated reactions and is essential for visualizing reaction time differences between negative control and CA-incubated reactions. (Medina Ferrer et al., 2020).

2.2.7. *Lysinibacillus sphaericus:*

The *Lysinibacillus sphaericus* belongs to a group of aerobic, spore-forming, mesophilic, Gram-positive, rod-shaped bacteria that can thrive in mildly alkaline conditions and temperatures as low as 30°C (Wang et al., 2020).

In the article, Efficient bioconversion of sugarcane bagasse into polyhydroxybutyrate (PHB) by *Lysinibacillus* sp. and its characterization, it was stated that the growth and poly-**β**-hydroxybutyrate of *Lysinibacillus* was found to be the highest when glucose was used as a carbon source and its growth decreased when sucrose was used as carbon source, indicating that the particular bacteria has certain nutritional parameters which need to be met for its efficient working to continue the processes. From 30 to 37 C, *Lysinibacillus sp.* exhibited a continuous rise in cell growth. Agitation increased the oxygen mass transfer rate, which caused

oxygen to become soluble and increased in bacterial cell oxygen consumption (Saratale et al., 2021).

In an article, by it was stated that with a 17-E2 degradation rate of roughly 97%, *Lysinibacillus* bacteria have a great capacity to break down oestrogen, and they also create the secondary metabolite estrone. It implies that the bacteria are widely used for biodegradation (Wang et al., 2020).

2.2.8. Temperature optimization for biotransformation:

The temperature plays a significant role in the biotransformation mechanism as the culture should be able to adapt and perform its biochemical process. The optimum temperature for the growth of *Lysinibacillus* was found to be 37ºC. The most typical environmental stress is temperature variation, which can have an impact on bacterial growth and energy transfer. Increased heat shock protein (HSP) synthesis, which can function as chaperones or proteases, is a component of the stress response in microorganisms. In contrast to proteases, chaperons aid in the proper folding and assembly of proteins. The regulation of mesophiles development and functional activity is greatly influenced by temperature. High temperatures damage the cell membranes; low temperatures reduce cellular activity due to cellular freezing; and at an ideal temperature of 30°C, the metabolic processes of the cell operate to their fullest potential (Ghosh et al., 2021).

2.2.9. SDS-PAGE:

SDS-PAGE can show how protein synthesis changes in response to a particular metal (Ghosh et al., 2021). The molecular sizes and charges of proteins can be used to separate them since these elements affect how quickly they move through a gel. Proteins are denaturized using the detergent sodium dodecyl sulphate (SDS). In the SDS-PAGE method, the SDS pulls the denaturated proteins along a polyacrylamide gel using an electric current (PAGE). When SDS is present at 0.1%, it binds to proteins very tightly, with one detergent molecule often attaching to two amino acids. Proteins acquire a negative charge when heated with SDS in proportion to their molecular size, which causes them to move through the acrylamide gel at different rates

depending on their molecular sizes (Abdulhamid A. Al-Tubuly, n.d.). The polymer of acrylamide monomers known as polyacrylamide acts as a sieve by forming a gel matrix that slows the movement of larger molecules more than smaller molecules. An electrical field causes proteins to move through the matrix's pores. The amount of acrylamide present affects how big the pores are. The size of the pores in the gel matrix decreases with increasing acrylamide concentration. The gel pore size, together with the protein's charge, size, and shape, all affect how quickly the protein migrates. The proteins will migrate differently after a certain length of time, depending on the voltage across the gel; smaller proteins will go further down the gel while larger proteins will stay nearer to the source (Joanne M. Manns, 2011).

2.3. Gaps in research

1. The role of urease and carbonic anhydrase in the biotransformation mechanism of phosphogypsum has not been reported.

2. The amount of protein production during the biotransformation of PG by *L. sphaericus* is not known.

2.4. Aim and objectives

2.4.1. Aim

To understand the mechanism of biotransformation of phosphogypsum in the presence of urease and carbonic anhydrase produced by *L. sphaericus.*

2.4.2. Objectives

1. Optimization of the pH for the biotransformation of phosphogypsum by *L. sphaericus*
2. Growth curve of *L. sphaericus* during the biotransformation of PG.
3. To study the activity of urease and carbonic anhydrase production by *L. sphaericus* during the biotransformation of phosphogypsum to calcium carbonate crystals.

3. MATERIALS AND METHODS

3. Materials and methods:

3.1. Gram staining:

The culture of *Lysinibacillus sphearicus* was kindly provided by the guide. It was isolated from soil contaminated with PG (Patil et al. 2021). A thin smear of the culture grown on nutrient agar was prepared on a clean, air-dried slide, using 0.85% saline. The crystal violet stain was used to flood the slide for 1 minute. The slide was rinsed with tap water. Gram's iodine was added to the slide and kept for 1 minute, followed by rinsing with tap water and also rinsing with alcohol for 15 seconds. The slide was again rinsed with tap water. The counterstain, safranin, was added to the slide for 45 seconds which was later followed by rinsing with tap water. The slide was air-dried observed under oil immersion lens of 100X objective using a light microscope.

3.2. Endospore staining:

A thin smear of one week old bacterial culture was prepared using 0.85% of saline on a glass slide and allowed it to air dry. The smear was then covered with a piece of blotting paper and flooded with 0.5% malachite green dye. Slide was steamed for 10 min using burner by keeping the slide moist. After 10 min, slide was rinsed under running water and then counter stained with safranin for 1 min slide and then air dried and observed under oil immersion lens of 100X magnification.

3.3. Urease test:

The culture of *L. sphearicus* was streaked on Christenson's urea agar slant and incubated at 37ºC for 24 hours. The urease activity was detected by the colour change from yellow to pink in the agar.

3.4. Motility test:

SIM medium was used to detect sulfur reduction, indole production and motility. The SIM agar stab were prepared in test tube and a loop of the culture was stabbed in the agar and incubated at 37ºC for 24 hours.

3.5. Carbonic anhydrase test:

Carbon dioxide sensitive strips were prepared by soaking the cellulose paper with fresh 0.00065M Na_2CO_3, 0.01% metacresol purple, 50% glycerol diluted with nitrogen purged distilled water. The papers were dried immediately using a steam of hot air and circles were cut and placed in the cap seals of the tubes. Cold 1M $NaHCO_3$ (80 **µ**l) and 15 **µ**l of sample was added in the tubes. The tubes were closed and incubated on ice.

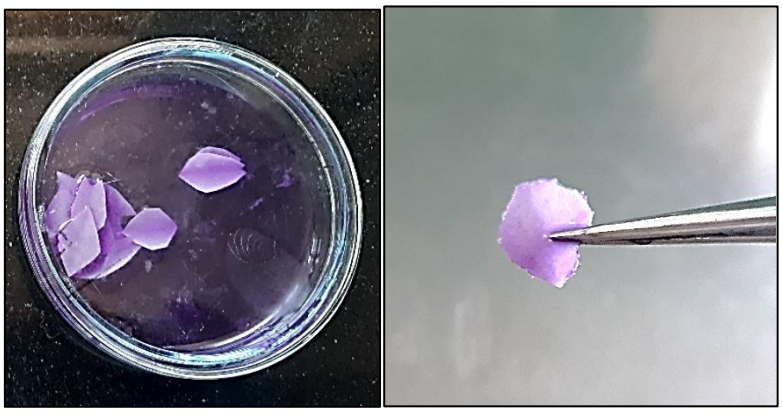

Fig. 3.5.: Carbon dioxide sensitive strips dyed with m-cresol purple

3.6. pH determination of the phosphogypsum biotransformation flasks:

Three flasks were prepared, one with only SF medium, the second with SF medium plus culture, and the third with SF medium containing phosphogypsum and culture. The pH was initially adjusted to 7.5 ± 0.2 before autoclaving. After autoclaving the readings were taken as day 0 till day 7 using the pH meter. The experiment was done in triplicates.

3.7. Growth of *L. sphearicus* in different media and study of biotransformation mechanism*:*

Eighteen flasks were prepared with modified SF medium; the pH of the media was adjusted to 7.5 before autoclaving. The experimental setup was made as per the following table no. 3.6:

Table no. 3.7: Setup of the experiment.

Components	**1. Blank (Media)**	**2. Culture control**	**3. Test (PG)**	**4. Control (PG)**	**5. Test ($CaCl_2$)**	**6.Control ($CaCl_2$)**
SF media	+	+	+	+	+	+
Culture	-	+	+	-	+	-
PG/$CaCl_2$	-	-	PG	PG	$CaCl_2$	$CaCl_2$

Triplicates of the culture control, test flasks (phosphogypsum and calcium chloride) and control flasks (phosphogypsum and calcium chloride) were prepared and were kept on shaker conditions for incubation at room temperature. The absorbance was taken at 600 nm for each sample for a period of 8 days starting from day 0 to day 7 and data was analysed.

3.8. Ammonium sulphate precipitation test:

The Barium chloride (1 ml of 10%) was added to the *L. sphaericus* culture supernatant (1 ml) from test tube number 3 from the experimental set up above in section 3.6. This would lead to the formation of a white precipitate which will confirm the presence of $(NH_3)_2SO_4$ that may be formed during biotransformation.

3.9. Acid test for precipitation test:

The precipitate from test tube no. 3 from the experimental setup in section 3.7 was taken on a clean glass slide and a few drops of 2M Hydrochloric acid was added to it, to check for the presence of effervescence.

3.10. Protein estimation by Folin-Lowry method:

In clean test tubes, different concentration (starting from 0.1 to 1 ml) of the Bovine serum albumin solution was taken with the total volume of 1 ml adjusted by using distilled water. Alkaline copper sulphate solution (Reagent A) (5 ml was added to each test tube, mixed well and kept at room temperature for 10 minutes. A blank was prepared with 1 ml of distilled water instead of the protein solution and the abovementioned steps were followed. The samples of the culture grown in flasks containing PG, calcium chloride and only culture control (see section 3.6) and the pellet and supernatant test samples of PG, $CaCl_2$ and the control culture samples obtained the rom the protein extraction method 1 (section 3.11.1) were prepared separately using 1 ml of the respective culture sample without any dilution. Folin's reagent (0.5 ml) was added to the test tubes after the incubation of 10 minutes and mixed. The test tubes were kept in dark for a 30 minutes incubation period. The absorbance was measured at 660 nm using the spectrophotometer and protein concertation was determined by comparing against standard BSA concertation.

3.11. Protein extraction:

Samples (2 ml) from each flask from the experimental setup 3.6. were taken after every 24 hours for the period of 7 days and stored at -20ºC. Two methods were used for protein extraction for the samples as follows.

3.11.1. Method 1:

The sample (1ml) was taken in the tube and centrifuged at 14000 rpm for 5 minutes at 4ºC. The supernatant which was approximately 1 ml was taken in a sterile tube and 2 ml of 99% ethanol was added to it. The contents in the tube were mixed well and incubated at 4ºC for 30 minutes. After the incubation period was over, the tube was centrifuged at 14000 rpm for 10 minutes. The supernatant was discarded and the pellet was air dried and later resuspended in 100 μl sterile distilled water.

The pellet which was obtained at the start after the centrifugation of the 1 ml sample at 14000 rpm for 5 minutes was suspended in 1 ml of 20 mM Tris buffer with 5 mM EDTA and 5 mM $MgCl_2$. The samples were then sonicated in the sonicator (Model no: LMUC-3) for 3 times for 2 minutes with a break of 3 minutes on ice each time after each cycle. After sonication, the tubes were centrifuged at 14000 rpm for 10 minutes at 4ºC. The pellet was discarded and the supernatant was stored in the -20ºC freezer.

3.11.2. Method 2:

The samples (750 µl) were taken in the tubes and centrifuged at 10000 rpm for 4ºC for 10 minutes. Lysozyme (50 μl) was added in each pellet. The tubes were incubated at 37ºC for 45 minutes, while tapping the pellet after every 10 minutes. After the incubation was over, the tubes along with the sample were centrifuged at 10000 rpm at 4ºC. The supernatant was transferred into sterile tubes. 500 μl of 20% Trichloroacetic acid was added to the supernatant and the protein was made to precipitate by vortex the tubes for 5 to 7 minutes. After vortexing, the tubes were centrifuged at 10000 rpm for 10 minutes at 4ºC. The pellet was later dissolved in 100 μl of sample buffer and the supernatant was discarded.

3.12. SDS-PAGE:

The electrophoresis assembly was set by adding the spacers and tapping the edges with the help of clamps for support. Agar (1%) was used to seal the three sides of the gel trays to prevent leaking of the gels. Resolving gel matrix (12%) was prepared, mixed thoroughly and poured in between the plates till the level was below 2 cm from the edge

of the notch plate. Ethanol was added above the resolving gel while it was solidifying to prevent the entry of oxygen which would hamper the polymerization process. After the gel was set, ethanol from the top of the gel was drained off. Stacking gel of 5% concentration was prepared and mixed properly before pouring above the resolving gel. The comb was inserted in the stacking gel immediately after pouring. Once the gels, were set the combs were removed and the well were washed with sterile distilled water to remove the non-polymerized acrylamide. Drain off the excess water using a Whatman filter paper. The gel trays were assembled on the electrophoresis unit and the bottom and top reservoir of the unit was filled with 1X Tris-Glycine buffer, the running buffer for SDS-PAGE. The samples to be loaded into the wells were prepared by the protein extraction method. Each of the samples (20 **µ**l) were added in the tubes and 20 **µ**l of the 2X sample loading buffer was added in the tubes. The tubes were kept in the water bath at 80ºC for 5 minutes. The protein sample (20 **µ**l) from the tubes were loaded into each well. The cables were connected to the power supply as per the conventional, i.e., red for anode and black for cathode. After connecting, the run was started at 50V for half an hour and later increase the voltage to 100V till the run is over. The gel was stained overnight using the staining solution. The gel was destained by replacing the staining solution with the destaining solution till clear bands are visible. The bands were observed under the light transilluminator and compare it using the ladder marker.

4. RESULTS AND DISCUSSIONS

4. Results and discussion:

4.1. Gram character:

Upon gram staining and observation under 100X oil immersion objective lens of the light microscope, the gram character of the culture of *Lysinibacillus sphaericus* was found to be Gram positive rods as they were observed as rod in shape and stained purple in colour (fig.4.1).

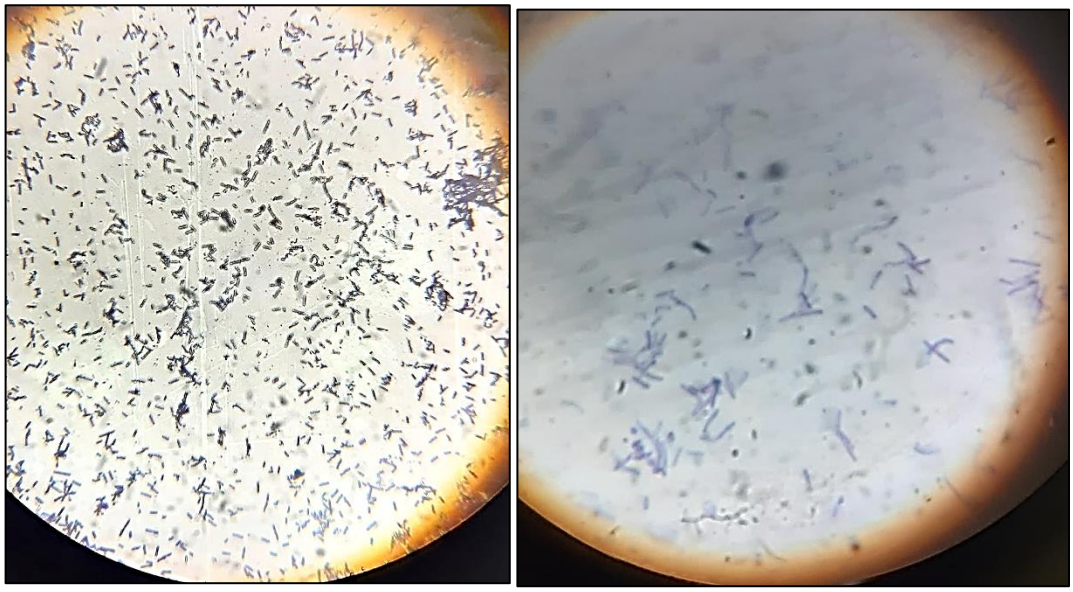

Fig 4.1: Gram character observed under 40X (left) and 100X (right)

Fig.4.1: Gram staining of *Lysinibacillus sphaericus.* Purple color-stained spherical structures were observed when viewed under 100X oil immersion

4.2. Endospore staining:

The *L. sphearicus* was incubated for 48 to 72 hours and stained by the Shaeffer and Fulton method. Endospores were observed as green, oval spore shape whereas the vegetative cells were pink in color (fig. 4.2).

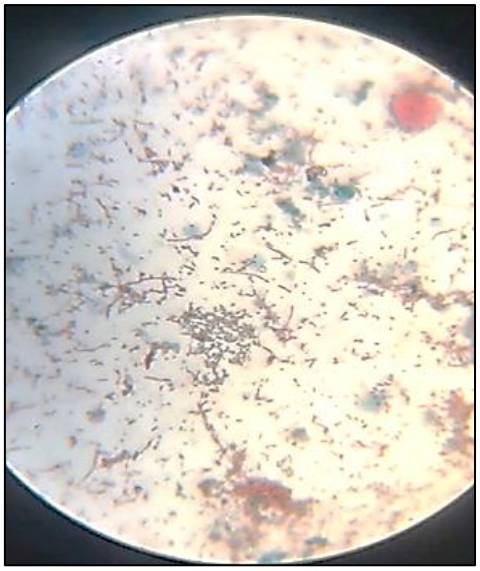

Fig. 4.2. Endospore staining of *L. sphaericus.* The endospore is visible as green dots and the vegetative cells are seen as pink colored rod shaped structures.

4.3. Urease test:

The colour of the slant changed from yellow to brilliant pink after streaking the culture on Christensen's urea agar slant. This demonstrated the presence of the urease in *L.sphaericus*, which spilt urea into ammonia and carbon dioxide when water was present. Ammonia and carbon dioxide combine to generate ammonium carbonate, which raises the pH of the medium to an alkaline level and gives it a bright pink color (fig. 4.3).

Fig. 4.3.: *L. sphaericus* streaked on Christenson urea agar changes color from yellow (control tube on left) to bright pink (tube on right).

4.4. Motility test:

The culture was stabbed in the SIM stab agar. After incubation for 24 hours, no haziness was observed around the stab line, which indicated that the culture was not motile (fig. 4.4).

Fig. 4.4.: Motility test: No haziness around the stab line

4.5. Carbonic anhydrase test:

The carbonic anhydrase of the culture was observed by using the indicator meta-cresol purple which was initially stained bluish purple and in the presence of carbon dioxide gets converted to purplish yellow color (fig.4.5).

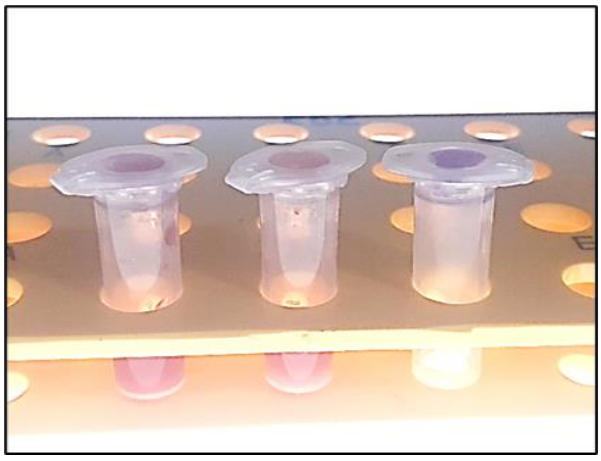

Fig. 4.5.: Shows the results of the carbonic anhydrase test. The paper strip on top of the control tube (right) that contains only distilled water, no colour change from purplish blue is observed. The paper strip on top of tube (to the left) contains the culture, the color of the paper

strip has changed from bluish purple to light yellowish purple indicating the presence of carbonic anhydrase enzyme. The paper strip in the middle tube containing the culture and phosphogypsum, has changed the color from bluish purple to purplish yellow. This colour change when compared with the paper strip of the control culture tube was more yellowish, implying that the bacteria produces higher amount of carbonic anhydrase in the presence of phosphogypsum.

4.6. Acid test for the precipitate formed:

When hydrochloric acid reacts with calcium carbonate it forms calcium chloride, carbon dioxide and water, leading to the formation of effervescence. The precipitate produced by the culture grown in PG, upon reaction with HCl, on a clean glass slide produced effervescence indicating presence of calcium carbonate in the precipitate (fig. 4.6).

Fig. 4.6.: Shows results of the acid test. The effervescence on the slide indicates the presence of calcium carbonate.

4.7. Ammonium sulphate precipitation test:

When the suphate from phosphogypsum is mixed with the ammonia created as a result of urea hydrolysis by the *L. sphaericus* that produces urease, ammonium sulphate is formed. The ammonium sulphate reacts with barium chloride, and forms barium sulphate and ammonium chloride. Barium sulphate is the white precipitate formed can be seen in fig. 4.7.

Fig. 4.7: The white test tube (left) indicates the presence of barium sulfate and the transparent coloured test tube is a control test tube (right).

4.8. pH optimization:

There was an increase in the pH in all the three flasks from the initial day 0 till day 7. The pH of the medium in the phosphogypsum flask during the start of the experiment (for the initial 2 days) was around 9 which later dropped to 8 and stabilized at 8.4 for the remaining days. The pH in the flask containing culture and SF media kept increasing until it reached 9.25. The pH of the control flask (SF media) was around 9 during the initial day and later over the incubation period it dropped down to 8. From the fig. 4.8, it can be stated that the culture preferred a alkaline environment to live and on the addition of phosphogypsum its pH decreased by 1 unit and it would still survive and perform its biochemical processes efficiently (fig. 4.8).

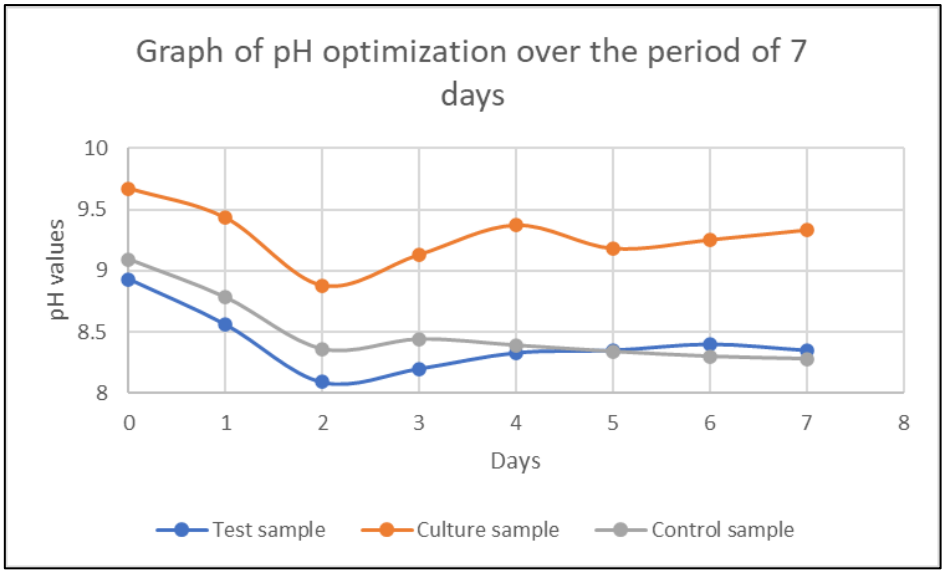

Fig. 4.8: Graph of pH optimization over the period of 7 days

4.9. Growth behaviour of *L. sphaericus* under various growth conditions:

The average OD of the cultures from the triplicates of the experimental setup (section 3.7) were taken and compared with the control set up, to know the difference in the growth pattern. From fig. 4.9.1., it can be seen that there was an increase in the growth curve of PG and $CaCl_2$ as compared to the growth seen in the culture control. The OD of the PG test samples went from 0.639 units (day 0) to 3.065 units (day 7), an increase of 2.426 units was seen. The OD of the $CaCl_2$ samples was 0.508 at day 0 and it increased to 2.358 units at day 7, an overall increase of 1.85 units was seen. The OD of the culture control on day 0 was 0.098 units and at the end of the incubation period (day 7), it was 0.65, a total increase of 0.552 units was seen. It implies that the *L. sphaericus* requires an additional calcium source to carry out its biochemical processes efficiently. The growth in the culture control was the least as compared to PG and $CaCl_2$ sample. The growth of *L. sphaericus* was higher (by 0.576 units) in the PG test samples as compared to the $CaCl_2$ test samples (Fig. 4.9.1.).

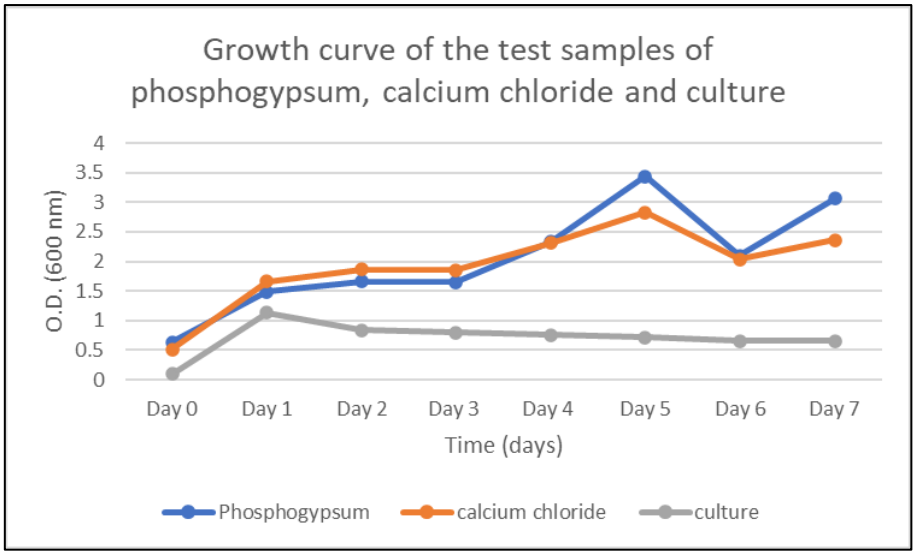

Fig. 4.9.1. Growth curve of the test samples of phosphogypsum, calcium chloride and culture control.

As seen in Fig. 4.9.2., there is an increase in the growth of all the samples starting from day 0, except for the culture in control flask. The OD of the culture control at day 0 was 0.098 units and it rose to 1.127 units on day 1, later reduced by 0.284 units on day 2 and eventually reduced to 0.65 units on day 7. An increase of 0.552 units was seen in the culture control flasks during the incubation period. The OD of the $CaCl_2$ test sample was 0.51 units on day 0, a sudden peak of 2.83 units was seen in the growth curve on day 5, and later on day 7 the OD dropped down to 2.359 units. The total increase of OD seen during the incubation period was 1.849 units. The absorbance of the PG test samples at day 0 was 0.64 which increased to 3.44 units on day 5 and reduced to 2.1 units on day 6. A drop in day 6 might be the indication of the death phase. There is growth from day 7 (OD 3.07) implying that the culture shows delayed and uneven growth patterns during the biotransformation process. Total increase of 2.43 units was seen in the absorbance from day 0 to day 7. In the control flask of PG, the initial absorbance was 0.56 on day 0 and 2.44 on day 7, a total increase of 1.88 units was seen. The OD of the control flasks of $CaCl_2$ increased from 0.36 units on day 0 to 2.18 units on day 7, showing a total increase of 1.82 units (Fig 4.9.2.). There was growth in the control flasks probably due to contamination.

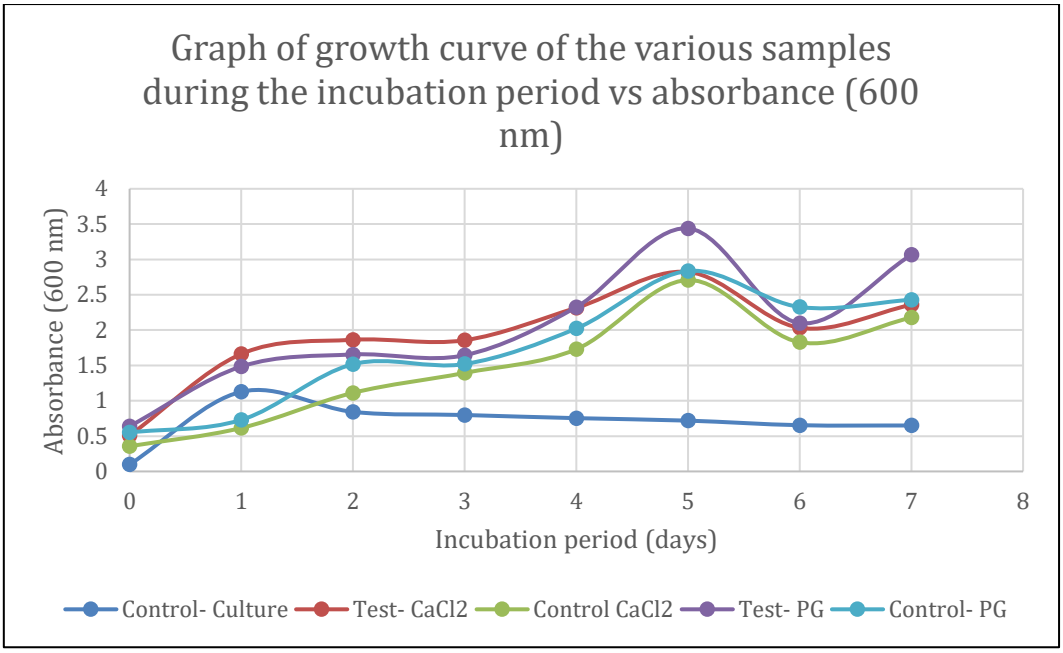

Fig. 4.9.2.: Growth curve of the test samples (PG and $CaCl_2$) and control samples (PG, $CaCl_2$ and culture).

4.10. Protein estimation by Folin Lowry's method:

4.10.1. Standard protein solution (Bovine serum albumin):

The Fig 4.10.1. shows the graph obtained from the protein estimation of the standard Bovine serum albumin by the Folin Lowry's method at 660 nm absorbance (Fig. 4.10.1.).

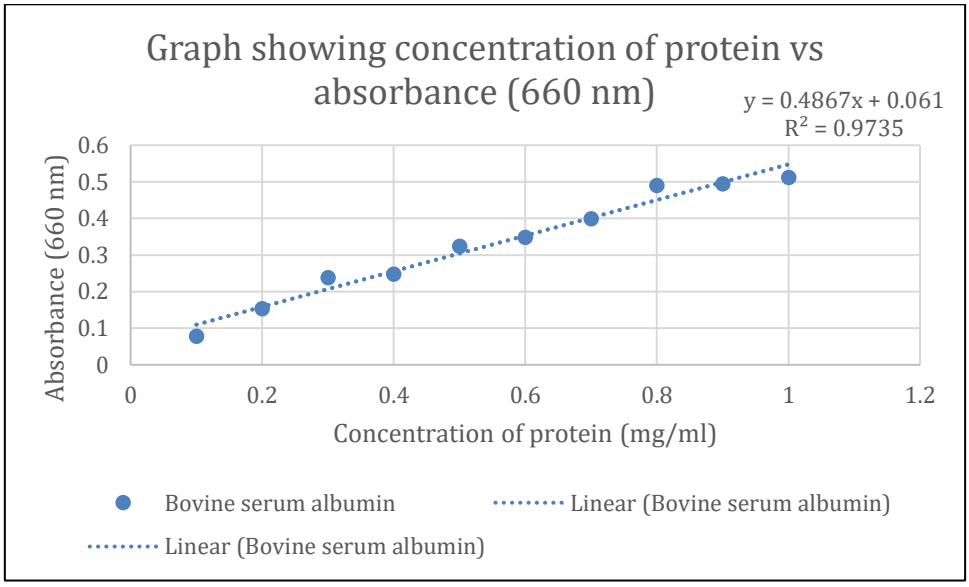

Fig. 4.10.1.: Graph of estimation of Bovine serum albumin protein by Folin Lowry's method

4.10.2 Test samples of PG, $CaCl_2$ and culture control

The average protein concentration in the PG test samples was 1.79 mg/ml. The highest protein concentration was 2.23 mg/ ml on day 1 and the lowest protein concentration was 1.29 mg/ml. The protein concentration has decreased from day 0 to day 7 by 0.66 mg/ml which may the nutrients in the media were depleted over time.

The average protein concentration in the culture control samples was 1.27 mg/ml. The highest protein concentration was 1.59 mg/ ml on day 6 and the lowest protein concentration was -0.13 mg/ml on day 2. The protein concentration has increased from day 0 to day 7 by 0.15 mg/ml. There is not much increase observed in the protein content, this may be because of the lack of a calcium source in the media.

The average protein concentration in the $CaCl_2$ test samples was 3.04 mg/ml. The highest protein concentration was 3.7 mg/ ml on day 2 and the lowest protein concentration was 2.23 mg/m on day 0. The protein concentration has increased from day 0 to day 7 by 1.06 mg/ml (Fig. 4.10.2.). This might be because the bacteria prefer $CaCl_2$ over PG as a calcium source.

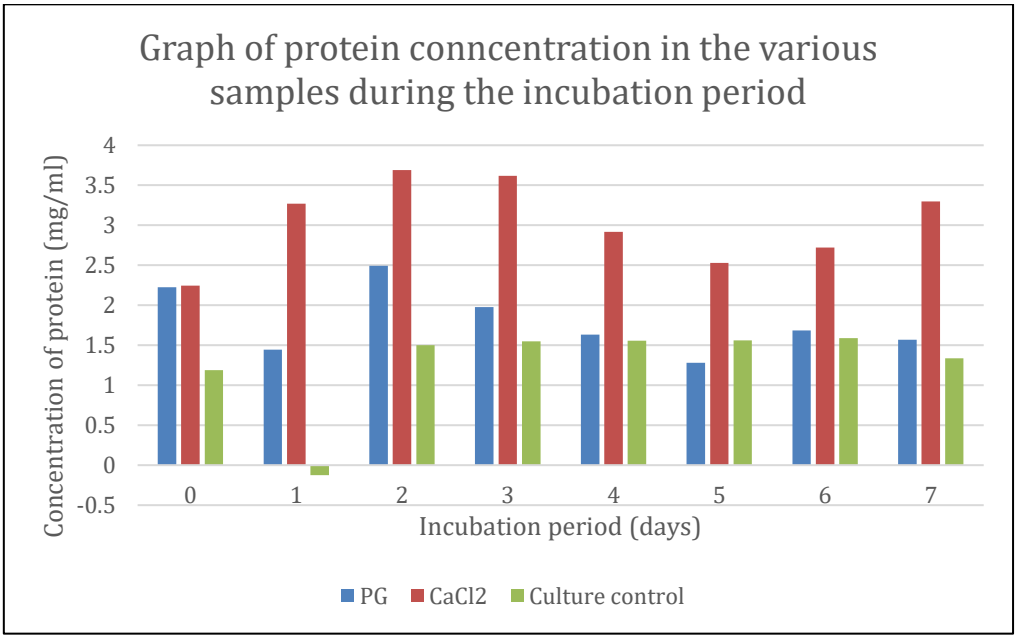

Fig. 4.10.2: Graph of the protein concentration in the test samples of PG, $CaCl_2$, and culture control during the incubation period

4.10.3. Pellet from the PG test samples

In Fig. 4.10.3., the average protein concentration in the pellet of the PG test samples obtained from the protein extraction method was 0.24 mg/ml. The highest protein concentration was 0.08 mg/ ml which was on day 6 and the lowest protein concentration was -0.023 mg/ml on day 1. The protein concentration has increased from day 0 to day 7 by 0.031 mg/ml (fig. 4.10.3). The protein concentration in the pellet has increased from day 0 to day 7.

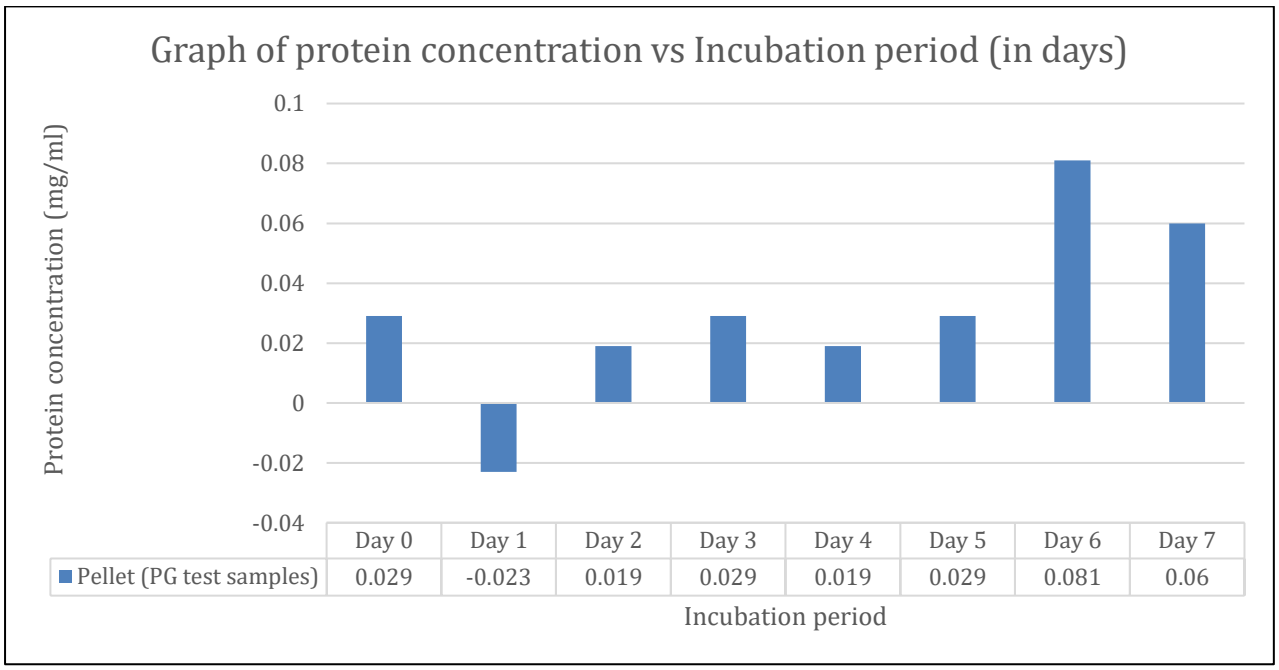

Fig. 4.10.3.: Graph of protein estimation of the pellet from the phosphogypsum test samples by Folin Lowry's method

4.10.4. Supernatant from the test samples of $CaCl_2$

The average protein concentration in the supernatant of the $CaCl_2$ test samples was 0.67 mg/ml. The highest protein concentration was 2.69 mg/ ml on day 0 and the lowest protein concentration was 0.19 mg/ml on day 6. The protein concentration increased by 2.42 mg/ml from day 0 to day 7 (fig. 4.10.4). The protein concentration was the highest on day and later decreased over the time period.

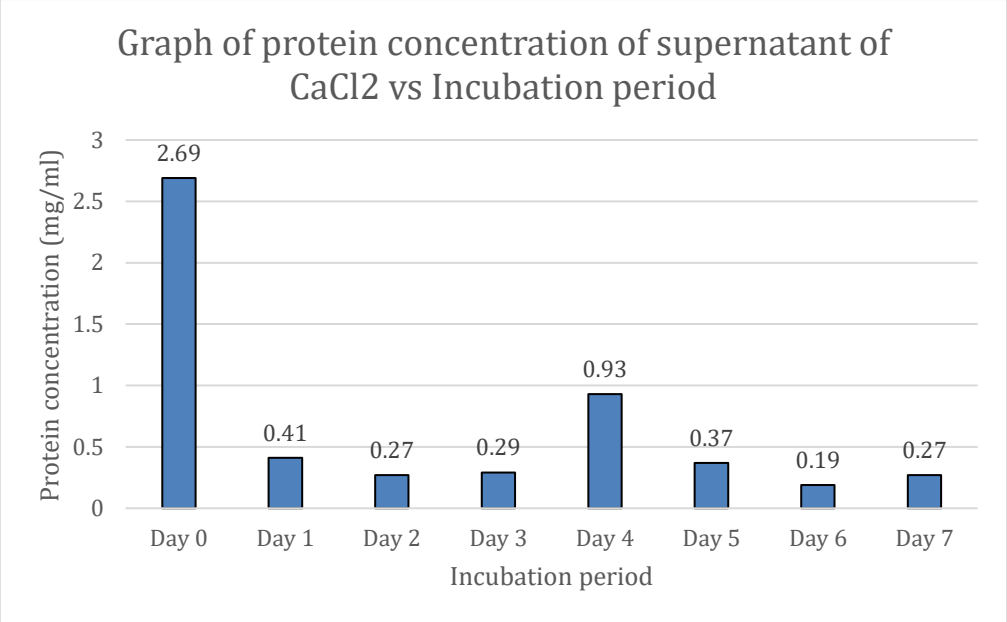

Fig. 4.10.4: Graph of the protein concentration of the supernatant of $CaCl_2$ obtained from the protein extraction method

4.11. SDS-PAGE:

4.11.1. Using method 1:

In fig. 4.11.1., 1 band was observed in the SDS-PAGE gel upon destaining and viewing under the light transilluminator. The band corresponds to the marker band (on the right) having molecular weight around 25 kDa which may indicate the presence of carbonic anhydrase which has a molecular weight of approx. 29 kDa. The samples loaded in the wells were of the phosphogypsum culture supernatant obtained from the method 1 of protein extraction (fig. 4.11.1.1). Ramazan Demirdağ et al. in an article, Purification and characterization of carbonic anhydrase from sheep kidney, stated that molecular weight of carbonic anhydrase is 29 kDa (Ramazan Demirdağ et al., 2012).

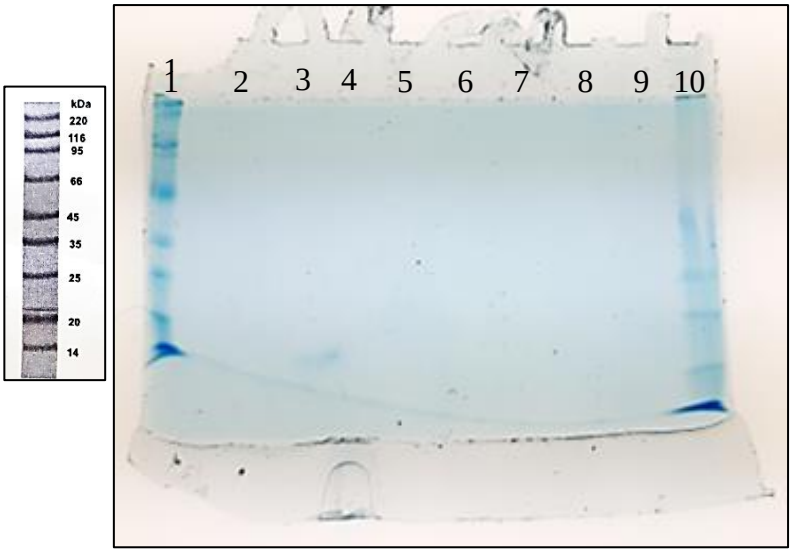

Fig. 4.11.1.1.: Gel 1 of SDS PAGE. The marker on the extreme left and right is a hi-range marker having a Molecular weight of 14-220 kDa. The samples loaded from left to right in the SDS-PAGE are the supernatant of the test samples of phosphogypsum starting from day 0 to day 7.

In the fig 4.11.1.2., four bands were observed in the gel after destaining and viewing under the light transilluminator. All the four bands were formed from the pellet of the culture grown. Mid-range marker was used to detect the molecular weights of the unknown bands (fig. 4.11.1.2.). The second band corresponds to the marker band of 95 kDa which would be approx. 90 kDa. Varsha Pawar et al (2022) in the article, 'Investigating purification and activity analysis of urease enzyme extracted from Jack bean source' stated that the urease enzyme is obtained as a single band at 90 kDa on a SDS-PAGE (Varsha Pawar et al., 2022).

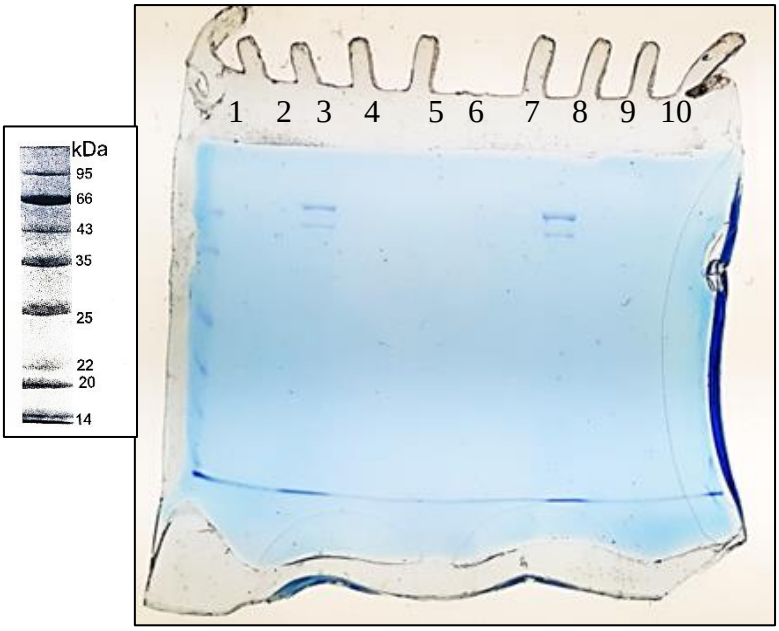

Fig. 4.11.2: Gel 2 of SDS-PAGE. On the left of the SDS-PAGE, the midrange protein marker having a molecular weight of 14-95 kDa is shown to correspond with the bands on the gel. The samples for protein estimation were taken from the supernatant and pellet obtained in the protein extraction method. The protein extraction method was conducted in 2 steps and from each step the supernatant and pellet are estimated. The samples in the wells of the SDS-PAGE from the extreme left to right are as follows:
1: Mid-range protein marker
2: culture sample- supernatant (step 1)
3: culture sample- pellet (step 2)
4: culture sample- supernatant (step 2)
5: culture sample- pellet (step 1)
6: phosphogypsum sample- supernatant (step 1)
7: phosphogypsum sample- supernatant (step 2)
8: phosphogypsum sample- pellet (step 2)
9: phosphogypsum sample- pellet (step 1)
10: midrange protein marker

4.11.2. Using method 2:

In the fig. 4.11.2., a smear of band was seen in the last well with prominent bands at the bottom. Faint bands were observed in wells 5 and 6 and prominent bands were observed in well 4, 5, 6 and 7 at the bottom (fig. 4.11.2). A mid-range marker was used. The bands corresponds to the marker bands at approx 29 kDa, indicating the presence of carbonic anhydrase production in the culture and phosphogypsum samples which states that the carbonic anhydrase enzyme was used in biotransformation mechanism and the formation of dark coloured bands states that the enzyme was produced in huge amount.

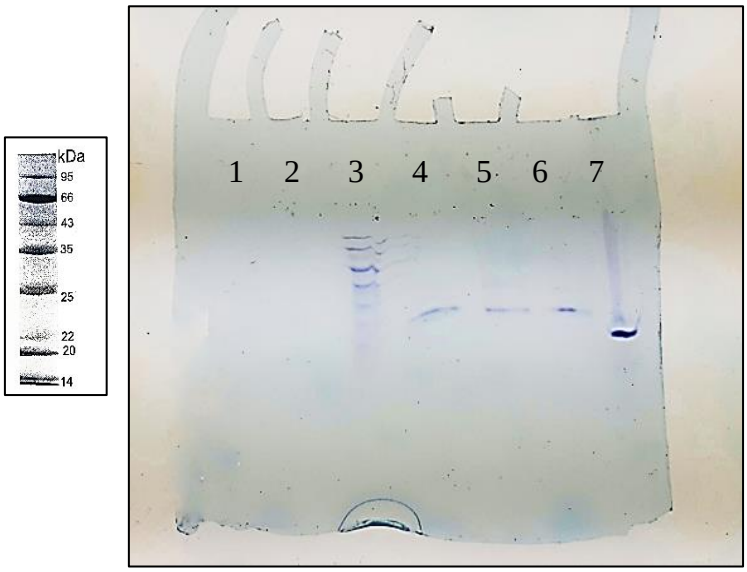

Fig 4.11.2.: shows the SDS-PAGE gel from method 2. The samples loaded in the wells of the SDS-PAGE from the extreme left to right are as follows:
1: phosphogypsum sample: supernatant from day 7
2: culture sample: supernatant from day 7
3: midrange marker (14-95 kDa)
4: phosphogypsum sample: pellet from day 7
5: culture sample: pellet from day 7
6: phosphogypsum sample: pellet from day 0
7: culture sample: pellet from day 0

5. CONCLUSION

5. Conclusion:

The *L. sphaericus* is a gram-positive, rod-shaped, non-motile bacteria. It contains the activity of urease as it was able spilt urea into ammonia and carbon dioxide when water was present. The urease activity of the culture was confirmed by two tests, the first is the color change of Christenson's urea agar from yellow to pink when streaked with the culture and the second is ammonium sulphate precipitation as ammonium sulphate is produced from the sulphate from PG and the ammonium is produced by the culture as a result of urea hydrolysis by urease. The pH of the medium increases when ammonium carbonate is formed after ammonia and carbon dioxide combines, increasing the pH of the medium. The colour change of the m-cresol purple indicator from bluish purple to purplish yellow indicated that the bacteria was able to produce carbonic anhydrase and the colour change was more when PG was added in the media. The formation of effervescence in the acid test confirmed the presence of calcium carbonate in the precipitate formed at the bottom of the flasks during incubation.

The pH of the PG, culture control and media control flasks on optimization over the incubation period was found to be 8.4, 9.25 and 8, respectively. An uneven growth pattern was observed in all the flasks of the experimental setup 3.6, where there was an increase in the growth pattern of the test and control flasks of PG and $CaCl_2$ and then it reduced on day 6 on the incubation period and started increasing again from day 7. The culture control flask had an increase in the growth pattern during the initial days and later it started decreasing.

The Folin-Lowry's method of protein estimation showed the highest protein concentration in the test samples of $CaCl_2$ followed by the PG test samples. The difference in the protein concentration between $CaCl_2$ test samples (1.06 mg/ml) and PG test samples (0.66 mg/ml) was 0.4 mg/ml. The protein concentration in the culture control (0.15 mg/ml) was the least amongst the other two. The protein concentration of the supernatant (2.42 mg/ml) obtained from the protein extraction method was higher than the protein concentration of the pellet (0.037 mg/ml) obtained by 2.38 mg/ml. The protein concentration in the pellet increased from day 0 to day 7 whereas the protein concentration in the supernatant decreased from day 0 to day 7.

SDS-PAGE was conducted using two protein extraction methods and protein bands were observed in both methods. The protein bands from the supernatant and pellet were obtained from method 1 but from method 2 protein bands were obtained only from the pellet. The protein bands obtained on the gel corresponded to the protein marker having the molecular weight band range of 29 kDa and 90 kDa which is also the molecular weight of carbonic anhydrase

and urease, respectively, indicating the production of urease and carbonic anhydrase by *L. sphaericus.*

6. FUTURE PROSPECTS

6. FUTURE PROSPECTS:

1. The contaminants in the phosphogypsum powder and its role in biotransformation has not been studied.
2. The enzymes produced by the *L. sphaericus* for biotransformation are extracellular or intracellular was not determined.
3. The estimation of the SDS-PAGE protein bands on the NATIVE-PAGE

7. APPENDIX

7.1. APPENDIX I- MEDIA

Components	grams/litre
1. Nutrient agar	
Peptic digest of animal tissue	5.000
Sodium chloride	5.000
Beef extract	1.500
Yeast extract	1.500
Agar	15.000
Final pH (at 25ºC)	7.4±0.2
2. Christenson's urea agar	
Peptic digest of animal tissue	1.000
Dextrose	1.000
Sodium chloride	5.000
Disodium phosphate	1.200
Monopotassium phosphate	0.800
Phenol red	0.012
Agar	15.000
Final pH (at 25ºC)	6.8±0.2

3. SF Medium

Nutrient broth	3.00
Urea	20.00
$CaCl_2.2H_2O$	1.4-5.6
NH_4Cl	10.00
$NaHCO_3$	2.12

4. Modified SF Medium

Nutrient broth	3.00
Urea	20.00
Phosphogypsum	5.00
$NaHCO_3$	2.12

5. Nutrient broth

Peptic digest of animal tissue	5.000
Sodium chloride	5.000
Beef extract	1.500
Yeast extract	1.500
Final pH (at 25ºC)	7.4±0.2

6. SIM Medium

Pancreatic digest of casein	20.000
Peptone	6.100
Peptonized iron	0.200
Sodium thiosulphate	0.200

7.2. APPENDIX II- CHEMICALS AND REAGENTS

1. **20% urea solution-** 2g of urea was dissolved into 10 ml of distilled water and the urea solution was filter sterilized.
2. **2M HCl-** 0.7292 ml HCl into 10 ml of distilled water.
3. **10% $BaCl_2$-** 0.1g of barium chloride was dissolved into 10 ml of distilled water.
4. **Gram staining reagents:**

. Crystal violet (1%)

Crystal violet	2.0 g
Absolute ethanol	20.0 ml
Ammonium oxalate	0.8 g
Distilled water	100 ml

b. Grams iodine

Iodine crystals	1.0 g
Potassium iodide	2.0 g
Distilled water	100 ml

c. Decolorizer- 70% ethanol

d. Safranin (0.25%)

Safranin	0.25 g

Absolute ethanol	10 ml
Distilled water	100 ml

5. Endospore staining reagents

- Malachite green (0.5% w/v)

Malachite green	0.5 g
Distilled water	100 ml

- Safranin

Safranin	2.5 g
95% ethanol	100 ml

6. Carbonic anhydrase assay

Na_2CO_3	6.89 mg
$NaHCO_3$	8.40 mg
m-cresol purple indicator dye	3.83 mg
Glycerol	50 ml
Distilled water	100 ml

7. Folin-Lowry method

- Preparation of reagent A:

  Reagent A= solution 1 + solution 2

Solution 1 = 2% alkaline sodium carbonate in 0.1M NaOH

Solution 2 = 0.5% copper sulphate in 1% sodium potassium tartarate

Reagent A = 50 ml solution 1 + 1 ml solution 2

- Bovine albumin serum = 1 mg/ml
- Folin's reagent

8. SDS-PAGE

I. Acrylamide-bisacrylamide stock solution (30%)

Acrylamide	2 g
N,N-bisacrylamide	1 g
Deionized water	100 ml

(store in amber coloured bottle)

II. Resolving gel buffer (pH 8.8) (100 ml)

1.5 M Tris	18.75 g
Deionized water	100 ml

III. Stacking gel buffer (pH 6.8) (100 ml)

1 M Tris	12.14 g
Deionized water	100 ml

IV. 10% Ammonium persulfate solution (APS) (100 ml)

Ammonium persulfate solution	10 g
Deionized water	100 ml

V. 10% sodium dodecyl sulphate (SDS) (100 ml)

SDS	10 g
Deionized water	100 ml

VI. 1% bromophenol blue (100ml)

Bromophenol blue	1 g
Deionized water	100 ml

VII. 1X Tris-glycine buffer (pH 8.3) (100 mll)

25 mM Trisbase	0.302 g
250 mM Glycine	18.755 g
0.1% SDS	100 ml of 10% stock solution

VIII. 2X sample/gel loading buffer (pH 6.8) (10 ml)

50 mM Tris-chloride	1 ml of Tris stock solution

200 mM **β**-mercaptoethanol	250 **µ**l
2% SDS	4 ml of 10% SDS stock solution
0.1% bromophenol blue	2 ml of 1% stock solution
10% glycerol	2 ml

IX. Staining solution (100 ml)

Coomassie brilliant blue	0.25 ml
Methanol	45 ml
Glacial acetic acid	10 ml
Deionized water	45 ml

X. Destaining solution (100 ml)

Methanol	45 ml
Glacial acetic acid	10 ml
Deionized water	45 ml

XI. 6N HCl

Molecular weight	36.46 MW
Assay	35.38%
Density	1.18 g/ml

XII. Preparation of gels

Components (total volume)	12% resolving gel (10 ml)	5% stacking gel (4 ml)
Deionized water	3.3 ml	2.7 ml
30% acrylamide mix	4 ml	0.67 ml
1.5 M Tris (pH 8.8)	2.5 ml	-
1.5 M Tris (pH 6.8)	-	0.5 ml
10% SDS	0.1 ml	0.4 ml
10% APS	0.1 ml	0.4 ml
TEMED	0.004 ml	0.004 ml

8. REFERENCES

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1. Abdulhamid A. Al-Tubuly. (n.d.). SDS-PAGE and Western Blotting. *Diagnostic and Therapeutic Antibodies*, 391–405.
2. Arianna Rath, Mira Glibowicka, Vincent G. Nadeau, Gong Chen, & Charles M. Debe. (2009). Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. *PNAS*, *106*(6), 1760–1765.
3. Dorota Wolicka, & Wlodzimierz Kowalski. (2006). *Biotransformation of phosphogypsum on distillery decoctions (preliminary results)*. *55*(2), 147–151.
4. Dorota Wolicka, Wlodzimierz Kowalski, &HankaBoszczyk-Maleszak. (2005). *Biotransformation pf phosphogypsum by bacteria isolated from petroleum-refining wastewaters*. *54*(2), 169–173.
5. EzzeddineSaadaoui, Naziha Ghazel, Chokri Ben Romdhane, Nouman, & Nouman Massoudi. (2017). *Phosphogypsum: Potential uses and problems – a review*. *74*(4), 558–567.https://doi.org/10.1080/00207233.2017.1330582
6. Ghosh, S., Gandhi, M., Van Hullebusch, E. D., & Das, A. P. (2021). Proteomic insights into Lysinibacillus sp.-mediated biosolubilization of manganese. *Environmental Science and Pollution Research*, *28*(30), 40249–40263.https://doi.org/10.1007/s11356-020-10863-4
7. Guo, Tingzong, Malone, Ronald F., &Rusch, Kelly A. (2001). Stabilized phosphogypsum: Class C fly ash: Portland II cement composites for potential marine applications. *Scopus*, *35*(15), 3967–3973.https://doi.org/10.1021/es010520+
8. Ines Hammas-Nasri, Karima Horchani-Naifer, Mokhtar Férid, & Donatella Barca. (2016). Rare earths concentration from phosphogypsum waste by two-step leaching method. *International Journal of Mineral Processing*, *149*, 78–83.https://doi.org/10.1016/j.minpro.2016.02.011
9. Joanne M. Manns. (2011). SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Proteins. *John Wiley & Sons, Inc.*https://doi.org/10.1002/9780471729259.mca03ms22
10. Kamble, P., & Prabhu, Dr. M. (2020). *Biotransformation of phosphogypsum to calcium carbonate using by Lysinibacillussphaericus strain GUMP2*. Goa University.
11. Kozicki, C., & Carlson, C. (n.d.). *Phosphogypsum – An Industrial ByproductWith Value*. FEECO INTERNATIONAL.https://feeco.com/phosphogypsum-an-industrial-byproduct-with-value/

12. Liu, M., Liang, Z., Ma, H., Huang, L., & Wang, M. (2010). *Responses of rice (Oryza sativa L.) growth and yield to phosphogypsum amendment in saline-sodic soils of North-East China. J+*}ik<*
13. Lozano, L. C., Ayala, J. A., &Dussan, J. (2011). Lysinibacillussphaericus S-layer protein toxicity against Culex quinquefasciatus. *Biotechnol Lett*.
14. Mahmoud, E., & Abd El-Kader, N. (2015). *Heavy metal immobilization in contaminated soils using phosphogypsum and rice straw compost.26*(8), 819–824.
15. Márcia Aiko Shirakawa, Maria Alba Cincotto, Daniel Atencio, Christine C.Gaylarde, &Vanderley M. John. (2011). *Effect of culture medium on biocalcification by Pseudomonas Putida, LysinibacillusSphaericus and Bacillus Subtilis*. 499–507.https://doi.org/10.1590/S1517-83822011000200014
16. Medina Ferrer, F., Hobart, K., & Bailey, J. V. (2020). Field detection of urease and carbonic anhydrase activity using rapid and economical tests to assess microbially induced carbonate precipitation. *Microbial Biotechnology*, *13*(6), 1877–1888.https://doi.org/10.1111/1751-7915.13630
17. Nascimento, R., Souza, J.A, Moreira, A., &Moraes, L.A.C. (2017). *Phosphogypsum and vinasse application: Soil chemical properties and alfalfa productivity and nutritional characteristics*. *30*(1), 213–219.
18. Ortega-García, J.A., López-Hernández, F.A, Cárceles-Álvarez, A., Fuster-Soler, J.L., Sotomayor, D.I., &Ramis, R. (2017). *Childhood cancer in small geographical areas and proximity to airpolluting industries. Environmental Research,.156,* 63–73.
19. Pandey, S., & Prabhu, Dr. M. (2019). *Mcirobially induced phosphogypsum biotransformation using urolytic bacteria Lysinibacillus sp.* Goa University.
20. Patil, P.P., Prabhu, M., & Mutnuri, S. (2023). A novel and sustainable approach for biotransformation of phosphogypsum to calcium carbonate using urease producing Lysinibacillus sphaericus strain GUMP2. *Environmental Technology*,44(2), 226-269.
21. *Radioactive Material From Fertilizer Production*. (2021, November 30). US EPA.https://www.epa.gov/radtown/radioactive-material-fertilizer-production#:~:text=Phosphogypsum%2C%20a%20waste%20product%20from,elements%20uranium%2C%20thorium%20and%20radium.
22. Ramazan Demirdağ, VeyselÇomaklı, Murat Şentürk, Deniz Ekinci, Ö İrfan Küfrevioğlu, &Claudiu T Supuran. (2012). Purification and characterization of carbonic anhydrase from sheep kidney and effects of sulfonamides on enzyme activity. *Bioorg Med Chem*.https://doi.org/10.1016/j.bmc.2012.08.018

23. Sangeetha, M., Sivarajan, A., Radhakrishnan, M., Siddharthan, N., &Balagurunathan, R. (2022). Biosequestration of carbon dioxide using carbonic anhydrase from novel Streptomyces kunmingensis. *Archives of Microbiology*, *204*(5), 270.https://doi.org/10.1007/s00203-022-02887-w
24. Saratale, R. G., Cho, S. K., Saratale, G. D., Ghodake, G. S., Bharagava, R. N., Kim, D. S., Nair, S., & Shin, H. S. (2021). Efficient bioconversion of sugarcane bagasse into polyhydroxybutyrate (PHB) by Lysinibacillus sp. And its characterization. *Bioresource Technology*, *324*, 124673.https://doi.org/10.1016/j.biortech.2021.124673
25. Shaw, D. R., &Dussan, J. (2018). Transcriptional analysis and molecular dynamics simulations reveal the mechanism of toxic metals removal and efflux pumps in Lysinibacillussphaericus OT4b.31. *International Biodeterioration & Biodegradation*, *127*, 46–61.https://doi.org/10.1016/j.ibiod.2017.11.016
26. Supuran, C. T. (2016). How many carbonic anhydrase inhibition mechanisms exist? *Journal of Enzyme Inhibition and Medicinal Chemistry*, *31*(3), 345–360.https://doi.org/10.3109/14756366.2015.1122001
27. Suvra Roy, & Vikash Kumar. (2014). A Practical Approach on SDS PAGE for Separation of Protein. *International Journal of Science and Research (IJSR)*, *3*(8).https://d1wqtxts1xzle7.cloudfront.net/77872949/MDIwMTU0MDk_-libre.pdf?1641099262=&response-content-disposition=inline%3B+filename%3DA_Practical_Approach_on_SDS_PAGE_for_Sep.pdf&Expires=1682790364&Signature=CLu4bDHnYuWK1OScaQsHPjR26ZIiH6J0XzY54Xf9kK7Jw2zCbY3yRd0jVKqi25zpaFEPlC0NoC~4Eu4W6sIN9jkSh-T~xIsM0DL81pGO45F1IAZbJJx2JHIJOpGFvaTMIdOHERD5MFXnu-8kj4FzdCOqvyBQxwI5x3jcmufni3EyICM-A3EaPdkX8NhYyRlSX-CpSvKLTz-9Rm3hGXzVEVKABnvjIPbq~V7nAK7eUN0LnS9j-SgpKF7Rjwfpft~pWX3bE4Y8BDRvaVwMEcsUhfhmcOgWYALAo6yQtTUNphfn~IClXPEATQzn-IvNd-ACIlrOBxfnBh1E9QAt4IscMw__&Key-Pair-Id=APKAJLOHF5GGSLRBV4ZA
28. Varsha Pawar, DhanashreeBhande, Shvaji Pawar, HarsihMudila, Ajeet Kaushik, & Anil Kumar. (2022). Investigating purification and activity analysis of urease enzyme extracted fromjack bean source: A green chemistry approach. *Analytical Biochemistry*, *659*(15).https://doi.org/10.1016/j.ab.2022.114925
29. Wang, Y., Zhao, X., Tian, K., Meng, F., Zhou, D., Xu, X., Zhang, H., & Huo, H. (2020). Identification and genome analysis of a novel 17β-estradiol degradation bacterium,

Lysinibacillussphaericus DH-B01. *3 Biotech*, *10*(4), 166.https://doi.org/10.1007/s13205-020-2155-0

30. Wu, F., Ren, Y., Qu, G., Liu, S., Chen, B., Liu, X., Zhao, C., & Li, J. (2022). Utilization path of bulk industrial solid waste: A review on the multi-directional resource utilization path of phosphogypsum. *Journal of Environmental Management*, *313*, 114957.https://doi.org/10.1016/j.jenvman.2022.114957
31. Xiaoyu Lin, Li Gan, Gary Owens, &Zuliang Chen. (2023). *Removal of cadmium from wastewater using biofunctional reduced graphene oxide synthesized by Lysinibacillussphaericus*. *383*.https://doi.org/10.1016/j.jclepro.2022.135369
32. Yassine Ennaciri, & Mohammed Bettach. (2021). *Comparative study of the transformation of phosphogypsum and pure gypsum into valuables products*. *4*(3), 165–174.https://doi.org/10.22034/AJCA.2021.273885.1242
33. Zheng, T., & Qian, C. (2020). Influencing factors and formation mechanism of CaCO3 precipitation induced by microbial carbonic anhydrase. *Process Biochemistry*, *91*, 271–281.https://doi.org/10.1016/j.procbio.2019.12.018

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INTRODUCTION:

Hazardous waste, particularly radioactive waste, is a distinct type of industrial wastes. Chemical industries (organic and inorganic); oil refining; and thermal processes are the major sources of hazardous waste (Ines Hammas-Nasri et al., 2016). The primary by-product of the production of phosphoric acid is the acidic digestion of phosphate rock, which results in the formation of phosphogypsum. It has a number of impurities, including radioactive and rare earth elements, fluorine compounds, residual acid (P_2O_5), and trace elements. During the process of making phosphoric acid, the rare earths are concentrated in phosphogypsum. Phosphate gypsum contains between 70% and 85% of the phosphate rock's original contents. The remaining portion remains dissolved in the phosphoric acid-containing leaching solution. Phosphate gypsum is an excellent source of rare earths due to the fact that rare earths are merely a by-product of the production of phosphoric acid and the enormous quantities of phosphate rock that are processed annually. When its large volumes are taken into consideration, phosphogypsum, which has low concentrations of rare earths, may therefore provide significant quantities of these elements (Ines Hammas-Nasri et al., 2016).

Phosphorus is a mineral found in phosphate rock that is used in some fertilisers to aid in the development of robust roots in plants. Small levels of naturally occurring radionuclides, primarily uranium and radium, can be found in phosphate rock. By dissolving the rock in an acidic solution, the phosphorous is extracted from phosphate rock while processing it to generate fertiliser. Phosphogypsum is the name given to the waste that is produced. This waste is where the majority of the naturally occurring uranium, thorium, and radium contained in phosphate rock ends up. Radium, a radioactive gas, is produced when the elements uranium and thorium decay into it. Phosphogypsum is more radioactive than the natural phosphate rock because of the concentrated wastes. In huge piles known as stacks, the waste generated during fertiliser manufacture is kept. Some stacks are hundreds of feet high and span hundreds of acres.