Biomineralization of PET (Polyethylene terephthalate) plastic through Sporosarcina species for plastic waste management

Author

Muhammad Kamran Shahzad

Reg. No. 00000327109

Supervisor

Dr. Adeeb Shehzad

Department of Biomedical Sciences and Engineering

School of Mechanical & Manufacturing Engineering (SMME)

National University of Sciences and Technology (NUST)

Islamabad, Pakistan

September 2023

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Author

Muhammad Kamran Shahzad

Reg. No. 00000327109

A thesis submitted in partial fulfillment of the requirements for the degree of

MS Biomedical Sciences

Thesis Supervisor: Dr. Adeeb Shehzad

Thesis Supervisor's Signature: _____

Biomedical Sciences

School of Mechanical & Manufacturing Engineering (SMME)

National University of Sciences and Technology (NUST)

Islamabad, Pakistan

September 2023

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Signature (HOD): 

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Name: Dr. Adeeb Shehzad

Signature:

Dr. Adeeb Shehzad
Associate Professor
Departmen of Biomedical Engg. & Sciences
School of Mechanical & Manufacturing
Engineering (SMME), NUST
Islamabad

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بسم الله الرحمن الرحيم

Acknowledgement

All praise is to ALLAH Almighty, the most Beneficent, the most merciful who bestowed upon me with the sight to observe, the mind to think and the courage to work more. I could complete my research work only because of Allah's help, guidance, and blessing.

My deepest and sincerest gratitude goes to my Supervisor, Dr. Adeeb Shehzad, whose support and guidance helped me through every problem I faced in research as well as in my life. Thank you for being the best mentor.

I am deeply indebted to my father Mumtaz Hussain and my mother Zahra Bibi. I am grateful to those who gave me my name and my life, and everything in between. Though I am skilled with words, I struggle to express the extent of their efforts in giving me the life I have now. Their contributions are the reason I was able to accomplish this. I would like to acknowledge my siblings Salama Bibi, Fauzia Bibi, Haseeb Ur Rehman, Summar Mumtaz, Abu Bakar, and Daniyal Mumtaz who always inspired me to achieve more and more in life.

I would like to express special thanks to my friends Sadia Manzoor, Faiza Tariq, Nabeel Ahmed and Muzammal Raza who are the reason I thrive to be better and without whom I would be deprived of the love, support, and advice that helped me through all. I will forever be grateful for their constant support and care throughout my research work.

I would like to express my gratitude to Dr. Iftikhar Ahmed (PSO, NARC) for being a listener to my problems, for helping me out wherever possible, and for inculcating positivity in me at every hurdle. In conclusion, I would like to extend my sincere appreciation to all those who have provided valuable support throughout my research.

Muhammad Kamran Shahzad

Dedication

I would like to Dedicate this thesis to my parents and adored siblings and friend Sadia Manzoor and Faiza Tariq, as they inspire me to be strong in every path of life and criticizes me to see me grow and face all the challenges in life to achieve every success that come along. All that I have done is because of their tremendous support, unfailing love and cooperation who led me to this wonderful accomplishment.

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List of Acronyms

MICP	Microbial induced calcium carbonate precipitation
Mt	Metric tonnes
MIT	Massachusetts Institute of Technology
NCCP	National cell culture collection Pakistan
NARC	National Agriculture Research Center
SMME	School of mechanical and manufacturing engineering
P.R China	People Republic of China
UV	Ultraviolet
PCBs	Polychlorinated biphenyls
DDT	Dichlorodiphenyltrichloroethane
SP	*Sporosarcina pastuerii*
BS	*Bacillus subtillus*
pH	Potential hydrogen
RPM	Revolution per minute
PET	Polyethylene terephthalate
CPM	Calcium carbonate precipitation media
PRC	Plastic reinforced cementitious materials
PRM	Plastic reinforced matrix
TSA	Tryptic soya agar
TSB	Tryptic soya broth
XRD	Xray diffraction
FTIR	Fourie transform infrared microscopy.
SEM	Scanning electron microscopy

Abstract

Purpose: This research work was done as an essential assignment to complete my thesis. It was aimed at finding an indigenous Sporosarcina. species that can produce calcium carbonate precipitates both in absence and presence of PET flakes through ureolysis. So that this strain in future can be used in treating PET flakes that make these flakes compatible with cement concrete (when added in concrete) and decrease the gaps between the PET flakes and cement matrix.

Materials and Methods: Three Sporosarcina closely related species bacteria strains (NCCP-2331, NCCP-2222 and NCCP-2716) and one already reported of producing calcium carbonate precipitates through ureolysis *Bacillus subtilus* were provided by National culture collection of Pakistan. The experiment that was designed included mixing of these strains overnight grown culture, CPM (Calcium carbonate precipitation media) that included ammonium chloride, sodium bicarbonate, calcium chloride and urea and in one case 1g PET flakes were added while in other case no flakes. Negative control was also run along the experiment in that flask there was not any bacteria colony inoculated. Then the precipitates were analyzed by XRD, FTIR and SEM to confirm their structure.

Results: All three strains had shown ureolytic activity and had produced calcium carbonate precipitates both in the absence and presence of PET flakes. Among these three strains NCCP-2331 had shown the more capability to produce calcium carbonate precipitates and its precipitates were more crystalline while NCCP-2716 and NCCP-2222 precipitates were more amorphous. XRD, FTIR and SEM analysis confirmed the structure of calcium carbonate crystals. SEM analysis also confirmed the formation of biofilm and calcium carbonate layer over the PET flakes.

Conclusion: Among three strains NCCP-2331 had shown the best capability to produce calcium carbonate precipitates when it was provided calcium carbonate precipitation media. That indigenous strain can be used in future for biotreatment of plastic flakes while adding these flakes in cement matrix.

Keywords: NCCP (National culture collection of Pakistan), PET (Polyethylene terephthalate), Calcium carbonate, cement matrix

CHAPTER 1

1 INTRODUCTION

1.1 Research background:

Plastic is a significant and rapidly expanding waste stream on a global scale, as evidenced by the generation of 368 million tons of plastic waste in the year 2019. (Ritchie & Roser, 2018). The current rates of plastic recycling are characterized by a persistent low level, as a mere 9% of plastics undergo the recycling process, despite the presence of significant public interest in this matter. (Meidl, 2018). The economic feasibility of plastic recycling is constrained by the relatively inexpensive nature of virgin plastic, juxtaposed with the substantial expenses incurred during recycling procedures, including transportation, sorting, cleaning, and extrusion. This constraint results in the disposal of low-value type 3–7 plastics predominantly in landfills or through inadequate management practices, thereby causing environmental contamination. There is an urgent need for the implementation of novel strategies aimed at mitigating the quantity of plastic waste that is disposed of in landfills and subsequently released into the environment (Kane et al., 2021).

My research work was aimed at finding a solution for the waste of plastic pollution. Although it was a little effort as it was founded and was a requirement to attain master's degree, but that journey was hard working and fantastic. The major source of my research work was a research paper published by an MIT university researcher who made the brilliant effort to find a way to lower the burden of plastic pollution (Kane et al., 2021). Their idea was to add the low valued plastic s type 3-7 in the concrete and prepare plastic reinforced mortar that have the similar tensile strength as of conventional concrete. No doubt the addition of plastic waste in concrete is a unique idea and has been tried and tested but one major limitation is the gaps that are created between the plastic flakes and cement mortar that ultimately reduce the tensile strength of the PRC.

In solution of that they come up with the idea of microbially induced calcium precipitation (MICP) and enzymatically induced calcium carbonate precipitation. MICP is the process in which the microbes have the capability to produce calcium carbonate crystals when they are provided with the urea (Gebru et al., 2021; Stocks-Fischer et al., 1999; Henze & Randall, 2018). These microbes produce urease enzymes that hydrolyze the urea and then produce ammonium

ions and bicarbonate ions. When they are provided with calcium sources like calcium chloride, these microbes then produce calcium carbonate crystals. That technique is also known as biomineralization (Gebru et al., 2021; Stocks-Fischer et al., 1999; Henze & Randall, 2018). These scientists came up with the solution of biomineralization of plastic flakes before their addition in concrete. *Sporosarcina pastuerii* is the bacteria strain that has the capability to produce the urease enzyme and when it is provided with calcium and urea it produces the calcium carbonate crystals (Kane et al., 2021; Henze, 2018). When they added biomineralized plastic flakes in concrete then the PRCs prepared have the similar strength as of the conventional concrete (Kane et al., 2021). My research work was reproduced from these scientist work where first I found the Sporosarcina closely related species that can produce high amount of calcium carbonate crystals both in the presence and absence of plastic flakes. As my work was for thesis requirement so I kept my study limited to only one plastic type that polyethylene terephthalate (PET) or plastic bottles. For this purpose, three closely related species of sporosarcina were tested NCCP-2222, NCCP,2716, NCCP-2331 and along with these species *Bacillus subtilus* was used as positive control as it was easily available to us and reported in literature (Nasser et al., 2022) in its capability to produce calcium carbonate crystals. We were aiming to identify sporasarcina species that can biomineralize the PET flakes and the results of our study are impressive as among these three sporosarcina species NCCP-2331 performed a leading role in producing calcium carbonate crystals. The calcium carbonate crystals produced by NCCP-2331 were not only high in quantity compared with other bacterial strains but also showed fine peaks when observed by XRD. These calcium carbonate crystals were also confirmed by FTIR spectroscopy and SEM images as well. Another impressive thing was that the presence of PET flakes did not hinder our bacteria strains from producing calcium carbonate crystals. So, SEM images confirmed the presence of not only bacteria strain colonies but also calcium carbonate crystals.

1.2 Research objectives:

- To find out an indigenous sporosracina species strain that show the capability of producing calcium carbonate precipitates.
- To find out whether that strain is capable of producing calcium carbonate precipitates even in the presence of PET flakes.

- To confirm that the precipitates produced are calcium carbonate crystals through XRD and FTIR analysis.
- To confirm the formation of biofilm and calcium carbonate crystals over PET flakes through SEM analysis.

CHAPTER 2

2 LITERATURE REVIEW

2.1 Plastic pollution:

The production of Bakelite, the initial synthetic plastic, commenced in 1907, signifying the birth of the worldwide plastics industry. Nevertheless, the significant expansion of worldwide plastic manufacturing only became apparent during the 1950s. Over the course of the subsequent seven decades, there was a significant surge in the yearly output of plastics, experiencing an exponential growth of around 230 times, ultimately reaching a staggering 460 million tons by the year 2019 (Ritchie & Roser, 2018).

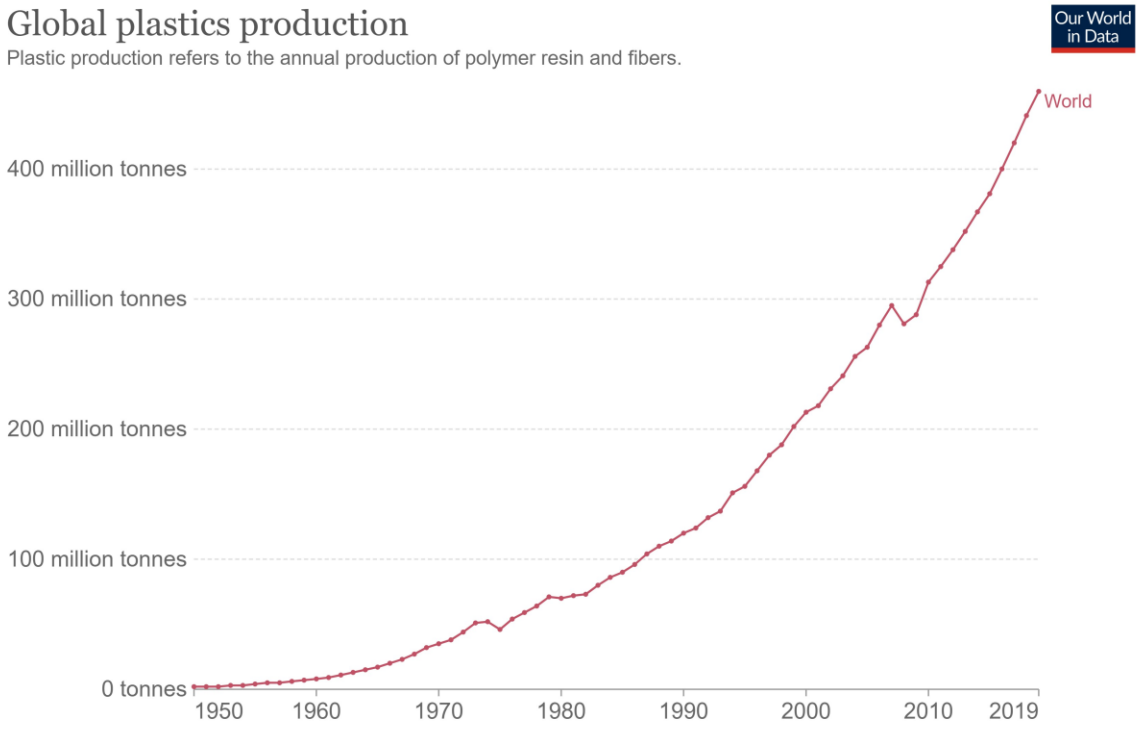

Figure 1: Global plastic pollution from 1950-2019 (Ritchie & Roser, 2018).

2.2 Plastic waste as global burden:

Since the year 2004, global plastic production has reached a level equivalent to that of the preceding 50 years. It has been estimated that the cumulative weight of newly produced polymers stands at 8.3 billion metric tonnes, predominantly sourced from natural gas and crude oil. These materials serve as both chemical feedstocks and energy sources (Ritchie &

Roser, 2018). In the year 2015, a total of 407 million metric tons (Mt) of plastics were manufactured, with packaging accounting for 164 Mt (equivalent to 36% of the overall production). Based on available data, it is evident that approximately 30% of the total plastic production, which amounts to 2.5 billion tonnes, is currently being utilized. Furthermore, the cumulative generation of primary and secondary plastic waste from 1950 to 2015 reached 6.3 billion tonnes. Among this waste, only 9% has undergone recycling, with a mere 10% of that being recycled more than once. Additionally, 12% of the plastic waste has been subjected to incineration, while the remaining 79% has been disposed of in landfills or has entered the natural environment (Ritchie & Roser, 2018).

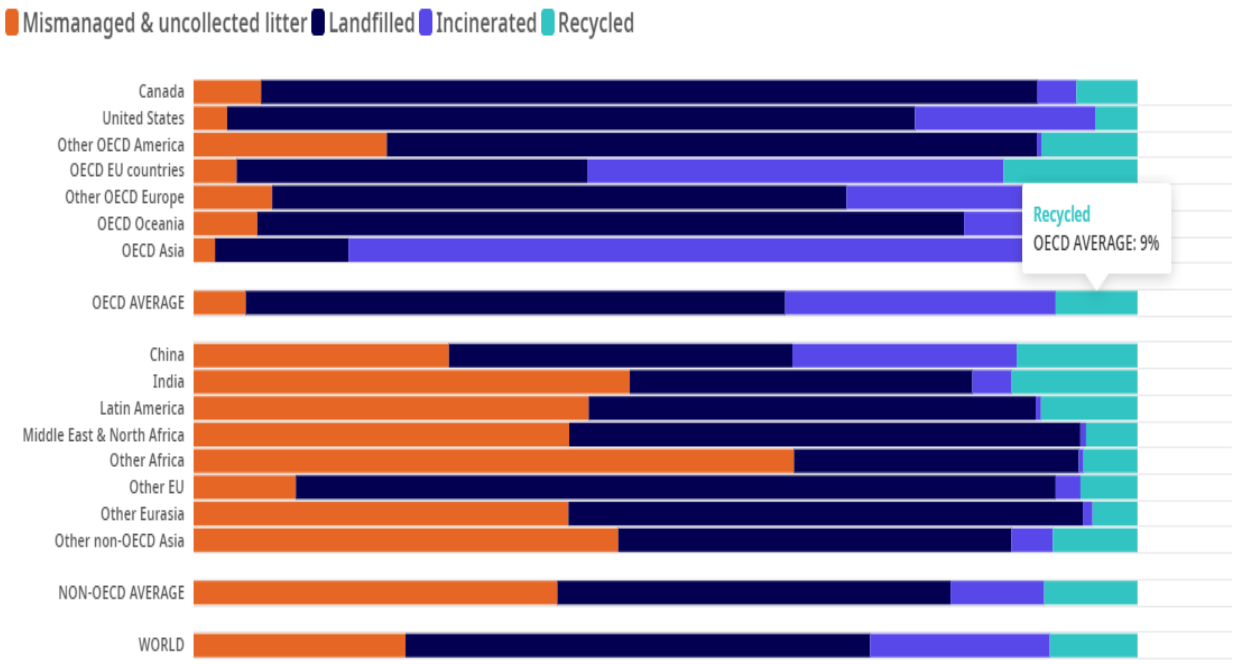

Figure 2: OECD countries management of plastic (Major chunk is either mismanaged or landfilled by each country. Very small quantity of plastic is recycled) (Ritchie & Roser, 2018)

The estimated proportion of plastic usage attributed to packaging ranges across different sources, but it is generally accepted that packaging constitutes approximately one third of total plastic consumption. Out of this amount, roughly 40% is disposed of in landfills, while 32% evades proper collection mechanisms. This evasion can occur through either non-collection or illegal dumping and mismanagement, resulting in direct environmental contamination (Ritchie & Roser, 2018) A mere 28% of packaging waste had been

successfully gathered, with half of this amount being subjected to incineration for the purpose of energy generation, while the remaining half underwent recycling processes. Based on current trends, it is projected that by the year 2050, there will be around 12 billion tonnes of plastic garbage present in landfills or dispersed in the natural environment. Furthermore, it is anticipated that the total mass of plastic in the oceans would surpass that of fish (Ritchie & Roser, 2018) The estimated quantity of plastic that infiltrated the oceans in 2010 was approximately 9 million metric tons (Mt). This influx of plastic was primarily attributed to the improper management of garbage. Additionally, an approximate amount of 0.5 Mt of microplastics originated from the laundering of synthetic textiles and the wear and tear of tires on roadways (Ritchie & Roser, 2018; MacLeod et al., 2021)

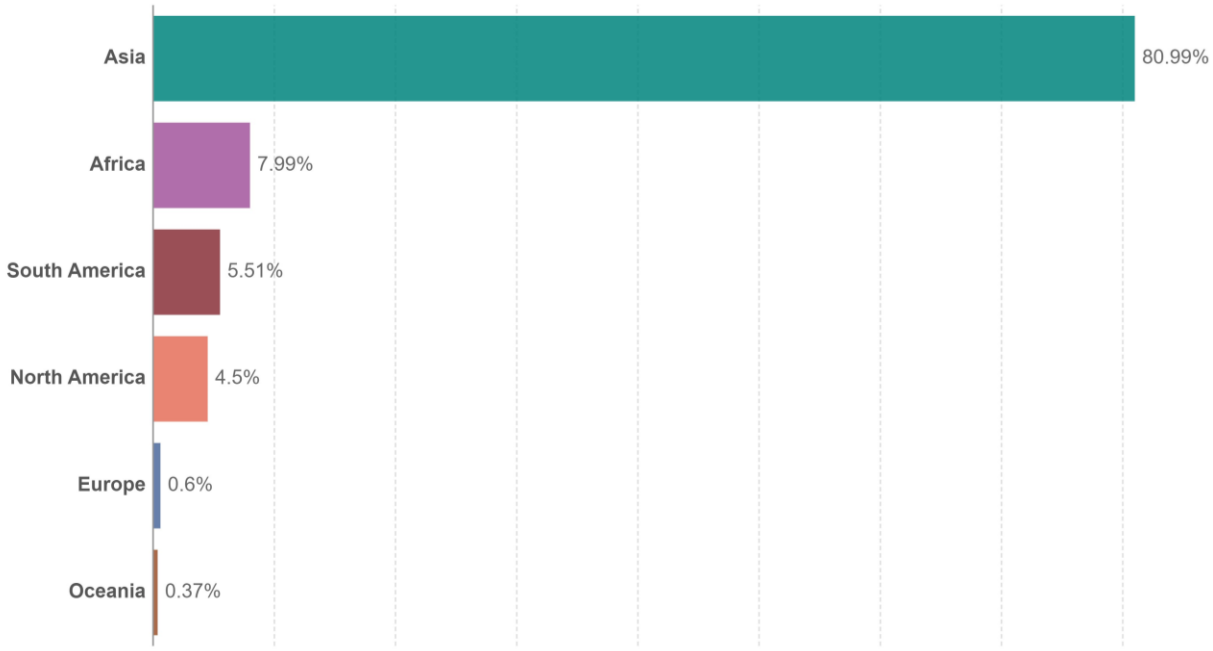

Figure 3: Graph showing percentage of plastic dumped into ocean by each continent Source (Ritchie & Roser, 2018)

The annual delivery of plastic waste to the oceans through rivers is estimated to be approximately 2 million metric tons. Nevertheless, the quantity of plastic identified in the Earth's oceans constitutes but a minute proportion of the overall amount, accounting for less than 1% of the estimated total mass of plastics that have been discharged into the oceans throughout history, which is believed to be at least 150 million metric tons (Mt). There is speculation regarding the whereabouts of the 'missing plastic'. It is suggested that a portion of

this plastic may exist in the form of microplastics, which could have either settled on the seafloor or been deposited on beaches. Another possibility is that these microplastics have been absorbed into seafloor sediments, dispersed and suspended throughout the ocean column, ultimately reaching the deep oceans, or consumed by marine organisms such as fish, other marine organisms, or microbes. However, the exact location of the sink for this plastic remains uncertain at present. From a geographical standpoint, it is noteworthy that the five nations with the highest plastic pollution rates are P.R. China, Indonesia, Philippines, Vietnam, and Sri Lanka. Collectively, these countries account for a significant 56% of the total worldwide plastic garbage generated.

2.3 Addition of plastic waste in concrete (Plastic reinforced mortar) and its limitations

The integration of waste materials into concrete presents an opportunity to effectively repurpose substantial quantities of various waste streams, such as glass, plastic, and industrial waste. (Babafemi et al., 2018; Jani & Hogland, 2014; Tomar et al., 2021). The incorporation of waste materials into concrete offers a dual advantage by diverting garbage from landfills and mitigating the greenhouse gas emissions linked to cement manufacturing. Plastic-reinforced cementitious materials (PRCs), exemplified by plastic-reinforced mortar (PRM), have the potential to facilitate the utilization of mixed-type plastic trash with diverse geometries, hence circumventing the expensive sorting procedure typically associated with traditional plastic recycling methods (Babafemi et al., 2018; Yin et al., 2016; Kim et al., 2010). The utilization of Polymer Reinforced Composites (PRCs) is presently constrained by their comparatively lower strength in comparison to traditional concrete. (Babafemi et al., 2018; Sharma & Bansal, 2016). The decrease in strength is exacerbated as the amounts of plastic addition increase, hence limiting the capacity for incorporating plastic waste into the PRC. There have been three hypothesized theories put forth to elucidate the observed decline in strength: firstly, stress concentrations arising from the relatively low modulus of plastic in comparison to the cement matrix; secondly, augmented porosity resulting from the addition of plastic; and thirdly, inadequate bonding between the plastic and cement matrix.

2.4 Microbially induced calcium carbonate precipitation:

Microorganisms occur naturally in soils and sediments and can facilitate the precipitation of minerals in two distinct manners: by acting as nucleation sites on their cell surfaces and

through metabolic activity that perturbs aqueous geochemistry and shifts mineral equilibria. The isoelectric point is the point at which charged surfaces carry no net charge – above this point the surface-bound functional groups are deprotonated and negative, while below the point the groups are positive. Therefore, at higher solution pH the calcium ion (Ca2+) tends to bind to cell surfaces more readily than at lower solution pH. The binding of cations to microbial cell surfaces acts as potential nucleation points for the nucleation and growth of minerals (Nasser et al., 2022; De Yoreo & Vekilov, 2003). Microorganisms are notably important in the microenvironment and because of their size and high surface area to volume ratio are adapted to interacting with metal ions in the environment. Microbially Induced Calcite Precipitation is a ubiquitous process in the environment, resulting in large-scale precipitation of biominerals (Liang et al., 2018). Although several metabolic pathways can lead to calcite precipitation, ureolysis, possesses exceptionally fast reaction kinetics and has been investigated due to its ability to precipitate large quantities of calcite in a relatively short period of time (Boling, 2015; Henze & Randall, 2018; Nasser et al., 2022). Ureolytic bacteria, such as Sporosarcina pasteurii, are frequently found in underground environments and exhibit a high capacity for utilizing urea as an electron donor in conjunction with oxygen as an energy source (Nasser et al., 2022).

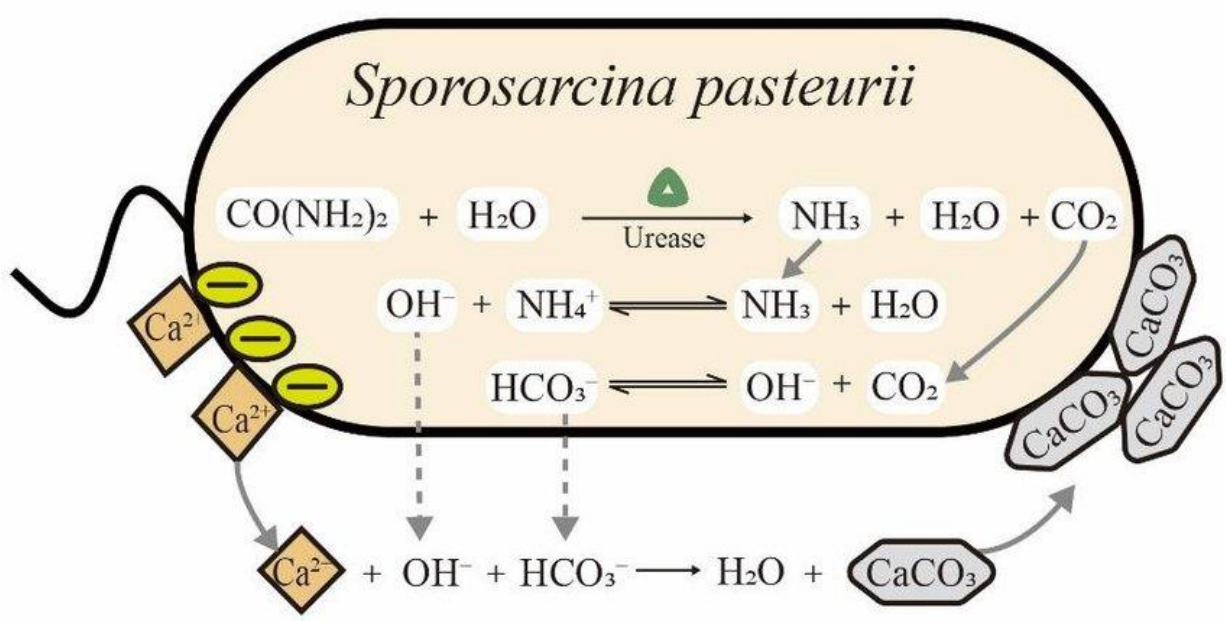

Figure 4: MICP (Microbial induced calcium carbonate precipitation) process

The bacteria enzymatically break down urea, resulting in the production of carbonic acid (H2CO3), which then undergoes equilibration in aqueous solution to form carbonate ions (CO3 2-). During the progression of the process, the hydroxide ion (OH-) is generated, leading to a rise in pH and simultaneous enhancements in the activity of the carbonate ion (CO3 2-). The condition under consideration is a metabolic process known as ureolysis. Microbial ureolysis has been observed to take place in various environments, including marine, soil, and karst settings (Boquet, 1973). Bacteria employ a generic geochemical route for the hydrolysis of urea. Bacterial metabolism of urea in aqueous environments leads to the generation of ammonia and carbonic acid, hence providing a source of energy for the bacteria. The carbonic acid subsequently reaches equilibrium in the solution, resulting in the production of carbonate. The phenomenon of carbonate mineral supersaturation, in the presence of Ca2+ or other metallic ions, arises due to the elevation in pH levels caused by the release of hydroxide ions during the ammonium synthesis process from ammonia. The outcome of this metabolic process entails the deposition of calcite onto the cellular membrane of bacteria. The kinetics of the reaction involved in the bioprecipitation of calcite through ureolysis exhibit a notably high rate. The reaction rate of Ureolytic Calcite Precipitation (UCP) at the point of critical saturation is approximately 0.8 mmol L-1 d -1(Ferris et al., 2004).

2.5 Sporosarcina pasteurii:

This bacterium can precipitate calcite and solidify sand given a calcium source and urea through the process of microbiologically induced calcite precipitation (MICP) or biological cementation (Henze & Randall, 2018). It has been proposed to be used as an ecologically sound biological construction material and can be used in bio self-healing of concrete to heal micro cracks (Gebru et al., 2021). In one study, biomineralization techniques were applied to coat plastic waste and improve the compressive strength of plastic-reinforced cement mortar using S. pasteurii (Kane et al., 2021). Based on the information Sporosarcina pasteurii is a suitable species for plastic biomineralization to use plastic waste in concrete.

2.6 Optimal Conditions for the Growth of Sporosarcina species:

Based on the search results, the optimal conditions for biomineralization using Sporosarcina pasteurii are:

1. Calcium concentration: Calcium is an essential factor for biomineralization, and its concentration affects the efficiency of the process. The optimal calcium concentration for biomineralization using S. pasteurii is reported to be 20 mM (Han et al., 2021).
2. Urea concentration: Urea is the substrate for urease, which catalyzes the hydrolysis of urea and produces ammonium and carbonate ions. The optimal urea concentration for biomineralization using S. pasteurii is reported to be 100 mM (Han et al., 2021).
3. pH: The pH of the environment affects the activity of urease and the solubility of calcium carbonate. The optimal pH for biomineralization using S. pasteurii is reported to be 9.25 (Lapierre et al., 2020) .
4. Temperature: The temperature affects the growth rate and activity of S. pasteurii. The optimal temperature for biomineralization using S. pasteurii is reported to be 30°C (Han et al., 2021).
5. Nucleation sites: The presence of nucleation sites such as sand or plastic waste can enhance the efficiency of biomineralization by providing a surface for calcium carbonate precipitation (Han et al., 2021; Wu et al., 2021).
6. Nutritional requirements: S. pasteurii has specific nutritional requirements for growth and biomineralization. The addition of L-methionine, L-cysteine, thiamine, nicotinic acid, phosphate, and trace elements can improve the growth of S. pasteurii (Lapierre et al., 2020).

2.7 Biomineralization bacteria strains:

Several bacterial strains have been identified for the biomineralization of plastic, including:

Table 1 Biomineralization bacteria strains reported in literature:

Bacteria Stains	Literature
Chytridiomycota	(Lear et al., 2021)
B. subtilis strain V8	
Pseudomonas aeruginosa PAO-1	(Ru et al., 2020)

Sporosarcina pasteurii	(Kane et al., 2021)
Bacillus spp.	
Enterobacter sp.	
Ideonella sakaiensis	
Pseudomonas spp.	
Streptococcus sp.	
Proteobacteria	
Bacteriodetes	
Cyanobacteria	
Brevibacillus borstelensis	
Rhodococcus ruber	
Ideonella sakaiensis	
Serratia sp.	

CHAPTER 3

3 METHODOLGY

3.1 Process of growing bacteria in petri dishes

3.1.1 Media Preparation:

The available medium in our lab was tryptic soya agar to prepare the petri plates for bacteria growth. We prepared the media for 20 plates so that we have enough of the plates for later use.

- First, we washed a 1000 ml conical flask and measured 24 g tryptic soya agar powder in this flask. After that we added 600 ml distilled water.
- We added a magnet stirrer in the conical flask and placed it on a hot plate at 60 C to mix the media thoroughly. As we ensured that our media was mixed completely, we placed in autoclave at 121 C for 15 minutes.
- After 2 hours when our media was autoclaved, and conical flask temperature was near 50 C we removed it from the autoclave and placed it in biosafety cabin for pouring of the media in petri plates.
- While pouring we gently keep whirling flask so that media would be distributed equally in all plates. After that we placed these plates in biosafety cabin for overnight for solidification and turn on UV light to make sure there is no contamination and our plates remained contamination free.
- Next day we collected these plates from biosafety cabin and placed these plates in 4 C refrigerators for preservation and future use.

3.1.2 Glycerol stock:

- Four bacteria strains permission was acquired from the National culture collection of Pakistan head Dr Iftikhar Ahmad. Thanks to him he was kind enough to give us authority to streak these strains from glycerol stock. These four bacteria strains were NCCP-2716, NCCP-2222, NCCP-2331 and NCCP-3001 (*Bacillus subtilus)*.
- In these strains first three are closely related to *the Sporosarcina pastueriii* the one that was used previously in our source paper and *Bacillus subtilus* was used as

positive control as it was already reported to produced calcium carbonate precipitates and is easy to grow in laboratory.

3.1.3 Streaking of the plates:

Once we prepared our plates the next step was the growth of bacteria strains on these plates for growth and culture preparation. For this purpose, the glycerol stock of the respective strains was streaked on the petri plates in aseptic environment and after streaking these plates were placed in incubator at 37 C (optimum temperature) for growth.

- On the next day we observed the plates, and we could easily see growth on the plates of the respective strains. These bacteria plates were then cross attached with already prepared plates that were preserved and then we confirmed their morphology was like the already preserved plates.

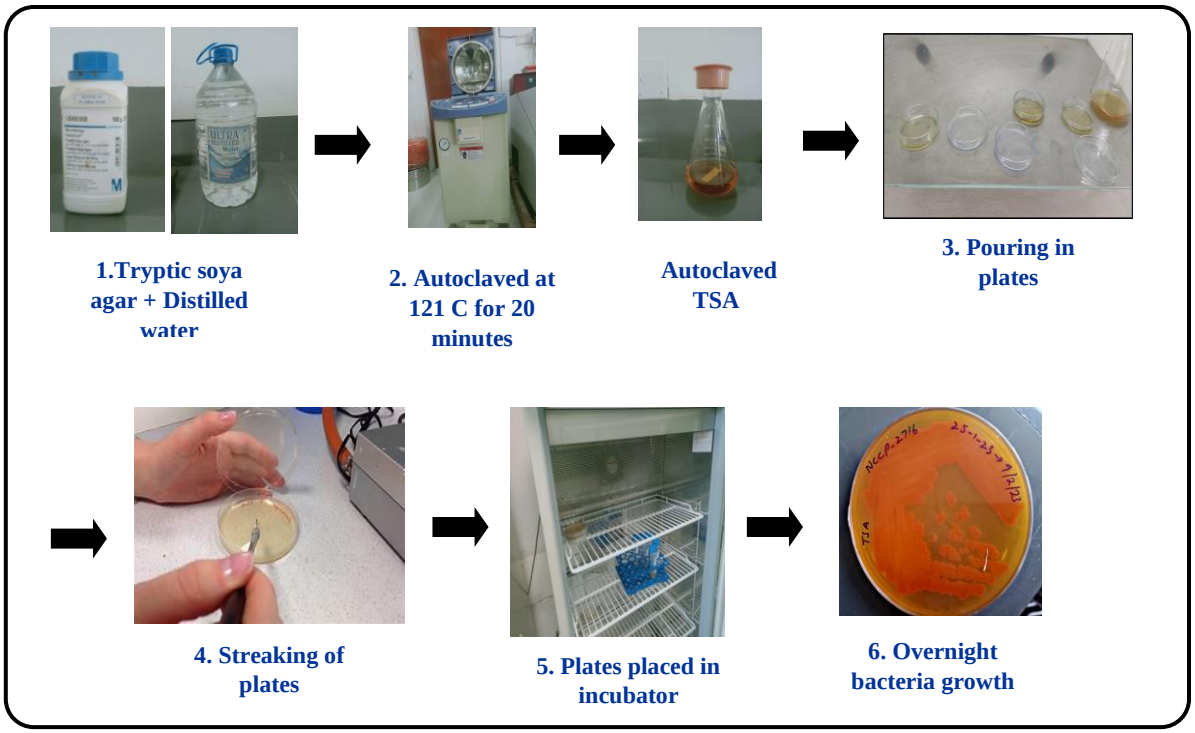

Figure 5: Bacteria plates preparation for culture and short time preservation purpose; 1. Addition of tryptic soya agar and distilled water in a conical flask and then mix the media on hot plate **2.** Autoclaving at 121 C for 15 minutes **3.** Pouring of media in petri plates **4.** Streaking glycerol stock on the plates **5.** Placing in incubator for overnight growth **6.** Overnight grown bacteria plates

3.2 Process of preparing bacteria culture:

As in our experiment we needed bacteria culture to make sure that we had enough bacteria colony and our desired bacteria strain dominated others so for all bacteria strains we prepared their cultures.

3.2.1 Media preparation:

The process of media preparation is like the media preparation for petri plates. However, one difference is that instead of using tryptic soya agar we use tryptic soya broth for broth media. The reason for this is that we do not want the media to solidify.

- So, like in petri plates media preparation we added 4.5g of tryptic soya broth in 150 ml distilled water in five conical flasks (for each 4.5 g TSB and 150 ml distilled water).
- After mixing we autoclaved these media at 121 C for 20 minutes.
- After autoclaving we left these broth containing flasks for cooling because the moment, we removed these flasks from the autoclave they were hot, and we needed to cool these flasks for inoculation purpose.

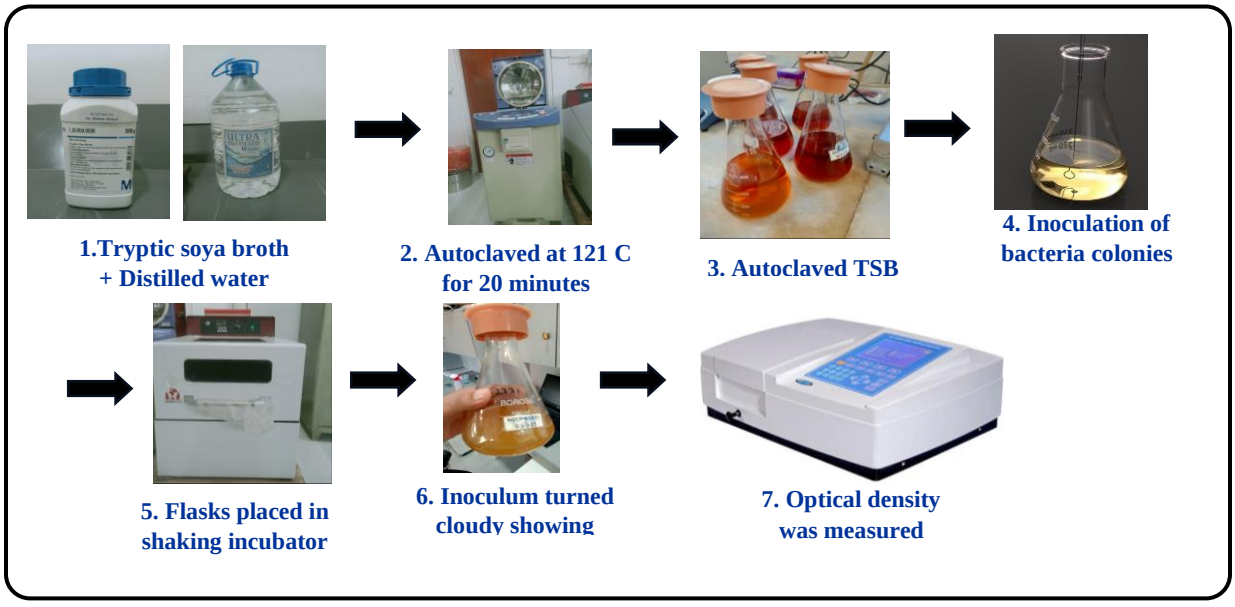

Figure 6: Bacteria culture preparation; 1. Addition of tryptic soya broth and distilled water in conical flask and then mixing of the media **2.** Autoclaving at 121 C for 15 minutes **3.** Inoculation of bacterial colonies in respective tagged flask **4.** Flasks p=in shaking incubator at 30 C and 60 RPM **5.** Placing in incubator for overnight growth **6.** On next day observation of flasks and if they turned cloudy then measuring culture optical density

3.2.2 Inoculation:

- After making sure that the temperature of these flasks is near to room temperature, we inoculated respective bacterial strains in tagged conical flasks in aseptic environment (Biosafety cabinet or laminar flow).

3.2.3 Shaking incubator:

- After that we placed these flasks in shaking incubator for overnight growth at 30 C temperatures and 60 RPM (round per minute).

3.2.4 Bacteria culture observation and optical density measurement:

- Next day morning we observed the flasks and on observation we observed that broth in the flaks turned cloudy making sure that bacteria have grown in maximum yield in their respective flasks.

- After that we measure the optical density of all these cultures and make sure then adjusted all the cultures optical density approximately 2 through fresh autoclaved broth.

3.3 Calcium Carbonate precipitation Media

This media was necessary to feed the bacteria strains so that these were able to hydrolyze urea through the urease enzyme that they produce. Basic ingredients of this media are mentioned below in table 3.1. These ingredients are urea, ammonium chloride, sodium bicarbonate and calcium chloride.

Table 2: The ingredients and their quantity that were used while preparing CPM.

Chemical	Concentration
Ammonium chloride	10g/l
Sodium bicarbonate	2.12g/l
Calcium chloride	0.25M or 4.41g/l
Urea	0.25M or 20g/l

Note: One thing that was kept in mind that urea was not added in this media before autoclaving as the urea degrades at 50 C. After autoclaving the urea was added to the media using 0.25 µm filter through 10 ml syringe to avoid the contamination.

3.4 Preparation of polyethylene terephthalate flakes:

We prepared the polyethylene terephthalate (PET) flakes for experiment use as we were eager to make sure that our strains were either capable of producing calcium carbonate crystals in the presence of plastic flakes or not. These flakes were prepared manually where through scissors we cut the mineral water and prepare almost 15-20 g PET flakes for experiment use.

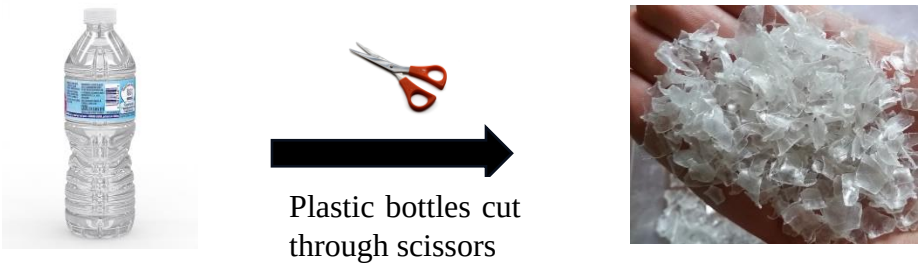

Figure 7: This figure explains how we prepared PET flakes for experiment purposes from the plastic bottles.

3.5 Experiment protocol:

The experiment was done in the aseptic environment, and it was made sure that by one reason or another we don't contaminate our media and we don't lose our interest strain. The procedure through which we conducted our experiment was as followed:

1. First, we tagged five conical flasks as Negative control, NCCP-2331, NCCP-2222, NCCP-2716 and *Bacillus subtillus.*
2. These tagged conical flasks were autoclaved at 121 C for 20 minutes to make sure these flasks were free of any life.
3. After tagging these flasks as we already had prepared these bacteria strains culture and the calcium carbonate precipitation media and plastic flakes.
4. We ran two experiments simultaneously, one without containing plastic flakes while the other containing plastic flakes. So, in this case we had a total of 10 conical flasks for our experiment.
5. After that we mixed 150 ml of bacteria culture, 150 ml CPM and 1g of PET flakes and in negative control we only added 150 ml tryptic soya broth, 150 ml CPM and 1 g of PET flakes.
6. The same was done for the flasks that we used for without plastic flakes experiment exception was that plastic flakes were not added while the other steps were same as of step 5.

Table 3: It shows quantity of the ingredients added in the flasks.

	Negative Control	NCCP-2222	NCCP-2331	NCCP-2716	B. subtilus as positive control
Calcium precipitating media	150 ml	150 ml	150 ml	150 ml	150 ml
Tryptic soya broth or bacteria culture	150 ml TSA	150 ml culture	150 ml culture	150 ml culture	150 ml culture
PET flakes	1g	1g	1g	1g	1g

7. These flasks were then placed in incubator for seven days so that bacteria could grow to maximum of their potential and could express their MICP activity.
8. After 7 days observation of flasks showed clear precipitate formation at the bottom of flasks in both cases (PET flakes containing flasks and in without PET flakes flasks).
9. These cultures were then harvested by centrifuging at 500 RPM for 10 minutes at 4 C and the precipitates and bacteria spores were collected as pellets that stayed at the bottom of 50 ml centrifuge tubes.
10. In aseptic environment we added 1 ml of T.E buffer containing lysozyme enzyme to breakdown the bacteria cells and made sure that we had maximum calcium carbonate precipitates. These pellets were then incubated for 1 hour and then centrifuged gain at 5000 RPM for 10 minutes at 4 C.
11. These pellets were then dried at 50 C oven for further microscopic observation by XRD, FTIR and SEM analysis.
12. As in XRD and FTIR observation we needed these samples in powder form for better results these pellets were then converted into powder form.

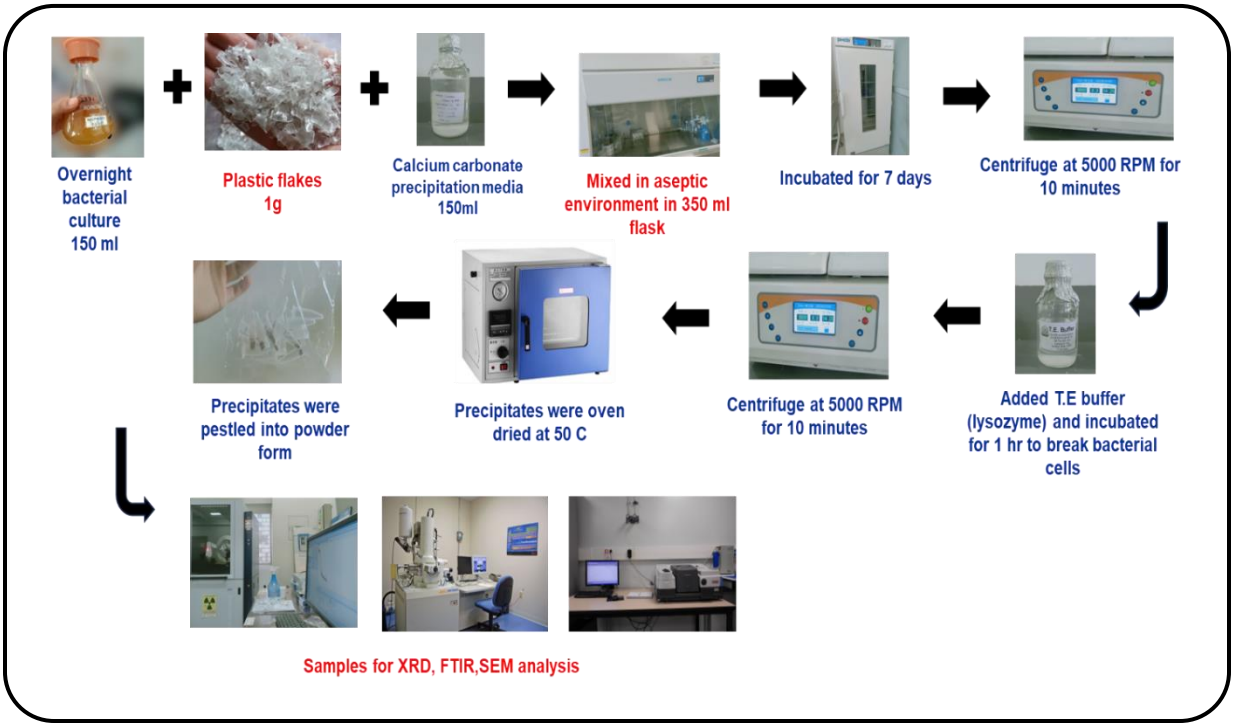

Figure 8: This figure explains the steps of conducting experiment 1. Mixing of bacteria culture or TSB, CPM and PET flakes **2.** Incubation and centrifugation **3.** Addition of T.E buffer and again centrifugation **4.** Oven drying **5.** Preparation of samples for XRD, FTIR and SEM analysis

3.6 Fourier transforms infrared spectroscopy.

FTIR spectroscopy is an analytical technique that employs infrared radiation to generate an absorption spectrum, which can be used to analyze the functional groups, chemical bonding, and molecular structure of both organic and inorganic substances. In this study, FTIR spectroscopy (PerkinElmer, SpectrumTM100) was utilized to identify the calcite and vaterite crystals of calcium carbonate present in the pellets produced by bacteria strains NCCP-2222, NCCP-2331 and NCCP-2716. The samples were analyzed using KBr pellets, with a wavelength range of 4000-400 cm^{-1} (Reig et al., 2002).

3.7 X-ray diffraction

By using an X-ray diffractometer structural characteristics and crystalline nature of dried samples can be discovered. The STOE (Germany) Powder X-ray diffractometer (operating voltage 40 kV and current 40 mA) was used for the analysis of structural characteristics and

crystalline nature of dried samples. The source used was Cu Kα radiations (λ = 1.54 A^{o}) with 2 θ range 05-80^{o} at a scan rate of 2 degrees/min (Amidi & Wang, 2015).

3.8 Scanning Electron Microscopy

To confirm the presence of the calcium carbonate crystals on PET flakes, SEM (JEOL JSM-M64900) was employed. In addition, the surface PET flakes were also analyzed using SEM. For this analysis, a secondary electron beam detector and a 20 kV accelerating voltage electron beam were utilized. Furthermore, Energy Dispersive Spectroscopy (EDS) was employed to determine the elemental composition of the samples (Achal & Pan, 2014).

CHAPTER 4

4 RESULTS

4.1 Mass change means calcium carbonate produced by bacterial strains:

To compare which of the bacteria strains has produced more calcium carbonate crystals we measured precipitates produced by each strain. In this experiment design we only added bacteria culture and calcium carbonate precipitation media and there weren't any PET flakes were added in fear of that these flakes might hinder these strains from producing calcium carbonate precipitates. In the negative control flask instead of any bacteria culture we only added autoclaved 150 ml of tryptic soya broth. *Bacillus subtilus* was taken as positive control as in previous studies it was reported that this strain has the capability to produce calcium carbonate precipitates. So, as we measure the precipitates produced by these strains among all the strains NCCP-2331 was the leading strain producing a higher quantity of calcium carbonate crystals than all other strains. After that NCCP-2716 and then NCCP-2222 produced more calcium carbonate precipitates respectively. The trend that these strains followed in producing calcium carbonate precipitates was NCCP-2331>NCCP-2716>NCCP-222, *Bacillus subtilus*>Negative control.

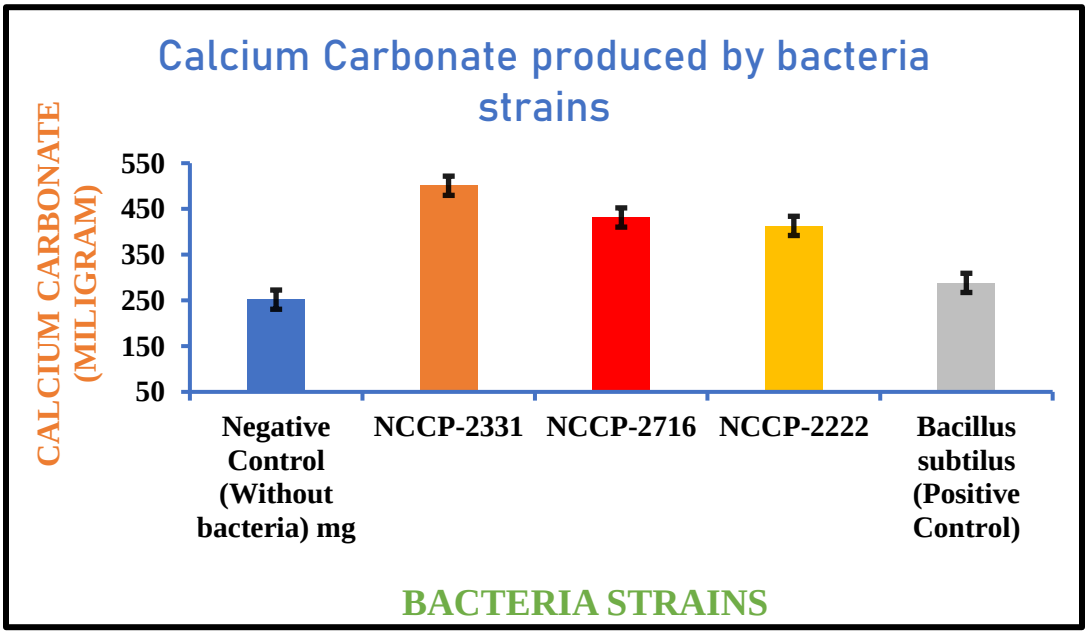

Figure 9: This graph shows the amount of calcium carbonate precipitates produced by each strain. NCCP-2331 was the leading strain in producing precipitates.

After this experiment to confirm that presence of PET flakes did not hinder the microbial activity and allow these strains to produce calcium carbonate precipitates, we added 1g of PET flakes in along with calcium precipitation media and bacteria culture. Luckily presence of PET flakes did not hinder these strains from producing calcium carbonate precipitates and these strains followed similar trend in producing calcium carbonate precipitates in the presence of PET flakes. Both these experiments were done in triplicates to confirm and make a valid statement. Like in previous experiment NCCP-2331 was the leading strains and after that NCCP-2716 and then NCCP-2222 produced more quantity of calcium carbonate precipitates. The trend that these strains followed in producing calcium carbonate precipitates was NCCP-2331>NCCP-2716>NCCP-222, *Bacillus subtilus*>Negative control.

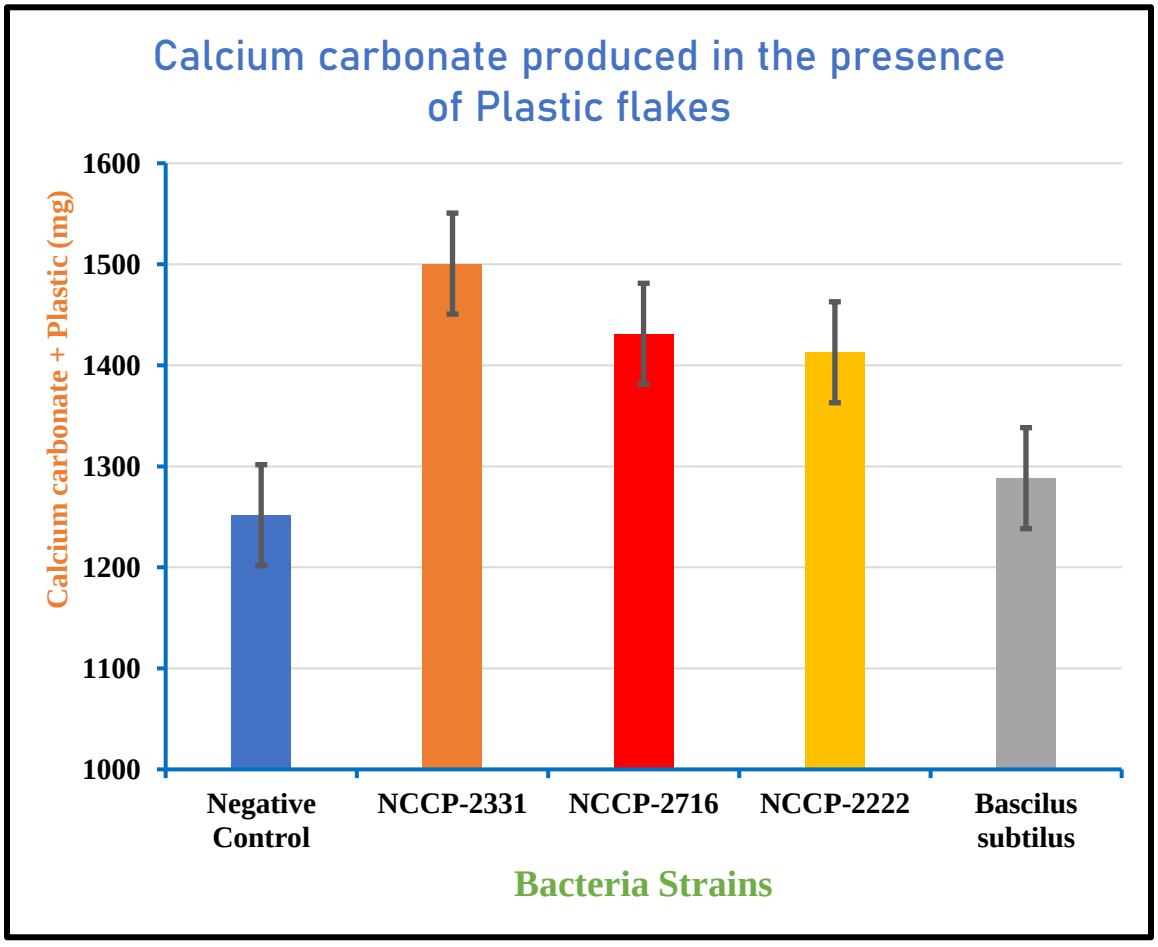

Figure 10: This graph shows the amount of calcium carbonate precipitates produced by each strain in the presence of PET flakes. NCCP-2331 was the leading strain in producing precipitates.

4.2 XRD analysis of biomineralized samples of NCCP-222, 2331, 2716:

In order to verify the presence of calcium carbonate crystals, an X-ray diffraction (XRD) examination was conducted on the precipitates generated by the aforementioned bacterial strains. The X-ray diffraction (XRD) patterns of the mineralized samples present on the surface of three different types of microbial cells are depicted, providing unambiguous evidence that all the mineralized samples consisted of calcite and vaterite. Based on the pertinent data from the PDF standard cards (JCPDS: 05-0586-JCPDS: 33-0268), it can be observed that the diffraction 2θ angle positions of approximately 24.87°, 27.03°, 32.72°, 43.80°, and 49.92° correspond to the crystal planes (1 0 0), (1 0 1), (1 0 2), (1 1 0), and (1 0 4) of vaterite, respectively. According to the study conducted by Sanchez-Moral et al. in 2003, The observed diffraction angles, namely 29.45°, 35.96°, 39.42°, 43.17°, and 48.57°, can be associated with the crystal planes (1 0 4), (1 1 0), (1 1 3), (2 0 2), and (1 1 6) of the calcite mineral, respectively (Sanchez-Moral et al., 2003:, Kontoyannis & Vagenas, 2000).

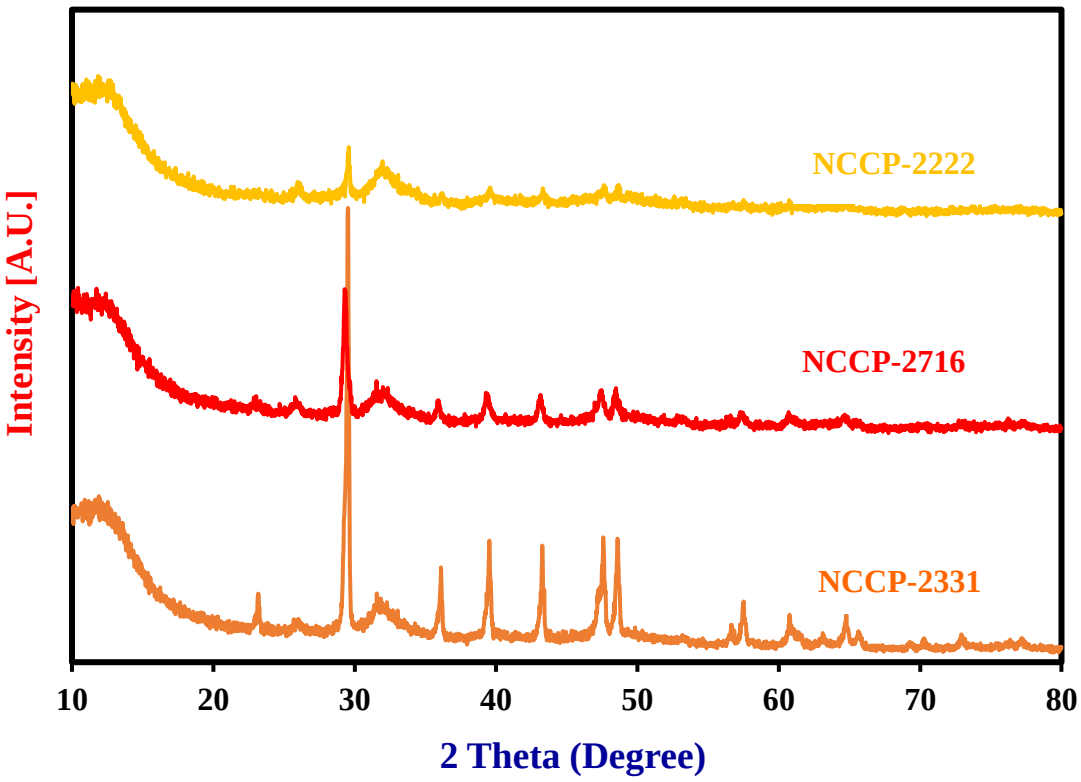

Figure 11: This graph shows the XRD pattern of biomineralized samples of three strains (NCCP-2222, NCCP-2716 and NCCP-2331) clearly indicating all three samples were composed of calcite and vaterite crystals.

4.3 Fourier Transforms infrared spectroscopy analysis:

Several absorption bands were identified in the mid-IR spectra of NCCP-2222, 2331 and 2716 (Fig. 13). The spectral band assignment for the NCCP-2222, 2331, and 2716 strains was determined by comparing and cross-referencing them with relevant FTIR literature on phototrophic bacteria. The results of this analysis are presented in Table 4 (Reig et al., 2002). The bands (1, 2, 3, 4, 5, 6, 7) were observed in all three NCCP-2222, 2331, and 2716 samples and were associated with non-ACC (alternating current coupling) components. The observed components encompassed various vibrational modes, each associated with specific molecular groups.(Mehta et al., 2022; Kontoyannis & Vagenas, 2000; Mehta et al., 2022).

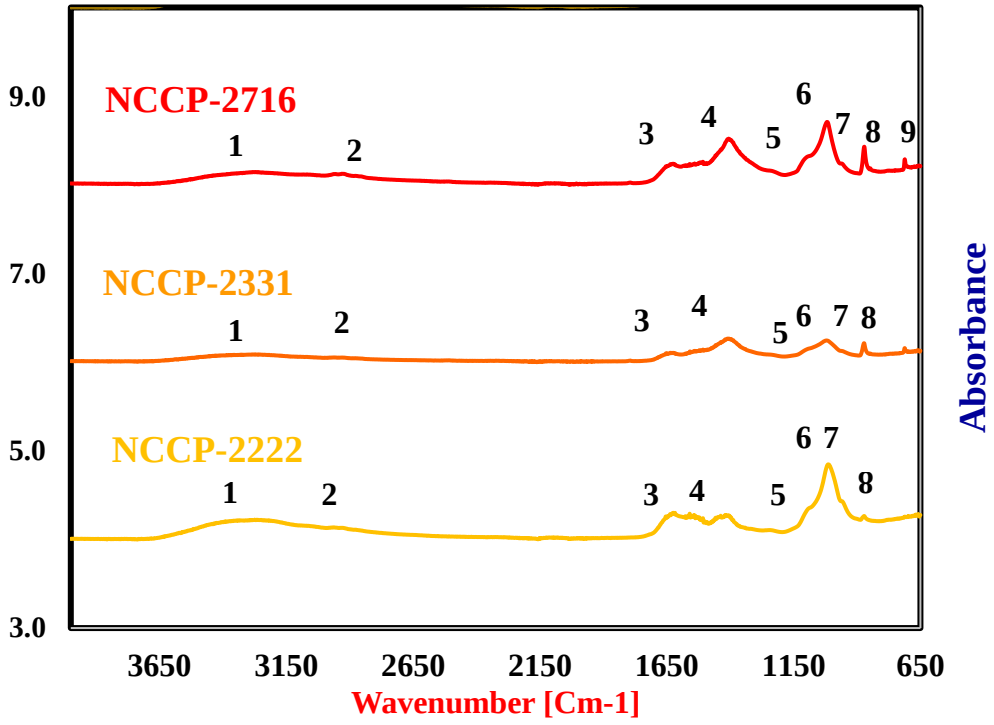

Figure 12: This graph shows the FTIR spectra of all three biomineralized samples NCCP-2331, 222, 2716. Here we can see that in all three spectra bands from 1 to 8 are present while band 9 is missing in NCCP-2331 and NCCP-2222 spectra.

Band 1 corresponded to the O-H vibrations originating from water. Band 2 represented the vibrations of methyl and methylene groups present in lipids and fatty acids. Bands 3 and 4 were attributed to the vibrations of amide I and amide II, respectively, which are characteristic of

proteins. Bands 5 and 6 were associated with the asymmetric bending of methyl groups in lipids and the symmetric stretching of the C-O group in carboxylic acids. Finally, bands 7, 8, 9, and 10 were a combination of the asymmetric stretching of the P = O group found in nucleic acids and/or polyphosphates, as well as the C-O stretching vibrations originating from polysaccharides. The spectral bands observed within the wavenumber range of 2500 to 2000 cm−1 can be attributed to the presence of ambient carbon dioxide (CO2) and inadequate purging of the sample chamber. The compound NCCP-2331 exhibited an extra absorption band at approximately 860 cm−1 (designated as band 11), which was not observed in NCCP-2716 and NCCP-2222. The highest point of band 11 aligns with the apex of the ACC at a wavenumber of 860 cm−1. (Fig. 13).

Table 4 FTIR peak position and peak assignment (Mehta et al., 2022)

Peak position (cm^{-1})		**Peak assignment**
ACC	Calcite	
3700-2950	-	O-H stretching
1640	-	O-H bending
1474	-	antisymmetric stretching of carbonate (U_3)
1395	1386	antisymmetric stretching of carbonate(U_3)
1070	1090	symmetric stretching of carbonate (U_1)
860	870	out-of-plane asymmetric bending of carbonate (U_2)
-	712	symmetric in-plane bending of carbonate (U_4)
-	361	Lattice vibrations
306	310	
212	280	
	202	
	107	
	96	
1450	2320	overtones and combination of fundamental modes of carbonate ion
1950	2550	
2500	2800	
	3100	
	3350	
	3500	

4.4 Scanning Electron Microscopy

To confirm the presence of the calcium carbonate crystals on PET flakes, SEM (JEOL JSM-M64900) was employed. In addition, the surface PET flakes were also analyzed using SEM (Chahal et al., 2011) Our two strains NCCP-2331 and NCCP-2716 produced more quantity of calcium carbonate precipitates and their produced precipitates had shown more calcite crystals (XRD and FTIR analysis confirmed it). So, for Scanning electron microscopy we selected these two strains treated PET flakes to observe their surfaces and to confirm calcium carbonate crystals formation on their surface (Dhami et al., 2013). SEM images of NCCP-2331 and NCCP-2716 treated PET flakes confirmed the presence of both bacteria colonies and calcium carbonate calcite and vaterite crystals as shown below in figure 14. As NCCP-2331 has the capability of producing more calcium carbonate crystals we can easily see the presence of these crystals on PET flakes from figure 14 (b). Similarly figure 14 (a) shows the presence of NCCP-2716 colonies and calcium carbonate crystals. While figure 14 (a) is the SEM image of untreated PET flake and on that PET flakes image we cannot observe the presence of any bacteria colony or calcium carbonate crystals either.

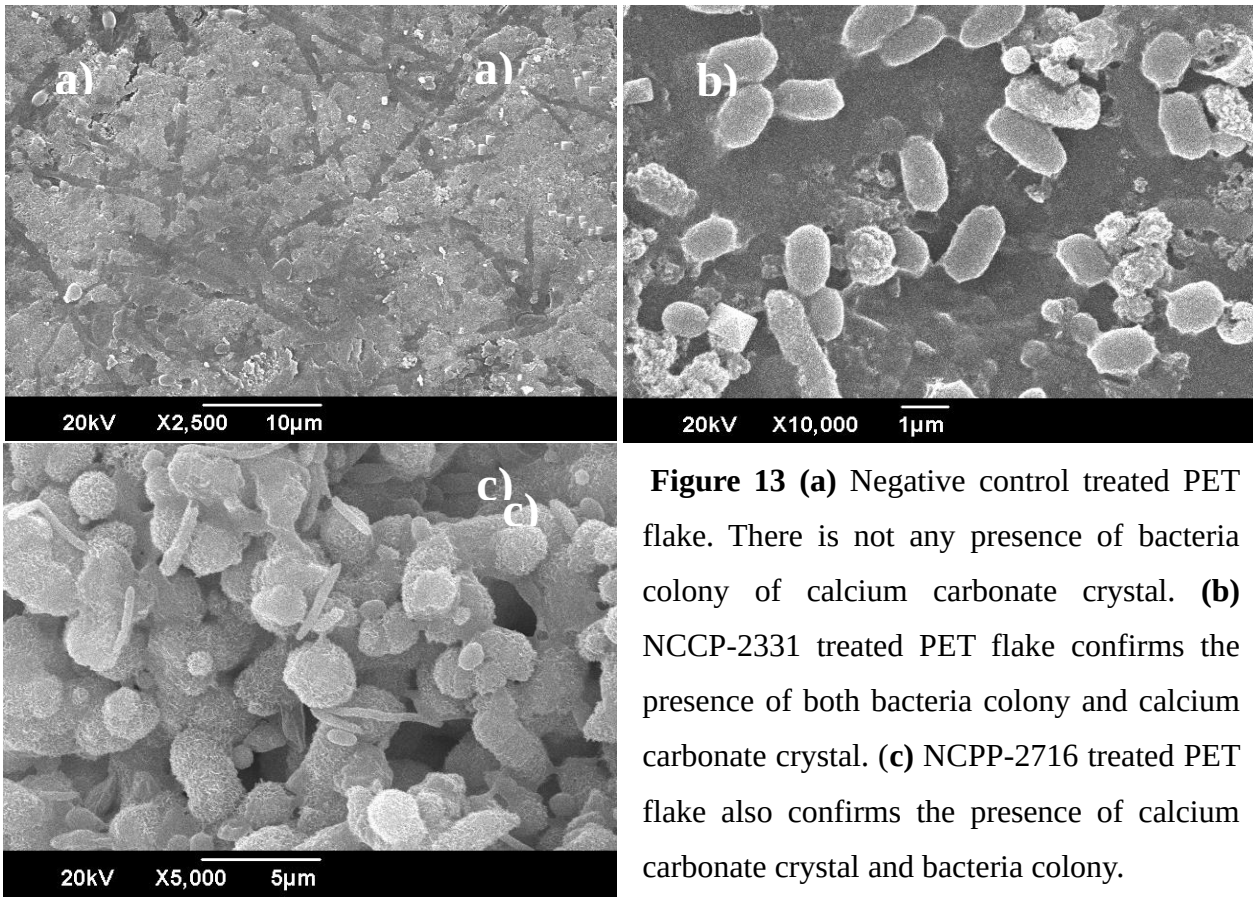

Figure 13 (a) Negative control treated PET flake. There is not any presence of bacteria colony of calcium carbonate crystal. **(b)** NCCP-2331 treated PET flake confirms the presence of both bacteria colony and calcium carbonate crystal. (**c)** NCPP-2716 treated PET flake also confirms the presence of calcium carbonate crystal and bacteria colony.

DISCUSSION

In this research work that was necessary to complete my thesis we tried to find an indigenous bacteria strain that belongs to sporosarcina species and do the same job as was done by *Sporosarcina pastuerii* in (Kane et al., 2021) Work. In this research we designed two experiments, one without PET flakes and the other including 1g of PET flakes. The aim was to observe the capability of our sporosarcina species strains to produce calcium carbonate precipitates when provided with calcium carbonate precipitation media. And the precipitates produced were then confirmed by chemical testing through XRD, FTIR and SEM analysis. And these precipitates had shown the same peaks as was observed in standard calcium carbonate precipitates. Among these three strains NCCP-2331, NCCP-2222 and NCCP-2716 NCCP-2331 had shown exceptional microbial activity. Through ureolysis it had produced the highest quantity of calcium carbonate precipitates and these precipitates were more crystalline as their peaks were more fine compared to NCCP-2716 and NCCP-2222.

One thing that made these strains unique and could help us in solving the problem of plastic pollution was their capability to produce calcium carbonate crystals even in the presence of PET flakes. As was reported in (Kane et al., 2021) like *Sporosarcina pastuerii* we could use NCCP-2331 in treating these PET flakes and then these flakes could be added in cement matric to prepare plastic reinforced mortar that will show similar ultimate tensile strength like conventional cement. Like we conducted the experiment by adding PET flakes we could test other types of plastic and other types of plastic can also be added in cement matrix.

In our experiment the bacteria strains had shown similar trends in increase in pH in 24h and had shown maximum of their microbial activity within 24 hours and produced maximum amount of calcium carbonate precipitates. So, all these activities were same as were reported in these studies (Gebru et al., 2021; Henze & Randall, 2018; Kontoyannis & Vagenas, 2000; Nasser et al., 2022) That discussed the microbial induced calcium carbonate precipitation (MICP process). The concentration of ingredients that we used were optimum i.e., sodium bicarbonate 2.12 g/L, Calcium chloride 0.25 M/4.41 g/L, Ammonium chloride 10g/L and urea 25 M/20g/L so that we could get the maximum amount of calcium carbonate precipitates. In other studies, different concentration of these ingredients was reported. We only used one resource of calcium rather than testing multiple resources due to limited resources and limited time. So, overall, our bacteria

strains had shown their capability to produce calcium carbonate precipitates both in the presence of PET flakes and in the absence of PET flakes.

XRD and FTIR pattern of the calcium carbonate precipitates had shown similar peaks as by standard. Among these three strains NCCP-2716 and NCCP-222 precipitates were more amorphous while NCCP-2331 strain precipitates were crystalline as these precipitates had shown more crystallite crystals than vaterite. The XRD peaks of NCCP-2716 and 2222 strain precipitates were not as fine confirming the amorphous nature of these crystals. (Sanchez-Moral et al., 2003), (Kontoyannis & Vagenas, 2000) FTIR analysis of these strains precipitates had shown all 1,2,3,4,5,6,7,8 bands while NCCP-2331 and NCCP-2716 also had shown 9^{th} band confirming more crystalline nature of these precipitates. (Kontoyannis & Vagenas, 2000; Mehta et al., 2022)

SEM (Scanning electron microscopy) images also confirmed the formation of calcium carbonate crystals. SEM images of NCCP-2331 and NCCP-2716 treated PET flakes confirmed the presence of both bacteria colonies and calcium carbonate calcite and vaterite crystals as shown below in figure 4.3. As NCCP-2331 has the capability of producing more calcium carbonate crystals we can easily see the presence of these crystals on PET flakes from figure 14 (Dhami et al., 2013).

Conclusion

In conclusion, among all three bacteria strains that were tested for their capability to produce calcium carbonate precipitated when they are provided with calcium carbonate precipitation media, they had shown that capability and all of them had produced calcium carbonate precipitates. To our best luck that all these strain NCCP-2331, NCP-2716 and NCCP-2222 also had produced calcium carbonate precipitates in the presence of PET flakes as well. Among these three strains NCCP-2331 was leading in producing calcium carbonate precipitates both in the presence of PET flakes and in flakes absence. So, in future if anyone would be interested in working on addition of plastic flakes in concrete can collect this strain from NCCP to treat these flakes to decrease the gaps between plastic flakes and cement matrix. Through microstructure analysis we also confirmed the structure of calcium carbonate precipitates. XRD pattern had shown that NCCP-2222 and NCCP-2716 precipitates are more amorphous while NCCP-2331 precipitates were crystalline. FTIR analysis had also confirmed the presence of all 1,2,3,4,5,6,7,8 bands in all three biomineralized samples confirming the samples were of calcium carbonate. SEM analysis also confirmed the structure of calcium carbonate crystals and the formation of biofilm over PET flakes.

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