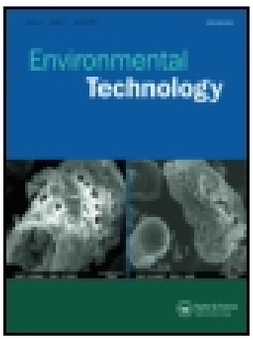

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A novel and sustainable approach for biotransformation of phosphogypsum to calcium carbonate using urease producing *Lysinibacillus sphaericus* strain GUMP2

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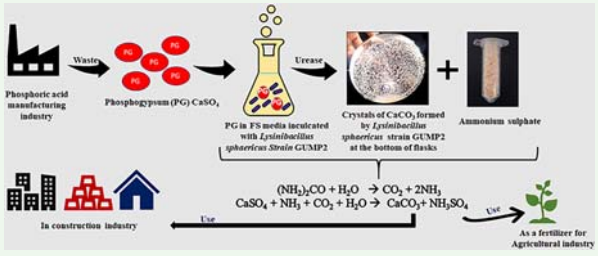
ABSTRACT

Phosphogypsum ($CaSO_4$) is produced as a waste by-product during phosphoric acid production in the fertilizer industry. Only 15% of worldwide phosphogypsum production is recycled, while 85% is stored in the vicinity of factories as huge piles resulting in environmental and health hazards. An extensively studied biotransformation of phosphogypsum to calcium carbonate or calcite ($CaCO_3$) using sulfate reducing bacteria (SRBs) is a prolonged process and results in the formation of extremely hazardous H_2S gas. Here we report for the first time a novel approach for biotransformation of phosphogypsum to $CaCO_3$ using urease producing *Lysinibacillus sphaericus* strain GUMP2. The strain could effectively transform phosphogypsum to crystalline, bead-shaped $CaCO_3$ precipitates. In a batch reactor with the PG loading rate of 60 g/L, 100% biotransformation was observed within seven days. After calcite recovery, the ammonium sulfate formed in the supernatant was recovered by precipitation. Urease-producing *L. sphaericus* strain GUMP2 could be used to remove the hazardous phosphogypsum from the environment by converting it to the industrially useful $CaCO_3$ and ammonium sulfate, a valuable agricultural fertilizer. This novel and sustainable approach could be a promising solution for the hazardous phosphogypsum in the phosphoric acid industries.



Abbreviation: PG: Phosphogypsum

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KEYWORDS
Environmental hazard; phosphogypsum; transformation; crystallization; calcite

Highlights of the present work

- Untreated phosphogypsum causes serious environmental and health hazards
- Urease producing *Lysinibacillus sphaericus* transformed phosphogypsum
- Hazardous phosphogypsum was converted into useful calcite and ammonium sulfate
- In a batch mode, the transformation rate was 60 g/L of phosphogypsum to calcite in 7 days
- End products have potential application in building construction and in agriculture

1. Introduction

Phosphogypsum (PG) is an industrial by-product of phosphoric acid manufacturing plants. The general scheme of the reaction process for phosphoric acid production is:

$$Ca_{10}F_2(PO_4)_6 + 10H_2SO_4 + 10nH_2O \rightarrow 6H_3PO_4 + 10(CaSO_4.nH_2O) + 2HF$$

Rockphosphate Sulphuricacid Water
Phosphoricacid Gypsum HydrogenFluoride

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where $n = 0$, ½, or 2 depending on the hydrate form in which the calcium sulfate is crystallized [1,2].

This economic process generates a large quantity of PG 5 tons of PG is generated for every ton of phosphoric acid production [3,4]. The worldwide generation of PG is estimated to be around 100–280 MT.yr^{-1} [5,6]. PG is mainly composed of calcium sulfate or gypsum (CaSO4.2H2O) and contains impurities such as phosphoric acid, calcium phosphate, monocalcium phosphate, dicalcium phosphate, residual acids, fluorides (NaF, Na_3AlF_6, Na_2SiF_6, Na_3FeF_6, and CaF_2), sulfate ions. PG also contains trace metals (e.g. Cu, Cr, Zn, and Cd) and organic matter adhered to the surface of gypsum crystals, as aliphatic compounds of carbonic acids, amines, and ketones [7,8]. This low-cost by-product (usually regarded as waste) is commonly used in agriculture as a fertilizer and soil stabilizer and utilized for commercial applications, such as gypsum board production and Portland cement [9–11]. Nevertheless, such practices are limited due to the high content of toxic substances for human health, and only 15% of the worldwide PG production is recycled. The remaining 85% requires huge storage or disposal area and causes health and environmental problems viz. contamination of soil, surface and groundwater, and atmospheric contamination [12,13]. Radioactive elements are the primary concern regarding the storage of PG and have an adverse environmental impact. As a result, environmental organizations such as the USEPA have imposed numerous limitations on using this residue for any purpose [3].

Biotransformation of phosphogypsum to $CaCO_3$ using sulfate-reducing bacteria (SRBs) *viz*., *Desulfomonas, Desulfococcus, Desulfobacter, Desulfosarcina,Desulfotomaculum, and Desulfovibrio* have been studied extensively [1,14–18]. This approach is based on the principle of reducing sulfate ions (that functions as a final electron acceptor) in the presence of organic molecules by SRB under anaerobic conditions [19]. The reaction of this process of sulfate reduction by SRB is as follows [20]:

$$SO_4^{2-} + 2[CH_2O] + OH^- + Ca^{2+} \rightarrow CaCO_3 + CO_2 + 2H_2O + HS^-$$

Currently, the biotransformation of PG using SRB is the most extensively studied method. However, slow reaction process and generation of foul-smelling, hazardous H_2S gas are some of the demerits associated with this process [1,14–16,19,21,22]. In few other studies, combined degradation of mixed industrial wastewater from multiple industries containing PG for microbial growth were attempted [14,17]. *In-situ* studies have been performed to remove sulfate from soil contaminated with gypsum [15]. Few fungi such as *Cladosporium* sp., *Aspergillus niger,* and *Trichoderma* sp. have been reported to grow on PG, but their efficiency seems very low [23].

A nickel-containing metalloenzyme, urease, producing aerobic bacteria are known to biomineralize calcium carbonate [24–26]. Urease plays a vital role in the nitrogen cycle of various bacteria, fungi, algae, and plants by catalyzing the hydrolysis of urea into ammonia and CO_2 and assist in biomineralization. The mechanism of biomineralization was explained by Varalakshmi and Archana [26]. In this study, for the first time, we demonstrate the use of urease-producing *Lysinibacillus sphaericus* strain GUMP2 for the biotransformation of PG to calcite and ammonium sulfate.

Research has been carried out to demonstrate the bioremediation potential of *L. sphaericus* strains with chromate reduction capacity isolated from different contaminated environments and naturally metal-rich soils [27–29]. The strain JG-A12 isolated from uranium-mining waste piles in Germany could reversibly bind Al, Cd, Cu, Pb, and Ur [30]. In another study, *L. sphaericus* strain CBAM5 with an arsenate reductase gene showed resistance to 200 mM arsenic [31]. Biomineralization of calcium carbonate by urease-producing *L. sphaericus* was reported recently [32]. However, to the best of our knowledge, the role of *L. sphaericus* in the biotransformation of PG to calcite and ammonium sulfate was never studied before. We also elucidate the probable mechanism behind the biomineralization of calcium carbonate by urease-producing bacteria. Urease-producing microbes hydrolyze urea in the presence of urease into ammonia and carbon dioxide (Equation 1).

The carbon dioxide thus formed will bind to the calcium of PG and form $CaCO_3$. At the same time, ammonia will bind to the sulfate to form ammonium sulfate (Equation 2).

$$(NH_2)_2CO + H_2O \rightarrow CO_2 + 2NH_3 \quad (1)$$

$$CaSO_4 + NH_3 + CO_2 + H_2O \rightarrow CaCO_3 + NH_3SO_4 \quad (2)$$

2. Materials and methods

2.1. Collection and characterization of phosphogypsum waste

Phosphogypsum sample and soil sample were kindly supplied by fertilizer company Paradeep Phosphates Limited, Bhubaneshwar, Odisha. It was characterized for chemical parameters including pH, Chemical Oxygen Demand (COD) using the titrimetric method; Total Kjeldahl

Nitrogen (TKN) and Total Ammonical Nitrogen (NH_3-N) using Kjeldahl method; Sulfate (SO_4^{2-}) using the turbidometric method, Sulfide (S^{2-}) by Iodometric way, and Nitrogen, Total Carbon (C), Hydrogen and Sulfur (S) by using CHNS analyzer. CHNS content in PG was measured using an elemental analyzer (Elementar, Hesse, Germany; Model: Vario Micro cube) mounted with Thermal Conductivity Detector (TCD) and Infrared Detector for sulfur and oxygen. For CHNS analysis, the sample was dried in an oven at 105°C and ground to a fine powder. Five hundred milligram of this powder was weighed in a tin foil container, sealed, and subjected to combustion at 1200°C furnace temperature. Helium and oxygen gases were used during the combustion.

2.2. Isolation of urease producing bacteria and its biochemical and molecular characterization

The soil sample (1 g) was suspended in 9 ml of distilled water. Serial dilutions up to 10^{-5} were carried out. The last three dilutions were spread plated (0.1 ml volume) on nutrient agar and incubated at 30 ± 2°C for 48 h. After incubation, different colonies were selected and tested for urea hydrolysis activity. Isolates were streaked on Christenson's urea agar and incubated at 30 ± 2°C for 24 h.

The colony of a bacterium showing urease activity on Christenson's urea agar was quadrant streaked on nutrient agar. After 24 h incubation at 30 ± 2°C, the colony was studied for its morphological characteristics and Gram staining nature. Endospore staining was performed on 72 h old grown culture. Cell morphology of 24 h old culture was also studied using scanning electron microscopy (SEM) (Calr-Zeiss, Avo 18, Germany). This bacterial culture was further characterized by studying its biochemical characteristics. Molecular identification was carried out using 16S rDNA partial sequencing. The DNA was isolated, and its quality was evaluated on 1.0% Agarose Gel to see a single band of high-molecular-weight DNA. Fragment of the 16S rDNA gene was amplified by 27F and 1401R primers. Upon doing PCR, the amplicon was resolved on agarose gel, and the amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with forward primer and reverse primers using the BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer using Sanger sequencing method. Using aligner software, the consensus sequence of the 16S rDNA gene was produced from forward and reverse sequence data. The 904-bp long 16S rDNA partial gene sequence was used in BLAST searches in the NCBI gene bank database. The top ten sequences were chosen based on their maximum identity score and aligned using Clustal W, a multiple alignment software application.

The evolutionary history was deduced based on the Kimura 2-parameter model and the Maximum Likelihood technique. The evolutionary history of the taxa studied was represented by a bootstrap consensus tree generated from 1000 replicates. Branches that corresponded to partitions that were replicated in less than 50% of bootstrap replicates were collapsed.

The initial tree(s) for the heuristic search were automatically generated by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances calculated using the Maximum Composite Likelihood (MCL) method and then picking the structure with the best log-likelihood value. Eleven nucleotide sequences were analyzed. 1st + 2nd + 3rd + Noncoding codon locations were included. Gaps and missing data were removed, and in the final dataset, the total number of positions remained was 1434 [33]. The obtained 904 bp long sequence was deposited in the NCBI gene bank library database with accession number MZ292988 and designated as *Lysinibacillus sphaericus* strain GUMP2. A Detailed explanation about culture characterization results is presented in the result and discussion section.

2.3. Biotransformation of PG to calcite by culture GUMP2

A monoculture of GUMP2 having OD of 1 was inoculated at the rate of 5% in a 250 ml flask containing 100 ml of modified SF medium. Modified SF medium contained nutrient broth 3 g/l, urea 20 g/L, NH_4Cl 10 g/L, $NaHCO_3$ 2.12 g/L, and 0.5% PG (in place of $CaCl_2{\cdot}2H_2O$ 1.4 g/L) [34,35]. The flask was incubated at 30 ± 2°C for seven days. After seven days of incubation, the supernatant was collected by centrifugation at 4000 rpm for 15 min, and the precipitate was harvested from the bottom. The residue was dried at 30 ± 2°C and ground to a fine powder using mortar and pestle. This powdered sample was used for further characterization. The presence of carbonate in the precipitate was checked by carrying out an acid test; approximately 20 mg of precipitate was taken on a glass slide, and two drops of 2M hydrochloric acid (HCl) were added. Effervescence formed after the addition of HCl indicated the presence of carbonate. The presence of ammonium sulfate formation was checked qualitatively. To 5 ml of the supernatant, 5 ml of 10% barium chloride $BaCl_2$ was added. The formation of a white precipitate of ammonium sulfate was checked. For quantitative estimation of ammonium sulfate,

calcite, and cell-free culture supernatant (50 ml) obtained after centrifugation was taken into 250 ml flask, and 50 ml of acetone was added to it. The flask was kept undisturbed for 2 h to allow the formation of ammonium sulfate precipitate. It was then centrifuged at 4000 rpm for 15 min. The supernatant was discarded carefully, and the pellet of ammonium sulfate was dried and weighed gravimetrically.

2.4. Characterization of the precipitate obtained in the biotransformation of PG

2.4.1. X Ray Diffraction (XRD) analysis

Room temperature XRD spectra of obtained powder was recorded with a powder X-ray diffractometer (Mini Flex II, Rigaku, Japan) with Cu Ka ($k = 0.15405$ nm) radiation. The crystalline nature and quality of calcite was determined by comparing the XRD patterns obtained with that of the standard XRD pattern of calcite [International Center for Diffraction Data (ICDD) Card no. 05-0586], [36].

2.4.2. Fourier Transform Infrared Spectroscopy (FTIR) analysis

FTIR analysis of the powder was recorded on Shimadzu FTIR-8900 (Kyoto, Japan). To obtain the FTIR spectra 1% crystalline powder was mixed and ground with 99% Potassium Bromide. Tablets of 10 mm diameter were prepared. They were prepared by pressing the powder mixture at a load of 5 tons for two min. The spectrum of powder was taken in the range of 400–4,000 cm^{-1} with 4 cm^{-1} resolution. The spectrum obtained was compared with the standard calcite FTIR pattern from the literature [37].

2.4.3. Scanning Electron Microscopy (SEM)

The shape and morphology of the calcite crystal formed were studied using SEM (Calr-Zeiss, Avo 18, Germany). The powdered sample was deposited on the carbon tape and mounted on the sample holder. Then the gold coating of the sample was done using the sputter coating device and observed under Scanning Electron Microscope.

SEM analysis of bacterial cells in SF medium with phosphogypsum was carried out to study the cell shape and morphology in the absence and the presence of phosphogypsum. The culture was inoculated in the 250 ml capacity flask containing 100 ml of modified SF medium, a glass coverslip, and 0.5% PG. The flask was then incubated at 30 ± 2°C for three days at static conditions. On the fourth day, the coverslip was removed from the flask. The coverslip was air-dried and pretreated as mentioned above before analyzing under SEM.

2.5. Optimization of PG biotransformation using GUMP2

2.5.1. PH optimization

Culture GUMP2 was inoculated (5%) in 6 different flasks containing 100 ml of modified SF medium with 0.5% phosphogypsum (PG) and pH 5, 6, 7, 8, 9, and 10. The flasks were incubated at 30 ± 2°C for seven days at static conditions. The pH of the flask was measured each day. After incubation, entire precipitate was harvested by scraping and weighed.

2.5.2. Temperature optimization

For temperature optimization, the culture was inoculated by the ratio of 2% into a 250 ml flask containing 100 ml of modified SF medium along with the control flask. One flask each was incubated at temperature 25°C, 30°C, 35°C, 37°C and 45°C for eight days. OD was taken at 600 nm, and a graph of OD v/s time (days) was plotted.

2.5.3. Time-dependent study of biotransformation

In another experiment, to determine the time of initiation of biotransformation of PG, GUMP2 culture (5%) was inoculated in a 250 ml flask containing 100 ml of modified SF medium and 0.5% PG. The flask was incubated at 30 ± 2°C for seven days and observed every day for calcite crystals in the form of precipitate at the bottom of the flask by doing an acid test. Ammonium sulfate was tested using $BaCl_2$, as mentioned in section 2.3. All the tests were carried out in triplicate, and results were calculated as mean with standard error.

The time required for biotransformation and crystal formation was also studied by a time-lapse study of crystal formation. Five percent culture GUPM2 was inoculated into seven flasks of 250 ml capacity containing 100 ml of modified SF medium and 0.5% phosphogypsum. One coverslip was placed in each of the flasks. The flasks were incubated at 30 ± 2°C for seven days at static conditions. The crystal formation on the coverslip and the growth of bacterial cells were observed daily. On each day, the coverslip was removed from one flask and observed under 45 X magnifications.

2.5.4. Optimization of biotransformation at different concentration of phosphogypsum at static and shaker conditions

GUMP2 culture (5%) was inoculated in 100 ml of SF medium, pH 7, containing 0.5%, 1%, 2%, 4%, 6%, 8% and 10% PG and in 250 ml capacity flasks in two different setups. They were incubated at 30 ± 2°C for seven days to obtain carbonate powder in the flask. One set of the flasks was incubated at static conditions, and another set

Table 1. Chemical characterization of phosphogypsum.

Parameter	Value (Unit)	Method Used
pH	2.8	pH metre
COD	0 (mg O_2/g)	Titrimetric
TKN	0.28 (%)	Kjeldahl
NH_3-N	84 (mg/kg)	Kjeldahl
SO_4^{-2}	789.22 (mg/g)	Turbidometric
S^{2-}	0 (mg/g)	Iodometric
N	0 (%)	CHNS analyser
C	0.02 (%)	CHNS analyser
H	2.33 (%)	CHNS analyser
S	13.58 (%)	CHNS analyser

of flasks was incubated on a shaker at 140 rpm. After seven days of incubation, the calcite formed in the medium was harvested by centrifugation at 4500 rpm for 10 min. The precipitates were dried at 37°C until a constant weight was seen and quantified gravimetrically.

2.6. Statistical analysis

A data analysis package in the Excel programme (ver. 13, Microsoft, USA) was used for data analysis and for calculating the mean and standard deviation. All the experiments were prepared and measured at least in triplicates, and the average calculated was used for graphical representation of the data.

3. Results and discussion

3.1. Characterization of PG

Various chemical parameters analyzed for PG are shown in Table 1. The PG sample did not show the presence of any organic carbon when analyzed using COD method; however, using CHNS analyzer, 0.02% C was observed. Similarly, 0.28% total N and 84 mg/Kg of NH_3-N was observed in PG sample when analyzed using the Kjeldahl method; however, N was nil in CHNS analyzer. A large quantity of sulfur (789.22 mg/g), in the form of SO_4^{-2}, was present. In India more than 6.0 million tons of phosphogypsum is produced per annum, which is produced out of the Morocco phosphate rock. As a result, the level of radioactivity does not exceed 13.5 pCi/g which is under safe level as specified by Euratom [38], hence radioactivity of the PG was not tested.

3.2. Isolation, characterization and identification of urease producing bacteria

Two different bacterial cultures having different morphology were initially observed on nutrient agar. These were selected, purified, and designated as GUMP1 and GUMP2. After streaking the two cultures on Christenson's urea agar slant separately, GUMP2 showed urease activity by hydrolyzing urea into ammonia plus CO_2 showing the pink colour formation on Christenson's urea agar slant (Figure S1a).

Culture GUMP2 was motile, utilized sugars such as glucose, maltose, and dextrose producing acid, and was positive for the enzyme catalase. These colony characteristics were of typical *Bacillus* sp. [39]. Upon Gram staining the culture, Gram-positive rods were observed under 100X oil immersion objective of the light microscope. These Gram-positive rods were seen as single and in pairs of two in 'V' shape arrangement (Figure S1b). After 48 h incubation, bacterial cell morphology changed from rod-shaped to spherical shape with bulging at the one end of rod indicating the spore-forming nature of the culture (Figure S1c). After further incubation, the cells changed their shape into an oval shape. Endospore staining of GUMP2 culture by Schoeffer- Fultons method showed green, spherical spore located in a terminal position within a swollen pink, oval, or round shape sporangium [40]. Culture GUMP2 growing in nutrient broth (without PG), clearly revealed the rod-shaped morphology as seen under Scanning Electron Microscopy. Rod-shaped bacterial cells in singles and in pairs, having a length of 4.49 µm and diameter of 0.23 µm were seen in SEM (Figure 1a). Upon extraction of genomic DNA from GUMP2 culture, a single genomic DNA band was observed in 1% agarose gel electrophoresis as visualized by gel documentation. A single discrete PCR amplicon band of 1500 bp was observed when resolved on Agarose gel.

Based on Gram staining, endospore staining, SEM, biochemical characteristics following Bergey's manual of systematic bacteriology, and 16S rDNA sequencing followed by NCBI-BLAST search, the bacterial isolate GUMP2 was identified as *Lysinibacillus*. Phylogenetic analyses using the maximum likelihood method clearly revealed its evolutionary relatedness with other reported strains as shown in figure (Figure 1b) and closely resembled *Lysinibacillus sphaericus*, (previously named as *Bacillus sphaericus* [28]) hence the culture was designated as *Lysinibacillus sphaericus* strain GUMP2. The 16S rDNA sequence was deposited in the NCBI nucleotide sequence database with NCBI accession number MZ292988 [41].

3.3. Biotransformation of PG to calcite by Lysinibacillus sphaericus *strain GUMP2*

3.3.1. Calcite crystals formation

Microorganisms producing urease hydrolyzes urea into ammonia and carbon dioxide in the presence of urease (Equation 1).

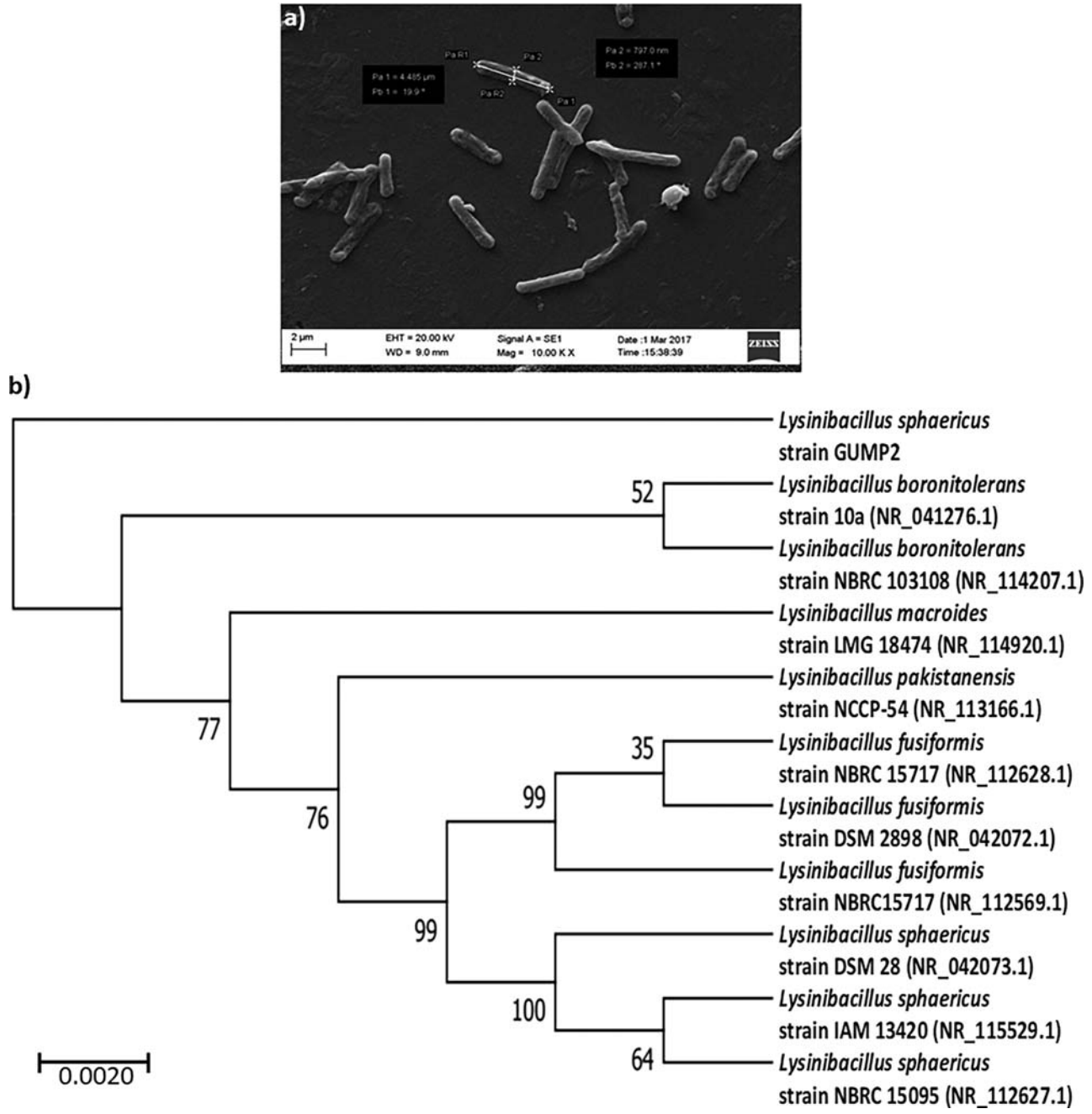

Figure 1. (a) SEM of *Lysinibacillus sphaericus* strain GUMP2, (b) Molecular Phylogenetic analysis by Maximum Likelihood method. Different *Lysinibacillus* culture strains are compared with *Lysinibacillus sphaericus* strain GUMP2 using 16S rRNA gene sequences in the NCBI genebank database with accession number MZ292988. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

The carbon dioxide binds to the calcium of PG and form $CaCO_3$, and at the same time, ammonia will bind to the sulfate to form ammonium sulfate (Equation 2).

Upon incubation and growth for seven days in a modified SF medium with 0.5% PG, precipitation of the calcite was observed at the bottom of the flasks in the form of crystals (Figure 2a). Crystal formed were visible to the naked eyes from day 3 of the incubation. Crystals were not observed in the control flask where the culture GUMP2 was absent (Figure 2b). Complete biotransformation of PG was achieved in seven days of incubation in the static phase, which was indicated by the absence of PG at the bottom of the flask at the end of day 7. This was confirmed by the lack of any solid residue upon acid test performed for precipitate obtained on day 7. In an acid test, effervescence is formed due to the

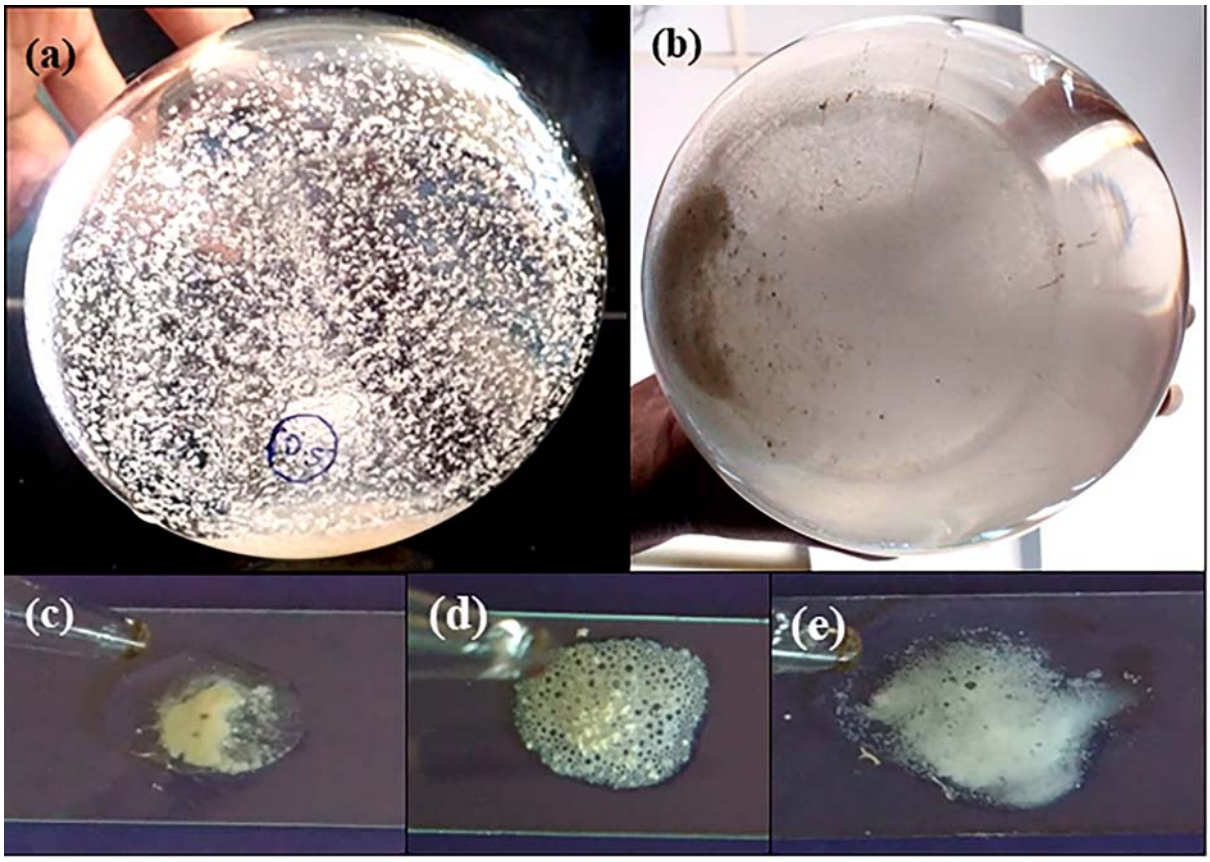

Figure 2. (a) Crystal formed by GUMP2 can be seen at the bottom of the flask, (b) crystals did not form in control flask, (c) acid test of control (mainly PG), (d) acid test of precipitate, (e) acid test of standard $CaCO_3$.

reaction between HCl and $CaCO_3$ forming $CaCl_2$, CO_2, and H_2O as per the following Equation 3:

$$CaCO_3 + 2HCl \rightarrow CaCl_2 + CO_2 + H_2O \quad (3)$$

The effervescence is seen solely due to the release of CO_2 from the $CaCO_3$ (calcite precipitate). In control, the PG powder remained unaffected, and the acid test was negative, indicated by the absence of effervescence (Figure 2c, d, and e).

3.3.2. Ammonium sulfate precipitation

The white precipitate formed when 1 ml of 10% $BaCl_2$ was added to 1 ml supernatant obtained after biotransformation indicated the presence of $(NH_3)_2SO_4$. The white precipitate of Barium sulfate was observed in the test tube (Figure 3). The SO_4 from PG combined with the ammonia (NH_3) produced by GUMP2 and formed ammonium sulfate as per the following Equation 4:

$$BaCl_2 + (NH_4)2SO_4 \rightarrow BaSO_4(\text{white ppt.}) + 2NH_4Cl \quad (4)$$

Upon acetone addition to the supernatant, the white-colored precipitate of ammonium sulfate was formed, and upon drying, the weight of the ammonium sulfate was 1.15 g (Figure 3).

3.4. Characterization of the precipitate obtained in the biotransformation of PG

3.4.1. X-Ray Diffraction (XRD) analysis

XRD analysis of the calcite precipitate showed a diffraction pattern that matched with that of pure Calcite [International Center for Diffraction Data (ICDD) Card no. 05-0586], [36]. The Brag's reflection angles were seen at 29.42°(104), 23.1°(012), 36.05°(110), 39.03°(013), 43.23°(202), 48.16°(018), 48.54°(116), 58.04°(1010). These peaks do not correspond to that of the PG XRD diffraction peaks from the control flask (Figure 4).

Since the XRD peaks pattern showed by the precipitate matched with that of calcite and was different from that of the XRD pattern of phosphogypsum, it further confirms the successful biotransformation of PG to calcite took place in the presence of culture *L. sphaericus* strain GUMP2. The slight difference in the diffraction peaks of precipitated calcite with respect to standard calcite may be due to the minor organic impurities from the bacterial cells present in precipitated calcite. A similar pattern of

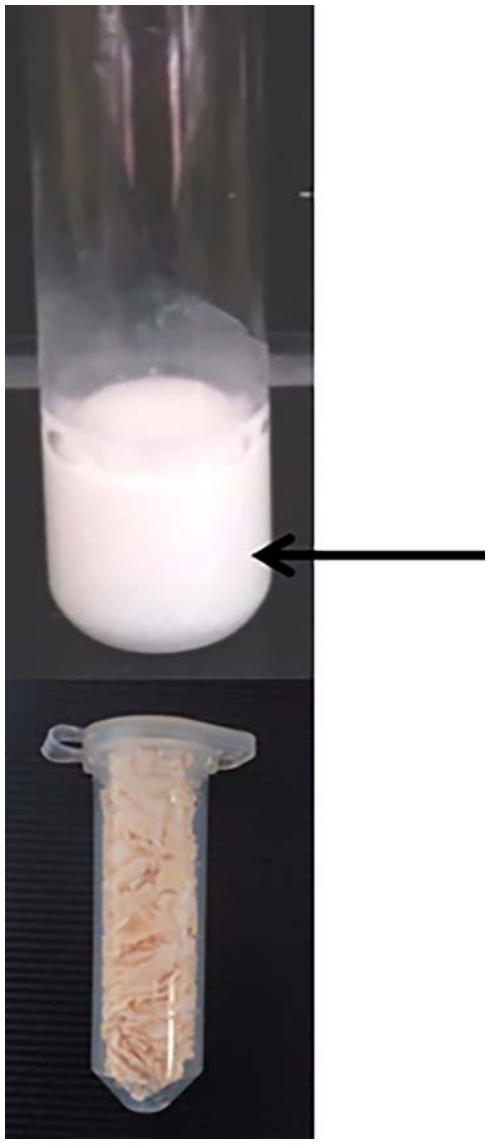

Figure 3. White precipitate of barium sulfate observed in a test tube (top image) (indicated by an arrow). Precipitate ammonium sulfate (bottom photo).

XRD was also seen in the product of the chemical transformation of PG to calcite [42].

3.4.2. Fourier Transform Infrared Spectroscopy (FTIR) analysis

FTIR analysis of the precipitate showed various bonding present in hydroxylated Calcite. The FTIR spectrum showed the presence of a strong band centred around 1431.18 cm^{-1}, characteristics of the C–O stretching mode of carbonate together with a narrow band around 875.68 cm^{-1} of the bending mode (Figure 5). Peaks at 873.75, 711.73 also matched with standard calcite spectrum peaks [37]. The stretching vibrations of the H_2O molecules were seen between 3100 and 3500 cm^{-1}. This may be due to the presence of water of hydration bonded with the calcite. The peaks of FTIR analysis were matching with the standard calcite except for the peaks of the H_2O molecule.

3.4.3. Scanning Electron Microscopy (SEM)

As can be seen from the SEM images, the size of the crystals formed on the glass coverslip ranged from 91.9 to 304.5 µm (Figure 6a). These bead-shaped calcite, when powdered, showed the presence of irregularly shaped crystals (Figure 6b). SEM images can give some insight into the mechanism of crystal formation. At 1000X magnification in SEM, bacterial rod-shaped hollow spaces were observed on the crystals (Figure 6c and d), indicating the rod-shaped cells of *L. sphaericus* strain GUMP2 played direct (acting as a seed for crystal growth) or indirect (supplying necessary ions, etc.) role in the formation of crystals. The cells may have later got detached from the crystal once it was formed or maybe got died, leaving the mark behind. Similar marks of bacterial cells were reported by Anbu et al. [28]. Different studies have shown that this ability was due to the presence of the S-layer covering the cells, it is able to bind high quantities of heavy metals in saline solutions [43]. Another possible mechanism could be that the $CaCO_3$ deposition starts on the bacterial cell surface [28], and a group of bacterial cells (colony) together leads to the formation and characteristic circular, bead-shaped growth of the $CaCO_3$ crystals. During this event, cells get trapped inside the crystals, and as the crystal becomes hard, the cells can no longer move away from the crystal. However, further studies on the exact mechanism of calcite crystal formation from PG need to be carried out.

3.5. Optimization of PG biotransformation using GUMP2

3.5.1. PH optimization

Upon monitoring the pH of all the flasks every day, it was seen that the pH was different every day starting from day 3. The overall trend was that the pH was rising towards 9 from the lower pH values, as well as declining from pH 10 towards pH value 9. In contrast, pH value of the pH 9 flask remained stable at pH 9 from the beginning till the end. On the last day (day 7) of the incubation, the pH of all the flasks reached to pH 9 (Figure S2a). This could be due to the hydrolysis of urea by the urease enzyme forming ammonia and carbamic acid. The two products later combine with water forming bicarbonate and hydroxide ions that result in an increase of pH of the medium and also assist bacterial

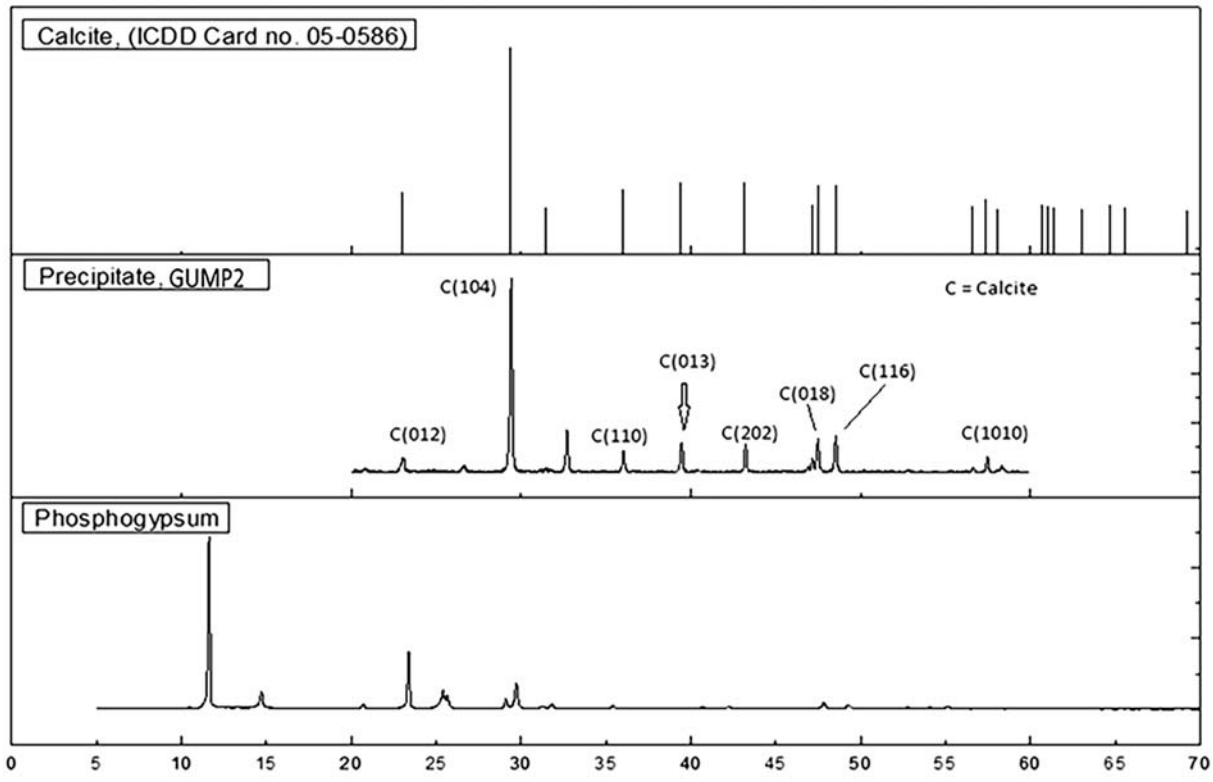

Figure 4. XRD patterns of Precipitate obtained from GUMP2 (middle) and Phosphogypsum (bottom) compared with the standard calcite (top) [36].

cell in precipitating metals. The mechanism was explained in detail by Anbu et al. [26,28,44]. Maximum calcite precipitation was obtained at pH 7 i.e. 0.37 g, followed by at pH 6 and 9 (0.33 g). The least calcite was formed at pH 5 and 10. This could also be related to the growth pattern of the culture during the pH experiment, where the growth of the culture was least at pH 5 and 10 (Figure S2b). This shows that the *L. sphaericus* strain GUMP2 has the ability to grow and biotransform the PG at varying pH ranges. However, with the

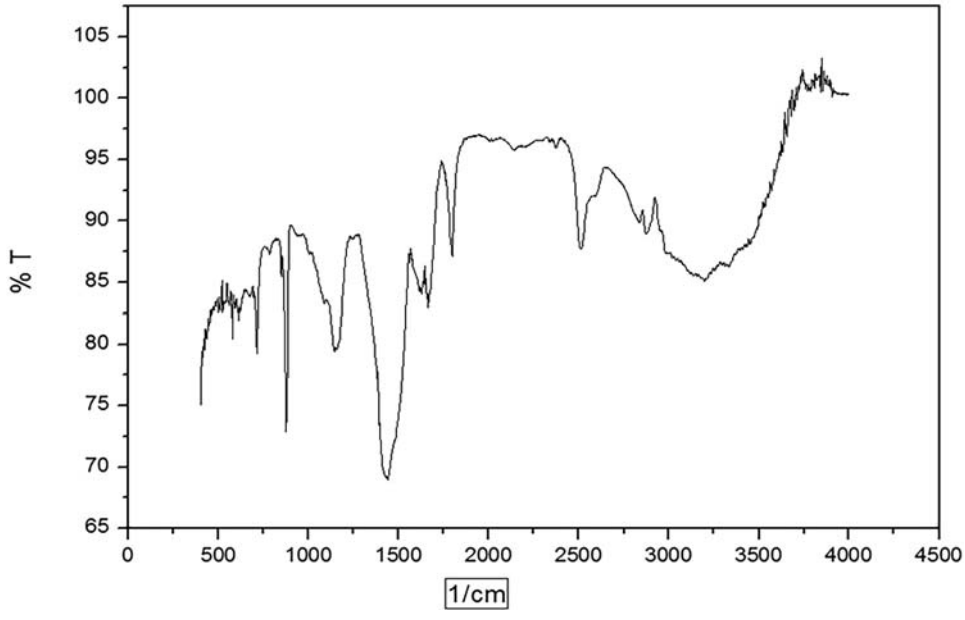

Figure 5. Infrared spectrum of precipitated calcite (GUMP2). The spectrum was compared with standard calcite [37].

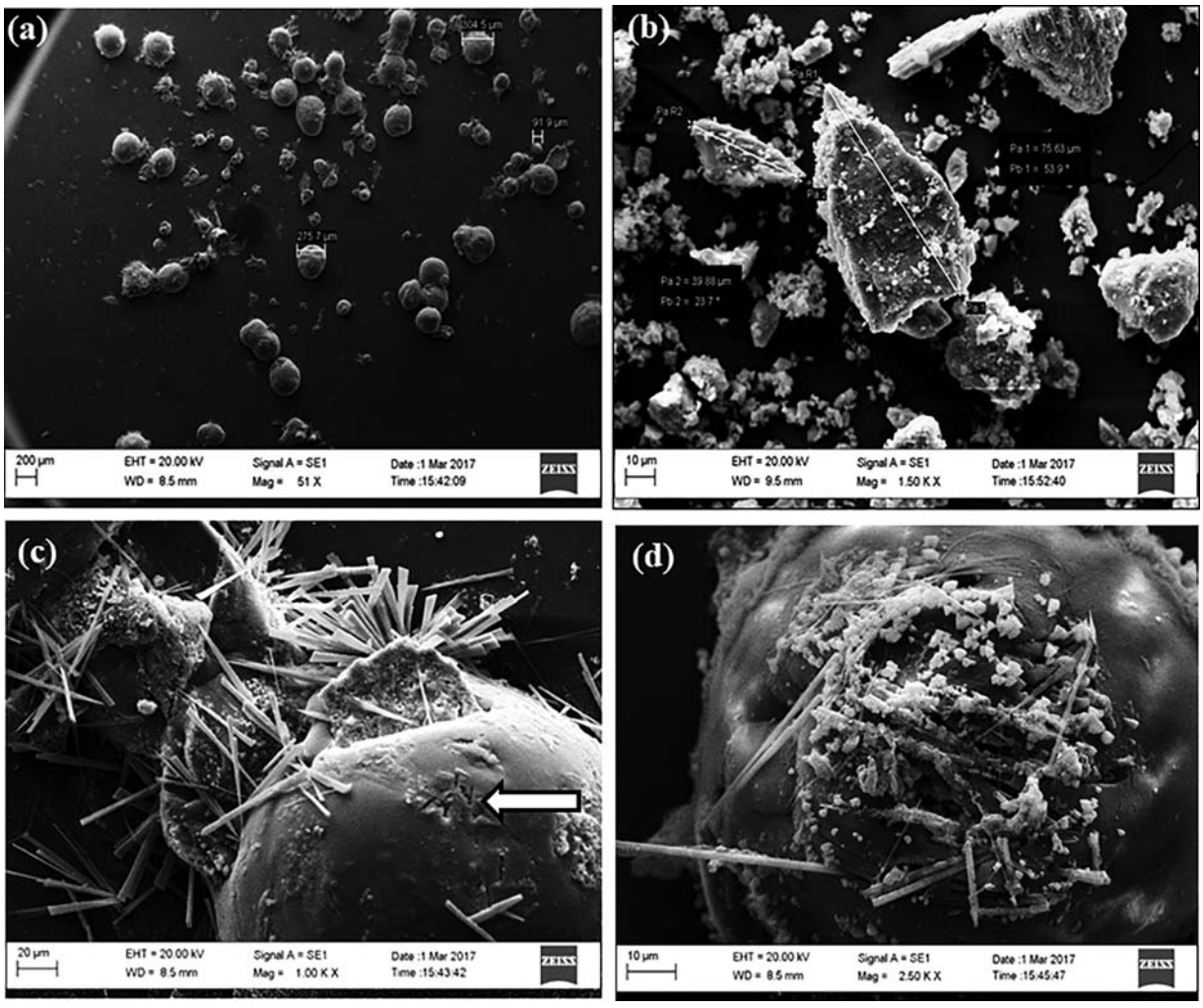

Figure 6. SEM images of calcite crystals. (a) Crystals formed on the glass coverslip (under 51 X mag). (b) Obtained calcite powder (1.5 KX mag). (c) Calcite crystal (1 KX mag) where the imprint of the rod-shaped bacilli can be seen on the bead-shaped crystal (indicated by an arrow). (d) Image of calcite crystal taken from the surface (2.50 KX mag).

observed results in this study, the optimum pH for biotransformation is 7.

3.5.2. Temperature optimization

The culture showed a similar growth pattern at different temperatures. However, it showed maximum growth at 35°C on day 3, followed by at 37°C. Similar growth patterns were observed by Singh et al. [45] where the maximum growth of the culture was seen at 37°C. Cultures showed good growth at 30°C as well; however, at 45°C slight growth was observed on day one and no growth from day two onwards (Figure S2c), indicating the culture could not resist high temperature and died after day 1.

3.5.3. Determination of initiation time for calcite crystal formation

In a time-lapse study for crystal formation, only bacterial cells were observed on the coverslip on the first two days, when observed under a 100X objective lens of the light microscope. Crystal formation was seen from the third day of incubation at 45X magnification Figure 7. This indicates that a minimum of 3 days is required for the sizable crystals to form that can be seen at 45X magnification. Correlating the crystals formation event to the pH experiment, both pH increase and crystal formation started on day 3. From these events, it may be further concluded that the alkaline pH environment is necessary for the crystals to form.

3.5.4. Optimization of biotransformation at different concentrations of phosphogypsum at static and shaker conditions

3.5.4.1. At static condition. Calcite precipitation was observed in all the flasks, but complete biotransformation of PG was observed with 0.5% and 1% of PG concentration upon seven days of incubation. Free and non-transformed PG was still present at the end of day 7 in

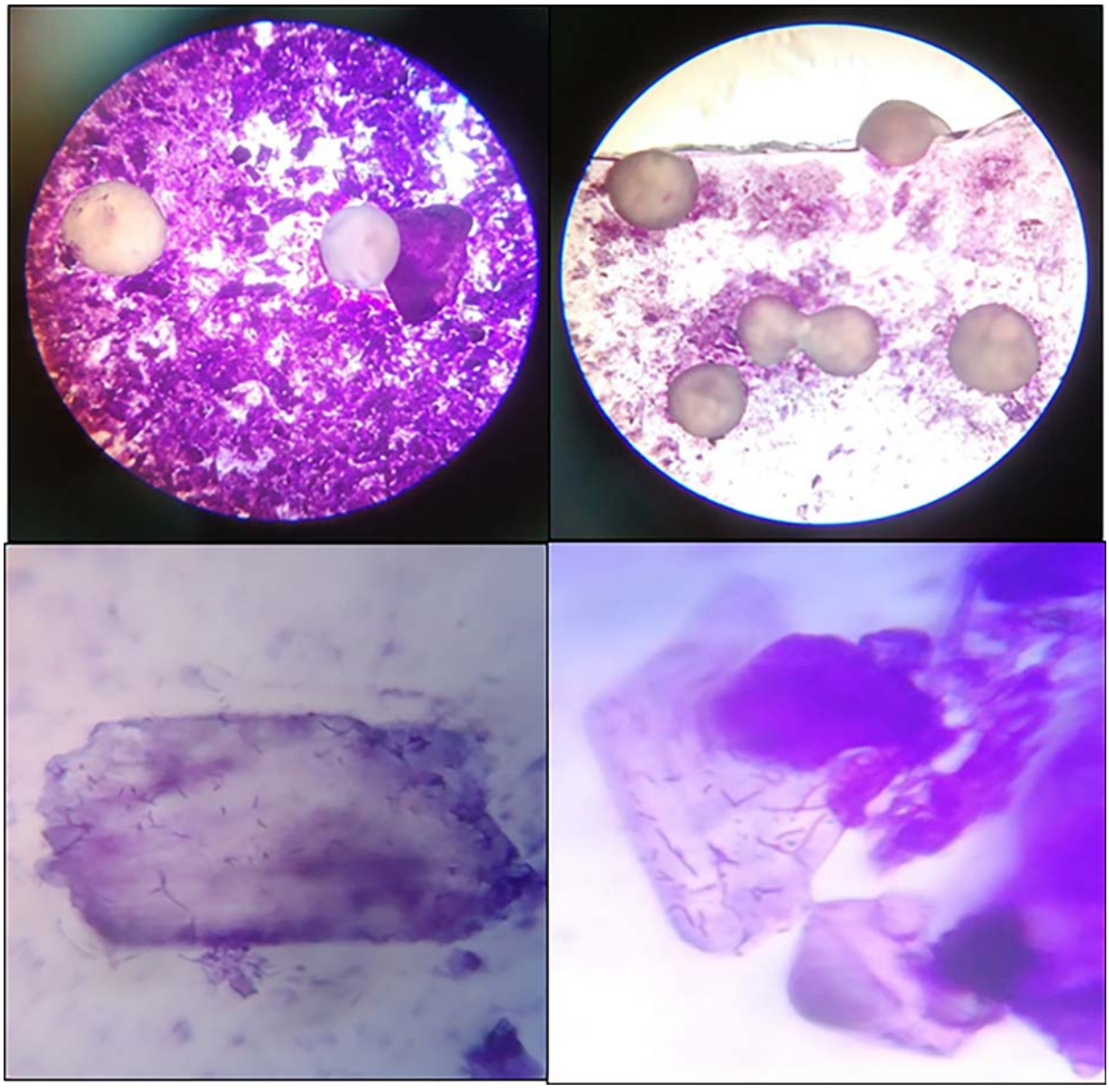

Figure 7. Calcite crystal formation on coverslip under 10X magnification (top two images). Rod-shaped bacterial cells can be seen on the crystals under 45X magnification (bottom two images).

the flasks containing 2.5%, 4%, and 5% of the PG. Hence the optimum concentration of PG for biotransformation within seven days and at static condition was 10 g L^{-1} which is more than that of 6 g L^{-1}, previously reported for sulfate-reducing bacteria [1,16].

3.5.4.2. At shaker condition. Under shaker conditions, flasks containing 1% and 2% PG showed a positive acid test (supernatant along with the precipitate) on day three of the incubation. Sample from the flask containing 4% of PG showed a positive acid test on day four of the incubation. Complete biotransformation was seen on day 7, and a negative acid test was observed for the control flask. At the end of 7 days, the samples from the flasks with PG concentrations 1%, 2%, 4%, and 6% gave a positive acid test. Although effervescence was seen on day 7 with samples from flasks containing higher PG concentrations (8% and 10% PG), complete hydrolysis of the precipitate in the acid test did not occur even after further incubation up to 10 days. Hence it is clear that up to 60 g L^{-1} of PG was completely biotransformed within seven days of incubation at shaker conditions.

L. sphaericus, a spore-forming bacterium, can be commonly found in soil, especially in metal contaminated soil, and can be isolated from such soils [27]. The culture can be easily isolated using microbiological isolation methods. Tryptone soya broth can be as an enrichment medium, and for isolation, tryptone soya agar [46] or nutrient agar (as used in this study) can be used. Further, pure culture

can be obtained using a suitable microbial purification technique. From the results of this study, *L. sphaericus* mediated PG biotransformation is a promising method for large-scale biotransformation of PG. In static mode, the process can be very economical; however, the efficiency would be limited. In static condition (limited oxygen environment), a maximum concentration of PG that was completely biotransformed to calcite within seven days was 10 g $L^{-1,}$ whereas this was up to 60 g L^{-1} at shaker conditions (oxygen saturation environment). From this it can be concluded that the culture *L. sphaericus* strain GUMP2 is capable of transforming up to 60 g L^{-1} PG to calcite under oxygen rich environment. However at a higher scale, one will have to bear additional cost of higher aeration. Animals are very good source of urea. Humans excrete on a average about 25–30 g of urea per day, cows excrete 69% of urea in their urine [47]. Such urine, if collected separately, could be a potential, economic and sustainable source of urea for the large-scale biotransformation of PG using *L. sphaericus* strain GUMP2. A detailed economical analysis of using such a urea source would explain the feasibility of the large-scale biotransformation process. However, it is out of the scope of this current study and will be dealt in future research.

4. Conclusion

In this study, phosphogypsum, a solid by-product of phosphoric acid manufacturing, was entirely transformed to calcite using urease-producing *Lysinibacillus sphaericus* strain GUMP2. We used a modified SF medium for the growth of *L. sphaericus* strain GUMP2. *L. sphaericus* utilized urea as the substrate for the urease enzyme and PG as the sole calcium source to produce calcite. The produced $CaCO_3$ was precipitated as the visible bead-shaped crystal in the medium, while ammonium sulfate was produced in a soluble form. This is the first report on the use of urease-producing aerobic bacteria for biotransformation of PG to $CaCO_3$ and ammonium sulfate. The method is efficient over other biological processes of PG transformation involving SRB. Such calcite precipitation method will act as a promising and eco-friendly remediation technology to immediately solve the problem of phosphoric acid manufacturing industries along with the significant application in other areas, including construction and agricultural sectors.

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Data availability statement

The authors confirm that the analyzed data supporting the findings of this study are available in the supplementary material. Raw data generated at Goa University supporting the findings of this study are available from the corresponding author [MP] on request.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Authors' contributions

PPP and MP contributed equally to this manuscript. PPP carried out material preparation, data collection, and analysis; wrote the first manuscript draft. MP conceived the idea, designed the experiments, did data analysis, wrote the manuscript draft, and edited the manuscript. SM contributed to the designing of some of the experiments and edited the manuscript.

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