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# Boosting nitrogen fertilization by a slow releasing nitrate-intercalated biocompatible layered double hydroxide–hydrogel composite loaded with *Azospirillum brasilense*†

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Indiscriminate use of chemical fertilizers leads to soil environmental disbalance and therefore, preparation and application of environment-friendly slow-release multifunctional fertilizers are of paramount importance for sustainable crop production in the present scenario. In this study, we propose a slow-release multifunctional composite nitrogen (N) fertilizer, which possesses the ability to supply plant accessible N in the form of ammonium ( $\text{NH}_4^+$ ) and nitrate ( $\text{NO}_3^-$ ) to improve nitrate assimilation coupled with zinc (Zn, a major micronutrient for plants in the soil) after its degradation. For this purpose,  $\text{NO}_3^-$ -intercalated zinc–aluminum (Zn–Al) layered double hydroxide (LDH) was synthesized using a co-precipitation protocol. The prepared LDH was added as 25.45% of total polymer weight to a sodium carboxymethyl cellulose/hydroxyethyl cellulose citric acid (NaCMC/HEC-CA) biodegradable hydrogel. *A. brasilense*, commonly used nitrogen-fixing bacteria in soils, was added to the LDH–hydrogel composite along with LDH alone to augment the availability of  $\text{NH}_4^+$  and  $\text{NO}_3^-$ . Adjusting the pH under acidic (pH 5.25) and neutral (pH 7) conditions, the release pattern of  $\text{NO}_3^-$  from LDH and the composite was monitored for 30 days at normal temperature. The pH was selected based on the soil analysis data of North East India. The LDH-composite released 90% (w/w) and 85.45% (w/w) of intercalated  $\text{NO}_3^-$  at pH 5.25 and 7.00 respectively in 30 days. However, 100% (w/w) and 87% (w/w) of intercalated  $\text{NO}_3^-$  at pH 5.25 and 7.00 respectively were released in 30 days when only LDH was applied, which indicated the lower performance of LDH alone in comparison to the LDH-composite for the nitrate holding pattern. The pH of the bacteria-loaded system was observed to be acidic (pH = 5–6) during the study of nitrate assimilation and  $\text{Zn}^{2+}$  release. *A. brasilense* improved nitrate assimilation and increased the  $\text{NH}_4^+$  ion concentration in the studied system. A significant increase in  $\text{Zn}^{2+}$  release was observed from day 5 in the presence of *A. brasilense* in the LDH-composite compared with that in the absence of *A. brasilense*. In conclusion, the prepared LDH–hydrogel–*A. brasilense* composite fertilizer system increases the availability of plant accessible N form (both  $\text{NO}_3^-$  and  $\text{NH}_4^+$ ) and can potentially improve soil fertility with the addition of Zn and bacteria to the soil in the extended course.

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## 1. Introduction

The concept of slow-release fertilizers emerged for the limitation of crises erupting from various dimensions of applying conventional chemical fertilizers including nutrient burn and ecological contamination, following leaching of nutrient ions.<sup>1</sup> Llive *et al.*<sup>2</sup> and Gungula *et al.*<sup>3</sup> have recently reported that the slow-release fertilizers prevent nutrient loss as well as control

the uptake of nutrients by plants to increase the fertilizer use efficiency. An excellent review by Vejan *et al.*<sup>4</sup> highlighted that slow-release fertilizers reduce nutrient ion runoff to water streams and rich nutrient use results in reduced environmental pollution, offering sustainability in agriculture and ecological hazard control compared to conventional fertilizers.

Recently, layered double hydroxide (LDH) materials have received tremendous attention as a very suitable agent for the formulation of controlled-release fertilizers.<sup>5,6</sup> Their structure comprises a ‘two-dimensional lamellar structure’ having dipositive and tripisitive ions at the center of octahedra, which form an infinite sheet by joining edge to edge.<sup>7–9</sup> Hydrated anions such as  $\text{NO}_3^-$  and  $\text{PO}_4^{3-}$  are present in the interlayer gallery region of the positively charged sheets for charge

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compensation.<sup>9,10</sup> The general formula of LDH materials is  $[M^{II}_{(1-x)}M^{III}_x(OH)_2][A^{n-}]_{x/n} \cdot zH_2O$ , where  $M^{II}$  and  $M^{III}$  are dipositive and tripositive metal ions.  $A^{n-}$  is the intercalated charge compensating anion and  $x$  represents the molar ratio  $M^{3+}/(M^{3+} + M^{2+})$ .<sup>7</sup> Due to their exceptional anion exchange property, versatility in intercalating a broad class of anions and non-toxic character, LDH materials and their polymer composites have been effectively used as slow-release agents in the agricultural and biomedical applications.<sup>11–13</sup>

Acidic soil is very much prone to plant nutrient dynamics, and under such conditions, LDH materials in the soil supply need-based nutrients due to their structural stability. The rupture of the lamellar structure of the LDH material and faster nutrient release caused by a lower soil pH are prevented to certain degree by the alkaline buffering of hydroxyl groups liberating from the LDH layered structures.<sup>14</sup> When the soil acidity predominates the alkaline buffering, the LDH material gradually loses its lamellar structure and the original slow release character. To overcome this, a composite formulation of the LDH material with cellulose and alginate-based biodegradable hydrogel materials had been documented by several researchers.<sup>11,14–18</sup> Carboxymethyl cellulose (CMC), hydroxyethyl cellulose (HEC) and alginate-based biodegradable hydrogels have already established their property of nutrient uptake and delivery, along with excellent water holding capacity, non-toxicity, and renewability.<sup>19–22</sup> However, their versatile applicability is hindered by the low mechanical strength of the materials. The composite formulation of the LDH material with biodegradable hydrogels presents a mutual complementary system, where the LDH material induces mechanical stability in the composite, while the hydrogel polymer provides structural barrier with soil acidity, along with water retention property. Several works improvising this concept have been reported.<sup>19–22</sup>  $[Zn-Al-NO_3^-]$ -LDH encapsulated with biodegradable alginate beads for enhanced nitrogen fertilization,<sup>23</sup> composites of norfloxacin-intercalated Mg-Al LDH with carboxymethyl cellulose, cross-linked by citric acid for biomedical applications,<sup>24</sup> carboxymethyl cellulose-based nanocomposite hydrogel beads with Cu-Al LDH for bacterial infection treatment<sup>18</sup> and several other layered nanocomposites are reported to have shown controlled release property.<sup>25–28</sup>

Furthermore, soil existing microbes convert the released nutrients into simpler plant-useable forms.<sup>29</sup> Plants take up N in the  $NO_3^-$  or  $NH_4^+$  form depending upon the various constraints related to the plant environment. Some plants prefer a mixture of  $NO_3^-$  and  $NH_4^+$  forms as a source of N from the growing medium.<sup>30,31</sup> Nitrogen taken up as  $NH_4^+$  directly does not need any further conversion post intake and can be directly used in amino acid production, termed as ammonium assimilation.  $NO_3^-$  after being absorbed from the soil follows two paths. The first involves immediate reduction of  $NO_3^-$  in the roots and the second involves  $NO_3^-$  reduction in leaves after the root-to-shoot transportation *via* the xylem. The assimilation of N taken up as  $NO_3^-$  proceeds *via* the reduction of  $NO_3^-$  into  $NO_2^-$  by a nitrate reductase enzyme, followed by the reduction of  $NO_2^-$  into  $NH_4^+$  by a nitrite reductase enzyme. The enzyme nitrate reductase is a homodimer possessing flavin adenine dinucleotide (FAD),

haem, and a molybdenum cofactor (MoCo).<sup>32</sup> The nitrite reductase enzyme contains sirohaem and a FeS cluster. The reduction of  $NO_3^-$  into  $NH_4^+$  requires 8 moles of electrons per mole of  $NO_3^-$ , proving to be an energy-consuming process for plants. Thus, during the reduction of  $NO_3^-$  in the roots, the microbes existing therein, possessing the required enzymes, can offer assistance in nitrate assimilation.<sup>30,31</sup>

Apart from the mentioned benefits of using LDH and its composite materials for the loading and release of different essential nutrient ions from the LDH gallery regions, the LDH material could also serve as a source for additional nutritional elements for the plants. Hatami *et al.*<sup>33</sup> reported increased availability of zinc in  $[Zn-Al-PO_4]$ -LDH-applied soil samples. Lopez-Rayó *et al.*<sup>34</sup> also reported increased zinc uptake by plants on the application of Zn-doped Mg-Al-LDH materials to plants. This was attributed to the partial dissolution of Zn from the LDH sheet structure due to various soil stimuli, which may be soil acidity or action of different microorganisms present already in the rhizosphere.

From all the above-mentioned information, we herein report the synthesis of a multifunctional N fertilizer system. The Zn-Al-LDH material was used as a basal source for the intercalation of  $NO_3^-$  ions. To restrict the faster release of  $NO_3^-$  from LDH and to induce water retention properties, the CMC/HEC hydrogel was incorporated into the system. Further to this system, a soil existing bacterial species, *Azospirillum brasilense*, was added to enhance nitrate assimilation and  $N_2$  fixation ( $NH_4^+$  source) from the atmosphere. Eventually, the degradation of the LDH material was also anticipated, and the release of plant essential Zn from LDH in the presence of the bacteria was carried out. The purpose of this study was to synthesize this highly efficient and multifunctional composite system and analyze its efficacy for providing plant essential nutrients N ( $NO_3^-$  and  $NH_4^+$ ), Zn ( $Zn^{2+}$ ), and the bacteria for improving the overall soil fertility regime.

## 2. Materials and methods

### 2.1 Reagents

Zinc nitrate hexahydrate  $[Zn(NO_3)_2 \cdot 6H_2O]$ ; CAS Number: 1019618-6], aluminium nitrate nonahydrate  $[Al(NO_3)_3 \cdot 9H_2O]$ ; CAS Number: 7784-27-2], dipotassium hydrogen phosphate  $[K_2HPO_4]$ ; CAS Number: 7758-11-4], and magnesium sulphate  $[MgSO_4]$ ; CAS Number: 7487-88-9] were purchased from Merck KGaA, Darmstadt, Germany. Sodium molybdate and manganese sulphate were purchased from FINAR, Ahmedabad, Gujrat, India. Sodium nitrate was obtained from MERCK, Maharashtra, India. Sodium hydroxide and citric acid were obtained from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Carboxymethyl cellulose sodium salt (high viscosity) with an average molecular weight of  $\sim 900\,000$  Da and hydroxyethyl cellulose (high viscosity) with an average molecular weight of  $\sim 380\,000$  Da, and ferrous sulphate were purchased from Research Lab Fine Chem Industries Mumbai, India. Malic acid and bromothymol blue were obtained from CDH, New Delhi, India. Sodium chloride and potassium hydroxide were purchased from RANKEM, New Delhi, India. For all synthesis



purposes, ultra-pure water or Milli-Q (18.2 M $\Omega$  cm) water was used throughout the experiment.

## 2.2 Synthesis of [Zn/Al-NO<sub>3</sub>]-LDH, CMC/HEC-CA hydrogels, and LDH-hydrogel composites

[Zn-Al-NO<sub>3</sub>]-LDH (ZLDH) was synthesized by a co-precipitation method.<sup>8,13,35</sup> A 30 mL solution of Zn(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O and Al(NO<sub>3</sub>)<sub>3</sub>·9H<sub>2</sub>O in 3 : 1 ratio was added to 30 mL of 1 M solution of NaNO<sub>3</sub> in Milli-Q water, with constant addition of a 2 M NaOH solution to control the pH and precipitate LDH, keeping the temperature at 75 °C. The nitrogen atmosphere was maintained throughout to minimize CO<sub>3</sub><sup>2-</sup> intercalation. The solution was aged for 24 hours in a nitrogen atmosphere and the same temperature condition. The white precipitate of LDH formed was separated by high-speed centrifugation and repeatedly washed with Milli-Q water. The product was then allowed to dry in a vacuum desiccator for 2–3 days and stored for further analyses.

The CMC/HEC-CA (CMCH)-based hydrogel from the sodium salt of carboxymethyl cellulose (NaCMC) and hydroxyethyl cellulose (HEC), cross-linked with citric acid in Milli-Q water was synthesized as reported.<sup>20</sup> The sodium salt of carboxymethyl cellulose (NaCMC) and hydroxyethyl cellulose (HEC) was mixed in Milli-Q water in a 3 : 1 ratio with constant stirring. After attaining a clear solution, 3% (w/w polymer) citric acid cross-linker was added. The resulting gel-like mixture was transferred to a Petri dish and dried in a hot air oven at 70 °C temperature. The LDH-hydrogel composite was prepared by adding the synthesized ZLDH sample as 25.45% of total polymer weight to the solution mixture containing NaCMC (1.5 g) and HEC (0.5 g), along with the cross-linker citric acid (0.06 g), as described by Nia *et al.*<sup>18</sup> and Demetri *et al.*<sup>20</sup> The resulting mixture was stirred, followed by ultra-sonication to obtain a homogeneous solution. The solution was then transferred to a Petri dish and heated in a hot air oven at 45–50 °C for 12 hours. The sample obtained was named C-3 and stored for further analyses.

## 2.3 Preparation of *Azospirillum* culture media

Nitrogen-free malate media were prepared for *Azospirillum brasilense* by mixing malic acid (5.0 g), K<sub>2</sub>HPO<sub>4</sub> (0.5 g), FeSO<sub>4</sub> (0.05 g), MnSO<sub>4</sub> (0.01 g), MgSO<sub>4</sub> (0.1 g), NaCl (0.02 g), CaCl<sub>2</sub> (0.01 g) and Na<sub>2</sub>MoO<sub>4</sub> (0.002 g) in 1.0 L deionized water. To this, 2 mL of 0.5% alcoholic solution of bromothymol blue and 4.0 g KOH were added. The pH of the solution was kept around 6–7. The solution was autoclaved at 120 °C and 15 lbs. pressure for 15 minutes and put under a UV light inside a laminar airflow cabin for complete sterilization. This sterile culture media solution was then inoculated with *A. brasilense* bacteria. The cultured bacterial broth solution was then incubated at 30 °C for 72 hours.<sup>36</sup>

## 2.4 Characterization of [Zn-Al-NO<sub>3</sub>]-LDH, CMC/HEC-CA hydrogels, and LDH-hydrogel composite

Fourier transform infrared (FTIR) spectra of the samples were recorded using an FTIR spectrometer (Agilent Model no: Cary

630, USA; SL. no. MY20192018) in the range of 400–4000 cm<sup>-1</sup>. Thermogravimetric (TGA) analyses of the samples were carried out using a PerkinElmer JS-1 TGA/DSC thermogravimetric analyzer, operating in a nitrogen environment in the temperature range of 25–900 °C at a heating rate of 10 °C min<sup>-1</sup>. The PXRD patterns of the samples were obtained using a Bruker D8 Advance X-ray diffractometer by irradiating with monochromatic Cu K $\alpha$  radiation ( $\lambda = 1.54056$  Å). The surface morphology of the samples was analyzed using a JEOL JSM-IT300 Scanning Electron Microscope. The concentration of Zn present in test samples was determined using an atomic absorption spectrometer (AAS) (Agilent, Model no. AA240, Malaysia). For Zn estimation, two standard reference materials (SRMs), namely, tomato leaves (SRM-1573a) and Montana soil (SRM-2710a) were used for quality assurance. The estimated values of Zn were in excellent agreement with the certified values in SRM1573a and SRM-2710a. All the samples were analyzed with three replications.

## 2.5 Species detection test for inoculated *A. brasilense* bacterial species

The growth of *A. brasilense* was first tested by streaking from the incubated culture into an agar plate. The constituents of agar medium are the same as the constituents of the culture media for *A. brasilense* with the addition of agar-agar powder for solidification of the media. Malic acid (5.0 g), K<sub>2</sub>HPO<sub>4</sub> (0.5 g), FeSO<sub>4</sub> (0.05 g), MnSO<sub>4</sub> (0.01 g), MgSO<sub>4</sub> (0.1 g), NaCl (0.02 g), CaCl<sub>2</sub> (0.01 g), Na<sub>2</sub>MoO<sub>4</sub> (0.002 g), 4.0 g KOH and 2 mL of 0.5% alcoholic solution of bromothymol blue were added to 1.0 L deionized water. To this solution, 13 g of agar-agar powder was added and then the solution was autoclaved and then poured on Petri plates. On cooling, the media solidified and the agar plates were ready to use.

The catalase test was performed to confirm the presence of the species. For this, the pre-cultured bacterial broth was streaked onto an agar plate and incubated for 24 hours at 30 °C. To the plate, 3–4 drops of 3% H<sub>2</sub>O<sub>2</sub> solution were allowed to drip. The formation of bubbles shows the presence of *A. brasilense* species.<sup>37</sup>

## 2.6 Investigation of water retention property

The water retention property of the synthesized ZLDH and the composite sample C-3 was investigated by teabag method at pH 5.25 and 7.0 at 25 °C for 24 hours. The swollen weight was recorded. Following this, the weight of the sample was recorded for the next six consecutive days to observe the water retention ability.

To observe the recyclability in swelling and water retention capacity, the C-3 sample was put for four consecutive cycles of swelling and drying at pH 5.25. Accurately, 0.3553 g of the C-3 sample was weighed and allowed to swell at pH 5.25 for 24 hours. The swollen weight of the sample for consecutive days was recorded and the cycle was repeated with the remaining dry sample after weighing at a temperature varying from 25 to 35 °C. The data for four consecutive cycles were recorded in triplicates.



## 2.7 Nitrate release study

The percentage loading and slow nitrate release studies for ZLDH and composite C-3 were performed by a spectrophotometric method<sup>38,39</sup> using a SHIMADZU UV-VIS Spectrophotometer within the range of 200–800 nm.  $\text{KNO}_3$  was taken as the standard for the calibration curve. Milli-Q water was used as a blank throughout the experiments. Each observation was made in triplicates.

For a comparative study of nitrate release from ZLDH in distilled water, Milli-Q water, and carbonated water, 350 mg of the ZLDH sample was added to 250 mL each of distilled water, Milli-Q water, and 0.08%  $\text{Na}_2\text{CO}_3$  solution, and the solutions were stirred. From each solution at specific time intervals, test solutions were withdrawn and centrifuged and the volume of the parent solutions was made up. Required volume was taken from the supernatant for spectroscopic analyses. Linearly, for comparative studies, 350 mg of C-3 sample was taken in 250 mL distilled water and stirred. Test solutions were taken out and analyzed as mentioned above. To compare the nitrate release rate from ZLDH and C-3 samples with conventional nitrate fertilizer  $\text{KNO}_3$ , 1.4 mM solution  $\text{KNO}_3$  was prepared, test solutions were withdrawn at similar time intervals and spectrophotometric analyses were carried out.

## 2.8 *A. brasilense*

**2.8.1 Incorporation of *A. brasilense* into the composite system.** First, 5 mL of bacterial broth of *A. brasilense* was added to a solution containing 35 mg of C-3 composite sample in 25 mL distilled water. To compare the relative growth and action of the bacterial species on the C-3 composite, an equal amount of bacterial broth was added to 25 mL of a solution containing 35 mg of ZLDH sample, 25 mL solution containing 35 mg of CMCH sample, and 25 mL of 0.4 mM  $\text{KNO}_3$  solution and put on a shaker incubator at 25 °C. For control studies, a solution with 5 mL of bacterial culture in 25 mL of deionized water and 25 mL of  $\text{NH}_4\text{Cl}$  solution were also taken. The presence of the bacteria in the samples was verified by streaking from the bacteria-loaded samples onto agar plates and the growth was observed on the agar plates on the day of addition of the bacterial culture to the test samples. The same experiment was repeated at an interval of days to verify the presence of the bacteria in the test solutions.

**2.8.2 Study of nitrate assimilation by *A. brasilense*.** Particular volumes were withdrawn from the bacteria-inoculated samples of ZLDH, C-3 and  $\text{KNO}_3$  at different time intervals. The supernatant obtained after centrifugation was used for testing the presence of  $\text{NO}_3^-$  ions according to the above-mentioned spectrophotometric analysis. The presence of  $\text{NO}_2^-$  ions converted by the bacterial reduction from  $\text{NO}_3^-$  ions was established by the spectrophotometric method mentioned in Section 2.7. For a clear indication of the increase in  $\text{NO}_2^-$  ion concentration in test solutions, a Red Sea MCP Nitrite/Nitrate ( $\text{NO}_2^-/\text{NO}_3^-$ ) Test Kit with a test range of 0–1  $\text{mg L}^{-1}$  was used.<sup>40</sup> ZLDH, C-3 and  $\text{KNO}_3$  samples without loading with the bacterial broth were taken as control.

**2.8.3 Presence of  $\text{NH}_4^+$  in test solutions.** Traces of  $\text{NH}_4^+$  ions were also expected to be present in solutions loaded with

*Azospirillum* broth due to atmospheric nitrogen fixation and possible conversion from nitrite by the bacteria *A. brasilense*. The presence of  $\text{NH}_4^+$  ions was confirmed by testing with Nessler's reagent, and the concentration was estimated using a Tetra Ammonia test kit ( $\text{NH}_3/\text{NH}_4^+$ ) with a test range of 0–8  $\text{mg L}^{-1}$ .<sup>41,42</sup>

**2.8.4 Presence of Zn in bacteria-loaded samples.** The estimation of Zn was carried out by withdrawing equal volumes from the test solutions containing ZLDH and C-3 composite materials, loaded with *Azospirillum* (mentioned in Section 2.8.1), at certain intervals and centrifuged. From the supernatant, 1 mL of solutions were taken for the investigation of traces of  $\text{Zn}^{2+}$ , if any. As control, samples were collected from *Azospirillum* broth (alone), ZLDH (without *Azospirillum*), and C-3 (without *Azospirillum*). The investigation and quantification of  $\text{Zn}^{2+}$  in the test samples were carried out using an atomic absorption spectrometer (Agilent, Model no. AA240, Malaysia).

## 3. Results

### 3.1 FTIR analysis

The FTIR spectra (Fig. 1a) of ZLDH exhibited a broad band around 3400–3600  $\text{cm}^{-1}$  due to the O–H stretching vibration of  $\text{H}_2\text{O}$  molecules and physically adsorbed hydroxyl groups in the LDH layers. Bands appearing at 1642  $\text{cm}^{-1}$  and 1385  $\text{cm}^{-1}$  are assigned to the bending mode of  $\text{H}_2\text{O}$  molecules and the characteristic stretching vibration for intercalated  $\text{NO}_3^-$  ions in the LDH layers. CMCH (Fig. 1b) shows its characteristic absorption peak at 1715  $\text{cm}^{-1}$  due to ester bond formation, resulting after the loss of water molecules and a strong characteristic absorption peak at 1600  $\text{cm}^{-1}$ . The band at 1065  $\text{cm}^{-1}$  is due to the C–O–C stretching vibration. A broad peak around 3346  $\text{cm}^{-1}$  is due to the O–H stretching vibration, and the peak at 2927  $\text{cm}^{-1}$  is due to the C–H stretching vibration. C-3 (Fig. 1c) exhibited a broad peak around 3436  $\text{cm}^{-1}$  due to the O–H stretching vibration of physically adsorbed water

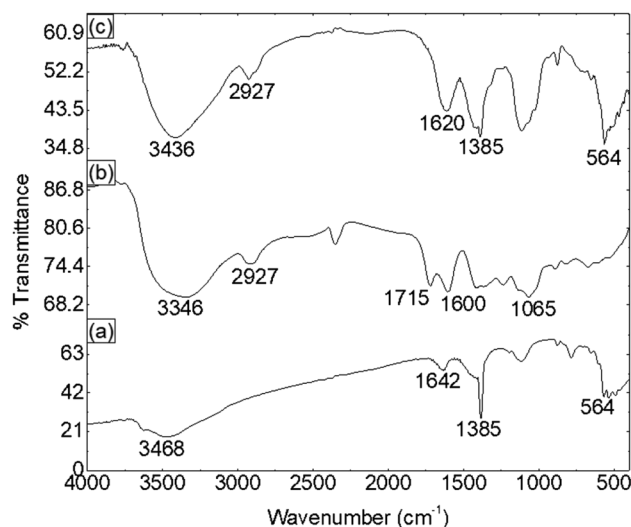


Fig. 1 FTIR spectra of (a) ZLDH, (b) CMCH, and (c) C-3 samples.



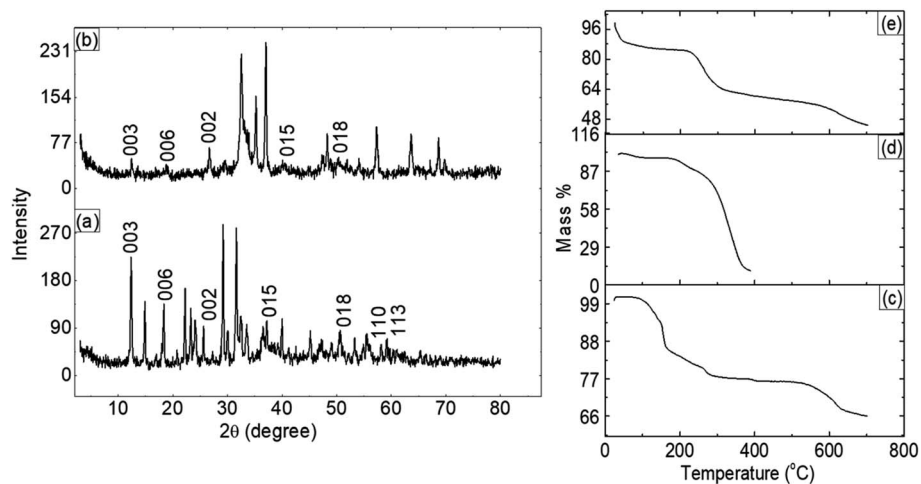


Fig. 2 PXRD patterns for (a) ZLDH and (b) C-3. TGA patterns for (c) ZLDH, (d) CMCH and (e) C-3 samples.

molecules and hydroxyl groups present in the basal layers. The peak around  $1385\text{ cm}^{-1}$  shows  $\text{NO}_3^-$  ion intercalation in the LDH layers. The peak at  $2927\text{ cm}^{-1}$  was obtained due to C-H stretching, which comes from the hydrogel part.

### 3.2 PXRD analysis

The PXRD patterns of ZLDH (Fig. 2a) showed sharp and symmetric characteristic peaks corresponding to the 003, 006 planes, characteristic of clay minerals having a layered structure. The same peak appears in the spectra of the C-3 (Fig. 2b)

composite sample at a  $2\theta$  value of  $12.39^{\circ}$ , with a basal spacing of  $7.1442\text{ \AA}$ , but with a lower intensity.

### 3.3 TGA analysis

In the TGA thermogram (Fig. 2c) for ZLDH, the first break was observed in the temperature range of  $110\text{--}147\text{ }^{\circ}\text{C}$ , due to the elimination of physically adsorbed water molecules from the layered surface. The second break was obtained in the range of  $170\text{--}274\text{ }^{\circ}\text{C}$ , whereas the third break was observed in the range of  $570\text{--}640\text{ }^{\circ}\text{C}$ . From the TGA thermogram of CMCH (Fig. 2d), it

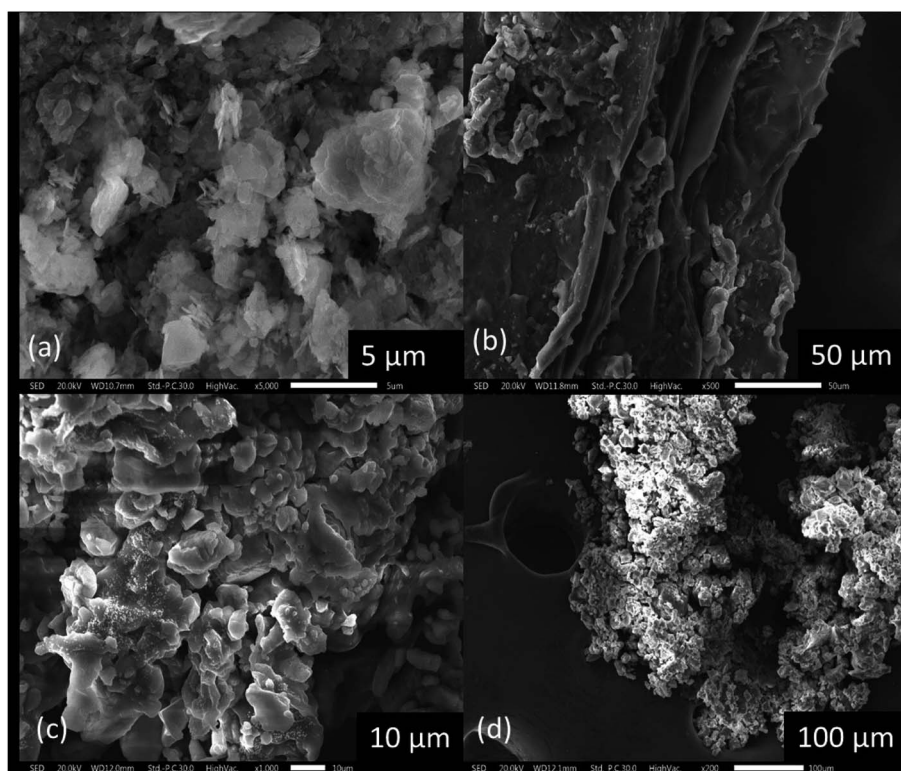


Fig. 3 SEM micrographs for (a) ZLDH, (b) CMCH, (c) C-3 sample and (d) C-3 sample.



is observed that the first break was obtained near 100 °C due to the loss of water molecules. Around 300 °C, the sample decomposes. In case of composite sample C-3 (Fig. 2e), the first weight loss was obtained in the temperature range of 20–110 °C. The second break was obtained around 235–296 °C and the third break was observed around 632 °C.

### 3.4 Surface morphology analysis

In the SEM images (Fig. 3a) of ZLDH, an aggregated mass of irregularly shaped particles, characteristic of LDH materials was obtained. LDH surfaces are observed as agglomerated and flake-like with a crystalline outer structure. The SEM micrograph of CMCH (Fig. 3b) shows ripped portions due to cross-linking. The composite C-3 (Fig. 3c and d) shows the aggregated mass, but not as flakes.

### 3.5 Study of the water retention property

The water retention and release profiles were studied at pH 7 and 5.25 for the samples as mentioned in Section 2.6.

The swelling behavior of the different samples in the respective pH media is expressed by the percentage swelling as follows:

$$\text{Swelling \%} = \frac{\text{released mass of sample (g)}}{\text{actual material mass (g)}} \times 100$$

where released mass = swollen mass of samples – dry mass of samples.

The water swelling and retaining capacity of the CMCH sample was observed to be highest at both the pH values (Fig. 4). The C-3 sample exhibited decent water swelling and retention behavior in both pH media. The swollen weight and the water-retaining capacity of the CMCH and the composite C-3 were observed to be highest in a neutral medium (Fig. 4a, b and 5a). At an acidic pH value, water molecules were released from the samples at a faster rate.

The recyclability in swelling and water-retaining capacity of the C-3 sample were studied for four consecutive cycles, and it was observed that the sample indeed exhibited profound water retention behavior till the fourth cycle, extending to the fourth week (Fig. 5b). However, the original mass of the sample could

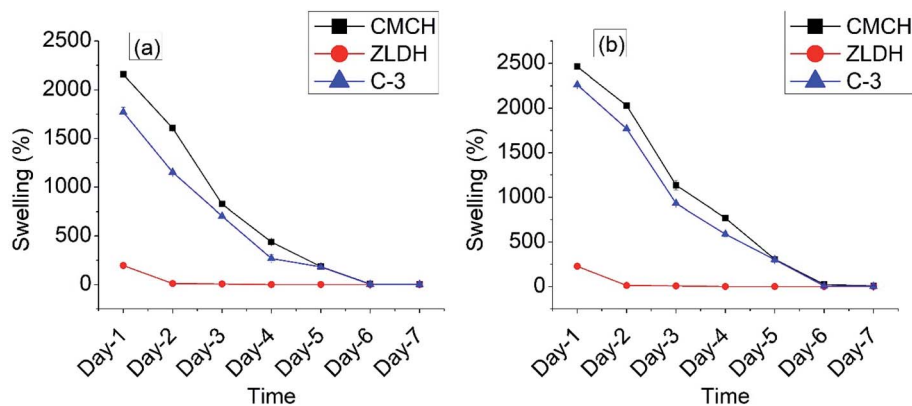


Fig. 4 Comparative water release profile for ZLDH, CMCH and C-3 at (a) pH 5.25 and (b) pH 7.

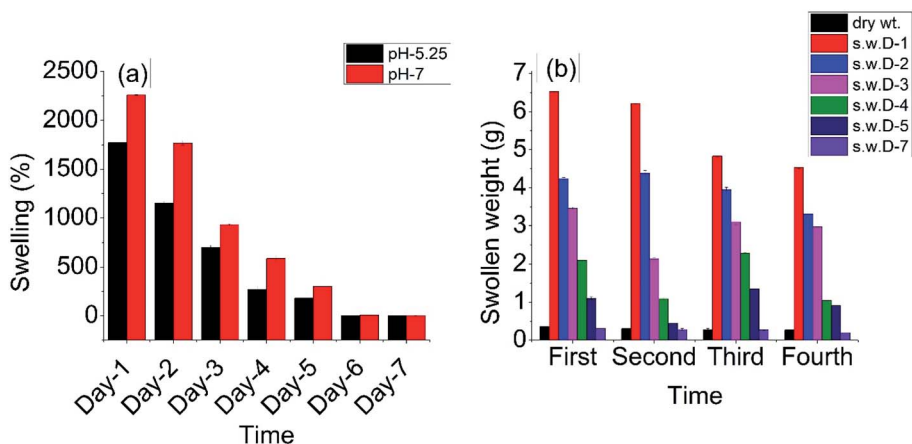


Fig. 5 (a) Comparative water release profile of composite C-3 at pH 7 and pH 5.25 for 7 consecutive days (S.W denotes swelling weight). (b) Comparative swelling for the C-3 composite at pH 5.25 for four consecutive cycles (four consecutive days).



not be recovered. With each cycle, mass loss of the sample was observed, and a comparative degree of swelling was observed to get reduced as well.

### 3.6 Release studies

The  $\text{NO}_3^-$ -N content in the synthesized ZLDH and C-3 was estimated by the Kjeldahl method.<sup>43,44</sup> The percentage loading of  $\text{NO}_3^-$  in the ZLDH and C-3 sample was observed to be 11.83 (w/w) and 5.39 (w/w) respectively. Following this, 350 mg of the ZLDH and C-3 sample taken for experiment contained 41.405 mg and 18.86 mg of intercalated nitrate. The nitrate release studies were carried out in different media and the nitrate release percentage for the samples is expressed as follows:

$$\% \text{ nitrate release} = \frac{\text{nitrate released from sample (mg)}}{\text{nitrate intercalated in the sample (mg)}} \times 100$$

From the nitrate release studies of ZLDH (Fig. 6a) in Milli-Q water, distilled water, and carbonated water at pH 7 and temperature 25–30 °C, the nitrate release rate was observed to be highest in the case of carbonated water. The LDH material released almost 98% of intercalated  $\text{NO}_3^-$  in 3 days in abundance of carbonate ions. In distilled water, around the same

time, almost 91% and in Milli-Q water, almost 86% of the intercalated  $\text{NO}_3^-$  anions were released. For a duration of 30 days, ZLDH retained 1.218% (0.504 mg) in carbonated water, 8.227% (3.407 mg) in distilled water and 13.995% (5.767 mg) of the intercalated nitrate in Milli-Q water.

Comparative nitrate release rate for ZLDH, composite C-3 and  $\text{KNO}_3$  solutions in a neutral pH medium exhibited slower nitrate release from ZLDH and C-3 composites. The  $\text{KNO}_3$  solution released 99% of its  $\text{NO}_3^-$  content in merely 5 minutes (Fig. 6b). The LDH material retained ~12% (5.148 mg) of the loaded  $\text{NO}_3^-$  ions up to 7 days, whereas the C-3 composite sample retained ~20% (3.860 mg) of the intercalated  $\text{NO}_3^-$  ions in the identical duration. The comparative nitrate release profile (Fig. 6c and d) for the synthesized ZLDH and C-3 composite samples at pH 7 and pH 5.25 showed the nitrate release rate to be faster at acidic pH for both the samples (Fig. 6d). In neutral medium, the ZLDH material released almost 91% of intercalated  $\text{NO}_3^-$  in 30 days, in acidic medium, and it released almost 100% of the intercalated  $\text{NO}_3^-$  ions. It retained 8.227% (3.466 mg) in a neutral medium and 0% of the intercalated nitrate in an acidic medium. The C-3 composite retained greater net contents of  $\text{NO}_3^-$  ions for the identical time period in both neutral and acidic media. It retained 14.545% (2.744 mg) in a neutral medium and 9.53% (1.798 mg) of the total intercalated nitrate in an acidic medium for 30 days.

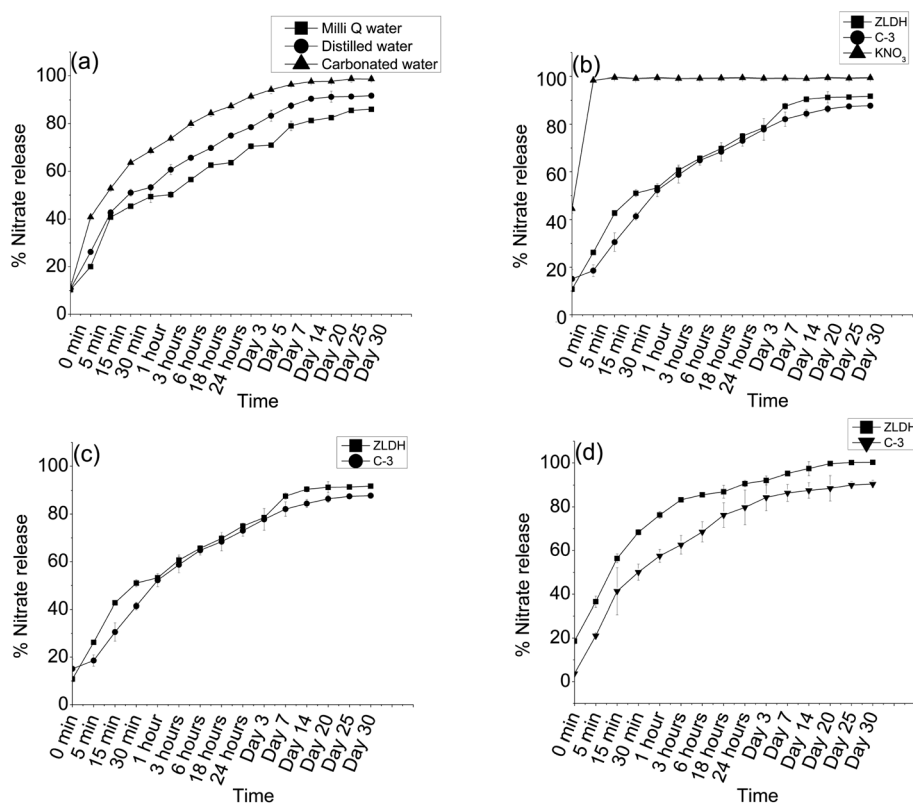


Fig. 6 Comparative  $\text{NO}_3^-$  release profiles for (a) ZLDH in Milli-Q water, distilled water and carbonated water and (b) ZLDH, composite C-3 and  $\text{KNO}_3$  solution in distilled water at pH 7. (c) and (d) Comparative  $\text{NO}_3^-$  release profiles for ZLDH material and C-3 composites in neutral and acidic media, respectively.



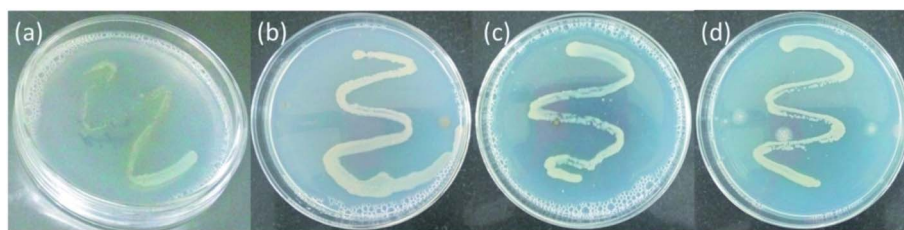


Fig. 7 Growth in agar plates for (a) *Azospirillum brasilense* alone before loading onto the samples, (b) *Azospirillum brasilense* in the C-3 composite sample on the day of loading, (c) after 15 days of loading and (d) after 30 days of loading.

### 3.7 Study of growth of *A. brasilense* after inoculation in LDH-composite samples

Observations from the catalase test mentioned in Section 2.5 showed the formation of bubbles after applying 3%  $\text{H}_2\text{O}_2$  onto the *Azospirillum*-streaked agar plates confirming the bacterial species to be *A. brasilense*.<sup>37</sup> The presence and growth of *A. brasilense* in the C-3 composite sample were established by streaking sample solutions loaded with *A. brasilense* into malate-agar plates on the day of loading and the plates were incubated for 24 hours at 30 °C. The similar streaking process was repeated after 15 and 30 days of loading *Azospirillum* broth to the sample solution. The growth of *A. brasilense* in the agar plates confirmed the presence of the bacteria in the sample on the first day of streaking, after 15 days, and after 30 days (Fig. 7).

### 3.8 Nitrate assimilation studies of *A. brasilense*-loaded samples

The nitrate assimilation by the bacteria *A. brasilense* was established by confirming the presence of  $\text{NO}_2^-$  ions in *Azospirillum*-loaded samples of ZLDH, composite C-3, and  $\text{KNO}_3$  (Table 1) with the test kit mentioned in Section 2.8.2.<sup>40</sup> Control solutions without the presence of the bacterial broth showed no traces of  $\text{NO}_2^-$  ions, and only  $\text{NO}_3^-$  ion was detected. It was observed that in the bacterial presence, all the  $\text{NO}_3^-$  ions

present in the  $\text{KNO}_3$  solution and released from ZLDH and C-3 samples were not converted into  $\text{NO}_2^-$  ions. Spectrophotometric tests and the kit tests showed the presence of both  $\text{NO}_2^-$  and  $\text{NO}_3^-$  ions in the test solutions.

*A. brasilense* is reported to have various useful plant functions other than nitrate assimilation, including phytohormone production and atmospheric nitrogen fixation. In culture media reach with a carbon source, fixation of atmospheric nitrogen and subsequent conversion into  $\text{NH}_3/\text{NH}_4^+$  by nitrogen-fixing bacteria were established by Kanimozhi *et al.*<sup>44</sup> The presence of  $\text{NH}_3/\text{NH}_4^+$  in the test solutions was confirmed by testing with Nessler's reagent, and quantitative detection was performed using the test kit mentioned in Section 2.8.3.<sup>41,42</sup> The pH of all the samples post-loaded with *A. brasilense* was found to be in the range of 5–6 during the observation period. The amount of  $\text{NH}_3/\text{NH}_4^+$  present in all the samples on the day of bacterial inoculation was observed to be in the range of 0–0.25  $\text{mg L}^{-1}$ . After three days, the amount raised to 0.25–0.50  $\text{mg L}^{-1}$  range, which remain unchanged in the following period for non-nitrogenous samples. The value increased to 0.5–1.0  $\text{mg L}^{-1}$  range in solutions containing ZLDH, C-3 and  $\text{KNO}_3$  (Fig. 8). This could be attributed to the fact that a particular volume of bacterial broth containing particular colony-forming units of the bacteria can only convert a certain amount of atmospheric nitrogen.<sup>37</sup> The additional quantity of  $\text{NH}_3/\text{NH}_4^+$  present in

Table 1 Estimation of concentration of  $\text{NO}_2^-$  ions in solutions loaded with *Azospirillum brasilense* and control solutions without loaded *Azospirillum brasilense*<sup>a</sup>

Interval	Samples						
	ZLDH (+)	C-3 (+)	$\text{KNO}_3$ (+)	CMCH (+)	(+)	ZLDH (–)	C-3 (–)
Day-1	*	*	*	*	*	*	*
Day-2	*	*	*	*	*	*	*
Day-3	**	*	**	*	*	*	*
Day-4	**	**	***	*	*	*	*
Day-5	**	**	****	*	*	*	*
Day-7	***	**	****	*	*	*	*
Day-10	****	**	****	*	*	*	*
Day-13	****	**	****	*	*	*	*
Day-15	****	****	****	*	*	*	*
Day-16	****	****	*****	*	*	*	*
Day-17	*****	*****	*****	*	*	*	*
Day-30	*****	*****	*****	*	*	*	*

<sup>a</sup> (+) loaded with *Azospirillum*; (–) no *Azospirillum*; 0 ppm = \*, 0–0.05 ppm = \*\*, 0.05–0.1 ppm = \*\*\*, 0.1–0.2 ppm = \*\*\*\*, 0.2–0.5 ppm = \*\*\*\*\*, 0.5–1.0 ppm = \*\*\*\*\*, 1.0 ppm = \*\*\*\*\*.



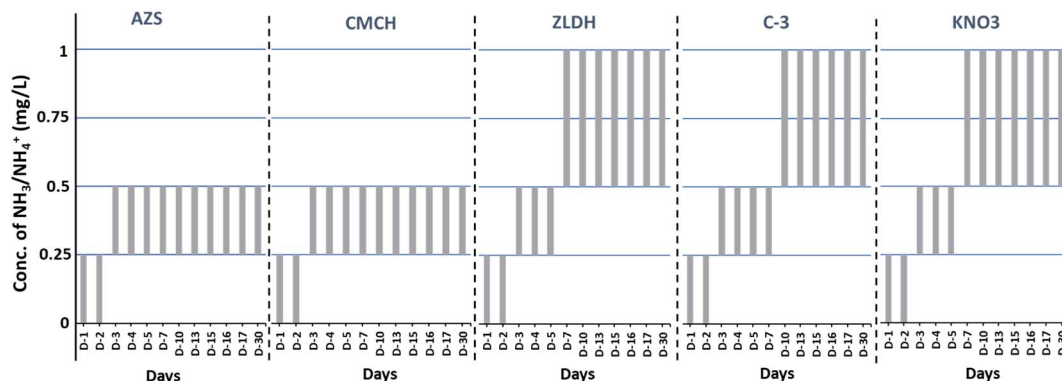


Fig. 8 Amount of  $\text{NH}_3/\text{NH}_4^+$  present in *A. brasilense*-loaded (+) and non-loaded (–) samples; AZS signifies *A. brasilense* alone.

ZLDH, C-3 and  $\text{KNO}_3$  samples contributes to the nitrate–nitrite–ammonium conversion occurring in the systems. Control solutions without *Azospirillum* incorporation showed no traces of  $\text{NH}_3/\text{NH}_4^+$  in the solution.

### 3.9 Study of release of Zn from bacteria-loaded C-3 samples

C-3 composite and ZLDH sample solutions without loading with *A. brasilense* exhibited  $0 \text{ mg L}^{-1}$  Zn content from day-1 to day-30. *Azospirillum* broth itself showed no traces of  $\text{Zn}^{2+}$ . Only ZLDH and C-3 samples loaded with *A. brasilense* showed the presence of  $\text{Zn}^{2+}$  from day-5 onwards after inoculation (Fig. 9). The amount of  $\text{Zn}^{2+}$  was observed to increase from day-5 to day-30 for LDH. The C-3 sample first exhibited an increase in Zn content from day-5 to day-15, and then again decreased on day-20, and the value was raised on day-30.

## 4. Discussions

The FTIR spectra (Fig. 1) of the ZLDH, CMCH and C-3 samples present the composite sample C-3 exhibiting characteristic peaks

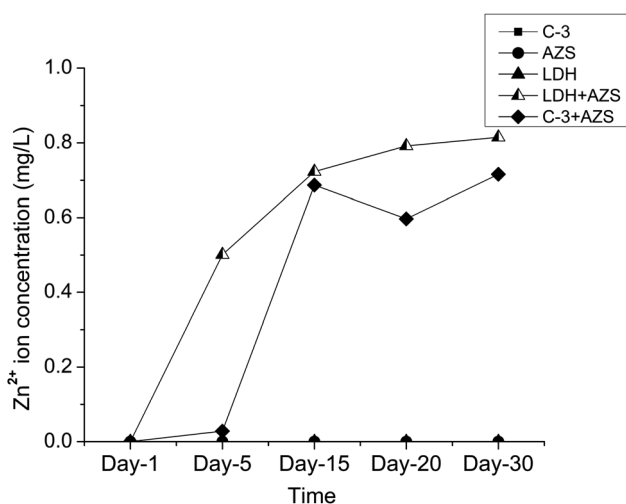


Fig. 9 Amount of  $\text{Zn}^{2+}$  ions in solutions with and without *Azospirillum brasilense* broth (AZS: *Azospirillum brasilense*; ZAZS: ZLDH sample loaded with *Azospirillum brasilense*; C-3AZS: C-3 sample loaded with *Azospirillum brasilense*).

for both LDH and hydrogel part. A band at  $564 \text{ cm}^{-1}$  is due to the superimposition of characteristic bands of hydroxalite due to Zn–Al–OH translational modes in LDH layers.<sup>13,14,37,45</sup> The PXRD (Fig. 2a and b) patterns exhibit peaks appearing in the ZLDH spectra also appearing in the C-3 spectra, having a lower intensity, predicting the loss of crystallinity from original LDH while forming a composite with the hydrogel material, having amorphous nature. The peak at  $2\theta$  value of  $12.3^\circ$  demonstrates the intercalation of  $\text{NO}_3^-$  in the LDH layers, with a basal spacing distance of  $7.196 \text{ \AA}$  for the 003 plane.<sup>14</sup> Thermal analysis (Fig. 2c) shows the thermal stability of the C-3 sample in the intermediate range of the parent LDH and hydrogel material, confirming the formation of the composite between the two materials. The second break corresponds to desorption of strongly held water molecules in the gallery region and dehydroxylation of LDH octahedral layers. However, the third break is observed in the range of  $570\text{--}640^\circ \text{C}$  due to major mass loss in connection with the elimination of intercalated anions from the gallery regions.<sup>11,13</sup> The first weight loss for C-3 samples (Fig. 2e) is due to the elimination of adsorbed water molecules from the system. The second break depicting major mass loss due to the decomposition of the hydrogel sample and dehydroxylation of LDH interlayers. The third break is due to the elimination of intercalated anions from LDH gallery regions, as suggested by Allou *et al.*<sup>11</sup> and Sharif *et al.*<sup>13</sup>

The composite formulation of these two materials led to some morphological modifications to their respective surfaces observed in the SEM micrograph (Fig. 3). The composite formulation caused the LDH material to compress and become more compact, unlike its previous flake-like structures.<sup>13</sup> The water retention studies (Fig. 4) show the C-3 sample exhibiting decent water swelling and retaining property in both acidic and neutral media. This can be governed by the formation of composites between CMCH and ZLDH. LDH without possessing water-retaining property itself on composite formulation with hydrogel inherits this ability. The faster water release in an acidic medium could be attributed to the possibility of the formation of hydrogen bonding in the samples. The hydrogen bonding acts as a physical cross-linker, constricting the hydrogel structure, and thus, preventing water absorption and retention in the sample matrices.<sup>18</sup>



At a neutral pH, nitrate release from the ZLDH sample was observed to be fastest in carbonated water (Fig. 6). This can be correlated with the high affinity of  $\text{CO}_3^{2-}$  ions for the LDH layers, displacing the intercalated nitrates faster when present in abundance.<sup>44</sup> Due to dissolved  $\text{CO}_2$  in distilled water and the presence of atmospheric  $\text{CO}_2$ ,  $\text{NO}_3^-$  ions were also displaced from LDH layers in distilled water and Milli-Q water, yet the displacement is slower, leading to slower nitrate release. The comparative nitrate release from ZLDH, C-3 and  $\text{KNO}_3$  makes ZLDH and C-3 to be slower nitrate release sources than the potential commercial nitrate fertilizer  $\text{KNO}_3$ , which releases almost 100% nitrate content merely during 5 minutes. C-3 performed slower nitrate release than the ZLDH sample, establishing the composite formulation inducing the slower nutrient ion release nature in the LDH material. Nitrate release profiles of ZLDH and C-3 at neutral and acidic pH exhibits greater retaining and slower nitrate release from the C-3 sample, demonstrating the protection offered by the polymer matrix to the LDH counterpart in the composite sample, retaining greater  $\text{NO}_3^-$  content in its matrix and performing slower nitrate release. For improved nitrate assimilation, *A. brasilense* bacterial broth was cultured and added to the C-3 composite sample and the differences were observed by testing the presence of  $\text{NO}_2^-$  ions. The growth of *A. brasilense* streaked from the loaded samples onto the agar plates shows the active presence of the bacteria up to 30 days post loading (Fig. 7). The presence of  $\text{NO}_2^-$  ions only in bacteria-loaded samples releasing  $\text{NO}_3^-$  ions shows reduction of released  $\text{NO}_3^-$  ions facilitated by the bacterial activity. The conversion of  $\text{NO}_3^-$  ions released from the ZLDH and C-3 composite to  $\text{NO}_2^-$  ions by the bacteria (nitrate reductase enzyme present in the bacteria is responsible for the function) establishes its ability in promoting nitrate assimilation and enhancing nutrient uptake in plants<sup>30,31,46</sup> (Table 1). The presence of  $\text{NH}_3/\text{NH}_4^+$  in bacteria-loaded samples indicates the conversion of atmospheric nitrogen into ammonia by the bacteria, containing the nitrogenase enzyme responsible for this function. The additional quantity of  $\text{NH}_3/\text{NH}_4^+$  present in bacteria-loaded ZLDH, C-3 and  $\text{KNO}_3$  samples demonstrates the complete conversion of  $\text{NO}_3^-$  into  $\text{NH}_4^+$ . In the presence of the bacteria, a fraction of  $\text{NO}_3^-$  ions were reduced to  $\text{NO}_2^-$  and  $\text{NO}_2^-$  ions were reduced to  $\text{NH}_4^+$  (Fig. 8).

The presence of  $\text{Zn}^{2+}$  in bacteria-loaded ZLDH and C-3 samples (Fig. 9) attributes to the partial dissolution of  $\text{Zn}^{2+}$  from ZLDH and composite structures in an *Azospirillum* broth environment with pH ranging from 5 to 6.<sup>43</sup> The enzymes present in *A. brasilense* along with acidic pH may also be a governing factor in these findings. The released  $\text{Zn}^{2+}$  could be utilized by the plant as an added nutrient apart from the targeted N fertilization. This further improves the multifunctionality of the composite system.

## 5. Conclusions

In this work, a multifunctional nitrogen fertilizer has been formulated for the dual delivery of  $\text{NO}_3^-$  and  $\text{NH}_4^+$ . Nitrate-intercalated Zn-Al-layered double hydroxide was utilized as a slow-release  $\text{NO}_3^-$  source, which was incorporated onto citric

acid cross-linked carboxymethyl cellulose and hydroxyethyl cellulose-based hydrogel for the preparation of the composite system. The water retention studies of the composite were carried out in neutral and acidic media taking the parent hydrogel and layered double hydroxide material as positive and negative controls. The composite exhibited a decent water retention property in both the media. Nitrate release studies demonstrated the composite sample to be capable of holding greater net nitrate content in both neutral and acidic media, establishing slower nitrate release from the composite compared to the layered double hydroxide material alone. Additional incorporation of *A. brasilense* into the composite system aided enhanced nitrate assimilation by converting a portion of  $\text{NO}_3^-$  ions released from the gallery regions to  $\text{NO}_2^-$  supported by the nitrite test and increased  $\text{NH}_3/\text{NH}_4^+$  availability via atmospheric  $\text{N}_2$  fixation and complete reduction of a fraction of nitrate released, established by the ammonium test. The degradation study of the composite further showed the availability of  $\text{Zn}^{2+}$  in the solution that plants can utilize as an essential nutrient. Thus, the formulated multifunctional fertilizer system possesses the ability to supply plant-accessible N in dual forms ( $\text{NO}_3^-$  and  $\text{NH}_4^+$ ), improve nitrate assimilation, and provide Zn upon degradation. This biodegradable multifunctional fertilizer composite with the residual bacteria could also uplift the overall soil fertility regime.

## Author contributions

Rimjim Gogoi: Data curation, Formal analysis, Investigation, Methodology, Writing - original draft; Arup Borgohain: Data curation, Formal analysis, Investigation, Methodology, Writing - original draft; Madhusmita Baruah: Data curation, Formal analysis, Investigation, Methodology, Writing-original draft; Tanmoy Karak: Methodology, Project administration, Resources, Validation, Visualization, Writing - review & editing. Jiban Saikia: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Validation, Visualization, Writing - review & editing.

## Conflicts of interest

There are no conflicts to declare.

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