



Research article

Nano-carriers effects on the viability and efficiency of *Pseudomonas* strains as phosphate solubilizing bacteriaMarzieh Safari^a, Elaheh Motamedi^b, Hossein Kari Dolatabad^c,
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ABSTRACT

In order to develop nanotechnology application in the agricultural systems achieving more sustainability in the environment, we have used different nano-carriers for phosphate solubilizing bacteria. The viability and efficacy of two bacterial species; *Pseudomonas putida* (PP20) and *Pseudomonas kilonensis* (PK11) in solubilizing phosphate sources (i.e., tricalcium phosphate and hydroxyapatite) with different nano-carriers including nanoclay, natural char micro-particles (NCMPs), nanoclay + alginate, NCMPs + alginate, and natural char nano-particles (NCNPs)+ alginate were investigated. Clay, talc powder, and natural char (NC) were included for comparison. The synthesized NCNPs and NCMPs were characterized using FTIR, SEM and Boehm titration analyses. The results confirmed that the chemical oxidation of pristine char made many oxygenated functional groups on the surface of tiny and spherical NCNPs (14.8 nm) which caused their effective incorporation in the matrix of alginate beads. Results of phosphate solubilizing study showed that *P. kilonensis* was the superior species for viability and stability of its performance on solubilizing phosphorus. The six months evaluation showed that NCNPs + alginate and nanoclay + alginate carriers at both temperatures (4 °C and 28 °C), were the proficient carriers for preserving both bacteria. The results of solubilizing phosphorus sources revealed that both bacteria solubilized tricalcium phosphate more than hydroxyapatite and PK11 showed more privilege in this regard. In addition, the solubilizing index determined after storage for 6 months at 4 °C was higher for all the carriers. Analysis of variance for phosphatase activity revealed that embedding both bacteria in nanoclay + alginate carrier guaranteed the highest phosphatase activity, even though differences between this carrier and NCNPs + alginate and NCMPs + alginate were not significant for the PK11.

1. Introduction

Phosphorus is the second essential element in plant nutrition, therefore providing the proper amount of it would be necessary for plant metabolism and performance. Poor availability of phosphorus in many agricultural soils has led to the annual application of phosphate fertilizers, which induce environmental pollution and disorders in systems such as eutrophication, soil degradation, and carbon footprint (Sharma et al., 2013). Sustainable management of phosphorus in the agricultural systems requires development in proper management in phosphorus application along with preserving the usefulness of the element in increasing yield, which could also reduce the environmental impacts

(Mullins, 2018). The alternative way to fulfill the plants need is the application of biofertilizers. Microorganisms have no negative impact on the environment and play significant roles in increasing phosphorus mobilization and availability in the soil (Subramanyam, 2015), and promoting plant growth. Nevertheless, the advancement of effective formulation of microbial inoculants remains a major scientific challenge (Alikhani et al., 2006) as most carriers do not assure bacterial viability and efficacy in long-term storage. Many bacterial strains that isolated from soils in Iran are able to mobilize P from organic and inorganic sources (Alikhani et al., 2006). Hence, it is important to enhance the longevity and viability of them in their carriers, which could reduce our reliance on chemical P fertilizers. Enhancement of biofertilizers with

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promising materials would be essential for their consistent performance and supplying a reliable amount of phosphorus to plants.

The distinguishing features of carriers are their ability to provide a suitable microenvironment for the prolonged survival of microorganisms (Rekha et al., 2007) and their capacity to deliver the proper amount of viable cells at the right time (Sahu and Brahmprakash, 2016), but other beneficial characteristics for a good carrier are included: having high water holding capacity, being cost-effective, available and uniform, having no lump-forming material, being non-toxic in nature, easily biodegradable and sterilizable, having good buffering capacity, supporting the growth and survival of bacteria, amenable to nutrient supplement, manageable in mixing, and packaging operations (Sahu and Brahmprakash, 2016). Different carriers are being used in the formulation process, which have organic or inorganic origins. The positive qualities and drawbacks of each carrier have an influence on the overall quality and efficacy of biofertilizers (Bashan et al., 2014). It has been stated that liquid bioinoculants are preferred for economical production of biofertilizers, they are easy to distribute, and has longer shelf-life (Goljanian-Tabrizi et al., 2016). Although in another study, long shelf-life, easy transportation, and storage gave preference to the solid formulation (granule and powder), they also showed better results under harsh environment compared to liquid ones (Sabaratnam and Traquair, 2002). The organic carriers include peat, turf, talc, lignite, kaolinite, montmorillonite, zeolite, alginate, sawdust, biochar, and vermiculite, etc. Addition of biochar to soil improved the soil function and fertility (Hale et al., 2015). Different physicochemical characteristics have been reported for biochars depending on their origin of feedstock and the pyrolysis process, which could influence their ability as a carrier for bacteria (Hale et al., 2015). The biochar large-scale production provides an inoculum carrier to deliver plant-growth-promoting rhizobacteria into agricultural soils (Hale et al., 2014). Talc is a mineral composed of hydrated magnesium silicate (Sahu and Brahmprakash, 2016), and available in powder form suited for a wide range of applications. It has very low moisture equilibrium, relative hydrophobicity, chemical inertness, and reduced moisture absorption, but owing to its inert nature and its availability, it is used as a carrier (Nakkeeran et al., 2005). Common carriers have some drawbacks for instance: peat as the most popular carrier worldwide is unavailable with good quality; lignite and coal form hard clumps after drying and during storage period which negatively influence the inoculant population (Subramanyam, 2015); perlite could not be considered as favorable carrier as it has low organic matter and low buffering capacity; bentonite in addition to low organic matter gets sticky after water absorption thereby does not provide a suitable ventilation for aerobic bacteria; and rock phosphate powder would not be good carriers for bacteria as it has low water retention capacity and low nutrients (Sahu and Brahmprakash, 2016). The optimal activity of microorganisms in the biofertilizers for solubilizing phosphorus could be guaranteed with choosing the appropriate carrier. The best formulation would support the bacterial growth, and their ability to solubilize phosphorus. Hence, it will decrease the amount of applied phosphate fertilizers in agricultural lands. In this path, several works have been done to assess different kinds of carriers for their efficacy in delivering the rightful amount of microorganisms with desirable function. By investigating in the existing carrier materials and formulation techniques, alginate bead formulations are considered to be the best choice in developing formulation as the sufficient number of bacterial cells could be loaded in alginate beads (Subramanyam, 2015). Encapsulation of living cells in alginate beads is a well-established technology due to their non-toxic nature and biodegradability (Young et al., 2006). Encapsulation of microorganisms in alginate beads protects them against the harsh environment, and could release microorganisms gradually as it gets degraded (Bashan et al., 2016; Gagné-Bourque et al., 2015; Young et al., 2006) thus may serve as a continuous source of inoculum in the rhizosphere. It also helps to preserve bacterial catalytic ability for a longer duration (Young et al., 2006). Alginate beads are three-dimensionally cross-linked networks, which have high water content (Sun and Tan,

2013). Encapsulation of microorganism into polymers has been mainly limited in practice to alginate beads, which still has some limitations for the industrial production (Malusà et al., 2016). The major drawback of polymeric inoculants is that the raw materials are relatively expensive (Bashan et al., 2016). Although due to the massive production of alginate in the Far East, it has got the attention of the inoculant industry (Bashan, 1998; Chandra, 2012). Storage of alginate beads at ambient temperature has been found to retain the viable inoculum for a prolonged period (Malusà et al., 2012). Amendments in alginate beads would also promote the stabilization and protection of the microbial cells during storage. Material such as starch or clay as fillers may increase the dry matter of bead, and its mechanical strength allows for a progressive release of cells into the soil (Schoebitz et al., 2012). Alginate beads supplemented with humic acid supported the bacterial survival and performance, and the efficacy of beads (Young et al., 2006). Most of the alginate formulations which have been recently used were not commonly accompanied with organic additives, except for humic acid (Bashan et al., 2016; Reetha et al., 2014; Rekha et al., 2007). Therefore, examining the possible advantages of other organic additives especially in nanometric sizes would be preferred. The survival rate of *P. fluorescens* (2-79RN10, W4F393) in montmorillonite, zeolite and vermiculite with smaller particle size was increased compared to kaolinite, pyrophyllite, and talc with bigger particle sizes. The carriers with smaller particle size have a higher surface area, thereby increasing the resistance of bacteria to desiccation by increasing the coverage of bacterial cells (Nakkeeran et al., 2005), hence nano-carriers would have superiority over other carriers in this regard.

Nano-carriers have been rarely used for the phosphate solubilizing microorganisms. Bio-based nano-carriers would have beneficial characteristics including physical stability, high surface area, and ease of use. Supplementing the alginate beads with nanostructure materials would be a novel attempt in producing biofertilizers, thereby reducing the phosphate chemical fertilizer which leads to more sustainability. Hence, in the present study, we investigated the potential capability of different nano-fillers in alginate beads and compared them with the common carriers for the viability and efficiency of two *Pseudomonas* species in solubilizing phosphate sources. This research explored whether nano-carriers could enhance the longevity and the bacterial efficiency in solubilizing phosphorus. The ultimate objective was the identification of the best formulation that could guarantee the bacterial maintenance in the field as well as inducing their ability to solubilize phosphorus.

2. Materials and methods

The effects of nanostructure material as carriers on the survival and capability of two bacterial species including *Pseudomonas putida* (PP20) and *P. kilonensis* (PK11) in solubilizing phosphate were investigated and compared with the common carriers like talc powder. The bacterial species were taken from the Soil and Water Research Institute, Karaj, Iran. These bacterial species have registered as CCSM-B00525 and CCSM-B00527 respectively for *Pseudomonas putida* (PP20) and *P. kilonensis* (PK11) in the Global Catalogue of Microorganisms for Soil and Water Research Institute, Karaj, Iran. The applied carriers in this study were included clay, talc powder, natural char (NC), nanoclay, natural char micro-particles (NCMPs), natural char nano-particles (NCNPs)+alginate, NCMPs + alginate and nanoclay + alginate. Each carrier loaded with each bacterial species in the sterile glass jars in aseptic condition, bacterial population and ability to solubilize phosphate with two phosphate sources (tricalcium phosphate and hydroxyapatite) were evaluated after one-day incubation. Each loaded carrier was incubated for 6 months at 4 °C or 28 °C. After the 6-month incubation, the bacterial population and their capability to solubilize phosphate media were assessed again.

2.1. Preparation of NCMPs and NCNPs

The raw NC mass used in this study was collected from Kuhbanan (MASI; 31 27 37 N and 56 16 22 E), Kerman province, Iran (August 24,

2017). NCMPs were prepared using ball-milling technology in a planetary ball-mill machine (NARYA-MPM; Amin Asia Co.) with two 250 mL Teflon (PTFE) mill jars, at room temperature. Each milling run conducted at 350 g and total working time of 10 h. Afterwards the products were collected, washed with deionized water, then centrifuged at 5000 g and finally dried in 70 °C overnight. To prepare NCNPs, chemical oxidation of NC powder was performed using modified Hummer's method where NC powder was oxidized in the mixture of concentrated H₂SO₄, and of KMnO₄ in an ice bath (Hummers and Offeman, 1958). Briefly, NC powder (0.5 g) was added to the 50 mL of the concentrated H₂SO₄, and then 2 g of KMnO₄ was slowly added and mixture stirred in an ice bath. After 2 h, the temperature of the reaction was raised to 35 °C while mixture was stirred gently for another 1 h. Afterward, the ice bath was provided again and deionized water (150 mL) was added to the reaction mixture gradually. After 30 min additional stirring, 30% H₂O₂ solution (10 mL) was dropped to the reaction vessel. Next, the precipitates were centrifuged and washed with deionized water for several times till neutralization of the supernatant's pH and the resultant was dried at 70 °C and the beige color fine powder was collected.

2.2. Nanoclay characteristics

The applied nanoclay in this study was montmorillonite with CEC 130 meq 100g⁻¹, which was supplied from the US Research Nanomaterials Inc. Nanoclay, which was water dispersible is a natural material and contains nutritional minerals, it is safe and environmentally friendly with the following characteristics: average particle size 18 nm, pH 7, yellow in color, and 10 wt % clay concentration.

2.3. Preparation inoculums

Every solid carrier (including clay, talc powder, nanoclay, NC and NCMPs) was placed in the glass jar and autoclaved twice. Bacteria were inoculated in the sterilized (121 °C for 20 min) nutrient broth (NB; Beef extract 1 g L⁻¹, yeast extract 2 g L⁻¹, peptone 5 g L⁻¹, sodium chloride 5 g L⁻¹), then incubated for 24 h in a shaker (120 g min⁻¹) at room temperature. After one-day, each carrier was inoculated with the bacterial suspension. The bacterial population in serial dilution and phosphate solubilization were measured at room temperature in each carrier after one-day incubation. Then solid inoculums were incubated for 6 months at 4 °C and 28 °C.

For the carriers with alginate; after preparing the nutrient broth in 50 mL distilled water, sodium alginate 3% and nanomaterials in the amount of 0.25 g (individually for each nanomaterial) were dispersed in the nutrient broth solution and autoclaved afterward. A loop of bacteria from the nutrient agar cultivated plate was added to the solution in aseptic condition and placed in the shaker (120 g min⁻¹) for one day at room temperature. Solutions containing alginate, bacteria, and nanomaterials were added to the autoclaved stirring CaCl₂ 0.5 M solution with a 5-mL sterile syringe in drop way to form the beads with 2–3 mm diameter. After 30 min when the formed beads hardened, they were removed from the CaCl₂ solution and washed 2–3 times with sterile distilled water and placed in the laminar hood to be air-dried. One gram of the formed beads was dissolved in the potassium phosphate buffer (pH 7.2) and bacterial population (serial dilution) and phosphate solubilization index in Sperber media (drop plate method) were evaluated. The remained beads were transferred to the sterile glass jars and incubated for 6 months at 4 °C and 28 °C. The bacterial population and solubilizing index were measured in alginate beads after dissolving them in the potassium phosphate buffer after 6 months. Some of the beads after formation were also placed at freezer –80 °C for 6 h before lyophilization at –45 °C for 24 h with ScanVac coolsafe 55-pro. Bacterial population was also evaluated in the lyophilized beads after 9-month storage in sterile glass bottles by dissolving 0.1 g of freeze-dried beads in 9.9 mL of sterile potassium phosphate buffer. Bacteria entrapped in the beads were serially diluted and counted by the plate count method.

2.4. Preparation Sperber plates and solubilization index

Sperber medium was prepared in distilled water by adding glucose (10.0 g L⁻¹), yeast extract (0.5 g L⁻¹), CaCl₂ (0.1 g L⁻¹), MgSO₄·7H₂O (0.25 g L⁻¹), agar (15.0 g L⁻¹) and 2.5 g L⁻¹ tricalcium phosphate (TCP) or hydroxyapatite, to appraise the ability of two strains in solubilizing phosphate. The pH of the medium was adjusted to 7.2 before autoclaving. The media were distributed in 9-cm diameter Petri plates. Using the pinpoint inoculation, 5 µL of inocula was placed in each quarter of the Sperber plate under aseptic conditions. Inoculated plates were incubated in dark at 28 °C and the diameter of the clear zone (halo) and the bacterial growth diameter were measured after 8 days. All assays were replicated three times and solubilization index (SI) calculated by the following formula:

$$SI = \text{Colony diameter} + \text{Halo zone diameter} / \text{Colony diameter} \text{ (Gandhi et al., 2014)}$$

2.5. Phosphatase activity

P-nitrophenyl phosphate disodium (PNPP, 0.115 M) was used as a substrate in determining phosphatase activity. Two mL sodium acetate buffer 0.5 M (pH 6.5), and 0.5 mL of substrate were added to 0.5 mL of PVK medium incubated at 37 °C. The reaction was stopped by cooling the solution at 2 °C for 15 min. Afterward, 0.5 mL of CaCl₂ 0.5 M and 2 mL of NaOH 0.5 M were added to the mixture and centrifuged at 5000 g min⁻¹ for 5 min. The released p-nitrophenol (PNP) was estimated by spectrophotometry at 405 nm and the phosphatase activity was expressed in µ moles of PNP released mL⁻¹ of filtrate h⁻¹ (Schoebitz et al., 2013). Phosphatase activity investigation was done in triplicates.

2.6. Statistical analysis

Two-way ANOVA was carried out through the general linear model (GLM) procedure using SAS 9.4 software for evaluating the population size of bacterial species in different carriers. Analysis of variance (two-way ANOVA) was done to assess the bacterial abilities in solubilizing phosphate at initial evaluation and after 6 months incubation for each phosphate sources (tricalcium phosphate and hydroxyapatite) in different carriers. Analysis of variance for phosphatase activity data was also done separately for each bacterium. Mean comparisons were carried out using least significant difference (LSD) tests when F test indicated statistical significance ($p \leq 0.05$). Pearson Correlation between the measured traits were also evaluated.

2.7. Scanning electron microscopy (SEM)

The external morphology of the beads was examined by using a field emission scanning electron microscopy (FESEM, TESCAN MIRA II microscope; 20 kV accelerating voltage).

2.8. FTIR analysis

FTIR spectra of nano-carriers were recorded using KBr pellets in a Thermo spectrometer to detect the chemical differentiation of tested nanomaterials due to the addition of sodium alginate within the range of 4000–400 cm⁻¹.

The average particle sizes of NCMPs and NCNPs samples evaluated based on SEM images histograms. Besides, the Boehm's titration method (Boehm, 1966, 2002) was utilized to calculate the surface oxygenated groups of as-synthesized NC samples.

This test is based on the fact that bases with various strengths (NaHCO₃, Na₂CO₃, NaOH) could neutralize different acidic oxygen functionalities on the surface of carbon materials (Boehm, 1966, 2002).

The strongest acidic groups (carboxylic groups) were neutralized with the weakest base (NaHCO_3), both carboxylic and lactonic functionalities were neutralized with the Na_2CO_3 , and finally, the strongest base (NaOH) neutralized all the carboxylic, lactonic and phenolic groups. The amount of each acidic functionality could be calculated by the difference between the uptake of each titrating base (Boehm, 1966, 2002).

3. Results

3.1. Characterization of as-prepared NCMPs and NCNPs fillers

To determine the functionalities of NC, NCMPs, and NCNPs samples, the FT-IR spectroscopy was employed (Figure 1). In the IR spectrum of NC, the peak at 800 cm^{-1} corresponded to the aromatic C—H out-of-plane deformation, and the intense band about 1075 cm^{-1} was assigned to aliphatic ethers (C—O—C) and alcohols (—OH). In addition, the band at 1659 cm^{-1} was attributed to C=C stretching in the aromatic ring as well as C=O stretching, and the broad peak at 3451 cm^{-1} along with a peak at 3632 cm^{-1} represented the hydroxyl groups (—OH) stretching vibration (Chen et al., 2008). The NCMPs spectrum differed from that of NC as evidenced by the strengthening of the above mentioned vibrational peaks originated from oxygenated functionalities. Besides, the peak for C—H bending vibrations which presented at 1418 cm^{-1} in the NC spectrum, appeared only as the shoulder in the spectrum of NCMPs sample. These all could be attributed to the more abundance of oxygen functionalities in NCMPs compared to NC, which might be introduced in the carbonaceous backbone of char through high energy ball-milling process (Lyu et al., 2018). Similarly, in the NCNPs spectrum, the much enhanced intensity for hydroxyl and etheric vibrations compared to NC and NCMPs along with the presence of a sharp band of C=O stretching (carboxylic moieties) at 1715 cm^{-1} were indicative of the effective chemical oxidation of pristine char using permanganate reagent and accordingly presence of more hydrophilic oxygen containing groups attached to the surface of NCNPs which turned it a possibly suitable nano-filler for modification of alginate beads (Saxena et al., 2014).

SEM images of produced NC, NCMPs and NCNPs samples at 50 and 200 KX magnifications were displayed in Figure 2. The SEM images of NC indicated micrometric stacked smooth hunks with various shapes that their edges were sunk together in a way that a particular separated particle could be hardly seen in the images. In the NCMPs images, the dense polygonal shape particles were observed which mostly aggregated to each other and made larger particles with several hundred nanometer sizes. In contrast, the SEM image of NCNPs portrayed close-packed tiny spherical nanoparticles, which appeared as bright dots particularly at higher magnifications (Figure 2). Moreover, size distribution histograms

of samples were prepared from measuring about 100 particles through SEM images of NCMPs and NCNPs samples (Figure 2). The average particle sizes based on SEM images histograms were to be $157.9 \pm 34.3\text{ nm}$ and $14.8 \pm 3.0\text{ nm}$ for NCMPs and NCNPs samples, respectively. The Boehm titration results were consistent with FT-IR and elemental mapping findings and showed that the total acidic groups of the samples followed the following descending order of NCNPs > NCMPs > NC (Table 1).

3.2. Characterization of as-prepared NCNPs enriched alginate bead nano-carriers

Scanning electron micrograph of NCNPs enriched alginate beads exhibited in Figure 3. At lower magnifications (Figure 3a), the image showed the surface of an intact bead, the puffy structure of a bead could be seen with increasing the magnification (Figure 3b), and finally, Figure 3c revealed evidently the distribution of NCNPs on the bead surface appeared as bright dots which well-dispersed in the matrix of polymer.

The enrichment of alginate beads with NCNPs was further confirmed by the FTIR (Figure 3d). The spectrum of NCNPs + alginate showed a sharp peak in the range of $3000\text{--}3600\text{ cm}^{-1}$, which is indicative of acidic OH (hydroxyl) stretching band, originated from alginate (Kusuktham et al., 2014; Larosa et al., 2018) and loaded NCNPs. Another peak that observed in 2920 cm^{-1} was attributed to the asymmetric CH stretching of aliphatic groups (methyl and ethyl) of alginate beads (Kusuktham et al., 2014; Larosa et al., 2018). The signals at 1631 and 1407 cm^{-1} were typically observed due to asymmetric and symmetric stretching vibrations of the carboxylate ions of calcium alginate beads (Kusuktham et al., 2014; Larosa et al., 2018). A weak shoulder at 1722 cm^{-1} could be attributed to the presence of C=O stretching groups of NCNPs. This signal could be considered as an evidence of loading of NCNPs in the alginate polymer. Its lower intensity compared to pristine NCNPs, was due to the little amount of the applied NCNPs in the alginate beads. Moreover, loading of NCNPs in the alginate beads could be confirmed again by presence of a peak at 1085 cm^{-1} which was one of the characteristic signals of NCNPs in the IR spectrum (Figure 3d).

3.3. Bacterial population

Investigating the bacterial population in different carriers initially and after 6-month incubation showed that the PK11 was the superior strain in all carriers. The initial evaluation of inoculums displayed that both bacteria in NCNPs + alginate and nanoclay + alginate carriers had the highest population. NCMPs + alginate was placed in the third rank for the bacterial population. On the other hand, the bacterial population

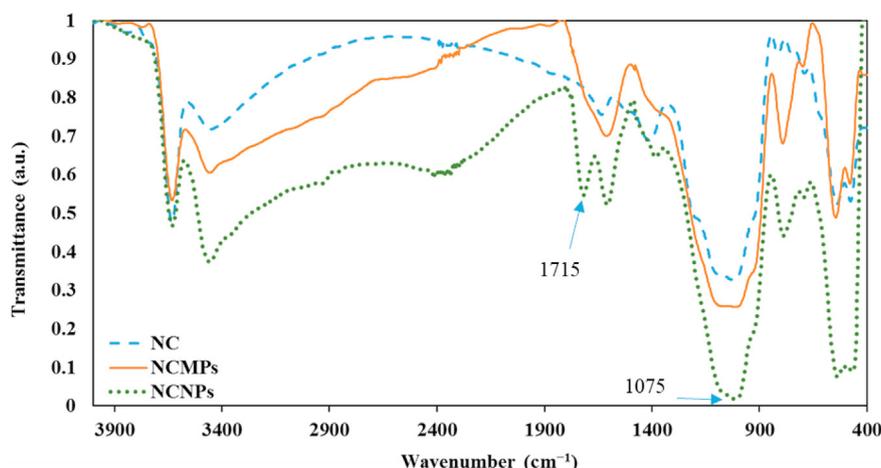


Figure 1. FT-IR spectra of NC, NCMPs, and NCNPs filler particles.

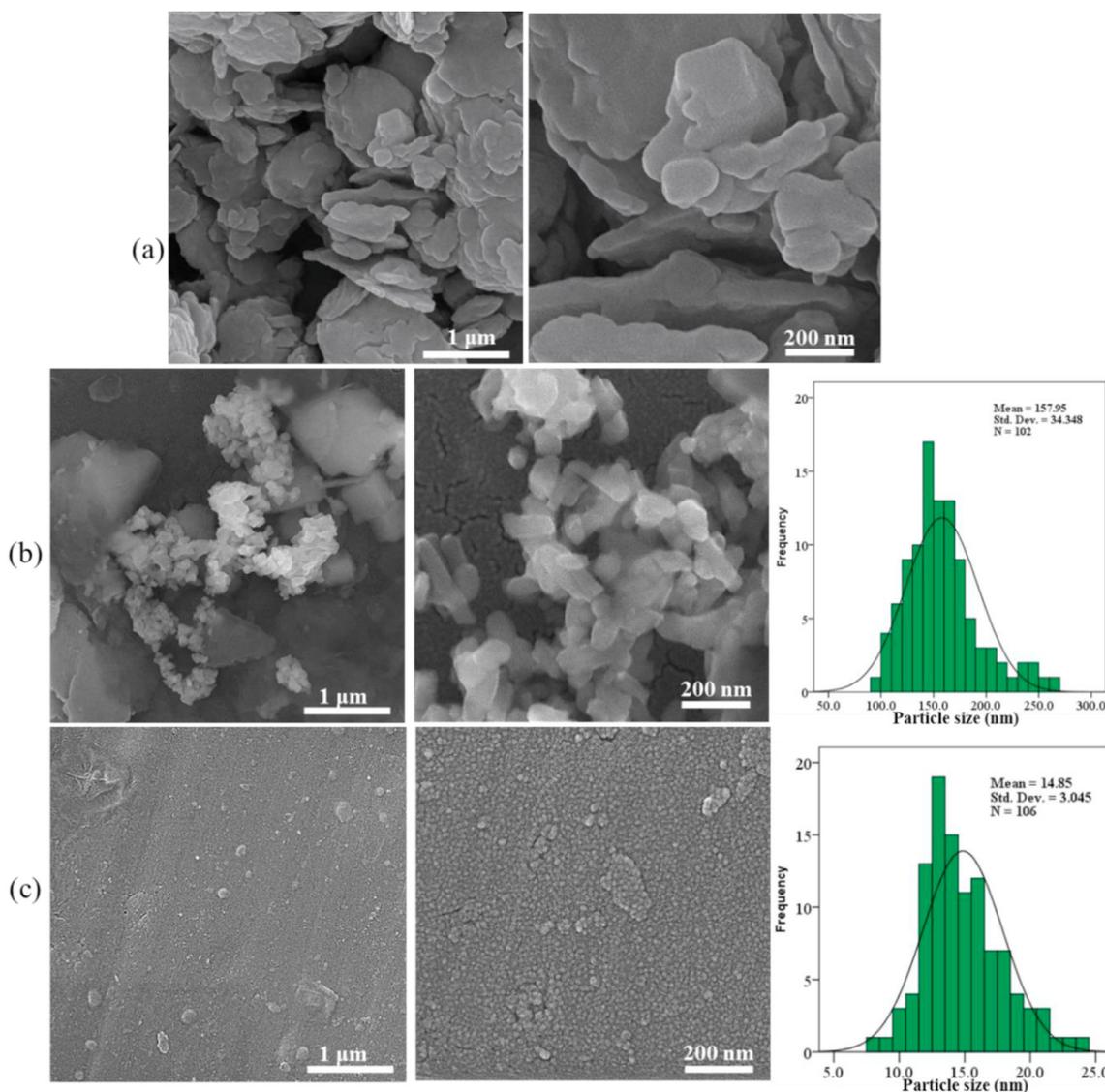


Figure 2. SEM images of (a) NC, (b) NCMPs, and (c) NCNPs samples in three different magnifications and the particle size distribution histograms of NCMPs and NCNPs samples.

in talc powder had exhibited the lowest number among the carriers (Table 2).

At the 6th month evaluation, again PK11 showed higher population in comparison to PP20 in different carriers. Both species had more population at 4 °C than 28 °C in most of the carriers. Interestingly, nanoclay + alginate and NCNPs + alginate carriers at both 4 °C and 28 °C were the proficient carriers in preserving both bacteria. The lowest population of two bacterial species at 4 °C and 28 °C were observed in talc powder (Table 2).

3.4. Phosphate solubilizing index

The investigation of bacterial ability to solubilize phosphate sources (tricalcium phosphate and hydroxyapatite) showed that at the initial evaluation the PK11 on NCNPs + alginate carrier had the highest ability to solubilize tricalcium phosphate Sperber plates even though difference with Nanoclay + alginate was not significant. NCMPs + alginate was the other carrier for PK11 with high solubilization index in both tricalcium phosphate and hydroxyapatite sources. *P. putida* (PP20) only showed the high solubilization index in the nanoclay + alginate carrier for tricalcium phosphate source (Table 3). Talc powder and NC were the inferior carriers at the initial evaluation.

The 6th month analysis of bacterial strains in different carriers for two phosphate sources at two temperatures showed that PK11 in nanoclay + alginate carrier had the highest solubilization indices at 4 °C and 28 °C respectively in tricalcium phosphate plates (Table 3). The PP20 in this carrier was placed at the second rank in similar condition. The highest solubility index in hydroxyapatite plate were observed in nanoclay + alginate, NCNPs + alginate and NCMPs + alginate for both bacteria at 4 °C. The lowest solubilizing index was seen in PP20 in talc powder carrier at 28 °C in hydroxyapatite plate. In overall, both bacteria in all carriers had shown higher solubilization index at 4 °C (Table 3).

3.5. Freeze-dried beads

The investigation of the bacterial population in the freeze-dried beads after 9-month incubation in different nano-carriers showed that nanoclay + alginate carrier had the highest population size in comparison to two other alginate based carriers in both bacterial species (Table 4), and the PK11 was the superior strain. NCMPs + alginate although showed the lowest population size. By the comparison between these results and the results of bacterial population after 6-month incubation (Table 2), it turns out that nanoclay + alginate carrier had a relatively steady trend in preserving bacterial species.

Table 1. The concentration of surface functional groups of as-prepared NCNPs, NCMPs, and NC samples.

Sample	Total acidic groups (mmol g ⁻¹)	Carboxyl groups (mmol g ⁻¹)	Lactonic groups (mmol g ⁻¹)	Phenolic Hydroxyl groups (mmol g ⁻¹)
NC	0.58	0.42	-	0.16
NCMPs	1.04	0.69	0.1	0.25
NCNPs	2.69	1.93	0.18	0.58

3.6. Phosphatase activity

Analysis of variance of phosphatase activity after 6-month incubation showed that the PK11 was the superior strain for producing phosphatase in all carriers. Results revealed that nanoclay + alginate carrier in both bacteria induced the highest phosphatase activity even though differences between this carrier and NCNPs + alginate and NCMPs + alginate were not significant for the PK11. Both bacteria in talc powder carrier showed the lowest phosphatase activity (Figure 4). Nevertheless, the differences between talc powder and NC in both bacteria were not significant ($p \leq 0.05$).

4. Discussion

According to the results of our experiments, NCNPs + alginate, NCMPs + alginate and nanoclay + alginate carriers after 6-month incubation showed better performance among the investigated carriers for bacterial population. The PK11 and PP20 strains after incubation for 6 months at 4 °C had 27.4×10^8 , 25.3×10^8 , and 22.7×10^8 , 21.4×10^8 CFUs respectively at NCMPs + alginate and nanoclay + alginate carriers. The addition of alginate to the nanostructure materials promoted more viability in both bacterial species in comparison to the application of these nanostructure materials alone. Nevertheless, the lowest population size was observed in talc powder for both bacteria at 28 °C 0.82×10^8 , 0.55×10^8 for PK11 and PP20, respectively. A study showed a decline in the population of *P. fluorescens* (Pf1) in talc-based formulation from 37.5

$\times 10^7$ CFU g⁻¹ to 1.3×10^7 CFU g⁻¹ after 8-months of storage (Nakkeeran et al., 2005). *P. putida* in talc-based formulation after 45 days in another study also had 1.0×10^3 CFU g⁻¹ (Amer and Utkhede, 2000).

Our results confirmed that type of nano-fillers in the matrix of alginate beads could control the temperature preference of the preserved bacterial species. A comparison between two bacterial species of *Bacillus subtilis* and *P. putida* in various formulations with organic, inorganic, and polymeric basis, revealed that *B. subtilis* survived at room temperature after 45 days, but *P. putida* depending on which carrier is used required refrigeration for survival (Amer and Utkhede, 2000). Nonetheless, it was reported that storage at 4 °C assists the maintenance of encapsulated cells with longer viability (Bashan, 1998). The viability of *B. subtilis* and *P. corrugata* in another study did not reduce after storing at 4 °C for 3 years in wet alginate beads (Bashan et al., 2016). Although, storing the *R. terrigena* as a gram-negative bacteria for 100 days at 4 °C induced a gradual decline in cell survival from 10^9 CFU g⁻¹ to 10^8 CFU g⁻¹ of dried beads (Schoebitz et al., 2012).

The bacterial population has a close association with the phosphate solubilizing ability, which is indicative of active metabolism (Malboobi et al., 2009). The bacterial ability for solubilizing phosphate in this experiment were also directly proportional to the bacterial population ($r = 0.772^{**}$), as we observed the highest solubilizing index for both bacterial species in nanoclay + alginate and NCNPs + alginate carriers after storing at 4 °C. The highest solubilizing indices for PK11 and PP20 were 2.935 and 2.870 both observed within the nanoclay + alginate carrier. Previous reports have revealed that the maximum and minimum colony

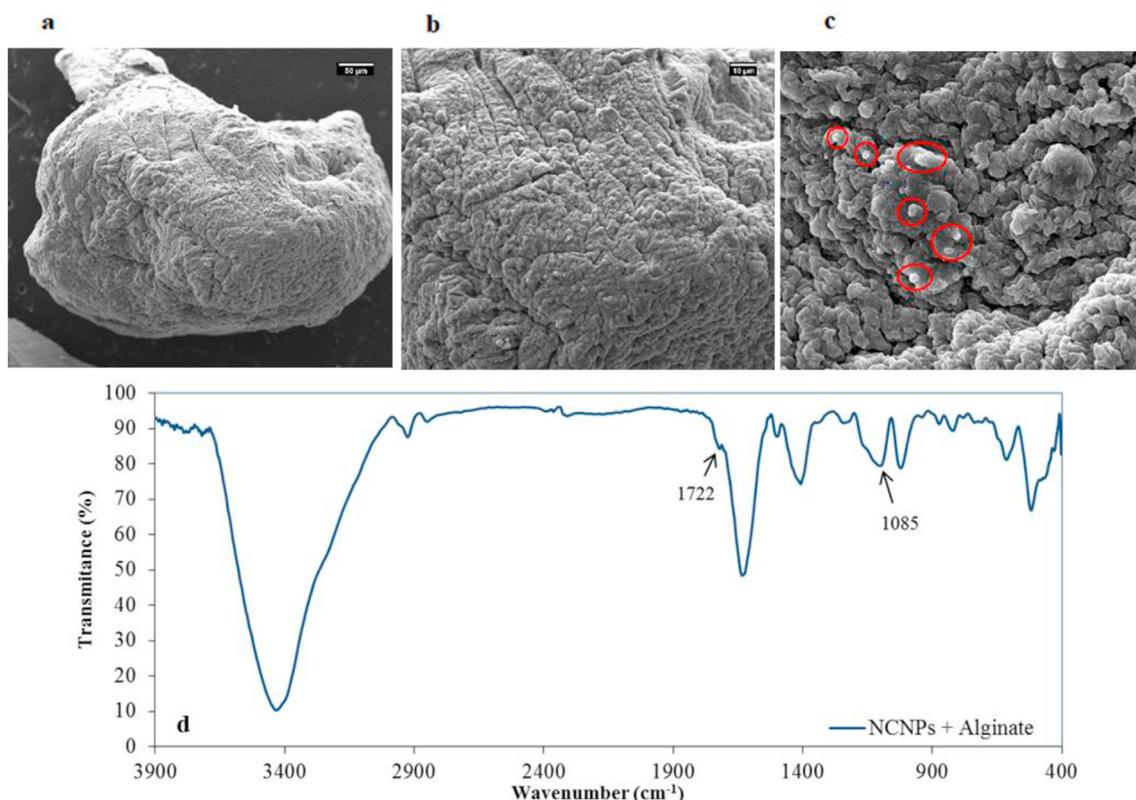


Figure 3. (a–c) SEM of NCNPs enriched alginate beads at different magnifications. (d) FTIR spectrum of NCNPs enriched alginate bead nano-carriers.

Table 2. The effect of storage at 4 °C and 28 °C on the population size of bacterial species in different carriers (data expressed in CFU g⁻¹).

Bacterial species	Carriers	Initial evaluation	6 th month evaluation	
			4 °C	28 °C
<i>Pseudomonas putida</i> (PP20)	Clay	17.80 × 10 ⁸ g	4.45 × 10 ⁸ fg	1.05 × 10 ⁸ g
	Nanoclay	55.10 × 10 ⁸ e	15.40 × 10 ⁸ e	1.23 × 10 ⁸ fg
	Nanoclay + alginate	99.20 × 10 ⁸ b	21.40 × 10 ⁸ bcd	3.21 × 10 ⁸ b
	Talc powder	7.80 × 10 ⁸ h	0.55 × 10 ⁸ g	1.04 × 10 ⁸ g
	NC	14.90 × 10 ⁸ g	3.40 × 10 ⁸ fg	1.15 × 10 ⁸ fg
	NCMPs	35.60 × 10 ⁸ f	14.10 × 10 ⁸ e	1.31 × 10 ⁸ ef
	NCMPs + alginate	79.60 × 10 ⁸ d	22.70 × 10 ⁸ bc	1.28 × 10 ⁸ fg
	NCNPs + alginate	91.90 × 10 ⁸ c	20.50 × 10 ⁸ cd	3.23 × 10 ⁸ b
<i>Pseudomonas kilonensis</i> (PK11)	Clay	29.90 × 10 ⁸ f	7.23 × 10 ⁸ f	1.07 × 10 ⁸ fg
	Nanoclay	78.00 × 10 ⁸ d	17.10 × 10 ⁸ de	1.64 × 10 ⁸ d
	Nanoclay + alginate	120.40 × 10 ⁸ a	25.30 × 10 ⁸ ab	3.50 × 10 ⁸ a
	Talc powder	12.80 × 10 ⁸ gh	0.82 × 10 ⁸ g	1.05 × 10 ⁸ g
	NC	15.40 × 10 ⁸ g	3.44 × 10 ⁸ fg	1.30 × 10 ⁸ ef
	NCMPs	49.30 × 10 ⁸ e	14.70 × 10 ⁸ e	1.54 × 10 ⁸ de
	NCMPs + alginate	98.40 × 10 ⁸ bc	27.40 × 10 ⁸ a	2.01 × 10 ⁸ c
	NCNPs + alginate	120.00 × 10 ⁸ a	22.60 × 10 ⁸ bc	3.44 × 10 ⁸ ab

NC: Natural char; NCMPs: Natural char micro-particles; NCNPs: Natural char nano-particles.

Data reported in CFU (colony forming units).

Different letters show significant differences among treatments.

diameter were found with *Bacillus* species P6 (1.91 mm) and P8 (0.61 mm), which also had the maximum and minimum of halo zone diameters of 5.89 mm and 2.1 mm, respectively (Gandhi et al., 2014). The solubilizing efficiency of two *Pseudomonas* species (P1 and P2) among different organisms were reported to be 44.44 and 71.43% (Selvi et al., 2017). Isolation of phosphate solubilizing bacteria with tricalcium phosphate sources produces numerous candidates; hence, it would not be the best strategy. There are less soluble phosphate sources such as fluorapatite and hydroxyapatite (Bashan et al., 2013), which should be considered to predict the microorganism capability in phosphate solubilizing with certainty. In the current study, both bacteria also more solubilized tricalcium phosphate than hydroxyapatite.

Investigating the phosphatase activity in both bacterial species in this experiment also displayed the better performance of nanoclay + alginate beads with the amounts of 36.8 and 32.7 μ moles of PNP released mL⁻¹ of filtrate h⁻¹ respectively for PK11 and PP20 strains. The positive correlation between phosphate solubilizing capacity and phosphatase activity reported, the scientists believed that the availability of the high amount of P in the medium and the ability of the strains are the reason for this event (Ponmurugan and Gopi, 2006). Results of our study were also in accord with their findings, which showed the positive relation between phosphatase activity and solubilizing index ($r = 0.825^{**}$).

Among all the investigated carriers for both bacterial species, alginate base carriers showed the highest population, phosphatase activity, and

Table 3. Effect of different carriers on the phosphate solubilization ability of two bacterial species after 8 days in two phosphate sources (tricalcium Phosphate and hydroxyapatite) at 4 °C and 28 °C.

Bacterial species	Carriers	Solubilization Index					
		Initial evaluation		6 th month evaluation			
		Tricalcium Phosphate	Hydroxyapatite	4 °C		28 °C	
<i>Pseudomonas putida</i> (PP20)	Clay	1.885 hi	2.080 gh	1.925 fg	1.840 d	1.345 ef	1.215 de
	Nanoclay	2.335 de	2.300 de	2.100 def	2.040 bc	1.545 cd	1.280 cde
	Nanoclay + alginate	2.530 b	2.390 cd	2.870 a	2.085 ab	2.275 a	1.380 abc
	Talc powder	1.890 hi	1.880 jk	1.520 i	1.595 e	0.985 g	0.930 f
	NC	1.800 hi	2.020 hi	1.865 gh	1.840 d	1.260 f	1.170 e
	NCMPs	2.240 ef	2.040 hi	2.140 cde	2.060 bc	1.420 def	1.260 cde
	NCMPs + alginate	2.490 bc	2.380 cd	2.215 cd	2.020 bc	1.545 cd	1.335 bcd
	NCNPs + alginate	2.395 cd	2.170 fg	2.509 b	2.100 ab	1.899 b	1.400 abc
<i>Pseudomonas kilonensis</i> (PK11)	Clay	2.180 f	2.205 ef	2.065 def	2.040 bc	1.445 de	1.265 cde
	Nanoclay	2.370 d	2.025 hi	2.320 bc	2.000 bcd	1.675 c	1.360 bcd
	Nanoclay + alginate	2.580 ab	2.580 a	2.935 a	2.145 ab	2.330 a	1.450 ab
	Talc powder	1.970 gh	1.950 ij	1.710 hi	1.665 e	1.045 g	0.930 f
	NC	2.020 g	1.850 k	2.005 efg	1.920 cd	1.410 def	1.190 e
	NCMPs	2.240 ef	2.420 bc	2.010 efg	2.010 bc	1.530 cd	1.410 abc
	NCMPs + alginate	2.575 b	2.490 ab	2.215 cd	2.100 ab	1.685 c	1.365 bcd
	NCNPs + alginate	2.685 a	2.435 bc	2.511 b	2.240 a	1.921 b	1.540 a

NC: Natural char; NCMPs: Natural char micro-particles; NCNPs: Natural char nano-particles.

Different letters show significant differences among treatments.

Table 4. Bacterial population size in freeze-dried alginate beads after 9-month incubation (CFU g⁻¹).

Carriers with alginate base	Bacterial species	
	<i>Pseudomonas kilonensis</i> (PK11)	<i>Pseudomonas putida</i> (PP20)
Nanoclay + alginate	16.38 × 10 ⁸ a	10.66 × 10 ⁸ b
NCMPs + alginate	0.60 × 10 ⁸ c	0.85 × 10 ⁸ c
NCNPs + alginate	1.83 × 10 ⁸ c	1.67 × 10 ⁸ c

NCMPs: Natural char micro-particles; NCNPs: Natural char nano-particles.

solubilizing activity. Previous research also showed the longer survival of bacteria in the alginate beads, which could make these formulations extremely attractive from the agricultural and commercial perspectives (Bashan et al., 2016). The longest survival time was reported 14 years for *Azospirillum brasilense* and *P. fluorescens* without losing efficacy in dry alginate beads stored at ambient temperature. Even though bacterial populations had decreased, but significant numbers survived (10⁵–10⁶ CFU g⁻¹ beads) (Bashan et al., 2016) it is due to the fact that alginate beads are capable of entrapping the sufficient number of bacteria in an environment with sufficient mechanical strength. It has been stated that encapsulated bacteria in the alginate beads have several advantages over free cell formulations including, protection from biotic and abiotic stresses, enhanced survival and improved physiological activity, and capability to supply nutritional additives to the encapsulated bacteria (Young et al., 2006). These good characteristics of alginate beads guarantee the bacterial survival. Alginate beads that contained *A. brasilense* had >10⁹ CFU g⁻¹ after air-drying at 38 °C (Bashan et al., 2016). Fresh beads for *Azotobacter chroococcum*, *Acinetobacter* sp. and *P. fluorescens* strains had 91 × 10⁹, 83 × 10⁹, and 88 × 10⁹ CFUs g⁻¹ of beads

respectively, after storing for 300 days at 4 °C and room temperature the population declined to 56 × 10⁹, 39 × 10⁹, and 82 × 10⁸; 53 × 10⁸, 44 × 10⁹, and 51 × 10⁸ CFUs g⁻¹ of beads respectively, but still large number of bacteria survived (Subramanyam, 2015).

Amendment of alginate bead with the materials like humic acid has led to high viability of the encapsulated *B. subtilis* CCpg104 with minimum cell loss after 5 months, also preserved the bacterial ability for solubilizing calcium phosphate in *in vitro* condition (Young et al., 2006). Humic acid addition into the alginate bead had beneficial effects on the encapsulated *P. putida* and *B. subtilis* as it served as a carbon source that promoted survival of the encapsulated microorganisms during storage (Rekha et al., 2007). It was revealed that humic acid has dual benefits for microorganism and plant and due to its chemical properties mixing it with alginate is easy, thereby does not interfere with the formation of the alginate beads (Young et al., 2006). Results of a study showed that immobilization of *P. fluorescens* and *Serratia* sp. in alginate-starch beads (starch as a carbon source for bacteria) was effective in dissolving insoluble phosphate (Schoebitz et al., 2013). Addition of other supplements like clay and skim milk to the beads also induced the increment in bacterial survival significantly compared to alginate beads alone (Bashan, 1998; Chandra, 2012; Gagné-Bourque et al., 2015). In the current study, no deleterious effect by the supplementation of nanoclay, NCMPs and NCNPs on the bacteria was observed. Even, we detected the maintenance of bacterial cells in the beads upon storage compared to other formulations, which might be due to the porous matrix of alginate beads and the addition of NC and nanoclay as nutrients resource that subsequently facilitate the bacterial multiplication within the bead environment. Carriers with good porosity would be able to supply microorganisms with good ventilation which would result in sustainability and better performance of bacteria. The puffy structure of the alginate beads formed with the addition of NCNPs (Figure 3a–c) could be the reason for the high viability of bacterial species in these carriers due to higher ventilation compared to the solid carriers (Sahu and Brahmprakash, 2016). Moreover, it could be assumed that NCNPs in our study provided C and N sources more than NCMPs, for the encapsulated bacteria. As it is confirmed before when particle size of carriers became smaller, a higher surface area would be provided, which accordingly resulted in increasing the coverage of bacterial cells (Nakkeeran et al., 2005).

Nanoclays have been studied and developed for different applications, due to its availability and relatively low cost and environmental impact (Guo et al., 2018). Montmorillonite (MMT) has been widely studied because of its high cation exchange capacity, high interior surface area (Sarmah et al., 2015), and swelling behavior (Guo et al., 2018). Substances such as organic or biological molecules could integrate into montmorillonite surface because of its high cation exchange capacity (Guo et al., 2018). Montmorillonite could be modified easily with organic molecules like alginate (Chanra et al., 2018). It could be dispersed in alginate beads as fillers or additives to form alginate + nanoclay composites, which enhance the mechanical strength, water and gas permeability of the beads (Guo et al., 2018). Our results were supported by these mentioned features for nanoclay + alginate composites, as we observed higher population size and solubilizing index in this carrier in comparison to others. Clay (MMT) modification which is known as “organophilization”, initiates intercalation of the organic compounds between the networks thus increases the distance between the individual layers. The initial distance between the plates is reported 9–12 Å (Tesaříková Svobodová, 2018). Alginate probably increases the distance between layers in clay, which perhaps would be the reason for preserving more bacteria in this kind of carrier.

The high viability of the cells (1 × 10¹⁰ CFU g⁻¹) guaranteed with lyophilization in the humic acid enriched alginate beads (Young et al., 2006). Freeze-drying of the nanoclay + alginate in this study also guaranteed the viability of both bacterial species, even though population size were declined in NCNPs + alginate and NCMPs + alginate freeze-dried beads after 9-month storage (Table 4). It was reported that beneficial

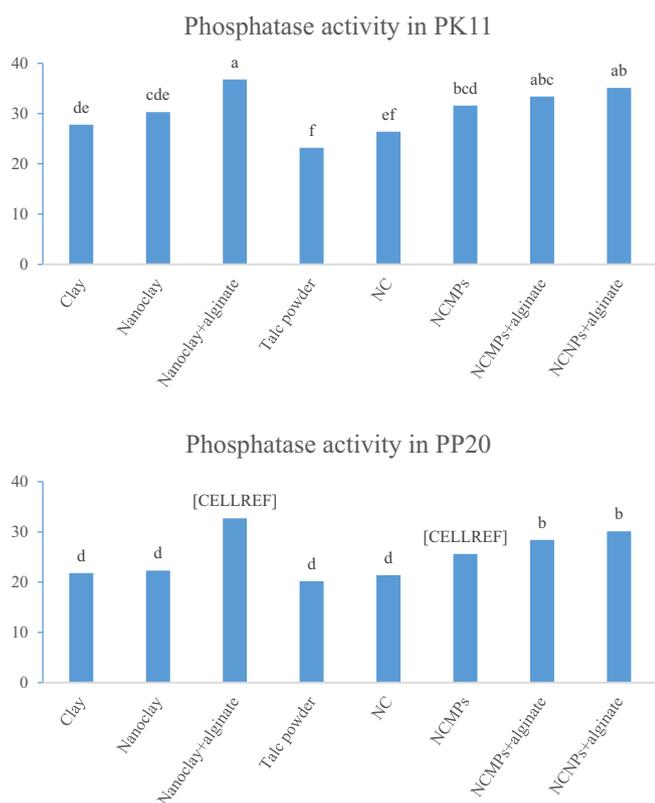


Figure 4. Phosphatase activity in bacterial strains after 6-month incubation in different carriers (data was expressed in μ moles of PNP released mL⁻¹ of filtrate h⁻¹). NC: Natural char; NCMPs: Natural char micro-particles; NCNPs: Natural char nano-particles. Different letters show significant differences among treatments.

characteristics of natural char such as high specific surface area, porosity, and its ability to absorb organic compound and microorganism supply bacteria with a perfect environment for their growth and proliferation so it has been considered to be a favorable carrier (Schoebitz and López Belchí, 2016). However, all the amendments have not advantageous effects on the microbial cells. For instance, the initial cell loading and survival of P-solubilizing bacteria were adversely affected by charcoal-soil mixed with alginate (Young et al., 2006). As the origin of natural char could have an impact on the bacterial population and their ability in solubilizing phosphate, so it should be examined before the application. The nitrogen content and pH were particularly identified as important characteristics to be considered when focusing on initial inoculum density and shelf life (Hale et al., 2015). It is exhibited that natural char effects on rhizosphere organisms could be positive or negative depending on the origin of raw material, pyrolysis conditions, method and frequency of applications, and doses (Gluszek et al., 2017). Deleterious effects of different kinds of natural char on microbial activity in soil were reported. Natural char with the origin of corn stover (CS), switchgrass (SG), and ponderosa pine wood residue (WC) had decreased the microorganisms activity (Gluszek et al., 2017). In a study natural char with coconut-shell origin was found to increase the survival of *Azospirillum lipoferum* up to 180 days ($\log 10.79$ CFU g^{-1} of carrier) compared to acacia wood-based natural char and lignite (Saranya et al., 2011). Our results for 6th and 9th month evaluations showed that NC had the ability for preserving bacterial population until the 6th month but afterward both bacterial population had decreased in beads enriched with NCMPs and NCNPs in the freeze-dried beads.

5. Conclusions

For the first time ever, application of nano-carriers for phosphate solubilizing bacteria proposed here as promising carriers to maintain bacterial viability and efficacy in solubilizing phosphate. Nanoclay and NCNPs were the best carriers; these nanomaterials were more proficient in the alginate beads. Bacterial population, solubilizing index and phosphatase activity were directly proportional, which were mediated by the positive characteristics of these carriers. Of the two bacterial species in this research, *P. kilonensis* (PK11) showed more viability and stability in its performance on solubilizing phosphorus. Hence, bioinoculants with the formulation of these nano-carriers would have more privilege in phosphate solubilizing in the field thereby reduce the amount of phosphate fertilizers. Further experiments should be done to appraise nanotechnology application in all aspects of agriculture in parallel to the current advancements in order to achieve more sustainability in the agricultural systems. For further work we suggest to apply these loaded carriers with these bacterial strains in the pot and field experiments to confirm their suitability.

Declarations

Author contribution statement

M. Safari: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

E. Motamedi: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

H. Kari Dolatabad: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

S. A. M. Modarres Sanavy: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

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The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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