CI&CEQ
Chemical Industry & Chemical Engineering Quarterly

Available online at
Association of the Chemical Engineers of Serbia AChE
www.ache.org.rs/CICEQ

Chem. Ind. Chem. Eng. Q. 32 (2) xxx–xxx (2026) CI&CEQ

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SCIENTIFIC PAPER

UDC

SPRAY DRYING OF PHOSPHATE-SOLUBILIZING BACTERIA FOR THE PRODUCTION OF NEW BIOFERTILIZERS

Highlights

- Phosphate-solubilizing strains of *Azotobacter*, *Pseudomonas,* and *Bacillus* were characterized.
- Selected strains showed multifunctional plant growth-promoting traits.
- The spray drying method was successfully applied for bacteria encapsulation.
- Encapsulates with particle size below 5 μm were obtained, suitable for seed inoculation.
- The encapsulation procedure requires further optimization to achieve higher cell numbers.

Abstract

Phosphate-solubilizing bacteria represent a sustainable solution to cope with phosphorus unavailability in agricultural soil. However, the success of their application is highly dependent on multiple environmental factors, and a novel approach is needed for bioformulations. The present study aimed to evaluate the suitability of the spray drying method for encapsulation of phosphate-solubilizing bacteria Azotobacter chroococcum F14/2, Bacillus megaterium 11/3, and Pseudomonas putida P1. Three strains were characterized, grown under optimal conditions, and encapsulated as a whole medium-cell system using the spray drying method and maltodextrin as carrier material. The described procedure provided encapsulates with an average particle size below 5 μm, moisture content under 10%, and satisfactory powder properties. The cell viability of encapsulates (after storage) was in the following order: Bacillus megaterium 11/3 > Azotobacter chroococcum F14/2 > Pseudomonas putida P1. Additional protection during spray drying was most probably achieved by the presence of microbial exopolysaccharides, which opened the possibilities for further optimization of encapsulation procedures.

Keywords: Azotobacter, Pseudomonas, Bacillus, encapsulation, soil.

INTRODUCTION

Plants require phosphorus (P) for their biological cycle and it is considered as one of the main nutrients for efficient crop production [1]. Despite the abundance of P in the soil, it generally remains unavailable for the plants, especially under unfavorable soil pH [2]. Modern agriculture is mainly based on P, which is implemented into the soil via mineral fertilizers. The implementation of P by fertilizers is one of the main issues in modern agriculture, especially regarding the accelerated depletion of P minerals used in fertilizer production [1]. Hence, there is a rising interest in new solutions for P management in agriculture.

Despite the important role of phosphate-solubilizing microbes in plant nutrition and the P biogeochemical cycle, the research on those microbes is far behind studies on nitrogen-fixing microbes [3]. Inoculation of plant seeds by phosphate-solubilizing bacteria could potentially improve the P soil dynamic, reduce the need for the application of commercial fertilizers, and generally improve the ecological aspect of modern agriculture [4]. Phosphate-solubilizing microbes are identified among various species of bacteria [5]. Two main mechanisms are known regarding the increase of P availability by beneficial microbes. One of them is the solubilization of insoluble phosphates due to local acidity increase by acid production, and the other mechanism includes the activity of phosphatases,

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Paper received: 16 December, 2024
Paper revised: 17 April, 2025
Paper accepted: 11 June, 2025

https://doi.org/10.2298/CICEQ241216017L

important for the mineralization of P contained in the form of organic compounds [6].

Bacteria exhibiting one or both mechanisms affect plant nutrition, thus belonging to the group of Plant Growth Promoting Bacteria (PGPB). Those microbes may additionally exhibit other plant growth-promoting (PGP) traits such as the production of siderophores, indole-acetic acid (IAA), and exopolysaccharides, which represent not only an important added value, but it was also confirmed that they show synergistic effects which leads to a better phosphate solubilization [7,8]. *Azotobacter* representatives are recognized as beneficial inoculants in agriculture mainly because of their nitrogen-fixing ability [9]. *Bacillus* and *Pseudomonas* species, particularly *B. megaterium* and *P. putida* are known in terms of increasing P availability to plants [10]. Besides well-described beneficial effects, the success of the promising strains' application is highly dependent on multiple environmental factors.

The main issue regarding the implementation of plant-beneficial microbes is related to their susceptibility to unfavorable environmental conditions that may hinder microbe's positive effects on plant growth. To overcome these limitations of as-prepared biomass and its further implementation in the agro sector, especially as PGP agents, encapsulation may be included in biomass preparation steps [11]. Encapsulation is usually defined as the protection of an active compound by the formation of layer(s) of selected carrier material(s) suitable for specific applications. In this regard, by applying an adequate encapsulation method, encapsulates could be formed into various shapes and sizes. Spray drying remains the main encapsulation technique accepted by many industries as a cost-effective and very efficient method for the production of encapsulates in the form of fine powders and with low water content [12]. Also, spray drying showed its potential for encapsulation of soil-beneficial microbes, such as P-solubilizing microorganisms [13].

As far as we know, this is the first study dealing with the encapsulation of non-spore-forming bacterial strains by spray drying (with maltodextrin as carrier material) and with the bacterial products formed during cultivation to be used as biofertilizers. In addition, there is a lack of studies that cover the entire pathway of processing PGPB into biofertilizers, i.e. isolation, identification, cultivation of the biomass, encapsulation, and characterization of the encapsulates. Taking into account all the points mentioned above, the aim of the present study was to evaluate the suitability of the spray drying method for encapsulation of beneficial phosphate-solubilizing bacteria belonging to different genera of PGPB. The bacterial strains *Azotobacter chroococcum* F14/2, *Bacillus megaterium* 11/3, and *Pseudomonas putida* P1 were characterized, grown under optimal conditions, and encapsulated as whole medium-cells system using spray drying method and maltodextrin as carrier material. The obtained encapsulates were analyzed in terms of morphological, physical, and chemical properties. Also, the viability of encapsulated phosphate-solubilizing bacterial strains was tested following the encapsulation procedure and after the storage period.

MATERIALS AND METHODS

Bacterial Isolation and Identification

Bacterial isolates used in this study originate from agricultural soil and were obtained using the standard dilution method and selective media. *A. chroococcum* F14/2 was isolated on the Fiodorov agar medium [14], and *B. megaterium* 11/3 was isolated on the National Botanical Research Institute's phosphate medium with $Ca_3(PO_4)_2$ (NBRIP; [15]). The NBRIP medium was used to search for isolates that can solubilize P, thus forming halo zones on the media. *A. chroococcum* F14/2 and *B. megaterium* 11/3 were identified and characterized within the research being presented; *P. putida* P1 was identified to the species level and characterized as PGPB in a previous research by Karličić *et al*. [16].

DNA isolation of the bacterial strains was performed using the Soil Mini-Prep Kit (Zymo Research, USA). The 16S rRNA region of *A. chroococcum* F14/2 was amplified with 63F and 1387 primers. A PCR reaction volume of 50 µL consisted of 5 µL 10xBuffer, 1 µL dNTP mix, 2 µL of each primer, 0.2 µL Taq polymerase, 1 µL template DNA, and RNAse free water up to 50 µL. The applied PCR program was: 95 °C/5 min, 35 cycles (95 °C/30 s, 55 °C/30 s, and 72 °C/1 min), 72 °C/1 min. The 16S rRNA region of *B. megaterium* 11/3 was amplified using the primer pair UNI 16SR/UNI 16SF. The Fast Gene Taq HotStart PCR Kit (Kapa Biosystems) was used for the PCR mixture consisting of: 10 µL 10xBuffer, 3 µL 25 $MgCl_2$, 1 µL dNTP mix, 2 µL of each primer, 0.2 µL Taq polymerase, 1 µL DNK, and RNA free water until 50 µL. The mixture was subjected to the following regime: 95 °C/5 min, 35 cycles (95 °C/30 s, 55 °C/30 s, and 72 °/1 min) and 72 °C/1 min. PCR products were sequenced in both directions by the Macrogen Sequencing Service (Macrogen Europe, Amsterdam, The Netherlands), using the same primer pairs as in the PCR reaction. Consensus sequences were compared with representative sequences from the Gene Bank database. 16S rRNA sequences of the strains included in the research are deposited to the Gene Bank database under the following accession numbers: OP975801 and ON478151 for *A. chroococcum* F14/2 and *B. megaterium* 11/3, respectively.

Characterization of Plant Growth Promoting Activity

Phosphate-solubilization capacity of the strains was tested on an NBRIP medium as described by Nautiyal [15]. The NBRIP medium was spot-inoculated and incubated at 30 °C for two weeks. The test was performed in three replicates. After measurement of halo zones and colony diameter, the solubilization index (*SI*) was calculated as follows:

SI = (colony diameter + halo zone diameter) / colony diameter.

The capability of tested strains to produce acid and alkaline phosphatase as well as naphthol-AS-BI-phospho-hydrolase was tested by API ZYM test (bioMérieux, France) according to the manufacturer's instructions.

The siderophore production was tested on a modified Chrome Azurol S (CAS) medium as described by

Lakshmanan *et al.* [17]. The appearance of a yellow-orange zone around the colony was considered a positive result. The production of exopolysaccharides (EPS) was tested on a medium for stimulation of the EPS production, according to Paulo *et al.* [18]. Sterile filter paper discs (5 mm Ø) were placed on the medium, inoculated with 5 µL of overnight bacterial cultures, and incubated for 48 h at 30 °C. The presence of slime around the discs indicated EPS production, which was confirmed by the formation of precipitates after mixing the slime with 96% ethanol. The colorimetric method described by Patten and Glick [19] was used to determine the indole-acetic acid (IAA) production by bacterial strains. Briefly, the strains were grown in a medium amended with 100 µg/mL of L-tryptophan (Sigma Aldrich, USA) for 72h at 30 °C and 150 rpm (Environmental Shaker-Incubator ES-20, BIOSAN, Latvia), and the supernatant mixed with the Salkowski reagent was used for the absorbance measurement at 530 nm (T70 UV/VIS Spectrometer, PG Instruments Ltd). A calibration curve was obtained by measuring the absorbance of solutions in the concentration range of 1-100 µg/mL of IAA (Sigma Aldrich, USA).

Encapsulation

Cultivation of Bacterial Strains

Bacterial strains were grown separately, by gradually increasing the volume of the growth media, until 1.5 L. *A. chroococcum* F14/2 was grown using a medium consisting of 10 g peptone, 10 g glucose, and 5 g NaCl per 1 L of distilled water, while *B. megaterium* 11/3 and *P. putida* P1 were grown in the Tryptic Soy Broth (Torlak, Serbia). The cultures were grown in a temperature-controlled rotary shaker (150 rpm, 30 °C) for 48 h, except for *A. chroococcum* F14/2 which was grown for 72 h. The CFU/mL in the final liquid culture was determined using a McFarland densitometer (Grant Instruments, Ltd. England).

Encapsulation Procedure

For encapsulation of the selected microorganisms, the spray drying technique and maltodextrin as a carrier material were used. After the cultivation period, the cell suspension (whole broth with the cells) was mixed with maltodextrin (10 g of maltodextrin/100 mL of cell suspension; maltodextrin (C*MD 01915), Palco, Serbia) on the magnetic stirrer for about 30 min. The encapsulation procedure was carried out using a spray drier B-290 (BÜCHI, Switzerland) under the following conditions: inlet temperature 130 °C; outlet temperature ~65 °C; liquid flow 8 mL/min. After spray drying, the samples in the powdered form were packaged in hermetic packaging and stored at room temperature and 4 °C. The yield of the encapsulation process (%) was calculated as the weight ratio of encapsulates and dry weight of feed material [20].

Characterization of the Encapsulates

Scanning Electron Microscopy (SEM)

The morphology of encapsulates was examined using scanning electron microscopy (SEM). Prior to the analysis, the samples were placed on the SEM sample stubs with a two-sided adhesive tape and plated with gold using an instrument BALTEC SCD 005. The SEM analysis was performed by a microscope JEOL JSM-6390LV (JEOL, Japan). The SEM images (of at least 100 particles) were used for the particle size analysis by the ImageJ program.

Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (ATR-FTIR)

The chemical properties of encapsulates and the potential chemical interactions between encapsulates' constituents were investigated using an IR-Affinity-1 infrared spectrometer (Shimadzu, Japan). The measurements were performed with the attenuated total reflection (ATR) technique in the spectral range of 4000–600 cm^{-1} and at a resolution of 4 cm^{-1}. The spectra processing and presentation were realized using Spectragryph software [21]. Spectra were baseline corrected, smoothed using Savitzky-Golay filters with 5 points and the second-order polynomial function, and normalized (Standard Normal Variates, SNV).

Moisture Content

The moisture content of encapsulates was analyzed using a procedure described by Kalušević *et al.* [20]. Briefly, samples were measured before and after drying at 105 °C in the drying oven to constant mass, and the moisture content (in %) was calculated from the mass loss.

Hygroscopicity

The hygroscopicity of obtained encapsulates was measured according to the procedure described by Tomsone *et al.* [22]. Briefly, samples (1 g each) were exposed to air with ~75% relative humidity created by a saturated NaCl solution in a closed vessel. After one week, samples were weighed and hygroscopicity was calculated based on the amount of the adsorbed water (expressed as mass of adsorbed moisture/100 g of dry matter).

Bulk and Tapped Densities, Flowability, and Cohesiveness

Bulk and tapped densities of encapsulates were measured following the method recommended by European Pharmacopoeia 8.0 [23]. In short, the bulk density was calculated from the ratio between the mass of the sample and the volume it occupies in the measuring cylinder. The tapped density was determined after manual tapping of the sample in the measuring cylinder. The value of the tapped density was calculated as the ratio between the sample mass and the volume after 500 taps. The flowability of samples was calculated as a Carr index (CI, %), while the cohesiveness was expressed as the Hausner ratio (HR), based on the values of bulk and tapped densities [24].

Survival of Bacterial Strains during and after Encapsulation

Survival of bacterial strains was determined right after encapsulation and after storage for two months at room temperature and 4 °C. The encapsulates were crushed in a sterile mortar and mixed with 1% Na-citrate solution at a ratio of 1:10. Further serial dilutions were prepared with

sterile 0.9% NaCl solution, and the following selective media: Fiodorov agar [14], Nutrient agar (Torlak, Serbia), and Cetrimide agar (HiMedia Laboratories Pvt. Ltd., India) for Azotobacter, Bacillus and Pseudomonas strains, respectively.

Statistical Analysis

Analyses were performed in triplicate and data were presented as mean ± standard deviation (unless otherwise stated), using PAST software [25]. One-way ANOVA was applied to determine significant differences between the physical parameters of encapsulates. The Tukey test was used at $p \leq 0.05$ to identify significant differences among the means (or the Kruskal-Wallis test and Mann-Whitney test for the non-normally distributed data).

RESULTS AND DISCUSSION

Molecular Identification

The obtained 16S rDNA sequence of strains 11/3 and 14/2 showed a high percentage of identity (above 99% and 96%, respectively) as *Bacillus megaterium* and *Azotobacter chroococcum* strains from a database, thus confirming their belonging to these species.

Characterization of Bacterial PGP traits

All of the studied strains manifest at least one of the mechanisms to improve the P availability in soil, simultaneously showing some other PGP traits. *A. chroococcum* F14/2 and *P. putida* P1 showed the ability for both solubilization of P salts and mineralization of organic P compounds. On the other side, *B. megaterium* 11/3 produces enzymes involved in mineralization with a neglectful ability to solubilize inorganic forms of P (Table 1).

The ability to mineralize organic P compounds was observed in all three isolates through the activity of acid and alkaline phosphatase and naphthol-AS-BI-phosphohydrolase. *A. chroococcum* F14/2 and *B. megaterium* 11/3 showed the ability to produce EPSs, which was important for the successful root colonization and establishment of plant-microbe interaction. The production of IAA was observed in *P. putida* P1, which was at the same time characterized by the most pronounced phosphate solubilization activity. Our results showed that *A. chroococcum* F14/2 exhibited several mechanisms beneficial for plant nutrition, in addition to its ability to fix nitrogen (Table 1). *P. putida* P1 could also produce siderophores, while the tested *Bacillus* strain did not show this ability. Many soil bacteria usually exhibit various plant growth-promoting traits, which are also important for the successful phosphate solubilization and uptake by plants. However, these traits such as the production of siderophores, indole-acetic acid, and exopolysaccharides (Table 1) vary depending on bacteria strain and environmental conditions. For example, *Azotobacter* representatives are known for their ability to dissolve phosphates, although this cannot be considered a common PGP trait, even at the species level. While *A. chroococcum* F14/2 phosphate SI was 1.94, Kerečki *et al.* [9] reported that the positive effects of *A. chroococcum* F8/2 on sugar beet growth were related to several PGP traits, but excluding phosphate solubilization, which was tested but not confirmed. Nosrati *et al.* [26] also observed variations in the phosphate solubilization potential of multiple *Azotobacter* isolates collected from diverse soil types. *Pseudomonas*, including *P. putida* as its representative, has been recognized as a microbe involved in phosphate solubilization and mineralization [27]. It should be pointed out that soil pH can vary, which also affects the solubility of inorganic P salts; the ability to produce both acidic and alkaline phosphatase is of crucial importance for the strains' selection. Climate conditions significantly affect the activity of phosphatases, but nitrogen availability can be of specific importance when temperature and water accessibility are not limiting factors [28]. Thus, the combination of nitrogen-fixing and phosphate-solubilizing properties could be a promising tool for plant nutrition improvement.

Furthermore, EPS production has been described as an important factor in the solubilization of tricalcium phosphate, as bacterial EPSs showed the P-holding capacity which additionally contributes to the P availability [29]. Although there are literature findings about EPS production by *P. putida* strains [30], the tested *P. putida* P1 did not show the ability to produce EPS. According to Sandhya and Ali [31], this trait can be highly dependent and triggered by the presence of some stressors in the environment, so the ability to produce EPS cannot be completely excluded under environmental conditions.

Regarding P, some findings show not only the indirect effect of bacterial IAA on root development and nutrient uptake but also the promotion of organic acids secretion by IAA, which directly affects phosphate solubilization [32]. We detected IAA production by *P. putida* P1, which was consistent with the previously described *P. putida* strains that exhibit both phosphate solubilization and IAA production activity [33]. Pyoverdine is a widely studied siderophore produced by bacteria from the *Pseudomonas* genus, whose primary role is in iron uptake, but other functions include improvement of access to phosphates in nutrient-depleted soil [34]. Thus, in addition to phosphate solubilization and mineralization, the demonstrated PGP traits identified among the tested bacterial strains in the present study could contribute significantly to the potential of bacterial strains to improve P uptake efficiency. The set of traits indicates the potential of tested strains to be used as microbial inoculants in modern agriculture.

Encapsulation of Phosphate-Solubilizing Bacteria

As we showed above, selected bacterial strains exhibit promising phosphate-solubilizing and plant growth-promoting properties. However, after cultivation, bacterial biomass is unstable for a long period of storage and also difficult to apply, especially for plant seed covering. Namely, to fulfill its role as a growth-promoting agent, it is necessary to introduce the bacterial biomass close to the plant root system. Hence, covering the plant seeds prior to planting is a promising strategy for inoculation of crops. The important issue in the use of beneficial soil microbes is providing a stable product until the expected sowing season [35]. In this regard, spray drying is a technique that

provides fast water removal from bacterial biomass which is essential for the preservation of bacterial cells, thereby reducing shipping and storage costs.

Therefore, in the present study, bacterial biomass, together with growth medium, was encapsulated using a spray drying technique and maltodextrin as a carrier material. This approach should provide encapsulates suitable for plant seed inoculation with phosphate-solubilizing microbes aimed to enhance P availability for plants and reduce the use of industrial fertilizers. Additionally, we applied encapsulation of the whole cell suspension (broth with the cells), which eliminated the biomass separation step, thus reducing the waste and simplifying the production procedure, while preserving most of the valuable extracellular metabolites.

Morphological Properties of Encapsulates

As a result of spray drying encapsulation of whole cells/media suspensions, fine powders were obtained. The color of the encapsulates (i.e. powders) was white (*A. chroococcum* F 14/2 encapsulate) or pale brown (*B. megaterium* 11/3 and *P. putida* P1 encapsulates) due to differences in the color of the media; the *Azotobacter* growth medium was white, while the *Bacillus* and *Pseudomonas* growth media exhibited brown color as a result of different chemical compositions. Detailed data on the physical properties of encapsulates (powders) are provided in Table 2. The encapsulation process yield was also affected by the growth media composition; the yield of *A. chroococcum* F 14/2, *B. megaterium* 11/3 and *P. putida* P1 encapsulates was 60.9±5.0%, 79.4±7.2%, and 76.3±4.7%, respectively.

The obtained encapsulates' particles (Fig. 1) were spherical or irregularly shaped, depending on the sample that was subjected to spray drying. The encapsulates showed micromorphological properties that are typical for particles obtained by spray drying and maltodextrin as a carrier material [36]. Maltodextrin is a suitable carrier for the encapsulation processes by the spray drying technique and provides encapsulates with suitable morphological and physical properties. In addition, the yield of maltodextrin-based encapsulates is high, making them suitable for the encapsulation of complex mixtures of active ingredients. Although other carriers, such as gum Arabic, may be even more suitable for encapsulation by spray drying [20], maltodextrin is cheaper and more readily available compared to many other carrier materials. Here should be mentioned that the initial spray drying trials without maltodextrin resulted in high losses, i.e., low product yield. Also, the spray-dried encapsulates without maltodextrin were sticky and inappropriate for further analysis and handling.

However, the observed surface properties of encapsulates were different, depending on the bacterial species used for spray drying. As can be seen, encapsulates of *A. chroococcum* F14/2 show smoother surfaces (which are even more pronounced in the case of smaller particles) compared to the other two formulations. Namely, the smoother surface of *A. chroococcum* F14/2 encapsulates could be explained by the presence of exopolysaccharides produced during cell cultivation. According to literature data, *Azotobacter* species produce exopolysaccharides that belong to the group of alginates [37]. The presence of alginate and generally polysaccharides in the formulations for spray drying could provide more structurally stable particles [38]. As our results showed (Table 1), *A. chroococcum* F14/2 and *B. megaterium* 11/3 produced exopolysaccharides, consequently providing an additional carrier material and encapsulates with more desirable surface properties. In this context, strains should be tested for the presence of EPS or even grown under conditions that promote EPS production. The significant presence of the EPS could reduce the need to add commercial polysaccharides (such as alginates) in formulations for spray drying encapsulation, reducing process costs and providing better protection of the biofertilizer. The encapsulate size (Table 2) is generally in the ranges common for formulations based on maltodextrin as a carrier material, while the formation of poly-dispersed particles is also noticed, which is expected during spray drying [36].

Physical Properties of Encapsulates

The main physical properties of encapsulates are shown in Table 2. Hygroscopicity of samples was in the range of ~8–17 g H_2O/100 g, depending on encapsulated bacterial species.

The hygroscopicity of encapsulates obtained in this study was in accordance with the literature data for spray dried-maltodextrin/alginate-based encapsulates (11-15 g H_2O/100 g) [38]. Generally, the higher hygroscopicity is an obstacle to storage, manipulation, and potential application of encapsulates, especially for seed coating. Yonekura *et al*[38] showed that the samples with low moisture contents exhibited low hygroscopicity. From this, one could conclude that increased moisture content (and hygroscopicity) could lead to undesirable biochemical reactions at the cellular level and consequently to a reduction in cell number during storage. This may partly explain the lower cell count in the *Pseudomonas putida* P1 encapsulates after storage (i.e. the encapsulates with the highest moisture content and hygroscopicity). On the other hand, *Azotobacter chroococcum* F14/2 encapsulates exhibited much lower hygroscopicity compared to the other two bacterial encapsulates. As we showed above, the particles with encapsulated *Azotobacter* cells have smoother surfaces, which probably provide a better barrier for moisture. However, these results pointed out that to preserve the physical properties of encapsulates and to maintain high cell viability, proper packaging of encapsulates should be considered. Further improvement of the spray drying process could be aimed at optimizing the encapsulation parameters, especially in reducing the moisture content in the encapsulates. In general, the moisture content of spray-dried powders containing live cells should be below 4% for prolonged storage [38]. It could be achieved by increasing the drying temperature. However, higher drying temperatures can lead to thermal damage to the cells. In this regard, spray drying encapsulation of phosphate-solubilizing bacteria requires optimization of various process parame-

ters such as growth conditions, drying temperature, carrier composition, and storage conditions.

To evaluate the handling properties of encapsulates, bulk, and tapped density, the Carr index (CI) and Hausner ratio (HR) were measured or calculated (Table 2). The values for the Carr index (CI) and Hausner ratio (HR) were in ranges of 27–34% and 1.38–1.51, respectively. According to Jinapong *et al.* [24], the values for the CI below 15% indicate a very good flowability of powders, while with CI values above 45%, the flowability is considered very bad. Also, according to the same authors, cohesiveness expressed as the Hausner ratio could be classified as low (HR < 1.2), intermediate (HR = 1.2-1.4), and high (HR > 1.4). Spray-dried encapsulates of phosphate-solubilizing bacteria obtained in this study can be classified as powders with fair flowability and intermediate cohesiveness. Here also *A. chroococcum* F14/2 encapsulates showed better flowability and cohesiveness in comparison with *B. megaterium* 11/3, and *P. putida* P1 encapsulates. Hence, handling, storage, and general manipulation of *A. chroococcum* F14/2 encapsulates should be more convenient, especially for potential application on seeds and further encapsulate processing, such as agglomeration.

Chemical Properties of Encapsulates

FTIR spectroscopy was used to test the chemical stability of encapsulates and to identify changes in their chemical properties as a result of chemical interactions between constituents (Fig. 2).

Table 1. Plant growth-promoting (PGP) properties of *Azotobacter chroococcum* F14/2, *Bacillus megaterium* 11/3, and *Pseudomonas putida* P1 (the latest characterized within Karličić *et al.* [16]).

	Bacterial strain		
Plant Growth Promoting Characteristic	*Azotobacter chroococcum* F14/2	*Bacillus megaterium* 11/3	*Pseudomonas putida* P1
Phosphate solubilization index (SI)	1.94	1.11	2.70
Production of alkaline phosphatase	+	+	+
Production of acidic phosphatase	+	+	+
Production of naphthol-AS-BI-phosphohydrolase	+	weak	+
Production of exopolysaccharides (EPS)	+	+	-
Production of indole-acetic acid (IAA)	-	-	1.2 μg/ml
Production of siderophores	+	-	+

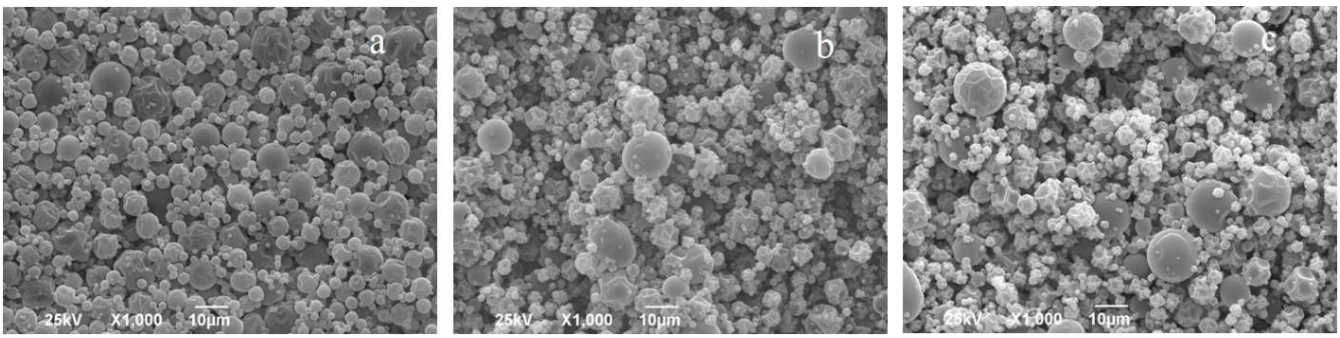

Fig. 1 SEM micrographs of Azotobacter chroococcum F14/2 (a), Bacillus megaterium 11/3 (b), and Pseudomonas putida P1 (c) spray-dried encapsulates.

Table 2. Physical properties of encapsulates.

Sample (encapsulates)	Mean particles size±s.d.* (μm)	Moisture content (%)	Hygroscopicity g H_2O/100 g of sample	Bulk density (g/mL)	Tapped density (g/mL)	CI (%)	HR
Azotobacter chroococcum F14/2	3.4±2.8^{a}	5.62±0.07^{a}	8.93±0.02^{a}	0.28 ± 0.00^{a}	0.39 ± 0.01^{a}	27.51 ± 1.86^{a}	1.38 ± 0.03^{a}
Bacillus megaterium 11/3	4.7±3.8^{b}	7.73±0.04^{b}	18.68±0.01^{b}	0.29 ± 0.01^{ab}	0.44 ± 0.01^{bc}	33.79 ± 1.75^{b}	1.51 ± 0.04^{b}
Pseudomonas putida P1	4.5±3.7^{b}	8.04±0.05^{c}	16.97±0.06^{c}	0.27 ± 0.01^{ac}	0.41 ± 0.01^{ad}	33.64 ± 1.05^{b}	1.51 ± 0.02^{b}

* s.d.-standard deviation

$^{a-d}$ The values in the same column superscripted with the same letter are not significantly different ($p \leq 0.05$)

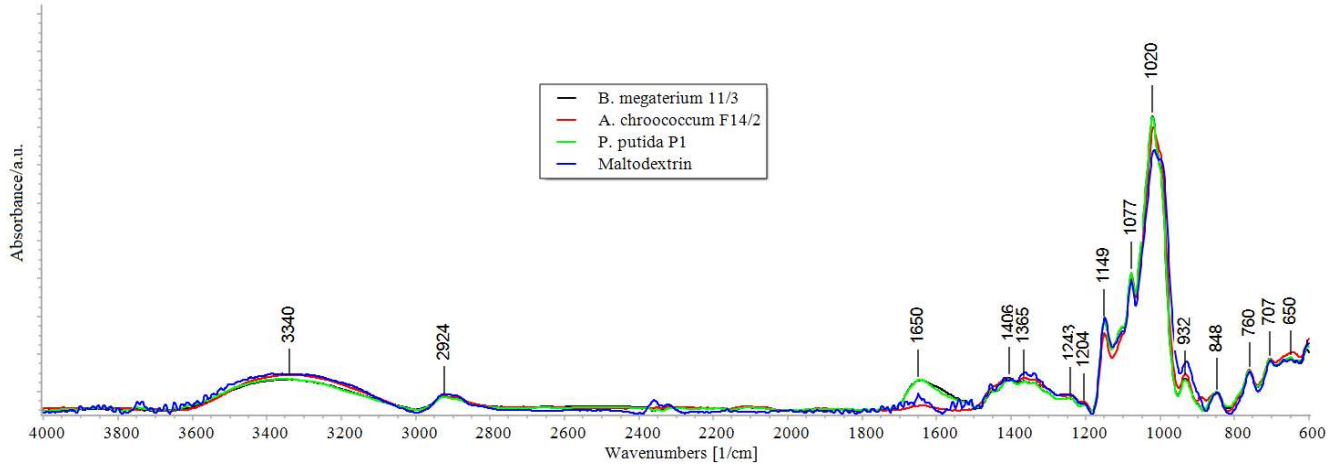

Fig. 2 FTIR spectra of encapsulates and maltodextrin.

The bands at around 3340 cm^{-1} are due to OH groups stretching vibrations and at ~ 2920 cm^{-1} is the band of C-H bonds. At around 1650 cm^{-1} are bands of water present in the particles, while at 1149 cm^{-1}, 1077 cm^{-1}, and 1020 cm^{-1} are bands identified as typical for carbohydrates, with low-intensity bands below 1000 cm^{-1} related to pyranose ring of sugars [39]. In the FTIR spectra of the encapsulates, the band positions associated with major encapsulate constituents, i.e., carbohydrates and primarily maltodextrin are very close, regardless of encapsulated bacterial strain. Moreover, the maltodextrin bands are dominant in all spectra of encapsulates, overlapping bands from growth media constituents and bacterial metabolic products, indicating sufficient cover-protection of cells. The similarities observed in the FTIR spectra suggest that the encapsulation procedure provides encapsulates without strong chemical interactions between constituents. Kalušević *et al.* [36] reported a similar absence of chemical interactions for spray-dried encapsulates of grape skin extract and maltodextrin. In this regard, the thermal and chemical stability of maltodextrin makes it preferable as a carrier material for spray drying encapsulation of complex liquid media and cells.

Storage Stability of Encapsulated Cells

Besides the simplified handling and application on plants, one of the most important benefits of dried microbial biofertilizers encapsulates is the possibility of their prolonged storage. Our results show that the cell viability of all tested strains was not significantly changed during encapsulation (Table 3).

However, storage significantly reduced the number of viable cells, making the applied encapsulation technique inadequate for *P. putida* P1 long storage. The number of viable *P. putida* P1 cells after two months of storage was below 3 log CFU/g, regardless of the storage temperature. On the contrary, *B. megaterium* 11/3 showed a favorable survival rate, while *A. chroococcum* F14/2 cell number declined, which was more pronounced when it was stored at room temperature.

The results for the cell number in encapsulates emphasize the importance of microbe properties, especially spores formation, for the survivability of spray-dried microbial biofertilizers. Encapsulates of *B. megaterium* 11/3 had a log CFU of 7.34 after encapsulation and 6.06 after two months of storage at room temperature. Stamenković Stojanović *et al.* [40] showed that a spore-forming *Bacillus subtilis* (after thermal shock procedure) and spray drying using maltodextrin as a carrier exhibited a very high survival rate of 8.45 log CFU/g even after storage at room temperature for one year. In this study, the lower cell number of all three encapsulated bacterial strains could be due to the lower initial cell number in the growth media. We used a rotary shaker for the production of bacterial biomass prior to encapsulation, which resulted in an overall lower cell number in the growth medium and consequently in the encapsulates compared to the literature [40]. Bioreactors with control of agitation and aeration could be used for increasing cell numbers aimed for encapsulation. On the other hand, *Azotobacter chroococcum* F14/2 encapsulates obtained in this study exhibited a relatively high number of cells compared to *Pseudomonas putida* P1. Since both species do not produce spores, and both belong to the group of Gram-negative bacteria, known as unstable as dry encapsulates [35], one of the explanations for a higher number of *Azotobacter* live cells in encapsulates could be the presence of exopolysaccharides. Namely, besides maltodextrin, exopolysaccharides may provide additional protection for cells. Polysaccharides have been used for the encapsulation of nitrogen-fixing microorganisms and effectively preserve relatively high numbers of live cells during the spray-drying process [35]. Since both *Azotobacter chroococcum* F14/2 and *Bacillus megaterium* 11/3 produce exopolysaccharides (Table 1), it could be expected that these macromolecules increase the possibility for cell survival at elevated temperatures and rapid dehydration through spray drying. On the other hand, the low number of *Pseudomonas putida* P1 cells in the encapsulates after storage could be explained by a higher moisture content of encapsulates compared to the other two bacterial species. High moisture content is the basis for more intense biochemical reactions on the cell level, which may cause increasing in the cell death rate in the case of *Pseudomonas putida* P1 encapsulates.

To overcome the problems related to the high moisture content of the encapsulates, and especially the decreasing of cell numbers during storage, an alternative encapsulation process could be considered. For example, the 'CLAM'

process (one-step, spray-dry cross-linked alginate micro-encapsulation process) that combines spray drying and gelling of carrier showed promising results in the preservation of nitrogen-fixing bacteria, enabling a high number of seed inoculants [35]. Such modification of spray drying encapsulation could be of interest for the preservation of non-spore-forming bacteria tested in this study, especially *Pseudomonas* strains, and potentially could provide biofertilizers with enhanced storage properties.

Encapsulates of the phosphate-solubilizing bacteria obtained in this study could be used in the direct seed covering. Additionally, since spray drying produced encapsulates with good physical properties, the agglomeration of the encapsulates could be applied. Agglomerated encapsulates are a more convenient form for the usual methods of fertilizer application in the fields. Also, the plant growth-promoting traits of the one bacterial strain may not be sufficient to cover the broader demands required from biofertilizers. Hence, future work could be oriented toward the creation of mixtures of various biofertilizers and the study of their effects on plant growth.

Table 3. Survival of *Bacillus megaterium* 11/3, *Azotobacter chroococcum* F14/2, and *Pseudomonas putida* P1 during and after encapsulation.

	Number of bacteria in liquid culture before encapsulation	Number of bacteria after encapsulation	Number of bacteria in encapsulates after 2 months of storage at 4 °C	Number of bacteria in encapsulate after 2 months of storage at room temperature
Bacterial strain	log CFU/mL		log CFU/g	
Bacillus megaterium 11/3	7.34±0.05	7.36±0.05	6.32±0.07	6.06±0.06
Azotobacter chroococcum F14/2	7.06±0.13	7.43±0.04	5.69±0.39	4.47±0.33
Pseudomonas putida P1	8.69±0.06	8.65±0.02	< 3	< 3

CONCLUSIONS

Three bacterial strains *B. megaterium* 11/3, *A. chroococcum* F14/2, and *P. putida* P1, characterized as beneficial PGPB, were encapsulated using spray drying technique and maltodextrin as carrier material. Variations in the properties of the obtained encapsulates were observed, and the strains showed different survival rates. The cell count after storage was highest for *B. megaterium* 11/3 (6.32 log CFU/g), followed by *A. chroococcum* F14/2 (5.69 log CFU/g) and *P. putida* P1 (<3 log CFU/g). Storage at 4 °C had a more positive effect on the preservation of the encapsulated cells compared to storage at room temperature. *A. chroococcum* F14/2 and *B. megaterium* 11/3 produced the exopolysaccharides, which could be a promising feature for the preservation of the cells and successful root colonization.

Encapsulates obtained by spray drying showed promising physical properties that open up possibilities for the application of encapsulated PGPB strains directly on the seeds i.e. by direct seed inoculation. The direct application of the encapsulated PGPB strains on seeds would overcome the relatively low cell number after storage and could allow successful root colonization and establishment of plant-microbe interaction in the early stages of plant growth.

Here should be pointed out some observed limitations of the encapsulated PGPB strains as well as the recommendations for further development. The low cell number of the encapsulates after storage could be the result of an initial lower cell count in the medium for cell cultivation. This problem could be overcome by cell cultivation in the bioreactors with controlled aeration and agitation. Optimization of growth conditions could increase the production of exopolysaccharides and other PGP traits, enabling more successful root colonization. Also, the cells could be more effectively preserved by optimization of the spray drying procedure and reduction of moisture content in encapsulates as well as by the introduction of new innovative spray drying encapsulation procedures and new carrier materials.

Acknowledgments

This work was supported by the Ministry of Science, Technological Development and Innovations, Republic of Serbia (Contract No. 451-03-137/2025-03/200116), and The Innovation Fund of the Republic of Serbia (Contract No. 244/2).

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NAUČNI RAD

SPREJ SUŠENJE FOSFAT-SOLUBILIZIRAJUĆIH BAKTERIJA ZA PROIZVODNJU NOVIH BIOFERTILIZATORA

Fosfat-solubilizirajuće bakterije predstavljaju održivo rešenje za suočavnje sa nedostatkom fosfora u poljoprivrednom zemljištu. Međutim, uspeh njihove primene u velikoj meri zavisi od velikog broja faktora životne sredine i potreban je novi pristup za dobijanje bioformulacija. Cilj ovog istraživanja je da se proceni pogodnost metode sprej sušenja za inkapsulaciju fosfat-solubilizirajućih bakterija: Azotobacter chroococcum F14/2, Bacillus megaterium 11/3 i Pseudomonas putida P1. Ova tri soja su okarakterisana, uzgajana pod optimalnim uslovima i inkapsulisana kao ceo sistem koji sadrži podlogu i ćelije korišćenjem metode sprej sušenja i maltodekstrina kao nosača. Opisanim postupkom dobijeni su inkapsulati sa prosečnom veličinom čestica ispod 5 µm, sadržajem vlage ispod 10% i zadovoljavajućim svojstvima za praškaste uzorke. Ćelijska vijabilnost inkapsulata (nakon čuvanja) bila je sledećim redom: Bacillus megaterium 11/3 > Azotobacter chroococcum F14/2 > Pseudomonas putida P1. Dodatna zaštita tokom sprej sušenja najverovatnije je postignuta prisustvom mikrobnih egzopolisaharida, što otvara mogućnosti za dalju optimizaciju postupaka inkapsulacije.

Ključne reči: Azotobacter, Pseudomonas, Bacillus, inkapsulacija, zemljište.