JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

STRUVITE PRODUCTION BY *PSEUDOMONAS SYRINGAE PV PHASEOLICOLA*

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doi: 10.15414/jmbfs.2018.8.2.812-814

ARTICLE INFO

Received 2. 5. 2018
Revised 21. 8. 2018
Accepted 7. 9. 2018
Published 1. 10. 2018

Regular article

OPEN ACCESS

ABSTRACT

Struvite is a biogenic mineral of low solubility. For many years, it has been considered as a fertilizer, but due to the additional cost of manufacture, its use has been limited to only high-value crops. Struvite production by some bacterial strains have been previously reported. However, this is the first study that reports struvite production in *Pseudomonas syringae pv phaseolicola* strain. Crystal formation was observed within four days of incubation on solid media. Microscopy, X-ray diffraction, Scanning electron microscope and Energy dispersive x-ray spectroscopy analysis confirmed the crystal structure as Struvite. Moreover, this study suggests a possible biotechnological use of *P. syringae pv phaseolicola* for struvite production for agricultural applications.

Keywords: Struvite, *Pseudomonas syringae pv phaseolicola*, Crystallization

INTRODUCTION

Crystal formation by bacteria is a widely studied phenomenon (**Robinson, 1889; Han *et al.*, 2015**). Reports during the past decades include the formation of struvite in different bacterial genus such as *Bacillus, Staphylococcus, and Myxococcus*, among others (**Beavon and Heatley, 1962; Nelson *et al.*, 1991; Rivadeneyra *et al.*, 1992**). Some of these structures are formed even under impaired physiological conditions. For instance, the formation of struvite by *Proteus mirabilis* in urinary tract infections (**Prywer *et al.*, 2012**).
Struvite [$NH_4MgPO_46H_2O$] is a hydrous magnesium-ammonium phosphate, relatively abundant in soils and lakes; it is a biogenic material that presents a solubility of 0.2 g/L in water (**Barak *et al.*, 2006**). This crystal structure is rare in nature; however, it has been reported in specific environments that involve organic matter decomposition (**Sánchez-Roman *et al.*, 2007**). Struvite can be found frequently in wastewater treatment lines and under anaerobic conditions in parts of the water treatment process (**Ariyanto *et al.*, 2014**). The struvite precipitation has lately been regarded as a plausible source for phosphate recovery, with interesting advantages over conventional methods (**Kataki *et al.*, 2016**). The *Pseudomonas* genus comprises one of the most important ecological groups in nature, which includes species with diverse characteristics. Within this group, plant pathogens such as *P. syringae*, has been extensively studied for its role in plant diseases and quorum sensing (**De Smet *et al.*, 2017**). *P. syringae pv phaseolica* is a specific strain that is related with the onset of halo blight and the production of the phytotoxin "phaseolotoxin" in *Phaseolus vulgaris* (**Aguilera *et al.*, 2017; De Ita *et al.*, 1998**). Also, its particular role in ice nucleation has also been described (**Morris *et al.*, 2013**). Some bacterial strains within the *Pseudomonas* genus have been associated with struvite precipitation (**Rivadeneyra *et al.*, 1992; Da Silva *et al.*, 2000**); however, to the best of our knowledge, the report of *P. syringae pv phaseolicola* as a struvite producer has not yet been reported. The main objective of the present study was to confirm the crystal formation capacity of *P. syringae pv phaseolicola*.

MATERIAL AND METHODS

Bacterial culture development

A bacterial strain of *Pseudomonas syringae pv phaseolicola* (NPS3121-120905) kindly provided by Dr. Ariel Alvarez-Morales (CINVESTAV, Campus Guanajuato) was cultivated in BD-King solid media (Sigma-Aldrich, Darmstadt, Germany) according to Lelliott and Stead, (1987) and placed in an incubator (IKA KS4000i, Staufen, Germany) at 28 °C and monitored every 4 h until crystal formation was noticed. Bacterial culture growth was obtained by solid BD-King medium, and crystal formation was analyzed every 4 h under a bright field microscope (Olympus, CX31 Tokyo, Japan).

Solubility test

Solubility test was conducted using distilled water, HCl and glacial acetic acid. In addition, the effect of temperature (30, 60, 70, 80 °C) in the crystal solubility was evaluated. Finally, the crystals were properly washed in hot distilled water (70 °C) to allow dissolution of the medium, and placed on a filter paper in a laminar flow cabinet for further analysis.

X-ray diffraction (XRD) analysis

The crystals structure produced by *P. syringae pv phaseolicola* were ground to powder in an agate mortar and analyzed by X-ray diffraction (XRD) methods. Targeted particles were selected and submitted to X-ray analysis to determine their mineralogical composition, which was assessed using a Rigaku MiniFlex X-ray diffractometer equipment with a scintillator detector. Data were collected for a 0.4 s integration time in 0.02° 2 steps at 40 kV and 40 mA in a 2 interval between 5–80°.

Scanning Electron Microscopy (SEM) Analysis

Scanning Electron Microscopy (SEM) images were obtained using a Scanning Electro Microscope (JEOL JMS-6060 LV with an Oxford spectrometer Inca X-Sight and a silicon doped with lithium WAFER detector) and various magnifications were tested: 37 x, 50 x, 100 x, 500 x, 1,000 x, 2,500 x, 5,000 x, 10,000 x, using gold as coating material.

Energy Dispersive X-Ray Spectroscopy (EDS) Analysis

For the Energy Dispersive X-Ray Spectroscopy (EDS) the amplification was 2,000x with no coating material and an acquisition time of 50s. Both EDS and SEM were analyzed in different sample zones to give more accurate results. The combined analysis of SEM-EDS and XRD confirmed the crystal structures as struvite.

RESULTS AND DISCUSSION

Struvite formation depends on the bacterial strain and culture conditions (**Rivadeneyra *et al.*, 1992**). Thus, we analyzed the effect of BD King culture media in struvite formation in by *P. syringae pv phaseolicola*. Crystal formation was observed 4 days after inoculation in solid growth media (Fig. 1a, and 1b).

Crystal formation was observed in a bright field microscope (Fig. 1). They were a colorless, transparent vitreous luster and hexagonal structure with 3 or more symmetric perpendicular axes.

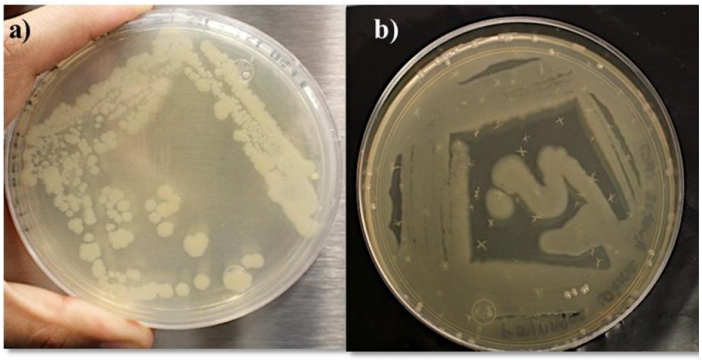

Figure 1 Effect of culture media on crystal formation in *P. syringae pv phaseolicola*, a) Bacterial growth before the crystal formation b) bacterial culture with crystal formation.

Figure 2 Microscopic observation of crystal formed by *P. syringae pv phaseolicola*.

Struvite precipitation can take place due to the adsorption of Mg^{2+} and $PO4^{3-}$ ions, along with NH_4^+ liberation (**Rivadeneyra *et al.*, 1992**). In our experiment, phosphate in the BD King solid medium (**King *et al.*, 1954**) came in the form of K_2HPO_4, and the magnesium was obtained in the form of $MgSO_4 \cdot 7H_2O$; thus, our bacterial strain could be using the mechanism for struvite precipitation reported by **Rivadeneyra *et al.*, (1992).** In fact, the chemical requirement for these ions in our study resembles the ones reported in the B-41 medium that is proposed as the most suitable culture for struvite precipitation in few *Pseudomonas* spp strains (**Rivadeneyra *et al.*, 1992**). Furthermore, the overall composition of BD King medium and B-41 medium are very similar regarding both $KHPO_4$ and $MgSO_4$ concentrations (0.15 % for BD King Medium and 0.2 % for B-41 medium). Solubility test results revealed no solubility of the crystal structure formed, under any of the conditions evaluated when water was used as a solvent. However, when HCl was used as a solvent, crystals disintegrated completely after 5 min of exposure. While with the use of glacial acetic acid, crystal samples turned into black and dark in color. Crystal formation depends on a set of physicochemical parameters, namely: pH, mixing, temperature, crystal size, supersaturation degree and presence of impurities. In these parameters, supersaturation and pH are considered the most important for struvite crystallization. Crystal precipitation can be controlled by adjusting the pH in culture conditions (**Ariyanto *et al.*, 2014**). Different authors have stated that pH controls the ion activity of the ionic forms of phosphorus and ammonium, especially in NH_4^+ and PO_4^{3-} (**Matynia *et al.*, 2006; Ariyanto *et al.*, 2014**). In our study, both pH and temperature were constant during culture conditions; as for the solubility analysis, the crystal structures were dissolved completely in the presence of HCl, and struvite is reported to be soluble at low pH (**Matynia *et al.*, 2006**).

Crystal structure identification was assessed using SEM and X-ray diffraction analysis. As a result, the crystal structures formed in BD King solid media were identified as struvite according to the reference pattern (Fig. 3). This struvite conformation is also reported by **Rivadeneyra *et al.*, (2014**) when analyzing crystal production by different bacterial genus in biofilm formation.

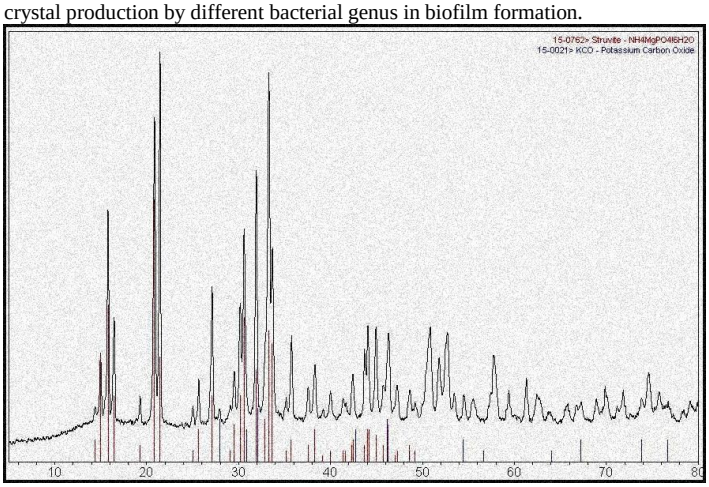

Figure 3 X –Ray powder diffraction with an angular range of 5 to 80º (in 2-theta), with a velocity of 2º per minute and a sampling every 0.02 seconds for the crystals formed by *Pseudomonas syringae pv phaseicola* that indicated Struvite component.

According to the results obtained from the SEM analysis, the main components found in all the samples analyzed were: Carbon (32.2 %), Oxygen (52.4 %), Mg (7.47 %), P (6.9 %) (mean weight values expressed as a percentage). The main structure of the samples analyzed is shown in Figure 4. Different sites of analysis were chosen for the struvite samples which were analyzed at different magnifications (Figure 4 a to d), and the composition of analyzed crystals has been shown in **Table 1**.

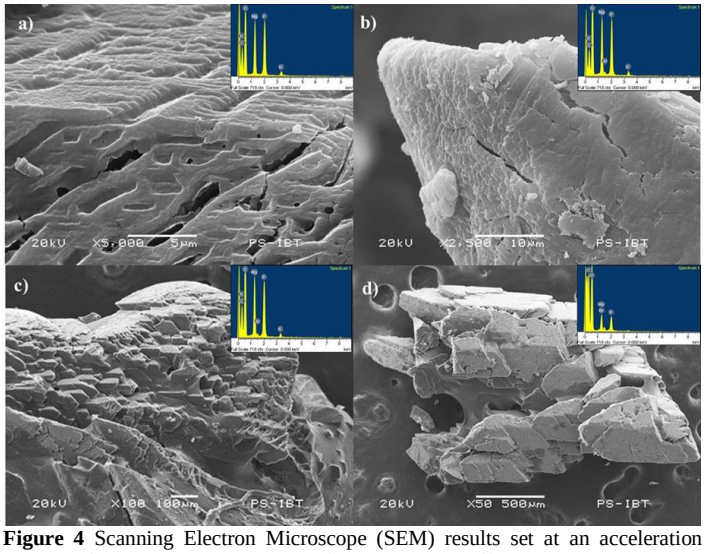

Figure 4 Scanning Electron Microscope (SEM) results set at an acceleration voltage of 20kV with a spot size of 50 a) to d). Different sample sites were chosen for the SEM analysis. In the upper right box, the general composition diagram can be observed.

Pseudomonas syringae pv phaseolicola is related with plant pathology and also with ice formation (ice nucleation) in the water cycle, in fact, some proteins related with this process are already reported (**Morris *et al.*, 2013**). Even though there are few reports on *Pseudomonas* genus and struvite production none of them demonstrate clear evidence for *P. syringae pv phaseolicola* and struvite precipitation; therefore, this constitutes the first report.

Struvite crystals contain 39 % phosphate, 10 % magnesium and 7 % ammonium (**Gell *et al.*, 2011**). Hence, due to its high phosphate content, several reports highlight the feasibility of using the struvite precipitation process as a way of recapturing phosphate from different sources (**Kataki *et al.*, 2016**). This is interesting since global demands for phosphate are increasing and phosphate deposits are diminishing worldwide (**Kataki *et al.*, 2016**). Therefore, finding bacterial strains that are able of phosphate precipitation in the form of struvite crystals like the one we describe here, opens the possibility of biotechnological applications that could help in the recapturing of phosphate in the environment. Furthermore, several reports indicate the relationship between phosphate solubilization and the *Pseudomonas* genus (**Oteino *et al.*, 2015**).

However, a clear mechanism for phosphate recovery or a possible re-introduction or fixation of phosphate in soils due to these microorganisms and their struvite production has not been fully elucidated to date. Therefore, we propose that this might also be a promising use for *P. syringae pv phaseolicola*; however, the exact mechanism by which this occurs remains to be fully investigated.

Table 1 SEM analysis results for struvite crystal structures

	Element	App conc.	Intensity corrn	Weight %	Weight sigma %	Atomic %
1	C, K,	47.03	0.4987	33.33	1.58	42.75
	O, K	96.39	0.6891	49.45	1.22	47.62
	Mg, K	18.01	0.7542	8.44	0.25	5.35
	P, K,	28.10	1.2454	7.98	0.24	3.97
	K, K	2.29	1.0132	0.8	0.05	0.32
	Totals	100				
2	C, K,	47.30	0.4973	31.03	1.49	40.02
	O, K	116.90	0.7331	52.04	1.17	50.39
	Mg, K	19.25	0.7455	8.43	0.23	5.37
	Al, K	0.71	0.7396	0.31	0.05	0.18
	P, K,	28.86	1.2367	7.61	0.21	3.81
	K, K	1.81	1.0134	0.58	0.05	0.23
	Totals	100				
3	C, K	35.52	0.4642	28.14	1.70	36.94
	O, K	110.06	0.7628	53.09	1.30	52.30
	Mg, K,	18.46	0.7458	9.11	0.27	5.90
	Al, K	0.65	0.7328	0.32	0.06	0.19
	P, K	28.61	1.2318	8.55	0.25	4.35
	K, K	2.17	1.0108	0.79	0.05	0.32
	Totals	100				
4	C, K	63.86	0.7427	35.95	0.83	44.04
	O, K	100.95	0.7395	57.10	0.79	52.51
	Mg, K	5.52	0.7049	3.27	0.12	1.98
	P, K	8.15	1.2476	2.73	0.10	1.30
	Br, L	1.57	0.6941	0.95	0.12	0.17
	Totals	100				

Table1 Comparative results of Scanning Electron Microscope observations for all the samples taken. Each column shows the concentration, weight and atomic proportion of the components present in struvite crystals

CONCLUSION

Struvite formation has extensively been reported in different microorganisms. Here we report struvite precipitation induced by *P. syringae pv phaseolica* strain in laboratory conditions. The crystal structure was validated through SEM and XRD analysis, and that confirms that these crystals belong to struvite mineral. Struvite has been considered as an eco-friendly form for Phosphate recovery from different disposal sources in the environment, namely wastewaters. Furthermore, it is now commercially produced. Therefore, we propose that *P. syringae pv phaseolicola* can be used to produce struvite through biotechnological approaches.

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