



Short Communication

Soil extract calcium phosphate media for screening of phosphate-solubilizing bacteria

Manoharan Melvin Joe,^{a, d, *} Shalini Deivaraj,^a Abitha Benson,^{b, d} Allen John Henry,^a G. Narendrakumar^c



^a Department of Microbiology, School of Life Sciences, VELS University, Chennai, 600117, India

^b School of Biotechnology, School of Life Sciences, VELS University, Chennai, 600117, India

^c Department of Biotechnology, Faculty of Bio and Chemical Engineering, Sathyabama Institute of Science and Technology, 600119, India

^d S. Thangapazham Agricultural College, Vasudevanallur, Tirunelveli, 627758, India

ARTICLE INFO

Article history:

Received 26 November 2016

Accepted 25 October 2017

Available online 27 September 2018

Keywords:

Aluminum phosphate *Bacillus* spp.

Calcium phosphate

Rock phosphate

Phosphate solubilization

ABSTRACT

Media development for screening bacteria capable of solubilizing inorganic complex forms of phosphorous to a soluble form is a major concern in agricultural research. This study formulated an effective, alternative solid and liquid media to the currently available Pikovskaya (PVK) medium, which relies on the use of calcium phosphate and numerous inorganic components for the screening of phosphate-solubilizing bacteria. In this present study, soil extract calcium phosphate (SECP) broth was formulated with simple medium requirements. SECP broth performed better than PVK broth as evidenced by its ability to support a higher bacterial population, higher reduction in broth pH and higher organic acid production and ultimately a better phosphate solubilization, based on the zone of clearance using bromocresol green or congo red in addition to bromothymol blue. Apart from calcium phosphate, rock phosphate and aluminum phosphate were also used as phosphate sources for the screening of different phosphate-solubilizing strains. This solid SECP medium could be used effectively with other phosphorous sources such as rock phosphate and aluminum phosphate. A high correlation between solid-based assay and broth-based assay for screening of phosphate-solubilizing bacteria was observed in the study.

Copyright © 2018, Kasetart University. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Phosphorus is one of the major essential macronutrients for growth and is involved in key plant metabolic processes such as energy transfer and photosynthesis (Plaxton and Tran, 2011). However, since the concentration of soluble phosphorous in soil is usually very low (400–1260 mg/kg; Fernández et al., 2014), it is applied to soil in the form of chemical fertilizers such as tri-superphosphate, mono-ammonium phosphate, di-ammonium phosphate and ammonium polyphosphate.

The major problem with the application of chemical fertilizer is that a large part of the soluble form of inorganic phosphate applied to the soil is rapidly immobilized and becomes unavailable to plants (Goldstein, 1986). Interestingly, these inorganic forms of phosphorous are solubilized by a group of heterotrophic microorganisms through organic acid production that dissolves

the phosphates through H^+ excretion, organic acid production and acid phosphatase biosynthesis (Arcand and Schneider, 2006).

Several media have been formulated to screen the phosphate-solubilizing bacteria (PSB) based on the visual detection of clear zones in media containing insoluble mineral phosphates. Pikovskaya (1948) was the first person to develop a medium for the detection of PSB using calcium phosphate and dextrose based on the visual detection of clear zones. An improved Pikovskaya (PVK) solid medium containing bromophenol blue, which produced yellow-colored halos around the colonies due to organic acid production, was developed by Gupta et al. (1994). However, no correlation of results between plate halo detection and liquid-culture based phosphate solubilization was found (Das, 1963).

Nautiyal (1999) developed National Botanical Research Institute Phosphate (NBRIP) liquid medium comprising glucose 10.0 g/L, tricalcium phosphate 5.0 g/L, magnesium chloride hexahydrate 5.0 g/L, magnesium sulphate heptahydrate 0.25 g/L, potassium chloride 0.2 g/L, ammonium sulphate 0.1 g/L in 1000 mL of distilled water, and this medium is widely used for the estimation of phosphate solubilization.

* Corresponding author. Department of Microbiology, School of Life Sciences, VELS University, Chennai, 600117, India.

E-mail addresses: micromelvin@gmail.com, melvin.sls@velsuniv.cin (M.M. Joe).

Later, [Bashan et al. \(2013\)](#) reported tri-calcium phosphate as an inappropriate universal selection factor for isolating and testing phosphate-solubilizing bacteria. Furthermore, these authors recommended the use of rock phosphate for alkaline soils and iron phosphate and aluminum phosphate compounds for acidic soils.

The current study reports the development of a new and simple SECP solid and liquid media for the isolation and screening of phosphate-solubilizing bacterial strains.

Materials and methods

Culture and growth conditions

Bacillus sp. PVMX4 was isolated from the medicinal plant *Phyllanthus amarus* ([Joe et al., 2016](#)) and the standard strain *B. megaterium* MTCC2444 was obtained from the Institute of Microbial Technology, Chandigarh, India. These strains were incubated on nutrient agar slants at 30 °C with the monthly subculture. Growth-promoting strains used in this study were *Achromobacter xylosoxidans* AUM54, *Acinetobacter* sp. ACMS25, *Bacillus subtilis* MUV449, *Pseudomonas fluorescens* PF25, *Pseudomonas aeruginosa* AUF24 and *Pseudomonas* sp. AU6 and were obtained from the Department of Microbiology, VELS University, Chennai, India. These strains were maintained as glycerol stocks at -20 °C. The strains were thawed at room temperature prior to the experiment. For experimental purposes, the inoculum was harvested from nutrient broth during log phase by centrifugation at 5000 × g for 10 min at 30 °C. The cells were harvested

and washed thrice with equal volumes of 10 mM phosphate buffered saline (PBS) and the cells were adjusted to a final concentration of 9 log colony forming units per millimeter (cfu/mL) by measuring the optical density at 600 nm (OD₆₀₀) to get a value of 0.9.

Formulation of SECP broth

The SECP broth contained the following components: dextrose 5.0 g/L, Ca₃(PO₄)₂ 5.0 g/L, KH₂PO₄ 0.0584 g/L, K₂HPO₄ 0.1547 g/L and 200 mL of soil extract. The final volume of the medium was made to 1 L, and the pH was adjusted to 7.0. The available nitrogen, phosphorous and potash contents and the organic carbon of the soil used in the experimental study was 106.4 mg/kg, 8.6 mg/kg, 159.6 mg/kg and 0.32%, respectively. The soil extract was prepared as per the protocols of [Provasoli et al. \(1957\)](#), which included dissolving 200 g of soil in 1000 mL of distilled water, followed by autoclaving at 105 °C (twice followed by cooling). The contents were cooled and filtered using Whatman No 44 filter paper. Compared to the PVK broth, apart from the dextrose and buffering salts, the SECP broth eliminated the need for the addition of other chemicals.

Growth and organic acid production

The bacterial growth, culture broth pH, organic acid production and phosphate solubilization were studied in SECP broth inoculated with 8 log cfu/mL of bacterial culture and incubated at 30 °C, 200 rpm for a period of 96 h, with observations recorded for the

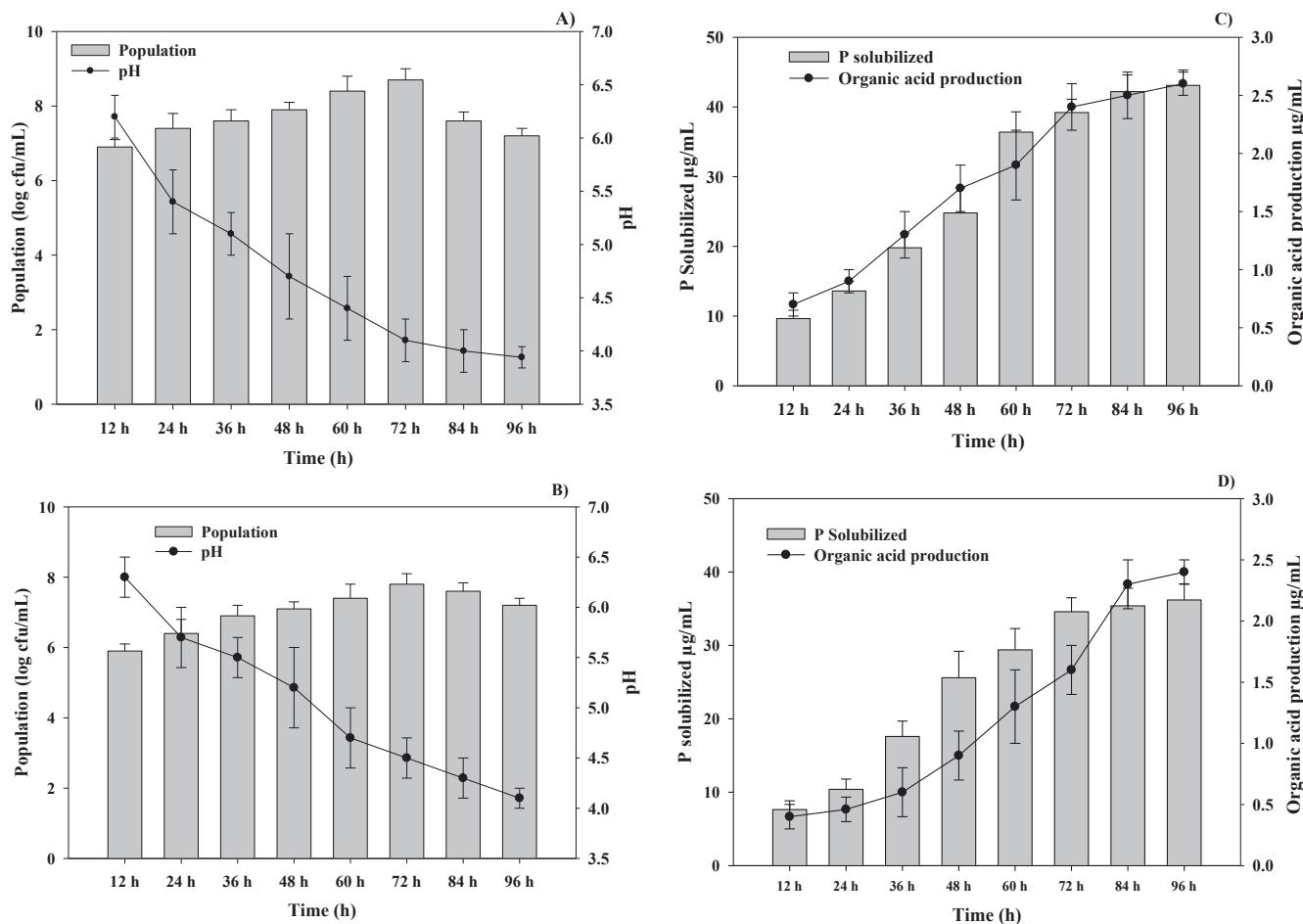


Fig. 1. Changes in growth pattern expressed as a population in cfu/mL and culture pH of (A) *Bacillus* sp. PVMX4; (B) *B. megaterium* 2444 in soil extract calcium phosphate broth. Organic acid (2-keto gluconic acid) and phosphate solubilization of (C) *Bacillus* sp. PVMX4; (D) *B. megaterium* 2444. Values are means of 3 replications \pm SD.

above-mentioned parameters every 12 h. Bacterial growth at different time periods was estimated from samples drawn using serial dilution and plating in nutrient agar plates incubated at 28 ± 2 °C for 72 h. Changes in pH were measured from the samples drawn at different time intervals using a pH meter equipped with a glass electrode (LI120, Elico, Hyderabad, India). For high-performance liquid chromatography (HPLC) analysis to determine organic acid 2-ketogluconic acid, the supernatant was filtered using a Millipore filter and concentrated using a rotary vacuum evaporator. The filtrate (20 μ L) was subjected to HPLC with a C18 column (1.5 mm \times 250 mm). The operating conditions consisted of 0.1% (volume per volume) H_3PO_4 as the mobile phase, a constant flow rate of 0.5 ml min^{-1} and retention time of the signal was recorded at a wavelength of 210 nm. The organic acid (2-ketogluconic acid) content was quantitatively determined after comparing peak heights of chromatograms with the standards. For determination of the soluble phosphate, 50 mL of the culture supernatant was centrifuged at 10,000 $\times g$ for 10 min to remove the bacterial cells. This supernatant was then filtered using Whatman No 44 filter paper. To 40 mL of the supernatant mentioned above, 8 mL of reagent mixture (comprising sulfuric acid, ammonium molybdate, ascorbic acid solution and potassium antimonyl tartrate solution) was added, and the amount of soluble phosphorous was determined spectrophotometrically at 882 nm as described by Murphy and Riley (1962).

Halo zone detection for phosphate solubilization

For halo zone detection for phosphate solubilization, 1.5% agar was added to the SECP solid medium, with the PVK solid medium (Pikovskaya, 1948) used for comparative purposes. Phosphate solubilization was observed based on the clear zone using 100 μ L of bromothymol blue (0.4%), bromocresol green (0.04%) or congo red (0.5%). For comparative plate assay, different phosphate-solubilizing plant growth-promoting rhizobacteria strains mentioned earlier were point inoculated (5 μ L) using a micropipette onto SECP agar as mentioned earlier before being

supplemented with calcium phosphate/rock phosphate or aluminum phosphate for the screening. For point inoculation, 24 h grown bacterial cultures were pelleted by centrifuging at 5000 $\times g$, and the pellets were dissolved in PBS with necessary adjustments made to attain an inoculation load of 8 log cfu/mL. The plates were incubated at 28 °C, for 14 d. Halo zone size was determined by subtracting the total zone diameter from the colony diameter as described earlier (Nautiyal, 1999). The Pearson correlation coefficient (<http://www.socscistatistics.com/tests/pearson/>) was used for the measurement of linear association between two variables (that is plate-based assay and broth-based assays).

Results and discussion

Growth and organic acid production

In this study, when both the phosphate-solubilizing *Bacillus* strains were grown in SECP broth incubated at 30 °C, 200 rpm, the highest bacterial population of 8.7 log cfu/mL, culture broth pH of 3.8, 2-keto gluconic acid content of 2.5 μ g/mL and phosphate solubilization of 37 μ g/mL were observed in *Bacillus* sp. PVMX4 at 72 h (Fig. 1A–C). The highest bacterial growth of 8.2 log cfu/mL, culture broth pH of 4.2, 2-keto gluconic acid content of 2.7 μ g/mL and phosphate solubilization of 32.7 μ g/mL was observed in *B. megaterium* MTCC2444 at 72 h (Fig. 1B and D). These changes in bacterial growth and the reduction in culture broth pH, 2-keto gluconic acid content and phosphate solubilization in this study were in line with Rodriguez et al. (2004), who observed changes in the broth culture pH and gluconic acid production as the major factors responsible for the release of soluble phosphate by a mutant of *Azospirillum* sp. It was observed that SECP broth performed better than the PVK broth for the studied parameters such as bacterial growth, culture pH, organic acid production and phosphate solubilization (Table 1). Scervino et al. (2011) reported that the medium pH, carbon, and nitrogen sources played a major role in

Table 1

Comparison of the bacterial population, cultural pH, organic acid production and P solubilization of *Bacillus* sp. PVMX4 and *Bacillus megaterium* MTCC2444 in soil extract calcium phosphate (SECP) medium with Pikovskaya (PVK) medium.

Parameter	<i>Bacillus</i> sp. PVMX4		<i>B. megaterium</i> MTCC2444	
	SECP medium	PVK medium	SECP medium	PVK medium
Population (log cfu/mL)	8.7 \pm 0.3 ^a	7.8 \pm 0.3 ^{a,b}	7.8 \pm 0.3 ^{a,b}	7.1 \pm 0.2 ^b
Cultural pH	3.9 \pm 0.3 ^{a,b}	3.4 \pm 0.2 ^b	4.1 \pm 0.1 ^a	4.5 \pm 0.2 ^a
Organic acid production (μ g/mL)	2.6 \pm 0.1 ^a	2.0 \pm 0.1 ^b	2.4 \pm 0.2 ^a	1.9 \pm 0.2 ^b
P solubilization (μ g/mL)	43.1 \pm 2.2 ^a	32.1 \pm 2.1 ^{b,c}	36.2 \pm 2.4 ^b	29.2 \pm 2.4 ^c

Values are means of 6 replications \pm SD. Different lowercase, superscript letters after values indicate significant difference at *P* value of 0.05, according to Duncan's mean range test.

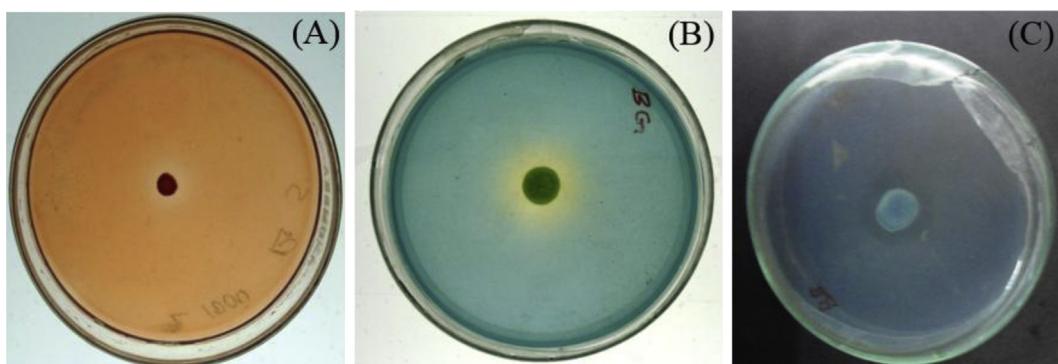


Fig. 2. Clear zone in soil extract calcium phosphate medium supplemented with: (A) Congo red; (B) Bromocresol green; (C) Bromothymol blue with the plates incubated at 28 ± 2 °C, for an incubation period of 7 days. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2

Comparison of soil extract calcium phosphate amended with calcium phosphate, rock phosphate, and aluminum phosphate in agar and broth assay using different plant growth-promoting rhizobacteria strains.

Bacteria	Zone (mm)			P solubilization (µg/mL)		
	CP	RP	AP	CP	RP	AP
<i>A. xylosoxidans</i> AUM54	4.2 ± 0.2 ^b	4.2 ± 0.3 ^a	3.5 ± 2.4 ^a	34.2 ± 2.1 ^a	20.2 ± 3.2 ^a	16.8 ± 3.1 ^b
<i>Acinetobacter</i> sp. ACMS25	4.6 ± 0.7 ^b	3.4 ± 0.6 ^a	4.6 ± 0.7 ^a	27.8 ± 2.4 ^b	22.6 ± 2.7 ^a	26.4 ± 3.1 ^a
<i>B. megaterium</i> MTCC2446	6.7 ± 1.1 ^a	2.4 ± 0.6 ^b	—	36.2 ± 3.1 ^a	24.4 ± 3.1 ^a	—
<i>Bacillus</i> sp. PVMX4	6.2 ± 0.7 ^a	3.5 ± 1.4 ^a	—	33.2 ± 3.1 ^a	15.4 ± 4.2 ^b	—
<i>B. subtilis</i> MUV449	7.4 ± 0.7 ^a	—	—	36.4 ± 2.4 ^a	6.6 ± 2.7 ^c	—
<i>P. fluorescens</i> PF25	7.5 ± 1.1 ^a	—	—	28.6 ± 2.3 ^b	—	—
<i>P. aeruginosa</i> AUF24	—	—	—	12.1 ± 2.5 ^d	—	—
<i>Pseudomonas</i> sp. AU6	2.1 ± 1.5 ^c	—	—	19.6 ± 2.1 ^c	—	—

CP, calcium phosphate; RP, rock phosphate; AP, aluminum phosphate.

Values are means of 6 replications ± SD. Different lowercase, superscript letters after values indicate significant difference at P value of 0.05, according to Duncan's mean range test.

Table 3

Pearson correlation coefficient between agar and broth assay for selected phosphate-solubilizing bacteria.

Medium	R value	R ² value
SE-CP	0.8704	0.7576
SE-RP	0.8841	0.7816
SE-AP	0.9954	0.9908

CP, calcium phosphate; RP, rock phosphate; AP, aluminum phosphate.

phosphate solubilization efficiency of the fungal strain *Penicillium purpurogenum* through organic acid production.

Detection by halo zone of clearance using indicator dyes bromocresol green or Congo red and different phosphate sources for phosphate solubilization in SECP solid medium

Bromocresol green and congo red were tried as indicator dyes instead of the widely used bromothymol blue. No marked improvement could be observed with congo red dye when used in lieu of bromothymol blue (Fig. 2A). However, bromocresol green gave a clear visible zone with a yellowish background and the visibility of this dye was better compared to the bromothymol blue used in the medium (Fig. 2B, C). When calcium phosphate was replaced with other sources of phosphate such as rock phosphate and aluminum phosphate, phosphate solubilization was observed in only some of the bacterial strains (Table 2). The reason for this can be explained based on the report of Bashan et al. (2013) that aluminum phosphate and several forms of calcium phosphate such as rock phosphate are less soluble than tricalcium phosphate in water.

Correlation studies between plate- and broth assay

A positive correlation between the plate-based and broth-based assays was observed with all three sources of phosphate with the majority of phosphobacterial strains evaluated (Table 3). This effectiveness of SECP either as a broth or solid media is due to the usage of soil extract as a natural medium for the cultivation of soil organisms, since the soil is the source of the carbon, nitrogen, minerals, and vitamins required for the growth of a diverse group of microorganisms (Subba-Rao, 1977).

The present study formulated SECP liquid and solid media for the screening of the phosphate-solubilizing bacteria. Bromocresol green was advocated as an indicator dye for the screening of phosphate-solubilizing bacteria as an alternative to bromothymol blue. In this study, a positive correlation was also observed between the plate-based and broth-based screening methods for phosphate-solubilizing bacteria. SECP solid and liquid media could be used

effectively for the screening of phosphate-solubilizing bacteria before undergoing for *in planta* evaluations for phosphorous nutrition in plants.

Conflict of interest

We declare no conflict of interest.

Acknowledgments

M.M.J and S.D thank Department of Science and Technology-Science and Engineering Board DST-SERB for the Grant No. SB/YS/LS- 79/2013 titled "Development of endophytic bacterial consortium from selected medicinal plants of Western Ghats of India".

References

- Arcand, M.M., Schneider, K.D., 2006. Plant- and microbial-based mechanisms to improve the agronomic effectiveness of phosphate rock: a review. *An Acad. Bras Ciências* 78, 791–807.
- Bashan, Y., Kamnev, A.A., de-Bashan, L.E., 2013. Tricalcium phosphate is inappropriate as a universal selection factor for isolating and testing phosphate-solubilizing bacteria that enhance plant growth: a proposal for an alternative procedure. *Biol. Fertil. Soils* 49, 465–479.
- Das, A.C., 1963. Utilization of insoluble phosphates by soil fungi. *J. Indian Soc. Soil Sci.* 11, 203–207.
- Fernández, V.P., Guzmán, C.A., Peirce, T.M., McBeath, M., Khayet, L., McLaughlin, M.J., 2014. Effect of wheat phosphorus status on leaf surface properties and permeability to foliar-applied phosphorus. *Plant Soil* 384, 7–20.
- Goldstein, A.H., 1986. Bacterial solubilization of mineral phosphates: historical perspectives and future prospects. *Am. J. Altern. Agric.* 1, 57–65.
- Gupta, R., Singal, R., Sankar, A., Kuhad, R.C., Saxena, R.K., 1994. A modified plate assay for screening phosphate-solubilizing microorganisms. *J. Gen. Appl. Microbiol.* 40, 255–260.
- Joe, M.M., Devaraj, S., Benson, A., Sa, T., 2016. Isolation of phosphate-solubilizing endophytic bacteria from *Phyllanthus amarus* Schum & Thonn: evaluation of plant growth promotion and antioxidant activity under salt stress. *J. Appl. Res. Med. Aromat. Plants* 3, 71–77.
- Murphy, J., Riley, J., 1962. A modified single solution method for the determination of phosphate in natural waters. *Anal. Chim. Acta* 27, 31–36.
- Nautiyal, C.S., 1999. An efficient microbiological growth medium for screening phosphate-solubilizing microorganisms. *FEMS Microbiol. Lett.* 170, 265–270.
- Pikovskaya, R.I., 1948. Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. *Mikrobiologiya* 17, 362–370.
- Plaxton, W.C., Tran, T.T., 2011. Metabolic adaptations in phosphorous-starved plants. *Plant Physiol.* 156, 1006–1015.
- Provost, L.M., McLaughlin, J.J.A., Droop, M.R., 1957. The development of artificial media for marine algae. *Arch. Mikrobiol.* 25, 392–428.
- Rodriguez, H., Gonzalez, T., Goire, I., Bashan, Y., 2004. Gluconic acid production and phosphate solubilization by the plant growth-promoting bacterium *Azospirillum* spp. *Naturwissenschaften* 91, 552–555.
- Scervino, J.M., Papinutti, V.L., Godoy, M.S., Rodriguez, M.A., Della, M.I., Recchi, M., Godeas, A.M., 2011. Medium pH, carbon and nitrogen concentrations modulate the phosphate solubilization efficiency of *Penicillium purpurogenum* through organic acid production. *J. Appl. Microbiol.* 110, 1215–1223.
- Subba-Rao, N.S., 1977. *Soil Microorganisms and Plant Growth*. Oxford and IBH Publishing Co., New Delhi.