Scientific Bulletin. Series F. Biotechnologies, Vol. XX, 2016
ISSN 2285-1364, CD-ROM ISSN 2285-5521, ISSN Online 2285-1372, ISSN-L 2285-1364

EVALUATION OF THE CAPACITY OF DIFFERENT MICROORGANISMS TO SOLUBILIZE SEVERAL COMPOUNDS OF PHOSPHOROUS AND ZINC

Andreea DOBRE[1], Laura Alexandra MARIN[1], Carmen MANOLE[1], Nicoleta ANDREI[1], Călina Petruța CORNEA[2]

[1]National Institute of Research and Development for Biological Sciences, 296 Spaiul Independenței, District 6, Bucharest, Romania
[2]University of Agronomic Sciences and Veterinary Medicine of Bucharest, 59 Marasti Blvd, District 1, Bucharest, Romania

Corresponding author email: dobre_mc_andreea@yahoo.com

Abstract

Microorganisms are the main responsible for bioremediation processes. They have the capacity to convert toxic substances in lesser or untoxic ones and also can solubilize compounds in order to make them available to plants. This article emphasizes the capacity of several microorganisms, isolated from different sources, to solubilize P_{in}, P_{org} and Zn based compounds. The screening methods comprised the use of several culture media (PVK, NBRIP, PSM and Bunt&Rovira) with added dyes used as pH markers (bromphenol blue and bromcresol purple). The used bacteria were three strains of Bacillus amyloliquefaciens (BIR, BW, OS15, OS17), four strains of Bacillus subtilis (BPA, ICPA, ATCC6633, 10), two strains of Bacillus spp. (B3, B4), one strain of Kluyvera ascorbata(35) and one unindentified strain isolated from soil contaminated with swines manure. After 1 to 7 days of incubation, the halos formed around the colonies showed the bacteria capacity to degrade the compounds of interest. Depending on the screening assay it was determined the eventual mechanisms involved, such as organic acids production, phytase or phosphatases synthesis, etc. Bacteria which had the capacity to solublize P_{in} based compounds were B3, B4, OS15, BPA, BIR, BW, OS17, ATCC6633, 35 and 10. The ones which were able to degrade phytate (P_{org}) were BIR, BPA, ICPC, OS15, OS17, BW, 10 and 35. The strains that solublized ZnO were BPA, B4, ATCC 6633, 32 and 35. The isolation sources of bacteria were mainly different types of soil, fact that strengthens the teory that soil bacteria are able to secret metabolites which could be involved in bioremediation and crop protection because they are already adapted to soil contaminants from environment.

Key words*: Bacillus spp., bioremediation, phytase, phytic acid.*

INTRODUCTION

The development of intensive agriculture and of industries determined the growth of xenobiotics concentration in water, soil and air. Shukla and Sharma (2010), stated that the most advanced technology for the remediation of the damages produced by polluting agents is bioremediation. Bioremediation, according to Kamaludeen et al. (2003), consists in using the biological agents, mostly microorganisms, in order to remediate the sources which are essential for life maintaining. The degradation of pollution agents requires the actual contact between microorganisms and contaminants, which is relatively difficult to happen because of the uneven spreading in natural conditions at soil level of both microorganisms and pollutants. However some bacteria are mobile, having a chemotactic response which is responsible for their capacity to move toward contaminant. Although some plants can be used in bioremediation processes, microorganisms have a lot more potential because of the shorter time required for their development, the adaptative mutagenesis necessary for pollutant biodegradation, the specific degradation of some pollutants or degradation of various pollutants all at once. Also different microorganisms species cand be used in clusters and, by symbiosis, are able to bioremediate more efficiently a polluted site and also to enhance the vegetation development on that site, this leading to the raising of the phytoremediation efficiency.

Phosphorus, along with potassium and calcium, is one of the macronutrients which are essential for the development, functioning and health of

all living creatures. Phosphorus, at cellular level, exists under the form of orto- and pyrophosphoric acids. It is found in nucleic acids, phospholipids, coenzymes, enzymes and hormones. Organic phosphorus (P_{org}) represents 30-50% from the entire soil phosphorus (Dai et al. 2011). Although phosphorus is one of the most important elements on Earth which ensure the development of living creatures, only 5% from the global amount is available in order to be used by plants (Maksimov et al., 2011). Phytic acid (mioinositol hexakisphosphate– PA – phytic acid (IUPAC-IUB 1978) and his derivatives, phytates, (phytic acid salts) are the phosphorus most common forms that are met at soil level. The international abbreviation PA is used both for phytic acid and phytates. PA being an inositol derivative, it has six molecules of phosphoric acid bound to six hidroxyl groups which can be assimilate by the animal body and microorganisms, but not by plants (Bohn et al., 2008). This is why the most common form met in nature is phosphoric acid molecules.

Once complete dissociation occurs, the six phosphate groups of phytic acid carry 12 negative charges. These charges will bond different bivalent or trivalent cathions (ex. Ca, Mg, Fe, Zn, Cu, Mn) in low acidic or neutral pH and form a stable complex (Frank, 2013). Due to its molecular structure, phytic acid has an anti-nutritive effect on certain living beings.

Interactions between phytic acid and proteins have also been reported. According to Munir and Maqsood (2013), in conditions of acidity, phytic acid will negatively influence the solubility of proteins by way of ionic bonding between phosphate groups of phytic acid and protonated amino acids (lysyl, histidyl and arginyl residues).

Zinc appears to be the element whose bioavailability is most influenced by phytic acid. Research done by Flanagan (1984) demonstrates that phytic acid not only reduces the bioavailability of zinc in alimentation, but also greatly reduces the intestinal reabsorption of endogenous zinc. Moreover, high concentrations of calcium increase the anti-nutritive effects of PA on zinc's bioavailability through the formation of Ca-Zn-PA insoluble complexes. A molar report of PA x Ca/Zn > 3.5 is regarded as a critical determining factor of using Zn (Munir & Maqsood, 2013).

According to Ritnbach et al. (2008), iron is difficult to assimilate by living beings because of its irreversible chelation in the digestive tract by certain fibers, polyphenols, as well as phytic acid. However, phytic acid inhibits the absorption of iron in its different forms via a high level of phosphorylation, such as IP5 and IP6, compared to IP1, IP2, IP3 and IP4.

Modern agriculture implies not using fertilizer supplementation, and focuses on the exploitation of soil resources with the help of microorganisms. One of the most important roles of microorganisms involved in development of crops is their capacity to solubilize phosphorus. The process consists of synthesizing metabolites, capable of chelating cations, such as calcium, from the structure of insoluble compounds. These phosphorous-based compounds release phosphorous molecules, which can be assimilated by plants (Vassilev et al. 2012).

Microbial phytases are actively secreted in the soil, where they participate in both the decay of vegetal detritus, as well as in releasing phosphorous from organic compounds found in soil. This makes microbial phytases the key enzymes of the organic phosphorous cycle in the soil. This enzyme is not secreted in the rhizosphere by the plant; consequently, it is incapable of assimilating the phosphorous which is bound to phytates in the soil. Thus, microorganisms play a unique role in degrading these compounds (Guimaraes et al. 2006). Of the many different types of bacteria which have the capacity to synthesize these enzymes, we mention: *Pseudomonas*, *Bacillus*, *Raoultella* and *Enterobacter* (Simon et al. 2002). Phytases produced by species of the genus *Bacillus*, which present optimal enzymatic activity in conditions of neutral pH, are substrate-specific with regards to calcium phytate and have a high thermic stability, according to Fu et al. 2008.

In its metallic form, zinc does not influence the environment because of the lack of its bioavailability. However, Radhika et al. (2006) claim that zinc can react with other elements, the result being toxic reaction products, which have a negative impact on ecosystems.

According to Perpetuo et al. (2011), there is a competition between zinc and cadmium for the

same cellular sites, which determines replication errors, mutagenesis, the destabilization of cellular structures, etc. The *Bacillus* spp. is recognized for its resistance in sites contaminated with heavy metals, including zinc (Krishna et al. 2013). The species of the *Bacillus* spp. have the capacity of assimilating, not only zinc, but other heavy metals such as copper, lead and cadmium, at the soil level (Issazadeh et al. 2011).

Specialty literature mentions that numerous microorganisms, capable of solubilizing otherwise insolvable forms of phosphate (or of other chemical elements), accomplish this through the generation of organic acids, which vary in type and quantity, according to the microbial species. Among the most frequent organic acids produced by bacteria which solubilize phosphate we find gluconic, ketogluconic, lactic, succinic, formic, malic, citric, oxalic, fumaric, tartaric, propionic, acetic, izobutiric, izovaleric, valeric and izocaproic acids (Khan et al. 2014). Also, the solubilization of otherwise insolvable forms of phosphate can be realized through biosynthesis of some specific enzymes, such as phosphatases and phytases. Phosphatases (acidic and alkaline) are eliminated outside of the cells as exo-enzymes. They utilize P_{org} as a substrate in order to transform it in its inorganic form, accessible to plants. Phytases have a more specific behavior, determining the liberation of phosphorous.

Having in mind these aspects, within the present experiments it was detected the capacity of several bacteria from various sources to solubilize different insoluble compounds.

MATERIALS AND METHODS

The used microorganisms

The evaluated microorganisms were mostly from the *Bacillus* genus, as follows in the Table 1:

The screening assay for detection of bacteria which have the capacity to solubilize the anorganic phosphorus based compounds

In order to identify the capacity of bacteria to synthesize organic acids involved in phosphate solubilisation, were used the following culture media: PVK and NBRIP, where the phosphorus source is tricalcic phosphate ($Ca_3(PO_4)_2$).

Table 1. The used microorganisms

No	Notation	Species	Isolation source
1	BIR	*B.amyloliquefaciens*	Soil (Microorganisms Collection of Biotechnologies Faculty USAMVB)
2	BPA	*B. subtilis*	Soil (M. C. of B. F. USAMVB)
3	Icpc	*B. subtilis*	M. C. of B. F. USAMVB
4	ATCC 6633	*B. subtilis* ATCC6633	M. C. of B. F. USAMVB
5	OS17	*B.amyloliquefaciens*	Onion ryzoshpere (Sicuia et al., 2012)
6	OS15	*B.amyloliquefaciens*	
7	BW	*B.amyloliquefaciens*	Soil (Sicuia et al, 2012)
8	B3	*Bacillus sp.*	Compost (M. C. of B. F. USAMVB)
9	B4	*Bacillus sp.*	
10	10	*Bacillus subtilis ss. subtilis*	*Microorganisms isolated from soils on which is deposited pig manure. The microorganisms are preservated in the Microbiology Laboratory of the Biotechnologies Department, NIRDBS*
11	32	*Unindentified*	
12	35	*Kluyvera ascorbata*	

The PVK ingredients for 1000 ml are: 10g glucose, 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.01 g $MgSO_4 \cdot 7H_2O$, 0.5 g yeast extract, 0.0001 g $FeSO_4 \cdot 7H_2O$, 0.0001g $MnSO_4 \cdot H_2O$, 15 g agar, 5 g $Ca_3(PO_4)_2$, pH 7 (Kaur and Reddy, 2013). The NBRIP ingredients for 1000 ml are: 10 g glucose, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2 \bullet 6H_20$, 0.25 g $MgSO_4 \bullet 7H_20$, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$, pH 7.

To these media were added dyes as markers in order to detect the pH variations of culture media which, in case of acidification would semnalize the presence of organic acids synthesized by bacteria. The color markers used were blue bromphenol (0.1 g/L(Gupta et al., 1994) and bromcrezol purple 0.1 g/L (Agrawal et al., 2015).

The presence of the hydrolysis halos generated by the interaction bacteria - stained culture media and the colour migration from blue to yellow (in case of blue bromphenol) and from purple to yellow-orange (in case of bromcrezol purple) highlights the decrease of pH value from 7 to 6 or less. This fact shows the biosynthesis of organic acid by the bacteria, in

order to solubilize the anorganic phosphorus-base compunds.

The screening assay for detection of bacteria which have the capacity to solubilize the organic phosphorus based compounds

For emphasis of the involvement of organic acids in solubilisation of P_{org} (phytate), the bacteria strains were cultivated on PSM with or without the dyes mentioned before. The PSM ingredients for 1000 ml are: 15 g glucose 15 g, 5 g $(NH_4)_2SO_4$, 0.5 g KCl, 0.1g $MgSO_4.7H_2O$, 0.1 g NaCl, 0.1 g $CaCl_2.2H_2O$, 0.01 g $FeSO_4$, 0.01 g $MnSO_4$, 15 g agar, 5 g sodium phytate, pH 6.5 (Bae et al., 1999; Singh et al., 2013; Tungala et al., 2013). The observations were made after 48 h.

The screening assay for evaluation of bacteria's ability to solubilize certain zinc compounds

The cultivation medium used is Bunt & Rovira. The ingredients for 1000 ml are the following: 20 g glucose, 1 g peptone, 1 g yeast extract, 0.5 g $(NH_4)_2SO_4$, 0.4 g K_2HPO_4, 0.1 g $MgCl_2$, 0.01 g
$FeCl_3$, 250ml soil extract, ZnO 0.1% in 750 ml water, pH 6.6.-7.0. Zinc oxide (Abaid-Ullah et al. 2015), as well as the aforementioned dyes (Kumar et al. 2012) have been added to the medium. The bacteria have been incubated for 7 days.

RESULTS AND DISCUSSIONS

Detection of bacteria which have the capacity to solubilize the anorganic phosphorus based compounds

By using the PVK medium without dyes, the solubilisation halo was detected at the following bacterias: B3, B4, OS15, BIR and 10 (Figure.1).
Regarding the screening using PVK medium + bromcresol purple the cultivated bacteria determined the pH variation, by decreasing it, after 24 h (Figure 2).
When bromphenol blue was used as pH marker, the color intensity generated on PVK media was much lower then within the experiments when was used bromcresol purple for the same purpose. Slightly color modifications were observed after 24h from inoculation and as well after 48h.

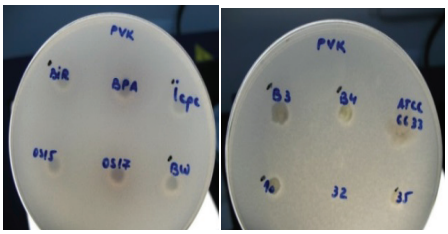

Figure 1. Highlighting hydrolysis halos on medium PVK agar

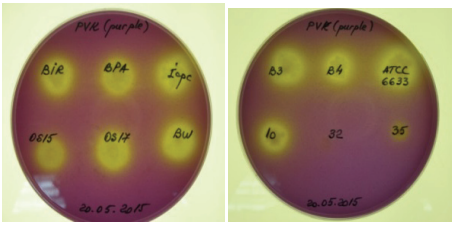

Figure 2. pH modification on PVK + bromocresol purple after 24 h from inoculation

After 48 h of cultivation, the decreasing of pH value was even more obvious and it was generated by all bacteria (Figure 3). This fact showed the microorganisms capacity to synthesize the organic acids required for phosphate solubilization.

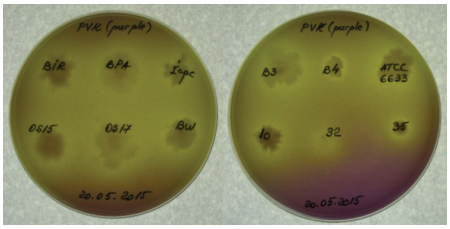

Figure 3. pH modification on PVK + bromocresol purple at 48 h after inoculation

After 24h, the bacteria that formed thin halos which showed a pH decrease were the following: BIR, BPA, ICPC, OS15, OS17, BW, B3, B4, ATCC 6633 and 10 (Figure 4).
Also, as in the previous case, when it was used, as pH marker, bromcresol purple, the strain 35 is the one who produces the smallest halo, showing its low capacity to synthesize organic acids (Figure 5).

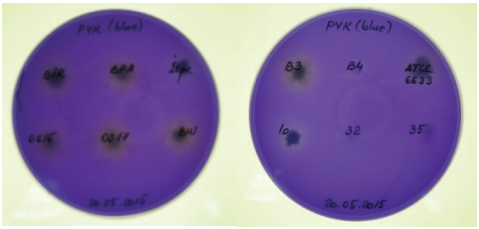

Figure 4. pH modification on PVK + bromphenol blue at 24 hours

By comparing the data obtained after using these two types of dyes as pH markers, it can be stated that the most bacteria were able to produce organic acids which modified the pH value and which also could be involved in the solubilisation of anorganic phosphorus.
Although, it needs to be mentioned that the pH decrease wasn't significant; most probably the bacteria strains synthesize organic acids which determine a pH decrease to the value of aprox. 5.0.

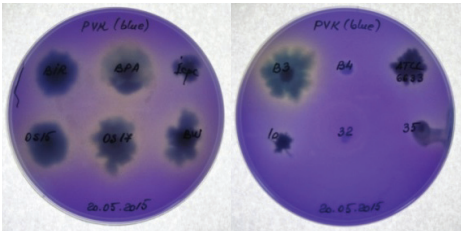

Figure 5. pH modification on PVK + bromphenol blue at 48 h

By concluding, it can be stated that the screening in which it was used PVK agar + bromcrezol purple it is the best assay for detecting the solubilisation of tricalcium phosphate by bacteria which synthesize organic acids for this purpose.
In order to validate the results obtained with this assay, there were realized similar experiments with other culture medium, NBRIP (National Botanical Research Institute's Phosphate growth medium). This medium is also used for the evaluation of microorganisms capacity to solubilize anorganic phosphate based compounds (Singh et al., 2014). The experiments comprised by using NBRIP within this article validated the observations made on PVK medium.
Because of the lack of contrast, the halos are slightly difficult to measure, same thing had happen also before, when PVK media was used. Although, by comparing the observations made on the two media (NBRIP and PVK), the NBRIP media showed greater halos (Figure 6).

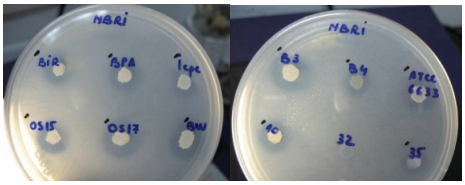

Figure 6. Highlighting hydrolysis halos on medium NBRIP

In order to detect the organic acids production which is associated to the bacteria ability to solubilize the phosphate, were added the same dyes as before in the NBRIP composition, but the results were different. Comparing with the results obtained on NBRIP, the observations made on NBRIP + bromcrezol purple lead to registration of a weak decrease of culture medium pH value. This fact suggests that, at least on this medium, the solubilisation of tricalcic acid is realized not only by organic acids production, but by other mechanisms, perhaps enzymatic ones (Figure 7).

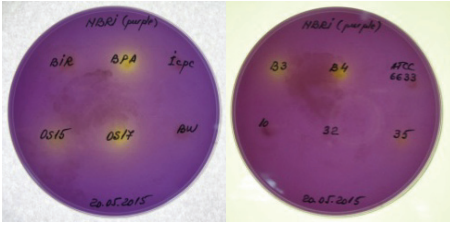

Figure 7. Highlighting hydrolysis halos on medium NBRIP agar + bromocresol purple

It needs to be mentioned the fact that the halos produced on NBRIP + bromcrezol purple are smaller than the ones generated on PVK + bromcrezol purple. NBRIP + bromphenol blue wasn't able to indicate the pH variations, although the bacteria developed optimaly.
The obtained results suggest that the concentration of organic acids produced by bacteria seems to be lower than the one produced by bacteria on PVK + pH markers. This fact could happen because of the media different composition. Also, the observations made are in conformity with the observations made by other researchers which stated that the

utilization of bromphenol blue as pH marker is not an efficiently way to highlight the solubilisation of some compounds (Gadagi and Sa, 2012).

Detection of bacteria which have the capacity to solubilize the organic phosphorus based compounds

On PSM culture medium the production of organic acids is very low (according to stained culture medium). Thus, on PSM + bromcresol purple, the strain 10 is the one that decrease the most the culture medium pH (figure 8). The strains 35, B3 și ATCC 6633 generated clear halos around their colonies, but with a color of intense violet, which is correlate with a basic pH value. This fact could be explained by the phytase and/or phosphatase synthesis which hydrolized the sodium phytate and did not decrease the pH value of the culture medium.

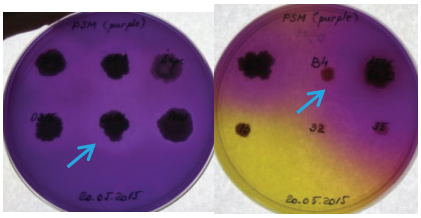

Figure 8. Solubilization of sodium phytate on PSM agar + bromocresol purple after 48 h of cultivation

Similar observations were made for BIR, BPA, ICPC, OS15, OS17 și BW (figure 8). For these ones were observed halos which weren't colored in yellow-orange, leading to the conclusion that the pH wasn't modified. Also, when blue bromphenol was used as pH marker, the results were similar. The phytate was solubilized without occuring a pH variation (Figure 9). Overall, the halos had a more intense color, suggesting an eventual increase of pH value. These increase is specific to *Bacillus* spp. The strain 10 cultivated on PSM + blue bromphenol did not decreased the culture medium pH, this leading to the conclusion that the bacteria did not biosynthesize organic acids on this culture medium. The obtained results confirmed the fact that on PSM, which contains sodium phytate, the phosphorus solubilisation is mainly realized by enzymatic mechanisms (phytase or phosphatases biosynthesis).

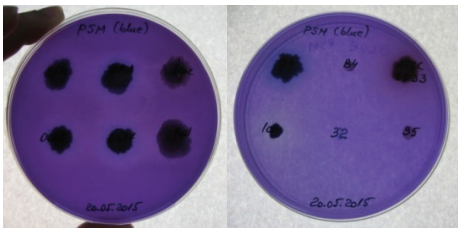

Figure 9. Highlighting the halos on PSM agar + bromphenol blue after 48 h from inoculation

By comparing the results obtained after using anorganic phosphorus or organic phosphorus it can be concluded that the solubilisation of phosphate requires the production of organic acids. Also, for phytate solubilisation are necessary enzymatic mechanisms. The data obtained emphasize that at least several strains have the capacity to solubilize phosphorus based compounds regardless its form. These bacteria have the potential to be used in bioremediation, or crop protection.

Evaluation of bacteria's ability to solubilize certain zinc compounds

Bacteria's involvement in the solubilization process of zinc on the ground level is an important attribute. This is due to the conversion of zinc-based compounds in compounds available for plants, zinc being an essential metal for their metabolic activities (Abaid-Ullah et al. 2015). Since previous experiments highlighted the capacity of certain bacterial strains to solubilize phosphate compounds, the following experiments are concerned with testing the capacity of the same bacteria to solubilize insolvable zinc compounds as well (ZnO).
After completing the experiments, the lack in development of certain bacteria was observed; this was due to a cultivation medium which was poor in nutrients, as well as the presence of zinc oxide in high concentrations. Also, we have noticed the development of strains B4, ATCC6633, 32, 35 and BPA, which generated a halo of clarification of the medium, around colonies (figure 10).

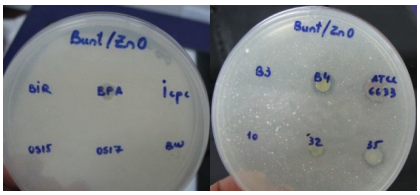

Figure 10. Evaluation of bacteria on medium Bunt& Rovira with ZnO

It should be noted that out of the 12 bacterial strains subjected to experiments, the B4 strain manifested the highest capacity to solubilize zinc oxide, the size of the clarification halo of the medium having grown constantly over the 7-day cultivation period (Figure11).

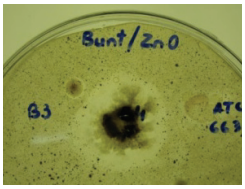

Figure 11. The ability of the strain B4 to solubilize the zinc from ZnO

Considering the fact that the B4 strain is isolated from compost, its capacity to solubilize both phosphorous from its insolvable compounds, as well as zinc oxide with a high yield, represents an important advantage with regards to its uses in different practical applications. With regards to the mechanisms involved in the zinc solubilization process, and taking into account the previous results obtained during phosphorous solubilization, the capacity to produce organic acids was also tested. After having used a purple bromcrezol dye as a pH indicator, we noticed a weak acidification of the medium, generated by strains BPA, B4 and 35 (Figure 12).

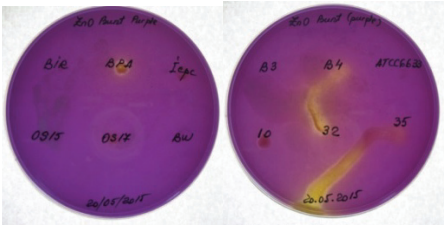

Figure 12. Evaluation of bacteria on medium Bunt and Rovira + bromcresol purple

After correlating these results with the ones obtained by using bromfenol blue (figure 13), we can conclude that the solubilization of zinc by the bacteria mentioned above is mainly due to the production of organic acids, even though the pH value of the medium does not decrease very much (~ 6.0). Moreover, the fact that the clarification halo of the medium has maintained on the plates inoculated with the bacteria of interest, even after a period of 1 month of storage at room temperature, suggests the possible implication of other mechanisms in the bio-solubilization process.

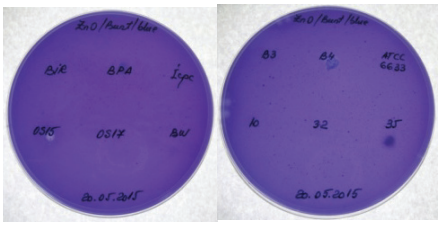

Figure 13. Evaluation of bacteria on Bunt&Rovira medium + bromphenol blue

The bacterial strains, identified as being potentially able to solubilize zinc, have different origins: compost - the *Bacillus* sp. B4 strain, a collection strain - *B. subtilis* ATCC6633, soil - *B. subtilis* BPA and soil contaminated with manure from swines - strain 32 (unidentified) and strain *Kluyvera ascorbata* 35. Considering these results, we can remark that soil represents a rich source for isolating microorganisms capable of solubilizing phosphate and zinc compounds, useful in the development process of plants. At the same time, these strains can be used in bio-remediation of contaminated sites (Issazadeh et al. 2011). The experiments were realized with a culture medium pH of 6.6 – 7, which confirms the study made by Hazarika et al. (2015), which claims that the solubilization of zinc-based compounds by bacteria was optimal in conditions of neutral pH.

CONCLUSIONS

During experiments performed on PVK and NBRIP media, the B3, B4, OS15, BPA, BIR, BW, OS17, ATCC6633, 35 and 10 strains were noted for generating specific halos, which emphasize their capacity to solubilize inorganic phosphorous.

The use of pH-indicator dyes (bromcresol purple and blue bromfenol) allowed us to highlight that solubilization of inorganic phosphorous can be done, in most cases, by the production of organic acids.

Clear solubilization halos of organic phosphorous have been highlighted on the PSM selective medium in the case of BIR, BPA, ICPC, OS15, OS17, BW, 10 and 35 strains, which suggests the production of enzymes.

Adding dyes to the PSM medium demonstrated that the mechanism for solubilization of organic phosphorous is, first of all, enzymatic, through the production of phytases; this, however, does not exclude the phosphatase synthesis (at least for some of the tested bacterial strains).

Equally, the production of organic acids can be associated with the solubilization of organic phosphorous but in a greatly reduced manner.

The solubilization of zinc from insolvable compounds (ZnO) was clearly highlighted in the case of BPA, B4, ATCC 6633, 32 and 35 strains. The use of pH-indicator dyes led to the conclusion that this solubilization ability can be largely due to the production of organic acids.

Taking the origin of the bacterial strains with phosphorous and zinc solubilization abilities into account we can appreciate that the soil represents a rich source of microorganisms, which, through their properties, can offer plants easy to assimilate nutritive compounds.

ACKNOWLEDGEMENTS

This research work was carried out under the frame of European Social Fund, Human Resources Development Operational Programme 2007-2013, project no. POSDRU/159/1.5/S/132765.

REFERENCES

Abaid-Ullah, M., Hassan, M., N., Jamil, M., Brader, G., Shah, M., K., N., Sessitsch, A., Hafeez, F., Y., 2015, Plant growth promoting rhizobacteria: an alternate way to improve yield and quality of wheat (Triticum aestivum), Int. J. Agric. Biol., vol. 17, pp. 51 – 60

Agrawal, T., Kotasthane, A., S., Kushwah, R., 2015, Genotypic and phenotypic diversity of polyhydroxybutyrate (PHB) producing Pseudomonas putida isolates of Chhattisgarh region and assessment of its phosphate solubilizing ability, 3 Biotech, vol. 5, pp. 45 – 60

Bae, H., D., Yanke, L., J., Cheng, K., J., Selinger, L., B., 1999, *A novel staining method for detecting phytase activity*, J Microbiol Methods, vol. 39, nr. 1, pp. 17 – 22

Bohn, L., Meyer, A., S., Rasmussen, S., K., 2008, Phytate: Impact on Environment and Human Nutrition, A Challenge for Molecular Breeding, vol. 9, nr. 3, pp. 165 – 191

Dai, F., Qiu, L., Ye, L., Wu, D., Zhou, M., Zhang, G., 2011, *Identification of a Phytase Gene in Barley (Hordeum vulgare L.)*, PLoS One, vol. 6, nr. 4, pp. 18 – 29

Flanagan, P., R., 1984, *A model to produce pure zinc deficiency in rats and its use to demonstrate that dietary pliytace increases the excretion of endogenous zinc*. The Joumal of Nutrition, vol. 114, pp. 493 – 502

Frank, A., W., 2013, Chemistry of Plant Phosphorus Compounds, ed. Elsevier, New York, pp. 16 – 17

Fu, S., Sun, J., Qian L., Li, Z., 2008, *Bacillus Phytases: Present Scenario and Future Perspectives*, Appl Biochem Biotechnol, vol. 151, pp.1 – 8

Gadagi, R., S., Sa, T., 2002, *New Isolation Method for Microorganisms Solubilizing Iron and Aluminum Phosphates Using Dyes*, Soil Sci. Plant Nutr., vol. 48, nr. 4, pp. 615 - 618

Guimaraes, L., H., S., Peixoto-Nogueira, S., C., Michelin, M., Rizzatti, A., C., S., Sandrim, V., C., Zanoelo, F., F., Aquino, A., C., Junior, A., B., Polizeli, M., 2006, *Screening of Filamentous Fungi for Production of Enzymes of Biotechnological Interest*, Brazil. J. Microbiol., vol. 37, pp. 474 – 480

Gupta, R., Mahapatra, H., 2003, Microbial biomass: An economical alternative for removal of heavy metals from waste water, Indian Journal of Experimental Biology, vol. 41, pp. 945 – 966

Hazarika, S., I., Paramasivam, S., Talukdar, S., Dutta, K., 2015, *Natural bioremediation of nickel (Ni) and zinc (Zn) in coal mine effluent using Bacillus megaterium and Pseudomonas aeruginosa*, International Journal of Emerging Technology & Research, vol. 2, nr. 2, pp. 5 - 17

IUPAC-IUB, 1978, *Nomenclature of phosphorus-containing compounds of biochemical importance. (Recommendations 1976). lUPAC-IUB Commission on Biochemical Nomenclature*, The Biochemical Joumal, vol. 7, pp. 1 – 19

Issazadeh, K., Pahlaviani, M., R., M., K., Massiha, A., 2011, bioremediation of toxic heavy metals pollutants by Bacillus spp. isolated from guilan bay sediments, North of Iran, International Conference on Biotechnology and Environment Management IPCBEE vol.18, ed. IACSIT, Singapore

Kamaludeen, S.,P.,B.,K., Arunkumar, K.,R., Avudainayagam ,S., Ramasamy, K., 2003, Bioremediation of chromium contaminated environments. Ind. J. Exp Bio., vol. 41, pp. 972 – 985

Kaur, G., Reddy, M., S., 2013, *Phosphate solubilizing rhizobacteria from an organic farm and their influence on the growth and yield of maize (Zea mays L.)*, J. Gen. Appl. Microbiol., vol. 59, pp. 295 - 303

Khan,M.D., Zaidi,A., Ahmad,E., 2014, Mechanism of Phosphate Solubilization and Physiological Functions of Phosphate-Solubilizing Microorganisms, în M.S. Khan et al. (eds.), Phosphate Solubilizing Microorganisms, Springer International Publishing Switzerland, p.31-62

Krishna, M,. P., Varghese, R., Babu, V., A., Jyothy, S., Hatha, A., A., M., 2013, *Bioremediation of Zinc Using Bacillus sp. Isolated from Metal-Contaminated Industrial Zone*, Prospects in Bioscience: Addressing the Issues, ed. Springer, New Delhi, pp. 11 – 18

Kumar, P., Dubey, R., C., Maheshwari, D., K., 2012, Bacillus strains isolated from rhizosphere showed plant growth promoting and antagonistic activity against phytopathogens, Microbiological Research, vol. 167, pp. 493 – 499

Maksimov, I., V., Abizgil'dina, R., R., Pusenkova, L., I., 2011, Plant Growth Promoting Rhizobacteria as Alternative to Chemical Crop Protectors from Pathogens (Review), Applied Biochemistry and Microbiology,vol. 47, nr. 4, pp. 333 – 345

Munir, K., Maqsood, S., 2013, *A review on role of exogenous enzyme supplementation in poultry production*, Emir. J. Food Agric., vol. 25, nr. 1, pp. 66 – 80

Perpetuo, E., A., Souza, C., B., Nascimento, C., A., O., 2011, Engineering Bacteria for Bioremediation, Progress in Molecular and Environmental Bioengineering - From Analysis and Modeling to Technology Applications, Prof. Angelo Carpi (Ed.), ISBN: 978-953-307-268-5, InTech, http://www.intechopen.com/books/progress-in-molecular-and-environmental-bioengineering-fromanalysis-and-modeling-to-technology-applications/engineering-bacteria-for-bioremediation

Radhika, V., Subramanian, S., Natarajan, K., A., 2006, Bioremediation of zinc using Desulfotomaculum nigrificans: Bioprecipitation and characterization studies, Water Research, vol. 40, pp. 3628– 3636

Ritnbach, G., Pallauf, J., Moehring, J., Kraemer, K., Minihane, A.,M., 2008, *Effect of dietary phytate and microbial phytase on mineral and the element bioavailability - a literature review*, Current Topics in Nutraceutical Research, vol. 6, nr. 3, pp. 131 - 144

Shukla, K., P., Singh, N., K., Sharma S., 2010, *Bioremediation: Developments, Current Practices and Perspectives,* Genetic Engineering and Biotechnology Journal, 2010, pp. 1 – 20, http://astonjournals.com/manuscripts/Vol2010/GEBJ-3_Vol2010.pdf

Sicuia, O., A., Oancea, F., Constantinescu, F., Dinu, S., Cornea, C., P., 2012, *Bacillus strains useful in improving vegetal mulch techology trough bio-activation*, Rom.Biotechnol. Letters, vol. 17, nr. 5, 7610 – 7619

Simon, O., Igbasan, F., 2002, *In vitro properties of phytases from various microbial origins*, Int. J. Food Sci. Technol., vol. 37, pp. 813 – 822

Singh, N., K., Joshi, D., K., Gupta, R., K., 2013, *Isolation of Phytase Producing Bacteria and Optimization of Phytase Production Parameters*, Jundishapur Journal of Microbiology, vol. 6, nr. 5, pp. 6419 – 6424

Tungala, A., Narayanan, K., A., Muthuraman, M., S., 2013, Izolation of phytase producing bacteria from poultry faeces and optimization of culture conditions for enhanced phytase production, International Journal of Pharmacy and Pharmaceutical Sciences, vol. 5, nr. 4, pp. 264 - 269

Vassilev, N., Eichler-Löbermann, B., Vassileva, M., 2012, *Stress-tolerant P-solubilizing microorganisms*, Appl Microbiol Biotechnol, vol. 95, pp. 851 – 859