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MINI-REVIEW

Multifunctional properties of phosphate-solubilizing microorganisms grown on agro-industrial wastes in fermentation and soil conditions

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Abstract One of the most studied approaches in solubilization of insoluble phosphates is the biological treatment of rock phosphates. In recent years, various techniques for rock phosphate solubilization have been proposed, with increasing emphasis on application of P-solubilizing microorganisms. The P-solubilizing activity is determined by the microbial biochemical ability to produce and release metabolites with metal-chelating functions. In a number of studies, we have shown that agro-industrial wastes can be efficiently used as substrates in solubilization of phosphate rocks. These processes were carried out employing various technologies including solid-state and submerged fermentations including immobilized cells. The review paper deals critically with several novel trends in exploring various properties of the above microbial/agro-wastes/rock phosphate systems. The major idea is to describe how a single P-solubilizing microorganism manifests wide range of metabolic abilities in different environments. In fermentation conditions, P-solubilizing microorganisms were found to produce various enzymes, siderophores, and plant hormones. Further introduction of the resulting biotechnological products into soil-plant systems resulted in significantly higher plant growth, enhanced soil properties, and biological (including biocontrol) activity. Application of these bio-products in bioremediation of disturbed (heavy metal contaminated and desertified) soils is based on another important part of their multifunctional properties.

Keywords Agro-wastes · Fermentations · Microbial properties · Phosphate solubilization · Soil-plant systems

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Introduction

Green chemistry and biotechnology are accepted as important tools in achieving sustainability. Their implementation, and the design of chemical products and microbial processes that reduce or eliminate the use and generation of hazardous substances and abundant waste materials, is an essential part of our efforts aimed at minimizing their negative impact (Kirchhoff 2005). The complete bio-recycling of agro-industrial wastes is now accepted as an important element of sustainable agriculture and agro-industry (Tengerby and Szakacs 1998). Microbially treated agricultural residues may restore soil fertility and soil microbiota. Alternatively, excess agro-industrial wastes may be converted microbiologically to valuable feed supplements and bio-industrial products (Ashworth and Azevedo 2009). In recent years, there has been an increasing trend towards more efficient utilization of agro-industrial residues based on processes which employed solid-state fermentation (Krishna 2005). This review will focus on research work employing microorganisms in solubilization of insoluble phosphates and particularly on their multifunctional properties demonstrated in organic matter-based solid-state fermentation and soil conditions.

In general, current management of phosphate-bearing resources (mainly rock phosphates (RP)), which in fact are finite non-renewable sources, is quite far from the principles of sustainability and may cause an escalating price increase mainly because of increases in RP-processing costs (Vassilev et al. 2009b). In order to reduce the depletion of global phosphorus reserves, research should be oriented towards more effective utilization of phosphates.

A biological approach for extracting phosphate from RP was proposed as a less expensive and lower energy technique compared with the conventional processes (Goldstein and Rogers 1999; Khan et al. 2007). Various techniques for RP solubilization have been proposed, with increasing emphasis on application of P-solubilizing microorganisms (Rodriguez and Fraga 1999; Whitelaw 2000; Vassilev et al. 2001a; Vassilev and Vassileva 2003) employing low-grade RP which is often considered as a waste material from the mining industry. The P-solubilizing activity is determined by the microbial biochemical ability to produce and release metabolites such as organic acids that, through their hydroxyl and carboxyl groups, chelate the cations (mainly calcium) bound to phosphate, the latter being converted into soluble forms (Sagoe et al. 1998).

Until recently, research on soil microorganisms was as a rule focused on a single (main) activity. At best, additional activities were studied individually and only during the last years simultaneously. During the last 15 years, in a number of studies, we have demonstrated the ability of P-solubilizing microorganisms to exert additional functions in fermentation and soil conditions. The aim of the present review paper is to analyze the achievements and suggest further research lines in this field.

Multifunctional properties of phosphate-solubilizing microorganisms in fermentation conditions (solid-state and immobilized-cell-based submerged fermentation processes)

Multifunctional properties of phosphate-solubilizing microorganisms in fermentation conditions

Pandey et al. (2001) defined solid substrate fermentation (SSF) as a process in which the substrate itself acts as carbon (and energy) source, occurring in the absence or near-absence of free water. Using SSF technology, we can bring the cultivated microorganisms into tight contact with the agro-industrial wastes and thus to achieve the highest substrate concentrations for fermentation. The major microbial groups used in SSF are fungi and bacteria. However, it is well established that fungi are the most important and the best adapted for SSF because of their mode of growth and high tolerance to low water activity (Himmel et al. 1999). All solid substrates, which include starch, cellulose, lignocellulose, pectin, and other polysaccharides in their structure, can be used in the SSF as substrates. Products, by-products, and wastes from agriculture and agro-industry provide excellent solid matrixes and substrates used directly or after pre-treatment in SSF (Pandey et al. 2000). In the field of formulation and preparation of microbial inoculants for environmental and agricultural soil application, SSF is the process of choice because of higher fermentation productivity and product stability, lower demand on sterility, low labor and technical difficulties, and generally does not need further formulation operations (Spadaro and Gullino 2005).

The use of SSF technology for RP fungal solubilization using agro-industrial wastes has been, for the first time, proposed by Vassilev et al. (1994) and further investigated with fungal microorganisms grown-on agro-industrial wastes derived from the sugar-producing and olive oil industries (Vassilev et al. 2006a, 2009a). Our first experiments with the fungal P-solubilizer *Aspergillus niger* grown on different agro-wastes in conditions of SSF processes showed at least three simultaneous activities—mineralization of lignocellulosic substrates, biosynthesis of organic acids and, as a consequence, solubilization of RP with a strict correlation between both last processes (Vassilev et al. 1995; Table 1). This observation was not surprising as we used the same strain in citric acid production on complex substrates (Vassilev et al. 1992b). SSF is accepted as a promising technology for production of organic acids on agro-industrial wastes (Pandey et al. 2000). Organic acids are excellent P-solubilizing agents and, therefore, bio-treatment of low-grade RP by fungal SSF can be accepted as an attractive approach within microbially mediated solubilization.

Sugar beet (SB) waste material was selected amongst various agro-wastes (Vassilev et al. 1998) as it provided acceptable mycelial growth, organic acid production, and a level of mineralization between 56% and 69% depending on the substrate concentration. This system was able to solubilize 76% of the RP supplemented to the medium at a concentration of 15 g/kg SB. The presence of RP affected the behavior of *A. niger*, particularly its growth and citric acid production (Vassilev et al. 1995). A similar biotechnological scheme has been applied using olive cake (OC)-based and dry olive waste (DOW)-based media and employing *A. niger* (OC) and *Phanerochaete chrysosporium* (DOW; Vassileva et al. 1998; Vassilev et al. 2002, 2006b). Depending on the olive oil industrial technology

Table 1 Multifunctional properties of P-solubilizing microorganisms in fermentation conditions

Microorganism/substrate	Fermentation substrate	Activities	References
A. niger/SB	SSF	Organic matter mineralization, citric acid release, P-solubilization	Vassilev et al. 1995
A. niger/OC			Vassileva et al. 1998
A. niger/SB+DOW	SSF	The above activities+phytase production+siderophore production	Vassilev et al. 2007a, 2008a
P. chrysosporium/DOC	SSF	Organic matter mineralization, oxalic acid release, P-solubilization	Vassilev et al. 2006b
P. chrysosporium/SB+DOW	SSF	The above activities+Mn peroxidase production	Vassilev et al. 2009c
P. chrysosporium/DOW+Glucose	IC, RBF	P-solubilization; Mn peroxidase production	Vassilev et al. 2007b
P. janthinellum/DOW+chitin	SSF	Organic matter mineralization, P-solubilization, chitinase production	Vassilev et al. 2008b
P. janthinellum/DOW+chitin	IC, RBF	P-solubilization, chitinase production	Vassilev et al. 2008b
B. thuringiensis/nutrient broth	IC, RBF	P-solubilization, indole-3-acetic acid production	Vassilev et al. 2007c

SB sugar beet wastes, *OC* olive cake, *DOW* dry olive wastes, *SSF* solid-state fermentation, *IC* immobilized cells, *RBF* repeated-batch fermentations

used, several types of wastes can be produced in different ratios and physico-chemical characteristics, such as wastewaters, OC, and DOW. The degree of OC and DOW mineralization, mycelial growth, and organic acid production were lower in comparison to the SB-based process, which resulted in lower solubilization of the total P present in the RP. Obviously, the insoluble and crystalline nature of cellulose, associated with a higher content of lignin, and the low content of oxidizable carbon in the OC/DOW experiments decreased the overall process efficiency. In addition, the presence of polyphenolic compounds in olive mill wastes could affect the physiological and biochemical characteristics of the fungal P-solubilizers.

Bearing the above considerations in mind, our recent experiments were based on a mixture of substrates containing both SB and DOW where additional microbial activities were demonstrated such as phytase, chitinase, and manganese peroxidase enzyme production (Vassilev et al. 2007a, 2008b, 2009c; Table 1). For example, DOW was used as a substrate for phytase production by *A. niger* in conditions of solid-state fermentation in the presence of RP (Vassilev et al. 2007a). Both enzyme production and phosphate solubilization depended on water medium content, type of nitrogen source, inoculum size, and the presence and initial concentration of phosphate in the medium. It was found that at optimized process conditions (moisture 70%, corn steep liquor as a nitrogen source, inoculum size of 3–4 ml, and presence of slow release phosphate), the filamentous fungal culture was able to produce 5,800 U phytase and 206 mg soluble phosphate per 100 g dry substrate. In another work, employing *P. chrysosporium* and DOW/SB/RP mixture, the white-rot fungus was able to produce 1,100 U/l manganese peroxidase after optimization of SSF parameters (Vassilev et al. 2009c).

Using DOW/RP-based medium supplemented with chitin, *Penicillium janthinellum* partially mineralized the lignocellulosic substrate, solubilized RP, and showed chitinolytic activity which reached 1,612.9 U per kilogram initial dry matter (DOW+chitin; Vassilev et al. 2008b). All fungal microorganisms used in the above experiments were also characterized by a high siderophore production demonstrated in plate assays. In summary, several simultaneous activities were proved by the fungal P-solubilizers such as lignocellulytic activity, organic acid production, solubilization of inorganic phosphates, enzyme (phytase, manganese peroxidase, and chitinase) activities, and release of siderophore-like substances at conditions of SSF experiments.

Multifunctional properties of phosphate-solubilizing microorganisms in immobilized state

During the last years, there has been an increased research interest on immobilization of P-solubilizing microorganisms and preparation and formulation of soil microbial inoculants (Bashan 1998; Vassilev et al. 1997b, 2001a, 2007d; Vassilev and Vassileva 2005; Vassileva et al. 1999). Immobilization is a term that describes the process of bounding enzymes or whole cells to a definite space applying support or carrier materials. Immobilized microbial cells offer a number of potential advantages over free cells, such as easier separation of the biocatalysts from the medium/products, higher stability of the biocatalyst, and much higher activity by one unit volume. In addition, there is no need for continuous replacement of the biocatalysts particularly in fermentation processes. As a result, immobilized biocatalysts are now employed in many bio-catalytic processes. Adsorption of cells onto a surface is the easiest and the oldest method of immobilization. In fact, SSF can be accepted as a process performed by microbial cells attached onto the solid surface of lignocellulosic particles. Entrapment in polymeric matrices tends to be a more controlled method which, in addition, allows immobilization of two or more beneficial soil microorganisms (Vassilev et al. 2001b, c, d). Detailed characteristics of immobilization methods and advantages of immobilized cells of P-solubilizing microorganisms in fermentation and soil conditions have been presented in the review paper of Vassilev et al. (2001a). Here, we will present our most recent studies with a special emphasis on the multifaceted properties of immobilized P-solubilizers (Table 1).

Fungal and bacterial microorganisms have been immobilized in various gel carriers to solubilize RP in the presence of agro-wastes bearing in mind the excellent results obtained in previous studies (Vassilev et al. 2001a). Multifaceted fungal metabolic behavior was observed when *P. chrysosporium* was encapsulated in alginate enriched with DOW powder and further cultivated (in repeated-batch process) in a medium with RP (Vassilev et al. 2007b). In this case, manganese peroxidase activity after the first batch cycle was sufficiently higher than the maximum values obtained in the SSF experiment independently of the presence of RP. It is also interesting to note the steady level of soluble P in the medium solution during the whole experiment and of the enzyme activity after the first batch. Despite the absence of agro-waste in the medium, the DOW powder, used as filler in the alginate beads, stimulated the enzyme synthesis. Both types of fermentation were compared (SSF vs repeated-batch with gel-immobilized mycelium) as possible biotechnological schemes for preparation of microbial inoculants in biocontrol experiments. Both processes produced viable cultures of *P. chrysosporium* with efficient biocontrol activity, which was capable of suppressing growth of *Fusarium oxysporum*, although gel-entrapped culture grew faster and, at the end of the studied period, invaded the colony of the pathogenic fungus in plate tests.

Applying the same experimental scheme, similar fungal metabolic behavior was observed when *P. janthinellum* was entrapped in alginate enriched with both DOW powder and chitin from crab shell and further cultivated in RP-supplemented medium (Vassilev et al. 2008b). Again, the enzyme (chitinase) activity was sufficiently higher than the maximum values obtained in the parallel SSF experiment independently of the presence of RP. It is also interesting to note the steady level of soluble P in the medium solution during the whole experiment. Despite the absence of agro-waste in the medium, the DOW powder and chitin, used as fillers in the alginate beads, stimulated the enzyme synthesis compared to combinations with only one or none of the fillers.

Simultaneous RP solutilization and production of indole-3-acetic acid (IAA) has been observed in *Bacillus thuringiensis* entrapped in k-carrageenan grown in repeated-batch mode of fermentation (Vassilev et al. 2007c). After five repeated batch fermentation experiments, an average of 6.9 mg/l IAA was recorded in the presence of 1.5 g/l RP and 4.7 mg/l IAA in the RP-free control. The bacterium simultaneously solubilized phosphates with a maximum soluble P of 115 and 150 mg/l after the fourth batch cycle in 1.5 and 3.0 g/l RP-amended media, respectively. Addition of tryptophan to the media increased IAA production to 13.9 and 20.7 mg/l per batch on RP-free and 1.5 g/l RP-supplemented media with poor bacterial growth and RP solubilization. These studies vitalized our earlier observations on IAA production by *B. thuringiensis* in tryptophan-free and tryptophan-amended media (Marulanda et al. 2002) and confirmed the statement that the immobilized microbial cells show high stability, metabolic activity, and catalytic longevity in comparison to the free cell forms, particularly related to biosynthesis of different organic acids (Kautola et al. 1990; Vassilev and Vassileva 1992; Vassilev et al. 1992a). In fact, there is not much progress in the field of application of immobilization technology in microbial P-solubilization except a recent work on scaling-up our shake-flask-based scheme for RP dissolution by passively immobilized fungal culture (Vassilev et al. 1997a) to higher volume airlift bioreactors (Ahuja and D'Souza 2009), although the advantageous effect of airlift fermentation systems for cultivation of fungal microorganisms producers of organic acids has been shown earlier (Vassilev et al. 1992b).

Effects of P-solubilizing microorganisms/agro-wastes on plant growth and health

Effects of P-solubilizing microorganisms/agro-wastes on plant growth

A variety of beneficial microorganisms are applied to agricultural soils in order to control plant pathogens, increase symbiotic or associative nitrogen fixation, increase plant nutrient availability, and promote plant growth through the production of plant hormones. The results of our first study demonstrate the viability of applying the biotechnological product *A. niger*/SB wastes/RP in improving the growth of plants (Vassilev et al. 1996) which was further proved with different P-solubilizers/agro-waste combinations. These experiments were recently reviewed (Vassilev and Vassileva 2003). Here, we will analyze other properties of the biotechnological approach related to the overall multifaceted characteristics of the P-solubilizers (Table 2).

In general, mineralization of lignocellulosic agro-industrial wastes by microbial processes and simultaneous

Table 2 Plant-growth-promoting and plant health-enhancing activities of P-solubilizing microorganisms

Microorganism/agro-wastes	Activities			References
	PS	PGP	BC	
A. niger/SB	+	+		Vassilev et al. 1995; Rodriguez et al. 1999; Medina et al. 2006
A. niger/OC	+	+		Vassileva et al. 1998; Vassilev et al. 2006b
A. niger/DOW	+	+		Medina et al. 2004b
A. niger/SB+DOW	+	+	+	Vassilev et al. 2008a
P. chrysosporium/DOW	+	+		Vassilev et al. 2006b
P. chrysosporium/SB+DOW	+	+	+	Vassilev et al. 2009c

PS P-solubilization, *PGP* plant growth promotion, *BC* biocontrol, *SB* sugar beet wastes, *OC* olive cake, *DOW* dry olive wastes

solubilization of inorganic insoluble phosphates provide the plants with an organic amendment rich in polysaccharide compounds and available P (when RP is applied in the fermentation process). In addition, this mineralized organic matter can be used as C and energy source for activities of soil microorganisms. This effect could be further extended to enhancement of the soil enzyme activities (see "Increase of soil and plant enzyme activities" section) which are considered as major factors contributing to overall soil microbial activity and soil quality (Garcia et al. 1997). The increase of enzymatic activities in soils is involved in an increase in the availability of nutrients to plants, which in turn have a positive influence on soil fertility thus completing the beneficial set of effects of the biotechnological products developed by our group.

It is important to note that P-solubilizer/agro-waste-amended treatments proved highly efficient in association with arbuscular mycorrhizal (AM) fungi. The differentiation in the AM fungal response in relation to the length of fermentation is an important characteristic of this system. For example, highest plant growth was observed when SB/RP, fermented with *A. niger* for 20 days, was introduced into the experimental soil compared to 15- and 30-day agro-waste/RP treatment process (Rodriguez et al. 1999). In this case, higher plant growth was also registered during the three crop periods in nearly all mycorrhizal treatments in comparison to the non-mycorrhizal control. Mycorrhized *Medicago sativa* L. cv Aragon grown in SB/RP/*A. niger*-amended soil resulted in 219% (first crop), 432% (second cropz), and 387% (third crop) plant growth increase over the non-mycorrhizal control containing untreated agro-waste. Plant uptake of N, and particularly P, was significantly increased by the addition *of A. niger*-treated agro-waste/RP and AM colonization. Similarly, high amounts of Ca, Mg, and K were found in the experimental plants grown in soil supplemented with microbially treated SB/RP.

Each component of the biotechnological product exerts additional effects within the overall beneficial activity. For example, even in heavy metal contaminated soil, the effect of microbially treated SB on shoot biomass of *Trifolium repens* ranged from 434% (without mycorrhiza) to 549% (with mycorrhiza) increase, while in unamended soil, the mycorrhizal effect on shoot biomass was 232% (Medina et al. 2006). However, treated SB/RP increased shoot growth over control by 1,006% (in absence of mycorrhiza) and by 1,192% (in presence of the AM fungus *Glomus mosseae*). Highly increased root development and the number of nodules formed were registered under the same conditions in mycorrhized plants.

The benefits of organic amendments are due also to the improvement of the physical characteristics of the soil, which in turn favor the establishment and viability of a stable plant cover (this effect will be discussed further). In this regard, fermented agro-wastes improved the structural stability of rhizosphere soil of plants to a statistically significant extent. The restoration of soil structure may depend on the amount and nature of the organic matter added. The latter was also found to affect the level of plant root mycorrhization. In AM-inoculated plants, the percentage of AM colonization was increased by microbially treated DOW, SB, and DOW+SB (Medina et al. 2004b). The highest percentage of AM colonization is reached in dually (P-solubilizing plus AM fungi) inoculated plants in amended soil. We also found in this study a great increase in AM spore population provoked by each one of the organic amendments used (values ranged from 319%, in DOW-, to 761%, in SB-amended plant-soil systems, respectively).

Effects of P-solubilizing microorganisms/agro-wastes on plant health

The beneficial effect of microbially treated agro-industrial wastes and partially solubilized RP was further extended to soil-plant systems infected with fungal pathogens (Table 2). The biocontrol potential of our biotechnological products was announced using SB as a substrate (Vassilev et al.

2005) and proved later in a series of studies where the phyto-pathogen was *F. oxysporum* (Vassilev et al. 2008a, 2009b). In these studies, a mixture of SB/DOW and RP was used in the fermentation experiments.

The introduction of *F. oxysporum* into the soil-plant system adversely affected the plant growth and development (Vassilev et al. 2008a). This effect was more pronounced in the control treatment when the fresh plant biomass of 3.1 g was 35% lower compared with 4.75 g obtained in the control non-infected with *F. oxysporum.* The AM fungus *Glomus intraradices* significantly reduced the effect of *F. oxysporum*, and fresh biomass (5.26 g) of mycorrhizal plants was 69.6% higher than that recorded in the pathogen-infected control treatments. The highest plant growth and nutrient uptake among all pathogen-infected treatments were measured in soil-plant systems amended with *A. niger*/SB/DOW/RP and inoculated with *G. intraradices*. In this treatment, differences between levels of plant growth, nutrient (particularly P) uptake, and root mycorrhization of plants grown in the presence and absence of the pathogen were found to be insignificant. Under these conditions, it was found that the presence of *G. intraradices* significantly reduced the effect of *F. oxysporum* on tomato plants accompanied by a significant decrease of 2.3×10^{3} in the number of colony-forming units compared with the control treatment. However, the AM symbiosis only partly limited the negative effect of the pathogen. Further introduction of *A. niger*/SB/DOW/RP into soil demonstrated higher capacity to control the pathogen effect.

Similar results have been achieved with SB/DOW/RP treated with *P. chrysosporium* which produced manganese peroxidase during the fermentation process (Vassilev et al. 2009c). Higher plant growth and nutrient uptake were registered in soil-plant systems amended with *P. chrysosporium*-treated agro-wastes and RP. It was found that the introduction of the biotechnological product into soil demonstrated high capacity to control the phytopathogen fungus (*F. oxysporum*), and only 2.61 log CFU were found per gram soil at the end of the experiment.

The biocontrol function of these products probably is based on more than one mode of action, but production of hydrolytic enzymes by the microbial P-solubilizers and competition for nutrients and space are the most likely mechanisms. In addition, DOW may contribute to the fungal antagonistic effect as olive mill residues are reported to suppress soil borne pathogens (Kotsou et al. 2004). Bearing in mind that micronutrients found in natural phosphates, such as zinc and cooper, are known to suppress pathogens (Duffy and Defago 1999) and the effect of mineralized organic matter and P microbial solubilizers on phyto-pathogenic fungi (Ros et al. 2005), we can explain the potential biocontrol properties described in the above-mentioned studies.

Effects of P-solubilizing microorganisms on improvement of soil properties

During the course of studies with microbially treated agro-wastes and RP by fungal microorganisms, we have demonstrated the multiple beneficial effects of the biotechnological products on a number of soil properties such as soil structure, soil enzyme activity, and soil microbial community (Table 3).

Improvement of aggregate stability

Agricultural wastes, by-products, and other waste disposal amendments of high organic matter content are applied to soils to enhance their fertility and improve their biological and physicochemical properties. Numerous studies, both in the field and under controlled conditions, have demonstrated the important role of organic matter in the formation and stabilization of soil aggregates. Many authors suggest that it is the production of polysaccharides which is responsible for the soil structure improvement (Bearden and Petersen 2000). The microorganisms participate mechanically (union by hyphae) or by the excretion of polysaccharides into the

Table 3 Effects of P-solubilizing microorganisms on soil aggregate stability, soil/plant enzyme activities, and soil microbiota

Microorganism/soil	Effects			References
	AS	S/PEA	SMC	
A. niger/SB/desertified soil	+	+	+	Medina et al. 2004a
A. niger/SB/Zn-contaminated soil	ND	+	ND	Medina et al. 2006
A. niger/SB/acidic mine tailings	+	+	ND	Carrasco et al. 2009a
A. niger/SB or DOW/desertified soil	ND	+	+	Medina et al. 2004b
A. niger/DOW/degraded soil	ND	+	ND	Caravaca et al. 2004
A. niger/SB/metal-contaminated soil	ND	+	ND	Azcon et al. 2009b
A. niger/SB sandy loam soil	ND	+	+	Medina et al. 2007

AS aggregate stability, *S/PEA* soil/plant enzyme activities, *SMC* soil microbial community, *SB* sugar beet wastes, *DOW* dry olive wastes, *ND* not determined medium. The symbiosis between AM fungi and plants has been shown to increase the stability of soil aggregates (Bearden and Petersen 2000).

In a number of publications, we have demonstrated that microbially treated agro-industrial wastes significantly improved soil properties. Aggregate stability, water-soluble C, and water-soluble carbohydrate C were increased in the rhizosphere when semiarid soil was supplemented with *A. niger*-treated DOW and SB alone or in combination with *Yarrowia lipolytica*, AM fungi, and *Rhizobium* using *Dorycnium* plants (Medina et al. 2004a). Root AM colonization was increased considerably by the amendments and by *Y. lipolytica* inoculation. The low nodulation found in this degraded soil was also increased by *A. niger*-treated DOW/SB/RP. The application of organic amendments to the soil increased enzyme (β-glucosidase and phosphatase) activities as well as the biodiversity of AM populations. These first results were further confirmed in other works which showed an increase in aggregate stability by the addition of the amendments (microbially treated SB- or SB+RP) to the soil, while no effect of AM inoculation was observed in heavy metal (Zn)-contaminated soil (Medina et al. 2006). It was suggested that microbial population developed after the addition of the SSF-processed wastes (that can be used as C and energy sources for activities of soil microorganisms) could be responsible for soil aggregate formation and stabilization.

Similar beneficial effect of our biotechnological product related to stabilization of soil aggregates was found in acidic semiarid mine tailings (Carrasco et al. 2009a). Particularly after soil drying, aggregate stability was 66% higher than the control soil. The fermentation mixture of mineralized organic matter and solubilized RP raised the soil pH and increased soil water-soluble carbon under either watering or drying conditions. It was concluded that the addition of microbially treated SB was more effective than inoculation with an autochthonous bacterium, for stabilizing the soil structure of acidic mine tailings during desiccation, although the overall effectiveness was dependent on the degree of acidity of the tailing. Soil surface structure stabilization of metal mine tailings could reduce erosion, protect soil against degradation, and limit the spread of metal contamination particularly when dealing with fragile, semiarid soils which are exposed to a high risk of water erosion. Soil structure has a prevailing role in soil infiltration and biogeochemical processes. Therefore, improved soil structure means increased water retention, nutrient uptake, drainage, aeration, and root growth.

Increase of soil and plant enzyme activities

Soil enzymatic activities were improved in heavy metal contaminated soil amended with microbially treated SB and RP when dehydrogenase, phosphatase, and β-glucosidase activities reached their highest values (Medina et al. 2005, 2006; Table 3). The increase of enzymatic activities in soils is involved in an increase in the availability of nutrients to plants, which in turn have a positive influence on soil fertility (Garcia et al. 1997). Values of β-glucosidase activity, that indicates carbohydrates transformation, showed that *A. niger*-treated SB+AM inoculation increased this hydrolytic activity, which is important as energy source provider for rhizospheric microorganisms. Similarly, dehydrogenase and phosphatase activities (indexes of microbial activity and phosphorus mineralization, respectively) were registered at maximal level when *A. niger*-treated SB/RP and AM fungus were applied, which is an indication of their mutual effect on nutrient cycling and energy flow. In addition, the biotechnological product was found to increase IAA production in rhizosphere Cd-contaminated soil more than AM-colonization.

It is interesting to note the enhanced antioxidant enzyme (ascorbate peroxidase (APX), glutathione reductase (GR), superoxide dismutase (SOD), and catalase (CAT)) activities in plants grown in heavy metal multi-contaminated soil enriched with our biotechnological product combined with autochthonous microorganisms (AM fungi and bacteria; Azcon et al. 2009b). As expected, *A. niger*-treated SB/RP mixture significantly increased micro- and macronutrients and reduced metal concentration, particularly Al and Ni, in plant shoot tissue. Moreover, enhanced antioxidant enzyme activity was observed in amended soil-plant systems. Degenerative reactions related to heavy metal stress are mediated by reactive oxygen species such as free radicals (superoxide and hydroxyl radicals) but also H_2O_2 and singlet oxygen. Hydroxyl radicals and singlet oxygen are so reactive that their production must be minimized. On the other hand, Cd and H_2O_2 are synthesized at very high rates even under optimal conditions and may trigger toxic reactions leading to lipid peroxidation, protein denaturation, and DNA mutations (Bowler et al. 1992). The ability of plants to increase antioxidative protection in order to combat the negative consequences of heavy metal stress appears to be limited as many studies have shown that exposure to elevated concentrations of redox reactive metals reduces rather than increases antioxidative enzyme activities (Schutzendubel and Polle 2002). However, results reported in this study proved that our biotechnological product alone or in combination with the autochthonous microorganisms makes a major contribution to plant antioxidant activities under the conditions of heavy metal stress. It is interesting to note that the effect of solid-state microbially treated agro-industrial wastes/simultaneously solubilized RP on antioxidant enzyme activities differed between the antioxidant enzymes: SOD and CAT activities were reduced by amendments while the opposite effect was observed for APX and GR activities.

In general, high enzymatic activities were related to the stimulating effect of root exudates. Root exudates have a variety of roles including that of metal chelators that may reduce the plant uptake of certain metals. The range of compounds exuded is wide and could play a role in plant metal tolerance (Hall 2002). These compounds are more abundant in rhizosphere of plants with a more developed root system as in our studies occurred with *A. niger*-treated SB/RP and AM inoculated plants. Thus, the role of such treatments alleviating heavy metal toxicity in plant, possibly via exudates chelation, seems to be important. An indication supporting this speculation is the fact that the metabolic (enzymatic) activity of particular groups of rhizosphere microorganisms, involved in nutrient cycling, increased with the specific amendments applied to heavy metal contaminated soil (Hall 2002). When assessing the overall effect of *A. niger*-treated organic matter/RP mixture, it should be bear in mind that in a recent study, we found high siderophores-like production (with metal-chelating properties) by *A. niger* grown in conditions of solid state fermentation processes (Vassilev et al. 2008a).

It is interesting to note that different levels of enzyme activity were observed depending on the waste material (DOW, SB, or both) used in the fermentation process and additional microbial inoculants (AM, *Y. lipolytica*) introduced into soil-plant systems (Medina et al. 2004b). For example, dehydrogenase activity increased in treated agrowaste-amended soil, particularly when microbially treated SB was added. However, in DOW-amended soil, non-significant responses to microbial treatments or dehydrogenase activity were found. Urease activity was highly increased in the rhizosphere of DOW- or SB-amended plants. The highest value was in the rhizosphere of mycorrhized plants growing in DOW treatments. The application of treated agro-wastes enhanced protease activity, and *Y. lipolytica* significantly increased such activity in DOW-amended soil.

It is widely accepted that soil enzyme activities are highly sensitive biochemical parameters indicating perturbations caused by soil treatments (Naseby and Lynch 1997). They give an indication of ecosystem function rather than just a measurement of perturbation. The increases observed in enzyme activities may be related mainly to reactivation of the rhizosphere microbial population as a consequence of the addition of our biotechnological product in combination or not with inoculation treatments.

Improvement of soil microbial community characteristics

The above-described studies showed that the effects of our biotechnological products on plant growth and health were more pronounced in combination with AM fungi. The question is how a product consisting of P-solubilizing microorganism/mineralized organic matter/partially solubilized RP affects the development and functionality of AM fungi and other soil microorganisms? An increased AM fungal growth and activity was found in the presence of *A. niger*-treated organic wastes introduced into plant-soil systems in compartmentalized growth units (Medina et al. 2007; Table 3). It was suggested that modification of soil microbial structure and production of exudates by the P-solubilizing microorganism could explain the hyphal growth increase of three AM fungi. On the other hand, it is well known that AM fungi affect microbial community in both direct and indirect ways (Johansson et al. 2004). In this work, however, the addition of the biotechnological product enhanced the amount of biomarker fatty acids of all groups of microorganisms as a result of the increase of carbon source, while the microbial community was unaffected by inoculation with AM fungi. It is important to note that biomarkers were measured in the hyposphere in the absence of the influence of roots.

A recent study evidenced that *A. niger*-treated SB/RP amendment is a suitable tool for increasing and changing the bacterial community in rhizosphere (Azcon et al. 2009a). In this work, the bacterial-community profiles were generated from DGGE of the amplified soil DNA. Soil microbial properties such as biodiversity and dominance index increased by the application of the treated SB agrowaste and concomitantly favored the plant development. An important result was that RP fertilization and single AM inoculation (used in parallel) similarly promoted plant biomass, but only AM inoculation increased microbial diversity in the presence of SB amendment. Therefore, the application of the biotechnological product seems to play a decisive role in improving soil properties.

Effects of P-solubilizing microorganisms on soil rehabilitation

Experiments aimed at soil rehabilitation have been oriented to reclamation of soils contaminated with heavy metals and aforestation of desertified Mediterranean sites.

Effect of P-solubilizing microorganisms on heavy-metal-contaminates soils

Elevated concentrations of heavy metals in soil from anthropogenic sources (fertilizers and amendments) or mining activities (Shetty et al. 1995) pose long-term risk to environmental and sustainable production. The deteriorated physical and biological characteristics of contaminated soils need to be improved to establish a vegetation cover. Heavy metal-contaminated soil can be treated by operations based on chemical, physical, or biological techniques. It is generally accepted that these remediation techniques may be grouped into two main categories: (1) ex situ techniques which are based on mechanical removal of the contaminated soil and subsequent disposal to a landfill site for further treatment and (2) in situ methods, which avoid excavation, transportation, and land-filling costs by treating the metal-contaminated soil in place. In situ techniques are easy to perform, favored over the ex situ techniques, and preferred due to their lower cost and reduced impact on the ecosystem (Khan et al. 2004; Kumpiene et al. 2008).

An interesting approach applied in in-situ remediation of heavy metal-contaminated soil includes a variety of phosphate amendments such as soluble phosphate and P-bearing insoluble sources, mainly RP. Ma et al. (1993) showed that hydroxyapatite is a very efficient metal immobilizer in laboratory conditions. Most of the further studies on heavy metal stabilization using this approach were carried out with a wide range of natural and synthetic P-bearing amendments, such as hydroxyapatites (Arnich et al. 2003), synthetic and natural apatites (Raicevic et al. 2009), phosphate rock (Chen et al. 2007), as well as their combinations (including other P-containing compounds).

On the other hand, it is well known that the-bioavailability of heavy metals in soil is affected by the presence of organic matter. Organic matter retains effectively heavy metals forming complexes and chelates of varying stability (Leita et al. 1999; Kiikila et al. 2002). Pascual et al. (1997, 1999) demonstrated that the addition of various forms of organic wastes to the soil positively changed some important soil characteristics such as biomass carbon, basal respiration, biomass C/total organic C ratio, and activated the metabolism of soil microorganisms. In particular, various microorganisms can mobilize metals through reactions such as autotrophic and heterotrophic leaching, chelation, and methylation (Gadd 2004). For example, acidification through organic acid production and siderophores can supply both protons and metal complexing anions thus leading to metal release (Gadd and Sayer 2000).

Bearing all the above in mind, our biotechnological scheme was employed for the first time in heavy metal-contaminated soil rehabilitation activities (Medina et al. 2005, 2006). In a microcosm experiment, 50 g/kg of the mixture produced after solid-state fermentation processes of SB treatment by *A. niger* in presence or absence of RP was introduced into a heavy metal (Zn or Cd) contaminated soil-plant system. The mixture of microbially mineralized agro-waste, simultaneously (partially) solubilized RP, and the fungal mycelium were the most effective amendment in improving growth and mineral nutrition of the white clover used as a test plant. In combination with the mycorrhizal fungus *(G. mosseae*), this treatment increased plant growth about 28 times more than in non-mycorrhized control plants. It is important to note that *Trifolium* plants failed to establish in non-amended Cd-contaminated soil (Medina et al. 2005). To illustrate the advantage of our biotechnological product, it should be noted that the effect of *G. mosseae* on plant biomass shifted from +86% (in the treatment without amendment) to +1,192% in SB+RP+*A. niger* amended Zn-contaminated soil (Medina et al. 2006). Improved nodule formation was observed in soil-plant systems inoculated with *Rhizobium trifoli* and amended with microbially treated SB and RP, while this process was highly depressed in Zn-contaminated control soil and was nearly zero in control Cd-contaminated soil. As consequence of an enhanced plant biomass, Zn phytoextraction increased by 1,832% over untreated ones. Similar results were registered in Cd-contaminated soil. In a separate phytoremediation experimental scheme using a natural heavy metal multi-contaminated soil, our biotechnological product was successfully tested in combination with autochthonous mycorrhizal fungi and bacteria such as *Bacillus cereus* (Azcon et al. 2009a, b). The bacterium was previously proved as a heavy-metal tolerant microorganism. The SB/RP/*A. niger* amendment increased the content of most of the analyzed elements (B, Cu, Mo, Cd, Cr, and Mn) except for AI. Mycorrhizal and bacterial addition improved this effect for whatever metal present in the multi-contaminated soil. Plant biomass (*T. repens*) highly increased compared to non-amended control, which is an important result particularly in phytoremediation experiments.

All experiments for application of our biotechnological products based on microbially treated agro-industrial wastes and RP in soil-plant systems contaminated with heavy metals showed higher efficacy in the presence of AM fungi. AM fungi, as an important part of the soil microbiota, are known for their ability to significantly improve plant nutrition providing mineral nutrients including heavy metals (Khan et al. 2000). Despite many contradictory studies, it is generally believed that these fungi may selectively be included in various biotechnological schemes for heavy metal land remediation. Some recent publications state that the beneficial effect of AM fungi on heavy metal accumulation in plants depends on both mycorrhizal and metal characteristics (Wang et al. 2005). According to Zhu et al. (2001), the level of heavy metal contamination in the soil has definitive influence on the AM plant-protective role (increasing or decreasing metal concentration in shoot tissues). If AM colonization protects the host plant against metal toxicity, the AM colonization and plant-available metal may be correlated. Nevertheless, the effects of AM colonization on host concentration of metals have been shown to vary with the host plant and fungal species (Kaldorf et al. 1999).

Results of these experiments showed that the combination of plant growth enhancement and reduced metal translocation caused by our biotechnological product combined with additional microbial inoculations could be regarded as a promising strategy for remediating heavy metal contaminated soil.

Effect of P-solubilizing microorganisms on desertified soils

In another series of experiments, our biotechnological products were successfully tested in aforestation of a desertified Mediterranean site (Caravaca et al. 2004). The establishment of a plant cover based on the use of seedlings with an optimized microbiological and physiological status is paramount in order to carry out successful re-aforestation activities in desertified ecosystems, particularly those developed under Mediterranean environments. Drought tolerant, native shrub species have been recommended for re-establishment of functional shrub lands and recovery of desertified Mediterranean ecosystems. The results of this study demonstrate the viability of applying the fermented DOW in the presence of RP in order to improve the growth of the woody legume *Dorycnium pentaphyllum*. This could be due to an improvement in the available nutrient supply in the soil, arising from the fermented DOW. During the course of *A. niger* fermentation, the RP was partially solubilized thus increasing the level of bio-available P in the DOW (Vassileva et al. 1998). Thus, plants grown in the amended soil had higher (NPK) nutrient contents in their tissues than non-amended control plants. The combination of fermented DOW/partially solubilized RP and mycorrhizal inoculation treatments considerably improved the performance of *D. pentaphyllum*. The rapid growth of inoculated seedlings, as compared with the un-inoculated seedlings, in the amended soil might be related to the capacity of the fungus to increase available P uptake from fermented DOW as observed in other experiments with our biotechnological product. Therefore, such a combined treatment employing microbiologically treated agro-industrial wastes/solubilized RP and AM fungi can be applied in re-vegetation strategies in typical Mediterranean conditions.

Conclusions and future trends

There is accumulating evidence that P-solubilizing microorganisms pose wide variety of functions which facilitate their implications in large number of agro-, bio-, and environmental technological strategies. These microbial features can be evidenced and studied in fermentation conditions and soil-plant system as well.

The results presented here emphasize the importance of studying simultaneously different metabolic features of soil microorganisms, including P-solubilizers. Enzyme synthesis, phytophormone and antibiotic production, and siderophore release are now well documented. However, these characteristics should be investigated further in different fermentation process parameters in order to establish well-defined conditions for metabolic control. The latter, combined with the utilization of other agro-industrial wastes, will permit wider possibilities for exploitation of the described biotechnological approach in agro-ecosystems.

Another advantageous feature that should be exploit is related with the fact that our laboratory SSF technological schemes gather both (in some sense) distinct operations such as fermentation and formulation in one single process. Here, a possible further progress can be expected in development and/or optimization of large-scale SSF processes with selected P-solubilizers with at least high organic matter mineralization and organic acid (and consequently solubilizing) activity. Any additional activity would enhance the economical (cost and application) significance of the overall technology for the preparation of microbial inoculants with complex (multiple) action modes.

Combination of soil microorganisms with well-defined P-solubilizing activity but which use different nutrient sources and/or demonstrate optimum metabolic capacity at different environmental parameters (temperature, pH, humidity, etc.) would probably enhance the multifaceted characteristics of all microbial mixture participants applied in different soil-plant systems. We have successfully tested such combinations between P-solubilizers, *Rhizobium*, and AM fungi in gel-based formulations (Vassilev et al. 2001b, c, d).

The application of our biotechnological product in bioremediation strategies is promising particularly in heavy metal-contaminated and desertified soils. In such sites, where soil microbiota is deteriorated and conventional physic-chemical techniques are unsuccessful, the multifunctionality of P-solubilizers/mineralized organic natter/partially solubilized RP seems crucial in improving soil characteristics and plant cover. Further research in this particular field should be concentrated on long-term experiments and application of traditional and novel plant phytoextractors.

Interactions with indigenous microbial populations should also be considered particularly in non-sterile, natural conditions. Reliable methods (polymerase chain reaction, restriction fragment analysis, and molecular techniques) for detecting colonization by the microbial P-solubilizers should be used to assess the efficacy of their introduction into soils and their long-term effect on the overall microbial community. Integration of the microbial products discussed in this review with agrochemicals and/or other agricultural practices can enhance the possibility of applications in wider set of environmental conditions and different soils-plant systems.

A close collaboration between soil microbiologists, fermentation specialists, and agronomists is a must to achieve acceptable results when applying similar bioproducts to avoid erroneous procedures such as the addition of agar to the fermentation mixture which makes no sense of the SSF processes based on agro-wastes as substrates and conclusions concerning improved soil properties due to increase microbial polysaccharide production (Caravaca et al. 2006; Alguacil et al. 2008; Roldan et al. 2008; Carrasco et al. 2009b). For this reason, these works are not discussed here.

Although further studies and careful validation of the laboratory/greenhouse-developed biotechnological schemes, described in this review, should be carried out in field conditions over extended time periods, the fermentation products based on the actions of multifunctional P-solubilizers can be accepted as feasible instruments in the sustainable agriculture and environmental microbiology.

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