Mansoura Journal of Biology Vol. 36 (2), Suppl. (1) Dec., 2009.

DEGRADATIVE CAPACITY OF *BACILLUS SUBTILIS* FOR ORGANIC PHOSPHORUS

Abou- Dobara, M. I., El –Katony, T. M., Hassan, N. M. and Ghozy, E. A.
Botany Department,Faculty of Science (Damietta branch), Mansoura University.

ABSTRACT

Sixteen bacterial isolates, which had the ability to tolerate detergent, were isolated after 3 days from soil sample incubated with detergent (persil). These isolates were found to be endospore-forming rods. Three of these bacterial isolates were found to have the ability to modify and release phosphorus from the detergent (persil) and one of them was identified as *Bacillus subtilis*.

Detergent solution (2 % persil) was incubated with *Bacillus subtilis* and the amount of released phosphorus was 116.2693 ppm and the major cations contents were also recorded, namely, potassium (0.329 mM), sodium (125.10 mM) and calcium (1.0317 mM).

Keywords: *Bacillus subtilis*, detergent, degradation.

INTRODUCTION

Phosphorus (P) is ranked second to nitrogen as the key plant nutrients. Phosphorus has important roles in energy transformation within the cell, cell division, and stimulation of early root growth, hastening plant maturity, fruiting and seed formation **[Schachtman *et al.*, (1998)]**.

Detergents are the major source of P in water bodies through sewage and drainage systems. The major part of detergents comprises builders containing polyphosphate salts as sodium tripolyphosphate, **[(Köhler (2006)]**. An environment friendly and effective synthetic builder is yet to be developed to replace existing P-containing builders of detergents **[Khan & Ansari (2005)]**. In addition to their use in industrial and domestic premises, surfactants are used in agriculture to enhance penetration of herbicides to control weed and for dispersing oil spills at sea and in firefighting's foams **[Hess & Foy (2000); Hartskeerl *et al.*, (2004); Madhou *et al.*, (2006) and Ezemonye *et al.*, (2006)]**.

In semiarid regions, wastewater irrigation is a valuable resource for agricultural productivity. The contamination of irrigated soils with surfactant is one of the ecological risks related to irrigation with untreated wastewaters. Increasing concentrations of alkylbenzen sulfonate led to a decrease in soil microbial biomass and

an increase in soil respiratory activity and denitrification rate [**Friedel *et al.*, (1999) and Elsgaard *et al.*, (2001)**].

The degradation of these chemicals is thus essential to save the environment. In addition, after treatment of sewage the P contained in these detergents can be utilized to promote growth of plants. The major factor contributing to triphosphate degradation in waste water treatment was shown to be biological in nature, with the most likely mechanisms being enzymatic hydrolysis [**Halliwell *et al.*, (2001)**].

The role of microorganisms in degradation of different surfactants, either anionic, amphoteric or non-ionic has been reported by [**Kloepper-Sams *et al.*, (1996) and Garland *et al.*, (2004)**].

Nevertheless, the breakdown products of surfactants might be more toxic than the original compound. In this respect, Scott and [**Jones (2000)**] reported that alkylphenol ethoxylates, one of the most currently used surfactants, showed little toxicity but their breakdown products, principally nonyl and octyl phenols adsorb readily to suspended solids and are known to exhibit oestrogen-like properties, possibly linked to a decreasing male sperm count and carcinogenic effects.

The aim of this work is to isolate some bacteria capable to tolerate detergent and degrade it to use the degradative detergent as a nutrient for plant growth.

MATERIALS AND METHODS

Collection of soil sample:

Soil sample was collected from Meet Assas, Mansoura, Dakhlyia Province, into clean plastic bags. The sample was used directly for bacterial isolation and then stored in cold conditions.

Isolation, counting of bacteria and actinomycetes from native soil:

The dilution plate technique described by [**Johnson *et al.*, (1959)**] was used for isolation of bacteria and actinomycetes. Under aseptic conditions, 10 gm of soil sample and 90 ml of sterile water, were shaken mechanically for 20 minutes on an orbital shaker before serial dilutions were made (10^{-1} to 10^{-6}) for isolation of bacteria on nutrient agar (beef extract, 3 g; peptone, 10 g; NaCl, 5 g; distilled water, 1000 ml, and the pH was adjusted to 7.0.) whereas actinomycetes were isolated on starch –nitrate agar medium (Waksman, 1959) (containing g/L: starch, 20 g; KNO_3, 2.0 g; K_2HPO_4, 1.0 g; $MgSO_4.7H_2O$, 0.5 g; NaCl, 0.5 g; $CaCO_3$, 3.0 g; $FeSO_4.7H_2O$, 0.01 g; trace salt solution, 1.0 ml, distilled water, 1000 ml, agar, 20 g, and the pH was adjusted to 7.2.). Trace salt solution contains $FeSO_4.7H_2O$, 0.1 g; $MnCl_2.4H_2O$, 0.1 g; $ZnSO_4.7H_2O$, 0.1 g per 100 ml of distilled water. A triplicate set of plates was used for each dilution and incubated at 30 ^{o}C for 24 hours or 48 hours for bacteria and for 7 days for actinomycetes. Plates were then examined and the isolated colonies were counted, picked and purified by sub-culturinng.

Isolation of bacteria and actinomycetes from detergent-treated soil:

100 gm of soil was mixed with 2 % (w/w) detergent (Persil) and then incubated at 30 °C., 1.0 gm of soil was withdrawn under aseptic condition after 3, 6, 9, 15 and 21 days for isolation of bacteria and actinomycetes on either starch-nitrate agar medium or nutrient agar medium as previously reported.

Bacterial degradative capability on detergent:

The degradation of detergent by three bacterial isolates were studied according to the following. Four treatments: 1) nutrient broth + equal amount of detergent (2 %) + inoculum, 2) nutrient broth + equal volume of H_2O + inoculum, 3) detergent (2 %) + equal volume of H_2O + inoculum, and 4) nutrient broth + equal amount of detergent (2 %).

An equal inoculum of each of the selected bacteria (4 X 10^3 CFU/ml) was used to inoculate 160 ml treatment in 250 ml conical flask, which were incubated shaken at 50 rpm at 30 °C. After 0, 1, 2, 4 and 7 days, aliquotes of 5 mls were withdrawn and phosphorus content was determined for each treatment. The most potent bacterial isolate was selected and its degradative ability was reaffirmed.

Determination of total phosphorus:

Total phosphorus was determined according to the method of John (1970) with some modifications.

A sample volume of about 1 ml or 0.5 ml was put in a labeled 50 ml volumetric flask. A deionised water blank and a range of standards were made by adding (0.5, 1, 2, 3, 4 or 5 ml) of the 5 ppm standard (KH_2PO_4) to 50 ml volumetric flask for 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 ppm phosphorus respectively. All the samples and standards were made up to approximately 40 ml with deionised water. Two drops of 2,4 dinitrophenol indicator were added to each flask, NaOH (5M) was added dropwise until the solution become yellow then HCl (2M) was added until the solution just becomes colorless again. Five mls of mixed reagent C (four parts of Reagent A (Acid Antimony Molybdate) with one part of Reagent B (2.5 % Ascorbic Acid) were added to each flask. The solution was shaken and made up to 50 ml with deionised water. After approximately 30 min, the absorbance of the samples was measured on a Spectronic 20 D spectrophotometer at 710 nm. The concentration of phosphorus was calculated as ppm using the standard curve.

Determination of the major cations:

Potassium, sodium and calcium were determined in the clear extract by using a Jenway PFP7 Flame Photometer according to the method described by [**Allen *et al.*, (1986)**] after digestion with sulphuric acid.

Identification of the selected bacterial isolates:

Isolated bacteria were identified depending on their growth characteristics and various morphological, physiological and biochemical activities according to Bergey's

Manual of Systematic Bacteriology [**Sneath (1986)**] and The Procaryotes [**Slepecky & Hemphill (1991) and Claus et al., (1991)**].

Statistical analysis:

Statistical analysis was done according to [**Snedecor & Cochran (1980)**] using Least Significant Difference (LSD) at 5 % level.

RESULTS

Degradative capability Of Bacteria and Actinomycetes from native and treated-detergent soils:

Large number of bacterial isolates and actinomycetes colonies were isolated from native soil collected from Met Assas, Mansoura, Dakhlyia Governorate. The count of bacteria and actinomycetes varied considerably. In the native soil, bacteria were found to be more abundant (4.1×10^4 CFU/ g dry soil) than actinomycetes (3.304×10^3 CFU/ g dry soil) after 24 hours and 7 days respectively.

When the detergent was added to the soil sample, the bacterial and actinomycetes counts were reduced to 5507 CFU/ g dry soil and 3304 CFU/ g dry soil respectively after 6 days incubation, almost ten fold after 9 days, the number of bacteria was still high (29736 CFU/ g dry soil). However, the lowest record of actinomycetes has been recorded after 9 days incubation (1101 CFU/ g dry soil). After 15 and 21 days, no actinomycetes were recorded.

Sixteen bacterial isolates were continuously recorded as high number and tolerant bacteria to the detergent after 3 days. These bacteria were subcultured and stained with Gram and endospore stains and all the sixteen bacterial isolates are gram-positive rods and endopsore forming bacteria with different shapes and characteristics features of the endospore. All the bacterial isolates were grouped into three types: type 1 (one isolate), type 2 (two isolates) and type 3 (thirteen isolates).

After 21 days, only three bacterial isolates were recorded as the most tolerant bacteria to the detergent. These three bacterial isolates were inoculated into medium containing detergent and the results of phosphorus degradation were recorded in (Figure 1.). After 7 days incubation, the amount of the released phosphorus from the detergent increased and the largest amount was recorded in the metabolite of the isolate 1 (116.27 ppm), followed by isolate 15 (113.99 ppm). Isolate 8 released the minimum amount of phosphorus (112.18 ppm) (Figure 1.).

The most potential phosphorus degradative bacteria (isolate 1) was selected for more experiments.

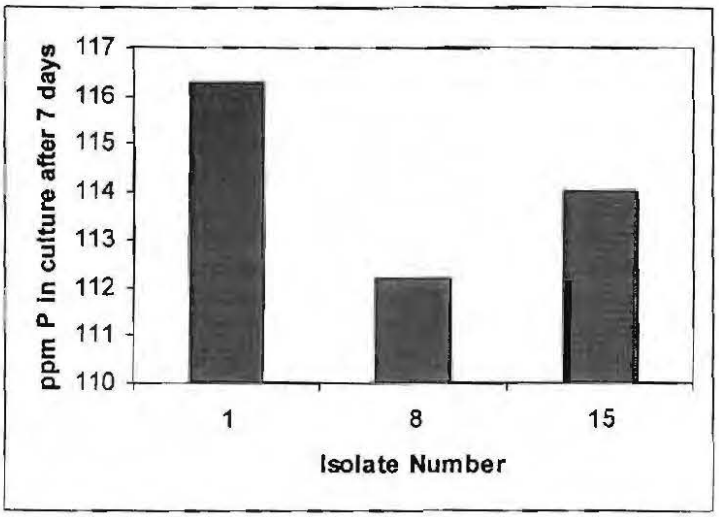

Fig. (1): Phosphorus release from the detergent ((2 %) by the most potential selected bacterial isolates.

Detergent solution was incubated with the selected isolate (isolate 1) of soil bacteria for 7 days and the amount of molybdate reactive P was assayed at intervals as shown in figure 2. From this figure, it is clear that P is released gradually and the concentration of P in the incubated detergent either with or without nutrient broth increased up to a certain limit compared with control value. The content of released P after incubation of selected bacterium with detergent including nutrient broth was highly significantly ($P < 0.05$) greater than those corresponding values without nutrient broth. In addition to that the growth of bacteria increased significantly ($P < 0.05$) with increasing the incubation period of the bacteria with the detergent either with or without nutrient broth increased up to a certain limit compared with control value (Figure 3).

K^+, Na^+ and Ca^{+2} were also measured after detergent incubation with the selected bacterium for 8 days, and the filtrate contained also the major cations that are needed for higher plants nutrition. The filtrate contained 0.329, 1.031 and 125.10 mM for K^+, Na^+ and Ca^{+2} respectively. This can be considered as a base for preparation of nutrient solutions in comparison with the standard solution.

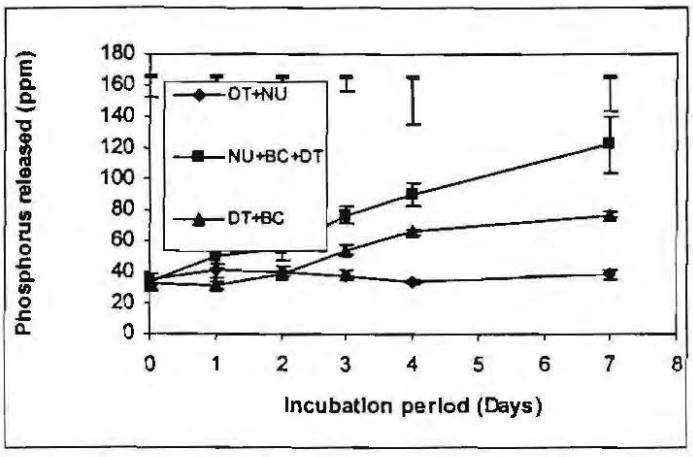

Fig. (2): Phosphorus release from the detergent incubated with the selected bacterium. Each value is the mean of 4 replicates ± SE. Vertical bars represent least significance difference (LSD) at 5 % level. BC indicates to bacterial culture; NU, nutrient broth; DT, detergent.

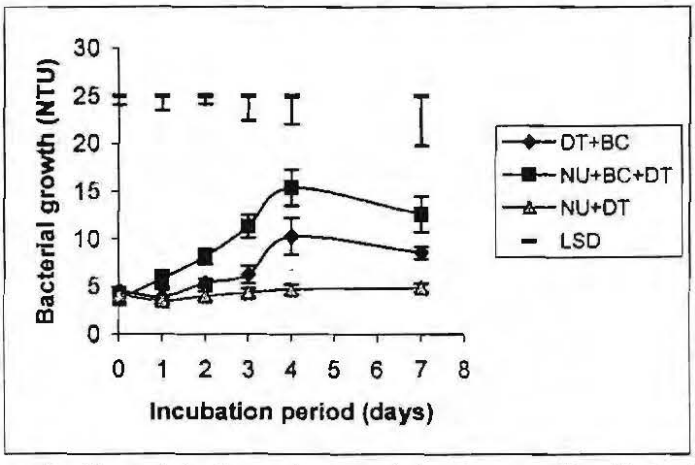

Fig. (3): Growth of bacteria in the nutrient-containing detergent (Persil). Each value is the mean of 4 replicates ± SE. Vertical bars represent least significance difference (LSD) at 5 % level. BC indicates to bacterial culture; NU, nutrient broth; DT, detergent.

Characterization and identification of the selected bacterial strain:

Bacterial isolate No. 1 was selected for more studies. Agar colonies of strain No. 1 are dull, yellowish, irregular, flat, opaque, highly spreading on nutrient agar medium.

Morphology and sporulation:

Isolate No. 1 had rod-shaped cells. The cell width is less than 1.0 μm and the length is about 1.0 μm .The isolate is Gram positive, and endospore forming and the sporangium is not swollen. Spore is Ellipsoidal in shape and terminal in position.

Biochemical and physiological properties:

The isolate produced acids from D-glucose, L-arabinose, D-xylose, and D-mannitol. The isolate coagulated casein, reduced nitrate, produced Di-hydroxy- acetone, and catalase. The isolate has the ability to hydrolyze starch, breakdown urea, and utilize citrate. The isolate was not able to liquefy gelatin, and failed to produce neither hydrogen sulphide nor indole and can not utilize neither propionate nor phenylalanine. Voges Proskauer test and proteolysis of egg were positive. The isolate can grow at 30 °C but not at 5°C and 50 °C, and can grow at pH 5.7 The isolate can grow in the presence of 2 % - 5 % and 7 % of NaCl.

Morphological and biochemical studies of strain 1 suggested its belong to the genus *Bacillus* as supported by Bergey's Manual of Systematic Bacteriology and was found to be closely related to *Bacillus subtilis* Ehrenberg, 1835 **[Sneath (1986); Slepecky & Hemphill (1991) and Claus *et al.*, (1991)].**

DISCUSSION

Phosphorus (P) is one of the major essential macronutrients for plants and is applied to soil in the form of phosphatic fertilizers. However, a large portion of soluble inorganic phosphate applied to the soil as chemical fertilizer is immobilized rapidly and becomes unavailable to plants **[Goldstein (1986)]**. Also, soil contains large amount of phosphorus that exists in insoluble forms **[Kim et al., (1998)]**. So, P is abundant in several soils and is one of the major nutrients limiting the plant growth. The overall P use efficiency following phosphate fertilizer application is low because of the formation of insoluble complexes **[Vassilev & Vassileva (2003)]**.

Microorganisms are involved in a range of processes that affect the transformation of soil P and are thus an integral part of the soil P cycle. In particular, soil microorganisms are effective in releasing P from inorganic and organic pools of total soil P through solubilization and mineralization **[Hilda & Fraga (1999)]**.

In this study, a large number of bacteria were isolated from soil incubated with detergent (Persil). This report represents one of only a few studies on the isolation and characterization of bacteria able to modify the detergent (Persil). Several isolates of bacteria, that able to grow in the presence of detergent (Persil) in the soil, were isolated. Sixteen bacterial isolates were found to be tolerant to the detergent. Most of these bacterial isolates were recorded as Gram-positive and endospore-forming rods. Bacterial endospores are highly resistant to chemical and physical agents including many disinfectants such as the detergent. This character of bacterial endospore is of great importance in the ecology of bacteria.

Furthermore, a combination of morphological and physiological properties were used to identify one isolate (the most tolerant and more degrading-detergent bacterium). The selected strain was identified as *Bacillus subtilis*. The wide spread

occurrence of *Bacillus* species in natural localities can be referred to physiological and genetical adptation. During this work it was noted that *Bacillus subtilis* was capable to degrade detergent and release phosphorus from the detergent and can be considered as a base of nutrient medium for higher plants.

Chen *et al.*, (2006) reported that the ability of a few soil microorganisms to convert insoluble forms of phosphorus to an accessible form is an important trait in plant growth-promoting bacteria for increasing plant yields. The use of phosphate solubilizing bacteria as inoculants increases the P uptake by plants. Isolation, screening and characterization of 36 strains of phosphate solubilizing bacteria (PSB) from Central Taiwan were carried out. Ten isolates belonging to genus *Bacillus*, nine to genus *Rhodococcus*, seven to genus *Arthrobacter*, six to genus *Serratia* and one each to genera *Chryseobacterium*, *Delftia*, *Ordonia* and *Phyllobacterium*. In addition, four strains namely, *Arthrobacter ureafaciens*, *Phyllobacterium myrsinacearum*, *Rhodococcus erythropolis* and *Delftia* sp. are reported for the first time as phosphate solubilizing bacteria (PSB). The removal of phosphorous compounds along with organic matters, by a denitrifying photosynthetic bacterium, *Rhodobacter sphaeroides* has been reported by **[Takeno *et al.*, (1999)]**.

Pseudomonas spp. and *Klebsiella* spp. could be regarded as phosphorus removal bacteria **[Lina et al., (2003)]**. Also, one of the isolates, strain NM-1 tentatively identified with the genus *Micrococcus* accumulated a large amount of phosphorus and its content reached 166 mg of phosphorus per g of cells. Some soil bacteria like *Enterobacter agglomerans* may have the capability to solublize insoluble P and hydrolyze organic P for plant growth **[Kim *et al.*, (1998)]**. Thus, identification and characterization of soil phosphate solubilizing bacteria (PSB) for the effective plant growth-promotion broadens the spectrum of phosphate solubilizers available for field application **[Chen *et al.*, (2006)]**.

Anionic surfactants degrading Citrobacter braakii was isolated and tested in bioaugmentation for industrial wastewater treatment. It was used for the continuous degradation of highly concentrated sodium lauryl ether sulfate (SLES) synthetic medium and cosmetic industry wastewater **[Dhouib *et al.*, (2003)]**.

In this study household detergent (Persil) was assessed as a potential alternative source of P, in comparison with the standard source of P in most hydroponic studies (KH_2PO_4). Detergents usually contain a considerable amount of P, added as builders in the form of sodium tripolyphosphate. Unfortunately, an environment-friendly and effective synthetic builder is yet to be developed to replace the existing P-containing builders **[Khan & Ansari (2005)]**. Therefore, huge amounts of P are discharged into sewage daily, which contributes to increasing eutrophication of water streams.

Detergent solution was incubated with selected isolates of soil bacteria for a specific time and the amount of molybdate reactive P (P_i) was assayed at intervals. Our results revealed that upon incubation of the detergent with bacteria P_i is released gradually and the concentration of Pi in the filtrate increased up to a certain limit specially when nutrient broth was used in combination with detergent. At the same time the filtrate contains the major cations K^+, Na^+ and Ca^{++}. In conclusion, this study may lead to friendly environmental process to convert detergent into available P and major cations for higher plants.

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ACKNOWLEDGEMENT

We thank Prof. M. M. Nematt Allah, Botany Department, Faculty of Science at Damietta, Mansoura Universitry for his help in statistical anaylsis.

الملخص العربى

القدرة التحليلية لباسيلس ستليس للفوسفور العضوي

محمد إسماعيل أبودبارة – طه محمد القاطوني – نعمت محمد حسن –
إيناس عبد اللطيف غزي
قسم النبات – كلية علوم دمياط – جامعة المنصورة – فرع دمياط

فى هذا البحث تم عزل مجموعة من البكتيريا التى لها القدرة على تكسير البرسيل وتحرر الفسفور منه.

وتم اختيار نوع واحد من هذه العزلات البكتيرية وتم تعريفها علي أساس دراسات مورفولوجية وبيوكيميائية علي أنها باسيلس ستليس ، وكانت الأكثر قدرة على التكسير وبهذه الطريقة يمكن تحويل هذه المركبات الكبيرة المعقدة إلى مركبات يمكن للنبات الاستفادة منها، وأنه بإمكاننا استخدام بعض الكائنات البكتيرية فى تكسير هذه المركبات وإعادة استخدامها والاستفادة منها وقد اتضح ذلك مع محلول البرسيل المحضن مع البكتيريا الذى تم استخدامه كمحلول مغذى .