Research Article

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Production of phosphorus biofertilizer based on the renewable materials in large laboratory scale

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Abstract: This paper discusses the effect of the utilization of *Bacillus megaterium* in the microbial solubilization process where poultry bones or ash were used as a source of renewable phosphorus. The process was performed in a large scale laboratory. The pH of the solution decreased during one-week solubilization, which had a direct influence on the increased concentration of phosphorus determined in the solution. It was proved that the phosphorus concentration in the solution was significantly correlated with the biomass concentration and pH. The trial allowed verification of the suitability of the method to prepare two P fertilizers: one based on poultry bones and one on ashes. The elemental analysis of their composition suggests that the bones are a P-bearing resource with properties better than ash. That enables for more efficient scaling-up the solubilization although the concentration of total phosphorus was comparable in both cases. The total amount (100%) of phosphorus was present in a form available to plants in the formulation based on the poultry bones, while 64% of plant available phosphorus was present in the formulation based on the ash. The concentration of Cd was significantly lower in the case of fertilizer based on bones with respect to ash.

Keywords: biofertilizers; phosphate solubilizing bacteria; animal bones; sewage sludge ash; large laboratory scale.

1 Introduction

The main branches of industry in the future will concentrate mainly on the reuse and recycling of by-products, since closing the loop by the elaboration of the new technologies that enable to use wastes from industry is the main assumption that stays in line with circular economy [1]. Nowadays this issue is related mainly to resources with a significantly high economic value that are considered as critical raw materials of which a stable and continuous supply is burdened with high risk [2].

The supply of phosphate rock is at risk due to limited natural resources available. The actual prediction reported in the literature indicated that in 100 [3]–150 years [4, 5] we will be deprived of this main substrate for phosphate fertilizers industry. In view of its importance in stable fertilizer production, steps to overcome the dependence of agricultural production from phosphate rock have to be made. As a result of this, a few European countries (Switzerland, Sweden, Austria and Germany) have introduced regulations that obligate recycling of phosphorus from wastes or residues deriving from food production process or wastewater treatment plants, for example in the form of struvite [6, 7, 8] that is considered a source of phosphorus widely available to plants and can be used for crop fertilization [9].

Another method of transforming P-bearing materials into valuable fertilizer products described in the literature utilizes the natural ability of soil microorganisms to solubilize scarcely available P sources by the production of low molecules of organic acids [10–16]. Many experiments have been undertaken on this topic [16–18]. The following renewable resources can serve as a source of P: poultry bones, fish bones and ash. And as a microbial agent, different kinds of microorganisms such as: *Acidithiobacillus ferrooxidans*, *Bacillus megaterium*, *Bacillus cereus* and *Bacillus subtilis* [19, 20]. The presence and concentration of different kinds of organic acids produced by bacteria were determined and correlated with the specific kind of P-bearing materials and the kind of bacteria that was used in the solubilization experiment

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[21, 22]. The utilitarian properties of obtained phosphorus fertilizer were evaluated in the pot experiments and field test, giving a promising outcome; the dry mass, the growth was higher when compared with the control group where the standard source of phosphorus was applied. The obtained results clearly indicate that apart from the advantages of using an alternative source of phosphorus in the production of phosphorus biofertilizer, a positive effect was observed as a result of the action of the beneficial bacteria with the properties of biostimulants [16, 17, 23-25]. Obtained fertilizers deliver to the soil phosphorus in a form available to plants as well as the beneficial microorganisms that after application to the soil can still solubilize not only phosphorus, but other nutrients and eventually increase their uptake. In this case, such products could be classified as a phosphorus fertilizer and biofertilizer at the same time.

Although the results of microbial solubilization are very promising in the laboratory, further investigation is still needed to optimize phosphorus solubilization, which is expected to lead to large scale exploitation. A few examples of chemically based recovery techniques were conducted on a technical and semi-technical scale, for example, AirPrex®, (Ostara) PEARLTM, AshDec®, and RecoPhos® [26]. According to our knowledge, in the literature there is a lack of information describing the production of phosphorus biofertilizer based on microbial solubilization of renewable raw materials in higher than laboratory scales.

The aim of the presented work was to obtain two formulations of phosphorus biofertilizers on a large laboratory scale in the reactor with a capacity of 30 L; first formulation based on the poultry bones and *B. megaterium* as a microbiological activator of phosphorus and the second based on ash and also with the utilization of *B. megaterium*. The parameters describing the efficiency of the performed solubilization process were measured and evaluated: pH, the concentration of soluble phosphorus and concentration of bacterial biomass. The obtained formulations were in the next stage tested in the field trials whose results were described elsewhere.

2 Materials and Methods

Cooked poultry bones or ashes originated from incineration (performed in sewage sludge incineration plant) of sewage sludge from a wastewater treatment plant applying a third stage of biological treatment (Olsztyn, Łyna) were used as a source of phosphorus. All phosphate substrates were

ground with a blender until they reached 1 mm particle size fractions for chemical and solubilization studies. The solubilization tests were conducted in eight batch cultures in a thermostatic reactor with a 30L capacity (Figure 1). In each batch 900 g of the different source (repetition: four times ash and four times bones) were mixed with 30 L of growth medium. The content of P_2O_5 in the bones was 19.6% and in ash 13.3% (determined by the ICP-OES techniques). Based on this and the mass of bones and ash used in the experiments, the mass of P_2O_5 introduced in each batch in the form of ash and bones was respectively 119.7 g and 176.4 g.

Phosphate sources were treated with *Bacillus megaterium* (PCM 1855) as a phosphate-solubilizing microorganism. Bacteria were obtained from the Polish Collection of Microorganisms located at the Institute of Immunology and Experimental Therapy in Wrocław. For the cultivation of bacteria, 1 L of growth medium contained 10 g glucose; 0.5 g $(NH_4)_2SO_4$; 0.2 g NaCl; 0.1 g $MgSO_4 \cdot 7H_2O$; 0.2 g KCl; 0.002 g $MnSO_4 \cdot H_2O$; 0.002 g $FeSO_4 \cdot 7H_2O$ and 0.5 g yeast extract; prepared with technical grade reagents (from POCh S.A. Gliwice, Poland). The content of P_2O_5 in the medium was 0.07 g/L (determined by the ICP-OES techniques). The 10% v/v of the *Bacillus* strain culture in the logarithmic growth phase, was added to the medium as an inoculum. The solubilization experiment was conducted for 7 days.

Every day during the process samples were taken and the reaction mixture was filtered through filter paper, and permeates were used for pH estimation and P_2O_5 concentration that was measured by the colorimetric vanadomolybdophosphoric acid method [18]. The biomass concentration of *Bacillus* was measured spectrophotometrically [14, 19]. The culture was sampled daily to determine its optical density. The optical density was the absorbance of samples at 550 nm (OD550) in a UV/Visible spectrophotometer (Varian Cary 50 Cone). Each sample was diluted to make an absorbance less than 1.0, if the optical density was greater than 1.0. The concentration of *Bacillus megaterium* was estimated by an equation describing the relationship between the absorbance A_{550} and the concentration of dry weight, Equation 1:

$$C_s = 0.00532 \cdot A_{550}, R^2 = 0.922, \text{ mg/L} \quad (1)$$

The biomass was dried at 60°C for three days (Manufacturing of medical and laboratory equipment, WAMED; Warsaw, Poland) and weighed.

The specific growth rate, μ , 1/day of *B. megaterium* was calculated using Equation 2 and 3:

Figure 1: Scheme of the conducted experiments.

* Extracted in the neutral ammonium citrate according to the PN-EN 15957:2011

** Soluble in the water according to the PN-EN 15957:2011

$$\mu = \frac{1}{c_s} \cdot \frac{dc_s}{dt} \quad (2)$$

$$\mu = \frac{\ln c_s^t - \ln c_s^0}{t} \quad (3)$$

where: t - time period (in days), after which the culture concentration was measured (assuming $t^0=0$), c_s^t - the culture concentration after time t (mg/L), c_s^0 - the initial concentration of the culture (mg/L). Relative growth rate was determined from the graphically depicted correlation of $\ln c_s = f(t)$. The linear regression for logarithmic phase of the growth was described by an Equation 4:

$$\ln c_s^t = \mu \cdot t + \ln c_s^0 \quad (4)$$

and parameter μ , 1/day is the slope.

In order to investigate the efficiency of the solubilization process and consequently the bioavailability of phosphorus (expressed as P_2O_5), two fractions of phosphorus present in the solids that remained after the solubilization process: ammonium citrate and water extracts, were determined according to Regulation (EC) No 2003/2003 of the European Parliament and of the Council relating to fertilizers (method 3.1.4 Extraction of phosphorus, which is soluble in neutral ammonium citrate, and 3.1.6 Extraction of water-soluble phosphorus). A full description of the procedure was published elsewhere [17, 27]. The multielement composition of raw

materials, as well as phosphorus fertilizer formulations was determined by the ICP-OES technique and elemental analysis (CN). A full description of the procedures was published elsewhere [27].

Decreasing pH as a result of the production of acids was described by the following Equation 5:

$$pH = f(c_{P_2O_5}) = \frac{A + pH_{min} \cdot c_{P_2O_5}}{c_{P_2O_5}} \quad (5)$$

where: A , mg/L is a value describing the decay of curve. Evaluated value of pH_{min} can be interpreted as the minimal value of pH [28].

To describe the changes in P_2O_5 concentrations during solubilization, the proposed model that describes the kinetics of releasing of phosphorus (expressed as the P_2O_5) was used (Equation 6):

$$c_{P_2O_5} = f(t) = \frac{c_{P_2O_5}^{max}}{1 + b \cdot e^{-k \cdot t}} \quad (6)$$

where the $c_{P_2O_5}^{max}$, mg/L is the maximum concentration of P_2O_5 , b is a value depending on the time when $c_{P_2O_5}$ is equal to half of $c_{P_2O_5}^{max}$ and k , 1/day constant is the variable slope, which is called the Hill slope. When k value is higher, the curve changes more sharply, which means that the solubilization process proceeds faster [28].

The arithmetic mean values, standard error (SE) and the model parameters of the equations describing the experimental data were determined using nonlinear model

and multiple regression modules of *Statistica* software ver. 13.1. The correlation was considered statistically significant at $\alpha < 0.05$. Chi-square test (χ^2 test) was also used, which was calculated from Equation 7, which more accurately described the fit of the model to experimental data compared to the determination coefficient R^2 .

$$\chi^2 = \frac{(\text{experimental value} - \text{model value})^2}{\text{model value}} \quad (7)$$

Ethical approval: The conducted research is not related to either human or animal use.

3 Results and Discussion

As a result of the performed experiments, depending on the used raw waste material, different growth rates of microorganisms and different acidity of the fertilizer suspension were obtained.

3.1 The growth of bacterial cell of *B. megaterium*

In the case of the two considered sources of renewable phosphorus used in the experiments, bones induced four times higher bacterial specific growth rate ($\mu = 0.026 \pm 0.0015$ g/h, $n=4$) when compared to the bacterial specific growth rate obtained for ash present in the medium ($\mu = 0.00597 \pm 0.00097$ g/h, $n=4$).

The introduction of a different source of nutrients into the growth medium is always related to the risk of bacteria growth inhibition. Bones can be categorized as an organic resource of phosphorus that delivers more nutrients in the available form that can enhance the growth of bacterial cells when compared with ash that is deprived of organic matter through its formation process [21]. The final concentration of the bacterial biomass for the culture with ash was two times lower (2.09 g/L - right OX axis) with respect to the culture with bones (4.72 g/L - right OX axis) (Figure 2). It is more beneficial to reach a higher concentration of bacterial cells as it enhances the chance of successful inoculation of soil environment with beneficial microorganisms after application; to ensure the function of biofertilizer, bacteria after application will grow in the soil environment and perform the solubilization process of phosphorus that is already present in the soil in the retrogradative - not available to plants form [29]. A statistically significant strong correlation between the biomass concentration (X , g/L) and the concentration

Figure 2: The kinetics of changes in pH and the biomass concentration during the solubilization process.

Figure 3: The correlation between the biomass concentration (X , g/L) and the concentration of P_2O_5 (mg/L), for ash and for bones.

of P_2O_5 (mg/L), for ash: $r=0.965$ ($p<0.05$), and for bones: $r=0.964$ ($p<0.05$) was found (Figure 3). That confirms the influence on how the concentration of microbial cells affects the effectiveness of solubilization. This is probably due to higher acid formation by a higher amount of bacteria in the solution. Obtained results agree with the previous findings of solubilization experiments at laboratory scale [16-18, 22].

3.2 Solubilization of phosphorus from ash and bones

The changes of pH were shown in Figure 2; ΔpH calculated for ash was 2.65, while for bones 2.32. The drop in pH

Figure 4: a) The changes of concentration of phosphorus (express as P_2O_5) during the solubilization process; b) The relationship between the concentration of phosphorus (express as P_2O_5) and pH.

Table 1: Evaluated parameters of models: describing the kinetics of changes of concentration of P_2O_5 and the changes in pH and P_2O_5 concentration during the solubilization process.

Phosphorus raw material	Model	Parameters	Value	SE	p-value	R ²	χ ²
Ash	$C_{P_2O_5} = f(t) = \frac{C_{P_2O_5}^{max}}{1 + b \cdot \exp^{-k \cdot t}}$	$C_{P_2O_5}^{max}, \text{mg/L}$	389	28	0.001	0.990	106
		b, day	37.4	26.1	0.247		
		$k, 1/\text{day}$	0.0516	0.0107	0.017		
	$pH = f(C_{P_2O_5}) = \frac{A + pH_{min} \cdot C_{P_2O_5}}{C_{P_2O_5}}$	$A, \text{mg/L}$	90.4	35.6	0.0638	0.765	0.389
		pH_{min}	4.01	0.50	0.00134		
Poultry bones	$C_{P_2O_5} = f(t) = \frac{C_{P_2O_5}^{max}}{1 + b \cdot \exp^{-k \cdot t}}$	$C_{P_2O_5}^{max}, \text{mg/L}$	1350	50	0.000	0.995	33
		b, day	33.5	21.3	0.213		
		$k, 1/\text{day}$	0.0846	0.0153	0.012		
	$pH = f(C_{P_2O_5}) = \frac{A + pH_{min} \cdot C_{P_2O_5}}{C_{P_2O_5}}$	$A, \text{mg/L}$	140.1	11.5	0.000260	0.987	0.0218
		pH_{min}	4.1	0.1	0.000001		

was higher for ash when compared with bones, which could suggest that the solubilization of ash should be better. However, the result was the opposite as the bones resulted in more efficient solubilization, probably due to a more complex composition of bones substrate that could neutralize formed organic acids. At the same time, the mechanism of microbial solubilization is not fully explained, being more complex than simple low molecules acids formation. The action of enzymes produced by cells as well could also be responsible for phosphorus liberation from hydroxyapatite [29]. The evaluated values of pH_{min} from Equation 5 were similar for ash and bones, and equal 4.01 and 4.1, respectively (Table 1). The most

probable mechanism of the phosphorus microbiological solubilization process is related to the production of low molecules organic acids. Their presence in the bacterial broth results in the lowering of the pH of the media that effects in the solubilization of phosphorus from the hydroxyapatite form that is present in ash and bones. A strong correlation between the pH and concentration of P_2O_5 was found, which was described by Equation 5 and presented in Figure 4b. A negative statistically significant correlation was found between the pH and the concentration of P_2O_5 ($r=-0.987$, $p<0.05$) for the solubilization process with bones, while for ash, the correlation was not statistically significant (Table 1).

Figure 5: Sankey diagram of P_2O_5 of the process of solubilization of phosphates from a) ash and b) poultry bones, performed by *Bacillus megaterium*.

Obtained results agree with the previous findings [16-22, 27].

The changes in the concentration of the phosphorus (express as P_2O_5) was described by Equation 6 and shown in Figure 4a while the evaluated parameters were presented in Table 1. The value of the evaluated parameter $C_{P_2O_5}^{max}$ for the growth solution, where a source of phosphorus bones were used (1350 mg/L), was 3.5 times higher when compared with ash (389 mg/L). The biomass of residual bones or ashes that remains after the solubilization process underwent the extraction in the water and in the citrate to evaluate the amount of phosphorus that remains in the biomass but is soluble (extracted in the water) or available (extracted in citrate). The obtained results were collected on the Sankey diagrams (Figure 5a and b). The primary calculated solubilization factor (SF) defined as a ratio between the amounts of phosphorus available to plants (present in the solution - blue bar on the Figure 5, 11.42 g for ash and 41.5 g for bones) after the solubilization process to the amount of phosphorus introduced (121.8 g for ash and 178.5 g for bones), was 2.5 times higher for bones (23%) when compared to ash (9.4%). But when the values of the amount of phosphorus available to plants present in the solids that remained after the solubilization process were taken into account, the SF for ash was equal to 63.8% ($SF = (58.24 + 8.05 + 11.42) / 121.8$ - Figure 5a) while for bones 100% ($SF = (41.5 + 23.6 + 113.4) / 178.5$ - Figure 5b). Obtained results demonstrate that within the evaluation of the solubilization process, remains solids should be also taken into account, as the significant amount of available phosphorus is still present in the solids as a result of microbiological action. In the most cases, described in the literature [16-18], the SF expresses only the amount of phosphorus present in the solution without the second step of extraction of solids.

These findings express the possibility of the total reuse of phosphorus bound in the form of bones via the solubilization process and to valorize it into the fertilizer. The composition of the two obtained fertilizer formulations: one based on ash and one based on poultry bones was collected in Table 2. The main difference between the two considered formulations (composed from solids residues and liquid medium) is the content of total phosphorus (express as P_2O_5), which is resulted from the higher content of phosphorus in used renewable resources in the experiment. A few differences considered crucial from the environmental point of view were also found. The content of Zn, Pb, Ni, Cu, Cr Al, and Cd was significantly higher in the formulation based on ash with respect to bones. In the case of bones that originate from the food industry, exceeding the applicable limits

Table 2: Elemental composition of two fertilizer formulation based on the ash and bones.

Component	Unit	Ash	Bones
N	% mas.	0.255	0.35
P_2O_5	% mas.	0.406	0.595
K_2O	% mas.	0.587	0.262
CaO	% mas.	0.97	0.521
MgO	% mas.	0.198	0.015
SO_3	% mas.	0.137	0.115
Na_2O	% mas.	0.0663	0.0494
C	mg/kg	0.59	1.65
Fe	mg/kg	1679	21.5
Al	mg/kg	1774	8.36
Cd	mg/kg	0.274	0.00965
Cr	mg/kg	5.94	0.218
Cu	mg/kg	55	0.433
Ni	mg/kg	2.45	0.212
Pb	mg/kg	10.4	1.04
Zn	mg/kg	117	6.85

of unwanted elements is very rare. Nowadays one of the most important environmental issues when considered by the phosphorus fertilizer industry, besides the significant amount of generated and difficult to utilize wastes such as phosphogypsum, is the Cd content, expressed as an mg Cd/kg P_2O_5 .

The calculated value of the ratio Cd/kg P_2O_5 for fertilizer from ash was 0.0675 mg Cd per kg P_2O_5 while for bones 0.00162 mg Cd per kg P_2O_5 . In the near future limits in mg Cd per kg P_2O_5 in fertilizers for EU will decrease to 20 mg Cd per kg P_2O_5 [30].

The significantly higher content of unwanted elements in the ashes and, as a result of this, in the fertilizer formulation based on the ash can influence not only the growth of bacteria but also the growth of plants. The result of its application in the field tests proves that biofertilizer based on bones shows better utilitarian properties as fertilizers when compared with ash at the same dose of phosphorus, the reason for that could be the lower concentration of bacterial cells acting as a biostimulant and a lack of unwanted elements such as aluminum and iron (published elsewhere).

When thinking about scaling up the microbiological solubilization process, another crucial issue arises in the form of odour emission. When bones are used as a

source of phosphorus, solutions to decrease the burden of released gases should be considered as the process is performed in a higher-than-ambient temperature (35°C) which has a direct influence on intensive odour emission. In the case of ash, it was not so significant since the odour emission was less burdensome.

4 Conclusions

The aim of the presented work was to obtain two phosphorus biofertilizer formulations based on ash and bones, which are considered nowadays as phosphorous significant renewable raw materials. Since obtained formulations contain, besides the phosphorus in available to plants forms, also living cells of the microorganism, they could be classified as a biofertilizer since after their application to the soils, the microorganism can stimulate the growth of plants. The use of the term biofertilizer is sometimes prone to misinterpretation: this should be used for products that are based on microorganisms, not on products that were obtained from a biotechnological process only. The two inorganic phosphorus fertilizers obtained and described in the paper underwent a biotechnological process, but also contain beneficial soil microorganisms that are very often present in commercially available biofertilizers.

The solubilization process performed with bones was less odour friendly than that performed with ash. Nevertheless, the elemental composition of fertilizer based on the bones was characterized by a higher total concentration of phosphorus, higher concentration of phosphorus available to plants and contained significantly less amount of unwanted elements such as heavy metals. These findings clearly demonstrate that it is possible to recycle the phosphorus present in bones via microbiological solubilization into a form that is available to plants and can be used as a fertilizer.

Conducted experiments are in the preliminary stage of scaling up the process. More experimental tests have to be undertaken to evaluate production efficiency and its effect on the environment, such as production on a semi-technical scale, odour emissions control, as well as the stability of the obtained formulation.

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Data Availability: The experimental data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest: The authors declare that there is no conflict of interest regarding the publication of this paper.

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