IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 8, Issue 6 Nov. – Dec. 2022), PP 19-25
www.iosrjournals.org

Thermo-anaerobic solubilization mechanism of a phosphate rock by *Bacillus subtilis*during a grassland biodegradation process

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Abstract
Bacillus subtilisisolated from the cellulosic fermentation process was previously selected for it phosphate rock (PR) solubilization ability under anaerobic thermophilic conditions [1]. The result shows a significant decrease of pH from 5.8 to 4.6, and solubilizing 38% of the phosphorus from the PR in the reactors after 30 days of incubation at 50°C. This solubilization is the consequence of the production of organic acids during the anaerobic fermentation of grassland substrate. The mechanisms involved in these weathering processes confirmed the production of organic acids which were identified and quantified. We also tested the dissolution of PR by leaching with organic acids trade predominantly present in the acidogenic stage during the anaerobic degradation of lignocellulosic substrate by Bacillus subtilis. The study focused on acetic, lactic and citric acids with varied concentration by meseauring pH and ortho-P released monitoring during PR leaching process. The result showed thatcitric acid exhibitedthe highest PR dissolution rate (98%, T_{23h}) compared to the other tested acids. By this way, we expect to lead to the development of novel, non-polluting farming practices by entering in the formulation of novel multi-functional biofertilizer by inoculating this thermo-tolerant phosphate-solubilizing bacterium into agricultural wastes as a practical and environmental strategy.

Keywords: *Grassland, phosphate rocksolubilization,Bacillus subtilis, thermo-anaerobic condition,organic acid*

Date of Submission: 02-12-2022 Date of Acceptance: 14-12-2022

I. Introduction

Phosphorus (P) among soil constituents is one of the most important plant nutrients next to nitrogen. Consequently, it requires a contribution of sufficient concentration of P to enhance the growth and development of plants[2]. However, only 0.1 % of the total soil P pool (0.5 %) is plant- available [3]and the remaining soil P is inaccessible to plants [4]due largely to its rapid fixation and precipitation ability [5]. Such P scarcity in agronomic practices is, however, corrected through the application of synthetic phosphatic fertilizers which their use and misuse has been questioned due in part to its cost and hazardous impact onto natural environment including soil ecosystems. Thus, it has become imperative to find an economically and environmentally promising strategy to chemical P fertilizers.

In many countries, ground rock phosphate (PR) reduced to fine particles was used in traditional agriculture. Morocco possesses the largest PR reserves worldwide. The Moroccan PR is a hydroxyapatite that is an insoluble form of phosphate [6]and, therefore, not available for plant growth. PR is a natural, cheap and clean compound but unfortunately it is a poor P fertilizer since its solubilization by chemical means is too slow to satisfy plant needs[7]thus doesn't allow its use as P fertilizers for agriculture. In order for PR to become an efficient P fertilizer, innovative methods must be found to free the P from its strong ionic interactions with the ion constituent's of the PR.

The naturally abundant yet functionally divergent phosphate-solubilizing microorganisms (PSM) have attracted greater attention of the farm practitioners due to its low cost and easy-to-apply approach [8,9]. Indeed, PSM offer a practicable alternative to hugely expensive chemical P fertilizers[10]and are indeed able to promote solubilization of PR and to increase crop yields[11]. Several genera and species of bacteria [12,13], fungi [14], and actinomycetes[14,15]have been described and characterized as belonging to the PSM phenotype and have been provided some solution to the P problems [8]. The solubilization process achieved by these PSM, involves

the excretion of organic acids[16]and chelating oxo acids from sugars[14,17]. However, PSM commonly studied and applied to date were mesophilic that could be observed only in mesophilic and aerobic conditions. Such types of microorganisms are not suitable for the preparation of multifunctional organic fertilizers to high temperatures that occur during the decomposition of complex organic waste such as cellulosic substrates [18]in an anaerobic environment. To date, few studies exist on anaerobic cellulolytic thermotrophic bacteria that could be an effective system for degradation of lingo-cellulosic substances [19]. However, the implication of these bacteria in the PR solubilization process is not well explored in anaerobic conditions [1].

The objective of this study is to evaluate the ability and the performance of *Bacillus subtilis*, previously identified as a thermo-tolerant PSM in vitro conditions [1], for it aptitude to solubilize a Moroccan PR during a thermo-anaerobic grassland waste biodegradation process in the reactor and to perform the mechanism of PR solubilization by *Bacillus subtilis* under the same conditions.

II. Material and Methods

1. The rock phosphate sample

Phosphate rocks originating from the Youssoufia phosphate mine (PR^Y, insoluble phosphate rock powder), was ground with pestle and mortar and passed through a 100 µm sieve. Its chemical characteristics were previously determined as follows: 31.4 g /100g P_2O_5, 50.2 g /100 g CaO and 2 g /100 g total organic carbon [20].

2. Substrate preparation

The grassland experiment was conducted in a greenhouse at 24°C under artificial light for 16 h and at 17°C for 8 h in darkness. The experiment was started on 12 June 2010 on a poorly drained Stagno-GleyicLuvisol (FAO classification) with rather wet and cool weather at the beginning of the investigation. After two weeks, the sward was maintained at a height of about 5 cm. The pasture was dominated by perennial ryegrass (*Loliumperenne*L.) and common bent (*Agrostis capillaries* L.). The grassland topsoil (0-0.15 m) was sterilized at 120°C for 30 min and used as organic substrate for the fermentation experiments. The C and N contents of the sterilized grassland were 1.6 and 0.17%, respectively, with soluble organic carbon (extracted with 0.05 M K_2SO_4) of 87.5% and the soluble P content was 19.6 mgl^{-1}.

3. Test of PRsolubilization by *Bacillus subtilis* during the grassland experiments

Sterilized dried substrate (16.5 g) was placed in 500 ml Erlenmeyer flasks containing 0.2 g of PR^Y as sole phosphate source and completed to 400 ml with sterile distilled water. Cultures were aseptically inoculated with 5ml of the selected thermo-tolerant PR solubilizing *Bacillus subtilis*[1]at 10^9CFU ml^{-1}. Cultures were grown in triplicate on a laboratory digester maintained at 50°C for 30 days in completely filled flasks with rubber stoppers and with shaking at 100 rpm in an incubation shaker to minimize aggregation of the selected bacterium. Anaerobic conditions were achieved through the consumption of residual oxygen. Similar experiments were carried out with no phosphate source incubated under the same conditions. Samples of 2 ml of each culture were collected periodically (every day). After then, the pH value of the medium was determined with a pH meter equipped with a glass electrode. The culture supernatant obtained by centrifugation (10,000 rpm, 15 min) was passed through a 0.45 mm Millipore filter. The inorganic phosphate content of culture filtrate and organic acids analysis were determined by high performance ion chromatography, HPIC, Type DIONEX Dx-120 (AS11-HC column, injection rate: 2.3$mlmin^{-1}$) equipped with aconductimetricdetector.

4. PR solubilization test by some organic acids trade

This test is performed in order to know the efficiency of some organic acids often found in anaerobic treatments (acetic or lactic acid) and others for their carboxylic multifunction (citric) to solubilize the PR^Y at different concentration. The acids used for this study are solutions or solid products of commercial origin. The characteristics of these products are described in Table 1. The study of the action of the acids on the PR solubilization was determined from ore dissolution by different solutions of these acids and compared to the performance of hydrochlorid acid (HCl, 37%) as reference (Prolabo-Normapur). Five grams of PR^Y (particle size <250 µm) were placed aseptically in solution with 500 ml of the four organic acids at respective concentrations: 1M, 0.5M, 0.1M, 0.05M and 0.01M. The mixtures were stirred continuously for 46 hours with the samples at T_{2h}, T_{23h}, T_{29h} and T_{46h}. Each reaction mixture was then filtered. The determination of phosphorus was conducted in the filtrates obtained in order to evaluate the action of each solution on dissolution of PR. A monitoring pH and PO_4^{3-} was performed on all samples.

5. Statistical analysis

All experiments were carried out in triplicate or more. All data are reported as means ± SD (standard deviation). The Independent-Samples t-test was used to compare means and the variance homogeneity

determination (ANOVA) was conducted with the General Linear Model using type II sum of squares and Tukey's Honestly Significant Difference (P = 0.05) using statistical analysis system software[21].

Table 1. Composition of organic acid solutions used for the PR solubilization test.

Acid	Origin	Purity (%)	Molar mass (g)	Density
Acetic acid **(CH3COOH)**	Prolabo-Normapur	99	60,50	1,12
Lactic Acid **CH3CH(OH)COOH**	Prolabo-Normapur	99	90,68	1,23
Citric Acid **Monhydrate Salt (C6H6O7-H2O)**	Jeulin	99	210,14	-

III. Results And Discussion

1. PR solubilization abilities of *Bacillus subtilis*in the fermentation experiments

The soluble phosphorus content increased significantly between 0 and 3 days from 105 ± 0.1 to 120 ± 0.2 mgl^{-1} and then decreased significantly from 3 to 7 days (Fig. 1A). This sharp drop in the amount of soluble P in the media could be related to the presence of the lignocellulosic substrate. Indeed, *Bacillus subtilis* could solubilized first organic P as P source to meet metabolic needs. It is shown that the *Bacillus subtilis* strains can grow under anaerobic conditions by respiration with nitrate as the terminal electron acceptor. In the absence of external electron acceptors uses the metabolic pathway for the fermentation of glucose or puryvate [22].

After we observed a decrease in the amount of P in the medium at 3 to 7 days of the growth phase and thus would probably due to the consumption of P solubilized by *Bacillus subtilis* under the fermentation lignocellulosic process (Fig. 1A). However, The P amount curves of the two reactors (treatment and control) have statistically the same allure (Fig. 1A). Thus could be due to the initial organic matter included in the grassland substrate that could influence the adsorption of phosphate ions allowing a high desorption. Many studies indicated that the glucose degradation products decrease the adsorption of phosphate ions and increase their desorption [23, 24]. These organic compounds on the one hand, would therefore fixed at the sites initially available for the phosphate ions oxyhydroxides and, secondly, have facilitated the creation of phosphorus compounds from which the phosphate ions are easily desorbed [23].

After the 7^{th} day of the culture, the soluble P content a re-increased significantly up to 106 ± 0.1 mgl^{-1} at the end of incubation period (30 days). In these times, *B. subtilis* could effectively solubilize the PR^{Y} that appear by the drop of soluble P in the medium. It was reported that the microbial activities were amplified more vigorously at early fermentation phase, resulting in the consumption of soluble P for microbial growth. These results indicate that the thermo-tolerant anaerobic *Bacillus subtilis* can increase the soluble P content and contribute to the solubilization of PR^{Y} during the fermentation process. *Bacillus subtilis* achieved the PR solubilization rate of 38% during the thermophilic anaerobic fermentation experiments (Fig. 1A). It was reported that *Bacillus smithii* F18 presented the highest soluble phosphorus percentage (5.3 ± 0.6%) of the total phosphorus after 56 days of composting under aerobic conditions[25].Similarly, a moderately thermophilic bacterium *Acidithiobacillus caldus* achieved a phosphorus solubilizing rate of 27.6% in shake flasks containing elemental sulfur (S0) as an energy substrate and only 2.19% for the same system without the additional S0[26].

2. PRsolubilization mechanism by *Bacillus subtilis*during the fermentation experiments

In order to compare the contribution of *B. subtilis* on the PRsolubilization process during the anaerobic degradation of the lignocellulosic substrate, *B. subtilis* was grown with the presence of grassland as sole carbon substrate in the fermentation experiments at 50°C (Fig.1B). The pH curves of the two reactors (treatment and control) have statistically the same allure (Fig. 1B). It is therefore the same metabolic profile and the presence of PR has no influence on the metabolic activity of *Bacillus subtilis* in the use of lignocellulosic substrate. A variation of the pH in the medium was observed and its decrease from the value of 5.8 to 4.8 after 24 h of the thermophilic anaerobic incubation. After from 2 to 4 days, pH reaches its minimum value of 4.6, a variation more than one unit in 4 days of treatment. The decrease in pH is negatively correlated ($r = -0.94$, $p < 0.05$) with the concentration of the soluble P in the presence of the selected strain during the anaerobic fermentation process (Fig.1A,B). The decrease in pH reflects an acidification of the medium by *B. subtilis* and confirms the implication of *B. subtilis* in the PRsolubilization by producing organic acids under anaerobic thermophilic conditions. At the same time, we revealed the presence of three organic acids (lactic, acetic and citric acids) at different concentrations during the anaerobic fermentation of the lignocellulosic substrate by *Bacillus subtilis* (Fig.1C). Lactic acid is the most produced from 200 mgl^{-1} to more than 1200 mgl^{-1} after 4 days of

incubation.The presence of formic, tartric and oxalic acids was signaled at only trace concentration (< 200 mgl^{-1}). Several studies [27,28]reported that these organic acids are involved in the solubilization of calcium phosphates.

After the 4th day, the pH gradually increases with time and reaches its initial value (pH= 5.8) at the end of incubation (Fig.1B). At this time, the increase in pH is negatively correlated with the concentration of organic acids in the medium during the anaerobic fermentation of the lignocellulosic substrate by *Bacillus subtilis*(Fig.1C). Acetic acid is the most produced with 180 mgl^{-1} after 4 days of incubation.The presence of citric, tartric and oxalic acids was signaled at only trace concentration (< 50 mgl^{-1}). In general, during the anaerobic digestion of the lignocellulosic material, a consortium of microorganisms is involved in the degradation of the carbon substrate. Chain reactions which follow include depolymerisation (hydrolysis), acidogenesis, acetogenesis and methanation. The main products of the enzymatic degradation of the carbohydrates are monosaccharides including glucose [29]which is transformed into acetate, hydrogen (H_2) and carbonic acid (H_2CO_3).Accordingly, the medium is acidified with doubling the production of organic acid and of carbonic acid.

After the 7th day, a significant increase of the P amount that correlate positively with the pH decrease (Fig.1A,B). This implies that *Bacillus subtilis* contribute significantly in the PR^Ysolubilization process under the thermotolerant anaerobic lignocellulotic conditions. Until then, the known anaerobic cellulolytic bacteria are essentially the genus *Clostridium*[30,31]. They are either mesophilic such as *Clostridiumpapyrosolvens* or thermophilic such as *Clostridiumthermocellum.*[30]studied the anaerobic digestion of cellulose by *Clostridium cellulotycum*and their mechanism of action on the lignocellulosic substrate. Initially, cells directly adhere to the cellulose and then the colonization continues with the formation of superposed layers of cells that are no longer in direct contact with the substrate. It is shown that the *Bacillus subtilis* strains can grow under anaerobic conditions by respiration with nitrate as the terminal electron acceptor. In the absence of external electron acceptors uses the metabolic pathway for the fermentation of glucose or puryvate[22]. The *Bacillus subtilis* strain, in addition to its ability to solubilize the PR^Y[1]is added to the list of the thermophilic anaerobic cellulolytic bacteria.

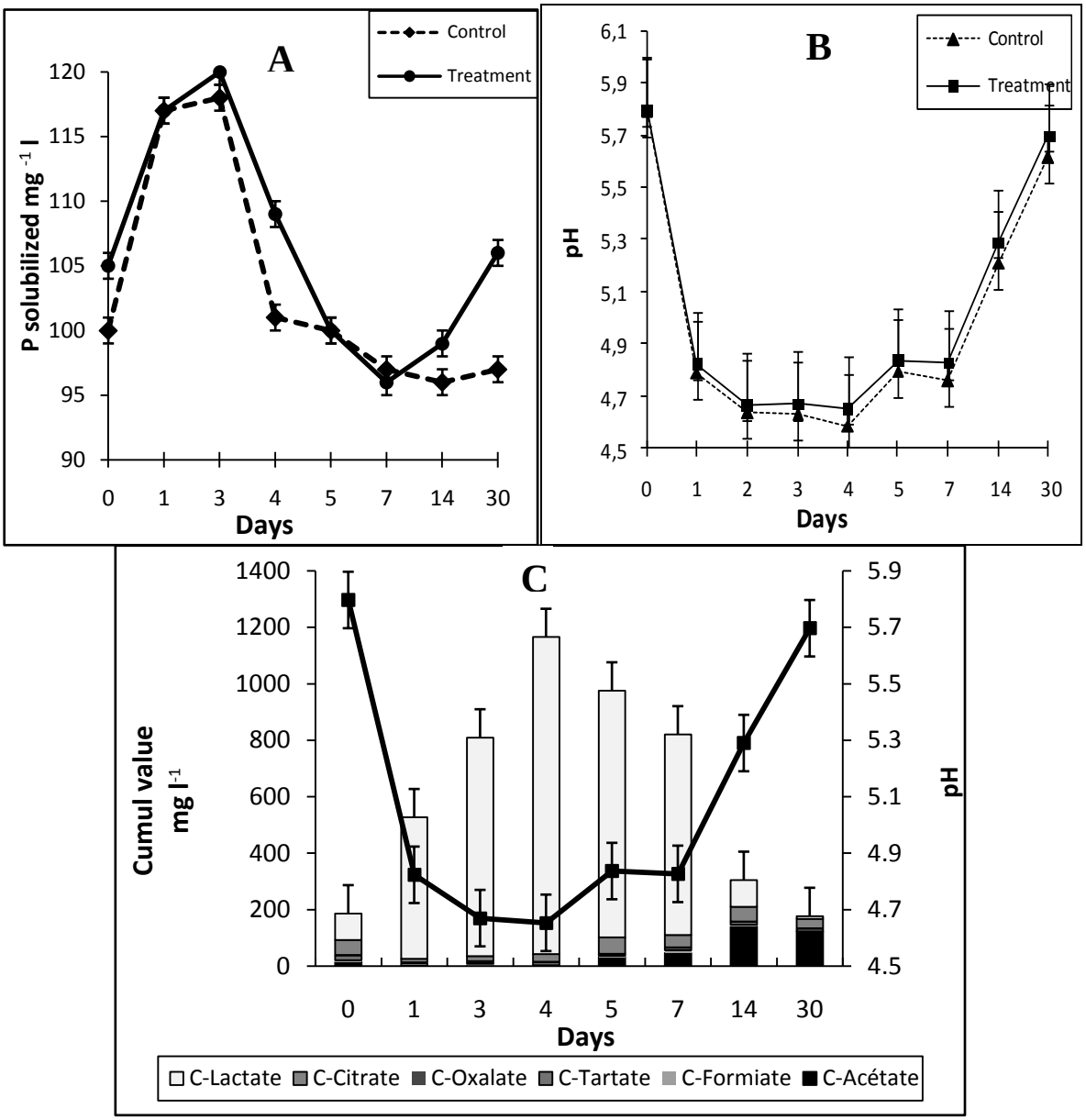

Fig.1.Fermentation experiments of *Bacillus subtilis* in bioreactor with sterilized grassland containing 0.5 gl^{-1} PR^{Y} (Treatment) or without PR^{Y} (Control) incubated for 30 days under the same conditions. **(A)** Concentration of soluble phosphate released from phosphate rock in the supernatant of cultures. **(B)** Evolution of the pH in the supernatant of cultures. **(C)** Concentration of organic acids produced in the supernatant of cultures. Data points are means and vertical bars are standard deviations (n=3).

3. PR solubilization mechanism by the selected organic acids trade

The action of different organic acid solutions on thePR dissolution was studied by pH measure and dosage of P_2O_5 present in the filtrates obtained after leaching of PR^{Y}. The results showed that pH changes in the reaction media in the presence of different acid solutionsthus indicate that there was a PR dissolution over time (Tables 2, 3, 4). This variation depends on the degree of dilution of the leaching solution. Thus, more acid is dilutedmore the amplitude of the pH change is important over time. After 23h of PRleaching, pH values reach a stationary phase whose value depends on the strength of the acids and its dilution level. We notice a buffer effect of the organic acids depending to the pH of the reactional solutionand to pKa (s) of each acid (Tables 2, 3, 4). At pH $\leq$pKa – 1, the solution contains more than 90% of the acid form and less than 10% of the anion. In this phase, the action of protons is predominant in the dissolution of PO_4^{3-} (case of 1M and 0.5M acid concentrations) (Tables 2,3,4).On the contrary, while pH $\geq$ pKa + 1the action of the anion is predominant by

complexing calcium (case of 0.1M, 0.05M and 0.01M acid solutions). So, we could concluded that the PRdissolution is guided by the pH and is correleted to the strength of the acid solution.

The acetic acid solutions have very little effect on the PR dissolution with a maximum of 8% P_2O_5 at 1M in the first two hours of the experiment while any dissolution is detected at lower concentrations to 0.5M (Table 2).The results obtained during the leaching solutions at varied concentration of lactic acid indicated a negative correlation between pH and dissolved PO_4^{3-} (Table 3). The highest concentration (1M) of the tested acids provides a maximum dissolution rate of 89% $P_2O_5$by citric acid after 2 h of PR leaching in comparison to that gived by HCl (37%) with the highest rate at 99% P_2O_5 in the same conditions (Table 4).

Table 2. Leaching test result with different acetic acid solutions at 25 °C

	Duration (h)	Aceticacid concentrations					
		1M	**0,5M**	**0,1M**	**0,05M**	**0,01M**	
pH	**0**	2,2	2,4	2,7	2,9	3,2	
	23	3,8	4,1	5,9	6,2	7,3	
	29	3,7	4,1	6,1	6,3	7,4	
	46	3,7	4,1	6,6	6,9	7,0	
							HCl (37%)
%P_2O_5solubilized	**2**	8%	3,4%	-	-	-	99%
	23	3,3%	0,8%	-	-	-	87%
	29	2,4%	0,4%	-	-	-	81%
	46	2,1%	0,3%	-	-	-	49%

(-): means not present or amount below the detection limit < 25µgl^{-1}

Table 3.Result of lixiviation test with lactic acid solutions at 25°C

	Duration (h)	Lacticacid concentrations					
		1M	**0,5M**	**0,1M**	**0,05M**	**0,01M**	
pH	**0**	1,7	1,8	2,1	2,2	2,6	
	23	2,9	3,4	6,1	5,9	6,8	
	29	2,9	3,4	6,0	6,2	7,0	
	46	3,0	3,4	5,9	6,1	6,9	
							HCl (37%)
%P_2O_5 solubilized	**2**	89%	40%	1,1%	-	-	99%
	23	76%	30%	0,03%	-	-	87%
	29	77%	30%	-	-	-	81%
	46	79%	28%	-	-	-	49%

(-): means not present or amount below the detection limit < 25µgl^{-1}

Table 4. Lixiviation test result of citric acid solutions at 25°C

	Duration(h)	Citricacid concentrations					
		1M	**0,5M**	**0,1M**	**0,05M**	**0,01M**	
pH	**0**	1,4	1,6	1,9	2,1	2,4	
	23	2,4	2,7	3,7	4,2	6,3	
	29	2,5	2,7	3,7	4,3	6,6	
	46	2,5	2,8	3,8	4,4	6,0	
							HCl (37%)
%P_2O_5solubilized	**2**	37%	55%	16%	8%	0,68%	99%
	23	98%	47%	14%	6%	0,10%	87%
	29	94%	39%	13%	6%	0,10%	81%
	46	66%	15%	12%	5%	0,09%	49%

Acid solutions at lower concentration to 0.5M, except citric acid, did not give positive results regarding to the PRdissolution (Tables 2,3,4). Whose indicate that citric acid exhibit a highest PRdissolution rate (Table 4). It is a tribasic acid (pKa_1 = 3.13; pKa_2 = 4.76 and pKa_3 = 6.40)which explains it leaching power compared to the otherstested organic acids.Acetic acid exhibit the lowest PR dissolution rate probably due to its low acidity (pKa = 4.75) (Table 2). These results showed that thetested organic acids presented different level of maximum efficienty on the PR dissolution as following order: Citric (98%, 2h) > Lactic (89%, 23h)> Acetic (8%, 2h) (Tables 2,3,4). Thus the results confirm the organic acids effiency on the PRdissolution that it increases with the number of carboxylic acid group. Similarly, [32]demonstrate that the dissolution of the apatite by citric acid and

EDTA solutions depends not only from the attack of hydrogen ions but they suggest the existence of other chemical processes that contribute to exacerbate the apatite dissolution. There would then forming acid-calcium complex whose stability depends on the pKa of the acid. It was also reported that the apatite dissolutionby organic acid solutions at low concentrations is largely due to complexation phenomena of the substrate by the carboxyl groups of such acids[33,34,35].Accordingly, other works reported the implication of organic acids trade on the dissolution of PR-Hahotoe' Kpogame' (Togo) and of PR- Pakistan [32,36]. However, we note a few points of difference with our results, especially regarding to the PRdissolution rate linked to the duration of leaching treatment who is probably related to the PRnature.

IV. Conclusions

During this study, we demonstrated that *Bacillus subtilis* is capable to solubilize the PR^Y in extreme conditions namely: the anaerobiosis, the thermophilic, the use of lignocellulosic substrate as sole carbon source and the inorganic phosphate solubilization. We showed that this bacterium produces mainly the lactic, acetic and citric acids under the same fermentation conditions of the lignocellulosic substrate. Secondly, we tested the solubility of PR^Y by leaching with organic acids trade mostly produced during the acidogenic phase of the anaerobic digestion of lignocellulosic substrate by *Bacillus subtilis*. The comparative study of different acetic, lactic and citric concentration varied with pH monitoring and ortho-P released during PR^Y leaching. Generally, the PR^Y dissolution by these organic acids will vary from acid to another and is very different from that observed in the presence of a strong acid such as hydrochloric acid. Moreover, the number of carboxylic acid functional group contributes to increase the PR^Y solubilization. Thus, citric acid exhibits a highest PR^Y dissolution rate compared to the lactic and acetic acids. We also concluded that the leaching of PR^Y by organic acids is a chemical reaction by protonation or complexation controlled by the pH of the solution. The results lead us to conclude that the acidity of the organic acids was not solely responsible for the PR^Y solubilization during the anaerobic degradation of the lignocellulosic substrate. The outcome of these studies might contributes to the development of novel, non-polluting farming practices by entering in the formulation of novel multi-functional biofertilizer by inoculating this thermo-tolerant phosphate-solubilizing *Bacillus subtilis* into agricultural wastes as a practical and environmental strategy.

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