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Characterization of Some Agricultural Soils: Presence and Activity of Tilemsi Rock Phosphate-Solubilizing Thiobacilli

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Research Article

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ABSTRACT

The bacteria *Thiobacillus thioparus, Thiobacillus thiooxidans* and *Thiobacillus ferooxidans* are known to be able to oxidize elemental sulfur in soils and influence phosphorus solubilization. These bacteria can solubilize the phosphorus of apatite rock by producing sulfuric acid from the oxidation of elemental sulfur and some sulphide. We think that the capacity of these bacteria can be exploited to set up a biophosphate from Tilemsi natural rock phosphate exploited in Mali. This work aims to identify, isolate and characterize Tilemsi rock phosphate (TRP) solubilizing *Thiobacillus* strains in various agricultural soils. Obtained results showed that the analyzed soils are poor in *Thiobacillus* and that the quantity of acid produced and TRP solubilized is directly proportional to the growth of these bacteria.

Keywords: Thiobacillus, sulfur, Tilemsi rock phosphate, biosolubilization, Mali;

1. INTRODUCTION

Interest in inorganic phosphate solubilization increased during the last twenty years, mainly because of the economic potential that can represent natural rock phosphates for agriculture (Bationo et al., 1997). Direct application of rock phosphate is desirable for acidic soils, however even if the application conditions are optimal, yields are under those obtained with chemical phosphate fertilizers (Bationo and al., 1997). An attractive approach for improving natural rock phosphates utilization as fertilizer is the inoculation with phosphate-solubilizing microorganisms (Vassilev, 1995; Schofield et al., 1981). *Thiobacillus thioparus, Thiobacillus thiooxidans* and *Thiobacillus ferrooxidans* are considered for being able to transform sulfurated compounds with reduced valence in soils and influence phosphorus's availability to plants (Wainwright, 1984; Lawrence and Germida, 1988). So, thiooxidant strains can make soluble the phosphorus from the apatite rock by producing sulfuric acid from the oxidation of elementary sulfur and of some sulfide (Swaby, 1975).

The capacity of these bacteria to produce sulfuric acid from the oxidation of sulfurs or sulfides can be exploited to formulate biological phosphate fertilizer from Tilemsi Rock phosphate (TRP granules containing sulfur). The peculiarity of this fertilizer would be that the phosphorus would be made available

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by the biological oxidation of the sulfur contained in the fertilizer. The oxidation of sulfur in soil, being a biological process, the solubilization of the natural rock phosphate depends on the nature of the sulfur and the sulfur-oxidizing bacteria strains. As, sulfur oxidation vary from soil to soil, we hypothesis that, the nature and number of soil microorganisms, more particularly some thiobacilli, vary according to soils.

The aim of this study was to evaluate and identify the population of Tilemsi rock phosphate-solubilizing *Thiobacillus* strains in some agricultural soils.

To reach this objective, we propose to: (i) study the presence of sulfur and iron-oxidizing bacteria in some agricultural soils, (ii) isolate and characterize the acidophilic and acidifying bacteria, which are largely responsible for the fertility of agricultural soils and, (iii) to verify the capacity of the selected bacteria to solubilize Tilemsi rock phosphate.

2. MATERIAL AND METHODS

2.1 SOIL SAMPLES

Surface soils were collected in plastic bags in Mali and Canada, 4 coming from Mali and 36 coming from Canada. Soils were stored in a refrigerator at 4°C, mostly for a few days. The Canadian soil samples and their main physicochemical characteristics are presented in Babana (1996). The main physico-chemical characteristics of Malian soil samples are presented in table 1.

Table 1: Physical and chemical characteristics of Malian soil samples used in this study

No	Sites	Texture	pH	% O.M.	CEC
S36	Tilemsi	Sablo-clayey loam	5.84	0.16	1.04
S37	Tilemsi	Sablo-clayey loam	5.89	0.1	0.72
S38	Tilemsi	Sandy loam	5.91	0.08	0.55
S39	Diré	Limono-clayey loam	6.37	0.17	1.21

2.2 ISOLATION OF SULFUR AND IRON-OXIDIZING BACTERIA

To isolate sulfur and iron-oxidizing bacteria, twenty (20) g of each soil and 180 ml of sterilized water, were placed in a 300 ml Erlenmeyer flasks containing 1% thiosulfate or ferrous sulfate. The flasks were loosely capped and shaken at room temperature for 30 min on a reciprocal shaker. The suspension was serially diluted 10-fold and 0.1 ml aliquots were spread on: (i) BS (Barton and Shively, 1964) agar plates which contained: $(NH_4)_2SO_4$, 0.4g; $CaCl_2$, 0.20g; KH_2PO_4, 3g; $MgSO_4.7H_2O$, 0.5g; $FeSO.7H_2O$, 0.01g; S^o, 10g and distilled water 1000 ml with the pH of the medium adjusted at 4 (BS4) or 7 (BS7), and (ii) modified 9K (Lundgren et Silverman, 1980) agar plates containing: $(NH_4)_2SO_4$, 3g; KCl, 0.1g; K_2HPO_4, 0.5g; $MgSO_4.7H_2O$, 0.5g; $Ca(NO_3)_2$; $FeSO_4.7H_2O$, 0.01g; agarose, 20g and distilled water 1000 ml. The pH of this medium was adjusted to 3.2 not 2.25.

For the isolation of acidophilic sulfur-oxidizing bacteria, the pH of the medium was adjusted to 3.5 with 0.05M sulfuric acid. To isolate the neutrophilic sulfur-oxidizing bacteria, the pH of the medium was adjusted to 6.5 with 0.1M sodium peroxide (Sallah et al., 1993, Ohba et al., 2003) and for acidophilic iron-oxidizing bacteria the pH was adjusted to 3.2. The salts medium agar plates were cultured at 30°C for 2 weeks.

After the two weeks, all morphologically different bacterial colonies were picked from each plate. Once the purity of each of these colonies had been verified, each colony was suspended in 10 ml of sterilized water and 0.5 ml of the suspensions were inoculated into 10 ml of liquid medium containing the same components as for the agar plates. After static cultivation at 30°C for 2 weeks, the bacteria were checked to see whether or not they were: (i) sulfur-oxidizing bacteria by determining the amount of elemental sulfur oxidize according to Linderman et al. (1991) and the final pH of the medium, or (ii) iron-oxidizing bacteria by dosing the ferrous iron (titration with potassium dichromate).

2.3 IDENTIFICATION AND CHARACTERIZATION OF THE ISOLATES

The cultivation of bacteria and the evaluation of their physiological and metabolic characteristics were carried out according to Bryant et al. (1983), Laishley et al. (1988) and Reynolds et al. (1981). The three new isolates and ATCC strains were routinely maintained by weekly transfer of 1 ml of the spent culture into 100 ml of 0.5% thiosulfate ($S_2O_3^{2-}$), 0.5% tyndalized elemental sulfur (S^o) synthetic salts medium (S_2O_3-SM, or S^o-SM) (Barton et Shively, 1968;) at either pH 4.0 (*Thiobacillus thiooxidans, Thiobacillus ferrooxidans* and strains AHB411 and AHB436) or pH 7.0 (*Thiobacillus thioparus* and strains AHB710 et AHB717), and ferrous sulfate synthetic salts medium ($FeSO_4$-SM) at pH 2.25 (*Thiobacillus ferrooxidans* and strain AHB411). The liquid cultures were incubated at 30°C in a gyratory incubator shaker (model G 25, New Brunswick Co.) set at 200 rpm. Reference bacteria *T. thiooxidans, T. thioparus* and *T. ferroxidans* used to inoculate the different control media were obtained from the American Type Culture Collection (ATTC; Rockville, Maryland, USA).

2.4 MORPHOLOGICAL CHARACTERIZATION

To determine colony characteristics (shape, color and diameter) and isolate sulfur and iron-oxidizing bacteria, modified 9K, BS7 and BS4 agar plates were used for bacterial growth. The isolates were plated in sterile Petri dishes by the pour plate method and the plates incubated at 30°C for 15 days. For cell and colony morphology determination, negative staining was done and the morphology (form, size, mobility and presence or not of spore) of the isolates was studied under a microscope as described by Reynolds et al. (1981). Gram staining of the isolates was performed as described by Bryant et al. (1983).

2.5 PHYSIOLOGICAL CHARACTERIZATION

The ability of the three isolates to utilize a carbon source for heterotrophic growth was tested by supplementing the basic synthetic salts medium (25 ml) with one of the following filter-sterilized organic compounds at a final concentration of 1%/ arabinose, fructose, glucose, lactose, maltose, mannitol, raffinose, ribose, sorbitol, xylose, lactate, glutamate, and 0.5% yeast extract. Growth on C1 compounds was tested using 0.5% methanol and formaldehyde.

The ability of the three isolates to oxidize iron as an alternate energy source was tested in synthetic salt medium cultures supplemented with 0.5% $FeSO_4$. In all cases, the flasks (50 ml) were incubated with 1% inoculum of 5-day culture, and the growth was monitored over a 2-week period. The three isolates were also examined for growth under anaerobic conditions in synthetic salts medium supplemented with 0.5% $NaHCO_3$ and 0.5% KNO_3 in stoppered bottles.

The assimilation of nitrogen sources was assayed by substitution of $(NH_4)_2SO_4$ in synthetic salt liquid medium with 0.1% of each compounds (KNO_3, KNO_2, urea, glutamate) and with only gaseous atmospheric nitrogen (Blais et al., 1991). The quantity of acid produced is an important criterion in the identification process of *Thiobacillus* (Hutchison et al., 1969; Kelly et Harrison, 1988). The limit of acidification was assessed in S^o synthetic salts liquid medium over the pH range 3.0 to 9.0 for strains AHB710 and AHB717 and 1.0 to 6.0 for strain AHB411, by monitoring the pH of the culture medium. The quantity of acid produced is represented by the decrease in pH observed after 24 hours. Bacterial growth and culture medium acidification was also assessed over a temperature range of 10 to 40°C by streaking 0.1 ml of culture on S^o synthetic salts agar medium (Linderman et al., 1991).

2.6 GROWTH, ACID PRODUCTION AND ROCK PHOSPHATE SOLUBILIZATION

The relationship between bacterial growth, sulfuric acid and phosphoric acid production was determined by inoculating liquid salts media containing elemental sulfur (S^o) and rock phosphate with the different isolates. The inoculated flasks were incubated under agitation at 30°C during 2 weeks. Once daily, for 12 days, a 5 ml subsample of growth medium was withdrawn from each flask. The number of cell was determined by direct counting after suitable dilution using a microscope. Sulfuric acid product by the isolated strains was determined by volumetric titration (American Public Health Association, 1989). A colorimetric dosage was used to determine the amount of phosphoric acid produce by the sulfuric acid in

the culture flasks. The dosage was performed on 1 ml aliquots, aseptically sampled from each salt culture medium, as described by Tandon et al. (1968).

3. RESULTS AND DISCUSSION

3.1 RESULTS

3.1.1 Isolation of sulfur and iron-oxidizing bacteria

The mineral salts agar BS7, BS4 and 9K were used to enumerate and isolate acidifying and acidophilic bacteria (*Thiobacillus thioparus, Thiobacillus thiooxidans and Thiobacillus ferrooxidans*) in the soil analyzed. The acidifying and acidophilic bacteria were isolated on the mineral salt agar BS7 and BS4. Results in table 2, shows that the acidifying sulfur-oxidizing bacteria isolated are almost double of the acidophilic sulfur-oxidizing bacteria.

Table2. **Number of sulfur and iron-oxidizing bacteria isolated from agricultural soils using various mineral salt media**

Culture media	Sulfur and iron-oxidizing bacteria		
	Acidophilic sulfur-oxidizing bacteria	Acidifying bacteria	Acidophilic iron-oxydizing bacteria
BS4	20	41	0
BS7	24	50	0
9K	0	0	0
Total	44	91	0

In fact, 91 acidifying bacteria, that are 1 to 3 bacteria per soil sample, were identified. For acidophilic bacteria, a total number of 44 bacteria were purified from soil samples (Table 2). No acidophilic iron-oxidizing bacterium was detected or isolated. 4 bacteria different in colony morphology were isolated. These selected isolates were named AHB710 (AHB = initials of the researcher if the soil used for isolation is a Canadian soil, 7 = initial pH of the isolate medium and 10 = the numerous of the soil sample used to isolate the bacteria), AHB717, AHB411 and AHB436 (AHB = name of the researcher if the soil used is a Malian soil).

3.1.2 Identification and characterization of the isolates

All the selected isolates form on agar media, round and yellowish colonies of diameter varying between 0.8 and 1.9 mm (Table 3).

Table3. Microscopic and macroscopic characteristics of the isolates

Isolates	Microscopic Characteristics					Macroscopic Characteristics		
	Length (mm)	Width (mm)	Gram	Motility	Spore	Form	Colour	Diameter (mm)
AHB710	1.6	0.6	Gram-	Motile	Absent	Rod-shaped	yellowish	1.9
AHB717	1.6	0.5	Gram-	Motile	Absent	Rod-shaped	yellowish	1.6
AHB436	1.8	0.5	Gram-	Motile	Absent	Rod-shaped	yellowish	0.9
AHB411	1.9	0.5	Gram-	Motile	Absent	Rod-shaped	yellowish	0.8

Isolates AHB710 and AHB717 are short, motile, rod-shaped, whereas isolates AHB411 and AHB436 consist of long, motile, rod-shaped cells (Table 3). Both strains have a typical Gram-negative cell wall.

Flagella were not observed in these preparations. All the isolates can produce sulfuric acid by oxidizing elemental sulfur (figure 1).

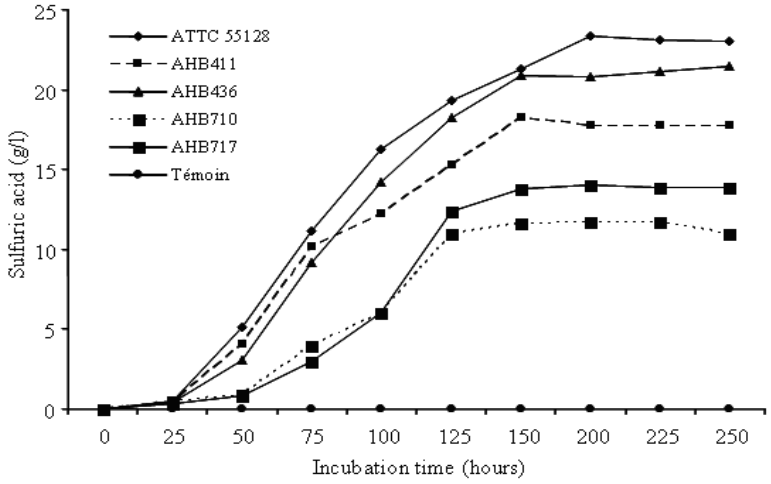

Figure 1. Sulfuric acid production per *Thiobacillus* isolates

The isolates AHB436 and AHB411 produce more sulfuric acid than the isolates AHB710 and AHB717. Growth of AHB710 and AHB717 occurred between pH 4 to 8, with maximal growth occurred at pH from 5.5 to 6.5. These bacteria cannot growth in a medium at initial pH from 0 to 3 (Figure 2). Growth of the isolates AHB411 and AHB436 occurred between 2 to 6, with maximal growth occurring at initial growth pH 4.0 (figure 2). Growth for both strains was observed at temperature ranging from 10 to 40°C, with maximal growth occurring between 25 to 30°C. The ammonium ion was the sole source of nitrogen for the strains AHB411 and AHB436, whereas, AHB710 and AHB717 can use glutamate as an alternative source of nitrogen. Neither strain could growth anaerobically, or in the presence of 5% NaCl. The three isolates could not metabolize any of the carbon sources tested. Autotrophic growth was supported by the oxidation of $S_2O_3^{2-}$, $S_4O_6^{2-}$, and S°, but not $FeSO_4$. The physiological and metabolic characteristics of the acidophilic strains AHB411 and AHB436 are compatible with the taxonomic description of *Thiobacillus thiooxidans*, while the isolates AHB710 and AHB717 correspond to *Thiobacillus thioparus* (Kelly and Harisson, 1988).

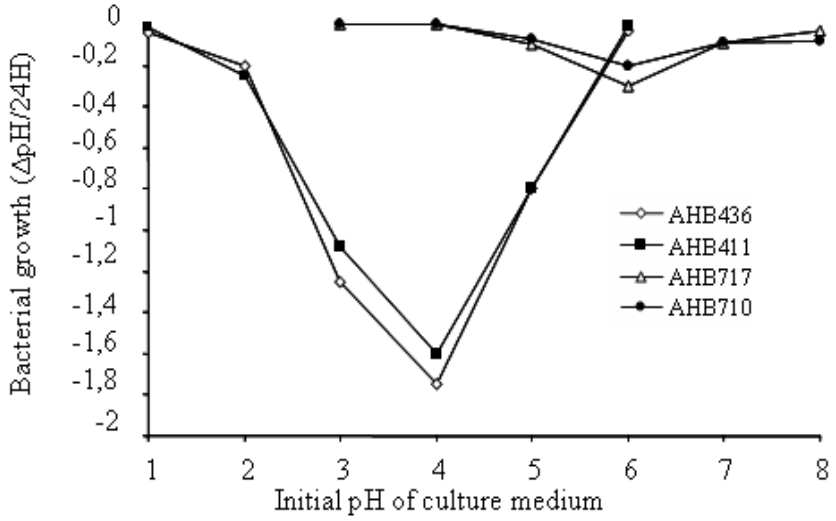

Figure 2: Influence of the initial pH of the culture medium on bacterial growth

3.1.3 Growth, sulfuric acid production and rock phosphate solubilization

AHB436, AHB411 and ATTC55128 grown on liquid salt medium were able to produce sulfuric acid by oxidizing the elemental sulfur contained in the culture medium (figure 3). However, the different isolates showed different levels of acid-producing activity on culture media containing rock phosphate (RP) as sole phosphorus source. In this study, with no RP, the highest sulfuric acid concentration (24.3 mg/100ml) was obtained with ATTC55128 followed by AHB436 (23 mg/100ml). The addition of rock phosphate to the growth medium decreased the sulfuric acid production by all the isolates (figure 3).

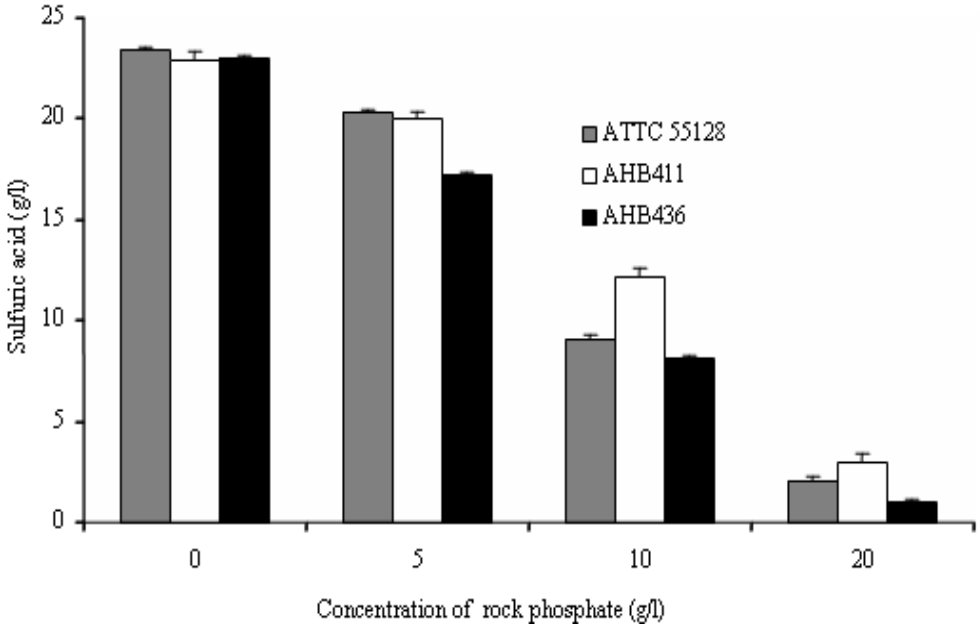

Figure 3: Influence of rock phosphate concentration on sulfuric acid production.

The amount of sulfuric acid decreased with increasing the rock phosphate concentration. At higher rock phosphate concentration (10 and 20 g l^{-1}), the strain AHB436 produce more acid than ATCC 55128 and AHB411.

The relation between the amounts of sulfuric acid produced, the number of bacterial cells and the quantity of PNT solubilized, is presented in figure 4A and figure 4B.

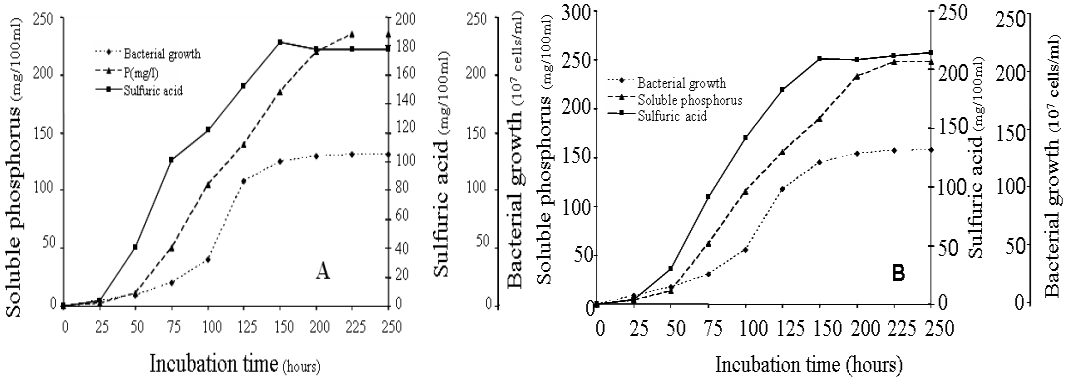

Figure 4: Growth, sulfuric acid production and rock phosphate solubilization by AHB411 (A) and AHB436 (B)

It appears that the more the number of bacterial cells increases, the more important are the quantities of sulfuric acid produced and the phosphorus solubilized in the salts culture medium. The quantity maximal of sulfuric acid produced and P solubilized, and the necessary time to solubilize the maximum of phosphorus, vary according to isolates. Here we find a gradual increase of the soluble phosphate, since the amount of sulfuric acid in the early part of the incubation period is small (figure 4A and 4B).

As soon as the amount of sulfuric acid reaches 50 mg/100 ml, the soluble phosphate increased rapidly. The quantities of phosphorus solubilized by the *T. thiooxidans* strains tested vary between 20 to 248 mg P/100 ml. The bacterium AHB436, which appears to be the most effective solubiliser, solubilized 248 mg P/100 ml in nearly 11 days (Figure 4B). These results also showed a relation between: (i) the bacterial growth and the amount of acid produced ($r = 0.97$ for AHB436 and $r = 0.95$ for AHB411), and (ii) the amount of sulfuric acid and the amount of phosphate solubilized ($r = 0.96$ for AHB436 and $r = 0.93$ for AHB411).

3.2. DISCUSSION

In this study, out of 40 soils samples analyzed, *Thiobacillus* strains were detected only in 3 soils. For soils were no *Thiobacillus* were detected, the results didn't inevitably mean their absence, but they are, perhaps, in insufficient number to be detected. These results are similar to those obtained by Swaby and Fedel (1973) who, using thiosulfate as energy source, detected the bacterium *T. thioparus* in 5 out of 56 Australian soils analyzed. *T. thiooxidans* was detected only in one of these soils, whereas, *T. ferroxidans* was absent in all the analyzed soils. Our results are in agreement with the general observation according to which *T. ferrooxidans* is absent in normal agricultural soils because of their pH being situated around the neutrality. In fact, acid soils are not appropriate for agriculture, because of the biological activity, in particular biological nitrogen fixation and nitrification (Dommergue et Mangenot, 1970). Soil pH didn't control only the biological activity of one microorganism but regularize also the activities of the other organisms and, the intensity of the competition between these organisms (Germida et Jansen, 1993). To correct the acidity of agricultural soils and improve agricultural productivity, farmers put a quantity of calcium carbonate ($CaCO_3$) to the acid soils. The activity of $CaCO_3$ improves the activity of some soil microorganisms by increasing the soil pH at an acceptable level (Germida et Jansen, 1993; Dommergue et Mangenot, 1970). But, by increasing acid soil pH, the $CaCO_3$ inhibits acidophilic bacteria as *Thiobacillus ferrooxidans*. Also, no *Thiobacillus* was discovered in the Saskatchewan soils analyzed (Lawrence and Germida, 1991), and similar results were observed by other researchers in New Zealand and Australia (Moser, 1953; Swaby and Fedel, 1973). An ascendancy of neutrophilic bacteria was observed in some New Brunswick soils analyzed by Chapman (1989).

In most of the soils analyzed, the percentage of organic matter is high (Table1). The high organic matter content prevents the contact between the thiobacilli and the sulfur particles (Wainwright and al, 1986), and inhibits the growth of the sulfur-oxidizing bacteria and constitutes one of the likely causes of the absence of thiobacilli in some of the soils analyzed.

The maximal quantity of sulfuric acid produced, the amount of phosphorus solubilized and the necessary time to solubilize the maximum of phosphorus appear to vary according to isolates. Also, the acid producing and the phosphate solubilizing capacity of the isolates decreased with increasing the rock phosphate in the growth medium. This activity inhibition is probably due to the presence of some elements (as the fluorine) in rock phosphate minerals. At high concentrations, these elements can reduce the microbial activity and so act on their acid producing capacity. In fact, the reactions involved in the conversion of rock phosphate into soluble forms by means of acids belong to the type of reactions of heterogeneous systems. The rock phosphate minerals have no definite composition and the products formed are not always definite. In such heterogeneous systems the speed of the reaction is a function of greater number of variables such as: the size of contact surface, the chemical composition of the solid phase; the physical properties of the solid phase; and the influence of a solid formation phase as result of the reactions. The transformation process of insoluble phosphates has been subject of study by a number of investigators other the world (Rajan, 1982; Swaby, 1975; Vassilev et al., 1995).

5. CONCLUSION

The isolation and identification of bacterial *Thiobacillus* strains reveal that: agricultural soils contain few thiobacilli, *Thiobacillus ferrooxidans* is generally absent in these soils and the production of acid is essentially caused by *Thiobacillus* strains.

The characterization of the *Thiobacillus* strains isolated from the agricultural soils used reveals that: acid production depends not only on the number of *Thiobacillus*, but also on the species of *Thiobacillus* present in the soil. In the rock phosphate biological solubilization processes, the initial acidification of soils is caused by *T. thioparus*, followed by the growth of *T. thiooxidans* accompanied with a strong pH decline.

Two thiobacilli, *T. thiooxidans* AHB411 and *T. thiooxidans* AHB436, able to solubilize Tilemsi rock phosphate (TRP), were isolated respectively from Canadian and Malian agricultural soils. At higher temperature, the isolate AHB436 appeared to be most effective in TRP-solubilization.

Because of, the importance of this work for poor farmers in Mali and the hopes that it arouses, I suggest to continue working in this research area to determine the effect of humidity, temperature, humectation, drought, exchangeable cations and the oxydo-reduction potential on the rate of the biological oxidation of sulfur and TRP solubilization.

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