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Phosphate, Nitrogen Fertlizer from Egyptian Ores Using *Azotobacter Vinelandai*

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Abstract

Phosphate obtained from microbial dissolution of phosphate ore consider one of the most vital recent methods, less expensive , environmentally friendly and energy saving. This process of dissolution do not need concentrate phosphate crude ore and not use specific strains of microorganism, where microorganisms were isolated from the phosphate ore as single organisms that have the most efficacy in phosphate dissolution or use mixure of microbial community of phosphate ore in dissolution to micic natural condition . The effect of different energy carbon and nitrogen sources on bacterial growth was evaluated during this work. Also the effect of *Azotobacter vinelandii* bacterial strain ,inoculum size was evaluated through this work for phosphate dissolution. production of (phosphate nitrogen) PN is cheap from local ores by harness the power of microorganisms as ore dissolution.

The optimum conditions of Abu Tartur phosphate ore dissolution were 28 hour incubation period, with growth on modified PVK medium as the best medium for dissolution of Abu Tartur phosphate ore, 2.9 log colony forming unit of *Azotobacter vinelandii* per 50 ml medium as inoculm size, 0.5% Abu Tartur phosphate ore concentration , incubation temperature at 30°C, ammonium oxalate as potential nitrogen source, glucose as potential carbon source. The leaching efficiency of phosphate content in Abu Tartur phosphate ore reaches to 52.6%.

Keywords: Bioleaching, Phosphate fertilizer, reducing bacteria, Gluconite NPK fertilizer.

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1. Introduction:

Phosphate is primary ingredients in the natural and synthetic fertilizers that utilized to provide food and feed for both mankind and animals. phosphate fertilizers application enhance agricultural production in soils with low phosphate availability, especially in the tropical and subtropical region. However, phosphate application in excess of plant requirements often results in contamination of aquatic systems. Natural rock phosphate is a complex raw material and is mainly used in the manufacture of phosphate fertilizer [1].

Nearlly 80% of all rock phosphate around the world is low-grade that not suitable for direct application to soils as a phosphate fertilizer because of its low phosphorus content and poor solubility [2]. Conventionally, rock phosphate is chemically processed with sulfuric acid or phosphoric acid into phosphate fertilizer. This process makes the fertilizer more expensive and contributes to environmental pollution [3].

Chemical methods of insoluble rock phosphate ore results in almost complete dissolution of the ore, as a result, undesirable ore contaminants are released. These contaminants, then must be dealt with as potential air and water environmental pollutants. However, bio-conversion processes of rock phosphate ore occurs at a low temperature and is more selective to phosphate extraction than chemical conventional process. The role of microorganisms in phosphate solubilization or enhanced phosphate availability has been related to production of organic acids [4], and to H^+ protonation [5]. Organic acids exuded by microorganisms form stable complexes with phosphorus adsorbents and thus maximize phosphate dissolution [5] hypothesized that the release of protons accompanying respiration or ammonium assimilation were related to phosphate solubilization by microorganisms that are not producing organic acids. microorganisms found in soil game high potential role in mobilizing P for the use of plants by bringing about changes in pH of the soil microenvironment and producing chelating substances which lead to native as well as added insoluble phosphates [6].

Some microorganisms, including bacteria and fungi, are known to be involved in the solubilization of rock phosphate [7-9]. Phosphate-solubilizing microorganisms used for industrial production of phosphate fertilizer lower the production cost. Their activity may also be exploited when an insoluble mineral phosphate is applied directly to soils.

The inoculation of P-solubilizing microorganisms is a

promising technique because it can increase P availability in soils fertilized with rock phosphates[10].

In this work, studying the factors affect on dissolution of phosphate content in Abu Tartur phosphate ore by using bacterium isolated from soil to reach to maximum dissolution of P_2O_5 from ore.

2. Material and methods:

Rock phosphate sample was collected in sterile plastic bags from phosphate mine in Safaga and Elkosir on the red sea cost in Egyptian eastern desert. Chemical composition of the studied phosphate sample is determined by using XRD analysis.

2.1. Isolation of bacterial species from soil:

Azotobacter vinelandii was idienfied according to El-Badry [11].

2.2. Culture media:

Different types of culture media are used through out the practical study of this work, which are:

Pikovskaya's medium(PVK medum):

It contains (g/l): 0.5 g/lYeast extract, 10 g/l Dextrose, 5 g/l Tri calcium phosphate, 0.5 g/l Ammonium sulphate, 0.2 g/l Potassium chloride, 0.1 g/l Magnesium sulphate, 0.0001 g/l Manganese sulphate and 0.0001 g/l Ferrous sulphate. Suspend 16.3 grams in 1000 ml distilled water. Heat if necessary to dissolve the medium completely and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Dispense as desired. This medium is solidified by adding 15 g agar per liter.

2.3. Modified Pikovskaya's medium:

It contains (g/l): 0.5 g/l Yeast extract, 10 g/l Dextrose, 0.5 g/l Ammonium sulphate, 0.2 g/l Potassium chloride, 0.1 g/l Magnesium sulphate, 0.0001 g/l Manganese sulphate and 0.0001 g/l Ferrous sulphate. Suspend 16.3 grams in 1000 ml distilled water. Heat if necessary to dissolve the medium completely and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Dispense as desired. This medium is solidified by adding 15 g agar per liter [12].

2.4. Nutrient medium:

It contains (g/l): 5g/l peptone, 3 g/l beef extract, 5g/l sodium chloride. Heat if necessary to dissolve the medium completely and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. This medium is solidified by adding 15 g agar per liter, [13].

2.5. Ashyb's medium:

It contains (g/l): 20 g/l mannitol, 0.2 g/l dipotassium phosphate, 0.2 g/l magnesium sulphate, 0.2g/l sodium chloride, 0.1 g/l potassium sulphate, 5 g/l Calcium carbonate,

15 g/l Agar. Final pH (at 25°C) 7.4±0.2, [14].

2.1.1. Testing phosphate solublization by isolated bacterium:

2.1.2. Pikovskaya's Agar Medium:

Azotobacter vinelandii was tested for phosphate solublization. Loopful sample of bacterium is placed in the center of Pikovskaya's medium agar plate and put in incubator at 30°C. The dissolution activity was screened by the presence of clear zone around the baterial colony.

2.1.3. Experiment method:

Prepare 50 ml of modified PVK broth medium in 100 ml conical flask and sterilized in autoclave for 15 min. at 121 °C and 1.5 atm. pressure and weigh 0.25 gm sterilized Abu Tartur phosphate ore per 50 ml of sterilized PVK medium and inoculants of *Azotobacter vinelandii* and also prepare another sample without bacterial strains as control then leave the flasks in shaking incubator at 30 °c and 160 rpm through the incubation period, take 5 ml from filtrate then centrifuged at 9000 rpm for 10 minuts . The amount of soluble phosphate in the culture filtrate is determined calorimetrically according to the method described by (Olsen *et al*.[15].

2.1.4. Effect of different growth parameter on phosphate solublization:

Azotobacter vinelandii is grown in 100 ml Erlenmeyer flasks containing 50 ml lots of PVK medium, modified PVK medium, Ashyb's medium and nutrient medium separately supplemented with 0.25g of Abu Tartur phosphate for 50 ml medium. Each flask is inoculated with 0.1 ml of fresh bacterial suspension and incubated at 30 °C. The amount of soluble phosphate in the culture filtrate was determined. The previous steps are conducted on modified pikovskaya's medium supplemented with Abu Tartur rock phosphate for *Azotobacter vinelandii* at different incubation periods, incubation temperatures, ore concentrations, bacterial concentration, carbon, and nitrogen sources, initial pH, addition of medium, bacterial and both of them during experiment, diameter of conical flask base.

Detection organic acid produced by *Azotobacter vinelandii***:**

Preparation of Pikovskaya's agar medium supplemented with 0.1% of bromocresol green as indicator at pH 6 then is inoculated with *Azotobacter vinelandii* at the center of plate then leave up to 3 days.

3. Results and discussion:

3.1. Chemical composition of Abu Tartur phosphate ore:

Chemical analysis of Abu Tartur phosphate ore as described in El-Barbary et al [16]

3.2. Detection of phosphate solublization by *Azotobacter vinelandii* on Pikovaskay's agar medium:

Azotobacter vinelandii has ability to dissolve phosphate which form clear zone around the bacterial colony on pikovaskay's agar medium and this refers to the solublization of $Ca_3(PO4)_2$ by bacterium and this may be due diffusion of organic acids and enzymes into the medium are recorded by formation clear zone around the bacterial colony on pikovaskay's agar medium.

3.3. Effect of incubation period on phosphate solublization by *Azotobacter vinelandii*:

Using PVK medium in presence 0.25 g Abu Tartur phosphate ore for 50 ml of medium and inoculated with 14.5 log colony forming unit of *Azotobacter vinelandii* and

measuring P_2O_5 each 1day and also pH and redox potential. The results revealed that maximum phosphate solublization is obtained after 3day which reaches to 25.3% with decreasing pH value and increasing redox potential value then P_2O_5 dissolution begins to decrease above this period (Figure (1)). Solubilization of rock phosphate depends on its structural complexity, particle size and metabolites of microorganism [17].

This agrees with Rahim Nosrati *et al*, [18] that show *A. vinelandii* delayed for 72 h and arrived at stationary phase and maximum phosphate solublizing index (~ 230mg/l) after 72 h and also noticeable reduction in phosphate solubilizing index during stationary phase of growth found in all cases supports the dependence of phosphate solubilizing index on bacterial metabolism. It was also shown that the phosphatase activity of bacterial strain could synergistically enhance the release of Pi in the acidified medium. The advantage of bacteria capable of phosphate solubilitizing with simultaneous secretion of organic acids and phosphatase activity on production and yield were shown in both green house and field trials [19].

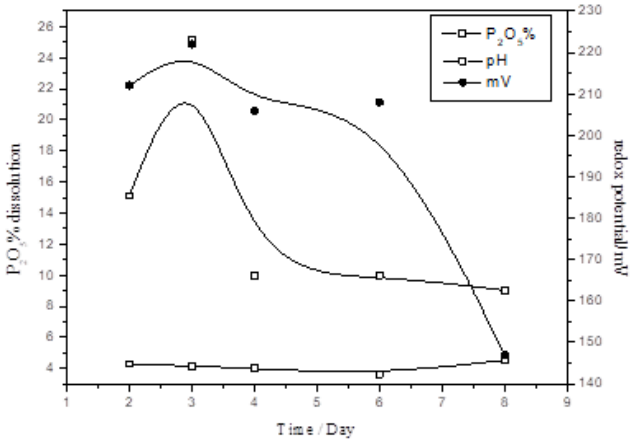

Fig (1): Effect Of Incubation Period on Dissolution of Phosphate Content of Abu Tartur phosphate Ore.

3.4. Effect of different liquid media on phosphate solublization by *Azotobacter vinelandii***:**

It is studied by using four different types of liquid media(PVK, ashyb's, modified PVK, nutrient medium) in presence 0.25 g Abu Tartur phosphate ore for 50 ml of medium and inoculated with 14.5 log colony forming unit of *Azotobacter vinelandii* and incubated at 30 °c and 160 rpm and measuring P_2O_5 each 1day and also pH and redox potential. The results revealed that maximum phosphate solublization is obtained with modified pikovasky's medium reaching to 25.3% while, the minimum phosphate solublization occurred with general liquid medium. The results are monitored with final pH, since the final pH is low with modified pikovasky's medium (3.2) and high with ashyb's medium and nutrient broth (above 7).

The solubilized phosphate may react with calcium or magnesium present in rock phosphate as soon as the pH of the growth medium increases and form insoluble phosphate (equation 1). As the dissolved phosphate concentration increases, the solution may become saturated and the re-crystallization of the mineral-phosphate species such as brushite can occur[20].

$$Ca^{+2} + HPO_4^{-2} + H_2O \longrightarrow CaHPO_4.2H_2O \quad (1)$$

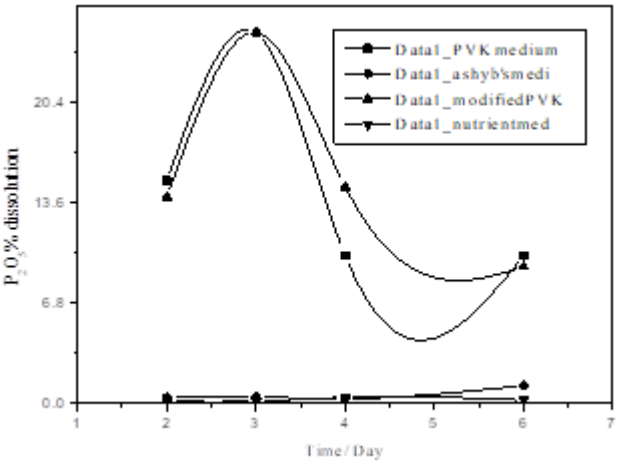

Fig (2): Effect of Type of Medium on Dissolution of Phosphate Content of Abu Tartur Phosphate Ore.

3.5. Effect of initial pH:

It is studied by using four different initial pH(4, 5, 6, 7, 8) of 50 ml of modified PVK medium in presence 0.25 g Abu Tartur phosphate ore and inculated with 14.5 log colony forming unit of *Azotobacter vinelandii* in 100 ml flask and incubated at 30 °c and 160 rpm evaluation of P_2O_5 dissolution percentage was assayed after 3day of incubation.

The growth of *Azotobacter vinelandii* was affected with the initial pH of the modified PVK medium as in the Figure (3).

The maximum growth of *Azotobacter vinelandii* on a medium containing rock phosphate is observ-ed at initial pH 7. At this pH value phosphate solublization exhibited high amounts it represented 34.7%. It is also observed that phosphate solublization at pH 5 was sharply decreased. The pH of the culture medium directly influences the growth of microorganisms and the biochemical processes they perform. In many cases, acidification is the main mechanism involved in phosphate solubilization [6 & 21 -23]. However, several studies have shown a lack of correlation between solubilized phosphorus and pH of the medium [24&9]. Therefore, a better understanding of the behavior of phosphate-solubilizing bacteria inoculated into culture media at different initial pH values may contribute to the production and management of inoculants that improve phosphate solubilization.

Several authors have suggested that a decrease in pH due to the production of organic acids and the release of protons is a basic principle of phosphate solubilization, (Sperber, 1958; Whitelaw, 2000)[25&23]. There are several solubilization mechanisms are involved at the pH of the

medium varies. These mechanisms can be: proton exclusion (via cellular respiration and ammonium absorption as N source [14], siderophores (Hamdali *et al.*, 2008)[8] and exopolisaccharide (EPS) production.

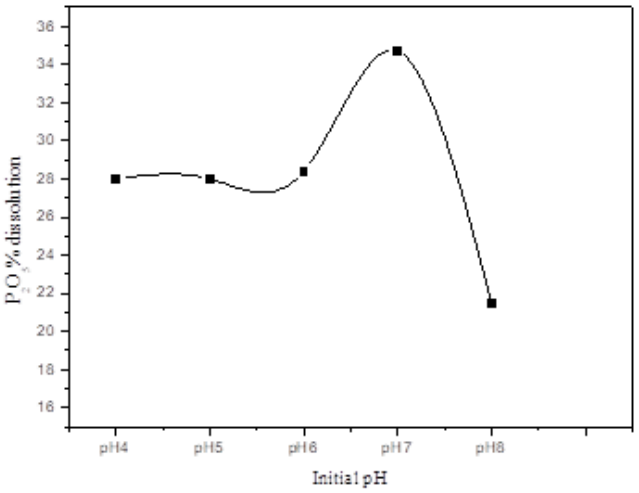

Fig (3): Effect of Initial pH on Dissolution of Phosphate Content of Abu Tartur Phosphate Ore.

3.7. Effect of bacterial inoculum size:

It is studied by using four different concentration of *Azotobacter vinelandii* (2.9, 14.5, 29, 87, 145) log colony forming unit of bacterium for 50 ml of PVK medium with intial pH7 in presence 0.25 g Abu Tartur phosphate ore and incubated at 30 °c and 160 rpm evaluation of P_2O_5 dissolution percentage was assayed after 3day of incubation.

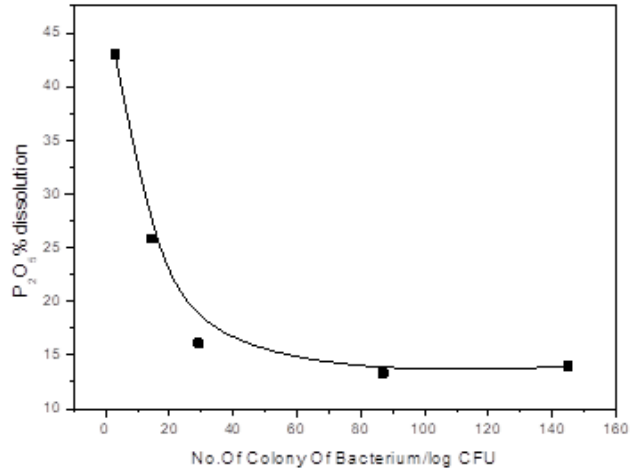

Fig (4): Effect of Bacterial Inoculum Size on Dissolution of Phosphate Content of Abu Tartur Phosphate Ore.

Concentration of bacterium affects on dissolution of phosphate content of the ore which study in the growth medium. The best phosphate solublization occurred at a concentration of 2.9 log colony forming unit of 0.25 and decrease at high concentration of bacterium with no highly change in final pH value and this may be due to competition between bacterial cells themselfes, decrease the aeration and also high growth which may consume phosphate. At a concentration of 2.9 log colony forming unit of bacterium, *bacterium* can solublize approximately 43% of phosphate content of the ore, Figure(4).

3.8. Effect of bulk density:

It is studied by using four different weights of ore(0.25, 0.5, 1, 2) g for 50 ml of modified PVK medium with initial pH 7 in presence 0.25 g Abu Tartur phosphate ore and inoculated with 2.9 log colony forming unit of bacterium and incubated at 30 °c and 160 rpm evaluation of P_2O_5 dissolution percentage was assayed after 3day of incubation.

Azotobacter vinelandii has varied growth in the presence of different concentrations of phosphate ore in the growth medium up to 4% (Figure(5). The optimum growth and best phosphate solublization occurred at a concentration of 0.5% of the Abu Tartur phosphate ore concentration and decreased above this concentration. It is also observed decrease pH value and no change highly between various concentration of ores; this may be due to the production of organic acids and acidic phosphatase enzymes. At a concentration of 0.5% ore, *Azotobacter vinelandii* can solublize approximately **43**% of phosphate content of the ore.

The dissolution of phosphate decreases with increasing phosphate ore concentration in the growth medium, that may be attributed to toxic effect of some metal ions which may be released into the culture medium such as Mn^{+2} and Na^{+1}, Ca^{+2} ions and these ions can react with soluble phosphate and form insoluble phosphate so decrease total soluble phosphate, these results found to be almost similar to that obtained by (Hefnawy *et al* [26]. Also, it may be due to the inhibitory effect on further phosphate solubilization[27], the negative effect of soluble P on microbial acid productivity might also be responsible for final soluble P concentration. Another explanation for this might be formation of an organo-P compound induced by organic metabolites released, which in turn, reduces the amount of available P[14].

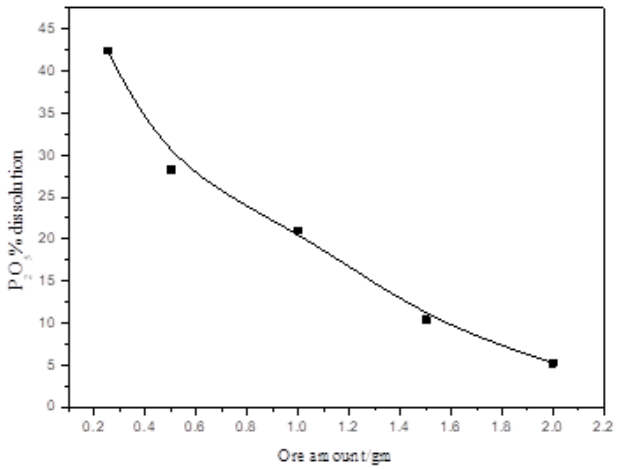

Fig (5): Effect of Bulk Density on Dissolution of Phosphate Content of Abu Tartur Phosphate Ore.

The adverse effect of increasing pulp density could be attributed to the inhibitory effect of increasing concentrations of ferric iron, the limited availability of nutrients and, O_2 and CO_2 with increasing pulp density and the mechanical damage to bacterial cells by solids, (Venkateshwarlu B. *et al*, (1984)[15].

3.9. Effect of different incubation temperatures:

It was studied by using four different temperature(20, 30, 40, 50 °c) in presence 0.25 g Abu Tartur phosphate ore for 50 ml of modified PVK medium with initial pH 7 and inoculated with 2.9log colony forming unit of bacterium with 160 rpm evaluation of P_2O_5 dissolution percentage was assayed after 3day of incubation.

The dissolution of phosphate content of ore increase with increase the temperature of incubation up to 30 °c then begins decrease (Figure 6) and dissolution of phosphate content of ore reach to 46.8% so the optimum incubation temperature for best phosphate solublization activity by *Azotobacter vinelandii* is 30 °C at which optimum growth for *Azotobacter vinelandii* and adapt to their indigenous environment so their metabolic activities are linked to the temperature of the environment {28].

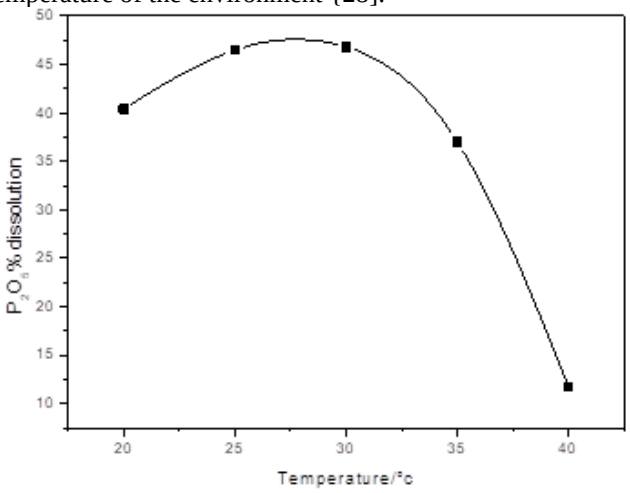

Fig (6): Effect of Incubation Temperature on Dissolution of Phosphate Content of Abu Tartur Phosphate Ore.

This agrees with Rahim Nosrati *et al* [18] which bacterial growth and consequently PSI of bacteria were reduced at both high and low temperatures. The growth of Bacterium at 30°c refers to mesophilic bacterium which grows best in moderate temperature, neither too hot nor too cold.

3.10. Effect of different nitrogen sources:

It is studied by using five different nitrogen source of (ammonium sulphate, ammonium chloride, ammonium oxalate, asparagine, glycine) in presence 0.25 g Abu Tartur phosphate ore for 50 ml of modified PVK medium with initial pH 7 in presence 0.25 g Abu Tartur phosphate ore and inoculated with 2.9log colony forming unit of bacterium and incubated at 30 °c and 160 rpm evaluation of P_2O_5 dissolution percentage was assayed after 3day of incubation.

Azotobacter vinelandii can solublize high amount of phosphorus from rock phosphate ore with all tested nitrogen sources, Figure (7). Ammonium oxalate is found to be the best nitrogen source utilized by *Azotobacter vinelandii* for maximum phosphate solublization which reaches to 50.8% followed by asparagine and lowest dissolution of phosphate content of the ore at using ammonium chloride as nitrogen source.

As a nitrogen source, ammonium oxalate was found to give maximum soluble P. Oxalate ions have the ability to form stable complexes with calcium, iron and aluminum to liberate phosphates (M.K. Saghir et al., 2009)[29] and are known to extract P from soils [30]. The phenomenon of P solubilization is correlated with the assimilation of both ammonium and chelation by oxalate ions in the culture medium and this observation may be attributed to the release of protons from the cytoplasm to the outer surface leading to dissolution of phosphate content of ore [5].

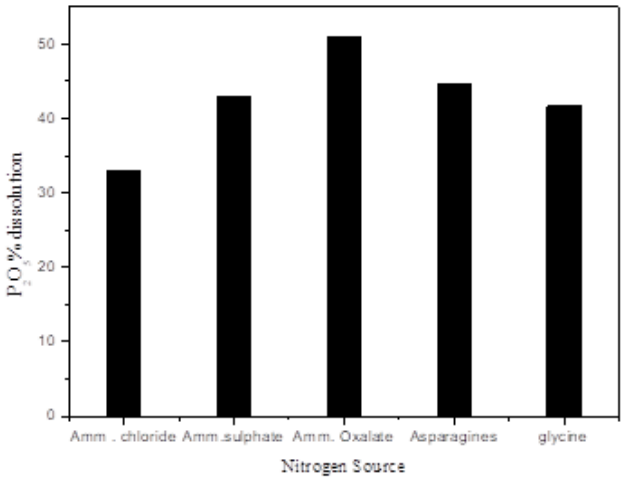

Fig (7): Effect Of Nitrogen Source On Dissolution Of Phosphate Content Of Abu Tartur Phosphate Ore.

3.11. Effect of different carbon sources:

It is studied by using four different carbon source of (glucose, starch, dextrose, sucrose) in presence 0.25 g Abu Tartur phosphate ore for 50 ml of modified PVK medium containing ammonium oxalate as nitrogen source with initial pH 7, inoculated with 2.9log colony forming unit of bacterium and incubated at 30 °c and 160 rpm evaluation of P_2O_5 dissolution percentage was assayed after 3day of incubation.

The results revealed that *Azotobacter vinelandii* grows well on modified PVK liquid medium containing different carbon sources. Whereas, high amounts of soluble phosphate is detected only in the culture filtrate of *Azotobacter vinelandii* with glucose which reaches to 52.8% then dextrose with low pH value, while starch and sucrose exhibited low amount of soluble phosphate with high pH value. The bacterial growth exhibited remarkable variation according to the utilized carbon source, the best bacterial growth to produce enzyme and organic acids reached when glucose is utilized as a carbon source (Figure 8).

The maximum amount of phosphorus solubilized corresponded to the highest value of the organic acid produced. The sugar consumption and organic acid liberation are seen to be most active up to 3 days. It is generally accepted that the release of insoluble and fixed forms of P carried out by the action of phosphate-solubilizing bacteria (PSB) via the secretion of low molecular weight organic acids mainly gluconic and keto-gluconic acids and phosphatases. These acids are produced in the periplasm of many Gram-negative bacteria through a direct oxidation pathway of glucose (DOPG, non-phosphorylating oxidation), consequently, the organic acids diffuse freely outside the cells and may release high amounts of soluble P from mineral phosphates, by supplying both protons and metal complex organic acid anions.

4. Conclusion

Production of cheap fertilizer that contains phosphate nitrogen (PN) essential element for plant from Egyptian phosphate ore by *Azotobacter vinelandii* dissolution that also as nitrogen fixation from Agriculture soil is prmising study as gate to apply microbial dissolution for egyption ore in agriculture sector as biofertilizer or as microbal fertilizer if we provide this products with active live cells of *Azotobacter vinelandii.*

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