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Isolation, occurrence, screening & characterization of rock phosphate solubilizing microbes

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Abstract
The present experiment is conducted on ‘isolation, Occurrence, Screening & Characterization of Rock phosphate solubilizing Microbes’. Phosphorus is the least mobile element in plant and soil contrary to other macronutrients. It precipitates in soil as orthophosphate or is absorbed by Fe and Al oxides through legend exchange. Phosphorus solubilizing bacteria play role in phosphorus nutrition by enhancing its availability to plants through release from inorganic and organic soil P pools by solubilization and mineralization Rock Phosphate is the cheapest and abundant Phosphatic fertilizer available but due to its sparse solubility. Most agricultural soils contain large reserves of phosphorus (P), a considerable part of which accumulates as a consequence of regular applications of P fertilizers. However, a greater part of soil phosphorus, approximately 95–99% is present in the form of insoluble phosphates and hence cannot be utilized by the plants combined application of rock phosphate with phosphate solubilizing microorganisms has emerged as a logical solution to this issue. The selection of microorganisms capable of solubilizing phosphorus (P) from rock phosphates (RP) may contribute to reduce the dependence of imported fertilizers in grain crops, reducing the costs of agricultural production, and also the environmental impacts. Principal mechanism in soil for mineral phosphate solubilization is lowering of soil pH by microbial production of organic acids and mineralization of organic P by acid phosphatase. Use of phosphorus solubilizing bacteria as inoculants increases P uptake. These bacteria also increase prospects of using phosphatic rocks in crop production. Greater efficiency of P solubilizing bacteria has been shown through co-inoculation with other beneficial bacteria and mycorrhiza In the present study bacterial and fungal strains were isolated from waste water, soil and sediment sample of ETP of Paradeep phosphate limited (PPL) and from Mahanadi river estuary (Paradeep), having potential to solubilize insoluble inorganic phosphates were characterized. Each isolates were tested for their tricalcium phosphate (TCP) and low grade rock phosphate (RP) solubilization efficiency in both solid and liquid medium. From the experiment it is found that out of 22 fungal isolates more than 50% were classified as efficient. There were significant differences in the availability of P among strains. From the Experiment it is also revealed that the solubilizing activity of both phosphates was associated with a reduction of pH which suggests that the acidification of the culture medium can be one of the mechanisms involved in the solubilization of P. Total number of 27 bacterial strains and 29fungal strains are taken for experiment and studied for maximum solubilization of insoluble inorganic phosphorous.. From the above study, it is concluded that More than 40% isolates of bacterial and fungal strains show the effect of phosphate solubilizing activity. The bacterial strain with 11B and fungal strain with 6F has highest phosphorous solubilization. From the maximum specific growth rate it can be concluded that the microbial isolates 6F and 11B are copiotrophs.

Keywords: isolation, screening, tricalcium phosphate, paradeep phosphate limited, rock phosphate biosolubilization, phosphorus

Introduction

In the soil, Phosphorus is one of the significant plant nutrients that are abruptly available. Phosphorus is imperative for morphological, physiological and biochemical growth of plants. It plays a vital part in plant germination. It holds an indispensable nutrient for plants which is an imperative integration of nucleosides, nucleotides, phospholipids etc. The plant will not accurately grow without an adequate quantity of phosphorus. A sufficient quantity of phosphorus in the initial stages of plant maturity improves physiological functions including initial root development and is essential for putting down the primordia for generative parts of plants. Externally phosphorus, crops do not transfer full yield, and animals do not succeed. Low Phosphorus levels in soils decrease crop yields by well over 50%. There are numerous animal illnesses associated with inadequate Phosphorus intake among which milk fever in high yielding cows. Lack of access to phosphorus (and other fertilizers) is one of the significant problems of agriculture in some areas.

In geological age, rocks and sediments especially phosphorous forms the largest reserve prior to minerals are concerned. The aspect of fixation and precipitation of P is extremely reliant on soil nature and pH. Therefore, iron fixes phosphorous in acid soils and Ca fixes it in alkaline soils. A broad scope of microbial Phosphate solubilization mechanisms endures in nature and many of the global cycling of soil phosphates are connected to bacteria and fungi.

Phosphate solubilising microorganisms (PSM) implies a collection of propitious organism’s proficient of hydrolyzing inorganic phosphorus from insoluble compounds. Pseudomonas, Bacillus, and Rhizobium are the usual dominant phosphate solubilizers. Mineral phosphate solubilization transpires essentially through the generation of organic acids, and acid phosphates play a very influential part in the mineralization of organic phosphorus in soil. The phosphate solubilizing microorganisms, in addition, to provide Phosphorus to plants

Further facilitate plant germination by other mechanisms.
According to the basic concepts of microbiology, the growth of bacteria and fungus is linearly formulated by Jacques Monod. Although the concept remains simple still few basic concepts remains unanswered. Monod's work in 1950's implicated the basis of growth for bacteria, fungus, and remains stringent for few decades. In Industries, the physiology of bacteria and fungus is greatly helpful is optimizing.
Growth curves reflects the maturity of an individual, particularly how the individual's growth takes periodically. The growth of bacteria and fungus is characterized by succession of phases like lag phase, exponential phase, stationary phase and decay phase. The growth of bacteria and fungus is strictly depended on few factors like temperature, pH, growth media, substrate concentration. Summing up, Monod model derived the interrelationship between growth rate and the substrate concentration.
Phosphate solubilising micro-organisms (PSMs) have the ability to convert organic or inorganic phosphorus into forms available for plant uptake, through the process of solubilisation and mineralization. They do this by producing organic acids which acidify their surrounding soil and lower the soil pH therefore, creating a favourable condition for phosphate solubilisation. Examples of common PSMs among the bacterial community are Pseudomonas, Bacillius, Rhizobium, Enterobacter. While among the fungal group Aspergilli and Penicillum are well known.
A study showed that PSMs integrated with rock phosphates, and compost increased crop yield and biological nitrogen fixation for two legume crops (chickpea and lentil) compared to inorganic/chemical phosphorus fertilizers. Phosphorus is the second most important essential element in plants nutrition, next to nitrogen (N) (Hamdali *et al*., 2012). It is involved in photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis, and respiration (Sharma *et al*. 2013), all of which are necessary for plant growth and development. Despite its importance in plant growth and metabolism, phosphorus is the least accessible macro-nutrient and hence the most frequently deficient nutrient in most agricultural soils because of its low availability and its poor recovery from the applied fertilizers.

Potential Role of Microorganisms in Solubilizing Phosphate Rocks

Some microorganisms are able to convert inorganic P present in phosphate rocks into the bioavailable form, thus facilitating uptake of P by plant roots. Some actinomycetes, bacterial and fungal species isolated from soils in the vicinity of different rock phosphate deposits around the world have shown positive results. Actinomycetes which have shown potential ability of solubilizing insoluble phosphorus are of the genera Micromonospora and Streptomyces, e.g. S. griseus and S. coelicolor. Similarly, members of the bacterial genus Serretia have been reported to display high P solubilizing ability (Hamdali *et al*., 2012). Other bacteria are from the genera Bacillus, Rhodococcus and Arthrobacter. Fungal species with the ability to solubilize phosphate rocks have also been reported. Omar (1997), for example, reported fungal species isolated from soil which have the ability to solubilize rock phosphate (RP) in agar plates; these include Aspergillus Niger and Penicillium citrinum. It is probable that these and other microorganisms with potential to solubilize insoluble P compounds could be widespread in soils, especially those in and around rock phosphate deposits.
Isolation, testing and identification of efficient P-solubilizing microorganisms (PSMs) could lead to the development of inoculants to be used with insoluble rock phosphate as sources of P to plants, akin to the use of rhizobia inoculants in inoculating legume seeds for increased nitrogen fixation. Zaidi *et al*. (2009) observed that PSMs could be isolated from rhizosphere and non-rhizosphere soils, rhizoplane, phyllosphere, soils in contact with rock P and even from P stressed soils. Historically, identification and classification of microorganisms were based on phenotypic characteristics. Currently, two fundamental molecular applications are being extensively utilized in identification and classification of microorganisms; these are based on hybridization and nucleotide sequencing molecular methods. These molecular methods are accurate in identifying organisms e.g. Chen *et al*. (2006), identified and phylogenetically analysed 36 phosphate solubilizing bacterial isolates using 16S rDNA sequencing. The studies cited above (e.g. Hamdali *et al*., 2008; Rodr gue and Fraga, 1999), of microbial solubilization of P have been largely laboratory based. There is need to extend them to field testing. However, in Tanzania there is a lack of basic studies on phosphate solubilizing microorganisms (PSMs). We must, therefore, start with laboratory tests (isolation, testing, and characterization) and then try to extend the results to the field. This is important because these PSMs may be widely spread in soil. Such microorganisms could then be used to develop inexpensive inoculants that would render feasible the use of unprocessed phosphate rocks by small-scale farmers.
In the present study bacterial and fungal strains were isolated from waste water, soil and sediment sample of ETP of Paradeep phosphate limited (PPL) and from Mahanadi river estuary (Paradeep), having potential to solubilize insoluble inorganic phosphates were characterized. Each isolates were tested for their tricalcium phosphate (TCP) and low grade rock phosphate (RP) solubilization efficiency in both solid and liquid medium. Among 22 fungal isolates more than 50% were classified as efficient. There were significant differences in the availability of P among strains. The solubilizing activity of both phosphates was associated with a reduction of pH which suggests that the acidification of the culture medium can be one of the mechanisms involved in the solubilization of P. Total number of 27 bacterial strain and 27 fungal strain are investigated from which the highest insoluble inorganic phosphorous concentration is considered. From Monod kinetics, an equation is designed to calculate out the maximum cell growth in batch culture using certain parameters and optimal conditions. The contribution of these strains for increasing the phosphorus nutrition of grain crops should be investigated *in vivo* experiments

Materials and methods

2.1 Soil and water sample collection

Low grade rock phosphate was collected from Hindustan Zinc limited, Udaipur. Marine soil. Water and sediment sample has been collected from the vicinity of Paradeep Phosphates Limited (PPL) Paradeep, Odisha for isolation of phosphate solubilizing microbes which is shown in Table 2. Prior to sampling the collection bottles has been rinsed well and then filled up to neck and stoppered immediately to prevent any accidental entry/escape as well as interaction with outside atmosphere. It is composed of 7.5% total P,30% total Ca,0.6% total Mg,3.82% total Fe,0.9% total Na,2.93% total Al,15.45% total Si,1.23% total D,0.16%total total K,0.23% total T;10.3 mg kg^{-1} total Mn,45 mg kg^{-1} total La respectively. The following chemical composition is shown in table 1. For soil and rock phosphate (RP)

2.2. Pure Culture of fungus
A pure culture is developed from a mixed culture, by transferring the culture into a new sterile medium in order to isolate unicellular cells. In the present study, the pure culture of the fungus was spread on Potato Dextrose Agar (PDA) plates for fungus growth. PDA plates were made by dissolving 39gm of PDA in 1000ml of distilled water. This was then sterilized by autoclaving at a pressure of 15psi and temperature 121 ^{0}C for the duration of 45 minutes and then poured into empty Petri plates. With the help of autoclave and UV sterilization, the glassware was sterilized. Once it reaches room temperature the plates were streaked and incubated for 2-3 days and was kept in inverted position in order to avoid water vapor

2.3.1. Microbes and media preparation
The organism isolated with the highest phosphorous solubilization efficiency are Fungus 6F and Bacteria 11B.The strain 6F fungus has a black color velvet morphology whereas the bacteria 11 B has a rod shaped white color morphology. The medium used was rock phosphate (RP) broth. The rock phosphate used was in the particle size of -75+45. The RP broth was prepared in the following manner.

2.3.2. Inoculation
The glassware's and the laminar air flow chamber was sterilized by autoclaving and UV sterilization respectively.50ml of the minimal media was added to each conical flask. Homogenous liquid aerobic cultures of the Fungus 6F and Bacteria 11B were obtained by growth in 50ml conical flask on a rotary shaker.1 ml of the respective suspension was added to the 50 ml minimal media flask and swirled to mix, sealed with cotton plug. The conical flask were incubated for 24,48,72,96 hours respectively on a rotary shaker at about 150 rpm and a temperature of 35 ± 3 ºC

2.3.3. Harvesting
Once the growth formation starts, the flask were harvested at respective time periods. The broth culture was found to be turbid. The cell mass concentration (mg/ml), pH, phosphorous concentration (ppm) were measured.

2.3.4. Cell mass concentration
The determination of cellular dry weight is the most commonly used direct method. Samples of culture broth were centrifuged at 10000 rpm, -4 ^{0}C for 10minutes and washed with distilled water. The washed wet cell mass was then dried at 60^0 C for 24 hours; then dry cell weight is measured.

2.3.5. PH
The pH of the culture broth samples were measured by water analyzer. The fungal pH varied in the range of 5-7 whereas the bacterial pH was in the range of 7-7.5.

2.3.6. Phosphorous concentration
The phosphorous standard was prepared by Bray's extraction method i.e. by using ammonium molybdate and stannous chloride solution. Samples of the culture broth were taken and phosphorous availability was calculated at 660nm with standard Monopotassium phosphate by using Spectrophotometer.

Results and Discussion
3.1 Pure Culture of Fungus

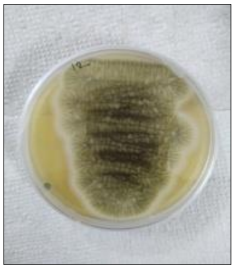

Fig 1: 6F

3.2. Pure culture of Bacteria

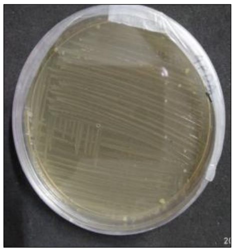

Fig 2: 11B

3.3. Effect of Particle Size

Table 3: Particle size of rock phosphate and solubilization

Particle Size(microns)	Phosphorous Concentration (ppm)
500	0
250	6.8
180	10.1
150	10.5
106	8.7
75	11.9
45	9.4

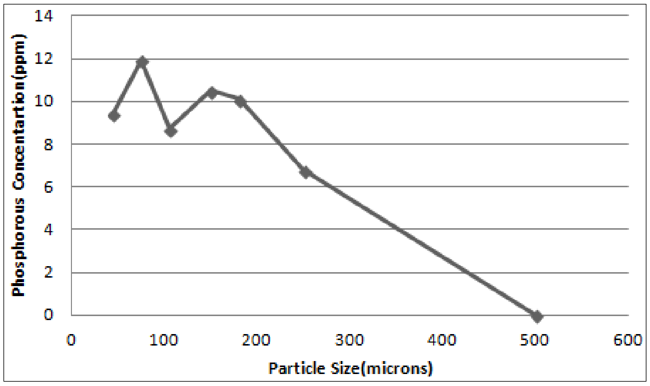

Fig 1: Particle size w.r.t. phosphorous concentration

The results of rock phosphate solubilized, are presented in Table 4. It is worth noted from figure 5 that with the increase in particle size the solubilization rate decreases and tends to increase the solubilization with the mono size fractions. Maximum solubilization of organisms was obtained when rock phosphate of -75+45 micron was used and the maximum phosphate solubilised amounted to 11.9 ppm.

3.4 Occurrence
Rock phosphate is found in all continents of the world. It is used as a raw material in the manufacture of inorganic phosphorus fertilizers and can be used as organic phosphorus fertilizers when applied directly to the soil. Direct application of rock phosphate increases crop yield and soil phosphorus levels. Other benefits to the direct use of rock phosphate are: It is relatively cheap compared to inorganic/chemical phosphorus fertilizers, It is environmentally friendly. As a natural source of phosphorus, it avoids the use of inorganic fertilizers which contribute to greenhouse gas emission during its manufacturing process, In addition to increasing soil phosphorus, it adds other nutrients to the soil. Studies show rock phosphate increases the soils exchangeable calcium and magnesium cations; it also increases carbon accumulation which in turn improves soil quality. Though it is a natural source, it is classified as a non-renewable resource. Continuous use will lead to depletion if no alternative is found. It is predicted that reserves will be depleted in about 500-600 years from now

Research has shown rock phosphate to be more beneficial in acidic soils than alkaline soils; this is because rock phosphate is more soluble in acidic soils (pH <5.5). Phosphorus in a soluble form is available for uptake by plant roots. To make phosphorus available in alkaline soils rock phosphate is partially acidified with inorganic acids before application. This approach is not applied in organic agriculture as it involves the use of inorganic compounds. An approach more welcomed in organic farming is the integration of rock phosphate with compost or the integration of rock phosphate, compost and phosphate solubilising microorganisms. This combination has proven to improve the availability of phosphorus to plants

Conclusion
The present investigation of bacterial and fungal strains were isolated from waste water, soil and sediment sample of ETP of Paradeep phosphate limited (PPL) and from Mahanadi river estuary (Paradeep), having potential to solubilize insoluble inorganic phosphates were characterized. More than 40% isolates of bacterial and fungal strains shows the effect of phosphate solubilizing activity. The bacterial strain with 11B and fungal strain with 6F has highest phosphorous solubilization. The present study also highlighted the growth and utilization of microbes using inorganic chemicals. Nowadays the most important prospect of bio industry is the control of optimum factors. For this particular reason an relationship is established to know the cell growth at each time period and the expression formulated will be used for further microbial processes. But the unreal structure makes the kinetic study difficult because of non-uniformity, use of heterogeneous medium. In the present study, the main objective was to find out the optimizing conditions influencing batch growth. The parameters were evaluated using optimum conditions.

- Different graphs were plotted of fungus and bacteria for PVK broth and RP broth and it is worth noted that with the decrease of pH value the solubilization rate decreases.
- The graph plotted for fungi and bacteria between dry weight w.r.t. to time assumes to be a sigmoid shape. Maximum dry weight is being observed at particular 72 hours.

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