Solubilization of Phosphate Rocks and Minerals by Wild Type and UV Induced Mutants of *Aspergillus tubingensis*

A
DISSERTATION

Submitted in partial fulfillment of the requirements
For the award of the degree of Masters of Science in Biotechnology

BY
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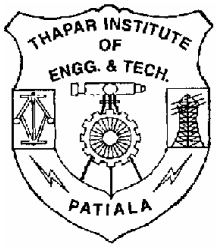

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MAY 2005

DEDICATED TO……MY GUIDE

Candidate's Declaration

I, hereby declare that the work which is being presented in the dissertation entitled **"Solubilization of Phosphate Rocks and Minerals by Wild Type and UV Induced Mutants of Aspergillus tubingensis",** in partial fulfillment of the requirement for the award of the degree of Masters of Science in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar Institute of Engineering and Technology, Patiala, Punjab; is an authentic record of my own work during a period of five months from January 2005 to May 2005, under the supervision of Dr. M. Sudhakar Reddy, Assistant Professor, Department of Biotechnology and Environmental Sciences, Thapar Institute of Engineering and Technology.

Place: Patiala VARENYAM ACHAL
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This is to certify that the above statement made by the candidate is correct and true to the best of my knowledge.

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Contents

Chapter 1

Introduction

1. INTRODUCTION

Phosphorus (The Morning star- Vallentyne, 1974) is an essential nutrient for plant growth and development. It is classified as a macronutrient because of the relatively large amounts of P required by plants. Despite its wide distribution in nature, it is a deficient nutrient in most soils. Many soils are defined as having high P-fixation capacity, since a substantial amount of any applied P fertilizer is rendered unavailable and frequent applications of soluble forms of inorganic P are needed to maintain adequate P levels for plant growth. With the current tendency for a reduced use of agrochemicals and efficient application of natural materials in agro ecosystems, a renewed interest in direct application of rock phosphate (RP) has arisen (Rajan *et al*., 1996).

Phosphorus plays a key role in the balanced nutrition of plants (Hayman, 1975). Phosphorus is second only to nitrogen as an essential macronutrient for plant growth and development (Scheffer *et al*., 1998).

Pierre (1938) referred to it as the 'Master Key' element in crop production. It is associated with several vital functions and is responsible for several characteristics of plant growth such as utilization of sugars and starch, photosynthesis, nucleus formation and cell division, fat and albumin formation, cell organization and the transfer of heredity (Arnon, 1956 and McVicker *et al*., 1963).

Phosphate is taken up by plants from soils, utilized by animals that consume plants, and returned to soils as organic residues decay in soils. Much of the phosphate used by living organisms becomes incorporated into organic compounds. When plant materials are returned to the soil, this organic phosphate will slowly be released as inorganic phosphate or be incorporated into more stable organic materials and become part of the soil organic matter. The release of inorganic phosphate from organic phosphates is called mineralization and is caused by microorganisms breaking down organic compounds. The activity of microorganisms is highly influenced by soil temperature and soil moisture. Soils are often high in insoluble mineral and organic phosphates but deficient in available orthophosphate (Pi) (Dadarwal *et al*., 1997).

Soil amendment with phosphatic fertilizer, produced via chemical processing of rock phosphate ore, is therefore an absolute requirement in order to feed the world's population. For over one hundred years, workers have recognized the ability of soil microorganisms to solubilize Pi from insoluble (*i.e.* nutritionally unavailable) organic and mineral phosphates (Whitelaw, 2000). Wide ranges of microbial biosolubilization mechanisms exist, so that much of the global cycling of insoluble organic and inorganic soil phosphates is attributed to bacteria and fungi. The genetic and biochemical mechanisms for this solubilization are as varied as the spectrum of P-containing soil compounds.

The limiting level of Pi in most soils provides the ecophysiological basis for positioning associations between plant roots and mineral phosphate solubilizing (MPS) and/or organic P solubilizing microorganisms. These associations are assumed to play an important role in phosphorus nutrition in many natural and agro-ecosystems. As a result, an enormous amount of research has been conducted involving isolation and characterization of MPS and organic P solubilizing microorganisms from a wide range of soils. In general, the goals have been to understand P cycling and/or to develop P biofertilizers analogous to biological nitrogen fixation.

With respect to agriculture, bioprocessing of rock phosphate ore (RPO) to inorganic phosphate may provide an energy efficient, environmentally desirable alternative to current technology for industrial P fertilizer production.

1.1 SIGNIFICANCE OF PHOSPHORUS

Phosphorus plays an indispensable biochemical role in photosynthesis, respiration, energy storage and transfer, cell division cell enlargement and several other processes in the living plant.

An adequate supply of phosphorus in the early stages of plant growth promotes physiological functions including early root formation, and is important for laying down the primordial for reproductive parts of plants. It is vital to seed formation and its content is higher in seeds than in any other part of the plant. It helps plants to survive winter rigors and also contributes to disease resistance in some plants. Also known to improve quality of many fruits, vegetables and grain crops. Biological nitrogen fixation depends appreciably on the available forms of phosphorus.

Phosphorus (P) is an important structural constituent of nucleic acids, phytin and phospholipids.

1.2 PHOSPHATE AVAILABILITY IN SOILS

Mineral forms of phosphorus constitute the biggest reservoirs of phosphorus, represented primarily by rocks and deposits formed during geological age. The principal characteristic of these primarily minerals (oxyapatite, hydroxyapatite, apatite) is their insolubility (Figure 1).

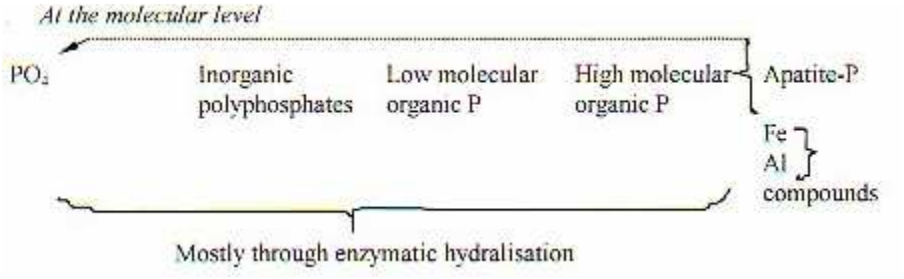

Figure 1: Diagram showing relative importance of phosphorus availability at the molecular level (Vollenweider, 1979)

A large portion of soluble inorganic phosphate applied to agricultural soil as chemical fertilizer is rapidly immobilized soon after application and becomes unavailable to plants (Dadarwal *et al*., 1997).

A second major component of soil P is organic matter, present largely in the forms of inositol phosphate (soil phytate), accounting for up to 50% of the total organic P. Other organic P in the soil is in the form of phosphomonoesters, phosphodiesters including phospholipids and nucleic acids, and phosphotriesters. A schematic representation of phosphorus cycle, found in nature is shown in Figure 2.

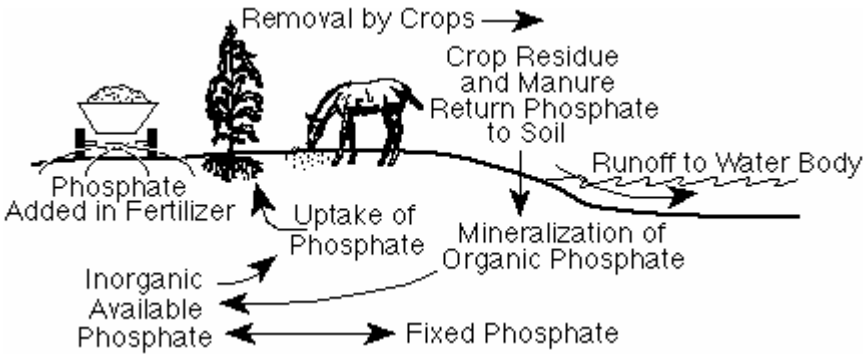

Figure 2: A Schematic Representation of Phosphorus cycle

Besides these, large quantities of xenobiotic phosphonates are released into the environment.

Despite of being so rich, the concentration of soluble P (*i.e.* bioavailable P) is usually very low in soils due to the phenomenon of chemical fixation of phosphate.

1.3 FORMS OF PHOSPHORUS IN SOILS

In soils P may exist in many different forms. In practical terms, however, P in soils can be thought of existing in 3 "pools":

The **solution P pool** is very small and will usually contain only a fraction of a pound of P per acre. The solution P will usually be in the orthophosphate form, but small amounts of organic P may exist as well. Plants will only take up P in the orthophosphate form. The solution P pool is important because it is the pool from which plants take up P and is the only pool that has any measurable mobility. Most of the P taken up by a crop during a growing season will probably have moved only an inch or less through the soil to the roots. A growing crop would quickly deplete the P in the soluble P pool if the pool was not being continuously replenished.

The **active P pool** is P in the solid phase which is relatively easily released to the soil solution, the water surrounding soil particles. As plants take up phosphate, the concentration of phosphate in solution is decreased and some phosphate from the active P pool is released. Because the solution P pool is very small, the active P pool is the main source of available P for crops. The ability of the active P pool to replenish the soil solution P pool in a soil is what makes a soil fertile with respect to phosphate. An acre of land may contain several pounds to a few hundred pounds of P in the active P pool. The active P pool will contain inorganic phosphate that is attached (or adsorbed) to small particles in the soil, phosphate that reacted with elements such as calcium or aluminum to form somewhat soluble solids, and organic P that is easily mineralized. Adsorbed phosphate ions are held on active sites on the surfaces of soil particles. The amount of phosphate adsorbed by soil increases as the amount of phosphate in solution increases and vice versa. Soil particles can act either as a source or a sink of phosphate to the surrounding water depending on conditions. Soil particles with low levels of adsorbed P that are eroded into a body of water with relatively high levels of dissolved phosphate may adsorb phosphate from the water, and vice versa.

The **fixed P pool** of phosphate will contain inorganic phosphate compounds that are very insoluble and organic compounds that are resistant to mineralization by microorganisms in the soil. Phosphate in this pool may remain in soils for years without being made available to plants and may have very little impact on the fertility of a soil. The inorganic phosphate compounds in this fixed P pool are more crystalline in their structure and less soluble than those compounds considered to be in the active P pool. Some slow conversion between the fixed P pool and the active P pool does occur in soils.

1.4 ROCK PHOSPHATES

To overcome the specific P nutrient deficiency, various forms of P, varying from processed rock phosphates (P-fertilizers) to ground phosphate rocks are applied. The use of commercial P-fertilizers is not cost effective. Among the alternative P sources, the most important are locally available Rock Phosphate (RP) resources (Rajan *et al*., 1996).

There are so many types of rock phosphate (Table 1), mainly based on their geographical region. Not all of the RP resources are readily plant available and agronomically reactive when applied directly to the soils. Reactivity is defined as the combination of RP properties that determines the rate of dissolution of RP in a given soil under given field conditions. The main factors influencing the agronomic effectiveness of rock phosphates are:

1) Mineralogy and chemistry of rock phosphates;
2) Reactivity/solubility of phosphate rocks;
3) Grain size and surface area;
4) Chemical and physical status of soil, especially pH and P fixing capacity of soil;
5) Type of crops and their nutritional requirements;
6) Management practices, including method and time of application and liming.

Table 1: Chemical composition of some of the rock phosphates:

S.No.	Type of Rock Phosphate	P_2O_5 (%)	Calcium as CaO (%)	Fluoride as F (%)	Acid Insoluble (%)
1.	Foskar	40.0	54.96	2.44	0.75
2.	Jordan	33.3	53.20	3.90	1.86
3.	Rajasthan	34.1	48.33	3.20	6.33
4.	Israel	32.9	52.60	3.85	0.95
5.	Morocco	33.2	51.90	4.20	2.80

Source: Bilt Chemicals, Karnataka.

1.5 PHOSPHATE SOLUBILIZING MICROORGANISMS (PSMs)

PSMs include different groups of microorganisms, which not only assimilate phosphorus from insoluble forms of phosphates, but they also cause a large portion of soluble phosphates to be released in quantities in excess of their requirements.

The species of *Aspergillus* and *Penicillium* are among fungal isolates identified to have phosphate solubilizing capabilities. Among the bacterial genera with this capability are *Pseudomonas*, *Azospirillum*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Arthrobacter*,

Alcaligenes, *Serratia*, *Enterobacter*, *Acinetobacter*, *Flavobacterium* and *Erwinia* (Rodriguez, 1996).

Seed or soil inoculation with PSMs is known to improve solubilization of fixed soil phosphorus and applied phosphates resulting in higher crop yields (Jones, 1994). PSMs are a low-cost solution that enriches the soil giving a thrust to economic development without disturbing ecological balance.

The production of organic acids by these microorganisms seems to be the main cause of phosphate solubilization. These microorganisms are found to produce monocarboxylic (acetic, formic); dicarboxylic (oxalic, succinic), tricarboxylic hydroxyl (citric) acids in liquid media. The role of organic acids in dissolving mineral phosphates and phosphorylated minerals can be attributed to the lowering of pH, which helps in the formation of stable complexes with such cations as Ca^{++}, Mg^{++}, Fe^{+++} and Al^{+++}. Phosphatase and phytase enzymes secreted by these microorganisms having important role in phosphate solubilization.

1.6 *Aspergillus tubingensis*

Aspergillus is a group of moulds, which is found everywhere world-wide, especially in the autumn and winter in the Northern hemisphere. Moulds are also called as filamentous fungi. Some of *Aspergillus* species are known as a pathogenic but this species i.e., *Aspergillus tubingensis* has not been found to be pathogenic. *Aspergillus* is a filamentous, cosmopolitan, black spore producing (Figure 3) and ubiquitous fungus found in nature. It is commonly isolated from soil, plant debris, and indoor air environment.

As previous work performed in this very laboratory, *Aspergillus tubingensis* (AT 1) was found to having better phosphorus solubilization capacity than any other microorganisms. So, this strain is used for further analysis.

Macroscopic morphology

Colonies on potato dextrose agar or Pikovskaya's agar medium at 30ºC are initially white, quickly becoming black with conidial production.

Microscopic morphology

Hyphae are septate and hyaline. Conidial heads are radiate initially, splitting into columns at maturity (Figure 4). The species is biseriate (vesicles produces sterile cells known as metulae that support the conidiogenous phialides). Conidiophores are long (400-3000 µm), smooth, and hyaline, becoming darker at the apex and terminating in a globose vesicle (25-65 µm in diameter). Conidia are brown to black, very rough, globose, and measure 4-5 µm in diameter (Sutton, D. A. *et al*., 1998).

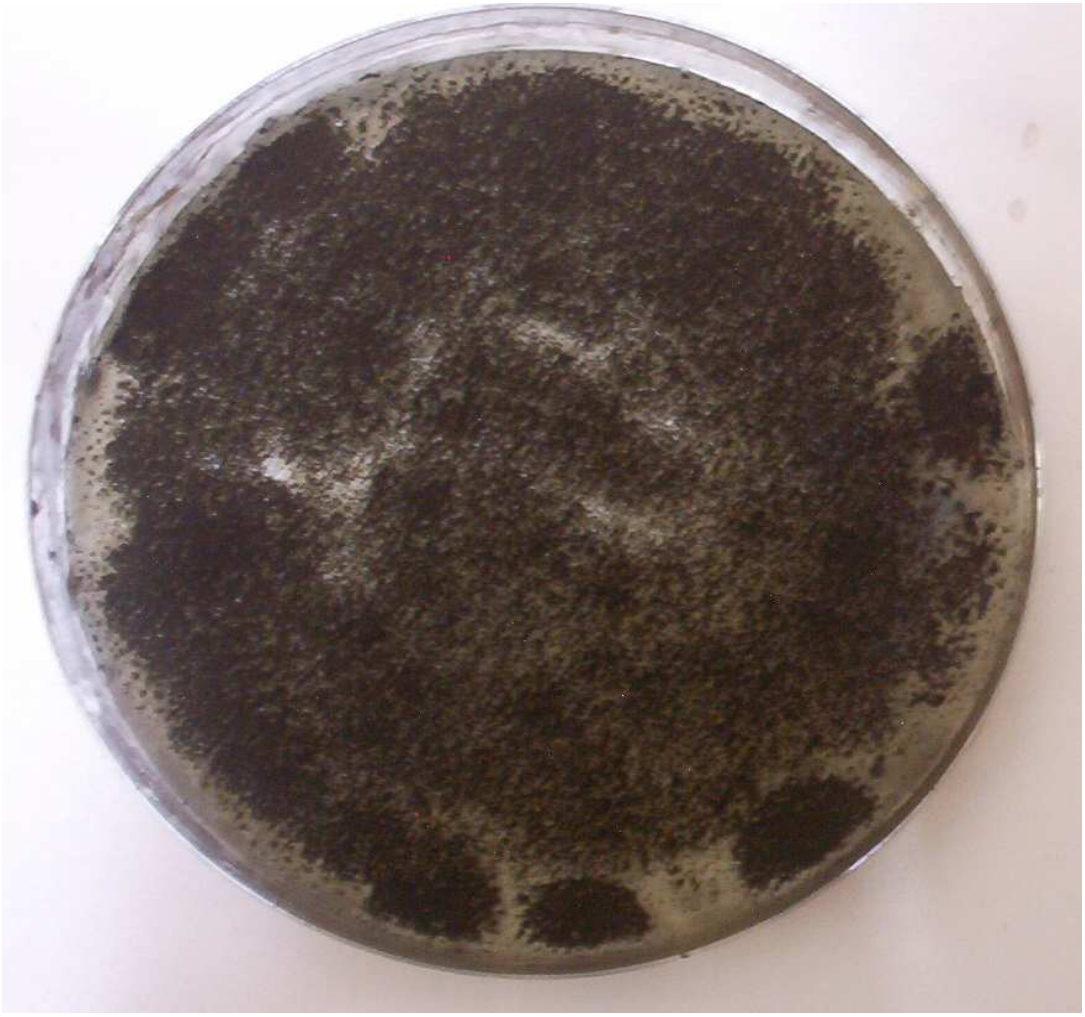

Figure 3: *Aspergillus tubingensis* (in PDA plate)

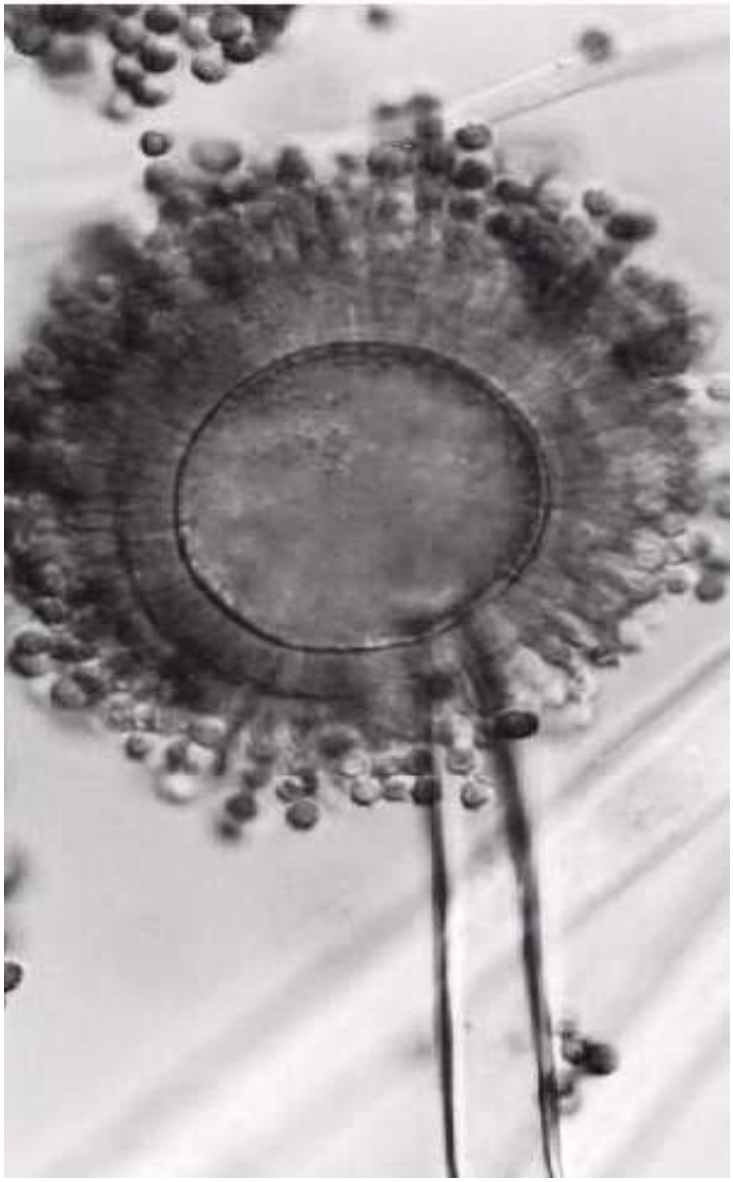

Figure 4: Microscopic structure of *Aspergillus tubingensis*

1.7 WILD TYPE AND UV INDUCED MUTANTS OF *Aspergillus tubingensis*

Mutation is regarded as an error during DNA replication that results in a change in the sequence of deoxyribonucleotide bases in the DNA. When phenotype of wild type is

changed using UV as a mutagen, it is UV induced mutant. (Forward mutation – change in phenotype from wild type to mutant).

Why this focus? Why are mutations important? There are several reasons: 1) they may have deleterious or (rarely) advantageous consequences to an organism (or its descendants); 2) they are important to geneticists: the most common way we study something is to break it--*i.e.*, we search for or make a variant (mutant) lacking the ability to perform a process which we want to study. These genetic variants possess mutant alleles of the genes we are interested in studying. 3) Mutations are important as the major source of genetic variation which fuels evolutionary change.

Ultraviolet rays are one of the widely used physical induced mutagen to make mutants. UV is normally classified in terms of its wavelength (Figure 5): **UV-C** (180-290 nm)--"germicidal"--most energetic and lethal, it is not found in sunlight because it is absorbed by the ozone layer; **UV-B** (290-320 nm)--major lethal/mutagenic fraction of sunlight; **UV-A** (320 nm--visible)--"near UV"--also has deleterious effects (primarily because it creates oxygen radicals) but it produces very few pyrimidine dimers.

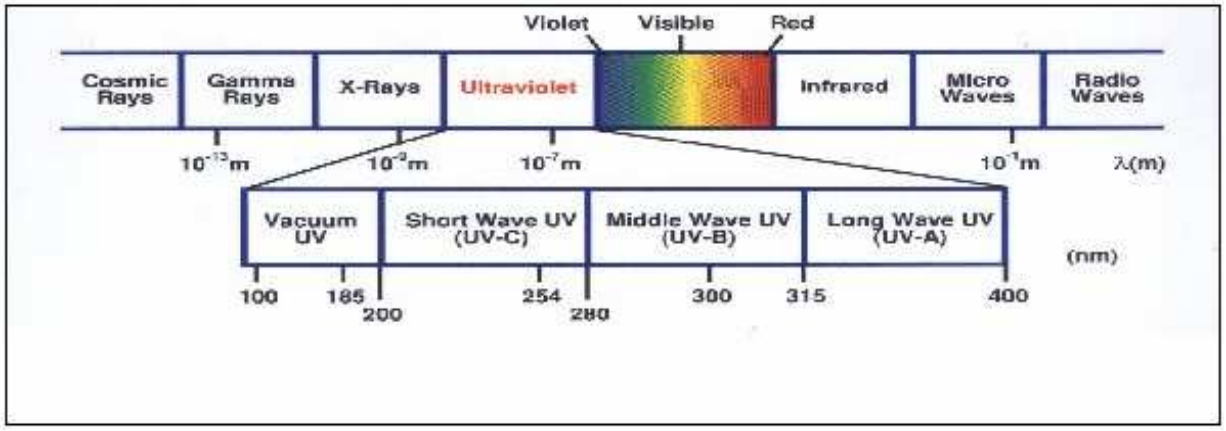

Figure 5: Ultraviolet wavelength range

In terms of its mode of action, UV light is absorbed by DNA and causes adjacent thymine bases on the same DNA strand to covalently bond together, forming what are called thymine-thymine dimers (Figure 6 and 7). UV light induces the condensation of two

ethylene groups to form a cyclobutane ring and forms cyclobutane pyrimidine dimers in cells (Figure 8). A second type of pyrimidine dimer, 6-4 photoproduct also formed during UV irradiation (Figure 9).

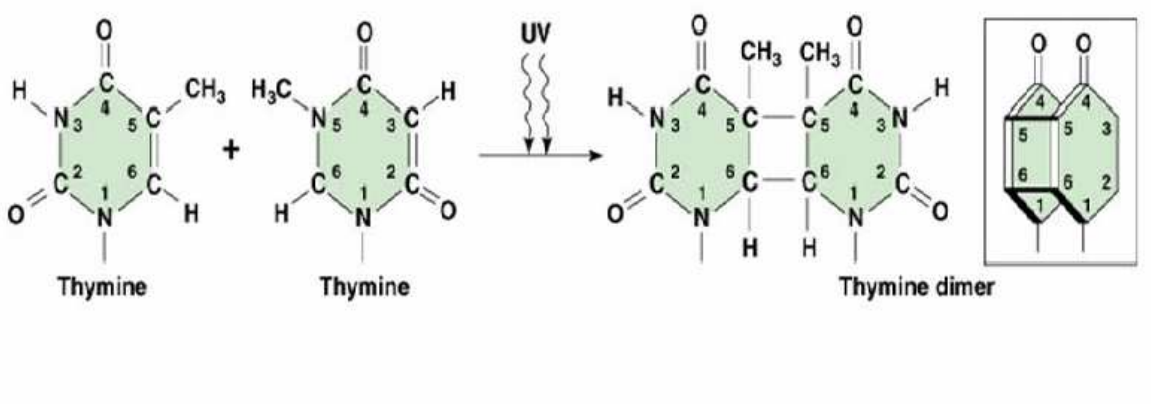

Figure 6: Formation of Thymine dimer upon exposure of UV rays

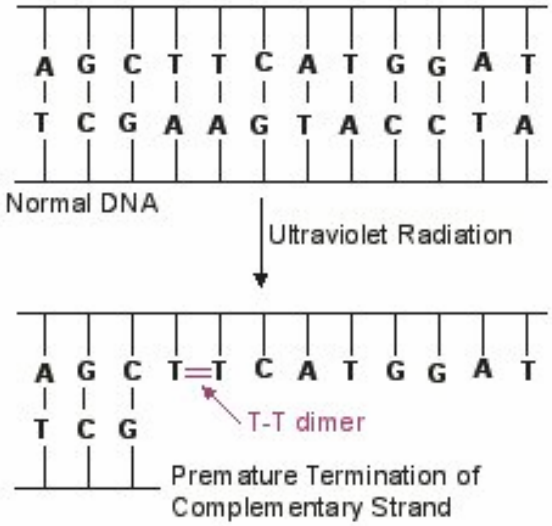

Figure 7: Formation of thymine-thymine dimer in a DNA strand

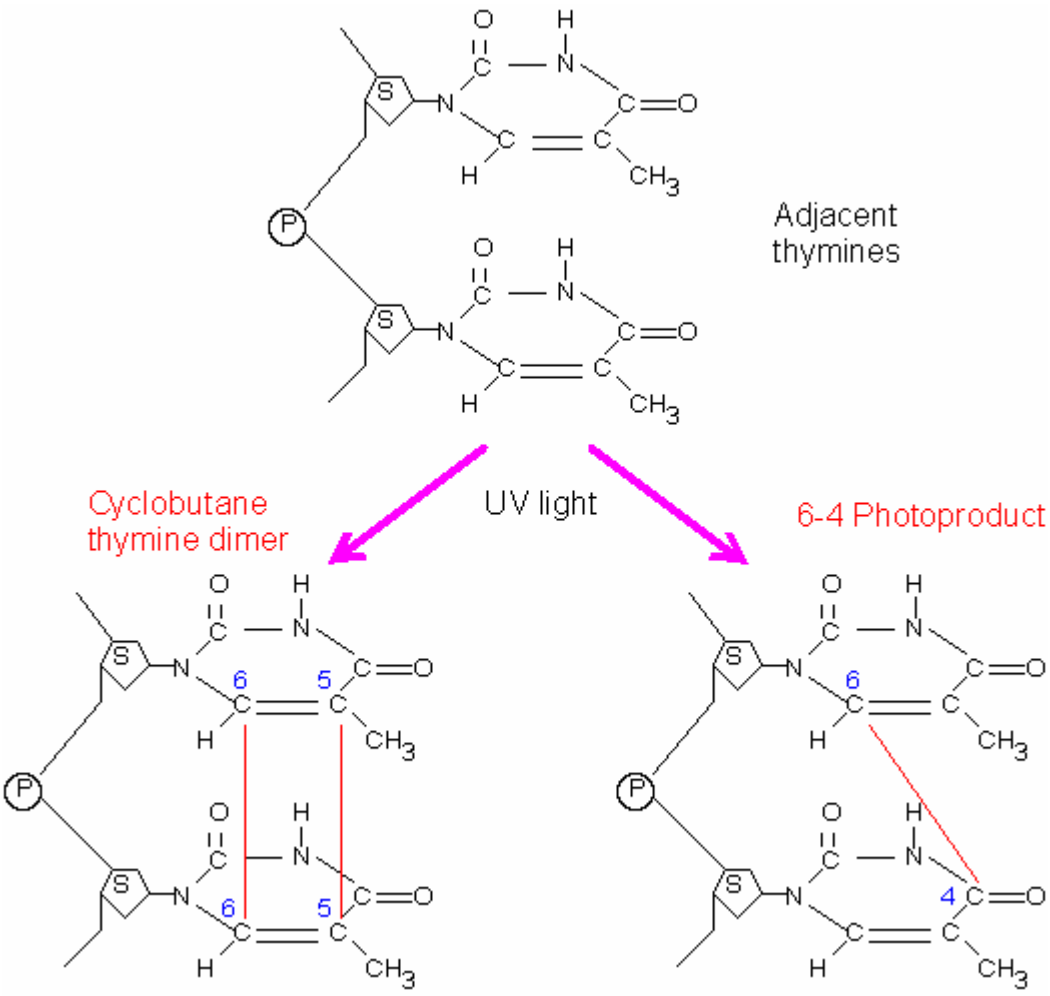

Figure 8: Mode of action of UV light on DNA, resulting in the formation of two Products

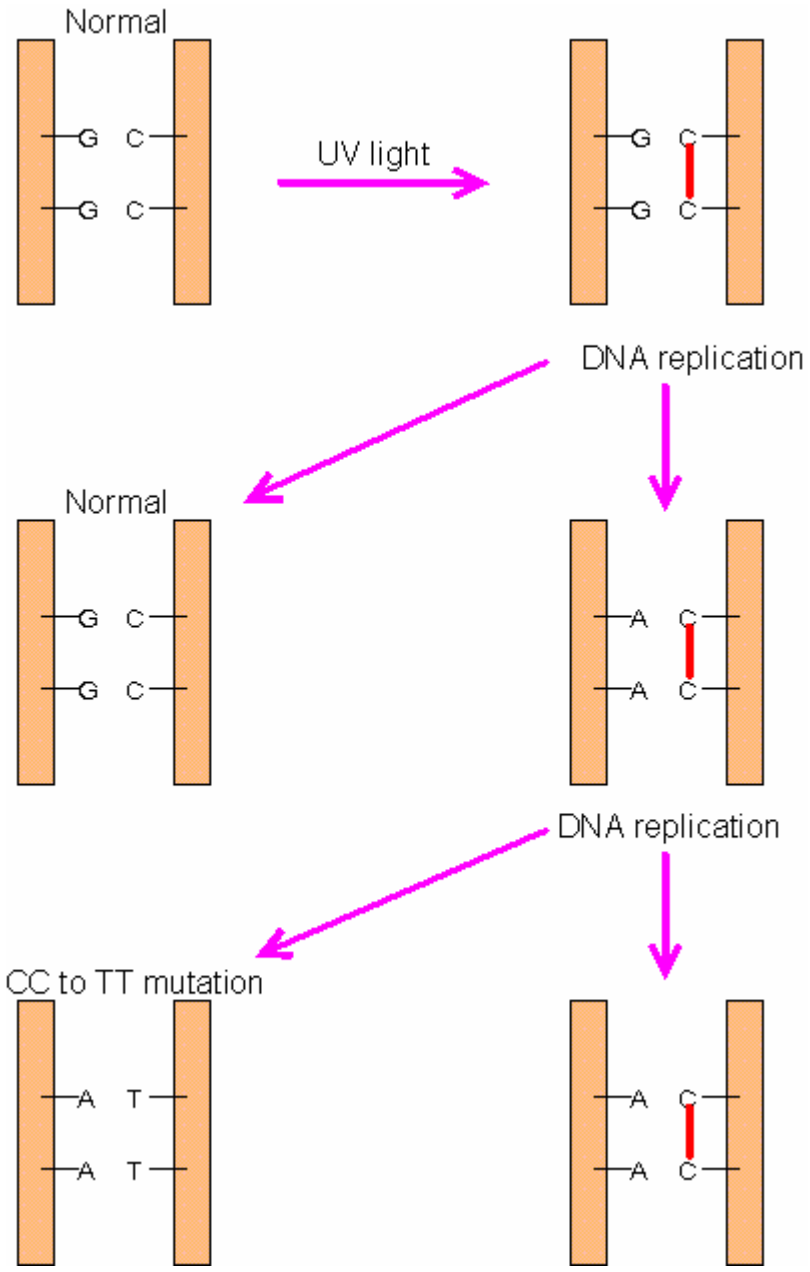

Figure 9: Action of UV light leading to Mutation

1.8 MECHANISMS OF PHOSPHATE SOLUBILIZATION AND MINERALIZATION

The phenomenon of fixation and precipitation of P in soil is highly dependent on soil type and pH. Thus, in acid soils, free oxides and hydroxides of aluminium and iron fix P while in alkaline soils, Ca fixes it. Organic acid metabolite production and decrease of medium pH appear to be the major mechanisms for RP solubilization.

1.8.1 ROLE OF PHOSPHATASE IN ORGANIC PHOSPHATE SOLUBILIZATION

Mineralization of most organic phosphorus compounds that may constitute up to 30-50% of the total phosphorus in most soils is carried out by means of phosphatase enzymes, primarily acid phosphatases. These catalyze dephosphorylating reactions involving the hydrolysis of phosphoester or phosphoanhydride bonds. These enzymes are nonspecific orthophosphoricmonoester phosphohydrolases (EC 3.1.3.2), cleaving Pi from ester linkage sites.

1.8.2 ROLE OF PHYTASE IN ORGANIC PHOSPHATE SOLUBILIZATION

Inositol phosphates (phytin, or phytic acid) are a major phosphate reserve in plants. However, this form of phosphate cannot be assimilated by monogastric animals. Many microorganisms possess the enzyme phytase which liberates phosphate groups from phytin.

Phytase (E.C. 3.1.3.8) catalyses the de-esterification of phytic acid (myo-inositol hexakisphosphate, IP6) to myo-inositol (Figure 10) and free ortho-phosphoric acid (hydrolysis of phosphate from phytic acid to inorganic phosphate and *myo*inositol phosphate derivatives). The phytic acid and its salts, the phytates, contain the major part (up to 90%) of the total phosphorus in the seeds of many plants (cereals, leguminous, oil-producing).

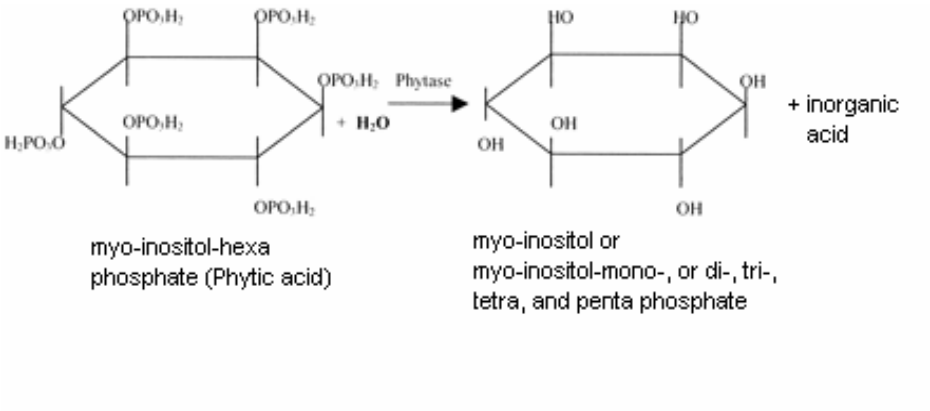

Figure 10: Action of Phytase

In the present study solubilization of rock phosphates and minerals by wild type and UV induced mutants of *Aspergillus tubingensis* was studied. *A. tubingensis* is known as one of the best phosphate solubilizer (Reddy *et al*., 2002), so an attempt was taken that if it gets maximum solubilization after making UV induced mutants of this species. Level of soluble P, phosphatase activity and phytase activity was determined and all the datas were compared between wild type and UV induced mutants of *Aspergillus tubingensis*.

Chapter 2

Review of Literature

Phosphorus (P) is essential macronutrient for plants but in most soils its content is about 0.05% of which only 0.1% is plant-available (Scheffer *et al.,* 1998). For this reason, the possibility of practical use of rock phosphate as a fertilizer has received significant interest in recent years.

The optimal development of crops demands a high, often costly, and input of P fertilizers. Current concepts in sustainability involve application of alternative strategies based on the use of less expensive natural sources of plant nutrients like rock phosphate. The beneficial effect of rock phosphate has made this material an attractive component for management in agriculture (Rajan *et al.,* 1996). Unfortunately, rock phosphate is not plant-available in soils with pH greater than 5.5-6.0 and, even when the conditions are optimal, plant yields are, as a rule, lower than those obtained with soluble phosphate (Khasawneh and Doll, 1978).

Rock phosphate is theoretically the cheapest P fertilizer but most phosphate rock deposits found in the world are classified as low reactive and, therefore, its direct application is not always effective without previous treatment. Depending on soil and climatic conditions, it could take up to four years of annual application before RP treatments became as effective as superphosphate (Sinclair and Dyson, 1988). Several methods have commonly been used to reduce this long lag-phase and increase P-rock availability. One traditional method of increasing P-availability is the acidulation of RP with small amounts of H_2SO_4 or H_3PO_4 to produce partially acidulated RP (Rajan and Watkinson, 1993). But this is uneconomical and environmentally nonviable. The conventional method for enhancing the rock phosphate availability is to increase its solubility by treating with inorganic acids, mainly sulphuric acid and phosphoric acid; but from the industrial point of view, this approach is not applicable because of high capital costs (Hammond *et al*., 1989). Other alternative methods include mixing of RP with various soil amendments or compaction of RP with water-soluble P fertilizers (Chien *et al*., 1987; Kpomblekou *et al*., 1991; Bolland, 1996). Finally, microbially-based approaches have

been proposed to improve the agronomic value of RP materials. Since the early work of Gerretsen (1948), the existence of soil microorganisms (mainly bacteria and fungi) capable of solubilizing inorganic phosphate materials has been reported by many researchers (Kucey *et al*., 1989; Whitelaw, 2000).

Thien and Myers (1992) indicated that by increasing soil microbial activities, bioavailability of P in a bioactive soil was remarkably enhanced. The fact that certain soil microbes are capable of dissolving relatively insoluble phosphatic compounds (Nahas *et al.,*1990; Bojinova *et al.,*1997) has opened the possibility for inducing microbial solubilization of phosphates in soils.

Some bacteria and fungi can promote selective and non-selective leaching of metals from ores or other rock by metabolic products such as organic acids (Ehrlich, 1997). Accordingly, the ability of low-molecular-weight organic acids to release P from raw materials, related to their ability to form stable metal complexes is well established (Kpomblekou and Tabatabai, 1994). Solubilization of phosphate-bearing inorganic materials by microorganisms excreting organic acids seems to be an attractive approach that has been actively studied during the last decade. Since the early work of Sperber (1958), microbially mediated solubilization was examined with bacteria and filamentous fungi (Kucey et al., 1989) in fermentation and soil conditions. A few workers reported RP solubilization in liquid medium by various fungi (Meyer and Konig, 1960; Ahmed and Jha, 1968; Gaur *et al*., 1973; Bardiya and Gaur, 1974; Khan and Bhatnagar, 1977; Arora and Gaur, 1979; Singh *et al*., 1984; Sattar and Gaur, 1985). It was also assumed that phosphate-solubilizing activity was greater for filamentous fungi than for bacteria.

Most rock phosphate deposits have low reactivity (Leon *et al*., 1986) and can not be used successfully as P sources for crop production (Kpomblekou and Tabatabai, 1994). It has been shown that organic acids can greatly increase the concentration of P in soil solution through chelation and an exchange reaction (Gadd, 1999). Therefore, the application of P solubilizing microorganisms (Kucey *et al*., 1989; Muchovej *et al*., 1989) is a promising approach for increasing P availability in rock phosphate amended soils. Production of carboxylic acids like citric and oxalic acids was associated with calcium phosphate solubilization by *Penicillium bilaii* (Cunningham and Kuiack, 1992). Gluconic acid has

been reported to be involved in the solubilization of Ca phosphate minerals by *Erwinia herbicola* (Liu *et al*., 1992), *Penicillium sp.* (Illmer and Schinner, 1995) and *Aspergillus niger* (Illmer and Schinner, 1995). It was also implicated in the solubilization of a rock phosphate by *Penicillium variabile* P16 (Vassilev *et al*., 1996), the solubilization of Ca and Al phosphate minerals by *Penicillium radicum* (Whitelaw *et al*., 1999), and the release of Ca^{2+}, Al^{3+} and Fe^{3+} from rock samples by *Penicillium frequentas* (De La Torre *et al*., 1993). However, release of toxic concentrations of some metals during rock phosphate solubilization can affect fungal growth, physiology and metabolism (Karamushka *et al*., 1996).

It should be noted that filamentous fungi are among the most active and studied solubilization agents and a typical process for RP solubilization in submerged (single batch, shake-flask) fermentation conditions involves glucose-based media and is performed for 7-20 days (Asea *et al.,* 1988; Cunningham and Kuiack, 1992; Illmer *et al.,* 1995; Nahas, 1996; Reyes *et al.,* 1999).

Filamentous fungi are widely used as producers of organic acids (Matty, 1992) and in particular *Aspergillus niger* and some *Penicillium* species have been tested in fermentation system or inoculated directly into soil in order to solubilize rock phosphate (Kucey, 1987 and Vassilev *et al.,* 1995).

Reddy *et al.,* (2002) found that al the isolates of *Aspergillus tubingensis* and *A. niger* isolated from rhizospheric soils were found to be capable of solubilizing all the natural forms of rock phosphates. This is the first report of solubilization of rock phosphates by *Aspergillus tubingensis* and showed that this fungus might serve as an excellent rock phosphate solubilizer when inoculated into soils where rock phosphate is used as P fertilizer.

Goenadi *et al.,* (2000) determined the optimum incubation period and the optimum level of rock phosphate for a Phosphate Solubilizing Fungus (PSF), *Aspergillus niger* BCCF 194, isolated from tropical acid soils. They conducted a simple, effective and environmentally sound process to improve P availability of phosphate rocks to crops by Phosphate solubilizing fungus.

Mechanism of Solubilization:

Phosphate solubilization by microbes is mediated by several different mechanisms including organic acid production and proton extrusion (Surange, 1995; Lapeyrie *et al.,* 1991; Burgstaller and Schinner, 1993; Cunningham and Kuiack, 1992; Dutton and Evans, 1996; Nahas, 1996). Increasing P concentration in the phosphate solubilizing fungal conataining medium is related to the production of organic- acid- type metabolites, which should correlate with pH of the medium (Illmer and Schinner, 1992; Illmer *et al.,* 1995; Narsian *et al.,* 1995). It is generally recognized that organic acids solubilize RP through protonation and/or chelation reactions (Sagoe *et al.,* 1998). Besides the acid strength, the type and position of the ligand determine the effectiveness of the organic acid in the solubilization process (Kpomblekou and Tabatabai, 1994).

It has been repeatedly shown that low molecular weight organic acids can strongly increase the concentration of phosphorus in solution by mechanisms involving chelation and exchange reactions (Earl *et al*., 1979; Fox and Comerford, 1990; Gerke, 1992).

Illmer *et al*., (1995) indicated the level of organic acids resulting in significant P dissolution were in order of 3-30 µM/ml, distinctly below the efficiency of biotic leaching. Thus, the production of organic acids is an important mechanism for solubilizing insoluble P, but not the only one.

The principal mechanism for organic phosphate solubilization is acid phosphatase activity (McGrath *et al.,* 1995). Arbuscular Mycorrhiza (AM) can make use of organic phosphate (Balaz and Vosatka, 1997) and are able to acidify the environment, which facilitates inorganic P dissolution (Bago *et al*., 1996). Phosphatases are required for the mineralization of organic forms of soil P to release phosphate (Raghothama, 1999). A wide range of phosphatases with differing substrate specificities have been characterized in plant roots (Bosse and Kock, 1998; Tandano and Sakai, 1991) and soil microorganisms (Richardson, 1994).

Phosphorus (P) deficiency is a major limitation to plant growth (Marschner, 1995; Vance *et al*., 2003). It is taken up by plants in its inorganic form, P_i or orthophosphate. Although a macronutrient for plants, the availability of soil P_i is often below that of micronutrients because P_i is immobilized within soil organic complexes, clay complexes, and

precipitated salts (Marschner, 1995). Sparingly available soil P_i can be ameliorated with P_i fertilizers, and applied fertilizers represent more than 80% of world P_i use (Steen, 1998). Modern agriculture relies on crops that provide maximum yields with these fertilizers; however, world resources of extractable P_i are limited, nonrenewable, and increasingly ecologically hazardous to obtain (Steen, 1998). As extractable world P_i stores become depleted, agriculture will be forced to adjust to a lack of P_i fertilizer while continuing to feed an expanding population. Alternatively, the organic P (P_o) component of agricultural soil is abundant, representing up to 50% of total soil P (Vance *et al*., 2003). Plants may mobilize P_o by secreting enzymes into the rhizosphere, including acid phosphatases (APases), ribonucleases, and deoxyribonucleases (Abel *et al*., 2002; Vance *et al*., 2003). While most soil enzymes are typically short-lived, APases can be immobilized on or within soil clays and humates that preserve their activity (Burns, 1986).

Phytases, *myo*-inositol hexakisphosphate phosphohydrolase (E.C. 3.1.3.8) are classified as the family of histidine acid phosphatases (Peddington *et al*. 1993). Phytates account for a large component of the organic P (some 20-50 % of the total soil organic P; Anderson, 1980; Dalal, 1977; Williams and Anderson, 1968), yet appear to be only poorly utilized by plants (Adams and Pate, 1992; Findenegg and Nelemans, 1993; Heyes *et al*., 2000; Richardson *et al*., 2000). Phytates may readily undergo physical and chemical reactions in soil environments, rendering them unavailable for plant uptake (Anderson, 1980; McKercher and Anderson, 1989). Phytases have been reported in bacteria (Kim *et al*. 1998), yeasts (Lambrechts *et al*. 1992), and fungi (Shieh and Ware 1968). Phytase-producing microorganisms comprise bacteria such as *Bacillus subtilis*, *Pseudomonas* sp., and *Escherichia coli*; yeasts such as *Schwanniomyces castellii* and *Saccharomyces cerevisiae*; and fungi such as *Aspergillus ficuum* and *Aspergillus terreus*. The phytase produced by *A. ficuum* NRRL 3135 has been isolated and well characterized by Ullah and Gibson (1987). In addition, the cloning and expression of the *phyA* gene have been reported for *Aspergillus awamori*, A. *ficuum* and *A. terreus* (Richardson *et al*., 2001). Recently, Kostrewa *et al*. (1999) reported the crystal structure of phytase from *A. ficuum*. The content of phosphorus in the medium is one of the basic factors which determine to a great degree the biosynthetic process of phosphatase and phytase enzymes (Gargova,

Roshkova and Vancheva, 1997). Inorganic phosphorus plays an important role as a regulator of the phytase and acid phosphatase production (Shieh and Ware, 1968; Howson and Devis, 1983; Chelius and Wodzinski, 1994). Recent studies have shown that phytase from *Aspergillus niger* can be expressed in plants and secreted as an extracellular enzyme when provided with an appropriate signal peptide (Brinch-Pedersen *et al*., 2000; Li *et al*., 1997; Verwoerd *et al*., 1995).

Extracellular phytases from a few filamentous fungi have been studied to some detail (Han and Gallagher 1987, Ullah and Gibson 1987, Piddington *et al.* 1993, Ehrlich *et al.* 1993) having significant role in phosphate solubilization and mineralization.

Aspergillus species, in particular, have been most extensively studied for commercial production of phytase (Ullah and Gibson 1987, Wyss *et al*. 1999). Other known fungi have been reported to produce phytases such as *Aspergillus terreus* (Dvorakova *et al*. 1997).

Aspergillus oryzae K1 (Shimizu 1993), *Aspergillus* spp. (Gargova *et al*. 1997), and *Aspergillus fumigatus* (Pasamontes *et al*. 1997). *Aspergillus ficuum NRRL* 3135 phytase which produces a high activity (Shieh and Ware, 1968) has been studied by many investigators (Howson and Davis, 1983; Gibson, 1987; Ullah and Gibson, 1987; Chelius and Wodzinski, 1994). Other known fungi that produce phytase: *Rhizopus oligosporus* (Howson and Davis, 1983), *A. oryzae* (Shimizu, 1993), *A. carbonarius* (Al-Asheh and Duvnjak, 1994), *A. niger* (Volfova *et al.*, 1994), have less activity.

Induction of Mutation in *Aspergillus tubingensis*

By using mutation using chemical and/or physical mutagens, genetic alterations can be achieved in *Aspergillus tubingensis.* Although it has been reported that in *Aspergillus spp.*, frequency of mutations falls down at higher UV doses (Sigurbjornsson and Micke, 1974).

Mutagenic effect of UV was discovered first time by Altenburg (1930). By making UV-induced mutants of *Penicillium rugulosum*, it has been found that there was drastic increase in solubilization capacity of phosphate rocks with wild-type strain (Reyes *et al*.,

2001). *Penicillium rugulosum* was selected for its high mineral phosphate solubilizing activity (Mps^{+}) with hydroxyapatite. Mutants with altered (Mps^{-}) or amplified activity (Mps^{++}) were obtained by UV irradiation of conidia (Reyes *et al*., 1999). These mutants allowed the elucidation of the mechanisms of action involved in the Mps^{+} activity of isolate. The Mps^{+} phenotype was mainly associated with the production of gluconic and citric acids, and is responded differently with calcium, iron and aluminium phosphate salts when used as P-sources and to ammonium, nitrate or arginine as nitrogen sources (Reyes *et al*., 1999). The well developed H pump mechanism involved in the solubilization of phosphate by the mutants allows the fungus to develop a high biomass, in media containing rock phosphates.

Induction of UV mutant in some of genera has been found to effective with respect to creation of new strains. New strains have been created using UV mutant *Hebeloma cylindrosporum* by reconstitution of dikaryons (Hebraud and Fevre, 1988). They exposed protoplasts of *Hebeloma cylindrosporum* under UV.

In some species of ectomycorrhiza induction of UV mutant has been performed to check mycorrhizal activity and found to be effective. IAA –over producer mutant has been created by exposing protoplasts of *Hebeloma cylindrosporum* under UV (Durand *et al*., 1992), which showed increased mycorrhizal activity (Gay *et al*., 1994).

Chapter 3

Materials and Methods

3.1 MATERIALS

3.1.1 Fungal strains used:

Aspergillus tubingensis (AT 1) (Reddy *et al*., 2002) was used for this study and maintained on Potato Dextrose Agar.

3.1.2 Media used:

Composition of Pikovskaya's medium (Pikovskaya, 1948)

Glucose	10.0 g
$Ca_3(PO_4)_2$	5.0 g*
$(NH_4)_2SO_4$	0.5 g
NaCl	0.2 g
$MgSO_4.7H_2O$	0.1 g
KCl	0.2 g
Yeast extract	0.5 g
$MnSO_4$	Trace
$FeSO_4.7H_2O$	Trace
Agar	15.0 g
Water	1000 ml
pH	7.0 ± 0.2

*Stock suspension of 2.5% $Ca_3(PO_4)_2$ was prepared in distilled water and was autoclaved for preparation of plates or broth, 10 ml of stock suspension was added aseptically to the 90 ml of sterilized medium.

3.1.3 Reagents for soluble P estimation in culture filtrate (Jackson, 1967)

♦ Chloromolybdic acid

Dissolved 15.0 g of ammonium molybdate in about 400 ml of warm distilled water. Filtered if it is necessary and added 342 ml of 12 N HCl, slowly with rapid stirring. Cooled and made the volume to one liter with distilled water and stored in amber glass bottle.

♦ Chlorostannous acid

Dissolved 10 g of $SnCl_2$ in 25 ml of conc. HCl. Kept it in a flask under airtight stopper. The solution was freshly prepared by taking 1 ml of above prepared stock solution and added 132 ml of distilled water.

♦ Standard phosphorus solution (100 ppm P)

Dissolved 0.4390 g of dried KH_2PO_4 in 400 ml of distilled water and added 25 ml of 7N H_2SO_4 and made up to 1 litre.

♦ Working phosphorus solution (10 ppm)

Dissolved 10 ml of standard P solution to 100 ml.

3.1.4 Reagents for Estimation of Phosphatase activity (Tabatabai and Bremner, 1969)

♦ Modified Universal Buffer 5X (Skujins *et al*., 1962)

Tris (hydroxyl methyl) amino methane	12.10 g
Maleic acid	11.60 g
Citric acid	14.00 g
Boric acid	6.28 g
NaOH (1N)	488 ml
Distilled water	up to 1L
pH	5.50

♦ **0.115 M p-nitro phenyl phosphate solution:** Dissolved 4.268 g of p-nitro phenyl phosphate disodium salt hexahydrate in 100 ml of modified universal buffer.

♦ **0.5 N NaOH:** Dissolved 20 g of NaOH pellets in distilled water and made the volume to one litre.

♦ **1 mg/ml p-nitrophenol (PNP)** solution in modified universal buffer.

3.1.5 Reagents for Estimation of Phytase activity

♦ **Tricarboxylic acid (10%):** Dissolved 1 gm of tricarboxylic acid in 10 ml of distilled water.

♦ **10 mM Ammonium molybdate:** Dissolved 1.2359 gm of ammonium molybdate in 100 ml of distilled water.

♦ **$FeSO_4$ solution:** Dissolved 2 gm of $FeSO_4$ in 20 ml distilled water and mixed with 0.5 ml of 0.9 N H_2SO_4. Finally made the total volume 25 ml with distilled water.

♦ **1.5 mM Sodium phytate:** Dissolved 0.138 gm of sodium phytate in 100 ml of distilled water.

♦ **AAM solution: (**Acetone: 10 mM Ammonium molybdate: 5 N H_2SO_4 = 2:1: 1**)**

Dissolved 0.2224 gm of ammonium molybdate in 18 ml of distilled water in one flask. In another flask mixed 2.5 ml of 36 N H_2SO_4 with 15.5 ml of distilled water. Finally kept these solutions in a colored bottle with 36 ml of acetone.

♦ **1 M Citric acid:** Dissolved 4.2 gm of citric acid in 20 ml of distilled water.

3.2 METHODOLOGY

3.2.1 To find the optimum time for maximum solubilization (Goenadi *et al.*, 2000)

- Grown *Aspergillus tubingensis* in YMG broth for 5-6 days and macerated with the help of homogenizer.

- Added 10 ml of autoclaved tri calcium phosphate (TCP) equivalent to 100 mg P_2O_5 separately in 90 ml of Pikovskaya's broth.
- Inoculated with equal amount of mycelia in all the flasks (*i.e.*, 1 ml).
- Incubated at 30ºC under shaking conditions (150 rpm) for different time intervals such as 1, 2, 3, 4 and 5 days.
- Filtered the mycelial mass using Whatman no. 42 filter paper.
- The mycelium was washed repeatedly with distilled water and dried at 70ºC for 48 hours. The dried biomass served as the parameter for growth determination.
- The culture filtrate was analyzed for soluble P by chlorostannous reduced molybdophosphoric acid blue method as described by Jackson (1967).
- This experiment was also repeated with rock phosphate instead of tri calcium phosphate equivalent to 100 mg P_2O_5.
- All the experiments were carried out in triplicates.

3.2.2 To study the various parameters with respect to solubilization of Rock Phosphates in a culture broth containing *Aspergillus tubingensis*

3.2.2.1 Growth determination

At the end of incubation, the contents of the flasks were filtered through Whatman no. 42 filter paper, washed repeatedly with distilled water and the mycelial mass was dried at 70ºC for 48 hours. Dry weight of the mycelium represents growth.

3.2.2.2 Phosphorus estimation and pH

Soluble P in the culture filtrate was estimated by the chlorostannous reduced molybdophosphoric acid blue method as described by Jackson (1967). The pH of the spent medium was measured by pH meter.

3.2.2.3 Procedure for estimation of soluble P in culture filtrate (Jackson, 1967)

- Transferred 100 µl of filtrate to 50 ml colored volumetric flask.
- Added 10 ml of chloromolybdic acid reagent along the sides of the flask.
- Diluted the contents of the flask to 40 ml.
- Then 1 ml of chlorostannous acid reagent was added and mixed well.
- After mixing made the volume to 50 ml as quickly as possible.
- The blue color intensity of the solution was measured at 600 nm against blank (without culture filtrate).
- To prepare standard curve, measured 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 ml of 10 ppm P solution in 50 ml volumetric flask and followed the steps as above.

3.2.2.4 Procedure for determination of Acid Phosphatase activity (Tabatabai & Bremner, 1969)

- The mycelium previously grown in Pikovskaya's broth with wild type and UV induced mutant strains of *Aspergillus tubingensis* was filtered and washed aseptically with sterile distilled water followed by a rinse with filter sterilized modified universal buffer.
- After an aseptic rinse with sterile modified universal buffer, mycelia were placed in sterile 30 ml screw cap tubes with 4 ml of sterile buffer solution.
- Then 1 ml of filter sterilized 0.115M disodium p-nitro phenyl phosphate solution was added.
- The contents were incubated at 30ºC in a water bath for 2 hours in the dark.
- After 2 hours of incubation, 5 ml of 0.5N NaOH was added to stop the reaction.
- The mixture was swirled and filtered through Whatman No 1 filter paper.
- Transferred the filtrate to glass cuvette and measured the yellow color intensity with UV-VIS spectrophotometer (Hitachi U-2001) at 410 nm.

- The filtered out mycelium from each vial was dried for 48 hours at 70ºC and the dry weight was recorded separately.
- Acid phosphatase activity was indicated as the amount of p-nitrophenol released in the filtrate from the p-nitro phenyl phosphate substrate per gram of the mycelium. The p-nitrophenol content was calculated with reference to a calibration graph plotted from the results obtained by standards containing 0, 10, 20, 30, 40 and 50 µg of p-nitrophenol.
- To perform controls, followed the procedure described for the assay but made the addition of 1 ml p-nitro phenyl phosphate after the addition of 0.5N NaOH (*i.e.* immediately before filtration).
- Phosphatase activity was calculated in the unit of µM PNP/g mycelium/hour.

Calculation:

$$\text{Phosphatase activity (µM PNP/g mycelium/hour)} = \frac{Conc.\ of\ \ PNP(\mu M)}{2 \times weight\ of\ mycelium}$$

3.2.2.5 Procedure for determination of Phytase activity (using mycelia)

- The mycelium grown previously in Pikovskaya's broth with wild type and UV mutant strains of *A. tubingensis* was filtered and washed aseptically with sterile distilled water followed by a rinse with 0.1 M sodium acetate buffer.
- After an aseptic rinse with sterile modified universal buffer, mycelia were placed in sterile 30 ml screw cap tubes.
- Added 0.5 ml of 1.5 mM sodium phytate in all the tubes including blank followed by addition of 1 ml tricarboxylic acid (10%) and 1 ml distilled water.
- Then 0.5 ml of 9N H_2SO_4 was added.

- The tubes were incubated at room temperature for 15 min and 0.5 ml of 6% ammonium molybdate was added and again kept at room temperature for 15 min. in dark.
- Finally added 0.5 ml of $FeSO_4$ solution in all the tubes and incubated in dark for 30 minutes.
- The mixture was swirled and filtered through Whatman No 1 filter paper.
- Transferred the filtrate to glass cuvette and measured the yellow colour intensity with UV-VIS spectrophotometer (Hitachi U-2001) at 660 nm.
- The filtered out mycelium from each vial was dried for 48 hours at 70ºC and the dry weight was recorded separately.
- The phytase activity was calculated with reference to a calibration graph plotted from the results obtained by standards containing 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 µg of potassium di-hydrogen phosphate (KH_2PO_4).
- Phytase activity was calculated in the unit of µM PNP/g mycelium/hour.

Calculation:

Phosphatase activity (µM PNP/g mycelium/hour) = $\frac{Conc.\ of\ \ PNP(\mu M)}{2X weight\ of\ mycelium}$

3.2.2.6 Procedure for determination of Phytase activity (using culture filtrate)

- Took 0.5 ml of culture filtrate from each sample in test tubes and 0.5 ml of 1.5 mM sodium phytate was added.
- For colour development 2 ml of AAM (Acetone: 10 mM Ammonium molybdate: 5 N H_2SO_4 = 2:1: 1) solution was added.
- Finally the reaction was stopped by adding 100 µl of 1M citric acid.
- Transferred the filtrate to glass cuvette and measured the yellow colour intensity with UV-VIS spectrophotometer (Hitachi U-2001) at 355 nm.

- One unit of phytase activity was defined as the amount of enzyme that liberates one nanomole inorganic phosphate per ml and per second under the assay conditions. The inorganic phosphate content was calculated with reference to a calibration graph plotted from the results obtained by standards containing 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 µg of potassium di-hydrogen phosphate (KH_2PO_4).

3.2.3 Irradiating Mycelia:

3.2.3.1 Culture Conditions:

The *Aspergillus tubingensis* culture was grown in a 250 ml flask containing 50 ml of liquid YMG broth at 30ºC under shaking condition (150 rpm) for 7 days. The mycelium was transferred into fresh YMG broth and grown under same conditions for 7 days. Finally, the mycelium was macerated using tissue homogenizer (Ultraturrax, T25 basic, Fisher Bioblock Scientific, and Germany).

3.2.3.2 Mutagenesis:

Mutagenic treatment was performed on mycelia obtained from maceration of *Aspergillus tubingensis*. Mutants were obtained from mycelia irradiated for 15 minutes at a distance of 17.5 cm with a UV germicidal lamp (where 90% of living mycelia died) and plated on potato dextrose agar (PDA) medium. To confirm mortality rate *i.e.* 90%, 5 ml of mycelia were irradiated in small Petri plates for different time periods viz. 2, 4, 6, 8, 10 and 15 minutes. Spreading was done on potato dextrose agar media using 10µl of mycelia from these plates followed by incubation at 30ºC for 24 hours including control (without exposing under UV light) in triplicates. Colonies were counted from these plates. Plates in which 90 % mortality were recorded, treated as UV mutant plates. Using bore plate method, randomly selected 10-12 colonies from that plate were transferred into Pikovskaya's agar plate and incubated at 30ºC for 24 hours. Finally, transferred all those colonies to Pikovskaya's agar media and incubated at 30ºC till good growth. Measured diameter zone in these plates and selected 8 different colonies with highest solubilization

zone along with one lowest solubilization zone and named the mutants as AtM-1, AtM-2, AtM-3, AtM-4, AtM-5, AtM-6, AtM-7 and AtM-8. The wild type was named as AtW.

3.2.4 Comparison of UV induced mutants of *Aspergillus tubingensis* with wild type in solubilization of phosphate rocks and minerals:

3.2.4.1 Estimation of soluble P:

- Inoculated mycelial discs from all the eight mutants plates (AtM-1, AtM-2, AtM-3, AtM-4, AtM-5, AtM-6, AtM-7, and AtM-8) and control *i.e.*, wild type (AtW) into YMG broth and grown under shaking condition (150 rpm) at 30ºC for 4-5 days.
- Mycelium was macerated for all these nine samples using tissue homogenizer in a broths and equal volume of mycelia was inoculated in Pikovskaya's broth containing 100 mg equivalent P_2O_5 tri calcium phosphate and rock phosphate (in triplicates). Inoculation was also done in Pikovskaya's broth containing 1 gm equivalent P_2O_5 of rock phosphate for all these nine samples.
- Estimated the level of soluble P by chlorostannous reduced molybdophosphoric acid blue method as described in 3.2.2.3.
- pH was measured from all the flasks using pH meter.
- Took dry weight of mycelia for growth determination.

3.2.4.2 Estimation of Phosphatase activity:

- Calculated Phosphatase activity as described in 3.2.2.4.

3.2.4.3 Estimation of Phytase activity:

- Calculated Phytase activity as described in 3.2.2.5 and 3.2.2.6.

3.2.5 Statistical Analysis

Datas were subjected to ANOVA and means were compared by Tukey's T-method ($P < 0.05$). Regression and correlation analyses were performed to determine the relationship between different isolates of *Aspergillus tubingensis* and soluble P, pH, biomass, acid phosphatase and phytase activity and/or to determine the optimum level of the parameters studied.

Chapter 4

Results and Discussions

All the mutants of *Aspergillus tubingensis* were capable of solubilizing all the natural forms of rock phosphates. The solubilization level of different rock phosphates varied with different mutants.

4.1 Optimum time for maximum solubilization:

In the experiment carried out to determine the optimum time for maximum solubilization using *Aspergillus tubingensis*, it was found that the levels of soluble phosphorus in culture filtrate increased significantly up to third day and remained high at fourth day. The maximum amount of P_2O_5 released by *A. tubingensis* from tri calcium phosphate was found at fourth day (33 mg/100 ml). However, later on, a significant drop in soluble phosphorus level was observed (Figure 11).

The biomass increased significantly up to day fifth (Figure 12). Excessive sporulation was observed in the following days, owing to deficiency of either of essential macronutrients that could explain no significant increase in biomass thereafter.

A plausible reason for such an observation could be attributed to the availability of soluble form of phosphate, which has an inhibitory effect on further phosphate solubilization (Narsian *et al*., 1995). The negative effect of soluble P on microbial acid productivity (Rohr *et al*., 1983) 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 (Illmer and Schinner, 1992).

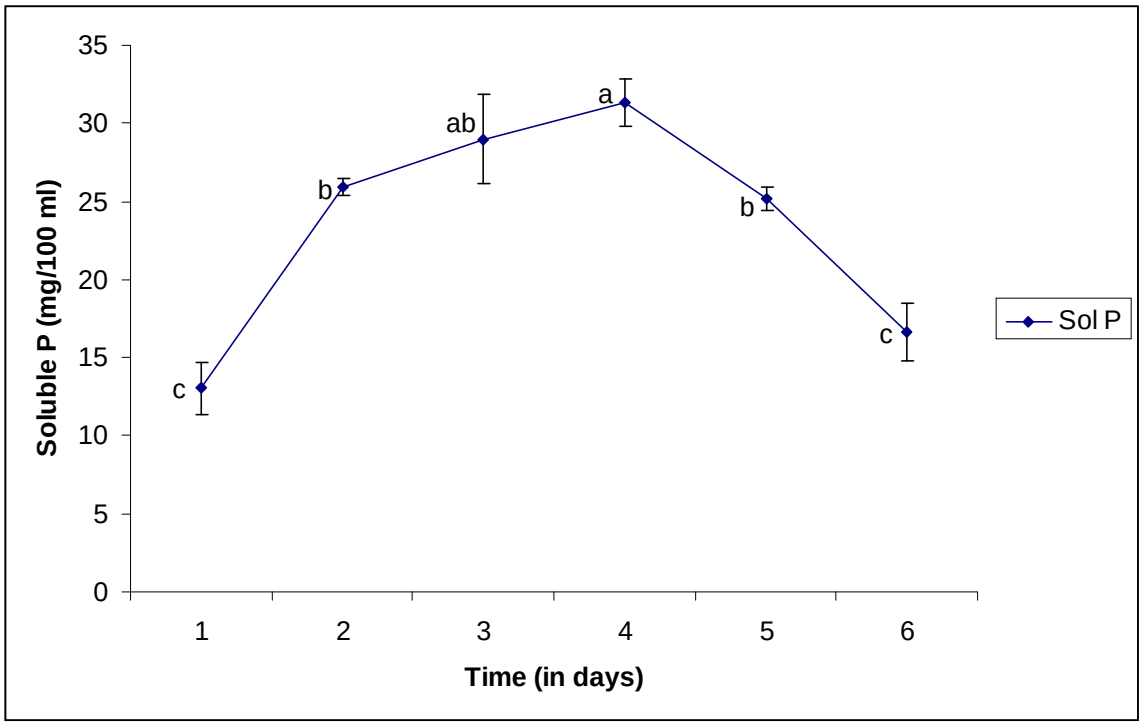

Figure 11: Solubilization kinetics of *Aspergillus tubingensis* in Pikovskaya's broth containing tri calcium phosphate (equivalent to 100 mg P_2O_5) with respect to optimum time for solubilization. Error bars are ± standard error (n = 3). Means sharing a common letter are non-significant at $P < 0.05$ level.

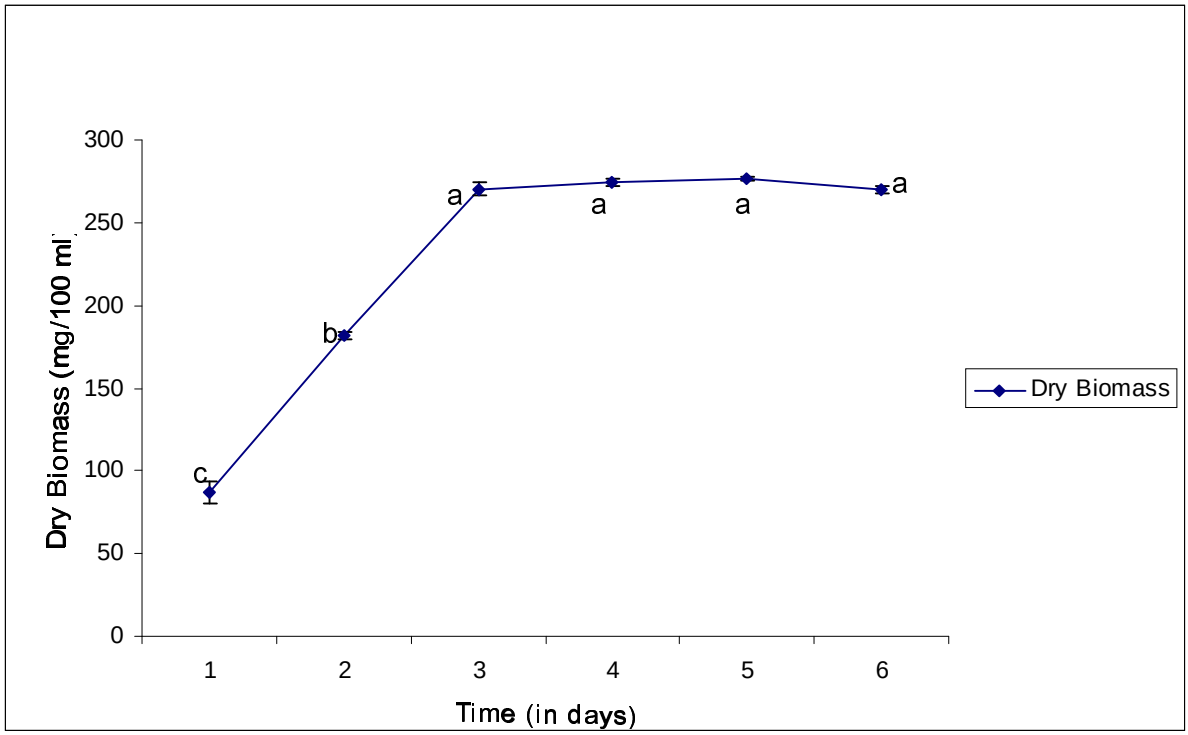

Figure 12: Biomass proliferation of *Aspergillus tubingensis* during optimum time for solubilization. Error bars are ± standard error (n = 3). Means sharing a common letter are non-significant at $P < 0.05$ level.

4.2 Development of UV mutants:

After spreading the 10 µl of UV exposed mycelia in Pikovskaya's agar plate and potato dextrose agar plate, numbers of colonies were counted with respect to time of UV exposure (Table 2). Some colonies were randomly selected from the plates having 90 %

mortality, which was found to be in plates which were exposed under UV for 15 minutes, and sub-cultured on Pikovskaya's agar plate (Figure 13).

4.3 Screening of mutants:

Diameter zone of solubilization was measured for all these colonies. The maximum solubilization zone was found to be 2.30 cm in one of the plate. Wild type showed solubilization zone of 1.5 cm. Similarly diameter zone of all the colonies were measured as mentioned in Table 3.

Table 2: Number of colonies observed in plates after exposing the mycelium to UV and spread them on to Petri plates containing PDA.

UV exposure time (in min.)	**No. of colonies (in triplicates)**		
Control (0)	400	365	400
2	400	380	387
4	258	280	265
6	210	200	215
8	115	125	122
10	90	88	98
15	35	40	44

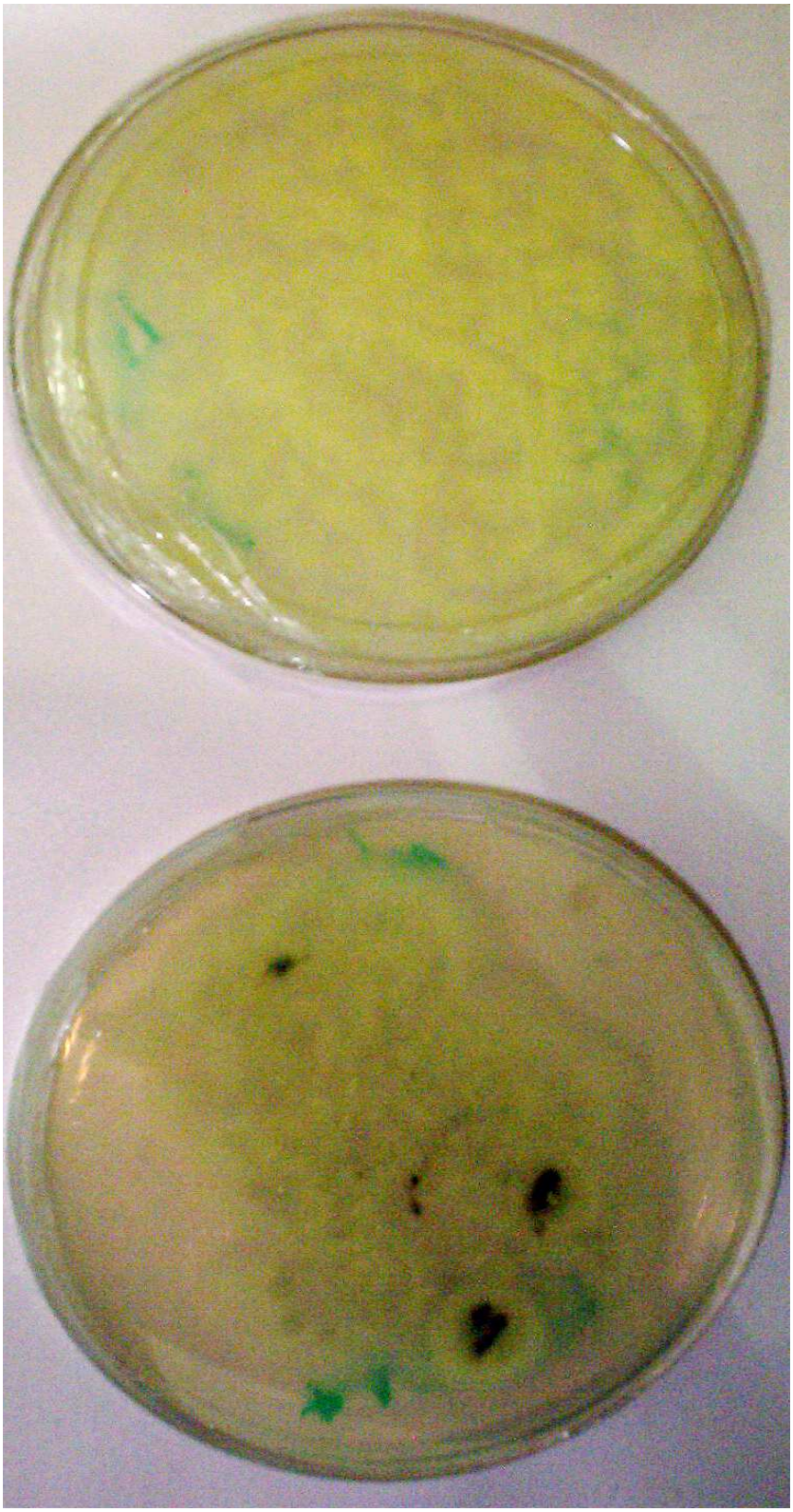

Figure 13: Wild type (upper plate) and UV exposed (lower plate) colonies of *A. tubingensis* on Pikovskaya's agar plate.

Total eight isolates of *Aspergillus tubingensis* were selected on the basis of solubilization zone (Figure 14), including one of the lowest solubilization zone (1.2 cm) and all these mutants were coded as AtM-1, AtM-2, AtM-3, AtM-4, AtM-5, AtM-6, AtM-7 and AtM-8. Wild type was named as AtW.

Table 3: Solubilization zone of different UV induced mutants of *Aspergillus tubingensis* in Pikovskaya's agar plate.

Name of Mutants	**Diameter zone (in cm)**
AtW	1.50
AtM-1	2.00
AtM-2	2.30
AtM-3	1.70
AtM-4	2.10
AtM-5	2.30
AtM-6	2.25
AtM-7	1.80
AtM-8	1.20

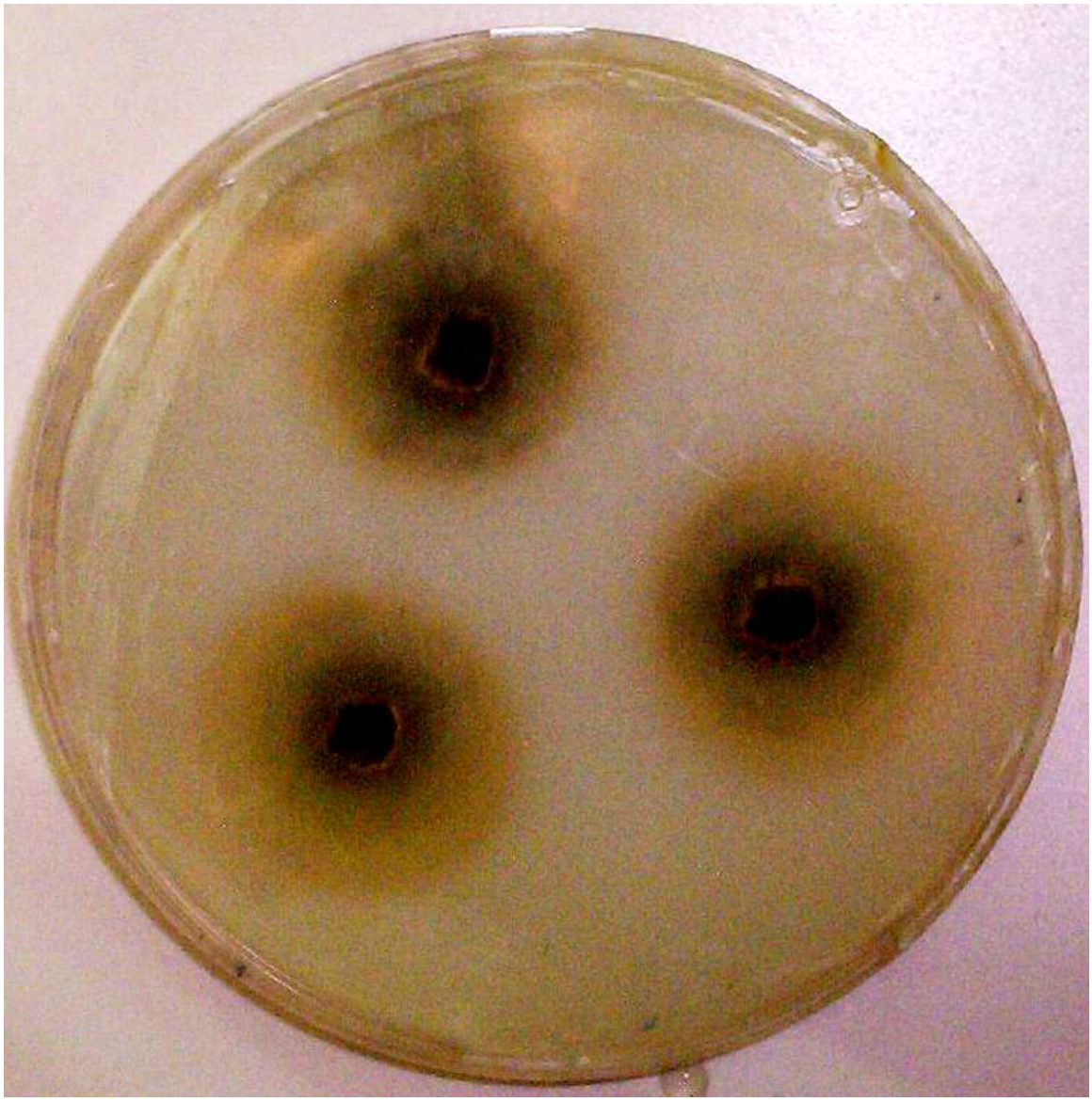

Figure 14: Different solubilization zones in Pikovskaya's agar plate shown by different mutants of *A. tubingensis*.

4.4 Solubilization of tri calcium phosphate and rock phosphates by wild type and UV induced mutants of *Aspergillus tubingensis*:

Inoculation of Pikovskaya's liquid medium containing tri calcium phosphate and rock phosphate equivalent to 100 mg P_2O_5 and also rock phosphate equivalent to 1 gm P_2O_5 with wild type and different other isolates of *A. tubingensis* after four days of incubation significantly altered the amount of P solubilized throughout the experiment (Figures 15, 17 and 19). AtM-5 showed maximum solubilization when compared to other isolates whereas AtM-8 showed the least solubilization (Tables 4 and 5). It was seen that biomass was not well correlated with other isolates with respect to solubilization (Figures 16, 18 and 20). Highest biomass was shown by AtM-7 (in case of tri calcium phosphate), AtM-5 (in case of 100 mg P_2O_5 equivalent rock phosphate) and AtM-8 (in case of 1 gm P_2O_5 equivalent rock phosphate).

A decrease in the pH of the medium with all the mutants of *A. tubingensis* was invariably observed after four days of incubation. The lowest pH was recorded in case of mutants AtM-2 and AtM-5, which showed almost highest phosphate solubilization, whereas mutant AtM-8 showed highest pH in liquid Pikovskaya's media containing tri calcium phosphate and rock phosphate (Figures 21 and 22) which may be due to complexity of the tri calcium phosphate and rock phosphate. The mutants AtM-2 and AtM-5 were almost same with respect to pH. However, no significant relationship could be established between the quantity of phosphate solubilized and drop in pH. This finding is in agreement with reports of Ahmed and Jha (1968), Das (1963), Sethi and Subba Rao (1968), Sperber (1958) and Narsian and Patel (2000).

Correlation studies (Tables 6 and 7) with the phosphate solubilization and change in pH for linear regression equations showed better relationship than with P solubilization and dry biomass weight, which suggests that lowering the pH having a significant role in phosphate solubilization. Correlation studies with the dry biomass weight and change in pH showed a positive relationship for most of the isolates. A fall in pH during the growth of *Aspergillus* species in liquid medium containing insoluble phosphates has often been reported due to production of dicarboxylic (oxalic, succinic); dicarboxylic hydroxyl (malic) and tricarboxylic hydroxyl (citric) acids from simple carbohydrates (Rose 1957, Sperber 1957,

Swaby and Sperber 1958, Louw and Webley 1959, Duff *et al*., 1963 and Taha *et al*., 1969). Assimilation of ammonium present in Pikovskaya's medium may be one of the causes of lower pH due to higher acid production. Higher acid production and P solubilization from ammonium assimilation has a been reported for the solubilization of fluoroapatite by *Aspergillus niger* (Cerezine *et al*., 1988) and tri calcium phosphate by *A. aculeatus* (Narsian *et al*., 1995).

Table 4: Soluble P, pH, acid phosphatase activity and phytase activity (mean values) observed by all the mutants of *Aspergillus tubingensis* in Pikovskaya's broth containing tri calcium phosphate (equivalent to 100 mg P_2O_5).

Mutants	**Soluble P (mg/100 ml)**	**pH**	**Acid phosphatase activity (µM/g/hr)**	**Phytase activity (µM/g/hr)**
AtW	35.13 cd	3.84 ab	45.0 cd	37.8 cd
AtM-1	34.00 bc	2.52 c	51.4 ab	43.0 b
AtM-2	46.63 ab	2.34 c	52.3 a	45.1 a
AtM-3	35.67 c	2.81 c	49.8 c	40.7 bc
AtM-4	41.23 bc	2.60 c	50.7 b	42.6 c
AtM-5	47.46 a	2.34 c	52.3 a	46.8 a
AtM-6	41.33 bc	2.65 c	50.0 c	41.5 c
AtM-7	37.23 c	3.55 b	49.2 bc	39.4 bc
AtM-8	29.07 d	4.10 a	29.0 d	33.2 d

Table 5: Soluble P, pH, acid phosphatase activity and phytase activity (mean values) observed by all the mutants of *Aspergillus tubingensis* in Pikovskaya's broth containing rock phosphate (equivalent to 100 mg P_2O_5).

Mutants	Soluble P (mg/100 ml)	pH	Acid phosphatase activity (µM/g/hr)	Phytase activity (µM/g/hr)
AtW	27.73 e	4.15 ab	33.0 cd	32.0 c
AtM-1	38.90 c	2.74 c	39.9 bc	36.7 b
AtM-2	44.00 ab	2.52 c	45.7 a	40.0 a
AtM-3	33.63 d	3.86 b	34.8 c	34.7 c
AtM-4	40.90 bc	2.56 c	42.5 b	35.9 c
AtM-5	46.00 a	2.52 c	46.0 a	42.4 a
AtM-6	39.50 bc	2.77 c	38.2 c	35.2 c
AtM-7	36.57 cd	3.81 b	35.0 c	34.0 c
AtM-8	26.37 e	4.30 a	25.0 d	30.9 c

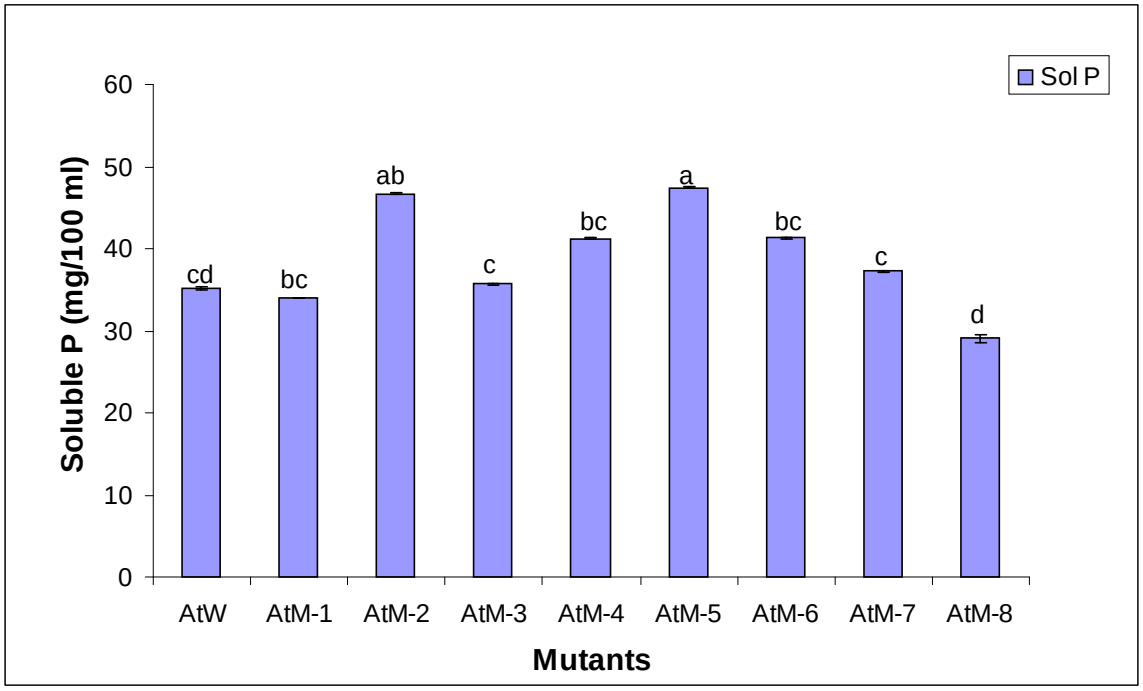

Figure 15: Solubilization of tri calcium phosphate by different mutants of *Aspergillus tubingensis*, after 4 days incubation in liquid cultures. Error bars are ± standard error (n=3). Means sharing a common letter are non-significant at $P < 0.05$ level.

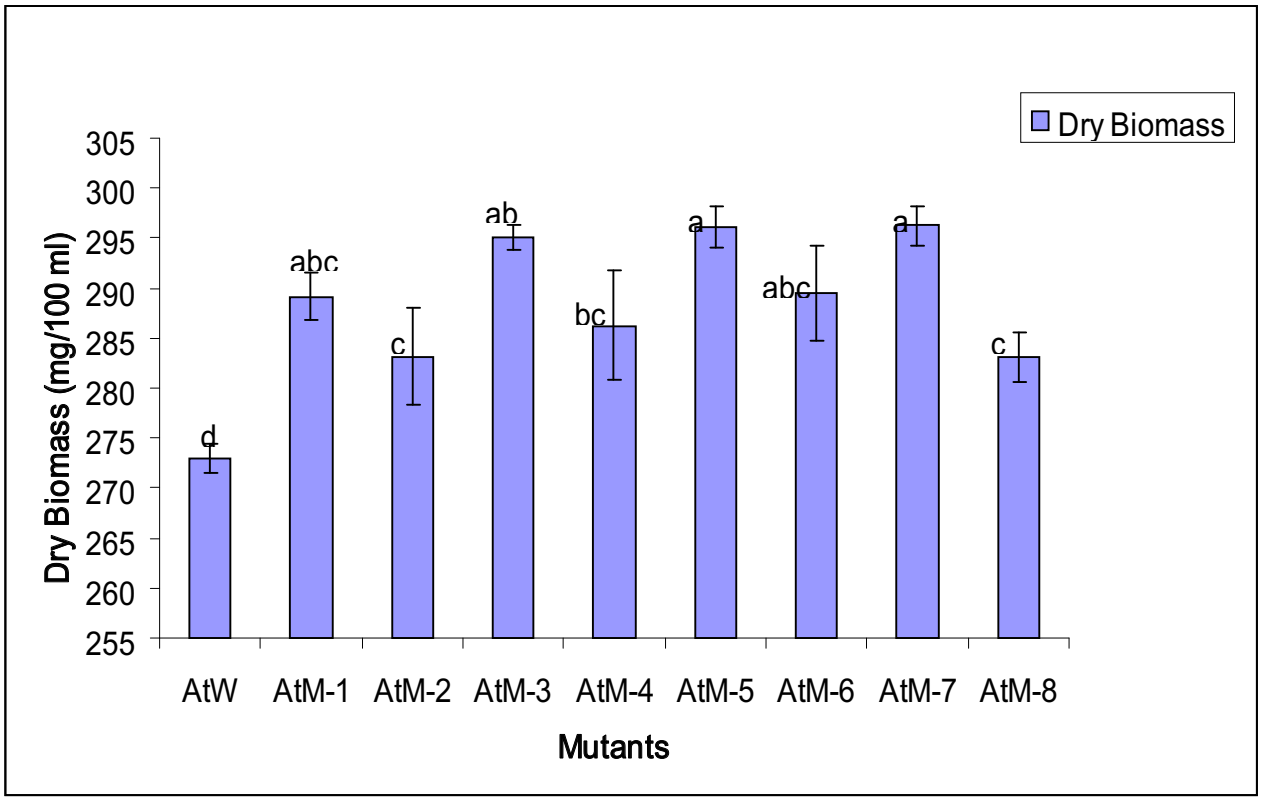

Figure 16: Biomass proliferation of different mutants of *A. tubingensis* in Pikovskaya's broth containing tri calcium phosphate (equivalent to 100 mg P_2O_5). Error bars are ± standard error (n=3). Means sharing a common letter are non-significant at $P < 0.05$ level.

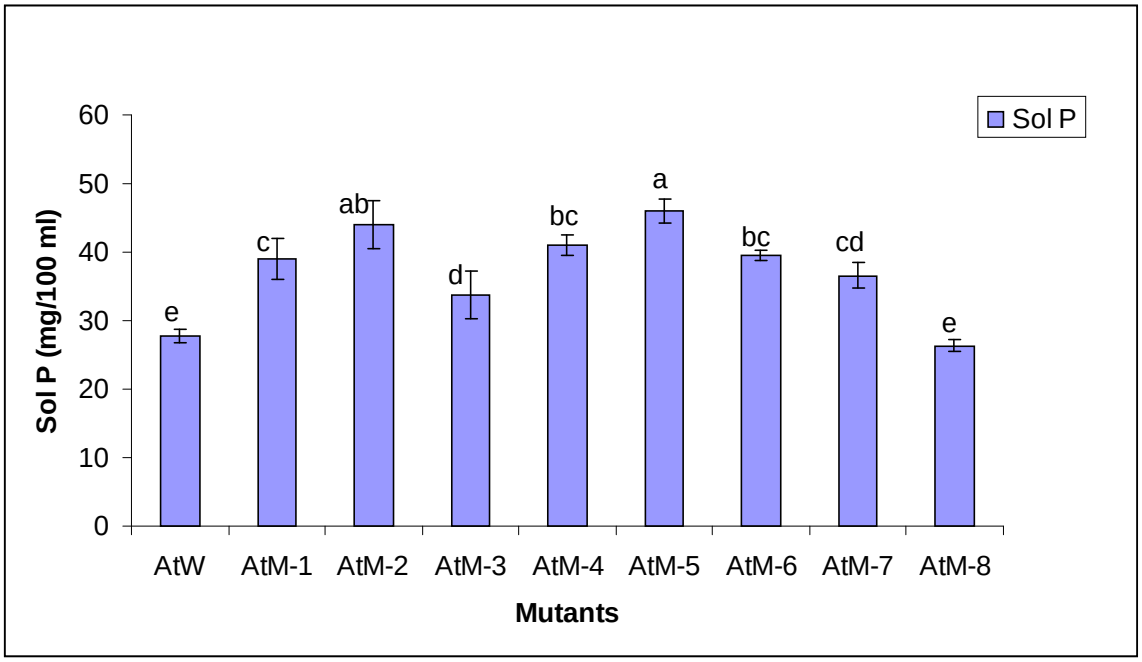

Figure 17: Solubilization of rock phosphate (equivalent to 100 mg P_2O_5) by different mutants of *Aspergillus tubingensis*, after 4 days incubation in liquid cultures. Error bars are ± standard error (n=3). Means sharing a common letter are non-significant at $P < 0.05$ level.

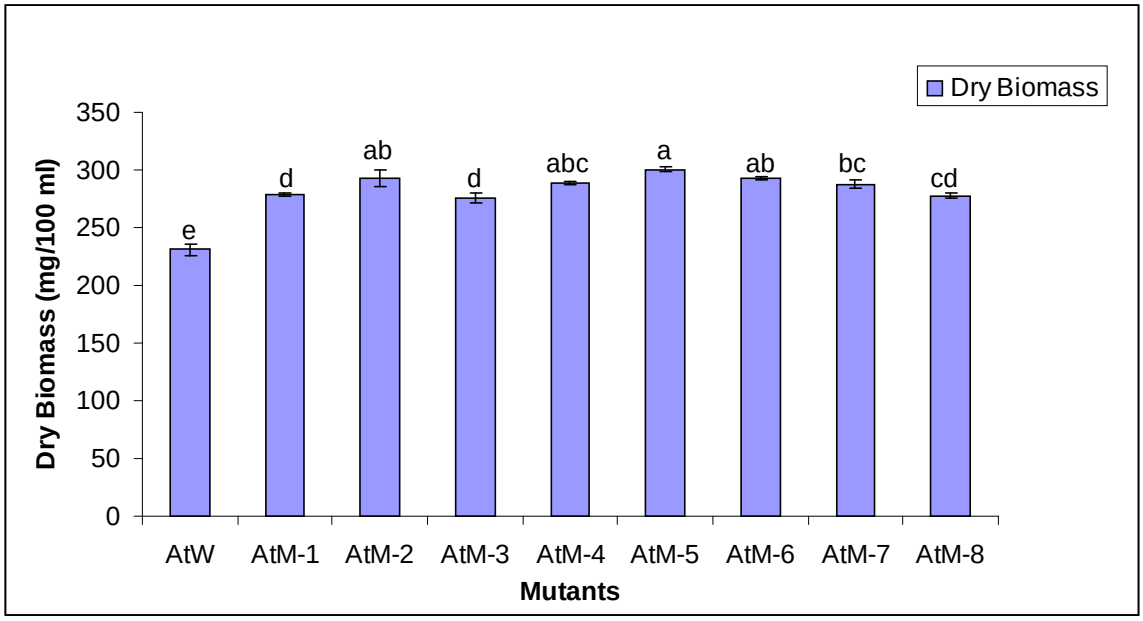

Figure 18: Biomass proliferation of different mutants of *A. tubingensis* in Pikovskaya's broth containing rock phosphate (equivalent to 100 mg P_2O_5). Error bars are ± standard error (n=3). Means sharing a common letter are non-significant at $P < 0.05$ level.

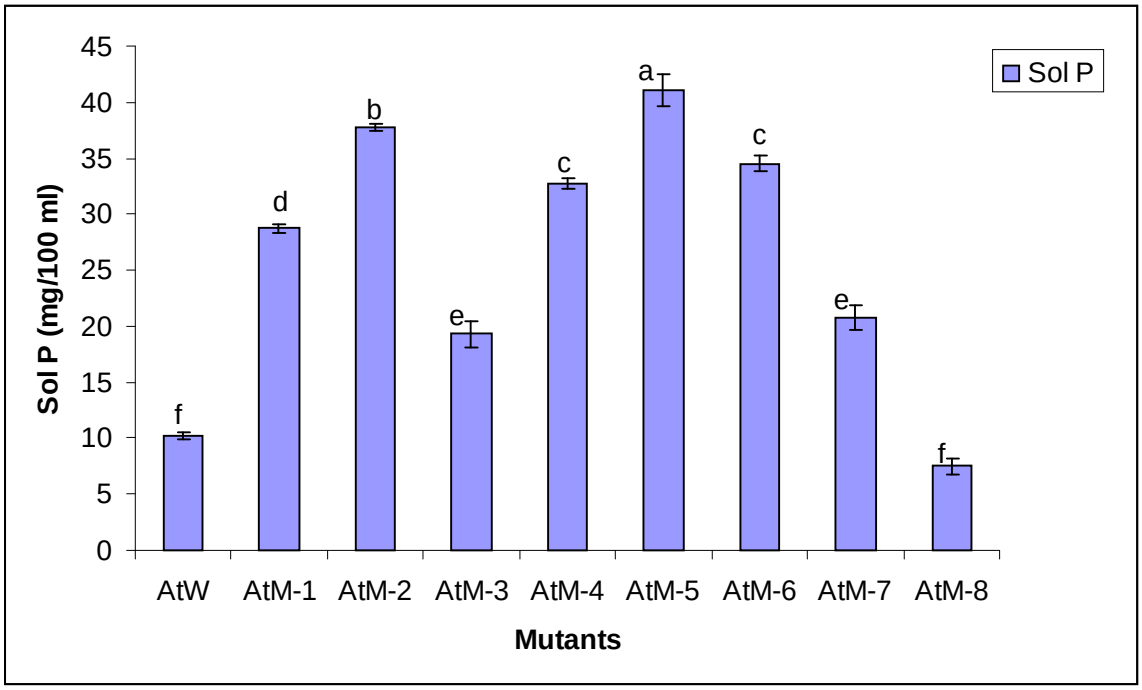

Figure 19: Solubilization of rock phosphate (equivalent to 1 gm P_2O_5) by different mutants of *Aspergillus tubingensis*, after 4 days incubation in liquid cultures. Error bars are ± standard error (n=3). Means sharing a common letter are non-significant at $P < 0.05$ level.

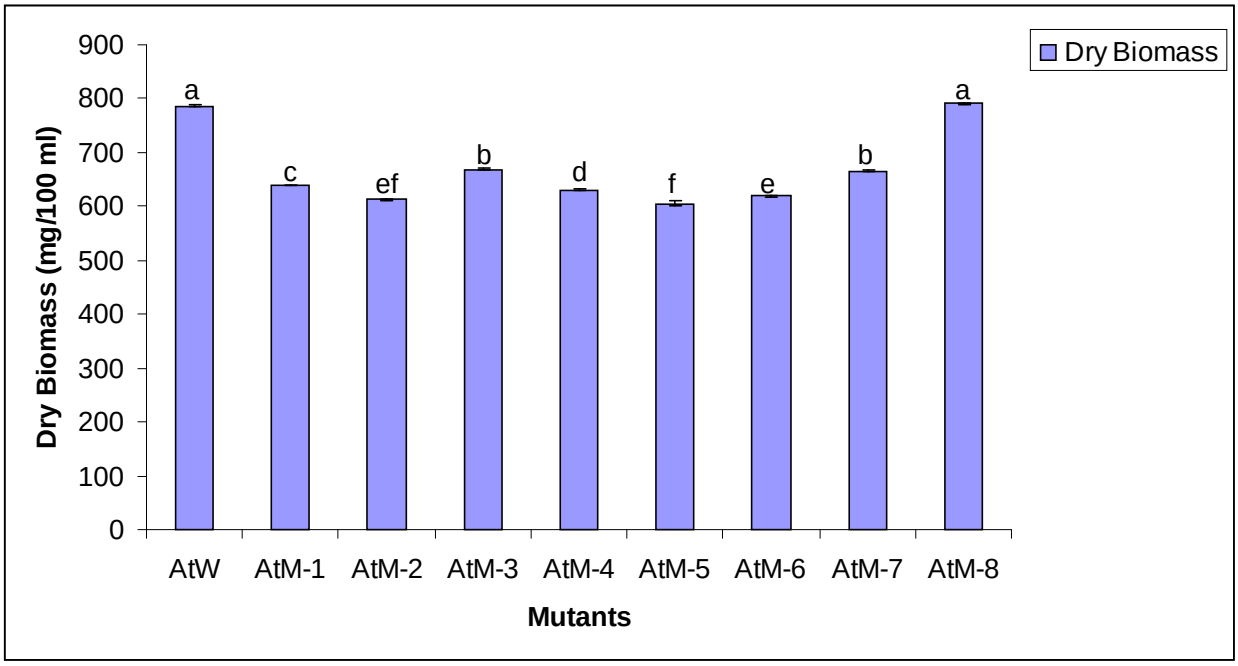

Figure 20: Biomass proliferation of different mutants of *A. tubingensis* in Pikovskaya's broth containing rock phosphate (equivalent to 1 gm P_2O_5). Error bars are ± standard error (n=3). Means sharing a common letter are non-significant at $P < 0.05$ level.

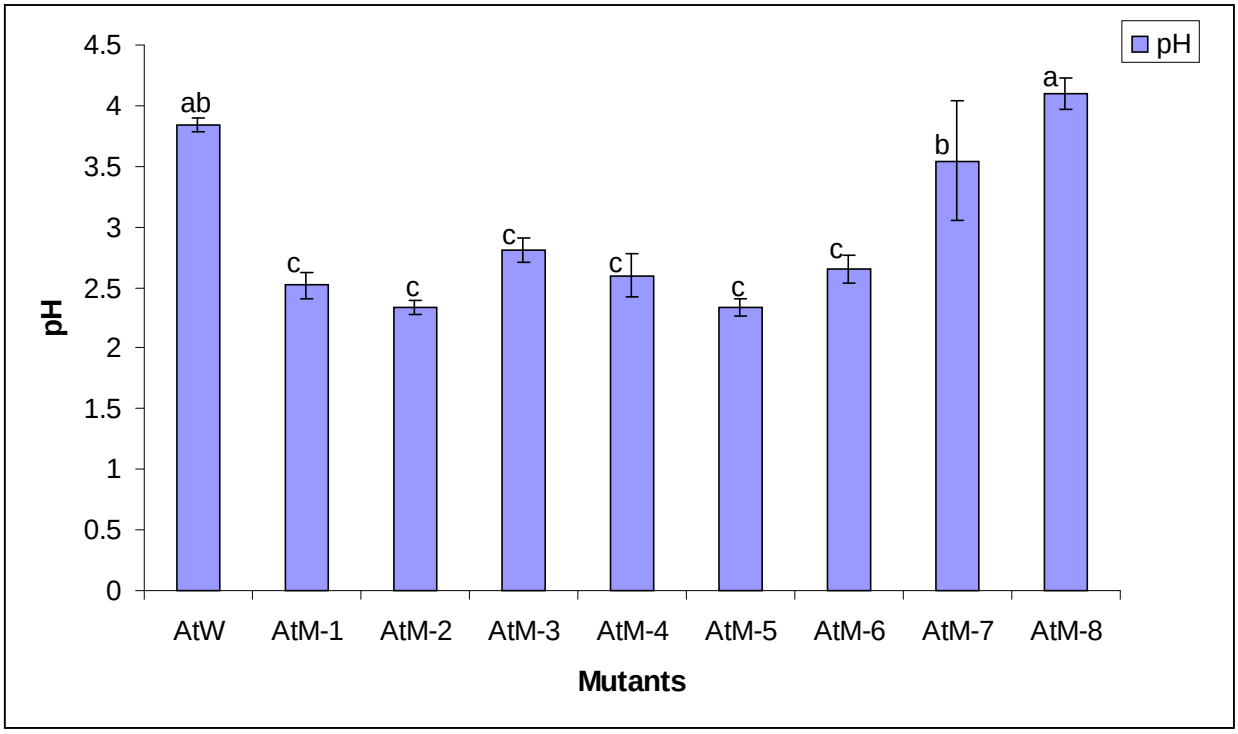

Figure 21: Change in pH by different mutants of *A. tubingensis* in liquid Pikovskaya's medium containing tri calcium phosphate (equivalent to 100 mg P_2O_5). Error bars are ± standard error (n=3). Means sharing a common letter are non-significant at $P < 0.05$ level.

Table 6: Correlation coefficients (r) for linear regression equations relating the amount of phosphate solubilized by different mutants of *A. tubingensis* with pH and dry biomass weight in case of Pikovskaya's medium containing tri calcium phosphate (equivalent to 100 mg P_2O_5). Significant correlations are shown in **bold** ($P<0.05$).

Mutants	**Correlation coefficients (r) for the entire incubation period**		
	P_2O_5 (mg/100 ml) vs. pH	**P_2O_5 (mg/100 ml) vs. dry biomass weight (mg/100 ml)**	**pH vs. dry biomass weight (mg/100 ml)**
AtW	**-0.994**	-0.904	0.854
AtM-1	-0.945	0.956	-0.809
AtM-2	**-0.994**	-0.922	0.957
AtM-3	-0.722	-0.979	0.846
AtM-4	**-0.999**	-0.952	0.942
AtM-5	-0.900	-0.830	0.990
AtM-6	-0.950	-0.800	0.946
AtM-7	-0.931	-0.940	0.752
AtM-8	**-0.999**	-0.960	0.962

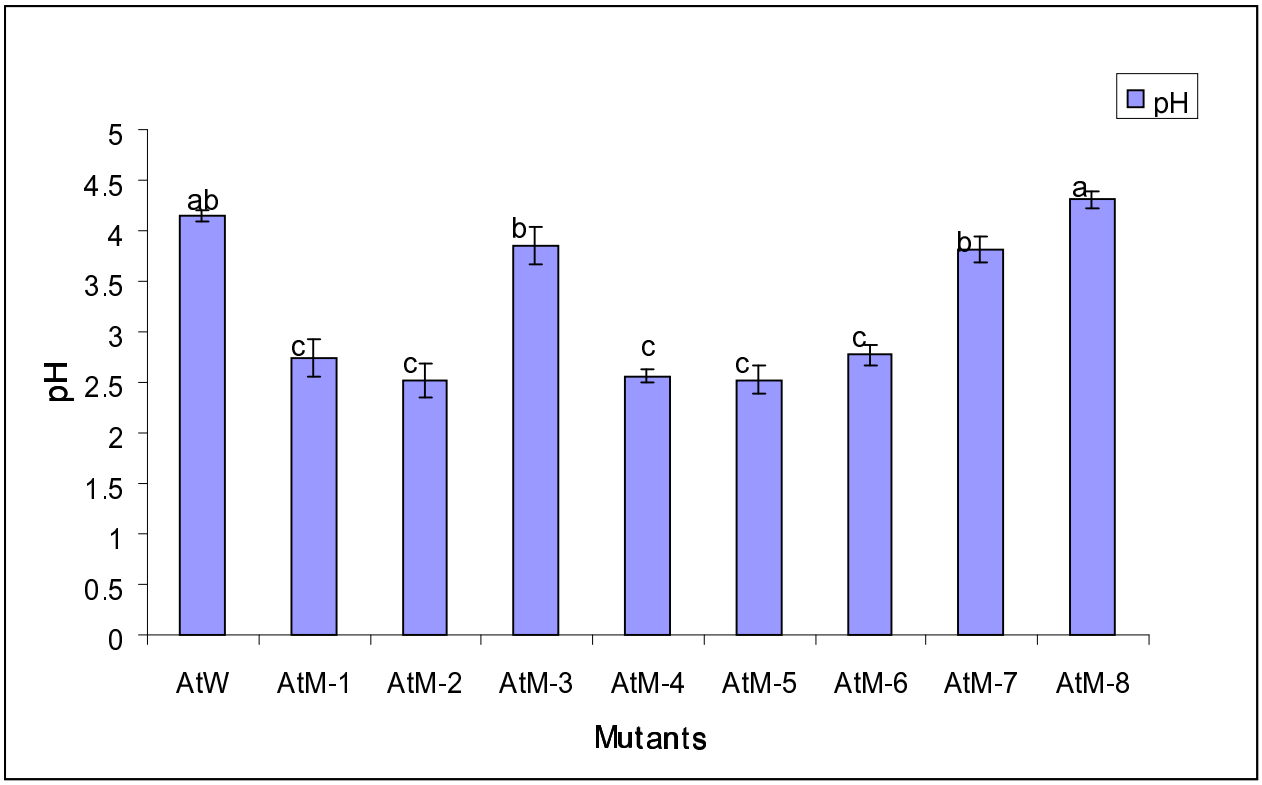

Figure 22: Change in pH by different mutants of *A. tubingensis* in liquid Pikovskaya's medium containing rock phosphate (equivalent to 100 mg P_2O_5). Error bars are ± standard error (n=3). Means sharing a common letter are non-significant at $P < 0.05$ level.

Table 7: Correlation coefficients (r) for linear regression equations relating the amount of phosphate solubilized by different mutants of *A. tubingensis* with pH and dry biomass weight in case of Pikovskaya's medium containing rock phosphate (equivalent to 100 mg P_2O_5). Significant correlations are shown in **bold** ($P<0.05$).

Mutants	**Correlation coefficients (r) for the entire incubation period**		
	P_2O_5 (mg/100 ml) vs. pH	**P_2O_5 (mg/100 ml) vs. dry biomass weight (mg/100 ml)**	**pH vs. dry biomass weight (mg/100 ml)**
AtW	-0.954	-0.894	0.987
AtM-1	**-0.999**	**-0.998**	**0.997**
AtM-2	**-0.997**	-0.522	0.990
AtM-3	-0.984	-0.971	0.915
AtM-4	-0.765	-0.347	0.909
AtM-5	**-0.995**	-0.805	0.857
AtM-6	-0.995	-0.885	0.837
AtM-7	-0.541	-0.882	0.873
AtM-8	-0.805	-0.956	-0.943

Acid production in the form of H^+ release in response to the assimilation of cations such as ammonium is a well known fungal phenomenon (Banik and Dey, 1983; Kucey, 1983; Roos and Luckner, 1984; Asea *et al*., 1988). The uptake of ammonium by fungi in a liquid medium commonly leads to a rapid drop in the pH of the medium (Cochrane, 1958). So, the lowering of pH may be the main P-solubilizing mechanism.

The majorities of organic P compounds constitutes a small component of the organic P in soil and are generally assumed to be readily mineralized in soil environments (Anderson, 1980; Dalal, 1977). On the contrary, phytate is a major component of soil organic P and is considered to be only poorly available in soil due to its propensity to undergo precipitation and adsorption reactions (Anderson, 1980; McKercher and Anderson, 1989). Isolates of *Aspergillus tubingensis* showed high specific activity towards phytate. This may be one of the causes of highest P solubilization in case of AtM-2 and AtM-5. Also, activity of this enzyme might play a role in lowering the pH of the media.

So, apart from acidification (or pH decrease), another mechanism which may aid microbial P solubilization is acid phosphatase and phytase activity.

4.5 Phosphatase Activity:

Maximum phosphatase activity was shown by mutants AtM-2 and AtM-5 in liquid medium containing tri calcium phosphate and rock phosphate at the end of four days of incubation (Figures 23 and 24). It was also related with lowering of pH. Acid phosphatase participates in the total dephosphorylating action of this enzyme group and due to the production of acids, pH lowers. AtM-8 and AtW showed lowest phosphatase secretion in the liquid medium.

4.6 Phytase Activity:

Since the assimilation of phytate is likely dependent on phytate dephosphorylating activities, phytate activity in culture filtrate and mycelia was determined. Some mutants of *A. tubingensis* were secreting clearly higher levels of phytate activity (AtM-2 and

AtM-5) than others (Figures 23 and 24). The phosphorus in the nutrient medium affects phytase production by *A. tubingensis*.

Using culture filtrate, little bit different kind of result was found for phytase activity. Phytase activity was found to be maximum in case of AtM-8 and AtW, which showed least P solubilization (Figure 25). When these datas were compared with pH, optimum pH for the activity of this enzyme in culture filtrate was 3.8 – 4.2. Acid pH values (3.8 - 4.2) were more favorable for phytase production in culture filtrate. This suggests that production of more amounts of organic acids (where pH value is lesser) may have inhibitory effect on phytase activity as activity of this enzyme in culture filtrate diminished at high acidic pH.

Acid phosphatase and phytase activity using mycelia was found to be maximum in case of AtM-2 and AtM-5, whereas least activity was showed by AtM-8 (Figures 23 and 24). Both the isolates AtM-2 and AtM-5 also showed maximum P-solubilization which suggests that there is direct relation between solubilization and activity of these enzymes. When the initial pH value of the nutrient medium is higher than 2.3 the development of the fungus is stimulated. The production of the acid phosphatase and phytase is favored by low pH values. The content of phosphorus in the medium is one of the basic factors which determine to a degree the biosynthetic process of the acid phosphatase and phytase. The conclusion about the role of the inorganic phosphorus as a regulator of the phytase and acid phosphatase production corresponds with that of other authors (Shieh and Ware, 1968; Howson and Devis, 1983; Chelius and Wodzinski, 1994).