GROWTH OF AGRICULTURALLY IMPORTANT *PSEUDOMONAS SPP.* AND *AZOTOBACTER CHROOCOCCUM* ON BEER WASTE AND OBSERVATION OF THEIR SURVIVAL IN PEAT

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

BENEK ABAT

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
FOOD ENGINEERING

SEPTEMBER 2006

Approval of Graduate School of Natural and Applied Sciences

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ABSTRACT

GROWTH OF AGRICULTURALLY IMPORTANT *PSEUDOMONAS SPP.* AND *AZOTOBACTER CHROOCOCCUM* ON BEER WASTE AND OBSERVATION OF THEIR SURVIVAL IN PEAT

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September 2006, 91 pages

In this study agriculturally important *Pseudomonas spp.* which may solubilize phosphate and *Azotobacter chroococcum* which can fix atmospheric nitrogen were grown on waste beer with 4 different concentrations and conditions for best growth were determined. Having potential of use as biofertilizers, they were put in the carrier material peat and survivals of them were observed for 3 months at three different temperatures.

Biofertilizer can be defined as a substance which contains living microorganisms which, when applied to seed, plant surface, or soil, colonizes the rhizosphere or

the interior of the plant and promotes growth by replacing soil nutrients or making nutrients more available or increasing plant access to nutrients.

In order to benefit from the biofertilizers, viable and active microorganisms in high numbers must be present which requires high quality inoculants. The carrier substrate is a critical part of the product formulation and must be capable of supporting high numbers of the intended microbe(s).

It was found that *Pseudomonas spp.* can solubilize phosphate. Furthermore, conditions for best growth for both bacteria were determined as 30 % of waste beer. Peat was found as an appropriate carrier due to preservation of viable cells for 3 months at 0 ºC, 20 ºC and 30 ºC. However, peat couldn't support high numbers of *Pseudomonas spp.* at 30 ºC.

Keywords: *Azotobacter chroococcum*, Biofertilizer, Peat, *Pseudomonas spp.*, Waste beer

ÖZ

ZİRAİ AÇIDAN ÖNEMLİ BİR *PSEUDOMONAS* TÜRÜ VE *AZOTOBACTER CHROOCOCCUM*'UN ATIK BİRADA BÜYÜTÜLMESİ VE TORFTA YAŞAM EĞRİLERİNİN GÖZLENMESİ

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Bu çalışmada zirai açıdan önemli olan fosfat çözme özelliğine sahip *Pseudomonas spp.* ve nitrojen çözme özelliğine sahip *Azotobacter chroococcum* türleri atık birada büyütülerek, en iyi büyüme şartları tespit edilmiştir. Biyolojik gübre olarak kullanılma potansiyeline sahip olan bu türler torf denen taşıyıcı madde içinde, 3 ayrı sıcaklıkta 3 aylık yaşam eğrileri çıkarılmıştır.

Biyogübreler içinde canlı mikroorganizmalar bulunduran, tohumlara, bitki yüzeylerine ya da toprağa uygulandıklarında bitkinin kök çevresine ya da içine tutunup, besinleri toprağa bağlayarak, topraktakileri bitkilerin kullanabilecekleri duruma getirerek ya da besinlerin alımın artırarak bitkinin gelişimine katkı sağlayan karışımlardır.

Biyolojik gübrelerden tam anlamıyla yararlanılabilmesi için, içinde yüksek rakamlarda ve canlı olan mikroorganizmalar bulunmalıdır. Bu da ancak kaliteli taşıyıcı malzeme kullanımıyla mümkündür. Bu durumda taşıyıcı madde önemli bir role sahiptir ve istenilen mikropları yüksek rakamlarda tutabilme kapasitesine sahip olmalıdır.

Yapılan deneyler sonucunda *Pseudomonas* türünün fosfatı çözebildiği bulunmuştur. Buna ek olarak %30 konsantrasyonlu atık bira *Azotobacter chroococcum* ve *Pseudomonas* türü için en uygun koşul olarak belirlendi. Torf 0 ºC, 20 ºC ve 30 ºC de yapılan 3 aylık deneyler sonucunda yüksek miktarlarda canlı sakladığı bakterilere bakılarak taşıyıcı madde olarak kullanılmaya uygun görüldü. Bu sonuca ters olarak torf 30ºC'de *Pseudomonas* türünün canlılığını fazla koruyamadı.

Anahtar Kelimeler: Atık bira, Biyolojik gübre, *Azotobacter chroococcum, Pseudomonas spp.,* Torf

To my Mom and Dad

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my thesis supervisor Prof. Dr. Haluk Hamamcı for his guidance, understanding, kind support, encouraging advices, criticism, and valuable discussions throughout my thesis. I am greatly indebted to Prof. Dr. Meral Yücel from Department of Biology for providing me every opportunity to use greenhouse, Prof. Dr. Aydın Güneş from Department of Soil Sciences in Ankara University for his corporation in this study.

I would sincerely thank to my brother Diren Abat for his endless friendship, support, and help in all parts of my life, making my stay in METU happy and memorable and being always right beside me. My deepest gratitude also goes to Lütfiye Kurt and her family who was a second family to me in Ankara.

I express my special thanks to Gül F. Sarıbay, for sparing her time and her all help during my experiments. I would also like to thank to my friends Şeyda Açar, A. Oğuz Büyükkileci, Fatma Işık, İlknur Çakar, Didem Dedeoğlu, Aslı Erdoğ for their cooperation and friendship, and helping me in all the possible ways.

It is with immense pleasure that I dedicate this dissertation to Güler and Nevzat Abat who always offered their advice, love, care and support. My family's absolute unquestionable belief in me, have been a constant source of encouragement and have helped me achieve my goals. I also express my sincerest love and thanks to Önder Şahinler for his love, patience, support and understanding and encouragement in every step of this study. It would not have been possible complete this study without their love and support to.

This study was supported by BAP-2005-07-02-00-46

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NOMENCLATURE

Abbreviations

AFM	Anti-fungal Metabolites
AMF	Arbuscular Mycorrhizal Fungi
APA	Acid Phosphatase Activity
BNF	Biological Nitrogen Fixation
BSA	Bovine Serum Albumin
EMS	Environmental Management System
EtOH	Ethanol
HPLC	High Pressure Liquid Chromatography
N	Nitrogen
NA	Nutrient Agar
NB	Nutrient Broth
NBRIP	Nutrient Botanical Research Institute's Phosphate Growth Medium
OD	Optical Density
OM	Organic Matter
P	Phosphate
PGPR	Plant Growth Promoting Rhizobacteria
PSM	Phosphate Solubilizing Microorganism
PVK	Pikovskaya Medium

CHAPTER 1

INTRODUCTION

In the food industry, the brewing sector holds a strategic economic position with the annual world beer production exceeding 1.34 billion hectoliters in 2002. Beer is the fifth most consumed beverage in the world behind tea, carbonates, milk and coffee and it continues to be a popular drink with an average consumption of 23 liters/person per year.

Water management and waste disposal have become a significant cost factor and an important aspect in the running of a brewery operation. Every brewery tries to keep waste disposal costs low whereas the legislation imposed for waste disposal by the authorities becomes more stringent. Use of factory wastes has possibly recently expanded. Those materials were traditionally burned, dumped at sea or stored in land-fills. However, in the last decades, there has been a trend to use those materials in agriculture due to an increasing demand to decrease environment pollution. As an alternative, waste can be used as growth medium for agriculturally used bacteria called biofertilizers.

Biofertilizer can be defined as a substance which contains living microorganisms which, when applied to seed, plant surface, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by replacing soil nutrients (e.g., by biological N_2 fixation (BNF)) or making nutrients more available (e.g., by solubilization of phosphates) or increasing plant access to nutrients. Some examples of biofertilizers are *Pseudomonas, Bacillus, Azotobacter, Rhizobium* and etc.

Pseudomonas species occur in and on plants, in water and soil and have important functions as chemical transformations, mineralization of organic compounds, and interactions with plants that affect their productivity. *Pseudomonas* which are aerobes could utilize a large number of organic compounds for growth and can degrade aromatic compounds to use in the TCA cycle. *Pseudomonas spp.* are phosphate solubilizing microorganisms (PSM) that can solubilize P in soil and make P, which increases the yield of plants, more available to them.

Azotobacters are widely distributed in nature. They have been found in soils with pH 6 or above in almost every instance where examinations have been made. The greatest limiting factor affecting their distribution in soil appears to be pH. These organisms are favored by pH 6 or more, good aeration, abundant organic matter, and suitable moisture. They depend on decomposable organic matter for their energy supply and thus, have limited activity in soils and contribute little nitrogen for plant growth in cultivated fields.

The carrier is the delivery vehicle of live microorganisms from the factory to field. Benefit from bio-pesticides and bio-fertilizers requires delivery to the field of viable, active microorganisms in high numbers, which requires high quality inoculants. The carrier substrate is a critical part of the product formulation and must be capable of supporting high numbers of the intended microbe(s).

In this study agriculturally important *Pseudomonas spp.* which can solubilize phosphate and *Azotobacter chroococcum* which can fix atmospheric nitrogen were grown on waste beer and concentration for best growth were determined as 30%. No growth was observed at 50%, 70% and 100% of waste beer. Species were also put in the carrier material peat and survival abilities of them were observed for 3 months at 3 different temperatures.

CHAPTER 2

BACKGROUND

2.1 ORGANISMS

2.1.1 *Azotobacter chroococcum*

In 1901, Dutch microbiologist M. W. Beijenirck, using an enrichment technique with a medium devoid of a combined nitrogen source, discovered an aerobic microorganism capable of fixing molecular nitrogen to which the name *Azotobacter chroococcum* was given. They are nonspore-forming short, thick rods with rounded ends. They are also gram negative and motile by means of a polar flagellum (*Brock and Madigan, 1991*)

Two characters serve to distinguish the *Azotobacters* from other bacteria. These are relatively large size of the individual cells, which may measure as much as 5µm in diameter as seen in wet preparations under the phase contrast microscope, and the ability of *Azotobacter* cultures to fix atmospheric nitrogen when provided with a suitable energy source. Nitrogen fixing ability is not, of course, restricted to the azotobacters, but few other groups of bacteria will fix amounts in excess of 10 mg of atmospheric nitrogen/g of carbohydrate consumed, as will the *Azotobacters*.

The cells of *Azotobacter chroococcum* measuring roughly 2.4µm x 5.0µm. They show considerable pleormorphism in old cultures. The formation of brown insoluble pigments is characteristic of the species, colonies of *A.chroococcum* frequently becoming very dark brown in color (*Gibbs and Shapton, 1968*).

Azotobacter is used for studying nitrogen fixation and inoculation of plants due to its rapid growth and high level of nitrogen fixation (cited in *Mrkovacki and Milic, 2001*). However, despite the considerable amount of experimental data concerning Azotobacteria stimulation of plant development, the exact mode of action by which Azotobacteria enhances plant growth is not yet fully understood.

Three possible mechanisms have been proposed: N_2 fixation, delivering combined nitrogen to the plant, the production of phytohormones-like substances that alter plant growth and morphology, and bacterial nitrate reduction, which increases nitrogen accumulation in inoculated plants (*Mrkovacki and Milic, 2001).*

Brown and Burlingham (1968) have demonstrated in their researches that the presence of *Azotobacter chroococcum* in the rhizosphere of tomato and cucumber is correlated with increased germination and growth of seedlings. Furthermore, *Bagyraj and Menga (1978), Barakart and Gabr (1998)* report that the dry weight of tomato plants inoculated with *Azotobacter chroococcum* and grown in phosphate deficient soil was significantly greater than that of noninoculated plants.

2.1.2 Bacillus spp.

In 1892, Ferdinand Cohn, a student of Robert Koch, recognized and named the bacterium *Bacillus subtilis*. The organism was made to represent a large and diverse genus of bacteria, *Bacillus*, and was placed in the family *Bacillaceae*. The family's distinguishing characteristic is the production of endospores, which is not a reproductive structure but rather a resistant, dormant survival form of the

organism. Since this time, members of the genus Bacillus are characterized as rod-shaped, gram-positive, aerobic and facultative anaerobic, endospore-forming bacteria (*Todar, 2004*). Numerous species of Bacillus are common in soil (*Bauman, 2004*).

Spore formation, universally found in the genus, is thought to be a strategy for survival in the soil environment, wherein the bacteria predominate.

Species of *Bacillus* usually grow well on defined media containing any of a number of sources. Many *Bacilli* produce extracellular hydrolytic enzymes that break down complex polymers such as polysaccharides, nucleic acids, and lipids, permitting the organisms use these products as carbon sources and electron donors. Many *Bacilli* produce antibiotics, of which bacitracin, polymyxin, tyrocidin, gramicidin, and circulin are examples. In most cases, antibiotic production is related to the sporulation process, the antibiotics released when the culture enters the stationary phase of growth and after it is committed to sporulation. Spore preparations derived from *B.thuringiensis* and *B. popilliae* are commercially available as biological pesticides (*Brock and Madigan, 1991*).

Members of the genus *Bacillus* are easy to isolate from soil or air and are among the most common organisms to appear when soil samples are streaked on agar plates containing various nutrient media (*Brock and Madigan, 1991*).

2.1.3 Pseudomonads

Bacteria called pseudomonads are gram-negative, aerobic, flagellated, and straight to slightly curved rods that catabolize carbohydrates by the Entner-Doudoroff and pentose phosphate pathways. These organisms are noted for their ability to break

down numerous organic compounds. *Pseudomonas, Burkholderia, Zymomonas*, and *Xanthomonas* are some examples of this group.

2.1.3.1 *Pseudomonas spp.*

The bacteriological criteria that distinguish the members of the genus *Pseudomonas* are given below in Table 2.1 (*Palleroni, 1975*)

Table 2.1 General Characteristics of Genus *Pseudomonas spp.* (*Palleroni, 1975*)

General characteristics of genus *Pseudomonas spp.*
• Gram- negative • Rod-shaped, 0.5-0.8 um x 1-3 um • Strictly aerobic; the only anaerobic activities may be denitrification and arginine degradation to ornithine • Motile by polar flagella; some strains also produce lateral flagella • Oxidative, chemoorganotrophic metabolism • Catalase-positive • Usually oxidase-positive • No organic growth factors are required • Diffusible and/or insoluble pigments may be produced • GC content of the DNA : 58-68 mol%

The aerobic pseudomonads are bacteria of considerable scientific and practical importance. They are among the most active participants in the process of mineralization of organic matter in nature, a role that can be easily inferred from their widespread occurrence in soil and water, and from the fact that many

members of the group are endowed with the capacity of attacking a large variety of organic compounds (*Clarke and Richmond, 1975*).

2.2 PLANT- MICROBE INTERACTIONS

Interactions between microbes and plants can be beneficial, neutral, or harmful to the plant. The conditions of the soil can influence these interactions, and neutral microbes may in fact become opportunistic pathogens. Rhizosphere bacteria often influence the root. Rhizobacteria that promote plant growth, directly or indirectly, are termed plant growth promoting rhizobacteria (*Lugtenberg and Weger, 1992)*

Inoculation of plants with beneficial bacteria can be traced back for centuries and recently interest in biological control has fuelled by public concerns over the use of chemicals in the environment in general, and need to find alternatives to the use of chemicals for disease control (*Whipps, 2000*).

2.2.1 Terms and Definitions

“Bacterial inoculant”- A formulation containing one or more beneficial bacterial strains (or more species) in an easy-to-use and economical carrier material, either organic, inorganic, or synthesized from defined molecules. The inoculant is the means of bacterial transport from the factory to the living plant. The desired effects of the inoculant on plant growth can include nitrogen fixation in legumes, biocontrol of (mainly) soil-borne diseases, the enhancement of mineral uptake, weathering of soil minerals, and nutritional or hormonal effects.

“Biofertilizers”- A misleading but widely used term meaning “bacterial inoculant”. Usually it refers to preparation of microorganism(s) that may be a partial or complete substitute for chemical fertilization (like rhizobial inoculants) (*Bashan, 1998*).

2.2.2 Rhizosphere

Rhizosphere is defined as the area influenced by the root system. In comparison with root-free soil, the rhizosphere forms a nutrient-rich niche for bacteria as a result of exudation of compounds such as organic acids, sugars and amino acids (*Lugtenberg and Dekkers, 1999)*

Rhizosphere includes,

i) the ectorhizosphere (area immediately surrounding the root)
ii) the rhizoplane (the root surface)
iii) the endorhizosphere (the area within the root that can be colonized by some rhizosphere)

The total rhizosphere environment is determined by an interacting trinity of soil, plant, and the organisms associated with the root. Quantitatively and qualitatively, the nutrients present in the rhizosphere are different from those in the bulk soil. Consequently, populations of bacteria, fungi, protozoa, and nematodes differ in those two environments.

Microbes in the rhizosphere may be attached to root cells, embedded in the mucigel, or not physically connected to the root at all. Their presence in the rhizosphere is assumed to be based on root exudates as nutrient source. The composition of exudate (primarily sugars, amino acids, and organic acids) varies with biotic factors (e.g.; plant species, age, and nutrient status) and abiotic factors (e.g., temperature, soil structure, pesticides, and supply of oxygen and water) (*Galli et al, 1992*).

Effective root colonization can contribute to the following processes;

- causing disease when pathogens are involved;
- disease control by microbes that produce antifungal metabolites (AFMs);
- phytostimulation, i.e. plant growth promotion, usually by the production of phytohormones;
- biofertilization, i.e. the process by which microbes increase the availability of nutrients, such as nitrogen, phosphate and micronutrients, to the plant;
- as more and more bacteria are isolated that are able to degrade potentially hazardous organic chemicals in nature, colonization can also play a prominent role in bioremediation promoted by plants, a process called phytoremediation (*Lugtenberg and Dekkers, 1999*).

2.2.3 Beneficial Microbe-Plant Interactions in the Rhizosphere

In the rhizosphere, bacteria are abundantly present, most often organized in microcolonies. Some of the rhizobacteria not only benefit from the nutrients secreted by the plant root but also beneficially influence the plant in a direct or indirect way, resulting in a stimulation of its growth. These plant-growth-promoting rhizobacteria (PGPRs) can be classified according to their beneficial effects. For instance, biofertilizers can fix nitrogen, which can subsequently be used by the plant, thereby improving the plant growth when the amount of nitrogen in the soil is limiting. Phytostimulators can directly promote the growth of plants, usually by the production of hormones. Biocontrol agents are also able to protect plants from infection by phyto-pathogenic organisms. (*Bloemberg and Lugtenberg, 2001*)

PGPR also increase plant growth indirectly either by the suppression of well-known diseases caused by major pathogens or by reducing the deleterious effect of minor pathogens (micro-organisms which reduce plant growth but without obvious symptoms) (*Whipps, 2000, Egamberdiyeva and Höflich, 2003*). Figure 2.1 shows the relation of the PGPR mechanisms in relation to the effect on the crop (*Tenuta*).

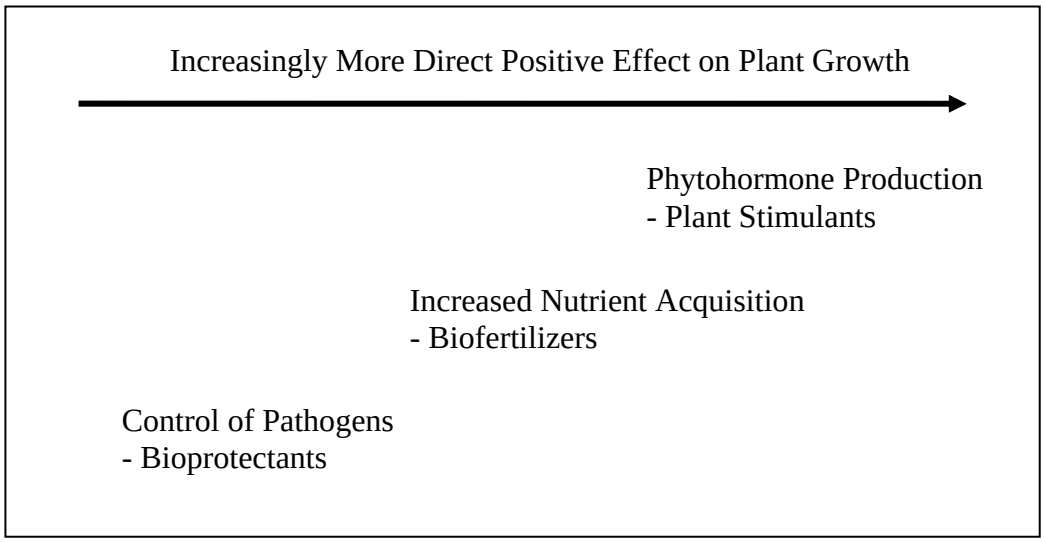

Figure 2.1 Spectrum of Mechanisms of Plant Growth Promotion by PGPR (*Tenuta*).

As shown in Figure 2.1 biofertilizers have a moderate effect on plants compared to bioprotectants and plant stimulators by means of nutrient acquisition increment.

2.3 BIOFERTILIZERS

Plant Growth Promoting Rhizobacteria (PGPR) represent a wide variety of soil bacteria which, when grown in association with a host plant, result in stimulation of growth of their host. Biofertilizer is a recently coined term whose exact definition is still unclear, but which commonly refers to the use of soil microorganisms to increase the availability and uptake of mineral nutrients for plants. Not all PGPR are biofertilizers.

Biofertilizer can be defined as a substance which contains living microorganisms which, when applied to seed, plant surface, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by replacing soil nutrients (e.g., by biological N_2 fixation (BNF)) or making nutrients more available (e.g., by solubilization of phosphates) or increasing plant access to nutrients (e.g., by increasing root surface area). This definition is based on the logic that the term biofertilizer is a contraction of the term biological fertilizer (*Vessey, 2003*).

The soil microorganisms used in biofertilizers are: Phosphate solubilizing microbes (*Pseudomonas spp., Bacillus spp*.), Mycorrhizae, Azospirillum, Azotobacter, Rhizobium, Sesbania, Blue Green Algae, and Azolla (*Vazquez et al, 1999, Canpolat et al, 2005, Egamberdiyeva, 2003*).

Fluorescent pseudomonads having phosphate solubilizing ability have been found to enhance peanut growth under potted conditions (*Pal et al., 1999*). Also certain *Pseudomonas spp*. have been studied as promising biocontrol agents against phytopathogenic fungi (*Khan and Khan, 2002, Jian-Hua Guo et al., 2003*). *P. fluorescens* was good at improving tomato growth and reducing galling and nematode multiplication (*Siddiqui, 2004*).

Azotobacter is a heterotrophic, aerobic micro-organism, fixing nitrogen as non-symbiotic (*Paul and Clark, 1989*) which is a good source of biofertilizer to improve the growth and yield of cereals and many other crops (*Idris, 2003*). It has been also reported that wheat yield increased up to 30 % with Azotobacter inoculation and up to 43 % with Bacillus inoculation (*Kloepper et al, 1991*).

Seed or soil inoculation with phosphate-solubilizing bacteria such as *Bacillus spp*. can solubilize fixed soil P and applied phosphates, resulting in higher crop yields (*Wong et al, 2004*, *Canbolat et al, 2005*).

2.3.1 Biofertilizers and Phosphorus

Microorganisms are important component of soil. Soil bacteria and fungi mediate soil processes such as decomposition, nutrient mobilization and mineralization storage release of nutrients and water, nitrogen fixation and denitrification. Phosphate solubilizing microorganisms (PSM) include different types of microorganisms that convert insoluble phosphatic compounds into soluble forms.

2.3.1.1 Phosphorus

Phosphorus is one of the major plant nutrients in the soil. It is often referred to as the “energizer” since it helps store and transfer energy during photosynthesis. It is also part of the genetic material of all cells – DNA and RNA. All plants require phosphorus during periods of rapid growth. Without phosphorus, plant growth is retarded. Deficiency symptoms include stunted roots, red pigment in leaf bases, dull grayish-green and dying leaves. Plants most often absorb phosphorus in the

form of phosphate ions $H_2PO_4^-$ and sometimes as HPO_4^{2-} (cited in *Rodriguez and Fraga, 1999*). These phosphate ions react readily with the soil and become part of the soil particles in a process called “fixation”. Fixation prevents the leaching of phosphorus, but also changes it to a form that plants can not use. The phenomena of fixation and precipitation of P in soil is generally highly dependent on pH and soil type (*Schulte and Kelling, 1996*).

Plants can use phosphorus from organic sources generally only after hydrolysis of the Carbon-Oxygen-Phosphate (C-O-P) ester bond by phosphatases and the release of P as inorganic phosphate. Therefore, phosphatases are important for P nutrition of plants especially when there is a shortage of inorganic P in the soil (*Tarafdar et al, 2001*). Phosphatases are either acid or alkaline phosphatase according to their pH optima. Phosphatases in the rhizosphere may arise from plant roots or from soil microorganisms (*Tarafdar and Jungk, 1987*). Microorganisms may produce both acid and alkaline phosphatase but plants can only secrete acid phosphatase. Plant phosphatase is very responsive to P stress, with the onset of P deficiency causing increased enzyme activity (*Tarafdar, 1989*). *McLachlan (1976*) showed that root exocellular acid phosphatase activities of cereals and clover were related to productivity and that the activities increased in all species as they became more P deficient.

In soils 30-80 % of the total P is in organic form, but plants can utilize this organic P after it is mineralized. However, the mineralized inorganic P is rapidly sorbed to the soil solid phase and becomes a part of the large pool of sorbed or precipitated P, and only a small fraction of which is available to plants. (*Tarafdar and Claassen, 2005*). A large portion of inorganic phosphates applied to soil as fertilizer is rapidly immobilized after application and becomes unavailable to plants. Thus, the release of insoluble and fixed forms of phosphorus is an important aspect of increasing soil phosphorus availability. Seed or soil inoculation with phosphate-solubilizing bacteria is known to improve solubilization of fixed soil phosphorus and applied phosphates resulting in higher crop yields (*Çakmakçı et al, 1999, Çakmakçı et al, 2001*).

2.3.1.2 Phosphorus Cycle

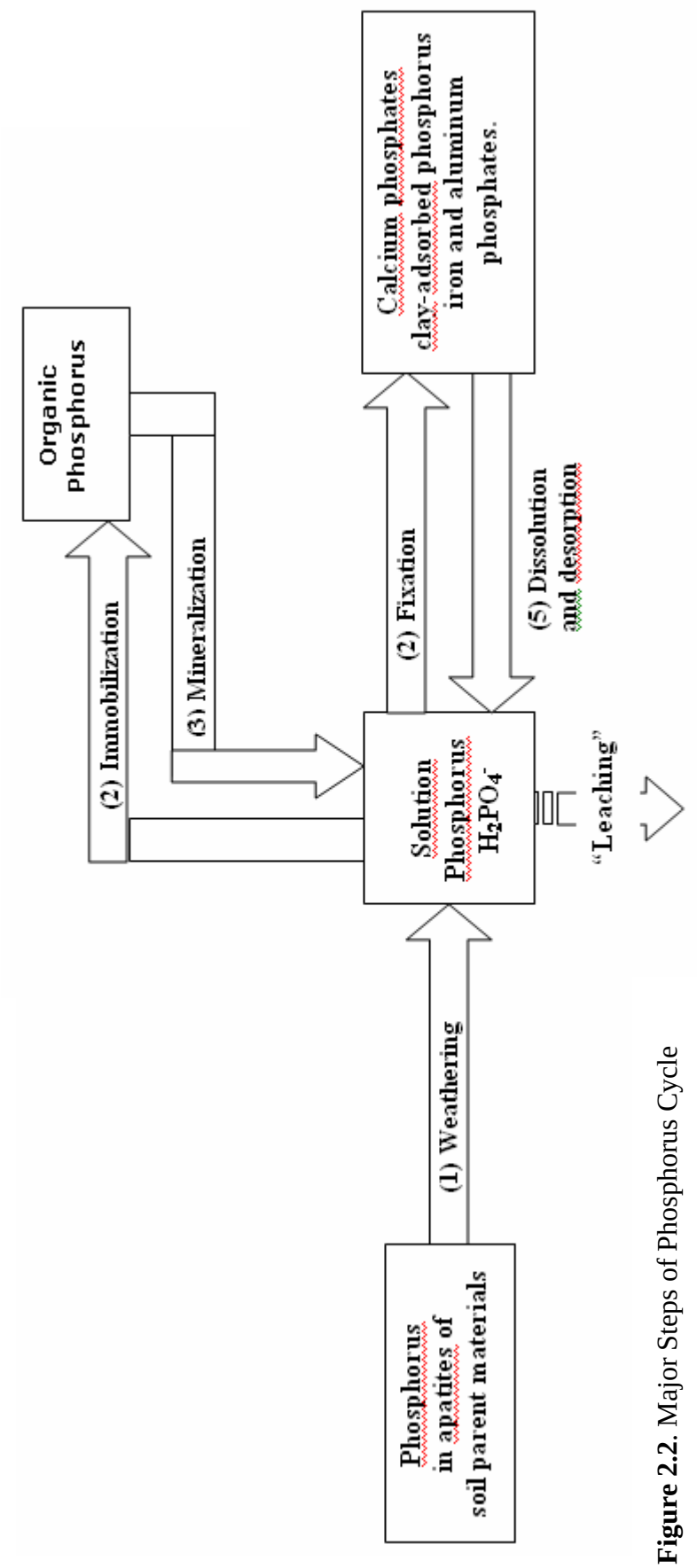

Figure 2.2. Major Steps of Phosphorus Cycle

Most phosphorus occurs in the mineral apatite in igneous rocks and soil parent materials. Apatite weathers slowly, producing the phosphate ion, $H_2PO_4^-$, which exists in the soil solution, as shown as step 1 of Figure 2.2. The $H_2PO_4^-$ is immobilized when roots and microorganisms absorb it and convert the phosphorus into organic compounds. This results in a significant amount of phosphorus in soils as organic phosphorus. Commonly, 20 to 30 percent of the phosphorus in plow layers of mineral soils is organic phosphorus. As with nitrogen, organic phosphorus is mineralized by microorganisms and is again released to the soil solution as $H_2PO_4^-$. The organic phosphorus cycle, a sub-cycle in the overall soil phosphorus cycle, where phosphorus is shuttled back and forth by mineralization and immobilization (steps 2 and 3 in Figure 2.2) (*Foth, 1990*).

A major difference between the nitrogen and phosphorus cycles is that the available form of nitrogen is mainly nitrate and is stable in the soil solution. Nitrate remains in solution and moves rapidly to roots by mass flow, unless denitrified or leached. The phosphate ion, by contrast, quickly reacts with other ions in the soil solution, resulting in precipitation and adsorption to mineral colloids that convert the phosphorus to an unavailable or fixed form (step 4 in Figure 2.2). As a consequence, most of the phosphate ions form mineralization of organic phosphorus or mineral weathering, may be converted to an unavailable form before plants have an opportunity to absorb the phosphorus and before loss by leaching can occur. The kinds of ions in the soil solution that render phosphate insoluble are related to soil pH (*Foth, 1990*).

2.3.1.3 Organic Phosphate Solubilization

The concentration of soluble P in soil is usually very low, normally at levels of 1 ppm or less. Several researches have been done on the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate *(Reddy et al, 2001, Goel et al, 2003, Goel and Katiyar, 2003*). Among the genera with this capacity are *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aereobacter, Flavobacterium* and *Erwinia*.

There are considerable populations of phosphate-solubilizing bacteria in soil and in plant rhizospheres. These include both aerobic and anaerobic strains, with prevalence of aerobic strains in submerged soils. A considerably higher concentration of phosphate solubilizing bacteria is commonly found in the rhizosphere in comparison with nonrhizosphere soil (cited in *Rodriguez and Fraga, 1999*).

Soil contains a wide range of organic substrate, which can be a source of P for plant growth. To make this form of P available for plant nutrition, it must be hydrolyzed to inorganic P. Mineralization of most organic compounds is carried out by means of phosphatase enzymes. Imported levels of microbial phosphatase activity have been detected in different types of soils.

In fact, the major source of phosphatase activity in soil is considered to be of microbial origin (*Tarafdar and Jungk, 1987).* In particular, phosphatase activity is substantially increased in the rhizosphere. (*Greaves and Webley, 1965).*

Table 2.2. Total P Accumulation in Cultures of Different Bacterial Species Grown on Insoluble Mineral Phosphate Substrates ($mg.l^{-}$)

*nd indicates not determined

Bacterial strain	**Substrate**		
	$Ca_3(PO_4)_2$	Hydroxyapatite	Rock Phosphate
Pseudomonas sp.	52	nd	nd
Pseudomonas striata	156	143	22
Rhizobium sp.	nd	300	nd
Rhizobium meliloti	nd	165	nd
Bacillus polymyxa	116	87	17
Bacillus megaterium	82	31	16
Bacillus circulans	11	17	6
Burkholderia cepacia	35	Nd	nd

Table 2.2 summarizes the solubilization ability of different insoluble P substrates by several bacterial species (taken from *Rodriguez and Fraga, 1999*). Phosphate solubilizing microorganisms are routinely screened by a plate assay method using Pikovskaya (PVK) agar (*Pikovskaya, 1948)*. However, modifications of this method or new methods are used recently because of their advantages such as exclusion of yeast extract which prevents the elucidation of role of microorganisms in phosphorus mineralization. For example, a novel defined microbiological growth medium, National Botanical Research Institute's phosphate growth medium (NBRIP), which is more efficient than Pikovskaya medium (PVK), was developed for screening phosphate solubilizing microorganisms (*Nautiyal, 1999*).

2.3.1.4 Phosphate Solubilizing Bacteria as Plant Growth Promoters

Although several phosphate solubilizing bacteria occur in soil, usually their numbers are not enough to compete with other bacteria commonly established in the rhizosphere. Thus, the amount of P released by them is generally not sufficient for a substantial increase in in-situ plant growth. Therefore, inoculation of plants by a target microorganism at a much higher concentration than that normally found in soil is necessary to take the advantage of the property of phosphate solubilization for plant yield enhancement. There have been many reports on plant growth promotion by bacteria that have the ability to solubilize inorganic and/or organic P from soil after their inoculation in soil or plant seeds (*Khan et al, 2002, Khan and Khan, 2002, Wong et al, 2004*).

The other metabolites produced by these strains which are beneficial to the plant, such as phytohormones, antibiotics, or siderophores, among others, has created confusion about the specific role of phosphate solubilization in plant growth and yield stimulation. However, there is evidence that several soil microorganisms, including bacteria, improve the supply of P plants as a consequence of their capability for inorganic or organic P solubilization (*Çakmakçı et al, 1999, Egamberdiyeva and Höflich, 2003*). Considering that P availability is a limiting step in plant nutrition, this evidence suggests a fundamental contribution of phosphate-solubilizing bacteria to plant nutrition and therefore, to the improvement of plant growth performance.

2.3.2 Biofertilizers and Nitrogen

As mentioned before, microorganisms are important components of soil. They contribute to nitrogen cycle by means of nitrogen fixation which is a very important step of the cycle.

2.3.2.1 Nitrogen

The atmosphere is made up of 79 percent nitrogen, by volume, as inert N_2 gas. Although a large quantity of nitrogen exists in the atmosphere, the nutrient that is absorbed from the soil in the greatest quantity and is the most limiting nutrient for food production is nitrogen. And also most plants cannot use nitrogen as it exists in the atmosphere. Plants can acquire N from two principal sources: (a) the soil, through commercial fertilizer, manure, and/ or mineralization of organic matter; and (b) the atmosphere through symbiotic N_2 fixation (*Bundy, 1998*).

2.3.2.2 Soil Nitrogen Cycle

The nitrogen cycle consists of a sequence of biochemical changes wherein nitrogen is used by living organisms, transformed upon death and decomposition of the organisms, and converted ultimately to its original state of oxidation (*Madigan et al, 2003*). The major segments of the soil nitrogen cycle are shown in Figure 2.3.

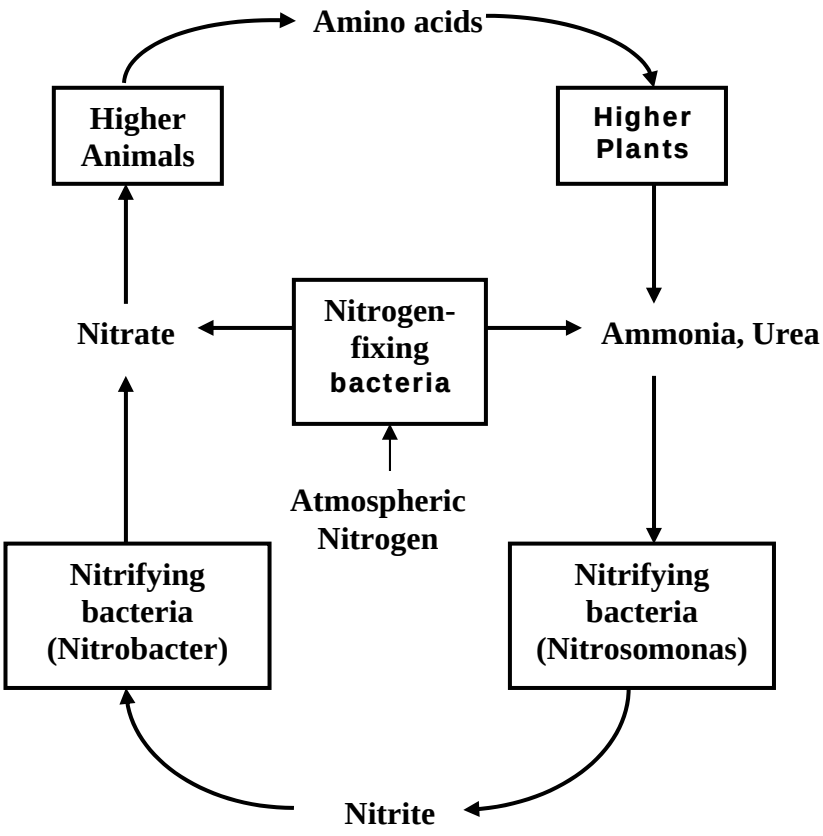

Figure 2.3. Basic Nitrogen Cycle

The reservoir of nitrogen for plant use is essentially that in the atmosphere, N_2. It takes about a million years for the nitrogen in the atmosphere to move through one cycle. The N_2 is characterized by both an extremely strong triple bonding between the two nitrogen atoms and a great resistance to react with other elements. The process of converting N_2 into forms that vascular plants can use is termed nitrogen fixation (*Madigan et al, 2003*). Key processes and prokaryotes in the nitrogen cycle were given in Table 2.3.

Table 2.3. Key Processes and Example of Prokaryotes in the Nitrogen Cycle

Processes	**Example of Organisms**
• Nitrification ($NH_4^+ \rightarrow NO_3$) $NH_4^+ \rightarrow NO_2^-$ $NO_2^- \rightarrow NO_3$	*Nitrosomonas* *Nitrobacter*
• Denitrification ($NO_3^- \rightarrow N_2$)	*Bacillus, Paracoccus* *Pseudomonas*
• N_2 Fixation ($N_2 + 8H \rightarrow NH_3 + H_2$)	
a) Free-living	
i) Aerobic	*Azotobacter* *Cyanobacteria*
ii) Anaerobic	*Clostridium*, purple and green bacteria
b) Symbiotic	*Rhizobium, Bradyrhizobium* *Frankia*

2.2.6.3 Nitrogen Fixation

Dinitrogen fixation is the conversion of molecular nitrogen (N_2) to ammonia and subsequently into organic forms utilizable in biological processes. Some N_2 is fixed by lightening and other ionizing phenomena of the upper atmosphere. This fixed nitrogen is added to soils in precipitation (*Madigan et al, 2003*)

2.3.2.4 Nitrogen Fixing Bacteria as Plant Growth Promoters

Most nitrogen fixation is biological, being either symbiotically (dinitrogen - fixing bacteria, such as *Rhizobium*, in conjunction with legumes) or non-symbiotically (free living organisms such as photosynthetic bacteria, blue-green algae, and free-living *Azotobacter* species). Nitrogen fixing organisms contain an enzyme, nitrogenase, which combines with a dinitrogen molecule (N_2) and fixation occurs in a series of steps that reduces N_2 to NH_3. It has been discovered that the effect of nitrogen fixation induced by nitrogen fixers is not only significant for legumes, but also non-legumes. (cited in *Wong et al, 2004*)

Azotobacter species are widely distributed in nature. They have been found in soils with pH 6 or above in almost every instance where examinations have been made. The greatest limiting factor affecting their distribution in soil appears to be pH. These organisms are favored by pH 6 or more, good aeration, abundant organic matter, and suitable moisture. They depend on decomposable organic matter for their energy supply and thus, have limited activity in soils and contribute little nitrogen for plant growth in cultivated fields. (*Deacon, 2003*)

2.4 CARRIER MATERIALS

There are various ways of producing a suitable inoculum, which is effective and can survive in the soil environment. For a bacterial inoculant to be effective in soil, optimal conditions for its survival after release must be provided. A widely accepted generalization is that the dynamic abiotic soil factors such as temperature, moisture content, pH and nutrient status all affect bacterial survival. There still is a gap between most of the more fundamental data on bacterial survival in soil, and the data and protocols used by the inoculant industry with respect to bacterial survival in the various carrier materials. This is mainly due to the use of carrier materials in commercial formulation.

The carrier is the delivery vehicle of live microorganisms from the factory to field. Benefit from bio-pesticides and bio-fertilizers requires delivery to the field of viable, active microorganisms in high numbers, which requires high quality inoculants. The carrier substrate is a critical part of the product formulation and must be capable of supporting high numbers of the intended microbe(s). To maintain the microorganisms in a viable state, commercially available carrier materials are typically based on milled peat, clays, rice, bran, seeds, or other complex organic matrices. (*Yardin et al, 2000*).

Variable results have been reported for all types of microbial products, with variation in their effectiveness attributed to three main causes:

(1) Presence of an already satisfactory level of the organism(s) prior to inoculation;
(2) Poor survival of the inoculant organism in the environment; and

(3) Low quality of the inoculant itself. Low quality inoculants contain insufficient viable cells of the intended microorganisms, high numbers of contaminating microorganisms, or both (*Yardin et al, 2000*).

To produce an inoculant, the target microorganism can be introduced into a sterile or non-sterile carrier. From a purely microbiological point of view, the sterile carrier has significant advantages but has not usually been cost effective from a commercial point of view. Table 2.4 summarizes the advantages and disadvantages of each carrier type (*Bashan, 1998*). The best conditions for the viability of strain UPM-Hc 3 and, in general for most *Rhizobium spp.* are the use of sterile peat and refrigerated storage (*Rodriguez-Navarro, 1991*).

Table 2.4. Comparison of Sterile and Non-Sterile Peat-Based Inoculants.

Inoculant Parameters	Sterile	Non-Sterile
Population of beneficial bacteria	High	Variable
Longevity	High, but depends on the carrier material	Relatively low
Addition of nutrients to the carrier to increase final population	Possible without sacrificing the final quality of the inoculant	Not always possible because many contaminants grow faster than many beneficial bacteria
Choice of materials to be used as carriers	Many materials are not easily sterilized or change their chemical and physical composition upon sterilization	Almost unlimited
Labor requirements	Skilled and expensive	Mostly unskilled and less expensive
Sterilization equipment required	Large autoclaves are costly to purchase and operate. Sterilization by irradiation not always available	Not needed
Sterile production space	Large and costly	Not needed
Monitoring of contamination	Essential for quality control of the product	Essential for quality control of the product
Total cost of production	High	Much lower than sterile preparations

Peat formulations have been the carriers of choice, and are the most commonly used in the rhizobia inoculation industry (*Bashan, 1998, Abd-Alla and Omar, 2001, Temprano et al, 2002*). Its popularity is primarily due to successful field results obtained under commercial cultivation. In this form, the bacteria are metabolically active, and in some inoculants, bacterial multiplication continues during the storage period, as long as sufficient nutrients, moisture, and the correct temperature are maintained (*Bashan, 1998*).

2.4.1 Peat

Peat is a soil composed mainly of water. The portion that is not water – usually only about 10-20% of the mass – is the partial decomposed residue of dead plants. Combined with remains of these plants are those of the decay microorganisms. Peat is a veritable graveyard of plants (*Fuchsman, 1980*).

2.4.1.1 Composition

Peat, not surprisingly, is variable in composition. It varies from material so slightly decomposed that the plants can be readily identified from bits of leaves and roots and steams, to soils so highly decomposed that is seems to be a structureless thick mud. Chemically, peats are largely organic material. The amount varies with type of peat, but ash contents of 2-10% can be regarded as typical. By contrast the ash content of the thick rich black soils of our best farmland is much more than 50% of the total, usually over 80% (*Fuchsman, 1980*).

Peat contains an enormously complex mixture of organic materials, in which the chemically more stable residues of plant tissues are prominent. Simple sugars and amino acids and other water-soluble components of the living tissues are present in low concentration and may entirely absent in peat. They presumably disperse into the surrounding water when the plant dies and quickly consumed before being covered over by new material and before the oxygen supply in the water is exhausted. More persistent, less reactive structural elements of plants contribute to the solid substance of peat (*Fuchsman, 1980*).

2.4.1.2 Degree of Peat Decomposition

The degree to which plants have undergone "humification" i.e., have lost their original character and become and organic soil, is of considerable importance the preliminary assessment of the possibly uses of the peat. However, the measurement of the degree of decomposition is inexact. Some chemicals methods for judging degree of decomposition have been proposed. The more widely accepted measurements consider that decomposition corresponds to replacement of the fibrous structure characteristic of plant tissue by exceedingly fine particles of no regular structure. A system, used in field testing, consists simply of squeezing a fresh sample of wet peat in the hand. The Post system (named after L. von Post, Swedish inventor), distinguished ten grades of "humification", H1-H10 (cited in *Fuchsman, 1980*). The system is described in Table 2.5

Table 2.5. The Post System of Peat Humidification Values, Based on Hand-Pressing a Sample of Field-Fresh Peat (*Fuchsman, 1980*)

Humification value	Quality of water pressed from the hand	Nature of residue left in the hand
H1	Clear colorless water	Characteristic plant structures
H2	Yellow-brown water	Identifiable plant residue
H3	Cloudy brown water	Plant residues, but no mud
H4	Very turbid, brown water	Muddy portions visible
H5	Very turbid with some coarser peat solids, brown water	Muddy, plant residues still identifiable
H6	One-third of peat is lost in the water	Very muddy, plant residues still identifiable
H7	One-half of peat is lost in the water	Very muddy, plant residues still identifiable
H8	Two-thirds of peat is lost in the water	Only roots, wood, coarse fibers
H9	Nine-tenths of peat is lost in the water	Only a few isolated plant residues
H10	Entire mass slips through fingers	No residue

2.5 APPLICATION METHODS FOR BACTERIAL INOCULANTS

Two main methods of inoculation are currently being used: seed inoculation and soil inoculation (*Hedge, 1992*). Soil inoculation is done by delivering the inoculant directly into the sowing furrow with the seeds (*Gault et al, 1982*). Seed inoculation is the most popular method worldwide, as long as the farmer willing to take the extra step of mixing the inoculant with the seeds immediately before sowing. The less common method, soil inoculation, is now being used successfully for rhizobia inoculation, but has several disadvantages which limit its future for the application of *Azospirillum*, which survives poorly in many soils (*Bashan et al, 1995*).

Microbial inoculants can be applied during three possible phases:

i. at the seed processing plant as a seed coating, months before the actual sowing
ii. "on site" , as a seed application just before sowing, or by inoculant delivery directly on to the seeds in the furrow, and
iii. after seedlings emerge

Soil inoculation is an alternative to seed inoculation. It is more convenient for the farmer than seed inoculation, but is sometimes not so effective. It is also more expensive because more inoculant is required. Soil inoculation can be done either with peat-based granules or with microgranulated forms of inert materials; sand, calcium carbonate, or marble powder. These materials have been previously mixed with the inoculum in the factory or can be mixed with the seeds by the farmer prior to sowing. A commercial product for *A. lipoferum* has successfully used this technology with corn for four years in 13 locations in France (cited in *Bashan, 1998*).

2.6 LIMITATIONS IN THE USE OF PLANT GROWTH PROMOTING RHIZOBACTERIA

There are several limitations in the use of PGPR for commercial use. Primarily, efficacy is not reliable for most PGPR. This is because the mechanism of action of the PGPR in promoting growth is not well understood. Research needs also to be conducted determining if and how variations of soil type, management practices (e.g. agrochemical use, rotations), and weather effect PGPR efficacy (*Bashan, 1998*).

2.7 BREWERY INDUSTRY

2.7.1 Process Description

Beer is a fermented beverage. The sugar needed for the fermentation process comes from the transformation of the starch contained into the grain through the effect of specific enzymes contained into the malt (*FAO/EBRD*). Flow chart of the brewery process was given in Figure 2.4.

Brewers are very concerned that the techniques they use are the best in terms of product quality and cost effectiveness. During production, beer alternately goes through three chemical and biochemical reactions (mashing, boiling, fermentation and maturation) and three solid-liquid separations (wort separation, wort clarification and rough beer clarification). Consequently water consumption, wastewater and solid-liquid separation constitute real economic opportunities for improvements in brewing (*Fillaudeau et al, 2005*)

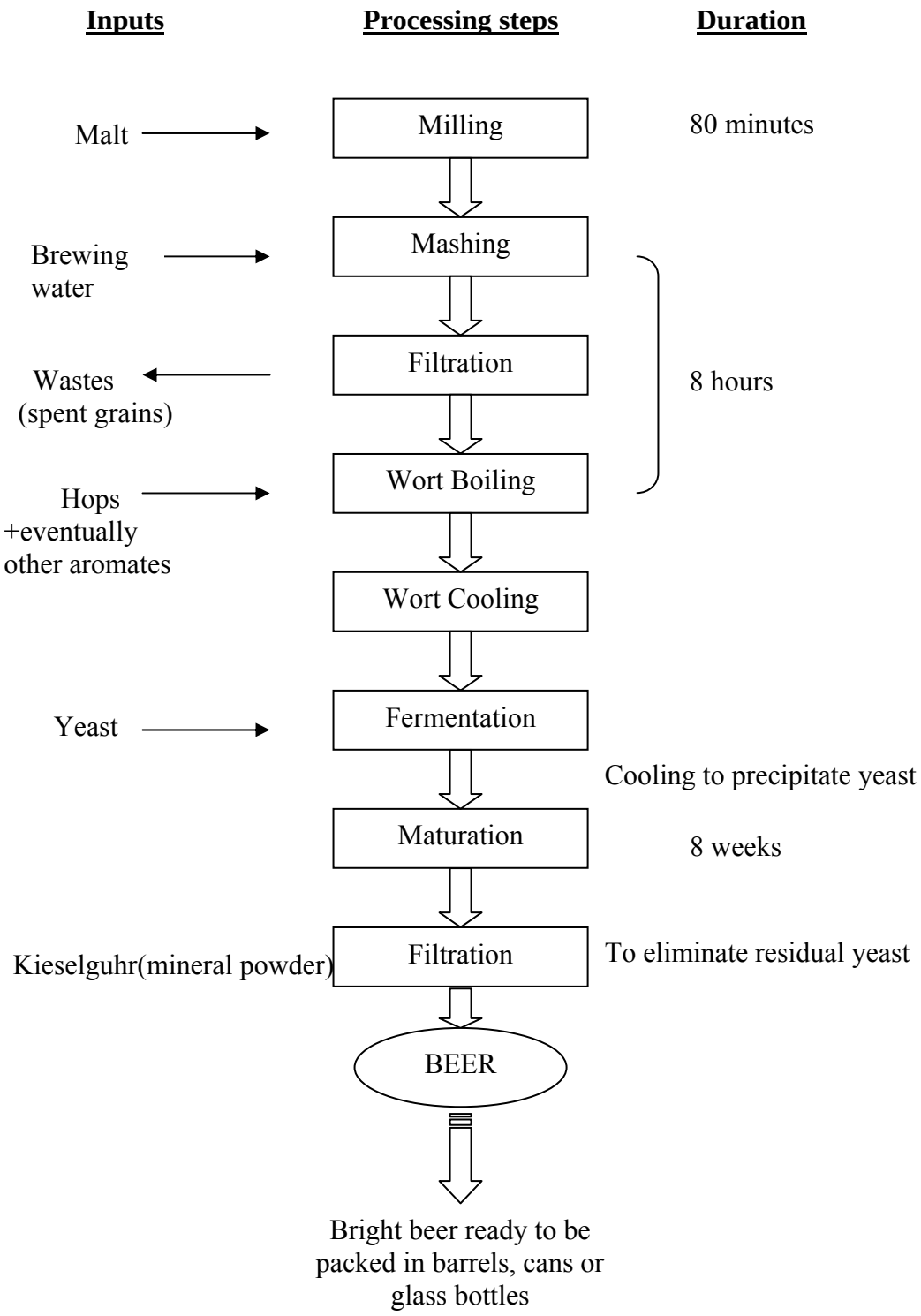

Figure 2.4. Step by Step Brewing Processing

2.7.2 Brewery Waste

In the food industry, the brewery sector holds a strategic economic position with the annual world beer production exceeding 1.34 billion hectoliters in 2002. The last 20 years environmental awareness of the brewing industry has grown significantly leading to increased investments in environmental protection measures. Important internal drivers for the brewing industry are implementation of environmental management systems (EMS) like ISO 14001 as well as the need for conducting of benchmarking studies for brewery process optimization. As a result there is a growing interest within the brewing industry in environmental pollution control systems (*Driessen and Vereijken, 2003*).

Factory wastes were traditionally burned, dumped at sea or stored in landfills. However, recently use of them expanded. Industrial wastes especially of organic origin have high potential for agricultural use. Using those wastes in arid and semi-arid regions, where the OM content in soils is rather low, will contribute on one hand reducing of environmental problems and on the other hand they will enrich the soil with OM (*Kütük et al, 2003).* Wastes also can be used as a carrier material instead of widely used peat (*Tyagi et al, 2001*).

The beer factory located in Kazan, Ankara has a capacity of 1.5 million hectoliter per year and produces 5 tones of sludge per day (*Kütük et al, 2003*).

CHAPTER 3

EXPERIMENTAL

3.1 MATERIALS

3.1.1 Organisms

In this study, initially four different species –*Pseudomonas spp.*, M-3, BA-140, *Azotobacter chroococcum*- were used in starting experiments. The further stages of the study were carried on with two of them which are *Pseudomonas spp.* and *Azotobacter chroococcum*.

3.1.1.1 *Pseudomonas spp.*

One of the bacterium used in this study was *Pseudomonas spp*. which was used for agricultural purposes for their phosphate solubilization ability. Stock cultures of *Pseudomonas spp*. was obtained from Tokat Gazi Osman Paşa University and maintained on Nutrient Agar and stored at 4°C.

3.1.1.2 *Azotobacter Chroococcum*

Other bacterium used in this study, *Azotobacter chroococcum* was also used for agricultural purposes by Catek, Havana, Cuba. Stock cultures of *Azotobacter chroococcum* was kept on Nutrient Agar and stored at 4°C.

3.1.1.3 Bacillus spp. (BA-140)

Bacillus spp.(BA-140) used in this study was obtained from Erzurum Atatürk University. It was used previously for agricultural experiments as N-fixing bacteria (*Çakmakçı et al, 2001*). Stock cultures of *BA-140* was maintained on NA and stored at 4°C.

3.1.1.3 Bacillus megaterium (M-3)

M-3 used in this study was obtained from Erzurum Atatürk University. It was used previously as nitrogen fixing bacteria for agricultural experiments (*Çakmakçı et al, 2001*). Stock cultures of *M-3* was maintained on NA and stored at 4°C.

3.1.2 Brewery Waste

Brewery Waste used in this study was obtained from continuously operating brewery factory at Kazan, Ankara in 2006, and it was stored at 4°C, before use.

Table 3.1 Some Characteristic Properties of Brewery Waste

	g/l
Protein	0.26
Ethanol	8.75
Dissolved Solid Content	1.22
Suspended Solid Content	1.64

3.1.3 Peat

Peat used in this study was bought from Praktiker Shopping Center, Ankara in 2005. It was imported from COMPO GmbH & Co. from Germany. It was stored at room temperature in a closed box. Contents of peat are given in Table 3.2.

Table 3.2 Contents of Peat

pH value ($CaCl_2$)	5.5 – 6.5
Salt amount (g/l)	1.0 – 2.0
Nutrient amounts	200 – 450 mg/l N – Nitrogen
	200 – 400 mg/l P_2O_5 – Phosphate
	300 – 500 mg/l K_2O - Potassium oxide
Contents	High-bog peat (Decomposition degree: H_5 – H_7) 95%
	Plant compost 5%
	AGROSIL
	Lime and all important materials for plants

3.1.4 Soil

The soil used for the pot experiments was collected from Ankara University Soil Sciences Department's garden. The most important point in soil collection is that no plant should exist on the soil surface.

3.2 METHODS

All experiments were carried out at three separate flasks, at the same conditions.

3.2.1 Cultivation

Lab M Nutrient Broth 'E' was used in this study. Its composition is given in Table 3.3. Broths were steam sterilized at 121°C for 15 minutes. Solid growth medium was obtained by adding 15 g/l agar-agar to the Nutrient Broth.

Table 3.3. Lab M Nutrient Broth 'E' Formulation

Formulation	g/l
Beef Extract	1.0
Yeast Extract	2.0
Peptone	5.0
Sodium Chloride	5.0

3.2.1.1 Inoculum

3.2.1.1.1 *Azotobacter chroococcum*

The growth medium was inoculated with 10^6 cell/ml approximately. *A. chroococcum* was grown in preculture medium for 17 hours. Preculture medium was NB and it was inoculated with one loopfull of bacterial cells transferred from the solid medium. Preculture was cultivated at 35°C and 150 rpm.

3.2.1.1.2 *Pseudomonas spp.*

The growth medium was inoculated with 10^{12} cell/ml approximately. *Pseudomonas spp.* was grown in preculture medium for 18 hours. Preculture medium was NB and it was inoculated with one loopfull of bacterial cells transferred from the solid medium. Preculture was cultivated at 30°C and 155 rpm.

3.2.1.1.3 *Bacillus Megaterium (M-3)*

The growth medium was inoculated with 10^{6} cell/ml approximately. M-3 was grown in preculture medium for 18 hours. Preculture medium was NB and it was inoculated with one loopfull of bacterial cells transferred from the solid medium. M-3 precultures were cultivated at 30°C and 155 rpm.

3.2.1.1.4 *Bacillus spp.(BA-140)*

The growth medium was inoculated with 10^{8} cell/ml approximately. BA-140 was grown in preculture medium for 24 hours. Preculture medium was NB and it was inoculated with one loopfull of bacterial cells transferred from the solid medium. BA-140 precultures were cultivated at 30°C and 155 rpm

3.2.1.2 Growth Conditions

Growth was carried out in 250 ml flasks containing 100 ml medium at specified temperature, specified rpm, at a rotary shaker. Used rpm values and optimum growth temperatures are given in Table 3.4.

Table 3.4. Growth Temperatures and Rpm Values

Bacterium	Temperature	Rpm
A. chroococcum	35	150
Pseudomonas spp.	30	155
M-3	30	155
BA-140	30	155

3.2.2 Growth Measurement

Growth curve for each bacterium was obtained by OD measurement during 24 hours after inoculation. OD measurement was performed by Shimadzu Spectrophotometer at 600 nm. Sterile distilled water was used as blank.

Growth was quantified with aerobic plate count method. The samples taken from the growth medium were diluted to appropriate concentrations. Sterile distilled water was used as the dilution medium. Diluted cell suspensions (25µl) were inoculated over the solid NA. Finally plates were incubated at optimum temperatures of each bacterium for 24 hours then the colonies were counted with colony counter device.

3.2.3 Phosphate Solubilization Experiments

A novel defined microbiological growth medium National Botanical Research Institute's phosphate growth medium (NBRIP) was used for screening phosphate solubilizing microorganisms. Its composition is given in Table 3.5. The pH of the medium was adjusted to 7.0 before autoclaving.

Table 3.5. National Botanical Research Institute's Phosphate Growth Medium (NBRIP)

Component	Concentration (g/l)
Glucose	10
$Ca_3(PO)_2$	5
$MgCl_2.6H_2O$	5
$MgSO_4.7H_2O$	0.25
KCl	0.2
$(NH_4)_2SO_4$	0.1

3.2.3.1 Qualitative Analysis

National Botanical Research Institute's Phosphate Growth Medium (NBRIP) media was supplemented with 1.5% Agar-agar. Three strains per plate were stabbed in triplicate using sterile toothpicks. The colony and halo diameters were measured after 15 days of the incubation of plates at 30ºC. Calculation of halo size was performed by subtracting the colony diameter from the total diameter.

Phosphate solubilization index = total diameter (colony + halo zone) / colony diameter

3.2.3.2 Quantitative Analysis

Quantitative estimation of phosphate solubilization in broth was carried out using 250 ml Erlenmeyer flasks containing 50 ml of medium inoculated in triplicate with the bacterial strains (0.5 mL inoculums) in the early stationary phase. Autoclaved uninoculated medium served as control. The flasks were incubated for 10 days at 30ºC on a shaker incubator at 200 rpm.

Growth mediums were centrifuged at 10 000 rpm for 10 minutes. Phosphate solubilization capacity of bacterium was quantified by measuring soluble phosphate amount in the supernatant by Fiske-Subbarow Method (*Fiske and Subbarow, 1925*).

The reagents used in Fiske-Subbarow Method are given in Appendix A and the standard curves preparation and procedure of this method is described in Appendix B. Standards were prepared with KH_2PO_4. Absorbances of the samples were read at 660 nm by taking the blank as the uninoculated medium itself.

3.2.3.3 Determination of pH

Change in pH was determined by measuring the pH of each Erlenmeyer flask containing 50 ml of medium inoculated with the bacterial strains (0.5 mL preculture) in early stationary phase. The flasks were incubated for 10 days at 30ºC in a shaker at 200 rpm and pH of each flask was measured on the even days.

3.2.4 Pot Experiments

3.2.4.1 Pot Preparation

Soil without a plant on its surface was obtained. It was dried for 15 days in greenhouse and then sieved. 2-3 kg of soil was put in each 48 pots.

3.2.4.2 Potting

Barley (10 seeds), Chickpea (10 seeds) and Barley-Chickpea (5+5 seeds) were seeded in every 16 pots. A handful of soil was taken from the pots. Seeds were put in an order and the handful of soil was put back and made flat with hand. 15-20 ml water was added in each pot and the pots were covered with newspaper in order to prevent moisture loss and light. Picture of the pots were given in Appendix H. Number of seeds were decreased to 3 and 5 for chickpea and barley respectively after one week to make each seed get enough nutrient from the soil.

3.2.4.3 Inoculation

Inoculations of plants with microbes were performed after 3 weeks. All four strains (BA-140, M-3, *Pseudomonas spp*, and *Azotobacter chroococcum*) were used in these experiments. Their precultures were grown in NB. They were applied to plants in their early stationary phase. 3 different applications were tested with four parallels in this study.

- Bacillus mixture (50% BA-140 and 50% M-3)
- *Pseudomonas spp*.
- *Pseudomonas spp*. and *Azotobacter chroococcum* mixture (%50/50)

4 pots were not inoculated for each kind of plant as controls. Pots were watered with 10 ml mixture (9 ml water and 1 ml precultures). Precultures plate counts are given in Table 3.6

Table 3.6. Precultures Used in Pot Experiments

Organism	**Cfu/ml**
Azotobacter Chroococcum	1.6E+08
BA-140	1.2E+08
M-3	4.0E+06
Pseudomonas spp.	1.2E+12

3.2.4.4 Leaf Analysis

Green and middle aged (not too old not too young) leaves were cut from each pot. They were sliced in to small pieces. 0.25 g of the leaf pieces was homogenized after they were added in 10 ml 0.1 N Sodium Acetate buffer. Buffer must be at refrigeration temperature (+4 ºC). Then homogenized samples were centrifuged at 15 000 g at 4 ºC for 10 minutes. 2 ml of aliquot was taken and 2 ml distilled water was added. Finally, acid-phosphatase activity was determined by the method given in Appendix E.

3.2.4.5 Microbiological Analysis of Soil

Soil on the root was used for microbiological analysis. It was conserved at refrigeration temperature until the analysis. Falcon tubes containing 9 ml distilled water was autoclaved for 15 minutes at 121 ºC. Then 1 g of soil samples from each pot was added to 9 ml water. It was vortexed for 1 minute. Finally they were diluted enough and 25 µL of each samples were spread on NA. Kept at 30 ºC for 2 days and colonies were counted (*Jones, 2000*).

3.2.5 Waste Beer Experiments

3.2.5.1 Waste Beer Analysis

Some characteristics of waste beer were determined prior to experiments. Methods for the characteristics determinations are given in Table 3.7.

Table 3.7. Methods for Determining the Characteristics of Waste Beer

Characteristic	Method	References
Protein	Bradford's Protein Assay	*Bradford, 1976*
Sugar	Nelson Smogyi's Method	*Nelson,1944,Somogyi,1952*
Phosphate	Fiske-Subbarow Method	*Fiske and Subbarow, 1925*
Ethanol	HPLC	

The reagents used in Bradford's Protein Assay are given in Appendix C and the standard curves preparation and procedure of this method is described in Appendix D.

3.2.5.2 Growth Experiments

Pseudomonas spp. and *Azotobacter chroococcum* were grown in 50 ml waste beer and water mixtures (%100, %70, %50, and % 30) as given in Table 3.8 for 10 days. The pH of each medium was adjusted to 7.0 before autoclaving. Their growths were observed by plate count on NA.

The pH of each medium was recorded during 10 days. The concentration of ethanol was measured before cultivation and after 10 day of incubation. Soluble phosphate amounts were also recorded before cultivation and after 10 day of incubation for *Pseudomonas spp*. by means of Fiske-Subbarow method.

Table 3.8. Growth Medium Concentrations for Waste Beer Experiments

Percent Beer	Beer(ml)	Water(ml)
%30	15	35
%50	25	25
%70	35	15
%100	50	0

3.2.5.3 Suspended Solids Content Measurement

Suspended solid content of waste beer was determined according to the procedures given in Standard Methods for the Examination of Water and Wastewater. Known volume of waste beer was filtered through rough filter paper of which dry weight was determined before. The residue retained on the filter paper was dried to a constant weight at 103-105 ºC. The increase in dry weight of the filter paper gives us the suspended solid content in the known volume (*Greenberg et al, 1989*).

3.2.5.4 Dissolved Solids Content Measurement

Dissolved solid content of waste beer was also determined according to the procedures given in Standard Methods for the Examination of Water and

Wastewater. Known volume of waste beer was filtered through filter paper, and the filtrate was dried in a dish; which was also dried before; in the ash oven at 180 ºC. The increase in the dry weight of the dish gives us the dissolved solid content in the known volume (*Greenberg et al, 1989*).

3.2.6 Peat Experiments

3.2.6.1 Preparation

15 grams of peat was weighed in 100 ml flasks. Mouths of the flasks were closed by cotton covered by aluminum foil to prevent the moisture loss. Flasks were steam sterilized at 121ºC for 1 hour.

3.2.6.2 Inoculation of *Azotobacter chroococcum*

Azotobacter chroococcum in the early stationary phase was inoculated to peat after 17 hours incubation of preculture. 100 ml preculture which was approximately 10^7 cfu/ml was poured into the 300 ml of 1/5 diluted NB. Then 5 ml of this mixture was inoculated to each flask. Only cottons were used to close the openings of flasks. Then flasks were vortexed for 1 minute.

3.2.6.3 Inoculation of *Pseudomonas spp.*

Pseudomonas spp. in the early stationary phase was inoculated to peat after 18 hours incubation of preculture. 100 ml preculture which was approximately 10^{12} cfu/ml was poured into the 300 ml of 1/5 diluted NB. Then 5 ml of this mixture was inoculated to each flask. Only cottons were used to close the openings of flasks. Then flasks were vortexed for 1 minute.

3.2.6.4 Incubation

Flasks of both species were put in to the incubator at 20 ºC and 30 ºC. Third set of experiment was incubated at refrigeration temperature. Water was put in the incubators to prevent the moisture loss.

3.2.6.5 Plate Count

Tree flasks were used as parallels. 40 ml of sterile distilled water was put in to each flask. Then they were shaken in the shaker for 30 minutes. 1 ml sample was taken from each flask and diluted to appropriate dilution. Then 25 µl was poured on the NA and spread over. *Azotobacter chroococcum* was incubated at 35 ºC and *Pseudomonas spp*. was incubated at 30 ºC overnight. Finally, the colonies were counted by means of colony counter device.

3.2.6.6 Moisture Loss Detection

Samples from each flask were dried in the oven at 100 ºC. Moisture of samples was calculated by subtracting the weight of dried peat from that of initial wet peat. Moisture contents at the beginning of the experiment were compared with the moisture contents at the end of the experiment.

CHAPTER 4

RESULTS AND DISCUSSION

Biofertilizers are products containing living cells of different types of microorganisms, which have an ability to convert nutritionally important elements from unavailable to available form through biological processes. Biofertilizers have emerged as an important component of the integrated nutrient supply system and hold a great promise to improve crop yields through environmentally better nutrient supplies. However, the application of microbial biofertilizers in practice, somehow, has not achieved constant effects. The mechanisms and interactions among these microbes still are not well understood, especially in real applications. In this study, mainly 2 potential biofertilizers were studied. *Pseudomonas spp.* was examined on NBRIP agar whether it can solubilize phosphate. Then all biofertilizers were examined by greenhouse trial if a positive effect could be seen.

As a preliminary study to further pot, survival on peat, and waste beer experiments, four bacteria were grown in NB to obtain their growth curves. Growth curve data were recorded until the microorganisms reached the stationary phase because all of the experiments were done in the early stationary phase. Growth was measured as optical density of the medium. This method was chosen for the growth curve determination because it does not take too much time and also it is simple.

Three different mixtures of *Pseudomonas spp.*, BA-140, M-3 and *Azotobacter chroococcum* as given in Section 3.2.4.3 were applied to soil of chickpea, barley and mixed potting of them as 4 parallels. Each application had 4 control pots

without any biofertilizer. Unsterile soil was used in this experiment. Experiment was performed in a greenhouse without a temperature and moisture controller. Leaf samples which were green and fresh were taken from each pot and analyzed for their acid-phosphatase activities to understand whether there was a P shortage or not.

Waste beer and survival observation in peat experiments were continued with only *Pseudomonas spp.* and *Azotobacter chroococcum* because they are nonspore-forming bacteria and *Pseudomonas spp*. is the one among them that can solubilize phosphate. Growth and survival of the bacteria were quantified as viable counts by plate counting. Although this method takes longer time, other two methods - counting cells with Thoma Chamber and Optical Density Measurement – were not appropriate. While counting with the Thoma Chamber both the viable and death cells are counted and some dilutions of brewery waste (%50, %70, %100) contains high number of death cells which cause errors during data collection. Also measurement of optical density is not appropriate due to formation of dark color and precipitate after steam sterilization of the waste beer at 121 ºC for 15 minutes.

4.1 Growth Curves

Growth profile of M-3 in NB which was loopfully inoculated and incubated for 24 hours in a rotary shaker at 30ºC and 155 rpm is shown in Figure 4.1. M-3 reached the exponential phase very late (12^{th} hour) because of being a spore-forming bacteria and reached the stationary phase after 20 hours.

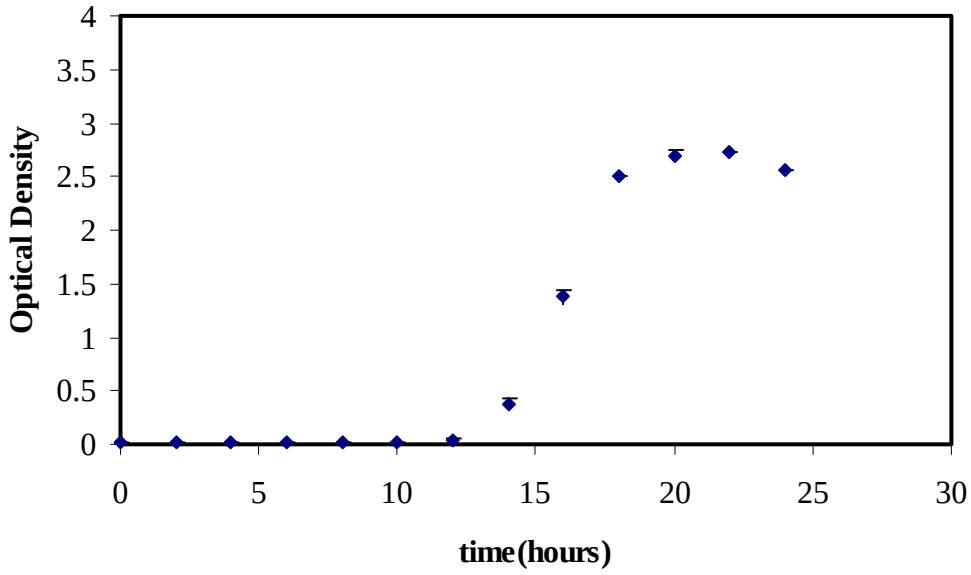

Figure 4.1 Growth profile of M-3 in NB

Growth profile of BA-140 in NB which was loopfully inoculated and incubated for 24 hours in a rotary shaker at 30ºC and 155 rpm is given in Figure 4.2. BA-140 reached the exponential phase at 8th hour and stationary phase after 24 hours as shown in Figure 4.2.

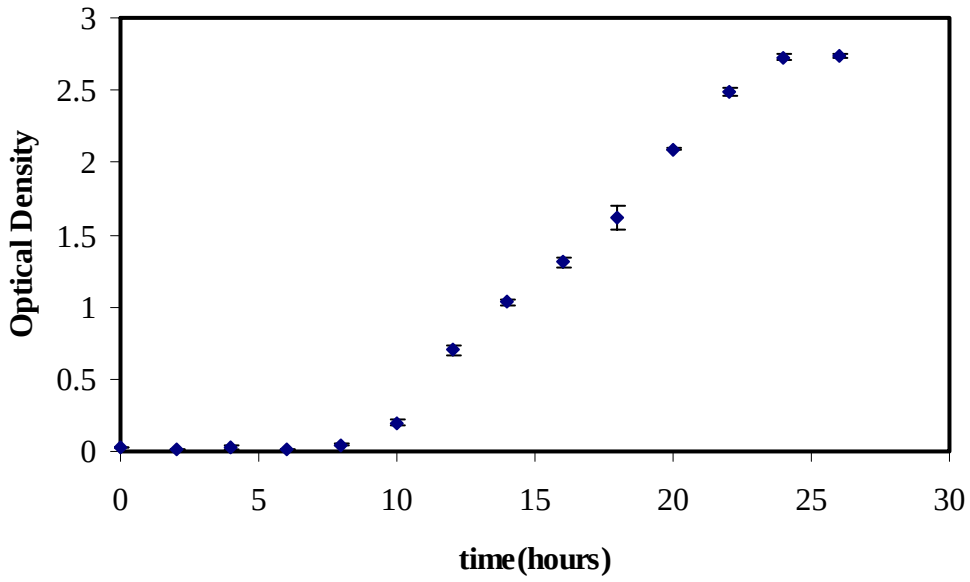

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Figure 4.2 Growth profile of BA-140 in NB

Pseudomonas spp. was also grown in NB at 30 ºC in a shaker of rpm 155. *Pseudomonas spp*. reached the exponential phase too early compared with the M-3 and BA-140. As seen in Figure 4.3 *Pseudomonas spp*. reached the stationary phase at 18th hour.

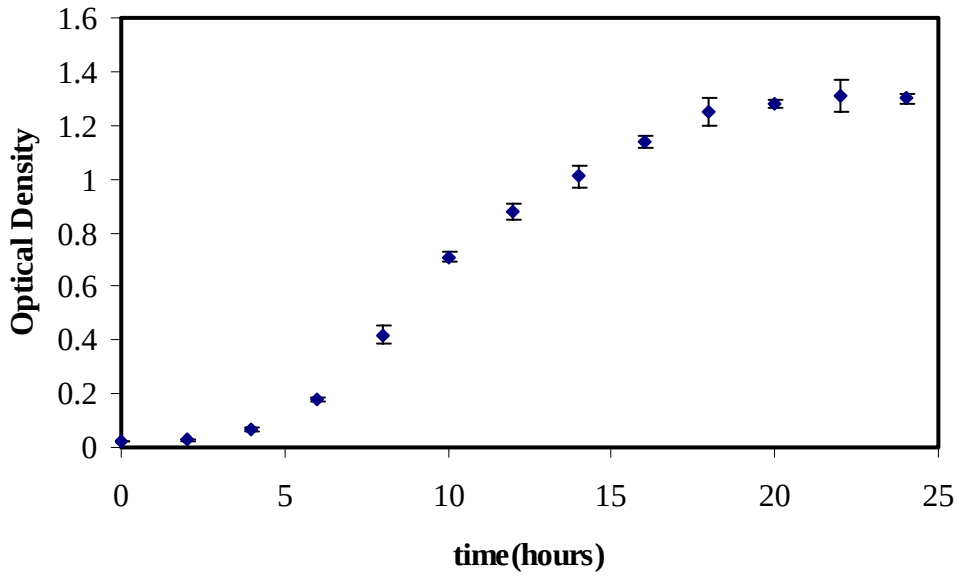

Figure 4.3 Growth profile of *Pseudomonas spp*. in NB

Azotobacter chroococcum was also grown in NB at 35 ºC in a shaker of rpm 150. *Azotobacter chroococcum* also reached the exponential phase earlier compared with the M-3 and BA-140. As seen in Figure 4.4 *Azotobacter chroococcum* reached the stationary phase at 17th hour.

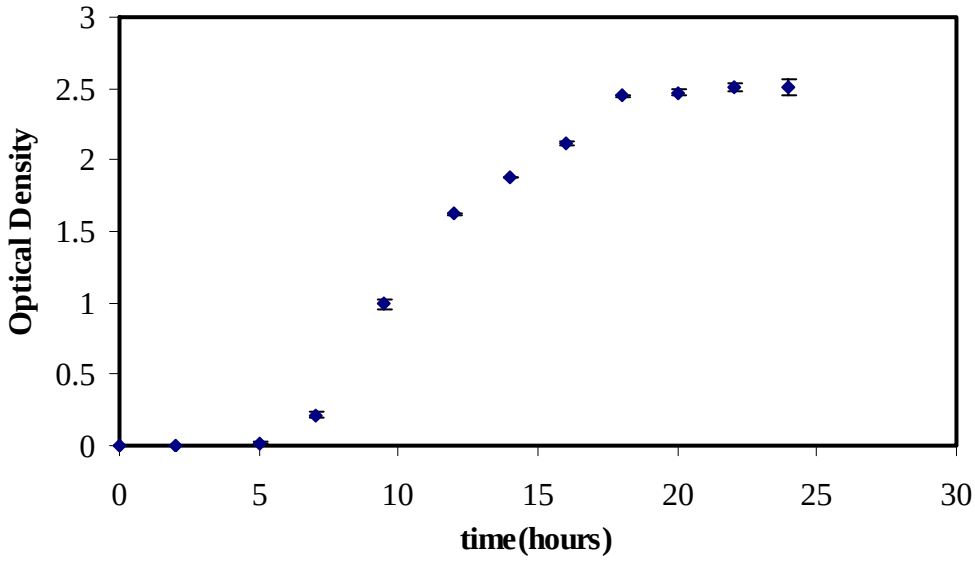

Figure 4.4 Growth profile of *Azotobacter chroococcum* in NB

4.2 Phosphate Solubilization Experiments

4.2.1 Solubilizing Index on Solid Media

For screening phosphate solubilization *Pseudomonas spp*., M-3 and BA-140 were grown on NBRIP agar for 15 days. Solubilization Index (SI) of *Pseudomonas spp*. was calculated as 1.86. In addition, a picture of a plate taken at the end of the experiment was given in Appendix H.

4.2.2 Phosphorus Solubilizing Capacity of PSM in Liquid Culture

Phosphorus solubilizing ability of *Pseudomonas spp*. was quantified at the end of 10 days by NBIRP broth instead of PKV broth because *Nautiyal (1999)* showed that phosphate solubilization abilities of *Pseudomonas spp*.1 and *Pseudomonas spp.* 2 were increased by about 30% and 12.5% respectively, in the absence of either yeast extract or $(NH_4)SO_4$. As shown in Figure 4.5 high amounts of insoluble phosphorus was solubilized by *Pseudomonas spp*. The amounts of phosphate solubilized by *Pseudomonas spp.* is % 0.44.

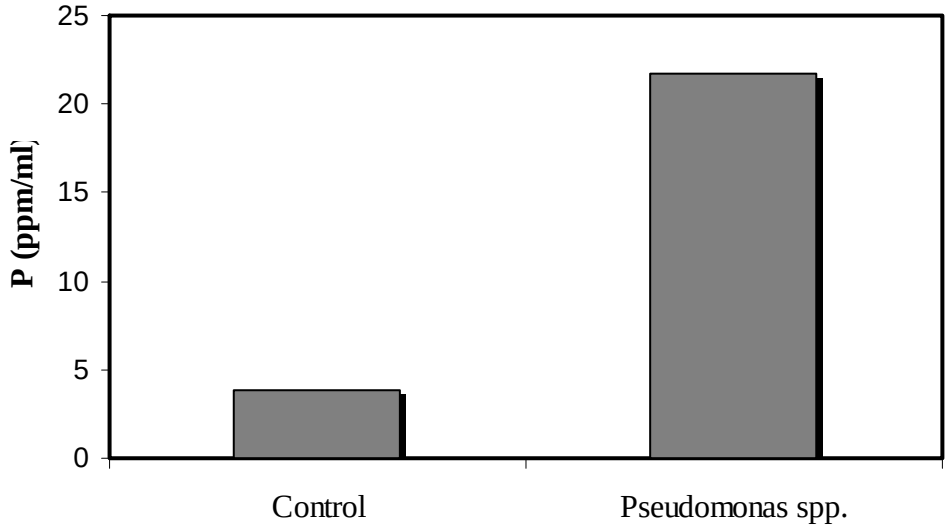

Figure 4.5 Phosphate Amount Solubilized by *Pseudomonas spp*.

4.2.3 Change of pH During Phosphate Solubilization

There may be several reasons that when phosphorus-solubilizing microbes are applied to the insoluble P, large amounts of unavailable P become available. The role of organic acid produced by PSM in solubilizing insoluble P may be due to the lowering of pH, chelation of cations and by competing with P for adsorption sites in the soil. It has also been investigated that organic acids may also form soluble complexes with metal ions associated with insoluble P (Ca, Al, Fe) and thus P is released (*Omar, 1998*). For pH determination 2 ml sample taken from the culture and pH was measured by pH meter during 10 days of incubation. pH of bacterial broth showed fluctuations as compared to control where it remained constant at 6.0. It can be said that most of the phosphate was solubilized in the first 3 days.

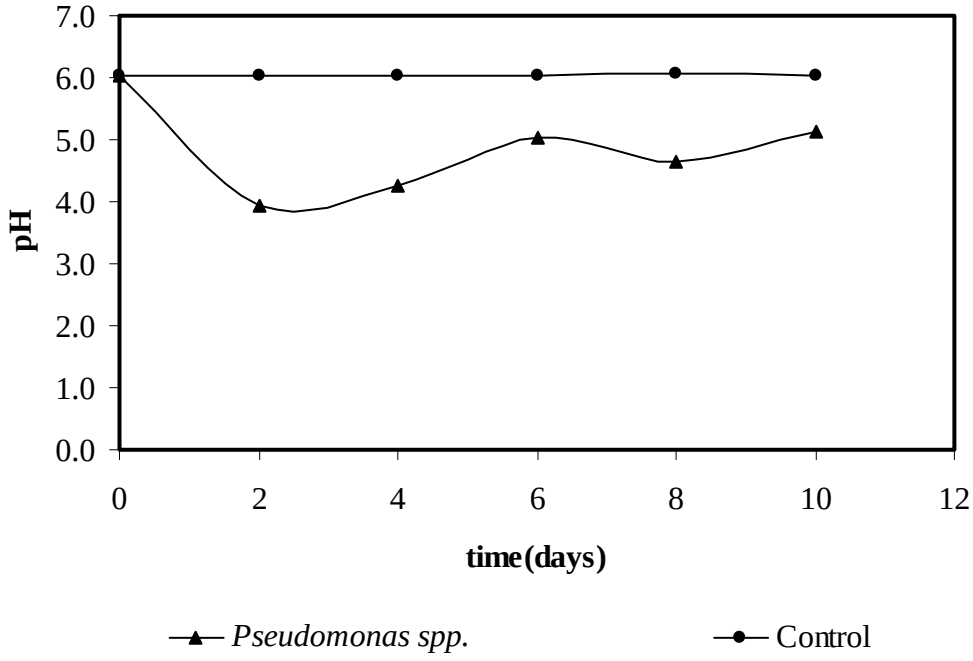

Figure 4.6 Change of pH for *Pseudomonas spp.* Against Control in NBRIP Broth

4.3 Survival Observation in Peat

Flasks containing *Pseudomonas spp.* and *Azotobacter chroococcum* were stored at 0, 20 and 30 ºC and the number of colony units were counted after one day of incubation and then data were taken for each treatment temperature. They were essentially kept away from light and also kept at constant temperature and moisture. To prevent the moisture loss a glass of distilled water was put in the incubator. The effects of storage temperature were evaluated. For growth and observation of survival, sterilized peat was preferred because *Hafeez et al (1989)* reported that the growth and survival of *Bradyrhizobia* during storage was much better in the sterilized carriers than in non-sterile carrier cultures. The advantage of a sterilized base for the preparation of inoculants is that it excludes the antagonistic effect of harmful microorganisms and ensures a more precise determination of the total number of viable target cells by means of plate counts than is possible with an unsterilized base.

4.3.1 Survival profile for *Pseudomonas spp.*

As shown in Figure 4.7 there is no significant change in the number of *Pseudomonas spp.* at 0 °C. Number of viable cells were conserved approximately at 10^9 cells/ml. Abrupt increases nor decreases were observed. Moisture of peat does not change significantly either. The favorable effect of low-temperature (0ºC) storage on the survival of *Pseudomonas spp.* may be due to maintenance of a suitable long-term moisture content. Further, at low temperatures bacterial cell division and activity slows down, resulting in a slower use of nutrients. *Pseudomonas spp.* stored at 0 ºC showed better survival than those stored at 20 and 30 ºC as shown in the following figures.

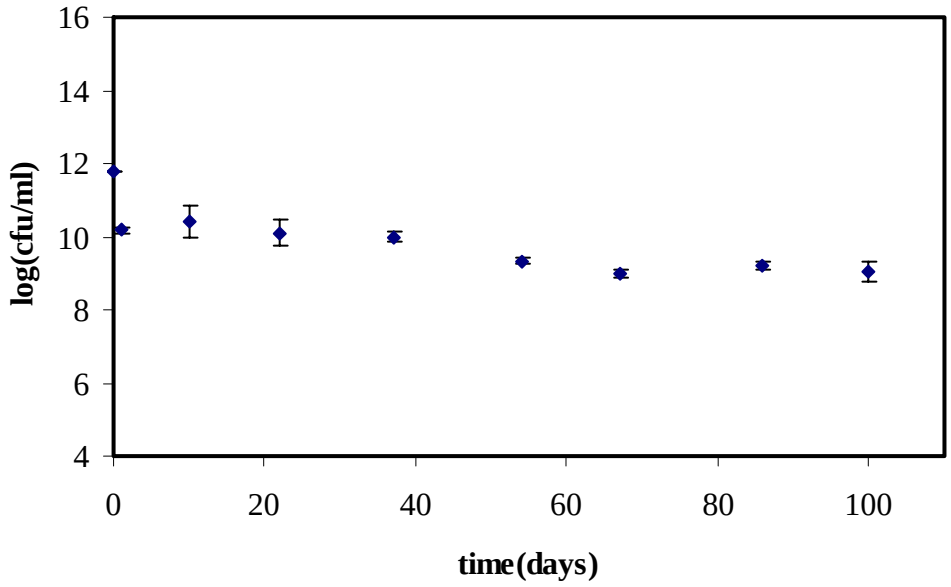

Figure 4.7 Survival curve for *Pseudomonas spp.* at 0ºC

It can be seen on Figure 4.8, *Pseudomonas spp.* could be conserved efficiently until the end of 2 months at 20 °C. Initial cell number of $1.6x10^{10}$ cells/ml increased abruptly in 1 month and then began to decrease and dropped to $2.2x10^7$ cells/ml at the end of 3 months. It can be said that storage at 20 ºC is not appropriate for long terms but it can be used for short term storages such as 2 months.

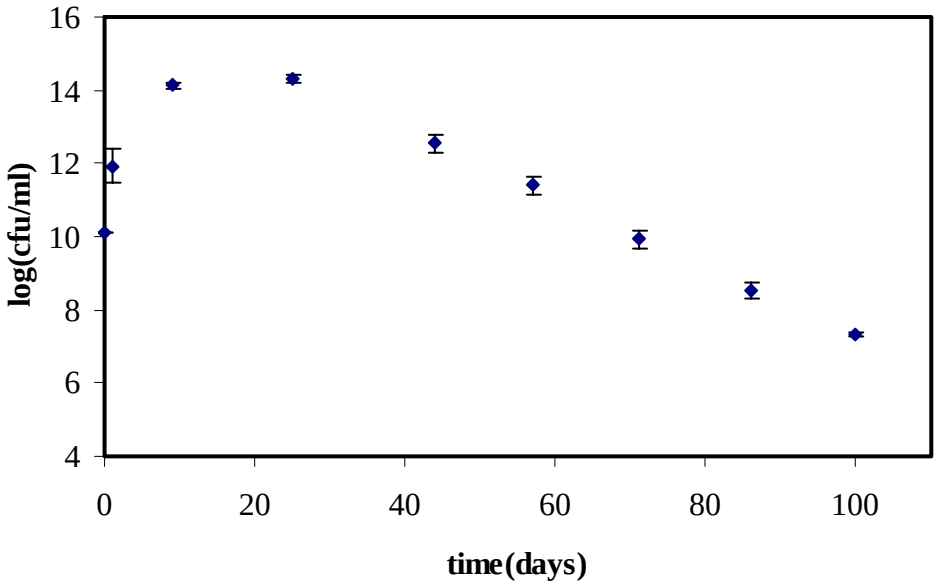

Figure 4.8 Survival curve for *Pseudomonas spp*. at 20 ºC

Like the storage at 20 ºC, 30 ºC storage caused *Pseudomonas spp.* to loose viability. As shown in Figure 4.9 initial cell concentration of $7x10^{11}$ cells/ml dropped to $1.68x10^{7}$ cells /ml after 3 months. Unlike the storage at 20 ºC, viable cells began to decrease at the end of first week. Therefore, storage at 30 ºC is not appropriate for short term storages either.

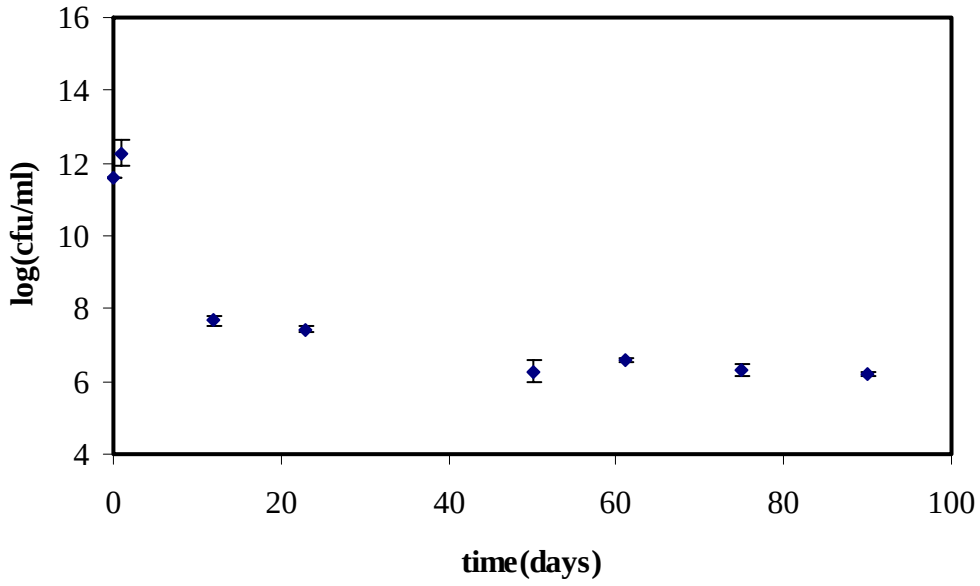

Figure 4.9 Survival curve for *Pseudomonas spp*. at 30 ºC

4.3.2 Survival profile for *Azotobacter chroococcum*

Azotobacter chroococcum stored at 0, 20 and 30 ºC showed similar survival profiles as shown in the following figures. As shown in Figure 4.10 there is no significant change in the number of *Azotobacter chroococcum* at 0 °C. Number of viable cells were conserved approximately at 10^6 cells/ml. Abrupt fluctuations were not observed. Moisture of peat does not change significantly either. Number of cells decreased to $7.2x10^5$ at the end of 3 months.

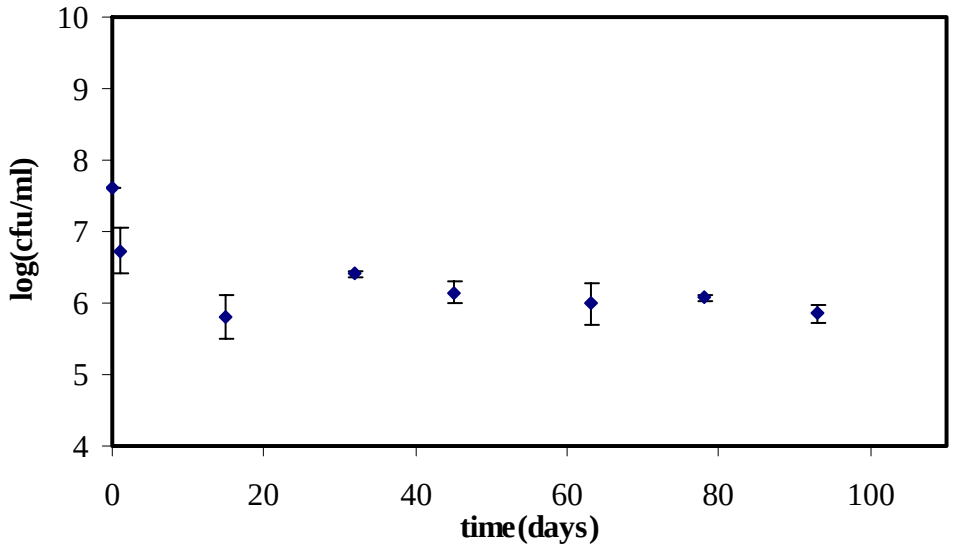

Figure 4.10 Survival curve for *Azotobacter chroococcum* at 0ºC

As shown in Figure 4.11 *Azotobacter chroococcum* was conserved efficiently at 20 ºC for 3 months. Initial cell load of $1x10^8$ was increased up to $6.4x10^9$ in the first 40 days then decreased to $1.2x10^9$ which is higher than the initial load.

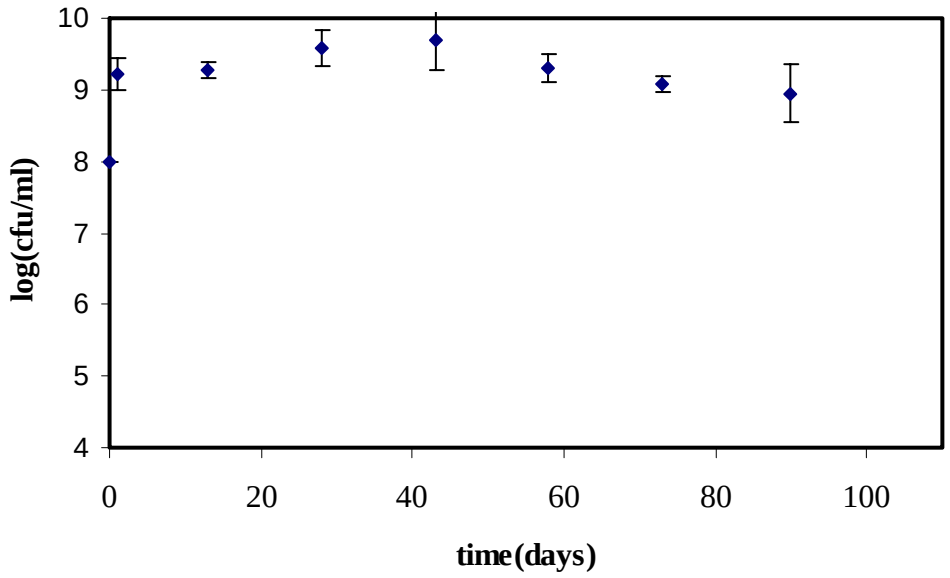

Figure 4.11. Survival of *Azotobacter chroococcum* at 20ºC

As seen in Figure 4.12 *Azotobacter chroococcum* was best survived in peat at 30 ºC. Initial load of $4x10^7$ was increased to $9.6x10^8$ after the first day and then fluctuations were observed. Final cell concentration was counted as $1.26x10^9$. As a conclusion, 30ºC is the best temperature value for conservation of *Azotobacter chroococcum* in peat for at least 3 months.

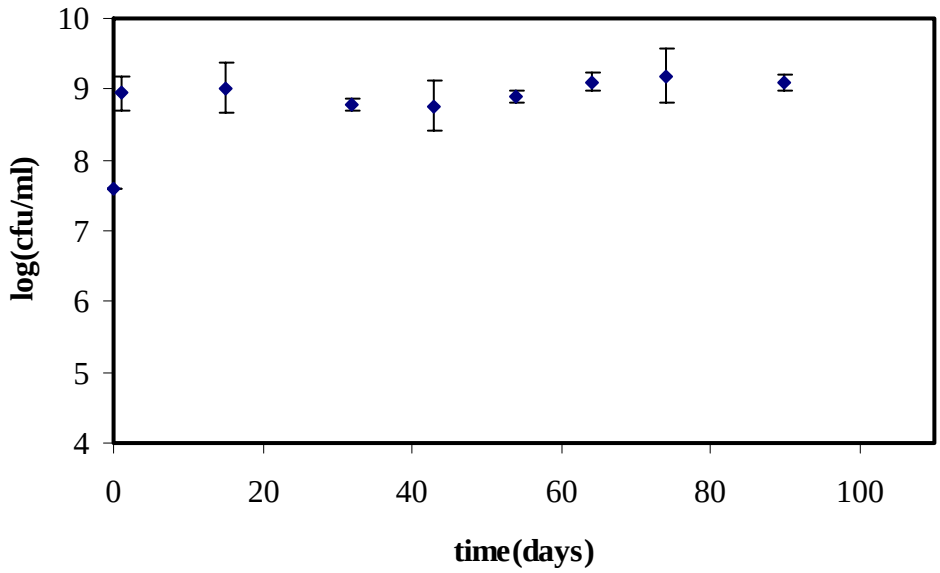

Figure 4.12. Survival of *Azotobacter chroococcum* at 30 ºC

4.3.3 Moisture Loss in Peat

Percent of Moisture of peat at 0ºC, 20 ºC and 30 ºC after 3 months of incubation compared with the initial percent of moisture. As seen in Figure 4.13 moisture loss increases as temperature increases. Abrupt decrease in number of *Pseudomonas spp*. at 30 ºC might be due to low moisture content. However, moisture loss did not affect *Azotobacter chroococcum* significantly.

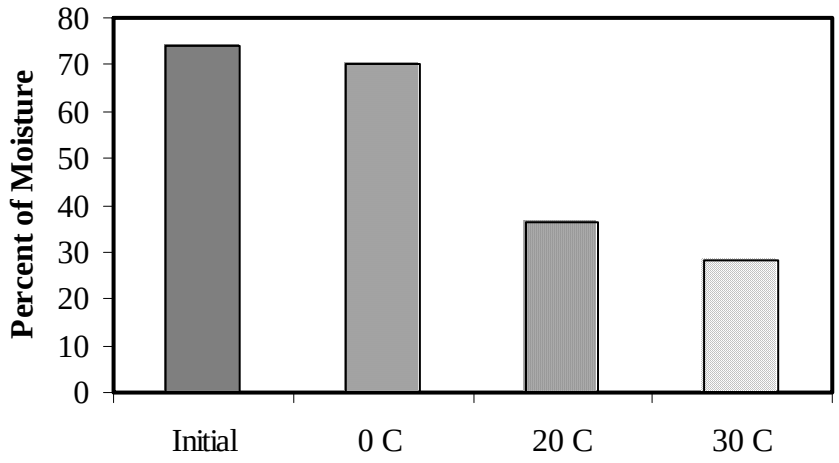

Figure 4.13 Percents of Moisture

4.4 Pot Experiments

After the application of biofertilizers APA was observed for 45 days. It was done to infer if the plant needed more phosphorus or not.

4.4.1 Chickpea

As given in Figure 4.14, APA was ranged between 35.69 and 60.12 Mmol/h.g in the first month and decreased to 10.25-21.73 Mmol/h.g range after 45 days for chickpea. However, this decrease was also observed in controls. Therefore, it cannot be said that inoculants are working in chickpea pots.

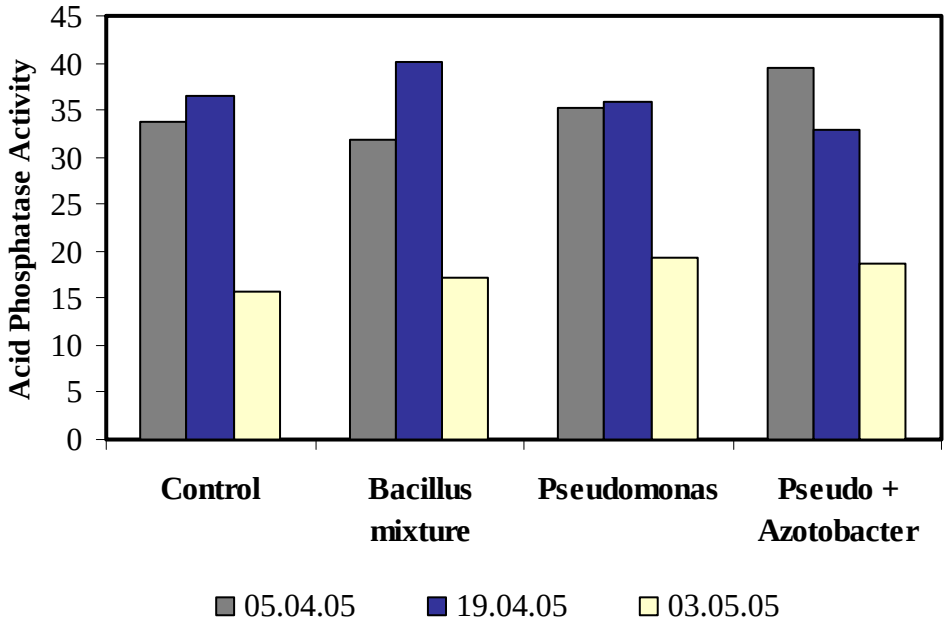

Figure 4.14 Acid Phosphatase Activities in Leaves for Chickpea

Pictures of the chickpea pots at the end of the experiment were given in Appendix H. As seen in Figure H.1 height of the chickpeas were significantly different from the control. If effects of inoculant applications on chickpea were compared according to height of plants then it can be said that, *Pseudomonas + Azotobacter chroococcum* mixture was better than *Pseudomonas* and *Bacillus* mixtures (BA-140 + M-3).

As seen in Figure 4.15 effect of inoculants on chickpea in barley+chickpea mixed potting was not significant either according to APA. Decrease in APA did not show any difference in inoculated pots compared with the control.

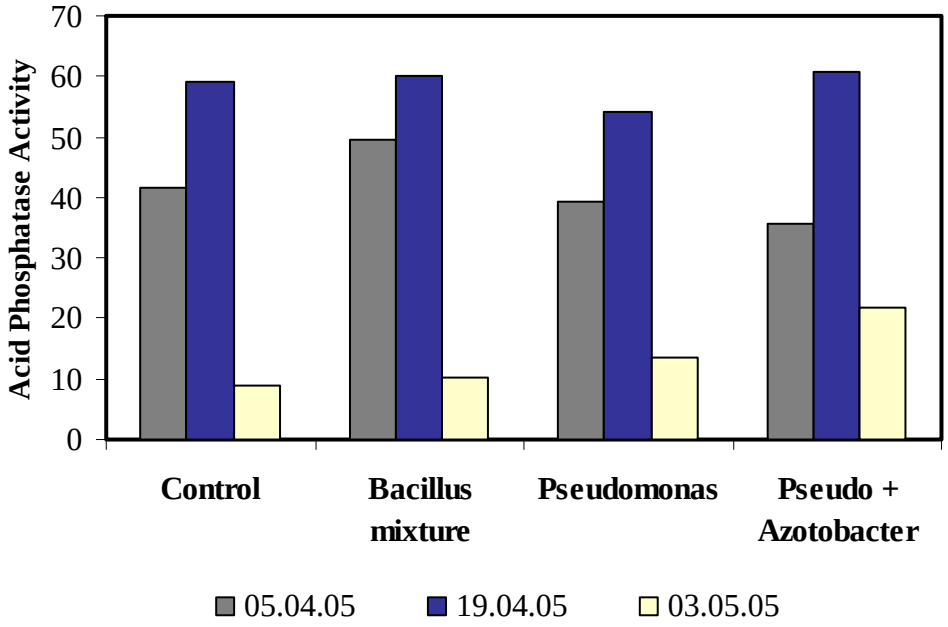

Figure 4.15 Acid Phosphatase Activities in Leaves of Chickpea in Mixed Potting

4.4.2 Barley

As given in Figure 4.16, APA was ranged between 29.31 and 38.28 Mmol/h.g in the first month and decreased to 5.80- 11.42 Mmol/h.g range after 45 days for barley. However, this abrupt decrease was also observed in control. Therefore, it cannot be said that inoculants are working in barley pots either.

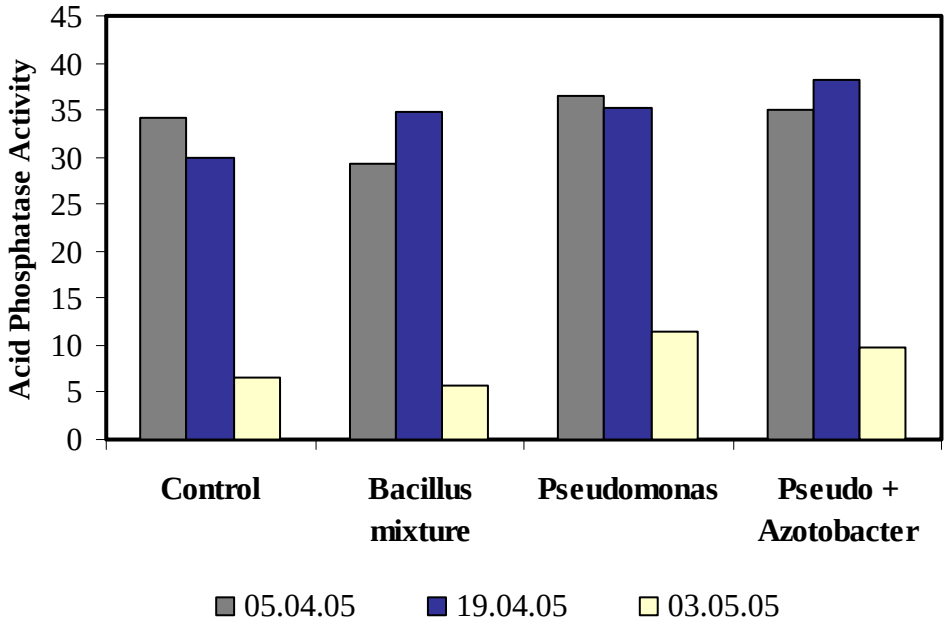

Figure 4.16 Acid Phosphatase Activities in Leaves of Barley

Pictures of the barley pots at the end of the experiment were given in Appendix H. As seen in Figure H.2 unlike the chickpea pots height of the inoculated barleys were shorter than the control. If effects of inoculant applications on barley were compared according to height of plants then it can be said that all inoculants had adverse affects on barley.

As seen in Figure 4.17, effect of inoculants on barley in barley + chickpea mixed potting was not significant either according to APA. Decrease in APA did not show any difference in inoculated pots compared with the control. As a conclusion, it can be said that effects of our inoculants could not be observed. This might be due to the use of unsterile soil in pots because the exact effect of the inoculants couldn't be observed due to unsterile conditions. As seen in Figure H.3 there is no significant difference between the height of control and the inoculated mixed pottings.

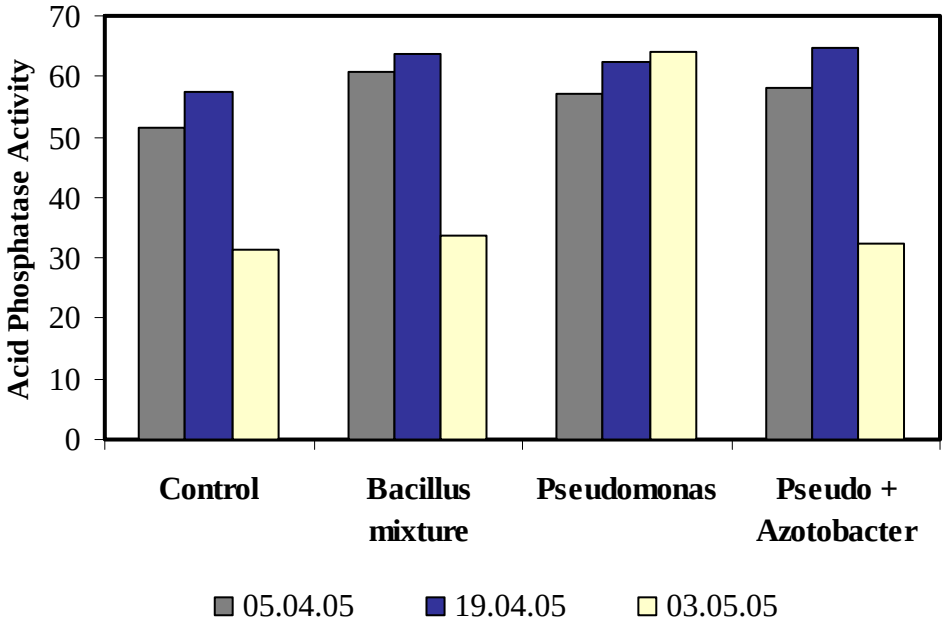

Figure 4.17 Acid Phosphatase Activities in Leaves of Barley in Mixed Potting

4.4.3 Total Plate Count for Soil

After harvesting of plants, rhizosphere soil was obtained from each pot and stored at refrigeration temperature for microbiological analysis. NB was used in order to count total viable cells in soil. As seen in Figure 4.18, total bacterial count was higher in *Pseudomonas* +*Azotobacter chroococcum* mixtures inoculated chickpea+barley mixed potting and barley. In addition, total bacterial count was also high for barley inoculated with Bacillus sp. Total bacterial count is very low for chickpea compared with the barley and barley + chickpea mixed potting. In addition, total bacteria reached high numbers in application of mixed cultures compared to control and *Pseudomonas spp.* application. Selective agar could not be used because of lack of enough information about the species we studied.

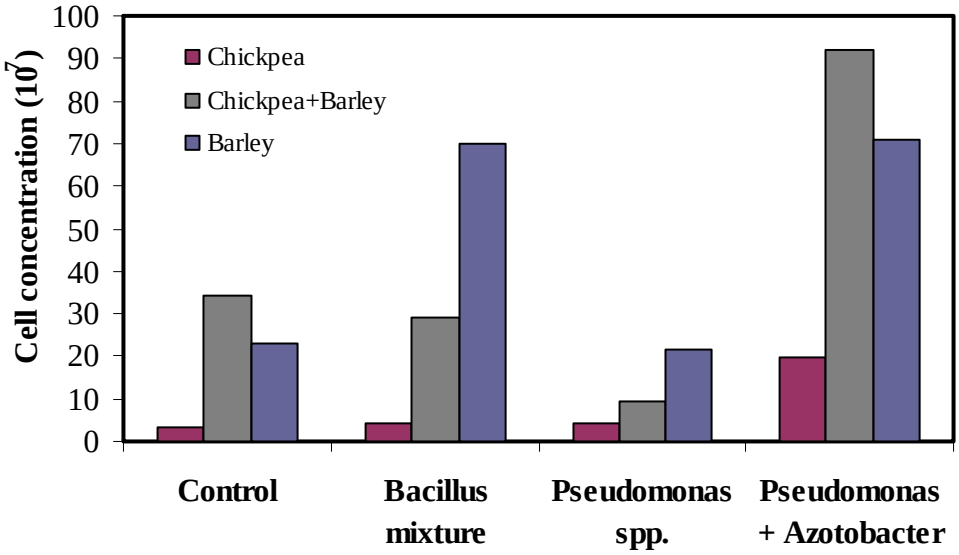

Figure 4.18. Total Plate Count Results for Pot Experiments

4.5 Waste Beer Experiments

Waste beer with concentrations of %30, %50, %70 and %100 were used as growth mediums for *Azotobacter chroococcum* and *Pseudomonas spp*. Their growths were observed for 10 days. *Azotobacter chroococcum* and *Pseudomonas spp.* could only survive in %30 waste beer. This might be due to decrease of ethanol concentration by means of dilution. No growth was observed in other dilutions for both bacteria.

As seen in Figure 4.19 *Pseudomonas spp.* reached the maximum number with 9.87 x 10^{10} cells/ml beginning from the initial cell concentration of 1.6 x 10^{10} at the 4^{th} day. In addition, no abrupt changes were observed in the pH of the waste beer medium. It was assumed that death of cells at 50%, 70% and 100% might be due to exposure of the cells to high ethanol concentrations. Therefore, initial and final ethanol concentrations of waste beer media were determined by HPLC method before and after the incubation as given in Figure 4.22.

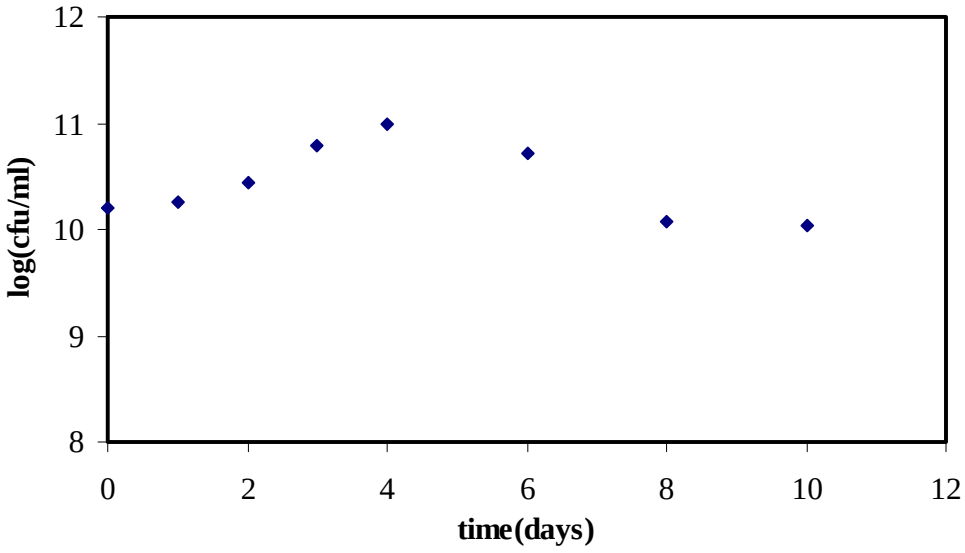

Figure 4.19 Growth Curve of *Pseudomonas spp*. in Waste Beer

Similar growth curve was obtained for 10 days incubation of *Azotobacter chroococcum* at 35 ºC. Initial cell number of 1.44 x 10^7 was increased to 2.13 x 10^9 after 4^{th} day of incubation. As seen in Figure 4.20 sharp increase and decrease were observed.

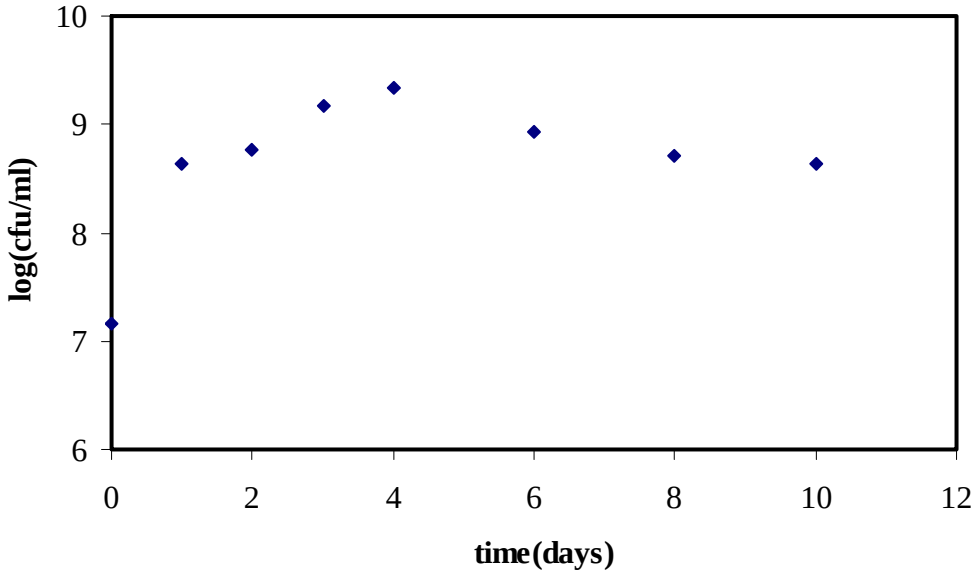

Figure 4.20 Growth Curve of *Azotobacter chroococcum* in Waste Beer

For all dilutions the pH of the waste beer was adjusted to 7.0 before steam sterilization. No significant pH difference was observed in 50%, 70% and 100 % waste beer mediums. The pH change of %30 waste beer media for both bacteria is given in Figure 4.21.

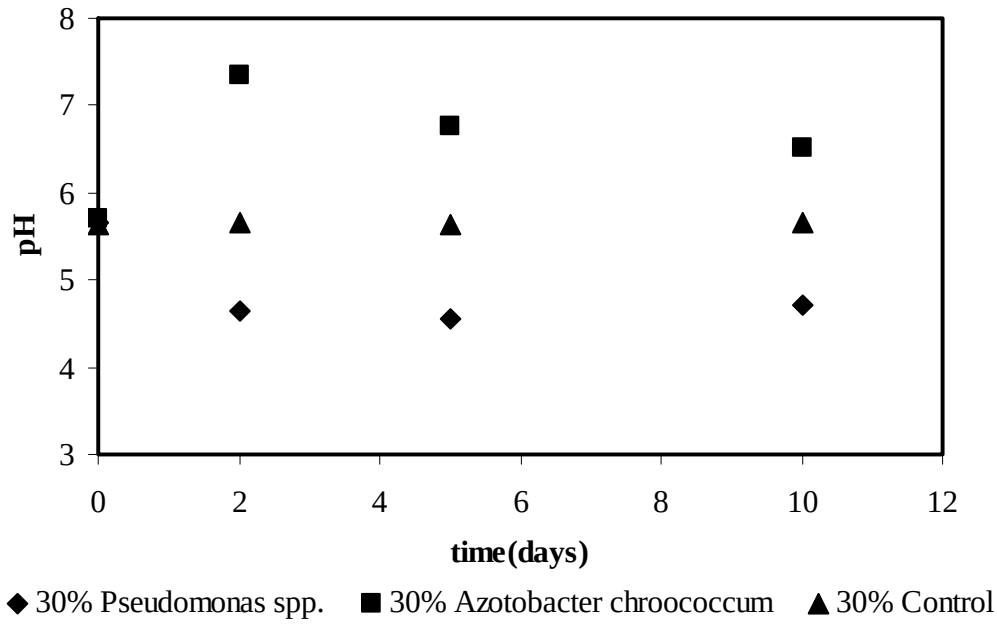

Figure 4.21 Change in pH of 30% Waste Beer

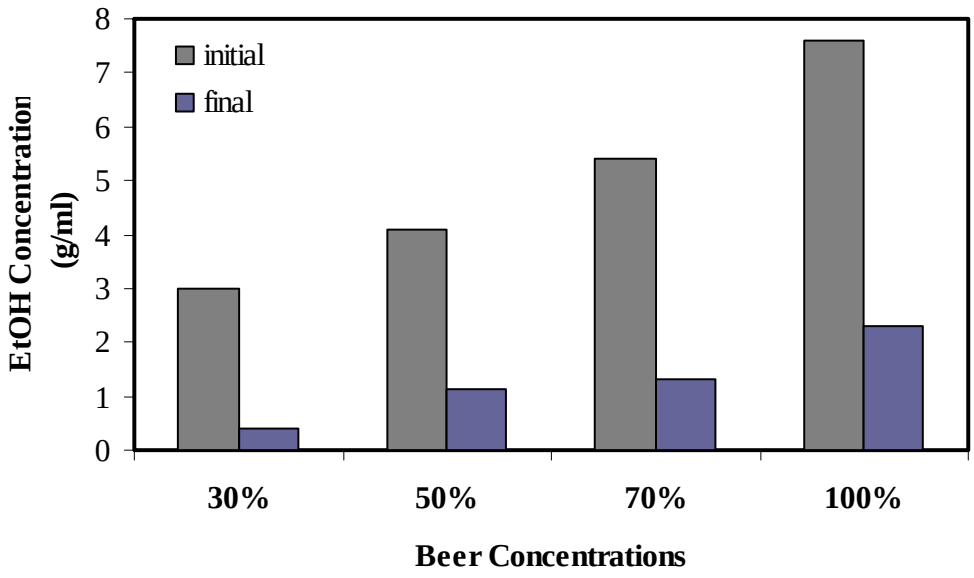

Figure 4.22 Initial and Final Ethanol Concentrations in Waste Beer

CHAPTER 5

CONCLUSIONS

In this study, several studies were done on biofertilizers, waste beer and peat. Waste beer was evaluated as a growth medium for agriculturally important bacteria such as *Pseudomonas spp*. and *Azotobacter chroococcum*. As a result, it was found that it might be valued by using as a growth medium for biofertilizers. However, pH adjustment might be a high cost factor for this process and also different and unwanted results may occur in unsterilized conditions.

Pseudomonas spp. was examined whether it can solubilize phosphate or not. First of all, qualitative and then quantitative analysis were performed and it was found that *Pseudomonas spp*. could solubilize phosphate in high percents. However, phosphate solubilizing ability as a biofertilizer could not be proved because acid-phosphatase activity of *Psudomonas spp.* inoculated and uninoculated control pots showed similarities.

Peat is a common carrier material used in the world. In this study, surviving abilities of *Azotobacter chroococcum* and *Pseudomonas spp*. were observed for 3 months and it was discovered that peat is more effective for low temperature storages and not appropriate for storage of *Pseudomonas spp.* at 30 ºC. As a further study, survival of bacteria may be examined in unsterile conditions. And they may be compared for the differences of survivals.

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APPENDIX A

REAGENTS OF FISKE-SUBBAROW METHOD

1. Acid Molybdate Stock Solution

Dissolve 75.25 g of ammonium molybdate, $(NH_4)_6Mo_7O_{24}.4H_2O$ in 500 ml of distilled water heated to 60 ºC.

Cool the solution and mix with 1500 ml HCl (sp.gr. 1.19, 37.5 percent).

Dilute the solution to 2000 ml with distilled water in a volumetric flask and store in a glass-stoppered brown bottle to which 100 g of boric acid (H_3BO_3) has been added.

2. Dry Reducing Agent: Aminonaphthol-Sulfonic Acid (ANSA)

Mix 5 g of 1-amino-2-napthol-4-sulfonic acid with 10 g of sodium sulfite (Na_2SO_3) and 292.5 g of sodium pyrosulfite ($Na_2S_2O_5$).

Grind the mixture to a fine powder.

If stored in a cool place in a sealed brown bottle, this reagent will keep for a year. Otherwise, discard after 6 months.

3. Dilute Reducing Agent

Dissolve 16 g of dry reducing agent in 100 ml of distilled water heated to 60 ºC.

Cool and store in brown bottle and make fresh every 3 weeks.

APPENDIX B

STANDARD CURVE PREPARATION TABLE AND STANDARD CURVE OF THE FISKE- SUBBAROW METHOD

Stock Standard Preparation

Dissolve 0.2197 g of oven-dried, reagent grade potassium dihydrogen phosphate (KH_2PO_4) in about 1000 mL of distilled water. (50 ppm)

Working Standard Solutions

Table B.1 Preparation of Working Standard with Stock Standard

Stock solution (µL)	Final volume (mL)	Concentration of working standard (ppm)
20	5	0.2
50	5	0.5
100	5	1.0
200	5	2.0
300	5	3.0
400	5	4.0
500	5	5.0
600	5	6.0

Procedure

- Transfer a 5 mL of aliquot to a test tube.
- Add 0.25 mL acid molybdate solution. Vortex then.
- Add 0.25 mL dilute-reducing agent.
- Allow color to develop 15 minutes before reading samples. Read optical density on the spectrophotometer set at 660 nm within 45 minutes after adding reducing agent.
- Determine the concentration in the medium using the standard curve (Figure B. 1)

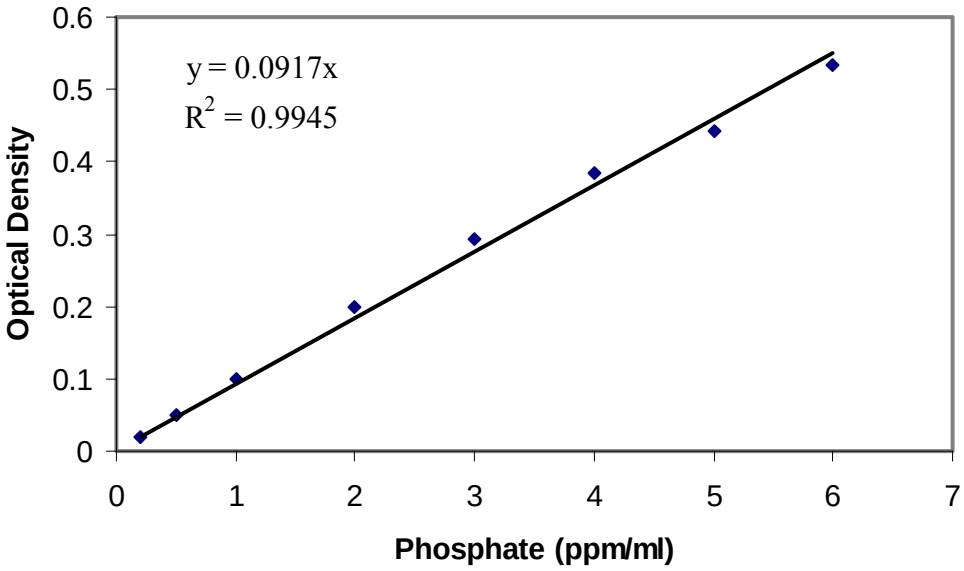

Figure B.1 Standard Curve for Fiske-Subbarow Method

APPENDIX C

BRADFORD'S DYE BINDING ASSAY

Preparation of Solutions:

A) Bradford's Reagent :

– 100 mg Coomassie Brilliant Blue G 250 (Eastman)
– 50 ml 95 % Ethanol
– 100 ml Phosphoric acid (85 % pure)

After the dye is completely dissolved, dilute the solution to 1 L with d H_2O. Filter through ordinary filter paper into a dark bottle (reagent is sensitive to light).

This solution can be kept at room temperature in tightly closed dark bottle up to 2 weeks.

B) BSA Protein Standard (1 mg/ml):

Weigh 104.17 mg of BSA (Sigma A2153) 96% and dissolve in 0.85 % NaCl and 0.1 sodium azide as preservative. This solution can be stored at +2 to +8 °C.

APPENDIX D

STANDARD CURVE PREPARATION TABLE AND STANDARD CURVE OF THE BRADFORD METHOD

Table D.1 Standard Curve Preparation with BSA

Tube #	**BSA Standard (μL)**	**Distilled Water (μL)**	**Bradford Solution (ml)**	**Protein Concentration (μL)**
1	-	500	5	0
2	5	495	5	0,01
3	10	490	5	0,02
4	15	485	5	0,03
5	20	480	5	0,04
6	25	475	5	0,05

** Bradford solution must be at room temperature before addition.

After addition of the Bradford's solution, samples were vortexed and mixed well,
Waited for 10 minutes,
Optical densities were measured at 595 nm within 1 hour.

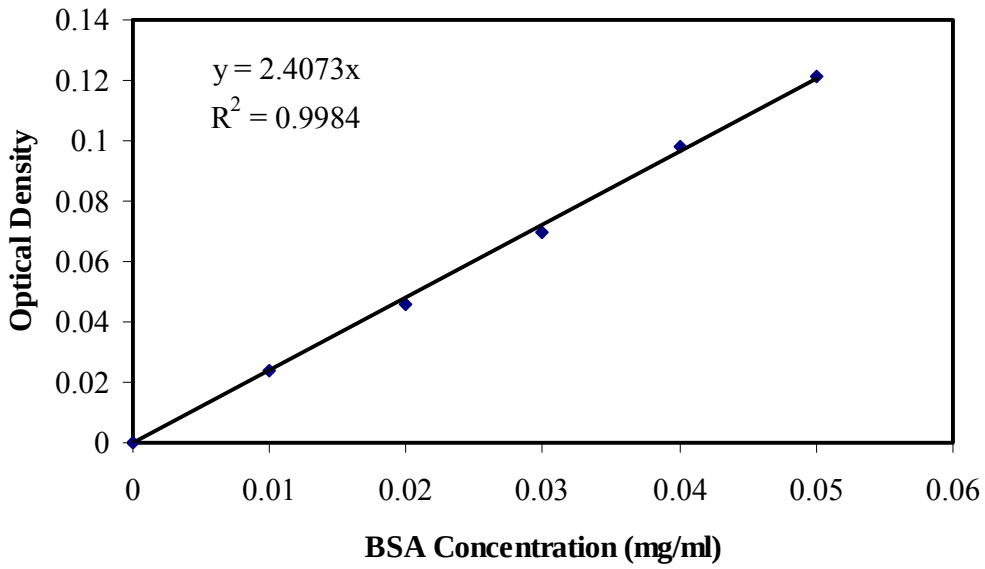

Figure D.1 Standard Curve for Bradford Protein Assay

APPENDIX E

ACID-PHOSPHATASE ANALYSIS

Reagents:

Sodium Acetate Buffer: 13.608 g Sodium Acetate (CH_3COONa) is dissolved in 1000 ml distilled water. (pH=5.5). Kept at refrigeration temperature.

P-Nitrophenol Phosphate Solution: 0.263 g P-Nitrophenol Phosphate is dissolved in 1000 ml distilled water.

Sodium Hydroxide Solution: 40 g Sodium Hydroxide (NaOH) is dissolved in 500 ml distilled water.

Procedure:

4 ml aliquot is mixed well with 0.2 mM p-nitrophenol.
Incubated in water bath at 35 ºC for 1 hour.
Add 2 ml 2N NaOH and mix well
Read absorbance at 405 nm after 4-5 minutes.

** Standard curve is prepared 0-10 mM p-nitrophenol ($C_6H_5NO_3$) solutions.

APPENDIX F

Figure F.1 Pot Experiment (Chickpea)

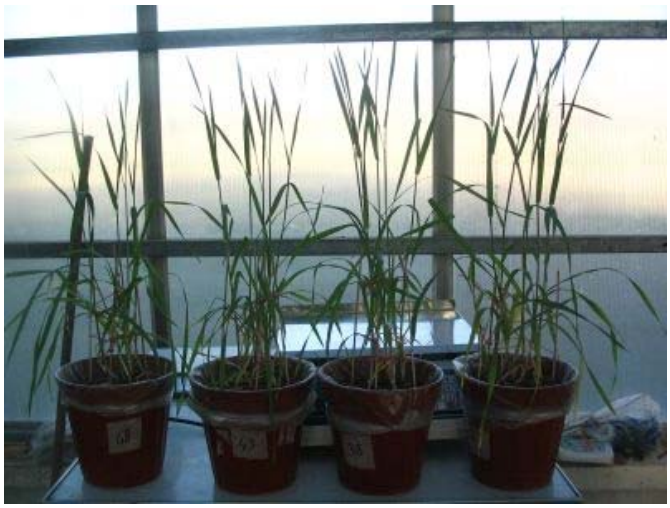

Figure F.2 Pot Experiment (Barley)

Figure F.3 Pot Experiment (Mixed Potting)

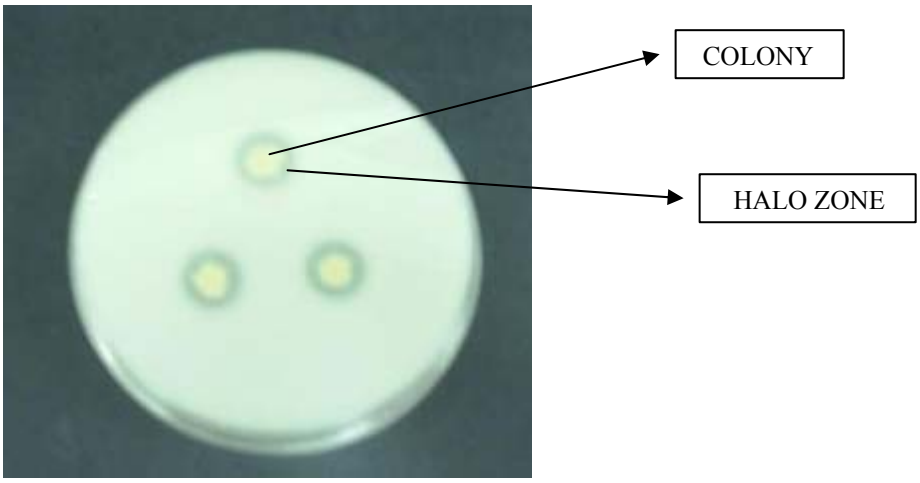

Figure F.4 Qualitative Analysis for *Pseudomonas spp*.

APPENDIX G

DATA OF THE FIGURES

Table G.1 Data of Figure 4.1

Time(hours)	Optical Density
0	0.012 ± 0.0012
2	0.013 ± 0.0065
4	0.013 ± 0.0015
6	0.013 ± 0.0006
8	0.014 ± 0.0012
10	0.016 ± 0.0021
12	0.045 ± 0.0060
14	0.380 ± 0.0481
16	1.380 ± 0.0625
18	2.510 ± 0.0006
20	2.700 ± 0.0481
22	2.720 ± 0.0161
24	2.560 ± 0.0100

Table G.2 Data of Figure 4.2

Time(hours)	Optical Density
0	0.024 ± 0.0000
2	0.020 ± 0.0000
4	0.026 ± 0.0116
6	0.020 ± 0.0000
8	0.043 ± 0.0058
10	0.200 ± 0.0208
12	0.700 ± 0.0346
14	1.030 ± 0.0200
16	1.310 ± 0.0360
18	1.620 ± 0.0866
20	2.090 ± 0.0058
22	2.490 ± 0.0231
24	2.730 ± 0.0200
26	2.740 ± 0.0153

Table G.3 Data of Figure 4.3

Time(hours)	Optical Density
0	0.026 ± 0.0000
2	0.027 ± 0.0015
4	0.066 ± 0.0065
6	0.180 ± 0.0058
8	0.420 ± 0.0306
10	0.710 ± 0.0208
12	0.880 ± 0.3055
14	1.010 ± 0.0416
16	1.140 ± 0.0208
18	1.250 ± 0.0503
20	1.280 ± 0.0153
22	1.310 ± 0.0577
24	1.300 ± 0.0208

Table G.4 Data of Figure 4.4

Time(hours)	Optical Density
0	0.001 ± 0.0000
2	0.002 ± 0.0007
5	0.018 ± 0.0043
7	0.213 ± 0.0200
9.5	0.990 ± 0.0350
12	1.620 ± 0.0014
14	1.880 ± 0.0052
16	2.120 ± 0.0120
18	2.450 ± 0.0052
20	2.470 ± 0.0230
22	2.510 ± 0.0026
24	2.510 ± 0.0600

Table G.5 Data of Figure 4.5

	Solubilized P Amount (ppm/ml)
Control	3.84 ± 0.0424
Pseudomonas spp	21.70 ± 0.2200

Table G.6 Data of Figure 4.6

Time(days)	pH for Control	pH for *Pseudomonas spp.*
0	6.03 ± 0.002	6.03 ± 0.006
2	6.02 ± 0.001	3.95 ± 0.020
4	6.04 ± 0.000	4.25 ± 0.046
6	6.03 ± 0.001	5.04 ± 0.101
8	6.05 ± 0.003	4.66 ± 0.025
10	6.04 ± 0.000	5.14 ± 0.076

Table G.7 Data of Figure 4.7

Time(days)	Log(cfu/ml)
0	11.78 ± 0.000
1	10.19 ± 0.078
10	10.41 ± 0.435
22	10.11 ± 0.374
37	9.98 ± 0.136
54	9.34 ± 0.073
67	8.98 ± 0.125
86	9.21 ± 0.097
100	9.05 ± 0.264

Table G.8 Data of Figure 4.8

Time(days)	Log(cfu/ml)
0	10.12 ± 0.000
1	11.92 ± 0.470
9	14.13 ± 0.092
25	14.31 ± 0.128
44	12.56 ± 0.239
57	11.39 ± 0.239
71	9.94 ± 0.241
86	8.52 ± 0.208
100	7.35 ± 0.056

Table G.9 Data of Figure 4.9

Time(days)	Log(cfu/ml)
0	11.58 ± 0.000
1	12.28 ± 0.355
12	7.67 ± 0.140
23	7.43 ± 0.079
50	6.28 ± 0.301
61	6.58 ± 0.055
75	6.30 ± 0.151
90	6.22 ± 0.057

Table G.10 Data of Figure 4.10

Time(days)	Log(cfu/ml)
0	7.60 ± 0.000
1	6.74 ± 0.314
15	5.81 ± 0.307
32	6.41 ± 0.042
45	6.14 ± 0.156
63	5.99 ± 0.291
78	6.07 ± 0.053
93	5.85 ± 0.130

Table G.11 Data of Figure 4.11

Time(days)	Log(cfu/ml)
0	8.00 ± 0.000
1	9.23 ± 0.223
13	9.27 ± 0.111
28	9.58 ± 0.256
43	9.70 ± 0.427
58	9.31 ± 0.191
73	9.09 ± 0.115
90	8.95 ± 0.396

Table G.12 Data of Figure 4.12

Time(days)	Log(cfu/ml)
0	7.60 ± 0.000
1	8.94 ± 0.241
15	9.01 ± 0.351
32	8.78 ± 0.089
43	8.77 ± 0.362
54	8.90 ± 0.094
64	9.11 ± 0.128
74	9.19 ± 0.379
90	9.09 ± 0.116

Table G.13 Data of Figure 4.13

Initial	0 ºC	20 ºC	30 ºC
74	69.95	36.43	28.04

Table G.14 Data of Figure 4.14

	05.04.05	19.04.05	03.05.05
Control	33.69	36.41	15.63
***Bacillus* mixture**	31.89	40.07	17.28
Pseudomonas spp.	35.18	35.88	19.41
Pseudomonas spp.* + *Azotobacter chroococcum	39.39	33.01	18.68

Table G.15 Data of Figure 4.15

	05.04.05	19.04.05	03.05.05
Control	59.96	57.39	31.49
***Bacillus* mixture**	60.67	63.83	30.28
Pseudomonas spp.	57.21	66.52	63.90
Pseudomonas spp.* + *Azotobacter chroococcum	68.78	64.70	32.29

Table G.16 Data of Figure 4.16

	05.04.05	**19.04.05**	**03.05.05**
Control	41.44	59.01	11.83
***Bacillus* mixture**	49.63	60.12	10.21
Pseudomonas spp.	46.41	54.04	13.65
Pseudomonas spp.* + *Azotobacter chroococcum	35.70	60.61	21.73

Table G.17 Data of Figure 4.17

	05.04.05	**19.04.05**	**03.05.05**
Control	34.26	29.92	6.62
***Bacillus* mixture**	29.31	34.91	5.8
Pseudomonas spp.	36.53	35.29	11.42
Pseudomonas spp.* + *Azotobacter chroococcum	34.95	38.28	9.77

Table G.18 Data of Figure 4.18

	Chickpea	**Chickpea+Barley**	**Barley**
Control	3.20E+07	3.42E+08	2.30E+08
***Bacillus* mixture**	4.19E+07	2.89E+08	7.01E+08
Pseudomonas spp.	4.12E+07	9.36E+07	2.16E+08
Pseudomonas spp.* + *Azotobacter chroococcum	1.97E+08	9.20E+08	7.11E+08

Table G.19 Data of Figure 4.19

Time(days)	Log(cfu/ml)
0	10.20 ± 0.000
1	10.26 ± 0.111
2	10.45 ± 0.385
3	10.79 ± 0.064
4	10.99 ± 0.332
6	10.72 ± 0.065
8	10.08 ± 0.180
10	10.03 ± 0.223

Table G.20 Data of Figure 4.20

Time(days)	Log(cfu/ml)
0	7.16 ± 0.000
1	8.64 ± 0.141
2	8.76 ± 0.341
3	9.16 ± 0.084
4	9.33 ± 0.362
6	8.94 ± 0.093
8	8.70 ± 0.108
10	8.63 ± 0.279

Table G.21 Data of Figure 4.21

days	pH for *A.chroococcum*	days	pH for *Pseudomonas spp.*
0	5.71	0	5.66
3	7.34	2	4.65
8	6.77	5	4.56
10	6.52	10	4.71

Table G.22 Data of Figure 4.22

	initial	final
30%	3	0.39
50%	4.1	1.12
70%	5.4	1.32
100%	7.6	2.31