GROWTH AND NITROGEN FIXATION DYNAMICS OF *AZOTOBACTER CHROOCOCCUM* IN NITROGEN-FREE AND OMW CONTAINING MEDIUM

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ABSTRACT

GROWTH AND NITROGEN FIXATION DYNAMICS OF *AZOTOBACTER CHROOCOCCUM* IN NITROGEN-FREE AND OMW CONTAINING MEDIUM

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Olive Mill Wastewater (OMW), by-product of oil industry, is a dark liquid with a characteristic fetid smell, bitter taste and bright appearance; having a high pollution potential, creating serious problems in countries producing olive oil. *Azotobacter chroococcum* as a Nitrogen-fixing bacteria can bioremediate OMW, by degrading its toxic constituents. With the help of this detoxification process OMW can be used as biofertilizer. In this study, the dynamics of growth and nitrogen fixation at different physiological conditions and nutrient requirements of *A. chroococcum* in chemically defined N-free medium was determined. These parameters were cultivation conditions such as pH, temperature and aeration and some additives such as inorganic salts, boric acid and nitrogen. Consequently, the

maximum cell concentration were obtained when *A. chroococcum* was grown at neutral pH, 35°C, 150 rpm and in medium supplemented with manganese salt at 0.01% concentration. The maximum nitrogen fixation products were attained when *A. chroococcum* was grown under the same conditions except at pH 8. Further, bioremediation of OMW by *A. chroococcum* was examined. When *A. chroococcum* was cultivated in OMW containing basal medium at 10% OMW concentration, a cell density 12 times higher than in the OMW free medium was achieved. Also, it was found to have maximum increase in extracellular protein concentration (112 mg/l) at 10% OMW containing medium and maximum increase in ammonia concentration (9.05 mg/l) at 5% OMW containing medium.

Key Words: *Azotobacter chroococcum*, Nitrogen Fixation, OMW, Bioremediation

ÖZ

AZOTOBACTER CHROOCOCCUM'UN AZOTSUZ VE ZEYTİN KARASUYU İÇEREN ORTAMDA BÜYÜME VE AZOT BAĞLAMA DİNAMİĞİ

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Zeytinyağı endüstrisinin atık suyu olan zeytin karasuyu, karakteristik bir pis kokusu olan, acı, parlak görünüşlü, koyu renk bir sıvıdır; kirlilik potansiyeline sahip olan bu sıvı, zeytinyağı üreten ülkelerde ciddi sorunlar ortaya çıkarmaktadır. *Azotobacter chroococcum* zeytin karasuyunu zehirli bileşenlerini parçalayarak biyolojik olarak işleyebilmektedir. Bu detoksifikasyon işlemi yardımıyla zeytin karasuyu biyolojik gübre olarak kullanılmaktadır. Bu çalışmada *A. chroococcum*'un kimyasal olarak belirlenmiş azotsuz besiyeri içerisindeki değişik fizyolojik koşullarında ve besin ihtiyacında büyüme ve azot bağlama dinamiği belirlenmiştir. Bu parametreler; büyüme koşulları olarak pH, sıcaklık, havalandırma ve bazı katkı maddeleri olarak anorganik tuzlar, borik asit

ve azottur. Sonuç olarak, en yüksek hücre konsantrasyonu *A. chroococcum* nötr pH, 35°C, 150 rpm ve %0.01 konsantrasyonda mangan tuzu ilave edilmiş besiyerinde elde edilmiştir. En yüksek azot bağlama ürünlerine de pH'ın 8 olması durumu hariç aynı koşullarda ulaşılmıştır. Daha sonra *A chroococcum*'un zeytin karasuyunu biyolojik olarak işlemesi incelenmiştir. *A chroococcum* zeytin karasuyu içeren temel besiyerinde geliştirildiğinde, %10 zeytin karasuyu içeren ortamda zeytin karasuyu içermeyen ortamda elde edilen hücre konsantrasyonunun 12 katına ulaşılmıştır. Ayrıca, en yüksek protein konsantrasyonu artışına (112 mg/l) %10 oranında zeytin karasuyu içeren besi ortamında, en yüksek amonyak konsantrasyonu artışına (9.05 mg/l) ise %5 oranında zeytin karasuyu içeren besi ortamında rastlanmıştır.

Anahtar Kelimeler: *Azotobacter chroococcum*, Azot Bağlama, Zeytin Karasuyu, Biyolojik İyileştirme

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CHAPTER 1

INTRODUCTION

1.1 Organism; *Azotobacter chroococcum*

In 1901, Dutch microbiologist M. W. Beijerinck, using an enrichment culture technique with a medium devoid of a combined nitrogen source, discovered an aerobic microorganism capable of fixing molecular nitrogen to which the name of *Azotobacter chroococcum* was given.

The genus *Azotobacter* comprises large, Gram-negative, primarily found in neutral to alkaline soils, obligately aerobic rods capable of fixing N_2 nonsymbiotically. *Azotobacter* is also of interest because it has the highest respiratory rate of any living organism. In addition to its ecological and physiological importance, *Azotobacter* is of interest because of its ability to form an unusual resting structure called a cyst. *Azotobacter* cells are rather large for bacteria, many isolates being almost the size of yeast, with diameter of 2-4µm or more. Pleomorphism is common and a variety of cell shapes and sizes have been described. Some strains are motile by peritrichous flagella. On carbohydrate containing media, extensive capsules or slime layers are produced by free-living N_2 fixing bacteria. *Azotobacter* is able to grow on a wide variety of carbohydrates, alcohols and organic acids. The metabolism of carbon compounds is strictly oxidative and acids or other fermentation products are rarely produced.

All members fix nitrogen, but growth also occurs on simple forms of combined nitrogen: ammonia, urea and nitrate (Brock *et al*., 1994). In their research Mishustin and Shilnikova (1969), list the characteristic sings of *A. chroococcum* as follows;

Size of cell (μ): 3.1x 2.0;

Forms cyst;

Motile, especially in young culture or if grown in ethanol;

With aging forms pigment, from dark brown to black, which does not diffuse into water;

Utilizes starch;

In some cases utilizes sodium benzoate;

Utilizes mannitol benzoate;

Utilizes rhamnose benzoate.

1.1.1 Effect of External Environmental Factors on the Growth of the Genus *Azotobacter*

1.1.1.1 pH

Azotobacter is able to develop on media with pH range from 4.5-5.5 to 9.0. Individual species of *Azotobacter*, and possibly even strains, differ in their sensitivity to an acid medium. It has been found, for example, that the minimum pH of the medium is about 5.5 for *A. chroococcum* and *A. beijerinckii* and about 4.6 for *A. macrocytogenes.* The optimum pH for *Azotobacter* lies within the pH range of 7.2 to 8.2 (Mishustin and Shilnikova, 1969) and observed at 7.5 (Dhanasekar, 2003). Furthermore, growth is decreased at both acidic and alkaline pH range (Dhanasekar, 2003).

1.1.1.2 Temperature

In relation to temperature, *Azotobacter* is a typical mesophilic organism. Most investigators regard 25-30ºC as the optimum temperature for *Azotobacter* (Mishustin and Shilnikova, 1969). The minimum temperature of growth of *Azotobacter* evidently lies a little above 0ºC.Vegetative *Azotobacter* cells cannot tolerate high temperatures, and if kept at 45-48ºC they degenerate and die (Mishustin and Shilnikova, 1969). According to the study of Dhanasekar(2003), *Azotobacter*, utilizing glucose and cultivated in a batch reactor, was reported to have optimum temperature at 30ºC furthermore, even at 28 and 32ºC growth was decreased.

1.1.1.3 Aeration

Owing to the fact that *Azotobacter* is an aerobe, this organism requires oxygen. As many investigators have noted, aeration encourages the propagation of *Azotobacter* (Mishustin and Shilnikova, 1969). Effect of different oxygen tensions on the biomass formation of *A. vinelandii* was studied and shown that biomass formation was optimum at pO_2 2-3% (air saturation) and decreased with increasing pO_2 (Sabra *et al*., 1999). In another study (Pena *et al*., 2000), both increasing dissolved oxygen tension and increasing agitation speed increased cell concentration of *Azotobacter* when grown diazotrophically. On the other hand, Dalton and Postgate (1969) reported that initiation of growth of nitrogen-fixing Azotobacter species was prevented by efficient aeration but proceeded normally with gentle aeration.

1.1.1.4 Inorganic Salts

Azotobacter needs some basic nutrient to proliferate in nitrogen-free medium. Beside the carbon source, it needs several salts to fix nitrogen so to propagate. Iron and molibdenum are the co-factors of the nitrogenase enzyme, responsible for the nitrogen fixation, so essential for growth (Brock *et al*., 1994). However, addition of $FeSO_4$ decreased the viable cell count of a mutant strain of *A. vinelandii* (Edwards *et al*., 2000), while in another study (Vermani *et al*., 1997) increased the growth. The propagation of *Azotobacter* is largely dependent on the presence of phosphorous and potassium compounds in the medium (Mishustin and Shilnikova, 1969). The absence or deficiency of phosphorus in the medium slows the development of the culture (Sabra *et al*., 1999). Calcium and magnesium play an important role in the metabolism of *Azotobacter*. A deficiency of calcium in the medium leads to prolongation of the lag phase, but its action is not regarded as specific in relation to process of nitrogen fixation. However, concentration of calcium salts must not exceed a certain optimum (Mishustin and Shilnikova, 1969). Although manganese is evidently not an essential element for nitrogen fixation, its favorable action was reported with the highest requirement of *A. chrooccocum* at the 20-30 ppm in the medium (Mishustin and Shilnikova, 1969). According to the information about the action of copper on *Azotobacter* is toxic even in very low concentrations (Becking, 1961).

1.1.1.5 Nitrogen

The experimental results obtained by Vermani *et al*., (1997) show that; although azotobacters in general are nitrogen fixers, addition of nitrogen in the medium decreases the lag phase and generation time and thus fermentation time. When nitrogen is supplied in the $NaNO_3$ form, up to 0.5 g/l concentration, there was an increase in growth, but further increases in concentration did not altered

the growth pattern. KNO_3 gave similar results. The best results are obtained with NH_4Cl form at 0.1 g/l.

Results of another investigation show that addition of NH_4Cl and $NaNO_3$ into the medium increased the growth of one wild and thirteen mutant types of *A. vinelandii* (Iwahashi and Someyo, 1992).

1.2 Biological Nitrogen Fixation

Nitrogen is an essential nutrient for all life on earth (Sylvia *et al*., 1999). Both organic and inorganic forms of nitrogen are being needed and recycled. Among the nitrogen cycle, biological nitrogen fixation takes the role of biological conversion of atmospheric dinitrogen to forms available for plant and microbial growth by a variety of prokaryotic microbes. From Table 1 (Brock *et al*, 1994), including the nitrogen-fixing organisms, it can be seen that both aerobic and anaerobic bacteria can fix nitrogen.

Table 1.1 Some Nitrogen-Fixing Organisms

Free-living				**Symbiotic**	
Aerobes		**Anaerobes**		**Leguminous plants**	**Nonleguminous plants**
Heterotrophs	**Phototrophs**	**Heterotrophs**	**Phototrophs**		
Bacteria: *Azotobacter* spp. *Klebsiella* *Beijerinckia* *Bacillus polymyxa* *Mycobacterium flavum* *Azospirilum lipoferum* *Citrobacter freundii* Methylotrophs (various, but not all)	Cynobacteria (various, but not all)	Bacteria: *Clostridium* spp. *Desulfovibrio* *Desulfoto maculum*	Bacteria: *Chromatium* *Chlorobium* *Rhodospirillum* *Rhodopseoudo-monas*	Soybeans, peas, clover, locust, etc., in association with a bacterium of the genus *Rhizobium* or *Bradyrhzobium*	*Alnus, Myrica, Ceanothus, Comptonia,* in association with actinomycetes of the genus *Frankia*

The N_2-fixing microbes can exist as independent, free living organisms, or in associations of differing degrees of complexity with other microbes, plants and

animals. These range from loose associations, such as associative symbiosis, to complex symbiotic associations in which the bacterium and host plant communicate on an exquisite molecular level and share physiological functions (Sylvia *et al*., 1999).

Mechanism of the reduction of N_2 to ammonia and the overall reaction is shown in Fig 1.1 (Brock *et al*, 1994).

The reduction process is catalyzed by the enzyme complex nitrogenase, which consists of two separate proteins called dinitrogenase and dinitrogenase reductase. The structure of nitrogenase complex from *A. vinelandii* was visualized at Fig 1.2. Both components contain iron and dinitrogenase contains molybdenum as well (Brock *et al*, 1994).

The N_2 fixation mechanism can be summarized as follows (Sylvia *et al*., 1999);

- Dinitrogenase reductase accepts electrons from a low-redox donor, such as reduced ferrodoxin or flavodoxin, and binds two MgATP.
- It transfers electrons, one at a time, to dinitrogenase.
- Dinitrogenase reductase and dinitrogenase from a complex, the electron is transferred and two MgATP are hydrolyzed to two MgADP+P_i.
- Dinitrogenase reductase and dinitrogenase dissociate and the process is then repeated.
- When dinitrogenase has collected enough electrons, it binds a molecule of dinitrogen, reduces it, and releases ammonium.
- Dinitrogenase then accepts additional electrons from dinitrogenase reductase to repeat the cycle.

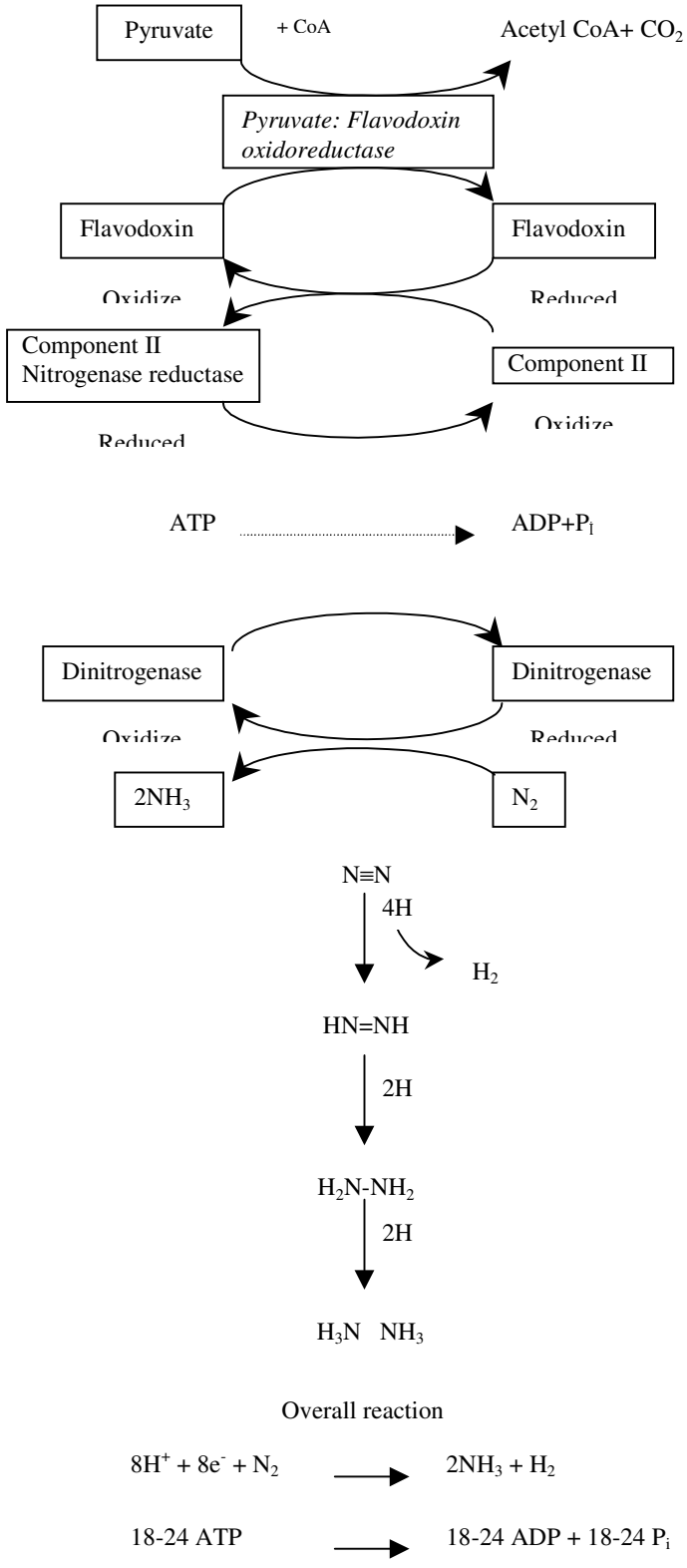

Fig. 1.1 Steps in Nitrogen Fixation; reduction of N_2 to $2NH_3$

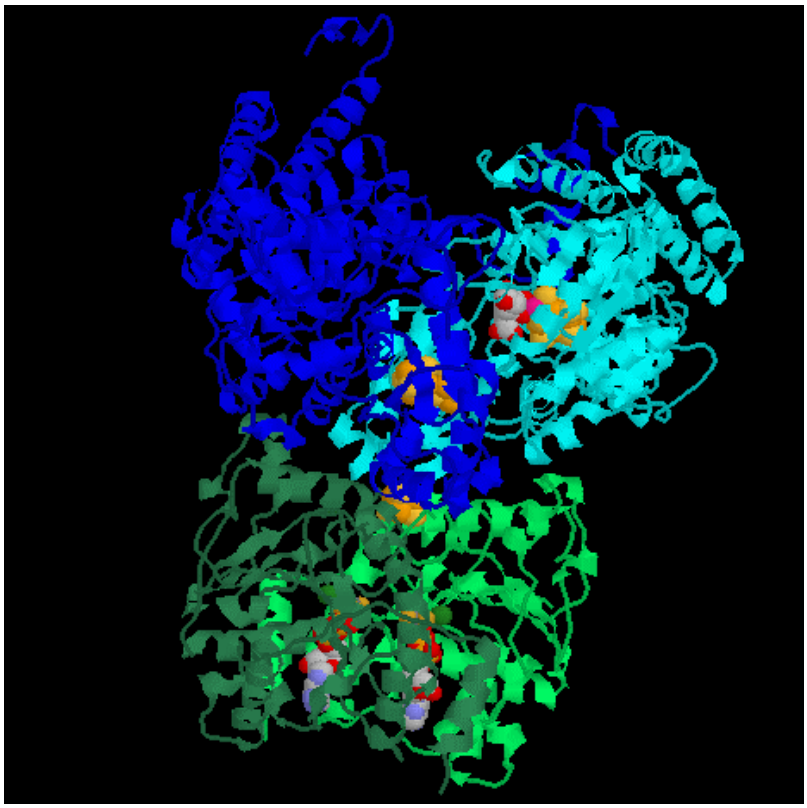

Fig 1.2 Structure of Nitrogenase from the free-living bacterium *Azotobacter vinelandii* stabilized by ADP-tetrafluoroaluminate.

The nitrogenase enzyme is actually a complex of two different proteins, the Fe-protein, or nitrogenase reductase (shades of green), and the MoFe protein (shades of blue) (also referred to itself as nitrogenase). These two proteins do not normally exist as a stable complex, but have been stabilized in the association shown here by the presence of 2 molecules of AlF_4-ADP bound to the Fe-protein (at bottom). ALF_4-ADP may mimic the transition state of ATP on hydrolysis. You can also see, shown in CPK coloring, the 4Fe:4S cluster in the Fe-protein, the 8Fe-7S cluster (also called the "P-cluster") and the 7Fe-1Mo-9S cluster in the MoFe protein. Note that

the 7Fe-1Mo-9S cluster is associated with one molecule of homocitrate, which is an obligate component of this cluster.

1.2.1 Significance of Biological Nitrogen Fixation

Because ammonia and nitrate levels often are low and only e few prokaryotes can carry out nitrogen fixation, the rate of this process limits plant growth in many situations (Prescott *et al*., 1999). In natural systems, nitrogen for plant growth comes from the soil, from rainfall or other atmospheric deposition or other biological N_2 fixation. Among these biological nitrogen fixation is reported to have the largest contribution. Biological processes contribute 65% of the nitrogen used in agriculture (Burris and Roberts, 1993). While much of this is through symbiotic N_2 fixation, nonsymbiotic and associative fixation are of some significance in crops, such as sugar cane and sorghum, which have a C_4 photosynthetic pathway, and in specific ecosystems where nitrogen for plant growth is a limiting factor (Sylvia *et al*., 1999). With this efficiency biological N_2 fixation offers an alternative to the use of expensive ammonium based fertilizer nitrogen, in other words biofertilizers compete with chemical fertilizers.

1.2.2 Factors Affecting Nitrogen Fixation

Diazotrophs while fixing nitrogen with the aid of their enzyme complex nitrogenase are affected by several factors such as acidity or alkalinity, temperature, oxygen, some inorganic salts, source of energy, and fixed nitrogen .

1.2.2.1 pH

Azotobacter favors neutral environments to proliferate. Both the growth and nitrogen fixation are affected by the pH. It is known that nitrogen fixing

activity of *Azotobacter* is weakened at acidic environments (Mishustin and Shilnikova, 1969).

1.2.2.2 Temperature

Nitrogenase is active over a fairly narrow temperature range. At the lower limits of 5 to 10ºC, nitrogenase activity is low, whereas at the upper limits, 37 to 40ºC, nitrogenase activity falls off rapidly because of the sensitivity of the enzyme to heat (Sylvia *et al*., 1999).

1.2.2.3 Oxygen

Nitrogen fixation is inhibited by oxygen since dinitrogenase reductase is rapidly and irreversibly inactivated by O_2. In aerobic bacteria, N_2 fixation occurs in the presence of O_2 in whole cells, but not in purified enzyme preparations and nitrogenase in such organisms is protected from O_2 inactivation either by removal of O_2 by respiration, the production of O_2-retarding slime layers, or by compartmentalizing nitrogenase in special type of cell, the heterocyst (Brock *et al*., 1994).

At 4 percent oxygen, *A. vinelandii* fixed 23.5 mg nitrogen and *A. chroococcum* 21.6 mg nitrogen per g sucrose supplied; at 20 percent oxygen the fixation was 8.1 mg and 7.4 mg nitrogen, respectively. These results showed greatly increased nitrogen-fixing efficiency with decreased partial pressures of oxygen (Parker, 1954).

High oxygen solution rates inhibited nitrogenase in whole bacteria (Yates, 1970), is a result indicating that nitrogen fixation by *A. chroococcum* is decreased with increasing shaking rates at continuos growth conditions.

1.2.2.4 Inorganic Salts

Some nutrients present in the growth medium are also takes part in the nitrogen fixation process.

Phosphorus compounds in the medium effect nitrogen fixation. The fixation of nitrogen by *Azotobacter* starts when the concentration of PO_4^{-3} anion reaches 4 mg/100 ml medium. If the concentration of PO_4^{-3} in solution is about 800 mg/100 ml medium, the assimilation of nitrogen stops altogether (Becking, 1961). Salmeron *et al*. (1990) investigated the effect of available phosphate on the nitrogen fixation and correlated them possitively, i.e. more nitrogenase activity is detected as the solubilization of phosphate increases.

Nitrogenase complex needs Mg^{2+} ions to be active (Sylvia *et al*., 1999), so magnesium requirement for nitrogen fixation is considerable.

Molybdenum is absolutely essential for most strains of *Azotobacter*. This need is shown both during fixation of molecular nitrogen and during development of nitrates (Mishustin and Shilnikova, 1969), is a consequence of the fact that molybdenum is needed for the expression of the nitrogenase enzyme complex.

It is known that vanadium stimulates N_2 fixation in a number of organisms including various species of *Azotobacter*, some cynobacteria and phototrophic bacteria and *Clostridium pasteurianum* (Brock *et al*., 1994). Vanadium causes the organism to express vanadium based nitrogenases in the molybdenum deficient medium.

1.2.2.5 Nitrogen

When the nitrogen fixation is evaluated as nitrogenase activity, in many cases it is evidenced that nitrogen fixation is depressed with the presence of

combined nitrogen in the medium as in the case of short term effect of ammonium chloride (Laane *et al.*, 1980). This inhibition is due to inhibition of electron transport system to nitrogenase. Further findings suggest that small doses of nitrogen containing mineral compounds (especially ammonium salts) stimulate nitrogen assimilation, but larger doses restrain this process (Mishustin and Shilnikova, 1969).

1.2.3 Methods for Measuring Nitrogen Fixation

Since the significance of biological nitrogen fixation have been recognized people tried to find ways to prove that the organism in concern can fix nitrogen.

1.2.3.1 Measurement of Nitrogen Fixation with the Increase in Growth in N-free Medium

The traditional method for detection of biological nitrogen fixation had been the ocular assay based on observation of growth of the agent on what is alleged to be a N-free medium. Though not a quantitative procedure, this method deserves some consideration because of its usefulness in screening agents and because historically it has led to the investigation of many agents known or alleged to fix nitrogen (Burris and Wilson, 1972).

1.2.3.2 Measurement of Nitrogen Fixation by the Nitrogen Difference Method

This method is adequate for active N_2 fixers, but it will not detect increases of less than about 1% in the total nitrogen even when uniform samples can be taken (Burris and Wilson, 1972). Increase in total nitrogen can be detected with the help of Kjeldahl method, in which all the forms of the nitrogen in the sample

are converted to ammonia by the digestion application followed by distillation of the ammonia to detect colorimetrically or by titration.

1.2.3.3 Measurement of Nitrogen Fixation by Ammonia Determination

Concern with the quantitative analysis for the intermediates in biological N_2 fixation is centered on ammonia because it is the only demonstrated intermediate in the process (Burris, 1972). Ammonia is determined colorimetrically with the Nessler's reagent.

1.2.3.4 Measurement of Nitrogen Fixation by Stable Isotope (^{15}N) Method

The most definitive measurements of biological N_2 fixation make use of the stable, heavy isotope, ^{15}N, and require access to a mass spectrometer. In this method, incorporation of $^{15}N_2$ (labeled dinitrogen) into plant or microbial cells is measured, being a straightforward method. Samples are exposed to an atmosphere of about 10% $^{15}N_2$, usually in a balance of argon or helium to eliminate competition from $^{14}N_2$. Following incubation, the samples are digested and the ^{15}N content of the materials is determined using a mass spectrometer. Detection of ^{15}N in tissues or cells provides definitive proof of N_2 fixation and allows very accurate quantification of the amount of dinitrogen fixation (Sylvia *et al*., 1999).

1.2.3.5 Measurement of Nitrogen Fixation by the Acetylene Reduction Assay

The ability of the nitrogenase complex to reduce acetylene (C_2H_2) to ethylene (C_2H_4) forms the basis for the acetylene reduction assay. In this method, the system to be measured is exposed to an atmosphere containing 11% acetylene and incubated under appropriate conditions. Samples of the gas phase are periodically removed and injected into the gas chromatograph for quantification of the ethylene production from acetylene (Sylvia *et al*., 1999). This method is far simpler and faster than other methods (Brock *et al*., 1994).

1.3 Olive Mill Wastewater (OMW)

One of the important industrial processes in Turkey has been the olive oil industry, achieving a production rate of 200000 tones per year. With this production rate, Turkey takes the fifth turn in olive oil processing. From the processing of 1 tone of olive oil, 0.4 - 0.5 m^3 of OMW is produced.

OMW is the liquid residue obtained by centrifugation or by sedimentation after pressing of olive tree fruits in the oil factory. Different names have been applied to this waste stream: vegatation waters, olive mill wastewaters, alpechins, etc. (Alcaide and Nefzaoui, 1996).

OMW is a dark liquid with a characteristic fetid smell, bitter taste and bright appearance; having a high pollution potential, have been creating serious problems in countries that produce olive oil. Along with other countries Spain, France, Italy, Portugal and Tunisia, Turkey is also affected by this problem (Garcia-Barrionuevo *et al*., 1993). As a pollution source, OMW has existed for thousands of years, but their effects on the environment are at present more noticeable because olive oil production has increased remarkably during the last 30 years (Rozzi and Malpei, 1996).

1.3.1 Disposal and Treatment of OMW

The difficulties in disposing of these wastewater are mainly related to their high concentration (in the range of 50-150g COD l^{-1}, i.e. two orders of magnitude higher than domestic sewage) (Rozzi and Malpei, 1996). Almost all of the water soluble constituents of the olives contribute to the organic fraction of the OMW, which contains phenolic substances, some nitrogenous substances, organic acids, sugars, tannins pectins, caratenoids and oil residues. And the inorganic fraction contains chloride, sulphate and phosphoric salts of potassium as well as calcium, iron, magnesium, sodium, copper and other trace elements in various chemical forms (Piperidou *et al*., 2000). Antibacterial activity (Ramos-Cormenzana, 1996), inhibition of seed germination (Perez *et al*., 1986) and phytotoxicity to herbage crops (Capasso *et al*., 1992) by OMW have been demonstrated. However, OMW is a pure vegetative by-product, containing no xenobiotics or heavy metals contaminants and its application to soils of low organic matter content, abundant in the Mediterranean basin, would be a sustainable recycling option, if its toxicity to microorganisms and plants was first eliminated (Ehaliotis *et al*., 1999). The detoxification processes can be classified as physical, physicochemical and biological ones. The physical and physicochemical ones can are considered together and they include: thermal processes (evaporation and incineration); flocculation/clarification; ultrafiltration; and reverse osmosis. The biological ones can be subdivided into anaerobic and aerobic (Rozzi and Mlpei, 1996).

In the aerobic biological treatment of the OMW, aerobic microorganisms degrade a fraction of the pollutants in the effluent by oxidizing them with oxygen which is provided by an external source (either as air or pure oxygen). These microorganisms use most of the remaining fraction of the pollutants to produce new cells (termed biomass or sludge) which have to be removed from the water. (Rozzi and Malpei, 1996). Several microorganisms succeeded to eliminate the toxicity of OMW such as; Pleurotus species (Flouri *et al*., 1996), *Bacillus pumilus* (Ramos-Cormenzana, 1996), *Arthrobacter* (Knupp *et al*., 1996), and

Azotobacter species (Ehaliotis *et al*., 1999, Piperidou *et al*., 2000, Balis *et al*., 1996, Garcia-Barrionuevo *et al*, 1992).

1.3.2 Utilization of OMW by *Azotobacter chrooccocum*

OMW because of their low content in nitrogenous organic components and richness in carbon sources offer a highly favorable environment for the growth of free living dinitrogen fixing microorganisms. Among the organisms that can detoxify OMW, the genus *Azotobacter* includes free living bacteria found in soil and water. They are distinguished by their large size and the ability to fix atmospheric nitrogen when supplied with a suitable energy source. *Azotobacter* was evidenced to use OMW as energy and carbon source (Balis *et al*., 1996), even though its high phenolic composition. *Azotobacter* is capable of achieving mean phenol degradation yields as high as 90 and 96% after 3 and 7 days of treatment (Piperidou *et al*., 2000). During degradation of OMW *Azotobacter* does not only reduce the phenolics, but also produce fertility promoting metabolites and exo- and capsular polysaccharides which provide a cementing action for soil aggregation (Fiorelli *et al*., 1996). Moreover, they suppress some soil-borne root pathogenic fungi (Balis *et al.*, 1996) and solubilize phosphate (Kumar *et al*., 2001).

1.3.3 Bioremediation of OMW

Due to the fact that OMW contain valuable substances, which could be recovered, bioremediation of this agroindustrial waste can serve for important beneficial uses. Mainly, its bioremediated product is used as a fertiliser (Tomati *et al*., 1996, Cegarra *et al*., 1996, Piperidou *et al*., 2000, Chatjipavlidis *et al*., 1996). Considering how important it is in agriculture to introduce parameters of

sustainability, particularly in the Mediterranean region, the nitrogen fixation process offers an economically attractive and environmentally sound system especially since it utilizes the notorious OMW. Repetitive addition of OMW to soil under aerobic conditions leads progressively to its enrichment with dinitrogen fixers, the activity of which is beneficial to soil fertility. The microbial consortium that develops in soil is dominated mostly by members of *Azotobacter* (Balis *et al*., 1996). In a study (Piperidou *et al*., 2000) where *Azotobacter vinelandii* was used to bioremediate the OMW in a biowheel reactor to be further used as a biofertilizer, 90 and 96% removal yields could be achieved after 3 and 7 days of treatment. The system eliminates the phytotoxic principles from OMW and concomitantly enriches it with an beneficial microbial consortium for agriculture along with useful metabolites of the latter. In another research (Cegarra *et al*., 1996), OMW used as compost for the cultivation of horticultural crops have shown that yields are similar and sometimes higher to those obtained with a balanced mineral fertilizer. In a pilot plant study (Chatjipavlidis *et al*., 1996), *Azotobacter vinelandii* is cultivated with OMW in a two stage bioreactor is further applied as a biofertilizer for the cultivation of olive trees, vines and potatoes showed promising results.

Second use of bioremediated OMW is as food and biomass and single cell protein food industry. Many kinds of edible mushrooms are observed to grow in composts fed with OMW (Ramos-Cormenzana *et al*., 1995). Recovery of feed additives is also possible from the treated OMW such as purification of anthocyanin (Codounis *et al*., 1983). Their utilization as a source of nutrients for animals is also reported (Alcaide and Nefzaoui, 1996).

OMW is also utilized as a growth medium for algae. In Spain logoons containing this effluent is inoculated with several algae such as *Dunaliella* and *Spirulina* (Ramos-Cormenzana *et al*., 1995).

Moreover, production of biopolymeric substances, focusing on polysaccharides and biodegradable plastics, such as polyhydrxyalkanoates by *Azotobacter chrooccocum* cultivated with OMW is reported (Pozo *et al*., 2002). Pollulan, used in both food and pharmaceutical industries, is another extracellular

polysaccharide produced as the product fermentations in which OMW is used as substrate (Ramos-Cormenzana *et al*., 1995).

The another common practice for the bioremediation of OMW is its utilization in biogas production, being a bioenergetic source (Ergüder *et al*., 2000).

Lastly, employment of OMW as a source of biopharmaceuticals is also reported (Ramos-Cormenzana *et al*., 1995).

1.4 Aim of the Study

The aim of this study can be introduced as follows; growth and nitrogen fixation conditions of *Azotobacter chroococcum* are optimized in chemically defined, nitrogen free medium. Parameters such as pH, temperature, aeration, organic salts and combined nitrogen are taken into account during optimization studies. Furthermore, *A. chroococcum* was cultivated in OMW containing nitrogen free medium at optimized conditions to verify the bioremediation capacity of this organism on OMW.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Organism

The bacterium used in this study was *Azotobacter chroococcum*, which was used for agricultural purposes by Catek, Havana, Cuba. Stock cultures of *A. chroococcum* was maintained on N-free agars and stored at 4°C.

2.1.2 Olive Mill Wastewater

OMW used in this study was obtained from a continuously operating olive oil factory at Morova, Atça, Aydın in 2002, and it was stored at 4°C, before use. Some characteristics of OMW used are given in Table 2.1.

Table 2.1 Some Characteristic Properties of OMW

Parameter	Amount
pH	5.00
Suspended Solids	14.3 g/l
Dissolved Solids	20.8 g/l
Protein Concentration	13.1 g/l
Ammonia Concentration	80.0 g/l

2.2 Methods

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

2.2.1 Cultivation of *A. chroococcum*

2.2.1.1 Growth Medium

The chemically defined basal N-free medium used in this study was modified from the Jensen's Medium (Kumar and Narula, 1999). Its composition is given in Table 2.2.

Table 2.2 N-free Medium

Component	Concentration (g/l)
Sucrose	20
$FeSO_4\ 7H_2O$	0.010
$MgSO_4\ 7H_2O$	0.50
$CaCl_2$	0.10
NaCl	0.20
Na_2MoO_4	0.0050
KH_2PO_4	0.50

In order to prevent the precipitation of the salt ingredients after sterilization, stock solutions of $MgSO_47H_2O$, $CaCl_2$, NaCl, Na_2MoO_4 and KH_2PO_4 were prepared and they were autoclaved separately. After sterilization they were combined in the written order. And the combined salts solution was added to the previously autoclaved sucrose and $FeSO_47H_2O$ solution. Then, pH of the representative medium solution was adjusted to 7 with 2N NaOH, so NaOH solution was added to the medium, in which cultivation will be carried out, at the amount needed for the representative one in order to maintain the sterility of the medium.

Solid growth medium was obtained by adding 20g/l agar-agar to the N-free medium.

Experiment Using Some Inorganic Salts and Boric Acid

The Boric acid and the salt ($ZnSO_47H_2O$, $MnSO_4H_2O$ and $CuSO_47H_2O$) were added to the N-free medium after all the ingredients of the basal medium were added (in the amount 0.005%, 0.002%, 0.005% and 0.003% (w/v), respectively).

Experiment Using Nitrogenous Medium

NH_4Cl was added to the basal medium by combining it with the sucrose and $FeSO_4\ 7H_2O$ solution at the amount 0%, 0.05% and 0.1% (w/v).

Experiments Using OMW

OMW was sterilized separately and added to the basal medium with its whole content that is; with its suspended and dissolved solids. Then pH of the medium was adjusted to 7 as described above.

2.2.1.2 Inoculum

The growth medium was inoculated with approximately 10^6 cell/ml *A. chroococcum* cells grown at preculture medium for 22 h. Preculture medium was the N-free medium and it was inoculated with one loopfull of bacterial cell transferred from the solid medium. Preculture was cultivated at 35°C and 150 rpm.

2.2.1.3 Growth Conditions

Unless specified, growth was carried out at 35°C; 150 rpm and pH 7.0 in 250 ml flasks containing 100 ml medium, at a rotary shaker.

2.2.2 Growth Measurement

Growth was quantified with aerobic plate count method. The samples taken from the growth medium was diluted to appropriate concentration. Dilution solution was the N-free medium except the carbon source. The diluted cell

suspensions were inoculated over the solid N-free medium. And the plates were incubated at 35°C for 40 h then the white colored colonies were counted.

2.2.3 Nitrogen Fixation Measurement

Nitrogen fixation capacity of *A. chroococcum* was quantified indirectly by measuring the products of nitrogen fixation activity; extracellular protein and ammonia concentrations.

2.2.3.1 Extracellular Protein Concentration Measurement

Extracellular protein concentration was measured with Modified Lowry Method (Hartree, 1972). The samples taken from growth medium were centrifuged at 15000 rpm for 15 min at 0°C and the supernatant was analyzed with Lowry Method. The reagents used in Lowry Method are given in Appendix A and the standard curve preparation and procedure of this method is described in Appendix B. Standards were prepared with bovine serum albumin (BSA). Absorbances of the samples were read by taking the blank as the uninoculated medium itself, since the salts contained in the medium interact with the reagents causing overestimation.

2.2.3.2 Ammonia Concentration Measurement

The ammonia concentration was measured with Nesslerization Method following the procedures described in Standard Methods for the Examination of Water and Wastewater. The reagents and the procedure of Nesslerization Method were described in Appendix C and D, respectively. Absorbances of the samples were measured at 400 nm against the blank solution prepared as the uninoculated

initial medium, since the salts contained in the medium interact with the reagent causing overestimation.

2.2.4 Characterization of OMW

2.2.4.1 Suspended Solids Content Measurement

Suspended solids content of OMW was determined according to the procedures described at Standard Methods for the Examination of Water and Wastewater. A well-mixed sample of known volume OMW was filtered through rough filter paper of which dry weight was determined previously. The residue retained on the filter paper was dried to a constant weight at 103-105°C. The increase in weight of the filter paper represents the suspended solids.

2.2.4.2 Dissolved Solids Content Measurement

Dissolved solids content of OMW was determined according to the procedures described at Standard Methods for the Examination of Water and Wastewater. A well-mixed sample of OMW was filtered through filter paper, and the filtrate was evaporated to dryness in dried dish, of which dry weight was determined previously, and dried to constant weight at 180°C. The increase in dish weight represents the dissolved solids content.

CHAPTER 3

RESULTS AND DISCUSSION

As a preliminary study to further bioremediate OMW with *A. chroococcum*, this nitrogen-fixing bacteria was cultivated in chemically defined nitrogen-free medium. Effect of several factors on growth and nitrogen fixation capacity of *A. chroococcum*, such as pH, temperature, aeration, inorganic salts and combined nitrogen was evaluated.

Growth is measured as viable counts by plate counting. Although this method takes longer time, the other two methods that were faster, counting cells with Thoma Chamber and optical density measurement, were not appropriate. While counting with Thoma Chamber both the viable and dead cells are counted and OMW contains a high dead cell load, the results obtained with these methods had lead into errors. Also measuring growth with optical density is not suitable for the OMW containing medium due to the dark color of OMW.

Ammonia and extracellular protein are the nitrogenous secretions of the nitrogen fixation activity of *Azotobacter* in nitrogen free or deficient medium, which are available to the plants or other organisms. That is why in this study nitrogen fixation is evaluated using the ammonia and extracellular protein concentrations as criteria in growth medium under diazotrophic conditions. However the fluctuating results of ammonia concentrations were probably due to the fact that ammonia is a

volatile gas and during the assays with Nesslerization ammonia can be lost and results can be underestimated.

3.1. Growth and Nitrogen Fixation in Chemically Defined Nitrogen-Free Medium

3.1.1. Effect of pH

In order to evaluate the effect of pH on growth and nitrogen fixation products, pH of N-free medium was adjusted to 5,6,7 and 8. Since the salt ingredients were added after sterilization and the addition of salts lowers the pH, pH was adjusted to intended value after sterilization with the help of 2N NaOH. The pH range (6-8) studied in this experiment was selected to obtain the optimum growth pH of *A. chroococcum*. Also the behavior of *A. chroococcum* at slightly acidic medium (pH=5) was studied, since pH 5 is a closer value to that of OMW. For this reason *A. chroococcum* was cultivated at specified pH values, 35°C and 150 rpm. At these conditions *A. chroococcum* had reached the stationary phase at 23rd h of incubation for all pH values (5,6,7 and 8). Maximum cell density ($4.8x10^7$ cfu/ml) was obtained at pH 7; similarly at pH 6 it was closer to the maximum value, $4.4x10^7$ cfu/ml. However at pH 8, a slight decrease was observed, where $4.1x10^7$ cfu/ml cell density could be attained. Minimum cell density ($3.9x10^{-7}$ cfu/ml) was obtained at pH 5 (Fig. 3.1.1.1). From these results it can be concluded that optimum growth pH value is around the neutral pH and cell density was lowered 15% and 19% at slightly alkaline pH (8) and at slightly acidic pH (5), respectively. Findings mentioned above are supporting the results claiming that the optimum pH for *Azotobacter* lies within the pH range of 7.2 to 8.2 (Mishustin and Shilnikova, 1969) and growth is decreased at both acidic and alkaline pH range (Dhanasekar, 2003).

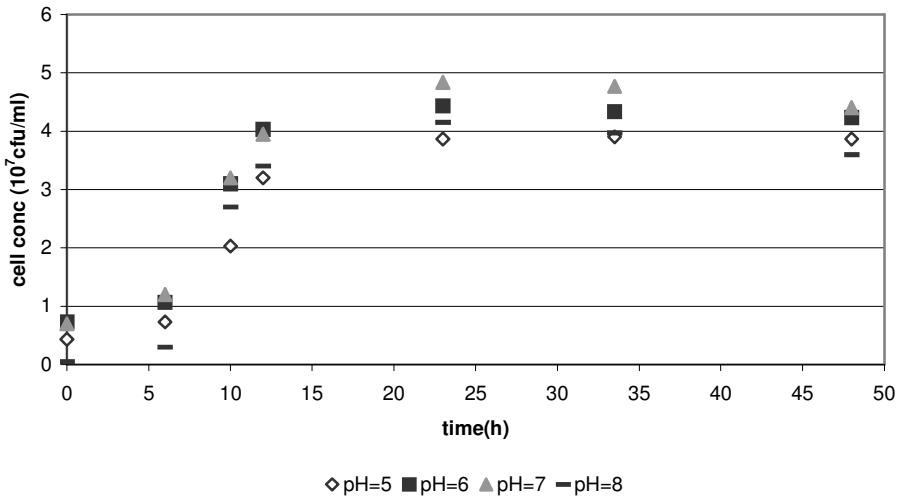

Fig. 3.1.1.1 Effect of pH on Growth. *A. chroococcum* was cultivated at different pH values as 5,6,7 and 8.

In Fig. 3.1.1.2 an increasing trend for extracellular protein levels were observed for incubation pH values of 5,6,7 and 8 during 48 h of incubation period, even at stationary phase extracellular protein levels seem to be increasing. Extracellular protein levels varied for different pH values, that is the maximum extracellular protein concentrations obtained were 6.20; 7.16; 8.61 and 10.40 mg/l at pH values 5,6,7 and 8, respectively. These results show that the maximum extracellular protein level was attained when *A. chroococcum* was cultivated at pH 8.

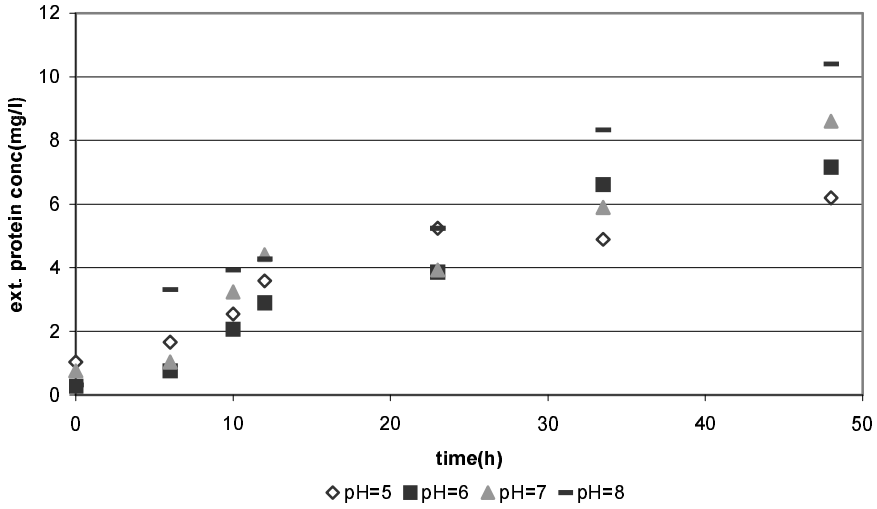

Fig. 3.1.1.2 Effect of pH on Extracellular Protein Levels. *A. chroococcum* was cultivated at different pH values as 5,6,7 and 8.

When the effect of pH on ammonia secretion is considered, maximum ammonia secretion value of 1.50 mg/l was observed when *Azotobacter* was grown at pH 8. At pH 8, a similar trend was observed with growth; ammonia concentration increases up to reaching stationary phase, however a slight decrease was observed during stationary phase. This decrease can be due to the fact that ammonia is released to the atmosphere at alkaline pH values. Also when *Azotobacter* was grown at pH 7, ammonia concentration had increased (to its maximum value of 1.07 mg/l) up to reaching stationary phase, but a sharp decrease was seen after 23rd h of incubation. That is not the case for pH 6 and 5; at these pH values a peak (0.95 mg/l and 1.3 mg/l, respectively) was observed at 10th h of incubation and then a sharp decrease was observed (Fig. 3.1.1.3).

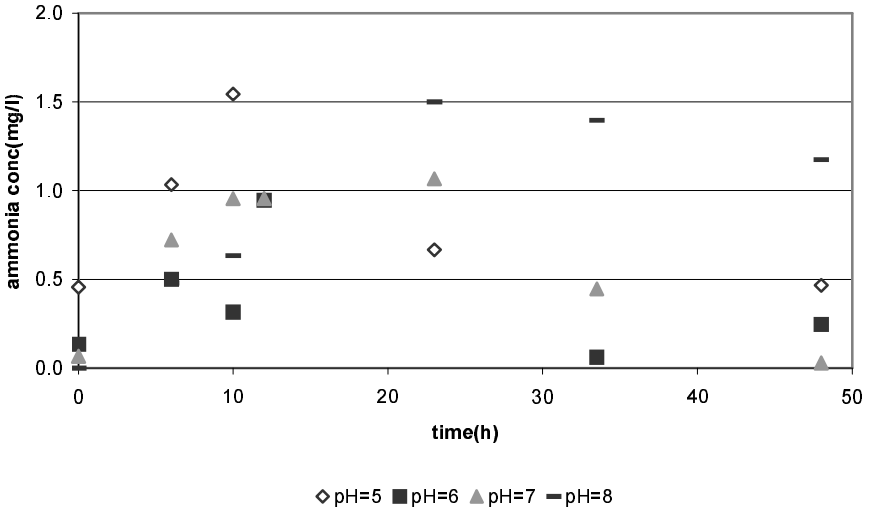

Fig. 3.1.1.3 Effect of pH on Ammonia Secretion. *A. chroococcum* was cultivated at different pH values as 5,6,7 and 8.

3.1.2. Effect of Temperature

A. chroococcum was cultivated in N-free medium at pH 7; 150 rpm and at three different temperatures of 30, 35 and 40°C. *A. chroococcum* had reached the stationary phase at 24th h of incubation at all the temperatures studied. At 30°C and 35°C growth profiles were similar and reached a maximum cell density of 3.4x10^7 cfu/ml. *A. chroococcum,* being a mesophilic organism, was reported to have the optimum growth temperature as 25-30°C (Mishustin and Shilnikova, 1969). However at 30°C and 35°C the cell density was raised to the same value (Fig 3.1.2.1). While at 40°C maximum cell density decreased to 2.8x10^7 cfu/ml (Fig 3.1.2.1), i.e. rising growth temperature had caused 18% decrease in the cell density.

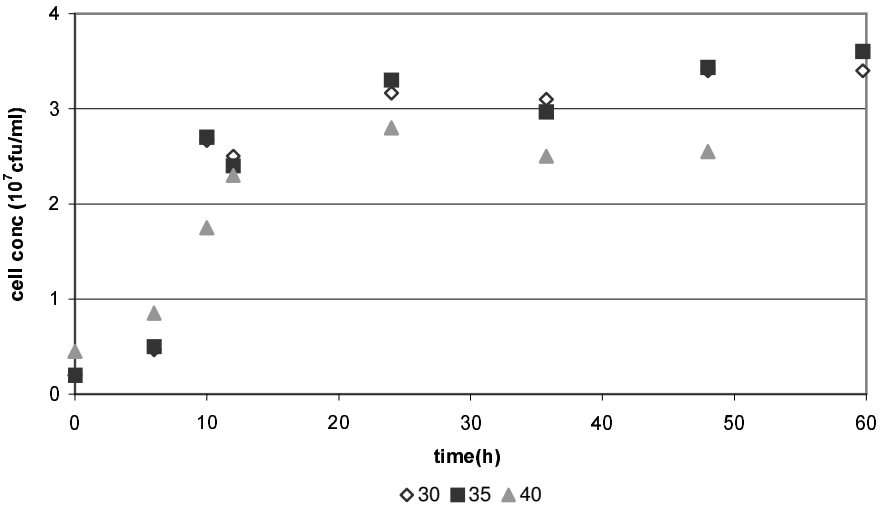

Fig. 3.1.2.1 Effect of Temperature on Growth. *A. chroococcum* was cultivated at different temperatures as 30°C, 35°C and 40°C.

In Fig. 3.1.2.2 the effect of growth temperature on the extracellular protein concentration is shown is shown. The maximum extracellular protein concentrations reached to 8.07 mg/l and 8.42 mg/l at 30°C and 35°C, respectively, and these values were reached at different incubation times; for 35°C it is 36^{th} h, while at 30°C it was reached at the end of incubation, 60^{th} h (Fig. 3.1.2.2). However when *A. chroococcum* was incubated at 40°C the maximum extracellular protein level decreased to 6.46 mg/l. From these results it can be seen that the maximum extracellular protein level had been reached when *A. chroococcum* was incubated at 35°C, furthermore this value was reached in shortest incubation time at that growth temperature.

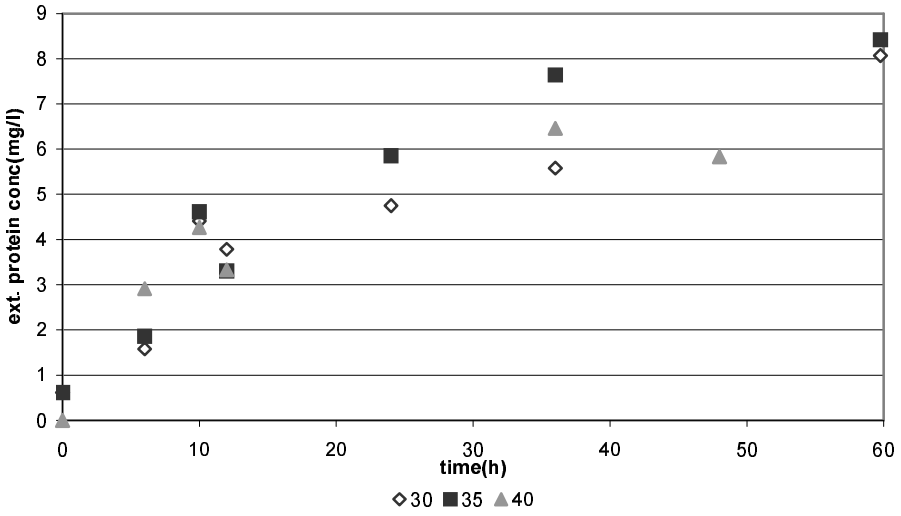

Fig. 3.1.2.2 Effect of Temperature on Extracellular Protein Levels. *A. chroococcum* was cultivated at different temperatures as 30°C, 35°C and 40°C.

Finally, the effect of growth temperature on the other nitrogen fixation product, ammonia, is seen at Fig.3.1.2.3. When *A. chroococcum* is grown at 30 and 35°C, the ammonia secretion profile shows a peak during 20-40h of incubation at the concentration of 4.6 mg/l and 6 mg/l, respectively, but it is decreased to 2 mg/l at 40°C growth temperature (Fig. 3.1.2.3). The maximum ammonia concentrations were attained at the region where the growth is having reached to stationary phase, after that time declines sharply even to the exhaustion levels.

Above results show that increasing the growth temperature, lowers both the growth, and the nitrogen fixation products; extracellular protein level and ammonia secretion concentrations. This result can be the reason of the decrease in activity of nitrogenase (Sylvia *et al*., 1999). In other words, the sensitivity of nitrogenase, the enzyme responsible from nitrogen fixation, to the heat caused to decrease the nitrogen fixation and consequently growth. Furthermore, the decrease of solubility of both the nitrogen and the oxygen gases with increasing

temperature could be probably the cause of the decreases in the growth and nitrogen fixing activity at higher temperatures, 40°C.

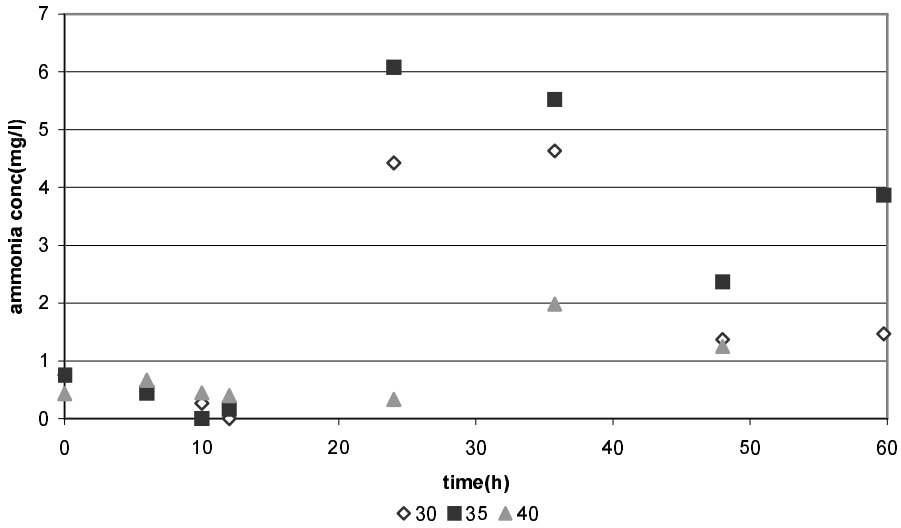

Fig. 3.1.2.3 Effect of Temperature on Ammonia Secretion. *A. chroococcum* was cultivated at different temperatures as 30°C, 35°C and 40°C.

3.1.3. Effect of Aeration

Although nitrogenase is inactivated with the presence of oxygen, nitrogen fixation by bacteria *A. chroococcum* continues at the aerobic conditions. Since in such organisms nitrogenase is protected from O_2 inactivation either by removal of O_2 by respiration, the production of O_2-retarding slime layers, or by compartmentalizing nitrogenase in special type of cell, the heterocyst (Brock *et al*., 1994). And since the oxygen transfer rate is increased with aeration the experiments below were carried out to validate the effect of aeration on the proliferation of *A. chroococcum* on N-free medium. For this reason, different headspace volumes and being agitated or not were tested. *A. chroococcum* was cultivated at 150 rpm in 100 ml N-free medium in 250 ml flask (Fig.3.1.3.1, 3.1.3.2, 3.1.3.3 (A)); at stationary conditions, in 100 ml N-free medium in 250 ml flask (Fig.3.1.3.1, 3.1.3.2, 3.1.3.3 (B)); and at 150 rpm in 100 ml N-free medium

in 500 ml flask (Fig.3.1.3.1, 3.1.3.2, 3.1.3.3 (C)). Growth temperature for this experiment was 35°C. This variation in aeration conditions did not alter neither the growth nor the nitrogen fixation products, extracellular protein, (Fig.3.1.3.1, 3.1.3.2), although improvement of growth with increasing agitation speed is a reported issue (Pena *et al*., 2000). However, maximum ammonia concentration was achieved at the 36th h of incubation when *A. chroococcum* was incubated at the stationary conditions (Fig. 3.1.3.3 B), while it was achieved at 24th h of incubation at the other incubation conditions described (Fig. 3.1.3.3 A and C).

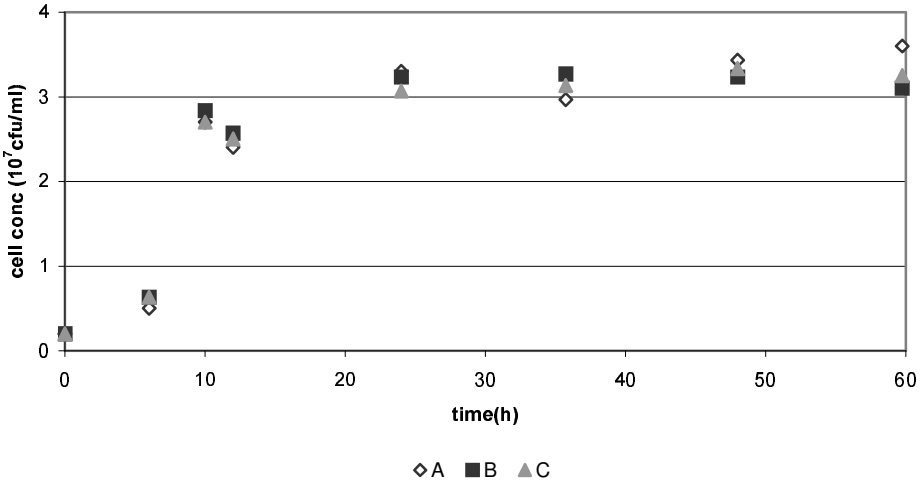

Fig. 3.1.3.1 Effect of Aeration on Growth. *A. chroococcum* A: is cultivated at 150 rpm in 100 ml N-free medium in 250 ml flask, B: at stationary conditions, in 100 ml N-free medium in 250 ml flask, C: at 150 rpm in 100 ml N-free medium in 500 ml flask.

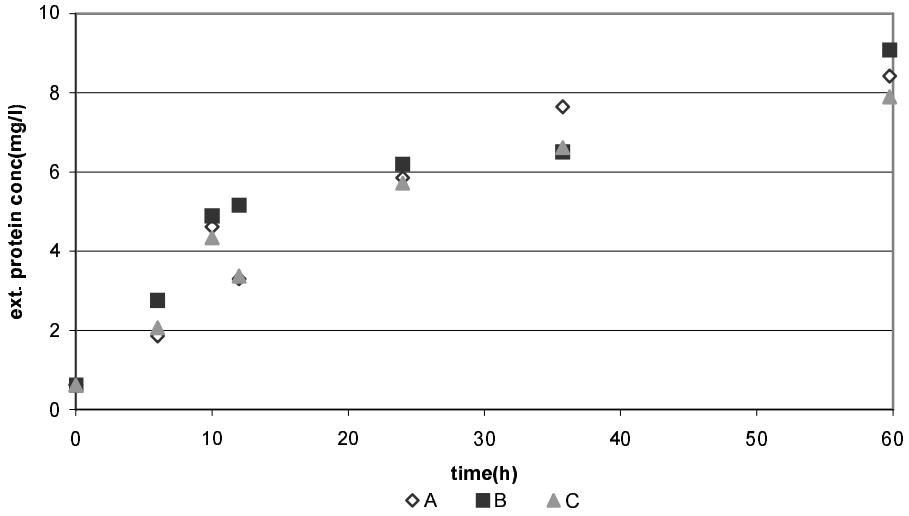

Fig. 3.1.3.2 Effect of Aeration on Extracellular Protein Levels. *A. chroococcum* A: is cultivated at 150 rpm in 100 ml N-free medium in 250 ml flask, B: at stationary conditions, in 100 ml N-free medium in 250 ml flask, C: at 150 rpm in 100 ml N-free medium in 500 ml flask.

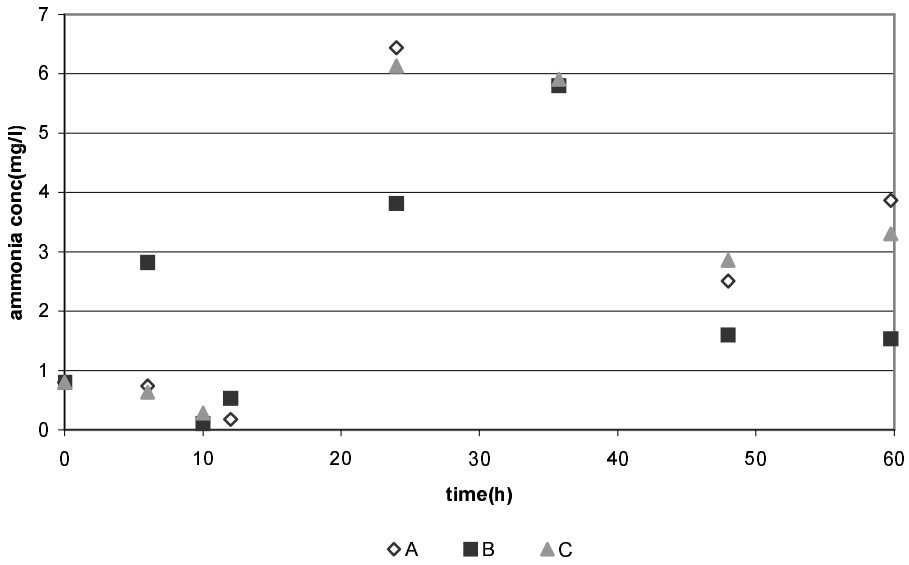

Fig. 3.1.3.3 Effect of Aeration on Ammonia Secretion. *A. chroococcum* A: is cultivated at 150 rpm in 100 ml N-free medium in 250 ml flask, B: at stationary conditions, in 100 ml N-free medium in 250 ml flask, C: at 150 rpm in 100 ml N-free medium in 500 ml flask.

3.1.4. Effect of Some Inorganic Salts and Boric Acid

Growth medium (N-free medium) used in this study contains several inorganic salts, all of which are used by *A. chroococcum* for distinct purposes. Some salts, present in the medium at any rate, iron and molybdenum containing salts (Brock *et al*., 1994), phosphorous, potassium, calcium and magnesium containing ones (Mishustin and Shilnikova, 1969) are essential for both growth and nitrogen fixation of *Azotobacter*.

In this study additive inorganic salts and boric acid were tested for their effect on growth and nitrogen fixation. In Fig. 3.1.4.1, 3.1.4.2, 3.1.4.3, H_3BO_3 at 0.005% concentration (B), $ZnSO_47H_2O$ at 0.002% concentration (Z), $MnSO_4H_2O$ at 0.005% concentration (M) and $CuSO_47H_2O$ at 0.003% concentration were added to N-free medium. Among the tested additives, when compared with the N-free basal medium, manganese salt showed the most effective increment in the cell density, up to $4.9x10^7$ cfu/ml (Fig.3.1.4.1), is a supporting result with the work of (Mishustin and Shilnikova, 1969) in which favorable action of manganese on *A. chroococcum* was reported. Zinc salt and boric acid addition into the basal medium were not effective on growth, although stimulatory effect of zinc was reported by Becking (1961) (Fig.3.1.4.1). Addition of copper salt into the medium was also tested and observed to inhibit the growth, is a similar result arguing that copper was toxic on *Azotobacter* (Becking, 1961).

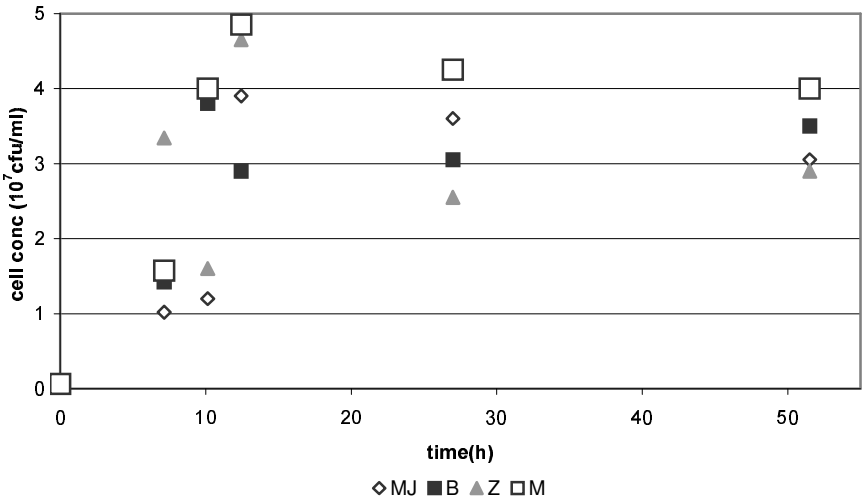

Fig. 3.1.4.1 Effect of Some Inorganic Salts and Boric Acid on Growth. *Azotobacter* is cultivated in MJ: N-free medium; B: N-free medium supplemented with 0.005% H_3BO_3; Z: N-free medium supplemented with 0.002% $ZnSO_47H_2O$; M: N-free medium supplemented with 0.005% $MnSO_4H_2O$.

In Fig. 3.1.4.2 effect of additives on protein concentration is illustrated. For all media tested extracellular protein concentration is parallel with growth and maximum values are obtained at 7-10 h of incubation, at logarithmic phase of growth. And all three additives; manganese and zinc containing salts and boric acid stimulated the extracellular protein concentration.

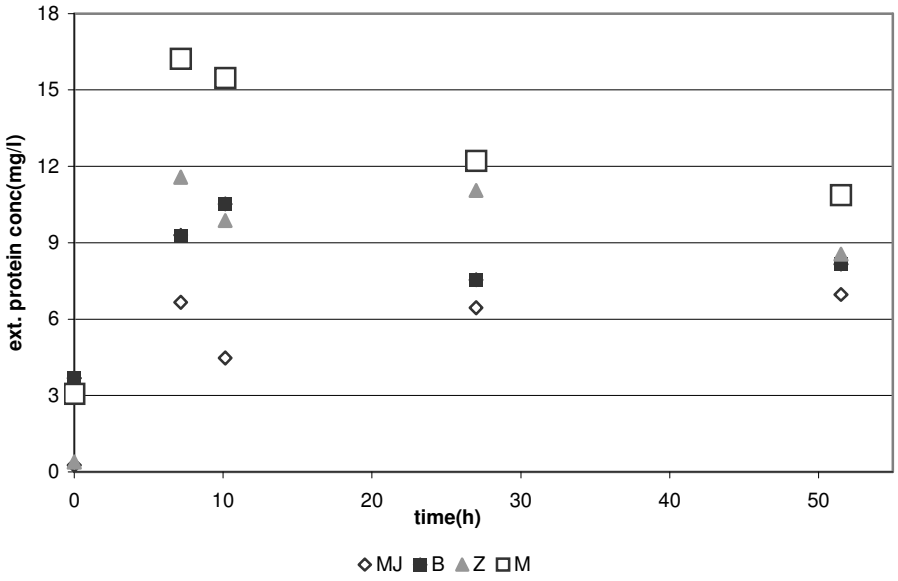

Fig. 3.1.4.2 Effect of Some Inorganic Salts and Boric Acid on Extracellular Protein Levels. *Azotobacter* is cultivated in MJ: N-free medium; B: N-free medium supplemented with 0.005% H_3BO_3; Z: N-free medium supplemented with 0.002% $ZnSO_47H_2O$; M: N-free medium supplemented with 0.005% $MnSO_4H_2O$.

And in Fig. 3.1.4.3, effect of additives on ammonia secretion, having a peak at 7h of incubation, is shown. At that time it can be seen that addition of manganese and zinc containing salts and boric acid had increased the ammonia concentration, as was with protein concentration.

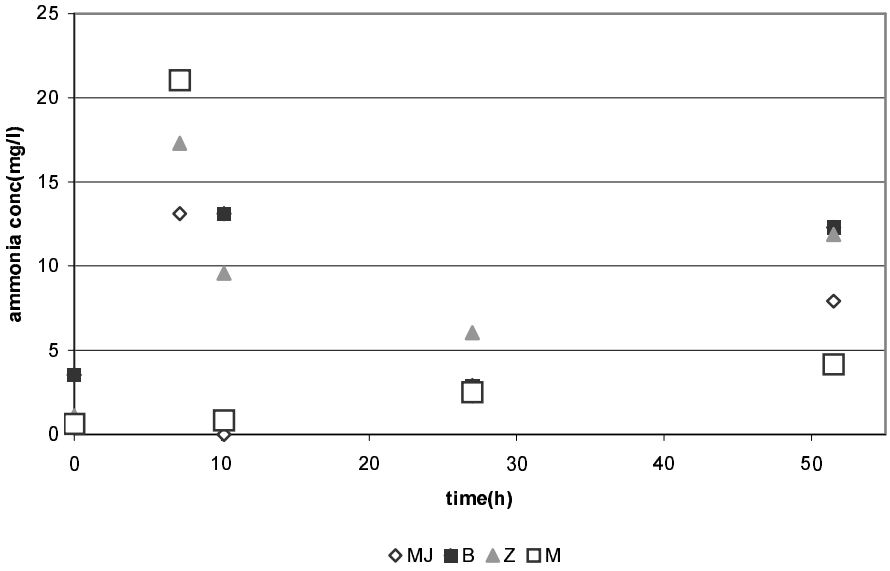

Fig. 3.1.4.3 Effect of Some Inorganic Salts and Boric Acid on Ammonia Secretion. *Azotobacter* is cultivated in MJ: N-free medium; B: N-free medium supplemented with 0.005% H_3BO_3; Z: N-free medium supplemented with 0.002% $ZnSO_47H_2O$; M: N-free medium supplemented with 0.005% $MnSO_4H_2O$.

3.1.4.1. Effect of Manganese Concentration

From the previous experiment (3.1.4), the highest increase at cell (Fig. 3.1.4.1), protein (Fig. 3.1.4.2) and ammonia (Fig. 3.1.4.3) concentrations were obtained with the supplementation of basal medium with manganese salt, so an experiment verifying the effect of $MnSO_4H_2O$ was added at 0%, 0.005% and 0.01% concentrations to the N-free medium. And the optimum concentration of MnSO4H2O was determined as 0.01%, since this addition rate showed the highest values for all parameters; growth (Fig.3.1.4.1.1), extracellular protein concentration (Fig.3.1.4.1.2) and ammonia concentration (Fig.3.1.4.1.3).

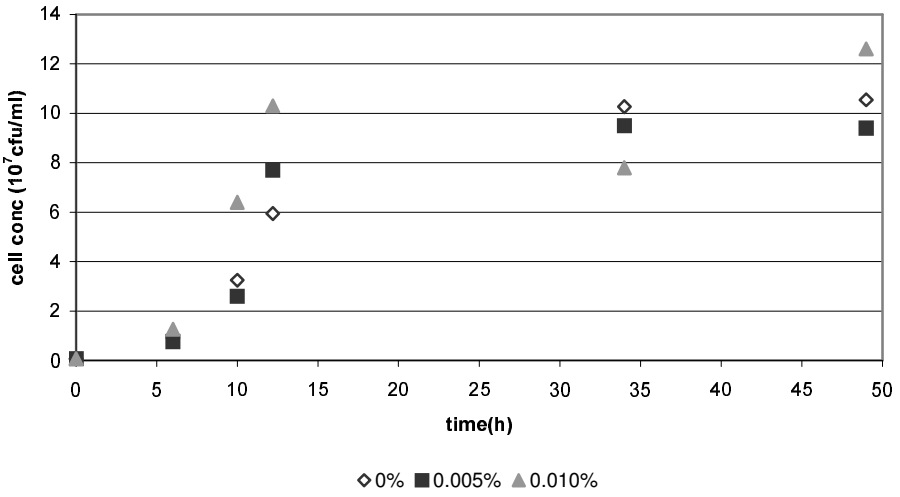

Fig. 3.1.4.1.1 Effect of Manganese Salt Concentration on Growth. *A. chroococcum* was cultivated at different $MnSO_4H_2O$ concentrations such as 0%, 0.005% and 0.010%.

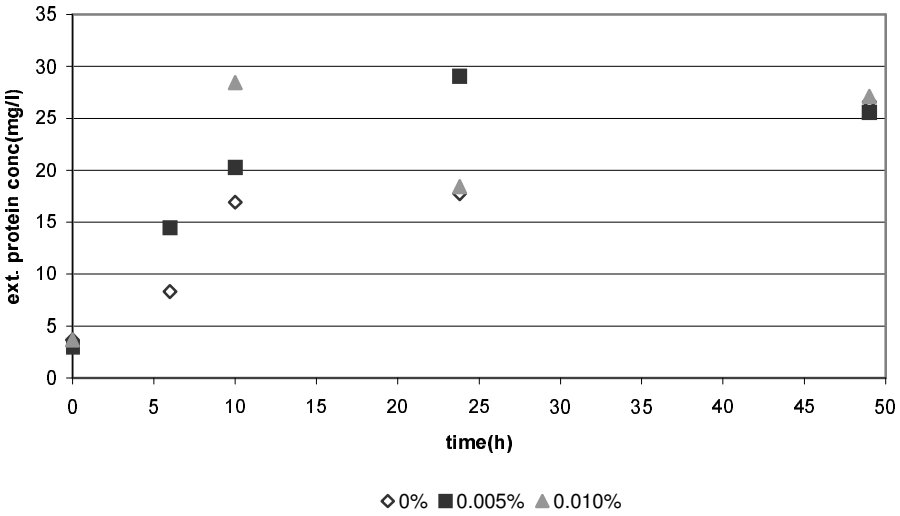

Fig. 3.1.4.1.2 Effect of Manganese Salt Concentration on Extracellular Protein Levels. *A. chroococcum* was cultivated at different $MnSO_4H_2O$ concentrations such as 0%, 0.005% and 0.010%.

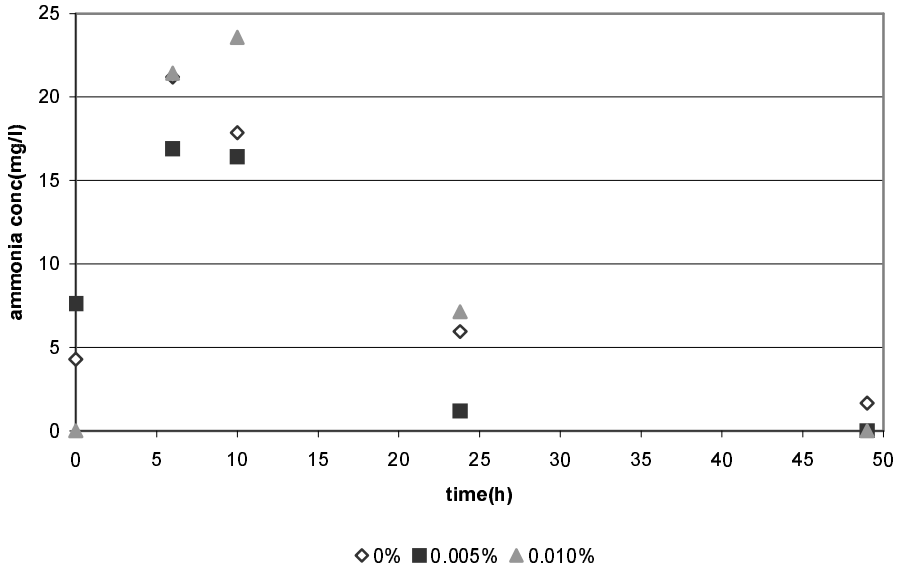

Fig. 3.1.4.1.3 Effect of Manganese Salt Concentration on Ammonia Secretion. *A. chroococcum* was cultivated at different $MnSO_4H_2O$ concentrations such as 0%, 0.005% and 0.010%.

3.2. Growth and Nitrogen Fixation in Nitrogenous Medium

The basal medium was supplemented with NH_4Cl to observe the effect of the presence of fixed nitrogen on the growth and as the evidence of nitrogen fixation; extracellular protein and ammonia concentrations. NH_4Cl was added to the growth medium at 0%, 0.05% and 0.1% concentrations and incubated at 35°C; 150 rpm and pH=7.

The presence of NH_4Cl at concentrations of 0.05% and 0.1% increased the cell density 10 fold when compared with N-free medium (Fig. 3.2.1). In other words, *A. chroococcum* with its nitrogenase enzyme, by fixing nitrogen, could only achieve the 1/10 of the cell density it can with nitrogenous medium.

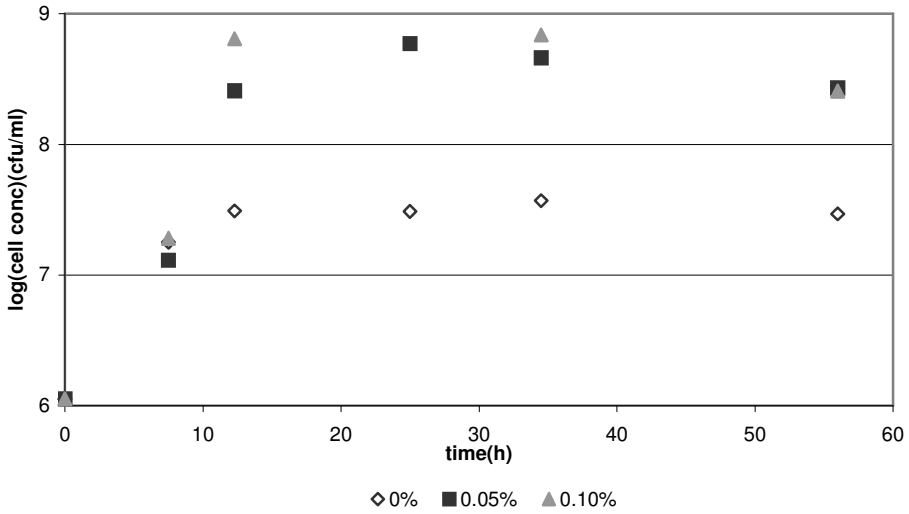

Fig. 3.2.1 Effect of Nitrogen Salt on Growth. *A. chroococcum* was cultivated at different NH_4Cl concentrations such as 0%, 0.05% and 0.10%.

In Fig.3.2.2 effect of fixed nitrogen on the protein production is illustrated, and the same profile with the growth was observed; a 10-fold increase with the addition of 0.05% and 0.1% NH_4Cl was observed.

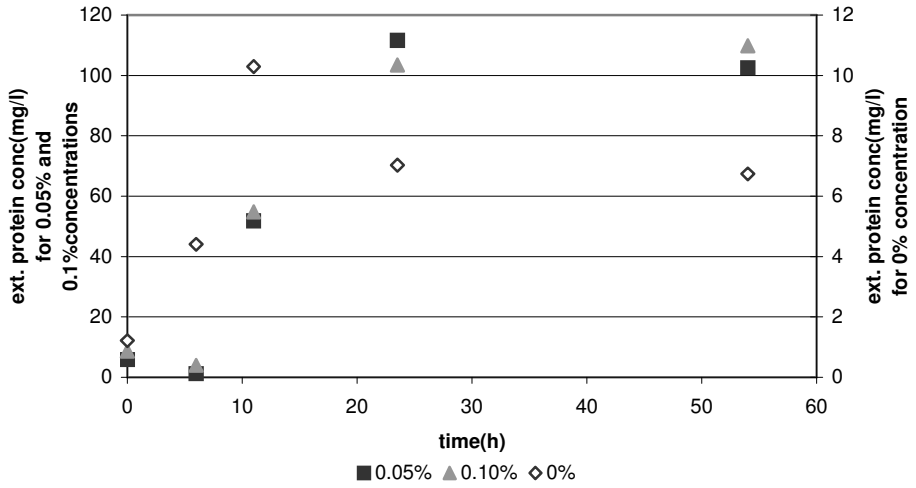

Fig. 3.2.2 Effect of Nitrogen Salt on Extracellular Protein Production. *A. chroococcum* was cultivated at different NH_4Cl concentrations such as 0%, 0.05% and 0.10%.

Ammonia secretion is constant during the incubation at nitrogenous medium, while in N-free medium it had showed a peak at 6^{th} h of incubation (Fig. 3.2.3). However, it can not be argued on the effect of fixed nitrogen on the nitrogen fixation, since although both the protein and ammonia concentrations in nitrogenous medium are higher than the that of N-free medium, it can not be decided that these products are results of nitrogen fixation or the growth with nitrogen assimilation.

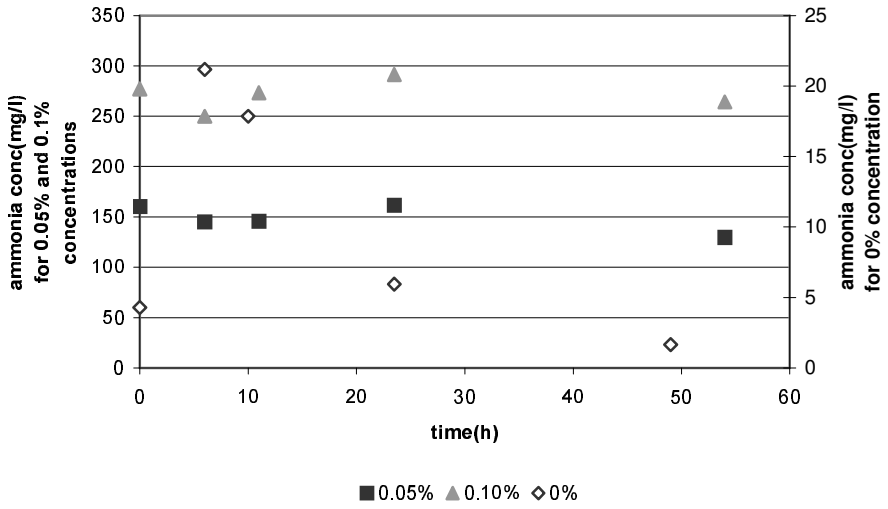

Fig. 3.2.3 Effect of Nitrogen Salt on Ammonia Secretion. *A. chroococcum* was cultivated at different NH_4Cl concentrations such as 0%, 0.05% and 0.10%.

3.3. Growth and Nitrogen Fixation in Olive Mill Wastewater (OMW) Containing Medium

A. chroococcum was incubated at the N-free medium supplemented with the OMW product of 2002. Two sets of experiments were done; one set with freshly received waste and the other set of experiments done after nine months of storage. OMW is a phenolic, nitrogenous and organic acid and oil residue containing substance. OMW was added to the medium without any treatment; it was added with its both suspended and dissolved solids contents. Since when the supernatant, formed after centrifugation, of the OMW was added to the N-free medium, *A. chroococcum* could not proliferate in such a medium. Furthermore, addition of OMW to the basal N-free medium lowered the pH to approximately 5. Although *A. chroococcum* could grow at pH 5 in chemically defined N-free medium (Fig. 3.1.1.1), it could not survive in the medium containing 5% OMW

without pH adjustment, that is at pH 5. So in this study, pH of the medium was adjusted with 2 N NaOH to 7 after sterilization.

3.3.1. Effect of Fresh OMW on Growth of *A. chroococcum*

In Fig. 3.1.1.1 N-free medium was supplemented with 0%, 5%, 10%, 15%, 20% and 30% (v/v) OMW. Fresh OMW was used as additive, i.e. experiments were performed immediately after the OMW was collected from the factory. The results plotted in Fig. 3.1.1.1 indicates that the maximum OMW concentration *A. chroococcum* can tolerate was between 20% and 30%, that is 30% OMW inhibited the growth of *A. chroococcum*. The maximum cell concentration achieved in this experiment was 5.2×10^8 cfu/ml, when *A. chroococcum* was incubated in 10% OMW containing N-free medium. In other words, cell concentration when *A. chroococcum* was incubated in 10% OMW containing N-free medium was 3 times that of the case when *A. chroococcum* was incubated in 0% OMW containing N-free medium.

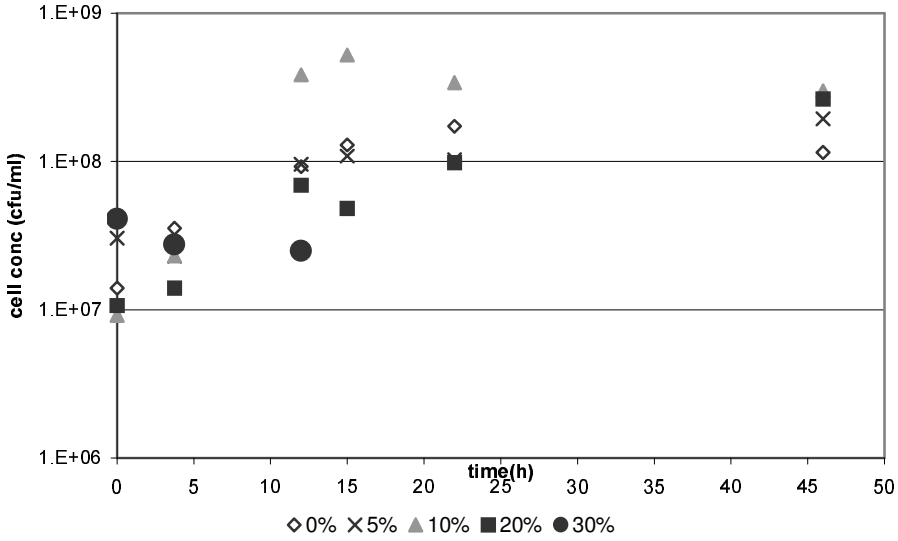

Fig. 3.3.1.1 Effect of OMW Concentration on Growth. *A.* chroococcum was cultivated in fresh OMW containing medium at concentrations of 0%, 5%, 10%, 20% and 30%.

A. chroococcum could not survive in 30% OMW containing medium (Fig. 3.1.1.1). Proliferation of *A. chroococcum* in 30% OMW was achieved by first incubating the organism in 20% OMW containing N-free medium for 24 h, and then transferring to 30% OMW containing N-free medium, at the 10% proportion. By this way, the maximum OMW concentration *A. chroococcum* can tolerate was increased to 30% from 20%. The cell concentration at the 144th h of incubation was 3.9x10^{8} cfu/ml (Fig 3.1.1.2). This result shows that *A. chroococcum* needs a pretreatment with a lower concentration of OMW in order to survive in higher concentrations.

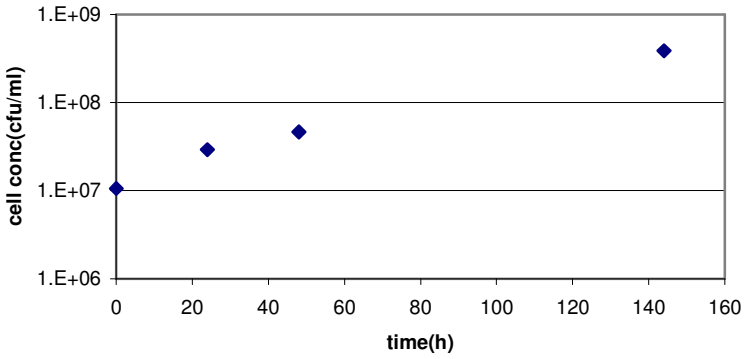

Fig. 3.3.1.2 Growth profile of *A. chroococcum* in 30% OMW containing N-free medium, which was inoculated at the 10% proportion from the 24 h incubated cell culture in 20% OMW containing N-free medium.

3.3.2. Effect of Stored OMW on Growth of *A. chroococcum*

In Fig. 3.1.1.3, N-free medium was supplemented with 0%, 5%, 10%, 15% and 20% (v/v) OMW, of which pH was adjusted to 7. The OMW used in this experiment was the same with the one used in the experiment whose results were presented at Fig. 3.1.1.1 and 3.1.1.2, but this one was stored at 4°C for about 9 months. These media were inoculated with the 24 h incubated preculture medium to obtain an initial cell load of approximately 10^6 cfu/ml. And then *A. chroococcum* was cultivated at 35°C, 150 rpm. The maximum OMW concentration *A. chroococcum* can grow in was determined as 10%, in other words 15% and 20% OMW containing medium had inhibited the growth of *A. chroococcum*. In Fig. 3.3.1.3 cell densities achieved at the 39^{th} h of incubation, at the stationary growth phase, for the 0%, 5% and 10% OMW containing medium were plotted. And the cell concentrations were $2.5x10^7$ cfu/ml, $1.5x10^8$ cfu/ml and $3.1x10^8$ cfu/ml in the media containing 0%, 5% and 10% OMW, respectively. The supplementation of N-free medium with OMW up to 10% stimulated the proliferation *A. chroococcum* in nitrogen deficient medium. When the results of Fig. 3.3.1.1 and Fig. 3.3.1.2 were compared with that of the Fig.

3.3.1.3, the effect of storage of OMW for longer time can be observed. The maximum OMW concentration that *A. chroococcum* can tolerate was lowered to 10% from 20% when the non-fresh OMW was used. With the fresh OMW used, the cell concentration of the 20% OMW containing culture was 3 times that of 0% OMW containing culture (Fig. 3.1.1.1) and with stored OMW the cell concentration of 10% OMW containing culture was 12 times that of 0% OMW containing culture (Fig. 3.1.1.3). The reason for variations in growth behavior can be contributed to the fact that possibly chemical composition of OMW changed during storage. Comparatively, in a similar study (Garcia-Barrioneuevo *et al*., 1993), in which growth of *A. chroococcum* was cultivated in glucose containing N-free medium supplemented with 0%, 1%, 5%, 10%, 15% and 20% OMW, was inhibited 71.5, 90.0, 91.5, 94.3 and 97.2%, respectively. In other words, OMW addition enhanced the growth while in the work of Garcia-Barrioneuevo (1993) and co-workers OMW addition inhibited growth although *A. chroococcum* caould survieve. It is obvious that these results are also different from our results in the maximum OMW concentration that the organisms can tolerate. The maximum OMW concentration that the organism can tolerate was 20% in the study of Garcia-Barrioneuevo (1993) and co-workers while that value was 10% in our experiments in Fig. 3.3.1.3 and 20% in Fig. 3.3.1.1 and increased to 30% in Fig. 3.3.1.2. These varieties can be attributed to the differences in the chemical compositions of the OMWs used in the different studies, since they were provided from different sources.

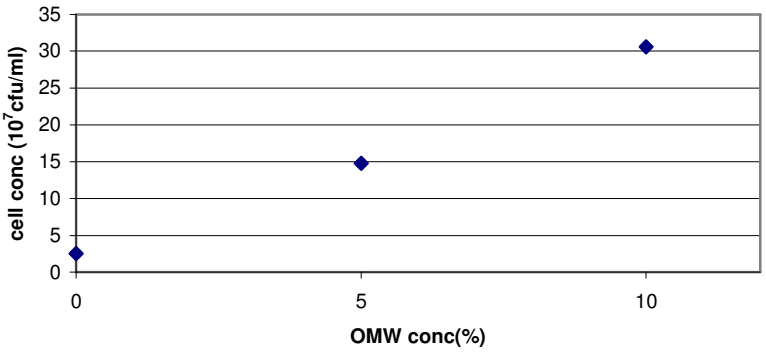

Fig. 3.3.1.3 Effect of OMW Concentration on Growth. *A.* chroococcum was cultivated in stored OMW containing medium at concentrations of 0%, 5% and 10%.Cell concentrations were measured at the 39th h of incubation, at the stationary growth phase.

Furthermore, *A. chroococcum* was cultivated in OMW containing medium was supplemented with manganese (0.01% $MnSO_4H_2O$), since the addition of manganese into the N-free medium improved the growth (3.1.4). However, addition of manganese in OMW containing medium inhibited growth instead.

3.3.3 Effect of Different OMW Concentrations on Nitrogen Fixation

In Table 3.3.1.1 initial protein concentration present in the medium, protein concentration at the end of incubation (at the 39th h of incubation) and the increase in protein concentration is introduced, where the highest protein concentration increase was observed when *A. chroococcum* was incubated in 10% OMW containing medium and in Table 3.3.1.2 initial ammonia concentration present in the medium, secreted at the end of incubation and the increase in ammonia concentration during the cultivation can be seen. And the highest increase in ammonia concentration was observed when *A. chroococcum* was incubated in 10% OMW containing medium. However, as was the case in

nitrogenous medium (3.2), the protein and ammonia produced at the end of cultivation can be either the products of nitrogen fixation process or the growth by using the nitrogen present in the medium.

Table 3.3.1.1 Effect of OMW Concentration on Extracellular Protein Levels

OMW Concentration (%)	Initial Protein Concentration (mg/l)	Final Extracellular Protein Concentration. *At the 39th of incubation* (mg/l)	Increase in Extracellular Protein Concentration (mg/l)
0	0.00	8.92	8.92
5	652.5	701.7	48.83
10	1148	1260	112.3

Table 3.3.1.2 Effect of OMW Concentration on Ammonia Concentrations

OMW Concentration (%)	Initial Ammonia Concentration (mg/l)	Final Ammonia Concentration. *At the 39th of incubation* (mg/l)	Increase in Ammonia Concentration (mg/l)
0	0.00	1.19	1.19
5	40.0	49.1	9.05
10	88.6	92.9	4.29

CHAPTER 4

CONCLUSIONS

The dynamics of growth and nitrogen fixation at different physiological conditions and nutrient requirements of *A. chroococcum* in chemically defined N-free medium was determined. The maximum cell concentration were obtained when *A. chroococcum* was grown at neutral pH, 35°C, 150 rpm and in medium supplemented with manganese salt at 0.01% concentration as $4.8x10^7$ cfu/ml. The maximum nitrogen fixation products were attained when *A. chroococcum* was grown at pH 8, 35°C, 150 rpm and in medium supplemented with manganese salt at 0.01% concentration as 10.4 mg/l extracellular protein and 1.5 mg/l ammonia.

Altering aeration conditions did not change the growth and nitrogen fixation activity, greatly.

Addition of manganese salt caused increase in growth (24%), extracellular protein concentration (133%) and ammonia concentration (60%). However, copper salt inhibited the growth. As a further work, the effect of combination of manganase and zinc salts and boric acid on growth and nitrogen fixation should be studied.

In bioremediation of OMW studies *A. chroococcum* could only grow at pH 7 rather than the usual pH of OMW. Also *A. chroococcum* could only grow when the OMW was added into medium with its both suspended and dissolved solids content.

The maximum OMW concentration *A. chroococcum* can tolerate was between 20% and 30% with fresh OMW and 10% and 15% with stored OMW. The cell density in 10% OMW containing medium was determined as 3 times (with fresh OMW) and 12 times (with stored OMW) higher than in the OMW free medium was achieved. When fresh OMW was used, 30% OMW concentration inhibited the growth of *A. chroococcum*. However when *A. chroococcum* was grown in 20% OMW and then transferred to 30% OMW, organism could tolerate 30% OMW.

Supplementation of growth medium with OMW and manganese salt, together, caused the growth inhibition of *A. chroococcum*.

Also it was found to have maximum increase in extracellular protein concentration (112 mg/l) at 10% OMW containing medium and maximum increase in ammonia concentration (9.05 mg/l) at 5% OMW containing medium.

These findings suggest that application of diluted OMW to growth medium is not likely to be detrimental to *A. chroococcum* up to a certain concentration of 20% (with fresh OMW) and 10% (with stored OMW), that is; *A. chroococcum* could survive in environments polluted with OMW. Additionally, during cultivation in such medium *A. chroococcum* secretes some substances available for plants, such as protein and ammonia. Also since OMW is a phenolic substance and *A. chroococcum* has grown in this medium, it should be tested that if *A. chroococcum* can degrade these phenolic substances, that may be toxic to some plants or other organisms, for further studies.

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APPENDIX A

REAGENTS COMPOSITION OF LOWRY METHOD

Reagent A

2 g potassium sodium tartrate ($C_4H_4KNaO_6.4H_2O$) and 100 g Na_2CO_3 are dissolved in 500 ml 1 N NaOH and diluted with water to 1 lt.

Reagent B

2 g potassium sodium tartrate ($C_4H_4KNaO_6.4H_2O$) and 1 g $CuSO_4.5H_2O$ are dissolved in 90 ml water and 1 N NaOH is added to this solution.

Reagent C

1 vol. Folin-Ciocalteu reagent is diluted with 15 vol. water. This reagent is prepared daily.

APPENDIX B

STANDARD CURVE PREPARATION TABLE AND STANDARD CURVE FOR LOWRY METHOD

Prepare a stock solution of 1 mg/ml BSA

Table B.1 Preparation of Standard Solutions of BSA

Tube #	dH_2O (ml)	0.02 mg BSA/ml	0.05 mg BSA/ml	0.10 mg BSA/ml	0.15 mg BSA/ml	0.20 mg BSA/ml
1	1	-	-	-	-	-
2	-	1	-	-	-	-
3	-	-	1	-	-	-
4	-	-	-	1	-	-
5	-	-	-	-	1	-
6	-	-	-	-	-	1

Procedure

- After preparing the standard tubes and samples at the appropriate dilutions, 0.9 ml of Reagent A is added to each tube and tubes are placed in a water bath at 50 ^{O}C for 10 min.
- The tubes are cooled to room temperature, and all tubes are treated with 0.1 ml of Reagent B.

- The solutions are left at room temperature for at least 10 min, then 3 ml of Reagent C is forced in rapidly to ensure mixing within 1 sec.
- The tubes are again heated at 50 ^{O}C for 10 min. and cooled to room temperature and read at 650 nm.
- Then the protein concentration was determined according to the standard curve (Fig B.1).

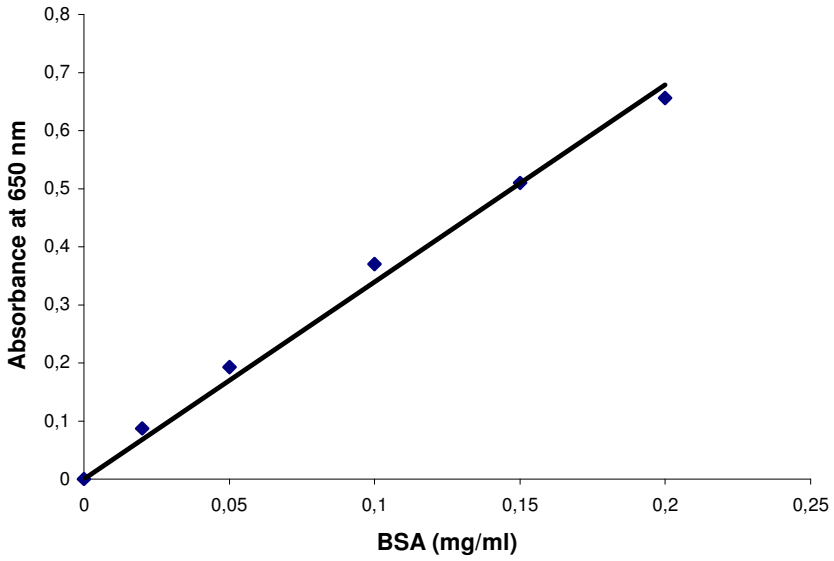

Fig. B.1 Standard Curve for Lowry Method

APPENDIX C

REAGENTS COMPOSITION OF NESSLERIZATION METHOD

Zinc Sulphate Solution

100 g $ZnSO_4$ $7H_2O$ was dissolved and to 1l with distilled water.

Nessler Reagent

100 g HgI_2 and 70 g KI was dissolved in a small quantity of distilled water and this mixture was added slowly, with stirring, to a cool solution of 160 g NaOH dissolved in 500 ml distilled water. Diluted to 1l. Reagent was stored in rubber-stoppered borosilicate glassware and out of sunlight to maintain reagent stability for up to a year under normal laboratory conditions.

Stock Ammonium Solution

3.819 g anhydrous NH_4Cl, dried at 100°C, dissolved in distilled water and diluted to 1l; 1.00ml = 1.00 mg N = 1.22 mg NH_3.

Standard Ammonium Solution

10.00 ml stock ammonium solution was diluted to 1000 ml with distilled water; 1.00 ml = 10.00 µg N = 12.2 µg NH_3.

APPENDIX D

PROCEDURE AND STANDARD CURVE PREPARATION FOR NESSLERIZATION METHOD

Procedure

Pretreatment

1 ml $ZnSO_4$ solution was added to 100 ml sample and mixed thoroughly. 0.4 to 0.5 ml 6N NaOH solution was added to obatain a pH of 10.5. Treated sample was let to stand for a few minutes, whereupon a heavy flocculent precipitate should fall, leaving a clear and colorless supernate. It was clarified by centrifugation or filtration. Filter paper should be pretested to be sure no ammonia was present as a contaminant. This was done by running water through the filter and testing the filtrate by nesslerization. Sample was filtered discarding first 25 ml filtrate.

Color Development

50.0 ml sample or a portion diluted to 50.0 ml with distilled water was used. 1 ml Nessler reagent was added and mixed by inverting the tubes. Such conditions as temperature and reaction time was kept the same in blank, samples and standards. Reaction was let to proceed for at least 10 min after adding Nessler reagent. If NH_3-N was very low, 30 min contact time for sample, blank and standards was used. Color was measured in sample and standards by using spectrophotometer. The yellow color characteristic of low ammonia nitrogen concentration (0.4 to 5 mg/l) can be

measured with acceptable sensitivity in the wavelength region from 400 to 425 nm. The reddish brown hues typical of ammonia nitrogen levels approaching 10 mg/l may be measured in the wavelength region of 450 to 500 nm. And a standard curve under the same conditions as the samples. And the concentration of ammonia present in the sample was determined from standard curve (Fig. D.1)

Standard Curve

A series of standards was prepared in tubes by adding the following volumes of standard Ammonium solution and diluting to 50 ml with distilled water: 0, 0.2, 0.7, 1.4, 2.0, 2.5, 3.0, 4.0, 5.0 and 6.0 ml. Standards are nesslerized by adding 1.0 ml Nessler reagent to each tube and mixing well.

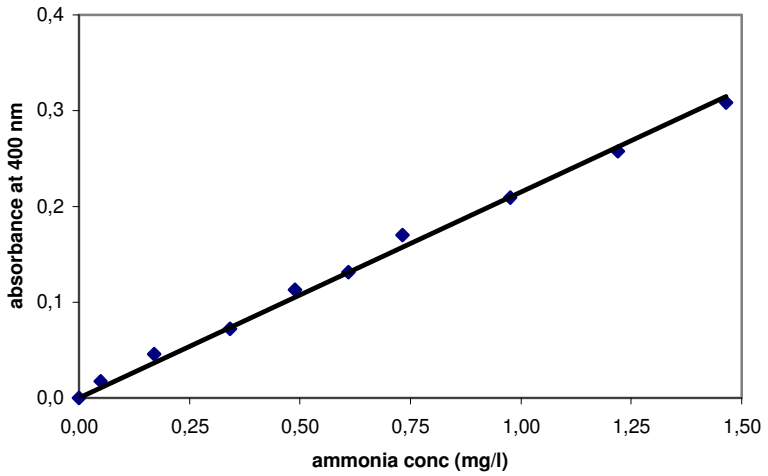

Fig. D.1 Standard Curve for Nesslerization

APPENDIX E

DATA OF THE FIGURES IN CHAPTER 3

Table E.1 Data of Fig. 3.1.1.1

time(h)	Cell concx10^{-7} (cfu/ml) at pH=5	Cell concx10^{-7} (cfu/ml) at pH=6	Cell concx10^{-7} (cfu/ml) at pH=7	Cell concx10^{-7} (cfu/ml) at pH=8
0.0	0.430 ± 0.060	0.730 ± 0.060	0.700 ± 0.100	0.050 ± 0.070
6.00	0.730 ± 0.150	1.07 ± 0.06	1.20 ± 0.00	0.300 ± 0.100
10.0	2.03 ± 0.76	3.10 ± 0.10	3.20 ± 0.26	2.70 ± 0.00
12.0	3.20 ± 0.56	4.03 ± 0.12	3.95 ± 0.07	3.40 ± 0.17
23.0	3.87 ±0.15	4.43 ± 0.15	4.83 ± 0.06	4.15 ± 0.07
33.5	3.90 ± 0.10	4.33 ± 0.12	4.77 ± 0.15	3.97 ± 0.23
48.0	3.87 ± 0.06	4.23 ± 0.29	4.40 ± 0.10	3.60 ± 0.00

Table E.2 Data of Fig. 3.1.1.2

time(h)	Extracellular Protein Conc (mg/l) at pH=5	Extracellular Protein Conc (mg/l) at pH=6	Extracellular Protein Conc (mg/l) at pH=7	Extracellular Protein Conc (mg/l) at pH=8
0.0	1.03 ± 5.27	0.280 ± 0.520	0.760 ± 0.520	0.310 ± 0.440
6.00	1.65 ± 0.74	0.760 ± 0.730	1.03 ± 1.15	3.31 ± 0.00
10.0	2.55 ± 0.12	2.07 ± 1.15	3.24 ± 1.25	3.93 ± 0.55
12.0	3.58 ± 0.66	2.89 ± 1.09	4.41 ± 0.84	4.27 ± 0.43
23.0	5.23 ± 1.49	3.86 ±.2.19	3.93 ±.1.35	5.23 ±.1.14
33.5	4.89 ± 1.14	6.61 ± 1.65	5.89 ± 1.31	8.33 ± 1.33
48.0	6.20 ± 1.46	7.16 ±.0.12	8.61 ± 0.78	10.40 ± 1.55

Table E.3 Data of Fig. 3.1.1.3

time(h)	Ammonia Conc (mg/l) at pH=5	Ammonia Conc (mg/l) at pH=6	Ammonia Conc (mg/l) at pH=7	Ammonia Conc (mg/l) at pH=8
0.0	0.460 ± 0.070	0.130 ± 0.860	0.070 ± 0.660	0.00 ±0 00
6.00	1.03 ± 0.62	0.500 ± 0.140	0.720± 0.620	0.480 ± 0. 010
10.0	1.54 ± 0.56	0.320 ± 0.290	0.950 ± 0.080	0.630 ± 0.000
12.0		0.950 ± 1.760	0.960 ± 0 460	
23.0	0.670 ± 0.020		1.07 ± 0.26	1.50 ± 0.00
33.5		0.0600 ± 0.0000	0.450 ± 0.260	1.40 ± 0.00
48.0	0.470 ± 0.020	0.250 ± 0.930	0.0300 ± 0.2000	1.17 ± 1.46

Table E.4 Data of Fig. 3.1.2.1

time(h)	Cell concx10^{-7} (cfu/ml) at T=30°C	Cell concx10^{-7} (cfu/ml) at T=35°C	Cell concx10^{-7} (cfu/ml) at T=40°C
0.0	0.200 ± 0.000	0.200 ±0.000	0.450 ± 0.070
6.00	0.470 ± 0.120	0.500 ±0 .100	0.850 ± 0.210
10.0	2.67 ± 0.06	2.70 ± 0.10	1.75 ± 0.21
12.0	2.50 ± 0.06	2.40 ± 0.10	2.30 ± 0.14
24.0	3.17 ± 0.15	3.30 ± 0.10	2.80 ± 0.42
35.8	3.10 ± 0.06	2.97 ± 0.36	2.50 ± 0.14
48.0	3.40 ± 0.17	3.43 ± 0.06	2.55 ± 0.21
59.8	3.40 ± 0.10	3.60 ± 0.00	

Table E.5 Data of Fig. 3.1.2.2

time(h)	Extracellular Protein Conc (mg/l) at T=30oC	Extracellular Protein Conc (mg/l) at T=35oC	Extracellular Protein Conc (mg/l) at T=40oC
0.0	0.620 ± 0.410	0.620 ± 0.410	0.00 ± 0.00
6.00	1.58 ± 0.55	1.86 ± 1.47	2.92 ± 1.17
10.0	4.41 ± 0.12	4.61 ± 0.73	4.27 ± 1.90
12.0	3.79 ± 0.72	3.31 ± 0.32	3.33 ± 0 00
24.0	4.75 ± 1.73	5.85 ± 0.55	
36.0	5.58 ± 0.72	7.64 ± 1.29	6.46 ± 0.00
48.0			5.83 ± 0.00
59.8	8.07 ± 0.79	8.42 ± 1.75	

Table E.6 Data of Fig. 3.1.2.3

time(h)	Ammonia Conc (mg/l) at T=30oC	Ammonia Conc (mg/l) at T=35oC	Ammonia Conc (mg/l) at T=40oC
0.0	0.760 ± 0.190	0.760 ± 0.190	0.430 ± 0.190
6.00	0.440 ± 0.220	0.470 ± 0.140	0.670 ± 0.380
10.0	0.00 ± 0.00	0.270 ± 0.000	0.450 ± 0.260
12.0	0.170 ± 0.000	0.00 ± 0.00	0.400 ± 0.420
24.0	6.08 ± 0.10	4.42 ± 0.43	0.330 ± 0.240
35.8	5.52 ± 0.98	4.63 ± 0.40	1.99 ± 0.00
48.0	2.37 ± 1.04	1.37 ± 0.33	1.25 ± 0.26
59.8	3.87 ± 0.75	1.47 ± 0.24	

Table E.7 Data of Fig.3.1.3.1

time(h)	Cell concx10^{-7} (cfu/ml) at A	Cell concx10^{-7} (cfu/ml) at B	Cell concx10^{-7} (cfu/ml) at C
0.0	0.200 ± 0.000	0.200 ± 0.000	0.200 ± 0.000
6.00	0.500 ± 0.100	0.630 ± 0.060	0.630 ± 0.060
10.0	2.70 ± 0.10	2.83 ± 0.06	2.70 ± 0.10
12.0	2.40 ± 0.10	2.57 ± 0.21	2.50 ± 0.36
24.0	3.30 ± 0 36	3.23 ± 0.15	3.07 ± 0.29
35.8	2.97 ± 0.06	3.27 ± 0 .06	3.13 ± 0.23
48.0	3.43 ±0.12	3.23 ± 0.06	3.33 ± 025
59.8	3.60 ± 0.00	3.10 ± 0.10	3.25 ± 0.07

Table E.8 Data of Fig.3.1.3.2

time(h)	Extracellular Protein Conc (mg/l) at A	Extracellular Protein Conc (mg/l) at D	Extracellular Protein Conc (mg/l) at C
0.0	0.620 ± 0.410	0.620 ± 0.410	0.620 ± 0.410
6.00	1.86 ± 0.55	2.75 ± 0.86	2.07 ± 0.55
10.0	4.61 ± 0.12	4.89 ± 0.98	4.34 ± 0.52
12.0	3.31 ± 0.72	5.17 ± 0.88	3.37 ± 0.32
24.0	5.85 ± 0.12	6.20 ± 1.07	5.72 ± 0.55
35.8	7.64 ± 0.72	6.51 ± 0.15	6.61 ± 1.09
59.8	8.42 ± 0.79	9.08 ± 0.19	7.89 ± 1.58

Table E.9 Data of Fig.3.1.3.3

time(h)	Ammonia Conc (mg/l) at A	Ammonia Conc (mg/l) at D	Ammonia Conc (mg/l) at C
0.0	0.800 ± 0.200	0.800 ± 0.200	0.800 ± 0.200
6.00	0.740 ± 0.000	2.82 ± 0.00	0.640 ± 0.110
10.0		0.110 ± 0.000	0.280 ± 0.000
12.0	0.180 ± 0.000	0.530 ± 0.010	
24.0	6.44 ± 0.11	3.81 ± 1.41	6.13 ± 0.21
35.8	5.85 ± 1.04	5.80 ± 1.12	5.91 ± 0.94
48.0	2.51 ± 1.11	1.60 ± 0.35	2.86 ± 0.37
59.8	3.87 ± 0.75	1.53 ± 0.98	3.30 ± 2.5

Table E.10 Data of Fig. 3.1.4.1

time(h)	Cell concx10^{-7} (cfu/ml) at MJ	Cell concx10^{-7} (cfu/ml) at B	Cell concx10^{-7} (cfu/ml) at Z	Cell concx10^{-7} (cfu/ml) at M
0.0	0.0600 ± 0.0000	0.0600 ± 0.0000	0.0600 ± 0.0000	0.0600 ± 0.0000
7.20	1.02 ± 0.30	1.43 ± 0.13	3.34 ± 0.00	1.57 ± 0.00
10.2	1.20 ± 0.28	3.80 ± 0.00	1.60 ± 1.91	4.00 ± 0 .00
12.5	3.90 ± 0.28	2.90 ± 0.14	4.65 ± 1.06	4.85 ± 0.35
27.0	3.60 ±1.98	3.05 ± 1.48	2.55 ± 1.06	4.25 ± 0.00
51.5	3.05 ± 0.21	3.50 ± 0.28	2.90 ± 0.57	4.00 ± 1.06

Table E.11 Data of Fig. 3.1.4.2

time(h)	Extracellular Protein Conc (mg/l) at MJ	Extracellular Protein Conc (mg/l) at B	Extracellular Protein Conc (mg/l) at Z	Extracellular Protein Conc (mg/l) at M
0.0	0.263 ±.1.860	3.68 ± 1.86	0.390 ± 3.720	3.07 ± 0.6
7.20	6.67 ± 2.19	9.30 ± 2.19	11.6 ± 5.2	16.2 ± 2.1
10.2	4.47 ± 0.74	10.5 ± 0.7	9.87 ± 5.43	15.5 ± 1.7
27.0	6.45 ± 1.61	7.54 ± 1.61	11.1 ± 5.6	12.2 ± 7.5
51.5	6.97 ± 3.35	8.16 ± 3.35	8.55 ± 3.63	10.9 ± 2.9

Table E.12 Data of Fig. 3.1.4.3

time(h)	Ammonia Conc (mg/l) at MJ	Ammonia Conc (mg/l) at B	Ammonia Conc (mg/l) at Z	Ammonia Conc (mg/l) at M
0.00	3.54 ± 0.43	3.54 ± 2.92	1.11 ± 1.27	0.625 ± 1.180
7.15	13.1 ± 1.8	20.8 ± 1.0	17.3 ± 2.7	21.0 ± 7.7
10.2	0.00 ± 0.00	13.1 ± 1.5	9.58 ± 1.91	0.83 ± 0.00
27.0	2.29 ± 0.12	2.92 ± 2.20	6.04 ± 1.34	2.50 ± 2.39
51.5	7.92 ± 0.07	12.3 ± 1.9	11.9 ± 0.51	4.17 ± 6.25

Table E.13 Data of Fig. 3.1.4.1.1

time(h)	Cell concx10^{-7} (cfu/ml) at 0%	Cell concx10^{-7} (cfu/ml) at 0.005%	Cell concx10^{-7} (cfu/ml) at 0.010%
0.0	0.0717 ± 0.0000	0.0673 ± 0.0000	0.0673
6.00	0.910 ± 0.340	0.767 ± 0.000	1.27
10.0	3.25 ± 0.49	2.60 ± 1.92	6.40
12.2	5.95 ± 0.07	7.70 ± 0.90	10.3
34.0	10.3 ± 1.5	9.50 ± 0.00	7.80
49.0	10.6 ± 0.9	9.40 ± 2.77	12.6

Table E.14 Data of Fig. 3.1.4.1.2

time(h)	Extracellular Protein Conc (mg/l) at 0%	Extracellular Protein Conc (mg/l) at 0.005%	Extracellular Protein Conc (mg/l) at 0.010%
0.0	3.60 ± 2.20	2.98 ± 3.35	3.68 ± 0.00
6.00	8.33 ± 4.06	14.5 ± 3.5	
10.0	16.9 ± 3.4	20.3 ± 10.3	28.4 ± 3.4
23.8	17.7 ± 4.1	29.0 ± 3.9	18.4 ± 0.0
49.0	26.6 ± 7.5	25.6 ± 3.0	27.1 ± 22.7

Table E.15 Data of Fig. 3.1.4.1.3

time(h)	Ammonia Conc (mg/l) at 0%	Ammonia Conc (mg/l) at 0.005%	Ammonia Conc (mg/l) at 0.01%
0.0	4.29 ± 6.06	7.62 ± 6.75	0.00 ± 0.00
6.00	21.2 ± 3.1	16.9 ± 2.9	21.4 ± 2.2
10.0	17.9 ± 2.6	16.4 ± 2.2	23.6 ± 7.07
23.8	5.95 ± 5.45	1.19 ± 5.02	7.14 ± 2.02
49.0	1.67 ± 5.02	0.00 ± 0.00	0.00 ± 0.00

Table E.16 Data of Fig. 3.2.1

time(h)	Cell Conc log(cfu/ml) at 0%	Cell Conc log(cfu/ml) at 0.050%	Cell Conc log(cfu/ml) at 0.10%
0.0	6.05 ± 0.15	6.05 ± 0.15	6.05 ± 0.15
7.50	7.25 ± 0.06	7.11 ± 0.10	7.28 ± 0.00
12.3	7.49 ± 0.09	8.41 ± 0.18	8.81 ± 0.00
25.0	7.49 ± 0.22	8.77 ± 0.14	
34.5	7.57 ± 0.14	8.66 ± 0.00	8.84 ± 0.19
56.0	7.47 ± 0.05	8.43 ± 0.05	8.41 ± 0.13

Table E.17 Data of Fig. 3.2.2

time(h)	Extracellular Protein Conc (mg/l) at 0%	Extracellular Protein Conc (mg/l) at 0.050%	Extracellular Protein Conc (mg/l) at 0.10%
0.0	1.22 ± 4.78	5.90 ± 4.83	8.71 ± 7.45
6.00	4.40 ± 3.26	1.22 ± 5.07	4.03 ± 5.04
11.0	10.3 ± 2.6	51.8 ± 12.5	54.8 ± 9.35
23.5	7.02 ± 5.62	112 ± 15	103 ± 19
54.0	6.74 ± 4.94	103 ± 15	110 ± 12

Table E.18 Data of Fig. 3.2.3

time(h)	Ammonia Conc (mg/l) at 0%	Ammonia Conc (mg/l) at 0.050%	Ammonia Conc (mg/l) at 0.10%
0.0	4.29 ± 0.06	160 ± 6	277 ± 18
6.00	21.2 ± 3.1	145 ± 7	250 ± 18
10.0	17.9 ± 2.6		
11.0		146 ± 10	273 ± 24
23.5	5.95 ± 5.4	161 ± 8	291 ± 16
49.0	1.67 ± 5.02		
54.0		130 ± 8	264 ± 16

Table E.19 Data of Fig. 3.3.1.1

time(h)	Cell Conc (cfu/ml) at 0%	Cell Conc (cfu/ml) at 5%	Cell Conc (cfu/ml) at 10%	Cell Conc (cfu/ml) at 20%	Cell Conc (cfu/ml) at 30%
0.0	1.40E+07	3.04E+07	9.20E+06	1.07E+07	4.10E+07
3.8	3.55E+07	2.30E+07	2.31E+07	1.40E+07	2.75E+07
12.0	9.22E+07	9.57E+07	3.83E+08	6.93E+07	2.50E+07
15.0	1.29E+08	1.09E+08	5.22E+08	4.83E+07	
22.0	1.73E+08	1.03E+08	3.40E+08	9.80E+07	
46.0	1.15E+08	1.94E+08	3.00E+08	2.64E+08	

Table E.20 Data of Fig. 3.3.1.2

time(h)	Cell Conc (cfu/ml)
0.00	1.06E+07
24.0	2.92E+07
48.0	4.62E+07
144	3.87E+08

Table E.21 Data of Fig. 3.3.1.3

OMW conc (%)	Cell concx10^{-7} (cfu/ml)
0	2.54 ± 0.75
5	14.8 ± 3.1
10	30.6 ± 5.5