89sh.

UTILIZATION OF NITROGEN FIXING ORGANISMS IN BIOLOGICAL TREATMENT OF NITROGEN DEFICIENT WASTEWATERS

A Thesis Submitted to the
Graduate School of Natural and Applied Sciences of
Dokuz Eylül University
In Partial Fulfilment of the Requirements for
the Degree of Master of Science in Environmental Engineering, Environmental Technology Program

by

Serpil ÖZMIHÇI

January, 2002
İZMİR

M.Sc THESIS EXAMINATION RESULT FORM

We certify that we have read this thesis and that "**UTILIZATION OF NITROGEN FIXING ORGANISMS IN BIOLOGICAL TREATMENT OF NITROGEN DEFICIENT WASTEWATERS**" completed by **Serpil ÖZMIHÇI** under supervision of **PROF. DR. Fikret KARGI** and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

119646

.....

Prof. Dr. Fikret KARGI

Supervisor

.....

Doç. Dr. Rengin ELTEM

Committee Member

.....

Yrd. Doç. Dr. Ilgi K. KAPDAN

Committee Member

Approved by the

Graduate School of Natural and Applied Sciences

Prof. Dr. Cahit HELVACI

Director

ACKNOWLEDGMENTS

I would like to thank to my supervisor *Prof. Dr. Fikret KARGI* for his guidance, motivation, valuable advises and for his patience.

I would like to thank to all of my friends, especially to Ezgi OKTAV and Öztan S. KÜÇÜK for their patience and help during the course of this study.

Special thanks to my family for their love and invaluable support.

I dedicate this thesis to my parents.

Serpil ÖZMIHÇI

ABSTRACT

Biological treatment of nitrogen deficient wastewaters are usually accomplished by external addition of nitrogen sources to the wastewater. The operational costs increase with the external addition of nitrogenous compounds such as ammonium, urea and nitrate salts. Also, externally added nitrogen may partly end up in the effluent of the wastewater treatment plants, which reduces the effluent quality.

As an alternative for effective biological treatment of nitrogen deficient and carbon rich wastewaters, nitrogen fixing bacteria can be used along with activated sludge culture. When nitrogen fixing bacteria was mixed with other activated sludge organisms, the performance of the system treating the nitrogen deficient wastewater may improve. The major objective of this thesis is to improve the treatment performance of nitrogen deficient wastewaters by using nitrogen fixing bacteria in an activated sludge unit. *Azotobacter vinelandii* was used as the nitrogen fixing bacteria since those organisms are obligate aerobes and can easily be adapted to an activated sludge system.

Biological TOC and COD removal efficiencies of nitrogen deficient synthetic wastewater were investigated in batch and continuous systems by using *Azotobacter vinelandii*–supplemented activated sludge culture. Performance of *Azotobacter* added and free cultures of activated sludge were compared at different initial TN/ COD ratio in batch experiments. The results indicated clear advantage of using *Azotobacter* in the activated sludge culture to improve TOC removal performance at low TN/ COD ratios. More than 90 % TOC removal efficiencies were obtained with

pure *Azotobacter* or *Azotobacter* added activated sludge culture from nitrogen deficient wastewaters.

Azotobacter vinelandii was used with activated sludge culture for treatment of nitrogen deficient wastewater in a continuously operating activated sludge unit. COD removal performance of *Azotobacter*-supplemented activated sludge was compared with *Azotobacter*-free activated sludge culture for biological treatment of nitrogen deficient synthetic wastewater. Effects of important process variables such as TN/COD ratio, sludge age, hydraulic residence time, feed COD concentration and the COD loading rate on the COD removal performance were investigated. Kinetic constants of the system were determined by using the experimental data. It was proven that *Azotobacter* addition to the activated sludge in biological treatment of nitrogen deficient wastewater (TN/ COD < 0.06) has improved the COD removal performance significantly. Nearly, 90% COD removal efficiency was obtained from nitrogen deficient wastewater (TN/COD< 3%) by using *Azotobacter* supplemented activated sludge culture at HRT of 14 hours and SRT of 10 days.

ÖZET

Azot içeriği düşük atıksuların arıtımında genellikle dışarıdan azot kaynağı eklenerek arıtma verimi arttırılır. Amonyum, nitrat tuzları ya da üre gibi azot kaynaklarının kullanımı işletme maliyetini artırmaktadır. Aktif çamur sistemlerinde bu tür azot kaynaklarının kullanımı, çıkış suyunda kalıntı azot oluşturmakta ve çıkış suyu kalitesini bozmaktadır.

Azot içeriği düşük atıksuların arıtımında alternatif arıtma yöntemi olarak havadan azot bağlayan bakterilerin kullanımı ile etkili biolojik arıtma sağlanabilir. Özellikle daha iyi arıtma performansları için bu tür bakteriler diğer kültürlerle birlike kullanılabilir. Bu tez, azot içeriği düşük atıksuların biyolojik arıtımında dışarıdan azot kaynağı eklenmesi yerine havadan azot bağlayan organizmaların kullanımını amaçlamaktadır. Bu amaçla azot bağlayan bakteriler aktif çamur kültürüyle karıştırılarak arıtma performansları değerlendirilmiştir. Deneylerde *Azotobacter vinelandii* kullanılmıştır. Zorunlu aerobik olan *Azotobacter* bakterileri aktif çamur kültürüne eklenmiştir.

Azot bağlayan bakteri *Azotobacter vinelandii* hem aktif çamurla birlikte hem saf olarak kullanılmıştır. Kesikli ve sürekli sistemlerde azot içeriği düşük sentetik atıksudan biolojik TOC ve KOİ giderme verimleri incelenmiş ve değişik TN/KOİ oranlarında kültürlerin performansları karşılaştırılmıştır. Değişik kültürlerin TOC giderme verimleri karşılaştırıldığında *Azotobacter vinelandii*'nin aktif çamurla birlikte kullanılması halinde daha avantajlı olduğu açıkça ortaya çıkmaktadır. Kesikli deneylerde saf *Azotobacter* yada *Azotobacter*'in aktif çamurla birlikte kullanılması ile azot içeriği düşük atıksulardan (TN/KOİ< %3) %90'ın üzerinde TOC giderme verimi elde edilmiştir.

Sürekli sistemde *Azotobacter vinelandii* aktif çamur kültürüne ilave edilerek sürekli işletilen bir aktif çamur ünitesinde kullanılmıştır. Azot içeriği düşük atıksuların biyolojik arıtımında, aktif çamurun *Azotobacter'li* ya da *Azotobacter'siz* olduğu durumlarda KOİ giderme verimleri karşılaştırılmıştır. Önemli proses değişkenlerinden TN/ KOİ oranı, çamur yaşı, hidrolik alıkonma süresi, giriş KOİ konsantrasyonu ve KOİ yükleme hızının, KOİ giderme verimi üzerine etkileri incelenmiştir. Deneysel veriler kullanılarak sistemin kinetik sabitleri belirlenmiştir. Sonuçta, *Azotobacter vinelandii*'nin aktif çamur kültürüne eklenmesi ile azot içeriği düşük atıksuların biyolojik arıtımında (TN/COD < 0.06) arıtma veriminin önemli ölçüde arttığı gözlenmiştir. *Azotobacter* ilaveli aktif çamur kültürü ile 14 saatlik hidrolik alıkonma süresi ve 10 günlük çamur yaşında, azot içerigi düşük atıksudan (TN/KOİ < 3%) yaklaşık %90 KOİ giderimi sağlanmıştır.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

1.1. PREFACE

Most of the industrial wastewaters are carbon rich but, nitrogen deficient. This characteristic of most of the industrial wastewaters requires external addition of nitrogen sources for effective biological treatment. Examples of these industrial wastewaters are olive-oil mill, landfill leachate, wine industry, beer industry, petroleum refinery, pulp and paper industry, etc. (Şengül F., 1991, pp. 83-315). However, external addition of nitrogen compounds such as ammonium, nitrate salts or urea increases the cost of treatment. Nitrogen compounds in high rate treatment systems like activated sludge requiring external addition of nitrogen may end up as residual nitrogen in the effluent, which is a disadvantage.

By monitoring flow and organic strength variations and matching nitrogen addition it may be possible to minimise the addition of nitrogen. This strategy needs accurate on-line measurements, effective control system, and knowledge of minimum nitrogen requirements. Any treatment, which could regulate nitrogen addition, even as maintaining effective treatment performance, would have considerable benefits, particularly when the treated effluent is discharged to a sensitive receiving environment. (Gapes D.J. et al., 1999, pp.85-92) However, this is not easy. As an alternative to the conventional approach of external nitrogen addition to the nitrogen deficient wastewaters, a rather novel approach was used in this study . This method was based on utilization of nitrogen fixing bacteria in activated sludge system for effective treatment of nitrogen deficient wastewater. Major advantages of

such approach are lower cost of treatment as compared to external addition of nitrogen and possibility of using the excess sludge as fertilizer additive due to nitrogen fixing capability.

Nitrogen fixing bacteria were used for biological treatment of olive mill wastewater, pulp and paper industry wastewater, leachate treatment, clean-up of oil polluted soil and alginate production.

1.2. LITERATURE SURVEY

1.2.1. FUNDAMENTALS OF NITROGEN FIXATION

Nitrogen is one of the most important sources used for bacterial growth. In a nutritionally balanced wastewater, the ratio of three essential elements should be approximately, C/ N/ P=100/ 14/ 2 or COD/ N/ P= 100/ 6/ 2. COD removal performance decreases with decreasing nitrogen content of the wastewater. Nitrogen deficiency occurs when biologically utilizable nitrogen concentration is below N/ C < 0.12 or N/ COD < 0.05. The alternative approach for external nitrogen addition to the nitrogen deficient wastewaters is utilization of nitrogen-fixing bacteria in activated sludge systems for effective treatment.

The nitrogen cycle gives a perspective for nitrogen fixation system as shown in Figure 1.1. The incorporation of atmospheric nitrogen (N_2) as a source of nitrogen for bacterial growth is termed as nitrogen fixation. Dinitrogen is reduced to ammonia, which is then converted to an organic form. (Emerich D.W.& Wall J. D., 1983, pp.75-107)

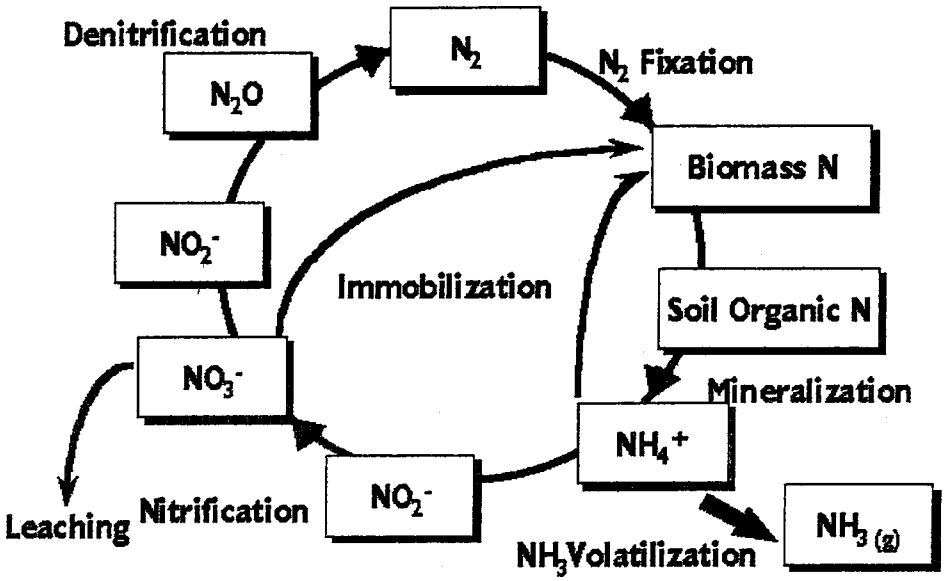


Figure 1.1. The nitrogen cycle

Nitrogen fixation; for aerobic, facultative anaerobic or anaerobic bacteria is an anaerobic or microaerophilic process. (Gapes D.J. et al., 1999, pp. 85-92) Biological nitrogen fixation is catalysed by the nitrogenase complex, which is formed only by prokaryotes and comprised up to 15% of soluble cell protein. (Oelze J., 2000, pp.321-333, Gapes D.J. et al., 1999, pp. 85-92) Nitrogenase, the enzyme catalysing the reduction of N_2 to NH_4, that requires large amounts of ATP is strongly inhibited by ADP, requires a constant supply of low potential electrons and is inactivated by oxygen. In the cell-free systems, nitrogenase activity can be measured by an exogenous ATP-regenerating system. Utilization of $Na_2S_2O_4$ as an electron donor is coupled with the ATP-regenerating system provides a readily available and reliable in vitro method. Nitrogenase requires the functioning of two distinct proteins for the reduction of atmospheric nitrogen. Neither of these component proteins separately displays any activity characteristic of nitrogenase itself. The smaller of the two proteins, dinitrogenase reductase (Fe protein, component 2) is reduced by an appropriate reductant and binds two molecules of ATP and the larger and more complex component of nitrogenase is dinitrogenase. Dinitrogenase reductase then transfers one electron to dinitrogenase (MoFe protein, component 1) with the

concomitants hydrolysis of both ATP molecules. The electrons are transferred within dinitrogenase to the various iron-sulfur centres and the iron-molybdenum cofactor (FeMoCo). (Emerich D.W.& Wall J. D., 1983, pp.75-107)

Dinitrogenase is a $\alpha_2\beta_2$ tetramer. Metal analyses indicate two molybdenum atoms and 24 to 32 iron atoms per molecule. The acid-labile sulfur content is approximately equivalent to the iron content. The metals appear to be arranged as four Fe_4S_4 clusters, two iron-molybdenum (FeMo) cofactors and possibly a Fe_2S_2 centre. (Emerich D.W.& Wall J. D., 1983, pp. 75-107)

The isolated component-2, FeMo cofactor contains 7-8 iron atoms per molybdenum atom. It contains no amino acids. FeMo cofactor-less mutants of *Azotobacter vinelandii* are able to reconstitute. In the presence of an appropriate reductant it is capable of binding and reducing acetylene. Dinitrogenase reductase is composed of two identical subunits. Two catalytically active binding sites for MgATP are possessed by dinitrogenase reductase. MgADP that strongly inhibits catalysis binds strongly to only one of the MgATP sites. The binding of MgATP causes a number of changes in dinitrogenase reductase indicative of a conformational change of the protein. MgATP binding decreases the midpoint potential from about –250 mV to -400 mV, increases the accessibility of the Fe_4S_4 centre to the iron chelators, increases the sensitivity to O_2. (Emerich D.W.& Wall J. D., 1983, pp. 75-107)

Kennedy C. determined that "Cultures need only 50 nanomoles per litre of Mo and 1 milimole per litre of Fe. In addition, if the Mo and Fe amounts were lower than this then one of the alternative nitrogenases carrying either V or Fe only might be active" (Kennedy, C., 24 July 2001)

An interesting study was carried out for *Azotobacter vinelandii* in which vanadium-dependent nitrogen fixation was not inhibited by molybdenum. A mutant of *Azotobacter vinelandii* grew profusely on an agar containing 1 μM Na_2MoO_4 alone or supplemented with 1 mM V_2O_5. *Azotobacter vinelandii* discriminated

against the ^{15}N of air to a similar extent and V-grown cultures discriminated more than Mo-grown ones. (Bageshwar U.K et al., 1998 pp. 161-167) In a study with 10 μM Na_2MoO_4, 10μM $VOSO_4$ or no added Mo or V at 30°C, organisms were grown in batch culture with a rpm of 120 and harvested by centrifugation. The discriminations against ^{15}N were greater in cells from all cultures grown in the absence of added Mo. Furthermore the V-grown cultures and –Mo-V cultures produced ethane and lower rates of acetylene was reduced than Mo-grown cultures. (Rowell P. et al., 1998, pp.2177-2180)

Dinitrogenase is capable of reducing a large number of doubly and triply bonded molecules. The substrates are reduced in two-electron steps or multiples. These are nitrogen, acetylene, protons, cyanide, nitrous oxide, azide, hydrazine and cyclopropane. N_2 is the natural substrate for the enzyme. The acetylene reduction technique is commonly employed as an index of nitrogenase activity. (Emerich D.W.& Wall J. D., 1983, pp. 75-107)

The nitrogenase activity with different carbon sources was observed. *Azotobacter vinelandii* was cultured in chemically defined media supplemented with p-hydroxybenzoic acid, either in the presence or absence of glucose. P-hydroxybenzoic acid was catabolized through protocatechuate dioxygenases by ring cleavage and, as these enzymes were strictly inducible, microorganisms grown in N-free glucose medium required a period of time to synthesize inducible enzymes degrading the aromatic compound. The results of this study suggested that p-hydroxybenzoic acid constitutes a more efficient carbon source for *Azotobacter vinelandii*, since specific nitrogenase activity was higher in N-free medium supplemented with this phenolic acid alone. In addition, the lower respiration activity observed in the medium, a higher ability to fix dinitrogen without a wasteful consumption of the carbon source. The study indicates that in habitats in which *Azotobacter vinelandii* was present, p-hydroxybenzoic acid would not reach high concentrations, hindering the undesirable effects that accumulation of this compound could imply. (J. Moreno, C. Vargas-Garcia, 1995, pp.2605-2610)

Energy requirement of nitrogen-nitrogen triple bond is remarkably high requiring 941 $kJmol^{-1}$ to completely dissociate the molecule. The large energy requirement for the reduction to the double-bonded intermediate (523 $kJmol^{-1}$) even though the free energy for the overall reaction is negative may explain the difficulty in reducing dinitrogen.

$$N_2\,(gas) + 3H_2\,(gas) \leftrightarrow 2NH_3\,(aq) \quad 25\ ^{\circ}C, \quad \Delta G_o = -53.34\ kJ\ mol^{-1}$$

ATP supplies the energy to force biological nitrogen fixation. A minimum of 12 ATP is needed to reduce N_2 since two ATP are hydrolysed for each electron transferred from dinitrogenase reductase to dinitrogenase. However, for each mole of N_2 reduced, a minimum of one mole of H_2 is evolved from nitrogenase. The evaluation of H_2 requires the same energy input as other substrates, thus raising the minimum number of ATP to 16. Also the eight low potential electrons (six for N_2, two for H^+) can be assumed. They possess energy as they could alternatively be used for oxidative phosphorylation or some other energy yielding reactions. The ATP for biological nitrogen fixation has to be originated from the metabolism of carbon compounds. Theoretically, 0.11 mol of glucose is needed to produce 1 mol of ammonia. Nitrogen fixation imposes a considerable energy metabolism of an organism. The ratio of ADP/ ATP is 0.3-0.5 in N_2-fixing cells and under non-fixing conditions the ratio is 0.8-0.9. (Emerich D.W.& Wall J. D., 1983, pp. 75-107; Krassimer K.S. & Zerner M.C., 1997, pp.141-145; Christiansen J. et al. 1999, pp. 195-204, Szilagyi R.K, 2000 pp. 131–146). In other words, the overall stoichiometry for the reduction of N_2 to ammonia is:

$$N_2 + 8H^+ + 8e^- + 16\ Mg^{-}ATP \rightarrow 2NH_3 + H_2 + 16\ Mg^{-}ADP + 16\ P_i$$

Addition of ammonia to the culture media causes nitrogenase not to be effective. The observed effects depend upon the organism the carbon and fixed nitrogen sources and the degree of membrane energization. (Emerich D.W.& Wall J. D., 1983, pp. 75-107)

Oxygen irreversibly inactivates both nitrogenase components. The half-lives of dinitrogenase and dinitrogenase reductase in air are 10min. and less than one min., respectively. Members of the *Azotobacter* represent aerobic bacteria, which are able to grow diazortophically at the highest dissolved O_2 concentrations about 230 μM O_2 (Oelze J., 2000, pp.321-333). The physiology of each N_2-fixing organism must be compatible with its respective ecological niches. *Azotobacter* possesses a unique mechanism to protect nitrogenase components during O_2 stress. Low O_2 tensions reduce ATP levels and lower the nitrogenase activity. (Emerich D.W.& Wall J. D., 1983, pp. 75-107; Oelze J., 2000, pp.321-333). 'A "high" dissolved oxygen concentration for a nitrogen fixing bacterium would be at a level of 2 mg/L in activated sludge operations ' (Gapes D.J. et al., 1999, pp. 85-92).

In an investigation with a nifU-deletion mutant of *Azotobacter vinelandii*, N_2 was fixed under low atmospheric O_2. The revertant's nitrogen activity was not accompanied by an increased respiration rate. This mutant had a 100 % fixed N_2 in air by introduction of another mutant (cydR) tranformants. (Hill S. et al. (1999) pp.185-191)

Oxygen, hydrogen and nitrogen fixation in *Azotobacter vinelandii* was investigated and it was observed that a mutant was capable of catalysing O_2-dependent H^3H uptake, indicating that H_2-dependent respiration linked a high affinity (O_2) oxidise system. (Yates M.G et al., 1996, pp.863-869)

1.2.2. CHARACTERISTICS OF NITROGEN FIXING ORGANISMS

Various organisms are known for their nitrogen fixing capabilities. The bacteria that can fix nitrogen are classified for special conditions, which are aerobes (*Azotobacter species, Beijerinckia, Derxia sp.*), facultative anaerobes (*Bacillus, Klebsiella, Rhodopseudomonas, Rhodomicrobium, Rhodospirillum*), anaerobes (*Clostridium*), microaerophiles (*Rhizobium sp., Enterobacteriaceae, Methylococcaceae, Pseudomonadaceae, Beggiatoaceae, azospirillum sp.,*

Xanthobacter sp., *Mycoplana sp.*). Those organisms may be important for wastewater treatment. (Williams&Wilkons, 1989, pp. 219-234)

Some facultative anaerobes such as *Bacillus, Klebsiella, Rhodopseudomonas* and some species of the genus *Clostridia* can also fix nitrogen under anaerobic conditions. Such organisms may be used in anaerobic treatment of nitrogen deficient, carbon rich, high strength wastewaters.

Certain blue-green algae (Cyanobacteria) such as *Anabena sp* can fix nitrogen under aerobic conditions which may have a potential use for treatment of nitrogen deficient wastewaters. *Rhizobium* species growing on the roots of leguminous plants also fix nitrogen under anaerobic conditions and make the fixed nitrogen available to the leguminous plants.

The nitrogen-fixing rate for nitrogen-fixing organism *Azotobacter vinelandii* is 2-5 kgN/ ha. (http://www.nap.edu/readingroom/books/bnf/contents.html , 28.07.2000)

The family of *Azotobacter* comprises of *Azotobacter vinelandii, Azotobacter chrococcum, Azotobacter nigrigans, Azotobacter armaniacus, Azotobacter beijerinekii, Azotobacter agilis, Azotobacter insignis, Azotobacter paspali (azorhizophilus p.) and Azotobacter macrocytogenes (azomontrichon m.)*. (Williams&Wilkons, 1989, pp. 219-234). Nitrogen fixation by these organisms are catalysed by the enzyme 'nitrogenase' which is inhibited by free oxygen.

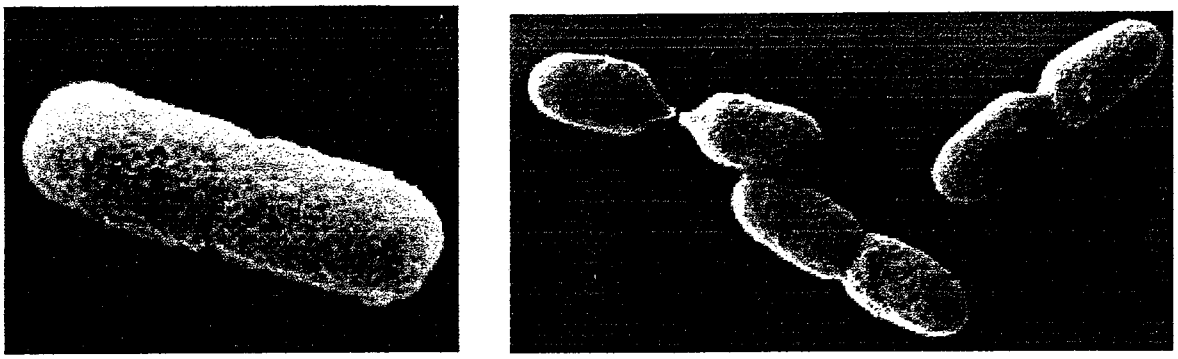


Figure1.2 View of *Azotobacter vinelandii* under microscope
(Dixon R. FRS, et al., 2001, pp.183-187)

Azotobacter vinelandii is an aerobic soil-dwelling organism with a wide variety of metabolic capabilities, which include the ability to fix atmospheric nitrogen by converting it to ammonia. It fixes nitrogen in the free-living state and does not enter into symbiosis with plants. *Azotobacter vinelandii* is capable of synthesising not only the molybdenum-containing nitrogenase enzyme. *Azotobacter vinelandii* has evolved a number of physiological mechanisms to allow it to fix nitrogen aerobically despite the inherent oxygen-sensitivity of nitrogenase. (Dixon R. FRS, et al., 2001, pp.183-187)

Also there are 126 strains of *Azotobacter* found by Kanitkar's (www.Promise, 1996). *Azotobacter vinelandii* is gram-negative *Azotobacter* genus, nitrogen fixing is obligate aerobe, distribute in soil (some species with the rhizosphere of tropical grasses) possibly due to the production of plant growth hormones. (Because of potential for coupling ammonia exertion from N_2 reduction with plant-hormone production in an organism that has an associative growth mode.) (Dixon R. FRS et al, 2000, www.nitrogen fixation) The bacteria are blunt rods to oval cells, 2μm or in diameter of various lengths. The morphology changes with various growth conditions. Cells are often in pairs also in chains. They are motile by peritrichouse or polar flagella or nonmotile. Intracellular poly-β-hydroxybutrate may be present. It's gram-negative to gram-variable. Endospores are not produced. (Williams S.T., 1989, pp.219-234) In nitrogen-free medium with glucose as the carbon source the shapes of the species were rods with rounded ends with a 1.3-1.7 μm in diameter and 3.0-7.0

forms become more common and metacromatic and sudanophilicgranules are observed. Also in peptone-yeast extract agar all members of the genus produce distored cells. Cysts are produced in only one genus and it's in *Azotobacter*. It forms only in old cultures grown with sugar as carbon source. For some species a medium containing butan-1-ol as the organic substrate enhances cyst formation. The cyst may be distinguished from an endospore by its characteristic structure. A cyst coat is surrounded a central body. It's consisted of an exocystrorium and an exine. The cell inside the cyst coat is similar to the vegetative form and is no cytological changes in the cell prior to its germination. The cyst exocystrorium is ruptured at one point and the cell, which emerges, may already be in a dividing state. It's chemoheterotrophic, catalyse positive, as told before capable of fixing molecular nitrogen. *Azotobacter* can fix 10mg of nitrogen/g of a suitable carbohydrate consumed on synthetic media with or without homopolysaccararides. Organic growth factors are not required. *Azotobacter vinelandii* rarely produces H_2S. Trace elements involved are required in nitrogenase activity. Can utilize various sources of combined nitrogen, some species do not, or poorly utilize nitrate. Water-insoluble and water-soluble pigments and fluorescent pigments are produced by some species. The habitat for the bacteria is soil, water, and the plant rhizosphere. The mol %G+C of the DNA ranges from 52-67.5. (Williams S.T., 1989, pp.219-234) The amount of nitrogen that *Azotobacter vinelandii* can fix is 8-18 mg/g. (www.promise, 1996)

Azotobacter requires a minimum temperature of 14°C for growth. In general, optimum temperature for growth is near 32 °C for *Azotobacter species*. The optimum growth temperature is 37°C for *Azotobacter vinelandii*. The optimum pH for nitrogen fixation is close to neutral pH 7-7.5. (Williams S.T., 1989, pp.219-234)

1.2.3. UTILIZATION OF NITROGEN FIXING ORGANISMS IN WASTEWATER TREATMENT

There are limited numbers of studies on utilization of nitrogen fixing organisms for biological treatment of nitrogen deficient wastewaters. Balis et al. used nitrogen-fixing bacteria for the treatment of oil-mill wastewaters. He carried out many studies

with different treatment types. Major investigations were carried out on "Improvements of treatments and validation of the liquid-solid waste from the two-phases olive oil extraction". He developed a treatment procedure for olive mill wastewaters and had a patent (Patent No: 93010266) originated from this study.

Olive mill wastewater (OMWW) is a high strength wastewater with high COD concentrations and low nitrogen content. It's very appropriate for treatment with nitrogen fixing organisms. *Azotobacter vinelandii* -strain A was used in a fermenter unit and in a field pilot plant of 5 m^3 capacity. *Azotobacter vinelandii* strain is the only one of three strains that can grow on OMWW; the others are *Azotobacter chroococcum* strain C and K. N_2 fixation reached peak of activity in 24 h and then gradually decreased to zero. (Balis C et al, 1996, pp. 169-174). *Azotobacter vinelandii* strain-A growth and nitrogenase activity showed that the maximum value of total acetylene reduction activity was obtained after 24 h of incubation as well as the maximum value of bacterial population. When nitrogen fixing capacity was expressed in reference to the bacterial population its maximum value was observed earlier, at the 7th hour of incubation (Papadelli M. et al, 1996 pp. 179-181). The pH rised from 6.9 to 8.6 during the incubation period. The behaviour of *Azotobacter* vinelandii under phenolic compounds were tested by Balis et al and in all cases the rate of N_2 fixation remained either unaffected or slightly enhanced. Also it's noticeable that the presence of caffeic acid has a stimulatory effect. The kinetics were determined for this system as follows:

$$y=y_o\,[1+(1+\lambda)\,kt]\,e^{-kt};$$

where yo=264.2, k=0.75, λ=5.7 , t=incubation period, y_o=initial N_2-fixing ratio, k=rate constant depending on units, strain and incubation conditions, λ=constant for physiological conditions of the culture. When λ=0, having conditions matching to the stationary stage of the culture, the equation expresses a downward S shaped curve; when $\lambda < 0$, exhausting substrate, end of stationary phase, the equation expresses a more and more rapidly descending curve; when $\lambda > 0$, exponential stage of the growth, the curve ascends first towards a maximum and thereafter declines to zero.

(Balis C., 1999, pp. 81-82). The bio-remediation process developed by Balis et al involved the cultivation of *Azotobacter vinelandii* (strain A) in a rotating-bio-wheel type of air conductor. The system was operated in fed batch mode. OMWW is pretreated with CaO and then is fed to the reactor for a period of 3 days. The disadvantage of the system is that feeding of fresh substrate suppresses N_2-fixing activity, but also enormous amount of slime production protects the system performance. The system was designed in two-stages for considering the performance of the bacteria. The system aimed achieving stable conditions in the second stage to enable the bacterial population to grow at high rates and complete their bioremediating effect by utilizing the more difficult components of OMWW. (Balis C et al, 1996, pp. 169-174)

The same system explained above was also used for a 5-day-period, under non-sterile conditions. Process product's phytotoxicity was reduced by over 90% at the end of both cycles. In the first stage the reduction performance was 70%. The *Azotobacter vinelandii* population dynamics were successfully monitored, with an initial adaptation period, having a maximum population on the fourth day of the both cycles. N_2 fixation rates were estimated using the acetylene reduction assay and reached a peak during the first 1-2 days of each cycle (36 and 29 nmol C_2H_2 $ml^{-1}h^{-1}$ respectively). The data were consistent with an initial physiological adaptation phase, where the presence of the phenolic compounds limits the growth of *Azotobacter vinelandii,* but stimulates N_2 fixation, followed by a rapid growth phase as phytotoxic declines. (Ehaliotis C et al., 1999, pp. 301-311)

A pilot plant was used for olive-mill wastewater treatment, suitable for agricultural use in Greece (Messinia). This method exploits OMWW as a substrate for the cultivation of nitrogen-fixing microorganisms (*Azotobacter vinelandii*) capable of transforming the material into an organic liquid of high fertilizing and soil conditioning value. The results obtained from the applications of olive trees, vines and potatoes were very promising. The stages of the pilot plant were pre-treatment with hydrogen peroxide under alkaline conditions. The material was treated first with calcium hydroxide to increase the pH to 11-12 and then with hydrogen peroxide

solution. The mixing process was carried out in a tank equipped with a suitable mechanical stirring system, for 6-12 h to allow the pH to stabilize at about 8.0. The second stage was bioconversion by a N_2-fixing microbial consortium under well aerated conditions. In this step the mixture was transferred into the bioreactor where an enriched diazotrophic microbial consortium had already established. A bioreactor with a volume of 5.5 m^3 consisting of a tank equipped with an air-conducting system was used.The process can be operated in repeated fed-batch mode with a residence time of 3-7 days according to the expected use of the final product. The product of the system was a thick yellow, non-phytotoixic liquid, with a pH of about 7.5-8.0. It was characterized as an "organic soil conditioner bio-fertilizer form". It can be used as an organic fertilizer by mixing it in various proportions with the irrigation water and in high doses as a soil conditioner. (Chatjipavlidis I., 1997, pp.183-186)

OMWW treatment studies were also carried out in Italy. A 2 L bioreactor with T=30°C, airflow of 1.4 l / min and stirring speed of 100 rpm was used. The reactor was inoculated with *Azotobacter vinelandii* isolated from soils and was used for the treatment of OMWW. Organisms developed capsular polysaccharides of 1 and 4 mg/ml concentrations. The respiration rate increased rapidly after inoculation and reached to the steady- state at the fourth day. The results obtained were similar to those obtained by Balis with CaO as explained above (Fiorelli F et al., 1996, pp. 165-167).

Also Prof. Dr. Fikret Kargı supervised a study on biological treatment of OMWW and landfill leachate (Diploma Project by Hidayet Argun and Serhan Sayın, 2001). Erlenmayer flask experiments were carried out with *Azotobacter vinelandii* and/or activated sludge culture. COD removal performances were observed and compared with each other. An increase of the removal efficiency was observed when dilution ratio of OMWW increased. The COD removal performance of *Azotobacter vinelandii* alone was 40% at a dilution rate of 1/40, whereas the COD removal efficiency obtained with the activated sludge culture alone was 81%. When a mixture of *Azotobacter vinelandii* and activated sludge culture was used a COD removal efficiency of 82% was obtained. Since OMWW is phytotoxic because of its

poliphenolic content biological treatability decreased with increasing OMWW's content. *Azotobacter vinelandii* was more effective in leachate treatment. At a dilution rate of 1/4 *Azotobacter vinelandii*'s COD removal performance was 92.5%, while 77% COD removal was obtained with the activated sludge culture alone. A mixture of *Azotobacter vinelandii* and activated sludge culture yielded 92% COD removal. COD removal efficiency decreased with increasing dilutions of the leachate (Diploma Project by Hidayet Argun and Serhan Sayın, DEÜ, 2001).

Onwurah investigated short-term effects of bony light crude oil on glucose uptake and BOD removal efficiency of *Azotobacter vinelandii* (1998). The results of this study showed that during the initial "lag" phase the BOD removed was primarily due to oxidation of adsorbed glucose and possibly some components of the crude oil. Although significant cell proliferation did not occur, *Azotobacter vinelandii* had the potential of surviving in crude oil polluted environment, even better when in "mutualistic" association with crude oil degrading bacteria. BOD_u taken as the index of growth, demonstrate that the growth of *Azotobacter vinelandii* decreased as the crude oil concentration increased. The toxicity of crude oil brings tendency of reducing dissolved oxygen level in aquatic solutions. (Onwurah I.N.E., 1998,pp. 464-471)

Pulp and paper industry wastewater treatment was also investigated by using nitrogen-fixing bacteria in New Zealand. Two activated sludge reactor systems were utilized. The first one was operated without nitrogen addition and the second with nitrogen addition. The volumes of the reactors were 2 L. Hydraulic retention times (HRT) were 0.5 days; solids retention times (SRT) with and without nitrogen additions were 2.3 d and 0.4 d, respectively. The first reactor was operated at low dissolved oxygen concentrations and at a temperature of 30°C. The results depict that nitrogen fixation only occurs in the first reactor. BOD removals were 97% in each reactor and COD removals were approximately the same as 65%. These results clearly have proven that that compounds present in the wastewater are not toxic to nitrogen fixing bacteria. The nitrogen-fixing activated sludge system achieved 0.5-0.7 mg/L of soluble organic nitrogen concentrations in the discharge, which was very

low. The second reactor with nitrogen addition had a higher concentration of nitrogen such as 2.1-4.4 mg/L. (Gapes D.J., 1999, pp.85-92)

Hunter and Slade (1999) carried out studies on kraft mill wastewater treatment by using a stabilization basin in New Zealand. The basin was operated at a hydraulic retention time of approximately 1.25 d., at low dissolved oxygen concentration of 0.3-0.5 mg/L. An influent wastewater with a BOD/ N/ P ratio of 100/ 0.8/ 0.5 was used. The BOD removal performance was 55% with an apparent nitrogen limitation. The results depicted that significant increases in total nitrogen (approximately 600 kg $N.d^{-1}$) occurred across the basin. (Gapes D.J., 1999, pp.85-92)

Azotobacter species can be used for production of some commercial polymers such as alginate and PHB. Alginate is a linear co-polymer of D-mannuronic and L-guluronic acids in varying proportions, owing to its gelling and viscosifying properties, it is widely used in food, textile, paper, and pharmaceutical industries as thickening, stabilizing and gelifying agents. In terms of abundance, location and uniform quality, only a few species of brown algae are suitable for alginate production. *Azotobacter vinelandii* is an alternative for bacterial alginate production since it's non-pathogenic. This bacterium has the ability to produce alginate under aerobic conditions, despite of the extreme sensitivity of the nitrogenase system against oxygen. An alginate capsule on the surface of *Azotobacter vinelandii* is present as determined by cell morphology and cell surface analysis. The composition, thickness and compactness of this alginate capsule also varied significantly. The production of alginate and formation of an alginate capsule on the cell surface forms an effective barrier for O_2 transfer into the cell. The quality, not the quantity of alginate is decisive for the protection of nitrogenase. (Sabra W. et al., 1999, 349-350) The alginate production by *Azotobacter vinelandii* is strongly influenced by the dissolved oxygen tension (DOT) and stirring speed of the culture. The bacteria produced more alginate (4.5 g/l) at a stirring speed of 300 rpm under high DOT (5% of air saturation) as compared to 1. g/L alginate which was obtained at low (0.5%) oxygen tension. In contrast, under constant DOT (3%), when higher stirring speed (from 300 to 700 rev./min) were used higher specific growth rate,

guluronic acid content and alginate production rates were obtained (Pen˜ a C. et al., 2000 pp. 390–398). The alginate capsule was accordingly thicker and more compact. Also, the formation of the alginate capsule was affected strongly by the shear rate in bioreactor. (Sabra W. et al., 1999, 349-350). A polymer of high molecular weight (680 000 g/g mol) was produced with low agitation speed (300 rev./min) whereas at high (700 rev./min) stirring speed a low molecular weight (352 000 g/g mol) alginate was isolated from the cultures used. When the culture was grown at 300 rev./min microscopic observations revealed the presence of cell aggregates, which were one order of magnitude larger than individual cells. (Peña C. et al., 2000 pp. 390–398)

Furthermore, mass distributions were investigated for alginate production. Shake flask experiments were carried out for the effects of different aeration conditions during the culture of *Azotobacter vinelandii* on the production and molecular mass of alginate. In baffled flasks (conical with three lateral indentations) the bacteria grew faster and produced 1.5 g/L alginate, which was less than (4.5 g/L) those obtained in conventional conical (unbuffled flasks) flasks. Higher final viscosities were attained in unbuffled flasks as compared to the baffled ones. The molecular mass increased with the culture age. At the end of the fermentation, the molecular mass of the alginate obtained in unbuffled flasks was five-fold higher than that obtained in buffled flasks. The cells of *Azotobacter vinelandii* grown in unbuffled flasks increased in diameter, whereas those cultured in buffled flasks decreased in size. (Peña C. et al., 1997 pp. 510–515)

Also as explained by the investigations on OMWW treatment, *Azotobacter vinelandii* was used for soil treatment. Symbiotic nitrogen fixers are mainly used for this purpose. As explained before the nitrogen fixing bacteria are isolated from the soil especially from polluted soils.

Inoculation of specific strains is the most effective to satisfy nitrogen hunger for legumes varieties. In West Region Ukraine the inoculation of vetch with the *Rhizobium* increased significantly the yield of the vetch gramineous mixture up to 55.0-65.0 t/ha. and the total and digestible protein content most as 1.5-2.0 %. It was

shown that under field conditions the inoculation with associative diazotrophs of cereal plants increased the green mass yield and grain yield. The highest influence on winter wheat yield has inoculation with *Enterobacter* and *Azospirillum* at the expense of the increased weight kernels. Instead of mineral nitrogen nearly 30% nitrogen may take place in nutriment of the maize. The highest nitrogen fixation at roots zone maize plants was found in variants with inoculation *Flavobacterium* sp. (5-6 times as large as of control). On the basis of the obtained results in trails with oat, it can be concluded that it is the cultivar sensibility to inoculation with diazotrophs. (Lisova N. et al., 1999, p 628).

1.3. OBJECTIVES AND SCOPE

As an alternative to the conventional approach of external nitrogen addition to the nitrogen deficient wastewaters, a rather novel approach was used in this study. Utilization of nitrogen fixing bacteria in activated sludge system for effective treatment of nitrogen deficient wastewater was developed.

In our country, treatment techniques with non-symbiotic nitrogen fixing bacteria are not used. However, investigations carried out in Europe especially in Mediterranean countries indicated that the use of nitrogen fixing bacteria was affective in treatment of nitrogen deficient wastewaters. Major advantages of such approaches are:

1) Lower cost of treatment as compared to external addition of nitrogen
2) Possibility of using the excess sludge as fertilizer additive because of its nitrogen fixing capability.
3) Lower levels of nitrogen in the effluent of the treatment plants

The aforementioned studies on treatment of nitrogen deficient wastewaters with nitrogen fixing bacteria constitute a basis for this study. The major objectives and scope are summarized as follows:

1) To develop an effective and low cost alternative for the treatment of nitrogen deficient wastewaters by using nitrogen fixing bacteria
2) To select an effective aerobic nitrogen fixing bacterial culture for treatment of nitrogen deficient wastewater
3) To compare the performances of the selected bacteria with activated sludge culture in treating nitrogen deficient wastewater.
4) To investigate the effects of nitrogen deficiency on activated sludge performance in the presence and absence of nitrogen fixing bacteria and to optimise the conditions.
5) To investigate the effects of operating parameters on COD removal from nitrogen deficient wastewaters in an activated sludge reactor in the presence and absence of *Azotobacter vinelandii.*
6) To investigate the kinetics of COD removal from nitrogen deficient wastewater in an activated sludge unit in the presence and absence of *Azotobacter* species.
7) To determine the kinetic constants of the biological treatment system used for nitrogen deficient wastewater with and without *Azotobacter* inclusion and to compare the results.
8) To prove the advantages of using nitrogen fixing bacteria in biological treatment of nitrogen deficient wastewater.

In the first part of the experiments carried out with shake flasks, TOC removal performances of nitrogen fixing bacteria *Azotobacter vinelandii* was compared with the activated sludge culture and *Azotobacter*-supplemented activated sludge to evaluate the treatment performance of *Azotobacter vinelandii*. The second set of investigations was planned in the light of the batch shake flask experiments. In the second part of the experiments, an activated sludge system was operated in the presence of *Azotobacter vinelandii* with different N/COD ratios to optimise the conditions for further experiments. Also, this set of experiments was carried out for *Azotobacter*-free activated sludge culture to compare the COD removal efficiencies

of both systems. Effects of important process variables on the COD removal performance were investigated.

Treatment of nitrogen deficient wastewater with nitrogen fixing bacteria is a rather novel approach investigated in this thesis. The results of this thesis provide valuable information for the design of treatment systems utilizing nitrogen-fixing bacteria for the treatment of nitrogen deficient wastewaters. Kinetic analysis and determination of the kinetic constants also provide valuable insight for scientific understanding of the behaviour of such systems.

CHAPTER TWO

MATERIALS AND METHODS

2. 1 EXPERIMENTAL SET-UP

Utilization of nitrogen fixing organisms in biological treatment of nitrogen deficient wastewater was studied for approximately 2 years in the laboratory. The experiments were carried out in two steps, which are classified as batch and continuous experiments.

Batch experiments were performed by using an incubator shaker (Gallenkamp) at 150 rpm and 28°C. 250 mL cotton plugged Erlenmayer flasks were filled with 200 mL semi-semi-synthetic wastewater and inoculated with appropriate culture (activated sludge and *Azotobacter vinelandii*).

Continuous experiments were carried out by using a laboratory scale activated sludge unit. The experimental set-up consisted of a wastewater tank, feed wastewater pump, activated sludge reactor, blower and an effluent tank. Activated sludge unit was used because the operation and control of this system are easy. The reactor was made of stainless steel with a 20x20x30 cm dimensions holding 9.3 L total liquid volume. The activated sludge reactor was divided into two parts with a stainless steel plate. This plate separates the aeration and settling tanks and allows passage of wastewater from the aeration to the sedimentation unit through the holes on it. The liquid volume in the aeration and sedimentation tanks were 7.6 L and 1.7 L, respectively.

Small diffusers and perforated tubes were used for aeration along with a blower. A schematic diagram of the experimental set-up is presented in Figure 2.1.

2.2. SEMI-SYNTHETIC WASTEWATER

2.2.1. Batch Experiments

In the batch experiments the inoculum cultures were either activated sludge and/or *Azotobacter vinelandii*. Depending on the experiment molasses or glucose was used as carbon source.

2.2.1.1. Semi-semi-synthetic Wastewater Composition with Molasses

The semi-semi-synthetic wastewater consisted of diluted molasses. Concentrated molasses were obtained from PAKMAYA Bakers Yeast Company, Izmir and was properly diluted to result in COD concentrations between 5000 and 2000 mg/L. The nutrient media for the activated sludge culture was prepared to obtain COD/ P ratio of 100/ 1.5. KH_2PO_4 was used as P source. $MgSO_4$ was used with a concentration of 0.05 g/L. The experiments were performed at different initial TN/ COD ratios, where urea was used as nitrogen source. TN/ COD ratios varied between 0/ 100 and 8/ 100 by changing the concentrations of urea in the medium. In the experiments carried out with *Azotobacter vinelandii*, the same semi-semi-synthetic wastewater composition was used and $FeSO_4$ (10 mg/L), H_2MoO_4 (5 mg/L), NaCl (0,2 g/L) and $NaHCO_3$ (0,1 g/L) were added to the medium as trace elements.

2.2.1.2. Semi-semi-synthetic Wastewater Composition with Glucose

The nutrient media contained glucose as carbon source to yield initial COD of 5000 and 2000 mg/L. $MgSO_4$ concentration was 0.05 g/L. KH_2PO_4 was used as P source and its concentration was varied with initial COD concentration to keep COD/P ratio constant at 100/ 1.5. The experiments were performed at different initial TN/ COD ratios, where urea was used as the nitrogen source. TN/ COD ratio varied

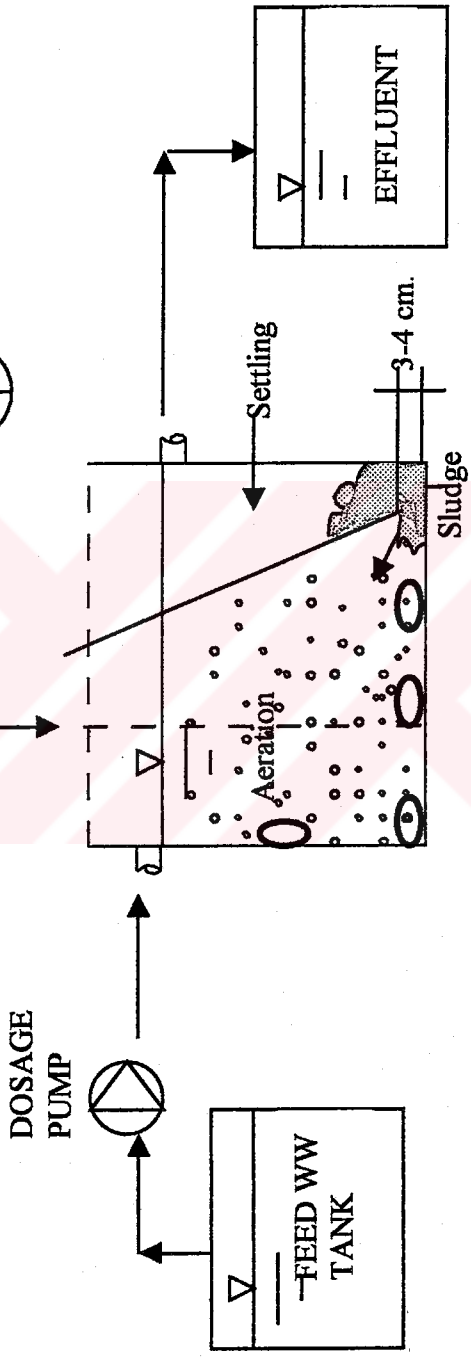


Figure 2.1: Schematic diagram of the activated sludge unit used in experimental studies.

between 0/ 100 and 8/ 100 by changing the concentrations of urea in the medium. In the experiments carried out with *Azotobacter vinelandii*. The same semi-semi-synthetic wastewater composition was used and $FeSO_4$ (10 mg/L), H_2MoO_4 (5 mg/L), NaCl (0,2 g/L) and $NaHCO_3$ (0,1 g/L) were added to the medium.

2.2.2. Continuous Experiments

Diluted molasses was used as carbon source in continuous experiments to yield initial COD of 2000 mg/L. KH_2PO_4 (0,130 mg/L), $MgSO_4$ (0,05 g/L) and different amounts of urea were added to the semi-synthetic wastewater to adjust COD/ N/ P ratio. Two sets of experiments were carried out. Activated sludge culture was used alone in the first set while a mixture of activated sludge and *Azotobacter vinelandii* was used in the second set of experiments. $FeSO_4$ and H_2MoO_4 were added to the feed wastewater when *Azotobacter vinelandii* was used in the culture. However, two sets of experiments, carried out with *Azotobacter* supplemented activated sludge, were performed without addition of iron and molybdenum salts. Those experiments were the ones performed with different initial COD/ N ratios and with different sludge ages. In order to avoid any contaminations while feeding the reactor, the feed wastewater was prepared daily and preserved at 4°C in the refrigerator. The pH of the semi-synthetic wastewater and activated sludge unit was adjusted to pH = 7-7.5 by adding diluted NaOH or diluted H_2SO_4, when necessary.

2.3. ORGANISMS

A pure culture of *Azotobacter vinelandii* (NRRLB-14643) was obtained from the USDA, Northern Regional Research Laboratories (NRRL), Peoria, Illinois, USA. *Azotobacter vinelandii* was cultivated in the laboratory by using an incubator shaker. Composition of the Burk's media used for cultivation of *Azotobacter* species is presented in Table 2.1. Cultures were grown at a temperature of 28± 2°C and pH of 7-7.5. The same procedure was used also for the preparation of a stock culture of *Azotobacter chrococcum* (NRRLB-14344), which was obtained from the same laboratory. (S. T. Williams, 1989, pp. 219-229).

Activated sludge culture was obtained from wastewater treatment plant of PAKMAYA Bakers Yeast Company, Izmir. It was cultivated in the laboratory on a shaker at 28± 2°C at pH7-7.5. The cultivation media contained 5 mL/L molasses, 326 mg/L KH_2PO_4, 0,05 g/L $MgSO_4$ and 870 mg/L urea. These cultures were observed under the microscope, for investigating their growing period and aliveness, before inoculation of the experimental flasks or reactors. Some stock cultures were preserved in the freezer in frozen form.

Table 2.1 Burk's media composition

Glucose	20	g/L
KH_2PO_4	1	g/L
$MgSO_4x7H_2O$	0,05	g/L
NaCl	0,2	g/L
$FeSO_4$	10	mg/L
H_2MoO_4	5	mg/L
$NaHCO_3$	0,1	g/L

2.4. EXPERIMENTAL PROCEDURE

2.4.1 Experiments with Erlenmeyer Flasks

Seven flasks with different initial TN/COD ratios were used in this phase of experiments. Three different sets of experiments were carried out. Activated sludge and *Azotobacter* cultures were used alone in the first two sets of experiments while a mixture of activated sludge and *Azotobacter* cultures (1/1) was used in the third step. The flasks were filled with semi-synthetic wastewater with different initial TN/ COD ratios of 8/100, 6/100, 4/100, 2/100, 1/100, 0/100. A control flask with no inoculation was also used. The flasks contained 180 mL wastewater and 20 mL culture and were incubated in an incubator shaker for five days at T = 28° C and rotational speed of 150 rpm.

2.4.2. Experiments with Activated Sludge System

2.4.2. Experiments with Activated Sludge System

Continuous experiments started by growing activated sludge culture and/or *Azotobacter vinelandii* in the aeration tank for three days. Each experiment lasted for five days. The system reached to steady state condition within several days and samples were analysed withdrawn at last three-days of steady-state condition. Small diffusers and perforated tubes were located at the bottom of the aeration tank to obtain vigorous aeration and agitation.

2.5. SAMPLING

In batch shake flask experiments, 10 mL samples were taken from each flask twice a day. The samples were centrifuged at 5000 rpm to remove the organisms and clear supernatant was kept for analysis. Small amount of diluted acid was added to the supernatant and preserved in the refrigerator. COD and TOC analysis were carried out on clear supernatant after centrifugation.

In continuous experiments, the system was operated until steady-state conditions were reached and the samples were taken at the steady state. Samples were removed once a day and centrifuged at 5000 rpm to remove the organisms. The supernatant was analyzed for COD, TOC and total nitrogen. Samples were preserved in the refrigerator by adding trace amount of diluted acid. Biomass concentrations were determined once in every steady state.

2.6 ANALYTICAL METHODS

2.6.1 Chemical Oxygen Demand (COD) Analysis

COD measurements were carried out according to Standard Methods. Open reflux and closed reflux colorimetric methods were used.

In open reflux method, standard potassium dichromate solution (prepared by dissolution of 12.259 g $K_2Cr_2O_7$ primary standard grade dried at 103°C for two hours diluted with 1000 mL distilled water), Sulfuric acid reagent (prepared by addition of 5.5 g Ag_2SO_4/kgH_2SO_4 to H_2SO_4 and stand for two days), ferroin indicator solution, standard ferrous ammonium sulphate (FAS) titrant (prepared by adding 98 g $Fe(NH_4)_2.6H_2O$ and 20 mL concentrated H_2SO_4 to distilled water to be 1000 mL) and mercuric sulfate was used. Two mL of wastewater was removed (depending on the strength of the sample it has to be diluted to obtain nearly 2000 mg COD/L) and completed to 20 mL with distilled water. Five mL of H_2SO_4, mercuric sulfate, 10 mL $K_2Cr_2O_7$ and 25mL H_2SO_4 was added to the sample and the samples were boiled for 2 hours under reflux. Then 80 mL of distilled water and 2 or 3 drops of ferroin indicator was added to the boiled sample and FAS was used for titration. The end point of the titration was realized when the first sharp colour changes from blue-green to reddish brown took place. A blank was also prepared in the same manner. (Greenberg A.E, 1989, pp. 5,7-8)

Calculations were made by using the following equation,

mg COD/ L= [(A-B). N.8000] / [mL sample]

where (A) is FAS used for blank; (B) is FAS used for the sample and (N) is the normality of FAS solution.

In closed reflux colorimetric method, borosilicate culture tubes with 10 mL capacity were used. A visible spectrophotometer was used to measure the absorbance

at 600 nm. Digestion solution (prepared by adding 10.216 g $K_2Cr_2O_7$, 167 mL conc. H_2SO_4 and 33.3 g $HgSO_4$ into distilled water to be 1000 mL and the solution was cooled to room temperature), sulfuric acid reagent (prepared as in open reflux method) and potassium hydrogen phthalate (KHP) standard was used. KHP was prepared for preparation of the calibration curve. KHP was lightly crushed and then dried to constant weight at 120°C. Then different initial KHP concentrations were dissolved in distilled water for different COD concentrations. KHP solution had a theoretical COD of 900 mg/L for 0.765 g KHP /L. At least five or more standards of KHP were prepared to obtain COD concentrations of 50 to 900 mg COD/L The calibration curve was used for determination of COD contents of the samples. The absorbances of the samples are placed to the equation for calculating the COD concentration. (Greenberg A.E, 1989, pp. 5,9-10)

2.6.2 Total Organic Carbon (TOC) Analysis

TOC analysis methods are based on oxidation of carbon content to carbon dioxide. Combustion with oxygen, ultraviolet or chemical oxidations and a combination of these methods may be used to convert organic carbon to carbon dioxide. Both inorganic and total carbon content of the samples were determined. The inorganic carbon (IC) content includes the carbonate, bicarbonate and dissolved CO_2. The difference between the total carbon and inorganic carbon contents is the total organic carbon content. A DOHRMAN, DC-190 type TOC analyzer was used for IC and TOC analyses. (Dohrman Manual)

2.6.3 Total Nitrogen Analysis

Organic and inorganic nitrogen compounds are transformed into nitrate according to Koroleff's method by treatment with an oxidizing agent in a thermoreactor. In concentrated sulfuric acid, this nitrate reacts with a benzoic acid derivative (Nitrospectral) to form a deep red nitro compound that is determined photometrically. The absorbance measurements are made at 517 nm, in a measuring range of 0.5-15 mg/LN. 10 mL of pretreated sample is taken. 1 microspoon of

Reagent N-1 is added to the sample and mixed perfectly by shaking. Then 6 drops of Reagent N -2 is added to the sample and mixed. The cell is heated at 100°C for 1 hour. The closed cell is allowed to cool to room temperature. The cell is briefly shaked after 10 min. 1.5 mL of sample is taken from the cell to the total nitrogen cell after adding 1 microspoon of Reagent N-3 to the measuring cell. After leaving the cell for 10 min. (reaction time) the absorbance value was read from the photometer. (Merck Spectroquant® Nitrogen (total) Cell Test Cat. No: 1.14537.0001)

2.6.4 Suspended Solid Measurements

Biomass (MLSS) concentrations were determined by filtering the samples through milipore filters (45 μm) and drying until constant weight in an oven at 105° C. The calculations were made by using the following equation (Greenberg A.E, 1989):

$$M=(A-B) \times 1000 / V$$

where, (A) is the weight of filter and residue after drying; (B) is the weight of filter after drying and (V) is the volume (mL) of the sample

Total suspended solids (TSS) measurements were carried out by using capsules with a volume of 50 mL. 25 mL sample was taken from the aeration basin and 25 mL sample from the influent synthetic wastewater and those samples were placed in separate capsules . The capsules were dried until constant weight in an oven at 105° C. Then the following equation was used for calculation of suspended solids concentrations,

$$TSS=[(B_2 - A_2) - (B_1 - A_1)] \times 1000/ V$$

where, A_1 is the weight of the capsule used for influent; A_2 is the weight of the capsule used for the samples from the aeration tank, B_1 is the weight of the influent capsule and residue; B_2 is the weight of the sludge capsule and residue and (V) is the volume (mL) of the sample

CHAPTER THREE

RESULTS AND DISCUSION

Major types of nitrogen fixing bacteria, which can be used for wastewater treatment, are summarized in section 1.2.2. *Azotobacter vinelandii* was selected and used in the experimental studies because of its favourable characteristics to develop and sustain in activated sludge culture. In the preliminary experiments the performances of two species of *Azotobacter* namely, *Azotobacter vinelandii* and *Azotobacter chrococcum* were compared. Both cultures were grown in Burk's media at 28°C and pH of 7.2-7.5 for stock culture preparation. The cultures were used for inoculation of the experimental flasks with an initial COD of nearly 5000 mg/L. TOC removal performances of both cultures were compared under the same conditions. At the end of the third incubation day *Azotobacter vinelandii* achieved nearly 44% TOC removal efficiency, while *Azotobacter chrococcum* removed only 32% of the initial TOC. Apparently, *Azotobacter vinelandii* grew faster than *Azotobacter chrococcum.* Microscopic observations indicated that flock formation by *Azotobacter vinelandii* was faster and the flocks were stronger then the flocks of *Azotobacter chrococcum*. The flocks of *Azotobacter chrococcum* disintegrated easily under vigorous aeration. Also, *Azotobacter chrococcum* was more sensitive to changes in environmental conditions. *Azotobacter chrococcum* require high dissolved oxygen concentrations; whereas *Azotobacter vinelandii* can tolerate low dissolved oxygen concentrations. On the basis of experimental results, *Azotobacter vinelandii* was preferred over *Azotobacter chrococcum* and used for further experiments.

3.1. BATCH SHAKE FLASK EXPERIMENTS

The first stage of the experimental studies was carried out in the incubator shaker. The experiments were performed to prove advantages of using *Azotobacter vinelandii*'s for treatment of nitrogen deficient wastewaters. Activated sludge culture, *Azotobacter vinelandii* species and their combinations were used. In the first set of experiments molasses and in the second set glucose was used as the carbon source. Experiments were performed with different initial TN/ COD ratio to determine performances of both cultures at different levels of nitrogen deficiency. Urea and KH_2PO_4 were the major nitrogen and phosphate sources in all experiments. COD/ P ratio in all experiments was 100/ 1.5. $FeSO_4$ and H_2MoO_4 were added to the semi-semi-synthetic wastewater when *Azotobacter* species were used as the culture. The incubation period was five or six days. TOC removal performances of the cultures were compared at the end of the incubation period. The temperature was controlled at 28°C and the flasks were shaked with 150 rpm. The initial pH was adjusted to 7.5 but dropped to 6.5 at the end of the experiments.

3.1.1. Shake Flask Experiments with Molasses as Carbon Source

In the first set of batch shake flask experiments, molasses was used as the carbon source. Figure 3.1 depicts variations of TOC with time in shake flasks at 5000 mg/L initial COD concentration where as activated sludge was used as the inoculum culture. TN/COD ratio was varied between 0/100 and 8/100 in this set of experiments. The data were taken daily through a five-day incubation period. The influent TOC concentration was 2488 mg/L and dropped to approximately 200 mg/L at the end of the incubation period in all flasks. However, the rate of TOC removal was different in each flask depending on initial TN/COD ratio. As seen from the figure, the TOC removal efficiency was 95 % at the end of the first day when TN/TOC ratio was 8/100, whereas the removal efficiency was only 40% when TN/COD ratio was 0/100. TOC removal performances of 92%, 92%, 93%, 93%, 95% were obtained in the flasks with different initial TN/ COD ratios of 0/100, 1/100, 2/100 4/100 and 6/100, respectively at the end of 5 days of incubation period.

TOC removal performance of activated sludge decreased with decreasing initial TN/COD ratio from 6/100 to 0/100. Although the final TOC removals were approximately the same in all flasks, TOC removal rate in nitrogen deficient flasks (low TN/COD ratio) were much slower as compared to sufficient nitrogen containing flasks. A control flask without any inoculum culture was used in experiments and no TOC removal was observed in this flask.

As explained in part 1.2.1, the ratio of the three essential elements, namely the C/ N/ P ratio, should be approximately 100/ 14/ 2 or COD/ N/ P ratio should be 100/ 6/ 2 in a nutritionally balanced wastewater. The nitrogen content of the molasses may affect the results since the reported TN/ COD ratios are based on externally added nitrogen concentration. Total nitrogen content of molasses was 74 mg/L N when COD was 2520 mg/L. That is, TN/ COD ratio was nearly 3% in molasses. However, only a fraction of total nitrogen content of molasses may be utilisable. That means the nitrogen content of molasses was not sufficient to balance the nutritional requirements of the organisms. However, high treatment performances obtained with the nitrogen deficient flasks may be because of the nitrogen content of the molasses.

Data obtained with pure *Azotobacter vinelandii* is presented in Figure 3.2 with an initial COD concentration of 2500 mg/L. Diluted molasses was used as carbon source. TOC concentration of influent was approximately 1000 mg/L and decreased to approximately 200 mg/L in the sixth day when TN/ COD ratio was 1/ 100. Significant TOC removal efficiencies, 80%, were obtained with TN/COD ratio of 0/ 100 and 1/ 100. TOC removal performance of pure *Azotobacter vinelandii* decreased with increasing initial TN/ COD ratio from 2/ 100 to 8/ 100. 77%, 83%, 51%, 74%, 62%, 5% TOC removal efficiencies were obtained when initial TN/ COD ratio were 0/ 100, 1/ 100, 2/ 100, 4/ 100, 6/ 100, 8/ 100, respectively. The results indicated that *Azotobacter vinelandii* was quite effective in TOC removal from nitrogen deficient wastewaters especially when TN/ COD ratio was extremely low. Molasses seemed to affect the treatment performance and some anomalies were observed in the results because of the nitrogen content of the molasses.

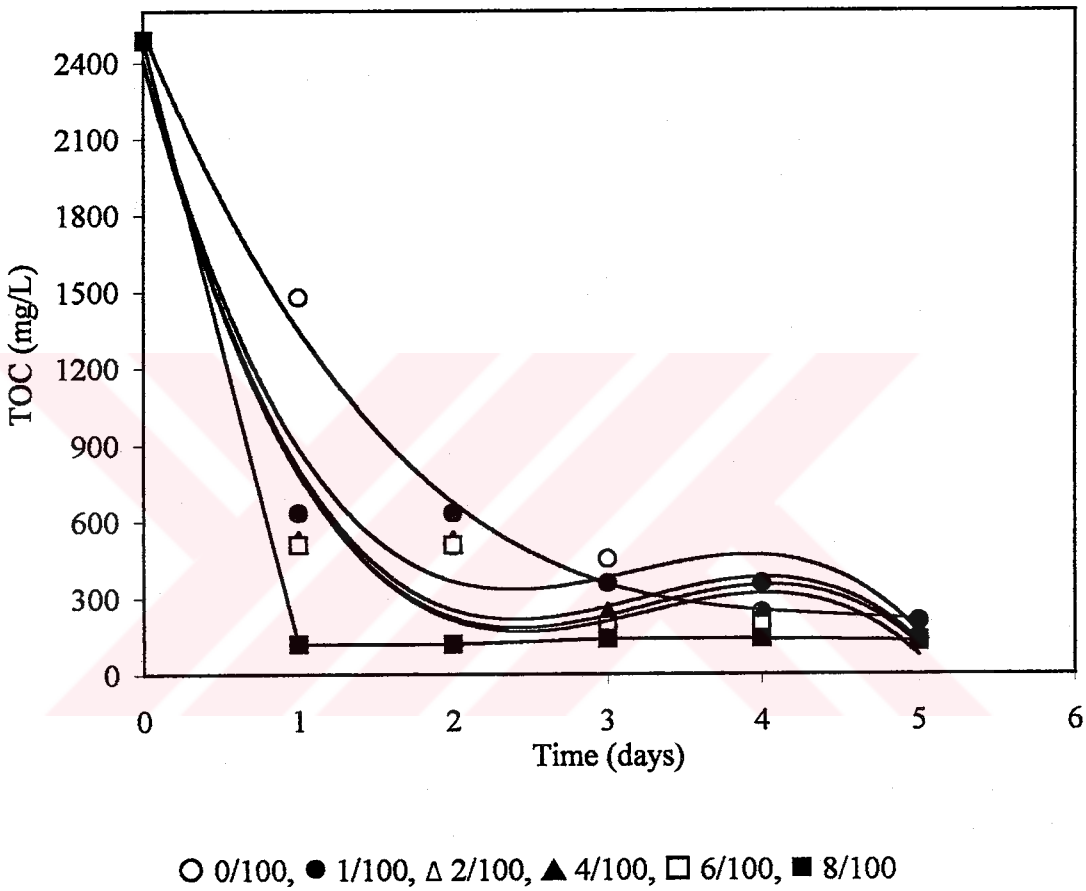


Figure 3.1 Variations of TOC concentrations with time at different TN/COD ratios with activated sludge culture

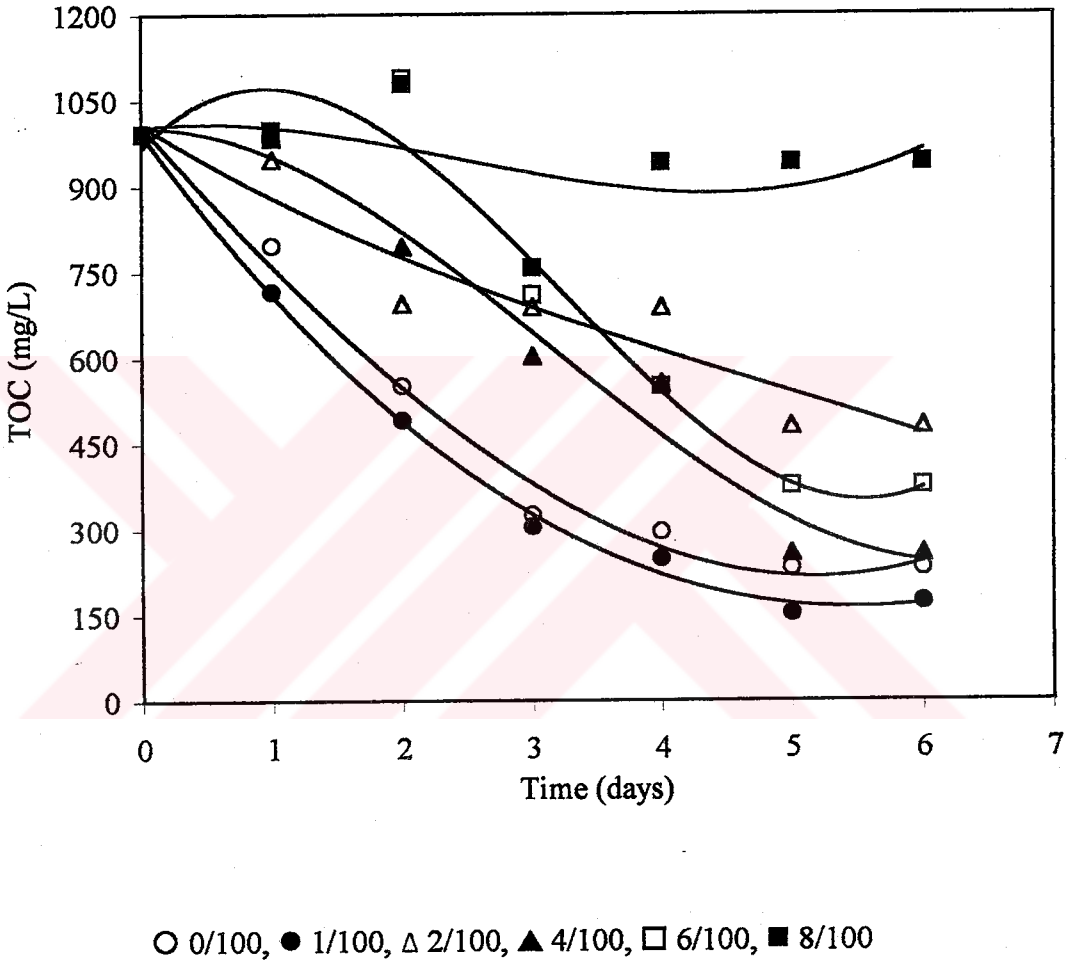


Figure 3.2 Variations of TOC with time at different TN/COD ratios with pure *Azotobacter vinelandii* culture

In order to minimise adverse effects of molasses nitrogen content on the results, further experiments were carried out with low molasses concentrations with an initial TOC of 500 mg/L. Figure 3.3 summarizes the results obtained with the activated sludge culture at different initial TN/ COD ratios. As seen from the figure the best TOC removal performance was obtained with initial TN/COD ratio of 8/100 where the TOC removal efficiency was 75% at the end of the first day. TOC removal performance decreased with decreasing TN/COD ratio. TOC removal efficiencies of 59%, 67%, 66%, 67% and 75% were obtained with initial TN/COD ratios of 0/100, 1/100, 2/100, 4/100 and 6/100, respectively. Because of the nitrogen content of molasses, anomalies were observed in some experimental results.

The last shake flask experiment with molasses as the sole carbon source was carried out with pure *Azotobacter vinelandii* culture. The results of this experiment obtained at different initial TN/ COD ratio are summarized in Figure 3.4 where the initial COD was nearly 2000 mg/L. Because of utilization of pure *Azotobacter* species in the culture media, the best TOC removal performance was obtained with the lowest initial TN/ COD ratio. TOC removal efficiencies of 83% and 77 % were obtained with initial TN/ COD ratios of 1/ 100 and 0/ 100, respectively. TOC removal performance decreased with increasing initial TN/ COD ratio. TOC removal efficiencies of 51%, 74%, 62%, 23% was obtained with initial TN/COD ratios of 2/100, 4/100, 6/100, 8/100. Again, because of nitrogen content of molasses some anomalies were observed in the results. However, it was clearly observed that *Azotobacter* species were effective in treating nitrogen deficient (low TN/COD ratio) wastewaters resulting in high TOC removal efficiencies.

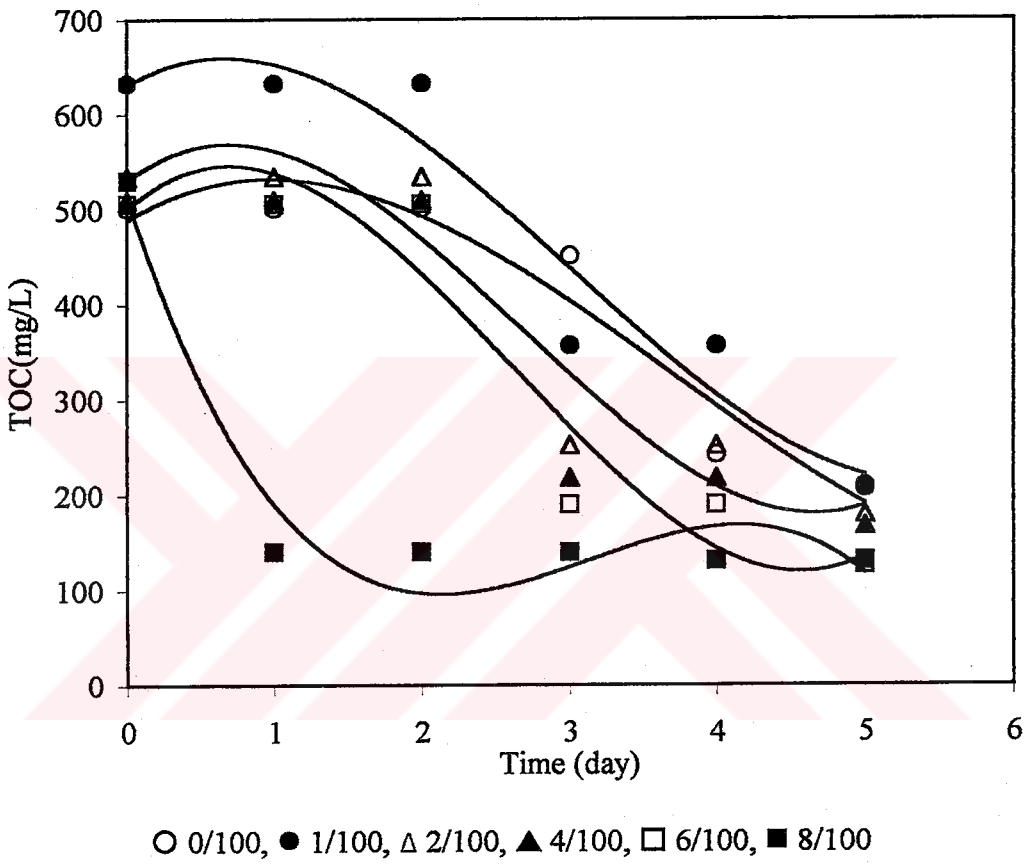


Figure 3.3 Variation of TOC with time at different TN/COD ratios with the activated sludge culture

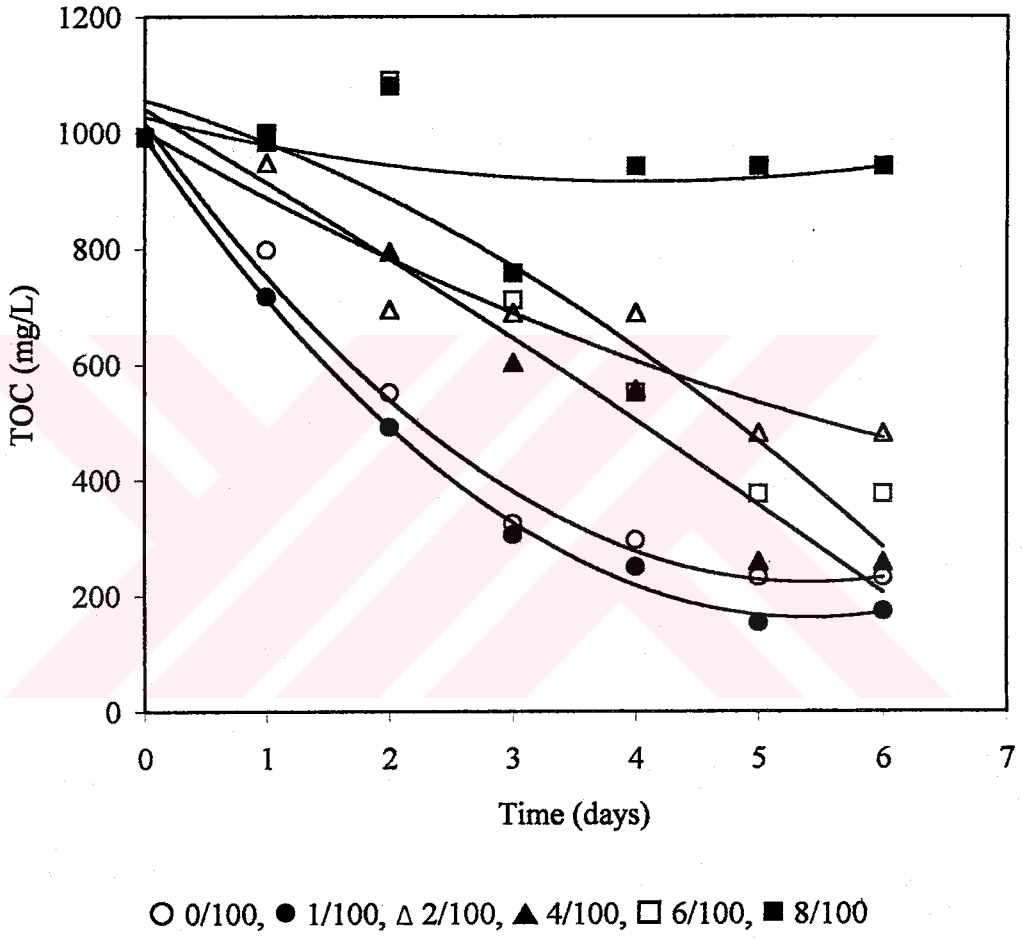


Figure 3.4 Variation of TOC with time at different TN/COD ratios with the *Azotobacter vinelandii* culture

3.1.2 Shake Flask Experiments with Glucose as Carbon Source

To eliminate the interference of molasses because of its nitrogen content, glucose was used as the sole carbon source in further experiments. The major objective of this set of experiments was to prove effectiveness of *Azotobacter* culture in biological treatment of nitrogen deficient wastewaters. For this purpose performance of *Azotobacter* and activated sludge cultures were compared at different initial TN/COD ratios. The initial COD was nearly 2000 mg/L and TN/ COD ratio was varied between 0/100 and 8/100 while the P/COD ratio was constant as 1.5/ 100. Pure *Azotobacter*, activated sludge and a mixture of both cultures were used in experiments to compare their performances.

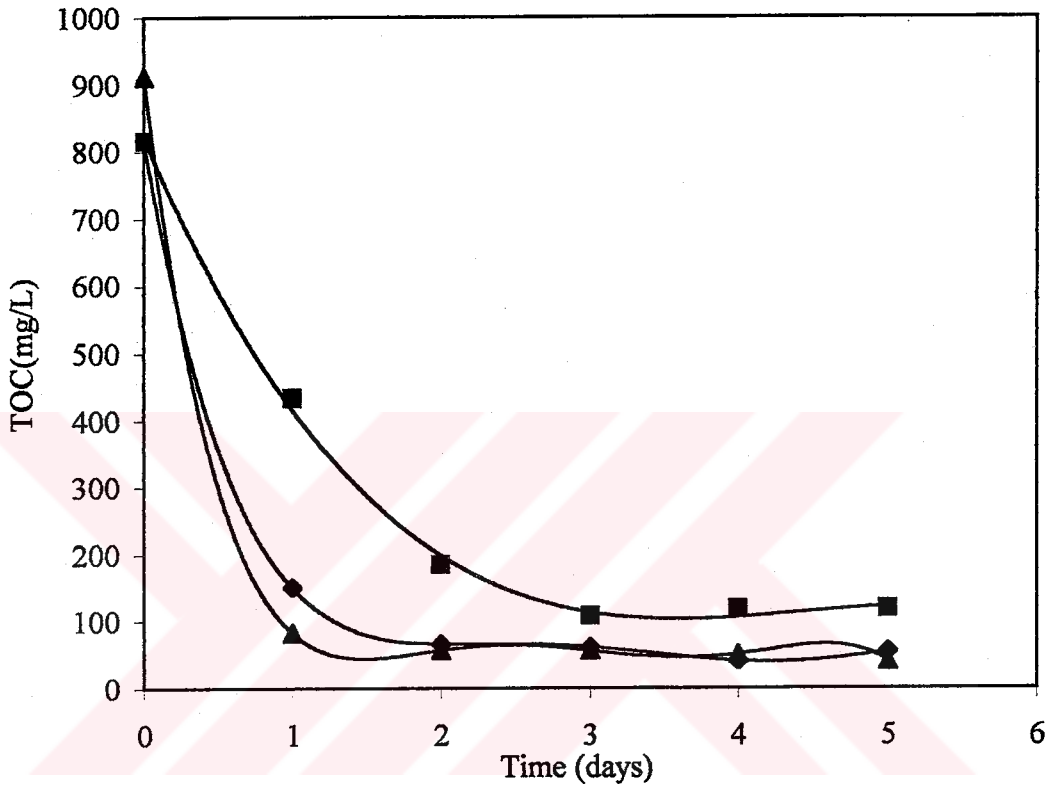
Figure 3.5 shows the variation of TOC with time for different cultures when TN/ COD ratio was 8/100. Because of high nitrogen content of the medium, pure *Azotobacter* culture performance was not as good as the other cultures. *Azotobacter* removed TOC rather slowly as compared to the activated sludge culture. The most effective TOC removal was obtained when *Azotobacter vinelandii* was used with the activated sludge culture. TOC removal efficiency of 96% was obtained with this culture after five days of incubation. Initial TOC of 912 mg/L dropped to 83 mg/L at the end of the first day resulting in nearly 90% TOC removal when *Azotobacter* was included in the activated sludge culture. Activated sludge culture alone had a TOC removal efficiency of 84% at the end of the first day. The results indicated that *Azotobacter* culture was not effective in TOC removal at high TN/ COD ratios such as 8/ 100 (no nitrogen limitations). However, activated sludge culture alone or in combination with *Azotobacter* were quite effective in TOC removal at high TN/ COD ratios.

Figure 3.6 depicts variations of TOC with time for TN/ COD ratio 6/ 100 for different cultures. Again the best TOC removal performance was obtained with *Azotobacter* containing activated sludge culture. TOC dropped from an initial value of 900 mg/L to 100 mg/L at the end of the first day resulting in nearly 90% TOC removal. Pure *Azotobacter* and activated sludge cultures alone removed TOC rather slowly. TOC removal efficiencies were nearly 95% for the pure *Azotobacter* and

Azotobacter containing activated sludge cultures at the end of five days of incubation period. However, only 56% TOC removal was obtained when activated sludge was used alone (free of *Azotobacter*) in five days. The results indicated that inclusion of *Azotobacter* in activated sludge culture is beneficial in treatment of even slightly nitrogen deficient wastewaters.

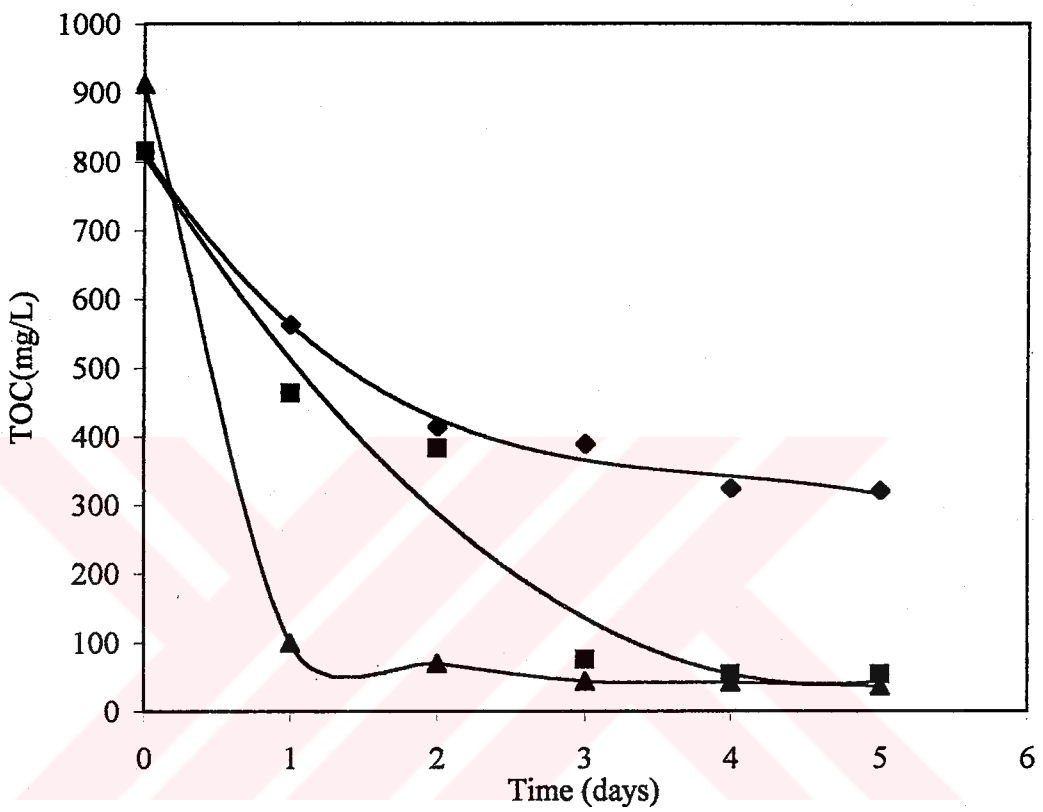
Variations of TOC with time for different cultures are shown in Figure 3.7 when TN/COD ratio was 4/100. The fastest TOC removal was obtained with *Azotobacter* containing activated sludge culture. TOC dropped from initial value of 900 mg/L nearly 80 mg/L resulting in nearly 91% TOC removal at the end of the first day in this flask. At the end of the 5 days of incubation period, 96%, 82% and 74% TOC removal efficiencies were obtained with *Azotobacter* containing activated sludge, pure *Azotobacter* and activated sludge cultures, respectively. Because of nitrogen deficiency in the medium, activated sludge culture performance was not as good as the *Azotobacter* containing cultures. The results clearly indicated that *Azotobacter* inclusion in the activated sludge culture in the presence of nitrogen limitation was beneficial.

Figure 3.8 shows the variations of TOC with time for different cultures when TN/COD ratio was 2/100. TOC removal efficiency obtained with the activated sludge culture was nearly 52% at the end of the 5 days incubation because of nitrogen deficiency. However, when *Azotobacter* culture *or Azotobacter* supplemented activated sludge culture was used nearly complete removal (98%) of TOC was achieved within 5 days. The rate of TOC removal obtained with *Azotobacter*-supplemented activated sludge culture was higher than that of the pure *Azotobacter* or activated sludge cultures resulting in nearly 95% TOC removal in a day. *Azotobacter* supplemented activated sludge performed much better under severe nitrogen limitations.



◆ Activated Sludge, ■ *Azotobacter vinelandii*, ▲ Activated Sludge & *Azotobacter vinelandii*

Figure 3.5 Variation of TOC with time for different cultures at initial TN/COD ratio of 8/100



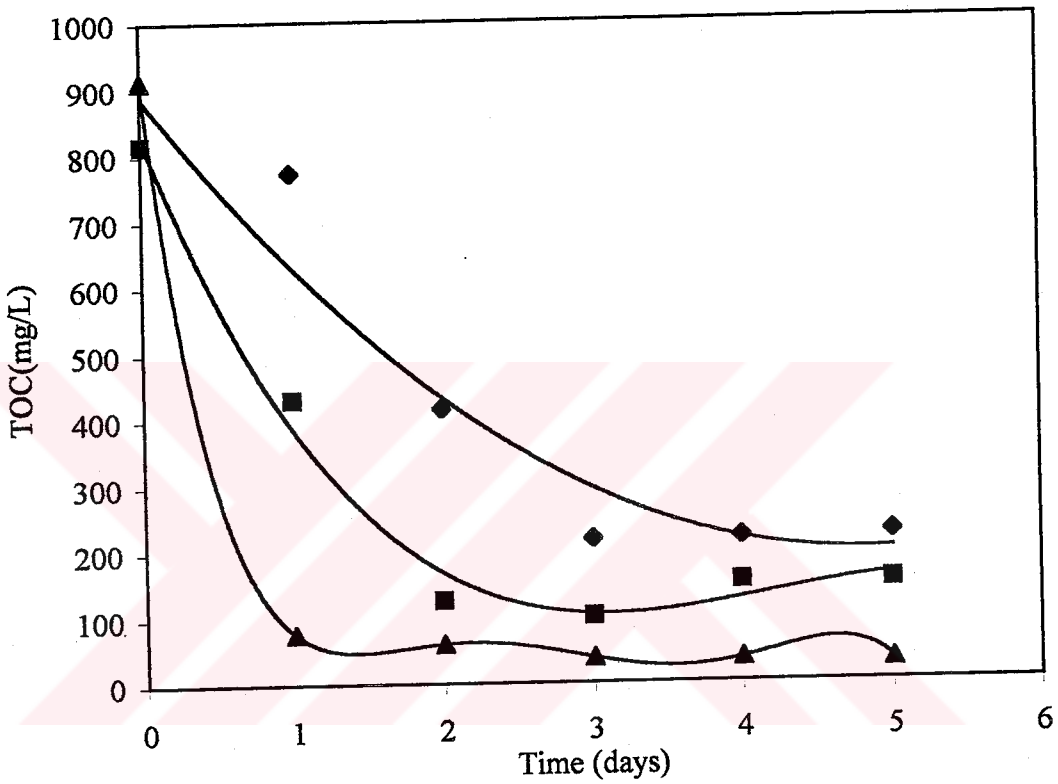
◆ Activated Sludge, ■ *Azotobacter vinelandii*, ▲ Activated Sludge & *Azotobacter vinelandii*

Figure 3.6 Variation of TOC with time for different cultures at initial TN/COD ratio of 6/100

TOC removal performances of the same cultures are compared in Figure 3.9 when TN/ COD ratio was 1/100. The most effective TOC removal was obtained when *Azotobacter* was used with activated sludge culture. TOC decreased from an initial value of 900 mg/L to less than 100 mg/L resulting in nearly 95% TOC removal within three days. Performance of the pure *Azotobacter* culture was comparable with the mixed culture in terms of TOC removal efficiency (85%), although the rate of TOC removal was rather slow. When Azotobacter-free activated sludge culture was used, TOC dropped from n initial value of 820 mg/L to 450 mg/L resulting in nearly 45% TOC removal at the end of five days. This is because of nitrogen deficiency in the medium. *Azotobacter* supplemented activated sludge culture resulted in higher rate and extent of TOC removal when initial TN/COD was 1/100 (severe nitrogen limitations) as compared to the other cultures tested.

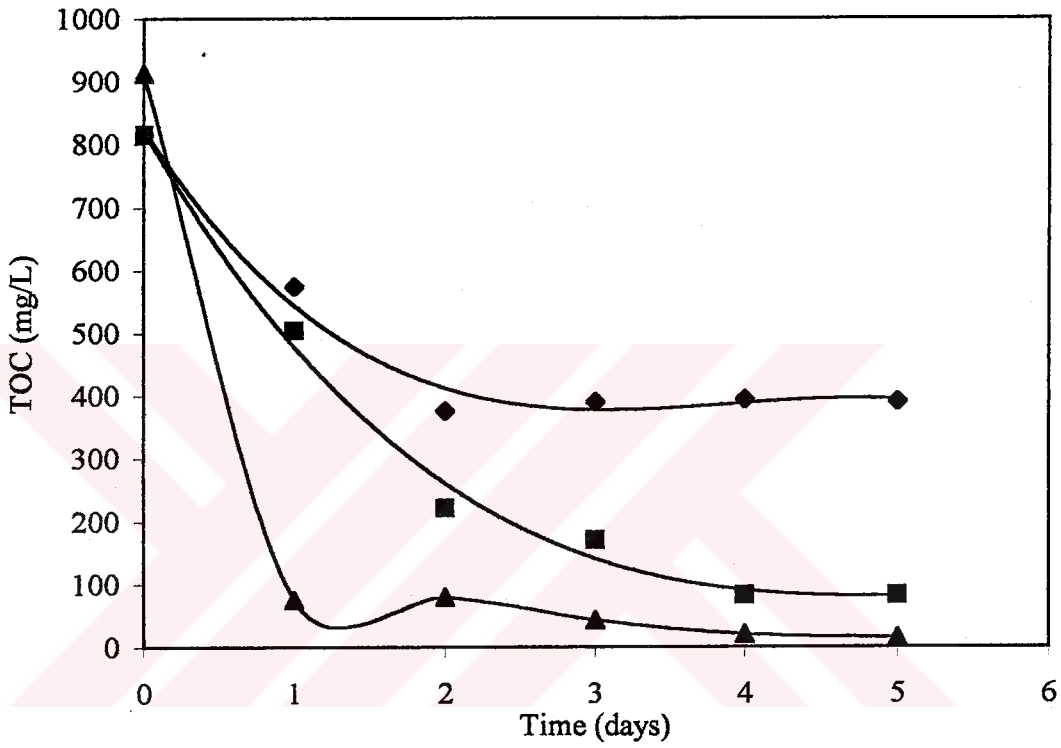
Figure 3.10 depicts variations of TOC with time for different cultures when TN/ COD ratio was 0/ 100. Because of severe nitrogen limitations, only 18% TOC removal was achieved with the activated sludge culture within five days. The best performance was observed with *Azotobacter*-supplemented activated sludge culture resulting in nearly 85% TOC removal. Pure *Azotobacter* culture also resulted in nearly 60% TOC removal within five days. The rate and the extent of TOC removal obtained with the *Azotobacter* supplemented activated sludge culture was superior to the others tested.

On the basis of the results of batch shake flask experiments, it can be said that *Azotobacter* supplemented activated sludge culture was very effective in treatment of nitrogen deficient wastewaters. Pure *Azotobacter* cultures did not perform as well as *Azotobacter*-supplemented activated sludge, which may be because of symbiotic interactions among *Azotobacter* and activated sludge organisms.



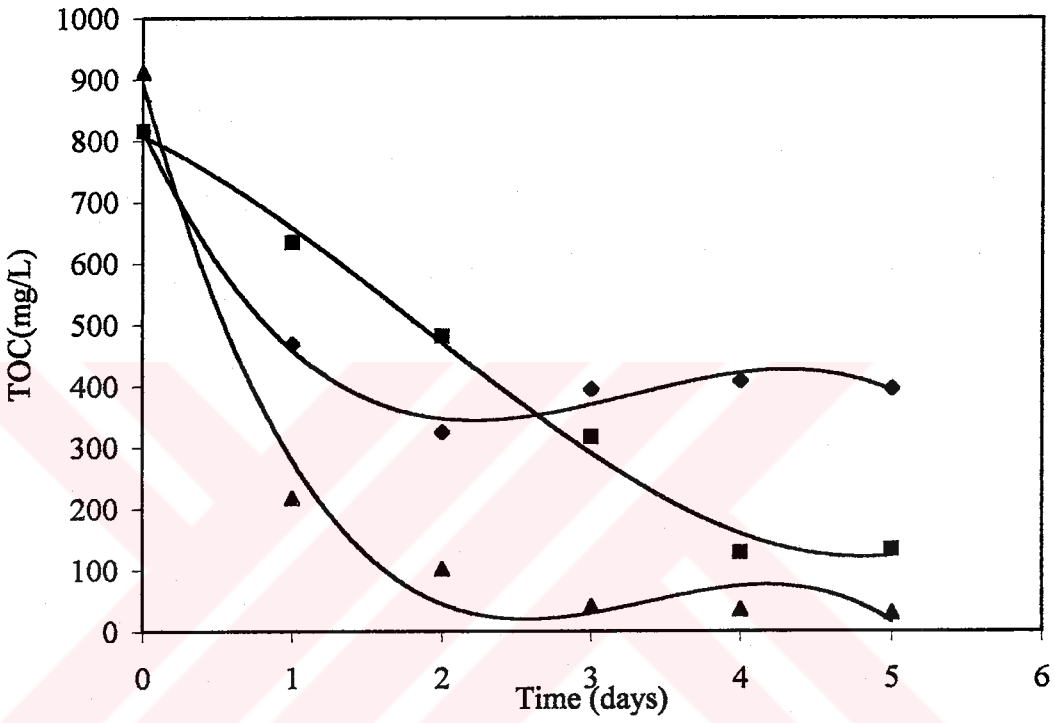
◆ Activated Sludge, ■ *Azotobacter vinelandii*, ▲ Activated Sludge & *Azotobacter vinelandii*

Figure 3.7 Variation of TOC with time for different cultures at initial TN/COD ratio of 4/100



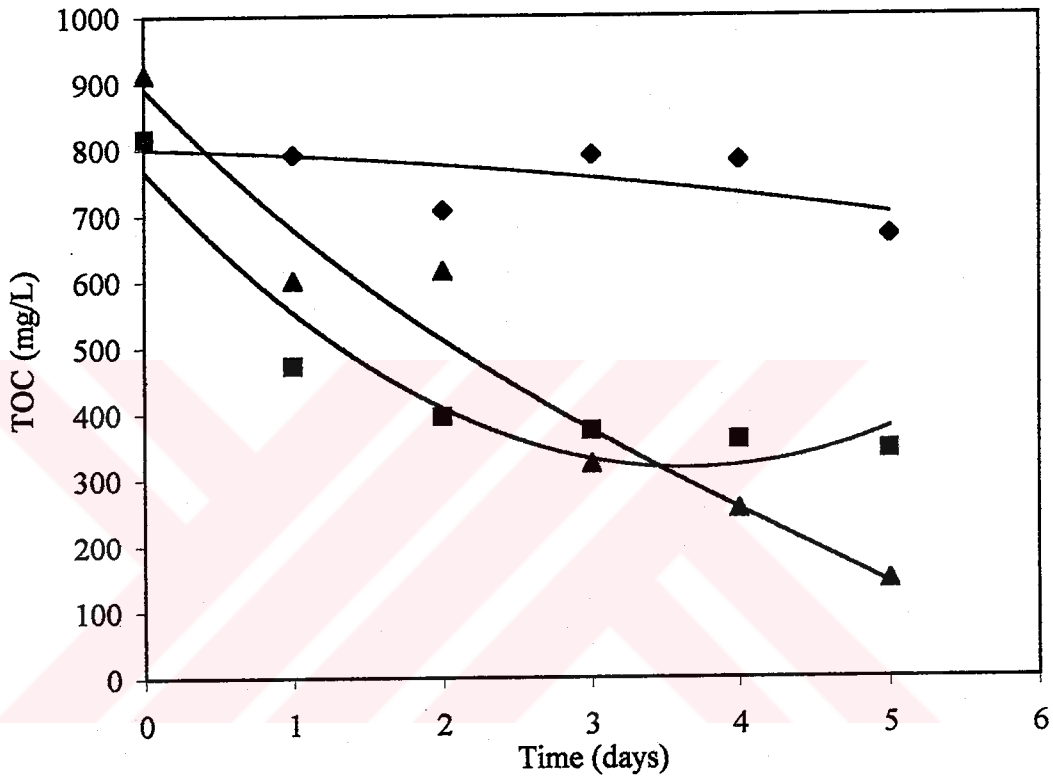
◆ Activated Sludge, ■ *Azotobacter vinelandii*, ▲ Activated Sludge & *Azotobacter vinelandii*

Figure 3.8 Variation of TOC with time for different cultures at initial TN/COD ratio of 2/100



◆ Activated Sludge, ■ *Azotobacter vinelandii*, ▲ Activated Sludge & *Azotobacter vinelandii*

Figure 3.9 Variation of TOC with time for different cultures at initial TN/COD ratio of 1/100



◆ Activated Sludge, ■ *Azotobacter vinelandii*, ▲ Activated Sludge & *Azotobacter vinelandii*

Figure 3.10 Variation of TOC with time for different cultures at initial TN/COD ratio of 0/100

3.2. CONTINUOUS EXPERIMENTAL STUDIES IN ACTIVATED SLUDGE UNIT

Continuous experimental studies were carried out in a laboratory scale activated sludge unit by using Azotobacter-supplemented activated sludge culture. Similar experiments were carried out by using activated sludge culture (Azotobacter-free) for comparison. No external nitrogen addition was realized. Molasses was used as the sole carbon source and the nitrogen content of molasses (TN/COD = 3/ 100) was the only nitrogen source. COD /TN /P ratio was 100 /0 /1.5 in terms of external nitrogen source. COD concentration in the feed was approximately 2000±200 mg/L. COD removal performance of the system was investigated for different feed TN/ COD ratios, hydraulic residence time (θ_H), sludge age (θ_c) and the feed COD concentration. HRT was chosen as 10 hours and SRT (θ_c) as 10 days for experiments with variable TN/ COD ratios.

3.2.1. Effect of Feed TN/ COD Ratio on COD Removal Efficiency

Continuous experiments were performed in an activated sludge unit in order to investigate the COD removal performance of the system at different feed TN/ COD ratio. Hydraulic residence time (O_H), sludge age (θ_c) and the feed COD content were θ_H = 10 h, θ_c = 10 d and throughout the experiments. Initial COD concentration was kept constant at S_o= 2000 mg/L. But TN concentration was varied by changing urea concentration to obtain TN/ COD > 0.03 in the feed. Fe and Mo salts were not added into the semi-synthetic media. The same experiments were performed with Azotobacter-supplemented and Azotobacter-free activated sludge cultures. pH varied between 7-7.8.

The results are depicted in Figure 3.11 in form of effluent COD and COD removal efficiency as a function of the feed TN/ COD ratio. Effluent COD and the COD removal efficiency were not affected significantly from the variations of TN/ COD ratio in the feed with *Azotobacter*-supplemented activated sludge culture. The COD removal efficiency varied between 84%-87%. This trend indicates a good level

of stability in COD removal efficiency when *Azotobacter vinelandii* was added into the activated sludge. However, the performance of *Azotobacter*–free activated sludge culture was not satisfactory, especially at low TN/ COD ratios in the feed. When TN/COD ratios in the feed were 0.03 and 0.05, COD removal efficiencies of 57% and 68% were obtained. The effluent COD decreased and COD removal efficiency increased with increasing TN/ COD ratio in the feed resulting in satisfactory COD removals at TN/ COD > 0.08. The performances of both systems were comparable at high feed TN/COD ratios such as TN/COD > 0.07 resulting in COD removal efficiency of above 70%. These results clearly indicated the advantage of using *Azotobacter*-supplemented activated sludge culture for effective COD removal from nitrogen deficient wastewaters (TN/COD < < 0.08).

Figure 3.12 shows variation of the effluent TOC and TOC removal efficiency with the feed TN/TOC ratio. The results are similar to Figure 3.11. TOC/ COD ratio was approximately 0.40-0.45 in the fed. The same trends were observed. *Azotobacter vinelandii*-supplemented culture had a TOC removal efficiency between 84%-87% and was almost constant for all TN/TOC ratios. Also in *Azotobacter* -free activated sludge, the TOC removal was 55% at TN/ TOC ratio of 0.08 and increased with increasing TN/ TOC ratio. The best TOC removal efficiency was 81% observed with TN/ TOC ratio of 0.292.

3.2.2. Effect of Sludge Age on COD Removal Efficiency

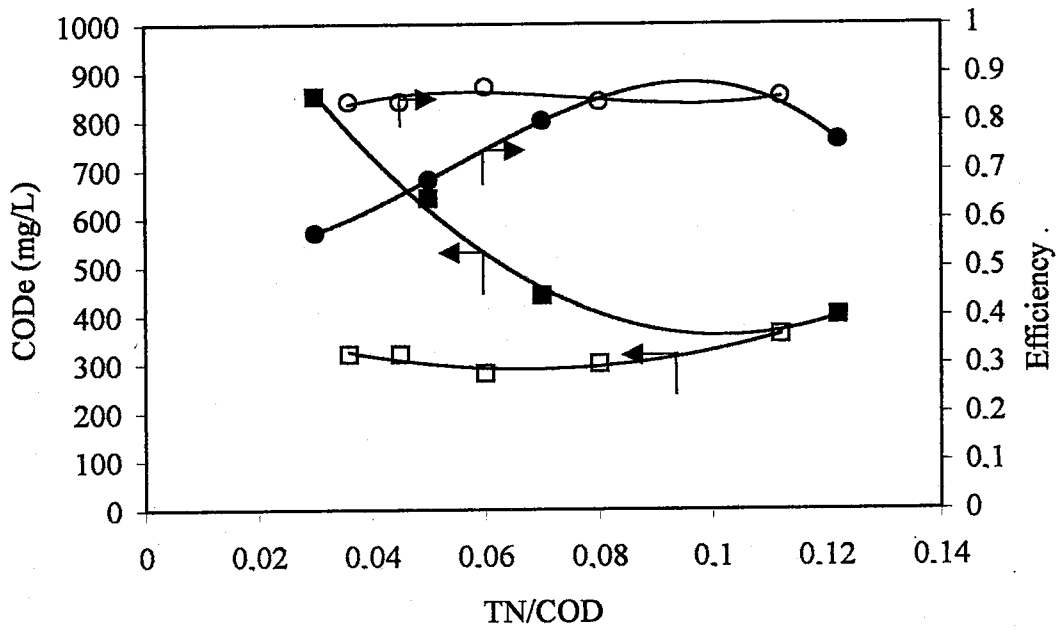
Sludge age is an important parameter affecting the effluent wastewater quality and the COD removal efficiency. In order to determine the effects of sludge age on the systems COD removal performance, continuous activated sludge experiments were performed at different sludge ages between 3-20 days. *Azotobacter*–supplemented activated sludge culture was used and no external nitrogen was added into the media. Therefore, TN/ COD ratio in the feed wastewater was nearly 0.03 which was because of the nitrogen content of molasses. Hydraulic residence time and the feed COD were θ_H = 10 h and CODo = 2,000 ± 200 mg/L throughout the experiments. In this set of experiments Mo and Fe was not added to the feed

wastewater. Sludge age was adjusted by removing certain amount of sludge from the aeration tank daily. TOC COD and nitrogen was measured. pH varied between 7-7.5. pH dropped below 7.0 at low sludge age's such as 6 and 3 days and it was adjusted to pH = 7 by adding diluted NaOH.

The results are depicted in Figure 3.13 in form of the effluent COD and COD removal efficiency as a function of the sludge age. The effluent COD decreased and COD removal efficiency increased with increasing sludge age. High sludge ages (≥10 days) resulted in large biomass concentrations in the aeration tank resulting in better COD removal performances. COD removal efficiency remained almost constant for sludge ages above 10 days. However, the efficiency increased sharply with the increasing sludge age for θ_C values between 3 to 10 days. COD removal efficiency was 11% when SRT was 3 days and increased to 44% at 6 days. These results indicated that the optimum sludge age for maximum COD removal efficiency was nearly 10 days. Further increases in sludge age above 10 days did not improve the system's performance significantly. The highest removal efficiency was 65% at SRT of 15 days. However, 64% and 63 % COD removals were obtained at SRT values of 10 days and 20 days, respectively.

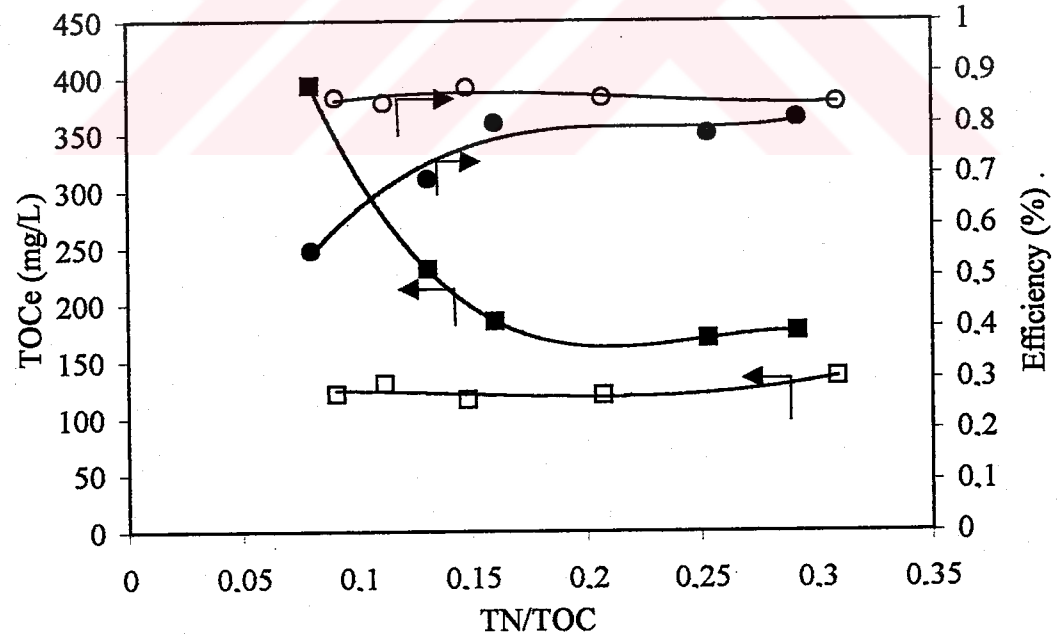
Figure 3.14 depicts variations of effluent TOC and TOC removal efficiency with the sludge age. The comparison of COD and TOC analysis indicated that the TOC/ COD ratio in the feed wastewater varied between 0.40-0.47. TOC removal efficiency increased with the sludge age and leveled off at sludge age values above 10 days. Th optimal sludge age was 10 days resulting in 65% TOC removal and 300 mg/L effluent TOC.

Bulking sludge formed at high sludge ages of 15 d and 20 d, which may cause of low DO concentrations. *Azotobacter vinelandii* formed polymeric substances such as alginate, which caused formation of large flocs and biofilm on the surfaces of diffusers and the separating plate.



■, ● Activated sludge; □, ○ Activated sludge &*Azotobacter vinelandii*

Figure 3.11 Variations of effluent COD and COD removal efficiency with the feed TN/ COD ratio (θ_c=10 d, θ_H=10 h, COD_o=2000±200 mg/L)



■, ● Activated sludge; □, ○ Activated sludge &*Azotobacter vinelandii*

Figure 3.12 Variations of effluent TOC and TOC removal efficiency with the feed TN/ TOC ratio (θ_c=10 d, θ_H=10 h)

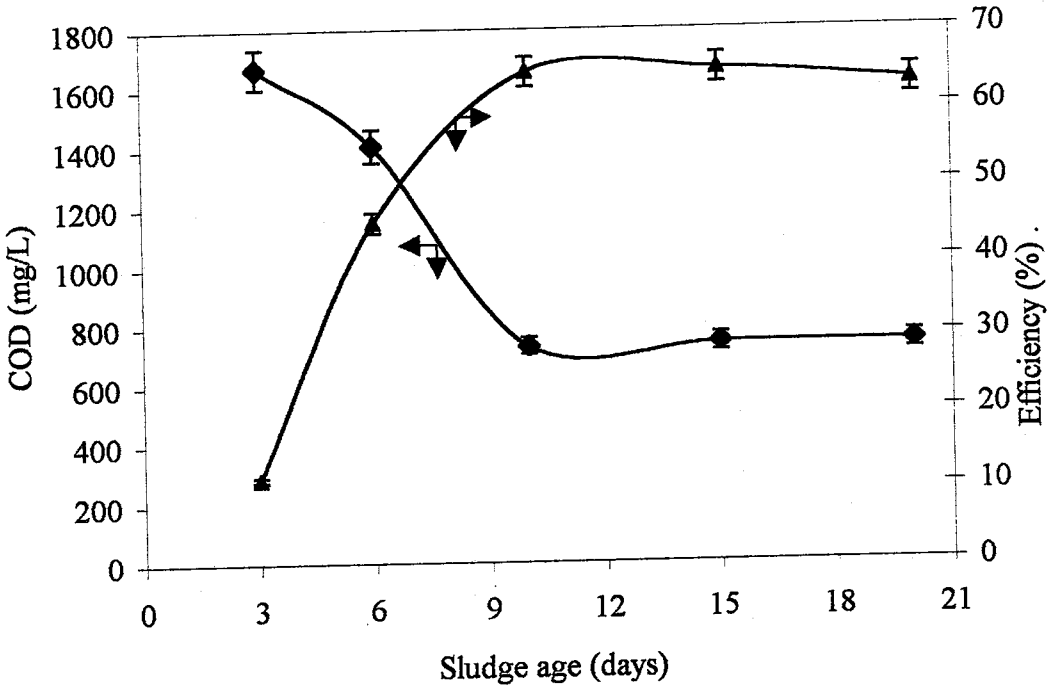


Figure 3.13 Variation of effluent COD and COD removal efficiency with sludge age. (θ_H=10 h, COD_o=2000±200 mg/L)

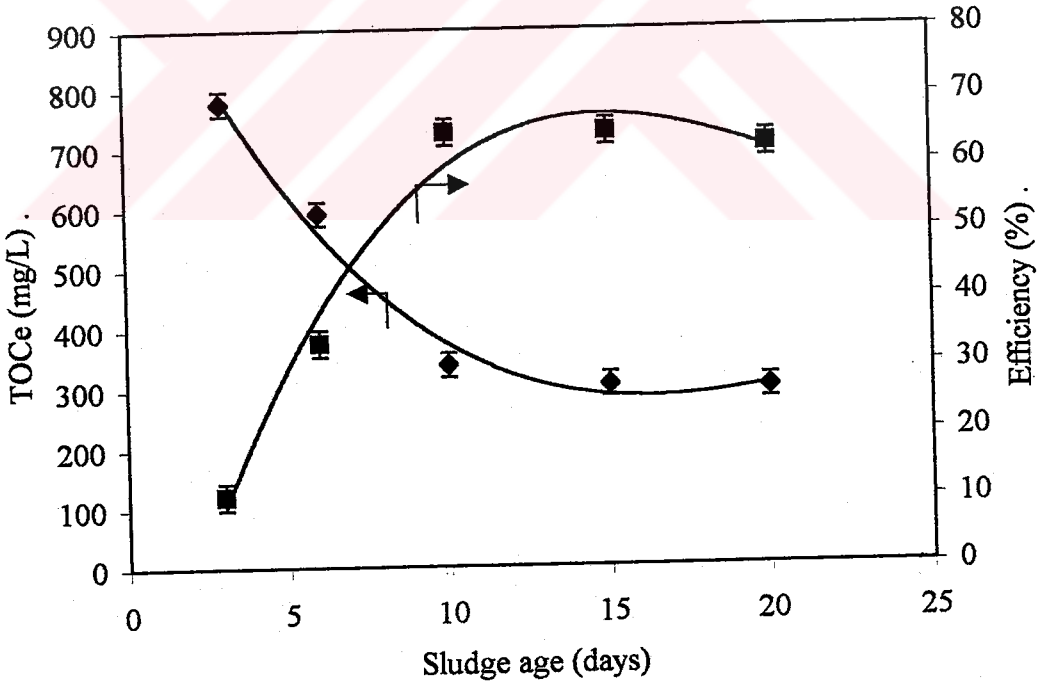


Figure 3.14 Variation of effluent TOC and TOC removal efficiency with sludge age. (θ_H=10 h, COD_o=2000±200 mg/L)

Results of nitrogen measurements are presented in Table 3.1. The feed COD concentrations of the samples varied between 2000± 300 mg/L. The results show that *Azotobacter*-supplemented activated sludge culture used the nitrogen content of molasses. Nitrogen content of the effluent decreased with increasing sludge age. Almost no nitrogen content of the molasses was used at the sludge age of 3 days. The effluent nitrogen dropped to 22 mg/L from the feed value of 65 mg/L at SRT of 10 days.

Table 3.1 Total nitrogen contents in the feed and the effluent at different sludge ages (*Azotobacter*- supplemented activated sludge culture)

Sludge Age (days)	20	15	10	6	3
N_{feed} (mg/L)	58	62	65	81	61
$N_{effluent}$ (mg/L)	27	20.7	22	48	61
N-removal efficiency (%)	53	67	66	41	-

Biomass concentrations at different SRT levels were also determined. Biomass concentration increased with the sludge age as expected. The results are given in Table 3.2. These results are not used in the kinetic analysis.

Table 3.2 Biomass concentrations in activated sludge unit at different sludge ages

Sludge age (days)	20	15	10	6	3
X (g/L)	-	2.853	1.775	1.078	0.381

3.2.3.Effect of Hydraulic Residence Time on COD Removal Efficiency

Hydraulic residence time was varied by changing the feed flow rate. This set of experiments were performed at constant sludge age of θ_C = 10 d and the feed COD of 2000± 200mg/L. Hydraulic residence time varied between 2-14 h. The same

experiments were performed with *Azotobacter*-supplemented and *Azotobacter*-free activated sludge cultures. pH of the media in this set of experiments varied between 7.8-5. Sharp drops of pH occurred when hydraulic residence time was lowered.

The results of this set of experiments are presented in Figure 3.15 in form of effluent COD and COD removal efficiency as a function of hydraulic residence time. COD removal efficiency increased and the effluent COD content decreased with increasing hydraulic residence time. The increases in COD removal efficiency was much sharper at low hydraulic residence times of between θ_H = 2-6 hours. Nearly 90% COD removal efficiency was obtained at θ_H= 14 h when *Azotobacter*-supplemented activated sludge culture was used. *Azotobacter*-free activated sludge culture could remove only 66% of COD at same θ_H. The same trend was observed when Azotobacter-free activated sludge was used. However, COD removal efficiencies obtained with Azotobacter-free activated sludge culture was significantly lower than those of *Azotobacter*-supplemented culture at all hydraulic residence times. At θ_H= 12.2 h, the COD removal efficiencies were 86% in *Azotobacter*-supplemented activated sludge culture and 62% in *Azotobacter*-free activated sludge culture. COD removal efficiency was 82 % at HRT of 10 hours with the Azotobacter- supplemented activated sludge culture which dropped to 53% with Azotobacter-free activated sludge culture. At θ_H =8.44 h the COD removal efficiency was 76% with the Azotobacter- supplemented activated sludge culture. At θ_H= 6.33 h, 4.22 h and 2.04 h, the COD removal efficiencies for Azotobacter containing cultures were 74%, 61% and 34%, respectively. Whereas at the same HRT values COD removal efficiencies were 33%, 25% and 20% for Azotobacter-free culture. This is mainly because of nitrogen deficiency (TN/COD < 0.03) in the feed wastewater. These results indicated that *Azotobacter* addition to the activated sludge is an effective solution for biological treatment of nitrogen deficient wastewaters.

Total nitrogen measurements were made in Azotobacter-free activated sludge culture. These results are given in the Table 3.3. The effluent total nitrogen contents varied between 40-50 mg/L, which were nearly the same.

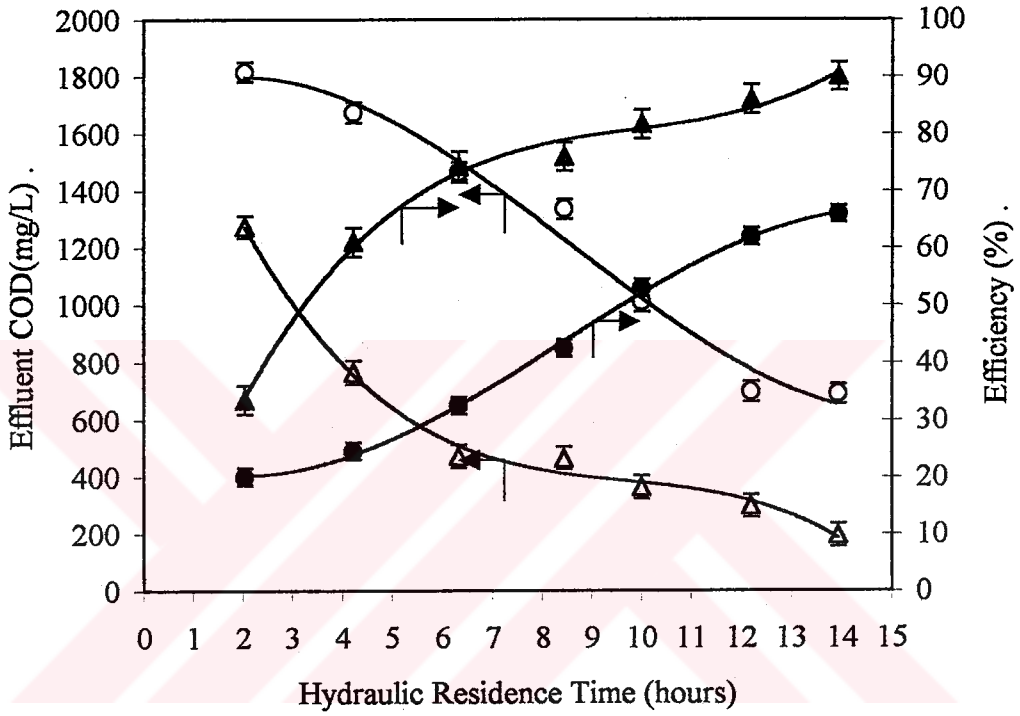
Table 3.3 Total nitrogen contents of the feed and the effluent at different HRT values (*Azotobacter*- free activated sludge culture)

θ_H (hours)	13.93	12.2	10	8.44	6.33	4.22	2.04
N_{feed} (mg/L)	60	49	62	105	97	90	90
$N_{effluent}$ (mg/L)	44	50	45	42	64	45	49
N-removal efficiency (%)	27	-	27	60	34	50	46

3.2.4. Variation of COD removal efficiency with feed COD concentrations

In this set of experiments, the optimum feed COD concentration was determined with *Azotobacter*-supplemented activated sludge culture. The feed COD content varied between COD_o=1000-7000 mg/L while θ_H was 10 hours and θc was 12 days. Mo and Fe sources were added to the feed wastewater. TN/ COD ratio was 0.03. because of molasses nitrogen content. pH varied between 7-7.8 in this set of experiments and increased with increasing influent COD concentration.

The results are depicted in Figure 3.16. When the feed COD concentration was below 4000 mg/L the COD removal performance was nearly 90%. At 2000 mg/L feed COD concentration, the COD removal efficiency was 88% and at 3000 mg/L it was 91%. But the performance dropped for the feed COD values above 4000 mg/L The efficiency decreased to 68% with the feed COD of 5000 mg / L. Further decreases were observed at higher feed COD values such as 66% with CODo= 6000 mg/ L and 64% with CODo =7000 mg/ L. As the feed COD increased from 2000 mg/L to 7000 mg/L the effluent COD increased from 250 mg/L to 2600 mg/L. In order to obtain nearly 90% COD removal efficiency, the feed COD should be below 4000 mg/L.



○●Activated sludge; ΔA Activated sludge &Azotobacter vinelandii

Figure 3.15. Variation of effluent COD and COD removal efficiency with hydraulic residence time (Θ_H=10 h, COD_o=2000±200 mg/L)

3.2.5. Effect of COD Loading Rate on COD Removal Efficiency

Figure 3.17 depicts variation of the effluent COD (S_s) and COD removal efficiency (E) with COD loading rate. COD loading rate was determined by the following equation:

$$L_s = Q\ S_o / V$$

Where, L_s is the COD loading rate (mg COD/ L h); Q is the flow rate (calculated for θ_H =12 h.); S_o is the feed COD concentration and V is the volume of aeration tank (7.6 L).

The effluent COD increased and the COD removal efficiency decreased with increasing COD loading rate especially for loading rates above 50 kg COD/m^3.h. COD removal efficiency (E) decreased with the increasing COD loading rates resulting in 62% COD removal at COD loadings of 85 kgCOD/m^3.h. COD loading rates should be below 50 kg COD/m^3.h in order to obtain nearly 90% COD removal efficiency.

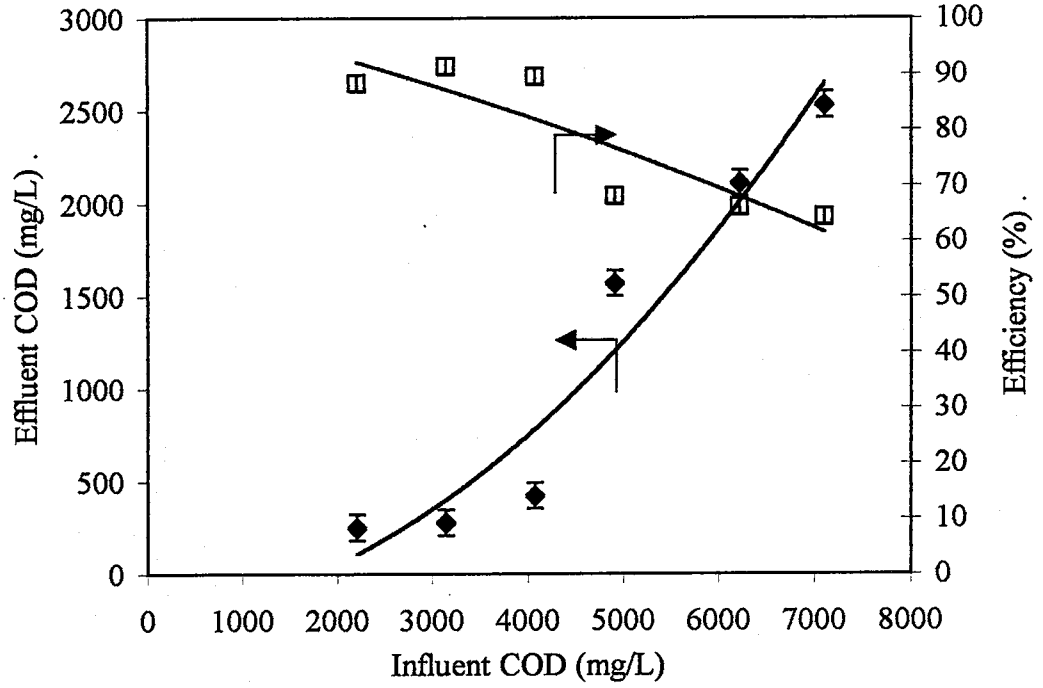


Figure 3.16 Variation of effluent COD and COD removal efficiency with the feed COD
(Θ_H=12 h, Θ_c=10 d)

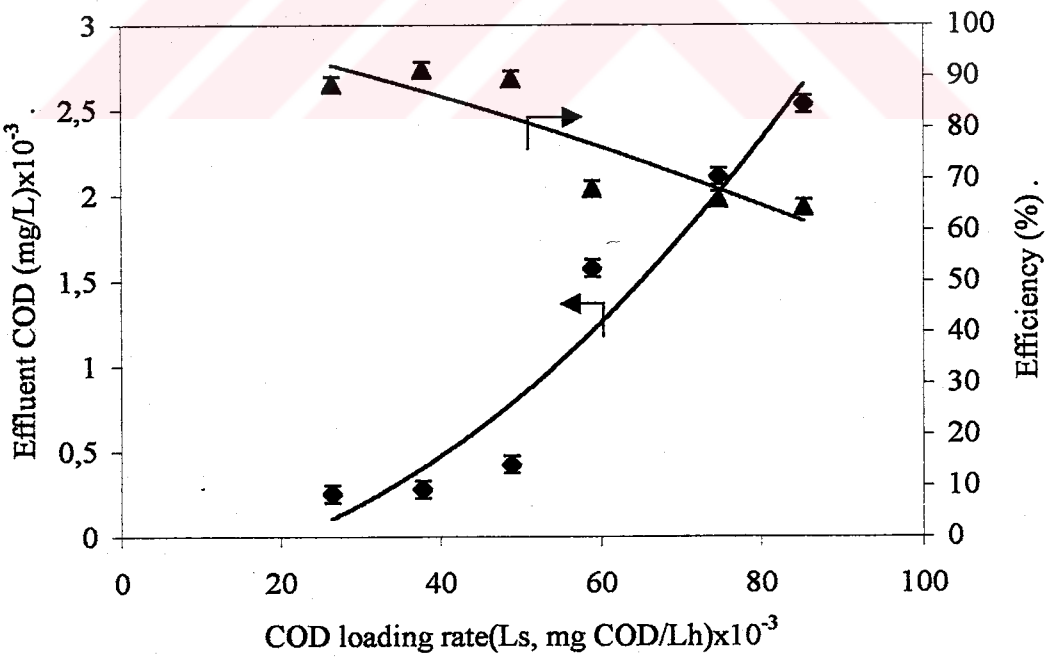


Figure 3.17 Variation of effluent COD and COD removal efficiency with COD loading rate (Θ_H=12 h, Θ_c=10 d)

3.2.6. Kinetic Analysis and Determination of Kinetic Constants

In a continuous culture, growth is limited by the rate limiting substrate. The effect of nutrient limitations are often characterized by the Monod kinetics. For biological COD removal from wastewater in an activated sludge unit, COD balance can be written as follows (Kargı F., 1995, pp.115-131):

$$Q(S_o - S) = \frac{k X S}{K_s + S} V = \frac{R_m S}{K_s + S} V \qquad (1)$$

$$\text{or} \quad R_s = \frac{Q(S_o - S)}{V} = \frac{R_m S}{K_s + S} \qquad (2)$$

In double reciprocal form eqn 2 can be written as follows,

$$\frac{1}{R_s} = \frac{\theta_H}{(S_o - S)} = \frac{Ks}{R_m} \frac{1}{S} + \frac{1}{R_m} \qquad (3)$$

where, Q is the wastewater flow rate (m^3/h); S_o and S are the feed and effluent COD content (kg/m^3) ; k is the maximum COD removal rate constant (kg COD/ kg X.h); X is the biomass concentration in the aeration tank at steady-state (kg/m^3); K_s is the saturation constant (kg/m^3); V is the wastewater volume in the aeration tank (m^3);R_m is the maximum rate of COD removal (= k X , $kgCOD/m^3.h$); R_s is the COD removal rate for a known hydraulic residence time (kg $COD/m^3.h$) ; θ_H is the hydraulic residence time (= V/Q , h).

A plot of $1/R_s$ versus 1/ S should yield a line with a slope of K_s/ R_m and y-axis intercept of $1/R_m$. Experimental data obtained at different hydraulic residence times were plotted in form of $1/R_s$ versus 1/S as shown in Figure 3.18. From the slope and

intercept of the best-fit line the following values were found for R_m and K_s. The details of the procedure are given in the appendix.

$R_m = 400$ mg / L.h, $\quad K_s = 470$ mg/L

Therefore, the rate expression takes the following form,

$$R_s = \frac{400\ S}{470 + S} \qquad \text{where } R_s \text{ is in mg/L h and S is in mg/L.} \qquad (4)$$

Similar data were obtained by using *Azotobacter*-free activated sludge culture at the same hydraulic residence times. The same model was used for the correlation of the experimental data. When experimental data was plotted in form of 1/Rs versus 1/S according to eqn. 3, following constants were found from the slope and intercept of the best-fit line.

$R_m' = 165$ mg/L h, $\quad K_s' = 475$ mg/L

Then the rate equation for Azotobacter-free activated sludge culture can be written as

$$R_s = \frac{165\ S}{475 + S} \qquad \text{where Rs is in mg/L h and S is in mg/L.} \qquad (5)$$

The R_m value for *Azotobacter*-supplemented activated sludge culture ($R_m = 400$ mgCOD/L.h) was significantly larger than that of the *Azotobacter*-free activated sludge (R_m=165 mgCOD/L.h). Also, the saturation constant with the *Azotobacter* was smaller then that of the *Azotobacter*-free culture. These results clearly indicated that, inclusion of *Azotobacter* in activated sludge culture for biological treatment of nitrogen deficient wastewater results in significant increases in the rate and extent of COD removal.

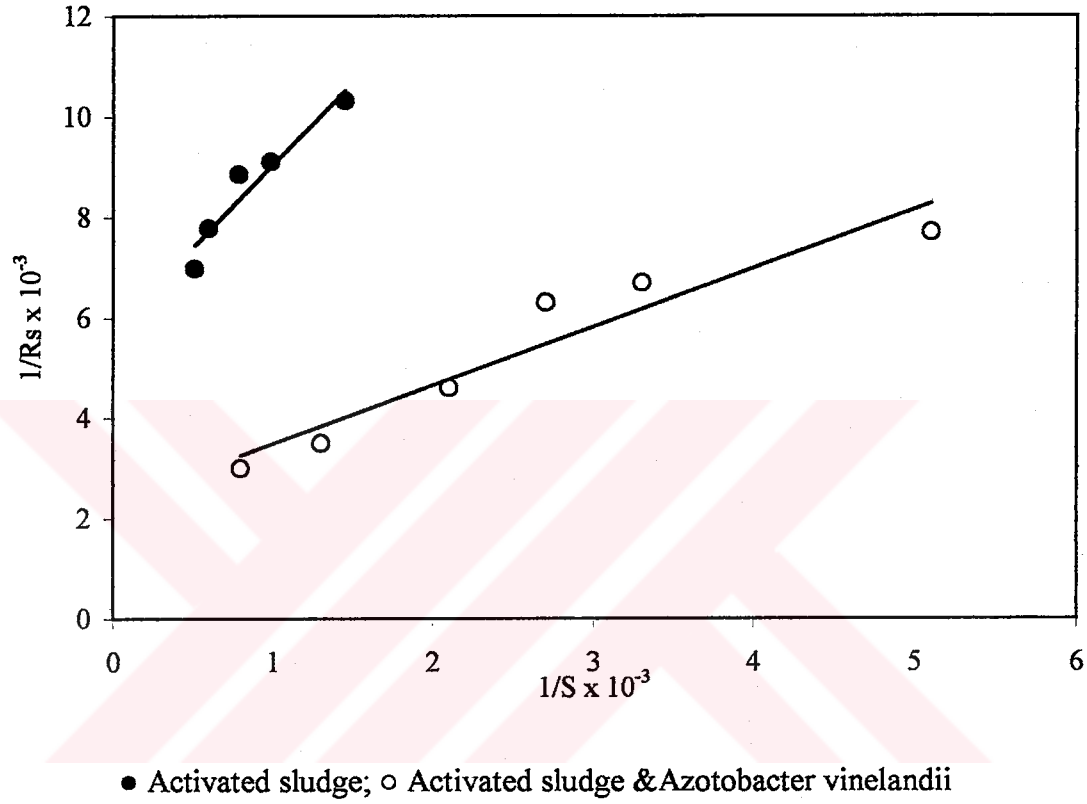


Figure 3.18 Double reciprocal plot of 1/ R_s versus 1/S for determination of kinetic constants

CHAPTER FOUR

CONCLUSIONS

Biological treatment performance of nitrogen deficient wastewaters was enhanced by using *Azotobacter* supplemented activated sludge culture. Experimental studies were carried out in shake flasks and laboratory scale activated sludge unit to investigate the treatment performance of nitrogen fixing bacteria in treatment of nitrogen deficient wastewaters. Up to 95 % TOC removal efficiencies were obtained with semi-synthetic media of TN/COD< 4, when *Azotobacter* was used in form of pure or mixed culture with activated sludge. The TOC removal performance was not as effective as at high TN/COD ratios (TN/COD > 6) as in low TN/COD ratios (TN/COD< 4). Therefore it has been proven that *Azotobacter* supplemented activated sludge cultures can be used for TOC or COD removal from nitrogen deficient wastewaters with high removal efficiencies. Similar results were optained in shake flasks and with *Azotobacter* culture in activated sludge unit. Activated sludge culture was used alone to compare the results. The results showed that activated sludge culture could not be effective in low TN/ COD ratios (TN/ COD< 4).

Also utilization of *Azotobacter vinelandii* in activated sludge improved the rate and the extent of COD removal from nitrogen deficient wastewater significantly as compared to the *Azotobacter*- free activated sludge.

Effects of important process variables on the performance of *Azotobacter* supplemented activated sludge culture treating nitrogen deficient wastewater were investigated. COD removal efficiency increased with increasing sludge age. Nearly

90 % COD removal efficiencies were obtained at hydraulic residence time of 10 h and sludge age of 10 d. Increase in hydraulic residence time improved the COD removal performance. Increasing feed COD content or COD loading rate resulted in decreases in percent COD removal. COD loading rate should be below 50 kg COD/ m^3.h in order to obtain nearly 90% COD removal at θ_H = 12 h and θ_c =10 d.

The optimum operating conditions for an *Azotobacter* supplemented activated sludge reactor were obtained as, sludge age of 10-15 days, hydraulic residence time of 10-14 hours, COD loading rate of maximum 50 g COD /L h.

Monod kinetic model was found to be suitable to correlate the experimental data. Kinetic constants were determined by using the experimental data obtained at different hydraulic residence times for both *Azotobacter*-supplemented and also for *Azotobacter*-free cultures. R_m was found 400 mg / L.h and K_s = 470 mg/L for *Azotobacter* supplemented activated sludge culture and the constants were R_m' = 165 mg/L h, K_s' = 475 mg/L for *Azotobacter* free activated sludge culture. The constants indicated that *Azotobacter* inclusion in the activated sludge culture improved the COD removal rate significantly.

CHAPTER FIVE

RECOMMENDATIONS

Following recommendations may be followed in order to extend investigations on utilization of nitrogen fixing bacteria in biological treatment of nitrogen deficient wastewaters.

1) Other nitrogen fixing bacteria can be used and their performances may be compared with the *Azotobacter* species used in this study.
2) Experiments can be carried out for treatment of different nitrogen deficient industrial wastewaters such as landfill leachate, petrochemical, olive mill, and pulp and paper wastewaters.
3) Different types of reactors can be utilized. Performances of biofilm systems or sequencing batch reactors may be investigated.
4) Different types of nitrogen fixing bacteria may be used in form of mixed culture in an activated sludge unit or in biofilm systems.
5) Nitrogen fixing bacteria and microalgea may be used in combination for treatment of nitrogen deficient wastewaters.
6) Experiments may be carried out within a wider range of COD such as 1000-10,000 mg/L to investigate the system performance.
7) Pure oxygen or oxygen enriched air may be used to meet oxygen requirements of *Azotobacter* for treatment of high strength wastewater .

REFERENCES

Bageshwar U.K., Raina R., Das H.K. (1998)"Characterization of a spontaneous mutant of Azotobacter vinelandii in which vanadium-dependent nitrogen fixation is not inhibited by molybdenum" Fems microbiology letters, V. 162 pp. 161-167

Balis C., Chatzipavlidis J., Flouri F.(1996); "Olive Mill Waste as a substrate for Nitrogen fixation". International Biodeterioration and Biodegredation pp. 169-174

Balis C. (1999); "reply to "An opinion on the kinetics of nitrogen fixation". International Biodeteration & Biodegradation, pp.81-82

Chatzipavlidis J., Antonacou M., Demou D., Flouri F, Balis C.(1996) "Bio-Fertilization of olive mills liquid waste. The Pilot-plant in Messinia,Greece". International Biodeterioration and Biodegredation (1996) pp. 183-186

Christiansen J,. Chan J. M,. Seefeldt L. C,. Dean D. R. (2000) "The role of the MoFe protein a-125 and b-125 residues in *Azotobacter vinelandii* MoFe protein–Fe protein interaction" Journal of Inorganic Biochemistry V:80 pp. 195–204

Dixon R. FRS, S. Austin, M. Drummond, (2001) "Characterisitcs of *Azotobacter vinelandii*" www.nitrogen fixation.com

" Total Organic Carbon Analyzer Manuel" Dohrman Manual

Ehaliotis C., Papadopoulou K., Kotsou M., Mari I., Balis C. (1999). "Adaptation and population dynamics of *Azotobactr vinelandii* during aerobic biological treatment of olive – mill wastewater". FEMS Microbiology Ecology V:30 pp. 301-311

Emerich D.W., Wall J.D. (1983) "Nitrogen Fixation; Specialized Activities and Potential Applications" pp. 75-107

Fiorelli F., Pasetti L., Galli E. (1996); "Fertility-Promoting metabolites produced by *Azotobacter vinelandii* grown on Olive-mill wastewater". International Biodeterioration and Biodegradation pp. 165-167

Gapes D.J., Frost N.M, Colark T.A., Dare P., Hunter R. G., Slade A.H.(1999) "Nitrogen Fixation in treatment of pulp and paper wastewaters". Water Science and Technology

V:40,No:11-12, pp. 85-92

Graham P.H., Vance C.P.(1999); "Nitrogen fixation in perspective: an overview of research and extension needs";Field Crops Research V:65(2000) pp. 93-106

Greenberg A.E., Trusell R.R, & Clesceri L.S., (1989),. "Standard Methods for the Examinations of Water and Wastewater", 17th edn, APHA (American Public Health Association, American Water Works Association and Water Pollution Control Federation)

J. Moreno, C. Vargas-Garcia (1995) "Growth and nitrogenase activity of Azotobacter vinelandii in chemically defined media containing glucose and p-hydroxybenzoic acid" Chemosphere, V. 31, No. 2, pp. 2605-2610

Hill S., He L., Kennedy C. (1999) "Physiological characterisation of anAzotobacter vinelandii nifU-deletion mutant and its spontaneous Nif‡ revertants that over-produce cytochrome" FEMS Microbiology Letters V: 175 pp.185-191

Kargı F. (1995) "Çevre Mühendisliğinde Biyoprosesler" Dokuz Eylül University, Engineering Faculty, Environmental Engineering pp. 115-131

Kargı F., Argun H., Sayıner S. (2001) "Biological treatment of Olive mill wastewater and Leachate". Dokuz Eylül University, Engineering Faculty, Environmental Engineering Department thesis, pp. 1-24

Kennedy C, (2001.07.24) (personal interview)

Krassimer K.S.& Michael C.Z., 1997, "On the reduced and oxidized forms of the FeMo-cofactor of Azotobacter vinelandii nitrogenase" Theoretical Chemistry Accounts V. 96 pp.141-145

Lisova N., Galan M., Patyka, V., Lorkiewicz Z. (1999). "Use of nitrogen fixation microorganisms in sustainable agriculture of West Region .Ukraine". Nitrogen Fixation Symposium Book, p 628

Oelze J., (2000) "Respiratory protection of nitrogenase in Azotobacter spoecies: is a widely held hypothesis unequivocally supported by experimental evidence?". FEMS Microbiology Reviews V: 24 pp. 321-333

Onwurah I.N.E. (1998) "Biochemical Oxygen Demand Exertion and Glucose uptake Kinetics of Azotobacter in Crude Oil Polluted :Medium" Environmental Contamination and Toxicology V:60,pp. 646-471

Papadelli M., Roussis A., Papadopoulou A., Venenieraki A., Chatzipavlidis I., Katinakis P., Ballis C. (1996). "Biochemical and molecular characterization of an *Azotobacter vinelandii* Strain with respect to its ability to growand fix nitrogen in olive mill wastewater".

International Biodeterioration and Biodegredation pp. 179-181

Pasetti. L, Fiorelli F,Tomati U. "Azotobacter biomass production from olive mill wstewater for heavy metal recovery" International Biodeterioration and Biodegredation (1996) pp. 163-164

Peña C., Compos N., Galindo E.(1997). "Changes in alginate molecular mass distribution, broth viscosity and morphology of *Azotobacter vinelandii* cultered in shake flasks". Applied Microbiolgy and Biotechology V:48 pp. 510-515

Peña C., Mauricio A. Trujillo-Rolda´n, Enrique Galindo (2000) "Influence of dissolved oxygen tension and agitation speed on alginate production and its molecular weight in cultures of *Azotobacter vinelandii*" Enzyme and Microbial Technology V.27 pp. 390–398

Rowell P., James W., Smith W., Handley L. L., Scimgeour C.M. (1998); "^{15}Ndiscrimination in molybdenum-and Vanadium-grown N_2-fixing *Anabaena variabilis* and *Azotobacter vinelandii*" Soil Biology and Chemistry V:30, No:14,pp. 2177-2180

Sabra W., Zeng A-P, Deckwer W-D. (1999). "Biological function and variation of alginate production in diazortophically growing Azotobacter vinelandii". Nitrogen Fixation Symposium Book, pp. 349-350

Szilagyi R.K., Musaev D.G., Morokuma K. (2000) "Theoretical studies of biological nitrogen fixation. Part II. Hydrogen bonded networks as possible reactant and product channels" Journal of Molecular Structure (Theochem) V:506 pp. 131–146

Şengül F (1991) "Endüstriyel atıksuların özellikleri ve arıtılması" Dokuz Eylül University Environmental Engineering Department Book pp. 83-315

Williams S.T., Ed. Williams& Wilkins (1989) "Family II Azotobacteraceae". Bergey's Manual of Systematic Bacteriology V. 2 pp. 219-234

www.promise.com created in (1996 June 10) "Biological nitrogen fixation"

http://www.nap.edu/readingroom/books/bnf/contents.html (2000.0728) "Biological Nitrogen Fixation-Research Challenges - A Review of Research Grants Funded by the U.S. Agency for International Development" Board on Science and Technology for International Development National Research Council National Academy Press Washington, D.C. 1994

Yates M.G., Souza E.M., Kahindi J.H. (1996). "Oxygen, Hydrogen and Nitrogen fixation in Azotobacter". Soil Biology and Chemistry V:29, No:5/6,pp. 863-869

APPENDICES

RAW DATA OF EXPERIMENTAL STUDIES

Table A.1 Raw data of shake flask experiments for Activated Sludge carried out with molasses as carbon source

Days	0	1	2	3	4	5
Feed TN/ COD ratio	TOC (mg/L)					
Control	2488.5					1890
0/ 100	2488.5	1478	501.4	451.5	242.3	206.9
1/ 100	2488.5	632.9	-	356.8	-	209.3
2/ 100	2488.5	534.8	-	252.35	-	179.7
4/ 100	2488.5	509.8	-	218.5	-	167.8
6/ 100	2488.5	506.3	-	189.7	-	126.2
8/ 100	2488.5	117.4	-	139.3	-	130.4

Table A.2 Raw data of shake flask experiments for *Azotobacter vinelandii* carried out with molasses as carbon source

Days	0	1	2	3	4	5	6
Feed TN/ COD ratio	TOC (mg/L)						
Control	1125					-	
0/100	990	795	549.8	325	298	235	230
1/100	990	714	485.6	306	254	155	174
2/100	990	946	690	684	-	480	-
4/100	990	984.8	790	603	556	260	-
6/100	990	980.4	1085	712	554	376	-
8/100	990	996.8	1075	759	940.5	-	-

Table A.3 Raw data of shake flask experiments for Activated Sludge carried out with molasses as carbon source

Days	0	1	2	3	4	5
Feed TN/ COD ratio	TOC (mg/L)					
Control	-					-
0/100	530	-	501.4	450.8	240	210
1/100	632.9	-	-	355	-	210
2/100	530	541.2	-	250	-	180
4/100	520	510.8	-	218	-	170
6/100	520	510.2	-	195	-	130
8/100	530	120.8	-	140	-	125

Table A.4 Raw data of shake flask experiments for *Azotobacter vinelandii* carried out with molasses as carbon source

Days	0	1	2	3	4	5	6
Feed TN/ COD ratio	TOC (mg/L)						
Control	992.2					-	
0/100	992.2	797.1	551.2	325	296.5	232.4	232.5
1/100	992.2	716.2	491.4	305.5	249.6	153.3	173.5
2/100	992.2	948	694.6	689.5	-	481.3	-
4/100	992.2	986.4	794.3	602.7	556.4	258.6	-
6/100	992.2	983.3	1089	710.6	550.6	375.6	-
8/100	992.2	998.4	1079	757.8	941.4	-	-

Table A.5 Raw data of shake flask experiments for Activated Sludge carried out with glucose as carbon source

Days	0	1	2	3	4	5
Feed TN/ COD ratio	TOC (mg/L)					
Control	815.85					780.7
0/ 100	2014	981	721.4	745.3	753.4	-
1/ 100	2014	-	436.1	132.3	70.67	-
2/ 100	815.85	574.5	375.7	390.3	393.7	390.6
4/ 100	815.85	772.6	415.4	217.3	219.01	225
6/ 100	815.85	563	414.5	389.3	324.19	320.2
8/ 100	815.85	150.4	66.32	61.85	40.92	55.6

Table A.6 Raw data of shake flask experiments for *Azotobacter vinelandii* carried out with glucose as carbon source

Days	0	1	2	3	4	5
Feed TN/ COD ratio	TOC (mg/L)					
Control	1489					1446.4
0/100	1489	1069	956	1068	1056	942
1/100	1489	857.9	650.4	427.6	173.4	180.2
2/100	1489	681.4	298.7	229.2	110.9	-
4/100	1489	581.4	169.4	135.2	205	-
6/100	1489	625.9	517.5	102.5	72.44	-
8/100	1489	582.6	248.8	145.6	159.3	-

Table A.7 Raw data of shake flask experiments for *Azotobacter vinelandii* carried out with glucose as carbon source

Days	0	1	2	3	4	5
Feed TN/ COD ratio	TOC (mg/L)					
Control	815.85					780.7
0/100	815.85	791.06	707.44	790.32	781.44	668.82
1/100	815.85	634.8	481.3	316.4	128.3	133.3
2/100	815.85	504.3	221.03	169.6	82.06	-
4/100	815.85	430.2	125.4	100.05	151.8	-
6/100	815.85	463.2	382.9	75.85	53.6	-
8/100	815.85	433.3	184.1	107.7	117.88	-

Table A.8 Raw data of shake flask experiments for *Azotobacter vinelandii* and activated sludge carried out with glucose as carbon source

Days	0	1	2	3	4	5
Feed TN/ COD ratio	TOC (mg/L)					
Control	912					875
0/100	912	601.4	615.8	323.8	254.6	147.3
1/100	912	217	101.8	40.22	35	30.5
2/100	912	75.6	79.4	42.38	20.14	15.2
4/100	912	75.96	60.05	37.71	35.4	32.3
6/100	912	-	69.63	43.81	41.6	36.8
8/100	912	82.86	57.12	56.28	51.3	40.4

Table A.9 Raw Data for COD and nitrogen removal studies at different initial TN/COD ratio in activated sludge

Run	COD/TN	θ_H (h.)	θ_c (d.)	COD_i (mg/L)	COD_e (mg/L)	TOC_i (mg/L)	TOC_e (mg/L)	pH (av.)	E (%) (COD)	E (%) (TOC)	N_{added} (mg/L)	Nt_{in} (mg/L)	Nt_{out} (mg/L)	Nt/COD_i	Nt/TOC_i
1	100/ 8	10	10	2400	360	930	176	7.8	85	81	160	272	200	0.113	0.292
2	100/ 6	10	10	1640	400	790	170	7.5	76	78	120	200	140	0.122	0.253
3	100/ 4	10	10	2160	440	907	185	7.3	80	80	80	146	58	0.07	0.16
4	100/ 2	10	10	2020	642	763	231	7.1	68	69	40	100	15	0.05	0.131
5	100/ 0	10	10	1960	850	881	393	7.1	57	55	0	68	8	0.03	0.08

Table A.10 Raw Data for COD and nitrogen removal studies at different initial TN/ COD ratios in Azotobacter supplemented activated sludge culture

Run	COD/ N	θ_H (h.)	θ_c (d.)	COD_i (mg/L)	COD_e (mg/L)	TOC_i (mg/L)	TOC_e (mg/L)	pH (av.)	E (%) (COD)	E (%) (TOC)	N_{added} mg/L	Nt_{in} mg/L	Nt_{out} mg/L	Nt/COD_i	Nt/TOC_i
1	100/ 8	10	10	2360	360	853	136	7.7	85	84	160	264	172	0.112	0.309
2	100/ 6	10	10	1920	300	788	120	7.6	84	85	120	163	81	0.08	0.207
3	100/ 4	10	10	2160	280	891	116	7.6	87	87	80	132	39	0.06	0.148
4	100/ 2	10	10	2080	320	840	130	7.3	84	84	40	94	8	0.045	0.112
5	100/ 0	10	10	2040	320	810	121	6.9	84	85	0	74	17	0.036	0.091

Table A.11 Average raw data for COD, TOC and nitrogen removal studies at different sludge ages in Azotobacter supplemented activated sludge culture

Run	θ_c (d.)	θ_H (h.)	COD_i (mg/L)	COD_e (mg/L)	TOC_i (mg/L)	TOC_e (mg/L)	pH (av.)	E (%) (COD)	E (%) (TOC)	Nt_i (mg/L)	Nt_e (mg/L)
1	3	10	1880	1670	895.7	778.6	7	11	10.7	61	61
2	6	10	2153	1408	890.3	592.3	7	44.7	33.3	81	48
3	10	10	2067	730	956.7	338	7.3	64.3	64.7	65	13
4	15	10	2083	740	859.2	304	7.5	64.7	64.7	62	20.7
5	20	10	1990	740	795.3	298.3	7.5	63	62.7	58	38

Table A.12 Avarage raw data for COD removal studies at different hydraulic residence times in Azotobacter- supplemented activated sludge culture

Run	θ_H (h.)	θ_c (d.)	S_o (mg/L)	S_e (mg/L)	pH (av.)	E (%)
1	13.93	12	1952	198	7.7	90
2	12.2	12	2128.6	298	7.4	86
3	10	12	1987.4	363.73	7.3	81.7
4	8.44	12	1940	465.5	7.2	76
5	6.33	12	1834	471.3	7.1	74.3
6	4.22	12	1962.3	765.3	7.1	61
7	2.04	12	1915.8	1274	5.8-6→7	33.5

Table A.13 Avarage raw data for COD removal studies at different hydraulic residence times in Azotobacter free activated sludge culture

Run	θ_H (h.)	θ_c (d.)	S_o (mg/L)	S_e (mg/L)	pH (av.)	E (%)
1	13.93	12	2031.8	690.8	7.7	66
2	12.2	12	1386.8	698	7.4	62
3	10	12	2153.2	1011.6	7.3	53
4	8.44	12	2325.2	1337	7.3	42.5
5	6.33	12	2170.4	1465	7.2	32.5
6	4.22	12	2217.2	1674.4	7.2	24.5
7	2.04	12	2271.25	1817	5.5-6→7	20

Table A.14 Average raw data for COD removal studies at different feed COD concentrations in Azotobacter supplemented activated sludge culture

Run	θ_H (h.)	θ_c (d.)	S_i (mg/L)	S_e (mg/L)	pH (av.)	E (%)
1	10	12	2203.9	250.6	7.3	88.3
2	10	12	3143.8	276.7	7.4	91.3
3	10	12	4064.95	422.3	7.5	89.5
4	10	12	4908.2	1570.2	7.5	68
5	10	12	6223.1	2109.57	7.65	66
6	10	12	7098.8	2533.6	7.8	64.3

Table A.15 Average raw data for COD removal studies at different COD loading rates in Azotobacter supplemented activated sludge culture

S_o (kg/m^3)	S_e (kg/m^3)	Ls (kgCOD/m^{3}h)	E (%)
2.2039	0.2506	26.45	88.3
3.1438	0.2767	37.73	91.3
4.06495	0.4223	48.78	89.5
4.9082	1.5702	58.9	68
6.2231	2.10957	74.68	66
7.0988	2.5336	85.19	64.3

DETERMINATION OF KINETIC CONSTANTS

Double reciprocal rate equation for COD removal has the following form.

$$\frac{1}{R_s} = \frac{\theta_H}{(S_o - S)} = \frac{K_s}{R_m}\frac{1}{S} + \frac{1}{R_m}$$

When $1/R_s$ versus $1/S$ is plotted, the slope is $1/R_m$ and intercept is K_s/R_m.

Table A.16 Raw Data for Azotobacter supplemented activated sludge culture used in Kinetic Analysis

θ_H (h)	S_o (mg/L)	S (mg/L)	1/S	(So-S)	$\theta_H/(S_o-S)$
13.93	1952	198	0.005	1754	0.007942
12.2	2128.6	298	0.0035	1830.6	0.006664
10	1987.4	363.73	0.00275	1623.67	0.006159
8.44	1940	465.5	0.00215	1474.5	0.005724
6.33	1834	471.3	0.0021	1362.7	0.004645
4.22	1962.3	765.3	0.0013	1197	0.003525
2.04	1915.8	1274	0.000785	641.8	0.003179

The equation for Azotobacter supplemented activated sludge culture is;

$Y = 1.1738X + 0,025 \qquad (R^2 = 0,93)$

$$\frac{K_s}{R_m} = 1.1738$$

$\frac{1}{R_m} = 0,0025$; so R_m is 400 mg/L and K_s is 469.5 mg/L

Table A.17 Raw data for Azotobacter- free activated sludge culture used in kinetic analysis

θ_H (h)	S_o (mg/L)	S (mg/L)	1/S	(So-S)	$\theta_H/(S_o-S)$
13.93	2031.7	690.8	0.00145	1340.9	0.01039
12.2	1386.8	698	0.001433	688.8	0.017712
10	2153.2	1011.6	0.000988	1141.6	0.00876
8.44	2325.2	1337	0.000748	988.2	0.008541
6.33	2170.4	1465	0.000683	705.4	0.008974
4.22	2217.2	1674.4	0.000597	542.8	0.007775
2.04	2271.25	1817	0.00055	454.25	0.004491

The equation for Azotobacter free activated sludge culture is;

$Y= 2.9002\ X+ 0.0061 \qquad (R^2=0.97)$

$$\frac{K_s}{R_m} = 2.9002$$

$$\frac{1}{R_m} = 0.0061;$$ so R_m is 163.9 mg/L and K_s is 475.4 mg/L