Addis Ababa University
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Enhancing nitrogen and organic matter removal from tannery wastewater using efficient proteolytic bacterial isolates and their enzymes in lab-scale anoxic-oxic process

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Table of contents

Acknowledgments ..... *ii*

Table of contents ..... *iii*

List of Tables ..... *v*

List of Figures ..... *vi*

Abbreviations ..... *vii*

Abstract ..... *viii*

1. Introduction *1*

2. Literature review *3*

2.1 Organic compounds in wastewater **3**

2.2 Characteristics of tannery wastewater **4**

2.2.1 Sources of organic nitrogenous waste in tannery process 5

2.3 Environmental impact of nitrogen containing waste **6**

2.4 An overview of biological nitrogen removal systems **8**

2.4.1 Nitrogenous compound transformations 9

2.4.2 Proteinaceous waste 10

2.4.3 Microbial processes in organic nitrogen removal 11

2.5 Enzymes in waste treatment **14**

2.6 Bio-augmentation in biological reactors **15**

3. Material and methods *17*

3.1 Sample collection **17**

3.2 Isolation and purification of proteolytic organisms **17**

3.2.1 Selection of isolates for further characterization 17

3.3 Enzymatic characterization of selected isolates **18**

3.3.1 Preparation of crude enzyme 18

3.3.2 Protease activity as a function of pH 18

3.3.3 Protease activity as a function of temperature 19

3.3.4 Preparation of standard curve 20

3.4 Lab-scale wastewater treatment **21**

3.4.1 Process configuration 21

3.4.2 Wastewater Composition 21

3.4.3 Operational conditions 22

3.4.4 Process monitoring and chemical analysis 23

3.4.5 Effect of protease on removal rate 23

3.4.6 Organic and inorganic characteristics of protease used for treatment 24

3.4.7 Effect of Bio-augmentation of proteolytic organisms 24
3.4.8 Production of extracellular enzyme by pure culture 25
3.4.9 Data analysis 25

4. Results and Discussions 26

4.1 Isolation and purification of potential proteolytic organisms 26

4.2 Properties of enzymes 27
4.2.1 Temperature optimum 27
4.2.2 Effect of pH on activity of protease 30

4.3 Lab-scale wastewater treatment process start-up 32

4.4 The effect of protease on treatment efficiency 34
4.4.1 Ammonium and nitrate production with and with out protease as supplement 34
4.4.2 Total Nitrogen removal efficiency without and with protease supplement 38
4.4.3 COD removal efficiency with and with out protease as supplement 41

4.5 Effect of Bio- Augmentation on treatment efficiency 43
4.5.1 Ammonium production before and after proteoytic bio-augmentation 43
4.5.2 Total Nitrogen removal efficiency for non-augmented and bio- augmentation 48
4.5.3 COD removal efficiency for non-augmented and bio-augmented systems 50
4.5.4 Pure culture enzyme production in effluent and laboratory media 52

5. Conclusions 54

6. References 55

List of Tables

Table 1. Nitrogen compound Source-based effluent characterization of tannery process 6

Table 2. Standard Curve for determination of unit of protease 20

Table 3. Lab-scale treatment plant operational parameters 22

Table 4. Different doses of protease added in different experimental runs.....23

Table 5. Characterization of crude protease from isolate YE-33 32

Table 6. Lab-scale treatment plant statr-up performances. 33

Table 7. Nitrate concentration in Anoxic, Oxic and effluent in enzyme supplemented and non supplemented experiments 38

Table 8. Nitrate concentration in different systems for non-augmented and bio-augmented systems.....47

Table 9. YE-33 cell density and enzyme productivity at 24h and 48h.....53

List of Figures

Figure 1. Microbial nitrogen cycle 10

Figure 2. Tyrosine standard curve 20

Figure 3. Schematic representation of lab-scale treatment plant..... 21

Figure 4. Isolates ETY-19, YE-33, and YE-25 on agar medium. 26

Figure 5. Effect of temperature on activity of protease in the presence and absence of Ca^{2+} for YE-33(A), YE-25(B) and ETY-19(C). 28

Figure 6. Effect of pH on the activity of protease YE-33 (A), ProteaseYE-25 (B) and ETY-19(C)..... 30

Figure 7. Change in ammonium concentration over time in anoxic reactor for enzyme supplemented and non supplemented experiments 35

Figure 8. Nitrate concentration Vs time for enzyme supplemented and non supplemented experiments..... 37

Figure 9. Total nitrogen removal efficiencies Vs time for enzyme supplemented and non supplemented experiments 39

Figure 10. Anoxic COD removal efficiency Vs time for enzyme supplemented an non supplemented experiments 41

Figure 11. Over all COD removal efficiency Vs time for enzyme supplemented and non supplemented experiments 42

Figure 12. (A), (B) and (C) Proteolytic cfu/ml counts in the anoxic tank Vs NH_4^+ concentration..... 45

Figure 13. Nitrate concentrations Vs time for bio-augmented and non augmented experiments..... 48

Figure 14. Total nitrogen removal efficiencies Vs time for none augmented and bio-augmented experiments 49

Figure 15. Anoxic step COD removal efficiency Vs time for bio-augmented and non bio-augmented experiments 50

Figure 16. Over all COD removal efficiency Vs time for bio-augmented and non bio-augmented experiments 51

Abbreviations

APHA	American Public Health Association
BOD	Biological Oxygen demand
COD	Chemical Oxygen Demand
cfu	Colony Forming Unit
DGGE	Denaturing Gradient Gel Electrophoresis
DO	Dissolved Oxygen
FISH	Fluorescence In-Situ Hybridization
F/M	Food to Microorganism
HRT	Hydraulic Retention Time.
mg	Milligram
ml	Millilitre
MLSS	Mixed Liquor Suspended Solid
MLVSS	Mixed Liquor Volatile Suspended Solid
pH	Potential Hydrogenation
PCR	Polymerase Chain Reaction
SVI	Sludge Volume Index
TCA	Tri Chloro Aceticacid
TN	Total Nitrogen
TGGE	Temperature Gradient Gel Electrophoresis
UNIDO	United Nations Industrial Development Organization

Abstract

Tannery wastewater contains high level of protein, which, as a macromolecule takes longer residence time for its microbial degradation. The objectives of this study were to see the effect of adding protease and bio-augmentation of proteolytic organisms on protein hydrolysis and on total nitrogen removal rate using anoxic/oxic lab-scale reactor feeded with synthetic wastewater. Eighty five proteolytic organisms were isolated from tannery wastewater sludge sample of which proteases extracted from three best isolates (ETY-19, YE-25 and YE-33). The temperature and pH characteristics of proteases of the three selected isolates showed optimum activity at around neutral pH range and at mesophilic temperature. Protease from YE-33 was used to see the effect of adding enzyme on protein hydrolysis and the same organism was used to test the effect of bio-augmentation on protein hydrolysis. Ammonium production, total nitrogen and chemical oxygen demand (COD) removal were determined for 120h experimental time. Increasing protease activity didn't considerably affect the overall COD removal where, removal efficiencies were 89-91%, 89-90% and 90-91% for no enzyme, 10 and 30U/ml protease supplements respectively. Anoxic system ammonium concentration increased from 65-70mg/l, 61-109mg/l and 74-153mg/l for the non enzyme supplemented, 10 and 30U/ml protease supplements respectively. The 120h average total nitrogen removal efficiencies were 55.4-56.8%, 54.5-61.3% and 54.7-73% in the non enzyme supplemented, 10 and 30U/ml protease supplemented experiments respectively. In the bio-augmentation experiments in addition to colony forming unit count Ammonium production, total nitrogen and COD removal efficiency were determined for 120h experimental time. There was very little variation in COD removal efficiencies where 90-93%, 89-91% and 90.5-91.5 % COD removal efficiencies observed in non augmented, 10 $x10^7$ and $10x10^9$ cfu/ml cell density augmentations respectively. Ammonium concentrations in the anoxic tank were 77-79mg/l, 78.2-100mg/l and 101-104mg/l in the non augmented, $10x10^7$ and $10x10^9$ cfu/ml augmentations respectively. Little improvement 56-58%, 58-64% and 65.68% in total nitrogen removal efficiencies were observed in the non augmented, $10x10^7$ and $10x10^9$ cfu/ml cell density augmentations respectively. In general exogenous protease and proteolytic bio-augmentation found to improve protein hydrolysis and nitrogen removal rates.

1. Introduction

For many years natural ecosystems used to deal with pollution with self-purification system because of the lower levels of pollutants discharged to the environment. In today's world as a result of increasing human population and industrialization, water pollution is becoming a common problem (Rudolf *et al*., 1998). Tanning industry is one of the major manufacturing processes which is responsible for tremendous pollution of water resources (Dereje, 2006; Lelissa, 2007; Seyoum *et al*., 2003).

The leather industry is a significant consumer of water. Untreated effluent from leather tanning industry is well known to have an adverse impact on health and environment. These characteristics come from its complex manufacturing process and variety of chemicals used in the process (Lofrano *et al*., 2006; Song *et al*., 2003). It can create heavy pollution from effluents that contain high levels of salt, organic load, inorganic matter, dissolved and suspended solids, ammonia, organic nitrogen, and specific pollutants such as (sulphide, chromium, and other toxic metal salt residues (Bosnic *et al*., 2000; Song *et al*., 2003).

The predominant N fractions in tanning industry effluent are ammonia nitrogen and organic N, linked to proteins from hides and skins (Seyoum, 2004). Bacteria cannot directly assimilate macromolecules like proteins and polysaccharides (with molecular size > 1,000 Daltons). That showed these macromolecules must first be hydrolyzed to monomers or small oligomers to enter in to the cell (David *et al*., 1998; Yin and Ryszard, 2006). In this process initial hydrolytic reaction catalyzed by hydrolases can cause longer residence time in biological treatment system (Amare *et al*., 2003b; Gonza *et al*., 2005; Yin and Ryszard, 2006).

In Ethiopia there are about 20 industries involved in leather processing. The annual production capacity is about 1.7 million tones of hides and annual liquid wastewater discharge varies between 2,000,000 -2,500,000 cubic meters. (Dereje, 2006; Seyoum, 2004).

Nitrogen pollution released from tanneries found in Ethiopia is becoming a problem (Seyoum *et al.*, 2004b). Different studies have been conducted on biological treatment of tannery waste water and different removal efficiencies have been reported. In the report of Seyoum *et al.* 2004a upto 98% and in a study by Yoon-jin *et al.*, 2004 74% total nitrogen removal efficiency from tannery wastewater were achieved using anoxic/oxic reactor. Despite high removal efficiencies achieved in biological reactors, the increasing stringent effluent criteria combined with increasing pollutant loading and limited land requires re-evaluation of existing treatment system (Chang and Ouyang, 2002).

Various investigators have shown that hydrolysis is a rate-limiting step in macromolecule degradation (Samantha *et al.*, 2006; Amare *et al.*, 2003b, Yun *et al.*, 2007). The principal form of nitrogen in tannery wastewater is organic nitrogen in the form of protein. Its degradation might take longer residence time in treatment plants. The need for longer residence time affects industries in terms of need for very large space and longer time to accommodate the large volume of the wastewater released from the process. Enhancing protein hydrolysis with different approaches might give faster nitrogen removal rate. Adding enzyme and selected group of organisms to biological waste treatment systems is getting attention to enhance removal efficiencies (Vasileva-Tonkova, and Galabova, 2003; Jean and James, 1997). In order to do away with the problem of long retention time of wastewater treatment system, this study was initiated with the following objectives.

General objective

To improve nitrogen and organic matter removal rate from tannery wastewater through enhancing protein hydrolysis using proteolytic microorganisms and their proteases.

Specific objectives

- To isolate protein degrading bacteria from tannery wastewater treatment sludge.
- To characterize protease from selected isolates.
- To test the effect of different doses of protease on protein hydrolysis and nitrogen removal rate.
- To test the effect of bio-augmentation of proteolytic bacterial isolates on protein hydrolysis and nitrogen removal rate.

2. Literature review

2.1 Organic compounds in wastewater

Organic matter in terms of COD or BOD is one concern in the treatment of any types of wastewater. Its disposal to water bodies might cause oxygen depletion that will have harmful effects to living resources like fishes, or eventually make the environment anaerobic(Fantahun, 2005; Hoshino *et al*., 2005). Typical organic composition of domestic wastewater is 40-60% proteins, 25-50% carbohydrates and 10% lipids (Lacina and Spiros, 2003).

A large percentage of dissolved organic matter (DOM) in wastewater can be composed of macromolecular substrates (David *et al.,* 1998). Hydrolysis of the organic matter in polluted sites is largely dependent on bacterial activity. Extracellular enzyme activity is a key step in degradation and utilization of organic polymers (Vasileva-Tonkova and Galabova, 2003). Organic matter in the wastewater must be enzymatically hydrolyzed through a series of hydrolytic reactions to smaller units that can be taken up by the bacterial cell uptake system (Amare *et al*., 2003b). Microbial hydrolysis is carried out by exoenzymes excreted by hydrolysing microorganisms. Activities of different exoenzymes have been reported in activated sludge. These include activities of proteases, lipases, and chitinases (Daniela *et al.,* 2006; Yun *et al.,* 2007).

Biological degradation of organic compounds during solid waste or wastewater treatment proceeds either in the presence of molecular oxygen by respiration, under anoxic conditions by denitrification, or under anaerobic conditions by methanogenesis or sulfidogenesis (Claudia and Josef, 2005). Aerobic degradation of organic compounds such as carbohydrates, proteins, fats, or lipids in activated sludge systems leads to the formation of carbon dioxide, water, and a significant amount of surplus sludge. Some ammonia and H_2S may be formed during degradation of sulfur-containing amino acids or heterocyclic compounds.

Under strictly anaerobic conditions, soluble carbon compounds of wastes and wastewater are degraded stepwise to CH_4, CO_2, NH_3, and H_2S via a syntrophic interaction of fermentative and acetogenic bacteria with methanogens or sulfate reducers(Claudia and Josef, 2005).

Microbial hydrolysis is a process through which macromolecules are hydrolysed to oligomers and monomers by microbial activity. It is the rate-limiting step for processes related to nitrogen removal (Yun *et al*., 2007). It plays an important role not only in natural biogeochemical cycles, but also in engineered systems such as activated sludge wastewater treatment plants (Yun *et al*., 2007). In order to understand the processes mechanisms and optimise their biodegradation, different of wastewater macromolecular substrates have been studied (Lacina and Spiros, 2003). According to Sotemann *et al*. (2005) the hydrolysis process acts separately on three main groups of complex organics, proteins, carbohydrates and lipids. These complex polymeric materials are hydrolyzed by extracellular enzymes to soluble products (amino acids, sugars and fatty acids) that are small enough to allow their transport across the cell membrane.

The particulate organic fraction and associated hydrolysis rates have a direct impact on the volume of nutrient removing treatment plants (Morgenroth *et al*., 2002). Knowledge of the hydrolases and the organisms producing the different enzymes, and factors affecting enzyme activity are important in optimizing organic matter removal in wastewater treatment plants (Amare *et al*., 2003b).

2.2 Characteristics of tannery wastewater

The characteristics of tannery wastewater vary widely depending on the nature of the tanning process adopted, the amount of water used, the process of hide preservation, the hide processing capacity, the in plant measures followed to reduce wastewater (Lidia and Santosh, 2004; Ugur *et al*., 2006). In general, suspended solids, settleable solids, Biological oxygen demand (BOD), chemical oxygen demand (COD), nitrogen, sulphide, salts, grease and chromium, are pollutants found in tannery effluent (Bosnic *et al*., 2000; Cooman, 2003; Lelissa, 2007).

There are three main stages in the processes of conversion of raw hides and skins into leather. The first is the beam house, the second is the tanning and the third is the finishing operations and water consumption is high in the process. Water consumption consists of two main components: *process water* (drum – float processes, vacuum drying, finishing, cleaning etc.) and *technical water* needed for energy generation, wastewater plant operations, sanitary purpose etc (UNIDO, 2000).

In *the beam house operation* which is the first step, skins and hides are socked, fleshed and de-haired, in the process highly loaded effluents are generated, containing organic matter, suspended solids because of unhairing and skinning. Sulphides used in the unhairing and chlorides from the salt present in the hides are released from this process (Cooman *et al*., 2003; Song *et al*., 2003). The second step is *tanning process*, where leather is treated using chromium salts and shaved. Thus in the tanning process the main pollutant is chromium, while other compounds could be used in the re-tanning process, such as tannins (Goltara *et al*., 2003).The third is the *finishing stage* where re-tanning, dyeing and drying activities are accomplished (Seyoum, 2004). Through these processes and complex stages animal hides are transformed into leather, consuming high quantities of water. Substantial quantities of such chemicals as lime, sodium sulfide, ammonium sulfate, sodium chloride, bactericides, vegetable tannins, and chromium salts which are used at different stages of the process are released in the effluent (Cooman *et al.*, 2003).

2.2.1 Sources of organic nitrogenous waste in tannery process

Nitrogenous compounds are the most abundant pollutants in tannery wastewater. They are mostly found in the form of ammonia and in the form of protein material (Bosnic *et al*., 2000; Buljan *et al*., 2000; Seyoum, 2004). Common organic nitrogenous compound emanate almost at each steps of tannery process (Table 1) (Kabdash *et al*., 2003). The source of the proteinaceous material is the leather (Yoon-jin *et al*., 2004). Raw leather is made up of three main layers (UNIDO, 2000).

Corium: is collagen containing the true leather-building substance is the one to react with the tanning agent and to constitute the product.

Epidermis: is mainly hair, cells and certain protein-like substances that are removed through chemical means.

Subcutis (subcutaneous tissue): collagen and certain other proteins and fats, which are removed by fleshing during beam-house processes.

Table 1. Nitrogen compound Source-based effluent characterization of tannery process (Kabdash *et al.*, 2003)

Source	M^3 tonhide^{-1}	Protein mg 1^{-1}	NH_3-N mg 1^{-1}
Soaking	0.9	3.135	95
Liming	0.6	135,665	70
Washing	0.3	21,710	101
Washing After fleshing	2.925	4,040	395
De-liming and bating	0.325	7,705	4,930
Washing	0.43	1,260	1,790
Pickling	0.36	2,935	1,560
Tanning	0.5	3,340	900

2.3 Environmental impact of nitrogen containing waste

Different nitrogen compounds represent important pollutants in water bodies. Several industries discharge significant amounts of nitrogen e.g. tanneries, slaughterhouses, fertilizer industries etc (Fousterova *et al*., 2004; Kabdash *et al*., 2003). The type of nitrogen compound in wastewater depends on the industrial discharges and nitrogen can exist in many forms like ammonia, nitrite, nitrate and organic nitrogen (Gerardi, 2002).

The uncontrolled releases of tannery effluents in natural water bodies increases the environmental pollution and pose health risks (Ugur *et al*., 2006). Ammonium is one of the

most important nitrogen compounds in surface waters and other ecosystems with concentration (>2mg/l) for three reasons: *(i)* It is the preferred nutrient form of nitrogen for most plant species and for autotrophic bacteria; *(ii)* it is chemically reduced and can therefore be readily oxidized in natural water, resulting in the consumption and decrease of dissolved oxygen; and *(iii)* non-ionized ammonia is toxic to many forms of aquatic life at concentrations as low as ≥ 0.2 mg/l (Claudia, *et al*., 2001; Paredes *et al*., 2007).

Availability of enough oxygen concentration is crucial for survival of higher life forms in aquatic ecosystem (Tasuo *et al*., 2006). According to Bosnic *et al*. (2000) about 40% of the oxygen requirements are spent on removing the nitrogen component of a typical tannery effluent. That can indicate how much untreated effluent can deplete the oxygen content of the receiving water body. All forms of nitrogen are taken up as a nutrient by photosynthetic bacteria and algae. The excessive growth of bacteria and algae due to increased amounts of nitrogen discharged into water, bring eutrophication on which contributes to the reduction of the oxygen level in water (Seyoum *et al*., 2003).

Although nitrate itself is not toxic, its conversion to nitrite is a concern to public health. Nitrite is a potential public health hazard in water consumed by infants. In the body, nitrite can oxidize the iron (II) in hemoglobin and form methemoglobinmia, which binds oxygen less effectively than normal hemoglobin. The resulting decrease in oxygen levels in young children leads to shortness of breath, diarrhea, vomiting, and in extreme cases even death(Carlos, 2004; Carrera *et al*., 2003).

Nitrite is toxic compound that has a lethal dose of around 22mg/Kg body weights, and can enter into the body due to ingestion of food or drink containing nitrate. Carcinogenic nitrosamines may be formed by the interaction of nitrite with compounds containing organic nitrogen (Carlos, 2004). Because of awareness increase about the health and environmental concern nitrogen is now rigidly controlled and wastewaters containing nitrogen are treated before discharge (Derin *et al*., 2000).

2.4 An overview of biological nitrogen removal systems

Many types of biological nitrogen removal systems such as activated sludge, anaerobic digestion, trickling filters and oxidation ditch have been developed so far (Joseph, 2005). In biological treatment process a wide variety of microorganisms participate in the process of organic matter removal (Gareth and Judith 2003; Joseph, 2005; Nicholas, 1996). According to Fantahun, (2005) bacteria are probably the most important group of microorganisms in wastewater treatment. The concentration of bacteria in an activated sludge is 10^7-10^9 cells/ml (Henze *et al*., 1997). Microbial metabolism of organic and inorganic nitrogen to dinitrogen gas is commonly employed by industrial and municipal wastewater treatment facilities to meet discharge limits for nitrogen compounds (Rick and Stuart, 2001; Xiao *et al*., 2007).

Combining anoxic and aerobic reactors with nitrate recycle has been widely used for nitrogen removal in full-scale wastewater treatment plants (Xiao *et al*., 2007). Such systems accomplish nitrogen removal through two steps: (a) Nitrification, the transformation of ammonium to nitrite or nitrate. (b) Denitrification, the transformation of nitrite or nitrate to nitrogen gas (Hoc *et al*., 2002; Claudia and Josef, 2005). Because of their different oxygen requirements, these two steps are separated in time or space. The most common configuration without carbon source is pre-denitrification (Heike *et al*., 2007).

The predenitrification-nitrification process is particularly suitable for the treatment of high concentrations of degradable organic carbon, nitrogen and ammonia (Seyoum *et al*., 2004a). It allows the optimal use of the incoming wastewater as carbon source for denitrification. The process relies on a dense microbial population mixed in suspension with the wastewater under anoxic and aerobic conditions (Seyoum, 2004). In an anoxic reactor the denitrifiers use the organic carbon in the influent as electron donor and the recycled nitrate as the electron acceptor (Xiao *et al*., 2007). With this configuration high total nitrogen removal efficiencies were reported from tannery wastewater (Seyoum *et al*., 2004a) and from abattoir wastewater (Fantahun, 2005).

Single sludge process is also widely used where nitrification and denitrification occur in one system but in different zones, and it can involve any number of tanks (David, 2001; Lidia and Santosh, 2004). Operational conditions such as temperature, pH and organic loading rate are important in biological nitrogen removal systems. Nitrification and denitrification rates decrease with decreasing temperature over a normal range of operating temperature (5-30^{0}C) (Joseph, 2005; Seyoum, 2004).

In most biological treatment systems, pH values 6-8 and 7-8 are found to be optimum for nitrification and denitrification respectively (Paredes ***et al.***, 2007). Dissolved oxygen less than 1-2mg/l induces reduction in nitrification (Joseph, 2005; Matthew2001). The other commonly used operational parameter is organic loading which indicate the F/M ratio in the treatment system. For conventional aeration tanks, the food to microorganism (F/M) ratio is 0.2-0.5 Kg/BOD/day/kg MLSS, but it can be as high as (1.5) for activated sludge using high-purity oxygen (Gerardi, 2002).

2.4.1 Nitrogenous compound transformations

Biological conversion of organic nitrogen is controlled by several biochemical reactions. These biochemical reactions are parts of the nitrogen cycle occurring in nature (Derin ***et al.***, 2000). Nitrogen gas (N_2) is the most stable form of nitrogen where the atmosphere is a major reservoir of it on earth. Reduction of nitrogen gas called nitrogen fixation is only possible for limited number of organisms. Due to this limited fixation ability most of the recycled nitrogen on earth is accomplished with more easily available compounds such as ammonia and nitrate (Sari, 2005).

As it is shown in (Fig.1), organic nitrogen is decomposed to ammonia, which on one hand is assimilated to bacterial cells, leading thus to net growth and on the other hand it is oxidized to nitrite and nitrate (Nicholas, 1996). The overall biochemical process of oxidation of NH_4^+ to NO_2^-, then finally to NO_3^- is known as nitrification.

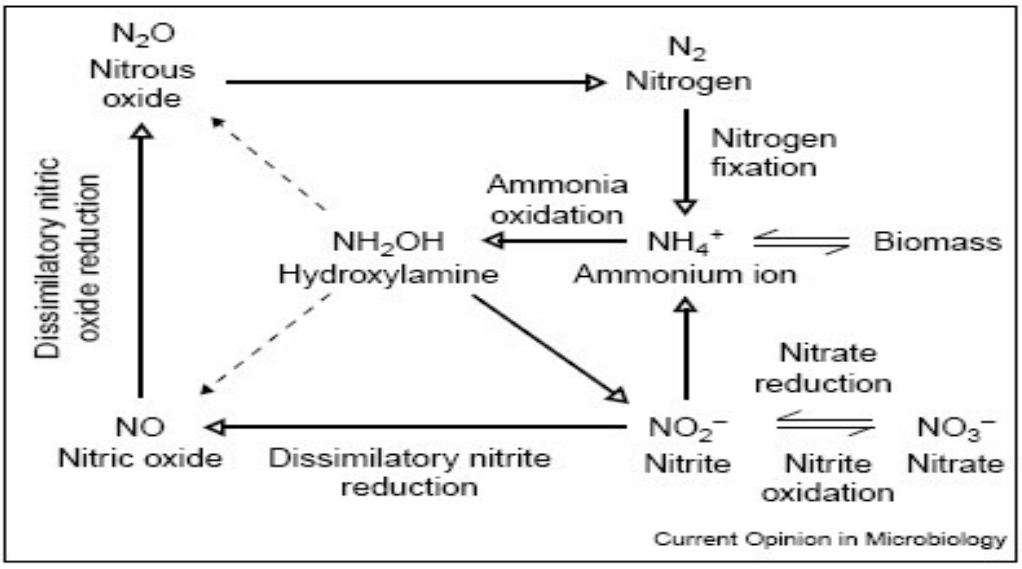


Figure 1. Microbial nitrogen cycle (Rick and Stuart, 2001).

The second step, denitrification is known to proceed as conversion of nitrates to nitrites and subsequent conversion of nitrites to nitric oxide, nitrous oxide and nitrogen gas (Matthew, 2001; Sotirakou *et al.*, 1999). In natural environments denitrification decreases readily available nitrogen compounds thus it is a detrimental process. However, in wastewater treatment systems denitrification is important as the final step in nitrogen removal (Sari, 2005).

2.4.2 Proteinaceous waste

Proteins are one of the most abundant nitrogenous organic macromolocules in wastewater (In-jae *et al.*, 2003). They are colloidal and complex in structure. As colloids they have a large surface area and are suspended in wastewater.

The first catabolic step in protein degradation is enzymatic hydrolysis of the peptide bond formed during protein synthesis resulting in the release of short pieces, or peptides, and after further degradation release amino acids (Gareth and Judith, 2003). Degradation of amino acids leads to the liberation of ammonia by the different mechanisms of

ammonification, including hydrolytic, oxidative, reductive, and desaturative deamination shown respectively in the following equations (Claudia and Josef, 2005; Fantahun, 2005).

$R–NH_2 + H_2O \rightarrow R–OH + NH_3$

$R–CHNH_2COOH + H_2O \rightarrow R–CO–COOH + 2\,(H) + NH_3$

$R–CHNH_2–COOH + 2\,(H) \rightarrow R–CH_2–COOH + NH_3$

$R–CH_2–CHNH_2–COOH \rightarrow R–CH=CH–COOH + NH_3$

Different studies have shown that it is very difficult to degrade protein based particulate substrate, and longer solids residence times are required (Gauri, 2006). Four experimental approaches can be used to evaluate hydrolysis (1) Measurement of specific hydrolytic enzymes; (2) Measurement of specific hydrolytic intermediates or specific end products; (3) Overall mass balances for bulk organic parameters, (4) Measurement of respiration rates to quantify bacterial activity(Morgenroth ***et al.,*** 2002).

2.4.3 Microbial processes in organic nitrogen removal

Organic nitrogen is made up of a variety of compounds including amino acids, amino sugars, urea and uric-acid and purines and pyrimidines (Paredes ***et al.***, 2007). Based on the form of nitrogen compound present in a typical wastewater, ammonification, nitrification and denitrification are the three biological nitrogen removal processes which take place in biological nitrogen removal systems (Seyoum, 2004).

2.4.3.1 Ammonification

Ammonification is a process where nitrogen containing organic compound is converted to ammonia by heterotrophic bacterial decomposition (Nicholas, 1996). These compounds are at first, oxidized to amino acids that contain the carboxylic (-COOH) and the amino group ($-NH_2$). During bacterial degradation of amino acids, the amino group is released by a process known as deamination. The process is either aerobic or anaerobic, but occurs much faster in oxygenated zones.

When the amino group is released in wastewater it is quickly converted to ammonia or ammonium (Gerardi, 2002). Both ammonium and ammonia are reduced forms of nitrogen and it is the ammonium not the ammonia oxidized during nitrification. The rate of mineralization is dependant on optimum temperature and pH (optimum range of 6.5–8.5) (Joseph, 2005).

In most activated sludge processes, temperature and pH ranges are 10-20^{0}C and 7.0 to 8.5, respectively that makes 95% of reduced form of nitrogen present as ammonium (Seyoum, 2004). Paredes *et al*. (2007) reported that at a normal condition (25^{0}C) and a pH of 7.0, non-ionized ammonia accounts to only 0.6 % of the total ammonia present. At a pH of 9.5 and a temperature of 30°C, the percentage of total ammonia present in the non-ionized form increases to 72 %.

2.4.3.2 Nitrification

Nitrification is the biological oxidation of NH_4^+ to NO_3^- through a two-step process by the bacteria of genera *Nitrosomonas* and *Nitrobacter* (David, 2001; Joseph, 2005).). There are autotrophic and heterotrophic nitrifiers. Several studies of heterotrophic nitrifiers have also been reported. Although it is not common some bacteria of the genera *Arthrobacter*, *Flavobacterium*, and *Thiosphaera* are able to catalyze heterotrophic nitrification of nitrogen-containing organic substances (Carrera *et al*., 2003). *Thiosphaera pantotropha and Alcaligenes faecalis* are capable of simultaneous heterotrophic nitrification and aerobic denitrification (Emiko *et al*., 2003). There are two steps in the autotrophic nitrification.

$$\text{Step 1: } NH_4^+ + 1.5O_2 \xrightarrow{\textit{Nitrosomonas}} NO_2^- + 2H^+ + H_2O$$

$$\text{Step 2: } NO_2^- + 0.5O_2 \xrightarrow{\textit{Nitrobacter}} NO_3^-$$

Although nitrification is common in oxic environments, it has also been reported under anoxic condition. The first experimental confirmation of anaerobic ammonia oxidation

(anammox) was obtained during experiments on denitrifying pilot plant (Christopher *et al*., 2007). They observed that the ammonia and nitrate disappeared from the reactor effluent with a simultaneous increase of nitrogen gas production. The microbial nature of the process was verified, and nitrite was shown to be the preferred electron acceptor (Ingo *et al*., 2002).

Because of different factors nitrification is rate-limiting step in many treatment systems (Derin *et al*., 2000). Nitrifying bacteria are sensitive that they have very low growth rate and they are extremely susceptible to a wide range of inhibitors present in wastewaters, such inhibitory pollutants include phenolic compounds, cyanide, thiourea, and heavy metals primarily originating from industrial processes (Seyoum, 2004). Extremely high concentrations of ammonia nitrogen and nitrous acid are reported to be inhibitory to the nitrification process (Gerardi, 2002). Similarly, high organic loading inhibits nitrification by promoting heterotrophic growth and activity which culminate in limited nitrifier growth and activity as a result of strong competition for the available oxygen and ammonia (Heike *et al*., 2007; Joseph, 2005).

2.4.3.3 Denitrification

Denitrification is a process by which certain species of bacteria under anoxic conditions reduce nitrate nitrogen to the gaseous end products of N_2, NO, or N_2O that can then escape from solution to the atmosphere. Unlike other nitrogen compounds, the gaseous forms of nitrogen are relatively unavailable for biological growth (Nicholas, 1996).

The ability to denitrify is considered to be a common property in the bacterial flora of activated sludge (Lena *et al*., 2001). The bacteria responsible for the denitrification are classified as chemoorganotrophic, lithoautotrphic, phototrophic bacteria, and facultative heterotrophs (Seyoum, 2004; Sotirakou *et al*., 1999). In wastewater treatment plant the most common denitrifiers belong to several genera such as *Pseudomonas, Paracoccus, Micococcus, Achromobacter, Bacillus, Brevibacterium, Flavobacterium,* and *Morxella* (Claudia,2001 ; Fantahun, 2005; Matthew,2001; Shiferaw, 2006).

During denitrification electrons are transferred from a donor, normally an organic substrate, to an acceptor, nitrate or nitrite. It is also critical to have a sufficient carbon source to serve as the electron donor for denitrification to occur (David, 2001). Practically any organic compound that can be biologically degraded under anaerobic conditions can be used for denitrification (Carrera *et al.*, 2003).

$$2NO_3^- + 2H^+ + 10(H^+) \longrightarrow N_2 + 6H_2O$$

In order for denitrification to occur, the absence of oxygen is a crucial factor. If oxygen is present, the organisms will preferentially use the oxygen rather than the nitrogen to oxidize the organic matter. Although denitrification takes place preferably under anoxic conditions, there is accumulating evidence however, that some bacteria denitrify aerobically or with low levels of dissolved oxygen (Claudia and Josef, 2005; Joseph, 2005; Paredes *et al.*, 2007).

2.5 Enzymes in waste treatment

Considerable research has been conducted to investigate the new possibilities offered by enzymes in waste treatment. Some of the reasons for these interests are (1) increasing rate of introduction of xenobiotics and recalcitrant organic pollutants into the environment. (2) there is a growing recognition that enzymes can be used to target specific pollutants for treatment and (3) recent biotechnological advances have allowed the production of cheaper and more readily available enzymes through better isolation and purification procedures (Jean and James , 1997).

Various research reports have described the use of microorganisms and/or enzyme pools developed for the biological waste treatment (Daniela *et al.*, 2006). The potential advantages of enzymatic treatment as compared to conventional treatment include: application to bio-refractory compounds; operation at high and low contaminant concentrations; operation over a wide range of pH, temperature and salinity; absence of shock loading effects; absence of delays associated with the acclimatization of biomass;

reduction in sludge volume and the ease and simplicity of controlling the process (Nicell *et al*., 1993).

Applications of different types of enzymes have been studied world wide. Lipases have been used to remove lipid from wastewater (Adriano and Heizir, 2005; Leal *et al.,* 2002). A study by Amare, (1998) showed the potential application xylanases in the hydrolysis of xylan-containing waste. According to Carlos (2004) a rapid and efficient process achieved for the removal of nitrate, using nitrate reductase from Zea mays (Corn) in combination with the nitrite reductase and the nitrous oxide reductase. Instead of using the whole bacterial culture only denitrification enzymes were used in a bioreactor and they observed complete conversion of nitrate to nitrogen gas without residues.

One of the most important extra cellular enzymes associated with the decomposition of bio-waste is protease (Takashi *et al*., 2004). Proteases are enzymes responsible for protein degradation. They have wide applications in pharmaceutical, leather, laundry, food and waste processing industries (Folasade and Joshua, 2005; Krishna *et al*., 2005). Proteases hydrolyze insoluble proteins through a multi-step process whereby the enzyme, which is initially adsorbed on the solid substrate, cleaves of polypeptide chains (Jean and James, 1997). Alkaline proteases from alkalophilic strains are recommended to facilitate processing of proteinaceous waste (Akbalik *et al*., 2004; Amare *et al*., 2003a).

2.6 Bio-augmentation in biological reactors

Several studies have been reporting on biodiversity of bacterial community isolated from activated sludge. Different applications of bacterial isolates from wastewater sludge are employed globally (Jalal *et al.,* 2006). Bio- augmentation is the application of indigenous or wild type or genetically modified organisms to polluted hazardous waste sites orbioreactors in order to accelerate the removal of undesired compounds (Limbergen, 1998; Yuanyuan *et al*., 2005; Lacina and Spiros, 2003).

In the field of wastewater treatment, the effectiveness of adding a selected species to a complex ecosystem is controversial. Some full-scale experiments with bio-augmentation have been reported using specific isolates of microorganisms is becoming successful strategy in degrading the dissolved and suspended organic substances in sludge others are not (Clure *et al*., 1991; Vasileva-Tonkova and Galabova, 2003).

Failure or success in bio-augmentation depends on many factors. These include problems concerning the adaptation of the inoculated microorganisms, the insufficiency of substrate, competition between the introduced species and indigenous biomass, and grazing by protozoa (Bouchez *et al*., 2000; Seyoum *et al*., 2004b). In spite of several successes of small-scale bioaugmentation in activated sludge and the low cost, the addition of specialized strains to activated sludge, (bio-augmentation) to enhance the removal of pollutants from wastewater is not yet widely applied (Limbergen, 1998).

Understanding the structure and function of microbial communities in activated sludge and bio-film reactors can be used to enhance the efficiency and stability of biological wastewater treatment systems (Seyoum *et al*., 2004b). Traditional and modern monitoring techniques have been used to accomplish this. Ttraditional methods like most probable method and selective plating were unreliable for assessing the fate of introduced microbial population. Consequently developing new strategies to recover the microbial population has become a real issue (Hoshino *et al*., 2005; Nathalie *et al*., 2003, Seyoum *et al*., 2004b). Molecular tools like DGGE/TGGE fingerprinting of 16S rDNA fragments has been used to examine the effects of bioaugmentation on indigenous bacterial community structures (Seyoum, 2004). Quantitative PCR assays targeting catabolic genes have successfully been used to monitor the fates of introduced bacteria in complex microbial communities e.g. those in activated-sludge and in soil (Clure *et al*., 1991; Kazuya, 2001).

3. Material and methods

3.1 Sample collection

A pilot treatment plant which is located in the premises of Addis Ababa University and Ethiopia tannery wastewater treatment plant located around Modjo town were the source of sludge for isolation of proteolytic microorganisms and source of seed for the oxic and anoxic compartments of the lab-scale treatment plant used in this study.

3.2 Isolation and purification of proteolytic organisms

Sludge Samples were taken from both anoxic and oxic tanks of the pilot treatment plant and from an aerated tanker in the case of Ethiopia Tannery Wastewater Treatment Plant. Serial dilutions prepared with series 10^{-1}-10^{-7} from the mixed tannery wastewater sludge using 0.85% saline solution.

From each dilution 100µl was taken and spread plated on agar plates with composition; Peptone (0.1%w/v), Bovine casein (0.5%wt/v), K_2HPO_4 (0.1%wt/v), $CaCl_2$ (0.1%wt/v), yeast extract (0.3%wt/v), $MgSO.7H_2O$ (0.02%wt/v) and agar (15 g/l) as described in (Amare *et al*., 2003a). After that incubated in aerobic condition at 28^0C with continuous checking for the growth of colonies. Colonies, which forms clear zones, were picked and re-streaked for a number of times on the agar medium until single and uniform colonies were obtained.

3.2.1 Selection of isolates for further characterization

From eighty-six isolates, three isolates which form relatively wider clear zone and with different colony characteristics were selected for further enzymatic characterization.

3.3 Enzymatic characterization of selected isolates

3.3.1 Preparation of crude enzyme

A loop full of the isolates from a slant culture were inoculated in 100 ml liquid media with composition of Peptone (0.1%w/v), Bovine casein (0.5%wt/v), K_2HPO_4 (0.1%wt/v), $CaCl_2$ (0.1%wt/v), yeast extract (0.3%wt/v) and MgSO.7H2O (0.02%wt/v) as described in (Amare *et al*., 2003a) and kept on a rotary shaker (120 revs/min) at ambient temperature for 24 h.

Two millilitres from the 24 h culture (starter culture) were transferred to fresh media with the same media composition and kept in similar condition as the starter culture. After 48 h, the whole content of the culture was centrifuged at 10,000 x *g* for 5 min to get cell free supernatant. The cell free supernatant was used as crude enzyme.

3.3.2 Protease activity as a function of pH

Different buffers were prepared to test effect of pH on the protease enzyme. The pH values were adjusted with citrate phosphate (pH 5-7) phosphate (pH 6-8), Tris-HCl (pH 7.5-9.0), glycine-NaOH (pH 9.0-11.0).1% casein substrate was prepared in the buffer solutions.

The reaction mixture in the assay (450 µl 1% casein substrate and 50 µl enzyme) was incubated at 40^0c for 30 min. After 30 min 10% Trichloroaciticacid added to stop the reaction and the tubes were left at room temperature to facilitate the unreacted protein precipitation. The precipitated protein was removed by centrifugation at 5000 × *g*, for 5 min. From the clear supernatant 500 µl was transferred to clean test tube, and 2.5 ml of 0.5M Na_2CO_3 and 1:10 diluted 2N folin Ciocalteau's phenol reagent added. Absorbance reading was measured at 660nm with spectrophotometer (JENWAY 6405 Uv/Vis).

3.3.3 Protease activity as a function of temperature

Temperature profile with out $CaCl_2$

The range of temperature for best activity of the enzyme was determined by incubating the reaction mixture at different temperatures ranging from 30°C to 80°C with 5°C interval. The optimum temperature was determined by exposing the reaction mixture containing 450 µl of 1 % casein prepared by using 100 mM Tris HCl buffer, pH 8.00 and 50 µl of enzyme (Amare et ***al.***, 2003a).

The reaction mixture was incubated at different temperatures for 30 min. After 30 min 10% Trichloroaceticacid was added to stop the reaction and the tubes were left at room temperature to facilitate the unreacted protein precipitation. The precipitated protein was removed by centrifugation at 5000 × ***g***, for 5 min. From the clear supernatant 500µl was transferred to clean test tube, and 2.5 ml of 0.5M Na_2CO_3 and 1:10 diluted 2N folin Ciocalteau's phenol reagent was added. Absorbance reading was measured with spectrophotometer at 660nm.

Temperature profile in the presence of 5mM $CaCl_2$

Temperature profile in the presence of $CaCl_2$ was done using 1% casein substrate containing 5mM $CaCl_2$. Except the addition of $CaCl_2$ in the substrate the assay temperature, all the reagents and procedure followed were the same as the temperature profile test in the absence of $CaCl_2$.

3.3.4 Preparation of standard curve

The standard curve for protease was prepared using the standard proportions of tyrosine (Amare *et al.*, 2003a). The final working concentration of tyrosine was 200µg/ml (Table 2).

Table 2. Standard Curve for determination of unit of protease

200µg/ml Tyrosine	Buffer	Concentration
-	500	0
25	475	20
50	450	40
100	400	80
125	375	100
150	350	120

In the above reaction mixture 2.5ml of 0.5M Na_2CO_3 and 1:10 diluted 2N folin Ciocalteau's phenol reagent added and vortexed. Absorbance reading measured at 660nm with spectrometer shown in (Fig.2).

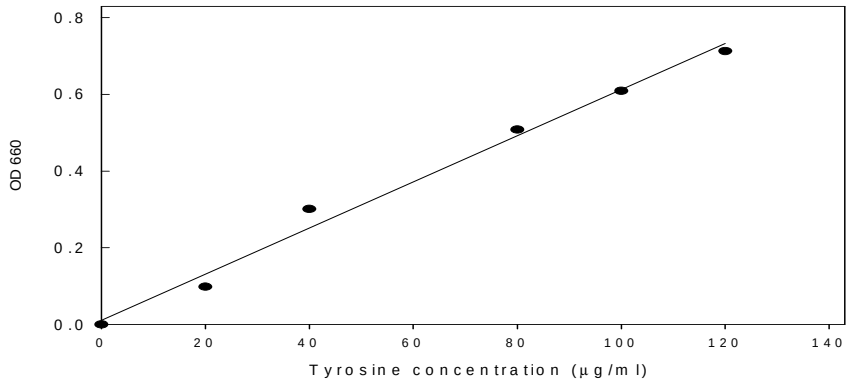


Figure 2. Tyrosine standard curve

The absorbance reading from the above concentration gave a regression equation.

Y= (164.515xOD)-1.117

Where Y= Unit of enzyme

OD = Absorbance reading times dilution factor if diluted.

One U (unit) is defined as that amount of the enzyme that catalyzes the conversion of 1 micromole of substrate per minute in a specific assay condition such as temperature and pH.

3.4 Lab-scale wastewater treatment

3.4.1 Process configuration

A lab-scale pre-denitrification/nitrification reactor shown in Fig. 3 designed by Seyoum, 2004 was used in this study. The system contains feed tank, anoxic reactor with a working volume of 1 L and oxic tank with working volume of 2 L. Air is supplied to oxic system using aerator pump. The feed tank, the anoxic and the oxic tank were stirred continuously using magnetic stirrer. Hundred percent sludge and mixed liquor recycle were done manually with regular time intervals.

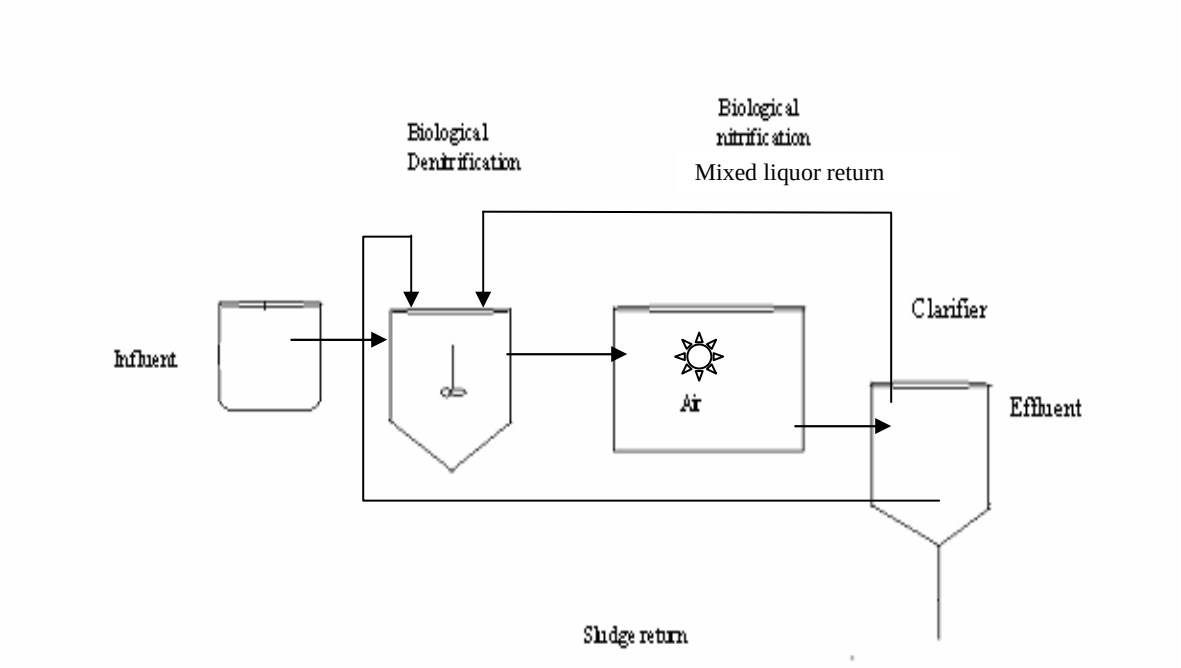


Figure 3. Schematic representation of lab-scale treatment plant

3.4.2 Wastewater Composition

Synthetic wastewater used throughout the study was composed of (gm/L) Sodium acetate,0.7; Casein,1.0; $Na_2S_2O_3$,0.5; $FeCl_2$,0.05; K_2HPO_4,0.2; KH_2PO_4,0.1; $CaCl_2$,0.05 and $MgSO_4,7H_2O$,0.02 as described by Shiferaw (2006).

3.4.3 Operational conditions

The set-up was operated at room temperature. The anoxic and oxic tank was initially seeded with 500 ml acclimatized sludge from pilot scale treatment plant which is found in the premises of Science Faculty Addis Ababa University. Table 3 shows the operational parameters in this study. MLSS and MLVSS were analyzed according to APHA (2001). The dissolved oxygen and pH was measured instrumentally. The influent from the feed tank pumped in to the anoxic tank with fixed flow rate (0.868ml/min) using peristaltic pump.

Table 3. Lab-scale treatment plant operational parameters

Operational Parameters	Anoxic tank	Oxic tank
DO	0.06 - 0.09mg/l	2.01 - 2.3mg/l
pH	6.7 - 7.5	7.4 - 7.9
MLSS g l^{-1}	2.2	2.7
MLVSS g l^{-1}	1.7	2.0
HRT	19.2h	38.40h
Flow rate	0.868ml/min	0.868ml/min

OLR is calculated after knowing the COD and flow rate of treatment system (Ronnachai, 2007).

$$\text{Organic loading rate (OLR)} = \frac{Q\,m^3/day \times COD\,kg/m^3}{Kg/m^3/day \qquad\qquad Vm^3}$$

Where, Q = flow rate

COD = Chemical Oxygen demand of the influent.

V = Volume of aerated tank

The organic loading rate used through out all the experiments was calculated to be, 1.16 $kg/m^3/day$.

3.4.4 Process monitoring and chemical analysis

Total N, NH^{+}_{4} –N, NO_3^{-} –N, and COD were determined colorimetrically, all according to HACH instructions using spectrophotometer (DR/2010 HACH Loveland, 2002). And removal efficiency for total nitrogen and COD were calculated as,

$$\% \text{ removal efficiency} = \frac{C_{inf} - C_{fin}}{C_{fin}}$$

Where, C_{inf} = Concentration initial (in the influent)

C_{fin} = Concentration final (in the effluent)

3.4.5 Effect of protease on removal rate

The synthetic wastewater was treated with different doses of crude protease extracted from the tannery sludge isolate YE-33. Enzyme concentrations tested in the anoxic tank were 10U/ml, and 30U/ml in two different experiments. The initial volume of enzyme added in the anoxic tank (Table 4) was based on the calculation for the whole content of the anoxic tank (for 1000 ml) then the successive addition of enzyme were done manually by calculating the enzyme lost per minute in relation to flow rate.

Table 4. Different doses of protease added in different experimental runs

Stock enzyme used for the experiment	Initial enzyme Added in ml	Enzyme compensation for 12h lose (ml)	Enzyme Compensation for 6h lose (ml)	Final Enzyme dose in the anoxic tank
420U/ml	24.5	15.38	7.67	10U/ml
438U/ml	68.43	42.76	21.38	30U/ml

All enzyme addition was done with regular time intervals. Samples were collected at intervals of 24 h, for 120 h and centrifuged at 3,000 rpm for 20 min (Daniela *et al*., 2006) and the supernatant was used to analyze the COD, total nitrogen, ammonium, and nitrate.

3.4.6 Organic and inorganic characteristics of protease used for treatment

The protease used for treatment was checked for the content of ammonia, ammonium, and total nitrogen and COD characteristics. The contributions of the two different doses used in the treatment (10u/ml, and 30u/ml) were analysed separately.

3.4.7 Effect of Bio-augmentation of proteolytic organisms

Abundance and removal efficiency relationship of proteolytic bacteria before augmentation.

Sludge sample was taken from the anoxic tank and serially diluted from 10^{-1}-10^{-10} with 0.85% saline solution. From each dilution spread plate was done on agar plates containing Peptone (0.1%w/v), Bovine casein (0.5%wt/v), K_2HPO_4 (0.1%wt/v), $CaCl_2$ (0.1%wt/v), yeast extract (0.3%wt/v), $MgSO.7H_2O$ (0.02%wt/v) and Agar (1% wt/v). Incubated at 28^oc and after 48 hour Plates were checked for growth. An indication of the original abundance of proteolytic bacteria was obtained by colony count. In parallel with the colony count ammonium production in the anoxic tank, nitrate production in the oxic tank and removal efficiency of the system for non augmented experiment were measured for total nitrogen and COD.

Abundance and removal efficiency of Proteolytic bacteria after augmentation.

Loop full of pure cultures of YE-33 which was isolated and characterized previously, were inoculated to 100ml of the synthetic wastewater which was composed of (in gm/L) Sodium acetate, 0.7; Casein, 1.0; $Na_2S_2O_3$, 0.5; $FeCl_2$, 0.05; K_2HPO_4, 0.2; KH_2PO_4, 0.1; $CaCl_2$, 0.05 and $MgSO_4$, $7H_2O$, 0.02. It was used as starter culture and 2 ml of this starter culture transferred to fresh synthetic wastewater which was used as inoculant. After 24 h serial dilution was prepared from the inoculant culture using 0.85% saline solution. The dilutions prepared were from10^{-1}-10^{-10} and spread plated on agar plates and incubated at 28^0C for 48h. The number of colonies counted to calculate colony-forming unit per milliliter (CFU/ml) of the inoculant. After standardizing the sample it was used as inoculum for bio-

augmentation of the wastewater treatment with the final cell densities in the anoxic tank of $1.5x10^7$ cfu/ml.

After inoculation Sludge samples were taken from the anoxic tank at 0h, 24h, 48h, 72h, 96h, and 120h serially diluted from 10^{-1}-10^{-10} with 0.85% saline solution. Then from each dilution streak plate were done on agar plates with the same composition with the non augmented system and incubated at 28^0C. After 48 h they were checked for growth. And indication of abundance or disappearance of the augmented proteolytic organisms was checked by colony count. In parallel with the colony count anoxic ammonium production, oxic nitrate production removal efficiency of the augmented system was measured for Total nitrogen, and COD.

Fresh inoculum culture was prepared to run the $1.5x10^9$cfu/ml cell density augmentation experiment. Other procedure is the same with the $1.5x10^7$cfu/ml experiment.

3.4.8 Production of extracellular enzyme by pure culture

YE-33 were grown on normal growth medium, the effluent + 0.5% casein and effluent only to see the enzyme productivity. Loop full of YE-33 was inoculated in media containing all the supplements and in effluent from the treatment plant supplemented with 0.5% casein. Cultures were kept on a rotary shaker under aerobic conditions at ambient temperature at 120rvs/min. Samples taken were at 24 and 48 h for plate count and enzyme productivity test. The plate count was done by preparing serial dilution of the sample with saline water. Spread plate was done from each dilution and incubated at 28^0Cfor 48 h to check for the number of colony forming unit. Portion of the same sample used for serial dilution was used to test for protease activity using tris-HCl pH 8.00 and assay was done at 40^0C.

3.4.9 Data analysis

Data analysis was done using Microsoft Excel program, Sigma plot and Microcal Origin 6.0 software.

4. Results and Discussions

4.1 Isolation and purification of potential proteolytic organisms

In this study 85 proteolytic isolates were isolated from tannery pilot treatment plant and from Ethiopia tannery waste treatment plant sludge sample. Clear zone formation on agar plates containing casein indicates for proteolytic activity. Based on the size of the clear zone and colony appearance three isolates designated as YE-33, YE-25, and ETY-19 were selected for further study.

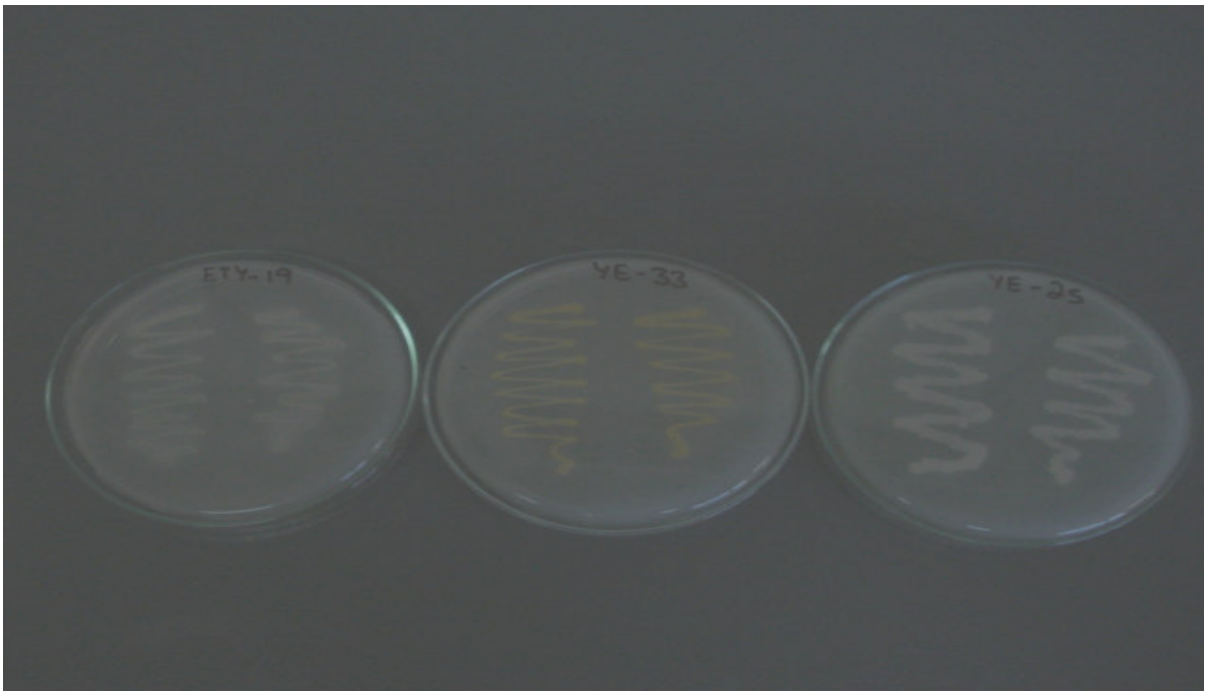


Figure 4. Isolates ETY-19, YE-33, and YE-25 on agar medium.

Isolate YE-33 was found to have raised, circular, mucoid and yellow colony while isolate ETY-19 had semi-translucent white, smooth and circular colony, whereas the third isolate YE-25 had rough, with irregular edge white colored colony characteristics. Of the three isolates YE-33 and YE-25 were isolated from the pilot treatment plant while ETY-19 was isolated from the Ethiopia tannery wastewater treatment plant.

Since all the isolates are part of the normal flora of a tannery wastewater treatment plant, the organisms and their extracellular enzymes are expected to be resistant to various toxic substances. Selecting for indigenous bacteria from contaminated environments is advantageous in minimizing the inhibitory effects from compounds that may be present along with a specific wastewater, since viable indigenous organisms develope some degree of resistance to these compounds (Shifaraw, 2006; Thiruneelakantan *et al*. 2001; Olaniran *et al*., 2005).

4.2 Properties of enzymes

4.2.1 Temperature optimum

Plate culture photographs of the three isolates selected for their protease characterization are shown in Fig 4. Three isolates ETY-19, YE-33 and YE-25 were studied for pH and temperature characteristics of their protease. The effect of temperature on the three proteases is shown in Fig. 5 A-C. Proteases from all the three isolates were active in a broad temperature range.

The optimum activity for proteaseYE-33 was observed at 35^0C - 40^0C in the absence of calcium and 35^0C - 50^0C in the presence of calcium respectively. While protease YE-25 showed optimum activity around 30^0C- 45^0C in the absence of Ca and from 30^0C - 50^0C in the presence of calcium respectively. And for protease ETY-19 the optimum activity was in the temperature range of 35^0C-70^0C and 35^0C-60^0C in the presence and absence of calcium, respectively.

For proteases YE-33 and YE-25 addition of calcium shifted the optimum temperature by at least 10°C (Fig. 5 A and B). This could show that Ca protect the enzyme against thermal denaturation and play a very important role in maintaining active conformation of the enzyme at high temperatures. Proteases from YE-33 and YE-25 showed similar properties while protease ETY-19 was different in that the optimum activity stayed up to 70^0C (Fig.5 C). YE-33 and YE-25 were isolated from the pilot treatment plant found where the

wastewater temperature ranges 18±3 during the day time. Seyoum, 2004 reported that the best temperature range for nitrification and denitrification process is 5-30^{0}C.

In wastewater treatment systems, together with hydrolytic organisms the nitrifiers and denitrifiers are group of organisms which are involved in organic nitrogen compound transformation to gaseous nitrogen. The temperature characteristics showed that the three enzymes could be applied in anoxic/oxic tannery wastewater treatment systems with out optimizing the temperature range specifically for the three proteases. ETY-19 was isolated from Ethiopia tannery wastewater treatment plant found around Modjo town.

Figure 5. Effect of temperature on activity of protease in the presence and absence of Ca^{2+} for YE-33(A), YE-25(B) and ETY-19(C).

Fig.5 A) Temperature profile of protease YE-33 in the presence (■) and absence (●) of Ca^{2+}

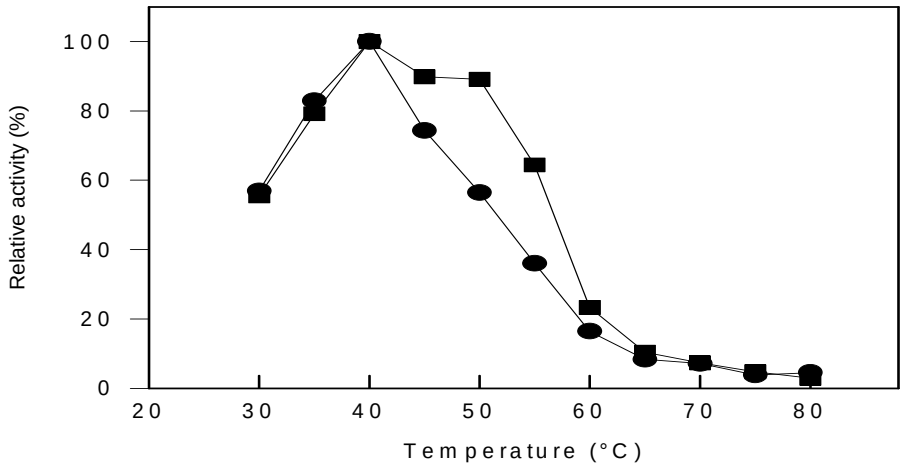


Fig.5 B) Temperature profile of protease YE-25 in the presence and absence of calcium.

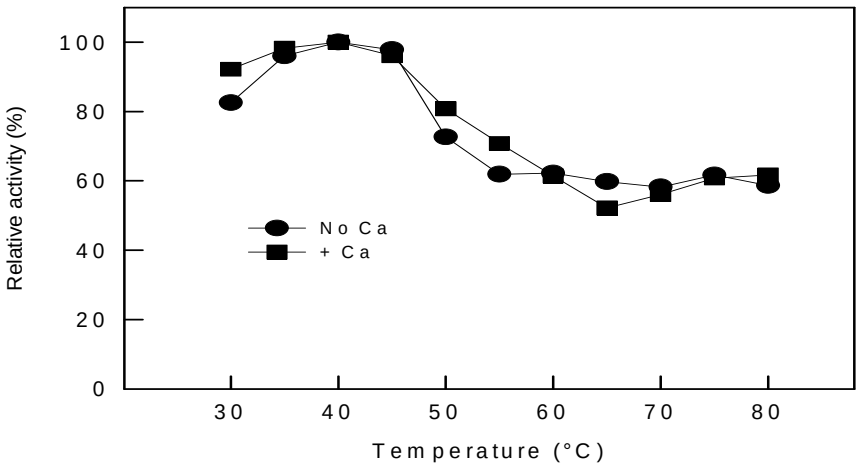
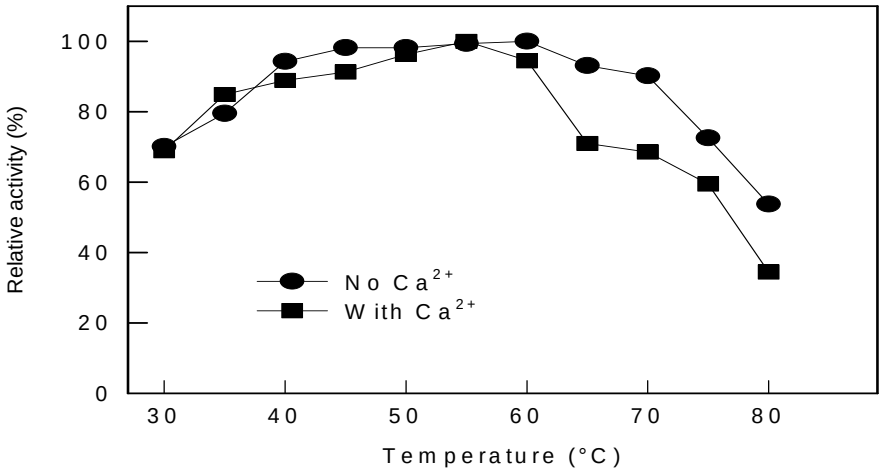


Fig.5 C) Temperature profile of protease ETY-19 in the presence and absence of calcium.



4.2.2 Effect of pH on activity of protease

The pH characteristics of the three proteases (YE-33, YE-25, and ETY-19) are presented in Fig.6. All the three enzymes were active in the pH range of 6.0 - 10.5. With optimum activity 7.5 -9.0 for protease YE-33, and 6.5-9.5 for both YE-25 and ETY-19. The pilot treatment plan where organisms YE-33 and YE-25 isolated from had a pH of 6.7-7.5 in the anoxic tank and 7.4- 7.9 in the oxic tank. And the pH of the sludge sample from Ethiopia tannery wastewater treatment plant had pH 9.5. The fact that these proteases are active in the same pH range to the anoxic/oxic plant shows their feasibility to use them in anoxic/oxic reactor. According to different authors pH around neutrality is favorable for nitrifiers and denitrifiers which are needed for complete nitrogen removal from wastewater (Carlos, 2004; Lidia and Santosh, 2004).

Figure 6. Effect of pH on the activity of protease YE-33 (A), ProteaseYE-25 (B) and ETY-19(C)

Fig.6 A) pH profile of YE-33 Citrate phosphate (●) Phosphate (■) Tris-HCL (▲) Glycine-NaOH (▼)

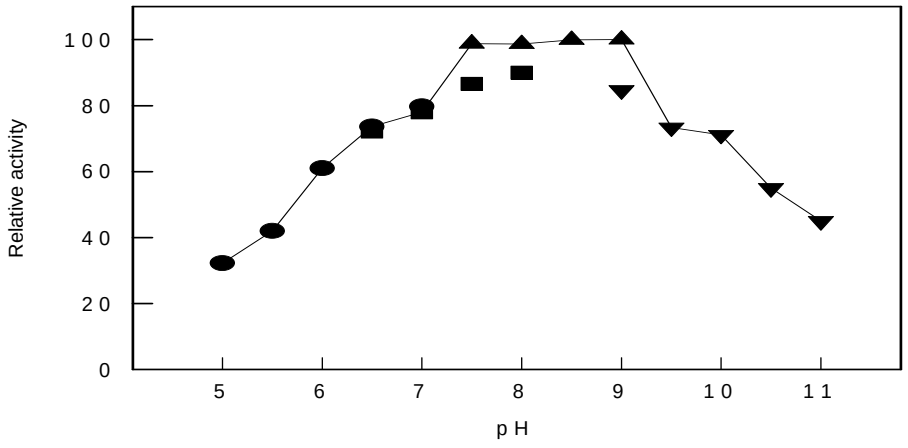


Fig.6 B) pH profile of YE-25 Citrate phosphate (●) Phosphate (■) Tris-HCL (▲) Glycine-NaOH (▼)

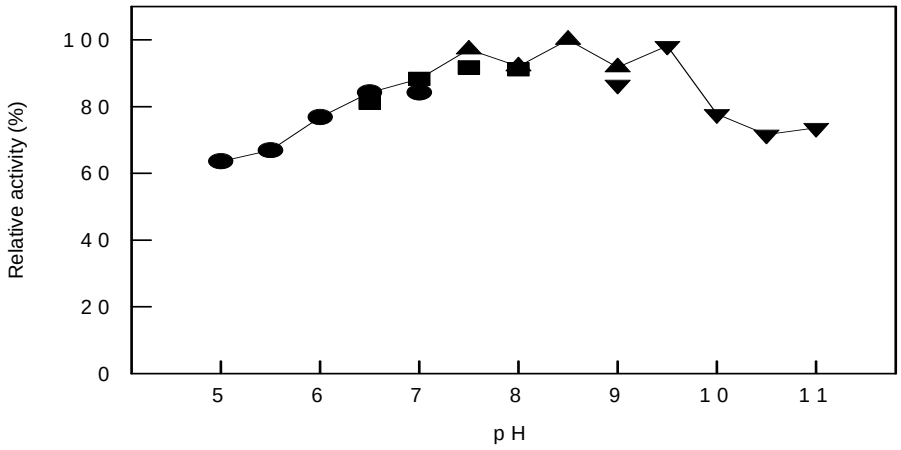
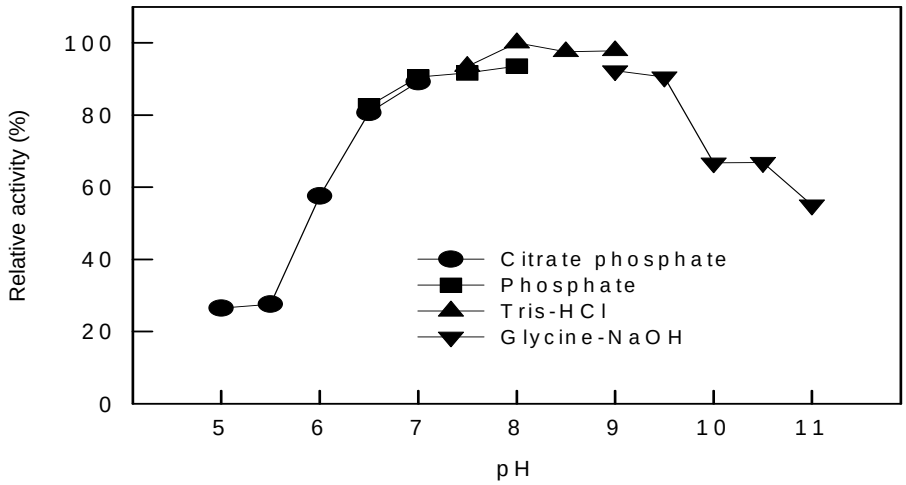


Fig. 6 C) pH profile of ETY-19



Biological reactors are not working under controlled process parameters like other manufacturing processes. The pH might vary because of different factors such as, variable influent characteristics and metabolic activities of different types of organisms which are found in the waste treatment plants. Therefore, the wide pH range tolerance exhibited by the three enzymes showed potential importance for their application for different purposes. Similar observation was also made by Shifaraw, (2006), that the temperature and pH characteristics of the protease extracted from the tannery sludge isolates were compatible for application in the tannery waste water treatment. The maximum protease activities for the three enzymes were 81.140U/ml, 101.49U/ml and 36.69U/ml for YE-33, ETY-19 andYE-25 respectively. From the three proteases YE-33 were selected for evaluating treatment efficiency based on optimum temperature for protease activity. Although ETY-19 had highest protease activity the optimum temperature range for protease activity was well above the working temperature of the anoxic/oxic reactor.

4.3 Lab-scale wastewater treatment process start-up

The effect of protein hydrolysis on nitrogen removal rate was studied using crude protease extracted from proteolytic isolate designated as (YE-33) which was isolated and characterized in the present study. The effect of the added enzyme and the augmented organisms were analyzed. The synthetic wastewater used to carry out all the experiments had a COD value of 1780 mg/l; ammonium 0.4 mg/l; Nitrate 40mg/l, total nitrogen 220 mg/l; BOD, 950 mg/l; and pH 6.4-6.7. The crude protease used to treat the proteinaceous component was characterized for different organic and inorganic parameters (Table 5).

Table 5. Characterization of crude protease from isolate YE-33

Dose of protease in anoxic tank.	NH_4^+mg/l	No_3^-mg/l	TN mg/l	COD mg/l
10u/ml	4.2	0.4	15.6	219
30u/ml	10.5	0.9	39.8	338

At the start up period variations on quality of wastewater in terms of COD and TN were measured to check the steady state removal efficiency of the seeded organisms. Monitoring of those specific operational parameters at the start-up and steady state condition were used to verify the effect of adding exogenous protease and proteolytic organisms. During the startup, the COD removal efficiency ranged from 35-90 % and remained constant around 90% for more than three weeks; the TN ranged from 10-55% and remained constant around 55% for more than three weeks (Table 6).

MLVSS were used to monitor the biomass quantity in the treatment system and it was monitored in parallel with the COD, TN removal efficiency. It was observed from the two reactors that the biomass production was highest at the aerobic condition. According to Nicholas, (1996) pure oxygen systems have been shown to operate effectively at MLVSS concentrations of 4g to 8g/l while conventional air activated sludge systems typically operate at 1.5g to 2.5g/l MLVSS (Table 6).

Table 6. Lab-scale treatment plant statr-up performances.

Parameter	First two week		Second two week		Third two week		Forth two weeks	
	Anoxic	Oxic	Anoxic	Oxic	Anoxic	Oxic	Anoxic	Oxic
p^H	6.8	7.5	6.7	7.4	7.2	7.6	7.5	7.9
MLSS g/l	1.27	1.4	2.2	2.4	2.2	2.7	2.2	2.7
MLVSS g/l	1.0	1.4	1.47	1.8	1.59	1.9	1.7	2.0
Parameter	First two weeks effluent characteristics		Second two weeks effluent characteristics		Third two weeks effluent characteristics		Forth two weeks effluent characteristics	
	% Removal Efficiency		% Removal Efficiency		% Removal Efficiency		% Removal Efficiency	
COD mg/l	35.2		70.0		85.0		90.0	
TN mg/l	15.0		35.3		55.20		55.0	

In this study substrate used to check hydrolysis was casein. Both casein and Bovine serum albumin were used widely in studies of protein hydrolysis in relation to wastewater systems and are assumed to represent the proteins present in wastewater fairly well (Yun , *et al*., 2007). The pH in both the reactors was > 6 which favor hydrolytic organisms. Pin-Jing *et al*., (2006) stated that optimal pH in the hydrolysis of primary sludge was 6.5 and reducing the pH from 5.1 to 4.5 did not influence the hydrolysis or rate of acidogenesis of primary

sludge, while an increase in pH from 5.1 to 6.1 increased the rate of hydrolysis but reduced the rate of acidogenesis.

In this specific study the COD was (1870mg/l), flow rate was (0.868ml/min or 0.001249m^3/day), and Hydraulic retention time were 19.2h for anoxic tank and 38.40h for oxic tank. These factors were kept constant to control their effect on the different experiments. Dissolved oxygen is the compulsory environmental factor for nitrification in complete biological nitrogen removal. The concentration of dissolved oxygen was less than 0.06 mg/l in the anoxic tank. During denitrification the presence of oxygen suppresses the synthesis of the enzyme needed for the substitution of nitrogen for oxygen as the terminal electron acceptor (Joseph, 2005). Dissolved oxygen was above 2.0mg/l in the oxic tank so that the nitrification process was not affected by dissolved oxygen concentration.

4.4 The effect of protease on treatment efficiency

4.4.1 Ammonium and nitrate production with and with out protease as supplement

In this study, the effect of adding exogenous protease on ammonia evolution, TN nitrogen removal, COD removal efficiencies was tested by adding 0, 10, and 30 U/ml of protease YE-33. The increase or decrease in ammonium production was measured only for the anoxic tank since the ammonium production in the oxic tank couldn't be known due to the continuous entering of ammonium from the anoxic tank and the simultaneous oxidation of ammonium by ammonia oxidizing bacteria in the oxic tank. Similarly, the ammonium production of the enzyme supplemented and none supplemented ones were compared only in the anoxic tank. When compared to the non enzyme supplemented system, the presence of 10U/ml and 30U/ml protease showed improvement in the production of ammonium. i.e as addition of protease dose increased ammonium concentration found to increase in the anoxic tank (Fig.7).

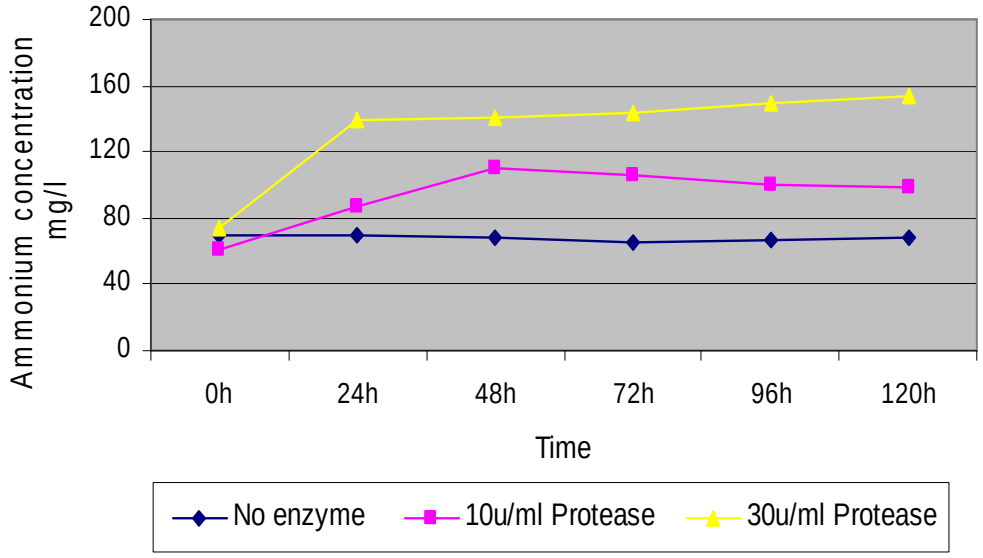


Figure 7. Change in ammonium concentration over time in anoxic reactor for enzyme supplemented and non supplemented experiments

As it seen in Fig.7 as experimental condition was changed from the non-enzyme supplemented to 10U/ml enzyme supplement the ammonium production increased by about 39mg/l at 48h and except little variation the increased amount of ammonium kept almost constant up to 120 h. When the enzyme is increased from 10 to 30u/ml, the increase in ammonium production was greater by 69mg/l at 24h experimental run. The increased concentration of ammonium in shorter time possibly attributed to improved hydrolysis processes that convert some of the total nitrogen into ammonium nitrogen.

The highest amount of ammonium production was observed when protease activity in the anoxic tank was 30u/ml Fig.7. This shows it is likely that before exogenous protease added enzyme activity was low compared to the substrate concentration found in the treatment tanks. Similar phenomena were observed in a study by Soo-Jung (2004) that at a highest ammonia accumulation and protease activities was found to increase in double. As ammonium production observed versus time, the ammonium production during adding 10u/ml protease was by far greater at 48h than the non-enzyme supplemented system. Similarly compared to non supplemented system addition of 30u/ml protease doubled the ammonium concentration at 24h. This indicates that adding the protease could help to get

shorter residence time in wastewater treatment systems. After hydrolysis the subsequent steps nitrification and denitrification are also important to complete the nitrogen removal process.

As it is exhibit by ammonium evolution, increasing the protease doses from 0 to 10 and 30U/ml has showed to enhance casein hydrolysis. The result only shows there was enzyme dose dependence of protein hydrolysis. Different studies used different concentrations of enzymes to degrade macromolecules present in wastewater. In a study by Shiferaw, (2006) synthetic wastewater containing casein were treated with 0.317U/ml protease and a TN removal efficiency of 80.2 and 57.1 and COD removal efficincies of 79.7 and 62.1 were attained for enzyme supplemented and non-enzyme supplemented respectively.

Crude lipase from ***P. aeruginosa*** (3.5 U/ mL) was tested on lipid-rich restaurant wastewater in a ratio of 1:1 and found to reduce the lipid content by 70% (Cammarota & Freire, 2006). Leal ***et al***. (2002) also reported that the use of 2.1U/ml lipase dose was found to have (80–95%) COD removal efficiencies which were higher than those obtained for raw effluents without enzymatic pre-hydrolysis; (19–55%) at different O&G concentrations.

In the anoxic/oxic reactor used in this study the ammonium produced in the anoxic tank enter to the oxic tank as it is the energy source for nitrifies during nitrification. Since nitrification is an aerobic process nitrate production was measured in the oxic tank to compare the improvement in nitrification in the presence and absence of protease. Fig.8 shows nitrate production with out enzyme and with 10U/ml and 30U/ml enzyme supplement. There was increasing trend in the nitrate production as the experimental condition was changed from no enzyme to 10U/ml and to 30U/ml enzyme supplement. However, more than 50% of the nitrate produced in the oxic tank was released in to the effluent (Table 7). This might be related to the manual mixed liquor recycling method used in or inefficient denitrification in the anoxic tank. The increase in concentration of ammonium (Fig. 7) as a result of enhanced hydrolysis might have favored nitrifieres in having additional available energy source during nitrification.

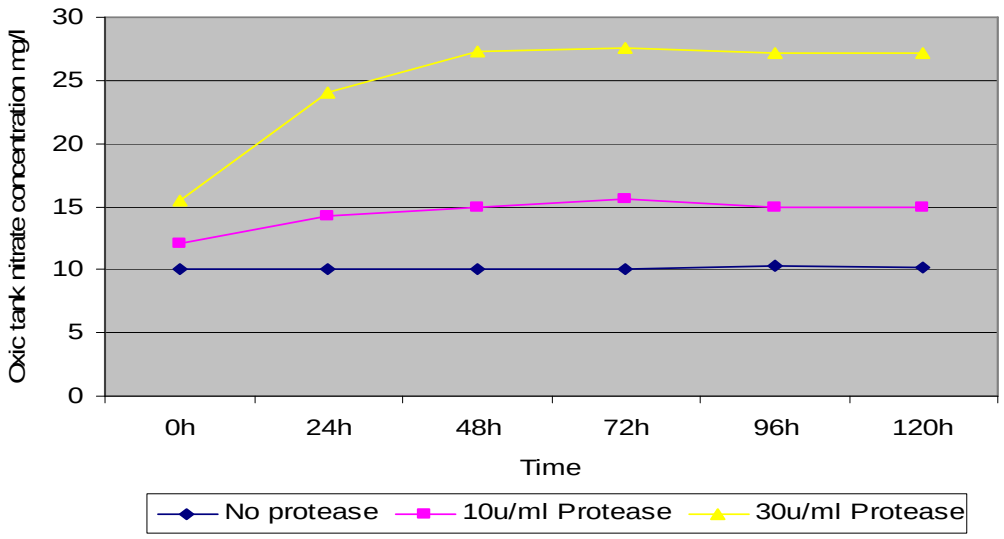


Figure 8. Nitrate concentration Vs time for enzyme supplemented and non supplemented experiments

Relatively larger amount of organic matter was entering the oxic tank during the experiments with out any enzyme treatment (Fig.10). Casein was the nitrogenous organic compound present in the synthetic wastewater used in this study. As it is evidenced by increased ammonium evolution, the added protease might have enhanced the casein hydrolysis which might be the main reason for decrease in the organic matter entering the oxic tank. There fore in addition to improvement in ammonium production, the improvement in protein hydrolysis could help to improve nitrification activity. Reduction in organic matter might also be improved by adding protease in the oxic tank so that the proteinaceous matter released from the anoxic tank will get another chance to be hydrolyzed.

A similar conclusion was also made by Xiao *et al.* (2007). They noted that in the presence of high concentrations of biodegradable COD, heterotrophic oxidizers out-compete autotrophic nitrifiers for dissolved oxygen and space. Heterotrophs have higher growth rates than nitrifier therefore they will consume the available oxygen. As a result there will be low production of nitrate.

Table 7. Nitrate concentration in Anoxic, Oxic and effluent in enzyme supplemented and non supplemented experiments

Time	Nitrate (mg/l) With out Enzyme			Nitrate (mg/l) 10U/ml Enzyme			Nitrate (mg/l) 30U/ml Enzyme		
	Anoxic	Oxic	Effluent	Anoxic	Oxic	Effluent	Anoxic	Oxic	Effluent
0h	5.00	10.0	5.64	5.46	14.10	6.50	9.15	15.5	13.43
24h	4.34	10.1	5.78	8.09	17.20	7.45	10.97	24.0	15.21
48h	5.20	10.1	5.87	8.70	18.00	6.67	14.53	27.3	14.00
72h	5.40	10.1	5.47	8.90	18.60	6.76	13.53	27.5	14.23
96h	5.00	10.3	5.69	7.60	17.00	7.65	14.40	27.2	14.77
120h	4.87	10.2	5.34	7.80	17.00	6.84	14.81	27.1	14.90

Seyoum, (2004) described that organic loading rate, dissolved oxygen concentration and toxic materials are some of the factors which inhibit nitrifiers and reduce nitrification. Similar organic loading rate, dissolved oxygen with very little variation and synthetic wastewater with same composition were used throughout the study.

4.4.2 Total Nitrogen removal efficiency without and with protease supplement

The total nitrogen removal (TN) efficiency improved at 30U/ml enzyme dose as compared to the non-enzyme supplemented and the 10U/ml enzyme supplemented experiments (Fig. 9). The highest total nitrogen removal efficiency achieved with out adding protease was 56.8%. Addition of 10U/ml enzyme has increased the total nitrogen removal to a maximum of 61.3%. Although adding 10u/ml enzyme has contributed 15.6mg/l total nitrogen, more than 5% removal efficiency improvement observed starting from the 48 h treatment time. Similar patterns were observed with the addition of 30U/ml protease. Adding 30U/ml crude protease has 39.8mg/l total nitrogen contribution. Despite the high total nitrogen contribution adding 30U/ml protease showed highest treatment performances which increased the maximum TN removal efficiency to 73.05 %(Fig. 9).

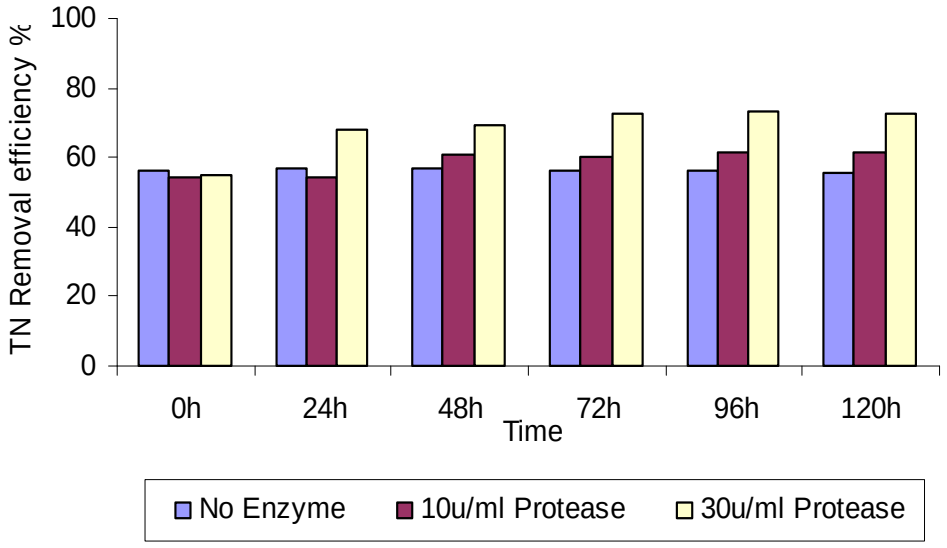


Figure 9. Total nitrogen removal efficiencies Vs time for enzyme supplemented and non supplemented experiments

The total nitrogen removal rate also showed improvement with increasing doses of exogenous protease. As it is presented in Fig. 9, in the presence of 10u/ml protease, the TN removal efficiency at 48h was 60.95% which was higher by about 5% than the TN removal efficiencies achieved even at 120h with out addition of enzyme. In experiments where the enzyme dose was 30U/ml the TN removal efficiency at 24 h was increased by greater than 10% from the 10U/ml enzyme experiment and by about 17% from the non-enzyme supplemented experiment (Fig.9). And at 30u/ml enzyme activity the removal efficiency achieved at 24 h was not reached even at 120h by the non-supplemented and the 10u/ml enzyme supplemented experiments.

Nitrate is electron acceptor for denitrifiers in the anoxic tank. As it is seen in (Fig.8) the concentration of nitrate in the oxic tank showed improvement as the protease doses increased from 0 to 10 and 30U/ml. Similar trend was observed in the total nitrogen removal efficiency where total nitrogen removal efficiency increased as the protease doses increased from 0 to 10 and to 30U/ml(Fig.9). In anoxic/oxic treatment system the denitrifiers get nitrate from the recycled mixed liquor.

The improvement in total nitrogen removal efficiency in the 30U/ml enzyme could be from the largest amount of nitrate recycled which could initiate additional denitrification in the anoxic tank. Unless there is anaerobic ammonia oxidizing organisms in treatment system the lesser the nitrate concentration in the anoxic tank the lesser will be the denitrification efficiency of the system. In this study 100% mixed liquor recycling rate was used. To overcome shortage of electron acceptor problem for denitrification some studies recommend recycling rate up to 400% (Xiao ***et al.***, 2007) for up to 80% NO_3^- removal.

Total nitrogen removal is cumulative effect of hydrolysis, nitrification and denitrification (Gerardi, 2002). Nitrogen removal efficiency will be affected if one of these processes failed or are sluggish in the treatment system. Assimilatory and dissimilatory processes are the system for nitrogen removal in biological reactors. Fig.9 shows that the total nitrogen removal efficiency was not more than 55% with out enzyme.

From the present result it can be deduced that enhancing hydrolysis might have caused dual effect on nitrogen removing pre-denitrification nitrification system. One providing enough hydrolyzed organic matter for denitrification other is decreasing organic matter entering the oxic tank. Both effects contributed for the total nitrogen removal from the system. And using enzymatic treatment associated with anoxic and oxic system could bring a reduction in hydraulic retention time and, consequently, in reactor volume, since it promotes faster hydrolysis of protein.

4.4.3 COD removal efficiency with and with out protease as supplement

The effect of the enzymatic treatment on the COD removal kinetics is shown in Fig. 10 and 11 which indicated the COD removal efficiency with time for anoxic and oxic tanks. The COD removal efficiency of the anoxic system and the overall COD removal efficiency were calculated considering the COD contribution from the crude enzyme extract depicted in Table 5.

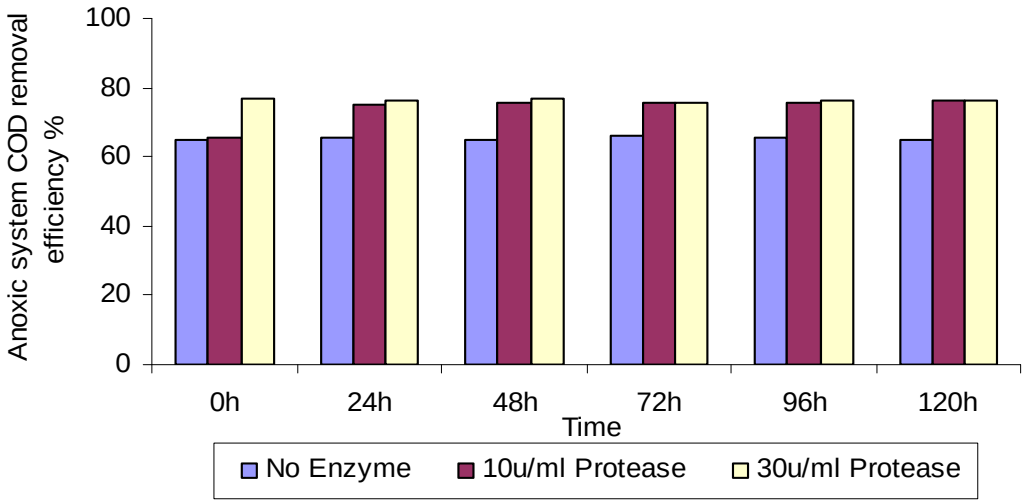


Figure 10. Anoxic COD removal efficiency Vs time for enzyme supplemented an non supplemented experiments

The COD removal efficiency of the anoxic tank is shown in Fig. 10. It is indicated that the greater portion of organic matter (65%) was degraded in the anoxic tank of the treatment system. In the absence of additional protease the maximum removal efficiency of COD for the anoxic tank was 66.2%. Whereas adding 10U/ml protease in the anoxic tank shifted the maximum removal efficiency to 76.16%. As the enzyme dose increased from 10U/ml to 30U/ml the maximum removal efficiency was about 76% for the 120h experimental time. Adding enzyme in the treatment system the COD removal efficiency improved on average by 10% in anoxic tank (Fig.10). This might be due to the degradation of the casein (organic nitrogen) left undegradaded.

It is shown in Table 5 that the characteristics of the crude protease contains high amount of organic content in the form of COD. Whereas the experimental results showed that despite the high chemical oxygen demand contributed by the crude enzyme, adding crude enzyme didn't affect the overall removal efficiency Fig.11. This might be due to the presence of biodegradable COD component found in the crude enzyme which might have served the organisms to use it as different nutrient source.

As it is indicated in Fig.11 although the over all COD removal was efficient which is (>90%) even in the absence of any additional treatment it doesn't go hand in hand with the nitrogen removal efficiency Fig.9. The removal of high overall COD might indicate the presence of group of the microbial consortia which consume the wastewater as different nutrient sources. Whereas the low TN removal efficiencies might indicate the microbial consortia present in the treatment system may not be efficient nitrogen removing ones. In order to get improvement in total nitrogen removal at least three different groups of organisms are need. In nitrogen removal systems the denitrifers are one group of organisms, which are supposed to use the organic matter, but if other heterotrophic organisms dominate the activity of denitrifiers will be affected (Gerardi, 2002).

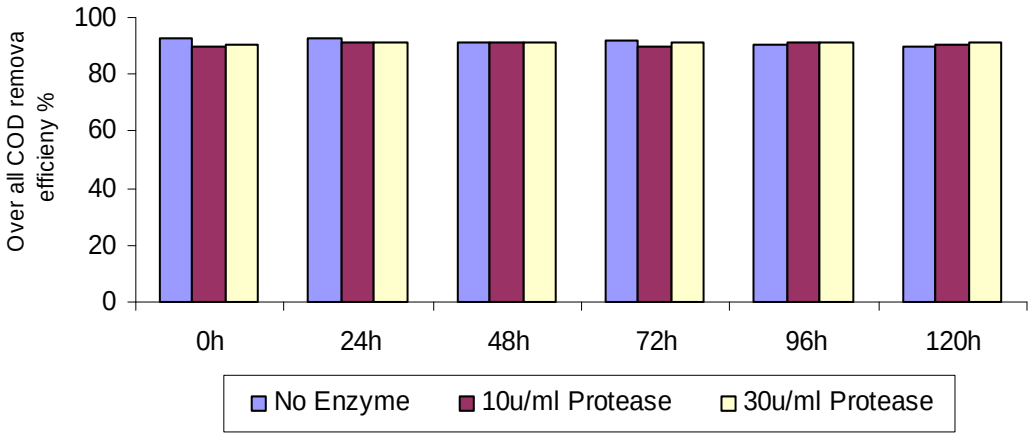


Figure 11. Over all COD removal efficiency Vs time for enzyme supplemented and non supplemented experiments

The nitrate concentration in oxic and anoxic tank showed increment with addition of more amount of protease (Table 7). And as recycled nitrate increased (Table 7) corresponding COD removal efficiency showed increment (Fig.10) this might be correlated with the consumption of nitrate together with the consumption of the organic matter by denitrifieres.

The relationship between COD removal and denitrification has been evaluated by several workers. According to Garrido, (2001) COD requirement for denitrification is around 5g COD/g N-NO_3. Similarly Claudia and Josef, (2005) reported that in wastewater treatment plants, more than 2.85 g COD is required for reduction of 1g NO_3^-–N. This means that it is necessary to guarantee a biodegradable COD concentration for denitrifiers to have successful denitrification. A study by Carlos, (2004) reported that insufficient amounts of organic carbon in groundwater limit the application of *in situ* heterotrophic denitrification unless organic substances are added as external carbon sources.

4.5 Effect of Bio- Augmentation on treatment efficiency

In the present study effect of bio-augmentation with proteolytic isolate (YE-33) with two different inoculum sizes of $1.5x10^7$cfu/ml and $1.5x10^9$cfu/ml was compared with the control (non augmented system).

4.5.1 Ammonium production before and after proteoytic bio-augmentation

Ammonium production together with the corresponding proteolytic count for the non-augmented and augmented systems in the anoxic system is presented in (Fig.12). Before augmentation the ammonium concentration in the anoxic tank ranged 77-79.1mg/l (Fig11 A). In the first bioaugmenation experiment where YE-33 were inoculated with $1.5X10^7$ cfu/ml cell density in the anoxic tank the ammonium evolution showed constant increment with a range 78.2-99.9mg/l. The corresponding viable cell count is also presented in (Fig.12) and compared to the non augmented cfu/ml small change in cell density observed in the augmented system. The increase in ammonium concentration is possibly due to the

enhanced protein hydrolysis caused by the inoculated organisms which might be efficient in hydrolytic characteristics.

The initial average proteolytic cell density of the anoxic tank was $1.17x10^8$cfu/ml. As it is seen in (Fig. 12 B) starting from the 24h around 20mg/l ammonium increase was observed when $1.5x10^7$ cfu/ml of proteolytic isolate YE-33 introduced in the anoxic tank and the increased ammonium remained in a similar trend for the 120h experimental time. To see the effect of increasing inoculum size of the augmented organism second round proteolytic bioaugmentation was at cell densities $1.5x10^9$cfu/ml (Fig.11 C). Despite inconsiderable change in cell number before and after augmentation the ammonium concentration showed increment by 20mg/l in the augmented systems. This might be related with the introduction of the efficient proteolytic bacteria.

The second bioaugmentation experiment was with cell density $1.5x10^9$cfu/ml. When the augmented cell density increased from $1.5x10^7$ to $1.5x10^9$cfu/ml, instead of showing increment in ammonium production in the anoxic tank, more stable ammonium production was observed for 120h (Fig.12 C). This might show that larger inoculum size helped the organism to better compete with other group of organisms and gave stable metabolic product.

In this study pure culture of have been augmented in the treatment system. However bioaugmentation with mixed culture of protelyotic organisms might bring more improved degradation performance in the treatment systems. As it is observed in this study, both adding the protease and the protelyotic bioaugmentation had showed their own enhanced treatment efficiencies. However simultaneous application of the protease enzyme and protelyotic bioaugmentation might be a potential alternative to be tested on the nitrogen removal rate.

Figure 12. (A), (B) and (C) Proteolytic cfu/ml counts in the anoxic tank Vs NH_4^+ concentration.

Fig.12 A) Before augmentation

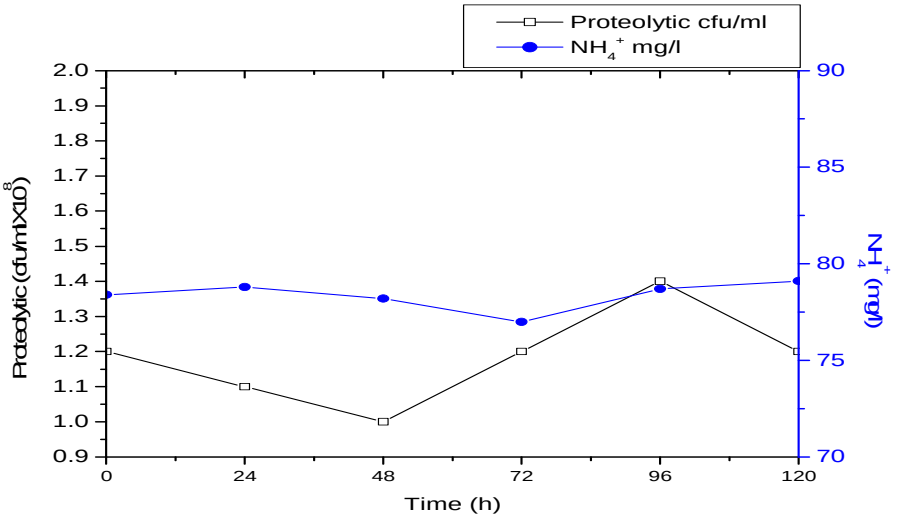


Fig.12 B) Augmentation with $1.5X10^7$cfu/ml inoculum size

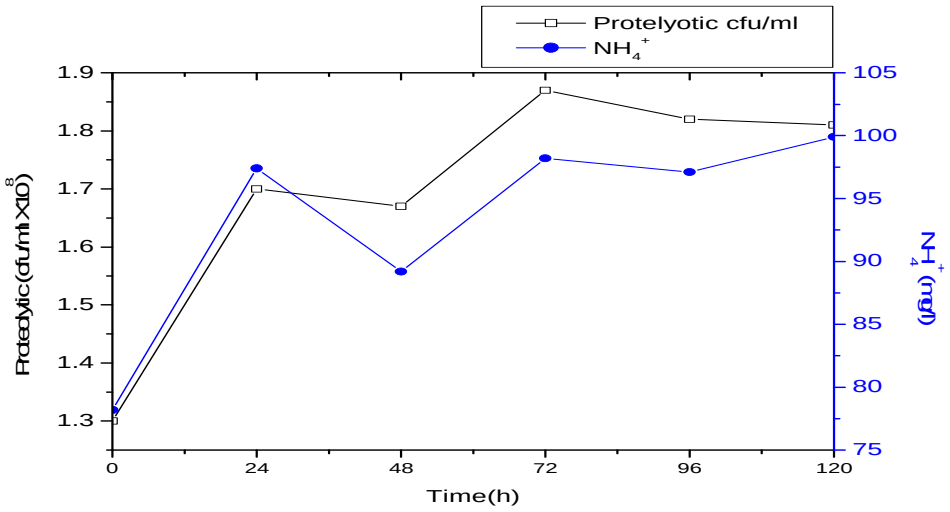
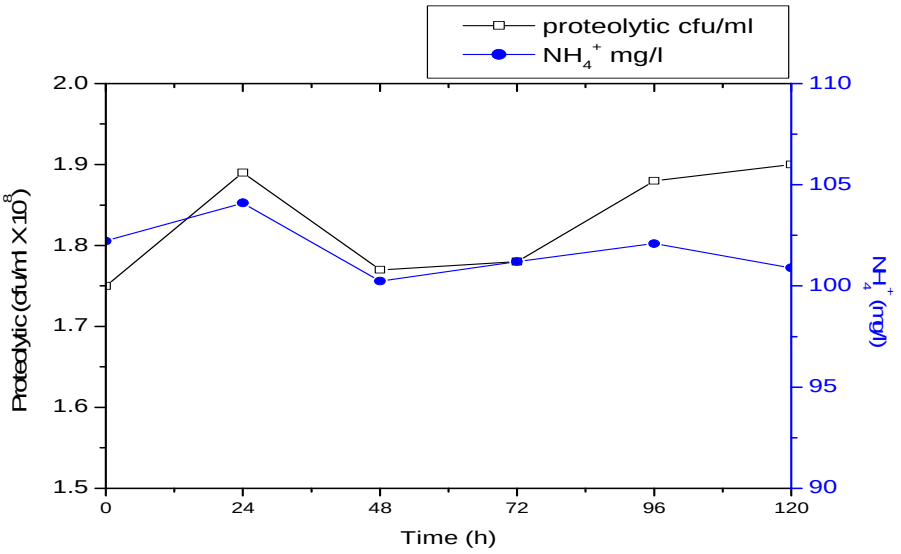


Fig.12 C) Augmentation with $1.5X10^9$cfu/ml inoculum size.



Different augmentation studies applied different cell densities of organisms for augmentation. According to Cammarota & Freire, (2006), they found that *Acinetobacter sp.* added to the wastewater at approximately 10^7 CFU (colony forming units)/mL, dominated the whole system, and typically degraded 60–65% of the fatty material, whose initial concentration had been 8 g/L. Another study by Fikret and Ahmet, (2005) reported that *Halobacter halobium* (ATCC 43214) obtained from the American Type Culture Collection (ATCC), cultivated in salt containing synthetic media in the laboratory was mixed with activated sludge culture in equal volumes (1/1, v/v). They further explained that *Halobacter*-added activated sludge resulted in 290 mg/l effluent COD and 73%COD removal compared to 600 mg /l effluent COD and 47% COD removal with the *Halobacter*-free activated sludge.

Ramadan *et al*. (1990) has also observed that a *pseudomonas cepacia* population of $4.3x10^3$ cells/ml inoculated in to lake water could multiply and degrade p-nitrophnol, whereas a population of $2.3x10^2$ cells/ml disappeared after 13h.

In a study on degradation of 3-chlorobenzaote and 4-methylbenzaote two genetically modified organisms *pseudomonas* sp. was introduced into an activated sludge unit at initial densities of 10^6-10^7cfu/ml which decreased to stable population of 10^4-10^5 cfu/ml and despite the colony forming unit decrease, three days after inoculation drastic decrease in the concentration of target pollutants was observed (Limbergen *et al*., 1998).

It is desirable that the fate of an introduced organism be monitored in order to prove its contribution to pollutant degradation and to assess its influence on the ecosystem. In this study the method used to get viable count was culture dependent. However, culture-dependent methods obviously do not show the real bacterial community structure because of the selective pressure imposed by the requirement for growth. The viable proteolytic counted in this study could be from the introduced one or the population already in the system. Counting colony forming unit was used only to indicate how the bio-augmentation had affected the bacterial abundance. The specific population density was correlated with the ammonia production, the total nitrogen and COD removal efficiencies.

Nitrate concentration in the three bio-augmentation experiments is shown on Table 8; the concentration more or less remained constant in the oxic tank among the three experimental conditions the none augmented, and the augmented with $1.5x10^7$cfu/ml and $1.5x10^9$cfu/ml cell densities. Fig.13 also shows the nitrification activity was similar in the augmented and non augmented systems. Therefore, there will be less variation in the recycled nitrate concentration.

Table 8. Nitrate concentration in different systems for non-augmented and bio-augmented systems.

Time	Non-bio Augmented system			Bio augmented system With $1.5X10^7$ cell density.			Bio augmented system With $1.5x10^9$ cell density.		
	Anoxic	Oxic	Effluent	Anoxic	Oxic	Effluent	Anoxic	Oxic	Effluent
0h	10.00	15.10	6.64	7.65	15.43	10.50	8.20	15.67	10.43
24h	10.29	14.21	6.78	7.65	15.41	10.45	7.40	15.30	9.87
48h	9.65	14.30	6.87	7.23	15.30	10.67	7.12	15.86	10.00
72h	9.67	15.22	5.47	7.45	15.22	10.76	7.24	15.43	11.23
96h	9.68	14.40	5.69	7.64	15.40	10.65	7.23	15.47	10.77
120h	9.87	14.64	6.34	6.98	15.64	10.84	7.42	15.36	10.90

It was seen in enzyme-supplemented experiments that the increase in nitrate concentration was related with at least two factors like ammonium accumulation and chemical oxygen demand reduction which favors nitrifies present in the oxic tank to. The lesser nitrate concentration in the oxic tank might be attributed to the effect of relatively higher organic matter entering the oxic tank. Similar conclusion was reached by Matthew, (2001) that nitrification begins immediately because of the absence of organics in the test solution.

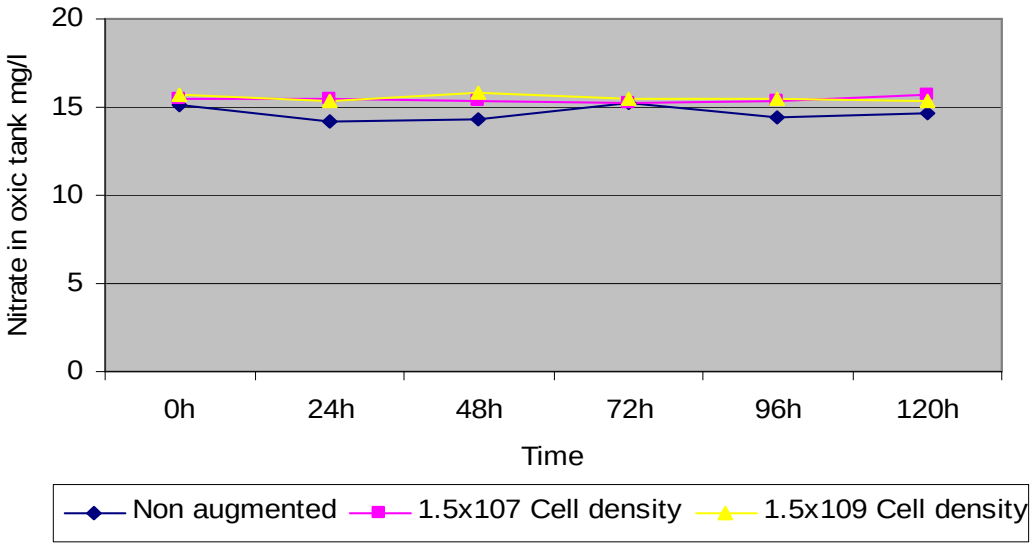


Figure 13. Nitrate concentrations Vs time for bio-augmented and non augmented experiments

4.5.2 Total Nitrogen removal efficiency for non-augmented and bio- augmentation

The total nitrogen removal efficiency showed slight increase when additional proteolytic organisms were bio-augmented to the system (Fig.14). The TN removal efficiency achieved before augmentation ranged 56.18-58.77%. While proteolytic bacteria augmented in the system at inoculum size of $1.5x10^7$cfu/ml, the TN removal efficiency showed increment with minimum removal efficiency of 58.5% and maximum removal efficiency 64.5%. Compared to none augmented and augmented with $1.5x10^7$cfu/ml systems highest nitrogen removal observed in the second augmentation with inoculum size of $1.5x10^9$ where the removal efficiency ranged from 65.4-65.8%.

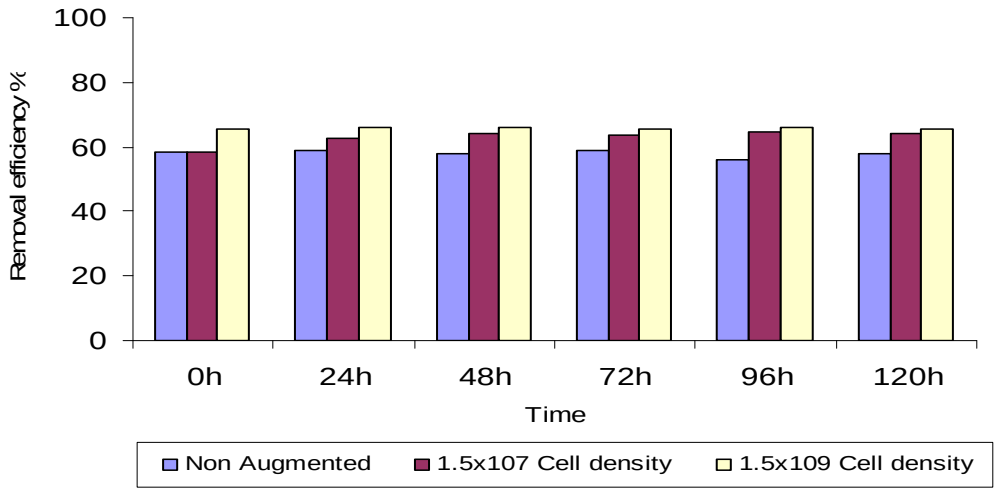


Figure 14. Total nitrogen removal efficiencies Vs time for none augmented and bio-augmented experiments

In addition to the increase in TN removal efficacy there is also improvement in total nitrogen removal rate. As it is seen in Fig.14 faster TN removal rate was observed with augmenting the treatment system. The maximum total nitrogen removal efficiency before augmentation was 58.77% for the 120h experimental time. However in the augmentation of $1.5X10^7$cfu/ml TN removal efficiency achieved at the 24h was 60.95% which is greater than the maximum removal efficiency in the non augmented system even at the 120h experimental time (Fig.14).

Although increase in the total nitrogen removal efficiency is not high, the interesting observation is that after augmenting the first batch of organisms in to the system the total nitrogen removal efficiency remained stable till the second augmentation carried out (Fig.14). This shows that the bio-augmentation strategy might be cost effective in terms of no need of repeated augmentation provided the augmented organisms can survive and remove the target pollutant efficiently.

During starting the second bio-augmentation experiment, the removal efficiency was 65.4% adding $10x1.5^9$cfu/ml cell density didn't show improvement in TN removal efficiency. The

denitrification activity increased in the anoxic tank of the bio-augmented system, this was shown by, the simultaneous increase of concentration of nitrate in the oxic tank (Table 8) and decrease in total nitrogen concentration in the effluent (Fig. 14).

4.5.3 COD removal efficiency for non-augmented and bio-augmented systems

The COD removal efficiency of the lab-scale reactor was high (>90%) even before any augmentation had started (Table 6). The result is possibly due to the microbial composition of the treatment system. It was shown that the degradation of organic matter in the form of COD was rapid. As the COD removal efficiency reached 90% the total nitrogen removal efficiency remained 55% (Table 6). The anoxic tank accounts for more than 65% COD removal efficiency (Fig 15). This might be due to the higher metabolic activity in the anoxic condition where COD is used as carbon and energy source for the microorganisms.

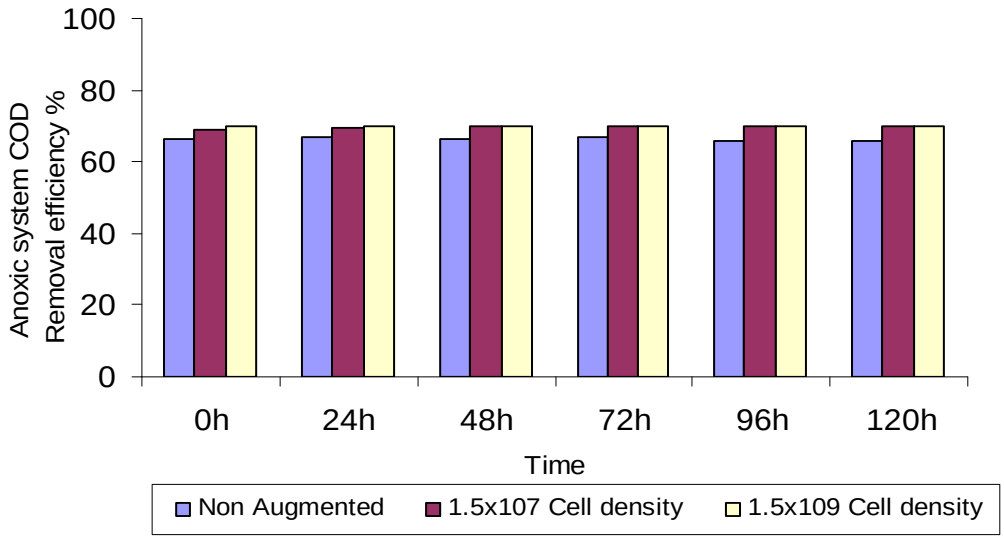


Figure 15. Anoxic step COD removal efficiency Vs time for bio-augmented and non bio-augmented experiments

As it is shown again in Fig 15, during augmentation of the organisms the COD removal efficiency of the anoxic tank changed by 3-4%. This slight change might be due to the activity of the introduced proteolytic organisms or higher denitrification activity caused by the stable nitrate supply during the augmented condition Fig.13. The remaining COD<35 % was removed in the oxic tank of the treatment system (Fig.16).

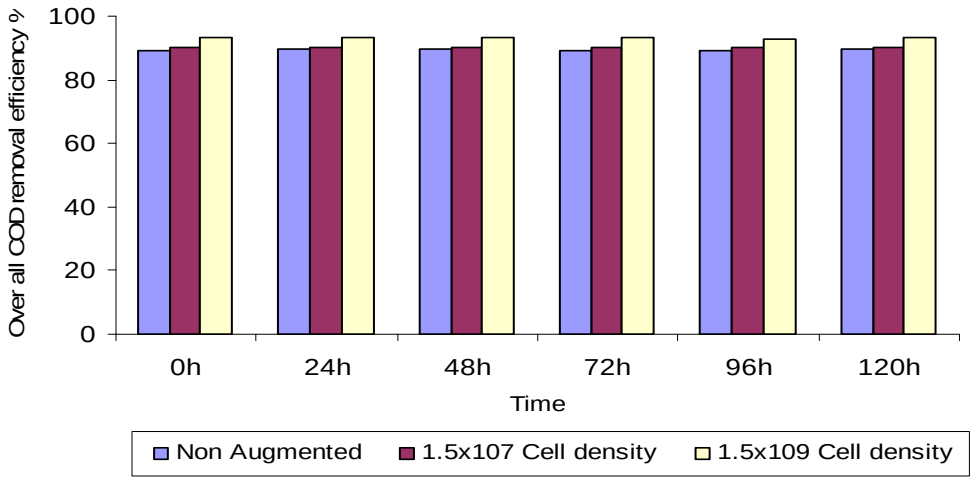


Figure 16. Over all COD removal efficiency Vs time for bio-augmented and non bio-augmented experiments

The change in over all COD removal efficiency ranged 89.1%-89.62, 90-90.19% and 93.02-93.71% in the none augmented, in the augmented with $1.5x10^7$cfu/ml, and in augmented with $1.5x10^9$cfu/ml respectively (Fig.16). This showed that the augmentation had slightly improved the over all COD removal efficiency which is possibly due to the additional hydrolytic activity from the augmented organisms.

The interpretations of the results of this study were not supported with molecular tool like FISH to monitor the fate and activity of the inoculated organisms and about the reaction of the indigenous microbial community. Therefore very little is known about characteristics the groups of organisms present in the system. Problems concerning the adaptation of the inoculated microorganisms, the insufficiency of substrate, competition between the introduced species and the indigenous biomass, and grazing by protozoa have been given as

possible reasons for the very little improvement or failure of bio-augmentation experiments (Bouchez, *et al*., 2000).

4.5.4 Pure culture enzyme production in effluent and laboratory media

In order to check the protease activity in the wastewater, pure culture of (YE-33) were grown on laboratory media mentioned in materials and methods section 3.3.1. The result of the protease activity and cfu/ml count is depicted in (Table 9), although protease activity was not as high as in the media supplemented with the casein or other nutrients, there was extra cellular protease production in the effluent without any type of additional nutrient addition. This shows there was no inhibition from the wastewater in the treatment system.

The presence of extra cellular protease indirectly shows the presence of protein (organic nitrogen) in the treatment system. This proves protein substrate availability can't be a factor for less proteolytic cell density change in the case of the bioaugmentation experiments. Further more since there was extra protein in the effluent to support growth of proteolytic organisms, the insignificant improvement in ammonium production (Fig.11) might not be caused by lack of enough protein substrate (Casein) to support the greater number of cells.

The enzyme activity observed (Table 9) in the effluent + casein media composition, better enzyme productivity was observed when it was compared with effluent used as a media. This indicates the additional casein added supported for more growth and better enzyme productivity. But when compared with the enzyme productivity in the laboratory media, the best enzyme productivity was obtained in the laboratory media.

Table 9. YE-33 cell density and enzyme productivity at 24h and 48h

Media	Flask	CFU/ml at 24h	Unit/ml protease at 48h	CFU/ml at 48h	Unit/ml protease at 48h
Laboratory Media	1	$1.20x10^{11}$	53.05±8.66	$2.69x10^{12}$	64.01±17.31
		$1.61x10^{11}$		$>3.00x10^{12}$	
	2	$1.37x10^{12}$		$>3.00x10^{12}$	
		$1.32x10^{12}$		$>3.00x10^{12}$	
	3	$1.07x10^{13}$		$2.46X10^{11}$	
		$1.25x10^{11}$		$>3.00x10^{12}$	
Effluent + 0.5% casein	1	$1.4x10^{12}$	28.97±1.58	$>3.00x10^{12}$	25.85±0.69
		$1.50x10^{11}$		$>3.00x10^{12}$	
	2	$1.1x10^{11}$		$2.00x10^{11}$	
		$1.5x10^{11}$		$2.78x10^{11}$	
	3	$1.2x10^{11}$		$2.10x10^{11}$	
		$1.9x10^{11}$		$>3.00x10^{11}$	
Effluent	1	$1.6x10^{4}$	10.44±1.74	$1.3x10^{6}$	11.79±1.5
		$1.6x10^{5}$		$1.3x\ 10^{7}$	
	2	$1.5x10^{6}$		$1.2x10^{6}$	
		$1.4x10^{5}$		$1.4x10^{6}$	
	3	$1.5x10^{6}$		$1.3x10^{6}$	
		$1.5x10^{6}$		$1.3x10^{5}$	

5. Conclusions

The following major conclusions have been drawn from this study

In this study the possibility of getting faster nitrogen removal rate was observed both in the case of adding protease and bio-augmentation with proteolytic organisms.

One of the factors that affect nitrification is concentration of organic matter. Improving hydrolysis found to have impact both on the ammonium production and indirect impact on decreasing organic matter which both are necessary for efficient nitrification.

Compared to the enzyme treatment the bio-augmentation is not easy to control since the organisms can be affected by more food and environmental factors than enzymes.

Once the augmentation of proteolytic organisms becomes successful, it has an advantage in that it doesn't need repeated inoculation of the organisms in a treatment system.

In general, because of enhanced hydrolysis higher nitrogen removal efficiencies obtained in shorter time both in enzyme treatment and in augmentation studies. This indicates both treatments can reduce hydraulic residence time in biological reactors. A reduction in residence time can help to have lower reactor volume and space so that treatment cost can be minimized.

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