DEVELOPMENT OF BIOREACTORS FOR NITRIFYING SEWAGE

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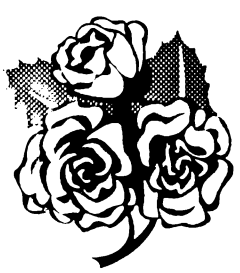

To My Parents

CERTIFICATE

This is to certify that the research work presented in this thesis entitled **'Development of Bioreactors for Nitrifying Sewage'** is based on the original work done by **Ms. Kavitha Ramachandran** under my guidance in the School of Environmental Studies, Cochin University of Science and Technology, in partial fulfilment of the requirements for the degree of Doctor of Philosophy and no part of this work has previously formed the basis of the award of any degree, diploma, associateship, fellowship or any other similar title or recognition ⊙

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DECLARATION

I, hereby declare that the work presented in this Ph.D thesis entitled **'Development of bioreactors for nitrifying sewage'** is based on the original work done by me under the guidance of Dr. I.S.Bright Singh, Reader in Microbiology, School of Environmental Studies, Cochin University of Science and Technology, Cochin -682 016 and no part of this work has previously formed the basis of the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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Abbreviations used

KCal/Mol.N^{-1}	=	Kilocalories per mole of nitrogen
ATP	=	Adenosine Tri Phosphate
NAD	=	Nicotinamide Adenosine
NAD(P)	=	Nicotinamide Adenosine Tri Phosphate
nm	=	nanometer
µg	=	Microgram
µmol	=	Micromole
mol	=	mole
APHA	=	American Public Health Association
BOD_5	=	Biochemical Oxygen Demand (mg per litre) at an incubation period of 5 days at 20°C.
COD		Chemical Oxygen Demand (mg per litre)
SS	=	Suspended Solids
lbs	=	Pounds
vvm	=	Volume of the air passed/volume of medium used / minute
UNA	=	Unit Nitrifying Activity
AVT	=	Ammonia Treated Vermiculate
µ	=	Specific growth rate
µmax	=	Maximum specific growth rate
S		Substrate concentration

Ks	=	Substrate concentration at half the value of μmax ($mg.L^{-1}$)
Km	=	Michaelis-Menton constant ($mg.L^{-1}$)
Vmax	=	Maximum velocity of an enzyme catalysed reaction
Y	=	Maximum yield co-efficient, measured as the ratio of the mass of cells formed to the mass of substrate consumed.
X	=	Concentration of cells (mass/unit volume) (MLSS)
V	=	reactor volume
g	=	generation time (hours)

CHAPTER 1

GENERAL INTRODUCTION

Chapter 1
GENERAL INTRODUCTION

Various compounds containing the element nitrogen are becoming increasingly important in wastewater management programmes because of the many effects that nitrogenous materials in the wastewater effluent can have on the environment. Nitrogen in its various forms, can deplete dissolved oxygen levels in receiving waters, stimulate aquatic growth, exhibit toxicity towards aquatic life, affect chlorine disinfection efficiency, present a public health hazard, and affect the suitability of wastewater for reuse (De Renzo, 1978). Biological and chemical processes which occur in wastewater treatment plants and in the natural environment can change the chemical form in which nitrogen exists. For example, by converting ammonia in raw wastewater to nitrate, the oxygen depleting and toxic effects of ammonia are eliminated.

The relationship between the various nitrogen compounds and the biological transformations which can occur are often presented in any typical representation of nitrogen cycle. The principal compounds of concern in the nitrogen cycle are nitrogen gas, ammonium, organic nitrogen, and nitrate. The toxicant ammonium is formed by the mineralization of dead animal and plant tissue and animal faecal matter by a process called ammonification.

1.1 Nitrification

The term 'nitrification' is applied to the biological oxidation of ammonium, first to nitrite and then to nitrate by two groups of aerobic, chemolithotrophic bacteria belonging to the family Nitrobacteriaceae. The bacteria responsible for these reactions are termed chemolithotrophic because they use inorganic chemicals as their source of energy. Generally, the *Nitrosomonas* are involved in the conversion of ammonium to nitrite under aerobic conditions as follows:

$$NH_4^+ + 1.5\ O_2 \xrightarrow{\text{bacteria}} NO_2^- + H_2O + 2H^+$$

$$G=\bar{6}5\ \text{KCal/Mol. N}^{-1}$$

The nitrites are in turn oxidized to nitrate generally by *Nitrobacter* according to the following reaction:

$$NO_2^- + 0.5\ O_2 \xrightarrow{\text{bacteria}} NO_3^-$$

$$G= -18.\text{K. Cal/Mol.N}^{-1}$$

The overall nitrification reaction is as follows:

$$NH_4^+ + 2O_2 \longrightarrow NO_3^- + 2H + H_2O.$$

The oxygen consumption ratios as per the above two equations are 3.22 mg O_2 mg.$^{-1}$ NH_4^+–N oxidized and 1.11 mg O_2 mg.$^{-1}$ NO_2^-–N oxidized which is in agreement with measured values (Gujer and Jenkins, 1974).

1.1.1 Biology of nitrifying bacteria

Scholesing and Muntz (1877) were the first to demonstrate the biological nature of nitrification by preventing conversion of ammonium to nitrite in soil percolation columns by addition of chloroform. Several years later Winogradsky (1890) isolated the bacteria responsible and classified them into two groups: the first converting ammonium to nitrite (ammonia oxidizers) and the second oxidizes nitrite to nitrate (nitrite oxidizers). No single species yet found oxidizes ammonia to nitrate. All strains are aerobic and with the exception of *Nitrobacter* species, all others are obligate chemolithotrophs, but some can grow mixotrophically.

Ammonia oxidizers are placed by Watson (1974) in four genera namely, *Nitrosomonas, Nitrosococcus, Nitrosospira* and *Nitrosolobus* and a fifth, *Nitrosovibrio* has been described by Harms *et al.*, (1976). Most work has been carried out on the genus *Nitrosomonas*, which consisted of ellipsoidal or short rod- shaped cells which are Gram negative. There are three genera of nitrite oxidizers, namely *Nitrobacter, Nitrospina* and *Nitrococcus* of which *Nitrobacter* is by far the most commonly isolated (Prosser and Cox, 1982). Cells are Gram negative, short wedge or pear shaped rods which may possess a single flagellum. The presence of cytochromes results in yellow or red suspensions.

1.1.2 Biochemistry of nitrification

Nitrifying bacteria are chemolithotrophs and obtain all their energy from the oxidation of inorganic compounds, while cellular carbon is obtained principally by the fixation of carbon dioxide. The nature of their energy sources leads to low cell yield compared to those of heterotrophs.

1.1.2.1 Ammonia oxidation

Work on the effect of pH on ammonia oxidation (Suzuki *et al.*, 1974, Drozd 1976) indicates that ammonia (NH_3) crosses the cytoplasmic membranes rather than ammonium (NH_4^+). The oxidation of ammonia occurs in two or more steps. The first step catalyzed by ammonia monooxygenase oxidizes ammonia to hydroxyl amine or a related enzyme-bound chemical species incorporating molecular O_2 (Watson, 1989). The second step, oxidation of hydroxyl amine via NOH to nitrite is thought to be the energy yielding portion of the reaction. This step is catalyzed by hydroxyl amine oxidoreductase. Although, all ammonia oxidizers oxidize hydroxyl amine to nitrite, this substrate does not support growth even when added continuously at 10^{-5}M. Cells of *Nitrosomonas* are red, reflecting the unusually high cellular content of cytochromes which are found in the periplasmic space and membrane (Hooper *et al.*, 1972, Watson, 1989).

Carbon dioxide fixed via the calvin cycle (Watson *et al.*, 1989.) serves as the primary carbon source. 18 mol ATP and 12 mol reduced

NAD(P) are required to fix 6 mol carbondioxide. Secondly, formation of the reducing equivalents also consumes energy such that in the order of 5 mol ATP are required for the reduction of 1 mol NAD(P). The specific activity of carbonic anhydrase of *N. europaea* is greater when the concentration of CO_2 in the medium are low, suggesting a role for the enzyme in promoting assimilation of carbon (Watson *et al.*, 1989,). Although most ammonia oxidizers can grow mixotrophically on several organic compounds such as acetate, formate, pyruvate, glucose and complex compounds (Smith and Hoare, 1977), heterotrophic growth has not been demonstrated (Watson *et al.*, 1989). The ability of cells of *Nitrosomonas* to oxidize ammonia is inactivated by ultraviolet light and by light of 410 nm (Hooper and Terry, 1974). Photoinactivation does not occur anaerobically or under conditions in which nitrite is being rapidly produced.

1.1.2.2 Nitrite Oxidation

The initial step is the hydration of nitrite followed by dehydrogenation with the reaction sequence (Aleem, 1977).

$$NO_2^- + H_2{}^{18}O \rightarrow NO_2^- \cdot H_2{}^{18}O$$

$$NO_2^- \cdot H_2{}^{18}O + 2\ Cyt.a_1.\ Fe^{3+} \rightarrow N^{18}O_3 + 2\ Cyt.a_1.\ Fe^{2+} + 2H^+$$

$$2Cyt.a_1.Fe^{2+} + 2Cyt.aa_3.Fe^{3+} \longrightarrow 2Cyt.a_1.Fe^{3+} + 2Cyt.aa_3.Fe^{2+}$$

$$2Cyt.aa_3.\ Fe^{2+} + 2H + \tfrac{1}{2}\ O_2 \rightarrow 2Cyt.aa_3.\ Fe^{3+} + H_2O$$

Oxidation of nitrite to nitrate, therefore, occurs by the addition of an oxygen atom from water and not from molecular oxygen. Electrons via Cytochrome oxidase are transferred with the generation of ATP. As in ammonia oxidizers, carbon dioxide fixation demands a high energy input and it has been calculated that 15 mol nitrite are required for fixation of 1 mol carbon dioxide to the level of a hexose sugar. Cellular carbon is obtained from carbon dioxide and ribulose bisphosphate carboxylase, the key enzyme in this process, believed to be residing in Carboxysomes (Bock *et al.,* 1974, Peters 1974). *Nitrobacter* strains grown chemolithotrophically oxidize nitrite to nitrate producing ATP and NADH (Watson *et al.,* 1989). In chemolithotrophically grown cells of *Nitrobacter,* only 2-11% of the free energy generated from the oxidation of nitrite is used for cell growth., 85-115 mol of NO^-_2 must be oxidized for the fixation of 1 mol of CO_2 (Watson, 1989). Here nitrite serves the following double function. (1) as the electron donor for oxidative phosphorylation and (2) as the electron donor for NADH synthesis.

Nitrobacter spp. are also capable of heterotrophic and mixotrophic growth. Smith and Hoare, (1968) demonstrated growth with acetate as the sole carbon and energy source and Bock (1976) found growth on pyruvate, formate and acetate in the presence of three different nitrogen sources. But most strains grow much slower and are much less efficient when grown heterotrophically than when grown

chemolithotrophically. The nitrite-oxidizing systems is inducible. Cells grown on acetate require 9 days before they regain their ability to grow chemolithotrophically. (Steinmuller and Bock, 1976). The nitrite oxidizing system is membrane-bound (Watson *et al.*, 1989, O'Kelley *et al.*, 1970). These membranes impart a brownish colour which is typically of all nitrite oxidizers.

1.1.3 Inhibition of nitrification

Nitrification of ammonia in sewage by *Nitrosomonas* and *Nitrobacter* is particularly susceptible to inhibition by a wide range of compounds, particularly thiourea and its derivatives (Tomlinson *et al.*, 1966). In order to protect this process in sewage treatment from the effect of trade effluents, it is necessary to identify acceptable dilutions at the work and the quality criteria for compounds contained there in (Watson *et al.*, 1989).

1.2 Heterotrophic nitrification

In addition to autotrophic nitrifying bacteria, many heterotrophic microorganisms can convert ammonium to nitrite and organic nitrogen compounds to nitrate. Species capable of such transformations are listed by Focht and Verstraete (1977) and include a wide range of bacteria (including actinomycetes) and fungi. Nitrification by these organisms occurs after growth has ceased and rates of nitrite and nitrate production are several orders of magnitude lower than those of autotrophs. Heterotrophic nitrification is important at temperatures, pH values and mois-

ture contents which are unfavourable for the growth of autotrophic nitrifying bacteria. Most of the transformed nitrogen (NO_2 and NO_3) in nature is reported to be due to autotrophic nitrification since the contribution of heterotrophic nitrifiers towards nitrification is very low.

1.3 Effects of nitrogen discharge and significance of biological nitrification

It was previously noted that nitrogenous compounds discharged from wastewater treatment facilities can have several deleterious effects. Although biostimulation of receiving waters was the most notable concern, other less publicized impacts can be of major importance in particular situations. These impacts include toxicity to fish life, an increase in the dissolved oxygen depletion in receiving waters, reduction of chlorine disinfection efficiency, and a reduction in the suitability of reuse (De Renzo, 1978).

1.3.1 Biostimulation of surface waters

A major problem in the field of water pollution is eutrophication, excessive plant growth and algal 'blooms' resulting from over-fertilization of rivers, lakes, and estuaries. Results of eutrophication include deterioration in the appearance of previously clear water, odour problems from decomposing algae, and a lower dissolved oxygen level which can adversely affect fish life. Eutrophication is of most concern in lakes because nutrients which enter tend to be recycled within the lake and build up over a period of time. In estuaries and oceans,

nitrogen compounds are often present in very low concentration and may limit the total biomass and types of species it contains. Thus, upwelling, which brings nutrient rich waters to the surface, may result in periodic blooms of algae or other aquatic life.

1.3.2. Toxicity

Unionized and free ammonia can adversely affect fish life in receiving waters. Since the pioneering work of Wuhrmann *et al.*,(Watson *et al.*, 1989) it has been generally accepted that the toxicity of ammonia is dependent on pH since this affects the equilibrium between the ionized (NH_4+) and unionized (NH_3) chemical species,

A slight increase in pH may cause a great increase in toxicity as the ammonium ion (NH_4^+) is transformed to ammonia in accordance with the following equation.

$$NH_4^+ + OH \longleftrightarrow NH_3 + H_2O$$

The toxicity of ammonia has been ascribed to the fact that the unionized form is readily soluble in lipid in cell membrane and so readily taken up by fish gills whereas the ionic form occurs as larger hydrated and charged entities which cannot readily pass through the charge lined hydrophobic micropores in cell membranes (Watson *et al.*, 1989,). Sublethal ammonia levels may reduce growth, damage gills and other organs and be a predisposing factor in bacterial gill disease (Burrows, 1964 Larmoyeux and Piper, 1973 and Robinette,

1973). Based on short term lethal toxicity tests, Watson et al., (1989) reported that only un-ionized ammonia is toxic to fish and that ionized ammonia has little or no toxicity.

Factors which may increase ammonia toxicity at a given pH are: greater concentrations of dissolved oxygen and carbon dioxide; elevated temperatures; and bicarbonate alkalinity (EPA, 1975). Reported levels at which acute toxicity is detectable have ranged from 0.01 $mg.L^{-1}$ to over 2.0 $mg.L^{-1}$. of molecular ammonia nitrogen (Brown and Caldwell, 1972).

The intermediate product of nitrification, nitrite (NO_2^-), is highly toxic to aquatic vertebrates (Watson *et al.,* 1989), causing threat to cultured inverebrates. The toxicity of nitrite may be due to the oxidation of hemoglobin to methemoglobin, a form incapable of transporting oxygen (Watson *et al.,* 1989).

1.3.3 Dissolved oxygen depletion in receiving waters

Ammonium can be biologically oxidized to nitrite and then to nitrate in receiving waters, by nitrification and thereby add to the oxygen demand imparted by carbonaceous materials. If either conventional biological treatment or physico-chemical treatment is utilized to provide 90 per cent BOD_5 removal, an effluent will be discharged which still contains over 100 $mg.L^{-1}$ of oxygen demand. This high level of oxygen demand may cause significant oxygen depletion in

the receiving water if insufficient dilution is available. Nitrification will reduce the total oxygen demand of the effluent to less than 40 $mg.L^{-1}$.

1.3.4 Effect on disinfection efficiency

When chlorine in the form of chlorine gas or hypochlorite salt is added to wastewater containing ammonium for disinfection, chloramines, which are less effective disinfectants, are formed. The major reactions are as follows:

$$NH_4^+ + HOCl \leftrightarrow NH_2Cl \text{ (monochloramine)} + H_2O + H^+$$

$$NH_2Cl + HOCl \leftrightarrow NHCl_2 \text{ (dichloramine)} + H_2O$$

$$NHCl_2 + HOCl \leftrightarrow NCl_3 \text{ (nitrogen trichloride)} + H_2O$$

Only after the addition of large quantities of chlorine does free available chlorine exist. If the effluent ammonia nitrogen concentrations were 20 $mg.L^{-1}$, about 200 mg. L^{-1} of chlorine would be required to complete the reactions with ammonium and organic compounds. Only rarely in wastewater treatment this level of chlorine addition (breakpoint chlorination) is used. Therefore, as a practical matter, the less effective combined chlorine residuals (monochloramine and dichloramine) must be relied upon for disinfection. This results in increased chlorine dose requirements for the same level of disinfection.

1.3.5 Water reuse

While direct wastewater for domestic water supply is not yet a reality because of public health considerations, plans for industrial reuse are being carried out in several areas. When reclaiming wastewater for industrial purposes, ammonia may need to be removed in order to prevent corrosion. Further, nitrogen compounds can cause biostimulation in cooling towers and distribution structures.

Biotreatment of wastewater utilizes the integrated application of microbiology and engineering. A wide array of biotreatment systems are in current use for industrial and domestic waste water purification. The system is based on the apparently simple process by which mixed population of microbes breakdown organic matter, using it as a source of nutrients which can be intensified and accelerated by chemical engineering techniques. Self purification of water takes place by this process in nature. Wastewater containing pollutants are brought into contact with a dense population of suitable microbes for a time sufficient enough for the breakdown and removal of pollutants to the desired level. The extent of purification depends on the treatment system adopted, its method of operation and the pollutants in the water undergoing treatment.

The bio-treatment process aims at the removal of specific pollutants by self selective microbes, but there are two major considerations, i.e., (i) Whether the cost of removal is justified by the result-

ant benefits, and (ii) What to do with the major contaminant after it has been removed.

Increasing environmental awareness in recent years of cumulative effects of pollution has led to public concern and strict legislation relating to the discharge of industrial wastes, effluents, which has led to the formulation of water quality standards by the Bureau of Indian Standards (BIS), Indian Council of Medical Research (ICMR), World Health Organisation (WHO) and American Public Health Association (APHA). Treatment of wastes, though uneconomical is essential to prevent water pollution and associated hazards.

1.4 Conventional Treatment Processes

Nitrogen in raw domestic wastewaters is principally in the form of organic nitrogen, both soluble and particulate, and ammonia. The soluble organic nitrogen is mainly in the form of urea and amino acids. Primary sedimentation acts to remove a portion of the particulate organic matter. This generally will amount to less than 20 percent of the total nitrogen entering the plant. Biological treatment will remove more particulate organic nitrogen and transform some to ammonium and other inorganic forms. A fraction of the ammonium present in the waste will be assimilated into organic materials of cells formed by the biological process. Soluble organic nitrogen is partially transformed to ammonium by microorganisms, but concentrations of 1 to 3 mgL^{-1} are usually found in biological treatment effluents (Parkin and Mc Carty, 1973). Through

these processes, an additional 10 to 20 percent of the total nitrogen is removed when biological treatment and secondary sedimentation follows primary sedimentation. Thus, total nitrogen removal for a conventional primary - secondary facility will generally be less than about 30 percent.

Primary treatment is employed to remove suspended solids and to certain extent colour and odor and to bring the pH in the neutral range. It includes screening, equalisation, neutralization, coagulation, sedimentation etc. Secondary treatment is a biological process applied to reduce BOD and COD. Dissolved solids like sugar, starch etc. can be removed by this treatment. This is important because organic matter comprises the major bulk of wastewater. Trickling filtration, activated sludge process, oxid ation ditch and oxidation ponds are some of the common secondary treatment processes.

Tertiary treatment is employed for further purification of wastewaters.

1.5 Major nitrogen removal processes

The major processes for ammonia removal are nitrification, breakpoint chlorination, (or super chlorination), selective ion exchange for ammonia removal and air stripping (ammonia stripping)

1.5.1 Biological nitrification

The principal effect of the nitrification in treatment process is to

transform ammonia-nitrogen to nitrate. The nitrification is usually used as a treatment method when requirements calls for ammonia oxidation. Nitrification can be carried out in conjunction with secondary treatment or a tertiary stage; in both cases, either suspended growth reactors (activated sludge) or attached growth reactors (such as trickling filters) can be used.

1.5.1.1 Activated sludge process

This is the most widely used biological process for the treatment of organic and industrial wastewater. A number of modifications have been done over the years which has made the process versatile and adoptable to a wide range of operational circumstances. The basic principle is that the wastewater is brought into contact with a mixed microbial population in the form of a flocculent suspension in an aerated, agitated system. Suspended and colloidal material is removed rapidly from wastewater by adsorption and agglomeration into the microbial flocs. This material and nutrients are then broken down slowly by microbial metabolism. In this process, part of the nutrients is oxidized to simple products such as carbon dioxide. Part of the microbial mass is also broken down by endogenous respiration. When the desired level of treatment has been achieved, the flocculent microbial mass known as the 'sludge' is separated from the treated wastewater by gravity settling referred to as 'clarifying/settling/sedimentation '. Most of the settled sludge is reused in the aeration stage to

maintain desired level of sludge concentration needed for effective treatment and to act as a microbial inoculum. Part of the sludge is removed for disposal which is known as waste or surplus activated sludge.

1.5.1.2 Advanced activated sludge process

The most recent advances in activated sludge process is the increase in rate of oxygen transfer from the gas phase by increasing the partial pressure of oxygen in the gas phase. This increases the saturation concentration of DO and the concentration of d riving force of mass transfer from the gas-liquid interphased into bulk of the liquid.

To increase the partial pressure of oxygen, two methods are usually adopted. (1) The "deep shaft process" (by increasing the total pressure of the system) and (2) the pure oxygen process, (by increasing the proportion of oxygen in the gas phase by enrichment or replacement with pure oxygen). The transfer rate achieved in the deep-shaft process is ten times that of conventional activated sludge plants and the contact time between the gas bubbles and the liquid is very much higher. The pure oxygen process is based on the fact that oxygen availability is a key feature control in general and the activated sludge process in particular.

1.5.1.3 Trickling filters

Here the wastewater is allowed to trickle or percolate through a

stationary bed made of stones, gravel, brick ballasts etc. When the water comes through the nozzle through the influent pipe, the sprinkler gets rotated and the water will be sprinkled all over the bed. The surface in contact with the nutrient rich wastewater containing microbes will develop a biologically active slime layer. Suspended and collodial materials in the wastewater are agglomerated and adsorbed into the microbial slime film. The dissolved oxygen is transferred to the microbial film and the oxygen and nutrients from the liquid diffuse into the microbes in the slime. In engineering terms, fixed bed medium system is a three-phased fixed-bed biological reactor for contacting gas, liquid and microbes. They are stable, simple in design and fairly fool-proof in design (Unni, 1995)

1.5.2 Breakpoint chlorination

Breakpoint chlorination (or superchlorination) is accomplished by the addition of chlorine to the wastestream in an amount sufficient to oxidize ammonia-nitrogen to nitrogen gas. After sufficient chlorine is added to oxidize the organic matter and other readily oxidizable substances present, a stepwise reaction of chlorine with ammonium takes place. The overall theoretical reaction is as follows:

$$3Cl_2 + 2NH_4^+ \longrightarrow 6HCl + 2H^+ + N_2$$

In practice, approximately 10 $mg.L^{-1}$ of chlorine is required for every 1 $mg.L^{-1}$ of ammonia-nitrogen. In addition, acidity produced

by the reaction must be neutralized by the addition of caustic soda or lime. These chemicals add greately to the total dissolved solids and result in a substantial operating expense. An important advantage of this method is that ammonia-nitrogen concentration can be reduced to near zero in the effluent. But the effect of breakpoint chlorination on organic matter is uncertain and nitrite is not removed by this method.

1.5.3 Selective ion exchange for ammonium removal

Selective ion exchange for removal of ammonium from wastewater can be accomplished by passing the wastewater through a column of clinoptilolite, a naturally occuring zeolite, which has a high selectivity for ammonium ion. Regeneration of the clinoptilolite is undertaken when all the exchange sites are utilized and breakthrough occurs. Filtration prior to ion exchange is usually required to prevent fouling of the zeolite. Ammonia removal of 90-97 percent can be expected. Nitrate and organic nitrogen are not affected by this process.

1.5.4. Air stripping for ammonia removal

Ammonia is in the form of a gas which dissolves in water to an extent controlled by the partial pressure of the ammonia in the air adjacent to the water. Reducing the partial pressure causes ammonia to leave the water phase and enter the air. Ammonia removal from

waste water can be effected by bringing small drops of water in contact with a large amount of ammonia-free air. This physical process is termed 'desorption', but the common name is ammonia stripping.

In order to strip ammonia from waste water, it must be in the molecular form (NH_3) rather than the ammonium ion (NH_4^+) form. This is accomplished by raising the pH of the waste water to 10 or 11, usually by the addition of lime. Again, nitrite, nitrate and organic nitrogen are not affected. The principal problems with ammonia stripping are its inefficiency in cold weather, required shut down during freezing conditions, pertinant to temperate countries and formation of calcium carbonate scales in air stripping tower.

1.6 Application of biological nitrification in municipal wastewater treatment

The application of biological nitrification in municipal waste water treatment is particularly applicable to those cases where an ammonia removal requirement exists, without need for complete nitrogen removal, (De Renzo, 1978). After completion of secondary waste treatment, about 85-90% of the nitrogen is in the form of ammonium (5-50 mg $N.L^{-1}$) with the remainder present as organic nitrogen, only trace amount of nitrite or nitrate is found (Mc Carty and Haug, 1971). Rapid removal of nitrogen from sewage involves separate treatments for reduction of BOD, nitrification and denitrification. Though all processes can occur simultaneously in a trickling filter bed in an activated sludge tank (Wuhrmann,

1964), it is the nitrification process that is rate limiting and thus necessitates a long retention time.

Separate carbon and nitrogen oxidation processes minimizes wash out of the nitrifiers and can be operated at shorter detention time, lower MLSS and sludge age (Rimer and Wood ward, 1972). The combined carbon oxidation -nitrification processes generally have low population of nitrifiers due to a high ratio of BOD_5 to total kjeldahl nitrogen (TKN) in the influent. The bulk of the oxygen requirement for this process comes from the oxidation of organics. But in separate stage nitrification, there is a lower BOD_5 load relative to the influent ammonia load. As a result, a higher proportion of nitrifiers is obtained resulting in higher rates of nitrification. The bulk of the oxygen requirements in this nitrification state derive from ammonia oxidation. Thus, to obtain separate stage nitrification, pretreatment is required to lower the organic load or BOD_5/TKN rates in the influent to the nitrification stage.

Eckenfelder (1976) calculated a sludge retention time of 2 days for adequate nitrification of domestic wastewater (10 -50 mg $N.L^{-1}$). Batch studies by Wong -Chong and Loehr (1975) showed that ammonium oxidation was the rate limiting step with a maximum removal rate of about 80 mg/N/litre/hour.

Both the combined carbon oxidation and separate stage nitrification processes can be further subdivided into suspended growth and attached

growth processes. Suspended growth processes are those which suspend the biological solids in a mixed liquor by some mixing mechanism. A subsequent clarification stage is required for returning these solids to the nitrification stage. Attached processes, on the other hand, retain the bulk of the biomass in the media and therefore do not require a solids separation stage for returning the solids to the nitrification reactor. In separate stage processes operated in the attached growth mode, a clarification step may not be required since solids synthesis is low and the sloughed solids are often low in concentration.

The bioreactors for effluent treatment works under non-sterile conditions, the concentrations of substrate are atleast one order of magnitude lower, and the volume handled are generally very large. Bioreactors are engineered under non-sterile conditions essentially by providing the right environmental conditions for the preferred microbial population to dominate over the unwanted competitors. The variables available for control include,

* Dissolved oxygen
* Temperature
* Biomass retention time
* Hydraulic residence time
* Hydrodynamic condition in the reactor
* Concentration of solids, volatile acids, dissolved gases, pH, alkalinity.

1.6.1 Combined carbon oxidation- nitrification in suspended growth reactors.

In activated sludge process, combined carbon-oxidation-nitrification takes places whereby the BOD is considerably lowered.

1.6.2 Attached growth reactors

Trickling filters used in waste water treatment is an example of attached growth in municipal wastewater treatment.

1.6.3 Separate stage nitrification with suspended growth process

By placing a carbon removal system ahead of the separate nitrification stage, the sludge would be enriched with nitrifiers as opposed to the marginal population present in combined carbon-oxidation-nitrification system.

1.6.4 Separate stage nitrification with attached growth process

The types of attached growth processes have been employed for separate stage nitrification. The differences lie in the type of medium provided for biological growth. The three types of processes are the trickling filter, the rotating biological disc and the packed bed reactor (PBR).

1.6.4.1 Nitrification with trickling filters

Initially, the two stage trickling filtration process was developed to increased the removal of organics in the effluents from the high rate trickling filters. Later, it was found that due to some operating conditions, the second stage produced a well nitrified effluent (Sor-

rels, and Zeller, 1956). Plastic media were used as the filter bed and the influent BOD_5, SS and ammonia -nitrogen values should range from 15-20, 15-20 and 8-18 respectively. BOD_5 removal in a pre-treatment stage is a requisite, which gave BOD_5 /TKN ratio as 1.1 (Duddles *et al.,* 1974 ; Duddles and Richardson, 1973).

1.6.4.2 Nitrification with rotating biological disc process

The RBD process consists of a series of large diameter plastic discs, which are mounted on a horizontal shaft and placed in a concrete tank. The discs are slowly rotated while approximately 40 percent of the surface area is immersed in the wastewater to be treated. Shortly after start up, organisms present in the wastewater begin to adhere to the rotating surfaces and grow until the entire surface area is covered with a layer of aerobic biomass. In rotation, the discs pick up a thin film of wastewater, which flows down the surface of the discs and absorb oxygen from the air. Shearing forces exerted on the biomass as it passes through the wastewater strip excess growth from the discs into the mixed liquor. The effluent from the secondary treatment stage can be nitrified which have less suspended solids by RBD.

1.6.4.3 Nitrification with packed bed reactors (PBR)

Packed bed reactors for nitrification are a comparatively recent development, having progressed from laboratory stage to pilot scale and commercial availability over a period of last two decades. A PBR consists of a bed of media upon which biological growth occurs.

Wastewater is distributed evenly across the floor of the PBR by baffles, nozzles or strainers. Similar to the way backwash water is distributed in rapid sand filters. Several means have been employed for supplying the necessary oxygen for nitrification. The earliest work used injection of air into the feed line entering the chamber (Mechalas *et al.*, 1970). A subsequent pilot-scale investigation used a similar procedure excepting that the air was distributed across the PBR floor (Young *et al.*, 1975). In another procedure, the liquid was pre oxygenated in a reaction chamber prior to entry into the PBR.

Several types of media have successfully performed in the PBR including 2.5 to 3.8cm stones, gravel, anthracite and plastic (Haug and Mc Carty, 1971, 1972, Mc Harness *et al.*, 1975, Gasser *et al.* 1974 and Young *et al*, 1975).

1.7 Pre-treatment for separate stage nitrification

In order to obtain a separate stage nitrification process, the influent to that process must be pretreated to remove organic carbon to ensure a significant fraction of nitrifiers in the biomass. Alternatives include chemical treatment in the primary, activated sludge, roughing filters and trickling filters. Treatment with activated carbon can also be effective when used in conjunction with primary chemical addition.

In the activated sludge system of any sewage treatment facility as Whurmann (1964) points out, bacterial activity is a function of the het-

erotrophs and not that of nitrifiers. The extreme differences in the population dynamics of heterotrophs and chemolithotrophic nitrifiers favour faster regeneration of heterotrophs resulting in the wash-out nitrifiers. In the activated sludge system, the constant st irring being carried out for aeration exposes the existing nitrifiers to light making it inefficient, as has been pointed out by Diab and Shilo (1989) and Vrba (1990) that large scale inhibition of ammonia is predominant in all wavelengths of light.

In a conventional trickling filter, as the sewage percolates towards lower layers, the high heterotrophic activity in the richly aerated top zones tend to consume the bulk of the oxygen, generating an almost anoxic environment towards the bottom. It has been reported that ammonia oxidation stops at ranges from 1.1 to 6.2 millimoles O_2 (Sugahara *et al.,* 1974. Jorgenson *et al.,*1984).

Studies have shown that the natural build up of nitrifiers in any system is very slow. This, coupled with the array of drawbacks experienced in activated sludge and trickling filter systems suggest the need for developing a novel device for accommodating large quantity of nitrifiers in a small unit volume to carry out nitrification of sewage at extremely rapid rate. It has been observed that *Nitrosomonas europaea* immobilized in calcium alginate (Van Ginkel *et al.,* 1983) and *Nitrosomonas* and *Nitrobacter* attached to particles of bentonite, cal-

cium carbonate, amberlite and fish pond sediment (Diab and Shilo, 1988) exhibited enhanced nitrification compared to freely suspended and detached cells.

These observations throw light on the scope of developing bioreactors based on immobilized cells of nitrifying bacteria on a suitable substratum.

CHAPTER 2

DEVELOPMENT OF NITRIFYING CONSORTIA

Chapter 2

DEVELOPMENT OF NITRIFYING CONSORTIA

2.1 Introduction

The aerobic, chemolithotrophic nitrifying bacteria as per conventional procedures can be enriched and isolated from treated sewage by inoculating an aliquot of the sample to a desirable medium to the final concentration of 1% (v/v) and by monitoring the production of nitrite from ammonia in the case of ammonia oxidizers and nitrite oxidizers (Prosser and Cox, 1982). But since nitrifiers are slow growers, it cannot be isolated directly from the source material and there fore enrichment cultures must be made (Prosser and Cox, 1982; Watson *et al.,* 1958). If the initial number of nitrifiers in the source material happens to be so small, there is all the possibilities for loss of organisms while sampling. In such instances, it is advisable to develop an enrichment system from which nitrifiers can be easily isolated either by plating out or by serial dilution techniques.

It has been observed by Kawai *et al.,* (1964) that nitrifiers in the filter bed of a conditioned biological filter can support hundred times more nitrifiers than that are available suspended in water.

The sand grains of a conditioned biofilter could then be a rich source of chemolithotrophic nitrifiers (Ramachandran and Singh, 1996). A biological filter system removes various nutrients from the water through bacte-

rial oxidation or transform them into other forms which are less toxic than the original species. These bacteria use the filter media or materials as a point of attachment forming biofilms so that they are not washed away from the surface (Liao and Mayo, 1972).

Biological treatment processes using attached cultures are attracting interest as an alternative to conventional treatment processes (suspended activated sludge process, for example) considering the advantages of small reactor volumes and high flowrate. Moreover, the immobilization of microorganisms can prevent them from being washed out, and a high sludge age can be more easily maintained (Moreau *et al.*, 1994).

The water circulates between the culturing vessel and filter bed, making it possible for the aerobic bacteria to carry out their essential role in eliminating toxic forms of nutrients (Hirayama, 1974). The passage of water through the sand bed of the filter maintain conditions that are favourable to aerobic bacterial growth. In this system, the filter bed function to increase the surface area for bacterial attachment.

This principle is utilized for the easy recovery of nitrifying bacteria from treated sewage in the present study by setting up a biological filter as the pre-enrichment of nitrifiers.

An air lift pump is the most trouble free means of moving water through a biological filter. An air lift is essentially a central pipe through which air-water mixture is moved upward. In culture applications, part of the pipe

extends below the sub gravel filter plate, a portion of it extends above the filter plate and the air is injected at the lower end of the pipe. As long as air is injected, the air-water mixture is spilled at the top of the pipe (Spotte, 1979).

A culture system is considered to be conditioned when the nitrifying bacteria in its biological filter can rapidly oxidize all the incoming ammonia to nitrate with no significant appearance of the intermediate nitrite (Bower and Turner, 1983). These sand grains of the filter bed of a conditioned biofilter could then be a rich source of chemolithotrophic nitrifiers which can be used for their further enrichment and isolation.

Nutritional requirements of nitrifying bacteria have been reviewed by Painter (1970) and Walker (1975). All media contain magnesium, calcium, phosphate, a trace quantity of iron and some incorporated other trace elements. Ammonium is usually provided as ammonium sulphate or ammonium choride and nitrite as the sodium or potasium salt. Growth of ammonia oxidizers is complicated by acid production which necessitates alkali addition such as sodium hydroxide as sodium carbonate. Ammonia oxidation is photosensitive (Hooper and Terry, 1974) and the maximum growth requires incubation in the dark . Hence the pH of the growth medium is adjusted to the range of 7.0 - 8.0 and cultures are incubated aerobically in dark at 25°C - 30°C.

Even though several media are found to have been used by earlier work-

ers for enrichment and isolation of nitrifiers, an appropriate one for the tropical organisms has not been recommended by any. Therefore in the present study, twelve media for ammonia oxidizers and six for nitrite oxidizers have been screened for selecting the best one for the enrichment and isolation of nitrifiers. The growth of ammonia oxidizers is assessed by the production of acid, nitrite and/or by the removal of ammonium. Growth of nitrite oxidizers is determined by the depletion of nitrite and is usually followed by spot tests for ammonium, nitrite and nitrate. Lewis and Pramer (1958) were the first to use particle-free media for the isolation of *Nitrosomonas* species. They prepared enrichment cultures either by successive transfers of fully grown cultures to fresh medium or by the addition of more ammonium ion after the oxidation of the initial amount present was exhausted. Using the latter method, they found an initial increase in heterotrophs, a decrease in numbers following the first addition of fresh ammonium and relatively constant numbers following the second addition. Ammonia oxidizers were found to outnumber heterotrophs by a factor of 50.

Although no organic compounds are added to the enrichment media, the nitrifying bacteria excrete organic compounds that can support the growth of heterotrophic bacteria , which usually outnumber the former in enrichment cultures because the generation time of the nitrifying organisms is 8 - 24 h, even under optional conditions (Watson *et al.,* 1981). For these reasons, isolation of nitrifying bacteria is a difficult and time con-

suming task. The study of biological aspects of nitrification is limited by difficulties in obtaining and maintaining pure cultures of nitrifying bacteria because of their slow growth and poor cell yields. Much early work was carried out with mixed cultures. Also, little is known of the adaptive ability of nitrifiers and the symbiotic effects of a mixed population on nitrifiers (Sharma and Ahlert, 1977). Pan (1971a) as part of her work on the basis of obligate autotrophy, showed that both *Nitrosomonas europaea* and *Nitrobacter agilis* grow favourably in conjunction with *Candida albicans*. Also, if pure cultures of nitrifiers are used for developing bioreactors for sewage treatment, the effluent itself may contaminate the immobilized pure cultures during the process. In view of all the above discussed factors, an integrated approach on culturing nitrifying bacteria and the associated heterotrophs as a mixed culture consortia or nitrifying consortia is justified in the light of our present understanding of applying nitrifiers in the field of sewage treatment .

Enrichment culture can be made from sewage with 1% inoculum and incubated in the dark at 25 - 30°C for a period of 1- 4 months. The oxidation of ammonia can be detected by an increase in the nitrite or nitrate concentration in cultures. Since lowering of pH may also be indicative of nitrification, this is taken as presumptive evidence for nitrification and a nitrite analysis is performed. If no nitrite was detected, a nitrate test (Strickland and Parsons, 1972) is done to determine if the culture contained both ammonia and nitrite oxidizers. It is seen that some organisms,

such as *Nitrospina gracilis* cannot tolerate more than 1mM nitrite in the medium and when higher concentrations of nitrite are used, *Nitrobacter winogradskyi* usually dominate the enrichment cultures. Thus, it is important to use low as well as high concentration of nitrite when enrichment cultures are made. Techniques for measuring growth for nitrifiers differ significantly from those used for heterotrophic organisms. Measurement of nitrifier biomass by absorbance techniques is difficult because of the low growth yields. The use of counting chambers to determine total cell concentration is tedious while viable cell counts are inaccurate and require several months of incubation. The availability of automated techniques, however facilitates measurement of substrate and product concentration, and growth can also be measured by uptake of ^{14}C labelled CO_2 or bicarbonate. Consequently, growth of nitrifying bacteria is usually measured by either substrate utilization or product formation (Prosser, 1989) In the present study depletion of ammonium and build up of nitrite along with the change of pH to acidic range was considered as indication of the growth of ammonia oxidizers and disappearance of nitrite and/or build up of nitrate was considered indicative of the growth of nitrite oxidizers.

Nitrifying bacteria can be grown in large volume (20 liters) culture; the ammonia oxidizing bacteria by semicontinuous culture, and the nitrite oxidizing bacteria by continuous culture. But these large mass cultures should be inoculated with at least 1 litre of a turbid culture. If a heavy inoculum is not used, the culture either will not grow at all or will undergo

a prolonged lag period (Watson *et al.,* 1981) In view of these disadvantages, a batch culture for enrichment and cell biomass generation was developed in the present study.

In nature, the growth rate of nitrifying bacteria is controlled by various parameters such as substrate concentration, pH, temperature and oxygen tension. Most strains of nitrifying bacteria grow optimally at substrate concentration of 1.25mM, pH between 7.5 and 8.0 and temperature of 25-30°C (Prosser and Cox, 1982). The maximum specific growth rate of nitrifiers is a strong function of the prevailing temperature and pH. However, many environmental factors with suboptimal condition still support the growth of nitrifying bacteria (Watson *et al.*, 1981).

Many contradiction appear in literature on the effect of pH on respiration rate of *Nitrosomonas* and for that matter a variety of pH curves have been published (Loveless and Painter, 1968). Typical of the extreme cases is that of Engel and Alexander (1958) which is relatively flat between pH 7 and 9, falling to about 50% values at 6.2 and 9.6 and that of Mayer ihof (1917) which has a sharp peak at 8.6 and 50% values at 7.9 and 9.3. The optimum temperature of *Nitrosomonas* isolated by Loveless and Painter (1968) was determined at 30°C. Nitrification reactions follow the Van't Hoff-Arrhenius law upto 30°C (Metcalf and Eddy, 1973). Thus, nitrification proceeds better in warmer seasons or climates. The overall optimum temperature for the growth of nitrifying bacteria appears to be in the range 28°C - 36°C although optimum temperature upto 42°C have been reported

for *Nitrobacter* (Painter, 1970). The growth constants of nitrifying bacteria are affected greatly by temperature. Knowles *et al.,* (1965) have estimated that the temperature co-efficient (increase in maximum specific growth rate constant) for *Nitrosomonas* was 9.5% per degree centigrade. Srna and Baggaley (1975) measured the effect of rapid temperature changes on nitrification via batch studies on a marine nitrifying filter system. Their studies revealed that a 4°C increase in temperature caused about a 50% increase in the rate of nitrification. Barritt (1933) found that the thermal death point of a pure culture of *Nitrosomonas* was between 54° and 58°C. Little or no growth of nitrifying bacteria is expected below 4°C (Buswell *et al.*, 1954, Painter[12], (1970).

Meyerhof (1917); Engel and Alexander (1958); Downing *et al.,* (1964); Hopwood and Downing (1965); Knowles *et al.,* (1965); Downing and Knowles 1966; Jenkins *et al.,* (1967); Loveless and Painter (1968); Hall (1974) and Painter and Loveless (1983) have shown that optimum nitrification rate is activated at a pH in the range 7.0 - 8.2 and is an increasing function of temperature in the range of practical interest (less than 30°C). However according to Engel and Alexander (1958), the pH of 8.5 is the optimum for autotrophic nitrification.

Both *Nitrosomonas* sp and *Nitrobacter* sp are sensitive to their own substrate and more so to the substrate of the other (Painter, 1970); According to Anthonisen (1974), the degree of inhibition depends upon the ammonia - ammonium and the nitrite -nitrous acid equilibria. Normal ammo-

nia and nitrite ion concentration in domestic waste waters are not in the inhibiting ranges (Schwinn and Dickson, 1972). These differing opinions and experiences by earlier workers necessitated the present study to determine (a) optimum substrate concentration, (b) optimum pH and (c) optimum temperature at which nitrifier displayed their full nitrifying potential.

Aeration of cultures of nitrifying bacteria is important not only to provide oxygen but also for the supply of carbon dioxide, unless carbonate is used to neutralize acid production (Prosser, 1982) Saturation constants for oxygen uptake are in the range 0.3 to 1 mg O_2 L^{-1}, with *Nitrobacter* sp. being slightly more sensitive to oxygen limitation than *Nitrosomonas* sp. The overall stoichiometric reactions in the oxidation of ammonia to nitrate can be written as follows (EPA, 1975).

$$NH_4^+ + 1.5\ O_2 \rightarrow 2H^+ + H_2O + NO_2^- + 58 - 84\ \text{k.cal}\ (1)$$

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^- + 15.4 - 20.9\ \text{k. cal}\ (2)$$

The stoichiometric quantities of oxygen required according to the above two equations are: 3.43 mg for oxidizing 1mg NH_4^+ - N and 1.14 mg for oxidizing 1 mg NO_2^- - N (Sharma and Ahlert, 1977).

The amount of air supplied to the reactor is an important design factor because energy consumption in air supply accounts for a high percentage of the total energy consumption in a wastewater treatment system. Keeping dissolved oxygen at a saturation level would ensure good nitrification, but it does, in turn, cost a lot. It is essential from the view point of process

economy to know the effect of low DO on nitrification and the acceptable DO (Hanaki *et al.*, 1990). Since it is believed that the extent of aeration also will be affecting the nitrifying potential, a series of experiments were carried out to assess the volume of air to be passed through for achieving maximum nitrification.

According to Watson *et al.,* (1981) liquid stock cultures of nitrifying bacteria are stored in the dark at 15°C and transferred every 4-6 months. Stock cultures of both ammonia and nitrite oxidizing bacteria are maintained in appropriate medium. Additional ammonia is added when the initial amount is exhausted. The concentration of nitrite in the culture is analysed periodically and increments of nitrite are added in the case of nitrite oxidizers when needed. Prior to subculturing, the storage cultures should be incubated at 25°C for several days (Watson *et al.,* 1981).

To facilitate the development of bioreactors for nitrifying sewage it is essential to have suitable nitrifying bacterial cultures preferably from sewage; mass culture them and make them available for further processes.

This chapter thus deals with the achievements made in

1. pre-enrichment of nitrifying bacteria in a biological filter;
2. selection of suitable media for the primary and secondary enrichment of nitrifiers;
3. development of nitrifying consortia in the selected media;

4. determination of optimum substrate concentration,pH, temperature and rate of air flow required for the consortia;
5. determination of nitrifying potential of the consortia (unit activity concept) and
6. maintenance of the nitrifying consortia.

2.2 Materials and Methods

2.2.1 Pre-enrichment of nitrifying bacteria in a biological filter

In a 10 litre plastic basin, a sand based *in situ* biofilter was set up with an air-lift pump operated by an aquarium aerator to facilitate recirculation of water through the filter bed (Figure2-1). To construct this biofilter, acid (O.1N HCl) washed coarse sand was taken in the inner small basin to a thickness of 3 cm over which a plastic funnel was kept inverted. A fritted glass bulb which functioned as the air sparger connected to an air tubing was inserted into the funnel through the bottom, both together functioning as the air lift pump. The space between the funnel and the small basin was filled with the above acid washed coarse sand leaving the tapering end of the funnel above the grains. The air tubing was connected to an aerator through an on-line sterilizable polypropylene air filter (Laxbro make) filled with cotton. The filter can be detached and sterilized as and when required.

The basin was filled with treated sewage and air lift pump was operated through a timer. Once in every 24 to 48 hours, NH_4^+-N, NO_2^--N, NO_3^-–N were estimated following Strickland and Parsons (1968) and APHA (1989). (Figure 2-6).

When the pH dropped to 5.5, it was adjusted to 8.0 using 10% sodium carbonate solution. When ammonia got exhausted, aliquots of ammonium sulphate was added to the final concentration of 0.3192g NH_4^+–N.L^{-1} to the treated sewage in the filter. The system was moni-

tored for attaining sufficient enrichment of nitrifiers in the filter bed so that further enrichment and isolation of the organism could be achieved early.

2.2.2 Scanning Electron Microscopic demonstration of adhesion of nitrifiers on sand grains in the biological filter.

A few grains from the conditioned biological filter were gently washed with sterile medium and fixed on 3% v/v glutaraldehyde for 24 hours at 4°C. The glutaraldehyde was then decanted gently without disturbing the grains. It was then passed through different grades of acetone such as 30%, 50%, 70%, 90%, 95% and dry (100%) acetone maintaining in each grade for about 15 minutes. The fixed and dehydrated grains were preserved at 4°C in dry acetone till used.

The sand grains were air dried and placed on EM stubs and gold coated for three minutes in a sputter coater. They were observed in a Scanning Transmission Electron microscope (H600, Hitachi Ltd., Japan) and photographed.

2.2.3 Selection of suitable media for the primary and secondary enrichment of nitrifiers.

Nitrifying bacteria can be isolated from sewage by enrichment techniques employing a variety of media formulated by the earlier workers. Eighteen such media were selected, out of which twelve were for ammonia oxidizers and six for nitrite oxidizers.

Media for ammonia oxidizing bacteria

Media Composition

1. Medium I : Medium of Aharon Abelovich (1987)

Na_2HPO_4	13.5g
KH_2PO_4	0.7g
$MgSO_4.7H_2O$	0.1g
$NaHCO_3$	0.5g
$CaCl_2.2H_2O$	0.18g
$FeCl_3.6H_2O$	0.014g
$(NH_4)_2S0_4$	0.5g
Distilled water	1000 mL
pH	8.2-8.4

pH was adjusted to 8.2-8.4 using a sterile K_2CO_3 solution

2. Medium II: Medium of Soriano and Walker (1968)

$(NH_4)_2 S0_4$	500 mg
$MgSO_4.7 H_2O$	40 mg
$CaCl_2.2H_2O$	40 mg
KH_2PO_4	200 mg
Ferric citrate	0.5 mg

Phenol red		0.5 mg
Distilled water	:	1000mL

pH was adjusted using sterile K_2CO_3 solution

3. Medium III : Medium of Watson (1971)

$(NH_4)_2 SO_4$	130 mg
$MgSO_4. 7H_2O$	200 mg
$CaCl_2. 2H_2O$	20 mg
K_2HPO_4	87 mg
Chelated iron	1 mg
(13% Geigy chemical)	
$Na_2MoO_4.2H_2O$	100 mg
$MnCl_2.4H_2O$	200 mg
$CaCl_2. 6H_2O$	2 mg
$CuSO_4.5H_2O$	20 mg
$ZnSO_4.7H_2O$	100 mg
Distilled water	1000 mL
pH	8.0

pH was adjusted to 8.0 using sterile K_2CO_3 solution.

4. Medium IV:Medium of Watson *et al.*, (1971)

$(NH_4)_2SO_4$	2000 mg
$MgSO_4.7H_2O$	200 mg
$CaCl_2.2H_2O$	20 mg
K_2HPO_4	15.9 mg
Chelated Iron	mL
(13% Geigy chemical)	
$Na_2MoO_4.2H_2O$	100 mg
$MnCl_2.4H_2O$	200 mg
$CaCl_2.6H_2O$	2 mg
$ZnSO_4.7H_2O$	1000 mL
pH	8.0

pH was maintained at 8.0 using sterile K_2CO_3 solution.

5. Medium V: Medium of Matulewich Strom and Finstein (1975)

$(NH_4)_2SO_4$	500 mg
$MgSO_4.7H_2O$	50 mg
$CaCl_2.2H_2O$	20 mg
K_2HPO_4	500 mg
$Na_2MoO_4.2H_2O$	2.4 Mg
Trace metal mix	1 mL

No.44

NaCl	:	500 mg
$KHCO_3$	:	20 mg
Distilled water		1000 mL
pH		8.0

pH was maintained at 8.0 using sterile K_2CO_3 or Na_2CO_3 solution.

6. Medium VI: Medium of Watson and Mandel (1971)

$(NH_4)_2SO_4$	1.7g
$CaCl_2.2H_2O$	20 mg
$MgSO_4.7H_2O$	200 mg
Chelated Iron (Atlas powder)	1 mL
$Na_2MoO_4.2H_2O$	100 mg
$MnCl_2.4H_2O$	200 mg
$CoCl_2.6H_2O$	2 mg
$ZnSO_4.7H_2O$	100 mg
K_2HPO_4	15 mg
$CuSO_4.5H_2O$	20 mg
Distilled water	1000 mL
pH	8.0

pH was maintained at 8.0 using sterile K_2CO_3 solution.

7. Medium VII: Medium for *Nitrosomonas europaea* Schmidt strain

Na_2HPO_4		93.6 mM
KH_2PO_4		6 mM
$NaHCO_3$		6 mM
$MgCl_2$	:	2.03 mM
$CaCl_2$		0.13 mM
$(NH_4)_2SO_4$		20 mM
Distilled water		1000 mL
pH		8.0

pH was maintained at 8.0 using sterile K_2CO_3 solution

8. Medium VIII: Growth Medium-19 IMTECH Microbial Type collection Catalogue

$(NH_4)_2SO_4$	:	23.5 mg
KH_2PO_4		20 mg
$CaCl_2.2H_2O$		4 mg
$FeSO_4.7H_2O$	:	0.05 mg
$Na_2EDTA.7H_2O$		0.05 mg
Phenol red		0.05 mg
Distilled water		100 mL
pH		8.0

9. Medium IX: Winogradsky Medium modification 2 (Rodina 1972)

$(NH_4)_2SO_4$	:	3 g
K_2HPO_4		0.5 g
$MgSO_4.7H_2O$		50 mg
$CaCl_2.6H_2O$	:	4 mg
Iron chelate	:	100 mg
Cresol red		50 mg
Double distilled water	:	1000 mL

pH of the medium is adjusted to 8.0 using a sterile solution of K_2CO_3.

10. Medium X: Winogradsky Medium modified Rodina (1972)

$(NH_4)_2SO_4$		2 g
K_2HPO_4		1 g
NaCl		0.5 g
$MgSO_4.7H_2O$	:	0.5 g
$MnSO_4.4H_2O$		trace
$Fe_2(SO_4)_3.9H_2O$		trace
Distilled water	:	1000 mL
pH		8.5

11. Medium XI: Medium for *Nitrosomonas europaea* (Anderson and Lewine, 1986)

$(NH_4)_2SO_4$		3 g
K_2HPO_4		0.5 g
$MgSO_4$		0.05 g
$CaCl_2.2H_2O$	:	0.004 g
Chelated Iron		
(Sequestrene		
138 Fe		0.1 mg
Ciba Geigy)		
Cresol red		0.05 mg
Distilled water		1000 mL
pH		8.0

12. Medium XII: Medium for nitrifying bacteria (Lewis and Pramer, 1958)

Na_2HPO_4	:	13.5 g
KH_2PO_4		0.7 g
$MgSO_4.7H_2O$		0.1 g
$NaHCO_3$		0.5 g
$(NH_4)_2SO_4$		2.5 g
$FeCl_3.6H_2O$		14.4 mg

$CaCl_2.2H_2O$	18.4 mg
Distilled water	1000 mL
pH	8.0

Media for nitrite oxidizing bacteria

13. Medium 1. Medium of Aleem and Alexander (1958)

KNO_2	:	300 mg
$MgSO_4.7H_2O$		187.5 mg
$CaCl_2.2H_2O$		12.5 mg
KH_2PO_4		500 mg
K_2HPO_4		500 mg
$FeSO_4.7H_2O$		10 mg
$KHCO_3$		1500 mg
NaCl		187.5 mg
Distilled water		1000 mL
pH		8.0

14. Medium 2. Medium for soil and fresh water nitrite oxidizers (Watson and Mandel, 1971)

$MgSO_4.7H_2O$	100 mg
$CaCl_2.2H_2O$	5 mg
Chelated Iron	
(Atlas powder)	1 mg

$Na_2MoO_4.2H_2O$	:	25 mg
$MnCl_2.4H_2O$		50 mg
$ZnSO_4.7H_2O$		25 mg
$CoCl_2.6H_2O$		0.6 mg
$CuSO_4.5H_2O$	:	5 mg
K_2HPO_4	:	3.4 mg
$NaNO_2$	:	690 mg
Distilled water		1000 mL
pH		7.5

pH of the medium was adjusted to 7.5 after antoclaving.

15. Medium 3. Medium 20 IMTECH type culture collection

$NaNO_2$	247 mg
KH_2PO_4	200 mg
$CaCl_2.2H_2O$	0.5 mg
$FeSO_4.7H_2O$	0.5 mg
Phenol red	0.5 mg
pH	8.0

16. Medium 4. Medium for nitrite oxidizers Winogradsky modification

Na_2HPO_4	13.5 g
$MgSO_4.7H_2O$	0.1 g

$NaHCO_3$		0.5 g
$CaCl_2.2H_2O$		18.4 mg
$FeCl_3.6H_2O$		14.4 mg
KH_2PO_4	:	0.7 g
$NaNO_2$	:	2.5 g
Distilled water	:	1000 mL
pH		8.0

17. Medium 5. Winogradsky medium

$NaNO_2$	1 g
Na_2CO_3	1 g
NaCl	0.5 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.3 g
$FeSO_4.7H_2O$	0.4 g
Distilled water	1000 mL
pH	8.0

18. Medium 6. Winogradsky medium modified Rodina (1972)

$NaNO_2$	2 g
K_2HPO_4	1 g
$MgSO_4.7H_2O$	0.5 g

NaCl		0.5 g
$MnSO_4.4H_2O$	:	Trace
$Fe_2(SO_4)_3.9H_2O$	:	Trace
Distilled water		1000 mL
pH		8.0

2.2.4 Preparation of media

The media were prepared as 100mL aliquot in 250mL Erlenmeyer flasks and autoclaved at 15 lbs for 15 minutes. The pH was maintained as suggested for each medium using sterile solution of 10% Na_2CO_3.

2.2.5 Inoculation of media

One spatula full of filterant grains (3.8g) from the middle portion of the filter bed of the conditioned biological filter in the laboratory, as discussed in section 2.2.1, was aseptically transferred to 100mL each of the above media prepared. All flasks were incubated in dark over a rotary shaker (Orbitek) at 100 rpm.

2.2.6 Maintenance of inoculated media

Periodically, drop in pH, depletion of ammonia and build up of nitrite in ammonia oxidizing media and depletion of nitrite in nitrite oxidizing media were monitored following Strickland and Parsons (1968) and APHA (1989). pH of the media were periodically adjusted to the desired level using sterile sodium carbonate solution.

When the substrates, i.e, ammonia for ammonia oxidizing media and nitrite for nitrite oxidizing media got depleted, fresh aliquots of ammonia and nitrite were added aseptically. The total consumption of ammonia, build up of nitrite by ammonia oxidizers and consumption of nitrite by nitrite oxidizers was calculated and recorded. This exercise was continued till no more consumption of substrates was observed.

At this point 1 mL culture and half the spatula of filterant grains (1.250g) from this primary enrichment sets were transferred to another set of the 18 fresh media of the same composition and the process continued till no more consumption of the substrates were noticed. The number of days taken to attain the stationary phase in each media was also noted.

2.2.7 Development of nitrifying consortia in the selected media in a batch fermentor.

The consortia of nitrifiers were mass cultured in 500mL selected media (Medium XII for ammonia oxidizers and medium 6 for nitrite oxidizers) in 1 litre flasks set up over a magnetic stirrer kept in darkness. An 1% culture from the secondary enrichment sets of both ammonia and nitrite oxidizers were used as the inoculum.

The cultures were maintained by the timely addition of substrate when exhausted along with manual adjustment of pH to 8.0 when it dropped below 5.5. Whenever the culture no more consumed the

substrate, about 50% of the broth was removed and replenished with an equal volume of fresh medium.

The removed consortia were centrifuged at 10,000 x g in a refrigerated centrifuge at an operating temperature of 4°C, the pellet was washed and re-suspended in fresh medium containing 100 µg. mL^{-1} substrate and preserved at 4°C.

2.2.8 Optimization of growth conditions

a. Substrate Concentration

A 1% strength of cell concentrate of ammonia and nitrite oxidizing consortia was inoculated into a series of flasks containing medium XII for ammonia oxidizers and medium 6 for nitrite oxidizers with the substrate (NH_4^+-N and NO_2^--N) concentration ranging from 0.1 to 5.0g.L^{-1}. The depletion of NH_4^+-N together with the build up of NO_2^--N in medium XII and depletion of NO_2^--N in medium 6 was estimated spectrophotometrically as described earlier in section 2.2.6 and represented as the indication of growth of nitrifiers. The rate of nitrifying activity against various ranges of substrate concentration was compared.

b. Hydrogen ion Concentration

A series of flasks containing medium XII and medium 6 having a wide range of pH from 4 to 10 were inoculated with 1% of cell concentrate of ammonia and nitrite oxidizing con-

sortia respectively. The NO_2^--N build up in medium XII and consumption of NO_2^--N in medium 6 were estimated at each pH spectrophotometrically as deseribed in section 2.2.6. The rate of nitrifying activity against various pH used was compared.

c. Temperature

The cell concentrates of ammonia and nitrite oxidizing consortia were inoculated into a series of flasks containing Medium XII and Medium 6 adjusted to their respective optimum pH 8.5 for ammonia oxidizers and pH 7.5 for nitrite oxidizers) to a final concentration of I% (v/v) and incubated at wide range of temperature from 4°C to 37°C. The NO_2^--N build up in medium XII and consumption of NO_2^--N in medium 6 were estimated at each temperature used and the rate of nitrification was compared.

d. Rate of air flow

A series of flasks containing medium XII having an NH_4^+-N concentrations of 339 $\mu g.L^{-1}$ and medium 6 having a NO_2^-–N concentration of 325 $\mu g.L^{-1}$ were inoculated with 1% cell concentrate of ammonia and nitrite oxidizing consortia respectively. These flasks were set at different air flow rates from static state culture (having zero air flow) up to 7 litres of air per minute (70 vvm). The air was drawn from an air compressor and the rate of air flow measured by an air flow meter (Oxytech Equipments,

Cochin). Air spargers were used to increase the aeration efficiency and the flasks were incubated at room temperatures (28±1°C) in the dark. The nitrifying activity in the flasks were assessed by determining the amount of NO_2^-–N produced in the case of ammonia oxidizing media and NO_2^-–N consumed in nitrite oxidizing media (Medium 6)during the incubation period, spectrophotometrically, following Strickland and Parsons (1968).

2.2.9 Unit Nitrifying Activity (UNA)

One unit nitrifying activity of a nitrifying consortium is defined as the nitrifying biomass which can bring about the generation of 1 µmole NO_2^--N $min.^{-1}L^{-1}$ in the case of ammonia oxidizers and the consumption of I µM NO_2^--N $min.^{-1}L^{-1}$ in the case of nitrite oxidizers under standard conditions.

1 UNA_a = 1 µ mole NO_2^--N $min.^{-1}L^{-1}$

(UNA_a = Unit nitrifying activity for ammonia oxidizing consortium)

1 UNA_n = 1 µ mole NO_2^--N $min.^{-1}L^{-1}$

(UNA_n = Unit nitrifying activity for nitrite oxidizing consortium)

To determine the nitrifying potential of ammonia and nitrite oxidizing consortia (Unit Activity) 0.1mL, 0.25mL 0.5mL and 0.75mL 1.0mL of ammonia and nitrite oxidizing consortia were inoculated into flasks con-

taining medium XII haveing a substrate concentration of 339 μg NH_4^+-N L^{-1} and medium 6 having 325 μg NO_2^--N.L^{-1}. These flasks were set at an air flow rate of 1 L· $min.^{-1}$ (10vvm) and the flasks were assessed by determining the amount of NO_2^--N built up in medium XII and the amount of NO_2^--N consumed during the incubation period, spectrophotometrically, by Strickland and Parsons(1968).

2.2.10 Maintenance of the ammonia and nitrite oxidizing consortia.

Both ammonia and nitrite oxidizing consortia thus developed were maintained in medium XII containing 0.1g.L^{-1} of NH_4^+–N and medium 6 containing 0.1g.L^{-1} of NO_2^--N respectively. Periodic additions of substrate was done when it was exhausted.

2.3 Results and Discussion

2.3.1 Filtrant grains of biological filter as source of nitrifiers:

The duration required for the onset of nitrification in various media which have received sewage and filtrant grains of a conditioned biological filter as the inocula are summarized in Table 2-1. It could be seen that when it required 17 to 23 days for the onset of nitrification in media which had received treated sewage as inoculum, the ones which were inoculated with filtrant grains exhibited nitrification within 4 days and in very few cases it required 23 days. This delay indicated that there was a remarkable difference in the number of nitrifiers in both these samples and the lesser time required for the onset of nitrification in media which received filtrant grains as inoculum indicated that it contained very high load of nitrifiers.

Scanning Electron Micrograph (SEM) of the filtrant grains used for inoculating various media are shown in Fig. 2-2 to 2-5. Bacteria which have colonized in the sand grains during the conditioning period of the filter are seen as dense mass forming a biofilm.

It has already been documented that the filter bed of a biological filter provides greater surface area for the nitrifiers to attach and grow making them 100 times more plentiful in the filter bed than suspended in water (Kawai *et al.*, 1964 and Spotte, 1979). This principle has been made use of in isolating nitrifying bacteria from treated sewage

through a biological filter. By just circulating treated sewage through a filter bed using an air-lift pump, sufficient attachment sites can be provided for the nitrifiers which are present initially suspended in very low numbers, facilitating active multiplication making use of the conducive conditions such as oxygenation, slightly alkaline pH(8.0) and sufficiently large quantity of energy sources such as ammonia and nitrite (Fig 6).

This was well reflected in the shorter period within which nitrification could be detected in media which received the filtrant grains as inoculum. Thus, by integrating this simple device into the procedure of isolating nitrifying bacteria from treated sewage, the whole process of enrichment of nitrifiers could be made fool proof and comparatively faster.

Another added advantage of setting up a biofilter for the enrichment and isolation of nitrifiers from treated sewage is that it is possible to maintain the filter for indefinite period by just adding sufficient quantity of ammonium sulphate periodically. Since accumulation of nitrate inhibit ammonia and nitrite oxidizers (Aleem and Alexander, 1958), as a management measure, one-third of the water has to be replaced with aged and autoclaved tapwater. Further, the air filter also has to be replaced with fresh, sterile units periodically.

2.3.2 Selection of suitable media for enrichment

In the primary and secondary enrichment systems set up to select the most suitable media for mass culturing nitrifiers, the maximum quantity of ammonia and nitrite consumed together with the nitrite built up are presented in Fig 2-7 to 2-9 and Tables 2-2 and 2-3. Generally it could be noticed that consumption of ammonia and nitrite stopped at a particular point which could be suggested as the beginning of stationary phase in the growth cycle of the consortium. But the point of attainment of this stationary phase varied with media despite the fact that all were inoculated uniformly from the same source.

In the primary enrichment system of ammonia oxidizing media as given in Fig 2-7 and Table 2-2, nitrifiers could consume 2.188 g.NH_4^+- N.L^{-1} with a nitrite build up of 0.420 g.NO_2^-–N.L^{-1} within 101 days in medium IX, 2.194 g NH_4^+-N.L^{-1} within 151 days in medium XII with a nitrite build up of 0.430 g.NO_2^--N.L^{-1} and 1.646 g.L^{-1} of NH_4^+-N.L^{-1} within 109 days in medium X with a nitrite build up of 0.32g.L^{-1}, all of which were the highest values recorded among the twelve media tested. However, among the secondary enrichment system of ammonia oxidizing media, which on receiving the inoculum from the first set (Fig. 2-8), the nitrifiers in medium XII alone could exhibit an increase in the overall consumption of NH^+_4-N with a nitrite build up of 0.71g.L^{-1} over a period of 97 days (Table 2-2).

Among the primary enrichment system nitrite oxidizers (Fig 2-9 and Table 2-3) the consortia in medium 6 could consume 1.8261 g.NO_2^- - N.L^{-1} during a period of 87 days followed by the consortium in medium 4 where it exhibited a maximum consumption of 1.021 g.NO_2^- - N.L^{-1} during 101 days of incubation before attaining stationary phase. On passaging the consortium to the secondary enrichment system, only in medium 6, an overall increase in the consumption of NO_2^- - N (5.9059 g.L^{-1}) was exhibited even though an extended period of 210 days were required. Meanwhile, the nitrite oxidizers in medium 4 entered into stationary phase within 89 days consuming 1.999 g NO_2^- - N.L^{-1}.

Accordingly, medium XII for ammonia oxidizers and medium 6 for nitrite oxidizers could be chosen as the most suitable ones for enrichment and mass production of nitrifiers. This selection was based on the fact that when the nitrifying consortia, (1mL aliquot along with filtrant grains) were transferred from the primary to the secondary enrichment media, remarkable enhancement in the extent of consumption of NH_4^+ - N and build up of NO_2^-–N in medium XII and consumption of NO_2^- – N in medium 6 respectively could be observed.

The use of filtrant grains as a rich source of nitrifiers was again confirmed by inoculating 1 gram filtrant grains from two filter beds and 1 mLeach of treated sewage both separately in 100 mL medium

XII, for ammonia oxidizers and in medium 6 for nitrite oxidizers. As shown in Table 2-4 the media which received the filtrant grains as inoculum required only a few days for complete exhaustion of NH_4^+–N and NO_2^-–N as compared to the ones inoculated with 1 mL of sewage.

One of the most important requirements to be considered in the study of bacterial ecology is the choice of a proper medium which ideally should be a chemically defined one (Sato *et al.*, 1985). Since the discovery of *Nitrosomonas* by Winogradsky in the latter part of the nineteenth century, a number of media have been developed and used for studying nitrifying bacteria. The availability of such a large number of media necessitated the selection of one medium each which could support higher metabolic activity of ammonia and nitrite oxidizers respectively. The medium XII found to be the most appropriate one for ammonia oxidizers is the one suggested by Lewis and Pramer, (1958) and the medium 6 recorded to be the most suitable one for nitrite oxidizers is the Winogradsky medium modified by Rodina (1972).

2.3.3 Development of nitrifying consortia.

Ammonia and nitrite oxidizing consortia developed in medium XII and medium 6 respectively could be mass cultured in a batch fermentor set up using 1 litre flask over a magnetic stirrer (Fig. 2-10 and 2-11 and Tables 2-5 to 2-8). This mode of cell generation has

several advantages such as lack of sophistication, minimal volume of the culture to be centrifuged at a time and total control over the possibility of any contamination over continuous and semicontinuous methods suggested by Watson *et al.*, (1981) where large volumes (20 litres) of the cultures were grown in carboy fitted with pH stat, air flow system, inlet and outlet ports. The overflow was directed to a continuous flow centrifuge for the separation of cells. But, considering the prolonged generation time of 8 hours (Watson *et al.*, 1981), it would be economical only if the nitrifiers are grown to a turbid culture possibly to stationary phase before the harvest, as achieved in the present study.

2.3.4 Optimum substrate concentration

By testing with various substrate concentrations ranging from 0.1 to 5.0g.L^{-1}, the maximum allowable substrate concentration for ammonia and nitrite oxidizing consortia was found to be 2.75 g.L^{-1} NH_4^+ -N and 2.5 g.L^{-1} NO_2^-- N respectively (Fig 2-12 and 2-13 and Tables 2-9 and 2-10) beyond which progressive inhibition of nitrification took place. Similar studies with consortia of nitrifying bacteria have not been made elsewhere. However, studies conducted by Srna and Baggaley (1975) in biological filter systems revealed that for a given initial concentration ranging from 0.035 to 1.96 g.L^{-1} of NH_4^+ - N, ammonia oxidation is a first order reaction as described in Michaelis - Menton kinetics. It implies that beyond 1.96 g.L^{-1}

NH_4^+ - N, inhibition of nitrification could take place. Eventhough similar comparable works could not be cited from literature with regard to nitrite oxidizers, Boon and Laudelout (1962) demonstrated that 1.4 $g.L^{-1}$ NO_2^- - N caused 40% inhibition in the activity of a pure culture of *Nitrobacter*. According to Painter (1970), both *Nitrosomonas* sp and *Nitrobacter* sp are sensitive to their own substrate. According to Anthonisen (1974), the degree for inhibition depends upon the ammonia - ammonium and the nitrite -nitrous acid equilibria. Other researchers (Prakasam and Loehr, 1972), Boon and Laudelout, (1962) support the suggestion that inhibition is due to free ammonia and undissociated nitrous acid; concentrations of these species have significance in inhibition of nitrification. Wide ranges of ammonium and nitrite ions can be oxidized by the nitrifiers; differences in conditions account for the apparent discrepancies (Sharma and Ahlert, 1977). Thus, influent NH_4^+ - N and NO_2^- - N influences the nitrifying microorganisms in a double manner, one by being the main energy source, and the other by being related to the concentration of inhibitory form, free ammonia (NH_3) and free acid (HNO_2) by the chemical equilibrium as given below (Anthonisen *et al.*, 1976 and Fdz - Polanco *et al.*, 1995)

$$NH_4^+ + OH^- \leftrightarrow NH_3 + H_2O$$

$$2NO_2^- + 2OH^- \leftrightarrow 2HNO_2$$

Nitrosocystis oceanus grow well on a wide range of ammonia concentrations (Watson 1975). When grown in ammonia concentrations greater than 1000 µg at. NH_4^+ - $N.L^{-1}$ the generation times were consistant from one experiment to another. Below this level, generation time varied between experiments and some times no growth was observed if these were less than 100 µg-at. NH_4^+–$N.L^{-1}$. While no lag phase was observed in ammonia concentration greater than 1000 µg - at. NH_4^+- $N.L^{-1}$, lag phases of a week or more took place in lesser concentration. In fermentors, nitrite was not noticeably toxic to cultures until it reached a concentration of 50 to 100 mg. at NO_2^- -$N.L^{-1}$. All these earlier observation tempt one to assert that the consortia of ammonia and nitrite oxidizers developed have a better tolerance to higher concentration of NH_4^+ - N and NO_2^- - N, a requisite for using them for ammonia removal from sewage where shock loading is often a reality.

2.3.5 Optimum hydrogen ion concentration

The maximum nitrifying potential of ammonia oxidizing consortium was exhibited at pH 8.5 and that of nitrite oxiding consortia at pH 7.5 (Fig 2-14 and 2-15 and Tables 2-11 and 2-12). Much controversies exist with regard to the pH optima of nitrifying bacteria. Engel and Alexander (1958) demonstrated that the pH curves of nitrifying bacteria are relatively flat between pH 7.0 and 9.0, falling to about 50% at 6.2 and 9.6 and that of Meyerhof (1917), has a sharp peak at

8.6 and 50% values at 7.9 and 9.3. The nitrifying consortia developed in the present study exhibited sharp peaks as optimum pH. Interestingly, the nitrite oxidizing consortium exhibit a pH optima peak one unit lesser than that of ammonia oxidizers. The pH optima for the overall nitrification reaction appear to be slightly on the alkaline side.

One mechanism by which pH affects the rate of nitrification has been proposed by Anthonisen (1974). His hypothesis is based on the fact that ammonia - ammonium and nitrite - nitrous acid equilibria depend on pH. According to Anthonisen, both 'free ammonia' (NH_3) and "free nitrous acid" (HNO_2) inhibit nitrifying organism. He postulated that, when the intracellular pH of a nitrifying organism is lower than the pH of the extra- cellular environment, free nitrous acid (FNA) permeates the cells and not the nitrite ion. Anthonisen proposed that the ability of FA and FNA to penetrate the nitrifying organism makes them more inhibitory than ammonium and nitrite ions.

2.3.6 Optimum Temperature

The optimum temperature for nitrification by ammonia oxidizing and nitrite oxidizing consortia lies in the range 28°C - 30°C with no significant growth below 4°C or above 37°C (Fig. 2-16 and 2-17 and Tables 2-13 and 2-14) reflected by the maximum nitrifying activity in the above temperatures.

Nitrification reaction follow the vant Hoff Arhenius law up to 30°C (Metcalf and Eddy, 1973). Thus, nitrification proceeds better in warmer seasons or climates. The overall optimum temperature for the growth of nitrifying bacteria appears to be in the range 28°C - 36°C although optimum temperature upto 42°C have been reported for *Nitrobacter* (Painter, 1970). The growth constants of nitrifying bacteria were affected greatly by temperature (Sharma and Ahlert, 1977). In experiments with marine nitrifying bacteria, Watson (1975) found that cells incubated at 35°C to 40°C had a shorter generation time for the first five generation, but subsequently, the generation time was prolonged to 36 hours. Therefore, 30°C proved to be the optimum temperature for growth.

2.3.7 Optimum airflow rate

The nitrite build up by ammonia oxidizing consortia at various rates of air flow at the end of 13 hours incubation period is given in Table 2-15 and Fig 2-18. It could be seen that the build up of NO_2^-–N was increased ten fold with the passage of 7 litres of air.minute^{-1}. Consumption of NO_2^-–N by nitrite oxidizing consortia at various rates of air flow at the end of one hour incubation period is given in Table 16 and Fig. 2-19. It was seen that the consumption of NO_2^-–N increased by a factor of 28.8 with the increase in the rate of air flow to 7 litres/minute.

It is reported that 3.43 mg O_2 was required for the conversion of 1 mg NH_4^+–N and 1.14 mg O_2 for the conversion of 1 mg NH_4^+–N (Sharma and Ahlert, 1977). It means that ammonia oxidation to nitrite demands 3 times more oxygen than required for the nitrite oxidation. Also, the ammonia oxidizing population had greater specific affinity for O_2 than the nitrite oxidizing bacteria (Laan broek and Woldendorp, 1995). Obviously, given the same air flow rate as per the present study, nitrite oxidizing activity is always greater than ammonia oxidising activity.

Studies conducted by Gunderson, *et al.*, (1966) showed that *Nitrosomonas oceanus* lost 25% of its nitrifying capacity when the O_2 concentration was reduced from 100% to about 10% of air saturation. Steady state culture of both *Nitrosomonas* and *Nitrobacter* gave complete conversion of ammonia to nitrate at DO concentration of about 100μ mol. L^{-1}. Below this, nitrate concentration went down and nitrite started accumulating indicating that the nitrite oxidizers were in trouble. At dissolved oxygen concentration below 50μ mol. L^{-1}, ammonia also began to accumulate and the culture was washed out.

Low concentration of oxygen reduce the rates of nitrification and the dual effects of substrate and oxygen limitation have been modelled using double substrate limiting kinetics (Sharma and Ahlert, 1977). Saturation constants for oxygen for pure cultures of ammonia and nitrite oxidizers lie in the range 0.25 to 2.5 mg DO L^{-1} (Painter, 1986) with similar values reported for mixed culture activated sludge

system. Mcgraw and Knowles (1987) found that at reduced O_2 levels, nitrifiers were out competed due to a combination of higher km value for lower specific growth rate and O_2 and lower growth yield. There is some evidence that ammonia oxidizers have greater saturation constants for O_2 than nitrite oxidizers. Helder and De-Vries (1983) suggest that ammonium oxidation is inhibited below 30µmol O_2 L^{-1} while nitrite oxidation is inhibited below 125µmol $O_2 \cdot L^{-1}$. However at higher concentration, oxygen is inhibitory, the free radical formation suggested as the mechanism for oxygen inhibition. Moreover the use of pure oxygen rather than air is not profitable where cost effectiveness is considered.Therefore, it is highly recommended to use air with increased flow rate to cope up with the Nitrogenous Biological Oxygen Demand (NBOD).

2.3.8 Unit activity of nitrifying consortia

The results of the experiment to find the unit activity of ammonia and nitrite oxidizing consortia are given in Table 2-17 and Fig. 2-20. Accordingly, 1mL of ammonia oxidising consortium exhibited a unit nitrifying activity of 0.702 x 10^{-3} UNA_a under standard conditions (pH 8.5; substrate concentration 339 µg NH_4^+-N $.L^{-1}$ and air flow rate of 1 litre of air $minute^{-1}$). One mL of the nitrite oxidizing consortium gave a unit nitrifying activity of 0.1729x10^{-1} UNA_n under standard conditions (pH 7.5, substrate concentration 325 µg. NO_2^--N $.L^{-1}$ and rate of air flow 1 litre $minute^{-1}$).

This novel concept of quantifying nitrifying bacteria by its nitrifying potential has many advantages. One of the methods such as MPN counts for nitrifiers even after 100 days of incubation could not reach a maximum as per the observation of Matulewich *et al.*, (1975). Further, measuring nitrifying biomass by absor bance techniques was difficult because of the low growth yields. The use of counting chambers to determine total cell concentration is tedious. Consequently, quantifying nitrifying consortia in terms of its nitrifying potential is the most suitable alternative. Throughout the study, the amount of nitrite produced by ammonia oxidizing consortium was used as a measure of growth and activity. This method was validated by Loveless and Painter (1968) who found out that the greater sensitivity, simplicity and accuracy of nitrite determination makes it a better choice than the estimation of cell carbon, mass or number.

2.3.9 Maintenance of consortia

Both ammonia and nitrite oxidizing consortia developed could be maintained in dark at 4°C in medium XII containing $0.1 g.L^{-1}$ of NH_4^+-N and in medium 6 containing $0.1 g.L^{-1}$ of NO_2^-– N respectively. At this temperature very little nitrification took place, (Fig. 2-16 and 2-17) but at the same time, cells remained viable for longer periods (more than one year) without any apparent loss of viability. Watson *et al.*, (1981) suggested the storage of liquid cultures of nitrifying bacteria at 15°C and their transference once in every 4-6 months,

with the addition of substrate. But by maintaning at 4°C, this addition of NH_4^+- N and NO_2^- - N could also be avoided for much longer period.

Thus two separate nitrifying consortia (viz. ammonia and nitrite oxidizing) were developed, and characterized for their optimum substrate concentration, pH and temperature and rate of air flow and after determining their nitrifying potential in terms of unit nitrifying activity they were maintained in dark at 4°C in their respective culture media containing 0.1g. L^{-1} of the respective substrates.

Heterotrophic bacteria usually outnumber the nitrifying bacteria in enrichment cultures because the generation time of the nitrifying bacteria is 8-24 hr, even under optimal conditions. For these reasons, isolation of nitrifying bacteria as such turned out to be a difficult and time consuming task. Further as nitrifying bacteria cannot be isolated directly from the source material, enrichment cultures had to be made. Precisely it took almost 3 years to develop both an ammonia and a nitrite oxidizing consortia in this laboratory.

Eventhough ammonia and nitrite oxidizers co-exist in nature, they have different pH and substrate requirement. And product inhibition. Interestingly, the ammonia oxidizing consortia developed in this laboratory does not contain any nitrite oxidizing bacteria and nitrite oxidizing consortia does not contain ammonia oxidizing bacteria too.

This observation was based on the fact that the nitrite built up as a result of oxidizing activity of ammonia oxidizing consortium was not further oxidized to nitrate. Since there was no further oxidation of nitrite to nitrate, it was confirmed that the ammonia oxidizing consortium did not contain any nitrite oxidizing counter part. The nitrate concentration in primary and secondary enrichment systems and also in the batch fermentor used for the development of ammonia oxidizing consortia were nil (Tables 2-5, 2-6 and 2-7).

Both ammonia and nitrite oxidizing bacteria were grown in their respective selective enrichment media (Medium XII for ammonia oxidizing bacteria and Medium 6 for nitrite oxidizing bacteria) and two separate consortia could be developed. Because of differing requirements of substrate concentration and pH optima for ammonia and nitrite oxidizing bacteria and because of product inhibition of nitrification, it is always feasible and highly practical to use two separate bioreactor systems, one with ammonia oxidizers and the other with nitrite oxidizers, for removing ammonia from sewage.

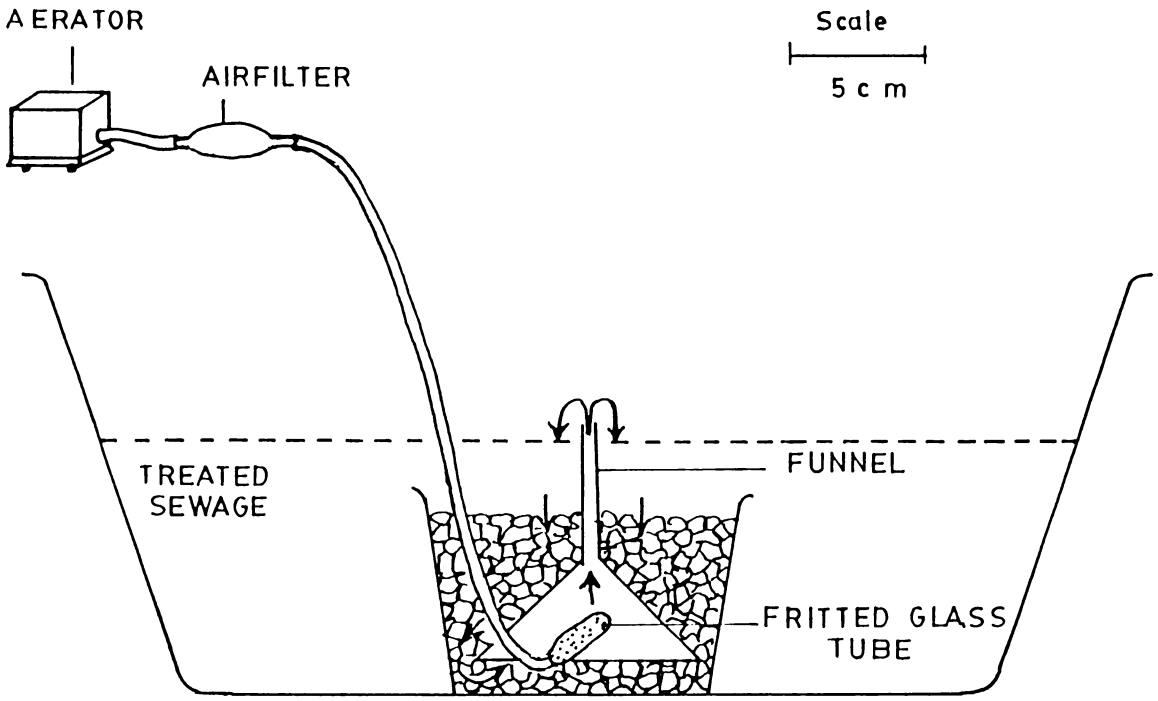


Fig. 2-1 Biological filter set -up for the pre-enrichment of nitrifying bacteria

Fig 2-2 Scanning Electron Micrograph of filterent grains with adsorbed nitrifying -like bacterial cells

Fig 2-3 Scanning Electron Micrograph of filterent grains with adsorbed nitrifying -like bacterial cells.

Fig 2-4 Scanning Electron Micrograph of filterent grains with a biofilm of nitrifying -like bacterial cells.

Fig 2-5 Scanning Electro Micrograph of filterent grains with biofilm of nitrifying-like bacteria cells.

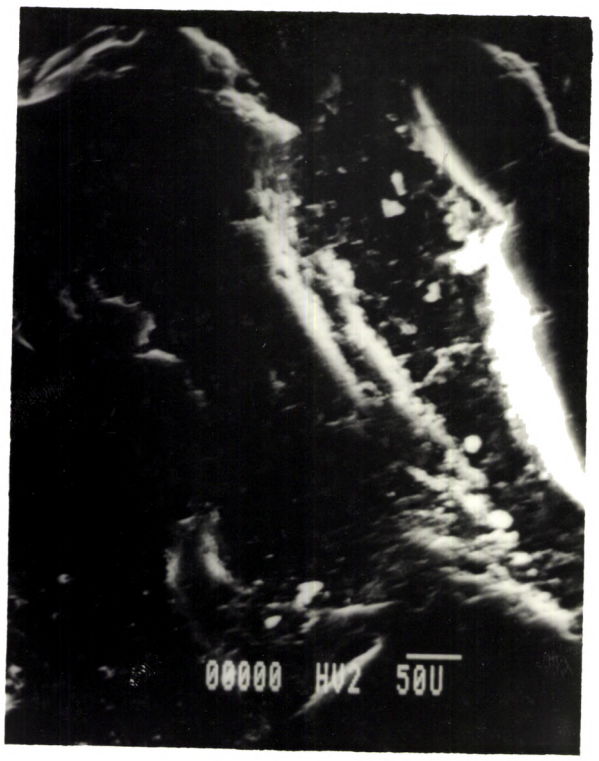


Fig 2-2

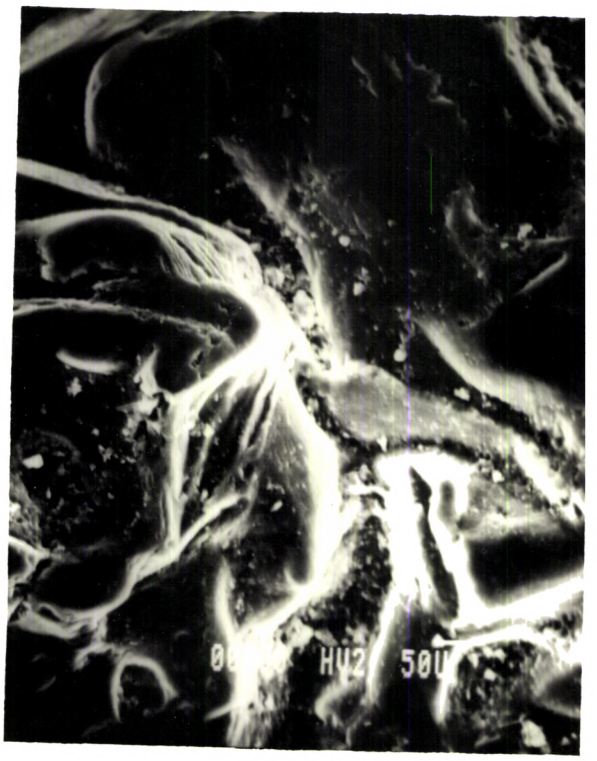


Fig 2-3

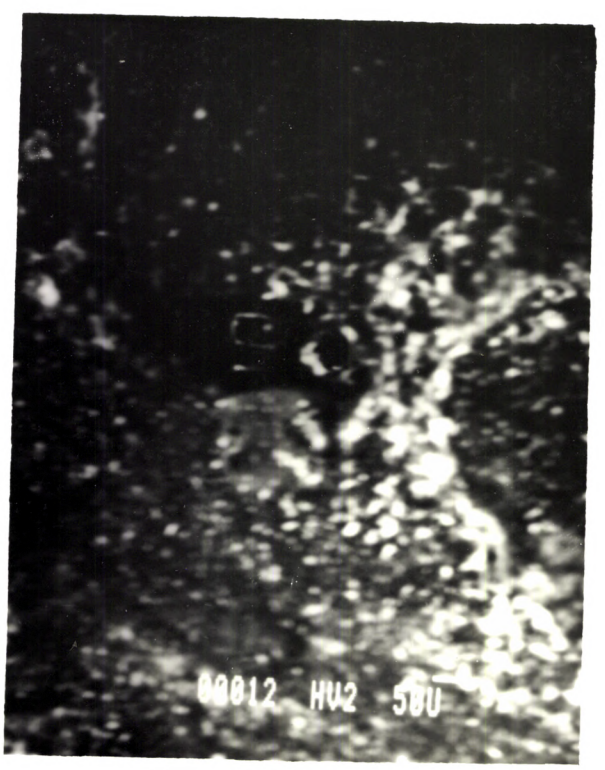


Fig 2-4

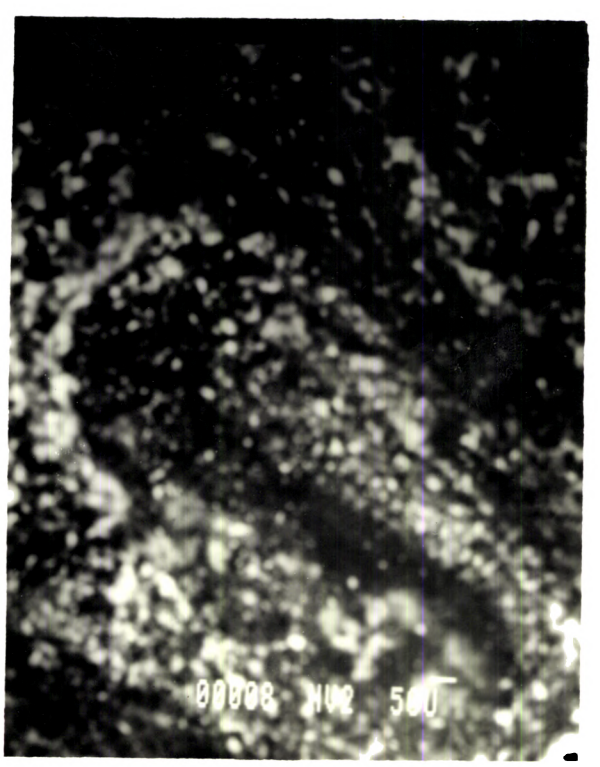


Fig 2-5

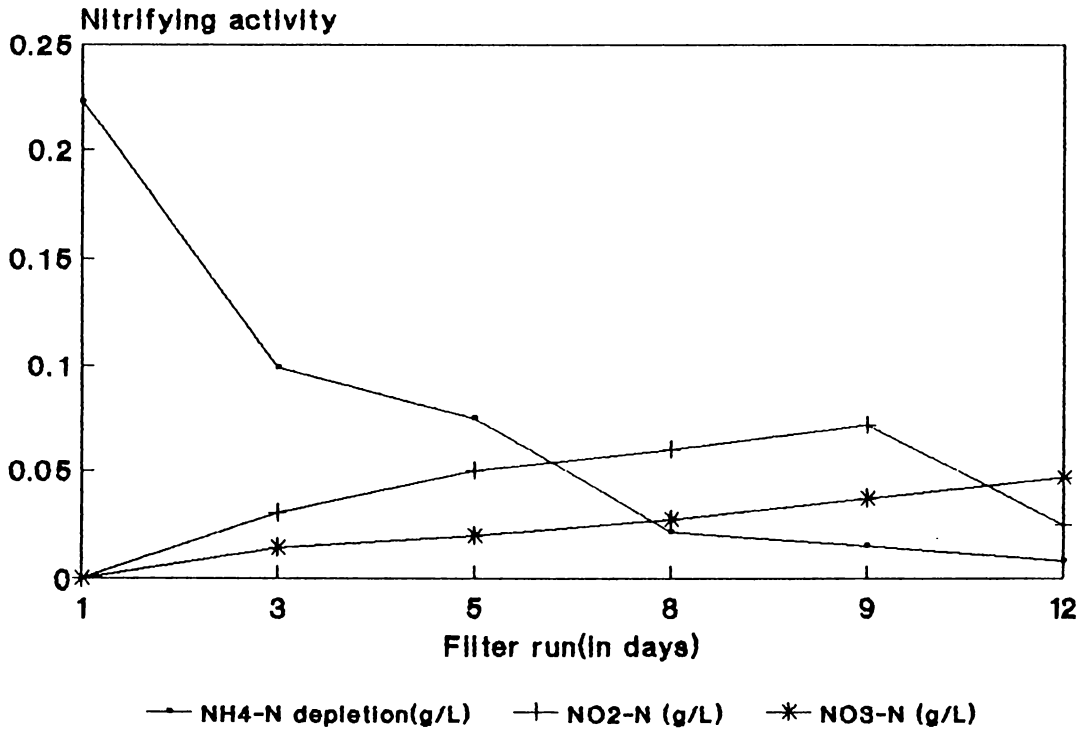


Fig 2-6 Nitrifying activity in a newly set biological filter.

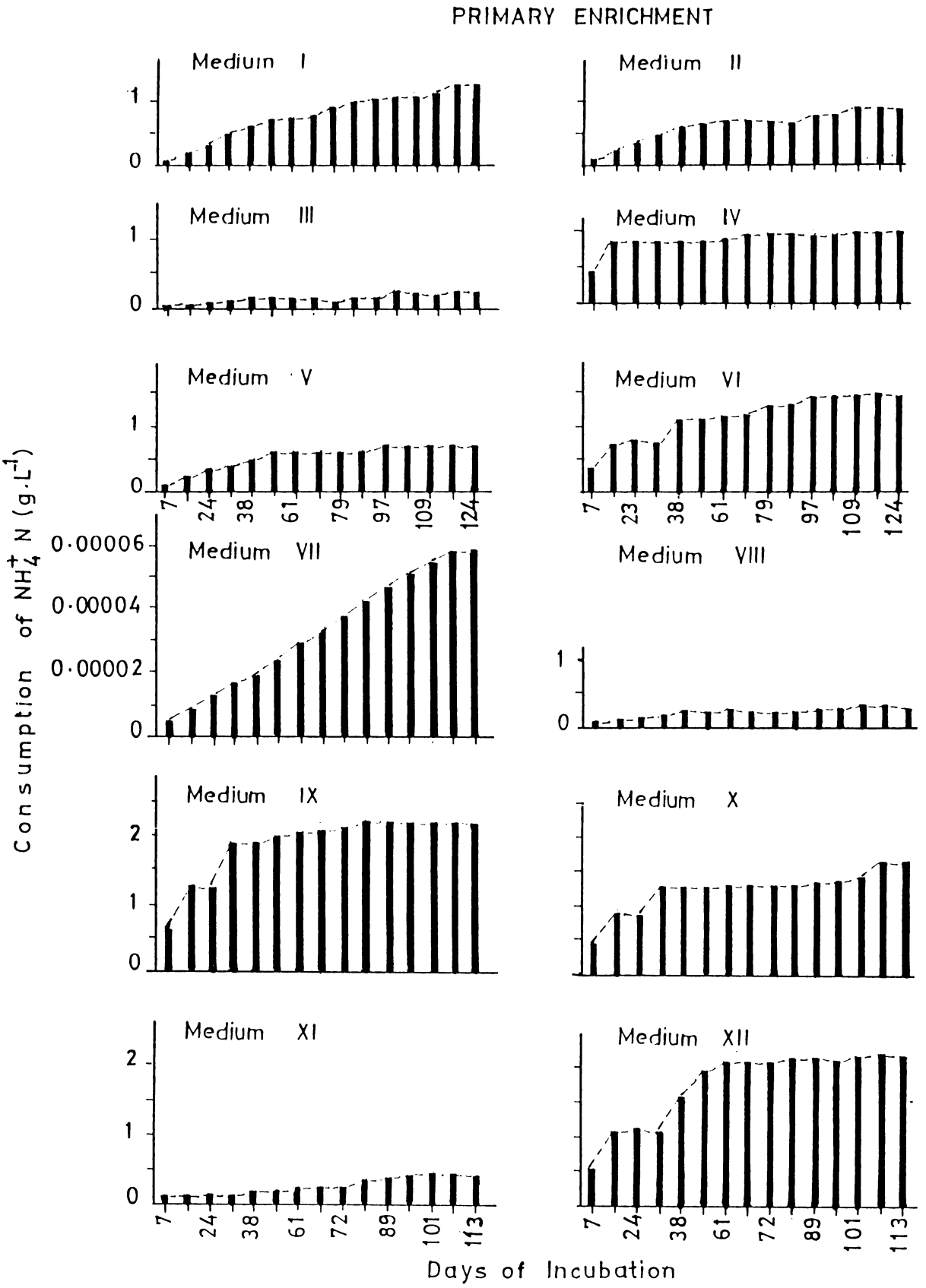


Fig 2-7 Consumption of ammonia nitrogen during primary enrichment in various ammonia oxidizing media.

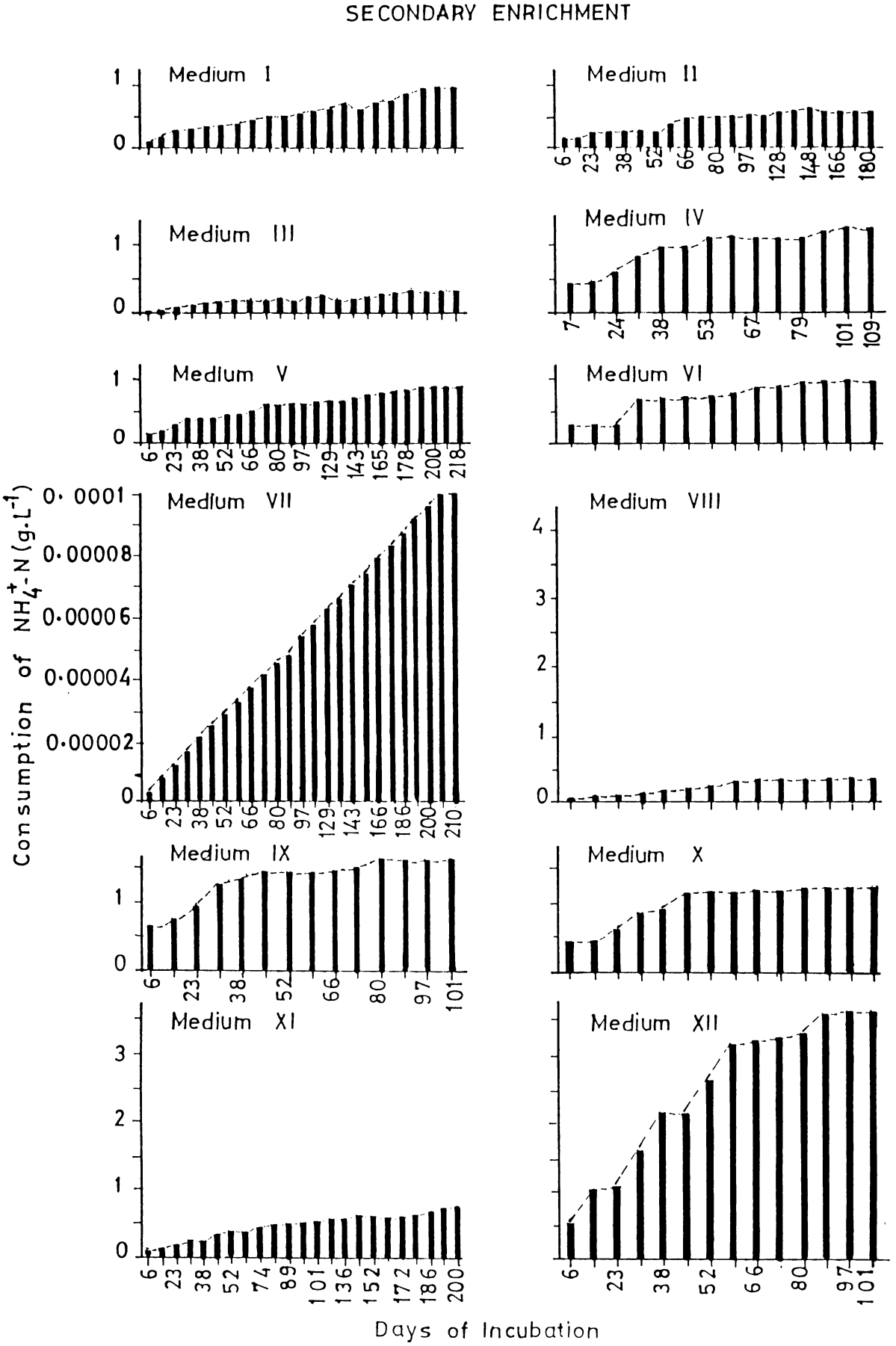


Fig 2-8 Consumption of ammonia nitrogen during secondary enrichment in various ammonia oxidizing consortia.

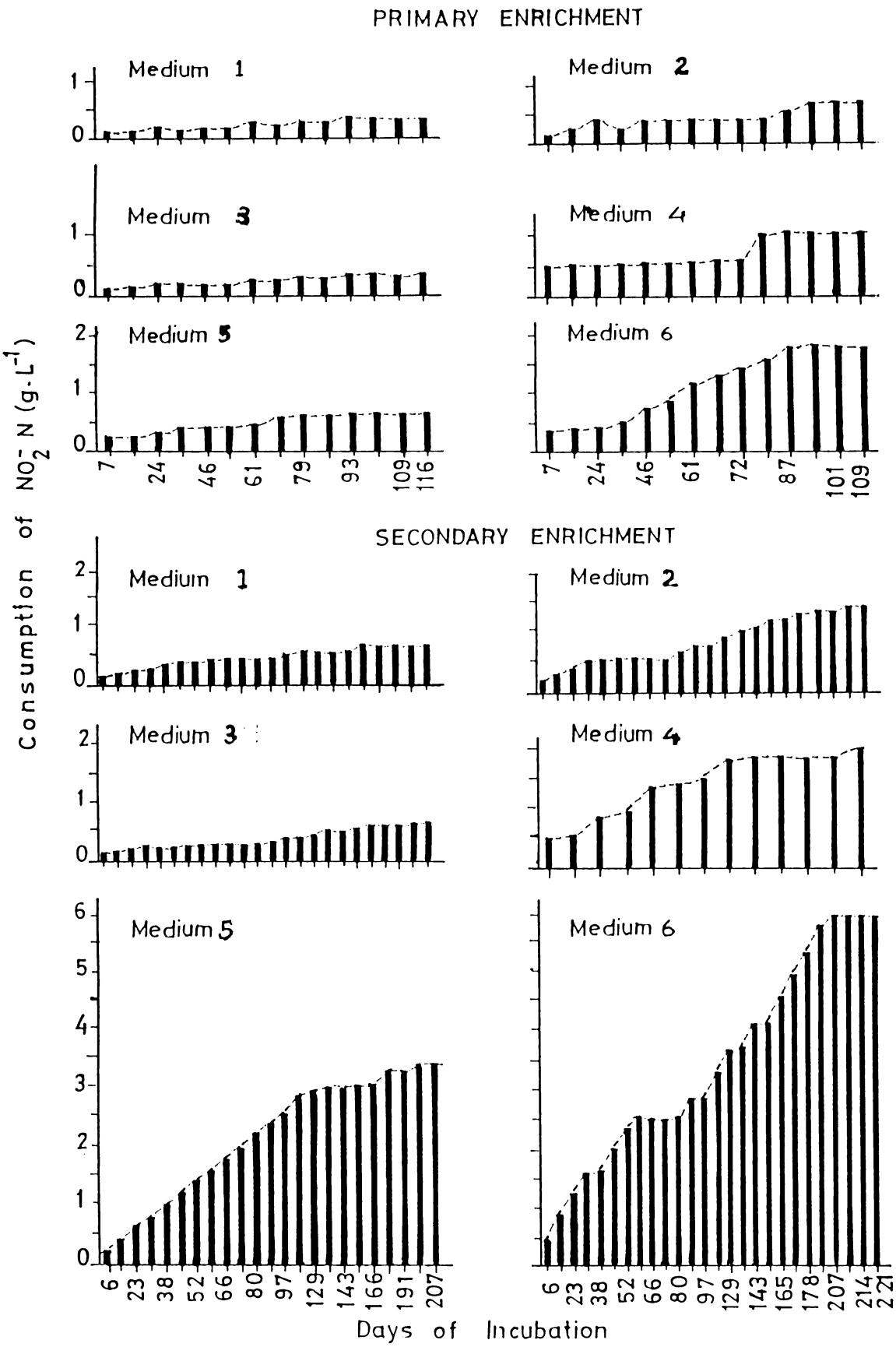
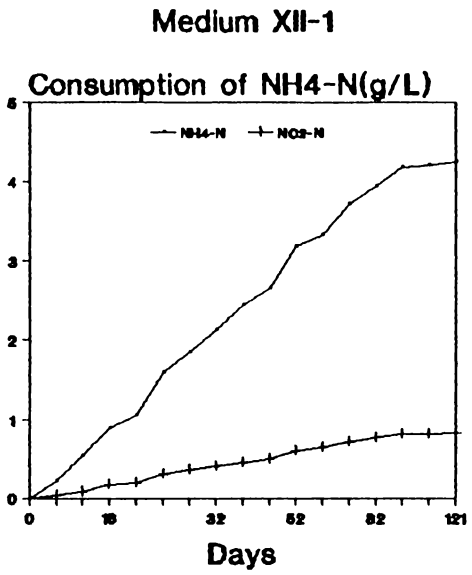
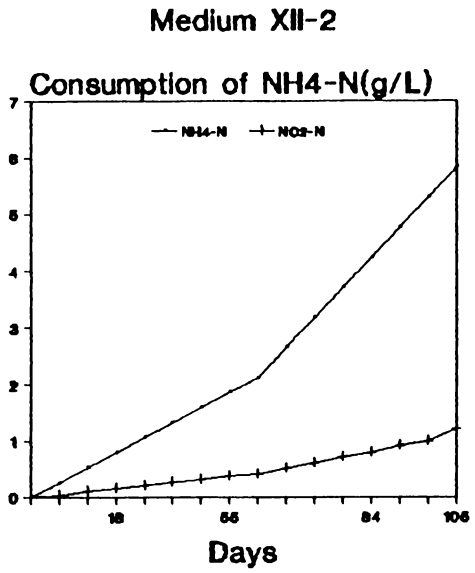


Fig 2-9 Consumption of nitrite nitrogen during primary and secondary enrichment in various nitrite oxidizing media.





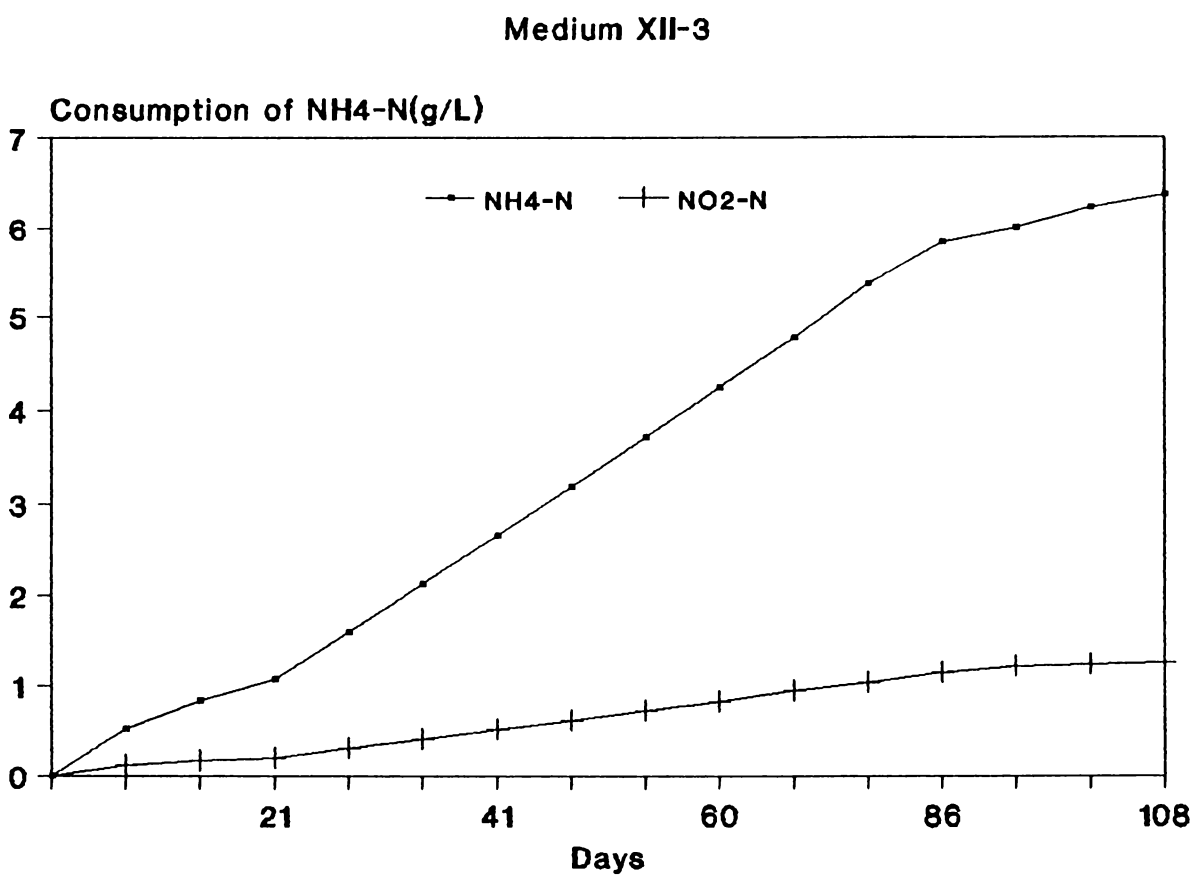


Fig 2-10 Activity of ammonia oxidizing consortium in batch process (XII -I, XII -2, XII -3)

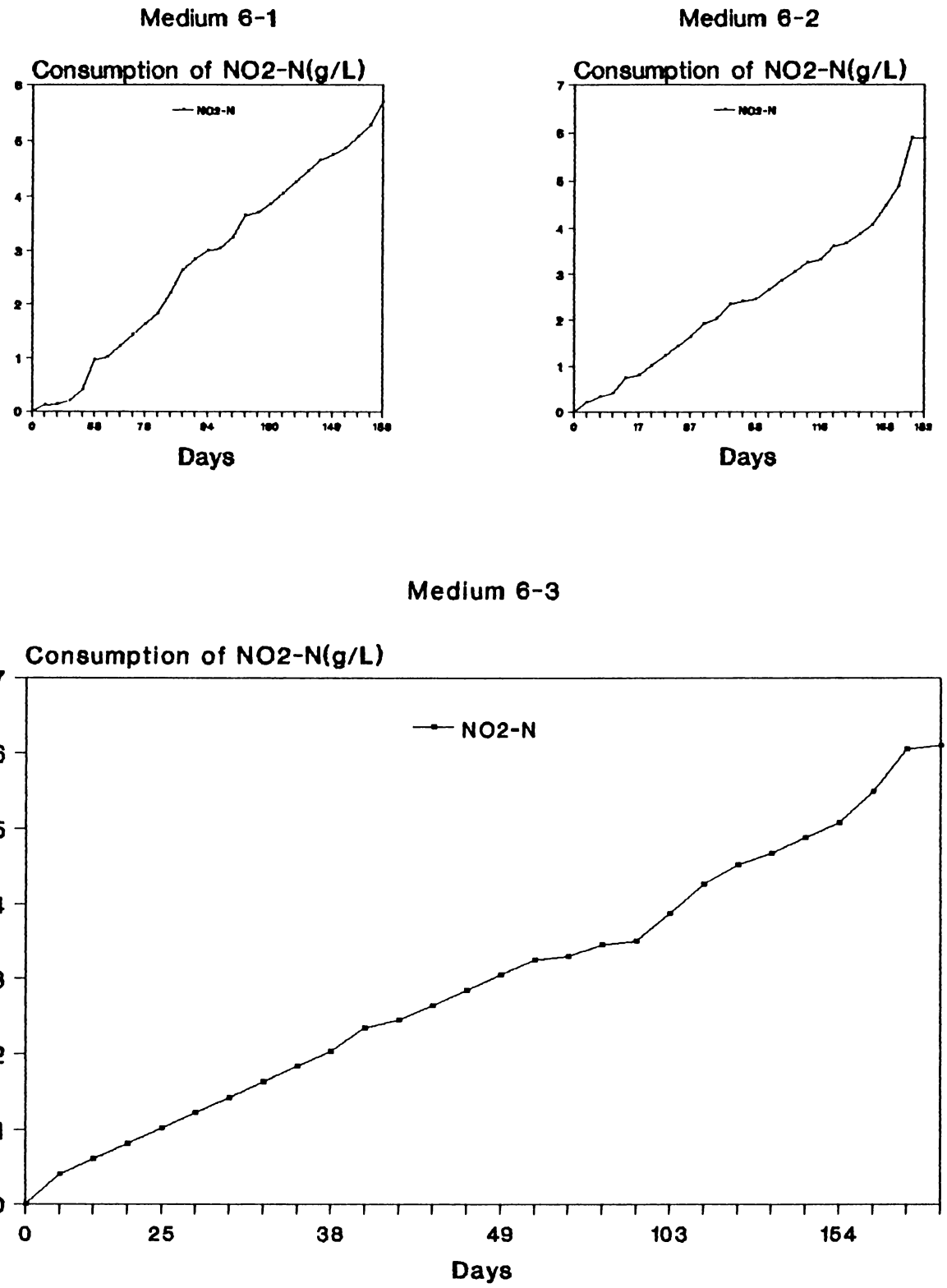


Fig 2-11 Activity of nitrite oxidizing consortium in batch process(6-1, 6-2, and 6-3)

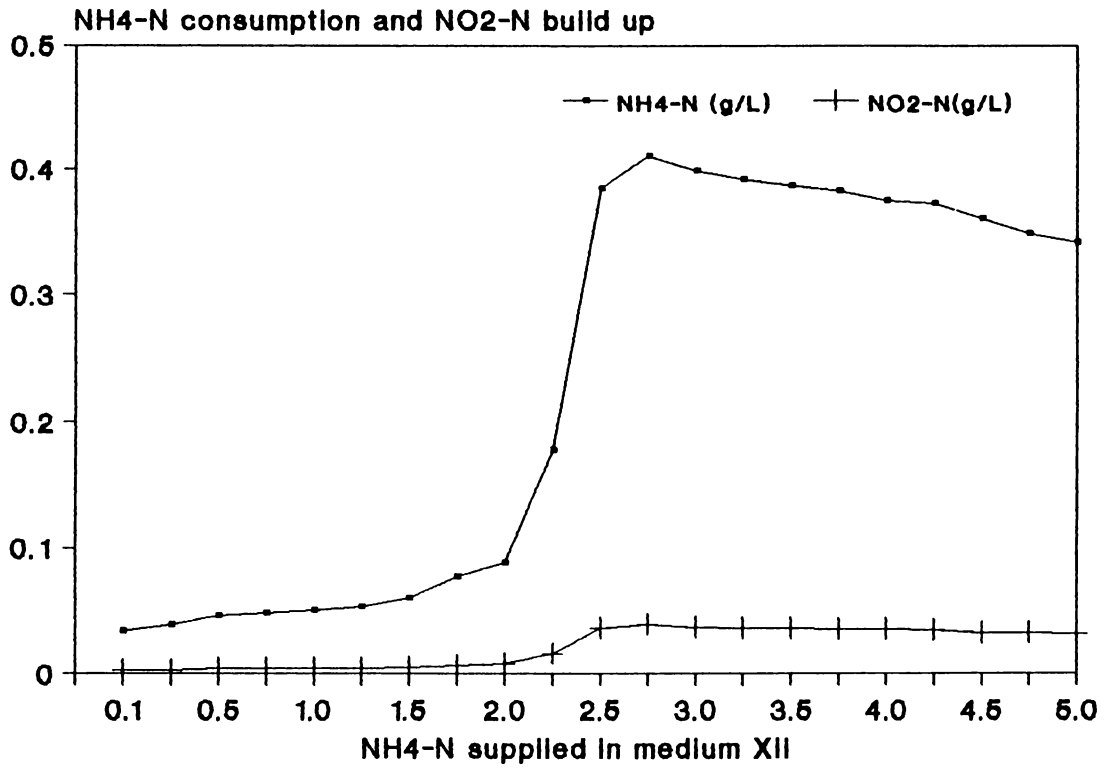


Fig 2-12 Optimum substrate concentration required for ammonia oxidizing consortium (n=3)

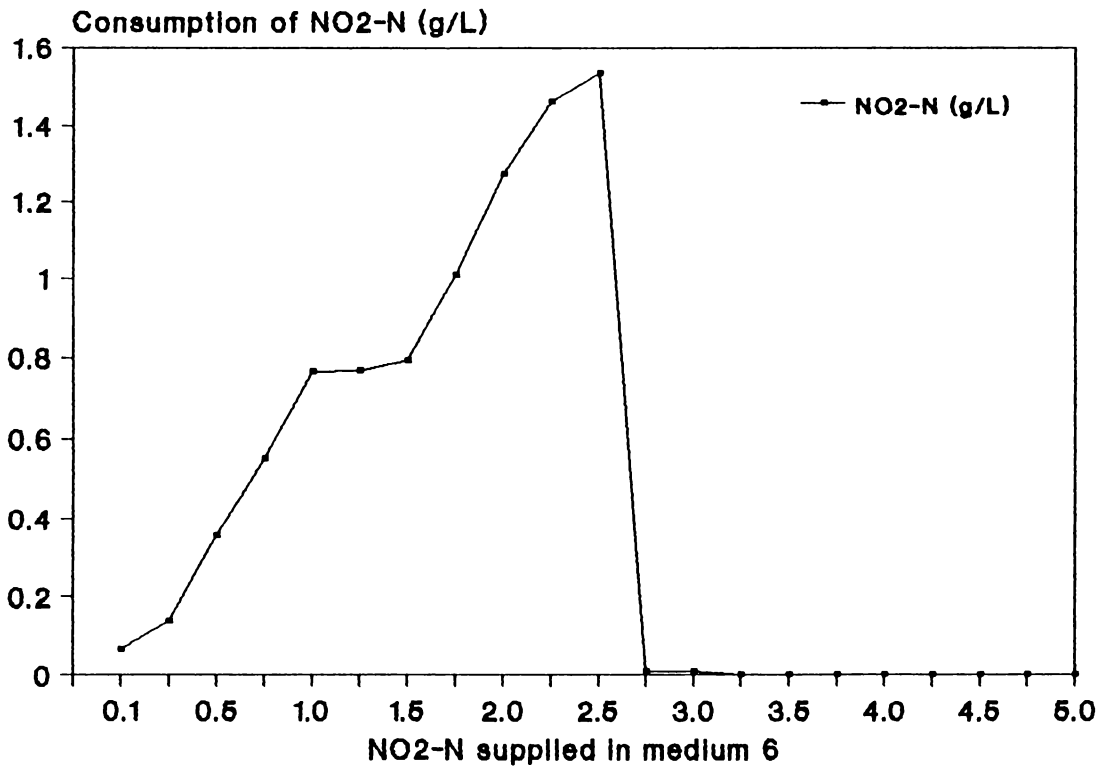


Fig 2-13 Optimum substrate concentration required for nitrite oxidizing consortium (n=3)

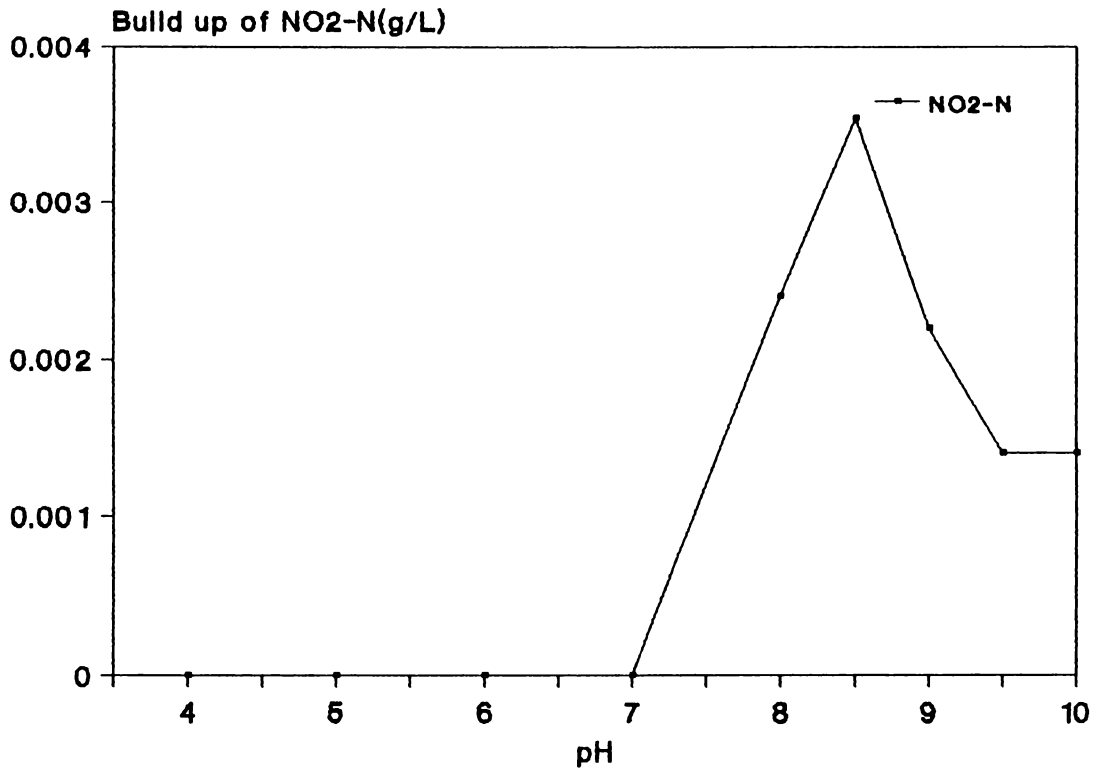


Fig 2-14 Optimum pH required for ammonia oxidizing consortium (n=3)

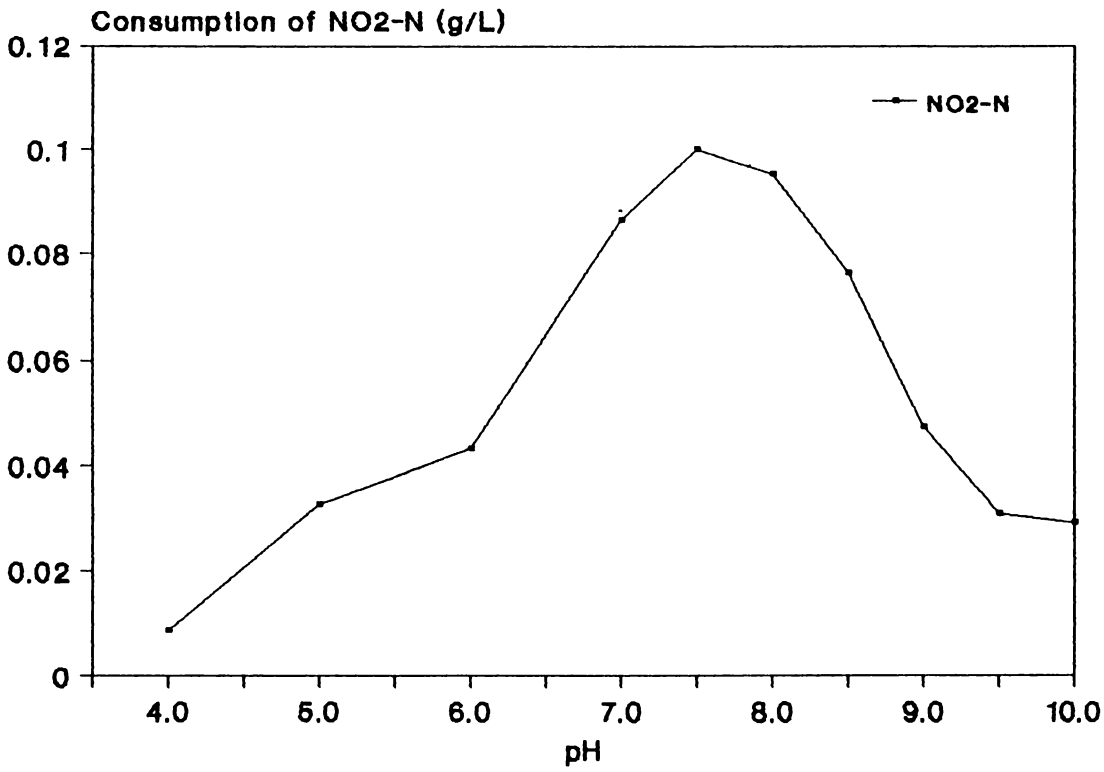


Fig 2-15 Optimum pH required for nitrite oxidizing consortium (n=3)

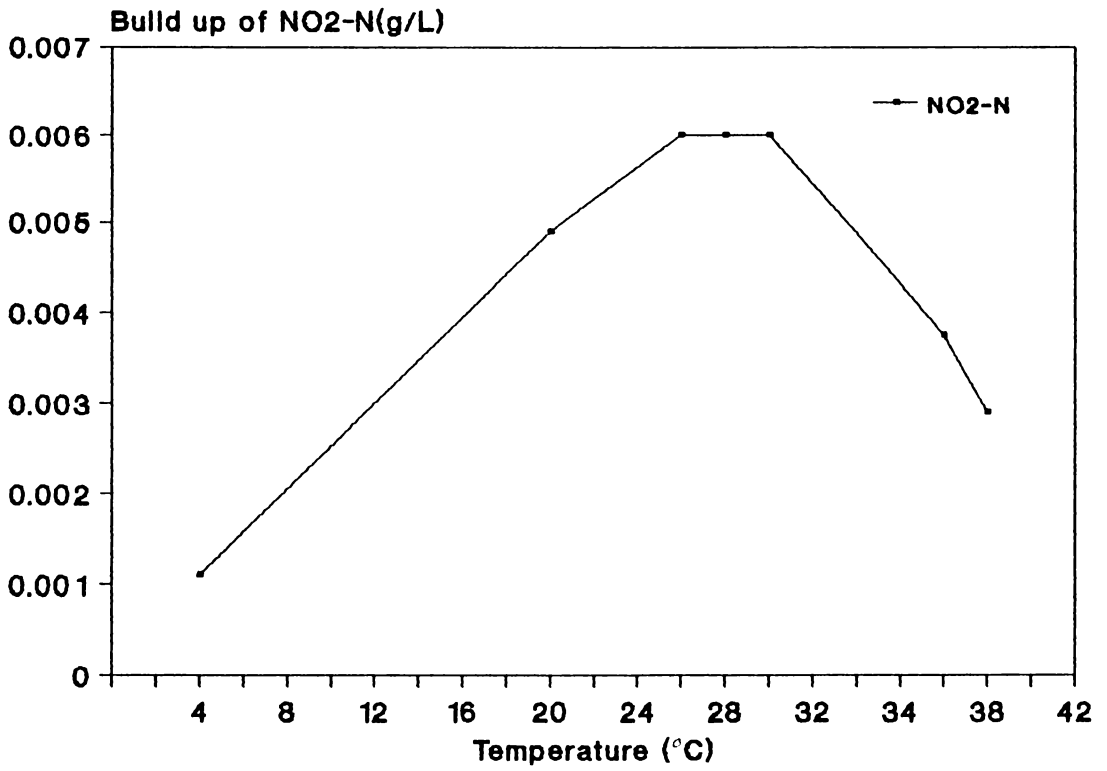


Fig 2-16 Optimum temperature required for ammonia oxidizing consortium (n=3)

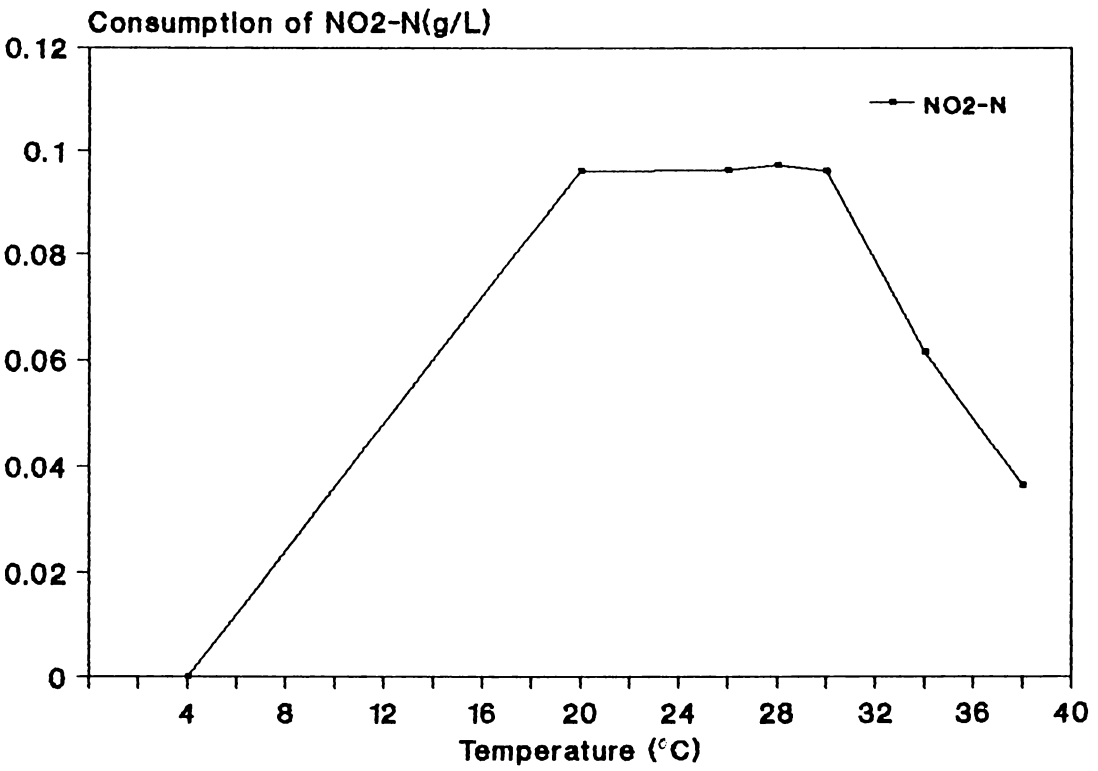


Fig 2-17 Optimum temperature required for nitrite oxidizing consortium (n=3)

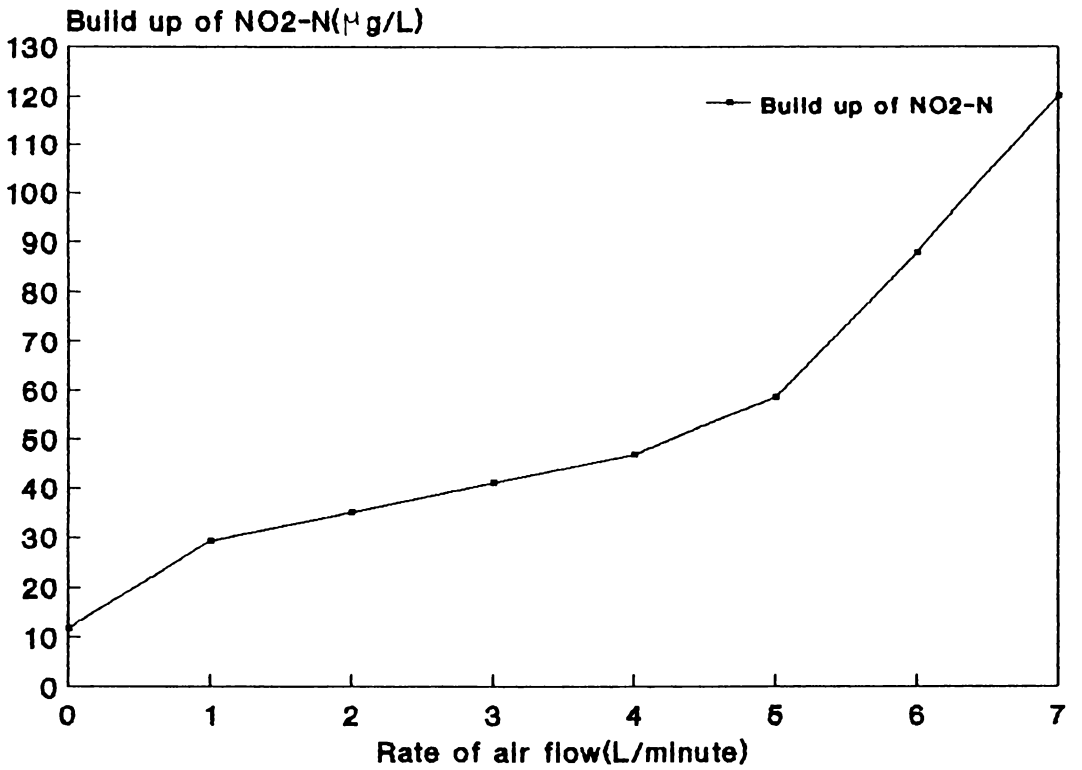


Fig 2-18 Optimum rate of air flow required for ammonia oxidizing consortium(n=3)

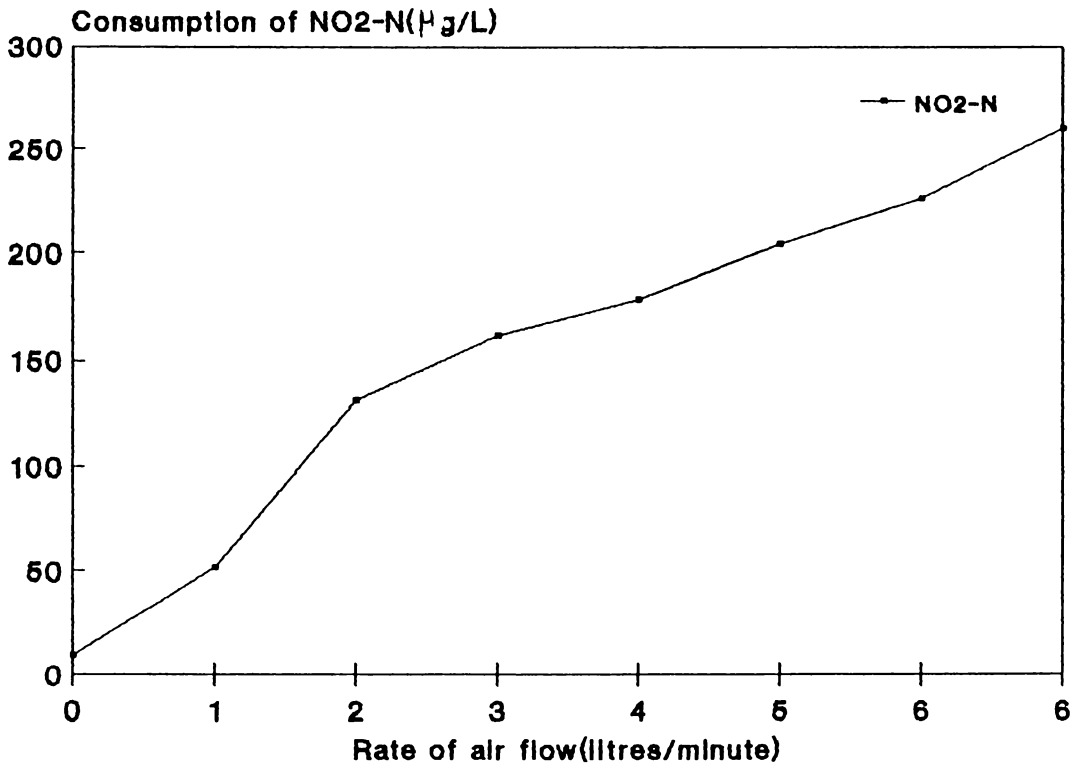


Fig. 2-19 Optimum rate of airflow required for nitrite oxidizing consortium

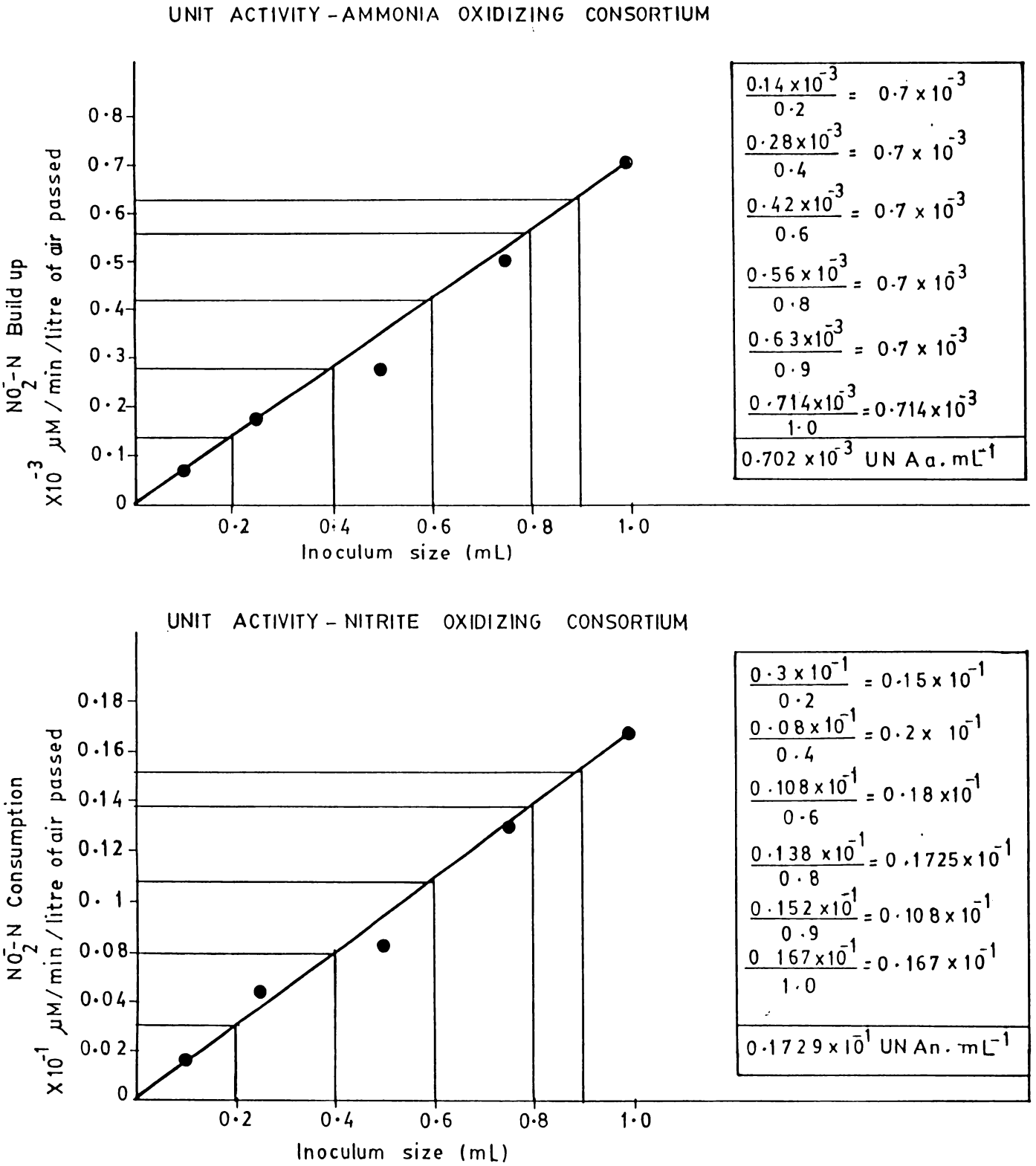


Fig.2-20 Determination of unit activity of ammonia and nitrite oxidizing consortia.

TABLE 2-1

Duration required for the onset of nitrification (in days) in ammonia and nitrite oxidizing media on receiving sewage and filtrant grains of a conditioned biofilter as the source

Code Number of media	Source	
	Sewage	Sand grains
I	32	19
II	32	4
III	32	4
IV	37	4
V	21	19
VI	17	4
VII	18	4
VIII	21	4
IX	18	4
X	22	4
XI	19	8
XII	21	4
1	33	19
2	33	19
3	33	19
4	33	23
5	33	21
6	33	19

TABLE 2-2

Total quantity of ammonia-nitrogen consumed in various ammonia oxidizing media before attaining stationary phase

Media used and initial composition of NH_4^+–N ($g.L^{-1}$)	Primary Enrichment			Secondary Enrichment		
	No. of days	Consumption of NH_4^+-N ($g.L^{-1}$)	Build up of NO_2^--N ($g.L^{-1}$)	No. of days	Consumption of NH_4^+-N ($g.L^{-1}$)	Build up of NO_2^--N ($g.L^{-1}$)
I (0.106)	113	1.192	0.231	180	0.992	0.190
II (0.106)	113	0.8776	0.172	143	0.609	0.11
III (0.0275)	113	0.2469	0.04	180	0.3365	0.06
IV (0.424)	109	1.014	0.197	101	1.261	0.24
V (0.106)	109	0.744	0.14	201	0.907	0.18
VI (0.3604)	109	1.436	0.27	89	0.9872	0.19
VII ($4.2x10^{-6}$)	109	$57.6x10^{-6}$	$0.11x10^{-5}$	207	$100.8x10^{-6}$	$0.21x10^{-6}$
VIII (0.0498)	109	0.3388	0.06	97	0.3745	0.07
IX (0.636)	101	2.188	0.42	89	1.6687	0.32
X (0.424)	109	1.646	0.32	89	1.247	0.24
XI (0.0636)	109	0.4237	0.08	200	0.7292	0.14
XII (0.530)	101	2.194	0.43	97	3.644	0.71

TABLE 2-3

Total quantity of nitrite-nitrogen consumed in various nitrite oxidizing media before attaining stationary phase

Media used and initial composition of NO_2^-–N ($g.L^{-1}$)	Primary Enrichment		Secondary Enrichment	
	No. of days	Consumption of NO_2^--N ($g.L^{-1}$)	No. of days	Consumption of NO_2^--N ($g.L^{-1}$)
1 (0.0608)	87	0.3578	200	0.6696
2 (0.139)	101	0.6995	200	1.5290
3 (0.050)	87	0.2 982	207	0.670
4 (0.507)	101	1.021	89	1.999
5 (0.2028)	101	0.6101	200	3.3736
6 (0.4056)	87	1.8261	210	5.9059

TABLE 2-4

Duration required for the onset of nitrification in two selected media on receiving treated sewage and filtrant grains of a biofilter

	Duration (in days)	
	1 mL sewage	1 gram filterant grains
Filter-1		
Medium XII	31	19
Medium 6	33	19
Filter-2		
Medium XII	42	14
Medium 6	42	14

TABLE 2-5

Consumption of ammonia-nitrogen and build up of nitrite-nitrogen in batch process(XII-I)of ammonia oxidizing consortium before attaining stationary phase

No. of days of incubation	Consumption of NH_4^+-N ($g.L^{-1}$)	Build up of NO_2^--N ($g.L^{-1}$)	Build up of NO_3^--N ($g.L^{-1}$)
1	-	-	-
8	0.218	0.042	-
13	0.530	0.091	-
16	0.891	0.174	-
20	1.06	0.200	-
27	1.59	0.310	-
29	1.84	0.361	-
32	2.12	0.409	-
36	2.43	0.458	-
43	2.65	0.504	-
52	3.18	0.600	-
64	3.33	0.647	-
73	3.71	0.708	-
82	3.94	0.764	-
94	4.18	0.811	-
112	4.205	0.817	-
121	4.24	0.823	-

TABLE 2-6

Consumption of ammonia-nitrogen and build up of nitrite-nitrogen in batch process(XII-2) of ammonia oxidizing consortium before attaining stationary phase

No. of days of incubation	Consumption of NH_4^+-N ($g.L^{-1}$)	Build up of NO_2^--N ($g.L^{-1}$)	Build up of NO_3^--N ($g.L^{-1}$)
1	-	-	-
8	0.243	0.035	-
12	0.53	0.100	-
18	0.79	0.158	-
25	1.06	0.204	-
33	1.31	0.259	-
46	1.59	0.304	-
55	1.86	0.371	-
67	2.12	0.406	-
72	2.65	0.509	-
76	3.18	0.601	-
80	3.71	0.711	-
84	4.24	0.796	-
90	4.77	0.914	-
96	5.3	1.218	-
105	5.83	1.218	-

TABLE 2-7

Consumption of ammonia-nitrogen and build up of nitrite-nitrogen in batch process (XII-3) of ammonia oxidizing consortium before attaining stationary phase

No. of days of incubation	Consumption of NH_4^+-N ($g.L^{-1}$)	Build up of NO_2^--N ($g.L^{-1}$)	Build up of NO_3^--N ($g.L^{-1}$)
1	-	-	-
11	0.52	0.1027	-
16	0.824	0.174	-
21	1.06	0.205	-
26	1.59	0.308	-
35	2.12	0.410	-
41	2.65	0.513	-
49	3.18	0.616	-
55	3.71	0.718	-
60	4.24	0.820	-
70	4.77	0.934	-
79	5.3	1.027	-
86	5.83	1.127	-
94	5.99	1.200	-
101	6.21	1.224	-
108	6.36	1.239	-

TABLE 2-8

Consumption of nitrite-nitrogen in batch process es(6-1,6-2 and 6-3) of nitrite oxidizing consortium before attaining stationary phase

Batch Fermentor 6-1		Batch Fermentor 6-2		Batch Fermentor 6-3	
No. of days	Build up of NO_2^--N ($g.L^{-1}$)	No. of days	Build up of NO_2^--N ($g.L^{-1}$)	No. of days	Build up of NO_2^--N ($g.L^{-1}$)
1	-	1	-	1	-
10	0.114	4	0.203	7	0.406
24	0.128	9	0.328	11	0.609
38	0.203	11	0.406	15	0.812
45	0.406	13	0.740	20	0.924
58	0.961	17	0.812	25	1.015
61	1.015	21	1.015	27	1.218
63	1.218	24	1.218	29	1.421
69	1.421	31	1.421	32	1.624
73	1.624	37	1.624	35	1.827
76	1.827	41	1.910	38	2.03
81	2.03	47	2.03	40	2.333
83	2.639	50	2.333	42	2.436
89	2.842	56	2.4	44	2.639
94	2.999	68	2.436	47	2.842
110	3.045	76	2.639	49	3.045
120	3.248	83	2.842	53	3.248
122	3.654	92	3.045	59	3.29
126	3.710	99	3.248	66	3.451
130	3.857	115	3.310	74	3.5
133	4.06	131	3.589	92	3.857
136	4.263	149	3.654	103	4.263
140	4.466	153	3.857	121	4.52
144	4.669	156	4.06	130	4.669
149	4.748	158	4.466	142	4.872
156	4.872	161	4.872	154	5.075
160	5.075	171	5.887	168	5.481
163	5.278	182	5.887	171	6.04
183	5.684			180	6.09

TABLE 2-9

Optimization of substrate concentration for ammonia oxidizing consortium

Concentration of NH_4^+–N used ($g.L^{-1}$)	NH_4^+–N consumed ($g.L^{-1}$)	Build up of NO_2^-–N ($g.L^{-1}$)
0.10	0.0336	0.0031
0.25	0.0384	0.00336
0.50	0.0456	0.004226
0.75	0.0480	0.00432
1.00	0.0504	0.00468
1.25	0.0528	0.00480
1.50	0.060	0.00552
1.75	0.0768	0.00696
2.00	0.0878	0.00816
2.25	0.1776	0.0163
2.50	0.3840	0.0355
2.75	0.4104	0.0384
3.00	0.3984	0.0369
3.25	0.3912	0.0362
3.50	0.3864	0.0357
3.75	0.3816	0.0352
4.00	0.3744	0.0348
4.25	0.3720	0.0345
4.50	0.360	0.0326
4.75	0.3480	0.0323
5.00	0.3408	0.0316

TABLE 2-10

Optimization of substrate concentration for nitrite oxidizing consortium

Concentration of NO_2^-–N used ($g.L^{-1}$)	Consumption of NO_2^-–N ($g.L^{-1}$)
0.1	0.0648
0.25	0.1368
0.50	0.3552
0.75	0.552
1.00	0.7656
1.25	0.7680
1.50	0.792
1.75	1.008
2.00	1.272
2.25	1.464
2.50	1.536
2.75	0.0096
3.00	0.0096
3.25	0
3.50	0
3.75	0
4.00	0
4.25	0
4.50	0
4.75	0
5.00	0

Table 2-11

Optimization of pH of ammonia oxidizing consortium

pH	Build up of NO_2^-–N ($g.L^{-1}$)
4.0	0
5.0	0
6.0	0
7.0	0
8.0	0.0024
8.5	0.0054
9.0	0.0022
9.5	0.0014
10.0	0.0014

TABLE 2-12

Optimization of pH of nitrite oxidizing consortium

pH	Consumption of NO_2^-–N ($g.L^{-1}$)
4.0	0.0088
5.0	0.0325
6.0	0.04319
7.0	0.08639
7.5	0.10
8.0	0.09526
8.5	0.07633
9.0	0.04733
9.5	0.03076
10.0	0.02899

TABLE 2-13

Optimization of temperature for ammonia oxidizing consortium

Temperature (°C)	Build up of NO_2^-–N ($g.L^{-1}$)
4	0.0011
20	0.0049
26	0.00576
28	0.00566
30	0.00572
35	0.00376
37	0.0029

TABLE 2-14

Optimization of temperature for nitrite oxidizing consortium

Temperature (°C)	Consumption of NO_2^-–N ($g.L^{-1}$)
4	0
20	0.09596
26	0.09610
28	0.09726
30	0.09598
35	0.06137
37	0.03629

TABLE 2-15

Ammonia oxidizing activity at various rates of air flow

Rate of air flow (Litres.Min^{-1})	Build up of NO_2^-–N (μg.L^{-1})
Static(0)	11.7
1	29.3
2	35.1
3	41.0
4	46.8
5	58.6
6	87.8
7	120

TABLE 2-16

Nitrite oxidizing activity at various rates of air flow

Rate of air flow (Litres.Min^{-1})	Consumption of NO_2^-–N (μg.L^{-1})
Static(0)	9
1	51.2
2	130.9
3	161.4
4	178.1
5	203.4
6	225.2
7	260

TABLE 2-17

Nitrifying Activity of Ammonia and Nitrite Oxidizing consortia at varying inoculum size under standard conditions

Volume of Ammonia oxidizing consortium used (in mL)	Nitrifying Activity (Build up of NO_2^-–N in $\mu M.Min^{-1}L^{-1}$ of air)	Volume of Nitrite oxidizing consortium used (in mL)	Nitrifying Activity (Consumption of NO_2^-–N in $\mu M.Min^{-1}L^{-1}$ of air)
0.1	0.742×10^{-4}	0.1	0.17×10^{-2}
0.25	0.174×10^{-3}	0.25	0.44×10^{-2}
0.5	0.272×10^{-3}	0.5	0.82×10^{-2}
0.75	0.502×10^{-3}	0.75	0.132×10^{-1}
1.0	0.714×10^{-3}	1.0	0.167×10^{-1}

CHAPTER 3

MASS PRODUCTION OF NITRIFYING CONSORTIA

Chapter 3

MASS PRODUCTION OF NITRIFYING CONSORTIA

3.1 Introduction

By comparing with the growth of most heterotrophic bacteria, growth of nitrifiers is a slow process. In liquid culture, maximum specific growth rates for both groups of nitrifiers are in the order of 1.0 to 2.0 d^{-1}, the fastest reported being 2.2 d^{-1} (doubling time 8h) for *Nitrosomonas europaea* (Skinner and Walker, 1961). Growth yields are also low. Skinner and Walker (1961) obtained a yield of 0.71 g dry weight. L^{-1} for *N. europaea* in continuous culture. Because of their autotrophic nature and the low energy yields obtained from the oxidation of ammonia and nitrite, the maximum cell yield is very low when compared to those of heterotrophs; and therefore biochemical studies on nitrification have been hindered by difficulties in obtaining sufficient cell biomass for analysis (Prosser and Cox, 1982).

Isolation of nitrifying bacteria in pure culture justifiably is recognized as a difficult matter (Clark and Schmidt, 1966). Contaminants build up rapidly and reach high population densities under conditions designed to favour the chemoautotrophic nitrifiers, especially in the case of *Nitrosomonas.* The maintainance of pure cultures once obtained is also very difficult and large-scale cultures are particularly susceptible to con-

tamination. It is an interesting consequence of mixed culture situation that nitrification reaction frequently are observed to proceed more rapidly in the presence of heterotrophic microorganisms than in pure culture (Gundersen, 1955). Organic metabolites formed by the chemosynthetic bacteria could readily account for any extensive development of heterotrophs in inorganic nitrification media, but the benefits of mixed culture to the autotroph are less obvious. The oft suggested possibility that organic compounds formed by the heterotrophs may stimulate growth of associated autotrophic nitrifiers is without experimental support (Clark and Schmidt, 1966).

The media used contained no available source of organic carbon, and as a result the growth of heterotrophic bacteria becomes limited and was dependent on the catabolic products of the nitrifying bacteria as a source of carbon (Jones and Paskins, 1982). In the light of all these findings, mass culturing of pure cultures of nitrifying bacteria was not attempted. Instead mass culturing of nitrifying bacterial consortia was conceived as a better alternative.

The nitrifying bacteria can be grown in large volume (20 litre); the ammonia - oxidizing bacteria by semicontinuous culture and the nitrite oxidizing consortium by continuous culture. These methods yield 0.5 g or more (wet weight) of cells daily (Watson *et al.*, 1981). But these large mass cultures should be inoculated with at least one liter of a turbid culture. If a heavy inoculum is not used, the culture either will not grow

or will undergo a very long lag period.

About 1 to 4 x 10^4 cells of *Nitrobacter* /µg. N are produced from the oxidation of nitrite (Belser, 1974, Ardakani *et al.*, 1974, Schmidt, 1974). Roughly 3 times this cell concentration has been shown to be supported from an equivalent ammonium-nitrogen oxidized by *Nitrosomonas* in soil with a continuous perfusion of ammonium (Volz *et al.*, 1975 a,b). The relationship of nitrite oxidation to cell density appears to be valid only for growing cultures since oxidation decreases as the cells become older (Hofman and Lees, 1952, Seeler and Engel, 1959, Gould and Lees, 1960).

The nitrifier cell activity in governed by Michaelis-Menton kinetics and the relationship between microbial specific growth rate and the concentration of a growth limiting substrate(s) is generally described by the Monod's(1949) equation (Prosser, 1989),

$$\mu = \frac{\mu max.S}{Ks + S}$$

where Ks = half rate saturation constant and equals the substrate concentration when the growth rate µ is equal to 0.5 µmax.

Short-term batch experiments are frequently used to determine kinetic constants and qualitative effects of substrate concentration, inhibitors etc and typically last for several hours (Prosser, 1989). The growth of nitrifying bacteria is usually measured by either substrate utilization or product formation. The benefits of measuring specific growth rate of

nitrifying bacteria by using changes in substrate and product concentration are thus enormous where alternative measurements based on enumeration or determination of biomass are not possible in nitrifying consortia. Under conditions of balanced growth, for example, during exponential phase in batch culture, product concentration increases exponentially.

Because of the narrow species diversity and the relative simplicity of the pathway of substrate oxidation, the effect of inorganic nitrogen compounds upon the growth and metabolism of the autotrophic nitrifiers is the most predictable aspect of the nitrogen cycle. The Km for ammonia oxidation by nitrifiers (*Nitrosomonas*) ranges between 0.06 - 5.6 while Km for nitrite oxidizers (*Nitrobacter*) falls between 0.06 - 8.4 (Sharma and Ahlert, 1977). According to Hoffman and Lees (1953) Ulken, (1963) Loveless and Painter (1968), Km for ammonium oxidation ranges between 1 and 10 mg $N.L^{-1}$, and, Km for nitrite oxidation ranges between 5 and 9 mg $N.L^{-1}$ in the same temperature range according to Lees and Simpson, (1959) Gould and Lees, (1960) Laudelout and Van Tichelen, (1960) and Ulken, (1963).

Gould and Lees (1960) found that greater cell densities could be maintained by continuous removal of nitrate by dialysis or by continuous culture. The intimate association of nitrification with carbon dioxide fixation may account for the greater rates of ammonium or nitrite oxidation and the lesser sensitivity to end product inhibition observed by growing

cells. The rate of nitrite oxidation is drastically reduced when the stationary growth phase is reached (Fliermans and Schmidt, 1975, Fliermans *et al.*, 1974).

One problem in the analysis of rate data for microbial systems involving nitrifying bacteria, with or without heterotrophic organisms is the estimation of nitrifier concentration for determination of specific growth rate, the yield co-efficient and the saturation constant. One approach to cultures involving both the nitrifiers and heterotrophs is to estimate the nitrifier fraction with the help of a yield co-efficient (Sawyer, 1940). Another approach for estimating the concentration of nitrifiers in a mixed culture has been proposed by Srinath *et al.,* (1974). They suggest that the specific activity of nitrifying bacteria is a constant at a given temperature and pH. A specific activity co-efficient (α = K mixed culture / K pure culture) is determined for a mixed sample culture and a pure culture of nitrifiers. It is argued that α equals the fraction of mixed culture TKN that is contributed by the nitrifiers. The concentration of the nitrifying bacteria is estimated by multiplying α by TKN.L^{-1} of the mixed culture. This approach has several drawbacks. It neglects substrate utilization for exogenous respiration or maintainance. The estimated generation time for *Nitrosomonas* sp. is in the range 8-36 hours and for *Nitrobacter* sp. in the range 12-59 hours (Sharma and Ahlert, 1977). It has been demonstrated (Belser and Schmidt, 1980, Keen and Prosser, 1987a) that product concentration increases exponentially dur-

ing batch growth of nitrifying bacteria. The slope of a semi-logarithmic plot of product concentration versus time is equivalent to the specific growth rate and provides similar values to those calculated as specific increases in cell concentration. Hoffmann and Lees (1952) and Koops (1969) have suggested that increases in NO_2^- - N concentration will decrease the efficiency with which ammonia oxidation is coupled to biomass formation as batch cultures age due to product inhibition.

The highest reported values for μmax is 0.087 h^{-1}, for *N.europaea* growing in a batch culture, although same strain in continuous culture had a μmax of 0.063 h^{-1} (Skinner and Walker, 1961). Values of μmax usually lie within the range 0.014 - 0.064 h^{-1}. Below the optimum pH value, specific growth rate falls off sharply. In the absence of substrate or product inhibition, exponential growth of ammonia oxidizers usually ceases when the medium becomes acidic unless the pH is re-adjusted, while that of nitrite oxidizers continues until nitrite is completely utilized. Both ammonia and nitrite oxidation follows first order kinetics.

When the growth of a batch culture is followed also by means of dry weight determinations, the growth parameters of primary interest are the cell yield (Y), the exponential growth rate and the duration of the lag phase. Sharma and Ahlert (1977) obtained cell yield 'Y' for ammonia oxidizers as 0.03 - 0.13 and 'Y' for nitrite oxidizers lies in the range 0.02 - 0.08. Estimated values of 'Y' from theoretical consideration on the thermodynamics of growth (Stratton and Mc Carty, 1967) are 0.29

for ammonia oxidation and 0.084 for nitrite oxidation.

Pure cultures of nitrifying bacteria can be stored in liquid nitrogen and can have a greater than 90% survival rate for an indefinite time period (Watson *et al.*, 1981). For storing the cultures in liquid nitrogen, Dimethyl Sulphoxide (DMSO) is added to an actively growing culture to a final concentration of 5%. An aliquot of the culture is then sealed in a 2 mL ampule, cooled at a rate of 4°C/minute in an acetone - dry ice bath to -20°C and then stored in a liquid nitrogen cryostat. Watson *et al.*, (1981)activated this cryopreserved culture by thawing the ampoule at 37°C and the culture was serially diluted into an appropriate medium. By serially diluting the culture, the DMSO concentration was diluted to a non-inhibitory level for growth, and any contaminants introduced during the freezing procedure were diluted out.

Lowering the temperature decreases the metabolic rate and in the extreme cases of liquid nitrogen at -196°C, to virtually nil. It is generally thought that the physiological age of cultures is also important in freezing preservation methods, with cells taken at the stationary phase being the more resistant to physical damage or cryoinjury. In the stationary phase, a proportion of cells will, of course, be lysed and it is possible that their cellular contents can act as protective agents for the survivors. Storage in liquid nitrogen (-196°C) is used to preserve bacteria and it appears to be widely applicable. There are several practical problems associated with liquid nitrogen storage. The

nitrogen continuously evaporates and will require a regular supply for replenishment. The room in which the containers are housed needs to be well ventilated and oxygen alarms should be mounted.

Liquid stock cultures of nitrifying bacteria can also be stored in the dark at 15°C and transferred every 4-6 months (Watson *et al.*, 1981.). The concentration of substrates in the cultures should be analysed periodically and increments added when needed. Prior to subculturing, the storage cultures should be incubated at 25°C for several days.

Freeze drying nitrifying bacteria is rarely attempted because of difficulties in obtaining sufficient quantities of biomass. Instead, cultures are usually stored in liquid medium, in sealed containers which are either refrigerated (Prosser, 1982) and subcultured every 4-6 weeks.

The three basic aims of maintainance and preservation of bacteria are to keep cultures viable, uncontaminated and unchanged in their properties. For very long term preservation, involving stocks of the strains, and where withdrawals from stocks are regularly made, a fourth aim is to have adequate stocks and appropriate systems for replenishing the stocks when necessary.

3.2. Materials and Methods

3.2.2 Mass culture of nitrifying consortia in a fermentor

3.2.2.1 Mass culture of ammonia oxidizing consortium

Mass culturing of ammonia oxidizing consortium was done

in medium XII selected as the best one for the ammonia oxidizing consortium (For more details see chapter 2). Using a 0.1% (v/v) strength inoculum from the cell concentrate obtained from the batch process XII-3 (See chapter 2 section 2.2.7 and 2.3.3) mass culturing of ammonia oxidizing consortium was done in a 2 litre capacity fermentor (New Brunswick Bioflo 2000). The pH was set at 8.5, temperature at 28°C, agitation at 200 rpm and aeration at 0.6 litres of air/minute. The initial substrate concentration at the time of inoculation was 0.120 $g.L^{-1}$ of NH_4^+ - N. The pH was maintained at 8.5 by addition of sterile 10% Na_2CO_3 solution by activating the base port. The fermentor culture vessel was covered with black cloth to protect the culture from light inactivation.

Nitrification in the system was followed by monitoring the depletion of NH_4^+ –N and the build up of NO_2^-–N, and NO_3^-–N (APHA, 1989, Strickland and Parsons, 1968). When the initial NH_4^+ - N was exhausted double the amount of initial NH_4^+ – N was aseptically added through the addition port. Each time when the substrate was totally consumed, fresh additions were made at an exponential rate until stationary phase was achieved, when, no more substrate was consumed further. Biomass was also estimated at regular intervals as described under section 3.2.3.

After attaining stationary phase (Fig.3-1), the culture was centrifuged at 10,000 xg in a cooling centrifuge (Remi Equipment, India) at a working temperature of 4°C for twenty minutes. The pellet was washed thrice and resuspended in fresh sterile medium XII and preserved at 4°C in a sterile saline bottle.

3.2.2.2 Mass Culture of nitrite oxidizing consortium

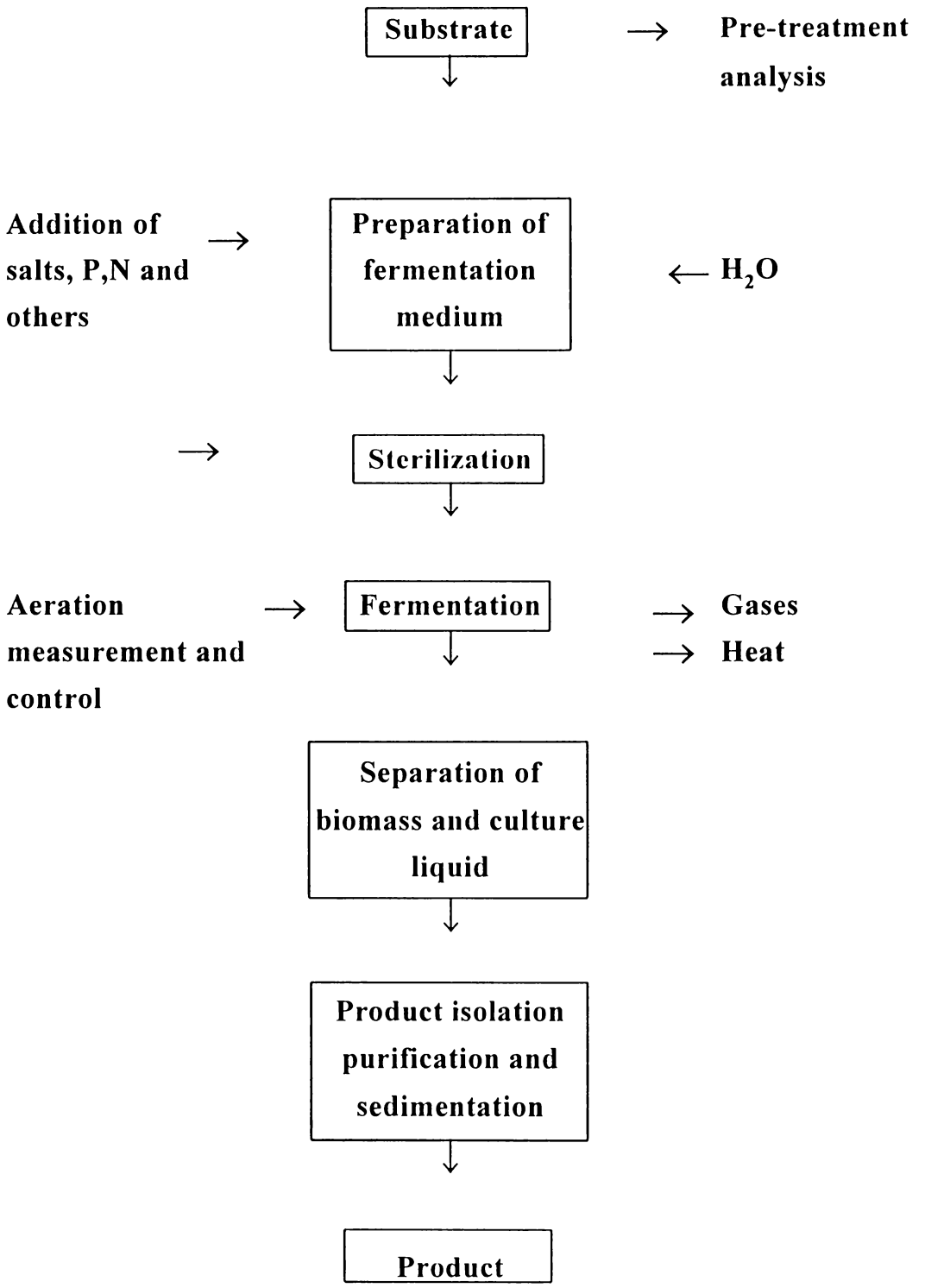
Mass culturing of nitrite oxidizing consortium was carried out in medium 6 selected as the best one for the nitrite oxidizing consortium (see chapter 2). Using a 0.1% (v/v) strength inoculum from the cell concentrate obtained from the batch process (see chapter 2 section2.2.7 and 2.3.3), mass culturing of nitrite oxidizing consortium was performed in a 2 litre capacity fermentor (New Brunswick, Bioflo 2000). The pH was set at 7.5, temperature at 28°C, agitation at 200rpm and aeration at 0.6 litres/minute. The initial substrate concentration at the time of inoculation was $0.01 g.L^{-1}$ of NO_2^- – N.Since there was no notable fall in pH, addition of 10% Na_2CO_3 was not required . The fermentor vessel holding the medium was covered with black cloth to prevent light inhibition.

Nitrite concentration in the fermentor was monitored daily to determine the degree of nitrification taking place following Strickland and Parsons (1968). When the initial NO_2^- – N was

exhausted, double the amount of the initial NO_2^-–N was aseptically added through the addition port. Each time when the substrate was totally consumed, fresh additions were made at an exponential rate until stationary phase was attained when no more substrate was consumed. Biomass was also estimated at regular intervals as described under section 3.3.3.

After attainment of stationary phase (Fig. 3-2), the culture was centrifuged at 10,000 x g in a cooling centrifuge (Remi Equipment, India) at a working temperature of 4°C for twenty minutes. The pellet was washed thrice and resuspended in fresh sterile medium 6 and preserved at 4°C in a sterile saline bottle.

3.2.2.3 Schematic representation of processes in a fermentation system



3.2.3 Determination of biomass

At different intervals of time, 1 mL of each of the culture from both ammonia and nitrite oxidizing consortia was drawn aseptically from the fermentor and filtered through a dried, preweighed membrane filter disc (Millipore Pvt. Ltd., Bangalore) having pore size 0.22 μm. The filter disc was then dried in an hot air oven set at 80°C till constant weight was obtained. The difference in weight gave the dry weight of biomass per mL of the culture and recorded as per litre of the culture.

3.2.4 Relationship between substrate uptake and product formed

The relationship between the percentage consumption of NH_4^+–N and buildup of NO_2^-–N and the percentage consumption of NO_2^-–N and build up of NO_3^-–N of ammonia and nitrite oxidizing consortia respectively were worked out from the overall consumption of substrate and build up of the products under optimum growth conditions in the fermentor.

3.2.5 Enumeration of nitrifying consortia developed in fermentor

Both ammonia and nitrite oxidising consortia developed in the fermentor were enumerated based on the following relationship (Belser, 1974; Ardakani *et al.,* 1974a, Schmidt, 1974; Volz *et al.,* 1975a, b) :

a. Ammonia oxidizers = $3 \times 10^4 - 1.2 \times 10^5$ cells/μg NH_4^+–N oxi-

dized.

b. Nitrite oxidizers = 1 – 4 x 10^4 cells/µg NO_2^-–N oxidized.

3.2.6 Alkalinity – pH relationship

Alkalinity–pH relationship of ammonia oxidizers is of great importance in their mass production. Nitrification stops when pH drops to 5.5 and it is adjusted to 8.5 using 10% sodium carbonate. Therfore the alkalinity destroyed due to growth of nitrifiers, espec ially ammonia oxidizers, is calculated based on the relationship: 6.0 – 7.4 mg alkalinity is destroyed per milligram NH_4^+–N oxidized to nitrate (EPA, 1975).

3.2.7 Determination of yield co-efficient or cell yield 'Y' of the consortia

Yield co-efficient can be defined as the ratio of weight of cells generated to the weight of substrate oxidized. In terms of product formed, yield co-efficient can be also defined as the ratio of weight of product formed to the weight of substrate utilized following Sharma and Ahlert (1977).

$$\text{Yield co-efficient 'Y'} = \frac{\text{Weight of cells generated}}{\text{Weight of substrate oxidized}}$$

or

$$\frac{\text{Weight of product formed}}{\text{Weight of substrate utilized}}$$

The yield co-efficient was calculated for the mass culture of both ammonia and nitrite oxidizing consortia in fermenter based on the weight of biomass generated, weight of product formed and weight of substrate oxidized.

3.2.8 Determination of generation time (tg) during the mass culturing of ammonia and nitrite oxidizing consortia

Generation time or doubling time during the mass culturing of consortia can be defined as the time taken for the biomass to double its initial amount and the substrate to double its consumption or the product to double its build up.

Thus, for ammonia oxidizing consortium, the generation time is the time taken for the doubling in the building up of NO_2^-–N or biomass to become double of its initial value. For nitrite oxidizing consortium generation time is the time taken for doubling the consumption of NO_2^-–N or biomass to become double of its initial value.

3.2.9 Specific Growth rate (μ)

The doubling time of bacteria is always inversely proportional to its specific growth rate or, the product of doubling time(g) and specific growth rate (μ) is a constant,

That is,

$$\mu g = 0.693$$

or

$$\mu = \frac{0.693}{g}$$

The specific growth rate μ of ammonia and nitrite oxidizing consortia were calculated from their generation time.

3.2.10 Batch experiment to determine Km and Vmax

A substrate concentration ranging from 100 - 1000μg.L^{-1} of NH_4^+–N and NO_2^-–N were prepared in medium XII for ammonia oxidizers and medium 6 for nitrite oxidizers with 1% v/v inoculum of ammonia and nitrite oxidizing consortia and flasks were incubated in dark in a shaker (Orbitek, India) at 100 rpm. The concentration of NO_2^-–N build up along with consumption of NH_4^+–N in medium XII and consumption of NO_2^-–N in medium 6 were determined by estimating NH_4^+–N and NO_2^-–N concentrations spectrophotometrically following Strickland and Parsons (1968) and APHA (1989). A Lineweaver – Burke plot was drawn for both the batch culture studies from which Km was found out.

The point at which the line intersected at Y axis was 1/Vmax from which Vmax was calculated. Slope was calculated as

$$\frac{Km}{Vmax}$$

3.3 Results and Discussion

3.3.1 Substrate consumption and product build up

Consumption of NH_4^+-N, built up of NO_2^--N and the production of biomass before attaining stationary phase of growth during the mass production of ammonia oxidizing consortium in the fermentor are summarized in Fig. 3-1 and Table 3-1. It could be seen that on consuming 4.29 $g.L^{-1}$ NH_4^+-N by ammonia oxidizers, only 0.834 $g.L^{-1}$ of the product, nitrite, was formed. The biomass build up was 0.80 $g.L^{-1}$ during a total incubation period of 30 days.

Consumption of NO_2^-–N and build up of NO_3^-–N and biomass for a total incubation period of 25 days before attaining stationary phase of growth during the mass production of nitrite oxidizing consortium are summarized in Fig. 3-2 and Table 3-2. On consuming 6.246 $g.L^{-1}$ of NO_2^-–N, by nitrite oxidizers, 1.242 $g.L^{-1}$ NO_3^-–N was produced and the biomass build up was 1.09 $g.L^{-1}$.

The relationship between percentage consumption of NH_4^+–N and the build up of NO_2^-–N by ammonia oxidizing consortium and the relationship between percentage consumption of NO_2^-–N and the build up of NO_3^-–N by nitrite oxidizing consortium are summarized in Table 3-3 and 3-4. It was observed that only 19.39 per cent of the ammonia consumed was oxidized to NO_2^-–N and the balance of 80.61 percent was used for some other purpose by ammonia oxidizing consortium. Similarly only 19.88 percent of NO_2^-–N consumed by nitrite oxidiz-

ing consortia was oxidized to NO_3^-–N and the rest was used up for some other purposes.

In the mass production of ammonia and nitrite oxidizing consortia in fermentor very low concentration of substrate (0.120 $g.L^{-1}$ NH_4^+–N) was initially given which was exponentially increased as and when they got exhausted till a stationary phase was attained. During this oxidation process, the build up of NO_2^-–N by the ammonia oxidizers was approximately 5.15 times less than the amount of NH_4^-–N consumed. In the same pattern biomass build up was also much lesser, 5.36 times lesser than the ammonia oxidized comparable to the quantity of nitrite generated. Similarly the genreation of NO_3^-–N by nitrite oxidizers was 5.02 times lesser than the NO_2^-–N consumed and in the same order the biomass built up was 5.73 times lesser than the substrate consumed. Analysis of the data thus reveals that the biomass of nitrifying bacterial consortia built up in a fermentor is receiprocal to the product of oxidiation such as NO_2^-–N in the case of ammonia oxidizers and NO_3^-–N in the case of nitrite oxidizers. This relationship is highly advantageous in the mass production of nitrifying consortia in a fermentor where the biomass can be indirectly measured by estimating the product of oxidation colourimetrically which can be done more accurately than the gravimetric estimation of the biomass.

Irrespective of the difference in the overall consumption of the substrate by the corresponding groups of nitrifying bacteria percentage of the product formed is the same (19.39–19.88) and the balance of 80.61 to 80.12 per cent of the substrate is used up for some other purpose. Prosser (1989) on working with pure cultures of *Nitrosomonas* and *Nitrobacter* observed that 76% of NH_4^+–N and 81% of NO_2^-–N oxidized were used up for maintainance and not for cell yield, and the same is found more or less applicable in the case of nitrifying consortia also.

3.3.2 Enumeration of nitrifying consortia

Both ammonia and nitrite oxidizing consortia mass cultured in the fermentor were enumerated and represented in Table 3-5. When the total ammonia oxidizers ranged from 2.496×10^{10} to 9.984×10^{10} cells per litre, the nitrite oxidizers ranged from 1.242×10^{10} to 4.968×10^{10} cells per litre. The difference in the cell output in both the consortia was marginal. According to Alexander (1965) *Nitrosomonas* oxidize about 35 atoms of nitrogen and *Nitrobacter* oxidizes 100 atoms of nitrogen (2.86 times more) for fixation of a molecule of CO_2. Considering this as the stoichiomentry of nitrification, the ammonia and nitrite oxidizing consortia in fermentor behave almost in the same pattern. To support more or less the same cell density/biomass, 1.5 times more of NO_2^-N had to be oxidized by nitrite oxidizing consortia than the ammonia oxidizers. Observations more or less compa-

rable to this have been found in literature. About 1 to 4 x 10^4 cells of *Nitrobacter* per kg N are produced from the oxidation of nitrite (Belser, 1974, Ardakani *et al.*, 1974 and Schmidt, 1974). Roughly 3 times this cell concentration has been shown to be supported from an equivalent of ammonia nitrogen oxidized by *Nitrosomonas* in soil with a continuous perfusion of ammonium (Volz *et al.*, 1975 a and b).

The marginal difference in the cell density between the consortia implies that the stationary phase is related to both cell number and the product accumulated as far as the present nitrifying consortia are concered.

3.3.3 Alkalinity – pH relationship

Based on the lowering of pH during the growth of ammonia oxidizers, the quantity of 10% sodium carbonate required for compensating the alkalinity destroyed on adjusting the pH to 8.5 was observed to be 25 mL (Table 3-6) during a 30d. period of incubation. When 4.29g NH^+_4-N was oxidized, 25.74-31.74 g. alkalinity was destroyed. This estimation has been made based on EPA(1975) where 6.0-7.5 mg alkalinity was determined to be destroyed per mg of NH^+_4–N oxidized to NO^-_3-N. These estimations are useful for predicting the requirement of Na_2CO_3 during mass production of the ammonia oxidizing consortium in fermentor by batch process. As has been estimated here 5.83 mL 10% Na_2CO_3 are required for every gm

NH^{+}_{4}-N oxidized. That is 25.74 to 31.74 gms of alkalinity was equivalent to 25mL of 10% $Na_2 CO_3$ solution.

3.3.4 Yield Co-efficient

The yield co-efficient 'Y' for ammonia and nitrite oxidizing consortia were determined and summarized in Table 3-7. It was observed that the yield co-efficient of ammonia oxidizing consortium ranged from 0.1864 to 0.1939 on calculating it both ways. Similarly, the yield co-efficient of nitrite oxidizers ranged from 0.1745-0.1988. But the estimated values of 'Y' from theoretical considerations on the thermodynamics of growth (Stratton and Mc Carty, 1967) are 0.2g from ammonia oxidation and 0.084 for nitrite oxidation. In the same fashion, Sharma and Ahlert (1977) obtained yield co-efficient 'Y' for ammonia oxidizers as 0.03 - 0.13 and for nitrite oxidizers as 0.02 to 0.08. Compared to these values, the yield co-efficient obtained here for ammonia and nitrite oxidizing consortia are very much higher. As NH_4^+–N and NO_2^-–N are the sole source of energy supplied in the media, the higher yield co-efficient obtained can be indicated as the better energy conversion efficiency and higher carbondioxide fixation potential of the consortium. Besides this can also be due to the fact that heterotrophs are also associated with the chemolithotrophs as integral part of the consortium. These heterotrophs live on the exudates/extrametabolites of chemolithotrophs as 'scavengers' and use them up as carbon and energy and probably as nitrogen source also.

This 'secondary process' adds to the total biomass generated and reasonably justifies the comparatively higher yield co-efficient obtained. This undoubtedly is a reflection of efficiency of consortium than pure cultures as far the energy assimilatory capability is concerned.

3.3.5 Generation Time

The Generation time of ammonia oxidizing consortium was calculated from Table 3-8 and Fig. 3-3 based on the quantity of NO_2^-–N built up over a period of time. Eventhough this can be worked out from the doubling time of biomass as far as the nitrifying consortia are concerned, the values determined may not be very much reliable as there is attached growth of organisms on the culture vessel and rotor which cannot be quantified *in toto*. From Table 3-8 and Fig. 3-3, it is clear that the duration required for doubling NO_2^-–N output increased from 24 hours in the initial phase upto 158.4 hours on attaining stationary phase. Therefore, the duration required for doubling the nitrite build up from 0.0006 to 0.0012 $g.L^{-1}$ was considered as the generation time of the ammonia oxidizing consortium for the reasons discussed later.

Similarly, generation time of nitrite oxidizing consortium was calculated from Table 3-9 and Fig. 3-4 based on the quantity of nitrite consumed over a period of time. Eventhough it can be worked out, as stated above, from the biomass build up, the attached growth of nitrifiers on the culture vessel and the rotor makes it an acceptable

method. From Table 3-9 and Fig. 3-4, it is apparent that the duration required for doubling the consumption of NO_2^-–N progressively increased from 16.8 hours in the initial phase of growth to 72 hours in the stationary phase. Therefore, the duration required for doubling the NO_2^-–N consumption from 0.0058 $g.L^{-1}$ to 0.0116 $g.L^{-1}$ was taken as the generation time of the culture which was found to be 16.8 hours for reasons discussed later.

The estimated generation time for *Nitrosomonas* species is in the range 8-36 hours and for *Nitrobacter* in the range 12-59 hours (Sharma and Ahlert, 1977). The generation time of both the consortia of nitrifying bacteria falls within this range. Meanwhile Skinner and Walker (1961) reported an extremely low generation time of 8 hrs for *N. europaea*.

Growth of nitrifying bacteria in batch systems follow the first order kinetics, ie., the rate is proportional to the substrate concentration at low substrate concentration, and later follow the zero order kinetics, ie., the initial rate is independent of substrate concentration at high substrate concentration (Downing *et al.,* 1964, Melamed *et al.,* 1920, Poduska and Andrews, 1975, Williamson and Mc Carty, 1975, Gujer 1977)

This is because of the product inhibition where the ammonia oxidizers are inhibited by NO_2^- and nitrite oxidizers by NO_3^-. Substrate

was never a limiting factor as it was added exponentially. This led to a declining in the rate of oxidation of NH_4^-–N and NO_2^-–N in the ammonia and nitrite oxidizing consortia respectively. Therefore the time required for doubling the nitrite build and nitrite consumption for the ammonia and nitrite oxidizing consortia respectively in fermentor was considered for working out the generation time of both the groups of nitrifying consortia.

3.3.6 Specific Growth rate (μ), Maximum specific growth rate (μmax) and Michaelis-Menton constant Km

The specific growth rate of ammonia and nitrite oxidizing consortia were determined and summarized in Table 3-10. Based on the generation time of these consortia in the fermentor, ammonia and nitrite oxidizing consortia have specific growth rate of 0.0288 h^{-1} and 0.041.h^{-1} respectively.

Using a batch experiment, Km and Vmax (μmax) of both ammonia and nitrite oxidizing consortia were determined. As summarized in Table 3-11 and Fig. 3-5, ammonia oxidizing consortium has a Km of 0.5 mg. L^{-1} and μmax 0.0082 hr^{-1}. At the same time, the nitrite oxidizers have a Km value of 0.370 mg.N.L^{-1} and μmax of 0.0232.h^{-1} as shown in Table 3-12 and Fig. 3-6.

Since the specific growth rate is dependant on generation time, the nitrifying consortia registered a very low specific growth rate in fermentor. The reason for a low specific growth rate and cell yield is

the small energy gain obtained from oxidation of ammonia on nitrite. (Prosser, 1989). On studying the growth parameters of *Nitrosomonas europaea* in the presence and absence of ammonia-treated vermiculate (AVT), Armstrong and Prosser (1988) noticed the specific growth rate ranging from 0.038 to 0.060 h^{-1}which is well comparable with the specific growth rate obtained with consortium in the present study.

According to Hoffman and Lees (1953), Ulken (1963). Loveless and Painter (1968), Km for ammonia oxidizers range between 1 and 10 $mg.N.L^{-1}$ and km for nitrite oxidizers ranges between 5 and 9 $mg.N.L^{-1}$. Later Sharma and Ahlert (1977) reported Km for *Nitrosomonas* between 0.06 - 5.6 $mg.N.L^{-1}$ and for nitrite oxidizers between 0.06-8.4 $mg.N.L^{-1}$. The Km values observed for the present nitrifying consortia are well with in the range of the earlier reported values and fall with in a lower order of magnitude. Since smaller Km values indicate maximal substrate conversion efficiency at low substrate concentration, these consortia can be designated more efficient than pure cultures. This is an added advantage of opting for consortia than for pure cultures for the bioreactor.

In liquid cultures, the maximum specific growth rate (μmax) for both the groups of nitrifiers are in the order of 0.04167 to 0.08333 h^{-1}. The fastest reported being $0.09167.h^{-1}$ (Skinner and Walker, 1961). Maximum specific growth occurs at high substrate concentrtion and it is a measure of its overall growth potential and lies with in the

range 0.014-0.064 h^{-1} (equivalent to doubling time of 50-11 h (Prosser, 1989). In the present situation, the maximum specific growth rate of both ammonia and nitrite oxidizing consortia are with in the range of pure cultures but with a lower order of magnitude of 0.0082.h^{-1} and 0.0232 h^{-1} respectively with ammonia and nitrite oxidizers. Difference in the maximum specific growth rate between the ammonia and nitrite oxidizing consortia is very high and is found to be influenced by the generation time as the generation time of nitrite oxidizing consortium was much lesser than that of the ammonia oxidizers.

When the values of μ and μmax were compared as given in Table 3-10, 3-11 and 3-12. μmax was found less than μ. This was because in the batch experiment carried out to find Km and Vmax, aeration was nil other than the agitation given by placing the flask on rotary shaker. Meanwhile, in the mass culture of the consortia in fermentor where from μ was calculated, 0.6 litre air per minute was given. It appeared that in the absence of aeration, Vmax decreased reflecting the importance of aeration in nitrifying kinetics. It implies that in an aerated system, Km values also could have been much less compared to the present values.

The foregoing discussion justifies the development of consortia of nitrifying bacteria in the place of pure cultures for the bioreactor. For all practical purposes, the behaviour of consortia was very much comparable to pure cultures recorded in literature. This includes the

percentage of substrate oxidized out of the total quantity consumed, total cell density on attaining stationary phase, alkalinity destruction by ammonia oxidizers, generation time, specific growth rate (μ) and maximum specific growth rate (μmax) and Km values. However, the consortia exhibited higher yield coefficient and thereby better efficiency.

Above all, isolation of nitrifying bacteria in pure cultures is justifiably recognized as a difficult process and contaminants build up rapidly and reach high population densities under conditions specifically meant for chemolithotrophs (Clark and Schmidt, 1966). Moreover, as Gundersen, (1955) pointed out during mixed culture situation, nitrification reaction are frequently observed to proceed more rapidly in the presence of heterotrophic microorganisms than in pure cultures.

The consortia thus developed could be maintained at 4°C for more than a year without apparent loss of viability, since relatively no growth at and below 4°C temperature occured.

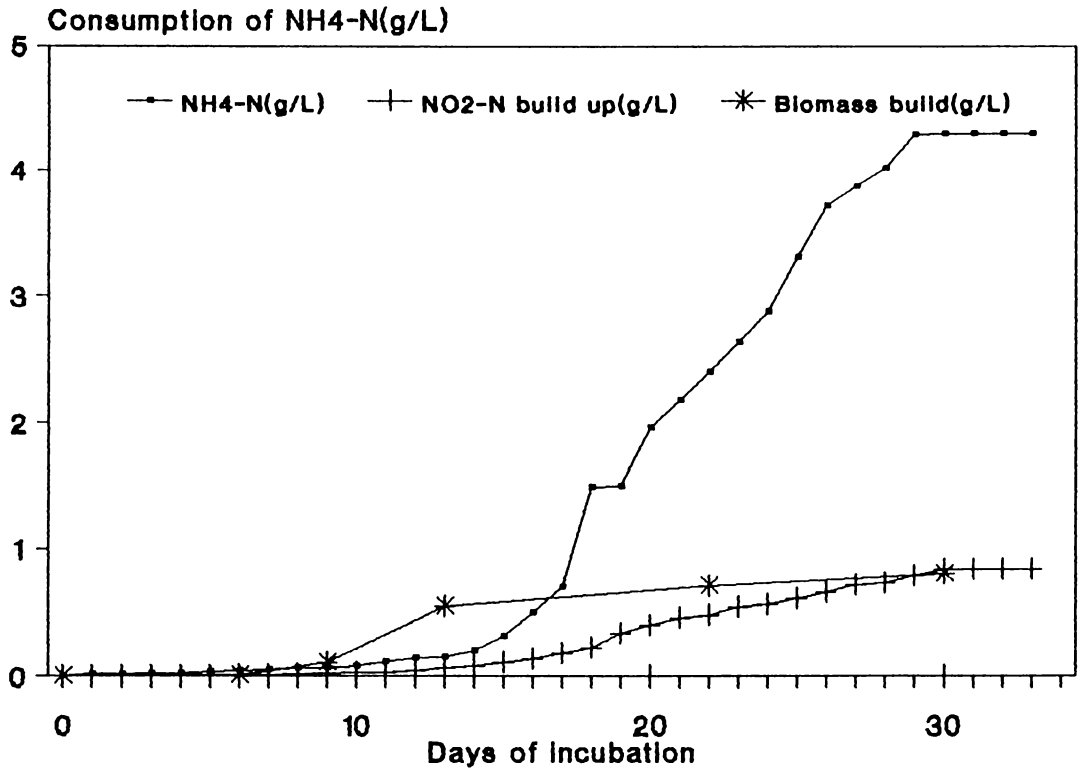


Fig 3-1 Ammonia consumption, nitrite build up and biomass generation during mass culture of ammonia in fermentor.

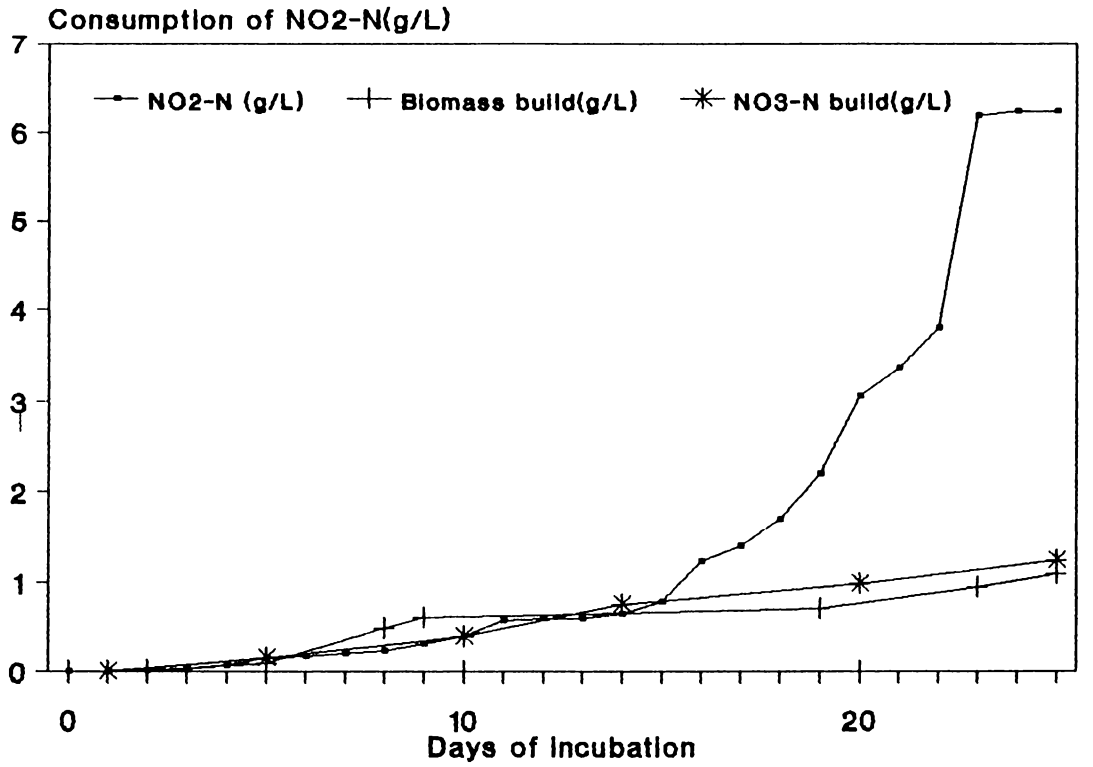


Fig. 3-2 Nitrite consumption, nitrate build up and biomass generation during mass culture of nitrite oxidizing consortium in a fermentor.

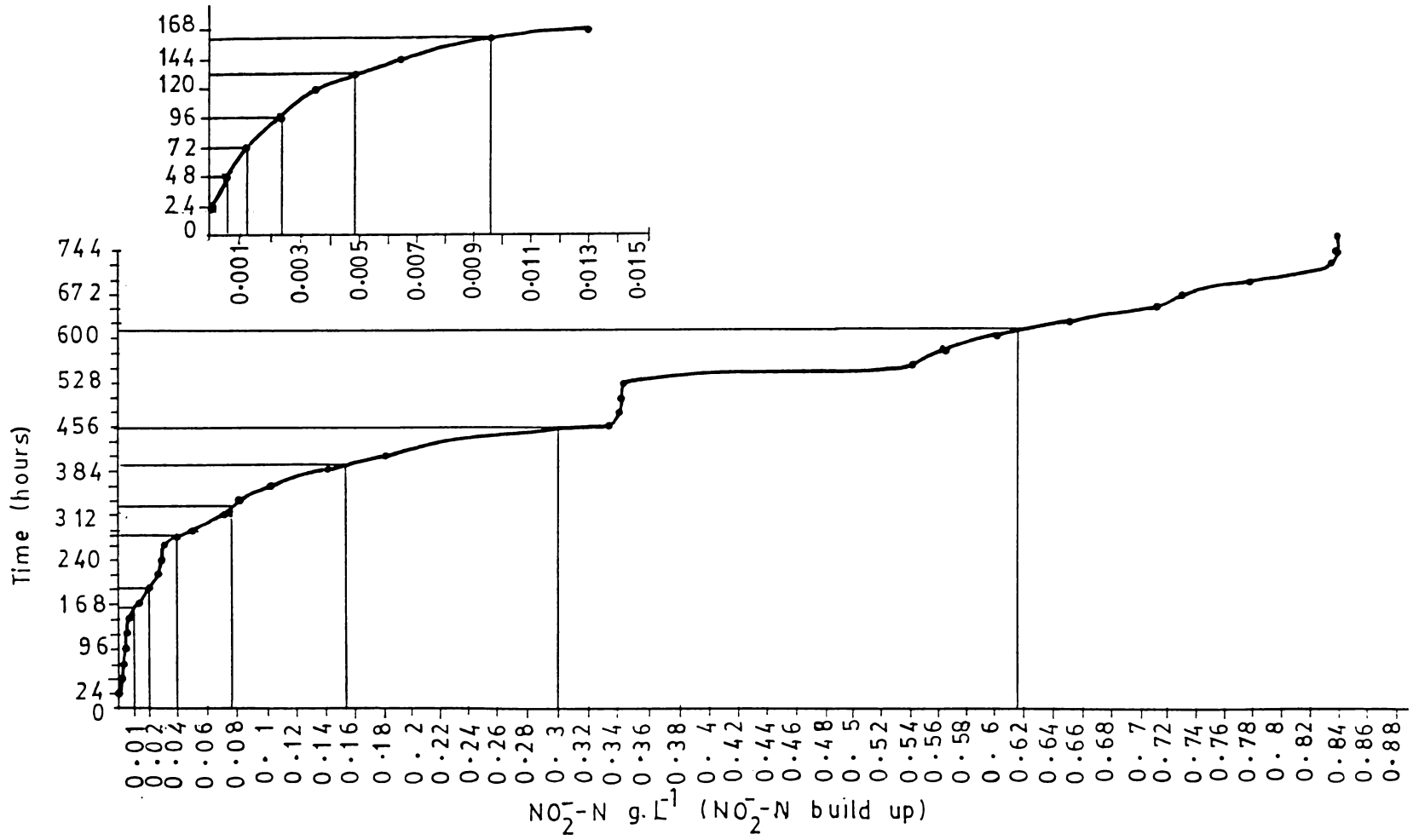


Fig. 3-3 Determination of generation time of ammonia oxidizing consortium.

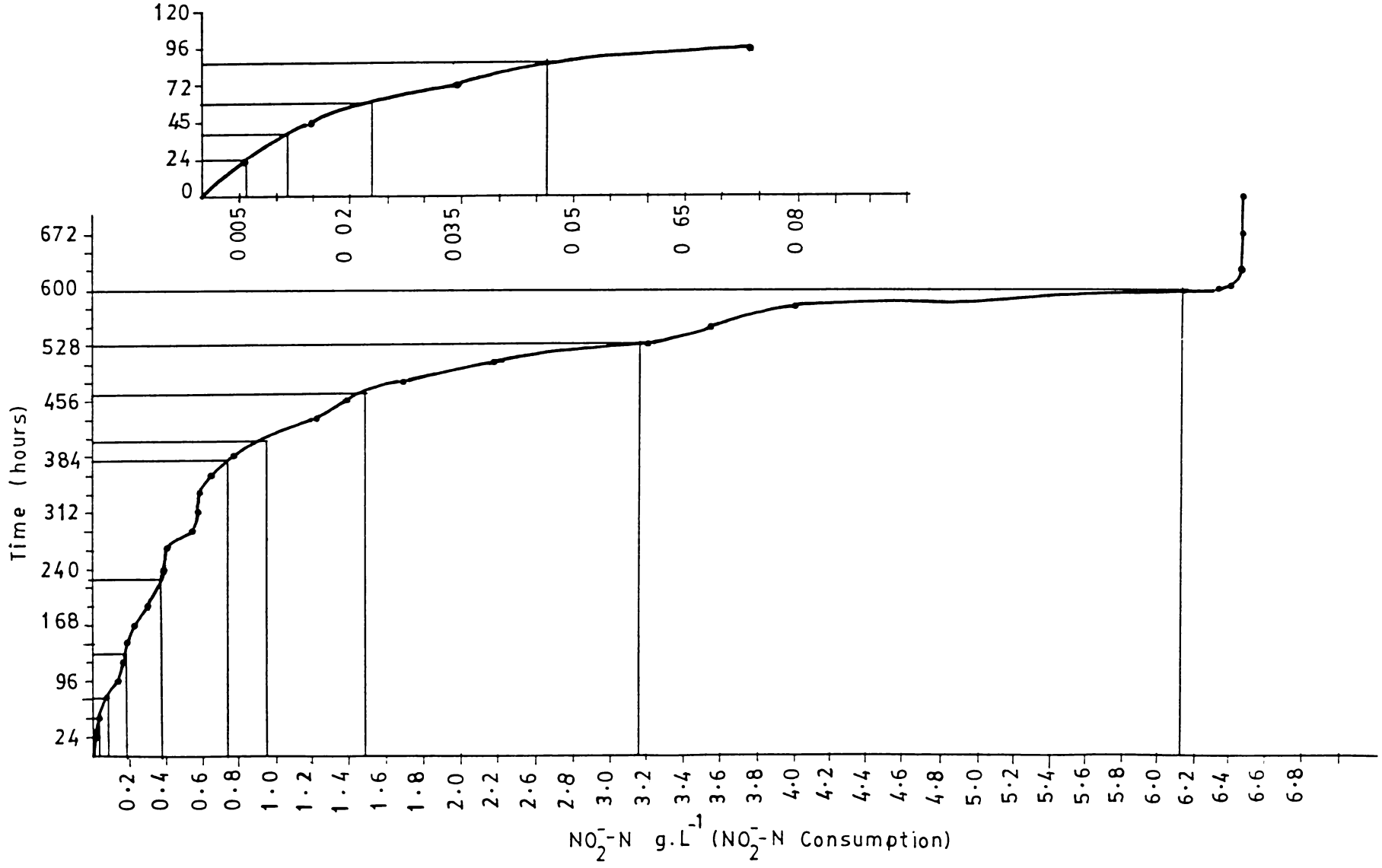


Fig. 3-4 Determination of generation time of nitrite oxidizing consortium.

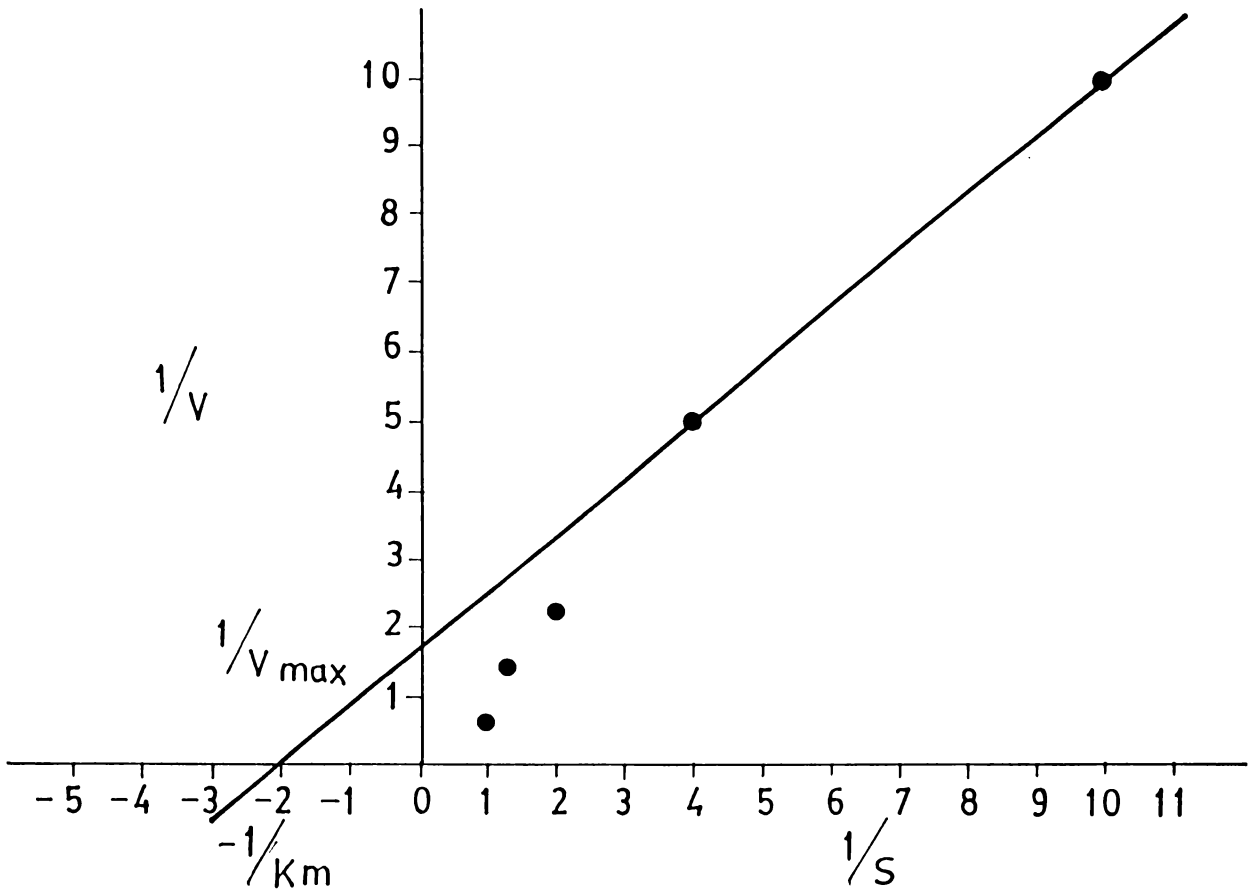


Fig. 3-5 Line weaver - Burke plot for substrate utilization by ammonia oxidizing consortium.

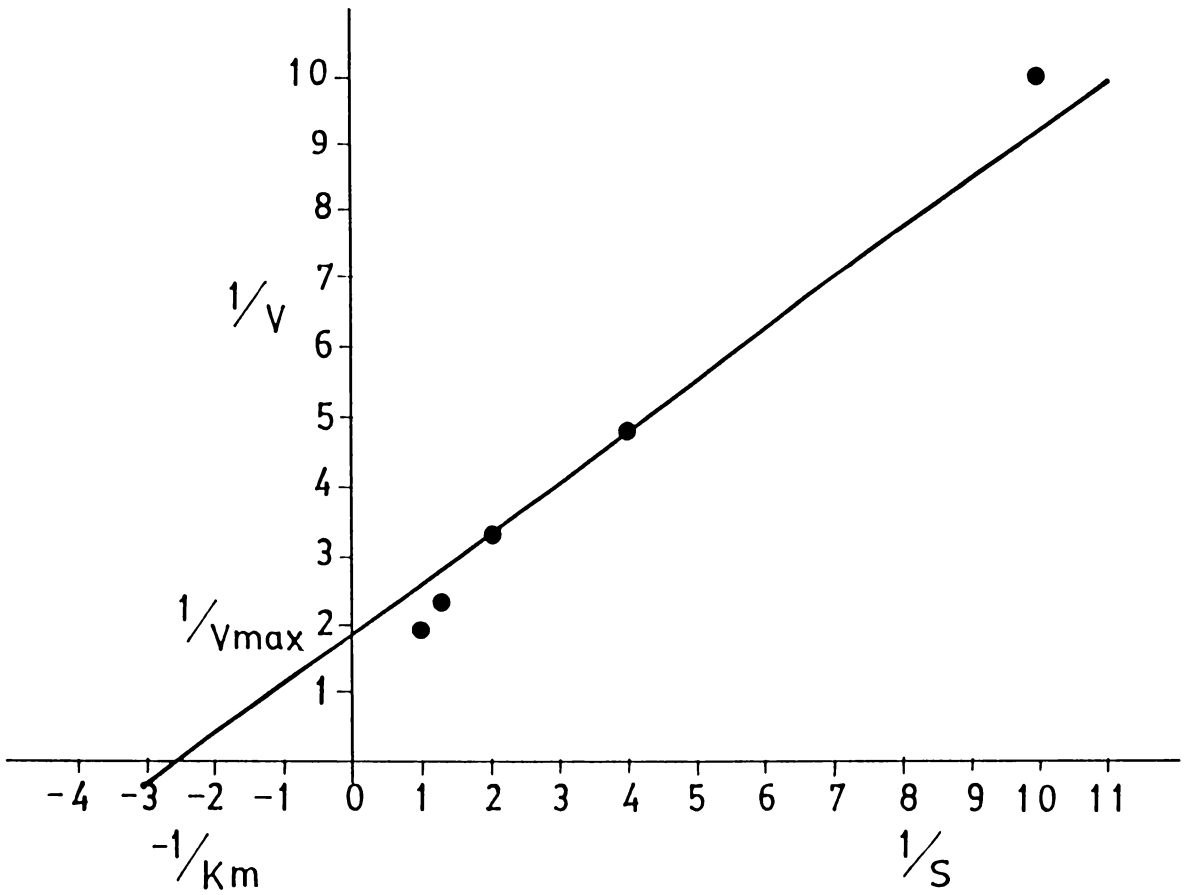


Fig. 3-6 Line weaver - Burke plot for substrate utilization by nitrite oxidizing consortium.

TABLE 3-1.

Substrate consumption and product build up during the mass production of ammonia oxidizing consortium in a 2 litre fermentor

Days of incubation	Consumption of NH_4^+–N ($g.L^{-1}$)	NO_2^-–N build up ($g.L^{-1}$)	Biomass build ($g.L^{-1}$)
0	0	0	0
1	0.016	0	
2	0.02	0.0006	
3	0.022	0.00118	
4	0.024	0.00235	
5	0.041	0.00353	0.01
6	0.046	0.00643	
7	0.053	0.01283	
8	0.064	0.02045	
9	0.066	0.0254	0.11
10	0.081	0.0292	
11	0.117	0.02975	
12	0.148	0.0495	
13	0.15	0.07045	0.55
14	0.2	0.08195	
15	0.319	0.11115	
16	0.499	0.14045	
17	0.7	0.18085	
18	1.481	0.2247	
19	1.489	0.33435	
20	1.96	0.4	
21	2.18	0.46	
22	2.4	0.48	0.71
23	2.632	0.543	
24	2.874	0.564	
25	3.3	0.604	
26	3.705	0.657	
27	3.863	0.713	
28	4.008	0.729	
29	4.281	0.788	
30	4.287	0.832	0.8
31	4.289	0.833	
32	4.29	0.834	
33	4.29	0.834	

TABLE 3-2.

Substrate consumption and product build up during the mass production of nitrite oxidizing consortium in a 2 litre fermentor

Days of incubation	Consumption of NO_2^-–N ($g.L^{-1}$)	NO_3^-–N build up ($g.L^{-1}$)	Biomass build ($g.L^{-1}$)
0	0	0	0
1	0.0058	0.0051	
2	0.01415		
3	0.03415		
4	0.07415		
5	0.15415	0.149	0.09
6	0.17155		
7	0.20145		
8	0.23415		0.48
9	0.31415	0.389	0.6
10	0.39415		
11	0.40315		
12	0.57115		
13	0.58715		
14	0.59415		
15	0.64525	0.750	
16	0.77695		
17	1.23195		
18	1.39415		
19	1.69055		
20	2.19415	0.980	0.7
21	3.04615		
22	3.35825		
23	3.79415		0.94
24	6.18895		
25	6.24615	1.242	1.09
26	6.24615		
27	6.2.615		

TABLE 3-3

Relationship between percentage consumption of NH_4^+–N, build up of NO_2^-–N and biomass of ammonia oxidizing consortium in fermentor

Consortium	NH_4^+–N consumption ($g.L^{-1}$)	NO_2^-–N build up ($g.L^{-1}$)	Biomass generated ($g.L^{-1}$)	% oxidation of NH_4^+–N	% used up for other purposes
Ammonia oxidizing	4.29	0.832	0.80	19.39	80.61

TABLE 3-4

Relationship between percentage consumption of NO_2^-–N, build up of NO_3^-–N and biomass of nitrite oxidizing consortium in fermentor

Consortium	NO_2^-–N consumption ($g.L^{-1}$)	NO_3^-–N build up ($g.L^{-1}$)	Biomass generated ($g.L^{-1}$)	% oxidation of NO_2^-–N	% used up for other purposes
Nitrite oxidizing	6.426	1.242	1.090	19.88	80.12

TABLE 3-5

Enumeration of nitrifying consortia based on substrate consumption in the fermentor on attaining stationary phase

Relationship between substrate oxidized and the number of cells generated	Quantity of substrate consumed $\mu g.\ L^{-1}$	Quantity of substrate oxidized $\mu g.L^{-1}$	Number of cells generated (per litre)
1. Ammonia oxidizers 3×10^4–1.2×10^5 cells/ $\mu g\ NH_4^+$–N oxidized	4.29×10^6	0.832×10^6	2.496×10^{10}– $9.984 \times 10^{10}\ L^{-1}$
2. Nitrite oxidizers 1–4 x 10^4 cells/μg NO_2–N oxidized	6.426×10^6	1.242×10^6	1.242×10^{10}– $4.968 \times 10^{10}.\ L.^{-1}$

TABLE 3-6

Alkalinity destroyed during mass production of ammonia oxidizing consortium (6.0 - 7.4 mg alkalinity destroyed per mg NH_4^+–N oxidized to NO_3^-–N (EPA, 1975)

Total NH_4^+–N removed (g. L^{-1})	Total alkalinity destroyed (g)	Volume of 10% sodium carbonate used to neutralize acid production
4.29	25.74 - 31.74	25 mL

TABLE 3-7

Yield coefficient (Y) of ammonia and nitrite oxidizing consortia recorded in a fermentor

Consortium	Mass of cells for used (a)	Mass of substrate utilized (b)	Mass of Product formed (c)	$Y = \frac{a}{b}$	$Y = \frac{c}{b}$
Ammonia oxidizing	0.80g.L^{-1}	4.29 g.L^{-1}	0.832g.L^{-1}	0.1864	0.1939
Nitrite oxidizing	1.09g.L^{-1}	6.2461g.L^{-1}	1.242 g.L^{-1}	0.1745	0.1988

TABLE 3-8

Determination of generation time of ammonia oxidizing consortium

Quantity of NO_2^-–N on doubling (g.L^{-1}) its build up	Duration required for doubling NO_2 –N output (in hours)
0.0006 – 0.0012	24
0.0012 – 0.0024	24
0.0024 – 0.0048	36
0.0048 – 0.0096	28.8
0.0096 – 0.0192	28.8
0.0192 – 0.0384	86.4
0.0384 – 0.0768	48
0.0768 – 0.1536	67.2
0.1536 – 0.3072	62.4
0.3072 – 0.6144	158.4

TABLE 3-9

Determination of generation time of nitrite oxidizing consortium

Quantity of NO_2^-–N on doubling its consumption $g.L^{-1}$	Duration required for doubling its consumption (in hours)
0.0058 – 0.0116	16.8
0.0116 – 0.0232	19.2
0.0232 – 0.0464	26.4
0.0464 – 0.0928	28.8
0.0928 – 0.1856	52.8
0.1856 – 0.3712	96.0
0.3712 – 0.7424	139.2
0.7424 – 1.4848	86.4
1.4848 – 2.9696	62.4
2.9696 – 5.9392	72.0

TABLE 3-10

Specific growth rate of ammonia and nitrite oxidizing consortia

Specific growth rate $\mu = \frac{0.693.}{g}$ Where g = generation time

Consortium	Generation time (g)	$\mu = \frac{0.693}{g}$
1. Ammonia oxidizing	24.0	0.0288 h^{-1}
2. Nitrite oxidizing	16.8	0.041 h^{-1}

TABLE 3-11

Batch culture studies to determine the kinetic constant (Km) for substrate utilization by ammonia oxidizing consortium (3 days' incubation)

Substrate Concentration used NH_4^+–N $mg.L^{-1}$ (S)	Rate of substrate utilized NH_4^+–N $mg.L^{-1}$ /3 days (V)	(1/S)	(1/V)
0.1	0.1	10	1.0
0.25	0.20	4	5
0.5	0.45	2	2.22
0.75	0.680	1.33	1.47
1.0	0.85	1.0	1.17

From fig 3.5,

$$-\frac{1}{Km} = -2$$

$$\frac{1}{Km} = 2$$

$$Km = \frac{1}{2} = 0.5$$

$$\text{Slope} = \frac{Km}{Vmax}$$

$$\frac{1}{Vmax} = 1.7$$

$$Vmax = \frac{1}{1.7} \quad 0.588\ d^{-3}\ (0.0082.h^{-1})$$

$$\text{Slope} = \frac{0.5}{0.558} = 0.896$$

TABLE 3-12

Batch culture studies to determine the kinetic constant (Km) for substrate utilization by nitrite oxidizing consortium (24 hours incubation)

Substrate Conc. used NO_2^-–N $mg.L^{-1}$ (S)	Rate of substrate utilized NO_2^-–N $mg.L^{-1}.d^{-1}$ (V)	(1/S)	(1/V)
0.1	0.1	10	1.0
0.25	0.207	4	4.83
0.5	0.296	2	3.37
0.75	0.423	1.33	2.36
1.0	0.506	1	1.97

From fig 3-6,

$$-\frac{1}{Km} = -2.7$$

$$\frac{1}{Km} = 2.7$$

$$Km = 1/2.7 = 0.370$$

$$\text{Slope} = \frac{Km}{Vmax}$$

$$\frac{1}{Vmax} = 1.8$$

$$Vmax = \frac{1}{1.8} = 0.556\ d^{-1}\ (0.023\ h^{-1})$$

$$\text{Slope} = \frac{0.37}{0.5} = 0.740$$

CHAPTER 4

RESOLUTION OF NITRIFYING CONSORTIA

Chapter 4

RESOLUTION OF NITRIFYING CONSORTIA

4.1 Introduction

Nitrifying bacteria were first isolated by Winogradsky (1890) from colonies growing on silica gel solidified medium containing inorganic mineral salts and either ammonium or nitrite as the energy source. By his use of silica gel, Winogradsky was able to prepare media free from organic substances, thus minimising contamination by heterotrophic bacteria. When ammonia - oxidizing bacteria were grown on silica gel plates coated with a layer of chalk or magnesium carbonate, the insoluble carbonates were dissolved by the nitrous acid and so the tiny colonies, surrounded by clear zones could be recognized. Originally, Winogradsky isolated pure cultures by plating an enrichment culture on silica gel medium and making transfers from parts of the silica gel apparently devoid of colonies. This was the negative plating technique. Beijerinck (1896) adopted a similar approach, replacing silica gel with agar washed to remove soluble organic compounds. In both cases, heterotrophs failed to grow since the media lacked organic carbon. The nitrifiers which formed small colonies were visible only by low power light microscopy and might be difficult to remove and distinguish from contaminant colonies. Likely colonies can be picked off with a micropipette (Walker, 1975).

In the method suggested by Funk and Krulwich (1964), for the preparation of silica gel plates to be used for purification of nitrifiers, plates of silica gel containing growth medium were prepared by aseptically mixing equal

volumes of sterile double strength fluid growth medium and sterile potassium silicate to which an appropriate amount of sterile 20% phosphoric acid was rapidly added and mixed by rotation immediately.

Soriano and Walker (1968) described another method for isolating ammonia oxidizers. They solidified inorganic nutrient medium with commercially available purified (Merck) or special Noble (Difco) agar which reduced the number of contaminants without completely preventing their growth. Heterotrophs developed colonies within 1 to 2 days and microscopic ammonia oxidizer colonies appeared after 3 to 4 days. Within 7 to 8 days, nitrifier colonies reached a diameter of 20 to 30μm and could be subcultured. They also described a micromanipulator for removing colonies with glass capillary pipettes which enabled the transfer of one or several colonies to fresh liquid medium.

Gelrite, a new gelling agent with good thermal stability and clarity was shown to be superior to agar in culturing selected thermophilic micro organisms(Lin and Casida, (1984). Because of its excellent clarity, gelrite can be used for isolating and purifying minute colonies of nitrifiers.

The enrichment and dilution method was orginally hampered by the belief that growth of nitrifiers, particularly ammonia oxidizers, in liquid media required the presence of particulate matter to which the cells could adsorb. However, such particles reduced suspended growth to such an extent that nitrifiers in the liquid phase were usually outnumbered by heterotrophs

(Prosser, 1982).

Lewis and Pramer (1958) were the first to use particle-free media for the isolation of *Nitrosomonas* species. They prepared enrichment cultures by successive transfers of fully grown cultures to fresh medium or by the addition of more ammonium ions after the oxidation of the initial amount present. Using the latter method, they found an initial increase in heterotrophs, decrease in numbers following the first addition of the substrate and relatively constant numbers following a second addition.Serial dilution of enrichment cultures from 1×10^{-1} to 1×10^{-10} were made into fresh medium and tests for purity and nitrite production carried out after 30 days incubation.

Soriano and Walker (1968) reported that a period of 12 weeks was required for the isolation of nitrifiers from soil by this method but the use of liquid media were found to be more convenient. Watson *et al.,* (1981) isolated nitrifying bacteria by serial dilution almost exclusively, although some other investigators preferred plating techniques. When serial dilution techniques were employed, dilution were made from all cultures in which nitrification was detected. The highest dilution giving nitrification was again serially diluted, and this process was repeated till pure cultures were obtained. Serial dilutions were made when the pH of the media dropped which indicated that approximately 300μg NH_4^+–N had been oxidized. Cultures became turbid when buffered to pH 7.8 - 8.0 with 0.5 M HEPES. Ammonia oxidizing bacteria could be isolated from these turbid cultures by making additional serial dilutions or by streaking them on agar plates (Watson *et al.*, 1981) and

the plates stored in the dark at 25 - 30° C in a moist chamber for about 4 months. The microcolonies visible with a microscope may be observed after one week, but colonies visible to the naked eye took 1-4 months to develop.

These microcolonies were transferred to tubes containing appropriate liquid medium. Subcultures that produced nitrite were streaked out again and the process repeated until pure cultures were achieved. Nitrite oxidizers were also purified by the same procedure.

To ascertain if a culture is free of heterotrophic contaminants, 1 mL of the cultures is transferred to 15 mL of several kinds of organic media. If growth is not obtained after 2 weeks at 25–30°C in any of the organic media, then it can be presumed that the ammonia-oxidizing culture is free of heterotrophic contaminants. The organic media generally employed include 0.25 strength nutrient broth (Difco), 0.5 strength fluid thioglycolate (Difco), 0.25 strength Trypticase soy broth (BBL), 0.25 strength A.C. broth (BBL). If no growth is observed in these media, the cultures are examined visually to determine if all of the bacteria present are morphologically similar. If the culture consisted of only one morphological type of cell, it is assumed to be pure (Watson *et al.,* 1981)

Both ammonia and nitrite oxidizing bacteria have prolonged generation time and so it usually takes several months to obtain pure cultures.

Five genera of ammonia oxidizers have been reported namely *Nitrosomonas*, *Nitrosococcus, Nitrosospira, Nitrosolobus* and *Nitrosovibrio*

and four genera of nitrite oxidizers namely *Nitrobacter, Nitrospina, Nitrococcus* and *Nitrospira* (Watson *et al.*, 1971).

The ammonia oxidizing bacteria are categorized taxonomically by their shape, size and arrangement of membranes within their cytoplasm. Cells are rod-shaped, spherical, spirillar or lobular without endospores. Some, but not all, species possess intracytoplasmic membranes, which may occur as flattened lamellae arranged centrally, peripherally or randomly. Most cells have a typical Gram negative cell envelope. When cells are motile, flagella are polar to lateral or peritrichous. In enrichment cultures and in nature, ammonia oxidizing bacteria frequently occur in cell aggregates, referred to as zoogloea or cysts. A zoogloea contains loosely associated cells embedded in a soft slime layer, while a cyst contains closely packed and compressed cells firmly embedded in and surrounded by a tough slime layer. The genus *Nitrosomonas winogradsky*, are wide spread in soils, sewage systems, compost piles, fresh water and marine environments. However, only one species *Nitrosomonas europaea* (Watson, 1971, 1974) is currently recognized.

Nitrosomonas europaea (Winogradsky, 1892) are ellipsoidal or rod shaped cells, (0.8 - 0.9 x 1-2 μm)intracytoplasmic membrane arranged as flattened vesicles primarily in the peripheral region of the cytoplasm. They are Gram negative, aerobic, motile or non motile. Optimum growth temperature range from 25 to 30°C, pH ranges for growth is 7.5 to 8.0. Cells can grow mixotrophically but not heterotrophically. The mol % G + C of DNA is 51.0 ± 0.4

Nitrosococcus sp. have spherical cells and have intracytoplasmic membranes arranged centrally, peripherally or randomly in the cytoplasm. They are Gram negative and motile by means of a tuft of flagella or single flagellum. They occur singly, in pairs and as tetrads and often embedded in slime, forming aggregates and occur in soils, oceans, brackish water and industrial sewage disposal systems. The mol % G + C of the DNA ranges from 48 - 51.

In *Nitrosospira* sp. the cells are spiral, 0.3 - 0.4 μm in width, with 3-20 turns. Usually tightly coiled, spherical forms are seen in cultures, cells lack cytomembrane system, are Gram negative and aerobic, motile or non-motile obligate chemolithotrophs. The mol % G + C of the DNA ranges from 52.2 to 55.4

In *Nitrosolobus* sp., cells are pleomorphic and lobate, 1.0 - 1.5 μm wide and 1.0 - 2.5 μm long with cytomembranes that partially compartmentalize the cell. Gram negative and aerobic, cells are chemolithotrophs but can grow mixotrophically. Optimum temperature 25-30°C, Optimum pH-7.5. The mol % G+C of the DNA is 53 - 56.3. *Nitrosovibrio* sp. are slender curved rods, 0.3–0.4 x 1.1 to 3.0 μm. Extensive cytomembranes are lacking and are Gram negative and aerobic. Motile or non-motile, chemolithotrophs., cells can grow mixotrophically but not heterotrophically. The mol % G+C of the DNA is 53.9. Optimum growth temperature, 25-30°C. Optimum pH for growth is 7.5–7.8.

In the genera of nitrite oxidizing bacteria, *Nitrobacter* sp. are rod or pear shaped, 0.5–0.8 x 1.0– 2.0μm. Cytomenbranes occur in the form of polar cap

of flattened vesicles in the peripheral region of the cell. They are Gram negative and motile or non-motile and aerobic. Grows chemolithotrophically as well as chemoorgrano-trophically. The mol % G+C of the DNA is 60-62.

Nitrospina sp. are rod shaped cells 0.3–0.4 x1.7 -6.6 μ m. Cells lack an extensive cytomembrane system and are Gram negative and non-motile. They are aerobic, obligate chemolithotrophs. Optimal growth requires 70-100% seawater. The mol % G+C of the DNA is 58.

Nitrococcus sp. are spherical 1.5 μm or more in diameter. Cells possess tubular cytomembranes randomly arranged throughout the cytoplasm. Gram negative, motile and aerobic and obligate chemolithotrophs. The mol % G+C of the DNA is 61.2

Nitrospira sp. are helical to vibriod in shape, having a width of 0.3-0.4 μm and a spiral amplitude of 0.8-1.0 μm. Cytomembranes are lacking. They are Gram negative, aerobic, non motile and are chemolithotrophs and the cells can grow mixotrophically. The mol % G + C of the DNA is 50.

As an attempt to define the nitrifying consortium, the heterotrophic components constituting the consortia need to be assessed. These associated heterotrophs together with the nitrifiers is an entity and though, the nitrifying activity express itself as the activity of chemolithotrophic nitrifiers, it is defined as the nitrifying activity of the consortia as a whole. The heterotrophic organisms in the consortia are thus to be resolved into individual components and their contribution to nitrifying activity assessed.

This chapter deals with the isolation and purification of ammonia and nitrite oxidizing bacteria and the associated heterotrophs thus totally resolving both ammonia and nitrite oxidizing consortia.

4.2 Materials and Methods

4.2.1 Transmission Electron Microscopy of nitrifying consortia

1 mL suspension each of ammonia and nitrite oxidizing consortia were taken in eppendorf tubes and centrifuged at 10,000 x g for 10 minutes in a cooling centrifuge at an operating temperature of 4°C. The supernatent was discarded and the pellet was then fixed in 3% (v/v) gluteraldehyde for 24 hours at 4°C. After fixing, the pellet was resuspended and passed through different grades of acetone from 50%, 70%, 90%, 95% and dry (100%) acetone by maintaining in each grade for about 15 minutes and centrifuging at 10,000 x g for 15 minutes and passing on to the next grade. The final dehydration with dry acetone was done twice and the pellet resuspended in dry acetone and diluted suitably for TEM. The suspensions were placed on formvar coated copper grids of 100 mesh size and observed under Transmission Electron microscope. (H 600, Hitachi Ltd., Japan)

4.2.2 Standardization of solid media for the isolation and purification of nitrifying bacteria from the nitrifying consortia.

4.2.2.a Modification in the preparation of silica gel plates.

In the method suggested by Funk and Krulwich (1964) as given in section 4.1, the gelation occured within few seconds and pouring into plates soon after the addition of phosphoric acid become practically impossible. Further, the gel so prepared was not transparent enough to make the minute colonies of nitrifying bacteria visible,

Therefore, a series of standardizations were carried out to modify the procedure for preparing silica gel plates so as to get a clear transparent gel with uniform pH throughout the plates with an extended gelling period.

Media

Twelve growth media meant for ammonia oxidizers and six for nitrite oxidizers (See chapter-2 section 2.2.3) were employed for the standar dization and modification in the preparation of clear silica gel plates. They were prepared as double strength in 10 mL aliquots.

Sodium and Potassium silicates

Aqueous (7%) Sodium and Potassium hydroxide (AR Grade) were used independently for dissolving silica gel powder (SRL, Bombay, 100 - 200 Mesh size).

Phosphoric acid

Aqueous (20%) phosphoric acid was used for adjusting the pH of the preparation between 7.5 and 8.0 as suggested for each medium.

Determination of phosphoric acid required for the right pH

Double strength growth media (10 mL) were mixed with 10 mL aliquots of 7% KOH and 7% NaOH independently and were titrated against 20% aqueous phosphoric acid by noting the pH changes using an Elico model pH meter.

Percentage of silica gel required for optimum gelation.

Aliquots (10 mL) of double strength growth media were mixed with the same quantity of sodium and potassium silicates containing 1 to 15% silica gel powder. The silica gel powder was dissolved in 7% NaOH and 7% KOH by boiling it till it dissolved completely. The double strength growth media and sodium and potassium silicates were autoclaved at 15 lbs for 15 minutes in screw cap bottles. The required quantity of sterile (20 % aqueous) phosphoric acid was pipetted into petriplates, the medium poured and rotated the plates clockwise and anticlockwise. The extent of gelling achieved at each concentration of silica gel was recorded.

Time of gelation

Aliquots (10mL) of double strength growth media mixed with sodium and potassium silicates were poured into petriplates of 4 inches diameter containing the required amount of (20 % aqueous) phosphoric acid. The time taken for gelling in the plates were noted.

4.2.2.b. Standardization of phytagel (GELRITE) plates for the isolation and purification of nitrifying bacteria from nitrifying consortia.

Medium XII meant for ammonia oxidizers and medium 6 meant for nitrite oxidizers were selected for the study. Concentration of gelrite (phytagel -Sigma Chemicals Co., USA) ranging from 0.1% to 1.25% were prepared in 20mL aliquots of Medium XII and Medium 6, autoclaved at 15 lbs for 15 minutes and poured into petriplates and

the extent of gelling and clarity noted.

4.2.2.c Soft agar plates for the isolation and purification of nitrifying bacteria.

A 1.5% w/v of agar (HIMEDIA) (washed with distilled water and subsequently dried at 80°C for 48 hrs.) solidified in Medium XII meant for ammonia oxidizers and medium 6 meant for nitrite oxidizers were also used for isolating nitrifiers as a part of resolving the nitrifying consortia.

4.2.3 Isolation and purification of nitrifying bacteria from ammonia and nitriite oxidizing consortia

Isolation and purfication of nitrifying bacteria from the consortia developed was done by a combination of both serial dilution and plating.

Serial dilution

1 mL each of ammonia and nitrite oxidizing consortia were serially diluted upto 10^{-25} in 20 mL test tubes containing the medium XII meant for ammonia oxidizers and the medium 6 meant for nitrite oxidizers and NH_4^+-N and NO_2^- –N were analysed in these tubes following APHA (1959) and Strickland and Parsons(1968) incubated in dark for 3-4 weeks. Tubes which have shown positive for nitrification in terms of build up of NO_2^--N in serially diluted ammonia oxidizing consortia in medium XII and disappearances of NO_2^--N in serially diluted nitrite oxidizing consortia in medium 6, were serially diluted

further upto 10^{-75} and again incubated in dark for 3-4 weeks.

Tubes which were positive for nitrification were streaked on nutrient agar plates to test the purity. Since nitrite oxidizers can grow heterotrophically also the growth in nutrient agar could not be taken as the purity test.

Plating

The highest dilution at which nitrification was recorded (that is, 10^{-75}) for both ammonia and nitrite oxidizing consortia were swabbed and drop inoculated on to silica gel, phytagel and soft agar solidified in medium XII meant for ammonia oxidizers and medium 6 meant for nitrite oxidizers respectively and incubated in Environmental Test Chamber (Remi Equipment) in dark at 28°C.

Each of the different types of colonies developed on these plates were picked up and streaked on to phytagel plates respectively until pure cultures with single type of colony on each plate were obtained. Phytagel plates were preferred for purification because of its transparency which helped the microcolonies of nitrifiers to be distinguished from other types.

The colonies thus obtained were streaked and isolated on to phytagel slants. After sufficient growth was obtained on the slants, a loopful of the culture was inoculated into 10 mL of medium XII for ammonia oxidizers and 10 mL of medium 6 for nitrite oxidizers and

incubated in dark for 3-4 weeks. The build up of NO_2^-–N in ammonia oxidizing medium and disappearance of NO_2^-–N in nitrite oxidizing medium was monitored.

4.2.4 Characterization of nitrifying bacteria

Pure cultures which demonstrated build up of NO_2^-–N from NH_4^+–N and NO_3^-–N from NO_2^-–N were recognized as ammonia and nitrite oxidizers. They were further characterized as described below.

Gram's reaction

The cultures were streaked on phytagel stants prepared out of corresponding medium and as soon as visible growth observed, they were smeared and Gram stained.

Motility

Motility was examined by using both soft phytagel tubes and by the microscopic observation of suspension of the culture. Soft phytagel stants with 0.5% phytagel was prepared with the corresponding media and they were autoclaved at 15 lbs for 15 minutes. The cultures were stabbed and incubated at 28±0.4°C in an Environment Test Chamber. The tubes were observed for hyphae like growth for 10 days. The suspension for microscopic observation was prepared from fresh phytagel slants grown culture. Using sterile physiological saline, a thin suspension was prepared on microscope slide and covered with coverslip and observed immediately under oil immersion objec-

tive for displacement of cells.

Phase contrast microscopic observation

The above preparation made for motility was put under phase contrast objectives and the cellular morphology was observed and photographed.

Micrometry

The Gram stained smears were used for measuring the length and width of cells using a calibrated occular micrometer.

Heterotrophic growth

To test the capability of nitrifiers to grow heterotrophically they were streaked on nutrient agar stants composed of peptone, 5g; beef extract, 5g; yeast extract, 1g; NaCl, 5g; pH : 7.0 ± 0.2 and Agar 2g per 1000 mL distilled water

4.2.5 Isolation and identification of heterotrophic bacteria associated with the nitrifiers in the consortia

Isolation

Isolation of heterotrophic bacteria in both ammonia and nitrite oxidizing consortia was done by a combination of serial dilution and plating techniques.

1 mL each of ammonia and nitrite oxidizing consortia were serially diluted upto 10^{-12} dilution in 20 mL test tubes containing medium XII meant for ammonia oxidizers and medium 6 meant for ni-

trite oxidizers. From these dilutions, the cultures were swabbed and pour plated on to nutrient agar plates. The composition of nutrient agar is give below :

Nutrient Agar (Per litre)

Peptone	:	5 gm
Beef extract	:	5 gm
Yeast extract	:	1 gm
NaCl	:	5 gm
pH	:	7.0 ± 0.2
Agar		2 gm

The isolates were purified by repeated streaking and maintained in nutrient agar vials overlaid with liquid paraffin. They were identified to the genera following Oliver (1982), and Buchanan and Gibbons (1974). The core characters examined for the identification were Grams reaction, oxidation/fermentation reaction, motility, cytochrome oxidase, sensitivity to O/129, Arginine dihydrolase, DNase, Sensitivity to penicillin, Luminiscence, Catalase, Sensitivity to polymyxin B, pigmentation, H_2S production, spore staining and morphology following Manual of Microbiological methods (1957), Society of American Bacteriologists.

4.2.6 Test for nitrifying activity of the isolated heterotrophic compounds of the nitrifying consortia.

Isolated and purified heterotrophs from the ammonia and nitrite oxidizing consortia were inoculated into 10 mL aliquots of medium XII and 10 mL aliquots of medium 6 and incubated it for 3-4 weeks in Environment Test Chamber. Regular checks of NH_4^+–N, NO_2^-–N and NO_3^-–N were made in the inoculated tubes.

4.3 Results and Discussion

4.3.1 Transmission Electron Microscopy of ammonia and nitrite oxidizing consortia

Transmission Electron Microscopic demonstration of ammonia oxidizing consortium is given in Fig. 4-1 to 4-6. Generally in all cases cells were found aggregated, covered with a less electron dense slime secreted by the organisms. Through these slimy mass the cells could make interconnections and remained entangled, designated as the zoogloea formation. It was apparent that the binary fission of the cells occured within the slimy sheath. Slender long cells which were lightly curved in most of the cases were identified as nitrifying-like bacteria. An interesting feature observed was the close association of larger ovoid cells which are probable heterotrophs with the nitrifying lek bacterial cells. This association appeared to be very firm through the slime produced. Overall length of the cells in the consortium varied from 0.5 to 2.15 μm.

Transmission Electron microscopic demonstration of nitrite oxidizing consortia is given in Fig. 4-7 to 4-10. In this group of organisms also the slender slightly curved cells in most of the cases, remain in aggregate covered with a less electron dense slimy layer. Through this slime, the cells remain interconnected. Fig. 4-7 clearly indicate that the slimy layer is secreted by the cell itself. Prosser and Cox (1982) stated that the growth of nitrifiers in liquid culture often

give rise to a surface pellicle and in shaken cultures some strains grow as clumps of cells. Johnson and Sieburth (1976) on studying the morphology of nitrifying bacteria in aquaculture systems observed in EM sections oval-shaped cells embedded in a polysaccharide-like matrix. Watson *et al.,* (1981) observed in enrichment cultures cell aggregates, referred to as zoogloea or cysts. Although there is no clear distinction between these two types of aggregates according to Watson *et al.,* (1981 and 1989) a zoogloea contains loosely associated cells embedded in a soft slime layer, while a cyst contains closely packed and compressed cells firmly embedded in and surrounded by a tough slime layer. These aggregate according to him may consist of a few to hundred or more cells and are rarely found in pure cultures. Meanwhile De Boer (1991) on studying nitrification at low pH by aggregated chemolithotrophic bacteria observed that the aggregates remained intact even after the electron microscopic pretreatments (dehydration) giving strong evidence that the cells are embedded in a stabilizing polymer matrix. The consortia of ammonia and nitrite oxidizing bacteria studies here are very well embedded in a slimy layer and look more like zoogloea and some cases especially the nitrite oxidizing consortium shown in Fig. 4-9 and 4-10 appears to be cysts. The exact chemical nature of the slimy layer is not known. However it is postulated that the slime is composed of polysaccharide-like compounds.

4.3.2 Silica Gel Plates

The results of the experients conducted to check the quantity of 20% (aq) phosphoric acid required to adjust the pH for the preparation of silica gel plates are summarized in Table 4-1. It was seen that irrespective of the media used and the application of either sodium hydroxide or potassium hydroxide for dissolving silica gel, the quantities of phosphoric acid required for adjusting pH to 7.5 and 8.0 were 2.5 mL and 2.4 mL respectively.

The experiments conducted to find out the quantity of the silica gel required for obtaining clear silica gel plates for streaking (Table 4-2) revealed that 12% silica gel in 7% NaOH gave instant and uniform gelation and sufficient transparency to observe minute colonies of nitrifiers, when the pH was adjusted to 8.0 using 2.4 mL of 20% phosphoric acid.

On determining the time of gelation with various media when both NaOH and KOH were used for dissolving silica gel, it could be noticed that 12 to 13 seconds were available for the satisfactory rotation of the plates so that uniform gelation could be achieved (Table 4-3)

In short, clear silica gel plates could be prepared by mixing 12% silica gel prepared in 7% aquous NaOH with equal quantity of double strength fluid growth media and autoclaving at 15 lbs for 15 minutes

and pouring into a single petri plate of 4 inch diameter containig 2.4 or 2.5 mL of 20% (aqueous) sterile phosphoric acid to attain a final pH of 8.0 or 7.5 respectively. The plates had to be rotated immediately clockwise and anticlockwise for 12 or 13 seconds within which the gelation occured.

4.3.3 Phytagel/soft agar plates

The results of the standardization of phytagel are given in Table 4-4. Accordingly, 1.25% phytagel gave sufficient clarity and gel strength for streak plate method of isolation of nitrifiers. A 1.5% washed agar were also used for the isolation and purification of nitrifiers. On soft agar plates, it was observed that nitrifiers formed warty type colonies and was not possible to subculture by streaking. On the contrary, a 1.25% phytagel solidified in medium XII meant for ammonia oxidizers and medium 6 meant for nitrite oxidizers had excellent clarity and could be streaked and hence phytagel was used to isolate and purify nitrifiers.

4.3.4 Isolation and purification of nitrifying bacteria from consortia

By the combination of both serial dilution and plating techniques, 14 pure cultures of ammonia oxidizing bacteria and 40 pure cultures of nitrite oxidizing bacteria were obtained. The pure cultures were stocked in phytagel slants and vials. The vials were wax-sealed and stored an Environment Test Chamber at 28°C. Since it was seen that

at and below 4°C, there was no nitrifying activity, the phytagel stants and vials could also be preserved at 4°C.

4.3.5 Characteristics of the nitrifiers

On phytagel plates, both ammonia and nitrite oxidizers formed small, circular and transparent or glassy colonies, easily visible under a low power light microscope. They were all Gram negative rods, showing tumbling, rotating movement, with a long axis measurement of ammonia oxidizers around 1.25 μm and that of nitrite oxidizers ranging from 1.875 – 2.5 μm, (Fig. 4-11 to 4-14). Alike the stained preparations, under phase contrast also they appeared as short rods, (Fig. 4-15 to 4-18) having the same dimensions. All the isolates could grow equally well on nutrient agar. But on transferring the nutrient agar grown culture in to the respective mineral base medium containing 30μg.mL^{-1} NH_4^+–N and NO_2^-–N, they regained the nitrifying potential after a prolonged lag phase (Tables 4-5 and 4-6). Smith and Hoare (1968) had grown *Nitrobacter agilis* heterotrophically which thus confirms the above observation. But according Bock (1976), heterotrophic growth of *Nitrobacter* sp. is slower than growth on nitrite. According to Steinmuller and Bock (1977), heterotrophically grown cells possess lower levels of nitrite oxidoreductase requiring upto 3-4 weeks induction before regaining ability to grow autotrophically and in the present study also the heterotrophically grown nitrite oxidizers experience a lag of 30 days against 4 days for the autotrophi-

cally grown cultures provided with the same amount of substrate.

Based on the above level of characterization cultures could not be identified to any genera described. According to Bergey's Manual of Systematic Bacteriology none of the already described ammonia oxidizers have shown heterotrophic growth and all them are obligate chemolithotrophs. But in the case of nitrite oxidizers *Nitrobacter* sp and *Nitrospina* sp. have been shown to grow heterotrophically also besides growing chemolithotrophically. Therefore the isolated cultures of nitrite oxidizers can be designated as *Nitrobacter* or *Nitrospina*-like organisms which can only be ascertained based on the pattern of cytomembranes and % G+C ratio. Nitrifying bacteria obtain energy from the oxidation of reduced forms of inorganic nitrogen and derive most of their cellular carbon by fixing CO_2 but they are not still obligate chemolithotrophs (Spotte, 1992). If they are obligate Chemolithotrophs they would not grow except in the presence of their specific nitrogen source, ammonia or nitrite (Bishop *et al.*, 1976). But the capacity to assimilate organic compunds makes nitrifying bacteria facultative autotrophs. Under certain conditions nitrifiers can use a substantial amount of organic carbon in the environment, both as a source of cellular carbon and as an energy source during respiration (Bower and Bidwell, 1978).

Nitrifying bacteria in the tropics is an unstudied lot and there fore the heterotrophic growth of nitrifiers have to be looked in to with a

different perspective. It is reasonably envisaged that several unknown groups of nitrifiers could be present in this part of aquatic ecosystem.

4.3.6 Isolation and identification of heterotrophic bacteria from nitrifying consortia.

Generic composition of heterotrophic bacteria isolated from the ammonia oxidizing consortium is given in Table 4-7. Based on the phenotypic characters examined, four genera were identified such as *Alteromonas, Alcaligenes, Pseudomonas,* and *Acinetobacter* in the decreasing order of dominance. Generic composition of heterotrophic bacteria isolated from the nitrite oxidizing consortium is given in Table 4-8. The same genera were isolated from this consortium also but with a different order of decreasing dominance as *Acinetobacter, Alteromonas, Alcaligenes* and *Pseudomonas.*

The association of heterotrophs with chemolithotrophs, especially with nitrifying bacteria has been demonstrated earlier by several workers. Clark and Schmidt (1966) found that the growth of *N. europaea* were stimulated in the presence of heterotrophs and this could be reproduced by supplying pyruvate which accelerated the recovering of old cultures. Jones and Hood (1980) reported a mutualistic interaction between a *Nitrosomonas sp.* a *Pseudomonas sp.,* and *Nocardia atlantica* isolated from an estuarine environment. Ammonium oxidation increased by 150% and growth of the heterotrophs by a factor of 10, but the precise nature of the interaction was unknown.

The ammonia oxidizing and nitrite oxidizing consortia developed here have a closely associated heterotrophic flora. Generally, a culture of nitrifying bacteria get vey easily contaminated and the contaminants grow at the expense of the extrametabolites of the nitrifiers. But, it is quite unlikely to have the heterotrophs in these consortia as just contaminants. In transmission electron microscopic observations, the nitrifying-like bacteria were found to be covered with a polysaccharide-like slime and all the cells were entangled in to a mass. In every such assemblage of cells heterotroph-like bacterial cells were also found to be integrated and very firm interconnections between the two types of cells through the slime could be demonstrated. May be because of this, resolution of the nitrifying consortia was very hard and in every apparently pure culture with discrete colonial morphology a heterotroph was also associated which would never facilitated any differentiation at colonial level.

These heterotrophs in minimal base media without a carbon source were found not to assimilate either NH_4^+–N or NO_2^-–N or buildup of NO_2^-–N or NO_3^-–N implying that heterotrophs do not possess the nitrifying potential. On the other hand all isolated and purified ammonia and nitrite oxidizing cultures were able to grow heterotrophically also.

It is postulated that the heterotrophic bacteria associated with the consortia of nitrifiers are not mere contaminants but an integral part

both together forming one entity. The heterotrophs survive at the exudates of the nitrifiers which derive energy from NH_4^+–N and NO_2^-–N by oxidation and assimilate carbon from carbondioxide. These extrametabolites serve as carbon, nitrogen and energy source, and its continuous removal from the vicinity of nitrifiers eliminates the growth inhibition which otherwise would have limited their growth and nitrifying potential. But the suggestion put forth by De Boer *et al.*, (1991) that the aggregates of nitrifying bacteria provide the heterotrophs with the polymeric matrix as an energy source can not be accepted, as, such relationship may lead to depletion of the protective cover to the entire biological entity including the heterotrophs which otherwise keeps the different groups of organisms together to function as a single unit. The nitrifiers are further benefitted by certain unknown growth factors elaborated by the heterotrophs. But more experimental evidences have to be generated to prove this hypothesis.

However, it is resolved that the nitrifying consortia developed here have to be treated as a single entity for all practical purposes of mass production and their application in bioreactors.

Fig 4-1. Electoronmicrograph of aggregates of cells in the ammonia oxidizing consortium. Larger cells probable heterotrophs and slender ones nitrifying bacteria like. See the soft slime surrounding the cells attaching them together length range from 0.8 to 1.25μ Mag. 12000 X ; scale bar = 0.83μ

Fig. 4-2 Densely packed cells of nitrifying (ammonia oxidizing) consortium. All cells are interconnected through the soft slime Larger cells are probable heterotrophs. Length range from 0.6 to 1.25μ Mag 12000 X ; scale bar = 0.83μ

Fig.4-3 TEM of long slender nitrifying bacteria like (ammonia oxidizing) organisms freed from the slimy mass. Length ranges from 1.9 to 2.15 mag. 20, 000 X ; bar = 0.5μ

Fig. 4-4 TEM of short rods of ammonia oxidizing -like organisms covered with soft slime interconnected and attached to a probable heterotroph. Length 0.5μ; mag: 80,000 X ; scale bar 0.125μ

Fig 4-1

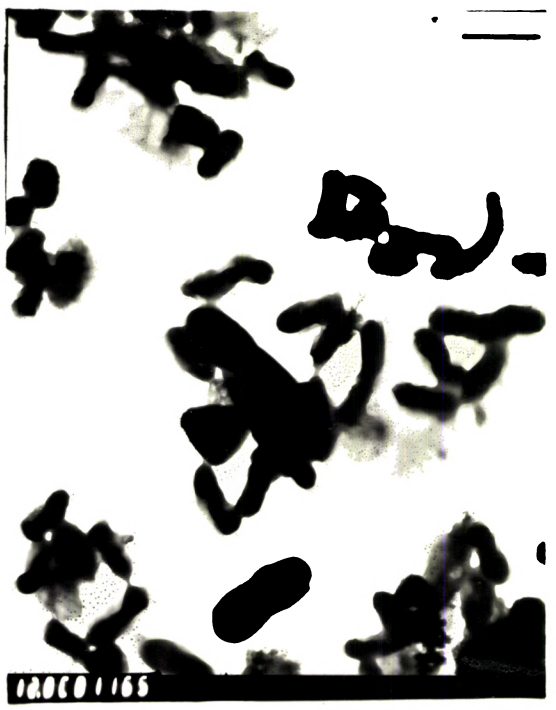


Fig 4-2

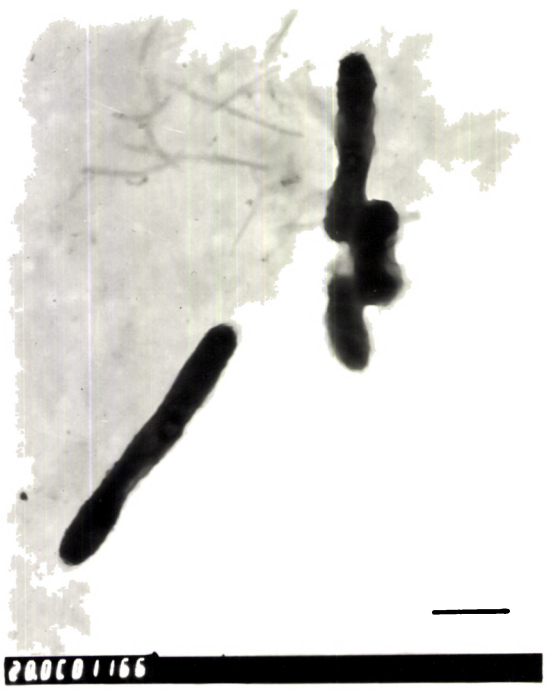


Fig 4-3

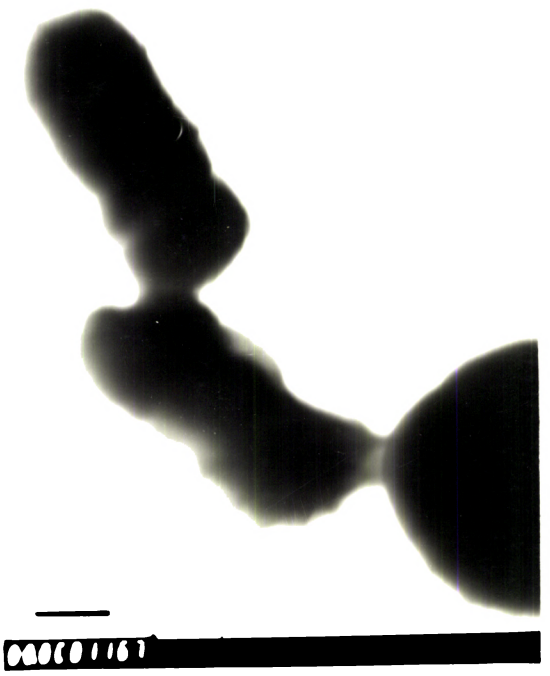


Fig 4-4

Fig. 4-5 TEM of an aggregate of ammonia oxidizing consortium. The long slender slightly curved cells are ammonia oxidizing -like aggregated together by the soft-slime. The larger cell, probable heterotroph is closely associated through the soft slime. Length ranges from 0.8 to 1μ; mag. 50000 X.; Scale : bar 0.2μ.

Fig 4-6 TEM of another aggregate of ammonia oxidizing consortium with profuse secretion of slime. A mass of less electron dense slime is seen separately. The large oval shaped cell seen at the centre is the probable heterotroph. Length ranges from 0.69 to 1.16 μ. Mag.30.000 X; scale: bar = 0.33 μ.

Fig. 4-7 TEM of slender slightly curved cell of nitrite oxidizing like organism see the secretion of slime from the cell surface. Length : 1.16 μ; mag :60000 X ; scale bar = 1.66μ.

Fig. 4-8 TEM of an aggregate of nitrite oxidizing consortium inter connected with the soft slime, but almost exposed out of the slimy mass. Length ranges from 0.8 to 1.15 μ; mag : 20000 X; scale : bar : 0.5 μ.

Fig4-5

Fig 4-6

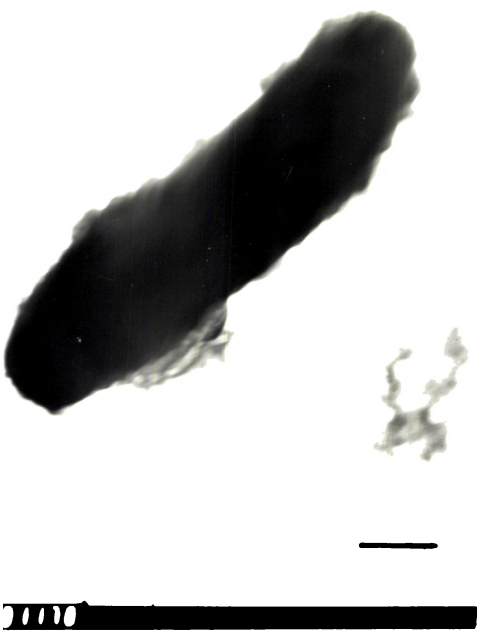


Fig 4-7

Fig 4-8

Fig. 4-9 TEM of an aggregate of nitrite oxidizing bacterial consortium fully embedded within a slimy sheath, all cells interconnected. Length ranges from 0.56 to 0.8 μ; mag: 50000 X; scale : bar = 0.2 μ

Fig. 4-10 TEM of another aggregate of nitrite oxidizing consortium fully embedded in a slimy layer all cells interconnected. Length ranges from 0.50 to 0.77 μ; mag: 30,000 X; Scale : bar = 0.33 μ.

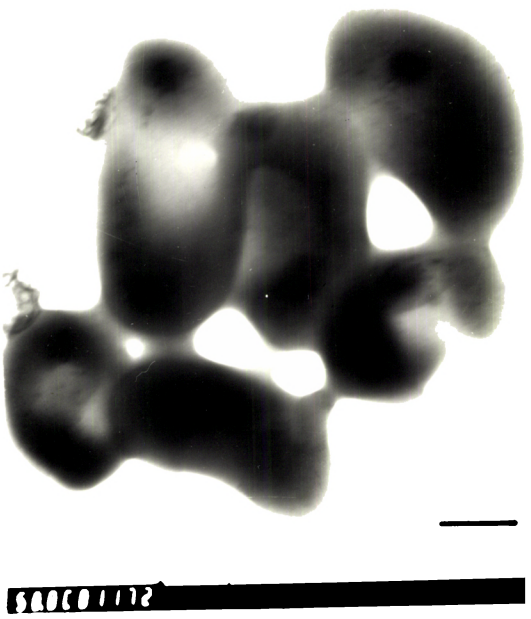


Fig 4-9

Fig 4-10

Fig. 4-11 Gram stained cells of ammonia oxidizing bacteria in a loosely aggregated mass. Mag. 1500 X.

Fig. 4-12 Gram stained cells of ammonia oxidizing bacteria fully dispersed. Mag. 1500 X.

Fig. 4-13 Gram stained cells of nitrite oxidizing bacteria seen in aggregate. Mag 1500 X.

Fig. 4-14 Gram stained cells of nitrite oxidizing bacteria fully dispersed. Mag. 1500 X.

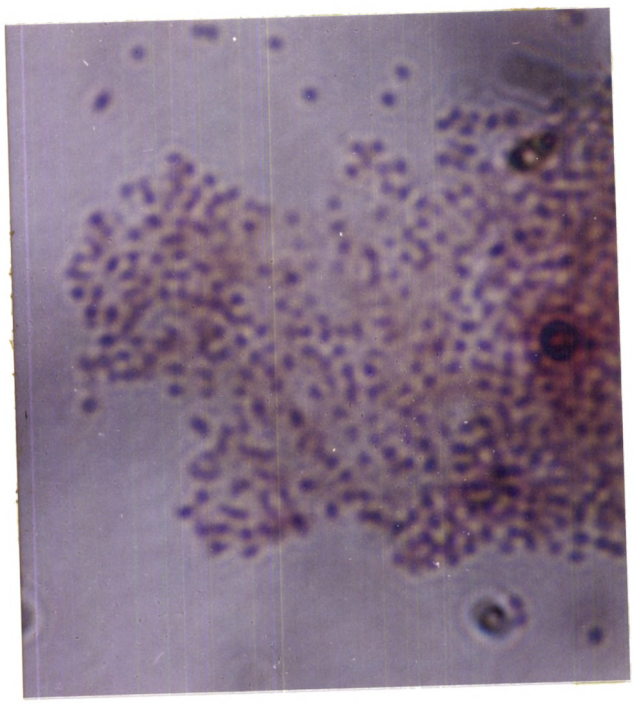


Fig 4-11

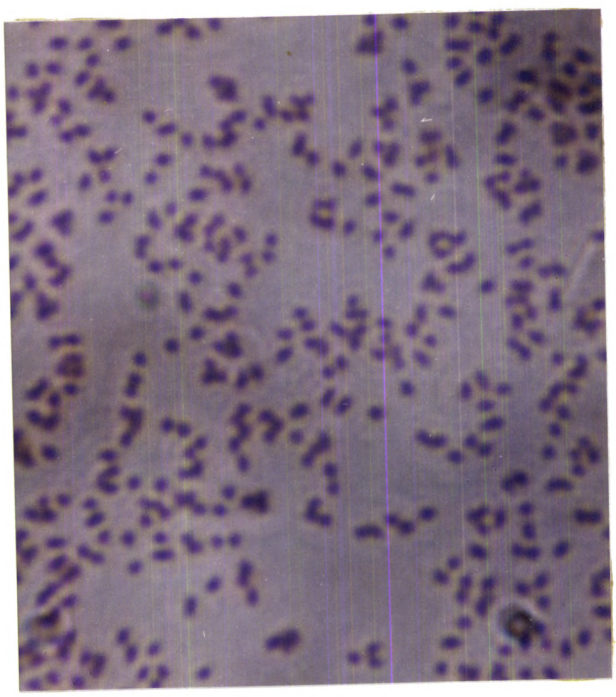


Fig 4-12

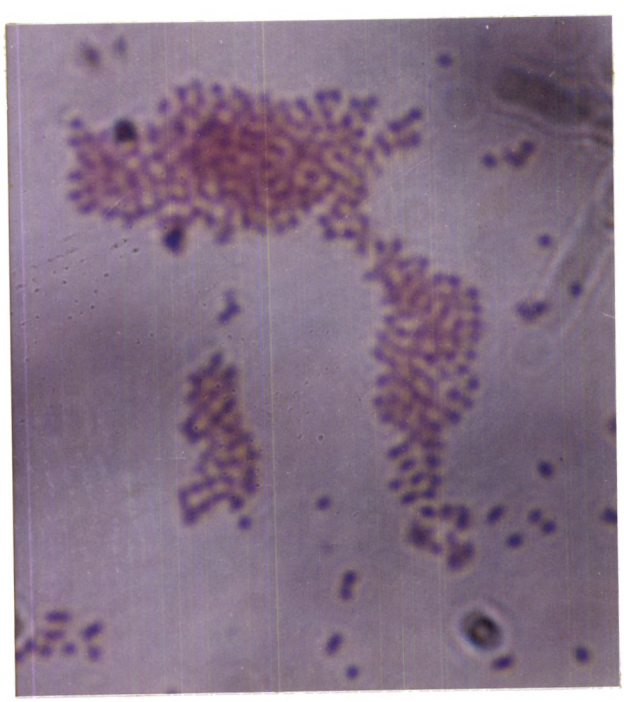


Fig 4-13

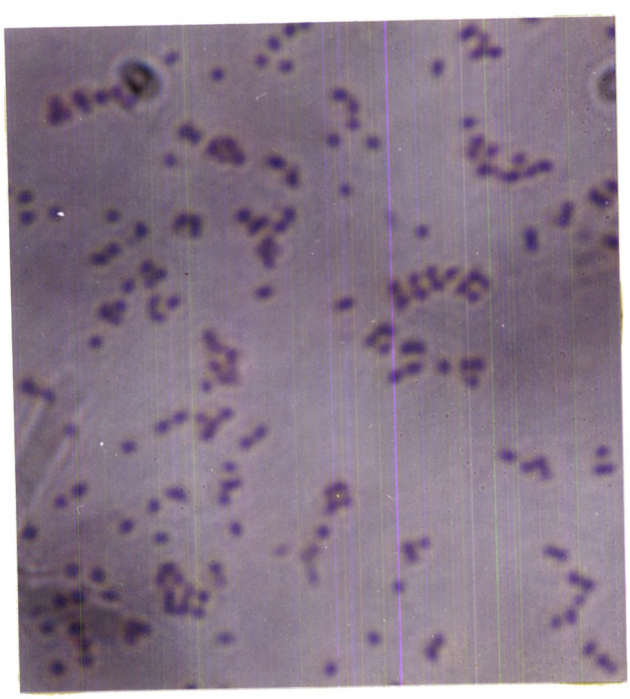


Fig 4-14

Fig. 4-15 Short rods of ammonia oxidizing bacteria as seen under positive phase contrast. Mag 1500 X.

Fig. 4-16 Short rods of ammonia oxidizing bacteria as seen under positive phase contrast. Mag 1500 X.

Fig. 4-17 Short rods of nitrite oxidizing bacteria as seen under positive phase contrast. Mag 1500 X.

Fig. 4-18 Short rods of nitrite oxidizing bacteria as seen under positive phase contrast. Mag. 1500 X.

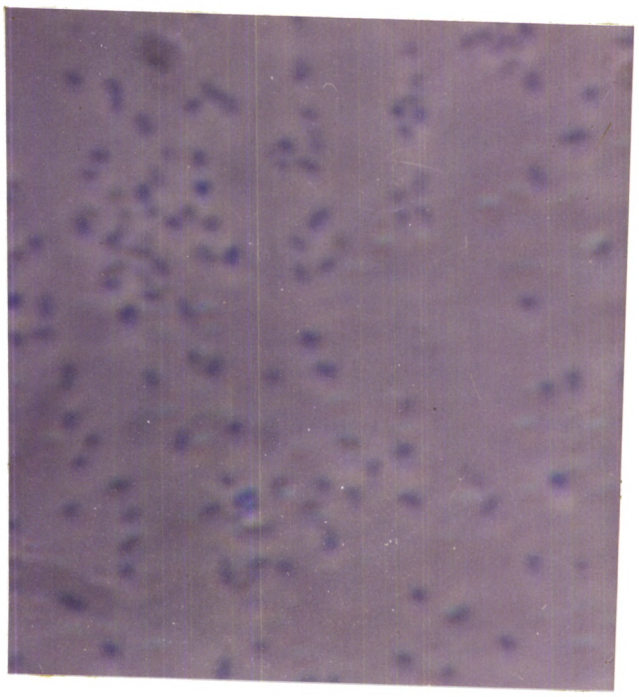


Fig 4-15

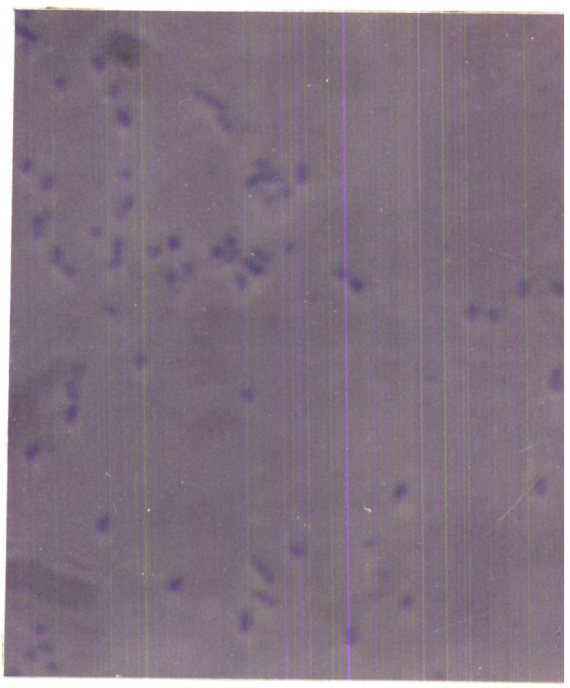


Fig 4-16

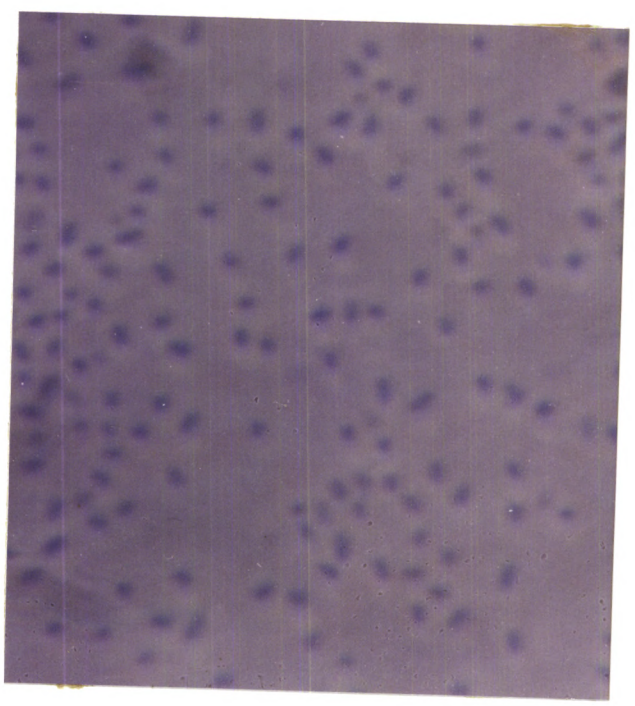


Fig 4-17

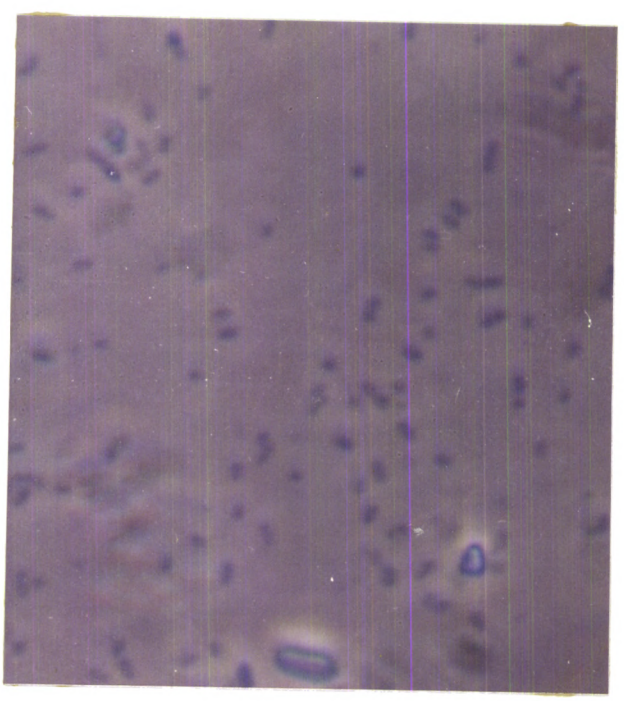


Fig 4-18

TABLE 4-1

Aqueous phosphoric acid required for various media when mixed with either 7% NaOH or KOH

Media used (Code No.)	Required pH	Volume of H_3PO_4 consumed (ml) when	
		7% NaOH was used	7% KOH was used
I, 1 and 4	7.5	2.5	2.5
II, III, IV, V, VI, VII, VIII, IX, X, XI, XII, 2, 3, 5 and 6	8.0	2.4	2.4

TABLE 4-2

Percentage of silica gel required for attaining optimum gelation and transparency

Percentage of silica gel	Extent of gelation and transparency obtained	
	when 7% KOH was used	when 7% NaOH was used
1	No gelation	No gelation
2-4	Loose gelation	Loose gelation
5-9	Gelation not uniform and sufficiently hard	Gelation not uniform and sufficiently hard
10& 11	Gelation uniform but turbid and not sufficiently hard	Gelation uniform with transparency but not sufficiently hard
12.	Gelation uniform and sufficiently hard but turbid	Gelation uniform, transparent and sufficiently hard
13 & > 13	Silica gel does not fully go into solution	Silica gel does not fully go into solution

TABLE 4.3

Time required for gelation for different media

Media used (Code No.)	Time(seconds) required for gelation after adjusting pH when mixed with	
	Potassium silicate	sodium silicate
I, II, III, V,VI, IX, X,XI, XII, 1,2, 3,4,5,6	13	13
IV, VII, VIII	12	12

TABLE 4.4

Percentage of phytagel (GELRITE) required for attaining optimum gelation and transparency

Percentage of phytagel used	Extent of gelation and transparency obtained	
	Medium XII	Medium 6
0.1 – 1.0	transparent gel but no gel strength	transparent gel but no gel strength
1.25	transparent and sufficiently good gel strength for streaking	transparent and sufficiently good gel strength for streaking

TABLE 4-5

Characteristics of Ammonia oxidizers

Culture No.	Colonial morphology	Grams reaction	Cellular morphology	Motility*	Observation under phase contrast	Micrometric measurement	Heterotrophic growth
AOB-1	Spherical, transparent, central slightly raised	-ve	Short rods	+	Short rods	1.25µM	+
AOB-2	do	-ve	Short rods	+	Short rods	1.25µM	+
AOB-3	do	-ve	Short rods	+	Short rods	1.25µM	+
AOB-4	do	-ve	Short rods	+	Short rods	1.25µM	+
AOB-5	do	-ve	Short rods	+	Short rods	1.25µM	+
AOB-6	do	-ve	Short rods	+	Short rods	1.25µM	+
AOB-7	do	-ve	Short rods	+	Short rods	1.25µM	+
AOB-8	do	-ve	Short rods	+	Short rods	1.25µM	+
AOB-9	do	-ve	Short rods	+	Short rods	1.25µM	+
AOB-10	do	-ve	Short rods	+	Short rods	1.25µM	+
AOB-11	do	-ve	Short rods	+	Short rods	1.25µM	+
AOB-12	do	-ve	Short rods	+	Short rods	1.25µM	+
AOB-13	do	-ve	Short rods	+	Short rod	1.25µM	+
AOB-14	do	-ve	Short rods	+	Short rod	1.25µM	+

* rotating, tumbling movements of cells in suspension.

TABLE 4-6

Characteristics of Nitrite oxidizers

Culture No.	Colonial morphology	Grams reaction	Cellular morphology	Motility*	Observation under phase contrast	Micrometric measurement	Heterotrophic growth
NOB-1	Spherical, transparent, central slightly raised	-ve	Short rods	+	Short rods	1.875µM	+
NOB-2	do	-ve	Short rods	+	Short rods	1.875µM	+
NOB-3	do	-ve	Short rods	+	Short rods	1.875µM	+
NOB-4	do	-ve	Short rods	+	Short rods	1.875µM	+
NOB-5	do	-ve	Short rods	+	Short rods	1.875µM	+
NOB-6	do	-ve	Short rods	+	Short rods	1.875µM	+
NOB-7	do	-ve	Short rods	+	Short rods	1.875µM	+
NOB-8	do	-ve	Short rods	+	Short rods	1.875µM	+
NOB-9	do	-ve	Short rods	+	Short rods	1.875µM	+
NOB-10	do	-ve	Short rods	+	Short rods	1.875µM	+
NOB-11	do	-ve	Short rods	+	Short rods	1.875µM	+
NOB-12	do	-ve	Short rods	+	Short rods	1.875µM	+
NOB-13	do	-ve	Short rods	+	Short rods	1.875µM	+
NOB-14	do	-ve	Short rods	+	Short rods	1.875µM	+
NOB-15	do	-ve	Short rods	+	Short rods	1.875µM	+
NOB-16	do	-ve	Short rods	+	Short rods	1.875µM	+
NOB-17	do	-ve	Short rods	+	Short rods	1.875µM	+
NOB-18	do	-ve	Short rods	+	Short rods	2.5µM	+
NOB-19	do	-ve	Short rods	+	Short rods	2.5µM	+
NOB-20	do	-ve	Short rods	+	Short rods	2.5µM	+
NOB-21	do	-ve	Short rods	+	Short rods	2.5µM	+
NOB-22	do	-ve	Short rods	+	Short rods	2.5µM	+
NOB-23	do	-ve	Short rods	+	Short rods	2.5µM	+
NOB-24	do	-ve	Short rods	+	Short rods	2.5µM	+
NOB-25	do	-ve	Short rods	+	Short rods	2.5µM	+
NOB-26	do	-ve	Short rods	+	Short rods	2.5µM	+
NOB-27	do	-ve	Short rods	+	Short rods	2.5µM	+
NOB-28	do	-ve	Short rods	+	Short rods	2.5µM	+
NOB-29	do	-ve	Short rods	+	Short rods	2.5µM	+
NOB-30	do	-ve	Short rods	+	Short rods	2.5µM	+
NOB-31	do	-ve	Short rods	+	Short rods	2.5µM	+
NOB-32	do	-ve	Short rods	+	Short rods	2.5µM	+
NOB-33	do	-ve	Short rods	+	Short rods	2.5µM	+
NOB-34	do	-ve	Short rods	+	Short rods	2.5µM	+
NOB-35	do	-ve	Short rods	+	Short rods	2.5µM	+
NOB-36	do	-ve	Short rods	+	Short rods	2.5µM	+
NOB-37	do	-ve	Short rods	+	Short rods	2.5µM	+
NOB-38	do	-ve	Short rods	+	Short rods	2.5µM	+
NOB-39	do	-ve	Short rods	+	Short rods	2.5µM	+
NOB-40	do	-ve	Short rods	+	Short rods	2.5µM	+

* rotating, tumbling movements of cells in suspension.

TABLE 4-7

Generic composition of heterotrophic bacteria isolated from Ammonia oxidizing consortium

Genera of heterotrophic bacteria	Percentage of heterotrophic bacteria isolated from Ammonia oxidizing consortium
Alteromonas	55.55
Alcaligenes	13.88
Acinetobacter	8.33
Pseudomonas	8.33
Total No. of isolates	**40**

TABLE 4-8

Generic composition of heterotrophic bacteria isolated from Nitrite oxidizing consortium

Genera of heterotrophic bacteria	Percentage of heterotrophic bacteria isolated from Nitrite oxidizing consortium
Acinetobacter	47.05
Alcaligenes	20.58
Alteromonas	20.58
Pseudomonas	2.94
Total No. of isolates	**40**

CHAPTER 5

DEVELOPMENT OF BIOREACTORS FOR NITRIFYING TREATED SEWAGE

Chapter 5

DEVELOPMENT OF BIOREACTORS FOR NITRIFYING TREATED SEWAGE

5.1 Introduction

For many decades, reactors with fixed biomass have been used in waste water treatment for the removal of organic matter, for nitrification and denitrification. Among various types of biofilm reactors, the following are considered to be the most feasible and economical for practical applications at the present time:

Trickling filters (TF) packed with volcanic rock or plastic media in the form of random packings or corrugated plastic sheets.

Rotating Biological Contactors (RBC) with different types of biomass carrier elements like discs or plastic media drum exerting different hydraulic flow patterns.

Biofilters (BF), or Biological Aerated Filters (BAF) with different granulated media or corrugated sheets in the up- and the down flow mode and mostly internally aerated with compressed air.

For a given degree of treatment, biofilters require 3 times less aeration volume than activated sludge units and 20 times less than trickling filters (Smith and Hardy, 1990)

In fluidized bed reactors (FBR) the biomass carrier material is suspended by the upflowing water and aerated by compressed air or pure oxygen.

These biofilm reactors can be applied in different process schemes enabling desired biological processes to occur (Boller *et al.*, 1994). Since the autotrophic nitrifying organisms show relatively low growth rates and are more delicate towards environmental conditions, an important requirement for nitrification to take place in biofilms is the absence or presence of very low levels of degradable organic matter. Therefore, in domestic waste waters, nitrification is always preceded by processes removing the organic materials by suspended or fixed film heterotrophic biomass systems. The removal of organic matter and nitrification can be performed in :-

- ✶ One biofilm reactor as one-stage treatment
- ✶ Two separate succeeding biofilm reactors with solid separation only after the second stage.
- ✶ Two stage treatment with intermediate solid separation and separate tertiary nitrification.

For reaching higher effluent quality, the effluent of a tertiary nitrifying biofilm reactor can be filtered directly (Boller and Gujer, 1986).

The different systems in use for maintaining aerobic nitrification reactors include trickling filter where the water percolates through a

bed with growing biomass (Focht and Chang, 1975), rotating discs to which the biomass is attached (Antonie, 1978) and fluidized beds in which the bacteria formed pellets around a carrier material (often sand) and are kept in suspension by the high flow rates used (Andrews, 1982, Heijnen, 1984).

Reactors in wastewater treatment employ immobilized bacteria in order to retain slow growing bacteria in high flow system or to provide dense biomass in a limited space (Robertson and Kuenen, 1992). In the past, a number of immobilization methods have been tried. These include attachment to a carrier such as sand (Gommers, 1987), cells trapped in liquid surfactant membranes (Mohan and Li, 1975), rotating discs and anaerobic filters (Winkler, 1981). In most of these cases, the natural abilities of microorganisms to attach to a variety of interfaces, supports and surfaces are exploited.

The immobilized cell systems (ICS) offer highly accelerated reaction rates due to increase in cell density. It is also possible to operate the systems at high dilution rate without wash out. The added beneficial feature of ICS is the inert solid supports in dilute media which may concentrate nutrients at the liquid solid interface and cells attached to these su pports are exposed to higher nutrient concentration than existing in the bulk liquid. Thus, higher cell population and faster growth rates may be achieved.

Use of immobilized cells has many other advantages also over the use of freely suspended cells such as the capability of re-using immobilized cells and the ease with which the cells can be separated from the reaction mixture, thus preventing contamination of the product stream.

Many methods namely adsorption, covalent bonding, crosslinking, entrapment and encapsulation are widely used for immobilization. The criteria imposed for cell immobilization technique usually determine the nature of the application. Immobilization by adsorption was apparently the first example of cell immobilization (Hattori and Furusaka, 1960, 1961). A variety of yeast cells were immobilized by adsorption on different supports like Kieselguhr, Wood, Glass, Ceramic,. Plastic materials etc. The adsorption phenomena is based on electrostatic interaction (Van der Waal's Forces) between the charged support and microbial cell. Several procedures of cell adsorption are based on pH dependence.

Adsorption of cells is generally considered to be one of the easiest methods of immobilization. (Woodward, 1988). The fixed film that attaches to the support material after inoculation of the bioreactor with municipal sludge can be used for the treatment of municipal wastewater (Genung et al, 1980). One of the disadvantages of employing adsorption for use in a bioreactor is the time required to build-up a microbial film sufficient for effecting a bioprocess.

The mechanism involved in immobilization by covalent bonding is based on covalent bond formation between activated inorganic support and cell in presence of a binding (cross-linking) agent. To introduce the covalent linkage, the chemical modification of the surface is necessary (Ramakrishna and Jamuna, 1990). Microbial cells can be immobilized by cross-linking each other with bi-or multifunctional reagents such as gluteraldehyde (Chibata *et al.*, 1974), toluene di iso-thiocyanate (Lartigue and Weetall, 1976) for diazotized diamine. Apart from chemical cross-linking, procedures employing physical processes such as flocculation (Lee and Long, 1974) and pelletization (Mcginis, 1975) also benefits the immobilization techniques because of strong mutual adherence forces of some microbial cell cultures.

The most extensively studied method so far in cell immobilization is the entrapment of microbial cells in polymer matrices. The matrices used are agar, alginate, carrageenan, cellulose and its derivatives, collagen, gelatin, epoxy resin, photocross linkable resins, polyacrylamide, polyester, polystyrene and polyurethane. The entrapment methods are based on the inclusion of cells within a rigid network to prevent the cells from diffusing into surrounding medium, while still allowing penetration of substrate. Entrapment of cells in alginate gel is popular because of its simplicity and mild conditions. Several reports are available employing alginate gel (Kierstan and Bucke, 1977). In this method, the cells are mixed in a solution of sodium alginate and cell-alginate sus-

pension is slowly extruded into 0.05 M $CaCl_2$ solution. The slurry droplets will instantly solidify as spherical beads. Apart from reversible network affected by certain calcium chelating agents like phosphates, Mg, K and EDTA, gel integrity is also poor.

Due to the very slow growth rate of nitrifying bacteria which results in wash-out of these cells and loss of nitrifying capacity, immobilization of nitrifiers is the only obvious solution and indeed the best nitrifying technical installations are biofilm reactors in which the nitrifying biomass is attached (naturally) to a solid support. However, the underlying mechanisms are difficult. To build up a better described and easier controlled systems, Tramper, (1989) suggested the immobilization of pure cultures of nitrifying bacteria in hydrocolloid gels like alginate and K-carageenan. Van Ginkel *et al.,* (1983), in their studies of the feasibility of nitrification by an immobilized biocatalyst, immobilized *Nitrosomonas eu ropaea*, cells in calcium alginate gel, though calcium alginate has certain disadvantages especially its instability in the presence of ions like phosphate and gluconate which form a complex with calcium . Yet it was chosen as a support material for immobilizing nitrifiers probably because of its simplicity and mild conditions. Van Ginkel *et al.,* (1983) observed that immobilized *N. europaea* in calcium alginate certainly had potential for nitrification, but applying a cheaper and a more stable support was a prerequisite. Tramper and Grootjen (1986). immobilized *Nitrobacter agilis* in carrageenan.

Kokufuta *et al.,* (1982) immobilized *Nitrosomonas europaea* (ATCC 25978) with polyelectrolyte complex prepared from strongly polyacidic and polybasic ions, where the insoluble polytectrolyte was formed in the culture broth itself. Nitrifiers could also be entrapped in polyethylene glycol gel as reported by Tanaka *et al.,* (1994). They immobilized activated sludge from a conventional nitrified liquor recycling activated sludge plant treating municipal wastewater in the elastic polyethylene glycol (PEG) gel and cut into pellets of 3 mm in size (PEG pellets). These PEG pellets were used to remove high levels of ammonium from the exhaust gas scrubber waste water of a municipal sludge drying facility.

However, in all these cases, most of the entrapped cells existed only in the narrow region near the surface of the gel beds due to the limited oxygen transfer rate inside them. Consequently, this resulted in the poor volume efficiency of the gel volume and thus lowered the maximum ammonium oxidation rate. Thus, to establish a practical system of rapid nitrification for small-scale point sources of pollution, Matsumura *et al.,* (1997) used a positively charged macroporous carrier 'AQUACEL', made of foamed cellulose with continuous macropores, in the immobilization of nitrifying bacteria by adsorption. To provide an ion-exchange capacity and for stabilization by cross linkage, the surface of the AQUACEL carrier was treated with polyethyleneimine (PEI). Inorganic synthetic waste water was used for the investigation.

In a recent work by Sousa *et al.,* (1997), the performance of different polymeric supports used in the adhesion of a consortium of autotrophic nitrifying bacteria were evaluated. The polymeric supports used were high density polystyrene, polyethylene, polypropylene, polyvinyl chloride and polymethyl methacrylate. The nitrifying biofilm formation on these polymeric supports was correlated with the hydrophobicity and surface charge of both bacteria and support media. They found that polypropylene being the most hydrophobic material had the best properties for biofilm formation, though other polymeric supports also gave good adsorption.

Polystyrene has been used as a support material in Biological Aerated Filter (BAF) and Trickling filters. Small polystyrene beads with an estimated specific surface close to 1200 m_2/m_3 was used in floating filters by Toetrup *et al.,* (1994). In an upflow biofilter, Biostyr (OTV), the filling material used by Vedry *et al.,* (1994) was expanded polystyrene in the form of 3.5 mm diameter spherical grains. In Aerated biofilters, Tschui *et al.,* (1994), the filter media consisted of granular polystyrene (Biostyr).

The development of immobilized cell system for potential commercial application involves a series of decisions on operating strategies and compromises on optimized condition due to economic viability. The process of decision making begins with the choice of the carrier material and ends with operating mode, each step is interdependent and all

the steps influence the overall economics of the final process flow sheet. Compromises need to be made at each level to make the process commercially feasible.

A number of configurations such as batch, continuous, fixed and fluidized bed are successfully employed in bioprocesses depending upon the need. In addition to these, there are several specialized reactor systems which are the combinations or modification of the basic types mentioned above. Fixed or packed bed reactor is one of the most common type of reactor used with immobilized biocatalysts. The construction is quite simple and the catalyst damage due to attrition can be totally eliminated in this reactor. Hence soft gel beads, such as calcium alginate and κarrageenan can be successfully used in this reactor. Although there are many forms of the fixed bed reactor, the most common is a packed bed of immobilized biocatalysts through which the substrate is either passed upwardly or downwardly. The shape and size of the packing is crucial because large particle size increases the diffusional resistances and smaller particle size results in high pressure drops, bed compaction and plugging problems. The flow direction of substrate solution is also important. In case where downward flow causes compression of the beds, upward flow is generally preferred for industrial application. Packed beds tend to be less expensive and because of high biomass loading per unit reactor volume than the more open reactor designs, fixed bed reactors will probably continue to dominate the large

scale industrial immobilized cell application.The packing provides large contact area for the organism, liquid and the gas phase.Typical packing materials used include pebbles, lava rock or plastic materials.

The fluidized bed reactor (FBR) in which the biocatalysts are fluidized by the upward flowing substrate solution has the advantage of low pressure drop and resistance to plugging. Here the microorganisms are present in the form of flocs or immobilized on to a carrier surface. The fluid flowing from the bottom of the reactor keeps the flocs or bioparticle in suspension. Because of the high surface area per unit reactor volume, the concentration of biomass in fluidized bed reactors are 10 times greater than that achieved in stirred reactors. Unfortunately the fluid velocities required for proper fluidization may lead to residence times insufficient to achieved the desired conversion.

The engineering of biological treatment processes using attached cultures requires a basic understanding of the kinetic bahaviour of the fixed microorganisms responsible for substrate removal from wastewaters. Although much research has been focussed on biofilm kinetics, particularly heterotrophic biofilms, (La Motta, 1976; Trulear *et al.*, 1982, Belkhadir,1986, Nguyen, 1989, Harremoes, 1978), little information is available on the dynamics of nitrifying biofilm development. For many years, the concept of active biofilm thickness has been studied to characterize biofilm behaviour. According to common diffusion-reaction theory, the biofilm accumulation continues until a critical thickness at

$Mb = Ma + Md$

The rate of accumulation of active bacteria on the support depends on the surface area available on the support. Thus, the rate of accumulation of active bacteria,

$(d\ Ma/dt) = \mu\ Ma.\ B.\ (A_0\text{-}a)/A_0$

Where a = the surface area covered by the microcolonies at the instant `t'

A_0 = the initial surface area of the support.

B = correction term for the difference between the surface area of the model and that of the physical reality of the phenomenon.

By writing substrate balance as developed in several papers (Bernard, 1990, NATO, 1992, Capdeville and Nguyen, 1990), modelling of aerobic and anaerobic biofilm leads to an expression of the volumetric substrate removal rate (Kov) which is dependent on initial substrate concentration (So) such as:

$$Kov = \frac{Kov\ max.\ So}{(So + K)}$$

According to Capdeville *et al.,* (1986,1993), based on their theory and confirmed by experiments, the volumetric substrate removal rate is a function of initial substrate concentration (So) and the growth rate μ is also closely bound to initial substrate concentration. But the maximal

concentration of active bacteria does not change with 'So.' Liu (1993) has shown that biofilm thickening is strongly dependent on initial substrate concentration. Thus, the autotrophic biofilm activity is independent of the thickness but dependent on the surface area to be colonized and only bacteria situated at the liquid/biofilm interface are concerned with substrate removal.

The research group of Harremoes (1978) demonstrated half-order/ zero-order kinetic models for fixed films. They found that the fixed biofilm samples from water recycle pilot plants showed a transition of the nitrification rate per unit area between half order kinetics and zero order kinetics (both in relation to the bulk concentration of total ammonia) respectively for relatively low and high concentration of total ammonia and a constant concentration of D.O. According to the kinetic model, half-order kinetics result from ammonia-diffusion limitation, while zero order kinetics result from oxygen-diffusion limitation or metabolism rate limitation.

With artificially established biofilms, pure culture nitrifiers closely follow biofilm kinetics corresponding to an intrinsic zero-order reactions (Williamson and Mc Carty, 1976). Nitrification is to be a zero-order reaction, as a sloping plane, where a half-order reaction is expected if ammonia is rate limiting. Nitrification in biofilms without interference from oxidation of carbonaceous BOD is rate limited by oxygen in the upper range of practical application (NH_4^+–N > 2-5 $mg.L^{-1}$)

unless aeration with pure O_2 is used. However, the removal rate per unit surface area increases with the concentration of ammonia at concentrations above 5mg.L^{-1} NH_4^+–N, though the effect is less pronounced than for concentration below 5 mg.L^{-1} NH_4^+–N. In order to increase the rate of reaction, either pure O_2 can be used with recycle to meet stoichiometry or bubble aeration can be used at the bottom of an upflow filter (Mc Harness, *et al.,* 1975; Haug and Mc Carty, 1971). The removal rates ranges from 0.07-0.15 Kg NH_4^+–N.M^{-3} per day.

The nitrifiers multiply exponentially only as long as they are in aerobic zone (Harremoes, 1978). About 50% nitrification is reached for 15-25 mg.L^{-1} effluent BOD, corresponding to an organic volume loading of approximately 0.3 Kg BOD_5/M^3/day (Parker, 1975). 90% nitrification requires 5-15 mg.L^{-1} effluent BOD. An important factor for biofilm nitrification is the combination of a large bacterial mass under aeration and a sufficient contact time between influent ammonia and the biomass.

The biofilm waste removal capacity can be determined from water samples taken at constant time intervals (0.5 or 1.0 hour) and analysing for the parameters involved (COD, total ammonia, nitrite and nitrate), D.O. can be measured in the inflow and outflow of the reactor vessel of the biofilm reactor unit with an oxygen sensor.

Under conditions of high organic matter loading rates, fixed biofilm nitrification is reduced by reduced oxygen penetration of the biofilm,

caused by oxidation of organic matter by heterotrophic bacteria. This infers that the influent BOD shouuld be very low to effect successful biofilm nitrification. On the influent BOD should lie in the range 5-15 $mg.L^{-1}$ thus lowering the organic load to the maximum.

In all practical application in waste water treatment, nitrifier growth takes place in waste water treatment processes when other types of biological growth occurs. In no case are there opportunities for pure cultures to develop. This fact has significant implications in process design for nitrification (De Renzo, 1978). In both combined carbon oxidation-nitrification systems and in separate stage nitrification systems, there is sufficient organic matter in the waste water to enable the growth of heterotrophic bacteria. In this situation, the yield of heterotrophic bacteria growth is greater than the yield of the autotrophic nitrifying bacteria. When this occurs, the slower growing nitrifiers will gradually diminish in proportion to the total population and be washed out of the system (Downing and Knowles, 1966). Thus, for consistant nitrification to occur, the following design conditions must be satisfied, assuming pH and D.O. do not limit nitrifer growth.

$$\mu N > \mu b$$

Where

μN = maximum growth rate of nitrifying population.

μb = growth rate of the heterotrophic population.

The rate of substrate removal is defined as:

$$qb = \frac{S0 - S1}{X1\ HT}$$

Where, S0 = influent total BOD (or COD), $mg.L^{-1}$

S1 = effluent soluble BOD (or COD), $mg.L^{-1}$

HT = hydraulic detention time, days and

X1 = MLVSS, $mg.L^{-1}$.

To reduce the food available to the heterotrophic bacteria and to lessen their dominance in controlling the solids retention time, an organic carbon removal step can be placed ahead of the nitrification stage, creating a "separate stage" nitrification process. Separate nitrification stages can have very long biological solids detention time (15 to 25 days). In biofilm nitrifications the DO concentration must be 2.7 times the ammonia-nitrogen concentrations to prevent oxygen transfer from limiting nitrification rates (Williamson and Mc Carty, 1973). Two operational procedures have been suggested to overcome this limitation: (1) dilution of the ammonia nitrogen through recirculation and (2) Increasing the oxygen transfer through the use of high purity oxygen (Williamson and Mc Carty,1973).

Taking into consideration, the various design criteria for successful biofilm nitrification, a prototype of a packed bed upflow aerobic bioreactor (PBAB) has been fabricated as given in fig.5-1, the description and working of which will be dealt with in section 5.2. and 5.3.

5.2 Materials and Methods

5.2.1 Selection of support material

Several materials such as sand grains, gravel, oyster shells, glass beads, plastics etc. were considered as the support materials for nitrifying consortia in the bioreactor. From among them plastic was finally chosen as the basic support material on the basis of the requirements such as:

1. Inertness in aquatic system;

2. Light weight;

3. Hydrophobicity;

4. Easiness to mould in to any shape and

5. Availability.

Among various types of plastics polypropylene and polystyrene are especially identified as good support materials for nitrifiers based on their hydrophobicity. (Sousa *et al.*, 1997). However, based on the availability and convenience to mould in to any shape, the High Density Polystyrene (HDPS) was selected here as the support material for nitrifiers in the bioreactor.

5.2.2 Preparation of polystyrene beads.

Clear transparent HDPS was moulded in to beads of 5mm diameter with rough surface and a hole of 2 mm at the centre.

5.2.3 Immobilization of nitrifiers on HDPS beads.

Two factors which determines the extent of immobilization of bacteria on solid surfaces are 1. Hydrophobicity and 2. Electrostatic force of interaction between the solid and the bacterial surfaces (Sousa *et al.,* 1997). HDPS has strong hydrophobicity, but to make the adsorption of nitrifiers effective positive charges also have to be imparted on to the surface. To achieve this end polyethylene imine, an ion exchanger (Matsumura *et al.,* 1997), was used.

5.2.3a Effect of polyethylene imine treatment of beads on immobilization.

To study the effect of polyethylene imine treatment of HDPS beads on the extent of immobilization of nitrifiers on their surface and also to standardize the whole process the following experiment was conducted.

Polystyrene beads (100 nos.) were dipped primarily in O.IN HCl for 3 hrs and washed with the detergent (Extran) (10% V/V) having neutral pH. The beads were subsequently rinsed with distilled water thrice and air dried. They were divided in to two lots and one of which was immersed in 0.2% aqueous polyethylene imine (Sigma Chemical Company, U.S.A.) at pH 7.0 over night (8 hrs.). The beads were washed thrice in distilled water to remove excess imine.

To evaluate the efficacy of the above treatment on the extent of immobilization of nitrifiers attained on beads, 25 beads treated with

imine and 25 untreated ones were immersed in 25mL cell suspension of ammonia oxidizing bacterial consortium having a cell density of 0.6 x 10^{-4}g.ml $^{-1}$ (dry weight) and nitrite oxidizing consortium with a cell density of 6.6 x 10^{-4} g.mL^{-1}(dry weight) on a rotary shaker at 100 rpm for 3 hours. After the exposure the cell suspension was decanted off and the cell density measured gravimetrically by passing through a membrane of 0.22 μ porosity. Beads were washed gently with sterile media containing 100μg. mL^{-} NH_4^+–N and 58 μg. mL^{-1} NO_2^- - N. The wash water also was collected and the cell biomass deterimined following the procedure described above. From these values the cell biomass adsorbed on to the surface of beads was estimated and expressed as the cell biomass (dry weight) per bead.

Following sets of beads were used further for the experiment.

1. Untreated beads not exposed to bacterial consortium.
2. Untreated beads exposed to bacterial consortium.
3. Treated beads not exposed to bacterial consortium.
4. Treated beads exposed to bacterial consortium.

The above category of beads were suspended in 25 mL medium XII containing 100 μg.L^{-1} NH_4^+–N and medium 6 containing 58 μg. L^{-1} NO_2^--N for ammonia and nitrite oxidizing bacteria respectively, and incubated in dark aerobically by passing air at a rate of 2 L.min^{-1}.

Periodically build up of NO_2^--N in the case of ammonia oxidizers and disappearance of NO_2^--N in the case of nitrite oxidizers were determined for 24 hrs. colourimentrically following Strickland and Parson (1968) and APHA (1989).

5.2.4 Reactor design and fabrication

A prototype of bench scale bioreactor designed is given in Fig.5-1. It is precisely a rectangular tank having 10 cms base and 45 cms overall height. The inlet of 2cms diameter is fixed on the side of the tank 35 cms from above the base. The outlet having the same dimension is fixed at the base and to maintain the water level, the outlet pipe is brought up to the same level of the inlet pipe. A perforated platform is fixed at a ridge 5cms from above the base. A 30 cms long pipe (2 cms dia) fixed at the centre of the perforated plate function as air lift pump.The tank along with the perforated plate were made of fibre glass and pipes with PVC. The effective volume of the reactor to be packed with the polystyrene beads immobilized with the nitrifying bacteria was estimated to be c= a-b where a: Volume of the tank; b: Volume of the central pipe (air lift pump). Volume of the tank beneath the perforated plate is estimated to be d (500 mL) which along with the void volume contributes to the total quantity of sewage which can be treated at a time, coming to 2.15 litres.

5.2.5 Reactor Kinetics

5.2.5.1 Ammonia oxidation

The reactor tank was filled with HDPS beads (12000 nos.) treated with polyethylene imine as described under section 5.2.3. Ammonia oxidizing consortium (14.33 mL) to attain a final biomass of 0.2×10^{-4}g. mL^{-1} was suspended in 2.15 litre medium XII containing 16.33 mg. L^{-1} of NH_4^+–N and added to the packed bed of polystyrene beads. The medium with cell suspension was circulated through the packed bed using the air left pump for three hours by passing compressed air at a rate of 1 litre per minute to effect immobilization. During this period the extent of immobilization attained was monitored indirectly by estimating the residual NH_4^+ -N and the build up of NO_2^- -N in the medium. After three hours the fluid was drained off.

The reactor was then filled with 2.15 litre synthetic wastewater (Tanaka *et al.,* 1994) containing per 1000 mL tap water (NH_4Cl, 76.4 mg.L^{-1}; $NaHCO_3$, 234.3 mg.L^{-1}; $Na_2HPO_4.12H_2O$, 23.1 mg.L^{-1}) having available ammonia concentration of 41.16 mg. L^{-1} NH_4^+-N at pH. 8.5 The fluid was kept under circulation by passing compressed air through the air lift pump at a rate of 1 litre per minute. Regular checks of NH_4^+ –N, NO_2^-–N and NO_3^- –N and pH at two hour intervals were made.

Whenever there was a fall in pH it was readjusted to 8.5 using 10% Na_2CO_3 solution. From the data generated with three consecutive runs the rate of activity in terms of substrate uptake, product formed, yield coefficient at varying intervals and alkalinity destroyed were estimated.

5.2.5.2 Nitrite oxid ation

The reactor tank was filled with HDPS beads (12000 nos.) treated with polyethylene imine as described under section.5.2.3. Nitrite oxidizing consortium (7.82 mL) to attain a final biomass of 0.6 x 10^{-4} mL^{-1} was suspended in 2.15 litre medium 6 containing 10.0 mg. L^{-1} NO_2^--N and added to the packed bed of polystyrene beads. The medium with cell suspension was circulated through the packed bed using the air lift pump for three hours by passing compressed air at a rate of 1 litre per minute to effect immobilization. During this period the extent of immobilization attained was monitored indirectly by estimating the residual NO_2^--N in the medium. After three hours the fluid was drained off. The reactor was then filled with 2.15 litre synthetic wastewater. (Tanaka *et al.*, 1994) containing 39.62 mg. L^{-1} NO_2^--N at 7.5 pH.

The fluid was kept under circulation by passing compressed air through the air lift pump at a rate of 1 litre per minute. Regular checks of NO_2^--N, NO_3^-–N and pH at one hour interval were

made. From the data generated with three consecutive runs the rate of activity in terms of substrate up take, product formed, yield coefficient at varying intervals and alkalinity destroyed were estimated.

5.2.5.3 Estimation of biomass on polystyrene beads in the reactor.

The biomass of nitrifying consortium built up on the polystyrene beads at the end of the experiment was determined. For this, beads from the bioreactor were gently removed and washed with the respective immobilization medium (Medium XII in the case of ammonia oxidizers and medium 6 in the case of nitrite oxidizers). They were dried over night at 80°C and weighed using an electronic precision balance. The difference in weight between the beads uncoated with nitrifiers and the ones removed from the reactor gave the biomass built on beads due to immobilization of cells and the subsequent growth during running of the reactor.

5.3 Results and discussion

5.3.1. Reactor Design and Support Material

This is a packed bed reactor with an upward flow of water through the central air lift pump. Reciprocal to this movement aerated water from the top flows down percolating through the bed during which nitrification takes place. The 2cm diameter central air lift pump is surrounded with a bed of 4cms on all the sides as the effective oxygenated area which function as an aeration cell. Size of the aeration cell is restricted to this much to assure avoidance of dead areas with in the reactor. Passage of compressed air through the air lift pump at a rate of 1 litre per minute facilitated effective circulation of water through the bed (Fig.5.1).

High density polystyrene beads with immobilized nitrifying consortia formed the effective surface biofilm for nitrification. The beads with rough surface and a hole at the centre were loosely packed which facilitated free flow of the wastewater. Because of the loose packing, possible clogging and cracking of the bed can be avoided. Even with this packing 2.15 litre wastewater can be treated at a time which is 71.67% of the total volume of the reactor. Only three category of materials are required for the construction of the reactor such as fibre glass, PVC and polystyrene all of which are available locally.

5.3.2 Effect of polyethylene imine treatment on immobilization of ammonia oxidizing consortium.

Results of the study on the effect of polyethylene imine treatment on the extent of immobilization of ammonia oxidizing consortium attained on HDPS beads are summarised in Table 5-1. It could be seen that out of the four categories of polystyrene beads used the PEI treated alone, when exposed to ammonia oxidizing consortium, showed marked adsorption of cells as manifested by the corresponding ammonia oxidizing activity and the removal of cells from the suspension. Each bead was found to immobilize 2.04 x 10^{-5}g cells on dry weight basis.

The stock culture used has a biomass of 0.003g. mL^{-1} and for 2150 mL immobilization medium, based on the biomass adsorbed on to the beads, a requirement of 14.33 mL culture is estimated.

5.3.3 Effect of polyethylene imine treatment on immobilization of nitrite oxidizing consortium.

Results of the study on the effect of polyethylene imine treatment on the extent of immobilization of nitrite oxidizing consortium attained on HDPS beads are summarised on Table 5-2. It could be seen that out of the four categories of polystyrene beads used, the PEI treated alone when exposed to nitrite oxidizing consortium showed marked adsorption of cells as manifested by the corresponding removal of cells from the suspension and nitrite oxidizing activity. Each bead was found to immobilize 1.224 x 10^{-4}g. cells on dry weight ba-

sis. The stock culture used has a biomass of 0.033 g mL^{-1} and for 2150 mL immobilization medium based on the biomass adsorbed on to the beads a requirement of 7.82 mL culture is estimated.

Application of polyethylene imine as an ion exchanger can be of both ways. Either it can be added to cell suspension imparting poly cationic characteristics to the cell surface which has a net negative charge. Such cells can adhere strongly as a monolayer to surfaces which bears a net negative charge (D' Souza and Kamath, 1988; and Matsumura *et al.,* 1997). On the other hand a solid substratum also can be treated with the polyethylene imine to impart polycationic characteristics to which the negatively charged cells would get adsorbed (Kamath, *et al.,* 1988, and Sankaran *et al.,* 1989). In the present work the polymeric support material was made poly cationic with the polyethylene imine.

Sousa *et al.,* (1997) on studying polymeric support for the adherance of a consortium of autotrophic nitrifying bacteria observed that besides glass, polypropylene, High density polystyrene and polyvinyl chloride are more hydrophobic whereas polymethyl - methacrylate and high density polyethylene presented an intermediate behaviour. Their observation lead to the conclusion that in the adhesion process of these microorganisms, not only interfacial energy should be considered but also the electrostatic interactions play a very important role.

Application of polyethylene imine has now made it possible to alter the surface charge of the polystyrene beads to make it adsorbable for the negatively charged bacteria.

5.3.4 Nitrifying biomass on polystyrene beads in the reactors.

The biomass of nitrifying consortium found to have built up on the polystyrene beads after three consecutive runs of the reactors are summarised in Table 5-3. As could be noticed an increase in biomass per bead by 3.9 times in the case of ammonia oxidizing consortium and 1.24 times in the case of nitrite oxidizing consortium had taken place when around 123.48mg. $NH_4^+ - N.L^{-1}$ and 118.86mg. NO_2^--$N.L^{-1}$ were consumed. This implies that as the running of the reactor continues a progression in the build up of biomass can be achieved which in turn favours better performance of the reactor.

5.3.5 Reactor Kinetics

The substrate (NH_4^+-N) utilization and the product (NO_2^--N) build up and the percentage consumption of $NH4^+$–N in the ammonia oxidizing bioreactor during its running at different HRT are summarized in Table 5-4. The rate of consumption of $NH4^+$–N and build up of NO_2^-–N are depicted in Fig. 5-2. Meanwhile the yield coefficients at different HRT are presented in Fig.5-3. It could be seen that 100% ammonia removal was attained at an HRT of 24 hours with an air flow rate of 1 litre per minute. The nitrification rate was found to be 41.16mg NH_4^+- $N\,L^{-1}$ in terms of ammonia removal for an HRT of 24

hours with a residual nitrite build up of 2.605mg. NO_2-N. L^{-1}. Yield coefficient, in terms of nitrite build up against ammonia consumed, was highest at 4th hour of treatment and gradually declined till 22nd hour. Subsequently it exhibited an upward trend at the 24th hour at which the entire ammonia had been consumed. A total alkalinity destruction of 304.58mg. equivalent to 5.2ml of 10% Na_2CO_3 was observed when the entire substrate had been consumed. (Table 5-5).

In the nitrite oxidizing bioreactor substrate (NO_2^--N) utilization and the product build up and the percentage consumption of NO_2^--N during its running at different HRT are summarized in Table 5-6. The rate of consumption of NO_2^--N and build up of NO_3^--N are depicted in Fig.5-4. Meanwhile the yield coefficients at different HRT are presented in Fig. 5-5. It could be seen that 100% nitrite removal was attained at an HRT of 5 hours with an air flow rate of 1 litre per minute. The nitrification rate was found to be 39.62 NO_2^-–N mg, $ml.^{-1}$ in terms of nitrite removal for an HRT of 5 hrs with a residual nitrate build up of 4.887 $mg.L^{-1}$. Yield coefficient in terms of nitrite build up against nitrite consumed was at the highest during the 1st hour of treatment (0.1443) and lowest at the 3rd hour (0.0967) and again showed an upward trend during which the entire NO_2^- – N had been consumed. There was no alkalinity destruction during the process.

It appears that the reduction in the yield coefficient during running of the reactor might be due to product accumulation since the system was run as batch process. However an upward trend in the yield coefficient at the end of nitrification cannot be explained at this stage. It implies that if the reactor is run as in a continuous process the product accumulation can be avoided resulting in higher yield coefficient throughout and overall better performance of the reactor.

In this reactor set up the time required for immobilization of the consortia has been reduced to 3 hours and the reactor can start functioning immediately after without manifesting any lag. Tanaka *et al.*, (1994) applied immobilized nitrifiers on gel to remove NH_4^+–N and Tschui *et al.*, (1994) made a comparision of aerated biofilters with varying support materials such as biocarbon, biostyr and biopur for tertiary nitrification. Since the configuration, mode and operation of the reactors developed here are different a true comparison could not be possible.

5.3.6 Conclusion

Prototype of two bioreactors , one for ammonia and the other for nitrite removal from sewage have been developed which function on a packed bed of polystyrene beads coated with a biofilm of nitrifying bacterial consortium. With the configuration of the reactor described the one meant of ammonia oxidation consumes 41.16mg.NH_4^{-1}-N.L^{-1} with in a HRT of 24 hours. Meanwhile the reactor meant for nitrite oxida-

tion consumes 39.62 mg NO_2^--N L^{-1} with in a HRT of 5 hours.

The reactor is meant for tertiary nitritification of sewage with very low BOD (<20mg. L^{-1}).The speciality and advantage of the reactors are that they can be set up with in 3 hours and can be readily integrated with the terminal part of the sewage treatment system without experiencing any lag in nitrification. A true comparison of this reactor with other similar systems could not be possible as the concept and design were quite novel and unique.

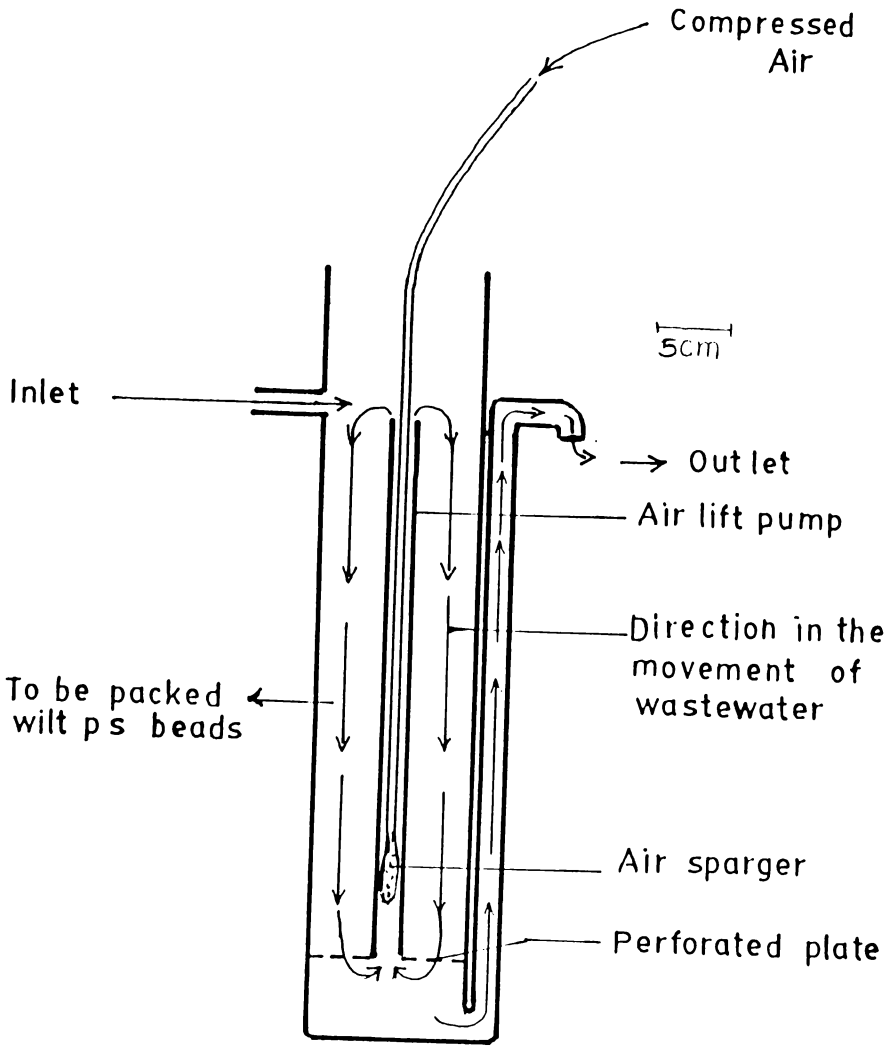


Fig. 5-1 Schematic diagram of the bioreactor.

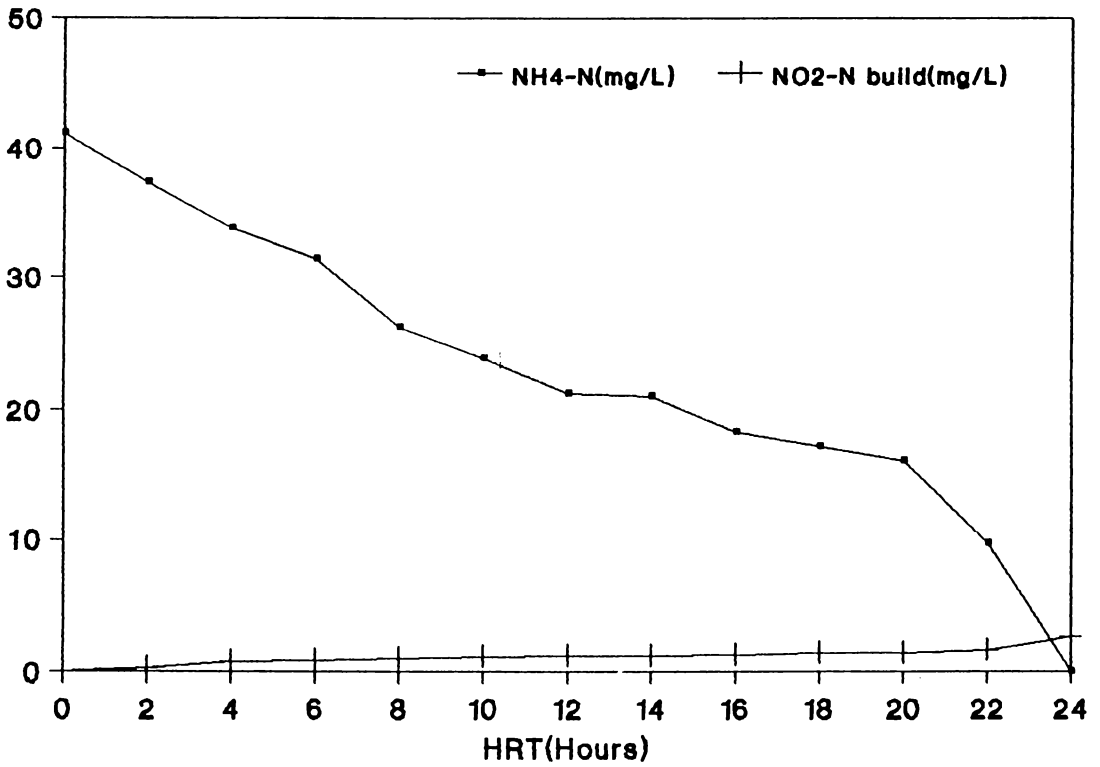


Fig. 5-2 Rate of ammonia removal and nitrite build up in the developed ammonia oxidizing bioreactor (n=3)

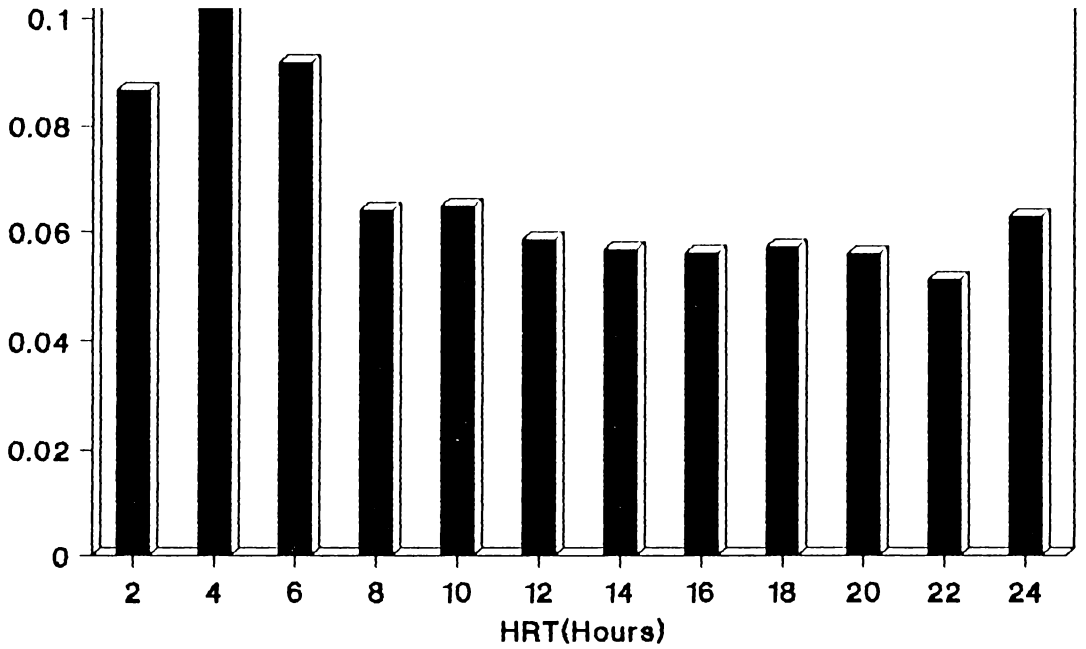


Fig. 5-3 Yield coefficient recorded in the developed ammonia oxidizing bioreactor (n=3)

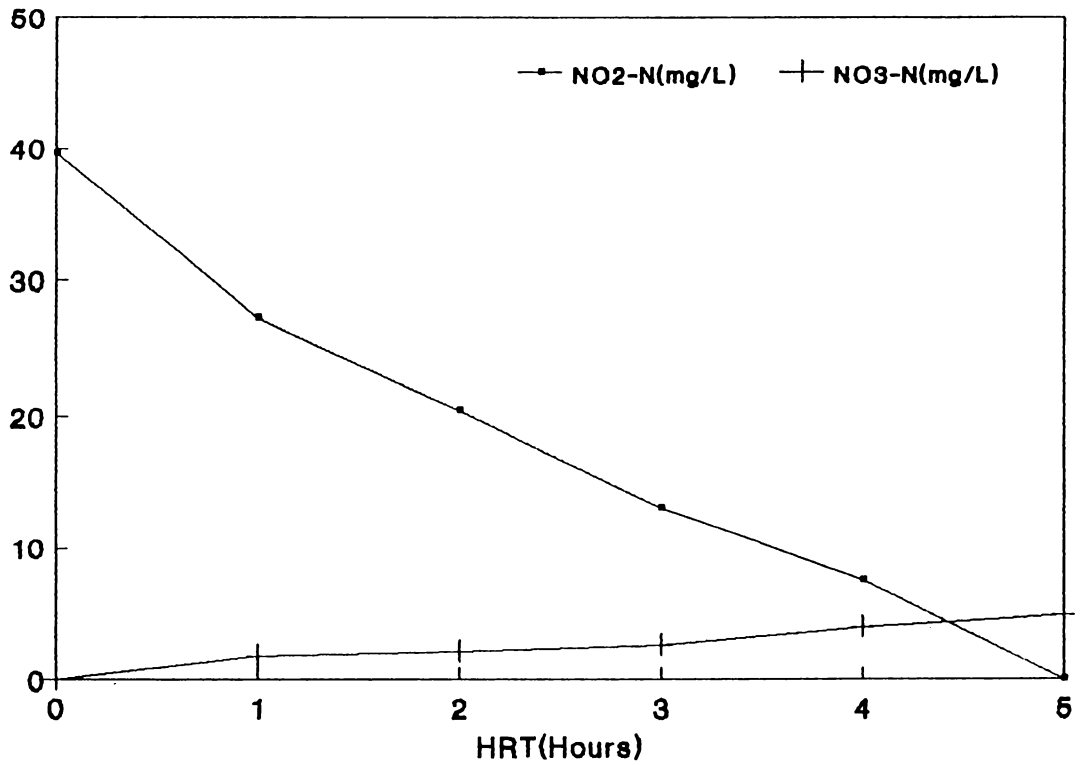


Fig. 5-4 Rate of nitrite removal and nitrate build up in the developed nitrite oxidizing bioreactor (n=3)

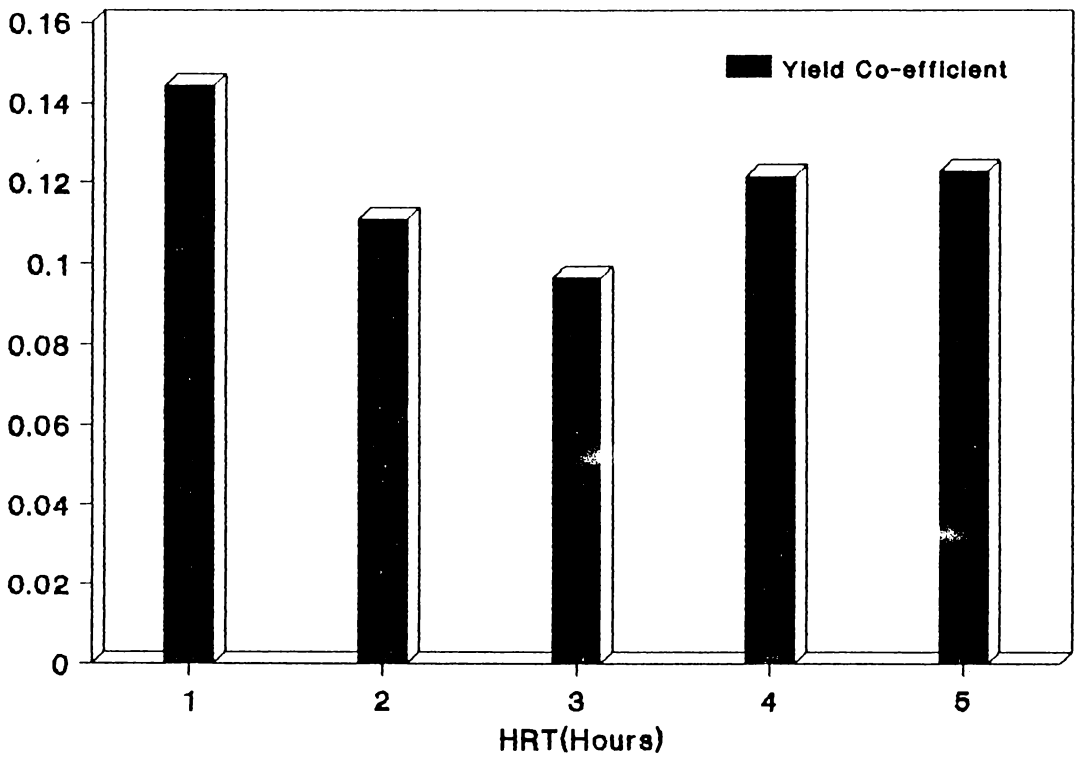


Fig. 5-5 Yield coefficient recorded in the developed nitrite oxidizing bioreactor (n=3)

TABLE 5-1

Effect of Polyethylene imine (PEI) treatment on immobilization of ammonia oxidizing consortium on polystyrene (PS) beads

	Total weight of ammonia oxidizing consortium used for immobilization ($g.mL^{-1}$)	Total weight of bacterial consortium remaining in the medium after 3 hrs. ($g.mL^{-1}$)	Total weight of bacterial consortium immobilized on PS beads (25 nos) ($g.mL^{-1}$)	Ammonia oxidizing activity	
				Consumption of NH_4^+–N $\mu g.mL^{-1}$	Build up of NO_2–N $\mu g.mL^{-1}$
PEI treated PS bead not exposed to bacterial consortium	—	—	—	—	—
PEI untreated PS beads exposed to bacterial consortium (0.5 mL stock culture in 25 mL medium)	0.00006	0.0006	—	—	—
PEI treated PS beads not exposed to bacterial consortium	—	—	—	—	
PEI treated PS beads exposed to bacterial consortium (0.5 mL stock culture in 25 mL medium)	0.00006	0.00004	0.00002 (2.04×10^{-5} $g.bead^{-1}$)	50	8

TABLE 5-2

Effect of Polyethylene imine (PEI) treatment on immobilization of nitrite oxidizing consortium on polystyrene (PS) beads.

	Total weight of nitrite oxidizing consortium used for immobilization ($g.mL^{-1}$)	Total weight of bacterial consortium remaining in the medium after 3 hrs. ($g.mL^{-1}$)	Total weight of bacterial consortium immobilized on PS beads (25 nos) ($g.mL^{-1}$)	Nitrite oxidizing activity Consumption of NO_2^-–N $\mu g.mL^{-1}$
PEI treated PS bead not exposed to bacterial consortium	—	—	—	—
PEI untreated PS beads exposed to bacterial consortium(0.5 mL stock, culture in 25 mL medium)	0.00066	0.00066	—	—
PEI treated PS beads not exposed to bacterial consortium	—	—	—	—
PEI treated PS beads exposed to bacterial consortium (0.5 mLstock culture in 25 mL medium)	0.00066	0.00054	0.00012 (1.224×10^{-4} $g.bead^{-1}$)	58

TABLE 5-3

Nitrifying biomass immobilized on PEI treated HDPS beads in the bioreactor

Reactor	Initial weight of biomass (g) per bead	Final weight of biomass (g) per bead	Times of increase
Ammonia oxidizing bioreactor	2.04×10^{-5}	8×10^{-5}	3.9
Nitrite oxidizing bioreactor	1.224×10^{-4}	1.52×10^{-4}	1.24

TABLE 5-4

Substrate utilization and product build up in ammonia oxidizing bioreactor for different HRT (n = 3)

HRT (hours)	NH_4^+–N concentration $mg.L^{-1}$	NO_2^-–N build-up $mg.L^{-1}$	Percentage consumption of NH_4^+–N (%)
0	41.16	0	0
2	37.35	0.3305	9.25
4	33.75	0.8085	18.0
6	31.395	0.8635	23.72
8	26.13	0.9655	36.51
10	23.845	1.127	42.06
12	21.23	1.171	48.42
14	21.06	1.220	48.83
16	18.29	1.288	55.56
18	17.20	1.376	58.21
20	16.11	1.405	60.86
22	9.80	1.610	76.19
24	0	2.605	100

TABLE 5-5

Alkalinity destroyed during ammonia oxidation in Packed Bed Reactor (6.0 - 7.4 mg alkalinity destroyed per mg NH_4^+–N oxidized to NO_3^-–N (EPA 1975)

Total NH_4^+–N removed (mg. L^{-1})	Total alkalinity destroyed (mg)	volume of 10% sodium carbonate used to neutralize acid production
41.16	246.96 — 304.58	5.2 mL

TABLE 5-6

Substrate utilization and product build up in nitrite oxidizing bioreactor for different HRT (n = 3)

HRT (hours)	NO_2^-–N concentration mg.L^{-1}	NO_3^-–N concentration mg.L^{-1}	Percentage consumption of NO_2^-–N (%)
0	39.62	0	0
1	27.22	1.79	31.29
2	20.39	2.134	48.53
3	13.07	2.57	67.01
4	7.51	3.91	81.04
5	0	4.887	100

CHAPTER 6
SUMMARY

Chapter -6

SUMMARY

Nitrification is the biological oxidation of ammonium, first to nitrite and then to nitrate by two groups of aerobic, chemolithotrophic bacteria belonging to the family Nitrobacteriaceae. The biological nitrification in municipal wastewater treatment is important in those cases were ammonia removal requirement specially exist. In a trickling filter or in an activated sludge system nitrification is rate limiting and thus necessitates longer detention time.

The combined carbon oxidation-nitrification processes generally have low population of nitrifiers due to a high ratio of BOD to total nitrogen in the effluent. This necessitates, separate carbon and nitrogen oxidation processes, which thus minimizes wash out of the nitrifiers. Therefore, a separate stage nitrification has become essential to achieve faster and efficient removal of ammonium from the wastewater. The present work deals with the development of bioreactor for nitrifying of sewage as the tertiary process so that the treated wastewater can be used for irrigation, algal culture or fish culture. The work carried out and achievements made are summarized as follows

- For the proposed bioreactors consortia of nitrifiers were required and for their development, pre-enrichment system based on sand based biofilter was employed. Treated sewage was passed through the filter bed using an airlift pump for a prolonged pe-

riod with the addition of increments of ammonium sulphate along with regular adjustment of pH. At the conditioning of the biofilter the sand grains were observed under scanning electronmicroscope and dense colonization of bacteria forming a biofilm was observed. These sand grains were used as source of nitrifiers.

- For the primary and secondary enrichment of nitrifiers grown as biofilm on the filtrant grains, twelve synthetic media were screened for ammonia oxidizers and six for nitrite oxidizers.Accordingly, the medium XII (Lewis and Pramer,1958) for ammonia oxidizers and the medium 6 (Winogradsky medium modified , Rodina,1972)for nitrite oxidizers were selected as the most ideal ones for all subsequent works

- By primary and secondary enrichment, a consortium of ammonia oxidizers and another one for nitrite oxidizers were developed in medium XII and 6 respectively and mass cultured by batch process set up using 1 litre flask over a magnetic stirrer.

- The maximum allowable substrate concentrations for ammonia and nitrite oxidizing consortia were found to be 2.75g.L^{-1}. NH_4-N and 2.5g.L^{-1} NO_2^- – N respectively, beyond which progressive inhibition of nitrification took place.

- The maximum nitrifying potential of ammonia oxidizing con-

sortium was exhibited at pH 8.5and that of nitrite oxidizing consortium at pH 7.5.

- The optimum temperature for nitrification by ammonia oxidizing and nitrite oxidizing consortia lies in the range of 28°C - 30°C, with no significant activity below 4°C or above 37°C.

- As the rate of air flow was increased, the nitrification was also found to increase exponentially. It could be seen that the build up of $NO_2^- - N$ by ammonia oxidizers was increased ten fold with the passage of 7 litres air per minute, and the consumption of $NO_2^- - N$ by nitrite oxidizers went up by a factor 28.8 with the passage of the same quantity of air.

- For quantifying nitrifying consortia, a new concept of Unit Nitrifying Activity (UNA) was introduced. One unit nitrifying activity of a nitrifying consortia is defined as the nitrifying biomass which can bring about the generation of 1μmol $NO_2^- - N$/min/L in the case of ammonia oxidizers and the consumption of 1μ mol $NO_2^- - N$/min/L in the case of nitrite oxidizers. Accordingly, 1mL of ammonia oxidizing consortium exhibited a unit nitrifying activity of 0.702 x 10^{-3} UNAa at pH 8.5, substrate concentration 339 μg NH_4-N.L^{-1} and air flow of 1 litre per minute and 1mL of nitrite oxidizing consortium exhibited a unit nitrifying activity of 0.1729 x 10^{-1} UNAnat pH 7.5, substrate con-

centration 325µg. $NO_2^- - N.L^{-1}$ and rate of air flow of 1litre per minute.

- The nitrifying consortia could be maintained at 4^0 C in a refrigerator for prolonged period without any apparent loss of viability

- Consumption of $NO_2^- - N$, build up of $NO_2^- - N$ and the production of biomass of ammonia oxidizers and the consumption of NO_3^--N and the production of biomass of nitrite oxidizing consortium before attaining stationary phase, was worked out in a fermentor. It could be seen that on consuming 4.29 $g.L^{-1}$ $NO_2^- - N$ by ammonia oxidizers, only $0.834g.L^{-1}$ of the product nitrite was formed, with a biomass build up of $0.80g.L^{-1}$ during a total incubation period of 30 days. In the same way, on consuming $6.246g.L^{-1}$ $NO_2^- - N$ by nitrite oxidizers, $1.242g.L^{-1}$ $NO_3^- - N$ was produced with a biomass build up of $1.09g.L^{-1}$. Only 19.39 per cent of the ammonia consumed was oxidized to $NO_2^- - N$ and the balance of 80.61 percent was used up for maintenance. Similarly 19.88 percent of $NO_2^- - N$ consumed by nitrite oxidizers was alone oxidized to NO_3^--N and the balance was used up for maintenance.

- During this oxidation process, the build up of $NO_2^- - N$ by the ammonia oxidizers was approximately 5.15 times less than the

amount of NH_4^+–N consumed. In the same pattern,biomass build up was also 5.36 times less than the ammonia oxidized,comparable to the quantity of nitrite generated. Similarly, the generation of NO_3^-–N by nitrite oxidizers was 5.02 times lesser than the NO_2^--N consumed and in the same order the biomass build up was 5.73 times leser than the substrate consumed. Thus the biomass of nitrifying bacteria in a fermentor can be indirectly measured by estimating the product of oxidation.

- The nitrifying consortium on attaining a stationary phase ranged from 2.496 x 10^{10} to 9.984 x 10^{10} cells.L^{-1} ammonia oxidizers and 1.242 x 10^{10} to 4.968 x 10^{10} cells.L^{-1} nitrite oxidizers. Apparently, to support more or less the same cell density/biomass, 1.5 times more of NO_2^- – N had to be oxidized by nitrite oxidizers than the ammonia oxidizers. This is in agreement with the stoichiometry of nitrification.

- Based on the lowering of pH during the growth of ammonia oxidizers, the quantity of 10% sodium carbonate required for compensating the 25.74-31.74 alkalinity destroyed was 25 mL.

- Yield coefficient of ammonia oxidizing consortium ranged from 0.1864 to 0.1939 and that of nitrite oxidizers from 0.1745-0.1938. Compared to the yield coefficients obtained with pure cultures

of nitrifiers, the values obtained here are very much higher. This is indicative of better energy conversion efficiency and higher carbon dioxide fixation potential of the consortium, and a reflection of the better assimilatory capability of the consortium.

- The generation time of ammonia and nitrite oxidizers under optimal conditions was found to be 24 hours and 16.8 hours respectively.

- Specific growth rate of ammonia and nitrite oxidizing consortium was found to be $0.0288h^{-1}$ and $0.041\ h.^{-1}$ respectively in the fermentor.

- Ammonia oxidizing consortium has a Km of 0.5 $mg.L^{-1}$ and μmax of $0.0082\ hr^{-1}$. At the same time, the nitrite oxidizers have a Km of 0.370 mg. $N.L^{-1}$ and μmax of $0.0232\ h^{-1}$.

- For all practical purposes, the behaviour of consortia was very much comparable to pure cultures recorded in literature and thus justifies the development of consortia of nitrifying bacteria in the place of pure cultures for applying in the bioreactor.

- Under transmission electron microscope, the cells of nitrifying consortium are found aggregated covered with a less electron dense slime secreted by the organisms. Through these slimy mass the cells could make interconnections and remain entangled designated as zoogloea formation. Even the binary fission of the

cells occured within the slimy sheath. Slender long cells which were slightly curved in most of the cases were identified as nitrifying -like bacteria. Overall length of cells in the consortium ranged from 0.5 to 2.15μ.

- By combining both serial dilution and plating techniques, 14 pure cultures of ammonia oxidizing bacteria and 40 pure cultures of nitrite oxidizers were segregated from the consortia, stocked in phytagel solidified slants and maintained at 28°C and 4°C.

- All the isolated pure cultures were Gram negative rods and all of them showing tumbling rotating movement. Ammonia oxidizers measured around 1.25 μm and nitrite oxidizers measures ranged from 1.875 - 2.5 μm. These measurements matches very well with that of the long slightly curved cells observed under TEM designated as nitrifying-like bacteria.

- All the pure cultures isolated could grow heterotrophically also. Based on the present level characterization, the ammonia oxidizers could not be identified. However, the nitrite oxidizers could be designated as *Nitrobacter* and *Nitrospira* -like organisms.

- The heterotrophic bacteria associated with the nitrifiers were found to belong to the genera *Alteromonas, Alcaligenes,*

Pseudomonas and *Acinetobacter*. It is postulated that these heterotrophs are not mere contaminants, but an integral part of the consortium. They survive on the exudates of the nitrifiers and the nitrifiers in turn are benefited by certain unknown growth factors elaborated by the 4 heterotrophs.

- For immobilizing these consortia of ammonia and nitrite oxidizers, polystyrene was chosen as the support material. It was moulded into 5mm diameter beads with rough surface and a hole of 2mm diameter at he centre.

- To make the surface of these beads polycationic, these were treated with 0.2% (v/v) aqueous polyethylene imine overnight and washed repeatedly to remove excess imine. The nitrifying consortia readily adsorbed on to the surface of these beads and started nitrification without exhibiting any lag. Each bead was found to immobilize on the surface 2.04×10^{-5} g of ammonia oxidizing consortium and 1.224×10^{-4}g nitrite oxidizers on dry weight basis.

- The prototype of the bench scale bioreactor designed and fabricated is a rectangular tank having 10 cms base and 45 cms overall height. A perforated platform is fixed at 5cms from bove the base. A 2cm diameter PVC pipe fixed at the centre functions as the air lift pump. The tank is filled with cationically charged

polystyrene beads. With this configuration 2.15 litres of sewage can be treated at a time by batch process.

- The rate of nitrification on the bioreactor build-up with ammonia oxidizers was found to be 41.16mg.NH_4^+–N.L^{-1} in terms of ammonia removal for an HRT of 24 hours with a residual nitrite build-up 0f 2.605 mg.NO_2^-–N.L^{-1}. Total alkalinity destruction was equivalent to 5.2 mL.

- The rate of nitrification in the bioreactor built-up with nitrite oxidizers was found to be 39.62mg.NO_2^-–N.L^{-1} in terms of nitrite removal for an HRT of 5 hours with a residual nitrate build up of 4.887 mg.L^{-1} with no alkalinity destruction.

- In both the reactors the yield coefficient was highest during the initial phase which subsequently declined during its running, indicating product inhibition on nitrification. This suggests that the performance of the reactor would be better if run in a continuous mode.

- The reactors developed here are meant for tertiary nitrification of sewage having low BOD (<20 mg.L^{-1}). The reactors can be set up with in 3 hours and can be readily integrated with the terminal part of the sewage treatment system without experiencing any lag in nitrification.

- For setting up and running the reactors, materials essentially required are fibreglass tanks, PVC pipes, polystyrene beads, polyethylene imine, nitrifying consortia , immobilization solution and compressed air.

REFERENCES

REFERENCES

Abelovich, A., 1987. Nitrifying bacteria waste water reservoirs. *Appl. Environ Microbiol,* **53 :** 754-755.

Aleem, M.I.H. and Alexander, M., 1958. Cell-free nitrification by *Nitrobacter. J.Bacteriol,* **76 :** 510 -514.

Alexander, M., 1965. Nitrification In: Soil nitrification (W.V. Bartholomev and F.E.Clark Eds.), 309-346. Amer.Soc. Argron No.10. Madison, Wisc.

Anderson, I.C. and Lewine, J.S.,1986. Relative rates of nitric oxide and nitrous oxide production by nitrifiers, denitrifiers and nitrate respirers *Appl. Environ. Microbiol,* **51 :** 938-945.

Andrews, G.G., 1982. Fluidized bed fermentors a steady state analysis. *Biotechnology Bioengineering,* **24** : 2013-2030.

Anon., 1989. Microbial type culture collection and gene Bank catalogue, Institute of Microbial Technology, India, 118.

Antonie, R.L., 1978. Nitrogen control with the rotating biological contactor. In *Advances in water and waste water treatment, Biological Nutrient Removal.* Eds., M.P. Wanielista, & W.W. Eckenfelder : 265-281. Michigan. Ann Arbor Science.

Anthonisen, A.C., 1974. The effects of free ammonia and free nitrous acid on the nitrification process, Ph.D.(Eng.) Thesis Corwell University , Ithaca, New York.

Anthonisen, A.C. Loehr, R.C. Prakasham, T.B.S. and Srinath, E.G., 1976. Inhibition of nitrification by ammonia and nitrous acid *J.W.P.C.F.* **48** : 835 852.

APHA., 1989. Standard Methods for the Examination of Water and Wastewater -17th Edn. Washington D.C.

Ardakani, M.S., Rehbock, J.T. and Mc Laren, A.D., 1974a. Oxidation of ammonium to nitrate in a solid column. *Soil Science Society of America Proceedings,* **38** : 96-99.

Ardakani, M.S., Schulz, R.K and Mc Laren A.D., 1974 b. A Kinetic study of ammonium and nitrite oxidation in a soil field plot. *Soil Science Society of America Proceedings,* **38** : 273-277.

Baritt, N.W., 1933. The nitrification process in soils and biological filters. *Ann. Appl. Biol* : **20** : 165-184.

Beijerink M.W., 1896. Kulturversuche mit Amoben aüf festem substrate. Zentrablatt für Bakleriologie Parasitenkunde, Infekionskrankheiten und Hygiene, **19** : 257-67.

Belkhadir, R., 1986. Etude fondamentale des biomasses fixees: description et modelisation des films biologiques anaerobies.Ph.D. thesis, N 18 INSAT Toulouse, France.

*Belser L., 1974 The ecology of nitrifying bacteria. Univ. California, Berkeley, Univ. Microfilms. *Ann. Arbor. Mich.*

Belser, L.W. and Schmidt, E.L., 1980. Growth and oxidation Kinetics of three genera of ammonia oxidizing nitrifiers. *FEMS Microbiology letters* **7** : 213-216.

Bergey's Manual of Systematic Bacteriology. Vol-3 . (James. T Staley, Williams and Wilkins, ed) 3rd edition . Baltimore.

Bernard, J., 1990. Technical advances in Biofilm reactors. Proc.Int. conf. Nice, France. *Wat. Sci. Tech.* **22** : 494.

Bishop.D.F., Heidman, J.A. and Stamberg, J.B.,1976. Single -stage nitrification-denitrification.*J.W.P.C.F.* **48** : 520-532.

Bock, E., 1976. Growth of *Nitrobacter* in the presence of organic matter II chemoorganotrophic growth of *Nitrobacter agilis. Archives of Microbiology,* **108** : 305-312.

Bock, E, Duvel, D & Peters, K.R.,1974.Charakteri Sierung eines Phagenahnlichen Partikels auszellen von Nitrobacter I. Wirts- Partikelbezie, **97** : 115-127.

Boller, M and Gujer, W., 1986. Nitrification in Tertiary Trickling Filters followed by Deep-Bed filtration.*Wat. Res.* **20** (11) : 1363 - 1373.

Boller, M. Gujer, W and Tschui, M., 1994. Parameters affecting nitrifying biofilms. *Wat. Sci. Tech.* **29** (11) : 1 - 11.

Boon, B and Laudelout, H., 1962. Kinetics of Nitrite oxidation by *Nitrobacter winogradskyi. Biochem. J.* **85** : 440.

Bower. C.E. and J.P. Bidwell., 1978. Ionizatian of ammonia in seawater: effects of temperature, pH and salinity. *J. Fish. Res. Board* Can. **35** : 1012 1016.

Bower, C.E. and Turner, D.T., 1983. Nitrification in closed sea water culture systems: effects of nutrient deprivation . *Aquaculture.***34** : 85-92.

Brown and Caldwell/Dewante and Stowell., 1972. Feasibility study for the North-east Central Sewerage Service Area. Prepared for County of Sacramento, Department of Public works.

Burrows., R.E., 1964. Effects of accumulated excretary products on hatchery reared salmonids. *Res. Rpt.* 66, U.S. Fish and Wild life Service, Washington : 12.

Buswell, A.M. Shiota, T., Lawrence. N and Meter, I.V. 1954. Laboratory studies on the kinetics of the growth of *Nitrosomonas* with relation to the nitrification phase of the BOD test. *Appl. Microbiol.* **2** : 21-25.

California Department of water Resources, Nutrients from Tile Drainage systems. EPA., 1971. Report No. 13030/71-3,

Campbell,A.E., 1966. Hellebust, J.A. and Watson, S.W., 1966. Reductive Pentose Phosphate cycle in *Nitrosocystics oceanus, J Bacteriol.* **91** : 1178-1185.

Capdeville, B and Nguyen, K.M., 1990. Kinetics and modelling of aerobic and anaerobic film growth. *Wat. Sci. Tech* : 149-170.

*Chibata, I, Tosa, T and Sato, T., 1974. Japanese patent , **74** : 189.

*Clark, C and Schmidt, E.L., 1966. *J. Bacteriol.* **91** : 367.

Collins, M.T. Gratzek, J.B Dawe, D.L. and Nemetz T.G., 1975. Effects of parasiticides on nitrification. *J. Fish Res. Board.* Can. **32** : 203-2037.

De Boer, W., Klein Gunnewick, P.J.A., Veenhuis, M. E. Bock, and Laan Broek, H.J., 1991. Nitrification at low pH by aggregated chemolithotrophic bacteria. *Appl. Environ. Microb,* **57** (12) : 3600-3604.

De Renzo, D J., 1978. Nitrogen control and phosphorus removal in Sewage Treatment. *Pollution Technology Review* No. **44** (Ed. De Renzo, D.J.), Noyes Data Corporation, New Jersey, U.S.A.

Diab, S and Shilo, M., 1988. Effect of adhesion to particles on the survival and activity of *Nitrosomonas* sp. *Arch. Microbiol,* **150** : 387-393.

Diab, S and Shilo, M., 1989. Effects of light on the activity and survival of *Nitrosomonas* sp. and *Nitrobacter* sp. isolated from fish ponds. *Fish breed.* ISR. **21** (4) : 3-8.

Downing, A.L. and Knowles G., 1966. Population dynamics in biological treatment plants. *3rd IAWPRC conference,* Munich (cited in EPA (1975) Process Design Manual for nitrogen control.)

Downing, A.L., Painter, H.A. and Knowles .G., 1964. Nitrification in the activated sludge process. *J.Proc. Inst. Sew Purif*: **130**.

Drozd, J.W., 1976. Energy coupling and respiration in *Nitrosomonas europaea. Archives of Microbiology* **110** : 257-262.

Drozd, J.W., 1980. In: Diversity of bacterial respiratory systems.(C.J. Knowles, Ed.). **2** : 87-111. R.C. Press Boca Raton, Florida.

*D'Souza, S.F. and Kamath, N., 1988. *Appl. Microbiol Biotechnol* : 136.

Duddles, G.A. Richardson, S.E. and Barth, E.F., 1974. Plastic Medium Trickling Filters for Biological Nitrogen Control.*J.W.P.C.F.*, **46** (5) : 937-946.

Duddles, G.A. and Richardson, S.E., 1973. Application of plastic media Trickling Filters for Biological Nitrification. Report prepared for the Environmental Protection Agency, EPA-R:2 - 73-199.

Eckenfelder, W.W., 1967. A design procedure for biological oxidation of N - formyl hydroxy amino acetic acid by *Penicillium. Mycologia.***55** : 211.

Engel, M.S. and Alexander M., 1958. Growth and autotrophic metabolism of *Nitrosomonas europaea, J. Bact.* **76** : 217.

Environment Protection Agency (Brochure), 1975. Process Design manual for Nitrogen control. Office of Technology Transfer, Washington, D.C.

Fdz. Polanco, F., Mendez, E. and Villaverde, S., 1995. Study of nitrifying biofilms in submerged biofilms by experimental design methods. *Wat. Sci.. Tech.* **32** (8) : 227-233.

Fliermans, C.B., Bohlool, B.B. and Schmidt, E.L., 1974. Autecological study of the chemoautotroph *Nitrobacter* by immunofluorescene. *Applied Microbiology,* **27** : 124-129.

Flierman, C.B. and Schmidt, E.L., 1975. Autoradiography and immunofluorescence combined for autecological study of single cell activity with *Nitrobacter* as a model system. *Appl. Microbiol,* **3** : 676.

Focht, D.D. and Chang, A.C.,1975. Nitrification and Denitrification process related to waste water treatment. *Advances in Applied Microbiology*, **19** : 153-86.

Focht, D.D. and Verstraete W., 1977. Biochemical ecology of nitrification and denitrification. *Advances* in *Microbial Ecology*,**1** : 135-214.

Funk, H.B. and Krulwich, T.A. ,1964. Preparation of clear silica gels that can be streaked .*J. Bacteriol*,,**80**:1200-1201.

Gasser, J.A., Chen, C.L. and Miele, R.P., 1974. Fixed film nitrification of secondary effluent presented at the EED-ASCE speciality conference, Penn. State University.

Genung, R.K. Pitt, W.W. Jr., Davis, G.M. and Koon, J.H. , 1980. Energy conservation and scale-up studies for a wastewater treatment system based on a fixed film anaerobic reactor. *Biotechnol. Bioeng. Symp.* **10** : 295-316.

Gommers, P.J.F., 1987. Microbial oxidation of sulphide and acetate in a denitrifying fluidized bed reactor. Ph.D Thesis, Delft University of Technology, The Netherlands.

Gould, G. W. and Lees, H., 1960. The isolation and culture of the nitrifying organisms: Part I, *Nitrobacter. Can. J. Microbiol.* **6** : 299.

Gujer, W & Jenkins, D., 1974. The contact stabilization process : Oxygen and Nitrogen mass Balances. University of California, Sanitary Engineering Research Lab., SERL Report 74-2,

Gunderson, K., 1955. Effects of B-vitamins and amino acids on nitrification *Physiol.plant.* **8** : 136-141.

Gundersen, K.A.F., Carlucci A.F. and Bostrum, F., 1966. Growth of some chemoautotrophic bacteria at different oxygen tensions. *Experimentia.* **22** : 229-230.

Hall, I.R. 1974. Some studies on the nitrification in the activated sludge process at pollution control, **73** : 538-547.

Hanaki, K.,Wantawin, C. and Ohgaki, S., 1990. Effects of the activity of heterotrophs on nitrification in a suspended growth reactor. *Wat. Res.* **24** (3) : 289-296.

Harms, H., Koops, H.P. and Wehrmann, H.,1976. An ammonia oxidizing bacterium *Nitrosovibrio tenuis nov. gen. nov. sp., Archives of Microbiology* **108** : 105-111.

Harremoes, P., 1978. Biofilm Kinetics. *Wat. Poll. Micro*, **2** : 71-109.

*Hattori, T and Furusaka., 1960. *J. Biochem,* **48** : 831-837.

*Hattori, T., and Furusaka, C., 1961. *J. Biochem,* **50** : 312-315.

Haug R. T and Mc Carty, P.L., 1971. Technical report No. 149, water quality office, EPA.

Haug, R.T. and Mc Carty, P.L.,1972. Nitrification with the submerged Fitter *J.W.P.C.F,.* **44** : 2086.

*Helder, W and, Devries, R.T.P., 1983. *Netherlands Journal of Sea Research.* **17** : 1.

Heijen J.J., 1984. Biological industrial waste water treatment minimising biomass production and maximizing biomass concentration. Ph.D. thesis. Delft University of Technology, The Netherlands.

Hirayama, K., 1974. Water control by filtration in close culture systems. *Aquaculture,* **4** : 369-385.

Hoehn R.C. and Ray, A.D.,1973. Effects of thickness on bacterial film. *J.W.P.C.F.* **45** : 2302-2320.

*Hoffman, T and Lees, H., 1952. *Biochem J.,* **52** : 140.

Hoffman, T. and Lees, H., 1953. The biochemistry of the nitrifying organisms. The respiration and intermediary metabolism of *Nitrosomonas. Biochem. J.*,**52** : 140.

Hooper, A.B, Erickson, R.H. and Terry, K.R., 1972. Electron transport system of *Nitrosomonas* : Isolation of a membrane envelope fraction. *J. Bact.* **110** : 430-438.

Hooper A, B and Terry, K.R., 1974. Photoinactivation of ammonia oxidation in *Nitrosomonas.J. Bacteriol.*,**119** : 899-906.

Jenkins, S.H; Keicht, D.G., Snaddon,X.W.M. and James, K., 1976. Large scale experiments on the treatment of sewage at Minworth works from 1961 to 1966. *J. Proc. Inst. Sewage Purif.*,**66** : 334-361.

Johnson, P.W. and Seiburth, J. Mc M., 1976. *In situ* morphology of nitrifying - like bacteria in Aquaculture Systems. *Applied and Environmental Microbiology,* **31** (3) : 423-432.

*Jones R.D. and Hood M.A. 1980. *Microbial Ecol.* **6** :.271.

Jones, G.L. and Paskins, A.R., 1982. Influence of high partial pressure of carbon dioxide and or oxygen on nitrification. *J. Chem.Technol. Biotechnol.* **32** : 213-223.

Jorgeson, S.K., Jensen, H.B. and Sorenson, J., 1984. Nitrous oxide production from nitrification and denitrification in marine sediments at low oxygen concentration. *Can. J. Microbiol.* **30** : 1073-1078.

*Kamath, N., Melo, J.S. and D'Souza, S.F., 1988. *Appl. Biochem Biotechnol.*

Kavitha Ramachandran and Bright Singh I.S. 1996. Filterant grains of a biofilter as source of nitrifying bacteria for developing bioreactors. *Proc. Eighth. Kerala Science Congress :* 544-546.

Kawai A., Yoshida, Y and Kimata, M., 1964. Biochemical studies on the bacteria in aquarium with circulating system - I. Changes of the qualities of breeding water and bacterial population of the aquarium during fish cultivation. *Bull. Jap. Soc. Sci. Fish.* **30** : 55-62.

*Keen, G.A. and Prosser, J.I.,1987a. *Archives of Microbiology,* **147** : 73.

*Kierstan, M. and Buck, C., 1977. *Biotechnol. Bioeng.* 29 : 387-397.

Klein, J. and Wagner, F., 1978. *Proceedings of the First European Congress of Biotechnology* : 142-164.

Knowles, G., Downing A.L. and Barrett, M.J., 1965. Determination of kinetic const a nts for nitrifying bacteria in mixed culture with the aid of an electronic computer. *J. Gen. Microbiol.,* **38** : 263.

Kokufuta, E., Matsumoto, W. and Nakamura, I., 1982. Immobilization of *Nitrosomonas europaea* cells with polyelectrolyte complex. *Biotech. Bioengg.* **24** : 1591-1603.

*Koops, H.P., 1969. Archiv fur *Microbiologie* 65 : 15.

Laanbroek, H.J. and Woldendorp, J.W., 1995. Activity of chemolithotrophic nitrifying bacteria under stress in natural soils. *Adv. Microb. Ecol.* (J.I. Prosser ed) **14**.

La Motta, E.J.,1976. Kinetics of growth and substrate uptake in a bacterial film system. *Applied. Environ. Microbiol.*,**31** : 286-293.

Laio P.B. and Mayo R.D., 1972. Salmonid hatchery water reuse systems. *Aquaculture*7: 317-336.

Larmoyeux, J.D. and Piper, R.G., 1973. Effects of water reuse on rainbow trout in hatcheries. *Prog. Fish-Cult.* **35** : 2-8.

Lartigue, D.J. and Weetall, H.,1976. US patent, **3** : 939, 041.

Laudelot, H. and Van Tichelen, L., 1960. Kinetics of nitrite oxidation by *Nitrobacter winograsdskyi. J. Bact.* **79** : 39-42.

*Lee, G.K. and Long M.E., 1974. U.S. patent, **3** : 821, 97086

Lees, H., and Simpson, J.R., 1957. The biochemistry of the nitrifying organisms. 5.Nitrate oxidation by *Nitrobacter. Biochem. J.,* **65** : 297-305.

Lewis, R.F. and Pramer D., 1958. In : methods for studying the ecology of soil microorganisms.D. Parkinson, T.R.G. Gray, Williams, S.T. (eds), Blackwell Scientific Publication. 109.

Lewis,R.F. and Pramer, D.,1958. Isolation of *Nitrosomonas* in pure culture. *Journal of Bacteriol.,* **76** : 524-528.

Lin, C.C. and Casida Jr. L.E.,1984 . GELRITE as a gelling agent in media for the growth of thermophilic microorganisms. *AEM.* **47** (2):427-429.

Liu, Y.,1993. Etude integrate sur la cinetique de croissance des films biologiques nitrifiants. Ph.D thesis. INSA Toulouse, France.

Loveless, J.E.and Painter, H.A.,1968. The influence of metal ion concentration and pH on the growth of a *Nitrosomonas* strain isolated from activated sludge. *J. General Microbiology.* **52** : 1-14.

Matulewich, V.A., Strom, P.F. and Finstein, M.S.,1975. Length of incubation for enumerating nitrifying bacteria present in various environments. *Applied Microbiology.* **29** : 265-268.

Matsumura, M., Yamamota, T., Wang, P., Shinabe, K. and Yasuda, K., 1997. Rapid nitrification with immobilized cells using macroporous cellulose carrier. *Wat. Res.* **31** (5) : 1027 - 1034.

Mc Carty, P.L. and Haug, R.T.,1971. Nitrogen removal from wastewater by biological nitrification and denitrification. In. *Microbial aspects of pollution* (G. Sykes and F.A. Skinner eds.) 215-232.

*Mcginis, R. 1975. Sugar, J. **38** : 8 (1985)

*Mc Graw, S. and Knowles, R., 1987. Biology and fertility of soils. 4 : 205.

Mc Harness D.D., Haug, R.T. and Mc Carty P.L., 1975. Field studies of nitrification with submerged filters. J.W.P.C.F. **47** (2):291-309.

Mechalas, B.J., Allen, P.M., Matyskiela, W.W., 1970. A study of nitrification and Denitrification. A report prepared for the Federal water quality Administration. WPCRS 17010 DRD 07/07.

Metcalf and Eddy Inc., 1973. Nitrification and Denitrification facilities. Wastewater treatment : 33. Technol. transfer seminar publ. USEPA Washington DC.

Meyerhof, O., 1917. Untersuchungen uber den Atmungsvorgang nitrifizierender Bakterien. IV. Die Atmung des Nitritbildners and ihre Beeinflussung durch chemische substanzen. Pflug Arch. ges. Physiol. **166** : 240.

Mohan, R.R. and Li, N.N., 1975. Nitrate and nitrite reduction by liquid membrane - encapsulated whole cells. *Biotech. Bioengg.* **17** : 1137-56.

Monod, J., 1949. The growth of bacterial cultures. *Ann. Rev. Microbiol.* **52** : 1-14.

Moreau, M, Lui, Y., Capdeville, B., Audie, J.M. and Calvez, L., 1994. Kinetic behaviour of heterotrophic and autotrophic biofilms in waste water treatment processes. *Wat. Sci. Tech.* **29** (10) : 385-391.

NATO ASI Series, 1992. Biofilms - Science and Technology. *Appl. Sci.* **223**.

Nguyen, K.M., 1989. Description et modilisation des films biologiques aerobies. PhD thesis n°96 INSA, Toulouse, France.

O'Kelley, J.C., Becker, G.E. and Nason, A., 1970. Characterization of the particulate in nitrite oxidase and its component activities from the chemoautotroph *Nitrobacter agilis*. *Biochem. Bio. phys. Acta.* **205** : 409-425.

Oliver J.D., 1982. Taxonomic scheme for the identification of marine bacteria. *Deep Sea Res.* 795-789.

Otah, J., Sharangi, N., and Datta N.C., 1986. City sewage ponds in Hungary and India. *Aquaculture,* **54** : 129-134.

Pan, P.C.,1971a. Basis of obligate autotrophy. PhD. thesis, Rutgers University, New Brunswick, New Jersey.

Painter, H.A., 1970. A review of literature on inorganic nitrogen metabolism in microrganisms.*Wat. Res.* **4** : 393.

Painter, H.P., 1986. In : Nitrification (J.I. Prosser eds.). 185 - 211, IRC Pres, Oxford.

Painter, H.A. and Loveless, J.E., 1983. Effect of temperature and pH value on the growth rate constants of nitrifying bacteria in the activated sludge process. Wat. Res. **17** : 237-248.

Parker, D., 1975. Process design manual for nitrogen control. USEPA. Technology Transfer.

Parkin, G.F. and P.L. McCarty, 1973. The nature ecological significance and removal of soluble organic nitrogen in treated Agricultural waste waters. Stanford University, prepared for the Bureau of Reclamation, Contract USDI 14-06-200-6090 A.

Peters, K.R., 1974.Charakterisierung eines phage nahnlichen Partikels aus Zellen von. *Nitrobacter* II. *Struktur und Grosse Archiv fur Microbiologie.* **97** : 129 - 140.

Prakasam, T.B.S. and Loehr, R. L.,1972. Microbial nitrification and denitrification in concentrated wastes. *Wat. Res.* **6** : 859.

Prosser, J.I. and Cox, D.J., 1982. Nitrification in Experimental Microbial Ecology (Eds., Richard G. Burns and J. Howard Slater) Blackwell. Scientific publication.

Prosser, J.I., 1989. Autotrophic nitrification in bacteria. *Adv. Microb. Physiol.* **3** : 125 - 182. (Eds. Rose, A.W. and Wilkinson, J.F.)

Ramakrishna S.V. & Jamuna, R., 1990. National symposium cum workshop on immobilized cells and enzymes, Regional Research Laboratory, (CSIR) Trivandrum, India.

Rimer, A.E. and Woodward R.L., 1972. Two stage activated sludge pilot plant operations at Fitchberg. J.W.P.C.F.. **44** : 101.

Robertson, L.A. and Kuenen, J.G., 1992. Nitrogen removal from water and water. In : Microbial control of pollution. (Eds. Fry J.C. Gadd, G.M., Herbert, R.A., Jones, C.W. and Watson - Craik, I.A.) The Bath press 227 - 267.

Robinette, H.R., 1976. Effect of selected sublethal levels of ammonia on the growth of channel catfish *(Ictalurus punctatus).Prog. Fish-cult.* **38** : 26-29.

Rodina, A.G., 1972. Methods in aquatic microbiology.(Colwell, R.R. and Zambruski, M.S. eds.) Butterworths, London.

*Sankaran, K., Godbole, S.S. and D'Souza, S.F., 1989. Enzyme. Microb. Technol. **11** : 617.

Sato, C., Schooner, J.L., Mc., Donald, D.B. and Huey, J., 1985. Test medium for the growth of *Nitrosomonas europeae.Applied Environmental Microbiology* . **49** : 1101 - 1107.

Sawyer, C.N., 1940. Activated sludge oxidation, V. The influence of nutrition in determining activated sludge characteristics. *Sewage Works Journal.* **12** (1) : 3-17.

Scholesing, T, and Muntz A.,1877. Sur la nitrification par les ferments organises. *Comptes Rendues de l' Acadamie des Sciences,* Paris. **84** : 301-303.

Schmidt, E.L., 1974. Quantitative autecological study of microorganisms in soil by immunofluorescene. *Soil Sci.* **118** : 141.

Schwinn, D.E. and Dickson, B.H.Jr., 1972. Nitrogen and Phosphorus variation in domestic waste water. J.W.P.C.F. **44** : 2059-2065.

Seeler, G.and Engel, J., 1959. Die Inaktivierung des oxydations vermaogens von *Nitrobacter winogradsky. Buch Arch. Microbiol.* **33** : 387.

Sharma,A.and Ahlert, C., 1997. Nitrification and nitrogen removal. *Wat. Res.* **11** : 897-925.

*Skinner, F.A. and Walker N.,1961. Archiv fiir Mirkrobilogie. **38** : 339.

Smith, A.T. and Hardy, J.P., 1992. High rate sewage treatment using Biological Aerated Filters : J.I.W.E.M., **6** (2) : 179-183.

Smith A.J. and Hoare, D.S., 1968. Acelate asisimilation by *Nitrobacter agilis* in relation to its obligate autotrophy. *J. Bact.* **95** : 844-855.

Smith, A.J. and Hoare, D.S.,1977. Specialist phototrophs,lithotrophs and methylotrophs : A unity among diversity of procaryotes ? *Bacteriological Reviews.* **41** : 419-448.

Soriano, S. and Walker, N., 1968. Isolation of ammonia oxidizing autotrophic bacteria. *J. Appl. Bacteriol.* **31** : 493-497.

Sorrels, J.H. and P.J.A. Zeller.,1956. Two-stage Trickling Filter Performance, *Sewage and Industrial wastes.***18** (8) : 943-954.

*Steimuller, W. and Bock E.,1976. *Archives of Microbiol.* **108** : 299.

Steinmuller W. and Bock E.1977. *Archives of Microbiol.* **115** : 51.

Stratton, F.E. and Mc Carty, P.L.,1967. Prediction of nitrification effects on the dissolved oxygen balance of streams. *Environmental science and Technology.* **1** (5) : 405-410.

Strickland, J.D.H. and T.R. Parsons, 1968. A practical hand book of sea water analysis. 2nd ed. Fish. Res. Board, Canada.

Strickland, J.D.H. and Parsons, T.R., 1972. Determination of reactive nitrate p. 71-76. In : A practical hand book of sea water analysis Ottawa : Fisheries Research Board of Canada.

Souza, M., Azeredo J., Fiejo, J and Oliveria.R., 1997. Polymeric supports for the adhesion of a consortium of autotrophic nitrifying bacteria. *Biotech. Tech.* : **10** : 751-754.

Spotte, S., 1979. Fish and invertebrate culture : water management in closed systems. 2nd ed. Wiley - New York, 179.

Spotte, S., 1992. Captive seawater fishes : Science and Technology, John Wiley and Sons, Inc., USA.

Srinath, E.G., Loehr, R.C., and Prakasam, T.B.S., 1976. Nitrifying organism concentration and activity. *J. Environ. Eng. Div.* **102** : 449-463.

Srna, R.F., and Baggaley, A., 1975. Kinetic response of perturbed marine nitrification systems. *J.W.P.C.F.* **47** : 472-486.

Sugahara, I., Sugiyaman, A., and Kawai, A., 1974. Distribution and activity of nitrogen cycle bacteria in water sediment systems with different concentrations of oxygen. In : Effects of Ocean Environment on microbial activities. Eds. Colwell, R.R. and Morita, R.Y. : 327-340.

Suzuki, I., Dular, U. and Kwok S.C.,1974. Ammonia or ammonium as substrate for oxidation by *Nitrosomonas europaea* cells and extracts. *J. Bacteriol.* 102 : 556-558.

Tanaka, K., Nakao, M., Mori, N., Emori, H., Sumino, T. and Nakamura, Y., 1994. Application of Immobilized nitrifiers gel to removal of high ammonium nitrogen. *Wat. Sci. Tech.* **29** : 241-250.

Toetrup, H., Rogalla, F., Vidal, A. and Harremoes, P., 1994. The treatment trilogy of floating filters from pilot to prototype to plant. *Wat. Sci. Tech.* **29** (10-11) : 23-32.

Tramper, J. 1989. Conversion by immobilized cells versus traditional fermentation. Proc. International Symposium. Physiology of Immobilized cells, Wagemingen. The Netherlands. Elsevier Sciences Publishers.

Tramper, J. and Grootjen, D.R., 1986. Operating performance of *Nitrobacter agilis* immobilized in carrageenan.*Enzyme. Microb. Technol.* **18** : 447-480.

Tomlinson, T.G., Boon, A.G. and Trotman, C.N.A., 1966. Inhibition of nitrification in the activated sludge process of sewage disposal. *J. appl. Bacteriol.* **29** : 226-291.

Trulear, M.G. and Characklis, W.G., 1982. Dynamics of biofilm processes. *J.W.P.C.F.*, **54** : 1288-1301.

Tschui, M., Boller, M., Gujer, W., Eugster, J., Mader, C. and Stengel, C., 1994. Tertiary nitrification in aerated pilot biofilters. *Wat. Sci. Tech.* **29** (10-11) : 53 - 60.

Ulken, A., 1963. Die Herkunft die Nitrites in der Elbe Arch. *Hydiobiol.* **59** : 486.

Unni, P.N., 1995. Biotreatment Techniques, Lecture delivered for the training course on Analytical Techniques and Instrumentation in Water Quality Management. CWRDM/IMTC.

Van Ginkel, C.G. Tramper, J., Luyben, K.C.A.M. and Klapwij, K.A., 1983. Characterization of *N. europaea* immobilized in calcium alginate. *Enzyme* and *Microbial Technology.* **5** : 297-303.

Vedry, B., Paffoni, C., Gousailles, M and Bernard, C., 1994. First months operation of two biofilter prototypes in the waste water plant of Acheres. *Wat. Sci. Tech.* **10-11** : 39-46.

Volz. M.G., Belser, L.W., Arkakani, M.S. and Mc. Laren, A.D., 1975a. Nitrate reduction and associated microbial population in ponded Handford sandy loam. *J. Environ. Qual.* **4** : 99.

Volz, M.G., Belser, L.W., Ardakani, M.S. and Mc Laren, A.D., 1975b. Nitrate reduction and associated microbial population in an unsaturated Handford sandy loam. *J. Environ. Qual.* **4** : 179.

Vrba, J., 1990. Effect of periodic illumination on nitrification in the continuous cultivation. In : Measurement of microbial activity in the carbon cycle in aquatic ecosystem. Proc. 4th Inter. Workshop. 145-150.

Walker, N., 1975. Nitrification and nitrifying bacteria. In : Soil Microbiology (Ed. N. Walker) 133-146. Butterworths, London.

Watson, S.W., 1965. Characteristics of a marine nitrifying bacterium, *Nitrosocystis oceanus* sp. nov. *Limnol. Oceanogr.* **10** : R 274-R-289.

Watson, S.W., 1971. Reisolation of *Nitrosospiria briensis. Arch. Mikrobiol.*75 : 179.

Watson, S.W., 1971a. Taxonomic consideration of the family *Nitrobacteriaceae Buchanan. International Journal of Systematic Bacteriology.* **21** : 254-270.

Watson, S.W., 1971b. Reisolation of *Nitrosospira briensis.* S. Winogradsky and H. Winogradsky 1933. *Archive fur Mikrobiologie.* **75** : 179-188.

Watson, S.W., 1974. Gram negative chemolithotrophic bacteria. In : Bergey's Manual of Determinative Bacteriology (Eds. R.E. Buchanan and N.E. Gibbons) 8th Edn. p. 450-456. Williams and Wilkins, Baltimore.

Watson, S.W., Bock, F., Harms, H. Koops, H.P. and Hooper A.B., 1989. Nitrifying bacteria p. 1808-1834. In. J. T. Staley M.T. Bryant, N. Pienning and J.G. Holt (ed.). Bergeys Manual of Systematic Bacteriology. Vol. 3. Williams and Wilkins Co. Baltimore.

Watson, S.W., and Mandel, M., 1971. Comparison of the morphology and deoxyribonucleic acid composition of 27 strains of nitrifying bacteria. *J. Bacteriol.* **107** : 563-569.

Watson, S.W., Valois, F.W. and Waterbury, J.B. 1981. The family *Nitrobacteriaceae.* In : The Prokaryotes Vol. I (Eds. Starr M.P., Stolp., H., Truper, H.G., Balows, A. and Schlegel, H.G.) Springer - Verlag. Vol. 1. p. 1006-1021.

Williamson, K.L. and Mc Carty, P.L.1973. A model of substrate utilization by Bacterial films. Presented at the 46th ann. Conf. of WPCF, Cinnannati, Ohio.

*Williamson, K. and Mc Carty, P.L.,1976. *J.W.P.C.F.*, **48** : 281.

Winkler, M., 1981. Biological treatment of waste water, Chichester. Ellis Horwood.

Winogradsky, S.,1890. Recherches sur les organismes de la nitrification. *Annales de l' Institute Pasteur, Paris.* **4** : 213 - 31.

Wong-Chong, G.M. and Loehr R.C., 1975. Kinetics of microbial nitrification as applied in the treatment of animal waste. Ammonium nitrogen oxidation. *Ai. Ch. E Symp. Ser.* **71** : 70-79.

Woodward, J., 1988. Methods of immobilization of microbial cells. *J. Microbiol. Methods.* **8** : 91-102.

Wuhrmann, K., 1964. Microbial aspects of water pollution control. In : *Advances in Applied Microbiology.* **6**. Ed. Wayne Wt Curb. 119-150.

Young J.C., Baumann, E.R and Wall, D.J., 1975. Packed - Bed Reactors for secondary effluent BOD and ammonia removal. *J.W.P.C.F.* **47** (1) : 46-56.

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