

**Ecology of fungi in the denitrification
zones of the Arabian Sea**

Thesis

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Goa University

For the award of
Doctor of Philosophy
in
Marine Sciences

By
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March 2010

STATEMENT

As per requirement, under the University Ordinance 0.19.8 (vi), I state that the present thesis titled “**Ecology of fungi in the denitrification zones of the Arabian Sea**” is my original contribution and the same has not been submitted on any previous occasion. To the best of my knowledge, the present study is the first comprehensive work of its kind from the area mentioned.

The literature related to the problem investigated has been cited. Due acknowledgements have been made whenever facilities or suggestions have been availed of.

Cathrine Sumathi Jebaraj

March 2010

CERTIFICATE

This is to certify that the thesis titled “**Ecology of fungi in the denitrification zones of the Arabian Sea**” submitted for the award of the degree of Doctor of Philosophy in the Department of Marine Sciences, Goa University, is the bona fide work of Ms Cathrine Sumathi J. The work has been carried out under my supervision and the thesis or any part thereof has not been previously submitted for any degree or diploma in any university or institution.

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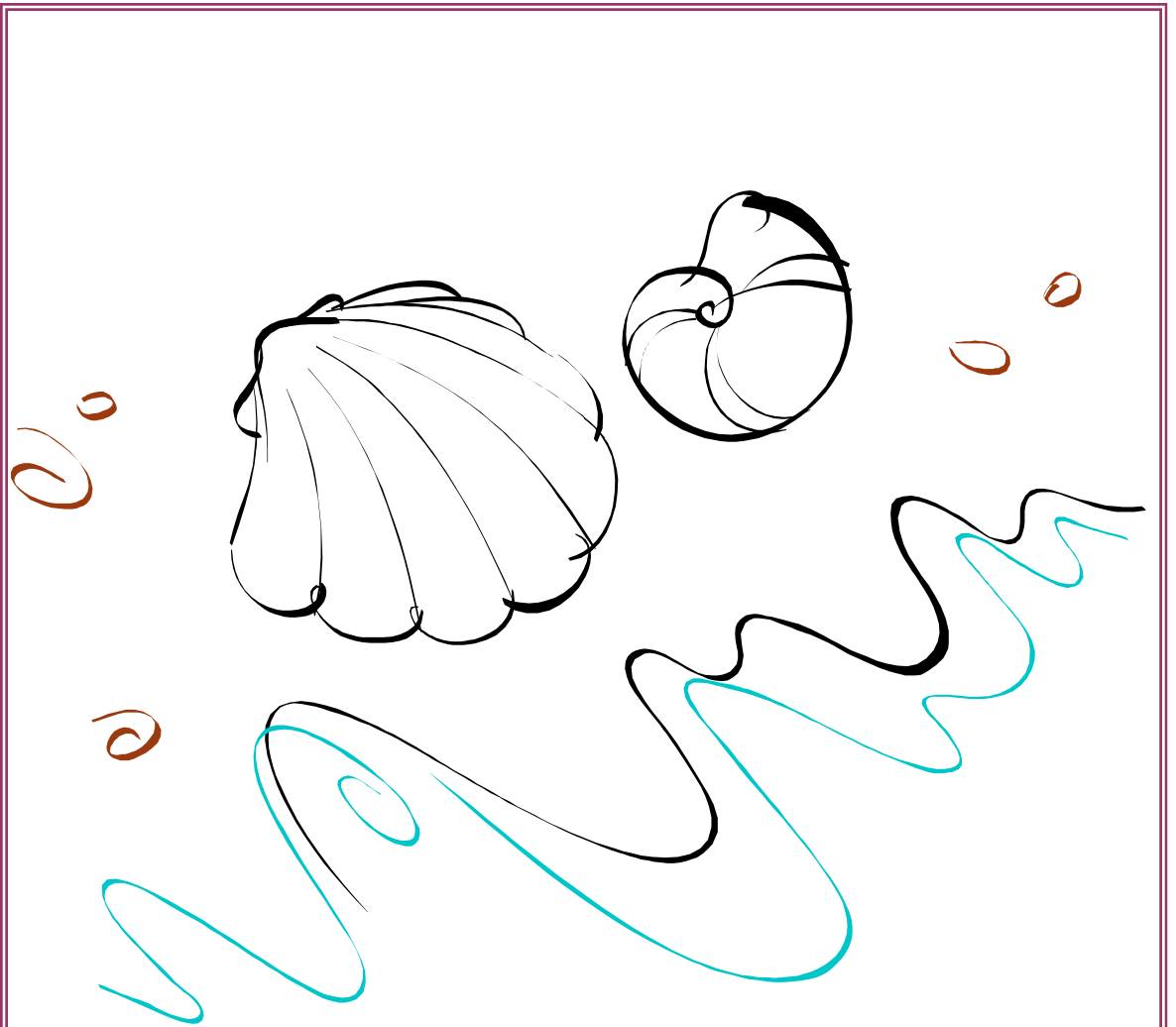
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*Dedicated to all the marvelous creations
and its magnificent Creator!!*

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PREFACE

The microbial process, denitrification was considered to be an inherent character of a few specialized groups of bacteria. These bacteria thriving in the denitrification zones contribute to the production of nitrous oxide, a major green house gas. The pathway for this process has been studied extensively especially in the marine ecosystem, as this process leads to the loss of the fixed nitrogen and causes an imbalance in the global nitrogen budget.

Molecular ecological studies from these regions have brought to light interesting and novel microbial diversity. Environmental sequences retrieved from these regions show that fungi are one of the major components in this ecosystem. Molecular enzymological studies of a few terrestrial fungi have shown that, fungi also have the physiological adaptations to denitrify.

In this study I have attempted to estimate the abundance, diversity and activity of fungi from the denitrification zones of the Arabian Sea, that occupies more than 50% of the world's oxygen depleted marine environment.

Cathrine Sumathi Jebaraj

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Chapter 1

*Fungal mediated denitrification process:
an introduction.*

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1.1 Fungal mediated denitrification process: an introduction.

The English word "fungus" is adopted from the Latin *fungus*, meaning "mushroom", which in turn has its origin from the Greek word *sphongos* "sponge", referring to the fruiting bodies produced by mushrooms and molds. Accordingly fungi are widely popular among the common man as mushrooms, rusts, puffballs, smuts, bread moulds etc. because of the macroscopic structures they produce. Only a small fraction of fungal groups grow these macroscopic structures and are known to occur only in the terrestrial environment. The majority of the fungal forms grow embedded in the detritus' and beneath the sediment layer. Fungi along with bacteria are the major heterotrophs that play a key role as decomposers and recyclers in a large number of ecosystems such as the tropical forests (Isaac *et al.*, 1993; Hyde, 1997), marine ecosystem (Hyde *et al.*, 1998) and mangrove regions (Cheng *et al.*, 2009). They are ubiquitous and almost occupy every niche.

The heterotrophic microorganisms lack the ability to fix atmospheric carbon dioxide and use them as their carbon source and are dependent on the phototrophs *en masse* for their survival. Because of their inability to synthesize their own organic compounds, they have acquired mechanisms to degrade complex organic substances for their growth and activity. The three basic metabolic strategies they have adapted are i) aerobic glycolysis, ii) anaerobic fermentation and iii) dissimilatory anaerobic respiration / reduction.

Glycolysis is the metabolic pathway that converts glucose, into pyruvate. The free energy released in this process is used to form the high energy compounds, adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH). Glycolysis is a sequence of ten reactions involving ten intermediate compounds and in aerobic

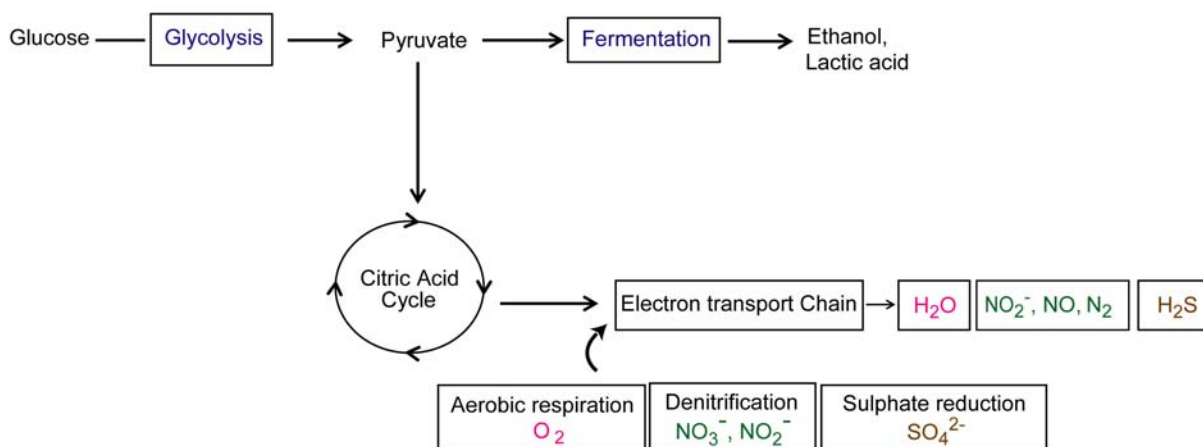


Fig.1.1 Schematic representation of the major metabolic pathways

organisms the pyruvate produced is converted to acetyl-CoA and Carbon-dioxide (CO₂) within the mitochondria in a process called pyruvate decarboxylation. The acetyl-CoA enters the citric acid cycle, where it is fully oxidized to carbon dioxide and water, producing yet more NADH. The NADH is oxidized to NAD⁺ by the electron transport chain, using oxygen as the final electron acceptor. This process creates a "hydrogen ion gradient" across the inner membrane of the mitochondria and the proton gradient generated is used to produce a large amount of ATP in a process called oxidative phosphorylation (Fig. 1.1). In situations when there is lack of oxygen, pyruvate is converted to a variety of other compounds through the fermentation process. For example pyruvate is converted to lactate in a process called lactic acid fermentation which causes the milk to curdle and yeasts convert pyruvate to acetaldehyde and carbon dioxide through ethanol fermentation. Some microbes can use a wide variety of compounds as the terminal electron acceptors in cellular respiration in the absence of oxygen. Specialised group of bacteria can utilize nitrogenous compounds such as nitrates and nitrites, sulfur compounds such as sulfates, sulfites, sulfur dioxide and elemental sulfur, carbon dioxide, iron compounds, manganese compounds, cobalt compounds and uranium compounds (Voet & Voet, 2004; Nelson & Cox, 2005).

1.1.2 Fermentation in fungi

Role of fungi in the fermentative process was known to man since the early days of civilization, the most popularly known example is the involvement of *Saccharomyces cerevisiae* popularly known as baker's yeasts in bread-making, brewing, and wine-making. During fermentation, organic compounds usually sugars such as glucose is oxidized and byproducts such as ethanol, lactic acid and butyric acid are formed. This is a low energy yielding process and is not affected by the oxygen concentration. Fermentation products of yeasts are known for their medicinal properties even from the first century as recorded in the traditional Chinese medicinal books. One of the well-known examples is the "red yeast rice" which is fermentative product of a specific fungus *Monascus purpureus* (Went, 1895). The grain of the red rice has the outer bran which is deep reddish brown color and the rice is known to have a number of medicinal properties which is imparted during the fermentative process (Heber *et al.*, 1999). The fermentative or the secondary metabolites produced by fungi are known to have a wide range of applications as antibiotics, anti-fungal agents and pharmaceutical products (Calvo, 2002; Pointing & Hyde, 2001) and they are also known to be used as dietary supplements in the nutraceutical industry (Agahar-Murugkar & Subbulakshmi, 2005). Though in many instances, the necessity of these metabolites to the organisms producing them are not clearly defined (Challis & Hoopwood, 2003). However the biotechnological industry has harnessed a great deal from these compounds and has found a wider application (Parekh *et al.*, 2000).

Though the ability of fungi as fermenters is well documented from the ancient times, fungi were always characterized as obligate aerobes with a requirement of oxygen for their growth. One of the salient features defining Kingdom Fungi was their requirement of oxygen environment for growth and survival. A major shift to this was brought about when a group of fungi, known as "rumen-fungi" first discovered inhabiting the rumen of cattles (Orpin, 1977) as

obligate anaerobes. They are chytridiomycete fungi found in the gastrointestinal tracts of sheep, cattle and goats, as well as in many other domesticated ruminant and non-ruminant herbivores and a wide variety of wild herbivorous mammals (Trinci *et al.*, 1994). They are principally found associated with the fibrous plant particles of digesta and aid in the fermentative degradation of their cellulose rich diet (Gordon & Philip, 1998).

The fermentative ability of fungi was known from ancient times and it had found a wide variety of applications. However, the role of fungi in oxygen depleted environments was known to be restricted and they were thought to have only a minor role in the ecosystem processes of anoxic environments (Mansfield & Barlocher, 1993; Dighton, 2003). The effects of low-oxygen conditions tested on the viability of wood-decay fungi *Coniophora puteana* and *Antrodia vaillantii* grown on artificial growth medium, showed that the anoxic treatment affected the regeneration of the cultures and this increased steadily with the prolongation of anoxic treatment (Tavzes *et al.*, 2001). Studies on the survival of *Sclerotinia* species in a combination of high temperature, high soil moisture, and reduced oxygen in irrigated fields contributed to the lower survival (Wu *et al.*, 2008). The survival and growth of fungi in anoxic conditions and the groups adapted to this are not yet very clear.

1.1.3 Anaerobic respiration: Denitrification

Anaerobic respiration or the dissimilatory reduction is an alternate respiratory process where the microorganism is capable of utilizing inorganic compounds such as nitrate, nitrite or sulphate as the final electron acceptor in the absence of oxygen. Oxygen is the most preferred electron acceptor which provides the maximum energy during the metabolic process, but in oxygen depleted environments, nitrate or nitrite is the alternate compound which could be utilized by a selected group of microorganisms the nitrate reducers belonging to archaeobacteria, proteobacteria and Gram-positive eubacteria (Knowles, 1982; Braker *et al.*, 2001).

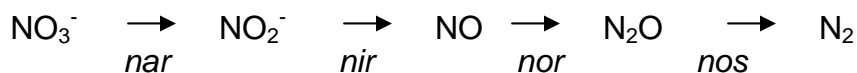


Fig. 1.2 Classical denitrification pathway

Denitrification is a microbial process (Fig.1.2), where nitrate or nitrite is reduced under anaerobic condition to gaseous nitrogen (N_2) or its intermediary products such as nitric oxide (NO) and nitrous oxide (N_2O) (Zumft, 1997). This is catalysed successively with the help of the enzymes dissimilatory nitrate reductase (*nar*), nitrite reductase (*nir*), nitric oxide reductase (*nor*) and nitrous oxide reductase (*nos*). This process is a major sink of the fixed nitrogen in the marine nitrogen cycle (Fig. 2.1). Studies on the denitrification process have evoked the interests of microbiologists and climatologists because of its direct impact on global warming, climate change and are vulnerable to anthropogenic inputs (Conrad, 1996).

Anaerobic ammonium oxidation (ANAMMOX) is another specialized metabolic process that leads to the N_2 gas formation in the anoxic conditions. This is identified to be carried out in a few bacterial groups belonging to Planctomyces (Kuypers *et al.*, 2003). Anammox reaction was first identified from the wastewater bioreactors and subsequently in oxygen depleted marine waters and sediments (Dalsgaard *et al.*, 2003). The anammox reaction is estimated to contribute 30 – 50 % of the N_2 production from the oceans (Devol, 2003). During this process the nitrate is initially reduced by the denitrifiers to nitrite, then the anammox bacteria uses this nitrite and oxidizes ammonia to form N_2 gas (Fig. 1.3). But this process has not yet been reported in any of the fungal isolates.

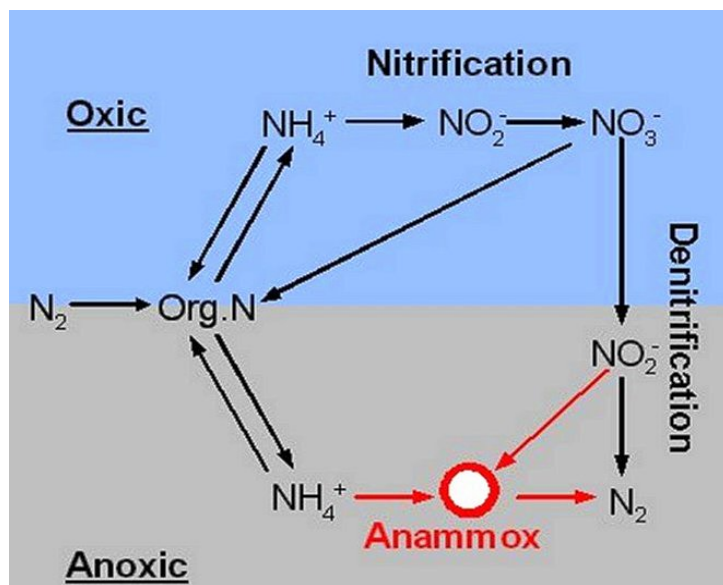


Fig. 1.3 Simplified marine nitrogen cycle including the anammox 'sink' (Kuypers *et al.*, 2003).

1.1.4 Anaerobic respiration in eukaryotes

Anaerobic or alternate respiratory process was thought to be restricted only to the specialized bacterial groups, but involvement of eukaryotes in this pathway was also reported. *Loxodes* sp. belonging to protozoa is known to be capable of living in the waters of anoxic lakes. Their capacity to participate in the denitrification pathway can be attributed to a dissimilatory nitrate reductase located within the inner mitochondrial membrane (Finlay, 2006). Recently, a benthic foraminifer *Globobulimina pseudospinenscens* has been demonstrated to show complete denitrification in marine sediments (Risgaard-Peterson *et al.*, 2006). Increased occurrence of foraminifers *Nonionella cf. stella* and a *Stainforthia* species within the oxygen minimum zone of the continental shelf off Chile and intracellular accumulation of nitrate and evolution of N_2 in these species have also been shown (Ingvarsdén *et al.*, 2006). Studies conducted using cultivation-independent, 18S rDNA-based survey in suboxic waters and

anoxic sediments has also shown large microeukaryotic diversity. The bulk of their sequences represent deep novel branches within green algae, fungi, cercozoa, stramenopiles, alveolates, euglenozoa, unclassified flagellate and a number of novel lineages that have no similarity with any of the known sequences (Stoeck & Epstein, 2003; Stoeck *et al.*, 2003; Stoeck *et al.*, 2006). Fungal sequences have also been retrieved from anaerobic sulfide and sulfur-rich springs of Zodletone Spring, Oklahoma (Luo *et al.*, 2005). Apart from this study Straminipiles (thraustochytrids) have also been reported from anoxic habitats (Raghukumar *et al.*, 2001; Kolodziej & Stoeck, 2007) but no studies have been attempted so far to study the denitrification pathway in the eukaryotic organisms. Though a large number of micro-eukaryotes have been studied from the anoxic habitats, only foraminiferas and fungi have been studied and proven for their denitrification abilities.

1.1.5 Denitrification in fungi

Involvement of fungi in anaerobic dissimilatory process was completely unheard till recent times. Initial studies that reported growth of fungi in anoxic environments suggested that they required supplementation of certain inorganic salts such as nitrate, selenite, or ferric iron in the culture media (Gunner & Alexander, 1964). Bollag & Tung (1972) were the pioneers to show N_2O production by fungi when grown under anaerobic conditions. They also showed growth of fungi under these conditions when supplemented with nitrate or nitrite and concluded that nitrate respiration may be a possibility in fungi.

Isolation, purification and characterization of an intracellular P-450 enzyme from *Fusarium oxysporum* was carried out for the first time in 1983 and was shown to have lipoxygenase activity (Shoun *et al.*, 1983). Subsequent studies on this cytochrome P-450 enzyme showed that they were induced by nitrate/nitrite and not directly related to lipid metabolism. Based on this, P-450 was also called P-450_{dNIR} representing that it was related to dissimilatory nitrite reduction in the fungus *F. oxysporum* (Nakahara *et al.*, 1993). The major

breakthrough came when the cDNA of the P-450_{dNIR} was sequenced by immunoscreening technique. The full length sequence had a very high homology to the P-450 of *Streptomyces* sp. and it came to be the first soluble, microsomal P-450 of eukaryote origin (Kizawa *et al.*, 1991). The novelty of the enzyme was further enhanced by its role in dissimilatory nitrate reduction activity and the gene sequence was called *CYP55*. But these studies were concentrated on a single fungus and the property of the denitrification in other fungal cultures was not known.

Screening of terrestrial fungal cultures for their denitrification activity showed that several fungi were involved in this process (Shoun *et al.*, 1992; Usuda *et al.*, 1995). This study showed that dissimilatory nitrate reduction was seen in fungal cultures such as *Fusarium oxysporum*, *Cylindrocarpon tonkinense* and *Aspergillus oryzae* and were shown to release N₂O under denitrifying conditions (Shoun *et al.*, 1992). Nitrate reductase activity and denitrification process was shown in another *Fusarium oxysporum* (11dn1) culture from Moscow (Kurakov *et al.*, 2000). Screening of cultures obtained from agricultural fields showed that the cultures varied widely in their denitrification ability and N₂O production (Morozkina *et al.*, 2005). Denitrification activity has been shown in fungi belonging to Ascomycota, selected Basidiomycota and Zygomycota (Kurakov *et al.*, 2008). This shows that the fungal denitrification activity is prevalent among all the major groups, but the molecular basis on how the members of Kingdom Fungi acquired the ability is yet to be studied

The fungal denitrification system is localized in the mitochondria and functions for anaerobic respiration, as seen in the denitrification system of bacteria (Kobayashi & Shoun, 1995). Dissimilatory *nar* partially purified from the mitochondrial fraction of the denitrifying fungus *F. oxysporum* was shown to be distinct from assimilatory *nar* (Uchimura *et al.*, 2002). The properties of this fungal *nar* were similar to those of the dissimilatory *nars* of *Escherichia coli* and other denitrifying bacteria. Analyses of mutants defective for dissimilatory *nar* or assimilatory *nar* have shown that *nar* is essential for fungal denitrification. *nir* is one of the key enzymes in the dissimilatory denitrification process. Two

structurally different *nirs* are found among denitrifying bacteria: one contains copper (*Cu-nir*) and is encoded by the *nirK* gene, and the other contains heme c and heme d1 (*cd1-nir*) and is encoded by the *nirS* gene (Zumft, 1997). No functionally significant differences between *Cu-nir* and *cd1-nir* have been reported. Fungal *nir* contains copper and is an ortholog of bacterial *nirK* (Kobayashi & Shoun, 1995). However, fungal *nor* is distinct from bacterial *nor*. Fungal *nor* has been classified in the cytochrome P450 superfamily on the basis of its primary and tertiary structures. All bacterial *nors* reported to date contain cytochromes *bc* in their catalytic activity.

The denitrification activities of bacteria are induced in the presence of either nitrate or nitrite under a limited O_2 supply and are suppressed by excess O_2 , whereas fungal denitrification activity is induced in the presence of nitrate or nitrite and significant amounts of O_2 , but not in excess O_2 (Zhou *et al.*, 2001). Two types of denitrifying fungi have been categorized according to their ability to reduce or in a dissimilatory manner. A few fungi, such as *F. oxysporum* and *Gibberella fujikuroi*, reduce both nitrate and nitrite to N_2O (Shoun & Tanimoto, 1991), whereas most other reported denitrifying fungi reduce only nitrite to N_2O (Shoun *et al.*, 1992). Thus, it appears that the minimal requirements in the fungal denitrification system are *nir* and P450*nor*. The homologies studied in the *nor* gene has shown that there are conserved regions between the bacterial and fungal *nor* gene. This shows that fungi could have acquired the denitrification ability through horizontal gene transfer from bacteria (Daiber *et al.*, 2005).

Many denitrifying fungi produce hybrid dinitrogen (N_2) or N_2O molecules by combining nitrogen atoms from nitrite and other nitrogen compounds (cosubstrates) under denitrifying conditions. This phenomenon was named “codenitrification” to indicate that nitrogen compounds (cosubstrates), such as azide and ammonia, are denitrified by the system induced by nitrite (or nitrate), but are incapable by themselves of inducing the denitrifying system (Shoun *et al.*, 1992; Tanimoto *et al.* 1992).

Ammonia fermentation is another mode of alternate respiration observed in fungi during complete anoxic conditions (Zhou *et al.*, 2002). In ammonia

fermentation, nitrate is reduced to ammonia and ethanol is simultaneously oxidized to acetate to generate ATP. In the reaction, nitrate acts as the terminal electron acceptor for fermentation, but not for anaerobic respiration. Ammonia fermentation is demonstrated to be common in many of the soil fungi. Ammonia fermentation supports fungal growth under conditions that are more anoxic than those of denitrification. This pathway is similar to the dissimilatory nitrate reduction to ammonia (DNRA) pathway known in some bacterial cultures (Tobias *et al.*, 2001). Studies on the denitrifying activities of *F. oxysporum* have shown that it expresses diversified pathways of nitrate metabolism in response to environmental O₂ tension, ammonia fermentation under anoxic conditions, denitrification when hypoxic, and oxygen respiration under aerobic conditions, thus demonstrating that a eukaryote can use a multimodal type of respiratory system to rapidly adapt to changes in the oxygen supply (Daiber *et al.*, 2005; Hayatsu *et al.*, 2008).

1.1.6 Fungal mediated denitrification process

The involvement of fungi as denitrifiers has been evaluated based on the effect of cycloheximide on fungal denitrification activity in woodland and arable soils Castaldi & Smith (1998). Cyclohexamide is widely known anti-fungal agent and the denitrification activity reduced on treatment with this anti-fungal agent. Recent studies from the grassland ecosystem based on substrate-induced respiratory inhibition studies have also shown that they account for nearly 80% of the N₂O production (Laughlin & Stevens, 2002). Contribution of fungi as the dominant gaseous producers has also been shown in semiarid soil (MacLain & Martens, 2006). Screening of fungal isolates has shown that all the major groups of fungi are capable of denitrification process (Shoun *et al.*, 1992; Kurakov *et al.*, 2008). It is interesting to study the ecological role of fungi as they predominantly form only nitrous oxide which directly affects the ecosystem.

As fungi have the ability to perform denitrification and O₂ respiration simultaneously in a range of O₂ stress conditions, the potential exists for fungi to

produce N_2O in a wider range of soil aeration conditions than bacteria. Fungi are widely distributed in soils and water; hence the potential exists for fungi to make a significant contribution to the global N_2O budget.

An understanding of these processes can give us insights into the role of fungi in the biogeochemical cycling of nitrogen and can also be used for biotechnological applications in sewage treatment plants. Till now nitrifying and denitrifying bacteria have been used for biological treatment of sewage wastes. But filamentous fungi which can perform both nitrifying and denitrifying process even under low oxygen conditions is proving to be a better alternative (Guest & Smith, 2002). These interesting studies on the fungal denitrification show that fungi could have an important ecological role in such special niches such as the denitrification zones of the marine environment. For this study I have chosen to study the fungal diversity and contribution in the denitrification zones of the Arabian Sea which occupies more than 60 % of the world denitrification zones. The bio-geochemical significance and the dynamics of the Arabian Sea oxygen depleted zone is explained in the subsequent chapter.

Author