

Chapter 4

*Denitrification ability of fungi isolated from the
marine denitrification zones.*

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4.1 Introduction

Fungi are primarily aerobic heterotrophs that play an essential role as decomposers of organic matter in a variety of environments. In marine ecosystems fungi are the major decomposers of woody and herbaceous substrates and their importance lies in their ability to aggressively degrade lignocellulose (Newell, 1996). Marine fungi also contribute to the degradation of dead animals and animal parts (Kushwaha & Gupta, 2008), and are important pathogens of animals (Lecampion-Alsumard *et al.*, 1995) and plants (Raghukumar, 1986), or partners in mutualistic symbioses (Mishra & Lichtwardt, 2000; Pivkin, 2000). Fungi have been isolated from diverse marine substrata such as marine algae (Raghukumar *et al.*, 1992a), mangrove plants (Kumaresan *et al.*, 2002), calcareous shells of marine organisms (Raghukumar *et al.*, 1992b), corals (Ravindran *et al.*, 2001, Golubic *et al.*, 2005) and deep sea sediments (Damare *et al.*, 2006). There was a strong conviction that all fungi required oxygen for growth and it was considered as one of the major metabolic differences between bacteria and fungi (Foster, 1949). This was disproved by the discovery of obligate anaerobic chytrid fungi from the gut of the herbivores (Orpin, 1977). However the ability of fungi to live in anaerobic conditions was restricted to fermentative rumen-fungi and studies have shown that colonization of fungi on wood in marine environment is slower or almost nil in oxygen minimum zones (Kohlmeyer & Kohlmeyer, 1979). Role of fungi in natural oxygen depleted environments was considered to be insignificant (Mansfield & Barlocher, 1993; Dighton, 2003) and there have been no attempts to study the diversity of fungi from oxygen-depleted marine environments.

Pioneering work on soil fungi capable of denitrification was shown in early 70s (Bollag & Tung, 1972). Denitrifying activity in fungi has been

studied in detail after 1991 when the involvement of cytochrome P450 in the fungal denitrification pathway was shown (Shoun & Tanimoto, 1991). Now several fungi like *Fusarium oxysporum*, *Aspergillus oryzae* and yeasts *Trichosporon cutaneum*, *Cylindrocarpum tonkinense* are shown to be capable of denitrification (Shoun *et al.*, 1992). The denitrification process in *Fusarium oxysporum* has been studied extensively and they have shown that fungi have an incomplete denitrification process. In bacterial denitrification process, nitric oxide (NO) is reduced to nitrous oxide (N₂O) by nitric oxide reductase, which is further reduced to the final product nitrogen gas (N₂). In fungi the denitrification process largely stops with the reduction of NO to N₂O. The enzyme involved in the NO to N₂O reduction is nitric oxide reductase (p450_{nor}), which is specific for fungi (Kobayashi *et al.* 1995, Daiber *et al.*, 2005). The role of fungi in the denitrification process of the grassland ecosystem has shown that the fungal denitrification accounts for nearly 80% of the nitrous oxide production based on substrate induced respiratory inhibition studies (Laughlin & Stevens 2002). Fungi are also known to be involved in co-denitrification and chemo-denitrification to produce N₂ gas (Laughlin & Stevens 2002).

Ammonia fermentation is another mode of alternate respiration observed in fungi during complete anoxic conditions. 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 respiration (or ATP-producing) system to rapidly adapt to changes in the oxygen supply (Zhou *et al.*, 2002).

Ammonia oxidation to N_2 by bacteria, through anammox reaction has been identified in the marine environment, both in the water column and in the sediments. The anammox reaction is estimated to contribute 30-50% of the N_2 production from the oceans (Devol, 2003). But much of the molecular biology, physiology and ecology of anammox are yet to be studied. Though the involvement of fungi in ammonia oxidation is not reported till now, an investigation on their ability to involve in anammox reaction and other alternate respiration modes are worth attempting.

Microeukaryotic diversity studied from the anoxic regions based on culture independent methods have always included a great number of sequences belonging to mycelial fungi and other lower fungal forms such as Stramenopiles (Dawson & Pace, 2002; Stoeck & Epstein 2003; Massana *et al.*, 2004). The thraustochytrid dynamics studied in the water column of the Arabian Sea OMZ shows that the total numbers were as high as that of bacterial numbers in the order of 10^6 and 10^9 respectively (Raghukumar *et al.*, 2001). Thraustochytrids have also been found associated in large numbers in the sediments of the continental shelf region. The preliminary studies in the sediments off Goa also showed large number of thraustochytrids, especially Labyrinthulomycetes during the post monsoon season (Jebaraj & Raghukumar, 2009). Studying fungi in the denitrifying environment can give us insights about their involvement in the biogeochemical cycling of nitrogen. An understanding of this process can be used for biotechnological applications in sewage treatment plants. Till now nitrifying and denitrifying bacteria are used for biological treatment of sewage wastes. But filamentous fungi can perform both nitrifying and denitrifying process even under low oxygen conditions. Hence the use of fungi can be beneficial in many ways (Guest & Smith, 2002).

Microbes involved in the denitrification pathway are studied by their ability to utilize nitrate or nitrite as an alternate electron acceptor in the absence of oxygen by studying the most probable numbers (especially of bacteria) from denitrifying zones (Michotey *et al.*, 2000) and studying the electron transport system activity and the enzymes involved in the denitrifying pathway (Devol *et al.*, 1976; Shailaja *et al.*, 2006). Functional markers coding for the key enzymes such as *nirK*, *nirS*, *norB*, *nos* of the denitrification process are used to study the community structure of the denitrifiers (Scala *et al.*, 1999; Braker *et al.*, 2000). Real time quantitative analysis of the genes involved such as nitrite reductase (*nirK* and *nirS*) provides us with the in-situ analysis to quantify bacterial denitrifiers in the natural ecosystems (Henry *et al.*, 2004). Apart from studying the natural oxygen depleted environments, isolation of cultures from these regions are also carried out and their utilisation of nitrate in the medium under anoxic condition has been used as the common and quick method to test the denitrifiers (Simbert & Kreig, 1981). Though this step is a basic for the denitrification process, identification of other complex microbial process involved in the nitrogen cycle has been brought to light (Zher & Ward, 2002), hence more detailed analysis of the denitrification process is required. Mahne and Tiedje (1995) has suggested that denitrifiers should satisfy two criteria to be called so which includes the production of nitrogen gases principally N_2O and N_2 , coupled with growth yield which can be quantified by the increase in biomass.

A selected set of cultures from our study isolated from the coastal station (CSt-26) during the anoxic and oxic season (Table 4.1) were screened for their ability to grow under anaerobic conditions to reduce nitrate. Measurement of nitrite accumulation, ammonia formation and respiratory electron-transport activity was carried out in these cultures.

Table 4.1 List of cultures used for experimental studies

Isolate	MTCC Acc No.	Identification	Isolated from
# An-2	MTCC 9376	<i>Fusarium</i> sp	CSt-26, Ft-1 (Oct 05)
#11	MTCC 9381	<i>Tritirachium</i> sp	CSt-26, Ft-1 (Oct 05)
# 31	MTCC 9375	<i>Myrothecium verrucaria</i>	CSt-26 FT-3 (Jan 06)
# 31a	MTCC 9388	<i>Penicillium namyslowskii</i>	CSt-26 FT-3 (Jan 06)
# MT-811	---	<i>Fusarium oxysporum</i>	Gift from Dr Shoun, Univ of Tokyo, Japan.

MTCC: Microbial Type Culture Collection, IMTECH, Chandigarh, India.

Activity of fungi and bacteria individually and in combination under *in-situ* condition was studied by setting up various microcosms. The different microcosm set-ups included incubation of fresh sediment collected from the coastal anoxic region, sediment supplemented with nitrate, sediment with antifungal or antibacterial agents and with fungal inoculum. Nitrate and nitrite formation, ETS activity and the Eh were measured from the microcosm after 7 days of incubation.

4.2 Materials and methods

4.2.1 Screening of fungi for their nitrate utilization capacity under aerobic and anaerobic conditions

For screening studies the growth of four different fungi was studied in an inorganic mineral medium (Appendix) supplemented with sodium nitrate. The final concentration of the nitrate supplement in the medium was 10 mM and the fungal cultures were studied for their ability to reduce nitrate by estimating the nitrite formed in the culture medium and their biomass formed. These were # An-2 (*Fusarium* sp.) isolated after anaerobic incubation of the sediment, # 11 (*Tritirachium* sp.) which was isolated from the sediment during anoxic condition and # 31 (*Byssoschlamys* sp.) and #

31a (*Paecilomyces* sp.) isolated from the sediments when the conditions were oxic (Table 4.1). These cultures were compared with a well studied denitrifier of terrestrial origin, *Fusarium oxysporum* # MT-811 (Shoun & Tanimoto 1991), a gift from Dr. Shoun, Tokyo University, Japan.

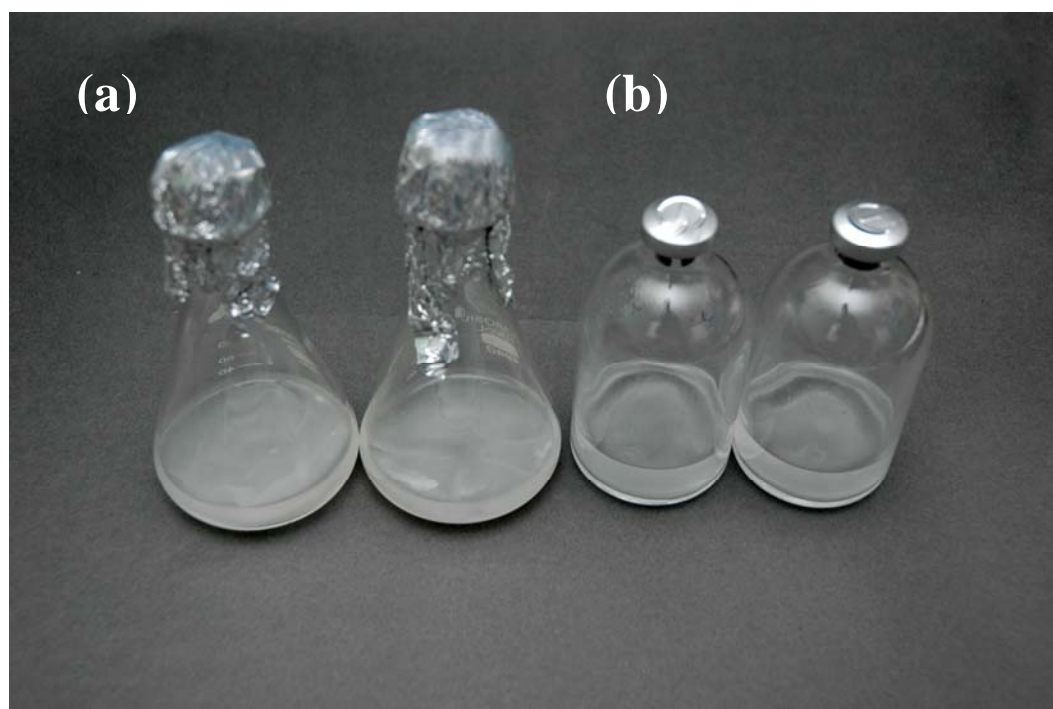


Fig. 4.1 Experimental set-up of fungal cultures grown in (a) aerobic and (b) anaerobic condition

Starter cultures of these fungi were grown in mineral medium supplemented with 10 mM of sodium nitrate for 3 to 5 days. Approximately 10-15 mg (dry weight) of the mycelial suspension was used as an inoculum. The cultures were maintained under aerobic conditions in 100 ml conical flasks (Fig 4.1a) plugged with cotton containing 20 ml of medium and under anaerobic conditions in 100 ml serum bottles (Fig 4.1b) sealed air tight with butyl rubber stoppers and steel crimps. The gas present in the medium was replaced with the inert nitrogen gas by flushing

nitrogen gas through the butyl septum for 2 min with a help of a 5 mm hypodermic needle.

The dissolved oxygen (DO) was determined by spectrophotometric method (Pai *et al.* 1993) at 0 hour and at the end of the experiment on the day 10 and on days when there was significant nitrite formation. Replicate bottles were used exclusively for DO measurement. The cultures were harvested every 48 hours up to 10 days and nitrite and ammonia formed were determined by spectrophotometric measurement (Strickland & Parsons, 1968).

The growth of the cultures was also measured on day 10 and biomass in mg dry weight was determined. The cultures grown in aerobic and anaerobic conditions were filtered on a pre-weighed filter paper to harvest the mycelia. The harvested mycelia on the filter paper was washed several times with distilled water and dried in an oven at 50 °C till it attained constant weight.

Ammonia was estimated in the culture filtrate, by treating it in an alkaline citrate medium with sodium hypochlorite and phenol in the presence of sodium nitroprusside which acts as a catalyzer. The blue indophenol color formed with ammonia was measured spectrophotometrically at 640 nm (Strickland & Parsons, 1968).

Nitrite in the culture filtrate was allowed to react with sulfanilamide in an acid solution. The resulting diazo compound is reacted with N-(1-naphthyl)-ethylenediamine which forms a highly colored azo dye that was measured at 543 nm (Strickland & Parsons, 1968). All chemicals used were of analytical grade.

Statistical analyses were carried out using Excel (Microsoft) programme. The data were transformed and tested for normality before analysis by Cochran Q test (Sokal & Rohlf 1981).

4.2.2 Determination of Electron transport system (ETS) enzyme activity in fungal cultures grown under aerobic and anaerobic conditions

Estimation of ETS activity in the aerobic and denitrifying condition is proportional to the respiratory activity and denitrification rates respectively (Packard *et al.*, 1983). ETS activity was determined for the four cultures ie., # AN- 2 and # 11, # 31, # 31a, # and compared with the terrestrial control culture (# MT-811).

The biomass of the fungal cultures grown under aerobic and anaerobic conditions was harvested and freeze dried. The freeze dried biomass (approximately 100 mg) was homogenized in 3 ml of 0.05 M phosphate buffer (pH 7.7) containing 9 mg of polyvinyl pyrrolidone and 0.5 mg of dithiothreitol. Precautions were taken to minimize bacterial contamination throughout the experimental procedure by preparing buffers in filter sterilized water and using sterilized glass wares. The crude homogenized mixture was centrifuged at 6000 g for 5 min at 4°C. The supernatant was immediately transferred to clean tubes and the volume obtained was recorded. One ml of the crude enzyme extract was incubated in dark for 20 minutes at room temperature with 1 ml of 4 mM 2(p-Iodophenyl)-3(p-nitrophenyl)-5phenyl tetrazolium chloride (INT) and 3 ml of substrate solution containing 0.6 g NADH, 0.2 g NADPH, 36 g sodium succinate hexahydrate and 2 ml Triton X 100 per liter of 0.05 M phosphate buffer (pH 8). During the incubation process INT is reduced to its formazan and this gives a pink coloration to the reaction mixture. The reaction was terminated by addition of 1 ml of 1:1 (V/V) solution of 1 M H₃PO₄ and concentrated formalin at the end of the 30 min incubation time. The quenched reaction mixture was centrifuged at 6000 g for 5 min at 4°C. The absorbance was recorded at 490 nm and 760 nm within 3 hours at

room temperature in a spectrophotometer. The peak at 490 nm is proportional to INT-formazan produced and the 760 nm reading serves as turbidity blank. Pigment blank consisting of an assay run without pyridine nucleotides or succinate on the clarified homogenate was run with each assay; in addition a reagent blank was also run.

The ETS activity was calculated from the equation:

$$\text{ETS (nq h}^{-1} \mu\text{g}^{-1} \text{ protein)} = 60 \cdot S \cdot (\text{COD-RB}) / 1.42 \cdot f \cdot t \cdot 5.6$$

where S: volume of quenched reaction mixture, COD: corrected absorbance, RB: reagent blank, f: conc. of protein in reaction mixture (μg), t: incubation time, the constants 60 and 1.42 conversion factor is based on the molar extinction coefficient of the INT formazan and the stoichiometry of oxygen consumption by the respiratory electron transport system. The constant 5.6 is used to convert the ETS activity obtained in $\mu\text{l O}_2 \text{ h}^{-1} \mu\text{g}^{-1}$ protein to $\text{neq h}^{-1} \mu\text{g}^{-1}$ protein. The total protein concentration in the crude homogenate was determined by Bradford method using the commercially available Bradford reagent (Sigma-Aldrich, Bangalore, India).

4.2.3 Determination of Electron transport system (ETS) enzyme activity under simulated *in-situ* condition (microcosm):

For this study, fresh sediment and near bottom water were collected from CSt-26 during the pre-monsoon season in April 09 (Table 2.2). Sediment samples were collected with the help of a Van-veen grab and a Niskin water sampler (5 L) was used to collect water samples. The samples were brought to the lab on ice and five different microcosms were set-up as described below.

For all the microcosms, sediment slurry was prepared with 15 - 20 g of sediment and ~ 100 ml of near bottom water. The slurry was added to

100 ml serum bottles and flushed with N₂ gas to remove the dissolved oxygen present in the sediment slurry. These bottles were closed with a butyl rubber stopper and sealed immediately with an aluminum crimp.

Table 4.2 List of microcosm set-ups studied

Microcosm	components	Purpose of the set-up
Mic-1a	Plain sediment slurry	To study the total microbial activity
Mic-2a	Sediment slurry with anti-bacterial agents	To study the fungal activity by suppressing the bacterial growth
Mic-3a	Sediment slurry with anti-fungal agents	To study the bacterial activity by suppressing the fungal growth
Mic-4a	Sediment slurry with culture suspension of # An-2	To study the activity of fungal culture # An-2 in the <i>in-situ</i> condition
Mic-5a	Sediment slurry with culture suspension of # MT-811	To study the activity of fungal culture # MT-811 in the <i>in-situ</i> condition

Note: Similar microcosms Mic-1b to Mic-5b were set-up with nitrate supplement.

In the first microcosm (Mic-1a) plain sediment was incubated in anaerobic condition without any supplementation. To study the activity of fungi, we fortified the sediment slurry with streptomycin (0.1 g in 100 ml medium) and penicillin (40,000 Units in 100 ml medium) to inhibit bacterial growth in microcosm-2 (Mic-2a). Likewise, the fungal activity was specifically suppressed with an anti-fungal compound Bavistin to a final concentration of 0.5% in microcosm-3 (Mic-3a). Bavistin is a commonly used benzimidazole fungicide containing methyl benzimidazol-2-yl carbamate (MBC). The concentration of the anti-bacterial and anti-fungal agent used was standardized by growing three isolates of bacteria and fungi in varying concentrations of both the inhibitors. The concentration

that did not show cross-inhibition (ie. fungicide not inhibiting bacteria and vice versa) were used in the study. In microcosm-4 and 5 (Mic-4a & Mic-5a) culture suspension with approximately 30 mg (dry weight) of two cultures # An-2 and # MT-811 was added to the sediment slurry respectively (Table 4.2).

As the initial nutrient concentration was very low in the fresh sediment collected, a set of experiments with nitrate (Sodium nitrate) supplementation to a final concentration of 10 mM was also carried out. The five microcosms with nitrate (Sodium nitrate) supplementation are Microcosm-1b to 5b (Mic-1b to Mic-5b). The experimental bottles were incubated for 7 days under controlled conditions at 30°C with mild agitation to prevent the slurry from settling to the bottom.

Concentrations of nitrate, nitrite, DO levels and Eh of the sediment in the slurry were determined at the start and at the end of the experiment. The DO levels were determined by spectrophotometric methods (Pai *et al.* 1993). Eh was recorded in mV using pH analyzer (PHAN, LabIndia, Thane, India). Nitrate in the sea water used in the experimental set-up was estimated by quantitatively reducing it to nitrite by passing the sample through the column containing cadmium fillings coated with metallic copper. The nitrate reduced to nitrite was estimated by measuring the azo dye formed and any nitrite present in the medium was corrected for (Strickland & Parsons 1968). Nitrite present in the sea water was estimated as described in section 4.2.1.

At the end of the experiment the incubated sediment was freeze dried in clean plastic containers. ETS activity in the freeze dried sediment was determined as mentioned in section 4.2.2 with slight modifications. Approximately 600 mg of freeze dried sediment was homogenized in 6 ml of 0.05 M phosphate buffer (pH 7.7) and incubated for 60 minutes at room temperature and all the experiments were carried out in triplicates.

4.3 Results

4.3.1 Screening of a few fungal cultures for their nitrate utilization capacity under aerobic and anaerobic conditions

The cultures # An-2, isolated after anaerobic incubation of sediments obtained from CSt-26 during the anoxic season in Oct 05 (Ft-1), # 11 isolated from CSt-26, during the anoxic season in Oct 05 (Ft-1). # 31 and # 31a isolated from CSt-26 during the oxic season in Jan 06 (Ft-3) were screened for their ability to reduce nitrate. The culture # MT-811 *Fusarium oxysporum*, a terrestrial isolate studied extensively for its denitrification abilities was included as a positive control (Table 4.1).

These cultures were grown in an inorganic medium supplemented with sodium nitrate to a final concentration of 10 mM. The cultures grew under aerobic and anaerobic conditions; their biomass was estimated after 10 days of incubation. Two cultures, # An-2 and # 11 showed equally good growth under aerobic and anaerobic conditions, the cultures # 31 and # 31a showed seven and fivefold less growth respectively under anaerobic condition and # MT-811 showed a tenfold decrease in biomass (Table 4.3).

Table 4.3 Fungal biomass mg (dry weight) 20 ml⁻¹ under aerobic and anaerobic culture conditions.

Isolate number	Biomass under aerobic culture condition	Biomass under anaerobic culture condition
# An-2	86.7	64.0
# 11	60.2	61.0
# 31	70.6	11.0
# 31(a)	250.3	46.0
# MT-811	130.2	29.3

Nitrate utilization by the cultures was studied based on their ability to accumulate nitrite by reducing the available nitrate. During denitrification process nitrate is reduced into nitrite and further reduced to produce gaseous products. To screen the cultures isolated from the marine habitats for their denitrification ability we estimated the nitrite accumulation under varying oxygen concentrations for 10 days. The initial dissolved oxygen DO level in the flasks was 115.2 μM signifying an oxic condition and as the cultures grew DO was utilized and suboxic conditions developed (18 - 97 μM) on day 4 or 6 depending on the increase in biomass. The DO was estimated every alternate day till the end of the incubation on the 10th day. The initial DO recorded in the anaerobic bottles was 9.5 μM on the first day and the amount of DO on the 10th day was almost undetectable in both the aerobic flasks and anaerobic bottles.

Fungal denitrification differs from bacterial denitrification because the former requires suboxic condition, whereas the latter takes place largely under strict anoxic conditions. Nitrite formation in fungi due to denitrification takes place during suboxic condition and they require about 690 and 250 $\mu\text{M O}_2 \text{ h}^{-1} \text{ g}^{-1}$ dry cell weight for reduction of nitrate and nitrite respectively (Zhou *et al.*, 2001). Accordingly nitrite accumulation could be recorded intermittently in cultures grown in flasks. As the conditions became suboxic the cultures started to utilize nitrate for respiration and nitrite accumulation was seen in the cultures maintained under aerobic conditions. The cultures widely varied in their nitrite accumulation capacity and in the time taken to produce nitrite (Fig.4.2). About 400 nM of nitrite was produced on the 8th day by #An-2 under aerobic condition and almost a similar amount of nitrite in the anaerobic condition. The culture # 11 produced a maximum of 605 nM on the 4th day and maximum nitrite formation in anaerobic condition was 356.17 nM. Among all the cultures, # 31 produced the maximum nitrite of about 904 nM on the 6th day and only

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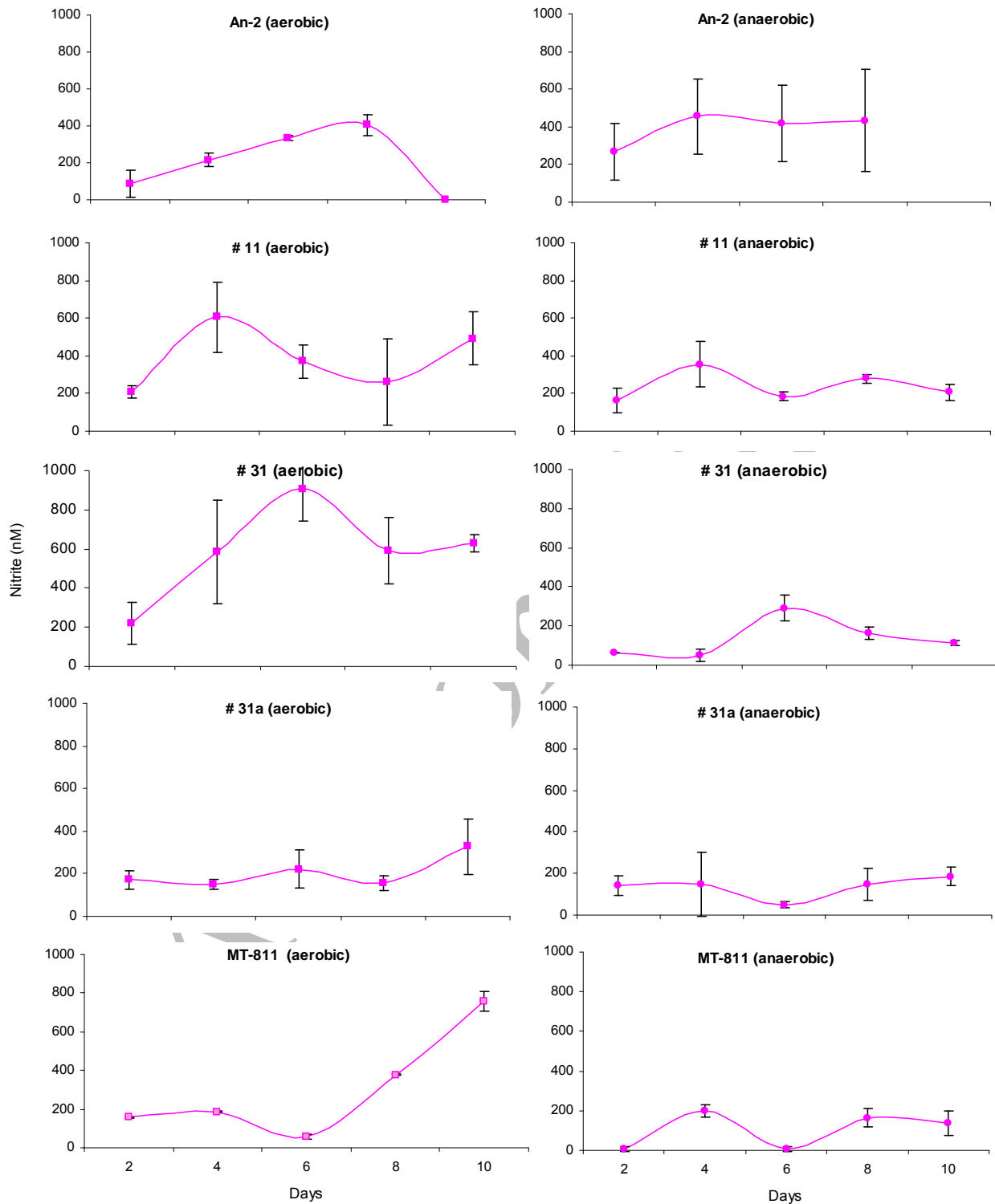


Fig. 4.2. Estimation of nitrite in the culture supernatant of fungi screened for their denitrification ability.

Table 4.4 Two factor Analysis of variance (ANOVA) to show the significance of nitrite accumulation by different cultures on different days

Variables	Df	F value	F critical value	P value
Different days				
(aerobic)	4	1.4	3	0.27
(anaerobic)	4	1.3	3	0.31
Different cultures				
(aerobic)	4	1.7	3	0.19
(anaerobic)	4	3.3	3	0.04*

Df= degrees of freedom, Df=4, n=5 (five sampling days or five cultures); F -critical value indicates statistical significance, * significant at 5 % level

Table 4.5 Two factor Analysis of variance (ANOVA) to show the significance of nitrite accumulation by different cultures in aerobic vs anaerobic conditions

Isolate number	Df	F value	F critical value	P value
# An-2	9	0.9	5.3	0.37
# 11	9	3.4	5.3	0.1
# 31	9	14.6	5.3	0.005**
# 31(a)	9	3	5.3	0.12
# MT-811	9	2.7	5.3	0.14

Df= degrees of freedom, Df=9, n=10 (five sampling of each culture in aerobic and anaerobic conditions); F value greater than F-critical value indicates statistical significance, ** significant at 1 %

about 292 nM was produced under anaerobic condition. Isolate # 31a produced a maximum of 327 nM of nitrite on the 10th day in aerobic condition and under anaerobic condition it was about 185 nM. Statistical analysis showed significant variation between cultures grown under anaerobic conditions (Table 4.4). Maximum nitrite accumulation was seen in # 31 and the nitrite production varied with a high significance (P= 0.005) between aerobic and anaerobic conditions (Table 4.5).

Fungi under complete anoxic condition are known to perform an ammonia fermentation process (Zhou *et al.*, 2002). To study this process

Table 4.6 Two factor Analysis of variance (ANOVA) to show the significance of ammonia accumulation by different cultures on different days

Variables	Df	F value	F critical value	P value
Different days				
(aerobic)	4	0.8	3	0.53
(anaerobic)	4	1.0	3	0.42
Different cultures				
(aerobic)	4	1.8	3	0.17
(anaerobic)	4	11.5	3	0.00014***

Df= degrees of freedom, Df=4, n=5 (five sampling days or five cultures); F value greater than F- critical value indicates statistical significance, *** significant at 0.1 %

Table 4.7 Two factor Analysis of variance (ANOVA) to show the significance of ammonia accumulation by different cultures in aerobic vs anaerobic conditions

Isolate number	Df	F value	F critical value	P value
# An-2	9	37	5.3	0.0003***
# 11	9	1.4	5.3	0.3
# 31	9	0.9	5.3	0.4
# 31(a)	9	0.1	5.3	0.7
# MT-811	9	3.5	5.3	0.09

Df= degrees of freedom, Df=9, n=10 (five sampling of each culture in aerobic and anaerobic conditions); F value greater than F-critical value indicates statistical significance, *** significant at 0.1 %

in the fungal cultures, ammonia accumulation in the culture supernatant maintained under aerobic and anaerobic condition was also determined. The four cultures studied and the positive control # MT-811 showed varying degree of ammonia formation during the incubation (Fig. 4.3). # An-2 produced about 26 μM of ammonia under anaerobic condition and only 6 μM under aerobic condition. The cultures # 11 and # 31 produced almost similar levels of ammonia in both the conditions, which were about 6 and 9 μM respectively. Ammonia formation in the positive control MT-

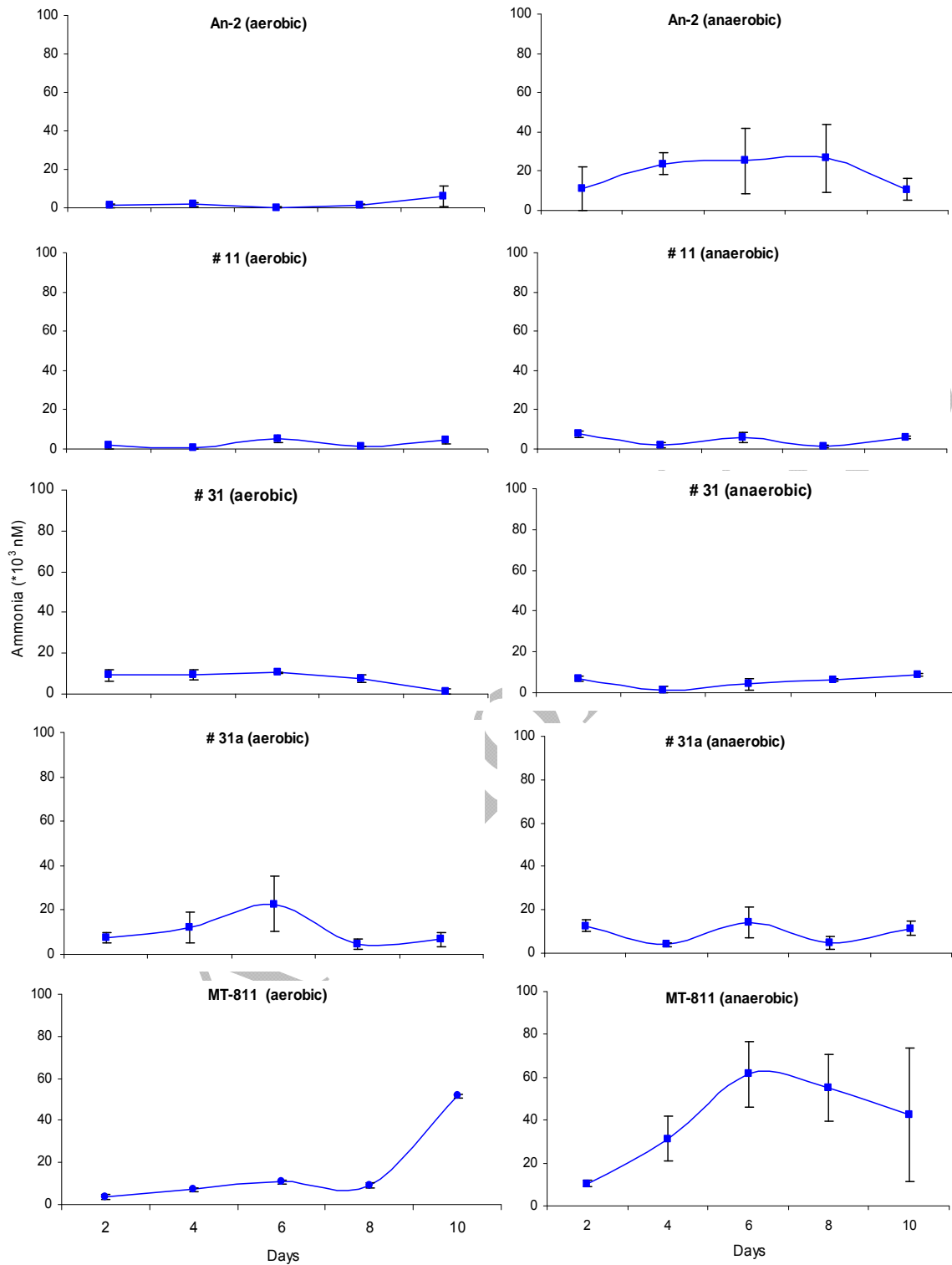


Fig. 4.3. Estimation of ammonia in the culture supernatant of fungi screened for their denitrification ability.

811 was about 50 and 51 μM under anaerobic and aerobic condition. Isolate # 31a produced a maximum of 22 μM under aerobic condition but was able to produce only 14 μM in anaerobic condition. A 2-way analysis of variance indicated significant difference between cultures in their capacity to accumulate ammonia (Table 4.6). Ammonia accumulation between aerobic and anaerobic cultures was significantly different in the culture # An-2 (Table 4.7).

4.3.2 Determination of Electron transport system (ETS) enzyme activity in fungal cultures grown under aerobic and anaerobic conditions

Electron transport system (ETS) activity was measured as an estimate of the respiratory activity under aerobic and anaerobic conditions. The ETS activity measured in the anaerobic condition show that the cultures were viable and active under anaerobic denitrifying conditions (Fig 4.4). The ETS activity measured in the aerobic cultures reached a maximum of about $719 \text{ neq h}^{-1} \mu\text{g}^{-1} \text{ protein}$ and in the anaerobic condition it reached a maximum of about $350 \text{ neq h}^{-1} \mu\text{g}^{-1} \text{ protein}$ only. ETS activity varied widely in the aerobic and anaerobic conditions in the cultures screened (Table 4.8) with a high significance. The cultures # MT-811, # An-2 and # 31 were significantly different in their ETS activity in the aerobic and anaerobic conditions (Table 4.9). On the other hand # 11 and 31a did not show any significant difference and the activity was uniform under both the conditions.

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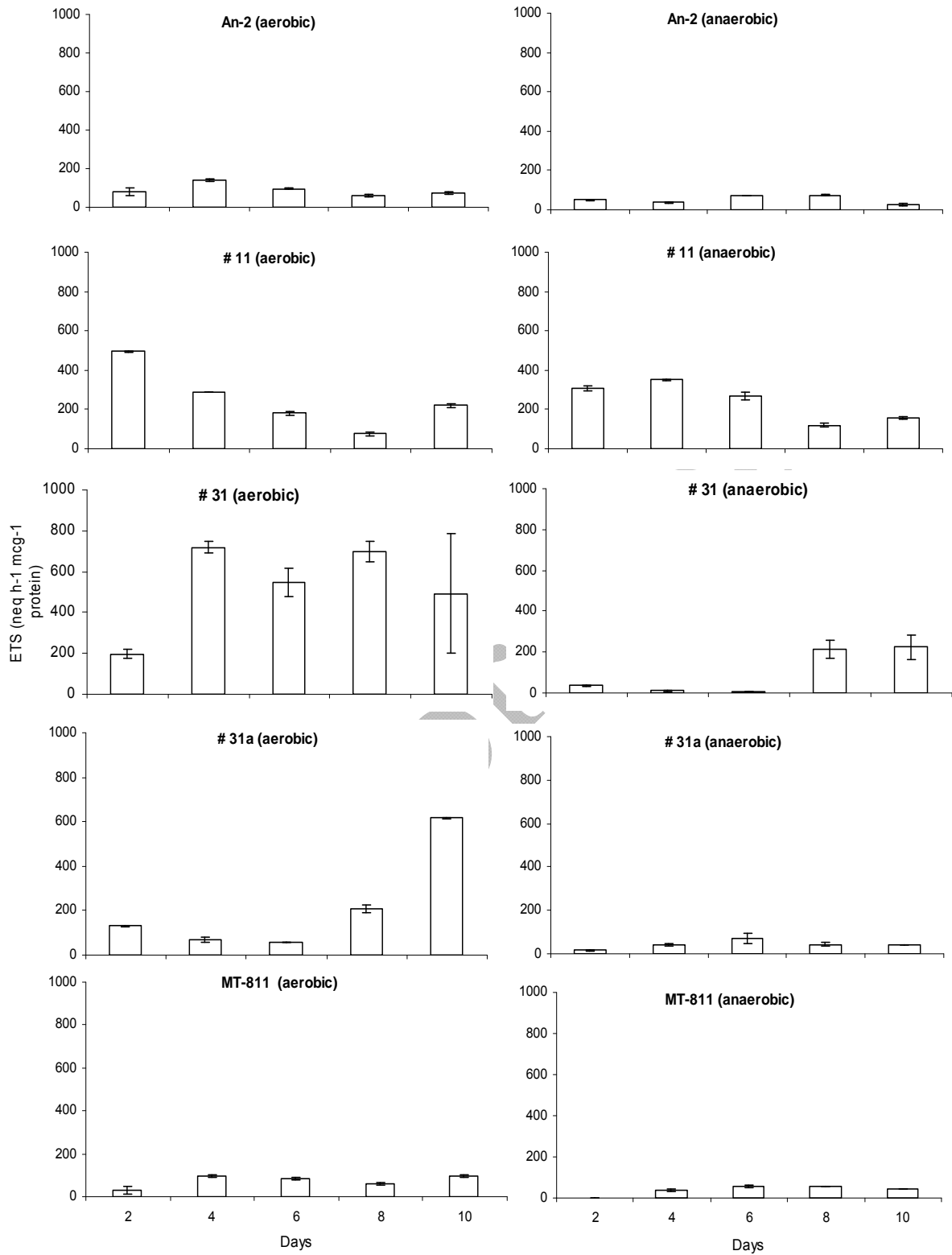


Fig. 4.4. Estimation of electron transport system (ETS) activity in cultures screened for their denitrification ability.

Table 4.8 Two factor Analysis of variance (ANOVA) to show the significance of ETS activity by different cultures on different days

Variables	Df	F value	F critical value	P value
Different days				
(aerobic)	4	0.4	3	0.8
(anaerobic)	4	0.05	3	0.99
Different cultures				
(aerobic)	4	6.0	3	0.003**
(anaerobic)	4	6.2	3	0.003**

Df= degrees of freedom, Df=4, n=5 (five sampling days or five cultures); F value greater than F- critical value indicates statistical significance, ** significant at 1 %

Table 4.9 Two factor Analysis of variance (ANOVA) to show the significance of ETS activity by different cultures in aerobic vs anaerobic conditions

Isolate number	Df	F value	F critical value	P value
# An-2	9	4.9	5.3	0.05*
# 11	9	0.01	5.3	0.9
# 31	9	16.5	5.3	0.004**
# 31(a)	9	2.8	5.3	0.13
# MT-811	9	4.5	5.3	0.07*

Df= degrees of freedom, Df=9, n=10 (five sampling of each culture in aerobic and anaerobic conditions); F value greater than F-critical value indicates statistical significance, ** significant at 1 % * significant at 5 %

4.3.3 Determination of Electron transport system (ETS) enzyme activity under simulated *in-situ* condition (microcosm)

The sediment samples were collected during the suboxic condition during the pre-monsoon season and the DO level in the near bottom water was 112 μM . The Eh of the sediment was -9 mV, the nitrate and nitrite value was less than 1 μM and ETS activity on day zero was about 90 $\text{neq h}^{-1} \mu\text{g}^{-1}$ protein. The sediment was incubated in anaerobic microcosms as described (in Table 4.2) for 7 days. At the end of the incubation period the DO level was 3.81 μM , and the Eh recorded was -19.4 mV in Mic-1a. In a

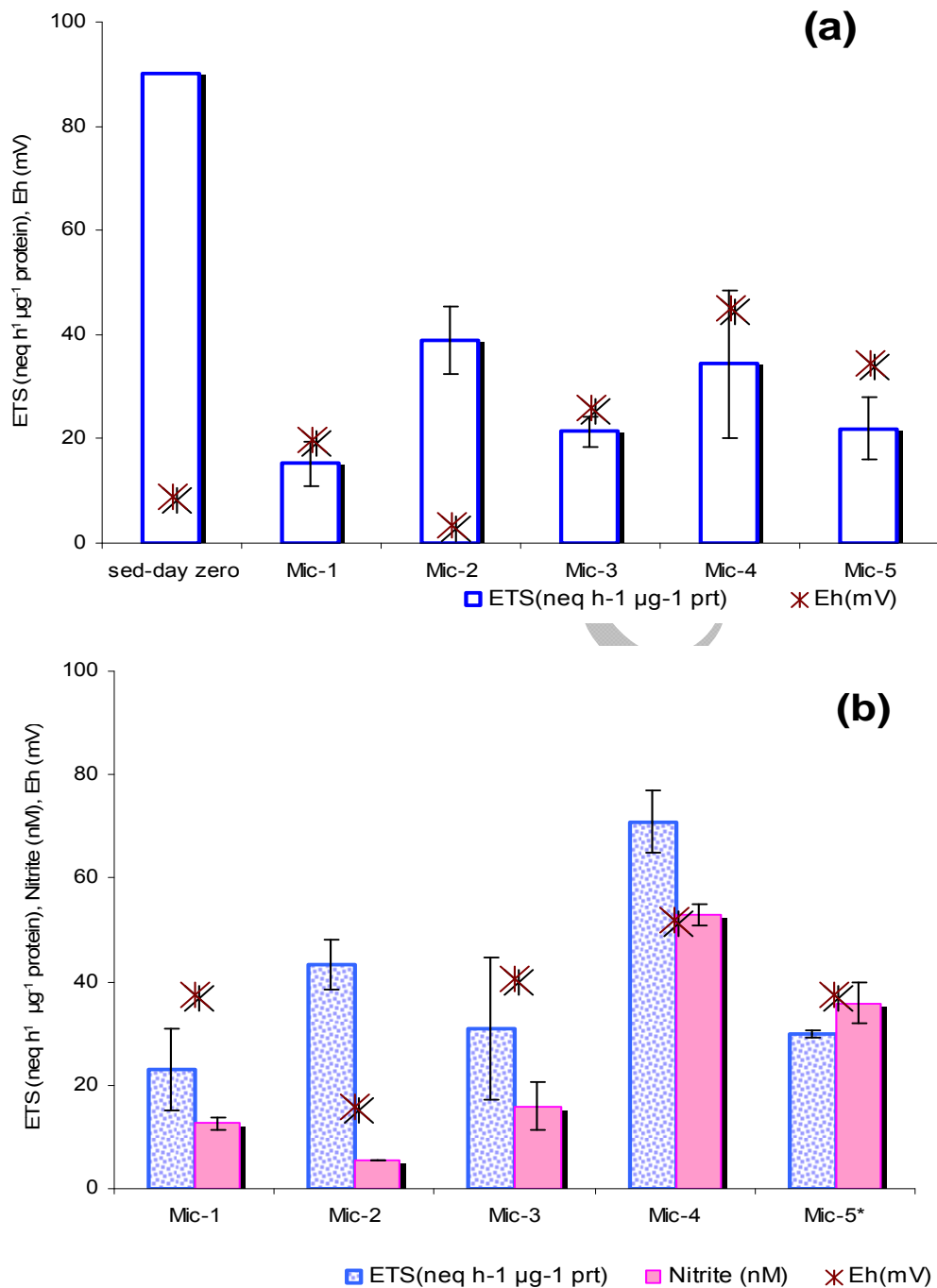


Fig. 4.5 ETS activity and nitrite formation in the (a) anaerobic microcosms without nitrate supplement and (b) with nitrate supplement (Table 4.2). * indicates the nitrite concentration was 10 times higher than shown in the graph.

similar set-up which was supplemented with antibiotics and anti-fungal compounds (Mic-2a and 3a), the Eh value was -2.5 mV and -25.8 mV respectively. In the microcosm set-up with addition of fungal inoculums (Mic-4a & 5a) the ETS activity was 34.3 and 21.9 neq h⁻¹ µg⁻¹ protein respectively. Nitrate or nitrite could not be detected on the initial day and in the experimental set-ups which was not supplemented with nitrate. Because the initial nitrate and nitrite levels were very low, sediments supplemented with 10 mM of sodium nitrate were also set-up (Mic-1b to 5b).

Nitrite accumulation could be seen in all the microcosm set-ups with nitrate supplement (Mic-1b to 5b) and ETS activity proportionately increased with the nitrate supplementation (Fig. 4.5). Increased activity was detected in the microcosm with external fungal inoculation and the ETS activity in Mic-4b and 5b was 70.9 and 29.8 neq h⁻¹ µg⁻¹ protein respectively. Nitrite accumulation recorded in the microcosms with nitrate supplementation was about 53 µM and 358 µM in Mic-4b and 5b respectively (Fig 4.5).

4.4 Discussion

4.4.1 Screening of a few fungal cultures for their nitrate utilization capacity and ETS activity under aerobic and anaerobic conditions

Besides bacteria, fungi are the major agents of decomposition and nutrient cycling in the ecosystem. Role of fungi in the marine ecosystem has been increasingly recognized over the past 50 years since the initial studies by Barghoorn and Linder (1944). Studies on fungi in the marine ecosystem are largely based on the isolation of fungi from various marine substrata

(Maria & Sridhar, 2002) and by studying the quantitative fungal biomass distribution from various habitats (Damare *et al.*, 2006). The role of fungi in the natural ecosystem was largely based on the various studies on the extracellular and intracellular enzymes produced by the fungal isolates. The physiology of fungi and their activity in the marine ecosystem has been studied based on their growth and enzyme production (Molitoris & Schaumann, 1986). The ability of fungal cultures to utilize nitrate in the aerobic conditions has been studied based on the the nitrate reductase activity (Bresinsky & Schnieder, 1975). A number of terrestrial isolates have also been tested for their ability to denitrify in anaerobic conditions (Shoun *et al.*, 1992). Screening of various isolates and studying their capacity under various oxygen concentrations has shown the ability of fungi to respond variously to the oxygen concentrations.

Here we have tested the ability of four cultures isolated from the anoxic coastal marine sediments for their growth under anaerobic conditions. While all the cultures grew much better under aerobic conditions, one culture (#11) produced almost the same amount of biomass under both aerobic and anaerobic conditions (see Table 4.2). These results indicate that fungi isolated from the anoxic sediments might have adapted to a facultative anaerobic mode of life. Shoun and Tanimoto (1991) have shown that # MT-811 can grow under anaerobic conditions and utilize nitrate and nitrite for dissimilatory purpose. They have also shown that there is a substantial amount of cell growth during this process, which shows that the dissimilatory nitrate or nitrite reduction is an energy yielding reaction.

Fungal denitrification process differs significantly from classical bacterial denitrification. Bacterial denitrification takes place only in the complete absence of oxygen and even a trace of oxygen could be toxic to the obligate anaerobes and inhibit denitrification. In fungi, reduction of

nitrate takes place under suboxic conditions, but excess oxygen ($> 900 \mu\text{M O}_2$) is shown to inhibit the process (Zhou *et al.* 2001). My study on the denitrifying ability of the cultures under varying oxygen concentrations has shown that all the cultures were able to grow and reduce nitrate to nitrite. The isolate # 31 showed the maximum nitrite accumulation on day 6 when suboxic conditions set in (Fig. 4.2). The cultures showed marked difference in their ability to reduce nitrate under the denitrifying conditions. The ability of the cultures was however much less in comparison with the positive control culture # MT-811. This may be due to the inability of the cultures to utilize nitrate, as all fungi are not capable of nitrate reduction, but can use nitrite more efficiently as an electron acceptor (Takaya, 2002). Hence experiments to screen isolates for their nitrite reducing capacity are to be carried out. Further fungi are also known to require supplements to aid their growth under anaerobic conditions (Bartnicki-Garcia & Nickerson, 1961). There was no organic nitrogen source in the medium during the present study, which may be the one of the reasons for the low efficiency of the cultures to utilize nitrate.

Fungi also follow another pathway to reduce nitrate under complete anoxic conditions, which is referred to as ammonia fermentation. This process in fungi appears to be widespread as 15 of 17 fungi tested by Zhou *et al.* (2002) showed ammonia formation under anaerobic condition. This process was studied in the same four cultures under both aerobic and anaerobic conditions. There was ammonia formation by all the cultures under anaerobic conditions and # An-2 produced the maximum ammonia under anaerobic culture condition (Fig 4.3). The fungal cultures screened varied widely in their capability to produce ammonia under anaerobic condition (Table 4.5). Ammonia formation in An-2 was distinctly different between aerobic and anaerobic condition (Table 4.6). The cultures were isolated from the seasonal anoxic zone from the coastal

waters, their ability to utilize nitrate and grow under suboxic and anoxic condition shows that the fungal cultures are adapted to a facultative mode of denitrification activity with varying oxygen concentration. Studies on the denitrifying activities of *Fusarium oxysporum* # MT-811 have shown that it expresses diversified pathways of nitrate metabolism in response to environmental O₂ tension (Takaya, 2002). Fungi show a multimodal type of respiration to rapidly adapt to changes in the oxygen supply, in anoxic conditions ammonia formation takes place, while denitrification process in suboxic and oxygen respiration under aerobic conditions (Takaya, 2002). This may be a survival strategy for mycelial fungi to thrive in extreme and dynamic environments.

Electron transport system (ETS) activity is a measure of the respiratory activity and is used as a measure of the denitrification rates under anoxic condition in the presence of nitrate as final electron acceptor. The ETS was active in the fungi under aerobic and anaerobic conditions and the potency of the cultures highly varied. Measuring the ETS activity directly shows that the respiratory system was active in fungal cultures in the anaerobic conditions also. The accumulation of nitrite, ammonia and the active ETS in the anerobic condition shows that the fungal cultures utilizes the alternate respiratory process or denitrification in the absence of oxygen.

4.4.2 Determination of Electron transport system (ETS) enzyme activity under simulated *in-situ* condition (microcosm)

Initial ETS activity in the natural sediment sample collected during suboxic condition in April 09 was about 90 neq h⁻¹ µg⁻¹ protein and the activity reduced approximately four times at the end of the incubation period in Mic-1a on the 7th day (Fig 4.5a). When the bacterial growth was

suppressed the ETS activity was higher in comparison to the plain sediment anaerobic microcosm. Microcosm study shows that the ETS activity due to fungi was higher in comparison to the bacterial activity (Fig 4.5a). Though ETS activity could be recorded, the nitrite formation was not seen, this may be due to the unavailability of nitrate in the natural condition. Nitrate or nitrite levels were undetectable on the day zero (data not shown).

Microcosms with nitrate supplementation (Mic-1b to Mic-5b) were set-up to study the denitrifying ability of the in-situ microbial population. Nitrite formation was recorded in all these microcosms and the ETS activity also proportionally increased with nitrate supplementation (Fig 4.5b). In natural system the fungal biomass C observed was 3 orders of magnitude lesser in comparison with the bacterial biomass C (Fig. 2.1). Enumeration of fungi from the forest soil capable of anaerobic growth accounts for only 0.01 to 0.1% in conifer and deciduous forest soil (Reith *et al.*, 2002). Though the abundance of fungal biomass in the leaf litter has shown to be high in comparison with the bacterial biomass, the denitrifying bacterial community may be dominating the natural oxygen depleted environment. A mix culturing of *Fusarium oxysporum* and *Pseudomonas stutzeri* in anaerobic condition was shown to be capable of faster nitrate and nitrite removal, than individual cultures (Liu *et al.*, 2006). Moreover the N₂O production recorded also reduced from the mix-culture because *P. stutzeri* were able to utilize the N₂O produced by *F. oxysporum*. This study is a preliminary attempt to screen a few marine fungal isolates and estimate the role of fungi in marine denitrification process. Detailed studies using labeled nitrate supplement and estimation of gaseous denitrifying products can improve our understanding of the fungal role in the oxygen depleted environments.