MARINE YEASTS FROM THE SLOPE SEDIMENTS OF ARABIAN SEA AND BAY OF BENGAL

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Dedicated to

my beloved father,

Late. S Narayanan Kutty,

the power and reminiscence to live with.

Declaration

I hereby do declare that the thesis entitled "**Marine Yeasts from the Slope Sediments of Arabian Sea and Bay of Bengal**", is a genuine record of research work done by me under the supervision of Dr. R Damodaran, Professor (Retd.) and Dr. Rosamma Philip, Senior Lecturer, School of Marine Sciences, Cochin University of Science and Technology, Kochi- 682016, and that no part of this work, has previously formed the basis for the award of any degree, diploma associateship, fellowship or any other similar title of any university or institution.

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Certificate

This is to certify that the thesis entitled "**Marine Yeasts from the Slope Sediments of Arabian Sea and Bay of Bengal**" is an authentic record of research work carried out by Ms. Sreedevi N Kutty under our supervision and guidance in the department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology in partial fulfilment of the requirements for the degree of Doctor of Philosophy and no part thereof has been presented before for the award of any degree, diploma or associateship in any university.

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Sreedevi N Kutty

CONTENTS

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Chapter 1

GENERAL INTRODUCTION

Marine ecosystem sprawls across more than 70% of the earth's surface. The inscrutable microorganisms flourish well in the hydrosphere and form a major component of marine fauna. This autonomous biotic entity is in fact the most important functional unit of the living world. Microbes are so powerful that play a decisive role in both the stability and functioning of ecosystems. Their ability to decompose countless organic substrates and quite a few xenobiotics puts them on the centre stage of nutrient cycling within ecosystems.

As the existence of microbial life was recognized only relatively recently in history, about 300 years ago, the understanding that we have gained about them is still little. The study of microbial diversity faces serious restrictions as only 0.01- 0.1% of these organisms could be cultured. In any case, microbes represent the largest reservoir of unrecorded biodiversity. Recently, fresh probes for revolutionary microbial products have converged on microorganisms from the marine environment. Powerful new tools, chiefly in molecular biology, remote sensing and deep sea explorations, have led to exciting discoveries of the profusion and heterogeneity of marine microbial life and its role in global ecology.

The world over, the benthic realm of the sea is unparalleled by virtue of harbouring multifarious, flora and fauna. However, our knowledge on the biodiversity and economical relevance of these organisms is inadequate. To this extent, benthic environment presents a pristine and largely untapped source of novel microorganisms with the potential to produce bioactive compounds.

Heterogeneity is evidently the earmark of a benthic environment; by the same token, benthic microfauna constitute diverse bacteria, fungi, yeasts and actinomycetes. Several enquiries have already been made into the distribution and intricate capabilities of bacteria, actinomycetes and fungi. Consequently, we are now well-informed on an appreciable number of microbial species, their uses and qualities, like the *Saccharomyces cerevisiae* that we deal with fairly

regularly in our daily lives. However, in our country, the domain of marine yeasts still remains largely unexplored. A researcher in the burgeoning branch of marine microbiology is bound to be spurred on by this nature's fortune that lies unexplored and the expectations it generates. Therefore, a prompt and exhaustive survey of the yeast species of our seas, not only in search of new life-saving drugs and leading edge bioactive compounds but also for a complete codification of its biodiversity and a better evaluation of the resources would be highly useful.

Yeasts in general are polyphyletic group of basidiomycetous and ascomycetous fungi with a unique characteristic of unicellular growth stage. The term 'yeast' was coined from the old Dutch word 'gist' and the German word 'gischt' which refers to fermentation. There are approximately 100 genera and 800 described species of yeasts and estimates suggest that these numbers represent only about 1% of the species that exist in nature, the rest being non-culturable (Fell, 2001). The pattern of their distribution is dependent on the concentration and type of organic material present. Yeasts as a eukaryotic microbe is of more importance for its nutritional quality and bioactivity.

Yeasts have been traditionally used in food industry principally for the production of ethanol and carbon dioxide, which are important to the brewing, wine distilling and baking enterprises. For commercial purposes, yeasts are recognized for their high nutritional value, relatively large size, convenience of cultivation, lack of toxic byproducts, lower content of nucleic acids, long history of use as food and therefore better public acceptance. Ability to grow on substrates of low pH reduces bacterial contamination in the medium.

Similar to many other fungi, the saprophytic endeavors of yeasts convert plant and animal organics to yeast biomass and some economically profitable by-products. Lipids, pullulans and enzymes are some of the industrially important extra cellular metabolites of yeasts. Immunostimulation is yet another compelling trait,

thanks to its complex carbohydrate and nucleic acid components. Certain species are also known to be pathogenic to plants and animals.

Yeasts are notably rich in proteins, lipids and vitamins. Hence the biotransformation of raw material into yeast biomass (single cell protein) has captured ample interest, for possible utilization as animal or aquaculture feed. In addition, yeasts can be produced efficiently and economically on a massive scale because of their relatively shorter generation time and the inexpensive culture media.

The present study is exclusively focused on marine yeasts; compared to their terrestrial or freshwater counterparts, studies on marine yeasts have been found to be very less. They are an important category of marine microorganisms and their saprophytic role plays a fundamental part in food webs. Different animals in the marine ecosystem devour detritus in various stages of decay with its associated microbes, formed from complex physical and chemical interactions mediated by the environmental conditions of the sediment and water column as well as the metabolism of micro and macro organisms closely associated with it (Kenworthy et al., 1989).

Marine yeasts are reported to be truly versatile agents of biodegradation. In the sea, they participate in a range of processes of ecological significance, especially in estuarine and near shore environments. Of these, decomposition of plant substrates, nutrient-recycling, biodegradation of oil/recalcitrant compounds and parasitism of marine animals are imperative. Biomass data and repeated observations of microhabitat colonization by various marine occurring yeasts support ancillary lab evidence for the contribution of this segment of the marine mycota to productivity and transformation activities in the sea. Terrestrial yeasts have transfixed the attention of science and industry for decades; but it is only during recent years that the potential applications of marine yeasts in different fields have been initiated.

Against this backdrop, the present study was undertaken with the following objectives:

- A detailed survey of the distribution of yeasts along the continental slope sediments of Arabian Sea and Bay of Bengal
- Isolation, characterization and identification of yeasts from sediment samples
- Estimating the hydrolytic enzyme production potential of the yeast isolates
- Effect of pH, temperature and NaCl concentration for the growth of the yeast isolates
- Potential of marine yeasts in oil degradation
- Characterization of black yeast isolates for bioactive materials

The results of the study are presented in four comprehensive chapters, following a broad introduction to the topic in Chapter 1. Chapter 2 gives an account of the hydrographical and geological conditions, besides the abundance of yeast population in the slope sediments of Arabian Sea and Bay of Bengal. Chapter 3 deals with the isolation, characterization and identification of yeasts. Crude oil degradation profile of selected yeasts and their characterization is delineated in Chapter 4, and Chapter 5 delves into the characterization of marine black yeasts for potential biomaterials. Summary, References and Appendices are followed.

Chapter 2

OCCURRENCE OF MARINE YEASTS IN THE SLOPE SEDIMENTS OF ARABIAN SEA AND BAY OF BENGAL

Contents

2.1 Introduction

Yeasts are distributed in almost every part of the aquatic environment, i.e. oceans and seas, estuaries, lakes and rivers (Fell, 2001). The discovery of marine yeasts goes back to 1894, when Fisher separated red and white yeasts from Atlantic Ocean and identified them as *Torula* sp. and *Mycoderma* sp. respectively. Following Fisher's discovery, many other workers such as Hunter (1920), Bhat et al. (1955a & 1955b), Suehiro (1960) and van Uden and Fell (1968) isolated marine yeasts from different sources viz. seawater, marine deposits, sea weeds, fish, marine mammals and sea birds. ZoBell and Feltham (1934) observed yeasts on most of their plates inoculated with samples of marine materials collected from land as well as from the open ocean. Russian microbiologists have reported the quantitative distribution of yeasts in the Black and Okhotsk seas, Pacific Ocean and the Arctic sea (Rukina and Novozhilova, 1952; Kriss et al., 1952; Kriss, 1955; Novozhilova, 1955).

The majority of reports on yeasts from marine environments are based on indirect collection methods, such as incubation of seawater and sediment with diverse substrates found in the sea. With such culture techniques, cells may grow *in vitro* which would have remained dormant and inactive in the marine habitats. Yeast species that have also been found in fruits, soil, domestic animals and man are most likely not native to estuaries and seas, even if they were isolated from such areas many times. It is more probable that they were washed into sea by way of rivers or sewage, or with dust blown seaward wind. Observations such as exceptionally high yeast densities following *Noctiluca* blooms in the North Sea (Meyers et al., 1967) could indicate the presence of indigenous species, but insufficient data did not allow these authors to draw definite conclusions. In addition, the area in question was polluted by sewage disposal and regular passenger traffic (Gors et al., 2006). Kriss and Rukina (1949) also found plankton blooms in the Black sea and the Pacific Ocean, to be locations of

greatest density of yeast populations in the sea. Direct examination of living marine invertebrates, however, has demonstrated the presence of parasitic and pathogenic yeasts (Roth et al., 1962; Seki and Fulton, 1969; Fize et al., 1970), and, if such species have grown *in situ* in the animal and its native habitat, they could rightly be called indigenous marine. So far, no physiological clues have been found to explain why marine-occurring yeast is able to live in this special habitat. Salinity tolerance does not distinguish marine species from terrestrial species because almost all yeasts can grow in sodium chloride concentrations exceeding those normally present in the sea. Certain distinctive metabolic attributes of yeasts are associated with environmental distribution. Yeasts found in aquatic environments are generally asporogenous and oxidative or weakly fermentative (Pitt and Miller, 1970).

2.1.1 Distribution of yeasts in the marine environment

Sea Water:

Yeast populations have been observed to decrease with increased distance from land (Ahearn et al., 1968) and certain yeast species frequently collected from seawater were obtained in the highest quantities from the vicinity of heavily polluted areas (Fell and van Uden, 1963) which could also indicate that the collected yeasts were merely contaminants from terrestrial sources, surviving passively in the sea. This may very well question the statement that there are truly indigenous marine yeasts. Near shore environments are usually inhabited by tens to thousands of cells per litre of water, whereas low organic surface to deep-sea oceanic regions harbour 10 or fewer cells per litre, although local nutrient rich areas may foster concentrations of yeast cells that reach 3-4 thousand per litre. Kriss and Novozhilova (1954) reported that budding yeasts were observed by direct microscopic examination of water samples down to depths of 2000 m. This fact would be an evidence for the growth of yeasts in seawater; however, the collection technique with Nansen bottles used by Kriss

and co-workers was questioned later when such containers were found to be easily contaminated (Sorokin, 1964).

During the cruise of the RV Vitiaz in 1957-1958, *Debaryomyces globosus* was isolated from a depth of 400 m in the central Pacific. Yamasato et al. (1974) conducted an ecological survey of yeasts from the Pacific Ocean and yeasts were isolated from the surface to a depth of 4000 m and were found belonging to the genera viz. *Rhodotorula, Cryptococcus, Debaryomyces and Candida*. *Cryptococcu*s and *Rhodotorula* species were predominant among yeasts isolated from deep-sea waters from Loma Trough, off San Diego, California. In samples collected off La Jolla, California, total yeast count varied from 0-1920 viable cells per litre (van Uden and Castelo- Branco, 1963).

From the open ocean waters of the Gulf Stream near Bimini, Bahamas, genera like *Candida, Rhodotorula, Cryptococcus, Debaryomyces* and Black yeasts were isolated. The distribution of species as well as their numbers and metabolic characteristics were found to be governed by existing environmental conditions. Fell et al. (1960) obtained a total of 179 yeast isolates from 45 sampling stations in the course of a qualitative yeast survey in Biscayne Bay, Florida of which *Candida tropicalis* and *Rhodotorula rubra* were the predominant species. Roth et al. (1962) and Fell (1965) made a quantitative study on the distribution of yeasts in the coastal areas of Southern Florida and in the Gulf Stream of Florida. Freshwater influx and heavy recreational bathing directly affected viable yeast count in these areas and *Candida tropicalis* and *Rhodotorula rubra* were predominant in the inshore region. A new species of basidiomycetous yeast, *Rhodosporidium diobovatum* was isolated from the marine and estuarine waters of south Florida by Newell and Hunter (1970). Yeasts were found to be widely distributed in the water and sediment of Chesapeake Bay and *Rhodotorula* sp. was frequently isolated from this region (Colwell, 1972).

Hagler and Mendonca (1981) studied the yeasts from marine and estuarine waters with different levels of pollution in the state of Rio de Janeiro, Brazil and found that yeast counts in clean sea water generally ranged from a few to several hundred per litre, but in the case of enrichments like pollution or algal blooms, the number may reach thousands per litre or more. In addition there is a shift from a prevalence of strictly aerobic yeasts in clean water to a presence of fermentative yeasts in polluted waters. Yeasts from polluted and unpolluted beaches in the southern area of Sao Paulo state "Baixada Santista", Brazil were isolated and studied by Paula et al. (1983). The isolates belonged to nine genera viz. *Candida, Cryptococcus, Rhodotorula, Torulopsis, Trichosporon, Debaryomyces, Hansenula, Pichia and Sporobolomyces*. The results point to the genus *Candida* as a probable pollution indicator for coastal seawater. Isolation and identification of yeasts from sand and seawater collected from two beaches of Olinda, Pernambuco state, Brazil was done by Loureiro et al. (2005). 292 strains of yeasts were obtained and they belonged to four genera and 31 species, of which *Candida* was the most prevalent genus.

Ahearn and Crow (1980) reported the species and densities of yeasts isolated from North Sea waters before and after the oil mining. *Debaryomyces hansenii* was the predominant species in both sets of samples, but after oil production, *Candida guilliermondii*, a hydrocarbonoclastic yeast, was more commonly isolated. Kriss (1960) found that yeasts were observed not only in the oxygenated zone but also in the H_2S zone of the Black sea. Further studies of Kriss revealed that the distribution of yeast in seawater is characterized by microzonation. In coastal waters, up to several thousand yeast cells per litre of water were found (Roth et al., 1962; Meyers et al., 1967). Yeasts are known to be the normal components of the biota of the world oceans (Fell, 1965; Kriss et al., 1967) and in heavily polluted waters there could be considerably more. Presence of some salt-tolerant yeast has been reported in the open ocean by van Uden and Fell (1968). Fungi and yeasts which are filamentous in nature are usual

inhabitants of marine environments (Norkrans, 1966b; Litchfield and Floodgate, 1975; Phaff et al., 1978; Hagler et al., 1979; Kohlmeyer and Kohlmeyer, 1979; Kirk and Gordan, 1988).

Fell (1967) found living yeasts in the Indian Ocean from the surface down to a depth of 200 m. Yeasts were collected from 16 stations during the cruise of RV Anton Brunn in Indian Ocean. The highest population of yeast was found in the Somali current and the species isolated were grouped according to their distribution. Ubiquitous species like *Rhodotorula rubra* and *Candida atmospheri*ca were seen in all water masses. Widely distributed species occurred in all water masses except from Red sea and it was represented by *Candida polymorpha* and *Rhodotorula glutinis*. Species like *Sporobolomyces hispanicus, S.odonus* and *Rhodotorula crocea* were of restricted distribution. Bhat and Kachwalla (1955) isolated yeasts from water samples collected from two to six miles off the coast of Bombay and they obtained species like *Saccharomyces italicus, S. chevalicri, S. rosei, Debaryomyces hansenii, Pichia guilliermondii, Candida tropicalis, Torulopsis glabrata, Torulopsis candida, Rhodotorula* sp., *Cryptococcus* sp. etc. Yeasts of the Indian Ocean waters were studied by Fell and van Uden (1963); D'Souza (1972) and Godinho et al. (1978). 33 strains of marine yeasts were isolated from the coastal and offshore waters off Cochin and *Candida* was the predominant genera obtained (Rhishipal and Philip, 1998). A marine hydrocarbon degrading yeast was isolated from Mumbai (India), which was identified as *Yarrowia lipolytica* (Oswal et al., 2002). Yeasts were isolated from seawater samples collected from the west and east coast of India up to 200 m depth in the EEZ (Sarlin, 2005). The most predominant genera were *Candida, Filobasidium and Leucosporidium*, most of which were fermentative with filamentous growth.

Various kinds of ethanol producing marine yeasts from coastal waters were isolated and characterized by Urano et al. (1998) and he found that most of them belonged to the genera *Candida and Debaryomyces*. Zhang et al. (1989) investigated the ecological distribution of marine microorganisms in the southern

ocean to the north west of the Antarctic Peninsula and isolated 6 genera of yeasts from seawater. A survey of the marine yeasts in the sub Antarctic region near South Georgia conducted by Connell and Rodriguez (1994), recovered 72 yeast isolates. Of these 72 isolates, 19% were psychrophilic (could not grow at or above 20^{o}C) and 43% grew more rapidly at 20^{o}C than at temperatures at which they were collected (<4^{o}C). Urano et al. (2001) studied halotolerant and fermentative yeasts living in various aquatic environments: upper stream of Arakawa river, middle and lower streams of Tamagawa rivers and sea coasts of Kemigawa in Chiba prefecture and of Chemigahama in Chishi city. Colony forming units of yeasts decreased with the increase of osmotic pressure or salt concentration and increased with increase of total organic carbon in aquatic areas.

Sediment:

Relatively high yeast densities, up to 2000 viable cells per gram have been reported from marine sediments, with most of the population in the top few centimeters (Fell et al., 1960; Lazarus and Koburger, 1974). About 99 yeast strains including 40 red yeasts were isolated from benthic animals and sediment collected from the deep sea floor in various areas in the northwest Pacific Ocean (Nagahama et al., 2001). Nagahama et al. (2003) isolated a novel species of yeast which belonged to the genus *Cryptococcus* from the sediment collected on the deep sea floor of Suruga Bay. They described the strain as *Cryptococcus surugaensis*.

Fell et al. (1960) isolated yeasts from Biscayne Bay at Florida and deep-sea sediments in the Bahamas. The most commonly isolated genera were *Rhodotorula, Debaryomyces, Torulopsis, Cryptococcus* and *Candida.* There was an abundance of yeasts in silty muds than in sandy sediments. The limited deep-sea collections showed a predominance of oxidative yeasts as compared to collections made in Biscayne Bay. In the investigations of Roth et al. (1962) sediments and surrounding waters of the grass beds showed higher cell counts and higher number of species than grasses and algae. Fell and van Uden (1963)

found that yeasts are confined to the upper 2 cm of the substrate at a depth of 540 m, in the Gulf Stream. In shallow Florida waters, however where strong wave action and rapid settling of sediments prevail, yeasts were found in depths up to 9 cm. The author concludes that the availability of oxygen is the limiting factor for growth process of yeasts within the sediments. They occur particularly in the top most centimeters and according to Suehiro (1963) they are more frequent in black zone than in sandy sediments. Meyers et al. (1971) observed very high concentrations of viable cells of *Spartina alterniflora* in marshes of Louisiana coast than adjacent water samples. Species of *Pichia and Kluyveromyces* were predominant and occurred most commonly in the culm-sediment region of the *Spartina* plants (Ahearn and Meyers, 1972). Several hundred living yeast cells per cm^3 were found in the damp mud from the Kiel Fjord (Hoppe, 1970).

The prevalent isolates from estuarine, littoral and deep-water marine sediments of Florida and the Bahamas have been mostly oxidative yeasts, including *Rhodotorula* and *Cryptococcus*, typical of sea water (Fell et al., 1960; Lazarus and Koburger, 1974; Volz et al., 1974). A new ascosporogenous yeast, *Lachancea meyersii* sp. nov. was isolated from mangrove regions in the Bahama Islands (Fell et al., 2004). Yeast abundance in the sediments of 13 coastal sites of Massachusetts was quantified by MacGillivray and Shiaris (1993). The most abundant genera identified included *Candida, Cryptococcus, Rhodotorula, Torulopsis and Trichosporon.* Few yeasts were isolated from greater depths (11000 m) and comparatively higher number from the shallower sites (1000 to 6500 m). The isolation frequency of yeasts fell as the depth of sampling site increased. The ratio of basidiomycetous yeasts to ascomycetous yeasts rose with increasing depth. Little diversity is observed among basidiomycetous isolates and *Rhodotorula* occupied 89% of all isolates. On the other hand, ascomycetous yeasts isolated at the shallower site than 2000 m showed a wide range of taxa such as *Candida, Debaryomyces, Kluyveromyces, Pichia, Saccharomyces and Willopsis* (Takami et al., 1998).

Hagler and Mendonca (1981) suggested that polluted littoral sediments are an unfavorable environment for strictly oxidative yeasts like *Rhodotorula* and *Cryptococcus* which are common in less polluted sediments. Hagler et al. (1982) studied the densities of some yeasts in intertidal sediments of a polluted subtropical estuary in Rio de Janeiro, Brazil. Highest yeast densities were found at the most polluted site, and at the upper 2 cm of sediments. *Candida krusei, Pichia membranefaciens* and similar species were the prevalent yeasts in these sediments, and species like *Rhodotorula rubra* related to basidiomycetous fungi were found in relatively low numbers. Diversity assessment of benthic yeast was done along a longitudinal gradient in Serra Do Cipo, Brazil to monitor organisms important in determining water contamination levels. These microbes usually feed on dissolved organic matter, multiplying rapidly under favorable conditions (Callisto et al., 2004).

Thirteen yeast strains were isolated from deep-sea sediment samples collected at a depth of 4500 m to 6500 m in the Japan Trench. Among them one of the strains, which belonged to the genus *Cryptococcus*, possessed high tolerance against Cu^{2+} (Abe et al., 2001). Yeasts and other fungi are prevalent in marine salt marsh and mangrove ecosystems where they play an important role in the detrital food web (Meyers et al., 1975; Hyde, 2002). Araujo et al. (1981) made studies on fungi and yeasts from the west coast of India. Fungi and yeasts were isolated from immersed timber test panels, drift wood, marine and estuarine sediments, mangroves and water samples. The estuarine sediments harboured several fungi and yeasts showing hydrocarbon degrading, nitrogen fixing and pectinolytic activities. The studies revealed that yeasts and fungi play significant role in the transformation of complex and persistent organic compounds and aid in the food chain in the sea. Prabhakaran and Ranu Gupta (1991) studied yeasts from sediment samples along the southeast coast of India. They found that *Candida* was the dominant group and next in abundance was *Rhodotorula.*

The *Cryptococcus vishniacii* (yeasts of basidiomycetous affinity), isolated from the soil samples of Dr. W V Vishniac's 1973 expedition is peculiar to the dry valleys of Antarctica, constituting the only heterotrophic biota demonstrably indigenous to the most severe cold desert on earth (Vishniac and Hempfling, 1979a; Vishniac and Hempfling, 1979b; Baharaeen and Vishniac, 1982). The *Cryptococcus vishniacii* complex appears to have undergone sub specific evolution in the Dry Valleys (Baharaeen et al., 1982).

The yeast community associated with deep-sea hydrothermal systems of Mid-Atlantic Rift was surveyed for the first time by Gadanho and Sampaio (2005). According to the survey, the non-pigmented yeasts were more abundant than the pink-pigmented ones. The authors state that this disproportion was not observed in studies of other marine systems and may be due to the unique conditions of hydrothermal vents, characterised by a rich animal and microbial diversity and therefore by the availability of organic compounds utilizable by yeasts. Twelve phylotypes belonged to ascomycota and seven phylotypes belonged to basidiomycota. They were identified as *Candida atlantica, C. atmosphaerica, C. lodderae, C. parapsilosis, Exophiala dermatitidis, Pichia guilliermondii, Trichosporon dermatis, Rhodosporidium diobovatum, R. sphaerocarpum, R. toruloides,* and *Rhodotorula mucilaginosa*. Some of the yeasts were found belonging to phylogenetic groups reported from other marine environments, and eight phylotypes represented undescribed species. The new species found at the Mid-Atlantic Ridge hydrothermal fields represented 33% of the total number of yeast taxa found.

Le Petit et al. (1970) found seven species which were able to metabolize hydrocarbon fractions from oil-polluted littoral marine areas in the Mediterranean. Ahearn et al. (1971) isolated yeasts from oil-polluted habitats and *Trichosporon* species was found to emulsify the oil. The responses of yeast populations to oil pollution were investigated by Ahearn and Meyers (1972). A considerable increase in yeast densities was noticed in the oil-soaked plots, and

the predominant yeasts of the marshland were replaced by hydrocarbonoclastic strains, especially *Pichia ohmeri* and *Trichosporon* sp. In the nutrient rich sediments of the estuary, populations of yeasts continued to increase in the presence of oil. In general, yeasts isolated from oil-polluted regions exhibited much higher hydrocarbonoclastic property than the same species from non-polluted areas. Yeast communities from heavily polluted sediments that received the discharge from oil refineries and other industries were studied by Romero et al. (2002). Yeast species were isolated from these sediments and their ability to degrade dibenzofuran was determined. Studies revealed that 13 strains of yeasts were able to hydroxylate dibenzofuran. The ecological relevance of this study was based on the fact that dibenzofuran is a xenobiotic not easily transformed, so the catabolic activities observed in autochthonous yeasts contribute to broadening the biodegradable substrate spectrum.

Estuaries:

In littoral zones of Crimea, Florida and California coasts, yeast population densities were generally higher than adjacent open seas (Kriss et al., 1952). The apparent dominance of some yeast species in estuaries and their apparent absence in open oceans may be due to a variety of reasons. One obvious possible source of yeast in estuaries is sewage pollution and terrestrial run-off. In fact two ecological groups encountered were yeasts like *Rhodotorula glutinis* which were wide spread in estuaries, the open oceans and inland waters and intestinal yeasts like *Candida tropicalis* and *Candida intermedia* from terrestrial substrates that were dominant in estuaries but rare in open seas (Cooke et al., 1960).

van Uden and Ahearn (1963) did quantitative studies on yeasts present in surface and deep water samples from a fresh water body (Douglas lake, Michigan) which revealed the presence of 12 species (*Candida parapsilosis, C. Pulcherrima, Cryptococcus albidus, Cr. diffluens, Cr. gastricus, Cr. laurentii, Rhodotorula glutinis, R. pilimanae, R. rubra, Trichosporon cutaneum,*

Debaryomyces sp. and Black yeasts). Taysi and van Uden (1964) found that higher number of yeasts was obtained from regions where there was relatively light pollution. It was found that with increase in distance from the estuaries, the number of species decreased. Ecological observations showed that estuaries had more dense yeast population than adjacent oceanic zones. The species common to both estuaries and oceanic regions were the genera *Debaryomyces* and *Rhodotorula,* the species exclusively or predominantly estuarine were *Candida intermedia, Candida lambica, Candida silvicola and Torulopsis candida*. Elevated yeast densities were observed at nutrient rich haloclines in estuaries (Norkrans, 1966a). Estuaries probably take an intermediate position with yeast population fluctuating between high levels in inland waters and low levels in non-estuarine regions. There are evidences that estuarine waters contain not only more yeast cells/volume, but also more species than adjacent sea (van Uden, 1967). This may be due to high organic load of the estuaries than the marine habitat. Numerous yeasts were identified from polluted water and sewage (Cooke and Matsura, 1963; Ahearn et al., 1968; Hagler and Mendonca, 1981).

Investigations on the yeast flora of the Suwannee estuary in Florida showed that *Candida* and *Rhodotorula* were the predominant genera, however the most frequently isolated strain was *Cryptococcus laurentii*. Nine ascosporogenous species were isolated, with *Hansenula saturnus* as the predominant form (Lazarus and Koburger, 1974). The microbial flora of the estuarine and inshore environments of the west coast of Taiwan was studied by Cheng and Lin (1977). Preliminary identification of the isolates revealed that they belong to the genera *Saccharomyces, Torulopsis, Debaryomyces, Endomycopsis, Pichia, Kloeckera and Rhodotorula.* Hagler et al. (1979) reported that *Candida* and *Rhodotorula* were the most frequently isolated genera from a polluted estuary. 112 yeast isolates were obtained from 31 samples of decaying vegetation in the rhizosphere of the mangrove plants, from 11 sites in Chapora,

Mandovi and Zuari estuaries of Goa, India (Da Costa and D'Souza, 1979). Yeast diversity in the estuary of Tagus River, Portugal was studied by Gadanho and Sampaio (2004). According to the analysis of the yeast community structure, the dominant populations belonged to *Debaryomyces hansenii, Rhodotorula mucilaginosa, Cryptococcus longus* and to an uncultured basidiomycetous yeast phylogenetically close to *Cr. longus*. Yeast community in the waters of Tagus estuary, Portugal, was studied by Almeida (2005) and the occurrence of some yeasts was partially correlated with faecal pollution indicators. *Candida catenulata, C. intermedia, C. parapsilosis, Clavispora lusitaniae, Debaryomyces hansenii, Pichia guilliermondii, Rhodotorula mucilaginosa* and *Rhodosporidium diobovatum* were the principal species found.

Yeast communities were isolated from water and sediment samples of two unpolluted natural lakes, located inside Rio Doce State Park and two rivers located outside this Park in Southeastern Brazil. A total of 134 yeast isolates were obtained and identified as belonging to 36 species. The genus *Candida* had the highest number of species (Medeiros et al., 2008).

Fishes:

Yeasts associated with fish were isolated from skin, gills, mouth, faeces and gut contents of animals. Of the various species of yeasts associated with fish, *Debaryomyces hansenii* was the most dominant species. This species is frequent in seawater, which may explain its high incidence in fish. Another important yeast species isolated from fish was *Metschnikowia zobelli.* High numbers of *Metschnikowia zobelli* were isolated from the gut contents of fish and it has been suggested that yeast flora of fish merely reflect their feeding habits (Fell and van Uden, 1963). In the Pacific, van Uden and Castelo-Branco (1963) found certain fish species containing significantly high numbers of yeast cells than the surrounding sea water, and the authors believe that these yeasts may be able to grow in the intestine of some fish. Ross and Morris (1965) reported that the greatest variety and highest number of yeasts

were obtained from fish skin while gill counts gave only less numbers. Yeasts were isolated from the intestine of farmed rainbow trout (*Salmo gairdneri*) by Andlid et al. (1995). The dominant species were *Debaryomyces hansenii, Saccharomyces cerevisiae, Rhodotorula rubra and Rhodotorula glutinis.* Red-pigmented yeasts dominated and composed about 90% of the isolates.

2.1.2 Sediment texture and Organic matter

The organic content of sediments, are often considered as an important trophic source for the benthos. This can be used as a measurement of the trophic status of marine ecosystems (Cocito et al., 1990). Animal sediment relationships are fundamental for studies on the distribution, development and maintenance of benthic communities. The grain size of sediment determines to an important degree the number of organisms within a community; therefore it functions as an environmental influence on biological diversity (Etter and Grassle, 1992). The organic contents also depend on the grain size of the sediment (Gremare et al., 2002). In sediment, organic matter quality rather than quantity can be related to benthic faunal abundance (Relexans et al., 1996; Fabiano and Danovaro, 1999; Danovaro et al., 2000; Gremare et al., 2002) and activity (Albertelli et al., 1999). Marine yeasts are reported to be truly versatile agents of biodegradation (De souza and D'Souza, 1979; Kobatake et al., 1992). They participate in a range of ecologically significant processes in the sea, especially in estuarine and near shore localities i.e. decomposition of plant substrates, nutrient-recycling phenomena, biodegradation of recalcitrant compounds etc. Biomass data and repeated observations of microhabitat colonization by various marine occurring yeasts reveal the contribution of this segment of the marine mycota to productivity and transformation activities in the sea (Meyers and Ahearn, 1974).

2.2 Materials and Methods

2.2.1 Study area

The study area was confined to the west and east coast of Indian peninsula, specifically the continental slope of Arabian Sea and Bay of Bengal. The area

covered was from Cape comorin to Porbander, in the Arabian Sea and from Karaikkal to Paradip in the Bay of Bengal. Collections were made from 200, 500 and 1000 m depth regions along 16 transect in the west and 12 transects in the east coast (Fig. 2.1).

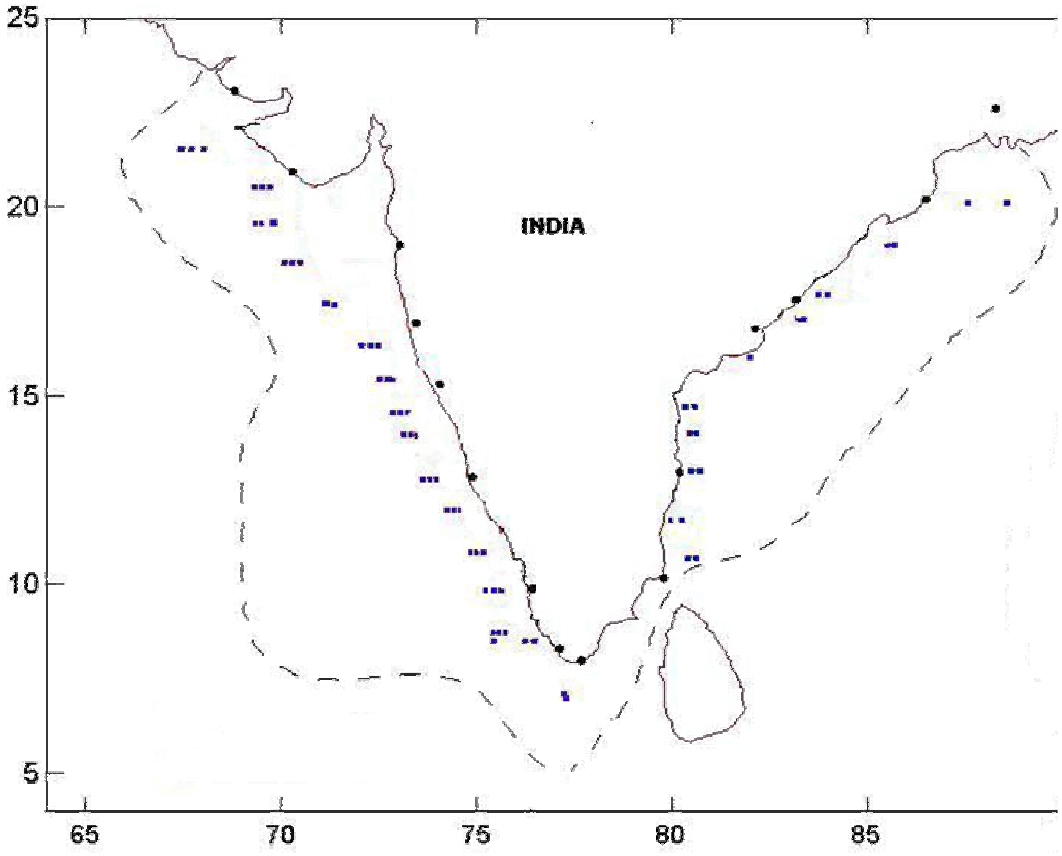


Fig. 2.1 Map showing the sampling stations in the Arabian Sea and Bay of Bengal

2.2.2 Sample collection

Sediment samples were collected from Arabian Sea and Bay of Bengal during 4 cruises (Cruise No. 228, 233, 236 and 245) of Fisheries and Oceanographic Research Vessel (FORV) Sagar Sampada of CMLRE, Ministry of Earth Sciences, Government of India using Smith McIntyre grab (Fig. 2.6) from three different depth ranges (200, 500 and 1000 m). Each transect was situated every one degree latitude apart. Sediment sample for

microbial analysis was transferred aseptically into sterile polythene bags. Temperature and pH of the sediment was also noted. Another fraction of sediment was stored at -20^{0}C in a freezer for biochemical analysis. Hydrographical parameters (Temperature, salinity, dissolved oxygen) were recorded using CTD (Sea Bird, USA) (Fig. 2.7).

Arabian Sea:

Collections were made from 16 transects in the Arabian Sea. Each transect consisted of 3 stations at 200, 500 and 1000 m depths. Stations were located between Cape comorin at the southern end and Porbander at the northern end. Samples were collected during cruises 228 (September, 2004) and 233 (April, 2005) (Fig. 2.2 and Fig. 2.3). The details of stations are given in table 2.1.

Table 2.1 Stations along the Arabian Sea (Cruise No. 228 and 233)

Transect	Station No.	Position		Depth (m)
		Latitude (N)	Longitude (E)	
Cape comorin (Cape)	0	7°10`	77°20`	207
	1	7°00`	77°20`	536
	2	6°57`	77°21`	999
Thiruvananthapuram (Tvm)	3	7°50`	76°41`	205
	4	7°50`	76°37`	584
	5	7°50`	76°37`	976
Kollam (Klm)	6	9°00`	75°54`	267
	7	8°55`	75°29`	490
	8	9°00`	75°26`	1050
Kochi (Kch)	9	9°54`	75°36`	180
	10	9°54`	75°33`	500
	11	9°51`	75°29`	1095
Ponnani (Pon)	12	10°59`	75°07`	226
	13	10°58`	75°05`	515
	14	10°59`	74°59`	992
Kannur	15	11°55`	74°26`	202

(Knr)	16	11°56`	74°22`	523
	17	11°56`	74°18`	958
Mangalore (Mngr)	18	12°51`	74°00`	200
	19	12°53`	73°56`	494
	20	12°53`	73°47`	1000
Coondapore (Cndr)	21	13°31`	73°25`	220
	22	13°29`	73°24`	520
	23	13°29`	73°17`	1040
Karwar (Kwr)	24	14°31`	73°12`	216
	25	14°31`	73°06`	416
	26	14°31`	72°58`	1000
Goa (Goa)	27	15°25`	72°51`	230
	28	15°26`	72°46`	520
	29	15°25`	72°37`	1050
Ratnagiri (Rtgr)	30	16°07`	72°27`	210
	31	16°08`	72°23`	470
	32	16°08`	72°19`	950
Dabhol (Dbl)	33	17°17`	71°46`	220
	34	17°16`	71°38`	588
	35	17°15`	71°30`	1010
Off Mumbai (Of Mmb)	36	18°30`	70°28`	200
	37	18°30`	70°23`	520
	38	18°31`	70°19`	1000
Off Mumbai (Of Mmb)	39	19°45`	69°30`	200
	40	19°49`	69°25`	490
	41	19°45`	69°18`	1010
Off Mumbai (Of Mmb)	42	20°44`	69°14`	202
	43	20°44`	69°10`	530
	44	20°44`	69°03`	1006
Porbander (Pbr)	45	21°31`	68°00`	215
	46	21°30`	67°49`	500
	47	21°29`	67°46`	1025

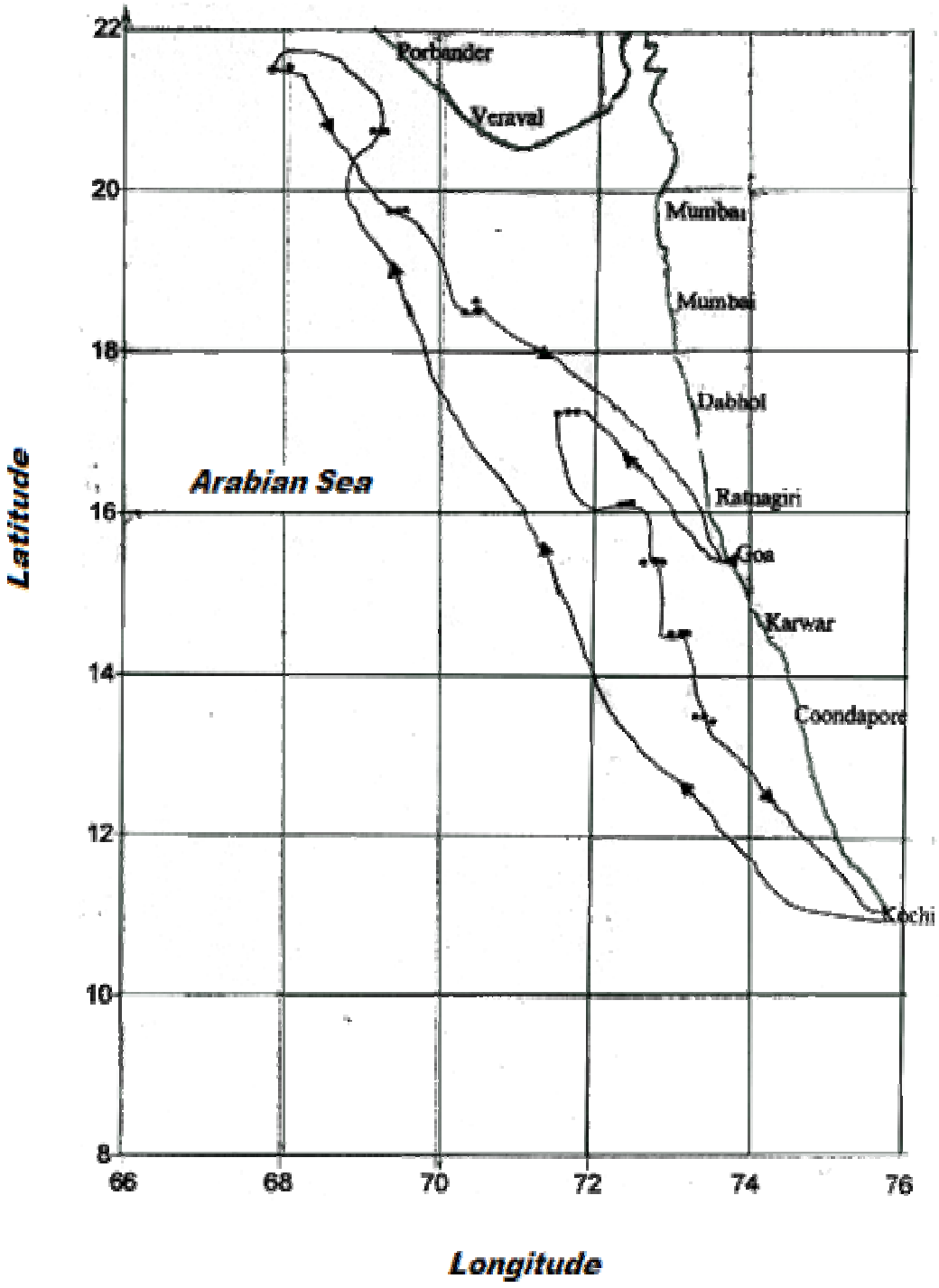


Fig. 2.2 Cruise track showing sampling stations - FORV Cruise No. 228

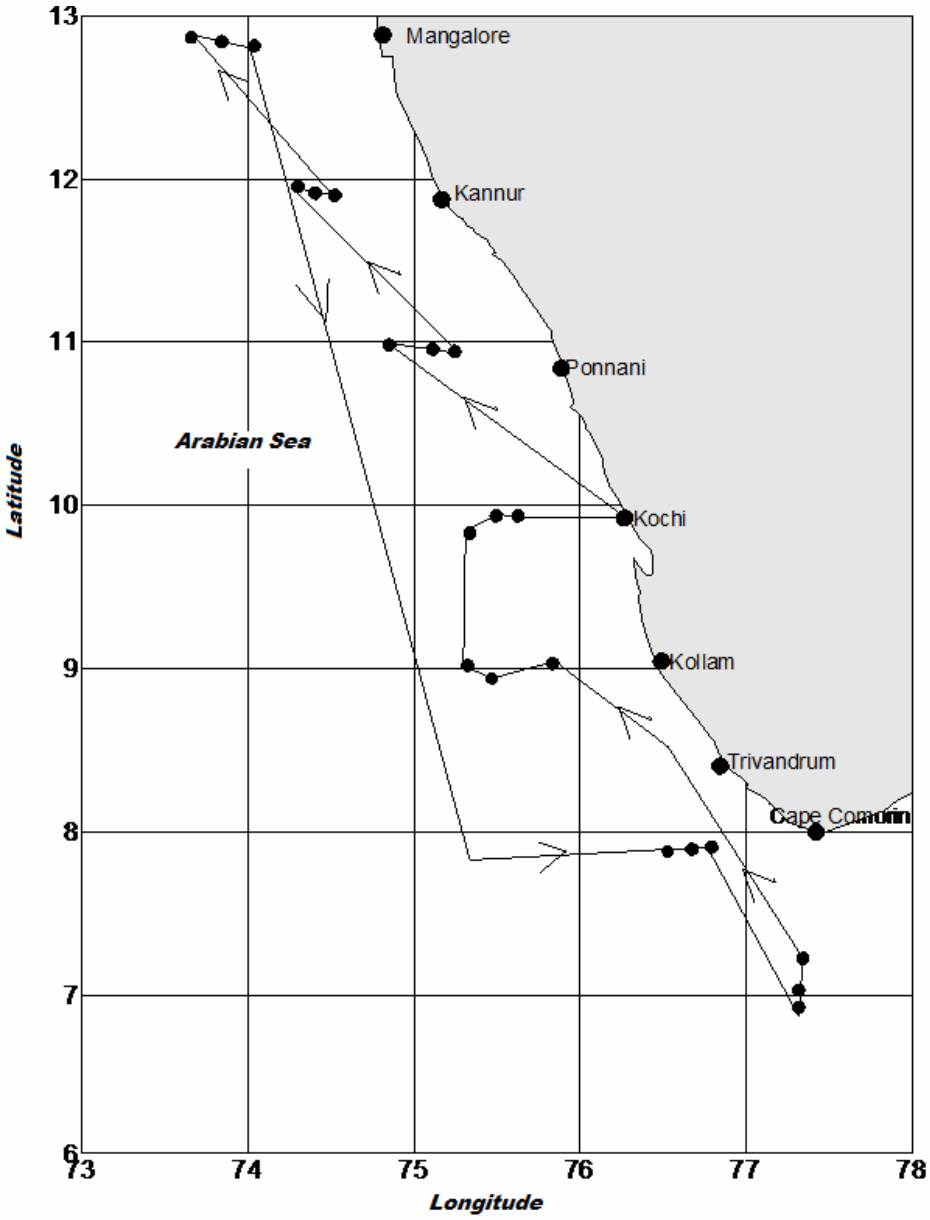


Fig. 2.3 Cruise track showing sampling stations -FORV Cruise No. 233

Bay of Bengal:

Sampling was done from 12 transects in the Bay of Bengal. Each transect consisted of 3 stations at 200, 500 and 1000 m depths. Stations were located between Karaikkal at the southern most end and Paradip at the northern end. Samples were collected during cruises 236 (July, 2005) and 245 (July, 2006) (Fig. 2.4 and Fig. 2.5). The details of stations are given in table 2.2.

Table 2.2 Stations along the Bay of Bengal (Cruise No. 236 and 245)

Transect	Station No.	Cr: 236			Cr: 245		
		Position		Depth (m)	Position		Depth (m)
		Latitude (N)	Longitude (E)		Latitude (N)	Longitude (E)	
Karaikkal (Kakl)	48	10°34`	80°26`	250	10°36`	80°23`	235
	49	10°36`	80°30`	500	10°39`	80°28`	570
	50	10°36`	80°32`	1108	10°42`	80°31`	803
Cuddallore (Cdlr)	51	11°31`	79°59`	221	11°32`	79°58`	200
	52	11°31`	80°02`	501	11°31`	80°01`	497
	53	11°32`	80°07`	951	11°32`	80°04`	770
Cheyyur (Chyr)	54	12°18`	80°29`	195	12°25`	80°34`	165
	55	12°21`	80°33`	521	12°24`	80°34`	400
	56	12°21`	80°36`	870	*	*	*
Chennai (Chni)	57	13°09`	80°56`	217	13°09`	80°36`	216
	58	13°09`	80°41`	515	13°09`	80°41`	490
	59	13°10`	80°43`	1039	13°09`	80°43`	1000
Thammera-pattanam (Tmpm)	60	14°10`	80°27`	221	14°09`	80°24`	180
	61	14°10`	80°26`	615	14°11`	80°26`	500
	62	14°10`	80°24`	925	14°10`	80°26`	836
Singaraya-konda (Sgkd)	63	15°15`	80°31`	215	*	*	*
	64	15°15`	81°33`	495			
	65	15°16`	80°35`	775			
Divi Point (Dvpt)	66	16°00`	81°29`	186	16°12`	82°02`	209
	67	16°04`	81°49`	527	16°09`	82°02`	426
	68	16°00`	82°03`	954	16°02`	82°00`	857
Kakinada (Knda)	69	17°00`	83°01`	198	17°06`	83°13`	207
	70	17°00`	83°12`	515	17°03`	83°16`	476
	71	17°00`	83°20`	849	17°02`	83°21`	850
Bheemuli (Beml)	72	18°01`	84°15`	181	*	*	*
	73	18°01`	84°16`	495			
	74	18°01`	84°20`	858			
Barua (Brua)	75	19°03`	85°25`	195	19°02`	85°19`	200
	76	19°06`	85°32`	445	19°02`	85°30`	482
	77	19°05`	85°39`	993	19°05`	85°33`	910
Chilka (Clka)	78	19°29`	85°46`	202	19°29`	85°45`	185
	79	19°31`	85°50`	610	19°30`	85°47`	537
	80	19°29`	85°53`	810	Nil	Nil	Nil
Paradip (Prdp)	81	20°05`	87°11`	214	20°02`	87°00`	150
	82	20°48`	87°13`	488	19°59`	86°58`	538
	83	20°01`	87°30`	952	*	*	*

***Collection could not be done due to rough weather conditions**

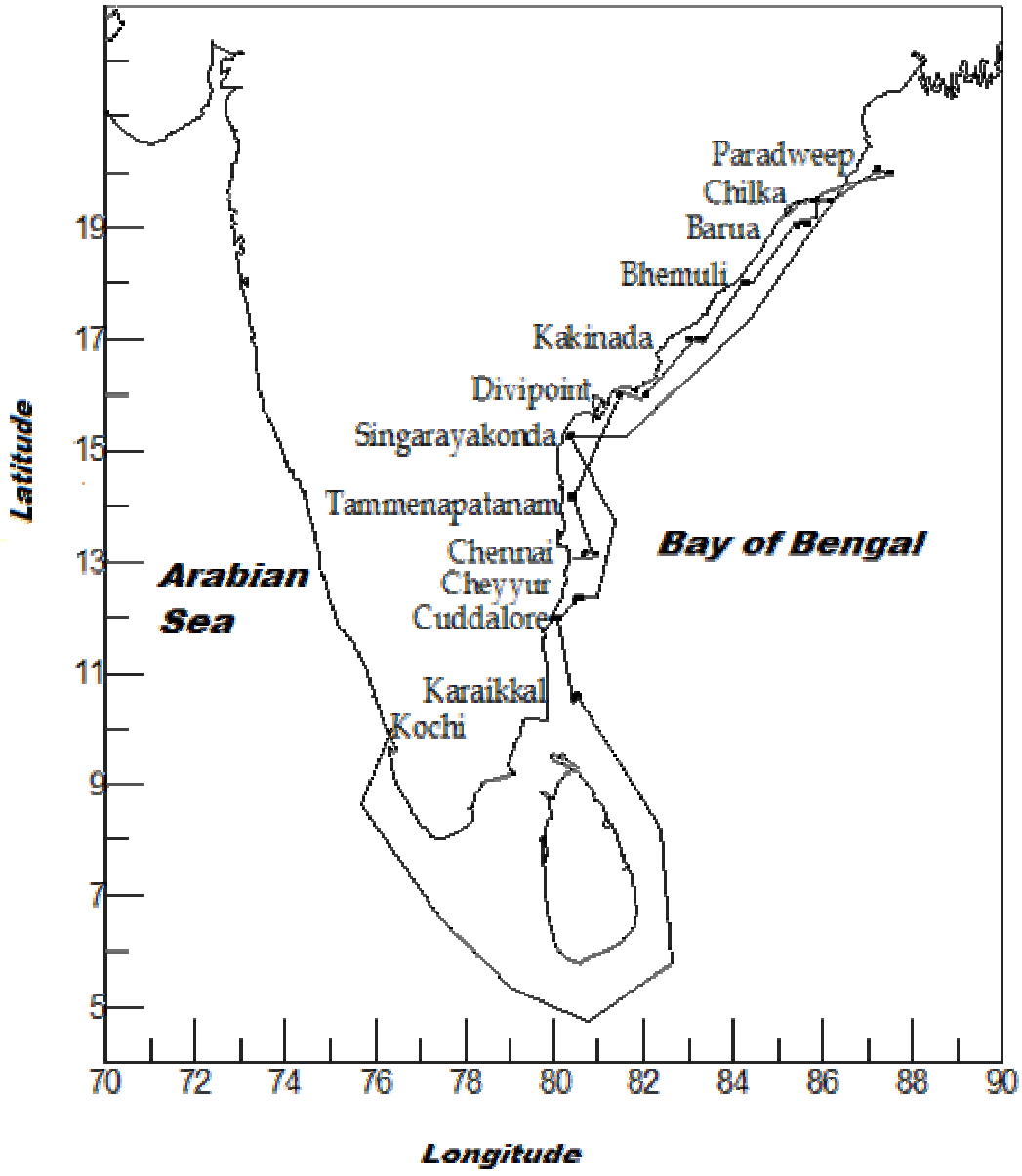


Fig. 2.4 Cruise track showing sampling stations - FORV Cruise No. 236

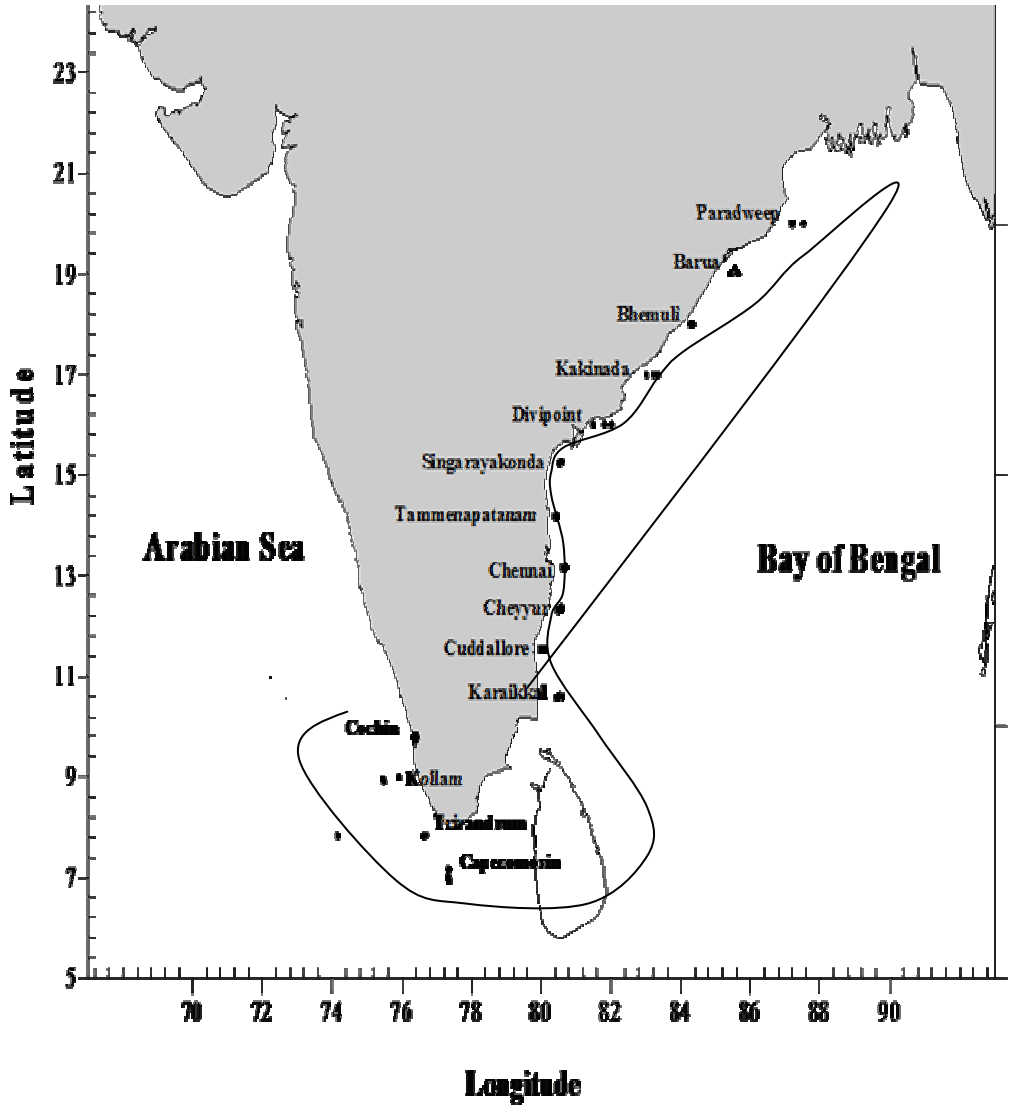


Fig. 2.5 Cruise track showing sampling stations FORV Cruise No. 245

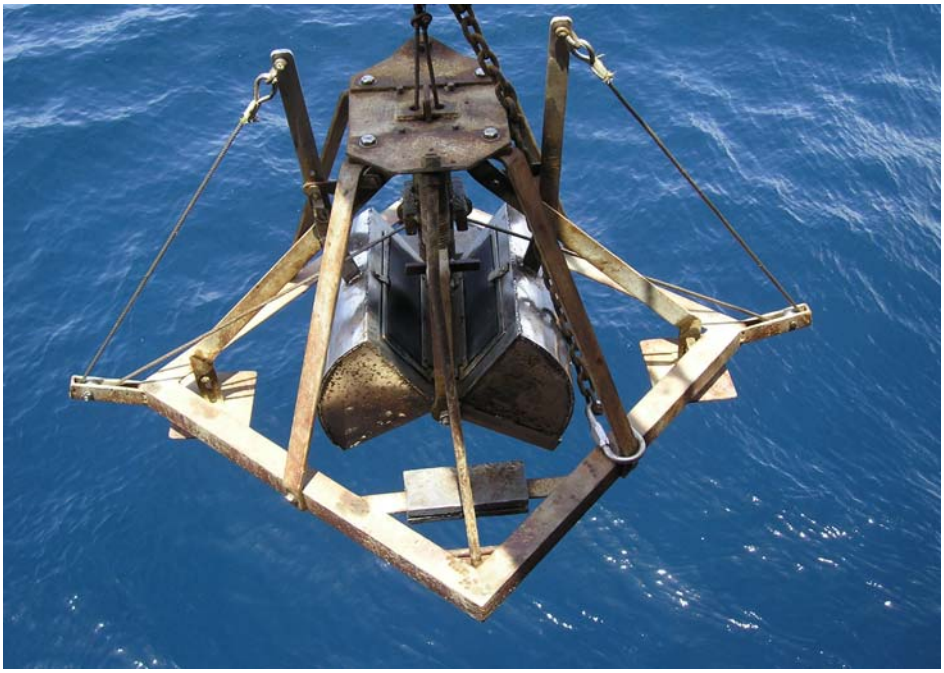


Fig. 2.6 Smith McIntyre Grab used for sediment sample collection

Fig. 2.7 CTD used for hydrographic data collection

2.2.3 Grain size analysis

Sediment was dried at 60°C for 48 hours and separated the fine fraction (<150 μm) by wet sieving. The size of the fine fraction was determined by using particle size analyser (Sympatec, Germany). The percentage of sand, silt and clay was calculated and the values were plotted in ternary diagram according to Shepard (1954).

2.2.4 Biochemical analysis

The sediment samples stored at -20^{0}C were dried and homogenized for the determination of organic matter. Organic matter was determined according to El-Walkeel and Riley's Wet Oxidation Method (1957) and the results expressed as percentage organic matter.

2.2.5 Isolation of marine yeast

For the enumeration of yeasts, plating of the sediment samples were done on-board employing spread plate method. About 10 g of the sediment sample collected was suspended in 30 ml sterile seawater, vortexed and used as inoculum. 1 ml of the inoculum was spread plated on malt-yeast-glucose-peptone agar (Wickerham, 1951) supplemented with 200 mg/l chloramphenicol.

Composition of Wickerham's medium

Malt extract	-	3 g
Yeast extract	-	3 g
Peptone	-	5 g
Glucose	-	10 g
Agar	-	20g
Sea water (35 ppt)	-	1000 ml
pH	-	7
Chloramphenicol	-	200 mg/l

The plates were incubated at 18±2°C for 14 days and the colonies developed were transferred to malt extract agar slants.

2.3 Results

2.3.1 Hydrography

Arabian Sea:

In Arabian Sea (Cr. No. 228 & 233) at 200 m stations, the temperature of the sediment ranged from 14.4 to 16.1°C. Along the 500 m stations it ranged from 9.8 to 12.6°C and at 1000 m stations from 6.8 to 10.2°C. Salinity ranged from 34.9 to 35.8 ppt at 200 m stations, 35.0 to 35.6 ppt at 500 m stations and 34.9 to 35.5 ppt at 1000 m stations. The dissolved oxygen ranged from 0.95 to 2.01 ml/l at the different stations (Fig. 2.8a-c).

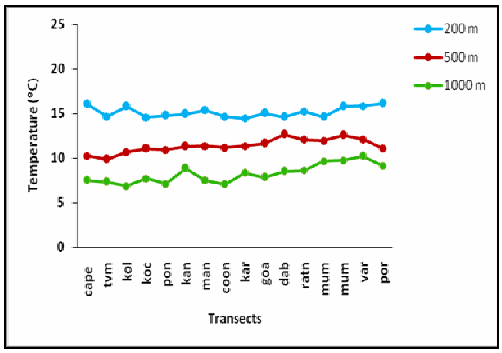


Fig. 2.8a Temperature

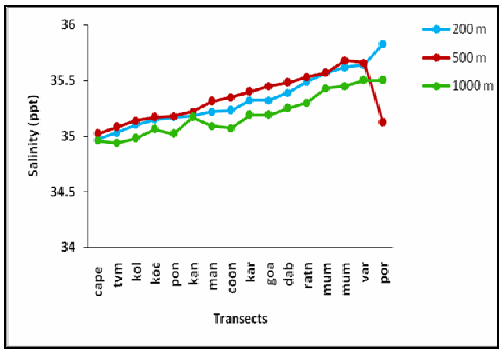


Fig. 2.8b Salinity

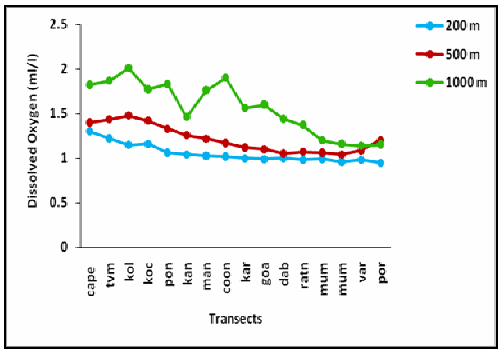


Fig. 2.8c Dissolved Oxygen

Fig. 2.8a-c Hydrographical parameters at different depths in the Arabian Sea (Cr. No. 228 & 233)

Bay of Bengal:

In Bay of Bengal (Cr. No. 236), temperature ranged from 12.1 to 22.7°C along the 200 m depth stations, from 8.6 to 11.3°C along 500 m depth zones and from 6.1 to 9.7°C at 1000 m stations. At 200 m stations the salinity ranged from 34.7 to 35.0 ppt, at 500 m it ranged from 34.9 to 35.0 ppt and along 1000 m depth zone from 33.4 to 35.0 ppt. The dissolved oxygen ranged from 0.17 to 1.34 ml/l at various stations (Fig. 2.9a-c).

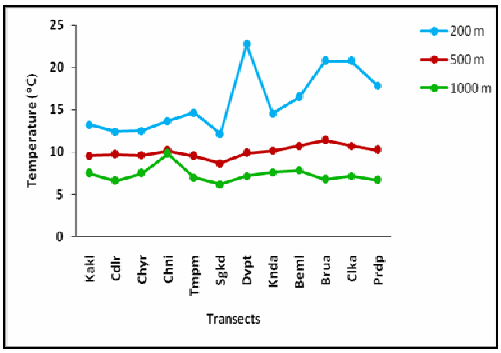


Fig. 2.9a Temperature

Fig. 2.9b Salinity

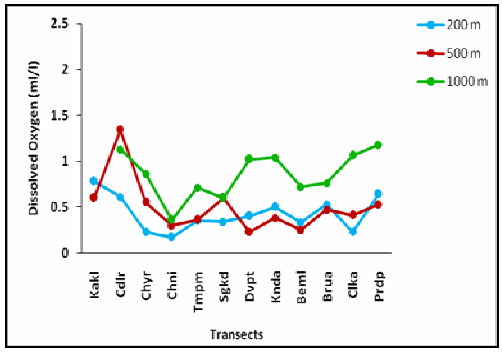


Fig. 2.9c Dissolved Oxygen

Fig. 2.9a-c Hydrographical parameters at different depths in the Bay of Bengal (Cr. No. 236)

At 200 m depths, the temperature ranged from 13.3 to 16.8°C, along 500 m depth stations it ranged from 9.5 to 10.9°C and from 7.6 to 9°C along the 1000 m depth zone. Salinity ranged from 34.8 to 35 ppt at 200 m stations, 35 to 35.02 ppt at 500 m depth zone and 34.95 to 35 ppt at 1000 m stations. The dissolved oxygen values ranged from 0.04 to 0.52 ml/l at different depth stations along Bay of Bengal (Cr. No. 245) (Fig. 2.10a-c).

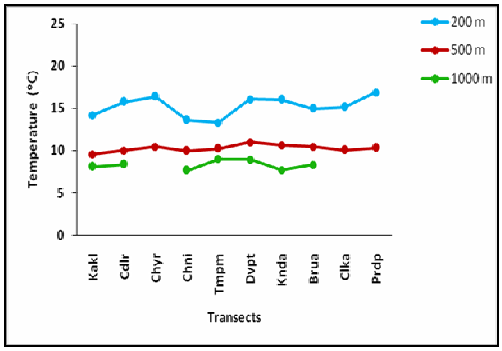


Fig. 2.10a Temperature

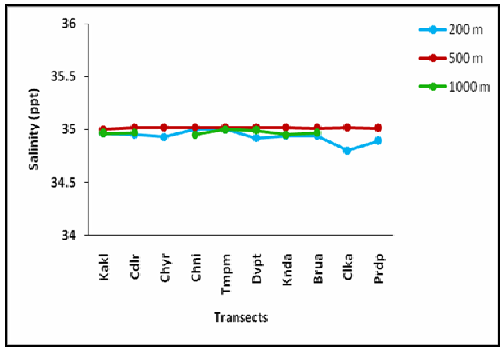


Fig. 2.10b Salinity

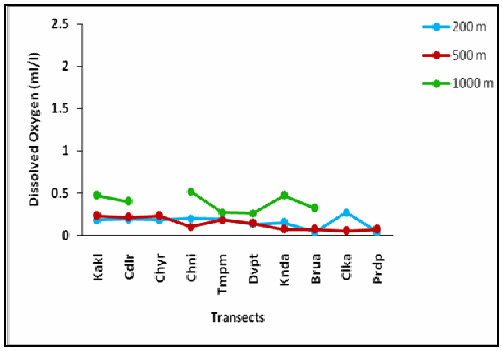


Fig. 2.10c Dissolved Oxygen

Fig. 2.10a-c Hydrographical parameters at different depths in the Bay of Bengal (Cr. No. 245)

2.3.2 Grain Size Analysis

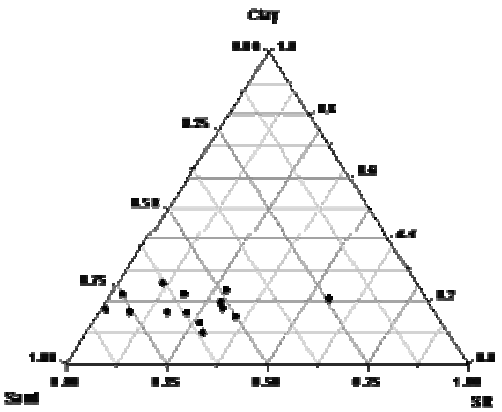
Arabian Sea:

Generally the sediment texture was sandy/clayey silt at various stations in Arabian Sea. At 200 m depth regions in Arabian Sea (Cr. No. 228 & 233), the sediment texture was sandy, except one station towards the central region of west coast (Fig. 2.11a). At 500 m depth zone both sandy and silty sediment texture was observed, but majority of the stations showed silty sediment texture (Fig. 2.11b). At 1000 m depth region, all the stations were clayey silt in texture except one showing sandy nature (Fig. 2.11c).

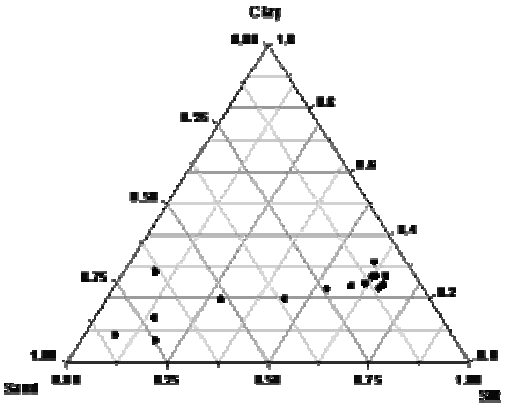
Bay of Bengal:

In Bay of Bengal the sediment texture was generally clayey silt. In Bay of Bengal (Cr. No. 236), the southern stations were sandy in texture and all others silty at 200 m depth region (Fig. 2.12a). However at 500 m depth, except station No. 49, all other stations were clayey silt /silty in nature (Fig. 2.12b). At 1000 m depth range, all the stations were clayey silt or silty (Fig. 2.12c). Generally the texture of the sediment was clayey silt or silt, except the three stations at 200 m and one station at 500 m depth regions.

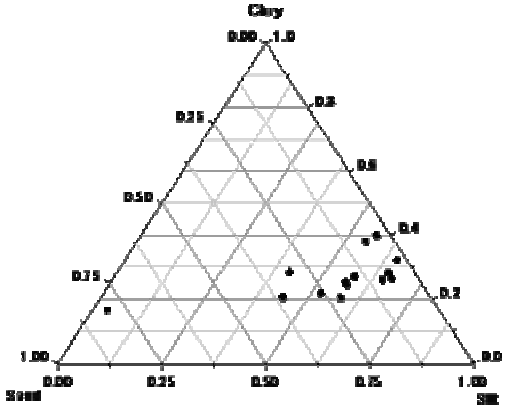
In Bay of Bengal (Cr. No. 245) the sediment was sandy in nature at two stations (Off Cuddallore and Cheyyur) and silty sand at one station (Off Karaikkal) in the southern region. Towards the northern region the proportion of sand decreased and the sediment was clayey silt or silty in nature, except for one station (Off Kakinada), which showed sand, silt and clay in almost equal proportions (Fig. 2.13a). At 500 m almost all the stations were clayey silt or silty in nature except two stations (Off Karaikkal and Off Cheyyur) which exhibited sandy silt texture (Fig. 2.13b). At 1000 m depth range, all the stations were clayey silt or silty in nature (Fig. 2.13c). Generally all the stations showed clayey silt or silty texture except three stations at 200 and 500 m depth zones.



2.11a 200 m

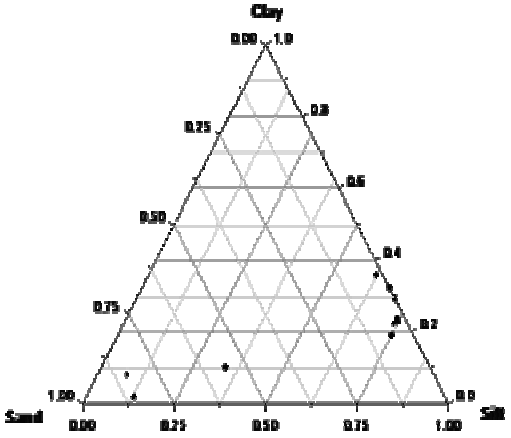


2.11b 500 m

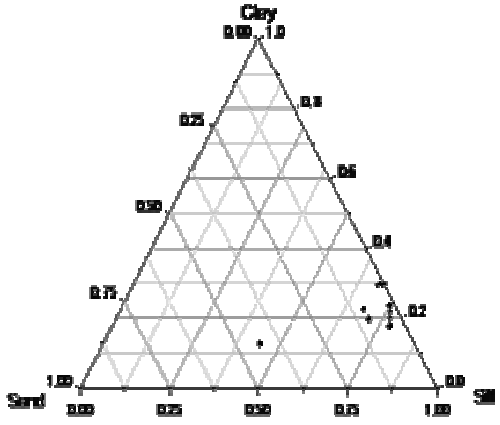


2.11c 1000 m

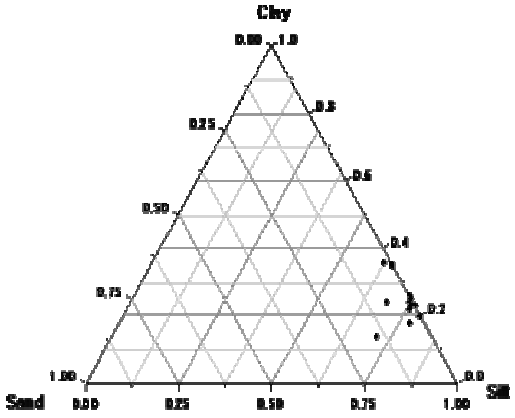
Fig. 2.11a-c Sediment texture at various depth regions in the continental slope region of Arabian Sea (Cr. No. 228 & 233) (200-1000 m depth)



2.12a 200 m

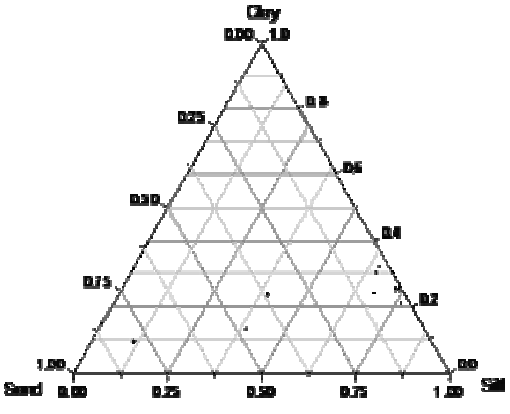


2.12b 500 m

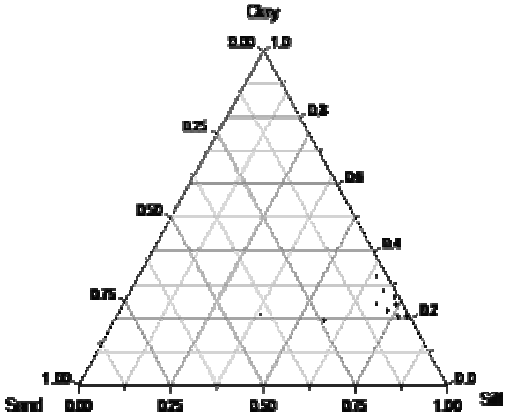


2.12c 1000 m

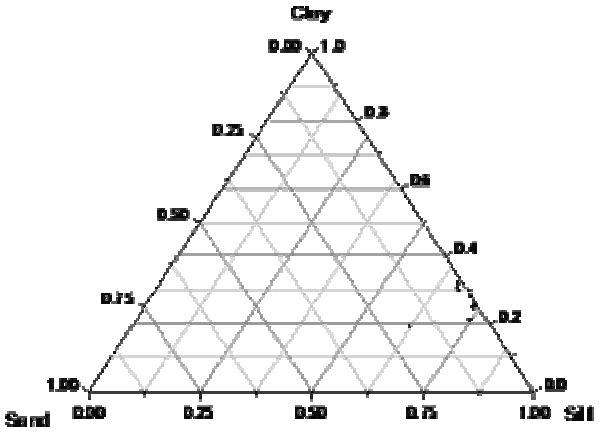
Fig. 2.12a-c Sediment texture at various depth regions in the continental slope region of Bay of Bengal (Cr. No. 236) (200-1000 m depth)



2.13a 200 m



2.13b 500 m



2.13c 1000 m

Fig. 2.13a-c Sediment texture at various depth regions in the continental slope region of Bay of Bengal (Cr. No. 245) (200-1000 m depth)

2.3.3 Biochemical analysis

Arabian Sea:

In Arabian Sea (Cr. No. 228 & 233) an increase in the organic matter content of sediment could be observed from 200 to 1000 m depth regions. Generally, the organic matter was comparatively high in the northern regions (Fig. 2.14a) at all the three depth regions with the highest being observed off Mumbai (Station No. 40) (Fig. 2.14b). Considerable amount of organic matter could be observed in almost all the stations along 1000 m depth. Lower values were observed off Porbander at 500 and 1000 m depths (Fig. 2.14c).

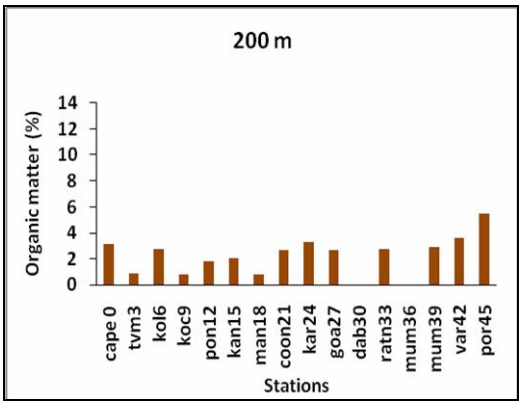


Fig. 2.14a

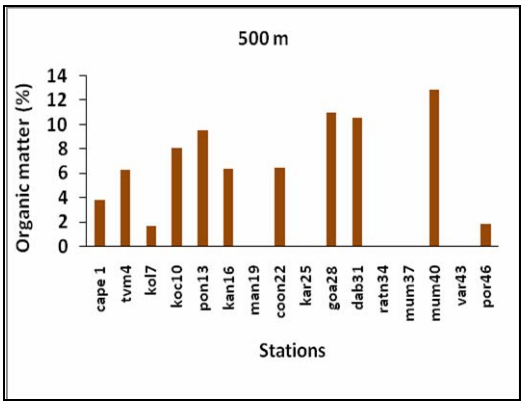


Fig. 2.14b

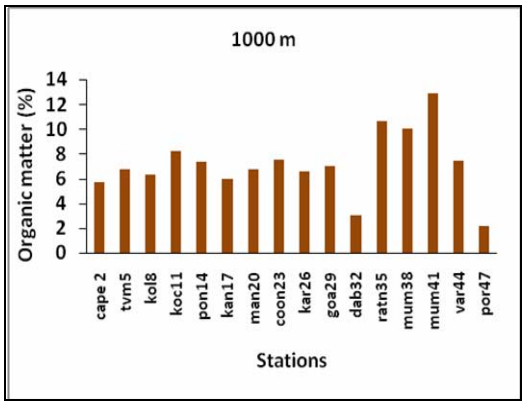


Fig. 2.14c

Fig. 2.14 a-c Percentage of organic matter in the sediment samples at various stations along the Arabian Sea (200-1000 m depth) (Cr. No. 228 & 233) *(Estimation was not done at station No. 30 and 36 due to lack of samples)*

The average values were 2.23%, 6.3% and 7.1% for 200, 500 and 1000 m depths respectively (Fig. 2.15). Stations at 200 m depths showed very low amount of organic matter compared to 500 and 1000 m stations. There was not much variation in organic matter between 500 and 1000 m stations.

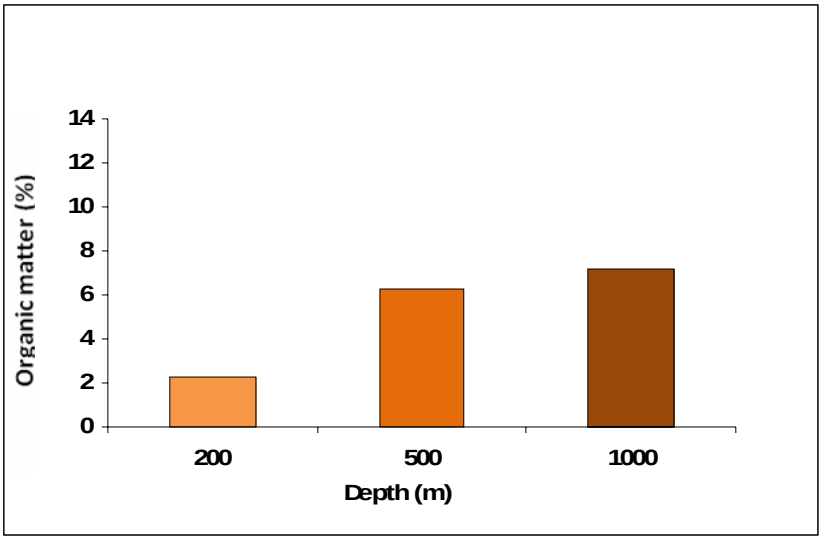


Fig. 2.15 Percentage of organic matter (average) at three depth regions in the continental slope sediments of Arabian Sea (Cr. No. 228 & 233)

Bay of Bengal:

In Bay of Bengal (Cr. No. 236) the percentage of organic matter was high along the northern region when compared to the southern region at all the three depth zones. The percentage organic matter ranged from 0.47% to 4.62% in the sediment samples at various stations. At 200 m stations a gradual increase could be seen towards the northern region except the southernmost station (Off Karaikkal) where the percentage was high (Fig. 2.16a). At 500 m depth regions there was a decrease in organic matter at the central stations when compared to the southern and northern regions (Fig. 2.16b). There was not much variation in organic matter at stations in the 1000 m depth ranges except for the slight increase in the northern stations (Fig. 2.16c). The average organic matter was more or less same at all the three depths (Fig. 2.17).

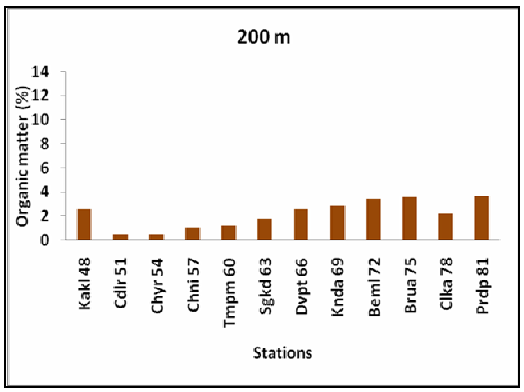


Fig. 2.16a

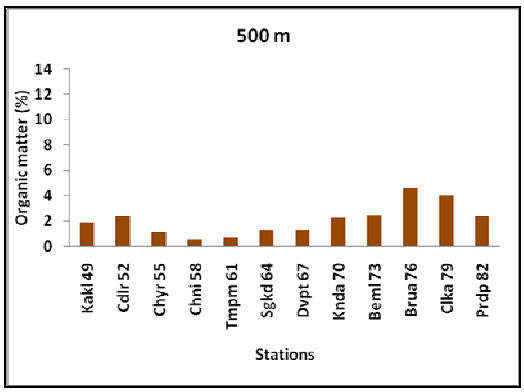


Fig. 2.16b

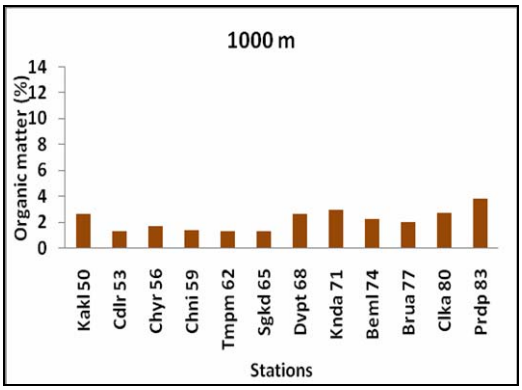


Fig. 2.16c

Fig. 2.16 a-c Percentage of organic matter in the sediment samples at various stations along the Bay of Bengal (200-1000 m depth) (Cr. No. 236)

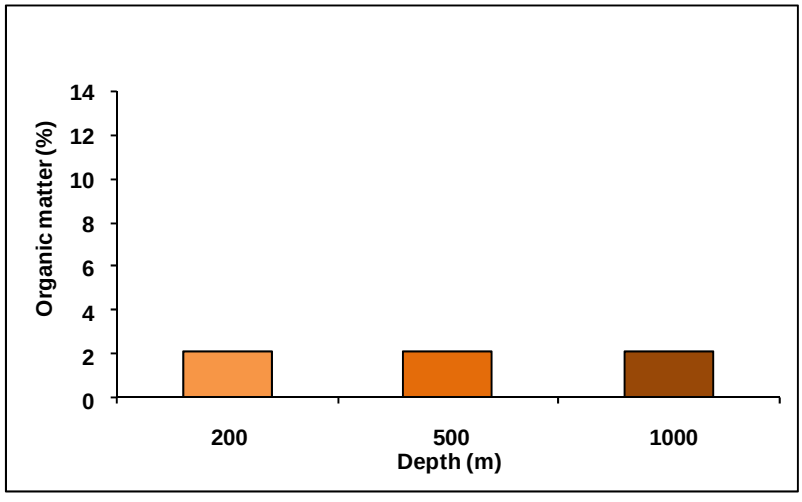


Fig. 2.17 Percentage of organic matter (average) at three depth regions in the continental slope sediments of Bay of Bengal (Cr. No. 236)

In the slope of Bay of Bengal (Cr. No. 245), the organic matter ranged from 1.24 to 4.33%. The organic matter content was higher at 500 and 1000 m compared to 200 m depth regions (Fig. 2.18a-c). There was not much variation in organic matter between the three depths and also between the various stations in the study area (Fig. 2.19).

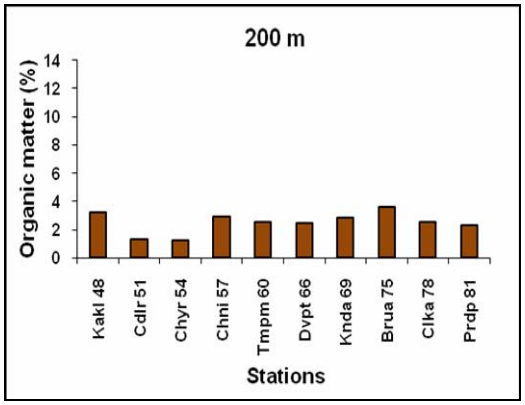


Fig. 2.18a

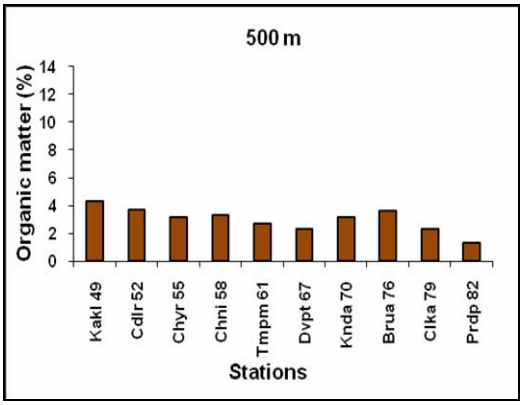


Fig. 2.18b

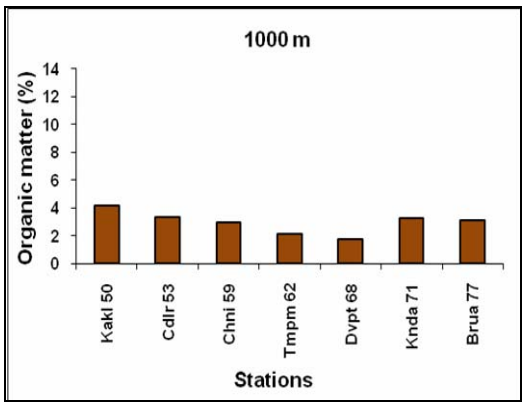


Fig. 2.18c

Fig. 2.18a-c Percentage of organic matter in the sediment samples at various stations along the Bay of Bengal (200-1000 m depth) (Cr. No. 245)

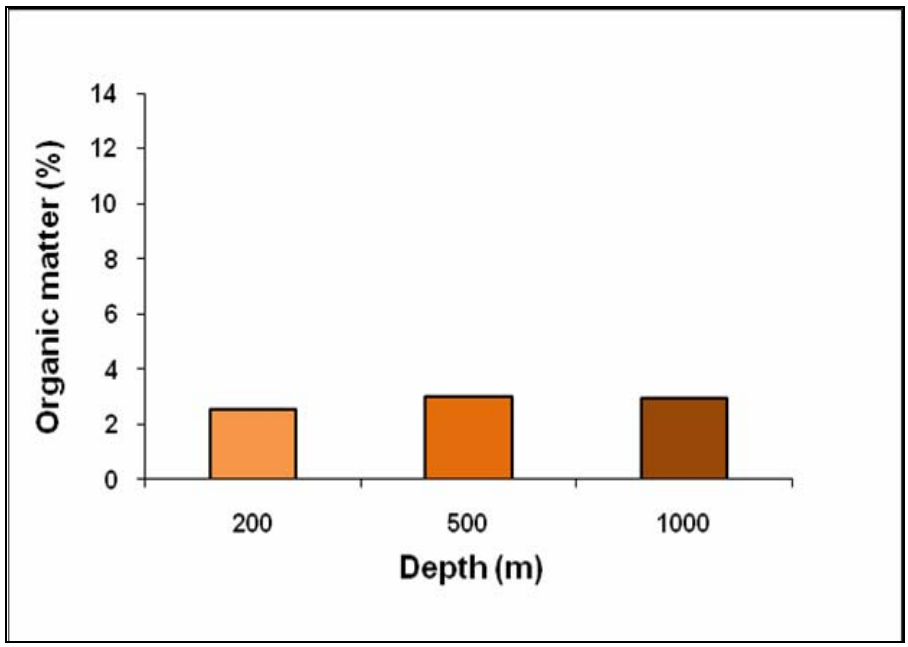


Fig. 2.19 Percentage of organic matter at three depth regions in the continental slope of Bay of Bengal (Cr. No. 245)

2.3.4 Microbiological analysis

Arabian Sea:

In Arabian Sea (Cr. No. 228 & 233), yeast population was found to be maximum at 500 m depth regions (92.8 cfu/g dry weight of sediment) followed by 1000 m (35.2 cfu/g dry weight of sediment) and 200 m (12.5 cfu/g dry weight of sediment) depth regions. At 200 m the population ranged from 0.35 to 120 cfu/g dry weight of sediment and the maximum was found off Dabhol (Fig. 2.20a). At 500 m depths the northern transects (Off Coondapore to Porbander) showed comparatively higher population. The population ranged from 23 cfu/g dry weight (Off Coondapore) to 556 cfu/g dry weight of sediment (Off Mumbai) (Fig. 2.20b). At 1000 m depth regions considerable yeast population could be observed off Thiruvananthapuram (68 cfu/g dry weight of sediment) to Ponnani (59 cfu/g dry weight of sediment) and off Dhabhol (3 cfu/g dry weight of sediment) to Porbander (5 cfu/g dry weight of sediment) with a maximum off Mumbai (91 cfu/g dry

weight of sediment). However the population was negligible in the middle transects (Off Kannur to Ratnagiri) (Fig. 2.20c). The average population was higher at 500 m depth followed by 1000 m and 200 m (Fig. 2.21).

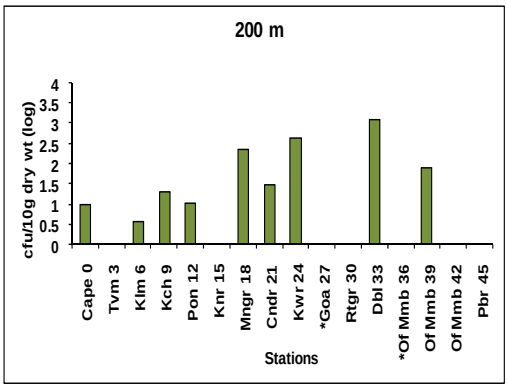


Fig. 2.20a

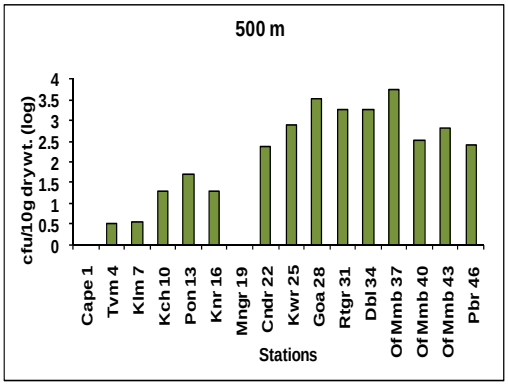


Fig. 2.20b

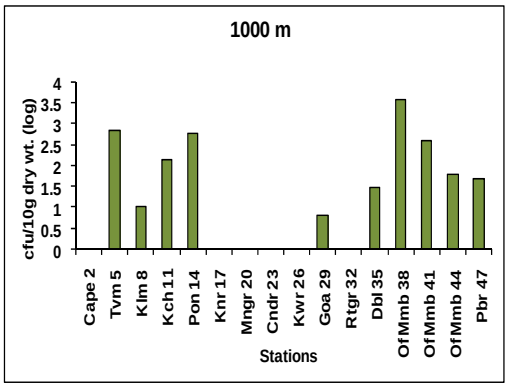


Fig. 2.20c

Fig. 2.20a-c Culturable yeast population at different stations in the continental slope region of Arabian Sea (200-1000 m depth) (Cr. No. 228 & 233) *(Sample collection not done at stations 30 and 36 due to adverse weather)*

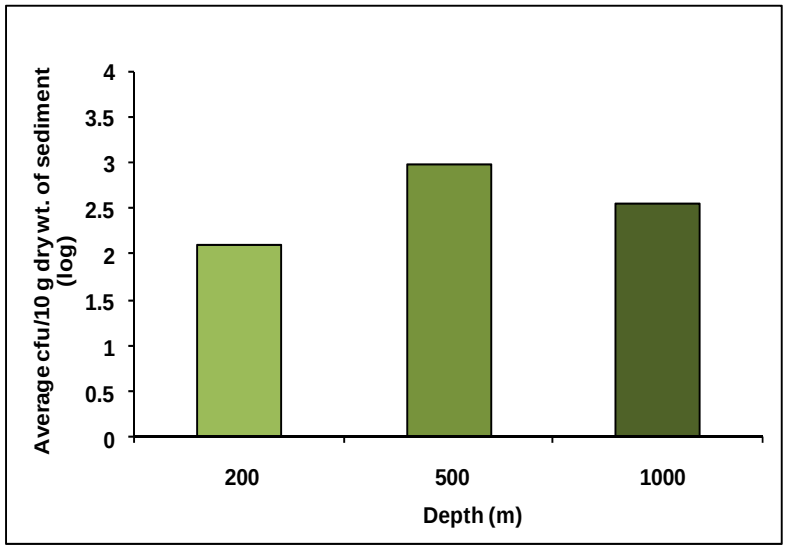


Fig. 2.21 Culturable yeast population (average) at three depth regions in the continental slope sediments of Arabian Sea (Cr. No. 228 & 233)

Bay of Bengal:

The yeast population in Bay of Bengal (Cr. No. 236) ranged from 0.60 cfu/g dry weight (Off Cuddallore) to 46.6 cfu/g dry weight of the sediment (Off Barua) at 200 m (Fig. 2.22a). At 500 m depth regions, the population ranged from 0.19 cfu/g dry weight (Off Thammerapattanam) to 53.09 cfu/g dry weight of the sediment (Off Divi Point) (Fig. 2.22b) and at 1000 m the population ranged from 0.2 cfu/g dry weight (Off Thammerapattanam) to 59.3 cfu/g dry weight of the sediment (Off Bheemuli) (Fig. 2.22c). Comparatively high population was observed off Singarayakonda (40.3 cfu/g dry weight of sediment), Barua (46.6 cfu/g dry weight of sediment) and Paradip (7.86 cfu/g dry weight of sediment) at 200 m depth regions and off Cheyyur (21.8 cfu/g dry weight of sediment) and Divi Point (53.09 cfu/g dry weight of sediment) at 500 m depth regions. At 1000 m the sample collected off Bheemuli exhibited considerably high yeast population (59.3 cfu/g dry weight of sediment). The population was very less or nil off Karaikkal and Thammerapattanam in the estimated sample size.

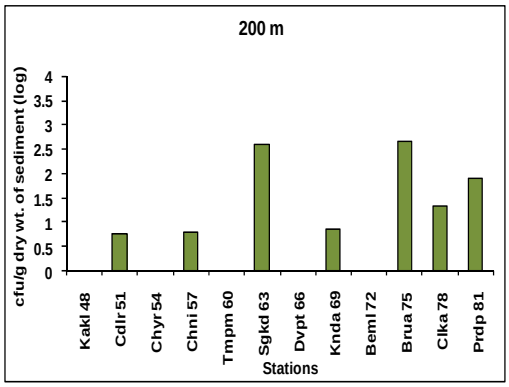


Fig. 2.22a

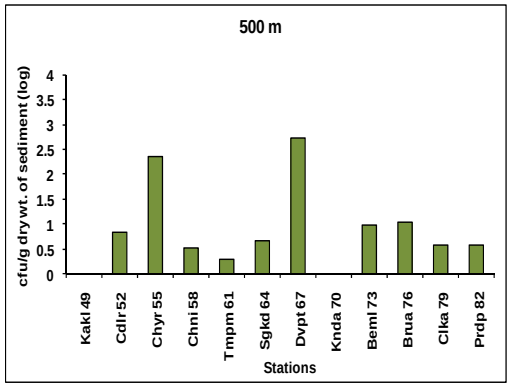


Fig. 2.22b

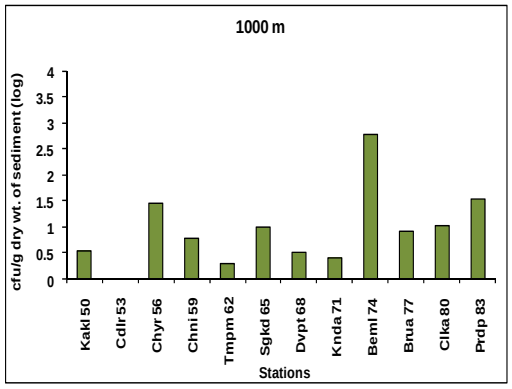


Fig. 2.22c

Fig. 2.22a-c Culturable yeast population at different stations in the continental slope sediments of Bay of Bengal (200-1000 m depth) (Cr. No. 236)

Yeast population was maximum at 200 m depth regions followed by 500 and 1000 m depth regions (Fig. 2.23). There was not much variation in average population between the various depth regions.

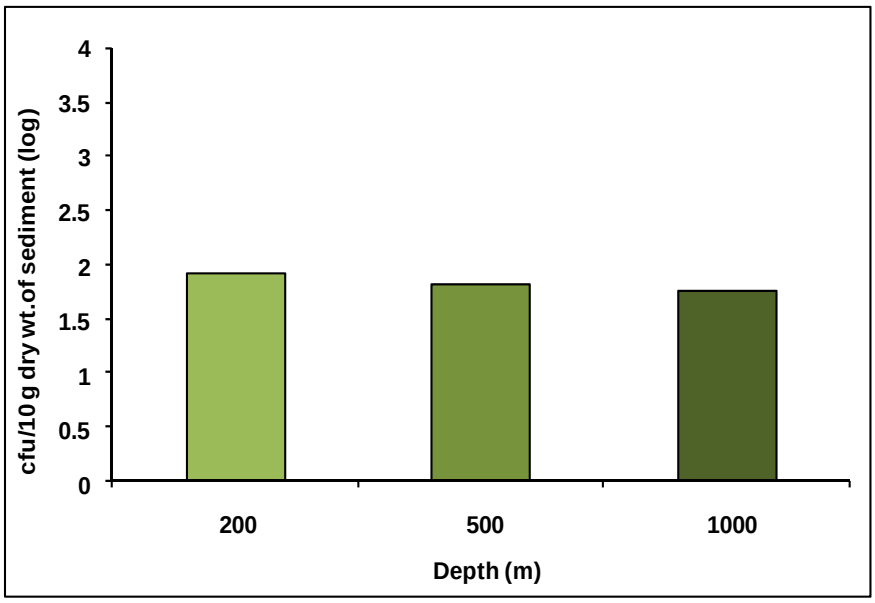


Fig. 2.23 Culturable yeast population (average) at three depth regions in the continental slope of Bay of Bengal (Cr. No. 236)

Total population of yeasts in Bay of Bengal (Cr. No. 245) ranged from 0.18 cfu/g dry weight (Off Cuddallore) to 49.31 cfu/g dry weight of sediment (Off Kakinada). At 200 m, the population ranged from 0.18 cfu/g dry weight (Off Cuddallore) to 7.82 cfu/g dry weight of sediment (Off Cheyyur) (Fig. 2.24a). The population ranged from 0.76 cfu/g dry weight (Off Cheyyur) to 49.31 cfu/g dry weight of sediment at 500 m (Off Kakinada) (Fig. 2.24b) and 0.65 cfu/g dry weight (Off Kakinada) to 1.61 cfu/g dry weight of the sediment (Off Karaikkal and Chennai) at 1000 m (Fig. 2.24c). Generally the yeast population was scanty in the northern transects.

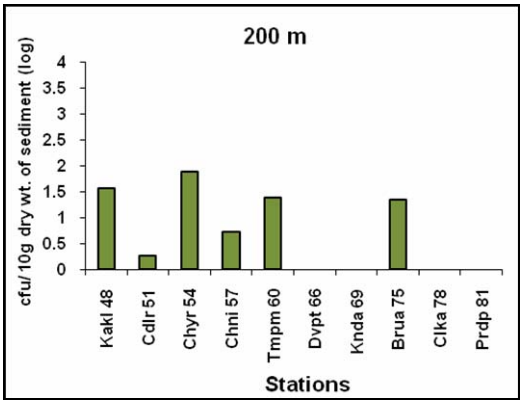


Fig. 2.24a

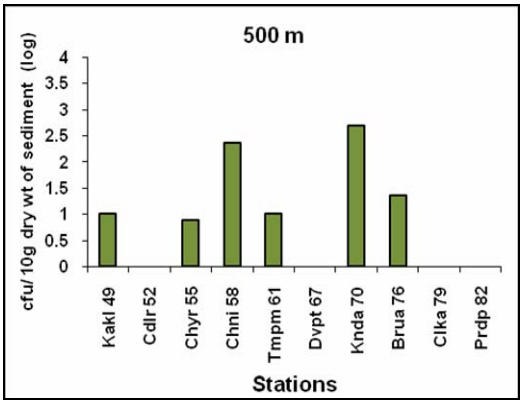


Fig. 2.24b

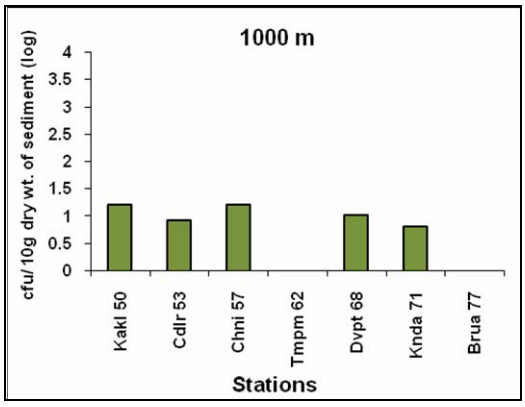


Fig. 2.24c

Fig. 2.24a-c Culturable yeast population at different stations in the continental slope region of Bay of Bengal (200-1000 m depth) (Cr. No. 245)

Yeast population varied considerably at different depth ranges and the population was maximum at 500 m depth followed by 200 and 1000 m depths (Fig. 2.25). Wickerham's agar plate with sediment spread on it, showing yeast colonies is given in fig. 2.26.

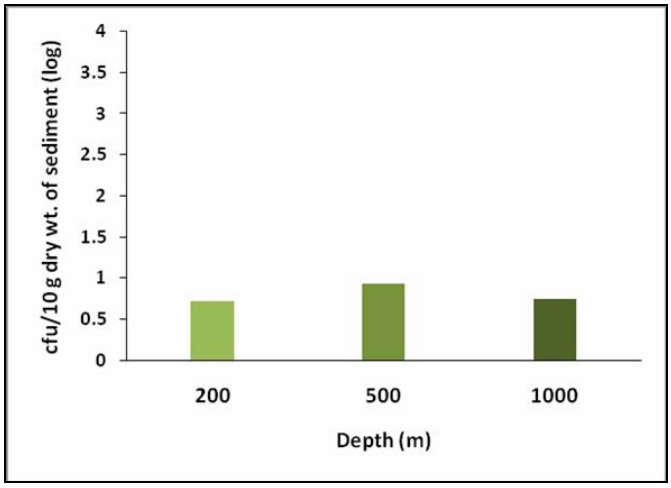


Fig. 2.25 Culturable yeast population (average) at three depth regions in the continental slope of Bay of Bengal (Cr. No. 245)

Table 2.3 Correlation of yeast population with organic matter in Arabian Sea and Bay of Bengal

Cruises	**Correlation coefficient (r value) Population with organic matter**
Arabian Sea (228 & 233)	0.076
Bay of Bengal (236)	0.019
Bay of Bengal (245)	0.111

The correlation is not significant at 0.05 level.

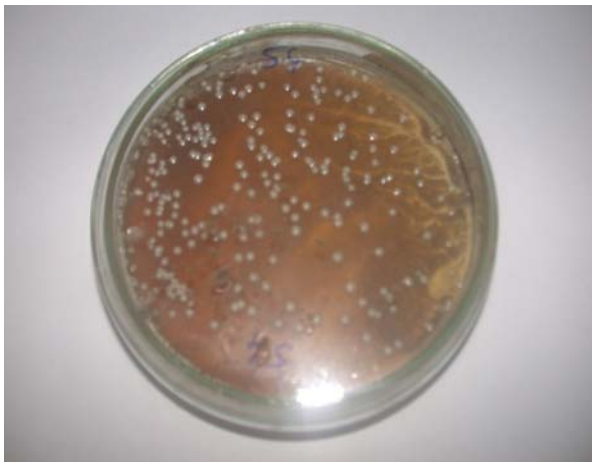


Fig. 2.26 Yeast colonies on Wickerham's agar plate

2.4 Discussion

The present study helped to generate a base line data of the culturable marine yeasts inhabiting 200, 500 and 1000 m depth zones in the slope sediments of Arabian Sea and Bay of Bengal. Wickerham's medium with spread plate technique was found to be fine for yeast isolation. At least 10 g sediment (wet weight) sample was to be used in order to get sufficient number for the enumeration and isolation of yeasts from the slope region. Suitable temperature of incubation was found to be 18-20ºC to retrieve the marine yeasts from the slope sediment samples curtailing the growth of fungi. The faunal- sediment relationships are fundamental for their distribution and the grain size of sediment largely determines the faunal diversity and abundance (Etter and Grassle, 1992). Even though the depth wise variation in temperature was remarkable, the spatial variation was negligible. However in the case of salinity a slight increase could be observed towards the northern region concomitant with a reduction in dissolved oxygen. This is due to the intrusion of high saline Red Sea water into the northern region of the west coast of India.

The unique features of the North Indian Ocean are caused by the diverse conditions prevailing in the area which include immense river runoff in the northeast (Bay of Bengal) and a large excess of evaporation over precipitation and run off in the northwest region (Arabian Sea, Persian Gulf and Red Sea), resulting in the formation of several low- and high salinity water masses. The occurrence of coastal upwelling seasonally makes the region highly fertile, and the existence of Asian landmass, forming the northern boundary, prevents quick renewal of subsurface layers. Consequently, dissolved oxygen gets severely depleted below the thermocline and reducing conditions prevail at intermediate depths (ca. 150-1200 m). Higher nutrients and lower oxygen concentrations occur in the bottom layer as compared to the overlying water column in deep waters of the Bay of Bengal and Arabian Sea, suggesting that considerable

quantities of organic matter reach the deep sea floor and get oxidized in the bottom layer (Gupta and Naqvi, 1984).

Sediment texture in the west coast was of mixed nature. At 200 m, the texture was sandy and the organic matter was comparatively lesser than the 500 and 1000 m depth zones. There was no distinct latitudinal variation in the amount of organic matter between the stations at 200 m depth. At 500 m stations the texture was sandy/silty and the organic matter was more in the central regions. At 1000 m depth, the sediment was clayey silt with more organic matter. Maximum amount of organic matter was observed off Mumbai at 500 and 1000 m depth stations. Also there was a steady increase in organic matter from 200 to 1000 m depth regions. Yeast population was comparatively less in 200 m stations, may be due to the sandy texture and low organic matter in that area. At 500 m stations, the population was comparatively higher than the 200 and 1000 m stations. The population was higher in the central and northern stations compared to the southern region. There were only two stations (Station No. 1 and 19), where yeast population was not detected at 500 m depth zone. At 1000 m depth region, yeast population was observed in the southern and northern stations, but absent or very less in central stations. Maximum population was obtained along off Mumbai region, at 500 and 1000 m depths, where the organic matter was also high. The number and distribution of yeasts were dependent on concentration and type of organic material present in the area (Fell, 2001). The absence of yeast population in the central region at 1000 m depth zone may be due to the presence of recalcitrant compounds not utilizable by the organisms.

In the Bay of Bengal, the yeast population was scanty in the southern stations where the sediment texture was sandy. The sediment texture and organic matter largely influenced yeast distribution. The average organic matter content of sediment was almost similar at all the three depths. A similar trend could be observed in the case of yeast population. The quality of organic matter is more important than quantity, and is related to faunal distribution and abundance

(Grémare et al., 2002). The sediment was generally soft and sticky in nature. The silty/clayey silt texture of the sediment and higher organic matter supported more yeast population in the northern stations. The transportation and accumulation of recalcitrant materials at higher depths might be the reason for high organic matter in the 1000 m depth region.

During cruise No. 245, sampling was not possible from all the stations due to rough weather conditions. Sediment texture was clayey silt in almost all the stations except a few along the 200 m depth zones. Population was found to be maximum at 500 m stations.

Generally the yeast population was higher in the sediment at 500 m depth regions compared to 200 and 1000 m depth, the reason for which could not be elucidated through this study. A progressive increase in organic matter could be observed with the increase in depth, in the slope region which might be due to the accumulation of refractory materials. Lack of a concomitant increase in yeast population may perhaps be due to the less bioavailability of organic carbon in the area. Generally the physicochemical factors did not seem to have any correlation with the yeast population in the area.

The sedimentary organic carbon maximum in the continental slope off western India is widely believed to be due to the preferential preservation of deposited organic matter at water depths where the intense oxygen minimum transects the sea floor. This region is considered to constitute one of the modern analogues for the environment of formation of rich sedimentary facies that are common in the geological record. The oxygen minimum in the eastern Arabian Sea is the site of enhanced organic matter accumulation and preservation. Organic carbon and nitrogen reach maximum concentrations between 200 and 1600 m depth, where as the lowest dissolved oxygen contents in the oxygen minimum lie between 200 and 800 m depth (Calvert et al., 1995). In the present study an increase in organic matter could be observed at 500 and 1000 m depth

region in Arabian Sea which might be due to the fact that this area come under the oxygen minimum zone. However, in Bay of Bengal the organic matter remained more or less same at various depths studied.

Sedimentary organic carbon concentrations reach a maximum on the upper continental slope off western India. Paropkari et al. (1992) have carried out an evaluation of the factors controlling the organic carbon content of the sediments of the Arabian Sea. They have concluded that bottom water anoxia in conjunction with a number of depositional parameters, such as sediment texture, sedimentation rates, the width of the shelf, the slope gradient, bottom currents and the adsorption capacity of clay minerals, are the most important factors determining the degree of preservation of sedimentary organic carbon in this area, and that the role of carbon supply (via productivity) is of secondary importance (Calvert et al., 1995).

Due to their small size and rapid turn over rates, micro and meio-benthic organisms respond promptly to changes in environmental constraints (Vezzulli et al., 2002; Boyd et al., 2000). The microbenthic loop encompasses organic matter, bacteria, microphytobenthos, protozoa and meiofauna and the state of its structure and dynamics has been recently proposed as a sensitive indicator of change occurring in the marine ecosystem (Lee et al., 2001; Dell' Anno et al., 2002).

Even though a positive correlation could be noted between yeast population and organic carbon, it was not significant. This may be due to the fact that we have estimated only a small portion of the microbial community i.e. yeasts and not accounted for other microbes including non-culturable forms, in this study. Also that numerous yeast cells may be attached to organic or inorganic particles and together will produce a single colony (Kriss, 1959). A comprehensive study taking into account other group of microbes including the non-culturable forms can only give a definite picture about the population dynamics.

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Chapter 3

ISOLATION AND CHARACTERIZATION OF YEASTS FROM THE SLOPE SEDIMENTS OF ARABIAN SEA AND BAY OF BENGAL

Contents

3.1 Introduction

Marine yeasts are considered to be an important category of marine microorganisms. Kohlmeyer and Kohlmeyer (1979) isolated yeasts from seawater, sediment, plants, animals and other organic matter in the marine habitat and divided them into two groups - 'obligate' and 'facultative'. "Obligate marine" yeasts are those that have never been collected from anywhere else but the marine environment, whereas, "facultative marine" yeasts are also known from terrestrial habitats. "Obligate marine" species may be confined to marine habitats, especially if they have been collected frequently and exclusively from the sea for several years.

Kohlmeyer and Kohlmeyer (1979) have compiled 177 species of yeasts, which were isolated from water, sediment, algae, animals and other organic matter in the marine habitat. Of these, only 26 species were regarded as obligate marine forms. The most important genera of true marine yeast are *Metchnikowia, Kluyveromyces, Rhodosporidium, Candida, Cryptococcus, Rhodotorula* and *Torulopsis.* These studies established that marine yeasts do not belong to a specific genus or group, but are distributed among a wide variety of well known genera such as *Candida, Cryptococcus, Debaryomyces, Pichia, Hansenula, Rhodotorula, Saccharomyces, Trichosporon and Torulopsis*. The isolation frequency of yeasts fall with depth and it was found that yeasts in the class Ascomycetes (eg. *Candida, Debaryomyces, Kluyveromyces, Pichia* and *Saccharomyces*) are common in shallow waters, whilst yeasts belonging to Basidiomycetes (*Cryptococcus, Rhodosporidium, Rhodotorula, Sporobolomyces*) are common in deep waters. For example *Rhodotorula* has been isolated from a depth of 11,000 m (Munn, 2004).

3.1.1 Isolation and cultivation of marine yeasts

Kriss (1959) found that the number of yeasts estimated by direct microscopic observation was higher than those obtained by plate count. This disparity can partly

be explained by the presence of non-viable and non-cultivable yeast cells. Another possible explanation is that numerous yeast cells may be attached to organic or inorganic particles and together will produce a single colony. Traditional methods of yeast isolation have specific limitations. The culture media and environmental growth conditions (particularly temperature) are selective, rapid growing strains will overgrow slower growing species and consequently rare species may not be represented. Also cell numbers obtained with plate cultivation techniques do not reflect factors such as turnover rates, hyphal fragmentation, spore release or rate of consumption by various invertebrates.

A variety of media and incubation conditions have been designed and employed by researchers. For water sampling, nitrocellulose filters, of diameter 47 mm and pore size 0.45 µm, are employed in an autoclavable glass or plastic filter apparatus. The filter is placed face up on a nutrient agar medium. A widely used medium is Wickerham's yeast malt medium that contains 0.3 g yeast extract, 0.3 g malt extract, 0.5 g peptone, 1 g glucose and 2 g agar prepared in 100 ml sea water at a salinity matching to that of the sampling site. Prior to autoclaving, chloramphenicol (200 mg/l) is added to the medium to inhibit bacteria. Alternatively, a mixture of antibiotics, penicillin G and streptomycin sulphate (each at 150-500 mg/l) is added dry to autoclaved and cooled (45^{o}C) medium. Sediment particles can either be placed directly on an agar medium, or known quantities of it can be added to a test tube containing a given volume of sterile sea water, vortexed and diluted 1:10 in sterile sea water series followed by preparation of standard spread plates from each of the dilution series. Suspected yeast colonies are picked and transferred to a microscopic slide for inspection. After confirmation they are transferred from the isolation medium to a growth medium (YM Sea water agar lacking antibiotics).

Selective media suitable for *Candida* species are chloramphenicol malt agar and chloramphenicol cycloheximide malt agar. Some *Candida* species grow in the presence of cycloheximide while most other species do not. So it has been used as a

differential medium for *Candida* species (Collins and Patricia, 1970). Broad-spectrum antibiotics are more effective in preventing bacterial growth and less harmful to yeast cells (Mossel et al., 1962; Flannigan, 1974; Beuchat, 1979; Thompson, 1984). Various compounds have been added to media to inhibit the growth of moulds, including rose bengal (Jarvis, 1973; King et al., 1979), dichloran (Jarvis, 1973) and propionate (Bowen and Beech, 1967). Oxytetracycline glucose yeast extract agar (OGYE) has been recommended for the selective isolation and enumeration of yeasts and moulds from food stuffs (Mossel et al., 1970). It was concluded that Rose Bengal Chloramphenicol agar is the medium of choice for samples heavily contaminated with moulds. Woods (1982) used various media, containing antibiotics, for the enumeration of yeasts and moulds in foods and worked out their comparative efficacy. The ability of media to suppress bacterial growth and to prevent excessive growth of fungal colonies was the two main factors considered. Where the main concern is enumeration of yeasts, malt extract agar containing oxytetracycline is recommended. Yeasts are usually maintained on slopes of malt extract agar while those of certain genera such as *Bensingtonia, Bullera, Cryptococcus, Leucosporidium, Rhodosporidium, Rhodotorula* and *Sporobolomyces*, generally survive longer on potato dextrose agar.

Plates are incubated at temperatures designed to simulate ambient environmental conditions. For example, polar and deep-sea samples should be incubated at ~5°C. Temperature required for temperate and tropical samples often result in overgrowth by filamentous fungi, which can be reduced by incubation at temperatures ~12°C (Fell, 2001). For taxonomic tests, yeasts are usually incubated at 25ºC (Buhagiar and Barnett, 1971), although optimum temperatures for growth are higher for some yeast and lower for others (Watson, 1987).

3.1.2 Identification

Yeasts were classified on the basis of their morphology and biochemical characteristics. The workers of the Dutch school were responsible for much of

the pioneering work on the classification of yeast species up to year 1950. These workers classified all the yeasts available to them on the basis of cellular morphology, spore shape/number and nature of conjugation process. At species level, they were classified based on the ability to ferment/assimilate 6 sugars, ability to use ethanol and nitrate and to hydrolyze arbutin. As judged by these criteria, the distinction between some species was rather fine.

Around the same time, Wickerham and Burton (1948) and Wickerham (1951) introduced a number of refinements to the Dutch system, especially the use of a much larger number of carbon compounds. These included additional hexoses, di-, tri-, and tetrasaccharides, 2 polysaccharides and a number of pentoses, polyhydric alcohols and organic acids. They also introduced tests for vitamin requirements.

The widely accepted practice is to use approximately 30 carbon compounds and to test for fermentation of at least 11 of these including inulin (Barnett et al., 1990). The ability to use nitrite as well as nitrate at depressed temperature and on media of high sugar or salt content is also noted. The type and number of additional reactions tested vary with the interests and preferences of the individual investigator.

Difficulties both major and minor accompany the use of these methods. One is a question of the stability of the biochemical criteria. For e.g. *Candida* and *Torulopsis* were separated solely on the ability of the former to produce pseudo hyphae until it was observed that the same species might produce two or more forms simultaneously or at different stages of growth. It has now become evident that different strains of the same species may differ in their ability to produce pseudo mycelium and the value of this criterion in distinguishing the two genera has approached vanishing point. Another obstacle encountered by an investigator is the instability of physiological characters. Scheda and Yarrow (1966) observed enough variability in the fermentation and carbon assimilation patterns of a number of *Saccharomyces* sp. causing difficulties in the assignment

of these yeast strains to different species. Yet another problem lies in the relationship of the biochemical tests to metabolism of the organisms. Formerly, it was not sufficiently appreciated that the various carbon compounds are not necessarily assimilated independently but may be metabolized by common pathways. Thus yeasts, which can use a particular compound can also use a structurally related one by the same metabolic pathway, Barnett (1968) noted that there was a small percentage of yeasts that were exceptions to this rule. In general the conclusions were valid, that the effective number of criteria for the number of substrates reduced distinguishing yeast species metabolized by such linked mechanisms. The metabolism of most or all of the compounds used involves a few distinct central pathways and depends on the ability of the cells to convert the substrates into intermediary metabolites of one of these pathways.

As per Barnett et al. (1990) the main characteristics used to classify yeasts are as follows

Microscopical appearance:

Taxonomists examine yeast cells microscopically and consider their size and shape, how they reproduce vegetatively (by multipolar, bipolar or unipolar filaments) and the form, structure and mode of formation of ascospores and teliospores.

Sexual reproduction:

Some yeasts reproduce sexually by ascospores, others by teliospores and yet others by basidiospores. For ascosporogenous yeasts, taxonomic significance is given to whether asci are formed from a) vegetative cells b) two conjugating cells or c) a mother cell, which has conjugated with its bud. For yeasts with asci borne on filaments, the arrangement of asci - whether in chains or bunches - may be used to distinguish between genera.The number of ascospores in each ascus, their shape and whether the ascospore walls are smooth or rough are relevant factors used in classification.

Physiological features:

Physiological factors used for classifying yeasts are chiefly their ability to:

a) Ferment sugars anaerobically

b) Grow aerobically with various compounds such as a sole source of carbon or nitrogen

c) Grow without an exogenous supply of vitamins

d) Grow at 37ºC

e) Grow in the presence of cycloheximide

f) Split fat

g) Produce starch like substances

h) Hydrolyze urea

i) Form citric acid.

Biochemical characteristics:

Studies of certain biochemical characters may influence taxonomic decisions. For e.g. the chemical structure of cell walls (Phaff, 1971), particularly the cell wall mannans (Gorin and Spencer, 1970; Ballou, 1974) and the kind of ubiquinone (coenzyme Q) present in different yeasts.

3.1.3 Hydrolytic enzyme production

Marine yeasts are reported to be truly versatile agents of biodegradation (De souza and D'souza, 1979; Kobatake et al., 1992). They participate in a range of ecologically significant processes in the sea, especially in estuarine and near-shore localities. These activities include decomposition of plant substrates, nutrient-recycling phenomena and biodegradation of oil and recalcitrant compounds. Biomass data and repeated observations of microhabitat colonization by various marine yeasts support ancillary lab evidence for the contribution of this segment of the marine mycota to productivity and transformation activities in the sea (Meyers et al., 1975).

Yeast enzymes were found to be useful in various industrial processes which emphasize their direct contribution to our day to day life. These enzymes are produced mostly extracellular by different metabolic reactions taking place inside the cell and participate in various transformation activities like mineralization of organic compounds. Studies by Paskevicus (2001) showed that almost all the yeast strains produce lipase enzyme. The most active lipase producers belonged to the genera *Rhodotorula, Candida, Pichia* and *Geotrichum*. Lipases catalyse a wide range of reactions like hydrolysis, esterification, alcoholysis, acidolysis, aminolysis etc. (Hasan et al., 2006). Lipases are mainly involved in detergent industry and biodegradation, especially oil residues. Wang et al. (2007) isolated a total of 427 strains from different marine substrates, and their lipase activity was estimated. They found that nine yeast strains obtained in this study when grown in a medium with olive oil could produce lipase. The optimal pH and temperature of the lipases produced by them were between 6.0-8.5 and 35-40ºC respectively. Some lipases from the yeast strains could actively hydrolyse different oils, indicating that they may have potential applications in industry.

A protease producing strain isolated from the sediments of saltern near Qingdao, China, had the highest activity at pH 9 and 45ºC (Chi et al., 2007). This principal enzyme, protease, has many applications in detergent, leather processing and feed industry besides waste treatment (Ni et al., 2008). Yeast amylases have many applications in bread and baking industry, starch liquefaction and saccharification, paper industry, detergent industry, medical and clinical analysis, food and pharmaceutical industries (Chi et al., 2003; Gupta et al., 2003). Amylolytic yeasts convert starchy biomass to single cell protein and ethanol (Li et al., 2006).

Cellulases have application in stone washing, detergent additives, production of SCP, biofuels and waste treatment (Zhang and Chi, 2007). The enzyme inulinase produce fuel ethanol, high fructose syrup and inulo oligosaccharides (Pandey et al., 1999). Sheng et al. (2007) isolated a marine yeast strain *Cryptococcus aureus* G7a from China South Sea sediment which

was found to secrete a large amount of inulinase into the medium. The crude inulinase produced by this marine yeast showed the highest activity at pH 5.0 and 50^{o}C. The enzyme phytase is a component of commercial poultry, swine and fish diets and animal/human nutrition (Haefner et al., 2005).

In a review article by Chi et al. (2009), the extracellular enzyme production, their properties and cloning of the genes encoding the enzymes from marine yeasts are overviewed. The extracellular enzymes include cellulose, alkaline protease, aspartic protease, amylase, inulinase, lipase, phytase and killer toxin. It was found that some properties of the enzymes from the marine yeasts are unique than that of the enzymes from terrestrial yeasts.

3.2 Materials and Methods

3.2.1 Isolation of marine yeast

For the isolation of yeasts plating of the sediment samples were done on-board employing spread plate method. About 10 g of the sediment sample collected was suspended in 30 ml sterile seawater, vortexed and used as inoculum. 1 ml of the inoculum was spread plated on malt-yeast-glucose-peptone agar (Wickerham, 1951) supplemented with 200 mg/l chloramphenicol.

Medium used for isolation (Wickerham's agar):

Malt extract	-	3 g
Yeast extract	-	3 g
Peptone	-	5 g
Glucose	-	10 g
Agar	-	20g
Sea water (35 ppt)	-	1000 ml
pH	-	7
Chloramphenicol	-	200 mg/l

The plates were incubated at 18±2°C for 14 days. The colonies developed were purified by quadrant streaking and transferred to malt extract agar slants for further studies.

Malt Extract Agar

Malt extract	-	15 g
Peptone	-	5 g
Agar	-	20 g
Sea water (35 ppt)	-	1000 ml
pH	-	7

Isolates were stocked in malt extract agar vials overlaid with sterile liquid paraffin.

3.2.2 Identification of the isolates

The isolates were identified up to genera as per Barnett et al. (1990). The characters studied were microscopic appearance of the cell, mode of reproduction and biochemical/physiological characteristics.

Microscopic appearance of cells:

a) Vegetative cells: Young growing yeast cultures were inoculated into sterile malt extract broth and incubated at 28±2^{o}C for 48 hrs. Wet mount preparations of the cultures were observed under oil immersion microscope for the following characteristics

- whether the yeast reproduce by budding, splitting or both
- the shape and sizes of the vegetative cells

b) Microscopic examination for filamentous growth: Malt extract agar plates were prepared. Four sterile cover slips dipped in melted malt extract agar (1% agar) were kept on the surface of the medium at 45^{o} angle position by gently piercing the agar, in each plate. These cover

slips were examined microscopically after 3-5 days incubation at 28±2°C. The cover slips were observed for the presence of filamentous growth and if present, whether it is true hyphae or pseudo hyphae.

Assessing the ability of isolates to use nitrogen compounds for growth:

This test is to check the ability of the isolates to use nitrate as a sole source of nitrogen. A mineral basal medium supplemented with glucose as carbon source and KNO_3 as nitrogen source was employed.

Beijerinck medium:

KH_2PO_4	-	2 g
$MgSO_4.\ 7H_2O$	-	0.5 g
Ca_2HPO_4	-	0.5 g
Glucose	-	20 g
KNO_3	-	1 g
NaCl	-	20 g
Distilled water	-	1000 ml

Cultures were inoculated in to the medium and incubated at 28±2^{o}C for one week. Results were recorded by observing the growth.

Assessing the ability to use sugars anaerobically/ aerobically:

Marine oxidation fermentation (MOF) medium was used for testing the ability of the yeast isolates to utilize dextrose aerobically (oxidative) or anaerobically (fermentative). When dextrose is utilized, acid is produced which changes the colour of the medium from pink to yellow. Yellow coloration at the slope region indicates an oxidative reaction, where as the whole tube turning yellow indicates a fermentative reaction.

Urea Agar:

Yeast extract	-	0.1 g
KH_2PO_4	-	9.1 g
Na_2HPO_4	-	9.5 g
NaCl	-	20 g
Urea	-	20 g
Agar	-	20 g
Phenol red	-	4 ml of 0.25% solution
Distilled water	-	1000 ml
pH	-	6.8

The above ingredients except urea were dissolved in 950 ml of distilled water and autoclaved at 15 lbs for 15 minutes. Urea was sterilized using solvent diethyl ether and dissolved in 50 ml sterile distilled water. This was then added to the sterilized basal medium, dispensed into sterile test tubes and slants were prepared. Cultures were inoculated and after incubation for 24 hrs, a change of colour in the medium from golden yellow to pink was noted as urea hydrolysis.

Production of starch like substances:

Certain yeasts produce starch like substances during metabolism. A mineral basal medium supplemented with glucose was used for this test.

Medium composition:

NH_4Cl	- 5 g
NH_4NO_3	- 1 g
Na_2SO_4	- 2 g
K_2HPO_4	- 3 g
KH_2PO_4	- 1 g
NaCl	- 20 g
Yeast extract	- 100 mg
Thiamine HCl	- 1 mg
*Trace metal mix	- 5 ml
Glucose	- 20 g
Distilled water	- 1000 ml

*Trace metal mix:

$FeCl_3$	- 16 mg
$MnCl_2$	- 18 mg
Co (NO_3)	- 13 mg
$MgSO_4$	- 25 mg
$ZnSO_4$	- 4 mg
$CuSO_4$	- 0.01 mg
$CaCl_2$	- 14.5 mg
Distilled water	- 1000 ml

The cultures were inoculated into the above medium and incubated for 1 week. After incubation, grams iodine solution (Iodine 1 g and potassium iodide 2 g in 300 ml distilled water) was added to each tube and change of colour to dark blue indicated the presence of starch like substances.

Diazonium Blue B (DBB) test:

The cultures were spot inoculated on Wickerham's agar and incubated for 10 days. After incubation these petridishes were held at 55^{o}C for several hours and then flooded with ice cold DBB reagent. The reagent must be kept ice-cold and used within a few minutes of preparation, before it discolours. It is prepared by dissolving diazonium blue B salt in cold 0.1 M- Tris-HCL buffer, pH 7.0, at 1 mg per ml. When the culture turned dark red within 2 minutes at room temperature, the result was recorded as positive

Based on the above tests the isolates were classified up to generic level except the black yeasts. This was due to lack of conventional identification procedures for black yeasts. These isolates were later identified by molecular methods.

3.2.3 Hydrolytic enzyme production

The isolates were tested for the production of enzymes viz. amylase, lipase, protease, urease, aryl sulfatase, ligninase, cellulase, DNAse, pectinase and chitinase.

Protease, Amylase, Lipase and Chitinase:

Nutrient agar medium (peptone 0.5 g; beef extract 0.3 g; agar 2 g; sea water (35 ppt) 100 ml; pH 7) supplemented with casein (2%), starch (1%), tributyrin (1%) and colloidal chitin (5%), were prepared for the detection of protease, amylase, lipase and chitinase respectively. Plates were spot inoculated and incubated at room temperature (28±2^{o}C) for 7 to 10 days. Presence of clearance zone was noted as positive and the diameter of the zone was recorded. In the case of amylase, plates were flooded with grams iodine solution (Iodine 1 g and Potassium iodide 2 g in 300 ml distilled water) and the presence of clearance zone was noted.

Pectinase:

Pectin agar (Pectin 0.5 g; $CaCl_2$ $2H_2O$ 0.02 g; NaCl 2 g; $FeCl_3$ $6H_2O$ 0.001 g; yeast extract 0.1 g; agar 2 g; distilled water 100 ml; pH 7) was used for testing the production of pectinase. The plates were spot inoculated and incubated at room temperature at 28±2ºC for 7 to 10 days. After incubation the plates were flooded with 1% cetavlon (cetyl trimethyl ammonium bromide) and the zone of clearance was noted.

Cellulase:

Cellulose agar (casein hydrolysate 0.05 g; yeast extract 0.05 g; $NaNO_3$ 0.1 g; cellulose powder 0.5 g; agar 2 g; sea water 100 ml; pH 7) was used for testing cellulase production. The plates were spot inoculated and incubated at room temperature (28±2ºC) for 7 to 10 days. The zone of clearance around the colonies was noted as positive.

DNAse:

The isolates were spot inoculated on DNAse agar (Tryptone 3 g; DNA 0.2 g; agar 2 g; sea water 100 ml; pH 7). After incubation at 28±2ºC for 10 days, the plates were flooded with 1N HCl. A clearance zone around the colonies was recorded as positive.

Aryl sulfatase:

For testing the production of aryl sulfatase, Zobell's agar (Peptone 0.5 g; yeast extract 0.1 g; ferric phosphate 0.002 g; agar 2 g; sea water 100 ml; pH 7) supplemented with 0.001M Tripotassium phenolphthalein disulfate (PDS) was used. The plates were spot inoculated and incubated at room temperature (28±2ºC) for 12 days. After incubation the agar plates were exposed to ammonia vapour, development of pink colour around the colonies due to the release of phenolphthalein from PDS was recorded as positive.

Ligninase:

Crawford's agar (Glucose 0.1 g; yeast extract 0.15 g; Na_2HPO_4 0.45 g; KH_2PO_4 0.1 g; $MgSO_4$ 0.002 g; $CaCl_2$ 0.05 g; agar 2 g; sea water (35 ppt)

100 ml; pH 7) was used as the basal medium for testing lignin degradation. The basal medium was supplemented with 0.5% tannic acid and the plates were spot inoculated and incubated at room temperature (28±2ºC) for 7 to 14 days. Formation of halo zone or brown colour around the colonies was considered as positive.

3.2.4 Growth assessment of the isolates at different temperature, salinity and pH

Preparation of inoculum:

Malt extract agar slants were prepared and sterilized at 121°C for 15 minutes in an autoclave. The yeast isolates were streaked on to malt extract agar slants. Incubation was done at room temperature (28±2°C) for 24 hours. The cells were harvested at logarithmic phase using 30 ppt sterile sea water. Optical density of the culture suspension was taken at 540 nm in a UV-VIS spectrophotometer (Shimadzu UV-1601). OD was adjusted to 1 by appropriate dilution and this suspension was used as the inoculum.

Preparation of medium:

Temperature

Malt extract broth prepared in sea water (35 ppt) was used for testing growth at different temperatures.

Salinity

Malt extract broth in triplicate was prepared using sea water of different salinities (0, 5, 10, 15, 20, 25, 30, 35, 40 and 45 ppt).

pH

Malt extract broth was prepared in sea water (35 ppt) at different pH 3, 4, 5, 6, 7, 8 and 9

Inoculation and incubation:

10 µl of 1 OD cell suspension was inoculated into the malt extract tubes prepared in triplicate so that the initial OD of the culture medium was 0.001.

Incubation was done at room temperature (28 ± 2°C) for 48 hours. In the case of temperature, the incubation was done at different temperatures (10, 20, 30, 40 and 50°C).

Determination of growth:

Yeast growth was estimated by measuring the optical density at 540 nm using Shimadzu UV-1601 spectrophotometer.

3.2.5 Statistical Analysis

The Shannon-wiener diversity, Peilou's evenness, Species richness and Species dominance were analyzed using PRIMER V5 (Clarke & Gorley, 2001). Diversity index provides a good measure of the community composition along with its survival strategy.

3.3 Results

3.3.1 Generic Composition

Arabian Sea:

Among the isolates obtained from Arabian Sea (Cr. No. 228 & 233), *Candida* (56.5%) was the predominant genus followed by *Lipomyce*s (17.03%), *Rhodotorula* (11.8%), *Yarrowia* (9.5%),), *Wingea* (1.7%), Black yeasts (1.3%), *Dekkera* (0.82%), *Debaryomyces* (0.67%) and *Pichia* (0.44%) (Fig. 3.1). About 84% of the isolates at 200 m belonged to *Candida* (Fig. 3.2a). This was followed by *Lipomyces* (13.5%), *Yarrowia* (0.96%), *Rhodotorula* (0.64%) and *Pichia* (0.32%). Diverse genera were identified from 500 m stations (Fig. 3.2b). *Yarrowia* (32.1%) was the predominant genera identified followed by *Candida* (22.4%), *Rhodotorula* (21.3%), *Lipomyces* (20.2%), *Debaryomyces* (1.24%), Black yeasts (0.97%), *Pichia* (0.83%), *Wingea* (0.69%) and *Dekkera* (0.69%). "Black yeasts" could be obtained only from 500 and 1000 m stations. At 1000 m depth zone *Lipomyces* (49.3%) and *Candida* (44.2%) were the dominant genera followed by *Wingea* (3.9%), Black yeasts (2.6%), *Dekkera* (1.9%) and *Rhodotorula* (0.48%) (Fig. 3.2c).

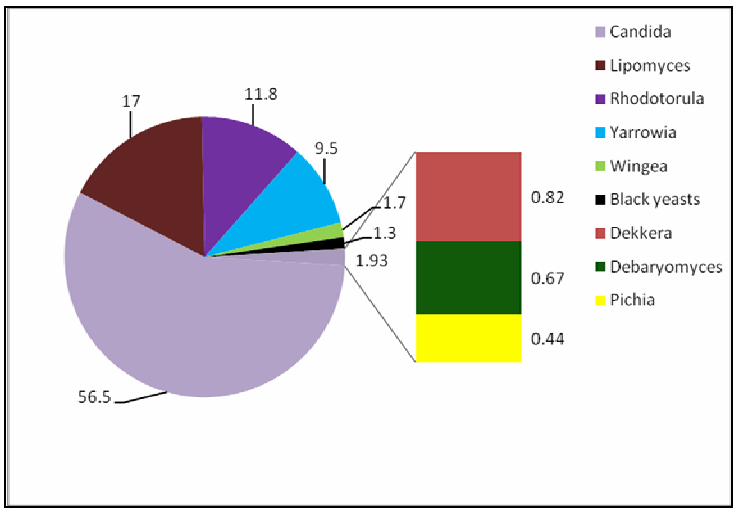

Fig. 3.1 Generic composition (average) of yeasts from the slope sediments of Arabian Sea (Cr. No. 228 & 233)

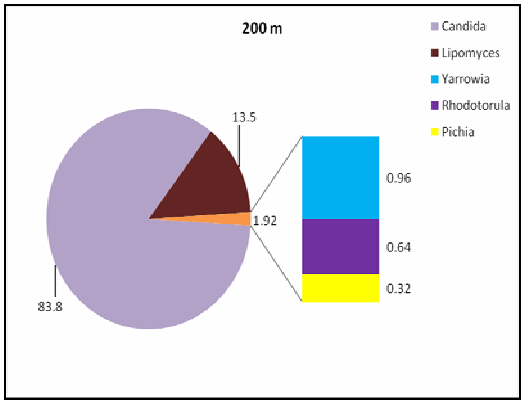

Fig. 3.2a

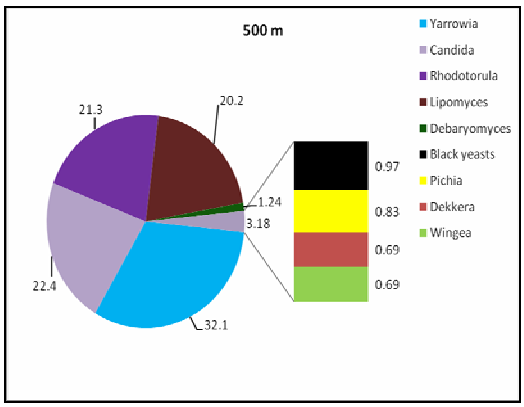

Fig. 3.2b

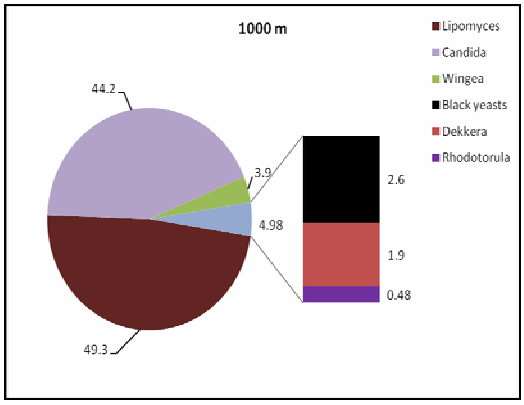

Fig. 3.2.c

Fig. 3.2a-c Generic composition of marine yeasts isolated from different depths in Arabian Sea (200-1000 m depth) (Cr. No. 228 & 233)

Bay of Bengal (Cr: 236):

Among the isolates of Bay of Bengal (Cr. No. 236), *Candida* (46.4%) was the predominant genera identified followed by Black Yeasts (23.5%), *Wingea* (20.5%), *Rhodotorula* (3.38%), *Cryptococcus* (2.3%), *Bullera* (0.99%), *Yarrowia* (0.59%), *Lipomyces* (0.59%), *Dekkera* (0.39%), *Pichia* (0.39%), *Oosporidium* (0.39%) and *Trichosporon* (0.19%) (Fig. 3.3). About 76% of the isolates at 200 m belonged to *Wingea* (Fig. 3.4a). This was followed by *Candida* (9.5%), *Cryptococcus* (7.1%), *Rhodotorula* (2.3%), *Bullera* (1.5%), *Lipomyces* (1.5%), *Oosporidium* (0.79%) and *Dekkera* (0.79%). Diverse genera were identified from 500 m stations (Fig. 3.4b). *Candida* (61.2%) was the predominant genera identified followed by Black Yeasts (32.4%), *Rhodotorula* (2.84%), *Cryptococcus* (0.85%), *Yarrowia* (0.85%), *Pichia* (0.56%), *Bullera* (0.28%), *Wingea* (0.28%), *Dekkera* (0.28%) and *Oosporidium* (0.28%).“Black yeasts” could be obtained only from 500 and 1000 m stations and formed a major group at these depths. Comparatively lesser genera were observed at 1000 m depth. (Fig. 3.4c). Here *Candida* (25%) and *Wingea* (25%) were the dominant genera followed by *Rhodotorula* (16.6%), Black yeasts (16.6%), *Bullera* (8.3%), *Lipomyces* (4.16%) and *Trichosporon* (4.16%).

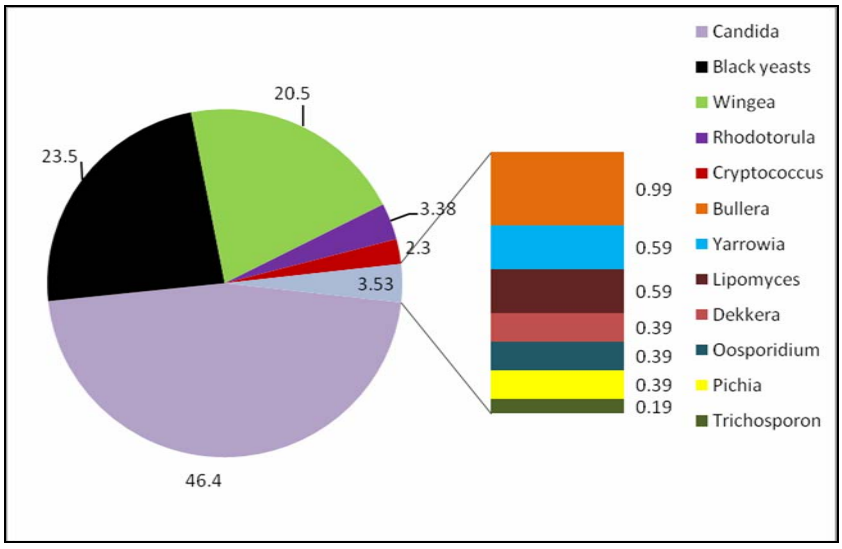

Fig. 3.3 Generic composition (average) of yeasts from the slope sediments of Bay of Bengal (Cr. No. 236)

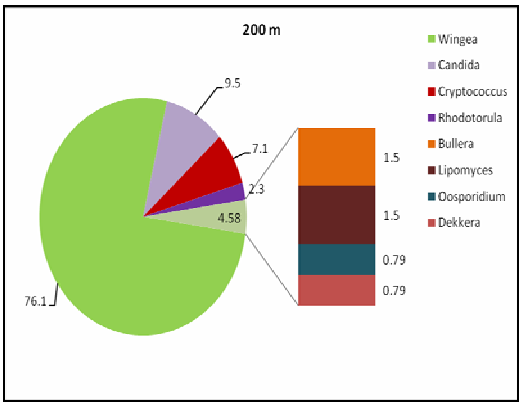

Fig. 3.4a

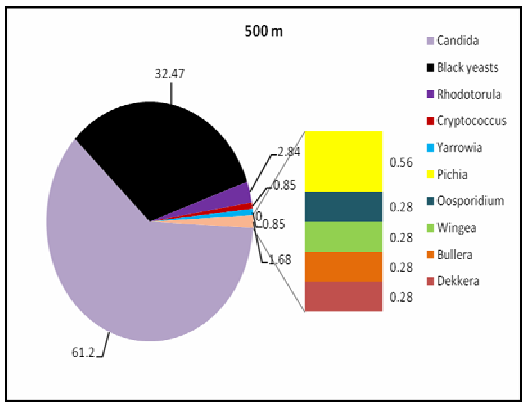

Fig. 3.4b

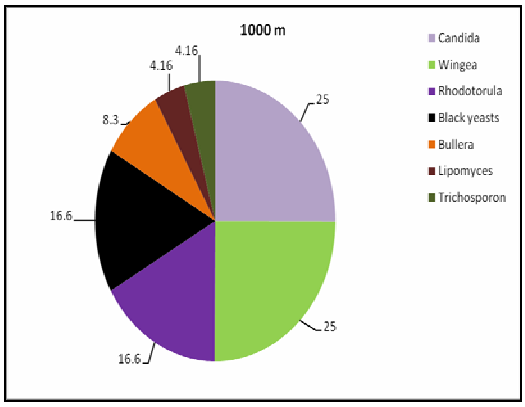

Fig. 3.4c

Fig. 3.4a-c Generic composition of marine yeasts isolated from different depths in Bay of Bengal (200-1000 m depth) (Cr. No. 236)

Cruise 245:

Among the Bay of Bengal (Cr. No. 245) isolates, *Yarrowia* (42.2%) was the predominant genera identified followed by *Candida* (31.7%), *Cryptococcus* (13.7%), Black yeasts (11.5%), *Debaryomyces* (1.33%), *Bullera* (0.88%) and *Lipomyces* (0.22%) (Fig. 3.5). At 200 m about 74% of

the isolates belonged to *Candida* followed by Black yeasts (21%), *Debaryomyces* (3%) and *Bullera* (2%) (Fig. 3.6a). About 70% of the isolates belonged to the genera *Yarrowia* followed by *Cryptococcus* (23%), *Candida* (4%) and Black yeasts (3%) at 500 m depth stations (Fig. 3.6b). At 1000 m depth, 50% of the isolates belonged to *Candida* followed by Black yeasts (39%), *Debaryomyces* (7%) and *Lipomyces* (4%). (Fig. 3.6c). Black yeasts were obtained from all the depths. Notably 39% of the isolates from 1000 m belonged to black yeasts. Microscopic view (100 x) of various yeast isolates are shown in fig. 3.7a-e.

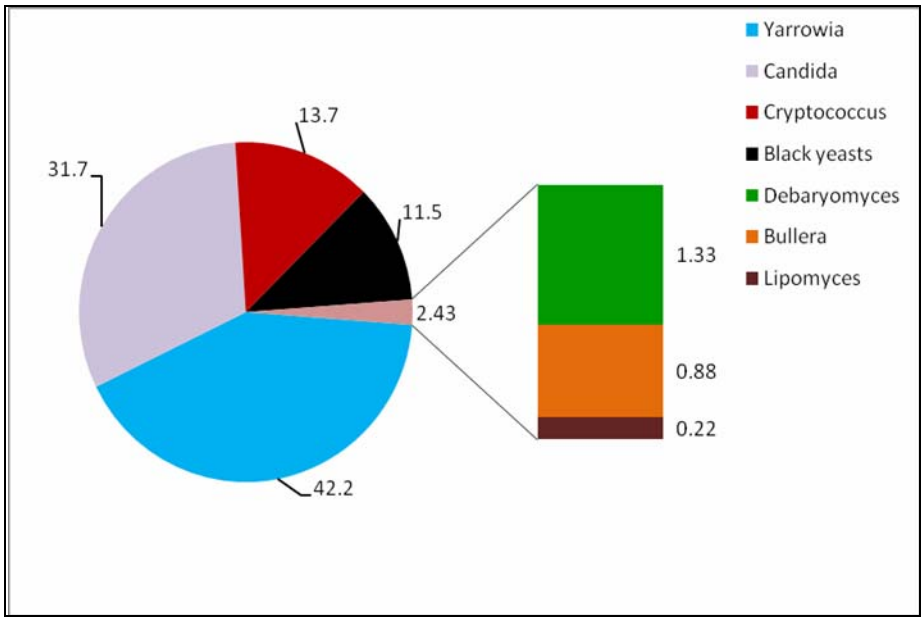

Fig. 3.5 Average generic composition of yeasts from the slope sediments of Bay of Bengal (Cr. No. 245)

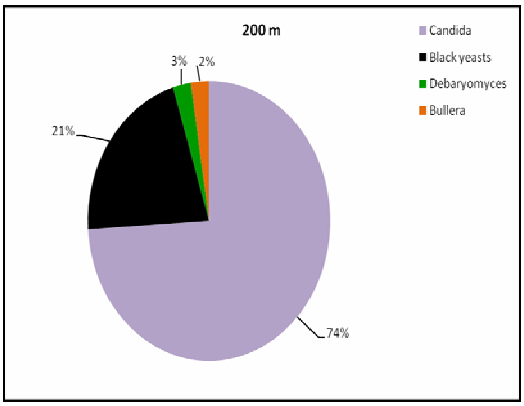

Fig. 3.6a

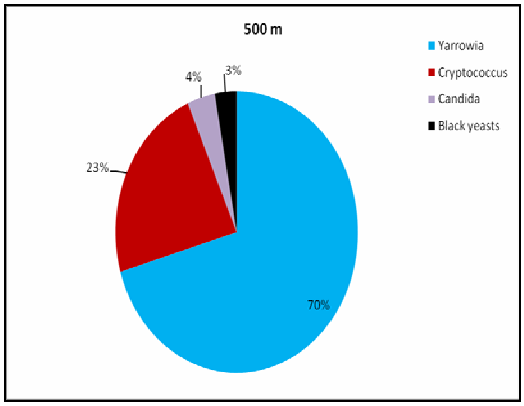

Fig. 3.6b

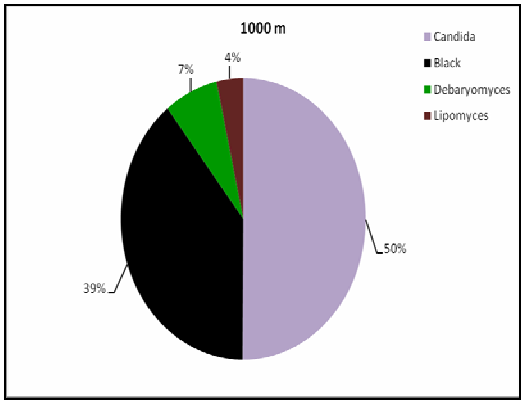

Fig. 3.6c

Fig. 3.6a-c Generic composition of marine yeasts isolated from different depths in Bay of Bengal (200-1000 m depth) (Cr. No. 245)

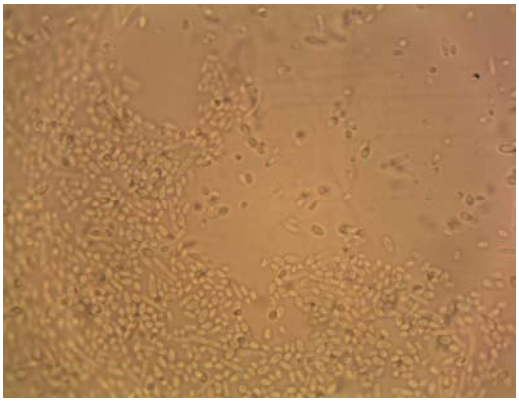

Fig. 3.7a *Candida*

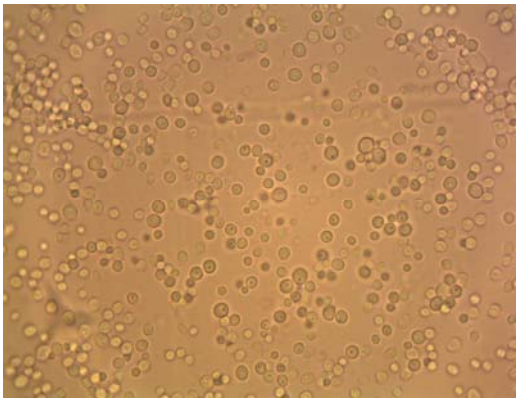

Fig. 3.7b *Yarrowia*

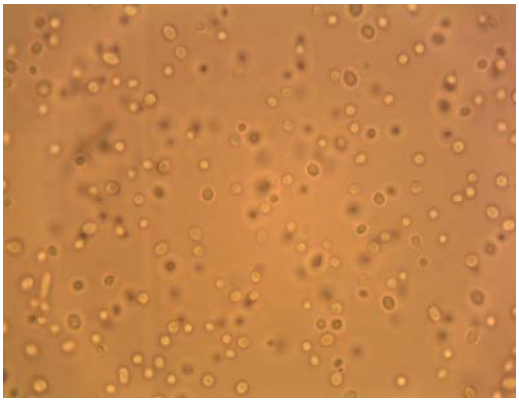

Fig. 3.7c *Cryptococcus*

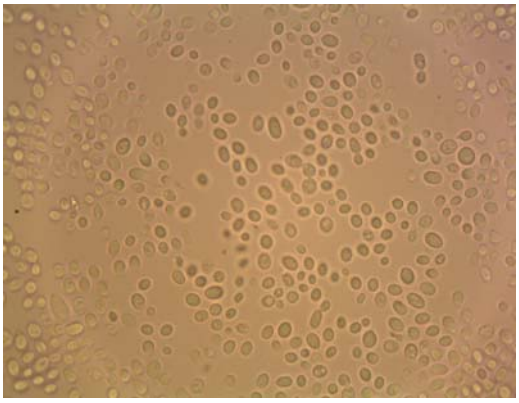

Fig. 3.7d *Rhodotorula*

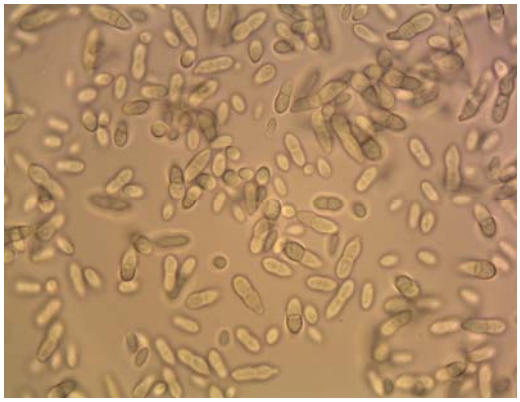

Fig. 3.7e Black yeast

Fig. 3.7a-e Microscopic view (100 x) of various yeast isolates

3.3.2 Diversity Indices

Diversity index gives a measure of the way in which individuals in an ecological community are distributed among species.

Arabian Sea:

In Arabian Sea (Cr. No. 228 & 233) Shannon-wiener diversity (H'(log2)), Peilou's evenness (J') and Species richness (d) were found to be higher at 500 m depth region. The diversity ranged from 0.73 to 2.24 and maximum was found at 500 m depth zone (Fig. 3.8a). Evenness was found to be in the range 0.31 to 0.70 (Fig. 3.8b) and richness in the range 0.87 to 1.7 (Fig. 3.8c). Dominance (λ) showed an inverse relationship with diversity and ranged from 0.23 to 0.72 (Fig. 3.d).

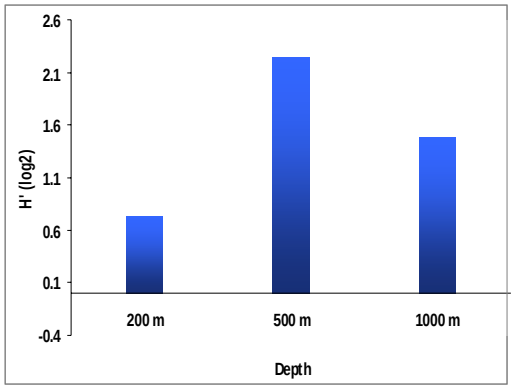

Fig. 3.8a Shannon-wiener diversity

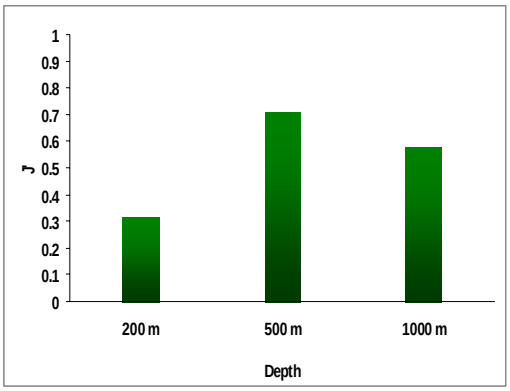

Fig. 3.8b Peilou's evenness

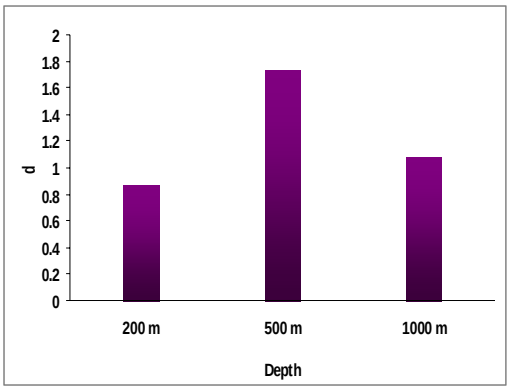

Fig. 3.8c Species richness

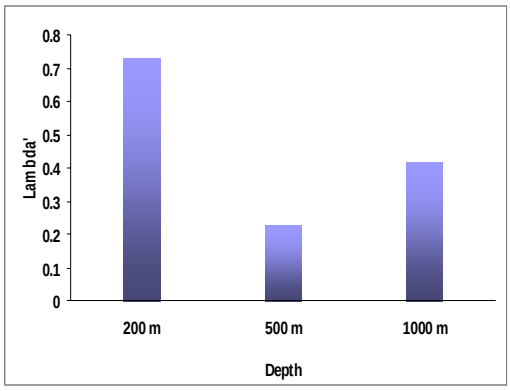

Fig. 3.8d Species dominance

Bay of Bengal (Cr. No. 236):

Shannon-wiener diversity (H'(log2)), Peilou's evenness (J') and Species richness (d) were found to be higher at 1000 m depth region of Bay of Bengal (Cr. No. 236). The diversity ranged from 1.32 to 2.54 and maximum was found at 1000 m depth zone (Fig. 3.9a). Evenness was found to be in the range 0.41 to 0.90 (Fig. 3.9b) and richness in the range 1.4 to 1.8 (Fig. 3.9c). Dominance (λ) showed an inverse relationship with diversity and ranged from 0.15 to 0.59 (Fig. 3.9d).

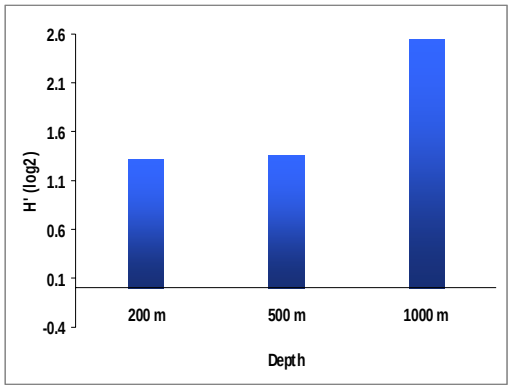

Fig. 3.9a Shannon-wiener diversity

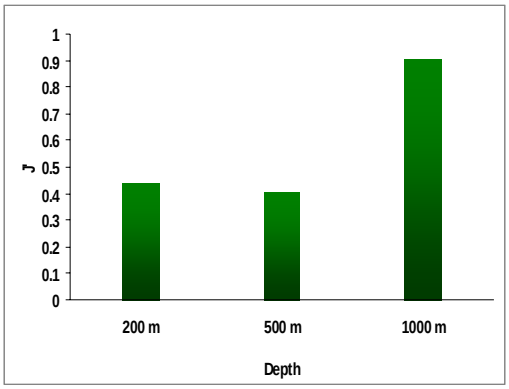

Fig. 3.9b Peilou's evenness

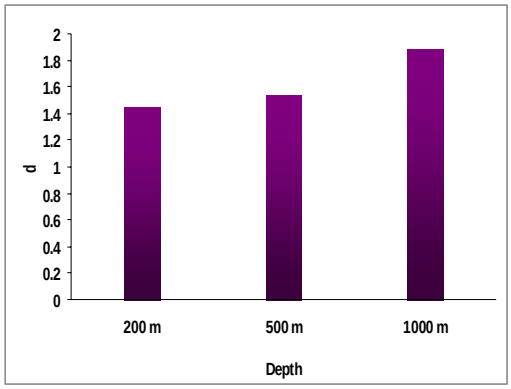

Fig. 3.9c Species richness

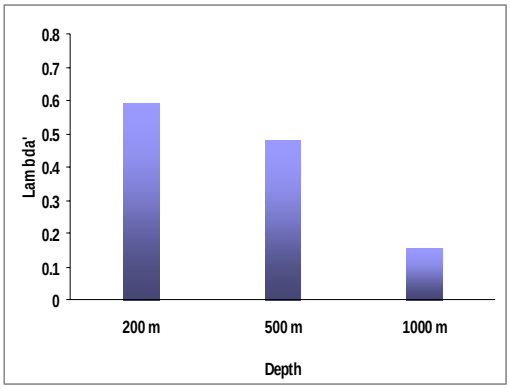

Fig. 3.9d Species dominance

Bay of Bengal (Cr. No. 245):

In Bay of Bengal during Cr. No. 245 also Shannon-wiener diversity (H'(log2)), Peilou's evenness (J') and Species richness (d) were found to be higher at 1000 m depth region. The diversity ranged from 1.06 to 1.47 and maximum was found at 1000 m depth zone (Fig. 3.10a). Evenness was found to be in the range 0.53 to 0.73 (Fig. 3.10b) and richness in the range 0.53 to 0.9 (Fig. 3.10c). Dominance (λ) showed an inverse relationship with diversity and ranged from 0.38 to 0.58 (Fig. 3.10d).

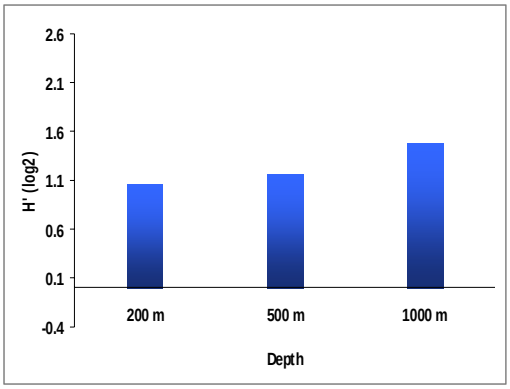

Fig. 3.10a Shannon-wiener diversity

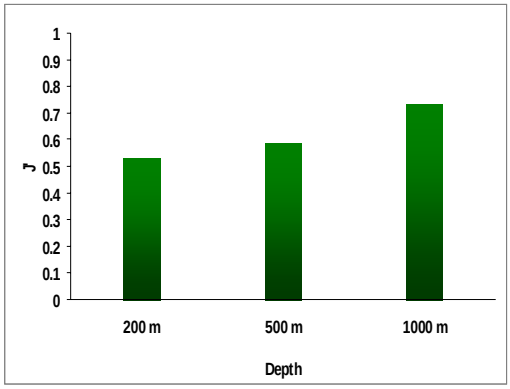

Fig. 3.10b Peilou's evenness

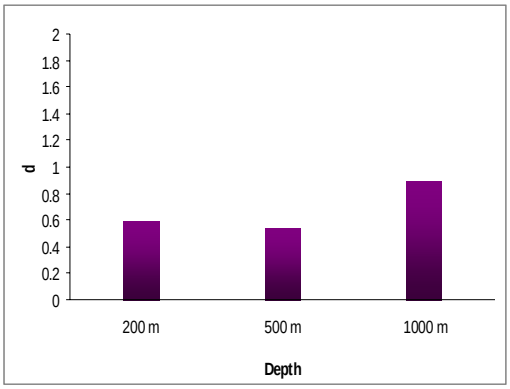

Fig. 3.10c Species richness

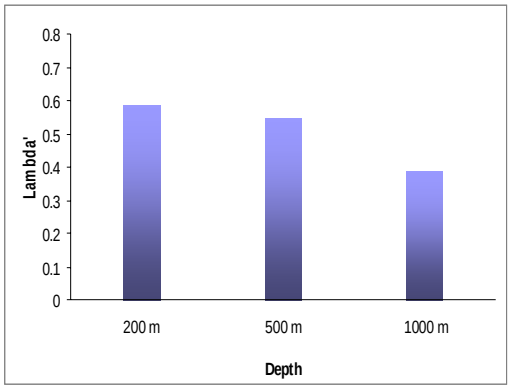

Fig. 3.10d Species dominance

3.3.3 Oxidative/fermentative nature of the yeast isolates

Arabian Sea:

Among the isolates of Arabian Sea (Cr. No. 228 & 233), only 7.4% were fermentative and the rest (92.5%) were oxidative (Fig. 3.11a). At all the three depth zones domination of oxidative forms could be noted i.e. 97.2%, 93.2% and 88.1% at 200, 500 and 1000 m depth respectively (Fig. 3.11b). Generic wise analysis of the oxidative and fermentative forms showed that isolates belonging to the genera *Candida*, *Lipomyces*, *Yarrowia*, *Rhodotorula*, *Debaryomyces* and Black yeasts were cent percent oxidative in nature. More than 95% of the *Wingea* spp. was oxidative and all the *Dekkera* spp. were fermentative (Fig. 3.12).

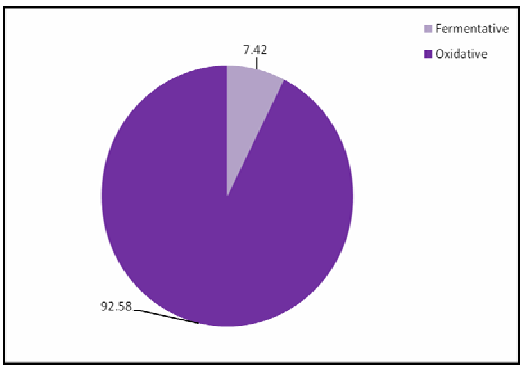

Fig. 3.11a Average percentage of fermentative and oxidative among the marine yeasts from the slope sediments of Arabian Sea (Cr. No. 228 & 233)

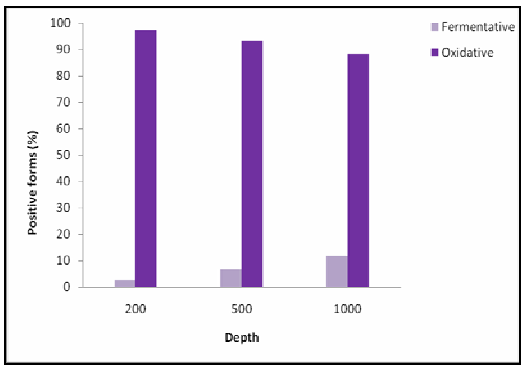

Fig. 3.11b Percentage of fermentative/oxidative yeasts at different depth regions in Arabian Sea (200-1000 m depth) (Cr. No. 228 & 233)

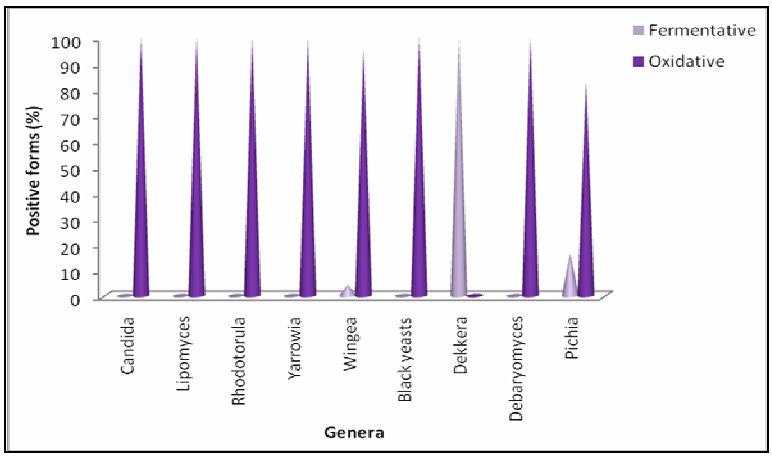

Fig 3.12 Percentage of fermentative and oxidative marine yeasts belonging to different genera isolated from the slope sediments of Arabian Sea (Cr. No. 228 & 233)

Bay of Bengal (Cruise 236):

Among the isolates of Bay of Bengal (Cr. No. 236) 23% were fermentative and 77% oxidative (Fig. 3.13a). At 200 m depth regions the fermentative (47%) and oxidative (52%) forms were in almost equal proportions. At 500 and 1000 m depth range oxidative forms were in high proportions and comprised about 87% and 76% respectively (Fig. 3.13b). Generic wise analysis of the oxidative and fermentative forms shows that isolates belonging to the genera *Bullera, Oosporidium, Cryptococcus, Pichia, Lipomyces, Yarrowia, Trichosporon* and Black yeasts were cent percent oxidative in nature. *Wingea* and *Dekkera* were cent percent fermentative. Isolates belonging to *Rhodotorula* (93.3%) and *Candida* (63%) were generally oxidative (Fig. 3.14).

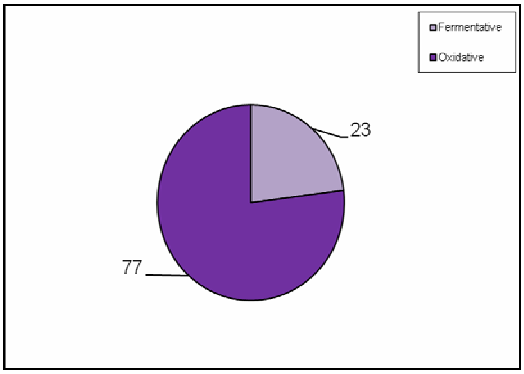

Fig. 3.13a Average percentage of fermentative and oxidative among the marine yeasts from the slope sediments of Bay of Bengal (Cr. No. 236)

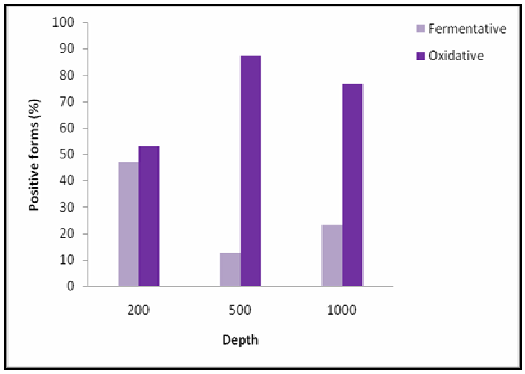

Fig. 3.13b Percentage of fermentative/oxidative yeasts at different depth regions in Bay of Bengal (200-1000 m depth) (Cr. No. 236)

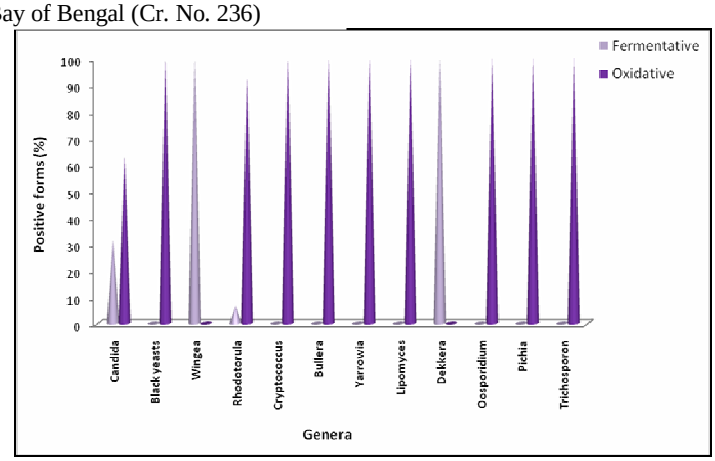

Fig 3.14 Percentage of fermentative and oxidative marine yeasts belonging to different genera isolated from the slope sediments of Bay of Bengal (Cr. No. 236)

Cruise 245:

Among the isolates 58.4% were fermentative and 42.8% oxidative (Fig. 3.15a). Fermentative forms dominated at 500 m depth regions, whereas at 200 and 1000 m depth zones the fermentative and oxidative forms were in equal proportions (Fig. 3.15b). Generic wise analysis of the oxidative and fermentative forms showed that isolates belonging to the genera *Bullera*, *Debaryomyces*, *Lipomyces* and Black yeasts were cent percent oxidative in nature, whereas *Candida* and *Yarrowia* were cent percent fermentative. Isolates belonging to *Cryptococcus* (83.3%) were generally oxidative (Fig. 3.16).

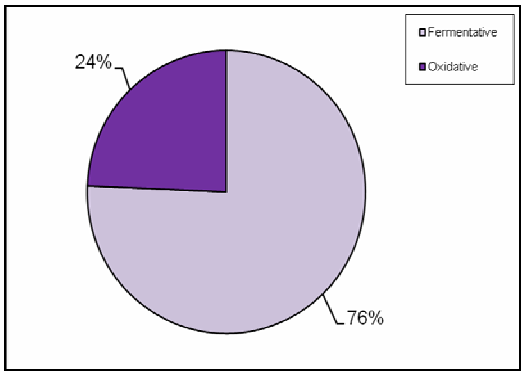

Fig. 3.15a Average percentage of fermentative and oxidative among the marine yeasts from the slope sediments of Bay of Bengal (Cr. No. 245)

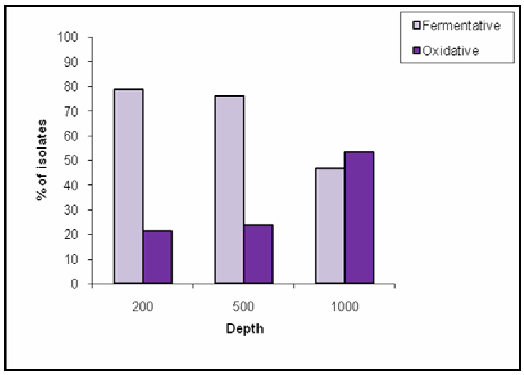

Fig. 3.15b Percentage of fermentative/oxidative yeasts at different depth regions in Bay of Bengal (200-1000 m depth) (Cr. No. 245)

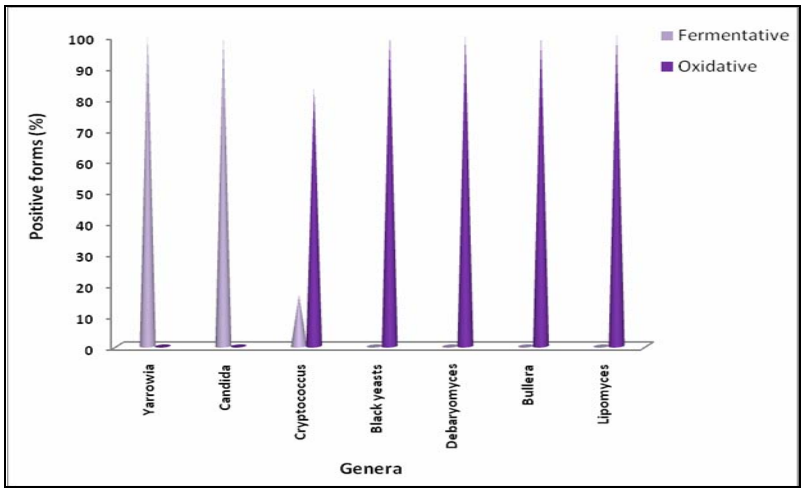

Fig 3.16 Percentage of fermentative and oxidative marine yeasts belonging to different genera isolated from the slope sediments of Bay of Bengal (Cr. No. 245)

3.3.4 Hydrolytic enzymes

All the isolates of the Arabian Sea (Cr. No. 228 & 233) were lipolytic, followed by ligninolytic (15.8%), ureolytic (13.3%), proteolytic (8.9%) and amylolytic (4.4%) forms (Fig. 3.17). None of the isolates produced aryl sulfatase, DNAse, pectinase, cellulase and chitinase. Percentage of isolates producing protease, amylase and urease was more in 500 m depth zones (Fig. 3.18b), where as ligninase producing forms were more in 200 m depth (Fig. 3.18a). Isolates producing protease, amylase and ligninase were meager at 1000 m depth. None of the isolates from 1000 m depth produced urease enzyme (Fig. 3.18c).

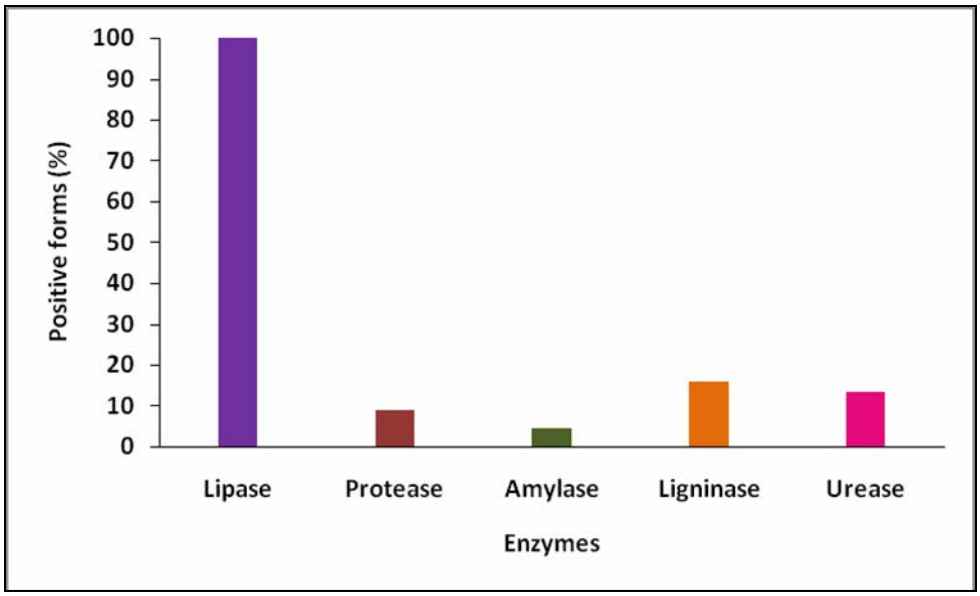

Fig. 3.17 Average hydrolytic enzyme production by marine yeasts from the slope sediments of Arabian Sea (Cr. No. 228 & 233)

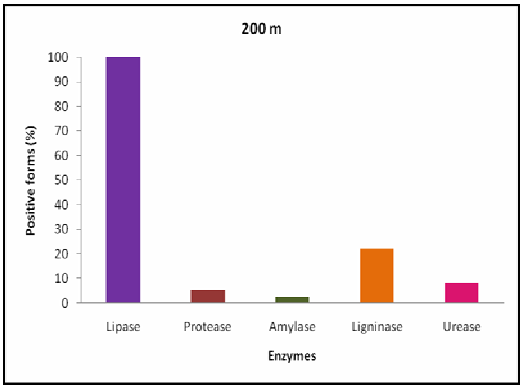

Fig. 3.18a

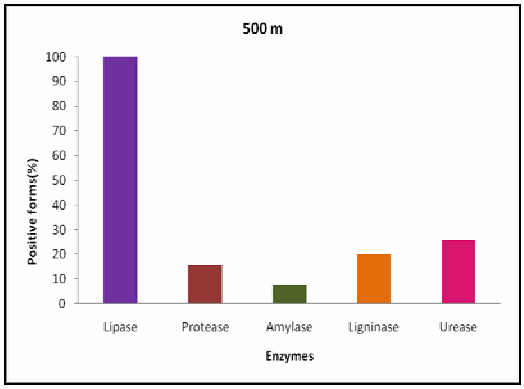

Fig. 3.18b

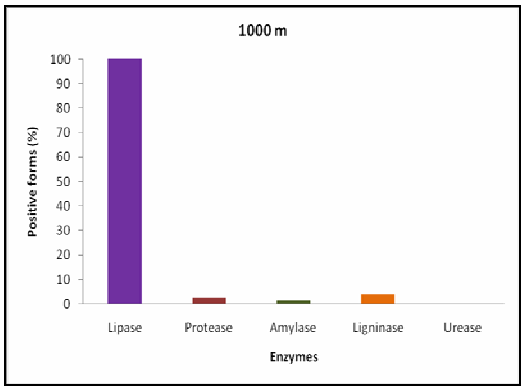

Fig. 3.18c

Fig. 3.18 a-c Hydrolytic enzyme production by marine yeast isolates at different depths along the Arabian Sea (200-1000 m depth) (Cr. No. 228 & 233)

Black yeasts were cent percent positive for lipase, protease, amylase and ligninase. They were found to be the most potent isolates in enzyme production (Fig. 3.19). Some of the isolates belonging to the genus *Yarrowia* were also able to produce all the enzymes. Generic wise hydrolytic potential of all the isolates are given in table 3.1.

Table 3.1 Generic wise hydrolytic potential of the isolates from the Arabian Sea

Genera/ Group	Lipase	Protease	Amylase	Ligninase	Urease
Candida	**100**	1.25	1.25	12.5	0
Lipomyces	**100**	0	0	16.2	0
Rhodotorula	**100**	0	0	37.5	37.5
Yarrowia	**100**	24.1	20.6	6.8	55.1
Wingea	**100**	16.6	0	33.3	16.6
Black yeasts	**100**	**100**	**100**	**100**	**0**
Dekkera	**100**	15.3	0	7.6	0
Debaryomyces	**100**	0	0	20	0
Pichia	**100**	0	0	60	0

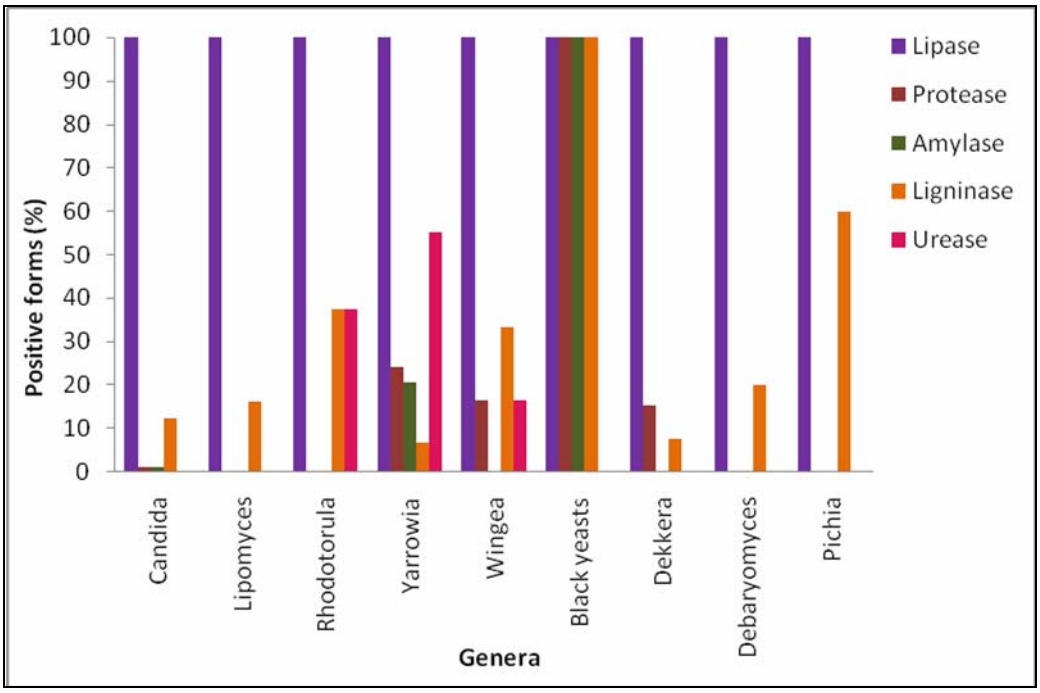

Fig. 3.19 Hydrolytic potential of different genera of marine yeasts isolated from the slope sediments of Arabian Sea (Cr. No. 228 & 233)

Bay of Bengal (Cruise 236):

All the isolates obtained from Bay of Bengal (Cr. No. 236) were lipolytic, followed by ligninolytic (63.7%), proteolytic (43.4%), ureolytic (36.2%), amylolytic (28.9%) and aryl sulfatase (1.45%) producing forms (Fig. 3.20). None of the isolates produced DNAse, pectinase, cellulase and chitinase. Other than lipase production, all other enzyme production was found to be less in isolates from 200 m depth (Fig. 3.21a). The only isolate which produced aryl sulfatase was obtained from 500 m depth (Fig. 3.21b). Protease producing isolates were maximum at 1000 m depth (Fig. 3.21c).

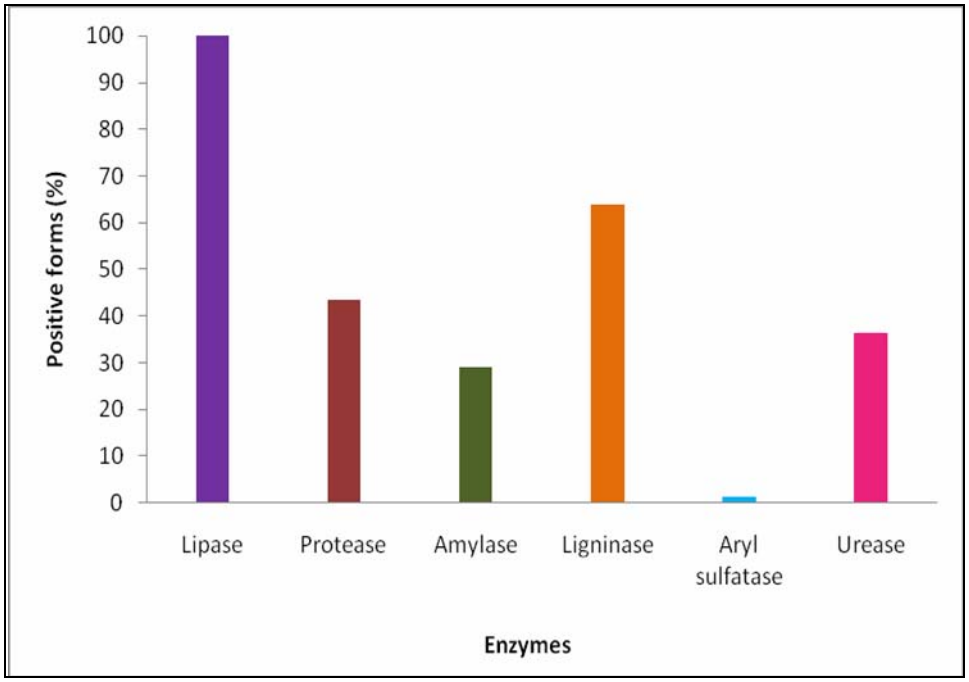

Fig. 3.20 Average hydrolytic enzyme production by marine yeasts from the slope sediments of Bay of Bengal (Cr. No. 236)

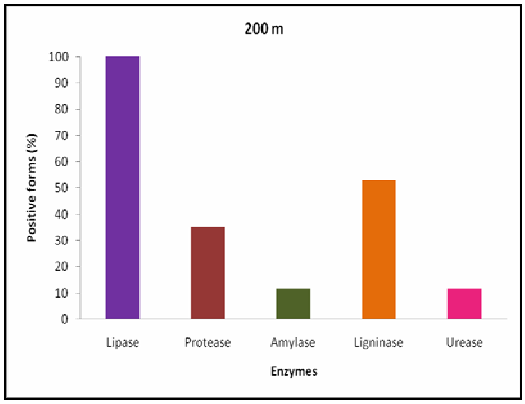

Fig. 3.21a

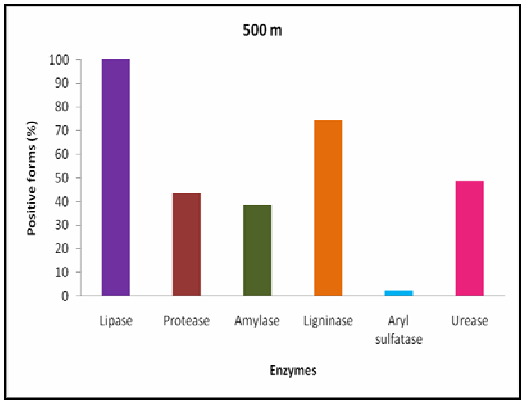

Fig. 3.21b

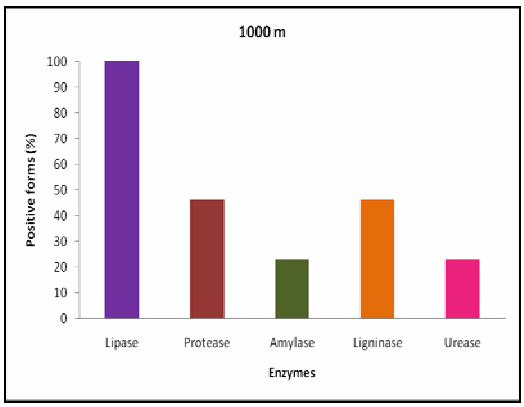

Fig. 3.21c

Fig. 3.21 a-c Hydrolytic enzyme production by marine yeast isolates at different depths along the Bay of Bengal (200-1000 m depth) (Cr. No. 236)

Among the whole isolates only one strain produced aryl sulfatase which belonged to the genus *Cryptococcus* isolated from 500 m depth station. Black yeasts were cent percent positive for lipase, protease, amylase, ligninase and 44.4% of them produced urease. They were found to be the most potent isolates in enzyme production (Fig. 3.22). Generic wise hydrolytic potential of all the isolates are given in the table 3.2.

Table 3.2 Generic/Group wise hydrolytic potential of the isolates from the Bay of Bengal (Cr. No. 236)

Genera/ Group	Lipase	Protease	Amylase	Ligninase	Aryl sulfatase	Urease
Candida	**100**	15.78	0	68.42	0	0
Black yeasts	**100**	**100**	**100**	**100**	**0**	**44.4**
Wingea	**100**	85.71	0	28.57	0	0
Rhodotorula	**100**	40	46.6	80	0	93.3
Cryptococcus	**100**	**50**	**50**	**50**	**25**	**75**
Bullera	**100**	20	0	60	0	0
Yarrowia	**100**	0	0	50	0	100
Lipomyces	**100**	50	0	0	0	0
Dekkera	**100**	0	0	100	0	0
Oosporidium	**100**	0	0	0	0	0
Pichia	**100**	100	100	0	0	100
Trichosporon	**100**	100	0	0	0	100

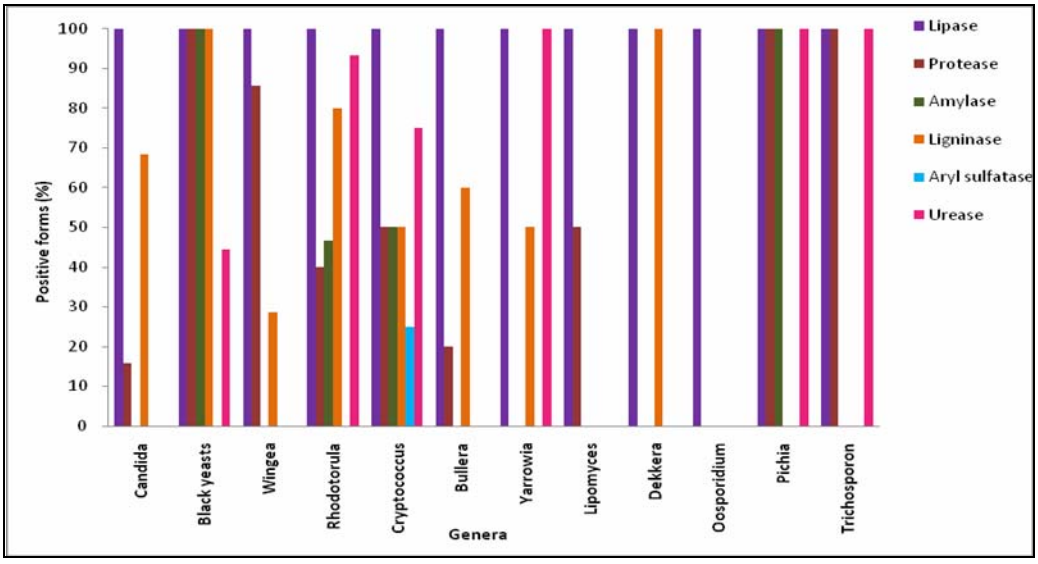

Fig. 3.22 Hydrolytic potential of different genera of marine yeasts isolated from the slope sediments of Bay of Bengal (Cr. No. 236)

Cruise 245:

All the isolates from Bay of Bengal (Cr. No. 245) were lipolytic, followed by proteolytic (28.5%), amylolytic (28.5%), ureolytic (18.1%) and ligninolytic (9.09%) forms (Fig. 3.23). None of the isolates produced aryl sulfatase, DNAse, pectinase, cellulase and chitinase. The isolates of 200 m depth had shown maximum percentage of protease, amylase and ligninase producing forms (Fig. 3.24a). Urease producing isolates were mostly obtained from 500 m depth (Fig. 3.24b). Hydrolytic enzyme production of isolates from 1000 m depth is shown in fig. 3.24c. The activity of various enzymes produced by the yeast isolates are given in fig. 3.26a-e.

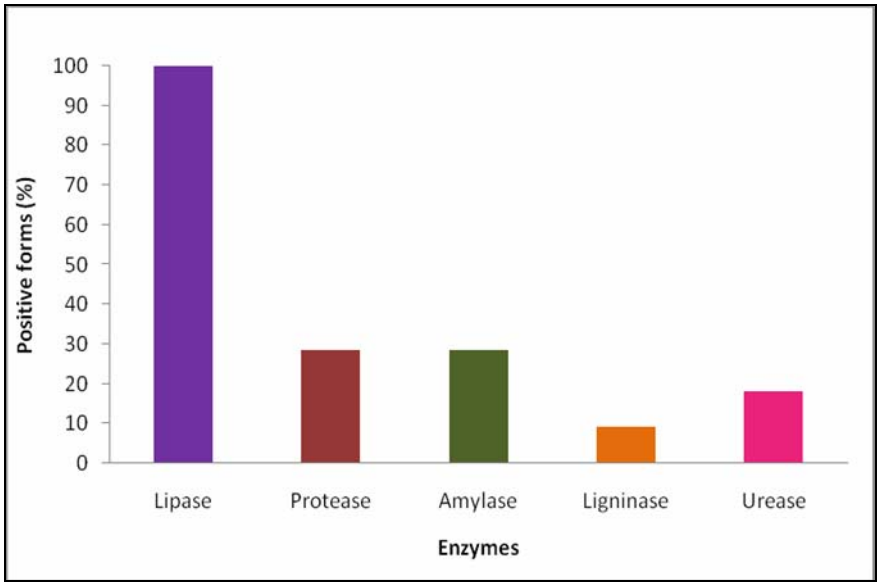

Fig. 3.23 Average hydrolytic enzyme production by marine yeasts from the slope sediments of Bay of Bengal (Cr. No. 245)

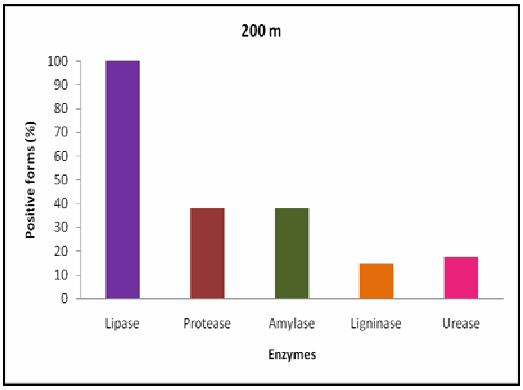

Fig. 3.24a

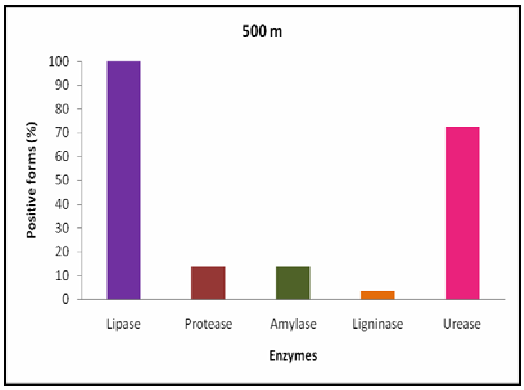

Fig. 3.24b

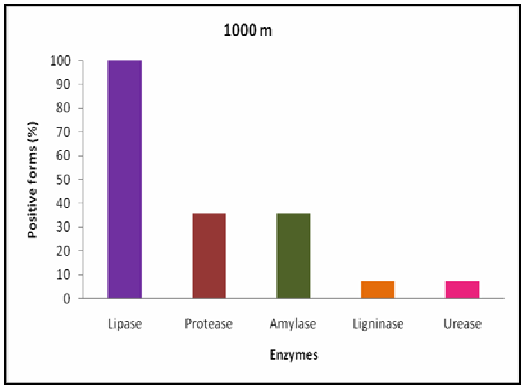

Fig. 3.24c

Fig. 3.24 a-c Hydrolytic enzyme production by marine yeast isolates at different depths along the Bay of Bengal (200-1000 m depth) (Cr. No. 245)

Black yeasts were cent percent positive for lipase, protease and amylase, 40.9% were ureolytic and ligninolytic 31.8%. They were found to be the most potent isolates in enzyme production (Fig. 3.25). Isolates belonging to *Yarrowia, Cryptococcus* and *Candida* exhibited urease production i.e. 100%, 33.3% and 18.75% respectively (Table 3.3).

Table 3.3 Generic/Group wise hydrolytic potential of the isolates from the Bay of Bengal (Cr. No. 245)

Genera/ Group	Lipase	Protease	Amylase	Ligninase	Urease
Yarrowia	**100**	0	0	0	100
Candida	**100**	0	0	0	18.75
Cryptococcus	**100**	0	0	0	33.33
Black yeasts	**100**	**100**	**100**	**31.8**	**40.9**
Debaryomyces	**100**	0	0	0	0
Bullera	**100**	0	0	0	0
Lipomyces	**100**	0	0	0	0

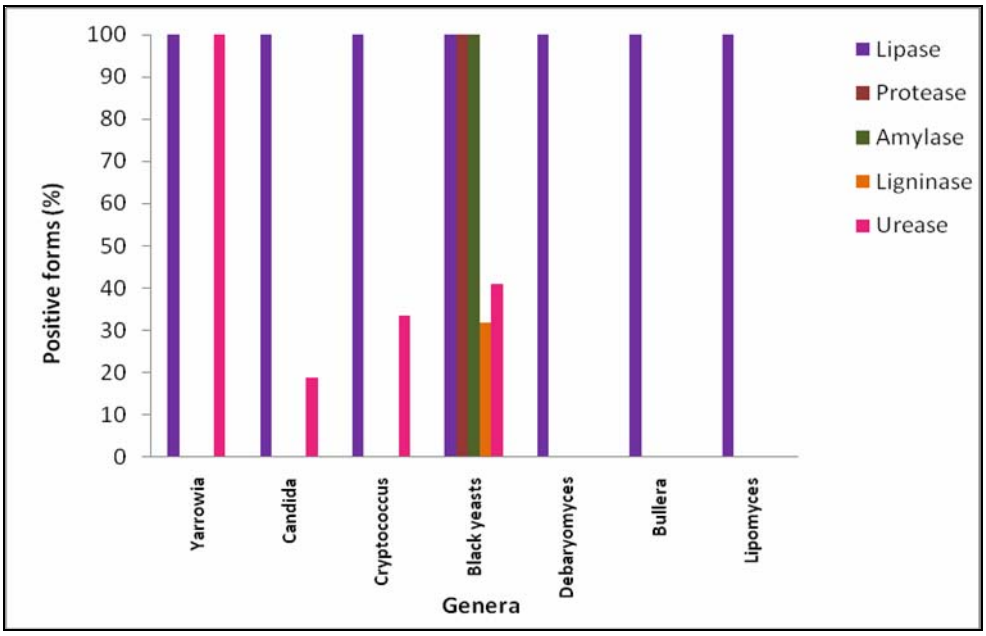

Fig. 3.25 Hydrolytic potential of different genera of marine yeasts isolated from the slope sediments of Bay of Bengal (Cr. No. 245)

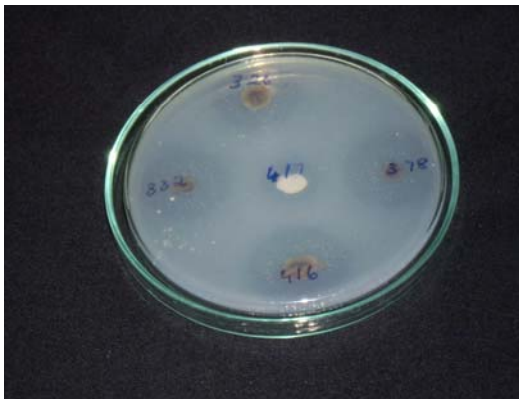

Fig. 3.26a Lipase activity

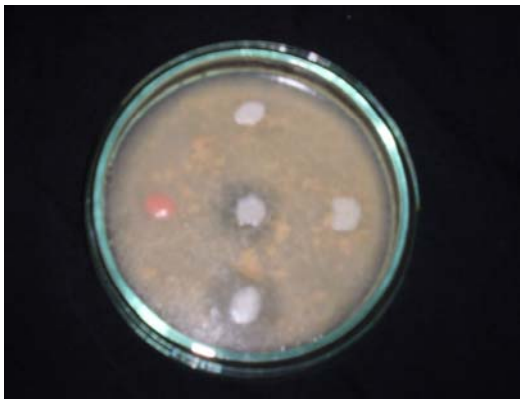

Fig. 3.26b Protease activity

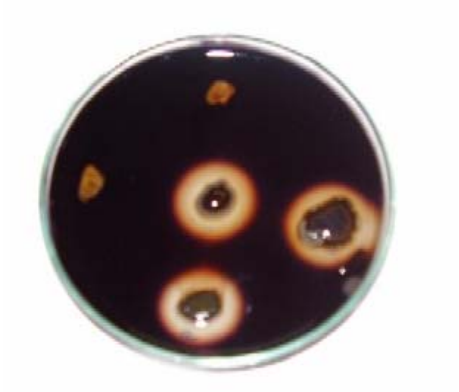

Fig. 3.26c Amylase activity

Fig. 3.26d Ligninase activity

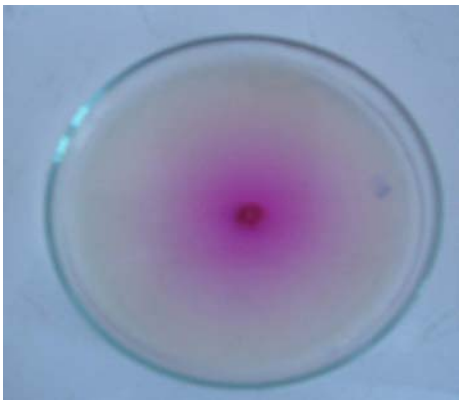

Fig. 3.26e Aryl sulfatase activity

Fig. 3.26 a-e Hydrolytic potential of the yeast isolates

3.3.5 Growth at different temperature, salinity and pH

Temperature:

Most of the isolates preferred 30°C (69%) for maximum growth followed by 20°C (18.18%) and 40°C (12.72%) (Fig. 3.27). The isolates did not show growth at 10 and 50°C (Appendix 1, table 3.7). Percentage of isolates having maximum growth at different temperature in three depths is given in table 3.4.

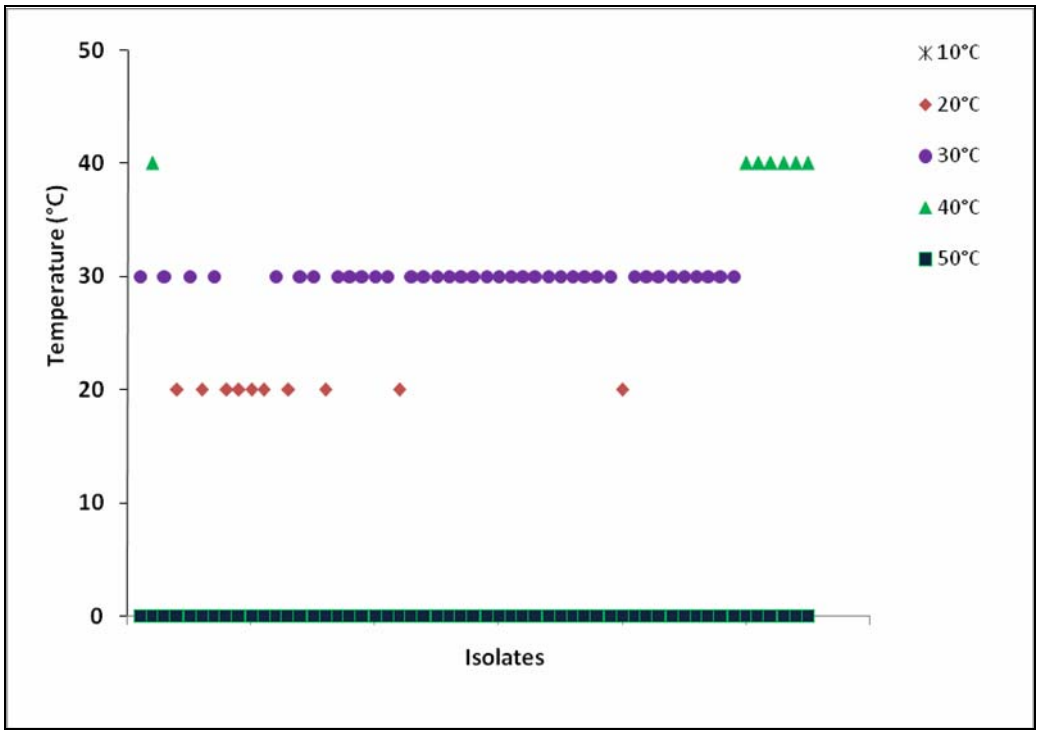

Fig. 3.27 Optimum temperature for the growth of various isolates

Table 3.4 Percentage of isolates showing maximum growth at different temperature in three depths

Depth (m)	10°C	20°C	30°C	40°C	50°C
200	0	14.3	57.14	28.6	0
500	0	26	70.37	13.7	0
1000	0	0	100	0	0
Total (%)	0	18.18	69.09	12.72	0

Salinity:

Considerable growth could be noticed for all the isolates from 0 to 45 ppt. However 15 to 25 ppt was found to be the most preferred range (Fig. 3.28 and table 3.5) (Appendix 1, table 3.8).

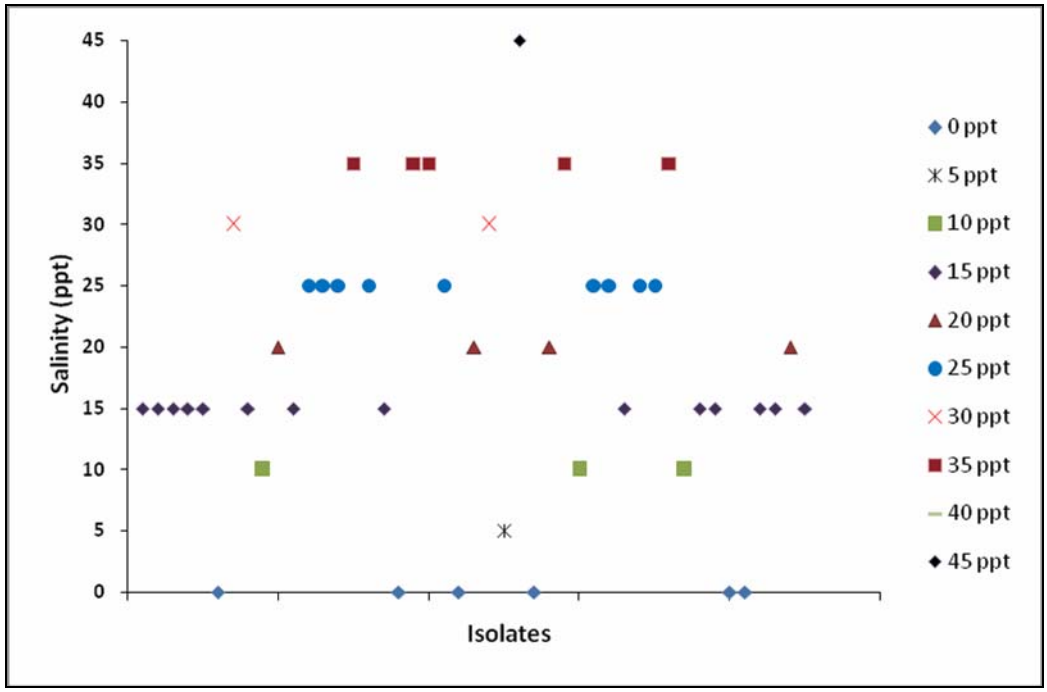

Fig. 3.28 Optimum salinity for the growth of the isolates

Table 3.5 Percentage of isolates showing maximum growth at different salinities in three depths

Depth (m)	0 ppt	5 ppt	10 ppt	15 ppt	20 ppt	25 ppt	30 ppt	35 ppt	40 ppt	45 ppt
200	6.25	0	6.25	18.75	12.5	31.25	0	25	0	0
500	16.6	4.16	8.3	37.5	4.16	16.6	8.3	0	0	4.16
1000	20	0	0	40	20	0	0	20	0	0
Total (%)	13.3	2.22	6.66	31.1	8.8	20	4.4	11.1	0	2.22

pH:

Most of the isolates showed maximum growth at pH 6 and 7 (Fig. 3.29 and table 3.6). However, considerable growth could be recorded at a pH range 4-9 (Appendix 1, table 3.9).

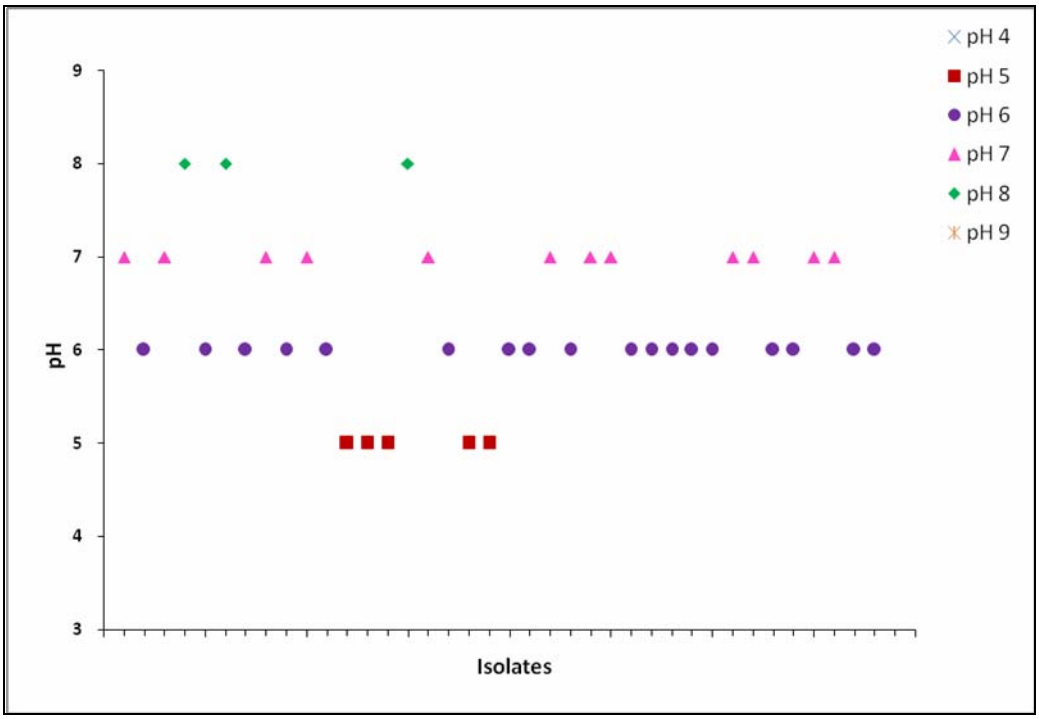

Fig. 3.29 Optimum pH for the growth of various yeast isolates

Table 3.6 Percentage of isolates having maximum growth at different pH in three depths

Depth (m)	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9
200	0	12.5	50	31.25	6.25	0
500	0	5.88	52.94	29.41	11.76	0
1000	0	40	20	40	0	0
Total (%)	0	13.15	47.36	31.57	7.89	0

3.4 Discussion

Candida exhibited a wide distribution, as it was found to be present in all the three depth regions and also it was the dominant genus in most of the cases. This agrees with the previous studies from different marine ecosystems where *Candida* was encountered in almost all the cases (Fell et al., 1960; Yamasato et al., 1974; Paula et al., 1983; Prabhakaran and Ranu Gupta, 1991; MacGillivray and Shiaris, 1993; Rishipal and Philip, 1998; Takami et al., 1998; Loureiro et al., 2005*;* Sarlin, 2005). The occurrence of *Rhodotorula*, which belonged to the class Basidiomycetes, increased as the depth increased. This finding is in agreement with the statement, yeasts in the class Ascomycetes (eg. *Candida, Debaryomyces, Kluyveromyces*, *Pichia* and *Saccharomyces*) are common in shallow waters, whilst yeasts belonging to Basidiomycetes (*Cryptococcus, Rhodosporidium, Rhodotorula, Sporobolomyces*) are common in deep waters. *Rhodotorula* had been isolated from a depth of 11,000 m by Munn (2004). Some of the common genera isolated during many studies include *Candida, Rhodotorula, Cryptococcus, Debaryomyces* etc. (Fell et al., 1960; Yamasato et al., 1974; Kohlmeyer and Kohlmeyer, 1979; Paula et al., 1983; Lakshmi, 2005). These genera were obtained from almost all the depths in this study. Diversity was found to be maximum at 500 m depth region in the Arabian Sea and 1000 m depth in Bay of Bengal. But dominance was found to have an inverse relationship with the diversity index. This shows the stability of the ecosystem at higher depth compared to shallower regions where pollution and anthropogenic alterations cause dominance of specific groups resulting in low evenness and diversity. Encountering newer genera as the depth increases shows possibilities of occurrence of novel organisms in greater depths. This increasing trend towards the bathy benthic region denotes scope for getting novel species from deep ocean regions.

Among the isolates oxidative forms were more in abundance than the fermentative forms. Studies by Fell (1965), revealed that yeasts found in aquatic environments are generally asporogenous and oxidative or weakly fermentative.

Hagler and Mendonca (1981) studied that oxidative yeasts are seen in clean waters and fermentative ones in polluted waters.

All the yeast isolates were lipolytic which indicate the presence of lipid matter and the cycling process of lipid moieties in the sampling region. Studies by Paskevicus (2001) showed that almost all the yeast strains produce lipase. Lipases are the most important biocatalysts and have wide variety of industrial applications. Yeast lipases draw special attention, as these organisms are considered very safe and are consumed by human population since decades (Vakhlu and Kaur, 2006). Lipases from *Yarrowia lipolytica* was found to have applications in bioremediation of environments contaminated with aliphatic and aromatic compounds, organic pollutants, 2,4,6-trinitrotoluene, and metals. Also they are industrially important in synthesis of β-hydroxy butyrate, l-dopa, and emulsifiers (Bankar et al., 2009). The extracellular enzymes play important role in various industrial processes and also in the environment. Crude amylase from *Saccharomycopsis fibuligera* A11 was found to convert cassava starch actively into monosaccharides and oligosaccharides (Chen et al., 2009). Yeast proteases have many applications in detergents, leather processing, feeds, chemical industry as well as waste treatment (Ni et al., 2008). Ligninolytic enzymes from yeasts are not commonly studied. Studies by Villas Boas (2002), shows that the yeast strain *Candida utilis* has lignocellulose degrading ability. Urease is a nickel containing enzyme that catalyses the hydrolysis of urea. Urease has many industrial applications like in diagnostic kits for determination of urea in blood serum, in alcoholic beverages as a urea reducing agent and in biosensors of haemodialysis systems for determining blood urea (Bakhtiari et al., 2006). The enzyme production potential showed that the isolates are truly versatile agents of biodegradation. Different enzymes from terrestrial microbes have been proved to have potential applications in various industries (Chi et al., 2009), yeasts from marine environments are also proved to be a good source of enzymes with

unique properties. As marine ecosystem is the largest in the world, this need to be explored for novel bioactive compounds.

This is the first report of isolation of black yeasts from Indian waters. They were found to be highly versatile agents of biodegradation since cent percent of them produced protease and lipase. They were also noted for the production of amylase, ligninase and urease. Their role in the biogeochemical cycling of elements would be worth investigating.

The present study highlights the importance of black yeasts as a potent source of extracellular enzymes. Further studies on the group of yeasts especially with regard to bioprocess technology for enzyme production followed by enzyme characterization will be highly rewarding. Phylogenetic analysis of these groups would also be highly important to derive evolutionary relationship with other groups of yeasts.

In general, the isolates were able to grow considerably at a temperature range of 20-40°C. But, for almost all the isolates the maximum growth was observed at 30°C. Even though these isolates were obtained from a marine realm where the temperature ranged between 6- 16°C, these organisms preferred the ambient room temperature (28±2°C) for their growth. Notably, the isolates were able to grow in a wide range of salinities, with the optimum between 15- 25 ppt for most of the isolates. Roth et al. (1962) stated that almost all the yeasts were able to grow at wide range of NaCl concentrations. Salinity tolerance does not distinguish marine species from terrestrial species because almost all yeasts can grow in sodium chloride concentrations exceeding those normally present in the sea. The isolates were all able to grow at a pH range of 4-9, but the optimum for most of the isolates was 6 and 7. Yeasts generally prefer a slightly acidic pH, which was evidenced in the case of these marine isolates also.

Generally *Candida* was the predominant organism in the slope sediments and black yeasts were encountered in considerable number. An increase in

diversity at 500 m depth region in the Arabian Sea was notable. However, in Bay of Bengal the diversity increased at higher depths. Hydrolytic enzyme production was higher among isolates from Bay of Bengal where the organic matter is reported to be low. Most of the isolates preferred 30^{o}C, pH 6 and 15 ppt salinity for maximal growth. The physico-chemical data for maximal growth points to the possibility of these isolates to be of terrestrial origin which got adapted to the marine habitat.

Chapter 4

DEGRADATION OF CRUDE OIL BY SELECTED MARINE YEASTS

Contents

4.1 Introduction

Environmental pollution by petroleum hydrocarbons and its adverse effects are among the most ominous problems that the world is grappling today. Past century witnessed a global surge in oil pollution due to industrial development, urbanization and so forth. Microbial degradation is one of the foremost routes in the natural removal of these oil products from contaminated environments. However, since these compounds are largely recalcitrant, they are not readily degraded by microbes in nature.

A rational approach to decontaminating an environment loaded with petroleum derivatives is the adoption of procedures based mainly on the metabolic activities of microorganisms (Leahy and Colwell, 1990; Galas et al., 1997; Yuan et al., 2000). Biodegradation is of crucial ecological significance as this contributes to bioremediation processes.

Advances in biotechnology have confirmed that several compounds present in petroleum hydrocarbons are consumed by microorganisms as sole source of carbon. These hydrocarbons are both a target and a product of microbial metabolism. It has been proved that the addition of nitrogen and phosphorous substantially enhances the growth of hydrocarbon degrading microbes (Atlas and Bartha, 1972).

Reducing the hydrocarbon in a contaminated environment is a significant challenge. Mechanical and chemical methods have limited effectiveness and they can be expensive. Several researchers have studied the use of microbes to decompose petroleum products and have demonstrated this to be a promising technological alternative (Grishchenkov et al., 2000; Kaluarachchi et al., 2000; Diaz et al., 2000; Bielicka et al., 2002; Gogoi et al., 2003; Lakha et al., 2005). Microbiological activity is affected by a number of environmental factors such as energy sources, donors and acceptors of electrons, nutrients, pH, temperature etc.

These parameters influence how quickly microorganisms adapt to the environment (Vieira et al., 2007).

4.1.1 Biodegradation of petroleum hydrocarbons

After an oil spill, hydrocarbons undergo certain physicochemical processes in nature, such as evaporation or photochemical oxidation which changes oil composition. But the most important process is biodegradation. Biodegradation is the process by which organic substrates are broken down by other living organisms. The term is often used in relation to ecology, waste management, environmental remediation (bioremediation), and to plastic materials due to their long life span. Organic material can be degraded aerobically (with oxygen) or anaerobically (without oxygen).

During oil degradation, the fluid properties of oil change because different classes of compounds in petroleum have different susceptibilities to biodegradation (Goodwin et al., 1983). The early stages of oil biodegradation are characterized by the loss of n-paraffins (n-alkanes or normal alkanes) followed by the removal of acyclic isoprenoids (eg., norpristane, pristine, phytane etc.). Compared with these compound groups, other compounds (eg., highly branched and cyclic saturated hydrocarbons as well as aromatic compounds) are more resistant to biodegradation. However, as biodegradation proceeds, even the more resistant compounds are eventually destroyed.

Microbial degradation targets the aliphatic or light aromatic fractions of oil. Several microbial species live on hydrocarbons and are responsible for the biodegradation of crude oil. Inoculating hydrocarbon degrading organisms and then adding nitrogen and phosphorous fertilizers to support their growth is being explored as an effective strategy in oil slicks. For microbes to act on oil, it needs to be brought to a soluble physical state. Fungi and bacteria are the key agents which decompose oil and oil products. Besides, cyanobacteria, yeast and algae have been shown to oxidize hydrocarbons. As oil is insoluble in water and is less

dense, it floats on the surface and forms slicks or oil films. Hydrocarbon oxidizing microbes develop rapidly in such films (Rajendran and Gunasekaran, 2006). Isolation and identification of microorganisms responsible for hydrocarbon transformations have long been acknowledged as pivotal from both fundamental and applied points of view and lists of hydrocarbon-degrading organisms like bacteria, yeasts, fungi and algae are available (Atlas, 1981; Leahy and Colwell, 1990; Rosenberg, 1992; Atlas and Cerniglia, 1995).

In addition to their chemical nature, the surface area and hydrophobicity of the compounds contained in crude oil affect bioavailability and hence biodegradability of oil. Owing to variability in the hydrocarbon composition of crude oil, microbes develop several adaptation mechanisms to utilize a range of hydrocarbons as substrate. Excretion of biosurfactants that emulsify crude oil is a major mechanism employed by microbes to enhance bioavailability.

Psychrophilic microbes have been shown to be adapted to degrade hydrocarbons, as the enzymes produced by these organisms are more thermolabile than those of mesophilic microbes and have higher activities at lower temperatures (Whyte et al., 1996). Distinct communities act in co-operation for the degradation of various contaminants, for e.g. one community may initiate the degradative reaction and transform or partially degrade the compound, while a second community completes the transformation (Budwill et al., 1997).

The first step in the biodegradation of hydrocarbons in oil is often specific to particular to the microbial species or strain. The preference for specific compounds and the mode of degradative action, i.e. primary or co-metabolic, are dependent on the microbial community and the organic compounds present. Some hydrocarbons are more readily biodegraded in the presence of other hydrocarbons than when present as sole carbon sources (Smith, 1990).

The microbial degradation of hydrocarbons is primarily an aerobic process, and anaerobic degradation is very slow (Jackson et al., 1996). The oxidation of

hydrocarbons takes place through the formation of fatty acids from alkanes and of hydroxyl derivatives from aromatic hydrocarbons (Hambrick et al., 1980). In aerobic conditions, the complete mineralization of organic compounds results in the formation of carbon dioxide and water, some organic carbon being incorporated into the biomass. In anoxic conditions, volatile fatty acid formation is the first indication of anaerobic degradation. In this case, complete mineralization results in the formation of carbon dioxide and methane.

Many studies have shown that low molecular weight alkane and aromatic compounds are readily biodegraded (Leahy and Colwell, 1990; Blackburn et al., 1993; Huesemann, 1995). Branching and an increase in ring structure decrease the biodegradability. The high molecular weight polycyclic aromatic hydrocarbons (PAHs) and cycloalkanes are highly recalcitrant to biodegradation (Blackburn et al., 1993; Chaineau et al., 1995).

Certain hydrocarbons are more readily degraded than others and environmental factors such as temperature and composition influence the ability of microbes to degrade petroleum hydrocarbons. Jobson et al. (1972) have reported that both low and high grade crude oils are subjected to microbial degradation at 4 to 30^{o}C.

Bioremediation refers to any process that uses microbes (microorganisms) to recycle organic materials and sequester inorganic ions. Because the primary responsibility of microbes is to recycle organic materials, they must be present in sufficient quantities and diversity in order to accomplish this. Under carefully controlled conditions, bioremediation can be a practical and cost effective method to remove hydrocarbons and other organics from contaminated surfaces and sub-surfaces. Bioremediation cleans up the environment by allowing living organisms to degrade or transform hazardous organic contaminants.

Several authors have studied hydrocarbonoclastic organisms and the occurrence of yeasts in oil spill sites. Le Petit et al. (1970) studied oil-polluted

littoral marine areas in the Mediterranean and found seven species which were able to metabolize hydrocarbon fractions. From non-polluted test sites, only one hydrocarbonoclastic species was isolated. Biodegradation was very slow and authors concluded that yeasts probably play only a minor role in the elimination of hydrocarbons from the sea. Ahearn et al. (1971) tested selected yeasts isolated from oil-polluted habitats for their ability to use hydrocarbons as sole source of carbon. *Trichosporon* species was found to emulsify the oil. The responses of yeast population to oil pollution were investigated by Ahearn and Meyers (1972). Plots of a *Spartina alterniflora* salt marsh in Louisiana were selected as test areas saturated with oil. Compared with adjacent control sites, a considerable increase in yeast densities was noticed in the oil-soaked plots, and the predominant yeasts of the marshland were replaced by hydrocarbonoclastic strains, especially *Pichia ohmeri* and *Trichosporon* sp. In the nutrient rich sediments of the estuary, population of yeasts continued to increase in the presence of oil. In offshore areas, however, yeast population declined after an initial increase, perhaps due to lack of nutrients and vitamins. It is suggested that the tested organisms may have relatively low capacity to decompose crude oil at oil spillage sites. In general, yeasts isolated from oil-polluted regions exhibited much higher hydrocarbonoclastic property than the same species from non-polluted areas.

Microorganisms are the key agents in the degradation of petroleum hydrocarbons. The organisms include bacteria, yeast, filamentous fungi and algae (Atlas, 1981). The ecology of hydrocarbon degradation by microbial population in the natural environment is reviewed, emphasizing the physical, chemical and biological factors that contribute to the biodegradation of petroleum and individual hydrocarbons. Rates of biodegradation depend greatly on the composition, state, and concentration of the oil or hydrocarbons, with dispersion and emulsification enhancing rates in aquatic systems and absorption by soil particulates being the key feature of terrestrial ecosystems. Salinity and

pressure may also affect biodegradation rates in some aquatic environments, and moisture and pH may limit biodegradation in soils. Adaptation by prior exposure of microbial communities to hydrocarbons increases hydrocarbon degradation rates (Leahy and Colwell, 1990).

Studies done by Lindstorm et al. (1991) shows that fertilizer application on oil spilled sites enhances the growth of hydrocarbon degrading microbes and in turn gives a better rate of oil biodegradation. Hydrocarbon utilizing microorganisms were isolated by enrichment techniques from soil and water samples collected from an oil spill site in the Niger delta area of Nigeria. The isolates included species of *Micrococcus, Pseudomonas, Bacillus, Aeromonas, Serratia, Proteus, Penicillium, Aspergillus, Candida* and *Geotrichum.* Oil biodegradation potentials of the isolates were evaluated using Nigerian Forcados blend crude oil as sole carbon source in mineral salts medium. There were quantitative changes in the oil content due to microbial degradative activities. Degradation was more pronounced with mixed cultures than with single cultures of the microorganisms (Benka-Coker and Ekundayo, 1997). Five microorganisms - three bacteria and two yeasts - capable of degrading Tapis light crude oil were isolated from oil-contaminated soil in Bangkok, Thailand. Crude oil degradation was measured by gas chromatography. The yeast strains were identified as *Candida tropicalis* strains 7Y and 15Y and found to be efficient oil degraders (Palittapongarnpim et al., 1998). The relative capabilities of two bacterial isolates *Serratia marcescens* OCS-21 and *Acinetobacter calcoaceticus* COU-27 from Ebubu oil polluted soil of Rivers state, Nigeria and a yeast isolate, *Candida tropicalis* PFS-95 from unpolluted soil of the University of Calabar campus, in degrading transniger pipeline crude oil were investigated. The yeast isolate showed more proficiency in degrading the crude oil than either of the bacterial counterparts. Results revealed that crude oil components of chain length C_{12} to C_{32} were extensively degraded by the yeast after 16 days of incubation (Ijah, 1998).

Hydrocarbon degraders from tropical marine environments were studied by Zinjarde and Pant (2002). Yeasts isolated from marine mud and water around Mumbai was able to degrade more than 10% of the supplied crude oil. The yeast strains were important degraders of the aliphatic fraction of crude oil, and all the isolates belonged to the genus *Candida*. None of the isolates degraded the aromatic or asphaltene fractions. All the isolates required aeration and nitrogen and phosphate supplementation for optimal degradation. Palm oil mill effluent treatment by tropical marine yeast was studied by Oswal et al. (2002). Palm oil mill effluent (POME), when released into local rivers or lakes without treatment, is an important cause of inland water pollution. POME contains lignocellulolytic wastes with a mixture of carbohydrates and oil. Treatment of this effluent using *Yarrowia lipolytica* NCIM 3589, a marine hydrocarbon-degrading yeast isolated from Mumbai, India, gave a COD reduction of about 95% with a retention time of two days.

Studies conducted by Jain et al. (2004) showed that *Yarrowia lipolytica* NCIM 3589, a tropical marine degrader of hydrocarbons and triglycerides transformed 2, 4, 6-trinitrotoluene (TNT) very efficiently. TNT is a man made explosive which also has industrial applications. TNT has been a priority pollutant with top recommendation for removal from contaminated sites since it is toxic to living forms (Letzel et al., 2003). *Yarrowia lipolytica* is a non-pathogenic aerobe isolated from environments rich in hydrophobic substrates. Though this yeast could not utilize TNT as a sole carbon or nitrogen source, it was capable of reducing the nitro groups in TNT to aminodinitrotoluene (ADNT). Awe et al. (2008) studied the degradation of phenyl alkanes, which constitute up to 28% of refined gasoline fuel oil, by the hydrocarbon utilizing yeast *Candida maltosa*. There is an accumulation of acidic intermediate products by the transformation of phenylalkanes, which the organism is unable to oxidize further. The toxic effects of these acidic products amplified by fallen pH values of the growth medium strongly inhibited the metabolism of phenyl alkanes.

4.1.2 Immobilized cells in bioremediation

Modern biotechnology is rapidly developing into a major process industry. Along the way it is confronted with the requirements of large scale industrial processes, mainly with economic and ecologic limitations. This calls for an overall process optimization, including upstream processes, conversion and downstream operations. One of the major possibilities of optimizing biotechnological processes lies in immobilization technologies, which may increase productivity, product concentration, and to some extent, conversion of raw materials.

Immobilization is the means, by which enzymes and cells are transformed into heterogeneous catalysts (Walker and Gingold, 1988). The biocatalyst is confined to a restricted region through which the substrate solution is passed, emerging as a catalyst free product. This approach makes it physically easier to control the environment in the immediate vicinity of the reaction and can dramatically increase the process intensity by concentrating all activity into a limited space.

Initial research into immobilization technology concentrated on single enzyme systems requiring no cofactors, the enzymes studied being predominantly those that catalyze degradative reactions (Hornby et al., 1968). The limitations of the practical applicability of such systems have led workers to study the immobilization of those enzymes requiring cofactors (Wilson et al., 1968), and interest has been shown in immobilizing cofactors (Weibel et al., 1971). Some research has also been reported on multistep cofactors (Mosbach and Mattiason, 1970; Mattiason and Mosbach, 1971). Such systems do not appear attractive as expensive purified cofactor preparations are required and, for a multistep reaction, a complex utilization procedure would be needed to achieve efficient interaction between components of the system. Immobilized whole cell preparations have been used for the catalysis of single step reactions, in particular for the isomerisation of glucose (Vieth et al., 1973), and research has been reported on the

utilization of immobilized cells to carry out a series of reactions requiring cofactors and it has obvious advantages in terms of the required isolation and purification procedures and in terms of interactive efficiency.

At present, cell immobilization technology is often studied for its potential to improve fermentation processes and bioremediation (Beshay et al., 2002; Abd-EL-Haleem et al., 2003). Immobilization of whole cells for the production of extracellular enzymes offer many advantages such as the ability to separate cell mass from the bulk liquid for possible reuse, facilitating continuous operation over a prolonged period and enhanced reactor productivity (Zhang et al., 1989; Galazo and Bailey, 1990).

Several important advantages of immobilized cell technology over traditional batch free-cell systems, such as possibility of continuous operation, high cell densities, improved resistance against contamination and high fermentation rates have been explained by Pilkington et al. (1998), Nedovic et al. (2001) and Virkajarvi (2002). Immobilization of microorganisms for enzyme production offers various advantages, such as decreasing contamination of the product by free cells, eliminating the contaminants from the stream in continuous fermentations without loss of biomass (Helmo et al., 1985), increasing the productivity and the operational stability or reducing the delays involved in enzyme production (Kokubu et al., 1981; Fujimura et al., 1984).

Cell immobilization has been applied to numerous food fermentations using a wide variety of bacteria, yeasts, molds and plant cells (Groboillot et al., 1994; Lacroix et al., 2003). Shapiro and Dworkin (1997) have demonstrated enhanced cell-to-cell signaling and cell-matrix interactions leading to coordinated behaviour of immobilized microorganisms. Immobilization of *Acinetobacter* spp. has been investigated using alginate (Muyima and Cloete, 1995) or ceramic (Kariminiaae-Hamedaani et al., 2003) carriers. Calcium and aluminium alginate gels have previously been used for the immobilization of cells and enzymes

(U.S. Patent 3,733,205, May 15th, 1973; Hackel et al., 1975). Besides the synthetic carriers, natural zeolite has been shown as a promising material for the immobilization of microorganisms due to its high porosity and large surface area (Shindo et al., 2001). The extent of bacterial colonisation depends on the chemical properties and particle size of zeolite.

In the immobilization of microbial biomass, porous and microporous carriers are frequently used. Their advantages include great porosity, mechanical stability, potentially high endurance of the carrier to immobilized mass and a short diffusion distance between the outer and the inner surface of the carrier. Carriers enhance the activity of their intracellular enzymes e.g. lipase and proteases. In addition, such enzymes retain the substrate specificity and enzymatic activity for a longer time and the immobilized biocatalysts are easier to handle in continuous processes. Mostly used carriers include DEAE cellulose, porous glass, silicon carbide and recently wood chips. These carriers provide simple immobilization procedure and good mechanical properties but are limited by relatively low cell concentrations and significant cell leakage. Porous matrices provide an alternative solution providing higher cell concentrations and better cell retention.

When immobilized cells are transferred into a growth medium, limitations on the diffusion of substrates and products result in the formation of a high-cell density layer extending from the bead surface to the radial depth where lack of substrate or accumulation of inhibitory product and local physicochemical conditions prevent growth (Champagne et al., 1994; Doleyres et al., 2002; Lacroix et al., 2003). Cell release from gel beads in the liquid medium occurs spontaneously because of active cell growth in the high biomass-density peripheral layer.

The most crucial stages in the process of obtaining immobilized biocatalysts include: the selection of strains with specified substrate activity, the selection of immobilization technique and the carrier properties e.g. porosity, charge etc. However, proper selection of immobilization techniques and supporting materials

is primarily required to minimise the disadvantages of immobilization. One of the most suitable methods for immobilization is entrapment in calcium alginate, since this technique is simple and cheap. Sodium alginate is readily available and it is a non-toxic biological material. Therefore, it is suitable as an immobilization matrix for biomolecules and microorganisms (Mattiasson, 1983). Beads of calcium alginate are prepared under mild conditions and have been used extensively for microencapsulating and entrapping cells.

Because of their high affinity for water, total lack of toxicity and the ability to form viscous solutions and gels, alginates have long been widely used in the food industry. More recently, their ability to form gels very rapidly in the presence of calcium ion under extremely mild conditions has been exploited for immobilization of particulate enzymes (Hussain et al., 1985) and many different kinds of living cells (Nilsson and Mosbach, 1980; Kopp and Rehm, 1983; Jain and Ghose, 1984; Draget et al., 1988; Smidsrød and Skjak-Braek, 1990). Alginates are glycuronans extracted from seaweeds. The molecules are linear chains of (1-4)-linked residues of β-D-mannuronic acid and α-L-guluronic acid in different proportions and sequential arrangements. Martinsen et al. (1989) and Smidsrød and Skjak-Braek (1990) have reported some relevant properties of calcium alginate gels, including mechanical rigidity, swelling and shrinking characteristics and resistance to interference by monovalent cation.

4.1.3 Molecular Characterization of Yeast

Fell and Kurtzman (1990) reported the nucleotide sequence analysis of a variable region of the large sub unit rRNA for identification of marine occurring yeasts. The data suggests that large subunit sequences can be used for yeast identification with possible exception of closely related homothallic species. The D1/D2 variable region of the large subunit rRNA was examined for nucleotide sequence signatures as potential taxonomic tools (Fell et al., 1992; Fell and Blatt, 1999; Diaz and Fell, 2000).

The isolation and purification of DNA is a key step for most protocols in molecular biology studies (Sambrook et al., 1989). Several DNA extraction methods are widely used to isolate DNA from yeast including phenol extraction but they often involve multiple, time consuming steps including the handling of toxic chemicals (Ausbel et al., 1995). An efficient, inexpensive method for obtaining yeast genomic DNA from liquid cultures or directly from colonies was developed by Harju et al. (2004). This protocol circumvents the use of enzymes or glass beads, and therefore is cheaper and easier to perform when processing large number of samples.

Coding regions of the 18S, 5.8S and 28S rRNA genes evolve slowly, are relatively conserved among fungi, and provide a molecular basis of establishing phylogenetic relationships. Between coding regions are the internal transcribed spacer 1 and 2 regions (ITS1 and ITS2 respectively) which evolve more rapidly and therefore vary among different species within a genus. From the conserved sequences of 18S and 28S rRNA genes at the ends of the ITS region two universal primers ITS1 (F) and ITS4 (R) were designed by White et al. (1990). This amplifies a fragment approximately 580 bp containing ITS 1, 5.8S and ITS 2 region are widely used for the purpose. PCR based detection of fungal DNA sequences can be rapid, sensitive and specific (Makimura et al., 1994). More recently, the differences in the rRNA internal transcribed spacer have been used to identify yeast species. Differentiation of closely related species requires analysis of both D1/D2 and ITS regions (Fell, 2001).

Gadanho and Sampaio (2004) studied the application of temperature gradient gel electrophoresis to the study of yeast diversity in the estuary of the Tagus River, Portugal. The molecular detection of yeast was carried out directly from the water samples and in parallel; a cultivation approach by means of an enrichment step was employed. A nested PCR was employed to obtain a fungal amplicon containing the D2 domain of the 26S rRNA gene. For identification the TGGE bands were extracted, re-amplified, and sequenced. Differentiation of

strains within a species can play a significant role in ecological population analysis. Phylogenetic analysis based on molecular sequencing of the D1/D2 domain of 26S rDNA (Botes et al., 2006; Lopandic et al., 2006), internal transcribed spacer (ITS) regions and 5.8S rRNA gene has been used to investigate the intraspecific relationships among the isolates (White et al., 1990; Fell et al., 2000; Nagahama et al., 2001; Porteous et al., 2003; Fell et al., 2004; Rodriguez-Tudela et al., 2005).

4.2 Materials and Methods

4.2.1 Yeast isolates used for the study

Isolates which showed good clearance zone on tributyrin agar plates (section 3.3.3) were selected for testing oil degrading potential. The isolates thus selected were SD 302, SD 328, SD 332, SD 337, SD 341a, SD 378, SD 398a, SD 416, SD 429, SD 430, SD 440, SD 449, SD 450, SD 454, SD 480 and SD 483 (16 Nos.).

4.2.2 Screening of oil degrading strains

A mineral basal medium incorporated with heavy crude oil (BPCL-Kochi refinery, Ambalamughal) was used for screening oil degrading isolates. The isolates were inoculated into mineral basal medium supplemented with heavy crude oil. The flasks were incubated at 28±2^{o}C in a shaker at 100 rpm for three weeks with frequent observation. A control flask with crude oil was also maintained.

Mineral basal medium:

$(NH_4)_2SO_4$	-	0.35 g
KH_2PO_4	-	0.0085 g
K_2HPO_4	-	0.0015 g
$MgSO_4.7H_2O$	-	0.005 g
Seawater (30 ppt)	-	100 ml
pH	-	7.8

Based on the visual observation of the medium for oil degradation, two potential isolates, *Candida* sp. SD 302 and *Pichia* sp. SD 337 were selected for further analysis.

4.2.3 Identification of the selected marine yeast isolates

Potential isolates were identified using molecular techniques by extracting genomic DNA and sequencing of ITS region as per Harju et al. (2004).

Yeast genomic DNA isolation:

Pure yeast colonies were inoculated into rich media containing 1% yeast extract, 2% peptone and 2% dextrose, and incubated for 18 hrs. Cells from 1.5 ml of the overnight cultures were pelleted in a microcentrifuge tube and the cell pellets were resuspended in 200 µl of lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)). The tubes were placed in a -80°C freezer for two minutes (until they were completely frozen), then immersed in a 95°C water bath for 1 minute to thaw quickly. The process was repeated twice, and the tubes were vortexed vigorously for 30 seconds. 200 µl of chloroform was added and the tubes were vortexed for 2 minutes and then centrifuged 3 minutes at room temperature at 20,000 x g. The aqueous layer was transferred to a tube containing 400 µl of ice-cold 100% ethanol. The samples were allowed to precipitate for 5 minutes at room temperature and then centrifuged at 20,000 x g. Supernatant was discarded and DNA pellets were washed with 0.5 ml of 70% ethanol followed by vacuum drying for 5 minutes at 60°C. DNA was resuspended in 20 µl TE (10 mM Tris and 1 mM EDTA, pH 8.0) and stored at 4°C for future use. DNA concentration and purity were assessed spectrophotometrically by comparing absorbance at 260 nm and 280 nm and 0.8 % agarose gel electrophoresis.

Optical density of the DNA was taken at both 260 nm and 280 nm and the concentration of DNA was found out from the following formula:

Conc. of DNA (µg/ml) = OD at 260 nm x 50 x dilution factor.

Amplification of the extracted DNA:

ITS (Internal Transcribed Spacer) sequences are considered to be the best tool for rapid and accurate identification of yeast isolates. ITS primers (Forward ITS 1-5' TCC GTA GGT GAA CCT GCG G 3' and Reverse ITS 4- 5' TCC TCC GCT TAT TGA TAT GC 3') by White et al. (1990) which amplify a fragment of approximately 580 bp containing the ITS 1, 5.8 S and ITS 2 regions was used for the purpose.

The amplification reaction was performed by using a DNA thermal cycler (Eppendorf). PCR was performed in a final volume of 25 µl containing 2.5 µl of 10X buffer, 1.5 µl 25 mM $MgCl_2$, 1.0 µl of 10 pmol of each oligonucleotide primer, 2 µl of 2.5 mM each deoxynucleoside triphosphate, 1 µl of the extracted DNA of concentration 600 ng/µl, 1 µl of Taq DNA polymerase and 16 µl of Milli Q water. After an initial denaturation at 95°C for 5 minutes, amplification was made through 30 cycles, each consisting of a denaturation at 94°C for 1 minute, annealing at 56°C for 45 seconds, extension step at 72°C for 1 minute and a final extension at 72°C for 10 minutes. The PCR products were analyzed by electrophoresis on 1% agarose gel prepared in 1X TAE buffer and stained with ethidium bromide.

Cloning into pGEM-T Easy vector:

Fresh PCR product of ITS gene was used for cloning into the pGEM-T Easy vector (Promega, USA). The ligation mix (10 µl) consisted of 5 µl ligation buffer (2X), 0.5 µl of the vector (50 ng/µl), 3 µl of PCR product and 1 µl of T4 DNA ligase (3U/µl). The ligation mix was incubated overnight at 4°C. The entire ligated mix was used to transform *Escherichia coli* JM 109 competent cells prepared using calcium chloride method. The ligation mix was added to 10 ml glass tube previously placed in ice to which 50 µl of competent cells were added and incubated on ice for 20 min, a heat shock at 42°C was given for 90 seconds, immediately the tubes were placed on ice for 2 min and then 600 µl of SOC medium was added and incubated for 2 hr at

37°C in an incubator shaker at 250 rpm. The transformation mixture (200 µl) was spread on Luria-Bertani (LB) agar plates supplemented with ampicillin (100 µg/ml), IPTG (100 mM), and X-gal (80 µg/ml). The plates were incubated at 37°C overnight. The clones were selected using blue/white screening. The white colonies were selected and streaked to purify on LB-Amp+X-gal+IPTG plates and incubated overnight at 37°C. To confirm the insert, colony PCR of the white colonies were carried out using the universal vector primers T7 (5'-TAATACGACTCACTATAG GG-3') and SP6 (5'- GATTTAGGTGACACTATAG-3'). White colonies (template) picked from the transformed plate were dispensed into the PCR reaction mix (25 µl) containing 2.5 µl 10X PCR buffer, 2.0 µl of 2.5 mM dNTPs, 1 µl of 10 p mol/µl of T7 and SP6 primers, 0.5 U of taq polymerase and the remaining volume was made up with Milli Q. The thermal cycling conditions were as follows: 95°C for 5 minutes; 35 cycles of 94°C for 15 seconds, 57°C for 20 seconds, 72°C for 60 seconds; 72°C for 10 minutes following which the temperature was brought down to 4°C.

Plasmid extraction & Purification:

Plasmid extraction and purification was done using Sigma plasmid miniprep kit.

Cells were harvested by centrifuging 2 ml of overnight recombinant *E.coli* culture at 16,000 x g. Resuspended the pellet in 200 µl resuspension solution with RNAse. Lysed the resuspended cells by adding 200 µl of the lysis buffer. Immediately mixed the contents by gentle inversion until the mixture becomes clear and viscous. Precipitated the cell debris by adding 350 µl of the neutralization buffer. Gently inverted the tube and the cell debris were pelleted by centrifuging at 16,000 x g for 10 minutes. Column was prepared by inserting a Gen Elute HP Miniprep Binding column into a provided microcentrifuge tube. Added 500 µl of the column preparation solution to miniprep column and centrifuged at 16,000 x g for 1 minute. Discarded the flow through liquid. Transferred the cleared lysate to the column and centrifuged at 16,000 x g for 1

minute. Discarded the flow through liquid. Added 500 µl of the wash solution 1 to the column and centrifuged at 16,000 x g for 1 minute. Discarded the flow through liquid. Added 750 µl of the wash solution 2 to the column and centrifuged at 16,000 x g for 1 minute. Discarded the flow through liquid. Centrifuged at 16,000 x g for 1 minute to remove excess ethanol. Transferred the column to a fresh collection tube. Added 100 µl of elution solution (10 mM Tris-HCl) to the column. Centrifuged at 16,000 x g for 1 minute. The DNA present in the eluate (plasmid DNA) was stored at -20°C.

Sequencing

Nucleotide sequencing was performed using ABI PRISM 3700 Big Dye Sequencer at Chromous Biotech, Bangalore. The primers used were T7 and SP6 (vector primers).

ITS gene sequence similarity & strain identification

Sequenced DNA data were compiled and analyzed. The sequence obtained was first screened for vector regions using 'VecScreen' system accessible from the 'National Centre for Biotechnology Information' (NCBI). After removing the contaminating vector regions, the sequences were matched with the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al., 1990) at NCBI (www.ncbi.nlm.nih.gov).

4.2.4 Optimum physico-chemical conditions for growth

Preparation of inoculum:

Malt extract agar slants were prepared and sterilized at 121.5°C for 15 minutes in an autoclave. The selected yeast strains were streaked on to malt extract agar slants. Incubation was done at room temperature (28± 2°C) for 24 hours. The cells were harvested at the logarithmic phase using 30 ppt sterile sea water. Optical density of the culture suspension was taken at 540 nm in a UV-VIS spectrophotometer (Shimadzu UV-1601). OD was adjusted to 1 by appropriate dilution and this suspension was used as the inoculum.

Preparation of medium:

Temperature

Malt extract broth was prepared in sea water (35 ppt) for studying the influence of temperature on the growth of the isolates.

Salinity

Malt extract broth in triplicate was prepared using sea water of different salinities (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90 and 100 ppt).

pH

Malt extract broth was prepared at different pH 3, 4, 5, 6, 7, 8 and 9.

Media were sterilized by autoclaving at 121°C for 15 min and used for the study.

Inoculation and incubation:

10 µl of 1 OD cell suspension was inoculated into the malt extract broth prepared in triplicate so that the initial OD of the culture medium was 0.001. Incubation was done at room temperature (28 ± 2°C) for 48 hours for pH and salinity. In the case of temperature, incubation was done at 10, 20, 30, 40 and 50°C.

Determination of growth:

Yeast growth was estimated by measuring the optical density at 540 nm using Shimadzu UV-1601 spectrophotometer.

4.2.5 Hydrocarbon utilization by yeast

Bushnell Haas agar (Bushnell and Haas, 1941) was used for studying the utilization of hydrocarbons. It reveals the ability of microorganisms to decompose hydrocarbons.

Bushnell Haas Agar

Magnesium sulphate	-	0.2 g
Calcium chloride	-	0.02 g
Monopotassium phosphate	-	1.0 g
Dipotassium phosphate	-	1.0 g
Ammonium nitrate	-	1.0 g
Ferric chloride	-	0.05 g
Agar	-	20 g
Sea water (35 ppt)	-	1000 ml
pH	-	7±0.2

It is formulated without a carbon source which allows the addition of alternative hydrocarbons. Except hydrocarbon this media contain all other nutrients necessary for the growth of microbes. 1% triphenyl tetrazolium chloride (TTC) was added to the media. This dye is colourless in oxidized form and turns red when reduced by microorganisms, due to the formation of formazan during their cellular metabolism. The isolates were spread plated on Bushnell Haas agar and allowed to absorb on to the agar. Crude oil was layered on the surface of the inoculated agar. After incubation, appearance of pink colonies confirms the utilization of crude oil as sole source of carbon.

4.2.6 Testing the efficacy of potential isolates for oil degradation

The potential isolates *Candida* sp. SD 302 and *Pichia* sp. SD 337 were checked for their ability to degrade crude oil by using suspended cells and immobilized cells.

Suspended yeast cell system:

In the case of suspended yeast cell bioreactor, the isolates were inoculated into mineral basal medium supplemented with heavy crude oil (refer session 4.2.2). The flasks were incubated at 28±2^{o}C in a shaker at 100 rpm for three weeks. A control flask with crude oil was also maintained.

Immobilized yeast cell system:

Cells were entrapped in alginate according to Johnsen and Flink (1986). Sodium alginate was dissolved in boiling water (1.5%, w/v) and autoclaved at 121°C for 15 min. Cells of marine yeast strains *Candida* sp. SD 302 and *Pichia* sp. SD 337 were harvested during the mid-logarithmic growth phase by centrifugation (5000 x g, for 10 min) and the cell pellet thus obtained containing approximately $4x10^{11}$ live cells/g was added to the sterilized alginate solution to give a final concentration of 5% yeast biomass (w/v). The resulting suspension was extruded drop by drop through a hypodermic syringe into a gently stirred 3% (w/v) cold sterile $CaCl_2$ solution to obtain gel beads of approximately 2 mm in diameter. The gel beads of calcium alginate with entrapped yeast cells were cured and hardened in the same solution for 30-60 min assuring the completion of gelling process. The beads were then thoroughly rinsed with sterile physiological saline to remove excess calcium ions and untrapped cells and, stored at 4°C in fresh sterile saline until used.

Oil degrading strains *Candida* sp. SD 302 and *Pichia* sp. SD 337 were immobilized by the above said process. Approximately 5 g of the immobilized beads were inoculated into 100 ml of mineral medium. After 24 hrs the growth and turbidity of the medium was checked and 1 ml of crude oil was added to each flask aseptically. The flasks were incubated at 28 ± 2^{o}C in a shaker at 100 rpm for three weeks. A control flask with crude oil and alginate beads was also maintained. After experiment the beads were recovered from the experimental medium by filtering through a sterile muslin cloth and maintained in fresh medium for testing the operational stability and growth of the organism.

4.2.7 Extraction of oil components from the medium for GC analysis

After 21 days of incubation, the hydrocarbon fractions in the medium was extracted (thrice) with 10 ml dichloromethane, and dried with anhydrous sodium sulphate (Norman et al., 2002). Subsequently, the extracts were

combined in round bottom flasks and evaporated to dryness under vaccum at 45^{o}C using a rotovap. The oil residues were then shaken with 5 ml of n-hexane to precipitate the asphaltene fraction. These samples were subjected to gas chromatography.

Gas Chromatographic analysis of hydrocarbon components

Extracted samples were analyzed using gas chromatograph

Conditions maintained

Detector temperature	-	550°C
Injector temperature	-	151°C
Sample size	-	1µl
Carrier gas	-	Helium

1 µl samples were injected and the composition of various petroleum hydrocarbons were recorded as peaks in the graph. The degradation was assessed by comparing the peak area in the chromatogram of the samples with that of control.

4.3 Results

4.3.1 Screening of yeast isolates for oil degradation

The oil degradation ability of the isolates was assessed by visual observation of the medium in the flasks. Flasks inoculated with *Candida* sp. SD 302 and *Pichia* sp. SD 337 were found with tiny oil particles dispersed in the medium resulting in the browning of the medium (Fig. 4.1). A few other isolates produced oil droplets which got dispersed in the medium on shaking and certain others with very low level dispersion in the medium. The growth of the potential isolates *Candida* sp. SD 302 and *Pichia* sp. SD 337 associated with oil droplets is given in Fig 4.2a-b. The isolates *Candida* sp. SD 302 and *Pichia* sp. SD 337 were able to utilize the hydrocarbon as being evidenced from the formation of formazan (pink coloration of the colonies) on Bushnell Haas agar plates (Fig. 4.3).

Table 4.1 Visual observation of the degradative property of yeast isolates

Isolates	Visual characteristics in medium	Rating
SD 302	**Tiny oil particles dispersed in the medium and browning of the medium**	+++
SD 328	Oil droplets which get dispersed in the medium on shaking	+
SD 332	Oil droplets which get dispersed in the medium on shaking	+
SD 337	**Tiny oil particles dispersed in the medium and browning of the medium**	+++
SD 341a	Oil droplets which get dispersed in the medium on shaking	+
SD 378	Oil droplets dispersed in the medium	++
SD 398a	Oil droplets which get dispersed in the medium on shaking	+
SD 416	Small oil droplets dispersed in the medium	++
SD 429	Small oil droplets dispersed in the medium	++
SD 430	Small oil droplets which get dispersed in the medium on shaking	+
SD 440	Oil droplets which get dispersed in the medium on shaking	+
SD 449	Oil droplets which get dispersed in the medium on shaking	+
SD 450	Small oil droplets which get dispersed in the medium on shaking	+
SD 454	Small oil droplets which get dispersed in the medium on shaking	+
SD 480	Oil droplets which get dispersed in the medium on shaking	+
SD 483	Small oil droplets and particles dispersed in the medium	++

Control ***Candida* sp. SD 302** ***Pichia* sp. SD 337**

Fig. 4.1 Crude oil degradation by selected marine yeast isolates *Candida* sp. SD 302 and *Pichia* sp. SD 337

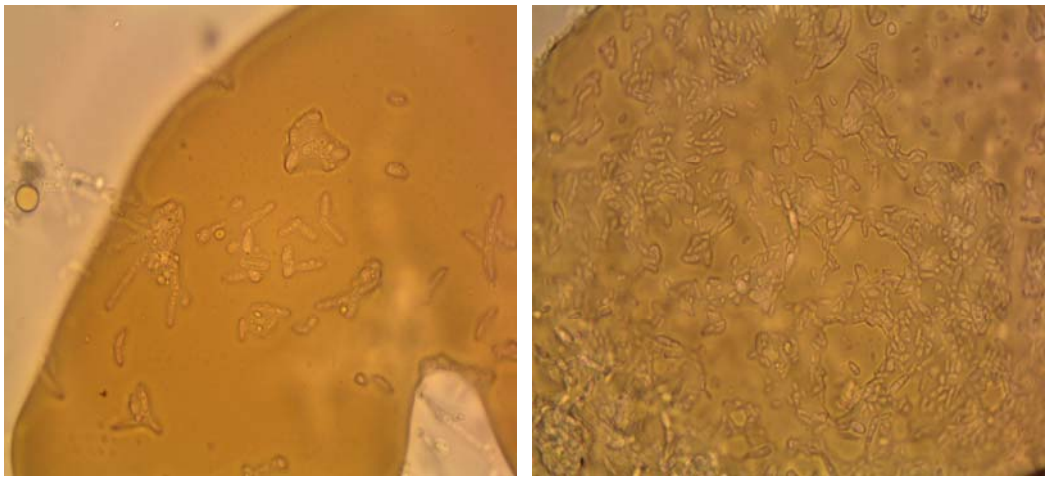

Fig. 4.2a *Candida* sp.SD 302 **Fig. 4.2b** *Pichia* sp. SD 337

Fig. 4.2a- b Microscopic view (100 x) of growth of selected yeast isolates *Candida* sp. SD 302 and *Pichia* sp. SD 337 within oil droplets

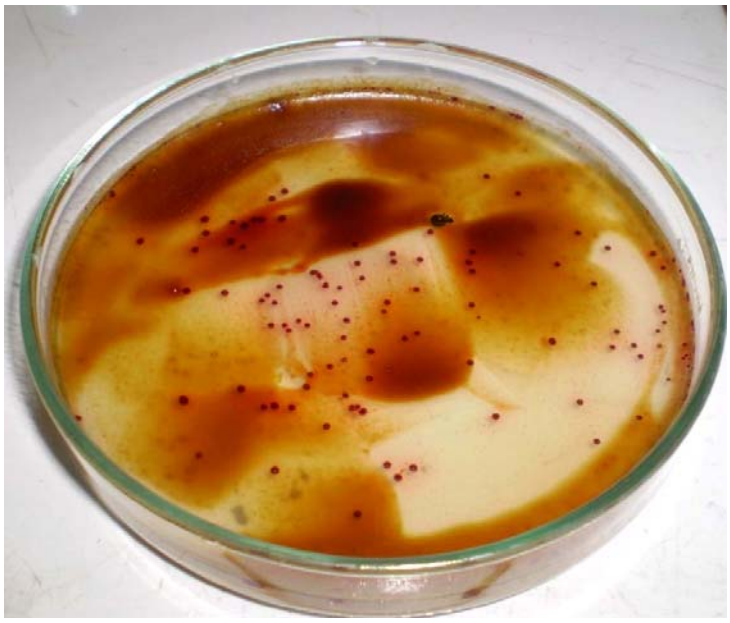

Fig. 4.3 Pink colonies indicating hydrocarbon utilization on Bushnell-Haas agar plate

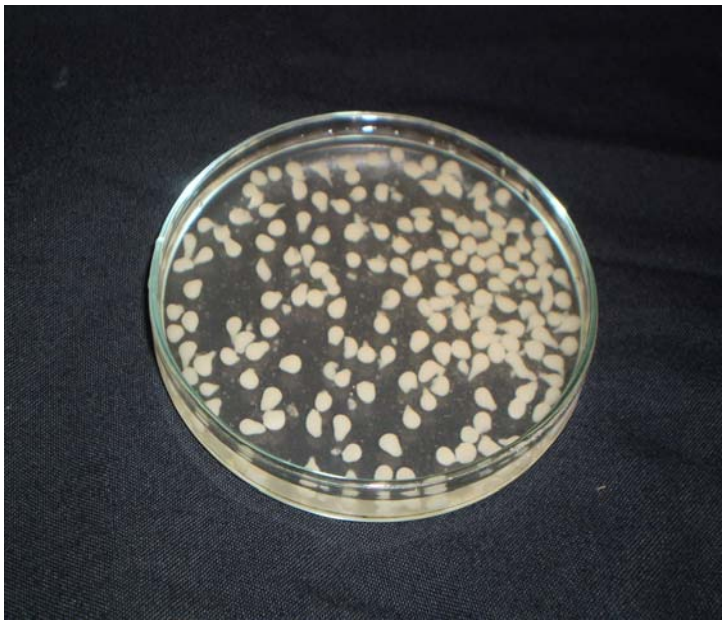

Fig. 4.4 Immobilized cells of the selected marine yeast isolate *Pichia* sp. SD 337

4.3.2 Characterization of potential isolates

The major characteristics of the potential isolates, *Candida* sp. SD 302 and *Pichia* sp. SD 337, are given in the table 4.2.

Table 4.2 Major characteristics of the potential oil degrading isolates

Characteristics	*Candida sp.* SD 302	*Pichia sp.* SD 337
Location of isolation	Arabian Sea	Bay of Bengal
Depth of isolation	1000 m	520 m
Shape of cells	Round	Oval
Asexual reproduction	Budding	Budding
Filamentous/Nonfilamentous	Nonfilamentous	Nonfilamentous
Pigmentation	Nil	Nil
MOF	Oxidative	Oxidative
Urea hydrolysis	Negative	Negative
Nitrate utilization	Negative	Positive
Production of starch like substances	Negative	Negative
DBB	Negative	Negative
Lipase	Positive	Positive
Protease	Negative	Positive
Amylase	Negative	Positive
Ligninase	Negative	Negative
Pectinase	Negative	Negative
Cellulase	Negative	Negative
Chitinase	Negative	Negative
DNAse	Negative	Negative
Aryl sulfatase	Negative	Negative

The nucleotide sequences have been deposited in GenBank (Appendix 3) SD 302 was having 97% similarity with *Candida tropicalis* and SD 337, 99% similarity with *Pichia guilliermondii*. The agarose gel electrophoresis of the genomic DNA (Fig. 4.5a), amplified ITS region (Fig. 4.5b) and the colony PCR product (Fig. 4.5c) of the isolates *Candida* sp. SD 302 and *Pichia* sp. SD 337 are given below. Based on ITS sequencing and BLAST similarity search the two isolates were identified as follows

Culture No.	Identity	GenBank Accession No.
SD 302	*Candida* sp.	GQ387379
SD 337	*Pichia guilliermondii*	GQ334393

Fig. 4.5a Agarose gel electrophoresis of yeast genomic DNA of the isolates *Candida* sp. SD 302 and *Pichia* sp. SD 337

Fig. 4.5b Amplified ITS region of the isolates *Candida* sp. SD 302 and *Pichia* sp. SD 337

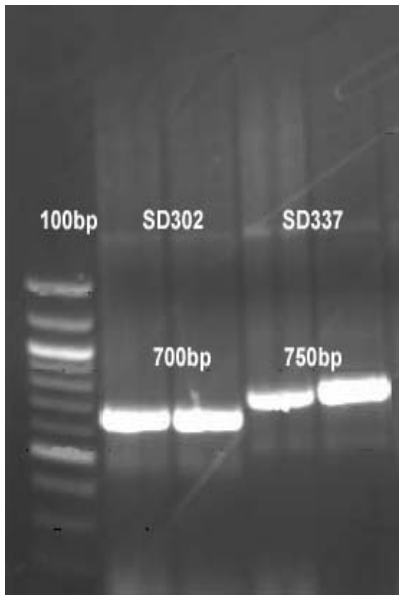

Fig. 4.5c Colony PCR of the ITS clones of *Candida* sp. SD 302 and *Pichia* sp. SD 337

4.3.3 Optimum physico-chemical conditions for growth

The growth of the potential isolates *Candida* sp. SD 302 and *Pichia guilliermondii* SD 337 were checked at different temperatures. Considerable growth was observed at temperatures 20°C, 30°C and 40°C. For *Candida* sp. SD 302 maximum growth was observed at 30°C and for *Pichia guilliermondii* SD 337 the maximum growth was at 40°C (Fig. 4.6a). In the case of growth at different salinities, *Candida* sp. SD 302 had maximum growth at 15 ppt and *Pichia guilliermondii* SD 337 at 25 ppt (Fig. 4.6b). Maximum growth of both the isolates was observed at pH 7 (Fig. 4.6c).

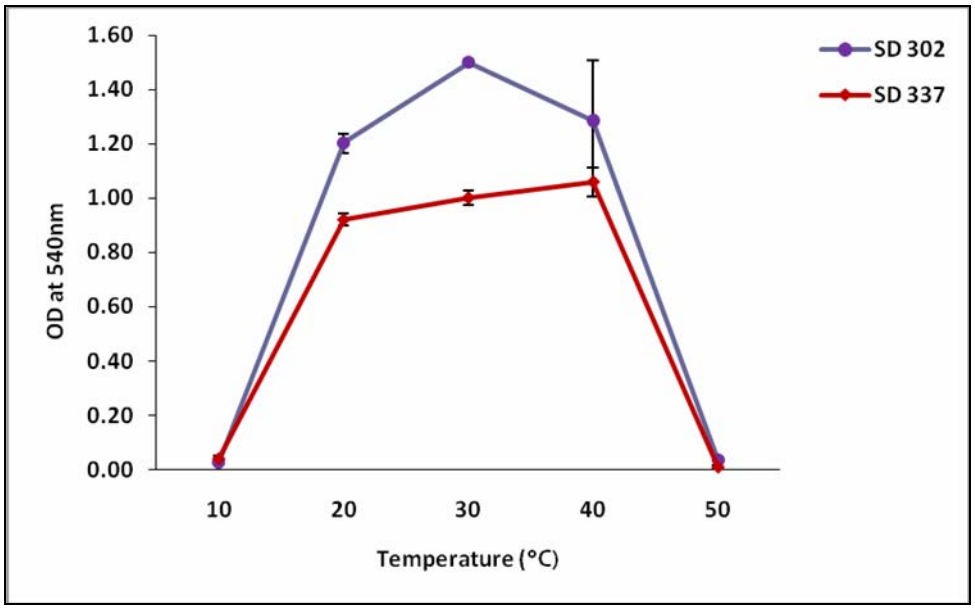

Fig. 4.6a Optimum temperature for growth of potential yeast isolates

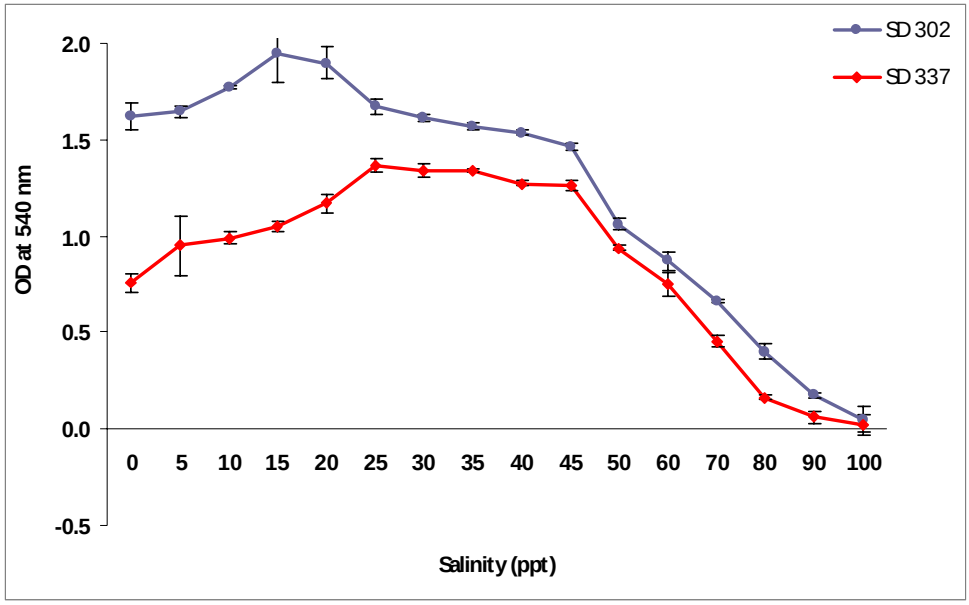

Fig. 4.6b Optimum salinity for growth of potential yeast isolates

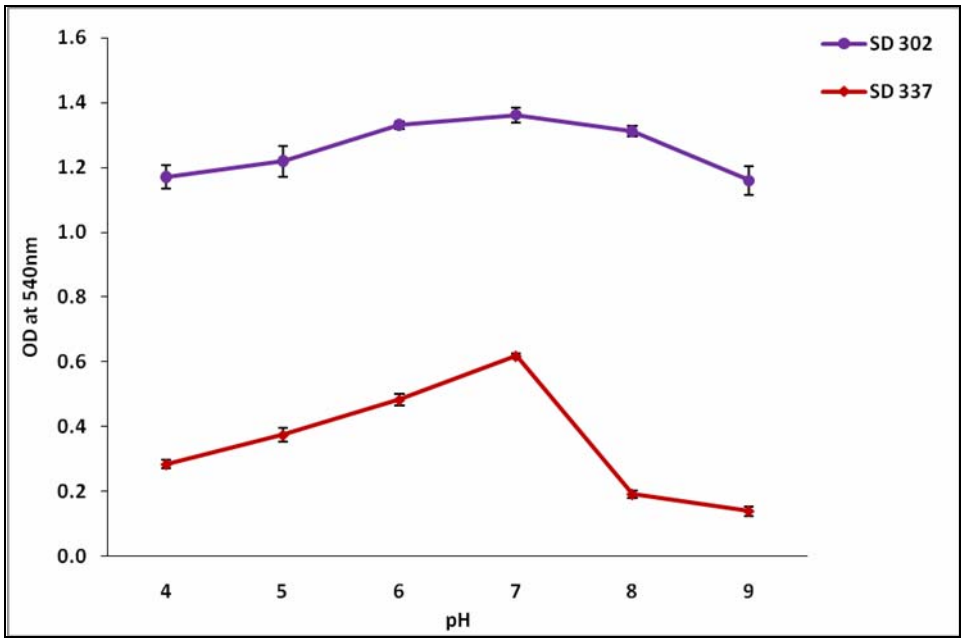

Fig. 4.6c Optimum pH for growth of potential yeast isolates

4.3.4 Gas Chromatographic analysis

The crude oil exhibited a distribution of n-alkanes between C_{12} and C_{40}. The lack of n-alkanes lighter than C_{12} may be due to weathering of the more volatile fraction. The proportion of hydrocarbons in the mixture was highly variable and ranged from as much as 97% by weight in the lighter oils to as little as 50% in the heavier oils and bitumen.

The control flask showed components with C_{12} to C_{40} (Fig. 4.7). Oil degrading ability of the isolates was assessed by comparing the chromatogram peak of the control with that of the samples.

Suspended cells of *Candida* sp. SD 302 showed degradation of components C_{12} to C_{24} and also C_{28}-C_{32} (Fig. 4.8). Immobilized *Candida* sp. SD 302 showed complete degradation of components C_{12} to C_{16}, C_{18} to C_{24} and C_{28} to C_{32} (Fig. 4.9). Components C_{17}, C_{18}, C_{24} to C_{28} and C_{32} to C_{40} showed nominal degradation.

Suspended cells of *Pichia guilliermondii* SD 337 showed complete degradation of components C_{12} to C_{16} and C_{18} to C_{24} (Fig. 4.10), whereas degradation of oil components C_{17}, C_{18} and C_{24} to C_{32} were meager. Complete degradation of components like C_{12} to C_{14}, C_{20} to C_{24} and C_{28} to C_{36} was shown by immobilized *Pichia guilliermondii* SD 337 (Fig. 4.11). Gradual degradation of components from C_{15} to C_{20} and C_{24} to C_{28} was observed by the immobilized *Pichia guilliermondii* SD 337 system.

The isolates *Candida* sp. SD 302 and *Pichia guilliermondii* SD 337 were found to be potential degraders of n-alkanes both as such and in immobilized forms. Some of them were completely degraded, while some partially (Table 4.3).

Table 4.3 Gas chromatographic analysis data showing percentage of crude oil degradation by selected isolates

Degradation of various fractions (%) compared with control				
Oil fractions	**SD 302**		**SD 337**	
	(Suspended)	**(Immobilized)**	**(Suspended)**	**(Immobilized)**
C_{12}	42	0	0	0
C_{12}-C_{14}	49.2	0	0	0
C_{15}	3.19	0	0	3.12
C_{16}	7.04	0	0	20.9
C_{17}	4.5	8.9	4.6	19.4
C_{18}	10.7	10.6	14.2	22
C_{18}-C_{20}	5.5	0	0	8.6
C_{20}-C_{24}	38.6	0	0	0
C_{24}-C_{28}	100	0.71	13.58	0.73
C_{28}-C_{32}	28.5	0	4.64	0
C_{32}-C_{36}	100	14.9	100	0
C_{36}-C_{40}	100	98.5	100	72.3

As per the results it could be noted that the rate of degradation by the immobilized isolates were higher compared to the suspended cells.

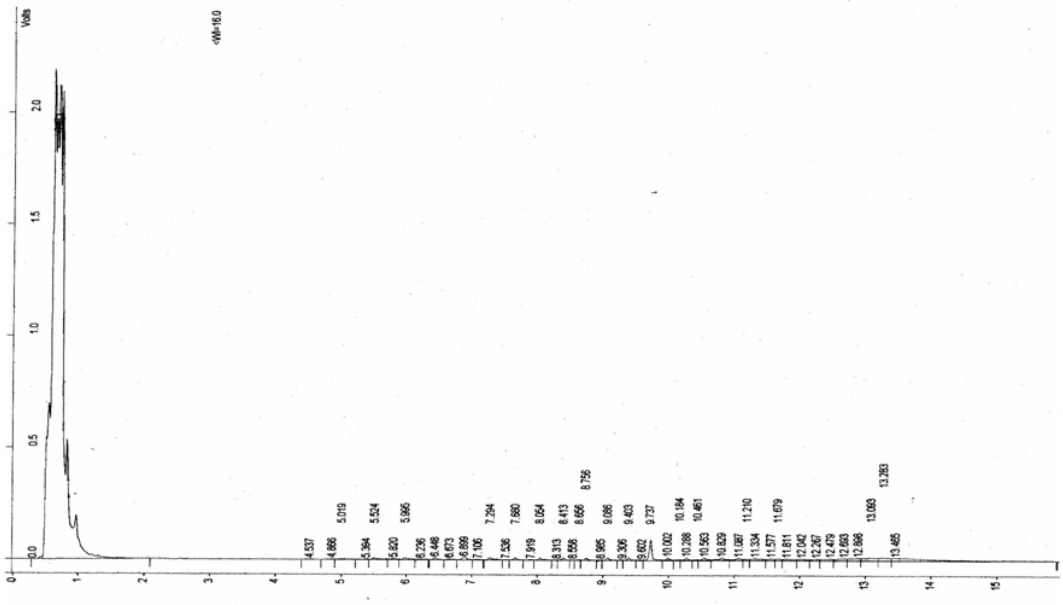

Fig. 4.7 Chromatogram of the crude oil components in the control flask

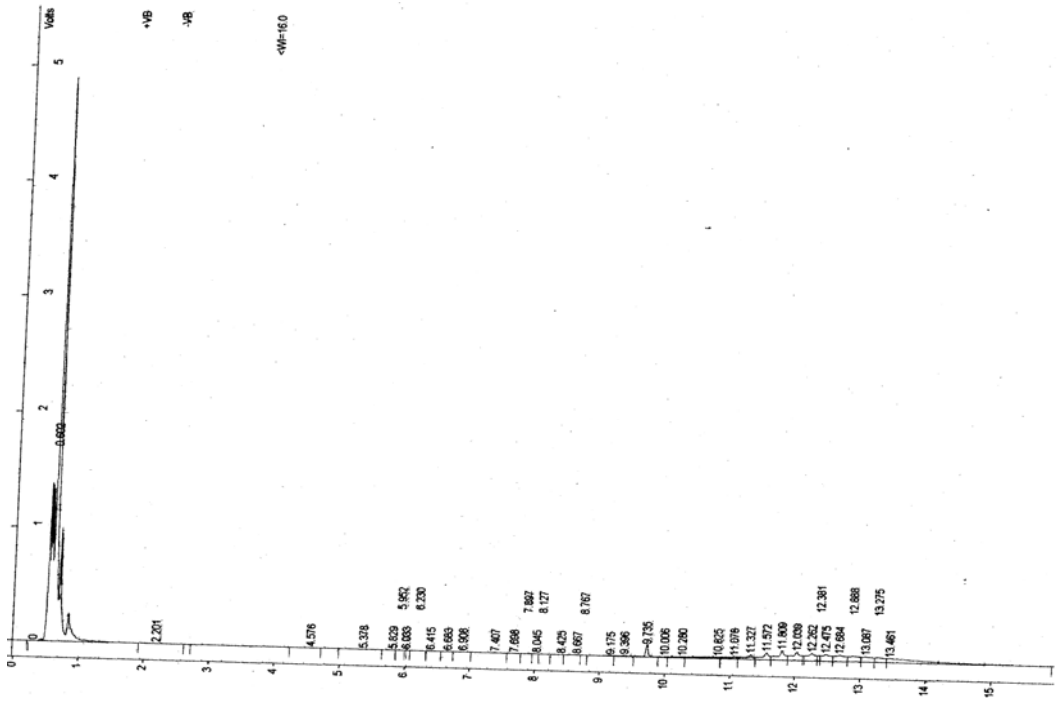

Fig. 4.8 Chromatogram showing the degradation products of the crude oil by suspended cells of *Candida* sp. SD 302

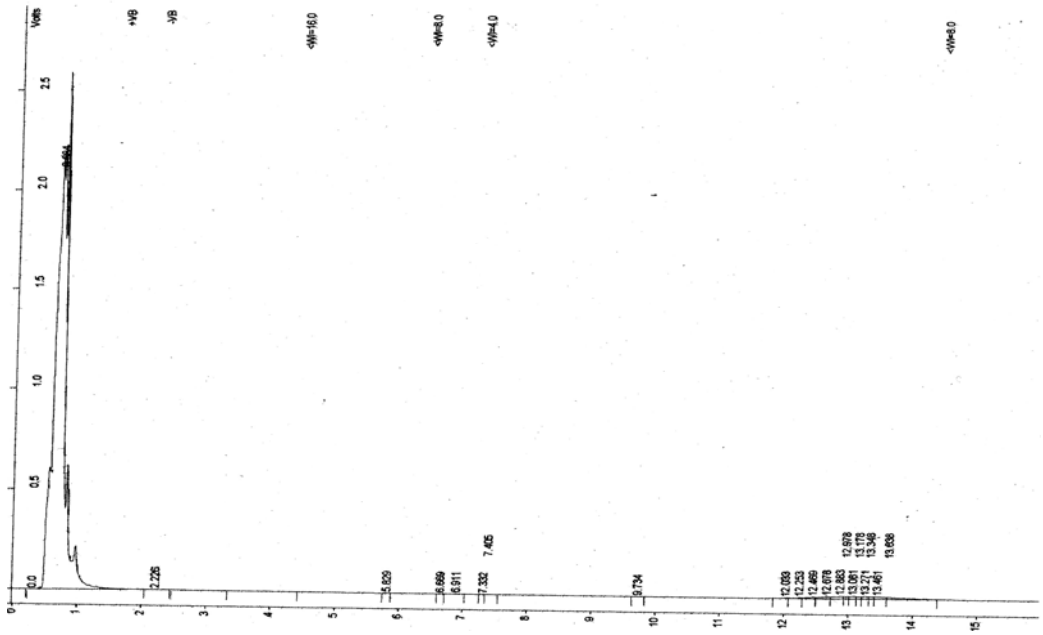

Fig. 4.9 Chromatogram showing the degradation products of the crude oil by immobilized cells of *Candida* sp. SD 302

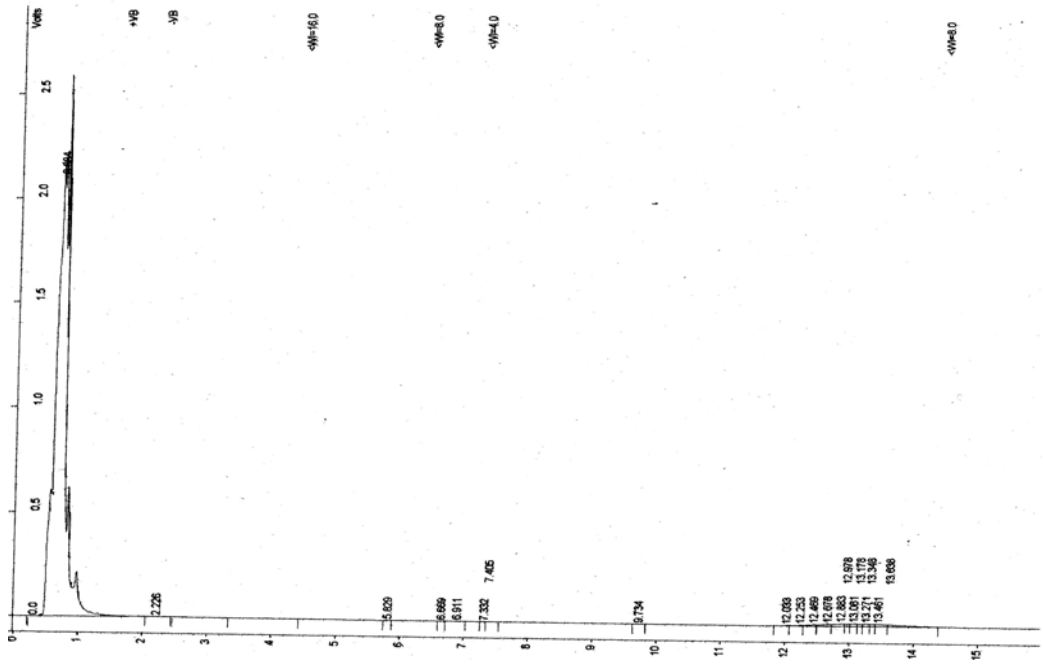

Fig. 4.10 Chromatogram showing the degradation products of the crude oil by suspended cells of *Pichia guilliermondii* SD 337

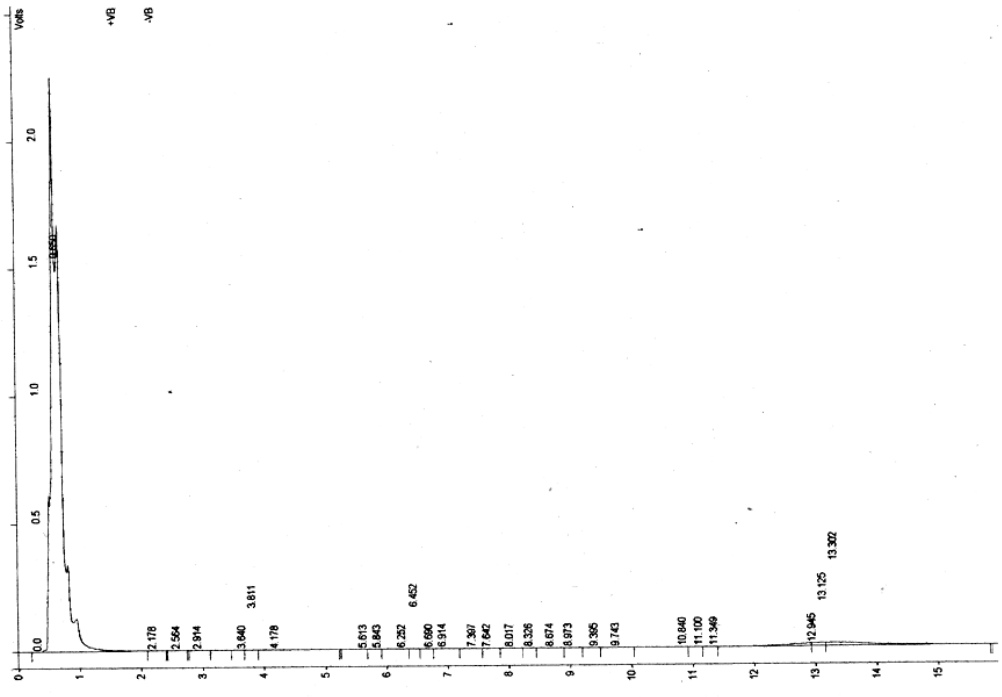

Fig. 4.11 Chromatogram showing the degradation products of the crude oil by immobilized cells of *Pichia guilliermondii* SD 337

4.4. Discussion

The hydrocarbons in crude oil are mostly alkanes, cycloalkanes and various aromatic compounds while the other organic compounds which contain nitrogen, oxygen, sulphur and trace amounts of metals such as iron, nickel, copper and vanadium are known as the asphaltenes. Asphaltenes are difficult to analyze with current methodology because of their complexity, these compounds are not well understood (Atlas, 1981).

The exact molecular composition varies widely from formation to formation but the proportions of chemical elements vary over fairly narrow limits. The alkanes, also known as paraffins, are saturated hydrocarbons with straight or branched chains which contain only carbon and hydrogen. They generally have 5 to 40 carbon atoms per molecule, although trace amounts of shorter or longer molecules may be present in the mixture.

Microbial degradation of crude oil is a very important post accumulation process in terms of influence on oil quality and composition. The biodegradation process results in the formation of increasingly heavier oils, leading to higher oil density, increased emulsion stability and polar acidic compounds. The higher content of polar compounds in biodegraded crude oils may be caused by (i) an increase in the relative concentration of heavy polar components resulting from removal of the light hydrocarbons by microbial activity or by (ii) products resulting from microbial processes.

Out of the 16 potential lipolytic yeast isolates, only two strains were found to be efficient in crude oil degradation. Immobilization was found to improve possible reuse of the strains and continuous operation for a prolonged period (Mattiasson, 1983; Zhang et al., 1989).

The control flask contained carbon components from C_{12} to C_{40}. The GC analysis showed a completely degraded profile of certain components, whereas

some components were only partially degraded. Both the strains as such and also in immobilized condition, showed different stages of degradation. The suspended cells of *Pichia* sp. SD 337 showed complete degradation of the alkane components (C_{16} and lesser) of the crude oil very efficiently than the suspended cells of *Candida* sp. SD 302. None of the components tested were completely degraded by the strain *Candida* sp. SD 302. So in a suspended system *Pichia* sp. SD 337 proved to be more efficient than *Candida* sp. SD 302. The suspended system of both the strains was unable to degrade fractions higher than C_{32}. It could be noted that the higher fractions like C_{18}-C_{24} and C_{28}-C_{36}, were degraded efficiently by both the immobilized yeast cells. The immobilized system of both the strains behaved almost in a similar way showing the complete degradation of lower carbon components as well as the higher fractions. Complete degradation of components C_{28}-C_{32} was observed by both the isolates in immobilized form. Maximum component degradation was observed in immobilized system of *Candida* sp. SD 302. So in the immobilized system *Candida* sp. SD 302 proved to be more efficient than *Pichia* sp. SD 337. At the same time it could be noted that the immobilized system of *Pichia* sp. SD 337 was able to degrade C_{32}-C_{36} completely. In the case of components above C_{32}, there is an increase in concentration of the components in the samples, than in the control, which may be due to an increase in the relative concentration of heavy polar components resulting from the removal of the light hydrocarbons by microbial activity or due to the products resulting from microbial processing of higher compounds.

According to Ijah (1998), yeasts showed more proficiency in crude oil degradation than their bacterial counterparts and also components C_{12} to C_{32} were extensively degraded by the yeasts. According to Prenafeta-Boldu et al. (2001), fungi are capable of degrading the hydrocarbons in engine oil to a certain extent, but they take longer periods to grow as compared to their bacterial counter parts.

The potential isolates, *Candida* sp. SD 302 and *Pichia* sp. SD 337 were isolated from different locations and different depths. Both the strains were oxidative in nature. According to De Hoog et al. (2006), if the strain shows 99% similarity with that of the already identified sequence, then it can be considered as the same species. So *Pichia* sp. SD 337 which was having 99% similarity with the NCBI sequence *Pichia guilliermondii*, was identified as the same organism. Whereas *Candida* sp. SD 302 which was having only 97% similarity with the NCBI strain *Candida tropicalis* was kept as a separate strain, *Candida* sp. SD 302. According to Paskevicus (2001), *Candida* and *Pichia* are among the most active lipase producers. *Candida tropicalis* has been already proven as a potential oil degrader (Palittapongarnpim et al., 1998; Ijah, 1998). Yeast strains isolated from oil polluted sites were cultured in laboratory and were found to utilize crude oil as a sole source of carbon (Chaillan et al., 2004). The strains were identified as *Candida, Yarrowia* and *Pichia*. Another yeast strain *Pichia anomala*, isolated from oil contaminated soil was capable of degrading naphthalene, phenanthrene and chrysene, singly, and benzo(a)pyrene in combination (Hesham et al., 2006). The strains *Candida tropicalis* and *Pichia membranifaciens* were found to degrade phenol in waste water (Rocha et al., 2007). It was reported that *Candida tropicalis* and *Pichia guilliermondii* strains, utilized hydrocarbons with chain length 8-10 as a sole source of carbon (Awe et al., 2008). From the previous reports and the current study it could be inferred that *Candida* and *Pichia* are versatile agents of oil degradation.

The rate of growth of *Candida* sp. SD 302 was high when compared with that of *Pichia* sp. SD 337 during all the experiments. Both the isolates were able to grow quite well at salinities ranging from 0 to 45 ppt and pH 4-9. This shows the efficiency of applying the isolates as crude oil degrading strains in fresh water as well as seawater. They had a temperature optimum of 30-40°C which shows their mesophilic nature. The optimal activity of lipase enzyme responsible for the oil degradation property was reported to be

between 35-40°C (Benjamin and Pandey, 2001). The growth and oil degrading potential of the isolates at different conditions need to be optimized further. Further characterization of the oil degrading property of these isolates might give a lead to its potential application in bioremediation.

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Chapter 5

CHARACTERIZATION OF MARINE BLACK YEASTS

Contents

5.1 Introduction

'Black yeasts' indicate those melanised group of fungi of which several representatives are able to reproduce by unicellular growth. Majority of black yeasts belong to divergent orders of ascomycetes (De Hoog & Mcginnis, 1987). An ascomycetous 'black yeast' may or may not have a yeast-like phase, depending on the ecological niche it inhabits. Closely related members of 'black yeasts' may be highly dissimilar in morphology. If the 'black yeasts' are taken as a taxonomic rather than a morphogenetic group, yeast-like growth is not a prerequisite for membership in the group. The occurrence of black yeasts is not ubiquitous. Only very few reports are there regarding the isolation of black yeasts from marine ecosystem. van Uden and Castelo-Branco (1963) have reported the presence of black yeasts among the different yeasts isolated from Pacific sub surface waters and deep waters of Loma Trough, off San Diego. Also van Uden and Ahearn (1963) did quantitative studies on yeasts present in surface and deep water samples from a fresh water body (Douglas Lake, Michigan) which revealed the presence of 12 species of yeasts including black yeasts.

5.1.1 Characteristics of black yeasts

Ascomycetous black yeasts show adaptations to a wide array of environmental conditions. Factors which are of ecological significance include the presence of melanin and carotene, formation of thick walls and meristematic growth, presence of yeast-like phase, presence of additional forms of conidiogenesis, thermo- and osmotolerance, adhesion, hydrophobicity, production of extracellular polysaccharides, siderophores and acidic or alkaline secondary metabolites (De Hoog, 1993).

Melanin:

The biological role of melanin has received attention only recently. Melanin is a common term used for dark brown to black pigments of high molecular mass formed by oxidative polymerization of phenolic compounds usually complexed with protein and carbohydrates. Fungal melanins occur in the

cell walls or as extracellular polymers formed enzymatically or auto oxidatively in the medium. The phenolic compounds from which the fungal melanins are derived include tyrosine via 3, 4-dihydroxyphenylalanine (DOPA) in various fungi and other microorganisms, γ-glutaminyl-3,4-dihydroxybenzene or catechol in basidiomycetes, and 1,8-dihydroxynaphthalene (DHN) in ascomycetes and related deuteromycetes. Melanins formed by DHN pathway are of particular interest, since they reportedly protect fungi against a number of environmental factors. These pigments are synthesised in organisms belonging to various taxonomic groups, which attests to their protective properties. Melanin preparations are widely used in dermatology and cosmetology. They also possess antioxidant and antiradical activities. Paramonov et al. (2002) studied the dependence of photoprotective activity of 1, 8-dihydroxynaphthalene melanin using the black yeast like fungus *Aureobasidium pullulans* as the source of melanin. The application of melanin to the skin produced dose dependent changes, which included photoprotection, photosensitization and photoburn. A study was made to determine if *Hortaea werneckii*, *Trimmatostroma salinum* and *Phaeotheca triangularis* make melanin from DHN. The systemic fungicide tricyclazole (5-methyl-1, 2, 4-triazolo (3, 4, b)-benzothiazole) was used as inhibitor. It was demonstrated that the three fungi synthesized DHN melanin under saline and non saline growth conditions (Kogej et al., 2004). Suryanarayanan et al. (2004) characterized the melanin pigment of a fungal endophyte, *Phyllosticta capitalensis* and demonstrated that it is 1, 8- dihydroxynaphthalene melanin. Melanized fungi are more resistant to environmental factors than are their non-melanized albino mutants or other normally non-melanized fungi (Butler et al., 2005). Melanin has diverse functions which have been reviewed by Nosanchuk and Casadevall (2006). They serve as energy transducers, affect cellular integrity, used for sexual display and camouflage, colouration in black and red hair, major role in innate immune system of insects which synthesize the polymer to damage and entomb microbial intruders, UV irradiation associated with virulence for a variety of pathogenic microbes etc. Isolation and

characterization of melanin pigment from *Pleurotus cystidiosus* was done by Selvakumar et al. (2008). The pigment was confirmed as melanin based on UV, IR and EPR spectra. The influence of ortho- and para-diphenoloxidase substrates on pigment formation in black yeast- like fungi was studied by Yurlova et al. (2008). The results indicate that the melanization process may involve more enzymes and more substrates than those commonly recognised. Black yeasts contain a multipotent polyphenoloxidase.

Halotolerance:

Zalar et al. (1999) studied about the halotolerant dothideaceous black yeasts. Black yeast strains isolated from salt pans at the Adriatic coast were identified as *Hortaea werneckii, Phaeotheca triangularis* and *Aureobasidium pullulans*. Salt tolerance were analyzed by comparing nutritional physiological profiles under standard conditions and in 5% additional NaCl. *Hortaea werneckii* was facultatively halophilic.

Until recently, it was believed that microbial communities at high salinities are dominated exclusively by archaea and bacteria and one eukaryotic species, the alga *Dunaliella salina*. Recently it became evident that melanized fungi, so far described only in the crystallization pond of Adriatic salterns within the season of salt production, can be considered as a new group of eukaryotic halophiles (Gunde-Cimerman et al., 2000). At the highest environmental salinities, melanized fungi represented 85-100% of the total isolated mycobiota, but with lowering salinities they were partially replaced by non-melanized fungi and, at the end of the season, with NaCl concentrations below 5%, they were detected only occasionally. Eukaryotic halophilic microorganisms are poorly investigated and only little is known about their adaptation to growth at extremely hypersaline conditions (Petrovic et al., 2002). Melanized fungi have been isolated from hypersaline waters on three continents, indicating that they are present globally in hypersaline waters of man-made salterns (Butinar et al., 2005). Molecular studies on

halophilic adaptations have focused on prokaryotic microorganisms due to a lack of known appropriate eukaryotic halophilic microorganisms. Black yeast-like fungi isolated from hypersaline waters of salterns as their natural ecological niche, have been previously defined as halophilic and halotolerant microorganisms (Kogej et al., 2005).

After the identification of the black yeast, *Hortaea werneckii* as the dominant fungal species in hypersaline waters on three continents; it represents a new model organism for studying the mechanisms of salt tolerance in eukaryotes. Ultrastructural studies of the *Hortaea werneckii* cell wall have shown that it synthesizes dihydroxynaphthalene (DHN) melanin under both saline and non-saline growth conditions. However, melanin granules in the cell walls are organized in a salt-dependent way, implying the potential osmoprotectant role of melanin. Even *Hortaea werneckii* grown in high NaCl concentrations maintains very low intracellular amounts of potassium and sodium, demonstrating the sodium-excluder characteristic of this organism (Gunde-Cimerman and Plemenitas, 2006). Studies by Kogej et al. (2006) revealed that hypersaline conditions induce changes in cell-wall melanisation and colony structure in a halophilic and a xerophilic black yeast species of the genus *Trimmatostroma*. They were able to adapt to hypersaline growth conditions, although their growth patterns show distinct adaptation of each species to their natural ecological niches. Kogej et al. (2007) showed that in *Hortaea werneckii,* melanization is effective in reducing the permeability of its cell wall to its major compatible solute glycerol, which might be one of the features that help it to tolerate a wide range of salt concentrations. *Hortaea werneckii* is an extremely halotolerant eukaryotic microorganism and thus a promising source of transgenes for improvement of osmotolerance in industrially important yeasts, as well as in crops (Plemenitas et al., 2008).

5.1.2 Identification of black yeasts

To overcome the confused physiological behaviour shown by the black yeasts, effort was put in to procure molecular data in order to cluster morphologically divergent strains, and determine their phylogenetic positions. The first method introduced was RFLP analysis of small ribosomal subunit. Today, owing to the emerging technical facilities, sequencing of DNA followed by homology search in the data banks of NCBI, alignment and phylogenetic tree construction is the general way for resolving the taxonomy and phylogeny of black yeasts.

Phylogenetic trees based on small sub unit (SSU) sequences showed that black yeasts are phylogenetically far away from the class Hemiascomycetes comprising classical yeast genera *Candida, Saccharomyces* and *Pichia* (Haase et al., 1995). Haase suggested the 1340-1389 positions of SSU as a promising region for species specificity, but SSU turned out to be too conserved to resolve the enormous intraspecies and interspecies variability existing in black yeasts. The sequencing of SSU places them to right classes; ITS 1 is a taxonomic tool for species identification. The 5.8S region sometimes shows higher heterogeneity than the ITS1 (De Hoog et al., 1999). For species determination ITS region 1 evolved as a suitable tool, because this region is nearly identical within a single species of black yeast (De Hoog et al., 2003). Lot of studies have been done for the identification of black yeasts using ITS sequencing.

5.2 Materials and Methods

5.2.1 Black yeast isolates

Black yeasts obtained from the sediment samples of the continental slope region of Arabian Sea and Bay of Bengal were used for the study. Most of the isolates lost viability during storage and only ten were used for further study.

5.2.2 Identification of black yeasts

These 10 isolates were identified using molecular techniques by extracting genomic DNA and sequencing of ITS region as per Harju et al. (2004).

Yeast genomic DNA isolation

Pure yeast colonies were inoculated into rich media containing 1% yeast extract, 2% peptone and 2% dextrose (in sea water) and incubated for 18 hrs. Cells from 1.5 ml of the overnight cultures were pelleted in a microcentrifuge tube and the cell pellets were resuspended in 200 µl of lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)). The tubes were placed in a -80°C freezer for two minutes (until they were completely frozen), then immersed in a 95°C water bath for 1 minute to thaw quickly. The process was repeated twice, and the tubes were vortexed vigorously for 30 seconds. 200 µl of chloroform was added and the tubes were vortexed for 2 minutes and then centrifuged 3 minutes at room temperature at 20,000 x g. The aqueous layer was transferred to a tube containing 400 µl of ice-cold 100% ethanol. The samples were allowed to precipitate 5 minutes at room temperature and then centrifuged at 20,000 x g. Supernatant was discarded and DNA pellets were washed with 0.5 ml of 70% ethanol followed by vacuum drying for 5 minutes at 60^{o}C. DNA was resuspended in 20 µl TE (10 mM Tris and 1 mM EDTA, pH 8.0) and stored at 4°C for future use. DNA concentration and purity were assessed spectrophotometrically by measuring absorbance at 260 nm and 280 nm by 0.8 % agarose gel electrophoresis.

Optical density of the DNA was taken at both 260 nm and 280 nm and the concentration of DNA was found out from the following formula:

Conc. of DNA (µg/ml) = OD at 260 nm x 50 x dilution factor.

Amplification of the extracted DNA

ITS (Internal Transcribed Spacer) sequences are considered to be the best tool for rapid and accurate identification of yeast isolates. ITS primers (Forward ITS 1-5' TCC GTA GGT GAA CCT GCG G 3' and Reverse ITS 4- 5' TCC TCC GCT TAT TGA TAT GC 3') by White et al. (1990) which amplify a fragment of approximately 580 bp containing the ITS 1, 5.8 S and ITS 2 regions was used for the purpose.

The amplification reaction was performed by using a DNA thermal cycler (Eppendorf). PCR was performed in a final volume of 25 µl containing 2.5 µl of 10X buffer, 1.5 µl 25 mM $MgCl_2$, 1.0 µl of 10 pmol of each oligonucleotide primer, 2 µl of 2.5 mM each deoxynucleoside triphosphate, 1 µl of the extracted DNA of concentration 600 ng/µl, 1 µl of Taq DNA polymerase and 16 µl of Milli Q water. After an initial denaturation at 95°C for 5 minutes, amplification was made through 30 cycles, each consisting of a denaturation at 94°C for 1 minute, annealing at 56°C for 45 seconds, extension step at 72°C for 1 minute and a final extension at 72°C for 10 minutes. The PCR products were analyzed by electrophoresis on 1% agarose gel prepared in 1X TAE buffer and stained with ethidium bromide.

PCR clean-up system

PCR clean-up for sequencing was done using Promega PCR clean-up system. An equal volume of membrane binding solution was added to the PCR product. Inserted SV minicolumn into collection tube. Transferred the prepared PCR product to the minicolumn assembly. Incubated at room temperature for 1 minute and centrifuged at 16,000 x g for 1 minute. Discarded the flow through liquid and inserted minicolumn into collection tube. Added 700 µl membrane wash solution (ethanol added). Centrifuged at 16,000 x g for 1 minute. Discarded the flow through liquid and reinserted minicolumn into collection tube. Repeated the above step with 500 µl membrane wash solution. Centrifuged at 16,000 x g

for 5 minutes. Emptied the collection tube and re-centrifuged the column assembly for 1 minute with the micro-centrifuge lid open to allow evaporation of any residual ethanol. Carefully transferred the minicolumn to clean 1.5 ml micro-centrifuge tube. Added 50 µl of nuclease free water to the minicolumn. Incubated at room temperature for 1 minute. Centrifuged at 16,000 x g for 1 minute. Discarded the medium and stored DNA at -20°C.

Sequencing

Nucleotide sequencing was performed using ABI PRISM 3700 Big Dye Sequencer at Chromous Biotech, Bangalore. The primers used were ITS1 and ITS4.

ITS gene sequence similarity & strain identification

Sequenced DNA data were compiled and analyzed. The sequences were matched with the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al., 1990) at NCBI (www.ncbi.nlm. nih.gov). The sequences were multiple aligned using the programme Clustal W (Thompson et al., 1994). Then the aligned ITS-rDNA gene sequences were used to construct a phylogenetic tree using the neighbour-joining (NJ) method (Saitou and Nei, 1987), contained within the MEGA 4.1 package (Tamura et al., 2007). Bootstrap analysis was based on 1000 replicates. Similarity matrix and genetic distance between sequences was calculated. The number of base substitutions per site of the sequence was analysed using BioEdit Sequence Alignment Editor Version 7.0.9.0 software. Similarity matrix of the sequence is scored using Maximum Composite Likelihood method in MEGA 4.1 software and the distance between each sequence was determined based on the pairwise analysis.

5.2.3 Hydrolytic enzyme production

The isolates were tested for the production of enzymes i.e. amylase, lipase, protease, urease, aryl sulfatase, ligninase, cellulase, DNAse, pectinase and chitinase as per section 3.2.3.

5.2.4 Optimum physico-chemical conditions for growth

Preparation of inoculum:

Malt extract agar slants were prepared and sterilized at 121.5°C for 15 minutes in an autoclave. The black yeast isolates were streaked on to malt extract agar slants. Incubation was done at room temperature (28± 2°C) for 48 hours. The cells were harvested at the logarithmic phase using 30 ppt sterile sea water. Optical density of the culture suspension was taken at 540 nm in a UV-VIS spectrophotometer (Shimadzu UV-1601). OD was adjusted to 1 by appropriate dilution and this suspension was used as the inoculum.

Preparation of medium:

Temperature:

Malt extract broth was prepared in sea water (35 ppt) was used for testing the growth of the isolates at different temperature.

Salinity:

Malt extract broth was prepared in sea water of different salinities (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 ppt).

pH:

Malt extract broth was prepared in sea water (35 ppt) with different pH 3, 4, 5, 6, 7, 8 and 9 using 1 N HCl and 1N NaOH.

Inoculation and incubation:

10 µl of 1 OD cell suspension was inoculated into the malt extract tubes prepared in triplicate so that the initial OD of the culture medium was 0.001. Incubation was done at room temperature (28 ± 2°C) for 48 hours in the case of pH and salinity.

Determination of growth:

Yeast growth was estimated by measuring the optical density at 540 nm using Shimadzu UV-1601 spectrophotometer.

5.2.5 Extraction and characterization of melanin from black yeasts

Melanin was extracted from black yeasts as per Gadd (1982).

Flow chart describing the extraction of melanin from black yeast

Added 10 ml of 1 N NaOH to harvested yeast biomass (5 g wet weight)

Autoclaved for 20 min at 121ºC

(Centrifuged for 10 min at 8000 rpm)

↓

Supernatant contains melanin

↓

Melanin precipitated by adding Conc. HCl until pH 2
(Centrifuged at 10,000 rpm for 10 min)

↓

Washed with distilled water twice
(Centrifuged at 10,000 rpm for 10 min)

↓

Melanin was finally dried in dehumidified atmosphere

Two-dimensional NMR spectroscopy analysis such as correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) were also done with the melanin extracted from the Black yeast isolates at Eastman Chemical Company, USA.

The samples were dissolved in 1M NaOD in D_2O with vortexing and heating to about 80ºC. The samples were dissolved by adding 0.75 ml of the solvent directly to the vials in which the samples were kept. The solvent was prepared by diluting 1 ml of 40% NaOD in D_2O with 15 ml D_2O. All the NMR spectra were obtained at probe ambient temperature on a JOEL Eclipse+ 600 NMR spectrophotometer operating at a proton observation frequency of 600.1723 MHz using 5 mm OD NMR tubes. 1D NMR spectra were collected with the following parameters: sweep width= 15 ppm, centre band= 5 ppm,

32768 complex points, acquisition time= 3.637 sec, spectral resolution= 0.275 Hz, scans= 64, 90º pulse and relaxation delay= 15 sec. The COSY 2D spectrum was obtained in absolute- value mode a pulse field gradient experiment using 521x 128 complex points zero filled 1- time in the y- dimension to give a final 512x 512 data matrix, sweep width= 10 ppm, centre band= 5ppm, pre scans= 4, scans= 8, X spectral resolution= 11.7 Hz, Y spectral resolution= 46.9 Hz and relaxation delay= 1 sec. The 2D TOCSY NMR spectrum was obtained using the same parameters except for the following: scans= 32, spin lock time= 100 m sec and relaxation delay= 2 sec. The 2D NOESY NMR spectrum was obtained in phase- sensitive mode using the same parameters except for the following: scans= 32, mixing time= 100 m sec and relaxation delay= 2 sec.

5.2.6 Antibacterial activity of melanin

Antagonistic activity of melanin against different pathogens was checked by Kirby- bauer disc diffusion method. Bacterial pathogens maintained at the National Centre for Aquatic Animal Health (NCAAH) were used for the study. The different human and fish pathogens checked were *Streptomyces lividans, Edwardsiella tarda, Aeromonas hydrophila, Vibrio harveyi, V. proteolyticus, V. fluvialis, V. cholerae, V. parahaemolyticus, E. coli, Pseudomonas aeruginosa, Bacillus cereus, Staphylococcus aureus* and *Arthrobacter* sp. Melanin extracted from the black yeast isolates were suspended in distilled water and used for testing the antibacterial property.

Nutrient agar (Peptone 0.5 g; beef extract 0.3 g; sea water (50%) 100 ml; pH 7.2) plates were prepared and swab inoculations of the pathogens were made on the surface to produce a lawn culture. Sterile filter paper discs impregnated with melanin were placed immediately on the agar surface. The plates were incubated at room temperature 28±2°C for 24 hours and observed for halo zone formation. Presence of halo zone around the discs was recorded as positive for antibacterial property.

5.2.7 Extraction of melanin degrading enzyme and its activity

Preparation of inoculum:

Malt extract agar (malt extract 1.5 g; peptone 0.5 g; agar 2 g; sea water (35 ppt) 100 ml; pH 7.2) slants were prepared and sterilized at 121.5°C for 15 minutes in an autoclave. The black yeast isolates were streaked on to malt extract agar slants. Incubation was done at room temperature (28± 2°C) for 48 hours. A loopful of this culture was inoculated in to malt extract broth (malt extract 1.5 g; peptone 0.5 g; sea water (35 ppt) 100 ml; pH 7.2), prepared in 500 ml flasks. The flasks were incubated at 26^{o}C in a shaker at 150 rpm for 48 hrs.

Extraction of enzyme:

The enzyme extract was collected by centrifuging the culture at 8000 rpm and resuspending it in buffer containing about 150 mM NaCl, 50 mM Tris and 1% Triton X 100. The mixture was sonicated for 30 minutes and centrifuged at 8000 rpm for 10 minutes to obtain the supernatant containing the crude enzyme.

Melanin degradation activity:

To check the activity of melanin degrading enzyme, petriplates were prepared using melanin dissolved in 100 ml alkaline distilled water with pH 8.5 and 2 g of agar. Wells of approximately 5 mm diameter were cut using gel cutter and about 200 µl of the crude enzyme was poured in each well. The plates were incubated at 37°C for 48 hrs. Zone of clearance was recorded as positive.

5.3 Results

5.3.1 Characterization of black yeasts

The black yeast isolates were studied in detail for their colony appearance, cellular morphology and filamentous growth. All the isolates showed filamentous growth (Table 5.1). The colony appearance on malt extract agar and cell morphology of all the isolates are given in the following figures (Fig. 5.1-5.11).

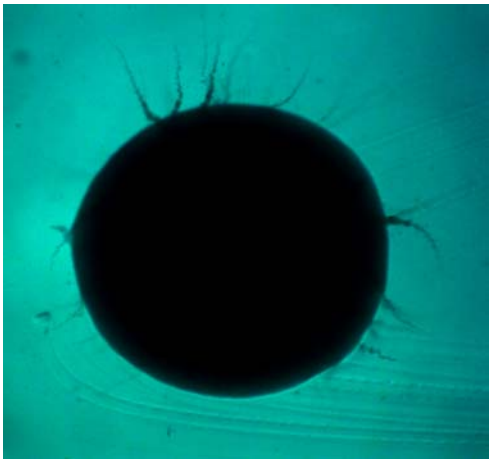

Fig. 5.1a SD 378 Single colony (4 x)

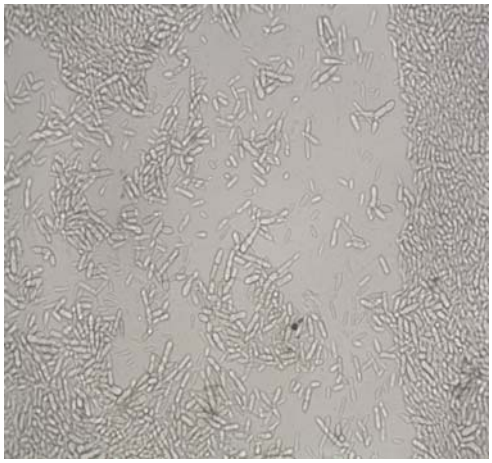

Fig. 5.1b SD 378 Wet mount (100 x)

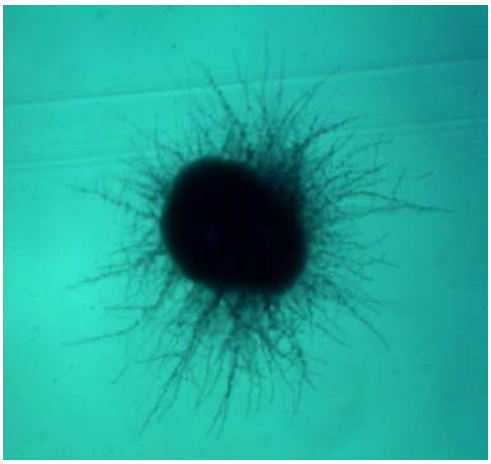

Fig. 5.2a SD 416 Single colony (4 x)

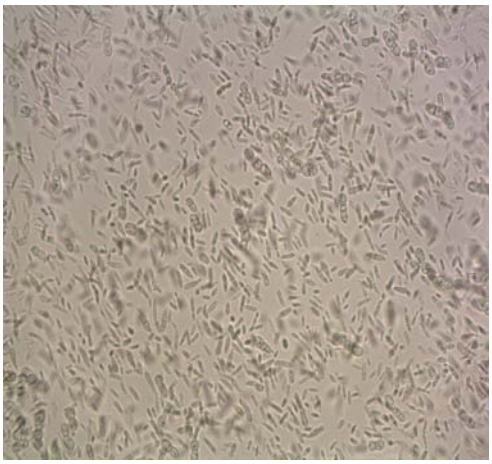

Fig. 5.2b SD 416 Wet mount (100 x)

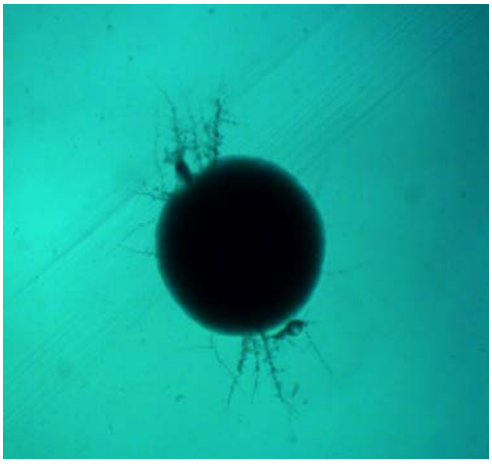

Fig. 5.3a SD 429 Single colony (4 x)

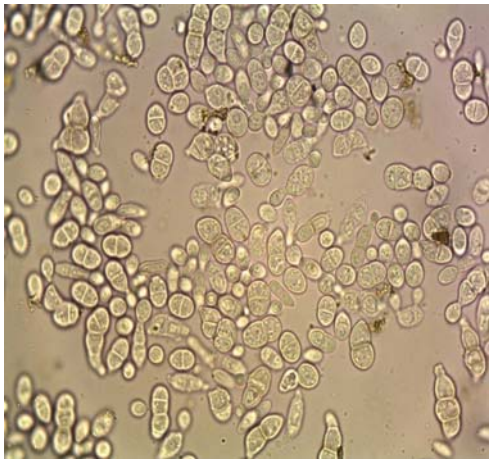

Fig. 5.3b SD 429 Wet mount (100 x)

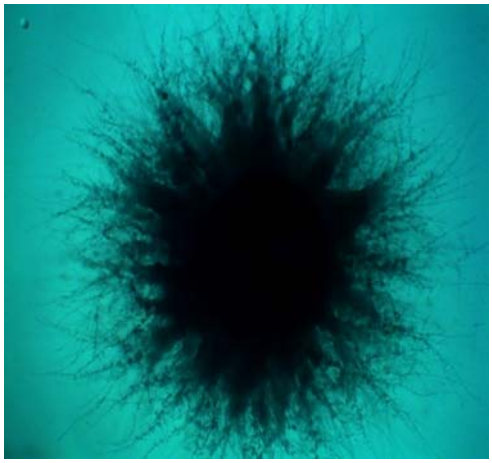

Fig. 5.4a SD 430 Single colony (4 x)

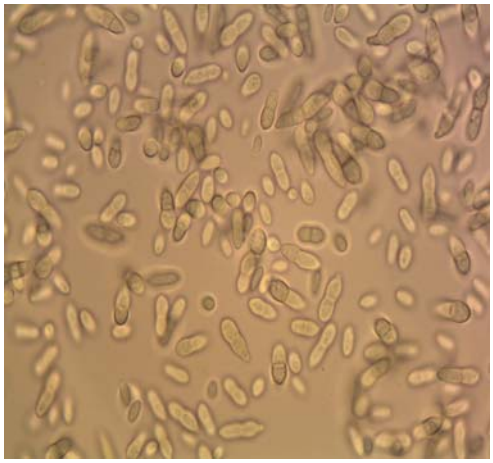

Fig. 5.4b SD 430 Wet mount (100 x)

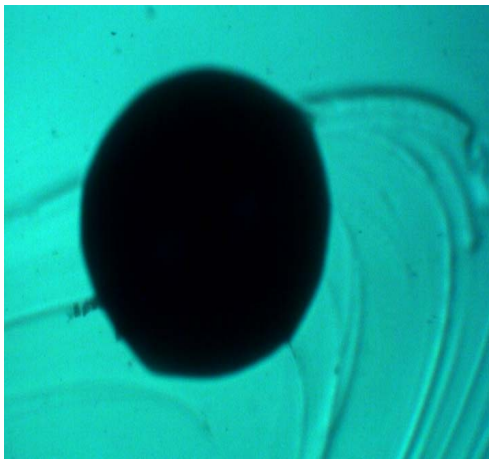

Fig. 5.5a SD 440 Single colony (4 x)

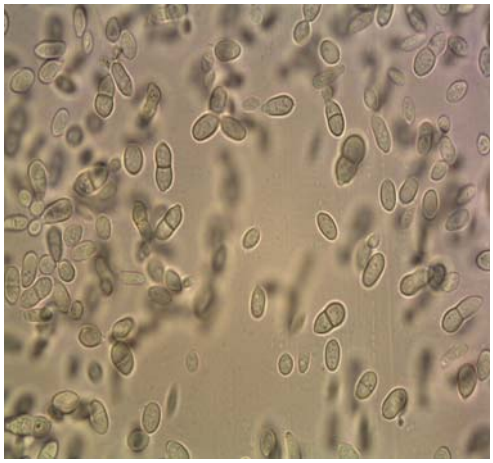

Fig. 5.5b SD 440 Wet mount (100 x)

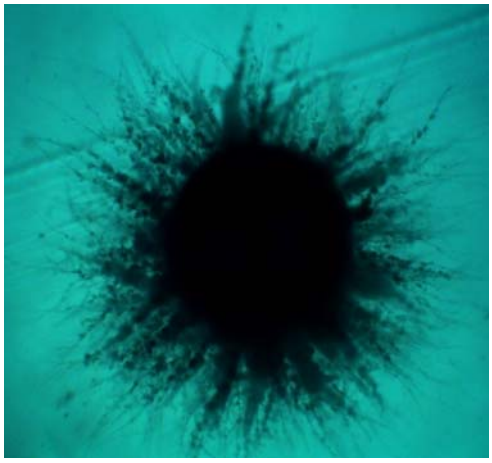

Fig. 5.6a SD 449 Single colony (4 x)

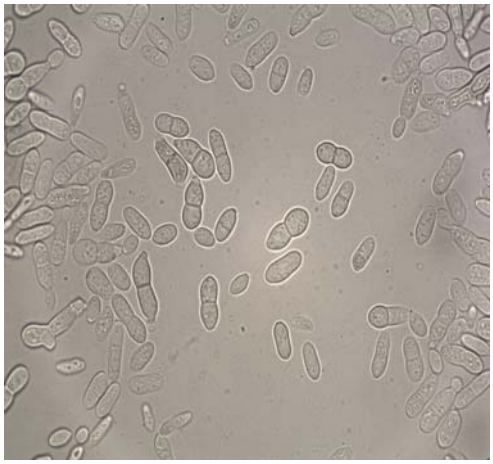

Fig. 5.6b SD 449 Wet mount (100 x)

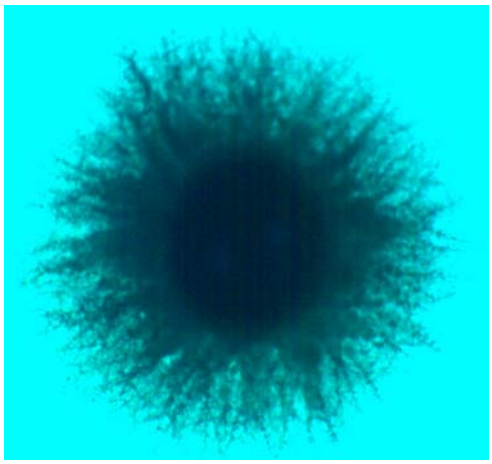

Fig. 5.7a SD 450 Single colony (4 x)

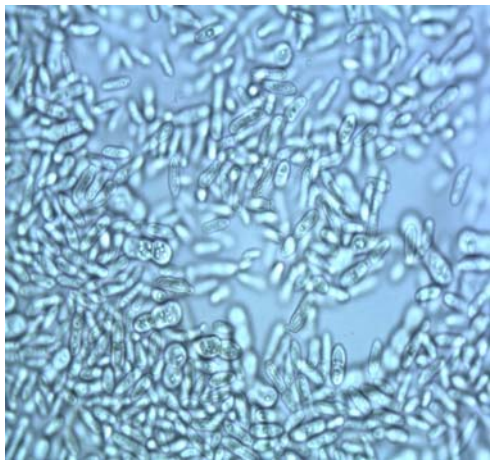

Fig. 5.7b SD 450 Wet mount (100 x)

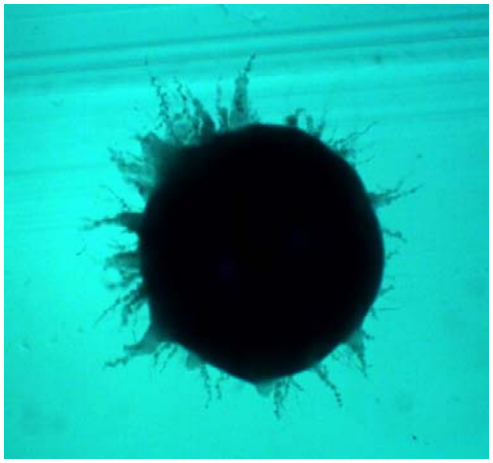

Fig. 5.8a SD 454 Single colony (4 x)

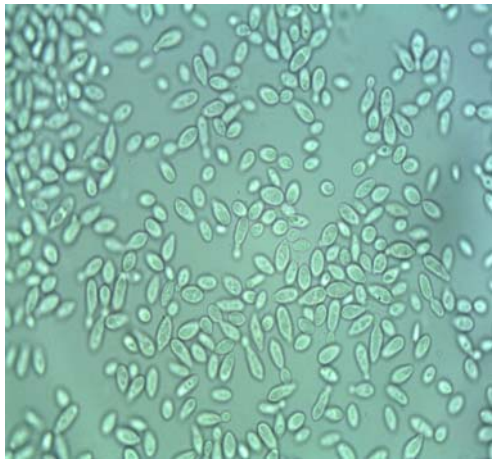

Fig. 5.8b SD 454 Wet mount (100 x)

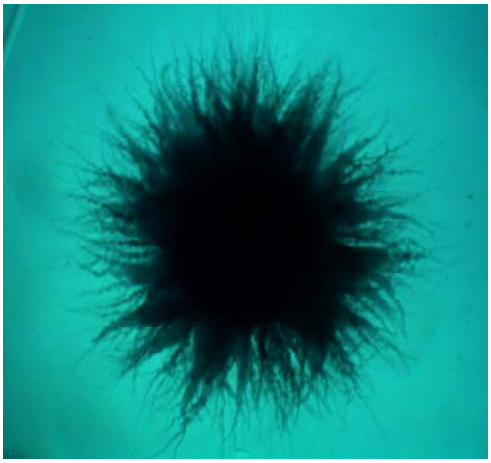

Fig. 5.9a SD 480 Single colony (4 x)

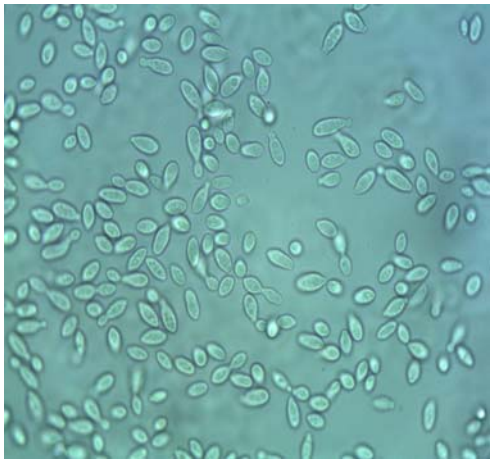

Fig. 5.9b SD 480 Wet mount (100 x)

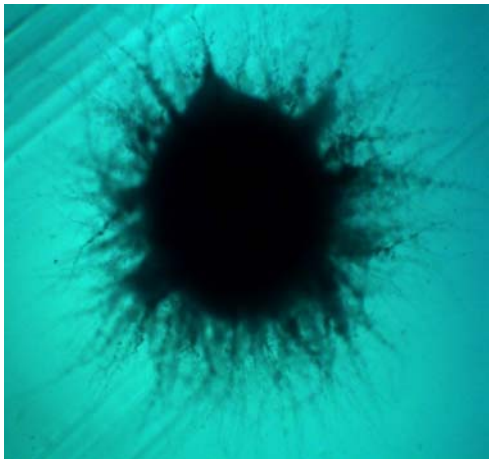

Fig. 5.10a SD 483 Single colony (4 x)

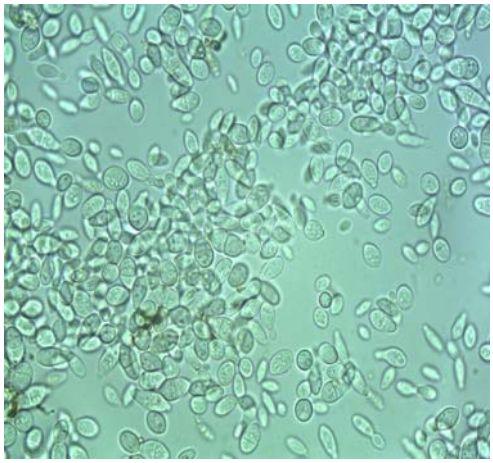

Fig. 5.10b SD 483 Wet mount (100 x)

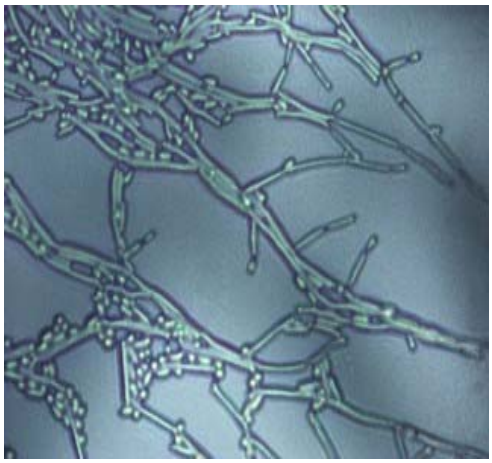

Fig. 5.11 Filamentous form of black yeast (SD 416)

Table 5.1 Major characteristics of black yeasts

Isolates	Station No.	Depth (m)	Asexual reproduction	Filamentous growth	MOF	Lipase	Amylase	Protease	Ligninase	Urease
SD 378	77	1000	Budding	+	Oxidative	+	+	+	+	-
SD 416	48	200	Budding/ Splitting	+	Oxidative	+	+	+	-	+
SD 429	48	200	Budding/ Splitting	+	Oxidative	+	+	+	+	-
SD 430	48	200	Budding/ Splitting	+	Oxidative	+	+	+	+	-
SD 440	51	200	Budding	+	Oxidative	+	+	+	-	-
SD 449	60	200	Budding/ Splitting	+	Oxidative	+	+	+	+	+
SD 450	60	200	Budding/ Splitting	+	Oxidative	+	+	+	-	+
SD 454	68	1000	Budding/ Splitting	+	Oxidative	+	+	+	+	-
SD 480	61	500	Budding/ Splitting	+	Oxidative	+	+	+	-	+
SD 483	58	500	Budding/ Splitting	+	Oxidative	+	+	+	+	-

3 µl each of DNA sample was loaded on 0.8% agarose gel and electrophoresis was done (Fig. 5.12). The ITS region was amplified using ITS primers and the PCR products (580 bp) were analysed by electrophoresis on 1% agarose gel (Fig. 5.13). The PCR products were purified and sequencing was done. The sequences obtained were subjected to BLAST at the NCBI and based on similarity index the isolates were identified as *Hortaea werneckii*. The nucleotide sequences determined in this study has been deposited in GenBank (Appendix 3). The identification details of the 10 isolates are as follows

Isolate No.	Genus/Species	GenBank Accession No.
SD 378	*Hortaea werneckii*	GQ334383
SD 416	*Hortaea werneckii*	GQ334384
SD 429	*Hortaea werneckii*	GQ334385
SD 430	*Hortaea werneckii*	GQ334386
SD 440	*Hortaea werneckii*	GQ334387
SD 449	*Hortaea werneckii*	GQ334388
SD 450	*Hortaea werneckii*	GQ334389
SD 454	*Hortaea werneckii*	GQ334390
SD 480	*Hortaea werneckii*	GQ334391
SD 483	*Hortaea werneckii*	GQ334392

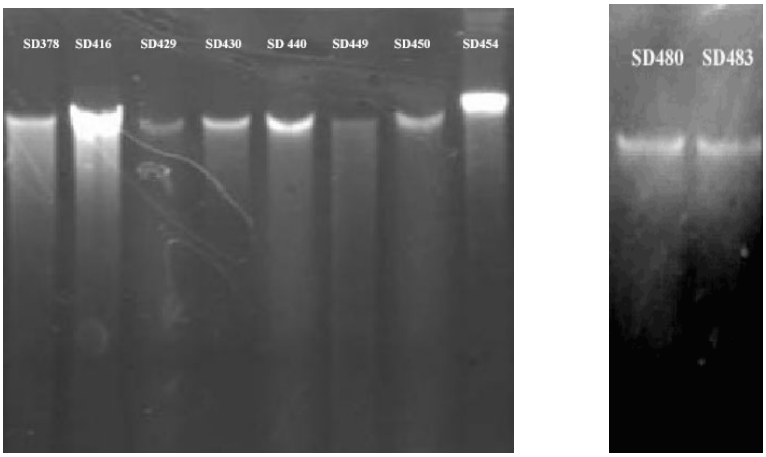

Fig. 5.12 Agarose gel electrophoresis of the genomic DNA of the 10 black yeast isolates

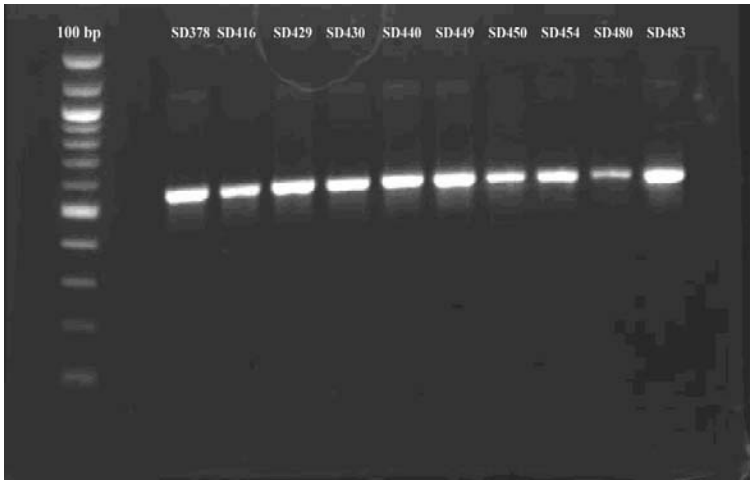

Fig. 5.13 Amplified ITS region of the 10 black yeast isolates

ITS sequences of five closely related black yeasts were downloaded from the NCBI GenBank and the Multple alignment was done with 15 black yeast ITS sequences i.e. 10 in the present study and 5 from GenBank (Fig. 5.14). A phylogenetic tree was constructed with the neighbor joining algorithm in the MEGA 4.1 package and 1000 bootstrap replicates (Fig. 5.15). The tree shows two monophyletic clades, one clade with 5 isolates SD 480, SD 440, SD 454, SD 378 and SD 429 having 99% similarity with that of the NCBI strains AB087201 *Hortaea werneckii* and FJ770076 Dothideales sp. HLS305. Another clade with remaining five isolates SD 483, SD 449, SD 416, SD 430 and SD 450 having 99% similarity with the NCBI starins FJ755827 *Hortaea* sp. F47 and EU497947 Dothideales sp. F6. A marine black yeast AJ238676 *Trimmatostroma salinum* belonging to different genus but same order i.e. Dothideales, was taken as an out-group to show the extent of similarity between the isolates. The consensus distance tree places these isolates SD 378, SD 416, SD 429, SD 430, SD 440, SD 449, SD 450, SD 454, SD 480 and SD 483, in the *Hortaea werneckii* monophyletic cluster with 99% similarity, strongly suggesting that all the isolates belong to *Hortaea werneckii*.

The analysis of similarity matrix shows that all the isolates share almost 100% similarity with a minor difference in the genetic distance ranging from 0.005-0.009, which is considered negligible when compared with the difference in the distance shown by the out group (*T. salinum*) of almost 0.3 (Appendix 2, table 5.5). This matrix shows that the 10 isolates share great genetic relatedness among themselves and also the GenBank strains, FJ770076 Dothideales sp. HLS305, AB087201 *H. werneckii*, FJ755827 *Hortaea* sp. F47 and EU497947 Dothideales sp. F6.

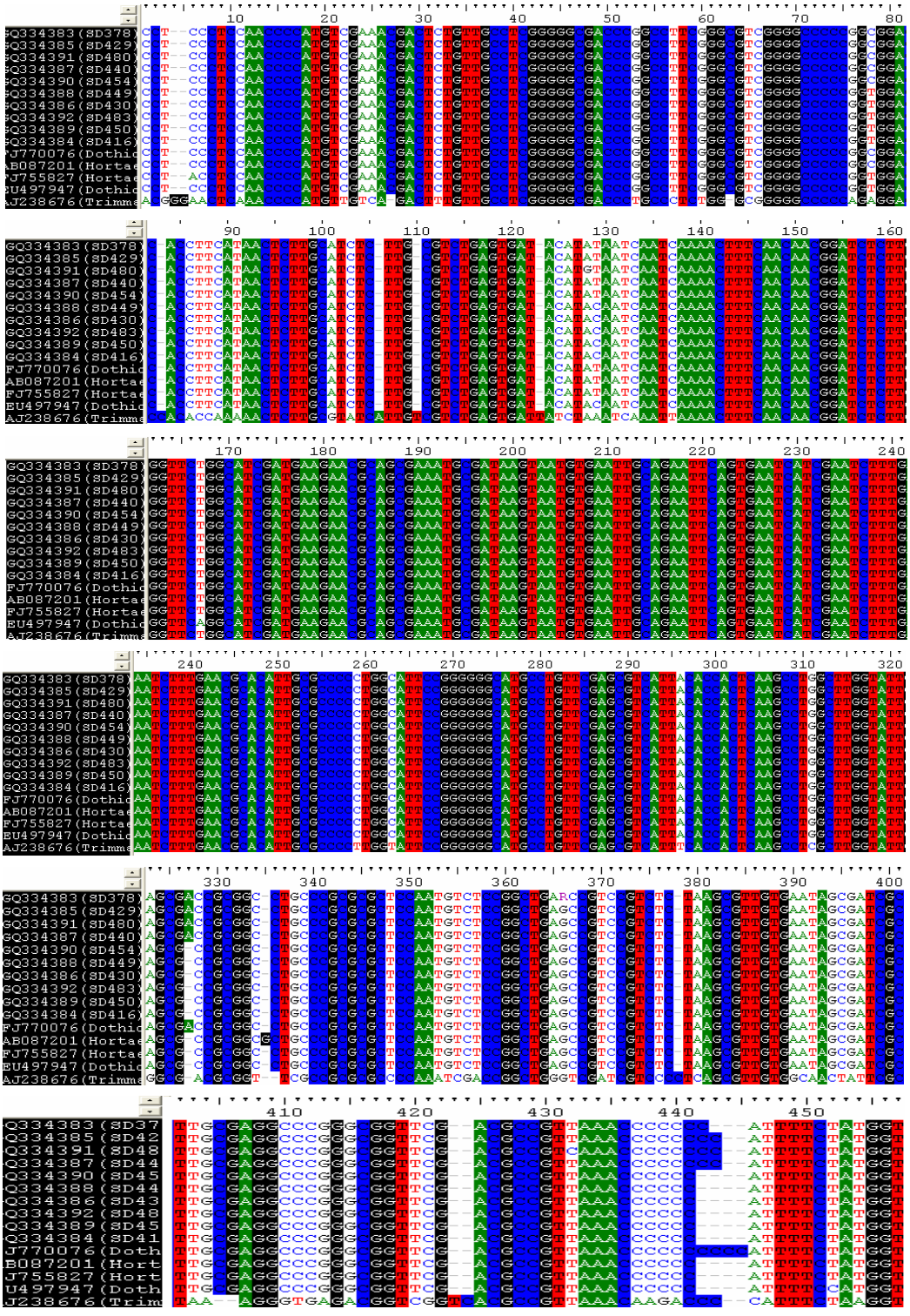

Fig. 5.14 Clustal W multiple alignment of the black yeast isolates and the NCBI strains. *(Conserved regions are indicated in colour)*

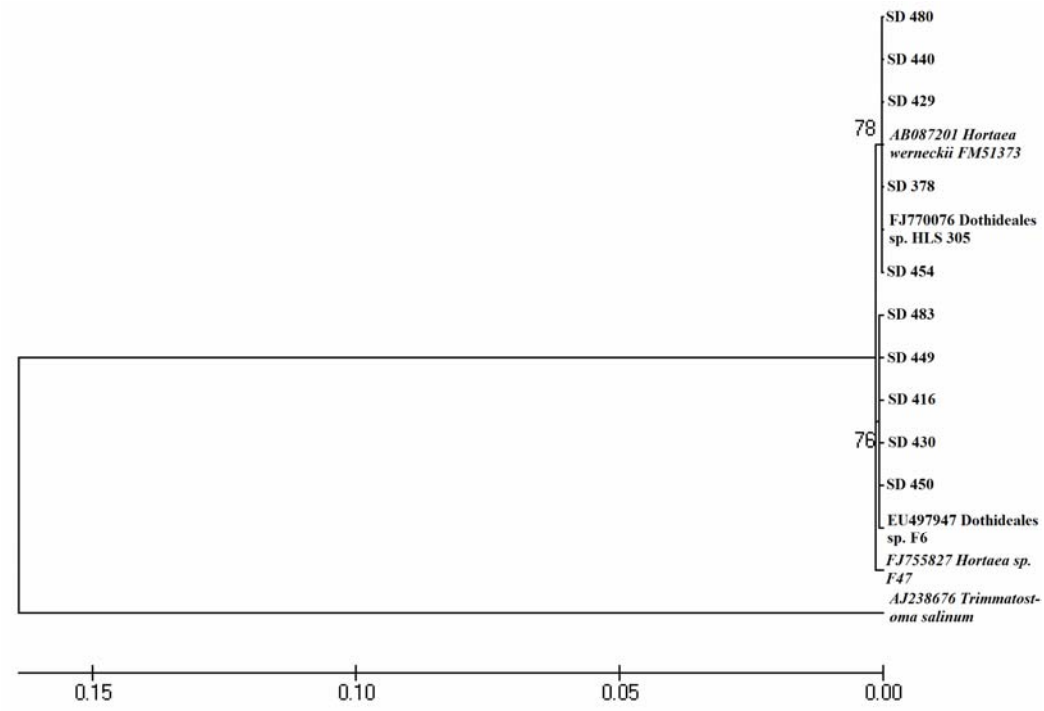

Fig. 5.15 Phylogenetic tree based on NJ method

5.3.2 Hydrolytic enzymes

All the isolates were able to produce lipase, protease and amylase. About 60% of the isolates were able to produce ligninase and 40% produced urease (Fig. 5.16). All the isolates were oxidative in nature.

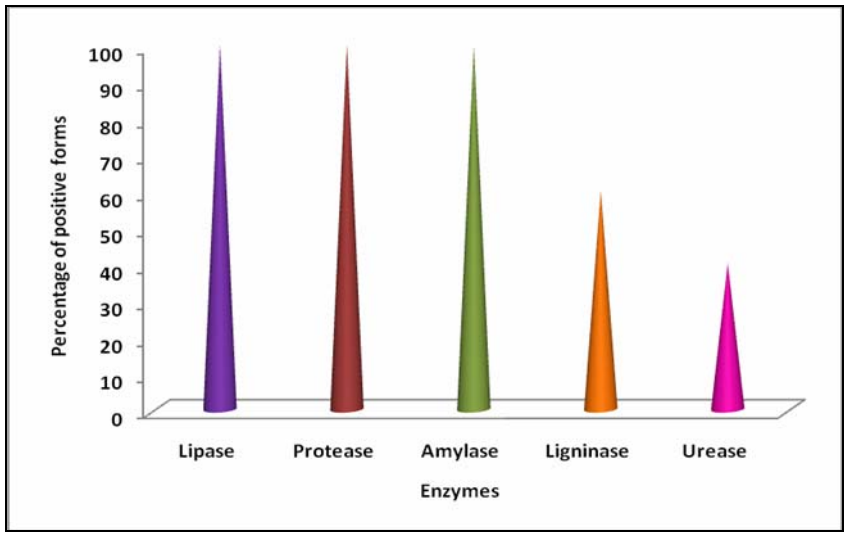

Fig. 5.16 Hydrolytic enzyme production by black yeasts

5.3.3 Optimum physico-chemical conditions for growth

The isolates had maximum growth at 30°C followed by 20, 40, 10 and 50°C (Fig. 5.17a) (Table 5.2a). All the isolates showed maximum growth at salinity between 30 and 60 ppt (Fig. 5.17b) (Table 5.2b). The isolates had maximum growth at pH 8 (Fig. 5.17c) (Table 5.2c).

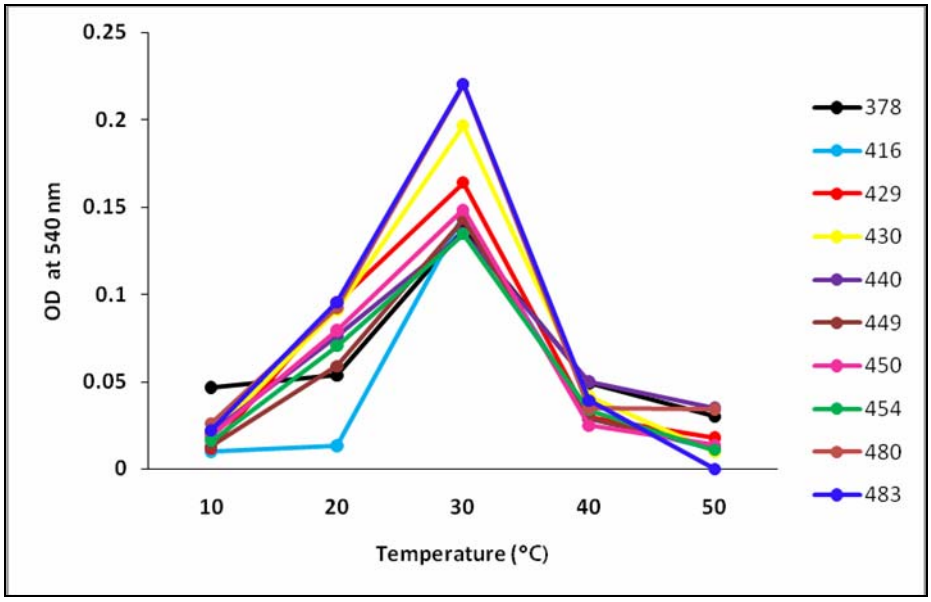

Fig. 5.17a Optimum temperature for growth of black yeast isolates

Table 5.2a Growth of the black yeasts isolates at different temperature

Isolates	10°C	20°C	30°C	40°C	50°C
378	0.047±0.004	0.089±0.002	0.137±0.009	0.049±0.003	0.010±0.003
416	0.025±0.001	0.098±0.001	0.146±0.060	0.029±0.001	0.011±0.003
429	0.012±0.001	0.096±0.001	0.164±0.008	0.033±0.001	0.010±0.001
430	0.019±0.001	0.091±0.006	0.197±0.019	0.042±0.001	0.011±0.022
440	0.021±0.001	0.076±0.008	0.135±0.010	0.045±0.002	0.012±0.006
449	0.024±0.001	0.075±0.007	0.143±0.003	0.030±0.001	0.014±0.005
450	0.019±0.008	0.080±0.005	0.149±0.009	0.034±0.004	0.014±0.002
454	0.017±0.006	0.071±0.001	0.135±0.018	0.034±0.003	0.011±0.003
480	0.027±0.001	0.093±0.002	0.220±0.003	0.035±0.027	0.011±0.009
483	0.022±0.004	0.096±0.008	0.221±0.028	0.040±0.003	0.010±0.001

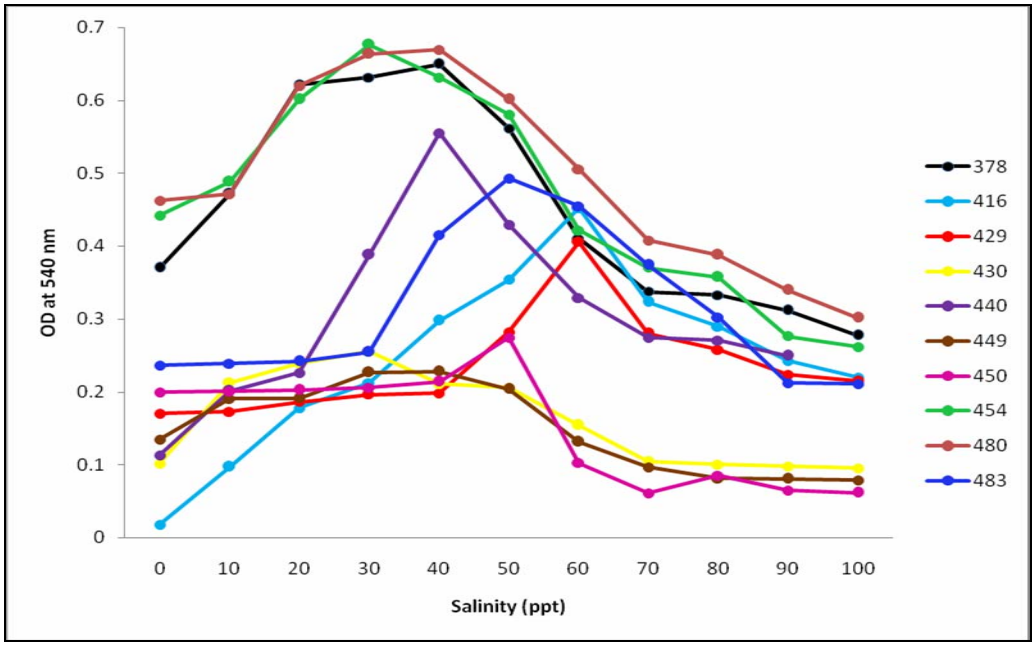

Fig. 5.17b Optimum salinity for growth of black yeast isolates

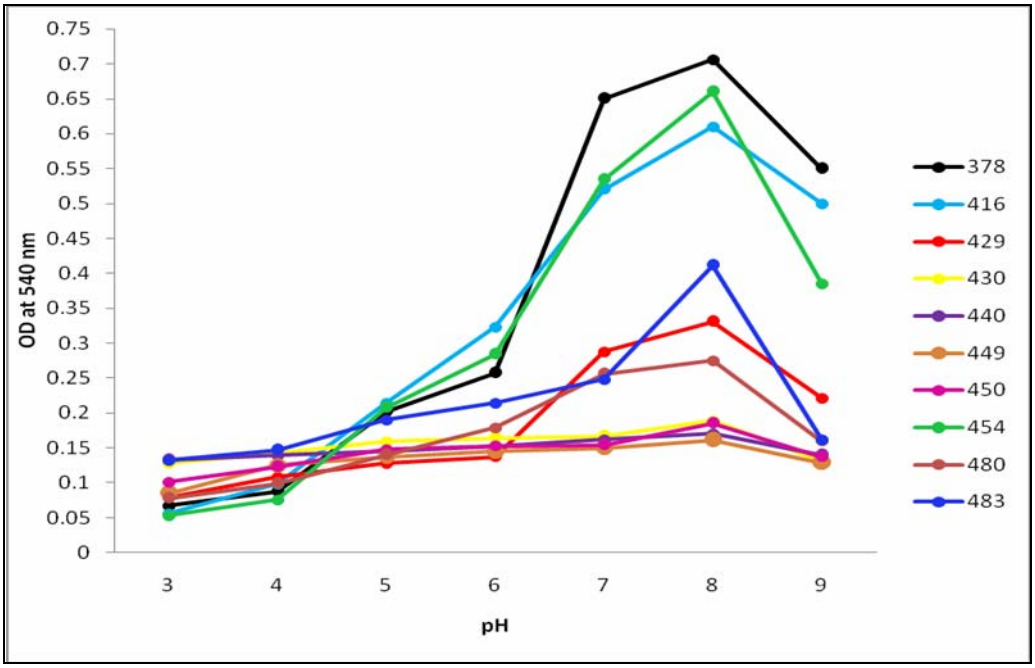

Fig. 5.17c Optimum pH for growth of black yeast isolates

Table 5.2b Growth of the black yeasts isolates at different salinity

Isolates	0 ppt	10 ppt	20 ppt	30 ppt	40 ppt	50 ppt	60 ppt	70 ppt	80 ppt	90 ppt	100 ppt
378	0.370±0.004	0.472±0.008	0.622±0.009	0.632±0.003	0.650±0.003	0.562±0.001	0.411±0.009	0.338±0.060	0.333±0.000	0.312±0.016	0.278±0.003
416	0.018±0.006	0.097±0.007	0.178±0.060	0.212±0.001	0.298±0.001	0.354±0.001	0.452±0.001	0.323±0.001	0.290±0.007	0.243±0.000	0.220±0.001
429	0.170±0.004	0.173±0.005	0.186±0.008	0.197±0.001	0.198±0.001	0.282±0.001	0.406±0.004	0.280±0.004	0.258±0.001	0.224±0.001	0.216±0.001
430	0.102±0.001	0.213±0.001	0.239±0.001	0.256±0.001	0.211±0.001	0.206±0.002	0.154±0.000	0.105±0.000	0.100±0.000	0.098±0.001	0.096±0.001
440	0.113±0.001	0.201±0.001	0.226±0.001	0.388±0.002	0.555±0.002	0.429±0.001	0.329±0.010	0.274±0.060	0.271±0.001	0.250±0.004	0.248±0.002
449	0.135±0.003	0.191±0.001	0.191±0.006	0.227±0.001	0.228±0.000	0.204±0.004	0.132±0.001	0.097±0.001	0.082±0.004	0.081±0.070	0.079±0.001
450	0.200±0.004	0.201±0.001	0.203±0.008	0.206±0.001	0.214±0.001	0.274±0.008	0.103±0.000	0.061±0.000	0.085±0.020	0.065±0.002	0.062±0.004
454	0.442±0.001	0.489±0.008	0.602±0.003	0.677±0.006	0.632±0.001	0.581±0.006	0.422±0.008	0.371±0.001	0.358±0.015	0.277±0.000	0.262±0.003
480	0.463±0.001	0.471±0.006	0.620±0.009	0.664±0.000	0.670±0.004	0.602±0.004	0.506±0.003	0.408±0.008	0.388±0.000	0.340±0.008	0.302±0.027
483	0.237±0.001	0.239±0.001	0.242±0.018	0.255±0.010	0.415±0.006	0.493±0.001	0.455±0.004	0.374±0.007	0.302±0.001	0.212±0.000	0.211±0.003

Table 5.2c Growth of the black yeasts isolates at different pH

Isolates	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9
378	0.067±0.006	0.088±0.023	0.202±0.007	0.257±0.007	0.65±0.005	0.706±0.016	0.55±0.006
416	0.056±0.012	0.098±0.010	0.214±0.005	0.323±0.000	0.52±0.008	0.61±0.000	0.5±0.006
429	0.079±0.005	0.108±0.005	0.128±0.003	0.137±0.012	0.288±0.001	0.331±0.008	0.221±0.000
430	0.128±0.017	0.142±0.006	0.159±0.006	0.164±0.002	0.167±0.006	0.189±0.007	0.131±0.019
440	0.133±0.004	0.139±0.006	0.145±0.029	0.152±0.009	0.162±0.006	0.17±0.000	0.14±0.001
449	0.085±0.001	0.125±0.019	0.137±0.018	0.145±0.015	0.15±0.002	0.161±0.008	0.129±0.014
450	0.101±0.004	0.123±0.010	0.148±0.039	0.153±0.016	0.154±0.016	0.185±0.001	0.137±0.006
454	0.053±0.039	0.076±0.020	0.208±0.014	0.284±0.000	0.534±0.023	0.66±0.006	0.385±0.009
480	0.078±0.006	0.099±0.004	0.14±0.012	0.179±0.008	0.256±0.041	0.275±0.008	0.16±0.007
483	0.132±0.004	0.147±0.017	0.19±0.007	0.214±0.019	0.248±0.008	0.411±0.019	0.162±0.000

5.3.4 Melanin extraction

NMR spectroscopy was done for all the melanin samples extracted from the 10 black yeast isolates. It was found that the melanin samples were of 1, 8-dihydroxynaphthalene (DHN) type (Fig. 5.18a). From these proton NMR spectra, each melanin isolate was found to contain varying amounts of lipids and carbohydrate. The lipid fraction is indicated by the COSY and NOESY cross peaks between the olefinic proton resonance at 5.3 ppm (Fig. 5.18c) and the aliphatic resonances between 2.5 and 0.5 ppm (Fig. 5.18b). These resonances are characteristic of a long chain aliphatic acid containing 1 olefinic bond. The two sharp aromatic resonances at 6.8 and 7.2 ppm are coupled from the COSY spectrum so are in the same para-substituted aromatic ring spin system. This assignment is supported by a correlation from the NOESY spectrum between the resonance at 6.81 ppm and the resonance at 4.09 ppm assigned to the alpha methylene group in the first ethylene oxide repeat unit of a polyethylene oxide chain attached to an aromatic ring. A weaker NOESY correlation connects to the beta methylene group of the first

ethylene oxide repeat unit. More evidence for the assignment comes from a correlation between the aromatic resonance at 7.23 ppm and aliphatic resonances at 1.3 and 1.7 ppm. The aromatic resonance region of each isolate spectrum shows the two rather sharp resonances just discussed and three broader resonances at 6.55, 6.95 and about 7.3 ppm (Fig. 5.18d). These resonances resemble resonances from indole or pyrrole structural units previously reported for melanin isolated from human hair.

Integral areas of the resonances at 6.95 and 6.55 ppm in melanins are shown in the Table 5.3. The resonance at 6.95 ppm is assigned to DHN while that at 6.55 ppm is assigned to indole structures. The relative amounts of DHN and Indole structures are shown in the columns labeled "DHN" and "Indole" below. Generally, these melanin isolates contain roughly 10-19% DHN with remaining 90-81% being Indole repeat units. The molar ratio of Indole to DHN is highest for SD 416 at 9.3, followed by SD 483 at 6.9, then SD 440 at 5.6, with 5 samples – SD 378, SD 429, SD 430, SD 454 and SD 480 – falling in the intermediate molar ratio range of 5.1 down to 4.7, and finally the lowest ratio for SD 449 and SD 450 with roughly equal ratios at 4.2.

Table 5.3 Integral areas of the resonances at 6.95 and 6.55 ppm in melanins and relative amounts of DHN & Indole

Sample	DFILE	6.95 ppm	6.55	DHN (%)	Indole (%)
SD 378	d57175	0.94	2.42	16	84
SD 416	d57176	0.36	1.68	10	90
SD 429	d57177	1.4	3.37	17	83
SD 430	d57178	2.06	4.8	18	82
SD 440	d57179	0.93	2.62	15	85
SD 449	d57180	0.95	1.99	19	81
SD 450	d57181	0.68	1.43	19	81
SD 454	d57182	1.31	3.05	18	82
SD 480	d57183	1.41	3.53	17	83
SD 483	d57186	0.49	1.69	13	87

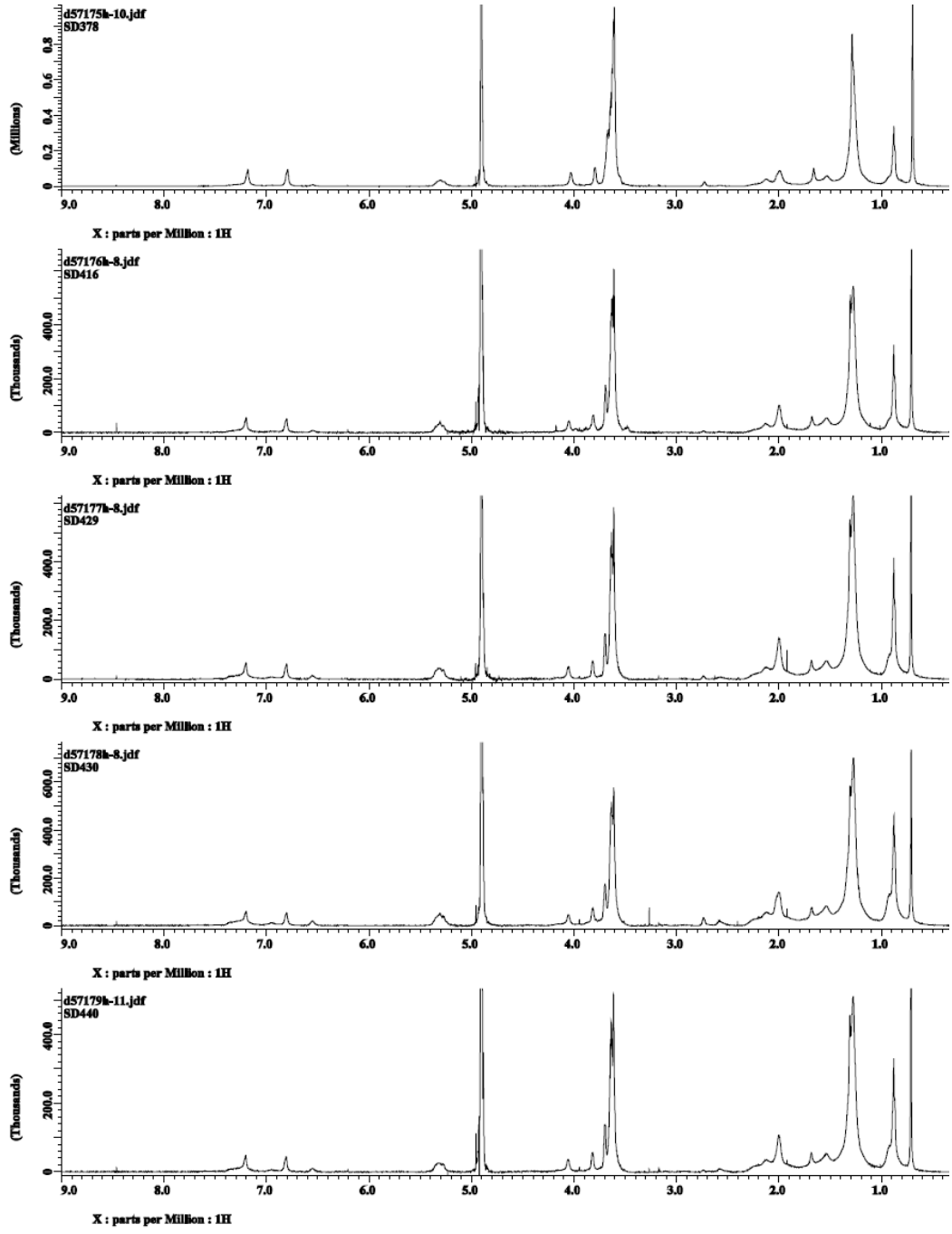

Fig. 5.18a Complete proton NMR spectra of 5 of the melanin samples, displayed from about 9.0 to 0.3 ppm (Cont…d)

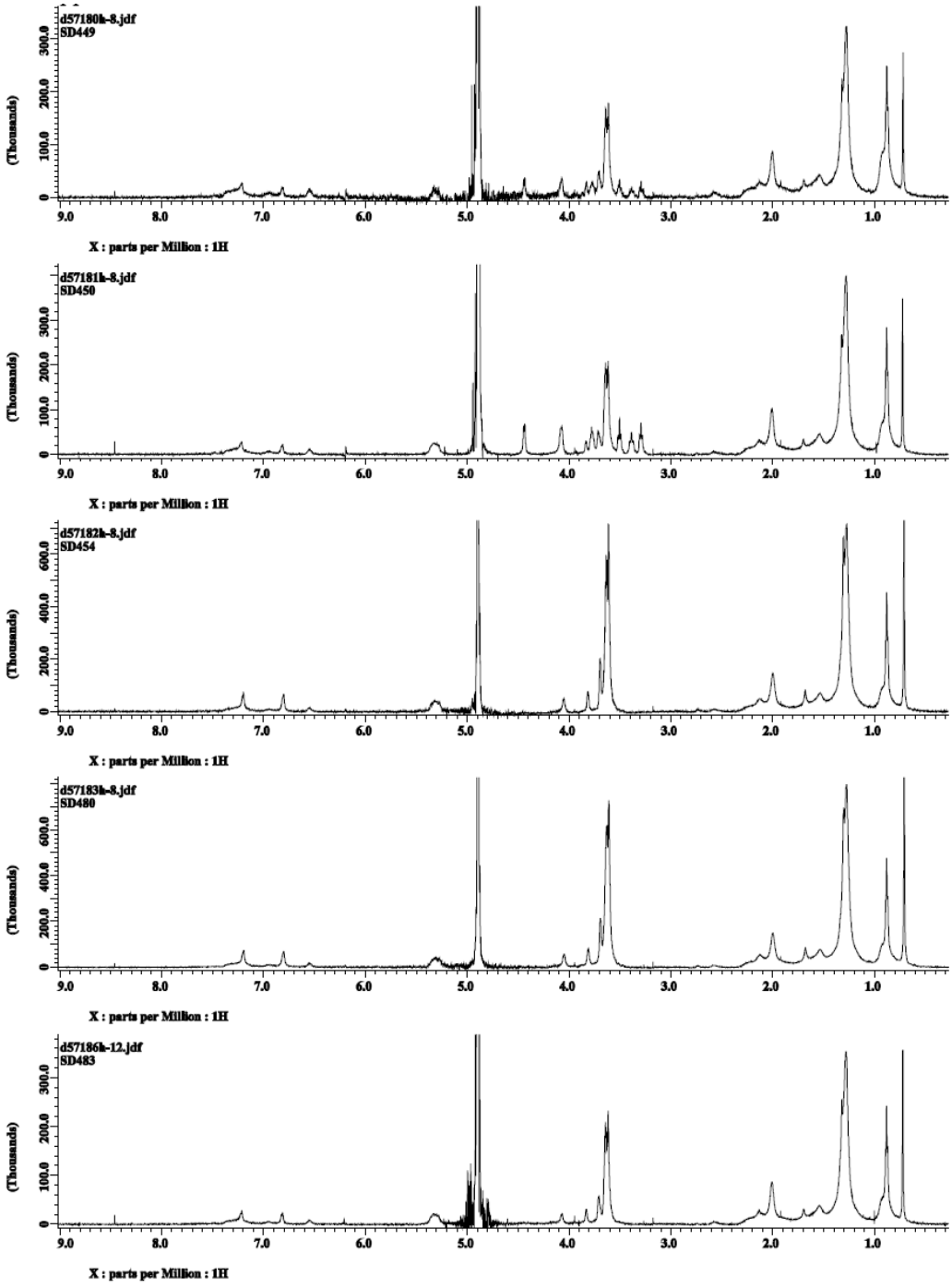

Fig. 5.18a (Cont…d) Complete proton NMR spectra of the remaining 5 melanin samples, displayed from about 9.0 to 0.3 ppm

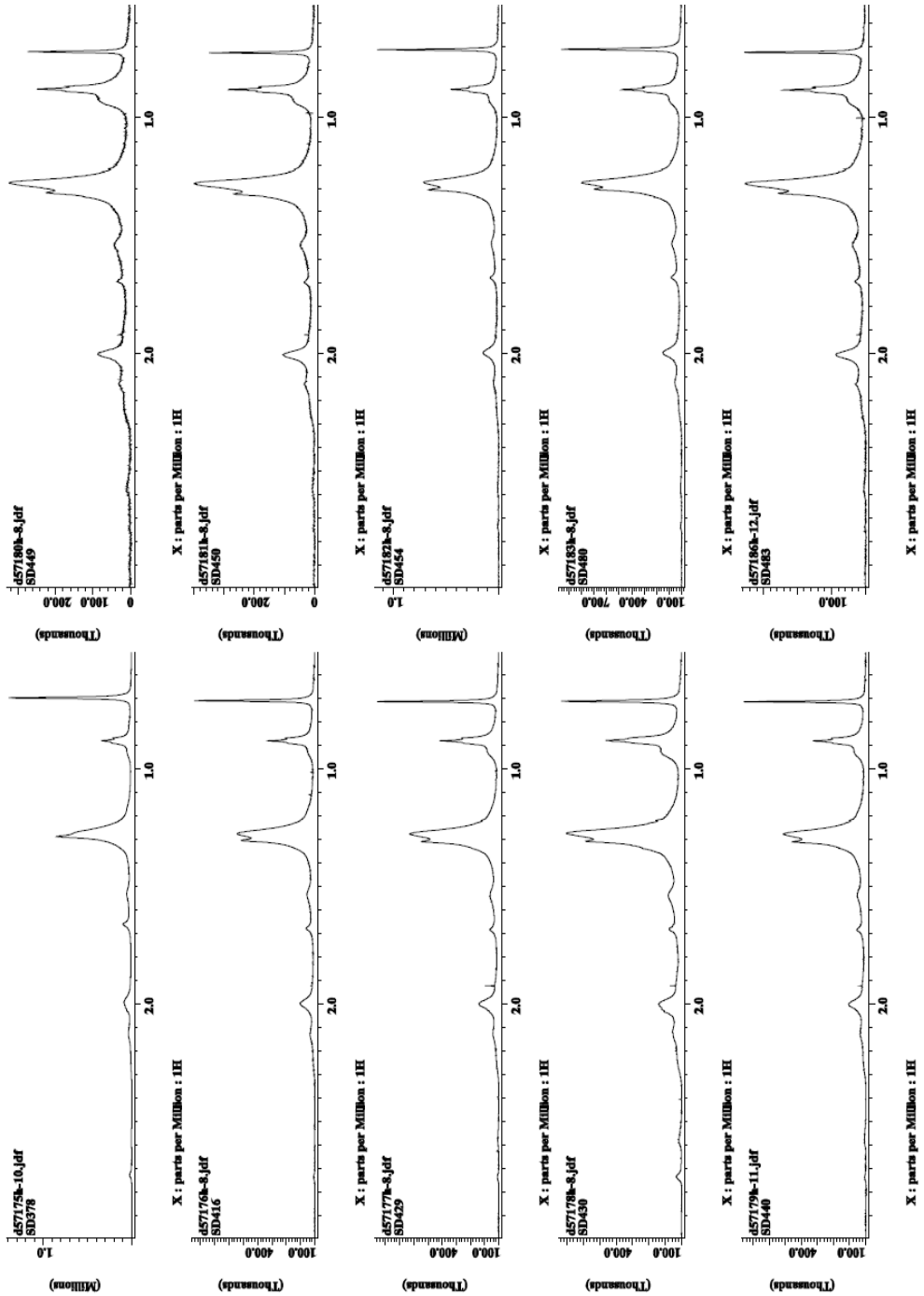

Fig. 5.18b X-axis expansion of the NMR spectra of the 10 melanin samples from about 3 to 0.5 ppm showing the aliphatic region

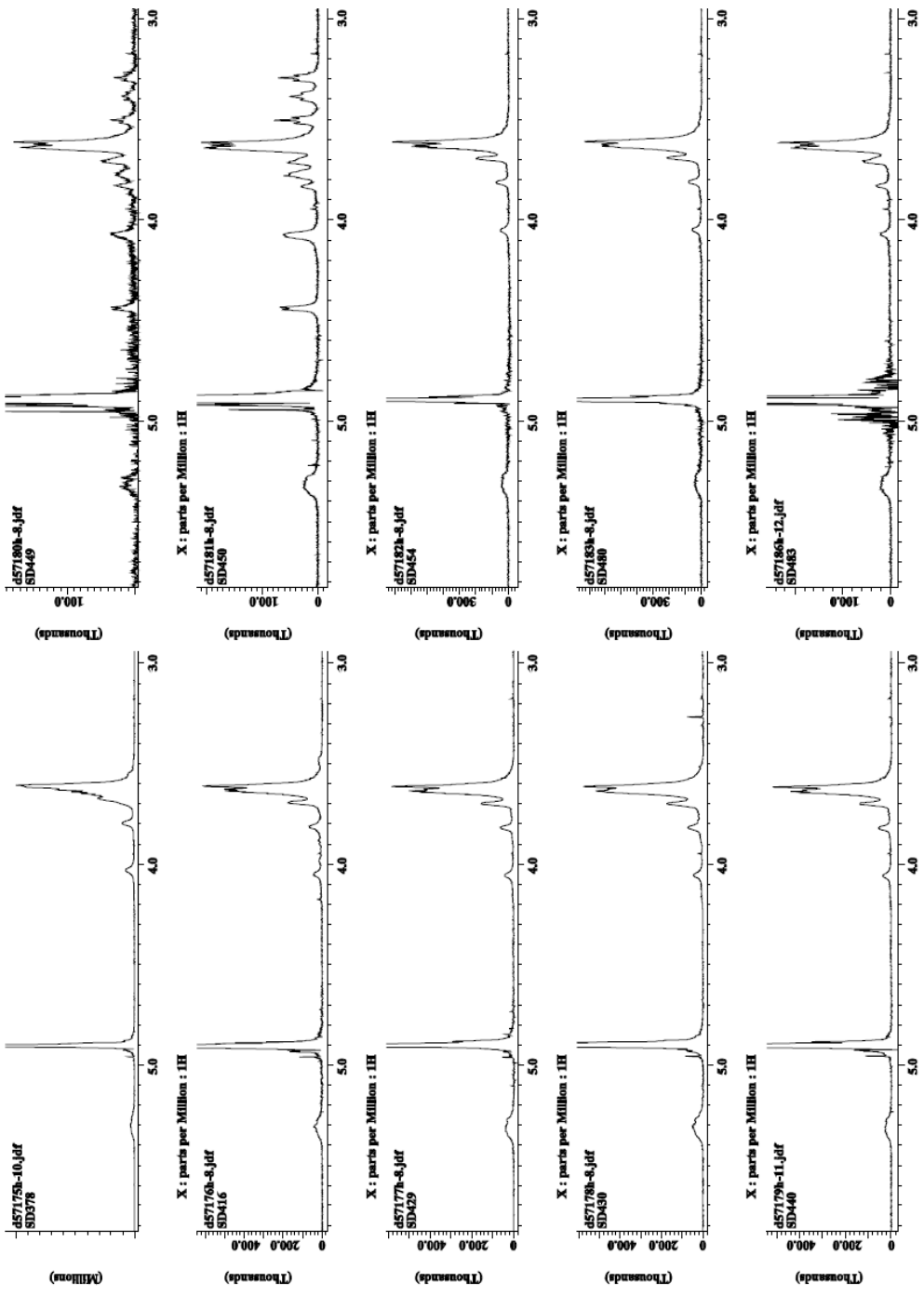

Fig. 5.18c X-axis expansion of the NMR spectra of the 10 melanin samples from about 6 to 3 ppm showing the ether and olefinic regions

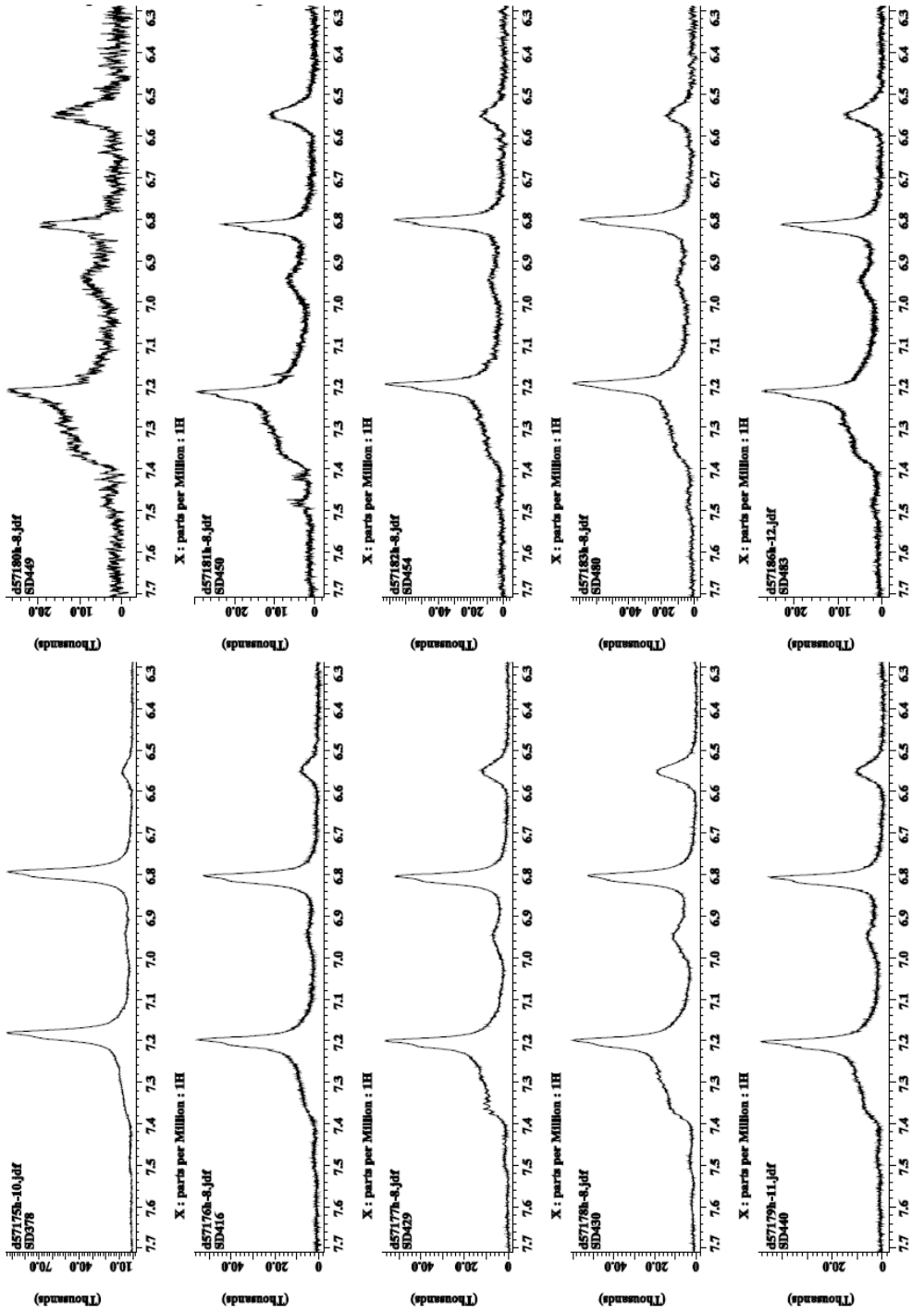

Fig. 5.18d X-axis expansion of the NMR spectra of the 10 melanin samples from about 7.7 to 6.3 ppm showing the aromatic region

5.3.5 Antibacterial activity of melanin

Melanin extracted from the black yeasts was found to have activity against almost all the human and fish pathogens tested (Fig. 5.19). Activity of melanin is given in table 5.4. Inhibitory activity was comparatively high against *Streptomyces lividans*, *Edwardsiella tarda*, *E. coli and Staphylococcus aureus*.

Table 5.4 Antibacterial activity of melanin against pathogens

Pathogens	SD378	SD416	SD429	SD430	SD440	SD449	SD450	SD454	SD480	SD483
Streptomyces lividans	++	++	++	++	++	++	+	++	++	+
Edwardsiella tarda	++	+	++	++	++	+	++	++	++	-
Aeromonas hydrophila	-	-	+	+	+	+	+	++	+	+
Vibrio harveyi	+	-	-	+	+	+	+	+	+	-
V. proteolyticus	+	-	-	+	+	+	+	+	+	-
V. fluvialis	+	-	+	+	+	+	+	+	+	-
V. cholerae	+	-	+	+	+	+	+	+	+	-
V. parahaemolyticus	+	-	+	+	+	+	+	+	+	-
E. coli	+	+	++	++	+	++	+	+	++	-
Pseudomonas aeruginosa	+	-	+	++	+	-	+	+	++	-
Bacillus cereus	-	-	-	+	-	+	-	+	+	+
Staphylococcus aureus	++	+	++	++	+	++	++	++	++	+
***Arthrobacter* sp.**	+	-	+	++	+	-	++	+	-	-

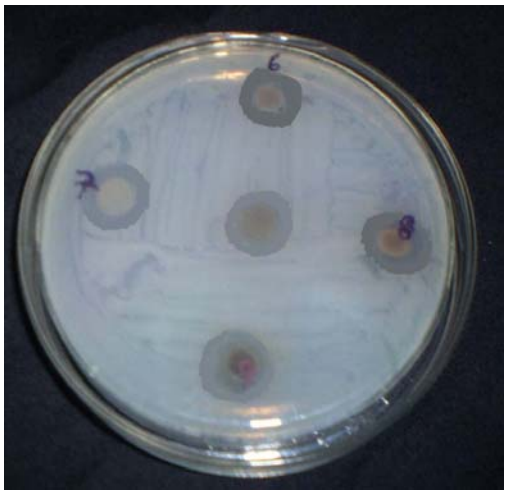

Fig. 5.19a *E. coli*

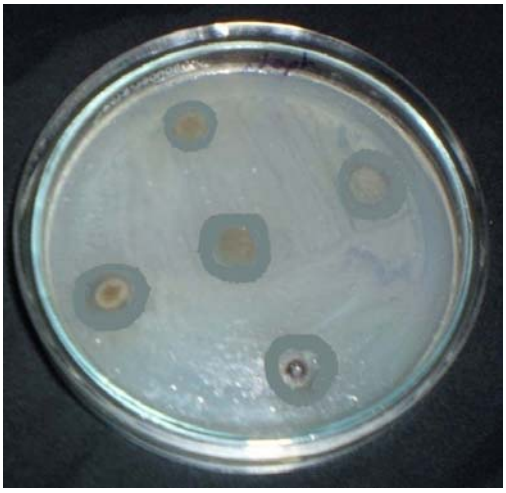

Fig. 5.19b *Staphylococcus aureus*

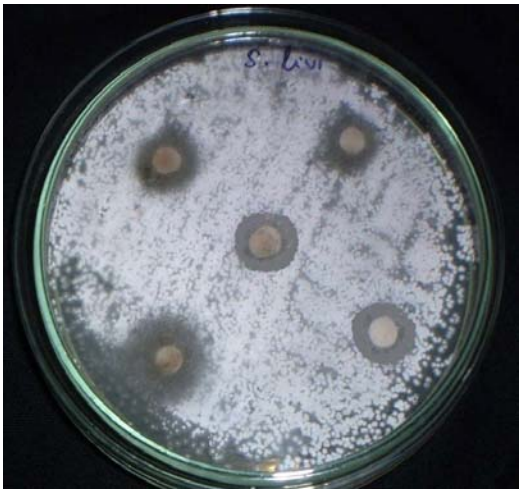

Fig. 5.19c *Streptomyces lividans*

Fig. 5.19a-c Antibacterial activity of melanin extracted from black yeasts

5.3.6 Melanin degrading enzyme

The crude enzyme extracted from black yeasts was found to have activity against the melanin extracted from the black yeasts. The enzyme was found to produce clearance zone in melanin agar plates (Fig. 5.20).

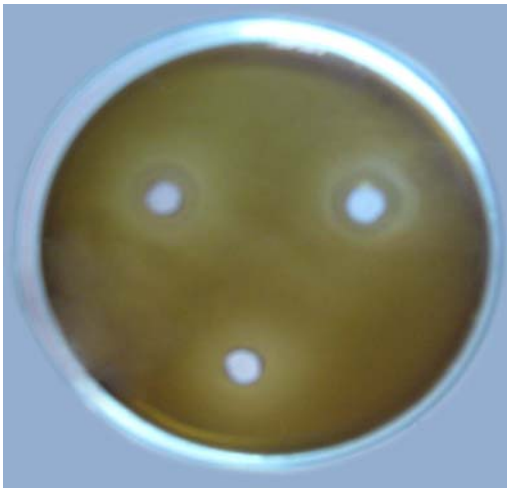

Fig. 5.20 Clearance zone showing the degradation of melanin by melanin degrading enzyme

5.4 Discussion

This is the first report of black yeasts both from the Arabian Sea and Bay of Bengal. It was difficult to identify these microbes using classical biochemical and physiological methods. The overall phylogeny of black yeasts is a complex task and needs a polyphasic approach (Rosa and Peter, 2006). So Internal Transcribed Spacer (ITS) sequencing was done for identification of black yeasts. Neighbour joining tree was constructed to determine the relationship between the isolates and the 5 other strains of black yeasts from NCBI including the strain, *Trimmatostroma salinum* which was taken as the out group. The NJ tree placed the black yeast isolates SD 378, SD 416, SD 429, SD 430, SD 440, SD 449, SD 450, SD 454, SD 480 and SD 483, in the *Hortaea werneckii* monophyletic cluster with 99% similarity. The analysis of similarity matrix in MEGA 4 package showed that all the isolates share almost 100% similarity.

Black yeasts were isolated from all the three depths: 200, 500 and 1000 m. They are characterized by slow growth rate. All the isolates showed filamentous growth and were oxidative in nature. It has already been proved that all black yeasts are aerobic; having an oxidative metabolism, no fermentation of sugars was ever observed (Rosa and Peter, 2006). All the isolates reproduced asexually by budding and they belonged to the class Ascomycetes and order Dothideales. The isolates were potent agents of biodegradation as they were able to produce lipase, protease, amylase, ligninase and urease. It has been found that black yeasts produce extra cellular glucans which is commercially used in human diet as immunostimulant. Japanese have commercialized a glucan from the black yeast like fungus, *Aureobasidium pullulans*, as AUREO (www.aureo.co.jp). The production of glucan like extra-cellular products needs to be studied in detail.

All the isolates had their maximum growth at 30ºC; however, they showed considerable growth at 20ºC also. The growth of black yeasts from nature is limited to temperatures below 32°C, whereas phylogenetically closely related species that are agents of human infections may grow at temperatures up to 37°C. For physiologically active and fully hydrated colonies, the lethal temperature is between 35 and 75°C, but dehydrated colonies can withstand up to 120°C which when transferred to fresh medium retains growth (Sterflinger, 1998). The isolates in the present study showed maximum growth at salinities between 30 and 60 ppt and considerable growth up to 100 ppt. This proves the halophilic nature of these organisms and the fact that they are excellent eukaryotic counter parts for studies regarding salt tolerance (Kogej et al., 2005).

The melanin extracted from the isolates was confirmed to be of di-hydroxynaphthalene (DHN) type. Bell and Wheeler (1986) reported that the members of the class Ascomycetes synthesize DHN type melanin. The melanin extracted was found to be complexed with lipid components. Melanins formed by DHN pathway are reported to protect these organisms against a number of environmental factors, which attests to their protective properties. The melanin

also confers an osmoprotectant role to the organisms enabling it to flourish in hypersaline conditions. Melanin preparations are widely used in dermatology and cosmetology and possess antioxidant activities. The melanin confers different properties to the organisms which prove their efficiency of action against the pathogenic microbes (Casadevall et al., 2000). The pigment was found to have antagonistic activity against different fish and human pathogens. This proves the importance of melanin in an organism to thrive under adverse and extreme conditions. Conditions for maximum production of the pigment need to be optimized for large scale production and commercial applications.

It was discovered that black yeasts, which produce melanin, also produces a melanin degrading enzyme, which has wide cosmetological and dermatological application like whitening of the skin, lightening the colour of hair etc. Experiments using the crude enzyme were successful in whitening of the skin (U.S. Patent 7291340). The crude enzyme extracted as per the patent protocol was tested for melanin degradation and it was found to degrade melanin in the melanin agar plates. The enzyme needs to be purified further and characterized for their application in cosmetics.

More studies need to be done on characterization of these organisms for industrial applications. Report on the isolation of these organisms from sea water or other environments are very few except those from hypersaline waters. According to the present study, these black yeast isolates identified as *Hortaea werneckii* are highly versatile in biodegradation besides being potent source of bioactive compounds like glucan, melanin and melanin degrading enzyme. Halophilic nature can be exploited for their utilization as eukaryotic model in salt tolerance and adaptation studies.

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Chapter **6**

SUMMARY AND CONCLUSION

Microbes constitute the invisible majority in the marine environment. Marine microorganisms include bacteria, fungi, yeasts, actinomycetes, viruses and protozoans. Eventhough there is considerable work on marine bacteria, study on marine yeasts is limited. Major properties of marine yeasts include mineralization of organic matter and mobilization of nutrients. Biotransformation of raw material into yeast biomass (single cell protein) is highly significant due to the nutritional quality of yeast and its possible utilization as animal or aquaculture feed supplement. They also have immunostimulatory property by virtue of their complex carbohydrate and nucleic acid components. Extra cellular metabolites from yeasts are also of considerable commercial importance.

The present work was focused on the occurrence and diversity of marine yeasts in the slope sediments of Arabian Sea and Bay of Bengal. Sediment samples were collected from 84 stations located at 200, 500 and 1000 m depths along the continental slope of Arabian Sea and Bay of Bengal. Hydrographical features and sediment characteristics were studied along besides the enumeration of the yeast population through plate count. The isolates were identified up to generic level based on morphological, physiological and biochemical characteristics. Growth of these isolates was examined at various pH, temperature and salinity to find out their optimal range. Hydrolytic enzyme production property of the isolates was also studied. Based on the very high lipolytic activity observed, the isolates were screened and subjected to crude oil degradation study. Black yeasts isolated during the study were subjected to detailed characterization focussing more on the bioactive compounds of commercial importance.

The salient findings of the study are as follows:

- In Arabian Sea at 200 m stations, the temperature of the sediment ranged from 14.4 to 16.1°C, at 500 m stations it ranged from 9.8 to 12.6°C and at 1000 m from 6.8 to 10.2°C. Salinity ranged from 34.9 to 35.8 ppt, and dissolved oxygen ranged from 0.95 to 2.01 ml/l in the study area.

- In Bay of Bengal, the temperature of the sediment ranged from 12.1 to 16.8ºC along the 200 m depth stations, 8.6 to 11.3ºC at 500 m depth zones and 6.1 to 9.7ºC at 1000 m stations. The salinity ranged from 33.4 to 35.0 ppt and dissolved oxygen 0.04 to 1.34 ml/l in the sampling stations of Bay of Bengal.
- In Arabian Sea the sediment texture was generally sandy at 200 m stations, silty at 500 m stations and clayey silt at 1000 m stations.
- In Bay of Bengal the sediment texture was generally clayey silt at different depth stations except a few stations in the southern region of the Bay of Bengal.
- In Arabian Sea the organic matter ranged from 0.83 to 12.8% at various stations. Maximum amount of organic matter was observed off Mumbai (12.8%). An increase in the organic matter content of sediment could be observed from 200 (2.23%) to 1000 m (7.1%) depth regions.
- In Bay of Bengal, the percentage of organic matter was high along the northern region when compared to the southern region at all the three depth zones. The percentage organic matter ranged from 0.47% to 4.62% in the sediment samples at various stations which was found to be much less when compared to that of Arabian Sea. Even though the organic matter content was higher at 500 and 1000 m compared to 200 m depth regions, the increase was not significant.
- In Arabian Sea (Cr. No. 228 & 233), the average yeast population was found to be maximum at 500 m (92.8 cfu/g dry weight of sediment) followed by 1000 m (35.2 cfu/g dry weight of sediment) and 200 m (12.5 cfu/g dry weight of sediment) depth regions. At 500 m depths, the northern transects (Off Coondapore to Porbander) showed comparatively higher population. However the population was negligible in the middle transects (Off Kannur to Ratnagiri).

- The population in Bay of Bengal (Cr. No. 236) ranged from 0.19 cfu/g dry weight (Off Thammerapattanam) to 59.3 cfu/g dry weight of the sediment (Off Bheemuli). The population was very less off Karaikkal and Thammerapattanam in the estimated sample size. There was not much variation in average population between the various depth regions. Also the population was comparatively less than that of Arabian Sea.

- Total population of yeasts in Bay of Bengal (Cr. No. 245) ranged from 0.18 cfu/g dry weight (Off Cuddallore) to 49.31 cfu/g dry weight of sediment (Off Kakinada). And generally the population was scanty in the northern transects. Yeast population varied considerably at different depth ranges and the population was maximum at 500 m depth followed by 200 and 1000 m depths.

- Among the isolates obtained from Arabian Sea (Cr. No. 228 & 233), *Candida* (56.5%) was the predominant genus followed by *Lipomyce*s (17.03%), *Rhodotorula* (11.8%), *Yarrowia* (9.5%), *Wingea* (1.7%), Black yeasts (1.3%), *Dekkera* (0.82%), *Debaryomyces* (0.67%) and *Pichia* (0.44%). Diverse genera were identified from 500 m stations.

- Among the yeast isolates from Bay of Bengal (Cr. No. 236), *Candida* (46.4%) was the predominant genera followed by Black Yeasts (23.5%), *Wingea* (20.5%), *Rhodotorula* (3.38%), *Cryptococcus* (2.3%), *Bullera* (0.99%), *Yarrowia* (0.59%), *Lipomyces* (0.59%), *Dekkera* (0.39%), *Pichia* (0.39%), *Oosporidium* (0.39%) and *Trichosporon* (0.19%). Diverse genera could be recorded from 500 m stations and comparatively lesser from 200 and 1000 m depths. Black yeasts could be obtained only from 500 and 1000 m stations and formed a major group at these depths.

- Among the Bay of Bengal isolates (Cr. No. 245), *Yarrowia* (42.2%) was the predominant genera identified followed by *Candida* (31.7%),

Cryptococcus (13.7%), Black yeasts (11.5%), *Debaryomyces* (1.33%), *Bullera* (0.88%) and *Lipomyces* (0.22%). Black yeasts were obtained from all the depths. Notably 39% of the isolates from 1000 m belonged to black yeasts.

- Among the isolates of Arabian Sea (Cr. No. 228 & 233), 92.5% were oxidative in nature and only 7.4% were fermentative. Genera wise analysis of the oxidative and fermentative forms showed that isolates belonging to the genera *Candida, Lipomyces, Yarrowia, Rhodotorula, Debaryomyces* and Black yeasts were cent percent oxidative. More than 95% of the *Wingea* spp. were oxidative and all the *Dekkera* spp. were fermentative.

- Among the isolates of Bay of Bengal (Cr. No. 236) 77% were oxidative and 23% fermentative. Genera wise analysis of the oxidative and fermentative forms showed that isolates belonging to the genera *Bullera, Oosporidium, Cryptococcus, Pichia, Lipomyces, Yarrowia, Trichosporon* and Black yeasts were cent percent oxidative in nature. *Wingea* and *Dekkera* were cent percent fermentative. Isolates belonging to *Candida* (63%) and *Rhodotorula* (93.3%) were mostly oxidative.

- Among the isolates of Bay of Bengal (Cr. No. 245) 58.4% were fermentative and 42.8% oxidative. Genera wise analysis of the oxidative and fermentative forms showed that isolates belonging to the genera *Bullera, Debaryomyces, Lipomyces* and Black yeasts were cent percent oxidative in nature whereas *Candida* and *Yarrowia* were cent percent fermentative. Isolates belonging to *Cryptococcus* (83.3%) were mostly oxidative.

- All the isolates of the Arabian Sea (Cr. No. 228 & 233) were lipolytic, followed by ligninolytic (15.8%), ureolytic (13.3%), proteolytic (8.9%), and amylolytic (4.4%) forms. None of the isolates produced aryl

sulfatase, DNAse, pectinase, cellulase and chitinase. Percentage of isolates producing protease, amylase and urease was more in 500 m depth zones. Black yeasts were cent percent positive for lipase, protease, amylase and ligninase. They were found to be the most potent isolates in enzyme production.

- All the isolates obtained from Bay of Bengal (Cr. No. 236) were lipolytic, followed by ligninolytic (63.7%), proteolytic (43.4%), ureolytic (36.2%), amylolytic (28.9%) and aryl sulfatase (1.45%) producing forms. None of the isolates produced DNAse, pectinase, cellulase and chitinase. Other than lipase production all other enzymes was found to be less in isolates from 200 m depth. The only isolate which produced aryl sulfatase was obtained from 500 m depth. Among the total isolates only one strain produced aryl sulfatase which belonged to the genus *Cryptococcus* isolated from 500 m depth station. Black yeasts were cent percent positive for lipase, protease, amylase, ligninase and 44.4% of them produced urease.
- All the isolates from Bay of Bengal (Cr. No. 245) were lipolytic, followed by proteolytic (28.5%), amylolytic (28.5%), ureolytic (18.1%) and ligninolytic (9.09%) forms. None of the isolates produced aryl sulfatase, DNAse, pectinase, cellulase and chitinase. Black yeasts were cent percent positive for lipase, protease and amylase. 40.9% were ureolytic and 31.8% ligninolytic.
- Most of the isolates preferred 30°C (69%) for maximum growth followed by 20°C (18.18%) and 40°C (12.72%). The isolates did not show growth at 10 and 50°C.
- Considerable growth could be noticed for all the isolates from 0 to 45 ppt salinity. However 15 to 25 ppt was found to be the most preferred range.

- Most of the isolates showed maximum growth at pH 6 and 7. However, considerable growth could be recorded at a pH range 4-9.
- Screening of lipolytic yeast isolates for oil degradation by visual observation showed that *Candida* sp. SD 302 and *Pichia* sp. SD 337 cause browning of the medium with dispersed tiny oil droplets.
- These two potential isolates were identified by ITS sequencing as *Candida* sp. SD 302 and *Pichia guilliermondii* SD 337.
- For *Candida* sp. SD 302, the growth was maximum at 30°C, 15 ppt salinity and pH 7. In the case of *Pichia guilliermondii* SD 337, the optimum growth conditions were 40°C, 25 ppt salinity and pH 7
- Suspended cells of *Candida* sp. SD 302 showed degradation of components C_{12} to C_{24} and also C_{28}-$C_{32.}$ Immobilized *Candida* sp. SD 302 showed complete degradation of components C_{12} to C_{16}, C_{18} to C_{24} and C_{28} to $C_{32.}$
- Suspended cells of *Pichia guilliermondii* SD 337 showed complete degradation of components C_{12} to C_{16} and C_{18} to $C_{24.}$ Complete degradation of components i.e. C_{12} to C_{14}, C_{20} to C_{24}, C_{28} to C_{36} and C_{32} to C_{36} was shown by immobilized *Pichia guilliermondii* SD 337.
- The isolates, *Candida* sp. SD 302 and *Pichia guilliermondii* SD 337 were found to be potential degraders of n-alkanes both as free and immobilized cells.
- The black yeast isolates showed radiating colonies on agar plates. The cells reproduced asexually by budding and fission. All the isolates showed filamentous growth. Molecular identification by ITS sequencing confirmed that the black yeast isolates belong to *Hortaea werneckii.*
- All the black yeast isolates were able to produce lipase, protease and amylase. About 60% of the isolates were able to produce ligninase and

40% produced urease. All the isolates were oxidative in nature. They were found to be versatile agents of biodegradation by virtue of the extracellular enzyme production.

- The black yeast isolates had maximum growth at 30ºC, 30 to 60 ppt salinity and pH 8.
- NMR spectroscopy of the melanins extracted from the 10 black yeast isolates showed that the melanins were of 1, 8-dihydroxynaphthalene (DHN) type.
- Black yeasts melanins exhibited inhibitory activity against fish / human pathogens tested.
- The black yeast extract (crude enzyme) was found to degrade the melanin extracted from the black yeasts.

The present study provides an account of the occurrence and diversity of marine yeasts in the slope sediments of Arabian Sea and Bay of Bengal. It also gives a clear idea about the role of yeasts in the benthic realm of marine ecosystem. The lipolytic potential of the organisms indicate the presence of rich lipid moieties in the study area. The isolates, *Candida* sp. SD 302 and *Pichia guilliermondii* SD 337 were proved to have potential oil degrading property and can be employed as bioremediators of oil spill after further characterization. The black yeasts isolated during the study area were found to have high commercial value by virtue of the by-products obtained from them. The melanin and the melanin degrading enzyme extracted from these organisms are potential bioactive materials for application in cosmetology.

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APPENDICES

Appendix 1

Table 3.7 Growth of selected marine yeasts at different temperature

Isolates	Depth (m)	10°C	20°C	30°C	40°C	50°C
SD 156	200	0.019 ± 0.009^{a}	**0.706 ± 0.025^{c}**	0.567 ± 0.068^{b}	0.034 ± 0.008^{a}	0.017 ± 0.012^{a}
SD 284	200	0.075 ± 0.005^{b}	**0.667 ± 0.0187^{d}**	0.519 ± 0.015^{c}	0.054 ± 0.002^{ab}	0.048 ± 0.004^{a}
SD 371	200	0.087 ± 0.008^{a}	0.761 ± 0.012^{b}	**1.016 ± 0.115^{c}**	0.063 ± 0.001^{a}	0.052 ± 0.004^{a}
SD 372	200	0.087 ± 0.003^{a}	0.744 ± 0.008^{b}	**0.944 ± 0.115^{c}**	0.063 ± 0.002^{a}	0.059 ± 0.005^{a}
SD 381	200	0.060 ± 0.011^{a}	0.672 ± 0.010^{b}	**0.903 ± 0.003^{c}**	**0.970 ± 0.141^{c}**	0.057 ± 0.004^{a}
SD 398a	200	0.044 ± 0.015^{a}	0.353 ± 0.014^{b}	**0.500 ± 0.027^{c}**	0.052 ± 0.005^{a}	0.046 ± 0.004^{a}
SD 398b	200	0.035 ± 0.013^{a}	0.497 ± 0.013^{c}	**0.884 ± 0.017^{d}**	0.195 ± 0.010^{b}	0.050 ± 0.006^{a}
SD 414	200	0.056 ± 0.008^{a}	0.593 ± 0.011^{b}	**0.908 ± 0.020^{c}**	**0.910 ± 0.048^{c}**	0.081 ± 0.005^{a}
SD 491	200	0.016 ± 0.012^{a}	**0.154 ± 0.039^{b}**	0.101 ± 0.009^{b}	0.027 ± 0.009^{a}	0.012 ± 0.003^{a}
SD 495	200	0.040 ± 0.012^{a}	0.920 ± 0.022^{b}	**1.001 ± 0.025^{c}**	**1.060 ± 0.052^{c}**	0.019 ± 0.005^{a}
SD 496	200	0.019 ± 0.009^{a}	**0.706 ± 0.025^{c}**	0.567 ± 0.068^{b}	0.034 ± 0.008^{a}	0.017 ± 0.012^{a}
SD 500	200	0.004 ± 0.006^{a}	**0.384 ± 0.010^{b}**	**0.393 ± 0.01^{b}**	0.029 ± 0.002^{a}	0.008 ± 0.004^{a}
SD 42	500	0.014 ± 0.005^{a}	**0.276 ± 0.016^{d}**	0.062 ± 0.006^{c}	0.051 ± 0.014^{bc}	0.030 ± 0.010^{ab}
SD 45	500	0.011 ± 0.006^{a}	0.624 ± 0.024^{b}	**0.966 ± 0.166^{c}**	**0.987 ± 0.074^{c}**	0.033 ± 0.007^{a}
SD 50	500	0.002 ± 0.004^{a}	**0.661 ± 0.023^{d}**	0.266 ± 0.003^{c}	0.027 ± 0.006^{b}	0.019 ± 0.011^{ab}
SD 73	500	0.000 ± 0.000^{a}	0.036 ± 0.008^{c}	**0.226 ± 0.013^{d}**	0.025 ± 0.005^{bc}	0.011 ± 0.010^{ab}
SD 77	500	0.000 ± 0.000^{a}	**0.524 ± 0.017^{c}**	0.451 ± 0.038^{b}	0.411 ± 0.019^{b}	0.034 ± 0.004^{a}
SD 90	500	0.008 ± 0.006^{a}	**0.681 ± 0.016^{c}**	0.401 ± 0.094^{b}	0.413 ± 0.013^{b}	0.044 ± 0.008^{a}
SD 105	500	0.015 ± 0.005^{a}	**0.685 ± 0.016^{c}**	0.289 ± 0.015^{b}	0.029 ± 0.005^{a}	0.020 ± 0.009^{a}
SD 112	500	0.016 ± 0.012^{a}	**0.154 ± 0.039^{c}**	0.101 ± 0.009^{b}	0.027 ± 0.009^{a}	0.012 ± 0.003^{a}
SD 141	500	0.040 ± 0.012^{a}	0.920 ± 0.022^{b}	**1.001 ± 0.025^{c}**	**1.060 ± 0.052^{c}**	0.019 ± 0.005^{a}
SD 265	500	0.004 ± 0.006^{a}	**0.384 ± 0.010^{c}**	**0.393 ± 0.016^{c}**	0.029 ± 0.002^{b}	0.008 ± 0.004^{a}
SD 267	500	0.001 ± 0.002^{a}	0.991 ± 0.004^{b}	**1.057 ± 0.009^{c}**	0.983 ± 0.020^{b}	0.004 ± 0.001^{a}
SD 382a	500	0.047 ± 0.001^{a}	**0.236 ± 0.013^{b}**	**0.221 ± 0.017^{b}**	0.053 ± 0.006^{a}	0.044 ± 0.014^{a}

SD 382b	**500**	0.048 ± 0.005^{a}	0.343 ± 0.015^{c}	**0.480 ± 0.011^{d}**	0.163 ± 0.004^{b}	0.055 ± 0.004^{a}
SD 384	**500**	0.068 ± 0.010^{a}	0.723 ± 0.012^{b}	1.076 ± 0.058^{c}	**1.217 ± 0.052^{d}**	0.057 ± 0.002^{a}
SD 386	**500**	0.064 ± 0.007^{ab}	0.663 ± 0.100^{c}	**0.824 ± 0.113^{d}**	0.204 ± 0.040^{b}	0.052 ± 0.003^{a}
SD 405	**500**	0.058 ± 0.001^{a}	0.241 ± 0.004^{b}	**0.393 ± 0.014^{c}**	0.068 ± 0.005^{a}	0.055 ± 0.000^{a}
SD 408	**500**	0.07 ± 0.015^{a}	0.996 ± 0.032^{b}	1.272 ± 0.020^{c}	**1.511 ± 0.045^{d}**	0.076 ± 0.004^{a}
SD 485	**500**	0.002 ± 0.004^{a}	**0.661 ± 0.023^{c}**	0.266 ± 0.003^{b}	0.027 ± 0.006^{a}	0.019 ± 0.011^{a}
SD 486	**500**	0.000 ± 0.000^{a}	0.036 ± 0.008^{a}	**0.226 ± 0.013^{b}**	0.025 ± 0.005^{a}	0.011 ± 0.010^{a}
SD 487	**500**	0.000 ± 0.000^{a}	**0.524 ± 0.017^{c}**	0.451 ± 0.038^{b}	0.411 ± 0.019^{b}	0.034 ± 0.004^{a}
SD 488	**500**	0.008 ± 0.006^{a}	**0.681 ± 0.016^{c}**	0.401 ± 0.094^{b}	0.413 ± 0.013^{b}	0.044 ± 0.008^{a}
SD 489	**500**	0.015 ± 0.005^{a}	**0.685 ± 0.016^{c}**	0.289 ± 0.015^{b}	0.029 ± 0.005^{a}	0.020 ± 0.009^{a}
SD 5	**1000**	0.026 ± 0.001^{a}	1.202 ± 0.035^{b}	**1.498 ± 0.013^{c}**	1.284 ± 0.224^{bc}	0.033 ± 0.002^{a}
SD 17	**1000**	0.002 ± 0.004^{a}	0.552 ± 0.027^{b}	**0.984 ± 0.044^{c}**	**1.027 ± 0.131^{c}**	0.029 ± 0.003^{a}
SD 25	**1000**	0.018 ± 0.006^{a}	0.791 ± 0.0208^{b}	**0.997 ± 0.061^{d}**	0.900 ± 0.020^{c}	0.046 ± 0.004^{a}
SD 302	**1000**	0.093 ± 0.004^{a}	0.782 ± 0.057^{b}	**0.846 ± 0.201^{b}**	0.168 ± 0.006^{a}	0.058 ± 0.001^{a}
SD 376	**1000**	0.049 ± 0.000^{a}	0.606 ± 0.013^{b}	**1.074 ± 0.071^{d}**	0.757 ± 0.038^{c}	0.050 ± 0.007^{a}
SD 387	**1000**	0.039 ± 0.002^{a}	0.573 ± 0.035^{b}	**0.903 ± 0.011^{c}**	**0.929 ± 0.091^{c}**	0.053 ± 0.001^{a}
SD 412	**1000**	0.062 ± 0.011^{a}	0.471 ± 0.017^{c}	**0.586 ± 0.003^{d}**	0.428 ± 0.019^{b}	0.068 ± 0.005^{a}

Table 3.8 Growth of selected marine yeasts at different salinity

Isolates	Depth (m)	0 ppt	5 ppt	10 ppt	15 ppt	20 ppt	25 ppt	30 ppt	35 ppt	40 ppt	45 ppt
SD 156	200	0.756 ± 0.049^{a}	0.951 ± 0.031^{b}	0.990 ± 0.026^{b}	1.053 ± 0.051^{bc}	1.171 ± 0.154^{cd}	$\mathbf{1.366 \pm 0.032^{e}}$	$\mathbf{1.332 \pm 0.038^{e}}$	$\mathbf{1.340 \pm 0.012^{e}}$	1.273 ± 0.010^{de}	1.262 ± 0.027^{de}
SD 284	200	0.047 ± 0.004^{abc}	0.051 ± 0.009^{ab}	0.057 ± 0.101^{bc}	0.069 ± 0.005^{abc}	0.087 ± 0.059^{abc}	$\mathbf{0.166 \pm 0.041^{c}}$	0.151 ± 0.050^{bc}	0.095 ± 0.038^{abc}	0.061 ± 0.010^{abc}	0.033 ± 0.031^{a}
SD 363	200	0.360 ± 0.033^{ab}	0.458 ± 0.014^{ab}	0.475 ± 0.036^{b}	0.488 ± 0.080^{b}	$\mathbf{0.608 \pm 0.077^{c}}$	0.444 ± 0.070^{ab}	0.386 ± 0.020^{ab}	0.371 ± 0.056^{ab}	0.372 ± 0.061^{ab}	0.333 ± 0.046^{a}
SD 371	200	0.708 ± 0.096^{a}	0.737 ± 0.041^{a}	0.763 ± 0.073^{ab}	0.767 ± 0.061^{ab}	0.792 ± 0.138^{ab}	0.832 ± 0.038^{ab}	0.849 ± 0.047^{ab}	$\mathbf{0.945 \pm 0.137^{b}}$	0.794 ± 0.034^{ab}	0.766 ± 0.061^{ab}
SD 372	200	0.558 ± 0.167^{ac}	0.633 ± 0.053^{ab}	0.646 ± 0.026^{ab}	0.681 ± 0.073^{abc}	0.715 ± 0.045^{abc}	$\mathbf{0.890 \pm 0.106^{c}}$	$\mathbf{0.880 \pm 0.131^{c}}$	0.860 ± 0.013^{bc}	0.850 ± 0.101^{bc}	0.807 ± 0.136^{bc}
SD 381	200	0.541 ± 0.029^{a}	0.566 ± 0.057^{a}	0.579 ± 0.027^{a}	0.590 ± 0.024^{ab}	$\mathbf{0.644 \pm 0.047^{b}}$	$\mathbf{0.614 \pm 0.035^{b}}$	0.590 ± 0.057^{ab}	0.589 ± 0.023^{ab}	0.543 ± 0.033^{a}	0.513 ± 0.065^{a}
SD 398a	200	0.401 ± 0.032^{a}	0.412 ± 0.014^{a}	0.468 ± 0.025^{a}	0.480 ± 0.055^{a}	0.493 ± 0.017^{ab}	0.508 ± 0.036^{abc}	0.520 ± 0.049^{abc}	$\mathbf{0.621 \pm 0.121^{c}}$	0.612 ± 0.072^{bc}	0.444 ± 0.002^{a}
SD 398b	200	0.719 ± 0.045^{a}	0.754 ± 0.038^{abc}	0.761 ± 0.047^{abc}	0.810 ± 0.031^{bc}	$\mathbf{0.822 \pm 0.025^{c}}$	0.735 ± 0.052^{ab}	0.723 ± 0.034^{a}	0.720 ± 0.019^{a}	0.700 ± 0.020^{a}	0.682 ± 0.042^{a}
SD 400	200	0.038 ± 0.009^{a}	0.040 ± 0.003^{a}	0.051 ± 0.004^{a}	0.067 ± 0.018^{a}	0.089 ± 0.027^{a}	$\mathbf{0.452 \pm 0.011^{b}}$	$\mathbf{0.448 \pm 0.008^{b}}$	$\mathbf{0.445 \pm 0.085^{b}}$	$\mathbf{0.436 \pm 0.011^{b}}$	$\mathbf{0.378 \pm 0.042^{b}}$
SD 414	200	0.858 ± 0.039^{abcd}	0.917 ± 0.010^{bcd}	0.921 ± 0.034^{cd}	$\mathbf{0.928 \pm 0.047^{d}}$	0.864 ± 0.043^{abcd}	0.852 ± 0.068^{abc}	0.833 ± 0.015^{abc}	0.831 ± 0.056^{abc}	0.827 ± 0.028^{ab}	0.813 ± 0.009^{a}
SD 417	200	0.053 ± 0.004^{a}	0.055 ± 0.010^{a}	0.061 ± 0.000^{a}	0.063 ± 0.004^{a}	0.117 ± 0.065^{a}	$\mathbf{1.020 \pm 0.033^{d}}$	0.788 ± 0.036^{c}	0.769 ± 0.043^{c}	0.756 ± 0.028^{c}	0.439 ± 0.020^{b}
SD 424	200	0.046 ± 0.006^{a}	0.055 ± 0.001^{a}	0.069 ± 0.011^{a}	0.070 ± 0.015^{a}	0.071 ± 0.013^{a}	$\mathbf{1.340 \pm 0.035^{e}}$	1.097 ± 0.012^{d}	0.940 ± 0.015^{c}	0.931 ± 0.053^{c}	0.538 ± 0.037^{b}
SD 491	200	0.994 ± 0.018^{a}	1.030 ± 0.032^{ab}	1.082 ± 0.013^{cd}	$\mathbf{1.095 \pm 0.023^{d}}$	1.051 ± 0.008^{bc}	1.050 ± 0.028^{bc}	1.048 ± 0.008^{bc}	1.027 ± 0.008^{ab}	1.024 ± 0.018^{ab}	1.046 ± 0.002^{bc}
SD 495	200	0.985 ± 0.020^{a}	1.076 ± 0.020^{cd}	1.084 ± 0.039^{cd}	$\mathbf{1.107 \pm 0.019^{d}}$	1.058 ± 0.019^{bc}	1.058 ± 0.021^{bc}	1.050 ± 0.016^{bc}	1.038 ± 0.020^{bc}	1.025 ± 0.013^{ab}	1.025 ± 0.006^{ab}
SD 496	200	$0.966 \pm 0.032a$	1.048 ± 0.024^{bc}	1.087 ± 0.009^{cd}	1.095 ± 0.025^{cd}	$\mathbf{1.103 \pm 0.020^{d}}$	1.068 ± 0.017^{bc}	1.046 ± 0.022^{b}	1.038 ± 0.014^{b}	1.025 ± 0.007^{b}	1.008 ± 0.026^{b}

SD 498	**200**	0.978±0.008a	1.064±0.020^{bc}	1.099±0.035^{cd}	**1.121±0.022^{d}**	**1.121±0.002^{d}**	1.061±0.023^{bc}	1.053±0.026^{b}	1.050±0.004^{b}	1.041±0.016^{b}	1.032±0.007^{b}
SD 500	**200**	0.954±0.004^{a}	1.033±0.025^{b}	1.062±0.023^{bcd}	**1.101±0.013^{d}**	1.087±0.013^{cd}	1.052±0.014^{bc}	1.052±0.011^{bc}	1.050±0.033^{bc}	1.037±0.032^{b}	1.017±0.012^{b}
SD 42	**500**	0.636±0.036^{bc}	0.686±0.060^{bc}	0.724±0.039^{c}	**0.854±0.058^{d}**	**0.849±0.029^{d}**	0.760±0.079^{cd}	0.747±0.012^{cd}	0.663±0.033^{bc}	0.580±0.061^{ab}	0.481±0.093^{a}
SD 45	**500**	0.934±0.031^{b}	0.990±0.016^{bc}	1.072±0.060^{cd}	**1.190±0.032^{e}**	1.163±0.014^{de}	1.086±0.041^{cd}	0.970±0.064^{b}	0.930±0.019^{b}	0.923±0.055^{b}	0.770±0.068^{a}
SD 50	**500**	0.330±0.126^{ab}	0.355±0.071^{abc}	0.416±0.171^{abcd}	0.448±0.159^{abcd}	**0.612±0.055^{d}**	0.558±0.034^{cd}	0.514±0.036^{bcd}	0.384±0.051^{abc}	0.377±0.009^{abc}	0.243±0.082^{a}
SD 73	**500**	0.578±0.061^{b}	0.593±0.030^{b}	0.630±0.055^{bc}	**0.720±0.038^{c}**	**0.728±0.023^{c}**	**0.728±0.013^{c}**	0.703±0.048^{c}	0.552±0.063^{ab}	0.460±0.025^{a}	0.456±0.054^{a}
SD 77	**500**	0.499±0.030^{b}	0.530±0.091^{bc}	0.613±0.018^{bcd}	**0.707±0.013^{d}**	0.632±0.076^{cd}	0.622±0.101^{bcd}	0.585±0.040^{bcd}	0.373±0.003^{a}	0.353±0.032^{a}	0.333±0.018^{a}
SD 90	**500**	0.555±0.008^{cd}	0.558±0.023^{cd}	0.568±0.025^{cd}	**0.572±0.027^{d}**	0.559±0.015^{cd}	0.538±0.013^{bcd}	0.522±0.002^{bc}	0.506±0.035^{ab}	0.471±0.020^{a}	0.496±0.009^{ab}
SD 105	**500**	0.327±0.030^{a}	0.411±0.003^{ab}	0.473±0.055^{bcd}	0.610±0.091^{de}	**0.653±0.027^{e}**	0.560±0.042^{cde}	0.504±0.020^{bcd}	0.485±0.123^{bcd}	0.480±0.008^{bcd}	0.452±0.087^{abc}
SD 112	**500**	0.309±0.028^{a}	0.385±0.039^{ab}	0.404±0.018^{ab}	**0.547±0.011^{c}**	**0.652±0.021^{c}**	**0.611±0.029^{c}**	**0.579±0.031^{c}**	0.514±0.018^{bc}	0.414±0.117^{ab}	0.389±0.13^{ab}
SD 141	**500**	0.228±0.001^{ab}	0.229±0.038^{ab}	0.247±0.020^{abc}	0.259±0.036^{abc}	**0.398±0.114^{d}**	0.368±0.028^{cd}	0.303±0.136^{bcd}	0.224±0.008^{ab}	0.186±0.011^{ab}	0.158±0.006^{a}
SD 265	**500**	0.771±0.022^{a}	0.789±0.025^{ab}	0.835±0.020^{bc}	0.841±0.052^{bc}	0.850±0.009^{c}	**1.322±0.017^{f}**	1.291±0.025^{ef}	1.265±0.006^{ef}	1.239±0.028^{e}	1.137±0.029^{d}
SD 267	**500**	0.426±0.108^{a}	0.534±0.041^{ab}	0.624±0.059^{abc}	**1.059±0.248^{d}**	0.862±0.007^{cd}	0.872±0.235^{cd}	0.750±0.022^{bc}	0.660±0.030^{abc}	0.624±0.004^{abc}	0.591±0.116^{abc}
SD 382a	**500**	0.161±0.136^{a}	0.235±0.206^{a}	0.351±0.141^{ab}	0.388±0.208^{ab}	0.542±0.054^{bc}	**0.805±0.024^{d}**	**0.832±0.045^{d}**	**0.815±0.034^{d}**	**0.811±0.008^{d}**	0.715±0.040^{cd}
SD 382b	**500**	**0.359±0.017^{b}**	**0.364±0.013^{b}**	**0.376±0.024^{b}**	**0.411±0.030^{b}**	**0.399±0.073^{b}**	**0.398±0.049^{b}**	**0.395±0.014^{b}**	**0.349±0.007^{b}**	**0.341±0.014^{b}**	0.236±0.060^{a}
SD 384	**500**	0.785±0.078^{cd}	0.802±0.040^{d}	0.805±0.034^{d}	0.808±0.083^{d}	0.854±0.055^{de}	**0.944±0.068^{e}**	0.744±0.040^{bcd}	0.678±0.025^{abc}	0.664±0.008^{ab}	0.610±0.043^{a}
SD 386	**500**	**0.701±0.026^{d}**	**0.696±0.120^{d}**	0.619±0.037^{cd}	0.560±0.050^{bc}	0.537±0.012^{abc}	0.520±0.018^{abc}	0.500±0.049^{abc}	0.486±0.060^{ab}	0.459±0.019^{ab}	0.422±0.062^{a}

SD 408	**500**	**1.812±0.115^{c}**	1.555±0.105^{b}	1.525±0.126^{b}	1.521±0.042^{b}	1.509±0.032^{b}	1.050±0.028^{a}	1.048±0.008^{a}	1.046±0.002^{a}	1.027±0.008^{a}	1.024±0.018^{a}
SD 443	**500**	0.561±0.049^{a}	0.574±0.038^{a}	0.581±0.020^{a}	0.618±0.025^{ab}	0.659±0.049^{bc}	0.687±0.034^{bcd}	0.726±0.049^{cd}	**0.757±0.008^{d}**	**0.753±0.024^{d}**	0.726±0.011^{cd}
SD 485	**500**	0.448±0.013^{cd}	**0.480±0.006^{d}**	**0.482±0.002^{d}**	**0.469±0.019^{d}**	0.424±0.021^{bc}	0.412±0.018^{abc}	0.405±0.045^{abc}	0.393±0.013^{ab}	0.394±0.004^{ab}	0.374±0.008^{a}
SD 486	**500**	0.476±0.028^{cde}	0.497±0.033^{de}	**0.521±0.020^{e}**	**0.527±0.052^{e}**	0.457±0.013^{bc}	0.439±0.013^{abc}	0.433±0.024^{abc}	0.426±0.023^{abcd}	0.412±0.008^{ab}	0.382±0.007^{a}
SD 487	**500**	0.539±0.022^{bc}	**0.558±0.009^{c}**	**0.562±0.027^{c}**	**0.568±0.015^{c}**	0.536±0.009^{bc}	0.531±0.008^{bc}	0.481±0.011^{ab}	0.453±0.009^{a}	0.442±0.010^{a}	0.438±0.095^{a}
SD 488	**500**	**0.573±0.012^{d}**	**0.565±0.016^{d}**	**0.555±0.024^{d}**	**0.555±0.019^{d}**	0.538±0.017^{cd}	0.510±0.006^{bc}	0.477±0.018^{ab}	0.456±0.015^{a}	0.447±0.005^{a}	0.443±0.021^{a}
SD 489	**500**	**0.618±0.023^{f}**	0.564±0.008^{e}	0.560±0.009^{e}	0.529±0.009^{de}	0.520±0.018^{de}	0.495±0.007^{cd}	0.488±0.035^{bcd}	0.474±0.037^{abc}	0.449±0.015^{ab}	0.438±0.003^{a}
SD 5	**1000**	1.617±0.012^{b}	1.644±0.033^{b}	1.666±0.073^{b}	**1.946±0.145^{c}**	**1.896±0.084^{c}**	1.570±0.035^{ab}	1.569±0.020^{ab}	1.546±0.019^{ab}	1.538±0.010^{ab}	1.463±0.016^{a}
SD 17	**1000**	0.718±0.069^{ab}	0.747±0.041^{ab}	0.812±0.022^{b}	**1.147±0.211^{c}**	0.874±0.017^{b}	0.872±0.084^{b}	0.812±0.116^{b}	0.748±0.036^{ab}	0.681±0.046^{ab}	0.560±0.091^{a}
SD 25	**1000**	1.347±0.038^{ab}	1.408±0.025^{b}	1.438±.102^{b}	**1.596±0.023^{c}**	1.453±0.060^{b}	1.329±0.068^{ab}	1.308±0.099^{ab}	1.228±0.041^{a}	1.217±0.036^{a}	1.213±0.107^{a}
SD 302	**1000**	1.032±0.009^{b}	1.060±0.026^{bc}	1.164±0.020^{cd}	**1.243±0.131^{d}**	1.154±0.037^{cd}	1.064±0.059^{bc}	1.063±0.021^{bc}	1.022±0.046^{b}	0.958±0.020^{ab}	0.879±0.028^{a}
SD 365	**1000**	0.776±0.020^{abc}	0.848±0.012^{abc}	0.899±0.032^{c}	**0.905±0.039^{c}**	**0.936±0.087^{c}**	0.895±0.088^{bc}	0.863±0.087^{bc}	0.787±0.134^{abc}	0.721±0.083^{ab}	0.684±0.071^{a}
SD 376	**1000**	1.279±0.031^{c}	**1.468±0.037^{d}**	**1.453±0.055^{d}**	1.112±0.096^{b}	1.088±0.129^{b}	0.926±0.045^{a}	0.912±0.052^{a}	0.905±0.057^{a}	0.871±0.005^{a}	0.804±0.042^{a}
SD 387	**1000**	**0.767±0.036^{b}**	**0.789±0.042^{b}**	**0.798±0.038^{b}**	**0.819±0.008^{b}**	**0.822±0.024^{b}**	**0.799±0.016^{b}**	**0.794±0.016^{b}**	**0.783±0.018^{b}**	**0.770±0.018^{b}**	0.609±0.132^{a}
SD 412	**1000**	0.450±0.028^{bc}	0.517±0.063^{c}	0.758±0.043^{d}	**1.120±0.037^{e}**	**1.109±0.033^{e}**	**1.121±0.030^{e}**	0.419±0.240^{abc}	0.300±0.038^{ab}	0.281±0.007^{ab}	0.253±0.020^{a}

Table 3.9 Growth of selected marine yeasts at different pH

Isolates	Depth (m)	pH 4	pH 5	pH 6	pH 7	pH 8	pH 9
SD 156	200	0.063 ± 0.017^{a}	0.343 ± 0.006^{b}	0.467 ± 0.025^{d}	0.514 ± 0.010^{e}	**0.732 ± 0.013^{f}**	0.430 ± 0.014^{c}
SD 284	200	0.283 ± 0.011^{c}	0.374 ± 0.018^{d}	0.483 ± 0.015^{e}	**0.517 ± 0.007^{f}**	0.195 ± 0.009^{b}	0.143 ± 0.012^{a}
SD 363	200	0.480 ± 0.018^{c}	0.482 ± 0.014^{c}	0.496 ± 0.002^{c}	**0.617 ± 0.016^{d}**	0.352 ± 0.017^{b}	0.254 ± 0.024^{a}
SD 371	200	0.370 ± 0.003^{cd}	**0.608 ± 0.007^{e}**	0.390 ± 0.013^{d}	0.347 ± 0.023^{c}	0.281 ± 0.011^{b}	0.218 ± 0.016^{a}
SD 372	200	0.455 ± 0.015^{d}	**0.507 ± 0.013^{e}**	0.424 ± 0.006^{d}	0.369 ± 0.022^{c}	0.311 ± 0.003^{b}	0.259 ± 0.019^{a}
SD 381	200	0.293 ± 0.005^{ab}	**0.522 ± 0.018^{e}**	0.461 ± 0.017^{d}	0.377 ± 0.016^{c}	0.336 ± 0.032^{bc}	0.259 ± 0.026^{a}
SD 398a	200	0.451 ± 0.006^{d}	**0.673 ± 0.013^{f}**	0.537 ± 0.006^{e}	0.328 ± 0.013^{c}	0.256 ± 0.019^{b}	0.212 ± 0.002^{a}
SD 398b	200	0.525 ± 0.006^{e}	**0.565 ± 0.024^{f}**	0.418 ± 0.009^{d}	0.330 ± 0.020^{c}	0.261 ± 0.026^{b}	0.197 ± 0.009^{a}
SD 400	200	**0.425 ± 0.019^{d}**	**0.426 ± 0.018^{d}**	**0.457 ± 0.011^{d}**	0.361 ± 0.007^{c}	0.328 ± 0.012^{b}	0.267 ± 0.015^{a}
SD 414	200	0.317 ± 0.004^{a}	0.366 ± 0.010^{b}	0.413 ± 0.001^{c}	**0.477 ± 0.015^{d}**	0.375 ± 0.021^{b}	0.318 ± 0.008^{a}
SD 417	200	0.265 ± 0.017^{a}	0.328 ± 0.012^{b}	0.401 ± 0.006^{c}	**0.629 ± 0.016^{e}**	0.447 ± 0.013^{d}	0.344 ± 0.019^{b}
SD 424	200	0.382 ± 0.003^{c}	0.413 ± 0.008^{d}	**0.494 ± 0.006^{e}**	0.387 ± 0.023^{c}	0.328 ± 0.014^{b}	0.285 ± 0.000^{a}
SD 491	200	0.305 ± 0.004^{c}	0.365 ± 0.000^{d}	**0.404 ± 0.007^{e}**	0.373 ± 0.021^{de}	0.254 ± 0.027^{b}	0.212 ± 0.013^{a}
SD 495	200	0.220 ± 0.018^{b}	0.338 ± 0.019^{d}	**0.408 ± 0.009^{e}**	**0.375 ± 0.022^{e}**	0.266 ± 0.014^{c}	0.112 ± 0.002^{a}
SD 496	200	0.218 ± 0.002^{b}	0.311 ± 0.001^{c}	0.408 ± 0.008^{d}	**0.447 ± 0.000^{e}**	0.402 ± 0.015^{d}	0.172 ± 0.020^{a}
SD 498	200	0.177 ± 0.015^{a}	0.236 ± 0.014^{b}	**0.348 ± 0.026^{d}**	**0.350 ± 0.036^{d}**	0.287 ± 0.016^{c}	0.171 ± 0.018^{a}
SD 500	200	0.233 ± 0.009^{a}	0.321 ± 0.006^{b}	**0.422 ± 0.015^{c}**	**0.409 ± 0.006^{c}**	0.345 ± 0.031^{b}	0.232 ± 0.012^{a}
SD 42	500	0.503 ± 0.068^{c}	0.560 ± 0.007^{c}	0.646 ± 0.022^{d}	**0.805 ± 0.007^{e}**	0.259 ± 0.028^{b}	0.182 ± 0.021^{a}
SD 77	500	0.064 ± 0.023^{a}	0.064 ± 0.005^{a}	0.128 ± 0.010^{b}	0.174 ± 0.005^{a}	**0.330 ± 0.025^{d}**	0.170 ± 0.006^{c}
SD 105	500	0.352 ± 0.003^{c}	0.355 ± 0.011^{c}	**0.467 ± 0.012^{d}**	0.368 ± 0.018^{c}	0.194 ± 0.013^{b}	0.150 ± 0.020^{a}
SD 265	500	0.361 ± 0.029^{c}	0.362 ± 0.001^{c}	**0.501 ± 0.016^{d}**	0.405 ± 0.005^{c}	0.224 ± 0.013^{b}	0.145 ± 0.033^{a}
SD 382b	500	0.524 ± 0.023^{a}	0.535 ± 0.024^{ab}	0.561 ± 0.008^{b}	**0.594 ± 0.006^{c}**	**0.622 ± 0.008^{c}**	0.520 ± 0.007^{a}
SD 384	500	0.076 ± 0.010^{bc}	0.092 ± 0.008^{c}	0.117 ± 0.002^{d}	**0.167 ± 0.017^{e}**	0.065 ± 0.008^{b}	0.025 ± 0.000^{a}

SD 408	**500**	0.471 ± 0.015^{b}	0.487 ± 0.005^{bc}	0.489 ± 0.010^{bc}	**0.512 ± 0.010^{c}**	**0.504 ± 0.005^{c}**	0.378 ± 0.016^{a}
SD 443	**500**	0.232 ± 0.010^{b}	0.346 ± 0.036^{c}	**0.416 ± 0.002^{d}**	0.364 ± 0.003^{c}	0.210 ± 0.009^{b}	0.133 ± 0.026^{a}
SD 485	**500**	0.137 ± 0.020^{a}	0.242 ± 0.019^{b}	**0.437 ± 0.016^{e}**	0.330 ± 0.002^{d}	0.288 ± 0.017^{c}	0.116 ± 0.005^{a}
SD 486	**500**	0.309 ± 0.005^{b}	0.422 ± 0.014^{c}	**0.636 ± 0.023^{e}**	0.588 ± 0.016^{d}	0.420 ± 0.007^{c}	0.252 ± 0.023^{a}
SD 487	**500**	0.229 ± 0.038^{a}	0.268 ± 0.030^{ab}	**0.453 ± 0.041^{d}**	0.383 ± 0.034^{c}	0.309 ± 0.003^{b}	0.259 ± 0.026^{ab}
SD 488	**500**	0.131 ± 0.017^{a}	0.251 ± 0.006^{b}	**0.358 ± 0.008^{c}**	**0.388 ± 0.017^{c}**	0.237 ± 0.031^{b}	0.134 ± 0.021^{a}
SD 489	**500**	0.312 ± 0.013^{c}	0.389 ± 0.006^{d}	0.441 ± 0.021^{de}	**0.474 ± 0.008^{e}**	0.256 ± 0.030^{b}	0.181 ± 0.048^{a}
SD 5	**1000**	1.219 ± 0.039^{b}	1.228 ± 0.029^{b}	**1.330 ± 0.009^{c}**	**1.361 ± 0.018^{c}**	**1.311 ± 0.013^{c}**	1.159 ± 0.037^{a}
SD 17	**1000**	0.541 ± 0.031^{c}	0.544 ± 0.009^{c}	**0.638 ± 0.040^{d}**	**0.624 ± 0.029^{d}**	0.428 ± 0.014^{b}	0.313 ± 0.009^{a}
SD 302	**1000**	0.216 ± 0.004^{c}	0.234 ± 0.039^{c}	**0.291 ± 0.006^{d}**	0.217 ± 0.004^{c}	0.090 ± 0.006^{b}	0.044 ± 0.013^{a}
SD 365	**1000**	0.042 ± 0.006^{c}	0.045 ± 0.012^{c}	**0.084 ± 0.005^{d}**	0.034 ± 0.017^{bc}	0.017 ± 0.004^{ab}	0.010 ± 0.001^{a}
SD 387	**1000**	0.217 ± 0.005^{c}	0.218 ± 0.004^{c}	**0.283 ± 0.010^{d}**	0.212 ± 0.006^{c}	0.157 ± 0.027^{b}	0.090 ± 0.012^{a}
SD 412	**1000**	0.244 ± 0.020^{a}	0.406 ± 0.009^{cd}	**0.434 ± 0.008^{d}**	0.378 ± 0.017^{c}	0.312 ± 0.003^{b}	0.257 ± 0.016^{a}

Table 5.5 Distance matrix of the 15 black yeast strains

Strains	SD 429	SD 480	SD 378	SD 440	SD 454	SD 449	SD 430	SD 483	SD 450	SD 416	FJ 770076	AB 087201	FJ 755827	EU 497947	AJ 238676
SD 429															
SD 480	0.005														
SD 378	0.000	0.005													
SD 440	0.000	0.005	0.000												
SD 454	0.000	0.005	0.000	0.000											
SD 449	0.005	0.009	0.005	0.005	0.005										
SD 430	0.005	0.009	0.005	0.005	0.005	0.000									
SD 483	0.005	0.009	0.005	0.005	0.005	0.000	0.000								
SD 450	0.005	0.009	0.005	0.005	0.005	0.000	0.000	0.000							
SD 416	0.005	0.009	0.005	0.005	0.005	0.000	0.000	0.000	0.000						
FJ770076	0.000	0.005	0.000	0.000	0.000	0.005	0.005	0.005	0.005	0.005					
AB087201	0.000	0.005	0.000	0.000	0.000	0.005	0.005	0.005	0.005	0.005	0.000				
FJ755827	0.005	0.009	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005	0.005			
EU497947	0.009	0.014	0.009	0.009	0.009	0.005	0.005	0.005	0.005	0.005	0.009	0.009	0.009		
AJ238676	0.264	0.275	0.264	0.264	0.264	0.264	0.264	0.264	0.264	0.264	0.264	0.264	0.258	0.275	

GenBank: GQ387379.1

Candida sp. strain SD 302 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
LOCUS       GQ387379    528 bp    DNA     linear   PLN   24-AUG-2009
DEFINITION  Candida sp. strain SD 302 18S ribosomal RNA gene,
            partial sequence; internal transcribed spacer 1, 5.8S
            ribosomal  RNA gene, and internal transcribed spacer 2,
            complete sequence; and 28S ribosomal RNA gene, partial
            sequence.

ACCESSION   GQ387379
VERSION     GQ387379.1  GI:256033039
KEYWORDS    .
SOURCE      Candida sp. SD 302
  ORGANISM  Candida sp. SD 302
Eukaryota;  Fungi; Dikarya; Ascomycota; Saccharomycotina;
            Saccharomycetes;,Saccharomycetales;mitosporic
            Saccharomycetales;Candida.
REFERENCE   1 (bases 1 to 528)
  AUTHORS   Kutty,S.N., Philip,R. and Bright Singh,I.S.
  TITLE     Marine Yeasts from the Continental Slope Sediments of
            Arabian Sea
  JOURNAL   Unpublished
REFERENCE   2 (bases 1 to 528)
  AUTHORS   Kutty,S.N., Philip,R. and Bright Singh,I.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (15-JUL-2009) Dept. of Marine Biology,
            Microbiology and Biochemistry, Cochin University of
            Science, Fine Arts Avenue, Kochi, Kerala 682016, India
FEATURES             Location/Qualifiers
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                     /mol_type="genomic DNA"
                     /strain="SD 302"
                     /db_xref="taxon:5482"
     misc RNA        <1..>528
                     /note="contains  18S  ribosomal  RNA,  internal
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2, and 28S ribosomal RNA"ORIGIN
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 61 ttttttattg aacaaatttt tttggtggcg ggagcaatcc taccaccaga ggttataact
121 aaaccaaact ttttatttac agtcaaactt gatttattat tacaatagtc aaaactttca
181 acaacggatc ttttggttct cgcatcgatg aagaacgcag cgaaatgcga tacgtaatat
241 gaattgcaga tattcgtgaa tcatcgaatc tttgaacgca cattgcgccc tttggtattc
301 caaagggcac ccctgtttga gcgtcatttc tccctcaaac ccccgggttt ggtgttgagc
361 aatacgctag gtttgtttga aagaatttac gtggaaactt attttaagcg acttagggtt
421 atccaaaacg cttatttttgc tagtggcccc cccaatttat ttcataactt ttacctcaaa
481 tcagggagga ctacccgctg aaattaagcc tatcaataag cggaggaa
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GenBank: GQ334383.1

Hortaea werneckii SD 378 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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LOCUS       GQ334383 512 bp    DNA     linear   PLN 29-JUL-2009
DEFINITION  Hortaea werneckii SD 378 18S ribosomal RNA gene, partial
sequence;internal transcribed spacer 1, 5.8S ribosomal RNA gene, and
internal transcribed spacer 2, complete sequence; and 28S ribosomal
RNA gene, partial sequence.
ACCESSION   GQ334383
VERSION     GQ334383.1  GI:254802556
SOURCE      Hortaea werneckii SD 378
  ORGANISM  Hortaea werneckii SD 378
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Dothideomycetes; Dothideomycetidae; Dothideales.
REFERENCE   1  (bases 1 to 512)
  AUTHORS   Kutty,S.N., Philip,R. and Bright Singh,I.S.
  TITLE     Marine yeasts from the continental slope sediments of Bay
of Bengal
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 512)
  AUTHORS   Kutty,S.N., Philip,R. and Bright Singh,I.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-JUN-2009) Dept. of Marine Biology,
Microbiology and Biochemistry, Cochin University of Science and
Technology, Fine Arts Avenue, Kochi, Kerala 682016, India
FEATURES             Location/Qualifiers
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/db_xref="taxon:661568"     misc_RNA        <1..>512
                     /note="contains 18S ribosomal RNA, internal
transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer
2, and 28S ribosomal RNA"ORIGIN
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 61 ggccttcggg cgtcggggcc cccggcggac accttcataa ctcttgcatc tcttgcgtct
121 gagtgataca tataatcaat caaaactttc aacaacggat ctcttggttc tggcatcgat
181 gaagaacgca gcgaaatgcg ataagtaatg tgaattgcag aattcagtga atcatcgaat
241 ctttgaacgc acattgcgcc ccctggcatt ccgggggggca tgcctgttcg agcgtcatta
301 caccactcaa gcctggcttg gtattgagcg accgcggcct gcccgcgcgc tccaatgtct
361 ccggctgarc cgtccgtctc taagcgttgt gaatagcgat cgcttgcgag gcccgggcgg
421 ttcgacgccg ttaaaccccc ccatttttcta tggttgacct cggatcaggt agggataccc
481 gctgaactta agcatatcaa taagcggagg aa
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GenBank: GQ334383.1

Hortaea werneckii SD 378 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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LOCUS       GQ334383 512 bp    DNA     linear   PLN 29-JUL-2009
DEFINITION  Hortaea werneckii SD 378 18S ribosomal RNA gene, partial
sequence;internal transcribed spacer 1, 5.8S ribosomal RNA gene, and
internal transcribed spacer 2, complete sequence; and 28S ribosomal
RNA gene, partial sequence.
ACCESSION   GQ334383
VERSION     GQ334383.1  GI:254802556
SOURCE      Hortaea werneckii SD 378
  ORGANISM  Hortaea werneckii SD 378
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Dothideomycetes; Dothideomycetidae; Dothideales.
REFERENCE   1  (bases 1 to 512)
  AUTHORS   Kutty,S.N., Philip,R. and Bright Singh,I.S.
  TITLE     Marine yeasts from the continental slope sediments of Bay
of Bengal
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 512)
  AUTHORS   Kutty,S.N., Philip,R. and Bright Singh,I.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-JUN-2009) Dept. of Marine Biology,
Microbiology and Biochemistry, Cochin University of Science and
Technology, Fine Arts Avenue, Kochi, Kerala 682016, India
FEATURES             Location/Qualifiers
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/db_xref="taxon:661568"     misc_RNA        <1..>512
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transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer
2, and 28S ribosomal RNA"ORIGIN
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121 gagtgataca tataatcaat caaaactttc aacaacggat ctcttggttc tggcatcgat
181 gaagaacgca gcgaaatgcg ataagtaatg tgaattgcag aattcagtga atcatcgaat
241 ctttgaacgc acattgcgcc ccctggcatt ccgggggggca tgcctgttcg agcgtcatta
301 caccactcaa gcctggcttg gtattgagcg accgcggcct gcccgcgcgc tccaatgtct
361 ccggctgarc cgtccgtctc taagcgttgt gaatagcgat cgcttgcgag gcccgggcgg
421 ttcgacgccg ttaaaccccc ccattttcta tggttgacct cggatcaggt agggataccc
481 gctgaactta agcatatcaa taagcggagg aa
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GenBank: GQ334384.1

Hortaea werneckii SD 416 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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LOCUS       GQ334384    551 bp    DNA     linear   PLN 29-JUL-2009
DEFINITION  Hortaea werneckii SD 416 18S ribosomal RNA gene, partial
sequence;internal transcribed spacer 1, 5.8S ribosomal RNA gene, and
internal transcribed spacer 2, complete sequence; and 28S ribosomal
RNA gene, partial sequence.
ACCESSION   GQ334384
VERSION     GQ334384.1  GI:254802557
SOURCE      Hortaea werneckii SD 416
  ORGANISM  Hortaea werneckii SD 416
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Dothideomycetes; Dothideomycetidae; Dothideales.
REFERENCE   1  (bases 1 to 551)
  AUTHORS   Kutty,S.N., Philip,R. and Bright Singh,I.S.
  TITLE     Marine yeasts from the continental slope sediments of Bay
of Bengal
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 551)
  AUTHORS   Kutty,S.N., Philip,R. and Bright Singh,I.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-JUN-2009) Dept. of Marine Biology,
Microbiology and Biochemistry, Cochin University of Science and
Technology, Fine Arts Avenue, Kochi, Kerala 682016, India
FEATURES             Location/Qualifiers
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/db_xref="taxon:661569"     misc RNA        <1..>551
/note="contains 18S ribosomal RNA, internal transcribed spacer 1, 5.8S
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121 ccccggtgga caccttcata actcttgcat ctcttgcgtc tgagtgatac atacaatcaa
181 tcaaaacttt caacaacgga tctcttggtt ctggcatcga tgaagaacgc agcgaaatgc
241 gataagtaat gtgaattgca gaattcagtg aatcatcgaa tctttgaacg cacattgcgc
301 cccctggcat tccgggggggc atgcctgttc gagcgtcatt acaccactca agcctggctt
361 ggtattgagc gccgcggcct gcccgcgcgc tccaatgtct ccggctgagc cgtccgtctc
421 taagcgttgt gaatagcgat cgcttgcgag gcccgggcgg ttcgacgccg ttaaaccccc
481 cattttctat ggttgacctc ggatcaggta gggatacccg ctgaacttaa gcatatcaat
541 aagcggagga a
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GenBank: GQ334385.1

Hortaea werneckii SD 429 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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LOCUS       GQ334385                554 bp    DNA     linear   PLN 29-JUL-2009
DEFINITION  Hortaea werneckii SD 429 18S ribosomal RNA gene, partial sequence;
            internal transcribed spacer 1, 5.8S ribosomal RNA gene, and
            internal transcribed spacer 2, complete sequence; and 28S ribosomal
            RNA gene, partial sequence.
ACCESSION   GQ334385
VERSION     GQ334385.1  GI:254802558
SOURCE      Hoetaea werneckii SD 429
  ORGANISM  Hortaea werneckii SD 429
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Dothideomycetes; Dothideomycetidae; Dothideales.
REFERENCE   1  (bases 1 to 554)
  AUTHORS   Kutty,S.N., Philip,R. and Bright Singh,I.S.
  TITLE     Marine yeasts from the continental slope sediments of Bay of Bengal
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 554)
  AUTHORS   Kutty,S.N., Philip,R. and Bright Singh,I.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-JUN-2009) Dept. of Marine Biology, Microbiology and
            Biochemistry, Cochin University of Science and Technology, Fine
            Arts Avenue, Kochi, Kerala 682016, India
FEATURES             Location/Qualifiers
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                     /db_xref="taxon:661570"
     misc_RNA        <1..>554
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                     spacer 1, 5.8S ribosomal RNA, internal transcribed spacer
                     2, and 28S ribosomal RNA"
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       61 aaccccatgt cgaaacgact ctgttgcctc gggggcgacc cggccttcgg gcgtcggggc
      121 ccccggcgga caccttcata actcttgcat ctcttgcgtc tgagtgatac atataatcaa
      181 tcaaaacttt caacaacgga tctcttggtt ctggcatcga tgaagaacgc agcgaaatgc
      241 gataagtaat gtgaattgca gaattcagtg aatcatcgaa tctttgaacg cacattgcgc
      301 cccctggcat tccgggggggc atgcctgttc gagcgtcatt acaccactca agcctggctt
      361 ggtattgagc gaccgcggcc tgcccgcgcg ctccaatgtc tccggctgag ccgtccgtct
      421 ctaagcgttg tgaatagcga tcgcttgcga ggcccgggcg gttcgacgcc gttaaacccc
      481 ccccattttc tatggttgac ctcggatcag gtagggatac ccgctgaact taagcatatc
      541 aataagcgga ggaa
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GenBank: GQ334386.1

Hortaea werneckii SD 430 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

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LOCUS       GQ334386  493 bp    DNA     linear   PLN 29-JUL-2009
DEFINITION  Hortaea werneckii SD 430 18S ribosomal RNA gene, partial
sequence;internal transcribed spacer 1, 5.8S ribosomal RNA gene, and
internal transcribed spacer 2, complete sequence; and 28S ribosomal
RNA gene, partial sequence.
ACCESSION   GQ334386
VERSION     GQ334386.1  GI:254802559
SOURCE      Hortaea werneckii SD 430
  ORGANISM  Hortaea werneckii SD 430
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Dothideomycetes; Dothideomycetidae; Dothideales.
REFERENCE   1  (bases 1 to 493)
  AUTHORS   Kutty,S.N., Philip,R. and Bright Singh,I.S.
  TITLE     Marine yeasts from the continental slope sediments of Bay
of Bengal
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 493)
  AUTHORS   Kutty,S.N., Philip,R. and Bright Singh,I.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-JUN-2009) Dept. of Marine Biology,
Microbiology and Biochemistry, Cochin University of Science and
Technology, Fine Arts Avenue, Kochi, Kerala 682016, India
FEATURES             Location/Qualifiers
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/mol_type="genomic DNA"/isolate="SD 430"
/db_xref="taxon:661571"
     misc_RNA        <1..>493/note="contains 18S ribosomal RNA,
internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed
spacer 2, and 28S ribosomal RNA"
ORIGIN
   1 ttccgtaggt gaacctgcgg agggatcatt accgagtgtg gcgctccggc gcctccctcc
  61 aaccccatgt cgaaacgact ctgttgcctc gggggcgacc cggccttcgg gcgtcggggc
 121 ccccggtgga caccttcata actcttgcat ctcttgcgtc tgagtgatac atacaatcaa
 181 tcaaaacttt caacaacgga tctcttggtt ctggcatcga tgaagaacgc agcgaaatgc
 241 gataagtaat gtgaattgca gaattcagtg aatcatcgaa tctttgaacg cacattgcgc
 301 cccctggcat tccgggggggc atgcctgttc gagcgtcatt acaccactca agcctggctt
 361 ggtattgagc gccgcggcct gcccgcgcgc tccaatgtct ccggctgagc cgtccgtctc
 421 taagcgttgt gaatagcgat cgcttgcgag gcccgggcgg ttcgacgccg ttaaaccccc
 481 cattttctat ggt
```

GenBank: GQ334387.1

Hortaea werneckii SD 440 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence LOCUS
GQ334387 549 bp DNA linear PLN 29-JUL-2009
DEFINITION Hortaea werneckii SD 440 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence.
ACCESSION GQ334387
VERSION GQ334387.1 GI:254802560
SOURCE Hortaea werneckii SD 440
ORGANISM Hortaea werneckii SD 440
Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetidae; Dothideales.
REFERENCE 1 (bases 1 to 549)
AUTHORS Kutty,S.N., Philip,R. and Bright Singh,I.S.
TITLE Marine yeasts from the continental slope sediments of Bay of Bengal
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 549)
AUTHORS Kutty,S.N., Philip,R. and Bright Singh,I.S.
TITLE Direct Submission
JOURNAL Submitted (23-JUN-2009) Dept. of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Fine Arts Avenue, Kochi, Kerala 682016, India
FEATURES Location/Qualifiers
source 1..549/organism="Dothideales sp. SD 440"
/mol_type="genomic DNA"
/isolate="SD 440"/db_xref="taxon:661572"
misc RNA <1..>549/note="contains 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 28S ribosomal RNA"
ORIGIN

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   1 taggtgaacc tgcggaggga tcattaccga gtgtggcgct ccggcgcctc cctccaaccc
  61 catgtcgaaa cgactctgtt gcctcggggg cgacccggcc ttcgggcgtc ggggccccg
 121 gcggacacct tcataactct tgcatctctt gcgtctgagt gatacatata atcaatcaaa
 181 actttcaaca acggatctct tggttctggc atcgatgaag aacgcagcga aatgcgataa
 241 gtaatgtgaa ttgcagaatt cagtgaatca tcgaatcttt gaacgcacat tgcgccccct
 301 ggcattccgg ggggcatgcc tgttcgagcg tcattacacc actcaagcct ggcttggtat
 361 tgagcgaccg cggcctgccc gcgcgctcca atgtctccgg ctgagccgtc cgtctctaag
 421 cgttgtgaat agcgatcgct tgcgaggccc gggcggttcg acgccgttaa acccccccca
 481 ttttctatgg ttgacctcgg atcaggtagg gatacccgct gaacttaagc atatcaataa
 541 gcggagaaa
```

GenBank: GQ334388.1

Hortaea werneckii SD 449 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
LOCUS    GQ334388      499 bp    DNA     linear   PLN 29-JUL-2009
DEFINITION  Hortaea werneckii SD 449 18S ribosomal RNA gene, partial
sequence;internal transcribed spacer 1, 5.8S ribosomal RNA gene, and
internal transcribed spacer 2, complete sequence; and 28S ribosomal
RNA gene, partial sequence.
ACCESSION   GQ334388
VERSION     GQ334388.1  GI:254802561
SOURCE      Hortaea werneckii SD 449
  ORGANISM  Hortaea werneckii SD 449
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Dothideomycetes; Dothideomycetidae; Dothideales.
REFERENCE   1  (bases 1 to 499)
  AUTHORS   Kutty,S.N., Philip,R. and Bright Singh,I.S.
  TITLE     Marine yeasts from the continental slope sediments of Bay
of Bengal
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 499)
  AUTHORS   Kutty,S.N., Philip,R. and Bright Singh,I.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-JUN-2009) Dept. of Marine Biology,
Microbiology and Biochemistry, Cochin University of Science and
Technology, Fine Arts Avenue, Kochi, Kerala 682016, India
FEATURES             Location/Qualifiers
     source          1..499/organism="Dothideales sp. SD 449"
     /mol_type="genomic DNA"                    /isolate="SD 449"
     /db_xref="taxon:661573"
     misc_RNA        <1..>499/note="contains 18S ribosomal RNA,
internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed
spacer 2, and 28S ribosomal RNA"
ORIGIN
   1 cttcgtaggt gaacctgcgg agggatcatt accgagtgtg gcgctccggc gcctccctcc
  61 aaccccatgt cgaaacgact ctgttgcctc gggggcgacc cggccttcgg gcgtcggggc
 121 ccccggtgga caccttcata actcttgcat ctcttgcgtc tgagtgatac atacaatcaa
 181 tcaaaacttt caacaacgga tctcttggtt ctggcatcga tgaagaacgc agcgaaatgc
 241 gataagtaat gtgaattgca gaattcagtg aatcatcgaa tctttgaacg cacattgcgc
 301 cccctggcat tccgggggggc atgcctgttc gagcgtcatt acaccactca agcctggctt
 361 ggtattgagc gccgcggcct gcccgcgcgc tccaatgtct ccggctgagc cgtccgtctc
 421 taagcgttgt gaatagcgat cgcttgcgag gcccgggcgg ttcgacgccg ttaaaccccc
 481 cattttctat ggtgacctc
```

GenBank: GQ334389.1

Hortaea werneckii SD 450 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
LOCUS       GQ334389                551 bp    DNA     linear   PLN 29-JUL-2009
DEFINITION  Hortaea werneckii SD 450 18S ribosomal RNA gene, partial sequence;
            internal transcribed spacer 1, 5.8S ribosomal RNA gene, and
            internal transcribed spacer 2, complete sequence; and 28S ribosomal
            RNA gene, partial sequence.
ACCESSION   GQ334389
VERSION     GQ334389.1  GI:254802562
KEYWORDS    .
SOURCE      Hortaea werneckii SD 450
  ORGANISM  Hortaea werneckii SD 450
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Dothideomycetes; Dothideomycetidae; Dothideales.
REFERENCE   1  (bases 1 to 551)
  AUTHORS   Kutty,S.N., Philip,R. and Bright Singh,I.S.
  TITLE     Marine yeasts from the continental slope sediments of Bay of Bengal
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 551)
  AUTHORS   Kutty,S.N., Philip,R. and Bright Singh,I.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-JUN-2009) Dept. of Marine Biology, Microbiology and
            Biochemistry, Cochin University of Science and Technology, Fine
            Arts Avenue, Kochi, Kerala 682016, India
FEATURES             Location/Qualifiers
     source          1..551
                     /organism="Dothideales sp. SD 450"
                     /mol_type="genomic DNA"
                     /isolate="SD 450"
                     /db_xref="taxon:661574"
     misc_RNA        <1..>551
                     /note="contains 18S ribosomal RNA, internal transcribed
                     spacer 1, 5.8S ribosomal RNA, internal transcribed spacer
                     2, and 28S ribosomal RNA"
ORIGIN
        1 tccgtaggtg aacctgcgga gggatcatta ccgagtgtgg cgctccggcg cctccctcca
       61 accccatgtc gaaacgactc tgttgcctcg ggggcgaccc ggccttcggg cgtcggggcc
      121 cccggtggac accttcataa ctcttgcatc tcttgcgtct gagtgataca tacaatcaat
      181 caaaactttc aacaacggat ctcttggttc tggcatcgat gaagaacgca gcgaaatgcg
      241 ataagtaatg tgaattgcag aattcagtga atcatcgaat ctttgaacgc acattgcgcc
      301 ccctggcatt ccgggggggca tgcctgttcg agcgtcatta caccactcaa gcctggcttg
      361 gtattgagcg ccgcggcctg cccgcgcgct ccaatgtctc cggctgagcc gtccgtctct
      421 aagcgttgtg aatagcgatc gcttgcgagg cccgggcggt tcgacgccgt taaaccccc
      481 attttctatg gttgacctcg gatcaggtag ggatacccgc tgaacttaag catatcaata
      541 agcggaggaa a
```

GenBank: GQ334390.1

Hortaea werneckii SD 454 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
LOCUS       GQ334390                536 bp    DNA     linear   PLN 29-JUL-2009
DEFINITION  Hortaea werneckii SD 454 18S ribosomal RNA gene, partial sequence;
            internal transcribed spacer 1, 5.8S ribosomal RNA gene, and
            internal transcribed spacer 2, complete sequence; and 28S ribosomal
            RNA gene, partial sequence.
ACCESSION   GQ334390
VERSION     GQ334390.1  GI:254802563
SOURCE      Hortaea werneckii SD 454
  ORGANISM  Hortaea werneckii SD 454
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Dothideomycetes; Dothideomycetidae; Dothideales.
REFERENCE   1  (bases 1 to 536)
  AUTHORS   Kutty,S.N., Philip,R. and Bright Singh,I.S.
  TITLE     Marine yeasts from the continental slope sediments of Bay of Bengal
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 536)
  AUTHORS   Kutty,S.N., Philip,R. and Bright Singh,I.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-JUN-2009) Dept. of Marine Biology, Microbiology and
            Biochemistry, Cochin University of Science and Technology, Fine
            Arts Avenue, Kochi, Kerala 682016, India
FEATURES             Location/Qualifiers
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                     /organism="Dothideales sp. SD 454"
                     /mol_type="genomic DNA"
                     /isolate="SD 454"
                     /db_xref="taxon:661575"
     misc_RNA        <1..>536
                     /note="contains 18S ribosomal RNA, internal transcribed
                     spacer 1, 5.8S ribosomal RNA, internal transcribed spacer
                     2, and 28S ribosomal RNA"
ORIGIN
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       61 cgactctgtt gcctcggggg cgacccggcc ttcgggcgtc ggggcccccg gcggacacct
      121 tcataactct tgcatctctt gcgtctgagt gatacatata atcaatcaaa actttcaaca
      181 acggatctct tggttctggc atcgatgaag aacgcagcga aatgcgataa gtaatgtgaa
      241 ttgcagaatt cagtgaatca tcgaatcttt gaacgcacat tgcgcccccc ggcattccgg
      301 ggggcatgcc tgttcgagcg tcattacacc actcaagcct ggcttggtat tgagcgccgc
      361 ggcctgcccg cgcgctccaa tgtctccggc tgagccgtcc gtctctaagc gttgtgaata
      421 gcgatcgctt gcgaggcccg ggcggttcga cgccgttaaa ccccccattt tctatggttg
      481 acctcggatc aggtagggat acccgctgaa cttaagcata tcaataagcg gaggaa
```

GenBank: GQ334391.1

Hortaea werneckii SD 480 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
LOCUS       GQ334391                503 bp    DNA     linear   PLN 29-JUL-2009
DEFINITION  Hortaea werneckii SD 480 18S ribosomal RNA gene, partial sequence;
            internal transcribed spacer 1, 5.8S ribosomal RNA gene, and
            internal transcribed spacer 2, complete sequence; and 28S ribosomal
            RNA gene, partial sequence.
ACCESSION   GQ334391
VERSION     GQ334391.1  GI:254802564
SOURCE      Hortaea werneckii SD 480
  ORGANISM  Hortaea werneckii SD 480
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Dothideomycetes; Dothideomycetidae; Dothideales.
REFERENCE   1  (bases 1 to 503)
  AUTHORS   Kutty,S.N., Philip,R. and Bright Singh,I.S.
  TITLE     Marine yeasts from the continental slope sediments of Bay of Bengal
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 503)
  AUTHORS   Kutty,S.N., Philip,R. and Bright Singh,I.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-JUN-2009) Dept. of Marine Biology, Microbiology and
            Biochemistry, Cochin University of Science and Technology, Fine
            Arts Avenue, Kochi, Kerala 682016, India
FEATURES             Location/Qualifiers
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                     /mol_type="genomic DNA"
                     /isolate="SD 480"
                     /db_xref="taxon:661576"
     misc_RNA        <1..>503
                     /note="contains 18S ribosomal RNA, internal transcribed
                     spacer 1, 5.8S ribosomal RNA, internal transcribed spacer
                     2, and 28S ribosomal RNA"
ORIGIN
        1 cttcgtaggt gaacctgcgg agggatcatt accgagtgtg gcgctccggc gcctccctcc
       61 aaccccatgt cgaaacgact ctgttgcctc gggggcgacc cggccttcgg gcgtcggggc
      121 ccccggcgga caccttcata actcttgcat ctcttgcgtc tgagtgatac atgtaatcaa
      181 tcaaaacttt caacaacgga tctcttggtt ctggcatcga tgaagaacgc agcgaaatgc
      241 gataagtaat gtgaattgca gaattcagtg aatcatcgaa tctttgaacg cacattgcgc
      301 cccctggcat tccgggggggc atgcctgttc gagcgtcatt acaccactca agcctggctt
      361 ggtattgagc gaccgcggcc tgcccgcgcg ctccaatgtc tccggctgag ccgtccgtct
      421 ctaagcgttg tgaatagcga tcgcttgcga ggcccgggcg gttcgacgcc gtcaaacccc
      481 ccccatttc tatggttgac ctc
```

GenBank: GQ334392.1

Hortaea werneckii SD 483 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence

```
LOCUS       GQ334392                491 bp    DNA     linear   PLN 29-JUL-2009
DEFINITION  Hortaea werneckii SD 483 18S ribosomal RNA gene, partial sequence;
            internal transcribed spacer 1, 5.8S ribosomal RNA gene, and
            internal transcribed spacer 2, complete sequence; and 28S ribosomal
            RNA gene, partial sequence.
ACCESSION   GQ334392
VERSION     GQ334392.1  GI:254802565
SOURCE      Hortaea werneckii SD 483
  ORGANISM  Hortaea werneckii SD 483
            Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;
            Dothideomycetes; Dothideomycetidae; Dothideales; mitosporic
            Dothideales; Hortaea.
REFERENCE   1  (bases 1 to 491)
  AUTHORS   Kutty,S.N., Philip,R. and Bright Singh,I.S.
  TITLE     Marine yeasts from the continental slope sediments of Bay of Bengal
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 491)
  AUTHORS   Kutty,S.N., Philip,R. and Bright Singh,I.S.
  TITLE     Direct Submission
  JOURNAL   Submitted (23-JUN-2009) Dept. of Marine Biology, Microbiology and
            Biochemistry, Cochin University of Science and Technology, Fine
            Arts Avenue, Kochi, Kerala 682016, India
FEATURES             Location/Qualifiers
     source          1..491
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                     /mol_type="genomic DNA"
                     /isolate="SD 483"
                     /db_xref="taxon:661567"
     misc RNA        <1..>491
                     /note="contains 18S ribosomal RNA, internal transcribed
                     spacer 1, 5.8S ribosomal RNA, internal transcribed spacer
                     2, and 28S ribosomal RNA"
ORIGIN
        1 cctccctcca accccatgtc gaaacgactc tgttgcctcg ggggcgaccc ggccttcggg
       61 cgtcggggcc cccggtggac accttcataa ctcttgcatc tcttgcgtct gagtgataca
      121 tacaatcaat caaaactttc aacaacggat ctcttggttc tggcatcgat gaagaacgca
      181 gcgaaatgcg ataagtaatg tgaattgcag aattcagtga atcatcgaat ctttgaacgc
      241 acattgcgcc ccctggcatt ccgggggggca tgcctgttcg agcgtcatta caccactcaa
      301 gcctggcttg gtattgagcg ccgcggcctg cccgcgcgct ccaatgtctc cggctgagcc
      361 gtccgtctct aagcgttgtg aatagcgatc gcttgcgagg cccgggcggt tcgacgccgt
      421 taaaccccc attttctatg gttgacctcg gatcaggtag ggatacccgc tgaacttaag
      481 catatcaata a
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