MARINE YEAST ISOLATES FROM ARABIAN SEA AND BAY OF BENGAL: BIOCHEMICAL, MOLECULAR AND PHYLOGENETIC ANALYSIS

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Dedicated to the Lords Divine Mercy

Declaration

*I hereby do declare that the thesis entitled "**Marine yeast isolates from Arabian Sea and Bay of Bengal :Biochemical, Molecular and Phylogenetic Analysis"** is an authentic record of research work done by me under the supervision and guidance of Dr. Rosamma Philip, Assistant Professor, Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, for the degree of Doctor of Philosophy in Marine Biology and that no part of this work has been presented before for the award of any other degree, diploma or associateship in any University.*

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November,2012. **Reema Kuriakose**

Certificate

*This is to certify that the thesis entitled "**Marine yeast isolates from Arabian Sea and Bay of Bengal :Biochemical, Molecular and Phylogenetic Analysis** is an authentic record of research work carried out by **Ms. Reema Kuriakose** under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology and that no part thereof has been presented before for the award of any other degree, diploma or associate ship in any University.*

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Chapter -1

GENERAL INTRODUCTION

Oceans harbour abundant biotic resources and the marine microbes represent the largest group of the biodiversity. Approximately 1500 species of yeasts belonging to 100 genera are described, which represent only a fraction of the yeasts that exist in nature, the rest being non culturable. Marine yeasts may be obligate or facultative. Obligate marine fungi are those that grow and sporulate exclusively in marine or estuarine habitat, where as facultative are those which originate from fresh water or terrestrial habitats but are able to grow and sporulate in the marine environment (Kohlmeyer and Kohlmeyer, 1979). Kohlmeyer and Kohlmeyer (1979) examined yeasts occurring in marine environments and gathered a list of 176 species isolated from diverse marine habitats. Of those, only 25 were obligate marine yeasts, widely represented by the genera *Metschnikowia, Rhodosporidium, Candida* and *Torulopsis*. Thus marine fungi are not a taxonomically, but an ecologically and physiologically defined group (Hyde et al., 1998).Yeasts are found in various regions like coastal, estuarine, and offshore regions in marine environment and they are present in open waters, sediments and bodies of marine plants/animals (Morris, 1968; Fell, 1976; Hagler and Ahearn, 1987; Spencer and Spencer,1997; Lachance and Starmer 1998). However yeast population is scarce in marine water than in fresh water and decrease with increasing distance from land (Hagler and Ahearn, 1987).

The English word yeast means "foam" and "to rise" referring to the fermentation processes that produce beer and bread. Yeasts are a group of basidiomycetous and ascomycetous fungi, that reproduce vegetatively by budding or fission, and that form sexual states which are not enclosed in a fruiting body (Boekhout and Kurtzman, 1996). Yeasts, which form one of the important sub classes of fungi are unicellular, eukaryotic, non motile and usually larger than bacteria. Their size varies from 5-30 µm in length and 1-5 µm in width and may be spherical, ovoid or elongated in shape depending on age and environment and they double every 1-3 hours in favorable media (Wayman and Parekh, 1990).Yeasts can reproduce both asexually and sexually. The characteristics used in traditional microbial identification schemes are all observable aspects of the organism's structure and function. In other words they are phenotypic characteristics and are the products of gene expression. By looking directly at the microbial genome itself it is possible to identify a species using its genotypic characteristics. The advent of molecular biology in the 1980s contributed a set of powerful new tools that have helped microbiologists to detect the smallest variations within microbial species and even within individual strains. This has added an entirely new dimension to a science that was in danger of becoming constrained by its reliance on traditional laboratory techniques. Morphological, physiological and biochemical tests have been commonly used for phenotypic characterization of yeast species. These methods are often unreliable, due to strain variability and, therefore, do not allow differentiation between yeast strains belonging to the same species. Genetic characterization using molecular techniques provides more powerful means of strain identification and differentiation among strains (Recek et al., 2002).

In molecular taxonomy, proteins and genes are used to determine evolutionary relationships. Here, the amount of DNA that is similar in different species is examined. Then specific genes or proteins can be used as molecular clocks. These clocks help to determine the divergence from a common ancestor, or more simply,

the relatedness of the species in question. Most of the experiments which have been carried out in this field so far are based on ribosomal sequences, which are used as phylogenetic markers (Woese, 1987). The ribosomal sequences are present in all organisms and they contain variable and highly conserved regions which allow to distinguish between organisms on all phylogenetic levels. In addition, a lot of data exist in the databases which can be used to compare the DNA-sequences of unknown microorganisms and allow a phylogenetic identification (Maidak et al., 1999). For identification based on genomic analysis, ribosomes are an indispensable component of the protein synthesis apparatus, and their structures are strictly conserved, the DNA component of the small subunit ribosome has proved to be an important and useful molecular clock for quantifying evolutionary relationships between organisms. Generally, the rate of base substitutions, deletions or insertions in various regions of the rRNA gene is not uniform; some areas are highly conserved and unchanged through millions of years, some are highly variable and others are semi conserved (Nishimura and Mikata, 2000). Application of gene sequence analyses to yeast systematics has shown conflict between the placement of species on gene trees and their classification from phenotype. For example, the genus *Wingea* had been described because of the uniqueness of its lenticular ascospores, but phylogenetic analysis of rRNA sequences has placed it in the genus *Debaryomyces*, where species are generally characterized by roughened, spheroidal ascospores (Kurtzman and Robnett, 1994). There is now a widespread pattern of disparity between phenotype and genotype as means for classifying yeasts, and these differences have been demonstrated from analyses of 18S ribosomal DNA (rDNA) (James et al., 1994; Cai et al., 1996; James et al., 1997), internal transcribed spacer (ITS) rDNA (James et al.,1996) and 26S rDNA (Kurtzman and Robnett, 1995, 1998), leaving little doubt that phenotype is a poor predictor of genetic relationships among species (Kurtzman and Robnett, 2003).

The composition and activities of microbial communities from diverse and often 'extreme' habitats have been the focus of intense research during the past decade, spurred on largely by advances in molecular biology. The contribution of microbial eukaryotes (protists and fungi) in this ecosystem is unknown, as these assemblages have been studied in deep-sea sediments only to a few centimeters into the seafloor (Edgcomb et al., 2011). In the natural ecosystems, both bacteria and fungi are active in decomposition and remineralization processes. Protists and fungi may impact carbon cycling in the marine subsurface through consumption of dissolved organic matter (fungi) and through bacterial/archaeal grazing. Protists may control bacterial and archaeal abundances and community composition, and thereby impact microbial production and nutrient cycling. Fungi and yeasts are more important in later stages of decomposition and they exploit the energy rich materials in decomposable organic matter and bring about the release of nutrients locked up in the decaying substances. They contribute to the energy flow and productivity of an ecosystem by their presence as a source for meeting the basic requirements of organic carbon at higher trophic levels (Das et al., 2008). Despite the pivotal roles played by these organisms, little is known regarding the presence, abundance, diversity and activities of these species in deep biosphere environments (Edgcomb et al., 2011). Yeast act as saprophytes converting plant and animal organic matter to yeast biomass (single cell protein) which is highly significant in aquaculture due to the nutritional quality and its possible utilization as feed for animals or in aquaculture. Yeasts are used in many industrial processes, such as baker's yeast (*Saccharomyces cerevisiae*) in bread production/ wine making and brewer's yeast (*Saccharomyces pastorianus*) in beer fermentation for the production of alcoholic beverages. Yeast Single Cell Protein (SCP) and metabolic products *viz.*, enzymes, vitamins, capsular polysaccharides, carotenoids, polyhydric alcohols, lipids, glycolipids, citric acid, ethanol, carbon dioxide are also important. Some of these products are produced commercially while others are potentially valuable in biotechnology. Certain yeast species have

also been used as prebiotic and probiotic agents for preventing or treating various intestinal, nutritional, and toxicological disorders. In particular, *S.boulardii*, originally isolated from fruit in Indo-China, has been used for treatment of intestinal diseases in children and adults since 1950 (Buts and Bernasconi, 2005; Edwards-Ingram et al., 2007).

The evaluation of diversity and ecology of the single-celled eukaryotic microbes (the protists and fungi) has been much slower despite their importance in marine microbial communities and expanding our understanding of the evolution of multicellular taxa (Caron et al., 2009a,b). Protistan lineages represent one and a half billion years of evolution and comprise the bulk of eukaryotic phylogenetic diversity and an astounding array of morphologies, physiologies and ecological activities. In the upper water column of the world's oceans protists play pivotal roles in global food webs as primary producers and consumers. Much less is known about the presence and activities of microbial eukaryotes in the deep ocean, although recent studies have provided preliminary insights into microbial eukaryote diversity in the bathypelagic (Countway et al., 2007; Not et al., 2007) and deep-sea sediments (Arndt et al.. 2003; Edgcomb et al., 2002). Recently, diversity of fungi from various extreme habitats such as deep-sea hydrothermal vents (Le Calvez et al., 2009; Burgaud et al., 2009, 2010), deep-sea waters and sediments (Bass et al., 2007; Nagano et al., 2010; Edgcomb et al., 2011) and methane hydrate-bearing deep-sea sediments (Takishita et al., 2006; Lai et al., 2007; Nagahama et al., 2011; Thaler et al., 2011) have been reported. Diversity was analyzed by either culture-dependent or culture-independent methods in the above studies resulting in the identification of highly novel phylotypes closely related to known fungi. However, a combination of these two approaches may provide deeper insight into the diversity assessment as reported by Jebaraj et al. (2010). Using culture-dependent approach, fungi were isolated and identified from the deep-sea sediments of the Central Indian Basin (CIB) (Raghukumar et

al., 2004; Damare et al., 2006; Singh et al., 2010). Further Singh et al. (2011a) analyzed the fungal diversity in deep-sea sediments using culture-independent approach by targeting universal 18S as well as fungal specific and universal ITS (internal transcribed spacers) regions of rRNA genes from three locations in the CIB study, four different primer pairs were used in amplifying sediment DNA to assess the fungal community (Singh et al., 2011b). Further Singh et al. (2012) analyzed diversity of fungi by culture-dependent as well as culture-independent approaches in the same sediment samples of two cores from the CIB.

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

1. Isolation and identification of yeasts from Arabian Sea and Bay of Bengal.
2. Molecular characterization of yeast isolates and phylogenetic analysis
3. Physiological and biochemical characterization of the isolates.
4. Proximate composition of yeast biomass and bioactive compounds.

The Thesis is comprised of six chapters. A general introduction to the topic is given in Chapter1. Isolation and identification of marine yeasts are presented in Chapter 2. Chapter 3 deals with molecular identification and physiological characterization of Non- pigmented yeasts. Molecular identification and physiological characterization of pigmented yeast is presented in Chapter 4. Proximate composition of yeast biomass of various genera and their bioactive compounds are illustrated in Chapter 5. A summary of the results of the present study is given in Chapter 6. References and Appendices are followed.

Chapter -2

ISOLATION AND IDENTIFICATION OF MARINE YEASTS

2.1. Introduction

Yeasts appear to be widespread throughout aerobic marine habitats, although their numbers and species distributions are dependent to a large extent on concentrations and types of organic materials. The presence of yeasts in oceans and coastal waters were first established by Fischer (1894), who isolated bacteria and yeasts on an Atlantic Ocean Cruise. He separated red and white yeasts and identified them as *Torula* sp. and *Mycoderma* respectively. Studies involving cultivation of yeasts from marine substrates have attracted increasing attention during the last 30 years. Kriss and Navozhilova (1954) and Meyers et al. (1967) studied the yeast fauna of the black sea. Yeasts in the Indian Ocean received attention from Bhat and Kachwalla (1955) and Fell (1967), those of Pacific Ocean by van Uden and Castelo Branco (1963). Yeasts from aquatic regions of Florida were isolated by Fell et al. (1960) and Ahearn et al. (1968). The yeast flora of the North Sea was studied by Meyers et al. (1967), that of the Baltic Sea by Nokrans (1966 a) and Hoppe (1970).

Most marine occurring yeasts can be identified on the basis of taxonomic study by Lodders et al. (1952), which includes mainly terrestrial forms. Majority of yeasts found in oceans and coastal waters are of terrestrial origin. Kohlmeyer and

Kohlmeyer (1979) named 177 species which were found in water, sediment, algae, animals or detritus of the sea. Of those, only 26 species, have been isolated exclusively from marine sources. The most important genera of true marine yeasts are *Metschnikowia, Candida*, *Torulopsis, Cryptococcus* and *Rhodotorula*, which are well adapted to the marine environment and perhaps autochthonous marine yeasts.

2.1.1. Distribution of yeasts in the aquatic habitats:

Yeasts exhibit excellent survival in water and they are widely distributed in almost every part of aquatic environment, such as oceans, seas, estuaries, rivers and lakes. Yeasts have been recovered from the deep igneous rock aquifers 200-400m below the surface of the Baltic Sea (Ekendahl et al., 2003) and also from deep ice cores of Greenland glaciers at extraordinary depths (200m below the glacial surface) (Starmer et al., 2005a). Yeasts found in aquatic environments are generally asporogenous and oxidative or weakly fermentative (Phaff et al., 1978).

Oceans and Seas:

The widespread occurrence of yeasts in diverse marine situations from the ocean surface to the deep sea has been well established. Marine yeasts are divided into obligate and facultative groups. Obligate marine yeasts are yeasts that have never been isolated anywhere other than marine environment whereas facultative marine yeasts are also known from terrestrial habitats (Kohlmeyer and Kohlmeyer 1979). Yeasts are frequently found in the digestive tract of marine organisms, in sea water, and beach sand (Van Uden and Branco, 1963; Taysi and Van Uden, 1964; Kawakita and Van Uden, 1965; Fell, 1976; Vogel et al., 2007; Kutty and Philip, 2008; Burgaud et al., 2010; Konishi et al., 2010; Liu et al., 2010; Galkiewicz et al., 2012). It is therefore considered that the factors affecting the distribution of marine yeasts include currents, migration of marine organisms and contamination

of terrigenous sources (Van Uden and Branco, 1963; Fell, 1967; Vogel et al., 2007; Kutty and Philip, 2008).

The discovery of marine yeasts goes back to 1894, when Fischer working on SMS Moltke, found yeasts in Atlantic water samples regardless of distance from land. Two yeast species were identified during the Chariot expedition to the Atlantic (Tsiklinsky, 1908). In 1934, ZoBell and Feltham observed yeasts on most of their plates inoculated with samples of materials collected from land as well as from the open ocean.

Russian microbiologists Kriss and Novozhilova (1954) have reported quantitative distribution of yeasts in the Black, Okhotsk Seas, the Pacific Ocean and the Arctic Sea. They separated the yeasts collected, by membrane filter technique and found that yeasts were found in all regions in the surface layers while in the inshore areas, yeasts could be found at all depths. Bhat and Kachwalla (1955) isolated yeasts from water samples collected from 2-6 miles off the coast of Bombay. The yeasts obtained were *Saccharomyces italicus, Saccharomyces chevalicri, Saccharomyces rosei, Debaryomyces hansenii, Pichia guilliermondii, Candida tropicalis, Torulopsis glabrata, Rhodoturula* sp. and *Cryptococcus* sp. During the cruise of the R/V Vitiyaz (1957*), Debaryomyces globosus* was isolated from a depth of 400 m in the Central Pacific. Kriss (1949) found that yeasts were observed not only in the oxygenated zone but also in the H_2S zone of the black sea. Fell et al. (1960) obtained a total 179 yeast isolates from 45 sampling stations in the course of qualitative yeast survey in Biscayne Bay, Florida. *Candida tropicalis* and *Rhodotorula rubra* were the predominant species. Van Uden and ZoBell (1962) obtained yeasts from 45 out of 62 samples collected from algal and coral growths in the Torres Strait region. Species like *Metschnikowia reukaufii*, *Pichia farinose, Kluyveromyces aestuarii, Candida marina, Torulopsis torresii* and *Torulopsis maris* were obtained.

Roth et al. (1962) and Fell (1965) made a quantitative study of yeast distribution in the coastal areas of Southern Florida and Gulf Stream of Florida. Fresh water influx and heavy recreational bathing directly affected viable yeast count in these areas. *Candida tropicalis* and *Rhodotorula rubra* were predominant in the inshore region.

In coastal waters, up to several thousand yeast cells per litre of water were found (Roth et al., 1962; Meyers et al., 1967). Fell (1967) found living yeasts in the Indian Ocean from surface down to a depth of 200 m. Yeasts of the Indian Ocean were studied by Fell and Van Uden (1963), D'Souza (1972) and Godinho et al. (1978). Thirty three strains of marine yeasts were isolated from the coastal and offshore waters of Cochin and *Candida* was the predominant genus obtained (Rishipal and Philip, 1998). A marine hydrocarbon degrading yeast was isolated from Mumbai (India) and was identified as *Yarrowia lipolytica* (Oswal et al., 2002). Yeasts were isolated from sea water samples collected from the west and east coast of India up to 200m depth in the Exclusive Economic Zone (EEZ) (Sarlin, 2005).

Cryptococcus and *Rhodotorula* were predominant among yeasts isolated from deep sea waters from Loma Trough, off San Diego, California. In samples collected off La Jolla California, total yeasts count varied from 0-1920 viable cells/L. (Van Uden and Branco, 1963). Yeasts collected by Fell (1967) from 16 stations during a cruise of R/V Anton Brunn in the Indian Ocean found highest population of yeasts in the Somali current and the isolates were grouped according to their distribution as 1) Ubiquitous species like *Rhodotorula rubra* and *Candida atmosphaerica*, which is seen in the water masses 2) Widely distributed species which occurred in all water masses except Red Sea water and it is represented by *Candida polymorpha* and *Rhodotorula glutinis* 3) Species of restricted distribution like *Sporobolomyces hispanicus, Sporobolomyces odorus* and *Rhodotorula crocea*. *Rhodosporidium diobovatum* in deep-sea vents seemed to be

able to colonize diverse fauna like shrimps, mussels and sponges (Burgaud et al., 2010). *Malassezia* sp. was recovered from methane hydrate-bearing deep-sea sediments (Lai et al., 2007) and marine subsurface (Edgcomb et al., 2011). Such methylotrophic yeasts may play a crucial role in converting methane into more accessible carbon and energy substrates. They are of great interest in physiological studies and industrial applications. *Malassezia* is also a known pathogen and its presence in deep-sea sediments suggests that it may be an opportunistic pathogen of marine mammals (Edgcomb et al., 2011). Several yeast morphotypes belonging to *Cryptococcus, Sporidiobolus, Rhodosporidium* and *Rhodotorula* were cultured from healthy cold–water deep sea coral *Lophelia pertusa* colonies collected from the northern Gulf of Mexico, the West Florida Slope, and the western Atlantic Ocean off the Florida coast (Galkiewicz et al., 2012).

Various kinds of ethanol producing marine yeasts from coastal waters were isolated and characterised by Urano et al. (1998), who 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 of the north west of Antarctic Peninsula and isolated 6 genera of yeasts from sea water. A survey of marine yeasts in the sub Antarctic region near south Georgia, conducted by Connell and Rodriguez (1994) recovered 72 yeast isolates of which 19 % were psychrophilic (could not grow at or above 20 ^{o}C) and 43 % grew more rapidly at 20 ^{o}C than at temp at which they were collected (<4 ^{o}C). A biosurfactant producing yeast strain, *Pseudozyma hubeiensis* SY62, was newly isolated from *Calyptogena soyoae* (deep-sea cold-seep clam, *Shirouri-gai*) at 1156 m in Sagami Bay (Konishi et al., 2010).

Filamentous fungi and yeasts are common in marine environments (Norkan, 1966b; Litchfield and Floodgate, 1975; Ahearn and Meyers, 1976; Hagler et al., 1979; Kohlmeyer and Kohlmeyer, 1979; Phaff et al., 1978). Allen and Leda

(1981) and Kirk and Gordon (1988) studied the yeasts from marine and estuarine waters with different levels of pollution in the state of Rio De Janeiro, Brazil. He found that yeast counts in clear sea water generally ranged from a few to several hundred per litre, but in the presence of enrichment like pollution or algal bloom, it can reach thousands per litre or more. In addition, there is a shift from strictly aerobic yeasts in clear water to the presence of fermentative yeasts in polluted waters. Studies by Paula et al. (1983) and Loureiro et al. (2005) in the polluted and unpolluted beaches of Southern area of Sao Paulo state, Brazil as well as in the 2 beaches of Olinda, Pernambico state, Brazil points to the fact that *Candida* was the most prevalent genus found in these areas. Studies on halotolerant and fermentative yeasts living in various aquatic environments found that 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 waters.

Estuaries:

Ecological observations showed that the estuaries had more dense yeast populations than adjacent oceanic zones. Total colony counts and number of species decreased with distance from the estuaries. The species common to both estuaries and oceanic regions were the genera *Debaryomyces* and *Rhodotorula*. 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 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 widespread in estuaries, the open ocean and inland waters and intestinal yeasts such as *Candida*

tropicalis and *Candida intermedia* from terrestrial substrates that were dominant in estuaries but rare in open seas (Cook and Matsura, 1963).

Taysi and Van Uden (1964) found that higher numbers of yeasts were obtained from regions where there is relatively light pollution. Elevated yeast densities were observed in nutrient rich haloclines in estuaries. 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).

Yeasts are encountered in many flowing waters and are particularly common in rivers which carry sewage (Rheinheimer, 1965). Numerous yeasts were identified from polluted water and sewage (Cook and Matsura, 1963; Ahearn et al., 1968; Hagler and Mendonca, 1981).

Studies on the yeast flora of the Suwanee estuary in Florida showed that *Candida* and *Rhodotorula* were the predominant genera; however the most frequently isolated strain was *Cryptococcus laurentii*. Nine ascosporogenous yeasts were isolated with *Hansenula saturnus* as the predominant form (Lazarus and Koburger, 1974). The microbial flora of the estuarine and inshore environment of the west coast of Taiwan was studied by Cheng and Lin (1977). Preliminary identification of the isolates revealed that they belonged to the genera *Saccharomyces, Torulopsis, Debaryomyces, Endomycopsis, Pichia, Kloeckera* and *Rhodotorula*. 112 yeast isolates were obtained from 31 samples of decaying vegetation in the rhizosphere of the mangrove plants from 11 sites in the Chapora, Mandovi and Zuari estuaries of Goa, India (Da Costa and D'Souza, 1979).

Sediments:

Yeasts have been found in marine sediments. 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*. They also reported that yeasts are abundant in silty muds than in sandy sediments, and the limited deep sea collections showed a predominance of oxidative yeasts as compared to collections made in Biscayne Bay. Roth et al. (1962) observed that sediments and surrounding waters of the grass beds showed higher cell count and higher number of species than grasses and algae. Fell and Van Uden (1963) found that yeasts were confined to 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. According to Suehiro (1963) yeasts occur particularly in the top most centimetres and they are more frequent in the black zone than in sandy sediments. Several hundred living yeast cells per cm^3 were found in the damp ground from the Kiel Fjord (Hoppe, 1970). 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). Nagahama et al. (2001b) reported that diversity of culturable fungi was dominated by ascomycetous yeasts such as *Candida, Debaryomyces, Kluyveromyces, Saccharomyces* and *Williopsis* in surface sediments of water depths, exceeding 200m. Inversely, the diversity was dominated by basidiomycetous yeasts like *Rhodotorula, Sporobolomyces, Cryptococcus* and *Pseudozyma* on the subsurface of sediments in water depths exceeding 2000 m and by deep sea clams, tube worms and mussels. *Cryptococcus* was the dominant genus sequenced from sediments collected at deep methane cold seeps (Takisheta et al., 2006, 2007; Bass et al., 2007). Yeasts mostly isolated

from deep sea sediments represented new species in the Ascomycota or Basidiomycota phyla (Nagahama et al., 1999, 2001a, 2003a, b, 2006, 2008, 2012).

Relatively high yeast densities up to 2000 viable cells/g, have been reported from marine sediments with most of the population present in the top few centimetres (Fell et al., 1973; Lazarus and Koburger, 1974). 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; Voltz et al., 1974). Yeasts are found in sediments of eutrophic lakes in numbers up to several thousands per gram (Niewolak, 1977). Niewolak (1977) found up to 82 yeast cells per ml in slightly polluted North Poland lakes but up to 2310 per ml in heavily polluted waters.

Rhodotorula rubra, was not isolated from sediments of the most polluted sites (Hagler and Mendonca, 1981) which suggests polluted littoral sediments are an unfavourable environment for strictly oxidative yeasts like *Rhodotorula* and *Cryptococcus* which are common in less polluted sediments. Hagler et al. (1982) studied the densities of 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 the sediments and species like *Rhodotorula rubra* related to basidiomycetous fungi were found in relatively low numbers. Diversity assessment of benthic yeasts 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 favourable conditions (Callisto et al., 2004).

The *Cryptococcus vishniacii* (yeasts of basidiomycetous affinity) isolated from the soil samples of Dr. W.V Vishniacs 1973 expedition is peculiar to the dry valleys of Antartica, constituting the only heterotrophic biota demonstratably indigenous to the coldest desert on Earth (Vishniac and Hempfling, 1979a and b; Baharaeen et al., 1982).

Prabhakaran and Ranu Gupta (1991), studied yeasts from sediment samples along the south east coast of India. They found that *Candida* was the dominant species and next in abundance was *Rhodotorula*. Isolation of yeasts was done at a depth range of 200-1000 m along the continental slope sediments of Arabian Sea and Bay of Bengal and the isolates belonged to the genera *Cryptococcus*, *Debaryomyces, Pichia* and *Trichosporon*. Araeyo et al. (1981) made studies on fungi and yeasts from the west coast of India. 12 yeasts were isolated from the deep-sea sediments of the Central Indian Basin from 20 sediment cores of 35 cm length. Of these 2 yeast species *i.e*., *Sporidiobolus johnsonii* and *Sagenomella* were recovered from the deepest part of the sediment core, namely 25–35 cm depth (Singh et al., 2010).

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 a significant role in the transformation of complex and persistent organic compounds and aid in the food chain in the sea.

13 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, 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 ecosystem where they play an important role in detritus food web (Meyers et al., 1975; Hyde, 2002).

Petit et al. (1970) found seven species which are 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* sp. 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 yeast of the marsh land were replaced by hydrocarbonoclastic strains especially *Pichia ohmeri* and *Trichosporon* sp. In general, yeasts isolated from oil polluted regions exhibited much higher hydrocarbonoclastic property than the same species from non–polluted areas. Yeasts communities from heavily polluted sediments that received the discharge from oil refineries and other industries were studied by Romero et al. (2002). Yeasts species isolated from these sediments were able to degrade dibenzofuran. 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.

The deep-sea is one of the most mysterious and unexplored extreme environments, characterised by the absence of sunlight, predominantly low temperatures (<4 ^{o}C but >400 ^{o}C close to hydrothermal vents) and high hydrostatic pressure (up to 110 MPa) (Nagano and Nagahama, 2012).The deep-sea normally refers to oceans greater than 200 m depth. With nearly three quarters of the Earth's surface area being covered by ocean, the average depth of which is 3800 m, the vast majority of our planet thus comprises deep-sea environments. Although once thought to be an uninhabitable milieu owing to its extreme conditions, the deep-sea environment is now recognized as highly dynamic, hosting a wealth of unique organisms. In particular, the discovery of hydrothermal vents, methane cold-seeps and surrounding ecosystems has resulted in completely

new concepts for considering energy sources available for sustaining life in deep oceans (Lonsdale, 1977; Cavanaugh, 1985; Grassle, 1985). Further, Culture-independent studies have produced an ever-growing body of knowledge regarding the diversity, distribution and ecology of deep-sea microbes (Thaler et al., 2012).

Although the presence of fungi in deep-sea environments was not well recognized until very recently, the isolation of deep sea fungi was first reported approximately 50 years ago from the Atlantic Ocean at a depth of 4450 m (Roth et al., 1964). Since then, many reports have been published on the isolation of fungi, including novel species (mostly yeasts) from several deep-sea environments, e.g. hydrothermal vents and the deepest of the seas, the Mariana Trench (Takami et al., 1997; Gadanho and Sampaio, 2005; Nagahama et al., 2006, 2008). Although the true role and diversity of deep-sea fungi remains largely unclear, the significance of fungi in deep-sea environments is starting to be recognized, with more intensive investigations in recent years. Fungal diversity in deep-sea environments has been investigated by both conventional culture-dependent methods (Nagahama et al., 2001; Raghukumar et al., 2004; Gadanho and Sampaio 2005; Damare et al., 2006; Le Calvez et al., 2009; Burgaud et al., 2009; Connell et al., 2009; Jebaraj et al., 2010; Singh et al., 2010) and culture-independent methods (Bass et al., 2007; Lopez-Garcia et al., 2007; Lai et al., 2007; LeCalvez et al., 2009; Jebaraj et al., 2010; Nagano et al., 2010; Sauvadet et al., 2010; Singh et al., 2011; Eloe et al., 2010; Quaiseret al., 2011; Nagahama et al., 2011, Nagano and Nagahama, 2012; Thaler et.al., 2012). Using culture-dependent approach, fungi were isolated and identified from the deep-sea sediments of the Central Indian Basin (Raghukumar et al., 2004; Damare et al., 2006; Singh et al., 2010). Further Singh et al. (2011) analyzed the fungal diversity in deep-sea sediments using culture-independent approach by targeting universal 18S as well as fungal specific and universal ITS (internal transcribed spacers) regions of rRNA genes from

three locations in the CIB and by multi primer approach from sea floor at a depth of 5000 m (Singh et al., 2012).

The yeast commonly 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 author's state that this disproportion was not observed in the 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 7 phylotypes to basidiomycota. They were identified as *Candida atlantica*, *C. atmosphaerica, C. lodderae*, *C. parapsilosis*, *Exophiala dermatidis, Pichia guilliermondii, Trichosporon dermatitis, Rhodosporidium diobovatum, Rhodosporidium sphaerocarpum, Rhodotorula toriloides* 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.

Fishes:

Skin, gills, mouth, faeces and gut contents of fishes harbour yeasts. *Debaryomyces hansenii* is the most dominant species associated with fish. *Debaryomyces* is frequent in seawater which may explain its high incidence in fish. *Metschnikowia zobelli* is another important yeast species and higher number of *Metschnikowia* isolated from the gill contents of the fish and it has been suggested that yeast flora of the fish merely reflect their feeding habits (Fell and Van Uden, 1963). Ross and Moris (1965) reported that the greatest variety and highest number of yeasts were obtained from the 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.

Weeds and Algae

Populations of yeasts in seaweeds are investigated by several scientists. Studies on zoo and phytoplankton revealed more than 20 associated yeast species. Bunt (1955) examined microbes present in the decomposing giant kelp at Macquaria island in Antarctica and found that large amount of yeasts were present in decomposing kelp tissue. According to Kriss (1959) the planktonosphere is richer in yeasts than other zones in the sea. Plankton catches from the black sea contained yeasts in 90% of the samples. Studies of Suehiro (1960) revealed that decomposing algae constitute a suitable substrate for yeast development. The predominant species of yeast isolates from the marine algae were *Torulopsis* sp., *Pichia albicans*, *Candida natalensis*, *Trichosporon cutaneum* and *Endomycopsis chodatii*. Van Uden and ZoBell (1962) obtained yeasts from samples collected from algal and coral growths in the Torres Strait region. Species such as *Metschnikowia reukaufii, Pichia farinose, Kluyveromyces aestuarii, Candida marina, Torulopsis torresii* and *Torulopsis mari*s were obtained. Fell et al. (1973) isolated several *Rhodosporidium* sp. from plankton samples at various water depths in the Pacific Ocean. Van Uden and Branco (1963) isolated yeasts from Giant Kelp in southern California and found *Metschnikowia zobelli* on all samples yielding yeasts except one. Suheiro et al. (1962) estimated that more than 50 % of algal biomass (phytoplankton) was transferred into yeast biomass. He also estimated that a mixed population of yeasts may be capable of degrading and

assimilating a large population of organic material released from decaying phytoplankton even in the absence of bacteria.

Patel (1975) found that living algae contained lower counts of yeasts compared to the surrounding seawater, but when decomposition starts, yeasts in algal material increased to higher numbers than those found in the surrounding sea water. Sieburth (1975) reported seaweeds as a reservoir of *Candida* in inshore waters. The authors considered the possibility that yeasts may utilize exudates of their living hosts. Meyers et al. (1975) studied the yeast population on living *Spartina alterniflora* plants in a Louisiana salt marsh. *Pichia spartinae* and *Kluyveromyces drosophilarum* were found on the outer surface of the culm. Yeast populations of *Sporobolomyces roseus* on marsh plants in England were investigated by Pugh and Lindsay (1975). Leaves of inland plants harboured much higher cell numbers than those near shore. Newell (1976) mentioned blooms of *Rhodotorula rubra* and *Debaryomyces hansenii* on submerged seedlings of *Rhizophora mangle*.

Invertebrates:

Studies on invertebrates have shown that they are either devoid of yeasts or support only a small density of the population. Phaff et al. (1952) obtained yeasts from the Mexican shrimp *Penaeus setiferus* and the yeasts isolated were *Trichosporon cutaneum, Rhodotorula glutinis, Pichia guilliermondii, Candida parapsilosis*, and *Pullularia pullulans*. Siepman and Hohnk (1962) sampled shrimp eggs, sponges and other invertebrate material collected from the North Atlantic Ocean and the yeasts isolated were *Debaryomyces hansenii*, *Torulopsis candida* and *Trichosporon cutaneum*. About half the number of species were from the internal parts of the animals and other half from surface water. In assimilation tests, they found strong formation of riboflavin by *Debaryomyces subglobosus* a yeast, they frequently isolated from the internal fluids of invertebrates, and the authors suggested that this yeasts may serve as a vitamin source for marine

animals. The whole body of the amphipod *Podocerus brasiliensis* was found to be invaded by *Rhodotorula minuta* (Roth et al., 1962). Seki and Fulton (1969) showed that the tissues of living marine copepods (*Calanus*) were attacked by *Metschnikowia* sp. Fize et al. (1970) reported a *Metschnikowia* sp. parasitizing living copepods (*Eurytemora velox)* in Southern France.

Yeast populations from conch and spring lobster on the Bahama Islands were studied by Voltz et al. (1974). They isolated fewer types of yeasts from the animals than from marine sand and sediment of the same habitat, and assumed that the isolates were probably ingested during feeding and did not seem to cause stress to their hosts. The commercially used brine shrimp, *Artemia salina*, was parasitized by *Metschnikowia bicuspidate var. australis*, a yeast that appears to be equipped with an active predatory mechanism attracting its host by forcible ascospore discharge (Lachance et al., 1976). Cheranoswski and Cowley (1977) found *Rhodotorula glutinis* and *Torulopsis ernobii* in the gut of fiddler crab, *Uca pugilator*. It was speculated that these yeasts might serve as food, but feeding experiments showed that they could not be utilized as a sole food source by the crabs. Buck et al. (1977) investigating on bivalve shellfish in Long Island found that, in general the liquid portion of the shell fish contained more yeasts than the internal viscera. The ascomycetous yeast communities associated with 3 bivalve molluscs and four crab spp. were studied in mangroves at Coroa grande or Sepetiba Bay in Rio De Janeiro, Brazil. The cultures obtained were classified into 84 species among which 44 species were novel. The ascomycetous yeast communities of the mangrove ecosystem included many new biotypes.

2.1.2. Isolation and Maintenance of 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. 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 malt sugar. *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 sugar (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.

Various other selective media used are *Debaryomyces hansenii* differential medium for selective detection of *Debaryomyces hansenii, Dekkera/ Brettanomyces* differential medium (DBDM) for isolation and identification of *Dekkera* and *Brettanomyces.* 18% glycerol (DG 18) agar for selective isolation of Xerotolerant yeasts, Dixon's agar modified (mDA) medium for *Malassezia* sp., *Kluyveromyces* differential medium (KDM), for *Kluyveromyces marxianus* and *K.lactis* and *Yarrowia lipolytica* differential medium for the isolation *Yarrowia lipolytica*, which develops a brown colour in the medium.

Plates are incubated at temperatures designed to stimulate ambient environmental conditions. For example, polar and deep-sea samples should be incubated at ~5^{o}C.Temperature required for temperate and tropical samples often result in overgrowth by filamentous fungi, which can be reduced by incubation at temperatures ~12^{o}C (Fell, 2001). For taxonomic tests, yeasts are usually incubated at 25^{o}C (Buhagiar and Barnett, 1968) although optimum temperatures for growth are higher for some yeasts and lower for others (Watson, 1987).

2.1.3. Identification of the isolates

Yeasts are 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 tetra saccharides, 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 example *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 metabolised 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 yeast 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 each species metabolised 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 Barnet et al. (1990) the main characteristics used to classify yeasts are as follows.

1. 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.

2. Sexual Reproduction:

Some yeasts reproduce sexually by ascospores, other 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) 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.

3. 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 37^{o}C e) Grow in the presence of cycloheximide f) Split fat g) Produce starch like substances h) Hydrolyze urea and i) Form citric acid.

4. Biochemical Characteristics:

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

2.1.4. 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 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 compounds. Studies by Paskevicus (2001) showed that almost all the yeast strains produce lipase. 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 35-40 ^{o}C 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 45 ^{o}C (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, medical and clinical analysis, food and pharmaceutical industries (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 at 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 described. The extra cellular enzymes include cellulase, 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.

2.2. Materials and Methods:

2.2.1a. Sample collection.

Sediment samples were collected from the west coast (Arabian sea) specifically the continental slope during Cruise No. 254 of Fisheries and Oceanographic Research Vessel (FORV) *Sagar Sampada* of CMLRE, Ministry of earth sciences, Govt of India, using Smith McIntyre grab from three different depth ranges (200,

500, 1000 m). Samples were collected from 16 transects in the Arabian Sea. Stations in the Arabian Sea were located between Cape Comorin at the southern end and Porbander at the northern end. Sediment samples were transferred aseptically in to sterile polythene bags for microbial analysis. The details of the stations are given in the Table 2.1.

Samples already collected and preserved in the microbiology Lab of CUSAT from 12 transects in the Bay of Bengal, located between Karaikkal at the southern end to Paradip at the northern end from Cruise No. 236 were also used for the study.

2.2.1b. Isolation of marine yeasts:

Spread plate method was employed for isolation of yeasts from marine sediments. About 10 g of the sediment obtained was suspended in 30 ml sterile seawater, vortexed and used as inoculum. 1ml of the inoculum was spread plated on malt–yeast-glucose-peptone agar (Wickerham, 1951) supplemented with 200 mg/l chloramphenicol. The plates were incubated at 18 ± 2 ^{o}C for 14 days. The colonies developed were purified by quadrant streaking and transferred to malt extract agar slants for further studies.

Medium used for isolation (Wickerham's agar)

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

Preservation:

Yeast isolates purified by quadrant streaking were stocked in malt extract agar vials overlaid with sterile liquid paraffin.

Malt Extract Agar (composition)

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

2.2.2. Identification of the Isolates:

The isolated yeast strains were identified up to genera as per Barnett et al. (1990). For this, microscopic appearance of the cell, mode of reproduction and certain biochemical and physiological characteristics were studied.

1. **Microscopic appearance of yeast 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: a) Whether the yeast reproduce by budding, splitting or both b) The shape and size of the vegetative cells c) If the yeast form buds where do they occur on the mother cell.

b) Microscopic examination for filamentous growth:

Slide cultures of isolated yeasts were prepared. For this, malt extract agar plates were prepared. In each plate four sterile cover slips dipped in malt extract agar (1 % agar) was kept on the medium surface at 45 ^{0} angle position by gently piercing the agar. These slides were examined microscopically daily or once in two days for up to about 2 weeks. Observations were done to ascertain whether or not there is filamentous growth. If so, what kind of cells grows from filaments (Fig 2.8).

2. Assessing the ability to use nitrogen compounds for aerobic growth

The test of ability to use nitrate as a sole source of nitrogen is a valuable aid to identify yeasts. A mineral basal medium supplemented with glucose as carbon source and KNO_3, as the sole nitrogen source was employed for the test.

Beijerinck medium: (Composition)

KH_2PO_4	-	2 g
$MgSO_4 7.H_2O$	-	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 into the medium and incubated at 28 ± 2 ^{o}C for one week. Ability to use nitrates as the sole nitrogen source was determined by observing the growth and turbidity.

3. Assessing the ability to use sugars anaerobically /aerobically.

Marine oxidation fermentation (MOF), medium was used for testing the ability of yeast to metabolize dextrose aerobically (oxidative) or anaerobically (fermentative). When dextrose is utilized, acid is produced which changes the

colour of the medium from pink to yellow. The pH indicator in the medium is phenol red. Yellow colouration at the slope region indicates an oxidative reaction, where as the whole tube turning yellow indicates a fermentative reaction.

4. Urea Hydrolysis:

Composition of the medium:

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 sea water and autoclaved at 15 lbs for 15 min. Urea was sterilised using solvent ether and dissolved in 50 ml sterile distilled water. This urea was added to the basal medium, dispensed into test tubes (3 ml each) and slants were prepared. Cultures were inoculated and after incubation a change of colour in the medium from yellow to pink was noted as urea hydrolysis.

5. Production of starch like substances:

Certain yeasts produce starch like substances during metabolism. For testing this property, a mineral basal medium supplemented with glucose was used.

Composition of medium:

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 medium and were incubated for a week. Gram's iodine was added to each tube. The change of colour to dark blue indicated the presence of starch like substances.

6**. Diazonium Blue B (DBB) Test:**

The cultures were spot inoculated on Wickerham's agar and incubated for 10 days. After incubation these Petri dishes were held at 55 ^oC 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 gets discoloured. It is prepared by dissolving diazonium blue B salt in cold 0.1M Tris-HCL buffer, pH 7.0, at 1 mg per ml. If the colour of the culture turned dark red within 2 min at room temperature, the result was recorded as positive.

7. Myo-Inositol test:

A mineral basal medium supplemented with myo-inositol was used for preparing the broth. Cultures were inoculated into this broth and after one week if the broth shows turbidity then the test is recorded as positive.

Composition of mineral basal 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

Based on the above mentioned biochemical tests the isolates were classified up to the generic level.

2.2.3. Hydrolytic Enzyme production:

The 192 isolates were tested for the production of enzymes, *i.e*. amylase, lipase, protease, urease, aryl sulfatase, ligninase, cellulase, DNase, pectinase and chitinase.

1. **Amylase, Protease, 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 7.0) supplemented with starch (1 %), gelatin (2 %), tributyrin (1 %) and colloidal chitin (5 %) were prepared separately. Plates were spot inoculated, incubated at room temp (28 ± 2 ^{o}C) for 7 to 10 days and observations were made. Presence of clearance zone was noted as positive and the diameter of

the zone was recorded. Starch agar plates were flooded with grams iodine solution (Iodine, 1 g and potassium iodide, 2 g in 300 ml distilled water) and gelatin agar plates with mercuric chloride solution (15 %) respectively. Lipid agar plates and chitin agar plates were noted for a clear zone around the colonies.

2. Pectinase.

Pectin Agar (Pectin 0.5 g; $CaCl_2$.$2H_2O$. 0.02 g; NaCl 2 g; $FeCl_3 6.H_2O$ 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 (28 ± 2 ^oC) 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. 1 % aqueous cetavlon was allowed to stand for 20-30 min. A clear zone around the colonies indicates positive result.

3. Cellulase:

Cellulase agar (Casein hydrolysate 0.05g; yeast extract 0.05g; $NaNO_3$ 0.01g; cellulose powder 0.5 g; agar 2 g; sea water 100 ml; pH 7) was used for testing cellulose production. The plates were spot inoculated and incubated at room temperature (28 ± 2 ^oC) for 7 to 10 days. The zone of clearance around the colonies was noted as positive.

4. 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 ± 2 ^oC for 10 days, the plates were flooded with IN HCl. A clearance zone around the colonies was recorded as positive.

6. Aryl sulfatase:

The medium used for the production of aryl sulfatase is 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 0.001 M Tripotassium phenolphathalein disulfate (PDS) was used. The plates were spot inoculated and incubated at room temperature (28 ± 2 ^{o}C) 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.

7. 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 ± 2 ^{o}C) for 7 to 14 days. Formation of halo zone or brown colour around the colonies was considered as positive.

7. Phosphatase

The medium used for the production of phosphatise is nutrient agar (Peptone 0.5 g; beef extract 0.1 g; agar 2 g, sea water 100 ml, pH 7) supplemented with 0.01M dipotassium phenolphathalein phosphate was used. The plates were spot inoculated and incubated at room temperature (28 ± 2 ^{o}C) for 12 days. After incubation the agar plates were exposed to ammonia vapour, development of pink colour around the colonies was recorded as positive.

2.2.4. Utilisation of different sugars:

Marine oxidation fermentation (MOF) medium was used for testing the ability of yeasts to metabolize various sugars. Sugars used for the study were

monosaccharides (glucose, galactose, xylose (pentose), disaccharides (maltose, sucrose, lactose, raffinose, melibiose), trisaccharides (trehalose,melezitose) and polysaccharides (starch, cellulose, inulin). 1 % of the respective sugar except for raffinose (2 %) was incorporated into the MOF medium and slants were prepared, surface dried and isolates were inoculated into the slants by stab and streak method. The pH indicator in the medium is phenol red. Yellow colouration at the slope region indicates an oxidative reaction and the whole tube turning yellow indicates a fermentative reaction.

2.2.5. Growth assessment of the isolates at different temperatures:

Malt extract agar plates were prepared. Isolates were streaked into agar plates and inoculated plates were incubated at 4 ^{o}C, 8 ^{o}C, 12 ^{o}C, 18 ^{o}C, 25 ^{o}C, 30 ^{o}C, 35 ^{o}C, 37 ^{o}C, 40 ^{o}C and 45 ^{o}C (± 1^{o}C) for 1 week and then observed for growth.

2.3. Results:

2.3.1. Generic Composition:

Of the total 192 isolates, 134 were from the Arabian Sea (Cruise no: 254) and 58 were from the Bay of Bengal (Cruise no: 236). Biochemically the isolates were categorised into 5 genera, the most predominant being the *Candida* (59.8 %) followed by *Debaryomyce*s (17.7 %), *Rhodotorula* (8.85 %), *Hortaea* (8.3 %) and *Pichia* (5.2 %) (Fig. 2.1).

2.3.2. Hydrolytic Enzymes:

All the isolates (100 %) were lipolytic followed by ligninolytic (18 %), proteolytic (17 %), ureolytic (17 %), amylolytic (12 %) and phosphatase producing forms (12 %). 1 % of the isolates produced aryl sulfatase and DNase. None of the isolates produced pectinase, cellulase and chitinase (Fig. 2.2).

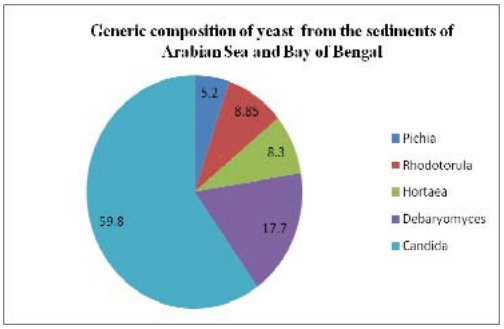


Fig.2.1. Generic composition of yeasts from the Arabian Sea and Bay of Bengal

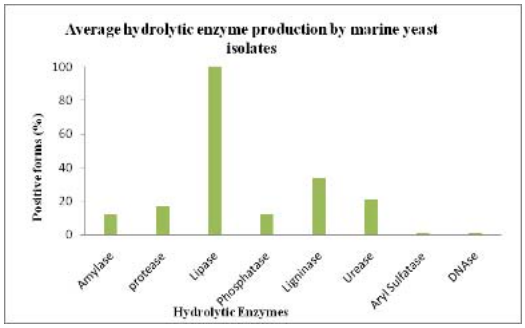
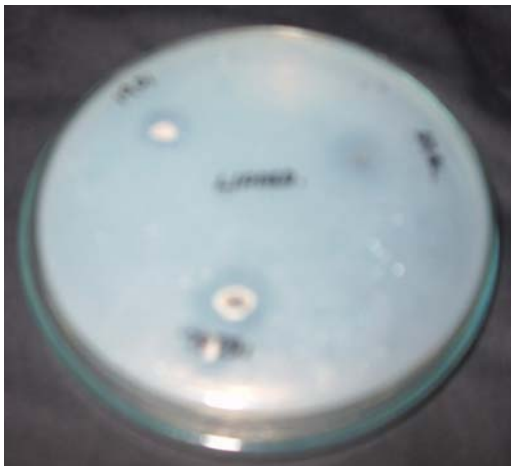


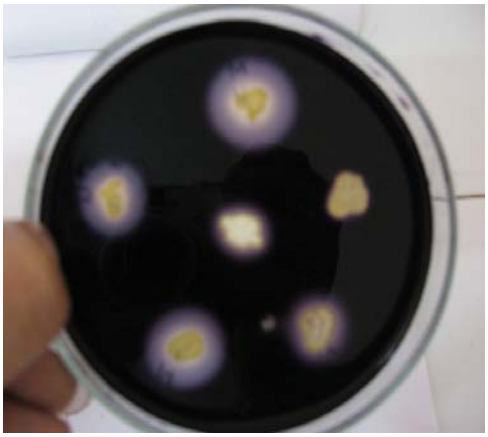
Fig: 2.2 Hydrolytic enzyme production by marine yeast isolates from Arabian Sea and Bay of Bengal

Black yeasts were a potent source of hydrolytic enzymes as they are all lipolytic and more than 65 % were ureolytic, proteolytic (71 %), amylolytic (71 %) and ligninolytic (80 %). The pink yeasts *i.e*., *Rhodotorula* were also highly lipolytic, both the pink and black yeasts produced large clearing zones, when compared to other isolates. The diameter of the zone varied between 2 mm to more than 1 cm in pink and black yeasts. 88 % of the pink yeasts were ureolytic and 28 % were ligninolytic. A single isolate of the genera *Pichia* exhibited amylolytic, proteolytic and ureolytic activity. 2 isolates of the genera *Candida* exhibited DNase activity and 7 % were phosphatase producers. Less than 2 % of the isolates of the genera *Debaryomyces* were amylolytic, proteolytic and ligninolytic (Fig. 2.3. a-f).

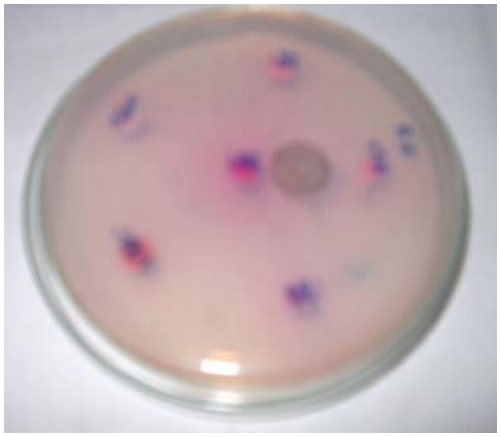
a) Protease activity



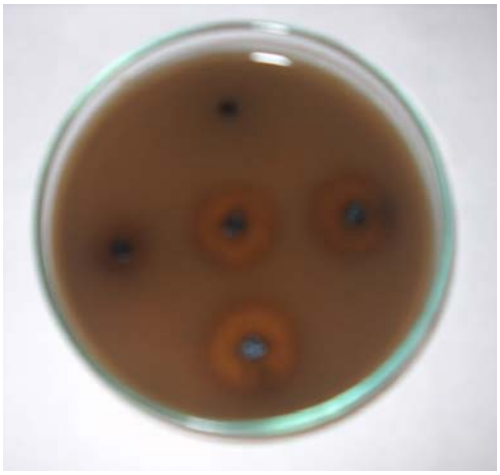
b) Lipase activity



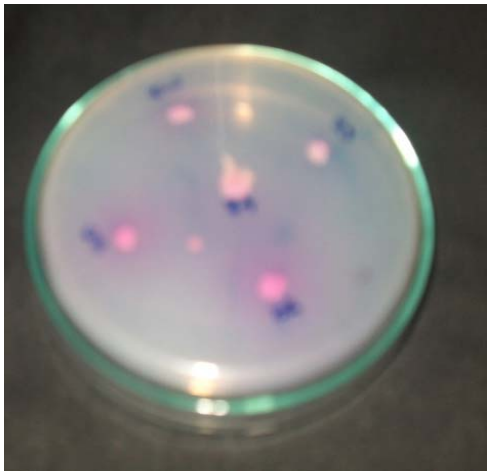
c)Amylase activity



d) Arylsulfatase activity



e) Ligninase activity



f) Phosphatase activity

Fig.2.3. (a-f) Hydrolytic enzyme activity of yeast isolates.

2.3.3. Ability to ferment different sugars:

Among the total 192 isolates obtained from marine sediments, all the isolates were able to either oxidise or ferment the glucose (monosaccharide), sucrose (disaccharide) and melezitose (trisaccharide). 27.60 % of the isolates were glucose oxidisers and 72.39 % glucose fermenters. 92.18 % of the isolates were able to oxidise or ferment xylose (5 carbon monosaccharide), followed by the monosaccharide galactose (91.14 %), disaccharides maltose (82.81 %), trehalose (37.97 %) and melibiose (26.02%). 38.96 % of the isolates were cellobiose (disaccharide) oxidising, 37.11 % were raffinose (trisaccharide) oxidising, 6.24 %

were inulin (polysaccharide) oxidising and 9.89 % of the isolates were lactose (disaccharide) fermenters (Fig.2.4).

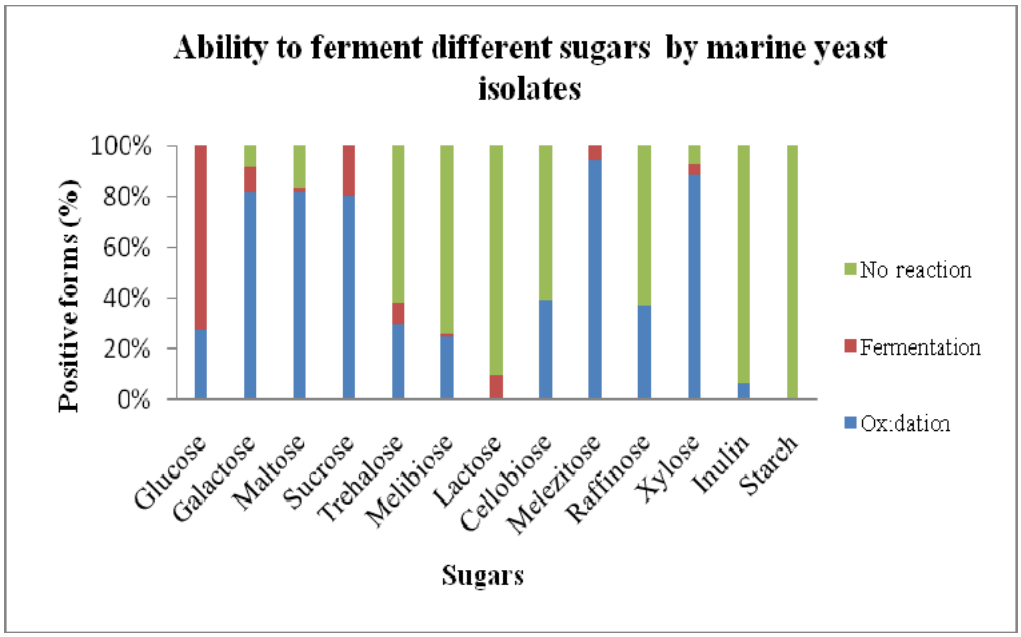


Fig.2.4. Utilization (Fermentative/Oxidative/ No reaction) of different Sugars by marine yeast isolates from Arabian Sea and Bay of Bengal

Isolates in the genera *Rhodotorula* was characterised by the inability to ferment glucose, galactose, maltose, lactose, raffinose, trehalose and melezitose. 100 % of isolates in the genera *Hortaea* was able to oxidise the sugars, sucrose, melezitose, galactose, and xylose. Only isolates belonging to *Hortaea* was able to ferment trehalose. Similarly maltose was fermented by few isolates of the genera *Candida* (2.6 %) and melibiose by few isolates of genera *Debaryomyces* (8.82 %). While none of the isolates was able to ferment the sugars, raffinose and inulin. All the isolates in the genera *Pichia* was able to ferment sucrose and oxidise melezitose, maltose and cellobiose.

The major glucose fermenters belonged to the genera *Candida* (57.29 %) followed by *Debaryomyces* (10.93 %) and the glucose oxidisers belonged to

genera *Rhodotorula* (8.85 %), *Hortaea* (7.29 %) followed by *Debaryomyce*s (6.77 %).The disaccharide sugar sucrose was oxidized by 85.37 % of the isolates, among which 50.52 % of the isolates belonged to *Candida* followed by *Debaryomyces* (14.06 %) and *Rhodotorula* (7.29 %). All the isolates belonging to *Hortaea* and *Pichia* were able to oxidize sucrose. Similarly all the isolates in the genera *Rhodotorula, Hortaea* and *Pichia* were able to oxidize melezitose, while only a small percentage of Candida (5.24 %) and *Debaryomyce*s (0.52 %) were able to ferment melezitose. The Pentose sugar xylose was oxidized by 88.49 % of the isolates, while only 3.64 % of the isolates were able to ferment xylose. All the isolates of the genera *Hortaea* were able to oxidize xylose (Fig 2.5a-l).

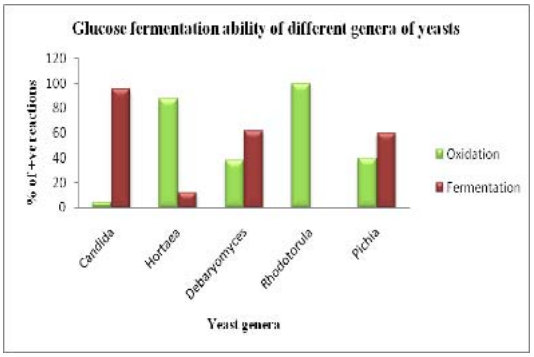


Fig.2.5.a) Glucose fermentation

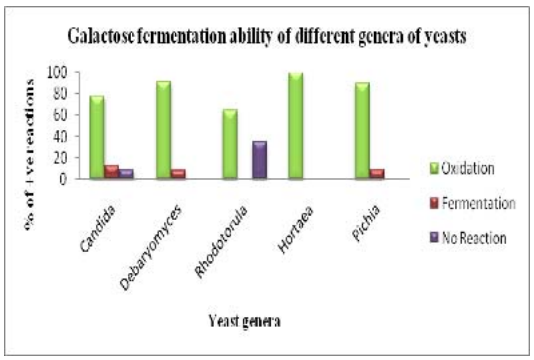


Fig.2.5.b) Galactose fermentation

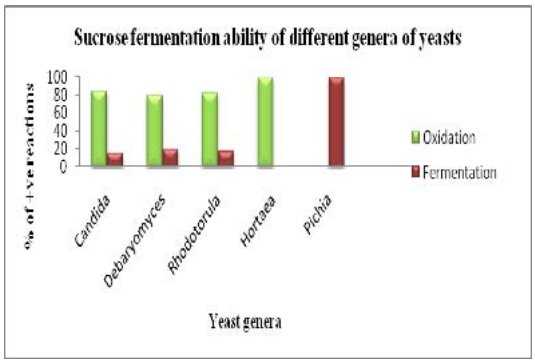


Fig.2.5.c) Sucrose fermentation

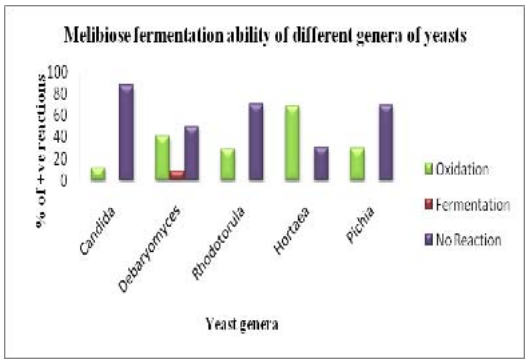


Fig.2.5.d) Melibiose fermentation

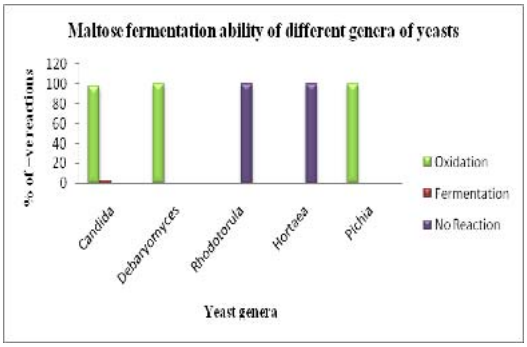


Fig.2.5.e) Maltose fermentation

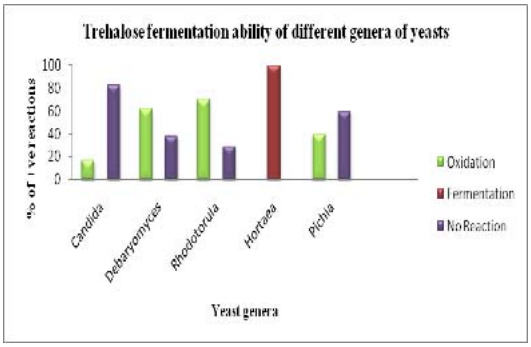


Fig.2.5.f) Trehalose fermentation

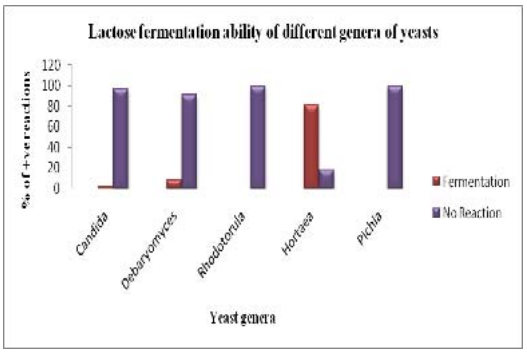


Fig.2.5.g) Lactose fermentation

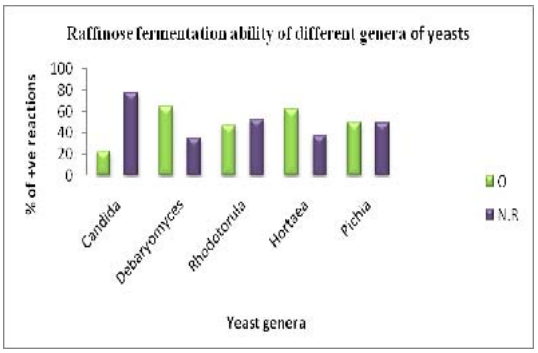


Fig.2.5.h) Raffinose fermentation

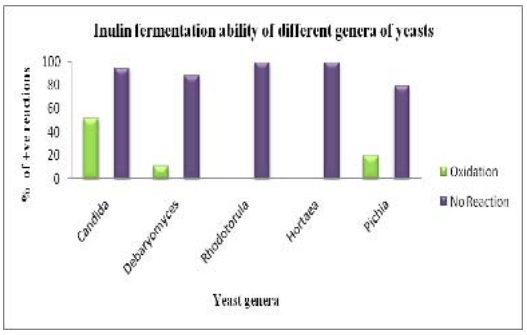


Fig.2.5.i) Inulin fermentation

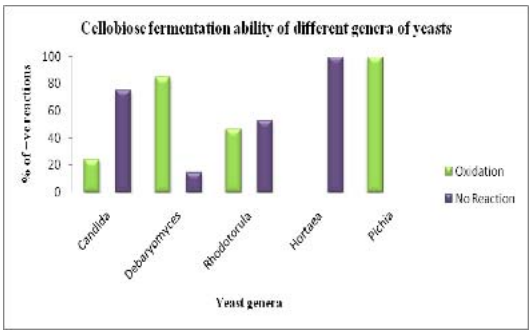


Fig.2.5.j) Cellobiose fermentation

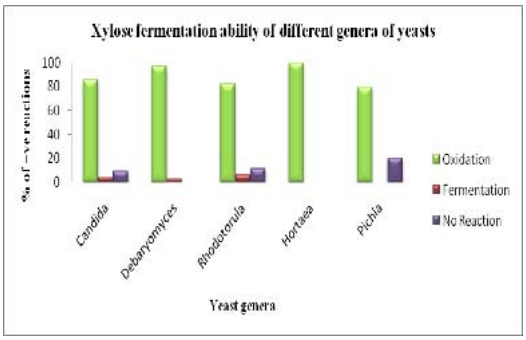


Fig.2.5.k) Xylose fermentation

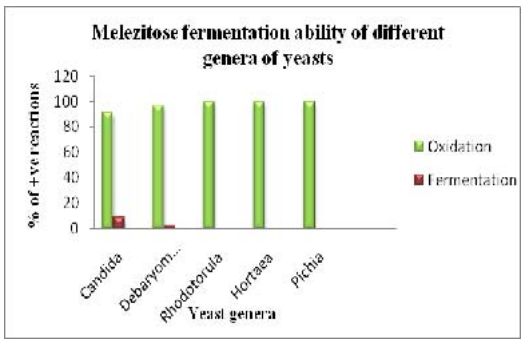


Fig.2.5. l) Melezitose fermentation

Fig.2.5(a-l). Utilization of different sugar sources by marine yeasts belonging to various genera

2.3.4. Growth of marine yeast isolates at different temperatures

Among the 192 isolates, 19.79 % were able to grow at 4 ^{o}C, 79.16 % at 8 ^{o}C and 85.93 % at 12 ^{o}C. All the isolates were able to grow at a temperature range of 18-30 ^{o}C and the growth decreased considerably above 35 ^{o}C. Only 83.33 % of the isolates were able to grow at 35 ^{o}C, 72.91 % were able to tolerate 37 ^{o}C and 54.16 % of the isolates 40 ^{o}C but none of the isolates tolerated 45 ^{o}C (Fig. 2.6.)

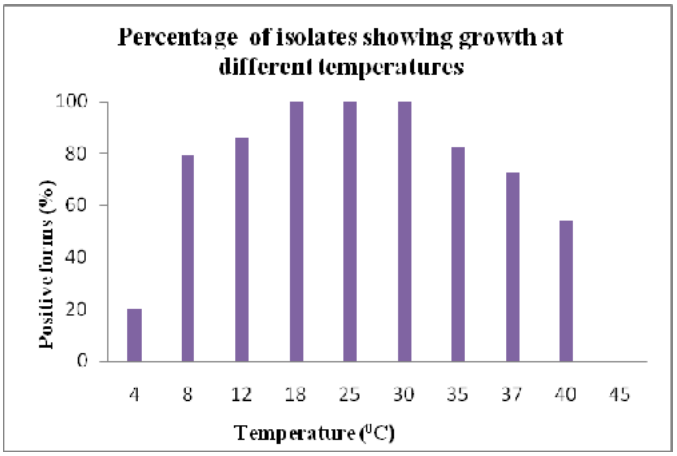
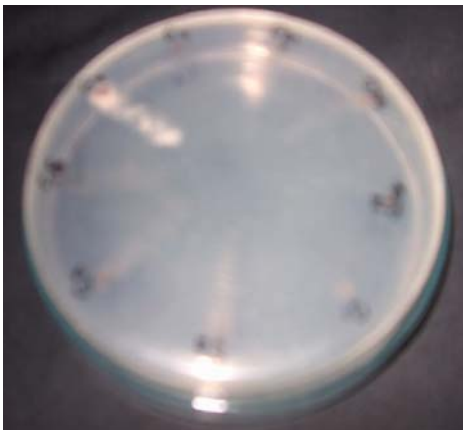


Fig. 2.6. Percentage of the marine yeast isolates showing growth at different temperatures

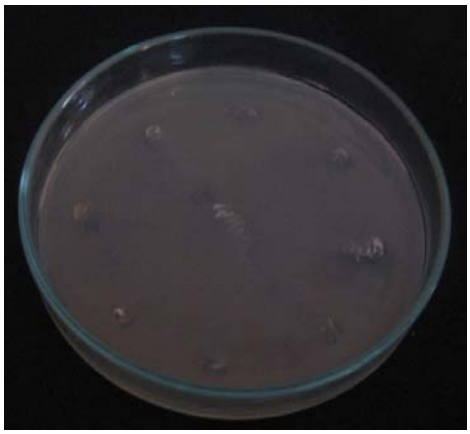
All the isolates in the genera *Candida, Hortaea* and *Rhodotorula* exhibited growth at 18-30 ^{o}C, while isolates of the genera *Debaryomyces and Pichia* exhibited growth at 12 ^{o}C itself. Similarly all the isolates of the genera *Pichia* and *Candida* exhibited growth at 35 ^{o}C. At the same time, isolates of the genera *Hortaea* was unable to grow at low temperatures of 4^{o}C and 8^{o}C as well as high temperature of 37 ^{o}C and 40 ^{o}C (Fig 2.7 a-d).

a) Growth at 8ºC

b) Growth at 35ºC



a) Growth at 40ºC



d) Growth at 45ºC

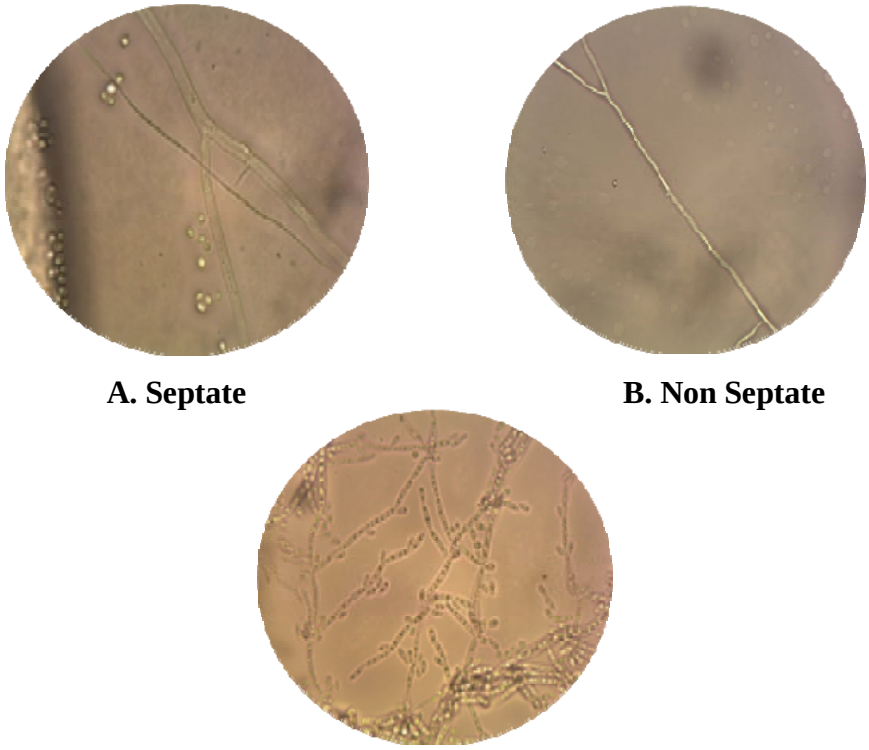
Fig.2.7. (a-d) Growth of the marine yeast isolates at different temperatures

Isolates belonging to different genera exhibited a variation in tolerance to temperature. In the genera *Candida*, only 14 of the 115 isolates were able to tolerate 4 ^{o}C, while it increased sharply to 100 at 8 ^{o}C, 104 at 12 ^{o}C, and all the

isolates were able to grow at 18-30 ^{o}C. With a further increase in temperature to 35 ^{o}C, all isolates exhibited growth which decreased to 90 at 40 ^{o}C and none of the isolates exhibited growth at 45 ^{o}C. While in the genera *Debaryomyces*, of the 34 isolates 13 were able to tolerate 4 ^{o}C which increased sharply to 31 at 8 ^{o}C and all the isolates were able to tolerate temperature in the range of 12-30 ^{o}C, while the temperature of 35 ^{o}C was tolerated by 18 isolates which decreased to 10 at 40^{o}C. The genera *Rhodotorula* exhibited a temperature tolerance similar to *Candida*. About 8 of the total 17 isolates were able to grow at 4 ^{o}C which gradually increased to 11 at 8 ^{o}C and 15 at 12 ^{o}C. Similarly all the 17 isolates in the genera *Rhodotorula* were able to grow at 35 ^{o}C and 10 at 37 ^{o}C, but none exhibited growth at 40 ^{o}C. The genera of black yeast was unique in that none of the isolates exhibited growth 4 ^{o}C and 8 ^{o}C, while they exhibited a poor growth at 12 ^{o}C. But all the isolates were able to grow at 18-30 ^{o}C which reduced steeply to 3 at 35 ^{o}C. Even though only 3 isolates belonging to *Pichia* exhibited growth at 4 ^{o}C all the isolates were able to tolerate temperature within a range of 8-35 ^{o}C, but it steadily decreased to 4 at 40 ^{o}C and 0 at 45 ^{o}C (Table 2.1).

Table 2.1 Percentage of growth exhibited by isolates of different genera at various temperatures

Genera	4^{o}C	8^{o}C	12 ^{o}C	30 ^{o}C	35 ^{o}C	37 ^{o}C	40 ^{o}C
Candida	12.17	86.95	90.43	100	100	93.04	78.2
Debaryomyces	38.2	91.17	100	100	52.94	44.1	29.41
Rhodotorula	47.05	64.70	88.23	100	82.35	58.82	0
Hortaea	0	0	12.5	100	18.75	0	0
Pichia	30	100	100	100	100	90	40



A. Septate **B. Non Septate**

C. Pseudo hyphae

Fig.2.8. Microscopic view of various types of hyphae in yeasts (100X)

2.4. Discussion

The average population densities of oceanic, coastal and estuarine waters are reported to be under 100 cells/l. Several thousand yeast cells per litre of water were observed in coastal waters (Meyers et al., 1967). Majority of yeasts were obligate aerobes that require oxygen for growth and reproduction and hence yeasts do not inhabit anaerobic waters and sediments. But Kriss (1961) reported that yeasts could be observed in the anaerobic zone of the black sea.

Candida exhibited a ubiquitous distribution, as it is the dominant genera in both Arabian Sea and Bay of Bengal and they are found in 200 m, 500 m and 1000 m depths. *Candida* is one of the most common genera in marine environments (Fell, 1967; Fell and Van Uden, 1963; Kriss et al., 1967; Fell and Uden, 1968). At the same time, Fell et al. (1963) also reported that *Candida* is found more often in

coastal waters often in close proximity to urbanized regions where waters are highly polluted by domestic water (Paula et al., 1983). The other common genera isolated were *Debaryomyces, Hortaea* and *Rhodotorula,* which is also in agreement with Roth et al. (1962). They reported that open ocean waters are generally dominant with *Candida, Debaryomyces* and estuaries with *Candida* and *Rhodotorula*. Black yeasts were also isolated from sediments by Fell et al. (1976). In Arabian Sea and Bay of Bengal, 90 % of black yeast isolates are from 200 and 1000 m depths and only 10 % from 500 m depth. Recent investigations in deep-sea environments by several scientists (Damare et al., 2006; Singh et al., 2010, 2011, Nagahama et al., 2011) also reported that the genera *Candida, Debaryomyces, Kodamaea, Metschnikowia, Aureobasidium, Cladosporium, Hortaea, Pichia* and their relatives have been frequently encountered under deep-sea conditions. Several culturable yeasts, like *Candida* and *Debaryomyces* belonging to Ascomycota and *Rhodotorula, Rhodosporidium* and *Cryptococcus* belonging to Basidiomycota were found in deep-sea hydrothermal vent fauna (Burgaud et al., 2010). These frequently encountered species have common characteristics of adaptation or resistance to low temperature and high osmotic pressure, which may be essential keys to survive under deep-sea conditions.

All the isolates were found to be lipolytic which indicate that yeasts and yeast like fungi were found to be able to adapt themselves to substrates rich in fat. The most active lipase producers were reported from the genera *Candida, Rhodotorula, Pichia*, *Kluyveromyces* and *Saccharomycopsis* (Paskevicius, 2001).

Wang et al. (2007) also reported that out of total 427 strains they have isolated from various marine environments lipase producing yeasts belonged to various genera of *Candida, Pichia, Lodderomyces, Yarrowia, Rhodotorula* and *Aureobasidium.* They also reported that optimum pH and temperature for lipase production were between pH 6 and 8.5 and temperature between 35 and 40 ^{o}C respectively. Some lipases from yeast strains (*C. intermedia, Lodderomyces*

elongisporus) could actively hydrolyse different oils, indicating that they have potential applications in industrial and biotechnological fields as they possess the unique feature of acting at the aqueous and non aqueous interface which distinguishes them from esterases (Verger, 1997; Schmidt and Verger, 1998). Lipases produced from yeasts *Candida rugosa* was used for the production of fatty acids from castor bean. Pandey et al. (1999), investigated the production of flavour in concentrated milk and cream, using microbial lipases. In detergent industry lipases found use as lipid stain digesters (lipase from *Yarrowia lipolytica*). Microbial lipase can be used as biosensors and lipases are used for the determination of lipids for clinical purpose. The basic concept is to utilize a lipase to generate glycerol from triglycerol and quantify the released glycerol. This principle enables physicians precisely to diagnose patients with cardio vascular complaints. *Candida rugosa* lipase biosensor, which optically conjugates to bio recognition in DNA, has been developed as probe by Pittner et al. (1974) and Pandey et al. (1995). Lipases are quite relevant to biotechnology industry and attract special attention as yeast products have been consumed by man since ages and are considered safe and natural. The last quarter of the 20^{th} century have witnessed unprecedented use of lipases in the manufacture of pharmaceuticals and in waste management (Torossian and Bell ,1991; Yadav et al., 1998; Jaeger et al., 1999; Saxena et al., 1999). Lipases from *Yarrowia lipolytica* was found to have applications in bioremediation of environments contaminated with aliphatic and aromatic compounds, organic pollutants, metals and 2,4,6 trinitrotoluene. The extracellular enzymes play an important role in various industrial processes and also in the environment. Crude amylases produced by terrestrial yeast *Saccharomycopsis fibuligera* are capable of converting raw starch and cassava into trehalose effectively (Chen et al., 2009). Chi et al. (2009) reported that gluco amylase produced by the marine yeasts was different from that produced by other yeasts and fungi. It may have great potential for use in direct digestion of raw potato starch in food and fermentation industries. The results also suggest that

marine yeast offer great potential for the production of novel enzymes which would not be observed from terrestrial yeasts and fungi (Gupta et al., 2003; Dariush et al., 2006). Black yeasts are the major amylolytic ureolytic, proteolytic enzyme producers of the study. Eventhough many terrestrial yeasts belonging to the genus *Candida, Yarrowia* and *Aureobasidium* is reported to produce protease, only a single marine yeast strain *Aureobasidium pullulans* is able to produce protease (Ni et al., 2008). Proteases have many applications in detergents, leather processing, silver recovery, medical purpose, feeds, chemical industry as well as waste treatment (Kumar and Tagaki, 1999, Anwar and Saleemuddin, 1998). Ligninolytic enzymes from yeasts are very few. Studies by Villas Boas et.al. (2002) showed that the yeast strain *Candida utilis* has lignocellulose degrading activity. Pink yeasts from marine environment have ligninase enzyme as well as urease enzyme. Cazin et al. (1969) reported that 13 different species of *Rhodotorula* and 5 different strains of *Tremella* were DNase and urease positive. Though *Rhodotorula* was isolated from marine sediments, none of them were positive for DNase.

Fermentation of various sugars like glucose, galactose, sucrose, lactose, raffinose etc. and assimilation of above mentioned sugars and others help in the species characterisation as well as to identify isolates having potential for biotechnological applications. Lachke (2002) reported that hemi cellulose abundant in plant biomass on hydrolysis yielded a 5 carbon sugar D-xylose, which is the most abundant sugar in nature and a potential feedback for generating food and fuel. D-xylose can be used for commercial production of valuable chemicals like ethanol, acetic acid, isopropanol acetone, and n-butanol using microorganisms and is thus important for enhancing the economic utility of ligno cellulose utilization. Yeasts like *Candida polymorpha* and *Pichia miso* can aerobically convert D-xylose to xylitol, sugar alcohol which is a natural sweetener present in small quantities in a wide variety of fruits and vegetables. It can be used

clinically as a sugar substitute for diabetic patients, frequently used in chewing gums and tooth paste. Yeasts like *Candida shehatae*, *Candida tropicalis* are also used for ethanol production from D-xylose. 4 % of isolates belonging to *Candida* is able to ferment xylose. Research on the role that marine MO play in the ethanologenesis from pentose sugars is still in the development stage, as researchers have developed genetically modified yeast which can effectively ferment cellulose to ethanol. Similarly lnulinase has received much attention as it can be widely applied to production of fuel ethanol and high fructose syrup from inulin and inulin rich materials. Inulin is a linear β-(2, 1) linked fructose polymer that occurs as a reserve carbohydrate in Jerusalem artichoke, dahlia tubers or chicory root. Inulin can be converted into fructose which is widely used in many food and beverages instead of sucrose. The best procedure for the enzymatic conversion of inulin into fructose is the use of microbial inulinase which yields 95 % pure fructose. Though many terrestrial yeasts belonging to the genus *Kluyveromyces, Candida* and *Debaryomyces* are reported to produce inulinase, very few marine yeasts like *Cryptococcus aureus, Pichia guilliermondii* are able to hydrolyse inulin. Of all the 192 isolates, only 6 % of the isolates were able to oxidise inulin and they belonged to the genera *Candida* and *Debaryomyces* (Sheng et al., 2007).

In general, most of the isolates are closely related to terrestrial genera and all the isolates were able to grow well within a temperature range of 18-30 ^{o}C, even though they were obtained from a depth where the temperature ranged between 6-16^{o}C. Growth rate of the isolates decreased from 35 ^{o}C onwards which indicated that most of them may be primarily terrestrial in origin which got washed off into the sea, along with running water and got secondarily adapted in the marine waters as suggested by Damare et al. (2006) that many fungi may be transported to the deep sea either as spores or hyphae associated with terrestrial organics. Several previous reports on fungi supports the hypothesis, that some of the fungal

isolates may have originated from terrestrial sources (Rypien et al., 2008, Singh et al., 2010, Galkiewicz et al., 2012). The presence of terrestrial fungi as a component of several culturable fungi in deep-sea sediments was reported by Singh et al. (2010). Fungi cultured from deep-sea reefs were often closely related to terrestrial species (Galkiewicz et al., 2012).

Similar observations were reported by Damare et al. (2006). He reported that isolates of fungi from deep-sea sediments did not have an absolute requirement of seawater for growth. Similarly, most of the recovered fungi from deep-sea environments are psychrotolerant, but isolates of fungi from deep-sea sediments grew more rapidly at 30 ^{o}C than 5 ^{o}C (Damare and Raghukumar 2008; Singh et al., 2010). Thus, it is possible that there are no true indigenous fungi in deep-sea environments but they gradually adapted to deep-sea extreme conditions from terrestrial environments.

To conclude *Candida* was the most dominant genera in the marine sediments. Both black and pink yeasts were encountered in considerable numbers in the sediments. The black yeasts *i.e*., *Hortaea* was the most potent one in terms of enzyme production, followed by *Rhodotorula* and *Candida.* More than 80 % of the isolates were able to ferment/oxidise the different sugars like the monosaccharides (glucose, galactose, xylose) disaccharides (maltose, sucrose) and the trisaccharide (melezitose) points to the fact that marine yeasts are potential source of enzymes like sucrase, maltase (glucase) and melezitose, and galactose degrading enzymes. Xylose as well as their fermenting ability can be used for commercial production of valuable chemicals like ethanol, acetic acid, acetone, isopropanol, n-butanol etc. Irrespective of the temperature of the marine habitat where from the yeasts were isolated, the isolates exhibited considerable growth within a temperature range of 18 - 30 ^{o}C, pointing to the fact that they are mesophiles, probably of terrestrial origin and got adapted to marine environments.

Chapter - 3

NON PIGMENTED YEASTS: MOLECULAR IDENTIFICATION AND PHYSIOLOGICAL CHARACTERIZATION

3.1. Introduction

Macroscopically the yeasts can be divided in to two groups based on colony pigmentation. One group includes the species that produce pink salmon or reddish colonies and with the exception of a few cases the vast majority belongs to the Basidiomycota. The other group includes species forming white or cream coloured colonies and its members are classified both in the Ascomycota and Basidiomycota.

It has long been known that yeasts occur in aquatic habitats, both in freshwater (Spencer et al., 1964; Van Uden and Ahearn, 1963) and in marine environments (Fischer and Brebeck, 1894; Van Uden and Fell, 1968). By conventional microbiological techniques, it was previously observed that yeasts are the dominant fungi in oceans (Sieburth, 1979). In marine waters, yeast populations normally decrease with increased depth and distance from land. However, plankton blooms, surface slicks, current boundaries, eddies and thermoclines may alter this pattern and yeast cell densities may rise above 10^3 per litre. The number of viable yeast cells per litre was found to vary between 13 (North Pacific off Japan) and 274 (off La Jolla, California) (Van Uden and Fell, 1968).

Non pigmented yeasts, which are represented mainly by ascomycetous species are found in environments rich in organic compounds utilizable by yeasts.

Conversely, in open sea or deep-sea benthic environments, where nutrients are probably scarce, pigmented yeasts (basidiomycetous yeasts) are found in large numbers than the non- pigmented ones (mostly ascomycetous) (Nagahama et al., 2001a,b). Besides nutrient availability, the factors that can influence the distribution of these two groups of yeasts is their possible unequal tolerance to increased values of hydrostatic pressure. Studies involving the enrichment of estuarine yeast populations prior to their molecular detection have indicated that the ascomycetous yeasts dominate the first 3 days of incubation, whereas normally basidiomycetous yeasts were only detectable a few days later (Gadanho & Sampaio, 2005).

Previously various non -pigmented yeasts were isolated from different marine environments and the frequently observed ones belong to the genera *Candida, Pichia, Debaryomyces, Williopsis, Trichosporon, Metschnikowia, Saccharomyces, Kluyveromyces* and *Kodomaea.* Nagahama et al. (2001b) reported that culturable fungal diversity was dominated by ascomycetous yeasts in surface sediments in water depths exceeding 2000m and the yeast isolates of the genera *Candida, Debaryomyces, Kluyveromyces, Saccharomyces* and *Williopsis* from deep sea environments around the northwest Pacific Ocean. Gadanho and Sampaio (2005) isolated yeasts from Mid-Atlantic Ridge and the non pigmented yeast isolates were identified as *Candida atlantica* (Burgaud et al., 2010), *C. atmosphaerica, C. lodderae, C. parapsilosis* (Coelho et al., 2010; Nagano et al., 2010; Singh et al., 2011) *Pichia guilliermondii* (Coelho et al.,2010; Burgaud et al., 2010; Edgcomb et al., 2011) and *Trichosporon dermatis*. Gadanho and Sampaio (2005) isolated *C. fluviatilis C. rancensis, Williopsis californica, D. hansenii.* (Coelho et al., 2010; Burgaud et al., 2010), *Glomerella lagenaria* (Nagano et al., 2010), *Bullera unica* and *Lecythophora hoffmannii* from the acidic environments of Iberian Pyrite Belt. Burgaud et al. (2010) obtained *C. viswanathii* and *Leucosporidium scottii,* from deep-sea hydrothermal vents. In addition to these, Coelho et al .

(2010) obtained *C. zeylanoides, C. oleophila, C. boidinii, Clavispora lusitaniae* from Tagus river estuary and Nagano et al. (2010) isolated different species of *Metschnikowia* i.e., *M. colocasiae, M. continentalis, M. kamakouana* and *Trichosporon mucoides* from deep sea sediments. Non pigmented yeasts obtained by Singh et al. (2011,2012) include *Debaryomyces yamadae, Pichia jadinii, Candida glucosophilaii, Trichosporon asahii, Malassezia pachydermatis, M. slooffiae* and *M. restricta* from deep sea sediments of Central Indian Basin. Edgcomb et al. (2011) isolated pathogenic forms viz., *Trichosporon aquatile, Malassezia furfur* along with non pathogenic *C. sagamina* .

A major factor that determines the validity of the studies in yeast ecology is the correct identification of species in the ecosystem. Before the present era of yeast taxonomy, which uses gene sequences and other molecular criteria, identifications were mainly based on phenotypic tests. Although phenotype can sometimes be used to identify species correctly, molecular comparisons have shown that many earlier identifications based on phenotype have been incorrect. While this does not mean that earlier work in yeast ecology is invalid, it does say that conclusions drawn may need to be re-examined following more accurate DNA sequence based identification of species.

3.1.1 Molecular Identification of Yeasts

Different molecular methods are currently used for identification of yeasts, their genetic resolution, impact on systematics, and a description of some of the molecular methods that are applicable to the large species populations often examined in ecological studies are given below:

3.1.2 Identification Methods

3.1.2.1 Nuclear DNA re-association studies

The transition from phenotypic identification of yeasts to molecular identification began with determination of the mole percent guanine (G) plus cytosine (C) ratios of nuclear DNA. These analyses demonstrated that ascomycetous yeasts range from approximately 28 to 50 mol% G+C, whereas basidiomycetous yeasts range from approximately 50 to 70 mol% G+C. Depending on the analytical methods used, strains differing by 1–2 mol% are recognized as separate species (Price et al.,1978; Kurtzman and Phaff,1987). The need for quantitative assessment of genetic similarity between strains and species was satisfied, in part, by the technique of nuclear DNA re association or hybridization. DNA from the species pair of interest is sheared, mixed, made single-stranded, and the degree of relatedness determined from the extent of re association. Many different methods are used to measure this process, which can be done spectrophotometrically or through use of radio isotopes or other markers (Kurtzman, 1993).

There are two basic methods. One is free solution in which both DNAs of the test pair react while dissolved in a buffer solution, and the second relies on binding of one of the pair as single stranded DNA to a matrix, such as a nitrocellulose filter, while the other strand of DNA remains free in the buffer surrounding the bound DNA. Free solution assays may be done spectrophotometrically or with the use of radioisotopes. The spectrophotometric method relies on separation of double stranded DNA by heating (melting)- at which time the reaction buffer containing the separated strands has maximum absorbance at 260nm (A260). DNA reassociates optimally at 25^{o}C below the midpoint of the melting curve (Tm-25) that results from DNA dissociation by heating. Consequently, after strand separation, the temperature is lowered to Tm-25, and the reassociation is monitored by measuring the decreasing A260. The reassociation process is typically conducted with a recording spectrophotometer equipped with thermally controlled cuvettes. Generally four of these are monitored, a blank, a mixture of DNAs from the two strains being compared and each of the DNAs alone. The

reaction is concentration dependent, and if the two strains are from the same species, the mixture of DNAs will reassociate as rapidly as the same concentration of DNA from single strains. If the strains are unrelated, the reassociation will take twice as long because each DNA reacts independently of the other, *i.e.*, two independent populations of DNA molecules that are at half the concentration of single strain DNAs. On the basis of shared phenotype, strains that showed 80% or greater nuclear DNA relatedness were believed to represent members of the same yeast species (Martini and Phaff, 1973; Price et al., 1978). Nuclear DNA reassociation studies have had a marked impact on recognizing yeast species, but the method is time consuming and the extent of genetic resolution goes no further than that of closely related species.

3.1.2.2 GENE SEQUENCE COMPARISONS

The ITS region is now perhaps the most widely sequenced DNA region in fungi (Peay et al., 2008). It has typically been most useful for molecular systematics at the species level, and even within species (e.g., to identify geographic races). Because of its higher degree of variation than other genic regions of rDNA (for small- and large-subunit rRNA), variation among individual rDNA repeats can sometimes be observed within both the ITS and IGS regions.

The rDNA transcription unit (TU) and intergenic spacer (IGS) region comprise a complete rDNA unit. Each rDNA TU consists of the 18S, 5.8S, and 28S genes, the external transcribed spacer (ETS) and internal transcribed spacers (ITS). ITS (for internal transcribed spacer) refers to a piece of non-functional RNA situated between structural ribosomal RNAs (rRNA) on a common precursor transcript. This polycistronic rRNA precursor transcript contains the 5' external transcribed sequence (5' ETS), 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA and finally the 3'ETS. Transcription units are separated by IGSs. The IGS comprises several arrays of tandem repeats, including five to ten copies of a 240-bp repeat located

immediately upstream of the rDNA TU in each rDNA repeat. Genes encoding ribosomal RNA and spacers occur in tandem repeats that are thousands of copies long, each separated by regions of non-transcribed DNA termed intergenic spacer (IGS) or non-transcribed spacer (NTS). Sequence comparison of the ITS region is widely used in molecular phylogeny as it is easy to amplify even from small quantities of DNA due to the high copy number of rRNA genes, and has a high degree of variation even between closely related species.

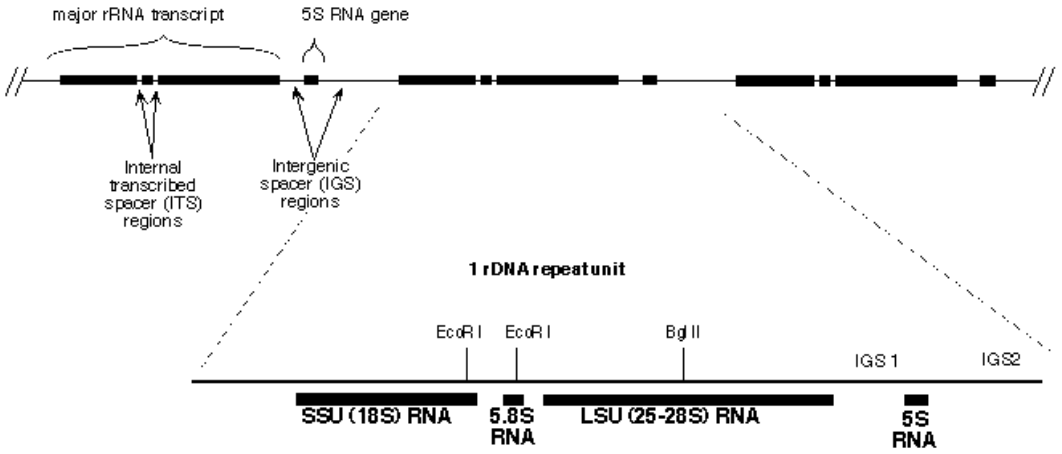


Fig.3.1. Genomic organization of the 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, IGS1, 5SrRNA, IGS2.

Ribosomal rRNA Genes

A limitation of DNA reassociation experiments has been that genetic resolution extends no further than to closely related species. In contrast gene sequence comparisons offer the opportunity to resolve closely related species, as well as more distantly related taxa, and a database of sequences can be developed and expanded for further use. The nucleic acid sequencing has created revolutionary advancements in biology and medicine. It has brought the possibility to know the primary structure of genes and to infer the encoded functions by comparison with well known sequences. Sequencing of the D1/D2 domains and the ITS regions

have been used for yeast identification. Gene sequencing offers a rapid method for recognizing species and resolution is not limited to closely related taxa.

Sequencing of D1/D2regions

The variable domain 2 (D2) from nuclear large subunit ribosomal RNA (LSU rRNA) was initially examined and found to resolve closely related species that had been circumscribed from genetic crosses and DNA reassociation experiments (Peterson and Kurtzman, 1991). This work was expanded to include domains 1 and 2 (D1/D2) and applied to all described species of ascomycetous yeasts, resulting in a diagnostic database (barcode) useful for rapid species identification (Kurtzman and Robnett 1998).Fell et al.(2000) developed a complementary D1/D2 database for known basidiomycetous yeasts. By comparing divergence among ascomycetous strain pairs with previously determined nuclear DNA reassociation values, it appeared that conspecific strains differed by no more than 3 nucleotides among the 500-600 nucleotides of the D1/D2 domains, whereas differences of 6 or more nucleotides (1%) indicated that the strains were different species. One impact of the D1/D2 database has been to permit detection of a large number of new species, which has resulted in a near doubling of known species. Another use is that the non taxonomist can now quickly and accurately identify most known species, as well as recognize new species, by sequencing approximately 600 nucleotides and doing a BLAST search in GenBank.

Sequencing of ITS1-ITS2 regions

The internal transcribed spacer regions ITS1 and ITS2, which are separated by the 5.8S gene of rDNA, are also highly substituted and often used for species identification, but for many species, ITS sequences give no greater resolution than that obtained from 26S domains D1/D2 (James et al. 1996; Kurtzman and Robnett, 2003). However, Fell and Blatt (1999) were able to resolve cryptic

species in the *Xanthophyllomyces dendrorhous* species complex that had been unresolved from D1/D2 sequence analysis, and Scorzetti et al. (2002) reported ITS sequences to provide somewhat greater resolution among many basidiomycetous species than was found for D1/D2, although a few species were less well resolved by ITS than by D1/D2. Consequently, it appears useful to sequence both D1/D2 and ITS when comparing closely related species.

Sequencing of IGS1-IGS2 regions

Of rDNA regions used for species identification, the intergenic spacer (IGS) appears to be the most suited and offers the greatest resolution of closely related species and sub specific lineages. The IGS is comprised of two regions, IGS1 and IGS2, which are often separated by the 5S rRNA gene. IGS sequences have been used to resolve lineages within *Cryptococcus neoformans* and closely related taxa (Diaz and Fell, 2005b, Diaz et al., 2000, 2005, Sugita et al., 2001a). The IGS has also been employed for resolution of closely related species of *Trichosporon* (Diaz and Fell 2004, Sugita et al., 2002), *Mrakia* (Diaz and Fell, 2000) and *Xanthophyllomyces* (Fell and Blatt, 1999; Fell et al., 2007). A characteristic of IGS is the diversity of length polymorphism. Sugita et al. (2001) reported that the IGS1 region among *Trichosporon* species ranged in length from 195 to 704 nucleotides. The IGS region includes a series of multiple repeat units, numerous deletions and insertions (indels).These repeat units and indels provide characteristics for defining strains and species, and may delineate geographical strain distributions (Fell et al., 2007; Libkind et al., 2007). In practice, however, the sequence alignments take considerable time and patience to prepare. Intragenomic sequence heterogeneity is another factor to consider for IGS analysis. Fell et al. (2007) reported sequence heterogeneity in the ITS and IGS regions among certain strains of *Xanthophyllomyces* required cloning prior to sequence analysis. Intragenomic variation in the rDNA spacer regions is not uncommon among fungi, and reports include ITS variation in *Fusarium*

(O`Donnell and Cigelnik ,1997) and IGS variability in hybrids of *Cryptococcus neoformans* (Bovers et al., 2006) which may be used as a tracking tool for investigations on the origin and distribution of strains and species.

Sequencing of genes other than rRNA

A major advantage of rDNA is that it is present in all living organisms, has a common evolutionary origin, occurs as multiple copies and is easy to sequence because primer pairs for conserved regions can generally be used for all organisms. However, gene sequences other than those of the rDNA repeat have been used for separation of species from many kinds of fungi (Geiser et al., 1998; O'Donnell et al., 2000), including the yeasts. Belloch et al. (2000) demonstrated the utility of cytochrome oxidase II for resolution of *Kluyveromyces* species, Daniel et al. (2001) successfully used actin-1 for species of *Candida*, and Kurtzman and Robnett (2003) showed the usefulness of elongation factor 1-α and RNA polymerase II for resolution of *Saccharomyces* species. At present, the main impediment to widespread use of gene sequences other than rDNA is developing sequencing primers that are effective for essentially all species, and construction of databases that include sequences from all known species. Daniel et al. (2001) and Daniel and Meyer (2003) have made considerable progress in development of an actin sequence database for species identification, although no primer set has been effective for all species, thus requiring additional primers to obtain these sequences. The need for multiple primers seems to be a problem common to sequencing of protein encoding genes because of frequent nucleotide substitutions. Resolution of taxa from actin is somewhat greater than from D1/D2, but not surprisingly, clear separation of closely related species is not always certain. Separation of species using single gene sequences is not always reliable. Different lineages may vary in their rates of nucleotide substitution for the diagnostic gene being used, thus confusing interpretation of genetic separation, and hybrids are common and appear to be part of the speciation process. Single

gene sequences are extremely useful for rapid species identification, but, caution in interpretation of species identity is required.

Recognition of species from Multigene analysis

Determination of whether strains are conspecific or members of separate species can be confused by hybridization events, by unexplained sequence polymorphisms and by differences in nucleotide substitution rates. Multigene analyses offer a means for detecting these changes, which would be signalled by lack of congruence for a particular gene tree. This approach was recommended by Goodman (1976) for vertebrates, Dykhuizen and Green (1991) for bacteria and by O`Donnell et al. (2000) for fungi. The paper by Taylor et al. (2000) provides an inclusive review of species concepts and the term Genealogical Concordance Phylogenetic Species Recognition (GCPSR) was introduced to describe the concept of multigene analysis for species recognition. Typing by the analysis of sequences of multiple loci i.e., Multilocus Sequence Typing **(**MLST**)** is an approach based on the amplification and sequencing of inner fragments of housekeeping genes. In the case of yeasts, different ribosomal and or virulence genes have been used with this purpose (Vazquez and Berron, 2004). MLST detects variations occurring in multiple loci by sequencing ca. 500 bp inner fragments of 7 different constitutive genes. For each gene, the different sequences detected within a species are assigned as different alleles and for each isolate the alleles in each of the 7 loci define its allelic profile or sequence type (ST) (Maiden et al., 1998). This typing procedure permits an exact assignation of the different isolates with the additional advantage that DNA sequences are not biased data easily interchanged by different laboratories and databases. MLST was originally described for haploid organisms, but a similar methodology has been used for typing *Candida*, which is diploid. Bougnoux et al. (2002) employed the method

by sequencing inner fragments of 6 different housekeeping genes. Tavanti et al. (2003) also obtained similar results using other housekeeping genes.

Many yeast species have been described almost exclusively from divergence in D1/D2 LSU rRNA gene sequences and/or from ITS sequences. However it is apparent that single gene analyses can lead to incorrect interpretations. Consequently, it appears that in addition to D1/D2 and/or ITS, one or more protein coding gene sequences should be utilized as well. This would also apply to rapid molecular detection method now being widely adopted.

3.1.2.3 Rapid molecular methods for species identification

Rapid molecular-based methods commonly used for species identification include species-specific primer pairs and probes, karyotyping, mitochondrial DNA polymorphisms, restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), micro satellite analysis, short sequence repeats, and amplified fragment length polymorphisms (AFLP).

Peptide Nucleic Acid Probes

Peptide nucleic acid (PNA) probes offer a means for detection and quantitation of species in clinical samples and food products, through fluorescence in situ hybridization (FISH). PNA probes have a peptide backbone to which is attached nucleotides complementary to a species-specific target sequence, and a fluorescent label is added for detection by fluorescence microscopy (Stender et al., 2001). If probes are complementary to rRNA, the whole cell of the target species will "glow" when visualized, which will also allow quantitation by cell counts. An advantage is that sample can be diluted and directly probed. One disadvantage is that probes must be developed for each species of interest, a problem common to most probe technology. PNA technology has been effective for detection of

Dekkera (Brettanomyces) bruxellensis in spoiled wine (Stender et al., 2001) and for detection of *Candida albicans* in blood samples (Rigby et al., 2002).

Karyotyping Electrophoresis Chromosomal length polymorphism

The advent of electrophoretic techniques for separating the intact chromosomal DNA molecules of lower eukaryotes has provided means of characterizing the chromosome sets of these organisms during last two decades. These techniques have provided fundamental new information about the basic organization of the genomes of many species of fungi. These approaches are based on the electrophoretic separation of undigested genomic DNA or comparison of genome macrorestriction patterns obtained by genome digestion with low frequency restriction endonucleases (Lopez-Ribot et al., 2000; Shin et al., 2004; Chen et al., 2001). Application of these protocols permits to obtain species or even strain specific profiles. Comparison of such profiles has been a great advance in the species differentiation within the genera *Candida*, *Saccharomyces*, *Kluyveromyces* and *Zygosaccharomyces*, anamorph- teleomorph relationships between *Candida*, *Kluyveromyces, Pichia* and *Saccharomyces* species, as well as for synonyms verification (Belloch et al., 1997).

Mitochondrial DNA polymorphism (mtDNA)

Yeasts are organisms showing a wide range of variability in the mitochondrial DNA size, from 6 to 25 μm in length. In most yeast species, mitochondrial genome has circular topology. The use of mtDNA in yeast taxonomy has several advantages as: (a) small size, (b) high number of mitochondrial DNA molecules per cell and (c) one single mitochondrial karyotype in each wild dikaryotic isolate (Belloch et al., 1997). For a long time, the main limitation of this technique was the difficult isolation of mitochondrial DNA. In 1990, Querol et al. designed a rapid method to overcome this problem and later, in 2001, this new protocol was slightly modified by López et al. (2001) which is fast and easy to perform. The method permits to analyse the mtDNA without previous isolation and purification requirements. The technique is based on the GC content differences between the

nuclear DNA (nDNA) and the mtDNA, being the GC content around 40% in the former but 20% in the latter. These differences show that when total fungal DNA is digested with restriction enzymes that recognize only GC rich regions, as for example *Msp*I, *Hae*III and *Cfo*I, all with 50% GC target site, the nDNA is over digested giving rise to a high number of short fragments, that are not detected by conventional agarose gel electrophoresis. This characteristic permits to assume that when total DNA digested with these endonucleases is subjected to agarose electrophoresis only the mtDNA fragments will be observed. These fragments will be ordered by size constituting species specific patterns (Fernandez-Espinar et al., 2000; Rycoyska et al., 2004) or even strain specific ones (Sabate et al., 1998).

Restriction Fragment Length Polymorphisms (RFLP)

This technique is based on the differentiation between microorganisms by the comparison of the restriction patterns obtained by digestion of a chosen target DNA with restriction endonucleases. The degree of similarity of the generated patterns allows to establish correlation between species, while the existence of unique patterns permits their use as identification markers. These methods have been successfully used to differentiate between the species of the genera *Candida*, *Cryptococcus* etc. (Esteve-Zarzoso et al., 1999; Sabate et al., 2002; Deak et al., 2004; Pinto et al., 2004).This technique has been successfully applied using the ribosomal DNA region including the intergenic spacers ITS1 and ITS2, and the 5.8S rRNA encoding gene (Kurtzman ,1993; Esteve-Zarzoso et al., 1999).

The 5.8S gene has a highly conserved sequence showing a low intra-specific variability which is not enough to delimit between nonspecific strains. However, the ITS regions, which are non coding hyper variable ones, could permit depending on the case the identification at the intra or interspecific levels. In several studies, this technique is used in combination with PCR *i.e.*, PCR-RFLP.

In this combinative method, specific DNA fragments are amplified by PCR and then, these amplicons are digested with restriction endonucleases to obtain specific patterns (Dendis et al., 2003; Llanos-Frutos et al., 2004).

Random Amplified Polymorphic DNA (RAPD)

This typing system is based on the PCR amplification of genomic DNA on the presence of a single short primer, often 10 nucleotides length. Due to the use of a low annealing temperature (35-39 ºC), a primer is likely to find many sequences within the template DNA to which it can anneal. Depending on the length and complexity of genome of an organism there can be numerous pairs of these sequences .In RAPD, PCR will amplify many random fragments that can vary in size when different species, sub species, populations, or individuals are analysed; and this will constitute the basis of identification. The amplified products are separated and visualized by gel electrophoresis. The use of RAPD permits to obtain the so called fingerprints which are combinations of different numbers of amplicons with different sizes, generating a pattern which is species or even strain specific (Orbera, 2004; Ergon and Gulay, 2005). RAPDs have been used to develop genetic markers within several species and to discriminate between varieties of pathogenic yeasts. By means of this technique, the different *Candida* spp. serotypes have been distinguished (Alonso-Vargas et al., 2000). Perurena et al. (2005) performed the genetic characterization of *C. albicans* strains recovered from the oral cavity of AIDS patients applying the PCR with 3 arbitrary primers (OPA-3, GACA 4 and M13); detecting different amplification patterns with each primer.

Microsatellite analysis

This molecular approach is based on the PCR amplification of fragments using oligonucleotides complementary to single repetitive sequences present in the

target DNA. These repetitive sequences are called microsatellites. Some of the most frequently used are $(GTC)_5$, $(GTG)_5$, $(GACA)_4$ and M13 phage (GAGGGTGGCGGTTCT).This technique differs from RAPD in the use of a higher annealing temperature (55ºC) in microsatellite analysis instead of 37ºC in RAPD. The application of a higher annealing temperature drives a more specific primer hybridization which consequently ensures a higher reproducibility (Botterel et al., 2001; Dalle et al., 2003).

Short sequence repeats (SSRs) and variable number of tandem repeat (VNTR) loci

Short Sequence Repeats (SSRs) are ubiquitous in eukaryotic genomes. Inter SSR (ISSR) fingerprinting is a typing technique developed such that no previous sequence knowledge is required for designing PCR primers. Primers based on a repeat sequence, such as (CA) n, can be made with a degenerate 3'-anchor, such as $(CA)_8RG$ or $(AGC)_6TY$. The resultant PCR reaction amplifies the sequence between two SSRs, yielding a multilocus marker system useful for fingerprinting, diversity analysis and genome mapping.

PCR products are radiolabelled with ^{32}P or ^{33}P *via* end-labelling or PCR incorporation, and separated on a polyacrylamide sequencing gel prior to autoradiographic visualisation. A typical reaction yields 20-100 bands per lane depending on the species and primer. Several investigators have demonstrated that ISSR analysis usually detects a higher level of polymorphism than that detected with Restriction Fragment Length Polymorphism (RFLP) or Random Amplified Polymorphic DNA (RAPD) analyses.

Short sequence repeats (SSRs), potentially representing Variable Numbers of Tandem Repeat (VNTR) loci, were identified for the human-pathogenic yeast species *C. albicans* by computerized DNA sequence scanning. The individual

SSR regions were investigated in different clinical isolates of *C. albicans*. Most of the *C. albicans* SSRs were identified as genuine VNTRs. They appeared to be present in multiple allelic variants and were demonstrated to be diverse in length among nonrelated strains. As such, these loci could provide adequate targets for the molecular typing of *C. albicans* strains. VNTRs encountered in other microbial species sometimes participate in regulation of gene expression and function as molecular switches at the transcriptional or translational level. Interestingly, the VNTRs identified in *C. albicans* often encode polyglutamine stretches and are frequently located within genes potentially involved in the regulation of transcription. DNA sequencing of these VNTRs demonstrated that the length variability was restricted to the CAA/CAG repeats encoding the polyglutamine stretches. For these reasons, paired *C. albicans* isolates of similar genotype, found as non invasive colonizers or encountered in an invasive state in the same individual, were studied with respect to potentially invasion related alterations in the VNTR profiles. However, none of the VNTRs analyzed thus far varied systematically with the transition from colonization to invasion (Van Belkum, 1999).

Amplified fragment length polymorphism (AFLP) analysis

Amplified fragment length polymorphism (AFLP) analysis has been shown to be a reliable method of reproducibly identifying medically important *Candida* species. Serial AFLP analysis of routine surveillance cultures for the identification and epidemiological examination of *Candida* sp. colonization has been successfully assayed (Ball et al., 2004). These findings show that colonization with yeasts during transplantation is a complex and dynamic interaction between the host and the microorganism. AFLP analysis of surveillance cultures have been demonstrated to allow more accurate and informative epidemiological evaluations of pathogenic yeasts. Moreover, genotyping of *Candida* spp. clinical isolates by

AFLP has revealed intraspecific genetic diversity among independent isolates and strain maintenance within patients.

In conclusion, the use of AFLP analysis as an identification method has shown very clear differences among medically important *Candida* species (Alcoba-Flórez et al., 1995). Furthermore, when screening a large collection of clinical isolates previously identified on CHROMagar as *C. albicans*, Alcoba-Flórez et al. (2005) found a misidentification rate of 6%. AFLP analysis is universally applicable, and the patterns can easily be stored in a general, accessible database. Therefore, AFLP might prove to be a reliable method for the identification of medically important *Candida* species.

3.1.2.4 Ascomycetous Yeasts

The distinction between yeasts and dimorphic filamentous fungi has often been uncertain. Some authorities have viewed the yeasts as primitive fungi, whereas others perceived them to be reduced forms of more evolved taxa (Cain, 1972; Red head and Malloch, 1977). Phylogenetic analyses of rDNA sequences demonstrated the ascomycetous yeasts, as well as yeast-like genera such as *Ascoidea* and *Cephaloascus*, to comprise a clade that is a sister group to the "filamentous" ascomycetes (euascomycetes). Some members of the yeast clade, such as certain species of *Ascoidea* and *Eremothecium,* show no typical budding, whereas budding is common among the so-called black yeasts in the genera *Aureobasidium* and *Phialophora*, as well as in certain other dimorphic euascomycete genera. Similarly, vegetative reproduction by fission is shared by *Dipodascus* and *Galactomyces*, members of the yeast clade, as well as by the distantly related genus *Schizosaccharomyces*. Consequently, yeasts cannot be recognized solely on the basis of the presence or absence of budding, but with a few exceptions, ascomycetous yeasts can be separated phenotypically from euascomycetes by the presence of budding or fission and the formation of sexual

states unenclosed in a fruiting body. During the past 10 years, the widespread use of molecular taxonomic methods has resulted in the discovery and description of a large number of new taxa, bringing the total of ascomycetous species to nearly 1,000. Many of these new species are readily detected by sequencing a single species-resolving gene, such as domains D1/D2 of large subunit rDNA. Eventhough only less than 1% of extant species are known and that current sequencing technologies allow rapid detection of new species, the limiting factor for presenting new species is the time required for formal description, which includes information on vegetative and sexual states, fermentation and assimilation reactions, and ecology. From single gene analyses, such as the D1/D2 phylogenetic trees presented by Kurtzman and Robnett (1998), it is apparent that many of the ascomycetous yeast genera are not well circumscribed, but actual boundaries are often not clear. Multigene sequence analyses have been applied to just a few genera, such as those of the "*Saccharomyces* complex", which includes *Saccharomyces*, *Kluyveromyces*, *Tetrapisispora*, *Torulaspora* and *Zygosaccharomyces*, as well as the neighboring genera *Eremothecium*, *Hanseniaspora* and *Saccharomycodes* (Kurtzman and Robnett, 2003). In this multigene study, approximately 80 species were compared from the combined signal of seven genes. The analysis gave 14 phylogenetically defined clades, most of which had strong bootstrap support. From this study, the major genera *Saccharomyces*, *Kluyveromyces* and *Zygosaccharomyces* were shown to be polyphyletic, leading to reclassification of certain of the species in the new genera *Naumovia*, *Nakaseomyces*, *Vanderwaltozyma*, *Zygotorulaspora* and *Lachancea* and expansion of the earlier described genus *Kazachstania* (Kurtzman ,2003). Lineages basal to the branches supporting the 14 clades generally had low bootstrap support, leaving uncertain the genetic relationships among the genera.

On the basis of single gene analyses, species of the *Lipomycetaceae* and genera such as *Yarrowia*, *Citeromyces* and *Saccharomycopsis* appear to be natural

groups. *Metschnikowia*, which is characterized by elongated needle like ascospores, is represented by a large number of phylogenetically divergent species, but molecular data are insufficient to determine if the genus is monophyletic. Consequently, multigene sequence analysis will be required to resolve relationships between the preceding genera as well as for determining relationships within the genera.

A major ecological problem is that estimates indicate that only 1% of the yeast species in nature have been described. Yeast ecology, therefore, is at a stage of discovery. The ability to undertake bio complexity studies, *viz*., environmental/population interactions, is difficult, if the individual species are unknown. The study of yeast populations in the Florida Everglades involves quarterly (seasonal) sampling in subtropical watershed that ranges from freshwater marshes to seawater mangrove habitats. The number of cells ranges from 100 to 2,700 per liter of water. These variations in density correlate with sample location and season of the year. Preliminary data demonstrate that the water samples comprised of 23 genera and 120 species of basidiomycetes and ascomycetes. The undescribed species in these collections represented 54% of the taxa.

Candida

Molecular sequence analysis widely used in different habitats of marine environments helped to categorize different species of eukaryotic microbes especially filamentous fungi and yeasts. ITS rDNA sequencing was carried out to classify yeasts in the coastal waters of Taiwan (Chen et al., 2009), waste water systems (Yang et al., 2011), deep sea sediments (Nagano et al., 2010; Connel et al., 2009; Singh et al., 2010, 11), deep sea hydrothermal vents (Burgaud et al., 2010) and methane seep in Gulf of Mexico (Thaler et al., 2012). However classification of microbes using a multi primer approach is an emerging trend in

diversity studies and is used by various researchers (Singh et al., 2010; Nagano et al., 2010; Burgaud et al., 2010; Gutierrz et al., 2010; Singh et al., 2010, 2011; Thaler et al., 2012) for the accurate identification of filamentous fungi and yeasts from various locations of marine environments.

Debaryomyces

Based on partial sequences of the nuclear large subunit ribosomal (LSU) DNA, Kurtzman & Robnett (1998) studied the phylogeny of ascomycetous yeasts including 15 *Debaryomyces* species. These 15 taxa were separated into four clades, exemplified by the species *D. hansenii*, *D. polymorphus*, *D. melissophilus* and *D. etchellsii*, respectively. The clade represented by *D. hansenii* included five more teleomorph species, *D. nepalensis*, *D. maramus, D. coudertii, D. robertsiae* and *D. udenii,* and is here referred to as the *D. hansenii* clade sensu Kurtzman & Robnett (1998). According to highly similar D1/D2 LSU sequences this clade also includes *D. prosopidis*, a species that resembles *D. hansenii* physiologically. Phaff et al. (1998) distinguished both by the inability of *D. prosopidis* to grow on cellobiose and salicin, low DNA reassociation values and opposed electrophoretic karyotypes.

Since then, it has become more and more obvious that *D. hansenii* is a complex of several, often cryptic, species. Prillinger et al. (1999), based on comparison of random amplified polymorphic DNA (RAPD) patterns, proposed to raise the two varieties of *D. hansenii* to species level, *i.e.,* the reinstatement of *D. hansenii* and *D. fabryi*. Their proposal was supported by the authors of subsequent studies applying many different methods (Groenewald et al., 2008; Nguyen et al., 2009; Jacques et al., 2009; Kurtzman and Suzuki, 2010). Groenewald et al. (2008) after a polyphasic re-examination of numerous strains suggested the reinstatement of *D. subglobosus*, treated among the synonyms of *D. hansenii var. fabryi* (Groenewald et al., 2008). Nguyen et al. (2009) analysed the *Alu*1 fingerprints of

the intergenic spacer (IGS) region of more than 170 *Debaryomyces* strains and described *D. vietnamensis* which belonged to the core group of the genus. Jacques et al. (2009) based on the investigation of the intron sequences of four housekeeping genes and the actin (ACT1) coding gene sequences proposed raising of *Candida famata* var. *flareri* to species level with the reinstatement of *Candida flareri* and the reinstatement of *D. tyrocola* (treated earlier as a synonym of *D. hansenii var. hansenii*). However, this latter species is still close to *D. hansenii* by ACT1 coding sequence comparison alone (Nguyen et al., 2009). Jacques et al. (2009) also described a new species *D. macquariensis* to accommodate the strain CBS 5572. Kurtzman and Suzuki (2010) investigated the combined sequences of the D1/D2 domains of the large subunit and the nearly complete small subunit rRNA genes of ascomycetous yeasts forming coenzyme Q-9. As a result of their analysis they proposed five phylogenetically circumscribed new genera and restricted *Debaryomyces* to those species that are phylogenetically closely related to *D. hansenii*, the type species of the genus. Dlauchy et al. (2011) recovered three yeast strains, which are phenotypically indistinguishable from *Debaryomyces hansenii*, from mineral deposits in the Crystal Eyes Cave, Mountain, Venezuela. Based on the sequence divergence along the nearly entire SSU rRNA gene, the ITS regions and the D1/D2 domains of the LSU rRNA gene confirmed the placement of these strains in the genus *Debaryomyces*, but relationship with all valid species of *D. hansenii* complex was distant. Based on the observed considerable sequence divergence, the three strains were proposed as a new species, *D. psychrosporus*.

Pichia

The genus *Pichia*, which comprises 91 species. (Kurtzman, and Fell 1998) currently represents one of the largest yeast genera and for this reason, rapid, easy and accurate methods to differentiate *Pichia* species are desirable. Recent years have seen a major revolution in yeast identification techniques and rapid

molecular techniques are replacing tedious physiological tests (Kurtzman et al., 2003). Different molecular identification techniques are tried in *Pichia* by several scientists. Some of them include DNA relatedness studies between *Pichia lindneri* and *Hansenula minuta* (Kurtzman, 1984), D1/D2 sequence analysis (Yamada et al., 1994, 1995a, 1995b), indicated the polyphyletic origin of the genus *Pichia* and enabled the reclassification of several *Pichia* spp. into the genera *Starmera* and *Phaffomyces* (Yamada et al., 1997, 1999). RFLP of the 5.8S – ITS rDNA have proved to be very suitable for discriminating between various yeast spp. from different genera (Belloch et al., 1998; Esteve – Zarzoso et al., 1999; Cadez et al., 2002; de Llanos et al., 2004) and environments (Guillamon et al., 1998; Fernandez Espinar et al., 2000; Arias et al., 2002; Villa Carvajal et al., 2004).

3.2 Materials and Methods:

3.2.1. Yeast Strains:

The non pigmented marine yeast isolates identified based on morphological and biochemical characteristics, were subjected to molecular identification following ITS sequencing. These isolates belonged to various genera *viz.*, *Candida* (59.8%), *Debaryomyces* (18) and *Pichia* (5.2%).

3.2.2. Molecular Identification

3.2.2.1 Yeast genomic DNA isolation:

Genomic DNA was isolated from 67 white or cream coloured isolates as per Harju et al. (2004). Pure yeast colonies were inoculated into rich media containing 1% yeast extract, 2% peptone and 2% dextrose (in seawater,30 ppt) and incubated for 18 hrs. Cells from 1.5ml of the overnight cultures were pelleted in a micro centrifuge tube and the cell pellets were resuspended in 200µl of the lysis buffer [2% Triton X-100, 1% SDS, 100mM NaCl, 10mM Tris – HCl (pH 8.0), 1mM EDTA (pH 8.0)]. The tubes were placed in a -80^0C freezer for 2 minutes (until they were completely frozen), then immersed in a 95^0C 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, vortexed 2 minutes and then centrifuged for 3 minutes (20,000xg) at room temperature. The aqueous layer was transferred to a tube containing 400 µl of ice cold 100% ethanol. The samples were allowed to precipitate 5 min at room temperature and then centrifuged at 20,000xg. Supernatant was discarded and DNA pellets were washed with 500µl of 70% ethanol followed by vacuum drying for 5 minutes at 60^{o}C. DNA was resuspended in 20µl TE (10mM Tris and 1mM EDTA, pH 8.0) and stored at 4^{0}C for future use. DNA concentration and purity was assessed spectrophotometrically by comparing absorbance at 260 and 280nm followed by 0.8% agarose gel electrophoresis. Concentration of DNA was found out from the following formula.

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

3.2.2.2 Amplification of ITS sequence

Most molecular yeast identification relies on the amplification and sequencing of the internal transcribed spacer (ITS) region of the yeast genome, which is highly variable among species or even populations of the same species. (Hibbet., 1992; Horton and Bruns, 2001).The ribosomal gene cluster is composed of 3 regions coding for the 5.8S, 18S and 28S ribosomal RNA genes. In yeast the ITS region is typically 650-900 bp in size, including the 5.8S gene. The ITS region was amplified by the universal primer pair V9G and LS266 designed by Hoog and Gerrits (1992).

Primers used:

Forward primer V9G – 5'TTA CGT CCC TGC CCT TTG TA3'

Reverse Primer LS266 – 5'GCA TTC CCA AAC AAC TCG ACTC3'

The Amplification reaction was performed using a DNA thermal cycler (Eppendorf). PCR was performed in a final volume of 25µl containing 2.5µl of 10x PCR buffer, 1.0µl of 10pmol $\mu l^{-1}$of each deoxyribonucleotide primer (V9G and LS266), 2µl of 2.5mM each deoxy ribonucleoside triphosphate, 1µl of the extracted DNA of concentration 600ng/ μl^{-1}, 1 µl of 1U $\mu l^{-1}$of Taq DNA polymerase and 16.5 µl of autoclaved MilliQ water. After an initial denaturation at 95ºC for 2 minutes, amplification was made through 35 cycles, each consisting of denaturation at 94ºC for 45 seconds, annealing at 52ºC for 30 seconds, extension step at 72ºC for 2 minutes and a final extension at 72ºC for 10min. The PCR products were analysed by electrophoresis on 1% agarose gel prepared in 1x TAE buffer and stained with ethidium bromide.

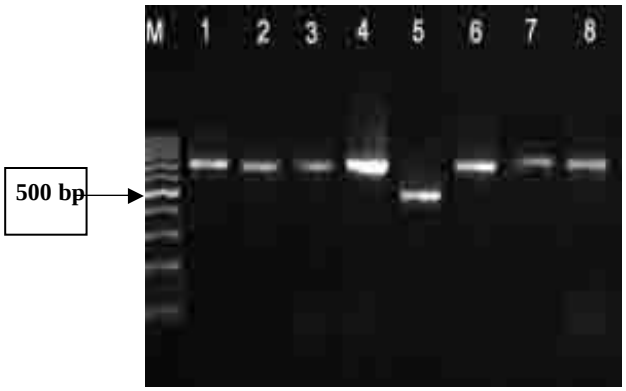
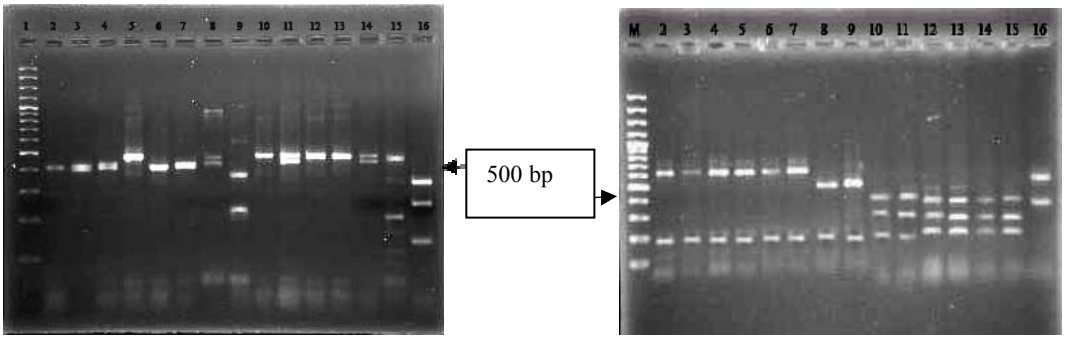


Fig 3.1 Amplified ITS regions of marine yeasts run on 1 % agarose gel

3.2.2.3 Restriction endonuclease digestion and analysis:

PCR products (10 µl or 0.5 – 1.0µg) were digested with 3 restriction endonucleases i.e., *Hinf*l, *Alu* 1 and *Taq*1 (Sigma) in separate reactions. *Alu*1 and *Taq*1 recognise 4-bp sequences and *Hinf*1 is a 5bp cutter. The digestions were performed for 3-4 hrs in 20µl reaction volume containing 8 µl of the PCR product, 2µl buffer (50mM NaCl, 10mM, Tris-Hcl, 10mM MgCl2, 1mM dithiothreitol) 8µl of milliQ and 2µl of each restriction enzymes. The restriction

fragments were separated by horizontal electrophoresis on 2% agarose gel in 1x TAE buffer for 1 hour at 100 volts. A100-bp ladder was used as the DNA marker (Fermentas). All electrophoresis were carried out with 15x7 cm gels on a mini sub cell unit (10x7cms tray with 16 wells). After electrophoresis the gels were stained with ethidium bromide and photographed under transilluminated U-V light. Digitized gel images were analysed to construct RFLP profiles for various yeast strains.



***a. Hin f* 1 *(L2-16)* restriction** **b. *Alu* 1*(*L2-9) *Taq*1(L10-16) restriction**

Fig 3.2 a&b. Different ARDRA patterns of ITS amplicons on 2% agarose gel

3.2.2.4 PCR clean up and Sequencing

The ITS amplicons of the segregated (based on RFLP pattern) yeast isolates were purified using Promega PCR clean-up system as per the manufacturer's instruction. An equal volume of membrane binding solution was added to the PCR product. Inserted SV mini column into collection tube. Transferred the prepared PCR product to mini column assembly. Incubated at room temperature for 1 minute and centrifuged at 16,000xg for 1 minute. Discarded the flow through liquid and inserted mini column into collection tube. Added 700µl membrane wash solution (ethanol added). Centrifuged at 16,000xg for 1 minute. Discarded the flow through liquid and reinserted mini column into collection tube. Repeated the above step with 500 ul membrane wash solution. Centrifuged at

16,000xg 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 mini-column to clean 1.5ml micro-centrifuge tubes. Added 50µl of nuclease free water to the mini column, incubated at room temperature for 1 minute and centrifuged at 16,000xg for 1 minute. Discarded the medium and stored DNA at.-20^{0}C. Nucleotide sequencing was performed using AB1 PRISM 3700 Big Dye Sequencer at M/s SciGenom, Kakkanad.

3.2.2.5 ITS gene sequence similarity & strain identification:

The nucleotide sequences obtained were assembled using Gene Tool software and the sequences were matched with the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al., 1990) at National Centre for Biotechnology Information (NCBI) USA (www.ncbi.nlm.nih.gov) and Centraalbureau voor Schimmelcultures (CBS) Netherlands (www.**cbs**.knaw.nl/ databases). The ITS Sequences were deposited at GenBank (NCBI, USA) The sequences were multiple aligned using the programme Clustal W (Thompson et al. ,1994). Then the ITS-rDNA gene sequence were used to construct a phylogenetic tree using the neighbour-joining (NJ) method (Saitou and Nei, 1987), with MEGA 4.1 package (Tamura et al., 2007). Bootstrap analysis was based on 1000 replicates.

3.2.3 Physiological Characterization of the strains

In the physiological characterization of the strains 94 characters were tested using a Biolog YT microplate™. Biolog YT Microplates are 96 well plates having 12 wells each in 8 rows (A-H). The first 3 rows (A-C) are for oxidation tests and the subsequent 5 rows (D-H) are for assimilation tests. The first well in the row A and D contains water and the observations are compared with A1 for oxidation tests and D1 for assimilation tests.

Among the 96 wells, used in Biolog plate, two wells are for control and 94 wells are for different carbon sources. These carbon sources can be categorised as carbohydrates (18 for oxidation, 31 for assimilation), carboxylic acids (5 each for oxidation and assimilation), amino acids (3 each for oxidation and assimilation), esters (1 for oxidation and 2 for assimilation) alcohols (5 for oxidation, 12 for assimilation), amide (1 for oxidation and 2 for assimilation), aromatic chemicals (1 for oxidation, 2 for assimilation), polymers (1 for oxidation, 2 for assimilation) and brominated chemical (1 for assimilation only) (Fig.3.4).

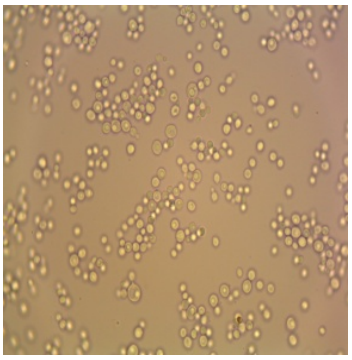


Fig. 3.3 a *Candida oceani*

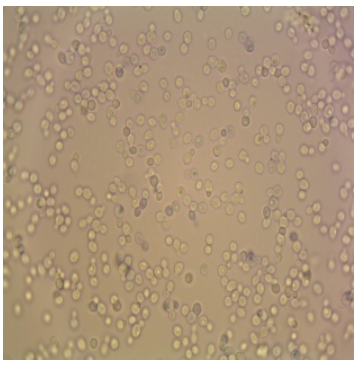


Fig. 3.3 b *Debaryomyces hansenii*

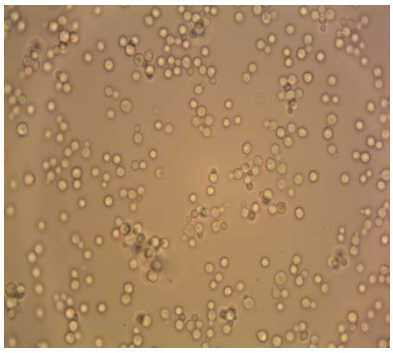


Fig. 3.3 c *Debaryomyces fabryi*

Fig.3.3 a-c. Micromorphology of different species of *Candida and Debaryomyces.*

Representative yeast isolates of each species were inoculated into malt extract agar plates. 48hr old culture was transferred to 15ml sterile sea water; optical density at 590nm was taken using Hitachi UV-2900 spectrophotometer. 100µl of 0.1OD cell suspension was inoculated into each of the 96 wells of Biolog microplate. OD of each of the microwell plates were taken initially, at 72 hrs and after 7 days at 590nm in a Microplate Reader (Tecan i-control). Reactions were scored based on absorbance at 590nm, noticeable absorbance was denoted as positive (+), slight increase in absorbance as borderline (/) and no absorbance as negative (-).

3.3 Results

3.3.1 RFLP Pattern of Yeast ITS Amplicons

The size of the yeast ITS amplicons varied between 800-900 bp in size. Among *Candida,* in species *Candida oceani* and *C. spencermartinsiae*, the amplicons were found to be 900 bp, while it was 800 bp in *C. haemulonii* and 850 bp in *C.tropicalis, C.parapsilosis, C.orthopsilosis* and *C. metapsilosis*. In the species of the genera *Debaryomyces* and *Pichia* there was no apparently significant variation in the size of the amplified products and it was approximately between 850-900bp in size.

The ITS amplicons of yeasts subjected to restriction digestion using *Taq*1, *Alu*1 and *Hinf*l, resulted in polymorphic patterns. Ten types of RFLP patterns were obtained for *Taq*1, nine for *Alu*1 and seven for *Hinf*l. *Taq*1 digested amplicons of all the genera, while *Alu*1 digestion resulted in lesser variation among the yeast isolates. The RFLP patterns produced by the restriction enzymes are given in Table 3.1.

YT MicroPlate BIO LOG

A1 Water	**A2** Acetic acid	**A3** Formic Acid	**A4** Propionic Acid	**A5** Succinic Acid	**A6** Succinic Acid Mono-Methyl Ester	**A7** L-Aspartic Acid	**A8** L-Glutamic Acid	**A9** L-Proline	**A10** D-Gluconic Acid	**A11** Dextrin	**A12** Inulin
B1 D-Cellobiose	**B2** Gentiobiose	**B3** Maltose	**B4** Maltotriose	**B5** D-Melezitose	**B6** D-Melibiose	**B7** Palatinose	**B8** D-Raffinose	**B9** Stachyose	**B10** Sucrose	**B11** D-Trehalose	**B12** Turanose
C1 N-Acetyl-D-Glucosamine	**C2** α-D-Glucose	**C3** D-Galactose	**C4** D-Psicose	**C5** L-Sorbose	**C6** Salicin	**C7** D-Mannitol	**C8** D-Sorbitol	**C9** D-Arabitol	**C10** Xylitol	**C11** Glycerol	**C12** Tween 80
D1 Water	**D2** Fumaric acid	**D3** L-Malic Acid	**D4** Succinic Acid Mono-Methyl Ester	**D5** Bromosuccinic Acid	**D6** L-Glutamic Acid	**D7** γ-Aminobutyric Acid	**D8** α -Keto glutaric Acid	**D9** 2 Keto-D-Gluconic Acid	**D10** D-Gluconic Acid	**D11** Dextrin	**D12** Inulin
E1 D-Cellobiose	**E2** Gentiobiose	**E3** Maltose	**E4** Maltotriose	**E5** D-Melezitose	**E6** D-Melibiose	**E7** Palatinose	**E8** D-Raffinose	**E9** Stachyose	**E10** Sucrose	**E11** D-Trehalose	**E12** Turanose
F1 N-Acetyl-D-Glucosamine	**F2** D-Glucosamine	**F3** α-D-Glucose	**F4** D-Galactose	**F5** D-Psicose	**F6** L-Rhamnose	**F7** L-Sorbose	**F8** α-Methyl-D-Glucoside	**F9** B-Methyl-D-Glucoside	**F10** Amygdalin	**F11** Arbutin	**F12** Salicin
G1 Maltitol	**G2** D-Mannitol	**G3** D-Sorbitol	**G4** Adonitol	**G5** D-Arabitol	**G6** Xylitol	**G7** i-Erythritol	**G8** Glycerol	**G9** Tween 80	**G10** L-Arabinose	**G11** D-Arabinose	**G12** D-Ribose
H1 D-Xylose	**H2** Succinic Acid Mono-Methyl Ester plus D-Xylose	**H3** N-Acetyl-L-Glutamic Acid plus D-Xylose	**H4** Quinic Acid plus D-Xylose	**H5** D-Glucuronic Acid plus D-Xylose	**H6** Dextrin plus D-Xylose	**H7** α-D-Lactose plus D-Xylose	**H8** D-Melibiose plus D-Xylose	**H9** D-Galactose plus D-Xylose	**H10** m-Inositol plus D-Xylose	**H11** 1,2-Propanediol plus D-Xylose	**H12** Acetoin plus D-Xylose

Ester
Carboxylic Acid
Sugars
Polymer
Alcohols
Amide
Aromatic
Amino Acid
Brominated Chemical
Water

Rows A to C Oxidation Test

Rows D to H Assimilation Test

Table 3.1 ARDRA pattern generated with the digestion using restriction enzymes *Hinf* 1,*Taq*1, *Alu*1 on ITS amplicons of various non-pigmented yeasts.

Isolate No.	Species	Size of amplicons	*In vitro* restriction fragment sizes		
			Hinf1	*Taq1*	*Alu1*
R 340	*Meyerozyma guilliermondi*	900	500+480	400+400	700+100
R 28 W	*Candidaspencer martinsiae*	800	500	400+150	700+200
R 89	*Candida oceani*	800	450	500+400	900
R 131	*C. oceani*	800	350+200+100	380+300+200	450+180+150
R 63	*C. haemulonii*	800	350+280	400+225	500
R 399	*C.haemulonii*	800	380+280+150	400+200+100	500+200+100
R 38	*C.metapsilosis*	800	480	400+280+200	600+200
R 56	*C.orthopsilosis*	800	425	400+250+200	600+200
R 302	*C. tropicalis*	800	500	400+125	700+100
R 76	*C. parapsilosis*	800	400+425	400+380+150	600+200
R 88	*C. parapsilosis*	800	400+425	400+280+200	600
R 305	*Debaryomyces nepalensis*	900	500	400+125	500
R 454	*D. nepalensis*	900	450	400+125	500
R 122	*D.subglobosus*	900	500	500+400	600+200
R 424	*D. fabryi*	900	425+450	400+100	800
R 81	*D. fabryi*	900	450	600	600
R100	*D. fabryi*	900	500	400+125	725
R 140	*D. hansenii*	900	500	500+400	700+100

All the 4 spp. of the genera *Debaryomyces* exhibited the same RFLP pattern when treated with the restriction enzyme *Hinf*1 and produced only a single fragment of 500-bp. But when treated with *Taq*1 *D. fabryii* and *D. nepalensis* had 2 fragments

of (400+125bp size) and *D. hansenii* and *D. subglobosus* also had 2 fragments but with different size *i.e*., (500+400bp). On treating with *Alu*1, *D. nepalensis* and *D. fabryii* had only a single fragment, of 500bp and 725bp respectively, while *D. hansenii* and *D. subglobosus* has 2 fragments of (700+100bp) and (600+200bp) in the former and latter respectively (Fig 3.5a-c).

Sequencing of representative isolates having dissimilar RFLP pattern resulted in 12 spp. representing 3 genera *i.e*., *Candida*, *Debaryomyces* and *Meyerozyma* (*Pichia*). Seven species could be identified from the genera *Candida*, four species from genus *Debaryomyces* and only one from *Meyerozyma* or *Pichia* (Table 3.2).

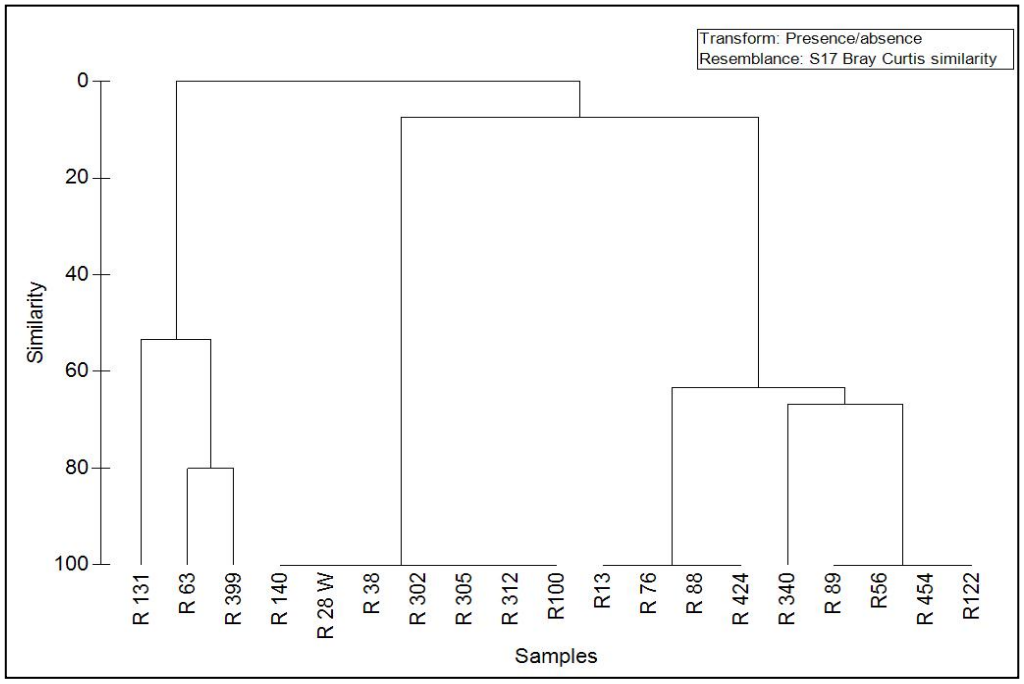


Fig3.5a.Dendrogram showing the clustering of the non pigmented yeast strains into various ARDRA types based onARDRA patterns developedby *Hin f*1

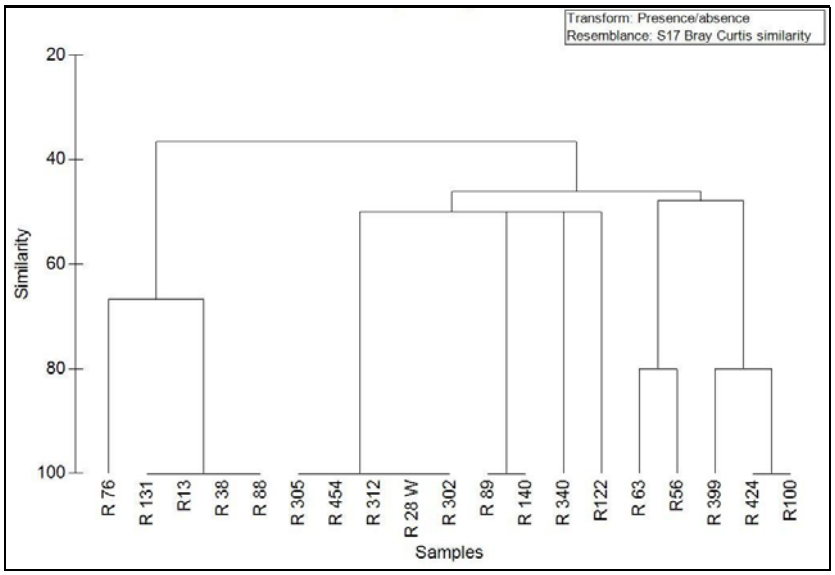


Fig3.5b. Dendrogram showing the clustering of the non pigmented yeast strains into various ARDRA types based onARDRA patterns developedby *Taq*1

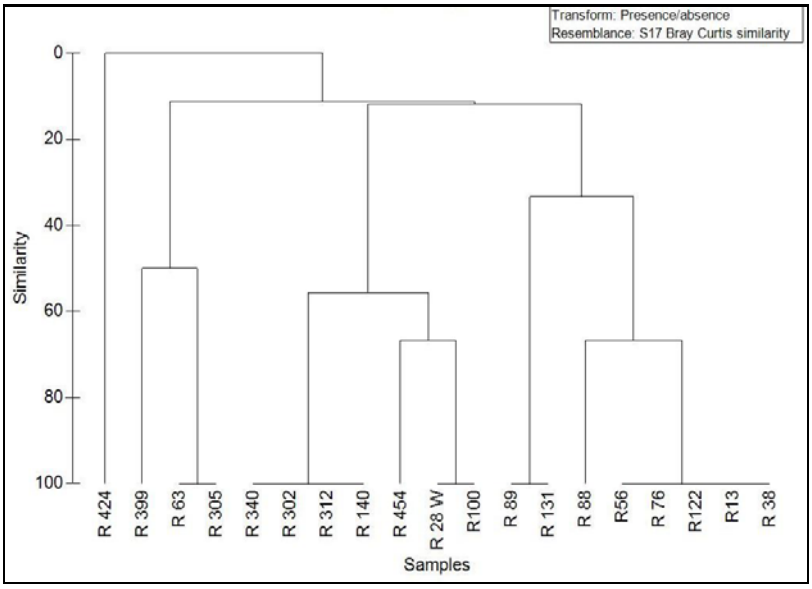


Fig3.5c Dendrogram showing the clustering of the non pigmented yeast strains into various ARDRA types based onARDRA patterns developed by *Alu*1

Table 3.2 NCBI/CBS Blast results

Isolate No.	Accession No.	Reference description	Score	Probability	Similarity (%)	Gaps	Coverage (%)
R13	EU564206 ATCC90018	*Candida parapsilosis*	678	0	100	0	100
	EU564204 , L8367	*Candida parapsilosis*	678	0	100	0	100
	GQ595610 A005	*Candida parapsilosis*	678	0	100	0	100
	EU564202, L8096	*Candida parapsilosis*	678	0	100	0	100
	EU564200	*Candida para psilosis*	L7936	0	100	0	100
R28w	CBS10894 12756910, FJ008050	*Candida spencermartinsiae*	792.48	0	99	0	70.280
	CBS10894 15922341	*Candida spencermartinsiae*	1066.68	0	99.155	0	100
	CBS10893 16078803	*Candida spencermartinsiae*	849.54	0	99.638	0	77.746
	CBS10894 16078815	*Candida spencermartinsiae*	835.27	0	98.913	0	77.746
	EU5263 15920833	*Candida atlantica*	808.33	0	98.54	0	77.606
R89	CBS11857 16074366	*Candida oceanii*	941.952	0	100	0	75.389
	GU 062885	*Candida Sps*	1230	0	100	0	100
	EU 871514	*Candida atlantica*	1040	0	99.75	0	84
	EU 871515	*Candida atlantica*	981	0	100		80
	EU 871513	*Candida atlantica*	972	0	100	0	78
R100	JN942657 JCM9913	*Debaryomyces prosopidis*	1068	0	100	0	86.398
	JN942651 JCM1989	*Debaryomyces subglobosus*	1068	0	100	0	86.398
	JN942672 JCM2104,	*Debaryomyces fabryi*	1068	0	100	0	86.398
	FN598876,	*Debaryomyces hansenii*	1038	0	99	0	86.398

R305	JCM2095	*Debaryomyces nepalensis*	897.089	0	100		80
	CBS5921 16097358	*Debaryomyces nepalensis*	879.65	0	100		78
	CBS7761 16073635	*Debaryomyces nepalensis*	897.089	0	100		78
R140	FR686594	*Debaryomyces hansenii.*	1210	0	100		100
	FN 675240	*Debaryomyces subglobosus*	1210	0	100		100
	JN 851033	*Debaryomyces subglobosus*	1050	0	100		86
	FR 686593	*Debaryomyces hansenii*	1205	0	99		100
R122	FR 686594	*Debaryomyces hansenii*	1206	0	100		100
	JN 675240	*Debaryomyces subglobosus*	1206	0	100		100
	GQ 458025	*Debaryomyces hansenii*	1206	0	100		99
	JN 851033	*Debaryomyces subglobosus*	1044	0	100		86
R340	AB480228	*Pichia guilliermondii*	1373	0	100		99
	AB305098	*Pichia guilliermondii*	1373	0	100		99
	AB160625	*Pichia guilliermondii*	1367	0	100		99
	AB188372	*Pichia guilliermondii*	1362	0	100		99
	AB158922	*Pichia guilliermondii*	1339	0	100		98
R 38	CBS 10907 15922345	*Candida metapsilosis*	900.259	0	99.65	0	100
	AY391849 MCO448	*Candida metapsilosis*	1038.	0	99	0	100
	EU564207, ATCC96143	*Candida metapsilosis*	1037	0	99	0	100
	EU484055	*Candida metapsilosis*	1031	0	99	0	100
	AY391844	*Candida metapsilosis*	1027	0	99	0	100
	EU557371	*Candida metapsilosis*	1022	0	99	0	100
R56	HE681725	*Candida orthopsilosis*	1277	0	99	0	98
	FN812686	*Candida orthopsilosis*	1277	0	99	0	98

	EU484057	*Candida orthopsilosis*	1131	0	99	0	87
	EU557371	*Candida orthopsilosis*	1129	0	99	0	86
R63	CBS5150 196941	*Candida haemulonii*-seawater	614.9	2.027E-175	99.75	0	97.567
	CBS5150 19695	*Candida haemulonii*-seawater	6 14.965	2.027E-175	99.75	0	97.567
	CBS5150 19696	*Candida haemulonii*-seawater	595.946	1.0742E-169	99.75	0	98.054
R399	CBS 5150 19695	*Candida haemulonii - seawater*	664 .099	0	100	0	62.952
	AM231725	*Candida haemulonii-seawater*	586	4e-164	99	0	78
	AM231724 A005	*Candida haemulonii-seawater*	586	4e-164	99	0	78

Of the total 192 isolates, 59.8% belonged to *Candida* which formed the dominant genera in the shelf sediments. Seven *Candida* species *i.e*., *C. haemulonii, C. oceani, C. orthopsilosis, C. metapsilosis, C. tropicalis, C. parapsilosis* and *C. spencermartinsiae* were obtained from the shelf sediments of Arabian Sea and Bay of Bengal, of which *C. oceani* and *C. spencermartinsiae* are reported for the first time from Arabian Sea. *Candida parapsilosis* formed the dominant species among *Candida* spp. and 48.6% of the isolates belonged to *C .parapsilosis*, followed *by C. orthopsilosis* (33.04%), *C. metapsilosis* (1.73%), *C. haemulonii* (2.56%) and *C tropicalis* (2.16%). Both *Candida spencermartinsiae* and *C. oceani* were obtained from 1000m depth. The former off the coast of Trivandrum (Cruise no.254, latitude- N 07 50 04, Longitude -E 76 38) and the latter (*C. oceani*) off the coast of Goa and Kochi. (Cruise no.254, Goa- latitude -N 15 25' 50, Longitude- E 72 37' 17" (Kochi –Latitude- N 95'06", Longitude E -75 29' 06"). Ten isolates (8.69%) belonging to *C. oceani* were obtained from both the stations together in Cruise No.254, of which 6 isolates were from Goa and 4 from Kochi station, while only 3 isolates (2.6%) belonging *C. spencermartinsiae*

was obtained in the present study, all of them were obtained off the coast of Trivandrum.

Restriction enzyme analysis (REA) using *Hinf*1 having two fragments (400+425) itself indicated that the isolate R13 belong to *C. parapsilosis*. On sequencing it had 100% similarity to *Candida parapsilosis* type strains with accession Nos. **CBS 2196, EU 564196, GQ 395610. FJ 872016, CBS 604.**

The isolate R56 which showed a single band (425-bp) with *Hinf*1, on sequencing were identified as *C. orthopsilosis*. It has 100% similarity to the type strain **AJ698048, ATCC 96139**, as well as to the other *C. orthopsilosis* strains having GenBank ID **EU557371** and **EU48057**. *C. metapsilosis* was represented by a single isolate (R38) which is 100% similar to type strain **ATCC 96143**. R38 also showed only a single band with only a *Hinf*1 restriction enzyme. Both *C. orthopsilosis* and *C. metapsilosis* were morphologically indistinct from *C. parapsilosis* and they were identified based on sequence analysis of ITS in the present work. ITS or multilocus sequence analysis was used for the identification of *C. parapsilosis, C. orthopsilosis* and *C. metapsilosis* in a number of previous works (Asad Zadeh et al., 2009; Borman et al., 2009; Johnson, 2009; Tavanti et al., 2005a).

The isolate R63 having 2 fragments (350+280bp) and R399 having 3 fragments with the enzyme *Hinf*1, on sequencing both were 99.7% similar to *Candida haemulonii* isolated from sea water with a query coverage of 97% for R63 and 78% for R399. Lower query coverage is due to V9G primers used for PCR amplification and the sequence of R399 is 664-bp when compared to the sequence given in the GenBank and it is only 379-bp (ID **AM231724, AM231725**). In the phylogenetic tree, both the isolates (R63 and R399), belonged to the same subclade of the major clade A. The other species in the subclade were *Candida pseudohaemulonii* and *Candida auris*.

R302 appeared as a single band (500bp size) with *Hinf*1. Sequences of R302 had 97% similarity to *C. tropicalis*. In the previous reports (Esteve -Zarzoso et al., 1999, Korabecna et al., 2003 and Llanos Frutos et al., 2004) *C. tropicalis* has 2 (240+240) fragments, while Korabecna et al. (2003) also reported 3 strains (1138, 1139, 1140) showing only a single band for *C. tropicalis*.

R28w showed only a single band of 500-bp size (*Hinf*1), and on sequencing, it was found to resemble *C. spencermartinsiae* (**FJ008050**), which have been isolated from coral reefs and hydrothermal vent habitats and it belonged to the *Debaryomyces / Lodderomyces* clade, which includes a cluster of species that includes *C. taylori, C. atlantica* and *C. atmosphaerica*, which have all been isolated from marine habitats. In the phylogenetic tree of R28w, the clade is represented by *C. spencermartinsiae* (**FJ0080050**) R28W and 2 strains of *Candida atlantica* (**AJ39368 and AJ539369**) (Fig.3.6a-b).

Sequences of R89, R131 (single band for R89, 3 fragments for 131 with *Hinf1*) resembled (100%) to *Candida* sp. (**GU0028850**) and also to *C. atlantica* (**EU87154**). *Candida* sp. was later on identified as *C. oceani*. In the phylogenetic tree R89, R131 *C. oceani*, and *C. atlantica*, all forms a single clade. *C. oceani* was first isolated from the mid-atlantic ridge hydrothermal vent by Burgaud et al. (2009). Both *C. spencermartinsiae* and *C. oceani* are first records from the Arabian sea.

The genus *Debaryomyces* is represented by 34 (18%) isolates and they constituted the second dominant genera of the present study. Four species from the genera *Debaryomyces*, i.e., *D. nepalensis*, *D. fabryi*, *D. hansenii* and *D. subglobosus* were identified by sequencing and physiological characterisation. *Debaryomyces hansenii* formed the dominant species among the genus *Debaryomyces*. About 50% of the isolates in the genus *Debaryomyces* belonged to *D. hansenii*, followed by *D. nepalensis* (9 isolates), *D. fabryi* and *D. subglobosus* (4 isolates each). This

is the first record of *D. nepalensis*, from the Bay of Bengal and the 9 isolates were obtained from various stations of Bay of Bengal.

Among the 4 species of *Debaryomyces* obtained in the present study, *D. subglobosus* and *D. fabryi* was obtained only from Arabian sea, whereas *D. nepalensis* was obtained from Bay of Bengal. But *D. hansenii* was obtained both from Arabian sea and Bay of Bengal.

On sequencing the strain R305 had 100% similarity to *D. nepalensis* (**JCM2095, CBS 5929** and **CBS 7761**) with a query coverage of 80%. The strain R100, had 100% similarity to *D. prosopidis*, (**JCM99130**, *D. subglobosus* (**JCM 1989**), *D. fabryi* (**JCM 2104**), and *D. hansenii* (**CBS 787**). Interestingly it has the same query coverage to all these spp. But it was able to grow at 37^0C, but not at 39^0C, hence it is identified as *D. fabryi*. The Isolate R140 also had 100% similarity and 100% query coverage to *D. hansenii* (**FR686594**) and *D. subglobosus* (**FN675240)** and it is 97% similar to *D. fabryi* (**CBS6066**), but its maximum growth temperature was 35^0C, hence it was identified as *D. hansenii*. The strain R122 was 100% similar to *D. hansenii* (FR686594) and *D. subglobsus* (FN675240) but it was able to grow at 39^0C hence its identity was confirmed as *D. subglobosus* (**Fig. 3.5** d,e).

The genera *Pichia* (synonym *Meyerozyma*) was represented by only one species *ie Meyerozyma guilliermondii* and 5% of the total isolates belonged to *P. guilliermondii*. The strain R340, has 99% similarity and 100 % QC coverage to *Meyerozyma guilliermondi* with **AB480228, AB305098.1**,. Both restriction patterns using *Hinf*1 and sequencing results confirms that the strain R340 belong to the species *Pichia guilliermondii*. (Fig 3.7a-b)

```
                    *        20         *        40         *        60         *        80
R105        : CTTAGT-GAGGCCTCCGGATTGGTTTAAAGAAGGGGGCAACTCCATCTTGGAACCGAAAAGCTGGTCAAACTTGGTCATTT :  80
R305        : ---------------------------------------------------------------------------- :   -
R454        : ---------------------------------------------------------------------------- :   -
R51         : --TAGT-GAGGCCTCCGGATTGGTTTAAAGAAGGGGGCAACTCCATCTTGGAACCGAAAAGCTGGTCAAACTTGGTCATTT :  78
R81         : ---------------------------------------------------------------------------- :   -
R372debfab  : ---------------------------------------------------------------------------- :   -
R100        : -TTAGTAGAGGCCTCCGGATTGGTTTAAAGAAGGGGGCAACTCCATCTTGGAACCGAAAAGCTGGTCAAACTTGGTCATTT :  80
R97         : --TAGTAGAGGCCTCCGGATTGGTTTAAAGAAGGGGGCAACTCCATCTTGGAACCGAAAAGCTGGTCAAACTTGGTCATTT :  79
r344        : ---------------------------------------------------------------------------- :   -
r122-3deha  : ---------------------------------------------------------------------------- :   -
R140        : ---------------------------------------------------------------------------- :   -
R312        : ---------------------------------------------------------------------------- :   -
R424        : ---------------------------------------------------------------------------- :   -
R447        : ---------------------------------------------------------------------------- :   -
D.hansenii  : GCTTAGTGAGGCCTCCGGATTGGTTTAAAGAAGGGGGCAACTCCATCTTGGAACCGAAAAGCTGGTCAAACTTGGTCATTT :  81
D.hansenii  : GCTTAGTGAGGCCTCCGGATTGGTTTAAAGAAGGGGGCAACTCCATCTTGGAACCGAAAAGCTGGTCAAACTTGGTCATTT :  81
.D.subglob  : ---------------------------------------------------------------------------- :   -
D.hansenii  : ---------------------------------------------------------------------------- :   -
D.hansenii  : ---------------------------------------------------------------------------- :   -
D.fabryi-A  : ---------------------------------------------------------------------------- :   -
D.nepalens  : ---------------------------------------------------------------------------- :   -
D.nepalens  : ---------------------------------------------------------------------------- :   -
C.psychrop  : ---------------------------------------------------------------------------- :   -
D.renaii-H  : ---------------------------------------------------------------------------- :   -
D.undenii-  : ---------------------------------------------------------------------------- :   -
D.polymorp  : ---------------------------------------------------------------------------- :   -
D.couderti  : ---------------------------------------------------------------------------- :   -

                    *       100         *       120         *       140         *       160
R105        : AGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACAGTATTCTTTTTGCCAGCGCTTA : 161
R305        : ---------------------------------------------------------------------------- :   -
R454        : ---------------------------------------------------------------------------- :   -
R51         : AGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACAGTATTCTTTTTGCCAGCGCTTA : 159
R81         : ---------------------------------------------------------------------------- :   -
R372debfab  : ---------------------------------------------------------------------------- :   -
R100        : AGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACAGTATTCTTTTTGCCAGCGCTTA : 161
R97         : AGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACAGTATTCTTTTTGCCAGCGCTTA : 160
r344        : ------------AGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACAGTATTCTTTTTGCCAGCGCTTA :  69
r122-3deha  : ---------------------------------------------------------------------------- :   -
R140        : ---------------------------------------------------------------------------- :   -
R312        : ---------------------------------------------------------------------------- :   -
R424        : ---------------------------------------------------------------------------- :   -
R447        : -----------------TAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACAGTATTCTTTTTGCCAGCGCTTA :  64
D.hansenii  : AGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACAGTATTCTTTTTGCCAGCGCTTA : 162
D.hansenii  : AGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACAGTATTCTTTTTGCCAGCGCTTA : 162
.D.subglob  : ------------------------------------------------AAGGATCATTACAGTATTCTTTTTGCCAGCGCTTA :  35
D.hansenii  : ---GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACAGTATTCTTTTTGCCAGCGCTTA :  78
D.hansenii  : ------------------------------------------------------------------TGCCAGCGCTT :  11
D.fabryi-A  : ------------------AACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACAGTATTCTTTTTGCCAGCGCTTA :  63
D.nepalens  : ------------------AACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACAGTATTCTTTTTGCCAGCGCTTA :  63
D.nepalens  : ------------------AACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACAGTATTCTTTTTGCCAGCGCTTA :  63
C.psychrop  : ------------------AACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACAGTATTCTTTTTGCCAGCGCTTA :  63
D.renaii-H  : --------------------CAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACAGTATTCTTTTTGCCAGCGCTTA :  61
D.undenii-  : ------------------------------------------TGCGGAAGGATCATTACAGTATTCTTTTTGCCAGCGCTTA :  40
D.polymorp  : ----------------------------------------------ATCATTACAGTATTCTTTTTGCCAGCGCTTA :  31
D.couderti  : ------------------------------------------AAGGATCATTACAGTATTCTTTTTGCCAGCGCTTA :  35
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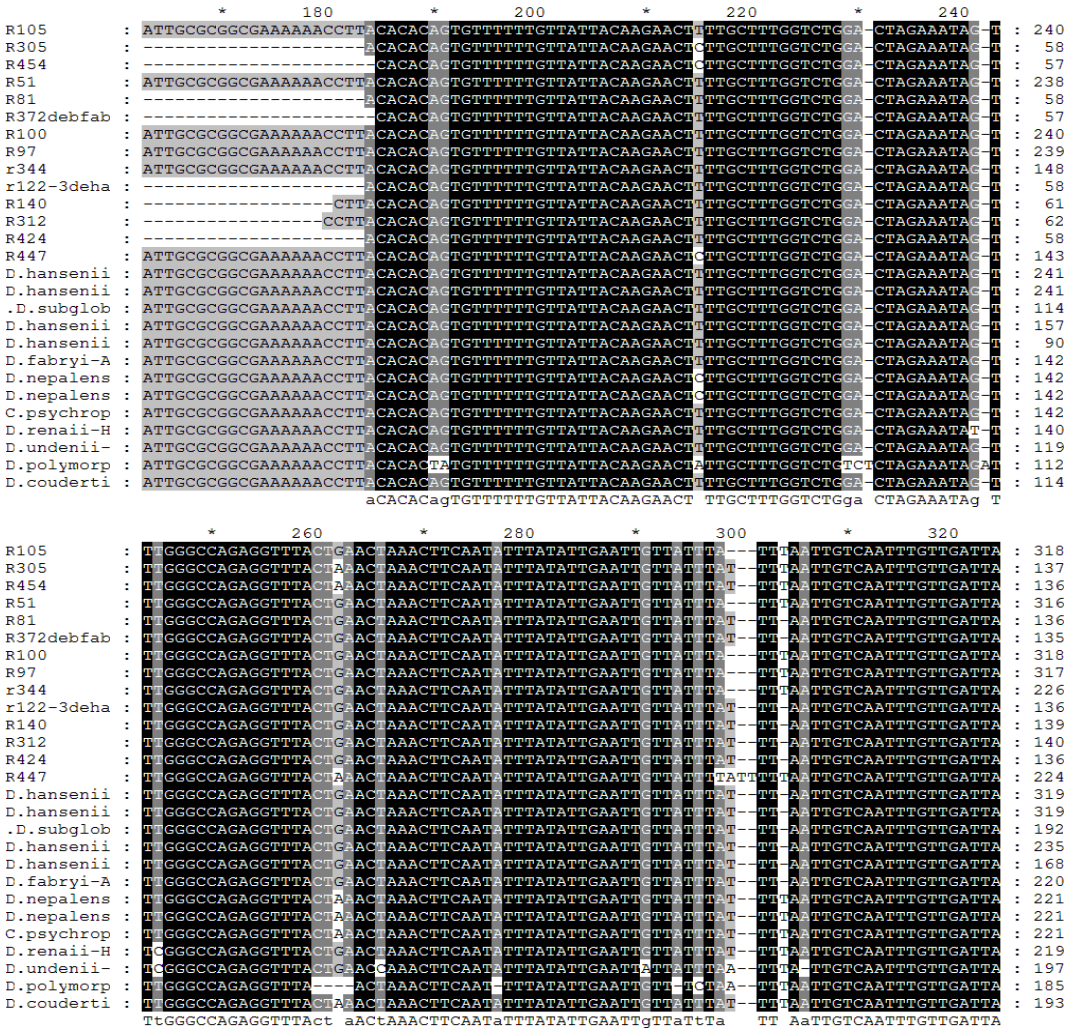
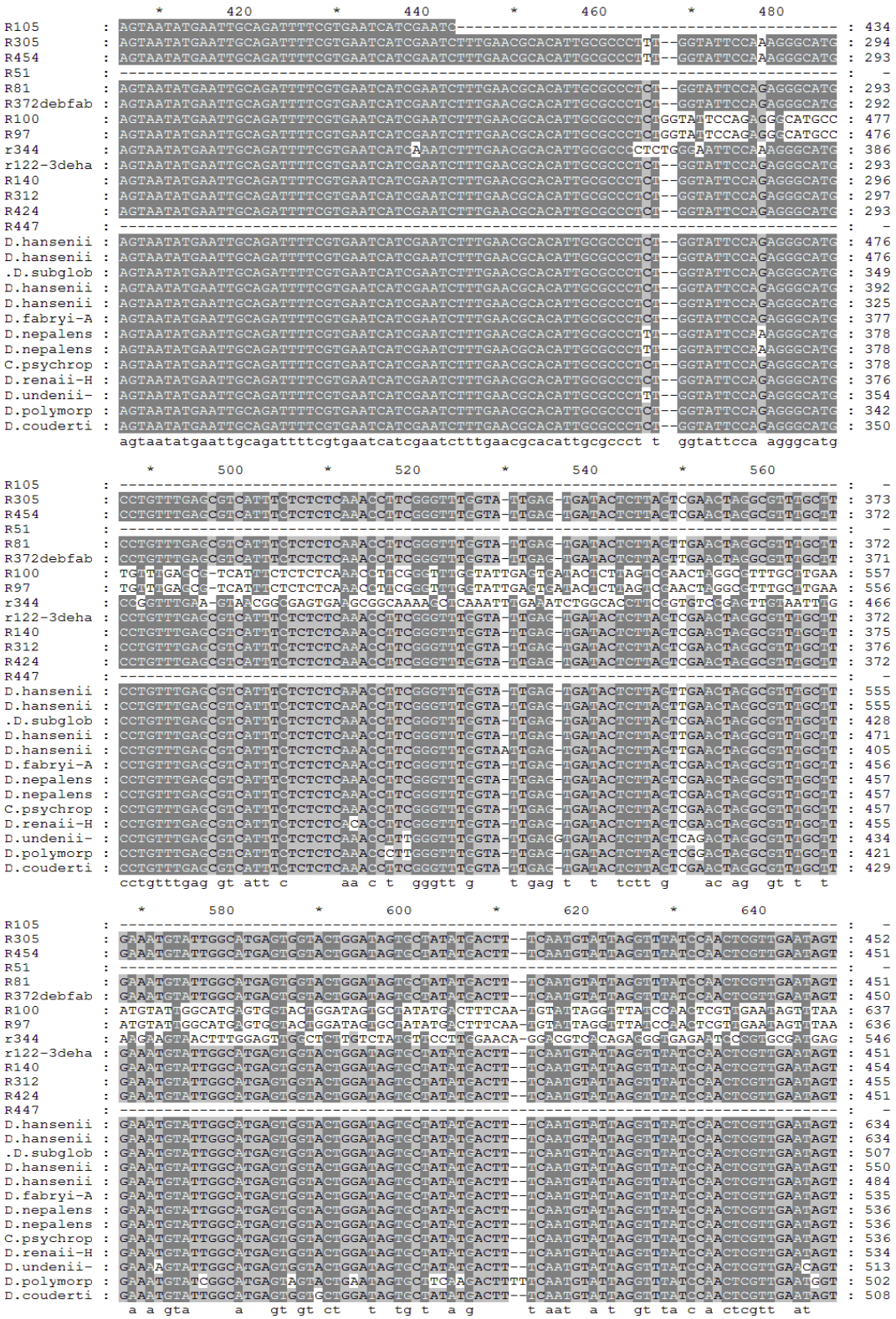




```
              *       660         *       680         *       700         *       720
R105       : --------------------------------------------------------------------------------- :   -
R305       : TTAATGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTTTGACCTCAAATCAGGTAGGACTACC : 533
R454       : TTAATGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTTTGACCTCAAATCAGGTAGGACTACC : 532
R51        : --------------------------------------------------------------------------------- :   -
R81        : TTAATGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTTTGACCTCAAATCAGGTAGGATTACC : 532
R372debfab : TTAATGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTTTGACCTCAAATCAGGTAGGATTACC : 531
R100       : TGGTATATTTCTCGGTATTCTAGG--------------------------------------------------------- : 661
R97        : TGGTATATTTCTCGGTATTCTAGG--------------------------------------------------------- : 660
r344       : ATG------------------------------------------------------------------------------ : 549
r122-3deha : TTAATGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTTTGACCTCAAATCAGGTAGGATTACC : 532
R140       : TTAATGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTTTGACCTCAAATCAGGTAGGATTACC : 535
R312       : TTAATGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTTTGACCTCAAATCAGGTAGGATTACC : 536
R424       : TTAATGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTTTGACCTCAAATCAGGTAGGATTACC : 532
R447       : --------------------------------------------------------------------------------- :   -
D.hansenii : TTAATGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTTTGACCTCAAATCAGGTAGGATTACC : 715
D.hansenii : TTAATGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTTTGACCTCAAATCAGGTAGGATTACC : 715
.D.subglob : TTAATGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTTTGACCTCAAATCAGGTAGGATTACC : 588
D.hansenii : TTAATGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTTTGACCTCAAATCAGGTAGGATTACC : 631
D.hansenii : TTA-TGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTTTGACCTCAAATCAGGTAGGATTACC : 564
D.fabryi-A : TTAATGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTTTGACCTCAAATCAGGTAGGATTACC : 616
D.nepalens : TTAATGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTTTGACCTCAAATCAGGTAGGACTACC : 617
D.nepalens : TTAATGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTTTGACCTCAAATCAGGTAGGACTACC : 617
C.psychrop : TTAATGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTTTGACCTCAAATCAGGTAGGACTACC : 617
D.renaii-H : TTAATGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTTTGACCTCAAATCAGGTAGGACTACC : 615
D.undenii- : TAAATGGTATATTTCCTGGTATTCTAGGCTCGGCCTTACAATACAACAAACAAGTTTGACCTCAAA--------------- : 579
D.polymorp : TTAGTAGTAAATTTTT-AGTATTATTGGCTCGGCCTTACAATACAACAAACAAGTTTGACCTCAAATCAGGTAGGACTACC : 582
D.couderti : TTAATGGTATATTTCTCGGTATTCTAGGCTCGGCCTTACAATATAACAAACAAGTTTGACCTCAAATCAGGTAGGACTACC : 589
             t      t t t   t

             *       740         *       760         *       780         *       800         *
R105       : --------------------------------------------------------------------------------- :   -
R305       : CGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCTTAGTAACGGCGAGTGAAGCGGCAAAA : 614
R454       : CGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCTTAGTAACGGCGAGTGAAGCGGC---- : 609
R51        : --------------------------------------------------------------------------------- :   -
R81        : CGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCTTAGTAACGGCGAGTGAAGCGGCAAAA : 613
R372debfab : CGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCTTA------------------------ : 588
R100       : --------------------------------------------------------------------------------- :   -
R97        : --------------------------------------------------------------------------------- :   -
r344       : --------------------------------------------------------------------------------- :   -
r122-3deha : CGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCTTAGTAACGGCGAGTGAAGCGGCAAAA : 613
R140       : CGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCTTAGTAACGGCGAGTGAAGCGGCAAAA : 616
R312       : CGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCTTAGTAACGGCGAGTGAAGCGGCAAAA : 617
R424       : CGCTGAACTTAAGCATATCAATAAGC------------------------------------------------------- : 558
R447       : --------------------------------------------------------------------------------- :   -
D.hansenii : CGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCTTAGTAACGGCGAGTGAAGCGGCAAAA : 796
D.hansenii : CGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCTTAGTAACGGCGAGTGAAGCGGCAAAA : 796
.D.subglob : CGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCTTAGTAACGGCGAGTGAAGCGGCAAAA : 669
D.hansenii : CGCTGAACTTAAGCATATCAATAAGCGGAGG-------------------------------------------------- : 662
D.hansenii : CGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCTTAGTAACGGCGAGTGAAGCGGCAAAA : 645
D.fabryi-A : CGCTGAACTTAAGCATATCAATAAGCGGAGGA------------------------------------------------- : 648
D.nepalens : CGCTGAACTTAAGCATATCAATAAGCGGAGGA------------------------------------------------- : 649
D.nepalens : CGCTGAACTTAAGCATATCAATAAGCGGAGGA------------------------------------------------- : 649
C.psychrop : CGCTGAACTTAAGCATATCAATAAGCGGAGGA------------------------------------------------- : 649
D.renaii-H : CGCTGAACTTAAGCATATCAT------------------------------------------------------------ : 636
D.undenii- : --------------------------------------------------------------------------------- :   -
D.polymorp : CGCTGAACTTAA--------------------------------------------------------------------- : 594
D.couderti : CGCTGAACTTAA--------------------------------------------------------------------- : 601
```

```
                820         *       840         *       860         *       880         *
R105        : ---------------------------------------------------------------------------------------- :   -
R305        : GCTCAAATTTGAAATCTGGCGCCTTCGGTGTCCGAGTTGTAATTTGAAGAA-GGTAACTTTGGAGTTGGCTCTTG-TCTAT : 693
R454        : ---------------------------------------------------------------------------------------- :   -
R51         : ---------------------------------------------------------------------------------------- :   -
R81         : GCTC------------------------------------------------------------------------------------ : 617
R372debfab  : ---------------------------------------------------------------------------------------- :   -
R100        : ---------------------------------------------------------------------------------------- :   -
R97         : ---------------------------------------------------------------------------------------- :   -
r344        : ---------------------------------------------------------------------------------------- :   -
r122-3deha  : GCTCAAATTTGAAATCTGGCACCTTCGGTGTCCGAGTTGT------------------------------------------------ : 653
R140        : GCTCAAATTTGAAATCTGGCACCTTCGGTGTCCGAGTTG------------------------------------------------- : 655
R312        : GCTCAAATTTGAAATCTGGCACCTTCGGTGTCCGAGTTGTAATTTGAAGAAAGGTAACTTTGGAGTTGGCTCTTG-TCTAT : 697
R424        : ---------------------------------------------------------------------------------------- :   -
R447        : ---------------------------------------------------------------------------------------- :   -
D.hansenii  : GCTCAAATTTGAAATCTGGCACCTTCGGTGTCCGAGTTGTAATTTGAAGAA-GGTAACTTTGGAGTTGGCTCTTG-TCTAT : 875
D.hansenii  : GCTCAAATTTGAAATCTGGCACCTTCGGTGTCCGAGTTGTAATTTGAAGAA-GGTAACTTTGGAGTTGGCTCTTG-TCTAT : 875
.D.subglob  : GCTCAAATTTGAAATCTGGCACCTTCGGTGTCCGAGTTGTA------------------------------------------- : 710
D.hansenii  : ---------------------------------------------------------------------------------------- :   -
D.hansenii  : GCTCAAATTTGAAATCTGGCACCTTCGGTGTCCGAGTTGTAATTTGAAGAA-GGTAACTTTGGAGTTGGCTCTTGGTCTAT : 725
D.fabryi-A  : ---------------------------------------------------------------------------------------- :   -
D.nepalens  : ---------------------------------------------------------------------------------------- :   -
D.nepalens  : ---------------------------------------------------------------------------------------- :   -
C.psychrop  : ---------------------------------------------------------------------------------------- :   -
D.renaii-H  : ---------------------------------------------------------------------------------------- :   -
D.undenii-  : ---------------------------------------------------------------------------------------- :   -
D.polymorp  : ---------------------------------------------------------------------------------------- :   -
D.couderti  : ---------------------------------------------------------------------------------------- :   -
```

```
                900
R105        : ------------ :   -
R305        : GTTCCTTGGAAC : 705
R454        : ------------ :   -
R51         : ------------ :   -
R81         : ------------ :   -
R372debfab  : ------------ :   -
R100        : ------------ :   -
R97         : ------------ :   -
r344        : ------------ :   -
r122-3deha  : ------------ :   -
R140        : ------------ :   -
R312        : GTTCC------- : 702
R424        : ------------ :   -
R447        : ------------ :   -
D.hansenii  : GTTCCTTGGAAC : 887
D.hansenii  : GTTCCTTGGAAC : 887
.D.subglob  : ------------ :   -
D.hansenii  : ------------ :   -
D.hansenii  : GTTCCTTGGAAC : 737
D.fabryi-A  : ------------ :   -
D.nepalens  : ------------ :   -
D.nepalens  : ------------ :   -
C.psychrop  : ------------ :   -
D.renaii-H  : ------------ :   -
D.undenii-  : ------------ :   -
D.polymorp  : ------------ :   -
D.couderti  : ------------ :   -
```

Fig 3.5d. Clustal W multiple alignment of the ITS Sequences from *Debaryomyces* strains isolated during the present study and phylogenetically related strain sequences from NCBI

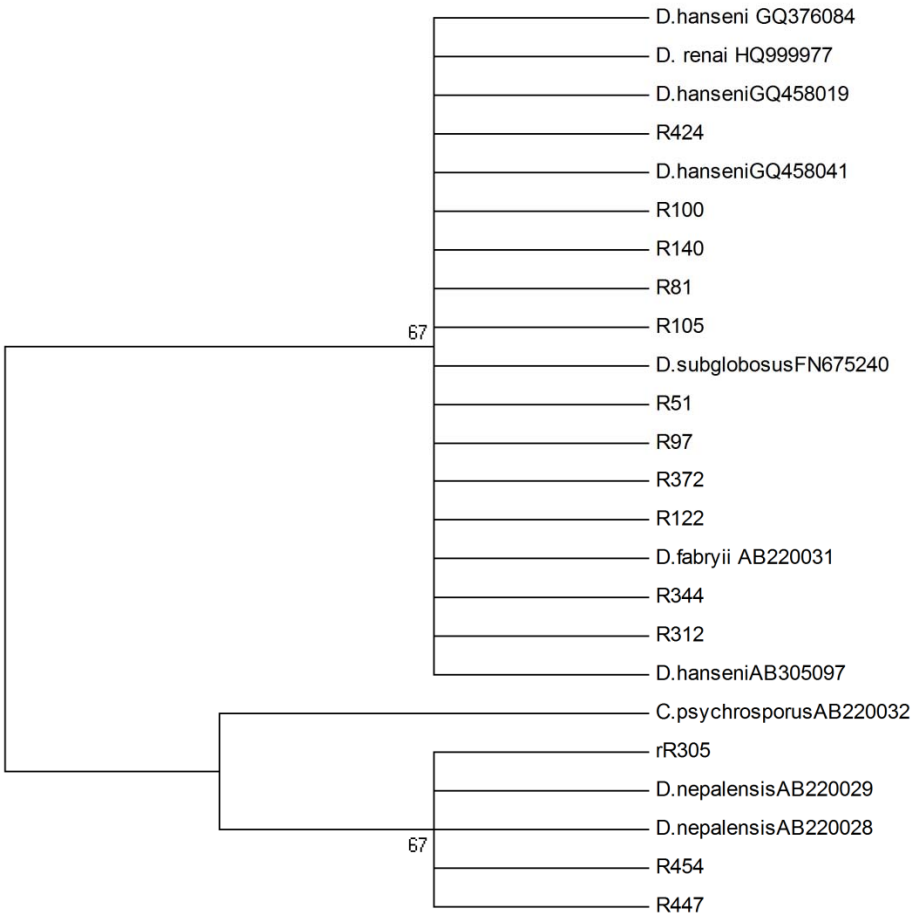
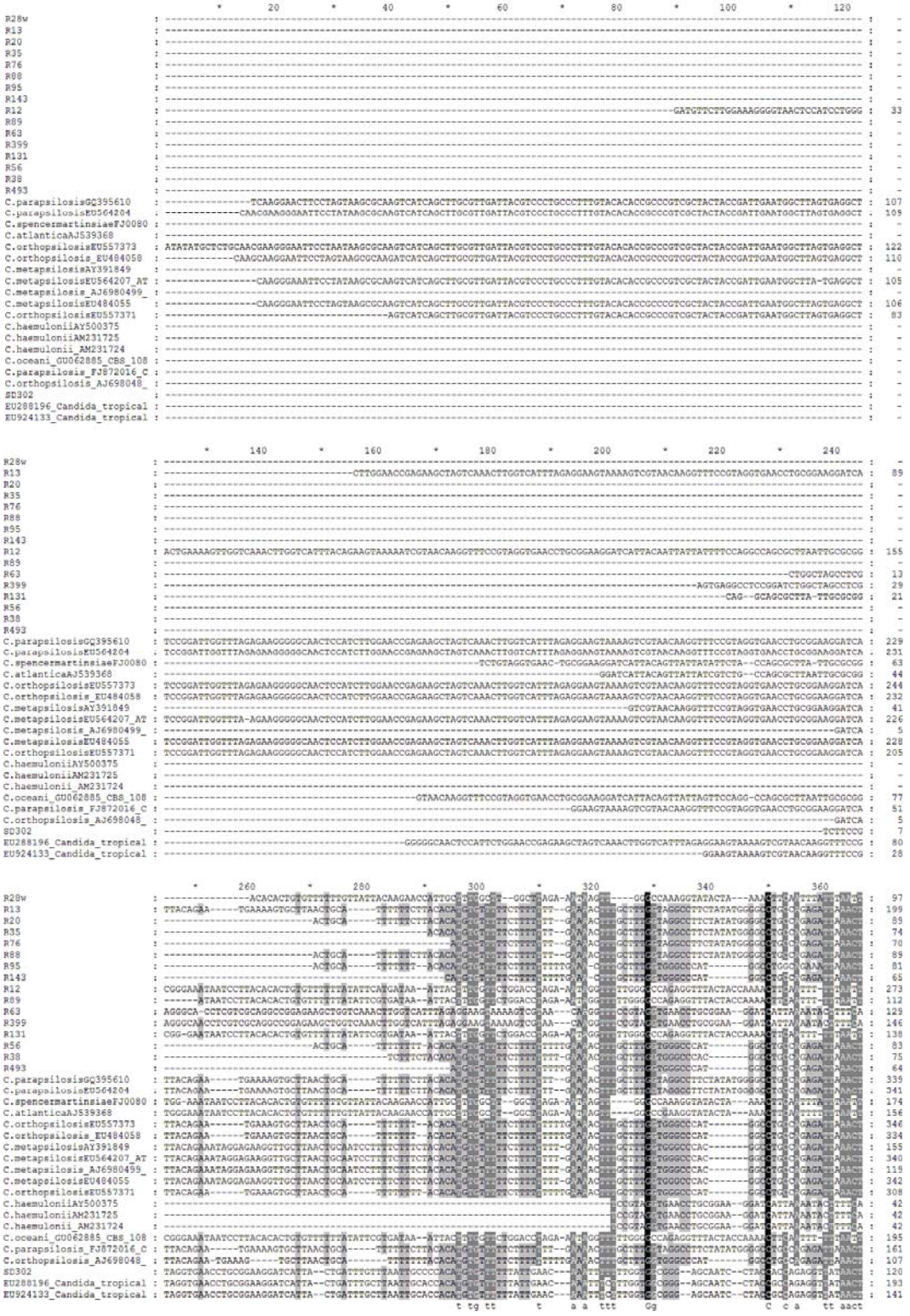
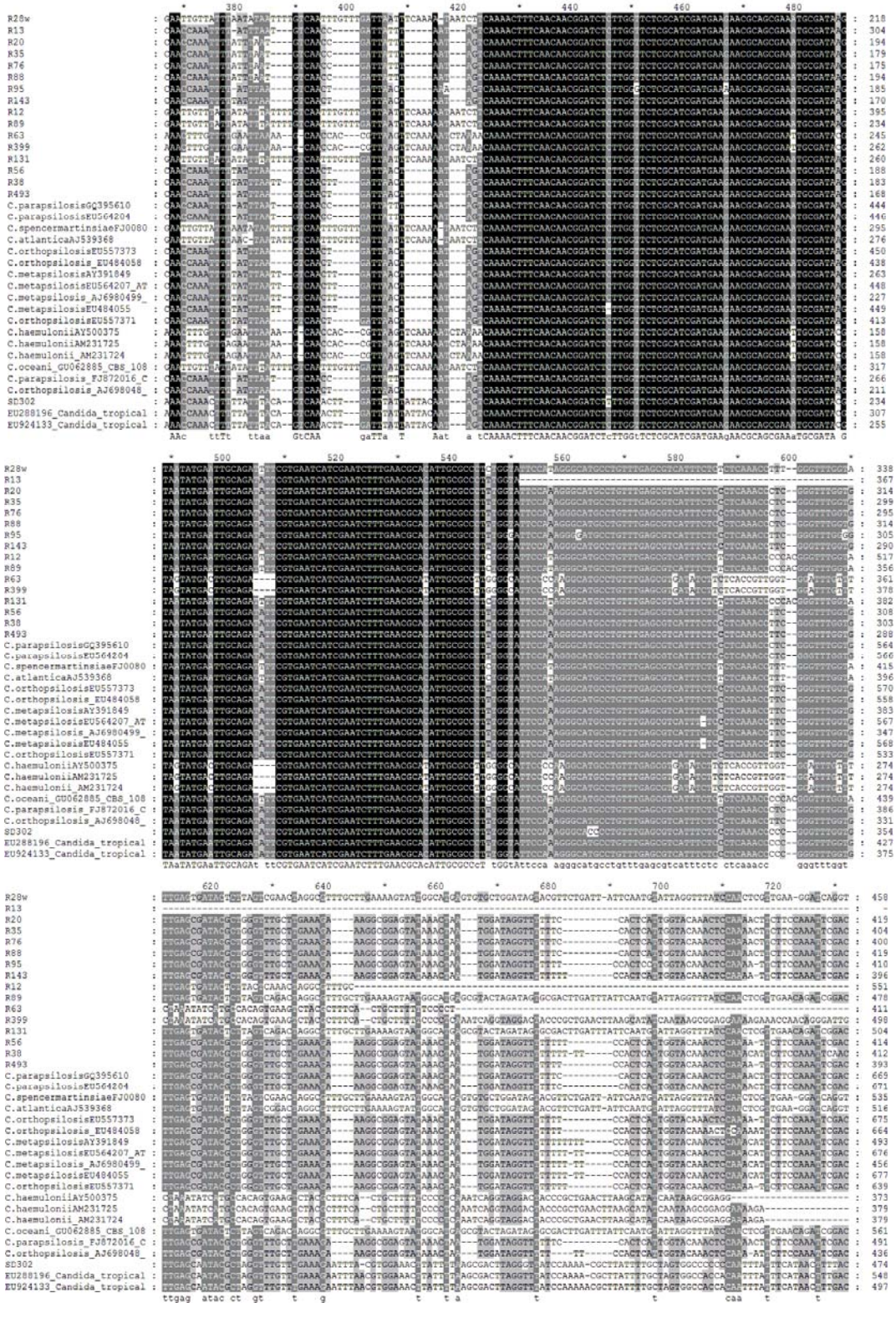


Fig 3. 5e Neighbour-Joining phylogenetic tree based on ITS sequences, showing the relationships among the species of the genus *Debaryomyces*. Bootstrap values are shown as percentages from 1000 replications as branch points




R28w
R13
R20
R35
R76
R88
R95
R143
R12
R89
R63
R399
R131
R56
R38
R493
C.parapsilosisGQ395610
C.parapsilosisEU564204
C.spencermartinsiaeFJ0080
C.atlanticaAJ539368
C.orthopsilosisEU557373
C.orthopsilosis_EU484058
C.metapsilosisAY391849
C.metapsilosisEU564207_AT
C.metapsilosis_AJ6980499_
C.metapsilosisEU484055
C.orthopsilosisEU557371
C.haemuloniiAY500375
C.haemuloniiAM231725
C.haemulonii_AM231724
C.oceani_GU062885_CBS_108
C.parapsilosis_FJ872016_C
C.orthopsilosis_AJ698048_
SD302
EU288196_Candida_tropical
EU924133_Candida_tropical

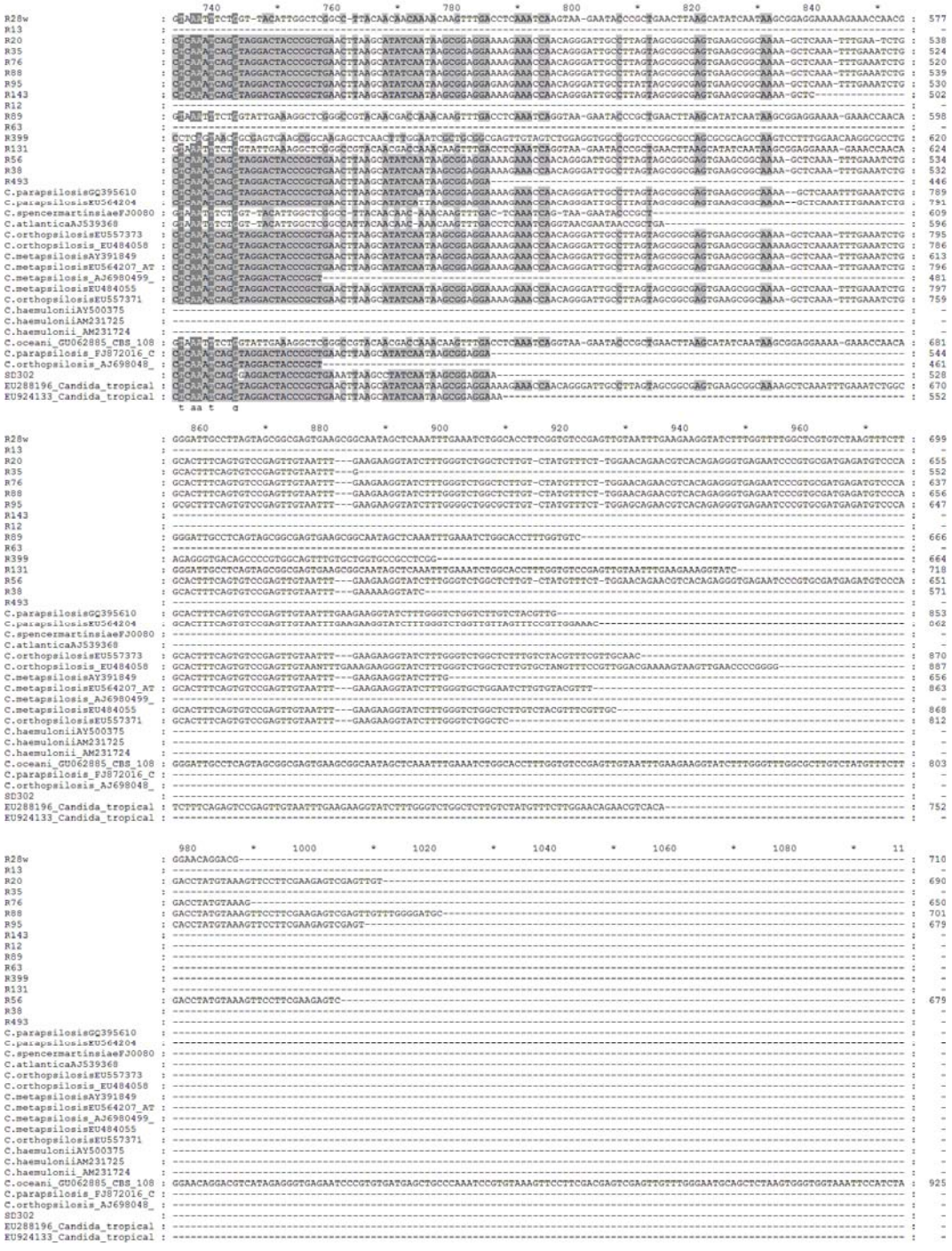
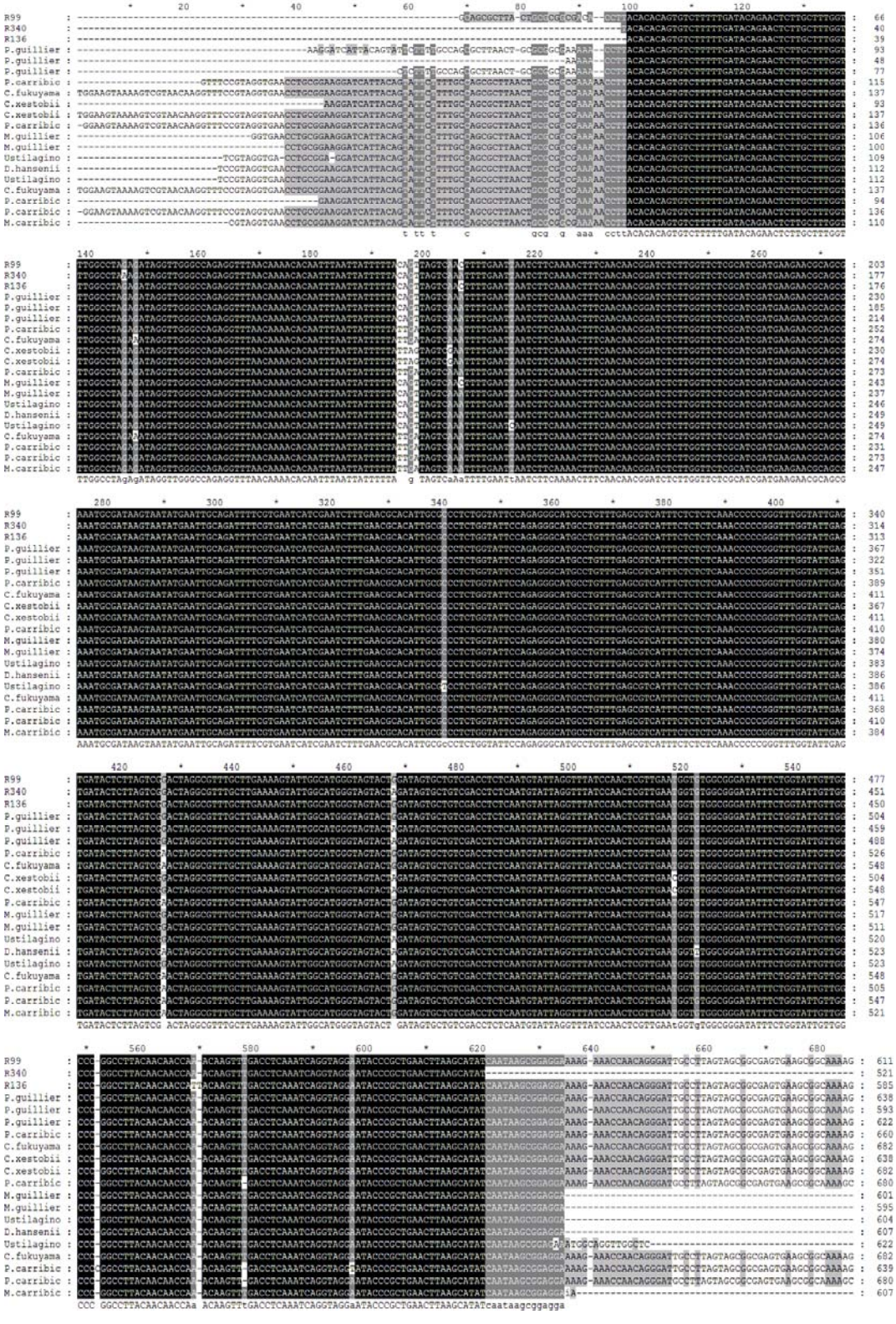


Fig 3.6a. Clustal W multiple alignment of the ITS Sequences from *Candida* strains isolated during the present study and phylogenetically related strain sequences from NCBI



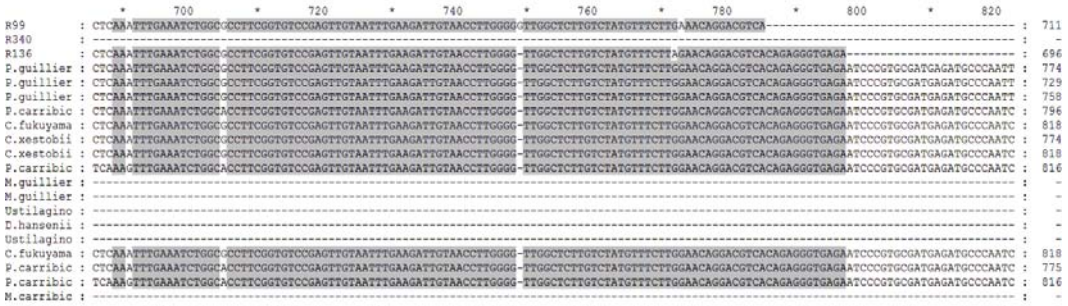


Fig 3.7a Clustal W multiple alignment of the ITS sequences from *Pichia* strains isolated during the present study and phylogenetically related strain sequences from NCBI

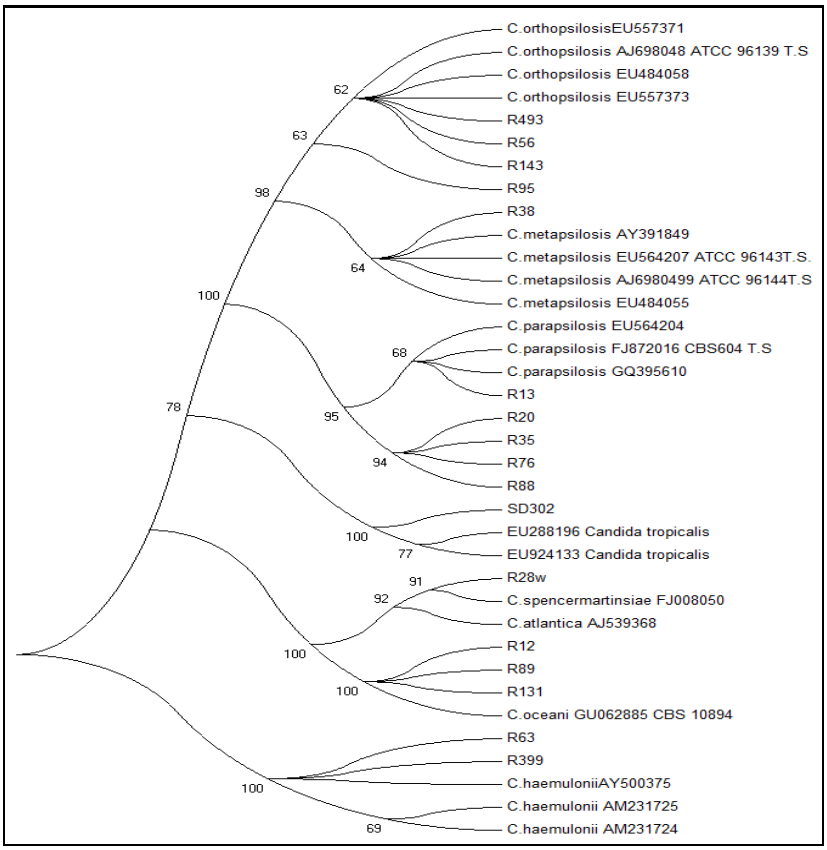


Fig 3.6b Neighbour-Joining phylogenetic tree based on ITS sequences, showing the relationships among the species of the genus *Candida*. Bootstrap values are shown as percentages from 1000 replications as branch points.

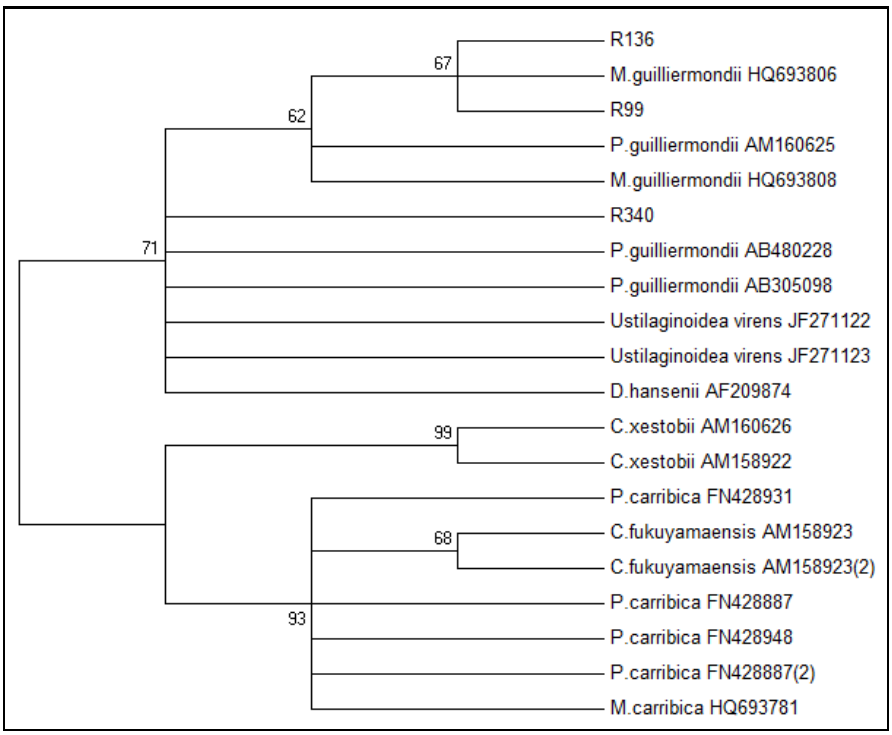


Fig 3.7b Neighbour-Joining phylogenetic tree based on ITS sequences showing the relationships among the species of the genus *Pichia*. Bootstrap values are shown as percentages from 1000 replications as branch points.

3.3.2 Physiological characterization

In the different species of *Candida*, maximum percentage of positive wells is exhibited by R56 (*Candida orthopsilosis*) and the minimum percentage of positive wells is shown by R302 (*Candida tropicalis*) but the maximum percentage of active growth is exhibited by R302 *i.e.*, (72.34%), followed by R56 (54.26%), R89 (48.93%), R38 (46.88%), R13 (45.74%), R28W (43.54%) and the lowest by R399 (41.49%). In the different species of the genus *Debaryomyces* highest % of the positive wells (95.74) as well as wells with active growth (90.45%) was found in the strain R122 (*D. subglobosus*) and the lowest % of positive wells (63.82) as well as the wells with active growth (17.02%) can be seen for the strain R140 (*D. hansenii*). Unlike the genera *Candida*, in the different species of genera

Debaryomyces various carbon sources oxidised /assimilated varied between 17.02% (*D. hansenii*) to 90.45 % (*D. subglobosus*). (Fig.3.8a-b). Among the 94 carbon sources used in Biolog YT microplate 30 of these characters were comparable with type strains. The reactions shown by different strains are given below.

Candida parapsilosis (R13: Acc. No. **JQ665413.1**): 86.6% of characters were similar to the type strain CBS 604 .The carbon sources inulin, cellobiose, salicin and erythritol showed a weak reaction in our study, but in the type strain showed a positive reaction. Except (6.23%) sugars and alcohols (11.8%), all carbon sources used in the well plate were assimilated by *C. parapsilosis* (Fig 3.9a).

C. metapsilosis (R 38: Acc. No. **JQ665419.1**): 90 % of characters were similar to the type strain ATCC 96144 (Tavanti et al., 2005a). The carbon sources the D-raffinose showed a weak reaction and D- glusosamine shows a positive reaction in our study. But both were negative in type strain. The sugar L-sorbose was negative in our study but positive in type strain. The strain R 38 was unable to oxidize sugars (44.5%), carboxylic acids (60%) and aromatic chemicals. It was also unable to assimilate sugars (22.6%), alcohols (8.4%) and aromatic chemicals (Fig 3.9 b).

C. orthopsilosis (R 56: Acc. No. **JQ665418.1**): 86.6% of characters were similar to the type strain ATCC 96139 (Tavanti et al.,2005a).The carbon sources D-glucosamine, L rhamnose, salicin and D- ribose showed a weak reaction in our study but positive in type strain. It was able to oxidize/assimilate all carbon types used in well plate, but only 54.26 % with active growth. *(*Fig 3.9 c)

C. spencermartinsiae (R28w: Acc. No. **JQ665412.1**): 80 % of characters were similar to type strain CBS10894 (Stadzell-Tallman et al.2010). D- melibiose and D- raffinose showed a weak reaction in our study but negative in type strain.

Salicin exhibited a negative reaction in our study but it showed a weak reaction in type strain. This strain was unable to oxidize (11.2%) and assimilate (6.6%) sugars and 50% of aromatic chemicals (Fig 3.9 d).

C. oceani (R 89: Acc. No. **JQ665415.1**): All the characters except D- arabinose were similar to the type strain CBS 53072. The type strain was positive for D-arabinose but our strain was negative. R89 was unable to oxidize sugars (3.3%) and carboxylic acids (40%) (Fig 3.9 e).

C. haemulonii (R 399: Acc. No. **JQ665417.1**): 86.6% of the characters were similar to the type strain CBS5149. D- Melibiose, erythritol and L – arabinose exhibited a weak reaction in our study but negative in type strain. It was unable to oxidize sugars (22.3%), carboxylic acids (60%) and alcohols (40% each). It was unable to utilize 32.3% of sugars (Fig 3.9f).

C. tropicalis (R 302): 80% of the characters were similar to the type strain CBS 94. The carbon sources D- Melibiose, D- raffinose, L- rhamnose, L- arabinose showed a positive reaction in our study but negative in type strain. D-arabinose exhibited a weak reaction and glycerol is positive in Biolog plates but glycerol stimulated a variable result and L- arabinose was negative for type strain. It was also unable to assimilate 9.7% of sugars (Fig 3.9 g).

Debaryomyces hansenii (R140: Acc. No. **JQ665432.1**): The carbon sources inulin, D- gluconic acid, L- rhamnose and L- sorbose induced a negative reaction in R140 but they were variable in type strain CBS 767 and it showed 86.6% similarity to the type strain (Fig 3.9 h).

D. subglobosus (R122): 90% of characters were similar to the type strain CBS792. Unlike the type strain, R122 was unable to ferment the sugars sucrose and raffinose (Fig 3.9 i).

D.nepalensis (R305:Acc. No. **JQ665426.1**): Except for D-arabinose and D-ribose all the carbon sources showed same type of reactions for both R305 and the type strain CBS 5921. At the same time in oxidation/ fermentation reaction *D. nepalensis* was found to be oxidative for glucose, galactose, sucrose, and maltose but the type strain CBS 5921 showed weak fermentation for glucose, sucrose, and maltose (Fig 3.9 j).

D. fabryi (R100: Acc. No. **JQ665429.1**): 93.3% of characters were similar to the type strain CBS789. The carbon sources D-Melibiose, D-cellobiose, L- rhamnose, L- arabinose stimulated a positive reaction in our study but negative in type strain. The strain R100 was characterised by the inability to oxidise any of the aminoacids, amides, aromatic chemical, polymer and the brominated chemical used in the well plate (Fig 3.9 k).

Pichia guilliermondii (R340:): 90% of the characteristics were similar to the type strain CBS 2030 (Kurtzman and Suzuki, 2010). The strain R340 in the present study was unable to assimilate glycerol and ferment galactose, sucrose while the type strain fermented both of them and assimilated glycerol (Fig. 3.9 l, Appendix Table 3.2, 3.3)

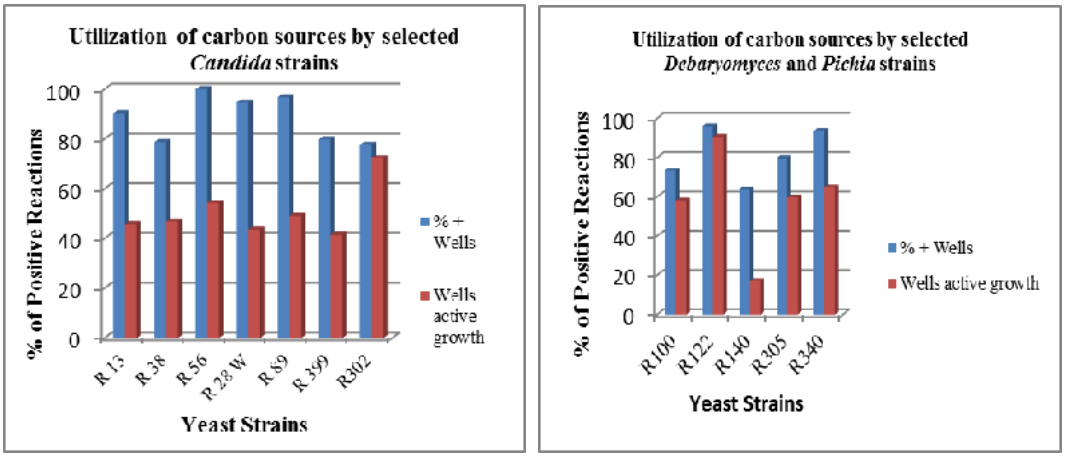


Fig.3.8a-b. Percentage Utilization of Carbon sources by various species of *Candida, Debaryomyces* and *Pichia*

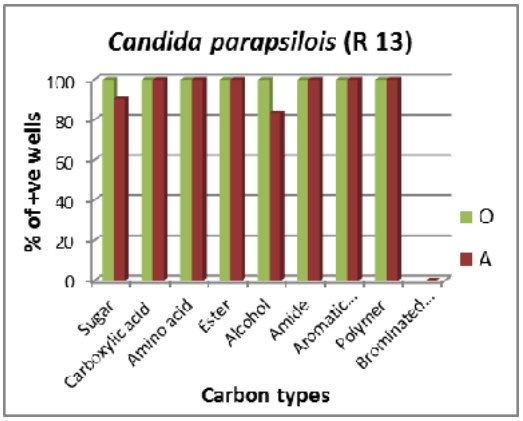


Fig 3.9a

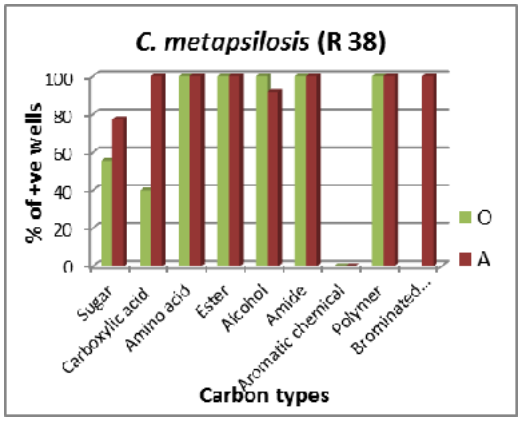


Fig 3.9b

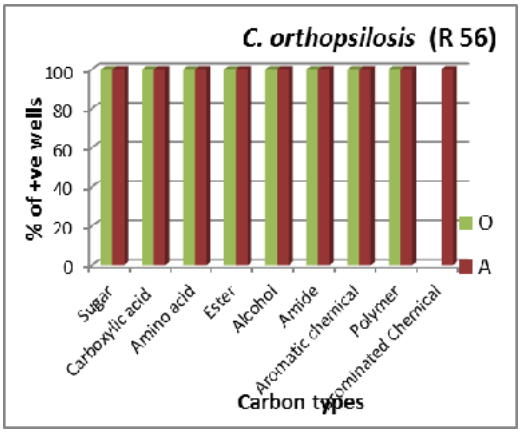


Fig 3.9c

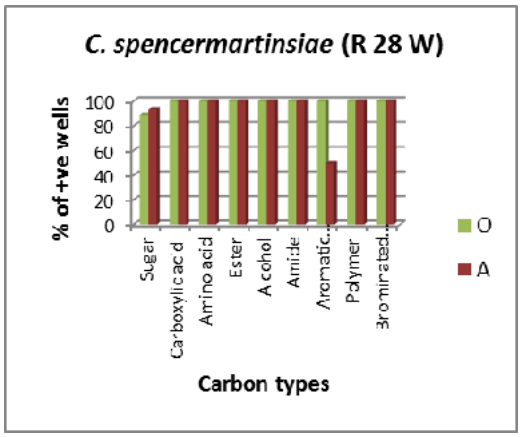


Fig 3.9d

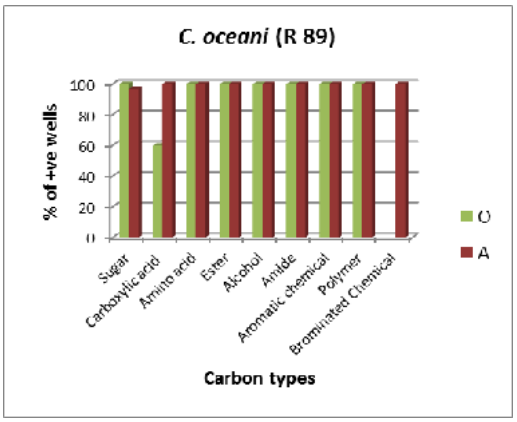


Fig 3.9e

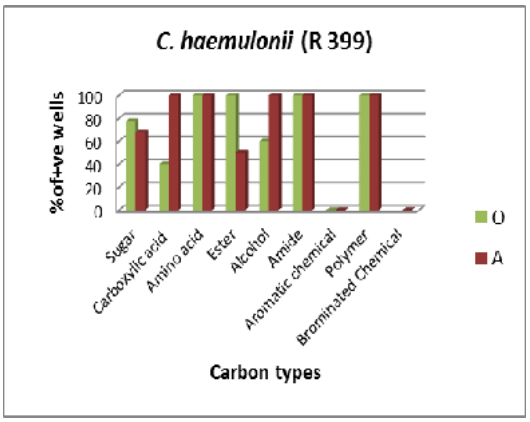


Fig 3.9f

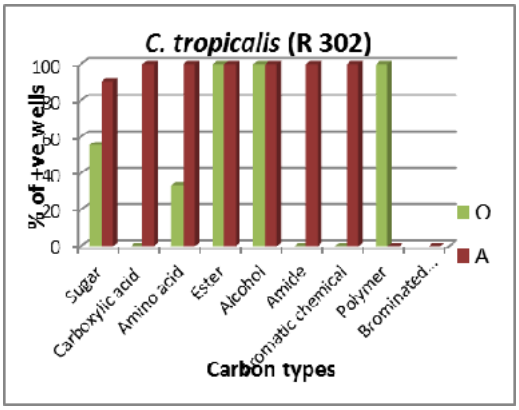


Fig 3.9g

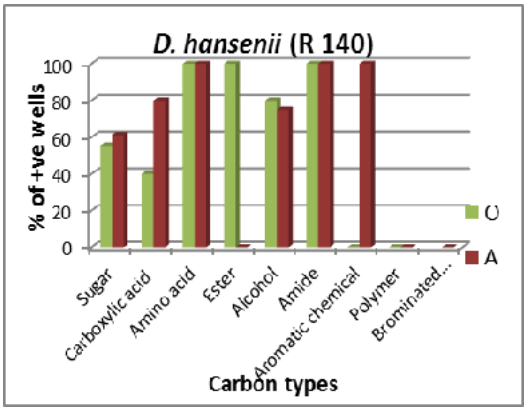


Fig 3.9h

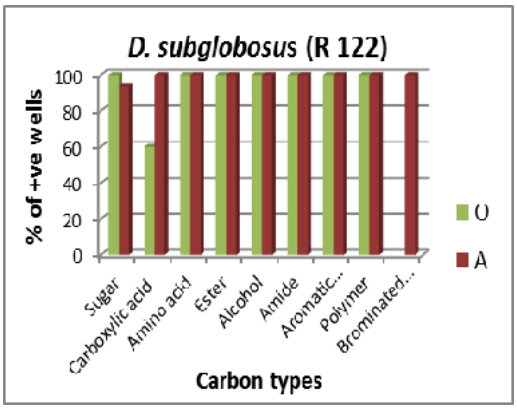


Fig 3.9i

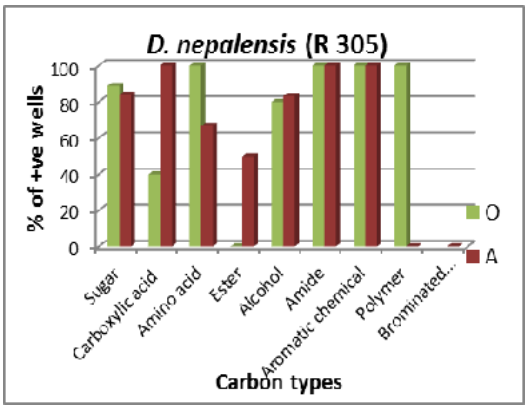


Fig 3.9j

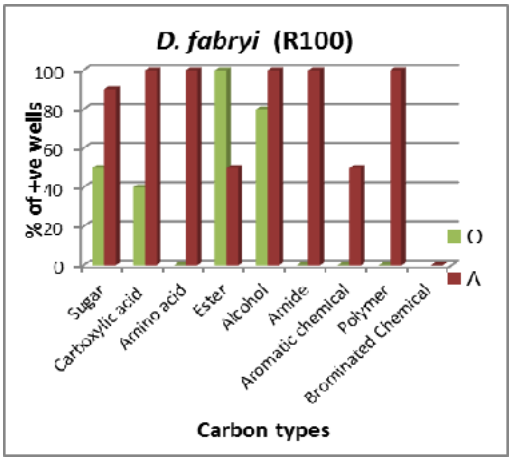


Fig 3.9k

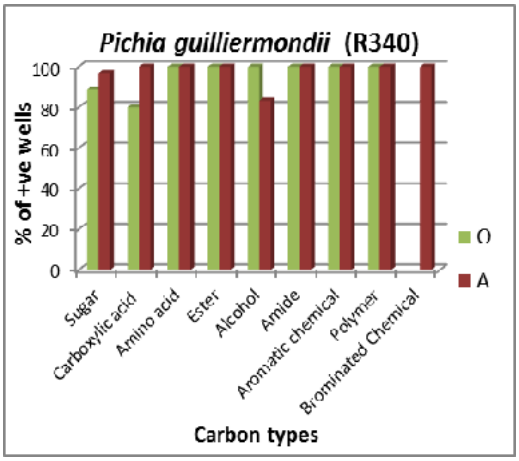


Fig 3.9 l

Fig 3.9a-l:Utilization of different carbon sources by various species of *Candida, Debaryomyces* and *Pichia.*

O – Oxidation, A - Assimilation

3.4. Discussion:

V9G and LS266 primers were used to amplify the regions of yeast DNA that includes partial 18S rDNA gene, 5.8S rRNA gene, 2 non-coding regions designated as the internal transcribed spaces (ITS1 and ITS2), and partial 28S rRNA gene. The size of the amplified product varies from 800-900bp in size. In fungi, as in the other eukaryotes, rRNA genes are repeated upto several hundred times in clusters. The ITS regions evolve rapidly and sequence differences in these regions are frequently found between closely related species or even among populations of the same sp. The heterogenity of this region has been documented in fungi.(Bernier et al., 1994; Buchko and Klassen, 1990; Irobi et al .,1991; Wang and Szmedt, 2000; Saed et al., 2001; Veleg ki et al., 2001) and in some higher eukaryotes (Karvonen et al., 1994, Reed and Philipps, 2000).The combined use of 2 molecular tools, restriction and sequence analysis of the amplified ITS region of the rRNA genes, was used as a means of yeast identification in the present study, but it was preceeded by conventional identification methods. Classical identification methods and restriction analysis helped to group the 159 non pigmented isolates into 12 species.

The genus *Candida* includes all yeast species that cannot be classified in other asexual ascomycetous yeast genera. As a result it is a highly heterogenous genus and the perfect state of most *Candida* species is still unknown. (Krejer – Van Rij, 1984). Different molecular techniques have been used to identify and characterise *Candida* species. However except in the case of the clinical species (Botelho and Planta, 1994; Iwaguchi et al., 1990; Jordan, 1994), a standard method of classification has not been developed. In this study about 115 isolates were from the genera *Candida* and they were analysed by RFLP of 5.8S – ITS region of 58 isolates using 3 restriction enzyme, *Alu*1, *Taq*1 and *Hinf*1.By RFLP patterns they were grouped into 7 categories and each of the 7 categories represented 7 spp. The

size of the amplified fragment of the various species of *Candida* varied from 800-850bp.

Among the 7 species of *Candida* obtained in the present study, data regarding 5.8S-ITS restriction pattern using *Hinf*1 of *Candida parapsilosis* and *Candida tropicalis* was reported from previous studies. (Esteve – Zarzoso et al., 1999; Korabecna et al., 2003). In the study of Esteve – Zarzoso et al., 1999, they have analysed by RFLP of 5.8S – ITS region of 56 strains belonging to 30 species. 56 strains showed a unique restriction pattern for each species with 3 endonucleases used (*Cfo*1, *Hae*111 *and Hinf*1). Restriction patterns provided by Esteve – Zarzoso et al. (1999) by *Hinf*1, helped to correctly identify *Candida parapsilosis*. RFLP of 5.8S – ITS also helped to cluster 115 isolates of *Candida* into 7 spp. Species identification was confirmed by sequencing because the samples were obtained from marine sediments, the data provided by Esteve-Zarzoso et al. (1999) was inadequate to make a correct identification upto the species level, for all environmental isolates. At the same time marine sediment isolates may have novel strains. The present study had 2 isolates (*C.spencermartinsiae* and *C.oceani*) and their RFLP pattern are not reported yet. For *C.parapsilosis*, Esteve – Zarzoso et al. (1999) got 2 fragments (290+260) using *Hinf*1 and *C.parapsilosis* from marine sediments also had 2 fragments (400+425). Variation in size is due to the variation in primers. (V9G and LS266) used for amplification. V9G and LS266 amplified a large portion when compared to ITS1 and ITS4 primers used by Esteve – Zarzoso et al. (1999) and Korabecna et al. (2003). On using *Taq*1 for *C.parapsilosis*, we have observed 3 fragments (400+280+200b.p.) and 2 fragments (600+200bp) for *Alu*1 which are in conformity with restriction patterns obtained *in silico*. Korabecna et al. (2003) got 2 types of restriction patterns for *C.tropicalis* for 2 different strains using *Hinf*1. R302 which had 97% similarity to *C.tropicalis* had only a single fragment of 500bp size when digested with *Hinf*1.This is in conformity with Korabecna's (2003) findings with the isolates

1138,1139,1140 while it is differing from the published restriction patterns by Esteve Zarzoso et al. (1999). *C. tropicalis* gave 2 fragments with both *Taq*1 (400+125) and *Alu*1 (700+100).

Candida orthopsilosis and *C. metapsilosis* represent group II and group III of *C. parapsilosis* (Tavanti et al., 2005a). Both the species cannot be distinguished morphologically from *C. parapsilosis*, but *C. metapsilosis* can be separated from *C. parapsilosis* based on multilocus sequence analysis (MLST) using 4 genes, comparison of amplicon patterns of 11 genes and ITS sequences (Asadzadeh et al., 2009; Berman et al., 2009; Johnson, 2009; Tavanti et al., 2005a). Similarly *C. orthopsilosis* can be separated from *C. parapsilosis* based on molecular markers such as nucleotide differences in the following genes SYA1, SADH as well as ITS (Asadzadeh et al., 2009; Borman et al., 2009; Tavanti et al., 2005a). On restriction digestion, *C. orthopsilosis* and *C. metapsilosis* were very similar to *C. parapsilosis* with *Taq*1 and *Alu*1. With *Hinf*1 *C. metapsilosis* had a 500bp (single fragment) and for *C. orthopsilosis*, it had a single band of 425 bp size, as if it was two fragments of equal size. Both the species were confirmed after sequencing and compared with the type strain sequences given in the GenBank using ClustalW (*C. orthopsilosis* **AJ698048**, **ATCC 96139**, *C. metapsilosis* **AJ698049**.1, **ATCC 96144**). No data was available regarding the restriction patterns of these two species, using the above mentioned restriction enzymes.

C. spencermartinsiae and *C. oceani* are recorded for the first time from the Arabian Sea, which is identified by ITS sequencing. *C. spencermartinsiae* showed only a single band (500bp) for *Hinf*1, 2 fragments each (400 + 150bp) for *Taq*1 and *Alu*1 (700+200bp). The *in vitro* restriction patterns of *Alu*1 are in conformity with that of *in silico* patterns, while the i*n vitro* restriction patterns of *Hinf*1 and *Taq1* is variable from that of *in silico* patterns. The isolates of *C. oceani* exhibited 2 types of restriction patterns. 4 of the isolates exhibited a single fragment (450bp) for *Hinf*1, 2 fragments (500+400bp) for *Taq*1 and a single fragment (900bp) for

Alu1, while the isolate 131 exhibited 3 fragments (350+200+100) for *Hinf*1, 3 fragments (380+300+200bp) for *Taq*1 and 2 fragments (600+200bp) for *Alu*1. Here also variations in RFLP patterns can be seen *in vitro* from that of *in silico*. According to Peter Raspor et al. (2007) absolute length of fragments is not as important as the pattern. Variations in length of restriction fragments in length can be ascribed to electrophoresis, where the concentration and the structure of the gel cannot always be accurate. *In vitro* digested fragments of rDNA smaller than 100base pairs are difficult to detect after staining and digitalization, and the restriction was often incomplete yielding misleading bands on electrophoregrams. The species *Candida haemulonii* also exhibited 2 types of restriction patterns. Even though *C. haemulonii* exhibited 2 types of patterns for the 3 restriction enzymes used for the study, both RFLP patterns were in conformity *in vitro* and *in silico* (Table 3.2.)

One species from the genera *Pichia i.e*., *Pichia guilliermondii* (synonym *Meyerozyma guilliermondii*) was found with two fragments on treating with *Hinf*1 (500+480bp) restriction enzyme. This is in conformation with previous studies (Esteve – Zarzoso et al., 1999; Korabecna et al., 2003; Villa – Crvajal et al., 2006). *Taq*1 and *Alu*1, restriction digestions on *Pichia* also resulted in 2 fragments for each enzyme. *Taq*1 yielded (400+400) fragments and *Alu*1 resulted in 700+100bp sized fragments. Both of them are in confirmation with *in silico* patterns.

Debaryomyces is a complex genus in which species previously identified as *Saccharomyces, Torulaspora Zygosaccharomyces* and *Pichia* have been included. Studies of Esteve – Zarzoso et al.(1999) found that restriction analysis of the 5.88 – ITS region of *Debaryomyces* spp. exhibited the same pattern with different endonucleases not only with the 3 general restriction enzymes (*Hae*111, *Cfo*1 and *Hinf*1) but also with *Alu*1, *Dde*1, *Scrf*1 and *Taq*1. Hence they were unable to contribute a correct RFLP patterns for the three spp. they have studied.

(*Debaryomyces polymorphus, D. hansenii, D. pseudopolymorprus*). At the same time Groenewald et al. (2008) reported that among several species of genus *Debaryomyces* including members of *D. hansenii* clade sensu (*D. hansenii, D. fabryi, D. subglobosus*) put forth by Kurtzman and Robnett (1998) highly conserved ITS and D1/D2 LSU sequences were reported (Martorell et al., 2005) while ACT1 sequences showed more variability and were considered suitable to differentiate these species. In the present study also ITS sequences and ARDRA patterns alone were insufficient to assign species in the *Debaryomyces* genera. MGT's (maximum growth temperature) have been considered a complimenting characteristic in yeast identification (Yarrow, 1998) and MGT's as well as growth rates at different temperatures have been used successfully in fungal identification (Groenewald et al., 2005). Differences in MGT's were recognised as an important criterion in the classification of *Debaryomyces* species and introduced by Nakase and Suzuki (1985b) to distinguish 2 varieties of *D. hansenii*. They studied 34 strains of *D. hansenii* and newly defined taxa delimited MGTS of *D. fabryi* as below 37°C, *D. subglobosus* as below 40ºC, while the MGT's *of D. hansenii* was the lowest and it is below 35°C. Hence a polyphasic approach or a multigene approaches or the actin gene sequence can be used to resolve closely related species of *Debaryomyces* such as *D. hansenii, D. fabryii* and *D. subglobosus*.

In general, Biolog YT microplates are used for rapid identification of clinical isolates. In the present study, YT plates were used for physiological characterisation of species after molecular identification by ITS sequencing. The sugars inulin, raffinose and melibiose were not assimilated by R 38, R 28W, R 399 and R302 which is in conformity with Kurtzman et al. (2011), but unlike Kurtzman et al.(2011) R13 and R56 exhibited a borderline positive growth for inulin. Similarly they also reported non-assimilation of the sugar raffinose for the different species of *Candida* obtained in the present study except *C. haemulonii*. But in the Biolog YT wells except for R 13 all the other species of *Candida*

exhibited a borderline growth and *C. haemulonii* and *C. tropicalis* exhibited an active growth. Unlike Kurtzman et al. (2011) cellobiose was utilized by R 13 and two strains (R 56 and R 38) exhibited borderline growth for cellobiose.

Of the 4 species of the genera *Debaryomyces* obtained in the study, except R122 (*D. subglobosus*), other 2 species (*D. fabryi, D. nepalensis*) were unable to assimilate the sugar inulin which was similar to the consolidated reports of Kurtzman et al. (2011), but according to them *D. hansenii* was variable in its ability to use inulin, but the isolate R140 identified as *D. hansenii* in the present study was able to assimilate inulin. Similarly they have given a variable result for the assimilation of melibiose and melezitose, but all the four species obtained except *D. hansenii* was able to assimilate both. The sugar L-rhamnose was not assimilated by any of the species in the genera *Debaryomyces*. The isolate R340 (*Pichia*) was unable to assimilate glycerol, gluconic acid and dextrin.

Of the twelve species of non pigmented yeasts isolated, *Candida parapsilosis* was previously isolated and identified using conventional and culture independent approach from mid Atlantic ridge (Gadanho and Sampaio, 2005), methane hydrate –bearing deep sea sediments (Lai et al., 2007) and deep sea sediments (Nagano et al., 2010). *Candida tropicalis* was obtained from the coastal sea waters of Taiwan (Chen et al., 2009) and waste water systems (Yang et al. 2011). Rapid molecular identification helped to identify *Pichia guilliermondii* from mid Atlantic ridge (Gadanho et al., 2005), methane hydrate –bearing deep sea sediments (Lai et al., 2007), deep sea volcano (Connel et al., 2009) deep sea sponge (Burgaud et al., 2010) waste water having biocalcitrants (Yang et al., 2011) and *Debaryomyces hansenii* from deep sea sponge (Burgaud et al., 2010).

To conclude, the RFLP analysis of 2 ribosomal internal transcribed spacers (ITS) has been proved to be a fast and simple method for species identification. (Belloch et al., 1998b; Fernandez – Espinar et al., 2000; Arias et al., 2002; Cadez et al.,

2002). For the present, as an alternative to conventional methodology, sequence analysis of the ITS region in combination with restriction analysis offers identification of yeast strains especially environmental isolates. In short, a polyphasic approach may be the best way to achieve proper microbial identification at the species level especially for the genera *Debaryomyces*.

Chapter -4

PIGMENTED YEASTS - MOLECULAR IDENTIFICATION AND PHYSIOLOGICAL CHARACTERIZATION

4.1. Introduction

Yeasts are among the economically and scientifically most important eukaryotic microorganisms known. At present, there are 1,500 recognized species, which are distributed between the ascomycetes and the basidiomycetes. For several decades, molecular biologists have focused on a small number of yeasts, mainly because of the limitations of available genetic and biochemical tools. However, during the last few years, with the onset of a variety of novel molecular biology approaches, additional species have undergone a thorough molecular analysis. This has recently culminated with the sequencing of over a dozen different yeast genomes (Piskur and Langkjaer, 2004). With this new information, yeasts are becoming increasingly interesting to molecular biologists because understanding the diversity and phylogenetic relationships is essential for comparative genomics studies.

Colours are vital to the sensing of the environment and have evolved in higher living organisms to guide their interaction with others. For *e.g.,* It is well appreciated that many birds exhibit brightly coloured plumage to attract members of the opposite sex, that a chameleon's adaptation to surrounding colour is an

important means of camouflage, and that the bright colouration of the poison dart frog warn potential predators to stay away. But such explanations cannot be offered to explain why certain microorganisms are pigmented. Because they lack colour perception one must assume evolutionary selective pressures behind the acquisition of pigments that promotes survival independent of their light absorbance, reflection or emission spectral properties. In microbes most pigments evolved initially as a mechanism to combat environmental reactive species, but overtime these compounds were adapted to serve divergent functions. Varied functions proposed for microbial pigments include acquisition of energy by photosynthesis as in Cyanobacteria (Chew and Bryant, 2007), acquisition of nutrients such as iron by *Cryptococcus*, (Nyhus et al., 1997), protection against ultra violet radiation as in several pigmented yeasts like *Phaffia, Rhodotorula* and *Sporobolomyces* (Martin Moline et al., 2009), protection against extremes of temperature (heat and cold) as in the black yeast *Wangiella* (*Exophiala*) (Paolo et al., 2006), and protection against natural antimicrobial compounds produced by other microbes as in *Cryptococcus neoformans* (Van duin et al., 2002). Pigmented yeasts belong to both ascomycota and basidiomycota. They generally grow poorly and accumulation of pigments occurs slowly. Pigmentation in yeast varies; it can be yellow, red, orange, pink or even black. Pigmentation in yeast affords protection from UV-B radiations. The most common pigments produced by yeasts include carotene and melanin.

4.1.1.Carotenoid producing yeasts

Carotenoids are liposoluble tetra terpene-type molecules which are generally red to yellow, due to the presence of several conjugated double bonds that act as chromophores (Britton, 1995). In fungi, their fundamental role is to provide protection against reactive oxygen species (ROS), specifically O_2. Furthermore they provide indirect protection against both UV-A (315–400 nm) and visible light (photosynthetically active radiation, PAR 400–700 nm) by means of ROS

quenching (Moore et al., 1989). Several yeast species are known to accumulate carotenoid pigments as secondary metabolites. In these microorganisms, carotenoid synthesis is associated with growth. Maximum carotenoid accumulation is observed in stationary phase in relation with cell ageing, and is probably a general mechanism of defense against oxidative stress (Bhosale and Gadre, 2001: Johnson, 2003) . The synthesis of torularhodin, torulene, and β-carotene is common in several genera of *Rhodotorula, Sporobolomyces*, and *Cystofilobasidium* (Simpson, et al., 1964). In these microorganisms, carotenoids may contribute to preserve the viability of ageing cells by quenching oxygen radicals, possibly compensating for their lack of antioxidant enzymes (Schroeder and Johnson, 1995). The genus *Rhodotorula* Harrison is a member of the basidiomycetous yeasts, occurring naturally in air and soil from which it can readily be isolated. In addition, this yeast can be isolated from human skin, stool and food. Although most species of the genus *Rhodotorula* are non-pathogenic, some species have been regarded as emerging yeast pathogens (Hazen, 1995) amongst which *Rhodotorula mucilaginosa* is the most frequently isolated from human infections. In addition, *Rhodotorula minuta* (Goldani et al., 1995) and *Rhodotorula glutinis* (Guerra et al., 1992: Casolari et al., 1992) are also isolated from human infections. The genus *Rhodotorula* has recently expanded markedly, from only eight species (Kreger-Van Rij, 1984) to 34 (Kurtzman and Fell, 1998). This increase in number is the result of several factors. The major one is redefinition of the genus *Candida* and segregation of basidiomycetous species into *Rhodotorula* and *Cryptococcus*, based on the composition of cell wall hydrolysates, particularly xylose which has only been found in the latter genus (Weijman et al., 1988). Use of improved methods, such as the application of molecular techniques to species identification, has also contributed significantly. A recent study by Fell et al. (2000) described seven more new species of the genus *Rhodotorula*, according to differences in the D1/D2 region of the large subunit rDNA. They are *Rhodotorula creatinivora*, *Rhodotorula cresolica*,

Rhodotorula laryngis, *Rhodotorula pallida*, *Rhodotorula slooffiae*, *Rhodotorula vanillica* and *Rhodotorula yarrowii.*

Differentiation of all species of the genus *Rhodotorula* based on morphological characters and physiological tests is potentially time-consuming, expensive and requires considerable expertise. Hence, a more efficacious method may be found through the presentation of an integrated view of the genus based on molecular data and this, though not an easy task, has been done by Fell et al.(2000).They have also shown that species of *Rhodotorula* are distributed in the *Microbotryum*, *Sporidiobolus* and *Erythrobasidium* clades, but not in the *Agaricostilbum* clade of the class Urediniomycetes. However, *Rhodotorula acheniorum*, *Rhodotorula bacarum*, *Rhodotorula hinnulea* and *Rhodotorula phylloplana* are members of the Ustilaginomycetes (Fell et al., 1995, 2000)

4.1.2.Melanin producing yeasts

Yeasts producing melanin are generally called black yeast. '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 and Mcginnis, 1987). All ascomycetous 'black yeasts' may or may not have a yeast-like phase, depending on the ecological niche it inhabits. Closely related members of the 'black yeasts' may be highly dissimilar in morphology. If the 'black yeasts' are taken as taxonomic rather than a morphogenetic group, yeasts 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 eco-system. 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.

Ecology of *Hortaea*

Eukaryotic microorganisms such as the ‘‘black yeasts’’ (De Hoog and Hermanides-Nijhof 1977), micro colonial fungi (Staley et al., 1982) and meristematic ascomycetes (Sterflinger et al., 1999) are also remarkably successful in adapting to certain extreme environments (Staley et al., 1982: Gorbushina et al., 1993: Nienow and Friedmann, 1993: Wollenzien et al., 1995: Gunde-Cimerman et al., 2000). Although they have been known since the end of the 19th century (de Hoog et al., 1999), difficulties in their morphological identification together with their slow growth and low competitive ability frequently hindered the isolation and identification of these fungi. The representatives of black yeasts belong to the ascomycetous *i.e*., Chaetothyriales, Dothideales and Pleosporales (Sterflinger et al., 1999). Several genera and species of black yeasts from the order Dothideales represent a group of rare halophilic eukaryotic microorganisms, highly appropriate for studying the mechanisms of salt tolerance in eukaryotes (Petrovic et al., 2002; Turk and Plemenitas, 2002). So far little is known about eukaryotic halophilic microorganisms, let alone the mechanisms of their adaptation to such conditions (Petrovic et al., 2002). Microscopy and histological studies of black yeasts have revealed that their morphological ecotype is important for their survival in various extreme environments. Black yeasts are characterized by melanized slowly expanding colonies, with reproduction by isodiametric enlargement of subdividing cells (Sterflinger et al., 1999: Wollenzien et al., 1995). Their distinctive features are polymorphism, meristematic growth, frequently muriform cells which develop by conversion from undifferentiated hyphae, and thick, melanized cell walls (de Hoog 1993: Zalar et al., 1999: Sterflinger et al., 1999: Wollenzien et al., 1995). *Hortaea werneckii*, the best described eukaryotic halophilic model organism to date, is characteristic black yeast. In the past, its

identification was based solely on morphological characteristics and has thus received many designations: *Cladosporium werneckii*, *Exophiala werneckii, Pullularia werneckii, Aureobasidium werneckii*, *A. mansonii*, *Sarcinomyces crustaceus* and *Phaeoannellomyces werneckii*. Currently the identification is mainly based on molecular methods and the nutritional physiology also contributes to it. The physiological tests include metabolism of different carbon and nitrogen sources with or without NaCl in high concentrations, as well as temperature tolerance tests. Molecular differentiation is based on the sequencing of the ITS rDNA region and RFLP markers from SSU rDNA and ITS rDNA regions (de Hoog et al., 1999). Until recently, the genus *Hortaea* consisted only a single species, *H. werneckii* (Horta) Nishimura and Miyaji, with no known sexual stage (Zalar et al., 1999). In 2004, another species was described and named *H. acidophila* (Holker et al.,2004). *Hortaea werneckii* was long known primarily as the etiological agent of human tinea nigra, a superficial infection of the human hand, particularly frequent in warmer areas of the world. Investigations have revealed that the fungus is strictly limited to the dead surface of the skin (stratum corneum), in particular to the grease on the skin. Since *H. werneckii* does not show any keratin-degrading activity, it does not invade the living tissue below and so the infection is only a cosmetic problem (Gottlich et al., 1995). Besides its involvement in tinea nigra, *H. werneckii* was also known as one of the few species of fungi capable of contaminating food preserved with high concentrations of NaCl (Mok and Barreto daSilva, 1981), without showing any obligate requirement for NaCl (Andrews and Pitt, 1987). In addition to human skin and salty food, the fungus has been isolated from seawater (Iwatsu and Udagawa, 1988), marine fish (Todaro et al.,1983), beach soil (de Hoog and Gueho, 1998) and arid inorganic and organic surfaces (Krumbein et al., 1996). On the basis of *H. werneckii* random isolations from different low water activity substrates and *in vitro* ecophysiological studies it was suggested that salt might be the decisive factor in its ecology and therefore in the etiology of tinea nigra. Furthermore, it

was thus speculated that *H. werneckii* might grow in drying salty ponds at the sea side (de Hoog and Gerrits van den Ende, 1992). However, the primary environmental ecological niche of *H. werneckii* remained unknown until investigations in the salterns along the Slovenian Adriatic coast for the potential presence of halophilic fungi. Their distribution was followed throughout two successive years in five different evaporitic ponds, covering the entire salinity range (3–32% NaCl) (Gunde-Cimerman et al., 2000: Butinar et al., 2005). This study revealed that hypersaline waters of the salterns harbor different species of melanized fungi from the order Dothideales. They appeared in three peaks, at the water salinities 5–8%, 10–20% and 18–25% NaCl, which correlated primarily with high environmental nitrogen values. At the highest environmental salinities, melanized fungi represented 85–100% of the total isolated mycobiota, but they were partially replaced by non-melanized fungi with lowering salinities and they were detected only occasionally at the end of the season, when NaCl concentrations were below 5%. *H. werneckii* was the dominant black-yeast species in the Adriatic salterns during the season of salt production (Gunde-Cimerman et al., 2000). Initially it was isolated from the crystallization ponds of the Adriatic salterns, but it was later identified in hypersaline waters of six salterns on three continents (Butinar et al., 2005). Besides being the dominant black yeast species in hypersaline water at salinities above 20%, *H. werneckii* was also isolated from various micro niches within the salterns: the surface and interior of wood submerged in brine, from biofilms on the surface of hyper saline water, and from dry ponds and microbial mats (Butinar et al., 2005: Zalar et al., 2005). However, it appears that *H. werneckii* survives in eutrophic thalassohaline waters of salterns in temperate climatic zones, since it was only occasionally retrieved from salterns in Puerto Rico, but never from the oligotrophic salterns in Eilat at the Red Sea in Israel, or the athalassohaline waters of the Dead Sea, or those of Salt Lake, Utah (Gunde-Cimerman et al., 2005).

In contrast to most prokaryotic halophiles, which display better growth in the presence of NaCl, the salinity range for *H. werneckii*, defined *in vitro*, was from 0% to 32% NaCl, with a broad optimum from 6 to 14% NaCl. Its complex polymorphic life cycle enables *H. werneckii* to show versatile ecotype adaptations in response to changing environmental concentrations of NaCl, UV intensity, nutrients and water availability. If sufficient nutrients are available; the hydrophilic yeast phase rapidly colonizes hypersaline water. At salinities above 15% NaCl, yeast cells begin to differentiate into meristematic budding cells, and at the highest salinities they form dormant meristematic sclerotial bodies with endogenous conidiation. Clustered growth allows sheltering of interior cells and minimizes the number of cells directly in contact with the hostile environment. Under conditions of drought, with no water in the ponds, the fungus changes into an aerophilic, hydrophobic hyphal stage, producing conidia which can be dispersed by air currents. These conidia can germinate in saline water, giving rise to actively propagating yeast cells (Butinar et al., 2005).

Molecular adaptations of *Hortaea*:

Molecular studies on halophilic adaptations have focused on prokaryotic microorganisms due to a lack of known appropriate eukaryotic halophilic microorganisms. However, the black yeast *Hortaea werneckii* has been identified 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. Ultra structural studies of the *H. werneckii* cell wall have shown that it synthesizes dihydroxynaphthalene (DHN) melanin under both saline and non-saline growth conditions. However, melanin granules in the outer part of cell walls are organized in a salt-dependent way, implying the potential osmoprotectant role of melanin. At the level of membrane structure, *H. werneckii* maintains a sterol-to-phospholipid ratio significantly lower than the salt-sensitive *Saccharomyces cerevisiae*. Accordingly, membranes of *H. werneckii* are more

fluid over a wide range of NaCl concentrations, indicating high intrinsic salt stress tolerance. Even *H. werneckii* grown in high NaCl concentrations maintains very low intracellular amounts of potassium and sodium, demonstrating the sodium-excluder characteristic of this organism. The salt-dependent expressions of two HwENA genes suggest roles for them in the adaptation to changing salt concentrations. The high similarity of these ENA ATPases to other fungal ENA ATPases involved in Na^+/K^+ transport indicates their potential importance of this, in ion homeostasis in *H. werneckii*. Glycerol is the main compatible solute which accumulates in the cytoplasm of *H. werneckii* at high salinity, although it seems that mycosporines may also act as supplementary compatible solutes. Salt dependent increase in glycerol synthesis is supported by the identification of two copies of a gene putatively coding for glycerol-3-phosphate-dehydrogenase. Expression of only one of these genes is salt dependent.

4.2. Materials and methods

4.2.1. Yeast strains

Among the 192 isolates from the Arabian Sea and Bay of Bengal, 33 isolates were pigmented, of which 17 were in various shades of pink and 16 brown or black in colour .Of the 17 pink ones 9 were deep pink in colour and 8 light pink. All the pink yeasts belonged to genus *Rhodotorula,* and black / brown isolates to genus *Hortaea*.

4.2.2. Yeast genomic DNA isolation

Genomic DNA was isolated from 17 pink and 16 black isolates as per Harju et al (2004).Refer section 3.2.2.

4.2.3. Amplification of ITS sequence

ITS (Internal transcribed spacer) sequences were amplified using V9G and LS266 primers in DNA Thermal Cycler (Eppendorf). PCR products were visualised on 1% agarose gels in 1x TAE buffer (Refer section 3.2.2).

4.2.4. Restriction endonuclease digestion and analysis

PCR products (8µl) without further purification, was digested with 3 restriction endonucleases *Hinf*1, *Alu*1 *and Taq*1 (Sigma) in separate reaction. Fragments were separated in 2% agarose gels in 1x TAE buffer and their sizes estimated by comparison against a 100bp DNA ladder.

Refer section 3.2.3

4.2.5. PCR clean up system

PCR clean up for sequencing was done using Promega PCR clean up system.

Refer section 3.2.5.

4.2.6. Sequencing

Refer section 3.2.6

4.2.7. ITS gene sequence similarity and strain identification

Refer section 3.2.7

4.2.8. Physiological characterisation of the isolates

Refer section 3.2.8

4.3. Results:

4.3.1. Size of PCR amplicons

In black yeasts, the size of the PCR product ranged between 850 to 900 bp in size, while in pink yeasts the size of the PCR products varied between 900 to 950- bp in size (Fig 4.1).

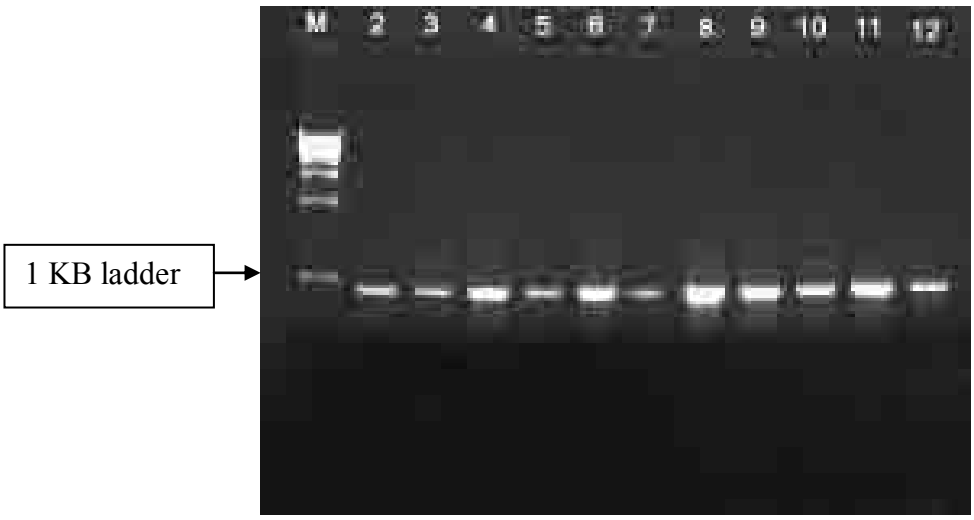


Fig.4.1 PCR amplicons of the ITS region of Black yeast isolates (*Hortaea)*

4.3.2.Restriction digestion - ARDRA

Restriction digestion using the enzyme *Hinf*1 resulted in 6 different patterns, which are either 2 fragments or 3 fragments. Two fragments were obtained for the pink yeasts R382 (425 + 200bp) and R28 (400 + 350bp), while 3 fragments were observed for R19 (425+200+180) and R149 (380+225+200bp). In the black yeast R23 also 2 fragments were seen (500+250).

In using the enzyme *taq*1, also 2 fragments were obtained for R382 (500+450bp) R19 (500 + 400 bp) and also for R149 (480 +180bp), while 3 fragments were obtained for R 28 (200+280+380). With the restriction enzyme *Alu*1, 2 fragments could be seen in all the isolates except R149, which has 3 fragments (500 +200+ 180bp). Though 2 fragments could be seen in R382, R19 and R28, there was variation in the size of the fragments of different isolates. For R382 it was 600+100 bp in size, for R19 it was 500+200 bp in size and for R28 it was 600+180 bp in size and for the black yeast R23, only a single fragment of 800 bp could be seen (Fig 4.2a, b,Fig 4.3a-c. and Table 4.1).

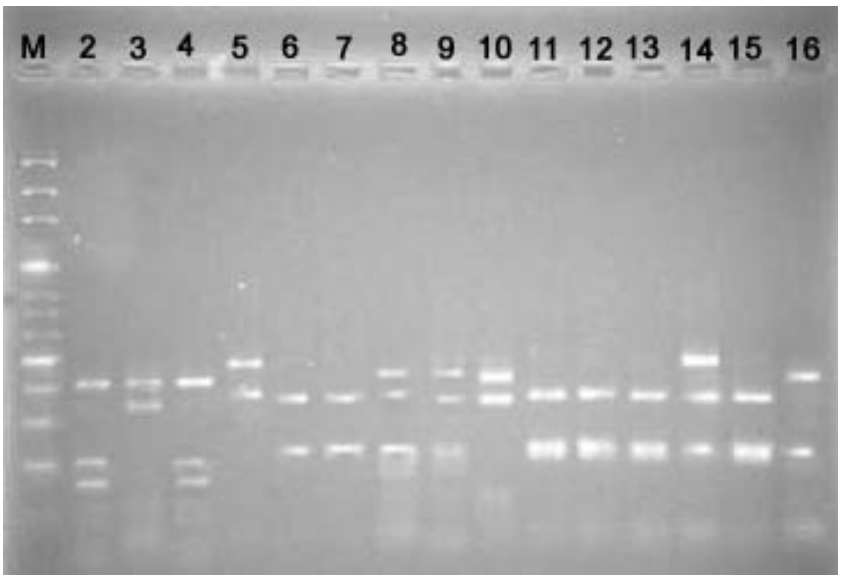


Fig. 4.2a ARDRA pattern generated using digestion with restriction enzymes *Hinf*1, on ITS amplicons of *Rhodotorula* isolates from Arabian Sea and Bay of Bengal

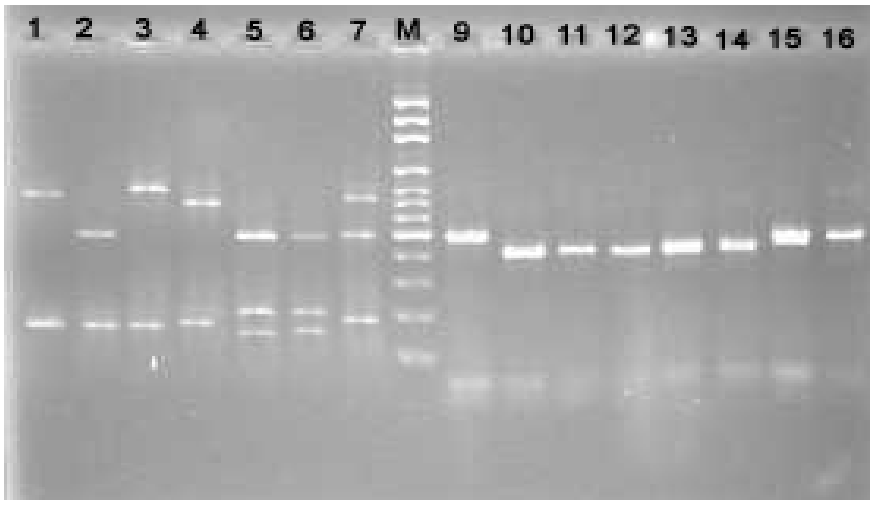


Fig. 4.2b ARDRA pattern generated with restriction enzymes *Alu*1 on ITS amplicons of *Rhodotorula* isolates from Arabian Sea and Bay of Bengal

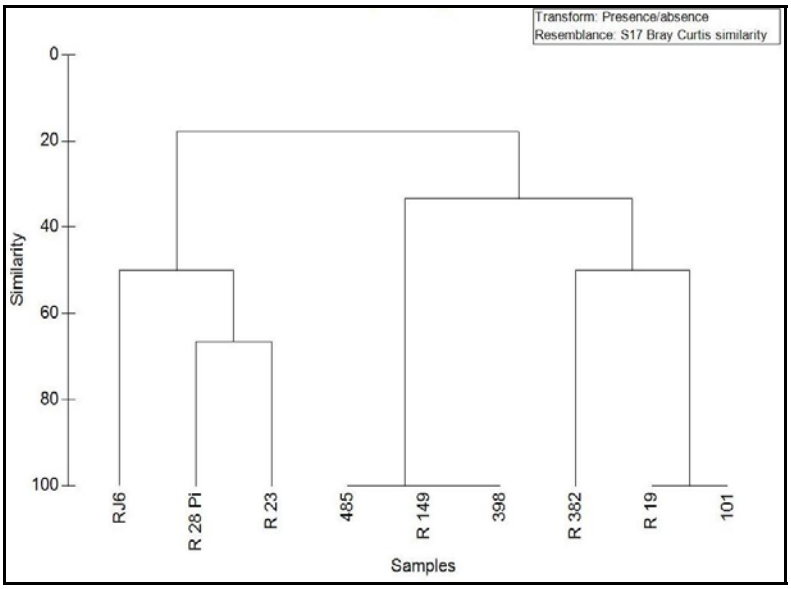


Fig.4.3a Dendrogram showing the clustering of the pigmented yeast strains into various groups based on ARDRA patterns developed by *Hin f*1

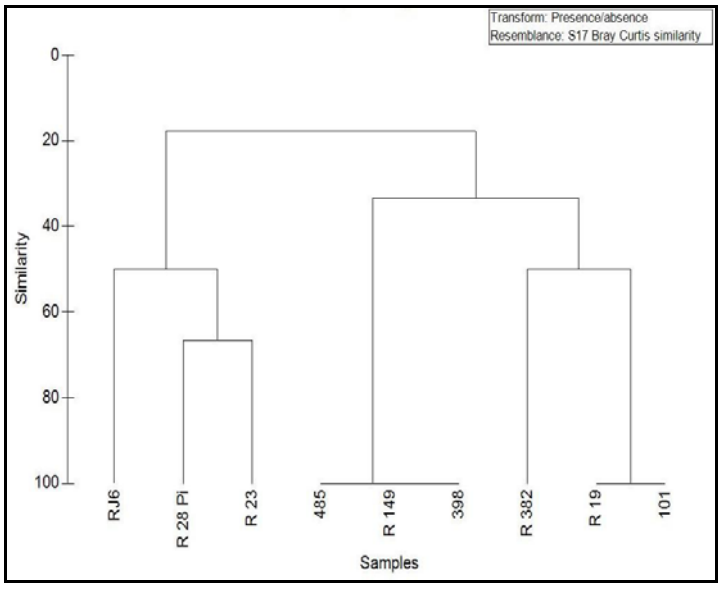


Fig.4.3b Dendrogram showing the clustering of the pigmented yeast strains into various groups based on ARDRA patterns developed by *Taq*1

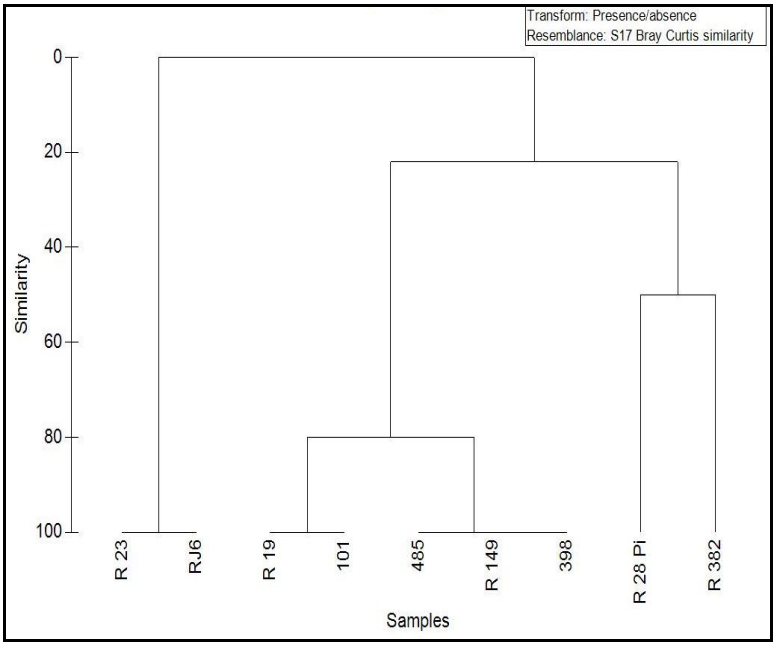


Fig. 4.3c Dendrogram showing the clustering of the strains into various groups based on ARDRA patterns developed by *Alu*1.

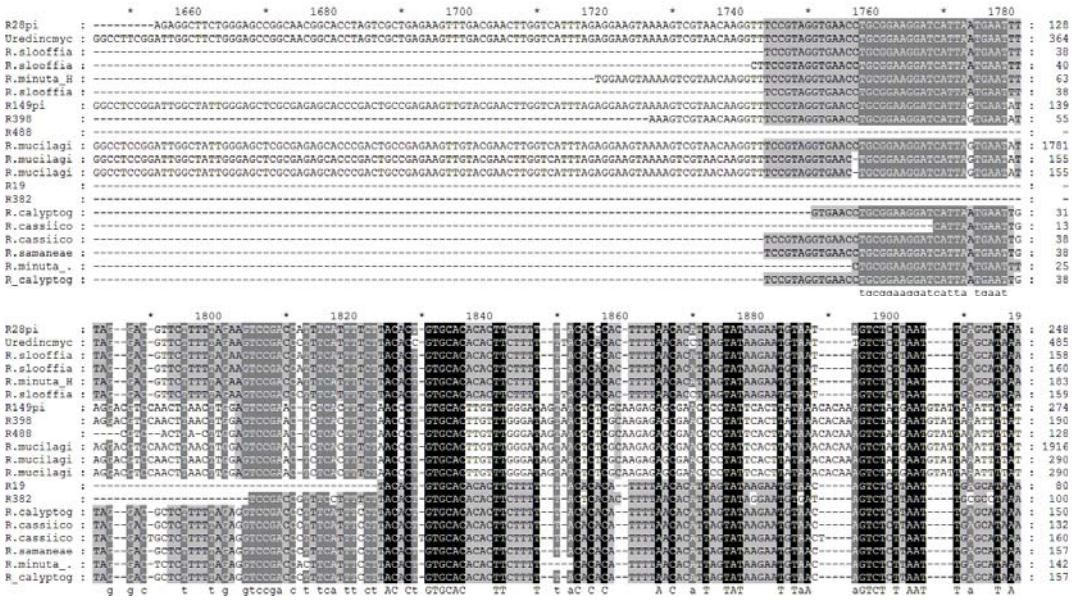
Table 4.1 ARDRA pattern generated using digestion with restriction enzymes *Hin f*1, *Taq*1, *Alu*1 on ITS amplicons of various pigmented yeasts from Arabian Sea and Bay of Bengal

Isolate No	Species	Size of ITS amplicons	*In vitro* restriction fragments		
			*Hin f*1	*Taq*1	*Alu*1
R 382	*Rhodotorula minuta*	900	425+200	550+400	600+100
R 19	*R. calyptogenae*	900	425+200+180	500 +400	500+200
R 28 Pi	*R. slooffiae*	900	400+350	380+280+200	600+180
R 149	*R.mucilaginosa*	950	380+225+200	380+180	500+200+180
R 23	*Hortaea werneckii*	800	500+250	300+280+180	800

4.3.3. Species composition of the pigmented yeasts

Pink yeast isolates having dissimilar patterns were sequenced and the sequences were blasted in both NCBl and CBS for the identification of the isolates. All the pink yeast isolates belonged to the same genera *Rhodotorula* representing 4 species *i.e., Rhodotorula mucilaginosa*, *R. minuta*, *R. slooffiae* and *R. calyptogenae*. The isolate R19 was 100% similar to *R. calyptogeneae* (ACC: No.FJ515209). R28Pi strain was 99% similar to *R. slooffiae* (Acc: No: FJ515213, FJ515193). The isolate R149 and R398 were 100% similar to *Rhodotorula mucilaginosa* (Acc: no: JQ 293997, DQ386306, ABO26017 and ABO26014). Among the pink yeast isolates *R. mucilaginosa* formed the dominant species as 9 out of the total 17 pink yeasts belonged to *R. mucilaginosa*, followed by *R. minuta* (4), *R. calyptogenae* (3) and *R. slooffiiae* (1)(Fig.4.4a,b;Fig 4.6a,b).

Among the 4 species of *Rhodotorula, Rhodotorula slooffiae* and *R. calyptogenae* were obtained from the Arabian sea alone, *and R. minuta* was obtained from the Bay of Bengal alone while *R. mucilaginosa* was obtained both from Arabian sea and Bay of Bengal.



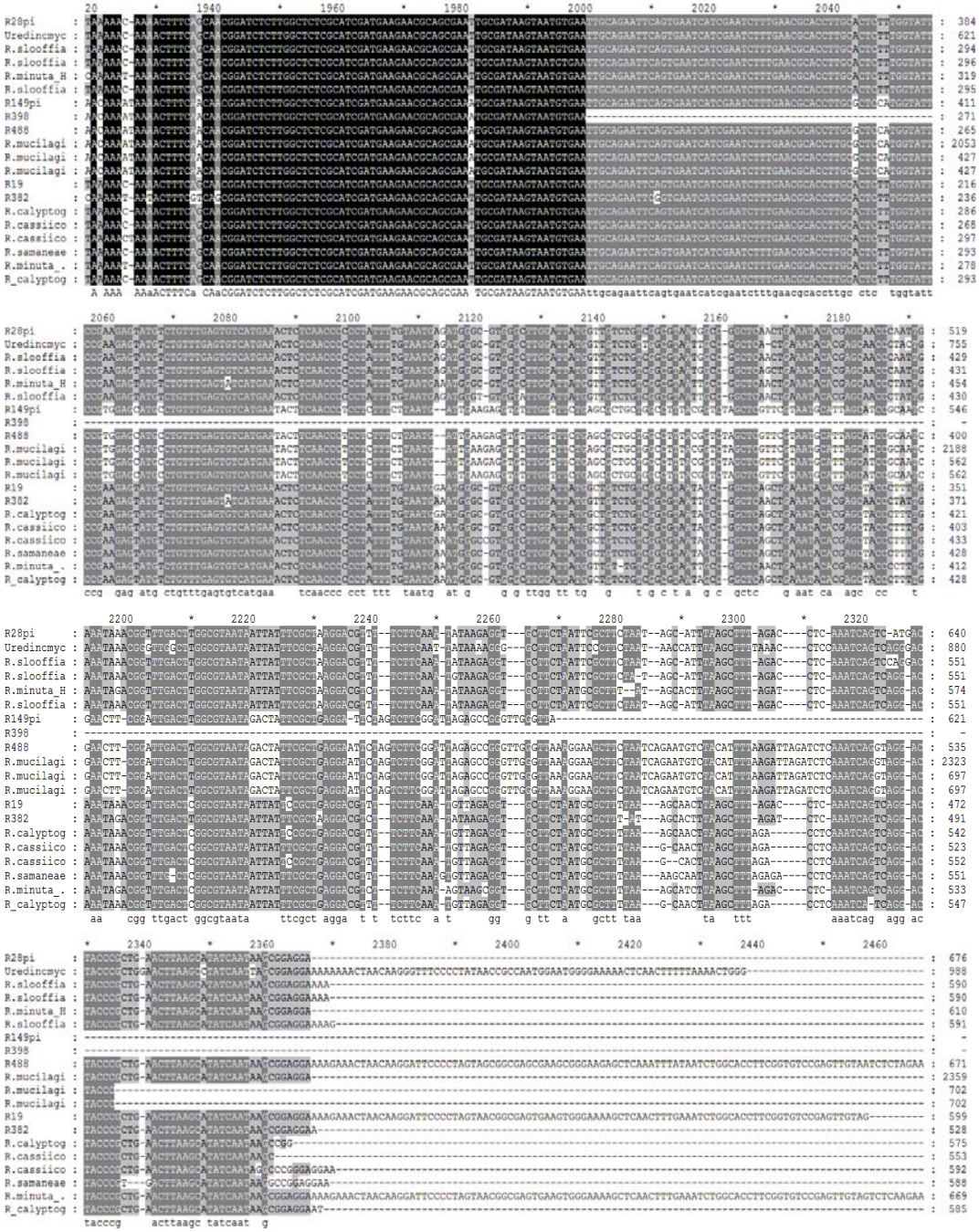
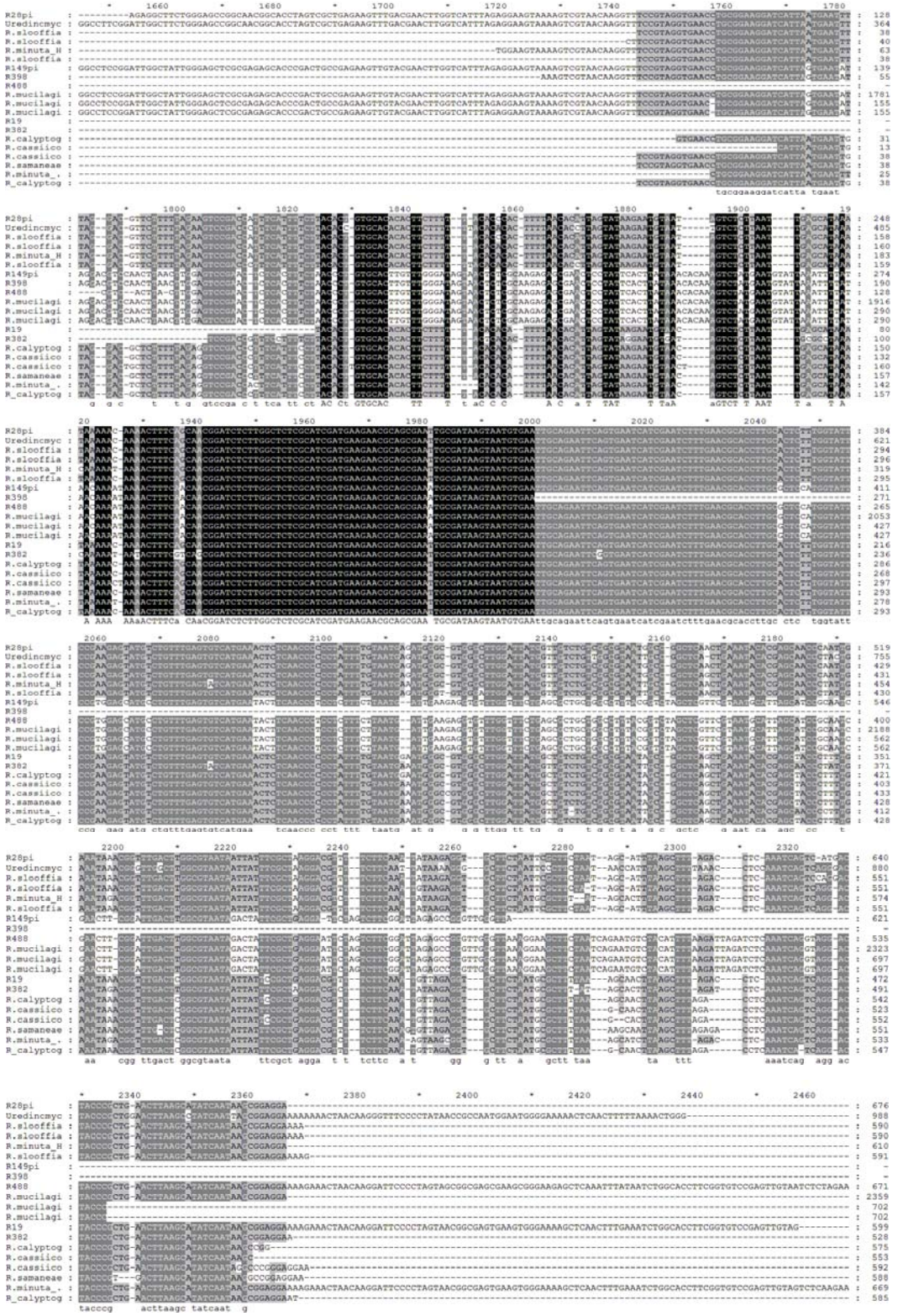


Fig.4.4a Clustal W multiple alignment of the ITS sequences from *Rhodotorula* strains and phylogenetically related strain sequences from NCBI



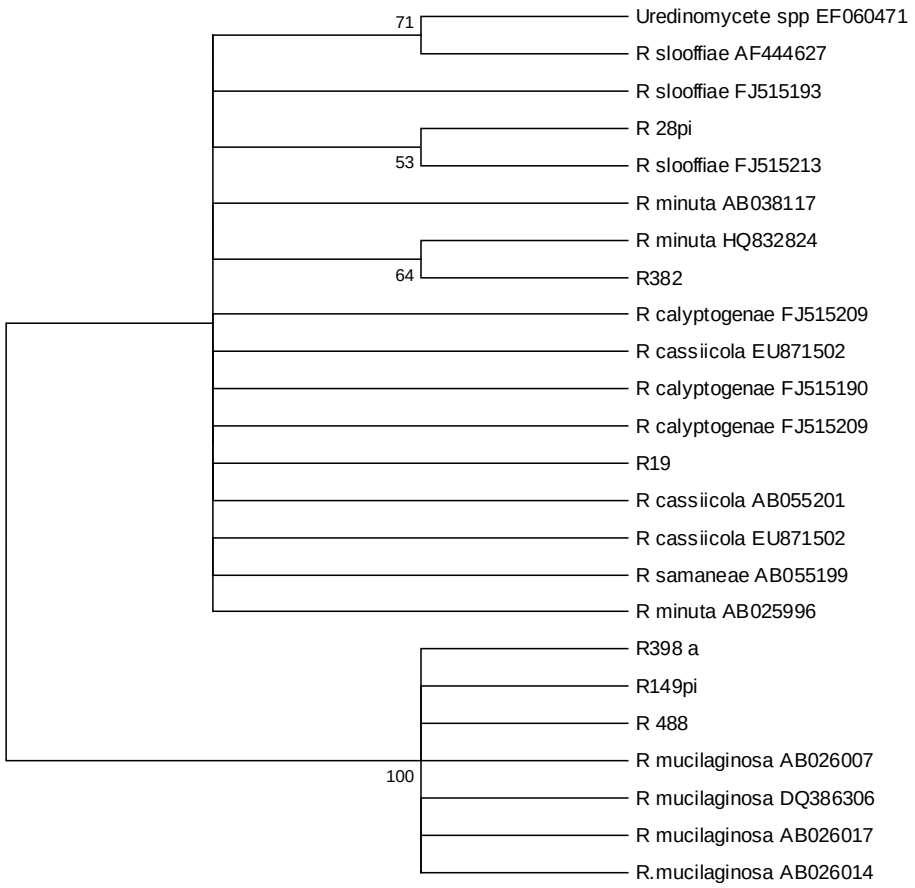
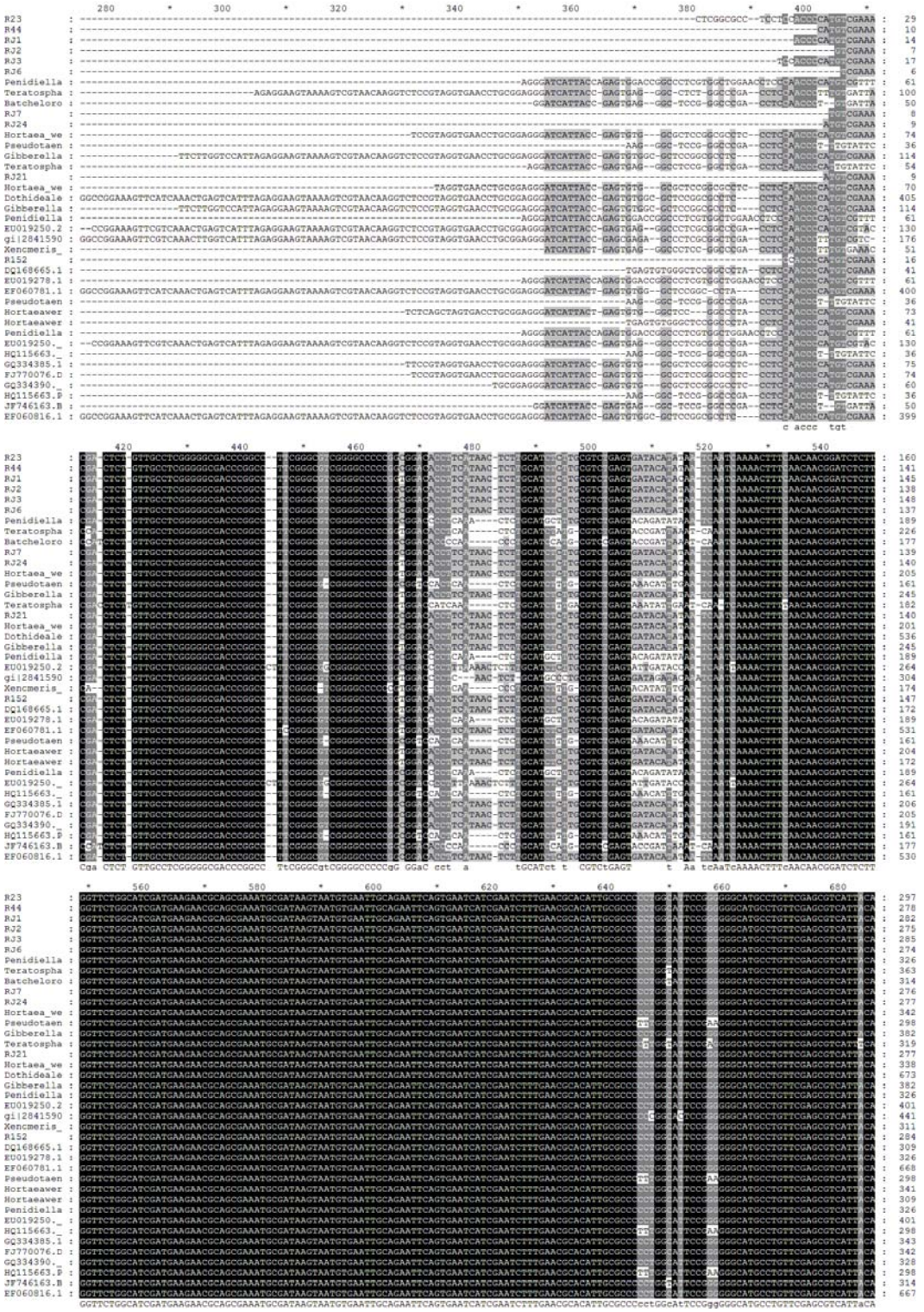
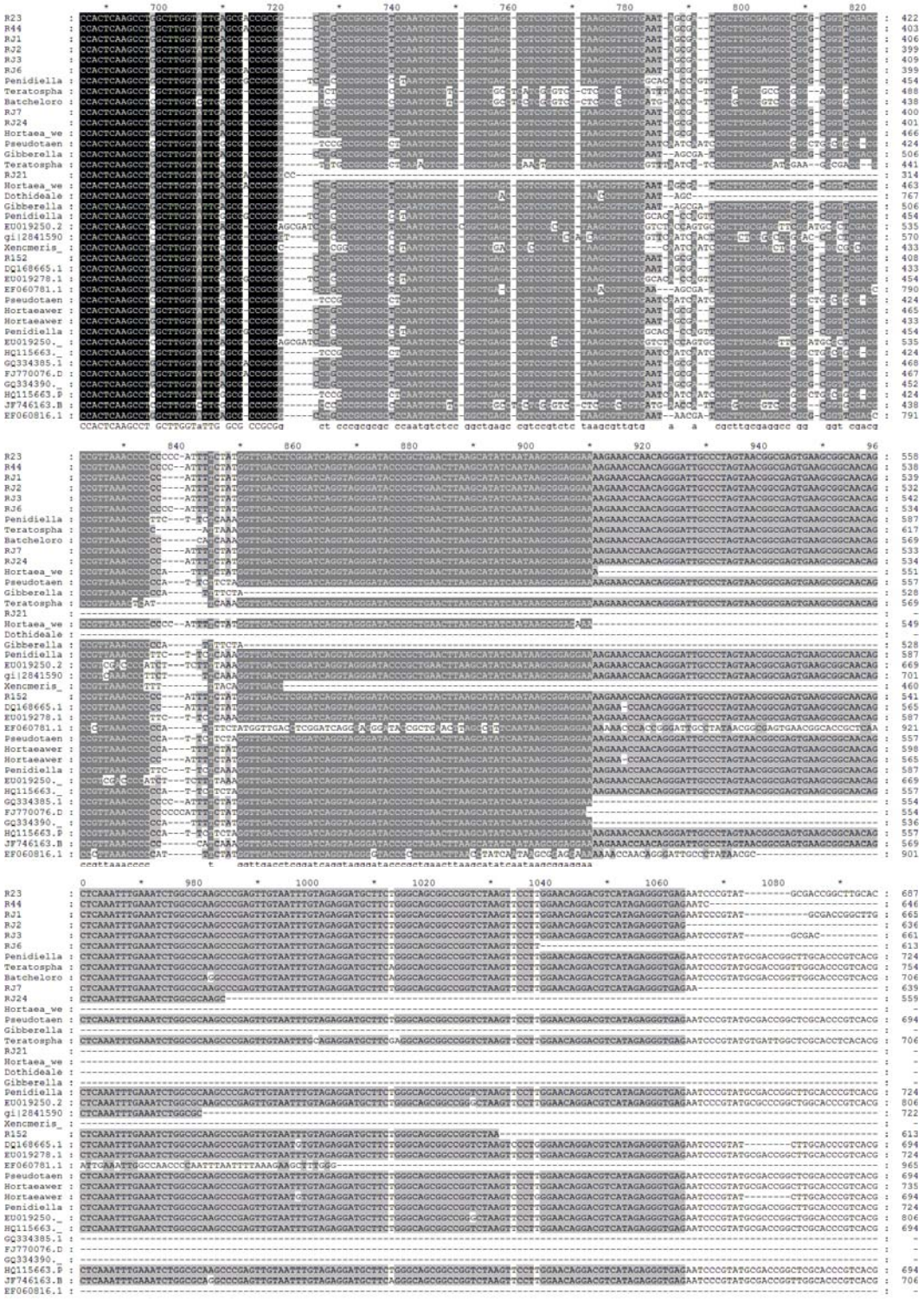


Fig.4.4b Neighbour joining phylogenetic tree based on partial sequences of ITS sequences, showing the relationship among the species of the genus *Rhodotorula.* Bootstrap values are shown as percentages from thousand replications at branch points

Even though 16 black yeast isolates were obtained in the study, 10 isolates were sequenced and all of them belonged to the same genera and species *i.e, Hortaea werneckii* and the isolates R23, R44, RJ2, RJ3, RJ6, RJ7, RJ21 had 100% similarity to the *Hortaea werneckii* (GQ334390, GQ334385, GQ334387) and 97% similarity to *Hortaea werneckii* type strain ATCC 36317 (DQ 168665). The isolates R152, RJ1 and RJ24 lie in the same clade to GQ334389 (Fig 4.5a,b; Fig 4.6c,d).





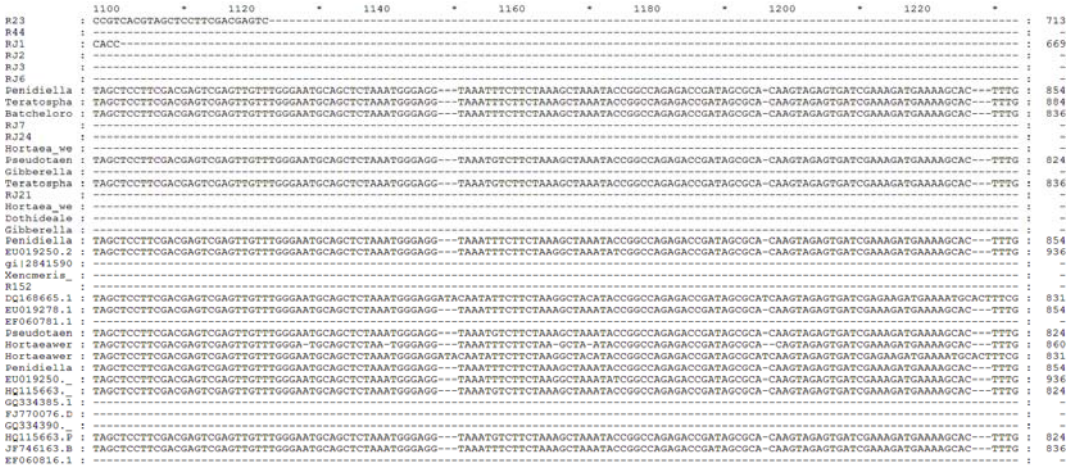


Fig.4.5a Clustal W multiple alignment of the ITS sequences from *Hortaea* strains isolated during the present study and phylogenetically related strain sequences from NCBI

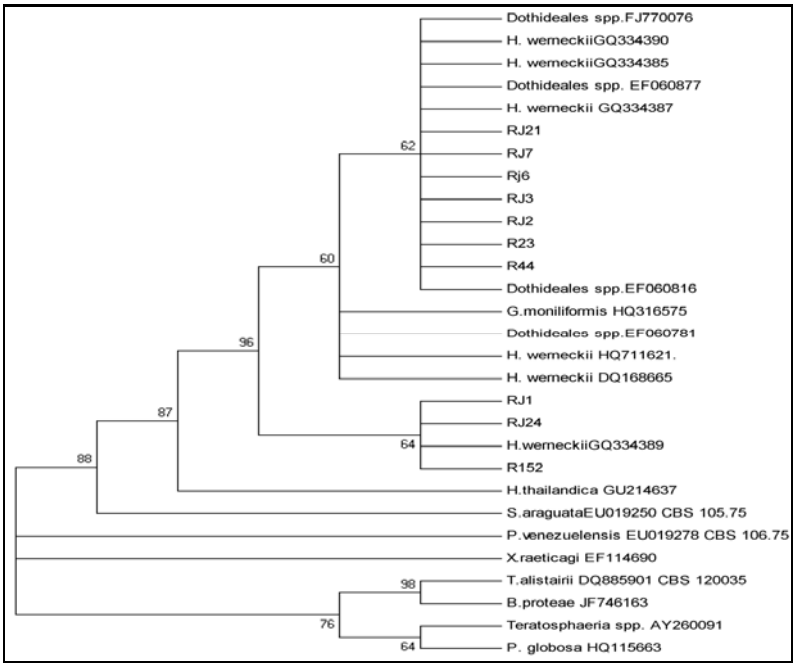


Fig.4.5b Neighbour joining phylogenetic tree based on partial sequences of ITS sequences, showing the relationship among the different isolates of the genus *Hortaea*. Bootstrap values are shown as percentages from thousand replications at branch points

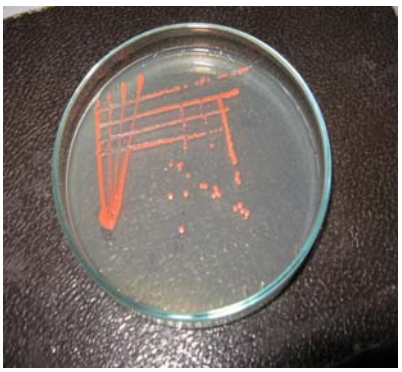


Fig.4.6a .*Rhodotorula* colony

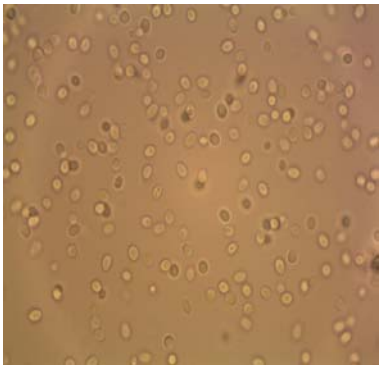


Fig.4.6b *Rhodotorula* – micromorphology

Fig 4.6c *Hortaea* colony

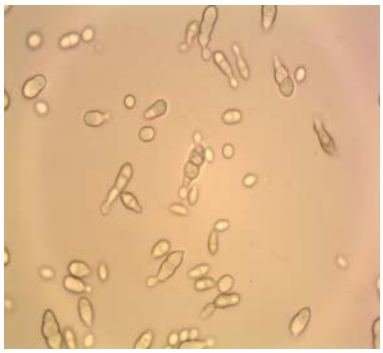


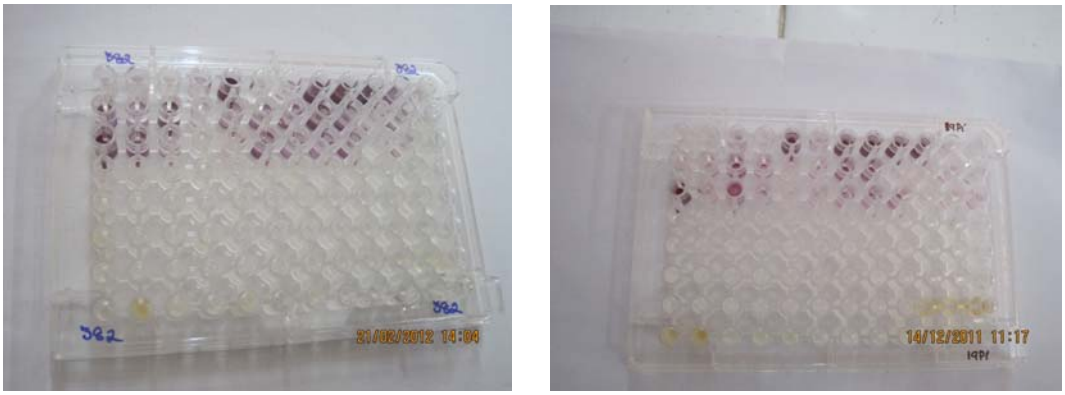
Fig 4.6d *Hortaea* – micromorphology

Fig.4.6(a-d) Different types of Pigmented yeasts

4.3.4. Physiological Characterisation:

Biolog YT microplates were used for the physiological characterisation of different species. In the characterisation using YT microplates maximum % of positive wells (82.97%) as well as wells with active growth (54.25%) was shown by R19 (*R. calyptogenae*) followed by R382 *i.e*., *R. minuta (R. minuta* was able to use 50% of the various carbon sources used in well plates with an active growth of 43.61%. *R. slooffiae* and *R. mucilaginosa* exhibited an active growth only in few wells *i.e*., 18.08% by the former and 10.63% for the latter respectively (Fig

4.7a,b). The only spp. in the black yeast, *i.e., Hortaea werneckii* (R23;Acc.No. JQ665422) was able to use 72.34% of carbon sources used in micro well plates.



R. minuta ***R. calyptogenae***

Fig. 4.7a Physiological characterization of *Rhodotorula* isolates using Biolog YT plates

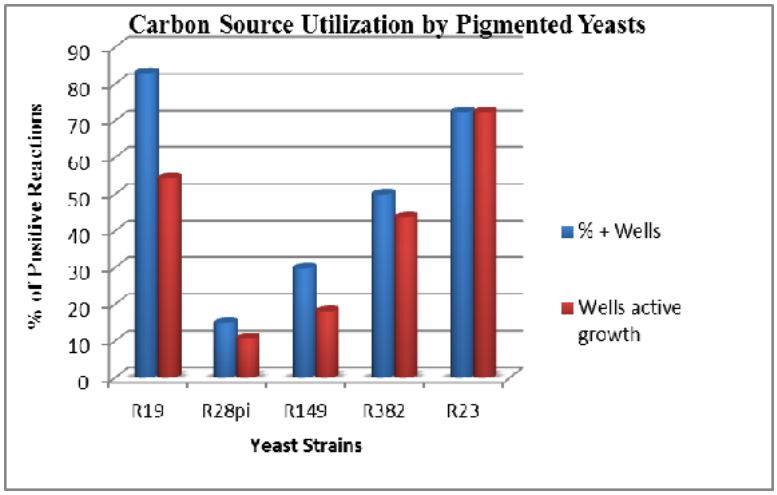


Fig. 4.7b Graph showing percentage of carbon source utilization by pigmented yeasts.

R. calyptogenae (R19pi; Acc. No. **JQ665424.1**): 90.5% of characters were similar to the type strain CBS 9125 and 77.65% to the strain 4107. The sugars D-

raffinose, α methyl-D- Glucoside and maltose were assimilated by R19 but not by the strain CBS 9125.

R. slooffiae (R28pi., Acc.No. **JQ665423.1**): Except for the sugars L-arabinose, D-arabinose, D- ribose all the other carbon sources utilization were similar to the type strain CBS5706.

R. mucilaginosa (R149pi., Acc.No. **JQ293997.1**): 83.3% of the characteristics were similar to the type strain CBS 316. But the carbon sources, *i.e.*,L-arabinose, D- arabinose, D- ribose., α methyl-D- glucose, and xylitol have shown a deviation from the type strain.

R.minuta (R382pi., Acc.No. **JQ665425.1**): 80% of the characters were similar to the type strain CBS 319. The carbon sources which exhibited a deviation of reaction from the type strain included maltose, raffinose, and trehalose.

In general, among *Rhodotorula* spp., *R. mucilaginosa* was unable to oxidise any of the alcohols used, but it was able to assimilate 4 alcohols (maltitol, D-mannitol, adonitol and glycerol) used in microwell plates, while *R. calyptogenae* , (maltitol), *R. slooffiae* (D. mannitol) and *R. minuta* (D-mannitol) was able to assimilate only one alcohol each, other than glycerol. The black yeast was able to assimilate all the 7 alcohols except adonitol (Appendix Table 4.2, 4.3)

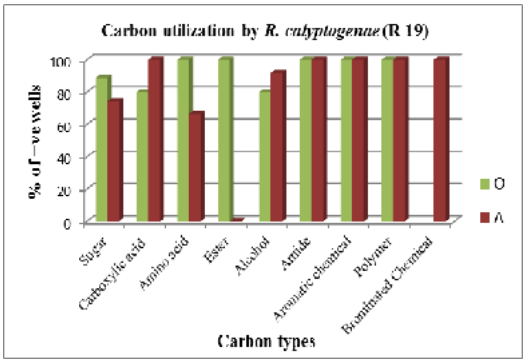


Fig.4.8 a

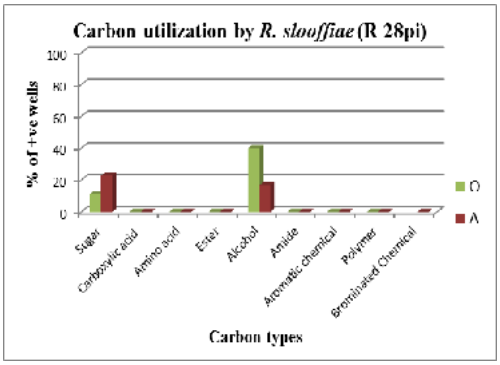


Fig.4.8 b

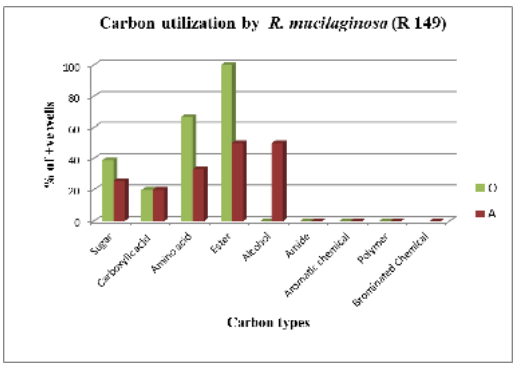


Fig.4.8 c

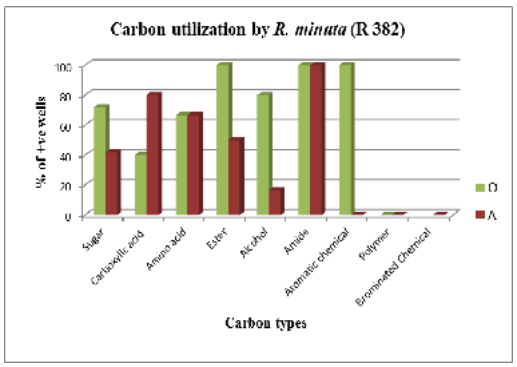


Fig.4.8 d

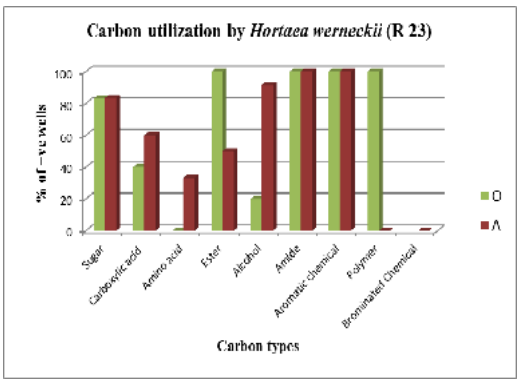


Fig.4.8 e

Fig.4.8 a-e Graph showing variation in utilization of different carbon sources by various pigmented yeasts

4.4. Discussion:

Pigmented yeasts producing pink, red or orange pigmented colonies generally belong to the genera *Oosporidium, Phaffia, Rhodosporidium, Rhodotorula, Sporidiobolus* and *Sporobolomyces*. Morphologically, out of the 17 isolates producing pink pigmentation, 9 of the isolates were deeply pigmented and 8 lightly pigmented. An important characteristic of *Rhodotorula* species was the inability to assimilate inositol, which distinguishes *Rhodotorula* from *Cryptococcus* (Krejer van Rij, 1987).

The size of the PCR amplicons varied between 900-bp in *Rhodotorula minuta* and R. *calyptogenae* to 950-bp in *R. slooffiae* and *R. mucilaginosa.* RFLP patterns using *Hin f*1 enzyme is a good indicator for species identification in the 4 species of *Rhodotorula* obtained in this study. Two fragments were observed for *R. minuta* (425+200) and *R. slooffiae* and 3 fragments for *R. mucilaginosa* and *R. calyptogenae*. RFLP patterns for *R. minuta* and *R. mucilaginosa* were already reported (Mokhtari et.al., 2011; Carvalho et al.,2010: Esteve-Zarzoso et al.,1999). Our findings are in conformity with the previous studies, the only difference being in the fragment size, which is due to the variation in the primers used for amplification and thereby variation in the amplicon size. At the same time the present study helped to add the restriction patterns of *R. slooffiae* and *R. calyptogenae* (Table 4. 1). *R. calyptogenae* is the first report from the Arabian sea. It was isolated from the sediments (200,500 and 1000m depths) off Ratnagiri, (Lat N 1607' 70", 7227' 62" Long E). Mangalore (Lat N 12 53' 30", Long E 73 56 40"). *R. calyptogenae* was first isolated from a depth of 1156 meters on the sea floor of Sagami bay, Japan by Nagahama et al. (2003). *R. calyptogenae* was isolated from the surface layers in Taiwan by Chih-Jen et al. (2008). Recently *R.calyptogenae (*NIOCC#Y7), *R.slooffiae* (OTU28*)* and *R. mucilaginosa* (OTU37), were obtained from deep sea sediments of Central Indian Basin at a depth of 5000m (Singh et al., 2010, 2012). *Rhodotorula minuta* was obtained from deep sea, Pacific Ocean (Nagahama et al., 2001b), coastal sea water and South Portugal (Gadanho and Sampaio 2003) and oligotrophic lakes of Patagonia (Libikind et al.,2003). *Rhodotorula minuta* is also described as an emerging yeast pathogen found particularly in immuno compromised patients with hip joint infection (Cerikcioglu et al., 2005, Curtrona et al., 2002). As *R. mucilaginosa* is a ubiquitous species, there are reports on the occurrence of these pigmented yeasts in deep sea hydrothermal vents (Burgaud et al., 2010), deep sea sediments of Central Indian Basin at a depth of 5000m (Singh et al., 2010, 2012), deep sea coral, *Lophelia pertussa* (Galkiewicz et al., 2012), Mid Atlantic Ridge

hydrothermal fields (Gadanho and Sampaio, 2005) and acidic environments of Iberian pyrite belt (Gadanho and Sampaio, 2005).

In the physiological characterization using YT micro plates, 90 % of the reactions were in conformity with that of Chih-Jen et al., (2008). 31 of these reactions were compared with that of type strain JCM 10899 L-sorbose and lactose stimulate a weak reaction in type strain of *R. calyptogenae* (Nagahama et al., 2003a) However, strain R19 showed negative reactions at both test which is in conformity with the strain 4107 of Chih-Jen (2008).

When physiological characters are compared with that of the type strain, 80% of the characters are in conformity with the type strain in the case of *R. minuta*, 85% in case of *R. mucilaginosa* and 88% in case of *R. slooffiae*. Variations in physiological tests can occur due to culture conditions, age of culture, size of the inoculum and strain differences. The trouble with the physiological test is that they are similar in numerous species and there is no specific biochemical test to identify one species. In this aspect none of the phenotypic methods could help to identify the yeast at the species level (Mokhtari et al., 2011)

The genus *Hortaea* includes only one species, that is *H. werneckii*. Even though 16 isolates of black yeasts were obtained, they have phenotypical differences in the development of substrate mycelia, brown to dark pigmentation, size of the cells, as well as physiological variations in the production of enzymes. Hence 8 isolates were sequenced, but all of them belonged to the same genera and species that is *Hortaea werneckii*, the only difference is in the percentage of similarity. The restriction fragment polymorphic pattern of black yeasts are not reported yet. The present study also report the restriction pattern of *Hortaea werneckii* using the *Hinf*1, (500+250 bp) *Taq*1 (300+280+180-bp) and *Alu*1 (800-bp).

In the phylogenetic tree, R19 is in the same clade with *R. calyptogenae* (ACC NO: FJ51590), *R. cassicola* (ABO55201) and *R. samaneae* (ABO55199). According to Kurtzman et al. (2011), the nomenclature of *R. samaneae* is invalid. Nagahama et al., (2003b) reported that *R. calyptogenae* is nutritionally similar to *R. benthica*, but the two species can be distinguished on sucrose assimilation (positive for *R. benthica*, negative for *R. calyptogenae*) and salicin (negative for *R. benthica* and positive for *R. calyptogenae*). *R. calyptogenae* can be grown at 37^{o}C. All these physiological characteristics of *R. calyptogenae* are in agreement with the isolate R19; hence it is physiologically, molecularly and phylogenetically identified as *R. calyptogenae*.

The pigmented yeasts are quiet easily identifiable by their distinctive colouration that is pink, orange, red and black. The distinctive colour is the result of pigments, the yeasts synthesise to block out certain wave length of light that would otherwise be damaging to the cell. Colony colour can vary from genera to genera, species to species as well as due to the number of organisms in a single colony. As phenotypic methods may lead to an unreliable result in the identification of yeast species, separation of isolates based on phenotype and biochemical tests followed by RFLP and sequencing will be the best way for the accurate identification of pigmented yeasts.

Both *Rhodotorula minuta* and *R. slooffiae* are closely related and for a long time since the discovery *R. slooffiae* in 1962, it was considered as a synonym of *R. minuta*. Fell et al. (2000) compared the D1/D2 sequences of *R. slooffiae* and *R. minuta* and found them to be sufficiently distinct to exclude the hypothesis of conspecificity and in the analysis of ITS regions. Six variable sites were found in the ITS regions. Nutritionally both of them were unable to utilize maltose, hence physiologically it was difficult to distinguish between *R. minuta* from *R. slooffiae*, but in our physiological characterisation using YT plates, it was found that the isolate R28pi, (identified as *R. slooffiae*) was unable to assimilate / oxidise any of

the alcohols used in the YT plates, while *R-minuta* was able to assimilate 6 of the 9 alcohols and oxidise 5 of the alcohols used. The cosmopolitan basidiomycetous yeast species, *Rhodotorula mucilaginosa* was found in a wide range of natural habitats including living or decomposing plant constituents ,soil (Inacio et al.,2004: Polyakova et al., 2001: Sampaio, 1994: Slovikova and Vadkertiova, 2000) and various aquatic environments including fresh water lakes (Libikind et al. 2003), estuaries (Almeida, 2005), coastal waters (Gadanho et al., 2003), mid Atlantic ridge hydrothermal fields (Gadanho and Sampaio, 2005) open ocean and deep sea environments (Nagahama et al., 2001a., Gadanho and Sampaio, 2005), deep sea hydrothermal vents (Burgaud et al., 2010), deep sea sediments of Central Indian basin at a depth of 5000 m (Singh et al., 2010,2012) and deep sea coral, *Lophelia pertussa* (Galkiewicz et al., 2012). Moreover *R. mucilaginosa* is also present in extreme environments, such as ultra acidic waters (Gadanho and Sampaio, 2006), mid Atlantic ridge hydrothermal fields and acidic environments of Iberian pyrite belt (Gadanho and Sampaio, 2005), uranium lechate (de Siloniz et al., 2002) and cold environments (Butinar et al., 2007, Starmer et al., 2005). Of the 17 pink yeast isolates, 9 were *R. mucilaginosa* isolated from 200 - 1000m depths in the present study. Coelho et al. (2010) also reported that *R. mucilaginosa* constitute the dominant pink yeasts in Tagus river estuary and they form 24.8% of the total yeast population in Tagus river estuary. The salient physiological attributes of *R. mucilaginosa* are the inability to assimilate nitrate which separates *R. mucilagnosa* from common members of the *Sporidiobolales*, such as *R. dairenensis, R. colostri* and *Rhodosporidium*. Both R149 and R398a were unable to assimilate nitrate, confirming physiological similarities of the isolates (R149 and R398a) to *R.mucilaginosa*.

PCR-RFLP based on 5.8S-ITS amplicon restriction analysis was found to be a rapid, reliable, and accurate tool for the identification of yeasts of the genera *Rhodotorula*. This technique provided good results especially with the restriction

enzyme *Hinf*1 compared to *Taq*1 and *Alu*1 in terms of time and accuracy, but the existent database is insufficient to provide an accurate identification of all species from different environments/habitats. Hence an updating of previous database with the profiles is required for rapid identification of new isolates. However as more profiles are added to the database, identification may become increasingly difficult due to no or slight differences between the 5.8S-ITS profiles (Arias et al., 2002). Furthermore, it has to be considered that one single mutation in the 5.8S-ITS region could lead to the loss or gain of a restriction site, resulting in a completely different pattern (Arias et al., 2002). One alternative to overcome such an occurrence would be to sequence either the 26S r DNA or the 5.8S-ITS region and compare them with the presently available databases (Arias et al., 2002). Even though the cost is high, the use of the 5.8S-ITS restriction analysis followed by sequencing of representative isolates is an ideal method for rapid and accurate identification of environmental yeasts having pigmentation.

Chapter -5

PROXIMATE COMPOSITION OF YEAST BIOMASS OF VARIOUS GENERA AND THEIR BIOACTIVE COMPOUNDS

5.1. Introduction

The terrestrial yeasts have been receiving great attention in science and industry as they are good source of bioactive substances. Yeasts are rich source of proteins, amino acids, vitamins, polysaccharides, fatty acids, phospholipids, polyamines, astaxanthines, beta- carotene, trehalose, glucans and mannans. They produce lipase, chitinase, amylase, phytase, protease, killer toxins etc. Beer and Wine industries which use the yeast *Saccharomyces cerevisiae* had an yearly turnover of 100 billion dollars and created 10 million jobs all over the world. At present bioactive substances mainly come from terrestrial yeasts (Kurtzman and Fell, 1998; Dijken, 2002). Till now little is known about the bioactive substances from marine yeasts. Recently, it has been found that marine yeasts could produce bioactive substances with potential application in mariculture, food, pharmaceutical, cosmetic, chemical industries and environmental protection (Zhenming et al., 2006).

5.1.1. Single cell protein (SCP) and Amino acids

Yeasts have long been cultivated as rich sources of proteins, minerals, vitamins (particularly B vitamins), and other nutrients for humans and animals (Anupama and Ravindran, 2000; Bekatorou et al., 2006; Boze et al., 1995). Several yeast

species have been used for biomass production especially, *C. utilis*, other non-methylotrophic *Candida* spp., *Saccharomycopsis (Endomycopsis) fibuligera, Kluyveromyces* spp., and *S. cerevisiae*. Methylotropic, ethanol-utilizing, and fat- and hydrocarbon-utilizing yeasts including species of *Candida, Ogataea, Pichia* and *Trichosporon* have also been used for biomass production (Bekatoruou et al., 2006; Anupama and Ravindra, 2000; Boze et al., 1995). Production of yeast SCP has certain advantages compared to plant, animal and other microbial sources of SCP including rapid growth and accumulation of biomass, high protein content, high content of vitamin and minerals, and ability to grow on a wide variety of substrates, including various industrial waste streams (Halasz and Lasztity, 1991; Klein and Favreau, 1995).

The mariculture industry of developing countries especially China has been developing very rapidly in the past 20 years and the China holds the highest position in the world in the field of cultured sea food yield. Hence the demand of food for animals cultured in the marine environments is increasing steadily. But, there is still a lack of food particularly those composed of SCP from microbes with high protein content and other nutrients (Chi et al., 2006).

Microalgae such as *Spirulina, Chlorella* and brown algae are used extensively as food for mariculture (Anupama and Ravindra, 2000; Chi et al., 2006). Algae have some limitations for animal consumption as they have rigid cell walls. Production of algae is generally done out doors and depending on climatic conditions, they are easily contaminated by pathogenic protozoa and bacteria. Cells of bacteria contain a higher amount of nucleic acids than yeasts cells (Anupama and Ravindra, 2000; Gao et al., 2007). SCP from yeasts have many benefits over algae and bacteria, as their protein content constitute 50 % of the dry cell weight. Food incorporated with yeast cells rich in B-Complex vitamins, minerals and other components could stimulate the disease resistance of marine animals. Moreover yeasts contain a low level of nucleic acid content (9.7%) in

comparison to that in microalgae and bacteria (and higher concentration of essential amino acids). Further, the digestion rate of SCP of yeast cells is generally above 80% (Ravindra, 2000) and generally, yeast cells can grow very quickly reaching a cell density of over 10^8 cells ml^{-1}. At the same time, it is easy to manage the large scale yeast production in the fermentor. Collection and concentration of yeast cells from the liquid culture is easy, because of their flocculation and bigger cell size than bacterial cells. The production of value added SCP from inexpensive substrates could help to alleviate world shortage in the food supply, particularly in developing countries (Anupama and Ravindra, 2000). Many yeasts can grow well in cheap products like molasses, starch, cassava, Jerusalem artichoke, whey products, sulfite waste liquor, Potato wastes, brewery wastes and other waste streams from agriculture processes, food processing and industrial processes (Boze et al., 1995; Halasz and Lasztity, 1991; Klein and Favreau, 1995; Ozyurt and Deveci, 2004).

However, previous reports indicate that nucleic acid safety in algae is better than that in fungi and bacteria (Anupama and Ravindra, 2000). Fungal sources can be exploited as nutritive SCP if the nucleic acid content is considerably reduced to levels comparable to nucleic acid content of algae. It has been reported that two polyunsaturated fatty acids (PUFA) 20: 5n-3 and 22:6n-3 are absent in yeast cells and thus if yeast strains are to be used in aquaculture they have to be supplemented with live algae rich in those two PUFAs (Brown et al.,1996).

Only very few researchers have worked on marine yeasts, hence only little is known about marine yeasts that have high protein content and could be used as aqua feed. 33 strains of marine yeasts were isolated by Rishipal and Philip (1998) from the coastal and offshore waters of Cochin in India and found that the isolates had the ability to degrade starch, gelatin, lipid, cellulose, urea, pectin, lignin, chitin. The isolate M15 of *Candida* sp. when inoculated into the prawn shell waste, resulted in the increase in protein content of the final products from 38.55

to 70.4% on transformation of prawn shell waste. This finding promises the microbial transformation of prawn shell, a waste in shrimp processing becoming a valuable raw material in a novel industry aiming at the production of aqua culture feed from shrimp processing waste. Such transformation will help to recycle the waste and reduce pollution. Brown et al. (1996) evaluated the possibility of using marine yeasts as feed for bivalve aqua culture and found that *D.hansenii* (ACM4784), *Dipodascus capitatus* (ACM 4779), *Dipodascus* sp.(ACM 4780), and *Candida utilis* (ACM4774) contained 23%, 32%, 36% and 42% of crude protein respectively. They concluded that marine yeasts in general had high protein, carbohydrate and good amino acid composition. Some of them had high levels of saturated fats. However all marine yeast strains lacked two polyunsaturated fatty acids (PUFA'S, 20: 5n-3 and 22:6n-3) making them unsuitable as a complete diet for larvae. Chi et al. (2006) determined the crude protein content of 327 marine yeast isolates and found that the strain YA03A isolated from the surface of Sargassum from Penglai coast had 56.38% of crude protein content on dry cell weight basis.

5.1.2. Carotenoids

Carotenoids are produced by yeasts, bacteria and fungi. Red yeasts, which accumulate carotenoids, belong to the basidiomycetous classes, such as *Rhodotorula*, *Rhodosporidium* and *Sporobolomyces.* The major pigments are β-carotene, γ-carotene, torulene and torularhodin (Simpson et al., 1971).

Carotenoids are natural pigments responsible for the pleasing yellow, orange and red color of many foods, *e.g.*, fruits, vegetables, egg yolk, fish like salmon and trout and crustaceans. Besides being natural pigments, carotenoids also have important biological activities. It is well known that some carotenoids are precursors of vitamin A. In more recent years, vitamin A-active and inactive carotenoids have been found to have other beneficial effects on human health;

enhancement of the immune system and reduction of the risk for degenerative diseases such as cancer, cardiovascular diseases, macular degeneration and cataract (Astorg, 1997; Bendich, 1994; Krinsky, 2001). Thus, carotenoids constitute one of the most valuable classes of compounds for industrial applications, *e.g.*, pharmaceutical, chemical, food and feed industries. Colour is an important attribute that determines consumer's acceptance of foods. The addition of coloring agents in processed foods has been a common practice for many years. Due to the possible toxicity of artificial coloring agents, natural coloring alternatives have been increasingly sought. The huge international market for carotenoids has been met mainly by synthetic carotenoids with structural identity to those of natural carotenoids, but there is growing demand for natural sources. Traditionally, carotenoids have been marketed as dried powder or extracts from plants, such as annatto, paprika and saffron. Natural colorants from plant sources, however, suffer from a diminishing or unstable supply of raw materials, subjected to climatic conditions, as well as varying colorant level and quality of the final product. Microbial carotenoids have attracted much attention in recent years (Johnson and Schroeder, 1995; Nelis and De Leenheer, 1991). The main reason for the interest in using microorganisms to produce compounds that can otherwise be isolated from plants and animals or synthesized is the ease of increasing production by environmental and genetic manipulation. The commercial utilization of microorganisms with biotechnological potential to produce carotenoids is presently limited by the high cost of production. However, the cost of carotenoid production by fermentation can be minimized by optimizing its process, using highly pigment-producing microorganisms cultured in cheap industrial by-products as nutrient sources (Aksu and Eren, 2005). Carotene from the microalga, *Dunaliella salina* is being produced and commercialized by Australia, Israel and the United States. Recently, astaxanthin has also been produced commercially in the United States by another microalga, *Haematococcus pluvialis* (Dufosse et al., 2005). Microorganisms have the

capacity to produce carotenoids, such as the red yeasts of the genera *Rhodotorula*, *Rhodosporidium* and *Sporobolomyces*. The carotenoid consist of carotene, torulene and torularhodin (Simpson et al., 1971). Torulene is a typical carotenoid found in yeasts. It is a very interesting carotenoid due to its structure, half - carotene and half lycopene, which is responsible for provitamin A activity and antioxidant property.

Of the major carotenoids found in the yeasts, torulene, the major carotenoid of *R. mucilaginosa*, is an interesting carotenoid for commercialisation. Having 13 double bonds, it has a nice reddish colour, in contrast to β-carotene, which has a yellow to orange colour, depending on the concentration. Probably because it has not been found in food, possible effect of torulene on human health has not been studied. Structurally, however, this compound fulfills the requirement for provitamin A, *i.e*., a β-ring without substituents and a lateral polyene chain of 11 carbons. Since the antioxidant property has been associated with the conjugated double bond system, and the efficiency being greater with a higher number of double bonds (Foote et al., 1970; Terao, 1989), this carotenoid should also be an efficient antioxidant. In fact, torularhodin, the carboxylated derivative of torulene, was found to be more potent in quenching singlet oxygen and scavenging peroxyl radicals than β-carotene through *in vitro* studies (Sakaki et al., 2001; Sakaki, et al., 2002). β-carotene is the principal carotenoid found in *R. glutinis*, but besides being marketed as nature identical synthetic carotenoid, commercial natural source of this carotenoid is already available, particularly from the micro alga, *Dunaliella salina*.

5.1.3. Ergosterol

Ergosterol is known to occur in both plant and animal kingdoms, but the fungi, and particularly the yeasts, remain its practical source. Ergosterol, a precursor of vitamin D_2 can be transformed into vitamin D_2 by U-V irradiation. It is also the

main precursor of cortisone and hormone progesterone and an additive of fodder to increase the laying and hatching rates of fowls.

Ergosterol is the primary sterol in the cell membrane of filamentous fungi and is either absent or present in very small quantities in higher plants. It is also present in the yeast cell wall, mitochondria and it is a constituent of membranes in mycelia, spores and vegetative cells. The major function of sterols in eukaryotic cells has been considered to be a structural influence on the dynamic state of membranes in acting on phospholipid-protein interactions, membrane permeability and membrane-bound enzyme activity. However, sterols occur both unesterified or esterified with fatty acids and may vary structurally in several cells or mutant strains. The level of sterol components has generally been determined to range from 0.03 to 4.6% of the cell dry weight, accounting for < 1 to 10% of the total cell lipid. Yeasts of the genus *Saccharomyces* are particularly rich in sterols; ergosterol has been identified as the major sterol in yeasts and can account for 90% of the total sterol (Rattray, et al., 1975; Elliot, 1977).Various factors influence the synthesis of sterols by yeasts. In particular, the composition of the growth medium and the stage of the growth cycle, and also the level of various environmental parameters in the course of cell cultivation have been noted to have an effect on the amount of sterol produced (Hunter and Rose, 1972; Pichova et al., 1995; Novotny et al., 1987). Bills et al. (1930) surveyed strains of 13 species in five genera, including *Endomyces, Nadsonia, Mycoderma, Saccharomyces, and Zygosaccharomyces.* Ergosterol content was approximately 0.2 to 0.3 per cent in all cultures except those of the genus *Saccharomyces* which varied greatly in their ergosterol content. The maximum ergosterol content found by these workers appears to be that of 2.4 percent in *Saccharomyces carlsbergensis* (Bills et al., 1930; Prickett et al., 1930). This group later investigated the effect of the carbohydrate source on ergosterol production by a Frohberg bottom type culture of *S. cerevisiae*. They concluded that the principal determinant of the ergosterol

content was sugar; ergosterol being primarily a product of carbohydrate metabolism. The ergosterol content bore no relation to non-ergosterol lipid nor to the protein content, or to the state of starvation or nourishment of the yeast (Massengale et al., 1931). Maguigan and Walker (1940) in studying baker's yeast concluded that sterol production was accompanied by a corresponding increase in total lipid. According to them, ergosterol formation was a feature of aerobic metabolism and arose early in fermentation, the ergosterol content increasing at a greater rate than growth.

Ergosterol content has been widely used as an estimate of fungal biomass in various ecosystems in soil and aquatic environments. Even though, a high correlation has been found between ergosterol content and fungal dry mass, the concentration of ergosterol does not always correlate with absolute fungal biomass since its amount in fungi depends on age of the culture, developmental stage, and growth conditions (Pasanen et al., 1999). Hence, application of this method to environmental samples is limited (Gors et al., 2006). Ergosterol is labile and undergoes a rapid degradation and cell death, hence a lot of environmental microbiologists use this molecule as an indicator of living fungal biomass, but not for total biomass. Ergosterol assay is considered as a promising method for the detection of fungi, because of the specificity of ergosterol to fungi, the fact that it indicates live fungal mass, (ergosterol becomes oxidized upon cell death) and the relative constancy of the conversion factors compared to other alternative methods (Parsi and Gorecki, 2006).

5.1.4 . Glucan and Mannan

β-Glucans (beta-glucans) are polysaccharides of D-glucose monomers linked by β-glycosidic bonds. They are a diverse group of molecules that can vary with respect to molecular mass, solubility, viscosity, and three-dimensional configuration. β-glucans occur in plants, bran of cereal grains, cell wall of

Saccharomyces cerevisiae, cell wall of certain fungi, mushrooms and bacteria. The most active forms of β-glucans are those comprising D-glucose units with (1, 3) links and with side-chains of D-glucose attached at the (1, 6) position. These are referred to as β-1, 3/1, 6 glucan. One of the most common sources of β (1, 3) D-glucan for supplement use is derived from the cell wall of *Saccharomyces cerevisiae*. However, β (1, 3) (1, 4)-glucans are also extracted from the bran of some grains such as oats and barley, and to a much lesser degree in rye and wheat. The β (1, 3) D-glucans from yeast are often insoluble. Those extracted from grains tend to be both soluble and insoluble. Other sources include some types of seaweed (Teas, 1983), and various species of mushrooms such as Reishi, Shiitake, and Maitake (Wasser & Weis, 1993).

Yeast and medicinal mushroom derived β-glucans are notable for their ability to modulate the immune system. Research has shown that insoluble (1, 3/1, 6) β-glucan, has greater biological activity than that of its soluble (1, 3/1, 4) β-glucan counterparts (Ooi and Liu, 2000). β-glucans are known as "biological response modifiers" because of their ability to activate the immune system (Miura et al., 1996). Immunologists at the University of Louisville discovered that a receptor on the surface of innate immune cells called Complement Receptor 3 (CR3 or CD11b/CD18) is responsible for binding to beta-glucans, allowing the immune cells to recognize them as "non-self"(Vetvicka,2007). Regular consumption of beta-glucans contributes to maintenance of normal blood cholesterol (Bresson et al., 2009). The tumoricidal properties of beta-glucans have been studied in several *in vitro* and *in vivo* animal models (Mansell et al., 1975; Morikawa et al., 1985b). Studies in mouse shows that that beta 1,3 glucan in conjunction with gamma interferon is able to inhibit tumors and liver metastasis. Further, it is also reported that, β -1,3 glucans are also able to enhance the effects of chemotherapy, but β 1,3 glucans did not reduce tumour incidence but it was associated with reduced mortality in combination with cyclophosphamide (Thompson et al., 1987).

Similarly, a study conducted by the Canadian Department of Defense showed that orally administered yeast β-glucan given with or without antibiotics protected mice against anthrax infection (Vetvicka, 2002). Yeast derived beta glucan significantly enhanced phagocytic activity in an experimental mouse and its potential role in the prevention of sepsis following surgery was reported by Browder et al. (1984;1990).

The immunostimulatory effects of yeast glucan in marine animals have been reported in several studies. The intraperitoneal injection of β 1, 3- immune glucan and β 1, 6-glucan from cell walls of *S. cerevisiae* into Atlantic salmon led to enhanced resistance to *V. anguillarum, V. salmonicida* and *Y. ruckeri* (Robertsen et al., 1990). Chen and Ainsworth (1992) reported that catfish injected with yeast glucan showed increased resistance to *E. ictaluri*. Oral administration of yeast glucan in Atlantic salmon can improve its protection against infection of *V. anguillarum and V. salmonicida* (Raa et al., 1992). Tiger shrimp immersed in yeast glucan solution (0.5 and 1 mg mL^{-1}) showed increased protection against *V. vulnificus* infection (Sung et al., 1994).

Yeast glucan also has adjuvant effects on marine animals and the abilities to enhance the lysozyme activity, complement activity and bacteria-killing activity of macrophages of marine animals and production of superoxide by macrophages or hemocytes in some marine animals (Sakai, 1999).They also found that β -1,3-glucan from *Schizophyllum commune* had similar function in marine animals (Sakai, 1999).

The yeast cell wall comprising 35 to 40% of the yeast cell, is made up of mannans and β glucans which surrounds the yeast cell. The outer layer is mannan protein which is also known as mannan oligosaccharides or MOS. Mannan is a non-digestible protein- carbohydrate, which can be added to an animal's total feed and it's complex nature prohibits it being digested by the animal. Hence it is available

as a nutrient for the resident bacteria in the gut which grow rapidly in the gut and provide an improved defense against harmful bacteria. As mannans assists in the prevention of the establishment of harmful bacteria in the gut it is called a prebiotic. The pathogenic bacteria have proteins on their surface called lectins that recognize sugar compounds on the animal's cells lining its gut. These sugar compounds allow the infectious bacteria to bind to the gut lining. To prevent the establishment of pathogenic bacteria, it is necessary to stop the binding process. The mannans prevent the establishment of infectious bacteria by getting between the lectins on the bacteria and the sugar compounds on the intestinal lining. The lectins attach to the mannans instead of the sugars on the cells of the gut wall lining. Mannans thus helps to maintain a healthy immune system, supports natural defences, promotes a healthy gut flora, maintains and promotes gut health, and they can acts as a macrophage as well. Hence mannan is an important component of many speciality animal feeds formulated overseas, particularly in quality dog feeds manufactured in the US since the 1990's. Mannan has also been recognized for its ability to protect and foster juvenile animals and subsequently is now included as a vital ingredient in a wide range of overseas formulated commercial milk replacers and animal health products. Yeast cell wall polysaccharides have been used as adjuncts for animal and fish feeds (Sauerwein et al., 2007). These polysaccharides have been proposed to promote animal growth and health by various mechanisms, including immuno modulation, oxidative status, binding of toxins and pathogens, and interactions with gut constituents (Kurtzman et al., 2011). 5-10 grams of mannans is recommended daily for cattle, 25 grams for horse, 500 grams for pigs, 250 grams to 500 grams for goats and sheep (www.fermex.com.au/img/File/YCWM).

Glucans and mannans have also been found to have a myriad of biological functions in animal models and potentially in humans, including modulation of

histamine release (Holck et al., 2007), and antitumor activities (Ghoneum et al., 2007).

5.1.5. Feed Enzymes from Yeasts

The primary enzymes used in animal feed are xylanases, β-glucanases, and phytases (Aehle, 2004; Kirk et al., 2002; Pandey et al., 2001). Xylanases and β-glucanases assist in digestion of polysaccharides in monogastric animals, which in contrast to ruminants, have limited ability to fully digest plant-based feeds containing high quantities of cellulose and hemicellulose. Commercial feed enzymes are produced by bacteria and filamentous fungi (Aehle, 2004, Kaur et al., 2007; Pandey et al., 2001). However, feed enzymes are increasingly being produced heterologously in yeasts with the advent of highly efficient expression systems (Aehle, 2004; Cregg, 2007).

Phytases have emerged as prominent feed enzymes to enhance the utilization of phosphate, which is 85-90 % bound in phytic acid. Phytase catalyses the release of phosphate from phytate (myco-inositol hexakiphosphate), which is the main form of phosphorus predominantly existing in cereal grains, legumes and oilseeds (Pandey et al., 2001). Phytases can be incorporated into commercial poultry, swine and fish diets and has a wide range of applications in animal and human nutrition as it can reduce phosphorous excretion of monogastric animals by replacing inorganic phosphate in the animal diet. They contribute significantly towards environmental protection as phytases can diminish the release of phosphate as a pollutant into the environment and leads to improved uptake of other nutrients, especially essential metals (Kirk et al., 2002), trace elements, amino acids and energy (Haefner et al., 2005). Since the recognition of bovine spongiform encephalopathy (BSE), certain countries have banned the use of bone

meals in feeds, which was a traditional source of phosphorous, and this policy has enhanced the use of phytases. In 1999, the annual sale of phytases as an animal feed supplement was estimated to be 500 million US dollars (Kaur et al., 2007). Phytases are also finding uses in non-traditional agriculture industries such as aquaculture (Kaur et al., 2007), in which feed components cost up to 70% of overall fish production costs. Phytases have attracted interest for improvement of human nutrition especially to counteract zinc and iron deficiencies (Kaur et al., 2007). Several yeast species, including *Blastobotrys adeninivorans*, *Candida* spp., *Cryptococcus* spp., *Kluyveromyces lactis*, *Wickerhamomyces anomalus*, *Rhodotorula gracilis, Sacchro*myces spp., *Schwanniomyces* spp., and *Torulaspora delbrueckii*, produce phytases; very few of the enzymes have been extensively studied (Kaur et al., 2007). Due to the increasing maricultured animal density in many regions of the world, the released phosphorus will result in accumulation of phosphate in marine environments, leading to eutrophication of the sea water. Microbial phytase has been supplemented to marine animal diets. It was found that supplementation of microbial phytase also showed effects on other nutrients. For example, the improvements of Ca, Mg, Zn, Cu and Mn availability could be achieved in many trials. Besides improving the availability of minerals and trace elements, microbial phytase is also able to enhance protein digestability by the degradation of phytate protein, phytate mineral protein complexes and phytate amino acid complex and by enhancement of protease activity. As phytate can also bind starch and inhibit amylase, phytate supplement is also able to increase energy utilization in marine animals as well. Recently 10 strains of marine yeasts which can produce phytase was isolated from the guts of marine animals and from various marine environments (Chi et al., 2009). The strains were identified as *Hanseniaspora uvarum*, *Candida* sp., *Yarrowia lipolytica*, *Issatchenkia orientalis* and *Kodamaea ohmeri*, of which the strain *Kodamaea ohmeri* BG3 was able to produce more phytase than other strains.

Antioxidants from yeasts

Antioxidant can be defined as "any substance that, when present at low concentration compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" (Halliwell and Gutteridge, 1999). The main characteristic of an antioxidant is its ability to trap free radicals. Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids or DNA and can initiate degenerative disease. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydro peroxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases.

It is to be emphasized here that there is a great difference between "antiradical" and "antioxidant" activity and that they do not necessarily coincide. According to Burlakova and coworkers (1975) the antiradical activity characterizes the ability of compounds to react with free radicals (in a single free radical reaction), but antioxidant activity represents the ability to inhibit the process of oxidation (which usually, at least in the case of lipids, involves a set of different reactions).

Consequently, all test systems using a stable free radical (for example, DPPH, ABTS etc) give information on the radical scavenging or antiradical activity, although in many cases this activity does not correspond to the antioxidant activity. In order to obtain information about the real antioxidant activity with respect to lipids or food stabilization, it is necessary to carry out the study on the real product (plant oil, lipoproteins etc.) (Tirzitis and Bartosz, 2010).

Antioxidant activity has been expressed in various ways including the percentage of the reagent used, the oxidation inhibition rate and so on. An easier way to present antioxidant activity of foods would be to reference a common reference

standard. One common reference standard, (S)-(-)-6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid, also known as Trolox, serves this purpose.

Carotenoids produced by pigmented yeasts and ergosterol in yeasts have the ability to scavenge free radicals (Maldonade et al., 2008).

5.2. Materials and methods

5.2.1. Strains used for growth /biomass and nutritional evaluation

Representative strains (seven isolates) of various genera *i.e*., *Candida, Debaryomyces*, *Pichia, Rhodotorula* and *Hortaea* were selected for testing growth /biomass production and nutritional quality evaluation, which are important criteria for the selection of a strain for single cell protein production. The strains subjected to these analysis were R23 (*Hortaea werneckii*), R28w (*Candida spencermartinsiae)*, R89 (*C. oceani)*, R100 (*Debaryomyces fabryi)*, R149 (*Rhodotorula mucilaginosa*), R305 (*D. nepalensis)* and R340 (*Pichia guilliermondii*).

5.2.2. Estimation of yeast growth/biomass production:

Starter culture was prepared by inoculating freshly grown yeast cells into 20ml culture tubes containing 10ml culture broth.1ml of 0.1 O.D culture broth was inoculated into 100ml of YM broth containing glucose (1g/L) in a 250ml Erlenmeyer flask, incubated at room temperature (28 ± 2^{o}C) in a shaker at 150 rpm for 5 days. Cells were harvested by centrifuging the culture broth at 10,000 x g for 7 minutes and washing twice with distilled water. Biomass obtained was transferred to preweighed petri plates and freeze dried. Yeast biomass was expressed as cell mass (dry wt) per litre of broth (g/L) based on triplicate analysis.

5.2.3. Proximate composition of yeast biomass

Biochemical composition of the yeast biomass was analysed to assess their nutritional quality. Protein was estimated by the method of Lowry et al. (1951), lipid by phospho vanillin method following chloroform methanol extraction of the sample (Barnes and Blackstock, 1973) and carbohydrate by anthrone method (Hodge and Hofreiter, 1962).

Preparation of yeast biomass:

The selected seven yeast cultures were swab inoculated into malt extract agar plates incubated at 28± 2^{o}C for 72 hours and harvested with sterile saline. The cell suspensions were centrifuged at 7000 rpm for 10 min in a refrigerated centrifuge (Remi C-30, Mumbai) and the yeast biomass was lypophilised (Lyolab DPG-001) and stored at 4^{o}C in a refrigerator.

5.2.3.1. Estimation of protein:

The protein content of the biomass of selected marine yeast isolates was estimated by the method of Lowry et al. (1951). Reagents required for the determination of protein by Lowry's method (1951) are

1. Reagent A: 2% solution of $Na_2 CO_3$ in O.IN NaOH.
2. Reagent B: 0.5% solution of $CuSO_4$. $5H_2O$, in 1% solution of sodium potassium tartarate.
3. Reagent C: A mixture of 1ml of reagent B and 50ml of reagent A.
4. Folin's reagent : Readymade Folin's reagent can be used.
5. Bovine serum albumin standard: 20 mg in 100ml of Bovine serum albumin in 0.1N of NaOH.

Procedure:

20 mg of finely ground lyophilised yeast biomass was taken in a volumetric centrifuge tube and added 5mlof 1N NaOH. The tubes were then heated in boiling

water bath for 1 min. After heating, the tubes were cooled and their volume adjusted to 10ml with the addition of distilled water. The samples were then centrifuged for 5minutes at 3000 rpm. 5ml of reagent C and 1ml of supernatant were taken in a 20ml tube. These were vigorously stirred and left for 10mins, after which, to each tube, 0.5ml of Folins reagent, diluted with an equal volume of water is added. The resulting solution was blue in colour and the intensity measured spectrophotometrically at 750nm. The protein content of the sample was obtained by applying the measured OD to a calibration curve. The standard used was Bovine serum albumin. The amount of protein was expressed as mg/100mg dry weight yeast biomass.

5.2.3.2. Estimation of Lipids:

The lipid content of the yeast biomass was analysed by Phosphovanillin method following chloroform – methanol extraction of the sample (Barnes and Blackstock, 1973).

Reagents:

1. 2:1 Chloroform – methanol solution
2. 0.9% NaCl
3. Phospho vanillin reagent – To 100 ml of distilled water 400ml of orthophosphoric acid was added and 1g of vanillin dissolved in that solution.
4. Cholesterol standard – 10mg in 10ml of (2:1) chloroform: methanol solution.

Procedure:

500 mg of the sample was mixed well with 10ml of chloroform methanol solution (2:1) in a homogeniser. Filtered the homogenate through Whatman no.1 filter

paper. Added 2ml of 0.9% NaCl and shaken well. Transferred the mixture to a separating funnel, allowed to stand overnight at 4°C. Removed the lower phase that contained all the lipids and adjusted the volume to 10ml, by the addition of chloroform. The extract (0.5ml) was taken in a clean tube, allowed to dry in vacuum desiccator over silica gel. Dissolved in 0.5ml of conc. H_2SO_4, mixed well, plugged and placed in a boiling water both for 10 min, and cooled to room temperature. To 0.2ml of the acid digest taken in a separate tube, added 5ml of vanillin reagent mixed and incubated for 30 minutes and measured the colour at 520nm. 0.2ml of chloroform served as blank and cholesterol (10mg in 10ml of (2:1) chloroform: methanol solution) as standard. A series of standards (cholesterol) were also run. The amount of lipid was expressed as mg/100mg dry weight yeast biomass.

5.2.3.3. Estimation of Total carbohydrates:

Total carbohydrates in the yeast biomass was determined spectrophotometrically by Anthrone method (Hodge and Hofreiter,1962). It is based on the reaction of Anthrone with furfural or furfural derivatives (formed by the hydrolysis of Oligo and polysaccharides into monosaccharides and subsequent dehydration) to produce a complex blue green coloured product with absorption maxima at 630nm.

Reagents

1. 2.5N HCl (21.8ml HCl+78.2ml distilled water)
2. Anthrone reagent (Dissolved 200mg Anthrone in 100ml ice cold (95%) Conc.H_2SO_4) .Prepared fresh before use.
3. Standard glucose
   (a) Stock: Dissolved 100mg glucose in100ml water.

(b) Working standard: 10ml of stock diluted to 100ml. with distilled water.

Procedure

100mg of the sample was hydrolysed in a boiling water bath for 3hrs with 5ml of 2.5 N HCl , cooled and neutralised with solid Na_2CO_3 until the effervescence ceased. The volume was made upto 100ml, centrifuged and the supernatant was collected. Different known volumes of standard were pipetted out. 1ml. distilled water was used as blank. Made up the volume to 1ml in all the tubes including sample tubes by adding distilled water. Added 4ml of Anthrone reagent, heated for 8 minutes in a boiling water bath, cooled rapidly and read the green colour at 630nm. Amount of carbohydrate was expressed as mg/100mg dry weight.

5.2.3.4. Estimation of ash in biomass:

The crucible containing a known amount of sample was kept in a muffle furnace at 550^0C for 5 hrs, when handling the crucible protect the sample from flies to avoid mechanical loss of sample. Carefully remove the crucible from the furnace directly into a desiccator and cool for a specific amount of time. Weighed the crucible plus ash and the value were recorded.

Calculation:

$$\% \text{ of ash: } \frac{(\text{wt of crucible + ash}) - (\text{wt of the crucible}) \times 100}{\text{Dry weight (sample).}}$$

5.2.4. Estimation of Carotenoids:

5.2.4.1. Organism used and preparation of yeast biomass:

Representative pigmented yeasts from each species, *i.e., Rhodotorula mucilaginosa, R. slooffiae, R.minuta* and *R.calyptogenae* were chosen for

carotenoid estimation. Selected yeast species were swab inoculated into Yeast Extract Sucrose (YES) (20g sucrose, 4g yeast extract, 1g KH_2PO_4, 0.5g $MgSO_4$. 7 H_2O, 2 g Agar, Sea water 1L, pH 7) agar plates, incubated at 28±2^oC for 7 days and harvested with sterile saline. The cell suspensions were centrifuged for 10 minutes at 12,000 rpm. The pellet was frozen at -20^oC.

5.2.4.2. Carotenoid extraction

The carotenoid extraction procedure was adopted from Martin et al. (1993) with modifications. 5gm wet cells were frozen at -20^oC, suspended (Merck) in 3-5 ml of pre-heated (55^oC) dimethyl sulfoxide (DMSO) by vortexing and incubated at room temperature (28±2^oC) overnight. Following centrifugation and collection of the DMSO phase, the pellet was suspended in 10ml acetone with a spatula by vortexing and centrifuged. The clear coloured acetone phase was decanted, and a further acetone aliquot was added, followed by suspension, centrifugation and decanting as before. If the pellet contained substantial residual pigmentation at this stage, a further DMSO incubation (1-3hrs) was carried, followed by two acetone washes as described above. All coloured DMSO and acetone phases were pooled and transferred to an extraction funnel containing an equal volume of light petroleum (b.p range 30-75^0C), 10ml distilled water, and in case of poor phase separation, 5-10ml saturated NaCl. Carotenoids were extracted into the light petroleum phase by gentle rotation, avoiding excessive agitation. The coloured light petroleum phase was washed 3 times with an equal volume of distilled water, adding saturated NaCl, if required for phase separation, dried over sodium sulphate and evaporated to dryness at 40^oC in dim light. Samples were kept on ice and light protected during the procedures.

Cell dry mass was determined after drying at 105^0C. The crude extract was taken to give a 100 fold volumetric concentration relative to the harvested culture broth. Freezing at -20^0C, resulted in the precipitation of colourless lipids which were

removed by filtration through a Whatman no. 4, filter paper, followed by freezing to –80°C and refiltration. The de-fatted crude extracts were filtered through 0.45µm syringe filter and the total carotenoids were assayed spectrophotometrically at 485nm (Roland et al., 2007).

5.2.5. Estimation of Ergosterol:

Ergosterol extraction was performed as described by Shawn and Jefferies (1953) with modifications. 100mg of lyophilized finely powdered yeast biomass was suspended in 3ml of methanol: KOH (40%) (25:10) and incubated at 90°C for 2 h previous to adding 3ml of distilled water. Petroleum ether (3ml) was used as solvent for ergosterol and the complete extraction was attained after 3 subsequent extractions. Solvent was evaporated and samples were suspended in petroleum ether (2ml) and the absorbance was measured at 283nm in a UV – Visible spectrophotometer (Hitachi-U-2900). Concentration of ergosterol was expressed as mg per g of dry yeast biomass (mg/g). The absorbance of ergosterol standard in series of concentrations was also measured (Moline et al., 2010).

5.2.6. Analysis of antioxidant / radical scavenging property

5.2.6.1. Scavenging of super oxide anion radicals

Scavenging of superoxide radicals was assessed according to Marklund and Marklund (1974). 3ml of 0.05 mol/L Tris HCl buffer and 1ml of sample solution of ergosterol at different concentrations were incubated at 25°C for 10 min and 500 µl of pyrogallol at the same temperature was added to the mixture, and the reaction was allowed to proceed at 25°C for 4 min. The reaction was terminated by the addition of 0.5ml of HCl. The absorbance of the mixture was measured at 420nm against the blank. Scavenging of superoxide anion radicals was calculated according to

$$\text{Scavenging ability (\%)} \quad 1 \quad - \quad \frac{\text{Ab sample x100}}{\text{Ab control}}$$

where Ab control is the absorbance of control (without the samples), and Ab sample is the absorbance in the presence of the samples.

5.2.6.2. Assay of 1, 1-Diphenyl – 2, picryl hydrazyl (DPPH) radicals scavenging ability:

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) is a stable free radical. On accepting hydrogen from a corresponding donor, the solutionlose the characteristic deep purple (λmax 515–517 nm) colour. DPPH is very popular for the study of natural antioxidants (Villano et al., 2007). The PubMed database shows that this radical has been employed in more than 850 studies since 1969 (Tirzitis & Bartosz, 2010). The antiradical activity of tested compounds is expressed as a relative or absolute decrease of concentration of DPPH or as IC_{50} (concentration of a compound decreasing the absorbance of a DPPH solution by 50 %). The rate of reaction of various antioxidants with DPPH differs (Janaszewska & Bartosz, 2002).

The scavenging effect of crude carotenoids extracted from *Rhodotorula mucilaginosa* and crude ergosterol extracted from *Candida oceani* was monitored according to the method described by Chen et al. (2008).

Reagents used:

1. 0.2mM solution of DPPH in 95% ethanol.
2. Samples (crude carotenoids and crude ergosterol)
3. 95% ethanol.

Procedure:

For the assay, 0.2mM solution of DPPH in 95% ethanol was freshly prepared. 1ml of samples (carotenoids and ergosterol) of different concentrations (0.06, 0.12, 0.18, 0.24, 0.30mg/ml) were thoroughly mixed with 2ml of freshly prepared DPPH and 2ml of 95% ethanol. After shaking vigorously, the mixture was kept for 30mins in the dark and then the absorbance was measured at 517 nm against a blank. The ability of the samples to scavenge the DPPH radical was calculated using the equation scavenging ability (I %) = $\left[1-\frac{Ai-Aj}{Ac}\right]\times 100$ where I% is percentage of inhibition. Ac is the absorbance of DPPH solution without sample (2ml DPPH + 3ml. 95% ethanol), Ai is the absorbance of the test sample mixed with DPPH solution (1ml sample + 2ml DPPH + 2ml 95% ethanol) and Aj is the absorbance of the sample without DPPH solution (1ml of sample + 4ml 95% ethanol). Assays were done separately for carotenoids and ergosterol.

5.2.6.3. Assay of ABTS (2, 2' – azinobis (3 – ethyl – benzothiazoline – 6 – sulphuric acid) Radical scavenging ability:

The peroxidase substrate 2, 2'-azino-bis (3-ethylbenzthiazoline- 6-sulphonic acid) (ABTS), forming a relatively stable radical (ABTS) upon one-electron oxidation, has become a popular substrate for estimation of total antioxidant capacity. Kinetic assays, including the commercialized TAS assay (Randox), are based on the inhibition of the formation of ABTS by one-electron oxidants (Bartosz & Bartosz, 1999; Bartosz, 2003). A simpler and more frequently applied approach is the decolorization of preformed ABTS (Re et al., 1999). An obvious drawback of ABTS-based assay is the promiscuity of reactions of ABTS which is a non physiological free radical (Tirzitis & Bartosz, 2010). ABTS radical scavenging assay was based on Miller and Rice Evans (1997).

Reagents:

1. ABTS stock solution: The ABTS stock solution was prepared by mixing 8mM of ABTS salt and 3mM of potassium per sulphate in 25ml of distilled water. The solution was held in the dark for 16h at room temperature.
2. Working Stock: The ABTS stock solution was diluted with 95% ethanol (approx 600µl of ABTS to 40ml of ethanol) to obtain an absorbance between 0.8 and 0.9 at 734 nm.
3. Samples of different concentrations (carotenoid and ergosterol)
4. 95% ethanol.

Procedure:

ABTS solution was prepared fresh for each analysis. 20µl of samples (carotenoids and ergosterol) were mixed with 1ml of diluted ABTS solution and incubated at 30^{o}C for 30 minutes. The absorbance was read at 734nm. Ethanol (95%) was used as blank. The free radical scavenging ability was calculated using the formula (I %) = $\left[1-\frac{Ai-Aj}{Ac}\right]\times 100$ where I% is percentage of inhibition. Ac is the absorbance of ABTS solution without sample (Control) (1ml of ABTS + 20µl ethanol). Ai is the absorbance of the test sample mixed with ABTS solution. (1ml of ABTS solution + 20µl sample) and Aj is the absorbance of the sample without ABTS solution (1ml ethanol + 20µl sample). ABTS scavenging ability was done separately for carotenoids and ergosterol.

5.2.6.4. Statistical Analysis

IC_{50} of the ergosterol and carotenoids were estimated for free radical scavenging/ antioxidant activity by probit analysis using SPSS 17.0.

5.3. Results:

5.3. 1.Yeast biomass and moisture content:

Biomass of yeast cells varied from 1.125g / L for *Rhodotorula calyptogenae* to 6.650 g/ L in *Rhodotorula mucilaginosa*. Unlike the pigmented yeasts, non-pigmented yeasts exhibited higher biomass and it varied from 4.262g/L to 7.868g/L. The moisture content of yeast biomass varied from 69.6% in *Debaryomyces fabryi* (R100) to 85.96% in *Rhodotorula calyptogenae* (R19). Among the 4 species of carotenoid producing yeasts, the amount of biomass produced in 5 days in 500 ml Erlenmeyer flasks varied greatly. *Rhodotorula mucilaginosa* is the highest producer of biomass (6.57 g/L) followed by *R. minuta* (3.62 g/L), *R. slooffiae* (2.06 g/L) and *R. calyptogenae* (1.125 g/L).

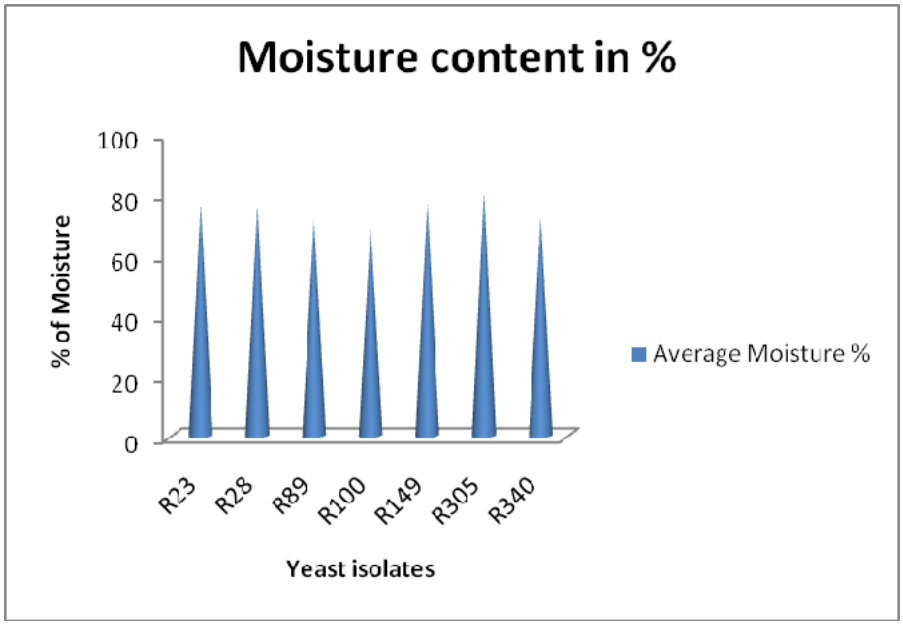


Fig 5.1 Graph showing the % of moisture content in the biomass of selected yeast isolates

5.3.2. Proximate Composition of yeast biomass:

Carbohydrate content of the yeast biomass of various strains belonging to 5 different genera was found to be in the range of 22 – 31%. 22% was observed in

Candida spencermartinsiae (R28w) and the maximum (31%) was found in *Candida oceani* (R89) (Fig 5.2a). The amount of protein in the different genera of yeasts varied between 25-43 %, the minimum in *Debaryomyces fabryi* (R100) and the maximum in *Rhodotorula mucilaginosa* (RI49). The amount of lipid in the various genera of yeasts was low compared to protein and carbohydrate content (5.2b). Lipid content ranged between 2-3%; maximum (3%) being observed in the strain R 89 belonging to *Candida oceani* (5.2c). The ash content of different genera varied from 3.71% in *Candida spencermartinsiae* to 5.9% in *Rhodotorula mucilaginosa*. *Pichia guilliermondii* (R340) and *Candida oceani* (R89) showed ash content of 5.42% and 5.2% respectively (Fig.5.2 a -d).

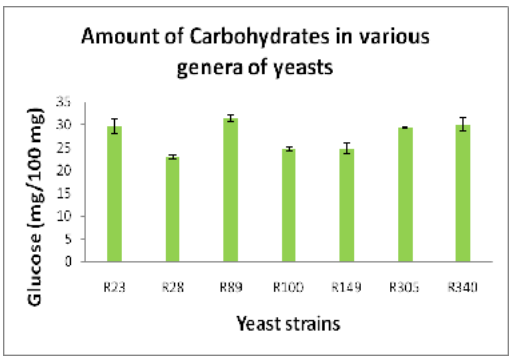


Fig.5.2a

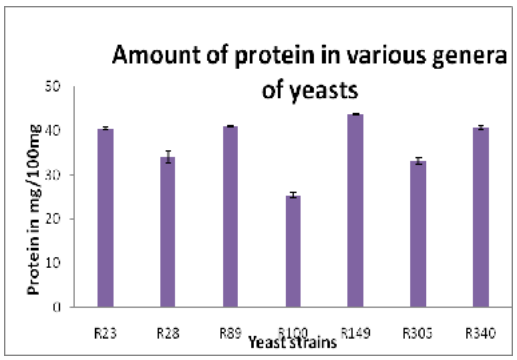


Fig.5.2b

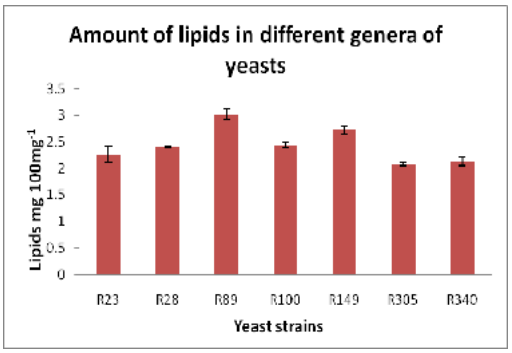


Fig.5.2c

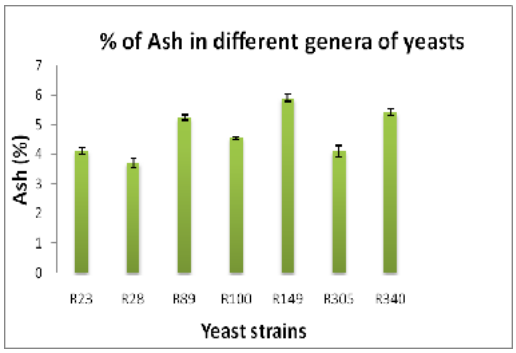


Fig.5.2d

Fig 5.2 a-d. Graphs showing the proximate composition of different yeast isolates

5.3.3. Carotenoids in *Rhodotorula*

Among the 4 species of *Rhodotorula* obtained from Arabian Sea and Bay of Bengal, the isolate *Rhodotorula slooffiae* produced the highest amount of carotenoids (322µg /gm dry weight). R19 belonging to *Rhodotorula calyptogenae* had the minimum amount of carotenoids (209.6µg/g dry weight). The carotenoid concentration in *Rhodotorula minuta* was about 225.6µg /g dry weight and in *Rhodotorula mucilaginosa* it was 315µg /g dry weight (Fig 5.3).

5.3.4. Ergosterol in yeast biomass

Ergosterol was found to be was highest in the isolate R 89 *(Candida oceani*) having 0.625 mg/100mg of sample and lowest in the black yeast R23 *i.e., Hortaea werneckii* (0.158mg/100mg). *Candida spencermartinsiae* (R28w) had an ergosterol content of 0.405mg/100mg of sample and *Pichia guilliermondii* about 0.338mg /100mg. The ergosterol content of the isolates R 100 and R305 was approximately 0.2mg/100 mg of sample (Fig. 5.4).

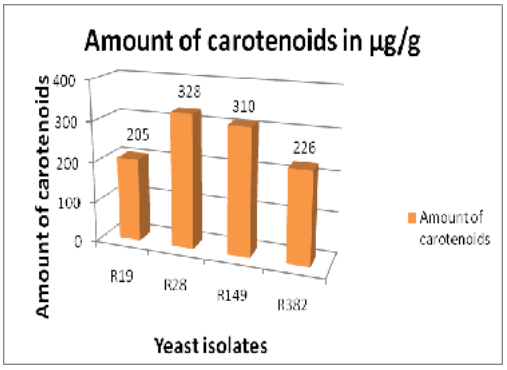


Fig 5.3

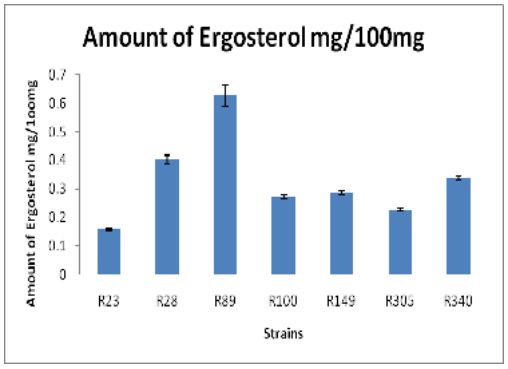


Fig 5.4

5.3.5. SCAVENGING CAPACITY OF ERGOSTEROL ON SUPEROXIDE ANION, DPPH and ABTS RADICALS.

The scavenging capacity of ergosterol on superoxide anion radicals was tested using pyrogallol. Crude extract of ergosterol from the isolate R89 belonging to *Candida oceani* was able to scavenge 25.75 ± 0.73 % of superoxide anion radicals generated by pyrogallol at a concentration of 0.06 mg/mL and the IC_{50} was 0.217 ± 0.03 mg/mL.

The crude extract of ergosterol dissolved in petroleum ether exhibited a dose dependent inhibition of DPPH radicals. At a concentration of 0.06mg/ml, the crude extract of ergosterol was able to inhibit 34.54 ± 0.68% of DPPH radicals which increased to 61.33 ± 0.95% as the concentration increased to 0.30mg/ml. Crude ergosterol from *Candida oceani* was able to scavenge ABTS radicals more effectively than DPPH radicals. At a concentration of 0.06mg/ ml crude ergosterol was able to scavenge 56.85 ± 0.63% of ABTS radicals which increased to 80.75 ± 1.11% as the concentration increased to 0.30 mg/ml (Table 5.1).

Table 5.1. Free Radical Scavenging / Anti oxidant activity

Concentration of Ergosterol (mg/ml)	Inhibition of DPPH radicals (%)	Inhibition of ABTS radicals (%)	Inhibition of Superoxide anion radicals (%)
0.04	nd	42.81 ± 1.48	nd
0.06	34.54 ± 0.68	56.85 ± 0.63	25.75 ± 0.73
0.12	39.55 ± 0.73	65.84 ± 0.80	35.61 ± 0.96
0.18	44.67 ± 0.58	71.31 ± 1.01	42.26 ± 0.73
0.24	51.16 ± 0.63	78.00 ± 0.59	51.77 ± 0.60
0.30	61.33 ± 0.95	80.75 ± 1.11	60.56 ± 0.74
IC_{50}	0.2 ± 0.039	0.051 ± 0.01	0.217 ± 0.03

nd – Not done

5.3.6. SCAVENGING ACTIVITY OF CAROTENOIDS ON DPPH, ABTS RADICALS

Carotenoids extracted from *Rhodotorula mucilaginosa* having a concentration of 302 µg/ml was used to assess the scavenging ability of carotenoids on DPPH radicals. The concentration used for the assay varied from 0.06 mg/ml to 0.30mg/ml. As the concentration of carotenoids increased from 0.06 mg/ml to 0.30mg/ml, there is a steady increase in scavenging of DPPH radicals. At a concentration of 0.06 mg/ml, the scavenging ability was about 38.66± 0.89 % which steadily increased to 81.93 ± 1.37 % at 0.30mg/ml. The IC_{50} was found to be 0.098 ± 0.011mg/ml.

As in the case of DPPH radicals, carotenoids was able to inhibit ABTS radicals. At a concentration of 0.06 mg/ml, the carotenoids exhibited 32.08 ± 1.29 % inhibition of ABTS radicals which slowly increased to 77.75 ± 1.03% at a concentration of 0.30mg/ml. 50% inhibition on ABTS radicals was found to be at a concentration of 0.116 ±.012 mg/ml (Table 5.2).

Table 5.2. Free Radical Scavenging Activity of Carotene (DPPH and ABTS)

Concentration of Carotenoid (mg/ml)	Inhibition of DPPH radicals (%)	Inhibition of ABTS radicals (%)
0.06	38.66 ± 0.89	32.08 ± 1.29
0.12	52.44 ± 0.92	51.96 ± 0.52
0.18	63.43 ± 0.80	59.34 ± 1.43
0.24	77.14 ± 1.40	69.59 ± 0.66
0.30	81.93 ± 1.37	77.75 ± 1.03
IC_{50}	0.098 ± 0.011	0.116 ± 0.012

5.4 DISCUSSION

Analysis of proximate biochemical composition of seven isolates belonging to different genera clearly indicated that the amount of protein was high (25-40%) in different species of yeasts examined and the amount of carbohydrate varied between 22-32%, whereas the amount of lipids was in the range 2-3%. High amount of protein in various species of yeasts have been reported in many previous works. Brown et al. (1996) reported proximate biochemical composition of seven different yeast stains belonging to the genera *Debaryomyces, Dipodascus*, and *Saccharomyces*. Brown et al. (1996) reported 25% protein in *Debaryomyces hansenii* (ACM4784) which was similar to the protein content obtained in the *Debaryomyces fabryi* (RI00) of the present study. At the same time, in the present study *Debaryomyces nepalensis* (R 305) showed a protein content of 33%. Chi et al. (2006) studied 327 yeast strains from various marine environments *i.e.,* sediment, mud of salterns, guts of marine fish, marine algae, and identified 8 strains of marine yeasts which contained more than 30.4 g protein per l00g of cell dry weight, and they belonged to *Metschnikowa reukaui, Cryptococcus aureus, Aureobasidium pullulan, Yarrowia lipolytica* and *Hanseniaspora uvarum*. *Hanseniaspora uvarum* (YA03A) isolated from the surface of sargassum had 56.38% of crude protein on the basis of dry cell weight. Of the 7 isolates used for the study, R149 belonging to *Rhodotorula mucilaginosa* had a high protein content (43%) which was also rich in carotenoids. Brown et al. (1996) also reported that the amount of carbohydrates in marine yeasts varied between 21-39%, lipids ranged from 2.5-9.0% and the ash content ranged between 4.7 to 14% of yeast dry weight. The amount of carbohydrates (22-32%), lipids (2-3%) and ash (3.5-5.5%) in the present study is in conformity with the above reports of Brown et al. (1996). Han et al. (1976) recorded a protein content of 44.3% in *Candida utilis* and Brown et al. (1996) have found 42% in the same species. But Kamel and Kawano (1986) reported the protein content of *Candida*

spp. as 34.9%. Sarlin (2005) reported that *Candida* contains 25-28% protein. However in the present study the protein content for *Candida spencermartinsiae* was found to be 33% and 40% for *Candida oceani*. Similarly a high protein content of 40% was observed for *Pichia* and the black yeast *Hortaea*. The amount of carbohydrates in the present study (22-32%) was closely similar to the earlier reports. High protein content and high carbohydrate content make yeast single cell protein a reliable nutrient source for animal feeds.

Unlike the previous reports (Kamel and Kawano,1986; Brown et al.,1996) on lipid content of yeasts, where a range of 1.05 to 7.7 was observed, the lipid content of the present study was low (2-3%). At the same time in the carotenoid producing yeast *Phaffia*, Sanderson and Jolly (1994) have reported a high lipid content of 23%. Similarly the ash content of yeasts in the present study varied from that of Brown et al. (1996) and Sarlin (2005) where the ash content was in the range of 4.7 to 14%; but in the present isolates studied, it was in the range 3.5-5.5%.

Marine yeasts with varied levels of proteins are ideal feed for Penaeid shrimps, as their protein requirements varies with species. Protein requirements of *Metapenaeus macleayi* appeared to be the lowest (27%) followed by *Litopenaeus setiferus* (28-32%) and *Penaeus monodon* (35-50%). The Kuruma shrimp, *Marsupenaeus japonicus*, one of the commercial shrimps in Japan requires 40-57% of protein in the diet. At the same time in general, shrimps require disaccharides and polysaccharides, hence diet rich in proteins and carbohydrates are ideal food for penaeid shrimps.

Ergosterol is a specific lipid of fungal membranes, absent in plants and animals, widely used as an index of fungal mass, particularly in the food agriculture industry. This special quality has also been exploited by pharmaceutical research as a specific target for antifungal drug development. Among the 7 isolates, from which ergosterol was extracted, the black yeast had the minimum quantity of

ergosterol (0.1%) and R89 belonging to *Candida oceani* had the maximum amount (0.6%) of ergosterol. The amount of ergosterol in *Pichia guilliermondii* and *Candida spencermartinsiae* was found to be 0.3% and 0.4% respectively. Isolates belonging to *Debaryomyces* (R100& R305) and *Rhodotorula mucilaginosa* (R149) had only 0.29% of ergosterol. This is in conformity with the previous works of Dulaney et al. (1954) and Moline et al. (2010). Dulaney et al. (1954) extracted ergosterol from 146 cultures belonging to 99 species and reported that ergosterol content of yeasts are generally low and rarely exceeded 0.4% except in *Saccharomyces*. Moline et al. (2010) quantified ergosterol from 15 strains of *Rhodotorula mucilaginosa* collected from Patagonia and the amount of ergosterol varied between 0.32 to 0.62% in different isolates. *Rhodotorula mucilaginosa* (R149) obtained in the present study had an ergosterol content of about 0.29%, which was slightly lower than the strains of *Rhodotorula mucilaginosa* from Patagonia but higher (0.11 ug/mg wet weight) than the clinical isolate *Rhodotorula mucilaginosa* obtained by Gomez-Lopez et al. (2011). Pasanen et al. (1999) reported that the average ergosterol content of yeasts were generally higher (37- 42 µg/mg) than the ergosterol content of filamentous fungi (2.6 to 14 µg/mg). However Gomez-Lopez et al. (2011) estimated the amount of ergosterol in 51 clinical yeast isolates of different genera and found that the amount varied between 0.06 µg/mg wet weight in *Dipodascus capitatus* to 0.23 µg/mg wet weight in *Candida tropicalis*. Recently ergosterol analysis has been suggested for quantitative monitoring of fungi in solid substrates, as there is a good co-relation between hyphal length and ergosterol concentration and mycelial mass, even with more than one fungal species present (Newell, 1992; Schnurer, 1993). In the studies conducted in indoor environments, the ergosterol content of house dust was used as a marker of fungal contamination (Axel son et al., 1995; Miller et al., 1998) and the ergosterol content of wood chip insulation, gypsum board and glass wool insulation was used as a marker of fungal contamination in bio contaminated building materials (Pasanen et al., 1999).

Carotenoid pigments are produced by all green plants and by a wide range of organisms including microbes. Several micro organisms, including bacteria, algae, molds and yeasts of the genera *Rhodotorula, Rhodosporidium, Sporobolomyces* and *Phaffia* are able to produce carotenoids naturally. Microbial carotenoids have attracted much attention in recent years, as they are easily grown and manipulated in the laboratory (Weber et al., 2007). Of the various species of *Rhodotorula* used for carotenoid extraction, *Rhodotorula calyptogenae* produced the minimum amount of carotenoid (209.6µg/gm) and *Rhodotorula minuta* produced a quantity of about 225.6µg/g dry weight. The quantity of carotenoid produced by *Rhodotorula minuta* (R382) was higher than the previous reports (Maldonade et al., 2008). Carotenoid content was higher in the strain R149 belonging to *Rhodotorula mucilaginosa* compared to earlier reports. In a study conducted by Moline et al. (2010) in 12 strains of *Rhodotorula mucilaginosa* obtained from different regions of Patagonia the amount of carotenoids varied from 8.7µg up to 496.3µg/g dry weight. Similarly *Rhodotorula mucilaginosa* strains from the Patagonia high altitude lake produced carotenoids in the range of 234-293 µg/g dry weight. In the present study, strain R28pi belonging to *Rhodotorula slooffiae* which has a light orange colour produced the maximum amount of carotenoids and it is approximately 322 µg/g dry weight and the amount of carotenoids produced by *R.mucilaginosa* was slightly lower (310 µg/g) than *R. slooffiae*. Previous literature indicates that total carotenoid values were variable even between the strains of the same species ranging from 60 to 496 µg/g dry weight, while dry biomass ranged between 3.2 to 8.32 g/L (Libikind et al ., 2006; Libikind and Broock, 2006; Maldonade et al., 2008).

In animals and humans, carotenoids are important to enhance immune response, conversion to vitamin A and the scavenging of oxygen radicals (Bast et al., 1998; Jimenez et al., 2000; Kiokias and Gordon, 2004; Lee et al., 2003). In humans, previous experimental results suggest that dietary carotenoids inhibit the onset of

many diseases in which free radicals are thought to play a role in initiation, such as arteriosclerosis, cataract, multiple sclerosis and cancer (Berset, 1999; Forman et al., 2004; Henneckens, 1997). Carotenoids are effective anti-oxidants and quenchers of singlet oxygen (Krinsky, 1979; Burton, 1989). In the present study, carotenoids showed a better IC_{50} value in terms of antioxidant / radical scavenging activity.

As animals cannot synthesize carotenoids, these pigments added to the feeds of aquaculture animals will improve the colour which in turn improves customer demands (Baker and Guenther, 2004)). At the same time beta carotene from *Rhodotorula* species is widely applied in mariculture in China, as it can increase the yields of mariculture animals and raise their ability to resist diseases (Misawa and Shimada, 1998).

Chapter -6

SUMMARY & CONCLUSION

Oceans which cover almost 70% of the earth's surface and over 90% of the volume of its crust are the host of diverse organisms ranging from microbes, the invisible majority to the large mammals. Organisms from these habitats may hold keys to nutrient cycling, metal detoxification and food-webs in extreme environments. They also provide unique bio molecules for industry and medicine (Conell et al., 2009). Following the first discovery of the marine yeasts from the Atlantic Oceans, yeasts were isolated from different sources, *viz*., seawater, sediment, seaweeds, fishes, marine mammals and sea birds. Yeasts are used in many industrial processes, such as the production of alcoholic beverages, biomass and various metabolic products which includes enzymes, vitamins, capsular polysaccharides, carotenoids, polyhydric alcohols, lipids, glycolipids, citric acid, ethanol, carbon dioxide and compounds synthesized by the introduction of recombinant DNA into yeasts. Some of these products are produced commercially while others are potentially valuable in biotechnology.

The present work was focused on the biochemical, molecular and phylogenetic characterization of marine yeast isolates from Arabian Sea and Bay of Bengal. Sediment samples collected from the slope regions of the Arabian Sea were used for the study. The marine yeast isolates were identified up to generic level based on morphological, physiological and biochemical characteristics. Ability of the isolates to grow at various temperatures, utilize different sugars like

monosaccharides, disaccharides, tri and polysaccharides were assessed. Hydrolytic enzyme production property of the isolates was also studied. Isolation of DNA, amplification of ITS region and restriction fragment pattern analysis followed by sequencing of the representative strains helped to identify the isolates up to species level. Estimation of proximate biochemical composition, ergosterol and carotene of selected yeast isolates were also carried out. Antioxidant/ free radical scavenging potential of both ergosterol and carotene was assessed. The salient findings of the study are as follows.

- Totally 192 yeast isolates were used in the present study, Employing conventional yeast taxonomy, the isolates were grouped into 9 genera. The most predominant being the *Candida* (59.8%) followed by *Debaryomyces* (18%), *Hortaea* (8.3%), *Rhodotorula* (8.85%) and *Pichia* (5.2%).

- Lipase producing yeasts (100%) were dominant in the marine environment followed by ligninase (34%), protease (17%), Amylase (12%), Urease (17%) and Phosphatase (12%) producing forms. Very few isolates (1%) were able to produce Aryl sulfatase and DNase.

- All the isolates were able to utilize the sugars *viz*., glucose, sucrose and melezitose. 92% of the isolates were able to utilize xylose followed by galactose (90%), maltose (88%), trehalose (42%), cellobiose (39%), raffinose (37%), melibiose (26%), lactose (10%), and polysaccharide inulin (6%).

- All the isolates were able to grow at a temperature range of 18 to 30ºC. None of the isolates exhibited growth at 45ºC. Only 19.79% of the isolates exhibited growth at 4ºC.

- Out of the 192 isolates 159 isolates were non-pigmented and 33 pigmented. The size of the amplicons (ITS) varied between 800 to 900 bp in both pigmented and non-pigmented yeasts. Restriction Enzyme Analysis (REA) of the ITS amplicons of non pigmented yeasts displayed various patterns *i.e*., 5 different patterns for *Hinf*1, 4 for *Taq*1 and 3 for *Alu*1.

- Restriction enzyme analysis patterns with *Hinf*1 were unique for *Candida parapsilosis* and *Pichia guilliermondii.*

- Twelve species of non pigmented yeasts belonging to three genera could be obtained. Seven species belonged to *Candida*, four to *Debaryomyces* and one species to *Pichia.*

- *Candida* spp. included *C. parapsilosis* (48.6%), *C. orthopsilosis* (33.04%), *C. metapsilosis* (2%), *C. haemulonii* (1.56%), *C. oceani (*5.2%), *C. spencermartinsiae (*1.5%) and *C. tropicalis (*1.56%). *Debaryomyces* spp. included *D. nepalensis* (28%), *D. fabryii* (11%), D. *subglobosus* (11%) and *D. hansenii* (50%). Only one species could be observed under the genera *Pichia i.e., P. guilliermondii*

- Among the pigmented yeasts, Restriction enzyme analysis of ITS amplicons produced five patterns with *Hinf*1 four patterns with *Taq*1 and five patterns with *Alu*1.

- REA patterns produced by *Hinf*1 helped to assign the identity of *Rhodotorula mucilaginosa* and *R. minuta* based on previous data from literature.

- All the 17 pink yeast isolates belonged to the same genera *Rhodotorula i.e., R. mucilaginosa* (53%), *R. minuta* (23.5%), *R. calyptogenae* (17.6%), and *R. slooffiae* (5.88%).

- All the black yeast isolates belonged to a single genus and species *i.e., Hortaea werneckii*

- Yeasts are a rich source of proteins, carbohydrates and ash. High protein content (above 40%) was found in *Rhodotorula mucilaginosa, Hortaea werneckii, Candida oceani* and *Pichia guilliermondii.*

- Carbohydrate content varied between 22-31mg/100mg dry yeast biomass in different genera. Maximum carbohydrate content was found in *Candida oceani* (31%) followed by *Pichia* (30%).

- The amount of lipid was generally low in the various genera studied. It varied between 2-3mg/100mg dry yeast biomass.

- Ash content of the different genera studied varied between 3.6 -5.8%. *Candida spencer martinsiae* had the minimum and *R. mucilaginosa* had the maximum amount of ash.

- Moisture content in the different genera varied between 69.6% in *D.fabryii* to 80.4% in *D. nepalensis.*

- Biomass (wet weight) production was highest in *Pichia* (7.87g/l*)* followed by *R. mucilaginosa* (6.57g/l). *Debaryomyces fabryii* (5.9g/l), *C. oceani* (5.4g/l), *R. slooffiae* (2.06 g/l*)* and lowest in *R. calyptogenae* (1.13g/l) when cultivated in malt extract broth for 5 days.

- The amount of crude ergosterol varied between 0.1 to 0.6mg/100mg yeast biomass (wet weight). Maximum amount of ergosterol was found

in *C. oceani (*0.6mg/100mg) and the minimum amount in the Black yeast, *Hortaea werneckii*.

- The amount of carotenoids varied in the different species of *Rhodotorula i.e., R. calyptogenae* produced 209. 6µg/g yeast wet weight, *R. slooffiae* 322µg/g yeast wet weight and *R. mucilaginosa*, 315µg/g yeast wet weight.

- The biomass produced by *R.mucilaginosa* (6.56g/l) was approximately three times higher than that of *R. slooffiae* (2.06 g/l). Hence *R. mucilaginosa* would be a better candidate species for production of carotenoids.

- Crude Ergosterol from *C. oceani* at 0.2 ± 0.039, 0.051 ± 0.01, and 0.217 ± 0.03 mg/mL were found to exhibit 50% inhibition of DPPH, ABTS radicals and superoxide anions respectively.

- Crude Carotenoids from *R. mucilaginosa* at 0.098 ± .011 and 0.116 ±0.012 mg/mL exhibited 50% inhibition of DPPH (1,1diphenyl 2-picryl hydrazyl) radicals and ABTS (2,2'azinobis 3-ethyl benzothiazoline 6- sulfonic acid) radicals respectively.

The present study provides an insight into the various yeast species occurring in the sediments of Arabian Sea and Bay of Bengal with *Candida* as the most predominant genus followed by *Debaryomyces*, *Hortaea*, *Rhodotorula* and *Pichia.* Conventional yeast taxonomy using phenotypic and biochemical methods helped to identify the isolates up to the generic level. Molecular identification by ITS amplification and restriction fragment pattern followed by sequencing helped to identify the yeast isolates up to species level. ITS sequencing was found to be a rapid and reliable method to identify yeast isolates belonging to the genera, *Rhodotorula, Hortaea, Candida* and *Pichia* but not for *Debaryomyces*. Hence it

can be concluded that conventional yeast taxonomy is also important and should go hand in hand/supplement the molecular methods in case of discrepancies with the identity of the isolates. Marine yeast isolates were found to be nutritionally rich and a good source of bioactive materials. Ergosterol in yeasts and the carotenoids in pink yeasts deserve special attention in terms of its bioactive potential. The potential of bioactive materials (ergosterol, carotenoids, glucans, mannans, melanins *etc*.) from marine yeasts would be a promising area of research in future for cosmetic, pharmaceutical and aquaculture applications.

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Table2.2: Biochemical and physiological characteristics of marine yeast isolates

St.No	Nitrite	MOF	Urease	Starch production	DBB	Myo-inositol	Amylase	Protease	Lipase	Phosphatase	Aryl sulfatase	Dnase	Lilgninase	Pectinase	Cellulase	Chitinase	Genera
R1	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R2	+	F	-	-	-	-	-	-	+	-	-	+	-	-	-	-	*Candida*
R4	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R5	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R6	-	F	-	-	-	-	-	-	+	-	-	+	-	-	-	-	*Candida*
R7	+ +	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R8	-	F	-	-	-	-	-	-	+	-	-	-	+	-	-	-	*Candida*
R9	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R10	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R11	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R12	-	o	-	-	-	-	-	-	+	+	+	-	-	-	-	-	*Candida*
R13	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R14	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R15	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R16	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R17	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R19W	+	F	-	-	-	-	-	-	+	-	-	-	+	-	-	-	*Candida*
R19P	+ +	F	-	-	+	-	-	-	+	-	-	-	+	-	-	-	*Rhodotorula*
R20	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*

R21	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
St.No	Nitrite	MOF	Urease	Starch production	DBB	Myo-inositol	Amylase	Protease	Lipase	Phosphatase	Aryl sulfatase	Dnase	Lilgninase	Pectinase	Cellulase	Chitinase	Genera
R22	+ +	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R23	-	o	+	-	-	-	+	-	+	-	-	-	+	-	-	-	*Hortaea*
R24	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R28W	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R28P	-	F	+	-	+	-	-	-	+	-	-	-	-	-	-	-	*Rhodotorula*
R29	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R30	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R31	+	F	-	-	+	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R32	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R33	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R34	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R35	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R36	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R37	+	F	-	-	+	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R38	+	F	-	-	+	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R39	+	F	-	-	+	-	-	-	+	-	-		-	-	-	-	*Candida*
R40	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R41	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R44W	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R44B	-	F	+	-	-	-	+	-	+	-	-	-	-	-	-	-	*Hortaea*
R46	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*

R49	+	F	-	-	-	-	-	-	+	+	-	-	-	-	-	-	*Candida*
R50	+	o	-	-	-	-	-	-	+	+	-	-	-	-	-	-	*Debaryomyces*
St.No	Nitrite	MOF	Urease	Starch production	DBB	Myo-inositol	Amylase	Protease	Lipase	Phosphatase	Aryl sulfatase	Dnase	Lilgninase	Pectinase	Cellulase	Chitinase	Genera
R51	+	F	-	-	-	-	-	-	+	+	-	-	-	-	-	-	*Debaryomyces*
R52	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R55	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R56	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R57W	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R57P	+ +	o	+	-	-	-	-	-	+	-	-	-	-	-	-	-	*Rhodotorula*
R59	+ +	F	-	-	-	-	-	-	+	-	+	-	-	-	-	-	*Debaryomyces*
R60	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Debaryomyces*
R61	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Debaryomyces*
R62	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R63	-	F	-	-	+	-	-	+	+	+	-	-	-	-	-	-	*Candida*
R64	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R65	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R66	-	F	-	-	-	-	-	-	+	-	-	-	+	-	-	-	*Debaryomyces*
R67	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R68	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R69	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R70	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R72	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R74	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*

R75	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R76	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R77	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Debaryomyces*
St.No	Nitrite	MOF	Urease	Starch production	DBB	Myo-inositol	Amylase	Protease	Lipase	Phosphatase	Aryl sulfatase	Dnase	Lignninase	Pectinase	Cellulase	Chitinase	Genera
R78	-	F	-	-	-	-	-	-	+	+	-	-	-	-	-	-	*Candida*
R79	-	F	-	-	-	-	-	-	+	+	-	-	-	-	-	-	*Candida*
R80W	-	o	-	-	-	-	-	-	+	+	-	-	-	-	-	-	*Candida*
R81W	-	o	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Debaryomyces*
R83	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R84	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R85	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R86	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R87	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R88	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R89	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R90	+	F	-	-	-	+	-	-	+	+	-	-	-	-	-	-	*Candida*
R91	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R92	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R93	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Debaryomyces*
R95	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R96	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R97	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	Debaryomyces
R99	+	F	-	-	-	+	-	-	+	-	-	-	-	-	-	-	Pichia

R100	+	F	-	-	-	+	-	-	+	-	-	-	-	-	-	-	*Debaryomyces*
R101P	+ +	o	+	-	-	-	-	-	+	-	-	-	-	-	-	-	*Rhodotorula*
R103	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R104	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
St.No	Nitrite	MOF	Urease	Starch production	DBB	Myo-inositol	Amylase	Protease	Lipase	Phosphatase	Aryl sulfatase	Dnase	Lilgninase	Pectinase	Cellulase	Chitinase	Genera
R105	+	F	-	-	-	-	-	-	+	-	-	-	+	-	-	-	*Debaryomyces*
R106	+	F	-	-	-	+	-	-	+	-	-	-	-	-	-	-	*Debaryomyces*
R107	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R109	+	F	-	-	-	+	-	-	+	-	-	-	-	-	-	-	*Candida*
R111	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R112	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Debaryomyces*
R113	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R114	+	F	-	-	-	+	-	-	+	-	-	-	-	-	-	-	*Candida*
R115	+ +	F	-	-	-	-	-	-	+	+	-	-	-	-	-	-	*Candida*
R116	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R117	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R118	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R120	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R121	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R122	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Debaryomyces*
R123	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R124	+	F	-	-	+	-	-	-	+	-	-	-	-	-	-	-	*Pichia*
R125	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*

R126	+	**F**	-	-	+	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R127	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R128	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R129p	-	o	+	-	+	-	-	-	+	-	-	-	-	-	-	-	*Rhodotorula*
R130	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
St.No	Nitrite	MOF	Urease	Starch production	DBB	Myo-inositol	Amylase	Protease	Lipase	Phosphatase	Aryl sulfatase	Dnase	Lilgninase	Pectinase	Cellulase	Chitinase	Genera
R131	-	F	-	-	-	-	-	+	+	-	-	-	-	-	-	-	*Candida*
R132	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R133	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R134	-	F	-	-	-	-	-	+	+	-	-	-	-	-	-	-	*Candida*
R135	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R136p	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Rhodotorula*
R136w	-	o	+	-	-	-	-	-	+	+	-	-	-	-	-	-	*Pichia*
R137	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R138p	-	F	-	-	-	-	-	+	+	-	-	-	-	-	-	-	*Candida*
R139	-	F	-	-	-	+	-	-	+	-	-	-	-	-	-	-	*Candida*
R140	+	F	-	-	-	+	-	-	+	-	-	-	-	-	-	-	*Debaryomyces*
R141	+	F	-	-	-	-	-	+	+	-	-	-	-	-	-	-	*Candida*
R142	+	F	-	-	-	+	-	+	+	-	-	-	-	-	-	-	*Candida*
R143	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R144	-	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R145	+	F	+	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R146	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Debaryomyces*

R147	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R148	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R149P	+	F	+	-	-	-	-	-	+	-	-	-	-	-	-	-	*Rhodotorula*
R149W	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R150	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R151W	+	F	-	-	-	-	+	+	+	-	-	-	-	-	-	-	*Candida*
St.No	Nitrite	MOF	Urease	Starch production	DBB	Myo-inositol	Amylase	Protease	Lipase	Phosphatase	Aryl sulfatase	Dnase	Lilgninase	Pectinase	Cellulase	Chitinase	Genera
R152W	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R152B	+	o	+	-	-	-	+	+	+	+	-	-	+	-	-	-	*Hortaea*
R154w	+	F	-	-	-	+	-	-	+	-	-	-	-	-	-	-	*Candida*
RJ1	-	o	+	-	-	-	+	+	+	+	-	-	+	-	-	-	*Hortaea*
RJ2	-	o	+	-	-	-	+	+	+	+	-	-	+	-	-	-	*Hortaea*
RJ3	-	o	+	-	-	-	+	-	+	+	-	-	+	-	-	-	*Hortaea*
RJ6	-	o	+	-	-	-	+	-	+	+	-	-	-	-	-	-	*Hortaea*
RJ7	-	o	+	-	-	-	+	-	+	+	-	+	+	-	-	-	*Hortaea*
RJ11	-	o	+	-	-	-	+	+	+	+	-	-	-	-	-	-	*Hortaea*
RJ21	-	o	+	-	-	-	+	+	+	+	-	-	-	-	-	-	*Hortaea*
RJ24	-	o	+	-	-	-	+	+	+	+	-	-	-	-	-	-	*Hortaea*
R302	+	F	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R303	+	F	-	-	+	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R304	+	F	-	-	+	-	-	-	+	-	-	-	-	-	-	-	*Debaryomyces*
R305	+	o	-	-	-	-	-	-	+	+	-	-	-	-	-	-	*Debaryomyces*
R306	+	o	-	-	-	-	-	-	+	+	-	-	-	-	-	-	*Candida*

R307	+	0	-	-	+	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R310	+	0	-	-	+	-	-	-	+	-	-	-	-	-	-	-	*Debaryomyces*
R312	+	0	+	-	-	-	+	-	+	-	-	-	-	-	-	-	*Debaryomyces*
R313	+	0	-	-	+	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R314	+	0	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Debaryomyces*
R317	+	0	-	-	-	-	-	-	+	+	-	-	-	-	-	-	*Debaryomyces*
R319	+	0	+	-	-	-	+	+	+	-	-	-	-	-	-	-	*Candida*
St.No	Nitrite	MOF	Urease	Starch production	DBB	Myo-inositol	Amylase	Protease	Lipase	Phosphatase	Aryl sulfatase	Dnase	Lilgninase	Pectinase	Cellulase	Chitinase	Genera
R322	+	0	-	-	+	-	-	-	+	-	-	-	-	-	-	-	*Debaryomyces*
R328	+	0	-	-	-	-	-	-	+	-	+	-	-	-	-	-	*Rhodotorula*
R328P	+	0	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Rhodotorula*
R337	+	0	+	-	-	-	+	+	+	-	-	-	-	-	-	-	*Pichia*
R340	+	F	-	-	-	-	-	-	+	+	-	-	-	-	-	-	*Pichia*
R344	+	F	-	-	-	-	-	-	+	+	-	-	-	-	-	-	*Debaryomyces*
R349	-	0	+	-	-	-	+	-	+	+	-	-	-	-	-	-	*Pichia*
R365	+	0	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Debaryomyces*
R371	+	0	-	-	-	-	-	-	+	+	-	-	-	-	-	-	*Pichia*
R372	+	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*Debaryomyces*
R376	+	0	-	-	+	-	-	-	+	+	-	-	-	-	-	-	*Candida*
R381	-	0	-	-	-	-	-	-	+	+	-	-	-	-	-	-	*Pichia*
R382P	-	0	+	-	-	-	-	-	+	-	-	-	-	-	-	-	*Rhodotorula*
R382	+	0	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Rhodotorula*
R384	-	F	-	-	+	-	-	-	+	+	-	-	-	-	-	-	*Debaryomyces*

R386	-	0	-	-	+	-	-	-	+	-	-	-	-	-	-	-	*Debaryomyces*
R387	+	0	-	-	-	-	-	-	+	+	-	-	-	-	-	-	*Pichia*
R398	-	0	+	-	-	-	-	-	+	-	-	-	-	-	-	-	*Rhodotorula*
R399	-	F	-	-	-	-	-	+	+	+	-	-	-	-	-	-	*Candida*
R414	+	F	-	-	-	-	-	-	+	+	-	-	-	-	-	-	*Pichia*
R418	+	0	+	-	-	-	+	+	+	-	-	-	-	-	-	-	*Hortaea*
R424	-	F	-	-	+	-	-	-	+	-	-	-	-	-	-	-	*Debaryomyces*
R430	-	0	-	-	-	-	+	+	+	-	-	-	-	-	-	-	*Hortaea*
St.No	Nitrite	MOF	Urease	Starch production	DBB	Myo-inositol	Amylase	Protease	Lipase	Phosphatase	Aryl sulfatase	Dnase	Lilgninase	Pectinase	Cellulase	Chitinase	Genera
R431	-	0	-	-	-	-	+	+	+	-	-	-	-	-	-	-	*Hortaea*
R440	+	0	-	-	-	-	+	+	+	-	-	-	-	-	-	-	*Hortaea*
R447	-	0	-	-	+	+	-	-	+	-	-	-	-	-	-	-	*Debaryomyces*
R448	+	0	-	-	+	-	-	-	+	-	-	-	-	-	-	-	*Debaryomyces*
R454	+	0	-	-	-	-	+	+	+	-	-	-	-	-	-	-	*Debaryomyces*
R480	+	0	+	-	-	-	+	+	+	-	-	-	-	-	-	-	*Hortaea*
R485P	-	0	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Rhodotorula*
R486	-	0	+	-	-	-	-	-	+	-	-	-	-	-	-	-	*Rhodotorula*
R487	-	0	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Rhodotorula*
R488	-	0	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Rhodotorula*
R489	-	0	-	-	-	-	-	-	+	-	-	-	-	-	-	-	*Rhodotorula*
R491	-	F	-	-	+	-	-	-	+	-	-	-	-	-	-	-	*Candida*
R493	-	F	-	-	+	-	-	+	+	-	-	-	-	-	-	-	*Candida*
R495	-	F	-	-	+	-	-	-	+	-	-	-	-	-	-	-	*Candida*

R496	-	F	-		+	-	-	+	+	-	-	-	-	-	-	-	*Debaryomyces*

Appendix – 1

Table : 3.2
Physiological Characterization of non pigmented yeast isolates using Biolog Micro plate (Oxidation)

Oxidation Test	R 13	R 38	R 56	R 28W	R 89	R 399	R 100	R 140	R 344	R 305	R 340
Acetic acid	/	-	/	/	-	/	-	-	-	-	/
Formic acid	/	-	/	/	/	-	-	-	-	-	/
Propionic acid	/	-	/	/	-	-	-	-	-	-	-
Succinic acid	+	/	+	/	+	+	/	+	/	+	+
Methyl succinate	+	/	+	/	/	+	/	/	/	-	/
L- Aspartic acid	+	/	/	/	+	/	+	+	/	+	+
L-Glutamic acid	+	/	+	/	+	/	-	+	/	+	+
L-proline	+	/	+	+	+	+	-	+	/	+	+
D-Gluconic acid	+	+	+	/	+	+	-	+	/	+	+
Dextrin	/	-	/	-	/	-	-	-	-	/	-
Inulin	/	-	/	-	/	-	-	-	-	-	-
Cellobiose	+	-	/	+	+	/	-	/	+	+	+
Gentiobiose	/	-	/	+	+	/	-	-	+	+	+
Maltose	+	+	+	+	+	+	/	/	+	+	+
Maltotriose	+	+	+	+	+	+	/	-	+	+	+
D-Melezitose	+	+	+	+	+	+	-	/	+	/	+
D-Melibiose	/	-	/	/	+	-	-	-	+	+	+
Palatinose	/	+	+	+	+	+	-	+	+	+	+
D-Raffinose	/	-	/	/	+	+	/	+	/	+	+
Stachyose	/	-	/	/	/	/	-	-	/	+	+
Sucrose	+	+	+	+	+	+	+	/	+	+	+
D-Trehalose	+	+	+	+	+	+	+	/	+	+	+
Turanose	+	+	+	+	+	+	-	/	+	+	+
N-Acetyl-d-glucosamine	+	+	+	/	+	+	-	+	+	+	+
α -D-Glucose	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	/	+	+	+	+	+
D-psicose	+	/	/	/	/	/	/	-	-	-	/
L-sorbose	+	/	+	+	/	-	/	-	-	+	/
Salicin	/	-	/	/	/	-	-	-	+	/	/
D-mannitol	+	+	+	+	+	+	/	/	+	+	+
D-sorbitol	+	+	+	+	+	+	/	/	+	+	+
D-arabitol	/	+	+	+	+	+	/	/	+	+	+
Xylitol	+	/	/	/	+	/	-	-	+	-	+
Glycerol	+	+	+	/	+	/	/	+	+	+	+
Tween-80	+	/	+	+	+	/	-	-	-	/	/

(+ >0.20D (/) <0.1OD (-) <0.05OD

Appendix – 2

Table : 3.3
Physiological Characterization of non pigmented yeast isolates using Biolog Micro plate (Assimilation)

Assimilation Test	R 13	R 38	R 56	R 28W	R 89	R 399	R 100	R 140	R 344	R 305	R 340
Fumaric acid	+	/	+	/	/	/	/	/	/	/	+
L-malic acid	+	+	+	/	+	/	/	/	/	/	+
Methyl succinate	+	/	+	/	/	/	-	-	-	-	/
Bromo succinic acid	-	/	+	/	/	-	-	-	-	-	/
L-glutamic acid	/	+	+	/	+	/	/	+	/	/	+
ɣ-amino butyricacid	+	+	+	/	/	+	+	/	+	/	+
a-ketoglutarc acid	/	/	/	+	/	/	/	/	-	-	/
2-keto- D-gluconic acid	+	-	+	/	/	+	+	+	/	/	/
D-gluconic acid	+	+	+	/	/	+	+	-	/	+	/
Dextrin	-	-	/	/	/	-	/	-	-	/	-
Inulin	/	-	/	-	/	-	-	-	-	-	/
cellobiose	+	/	/	+	+	/	+	/	+	+	+
Gentiobiose	/	/	/	+	/	-	+	+	+	+	+
Maltose	/	+	+	+	+	+	/	/	+	+	+
Maltotriose	+	+	+	+	+	+	+	/	+	+	+
D-Melezitose	+	+	+	+	/	/	+	/	+	+	+
D-Melibiose	-	-	/	/	/	/	+	/	+	+	+
Palatinose	/	+	+	+	+	+	+	/	+	+	+
D-Raffinose	-	/	/	/	/	+	+	/	/	+	+
Stachyose	/	/	/	/	/	/	+	-	/	+	+
Sucrose	/	+	+	+	+	+	+	+	+	+	+
D-Trehalose	/	+	+	+	/	/	+	/	/	+	+
Turanose	/	+	+	+	+	+	+	/	+	+	+
N-Acetyl-d-glucosamine	+	+	+	/	+	/	+	/	+	/	+
D-Glucosamine	/	+	/	/	/	+	/	-	-	/	+
α-D-Glucose	/	+	+	+	+	+	+	/	+	+	+
D-Galactose	/	+	+	+	+	/	+	/	+	+	+
D-psicose	-	/	/	/	/	/	/	-	-	/	+
L-Rhamnose	/	-	/	+	/	/	-	-	/	-	/
L-sorbose	/	-	+	/	/	-	/	-	-	+	+
α-Methyl D-Glucoside	/	+	+	+	/	-	+	/	+	+	+
β-Methyl D-Glusoside	/	-	/	/	/	-	+	/	+	+	/
Amygdalin	/	/	/	/	/	-	/	-	-	-	/
Arbutin	/	-	/	/	/	-	+	/	+	/	+
Salicin	/	-	/	-	/	-	+	/	+	/	+
Maltitol	/	+	+	+	+	+	+	/	+	+	+
D-mannitol	/	+	+	+	+	+	+	/	+	+	+
D-sorbitol	+	+	+	+	+	+	+	/	+	+	+
Adonitol	-	+	+	+	+	/	+	/	+	+	+
D-arabitol	/	+	+	+	+	+	+	/	+	+	+
Xylitol	/	+	/	/	/	+	+	/	+	+	+

i-erythritol	/	-	/	+	/	/	+	/	+	+	-
Glycerol	+	+	+	+	+	+	/	-	+	+	-
Tween-80	+	/	/	/	/	/	/	-	+	-	/
L-Arabinose	/	+	+	+	/	/	+	/	+	+	+
D-Arabinose	-	-	/	/	-	-	/	-	-	-	/
D-Ribose	+	+	/	/	/	-	+	-	-	-	/
D-Xylose	+	/	+	+	+	/	/	-	+	+	+
Succcinic acid monomethyl ester + D-Xylose	+	/	+	/	/	+	/	-	/	/	+
n-Acetyl-L-Glutamic acid + D-Xylose	/	/	/	/	/	+	/	/	-	/	/
Quinic acid +D-Xylose	-	/	/	/	/	+	+	/	+	+	/
D-Glucornic acid + D-Xylose	/	/	/	/	/	/	+	/	+	+	+
Dextrin + D-Xylose	/	/	/	-	/	+	+	/	-	/	/
α-D-lactose + D-Xylose	-	/	/	/	/	/	+	-	-	/	+
D-Melibiose +D-Xylose	/	+	/	/	/	/	+	/	+	+	+
D-Galactose +D-Xylose	/	+	+	+	+	+	+	/	+	+	+
M-inositol+D-Xylose	+	/	/	/	+	/	/	-	/	/	/
1,2- Propanediol +D-Xylose	/	/	/	/	/	/	/	-	-	-	/
Acetoin + D-Xylose	/	/	/	/	/	/	/	-	/	-	/

(+ >0.20D (/) <0.1OD (-) <0.05OD

Table 4.2 a Results of Oxidation test in Biolog plates of pigmented yeast isolates from Arabian Sea and Bay of Bengal

Oxidation Test	R19	R28	R149	R382	R23
Acetic acid	-/	-	-	-	-
Formic acid	/	-	-	-	-
Propionic acid	-	-	-	-	-
Succinic acid	-	-	/	+	+
Methyl succinate	+	-	+	/	+
L- Aspartic acid	+	-	/	-	-
L-Glutamic acid	+	-	+	+	-
L-proline	+	-	-	+	-
D-Gluconic acid	+	-	-	+	+
Dextrin	/	-	-	+	+
Inulin	+	-	-	-	-
Cellobiose	+	+	-	+	-
Gentiobiose	+	-	-	+	+
Maltose	+	-	/	+	+
Maltotriose	+	-	-	-	+
D-Melezitose	/	/	-	+	+
D-Melibiose	/	-	-	+	+
Palatinose	+	-	+	-	+
D-Raffinose	+	-	+	+	+
Stachyose	-	-	/	+	+
Sucrose	+	-	/	+	+
D-Trehalose	+	-	/	+	+
Turanose	/	-	+	-	+
N-Acetyl-d-glucosamine	+	-	-	+	+
α -D-Glucose	+	-	-	+	+
D-Galactose	+	-	-	+	+
D-psicose	+	-	-	-	-
L-sorbose	+	-	-	/	-
Salicin	+	-	-	+	+
D-mannitol	+	+	-	+	+
D-sorbitol	+	+	-	+	-
D-arabitol	+	-	-	+	-
Xylitol	-	-	-	-	-
Glycerol	+	-	-	+	-
Tween-80	+	-	-	-	+

(+ >0.20D (/) <0.1OD (-) <0.05OD

Table 4.2 b. Results of Assimilation test in Biolog plates of pigmented yeast isolates from Arabian Sea and Bay of Bengal

Assimilation Test	R19	R28	R149	R382	R23
Fumaric acid	+	-	-	+	-
L-malic acid	+	-	-	+	-
Methyl succinate	-	-	/	-	-
Bromo succinic acid	+	-	-	-	-
L-glutamic acid	+	-	+	+	+
-amino butyricacid	-	-	-	+	-
a-keto glutarc acid	/	-	-	-	-
2-keto- D-gluconic acid	+	-	/	+	+
D-gluconic acid	+	-	-	+	+
Dextrin	+	-	-	+	-
Inulin	-	-	-	+	-
cellobiose	+	+	-	+	+
Gentiobiose	+	-	-	+	+
Maltose	+	-	-	+	+
Maltotriose	/	-	-	+	+
D-Melezitose	/	-	-	/	+
D-Melibiose	-	-	-	-	+
Palatinose	/	-	-	/	+
D-Raffinose	+	-	+	+	+
Stachyose	+	-	-	-	+
Sucrose	+	+	+	+	+
D-Trehalose	+	+	+	-	+
Turanose	/	-	-	-	+
N-Acetyl-d-glucosamine	+	-	-	+	+
D-Glucosamine	+	-	-	-	+
α-D-Glucose	+	+	+	+	+
D-Galactose	+	-	+	-	+
D-psicose	+	-	-	-	+
L-Rhamnose	-	-	-	-	-
L-sorbose	-	/	+	-	-
α-Methyl D-Glucoside	+	-	-	-	+
β-Methyl D-Glusoside	-	-	-	-	+
Amygdalin	-	-	-	-	+

Arbutin	/	-	-	-	+
Salicin	+	/	-	-	+
Maltitol	+	-	+	-	+
D-mannitol	/	+	+	+	+
D-sorbitol	/	-	-	-	+
Adonitol	/	-	+	-	-
D-arabitol	/	-	-	-	+
Xylitol	/	-	-	-	+
i-erythritol	-	-	-	-	+
Glycerol	/	+	+	+	+
Tween-80	+	-	-	-	-
L-Arabinose	/	-	-	-	+
D-Arabinose	-	-	-	-	-
D-Ribose	/	-	-	/	+
D-Xylose	/	+	/	-	+
Succcinic acid monomethyl ester + D-Xylose	-	-	-	+	+
n-Acetyl-L-Glutamic acid + D-Xylose	+	-	-	+	+
Quinic acid +D-Xylose	+	-	-	-	+
D-Glucornic acid + D-Xylose	+	-	-	-	+
Dextrin + D-Xylose	/	-	-	+	+
α-D-lactose + D-Xylose	/	-	-	/	+
D-Melibiose +D-Xylose	/	-	-	-	+
D-Galactose +D-Xylose	+	-	+	+	+
M-inositol+D-Xylose	/	-	/	-	+
1,2- Propanediol +D-Xylose	/	-	-	-	+
Acetoin + D-Xylose	/	-	/	-	+

(+ >0.20D (/) <0.1OD (-) <0.05OD

Candida spencermartinsiae strain R28w 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

GenBank: JQ665412.1

FASTAGraphicsPopSet

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ORGANISM Candida spencermartinsiae
Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina;
Saccharomycetes; Saccharomycetales; mitosporic Saccharomycetales;
Candida.REFERENCE 1 (bases 1 to 710) AUTHORS Kuriakose,R., Bright Singh,I. and Philip,R.
TITLE Molecular identification of Candida from Arabian Sea
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 710)
AUTHORS Kuriakose,R., Bright Singh,I. and Philip,R.
TITLE Direct Submission
JOURNAL Submitted (09-FEB-2012) Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Fine
Arts Avenue, Kochi, Kerala 682016, India
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Candida parapsilosis strain R13 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

GenBank: JQ665413.1

FASTAGraphicsPopSet

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VERSION JQ665413.1 GI:387230656
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ORGANISM Candida parapsilosis
Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; mitosporic Saccharomycetales; Candida.
REFERENCE 1 (bases 1 to 367)
AUTHORS Kuriakose,R., Bright Singh,I. and Philip,R.
TITLE Molecular identification of Candida from Arabian Sea
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 367)
AUTHORS Kuriakose,R., Bright Singh,I. and Philip,R.
TITLE Direct Submission
JOURNAL Submitted (09-FEB-2012) Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Fine Arts Avenue, Kochi, Kerala 682016, India
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181 gcctgccaga gattaaactc aaccaaattt tatttaatgt caaccgatta tttaatagtc
241 aaaactttca acaacggatc tcttggttct cgcatcgatg aagaacgcag cgaaatgcga
301 taagtaatat gaattgcaga tattcgtgaa tcatcgaatc tttgaacgca cattgcgccc
361 tttggta


Candida parapsilosis strain R20 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

GenBank: JQ665414.1

FASTAGraphicsPopSet

Go to: LOCUS JQ665414 690 bp DNA linear PLN 16-MAY-2012

DEFINITION Candida parapsilosis strain R20 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 JQ665414

VERSION JQ665414.1 GI:387230657

KEYWORDS .

SOURCE Candida parapsilosis

ORGANISM Candida parapsilosis

Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; mitosporic Saccharomycetales; Candida.

REFERENCE 1 (bases 1 to 690)

AUTHORS Kuriakose,R., Bright Singh,I. and Philip,R.

TITLE Molecular identification of Candida from Arabian Sea

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 690)

AUTHORS Kuriakose,R., Bright Singh,I. and Philip,R.

TITLE Direct Submission

JOURNAL Submitted (09-FEB-2012) Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Fine Arts Avenue, Kochi, Kerala 682016, India

FEATURES Location/Qualifiers source 1..690

/organism="Candida parapsilosis"

/mol_type="genomic DNA"

/strain="R20"

/isolation_source="marine sediment"

/db_xref="taxon:5480" misc_RNA <1..>690

/note="contains 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 28S ribosomal RNA"

ORIGIN

1 actgcatttt ttcttacaca tgtgtttttc ttttttgaa aactttgctt tggtaggcct

61 tctatatggg gcctgccaga gattaaactc aaccaaattt tatttaatgt caaccgatta

121 tttaatagtc aaaactttca acaacggatc tcttggttct cgcatcgatg aagaacgcag

181 cgaaatgcga taagtaatat gaattgcaga tattcgtgaa tcatcgaatc tttgaacgca

241 cattgcgccc tttggtattc caaagggcat gcctgtttga gcgtcatttc tccctcaaac

301 cctcgggttt ggtgttgagc gatacgctgg gtttgcttga aagaaaggcg gagtataaac

361 taatggatag gttttttcca ctcattggta caaactccaa aacttcttcc aaattcgacc

421 tcaaatcagg taggactacc cgctgaactt aagcatatca ataagcggag gaaaagaaac

481 caacagggat tgccttagta gcggcgagtg aagcggcaaa agctcaaatt tgaatctggc

541 actttcagtg tccgagttgt aatttgaaga aggtatcttt gggtctggct cttgtctatg

601 tttcttggaa cagaacgtca cagagggtga gaatcccgtg cgatgagatg tcccagacct

661 atgtaaagtt ccttcgaaga gtcgagttgt

Candida oceani strain R89 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

GenBank: JQ665415.1

FASTAGraphicsPopSet

Go to:

LOCUS JQ665415 666 bp DNA linear PLN 16-MAY-2012
DEFINITION Candida oceani strain R89 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 JQ665415
VERSION JQ665415.1 GI:387230658
KEYWORDS .SOURCE Candida oceani
ORGANISM Candida oceani Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina;
Saccharomycetes; Saccharomycetales; mitosporic Saccharomycetales;
Candida.
REFERENCE 1 (bases 1 to 666)
AUTHORS Kuriakose,R., Bright Singh,I. and Philip,R.
TITLE Molecular identification of Candida from Arabian Sea
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 666)
AUTHORS Kuriakose,R., Bright Singh,I. and Philip,R.
TITLE Direct Submission
JOURNAL Submitted (09-FEB-2012) Department of Marine Biology, Microbiology
and Biochemistry, Cochin University of Science and Technology, Fine
Arts Avenue, Kochi, Kerala 682016, India
FEATURES Location/Qualifiers
source 1..666
/organism="Candida oceani"
/mol_type="genomic DNA"
/strain="R89"
/isolation_source="marine sediment"
/db_xref="taxon:1186037"
misc_RNA<1..>666
/note="contains 18S ribosomal RNA, internal transcribed
spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 28S ribosomal RNA"
ORIGIN
1 ataatcctta cacactgtgt tttttatatt cgtgataaat tactttggtt ctggacctag
61 aaataggttt ttggggccag aggtttacta ccaaaacttc aatttttaa ttgaattgtt
121 tattatattt attttgtcaa tttgtttgat taatttcaaa aataatcttc aaaactttca
181 acaacggatc tcttggttct cgcatcgatg aagaacgcag cgaaatgcga taagtaatat
241 gaattgcaga ttttcgtgaa tcatcgaatc tttgaacgca cattgcgccc tctggtattc
301 catagggcat gcctgtttga gcgtcatttc tctctcaaac ccccacgggt ttggtattga
361 gtgatactct tagtcagact aggcgtttgc ttgaaaagta atggcatgag cgtactagat
421 agtgcgactt gatttattca atgtattagg tttatccaac tcgttgaaca gatcggacgt
481 aaatttctgg tattgaaagg ctcgggccgt acaacgacca aacaagtttg acctcaaatc
541 aggtaagaat acccgctgaa cttaagcata tcaataagcg gaggaaaaga aaccaacagg
601 gattgcctca gtagcggcga gtgaagcggc aatagctcaa atttgaaatc tggcaccttt
661 ggtgtc

Candida haemulonis strain R63 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

GenBank: JQ665416.1

FASTAGraphicsPopSet

Go to: LOCUS JQ665416 411 bp DNA linear PLN 16-MAY-2012

DEFINITION Candida haemulonis strain R63 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 JQ665416

VERSION JQ665416.1 GI:387230659

KEYWORDS .

SOURCE Candida haemulonis

ORGANISM Candida haemulonis

Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; mitosporic Saccharomycetales; Candida. REFERENCE 1 (bases 1 to 411)

AUTHORS Kuriakose,R., Bright Singh,I. and Philip,R.

TITLE Molecular identification of Candida from Arabian Sea

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 411)

AUTHORS Kuriakose,R., Bright Singh,I. and Philip,R.

TITLE Direct Submission

JOURNAL Submitted (09-FEB-2012) Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Fine Arts Avenue, Kochi, Kerala 682016, India

FEATURES Location/Qualifiers

source 1..411

/organism="Candida haemulonis"

/mol_type="genomic DNA"

/strain="R63"

/isolation_source="marine sediment"

/db_xref="taxon:45357"

misc_RNA <1..>411

/note="contains 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 28S ribosomal RNA"

ORIGIN

1 ctggctagcc tcgagggcac ctcgtcgcag gccggagaag ctggtcaaac ttggtcattt
61 agaggaagta aaagtcgtaa caaggtttcc gtaggtgaac ctgcggaagg atcattaaaa
121 tacttttcaa aactttgttt tgaattaaaa gcaaccaccg ttaagttcaa aaatctaaaa
181 caaaactttc aacaacggat ctcttggttc tcgcatcgat gaagaacgca gcgaattgcg
241 atacgtagta tgacttgcag acgtgaatca tcgaatcttt gaacgcatat tgcgccttgg
301 ggcattcccc aaggcatgcc tgtttgagcg tgatatcttc tcaccgttgg tggatttgtt
361 tctaaatatc atgccacagt gaagtctacg ctttcactgc ttttttcccc t

Candida haemulonis strain S399 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

GenBank: JQ665417.1

FASTAGraphicsPopSet

Go to:LOCUS JQ665417 664 bp DNA linear PLN 16-MAY-2012
DEFINITION Candida haemulonis strain S399 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 JQ665417
VERSION JQ665417.1 GI:387230660
KEYWORDS .
SOURCE Candida haemulonis
ORGANISM Candida haemulonis Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; mitosporic Saccharomycetales; Candida.
REFERENCE 1 (bases 1 to 664)
AUTHORS Kuriakose,R., Bright Singh,I. and Philip,R.
TITLE Molecular identification of Candida from Arabian Sea
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 664)
AUTHORS Kuriakose,R., Bright Singh,I. and Philip,R.
TITLE Direct Submission
JOURNAL Submitted (09-FEB-2012) Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Fine Arts Avenue, Kochi, Kerala 682016, India
FEATURES Location/Qualifiers
source 1..664
/organism="Candida haemulonis"
/mol_type="genomic DNA"
/strain="S399"
/isolation_source="marine sediment"
/db_xref="taxon:45357"
misc_RNA<1..>664
/note="contains 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed space 2, and 28S ribosomal RNA"
ORIGIN
1 agtgaggcct ccggatctgg ctagcctcga gggcaacctc gtcgcaggcc ggagaagctg
61 gtcaaacttg gtcatttaga ggaagtaaaa gtcgtaacaa ggtttccgta ggtgaacctg
121 cggaaggatc attaaaatac ttttcaaaac tttgttttga attaaaagca accaccgtta
181 agttcaaaaa tctaaaacaa aactttcaac aacggatctc ttggttctcg catcgatgaa
241 gaacgcagcg aattgcgata cgtagtatga cttgcagacg tgaatcatcg aatctttgaa
301 cgcatattgc gccttggggc attccccaag gcatgcctgt ttgagcgtga tatcttctca
361 ccgttggtgg atttgtttct aaatatcatg ccacagtgaa gtctacgctt tcactgcttt
421 ttcccctcaa atcaggtagg actacccgct gaacttaagc atatcaataa gcggaggaaa
481 agaaaccaac agggattgcc tcagtaacgg cgagtgaagc ggcaagagct caactttgga
541 atcgctgcgg cgagttgtag tctggaggtg gccggtcccg gcgccagcgc gcagccaagt
601 cctttggaac aaggcgcctg agagggtgac agccccgtgg cagtttgtgc tggtgccgcc
661 tcgg

Candida orthopsilosis strain R56 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

GenBank: JQ665418.1

FASTAGraphicsPopSet

Go to:

LOCUS JQ665418 679 bp DNA linear PLN 16-MAY-2012

DEFINITION Candida orthopsilosis strain R56 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 JQ665418

VERSION JQ665418.1 GI:387230661

KEYWORDS .

SOURCE Candida orthopsilosis

ORGANISM Candida orthopsilosis

Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; mitosporic Saccharomycetales; Candida.

REFERENCE 1 (bases 1 to 679)

AUTHORS Kuriakose,R., Bright Singh,I. and Philip,R.

TITLE Molecular identification of Candida from Arabian Sea

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 679)

AUTHORS Kuriakose,R., Bright Singh,I. and Philip,R.

TITLE Direct Submission JOURNAL Submitted (09-FEB-2012) Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Fine Arts Avenue, Kochi, Kerala 682016, India

FEATURES Location/Qualifiers

source 1..679

/organism="Candida orthopsilosis"

/mol_type="genomic DNA"

/strain="R56"

/isolation_source="marine sediment" /db_xref="taxon:273371"

misc_RNA <1..>679

/note="contains 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 28S ribosomal RNA"

ORIGIN

1 actgcatttt ttttacacat gtgtttttct ttttttttga aaactttgct ttggtgggcc

61 catggcctgc cagagattaa actcaaccaa atttttattt aagtcaactg attaactaat

121 agtcaaaact ttcaacaacg gatctcttgg ttctcgcatc gatgaagaac gcagcgaaat

181 gcgataagta atatgaattg cagatattcg tgaatcatcg aatctttgaa cgcacattgc

241 gccctttggt attccaaagg gcatgcctgt ttgagcgtca tttctccctc aaaccttcgg

301 gtttggtgtt gagcgatacg ctgggtttgc ttgaaagaaa ggcggagtat aaactaatgg

361 ataggttttt tttccactca ttggtacaaa ctccaaaatt cttccaaatt cgacctcaaa

421 tcaggtagga ctacccgctg aacttaagca tatcaataag cggaggaaaa gaaaccaaca

481 gggattgcct tagtagcggc gagtgaagcg gcaaaagctc aaatttgaaa tctggcactt

541 tcagtgtccg agttgtaatt tgaagaaggt atctttgggt ctggctcttg tctatgtttc

601 ttggaacaga acgtcacaga gggtgagaat cccgtgcgat gagatgtccc agacctatgt

661 aaagttcctt cgaagagtc

Candida metapsilosis strain R38 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

GenBank: JQ665419.1

FASTAGraphicsPopSet

Go to:

LOCUS JQ665419 571 bp DNA linear PLN 16-MAY-2012

DEFINITION Candida metapsilosis strain R38 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 JQ665419

VERSION JQ665419.1 GI:387230662

KEYWORDS .

SOURCE Candida metapsilosis

ORGANISM Candida metapsilosis

Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; mitosporic Saccharomycetales; Candida.

REFERENCE 1 (bases 1 to 571)

AUTHORS Kuriakose,R., Bright Singh,I. and Philip,R.

TITLE Molecular identification of Candida from Arabian Sea

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 571)

AUTHORS Kuriakose,R., Bright Singh,I. and Philip,R.

TITLE Direct Submission

JOURNAL Submitted (09-FEB-2012) Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Fine Arts Avenue, Kochi, Kerala 682016, India

FEATURES Location/Qualifiers

source 1..571

/organism="Candida metapsilosis"

/mol_type="genomic DNA"

/strain="R38"

/isolation_source="marine sediment"

/db_xref="taxon:273372"

misc_RNA <1..>571

/note="contains 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 28S ribosomal RNA"

ORIGIN

1 tctttctaca catgtgtttt tctttttttt gaaaactttg ctttggtggg cccacggcct

61 gccagagatt aaactcaacc aaatttttat ttaattgtca acttgattaa ctaatagtca

121 aaactttcaa caacggatct cttggttctc gcatcgatga agaacgcagc gaaatgcgat

181 aagtaatatg aattgcagat attcgtgaat catcgaatct ttgaacgcac attgcgccct

241 ttggtattcc aaagggcatg cctgtttgag cgtcatttct ccctcaaacc ttcgggtttg

301 gtgttgagcg atacgctggg tttgcttgaa agaaaggcgg agtataaact aatggatagg

361 tttttttttt ccactcattg gtacaaactc caaacattct tccaaattca acctcaaatc

421 aggtaggact acccgctgaa cttaagcata tcaataagcg gaggaaaaga aaccaacagg

481 gattgcctta gtagcggcga gtgaagcggc aaaagctcaa atttgaaatc tggcactttc

541 agtgtccgag ttgtaatttg aaaaaggtat c

Candida orthopsilosis strain R95 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

GenBank: JQ665420.1

FASTAGraphicsPopSet

Go to:

LOCUS JQ665420 679 bp DNA linear PLN 16-MAY-2012

DEFINITION Candida orthopsilosis strain R95 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 JQ665420

VERSION JQ665420.1 GI:387230663

KEYWORDS .

SOURCE Candida orthopsilosis

ORGANISM Candida orthopsilosis

Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; mitosporic Saccharomycetales; Candida.

REFERENCE 1 (bases 1 to 679) AUTHORS Kuriakose,R., Bright Singh,I. and Philip,R.

TITLE Molecular identification of Candida from Arabian Sea JOURNAL Unpublished

REFERENCE 2 (bases 1 to 679)

AUTHORS Kuriakose,R., Bright Singh,I. and Philip,R.

TITLE Direct Submission

JOURNAL Submitted (09-FEB-2012) Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Fine Arts Avenue, Kochi, Kerala 682016, India

FEATURES Location/Qualifiers

source 1..679

/organism="Candida orthopsilosis"

/mol_type="genomic DNA"

/strain="R95"

/isolation_source="marine sediment"

/db_xref="taxon:273371" misc_RNA <1..>679

/note="contains 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 28S ribosomal RNA"

ORIGIN

1 actgcatttt tttacacatg tgtttttctt ttttttgaa actttgcttt ggtgggccca

61 tggcctggca gaaattaaac tcaaccaaat tttatttaag tcaactgatt aactaaaagt

121 caaaactttc aacaacggat ctcttgggtc tcgcatcgat gaaaaacgca gcgaaatgcg

181 ataagtaata tgaattgcag atattcgtga atcatcgaat ctttgaacgc acattgcgcc

241 ctttgggatt ccaaagggga tgcctgtttg agcgtcattt ctccctcaaa ccttcgggtt

301 tggggttgag cgatacgctg ggtttgcttg aaagaaaggc ggagtataaa ctaatggata

361 ggtttttttc cactccttgg tacaaactcc aaaattcttc caaattcgac ctcaaatcag

421 gtaggactac ccgctgaact taagcatatc aataagcgga ggagaagaaa ccaacaggga

481 ttgccttatt agcggcgagt gaagcggcaa aagctcaaat ttgaaatctg gcgctttcag

541 tgtccgagtt gtaatttgaa gaaggtatct ttggggctgg cgcttgtcta tgtttcttgg

601 agcagaacgt cacagagggt gagaatcccg tgcgatgaga tgtcccacac ctatgtaaag

661 ttccttcgaa gagtcgagt

Candida orthopsilosis strain S493 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

GenBank: JQ665421.1

FASTAGraphicsPopSet

Go to:

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 LOCUS       JQ665421                446 bp    DNA     linear   PLN 16-MAY-2012
DEFINITION  Candida orthopsilosis strain S493 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   JQ665421
VERSION     JQ665421.1  GI:387230664
KEYWORDS    .
SOURCE      Candida orthopsilosis
ORGANISM  Candida orthopsilosis
          Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina;
          Saccharomycetes; Saccharomycetales; mitosporic Saccharomycetales;
Candida.
REFERENCE   1  (bases 1 to 446)
  AUTHORS   Kuriakose,R., Bright Singh,I. and Philip,R.
  TITLE     Molecular identification of Candida from Arabian Sea
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 446)
  AUTHORS   Kuriakose,R., Bright Singh,I. and Philip,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (09-FEB-2012) Department of Marine Biology, Microbiology
and Biochemistry, Cochin University of Science and Technology, Fine
          Arts Avenue, Kochi, Kerala 682016, India
FEATURES             Location/Qualifiers
source          1..446
                /organism="Candida orthopsilosis"
                /mol_type="genomic DNA"
                /strain="S493"
                /isolation_source="marine sediment"
                /db_xref="taxon:273371"
     misc_RNA       <1..>446
                /note="contains 18S ribosomal RNA, internal transcribed
spacer 1, 5.8S ribosomal RNA, internal transcribed spacer
2, and 28S ribosomal RNA"
ORIGIN
        1 atgtgttttt cttttttttt gaaactttgc tttggtgggc ccatggcctg ccagagatta
       61 aactcaacca aattttattt aagtcaactg attaactaat agtcaaaact ttcaacaacg
      121 gatctcttgg ttctcgcatc gatgaagaac gcagcgaaat gcgataagta atatgaattg
      181 cagatattcg tgaatcatcg aatctttgaa cgcacattgc gccctttggt attccaaagg
      241 gcatgcctgt ttgagcgtca tttctccctc aaaccttcgg gtttggtgtt gagcgatacg
      301 ctgggtttgc ttgaaagaaa ggcggagtat aaactaatgg ataggttttt ttccactcat
      361 tggtacaaac tccaaaattc ttccaaattc gacctcaaat caggtaggac tacccgctga
      421 acttaagcat atcaataagc ggagga
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Hortaea werneckii strain R23 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

GenBank: JQ665422.1

FASTAGraphics

Go to:

LOCUS JQ665422 713 bp DNA linear PLN 16-MAY-2012
DEFINITION Hortaea werneckii strain R23 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 JQ665422
VERSION JQ665422.1 GI:387230665
KEYWORDS .
SOURCE Hortaea werneckii
ORGANISM Hortaea werneckii Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetidae; Dothideales; mitosporic Dothideales; Hortaea.
REFERENCE 1 (bases 1 to 713)
AUTHORS Kuriakose,R., Bright Singh,I., Antony,S.P. and Philip,R.
TITLE RFLP in yeast JOURNAL Unpublished
REFERENCE 2 (bases 1 to 713)
AUTHORS Kuriakose,R., Bright Singh,I., Antony,S.P. and Philip,R.
TITLE Direct Submission
JOURNAL Submitted (10-FEB-2012) Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Fine Arts Avenue, Kochi, Kerala 682016, India
FEATURES Location/Qualifiers
source 1..713
/organism="Hortaea werneckii"
/mol_type="genomic DNA"
/strain="R23"
/isolation_source="marine sediment"
/db_xref="taxon:91943"
misc_RNA <1..>713
/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 ctcggcgcct cctccacccc atgtcgaaac gactctgttg cctcgggggc gacccggcct
      61 tcgggcgtcg gggcccccgg cggacacctt cataactctt gcatctcttg cgtctgagtg
     121 atacatataa tcaatcaaaa ctttcaacaa cggatctctt ggttctggca tcgatgaaga
     181 acgcagcgaa atgcgataag taatgtgaat tgcagaattc agtgaatcat cgaatctttg
     241 aacgcacatt gcgccccctg gcattccggg gggcatgcct gttcgagcgt cattacacca
     301 ctcaagcctg gcttggtatt gagcgaccgc ggcctgcccg cgcgctccaa tgtctccggc
     361 tgagccgtcc gtctctaagc gttgtgaata gcgatcgctt gcgaggcccg ggcggttcga
     421 cgccgttaaa ccccccccca ttttctatgg ttgacctcgg atcaggtagg gatacccgct
     481 gaacttaagc atatcaataa gcggaggaaa agaaaccaac agggattgcc ctagtaacgg
     541 cgagtgaagc ggcaacagct caaatttgaa atctggcgca agcccgagtt gtaatttgta
     601 gaggatgctt ctgggcagcg gccggtctaa gttccttgga acaggacgtc atagagggtg
     661 agaatcccgt atgcgaccgg cttgcacccg tcacgtagct ccttcgacga gtc
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Rhodotorula slooffiae strain R28Pi 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

GenBank: JQ665423.1

FASTAGraphicsPopSet

Go to:

LOCUS JQ665423 676 bp DNA linear PLN 16-MAY-2012
DEFINITION Rhodotorula slooffiae strain R28Pi 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 JQ665423
VERSION JQ665423.1 GI:387230666
KEYWORDS .
SOURCE Rhodotorula slooffiae
ORGANISM Rhodotorula slooffiae
Eukaryota; Fungi; Dikarya; Basidiomycota; Pucciniomycotina; Cystobasidiomycetes; Erythrobasidiales; Erythrobasidiaceae; Rhodotorula.
REFERENCE 1 (bases 1 to 676)
AUTHORS Kuriakose,R., Bright Singh,I. and Philip,R.
TITLE RFLP in Rhodotorula
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 676)
AUTHORS Kuriakose,R., Bright Singh,I. and Philip,R.
TITLE Direct Submission
JOURNAL Submitted (10-FEB-2012) Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Fine Arts Avenue, Kochi, Kerala 682016, India
FEATURES Location/Qualifiers
source 1..676
/organism="Rhodotorula slooffiae"
/mol_type="genomic DNA"
/strain="R28Pi"
/isolation_source="marine sediment"
/db_xref="taxon:106018"
misc_RNA<1..>676
/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 agaggcttct gggagccggc aacggcacct agtcgctgag aagtttgacg aacttggtca
     61 tttagaggaa gtaaaagtcg taacaaggtt tccgtaggtg aacctgcgga aggatcatta
    121 atgaatttta ggacgttctt tttagaagtc cgaccatttc attttcttac actgtgcaca
    181 cacttctttt tacacccact tttaacacat tagtataaga atgtaatagt ctcttaattg
    241 agcataaata aaaacaaaac tttcagcaac ggatctcttg gctctcgcat cgatgaagaa
    301 cgcagcgaat tgcgataagt aatgtgaatt gcagaattca gtgaatcatc gaatctttga
    361 acgcaccttg cactctttgg tattccgaag agtatgtctg tttgagtgtc atgaaactct
    421 caacccccct attttgtaat gagatgggcg tgggcttgga ttatggttgt ctgtcggcgt
    481 aatggccggc tcaactgaaa tacacgagca acccaattga aataaacggt ttgacttggc
    541 gtaataatta tttcgctaag gacgttttct tcaaatataa gaggtgcttc taattcgctt
    601 ctaatagcat ttaagcttta gacctcaaat cagtcatgac tacccgctga acttaagcat
    661 atcaataagc ggagga
```

Rhodotorula calyptogenae strain R19Pi 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

GenBank: JQ665424.1

FASTAGraphicsPopSet

Go to:

LOCUS JQ665424 599 bp DNA linear PLN 16-MAY-2012
DEFINITION Rhodotorula calyptogenae strain R19Pi 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 JQ665424
VERSION JQ665424.1 GI:387230667
KEYWORDS .
SOURCE Rhodotorula calyptogenae
ORGANISM Rhodotorula calyptogenae
Eukaryota; Fungi; Dikarya; Basidiomycota; Pucciniomycotina; Cystobasidiomycetes; Erythrobasidiales; Erythrobasidiaceae; Rhodotorula.
REFERENCE 1 (bases 1 to 599)
AUTHORS Kuriakose,R., Bright Singh,I. and Philip,R.
TITLE RFLP in Rhodotorula
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 599)
AUTHORS Kuriakose,R., Bright Singh,I. and Philip,R.
TITLE Direct Submission
JOURNAL Submitted (10-FEB-2012) Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Fine Arts Avenue, Kochi, Kerala 682016, India
FEATURES Location/Qualifiers source 1..599
/organism="Rhodotorula calyptogenae"
/mol_type="genomic DNA"
/strain="R19Pi"
/isolation_source="marine sediment"
/db_xref="taxon:255702"
misc_RNA<1..>599
/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 tacactgtgc acacacttct ttttacacac attttaacac attagtataa gaatgtaaca
     61 gtctcttaat tgagcataaa taaaaacaaa actttcagca acggatctct tggctctcgc
    121 atcgatgaag aacgcagcga attgcgataa gtaatgtgaa ttgcagaatt cagtgaatca
    181 tcgaatcttt gaacgcacct tgcactcttt ggtattccga agagtatgtc tgtttgagtg
    241 tcatgaaact ctcaaccccc ctattttgta atggaatggg cgtgggcttg gattatggct
    301 gtctgtcggc gtaatagccg gctcagctga aatacacgag ctacccttt gaaataaacg
    361 gtttgactcg gcgtaataat tattccgctg aggacgtttt cttcaaatgt tagaggtgct
    421 tctaatgcgc ttttaaagca acttaagctt tagacctcaa atcagtcagg actacccgct
    481 gaacttaagc atatcaataa gcggaggaaa agaaactaac aaggattccc ctagtaacgg
    541 cgagtgaagt gggaaaagct caactttgaa atctggcacc ttcggtgtcc gagttgtag
```

Rhodotorula minuta strain R382Pi 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

GenBank: JQ665425.1

FASTAGraphicsPopSet

Go to:

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 LOCUS       JQ665425                528 bp    DNA     linear   PLN 16-MAY-2012
DEFINITION  Rhodotorula minuta strain R382Pi 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   JQ665425
VERSION     JQ665425.1  GI:387230668
KEYWORDS    .
SOURCE      Rhodotorula minuta
ORGANISM  Rhodotorula minuta
          Eukaryota; Fungi; Dikarya; Basidiomycota; Pucciniomycotina;
          Cystobasidiomycetes; Erythrobasidiales; Erythrobasidiaceae;
Rhodotorula.
REFERENCE   1  (bases 1 to 528)
  AUTHORS   Kuriakose,R., Bright Singh,I. and Philip,R.
  TITLE     RFLP in Rhodotorula
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 528)
  AUTHORS   Kuriakose,R., Bright Singh,I. and Philip,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (10-FEB-2012) Department of Marine Biology, Microbiology
and Biochemistry, Cochin University of Science and Technology, Fine
          Arts Avenue, Kochi, Kerala 682016, India
FEATURES             Location/Qualifiers
source          1..528
                /organism="Rhodotorula minuta"
                /mol_type="genomic DNA"
                /strain="R382Pi"
                /isolation_source="marine sediment"
                /db_xref="taxon:29899"    misc_RNA       <1..>528
                /note="contains 18S ribosomal RNA, internal transcribed
spacer 1, 5.8S ribosomal RNA, internal transcribed spacer
2, and 28S ribosomal RNA"
ORIGIN
        1 tccgaccgtt tccttttctt acactgtgca cacacttctt tttactcaca cttttaacac
       61 attagtatag gaatgtgata gtctcttaat tgcgcctaaa caaaaataat actttcgtca
      121 gcggatctct tggctctcgc atcgatgaag aacgcagcga attgcgataa gtaatgtgaa
      181 ttgcagaatt cggtgaatca tcgaatcttt gaacgcacct tgcactcttt ggtattccga
      241 agagtatgtc tgtttgagta tcatgaaact ctcaaccccc ctattttgta atgaaatggg
      301 cgtgggcttg gattatggtt gtctgtcggc gtaattgccg gctcaactga aatacacgag
      361 caaccctatt gaaatagacg gtttgacttg gcgtaataat tatttcgcta aggacgtctt
      421 cttcaaatat aagaggtgct tctaatgcgc tttatagcac tttaagcttt agatctcaaa
      481 tcagtcagga ctacccgctg aacttaagca tatcaataag cggaggaa
```

Debaryomyces nepalensis strain R305 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

GenBank: JQ665426.1

FASTAGraphicsPopSet

LOCUS JQ665426 705 bp DNA linear PLN 16-MAY-2012

DEFINITION Debaryomyces nepalensis strain R305 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 JQ665426

VERSION JQ665426.1 GI:387230669

KEYWORDS .

SOURCE Debaryomyces nepalensis

ORGANISM Debaryomyces nepalensis

Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Debaryomycetaceae; Debaryomyces.

REFERENCE 1 (bases 1 to 705)

AUTHORS Kuriakose,R., Kutty,S.N., Bright Singh,I. and Philip,R.

TITLE Molecular identification of Marine yeast

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 705)

AUTHORS Kuriakose,R., Kutty,S.N., Bright Singh,I. and Philip,R.

TITLE Direct Submission

JOURNAL Submitted (10-FEB-2012) Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Fine Arts Avenue, Kochi, Kerala 682016, India

FEATURES Location/Qualifiers source 1..705

/organism="Debaryomyces nepalensis"

/mol_type="genomic DNA"

/strain="R305"

/isolation_source="marine sediment"

/db_xref="taxon:27299" misc_RNA <1..>705

/note="contains 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 28S ribosomal RNA"

ORIGIN

1 acacacagtg ttttttgtta ttacaagaac tcttgctttg gtctggacta gaaatagttt

61 gggccagagg tttactaaac taaacttcaa tatttatatt gaattgttat ttattttaat

121 tgtcaatttg ttgattaaat tcaaaaaatc ttcaaaactt tcaacaacgg atctcttggt

181 tctcgcatcg atgaagaacg cagcgaaatg cgataagtaa tatgaattgc agattttcgt

241 gaatcatcga atctttgaac gcacattgcg ccctttggta ttccaaaggg catgcctgtt

301 tgagcgtcat ttctctctca aaccttcggg tttggtattg agtgatactc ttagtcgaac

361 taggcgtttg cttgaaatgt attggcatga gtggtactgg atagtgctat atgactttca

421 atgtattagg tttatccaac tcgttgaata gtttaatggt atatttctcg gtattctagg

481 ctcggcctta caatataaca aacaagtttg acctcaaatc aggtaggact acccgctgaa

541 cttaagcata tcaataagcg gaggaaaaga aaccaacagg gattgcctta gtaacggcga

601 gtgaagcggc aaaagctcaa atttgaaatc tggcgccttc ggtgtccgag ttgtaatttg

661 aagaaggtaa ctttggagtt ggctcttgtc tatgttcctt ggaac

Debaryomyces sp. R344 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

GenBank: JQ665427.1

FASTAGraphicsPopSet

Go to:

LOCUS JQ665427 549 bp DNA linear PLN 16-MAY-2012

DEFINITION Debaryomyces sp. R344 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 JQ665427

VERSION JQ665427.1 GI:387230670

KEYWORDS .

SOURCE Debaryomyces sp. R344

ORGANISM Debaryomyces sp. R344

Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Debaryomycetaceae; Debaryomyces.

REFERENCE 1 (bases 1 to 549)

AUTHORS Kuriakose,R., Kutty,S.N., Bright Singh,I. and Philip,R.

TITLE Molecular identification of Marine yeast

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 549)

AUTHORS Kuriakose,R., Kutty,S.N., Bright Singh,I. and Philip,R.

TITLE Direct Submission

JOURNAL Submitted (10-FEB-2012) Department 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="Debaryomyces sp. R344"

/mol_type="genomic DNA"

/strain="R344"

/isolation_source="marine sediment"

/db_xref="taxon:1186041" 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

1 agtcgtaaca aggtttccgt aggtgaacct gcggaaggat cattacagta ttctttttgc
61 cagcgcttaa ttgcgcggcg aaaaaacctt acacacagtg ttttttgtta ttacaagaac
121 ttttgctttg gtctggacta gaaatagttt gggccagagg tttactgaac taaacttcaa
181 tatttatatt gaattgttat ttatttaatt gtcaatttgt tgattaaatt caaaaaatct
241 tcaaaacttt caacaacgga tctcttggtt ctcgcatctt ttttttacg cagcgaaatg
301 cgataagtaa tatgaattgc agattttcgt gaatcatcaa atctttgaac gcacattgcg
361 cccctctggg aattccaaag ggcatgccgg tttgaagtaa cggcgagtga agcggcaaaa
421 gctcaaattt gaaatctggc accttcggtg tccgagttgt aatttgaaga agtaactttg
481 gagttggctc ttgtctatgt tccttggaac aggacgtcac agagggtgag aatcccgtgc
541 gatgagatg

Debaryomyces sp. R126 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

GenBank: JQ665428.1

FASTAGraphicsPopSet

LOCUS JQ665428 653 bp DNA linear PLN 16-MAY-2012

DEFINITION Debaryomyces sp. R126 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 JQ665428

VERSION JQ665428.1 GI:387230671

KEYWORDS .

SOURCE Debaryomyces sp. R126

ORGANISM Debaryomyces sp. R126

Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Debaryomycetaceae; Debaryomyces.

REFERENCE 1 (bases 1 to 653)

AUTHORS Kuriakose,R., Kutty,S.N., Bright Singh,I. and Philip,R.

TITLE Molecular identification of Marine yeast

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 653)

AUTHORS Kuriakose,R., Kutty,S.N., Bright Singh,I. and Philip,R.

TITLE Direct Submission

JOURNAL Submitted (10-FEB-2012) Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Fine Arts Avenue, Kochi, Kerala 682016, India

FEATURES Location/Qualifiers

source 1..653

/organism="Debaryomyces sp. R126"

/mol_type="genomic DNA"

/strain="R126"

/isolation_source="marine sediment"

/db_xref="taxon:1186039" misc_RNA <1..>653

/note="contains 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 28S ribosomal RNA"

ORIGIN

1 acacacagtg ttttttgtta ttacaagaac ttttgctttg gtctggacta gaaatagttt

61 gggccagagg tttactgaac taaacttcaa tatttatatt gaattgttat ttatttaatt

121 gtcaatttgt tgattaaatt caaaaaatct tcaaaacttt caacaacgga tctcttggtt

181 ctcgcatcga tgaagaacgc agcgaaatgc gataagtaat atgaattgca gattttcgtg

241 aatcatcgaa tctttgaacg cacattgcgc cctctggtat tccagagggc atgcctgttt

301 gagcgtcatt tctctctcaa accttcgggt ttggtattga gtgatactct tagtcgaact

361 aggcgtttgc ttgaaatgta ttggcatgag tggtactgga tagtgctata tgactttcaa

421 tgtattaggt ttatccaact cgttgaatag tttaatggta tatttctcgg tattctaggc

481 tcggccttac aatataacaa acaagtttga cctcaaatca ggtaggatta cccgctgaac

541 ttaagcatat caataagcgg aggaaaagaa accaacaggg attgccttag taacggcgag

601 tgaagcggca aaagctcaaa tttgaaatct ggcaccttcg gtgtccgagt tgt


Debaryomyces sp. R100 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

GenBank: JQ665429.1

FASTAGraphicsPopSet

Go to:

LOCUS JQ665429 661 bp DNA linear PLN 16-MAY-2012

DEFINITION Debaryomyces sp. R100 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 JQ665429

VERSION JQ665429.1 GI:387230672

KEYWORDS .

SOURCE Debaryomyces sp. R100

ORGANISM Debaryomyces sp. R100

Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Debaryomycetaceae; Debaryomyces.

REFERENCE 1 (bases 1 to 661)

AUTHORS Kuriakose,R., Kutty,S.N., Bright Singh,I. and Philip,R.

TITLE Molecular identification of Marine yeast

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 661)

AUTHORS Kuriakose,R., Kutty,S.N., Bright Singh,I. and Philip,R.

TITLE Direct Submission

JOURNAL Submitted (10-FEB-2012) Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Fine Arts Avenue, Kochi, Kerala 682016, India

FEATURES Location/Qualifiers source 1..661

/organism="Debaryomyces sp. R100"

/mol_type="genomic DNA"

/strain="R100"

/isolation_source="marine sediment"

/db_xref="taxon:1186038"

misc_RNA <1..>661 /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 ttagtagagg cctccggatt ggtttaaaga agggggcaac tccatcttgg aaccgaaaag
       61 ctggtcaaac ttggtcattt agaggaagta aaagtcgtaa caaggtttcc gtaggtgaac
      121 ctgcggaagg atcattacag tattcttttt gccagcgctt aattgcgcgg cgaaaaaacc
      181 ttacacacag tgtttttgt tattacaaga acttttgctt tggtctggac tagaaatagt
      241 ttgggccaga ggtttactga actaaacttc aatatttata ttgaattgtt atttatttaa
      301 ttgtcaattt gttgattaaa ttcaaaaaat cttcaaaact ttcaacaacg gatctcttgg
      361 ttctcgcatc gatgaagaac gcagcgaaat gcgataagta atatgaattg cagattttcg
      421 tgaatcatcg aatctttgaa cgcacattgc gccctctggt attccagagg gcatgcctgt
      481 ttgagcgtca tttctctctc aaaccttcgg gtttggtatt gagtgatact cttagtcgaa
      541 ctaggcgttt gcttgaaatg tattggcatg agtggtactg gatagtgcta tatgactttc
      601 aatgtattag gtttatccaa ctcgttgaat agtttaatgg tatatttctc ggtattctag
      661 g
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Debaryomyces sp. R99 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

GenBank: JQ665430.1

FASTAGraphicsPopSet

Go to:

LOCUS JQ665430 711 bp DNA linear PLN 16-MAY-2012

DEFINITION Debaryomyces sp. R99 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 JQ665430

VERSION JQ665430.1 GI:387230673

KEYWORDS .

SOURCE Debaryomyces sp. R99

ORGANISM Debaryomyces sp. R99

Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Debaryomycetaceae; Debaryomyces. REFERENCE 1 (bases 1 to 711)

AUTHORS Kuriakose,R., Kutty,S.N., Bright Singh,I. and Philip,R.

TITLE Molecular identification of Marine yeast

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 711)

AUTHORS Kuriakose,R., Kutty,S.N., Bright Singh,I. and Philip,R.

TITLE Direct Submission

JOURNAL Submitted (10-FEB-2012) Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Fine Arts Avenue, Kochi, Kerala 682016, India

FEATURES Location/Qualifiers

source 1..711

/organism="Debaryomyces sp. R99"

/mol_type="genomic DNA"

/strain="R99"

/isolation_source="marine sediment"

/db_xref="taxon:1186043" misc_RNA <1..>711

/note="contains 18S ribosomal RNA, internal transcribed spacer 1, 5.8S ribosomal RNA, internal transcribed spacer 2, and 28S ribosomal RNA"

ORIGIN

1 gcagcgctta ctgcgcggcg acaccttaca cacagtgtct ttttgataca gaactcttgc
61 tttggtttgg cctagagata ggttgggcca gaggtttaac aaaacacaat ttaattattt
121 ttacagttag tcaacttttg aattaatctt caaaactttc aacaacggat ctcttggttc
181 tcgcatcgat gaagaacgca gcgaaatgcg ataagtaata tgaattgcag attttcgtga
241 atcatcgaat ctttgaacgc acattgcgcc ctctggtatt ccagagggca tgcctgtttg
301 agcgtcattt ctctctcaaa cccccgggtt tggtattgag tgatactctt agtcggacta
361 ggcgtttgct tgaaaagtat tggcatgggt agtactggat agtgctgtcg acctctcaat
421 gtattaggtt tatccaactc gttgaatggt gtggcgggat atttctggta ttgttggccc
481 ggccttacaa caaccaaaca agtttgacct caaatcaggt aggaataccc gctgaactta
541 agcatatcaa taagcggagg aaaagaaacc aacagggatt gccttagtag cggcgagtga
601 agcggcaaaa gctcaaattt gaaatctggc gccttcggtg tccgagttgt aatttgaaga
661 ttgtaacctt gggggttggc tcttgtctat gtttcttgaa acaggacgtc a

Debaryomyces sp. R81 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

GenBank: JQ665431.1

FASTAGraphics

Go to:

LOCUS JQ665431 617 bp DNA linear PLN 16-MAY-2012

DEFINITION Debaryomyces sp. R81 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 JQ665431

VERSION JQ665431.1 GI:387230674

KEYWORDS .

SOURCE Debaryomyces sp. R81

ORGANISM Debaryomyces sp. R81

Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Debaryomycetaceae; Debaryomyces.

REFERENCE 1 (bases 1 to 617)

AUTHORS Kuriakose,R., Kutty,S.N., Bright Singh,I. and Philip,R.

TITLE Molecular identification of Marine yeast

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 617)

AUTHORS Kuriakose,R., Kutty,S.N., Bright Singh,I. and Philip,R.

TITLE Direct Submission

JOURNAL Submitted (10-FEB-2012) Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Fine Arts Avenue, Kochi, Kerala 682016, India FEATURES Location/Qualifiers source 1..617

/organism="Debaryomyces sp. R81"

/mol_type="genomic DNA"

/strain="R81"

/isolation_source="marine sediment"

/db_xref="taxon:1186042" misc_RNA <1..>617

/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 acacacagtg ttttttgtta ttacaagaac ttttgctttg gtctggacta gaaatagttt
     61 gggccagagg tttactgaac taaacttcaa tatttatatt gaattgttat ttatttaatt
    121 gtcaatttgt tgattaaatt caaaaaatct tcaaaacttt caacaacgga tctcttggtt
    181 ctcgcatcga tgaagaacgc agcgaaatgc gataagtaat atgaattgca gattttcgtg
    241 aatcatcgaa tctttgaacg cacattgcgc cctctggtat tccagagggc atgcctgttt
    301 gagcgtcatt tctctctcaa accttcgggt ttggtattga gtgatactct tagttgaact
    361 aggcgtttgc ttgaaatgta ttggcatgag tggtactgga tagtgctata tgactttcaa
    421 tgtattaggt ttatccaact cgttgaatag tttaatggta tatttctcgg tattctaggc
    481 tcggccttac aatataacaa acaagtttga cctcaaatca ggtaggatta cccgctgaac
    541 ttaagcatat caataagcgg aggaaaagaa accaacaggg attgccttag taacggcgag
    601 tgaagcggca aaagctc
```

Debaryomyces sp. R140 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

GenBank: JQ665432.1

FASTAGraphicsPopSet

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LOCUS JQ665432 655 bp DNA linear PLN 16-MAY-2012

DEFINITION Debaryomyces sp. R140 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 JQ665432

VERSION JQ665432.1 GI:387230675

KEYWORDS .

SOURCE Debaryomyces sp. R140

ORGANISM Debaryomyces sp. R140

Eukaryota; Fungi; Dikarya; Ascomycota; Saccharomycotina; Saccharomycetes; Saccharomycetales; Debaryomycetaceae; Debaryomyces. REFERENCE 1 (bases 1 to 655)

AUTHORS Kuriakose,R., Kutty,S.N., Bright Singh,I. and Philip,R.

TITLE Molecular identification of Marine yeast

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 655)

AUTHORS Kuriakose,R., Kutty,S.N., Bright Singh,I. and Philip,R.

TITLE Direct Submission

JOURNAL Submitted (10-FEB-2012) Department 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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/strain="R140"

/isolation_source="marine sediment"

/db_xref="taxon:1186040" misc_RNA <1..>655

/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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