Summary and Conclusions

Marine yeasts are an important category of marine microorganisms because they are the active producers of different kind of metabolites, which cannot be produced by the terrestrial microorganisms. Among these, polysaccharides are widely used for biomedical, pharmaceutical and many other industrial purposes. The most promising biological activities of these polysaccharides are their antioxidant and anticancer effects.

Marine yeasts were isolated and characterized from sea water and sediments at different depth regions of Arabian Sea. 109 different species were distributed among the genera Candida, Debaryomyces, Pichia, Rhodotorula, Trichosporon and Cryptococcus. Candida was found to be the dominant genera. Furthermore, the oxidative forms were more predominant than the fermentative forms. All the 109 yeast isolates were potential producers of industrially important enzymes such as amylase, gelatinase, lipase and urease. Investigations of the biodiversity of marine yeasts at different stations and depth regions have suggested possible interactions among species. However, by considering the different diversity indices, Shannon-wiener diversity (H’ (log2)), Pielou’s evenness (J’), Species richness (d) and Species dominance (λ)) were found to be higher at 500m depth region. The maximum similarity in the distribution and abundance was observed between station 19, station 10 and station 12, at 800, 700 and 900m depth regions.

Microorganisms are the best sources for the commercial production of polysaccharides. A potential isolate (MBTU_MYW1 (TW1)) isolated from seawater at a depth of 1000m, tentatively identified as Meyerozyma
**guilliermondii** was very active in production of exomannan and cell wall mannan was selected for further study.

A suitable media was optimized for maximum yield of exomannan by one-factor-at-a-time method. The optimal medium for maximum yield of biomass and exomannan production was sea water containing 5% sucrose and 0.25% ammonium sulphate with the optimum cultivation conditions of initial pH 5.6, temperature 22°C and an incubation period of 120 hrs. Under the optimized conditions, exomannan production was highest and 4 g/L was obtained by ethanol precipitation from 10.24 g/L biomass. 4.25 g/L cell wall mannan was obtained from 10.27 g/L biomass of *M. guilliermondii* cells was obtained by alkali extraction. These mannans were of exceptionally high purity: 98.8% and 95% for exomannan and cell wall mannan respectively. The purified mannans contain mannose alone. So *M. guilliermondii* can be used extensively for large-scale industrial production of mannan and has found a number of applications in different industries.

The SEM analysis revealed that the morphology of the yeast cells during exomannan biosynthesis in a continuous culture at 0, 24, 48, 72, 96, 120 and 144 hrs of incubation was markedly different from that of the normal cells. The study revealed that the release of exomannan into the culture medium may also be by the autolysis of mature cells. The cells had an irregular, uneven and perforated shape and an extensive lysis of cells occurred at 120 hrs of incubation. The release of exomannan may be a consequence of cell wall-controlled hydrolysis of the mother cells.

In the SEM analysis, both the cell wall mannan and exomannan appeared as loose flaky curly aggregations and this demonstrated that these polysaccharides were a type of amorphous solid. Elemental analysis of both
the mannans by SEM-EDX revealed that exomannan contain only 40.369% carbon and 59.631% oxygen and cell wall mannan 41.924% carbon and 58.076% oxygen. In addition to SEM, the ultrastructure of yeast mannans were analyzed by TEM and the results revealed that these mannans consist of agglomerates of small grains with a diameter of 100 nm. The TEM analysis was in good agreement with the potential application of these mannans in nanotechnology and biomedical industry as an organic nanomaterial.

The monosaccharide composition of both the yeast mannans was identified as mannose by TLC analysis. The UV-visible spectrophotometer showed the characteristic UV absorption peak for a polysaccharide at 190 nm only. The purity, homogeneity and molecular weight of the yeast mannans determined by HPLC-GPC showed a symmetrical peak, indicating a homogeneous fraction. According to HPLC-GPC analysis the molecular weight of the exomannan and cellwall mannan were 107713 Da and 161355 Da respectively. MALDI-TOF MS analysis in [M+H]+ mode gave a single peak at a m/z value of nearly the molecular weight obtained by HPLC-GPC.

The GC analysis of both mannans showed the peak for mannose as the predominant monosaccharide. GC-MS using the combination of methylation, a powerful tool for linkage analysis showed the predominant structural features such as the 1,2- and 1,6-linked mannopyranose units in the polymer. The FT-IR and FTR analysis showed characteristic absorption bands for $\alpha$-type glycosidic linkage. The chemical structure of both mannans assigned by 1D ($^{13}$C NMR and $^1$H NMR) and 2D (HSQC, HMBC and COSY) NMR experiments revealed a highly branched structure of the mannans with a backbone composed of 1,6 linked mannopyranose residues branched at the position O-2 by side oligosaccharide chain composed mostly 1,2-linked mannopyranose residues and small amount of 1,6-linked internal
mannose residues. Therefore, these results confirmed the monosaccharide types, glycosidic bonds and functional groups of the mannans produced by the *M. guilliermondii*. The cell wall mannan and exomannan isolated from *M. guilliermondii* had different molecular weights but similar structural configuration. These promising structural characteristics could be regarded as initiatory steps towards the utilization and modification of yeast mannans as future sources for the production of valuable drugs in the biomedical fields.

Taking all the *in vitro* antioxidant activity into consideration, the cell wall mannan and exomannan from *M. guilliermondii* was found to possess good antioxidant activity in a concentration dependent manner. These findings suggested that the yeast mannans were the potential source of natural antioxidants. The present findings confirm that the antioxidant activities of polysaccharides were related to the chemical characteristics such as molecular weight, monosaccharide composition and configuration. The exomannan having lowest molecular weight exhibited strong antioxidant activity compared to cell wall mannan. The antioxidant activity of the exomannan was nearly equal to that of the standard antioxidants.

The *in vitro* anticancer activity of yeast mannans by using Estrogen-receptor-positive MCF-7 cell line revealed that both the yeast mannans hold promise as potential sources of natural anticancer agents which are not cytotoxic to normal cells. The cell wall mannan and exomannan inhibited the cell growth of MCF-7 in a concentration dependent manner. The cells lost their characteristic appearance, became rounded, cell shrinkage and loss of cell adhesion of the culture surface, the characteristic of the apoptotic induction of cell death were seen in morphological studies. Caspase 3 activation cascade plays a central role in several apoptotic mechanisms. The
exomannan showed more Caspase activity compared to cell wall mannan. The accuracy of apoptosis results by DNA fragmentation which showed that DNA is cleaved into fragments of 180-200 nucleosomal units. No DNA fragmentation was detected in untreated cells. TUNEL assay was done to understand whether apoptotic nuclear morphology observed in MCF-7 cells after yeast mannan treatment correlated with the generation of 3’-OH DNA ends. The yeast mannan treated cells containing fragmented nuclear chromatin, characteristic of apoptosis exhibiting brown nuclear staining was observed in TUNEL assay. MTT, SRB, LDH, Caspase 3 activity, DNA fragmentation and TUNEL assay revealed the cytotoxic activity and induction of apoptosis by yeastmannans.

Based on the results obtained from the present study following conclusions are made. Biodiversity study of marine yeast from 21 different stations in the Arabian Sea coast at south west region of India revealed that among the six genera, genus Candida was the most prevalent both depth wise and station wise analysis. MBTU_MYW1 isolated from seawater at 1000m depth region is a potential yeast, which could be harnessed for large scale production of both cell wall mannan and exomannan. Under optimized condition MBTU_MYW1 can produce exomannan 4g/L from 10.24g/L biomass and 4.25g/L cell wall mannan from 10.27g/L biomass. To our knowledge this is the first report that a marine yeast can produce highest level of this important biopolymer. So we can propose that this strain can be developed as an industrial strain for the production this important biopolymer. MBTU_MYW1 was identified as Meyerozyma guilliermondii based on both biochemical and molecular level identification. Morphological study by SEM analysis during the biosynthesis of exomannan revealed that release of mannan may be the consequence of cell wall controlled hydrolysis.
of the mother cell during the emergence of bud and also may be autolysis of mature cells. The ultra structure of yeast mannans by TEM analysis indicated that these mannans consist of agglomerates of small grains with a diameter of 100nm. These results points out potential application of these mannans in nanotechnology. The molecular weight and homogeneity of these mannans were determined by HPLC-GPC and also confirmed by MALDI-TOF MS analysis. It was found to be 107713 Da for exomannan and 161355 Da for cell wall mannan respectively. The monosaccharide component was identified as mannose by TLC analysis. Polysaccharides are well known for their diverse biological activities. Their biological activity is mainly determined by molecular weight and structural complexity. In the present work we have tried to elucidate the structure based on GCMS, FT-IR and FTR spectroscopy and also by 1D and 2D NMR experiments. Based on these results we have proposed a possible chemical structure of the mannans and have come to the conclusion that both the mannans are of the same structural configuration. Under in vitro conditions both the mannans have potential antioxidant and antitumor activity against MCF-7 cells in a dose and time dependent manner. Among the mannans low molecular weight exomannan exhibited high degree of antioxidant as well as antitumor activity. SRB assay, LDH activity, Caspase 3 assay, DNA fragmentation and further TUNEL assay on treated MCF-7 cells showed cytotoxicity and apoptotic induction of these mannans. These results also suggested that both the mannans from M. gulliermondii are potential source of natural antioxidant and anticancer agents that could have great importance as therapeutic agents in preventing or slowing the prossess of aging and age associated oxidative stress related degenerated diseases such as cancer and various other human ailments. However further in vivo studies are required as this proof of
concept and also the molecular mechanism of apoptosis has to be elucidated. Taking into consideration the overall therapeutic potential of *M. guilliermondii* yeast mannans, it can be summerised that they are natural biopolymers with promising applications in nanotechnology and biomedical industry.