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Black yeasts from the slope sediments of Bay of Bengal: phylogenetic and functional characterization

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Abstract

Occurrence of black yeasts in the slope sediments of Bay of Bengal was investigated during FORV Sagar Sampada cruises 236 and 245. The black yeast population was found to be very scanty in the area and the isolates could be obtained from 200m to 1000m depth regions in the slope sediments. The isolates were identified as *Hortaea werneckii* by Internal Transcribed Spacer (ITS) sequencing. The biodegradation potential of these strains was found to be very high with all the strains exhibiting protease, lipase and amylase production. The optimum growth conditions were pH 8, salinity 30 ppt and temperature 30^{o}C. The pigment melanin, in these organisms was identified to be of dihydroxynaphthalene type by NMR. The melanin was found to exhibit inhibitory activity against different human and fish pathogens. Melanin degrading enzyme could also be extracted from these organisms.

Key words – Antibacterial – Black yeasts – Dihydroxynaphthalene – *Hortaea werneckii* – Marine – Melanin

Introduction

Black yeasts are a melanised group of fungi belonging to divergent orders of Ascomycetes (De Hoog & Mcginnis, 1987). Van Uden and Castelo-Branco (1963) have reported the presence of black yeasts 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 black yeasts. Ascomycetous black yeasts show adaptations to a wide array of environmental conditions. Factors which are of ecological significance include the presence of melanin and carotene, formation of thick walls and meristematic growth, presence of yeast-like phase, additional forms of conidiogenesis, thermo and osmotolerance, adhesion, hydro-phobicity, production of extracellular polysaccharides, siderophores and acidic or alkaline secondary metabolites (De Hoog, 1993).

Melanin is a common term used for dark brown to black pigments of high molecular mass formed by oxidative polymerization of phenolic compounds usually complexed with protein and carbohydrates. Fungal melanins occur in the cell wall or as extracellular polymers formed enzymatically or by auto oxidation (SN Kutty, 2010). The phenolic compounds from which the fungal melanins are derived include tyrosine *via* 3, 4-dihydroxyphenylalanine (DOPA) in some

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groups of fungi, γ-glutaminyl-3,4-dihydroxy-benzene or catechol in Basidiomycetes, and 1,8-dihydroxynaphthalene (DHN) in Ascomycetes and related asexual fungi. Melanins formed by DHN pathway are of particular interest, since they reportedly protect fungi against a number of environmental factors. Melanin preparations are widely used in dermatology and cosmetology. Paramonov et al. (2002) studied the dependence of photoprotective activity of 1, 8-dihydroxynaphthalene melanin using the black yeast like fungus *Aureobasidium pullulans* as the source of melanin. The application of melanin to skin included photoprotection, photosensitization and photoburn. Studies on *Hortaea werneckii, Trimmatostroma salinum, Phaeotheca triangularis* and *Phyllosticta capitalensis* showed the presence of DHN melanin (Kogej et al., 2004 and Suryanarayanan et al., 2004). Melanized fungi are more resistant to environmental factors than their non-melanized albino mutants or other normally non-melanized fungi (Butler et al., 2005). Melanin has diverse functions (Nosanchuk and Casadevall, 2006) *i.e*., sexual display and camouflage, colouration in black/ red hair and defence. They also serve as energy transducers and affect cellular integrity. Black yeast strains isolated from salt pans at the Adriatic coast were identified as *Hortaea werneckii, Phaeotheca triangularis* and *Aureobasidium pullulans*. Recently it became evident that melanized fungi, so far described only in the crystallization pond of Adriatic salterns within the season of salt production can be considered as a new group of eukaryotic halophiles (Gunde-Cimerman et al., 2000, Butinar et al., 2005). At the highest environmental salinities, melanized fungi represented 85-100% of the total isolated mycobiota, but with lowering salinities they were partially replaced by non-melanized fungi and, at the end of the season, with NaCl concentrations below 5%, they were detected only occasionally. Eukaryotic halophilic microorganisms are poorly investigated and only little is known about their adaptation to growth at extremely hypersaline conditions (Petrovic et al., 2002).

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

Materials and Methods

Study area and collection of sediment samples

The study area was confined to the east coast of Indian peninsula, specifically the continental slope of Bay of Bengal. The area covered was from Karaikkal to Paradip in the Bay of Bengal. Collections were made from 200, 500 and 1000 m depth regions. Sediment samples were collected onboard Fisheries and Oceanographic Research Vessel (FORV) Sagar Sampada of CMLRE, Ministry of Earth Sciences, Government of India using Smith McIntyre grab. Sample collection was done during cruises Cruises ≠ 236 (July, 2005) and 245 (July, 2006) from Bay of Bengal. Sediment sample for microbial analysis was transferred aseptically into sterile

polythene bags.

Isolation of black yeasts

Approximately 30 g sediment sample was transferred into 100ml sterile sea water and kept on a magnetic shaker for 10 minutes. 1 ml slurry was spread plated onto malt- yeast- glucose-peptone agar (Wickerham, 1951) supplemented with chloramphenicol (200 mg/l). The plates were incubated at 17± 2°C for 14 days. Enumeration was done and all the colonies were isolated into malt extract agar slants. Isolates were purified by repeated streaking on malt extract agar plates and stored in soft agar vials overlaid with sterile liquid paraffin. Besides, morphological analyses, the isolates were tested for fermentation/oxidation, production of starch like substances, nitrate assimilation, Diazonium Blue B reduction, urea hydrolysis, presence of pigments, casein and tyrosine decomposition and potassium nitrate assimilation. Based on morphological and biochemical characteristics the isolates were identified up to generic level.

Molecular identification of black yeasts

The isolates were identified using molecular techniques by extracting genomic DNA and sequencing of Internal Transcribed Spacer (ITS) region as per Harju et al. (2004). ITS primers (Forward ITS 1-5' TCC GTA GGT GAA CCT GCG G 3' and Reverse ITS 4- 5' TCC TCC GCT TAT TGA TAT GC 3') by White et al. (1990) which amplify a fragment of approximately 580 bp containing the ITS 1, 5.8 S and ITS 2 regions were used. The amplification reaction was performed by using a DNA Thermal cycler (Eppendorf). After an initial denaturation at 95°C for 5 minutes, amplification was made through 30 cycles, each consisting of a denaturation at 94°C for 1 minute, annealing at 56°C for 45 seconds, extension at 72°C for 1 minute and a final extension at 72°C for 10 minutes. Nucleotide sequencing was performed using ABI PRISM 3700 Big Dye Sequencer at Chromous Biotech, Bangalore. The sequences were matched with the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al., 1990) at NCBI (www.ncbi.nlm.nih.gov). The sequences were multiple aligned using Clustal W (Thompson et al., 1994) and the aligned ITS- rDNA gene sequences were used to construct a phylogenetic tree using the neighbour-joining (NJ) method (Saitou and Nei, 1987) of MEGA 4.1 package (Tamura et al., 2007). Bootstrap analysis was done based on 1000 replicates. Similarity matrix and genetic distance between sequences was calculated. The number of base substitutions per site of the sequence was analysed using Bio Edit Sequence Alignment Editor. Similarity matrix of the sequence was scored using MEGA 4.1 and the distance between the strains was determined based on the pair wise analysis.

Hydrolytic enzyme production

The isolates were tested for the production of enzymes *i.e.*, amylase (starch 1%), lipase (tributyrin 1%), protease (gelatin 2%), urease (urea 2%), aryl sulphatase, ligninase (methylene blue 2%), cellulase (carboxy methyl cellulose 1%), DNAse (DNA 2 %), pectinase (pectin 1%) and chitinase (colloidal chitin 5% w/v). Nutrient agar supplemented with the corresponding substrates was used for the enzyme assay. Clearing zone on the plates was regarded as positive except for urease and aryl sulfatase. In the case of protease and pectinase, plates were flooded with mercuric chloride and cetavlon respectively and the clearing zone was noted. For urease, development of alkaline pH due to the release of ammonia was noted by incorporating phenol red in the medium. In the case of aryl sulfatase a pink coloration of the medium around the colony due to the release of phenolphthalein from phenolphthalein sulfate was noted as positive.

Optimum physico-chemical conditions for growth

Preparation of inoculum

The black yeast isolates were streaked on to malt extract agar slants, incubation was done at

room temperature (28± 2°C) for 48 hours and the cells were harvested at logarithmic phase using 30 ppt sterile sea water. Optical density of the culture suspension was taken at 540 nm in a UV-VIS spectrophotometer (Shimadzu UV-1601).OD was adjusted to 1 by appropriate dilution and this suspension was used as the inoculum.

Preparation of media 1) **Temperature**

Malt extract broth prepared in sea water (35 ppt) was used for testing the growth of the isolates at different temperature. 2) **Salinity**: Malt extract broth was prepared in sea water of different salinities (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 ppt). 3) **pH**: Malt extract broth was prepared in sea water (35 ppt) with different pH 3, 4, 5, 6, 7, 8 and 9.

Inoculation, incubation and measurement of growth:

Ten µl of 1 OD yeast cell suspension was inoculated into the malt extract agar tubes prepared in triplicate so that the initial OD of the culture medium was 0.001. Incubation was done at room temperature (28 ± 2°C) for 48 hours in the case of pH and salinity. Growth was estimated by measuring the optical density at 540 nm using Shimadzu UV-1601 spectrophotometer

Extraction of melanin

Melanin was extracted from black yeasts as per Gadd (1982). 1N NaOH was added to the harvested yeast biomass and autoclaved for 20 min at 121°C. Then it was centrifuged at 8000 rpm for 10 min and the supernatant (melanin) was separated. Melanin was then precipitated by adding conc. HCl until pH 2 and again centrifuged at 10,000 rpm for 10 min. This was washed repeatedly with distilled water and dried in a lyophilizer.

Characterization of melanin from black yeasts

Two-dimensional NMR spectroscopy analysis *i.e.*, Correlation spectroscopy (COSY) and Total correlation spectroscopy (TOCSY) of the melanin extracted from the Black yeast isolates were done at Eastman Chemical Company, USA. The melanin samples were dissolved in 1M NaOH by heating to 80ºC. All the NMR spectra were obtained at ambient temperature on a JOEL Eclipse+ 600 NMR spectrophotometer operating at a proton observation frequency of 600.1723 MHz using 5 mm OD NMR tubes. 1D NMR spectra were collected with the following parameters: sweep width= 15 ppm, centre band= 5 ppm, 32768 complex points, acquisition time= 3.637 sec, spectral resolution= 0.275 Hz, scans= 64, 90º pulse and relaxation delay= 15 sec. The COSY 2D spectrum was obtained in absolute-value mode a pulse field gradient experiment using 521x 128 complex points zero filled 1- time in the y- dimension to give a final 512x 512 data matrix, sweep width= 10 ppm, centre band= 5ppm, pre scans= 4, scans= 8, X spectral resolution= 11.7 Hz, Y spectral resolution= 46.9 Hz and relaxation delay= 1 sec. The 2D TOCSY NMR spectrum was obtained using the same parameters except for the following: scans= 32, spin lock time= 100 m sec and relaxation delay = 2 sec. The 2D NOESY NMR spectrum was obtained in phase- sensitive mode using the same parameters except for the following: scans= 32, mixing time= 100 m sec and relaxation delay= 2 sec.

Antibacterial activity of melanin

Inhibitory activity of melanin against bacteria was checked by Kirby-Bauer disc diffusion method. Bacterial strains maintained at the National Centre for Aquatic Animal Health (NCAAH) were used for the study. The human and fish pathogens used for the study were *Edwardsiella tarda, Aeromonas hydrophila, Vibrio harveyi, V. proteolyticus, V. fluvialis, V. cholerae, V. parahaemolyticus, E. coli, Pseudomonas aeruginosa, Bacillus cereus, Staphylococcus aureus, Streptomyces lividans* and *Arthrobacter* sp. Melanin extracted from the black yeast isolates were suspended in distilled water and used for testing the antibacterial property. Nutrient agar (peptone 0.5 g; beef extract 0.3 g; sea water (50%) 100 ml; pH 7.2) plates were prepared and swab

inoculation of the pathogens was made on the surface to produce a lawn culture. Sterile filter paper discs impregnated with melanin were placed immediately on the agar surface; the plates were incubated at room temperature 28±2°C for 24 hours and observed for clearing zone formation.

Extraction of melanin degrading enzyme

The culture was inoculated in to malt extract broth (malt extract 1.5 g; peptone 0.5 g; sea water (35 ppt) 100 ml; pH 7.2), and incubated at 28 ± 2^{o}C in a shaker at 150 rpm for 48 hrs. The cells were harvested by centrifuging the culture at 8000 rpm and resuspended in a sonicating buffer containing 150 mM NaCl, 50 mM Tris and 1% Triton X 100. The mixture was sonicated for 30 minutes and centrifuged at 8000 rpm for 10 minutes to obtain the supernatant containing the crude enzyme.

Melanin degradation activity:

To check the activity of melanin degrading enzyme, melanin agar plates (melanin, 1% agar, 2%, Distilled water, pH 8.5) were prepared. Wells of approximately 5 mm diameter were cut using gel cutter and about 200µl of the crude enzyme was transferred into each well. The plates were incubated at 37°C for 48 hrs and the appearance of clearance zone was recorded as positive.

Results

Occurrence of black yeasts in Bay of Bengal

Black yeast populations were generally sporadic in the study area. They were found in all depth zones. The population was maximum at Cheyyur *i.e.*, 1.82 cfu/g dry weight of sediment at 200m depth and 1.65 cfu/g dry weight of sediment at 500m depth. Considerable populations were recorded off Karaikkal also at 200m depth regions (0.957 cfu/g dry weight of sediment). Totally 98 cultures were isolated, streaked on malt extract agar plates for testing purity and maintained in soft agar (malt extract) vials overlaid with sterile liquid paraffin at 4^{0} C (Fig. 1).

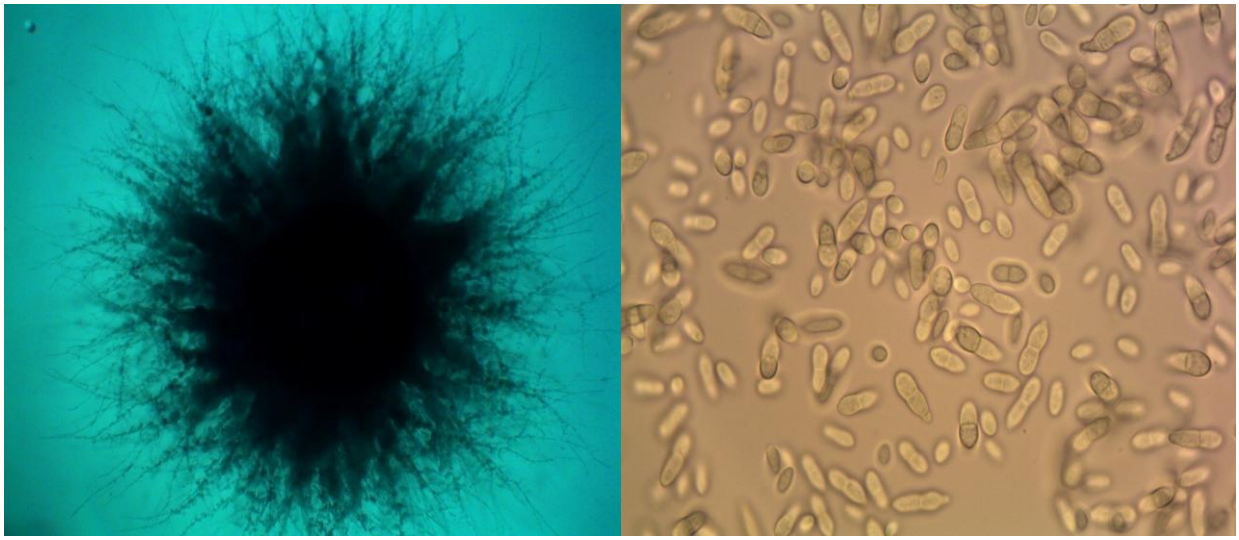


Fig. 1 – Single colony of Black yeast (4X) Wet mount (100 X)

Identification of black yeasts

Based on the morphological and biochemical characteristics the black yeasts were identified as belonging to the Genus *Hortaea*. All the isolates showed filamentous growth (Table 1). ITS sequencing and NCBI- BLAST analysis showed that the 10 isolates belonged to *Hortaea wernickii* (Table 2). ITS sequences of five closely related black yeasts were downloaded from the NCBI GenBank and the multiple alignments were done. A phylogenetic tree was constructed with the

neighbor joining algorithm with 1000 bootstrap replicates in the MEGA 4.1 package (Fig. 2). The tree shows two monophyletic clades, one clade with 5 isolates SD 480, SD 440, SD 454, SD 378 and SD 429 having 99% similarity with that of the NCBI strains AB087201 *Hortaea werneckii* and FJ770076 *Dothideales* sp. HLS305. Another clade with remaining five isolates SD 483, SD 449, SD 416, SD 430 and SD 450 having 99% similarity with the NCBI strains FJ755827 *Hortaea* sp. F47 and EU497947 *Dothideales* sp. F6. A marine black yeast AJ238676 *Trimmatostroma salinum* belonging to different genus but same order *i.e.*, Dothideales, was taken as an out-group to show the extent of similarity between the isolates. The consensus distance tree places these isolates SD 378, SD 416, SD 429, SD 430, SD 440, SD 449, SD 450, SD 454, SD 480 and SD 483, in the *Hortaea werneckii* monophyletic cluster with 99% similarity, strongly suggesting that all the isolates belong to *Hortaea werneckii*. The analysis of similarity matrix showed that all the isolates shared almost 100% similarity with a minor difference in the genetic distance ranging from 0.005- 0.009, which is considered negligible when compared with the difference in the distance shown by the out group (*T. salinum*) of almost 0.3. This matrix shows that the 10 isolates share great genetic relatedness among themselves and also the GenBank strains, FJ770076 *Dothideales* sp. HLS305, AB087201 *H. werneckii*, FJ755827 *Hortaea* sp. F47 and EU497947 *Dothideales* sp. F6.

Characteristics of the black yeasts

All the isolates were able to produce lipase, protease and amylase. About 60% of the isolates were able to produce ligninase and 40% produced urease (Fig. 3).The isolates had maximum growth at 30°C followed by 20, 40, 10 and 50°C (Fig. 4.a). All the isolates showed maximum growth at salinity between 30 and 60 ppt (Fig. 4.b). The isolates had maximum growth at pH 8 (Fig. 4.c).

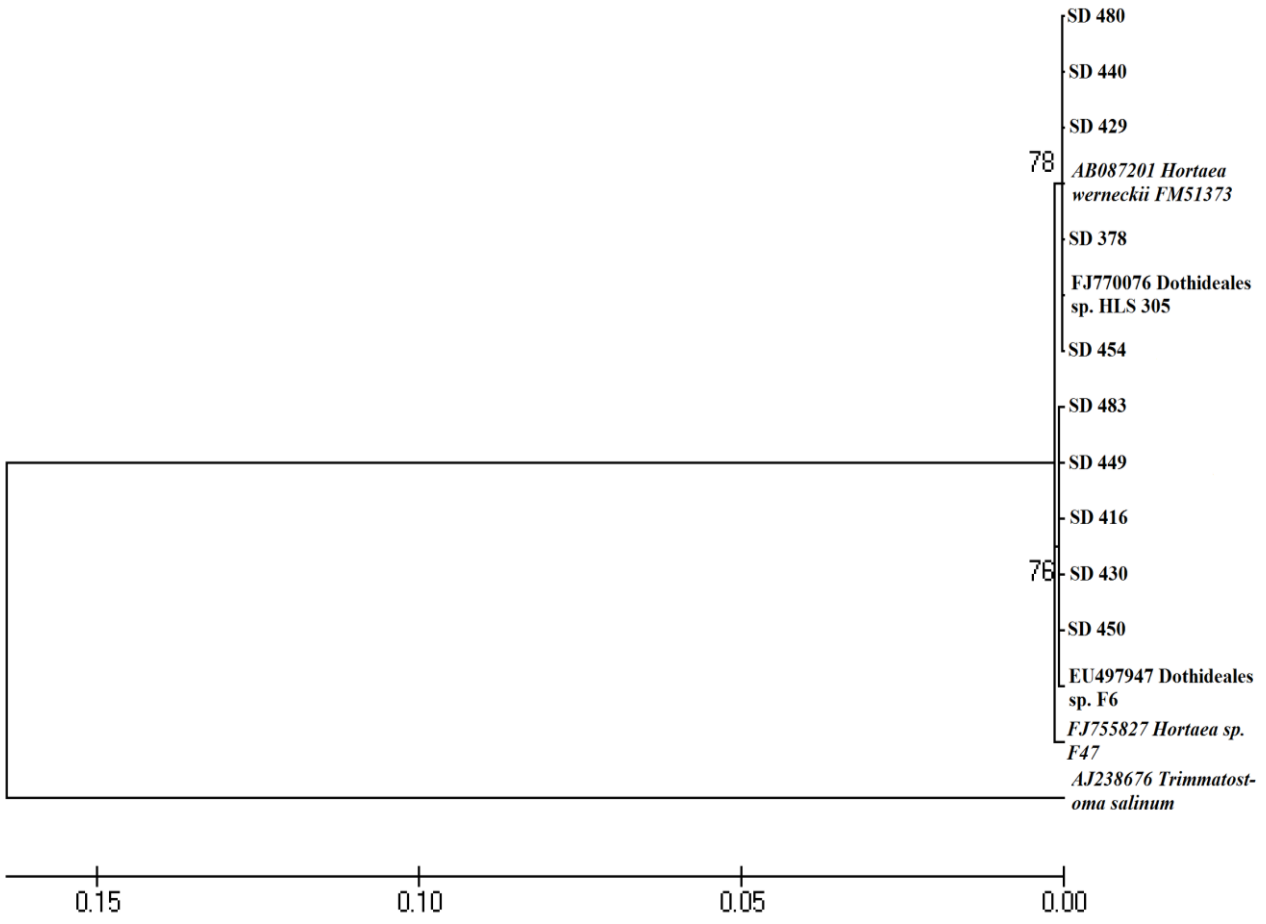


Fig. 2 – Phylogenetic tree based on NJ method

Table 1 Characteristics of black yeasts

Isolates	Location		Depth (m)	Asexual reproduction	Filamentous growth	MOF	Lipase	Amylase	Protease	Ligninase	Urease
	Lat	Long									
SD 378	19°05’	85°39’	1000	Budding	+	Oxidative	+	+	+	+	-
SD 416	10°34’	80°26’	200	Budding/ Splitting	+	Oxidative	+	+	+	-	+
SD 429	10°34’	80°26’	200	Budding/ Splitting	+	Oxidative	+	+	+	+	-
SD 430	10°34’	80°26’	200	Budding/ Splitting	+	Oxidative	+	+	+	+	-
SD 440	11°31’	79°59’	200	Budding	+	Oxidative	+	+	+	-	-
SD 449	14°10’	80°27’	200	Budding/ Splitting	+	Oxidative	+	+	+	+	+
SD 450	14°10’	80°27’	200	Budding/ Splitting	+	Oxidative	+	+	+	-	+
SD 454	16°00’	82°03’	1000	Budding/ Splitting	+	Oxidative	+	+	+	+	-
SD 480	14°10’	80°26’	500	Budding/ Splitting	+	Oxidative	+	+	+	-	+
SD 483	13°09’	80°41’	500	Budding/ Splitting	+	Oxidative	+	+	+	+	-

Table 2 GenBank accession of ITS sequence data

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

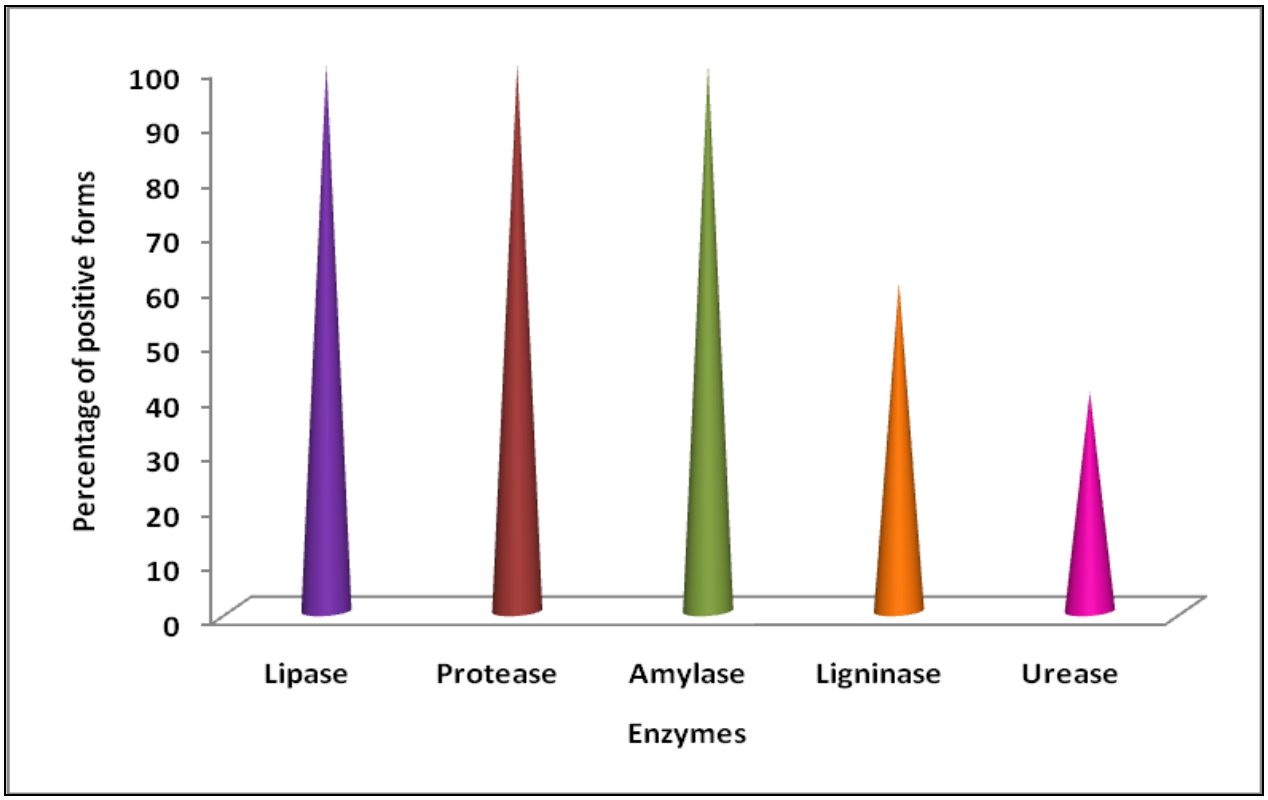


Fig. 3 – Hydrolytic enzyme production by black yeasts

Melanin characterization

NMR spectroscopy was done for all the melanin samples extracted from the 10 black yeast isolates. It was found that the melanin samples were of 1,8-dihydroxynaphthalene (DHN) type (Fig. 5). From these proton NMR spectra, each melanin isolate was found to contain varying amounts of lipids and carbohydrate. The lipid fraction is indicated by the COSY and NOESY cross peaks between the olefinic proton resonance at 5.3 ppm and the aliphatic resonances between 2.5 and 0.5 ppm. These resonances are characteristic of a long chain aliphatic acid containing 1 olefinic bond. The two sharp aromatic resonances at 6.8 and 7.2 ppm are coupled from the COSY spectrum so are in the same para-substituted aromatic ring spin system. This assignment is supported by a correlation from the NOESY spectrum between the resonance at 6.81 ppm and the resonance at 4.09 ppm assigned to the alpha methylene group in the first ethylene oxide repeat unit of a polyethylene oxide chain attached to an aromatic ring. A weaker NOESY correlation connects to the beta methylene group of the first ethylene oxide repeat unit. More evidence for the assignment

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

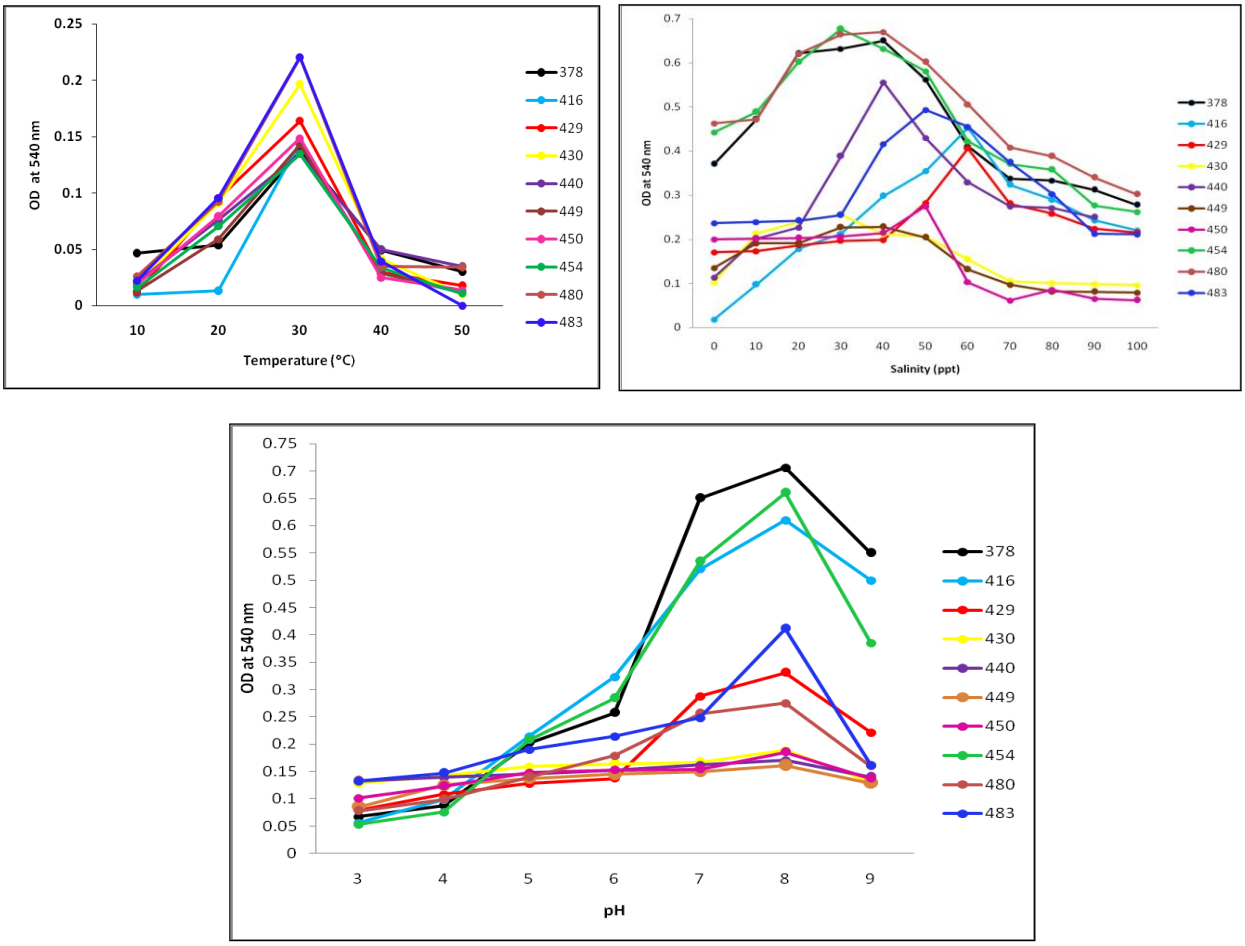


Fig. 4 – Optimum temperature, salinity and pH for the growth of black yeast isolates

Integral areas of the resonances at 6.95 and 6.55 ppm in melanin are shown in the Table 3. The resonance at 6.95 ppm is assigned to DHN while that at 6.55 ppm is assigned to indole structures. The relative amounts of DHN and Indole structures are shown in the columns labeled "DHN" and "Indole" below. Generally, these melanin isolates contain roughly 10-19% DHN with remaining 90-81% being Indole repeat units. The molar ratio of Indole to DHN is highest for SD 416 at 9.3, followed by SD 483 at 6.9, then SD 440 at 5.6, with 5 samples – SD 378, SD 429, SD 430, SD 454 and SD 480 falling in the intermediate molar ratio range of 5.1 down to 4.7 and finally the lowest ratio for SD 449 and SD 450 with roughly equal ratios at 4.2.

Antibacterial activity of melanin

Melanin extracted from the black yeasts was found to have activity against almost all the human and fish pathogens tested (Fig. 6). Inhibitory activity was comparatively high against *Streptomyces lividans*, *Edwardsiella tarda*, *Escherichia coli* and *Staphylococcus aureus* (Table 4).

Table 3 Integral areas of the resonances at 6.95 and 6.55 ppm in melanins and relative amounts of DHN & Indol

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

Melanin degrading enzyme

The crude enzyme extracted from black yeasts was found to have activity against the melanin extracted from the black yeasts. The enzyme was found to produce clearance zone in melanin agar plates (Fig. 7).

Table 4 Antibacterial activity of melanin against pathogens

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

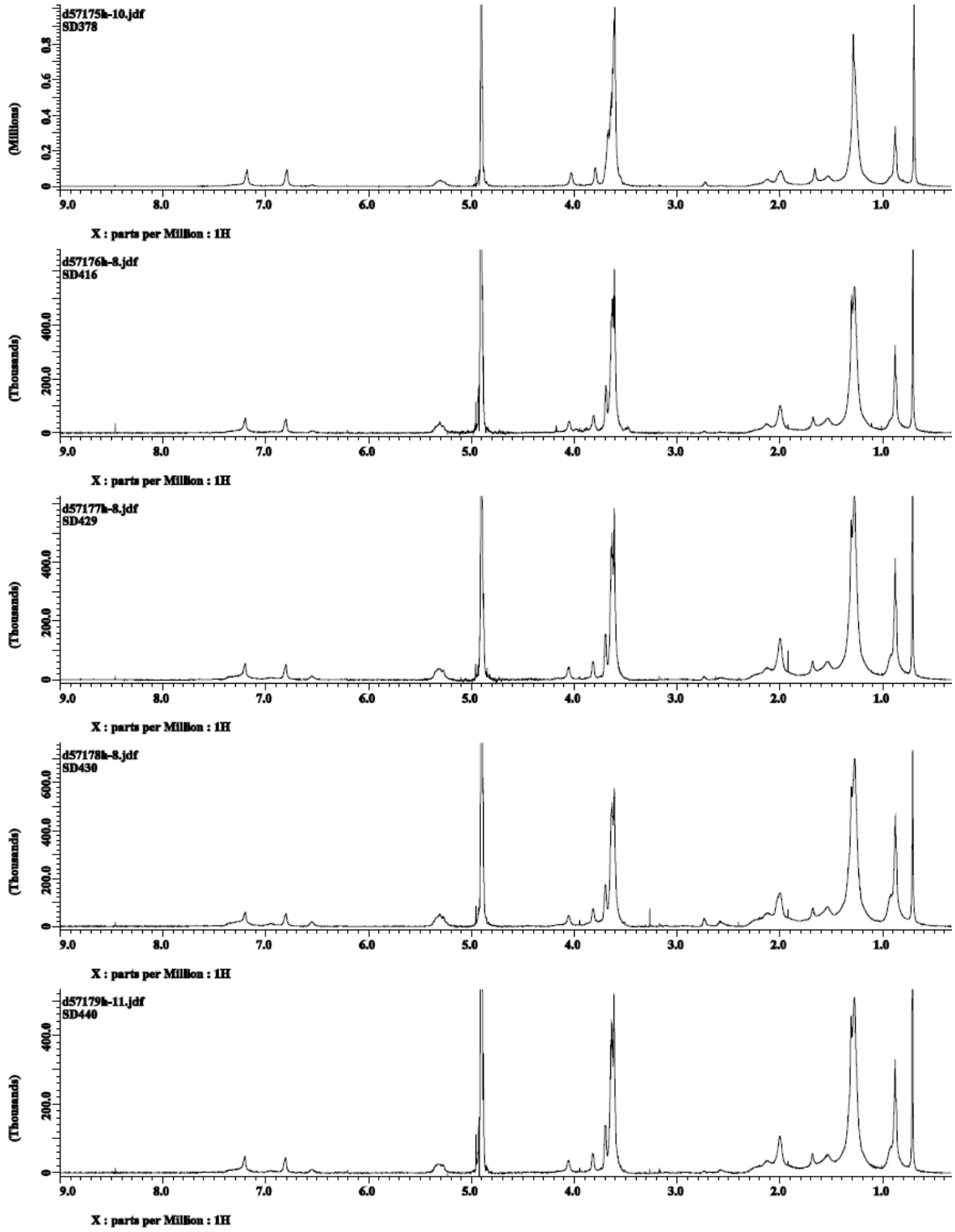


Fig. 5 – Complete proton NMR spectra of the melanin samples, displayed from about 9.0 to 0.3 ppm

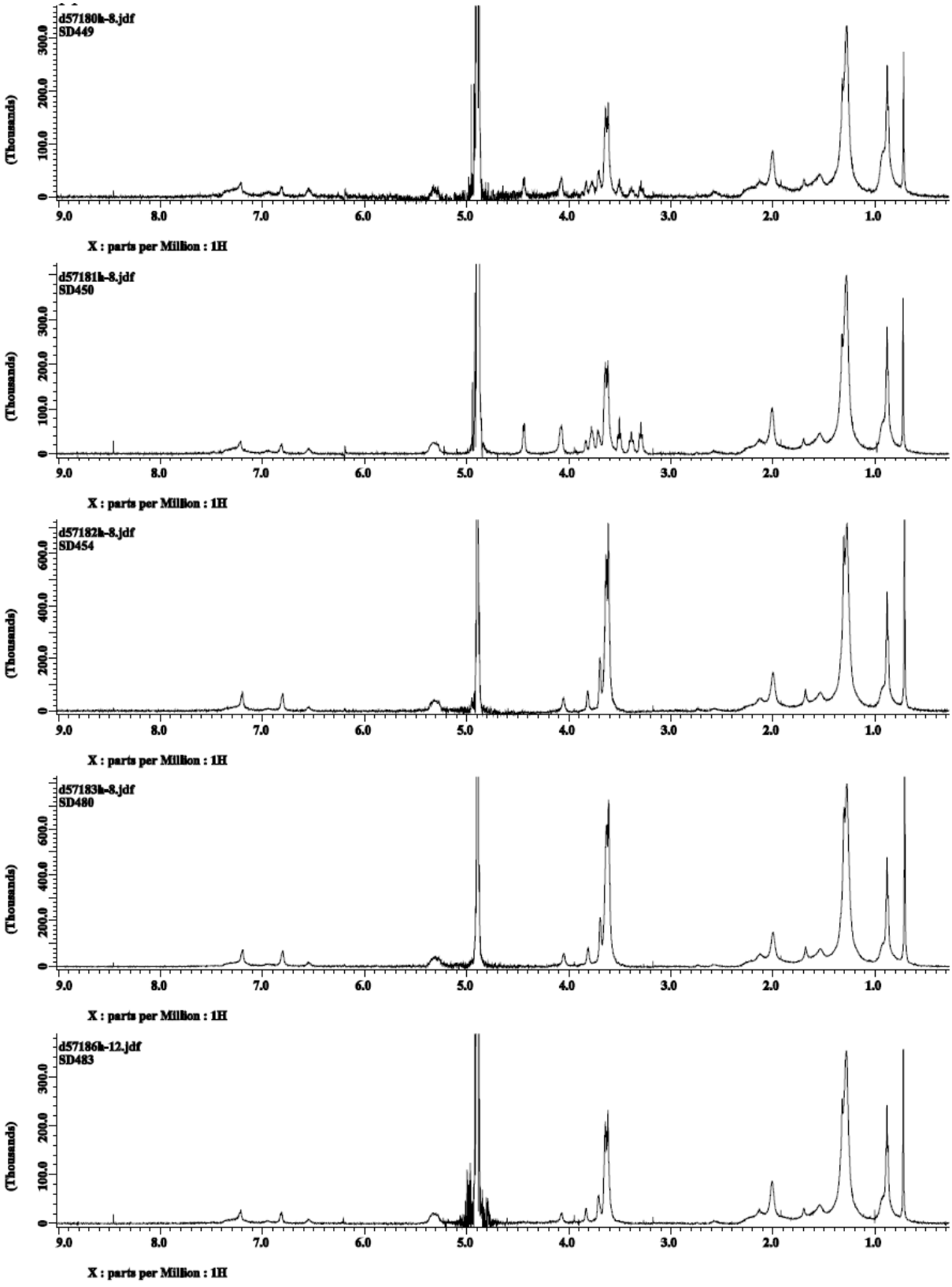
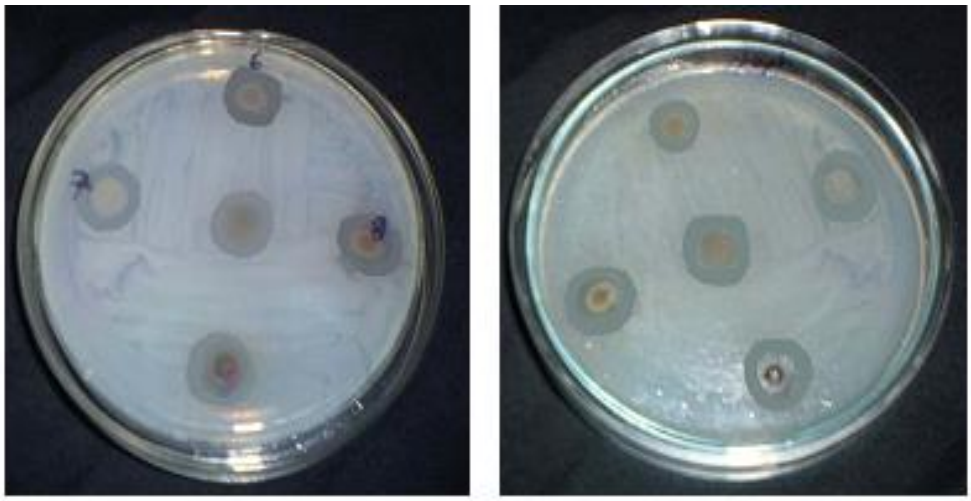


Fig. 5 – cont. Complete proton NMR spectra of the remaining 5 melanin samples, displayed from about 9.0 to 0.3 ppm



a *E. coli* **b** *Staphylococcus aureus*

Fig. 6 – Antibacterial activity of melanin extracted from black yeasts

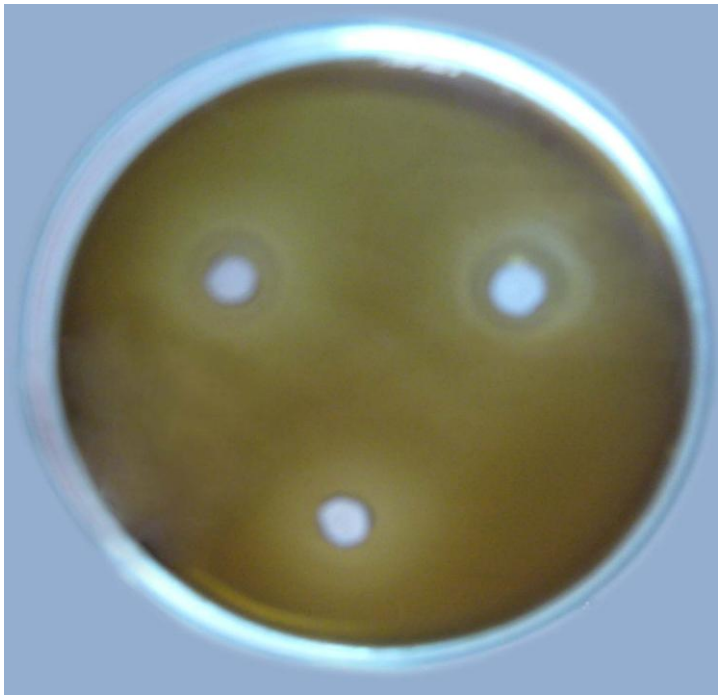


Fig. 7 – Clearance zone showing the degradation of melanin by melanin degrading enzyme

Discussion

Black yeasts are characterized by very slow growth at ambient conditions. All the isolates showed filamentous growth and were oxidative in nature. It has already been shown that all black yeasts are aerobic; having an oxidative metabolism, no fermentation of sugars has been observed (Rosa and Peter, 2006). All the isolates reproduced asexually by budding and they belonged to the Division Ascomycota and Order Dothideales. The overall phylogeny of black yeasts is a complex task and needs a polyphasic approach (Rosa and Peter, 2006). So Internal Transcribed Spacer (ITS) sequencing was used for identification of black yeasts. Neighbour joining (NJ) tree was constructed to determine the relationship between the isolates and five other strains of black yeasts from NCBI

including the strain, *Trimmatostroma salinum* which was taken as the out group. The NJ tree placed the black yeast isolates SD 378, SD 416, SD 429, SD 430, SD 440, SD 449, SD 450, SD 454, SD 480 and SD 483, in the *Hortaea werneckii* monophyletic cluster with 99% similarity. The analysis of similarity matrix in MEGA 4.1 package showed that all the isolates shared about 100% similarity.

Enzymes from yeasts are found to be useful in various industrial processes which emphasize their contribution (Kutty and Philip, 2008). These enzymes are produced mostly extracellular by different metabolic reactions taking place inside the cell and participate in various transformation activities like mineralization of organic compounds. Studies by Paskevicus (2001) showed that almost all the yeast strains produce lipase. Lipases catalyse a wide range of reactions like hydrolysis, esterification, alcoholysis, acidolysis and aminolysis *etc*. (Hasan et al., 2006). Lipases are mainly involved in detergent industry and biodegradation, especially oil residues. Protease has many applications in detergent, leather processing and feed industry besides waste treatment (Ni et al., 2008). Yeast amylases have many applications in bread and baking industry, starch liquefaction and saccharification, paper industry, detergent industry, medical and clinical analysis, food and pharmaceutical industries (Chi et al., 2003; Gupta et al., 2003). Amylolytic yeasts convert starchy biomass to single cell protein and ethanol (Li et al., 2006). The isolates were potent agents of biodegradation as they were able to produce lipase, protease, amylase, ligninase and urease. There are reports on black yeasts producing extra cellular glucans which is commercially used in human diet as immunostimulant. Japanese have commercialized a glucan from the black yeast like fungus, *Aureobasidium pullulans*, as AUREO (www.aureo.co.jp). The production of glucan like extra-cellular products needs to be studied in detail.

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

The melanin extracted from the isolates was confirmed to be of di-hydroxynaphthalene (DHN) type. Bell and Wheeler (1986) reported that the members of the class Ascomycetes synthesize DHN type melanin. The melanin extracted was found to be complexed with lipid components. Melanin formed by DHN pathway is reported to protect these organisms against a number of environmental factors, which attests to their protective properties. The melanin also exhibits an osmoprotectant role enabling the organism to flourish in hypersaline conditions. Melanin preparations are widely used in dermatology and cosmetology and possess antioxidant activities. The melanin confers different properties to the organisms which prove their efficiency against pathogenic microbes (Casadevall et al., 2000). The pigment was found to have antagonistic activity against different fish and human pathogens. This proves the importance of melanin in defence apart from their protective effect to thrive under adverse and extreme conditions. Conditions for maximum production of the pigment need to be optimized for large scale production and commercial applications.

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

tolerate high temperature retaining the physiological activities; being endowed with wide spectrum hydrolytic potential, these are potential candidates for various industrial applications.

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