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Deciphering the salt induced morphogenesis and functional potentials of *Hortaea werneckii*; a black pigmented halotolerant yeast isolated from solar saltern

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ABSTRACT

An intense black pigmented halotolerant yeast GUBPC1, was obtained from the solar salterns of Nerul, Goa-India. The isolate could tolerate 0 to 20 % NaCl. FE-SEM analysis revealed its polymorphic nature, exhibiting oval cells at higher salt concentrations and filamentous spindle like shapes at lower concentrations. Initially, the cells appear oval, yeast like in shape but gradually after 15 days of incubation, it becomes elongated and undergoes budding, exhibiting various budding patterns, from single polar bud to bipolar buds with annellidic ring, to lateral buds and eventually forming filamentous hyphae. The intracellular black pigment was identified as melanin based on ultraviolet–visible spectroscopy analysis. The molecular identification of the culture showed closest similarity with *Hortaea werneckii*. Plant polymer-degrading enzymatic activities such as cellulase, laccase, chitinase, xylanase, pectinase, amylase and protease were exhibited by the isolate GUBPC1. To further understand and explore its biotechnological potential, we performed whole-genome sequencing and analysis. The obtained genome size was 26.93 Mb with 686 contigs and a GC content of 53.24 %. We identified 9383 protein-coding genes, and their functional annotation revealed the presence of 435 CAZyme genes and 16 functional genes involved in secondary metabolite synthesis, thus providing a basis for its potential value in various biotechnological applications.

1. Introduction

Saltern ecosystems are highly diverse due to their high salt content and unique physiochemical parameters, making them distinct from other ecosystems (Kajale et al., 2020a). Utilized commercially for salt production, salterns are predominantly inhabited by halophilic and halotolerant microorganisms (Ma et al., 2010). These extreme environments impose various stressors such as ultraviolet (UV) radiation, fluctuating temperatures, osmotic stress and metal salts like KCl, $MgSO_4$ and $MnCl_2$, favouring the growth of halophilic, halotolerant, UV-resistant and thermotolerant microorganisms (Kajale et al., 2020a; Ma et al., 2010). Microorganisms thriving in such extreme habitats are unique in their metabolic functioning and also represent a wide array of processes (Martínez et al., 2022).

These hypersaline environments harbor extremophilic microorganisms from all three domains of life: Archaea, Bacteria and Eukarya. Halophilic yeasts from hypersaline regions are usually extremotolerant, exhibiting tolerance to multiple stresses such as UV, halo, thermo, osmo and xero (Gunde-Cimerman et al., 2000). The solar salterns and associated salty regions serve as the natural ecological niche for halophilic/halotolerant black yeast, *Hortaea werneckii* (Gunde-Cimerman et al., 2000). *H. werneckii*, an extremely halotolerant melanized black yeast, is a model organism for studying halotolerance mechanisms (Plemenitaš et al., 2008). It is found in saline regions worldwide, including salterns, mangroves, coastlines and sea-associated gadgets like scuba diving equipment and macroalgae surfaces (Elsayis et al., 2022a;

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Nazareth et al., 2011; Cabañes et al., 2012; Goshima, 2022).

Apart from *H. werneckii*, other examples of extremotolerant yeasts include *Debaryomyces hansenii, Trimmatostroma salinum, Aureobasidium pullulans* and *Phaeotheca triangularis* (Prista et al., 2005; Kogej et al., 2006; Chi et al., 2007; De Hoog et al., 1997). These microorganisms produce melanin pigment, which acts as UV-protectant shields (Oren, 2013; Gunde-Cimerman et al., 2009). Although *H. werneckii* strains are found in salty regions, the organism can grow in the absence of NaCl, classifying it as halotolerant and rendering NaCl as a non-obligate requirement for growth. However, *in vitro* laboratory studies have reported the growth of *H. werneckii* up to 32 % (w/v) NaCl concentration (Plemenitaš and Gunde-Cimerman, 2005). *H. werneckii* exhibits pleomorphic characteristics, alternating between oval cell yeast and filamentous phases with spindle-like elongated cells (Rizk and Magdy, 2022; Elsayed et al., 2016).

Halophilic microorganisms are known for their ability to thrive in extreme environments, and are valuable sources of commercially important biomolecules (Corral et al., 2020). However, efforts and multiple approaches are required to culture these unique organisms which are not yet cultured (Jiya et al., 2024; Kajale et al., 2020b). In our study, we isolated intense black-pigmented yeast from a solar saltern of Goa, India. We conducted detailed investigations into the colony and cell morphogenesis induced by sodium chloride, documenting various budding and cell patterns along with the isolate's enzymatic profile. Additionally, to explore its biotechnological potential further, we employed whole-genome sequencing to uncover adaptive strategies, secondary metabolite synthesis, and other genes relevant toindustrial applications. This comprehensive approach helps us to understand the genetic information of *H. werneckii* resilience, enabling its use for various biotechnological purposes.

2. Materials and methods

2.1. Sampling site: solar salterns of Nerul, Goa-India

Sampling was done at the solar saltpan located at Bhatti waddo, nestled amidst the undulating fields of Nerul village, North- Goa, India (Latitude15°31′11.02″N, Longitude 73°47′22.33″E). Brine samples were collected in clean glass bottles from the crystallizer pans of the solar saltern to isolate halophilic microorganisms. The samples were transported to the laboratory until processed further (Salgaonkar et al., 2013). The physicochemical parameters such as the temperature, pH and salinity were recorded onsite using thermometer, pH paper and hand-held refractometer, respectively (Mani et al., 2012a). The samples were processed in the Microbiology Research Laboratory 1, School of Biological Sciences and Biotechnology, Goa University for obtaining the culturable halophilic microorganisms as described in section 2.2.

2.2. Isolation, morphological and molecular characterization of culture GUBPC1

2.2.1. Isolation of culture GUBPC1

Black yeast was isolated from brine samples by surface spread plating method on Tomlinson's medium (TM) having composition (g/L) NaCl – 250.0, KCl - 6.0, $MgCl_2.6H_2O$ - 20.0, $CaCl_2.6H_20$–0.2, yeast extract - 5.0, peptone - 5.0, with pH adjusted to 7.2 using 1 M NaOH (Tomlinson and Hochstein, 1972). Briefly; fifty microliters (50 μl) of the brine sample was spread onto TM agar plate and incubated at an ambient temperature (~32 °C) for 30 days. Following incubation, yellow, orange, off-white and black pigmented colonies appeared on the plate. The black colony was carefully picked using nichrome loop and streaked on fresh plates of TM agar until pure culture was obtained. The pure culture was designated as GUBPC1 (Goa University Black Pigmented Culture 1) and maintained by periodically subculturing on TM agar after every 15–20 days.

2.2.2. Preliminary morphological characterization and optimization of growth medium for GUBPC1

The isolated black yeast was identified primarily by microscopic observation using wet mount and lactophenol cotton blue stain as described by Anteneh et al. (2019). The slide was observed under 40x magnification using laboratory optical Microscope (Radical, RXL-4).

To determine the optimum growth medium for GUBPC1, various commercially available fungal media (HiMedia Laboratories Pvt. Ltd.) which have been previously reported for studying black yeast were employed. The media reported includes (a) Malt Extract Agar (MEA) by Elsayed et al. (2016), (b) Potato Dextrose Agar (PDA) by Zalar et al. (2019), (c) Sabouraud Dextrose Agar (SDA) by Cabañes et al. (2012) and (d) Czapek Dox Agar (CDA) by Nazareth et al. (2011). These media were supplemented with 18 % NaCl (w/v) to retain the salt requirement of halophilic isolate GUBPC1. A loopful of the GUBPC1 culture was streaked on all the above-mentioned fungal media using quadrant streak technique. The plates were incubated for 5–7 days at an ambient temperature (~32 °C) to determine which media supports and promotes the best growth of the isolate GUBPC1. The growth and pigmentation were monitored visually every day and the medium on which the growth appeared fastest was selected as the best medium and used for further studies.

2.2.3. Molecular characterization of the GUBPC1 isolate

Genomic DNA extraction was carried out using the phenol-chloroform method described previously by Jani et al. (2019). DNA integrity and purity were checked using Nanodrop and 1.2 % (w/v) agarose gel electrophoresis. High quality DNA ($A_{260/280} = 1.8$) was used for molecular identification and whole genome sequence (WGS) analysis. The extracted DNA was stored at −20 °C until further use. The molecular identification process involved amplification, purification and sequencing of the 28S rRNA gene and internal transcribed spacer (ITS) region. The ITS and LSU regions were amplified using the ITS1/ITS4 (White et al., 1990) and LROR/LR5 (Porter and Golding, 2012) primer pairs, respectively. Taxonomic classification of the isolate by 28S rRNA gene and ITS region was done against the NCBI database using the BLAST search tool. The sequences of the concatenated neighbours were retrieved from NCBI database (rRNA/ITS databases) and were subsequently used for phylogenetic analysis. Multiple sequence alignment was performed using the ClustalW within MEGA software, version 11 (MEGA11) (Tamura et al., 2021). A Phylogenetic tree was constructed using the concatenated sequences of the D1/D2 domains of the LSU rRNA gene. The Neighbour-joining (NJ) method (Saitou and Nei, 1987), Maximum Likelihood (ML) method with Kimura-two- parameter model (Kimura, 1980), and the Maximum Parsimony (MP) method were employed. Confidence levels of the clades were estimated through Bootstrap analysis (1000 replications) (Felsenstein, 1985) and *Cladosporium cladosporioides* (KX664415) was used as outgroup.

2.3. Salt tolerance and its effect on morphogenesis of the isolate GUBPC1

2.3.1. Colony morphology at various NaCl concentrations

Among the various fungal media used, the isolate GUBPC1 grew fastest on MEA medium. Therefore, MEA medium supplemented with various NaCl concentrations was used to determine the salt tolerance activity. Briefly; MEA medium plates supplemented with 5 %, 10 %, 15 %, 20 %, 25 %, 30 % NaCl (w/v) concentration and without NaCl (0 % NaCl) were inoculated with freshly grown GUBPC1 culture (~5 days old). The plates were incubated at an ambient temperature (~32 °C) for 10–15 days and visible growth and pigmentation was monitored and documented on day-to-day basis. The colony morphological characteristics such as the colony shape, size, margin, pigmentation and other features were considered for morphological identification and were documented at various NaCl concentrations.

2.3.2. Cell morphology at various NaCl concentrations

The cell morphology of GUBPC1 at different NaCl concentrations was determined by lactophenol cotton blue staining method using laboratory optical microscope (Radical, RXL-4) as described in section 2.2.2. Cell morphology was further analysed using field emission scanning electron microscopy (FE-SEM). Briefly, smears of the culture grown at various NaCl concentrations i.e., 0 %–30 % were prepared on a clean grease free slide, air dried and heat fixed. Desalting was done using 2 % acetic acid solution to remove excess salt (Mani et al., 2012a). The culture smears were flooded with 2 % (v/v) glutaraldehyde for 6–8 h, which acts as a fixative. The culture smears were treated with a series of increasing concentrations of acetone gradient i.e., 10 %, 30 %, 50 %, 70 % and 90 % for 10 min each. The smear was lastly exposed to 100 % acetone for 30 min to ensure complete drying. The samples were dried and sputter coated with gold particles using LEICA EM ACE 200 sputter coater and were viewed under FE-SEM model Quanta FEG 250.

2.4. Study on the morphological characteristics differentiating the yeast phase and hyphal phase of GUBPC1 isolate

Among the various NaCl concentrations used, the isolate GUBPC1 exhibited optimum growth on MEA medium supplemented with 5 % (w/v) NaCl. Therefore, the colony and cell morphological characteristics of the isolate GUBPC1 were documented on MEA medium with 5 % NaCl after 5 days and 15 days of incubation. The colony characteristics were studied by using macro lens (SKYVIK SIGNI X 20x Macro Lens), whereas the cell morphology was documented using FE-SEM analysis.

2.5. Extraction and characterisation of pigment using UV–visible spectroscopy

The culture GUBPC1 was grown in Malt Extract Broth (MEB) containing 5 % (w/v) NaCl until the culture biomass turned intense black (~25–30 days of incubation). The intense black lipid soluble pigment from the culture GUBPC1 was extracted according to the method reported by Gadd (1982) and Suwannarach et al. (2019) with slight modifications. The cells of the isolate GUBPC1 were centrifuged at 8000 rpm for 10 min. The intense black pigmented cell pellet was boiled in distilled water for 5 min, followed by re-centrifugation at 8000 rpm for 20 min. The supernatant was discarded and the cell pellet obtained was resuspended in NaOH (1M) and autoclaved at 121 °C for 20 min. The autoclaved suspension of cell residues in NaOH was cooled and re-centrifuged at 8000 rpm for 10 min. The pellet containing the cell debris was discarded and the pigment was precipitated and recovered from the supernatant by acidifying to pH 2 using concentrated HCl. The tubes were allowed to stand for 5–10 min to ensure complete pigment precipitation, followed by centrifugation at 10,000 rpm for 30 min. The supernatant was carefully decanted and the obtained pigment pellet was resuspended in distilled water and centrifuged at 10,000 rpm for 30 min. This step of resuspending in distilled water was repeated thrice in order to wash the precipitated pigment and neutralize the pH. The extracted pigment was dried for 4–5 h in a hot air oven at 50 °C. The pigment was later dissolved in NaOH solution (1M) and the absorption spectra of the pigment was recorded by scanning from wavelength range of 190–800 nm using UV–Visible spectrophotometer (Analytical Technologies Ltd., UV2080TS).

2.6. Screening of GUBPC1 isolate for extracellular hydrolytic enzymes

The extracellular enzyme production by the GUBPC1 isolate was screened using minimal medium by agar plate assay method. The screening for various enzymes was done using Norberg and Hofsten (NH) medium containing (g/l) NaCl - 50.0, $MgSO_4.6H_2O$ - 10.0, KCl - 5.0, $CaCl_2.2H_2O$ - 0.2, yeast extract - 1.0, agar - 18.0 and supplemented with various polymers (substrates) of plant and animal origin as sole source of carbon (Norberg and Hofsten, 1969). The pH of the medium was adjusted to 7.0–7.2 using 1M NaOH. Freshly grown culture from malt extract broth containing 5 % NaCl was spot inoculated (10 μl) at the centre of the plates followed by incubation at an ambient temperature (32 °C) for 12 days. The diameter of the zone of clearance/precipitation was directly proportional to the amount of hydrolysis. Screening for all the enzymes was done in triplicates to ensure reproducibility. The total as well as the colony zone diameter was measured, and the enzymatic index (EI) was calculated as given below (Saroj and Narasimhulu, 2018). A value of this ratio ≥2 indicates a satisfactory enzyme production.

$$Enzymatic\ Index\ (EI) = \frac{Diameter\ of\ zone\ of\ hydrolysis\ (in\ cm)}{Diameter\ of\ culture\ growth\ (in\ cm)}$$

The screening in brief was done as follows: Laccase activity was detected by supplementing NH media with 0.5 % glucose, 1 mM ABTS and 0.4 mM $CuSO_4$ (Gogotya et al., 2021). A bluish green coloured zone around the culture growth indicated positive laccase activity. Amylase, cellulase, xylanase, pectinase and chitinase activities were determined by supplementing NH media with 0.5 % (w/v) amylum (starch soluble), sodium salt of carboxy methyl cellulose (CMC), xylan from beechwood, pectin (Poly-D-galacturonic acid methyl ester) and colloidal chitin (HiMedia), respectively. The detection was done by flooding the plates with I_2 (0.3 % I_2–0.6 % KI) solution; Zone of clearance around blue-brown background depicted positive amylase, cellulase, xylanase, pectinase and chitinase activity (Salgaonkar et al., 2019; Mmango-Kaseke et al., 2016; Rehman et al., 2015; Gherbawy et al., 2012). Proteolytic activity was determined using 0.5 % (w/v) skimmed milk and a zone of clearance around the culture growth against white background indicated positive protease activity. Esterase and lipase activity was screened using 0.1 % (v/v) tween 80 (Thomas Baker) and olive oil, respectively. A turbid zone of precipitation around the culture growth indicated positive esterase and lipase activity. Gelatinase activity was screened by using 0.5 % (w/v) Gelatin Purified (Merck) and detected using 15 % (w/v) mercuric chloride acidified with 20 % (v/v) concentrated HCl solution; wherein zone of clearance around white background indicated positive gelatinase activity.

2.7. Whole genome sequencing (WGS) analysis of the isolate GUBPC1

2.7.1. Library preparation, sequencing and genome assembly

The whole-genome sequencing (WGS) of *H. werneckii* strain GUBPC1 was carried out on the Illumina Novaseq6000 platform (Illumina). A sequencing library was prepared with insert sizes of approximately 348 and sequenced using 150 x 2 paired-end (PE) chemistry. Upon sequencing, raw reads underwent quality assessment using the FastQC tool (Andrews, 2010). Subsequently, Cutadapt (Martin, 2011) was employed to eliminate adapters and Illumina technical sequences, ensuring the reliability for downstream analysis. The resulting trimmed and cleaned reads were then subjected to *de novo* genome assembly using the SPAdes v.3.13.0 assembler (Bankevich et al., 2012). The quality of the genome assembly was evaluated using QUAST v.5.0.2.

2.7.2. Gene prediction and functional genome annotation

For gene prediction, structural annotation and functional genome annotation, the funannotate pipeline (v.1.8.15) was employed as described in (https://funannotate.readthedocs.io/en/latest/tutorials.html). This pipeline is integrated with ab initio gene predictors such as SNAP (v.2.39) and AUGUSTUS (v.3.5.0). Furthermore, the structural prediction was performed with a comprehensive set of reference proteins from *Capnodiales* (NCBI: txid134362) obtained from the NCBI Protein database. tRNAs were identified with tRNAscan-SE v2.0.12. Functional annotation of the predicted genes was conducted using several bioinformatics tools and databases. Firstly, EggNOG mapper was utilized to obtain functional information based on the evolutionary genealogy of genes from the EggNOG database (Cantalapiedra et al., 2021). A standalone run of InterProScan (Jones et al., 2014) was performed to identify protein domains, families, and functional sites.

Furthermore, the fungal version of antiSMASH v7.0 (Blin et al., 2023) was employed to predict secondary metabolite biosynthetic gene clusters (BGC) in the genome. The functional annotation obtained with funannotate includes annotation from Interpro terms, Pfam domains, CAZYmes, secreted proteins, proteases (MEROPS), BUSCO groups, EggNOG annotations, Clusters of Orthologous Groups (COGs), GO ontology and antiSMASH (fungi version). These analyses collectively provide comprehensive insights into the predicted genes, facilitating the elucidation of their putative biological roles and pathways.

3. Results and discussion

3.1. Sampling site and physicochemical analysis

Located on the west coast of India, the state of Goa is bordered by the Arabian Sea and has a long coastline stretching ~104 kms. Being a coastal state, salt making is one of the age-old traditions practiced by the people of Goa (Mani et al., 2012b). Fig. 1a–c represents the location of the study site on the map of Goa. It also clearly demarcates the salt crystallizer pans of the solar salterns at Nerul. Halophilic microorganisms thrive in areas having high salinity like salt pans, salt marshes, saline soils, etc. In order to isolate halophilic microorganisms, brine samples were collected from a salt pan located at Bhatti Waddo at Nerul, Bardez Taluka, North Goa District, Goa India (Latitude 15°31′11.02″N, Longitude 73°47′22.33″E). The physicochemical parameters such as pH, temperature and salinity of the samples were neutral (7.0–7.2), 42–45 °C and 25–27 %, respectively.

3.2. Isolation, morphological and molecular characterization of the black pigmented isolate

3.2.1. Isolation of black pigmented isolate

The Tomlinson's medium (TM) comprising of the following mixture of salts such as 25 % NaCl, 6 % KCl, 20 % $MgCl_2.6H_2O$ and 0.2 % $CaCl_2.6H_2O$ was used for the isolation of halophilic microorganisms. Upon incubation of the plates for 5–7 days few colourless colonies appeared, however on further incubation of the TM plates for 20–25 days diverse pigmented and non-pigmented colonies appeared as shown in Fig. 1d. The counts of the white or non-pigmented, orange, pale-yellow and black colonies were 3.4×10^{-2}, 3.8×10^{-2}, 60 and 20 Colony Forming Units per mL (CFU/mL), respectively. The black pigmented colony was purified by repeated streaking on TM plates (Fig. 1e) and was designated as GUBPC1 (Goa University Black Pigmented Culture 1).

3.2.2. Preliminary morphological characterization and growth of isolate GUBPC1 on fungal media

The morphological examination of the isolate GUBPC1 grown on TM medium revealed that the cells appeared as oval shaped, single, depicting typical yeast like cell morphology. Furthermore, the isolate GUBPC1 was examined for its growth on different fungal media such as MEA, PDA, SDA and CDA supplemented with 18 % (w/v) NaCl at an ambient temperature (~32 °C). The isolate GUBPC1 exhibited prominent growth after 7–10 days of incubation on the MEA, PDA and SDA media with the colony size ranging from 1.5 mm ± 0.31, 1.0 mm ± 0.28 and ≤1 mm (pinpoint), respectively. However, isolate GUBPC1 exhibited negligible growth on CDA plate, even after 15 days of incubation. The pigmentation exhibited by the colonies was intense black on MEA and PDA media, whereas brown-black pigmentation was observed on SDA medium. Among all the fungal media used, MEA medium was found to support best and fastest growth with intense black pigmentation. Therefore, MEA medium was selected as the best medium for further studies. However, reports of *Hortaea werneckii* by Cabañes et al. (2012) indicated that the culture exhibited best growth on PDA followed by oatmeal agar and SDA.

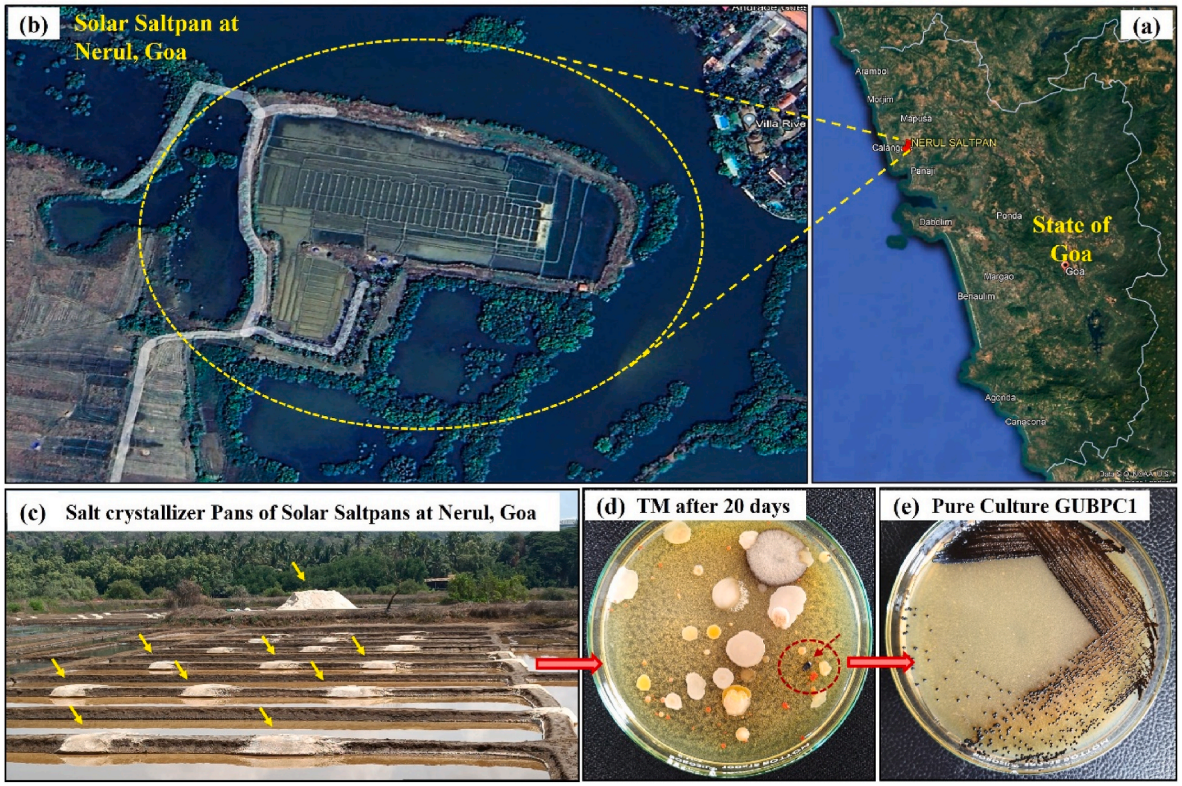

Fig. 1. (a) Map of Goa showing the location of the study site (b) the solar saltpans at Nerul, Bardez, North Goa-India (Google Earth Pro). (c) The salt crystallizer pans of the solar salterns (d) Tomlinson's medium (TM) used for the isolation of halophilic microorganisms and (e) The pure culture of black pigmented isolate GUBPC1.

3.2.3. Molecular characterization of the GUBPC1 isolate

Molecular identification using the 28S rRNA gene and ITS region against BLAST search identified the isolate as "*Hortea werneckii*". The generated sequences have been submitted to the NCBI database. The sequences are now available under the following accession numbers: (PP320571) for the LSU rRNA gene and (PP724654) for the ITS region. Furthermore, estimation of evolutionary distances by phylogenetic analysis on the basis of 28S rRNA gene demonstrated that isolate GUBPC1 clustered with *Hortaea werneckii* CBS 107.67 (NG 057773) with high bootstrap consensus (Fig. 2). The results of tree topology reconstruction using another method (ML, MP) yielded consistent topology (Supplementary Figs. 1 and 2).

Melanized black halophilic microorganisms represent ≥85 % of the total mycobiota of hypersaline waters and has been reported to inhabit the solar salterns around the globe (Musa et al., 2018). A study by Plemenitaš and Gunde-Cimerman (2011) reported that the black yeast *Hortaea werneckii* is a dominant fungal species in hypersaline waters. However, other dominant halophilic black yeast species reported are *T. salinum* (Zalar et al., 1999), *A. pullulans* (Gunde-Cimerman et al., 2000), *P. triangularis* (De Hoog et al., 1997) and *Cladosporium* species (Gunde-Cimerman et al., 2000). To date, very few studies have been carried out on the occurrence of halophilic Black Yeast *Hortaea werneckii* from India. Zhang et al. (2014) revealed the presence of *H. werneckii* by phylogenetic analyses using targeted environmental sequencing of fungal communities from the deep-sea sediment of the East Indian Ocean. Kutty et al. (2013), isolated *H. werneckii* from the continental slope of Bay of Bengal, East Coast of Indian peninsula. Rani et al. (2013) reported *H. werneckii* from the brine samples of solar salterns from Marakkanam near Pondicherry, India. Singh et al. (2011) studied the fungal diversity of the deep-sea sediments from the central Indian basin and reported *H. werneckii* using both culture dependent and independent approach. Another report by Nazareth et al. (2011) documented the occurrence of *H. werneckii* in brackish waters of mangroves and hypersaline salterns of Santa Cruz and Ribandar Goa, India.

3.3. Salt tolerance and its effect on cell morphology of isolate GUBPC1

The isolate GUBPC1 was examined for its growth on MEA medium supplemented with various concentrations of NaCl after 15 days of incubation (Fig. 3 and Table 1). To determine the true halophilic nature of the isolate, it was grown on MEA medium devoid of NaCl (0 %). The isolate GUBPC1 exhibited prominent growth with intense black pigmentation on the MEA medium supplemented with 5 %, 10 % and 15 % of NaCl, with the colony size ranging from 4 mm ± 0.025, 3 mm ± 0.045, 1 mm ± 0.02, respectively. The isolate grew well on MEA medium without NaCl (0 % NaCl) with colony size of 2 mm ± 0.03, indicating its halotolerant nature. The colonies grown in the presence of 0 %–15 % NaCl exhibited typical rhizoid margin and appeared filamentous in shape with butyrous consistency. It was noted that the culture GUBPC1 grew on the above mentioned NaCl concentrations after 5–7 days of incubation and moreover, as the NaCl concentration in the medium increased to ≥15 % the growth of the culture decreased. The culture did not exhibit any visible growth at 20 % NaCl after 5–7 days of incubation but grew well when the plates were incubated further for 15 days with colony size of ≤1 mm (pinpoint) with brown-black pigmentation. The colonies of the culture appeared circular in shape with entire margin and butyrous consistency at 20 % NaCl concentration (Table 1).

Fig. 3 represents the cell morphology of the isolate GUBPC1 grown on MEA with varying NaCl concentrations. The cell morphology under light microscopy revealed the cells to be yeast form at higher NaCl concentrations (15 % and 20 % NaCl) and both yeast and filamentous form with septate hyphae at NaCl concentration of 5 %, 10 % and without NaCl (0 %). This was further confirmed using FE-SEM analysis which revealed the cells to be oval yeast-type to hyphal-type or mixture

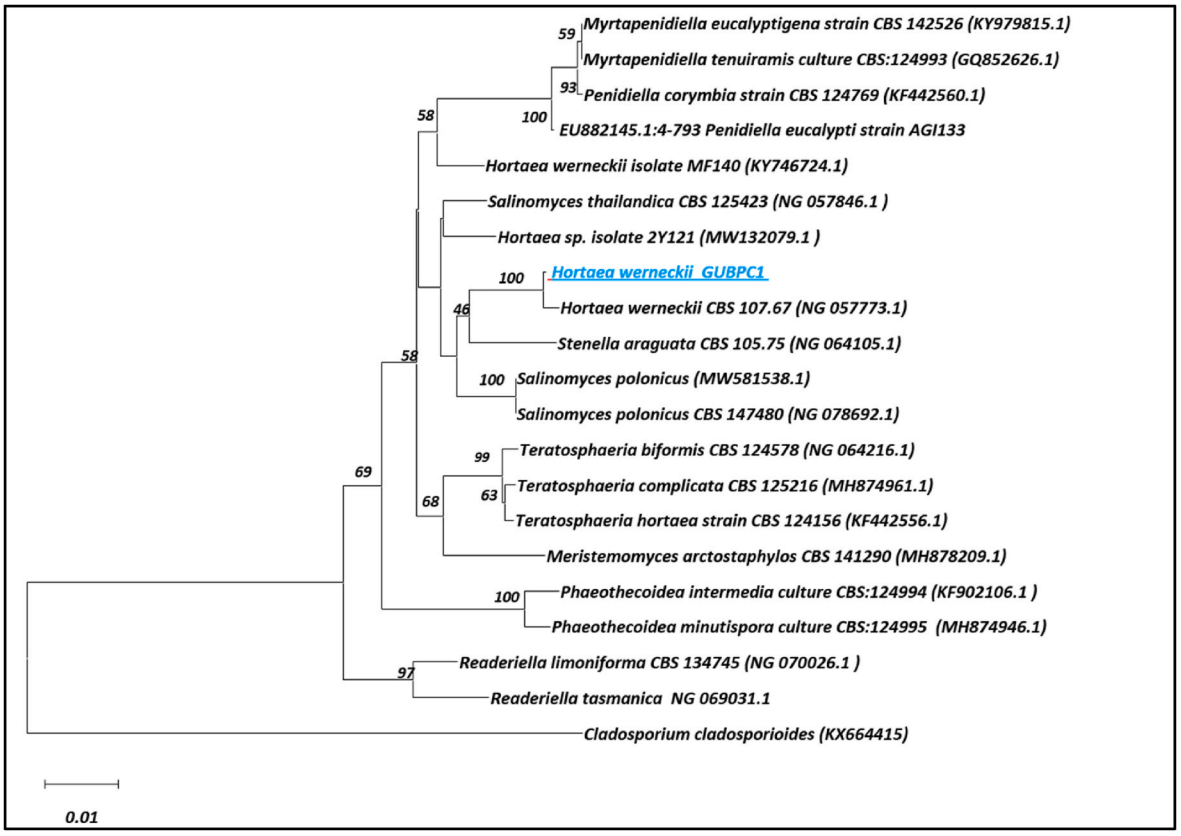

Fig. 2. Neighbour-joining phylogenetic tree based on the D1/D2 domains of the LSU rRNA gene sequences showing the position of isolate GUBPC1. Bootstrap values (1000 replications) of above 50 % are shown at the branch points.

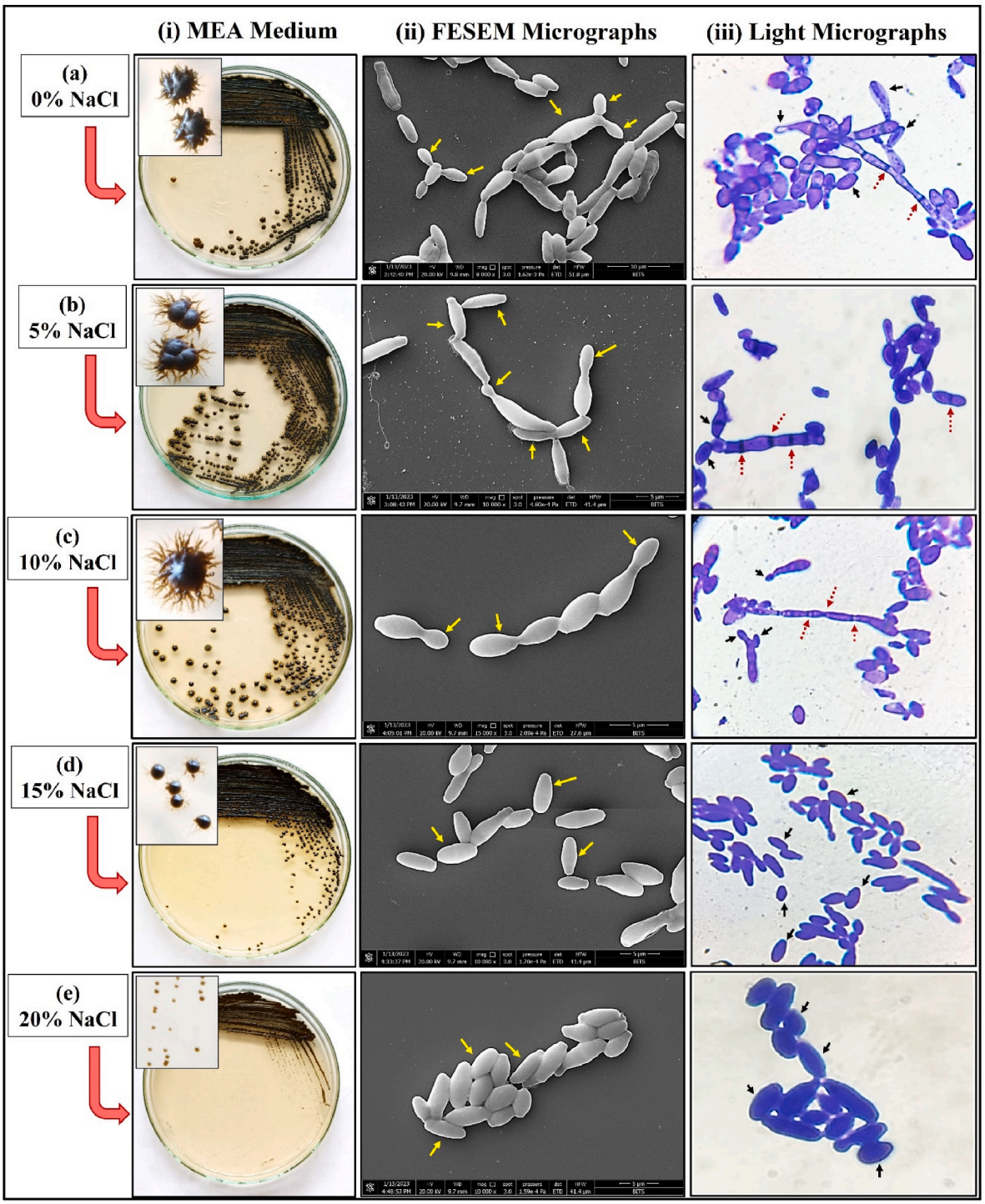

Fig. 3. The variation in the morphological colony characteristics of the culture GUBPC1 grown on (i) MEA medium supplemented with various concentrations (w/v) of NaCl (a) 0 %, (b) 5 %, (c) 10 %, (d) 15 % and (e) 20 %. Inset depicting the colonies on the respective NaCl concentration observed under the Macro Lens. The Micrographs captured under (ii) FE-SEM and (iii) Light microscopy depicts the variation in the cell morphology at different NaCl concentrations.

of both types.

3.4. Morphological characteristics differentiating the yeast phase and hyphal phase

Among all the NaCl concentrations tested, the culture GUBPC1 exhibited excellent growth in MEA supplemented with 5 % (w/v) NaCl. Therefore, this medium and NaCl concentration was used to study the morphogenesis pattern exhibited by the cells of the culture GUBPC1 after initial growth phase (5 days of incubation) and mature growth phase (15 days of incubation). The culture exhibited prominent growth after 5 days of incubation with the colonies being black pigmented, circular in shape with entire margin and butyrous consistency (Fig. 4a). Interestingly, the colony characteristics drastically changed after further incubation for 15 days with the pigmentation being intense black, typical rhizoid margin and filamentous shape having butyrous consistency (Fig. 4b). The microscopic observations using FE-SEM revealed that initially after 5 days incubation, the culture cells appeared yeast-

Table 1
Colony and cell morphological characteristics of the isolate GUBPC1.

Characteristics	Various NaCl Concentrations (w/v)				
	0 %	5 %	10 %	15 %	20 %
Colony characteristics					
Media	MEA	MEA	MEA	MEA	MEA
Time	15 days	15 days	15 days	15 days	15 days
Temperature	RT (~32 °C)	RT (~32 °C)	RT (~32 °C)	RT (~32 °C)	RT (~32 °C)
Colony size (mm)	2 ± 0.03	4 ± 0.025	3 ± 0.045	1 ± 0.02	Pinpoint (≤1)
Colony shape	Filamentous	Filamentous	Filamentous	Filamentous	Circular
Margin	Rhizoid	Rhizoid	Rhizoid	Slight Rhizoid	Entire
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque
Consistency	Butyrous	Butyrous	Butyrous	Butyrous	Butyrous
Elevation	Convex	Convex	Convex	Convex	Raised
Pigmentation	Black	Black	Black	Black	Brown-Black
Cell Characteristics (FE-SEM & Light Microscopy)					
Cell Phase	Yeast & Hyphal Phase	Yeast & Hyphal Phase	Yeast & Hyphal Phase	Yeast Phase	Yeast Phase
Style of Budding	Yeast like cells with Sympodial producing daughter cells	Polar bud & Lateral buds	Polar bud	Unbudded single oval cell	Unbudded single oval cell
Cell types observed (Arrangement)	Elongated cells (Chain) Oval cells (single)	Elongated cells (Chain) Oval cells (single)	Oval cells (single and Chain)	Oval cells (single)	Oval cells (single)
Hyphae	Present (septate)	Present (septate)	Present (septate)	Absent	Absent
Cell size: (FE-SEM)					
Cell Length (μm)	7.36 ± 3.03	6.18 ± 2.08	4.47 ± 0.35	4.79 ± 0.71	5.76 ± 0.43
Cell Width (μm)	2.71 ± 0.40	1.87 ± 0.55	2.23 ± 0.16	2.22 ± 0.52	2.15 ± 0.31
Bud size:					
Bud Length (μm)	3.68 ± 0.45	3.75 ± 1.78	3.09 ± 0.92	Unbudded cells	Unbudded cells
Bud Width (μm)	2.01 ± 0.15	1.59 ± 0.31	1.80 ± 0.16		

RT: Room temperature; **%** Percentage; **MEA:** Malt extract agar; **mm** = millimetre; **μm** = micrometre.

type with oval cell morphology with cell length of 4.34 μm ± 0.5 and cell width of 2.85 μm ± 0.7 (Fig. 4a). However, after prolonged incubation for 15 days, drastic change in the cell morphology was observed wherein the yeast-like cells became elongated with cell length of 6.91 μm ± 0.54 and cell width of 2.11 μm ± 0.12, showed chain like arrangement and underwent budding (Fig. 4b).

Fig. 4c represents the various budding patterns and cell types of the isolate GUBPC1 grown in MEA supplemented with 5 % NaCl. The micrographs captured under the FE-SEM indicates that the culture exhibited a spindle-like elongated shape and various patterns of budding. The cells varied from single oval to elongated shape with single polar bud. The cells also exhibited bipolar buds with annellidic ring at the centre of the cells. Some cells further elongated as filaments and exhibited lateral buds. Studies by Mitchison-Field et al. (2019) revealed that *H. werneckii* alternates between fission and budding. Although, they observed septum formation it was not followed by separation. This is in analogy to our findings which have also showed septa formation but lacked fission. While most of the cells displayed division by budding, only a small proportion was reported to exhibit fission (Mitchison-Field et al., 2019). The isolate GUBPC1 exhibited all the morphological features and characters as recorded previously by Elsayed et al. (2016) for black yeast *Hortaea werneckii* using light microscopy. The FE-SEM micrographs clearly indicates the various cell forms of the pure culture GUBPC1, thereby indicating its polymorphic nature. Our study is well supported by the research investigation of Petrovič (2006) who studied the cell morphology of *H. werneckii* at different NaCl concentrations and reported that the cell morphology appeared as spindle-like elongated shape when grown in media supplemented with 0 %–10 % NaCl. At higher salinity of ≥17 % NaCl, the cells appeared oval, non-elongated with thicker cell wall. Gunde-Cimerman et al. (2009) reported, the halotolerant black yeast *H. werneckii* to exhibit polymorphism at lower salinities. Zalar et al. (2019) reported that the cells of *H. werneckii* initially exhibited yeast like morphology on MEA medium containing 10 % NaCl that later developed into marginal immersed budding mycelium showing filamentous growth.

3.5. Identification of the pigment from the culture GUBPC1

The intracellular black pigment from the culture GUBPC1 was extracted and spectrophotometrically analysed in the UV–visible wavelength range of 190–800 nm (Fig. 5). The extracted black pigment exhibited absorption peak at UV region with the wavelength of maximum absorbance recorded at 220 nm, which declined towards the visible region (Fig. 5c.). This was compared well with the extracted fungal melanin pigment from *Spissiomyces endophytica* SDBR-CMU319 and synthetic DOPA-melanin standard which exhibited maximum absorbance at 215 nm (Suwannarach et al., 2019). Elsayis et al. (2022a) reported the maximum absorbance at 240 nm for melanin extracted from *H. werneckii* strain AS1, thereby verifying the pigment produced by halotolerant black yeast GUBPC1 as melanin. The UV–Visible scan of the melanin produced by various microorganisms from the previous studies exhibits maximum absorbance in the UV region ranging from 200 to 300 nm (Suwannarach et al., 2019).

3.6. Extracellular hydrolytic enzyme profiling

The capability of the black pigmented halotolerant yeast GUBPC1 to produce extracellular hydrolytic enzymes was qualitatively assayed using minimal NH medium supplemented with 5 % NaCl and various substrates of plant and animal origin. The enzymes screened were amylase, xylanase, protease, pectinase, esterase, lipase, cellulase, chitinase, gelatinase and laccase (Fig. 6b). The screening of GUBPC1 for extracellular hydrolases revealed the culture to hydrolyse 7 out of the 10 substrates. Positive hydrolytic activities of cellulase, laccase, chitinase, xylanase, pectinase, amylase and protease were exhibited by the culture with enzymatic indices corresponding to 3.37 ± 0.36, 3.32 ± 0.12, 2.5 ± 0.2, 2.21 ± 0.08, 2.09 ± 0.35, 1.62 ± 0.07 and 1.76 ± 0.07 cm, respectively (Fig. 6a). Zalar et al. (2005) studied the enzyme profile of *H. werneckii* EXF-1956 at varying NaCl concentrations and reported that the culture produced amylase, esterase, β-glucosidase, laccase, ligninase, lipase, mangan peroxidase, protease and xylanase in media supplemented with 5 % NaCl. Elsayed et al. (2016) studied enzymatic activities of three strains of *H. werneckii* EGYNDA08, EGYNDA16 and

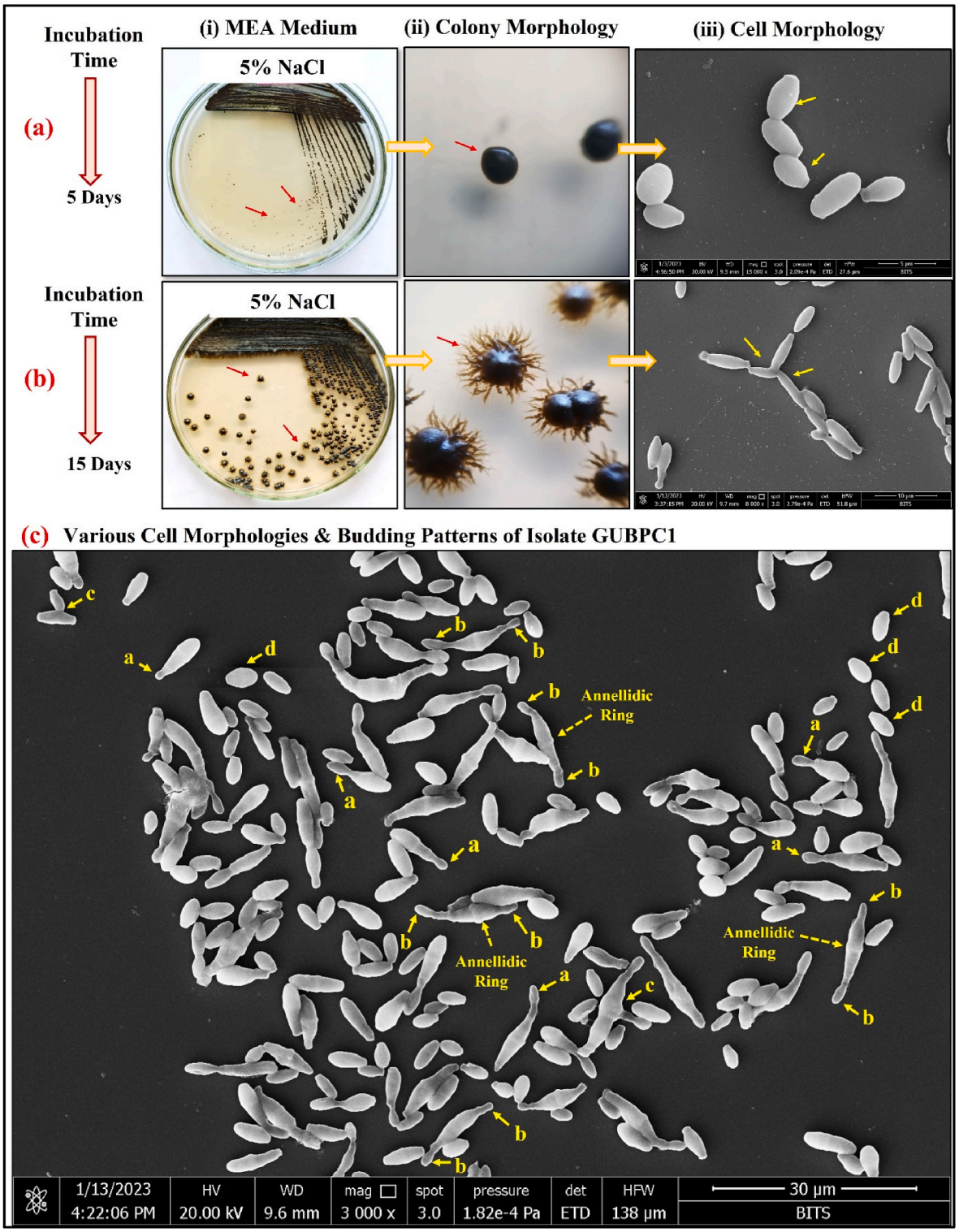

Fig. 4. (a) The morphological colony characteristics observed under the Macro Lens, depicts the progressive growth of the culture GUBPC1 on MEA medium supplemented with 5 % (w/v) NaCl from (a) oval cells after 5 days incubation to (b) meristematic hyphae after 15 days incubation. (c) The Micrographs captured under the FE-SEM representing a-Polar bud, b-bipolar buds with annellidic ring, c-lateral buds and d-oval cells.

EGYNDA90 isolated from salt marshes of Egypt and reported them to be positive for catalase, amylase, protease, cellulase, lipase and urease. A recent study by Rizk and Magdy (2022) on *H. werneckii* strain GPS5 reported the dominance of enzymes degrading substrates of animal origin such as phospholipase, caseinase and gelatinase and few enzymes such as amylase and esterase degrading substrates of plant origin. However, the substrates which were hydrolysed by our isolate GUBPC1 were majorly plant polymers. This suggests that the organism produces enzymes which have the ability to degrade plant biomass. Our results showed similarities to that of Formoso et al. (2015) who mentioned that *H. werneckii* was more adapted to the degradation of plant constituents than of substrates of animal origin. The study also concludes that in saline environment which is its natural habitat, the organism may live as a saprophyte and decomposer (Formoso et al., 2015).

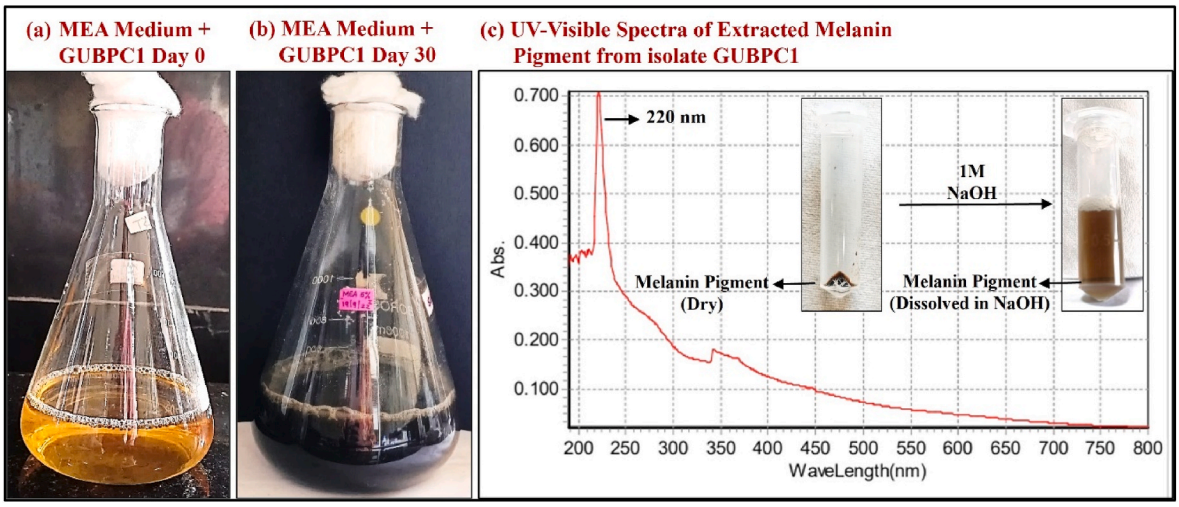

Fig. 5. (a) MEA medium inoculated with the culture GUBPC1 at (a) 0 day incubation (b) after 30 days of incubation and (c) The melanin pigment extracted from the isolate GUBPC1 and the UV–Visible spectrum obtained of the pigment.

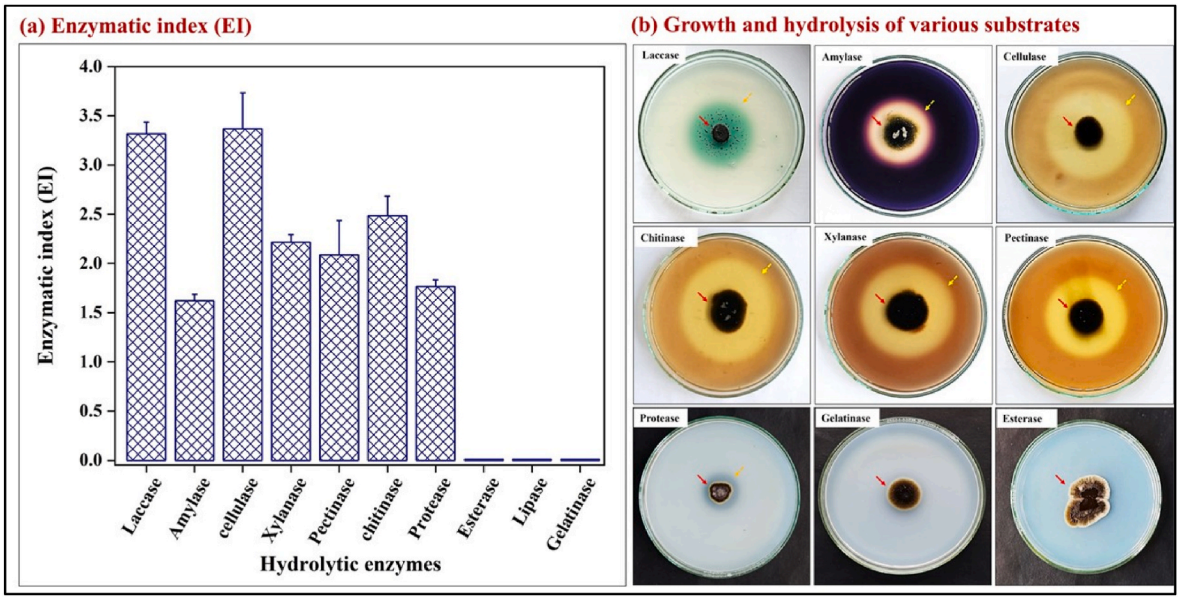

Fig. 6. (a) The bar graph representing the enzymatic index of various enzymes produced by the culture GUBPC1. (b) Production of extracellular hydrolytic enzymes such as laccase, amylase, cellulase, chitinase, xylanase, pectinase and protease by the culture GUBPC1 grown on NH medium supplemented with various substrates. The zone of hydrolysis and culture growth is indicated by yellow and red arrow, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.7. Whole genome sequencing (WGS) of H. werneckii GUBPC1

The length of the genome obtained from *H. werneckii* GUBPC1 strain is 26,932,718 bp comprising 686 contigs. The longest contig is 512,409 bp, and the guanine-cytosine (GC) content is 53.24 %. The basic statistics of assembly are given in Table 2. The assembled genome size is approximately 26.93 Mbp. The structural annotation of GUBPC1 genome revealed a total of 9383 protein coding genes, with an average gene length of 1610.46 base pairs. Among these, 9310 mRNA sequences and 73 tRNA sequences were predicted. Additionally, 6097 transcripts contained multiple exons, while 3213 were single-exon transcripts. The average exon length was calculated to be 529.74 base pairs, and the average protein length was 498.39 amino acids. Furthermore, functional annotation revealed the presence of 6271 GO terms, 7471 InterProScan annotations, 7917 EggNOG annotations, 6449 Pfam annotations, 435 CAZyme annotations, 299 MEROPS annotations, and 1281 BUSCO annotations. These comprehensive annotations provide valuable insights into the genomic features and functional potential of *H. werneckii*, facilitating further studies on its biology and adaptive mechanisms.

Table 2
Statistics of the assembly results.

Content	Value
Total length	26932718
Contigs	686
Largest contig	512409
Average (bp)	39,260.521
GC (%)	53.24
N50	159851
N90	38930
L50	53
L90	188

3.7.1. Kyoto Encyclopedia of genes and genomes (KEGG) pathway

The KEGG pathway analysis revealed a diverse range of biological processes and metabolic pathways in the genome of *H. werneckii* GUBPC1. 3567 genes were annotated using KEGG blastKOALA (Version 3.0). The majority of pathways were associated with genetic information processing, including protein families involved in transcription, translation and replication (Fig. 7a). A significant proportion was attributed to metabolism pathways. Pathways related to carbohydrate metabolism appear to be dominant followed by amino acid, lipid, energy metabolism, metabolism of cofactors and vitamins and nucleotide metabolism. Interestingly, pathways related to xenobiotic biodegradation and metabolism were also identified, suggesting that the *Hortaea* strain may contribute to the degradation of xenobiotic compounds thus it can be used as a potent agent for bioremediation purposes in xenobiotic-polluted areas (Elsayis et al., 2022b). The relatively low percentage of metabolism of terpenoids and polyketides and biosynthesis of other

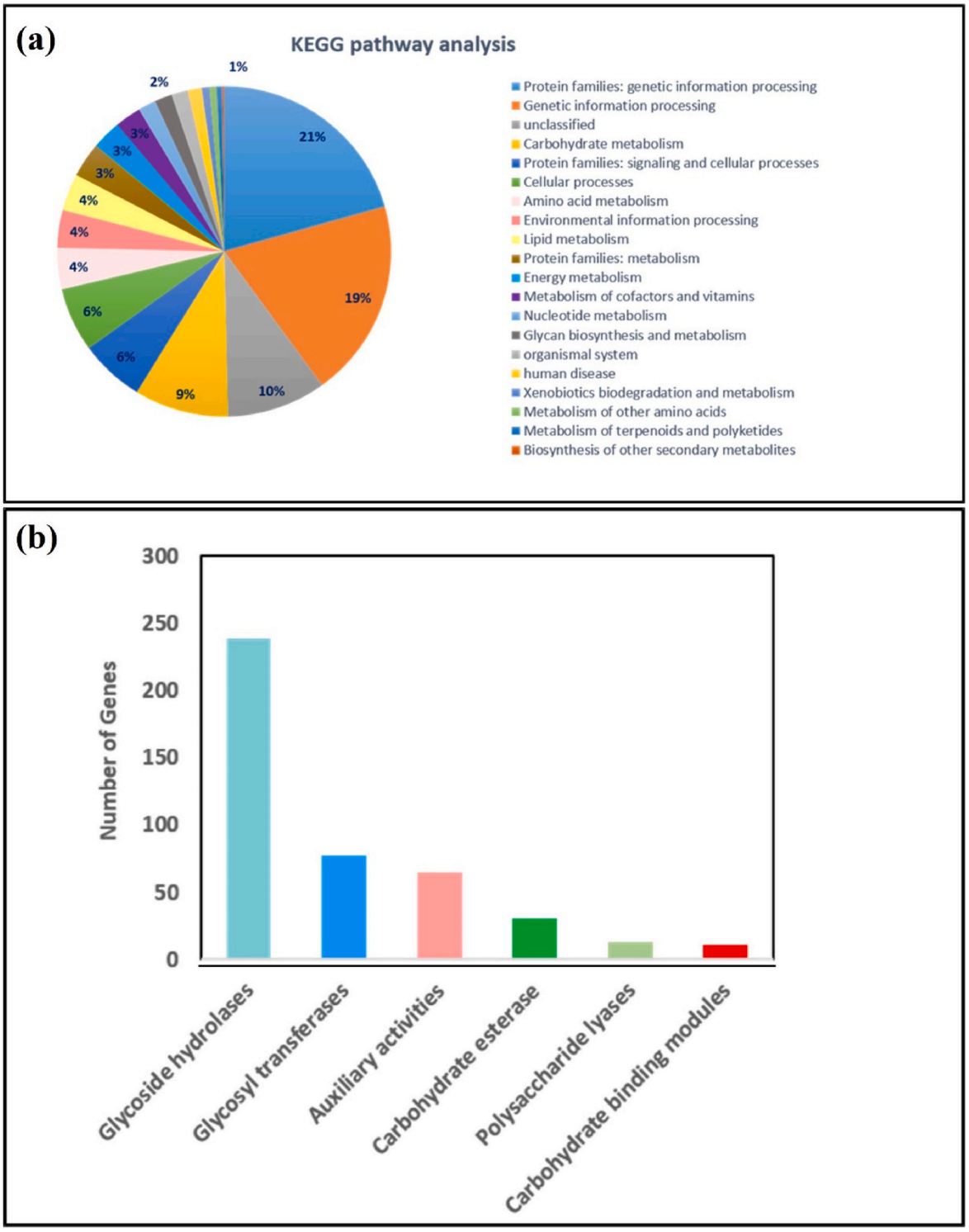

Fig. 7. Functional annotation of the *Hortaea werneckii* genome. (a) KEGG Pathway functional classification pie chart (b) Carbohydrate activity enzyme annotation results.

secondary metabolites were also observed, indicating the potential metabolic capabilities of this strain GUBPC1. Further deep investigation into these pathways could reveal unique biochemical features and biotechnological applications of the strain.

3.7.2. Antibiotics and secondary metabolites analysis Shell (AntiSMASH)

AntiSMASH results showed that strain GUBPC1 has 16 Secondary Metabolites (SMs) Biosynthetic Gene Clusters (BGCs), 8 NonRibosomal Peptide Synthetases like (NRPS-like), and 3 NonRibosomal Peptide Synthetases (NRPSs), 3 terpenes, and 2 T1PKS (Type I Polyketide Synthase) (Table 3 and Fig. 8). Notably, regions 38.1 and 144.1 were classified as T1PKS, associated with T1PKS gene clusters. Region 38.1 in the *H. werneckii* GUBPC1 genome exhibits 100 % similarity to melanin and polyketide biosynthetic pathways. Melanin, a complex pigment known to have a dark brown to black colour, protects the fungal cell against various environmental stress conditions such as UV protection, extreme temperature, enzymatic action, oxidizing agent and osmotic stress (Elsayis et al., 2022a; Kejzar et al., 2013). The synthesis of melanin by black fungi in extreme environments suggests that it may serve as a survival and/or adaptive strategy for these fungi. Melanin pigment extracted from halotolerant black yeast *H. werneckii* isolated from solar salterns in India exhibited antibacterial properties (Rani et al., 2013). Another study has also reported the antimicrobial capability of the melanin pigment synthesized by *H. werneckii* against certain bacterial and fungal strains (Elsayis et al., 2022b). The presence of NRPS-like, NRPS and terpenes clusters suggests that, GUBPC1 is able to synthesis wide range of bioactive compounds with pharmacological and biotechnological importance (Abo Nouh et al., 2021; Thompson and Gilmore, 2023). Synthesis of these peptides may also contribute to defense mechanisms against harsh environmental conditions.

Table 3
The analysis of biosynthetic pathways by fungi antiSMASH 6.1.1.

Cluster No	Region	Type	From	To	Most similar known cluster	Similarity
1	Region 1.1	NRPS-like	55074	98226		
2	Region 11.1	NRPS-like	62517	121984		
3	Region 11.2	NRPS	180131	235182		
4	Region 14.1	NRPS-like	39589	82792		
5	Region 21.1	NRPS-like	104201	148079		
6	Region 34.1	terpene	82086	102859		
7	Region 38.1	T1PKS	8844	55463	melanin	100 %
8	Region 44.1	NRPS-like	127283	172526		
9	Region 45.1	NRPS-like	135862	175106		
10	Region 48.1	terpene	153723	168893		
11	Region 54.1	NRPS	51489	95061		
12	Region 72.1	terpene	66495	88080		
13	Region 96.1	NRPS-like	1	33953		
14	Region 111.1	NRPS	35488	77919		
15	Region 144.1	T1PKS	7067	55228		
16	Region 169.1	NRPS-like	3727	45004		

3.7.3. Carbohydrate–active enzymes (CAZymes)

CAZymes are involved in the degradation of complex carbohydrates (Yang et al., 2019). We annotated the *H. werneckii* protein sequences based on the CAZyme database with HMMER. Analysis results showed the presence of 435 CAZymes belonging to 6 different CAZyme families (Fig. 7b). Genes belonging to the glycoside hydrolases family were the most abundant, with 239 genes, followed by glycosyl transferases with 77 genes and auxiliary activities with 65 genes. Furthermore, groups of carbohydrate esterases, polysaccharide lyases, and carbohydrate-binding module families were also represented with 30, 13 and 11 genes. Our study delved into the CAZymes of our fungal strain GUBPC1, revealing promising enzymatic capabilities. We identified genes responsible for the hydrolysis of starch, pectin, xylan and glucose, which compared well with the culture based hydrolytic enzyme studies of the isolate GUBPC1, suggesting its capacity for efficient degradation of polymer of plant origin. Additionally, we found genes encoding for chitinase enzymes, which could contribute to biocontrol properties against various fungal strains. These findings suggest the potential agricultural and pharmaceutical applications of CAZyme-producing fungi, highlighting their value in biotechnological applications (Ali et al., 2020).

The detailed study of annotation depicted the functional capabilities of *H. werneckii* GUBPC1. AntiSMASH analysis uncovered intriguing biosynthetic gene clusters, including those associated with melanin and other polyketide biosynthesis. The identification of BGC associated with melanin and polyketides further underscores its adaptive strategies to environmental stresses and potential bioactive properties (Elsayis et al., 2022b). The presence of diverse CAZymes suggests its capability to degrade complex carbohydrates, aiding in nutrient acquisition and survival in extreme conditions (Segal-Kischinevzky et al., 2022). KEGG pathway analysis identified a range of metabolic pathways, with carbohydrate metabolism being the most prominent. Some genes involved in xenobiotic degradation of various pollutants like benzoate, aminobenzoate, chloroalkane, chloroalkene and styrene were found, suggesting future application of this strain in bioremediation. Furthermore, we have identified genes encoding for the synthesis of enzymes that are crucial when dealing with oxidative stress, including superoxide dismutases (SOD), catalases (CAT), glutathione peroxidases (GPX), and peroxiredoxins (PRX). These enzymes play pivotal roles in scavenging reactive oxygen species (ROS), thereby helping to maintain cellular balance, especially under oxidative stress conditions (Segal-Kischinevzky et al., 2022). Moreover, interproscan shows an arsenical resistance gene. The presence of the arsenic resistance gene (ARR3) is particularly noteworthy, suggesting the ability of yeast to tolerate and detoxify potentially toxic compounds present in its environment (Gupta and Chatterjee, 2017).

4. Conclusion

This study underscores the resilience of melanized yeast, *Hortaea werneckii* GUBPC1, isolated from saltpans, showcasing its halotolerant nature. The isolate exhibits diverse colony and cell morphologies in response to varying NaCl concentrations, indicating its adaptability to extreme conditions. Notably, GUBPC1 demonstrates significant synthesis of plant polymer-degrading enzymes, highlighting its potential in biomass degradation. Genomic analysis reveals valuable insights into the adaptive strategies of *H. werneckii,* emphasizing its importance in challenging environments and its promising applications in biotechnology and environmental contexts.

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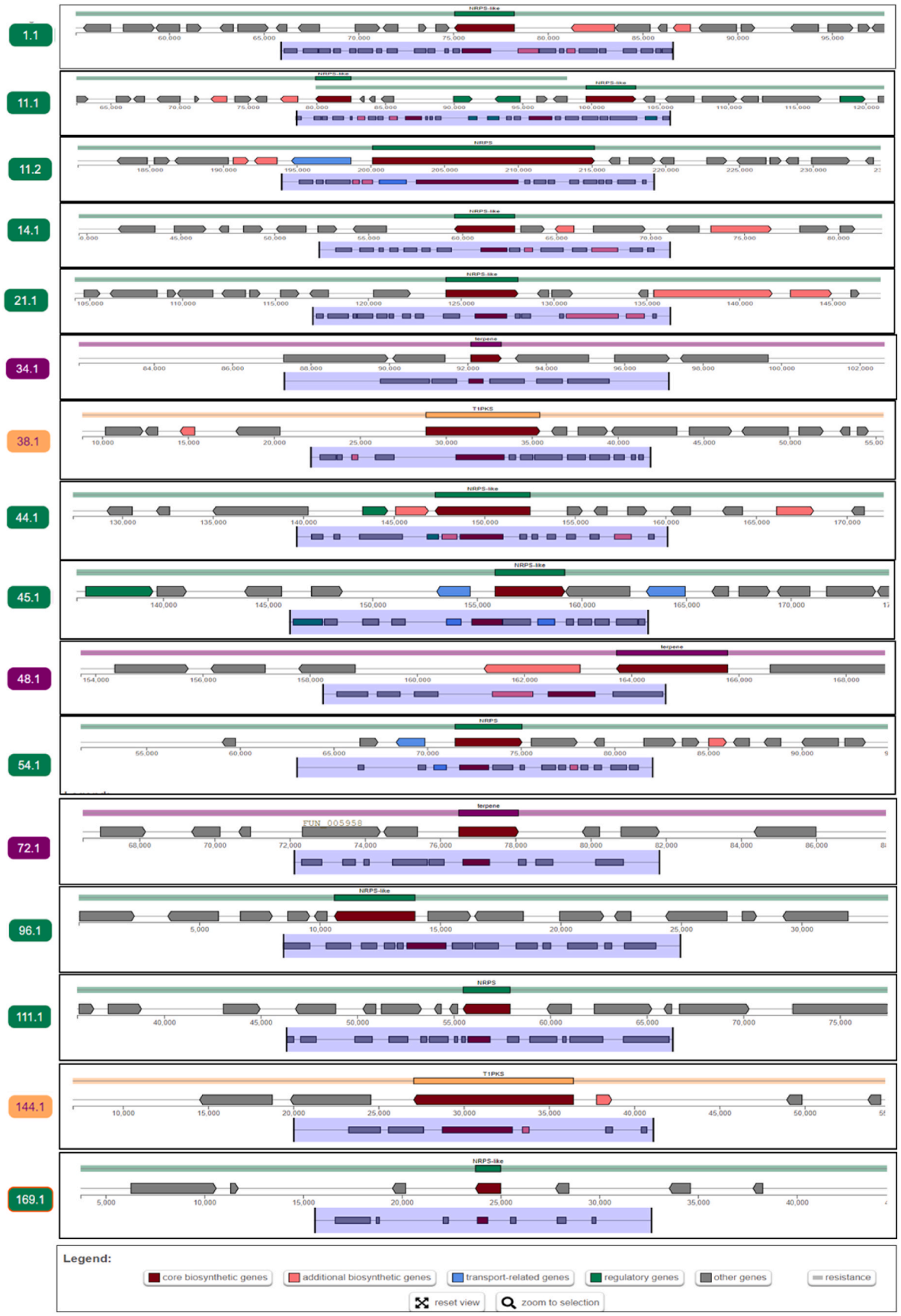

Fig. 8. Localisation of biosynthetic gene clusters (BGC) in the genome of *H. werneckii* GUBPC1.

Government of India, file. no: SRG/2021/001100. Dr. AS acknowledges Department of Biotechnology for their funding support under the project BT/PR41474/NDB/39/760/2020.

Data availability

Our draft genome obtained from Whole Genome Sequencing (WGS) has been submitted to NCBI with accession number (JBDIUT000000000), under BioProject ID (PRJNA1099926) and BioSample ID (SAMN40951498).

CRediT authorship contribution statement

Siddhi Deelip Nadodkar: Data curation, Investigation, Methodology, Resources, Writing – original draft. **Mrunal Karande:** Data curation, Methodology, Software, Writing – original draft. **Gandisha Masso Pawar:** Data curation, Formal analysis, Validation, Writing – original draft. **Aishwarya Vinayak Dhume:** Conceptualization, Data curation. **Avinash Sharma:** Data curation, Supervision, Validation, Visualization, Writing – review & editing. **Bhakti Balkrishna Salgaonkar:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2024.08.010.

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