Do yeast host bacteria? Insights into the bacterial communities of yeast

Thesis submitted for the degree of

Doctor of Philosophy

By

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CERTIFICATE

This is to certify that **Ms. Indu** has carried out the research work embodied in the present thesis under the supervision and guidance of Prof. Ch. Venkata Ramana for a full period prescribed under the Ph.D. ordinances of this University. We recommend her thesis entitled **"Do yeast host bacteria? Insights into the bacterial communities of yeast"** for submission for the degree of Doctor of Philosophy of the University.

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DECLARATION

I, **Indu** hereby declare that this thesis entitled **"Do yeast host bacteria? Insights into the bacterial communities of yeast"** submitted by me under the guidance and supervision of **Prof. Ch. Venkata Ramana** is an original and independent research work. I hereby declare that this work is original and has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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This is to certify that this thesis entitled **"Do yeast host bacteria? Insights into the bacterial communities of yeast"** is a record of bonafide work done by **Ms. Indu**, research scholar bearing registration number 15LPPH08 for award of Doctor of Philosophy in Department of Plant Sciences, School of Life Sciences, University of Hyderabad, under my guidance and supervision. This thesis is free from plagiarism and has not been submitted in any part or to full to this or any other University or Institute for the award of any degree or diploma.

Parts of the thesis has been:

A. Published in following publication:

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Further, the student has passed the following courses toward the fulfillment of the coursework requirement for Ph.D. degree.

S.no	Course	Name	Credits	Pass/Fail
1.	AS801	Analytical Techniques	4	Pass
2.	AS802	Research Ethics, Data Analysis and Biostatistics	3	Pass
3.	AS803	Lab work and record	5	Pass

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Indu*….*

Dedicated to my beloved parents
and
my supervisor

“When we try to pick out anything by itself, we find it is tied to everything else in the universe”

— John Muir

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LIST OF ABBREVIATIONS

Abbreviations	Expansion
AAI	Average amino acid identity
AAPB	Aerobic anoxygenic phototrophic bacteria
AL	Aminolipid
AMF	arbuscular mycorrhizal fungi
ANI	Average nucleotide identity
BFI	Bacterial-fungal interactions
BLAST	Basic Local Alignment Search Tool
BLASTn	Nucleotide Basic Local Alignment Search Tool
BLASTP	Protein Basic Local Alignment Search Tool
BLBs	bacteria like bodies
bp	Base pair
DAPI	4',6-Diamidino-2-Phenylindole
DBB	Diazonium Blue B
DRISEE	Duplicate Read Inferred Error Estimation
*d*DDH	Digital DNA-DNA hybridization
DDH	DNA- DNA hybridization
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra acetic acid
EF	Elongation factor
FISH	Fluorescence in situ Hybridization
FAME	Fatty acid methyl esters
g	Gram
$g.l^{-1}$	Gram per liter
G+C	Guanine+cytosine
GC	gas chromatography
GC-MS	Gas chromatography mass spectrophotometry
GL	Glycolipid
GPS	Global positioning system
h	Hour
HPLC	High-performance liquid chromatography
ITS	internal transcribed spacers
KCTC	Korean collection for type cultures
L	Litre
MAT	Mating-type
MG-RAST	Metagenomic Rapid Annotations using Subsystems Technology
ME	Minimum Evolutionary
MEGA	Molecular Evolutionary Genetics Analysis

Mg	Milligram
MK	Menaquinone
min	Minute
ml	Milliliter
ML	Maximum Likelihood
MLSA	Multilocus sequence analysis
mM	Millimolar
MUSCLE	MUltiple Sequence Comparison by Log-Expectation
NaCl	Sodium chloride
NBRC	NITE Biological Resource Center
NCBI	National Center for Biotechnology Information
NJ	Neighbor-Joining
nm	Nanometer
nt	Nucleotide
OD	Optical density
OrthoANI	Orthologous average nucleotide identity
PATRIC	Pathosystems Resource Integration Center
PCoA	Principal Coordinates Analysis
PCR	Polymerase chain reaction
QIIME	Quantitative Insights Into Microbial Ecology
QS	Quorum Sensing
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
s	Seconds
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SRA	Sequence Read Archive
SSC	saline-sodium citrate
TEM	Transmission electron microscopy
TLC	Thin layer chromatography
UV	Ultraviolet
WGA	wheat germ agglutinin
WGS	Whole Genome Sequencing
YCB	Yeast Carbon Base
YND	Yeast Nitrogen Base
µg	Microgram
$\mu g.l^{-1}$	Microgram per liter
(w/v)	Weight/volume
≤	Less than or equal to
°C	Degree Celsius

µl	Microliter
µM	Micromolar
^{0}C	Degree Celsius
2D	Two dimensional
1D	One dimensional

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Introduction

Introduction

1.1. Taxonomy of yeasts

Yeasts are the members of kingdom Fungi and often used as a synonym of *Saccharomyces cerevisiae*. They are classified into two separate phyla, i.e., Ascomycota and Basidiomycota and together form dikarya. Phylum Ascomycota is also known as sac fungi and is the largest phylum of kingdom Fungi containing more than 64000 species (Bennet et al., 2017). Phylum Basidiomycota is known as club fungi and comprises 37% of described species of true fungi (Hibbett et al., 2007).

Yeasts like *Saccharomyces cerevisiae* and *Saccharomyces pombe* served as model systems in the field of genetics and molecular biology as their cellular architecture and the basic life cycle pattern including the processes like cell division, DNA replication, recombination and other fundamental processes are similar to the higher eukaryotic organisms. Yeast being unicellular eukaryote, can easily be manipulated and cultivated under laboratory conditions (Brückner et al., 2009). They are instrumental in wine making, baking and brewing industries (Bond and Blomberg, 2006).

1.1.1. Characterization of yeast

Yeasts are the unicellular eukaryotes. Although they are unicellular, their cellular organization resembles higher eukaryotic organisms. The size varies in between 3-4 µm in diameter; sometimes grow up to 40 µm (Walker et al., 2002). They reproduce asexually via budding and occasionally by fission. Some yeast forms string of attached budding cells called pseudohyphae or false hyphae (Mukaremera et al., 2017). Yeasts are characterized based on their cell morphology, physiology and by molecular techniques. They are ubiquitous and found in soil, water, fruit, vegetables, insects several other sources (Cooke, 1968). They have a thick cell wall of chitin, glucan and glycoprotein that protects the cell against the mechanical and osmotic stress (Garcia-Rubio et al., 2020).

1.1.1.1. Asexual reproduction

Yeasts reproduce asexually by budding, fission and by producing blastoconidia. They also reproduce by forming pseudohyphae or false hyphae (Mukaremera et al., 2017). In budding, a small outgrowth or evagination on the surface of parent cell formed which increase in size, separate themselves from the parent and form a new cell. In fission, yeast duplicates by inward growing septum, thereby forming fission cells. Reproduction by means of blastoconidia is uncommon where the cells are short oval formed at the end of the pseudomycelial cells called as blastospore and those formed at the sides of pseudomycelial are known as blastoconidia (Becze, 1955).

1.1.1.2. Sexual reproduction

Many of the ascomycetous yeast reproduces sexually (Trail, 2007). They propagate sexually by producing ascospores within the ascus. Typically, a single ascus contains eight ascospores. Basidiomycetous yeast also shows sexual reproduction (Rivera-Mariani and Bolaños-Rosero, 2012). They form reproductive spores within mycelium which have club shaped end structures harboring external meiospores or basidiospores. Reproduction in many basidiomycetous yeast occurs in two phases. These are budding haplophase and self-sporulating diplophase or dikaryotic hyphal phase (Lin et al., 2015).

1.1.1.3. Fermentation of carbohydrate

The ability of yeast to ferment sugar varies from species to species, they produces carbon dioxide on fermentation (Kurtzman et al., 2011). There are several methods for the detection of carbon dioxide produced from carbohydrate. Of these methods, the use of Durham tubes was the most effective. Durham tubes containing 2% solution of sugars is placed inverted in medium containing tubes to collect gas produced by yeast during fermentation of sugars. The basal medium used for the analysis contains all essential nutrients and minerals required for yeast growth. Commonly used sugars for routine identification include glucose, galactose, trehalose, maltose, raffinose, lactose, xylose and sucrose.

1.1.1.4. Assimilation of carbon compounds

Yeast can grow aerobically on various carbon sources as their sole carbon source. To test the ability of yeast for assimilation of the carbon sources, they are grown on solid and liquid media where the latter gives better reproducible results thus suggested by most of the taxonomists. Rimless boiling tubes are used to conduct these tests. Commonly used carbon sources include hexose-based carbon sugars i.e., inulin, sucrose, raffinose, melibiose, galactose, lactose etc.; pentose carbon sources i.e., xylose, arabinose, ribose; alcohols i.e., methanol, ethanol, glycerol, ribitol etc.; amines i.e., D-glucosamine, N-acetyl-D- glucosamine and acids i.e., succinate, citrate.

1.1.1.5. Assimilation of nitrogen compounds

A wide variety of nitrogen sources were utilized by yeast for their growth. These sources include nitrate, ethylamine hydrochloric acid, cadaverine dihydrochloride, imidazole, glucosamine, creatinine and creatine. The methods and principle used are the same as used to test the carbon utilization except for the media. The media used is yeast carbon base media which is devoid of nitrogen and contain dextrose as the carbon source. The medium is enriched with the desired source of nitrogen, inoculated with culture and incubated for 1-3 weeks at room temperature and observed for the growth.

1.1.1.6. Other growth tests

i) **Growth in Vitamin free media**: To test the yeast ability to grow in vitamin free media was introduced by Wickerham in 1951 (Wickerham, 1951) and extended to check the discrete requirement of vitamins by Van Uden and Farinha 1958 (Van Uden and Farinha, 1958).

ii) **Growth in media of high osmotic pressure**: Yeasts which are isolated from sugar and salt-rich sources generally tolerate high osmotic pressure. They can grow well in glucose concentration by up to 40% and can sometimes also grow in the range of 50% to 70% of glucose. The tolerance is checked by growing it in the media containing high sugar and salt concentrations.

iii) **Growth at different temperatures**: Yeast grows optimally between 20 to 28 °C. Growth of yeasts is accessed in liquid or agar medium at various temperature. Yeasts are inoculated in dextrose medium, incubated at a different temperature ranging from 4-40 °C and observed for the turbidity.

iv) **Diazonium blue B color reaction**: Diazonium Blue B (DBB) test is used to differentiate between basidiomycetous and ascomycetous yeasts. Basidiomycetous yeast gives dark red color reaction when treated with DBB whereas no coloration or reaction is be observed in ascomycetous yeasts.

1.1.1.7. Molecular characterization and phylogeny

Prior to the existence molecular phylogeny analysis, identification and placement of yeast species was based on the phenotypic traits. After the advent of genomic sequencing, the comprehensive information about the yeast enabled to establish functional relationship between the sequences and the gene products in fungi and higher organisms. Advancement in the sequencing techniques and molecular comparison have shown that many of the earlier identified yeast taxa were placed inappropriately and there was a need for reclassification (Kurtzman et al., 2015). Sequencing of 5.8S rRNA gene, internal transcribed spacers ITS1, ITS2 and D1/D2 domain of 26S rRNA gene are used for the classification of fungi. Sometimes, sequence analysis of elongation factor alpha (EF-alpha) is also necessary.

With the widespread use of molecular taxonomic methods, the improvement in identification and description of new taxa of ascomycetous and basidiomycetous species have been phenomenal. Yeast identification is performed routinely from sequence divergence in ITS region and D1/D2 domain of large subunit rRNA gene. Species delineation for both ascomycetous and basidiomycetous yeast is based on the prediction that strains of a species diverge in ITS and D1/D2 sequences by no more than 1% (Kurtzman and Robnett, 1998; Scorzetti et al., 2002; Sugita et al., 1999). The ITS and D1/D2 databases are the powerful tools for the species identification and for species delineation of hitherto unknown lineages. The phylogenetic placement of ascomycetous and basidiomycetous yeast are executed by constructing phylogenetic trees based on ITS, EF-alpha and D1/D2 domain of large subunit.

1.1.1.8. Maintenance of yeast cultures

Glucose in yeast nitrogen base medium is used for the maintenance of yeast cultures. Glucose is used as the sole carbon source to reduces the risk of alteration in growth and fermentation patterns of yeasts (Wellman and Stewart, 1973). Generally, yeast cultures are preserved between 4 °C and 12 °C and sub-cultured repeatedly at regular intervals. Many yeasts lost their capability to reproduce sexually when preserved through repeated sub-culturing.

Several techniques are used to preserve the yeast for a longer duration. These are:

i) Lyophilization: Culture is grown at room temperature for 1-2 days. Actively growing yeast cells are precipitated and freeze-dried to inactive, low moisture state.

ii) Liquid nitrogen preservation: Several yeasts do not endure lyophilization. In this technique, culture is preserved in liquid nitrogen vapor which usually remains viable for several years.

While cataloging the yeast diversity from different habitats during this study, we have isolated few novel species belonging to genera *Candida* and *Suhomyces*. The general characteristic of both the genera are further expanded.

1.1.2. General characteristics of the genus *Candida*

The genus *Candida* encompasses several anamorphic fungi which are ubiquitous in a vast range of natural and artificial habitats (Kurtzman et al., 2011). These fungi are phylogenetically disparate as they comprise of many common evolutionary ancestors. Due to the lack of distinguishing typeset, they are not suitable to be placed under the presently acknowledged genus and at the same time, all of them can't be assigned under the same taxon. Thus, these were grouped into one or more non-natural genera which are phylogenetically restricted (Zhang et al., 2013). According to International Code of Nomenclature for algae, fungi, and plants (ICN), presently existing species of *Candida* along with some other anamorphic yeast; requires reconsideration to be a part of the genus comprising of species with reliable similarities (Turland et al., 2018). The aim is to classify *Candida* species into well-sustained clades.

Nucleotide sequences of RNA polymerase II large subunit genes were used for investigating phylogenetic relationships. Actins nuclear sequence also played a crucial role to elucidate phylogeny amongst *Candida* species and it offered a better phylogenetic distinction (Daniel et al., 2001). Melbourne Code has revised the fungal taxonomy and recommended one valid single name for a taxon irrespective of their reproductive stages (anamorph or teleomorph). This was possible by using molecular markers (SSU, D1/D2 LSU, ACT1, EF1, Mcm7, and RPB2) which helped in the reclassification of several yeasts (Daniel et al., 2014). Most of the yeast belongs to the subdivision *Saccharomycotina*. The genus *Candida* belongs to the *Saccharomycetaceae* family, comprising 430 species (http://genome.jouy.inra.fr/yeastip/) (Weiss et al., 2013). The placement of these anamorphic species was based on twelve different clades which were formed using five concatenated gene sequences (Daniel et al., 2014).

1.1.3. General characteristics of the genus *Suhomyces*

Species of *Suhomyces* are found to be present vastly all over the world and they have been isolated from fungus feeding insects (Suh et al., 2004), rotten mushroom (Middelhoven and Kurtzman, 2007), moss (Nakase et al., 1988) and soil (Middelhoven and Kurtzman, 2007). The discovery of the genus *Suhomyces* as a group of species sharing important biological traits can be traced back to the detection of five new species related to *Candida tanzawaensis* (Nakase et al., 1988, Kurtzman et al., 2016) and the study of yeasts from the guts of basidiocarp-feeding beetles (Suh et al., 2004, 2006). About 30% of the 650 yeasts isolated from beetle guts, formed a cohesive phylogenetic clade of 16 new species with *C. tanzawaensis* and its relative species. The number of yeast isolates, from beetles clustering with *C. tanzawaensis* was already remarked by Kurtzman (2001), but the significance for an insect-associated habitat of later diverging *Suhomyces* species accumulated largely by the work initiated in the Blackwell lab (Kijpornyongpan et al., 2019). Sexuality has never been observed in *Suhomyces* species and the decline of sexual reproduction was inferred from the repeated disintegration of MAT loci (Kijpornyongpan et al. 2019).

1.2. Taxonomy of bacteria

Ferdinand Cohn was the first scientist to propose bacterial taxonomy based on the morphological characters of bacteria in 1872. Later, numerical taxonomy also played an important role in phenotypic characterization by comparing several phenotypic characters of various strains (Sneath, 2015). With the advancement of emerging techniques, several biochemical tests were incorporated in bacterial identification and its classification.

1.2.1. Polyphasic taxonomy

The term "polyphasic taxonomy" was coined by Colwell in 1970 and defined it as a taxonomy that gathers and incorporates many levels of information from molecular to morphology to ecology thereby refers to as a consensus approach to bacterial systematics (Colwell, 1970). This taxonomy includes classification based on phenotypic, genotypic and phylogenetic analysis.

1.2.1.1. Phenotypic characterization

1.2.1.1.1. Morphological characterization

Bacterial colony morphology varies in their shape, size, color, margin and texture. They may be smooth to rough, circular to irregular, filamentous to rhizoids. Their elevation varies from raised to convex, flat, umbonate and crateriform. Their margins might be entire, undulate, filiform, curled and lobate. Shape of the bacteria varies from circular to rod to pleomorphic. Color of the colony depends on the type of pigmentation bacteria possess for example flexirubin pigment imparts an intense yellow color to the colonies in the members of family *Flavobacteriaceae*. Textures of colonies varies from dry, mucoid (thick and wet), moist, smooth, rough or rugose.

1.2.1.1.2. Physiological and biochemical characterization

Several experiments need to be performed to decipher physiological and biochemical characterization. These experiments include growth modes, growth at different temperatures, pH and salinity. Biochemical tests include hydrolysis of gelatin, casein, chitin, urea, starch, tween-20, tween 80 and several enzymatic activities like catalase, oxidase activity and antibiotics sensitivity

1.2.1.1.3. Chemotaxonomic characterization

i) Polar lipids

Identification of polar lipids is significant in the classification of bacteria as they are the unique and essential component of the bacterial membranes (Lechevalier and Moss, 1977). Phosphatidylethanolamine, phosphatidylglycerol, phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol and sphingolipids are the major polar lipids of phylum Proteobacteria whereas in Bacteroides, phosphatidylethanolamine is the only major polar lipids (Costa et al., 2011). In addition to these major polar lipids, bacterial polar lipids also constitute to several unidentified minor polar lipids which aid in differentiation up to the species level.

ii) Isoprenoid quinones

Quinones are the lipid molecules that act as a biomarker or chemotaxonomic marker (Hiraishi, 1999). They are present in the cell membrane of all the photosynthetic and respiratory bacteria and plays a vital role in electron transport and oxidative phosphorylation. Their structure varies depending on the microbial taxon they are associated with (Collins and Jones, 1981). Prokaryotic quinones are categorized into two structural classes i.e., naphthoquinones (menaquinones and phylloquinones) and benzoquinones (ubiquinone and plastoquinone) (Nowicka and Kruk, 2010). Later, two more types of quinones i.e., caldariellaquinone and rhodoquinone were also reported in bacteria (Brajcich et al., 2010; De Rosa et al., 1977; Glover, 1962). The side chains of all these quinones vary depending on the associated bacteria and thus plays an essential role in taxonomic classification.

1.2.1.2. Molecular characterization and phylogeny

The advancement of molecular systematics led to the use of genotypic and phenotypic data in the classification of organisms at each levels of the hierarchy. 16S rRNA gene sequence analysis and DNA-DNA hybridization methods are considered as gold standard for the proposed new species in bacterial taxonomy.

1.2.1.2.1. 16S rRNA gene sequence analysis

16S rRNA gene is the most common and conserved housekeeping marker present in all the bacterial species. The sequence length of the gene is around 1500 bp which is large enough with sufficient interspecific polymorphisms for species delineation and informatic purpose and hence used to study the bacterial phylogeny and taxonomy. The conservation of 16S rRNA gene was initially observed by Dubnau in *Bacillus* members (Dubnau et al., 1965). Later, the importance of 16S rRNA gene in taxonomy was proposed by Carl Woese and subsequently this strategy was incorporated into the bacterial taxonomy (Woese et al., 1985). In 1994, Stackebrandt and Goebel recommended 97% identity of 16S rRNA gene and 60% re-association values among the species for the species delineation (Stackebrandt and Goebel, 1994). Later, the recommended cut-off shifted from 97% to 98.7% 16S rRNA gene similarity among the species (Stackebrandt and Jonas, 2006). Irrespective of serving as a universal phylogenetic marker in higher ranks of bacterial taxonomy, 16S rRNA gene cannot provide sufficient resolution at species level. To resolve this concern, multilocus sequence analysis (MLSA) is performed.

1.2.1.2.2. Multilocus sequence analysis (MLSA)

Multilocus sequence analysis (MLSA) refers to the simultaneous phylogenetic analysis of house-keeping genes. It is implemented to ensure higher resolution of phylogenies of species within a genus or a genus within a family (Glaeser and Kämpfer, 2015). Sometimes, single gene-based analysis inaccurately deduces the phylogenetic placement of the strain and exhibit wrong evolutionary relationships. This problem is resolved by multilocus sequence analysis where several protein coding genes (housekeeping genes) are considered to achieve the proper phylogenies (Gevers et al., 2005; Glaeser and Kämpfer, 2015).

MLSA is a two-step identification process. In the first step, analysis of 16S rRNA gene is used to initially assign a new strain to the genus or a family. Second step is based on the initial assignment of the strain where several house-keeping genes (rpoB, recA, atpD etc) are used to more accurately assigned the strain within a genus more precisely. Since, MLSA approach used the analysis of few genes, more advance method i.e., genome-based tools are further used for species delineation and its phylogenetic assignment.

1.2.1.3. Genome-based classification of bacteria

The role of comparative genomics is important in delineating different species at taxonomic levels. Average Nucleotide Identity (ANI), *In-silico*/digital DNA-DNA hybridization (*d*DDH), and Average Amino acid Identity (AAI) are the new genomic indexes used in the classification of bacteria.

1.2.1.3.1. Average Nucleotide Identity (ANI)

ANI is a measure of genomic similarity at the nucleotide level between the coding regions of two genomes (Konstantinidis and Tiedje, 2005). It is also referred to as the mean identity value between the homologous genome of query and the reference genome. In ANI, the query genome splits into smaller fragments. These small fragments are generally of 1020 bp which is later searched against the reference genome. The search is performed with algorithms like BLASTn and MUMmer. The ANI values are the mean identity of BLASTn matches where the fragments align at least 70% in length with more than 30% identity (Goris et al., 2007; Yoon et al., 2017).

1.2.1.3.2. *In-silico*/digital DNA-DNA Hybridization (*d*DDH)

Another gold standard used for species delineation is DNA-DNA hybridization (DDH) between the query and the reference genome. Earlier, this method was based on denaturation-renaturation between the query and the reference genome using isotopic studies. With the advancement of techniques, *in silico* DNA-DNA Hybridization technique is used for species delineation (Auch et al., 2010a). This method involves genome to genome comparison among the species. The threshold range of ANI lies between the 95-96% for species delineation and is correlated with the DDH cut off values of 70% (Auch et al., 2010a, b). The requirement of a draft genome or complete genome is mandatory for the calculation of ANI score and DDH values.

1.2.1.3.3. Average Amino acid Identity (AAI)

Unlike ANI which is used for evolutionary noise, AAI is suitable for comparing distantly related genomes because resolution is progressively lost at nucleotide level (Rodriguez and Konstantinidis, 2014). In cases where the ANI score among the species is 80% or less and shares less than or equal to 30% of gene content, AAI provide a much better resolution than ANI. The AAI score between 45-65% among the two strains delineate them in different genera and if the AAI score is less than 45% then they are considered as the member of different families

(Goris et al., 2007; Konstantinidis et al., 2017). However, recent reports claimed that AAI values between members of related but different genera can vary between 60-80% (Luo et al., 2014; Orata et al., 2018). Apart from genus delineation, it can also be used for species delineation (Thompson et al., 2013).

1.3. Microbial interactions

There is a saying among microbial ecologists that no organism lives in isolation which highlights the importance of interactions and relationships among different organisms, even if it is a unicellular one like protists (Nowack and Melkonian, 2010). No species exist as autonomous entity but comprises of a host and its associated microbes i.e., holobiont (Bordenstein and Theis, 2015). The interaction of these associated organisms with host varies from mutualistic to antagonistic. Microbe-microbe interactions should not be ignored as they play a crucial role in host-microbiota equilibrium and help in understanding the phenomenon of co-evolution (Simon et al., 2019).

The interactions are of two types:

1. Positive interactions.
2. Negative interactions.

1.3.1. Positive interactions

Positive interactions are further classified into two groups:

1.3.1.1 Mutualism

It is a kind of interaction where both the organisms are getting benefited. Protozoan termite relationship possess the mutualism where protozoa live in the guts of insects that ingest but cannot metabolize cellulose; the protozoa secrete cellulase which metabolizes cellulose, releasing nutrients that the insect can use (Poinar, 2009).

1.3.1.2 Commensalism

In this type of interaction, one organism is getting benefits from the other without providing any benefit nor causing harm to other. For example, the strain *Escherichia coli* resides in human colon utilizes oxygen and hence creates an anaerobic environment where obligate anaerobes can grow (Maier et al., 2014).

Since microorganisms engage themselves in varieties of interactions, they may be physically associated with others via the phenomenon of ectosymbiosis and endosymbiosis. In ectosymbiosis, one organism lives at the surface of others whereas, in endosymbiosis, one organism or species is found inside the other organism (Martin and Schwab, 2012).

1.3.2. Negative interaction:

Negative interactions are further grouped into three categories:

1.3.2.1 Competition

It is a type of interaction between the organism or species for the common resources present in the limited quantity and hence cause negative impact on both the individuals (Lang and Benbow, 2013).

1.3.2.2 Ammensalism

In Ammensalism, one organism is getting harmed whereas other is neither benefitted nor harmed. For example, the bread mould *Penicillium* secretes the antibiotic Penicillin which cleaves the beta-lactamase of bacteria thereby inhibit the bacterial growth (Kong et al., 2010).

1.3.2.3 Antagonism

Here, one organism is benefited at the expense of other. This interaction is further classified into parasitism where one organism depends on other organism and predation where the predatory organism causes engulfment followed by lysis of their prey (Long and Azam, 2001).

1.3.3. Bacterial fungal interactions

Bacterial-fungal interactions are widespread and co-exist for more than a billion year. These bacterial fungal interactions (BFI) are complex and influences the biology of each other at various levels i.e. they may affect each other's growth pattern, reproduction, nutrition requirement, transport/movement, stress-resistance and pathogenicity (Deveau et al., 2018; (Fig 1.1)). Their role is crucial in many ecosystems and has many applications in improving the soil fertilization, nutrient cycle, bioremediation, plant development and food production.

Fig. 1.1 Diagram representing the role of bacterial fungal interactions. Role of bacterial–fungal interaction in various ecological and biotechnological phenomenon (Deveau et al., 2018).

Bacterial-fungal interactions vary from mutualism to antagonism. For example, *Candidatus* 'Glomeribacter gigasporarum' is an endobacterium of fungus *Gigaspora margarita* BEG34. It forms a homogenous population of 10^7 individuals per fungal spore and promotes the growth of their fungal host (Bianciotto and Bonfante, 2002).

In some cases, bacterial-fungus interaction helps the fungus to maintain its virulence against mammalian hosts; Type III secretory system and Quorum Sensing (QS) also play a potential role in these interactions. Many eukaryotes can sense bacterial QS molecules (e.g. farnesol, tyrosol, phenylethanol etc.) for example, QS molecule sesquiterpene farnesol was perceived by *Candida albicans* which inhibits filamentation, germination and biofilm formation by *C. albicans* (Rodrigues and Černáková, 2020; Shirtliff et al., 2009). Several fungal genera harbored bacterial endosymbionts which performs beneficial functions in them ranging from enhancing the fungal growth to their germination but very little is known about their molecular aspects. In many ecological niches, secondary metabolites play an essential role in co-habitation of various microorganisms (Pawlowska et al., 2018). For example, *Rhizopus microspores,* have an endosymbiont *Burkholderia rhizoxinica* that produces virulence factor rhizoxin which is known as the causative agent of rice seedling blight (Lackner et al., 2011). Several researchers have specified the evolvement of certain pathogenic bacteria, such as *L. pneumophila, Vibrio mimicus,* and *H. pylori*, to establish themselves in the vacuoles of free-living amoebae, the inhabitants of soil, air and water which act as a niche and protect them from environmental stresses (Abd et al., 2005; Jules and Buchrieser, 2007; Siavoshi and Saniee, 2014).

1.3.4. Concept of holobiont and hologenome

The term 'holobiont' was given by Lynn Margulis in 1991 and is referred to a biological entity comprises of a host and its single inherited symbiont (Margulis, 1990).

Later it was extended and defined as the entity which comprises of multiple domains of life i.e., host and its associated microbiota, as well as virus (Bordenstein and Theis, 2015). Holobiont plays a pivotal role in host-microbiota interaction. Their research is now imperative in unravelling the role in host biology, ecology and evolution (Roughgarden et al., 2018). With the development of sequencing methods, hologenome came into the picture.

The term ‘hologenome’ was coined by Zilber-Rosenberg in 2007 and referred to the genome sequencing of host and its associated microbiota i.e., collective genome of holobiont (Roughgarden et al., 2018). Based on the evidences, ten principles of holobiont and hologenome were presented by the Bordenstein and Theis in 2015. These principles considered holobiont and hologenome as a unit of biological organisation which reboots the concept of Lamarckian evolution (Bordenstein and Theis, 2015). Microbes of holobiont are the dormant target of evolution and genetic variation in microbial genome of host append much more rapidly than in host genomes. If microbiome is considered as the major component of the DNA of holobiont, then variation in them can result in new adaptation and speciation.

Since several microbes of holobiont are unculturable, it is difficult to study their role in host biology. This problem is overcome with the hologenomic approach which help in understanding the role of microbiota on their host biology (Simon et al., 2019). Microbiota has a great impact on host health as their interactions within the host played an important role in maintaining the host-microbial homeostasis. The importance of host microbiota equilibrium in human was also addressed by the Van de Guchte and colleague (Van de Guchte et al., 2018) where the changes in microbiota leads to shift from healthy to pre-disease and disease state. They blamed western lifestyle for this shift to pre-disease state followed by shifting to diseased like obesity and inflammatory disease in western societies. Hence, holobiont research deals with the prevention and therapy of diseases based on treatment of restoring altered microbiota.

1.4. Metagenomics and data analysis

The diversity of microorganism is high on earth and ubiquitous. Even one microliter of seawater contains millions of bacteria, archaea and viruses (Fuhrman, 2009). Microorganisms are the key player and perform several tasks from causing diseases to fixing nitrogen in the environment. It means that microbes maintain the life balance on this planet, although they can’t be seen with naked eyes but plays an important part in everyone’s life (Anton, 2017). It is impossible to isolate, identify and classify all the microbes present on the earth and only 1% of all microbes are cultured so far because of their complexity and symbiotic nature (Vartoukian et al., 2010). The understanding of the microorganism mostly depends on the ‘laboratory knowledge’ attained by culturing the organisms on the artificial media. Thus, to study the microbes in their natural habitat, interaction with their partners is possible with the help of the science of metagenomics (Council, 2007). Metagenomics circumvents the genomic diversity and uncultivability of the most microbes. It is used to analyse the DNA from the environmental samples to gain knowledge at the gene level of the communities (Handelsman, 2004). It involves culture-independent studies of communities and members (Daniel, 2005; Handelsman, 2005). At present, different platforms are available for next-generation sequencing like Roche (454 pyrosequencing), Illumina, Ion Torrent and SOLiD are used for metagenomic samples.

1.4.1. Platforms used for metagenomic sequencing

454 pyrosequencing involves use of binding primers to template followed by sequential addition of nucleoside triphosphate with the help of DNA polymerase (Fakruddin et al., 2013; Kröber et al., 2009). Incorporation of nucleoside triphosphate to the next position after the primer will result in the release of diphosphate (pyrophosphate prPPi**).** This PPi is coupled by an enzymatic reaction involving an ATP sulfurylase and a luciferase to the production of a light signal that is detected through a charge-coupled device. In Ion torrent sequencing, protons are released during incorporation of nucleoside which is detected through semiconductor technology. In both the cases i.e., 454 pyrosequencing is an ion torrent sequencing where light or charge production is related to the incorporation of nucleoside.

Illumina sequencing includes the incorporation of fluorescently labelled nucleoside triphosphate to a template (Lazarevic et al., 2009; Qin et al., 2010). The chemical modification of 3' position of nucleoside triphosphate doesn't allow further chain extension therefore, primer get extended by only one position which was detected by different fluorescent colours of each of the four nucleosides. The fluorescent label is later removed through chemical reactions and 3' position is converted to hydroxyl group making them ready for the next round of nucleoside addition.

SOLiD sequencing is different from above platforms as it involves ligation of nucleotide to the template thereby extending the primers with fluorescently labelled oligonucleotide (Singh et al., 2013). The sequences obtained are then quality checked and assembled either by overlap-layout consensus (OLC) or by De Novo Metagenomics Assembly for Shotgun DNA Reads. As the sequences get assembled, they are then annotated using various online software's such as MG-RAST, PATRIC and INM/M. Data can be annotated using an offline tool such as MOTHUR. These online and offline softwares offer quality control, comparing the datasets by importing and exporting data tools such as QIIME. The taxonomic information and the number of different bacterial species are predicted using these softwares. Bacterial diversity of different samples are compared using different bioinformatics tools.

1.4.2. Sequencing Techniques

1.4.2.1. Amplicon based metagenomics

Amplicon based metagenomic refers to the sequencing of the various ribosomal RNA coding DNA (rDNA) conserved region of the genetic material (Uyaguari-Diaz et al., 2016). It is a targeted amplicon analysis of various conserved markers. 16S, 18S and ITS are the frequently used regions for the amplicon based sequencing. These variable regions are the excellent tools for the metataxonomic amplicon profiling. 16S rRNA gene sequencing is extensively used in phylogenetic and taxonomic studies of bacteria and archaea. This methods generally uses primers specific to the gene of interest to get genetic information of the amplified sequences.

1.4.2.2. Shotgun metagenomics

Shotgun metagenome sequencing involves random shearing of genetic material into the smaller fragments followed by amplification and sequencing of fragments using universal primers. The sequenced small fragments are then assembled to form the longer fragment. Shotgun metagenome not only aids in taxonomic analysis but also help in-depth study on genes and functions of the microbial communities present in the sample (Quince et al., 2017).

Three aspects of bacterial diversity are there, (a) alpha diversity where the diversity is analysed in the single system and this alpha diversity is measured using tools like Chao 1 index , (b) beta diversity is the comparative bacterial diversity in two or more system and is measured with the tools like bray curtis index where they are represented as PCoA plots, (c) gamma diversity is the measure of total species diversity across the multiple systems.

1.5. Origin of proposal

While cataloguing the yeast diversity and observing the morphology of yeast cells in microscope, we noticed some bacteria like moving bodies inside them. These moving bodies triggered our interest to know more about them and the relationship they possess with yeast. While reviewing insight into yeast interaction, we came across that the yeasts engage in a wide variety of interactions (Starmer and Lachance 2011) such as predation (Lachance and Pang, 1997) and processes like autophagy (Reggiori and Klionsky, 2013). Although there are numerous instances where yeasts and bacteria occur in the same environment (Belda et al., 2017), their interactions is still under research. Fungal microbiomes are the booming area of research, showing how distinct each microbiome is to the fungal host (Bonfante and Desirò, 2017; Deveau et al., 2018; Pawlowska et al., 2018). Till date, well-studied cases of fungal endobacteria have been mostly restricted to few fungal hosts like the AMF and other plant-associated filamentous fungi.

Although some researches related to bacterial endosymbionts of yeasts is still underway such cases are sparse in number and can be counted on the fingers. One of the earliest reports among these were of possible symbiotic relationship between *Candida tropicalis* and *Microbacterium* sp. (Kang et al., 2009). Other reports have demonstrated *Helicobacter* cells inside yeast vacuoles by immunofluorescence, wherein vacuoles may be considered as a specialized niche for such bacteria (Saniee et al., 2013). Localization of *Staphylococcus* in yeast vacuole has also been demonstrated (Tavakolian et al., 2019). Work on *Saccharomyces* and its endosymbiont *Wolbachia*, demonstrated the latter's infection in the yeast, allowing the scope of research regarding of host-endosymbiont dynamics (Uribe-Alvarez et al., 2019). Yeast-bacterial interactions, notably the concept of endobacteria was speculated as crucial to nectar research, where both these microorganisms play a vital role in the ecology and biochemistry of flower nectar (Álvarez-Pérez et al., 2019).

Similarly, the yeast *Rhodotorula mucilaginosa* is known to harbour endosymbiotic *Pseudomonas stutzeri* (Sen et al., 2019). This yeast-endobacteria system has been implicated in interactions with rice plant that hosts the yeast, wherein these microorganisms aid in nitrogen fixation (Paul et al., 2020). Most recently, bacteria like *Staphylococcus* were "excised" from yeast hosts (Heydari et al., 2020). Additionally, there has been growing interest to artificially generate yeast-endobacteria systems in order to understand the endosymbiosis theory (Mehta et al., 2018, 2019).

The present study is undertaken with the following objectives:

1.6. Objectives

1. To catalogue the yeast diversity from various sources.
2. To unravel the bacterial communities of the isolated yeast using culture dependent and culture independent approaches.
3. To unravel the holomicrobiome of *Candida tropicalis* and establish bacterial infection.

Material and methods

Materials and Methods

2.1. Glassware, chemicals and devices

2.1.1. Glassware, Plastic ware and cleaning

Glasswares used include test tubes, culturing flask, Petriplates, measuring cylinders, beakers, screw cap test tubes, boiling tubes, spreader, tips and pipettes (Merck, Eppendorf, Qualigens, Schott, Duran and Tarson made).

2.1.2. Chemicals

The chemicals used were obtained from Sigma-Aldrich, Himedia, Thermo Fisher Scientific and Merck.

2.1.3. Buffers and standard solutions

Deionised water was used for buffer solutions preparation. Standard operating procedure was used to prepare buffer solutions and pH was adjusted using 1N HCl or 1N NaOH solution at room temperature.

Phosphate buffer saline (PBS) buffer (composition $g.l^{-1}$): NaCl-8, KCl-0.2, Na_2HPO_4-1.44 and KH_2PO_4-0.24; pH was adjusted to 7.4 and sterilized by autoclaving.

2.1.4. Sterilization

Sterilization of culture media, glassware and microtips were performed by autoclaving at 121.1 °C for 15 min. Thermolabile solutions were filter sterilized through 0.22 µm membrane filter (Millipore). For reuse, glassware was decontaminated and soaked in diluted chromic acid solution (potassium dichromate and sulphuric acid) overnight. All the glassware was further cleaned with detergent and tap water. Before using, glasswares were autoclaved at 121.1 °C at 15 psi for 15 min.

2.1.5. Culture media

a) Yeast Peptone Dextrose broth (composition g.l^{-1})

Peptone-20.0, Yeast extract-20.0, and Dextrose-20.0; pH was adjusted to 6.5 and sterilized by autoclaving.

b) Yeast malt broth (composition g.l^{-1})

Yeast extract-3.0, peptone-5.0, malt extract-3.0 and glucose-10.0; pH was adjusted to 6.2 and sterilized by autoclaving.

c) Nutrient broth (composition g.l^{-1})

Peptone-5.0, yeast extract-1.5, beef extract-1.5, NaCl-5; pH was adjusted to 6.5 and sterilized by autoclaving.

d) Yeast Nitrogen base (YNB)

Ready-to-use media from Himedia with catalogue number-M139 was used.

e) Yeast Carbon base (YCB) media

Ready-to-use media from Himedia with catalogue number-M141 was used.

f) V8-juice agar media

Ready-to-use media from Himedia with catalogue number-M638 was used.

g) Beibl and Pfennigs media (composition g.l^{-1})

$MgSO_4.7H_2O$ - 0.2, KH_2PO_4 - 0.5, NH_4Cl - 0.6, NaCl - 20.0, $CaCl_2.2H_2O$ - 0.05 and 5 ml of ferric citrate solution (0.1%, w/v) with 22 mM pyruvate; pH was adjusted to 6.5 and sterilized by autoclaving.

2.1.6. Ready-to-use kits used in this study

Genetix plasmid isolation kit and NucleoSpin Tissue Kit for genomic DNA isolation were used. Both the kits were used as per the manufacturer's instructions.

2.1.7. Primers

Primers used for amplification of internal transcribed region (ITS) region and D1/D2 domain of LSU of yeast and 16S rRNA gene of bacteria were procured from Xceleris labs. These sequences of primers are given below:

Table 2.1: Details of primers used for amplification of conserved regions of yeast and bacteria.

Primer name	**Sequences (5'→3')**
ITS1	GTCGTAACAAGGTTTCCGTAGGTG
ITS4	TCCTCCGCTTATTGATATGC
NL1	GCATATCAATAAGCGGAGGAAAAG
NL4	GGTCCGTGTTTCAAGACGG
F27	GTTTGATCCTGGCTCAG
R1525	GGTTACCTTGTTACGACT

2.2. Sample collection

Samples were collected from different environmental sources including soil, water (sewage water, marine water), soil sediments, lichen, algae, mushroom, fermented fruits and vegetables (grapes, pomegranate, lemon watermelon etc). All these samples belonging to different geographical area such as Hyderabad, Gujarat, Western Ghats and Guwahati were collected in the sterilised falcon tubes and zip-lock covers. Their GPS locations and pH were noted and samples were kept for enrichment within 10 days of collection.

2.2.1. Enrichment and isolation of yeast strains

1 ml of water sample or 1 g of solid sample was inoculated in 100 ml of yeast peptone dextrose (YPD) broth containing 100 $\mu g.ml^{-1}$ of chloramphenicol to inhibit the growth of bacteria and incubated at room temperature for 3-5 days. Enriched samples were then spread onto YPD agar plate containing 100 $\mu g.ml^{-1}$ of chloramphenicol and incubated at room temperature. Colonies were purified by repetitive sub-culturing on YPD agar plates containing chloramphenicol.

2.2.2. Preservation of cultures

a) Glycerol stocks: Glycerol stocks of all yeast strains were made. Yeast were maintained in 50% of glycerol by adding 0.5 ml of yeast culture in 0.5 ml of autoclaved glycerol. They were first stored at 4 °C for overnight followed by storage at -80°C for long term preservation.

b) Agar slants: Yeast strains were also maintained in agar slants for short term preservation. In boiling tubes, 5 ml of YPD media containing 1.8% of agar was added. To the solidified media, cultures were streaked and incubated at room temperature. After 2 days of incubation, they were kept at 4 °C as stocks. These were sub-cultured once in every 3 months and checked for purity.

2.2.3. DNA isolation and molecular identification of yeast

Genomic DNA of all yeast strains was isolated using the method described by Hoffman, 1997. Briefly, 72 h grown yeast culture was centrifuged at 5000 rpm for 5 min. Pellet was washed in 0.5 ml of MilliQ water. Cells were lysed with Triton-X 100 and glass 425-600 mm glass beads (Sigma). DNA was extracted using phenol-chloroform-isoamyl alcohol and eluted with Tris EDTA buffer. The quality of DNA was checked on 1% agarose and was stored at -20 °C. For the identification of yeast, internal transcribed spacer (ITS) region was amplified with ITS1 and ITS4 primers and D1/D2 domain of 26S rRNA gene was amplified using NL1 and NL4 primers and PCR was executed under the following conditions: 95°C for 5 min followed by 33 cycles of 95 °C for 60 s; 53 °C for 90 s and 72°C for 90 s, then, last elongation step at 72 °C for 15 min was also incorporated. The sequencing of amplified products was outsourced to M/s. Agrigenome, India. For the outsourced sequences, BLAST analysis was carried out using NCBI database and identification of yeast was performed using YeastIP database and CBS database.

2.3. Characterization of yeast strains

2.3.1. Morphological observation

Colony morphologies (texture, colour, surface, elevation and margins) of all yeast strains were observed on YPD agar medium.

2.3.2. Microscopic observation

Cell morphologies like cell shape, cell size and cell division/reproduction were observed under confocal microscopy (Zeiss LSM 880) with total magnification of 630X.

2.3.3. Biochemical tests

2.3.3.1. Utilisation of carbon sources

Yeast Nitrogen base (YNB) medium was used in the presence of various carbon sources (0.5%). These include D-glucose, sucrose, lactose, starch, citrate, inulin, raffinose, melibiose, trehalose, maltose, D-ribose, rhamnose, cellobiose, salicin, xylose, D-arabinose, L-arabinose, citrate, gelatin, methanol, ethanol, ribitol, glycerol, myo-inositol, succinate, mannitol, 50% glucose and 10% NaCl with 5% glucose. The inoculum (primary culture) was starved where the yeast cells were grown in carbon free yeast nitrogen base medium for 24-48 h. This culture was taken as inoculum for carbon utilisation tests. The tests were conducted in boiling tubes with cotton plugs. Inoculum was added to each tube containing YNB medium with one test substrate except for control where no carbon source was added. Tubes were incubated at room temperature for 3-4 weeks and observed for the presence of turbidity.

2.3.3.2. Utilization of various nitrogen sources

Yeast Carbon Base (YCB) medium media was used to observe the utilization of various nitrogen source (glutamine, sodium nitrite, ammonium chloride, urea, nitrate, nitrite, glutamic acid, L-lysine, and imidazole and glutamate 0.07%, w/v). Culture was inoculated in each tube containing YCB medium with one nitrogen source except one where no nitrogen source was added to act as control. Increase in the optical density was considered as positive for utilisation of nitrogen source.

2.3.3.3. Hydrolysis of urea

Cells from fresh culture were inoculated in urea broth and incubated at room temperature. The tube was observed daily for up to 4 days. The presence of deep pink color was recorded as positive for urea hydrolysis.

2.3.3.4. Gelatin liquefaction

Yeast culture was inoculated in gelatin agar. The tubes were then incubated and examined daily for about 3 weeks for liquefaction.

2.3.3.5. Fermentation of carbohydrates

The fermentation of D-glucose, sucrose, lactose, D-galactose, maltose, α-α-trehalose and raffinose were tested. 2% (w/v) solution of sugars in the boiling tubes containing YNB medium with small inverted Durham tubes in it. Culture was then incubated and observed for the production of gas in the Durham tube.

2.3.3.6. Phylogenetic analysis

Phylogenetic study was performed using MEGA7 software. MUSCLE algorithm was used for the sequence alignment and Kimura's-2-parameter model was used for the distance calculation using pairwise deletion procedure (Kimura, 1980). The phylogenetic relationship with other existing yeast taxa based on ITS region, D1/D2 domain of LSU 26S rRNA gene and Elongation factor (EF) alpha regions were deduced using phylogenetic tree by neighbor-joining method in MEGA7 software with 1000 bootstrap value (Tamura et al., 2013).

2.4. Staining and microscopy

Cells were observed under phase contrast (Leica DFC295) microscope where moving bacteria like bodies (BLBs) in the yeast cells were seen. These BLBs were confirmed using staining and fluorescence in situ hybridisation technique.

2.4.1. ViaGram Kit Staining

To stain and observe the BLBs inside the yeast cells, ViaGram (Invitrogen, V7023) kit containing DAPI, SYTOX and Texas Red conjugated with wheat germ agglutinin (WGA) was used. Yeast cells were washed thrice with BSA-0.9% NaCl buffer and then incubated first with Texas red for 15 min. Excess stain was removed by washing the incubated cells with BSA-0.9% NaCl buffer. To the washed cells, 2 µl mixture of DAPI and SYTOX prepared according to the kit instructions (Invitrogen) was added and incubated for 15 min in the dark. Cells were then observed under confocal microscope (Zeiss LSM880) which showed fluorescent red stained BLBs under the excitation/emission maxima set to ~595/615 nm.

2.4.2. Fluorescence in situ Hybridisation (FISH)

Fluorescence in situ Hybridisation (FISH) is a powerful tool to enumerate and specify the bacteria in the environmental sample or mixed cultures (Parsley et al., 2010). This protocol uses the oligonucleotide probe targeting the 16S rRNA gene of bacteria. Briefly, 2 ml of overnight grown yeast cells were centrifuged at 13000 rpm for 5 min. Supernatant was discarded and cells were fixed in 1 ml of fixative solution (4% formaldehyde in PBS buffer). Fixed cells were incubated for 3 h at room temperature and then centrifuged at 13000 rpm for 5 min. The pellet obtained was resuspended in 50% ethanol and incubated for 5 min at room temperature.

Resuspended pellet was centrifuged at 13000 rpm for 5 minutes. Pellet was again washed with 80%, 95% ethanol and air dried the cell by placing it in speed vacuum for 10 min. 500 µl of hybridisation buffer (Solution of 20 mM Tris HCl, 0.9 M NaCl, 0.01 % SDS in 40 % formamide) was added to dried cells and incubated at 37 °C for 30 min thereby firming pre-hybridisation mixture. 10 µl of bacterial specific probe EUB338 (10 pmol.μl^{-1} of working stock with sulfoindocyanine dye Cy3) was added to the 50 µl of pre-hybridisation mixture and incubated for 24 h at 50 °C. After 24 h, cells were centrifuged at 13000 rpm for 5 min and washed thrice in 0.1X SSC buffer. The final pellet was dissolved in 20 µl of 0.1X SSC buffer. Slides were prepared and observed under 63X objective of confocal microscope.

2.4.3. Transmission electron microscopy (TEM)

TEM is used to observe the minor details or organelles in cell and tissues. In the protocol, chemical fixation of yeast was performed as illustrated by Wright, 2000 with few modifications (Wright, 2000). Briefly, 48 h grown culture of *Candida tropicalis* was harvested and fixed in 2.4% of glutaraldehyde and sectioning was outsourced to RUSKA lab, Rajendra Nagar, Telangana, India. Grids obtained from Ruska Lab were then observed under transmission electron microscope.

2.5. DNA extraction and pyrosequencing

DNA was extracted by the method described by Hoffman, 1997 (Hoffman, 1997) from equivalent biomass of twenty-five yeast strains grown in YPD broth harvested at optical density of 0.8. Briefly, cells were lysed with Triton-X 100 and glass beads. DNA was extracted using phenol-chloroform-isoamyl alcohol and eluted with Tris EDTA buffer. The extracted DNA was quantified using NanoDrop spectrophotometer. The extracted DNA was stored at -20 °C. As a control, samples without yeast biomass were also processed for DNA extraction. Further, amplification and sequencing of V1-V3 region of 16S rRNA gene of DNA extracted from all the yeast were outsourced to RTL genomics, USA and Eurofins Scientific, India. The amplification was performed using universal primers 16S rRNA F (5’-GCCTACGGGNGGCWGCAG-3') and 16S rRNA R (5’-ACTACHVGGGTATCTAATCC-3'). These primers were used to partially amplify the V1-V3 region of the 16S rRNA gene. The sequencing was performed on Illumina MiSeq platform. All the sequences obtained were submitted at SRA database of NCBI. In NCBI-SRA database portal, a bioproject was created. After the approval of bioproject, biosample details which include the sampling site coordinates, type of sample and date of sample were filled followed by the submission of raw reads in SRA database.

2.5.1. 16S rRNA gene metagenome analysis of V1-V3 region

The sequences obtained from RTL genomics, USA and Eurofins Scientific, India were analysed and taxonomy was assigned by using the free offline software mothur (Schloss et al., 2009). In mothur, minimum length of sequences was restricted to 350 bp to 550 bp in order to correspond to the size of V1- V3 region and the screened sequences were aligned by SILVA 16S rRNA database (Pruesse et al., 2007).

The commands used in mothur were as follows:

1. make.contigs(fastq=JY101_R$_1$.fastq, rfastq=JY101_R$_2$.fastq)
2. summary.seqs(fasta= JY101_R$_1$.brim.contigs.fasta)
3. screen.seqs(fasta=current, minimum length=350, maxhomop=8, maxambig=0, maxlength=550)
4. unique.seqs(fasta= JY101_R$_1$.trim.contigs.good.fasta)
5. count.seqs(name= JY101_R$_1$.trim.contigs.good.name)

6. summary.seqs(count= JY101_R$_1$.trim.contigs.good.count_table)
7. align.seqs(fasta=JY101_R$_1$.trim.contigs.good.unique.fasta,reference=silva.bacteria.fasta)
8. screen.seqs(fasta=current,minlength=350,maxlength=550,maxhomop=8,maxambig=0)
9. summary.seqs(fasta= current)
10. filter.seqs(fasta=JY101_R$_1$.trim.contigs.good.unique.good.align,verticle=T,trump=.)
11. unique.seqs(fasta=JY101_R$_1$.trim.contigs.good.unique.good.filter.fasta,count=JY101_R$_1$.trim.contigs.good.count_table)
12. pre.cluster(fasta=JY101_R$_1$.trim.contigs.good.unique.good.filter.unique.fasta,count=JY101_R$_1$.trim.contigs.good.unique.good.filter.count_table)
13. chimera.vsearch(fasta=JY101_R$_1$.trim.contigs.good.unique.good.filter.unique.precluster.fasta,count=JY101_R$_1$.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.acenos)
14. remove.seqs(fasta=JY101_R$_1$.trim.contigs.good.unique.good.filter.unique.precluster.fasta,accnos=JY101_R$_1$.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsearch.accnos)
15. classify.seqs(fasta=JY101_R$_1$.trim.contigs.good.unique.good.filter.unique.precluster.pick.fasta,reference=trainset9_032012.pds.fasta,taxonomy=trainset9_032012.pds.tax,cutoff=80)
16. remove.lineage(fasta=JY101_R$_1$.trim.contigs.good.unique.good.filter.unique.precluster.pick.fasta,taxonomy=JY101_R$_1$.trim.contigs.good.unique.good.filter.unique.precluster.pick.pds.wang.taxonomy,taxon=chloroplast_mitochondria_unknown_Archea_Eukaryota)

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17. dist.seqs(fasta=JY101_R1.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.fasta,cutoff=0.03)
18. unique.seqs(fasta=JY101_R1.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.fasta)
19. cluster(coloum=JY101_R1.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.dist,name=JY101_R1.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.name)
20. count.seqs(name=JY101_R1.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.names)
21. classify.otu(list=JY101_R1.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.opt_mcc.list,count=JY101_R1.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.count_table,taxonomy=JY101_R1.trim.contigs.good.unique.good.filter.unique.precluster.pick.pds.wang.pick.taxonomy,label=0.03)
```

After the completion of mothur analysis, aligned sequences obtained were clustered into operational taxonomic units (OTUs). Diversity and comparison of the data along with graphical representation was done using Microsoft Excel and web-based tools. Microbiome Analyst online tool (Dhariwal et al., 2017) was used to filter the data for low count, low variance and normalization by total sum-scaling for alpha and beta diversity measurements. Data was also rarefied as recommended to minimum library size. Chao-1 index was used to measure alpha diversity and species richness with statistical method set to T-test/ANOVA for significance testing. Bray-Curtis index calculated for beta diversity assessment among the samples was visualised as Principal Coordinates Analysis (PCoA) plots. Permutational multivariate analysis of variance (PERMANOVA) was used to test the significance of the index.

The common and unique features (phyla and genera) were evaluated visually in the form of Venn diagrams constructed using InteractiVenn web-based tool (Heberle et al., 2015). Heatmaps were created using Heatmapper online tool (Babicki et al., 2016) with the normalized data. Row and column clustering using average linkage were applied by Euclidean measurement of the distances. PAST V3.26 software was used to construct the rarefaction curves (Hammer, 2001).

2.5.2. Sub-culturing studies of *Candida tropicalis* JY101

DNA of first, fifth and tenth sub-culture of *Candida tropicalis* were extracted by the method described by Hoffman, 1997 (Hoffman, 1997) at optical density of 0.8. Briefly, the same protocol was used as described in the above for extraction of DNA of twenty five yeast strains. Further, amplification and sequencing of V1-V3 region of 16S rRNA gene of DNA extracted from first, fifth and tenth sub-culture were outsourced to Eurofins Scientific, India. The amplification was performed using universal primers 16S rRNA F (5’-GCCTACGGGNGGCWGCAG-3') and 16S rRNA R (5’-ACTACHVGGGTATCTAATCC-3'). The sequencing was performed on Illumina MiSeq platform. The sequences obtained from Eurofins Scientific, India were analysed and taxonomy was assigned by using mothur (Schloss et al., 2009). In mothur, minimum length of sequences was restricted to 350 bp to 550 bp in order to correspond to the size of V1- V3 region and the screened sequences were aligned by SILVA 16S rRNA database (Pruesse et al., 2007).

2.6. Cultivation experiments

To cultivate endobacteria, strain JY101 was taken as the model system. Several approaches were used to isolate bacteria from strain JY101.

2.6.1. Sphaeroplast formation

Spheroplasts are the fragile structures which are very sensitive and can be broken easily. Sphaeroplast were prepared as described by Ovalle et al., 1998 (ovalle, 1998). Briefly, strain JY101 was grown in nutrient broth for 96 h. Cells were centrifuged at 13000 rpm for 10 min. Supernatant was discarded and pellet was dissolved in 1.5 ml MilliQ water and again centrifuged at 5000 rpm for 5 min. Net weight of pellet was determined and resuspended in 0.1M of Tris sulphate/10 mM DTT and incubated for 15 min at 30 °C with slow shaking. After incubation, cells were centrifuged and pellet was resuspended in 1 ml of 1.2 M sorbitol/20 mM KPO_4 per 0.15 g of cells. 3 mg of zymolase per gram of cells was added and incubated at 30°C with slow shaking for 30 min. Once the sphaeroplast were ready they were centrifuged at 3500 rpm for 5 min and resuspended in 1.2 M sorbitol. Sphaeroplast were broken by vortexing and centrifuged at 5000 × g for 1 min to remove the cell debris. Supernatant was collected and then again centrifuged at 20000 × g for 60 min to remove other organelles like mitochondria. Supernatant was then passed through 0.8 µm membrane filter which retained the yeast cells at the membrane surface and allowed the bacteria to pass through. The filtrate was passed through the 0.22 µm membrane filter which retained the bacteria within the membrane. Membrane was placed on nutrient agar and incubated at room temperature for few days.

2.6.2. Physical shearing

Strain JY101 was grown in nutrient broth containing 100 $\mu g.ml^{-1}$ of chloramphenicol at room temperature for 96 h. To this, sterile glass beads were added and vortexed vigorously to shear the yeast cells. The sheared yeast cells were centrifuged at 6000 × g for 5 min for settling down of yeast cell debris. Being lighter in weight, bacterial cells were presumed to remain in supernatant. Hence, the supernatant was serially diluted up to 10^{-5} dilution and plated onto nutrient agar plate containing cycloheximide (200 µg.ml^{-1}). All the steps were carried out under aseptic conditions to minimize contaminations. Plates were then incubated at 30 °C for one week.

2.7. Characterisation of *Chryseobacterium* sp. JC507

2.7.1. Morphological observation

Colonies morphology of *Chryseobacterium* sp. JC507 was observed on nutrient agar plate under optimum growth conditions.

2.7.2. Microscopic observation

Cell morphologies like cell shape, cell size and cell division/reproduction were observed under 63X objective lens of confocal microscope (Zeiss LSM 880)

2.7.3. Gram staining

Gram staining was performed using the Himedia Gram staining kit as per the manufacturer's protocol. Briefly, dried smear of bacteria was formed on glass slide. The smear was first stained with basic dye crystal violet and then treated with Gram's iodine solution forming an insoluble crystal violet-iodine complex. Gram decolourizer i.e., acetone and ethyl alcohol were added which is a differential step where the gram-positive bacteria retains the crystal violet-iodine complex while gram-negative cells get decolorize. Finally, the counterstain safranin was added which binds the gram-negative bacterial cells. The cells were then observed under 100X objective of light microscope.

2.7.4. Biochemical tests

2.7.4.1. Carbon source utilisation

Strain JC507 was tested for the utilisation of different carbon sources where the culture was grown in the Beibl and Pfennigs media containing different carbon sources (0.3%). These sources include glucose, fructose, sucrose, galactose, mannose, rhamnose, raffinose, ascorbate, fumarate, glycerol, succinate, sorbitol, glutamate, mannitol, lactose, cellobiose, ethanol, cysteine and malate. Cultures in different carbon sources were incubated at room temperature and their turbidity was measured at 600 nm.

2.7.4.2. Nitrogen source utilisation

Different nitrogen sources including sodium nitrite, urea, ammonium chloride, sodium nitrate and glutamine were used to check their utilisation by strain JC507. Culture was grown in Beibl and Pfennigs media containing above nitrogen sources and incubated at room temperature. Their turbidity was measured at 600 nm.

2.7.4.3. Requirement of sodium chloride for optimum growth

Strain JC507 was inoculated in nutrient broth with different concentrations of sodium chloride (0, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0%) and incubated at room temperature. Turbidity was measured using colorimeter at 600 nm.

2.7.4.4. Different temperatures for optimum growth

Culture was incubated in nutrient broth at different temperatures (8, 16, 20, 25, 30, 35, 40 and 50 °C) and turbidity was measured.

2.7.4.5. Growth at different pH

Growth of strain JC507 was observed at different pH ranging from 5.0 to 12.0 with interval of 0.5 unit. Media was prepared with $CHCOONa$–CH_3COOH buffer for pH 5.0, $NaHCO_3$–$NaOH$ buffer for pH 8.5– 11.0, K_2HPO_4–KH_2PO_4 buffer for pH 5.5–8.0, and Na_2CO_3–$NaOH$ buffer for pH 11.5–12.0. Culture was incubated at 30 °C and growth was monitored.

2.7.5. Chemotaxonomic characterisation

2.7.5.1. Analysis of pigments

Culture was grown aerobically for 2-3 days in nutrient broth. It was centrifuged at 7000 rpm for 10 min. Pigment was extracted from wet weight (0.5 g) into mixture of acetone and methanol (7:2) by vortexing at high speed for 5-10 min. Culture was centrifuged and supernatant was concentrated using flash rotary evaporator and dissolved in 1 ml of HPLC grade methanol. The pigment was analysed using HPLC where acetonitrile, methanol and tetrahydrofuran in the ratio of 58:35:7 respectively was used as solvent system with flow rate 1.5 $ml.min^{-1}$ using C18 100A column and compounds were detected by PDA detector at 450 nm with retention time of 20 min (Ramaprasad et al., 2013).

2.7.5.2. Cellular fatty acid composition

Cellular fatty acid was analysed using biomass of strain JC507. Culture was grown aerobically in nutrient broth at 30 °C until their optical density reached 70%. It was pelleted down and fatty acid methyl esters were separated and identified according to the instructions for Microbial Identification System which was outsourced to Royal life sciences Pvt Ltd (Sasser, 2001).

2.7.5.3. Polar lipid analysis

Polar lipid were extracted as described in Oren et al in 1996 (Oren et al., 1996). 0.5 g of lyophilised culture was dissolved in 2 ml of 0.3% of sodium chloride solution and incubated at room temperature for 15 min. To this 10 ml of methanol was added and mixed well. The mixture was boiled till the methanol evaporates; it was then allowed to cool at room temperature. 10 ml of chloroform was added and mixed properly by vortexing. 6 ml of 0.3% of sodium chloride was added and mixed well on vortex. The sample was centrifuged for 6 min which resulted in appearance of two layers (one was of chloroform and other was aqueous layer of water). The chloroform layer i.e., the lower layer was transferred to round bottom flask and concentrated using rotary flash evaporator. 0.5 ml mixture of chloroform and methanol in the ratio of 2:1 was added to dissolve the components. 5 µl was spotted at the corner of the 9 × 9 TLC silica gel plate (Merck KGaA, 64271 Dermstadt, Germany) and two dimensional chromatography was carried out. In the first dimension, a mixture of chloroform, methanol, glacial acetic acid and water was taken in the ratio of 30:12:1:1.5 whereas in the second dimension, a mixture of chloroform, methanol, glacial acetic acid and water was taken in the ratio of 35:7:6:1. Spots of lipids on TLC plate were observed by spraying 5% ethanolic molybdophosphoric acid on TLC followed by drying.

2.7.5.3.1. Ninhydrin reagent (amino group containing lipids)

Amino group was detected by spraying 0.1% of ethanolic ninhydrin followed by drying using hot air gun. The presence of pink color spots on TLC plate confirmed the amino group of lipid.

2.7.5.3.2. Molybdenum blue reagent (Phosphate containing lipids)

25N H_2SO_4 was used for making the Molybdenum blue reagent.

Reagent 1: 40.1 g of molybdenum trioxide was dissolved in 1 litre of 25N H_2SO_4.

Reagent 2. 1.78 g molybdenum was added to 500 ml of 25N H_2SO_4. The mixture was boiled for 20 min. After cooling, the mixture was kept in bottle.

Molybdenum blue reagent was prepared by adding 20 ml of reagent 1, 20 ml of reagent 2 and 40 ml of MilliQ water. This was sprayed onto the TLC plate and observed for the presence of blue color spots.

2.7.5.3.3. Dragendorff's reagent (Choline containing lipids)

Preparation of solution A and B

Solution A: 1.7 g bismuth nitrate was dissolved on 100 ml of water/acetic acid (4:1).

Solution B: 40 g of potassium iodide was dissolved in water.

Dragendorff's reagent was prepared by mixing 5 ml of solution A, 5 ml of solution B, 20 ml acetic acid and 70 ml of water. The mixture was spread onto the TLC plate and the presence of orange color confirmed the choline group containing lipids.

2.7.5.3.4. Identification of glycolipids

3.2% (w/v) of alpha-naphthol was prepared in the solution of methanol, sulphuric acid and water (23:3:1.5). This mixture was sprayed on TLC plate and the plate was heated at 115 °C. Presence of purple color spots confirmed the presence of glycolipids.

2.7.5.4. Quinone identification

Culture was grown in nutrient broth for 48 h and centrifuged. 1 g of pellet was dissolved in 2 ml of autoclaved distilled water. Pellet was dissolved in 10 ml of acetone and sonicated for 1 h. Further, 180 ml of acetone was added to the sonicated cells. To extract the quinone, sonicated cells were incubated overnight at 4°C. Cells were centrifuged at 1000 × g for 20 min. Supernatant was filtered using filter paper and evaporated using flash rotary evaporator. A solution of 90 ml of MilliQ water and 180 ml of n-hexane was added to the evaporated filtrate and mixed properly in shaking incubator. The solution was then kept still for 30 min which formed two layers. The water layer was then removed and remaining n-hexane layer was evaporated and quinone was concentrated under vacuum. 1 ml of ethanol was added to dissolve the concentrated quinone. The quinone components of strain JC507 was analysed by the method of Xie and Yokota (Xie and Yokota, 2003).

2.7.5.5. Identification of indoles

Culture was grown in the presence of L-tryptophan (1 mM) for 48 h. Cells were harvested by centrifuging at 10000 rpm for 10 min. The supernatant was acidified with 5N hydrochloric acid and extracted in ethyl acetate as described earlier (Mujahid et al., 2010). The extract was concentrated (dried) by evaporating using flash rotary evaporator. The dried extract was dissolved in 1 ml of methanol and filtered through a 0.22 μm membrane filter. HPLC analysis was performed for the indole estimation where 1% of acetic acid (solvent A) and acetonitrile (solvent B) was used as mobile phase with a 1.5 $\mathrm{ml.min^{-1}}$ flow rate. Metabolites were detected using photodiode at 280 and 500 nm and indoles were identified based on the absorption spectra of HPLC peaks with signatures between 270-285 nm.

2.8. Genomic DNA isolation

Genomic DNA of strain $\mathrm{JC507^T}$ was isolated using nucleopore gDNA fungal/bacterial kit. Briefly, culture was grown aerobically in nutrient broth at room temperature. It was centrifuged, 50 mg wet weight was resuspended in water and added to thrashing beads. To it, 750 μl of lysis buffer was added. Tube was vortexed at high speed for 5 min. The thrashing beads containing culture was centrifuged at 10000 × g for 1 min. 400 μl of supernatant was transferred to FB shredder column and centrifuged. 1.2 ml of binding buffer was added to the filtrate in the collection tube. It was transferred to FB spin column and centrifuged at 10000 × g for 1 min. Filtrate was discarded and pre-wash buffer added and centrifuged. Column was washed with 500 μl of wash buffer and centrifuged. 50 μl of elution buffer was added to the washed FB spin column kept in 1.5 ml of microcentrifuge tube and centrifuged at 10000 × g to elute pure DNA in the microcentrifuge tube.

2.8.1. Molecular analysis

2.8.1.1. PCR analysis

DNA extracted was used for amplification of 16S rRNA gene and whole genome sequencing. Universal 16S rRNA gene was amplified using forward primer F27 and reverse primer R1525. The PCR conditions followed for amplification were as follows: initial denaturation at 94 °C for 10 min followed by 32 cycles of denaturation at 94 °C for 1 min, annealing temperature of 54 °C for 30 s, elongation temperature of 72 °C for 90 s. The Final elongation temperature of 72 °C was applied for 10 min. Agarose gel electrophoresis was used to check the amplification where 1.0% of agarose gel was prepared and the amplified product along with DNA marker were run onto the gel. Sequencing of the amplified product was outsourced to Agrigenome. The genomic DNA which was extracted using nucleopore kit was sent for whole genome sequencing to Agrigenome where the sequencing was performed using Illumina HiSeq 2500 platform and the sequence was deposited in NCBI database.

2.8.1.2. Phylogenetic analysis

MEGA7 software was used for the phylogenetic analysis. The 16S rRNA gene sequence obtained from Agrigenome was deposited in NCBI. Phylogenetic tree was drawn based on the 16S rRNA gene where the sequences of 16S rRNA gene of closely related strains were extracted from NCBI database. Kimura 2 model was used to calculate the distances. Neighbour joining (NJ), Maximum likelihood (ML) and Minimum evolution (ME) methods in MEGA7 software were used to draw the phylogenetic trees. Bootstrap analysis was implemented to assess the confidence limits of branching.

2.8.1.3. Digital/ *in-silico* DNA-DNA hybridisation (*d*DDH)

In silico DNA-DNA hybridisation between the strain JC507 and its closely related strains were calculated using Genome-to-Genome Distance Calculator 2.1 (Auch et al., 2010).

2.8.1.4. Calculation of Ortho ANI score

OrthoANI score between strain JC507 and its closely related species were calculated from their genome sequence using USEARCH online service in EzBioCloud (Yoon et al., 2017).

2.9. Infection studies of *Candida tropicalis* JY101

2.9.1. Transformation studies

2.9.1.1. Fluorescence labelling of *Escherichia coli* DH5α

The Inoue method was used for preparation of competent cells of *E. coli* DH5α (Im, 2011). For transformation of GFP tagged plasmid (pBSU101) in competent *E. coli*, 5 µl of Plasmid was added to 50 µl of competent cells of *E. coli* DH5α and incubated the mixture on ice. After 30 min, heat shock of 42 °C for 90 s was given. Cell were immediately transferred to ice for 3 min. After 3 min, 800 µl of Luria broth was added and incubated for 1 h on shaker incubator. Later, cells were centrifuged and 700 µl of supernatant was discarded and pellet was dissolved in the remaining LB. The dissolved cells were then spread on LB plate containing antibiotic spectinomycin (65µg.ml^{-1}) and incubated at 37 °C overnight and observe for the presence of transformed colonies. Transformed cell were further confirmed by colony PCR and GFP tagged transformed cells were observed for the fluorescence under confocal microscope.

2.9.1.2. Fluorescence labelling of *Pseudomonas stutzeri* JC703

Transformation of *P. stutzeri* JC703 was done using heat shock protocol as described by Feng and co-workers, 2013 (Feng et al., 2013) with few modifications. Briefly, 100 µl of overnight grown *P. stutzeri* JC703 was inoculated in 10 ml of Luria broth (HIMEDIA) and incubated at 30 °C for 2-3 h until the OD reached 0.4-0.5. Culture was then kept on ice for 30 min followed by centrifuging 2 ml of culture at 7000 × g for 10 min. Pellet was washed thrice in water and dissolved in 1 ml of 0.1 M $CaCl_2.2H_2O$ (Sigma). Cells were incubated on ice for 1 h and centrifuged at 7000 × g for 10 min. Pellet was dissolved in 1 ml of solution containing 0.7 ml of $CaCl_2.2H_2O$ and 0.3 mL of 50% glycerol. Above steps were performed to make the cells competent to receive foreign DNA. The competent *Pseudomonas* cells were then used for transformation with the pBBRMCS plasmid. Competent *P. stutzeri* JC703 cells were incubated with the plasmid on ice for 30 min followed by heat shock at 42 °C for 6 min. Cells were immediately transferred to ice for 3 min and then incubated in 0.9 ml of Luria broth (HIMEDIA) at 30 °C for 2 h. Cells were centrifuged and 0.9 ml of supernatant was discarded and the remaining was spread onto LB agar plates containing gentamycin (25 $\mu g.ml^{-1}$). The plates were incubated at 30 °C for 48 h and screened for the presence of transformed colonies.

2.9.1.3. Selection and observations of auto-fluorescent bacteria

Total ten bacterial species were screened for the presence of auto-fluorescence. Of these, three were the member of genus *Rhodobacter*, two strains of *Rhodopseudomonas* and each species of genera *Rubrivivax* (*R. benzoatilyticus*), *Rhodomicrobium* (*R. udaipurense*) and *Prochlorococcus*. All these species were isolated in our laboratory. All the bacterial strains were grown in Biebl and Pfening's media except for *Prochlorococcus* species which was grown in sea water media.

Cultures with turbidity of 0.6 were then observed under confocal microscopy of the presence of auto-fluorescence.

2.9.2. Infection of yeast with fluorescent bacteria

The transformed *P. stutzeri* JC703, *P. hibiscicola*, *E. coli* DH5α and *S. typhimurium* as well as auto-fluorescent *Prochlorococcus* sp. and *Rhodopseudomonas palustris* were used for infecting *C. tropicalis* JY101. The fluorescently labelled *P. stutzeri* JC703, *E. coli* DH5α, *S. typhimurium* were grown in Luria broth containing gentamycin (25 µg.ml^{-1}) for *P. stutzeri* JC703 and spectinomycin (125 µg.ml^{-1}) for *E. coli* DH5α, *S. typhimurium*. 1 ml of each culture was centrifuged and pellets were co-cultured with *C. tropicalis* JY101 cells in YCB medium devoid of additional nitrogen sources at room temperature. The auto-fluorescent *Rhodopseudomonas palustris* TIE-1 and *Prochlorococcus* sp. cells were co-cultured with *C. tropicalis* JY101 cells in YCB medium devoid of nitrogen sources at room temperature. Co-cultures were observed periodically under confocal microscope (Zeiss LSM880) with 63X objective lens.

2.9.3. Scanning electron microscopy (SEM)

0.5 ml of co-culture *C. tropicalis* JY101 and *P. stutzeri* was centrifuged at 10000 rpm for 10 min at 25 °C. Supernatant was then discarded and pellet was washed in 0.9% autoclaved saline and centrifuged at 10000 rpm for 10 min. The resulted pellet then suspended in 2.4% glutaraldehyde solution and incubated overnight at 4 °C. Cells were washed in 0.9% saline thrice before dehydration. Dehydration was performed by washing the pellet sequentially with increased concentration of ethanol with 10% interval ranging from 10-100%. After ethanol washes, cells were dissolved in 100% ethanol. 5-10 µl of sample was placed on the small size coverslips which in turn placed on clean 13 mm size carbon tape on the

SEM stab (Kaláb et al., 2008). SEM stabs were then subjected for gold sputtering for 15 min and viewed under the Scanning electron microscope (Philips XL3O).

2.10. Shotgun metagenome sequencing of *Candida tropicalis* strain JY101

Genomic DNA of yeast *Candida tropicalis* strain JY101 was isolated using NucleoSpin Kit for genomic DNA isolation as per the manufacture's instruction. Sequencing of whole genome DNA was outsourced to nucleome, Hyderabad where DNA libraries were constructed through the process of end pairing, adding A to tail, purification, PCR amplification etc. Libraries were sequenced by Illumina high throughput sequencer with paired-end sequencing strategy. The sequencing generated 83589608 paired end reads. The raw reads were then submitted in NCBI database.

2.10.1. Metagenome Rapid Annotation using Subsystem Technology (MG-RAST)

The FastQ raw files of strain JY101 was uploaded and processed in MG-RAST server (Meyer et al., 2008). Analysis of metagenome of strain JY101 was performed in the same MG-RAST server by using the default pipeline. The default pipeline used contains several steps like the removal of all the low quality reads, dereplication of filtered sequences and artificially duplicated reads using Duplicate Read Inferred Error Estimation (DRISEE) program (Keegan et al., 2012). Adaptor trimming was performed using a bit masked k-difference matching algorithm. The pre-processing steps include denoising and normalisation where the sequences were filtered based on the length and number of ambiguous bases. Sequencing artefacts and host DNA contamination were removed and the retained sequences were passed onto the next stage of analysis pipeline. The sequences were clustered at 97% as this percentage of rRNA identity is considered to be the same species. Coding region with the sequences (gene calling) were predicted using FragGeneScan software which is an ab-initio protein gene calling algorithm (Rho et al., 2010). Predicted genes and the annotated sequences were retrieved from the MG-RAST.

2.10.1.1. Taxonomic analysis

The taxonomic assignment of the annotated sequences was performed using contigLCA algorithm which finds a single consensus taxonomic entity on each individual sequence. This is the automated analysis generated by the MG-RAST automated processing pipeline.

2.10.1.1.1. Rare fraction curve

Rare fraction curve was drawn to show the annotated species richness. The plot was drawn to represent the distinct species annotation as a function of the number of sequences sampled.

2.10.1.1.2. Rank Abundance plot

Taxonomic richness and evenness of different genera were represented in the form of bar graph. The bar graph shows the richness ranging from most abundance to the least abundance. Abundance of annotation was represented in log scale on y-axis.

2.10.1.1.3. Taxonomic distribution of archaea and bacteria

Taxonomy distribution of archaea and bacteria at the phylum and genus level were represented in the form of pie-charts. Briefly, all the reads belong to archaea and bacteria were retrieved from the MG-RAST server and pie-charts were constructed using Microsoft excel.

Results

Cataloguing yeast diversity from various habitats

3.1. Isolation, identification and preservation of yeast

A total of fifty-five yeast and one yeast like fungus were isolated from different sources with various geographical locations (Table 3.1). These were identified based on the internal transcribed spacer (ITS) region and D1/D2 domain of large ribosomal unit sequences. Eleven strains (JY101, JY106, JY107, JY108, JY111, JY113, JY114, JY125, JY134, JY149, JY157) had highest (>99%) identity with *Candida tropicalis*. While other species of the genus *Candida* include; *Candida glabrata* (JY118), *Candida metapsilosis* (JY103), *Candida intermedia* (JY110), *Candida suratensis* (JY120, JY121, JY123, JY124) and *Candida ampae* (JY135). Six strains (JY105, JY112, JY116, JY131, JY129, JY136) showed maximum identity (99%) with the genus *Pichia* include; *Pichia kudriavzevii* (JY105, JY112, JY116, JY129, JY131), *Pichia kluyveri* (JY136).

Nine strains belonged to the genus *Rhodotorula* include; *Rhodotorula mucilaginosa* (JY109, JY122, JY127, JY132, JY143, JY147 JY154, JY155, JY156), Eleven strains of genus *Meyerozyma* include; *Meyerozyma guilliermondii* (JY104, JY137, JY138, JY139, JY140, JY141, JY142, JY146, JY148, JY158), *Meyerozyma carribicca* (JY117) and a single strain each belonging to the genera *Hanseniaspora* (JY102), *Trichosporon* (JY115), *Cryptococcus* (JY126), *Debaryomyces* (JY130), *Sporidiobolus* (JY133), *Papiliotrema* (JY144), *Rhodosporiobolus* (JY145) and *Gjaerumia* (JY153). The yeast-like fungus with strain number JY119 was identified as *Zalaria obscura*. All the strains were preserved in 50% glycerol at -20 °C.

Table 3.1. Strain, Source, GPS coordinates, Similarity with the nearest yeast, highest identity and accession numbers of D1/D2 or ITS region of yeasts used in this study.

Strain	Source of isolation	GPS coordinates	Similarity with the nearest yeast	% Similarity with the nearest yeast	GenBank accession number of deposited yeasts
JY101	Soil	N 17° 28’ 28”	*Candida tropicalis* CBS 94^{T}	100	LT999794
JY102	Grapes	E 78° 19’ 56”	*Hanseniaspora guilliermondii* CBS 465^{T}	99	LT999810
JY103	Sewage water		*Candida metapsilosis* CBS 10907 ^{T}	100	LT719069*
JY104	Idly batter		*Meyerozyma guilliermondii* CBS 2030^{T}	99	LT719070*
JY105	Gooseberry		*Pichia kudriavzevii* CBS 5147^{T}	100	LT719071*
JY106	Date fruit		*Candida tropicalis* CBS 94^{T}	100	LT719072*
JY107	Lichen	10.1667° N 77.0667° E	*Candida tropicalis* CBS 94^{T}	100	LT719073*
JY108	Leaf litter	25.3443° N	*Candida tropicalis* CBS 94^{T}	100	LT719074*
JY109	Soil	91.5335° E	*Rhodotorula mucilaginosa* CBS 316^{T}	100	LT795045*
JY110	Muskmelon	N 17° 28’ 28”	*Candida intermedia* CBS 572^{T}	99	LT795046*
JY111	Muskmelon	E 78° 19’ 56"	*Candida tropicalis* CBS 94^{T}	100	LT795047*
JY112	Idly batter		*Pichia kudriavzevii* CBS 5147^{T}	99	LT795048*
JY113	Citrus fruit		*Candida tropicalis* CBS 94 ^{T}	100	LT795049*
JY114	Decomposed wood	10.1667° N 77.0667° E	*Candida tropicalis* CBS 94^{T}	100	LT795050*
JY115	Idly batter	N 17° 28’ 28”	*Trichosporon asahii* CBS2479^{T}	100	LT795051*
JY116	Sugarcane	E 78° 19’ 56”	*Pichia kudriavzevii* CBS 5147^{T}	99	LT795052*
JY117	Lemon		*Meyerozyma carribicca* CBS 9966^{T}	99	LT795053*
JY118	Pomegranate		*Candida glabrata* CBS 138^{T}	99	LT840077*
JY119	Pine tree bark	25.3443° N 91.5335° E	*Zalaria obscura* DAOMC 250849^{T}	100	LT840078
JY120	Epilithic green fringes	N 20°54’ 8.07”	*Candida suratensis* CBS 10928^{T}	99	LT962956
JY121	Brown Gracillaria	E 70°21’51.7”	*Candida suratensis* CBS 10928^{T}	99	LS992563

JY122	Sea water		*Rhodotorula mucilaginosa* CBS 316^{T}	100	LT799390*
JY123	Sargassum		*Candida suratensis* CBS 10928^{T}	99	LT962660*
JY124	Brown Gracillaria		*Candida suratensis* CBS 10928^{T}	97	LT840079
JY125	Biofilm	25.3443° N	*Candida tropicalis* ATCC 750^{T}	100	LT838873*
JY126	Soil	91.5335° E	*Cryptococcus terrens* CBS 1895^{T}	99	LT838874*
JY127	Algae		*Rhodotorula mucilaginosa* CBS 316^{T}	100	LT799394
JY129	Mushroom	10.1667° N	*Pichia kudriavzevii* NRRL Y-5396^{T}	99	LT962960
JY130	Mushroom	77.0667° E	*Debaryomyces prosopidis* JCM 9913^{T}	99	LT962955
JY131	Lichen		*Pichia kudriavzevii* NRRL Y-5396^{T}	99	LT962958
JY132	Mushroom		*Rhodotorula mucilaginosa* CBS 316^{T}	99	LT962959
JY133	Soil	N 19° 51’ 54” E 86° 06’ 40”	*Sporidiobolus pararoseus* CBS 491^{T}	99	LT962957
JY134	Soil	25.3443° N 91.5335° E	*Candida tropicalis* CBS 94^{T}	100	LT995997
JY135	Neolamarckia fruit	26.7697° N	*Candida ampae* CBS 7872^{T}	99	LT996821
JY136	Neolamarckia fruit	80.9262° E	*Pichia kluyveri* NRRL y-11519^{T}	99	LT996820
JY137	Chilika lake sediment	N 19° 51’ 54”	*Meyerozyma guilliermondii* CBS 2030^{T}	100	LT996828
JY138	Chilika lake sediment	E 86° 06’ 40”	*Meyerozyma guilliermondii* CBS 2030^{T}	100	LT996825
JY139	Chilika lake sediment		*Meyerozyma guilliermondii* CBS 2030^{T}	100	LT996824
JY140	Chilika lake sediment		*Meyerozyma guilliermondii* CBS 2030^{T}	100	LT996822
JY141	Chilika lake sediment		*Meyerozyma guilliermondii* CBS 2030^{T}	100	LT996826
JY142	Chilika lake sediment		*Meyerozyma guilliermondii* CBS 2030^{T}	100	LT996823
JY143	Chilika lake sediment		*Rhodotorula mucilaginosa* CBS 316^{T}	99	LT996827
JY144	Chilika lake sediment		*Papiliotrema laurentii* CBS 139^{T}	99	LT996829
JY145	Soil	N 24° 30’ 21”	*Rhodosporidiobolus nylandii* CBS 9093^{T}	100	LT996831
JY146	Soil	E 93° 47’ 43”	*Meyerozyma guilliermondii* CBS 2030^{T}	100	LT996830
JY147	Mushroom	N 17° 27’ 24”	*Rhodotorula mucilaginosa* CBS 316^{T}	99	LR215658
JY148	Mushroom	E 78° 18’ 55”	*Meyerozyma guilliermondii* CBS 2030^{T}	99	LR135994
JY149	Mushroom		*Candida tropicalis* ATCC 750^{T}	100	LR215661
JY150	Mushroom		*Suhomyces bolitotheri* CBS 9832^{T}	96	LR215662
JY152	Mushroom		*Suhomyces bolitotheri* CBS 9832^{T}	96	LR215663
JY153	Soil sediment	Antarctica	*Gjaerumia minor* strain CBS 543^{T}	99	LR215664

JY154	Sponge	N 9° 9’ 55” E 78° 38’ 33”	*Rhodotorula mucilaginosa* CBS 316^{T}	99	LR215666
JY155	Sponge		*Rhodotorula mucilaginosa* CBS 316^{T}	100	LR215665
JY156	Sponge		*Rhodotorula mucilaginosa* CBS 316^{T}	100	LR215668
JY157	Sponge		*Candida tropicalis* ATCC 750^{T}	100	LR215667
JY158	Soil	N 19° 51’ 54” E 86° 06’ 40”	*Meyerozyma guilliermondii* CBS 2030^{T}	99	LR215669

*Indicates the ITS region sequencing

Based on the preliminary studies, ITS region and D1/D2 domain of LSU sequence similarities, out of total yeast, strains JY118, JY124 and JY150 were identified as novel and further characterized. Strains JY118 and JY124 belong to genus *Candida* and strain JY150 belongs to genus *Suhomyces*.

Characterization of novel yeast taxa

3.2. Polyphasic characterization of novel yeast

3.2.1. Characterization of strain JY118^T and strain JY124^T

3.2.1.1. Habitat, colony and cell morphology

Strain JY118^T was isolated from a rotten pomogranate collected from the fruit market at University of Hyderabad, Hyderabad, India (GPS position N 17° 28' 28" E 78° 19' 56") and strain JY124^T was isolated from an algal (*Gracillaria*) sample collected from Viraval, Gujarat, India (GPS position N20° 54' 8.079" E 70° 21' 51.794"). Both the strains (strain JY118^T and strain JY124^T) are members of ascomycetes with white color colonies on yeast dextrose peptone agar media.

Both the strains exhibit different colony characteristics on the nutrient medium used for growth. Colonies of strain JY118^T were smooth, shiny, white, circular with an entire margin (Fig. 3.1a), whereas strain JY124^T formed colonies with smooth, bright, white with entire margin (Fig. 3.1b). Cells of strain JY118^T were spherical to ovoidal (2.5-3.5 µm ×3-5 µm) and that of JY124^T were spherical to ovoidal with 3-4 µm×4-6 µm in size, occurs singly or in budding pairs. They divide asexually via budding (Fig. 3.1c and d) and ascospore formation were not observed when grown on YPD, malt extract agar and V8 juice agar in pure culture as well as in different mating combinations at 25 °C for 3-4 weeks.

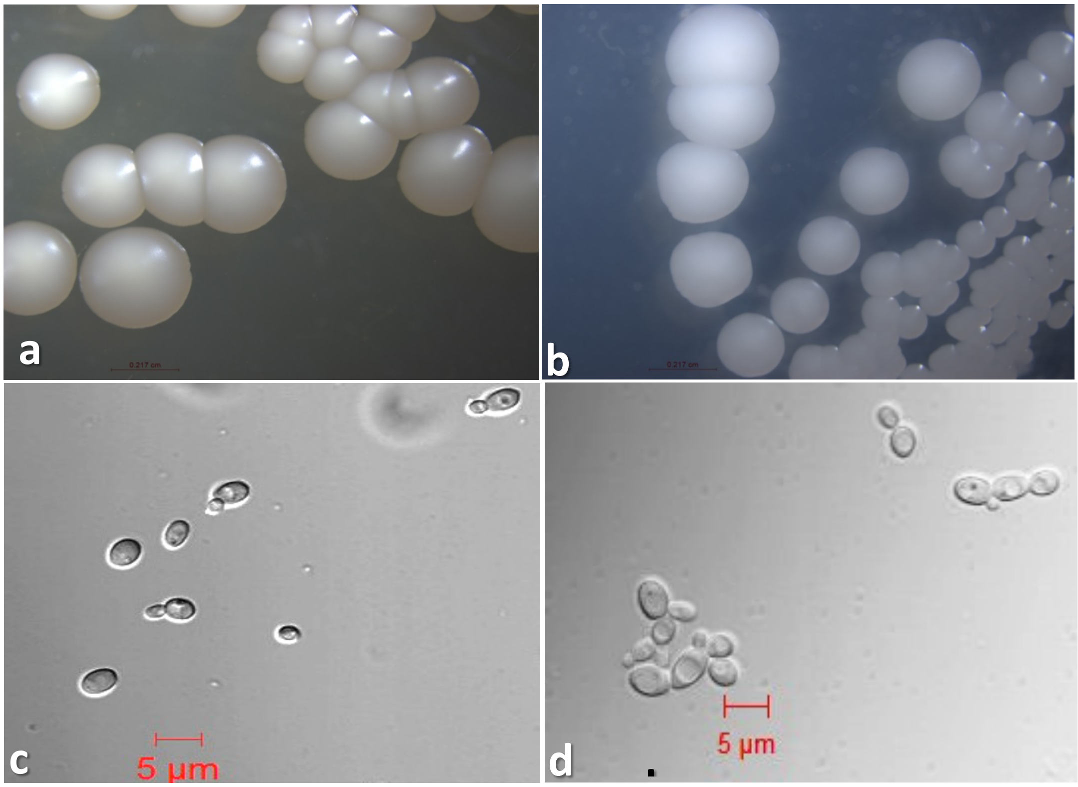


Fig. 3.1: Colony morphologies and budding of yeast strains JY118^T and JY124^T: (a) white color colony of strain JY118^T; (b) white color smooth colony of strain JY124^T when grow on nutrient agar plates. Budding in yeast strain JY118^T (c) and in strain JY124^T (d) when observed under 63X objective of microscope.

3.2.1.2. ITS region and D1/D2 domain of LSU sequence similarities and phylogenetic affiliation

NCBI BLAST analysis of ITS and D1/D2 region of both the isolates revealed that they belonged to the genus *Candida*. The NCBI BLAST of the ITS (836 nt) region of strain JY118^T exhibited 99% sequence similarity with ITS region of *Candida glabrata* CBS 138^T, 90% with *Candida bracarensis* CBS 10154^T and 86% with *Candida uthaithanina* CBS 10932^T, while D1/D2 region (631 nt) was 98% similar with *Candida glabrata* CBS 138^T, 95% with *Candida bracarensis* CBS 10154^T and 94% with *Candida uthaithanina* CBS 10932^T. Similar results were observed using the YeastIP database which is a gene database for molecular taxonomy and phylogeny of yeast (Weiss et al., 2013). ITS (383 nt) region of strain JY124^T showed 97% similarity with *Candida suratensis* CBS 10928^T, 89% with *Candida dosseyi* ATCC MYA-4359^T and *Candida blattae* ATCC MYA-4360^T. D1/D2 region (504 nt) of strain JY124^T showed 99% similarity with D1/D2 region of *Candida suratensis* CBS 10928^T, 85% with *Candida blattae* ATCC MYA-4360^T and 84% with *Candida dosseyi* ATCC MYA-4359^T.

Phylogenetic trees were constructed by the neighbor-joining (NJ), maximum likelihood (ML), minimum evolution (ME) methods using MEGA7 software with 1000 bootstrap value. The phylogenetic relationship of strains JY118^T and JY124^T with closely related species was represented as a combined tree of NJ, ML, ME (Fig. 3.2; Fig. 3.3). Strain JY118^T was found to clade with *Candida glabrata* CBS 138^T with notable evolutionary distance. Similarly, phylogenetic relationship for strain JY124^T showed cladding with *Candida suratensis* CBS 10928^T.

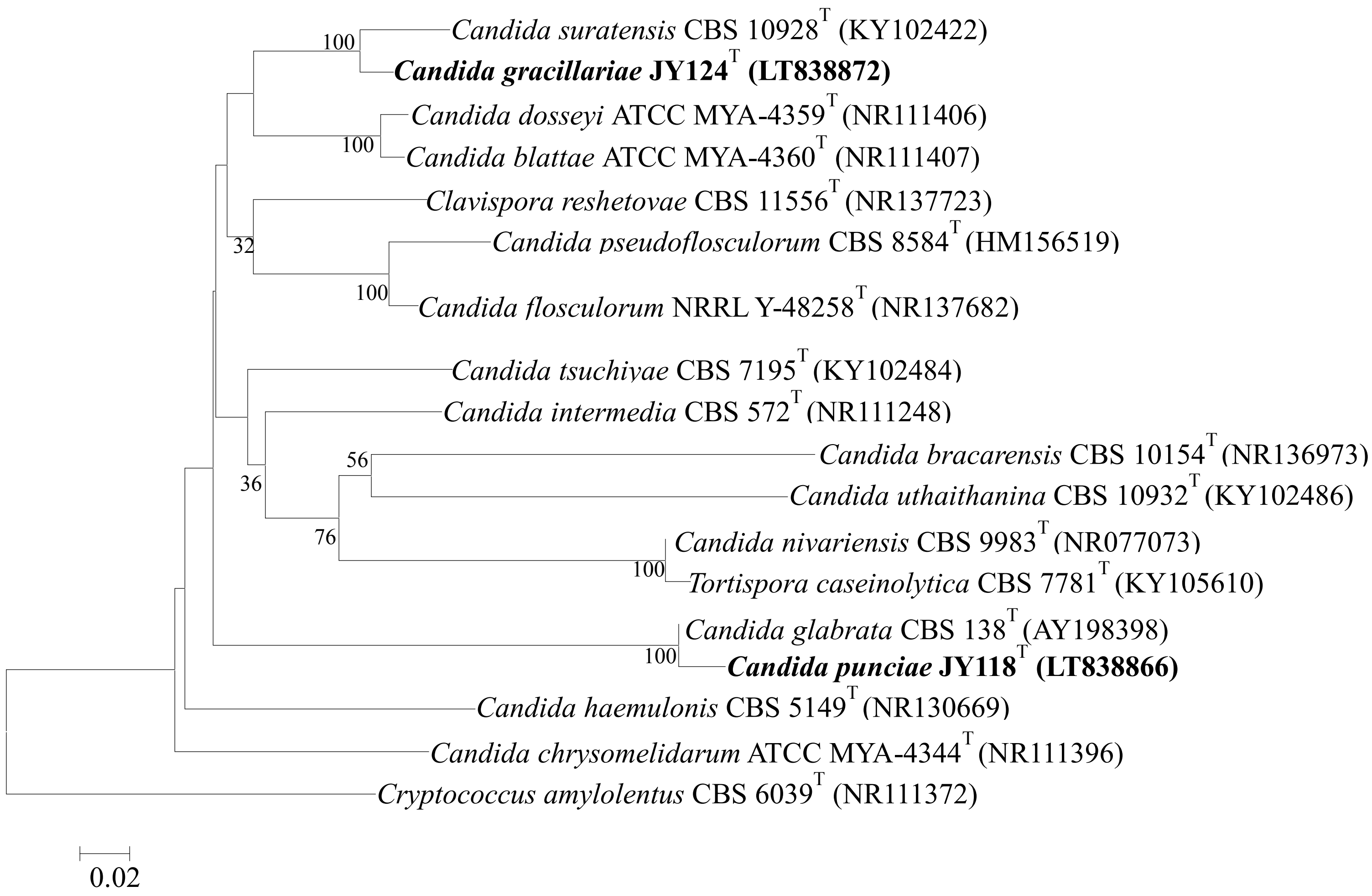


Fig. 3.2: Phylogenetic tree based on ITS region showing the relationship of strain JY118^{T} and JY124^{T} with the most closely related members of the genus *Candida*. The tree was constructed using neighbor-joining (NJ), using MEGA7 and rooted by using *Cryptococcus amylolentus*. Represented is the NJ tree with number at nodes represents the percentage bootstrap values of NJ (based on 1000 resampling). The EMBL accession number ITS are shown in parentheses.

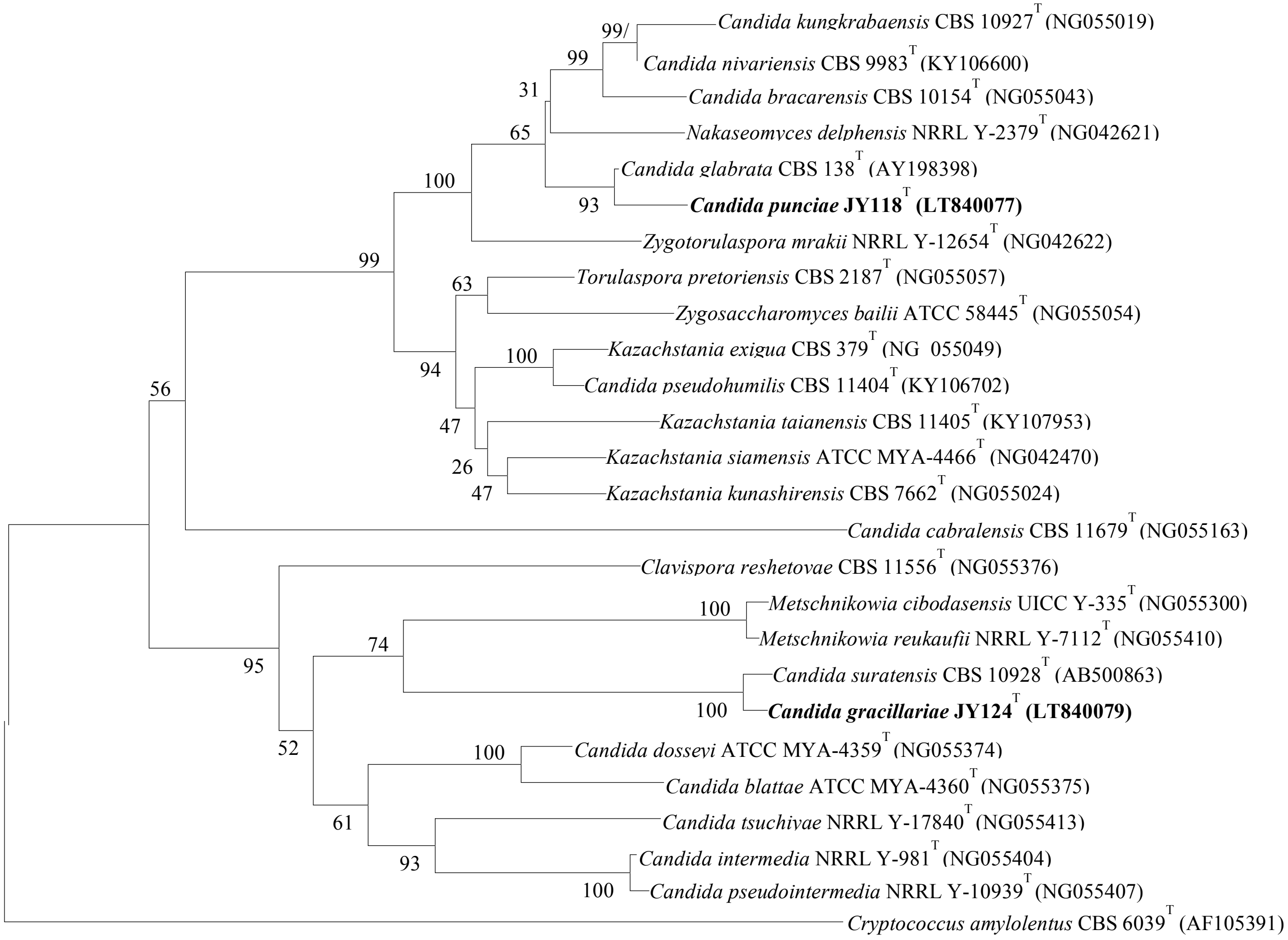


Fig. 3.3: Phylogenetic tree based on D1/D2 region showing the relationship of strain JY118^T and JY124^T with the most closely related members of the genus *Candida*. The tree was constructed using neighbor-joining (NJ), using MEGA7 and rooted by using *Cryptococcus amylolentus*. Represented is the NJ tree with number at nodes represents the percentage bootstrap values of NJ (based on 1000 resampling). The EMBL accession number for D1/D2 domain are shown in parentheses.

3.2.1.3. Physiological properties of strains JY118^T and JY124^T

Physiological properties of strains JY118^T and JY124^T were compared with the nearest phylogenetic species (Table 3.2). Strain JY118^T and *C. glabrata* CBS 138^T assimilated trehalose, glycerol, D-glucose whereas did not assimilate sucrose, raffinose, maltose, starch, rhamnose, cellobiose, salicin, methanol, ribitol, mannitol and succinate. Carbon sources which supported the growth of strain JY118^T were inulin, D-ribose, D-xylose, D-arabinose whereas, the nearest member *C. glabrata* CBS 138^T could not grow on them which differentiated strain JY118^T from *C. glabrata* CBS 138^T. Strain JY118^T and *C. glabrata* CBS 138^T showed fermentative growth on D-glucose whereas did not ferment sucrose, D-galactose, lactose maltose and raffinose. Strain JY118^T can grew in temperature ranging from 8 °C to 37 °C with optimum at 30 °C. Acid production was positive for strain JY118^T, whereas it could not grow in vitamin free media. GC-MS analysis showed the production of acetic acid, propionic acid, butanoic acid and citramalic acid as major fermentation products by strain JY118^T.

Strain JY124^T and *C. suratensis* CBS 10928^T assimilated sucrose, raffinose, salicin, D-xylose, ribitol, glycerol, mannitol, succinate, D-glucose and citrate. Inulin, trehalose, maltose, starch, rhamnose, methanol, ethanol and cellobiose supported the growth of strain JY124^T whereas *C. suratensis* CBS 10928^T did not grow on these carbon sources except cellobiose. Strain JY124^T and *C. suratensis* CBS 10928^T can ferment D-glucose, D-galactose, sucrose whereas do not ferment lactose. Only strain JY124^T shows fermentative growth on maltose, raffinose, D-xylose and cellobiose. Strain JY124^T grew in temperature ranging from 8 °C to 37 °C with 30 °C as optimum growth temperature. GC-MS analysis revealed the production of propionic acid, acetic acid, hexanoic acid and butanedioic acid as fermentative products by strain JY124^T.

Table 3.2. Differential characteristics of strains *Candida punciae* JY118^T and *Candida gracillariae* JY124^T with these respective phylogenetically nearest members.

Characteristics	**JY118^T**	**JY124^T**	***C. glabrata* CBS138^T**	***C. suratensis* CBS10928^T**
Cell size (µm)	2-3 ×3-5	3-4×4-6	1–4	2–4 × 2–6
Source of isolation	Pomegranate	*Gracillaria*	Human	Decaying plant
Carbon sources utilized for growth (0.5%)				
Sucrose	-	+	-	+
Inulin	+	+	-	-
Raffinose	-	+	-	+
Trehalose	+	+	+,-	-
Maltose	-	+	-	-
D-ribose	+	-	-	+
Starch	-	+	-	-
Rhamnose	-	+	-	-
Salicin	-	+	-	+
D-xylose	+	+	-	+
D-arabinose	+	-	-	+
Methanol	-	+	-	-
Ethanol	-	+	-,+	-
Ribitol	-	+	-	+
Glycerol	+	+	-,+	+
Mannitol	-	+	-	+
Succinate	-	+	-	+
50% glucose	+	+	-,+	+
Citrate	-	+	-	+
Nitrogen Sources				
Nitrate	-	+	-	-
Nitrite	-	-	-	-
L-Lysine	+	+	-	+

Fermentation				
D-galactose	-	+	-	+
Maltose	-	+	-	-
Sucrose	-	+	-	+
Lactose	-	-	-	-
Raffinose	-	+	-	-

*The data for *Candida glabrata* CBS 138^T and *Candida suratensis* CBS 10928^T were obtained from CBS database (www.westerdijkinstitute.nl). All the four strains showed assimilation of D-glucose whereas could not grow on lactose, melibiose, L-arabinose, myo-inositol as carbon source. All strains are susceptible to cycloheximide. Strain JY118^T, JY124^T, CBS 138^T and strain CBS 10928^T showed fermentative growth on D-glucose and trehalose.

3.2.2. Characterization of strain JY150^T

3.2.2.1. Habitat, colony and cell morphology

Strain JY150 and JY152 were identical strains isolated from the mushroom collected from the rock of University of Hyderabad (17° 27’ 24” E 78° 18’ 55”), Telangana. They are the member of ascomycetous fungus. Colonies appeared as yellow color on yeast dextrose peptone agar media (Fig. 3.4a). Cells were spherical to ovoid, 3-4 µm×4-6 µm in size, divides asexually by budding (Fig. 3.4b). No sexual state was observed. They were Mesophile (range from 25-35 ˚C).

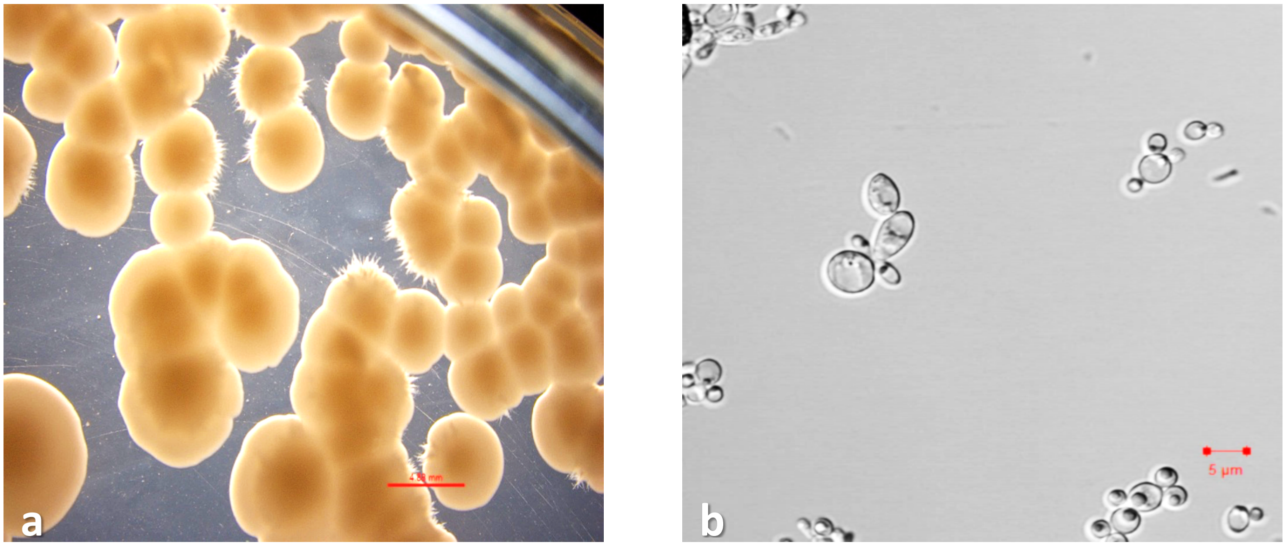


Fig. 3.4: Morphology of strain JY150^T: (a) Macroscopic Colonies of strain JY150^T on yeast peptone dextrose (YPD) agar; (b) Microscopic images showing the budding in strains JY150^T. Images are taken at 63X magnification with Zeiss LSM880 microscope.

3.2.2.2. ITS region and D1/D2 domain of LSU sequence similarities and phylogenetic affiliation of strain JY150^T

NCBI BLAST analysis of D1/D2 of LSU region of strains JY150 and JY152 showed 95.5% similarity with the type strain *Suhomyces bolitotheri* CBS 9832^T followed by 93.90% similarity with *Suhomyces terraborum* CBS 9826^T . JY150 also showed 99.3% similarity with the isolate ST431 isolated in Prof. Savitri Limtong's lab, Thailand. ITS regions of strain JY150 and JY152 were 100% identical and showed 92.8% similarity with *Suhomyces bolitotheri* BG-10-06-11-60A-5-1 followed by 89.3% similarity with the type strain *Suhomyces bolitotheri* CBS 9832^T. Phylogenetic tree was constructed by the neighbor-joining (NJ) method using MEGA7 software with 1000 bootstrap value. The phylogenetic relationship of three strains (JY150, JY152 and ST431) with phylogenetically related species showed distinct cladding from that of *Suhomyces bolitotheri* CBS 9832^T (Fig. 3.5 and Fig. 3.6) and were further characterized

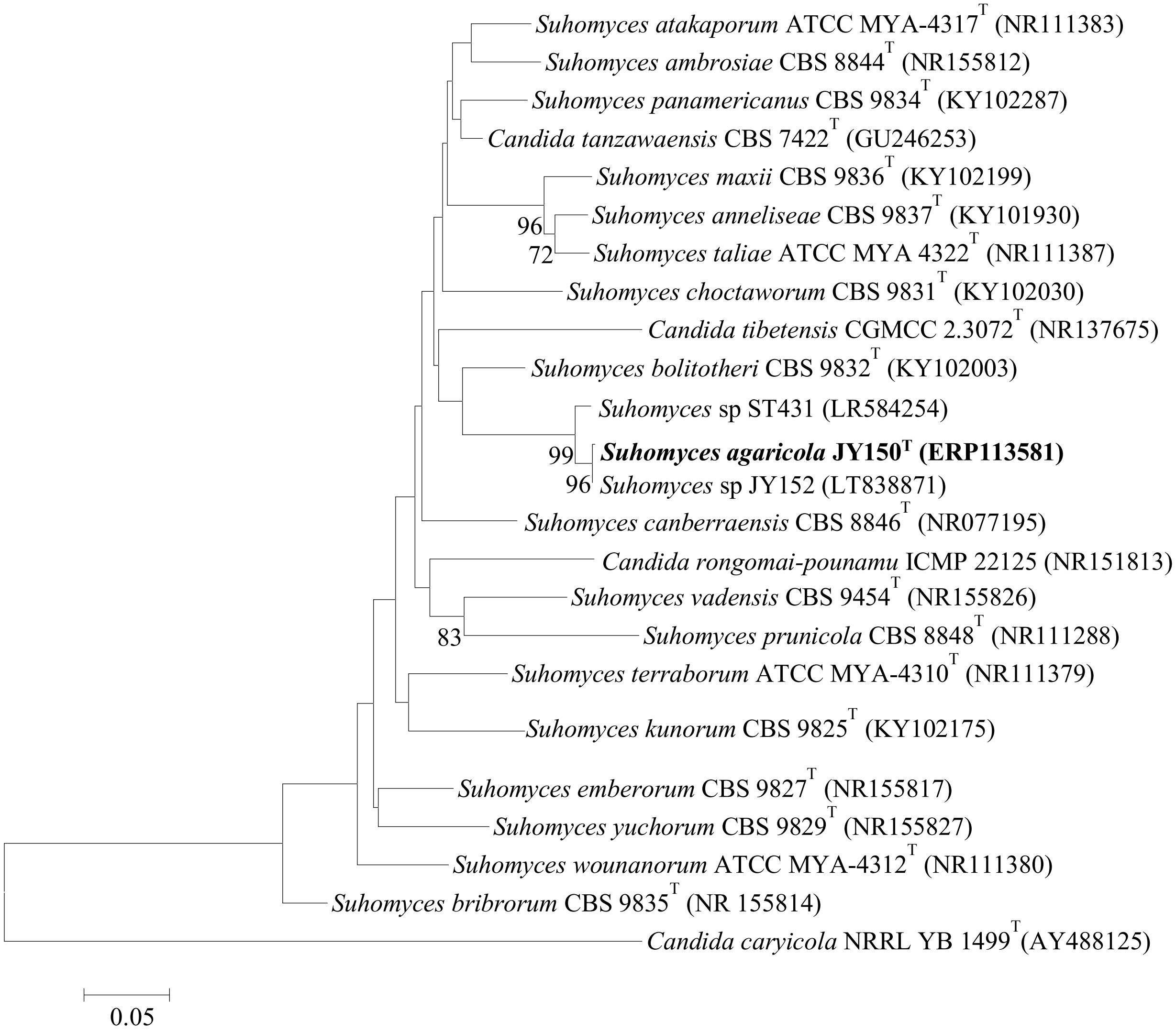


Fig. 3.5: Phylogenetic tree based on ITS region showing the relationship between strain JY150^T with the most closely related members of the genus *Suhomyces*. The tree was constructed using neighbor-joining (NJ), using MEGA7 and rooted by using *Candida caryicola*. Represented is the NJ tree with number at nodes represents the percentage bootstrap values of NJ (based on 1000 resampling). The EMBL accession number for ITS are shown in parentheses.

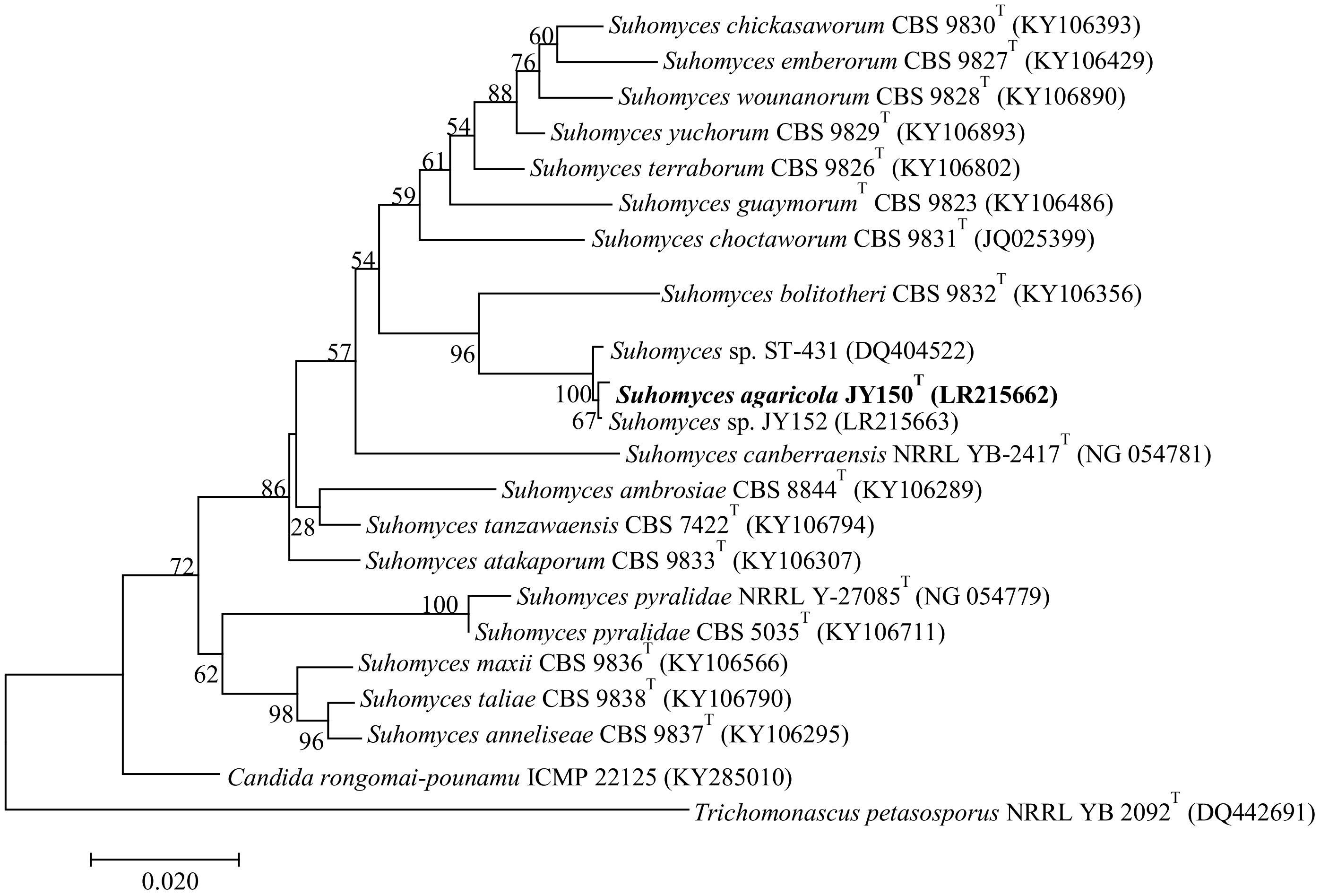


Fig. 3.6: Phylogenetic tree based on D1/D2 domain of large subunit of 26S rRNA gene showing the relationship between strain JY150^T with the most closely related members of the genus *Suhomyces*. The tree was constructed using neighbor-joining (NJ), using MEGA7 and rooted by using *Trichomonascus petasosporus*. Represented is the NJ tree with number at nodes represents the percentage bootstrap values of NJ (based on 1000 resampling). The EMBL accession number for D1/D2 domain are shown in parentheses.

3.2.2.3. Physiological properties of strains JY150^T

Physiological properties of strains JY150^T, JY152 nad ST431 were compared with the nearest phylogenetic species (Table 3.3). All three strains showed differences with their relative type strains in terms of utilization of L-lysine, D-ribose, D-arabinose, 60% glucose and growing in vitamin free media. Good growth occurred with D-glucose, D-galactose, D-glucosamine, D-ribose, D-xylose, D-arabinose, salicin, trehalose, glycerol, erythritol, ribitol, ethanol, mannitol and cadaverine. L-lysine was utilized for growth. Fermentation occurred with D-glucose. Did not ferment D-galactose, maltose, sucrose, lactose, and raffinose (Table 3.3).

Table 3.3. Differentiating characters of strain JY150^T and strain ST431 with phylogenetically nearest members

Characteristics	1	2	3	4	5	6	7	8
Assimilation of Carbon sources								
D-Glucose	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+	+
D-Sorbose	-	-	v	-	-	-	w/-	-
D-Glucosamine	+	+	+	w	d	d/v	+/w	d/w
D-Ribose	+	+	v	-	-	-	+/d	-
D-Xylose	+	+	+/d/w	w	+	+/d	+	+
L-Arabinose	-	-	-	-	-	-	+/d/w	--
D- Arabinose	+	w	-	-	w	-	-	-
Sucrose	+	-	-	+	+	+	+	+
Maltose	+	-	-	+	+	+	+	+
Salicin	+	+	+	+	+	+	+	+
Rhamnose	-	-	-	-	-	-	-	-
Trehalose	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+

Arbutin	+	+	+	+	+	+	+	+
Soluble starch	-		-	-	w	-	-	-
Glycerol	+	+	+	+	+	+	+	+
Erythritol	+	+	+	-	-	-	+	-
Ribitol	+	+	+	+	+	+/d	+	+
Citrate			+	+	d	+/d/w	+	+/d
Ethanol	+	+	+/d	d	+	+/d/w	+	+
Methanol	-	-	-	-	-	-	-	-
Inulin	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-
Mannitol	+	+	+	+	+	+	+	+
Myo-inositol	+	+	-	-	-	-	-	-
Creatinine	-	-	-	-	-	-	-	-
Cadaverine	+	+	+	+	+	+	+	+
Assimilation of nitrogen source								
Nitrite	-	-	-	-	-	-	-	-
L-Lysine	+	+	-	-	-	-	-	-
Nitrate	-	-	-	--	-	-	-	-
Fermentation occurs with								
D-galactose	+	+	+/d	w	-	+/d	+/d	+/d
Maltose	-	-	-	d	-	-	d/w	-
Sucrose	-	-	-	d	-	-	d/w	-
Lactose	-	-	-	-	-	-	-	-
D-Glucose	+	+	+	d	+	-	+	+
Lactose	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-
Vitamin free media	+	+	-	-	-	-	-	-
Growth at/on								

at 35˚C	+	+	v	-	+	-	+	+/w
0.01% cycloheximide	-	-	w/-	-	-	-	v	-
0.1% cycloheximide	-	-	-	-	-	-	-	-
50% D-Glucose	+	+	+/d	+	w	+/d	+	+/w
60% D-Glucose	+	+	v	w	-	w/-	+/d/w	-
10% NaCl	-	-	+/d/w	w	+	+/w	+	v
16% NaCl	-	-	-	-	w	-	w/-	-
1% acetic acid	-	-	-	-	-	-	-	-
Starch formation	-	-	-	-	-	-	-	-
Urea hydrolysis	-	-	-	-	-	-	-	-

1, strain JY150^T (strain JY152 had similar characters; data from this study); 2, strain ST431 (data from this study and from Prof. Savitree Limtong's lab); 3, *Suhomyces bolitotheri*; 4, *Suhomyces terraborum*; 5, *Suhomyces atakaporum*; 6, *Suhomyces chickasaworum*; 7, *Suhomyces ambrosiae*; 8, *Suhomyces emberorum*. The data of all type strains were taken from Sung et al. Abbreviations: +, growth positive; -, growth negative; w, weak positive reaction; v, variable reaction; d, delayed positive reaction

Amplicon based metagenome analysis of yeast

3.3. Microscopic observation of bacterial like bodies

While observing the yeast cells under confocal microscopy, rapidly moving bodies roughly the size of bacteria were seen in the vacuoles of yeast (Fig. 3.7a) . Bacterial fluorescent staining was performed to confirm the bacteria in yeast vacuole, where Texas Red conjugated with wheat germ agglutinin stained the peptidoglycan of bacterial cells and yeast DNA was counter stained with DAPI/SYTOX (Fig. 3.7b and c). Further, we observe more than one bacteria in the yeast cell which intrigued us to look into bacterial diversity of yeast through metagenome sequencing.

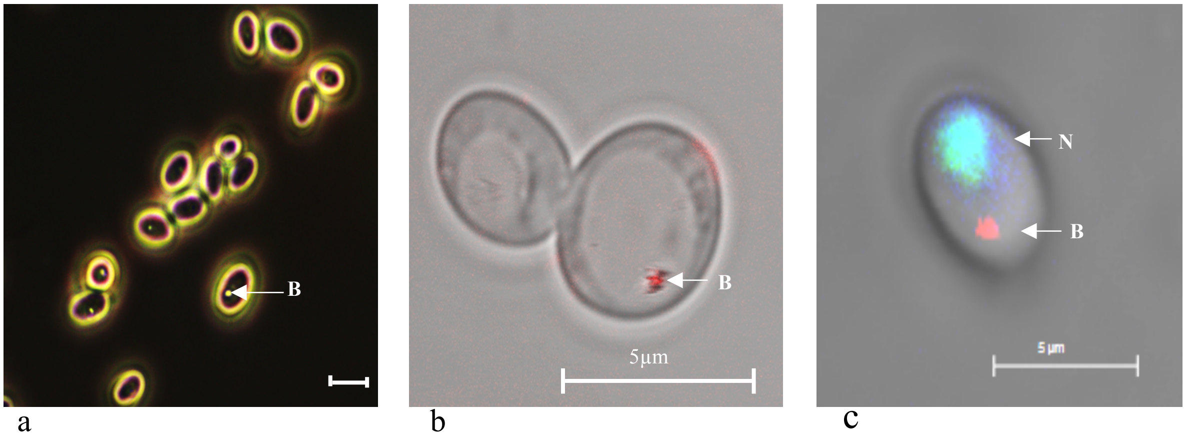


Fig. 3.7: Microscopic observation of bacterial like bodies in of yeast cells: (a) Phase contrast microscopy of yeast strain JY101 showing the presence of bacteria inside the vacuole; (b) Confocal microscopy of yeast strain JY101 and its endobacteria with viability staining using Texas red; (c) Confocal microscopy of yeast strain JY101 and its endobacteria with viability staining using DAPI, SYTOX and Texas red. Staining was done with ViaGram viability staining kit. Arrows: B, Bacteria like objects seen in vacuoles; N, Yeast nucleus.

3.4. 16S rRNA gene amplicon based metagenome analysis

Prior to the extraction of genomic DNA of yeast, we made sure that there were no external bacteria by staining them, as observed under confocal microscope. Genomic DNA was extracted from twenty-nine strains of yeast and 16S rRNA gene-based metagenome sequencing of the V1-V3 region was performed. Except for the yeast strains JY127, JY132, JY135 and JY143, remaining twenty-four yeast strains as well as yeast like fungal strain JY119 yielded good sampling depth as observed from the rarefaction curves (Fig. 3.8). The same four strains also yielded negative result for staining, suggesting that they may lack bacteria. The metagenome data of the yeasts is deposited with NCBI and the Sequence Read Archive (SRA) accession numbers are given in Table 3.4. Since *Candida* and *Pichia* were among the largest (represented by about 48% and 24%, respectively) of our collection of yeast in this study, we have presented the results separately.

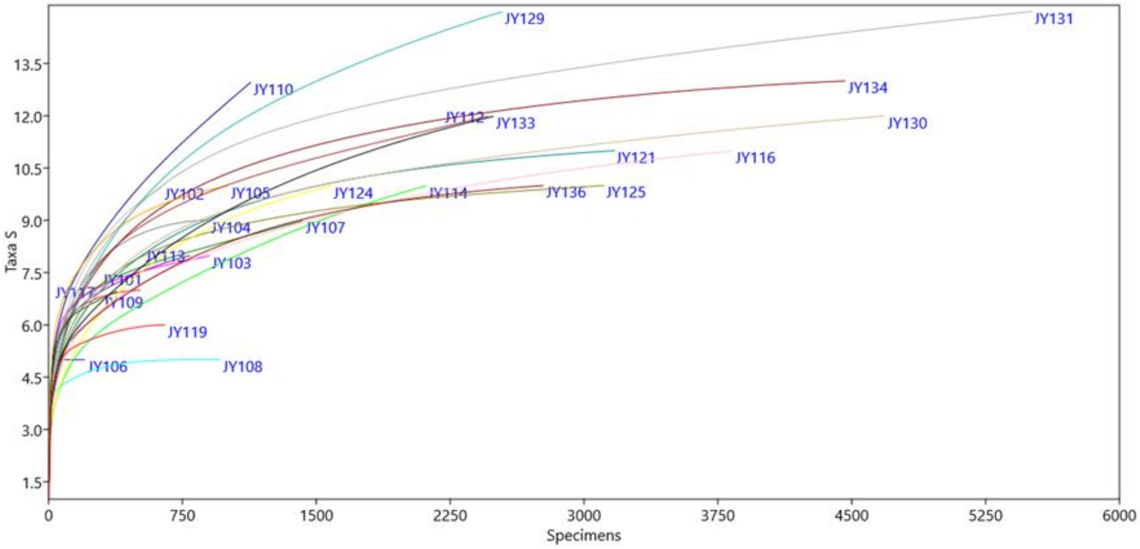


Fig. 3.8: Rarefaction curves of twenty five yeast strains for their V1-V3 region of 16S rRNA gene metagenomes showing the saturation of bacterial diversity and richness of each yeast strain. Data on bacterial taxa reads found with each yeast strain was obtained using mothur V.1.41.1 and the curves were constructed using PAST V3.26.

Table 3.4. Strain, Source and accession numbers of metagenome of yeasts used in this study.

Strain	**Source**	**Similarity with the nearest yeast**	**Metagenome accession number of the yeast**
JY101	Soil	*Candida tropicalis* CBS 94^{T}	SRR7746671
JY106	Date fruit	*Candida tropicalis* CBS 94^{T}	SRR7752355
JY107	Lichen	*Candida tropicalis* CBS 94^{T}	SRR7752303
JY108	Leaf litter	*Candida tropicalis* CBS 94^{T}	SRR7752354
JY113	Citrus fruit	*Candida tropicalis* CBS 94^{T}	SRR7757783
JY114	Decomposed wood	*Candida tropicalis* CBS 94^{T}	SRR7757789
JY125	Biofilm	*Candida tropicalis* ATCC 750^{T}	SRR7762712
JY134	Carve soil	*Candida tropicalis* CBS 94^{T}	SRR7774106
JY103	Sewage water	*Candida metapsilosis* CBS 10907^{T}	SRR7752356
JY110	Muskmelon	*Candida intermedia* CBS 572^{T}	SRR7757784
JY121	Brown *Gracillaria*	*Candida suratensis* CBS 10928^{T}	SRR7760802
JY124	Brown *Gracillaria*	*Candida suratensis* CBS 10928^{T}	SRR7762711
JY135	Kadam fruit	*Candida ampae* CBS 7872^{T}	-
JY105	Gooseberry	*Pichia kudriavzevii* CBS 5147^{T}	SRR7752353
JY112	Idly batter	*Pichia kudriavzevii* CBS 5147^{T}	SRR7757792
JY116	Sugarcane	*Pichia kudriavzevii* CBS 5147^{T}	SRR7757791
JY131	Lichen	*Pichia kudriavzevii* NRRL Y-5396^{T}	SRR7774038
JY129	Mushroom	*Pichia kudriavzevii* NRRL Y-5396^{T}	SRR7762710
JY136	Neolamarckia fruit	*Pichia kluyveri* NRRL y-11519^{T}	SRR7774105
JY104	Idly batter	*Meyerozyma guilliermondii* CBS 2030^{T}	SRR7752352
JY117	Citrus fruit	*Meyerozyma carribicca* CBS 9966^{T}	SRR7757782
JY102	Grapes	*Hanseniaspora guilliermondii* CBS 465^{T}	SRR7752479
JY109	Soil	*Rhodotorula mucilaginosa* CBS 316^{T}	SRR7752357
JY127	Algal growth	*Rhodotorula mucilaginosa* CBS 316^{T}	-
JY132	Mushroom	*Rhodotorula mucilaginosa* CBS 316^{T}	-
JY143	Lake sediment	*Rhodotorula mucilaginosa* CBS 316^{T}	-
JY119	Pine tree bark	*Zalaria obscura* DAOMC 250849^{T}	SRR7757780
JY130	Mushroom	*Debaryomyces prosopidis* JCM 9913^{T}	SRR7762713
JY133	Soil	*Sporidiobolus pararoseus* CBS 491^{T}	-

3.4.1. Microbiome studies of *Candida* spp.

Highest number of OTUs (3181) were observed from strain JY121 and lowest number (529) from JY101. Firmicutes constituting approximately 49% of the OTUs was the most abundant phylum in all strains of *Candida*. Other reads mapping to Actinobacteria (~20%), Bacteroidetes (~2%), Proteobacteria (~20%) and unclassified bacteria (~7%) were common to the four species (Table 3.5; Fig. 3.9). Further, differences in their bacterial composition are presented in beta diversity plot using Bray-Curtis index with permutational MANOVA (PERMANOVA) statistical method. The plot shows (Fig. 3.9b) the individuality of OTUs make-up in each strain, clearly displaying no correlation among them, despite sharing some common trends at the level of phylum (Fig. 3.9a) or genus (Fig. 3.10a). Further comparison at phylum level among these strains is given in Table 3.5 and Venn diagram (Fig.3.9). Varied results were observed at genus level where *Streptococcus* (~10%) was common among all the *Candida* spp. with maximum OTUs (210) in strain JY103. *Planococcus* reads were highest in strain JY121 with 446 OTUs. Genera like *Corynebacterium* (~2%), *Propionibacterium* (~3%), *Staphylococcus* (~7%), unclassified bacteria (~9%) and unclassified actinomycetales (~6%) were also common to all *Candida* spp. (Table 3.6). In addition, unclassified Pseudomonadaceae reads were unique to strain JY101 having 25 OTUs (Table 3.6; Fig. 3.10).

Table 3.5. Common and unique endobacterial taxa at phylum level between *Candida* spp.

Yeast Strain(s)	**Bacterial Phyla**
JY110	Armatimonadetes, TM7
JY121	Acidobacteria
JY101, JY103 & JY110	Fusobacteria
JY101, JY103, JY110 & JY121	Actinobacteria, Bacteria unclassified, Bacteroidetes, Firmicutes, Proteobacteria
JY103 & JY110	SR1
JY103, JY110 & JY121	Cyanobacteria
JY101 & JY121	Deinococcus-Thermus
JY110 & JY121	Chloroflexi, Planctomycetes, Spirochaetes

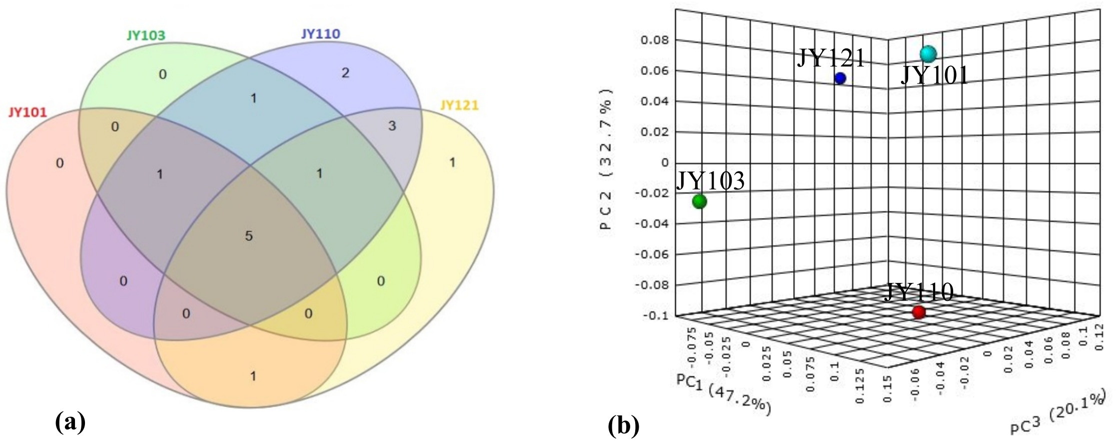


Fig. 3.9: Bacterial diversity among different *Candida* spp. for intra-genera comparison of yeast strains: Venn diagram (a) and PCoA (b) plot of bacterial phyla distribution in yeast, Principal Coordinates Analysis (PCoA) plots were constructed at phylum level using Microbiome Analyst web-based tool for the assessment of bacterial beta-diversity between *Candida* strains using Bray-Curtis index. Venn diagram constructed using InteractiVenn web-based tool to compare common and unique bacterial phyla and genera of the *Candida* strains.

Table 3.6. Common and unique endobacterial taxa at genus level between *Candida* spp.

Yeast Strain(s)	Bacterial Genera
JY103	*Fusobacterium*
JY110	Enterobacteriaceae unclassified, Bacilli unclassified, Proteobacteria unclassified, Gammaproteobacteria unclassified, *Actinomyces*
JY121	*Planococcus*, *Salinicoccus*, *Planomicrobium*, *Exiguobacterium*, *Microbacterium*, *Brachybacterium*, *Caulobacter*, *Kocuria*, *Bacillus*, *Brevundimonas*, *Anaerobacillus*, Planococcaceae unclassified, *Delftia*, *Jeotgalicoccus*, Rhizobiales unclassified, *Dietzia*, Micrococcaceae unclassified, Sphingomonadaceae unclassified, Microbacteriaceae unclassified, Alphaproteobacteria unclassified, *Methylobacterium*, *Sporosarcina*, Ruminococcaceae unclassified, Caulobacteraceae unclassified, Bacillaceae 1 unclassified, *Rhodococcus*, Bacteroidetes unclassified, *Ralstonia*, Comamonadaceae unclassified
JY101	*Gemella*, Pseudomonadaceae unclassified
JY101, JY103, JY110 & JY121	Actinomycetales unclassified, Bacteria unclassified, *Corynebacterium*, *Propionibacterium*, *Staphylococcus*, *Streptococcus*
JY103 & JY110	Firmicutes unclassified, *Granulicatella*, Lactobacillales unclassified, *Veillonella*
JY103 & JY121	Bacillales unclassified, *Brevibacterium*
JY101, JY103 & JY121	*Acinetobacter*
JY101 & JY121	Rhodobacteraceae unclassified
JY110 & JY121	*Stenotrophomonas*

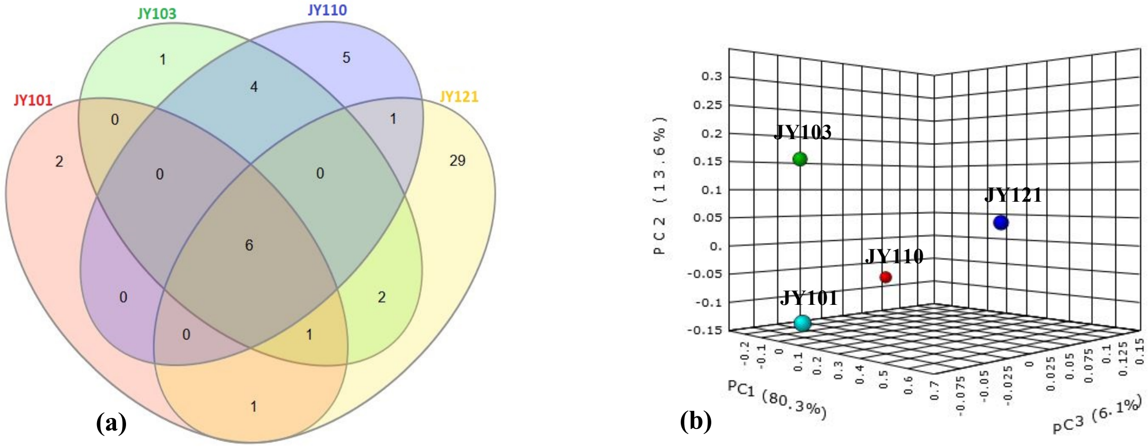


Fig. 3.10: Bacterial diversity among different *Candida* spp. for intra-genera comparison of yeast strains: Venn diagram (a) and PCoA (b) plot of bacterial genera distribution in yeast, Principal Coordinates Analysis (PCoA) plots were constructed at genus level using Microbiome Analyst web-based tool for the assessment of bacterial beta-diversity between *Candida* strains using Bray-Curtis index. Venn diagram constructed using InteractiVenn web-based tool to compare common and unique bacterial genera of the *Candida* strains.

We further compared six strains of *C. tropicalis* (JY101, JY107, JY108, JY113, JY114 and JY125) for intra-species analysis of endobacterial diversity. Yeast strain JY125 had the greatest number of OTUs (3125) and strain JY101 had the least (529). Deinococcus-Thermus, Spirochaetes, Gemmatimonadetes were unique phyla of endobacterial diversity of *C. tropicalis* strains JY101, JY107 and JY114 respectively (Table 3.7; Fig. 3.11). Firmicutes constituted approximately half the total read share (up to 46%) in these strains. Proteobacteria (~23%) and Actinobacteria (~22%) were the next abundant phyla with similar number of reads. Genus level analysis (Table 3.8; Fig. 3.12) showed the presence of the genus *Staphylococcus*, unclassified bacteria and unclassified actinomycetales in all the *C. tropicalis* strains. Although diversity was more varied at the genus-level, few taxa showed high read dominance. For example, *Staphylococcus* (~54%) was abundant in strain JY114 while *Streptococcus* and *Planococcus* reads were in similar proportions in the rest of the *C. tropicalis* strains. As mentioned previously, unclassified Pseudomonadaceae reads only showed up in strain JY101.

Table 3.7. Common and unique endobacterial taxa at phylum level between *Candida tropicalis*

Yeast Strain (s)	**Bacterial Phyla**
JY101	Deinococcus-Thermus
JY107	TM7
JY125	Spirochaetes, Synergistetes
JY114	Gemmatimonadetes
JY114 & JY125	Chloroflexi
JY107, JY114 & JY125	Acidobacteria
JY113 & JY125	Planctomycetes
JY101, JY107, JY113 & JY114	Fusobacteria
JY107, JY113 & JY114	Armatimonadetes
JY101, JY107, JY108, JY113, JY114 & JY125	Actinobacteria, Bacteria unclassified, Bacteroidetes, Firmicutes, Proteobacteria

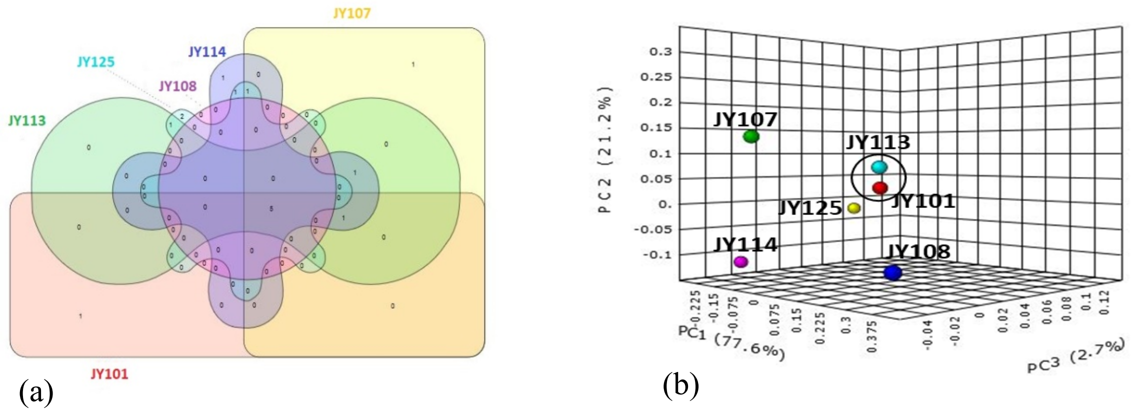


Fig. 3.11: Bacterial diversity among six *Candida tropicalis strains* showing intra-species comparison: Venn diagram (a) and PCoA plot (b) of bacteria at phyla distribution.

Table 3.8. Common and unique endobacterial taxa at genus level between *Candida tropicalis*

Yeast Strain(s)	**Bacterial Genera**
JY101	*Gemella*, Pseudomonadaceae unclassified
JY107	Firmicutes unclassified, *Veillonella*, *Finegoldia*
JY108	*Alcaligenes*, *Serratia*, Xanthomonadaceae unclassified, Proteobacteria unclassified, Gammaproteobacteria unclassified, *Achromobacter*
JY125	Comamonadaceae unclassified, *Dietzia*, *Methylobacterium*, Planococcaceae unclassified, *Janibacter*, *Micrococcus*, Microbacteriaceae unclassified, *Rhodococcus*, Bacteroidetes unclassified, Caulobacteraceae unclassified, Micrococcaceae unclassified, Bacillaceae 1 unclassified
JY113	*Actinomyces*, *Facklamia*
JY114	*Caulobacter*
JY108 & JY114	*Delftia*
JY114 & JY125	*Planococcus*, *Salinicoccus*, *Planomicrobium*, *Exiguobacterium*, *Kocuria*, *Brachybacterium*, *Variovorax*, *Microbacterium*, Sphingomonadaceae unclassified, *Brevundimonas*, *Anaerobacillus*, *Bacillus*, Rhizobiales unclassified
JY107 & JY125	*Brevibacterium*
JY107 & JY113	Lactobacillales unclassified, *Lactobacillus*
JY107 & JY108	*Granulicatella*
JY101 & JY108	Rhodobacteraceae unclassified
JY108 & JY113	Enterobacteriaceae unclassified, *Pseudochrobactrum*

JY101, JY107, JY108, JY114 & JY125	*Propionibacterium*
JY101, JY107 JY113 & JY125	*Corynebacterium*
JY101, JY107, JY108, JY113 & JY125	*Streptococcus*
JY101, JY107, JY108, JY113, JY114 & JY125	Actinomycetales unclassified, Bacteria unclassified, *Staphylococcus*
JY101, JY108, JY113, JY114 & JY125	*Acinetobacter*
JY108, JY113 & JY125	*Stenotrophomonas*
JY108, JY114 & JY125	Bacillales unclassified

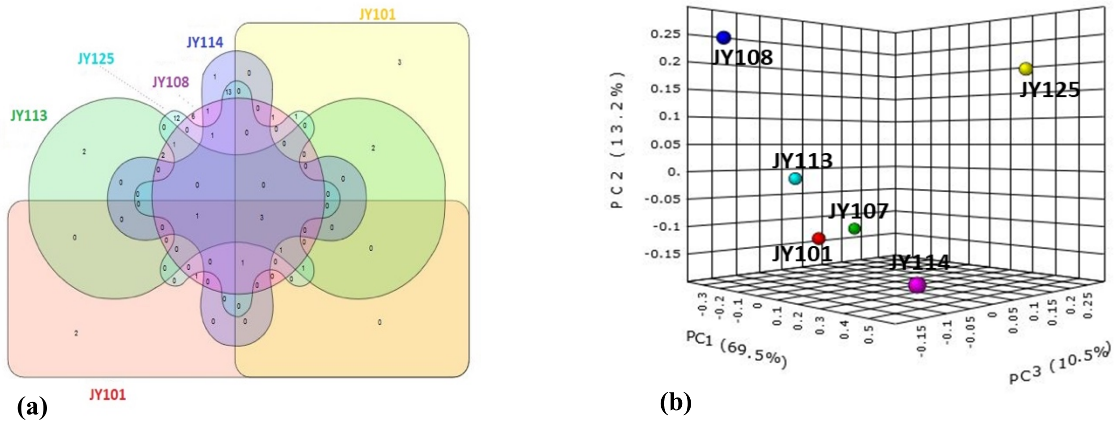


Fig. 3.12: Bacterial diversity among six *Candida tropicalis strains* showing intra-species comparison: Venn diagram(a) and PCoA plot (b) of bacteria at genus distribution

3.4.2. Microbiome studies of *Pichia* spp.

Endosymbionts of *Pichia kudriavzevii* (JY105, JY112, JY116, JY129 and JY131) and *Pichia kluyveri* (JY136) were compared similarly at bacterial phylum and genus level. Strain JY131 had maximum number of OTUs of 5524 and strain JY105 had minimum number of 1015. Diversity measures for *Pichia* spp. were calculated as for *Candida* spp. Firmicutes constituted 46% and was the most abundant reads among all *Pichia* strains. Acidobacteria (~0.3%), Actinobacteria (~23%), Bacteroidetes (~2%), Firmicutes (~46%), Planctomycetes (~0.2%), Proteobacteria (~25%) and unclassified bacteria (~3%) were the common phyla for all strains (Table 3.9; Fig. 3.13). Whereas Chlorobi and Deinococcus-Thermus were found to be specific for strain JY129. At genus level, *Staphylococcus* (~8%), *Propionibacterium* (~3%), *Streptococcus* (~2%) and *Corynebacterium* (~1%) OTUs were common in the total read share (Table 3.10; Fig. 3.14). Genus *Planococcus* was present in all strains except JY105 showing high read dominance and contributing a total of 13% of OTU count. Reads of *Weissella* were only found in yeast strain JY136. Reads of unclassified Actinomycetales*, Granulicatella, Gemella*, unclassified Lactobacillales and unclassified Pseudomonadaceae were present only in yeast strain JY105 (Table 3.10). Unclassified Micromonosporaceae OTUs were uniquely present in strain JY129. Yeast strain JY131 has the OTUs of genus *Siphonobacter*. Further diversity analysis of different strains of the genus *Pichia* are given in Table 3.10 and Venn diagram (Fig.3.14).

Table 3.9. Common and unique endobacterial taxa at phylum level between *Pichia* spp.

Yeast Strain(s)	**Bacterial Phyla**
JY129	Chlorobi, Deinococcus-Thermus
JY116 & JY129	SR1
JY129 & JY131	Armatimonadetes, TM7
JY105, JY112 & JY131	Spirochaetes
JY105, JY112, JY129 & JY131	Verrucomicrobia
JY105, JY112, JY116, JY129, JY131 & JY136	Acidobacteria, Actinobacteria, Bacteria unclassified, Bacteroidetes, Firmicutes, Planctomycetes, Proteobacteria
JY112, JY116, JY129, JY131 & JY136	Cyanobacteria
JY105, JY129, JY131 & JY136	Fusobacteria
JY112, JY116, JY131 & JY136	Chloroflexi
JY112, JY116 & JY131	Gemmatimonadetes

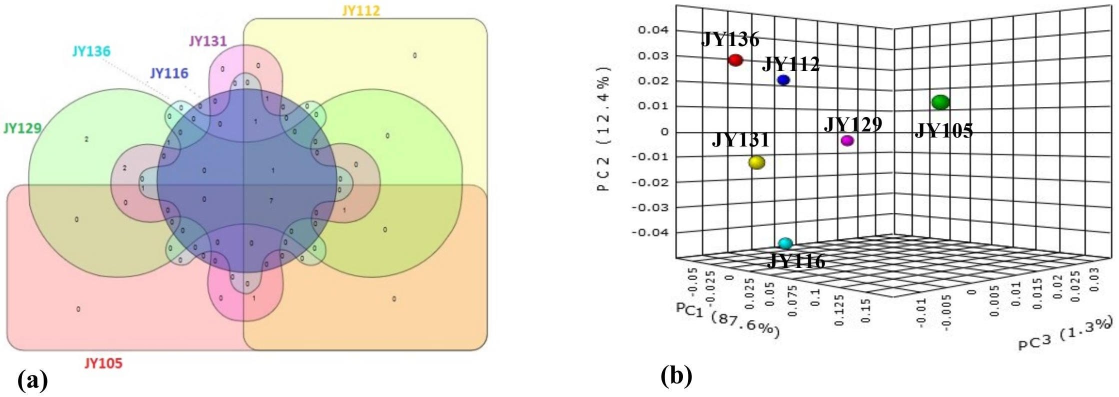
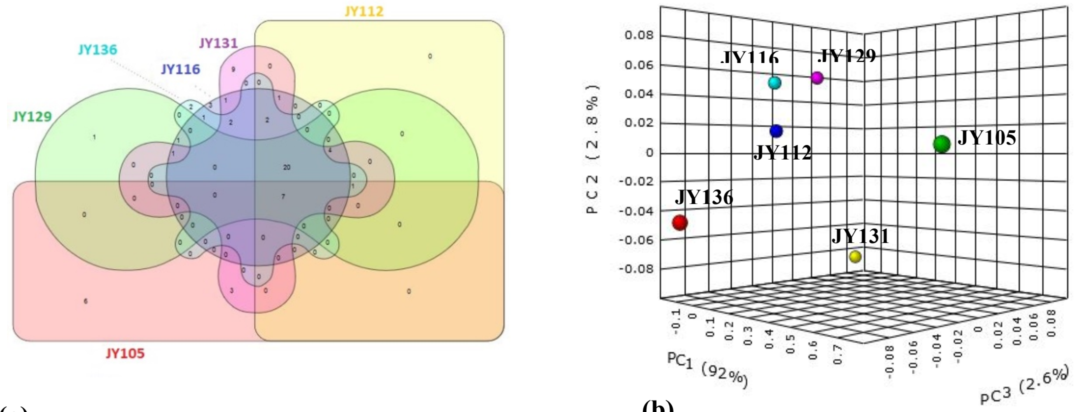


Fig. 3.13: Bacterial diversity among different *Pichia* spp. for intra-genera comparison of yeast strains: Venn diagram (a) and PCoA plot (b) of bacterial phyla distribution in yeast.

Table 3.10. Common and unique endobacterial taxa at genus level between *Pichia* spp.

Yeast Strain(s)	**Bacterial Genera**
JY105	*Actinomyces*, Gammaproteobacteria unclassified, *Granulicatella*, Lactobacillales unclassified, Pseudomonadaceae unclassified, Xanthomonadaceae unclassified
JY116	Neisseriaceae unclassified, Bacillaceae 1 unclassified, *Rothia*
JY136	*Weissella*, *Alcaligenes*
JY129	Micromonosporaceae unclassified
JY131	Lachnospiraceae unclassified, *Ralstonia*, Ruminococcaceae unclassified, Bacteroidales unclassified, Dermabacteraceae unclassified, *Oligella*, Clostridiales unclassified, Porphyromonadaceae unclassified, *Siphonobacter*
JY116 & JY131	*Sphingomonas*
JY116 & JY136	*Paracoccus*
JY116 & JY129	*Halomonas*
JY105 & JY131	Firmicutes unclassified, *Gemella*, Proteobacteria unclassified
JY112, JY116, JY129 & JY131	*Delftia*, Bradyrhizobiaceae unclassified, Planococcaceae unclassified, *Caulobacter*
JY116, JY129 & JY131	*Comamonas*
JY105, JY112, JY116, JY129, JY131 & JY136	Actinomycetales unclassified, Alphaproteobacteria unclassified, Bacteria unclassified, *Corynebacterium*,

	Propionibacterium, *Staphylococcus*, *Streptococcus*
JY112, JY116, JY129, JY131 & JY136	*Planococcus*, *Salinicoccus*, *Brachybacterium*, *Planomicrobium*, *Exiguobacterium*, *Microbacterium*, *Brevundimonas*, *Kocuria*, Sphingomonadaceae unclassified, *Anaerobacillus*, *Bacillus*, Bacillales unclassified, *Variovorax*, Rhizobiales unclassified, *Methylobacterium*, *Micrococcus*, *Stenotrophomonas*, Microbacteriaceae unclassified, *Acinetobacter*, Caulobacteraceae unclassified
JY105, JY112, JY129, JY131 & JY136	*Brevibacterium*
JY116, JY131 & JY136	Rhodobacteraceae unclassified, Bacteroidetes unclassified
JY112, JY116, JY131 & JY136	*Dietzia*, Comamonadaceae unclassified
JY112, JY116 & JY131	Micrococcaceae unclassified



(a)

(b)

Fig. 3.14: Bacterial diversity among different *Pichia* spp. for intra-genera comparison of yeast strains: Venn diagram (a) and PCoA plot (b) of bacterial genera distribution in yeast.

3.4.3. Microbiome diversity indices of all yeast strains

Comparative metagenomic diversity of all the 25 yeasts had shown endosymbiont heterogeneity among all the yeast as analysed by diversity indices- heatmaps (Fig. 3.15; Fig 3.16) and bar plots (Fig. 3.17; Fig. 3.18). Maximum number (5539) of OTUs were seen for *P. kudriavzevii* JY131 whereas the least (218 reads) was found in *C. tropicalis* JY106. Alpha diversity using Chao-1 index and T-test/ANOVA statistical method (Fig. 3.19a) and beta diversity using Bray-Curtis index and PERMANOVA statistical method were examined (Fig. 3.19b) to validate our diversity analysis. Several unclassified reads were also detected at various taxonomic levels, with unclassified bacterial reads at phylum level contributing to significant abundance in strains JY106 (27%), JY109 (13%), JY110 (18%) and JY119 (33%) which would either need further analysis or corroboration. Firmicutes (~45%) remain the dominant phylum of endosymbiotic bacteria followed by Proteobacteria (~25%) and Actinobacteria (~22%) for all the yeasts examined in this study.

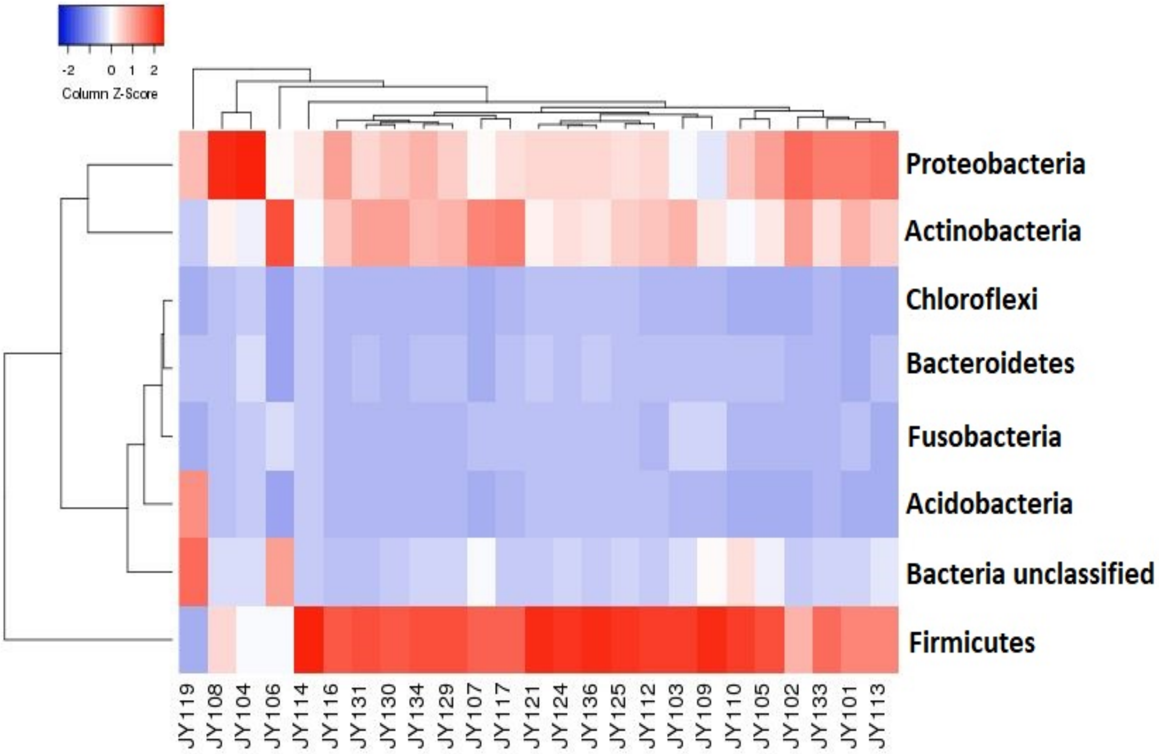


Fig. 3.15: Heatmaps on bacterial abundance in twenty-five yeast strains at phylum level: The heatmaps were constructed using Heatmapper web-based tool. Clustering was applied to both column and row data with average linkage method and distances were measured as Euclidean distances. Red colour indicates high abundance and blue colour indicates low abundance as given in the colour scale on the top of the maps.

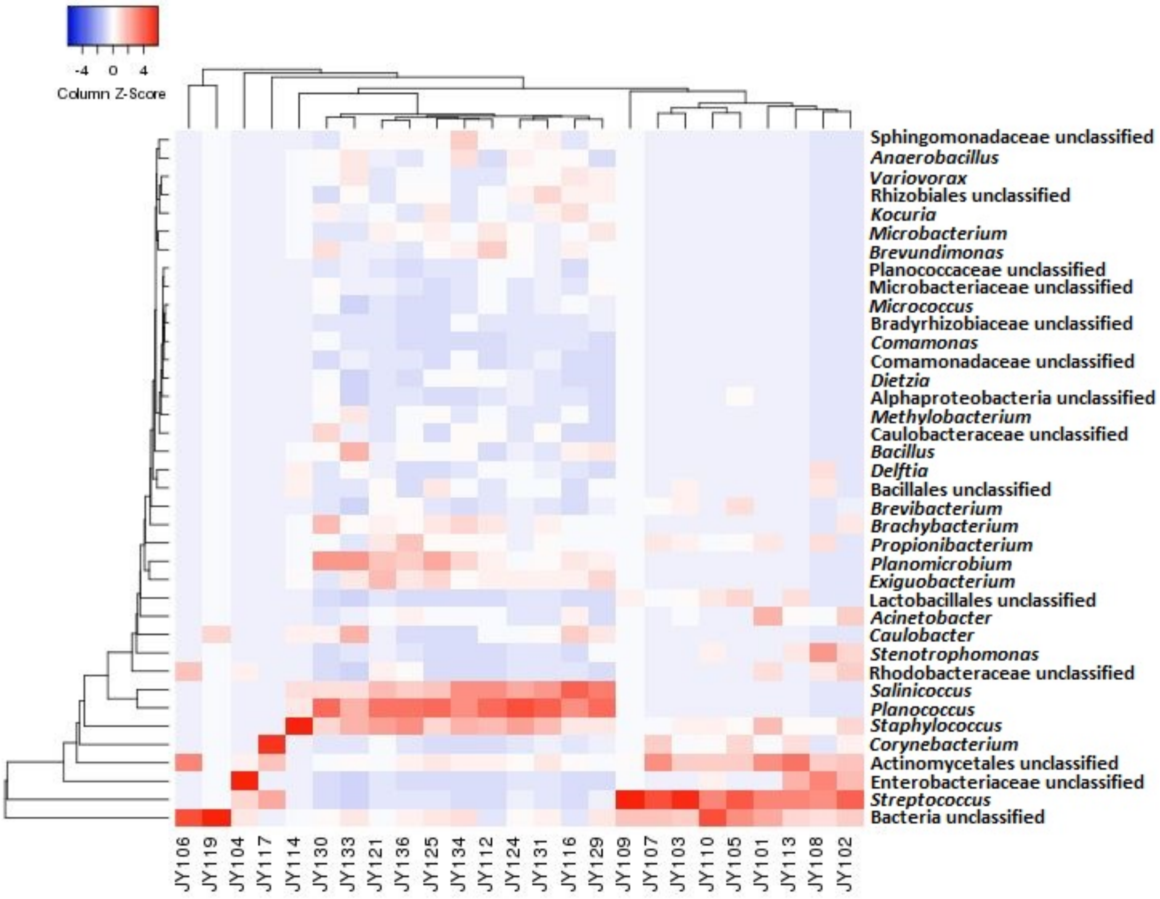


Fig. 3.16: Heatmaps on bacterial abundance in twenty-five yeast strains at genus level: The heatmaps were constructed using Heatmapper web-based tool. Clustering was applied to both column and row data with average linkage method and distances were measured as Euclidean distances. Red colour indicates high abundance and blue colour indicates low abundance as given in the colour scale on the top of the maps.

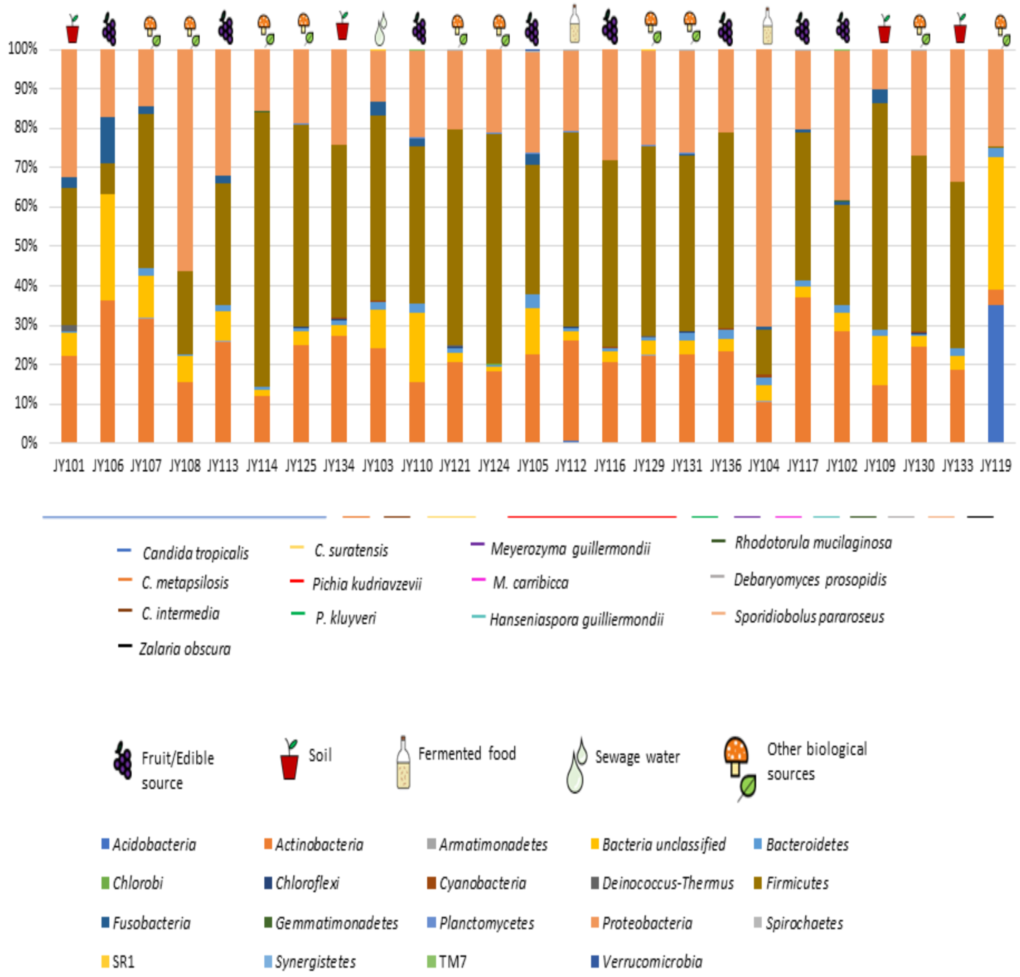


Fig. 3.17: Stacked bar plots showing the relative abundances of bacterial taxa reads distributed among the twenty-five yeast strains at phylum level: X-axis indicates yeast strains and Y-axis indicates the relative abundance of bacterial taxa calculated as percentage of Operational Taxonomic Units (OTUs). The plots are supported with legends showing the yeast strains, bacterial taxa as well as environmental sources of yeast isolation.

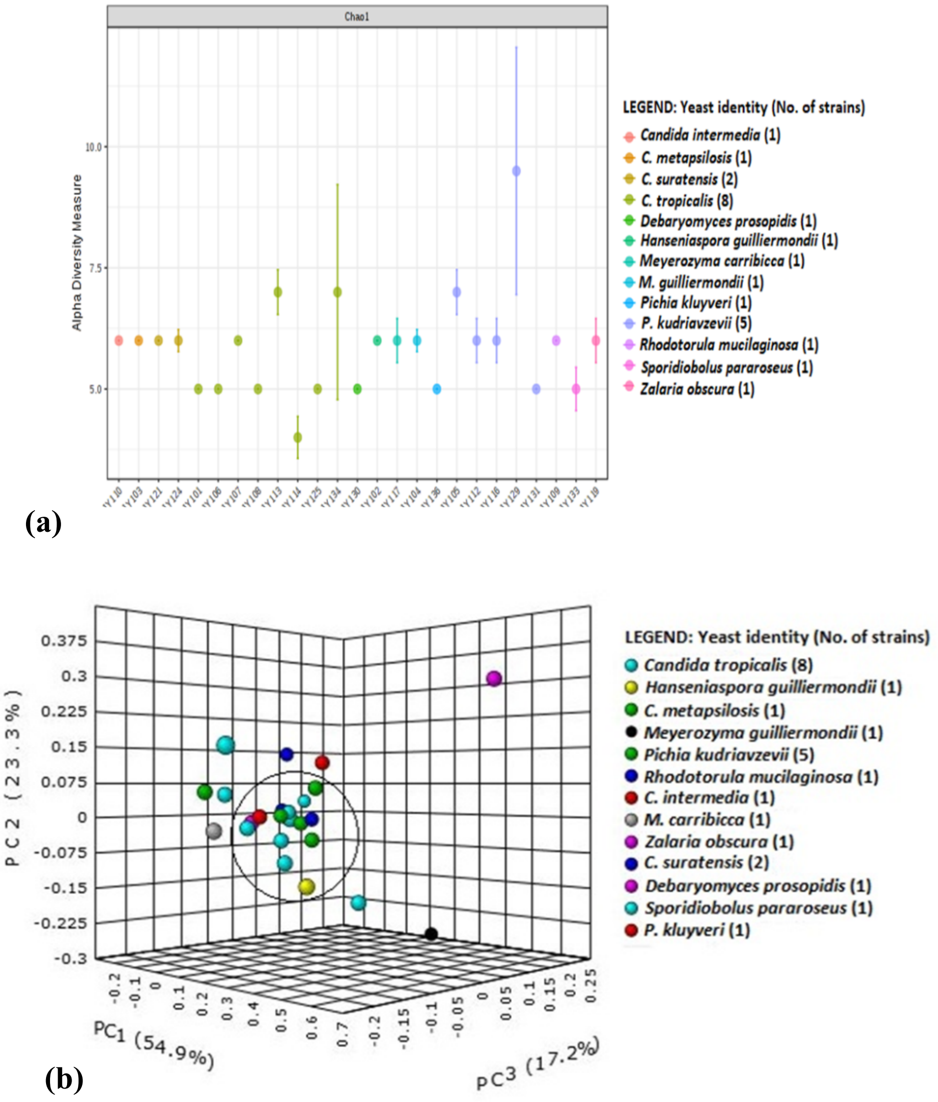


Fig. 3.18: Bacterial diversity among all twenty-five yeast strains: Alpha (a) and beta (b) diversity of bacterial phyla distribution in all yeast. Alpha diversity was measured by Chao-1 index and beta diversity with PCoA plots was measured with Bray-Curtis index at bacterial phylum levels for all yeast strains.

At the genus level, major differences in the abundance was observed. Only a few bacterial genera were common among certain groups of yeast strains, as shown by the clustering of these samples in the PCoA plots (Fig. 3.19). Reads of the genus *Streptococcus* contributed greatest number of OTUs in strains with maximum number in *C. metapsilosis* strain JY103 (~34% of their total OTUs) and minimum in *C. suratensis* strain JY121 (~4% of their total) (Fig. 3.20). Strain JY119 was the only strain which did not possess any *Streptococcus* reads. It had notably different read composition from the rest of the yeast strains where acidobacteria (236 OTUs) was the most dominant phylum. Also, JY119 showed lesser richness of bacterial diversity as against majority of the yeast strains. *Planococcus* reads dominated in strains JY112, JY116, JY121, JY124, JY125, JY129, JY130, JY131, JY133, JY134 and JY136 with maximum number of OTUs in *Candida suratensis* strain JY124 (~20% of their total) and minimum in *P. kudriavzevii* strain JY131 (~11% of their total). OTUs of unclassified Clostridiales were only observed in *P. kudriavzevii* JY131. Diversity and abundance of the endobacterial population in yeast were represented in the form of heat map and bar graph at phylum as well as at genus level (Fig. 3.15; Fig. 3.16; Fig. 3.17; Fig. 3.20).

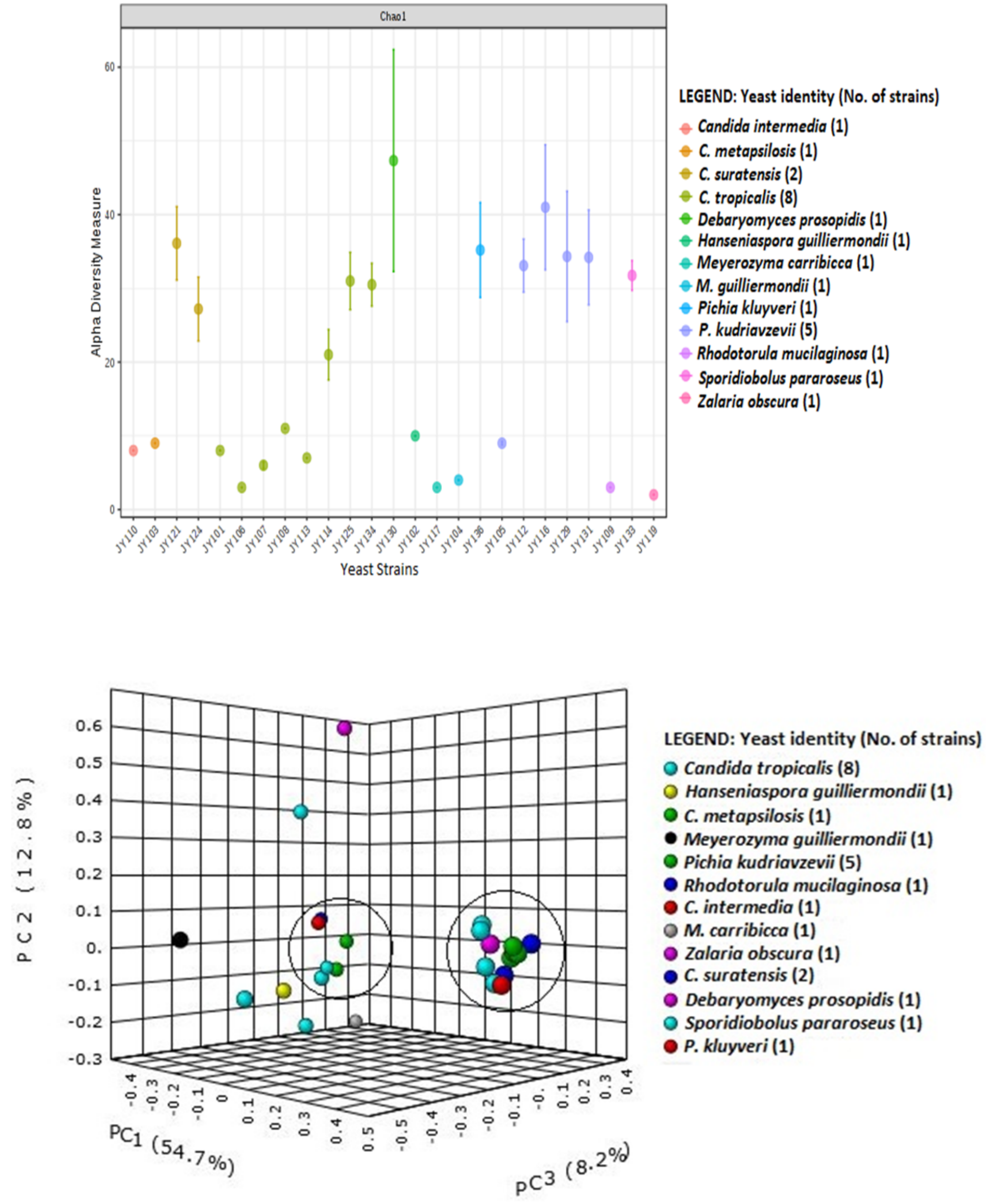


Fig. 3.19: Bacterial diversity among all twenty-five yeast strains: Alpha (a) and beta (b) diversity of bacterial phyla distribution in all yeast. Alpha diversity was measured by Chao-1 index and beta diversity with PCoA plots was measured with Bray-Curtis index at bacterial genera level for all yeast strains.

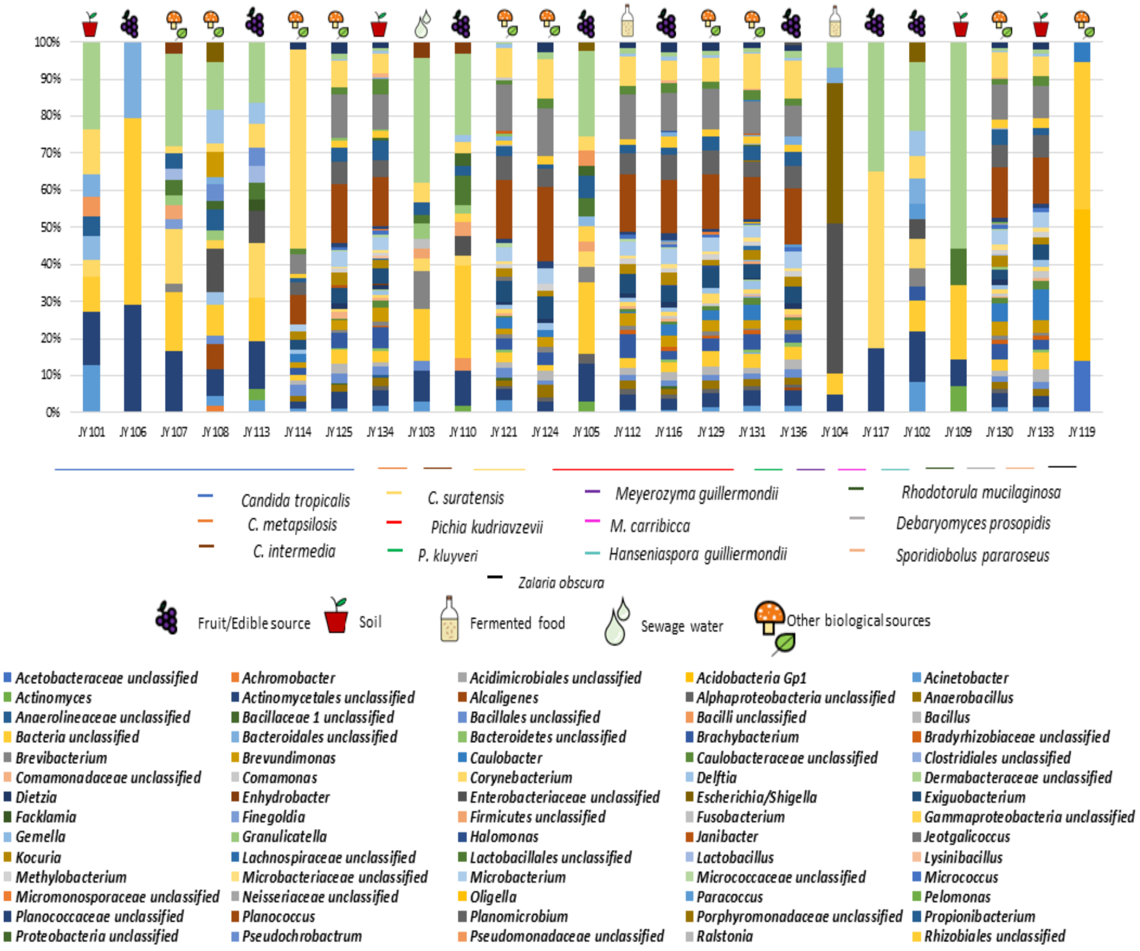


Fig. 3.20: Stacked bar plots showing the relative abundances of bacterial taxa reads distributed among the twenty-five yeast strains at genera level: X-axis indicates yeast strains and Y-axis indicates the relative abundance of bacterial taxa calculated as percentage of Operational Taxonomic Units (OTUs). The plots are supported with legends showing the yeast strains, bacterial taxa as well as environmental sources of yeast isolation

Insights into endobacteria of Candida tropicalis JY101

3.5. Microscopic studies

3.5.1. Phase contrast microscopic studies

Cells of *Candida tropicalis* JY101 showed the rapidly moving bodies roughly the size of the bacteria (Fig. 3.7a) when observed under phase contrast microscope.

3.5.2. Confocal microscopic studies along with staining with fluorescent dyes

Simple bacterial fluorescent staining was done to confirm bacteria in the vacuoles using Texas red conjugated with wheat germ agglutinin which stained the bacterial peptidoglycan (Fig. 3.7b) and also the yeast DNA was counter stained with DAPI/SYTOX (Fig. 3.7c).

3.5.3. Fluorescence In Situ Hybridization (FISH)

FISH was performed considering *Candida tropicalis* JY101 as a model system to observe the bacterial communities in the yeast cells. In FISH, EUB338-Cy3 probe was used to hybridized the bacterial rRNA which gave positive signals as numerous bacterial bodies in yeast cell imparted green fluorescence and hence confirmed the presence of bacterial communities inside the yeast cells (Fig. 3.21).

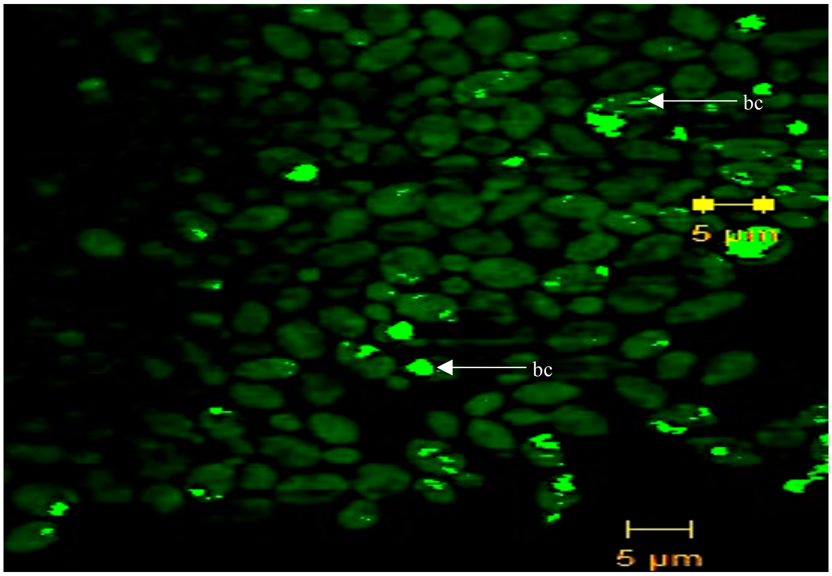


Fig. 3.21: Confocal microscopic image of native bacteria community (bc) of the yeast strain JY101 showing the green fluorescence inside the yeast cell.

3.5.4. Transmission electron microscopy (TEM)

Bacterial structure were also observed with the transmission electron microscopy (Fig. 3.22)

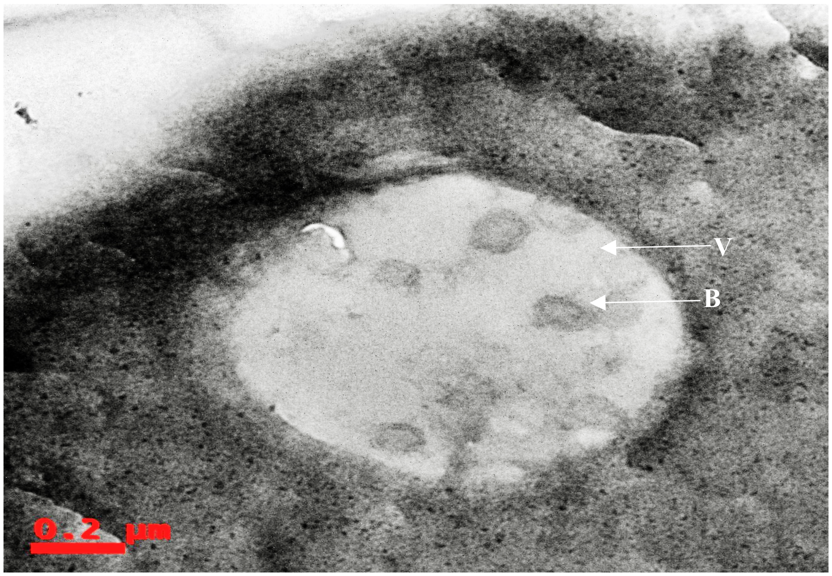


Fig. 3.22: Transmission electron microscopy (TEM) of strain JY101 showing the bacterial like bodies in the vacuole of yeast. Arrows: B, Bacteria like objects seen in vacuoles; V, Vacuole of yeast

3.6. Sub-culturing studies

We demonstrated the transmission of bacterial diversity from parent to daughter cells of yeast strain JY101. Interestingly, results of 16S rRNA amplicon based metagenomic analysis of first, fifth and tenth sub-cultures revealed the bacterial community homeostasis from one to next generation. Similar pool of microorganism were transmitted among generations constituting the specific cohort of bacteria. At the Phylum level, Proteobacteria were dominant even after the tenth sub-culture followed by the abundance of Firmicutes and actinobacteria (Fig. 3.23). At the genus level also, *Staphylococcus, Propionibacterium, Pseudomonas* and several other genera were retained among several generations of yeast strain JY101 (Fig. 3.24).

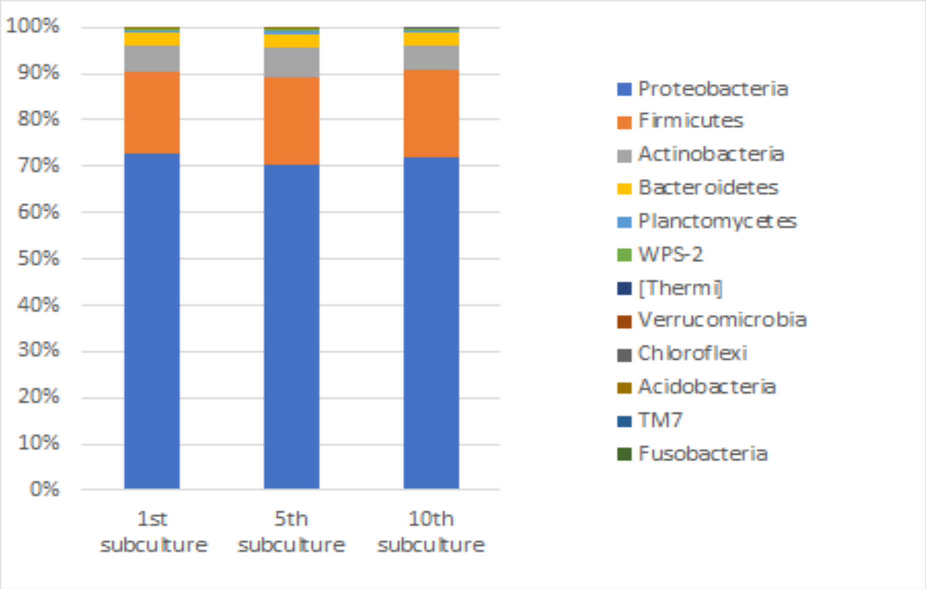


Fig. 3.23: Stacked bar plots showing the relative abundances of bacterial taxa reads of first, fifth and tenth subcultures of strain JY101 at phylum level X-axis indicates the different sub cultures and Y-axis indicates the relative abundance of bacterial taxa calculated as percentage of Operational Taxonomic Units (OTUs).

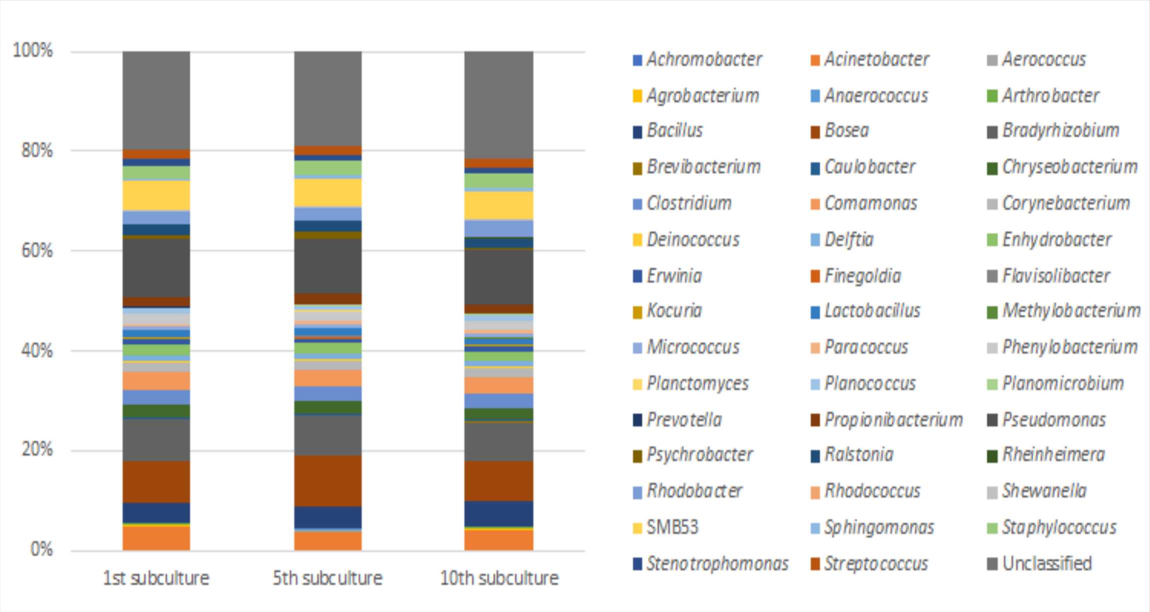


Fig. 3.24: Stacked bar plots showing the relative abundances of bacterial taxa reads of first, fifth and tenth subcultures of strain JY101 at genus level X-axis indicates the different sub-cultures of strain JY101 and Y-axis indicates the relative abundance of bacterial taxa calculated as percentage of Operational Taxonomic Units (OTUs).

3.7. Isolation and identification of yeast endobacteria

Candida tropicalis strain JY101 was used as model yeast for further studies to validate the endobacterial diversity by cultivating the bacteria from the yeast and reinfect the yeast strain using a fluorescently labelled bacterium to mimic Koch's postulates. 16S rRNA gene metagenome of *C. tropicalis* JY101 showed reads of unclassified Pseudomonadaceae, members of which can be cultured easily. Cells of strain JY101 ruptured with physical (glass beads) and enzymatic (zymolase) treatments when plated on nutrient broth yielded four different bacteria which were purified on nutrient agar (HIMEDIA, M002) and sequenced for 16S rRNA gene-based identification. The bacteria identified were members of genus *Pseudomonas, Chryseobacterium, Lysinibacillus* and *Propionibacterium*. EzTaxon BLAST search of the 16S rRNA gene sequence (1387 nt) of *Pseudomonas* strain JC703 had highest (99.9%) identity with *Pseudomonas stutzeri* ATCC 17588^T followed by other members of the genus *Pseudomonas* with <99% sequence identity. While the *Chryseobacterium* strain JC507 sequence (1428 nt) had highest (98.7%) identity with *Chryseobacterium indologenes* NBRC 14944^T followed by other members of the genus *Chryseobacterium* with <98.6% identity. *Lysinibacillus* strain JC1018 sequence (889 nt) had highest (100%) identity with *Lysinibacillus fusiformis* NBRC 15717^T followed by <98.7% with other members. *Propionibacterium* strain JC704 sequence (1396 nt) had the highest similarity (98.6%) with *Propionibacterium acne* DSM 1897^T followed by <95.7% with other members. The 16S rRNA gene sequences of the four endobacteria of *C. tropicalis* JY101 were deposited with NCBI with accession numbers LR735276, LT838865, LR735277 and LR743671 respectively for bacterial strains JC703, JC507, JC1018 and JC704. All the cultures are preserved as glycerol (50% v/v) stocks stored at -20 °C and *Chryseobacterium* sp. JC507 was deposited with KCTC (KCTC 52928) and NBRC (NBRC 113872) as *Chryseobacterium candidae* JC507^T. Our attempts failed to cultivate other endobacteria from *C. tropicalis* JY101 where we have used Marine agar media (HIMEDIA, M384) for marine samples, Mueller Hinton agar (HIMEDIA, M173) and Thiobacillus agar media (HIMEDIA, M788).

Characterization of the bacterial strain JC507^T isolated from C. tropicalis

3.8. Characterization of strain JC507^T

3.8.1. Habitat, colony and cell morphology

Strain JC507^T was isolated from the yeast *Candida tropicalis* JY101. Colonies on nutrient agar were slimy, round, had a smooth margin and pale yellow in colour. Cells were gram-stain-negative, rod-shaped, 0.2–0.4 µm wide, 1.0–2.0 µm long, non-motile, aerobic and divide by binary fission (3.25).

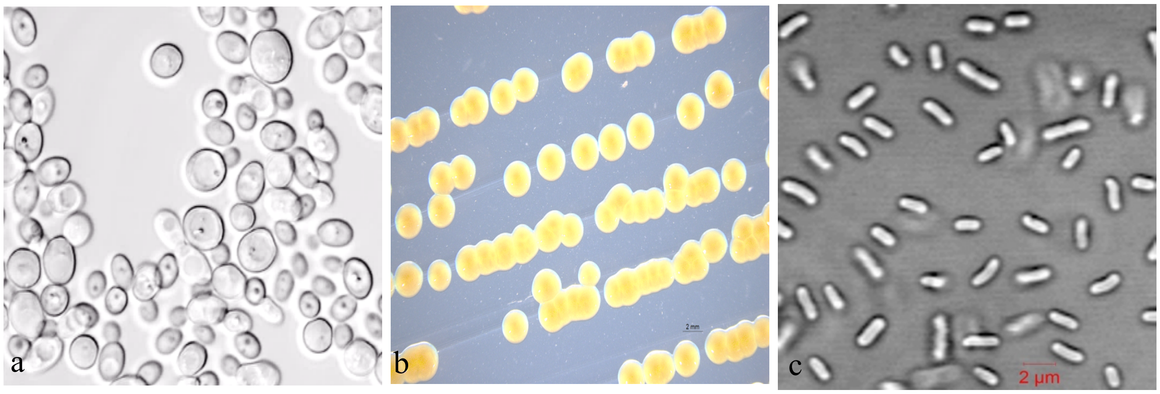


Fig. 3.25: Characteristics of bacterial strain *Chryseobacterium* JC507^T: (a) Bacterial like moving bodies (shown with arrows) observed within the vacuoles of the yeast (*Candida tropicalis* JY101); (b) Colony morphology of strain JC507^T; (c) Cell shape of strain JC507^T when observed under 63X objective.

3.8.2. 16S rRNA gene sequence similarities and phylogenetic affiliation

The EzBioCloud database used for the BLAST search analysis of the 16S rRNA gene sequence obtained (1423 nt) of strain JC507^T showed a similarity of 98.7% to *Chryseobacterium indologenes* NBRC 14944^T followed by 98.6% similarity to *Chryseobacterium arthrosphaerae* CC-VM-7^T and less than 98.6% to other species of the genus *Chryseobacterium*. A phylogenetic tree was constructed by using MEGA7 of strain

JC507^T clades along with the members of the genus *Chryseobacterium* and formed a separate branch along with *C. indologenes* followed by other taxa (Fig.3.26).

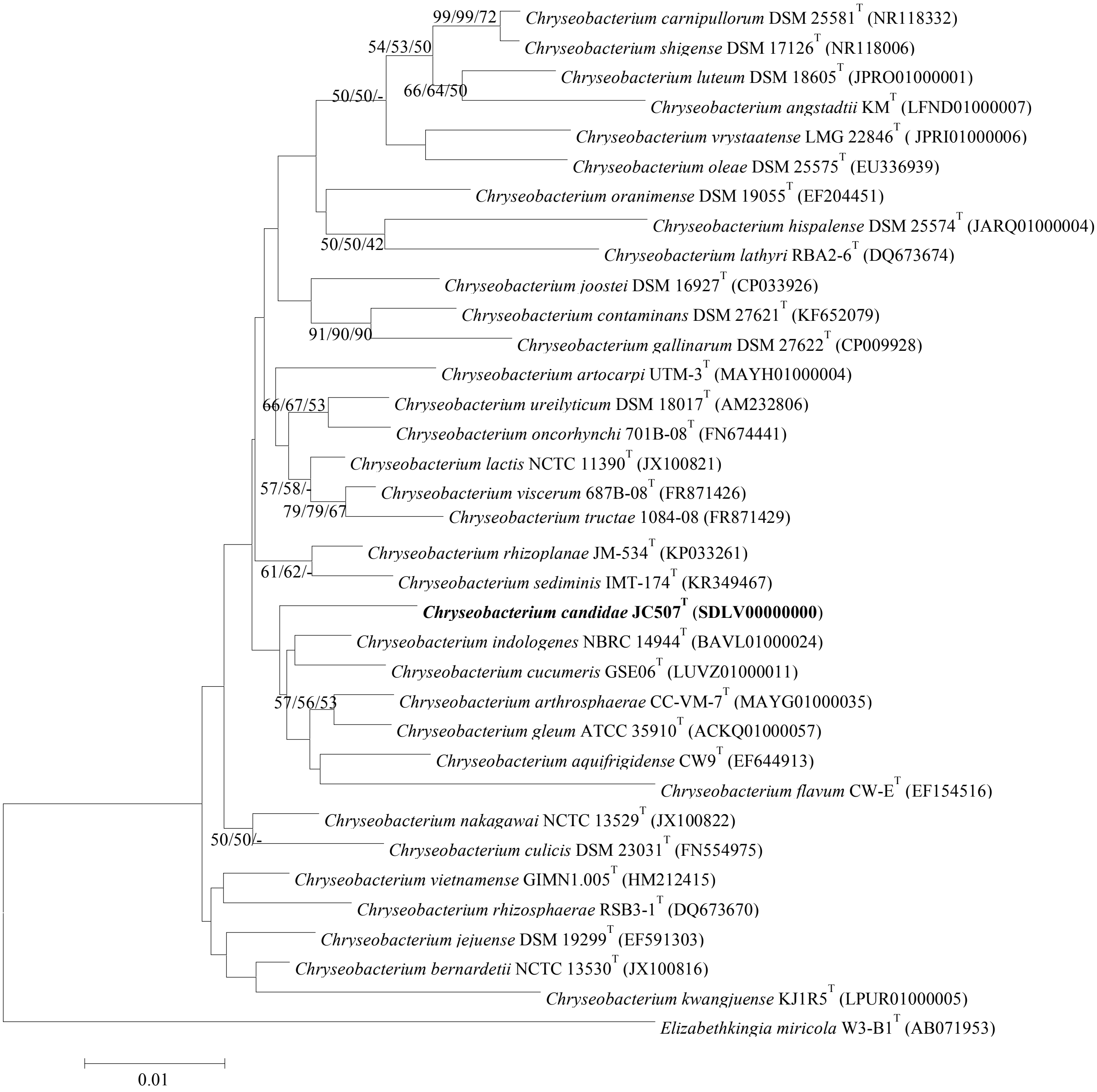


Fig. 3.26: Neighbour-joining tree based on 16S rRNA gene sequences showing phylogenetic relationships between strain JC507^T (1517nt) and representatives of some related taxa: The tree was computed with MEGA7 and rooted by using *Elizabethkingia miricola* as the out-group. The GenBank accession numbers for 16S rRNA gene sequences are shown in parentheses. Bootstrap percentages refer to NJ/ML/ME/MP analysis. Bar, 0.01 nucleotide substitution per position.

3.8.3. Genomic information

The draft genome size of strain JC507^T was 4,688,677 bp having 4399 protein coding genes and 68 RNAs. The genome was sequenced and consisted of total of 65 contigs in the genome assembly, with N_{50} of 177818 bp. The G+C content of strain JC507^T is 36.0 mol%.

3.8.3.1. *In-silico* DNA-DNA hybridization and OrthoANI values

The average nucleotide identity (ANI) values and *in-silico* DNA–DNA hybridization values between strain JC507^T and *C. indologenes* NBRC 14944^T, *C. arthrosphaerae* CC-VM-7^T and *C. gleum* ATCC 35910^T were 80.2, 83.0 and 87.0% and 24, 26.7 and 32.7%, respectively. The ANI and *d*DDH values of strain JC507^T with the type strains *C. indologenes* NBRC 14944^T, *C. arthrosphaerae* CC-VM-7^T and *C. gleum* ATCC 35910^T were far less than the recommended cut-off values for species delineation (95–96% for ANI and 70% for *d*DDH) and thus strain JC507^T represented a new species in the genus *Chryseobacterium*.

3.8.4. Physiological and biochemical analysis

Strain JC507^T is aerobic, showed growth between 10-40 °C, with optimum growth at 30 °C. pH range for growth was 6.0–9.0, with optimum growth at pH 7.0 and tolerated up to 3% NaCl (w/v) concentration with optimum growth at 1%. Strain JC507^T was catalase and oxidase positive. This strain hydrolysed aesculin, starch and carboxy methyl-cellulose, whereas did not hydrolyse urea and Tween compounds. Good growth was observed with trehalose, glycerol and sorbitol. Oxidation of cellobiose, sucrose, galactose, fructose, mannitol, salicin, inositol, mannose, maltose, lactose, L-arabinose, xylose, raffinose, dextrose, melibiose, inulin, sodium gluconate, adonitol, methyl a-D-glucoside, ribitol, melezitose, erythritol and xylitol were negative. Positive for glycerol, dulcitol, trehalose, fructose, sorbitol, galactose and methyl a-D-mannoside.

Table 3.11. Differentiating characteristics of strain JC507^{T} with the closely related species of the genus *Chryseobacterium.* 1, strain JC507^{T} and 2, *Chryseobacterium indologenes* NBRC 14944^{T}; 3, *Chryseobacterium arthrosphaerae* CC-VM-7^{T} and 4, *Chryseobacterium gleum* ATCC 35910^{T}

Characteristic	1	2	3	4
Cell size (µm; W x L)	0.2–0.4 x 1.0–2.0	0.3–0.6 x 2.0–4.0	1.0x2.0	0.3x1.0-3.0
Source of isolation	Yeast	trachea	Faeces of millipede	Human clinical specimen
Tolerance to 3% NaCl	+	+	-	+
Temperature range (optimum; ˚C)	10-40 (30)	5-35 (30)	5-37 (30)	5-37 (30)
Carbon Utilization (0.3% w/v)				
Mannitol	-	+	+	-
D-maltose	-	-	+	+
Xylose	-	-	+	
Fructose	-	-	-	+
Glycerol	-	+	-	+
Trehalose	+	+	-	+
Dulcitol	-	+	-	-
Sorbitol	-	+	-	+
Hydrolysis of				
Gelatin	-	-	+	-
Casein	-	-	+	+
Starch	+	-	+	+
Chitin	-	-	-	-
Esculin	+	-	+	+
CMC	+	-	-	-
Urea	-	+	+	+
Esterase	+	+	+	-
Esterase Lipase	+	+	-	+
Lipase	+	-	-	-
Leucine arylamidase	+	+	+	+
Valine arylamidase	+	-	+	+
Cystine arylamidase	+	-	-	-
Trypsin	-	-	+	-

Naphthol-AS-BI- phosphohydrolase	+	-	+	+
β-Galactosidase	-	-	-	+
α-Glucosidase	+	-	+	-
β-Glucosidase	-	-	-	+
N-acetyl-β-glucosaminidase	+	-	+	-
α-Fucosidase	-	+	-	-
G+C content	36.0	37.9	38.3	38.0
FATTY ACIDS				
anteiso-C11:0	13.4	7.8	-	-
anteiso-C12:0	2.0	1.4	-	-
anteiso-C13:0	9.0	6.0	TR	TR
anteiso-C15:0	8.0	6.5	1.14	1.0
anteiso-C17:0	5.0	3.0	TR	-
anteiso-C17:1ω9c	4.0	3.0	1.36	
iso- C13:0	-	-	1.07	1.0
iso- C15:0	10.0	19.0	33.68	34.13
iso-C16:0	2.0	3.0	1.43	1.13
iso-C17:0	-	-	1.58	1.64
C17:0-3OH	-	-	1.07	
iso-C17:0-3OH	11.0	14.0	20.23	18.30
iso-C15:0-3OH	2.7	3.0	3.36	3.69
iso-C16:0-3OH	2.0	3.0	1.21	1.0
C16:0	6.0	4.0	2.40	2.25
C18:0	3.0	-	-	-
C16:0-3OH	2.0	2.0	1.89	2.36
C18:1ω9c	2.0	1.0	1.0	0.54
Summed Feature 3	7.0	7.5	12.07	13.87
Summed Feature 9	6.5	10.0	11.73	14.43

All strains were catalase and oxidase positive. Acid production from glucose/sucrose, citrate utilization, esculin hydrolysis and nitrate reduction were not observed for all strains. All strains did not utilize galactose and inulin. β-Glucoronidase, α-chymotrypsin, α-galactosidase and α-mannosidase negative for all strains; positive for alkaline/acid phosphatase. CMC, Carboxy methyl-cellulose; Summed feature 3 was listed as $C_{16:1}\omega 7c$ and/or $C_{16:1}$ $\omega 6c$; summed feature 9 was listed as iso-$C_{17:1}$ $\omega 9c$ and/or $C_{16:0}$ 10-methyl.

3.8.5. Chemotaxonomic characters

3.8.5.1. Indole, pigment and quinone composition of strain JC507^T

Indole metabolites were detected at 280 and 500 nm and indole were identified based on absorption spectra of HPLC peaks between 270-285 nm. Seven putative indoles were identified in strain JC507^T through HPLC chromatograms (Fig.3.27). HPLC analysis confirmed the presence of flexirubin pigment produced by strain JC507^T and menaquinone-6 was the respiratory quinone of strain JC507^T which was analysed by the method of Xie and Yokota (Xie and Yokota, 2003).

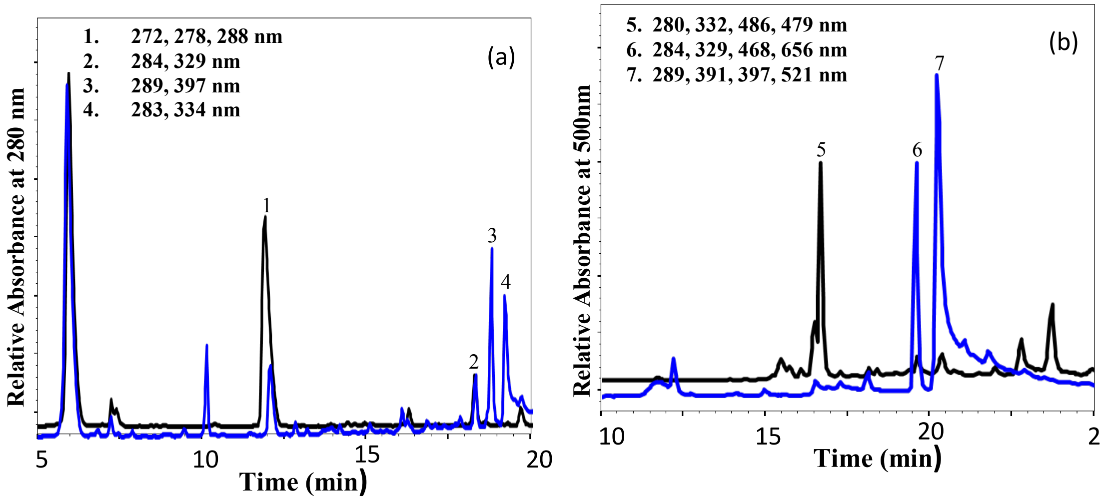


Fig. 3.27: HPLC chromatograms (a) at 280 nm and (b) at 500 nm] showing L-tryptophan catabolites produced by *Chryseobacterium indologenes* NBRC 14944^T (black line spectra) and strain JC507^T (blue line spectra).1-7, absorption maxima (nm) taken from the absorption spectra of the peak. Peaks which have not shown absorption spectra are not labeled.

3.8.5.2. FAME analysis

Major fatty acids (>10%) of strain JC507^T were iso-$C_{17:0}$ 3OH, anteiso-$C_{11:0}$ and iso-$C_{15:0}$. Minor (>1 but <10) amounts of anteiso-$C_{15:0}$, anteiso- $C_{13:0}$, anteiso-$C_{12:0}$, anteiso- $C_{17:1}\omega 9c$, anteiso-$C_{17:0}$, iso-$C_{15:0}$3OH, iso-$C_{16:0}$, iso-$C_{16:0}$3OH, $C_{16:0}$, $C_{18:1}\omega 9c$, $C_{18:0}$, $C_{16:0}$3OH, , summed feature 9 and summed feature 3 were present (Table 3.11).

3.8.5.3. Polar lipid profile

Strain JC507^T had phosphatidylethanolamine (PE), four unidentified amino lipids (AL1, 2, 3 and 4) and two unidentified phospholipids (L1 and 2) as the polar lipids (Fig 3.28).

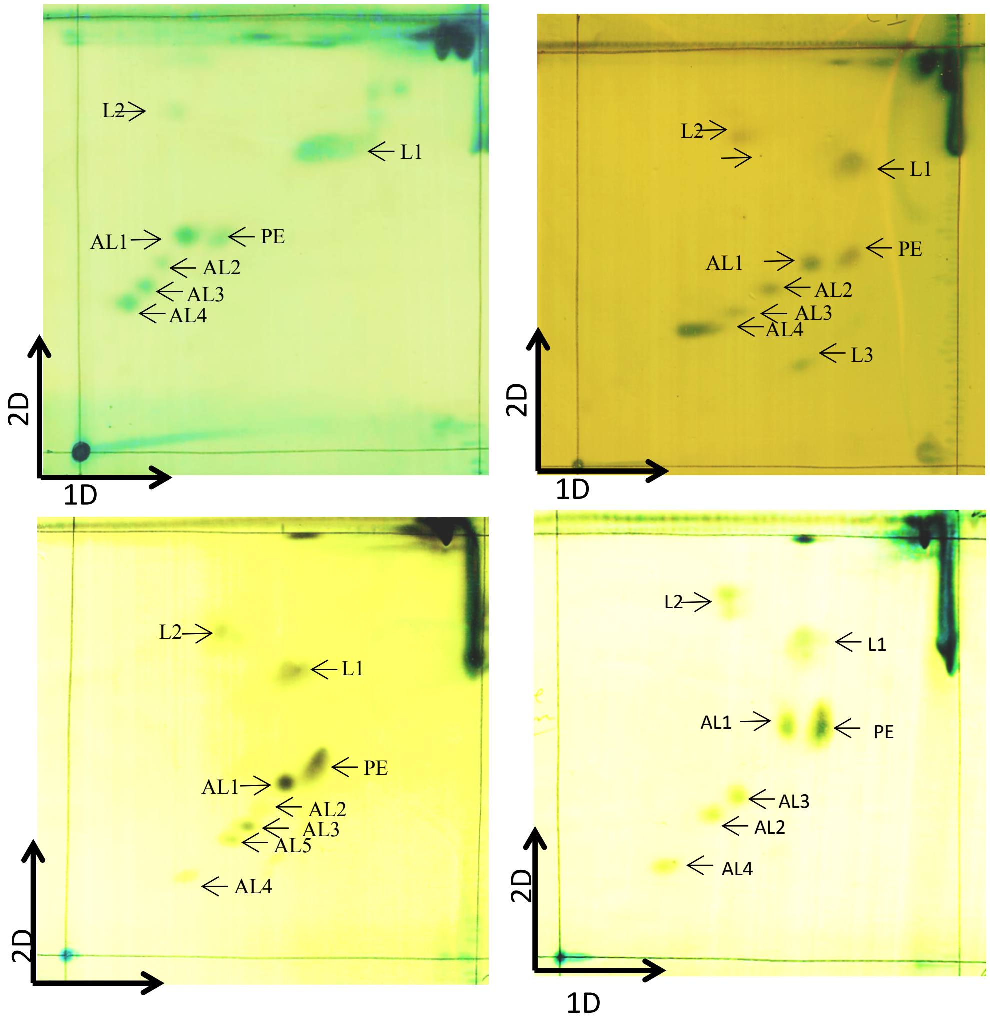


Fig. 3.28: Two-dimensional thin-layer chromatogram of whole cell polar lipids of strains JC507^T (a); *Chryseobacterium indologenes* NBRC 14944^T (b); *Chryseobacterium arthrosphaerae* CC-VM-7^T (c) and *Chryseobacterium gleum* ATCC 35910^T (d). PE, phosphatidylethanolamine; L1,2 uncharacterized lipids; AL1,2,3,4 unidentified amino lipids.

Infection studies of Candida tropicalis JY101

3.9. Infection studies of *Candida tropicalis* JY101

3.9.1. Screening and maintenance of fluorescently labelled bacteria.

Pseudomonas stutzeri JC703 and *Salmonella typhimurium* were successfully transformed with pBBRMCS plasmid containing mCherry gene and *E. coli* DH5α cells were transformed with GFP gene containing pBSU101 plasmid. The transformed colonies were picked on screening media containing gentamycin (25 $\mu g.ml^{-1}$) antibiotic for *Pseudomonas stutzeri* JC703 and *Salmonella typhimurium* and spectinomycin (65 $\mu g.ml^{-1}$) for *E. coli*. On antibiotic selective plates, the transformed colonies showed fluorescence at excitation/emission maxima of ~595/615 nm under confocal microscope. These fluorescent colonies were further sub-cultured and preserved in glycerol stocks and stored at -20 °C.

3.9.2. Selection and observation of autofluorescent bacteria

In addition to fluorescence labelling, naturally auto-fluorescent bacterial species were also studied and included in infection assays. Ten strains of anoxygenic phototrophic bacteria were screened for their auto-fluorescence. Of these, three species belonged to the genus *Rhodobacter*, one species of *Rubrivivax* (*R. benzoatilyticus*), one species of genus *Rhodomicrobium* (*R. udaipurense*), two strains of *Rhodopseudomonas* and one cyanobacterial species i.e., *Prochlorococcus* sp. Of the total species screened, only *Rhodopseudomonas palustris* and *Prochlorococcus* showed auto fluorescence and hence selected for the infection studies. The cells of *Prochlorococcus* were seen as green coloured cells under 470/500 wavelength/maxima and cells of *Rhodopseudomonas* were seen as red coloured cells under 595/615 wavelength/maxima.

3.9.3. Establishment and observation of infection studies

In order to study the interactions of bacteria with yeast, an experimental design was set up to co-culture the two microorganisms under certain conditions. For this, yeast *C. tropicalis* JY101 was used as a model for the design along with the above described bacterial species.

3.9.3.1. Infection of *C. tropicalis* JY101 with autofluorescent bacteria

Two autofluorescent bacteria i.e., *Prochlorococcus* sp. and *Rhodopseudomonas palustris* were used for the infection. Before proceeding with the infection, the purified yeast culture was first starved for two consecutive sub-cultures in order to deprive it of nutrients. This was done by growing the yeast cells in yeast carbon base (YCB) media lacking nitrogen or any other external nitrogen source at room temperature. The yeast cells were observed to exhibit poor growth in comparison to those grown in nutrient rich conditions. Secondly, autofluorescent bacteria were maintained in nutrient rich conditions while ensuring that fluorescence was retained. The two cultures, i.e., starved yeast and well-grown autofluorescent bacteria were co-cultured in tubes containing YCB without any external nitrogen source. The tubes were observed for at least 1 week, including observation of co-cultures under confocal microscopy. After 96 h of incubation, *Prochlorococcus* sp. and *Rhodopseudomonas palustris* were seen inside the yeast cell, specifically inside the vacuole where they exhibited rapid movements. *Prochlorococcus* sp. was observed as green fluorescent bacteria (Fig. 3.29a) and *Rhodopseudomonas palustris* TIE-1 cells were seen as red fluorescent bacteria (Fig 3.29b).

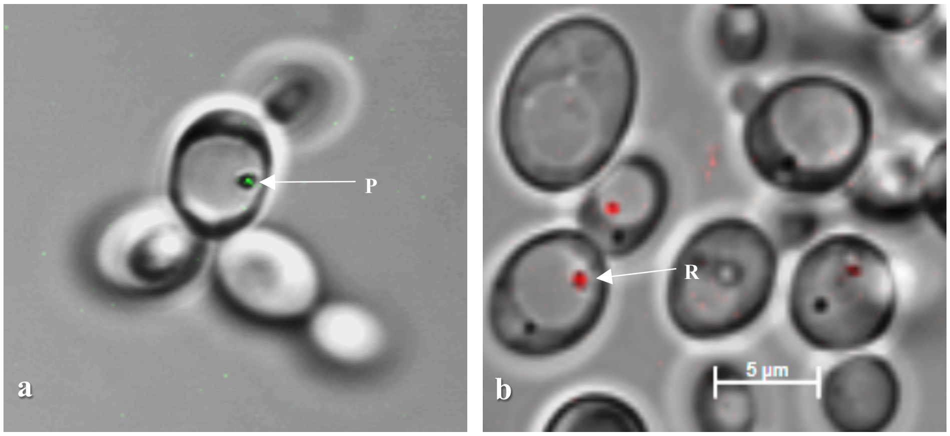


Fig. 3.29: Confocal microscopy images of yeast-autofluorescent bacteria co-cultures under nitrogen deficit: a- *Prochlorococcus* sp. as green fluorescent bacteria in *C. tropicalis* JY101 yeast vacuole, b- *Rhodopseudomonas palustris* TIE-1 as red fluorescent bacteria in *C. tropicalis*. Arrows: P, *Prochlorococcus* seen in vacuoles; R, *Rhodopseudomonas palustris*

3.9.3.2. Infection of *C. tropicalis* JY101 with fluorescently labelled bacteria

mCherry gene transformed *Pseudomonas stutzeri* JC703, *Salmonella typhimurium* and GFP transformed *E. coli* cell were used to infect *Candida tropicalis* JY101. The yeast cells were starved in a same way as mentioned in the infection with autofluorescent bacteria. The starved yeast cells were co-cultured with the above fluorescently labelled bacteria in tubes containing YCB without any external nitrogen source. These co-cultures were incubated at room temperature for 96 h. When observed under confocal microscope, successful entry of *Pseudomonas stutzeri* was observed inside the yeast vacuole (Fig. 3.30a; Fig. 3.30b) whereas on other hand, other fluorescent bacteria like *Escherichia* and *Salmonella* were not observed to infect or enter the yeast cells (Fig. 3.30c). Further, attachment of these bacteria could not be observed under microscope. In addition, a control experiment was also set up where the above steps were carried out without the addition/co-culturing with any other bacteria. Therefore, the control tubes consisted only of ‘starved’ yeast cells. Under confocal microscope, these control yeast cells had different cell morphology compared to those yeast cells co-cultured with bacteria. In particular, these control cells showed spore-like structures and a conspicuous lack of vacuoles even after 3 days of growth in the same media (Fig. 3.30d).

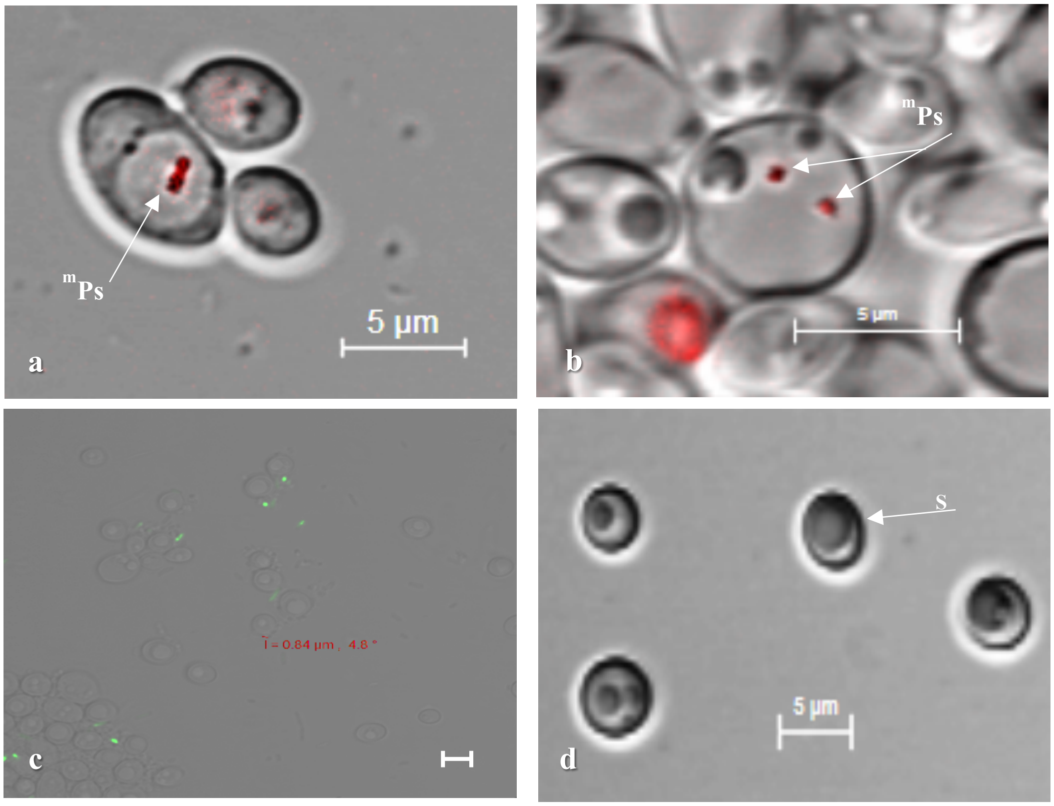


Fig. 3.30: Confocal microscopy images of yeast-bacteria co-cultures under nitrogen deficit: a and b- mCherry *tagged P. stutzeri* JC703 as red fluorescent bacteria in *C. tropicalis* JY101 yeast vacuole, c- *E. coli* DH5α as green fluorescent bacteria failed to enter in yeast when co-cultured with yeast strain JY101, d- Control *C. tropicalis* JY101 cells grown in nitrogen deficiency without bacteria forming spores

3.9.4. Hypothetical stages for infection of *C. tropicalis* JY101 with mCherry labelled *Pseudomonas stutzeri* JC703

Pseudomonas stutzeri JC703 which was isolated from the yeast *Candida tropicalis* JY101 was used as model bacteria to re-infect the same yeast strain. Time-dependent observations on the infection of *C. tropicalis* JY101 with *P. stutzeri* JC703 revealed different stages of bacterial entry into the yeast. The infection process was explored using scanning electron microscopy and confocal microscopy.

3.9.4.1. Scanning electron microscopy

Co-cultures of the yeast and bacteria were observed under scanning electron microscopy. For the 96-h grown co-culture of *C. tropicalis* JY101 with *P. stutzeri* JC703, *P. stutzeri* were seen to adhere onto the yeast cell in many numbers (Fig. 3.31a; counter supported by confocal image Fig. 3.31a1); particularly at the collar of the budding yeast (Fig. 3.31b; counter supported by confocal image Fig. 3.31b1, confocal microscopy was performed.

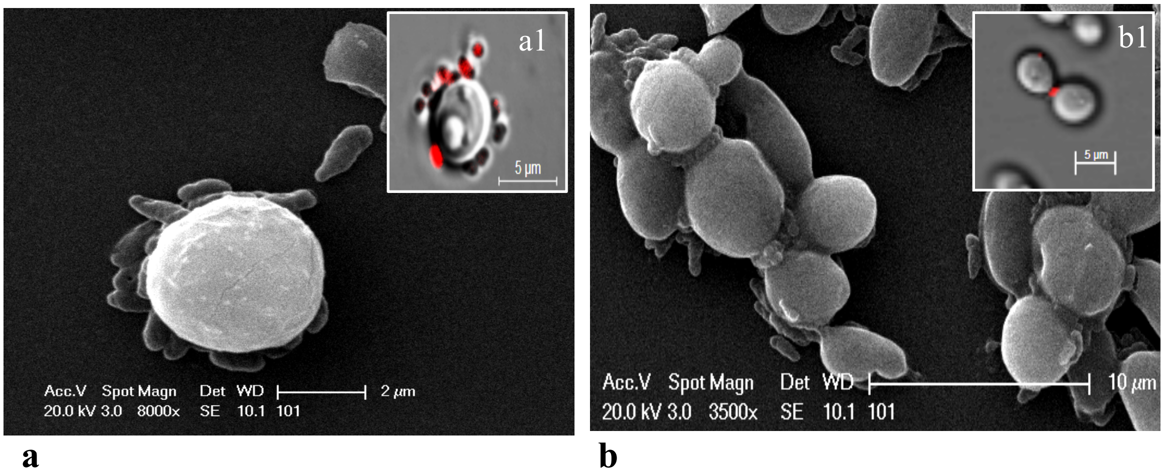


Fig. 3.31: Scanning Electron Microscopy (SEM) images: a- *P. stutzeri* JC703 bacterial attachments on a *C. tropicalis* JY101 cell, b- *P. stutzeri* JC703 bacterial attachments onto *C. tropicalis* JY101 bud collars. Inset a1- Confocal microscope image showing similar *P. stutzeri* JC703 attachment on a *C. tropicalis* JY101 cell, a2- Confocal microscope image showing similar *P. stutzeri* JC703 attachment onto budding *C. tropicalis* JY101 cells.

3.9.4.2. Confocal microscopic studies of infected cells of *C. tropicalis*

The infection process was further explored using confocal microscopy. Despite simultaneous attachment of more than ten bacteria to the exterior of *C. tropicalis* JY101, subsequent observations showed presence of just one or two bacteria inside yeast vacuole (Fig. 3.30). While measuring the cell size at each stage, a decrease in size (~0.8-0.6 µm) from (~1.8-1.6 µm) of the fluorescent bacterial cells present inside the yeast cell was noted. This indicated a reduction by close to half of the bacterial cell size. With these indications, we were able to capture the infection process and presented it diagrammatically (Fig. 3.32).

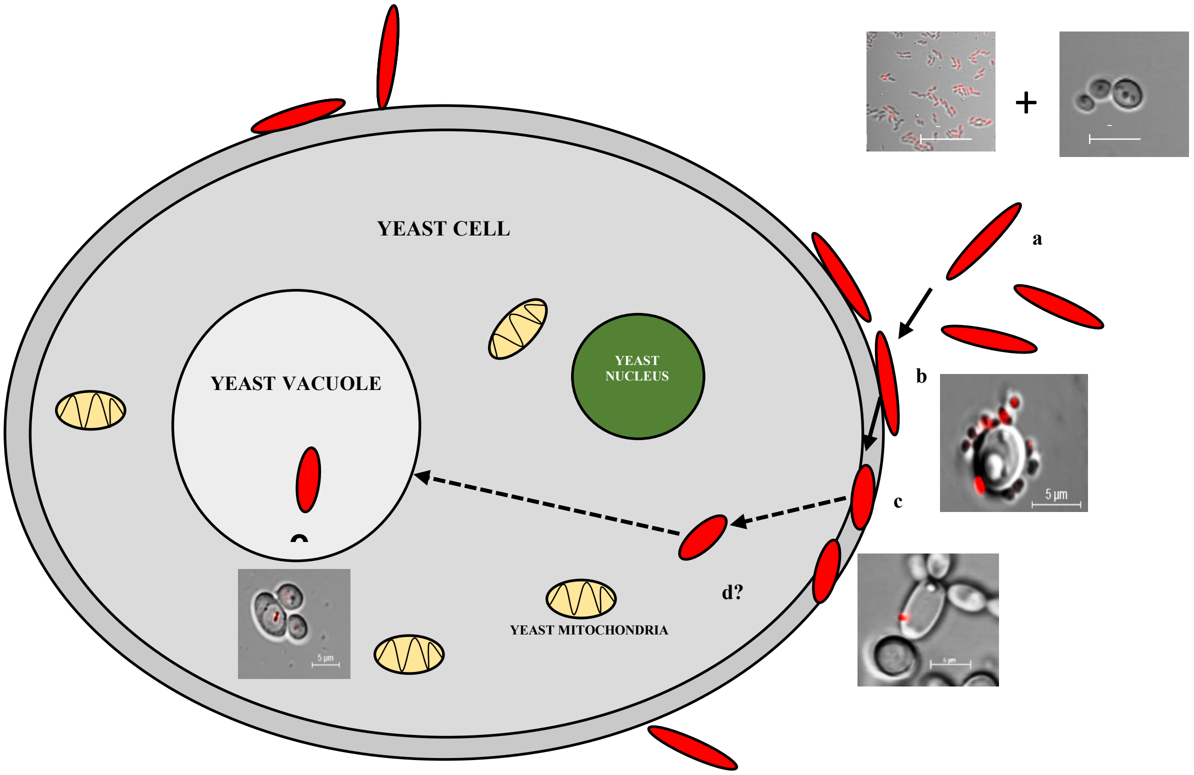


Fig. 3.32: Diagrammatic representation of stage-wise infection of fluorescent tagged bacteria to the yeast by co-cultivation: a- Co-culturing of bacteria and yeast, b- Attachment of bacteria to yeast cell, c- Reduction in bacterial cell size followed by entry into yeast periplasm, d- Entry of bacteria into cytoplasm (hypothetical; not observed) and e- Entry of bacteria into yeast vacuole, exhibiting rapid movements. Each stage is supported by confocal microscopic evidence observed for the infection of *P. stutzeri* JC703 in *C. tropicalis* JY101 as given inside the diagram.

Shotgun metagenomics of C. tropicalis

3.10. Holomicrobiome of *C. tropicalis* JY101

3.10.1. Sequencing and quality control

A total of 36,547,154 reads were obtained from the shotgun metagenome of *Candida tropicalis* with an average length of 196 ± 57 bp. Species richness and depth of the reads is represented in rare fraction curve (Fig. 3.33).

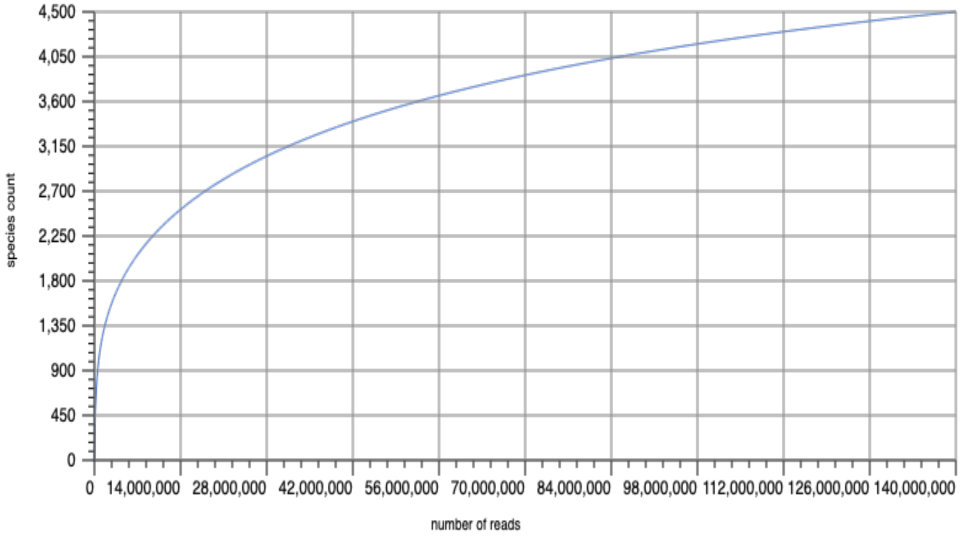


Fig. 3.33: Rarefaction curve of shotgun metagenome showing the saturation and read richness of *C. tropicalis*.
The curve represented the average number of different species annotations for subsamples of the complete data set and the curves were constructed using MG-RAST

3.10.2. Taxonomic distribution of holomicrobiome

A total 25,353,568 reads were assigned to different taxonomies when analysed using MG-RAST server. Eukaryotic sequences dominated among all the assigned reads accounting for 99.89% of the total reads (Fig. 3.34). Of total reads assigned, 25,198,452 reads were assigned to the domine eukaryote whereas 151,572 reads mapped bacterial domine which account for 0.11% of total reads followed by the 1011 and 2525 reads of viruses and archaea respectively (Fig. 3.34). Sequences from the shotgun metagenome of strain JY101 were assigned to 20 eukaryotic, 28 bacterial and 3 archaeal phyla. Several reads were also mapped to unclassified viruses. Ascomycota is the dominant phyla of domain Eukarya comprising of 99.75% of total reads followed by the members of phylum Chordata (Fig. 3.35a). Among the domine Bacteria, members of the phylum Proteobacteria contributed 52.8% of bacterial reads followed by the reads of Actinobacteria (13.0%) and Firmicutes (11.4 %) (Fig. 3.36a). Among three archaeal phyla, reads belonging to the Euryarchaeota dominated, followed by Crenarchaeota and Thaumarchaeota (Fig. 3.37a).

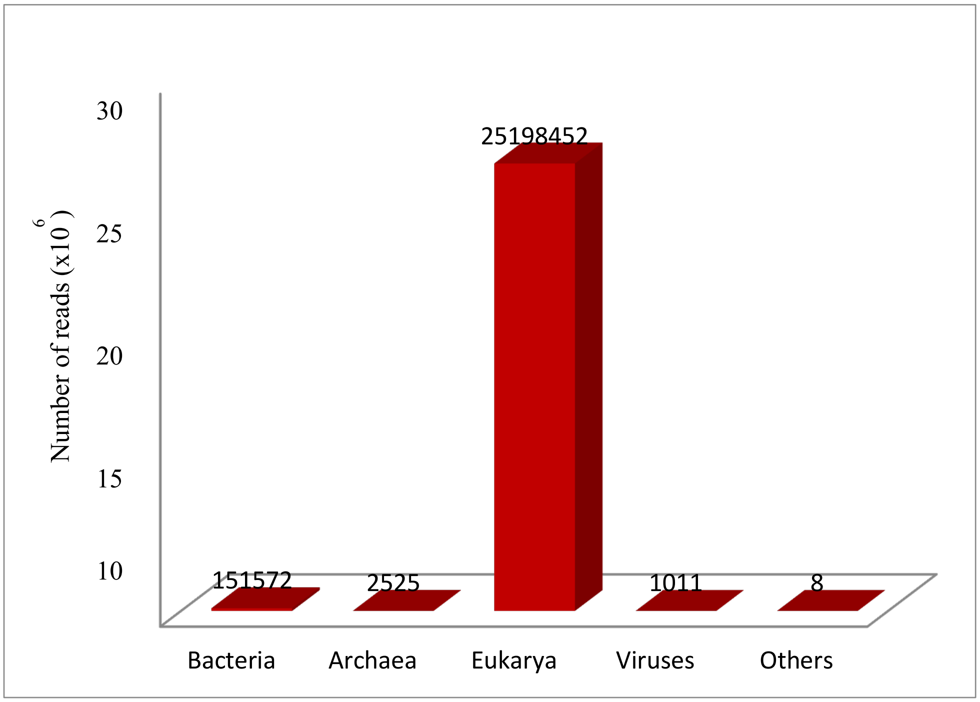


Fig. 3.34: Hologenome of *C. tropicalis* as represented by the number of sequence reads belonging to different domines obtained from the shotgun metagenome analysis .

Taxonomic distribution at genus level showed the dominance of reads belonging to the genus *Candida* with 99.53% of total reads followed by the reads of *lodderomyces* among eukaryotes (Fig 3.35b). Among bacteria, maximum number of reads were assigned to the genus *Staphylococcus* followed by reads of *Propionibacterium* and other bacterial genera represented in Fig 3.36b. Among the Archeaea, *Pyrococcus* and *Methanoculleus* were the dominant genera with 310 reads each (Fig 3.37b). The abundance of the reads at the genus level ordered from highest to least abundance were shown in Fig. 3.38.

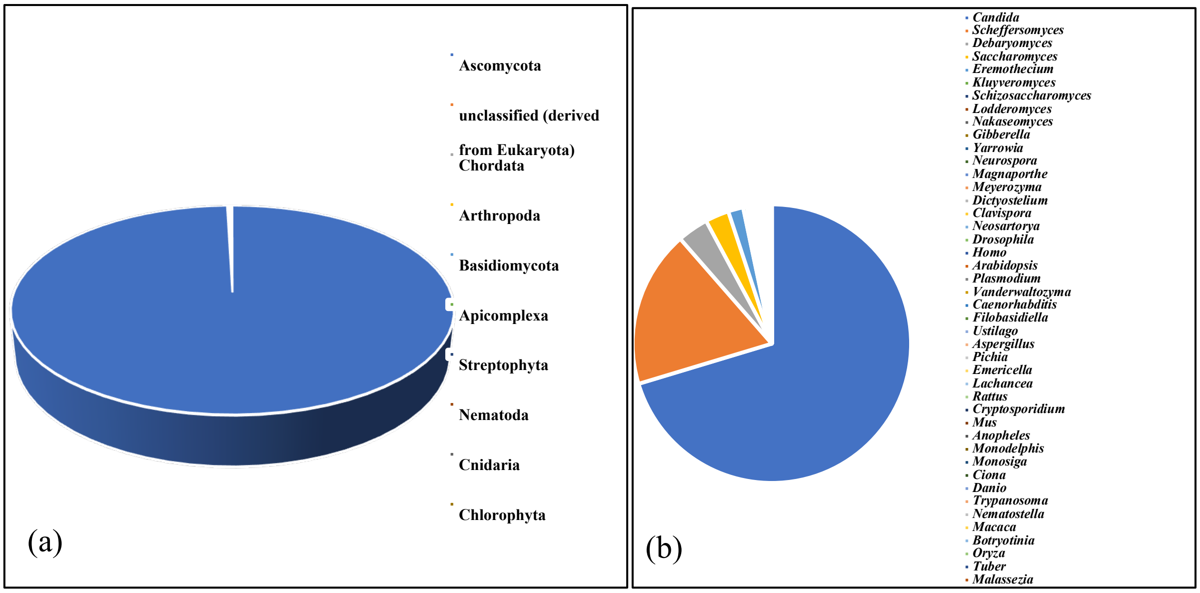


Fig. 3.35: Taxonomic abundances of the domine Eukarya showing at the level of phylum (a) and genus (b).

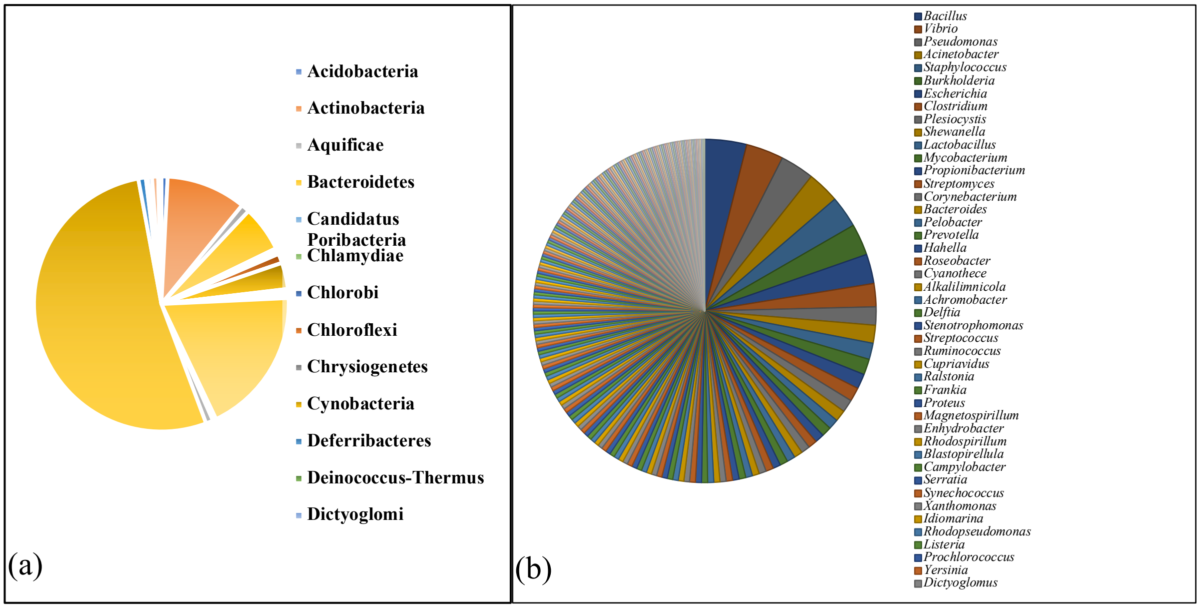


Fig. 3.36: Taxonomic abundances of the domine Bacteria showing at the level of phylum (a) and genus (b).

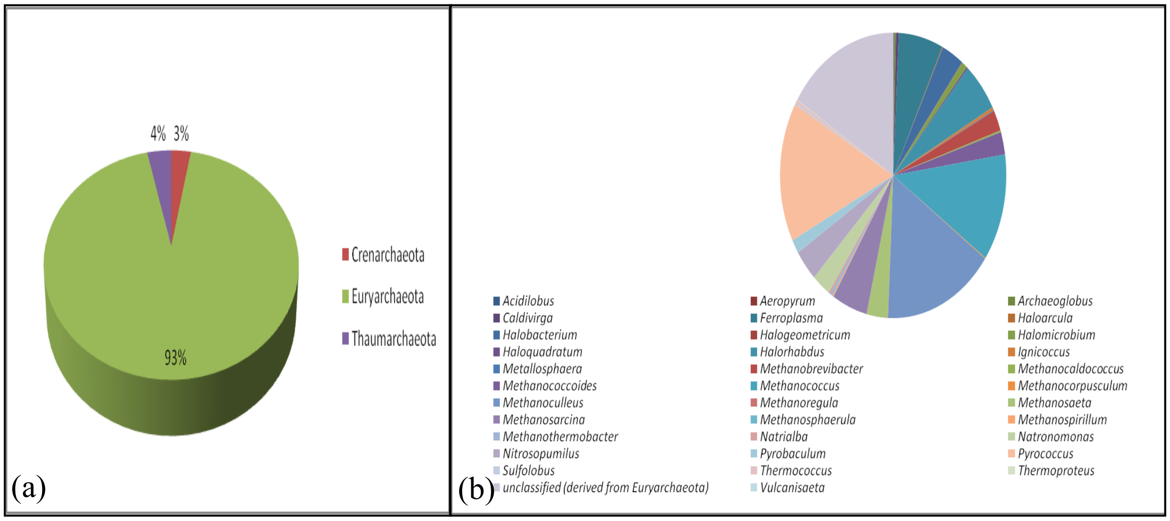


Fig. 3.37: Taxonomic abundances of the domine Archaea showing at the level of phylum (a) and genus (b).

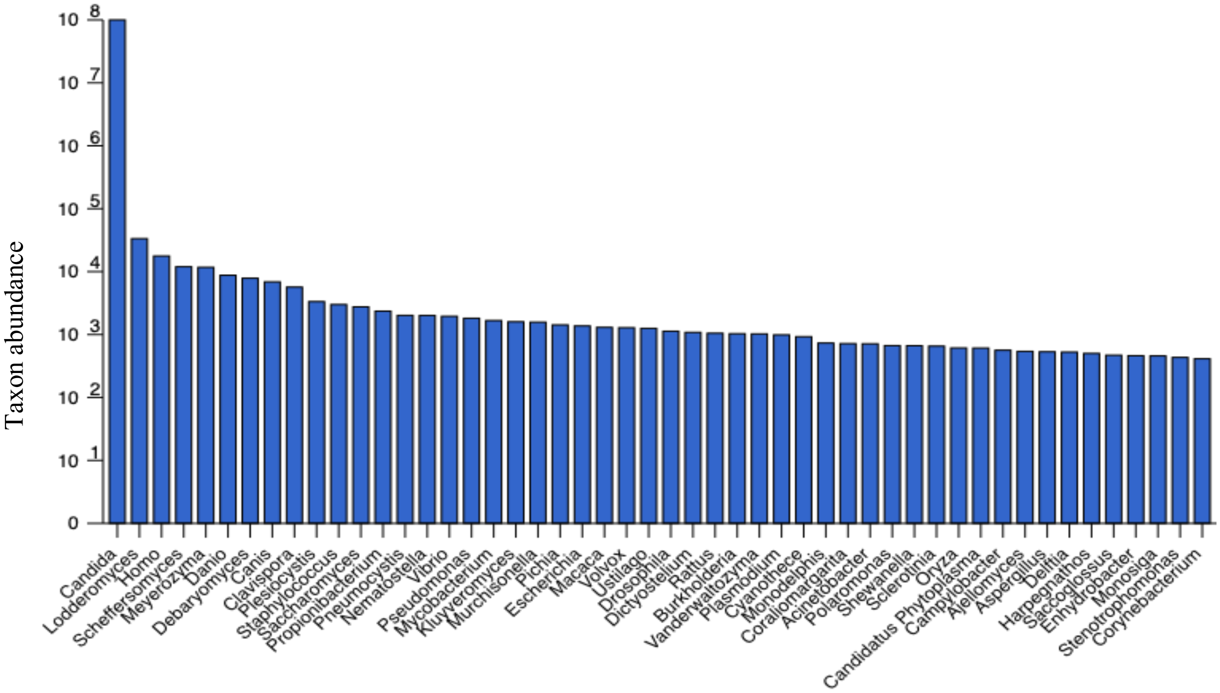


Fig. 3.38: The plots represent the taxonomic abundances ordered from the most abundant to least abundant. The y-axis represent the abundances of annotations on a log scale. The plot was constructed in MG-RAST server

Discussion

Discussion

Endosymbionts are the key life forms for evolution and prokaryotes play an important role in the formation of higher life forms. No organism including animals or plants are autonomous entities but are holobionts *i.e.*, a network of host with its associated microorganisms (Bordenstein and Theis, 2015). Endosymbiosis is a highly dynamic process as it imparts the benefit of sharing mutualism. In this process, some of the endosymbiotic bacteria became integral components of the host as organelles, while a few remained as endosymbionts of fungi (Bianciotto and Bonfante, 2002), plants (Santoyo et al., 2016) and animals (Perotti et al., 2006). In this context, the present study has observed the existence of bacteria-like-bodies (BLBs) moving inside vacuoles of yeast cells (Movie 1). To understand how BLBs can associate themselves with the yeast cells, it is indispensable to understand the physiology of yeast cells and their potential to harbor bacteria. Broadly, yeast acclimatizes itself to punitive environmental conditions such as temperature, desiccation, pH and salt. Since yeast cells can adapt to difficult environmental condition, it is possible that the yeast may serve as a niche that protects the bacteria against the environmental stresses. The way around could also be the possible that the endo-bacteria are protecting the yeast from stress. The recognition of endosymbionts and their characterisation is essential to understand the relationship between different life forms. A clear knowledge of yeast and endo-bacterial diversity is indispensable to probe deeper into yeast bacterial interaction.

4.1. Yeast Taxonomy

While screening for the presence of bacteria in the yeast cells, as many as fifty-six yeasts strains were isolated from different environmental sources. Of the total yeast isolated, 75% belong to the phylum Ascomycota and remaining to the phylum Basidiomycota (Table 3.1) with majority of the taxa having 99-100% ITS or D1/D2 sequence identity with the recognised type species of yeast. Majority of the yeast (90%) when observed under confocal microscope showed the presence of bacteria like bodies (Fig. 3.1). While cataloging the diversity of yeast across India (Table 3.1), three potential new species of yeast (represented as *Candida* sp. JY118^T, *Candida* sp. JY124^T and *Suhomyces* JY150^T) were charecterised using polyphasic taxonomy.

4.1.1. *Candida* sp. JY118

Even though the ITS region sequence identity was more than 99% and D1/D2 of LSU region identity of 98% with the nearest species of genus *Candida*, we still found differences in physiological properties including the utilisation of various carbon and nitrogen sources. Phylogenetic tree drawn based on the gene sequence of ITS and D1/D2 region showed the cladding of strain JY118^T with *Candida glabrata* CBS 138^T (Fig. 3.3).

4.1.1.1. Description of *Candida punciae* JY118^T sp. nov.

(pu'ni.cae. N.L. gen. n. *punciae* of *Punica*)

On YPD media, after three days of incubation, cells appear to be globose, shiny with entire margins. Colonies are either present singly or in pairs or small clusters. Cells are spherical, ovoid (2.5-3.5 µm ×3-5 µm), occur singly or in budding pairs. Sexual reproduction could not be demonstrated. Assimilate different carbon and nitrogen sources including trehalose, ethanol, glycerol, 50% glucose, inulin, D-ribose, D-xylose, D-arabinose and L-lysine and do not assimilate sucrose, lactose, raffinose, melibiose, starch, rhamnose, cellobiose, salicin, L-arabinose, methanol, ribitol, myo-inositol, mannitol, succinate, gelatin, cycloheximide (0.01% and 0.1%), citrate, nitrate and nitrite. Ferment glucose and trehalose but cannot ferment D-galactose, maltose, sucrose, lactose, and raffinose. Type strain is JY118^T (=KCTC 27783^T)) isolated from fermented pomegranate. ITS, D1/D2 gene sequences are deposited with NCBI with accession numbers; LT838866, and LT840077.

4.1.2. *Candida* sp. JY124

NCBI and YeastIP BLAST analysis of gene sequence of ITS region of strain JY124 showed 97% identity with *Candida suratensis* CBS 10928^T (Table 3.1). Different cladding patterns were observed when the phylogenetic trees were drawn based on the gene sequences of ITS and D1/D2 region with the nearest members belonging to the genus *Candida* (Fig. 3.2; Fig. 3.3). The molecular differences were supported by the phenotypic dissimilarities among the strain JY124 and its nearest member *C. surantensis* CBS 10928^T (Table 3.2) thereby warranted the description of strain JY124 into a new species of the genus *Candida* for which we propose the name *Candida gracillariae* sp. nov.

4.1.2.1. Description of *Candida gracillariae* JY124^T

(*gra.cil.la'ri.ae. N.L. gen. gracillariae of Gracillaria*)

Colonies appear as white with regular margin, present singly or in small clusters when grown on YPD media. Cells are spherical to ovoid with size 3-4 µm × 4-6 µm in size. Divides asexually via budding, assimilate glucose, sucrose, raffinose, salicin, D-xylose, ribitol, glycerol, mannitol, succinate, 50% glucose, L-lysine, inulin, trehalose, maltose, starch, rhamnose, ethanol, methanol, nitrate, and citrate. Sexual reproduction could not be demonstrated. Ferment raffinose and maltose; does not ferment D-galactose, maltose, sucrose, lactose and raffinose. Type strain is JY124^T (= KCTC 27784^T; = CCY 29-190-1^T) isolated from brown *gracillaria*. ITS and D1/D2 gene sequences were deposited with NCBI having accession numbers are LT838872, and LT840079 respectively.

4.1.3. *Suhomyces* sp. JY150

Two ascomycetous yeast strains (JY150 and JY152) were isolated from mushrooms (*Agaricus* sp.) collected from Hyderabad, India and strain ST431, isolated from insect frass in Pak Thong Chai District, Nakhon Ratchasima Province was given as a gratis by Prof. Savitree Limtong and group, Thailand. The D1/D2 domain gene sequences of strain JY150, JY152 and ST431 showed 95.5% identity with the type strain *Suhomyces bolitotheri* CBS 9832^T and less than 95% similarity with other strains of genus *Suhomyces*. The ITS region of the strain JY150 showed 89.4% similarity with the type strain *Suhomyces bolitotheri* CBS 9832^T. Thus, based on the phylogenetic analysis, physiological properties and their unique nucleotide sequences for the D1/D2 domains of the LSU gene and the ITS region (Table 3.3; Fig. 3.5; Fig. 3.6), we propose to designate this newly isolated strain as a new species in the genus *Suhomyces* as *Suhomyces agaricola* sp. nov.

4.1.3.1. Description of *Suhomyces agaricola* sp. nov. JY150^T

(a.ga.ri'co.la. N.L. n. *Agaricus*, a fungal genus; L. suff. -cola, an inhabitant, dweller; N.L. n. *agaricola*, an inhabitant of *Agaricus*)

Colonies appear as white colored with smooth and flat surface when grown on yeast dextrose peptone agar media when incubated for 3-4 days at room temperature. Cells are spherical to ovoid, 3-4 µm × 4-6 µm in size. Divides asexually by multilateral budding. Sexual state could not be demonstrated. Mesophilic growth range from 25-35 °C. Fermentation occurs with D-glucose, but not with D-galactose, maltose, sucrose, lactose, and raffinose. Growth occurs with the carbon sources D-glucose, D-galactose, D-glucosamine, D-ribose, D-xylose, D-arabinose, salicin, trehalose, glycerol, erythritol, ribitol, ethanol, mannitol, and cadaverine. L-Lysine is utilized for growth. Delineation of the species is primarily based on ITS and D1/D2 sequence analysis and is further strongly supported by phenotypic traits. Type strain is JY50^T (= KCTC 27885^T = CCY 96-1-1^T) isolated from mushroom, Telangana, India. ITS and D1/D2 gene sequences were deposited with NCBI and their accession numbers are ERP113581 and DQ404522.

4.2. Metagenomic studies

Commonly, culture dependent techniques are used to analyse and understand bacterial diversity of a sample. For this, different media are used to culture microorganisms and this method is limited to the isolated bacteria only. It is widely accepted in microbiology that less than 1% of total microorganisms have been cultivated so far (Vartoukian et al., 2010).

In the begining of the twenty first century, with the advancement of sequencing technologies, culture independent bacterial diversity analysis gained importance (Daniel, 2005; Handelsman, 2005). In culture independent methods, sequencing of minute amounts of the sample provides the details of the bacterial diversity of a sample. Metagenomic analysis was further revolutionised with high throughput sequencing methods like next generation sequencing techniques which can sequence the whole human DNA in a single day (Behjati and Tarpey, 2013) or mNGS which can sequence all the nucleic acid content of microorganisms present in a sample (Gu et al., 2019).

In this study, presence of more than one BLB in the yeast (Movie 1) led us to investigate and understand the bacterial diversity of yeast. The bacterial diversity was examined through 16S rRNA gene metagenome analysis. Prior to sequencing, the purity of yeast was confirmed by different methods. First, BLBs moving inside the yeast were confirmed using bacterial viability kit (Fig. 7 b and c). Next, we performed FISH, which is known as a powerful tool to detect bacteria without the need for cultivation. The rRNA bacterial probe EUB338-Cy3 has been routinely employed to detect the bacterial population in the sample. FISH analysis using EUB338-Cy3 probe resulted in successful hybridization of the probe inside the yeast cells (Fig. 3.21). Subsequent sub-culturing of yeast on different bacterial media (nutrient agar, thiobacillus agar, marine agar, Biebl and Penning media) did not yield any bacterial colonies. With these leads, the bacterial communities were analysed with both culture independent and dependent techniques.

In the culture independent techniques, the 16S rRNA gene metagenome analysis was performed for twenty nine yeast strains and the results obtained were astonishing. About 85% of the yeasts examined in this study possessed bacterial reads and also the presence of the rich diversity of bacteria associated with the yeast (Fig. 3.15; Fig. 3.16). Isolation of yeast-like fungus, *i.e., Zalaria obscura* JY119 presented the opportunity to utilize its system as an out-group. It was intriguing to note that the bacterial diversity of JY119 was markedly different from the rest of the yeast as can be seen from the bar plots and heatmaps (Fig. 3.15; Fig. 3.16; Fig. 3.17; Fig. 3.18). This evidently conveys the specificity of bacterial diversity among fungi.

Phylum and genera-specific diversity patterns were also analysed and spanned for all yeast strains. Members of the phylum Firmicutes were dominated in the yeast analysed in this study (Fig. 3.16 and Fig. 3.18). Dominant Firmicutes taxa of yeasts were *Streptococcus*, *Staphylococcus* and *Planococcus* (Fig. 3.16 and Fig. 3.20) which are coccoidal and are in co-relation with stained BLBs which were observed initially under confocal microscopy (Fig. 3.7). Also, reads of rod shaped unclassified

Pseudomonadaceae were obtained in the metagenome analysis. This heterogeneity in the morphology of bacteria is difficult to explain and needs further research.

Generally, the microbiota are linked to the properties of habitat from where the sample is taken or the DNA is isolated. But in our results, the bacterial diversity did not show any dependence on environmental conditions from which the yeast hosts were isolated (Table 3.1). Instead, the diversity was specific to the yeast host itself, as indicated by the comparison among *Candida* and *Pichia* species (Fig. 3.10; Fig. 3.14). This culture-independent method helped to explore the existence of a bacterial "community" as against studies reporting the presence of single bacterial species inside the yeast cell (Saniee et al., 2013; Siavoshi and Saniee, 2014; Tavakolian et al., 2019). Also, as many as 10^7 live bacteria were counted in a single spore of the Arbuscular Mycorrhizal Fungus, *Gigaspora margarita* (Bianciotto and Bonfante, 2002) which supports the existence of communities. Uncovering a wealth of bacterial reads from twenty-five different yeasts in this study reveals substantial numbers of bacteria that cannot be easily dismissed. Consequently, future studies should focus on understanding where and how the yeast hosts these bacteria.

"The time has come to replace the purely reductionist 'eyes-down' molecular perspective with a new and genuinely holistic, eyes-up, view of the living world, one whose primary focus is on evolution, emergence, and biology's innate complexity."—Carl Woese

4.3. Insights into bacteria associated with *Candida tropicalis* JY101

4.3.1. Cultivated and characterized bacteria from yeast

Candida tropicalis strain JY101 was selected as a model yeast to study and validate the bacterial diversity. Several reads of unclassified *Pseudomonadaceae* were observed in the metagenome analysis of strain JY101 which can be culturable. Methods like physical rupturing (using glass beads) and zymolase treatment yielded four different bacteria. They were the members of genus *Pseudomonas*, *Chryseobacterium*, *Lysinibacillus* and *Propionibacterium*. EzTaxon BLAST search of the 16S rRNA gene sequence (1387 nt) of *Pseudomonas* sp. JC703 had the highest (99.9%) identity with *Pseudomonas stutzeri* ATCC 17588^T. While the *Chryseobacterium* sp. JC507 sequence (1428 nt) had highest (98.7%) identity with *Chryseobacterium indologenes* NBRC 14944^T followed by other members of the genus *Chryseobacterium* with <98.6% identity. *Lysinibacillus* sp JC1018 sequence (889 nt) had the highest (100%) identity with *Lysinibacillus fusiformis* NBRC 15717^T. *Propionibacterium* sp. JC704 sequence (1396 nt) had the highest similarity (98.6%) with *Propionibacterium acne* DSM 1897^T (renamed as *Cutibacterium acnes*; Dreno B et al., 2018). *Propionibacterium* sp. JC704 lost its viability soon after its isolation. It is proven that many obligate endosymbionts are uncultivable such as "*Candidatus* Glomeribacter gigasporarum" (Salvioli et al., 2008). It can be similarly hypothesised that *Propionibacterium* sp. JC704 has become an obligate endosymbiont of yeast probably due to the loss of few essential genes making it dependent on the host. Such a dependency is frequently observed in the evolution of endosymbionts as shown by Mehta, 2018 (Mehta et al., 2018). Also, the reads of the genus *Propionibacterium* sp. were common to all the twenty-five yeast strains suggesting its status as an obligate endosymbiont of yeast. Interestingly, *P. acnes* has been observed to adapt to new organisms as an endosymbiont, displaying a flexibility for hosts (Campisano et al., 2014). Therefore, it may not be so unusual to note the presence of *Propionibacterium* inside yeast cells.

Out of 12 genera identified through the metagenome of *C. tropicalis* JY101, only members of three genera could be successfully cultivated in this study. Hence, a large number (~95%) remains yet-to-be cultivated or lost cultivability, indicating an unexplored black box of diverse microbiota of yeast. Among those that were sub-cultured, *Chryseobacterium* sp. JC507 was new species belonging to genus *Chryseobacterium* which was further described and validated.

4.3.1.1. *Chryseobacterium sp.* JC507

EzBiocloud BLAST analysis of 16S rRNA gene sequence yielded a similarity of 98.7% with the closest member of genus *Chryseobacterium*. Their low DNA-DNA hybridisation value between strain JC507 and *C. indologenes* NBRC 14944^T, *C. arthrosphaerae* CC-VM-7^T and *C. gleum* ATCC 35910^T of 24, 26.7 and 32.7%, respectively and ANI score between strain JC507^T and *C. indologenes* NBRC 14944^T, *C. arthrosphaerae* CC-VM-7^T and *C. gleum* ATCC 35910^T were 80.2, 83.0 and 87.0 % respectively which were far less than the recommended cut-off for species delineation (95-96%;Yoon et al., 2017) indicated that the strain JC507 represents a new species of genus *Chryseobacterium*. Strain JC507 had phosphatidylethanolamine (PE), two unidentified lipids (L1, L2) and four unidentified amino lipids (AL1-4) (Fig. 3.28). The major fatty acids (>10 %) were iso-$C_{15:0}$, anteiso-$C_{11:0}$ and iso-$C_{17:03}$OH (Table 3.11). Based on the phenotypic, chemotaxonomic and phylogenetic analysis, strain JC507 represent a new of the genus *Chryseobacterium*, for which we propose the name *Chryseobacterium candidae* sp. nov.

4.3.1.1.1. Description of *Chryseobacterium candidae* JC507^{T} sp. nov.

(can'di.dae. N.L. gen. n. candi- dae of the yeast *Candida*, from where the type strain was isolated)

Cells are rod shaped, Gram-stain-negative, 0.2–0.4 µm wide, 1.0–2.0 µm long, aerobic, non-motile and divide by binary fission. Colonies are slimy, round with smooth margin, and pale yellow in colour on nutrient agar plate. Flexirubin pigments are produced. Optimum growth is at 30 °C. Optimum pH for growth is 7.0. Tolerates up to 3% NaCl (w/v), oxidase and catalase positive. Produces indole derivatives from L-tryptophan. Acid is produced from glucose and sucrose. Nitrate reduction and citrate utilization are negative. Starch, aesculin, and carboxy methyl-cellulose are hydrolysed. Gelatin, Tween 40/80 and urea are not hydrolysed. Grows well with glycerol, trehalose and sorbitol. Oxidation of cellobiose, sucrose, galactose, mannitol, inositol, salicin, L-arabinose, maltose, mannose, xylose, lactose, fructose, dextrose, raffinose, inulin, melibiose, sodium gluconate, arabitol, adonitol, erythritol, melezitose, methyl a-D- glucoside and xylitol are negative. Positive for trehalose, glycerol, sorbitol, dulcitol, fructose, methyl a-D-mannoside and galactose. Enzyme activity (API ZYM) positive for: acid phosphatase, alkaline phosphatase, cysteine arylamidase, valine arylamidase, leucine arylamidase, esterase lipase (C4), lipase, N-acetyl-b-glucosaminidase and naphthol-AS-BI-phosphohydrolase. Negative for: trypsin, b-glucuronidase, a-fucosidase, a-chymotrypsin, b-galactosidase, a-glucosidase, b-glucosidase, a-galactosidase and a-mannosidase. Respiratory quinone Menaquinone 6 (MK-6) is present in strain JC507^{T}. Iso-$C_{15:0}$, anteiso-$C_{11:0}$ and iso-$C_{17:03}$OH are the major fatty acid. Minor amounts of anteiso- $C_{13:0}$, anteiso-$C_{15:0}$, anteiso-$C_{12:0}$, anteiso-$C_{17:0}$, iso-C1$_{6:0}$, iso-$C_{15:03}$OH, iso-$C_{16:0}$3OH, $C_{16:0}$, $C_{18:0}$, $C_{16:0}$3OH, summed feature 3 and summed feature 9 are present.

phosphatidylethanolamine (PE), two unidentified lipids (L1, L2) and four unidentified amino lipids are the polar lipid composition. DNA base composition is 36.0 mol% G+C.

Type strain is JC507^T (=KCTC 52928^T = NBRC 113872^T = MCC 4072^T), isolated from yeast *Candida tropicalis* JY101. NCBI accession number for 16S rRNA gene sequence is LT838865 and GenBank accession number for the whole genome is SDLV00000000. Culture is maintained as glycerol stock stored at -20 °C.

4.3.2. Infection studies of *Candida tropicalis* JY101

As mentioned above, total four bacteria were isolated from *C. tropicalis*. The function and role of these bacteria of yeast need to be elucidated, particularly concerning the host which bears the cost of harbouring them. The recent finding on interactions between the yeast *Rhodotorula mucilaginosa* harbouring N_2-fixing *P. stutzeri* and rice plants highlights the role of endosymbiotic bacteria in the nitrogen needs of plants (Sen et al., 2019). This example parallels the role of endobacteria of plant-associated filamentous fungi (Paul et al., 2020). Similarly, certain species of the isolated bacteria from our study as well as *Rhodopseudomonas palustris* used for infection assays, are known to harbour *nif* genes that help in nitrogen fixation (Cantera et al., 2004; Yan et al., 2008). We hypothesize that this capability could be of use to the yeast host and potentially form a bipartite system. A simple strategy was applied to infect a yeast host with the isolated bacteria.

Co-cultures of *C. tropicalis* along with fluorescently labelled *P. stutzeri* JC703 showed the establishment of *P. stutzeri* JC703 inside the yeast cell under nitrogen crisis (Fig. 3.30a). The control cultures of *C. tropicalis* without *P. stutzeri* formed spore like structures with poor growth (Fig. 3.30d). Devoid of a helpful partner, the control yeast cells possibly coped in the absence of an important nutrient like nitrogen by forming spores. These observations support our hypothesis that *P. stutzeri* JC703 and other bacteria aid the host under nitrogen deprivation, providing scope for further analysis. Although no reads of *Prochlorococcus* sp. were observed in the yeast 16S rRNA metagenome, its entry into the yeast under nutrition deficiency was seen (Fig. 3.29). This could demonstrate the ability of the yeast to acquire bacteria if the situation demands. In such cases, these bacteria could be temporary residents where they may be considered as beneficial guests to the yeast under stress. This is unlike the case where bacteria like *Rickettsia* that have, through obligate endosymbiosis, evolved into organelles like mitochondria.

Examination of co-cultures of *C. tropicalis* and *P. stutzeri* JC703 presented important observations portraying each stage of bacterial infection of the yeast (Fig. 3.32). Stage 1 showed the adherence of more than one bacterial cell to yeast surface. No specific site of adherence was observed yet most of the adherence was seen either on the collar of the dividing yeast cell or on young buds of yeast (Fig. 3.31). In stage 2, entry of only one or two bacteria could be viewed into the periplasmic space of the yeast with reduced bacterial cell size. Size reduction is speculated to be necessary for the bacteria to infect the yeast cell and may be a factor for permitting bacterial entry. Hence, large bacterial cells like *E. coli* or *S. typhimurium* unable to undergo size reduction could not infect *C. tropicalis*, while *P. stutzeri* could make its entry into the host. The importance of cell size for infection of yeast is also supported by the presence of dominating reads of endobacteria which are small cocci. In stage 3, these bacteria channel themselves from the yeast periplasm to the cytoplasm and finally reach the yeast vacuole, where they reside and exhibit rapid movements (Fig. 3.32). These interesting stages of infection should be carefully explained with supporting experimental designs that can unravel the molecular events controlling this phenomenon and critically probe the process of bacterial infection of yeast.

4.4. Holobiont analysis using shotgun metagenomic approach

Holobiont (host and its diverse microbial symbionts) are the well-defined interactors, reproducers/and manifestos of adaptation and hence considered as levels of selection in evolution (Roughgarden et al., 2018). The concept of hologenome considered all holobiont from obligatory to loosely defined state of symbiosis (Rosenberg and Rosenberg, 2008). The genomic and post-genomic era that today's science is undergoing helped to contribute to the concept of holobiont, with the advent of shotgun metagenomics. Apart from knowing the taxonomy (the "who is there"), it is now easy to get data on functional aspects (the "what are they doing?").

Although the holobiont concept is majorly associated with symbiosis, it also encompasses a larger picture of a well-oiled machine which houses associations between hosts and microorganisms that directly or indirectly impact each other. This definition has great impact on how we view an assemblage of organisms, if we hypothesize them to be a holobiont. The concept when applied to the associations seen between yeast and bacteria could help hypothesize the existence of an ecological interaction between the two, *i.e*, these organisms may not be merely co-occurring, they may bear importance on one another. From this point of view, if one considers the yeast cell as the holobiont, then the analysis of its hologenome will be greatly interesting to find out the taxonomy as well as functional properties of the holobiont. To this end, high throughput sequencing of *C. tropicalis* has demonstrated the rich bacterial, archaeal and viral diversity present inside this yeast (Fig. 3.33). As many as more than 28 bacterial and archaeal phyla reads were retrieved from the hologenome of *C. tropicalis*. It would be intriguing to analyse more such "hologenomes" of other yeast and find out common and differing trends. For the data at hand, it appears that there is a strong relationship between findings from 16S rRNA gene-based metagenome and shotgun metagenome analyses. Most of the taxa claimed to be abundant under the former approach, appeared in the shotgun analysis as well. Further, *C. tropicalis* also maintained the bacterial homeostasis for generations as the reads of same cohort of bacteria were present in the 16S rRNA amplicon based metagenome of first, fifth and tenth sub-culturing (Fig. 3.23). From Fig 3.33 (obtained through shotgun metagenomics), it can be seen that proteobacteria is dominant, which is comparable to data from Fig 3.18 (obtained through 16S rRNA gene metagenomics). This clearly indicates that the preliminary analysis for the discovery of bacterial diversity associated with *C. tropicalis* holds strong. Other abundant bacterial taxa include members of the phylum Firmicutes and Acidobacteria which were seen in initial analysis also.

The next stage in establishing yeast as a holobiont with its many associations with lower taxa like bacteria needs to be investigated at functional level as well. Results from this analysis, combined with more evidence on the infection process will go a long way in understanding the true relationship among the yeast microbiota. Such hypotheses could drive future research on these matters, for which the present study can act as a foundation.

Summary and Major findings

Summary and Major Findings

- Fifty-five yeast and one yeast like fungus were purified and identified based on ITS/D1D2 gene sequences BLAST search analysis. Of which 75% belong to Ascomycota, remaining with Basidiomycota.
- Strains JY118, JY124 and JY150 were identified as putative novel taxa. JY118 and JY124 were putative new species of the genus *Candida*, whereas JY150 was putative new species belonging to genus *Suhomyces* which were further characterized on the basis of their morphological, physiological and phylogeny.
- Amplicon based metagenome analysis revealed the presence of large ghost population of bacteria which reside in yeast.
- Firmicutes (~45%) remain the dominant phylum followed by Proteobacteria (~25%) and Actinobacteria (~22%) for all the yeasts examined.
- Passaging of yeast-maintained endo-microbial community homeostasis.
- Guestimates indicate 95% of endobacterial species of *C. tropicalis* as non-cultivable.
- *Pseudomonas stutzeri, Propionibacterium acne, Lysinibacillus fusiformis* and *Chryseobacterium* sp. were isolated from *Candida tropicalis.*
- *Chryseobacterium candidae* JC507^{T} was described as a new species based on phylogenomic integrated polyphasic taxonomic analysis.
- Successfully infected *C. tropicalis* with two autofluorescence bacteria; *Prochlorococcus* sp. and *Rhodopseudomonas palustris* in addition to mCherry tagged *Pseudomonas stutzeri.*
- Hologenome analysis exhibited a rich bacterial, archaeal and viral diversity associated with *C. tropicalis.*

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Publications

7. Publications

1. **Indu B.,** Kumar G., Smita N., Shabbir A., Sasikala Ch., and Ramana Ch. V. (2020). Chryseobacterium candidae sp. nov., isolated from a yeast (Candida tropicalis). *Int J Syst Evol Microbiol.* 70:93-99. doi.org.10.1099/ijsem.0.003716
2. **Indu B**, Keertana T, Ipsita S, Jagdeeshwari U, Sasikala Ch, and Ramana Ch. V. Do yeasts host bacteria? Uncovering the hidden bacterial ghost communities of yeast. *Scientific Reports*. (under revision)
3. Tushar L, **Indu B**, Sasikala Ch, Ramana Ch.V. (2018). Transcriptome analysis of hopanoid deficient mutant of *Rhodopseuodomonas palustris* TIE-1. *Microbiological Research Volume* 218, 108-117.
4. Anusha R, **Indu B**, N. Smita, G. Deepshikha, K. Gaurav, K. Dhanesh, G. Suresh, Ch. Sasikala, and Ch. V. Ramana (2019). Emerging Concepts in Bacterial Taxonomy. Microbial Diversity in Ecosystem Sustainability and Biotechnological Applications. Springer, Singapore.
5. Suresh G, Tushar L, **Indu B**, Sasikala Ch, Ramana Ch.V. (2019). Taxogenomics resolved the conflict of the genus *Rhodobacter*: A two and half decades pending thought to reclassify the genus *Rhodobacter*. *Frontiers in Microbiology*. Volume 10, 2480. DOI:10.3389/fmicb.2019.02480.

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6. **Indu,** Keertana T, Ch. Sasikala and Ch. V. Ramana (2018). Yeast endobacteria: Diversity and interaction. Poster presentation in **“**59th Annual Conference of Association of Microbiologists of India (AMI)**”** Dec 09-12 2018, Hyderabad, India.
7. **Indu**, Sasikala Ch and Ramana Ch. V. (2015). Insights of aromatic hydrocarbon metabolism of *Rhodomicrobium udaipurense* JA643^T. Poster 56th Annual Conference of Association of Microbiologists of India (AMI)” Dec 07-10 2015, Hyderabad, India.

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Chryseobacterium candidae sp. nov., isolated from a yeast (*Candida tropicalis*)

Indu B,[1] Kumar G.,[1] Smita N.,[1] Shabbir A.,[2] Sasikala Ch.[1,*] and Ramana Ch. V[1]

Abstract

A Gram-stain-negative, rod shaped, non-motile, aerobic bacterium (strain JC507^{T}) was isolated from a yeast (*Candida tropicalis* JY101). Strain JC507^{T} was oxidase- and catalase-positive. Complete 16S rRNA gene sequence comparison data indicated that strain JC507^{T} was a member of the genus *Chryseobacterium* and was closely related to *Chryseobacterium indologenes* NBRC 14944^{T} (98.7 %), followed by *Chryseobacterium arthrosphaerae* CC-VM-7^{T} (98.6 %), *Chryseobacterium gleum* ATCC 35910^{T} (98.5 %) and less than 98.5 % to other species of the genus *Chryseobacterium.*The genomic DNA G+C content of strain JC507^{T} was 36.0 mol%. Strain JC507^{T} had phosphatidylethanolamine, four unidentified amino lipids and four unidentified lipids. MK-6 was the only respiratory quinone. The major fatty acids (>10 %) were anteiso-C$_{11:0}$, iso-C$_{15:0}$ and iso-C$_{17:0}$3OH. The average nucleotide identity and *in silico* DNA–DNA hybridization values between strain JC507^{T} and *C. indologenes* NBRC 14944^{T}, *C. arthrosphaerae* CC-VM-7^{T} and *C. gleum* ATCC 35910^{T} were 80.2, 83.0 and 87.0 % and 24, 26.7 and 32.7 %, respectively. The results of phenotypic, phylogenetic and chemotaxonomic analyses support the inclusion of strain JC507^{T} as a representative of a new species of the genus *Chryseobacterium*, for which the name *Chryseobacterium candidae* sp. nov. is proposed. The type strain is JC507^{T} (=KCTC 52928^{T}=MCC 4072^{T}=NBRC 113872^{T}).

The family *Flavobacteriaceae* comprises the genus *Chryseobacterium* which was first established by Vandamme *et al.* [1]. At the time of writing, more than 100 species of bacteria (www.bacterio.net/chryseobacterium.html) have been placed in the genus *Chryseobacterium* and were isolated either from clinical samples [2], fish [3], fresh water [4], sewage and plants [5], soils [6, 7], meat [8] or waste water [9]. New species with valid names added during 2018–2019 included *Chryseobacterium salipaludis* [10] *Chryseobacterium aurantiacum* [11], *Chryseobacterium phosphatilyticum* [12], *Chryseobacterium glaciei* [13] and *Chryseobacterium populi* [14]. The typical characteristics of the genus *Chryseobacterium* include the presence of an aerobic type of metabolism, branched-chain fatty acids, iso-C$_{15:0}$ and iso-C$_{17:0}$3-OH as the major fatty acids, phosphatidylethanolamine as a major polar lipid, menaquinone-6 (MK-6) as the characteristic respiratory quinone, and production of flexirubin-type pigments [15–17]. Some of the members of this genus are associated as endophytes [12, 17] and during our study on yeast diversity, we have isolated an endobacterium (strain JC507^{T}) of a yeast (*Candida tropicalis*) which was characterized by a genomic and polyphasic taxonomic approach.

A yeast (strain JY101) was isolated from a soil sample collected from Hyderabad, India (22° 98′ N 71° 47′ E). The soil sample was serially diluted and plated onto a nutrient agar plate containing chloramphenicol (100 µg ml^{-1}). Several white-coloured colonies were observed which were further purified and sequenced using yeast-specific primers used for amplification of the ITS regions and the D1/D2 domain of the 26S rRNA gene. The Internal transcribed spacer (ITS) region was amplified with ITS1 (5′-GTCGTAACAAGGTTTCCGTAGGTG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers (31). The D1/D2 domain of the 26S rRNA gene was amplified using NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) primers. NCBI/CBS BLAST analysis of strain JY101 for D1/D2 (599 bp; NCBI accession numberLT999794) and ITS (340 bp; NCBI accession number LT795043) showed 100 % sequence similarity to the yeast *Candida tropicalis*. It was interesting to observe moving objects inside the vacuoles of *C. tropicalis* JY101 (Fig. S1, available in the online version of this article). There are a few reports [18, 19] of yeast hosting bacteria such as *Helicobacter pylori*. However, these reports were

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strainJC507^{T} is LT838865. The GenBank/EMBL/DDBJ accession for the whole genome shotgun sequence is SDLV00000000.
One supplementary table and three supplementary figures are available with the online version of this article.

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Transcriptome analysis of hopanoid deficient mutant of *Rhodopseuodomonas palustris* TIE-1

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ABSTRACT

All three domains of life have an ordered plasma membrane which is pivotal in the selective fitness of primitive life. Like cholesterol in eukaryotes, hopanoids are important in bacteria to modulate membrane order. Hopanoids are pentacyclic triterpenoid lipids biosynthesised in many eubacteria, few ferns and lichens. Hopanoid modulates outer membrane order and hopanoid deficiency results in the weakened structural integrity of the membrane which may in turn affect the other structures within or spanning the cell envelope and contributing to various membrane functions. Hence, to decipher the role of hopanoid, genome-wide transcriptome of wild-type and Δ*shc* mutant of *Rhodopseudomonas palustris* TIE-1 was studied which indicated 299 genes were upregulated and 306 genes were downregulated in hopanoid deficient mutant, representing ~11.5% of the genome. Thirty-eight genes involved in chemotaxis, response to stimuli and signal transduction were differentially regulated and impaired motility in hopanoid deficient mutant showed that hopanoid plays a crucial role in chemotaxis. The docking study demonstrated that diguanylate cyclase which catalyses the synthesis of secondary messenger exhibited the capability to interact with hopanoids and might be confederating in chemotaxis and signal transduction. Seventy-four genes involved in membrane transport were differentially expressed and cell assays also explicit that the multidrug transport is compromised in Δ*shc* mutant. Membrane transport is reliant on hopanoids which may explain the basis for previous observations linking hopanoids to antibiotic resistance. Disturbing the membrane order by targeting lipid synthesis can be a possible novel approach in developing new antimicrobials and hopanoid biosynthesis could be a potential target.

1. Introduction

Membrane lipids are hydrophobic biomolecules which play a vital role in bacterial cell physiology. Initially, it was thought that membrane lipids are the static barrier. But with advanced findings, it is recognized as a complex and dynamic component of the membrane which selectively connects cell with its extracellular environment. In eukaryotes, lipid raft organizes signal transduction proteins which are enriched in specific lipids such as cholesterols (López and Kolter, 2010). Lipid rafts can sense extracellular signals, and they support different cellular processes, including cell cycle, cytoskeleton rearrangement, signal transduction, vesicle trafficking and cellular homeostasis (Barak and Muchova, 2013; Bramkamp and Lopez, 2015). Sterol lipids (cholesterol and its derivatives) are widely distributed in a eukaryotic system and form a vital component of membrane lipids along with phospholipids and sphingolipids which play a key role in signal transduction and various membrane functions (Hannich et al., 2011). Prokaryotes contain lipid rafts (microdomains) but sterols are absent in prokaryotic members with few exceptions (Wei et al., 2016). But bacteria harbour hopanoid which is widespread of all complex natural products and analogous to sterols and are probably an essential constituent of many prokaryotes (Blumenberg et al., 2012, 2010; Doughty et al., 2009; López and Kolter, 2010; Rohmer et al., 1984; Saenz et al., 2012b; Wei et al., 2016).

Abbreviations: ATP, Adenosine triphosphate; BCAA, Branched chain amino acid; CARC, CRAC like motif; CCCP, Carbonyl cyanide m-chlorophenyl; CRAC, Cholesterol recognition amino acid consensus; DMSO, Dimethyl sulfoxide; GO, Gene Ontology; H33342, Bisbenzimide H33342; MPP1, Membrane palmitoylated protein; NPN, N-phenyl-naphthylamine; OD, Optical density; PBS, Phosphate buffer saline; PDB, Protein Data Bank; PDZ, Post-synaptic density; qPCR, Quantitative polymerase chain reaction; *shc*, squalene hopene cyclase; TBDT, TonB-dependent transport system; WT, Wild-type; YP, yeast extract, peptone; YPP, Yeast extract, peptone and pyruvate

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Taxogenomics Resolves Conflict in the Genus *Rhodobacter*: A Two and Half Decades Pending Thought to Reclassify the Genus *Rhodobacter*

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The genus *Rhodobacter* is taxonomically well studied, and some members are model organisms. However, this genus is comprised of a heterogeneous group of members. 16S rRNA gene-based phylogeny of the genus *Rhodobacter* indicates a motley assemblage of anoxygenic phototrophic bacteria (genus *Rhodobacter*) with interspersing members of other genera (chemotrophs) making the genus polyphyletic. Taxogenomics was performed to resolve the taxonomic conflicts of the genus *Rhodobacter* using twelve type strains. The phylogenomic analysis showed that *Rhodobacter* spp. can be grouped into four monophyletic clusters with interspersing chemotrophs. Genomic indices (ANI and *d*DDH) confirmed that all the current species are well defined, except *Rhodobacter megalophilus*. The average amino acid identity values between the monophyletic clusters of *Rhodobacter* members, as well as with the chemotrophic genera, are less than 80% whereas the percentage of conserved proteins values were below 70%, which has been observed among several genera related to *Rhodobacter*. The pan-genome analysis has shown that there are only 1239 core genes shared between the 12 species of the genus *Rhodobacter*. The polyphasic taxonomic analysis supports the phylogenomic and genomic studies in distinguishing the four *Rhodobacter* clusters. Each cluster is comprised of one to seven species according to the current *Rhodobacter* taxonomy. Therefore, to address this taxonomic discrepancy we propose to reclassify the members of the genus *Rhodobacter* into three new genera, *Luteovulum* gen. nov., *Phaeovulum* gen. nov. and *Fuscovulum* gen. nov., and provide an emended description of the genus *Rhodobacter sensu stricto*. Also, we propose reclassification of *Rhodobacter megalophilus* as a sub-species of *Rhodobacter sphaeroides*.

Keywords: *Rhodobacter*, taxogenomics, *Rhodobacter sensu stricto*, gen. nov., *Rhodobacter* reclassification, phylogenomics, proposal of 3 new phototrophic genera, photosynthetic gene cluster

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Emerging Concepts in Bacterial Taxonomy

1

Anusha Rai, Indu, N. Smita, G. Deepshikha, K. Gaurav, K. Dhanesh, G. Suresh, Ch. Sasikala, and Ch. V. Ramana

Abstract

Bacterial taxonomy has progressed over the years by virtue of the brisk and competent scientific developments. Ground-breaking molecular techniques have added an edge in the phylogenetic studies, resulting in the quality description of the taxa under studies. New avenues are rapidly developing whose validation has always been embraced and included, which will assist in resolution. It began with the simple application of objective procedures for classification, and now we have arrived at the genome-based taxonomy. This pedantic step has led to the meticulous examination and served to reconcile certain conflicts of the status of the taxa. This field is dynamic and is exploring more options like proteomics and metabolomics in gaining more insights into the lineal heritage. Even though there has been a significant change and addition, there is an ever-growing need for a comprehensive study, which would thread all the attributes together into one functional unit of classification. In this review, we examine the paradigm shift from traditional taxonomy to integrated taxonomy useful in the characterisation of bacteria which in addition aids in the identity of biotechnological targets.

Keywords

Bacterial taxonomy · Polyphasic · Phylogenomics · Integrated taxonomy · Average nucleotide sequence index (ANI)

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