Puja Saluja(2009). *Exploration of yeasts from selected niches of India and study of thermotolerance.* Ph.D. Thesis. CSIR-IMTECH, Chandigarh/ Jawaharlal Nehru University, New Delhi: India.
Supervisor: Dr. G.S. Prasad

Acc. No. : Th-205

8.1. Highlights of the work

Yeasts are probably one of the earliest domesticated organisms without people actually realizing them to be yeasts. They are well-known for wide-ranging roles in fermentation, as established model organisms, as hosts for heterologous protein-expression systems *etc.* Despite the realized and/or unrealized biopotential of yeasts, the rate of species discovery of yeasts has not kept pace with time. According to recent estimates only about 1000 yeast species were described till 2005. A great majority of the known yeast species comes from Western Europe, Japan and North America. The yeast diversity in India is largely unexplored except for a few recent studies (Rao *et al.*, 2008, Bhadra, 2008).

8.2. Diversity of Yeast Isolates from soil samples

In the present study an attempt has been made to examine the diversity of yeasts from soil and flowers collected from Ganganagar and Rawatbhata in Rajasthan and coal-belt region of Khammam district in Andhra Pradesh. Both soil and flower samples were collected from Ganganagar and Rawatbhata, while only soil samples could be collected from Khammam (Coal-belt region has very little flowering during summer months). Total 34 samples were collected from all the three regions.

The number of yeast isolates in soil ranged from 0 to 10^4 CFU/g. Total 85 yeast isolates were picked-up for examining their diversity. These isolates were initially grouped into 5 groups based on colony and cell morphologies, this was followed by sub-grouping them by using microsatellite fingerprinting patterns generated with two primers PMS1 $(GTG)_5$ and PMS2 $(GAC)_5$. The isolates showing identical patterns were considered as strains of one species and representative isolates were selected and examined by sequencing the D1/D2 domain of large subunit (LSU) rRNA gene and the internal transcribed spacer (ITS) region. Isolates that showed different patterns with any one of the primers were considered unique and examined by sequencing.

Sequence analysis of ITS and D1/D2 domains showed that grouping of isolates based on morphological characteristics mostly correlated with the sequence-

based identification of yeast isolates. Analysis of microsatellite fingerprinting patterns showed identical patterns for a group of isolates (irrespective of the primer used), they were found to be strains of same species. Isolates having minor differences in banding patterns mostly showed at least one base substitution or deletion in the sequences from related isolates. Most of the isolates of group-1 (total 59) were identified as members of the genus *Pseudozyma*. Similarly, among the 33 isolates of group-2, 27 were related to different species of the genus *Cryptococcus* that formed slimy colonies. All the 13 isolates of group-3 were identified as species of basidiomycetous genera *Rhodosporidium* or *Rhodotorula*. All the 3 isolates from group-4, and 3 isolates from group-2 were found to be related to *Aureobasidium pullulans*. All the ten members of group-5 were identified as ascomycetous yeasts.

Interestingly 81% of the soil isolates were basidiomycetous yeasts, and 19% were ascomycetous yeasts. These isolates were distributed into 15 genera, of which 7 were ascomycetous and 7 were basidiomycetous and one isolate was yeast state of a zygomycetous fungus *Benjaminiella.* Ascomycetous yeasts were represented by 9 different species and basidiomycetous yeasts by 16 different species. Among the basidiomycetous yeasts 20, 16 and 9 isolates were related to *Cryptooccus laurentii*, *Pseudozyma aphidis* and *Rhodosporidium kratochvilovae* respectively. The soil yeasts were dominated by basidiomycetous yeasts, which are thought to be more adaptive and capable of surviving under stressful conditions. The dominance of *Cryptococcus* especially *Cr. laurentii* and frequent occurrence of *Rhodosporidium* species is consistent with the other studies of yeast diversity from soil. Among the ascomycetous yeasts isolates related to *Aureobasidum pullulans* (6), *Torulaspora globosa* (4) and *Debaryomyces etchellsii* (3) were predominant.

Three of our soil yeast isolates have been described as strains of a novel species *Debaryomyces singareniensis* sp. nov. (Saluja & Prasad, 2007b). Combined sequence analysis of SSU rDNA, ITS and D1/D2 domains suggested that *D. singareniensis* and *D. etchellsii* may belong to a separate genus, but more work is needed to establish this possibility. Some of the other isolates that we proposed as novel species are *Cryptococcus solicola* sp. nov. (APSS 862), *Cryptococcus andhrapradeshensis* sp. nov. (APSS 864), *Cryptococcus khammamensis* sp. nov. (APSS 870), and *Rhodosporidium singareniensis* sp.nov. (APSS 849). In addition,

two other isolates related to *Rhodotorula crocea* that we proposed as new species are *Rhodotorula tapanii* sp. nov. (APSS 906) and *Rhodotorula shivajii* sp. nov. (APSS 907). *R. crocea* is presently described on the basis of two strains (CBS 2029^T and CBS 5950) with no other closely-related species known. Our two isolates APSS 906 and APSS 907 formed a separate clade with both the strains of *R. crocea*, supported by high bootstrap value. Our results also strongly suggested CBS 5950 as a new species rather than a strain of *R. crocea*. In soil total 16 isolates were found to be putative new species.

8.3. Diversity of Yeast Isolates from Flowers Samples

Flowers as habitat for yeast diversity is considered to be an intriguing area of research. Specific yeast communities are thought to be associated symbiotically with flowers and their visiting insects. Total 20 samples were examined in our study. The number of yeast isolates ranged from 10^2 to 10^6 CFU/g. A total of 130 yeast isolates were obtained from 20 flower samples collected from Ganganagar and Rawatbhata in Rajasthan. Sequence analysis of the flower isolates showed that basidiomycetous yeasts were dominant (54%) in comparison to ascomycetous yeasts (46%). Interestingly, the number of basidiomycetous yeasts in our flower isolates increased by a significant amount owing to the presence of anamorphs of yeast-like fungi (*Pseudozyma* sp.) in several samples (total 53 out of 130). Other basidiomycetous yeast isolates included the genera *Cryptococcus, Rhodosporidium* and *Sympodiomycopsis*. The flower yeast isolates consisted of 16 genera, of which 10 were of ascomycetous affinity and 6 were of basidiomycetous affinity. These isolates represented 29 different species, 14 of basidiomycetous and 15 of ascomycetous affinity. Among the asomycetous yeasts, eighteen isolates were related to *Kodamaea ohmeri* but exhibited only two different sequence types.

Phylogentic analysis of the ascomycetous yeast isolates from flower samples suggested that the diversity was similar to an earlier study on yeast diversity from ephemeral flowers and associated insects in which *Candida, Metschnikowia, Starmerella, Trichosporonoides,* and *Wickerhamiella* were isolated (Lachance *et al.*, 2001c) . In our study too, several isolates were found to be putative new species related to *Wickerhamiella* clade which we proposed as *Wickerhamiella lachancei*

sp.nov. (9E1, 9E2, 9G2, 9G3, 9A4,) and *Candida cucurbitacearum* sp. nov. (9A2). Another 5 isolates from Rawatbhata (1A1, 1B1, 1Br6, 1Br8, 1Br9) were all the strains of a putative new species related to *Candia tolerans* and they are together proposed as strains of a novel species *Candida imtechensis* sp. nov. Among our other novel isolates, 16S1 was found to be a sister species of *Candida riodocensis* and 3 other isolates were related to *C. vaccinii* of *Starmerella* clade. *Starmerella bombicol*a and *Starmerella meliponinorum* are the only two teleomorphic species among more than 30 described and undescribed species of this clade. Our isolate 16S1 was found to be homothallic and produced one-celled ascospores and is proposed as *Starmerella dubei* sp. nov. while the other isolates (2G3, 2B3, 2B4) are anamorphs and are proposed as *Candida sesami* sp. nov..

We described a novel species, *Candida ruelliae*, on the basis of two strains (16Q1 and 16Q3). It was recovered from flowers of *Ruelliae* plant, collected from Rawatbhata (Saluja & Prasad, 2008). The closest relatives of *C. ruelliae viz* C. *haemulonii*, *C. pseudohaemulonii* and *C. haemulonii* type II are associated with human infections and are resistant to amphoterecin B and fluconazole. Interestingly, our new isolates were sensitive to these drugs.

Three flower isolates from Ganganagar (S1B, S1C, S1D) represented three sister species of *Cryptococcus victoriae*, one of them is proposed as *Cryptococcus floricola* sp. nov.. Two isolates, S15L (from Ganganagar) and 3C1 (from Rawatbhata), were related to *Cr. laurentii* and were described as novel species *Cryptococcus rajasthanensis* (Saluja & Prasad, 2007a). *Cryptococcus rajasthanensis* was found to be the closest among the described species of *Cr. laurentii*. *Cryptococcus laurentii* is very frequently reported from soil but reports of this species from flowers are very rare. Interestingly, in our studies, two strains of *Cryptococcus rajasthanensis* were isolated. One came from a flower sample from Ganganagar while the other from a Rawatbhata flower sample. Remarkably four other isolates from flowers were found be closely related to *Sympodiomycopsis paphiopedili*, which is a monospecies genus even after 17 years of its initial description (Sugiyama *et al.*, 1991). Our isolates are proposed as novel species *Sympodiomycopsis ganganagarensis* sp. nov. (S6A, S6B, S1K) and *S. indica* sp. nov. (8A1).

The most interesting observation from phylogenetic analysis of our *Pseudozyma*-related species is that, our isolates represented all the major clades of Ustilaginales *viz Ustilago* sensu lato clade, Ustilago-Sporisorium clade, Sporisorium clade and even members basal to Ustilaginales were also represented in our isolates. Eight isolates from Rawatbhata flowers samples were found to be basal to Ustilago-Sporisorium clade. None of the earlier studies from flowers have ever reported such a high proportion of yeast-like fungi. Twenty five percent of our flower isolates (32 out of 130) belong to 18 putative new species of which nine each belong to ascomycetous and basidiomycetous yeasts.

8.4. Future Directions: Phylogeny and Prediction of Functionality

In the present study we obtained 215 yeast isolates of which more than 30 are novel species. Our isolates constitute a diverse repertoire of yeasts represented by ascomycetes and basidiomycetes. These isolates can be evaluated and exploited for their biotechnological potential. Phylogenetic analysis could be helpful in judiciously selecting and exploiting yeasts for a particular biotechnological application. Some of the following well-known examples can help in understanding this point better. Most *Candida* and *Pichia* species that are able to assimilate methanol as the sole carbon source, form a single clade in a phylogenetic tree. All the five species of the genus *Wickerhamiella* have been shown to produce extracellular lipase (Lachance *et al.*, 1998). Similarly several species of the genus *Pseudozyma* are known to produce Mannosyl erythritol, a glycolipid biosurfactant. This biosurfactant shows antimicrobial and antiviral activity, induces cell differentiation and apoptosis and dramatically increases gene-transfection efficiency (Kitamoto *et al.*, 2002).

In order to strengthen the hypothesis that phylogeny could be a predictor of functionality, we performed some preliminary experiments and found that two of our isolates 9A2 and 9E1 that belong to the genus *Wikerhamiella* were able to produce extracellular lipase. Since we isolated a large number of isolates related to *Pseudozyma*, we carried out a preliminary screening for production of biosurfactant and found that many of our *Pseudozyma* isolates were biosurfactant producers. Similarly, some of our *Aureobasidium* isolates produced pullullan, an extracellular

polysaccharide that was reported from *Aureobasidium pullulans*. The above examples suggest that our repertoire of yeasts can have vast potential which could be explored further for several different biotechnological applications.

8.5. Thermophily in Yeasts

Yeasts are mesophilic by nature. But there are some yeasts that are able to grow at temperatures as high as 50°C. Such yeasts are designated as thermotolerant or thermophilic yeasts. Such yeasts are subjects of continued research for exploiting their high-temperature growth characteristics for industrial applications. The mechanism which enables these otherwise mesophilic organisms capable of growing at higher temperatures remains a mystery. Research on other thermophilic organisms has suggested the possibility of several contributing factors to this phenomenon. We decided to address this question using a genetic approach. We chose the thermophilic yeast *Hansenula polymorpha* for asking which of its gene(s) when overexpressed in a mesophilic yeast (*Saccharomyces cerevisiae*) could enable budding yeast to grow at higher than its optimal growth temperature. We generated a genomic DNA library from *H. polymorpha* DNA and screened it in *S. cerevisiae* for growth at several temperatures between 40°C to 50°C. We screened approximately 1 lakh transformants but unfortunately we were unable to obtain a transformant capable of growing at higher temperature(s). Implications of our inability to obtain positive results are discussed already in the Chapter 7. The genomic DNA library that we generated has been found to be functional in relieving auxotrophy in budding yeast. Therefore this library is a useful reagent and can be utilized to ask more questions about the biology of *H. polymorpha* in future.