3. Isolation screening and characterization of n-3 polyunsaturated fatty acids producing yeasts

Yeasts represent a part of the natural micro-flora of soil, various fruits, fermented products *etc*. (Torok and King, 1991). Significant efforts have been made to discover and analyse the possible biotechnological applications of novel yeast strains as a source of economically important biochemicals, secondary metabolites, essential fatty acids *etc*. (Krivoruchko *et al*., 2011; Beopoulos and Nicaud, 2012; Xue *et al*., 2013).

Beneficial roles of essential n-3 PUFAs in maintaining proper human health are now well accepted. Several problems like bioaccumulation of toxic compounds, seasonal variations and cost associated with the presently used n-3 PUFAs sources have resulted in necessity of acquiring them from new sources. Microbial lipids are one of the promising sources which can be used as an alternative. Bacteria cannot compete with fungi as primary producers of PUFA-rich oils due to low yield (2% - 5% of total dry cell mass) and the presence of some undesirable lipids which include branched-chain fatty acid family, iso-, anteiso-, and omega-alicyclic fatty acids (Russell and Nichols, 1999). Few yeast species showed presence of n-3 PUFAs in their total fatty acid extracts (Subramaniam *et al*., 2010; Ageitos *et al*., 2011). Relatively high growth rate with high lipid content (20% - 40% of dry cell mass) in low-cost fermentation media such as nutritional residues from agriculture and industry, make yeasts as suitable candidate for microbial oil production (Meng *et al*., 2009; Ageitos *et al*., 2011).

Most of these are studied for microbial oil production regardless of their fatty acid composition (Meng *et al*., 2009; Ageitos *et al*., 2011). Discovery of novel oleaginous yeasts that produce essential n-3 PUFAs can open a new window for the development of microbial fermentation and/or enzymatic processes to obtain high yields of the desired PUFAs (Beopoulos *et al*., 2011). With this background, a systematic study for screening of novel yeast isolates producing n-3 PUFAs was undertaken.

3.1 Isolation of yeast from suitable sources

Present work was started with the main objective of isolation and screening of various yeast isolates for the presence of n-3 PUFAs and the genes involved in their biosynthesis ultimately to improve the nutritional status of our diet. PUFAs producing microorganisms occur in a wide variety of environments, typically in soil, fresh water, sea water, in association with plants or with food and in low temperature environments (Ageitos *et al*., 2011).

In all, 70 yeast isolates were purified from different sources from several ecological niches of India. The different ecological sources included oil socked soils from the premises of plant oil mills from different parts of Rajasthan, Maharashtra, Uttar Pradesh and Gujarat in India, decaying fruits (*Musa* spp., *Mangifera indica*, *Archa sapota*), and traditionally used drinks mainly fresh '*Neera*' (a sweet fresh juice of date or sago palm *Phoenix sylvestris*; a well-known beverage in India and has been accepted for its nutritional values). The screenings were limited to habitats that pose no health risks. Yeasts from these sources are likely to be non-pathogenic or are components of fermented foods.

These isolates were subdivided into twenty five independent clusters based on colony, and morphological characteristics. Representative isolates from each cluster were selected for further study.

Yeast isolates, their colony and morphological characteristics are listed in table 3.1. All isolates were grown in YPD medium with glucose as principal carbon source (Figure 3.1).

Table 3.1 Yeast Isolates from the various sources

Name	Source	Colony characteristics at 48 hrs incubation at 30 °C	Cell morphology
A-1	Oil mill soil, Sonipat, Haryana	2-4 mm, Cream white, smooth, convex, circular.	4-5 µm, oval / round cells, fission observed.
A-2	Sonipat	1-2 mm, Cream white, smooth, convex, circular.	3-4 µm, Oval cells, budding observed.
A-3	Varanasi	1-2 mm Off white, low smooth, convex, circular.	2 X 3 µm Elongated cells, fission and budding observed.
A-4	Varanasi	1-2 mm, White, smooth, Convex, circular.	3-4 µm, Oval cells, budding observed.
A-5	Varanasi	2-3 mm, Off white, low convex, smooth, circular.	4-5 µm, oval / round cells.
A-6	Varanasi	2-3 mm, Cream white, low convex, smooth, circular.	2 X 3 µm Elongated cells, budding observed.
A-7	*Neera*: drink of *Phoenix sylvestris*	2-4 mm, Cream white, convex, smooth, circular.	3 X 4 µm Elongated cells, fission and budding observed.
A-8	Oil mill soil, Tara. Maharashtra.	1-2 mm, White, low convex, smooth, circular,	1-2 µm, elongated rod-shaped cells.
A-9	Bhiwadi	2-4 mm, Reddish orange, convex, smooth, circular.	7-8 µm round cells budding observed.
A-10	Varanasi	2-3 mm, White, granular, low convex, rough, circular.	2-3 µm, round/ oval cells, budding observed.
A-11	Varanasi	1-2 mm, White, convex, circular, smooth, circular.	1-2 µm, elongated cells.
A-12	Gultekdi, Pune	1-2 mm, Off white, low convex, smooth, circular.	3-4 µm round/oval cells, budding observed.
A-13	Gultekdi, Pune	2-3 mm, Off white, low convex, smooth, circular.	2-3 µm, oval cells, budding observed.
A-14	Gultekdi, Pune	1-2 mm, White, flat, smooth, circular.	3-4 µm, oval cells, budding observed
A-15	Gultekdi, Pune	1-2 mm, White, low convex, Smooth, circular.	4-5 µm, oval, round cells

A-16	Gultekdi, Pune	2 mm, Off white, low convex, smooth, circular.	2-3 µm, elongated/oval cells, fission observed.
A-17	Gultekdi, Pune	2-3 mm Off white, convex, smooth, circular.	3-4 µm, Round to oval cells
A-18	Gultekdi, Pune	1-2 mm, Off white, low convex, smooth, circular.	1-2 µm, round cells, budding observed
A-19	Gultekdi, Pune	1-2 mm, Orange, low convex, smooth, circular.	2-3 µm, elongated cells, fission observed
A-20	Tara	2-4 mm, Cream white, low convex, smooth, circular.	2-3 µm, Elongated cells, fission observed.
MS-1	*Musa sapientum* (Banana)	1-2 mm, Off white, low convex, smooth, circular.	4-5 µm, oval, budding, chain formation
MS-2	*Musa sapientum* (Banana)	1-3 mm, Off white, low convex, rough, circular.	5-6 µm, round, cluster formation, nucleus seen.
MS-3	*Musa sapientum* (Banana)	3-5 mm, Off white, flat, smooth, circular.	2 µm, Round, small, cluster formation, fission and budding.
MS-4	*Musa sapientum* (Banana)	2-3 mm, Cream color, translucent, convex, smooth, convex, circular.	1-2 µm, fission and budding, cluster formation.
MS-5	*Musa sapientum* (Banana)	2-3 mm, Cream color, translucent, irregular margin, flat, circular.	4-5 µm, round, cluster formation, budding observed.
MI-1	*Mangifera indica* (Mango)	2-3 mm, White, low convex, rough, circular.	5 x 2 µm, elongated, bi-polar and axial budding, cluster formation
MI-2	*Mangifera indica* (Mango)	2-3 mm, White, low convex, rough, circular.	7 x 2 µm, axial budding, chain formation.
MI-3	*Mangifera indica* (mango)	2-3 mm, White, low convex, rough, circular.	7 x 2 µm, axial budding, chain formation
MI-4	*Mangifera indica* (Mango)	2-3 mm, White, low convex, rough, circular.	6 x 2 µm, elongated, binary fission, budding.
AS-1	*Achras sapota* (Chicoo)	1-2 mm, White, convex, rough, round.	5 x 2 µm, axial budding, binary fission, cluster formation.

AS-2	*Achras sapota* (Chicoo)	2-3 mm, Cream white, Translucent, convex, smooth, round.	4 x 2 µm, oval, axial budding.
AS-3	*Achras sapota* (Chicoo)	1-2 mm, Cream white, Translucent, convex, smooth, circular.	5 x 2 µm, oval, axial budding, chain formation
AS-4	*Achras sapota* (Chicoo)	1-2 mm, White, Convex, Rough, round.	6 x 2 µm, elongated, budding.
PS-1	*Neera*: drink of *P. sylvestris*	2-3 mm, Cream white, Translucent, convex, smooth, circular.	7 x 2 µm, axial and bipolar budding, cluster and chain formation.
PS-2	*Neera*: drink of *P. sylvestris*	2-3 mm, Off white, opaque, Convex, smooth, circular.	6 µm, oval, budding, chain formation.
PS-3	*Neera*: drink of *P. sylvestris*	1-2 mm, White, low convex, rough, circular.	7 x 2 µm, budding, chain formation, nucleus seen.
PS-4	*Neera*: drink of *P. sylvestris*	1-2 mm, Off white, opaque, low convex, rough, circular.	6 µm, round, budding, chain formation, fission
Z- 1	Aiyappa temple, Vadodara	2-3 mm, Red, smooth, high convex, smooth round.	3-4 µm, oval / hexagonal, cluster formation, budding/fission
Z-2	Gingili oil mill, Banswara	1-2 mm, Cream color, convex, translucent, smooth, circular	5 µm, oval, Binary fission /budding, cluster formation
Z-3	Gingili oil mill, Banswara	1-2 mm, Cream color, convex, translucent ,smooth, convex, circular	6 µm, oval, chain formation
Z- 4	Gingili and ground nut oil mill. Banswara	2-3 mm, Red, high convex, Smooth, round.	5-6 µm Oval/hexagonal, cluster formation

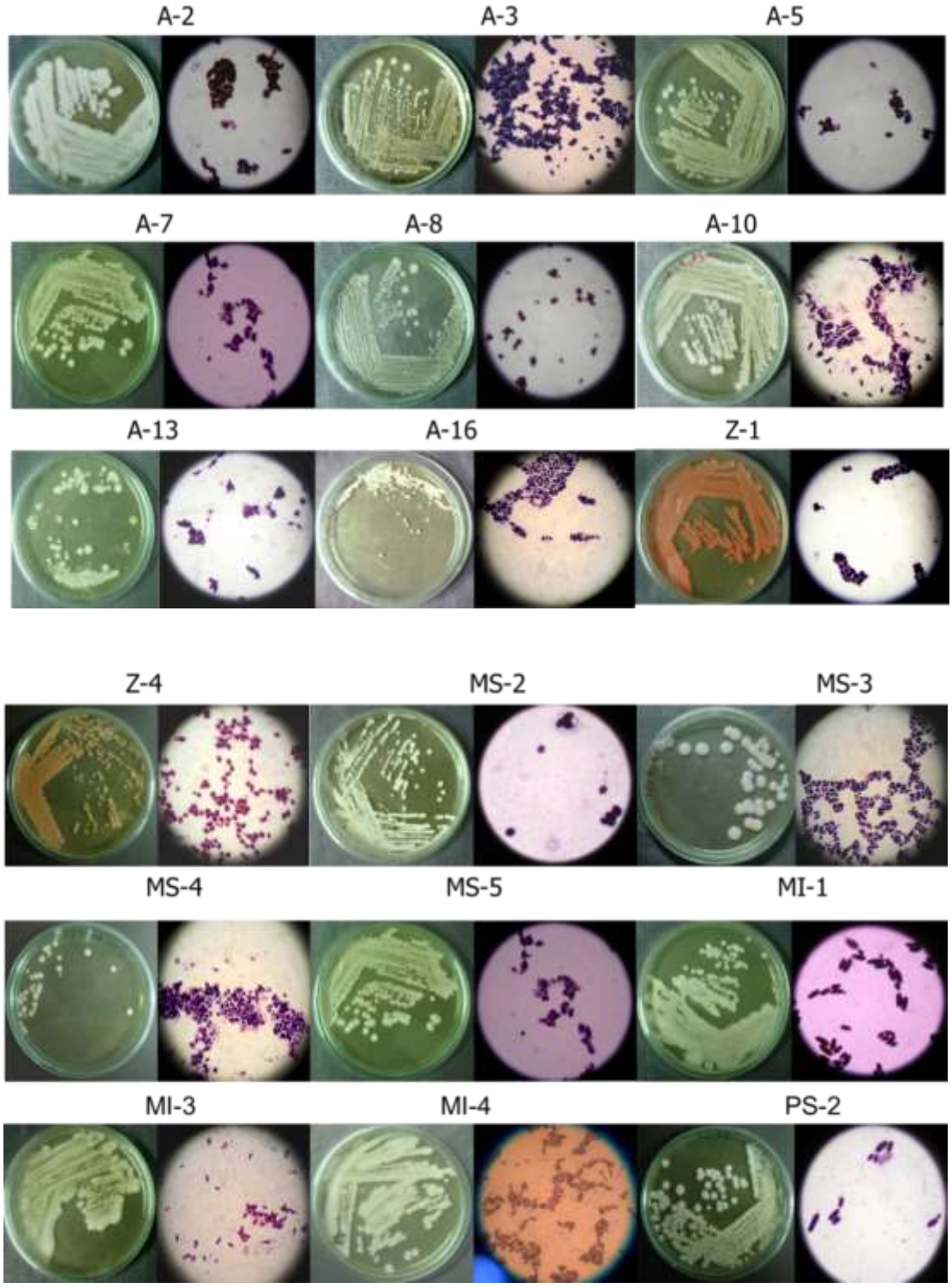


Figure 3.1 Colony and morphological characterization of yeast isolates. Yeast isolates differed in their colony and cellular morphology. Few isolates also showed pleomorphic colony morphology with flowery appearance (A-10, A-18, MI-1, MI-3 and MS-5). Few of them (A-9, Z-1 and Z-4) were red carotenoid pigmented in different shades, the rest having white to cream pigmentation. Microscopic examination revealed small to large (2-8 µm), round to oval shaped cells showing axial and / polar budding, fission dividing cells.

3.2 Screening of yeast isolates for *fad*-3 gene and FAD-3 activity

With advances in techniques it is now possible to rapidly screen and select for specific genes encoding particular enzymes or desirable characteristics within diverse groups. 'Microorganisms as n-3 PUFAs producers' is still a relatively new field of research, and research in this area have been growing significantly within the last few years.

3.2.1 PCR screening for *fad*-3 gene

3.2.1.1 Degenerate primer designing

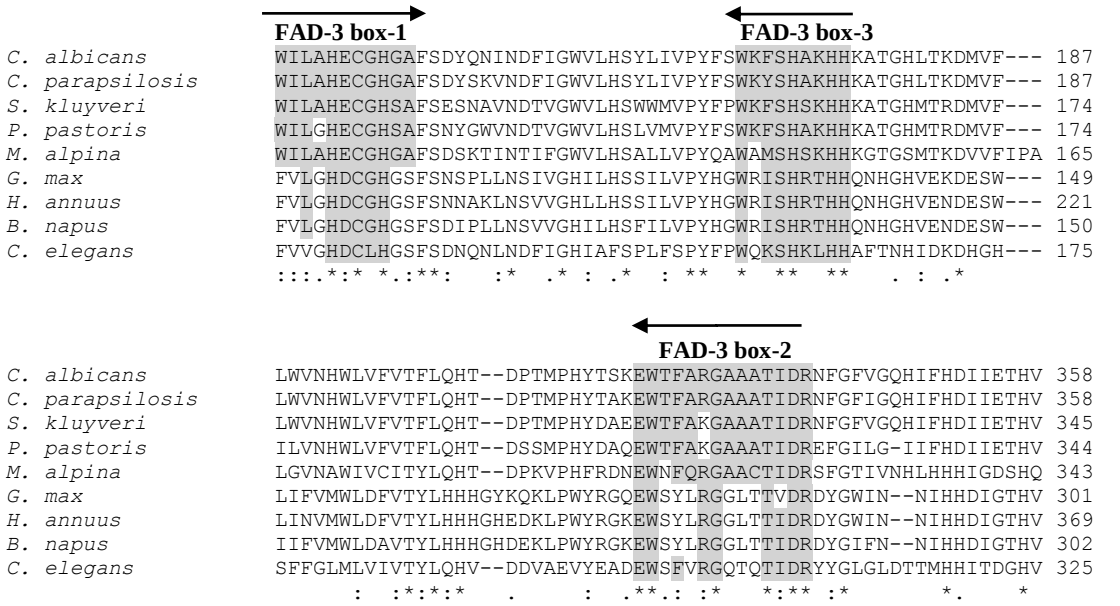
In present work PCR screening was done for selecting yeast isolates with *fad*-3 gene by using specially designed gene specific degenerate primers. A Large number of n-3 FADs have been characterized from plants, animals, microalgae but only in a few yeasts and fungi. Some of the reported yeasts genome sequences show ORFs annoted as the putative *fad*-3 gene but most of these are uncharacterised.

For designing of degenerate primers the amino acid sequences of characterized and annotated n-3 desaturases from yeast, algae, fungi and plants (Table 2.4) *viz. Arabidopsis thaliana, Bracica napus, C. albicans, C. parapsilosis* (putative) *Caenorhabditis elegans, Glycine max, Helianthus annuus, Mortriella alpina, P. pastoris, S. kluyveri and Saprolegnia diclina etc.* were aligned using ClustalW software; ClustalW multiple alignments showed 25% - 30% primary sequence similarity and five 16-22 amino acid long clusters (Figure 3.2), with 45% - 50% conservation across different phylogenetic groups. On the other hand the amino acid sequences of the two major methyl end desaturases (n-3 and n-6 FADs) show high similarities within the same species of *S. kluyveri* and *C. albicans* (Oura and Kajiwara, 2004; Murayama *et al*., 2006). Among the five conserved stretches, three included histidine cluster motifs that contain eight catalytically essential histidine residues *viz,* HXXX(X)H, HXX(X)HH, and HXXHH. These are proposed to be the ligands for the iron atoms (Shanklin and Cahoon, 1998). Three out of the five amino acid clusters namely, yeast n-3 FAD box-1(Histidine), FAD box-2 and yeast n-3 FAD box-3 (Histidine) (Figure 3.2) were used for designing degenerate primers. High degeneracy in the primers was contributed by the amino acids having high degenerate codons such as serine and other non-conserved variants with in the conserved regions.

Degenerate primers were designed with a two-part structure having a consensus clamp and a degenerate core using CODEHOP primer design strategies (Rose *et al*.,

1998). Inclusion of a single residue that was uniquely conserved for FAD-3 proteins at the 3'end of each gene specific primers OF1F and OF2R (Figure 3.2) was expected to impart specificity to the PCR by eliminating the *fad*-6 genes. First series of primers were designed with 64-128 degeneracy (Appendix II. C). High degeneracy and increased A+T% made PCR based screening complex.

A



B

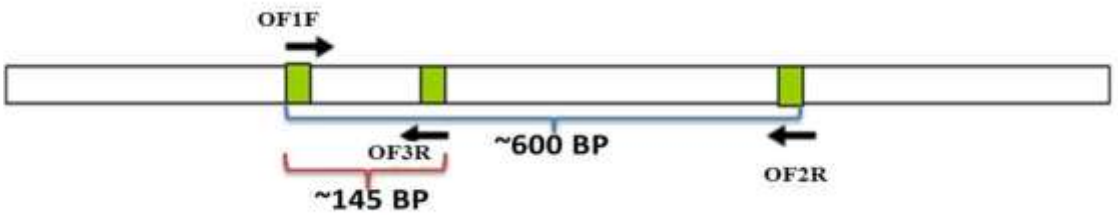


Figure 3.2 Comparison of the deduced amino-acid sequences of previously reported *fad*-3 genes from several fungi, plants, algae and cyanobacteria. The amino-acid sequences of omega-3 desaturases from *Candida albicans* (*C. albicans*), *C. parapsilosis*, *Saccharomyces kluyveri* (S. *kluyveri*), *Pichia pastoris* (*P. pastoris*), *Mortierella alpina* (*M. alpina*), *Glycine max, Helianthus annuus* (*H. annuus*), *Brassica napus* (*B. napus*) and *Caenorhabditis elegans* (*C. elegans*) are used. Sequences were aligned using the ClustalW algorithm (A). The positions of the three conserved boxes are indicated by boxes numbered 1, 2, and 3. The arrows indicate the amino-acid sequences (shaded with grey) used in the design of the primers OF1F, OF2R and OF3R (B). Amino acids representing maximum conservation were shaded with light grey.

Since *fad*-3 gene sequences from a few yeast such as *S. kluyveri*, *C. albicans etc*. were available, primers were designed with a bias to the FAD-3 sequences from these organisms. Ca-FAD-3 showed only 63.2% identity with that of Sk-FAD-3 (Murayama *et al*., 2006). Different primers were designed, with *S. kluyveri* NBRC 1893 bias

(OF1F5S) and with *C. albicans* bias (OF1F5C). This contributed to minimal degeneracy of primers (Table 2.5).

3.2.1.2 Primary PCR (~600 bp)

The volume of primers varied (50-200 pM) in the reactions according to the degeneracy of primers. PCR results showed inconsistency at the same annealing temperature. Even with different stringency conditions and modified sets of primers reproducibility was poor. Different sets of primers used in this study are listed in appendix II. C.

Out of the 73 yeast isolates, 25 yeast isolates gave an amplicon of ~600 bp (Figure 3.3 A and 3.3 B) by touchdown PCR 60-50 ºC using primers (Table 2.5) corresponding to the conserved yeast FAD-3 box-1 and FAD-3 box-2 respectively (Figure 3.2). Most of these 25 positive yeast isolates gave amplification with *Candida* biased primers.

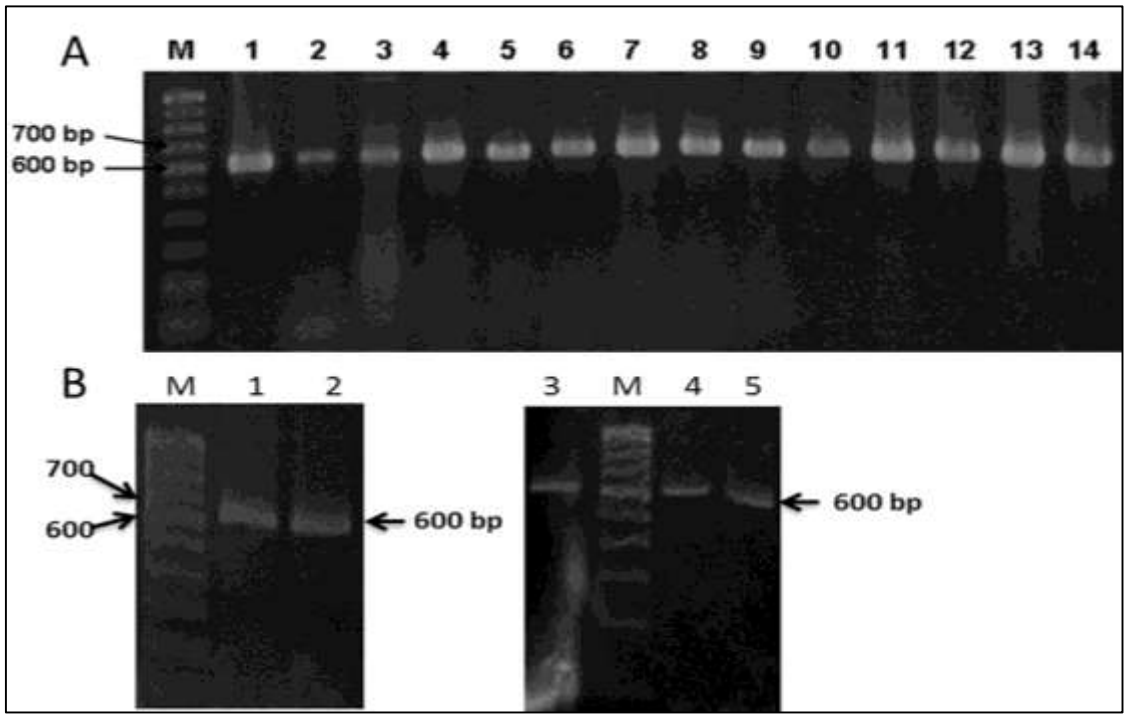


Figure 3.3 PCR based screening for *fad*-3 gene (~600 bp PCR). A: Lane 1: *S. kluyveri* (positive control), Lanes 2-14: Yeast isolates PS-4, PS-3, PS-2, MS-5, MI-4, MI-3, MI-1, A-19, A-18, A-16, A-10, A-7 and A-1 respectively, Lane M: 100 bp ladder DNA marker. B: Lane 1-5: Yeast isolates A-2, A-3, A-5, Z-1 and Z-4 respectively.

3.2.1.3 Semi-nested PCR (~145 bp)

The probability of getting false positive amplification was ruled out by a second semi-nested PCR with primers corresponding to FAD-3 box-1 and FAD-3 box-3 motifs (Figure 3.2). Seventeen of the 600 bp amplicons obtained in the first PCR could be validated by this second PCR that generated a ~145 bp product (Figure 3.4), while six were inconsistent and two were negative (Table 3.3). A similar approach of nested PCR has been used to increase the sensitivity of the screening (Akhavan *et al*., 2010).

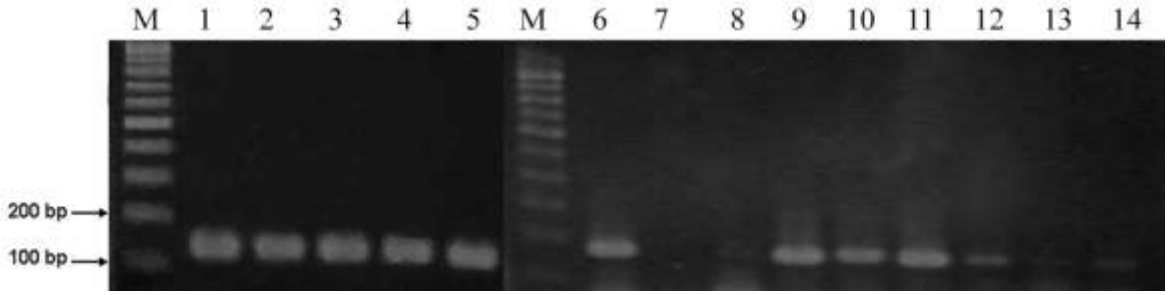


Figure 3.4 Semi-nested PCR for the confirmation of *fad*-3 gene using 600 bp amplicon as template. Lane 1: *S. kluyveri* (positive control), Lane 2-14: yeast isolates PS-2, A-10, MS-5, MI-1, MI-3, A-16, A-9, A-8, A-7, A-5, A-3, A-18, A-19, Z-4 respectively, Lane M: 100 bp ladder DNA marker.

The negative results of second PCR might reveal sequence variability in conserved regions of *fad*-3, while the inconsistency seen in few yeast isolates could suggest that the sequences corresponding to the 3' clamps of the primer is less conserved between species. A comprehensive screening of yeasts for *fad*-3 genes has not been yet reported. Most of the *fad*-3 genes characterised before, have been identified singly by direct genome mining (Kainou *et al*., 2006; Zhang *et al*., 2008; Murayama *et al*., 2006) or from constructed genomic library (Oura and Kajiwara, 2004). Recently a complete CDS of the Lk-*fad*-15 was obtained and characterized from oleaginous yeast *Lipomyces kononenkoae* using degenerate primer PCR and TAIL-PCR, (Yan *et al*., 2013).

3.2.2 Hybridization analysis

The PCR positive yeast strains were further screened by low stringency dot-blot hybridization and thereafter narrowed down by high stringency Southern hybridization using *fad*-3 (610 bp) sequence from *S. kluyveri* NBRC 1893. Out of these 25 isolates, 22 isolates showed positive results in dot blot analysis (Figure 3.5; Table 3.3).

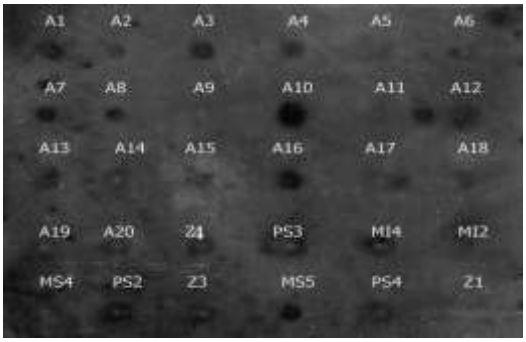


Figure 3.5 Dot blot analysis. Out of 25 PCR positive yeast isolates, 22 showed positive signals. The PCR product from internal conserved region of *fad*-3 gene of *S. kluyveri* (610 bp amplified by OF1F5S and OF2R3) was used as a probe.

In Southern blot analysis, most isolates presented a single band of hybridization after digestion with HindIII, which has no site in the Sk-*fad*-3. No positive signal was seen for a few dot blot positive isolates in southern hybridization (Figure 3.6); the results of analysis are summarised in table 3.3.

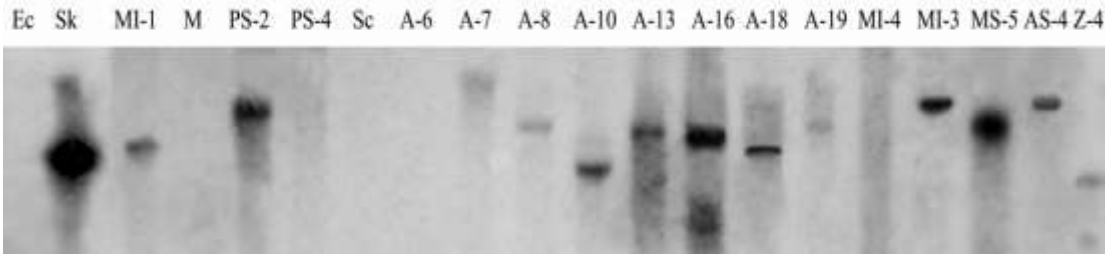


Figure 3.6 Southern hybridization analysis of yeast isolates. The PCR product from internal conserved region of *fad*-3 gene of *S. kluyveri* (610 bp amplified by OF1F5S and OF2R3) was used as a probe. Ec: *E. coli*, Sk: *S. kluyveri* (positive control), Sc: *S. cerevisiae*, M: 1 kb ladder, yeast isolates: MI-1, PS-2, PS-4, A-6, A-7, A-8, A-10, A-13, A-15, A-16, A-18, MI-4, MI-3, MS-5, AS-4, Z-4.

Out of 25 isolates which showed positive results in 600 bp PCR, 13 isolates gave strong signals in southern hybridization. Seven isolates which were PCR and Dot blot positive gave no signal while signals given by remaining isolates were weak. These differences in the results may be due to high degree of variations within the *fad*-3 gene sequences among the selected isolates. Sequence analysis showed that there is similarity between previously reported *fad*-3 and *fad*-6 sequences (Murayama *et al*., 2006). Homology between n-3 and n-6 *fad* gene sequences might result in false positive signal in hybridization studies. Hence functional expression was analysed in putative *fad*-3 positive yeast isolates.

3.2.3 Identification of selected yeast isolates

The FAD-3 positive yeast strains were identified and characterized by biochemical tests and -ITS1-5.8S-ITS-2- rDNA sequencing. The primary characterization of few yeast isolates was done by biochemical tests (Kurtzman *et al.*, 2011). These included sugar fermentations and other physiological tests as represented in table 3.2 A and 3.2 B. Fermentation of sugars varied amongst the isolates. All were able to ferment glucose whereas none fermented lactose. Growth at elevated temperature (37 ºC) was observed in all isolates, wherein some of the yeasts (A-10, A-16, A-18 and MS-5) gave flowery colony morphology.

The -ITS1-5.8S-ITS2- region of the FAD-3-positive isolates were amplified and sequenced with specific fungal primers (Table 2.5) (White *et al*., 1990). Blast analysis of obtained sequences was done and yeast isolates were identified as the strain of the closest similarity within a range 95% - 99% (Table 3.3). The GeneBank accession numbers of the sequences are given in table 3.3. Identification by BLAST analysis was in agreement with biochemical/morphologic results presented by these isolates.

Among the 19 finally projected *fad*-3 positive yeast isolates, 9 belonged to Genus *Candida* (*C. tropicalis, C. parapsilosis, C. metapsilosis* and *C. pararugosa*) and 7 were related genera which included *Issatchenkia orientalis, Kodamaea ohmeri, Meyerozyma caribbica, Meyerozyma guillermondii, Pichia kudriavzevi* and *Pichia anamola*. The remaining 3 were *Arxula adeninivorans* (A-3 and A-7), and *Rhodotorula mucilaginosa*. These were verified by growth on CHROMagar (Figure 3.7; Table 3.2 A) which differentiates *C. albicans* from other *Candida* spp. based on colour formation (Odds and Bernaerts, 1994; Baradkar *et al*., 2010).

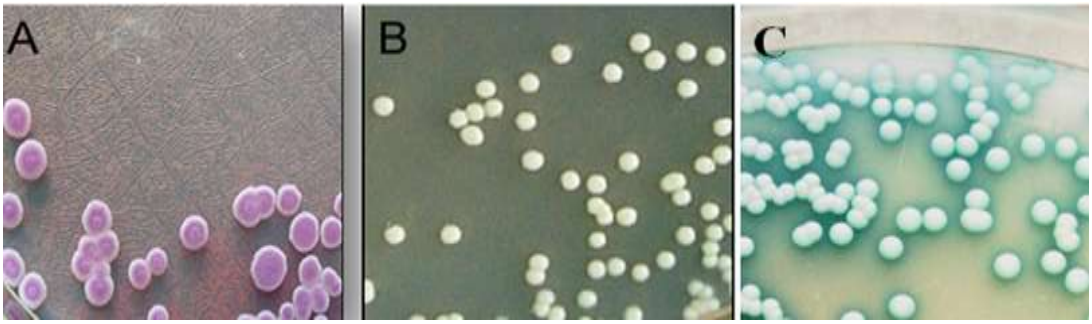


Figure 3.7 Yeast morphology on HiCHROMagar A: Pale pink colonies of *C. krusei* MI-3 with rough spreading pale edges, B: Cream colored colonies of *C. parapsilosis* A-16, C: Blue colored colonies of *C. tropicalis* PS-2.

Table 3.2 A Biochemical characteristics of selected yeast isolates

Yeast Isolate	$(NH_4)_2SO_4$	KNO_3	Catalase	Urease	HiChrom agar
A-2	+	–	+	–	Off white cream
A-3	+	–	+	+	Off white
A-5	+	+	+	–	Pale purple
A-7	+	–	+	–	Off white
A-8	+	–	+	–	Pale purple
A-10	+	+	+	+	Blue green
A-13	+	+	+	–	Blue purple
A-16	+	–	+	–	Cream
MI-1	+	+	+	–	Blue green
MI-3	+	+	+	+	Pink
MS-5	+	+	+	–	Dark green
Z-4	+	–	+	+	Red
PS-2	+	+	+	–	Blue
SK	+	+	+	–	Off white cream

'+': Positive test, '–': negative test.

Table 3.2 B Comparison of fermentation tests of the selected yeast strains isolates

Isolate	Glucose	Sucrose	Lactose	Galactose	Maltose	Xylose	Fructose	Raffinose	Arabinose
A-2	++	+	−	++	+	+	+	–	+
A-3	++	++	−	++	++	+	+	+	−
A-5	++	++	–	+	–	+	+	+	–
A-7	++	++	−	+	+	+	+	–	−
A-8	++	++	−	+	+	–	+	+	−
A-10	++	++	−	+	+	+	++	+	−
A-13	++	++	–	+	+	+	+	–	+
A-16	++	+	–	++	+	+	+	–	+
MI-1	++	+	−	+	–	–	+	+	–
MI-3	++	+	–	+	–	+	+	+	+
MS-5	++	+	–	–	+	+	+	+	+
Z-4	++	+	−	+	+	+	+	+	+
PS-2	++	+	–	–	+	+	+	+	+
SC	++	++	−	++	+	–	++	+	–
SK	++	++	–	+	+	–	+	–	–

‘++’: acid and gas production; ‘+’: acid production, ‘–’: no acid/gas production.

3.2.4 Taxonomic analysis of identified yeast strains:

Phylogenetic relationship among the strains was deduced by analysis of the -ITS1-5.8S-ITS2- nucleotide sequences by MEGA6 (Figure 3.8) (Tamura *et al*., 2013). Out of these, *Kodamaea* shares common branch point with that of *Candida; I. orientalis* MI-1, *P. kudriavzevii* MI-3 and *C. pararugosa* were separated from others early in the tree. *Rhodotorula* and *Arxula* shared common branch point. *I. orientalis* is considered as an anamorphic form of *C. krusei* (Kurtzman and Fell, 2001). *P. caribbica* is a basionym of *M. caribbica*, *P. guilliermondii* of *M. guilliermondii* (Kurtzman and Suzuki, 2010; Kurtzman *et al*., 2011). Genus *Pichia* which is a polyphyletic genus, its members closely resembles with the members of *Issatchenkia* and *Candida* genus (Kurtzman *et al*., 2008).

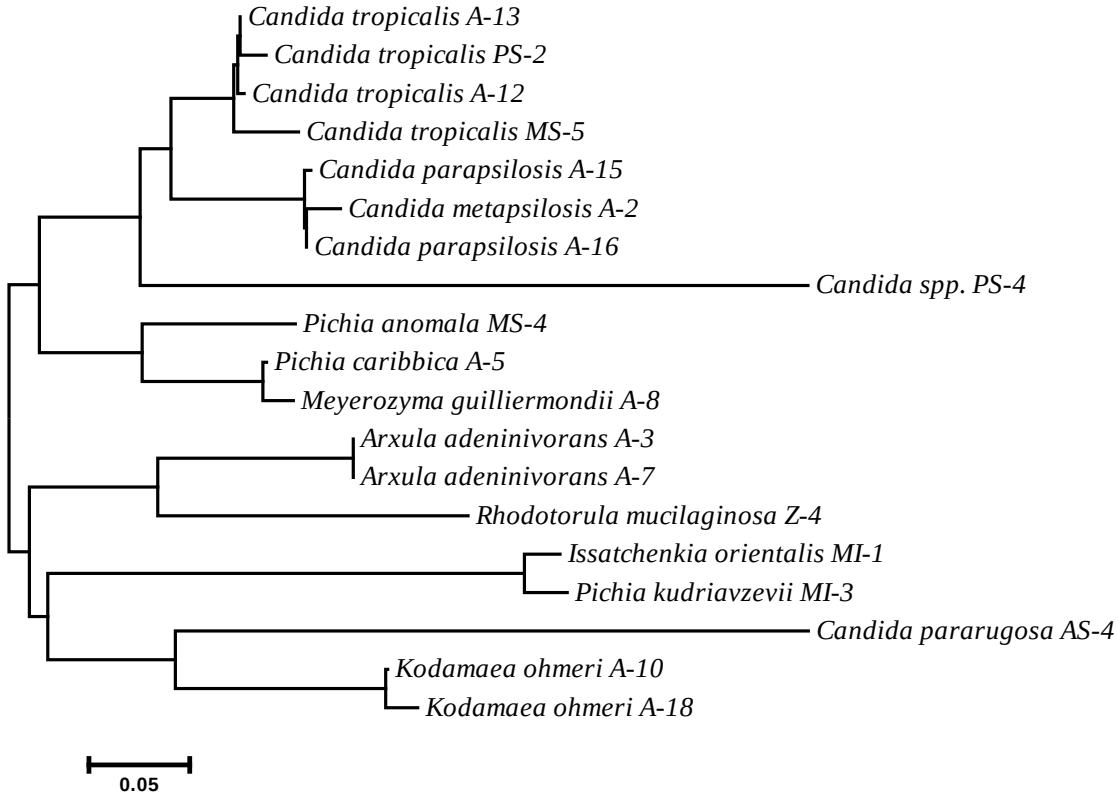


Figure 3.8 Evolutionary relationships of the identified *fad*-3 positive yeast isolates. Phylogenetic tree drawn from neighbor-joining analysis of the 19 nucleotide sequences of -ITS1-5.8S-ITS2- regions of the *fad*-3 gene containing yeast isolates. Evolutionary analyses were conducted in MEGA6. The optimal tree with the sum of branch length = 1.89257024 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

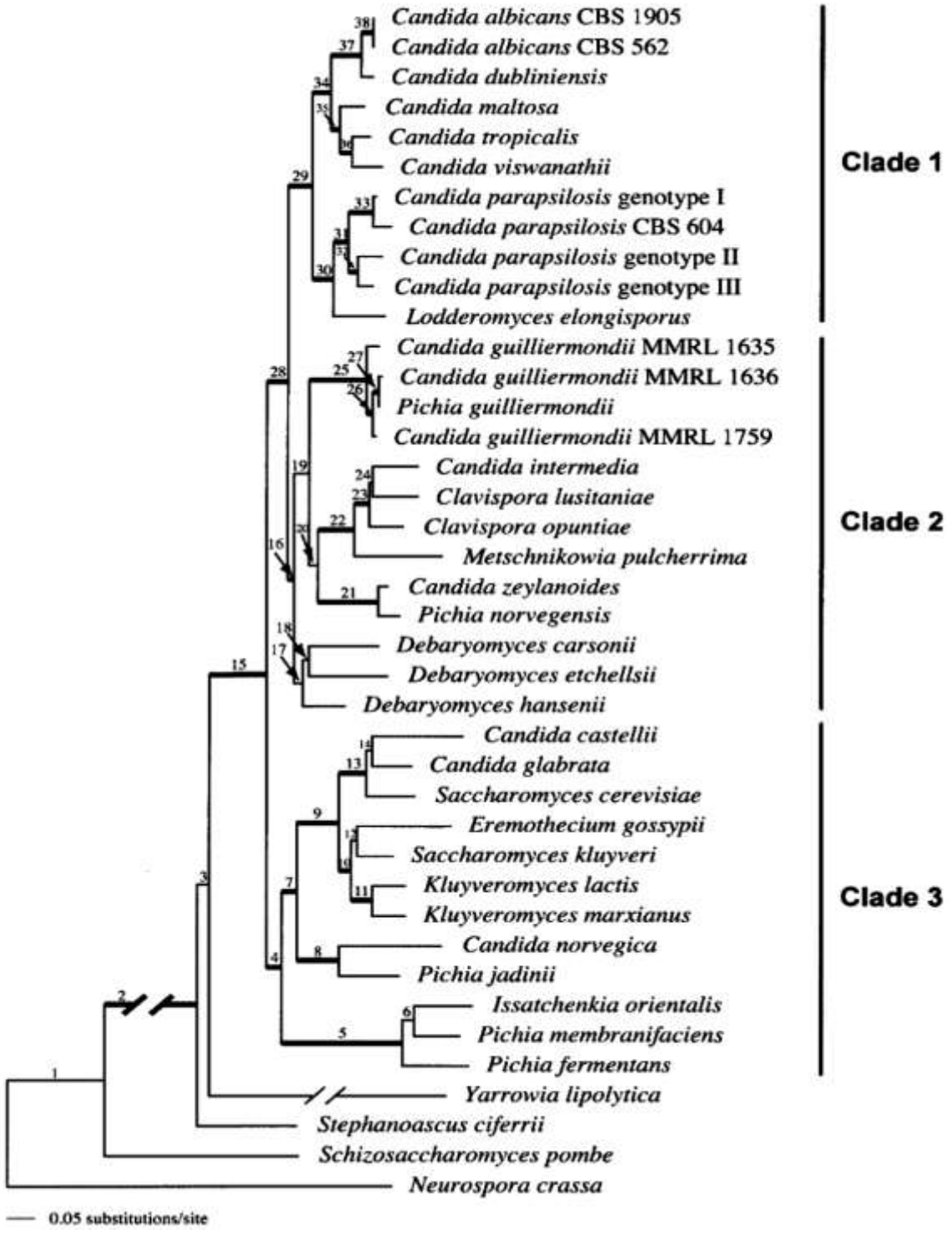


Figure 3.9 Combined maximum likelihood analysis of six genes (ACT1, EF2, RPB1, RPB2, 18S rDNA and 26S rDNA). 38 taxa of Hemiascomycetes and two out-group species, an Archiascomycete (*S. pombe*) and a Euascomycete (*N. crassa*) were analysed (Diezmann *et al*., 2004).

Our efforts to find an evolutionary correlation to rationalise the distribution of *fad*-3 gene among these isolates gave somewhat imprecise interpretations due to lack of consensus in yeast taxonomy. Phylogenetic classifications have distributed *Debaryomyces*, *Kodamaea*, *Candida*, *Pichia*, *Issatchenkia* and *Meyerozyma* into three major clades in Hemiascomycetes group (Figure 3.9). Strains of *Issatchenkia* or *Meyerozyma* have been interchangeably synonymous with *Candida* and *Pichia* respectively and there has been no clear distinction between *Candida* and *Pichia* and these are distributed among many closely related phylogenetically circumscribed clades (Kurtzman *et al*., 2008; Kurtzman and Suzuki, 2010).

The difficulty in finding common markers for species distinction of hemiascomycetous yeasts has been now addressed by molecular taxonomy by comparing a large number of genes such as 18S, 26S rDNA repeats, mitochondrial genes such as the COXII small subunit and single copy nuclear genes. These highlighted evolution and the existence of interspecific hybrids (Casaregola *et al*., 2011). *Meyerozyma*, comprising cryptic species that form hybrids, is a new genus among the Coenzyme Q forming ascomycetes (Kurtzmann and Suzuki, 2010; Casaregola *et al*., 2011). Species of *Issatchenkia* are now proposed as members of the *Pichia* clade based on divergence of rRNA genes and translation elongation factor-1α. *I. orientalis* is now *P. kudriavzevii* (Kurtzmann *et al*., 2008; Casaregola *et al*., 2011), while the *A. adeninivorans* is now termed *Blastobotrys adeninivorans*. Confinement of *fad*-3 to the above similar or related organisms in different and distant ecological niches indicates high conservation and minimal horizontal transfer.

3.2.5 Biotransformation of LA to ALA and analysis of fatty acids content

Most of the yeast and fungal species are adapted to growth under cold conditions, and correspondingly produce a high proportion of PUFAs, which are able to maintain membrane fluidity at low temperatures (Rodriguez-Vargas *et al*., 2007). PUFAs production is found to be increased at low temperatures (Watanabe *et al*., 2004; Oura and Kajiwara, 2004). Hence for detection of n-3 PUFAs, all PCR positive yeast isolates were grown at low temperatures (at 20 ºC) in a medium supplemented with 1 mM LA as a substrate for direct transformation by FAD-3 to produce ALA.

All the above identified hybridization positive strains, but not the randomly selected hybridization negative isolates transformed LA to ALA. All the strains metabolized linoleic acid completely and no residual LA was detectable by thin layer chromatography or gas chromatography analysis in culture supernatants. While ALA was detected inherently in many strains, some strains produced detectable levels of ALA only when 1 mM LA was supplemented as a substrate in the growth medium. Out of 25 PCR positive yeast isolates, 19 showed presence of ALA in their total fatty acid extract in Silver ion TLC analysis (Figure 3.13 B and C; table 3.3). Higher PUFAs were not detectable in these FAMEs. The conversion of LA to gamma linolenic acid (C18:3, n-6) by FAD-6 was not given emphasis in this study.

The ALA / LA content in the yeast cell extracts are given in table 3.4. A representation of comparative gas chromatography peaks for unsaturated fatty acids is shown in figure 3.11. Strains *C. parapsilosis* A-16, *C. tropicalis* PS-2, *I. orientalis* MI-1, and *P. kudriavzevii* MI-3 gave 2- to 4- fold high volumetric productivity of ALA (20-60 mg/lit; approximately 4-7 g dry weight of cells), compared to that of the standard strain *S. kluyveri* (10 mg/lit). The LA content of cells were in the range of 35-120 mg/g dry cell weight and the ratio of n-3: n-6 PUFAs ranged in between 1:4-8, the values better than that for *S. kluyveri* NBRC 1893 (1:10). Maximum substrate (LA) conversion and accumulation of ALA was obtained in MI-1.

The *fad*-3 gene was detected in a very limited number of closely related yeast genera. Strains expressing FAD-3, isolated from fruit sources belonged to *Pichia* and *Candida* clades while those from soil additionally included *Rhodotorula*, *Blastobotrys* spp. It was interesting to see that in a few yeast isolates presence of *fad*-3 gene was confirmed by PCR and hybridization analysis but functional expression of the same was not observed. Further a few isolates with very high lipid content didn't show presence of n-3 PUFAs in their total fatty acid extracts, indicating that the functional expression of *fad*-3 gene may not be related to the total fatty acid content. A comprehensive summary of the results obtained is given in table 3.3 and 3.4.

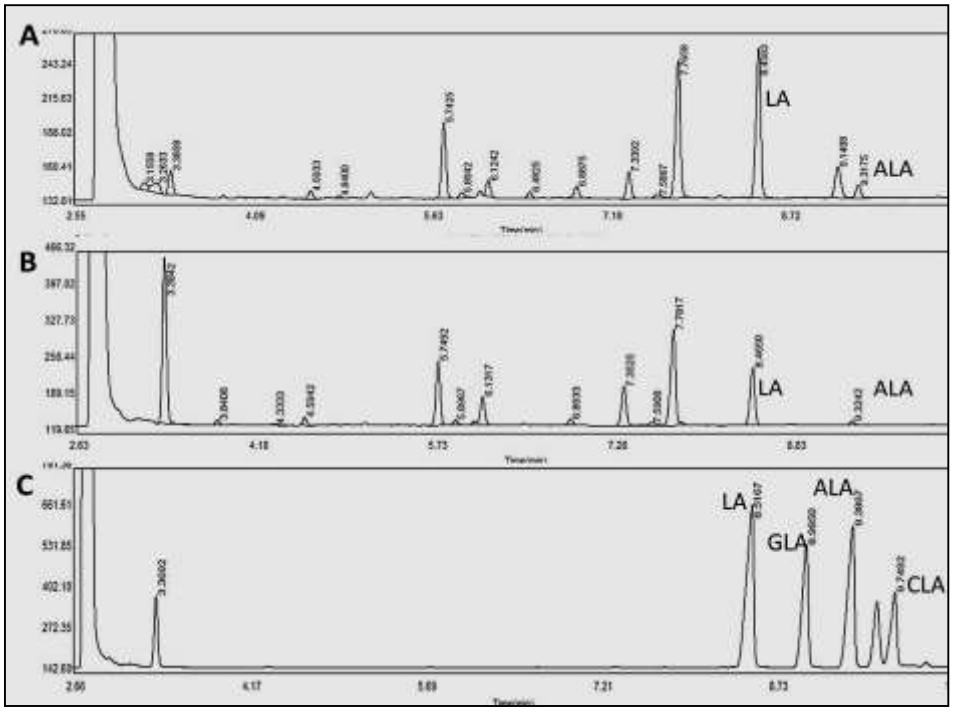


Figure 3.11 Representative GC chromatogram of FAMEs derived from yeast total fatty acids extracts. FAMEs derived from fatty acid extract of various yeast strains grown in medium supplemented with 1 mM LA. A to C: *K. ohmari* A-10, *P. kudriavzeli* MI-3 and Fatty acid standards respectively.

Many isolates including A-9, Z-1 with very high LA content did not show presence of n-3 PUFAs (Figure 3.12).

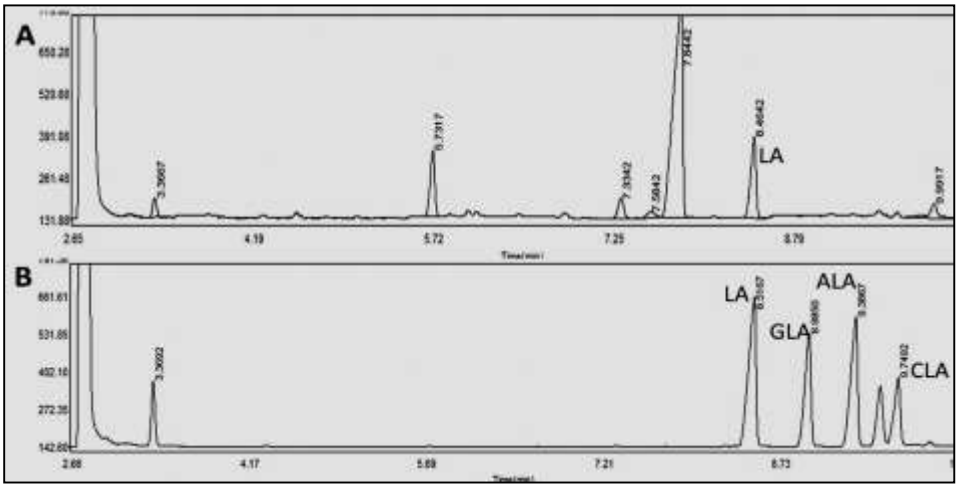


Figure 3.12 Representative GC chromatogram of FAMEs derived from yeast total fatty acids extracts (A-9) showing production of LA only. A: yeast isolate A-9 grown in YPD medium only. B: Standard fatty acids (LA, ALA, GLA and CLA) methyl esters.

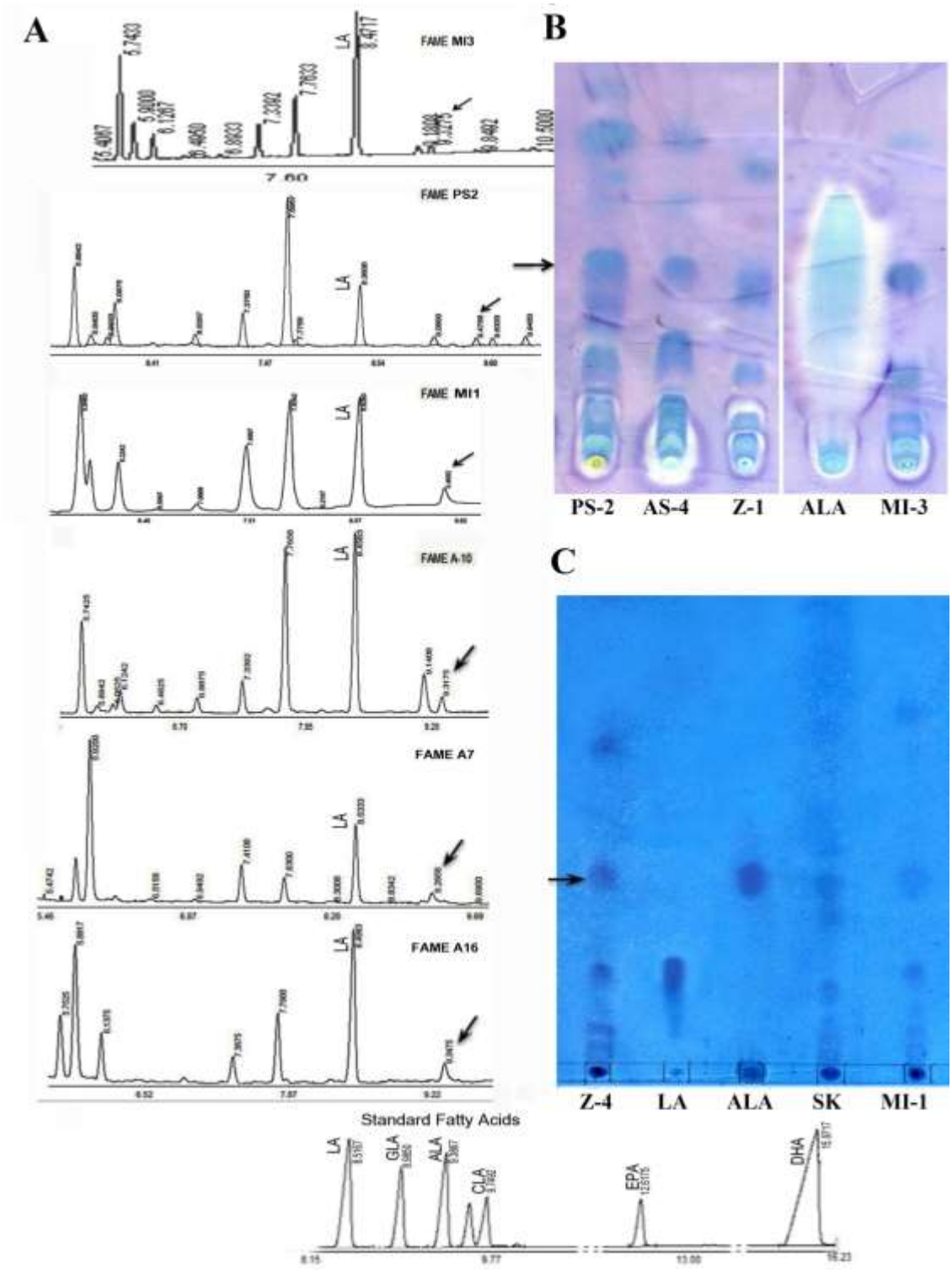


Figure 3.13 Comparative analysis of screening for FAD-3 activity. A: GC analysis of FAMEs derived from total fatty acid extracts of different yeast isolates, B and C; TLC analysis of FAMEs derived from total fatty acid extracts of different yeast isolates. Arrow indicates ALA peak in GC chromatogram and ALA band in TLC.

Table 3.3 Summary of screening of yeast isolates for *fad*-3 gene and its activity.

Yeast Isolate **GeneBank Accession number Of -ITS1-5.8S-ITS2- region sequence.**		**Source**	**PCR screening results** **600 bp**	**145 bp**	**TLC**	**Dot blot**	**Southern hybridization**
A-1	–	*Achras sapota*	+	–	–	+	–
A-2	*C. metapsilosis* A-2 KC556807	*Achras sapota*	+	**	+	+	+
A-3	*A. adeninivorans* A-3 KC556808	*Achras sapota*	+	+	+	+	+
A-5	*P. caribbica* A-5 KC556809	*Achras sapota*	+	+	+	+	+
A-7	*A. adeninivorans* A-7 KC556810	*Phoenix sylvestris* drink: *Neera*	+	+	+	+	+
A-8	*M. guilliermondii* A-8 KC556811	Oil mill soil (Maharashtra)	+	+	+	+	+
A-9	–	Oil mill soil (Maharashtra)	+	**	–	–	–
A-10	*K. ohmeri* A-10 KC556811	Oil mill soil (UP)	+	+	+	+	+
A-12	*C. tropicalis* A-12 KJ187753	*Ananas coosus*	+	+	+	+	–
A-13	*C. tropicalis* A-13 KC556813	*Ananas comosus*	+	+	+	+	+
A-15	*C. parapsilosis* A-15	*Citrus limetta*	+	+	+	+	+
A-16	*C. parapsilosis* A-16 KC556814	*Citrus sinensis*	+	+	+	+	+
A-18	*K. ohmeri* A-18 KJ187755	*Vitis vinifera*	+	+	+	+	+
A-19	–	*Vitis vinifera*	+	**	–	–	–
MI-1	*I. orientalis* MI-1 KC556817	*Magnifera indica*	+	+	+	+	+
MI-3	*P. kudriavzevii* MI-3 KC556818	*Magnifera indica*	+	+	+	+	+

MI-4	–	*Magnifera indica*	+	+	–	+	–
MS-4	*P. anomala* MS-4 KJ187757	*Musa sapientum*	+	–	+	+	+
MS-5	*C. tropicalis* MS-5 KC556819	*Musa sapientum*	+	+	+	+	+
PS-2	*C. tropicalis* PS-2 KC556815	*Phoenix sylvestris*	+	+	+	+	+
PS-3	–	*Phoenix sylvestris*	+	**	–	+	–
PS-4	*Candida spp.* PS-4 KJ187758	*Phoenix sylvestris*	+	**	+	+	+
Z-1	–	oil mill soil (Gujarat).	+	**	–	–	–
Z-4	*R. mucilaginosa* Z-4 KC556816	oil mill soil (Gujarat).	+	+	+	+	+
AS-4	*C. pararugosa* AS-4 KJ187756	*Achras sapota*	+	**	+	+	+
S. kluyveri NBRC 1893		NITE, Japan.	+	+	+	+	+

'+': positive amplification / signal; '–': No amplification / signal, '**': inconsistent amplification.

Few *fad*-3 PCR positive strains *viz*, A-1, A-9, A-19, MI-4 and PS-3, didn't show presence of ALA in their total fatty extracts. Finally 18 strains were identified by amplification and sequencing of -ITS1-5.8S-ITS2- region. One of the isolate PS-4 was identified up to genus level only.

Table 3.4 Lipid in cells and lipid yield of the selected yeast strains after 48 hrs

Yeast Isolate	**Media used** Y: YPD only; S: YPD + 1 mM LA	**Approx. Dry Cell mass** (g / lit)	**Fatty acid detected in the cell mass** (mg/lit)		**Relative ALA yield** (mg/g dry cell mass)	**Relative content of ALA/LA**
			LA	**ALA**		
C. metapsilosis A-2	Y	6.22 ± 0.30	21.40 ± 1.4	No peak	–	–
	S		41.36 ± 2.0	4.70 ± 1.2	0.75	0.114
A. adeninivorans A-3	Y	7.10 ± 0.20	26.10 ± 2.0	4.20 ± 1.0	0.59	0.161
	S		75.00 ± 3.0	9.70 ± 1.5	1.37	0.129
P. caribbica A-5	Y	5.17 ± 0.21	7.30 ± 0.2	No peak	–	–
	S		68.00 ± 2.0	17.10 ± 2.9	3.31	0.251
A. adeninivorans A-7	Y	6.56 ± 0.16	4.43 ± 1.5	No peak	–	–
	S		20.06 ± 4.0	7.00 ± 2.0	1.07	0.35
M. guilliermondii A-8	Y	5.27 ± 0.20	19.00 ± 0.5	No peak	–	–
	S		73.00 ± 2.0	11.00 ± 1.8	2.09	0.151
K. ohmeri A-10	Y	6.20 ± 0.23	14.60 ± 0.4	7.40 ± 1.2	1.19	0.507
	S		43.00 ± 1.2	9.80 ± 1.7	1.58	0.228
C. tropicalis A-12	Y	5.88 ± 0.38	5.85 ± 0.8	4.10 ± 0.1	0.70	0.701
	S		29.55 ± 1.9	6.02 ± 0.68	1.02	0.204
C. tropicalis A-13	Y	6.50 ± 0.18	18.90 ± 0.6	3.73 ± 0.6	0.57	0.197
	S		50.00 ± 1.5	9.52 ± 1.6	1.46	0.190

C. parapsilosis A-15	Y	6.70 ± 0.30	4.85 ± 0.6	No peak	–	–
	S		39.56 ± 0.8	12.88 ± 1.3	1.92	0.326
C. parapsilosis A-16	Y	6.46 ± 0.18	38.00 ± 1.1	3.25 ± 2.2	0.49	0.086
	S		122.07 ± 4.0	42.36 ± 3.2	6.59	0.344
K. ohmeri A-18	Y	6.80 ± 0.18	12.01 ± 1.2	5.46 ± 0.8	0.80	0.466
	S		24.28 ± 1.7	14.23± 1.4	2.09	0.586
C. tropicalis PS-2	Y	3.70 ± 0.08	14.45 ± 2.0	2.27 ± 1.2	0.61	0.157
	S		51.00 ± 1.5	19.12 ± 3.25	5.13	0.375
Candida spp. PS-4	Y	7.28 ± 0.28	6.36 ± 0.3	2.69 ± 0.4	0.37	0.423
	S		39.56 ± 2.3	8.62 ± 0.9	1.18	0.218
R. mucilaginosa Z-4	Y	4.10 ± 0.10	9.44 ± 0.75	No peak	–	–
	S		41.56 ± 1.6	13.00 ± 1.3	3.17	0.313
I. orientalis MI-1	Y	7.48 ± 0.27	70.00 ± 2.0	7.48 ± 0.3	1.00	0.107
	S		157.00 ± 4.7	58.32 ± 6.5	7.75	0.371
P. kudriavzevii MI-3	Y	5.12 ± 0.20	45.00 ± 1.3	5.12 ± 0.2	1.00	0.114
	S		109.00 ± 3.3	25.00 ± 4.2	4.88	0.230
C. tropicalis MS-5	Y	6.26 ± 0.30	9.36 ± 1.5	No peak	–	–
	S		38.70 ± 1.3	4.21 ± 0.7	0.67	0.109
C. pararugosa AS-4	Y	8.23 ± 0.50	3.21 ± 0.2	No peak	–	–
	S		11.46 ± 0.6	1.83 ± 0.3	0.23	0.160
S. kluyveri NBRC 1893 (Positive control)	Y	5.45 ± 0.10	9.30 ± 1.5	No peak	–	–
	S		25.50 ± 0.5	7.12 ± 0.5	1.31	0.279

3.2.6 Distribution of n-3 PUFA among yeasts and implications

A wide range of non-conventional yeasts with attractive characteristics can be exploited as resources of economically important biochemicals and secondary metabolites. Ability to produce and accumulate fatty acids is an inherent property of specific yeasts and depends upon the growth conditions e.g. *Rhodotorula* spp. and *Cryptococcus curvatus* can accumulate between 40% - 60% of lipids, whereas *S. cerevisiae* or *Candida* spp. only accumulate 5% - 10% when grown under the same conditions (Meng *et al*., 2009). Omega-3 desaturase has been expressed from a few yeasts *viz*. Sk-FAD-3, from *S. kluyverii* (Oura and Kajiwara, 2004), Kl-FAD-3 from *K. lactis* (Kainou *et al*., 2006), Pp-FAD-3 from *P. pastoris* (Zhang *et al*., 2008). Pp–FAD-3 exhibited broad spectrum desaturation ability for 18- and 20- carbon n-6 substrates and showed 35% higher conversion rate than the n-3 fatty desaturases from mycelial fungi, while Sk-FAD-3 and Kl-FAD-3 preferred the 18-carbon n-6 substrate only. Omega-3 PUFAs have been detected in a few basidiomycetes (Libkind *et al*., 2008). The ability of these organisms to convert LA to ALA could form the primary basis to use these genes in developing a strategy for improving the essential fatty acid nutrition in the diet. Expression of a fungal or yeast *fad*-3 in yeast may result in higher yields of ALA (Meesapyodsuk *et al*., 2000).

Previous studies have shown that along with many strains of the genus *Candida*, varieties of other yeasts are associated with fruits or vegetables (Torok and King, 1991; Chavan *et al*., 2009). An intense molecular and biochemical screening was undertaken in this study, with the main objective of exploring yeast strains as a source of n-3 PUFAs. The present efforts for exploring yeasts as a source of n-3 PUFAs identified several species of *Candida,* other genera such as *Issatchenkia, Meyerozyma* and *Kodameae* which are related to *Candida* and *Pichia* clades as major groups having FAD-3 activity, in addition to *Rhodotorula, Blastobotrys* and few other basidiomycetes. The distribution of FAD-3 activity among isolates studied here also implied the presence of n-3 desaturase to limited members of oleaginous yeasts. *A. adeninivorans* A-3 and A-7 showed relative high percentage of ALA. The screening of soil from the oil mill premises increased chances of identifying several oleaginous yeasts as *fad*-3 possessors since the fatty acids in these organism make up 25% - 40% of the cellular dry weight. Yet the most oleaginous yeast such as *Cryptococcus*, *Lipomyces* or *Yarrowia* were absent in the FAD-3 producers

identified. On the other hand all the n-3 PUFA producers identified here including the basidiomycete *Rhodotorula* have dimorphic nature of forming filaments or thallus, implicating a correlation of distribution of *fad*-3 seems with the developmental morphology. In present study we did not get any isolate belonging to genus *Saccharomyces*.

3.2.7 Importance of yeast having n-3 PUFAs producing ability

Alternate sources for n-3 PUFAs in the diet are desirable, one option is to modify oil-seed crops with efficient genes to produce n-3 PUFAs through genetic engineering technique and the other option is to produce them in well-studied microbial expression systems such as *S. cerevisiae*. *S. cerevisiae* is a well-established model system for the development of metabolic engineering strategies to produce several proteins and metabolites (Nevoigt, 2008; Hong and Nielsen, 2012). The beneficial probiotic properties of strains of some *Saccharomyces spp.* are well documented (Kelesidis and Pothoulakis, 2012; Hatoum *et al*., 2012). The concept of obtaining n-3 PUFAs from *S. cerevisiae* in sustainable quantities for human consumption purpose can be of special attraction and may be explored further for probiotic preparations. Study of the genetic diversity of yeasts with respect to essential FAs may lead to discovery of new alternatives for n-3 PUFAs which are deficient in presently used edible oils. The incorporation of the genes involved in biosynthesis of ALA from putative yeast isolates and there after EPA and DHA in *S. cerevisiae*, strategies can be designed for the applying yeast as a source of essential n-3 PUFAs.

Fatty acid profile of many yeast are similar to rape seed oil and soybean oil (Beopoulos and Nicaud, 2012). Most of the oleaginous yeast and fungi have been studied as source of microbial oil for biodiesel production (Ageitos *et al*., 2011; Beopoulos and Nicaud, 2012) and are being studied for microbial oil production with less emphasis on the fatty acid composition. The ability of these yeasts to accumulate high lipid content using sugar or related compounds as substrates has made them popular for promise of biodiesel production or for substituting high value exotic fats such as cocoa butter (Meng *et al*., 2009).

In the present study, screening for n-3 PUFAs producers was limited to certain habitats that pose no health risks. Many of these ascomycetous yeasts associated with food and

drink products could be explored as direct source of essential fatty acids. *Debaromyces* are found in cheese fermentations and many strains of the genus *Candida* including *C. tropicalis*, are associated with fruits or vegetables. The pigmented basidiomycetes of genera *Rhodotorula, Rhodospridium, Sporidiobolus, and Sporobolomyces* have been shown to contain 5% - 20% ALA in their fatty acid content (Libkind *et al*., 2008). *A. adeninivorans* is reported as nonpathogenic and is already used in several biotechnology applications (Wartmann and Kunze, 2000); single cell protein production (Wartmann *et al*., 2002) and animal feed improvement rendered by its phytase activity (Olstorpe *et al*., 2009). Therefore all the yeast strains identified in this study as n-3 PUFAs (ALA) producers including *A. adeninivorans* A-3, A-7 and the basidiomycete *Rhodotorula* can be explored for their usefulness as a precursor of essential n-3 fatty acids.