Materials and Methods

Chapter 2

2. Materials and Methods

2.1 Microbial strains and plasmids

The bacterial strains, yeast strains and plasmids used in this study are listed in Tables 2.1 and 2.2 respectively. The restriction maps of the plasmids and sequence of fad-3 gene are given in appendix II. *Saccharomyces kluyveri* NBRC 1893 was used as a source of standard *fad*-3 gene.

Other yeast strains used in this study were isolated from sources including soil near edible oil mills, over ripened fruits fermented drinks etc. are listed in table number 3.1.

Table 2.1 List of bacterial and yeast strains used in this study

Bacterial / yeast strains	Genotype and relevant characteristics	Reference-Source
S. kluyveri NBRC 1893	Wild type	NITE Biological Resource, Chiba, Japan.
S. cerevisiae W-9100	MATα ADE2 leu2-3, 112 his3-11, 15 Ura$^-$3-1 TRP1 lys2Δ RAD5	Dr. Rodney Rothstein, Genetics and Development, Columbia University.
Escherichia coli DH5α	F′/endA1 hsdR17 ($r_K^- m_K^+$) glnV44 thi-1 recA1 gyrA (Nal^r) relA1 Δ(lacIZYA$^-$ argF) U169 deoR (φ80dlacΔ(lacZ)M15)	Lab stock
E. coli BL21(DE3)	*E. coli* B F$^-$ *ompT gal [E. coli* B is naturally *dcm and lon] hsdS$_B$* with DE3, a λ prophage carrying the T7 RNA polymerase gene and lacI^Q	Lab stock

Table 2.2 List of plasmids used in this study

Plasmid	Features	Size (bp)	Reference
pBluescriptKS+	Cloning vector for *E. coli*; Amp^r	2958	Stratgene,USA
pET-28c(+)	Expression vector for *E. coli*; T7 promoter; Kan^r	5367	Novagen, USA
pGAL-MF	Yeast expression vector; Ura-3; GAL-L; Amp^r	6636	Dualsystems, Sweeszarland.
pBSCtN10	pBluescript KS(+) with N-terminal 1000 bp fragment of Ct-*fad*-3 gene; Amp^r	3962	This work
pBSCtC4	pBluescript KS(+) with C-terminal 400 bp fragment of Ct-*fad*-3 gene, Amp^r	3369	This work
pBS-*fad*-3	pBluescript KS(+) with Complete Ct-*fad*-3 gene, Amp^r	4266	This work
pSP-*fad*-3	pGAL-MF + complete Ct-*fad*-3 gene; Ura-3; Amp^r	7941	This work
pETSP28c	pET28c(+) with Complete Ct-*fad*-3 gene; Kan^r	6643	This work

Amp: Ampicillin; Kan: Kanamycim; r = resistant

2.2 Media, chemicals, enzymes, biochemicals and kits

Media and general chemicals were obtained from HiMedia Laboratories, India; Qualigens, India or Sisco Research Laboratories, India. Analytical grade chemicals were procured from Merck, India or Sigma Chemicals Pvt. Ltd., USA.

All enzymes and molecular biology grade biochemicals were from Bangalore Genei Pvt. Ltd, India (now Merck Lifesciences, India); New England Biolabs (NEB), USA; MBI Fermentas, Germany; Sigma Chemicals Pvt. Ltd., USA; Promega, USA; Roche Diagnostics, Germany and Applied Biosystems, USA.

High pure PCR product and plasmid purification kit from Roche Diagnostics, GenElute mini-prep kit for plasmid isolation from Sigma Chemicals Pvt. Ltd. were used for crucial experiments. GenElute Agarose spin columns from Sigma Chemicals

Pvt. Ltd. were used to obtain elution of DNA bands from LMP agarose. GenElute Yeast total RNA purification kit from Sigma Chemicals Pvt. Ltd. was used to isolate RNA and GeNei^{TM} AMV RT-PCR Kit was to synthesize cDNA. Dig DNA labeling and Detection Kit from Roche Diagnostics, Germany was used for labeling probe and Northern blot.

2.3 Media and Culture conditions

2.3.1 Media

2.3.1.1 Luria Broth

Luria broth (LB) obtained as a readymade medium from Hi-Media Laboratories was used at 2% (w/v) according to the manufacturer's instructions and 2% (w/v) agar was added to prepare solid Luria agar (LA) medium.

2.3.1.2 YPD broth and CSM broth

Yeast cells were grown in YPD medium (1% yeast extract, 2% peptone, 2% Dextrose, pH 7.0) containing Ampicillin (100 µg/ml) for preventing bacterial contaminants. For induction of gene expression in *S. cerevisiae*, yeast cells were cultivated in complete synthetic minimal medium without uracil (SD Ura$^-$) (Himedia, India) with appropriate induction conditions described in later section.

All the media and buffers were autoclaved at 15 pounds per square inch for 15 min while SD Ura$^-$ medium was autoclaved at 10 pounds per square inch for 20 min. Heat labile components like antibiotics, IPTG, X-Gal, Sugars, Amino acids etc. were filter sterilized and added freshly to cooled media (~45 °C). Antibiotics, IPTG and X-Gal were used at concentrations indicated in table 2.3.

Table 2.3 Concentration of antibiotics, IPTG, X-Gal

Biochemical	Concentration of stock solution (mg/ml)	Final concentration (µg/ml)
Ampicillin	100	100
Kanamycin	40	40
IPTG	20	20
X-Gal	20	40

2.3.2 Culture conditions:

All bacterial strains were cultured and maintained on Luria Agar (LA) at 37 °C and yeast cultures on YPD agar at 30 °C unless mentioned specifically. The cells were grown under shaker condition at 200 rpm. All the recombinants were grown in a media containing appropriate selection marker. Measurements of growth as optical density at 600 nm were carried out on Elico SL171 mini spectrophotometer. All strains were maintained as slants or stabs at 4 °C. For long term preservation, the cultures were maintained as 20% glycerol stocks and stored at -20 °C.

2.4 Isolation of yeast from various sources

Yeasts strains were isolated, from 17 soil samples from the premises of plant oil mills of different parts of India and from 13 samples of fruit pulp, on yeast peptone dextrose (YPD) agar, containing ampicillin (100 µg/ml) to prevent bacterial growth. The isolates were grown at 30 °C for 72 hrs on YPD agar, confirmed for cell morphology microscopically, maintained on YPD slant at 4 °C and sub-cultured at intervals.

2.5 Primary Classification and Identification of yeast isolates

Morphological and physiological characteristics were determined by the methods and keys described by Kurtzman *et al.*, (2011). Light microscopy was used to observe cell morphology, budding, pseudo-mycelium and true mycelium formation, if any. Nitrite utilization, Urease production, Catalase test, Ammonium sulphate utilization, Sugar fermentation and assimilation tests were performed. Fermentation and gas production in various sugars vis. glucose, sucrose, lactose, galactose, maltose, xylose, fructose, raffinose, arabinose etc. were analyzed. 2% sugar was added in a medium containing 0.45% yeast extract, 0.75% peptone and bromothymol Blue as an indicator of acid

production. The tubes were incubated at 30 °C for 24 hrs. Additional differentiation of selected yeast isolates was done by culturing them on the chromogenic medium-CHROMagar (HiMedia) *Candida* differential agar (Baradkar *et al.*, 2010; Odds and Bernaerts, 1994) The medium comprised (per liter) peptone (10 g), glucose (20 g), agar (15 g), chloramphenicol (0.5 g), and "chromogenic mix" (2 g); the yeasts were streaked out to single colonies from yeast suspensions, grown for over 3-7 days at 30 °C and 37 °C on CHROMagar *Candida*, and colony colors and unusual colony forms were recorded.

2.6 Molecular biology tools and techniques

2.6.1 Isolation of plasmid DNA

Plasmid DNA was isolated from overnight grown cultures by standard alkaline lysis/ boiling lysis method (Sambrook and Russell, 2001). For few crucial experiments plasmid isolation was done by the plasmid purification kit from Roche Diagnostics, Germany.

2.6.2 Yeast genomic DNA isolation and DNA quantification

Genomic DNA was isolated from the cells by enzymatic lysis using lyticase (Karakousis *et al.*, 2006) with minor modifications. Cells from 50 ml of log phase culture were re-suspended in 5 ml of 0.9 M sorbitol, 0.1 M EDTA, 0.1 M tris, 0.1% β-mercaptoethanol and 0.4 mg/ml lyticase solution. The reaction was incubated 37 °C at 50 rpm for 2.30-3hrs. Cell lysis was confirmed by microscopic observation. 350 µl of 10% SDS and 20 µg/ml proteinase K was added incubated further at 55 °C for 50 minutes. After sequential extraction by 0.85 ml 10% CTAB treatment and 6.6 ml of chloroform, genomic DNA was precipitated and dissolved in 1 ml TE buffer. The preparation was further treated with RNase and purified by phenol: chloroform extraction.

Absorption of suitably diluted DNA preparation was recorded on UV spectrophotometer at 260 nm. The purity was checked by the ratio of A_{260} /A_{280}. The DNA was quantified by using equation:

1 unit absorbance (A_{260}) at 260 nm = 50 µg DNA/ml.

2.6.3 Restriction enzyme digestion

Plasmid DNA to be analyzed was mixed with 5-10 U of restriction endonuclease (RE) per microgram of DNA in 1 X buffer supplied by the manufacturer in a final reaction volume of 20-100 µl. The reaction mixtures were incubated for 2-8 hrs at the appropriate temperature. In case of double digestion, a compatible buffer for the two restriction endonucleases was essentially checked. If not available, digestion with one enzyme was performed followed by purification and subsequent digestion with the other enzyme, using respective buffers.

2.6.4 Agarose gel electrophoresis

The DNA samples were mixed with appropriate volume of 6X loading buffer (0.25% bromophenol blue, and 40% sucrose in water) and subjected to electrophoresis through 0.8% - 2% agarose gel in 0.5X Tris-borate-EDTA (TBE) buffer at 4 V/cm for 1-3 hrs. The gels were then stained for 30-45 min in 0.5X TBE containing 5 µg/ml ethidium bromide (EtBr). The DNA bands were visualized by fluorescence under UV light using a UV transilluminator and subsequently photographed using gel docking apparatus.

2.6.5 Elution of DNA from gels and purification

For the elution of particular DNA fragment, sample DNA was size separated by agarose gel electrophoresis using low melting point agarose. Exposure of DNA fragment to the UV light and EtBr was avoided by running a parallel sample for EtBr staining and visualization. The DNA fragments of desired sizes were cut out from low melting point agarose. Agarose band was then minced into fine pieces and then digested with β-agarase (NEB) and DNA fragments were recovered from the supernatant obtained after digestion by ethanol precipitation. In some cases, the DNA fragment of interest was purified using GenElute agarose spin columns from Sigma.

2.6.6 Ligation

The ligation reaction was usually carried out with Quick ligase (NEB), T4 DNA ligase (Merck, India and MBI Fermentas) according to the manufacturers' instructions. The vector to insert molar ratio (molar concentrations calculated by the under mentioned formula) of 1:3 for cohesive end and 1:8 for blunt end ligations was maintained, with a total of 50-100 ng of vector DNA in each ligation system. The ligation mixtures were incubated at the temperatures and times recommended by the manufacturer.

$$\text{Molarity} = \frac{\mu g/\mu l}{(\text{base pairs of DNA x } 650 \text{ Daltons})}$$

2.6.7 Transformation of plasmid DNA in *E. coli*

E. coli DH5α competent cells were prepared by using the $CaCl_2$ method (Sambrook and Russell, 2001). Plasmid DNA was transformed into the competent cells by heat shock treatment. The transformants were selected by plating on selection medium containing appropriate antibiotic and/or by blue white selection using IPTG and X-Gal.

2.6.8 Yeast transformation

High efficiency Lithium Acetate (LiOAc) transformation modified method was used for transformation of recombinant plasmids in yeast expression host cells (Gietz and Schiestl, 2007). From overnight grown host yeast culture, fresh inoculation was done to at 0.1 OD_{600} and allowed to grow at 30 C to an OD of ~0.3. Cells from 10 ml were harvested, washed twice with distilled water and re-suspended in 100 µl 0.1 M LiOAc prepared in 1X TE for 15-30 min at room temperature. From that 50 µl of cell suspension was then transferred to an eppendorff tube containing 330 µl mix containing 50% PEG-3350; 0.1 M LiOAc solution, 2-5 µg plasmid DNA to be transformed and 50-100 µg ssDNA and incubated at 30 °C for 30 min. Heat shock was given at 42 °C for 20-25 min. Cells were pelleted down and re-suspended in a 1 ml selective liquid medium (SD Ura^-) for 30 min and 100 µl of re-suspended cells was plated onto the appropriate selective medium to recover cells containing the selectable marker.

2.6.9 PCRs

PCR amplifications were performed in Eppendorf master cycler personal or Eppendorf master cycler gradient thermal cycler.

2.6.9.1 Primer designing

For PCR based screening degenerate primers were designed based on the conserved amino acid sequences within the previously reported and few annoted FAD-3 proteins. Following amino acid sequences of n-3 desaturases from NCBI protein database were retrieved (listed in table 2.4) and aligned with each other using the program ClustalW for identification of conserved stretches of amino acids.

Sequence conservations seen in the multiple alignments of published and annotated yeast FAD-3 proteins with those of algae, fungi and plants using ClustalW were used to make BLOCKS in the program CODEHOP (Rose *et al.*, 2003). Yeast codon bias was taken into consideration for designing degenerate primers against conserved amino acid blocks. In order to standardize the PCR screening few modifications were done in these three primers. Oligonucleotides were custom-synthesized from Sigma Aldrich. All the primers used in this study are listed in appendix II.D. Primers that gave positive amplifications are only listed here in table 2.5. The PCR reaction set up was based on the guidelines given in Sambrook and Russell 2001. The assay system and temperature profile used for PCR amplifications are described in table 2.6.

2.6.9.2 PCR Protocols

2.6.9.2.1 Screening of *fad*-3 positive yeast by PCR

PCR was performed for screening of yeast isolates for *fad*-3 gene by using the 2 sets of degenerate primers (Table 2.4). For standardization of PCR using OF1F and OF2R as primer set, a touch down PCR was carried out.

Alternatively, A 610 bp PCR product was amplified by using primers OF1F5S and OF2R5 directed towards the internal conserved region of *fad*-3 gene from *S. kluyveri.*

2.6.9.2.2 rDNA amplification

The procedure used to amplify 18S rRNA genes was the procedure described by Cai *et al.* (1996), except that in this study 50 ng of genomic DNA rather than a single colony was used as the PCR template. The two pairs of PCR amplification primers used were P108 plus M2130, P1190 plus M3490, (Table 2.5). The -ITS1-5.8S-ITS2- regions of rDNA gene were amplified with ITS1 and ITS4 standard primer (Table 2.5) according to White *et al.*, (1990).

Table 2.4 FAD-3 protein sequences retrieved from NCBI protein database

Sr. No.	Organism	Accession number and length of FAD-3	Reference
1	*Mortierella alpine* 1S-4	BAD91495.1, 403 aa	Sakuradani *et al.*, 2005.
2	*Pichia pastoris*	ABL63813.1, 415 aa	Zhang *et al.*, 2008.
3	*Kluyveromyces lactis*	XP_451551.1, 415 aa	Kainou *et al.*, 2006.
4	*Candida albicans*	FAD-3 COORDS: Ca21chr1 SC5314:2845037-2843736C.	Murayama *et al.*, 2006. orf19.4933
5	*Saccharomyces kluyveri*	BAD11952.1, 419aa	Oura and Kajiwara, 2004.
6	*Candida parapsilosis*	CAY39363.1, 432 aa	Bucek *et al.*, 2009. (Unpublished work)
7	*Saprolegnia diclina*	AAR20444.1, 358 aa	Pereira *et al.*, 2004.
8	*Caenorhabditis elegans*	AAA67369.1, 402 aa	Spychalla *et al.*, 1997.
9	*Arabidopsis thaliana*	L22961.1, 1525 bp	Yadav *et al.*, 1993
10	*Brasicca napus*	AAT02410.1, 439 aa	Zhang *et al.*, 2004. (Unpublished work)
11	*Helianthus annuus*	AAP78965.1, 443 aa	Venegas-Calerón *et al.*, 2006.
12	*Glycine max*	AAO24265.1, 380 aa	Bilyeu *et al.*, 2003

Table 2.5 Primers used in this study

Primer	Sequence (5' ⟶ 3')	
A) Primers used for screening of yeast isolates for *fad*-3 gene		
OF1F 5S	GCTCAYGAATGYGGYCACTC	~360-379 (On *S. kluyveri fad*-3)
OF1F5C	GCYCAYGAATGYGGYCATGG	~400-419 (On *C. albicans fad*-3)
OF2R5KC	CCWYKRGCAAAAGTCCATTC	959-940 (On *S. kluyveri fad*-3) 998-979 (On *C. albicans fad-3*)
OF3R3	ATGGCCWGTWGCTTTATGRTGYTT	500-477 (On *S. kluyveri fad*-3)
		540-517 (On *C. albicans fad*-3)
D) Primers used in Amplification of Ct-*fad*-3 gene sequence		
OF5CtF	*ATG*AGYGTWGTTGARGCATCWTC	Jan-23
OF2CtR	CCWYKRGCAAAAGTCCATTC	1007-988
OF6CtF	TGGTTTRTTCCATGGTTRTGG	895-915
OF7CtR	*CTA*ATCTYTWGGTTTAACWGGWCC	1305-1281
OF5CtFN	AGC*CATATG*AGTGTTGTTGAAGCATCT	Jan-21
OF7CtRX	CA*CTCGAGCTA*ATCCTTTGGTTTGACAGG	1305-1284
C) Primers used in 18S rDNA characterization		
P108	ACCTGGTTGATCCTGCCAGT	2-21
M2130	CAATAAATCCAAGAATTTCACC	921-900
P1190	CAATTGGAGGGCAAGTCTGG	543-562
M3490	TCAGTGTAGCGCGCGTGCGG	1473-1454
D) Primers used in -ITS1-5.8S- ITS2- region amplification		
ITS1	TCCGTAGGTGAACCTGCGG	
ITS4	TCCTCCGCTTATTGATATGC	

Table 2.6 PCR conditions used in the present study

PCR reaction mixture	For *fad*-3 gene screening		Sk-*fad*-3 probe generation	PCR for yeast identification		Ct-*fad*-3 gene isolation
	First PCR (600 bp)	Semi-nested PCR (145 bp)		18S rDNA	ITS-1-5.8s-ITS-2 region	
Autoclaved RO water : To make up the volume to 20 µl						
DNA template	50 ng gDNA of yeast	5-10 ng 600 bp PCR amplicon	50 ng of *S. kluevary* gDNA	50 ng gDNA of yeast	50 ng gDNA of yeast	50 ng *C. tropicalis* PS-2 gDNA
10X buffer	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl
Forward primer	OF1F5S/ OF1F5C 0.1-0.3 µM	OF1F5S/ OF1F5C 0.1-0.3 µM	OF1F5S 0.1 µM	P108 and P1190 0.1 µM	ITS-1 0.1 µM	OF5CtF (1 kb) OF6CtF (0.4 kb) 0.1 µM
Reverse primer	OF2R5 1.2 µM	OF3R3 2 µM	OF2R5 2 µM	M2130 and M3490 0.1 µM	ITS-4 2 µM	OF2R5 (1 kb) OF7CtR (0.4 kb) 0.1 µM
dNTPs	200 µM	200 µM	200 µM	200 µM	200 µM	200 µM
$MgCl_2$*	1.5-2.5 mM	1.5-2.5 mM	1.5 mM	1.5 mM	1.5 mM	1.5 mM
Taq DNA polymerase	1.5 U	1.5 U	1.5 U (X-Taq#)	1.5 U (X-Taq#)	1.5 U (X-Taq#)	1.5 U (X-Taq#)
PCR program used for amplification						
94 °C (Initial denaturation)	270-300 sec	120 sec	270-300 sec	300 sec	300 sec	300 sec
94 °C (Denaturation)	45 sec	45 sec	45 sec	45 sec	45 sec	45 sec
Annealing temperature And time	60-50 °C, 45 sec, 10 cycles 50 °C, 45 sec, 20 cycles	60-50 °C, 45 sec, 10 cycles 50 °C, 45 sec, 20 cycles	60-50 °C, 45 sec, 10 cycles 50 °C, 45 sec, 20 cycles	54-52 °C, 45 sec, 30 cycles	54-52 °C, 45 sec, 30 cycles	55-52 °C, 45 sec, 6 cycles 52 °C, 45 sec, 24 cycles
72 °C (Extension)	50 sec	30 sec	50 sec	80 sec	70 sec	70 sec (1 kb) 40 sec (0.4 bb)
72 °C (Final extension)	10 min	10 min	10 min	10 min	10 min	10 min

'*': Mg^{++} Concentration was changed, '#': PCR amplification was done by using high fidelity X-Taq polymerase.

2.6.10 DNA sequencing and identification

The PCR products were purified by High Pure PCR product purification kit (Roche diagnostics, Germany) and customized DNA sequencing was done from Merck, India and Xleris Labs, Ahmadabad. DNA sequencing was done by primer walking or single pass analysis. Blast analysis of deduced sequences was done for identification of the respective yeast isolate. The sequences obtained were compared against the sequences in the GenBank nucleotide collection through the Basic Local Alignment Search Tool (BLAST) available on the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov/). Phylogenetic and molecular evolutionary analyses of obtained sequences were done by using MEGA6 (Tamura *et al.*, 2013).

2.6.11 Total RNA isolation and Reverse Transcriptase-PCR

Total RNA was isolated from *S. cerevisiae* W-9100 containing pSP-*fad*-3 yeast cells induced by 2% galactose (test) and *S. cerevisiae* W-9100 containing pGAL-MF yeast cell (control) by using Gene-elute yeast total RNA isolation kit (Sigma Aldrich). About 2-4 µg of RNA was then treated with 5 U of DNaseI (MBI Fermentas) at 37 °C for 30 min and DNaseI was inactivated by adding 1 µl of 0.25 M EDTA and incubating at 65 °C for 10 minutes. RNA was quantified using a Nanodrop ND-1000 spectrophotometer and was also run on 0.8% agarose gel to check the quality and integrity of the sample and used as template for confirmation of *fad*-3 mRNA transcripts by reverse transcriptase PCR by using GeNei AMV RT-PCR kit with *fad*-3 gene specific primers.

1 µl RNase inhibitor

1 µl 0.1 M DTT

4 µl Reverse transcriptase buffer (5X)

2 µl 30 mM dNTP mix

0.5 µl AMV Reverse transcriptase

1 µl sterile water

The solution was mixed well, incubated at 42 °C for 1 hour and further at 95 °C for 2 min to denature RNA-cDNA hybrids, spun briefly and quickly placed on ice. Further the c-DNA preparation was used as template for subsequent PCR with Taq-polymerase. Total RNA sample was used as control to confirm the amplification that

obtained was from c-DNA only and not from DNA contaminants in the total RNA preparation.

2.6.12 DNA - Hybridization studies

2.6.12.1 Probe labeling

The DNA fragment to be labeled (the 610 bp PCR product from the internal conserved region of *n*-3 desaturase gene from *S. kluyveri*) was amplified and purified by High pure PCR product purification kit from Roche Diagnostics, Germany. The purified PCR product was labeled and quantified using DIG High Prime Labeling and Detection Kit from Roche Diagnostics, Germany; that uses digoxigenin (DIG), a steroid hapten, to label DNA probes for hybridization and subsequent color detection by enzyme immunoassay. DIG-labeled DNA probes were generated with DIG-High Prime according to the random primed labeling technique. About 2 µg of DNA template and sterile water were added to a final volume of 16 µl in a reaction vial. The DNA was then denatured by heating in a boiling water bath for 10 min and then quickly chilled on ice. 4 µl of DIG-High prime mix was added to the DNA template and incubated overnight at 37 °C. The reaction was stopped by heating at 65 °C for 10 min. The DIG labeled probe was purified by Sephadex G-50 quick spin column (Roche); quantified and stored at -20 °C.

2.6.12.2 Dot-blot analysis

Nylon membrane (Hybond-N, Amersham Pharmacia) was spotted with 50 ng genomic DNA per sample and UV- crossed linked. DNA was denatured by soaking the membrane for 3 min in 0.4 M NaOH, washed in 2X SSC and then hybridized.

2.6.12.3 Southern Hybridization

Genomic DNA of selected yeast isolates was digested completely with *Hind* III and fractionated on 0.8% agarose gel for 5 hrs at 30 mV and blotted on nylon membrane as described by Sambrook and Russell, 2001. DNA of *S. kluyveri* and *S. cerevisiae* was used as positive control and negative control respectively.

The labeled probe DNA was then used to hybridize the blot according to the standard procedure described in 'Roche Molecular Biochemicals – Instruction Manual'. The blot was subjected to pre hybridization with DIG Easy Hyb (10 ml/100 cm^2

membrane) for 30 min at 37 °C. The DIG-labeled probe DNA (25 ng/ml DIG Easy Hybridized) was denatured by boiling for 10 min and rapidly cooling on ice. The blot was then removed from pre hybridization solution and hybridized with denatured hybridization mixture (3.5 ml/100 cm^2 membrane containing 100 ng probe) overnight at 37 °C with gentle shaking. Stringency washes with 0.5X SSC, 0.1% SDS at 65-68 °C (pre warmed to 65 °C) were given for 15 min under constant agitation to remove nonspecifically bound probe DNA. The membrane was later incubated in blocking solution for 30 min at room temperature with constant agitation. The immune-detection was done with anti-digoxygenin antibodies conjugated with alkaline phosphatase and developing the blot by reacting with a chemiluminscence substrate CSPD. The antibody was diluted as per the manufacturer's instruction and the blot was incubated in antibody solution for 30 min at room temperature. Enzymatic dephosporylation of CSPD by alkaline phosphatase that enables extremely sensitive and fast detection of biomolecules by producing visible light at λ_{max} of 477 nm, which was recorded on X-ray film.

2.7 Sub-cloning and Heterologous expression of *fad*-3 in *E. coli* BL21(DE3)

E. coli BL21(DE3) was used as the host for protein expression. Plasmid pET-28c(+) (Novagen); which carries the T7 promoter, a hexa-histidine tag coding sequence and kanamycin resistance gene was used as expression vector for the *fad*-3 gene.

The *fad*-3 gene was amplified from pSP-*fad*-3 and cloned in the expression vector at NdeI and XhoI sites. Recombinant plasmid construct (pETSP28c) was then transformed in *E.coli* BL-21(DE-3) expression host.

2.8 Biotransformation studies

2.8.1 In vivo transformation of standard fatty acids and edible oil

Selected yeast isolates were grown in 50 ml YPD up to early log phase, then supplemented with 1 mM LA pre-emulsified with 1% tergitol type NP-7 (final concentration of tergitol in the medium not more than 0.005%) and further grown to late log phase (~6-8 hrs) at 25 °C with 200 rpm. Same protocol was followed for recombinant yeasts generated in present study using SD Ura^- medium with galactose induction step (2.8.1.2).

S. cerevisiae culture supplemented with LA was used as negative control and *S. kluyveri* culture supplemented with LA was served as a positive control in all experiments. The yeast isolates not supplemented with LA was used for testing inherent content of n-3 PUFAs. Isolates under study were supplemented with LA to test whether the inherent n-3 fatty acids could be further increased. Three independent analyses were performed in YPD for each yeast strain.

Commercially available sunflower oil which contain ~65% - 70% LA was used as substrate for transformation by the selected and the recombinant yeasts. Sunflower oil was added to the medium to a final content of 1 mM LA.

2.8.2 Induction of FAD-3 expression

S. cerevisiae W-9100 yeast cultures containing (a) pSP-*fad*-3(recombinant); (b) pGAL-MF (vector control) were grown in SD Ura$^-$ medium containing glucose up to early log phase. Cell mass was harvested by centrifugation and grown in 25 ml of SD Ura$^-$ medium containing 1% raffinose for 2-3 hrs. Cells were then collected and grown in 50 ml 2% galactose containing SD Ura$^-$ medium up to late log phase. Yeast cell mass was harvested from culture broth by filtration through Whatman No. 4 filter paper. The cell mass was washed with distilled water twice and dried till constant mass was obtained.

In *E. coli* BL21(DE3) (pETSP28c), FAD-3 expression was standardized for appropriate induction conditions of IPTG concentration and temperature of induction. Different combinations listed in table 2.7 were used for standardization of FAD-3 induction. Finally appropriate condition selected and subsequent inductions were given by 0.5 mM IPTG at 30 °C.

Table 2.7 Standardization of FAD-3 induction in *E. coli* BL21(DE3)

Temperature / IPTG (mM)	25 °C	30 °C	37 °C
0.1	0.1 mM, 25 °C	0.1 mM, 30 °C	0.1 mM, 37 °C
0.5	0.5 mM, 25 °C	0.5 mM, 30 °C	0.5 mM, 37 °C
1.0	1.0 mM, 25 °C	1.0 mM, 30 °C	1.0 mM, 37 °C

2.8.3 Total Fatty acid extraction and their derivatization in methyl esters

Fatty acids in cells were analyzed after extraction and derivatization into Fatty Acid Methyl Esters (FAME) according to the protocol described by Knutzon et.al (1998) with slight modification. Pellet from 50 ml culture was thoroughly washed in deionized water, vortexed in 3 ml of methanol, followed by addition of 6 ml of chloroform containing 0.005% butylated hydroxytoluene as antioxidant. The mixture was incubated at 4 °C overnight after flushing with inert gas (nitrogen), chloroform layer separated and filtered through a Whatman filter with 1 g of anhydrous sodium sulphate to remove particulates and residual water. The organic solvents were evaporated to dryness at 45-50 °C, and the extracted lipid residue was dissolved in minimum amount of hexane (1 ml) and then vortexed with 2 ml of 0.5 N KOH in methanol, heated to 95-100 °C for 30 min, cooled to room temperature. 2 ml of 14% boron tri-fluoride in methanol was added and heating was repeated and then cooled to room temperature. The resulting FAMEs were extracted by adding 1 ml of water and 0.2 ml of hexane, and stored at -20 °C after flushing with inert gas in screw cap vial. Methyl esters of pure fatty acids ALA, LA, Arachidonic acid (AA) (C20:4 n-6), conjugated linoleic acid (CLA) (isomer of LA; C18:2), gamma linolenic acid (GLA) (C18:3 n-6), EPA and DHA were used as standards.

2.8.4 Thin layer Chromatography (TLC)

The preparations of FAME were resolved by thin layer chromatography using silica gel (thickness 0.25 mm) coated on TLC PET Foil plates with fluorescent indicator (Sigma Fluka). These plates were pre-impregnated with silver nitrate by dipping rapidly in a 5% solution of silver nitrate in acetonitrile and heating at 80 °C for one

hour prior to sample application. TLC tank was equilibrated with developing solution toluene: ethyl acetate (95/5; v/v). Samples were spotted 2.5 µl at a time along the 0.5 cm marked line. The plate was gently put in to the tank sample down. Plate was removed when the solvent overrun for one and half hour from the solvent front line. Fatty acids separated on the chromatogram were visualized with 0.2% Amido Black in 1 M NaCl (Plekhanov, 1999).

2.8.5 Gas Chromatography

The FAMEs were analysed by gas chromatography and gas chromatography–mass spectrometry. Analysis of FAMEs by gas chromatography was performed using an Acme6100 Gas Chromatograph (Young Lin Instrument, Korea) with 30 m x 0.22 mm x 0.25 µm Forte GC capillary column (BPX70) (SGE analytical science, Australia) and FID detector. The initial column temperature of 180 °C was raised at 4 °C per min to 230 °C and then held for 10 min at 230 °C. Temperature of injection port and detector was set at 260 °C. 1 µl FAME sample was injected with split ratio 20:1. Fatty acids form the test samples were identified and quantified by comparing by their retention time, the area of peaks to that of the FAME standards. The GC–MS (GC–MS; Trace GC Ultra-ITQ1100, Thermo Fisher) conditions were as follows: a HP- 5 ms column (30 m length, internal diameter 0.25 mm and film thickness 0.25 µm); an oven temperature program composed of an initial hold at 100 °C for 2 min, ramping at 10 °C per min to 280 °C, and a final hold at 280 °C for 5 min; an ion source temperature of 220 °C and EI ionization at 70 eV.

2.9 Protein expression and detection

2.9.1 Whole cell protein fraction

For whole cell protein, 50 µl of sterile D/W was added in the pellet and mixed by vortex. 100 µl of 0.5 M NaOH was added and incubated at RT up to 10 min for lysis. Samples were centrifuged at 10,000 rpm for 2 min, 100 µl deionized water and 20 µl 6X SDS loading buffer were added, mixed well and centrifuged again at 8000 rpm for 3 min. Boiled for 2 min and 10 µl of sample was loaded onto 12% SDS PAGE gel and electrophoresis was carried out.

The samples for recombinant *E. coli* (pETSP28c) were prepared by adding to the cells equal volume of 2X Laemmli buffer with freshly prepared 100 mM DTT incubating at

95 °C for 5 min in water bath. Concentration of protein in each sample was quantified by Bradford's protein assay (Bradford, 1976). Appropriate amount of protein of each sample was loaded onto gel with 12% separating gel. Gels were visualized with Coomassie Brilliant Blue/silver staining procedure.

2.9.2 Culture supernatant protein fraction precipitation and dialysis

2.9.2.1 Ammonium sulphate precipitation

The culture supernatants of galactose induced *S. cerevisiae* W-9100 yeast cultures containing (a) pSP-*fad*-3(recombinant); (b) pGAL-MF (vector control) was partially purified by 25% - 70% ammonium sulphate precipitation. Proteins were precipitated and pelleted as before and the protein pellet resuspended in minimal amount of dialysis buffer (50 mM Tris pH 7 + 7.26 mM NaCl and 1.5 mM $CaCl_2$) and stored at 4 °C until dialysis. The protein solutions were dialyzed three times against 500 times the volume of dialysis buffer each time, with stirring in the cold for 3 hrs, 3 hrs and 16 hrs respectively. The dialyzed ammonium sulphate precipitated enzyme (appendix I) solution was concentrated by vacuum evaporation at -20 °C.

2.9.2.2 Acetone precipitation

As an alternative to ammonium sulphate precipitation, equal volume of acetone was added to the galactose induced yeast culture supernatant and kept at 4 °C overnight. The precipitated proteins were then separated by centrifugation at 8000 rpm for 20 min. The protein pellet was air dried, resuspended in dialysis buffer and stored at 4 °C.

Total protein precipitated from culture supernatant was attempted for solubilization in two separate buffers *viz.* buffer A containing 20 mM Tris-Cl buffer pH 7.5, 0.33 mM Triton X-100, 10 mM KCl, 20 mM $MgCl_2$ and 5% glycerol; buffer B containing 20 mM Tris-Cl pH 7.5, 0.30 mM DTT, 0.33 mM Triton X-100, 20 mM NaCl and 5% glycerol. Protein was estimated by Bradford's protein assay (Bradford, 1976) using bovine serum albumin as the standard.

2.9.3 Solubilization of membrane proteins

Galactose induced yeast cells were used for preparation of the samples for SDS-PAGE. Yeast cells from 5 ml culture, were resuspended in 0.5 ml working sorbitol solution (0.9 M sorbitol, 0.1 M EDTA, 0.1 M Tris, 0.1% β-mercaptoethanol)

containing lyticase (final concentration of 0.4 mg/ml) enzyme to prepare spheroplasts. Speroplasts were pelleted at 10000 rpm and membranes proteins were solubilized by different detergents e.g. Triton X-100 (1% - 5%), SDS (1% - 5%), Sarcosyl (0.5% - 2.5%) (n-lauroyl sarcosine), Tween 20, Nonidet P-40, Dodecyl Maltoside, Brij-58, Tergitol etc. These treatments were given at different concentrations of detergents and at different temperatures. Soluble fractions were recovered by centrifugation.

Both soluble and insoluble fractions were then treated with equal volume of 2X Sample loading Buffer and incubated at 70 °C for 30 min. SDS-PAGE was run as per normal protocol with soluble and insoluble fractions separately.

2.9.4 Purification of Ct-FAD-3 protein expressed in *E. coli*

For purification of FAD-3 protein from *E. coli* BL-21(DE3) harbouring the expression plasmid, pETSP28c, cells were grown to an OD_{600} of approximately 0.4 to 0.5, induced by 0.5 mM IPTG and cells were further incubated for 3 hrs at 30 °C. Cell mass was harvested and washed twice with Phosphate Buffer Saline (PBS; containing 137 mM NaCl, 2.7 mM KCl, 10 mm Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.4). Cells were stored as frozen pellet at -20 °C, thawed later and disrupted by sonication in PBS, centrifuged at 12,000 g for 10 min. The supernatant was collected, protein was estimated and analysed further on SDS-PAGE. Expressed His tagged fusion protein was purified by His tag-Nickel ion affinity chromatography using GeNei His-tag fusion protein purification kit (Merck, India). Purified FAD-3 protein was confirmed by SDS-PAGE.

2.9.5 Polyclonal anti-Ct-FAD-3 antibody generation and purification

Two New Zealand white Rabbits were immunized with 500 µg/kg body weight purifird Ct-FAD-3 protein. Pre-immune serum from the rabbits, collected before initiation of the immunization with FAD-3 protein. One rabbit was injected with purified FAD-3 protein while other one was kept as control. Polyclonal anti-FAD-3 antibodies were generated in rabbit against purified FAD-3 protein expressed in the heterologous host *E. coli* BL21(DE3). Standard regime of antigen injections was followed as per given bellow:

Day 1: 1st Injection was given with Complete Freund's adjuvant (CFA) + Ct-FAD-3 protein preparation

Day 30: 2nd Injection was given with Freund's adjuvant incomplete (ICFA) + Ct-FAD-3 protein preparation

Day 40: 5-10 ml blood was collected from rabbit (1st serum)

Day 60: 3rd Injection was given with ICFA + Ct-FAD-3 protein preparation

Day 70: 5-10 ml blood was collected from rabbit (2nd serum)

Day 74-75: Terminal 10-15 ml blood collection was done.

The final blood was collected one week after the last booster dose and immediate testing was done by ELISA for estimating the titer.

Rabbit serum containing polyclonal anti-FAD-3 antibodies was separated from whole blood. Anti-Ct-FAD-3 IgG antibodies were purified from that serum by using 'Protein-A based affinity purification column kit (Merck, India) and analyzed by loading on SDS-PAGE.

2.9.6 Indirect ELISA

Antigen preparations were diluted to a final concentration 20 µg/ml in 1X PBS. The wells of a micro-titer plate were coated with diluted 1-2 µg of antigen and incubated at 4 °C overnight. The coating solution was removed and the plate was washed three times with 200 µl washing buffer (PBST; 1X PBS containing 0.05% v/v Tween 20). The remaining protein binding sites in the coated wells were blocked by adding 200 µl of blocking buffer, 3% BSA in 1X PBS and incubated for 2 hrs at room temperature. The plate was washed twice with of washing buffer. Primary antibody was diluted at three different dilutions i.e. 1:5000, 1:10000 and 1:15000 in blocking buffer and 100 µl was added to each well and incubated for 2 hrs at room temperature. The plate was washed four times with washing buffer. Secondary antibody (HRP conjugated goat anti-rabbit IgG antibody; Merck, India) was diluted 1:1000, added to wells and incubated for 1-2 hrs at room temperature. The plate was washed four times with washing buffer. The detection was done by adding 100 µl of 1X substrate, TMB/H_2O_2 (Tetramethybenzidine/Hydrogen peroxide). After sufficient color development the reaction was stopped by adding 100 µl of 2 M of H_2SO_4. The micro titer plate was placed inside the ELISA plate reader and absorbance was measured at 450 nm.

2.9.7 SDS Polyacrylamide gel electrophoresis (SDS-PAGE)

S. cerevisiae W-9100 yeast cultures containing (a) pSP-*fad*-3(recombinant); (b) pGAL-MF (vector control) were grown in 50 ml SD Ura^- medium and subsequently 2% galactose induction was given. Supernatant was separated and used for acetone precipitation of total secreted proteins. Cell mass (from 5 ml culture) induced for FAD-3 expression was harvested. Both cell mass and precipitated total protein fractions were used for further detection of the FAD-3 protein and desaturase activity.

The electrophoretic separation of proteins was done by 12% SDS-polyacrylamide (SDS-PAGE) gel. The compositions of the reagents used are given in appendix I.A. 5-10 µg protein of the protein sample was boiled for 5 min (unless mentioned otherwise) with an equal volume of 2X gel loading buffer containing 4% SDS and 5% β-mercaptoethanol and run on an SDS-polyacrylamide gel with 4% stacking (pH 6.8) and 12% resolving gels (pH 8.8) at 20 mA constant current till the bromophenol blue dye reached near the edge of the gel. Afterwards, proteins were visualized by silver staining or by Coomassie Brilliant Blue G-250 staining. The protein molecular weight marker used was obtained from Merck, India with following size markers 205 kDa, 97.4 kDa, 66 kDa, 44 kDa and 29 kDa. About 2-20 µg of protein was applied to the gels depending upon the sample source and subsequent visualization method used.

Coomassie™ Brilliant Blue G-250 staining

Coomassie staining of protein gels is based on binding of the dye Coomassie Blue G-250, which binds nonspecifically to virtually all proteins. The gel was first washed with double distilled water (D/W) for ten minutes and then kept overnight at room temperature (RT) in a dye solution (0.25% in a de-staining solution; 40:10:50 – Methanol: Acetic acid: Distilled water) with gentle shaking. Dye that is not bound to protein diffuses out of the gel during the de-staining steps with destaining solution. De-staining was carried out until proper visualization of bands was achieved.

Silver Staining

The gel was first washed with D/W for ten minutes and then kept overnight at room temperature with gentle shaking in fixative of methanol: glacial acetic acid: water (30: 10: 60). The gel was then incubated in 5 gel volumes of 30% ethanol or methanol and kept on gentle shaking at RT for 30 min. After this, gel was washed thrice with 5 gel volumes of deionized water for 5 min each time at RT with gentle shaking. The gel

was then incubated for about a minute in freshly prepared 0.02% sodium thiosulphate ($Na_2S_2O_3.5H_2O$) solution and washed briefly with deionized water for about 10 sec. This was followed by incubation with 5 gel volumes of 0.2% $AgNO_3$ solution with gentle shaking at RT for 30 min. The gel was then rinsed in deionized water for a few seconds and subsequently immersed in 5 gel volumes of 2.2% Na_2CO_3 + 0.05% formaldehyde + 2 ml of 0.02% $Na_2S_2O_3.5H_2O$ for every 100 ml solution, till the desired contrast was obtained (~10 min) The reaction was stopped by immediately incubating the gel in 10 gel volumes of 1% acetic acid solution.

Ponceau-S Staining

It is used for the detection of proteins on cellulose acetate, PVDF, and nitrocellulose membranes. This staining technique is reversible to allow further immunological detection. After electrophoresis, the blotted membrane was immersed in a sufficient amount of Ponceau S Staining Solution (0.1% in 5% Glacial Acetic acid) for 5-10 minutes. After staining, the membrane was kept in an aqueous solution containing 5% acetic acid (v/v) for 5 minutes and washed subsequently with D/W until protein bands were visible. After visualization membrane was washed completely with D/W and kept for blocking.

2.9.8 Western blot analysis

The reactivity of the recombinant protein with rabbit anti-FAD-3 antibodies was evaluated by Western blot analysis. For Western blot analysis, protein gels were transferred onto a nitrocellulose membrane (Thermo Fisher Scientific - Rockford, IL) by electro-blotting (35-40 V, overnight at 4 °C) using a Trans-Blot electrophoretic transfer apparatus. The membrane was blocked with 3% BSA in wash buffer (PBST), and probed rabbit anti-FAD-3 antibodies for detection of FAD-3 protein in yeast cells; followed by appropriate secondary antibodies and subsequently detection system.

2.9.9 Isoelectric focusing (IEF) and 2-D SDS-PAGE

Purified *E. coli* Ct-FAD-3 protein was subjected to isoelectric focusing; using immobilized pH gradient (IPG) strips (Ready Strip IPG strips, Bio-Rad). His tag affinity purified FAD-3 protein preparation was first re-suspended in 500 µL of distilled water. 2-5 µg, was diluted to 125 µl with rehydration solution (9 M Urea, 2% CHAPS, 30 mM DTT, 0.5% ampholytes pH 3-10, 0.002% bromophenol blue). IEF dry strip gels (pH 5-8), 7 cm, (Bio-Rad, CA, USA) were rehydrated with 125 µl of

mixture solution in 7 cm strip holders and electro-focused with Protean (IEF) Isoelectric Focusing System (Bio-Rad, CA, USA). The focusing was performed at 50 μA per strip at 20 °C; 250 V for 20 min; 4000 V for 2 hrs; and 15000 V/hrs.

Second dimension electrophoresis (2-DE) was performed according to the manufacturer's instruction (Bio-Rad, CA, USA). After isoelectric focusing, strips were equilibrated (10 min) with gentle shaking in SDS equilibration buffer I (0.375 M Tris-Cl buffer, pH 8.8, 6 M urea, 20% glycerol, 2% SDS, 2% DTT w/v), thereafter SDS equilibration buffer II (0.375 M Tris- Cl buffer, pH 8.8, 6 M urea, 20 % glycerol, 2% SDS, 2% DTT, 2.5% Iodoacetamide w/v). The second dimension SDS electrophoresis was performed using Mini-PROTEAN SYSTEM. (Bio-Rad, CA, USA). The protein spots were detected by silver staining (Sambrook and Russell, 2001).

2.10 Bioinformatics tools and websites

- *Primer 3*: The theoretical validation of the primers with respect to absence of intermolecular and intramolecular complementarities to avoid primer-primer annealing and hairpin structures and the calculations of G-C percentage was carried out with the help of online primer designing software Primer 3.
  http://biotools.umassmed.edu/bioapps/primer3_www.cgi
- CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) PCR primer design program was used for designing degenerate primers.
  http://blocks.fhcrc.org/codehop.html
- Oligo Calc program was used for analysis of the designed primer sequences.
  http://www.basic.northwestern.edu/biotools/OligoCalc.html
- *BLAST* (Basic Local Alignment Search Tool): The homology and similarity of the obtained sequences was predicted by using at BLASTn program at NCBI.
  http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome
- The Sequence Manipulation Suite is a collection of web-based programs was used for analyzing and formatting DNA and protein sequences.
  http://www.bioinformatics.org/sms/rev_comp.html
- ORF Finder was used to translate a nucleotide sequence to a protein sequence in different outputs (3 different reading frames).

http://www.ncbi.nlm.nih.gov/gorf/gorf.html

- SIB Bioinformatics Resource Portal was used for different DNA and protein analysis from ExPASy website.
  http://www.expasy.org/proteomics
- NEBcutter V2.0 was used to find restriction sites of enzymes within a sequence of interest and to determine the sizes of fragments obtained thereafter.
  http://tools.neb.com/NEBcutter2/
- ClustalW2 program was used for multiple sequence alignment of DNA or proteins (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and EMBOSS Needle that reads two input sequences and writes their optimal global sequence alignment to file was used to align nucleotide sequences for comparison or for predicting binding sites of primers.
  http://www.ebi.ac.uk/Tools/psa/emboss_needle/nucleotide.html
- MEGA6: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods was used for phylogenetic analysis of identified yeast strains.
  http://www.megasoftware.net/index.php