4. Isolation, cloning and heterologous expression of Ct-*fad*-3 gene from *Candida tropicalis* PS-2

Single Cell Oil (SCO) rich in n-3 PUFAs have been reported from *Schizochytrium* (high DHA) (Barclay *et al*., 2010) and genetically modified yeast *Yarrowia lipolytica* (high EPA) (Beopoulos *et al*., 2009; Xue *et al*., 2013). Bio-prospecting the yeasts screened for n-3 PUFAs from the local niche is desirable to assess these as an alternative source for essential PUFAs. In previous part of work, 19 locally isolated n-3 PUFAs producing yeasts were selected by extensive screening procedures. This part of the work mainly focused on the isolation of *fad*-3 gene from one of the putative isolate *C. tropicalis* PS-2.

4.1 Development of *C. tropicalis* PS-2 as a potential n-3 PUFA producer

In the present study, four strains of *C. tropicalis* (PS-2, MS-5, A-12 and A-13) were among the screened n-3 PUFAs producers (Table 3.3). *C. tropicalis* PS-2 was isolated from '*Neera*' which is a traditional drink obtained from palm, *Phoenix sylvestris* well accepted for its health benefits. Hence this isolate was selected for further study and *fad*-3 gene isolation. GC analysis of the fatty acid profile of *C. tropicalis* PS-2 grown in YPD at 25 ºC showed presence of ALA in its total fatty extract, accounting to 2.27 mg/lit of ALA without any n-6 PUFAs supplementation. The ratio of ALA: LA was found to be 1:8-10. Other higher n-3 PUFAs were not detected. On exogenous supply of LA (n-6), production of ALA (n-3) was increased up to 19.24 ± 3.25 mg/lit with the ALA: LA ratio up to 1:3-4 indicating incorporation of n-6 PUFAs in cell membrane and increased desaturation at n-3 position (Table 3.4). Hence *C. tropicalis* PS-2 was considered as a putative source of *fad*-3 gene and explored further for isolation and characterization. Ct-*fad*-3 sequence isolated from *C. tropicalis* PS-2 was subsequently submitted in GeneBank; Accession No. ADN42964.

Humans carry *Candida* throughout the gastrointestinal and genital tract as part of the normal commensal flora. Most yeast infections are due to endogenous strains of *Candida* and begin especially when combined with underlying immunosuppressed state, and the use of certain medications (Ozhak-Baysan *et al*., 2012; Arendrup, 2013; Kwamin *et al*., 2013).

On the other hand, different applications of *C. tropicalis* such as production of dicarboxylic acid (Picataggio *et al*., 1992; Gangopadhyay *et al*., 2006), xylitol fermentation (Wang *et al*., 2013, Ahmad *et al*., 2013), sterol concentration and production (Zhao *et al*., 2013), lipid accumulation for biodiesel production (Karatay and Donmez, 2010; Dey and Maiti, 2013), production of biosurfactant and polyhydroxybutyrate (Priji *et al*., 2013) *etc*. have been reported. With this in mind, n-3 PUFAs producing ability of locally isolated *C. tropicalis* PS-2 may find its application in improving health as nutraceutical.

4.2 Isolation of *fad*-3 gene from *C. tropicalis* PS-2

Analysis of amino acid sequences of previously characterized n-3 fatty acid desaturases (FAD-3) and n-6 fatty acid desaturases (FAD-6) among the several organisms, showed wide variations towards both N- and C-terminal amino acid residues (Table 4.1). This contributed to the difficulty in designing primers for the isolation of complete gene.

Table 4.1 Variations in FAD-3 protein sequences at N- and C-terminals.

Organism	N-terminal amino acid sequence	C-terminal amino acid sequence
C. albicans	**MS**VVEA**S**SSSVVED	DAK**GV**MM**M**F**RN**V**N**GW**G**P**VKP**KD----
C. Parapsilosis (p)	**MS**TVHA**S**TTTNLDN	DAK**GV**MM**M**Y**RN**V**N**GL**G**–**VKP**RN------
S. kluyveri	**MS**IETVG**S**SSGVAI	------ **GV**R**M** F**RN**T**N**GV**G**–**VKP**EDGSSQ
P. pastoris	**MS**KVTV**S**GSEILEG	------ **GV**Y**M**F**RN**C**N**NV**G**–**VKP**KDT----
K. lactis	**MS**KSTGVEHHISGV	-------**GV**L**M**F**RN**T**NG**V**G** ––A**P**CQE
M. alpina	**M**APPHVVDEQVRRR	-------D**V**VFYKH----------

(p): putative.

4.2.1 Strategy employed for amplification of *fad*-3 gene sequence and expression vector construction

Due to wide variations within both N- and C-terminal amino acids, a two-step strategy was employed for isolation of Ct-*fad*-3 gene, using 2 sets of degenerate primers (Table 2.5). The N-terminal (~1000 bp) and C-terminal (~400 bp) fragments with 100 bp overlap were separately amplified, cloned separately in pBlueScript KS (+) and recombined *in vitro* to get the complete Ct-*fad*-3 gene (Figure 4.1).

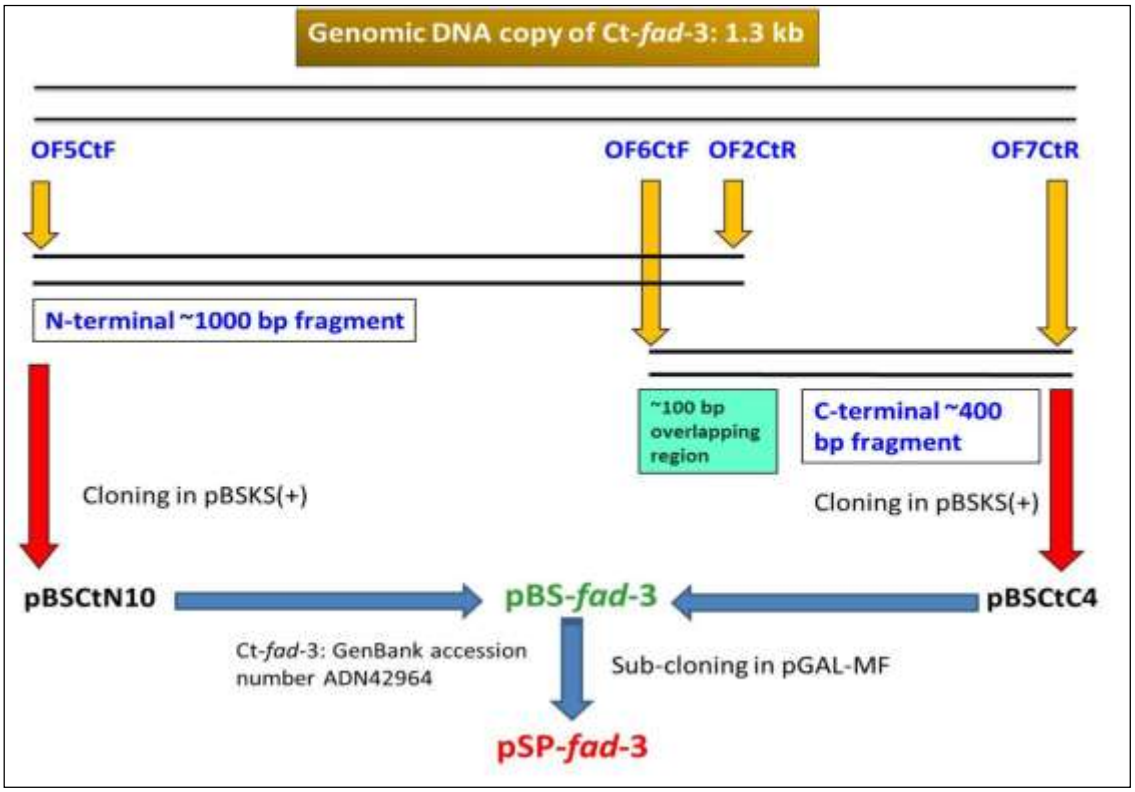

Figure 4.1 Schematic representations of Ct-*fad*-3 gene isolation and cloning. The *fad*-3 gene was amplified as separate N- and C-terminal fragments and cloned separately and subsequently recombined to give a complete gene Ct-*fad*-3 which is then sub-cloned in yeast expression vector pGAL-MF to produce the expression construct pSP-*fad*-3.

4.2.2 Cloning of Ct-*fad*-3

The first N-terminal ~1000 bp Ct-*fad*-3 gene fragment was amplified by using OF5CtF and OF2CtR and cloned into a pBSKS (+) vector at HincII site by blunt end ligation. The resulting construct was named pBSCtN10 (Figure 4.2 A); and sequence was obtained.

Based on the sequence of N-terminal 1000 bp fragment, a second forward primer (OF6CtF) was designed from the 3' end of the 1000 bp fragment and was used along with OF7CtR to amplify remaining stretch of *fad*-3 gene (C-terminal 400 bp fragment) in such a way that both N- and C-terminal fragments shared 100 bp overlap which contained a NcoI site.

The C-terminal 400 bp cloned at HincII site in pBSKS (+) was named as pBSCtC4 (Figure 4.2 B). Full length Ct-*fad*-3 gene was obtained by removing the 400 bp fragment by RE digestion at NcoI and XhoI site and its subsequent cloning in NcoI and XhoI

digested pBSCtN10 (Figure 4.3 A and B). The construct containing complete Ct-*fad*-3 was named pBS-*fad*-3.

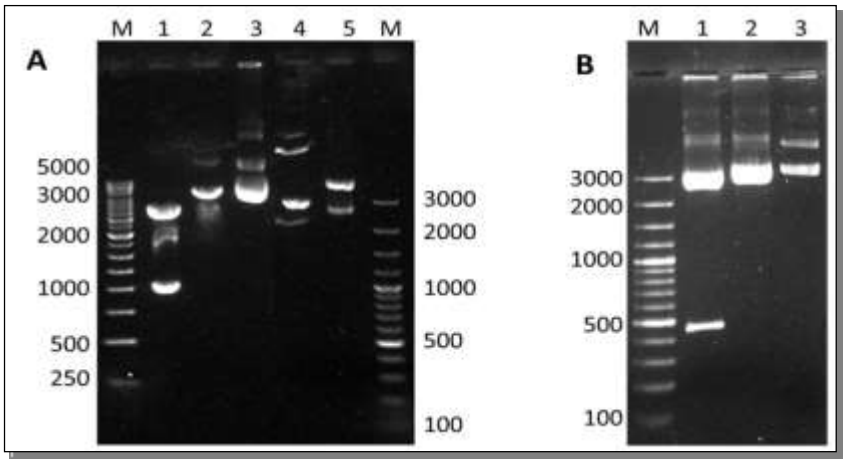

Figure 4.2 Cloning of N- and C-terminal fragments of Ct-fad-3. A; pBSCtN10; M: DNA Marker, Lane 1: Insert release from pBSCtN10 by HindIII and XhoI, Lane 2: Linearization of pBSCtN10 by HindIII, Lane 3: Undigested pBSCtN10, Lane 4: pBSKS (+) linearization by HindIII, Lane 5: Undigested pBSKS (+). B; pBSCtC4; M: DNA Marker, Lane 1: Insert release from pBSCtC4 by NcoI and XhoI, Lane 2: Linearization of pBSCtC4 by HindIII, Lane 3: Undigested pBSCtC4.

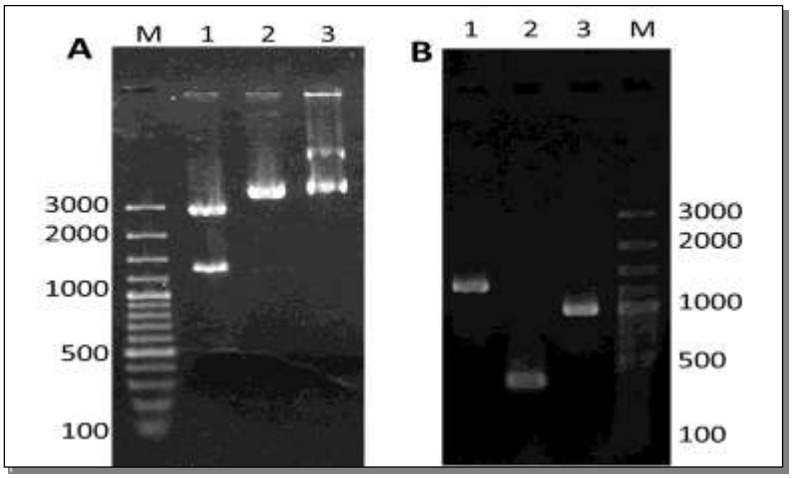

Figure 4.3 Generation of complete Ct-*fad*-3 gene in pBS-*fad*-3. A; M: DNA Marker, Lane 1: Insert release from pBS-*fad*-3 by HindIII and XhoI, Lane 2: Linearization of pBS-*fad*-3 by HindIII, Lane 3: Undigested pBS-*fad*-3. B; PCR confirmation of recombinant; M: DNA Marker, Lane 1: pBS-*fad*-3 1.3 kb PCR, Lane 2: pBS-*fad*-3 0.4 kb PCR, Lane 3: pBS-*fad*-3 1.0 kb PCR.

4.2.3 Sub-cloning of Ct-*fad*-3 in yeast expression vector pGAL-MF

The complete Ct-*fad*-3 gene (1.3 kb) was released from pBS-*fad*-3 by HindIII and XhoI digestion and then sub-cloned in to the yeast expression vector pGAL-MF at same sites

to generate the construct pSP-*fad*-3 (Figure 4.4). The presence of the inserts in the plasmid was confirmed by restriction analysis and sequencing. Recombinant construct was then transformed in yeast expression host *S. cerevisiae* W-9100.

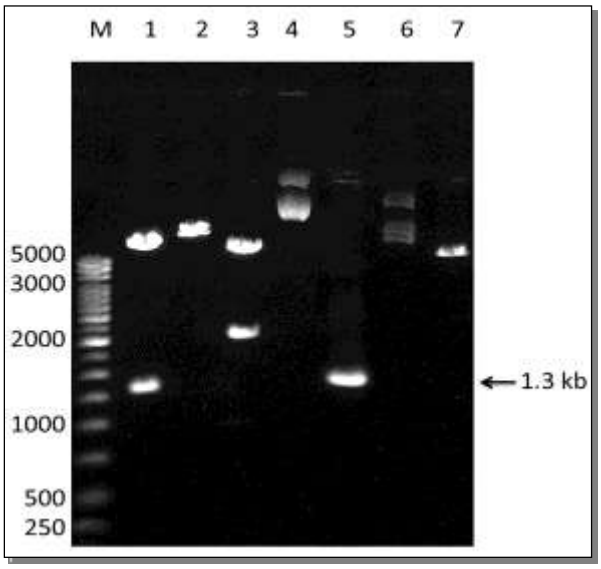

Figure 4.4 Construction of pSP-*fad*-3. M: DNA Marker, Lane-1: Insert release from pSP-*fad*-3 by HindIII and XhoI, Lane 2: Linearization of pSP-*fad*-3 by HindIII, Lane 3: Orientation confirmation by NcoI digestion of pSP-*fad*-3, Lane 4: Undigested pSP-*fad*-3, Lane 5: PCR confirmation of pSP-*fad*-3, Lane 6: Undigested pGAL-MF. Lane 7: pGAL-MF linearization by HindIII.

4.3 Bioinformatic analysis of the derived Ct-*fad*-3 sequence

The nucleotide sequence of Ct-*fad*-3 gene consisted of an open reading frame with 1305 bp, encoding for a protein with 434 amino acid residues, with a calculated molecular mass of 49.85 kDa and a pI of 6.4. The sequence has been submitted to GenBank database under accession number ADN42964. According to the primary structure, this enzyme belongs to Δ-12 domain super-family and has extensive hydrophobic regions that would be capable of spanning the membrane bilayer. These integral hydrophobic signal sequences might be involved in directing protein targeting by either co-translational (Signal Recognition Particle-dependent; SRP-dependent) or post-translational (SRP-independent) pathways. Comparison of amino acid sequence of Ct-FAD-3 with that of previously reported FAD-3 proteins also reveals that the Ct-FAD-3 protein is a membrane bound desaturase which contains the N-terminal cytochrome b5 domain as electron donor at the N-terminal end of the desaturase domain (Zdobnov and Apweiler,

2001). There are three regions of conserved histidine cluster motifs that contain eight histidine residues: HXXXH, HXXHH, and HXXHH in dasaturase domain (Appendix II.E), a characteristic feature of membrane-bound desaturases. These act as ligands for the di-iron cluster that are likely to be involved in the catalysis of the desaturation reaction (Shanklin and Cahoon, 1998). On the other hand soluble desaturases (Δ-9; stearoyl-ACP desaturase; EC 1.14.99.6) contain two conserved histidine motifs (D/EXXH) (Shanklin and Cahoon, 1998).

Hydropathy and topology analyses of the deduced amino acid sequence of Ct-FAD-3 were generated using tools available at expasy site (http://www.expasy.org/). Ct-FAD-3 sequence was analyzed by online web based tool 'The DAS transmembrane Predictor' available at; http://www.sbc.su.se/~miklos/DAS (Cserzo *et al*., 1997) which showed the presence of six transmembrane regions within the sequence (Figure 4.5).

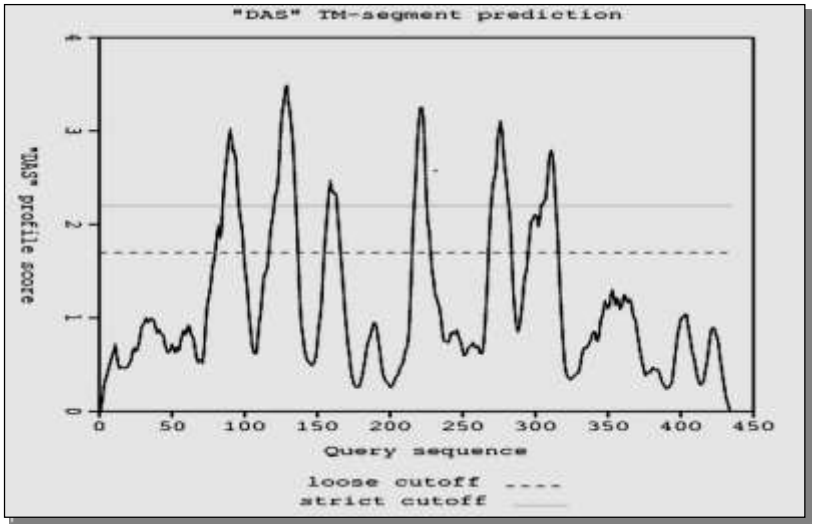

Figure 4.5 Hydropathy plot of Ct-FAD-3. Curves obtained in the hydropathy plot were deduced by pairwise comparison of the proteins in the test set in "each against the rest" fashion. The two cutoffs indicated on the plots; a "strict" one at 2.2 DAS score, and a "loose" one at 1.7. The hit at 2.2 is informative in terms of the number of matching segments, while width at 1.7 indicated actual location of the transmembrane segment.

Protein localization prediction by HMMTOP (http://www.enzim.hu/hmmtop/index.php) showed one putative and six certain trans-membrane spanning regions (Schematic representation as per figure 4.6) which account 30% - 35% of total amino acids in the protein (Tusnady and Simon, 2001) like other members of this superfamily (Los and Murata, 1998).

The catalytic pocket is formed at the interface of membrane and cytoplasm after anchoring to the membrane by virtue of these specialized transmembrane regions. All the histidine boxes are located at the hydrophobic regions of the protein that make them fall at the cytoplasmic side and the N-terminal termini with cytochrome B5 like domain was found to be exposed to the cytosol (Figure 4.6).

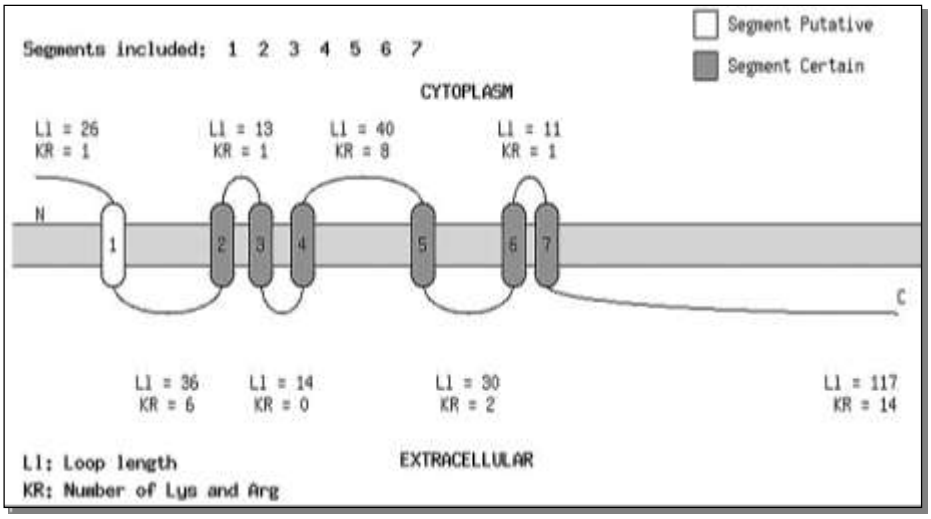

Figure 4.6 Schematic representation of predicted trans-membrane regions of Ct-FAD-3. It shows 1 putative (1, amino acid residues 24-42) and 6 certain (2-7, amino acid residues 78-100, 116-141, 154-170, 216-236, 266-284 and 291-312 respectively) trans-membrane regions.

The final nucleotide and deduced amino acid sequences of the Ct-*fad*-3 gene and protein are as per given in appendix II. Pairwise multiple alignment showed that the amino acid sequence of Ct-FAD-3 has 87% identity with Ca-FAD-3 (Murayama *et al*., 2006), 75% with Lk-FAD-3 (Yan *et al*., 2013), 72% with Kl-FAD-3 (Kainou *et al*., 2006), 64% with Sk-FAD-3 (Oura and Kajiwara, 2004) and 62% with Pp-FAD-3 (Zhang *et al*., 2008). Phylogenetic analysis of the deduced Ct-FAD-3 with these fungal FAD-3s is as per shown in figure 4.7.

Amino acid sequence comparison also revealed no conservation at N-terminal region while few residues were conserved at the C-terminal. Two small stretches amino acid residues were additionally found in Ct-FAD-3 as well in the reported Ca-FAD-3, out of which one -KEDEK- at C-terminal end was found to be conserved while the second (-IENHETL-) was at N-terminal and showed variations with that of Ca-FAD-3. Cytochrome B5 domain at N-terminal of Ct-FAD-3 extends from 22 to 131 while

desaturase domain is of 257 amino acid long while no signal peptide was detected at N-terminal region of the protein.

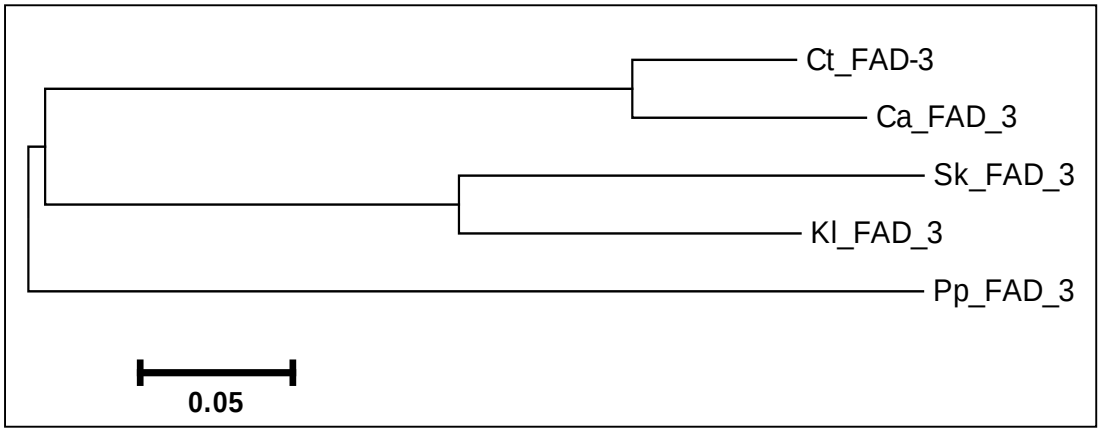

Figure 4.7 Molecular Phylogenetic analysis of deduced Ct-FAD-3 protein sequence.
The evolutionary history was inferred by using the Maximum Likelihood method conducted in MEGA6 (Tamura *et al*., 2013). The analysis involved 5 protein sequences from yeast origin [FAD-3 from *C. tropicalis* PS-2 (Ct-FAD-3), *C. albicans* (Ca-FAD-3), *S. kluyveri* (Sk-FAD-3), *K. lactis* (Kl-FAD-3), *P. pastoris* (Pp-FAD-3)]. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

4.4 Heterologous expression and functional identification

The full length Ct-*fad*-3 gene sub-cloned into yeast expression vector pGAL-MF under the strong control of GAL L promoter, fused with mating factor-α; the generated pSP-*fad*-3 recombinant construct was transformed into *S. cerevisiae* W-9100. *S. cerevisiae* has been well accepted as an excellent experimental system to study fatty acid desaturation, as it provides a eukaryotic endoplasmic reticulum, cytochrome b5, NADH and lacks inherent PUFAs in their membranes but can accumulate them if exogenous supplementation is given (Beopoulos *et al*., 2011).

4.4.1 Analysis of effect of *fad*-3 expression on growth of yeast cells

The influence of *fad*-3 expression on growth physiology at different temperatures in media supplemented with 1 mM LA was studied. The recombinant *S. cerevisiae* W-9100 (pSP-*fad*-3) showed growth characteristics similar to the control at 30 ºC (Figure 4.8 A). On the other hand, at low temperature (20 ºC) growth rate of recombinant yeast was comparatively higher (0.452 generations/hrs; generation time: 2.21 hrs) than that of control (0.345 generations/hrs; generation time: 2.90 hrs) (Figure 4.8 B).

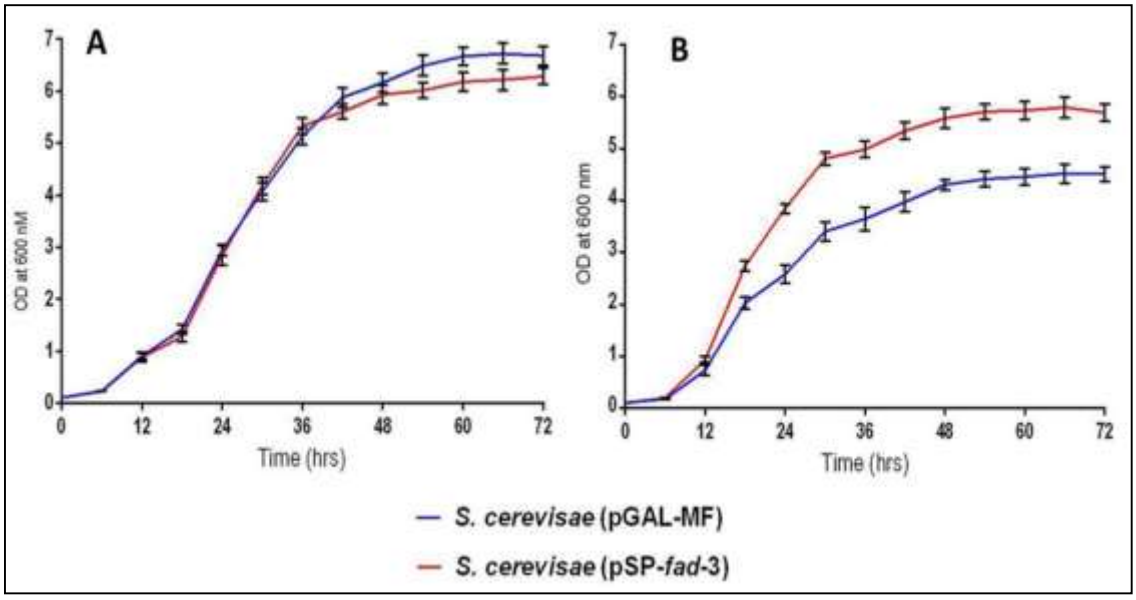

Figure 4.8 Analysis of effect of FAD-3 expression on growth of yeast cells. Yeast cultures were grown in a SD Ura$^-$ medium containing 2% galactose supplemented with 1 mM LA. A: Growth at 30 °C, B: Growth at 20 °C.

Expression of fatty acid desaturase genes is induced at low-temperature stress in most fungi (Oura and Kajiwara, 2004) which might be responsible in acclimatizing the cells at low temperatures.

Expression of *fad*-3 gene was analyzed in yeast cells induced by galactose, first for the presence of *fad*-3 mRNA transcripts by reverse transcriptase PCR, followed by expression of FAD-3 protein by SDS-PAGE and thereafter by substrate transformation.

4.4.2 Analysis of Ct-*fad*-3 mRNA transcripts by Reverse Transcriptase PCR

Total RNA was isolated from galactose induced yeast cell cultures (Figure 4.9 A). A two-step RT-PCR for c-DNA from total RNA samples using gene specific primers in galactose induced *S. cerevisiae* W-9100 (pSP-*fad*-3) (recombinant) confirmed the presence of 1.3 kb Ct-*fad*-3 transcripts (Figure 4.9 B). A PCR done directly from total RNA sample of *S. cerevisiae* W-9100 (pSP-*fad*-3) turned negative indicating any absence of DNA contamination in the sample, thus confirming expression.

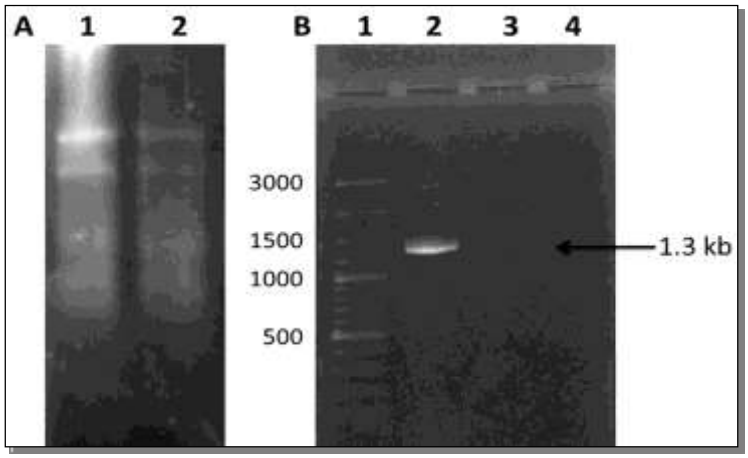

Figure 4.9 Reverse transcriptase PCR of Ct-*fad*-3. A; Total RNA isolated from galactose induced *S. cereviecae* W9100 cultures. Lane 1: Control vector pGAL-MF, Lane 2: Recombinant construct pSP-*fad*-3, B; Lane 1: 100 bp ladder, Lane 2: Reverse transcriptase PCR, *S. cerevisiae* W9100 (pSP-*fad*-3) total RNA, Lane 3: Normal PCR using Taq polymerase, *S. cerevisiae* W9100 (pSP-*fad*-3) total RNA, Lane 4: Reverse transcriptase PCR, *S. cereviecae* W9100 (pGAL-MF) total RNA.

4.4.3 Determination of desaturase activity of *fad*-3 gene on linoleic acid

In order to investigate whether the functional activity of the recombinant Ct-*fad*-3 is manifested at all, substrate transformation studies were carried out. Recombinant cells were grown in a medium supplemented with LA and extracellular (in vitro) or intracellular conversion (in vivo) of LA to ALA was analysed. In addition protein precipitated from the culture supernatant was used as an enzyme source in a buffered system where in cell extract of non-recombinant *S. cerevisiae* W-9100 was supplied as a source of necessary cofactors. No significant n-6 to n-3 transformation was observed in culture supernatant or protein precipitated from there in, while ALA was detectable in FAMEs preparations made from intact whole cells only (Figure 4.10).

Fatty acid methyl esters (FAME) of whole cells of *S. cerevisiae* W-9100 (pSP-*fad*-3) showed a new peak corresponding to ALA, that was absent in the control cells *viz*. *S. cerevisiae* W-9100 (pSP-*fad*-3) without LA supplementation and *S. cerevisiae* W-9100 (pGAL-MF) with LA supplementation which confirms the presence of cell associated Ct-FAD-3 activity (Figure 4.11). Detailed analysis of desaturation activity by expressed Ct-FAD-3 is presented in chapter 5.

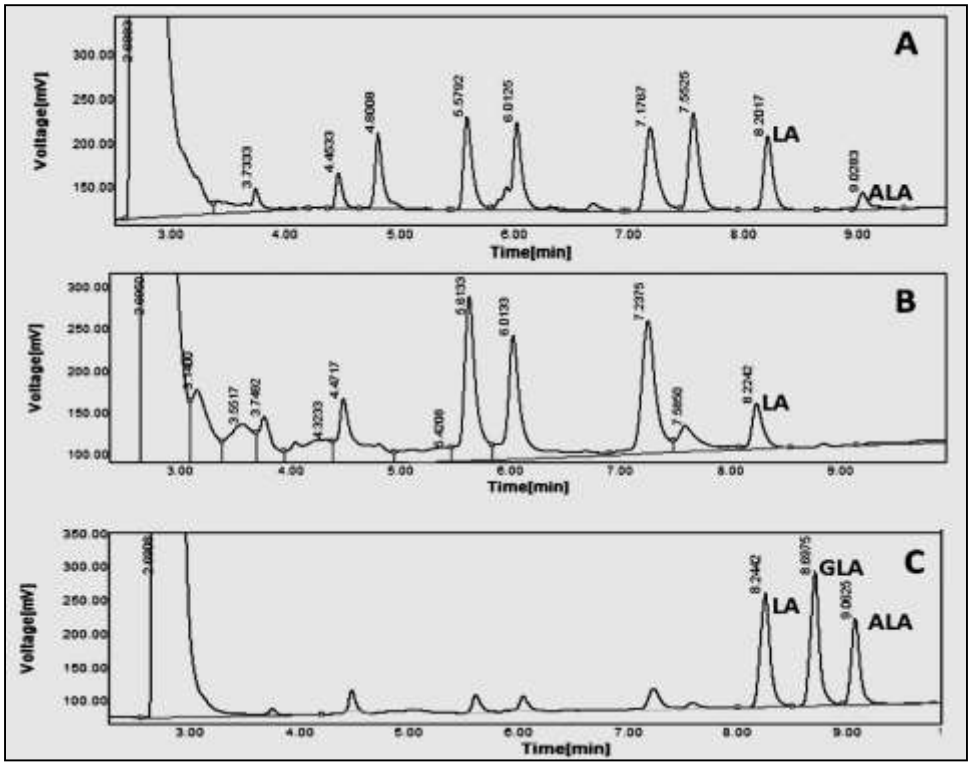

Figure 4.10 GC chromatograms of FAMEs derived from yeast total fatty acids extracts. A: *S. cerevisiae* W-9100 (pSP-*fad*-3)**,** B: *S. cerevisiae* W-9100 (pGAL-MF)**,** C: Standard fatty acids LA, GLA and ALA methyl esters**.** Yeast cultures were grown in media supplemented with 1 mM LA. An ALA peak at retention time 9.02 min was observed in *S. cerevisiae* W9100 (pSP-*fad*-3).

Lack of detection of FAD-3 activity in culture supernatant and positive substrate transformations by cells in vivo indicate that the FAD-3 activity is cell associated. This suggests that, recombinant Ct-FAD-3 protein remained within the cell despite its fusion with mating factor-alpha (secretory signal). The same observations have just been reported by Chen *et al*., (2013).

4.5 Analysis of yeast protein expression

The Ct-*fad*-3 gene was expressed under the strong control of GAL L promoter and fused with mating factor alpha in pSP-*fad*-3. Hence recombinant protein was expected to be expressed and secreted in the culture supernatant. Proteins from the supernatant of a 5 ml culture induced by galactose were precipitated by acetone or ammonium sulphate (60% saturation) and analyzed for FAD-3 protein expression by SDS-PAGE (Figure 4.11).

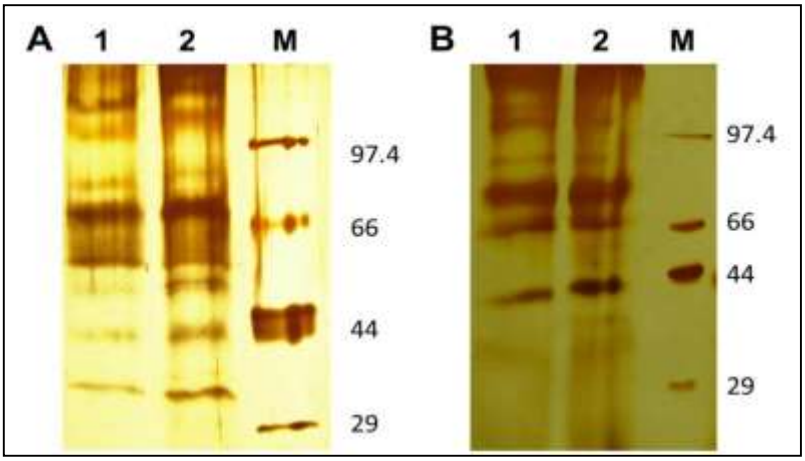

Figure 4.11 Analysis of culture supernatant proteins. A: Acetone precipitated protein samples, B: Ammonium sulphate precipitated protein samples, M: Protein molecular weight marker Lane 1; *S. cerevisiae* W9100 (pGAL-MF), Lane 2; *S. cerevisiae* W9100 (pSP-*fad*-3).

Proteins from galactose induced whole cells were also prepared and analyzed as per method described by Kushnirov (2000), (Figure 4.12 A and B).

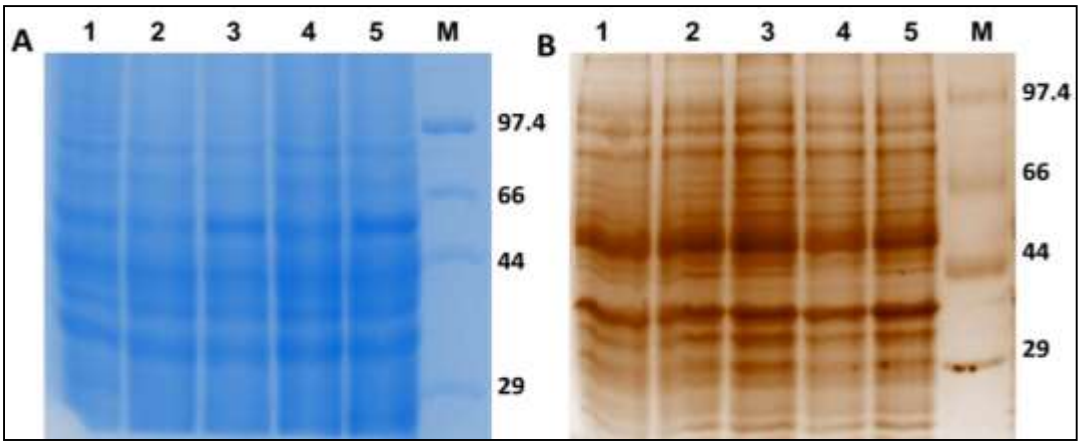

Figure 4.12 Analysis of whole cell proteins by SDS-PAGE. A: Coomassie brilliant blue staining; B: Silver staining; Lane 1 and 3: *S. cerevisiae* W9100 (pGAL-MF) (30 and 20 ºC resp.), Lane 2, 4 and 5: *S. cerevisiae* W9100 (pSP-*fad*-3) (30, 25 and 20 ºC respectively).

Several attempts were made to confirm the presence of Ct-FAD-3 in culture supernatant and whole cell extracts by SDS-PAGE gels. However no band corresponding to FAD-3 protein was seen in either case even by silver staining. Whether this was due to poor expression or problems in solubilization of FAD-3 protein is not clear. Overlapping of recombinant protein FAD-3 with endogenous background proteins in the gel has been lately reported (Chen *et al*., 2013).

4.6 Solubilization and analysis of yeast membrane proteins

In the present work, a membrane bound Ct-FAD-3 protein was fused with mating factor-α in order to achieve extracellular expression, but the expressed protein was neither detected in culture supernatant nor in the cell extracts by SDS-PAGE gel but the functional activity of the expressed FAD-3 was rather found to be cell associated.

The mating factor-α leader peptides have been used efficiently for over expression and secretion of soluble proteins without any potential N-glycosylation sites (Schuster *et al*., 2000a, 2000b). However, secretion can be inhibited even in presence of α-leader peptide if hydrophobic regions or transmembrane domains are present. Hence, though proteins are guided through the secretory pathway, they remain trapped in membrane compartments either in the ER, the Golgi apparatus, or the cytoplasmic membrane (Schuster *et al*., 2000a; Ton and Rao, 2004).

Hydropathy and topology analyses of Ct-FAD-3 also showed trans-membrane localization of the protein with six certain membrane spanning regions. Hence it might be possible that the protein stay docked within the membrane even in the presence of the signal sequence. FAD-3 proteins expressed in the cells may get efficiently targeted to and inserted into the ER membrane in yeast due to presence of the N-terminal domain and the central hydrophobic domain that is capable of interacting with the signal recognition particle (Wessels and Spiess, 1988; Zhang *et al*., 1998). They also showed conservation through evolution in ER targeting determinants and topogenic sequences (Beaudoin and Napier, 2002; Beaudoin *et al*., 2000). Hence expression of FAD-3 protein might be associated with cell membrane.

Several attempts were made to analyze it in the total yeast cell protein by SDS-PAGE but no band corresponding to molecular weight of FAD-3 protein was observed in the recombinant yeast. Hence solubilization of membrane proteins to detect expressed FAD-3 was done by using a number of combinations of different nonionic detergents, temperatures and time durations (Table 4.2).

Table 4.2 Detergent combinations used for solubilization of yeast cell membrane proteins

Detergent	Concentration used (%)				
	1	**2**	**3**	**4**	**5**
SDS	+	++	+	+	+
SDS + 0.1 mM DDM	+	+++	+	ND	ND
SDS + Triton X-100 (0.5%)	++	++	+	ND	ND
Sarcosyl	+	++	+	+	+
Sarcosyl + 0.1 mM DDM	++	+++	++	ND	ND
	0.5	**1**	**1.5**	**2**	**2.5**
Triton X-100	+	++	+++	++	+
Triton X-100 + 0.1 mM DDM	ND	++	+++	++	ND
Tergitol Type NP-7	+	++	++	+	+
Nonidet P40	+	++	++	+	+
Nonidet P40 + 0.1 mM DDM	ND	++	+++	+	ND
Tween 20	+	++	++	+	+
Brij-58	+	++	++	+	+
Btij-58 + 0.1 mM DDS	ND	+++	+++	ND	ND

SDS: Sodium dodecyl sulphate, DDM: Dodecyl Maltoside. '+'sign indicated extent of solubilization. +: Moderate; ++: good; +++: best, ND: Not Determined.

Along with SDS detergents such as triton X-100, NP-40, Tergitol, Tween-20, Brij-58, Dodecyl Maltoside *etc*. were used to dissolve the membrane protein but nevertheless no expressed protein was not detectable (Figure 4.13).

Use of 2% SDS with 0.1 mM Dodecyl Maltoside gave relative good solubilization of yeast total cell proteins but yet it was difficult to detect any band corresponding to FAD-3 protein by SDS-PAGE analysis.

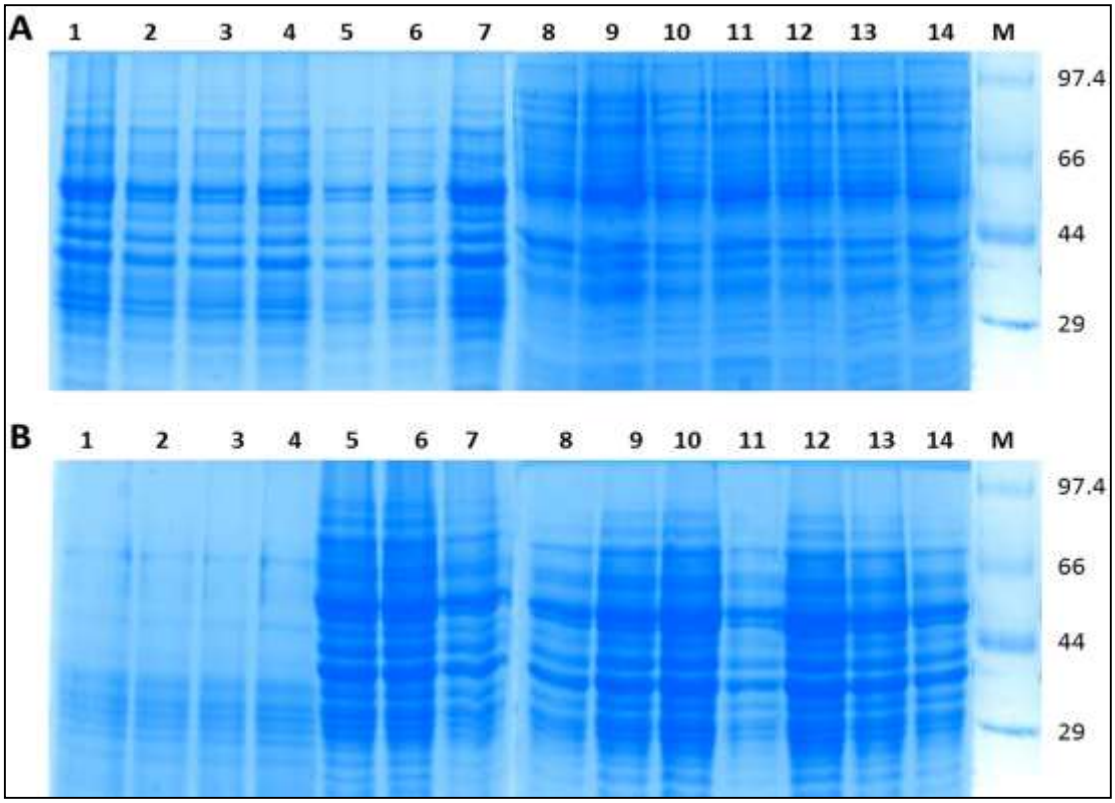

Figure 4.13 Analysis of galactose-induced *S. cerevisiae* W9100 total cell proteins by SDS-PAGE. Recombinant protein was not detectable on SDS-PAGE gels by any of the pretreatments. M: Protein molecular weight marker, Lanes 1, 3, 5, 7, 9, 11, 13: Control vector pGAL-MF, Lanes 2, 4, 6, 8, 10, 12, 14: Recombinant construct pSP-*fad*-3. A; Lanes 1-2, 3-4 and 5-6: 1, 2 and 3% SDS respectively, for 30 min, Lanes 7-8, 9-10 and 11-12: 1, 2 and 3% Sarcocyl respectively, for 30 min, Lanes 13-14; 2% SDS + 0.1 mM Dodecyl Maltoside; B; Lanes 1-2 and 3-4: soluble fractions after 1 and 2% SDS respectively, Lanes 5-6, 7-8 and 9-10: 0.5, 1 and 1.5% Nonidet P40 respectively, for 30 min, Lanes 11-12 and 13-14: 1 and 1.5% Nonidet P40 + 0.1 mM Dodecyl Maltoside.

This might be due to limited expression of FAD-3 or problems in solubilization of membrane proteins or due overlapping of recombinant protein with endogenous background proteins. Due to strong associations and hydrophobicity related problems, even after decades of research very few membrane bound protein are being studied till date (Rabilloud, 2009a; 2009b). In present work also, the predicted strong protein membrane association would have made it difficult to detect by SDS-PAGE analysis.

4.7 Heterologous expression of Ct-*fad*-3 in *E. coli* BL21(DE3)

Heterologous expression of Ct-*fad*-3 in *E. coli* BL21(DE3) was done using pET-28c(+) to study whether the functional Ct-FAD-3 is produced in *E. coli*. FAD-3 protein expression was induced by IPTG and purified by 'Nickel ion affinity chromatography'.

4.7.1 Cloning of Ct-*fad*-3 in pET-28c(+)

Full length Ct-*fad*-3 gene was amplified by using *fad*-3 gene specific primers from pBS-*fad*-3 so as to bring the gene in the correct reading frame with the His tag in pET-28c(+) vector. Size-separated and eluted gene was cloned in pET-28c(+) at NdeI and XhoI sites. The 1.3 kb insert in pETSP28c in *E. coli* DH5-α were confirmed by restriction analysis and PCR with Ct-*fad*-3 gene specific primers (Figure 4.14). The construct was transformed into the expression host *E. coli* BL21(DE3). Transformants were selected by Kanamycin as marker and then further confirmed by colony PCR.

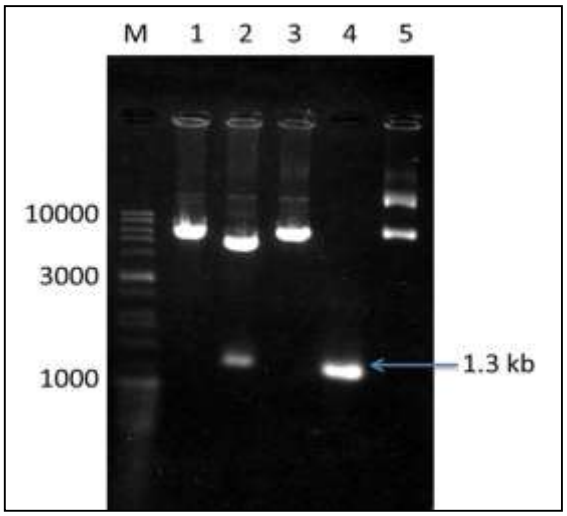

Figure 4.14 Cloning of Ct-fad-3 in pET-28c(+) expression vector. Lane M: DNA marker, Lane 1: pETSP28c NdeI linearization, Lane 2: pETSP28c NdeI & XhoI digest (Insert release 1.3 kb), Lane 3: pETSP28c XhoI linearization. Lane 4: pETSP28c Ct-*fad*-3 PCR (1.3 kb), Lane 5: pETSP28c undigested.

4.7.2 Analysis of protein expression in *E. coli* by SDS-PAGE

Initially, the expression of the fusion His tagged Ct-*fad*-3 protein was induced by using 1 mM IPTG at 30 ºC for 4 hrs. *E. coli* transformants containing empty plasmid (pET-28c(+)) served as control. A distinct protein band corresponding to the calculated

molecular weight of approximate 50 kDa of FAD-3 protein was observed in the extracts of *E. coli* harboring pETSP28c (Figure 4.15).

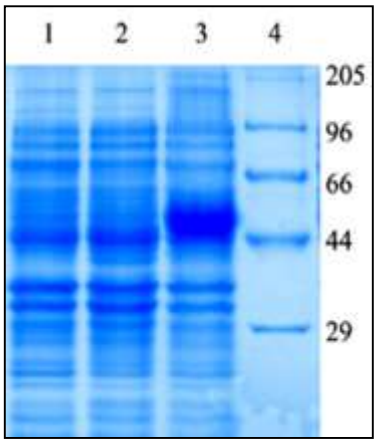

Figure 4.15 SDS-PAGE analysis of Ct-FAD-3 expression in *E. coli* BL21(DE3) IPTG induction was given at 1 mM concentration at 30 ºC for 4 hrs. Lane 1: Control vector pET-28c(+) (induced), Lane 2: Recombinant construct pETSP28c (un-induced), Lane 3: Recombinant construct pETSP28c (induced), Lane 4; Protein Molecular weight marker.

Different IPTG concentrations and induction temperatures were employed for testing optimal expression. Induction was carried out at different IPTG concentrations (0.1 to 1 mM) and temperatures (25, 30, and 37 ºC). There were no significant differences in protein expression when cells were induced under these conditions as represented in Figure 4.16 A and B. Further induction procedures were carried out at 25 ºC and at 0.5 mM IPTG concentration.

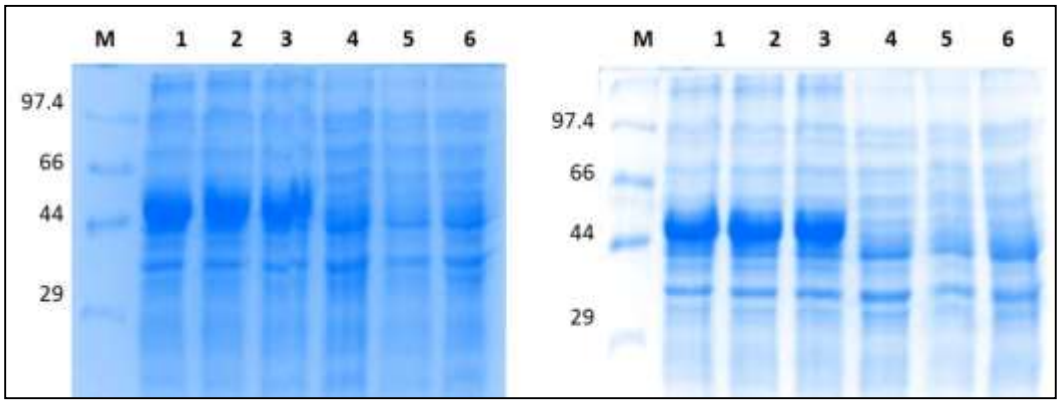

Figure 4.16 Induction of FAD-3 under different conditions in *E. coli* BL21(DE3). A: Lane 1-3: Recombinant construct pETSP28c induced with 0.3, 0.5 and 1.0 mM IPTG respectively, Lane 4-6: Control vector pET-28c(+) induced with 0.3, 0.5 and 1.0 mM IPTG respectively, Induction temperature used was 30 ºC. B; Lane 1-3: Recombinant construct pETSP28c induced at 25, 30 and 37 ºC temperature respectively, Lane 4-6: Control vector pET-28c(+) induced at 25, 30 and 37 ºC temperature respectively, Induction by 0.5 mM IPTG, Lane M: Protein Molecular weight marker.

4.7.3 Purification of FAD-3 protein from *E. coli* BL21(DE3) (pETSP28c)

The optimally expressed His-tagged Ct-FAD-3 protein accumulated up to approximately 10% - 15% of the total cell protein as judged by SDS-PAGE. The protein was purified to ~95% purity by one step purification with His tag-Nickel ion affinity chromatography (Figure 4.17). About 50% - 55% FAD-3 protein was recovered from *E. coli* cell lysate. Mild aggregation in the purified protein as observed on SDS-PAGE was decreased by diluting the enzyme preparations and adding with 0.1 mM Dodecyl maltoside.

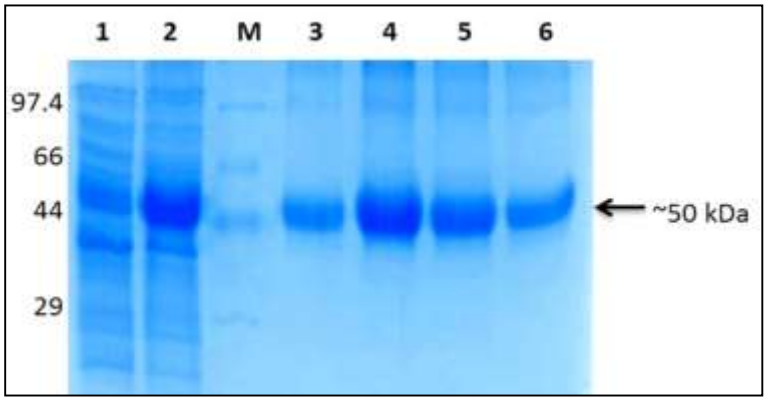

Figure 4.17 Purification of Ct-FAD-3 from *E. coli* BL21(DE3). Lane M: Protein Molecular weight marker, Lane 1: Control vector pET-28c(+), Lane 2: Recombinant construct pETSP28c, Lane 3-6: His tag-Nickel ion affinity chromatography 2nd, 3rd, 4th and 5th elution fraction respectively.

4.8 Determination of pI of Ct-FAD-3

The pI of the Ct-FAD-3 protein with the deduced amino acid sequence was calculated to be 6.4. A narrow pH gradient (pH 5-8) strip was used in isoelectric focusing. Three protein spots having pI 6.6, 6.8 and 7.2 were observed in isoelectric focusing (Figure 4.18). However 2D analysis with appropriate markers indicated that these three protein spots corresponded to identical molecular weight of ~50 kDa (Figure 4.18).

Proteins with several isoelectric point variants due to post-translational modifications have been reported (Green *et al*., 1986; Gygi *et al*., 2000). Amino acid oxidation reductions or other factors affecting the net charge might also be reasons behind these multiple pI of the protein (Rabillioud, 2000; Gorg *et al*., 2004).

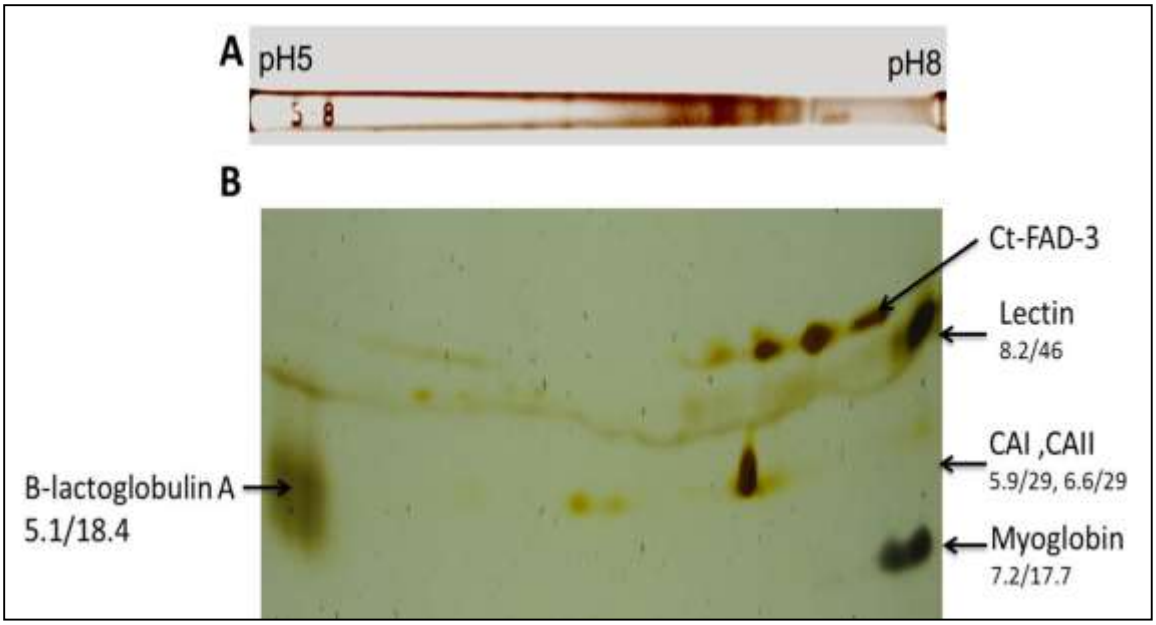

Figure 4.18 Isoelectric focusing and 2-D analysis of Purified FAD-3 protein A; Isoelectric focusing of Purified Ct-FAD-3 protein only, B; Isoelectric focusing and subsequent second dimensional SDS-PAGE analysis of purified Ct-FAD-3 along with IEF marker proteins (Myoglobin (17.7 kDa): pI-7.2, β-lactoglobulin A (18.4 kDa): pI 5.1, Carbonic Anhydrase Isozyme II from bovine erythrocytes (29 kDa): pI 5.4 and 5.9, Carbonic Anhydrase Isozyme I from human erythrocytes (29 kDa): pI 6.6, Lectin (46 kDa): pI 8.2).

4.9 Generation and purification of polyclonal anti-FAD-3 antibody

Polyclonal anti-FAD-3 IgG antibodies against purified FAD-3 protein were generated in rabbit and purified. The total IgG was estimated to 9.286 mg (extinction coefficient of IgG is 1.4). Purified IgG anti-FAD-3 antibodies were analyzed by loading on SDS-PAGE (Figure 4.19 A). The Titer of purified IgG antibodies was determined by using purified FAD-3 protein from *E. coli*. The optimal titer was found to be 1:15000 from among the dilutions tested (Figure 4.19 B). These anti-FAD-3 antibodies were used as primary antibodies for detection of expressed FAD-3 in yeast by ELISA and Western blot analysis.

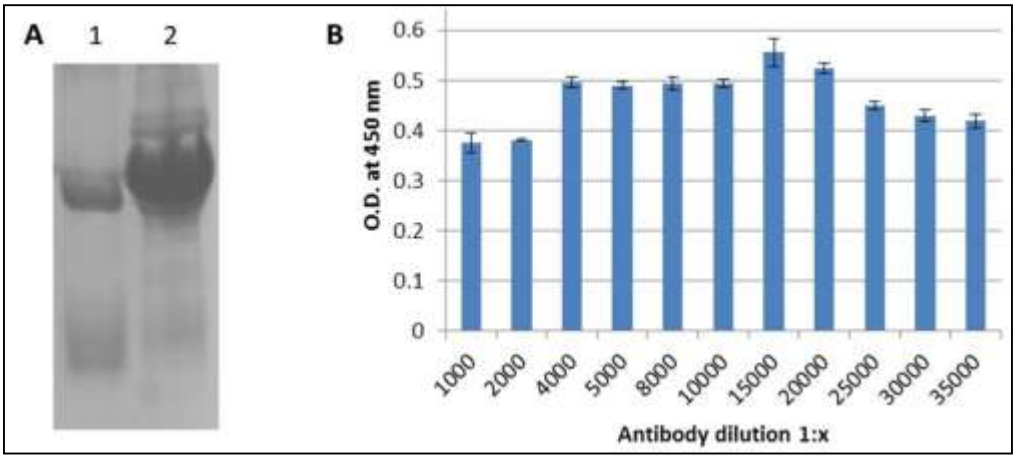

Figure 4.19 Purification and determination of optimal titer of anti-FAD-3 antibodies. A; IgG purification from rabbit serum Lane 1: Purified Rabbit IgG, Lane 2: Crude Rabbit Serum. B; Optimal titer determination of purified anti-FAD-3 antibodies against FAD-3 protein. It was found to be 1:15000.

4.10 Analysis of FAD-3 expression in yeast by indirect ELISA

ELISA tests using whole cell extracts of induced both *E. coli* BL-21(pETSP28c) and *S. cerevisiae* W-9100 (pSP-*fad*-3) as antigens gave strong positive signals at 1:15000 dilution of anti-FAD-3 antibodies, confirming the expression of the protein in yeast cells. There were weak positive signals in un-induced cell samples (Figure 4.20).

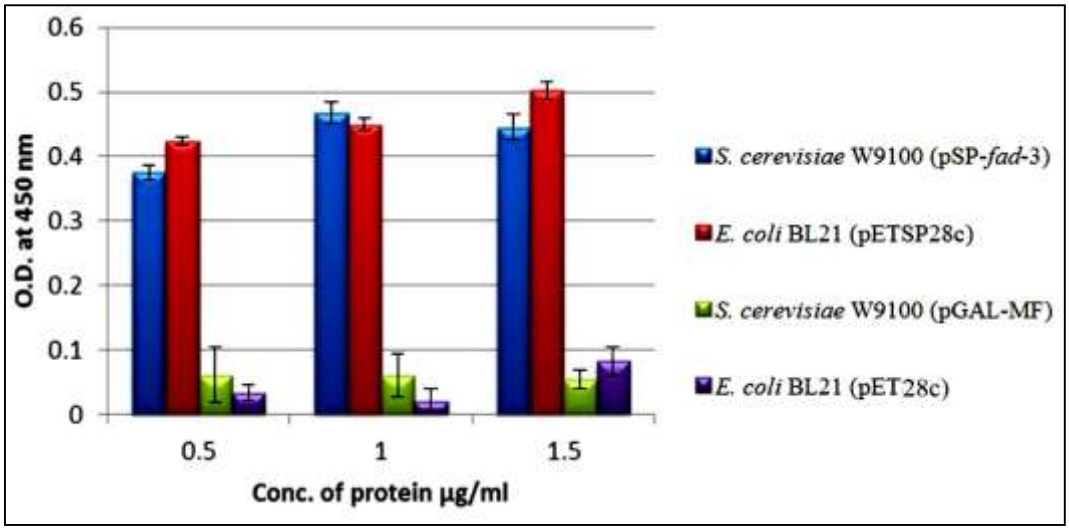

Figure 4.20 Analysis of FAD-3 protein expression by Indirect ELISA. Purified rabbit anti-FAD-3 IgG antibodies were used as primary antibodies, HRP conjugated goat anti-rabbit IgG antibodies were used as secondary antibodies and TMB-H_2O_2 was used as substrate.

4.11 Analysis of expression by Western blot

4.11.1 Analyzing specificity of generated anti-Ct-FAD-3 antibodies

Western blot analysis of induced *E. coli* BL21(DE3) (pETSP28c) revealed a protein band corresponding to ~50 kDa that was absent in control and un-induced cultures indicating high specificity of the generated antibodies (Figure 4.21).

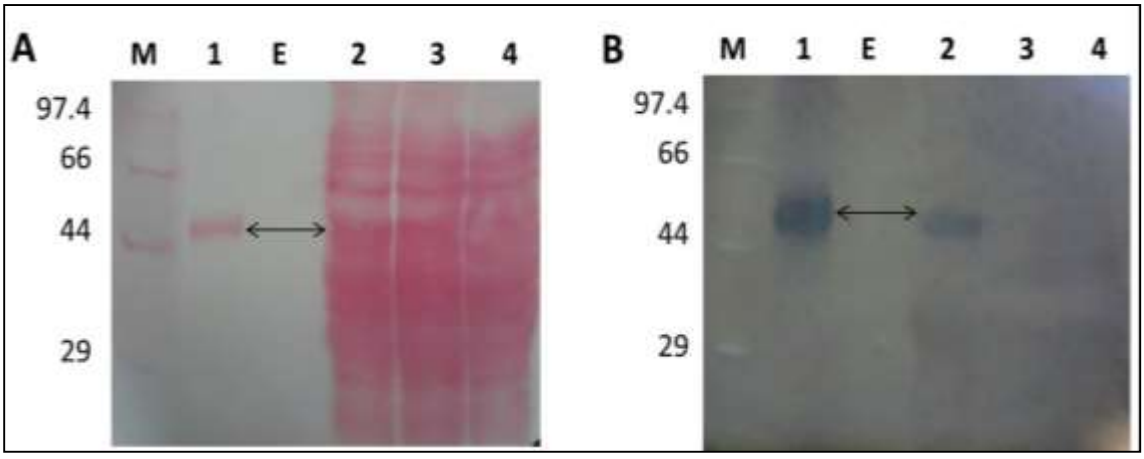

Figure 4.21 Western blot analysis of *E. coli* BL21(DE3) total proteins. A: Ponceau staining, B: Western blot. Lane M: Protein molecular weight marker, Lane 1: Purified Ct-FAD-3 protein, Lane E: Empty, Lane 2: Recombinant construct pETSP28c, IPTG induced, Lane 3: Recombinant construct pETSP28c, Un-induced, Lane 4: Control vector pET-28c(+),IPTG induced. Purified rabbit anti-FAD-3 IgG antibodies were used as primary antibodies, HRP conjugated goat anti-rabbit IgG antibodies were used as secondary antibodies and TMB-H_2O_2 was used as substrate.

4.11.2 Western blot analysis of Ct-FAD-3 expression in yeast

The expression Ct-FAD-3 protein in yeast was analyzed by western blot. Samples solubilized by adding 2% SDS along with 0.1 mM Dodecyl Maltoside as in table 4.2. A band corresponding to ~50 kDa protein was detected by the purified anti-FAD-3 antibodies (Figure 4.22), confirming the presence of expressed Ct-FAD-3 in the recombinant. The protein might have been overlapped by indigenous host cell protein hence was not seen in Coomassie or silver staining methods.

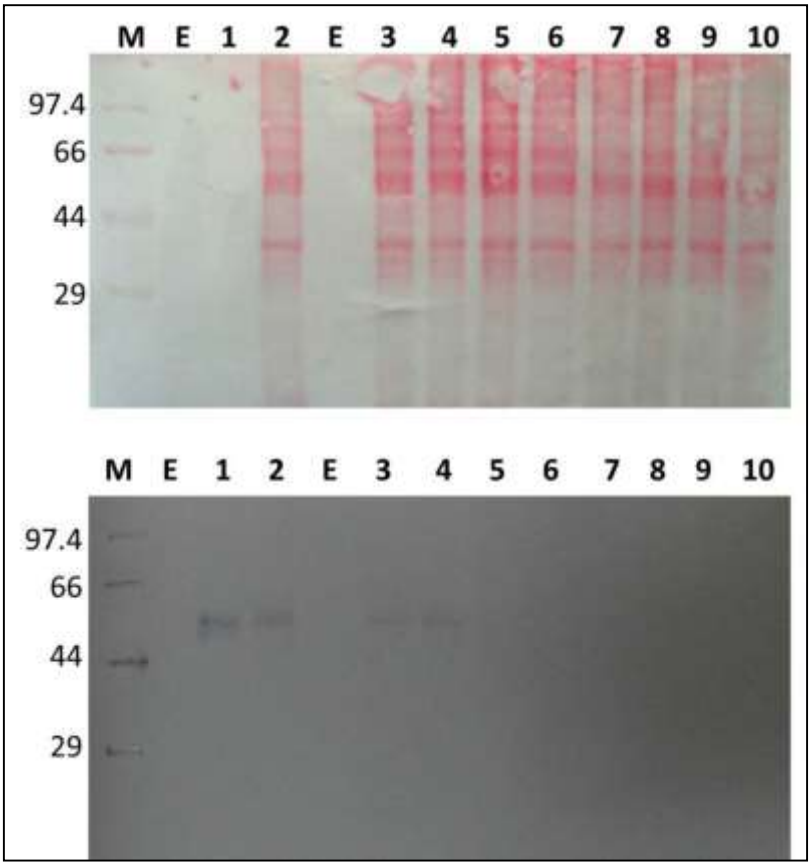

Figure 4.22 Western blot analysis of total cell proteins from yeast cultures expressing Ct-*fad*-3 gene constructs. A: Ponceau staining, B: Western blot. Lane M: Protein molecular weight marker, Lane E: empty lane, Lane 1: Purified Ct-FAD-3 protein from *E. coli*, Lane 2, 3 and 4: *S. cerevisiae* W-9100 (pSP-*fad*-3) (Galactose induced), Lane 5, 6 and 7: *S. cerevisiae* W-9100 (pSP-*fad*-3) (Un-induced), Lane 8, 9 and 10: *S. cerevisiae* W-9100 (pGAL-MF) (Galactose induced). All cultures were grown in a medium supplemented with 1 mM LA. Purified rabbit anti-FAD-3 IgG antibodies were used as primary antibodies, Biotin conjugated goat anti-rabbit IgG antibodies were used as secondary antibodies and Streptavidin ALP conjugate - BCIP/NBT detection system was used.

5. Functional analysis of recombinant Ct-FAD-3 in transgenic *E. coli* and *S. cerevisiae*

As discussed in section 4.4.3, GC analysis showed that functional expression of FAD-3 protein was associated with whole yeast cells only. No activity was detected in total proteins obtained from culture supernatant, despite the presence of mating factor-α (secretion signal).

5.1 Functional analysis of FAD-3 protein expressed in *E. coli*

Biotransformation studies using purified FAD-3 protein preparations of *E. coli* origin, did not yield ALA as a product either when present alone in the reaction system or when supplied with control yeast cell homogenate as a source of co-factors.

Biotransformation using whole cell was studied in *E. coli* BL21(DE3) (pETSP28c) grown in LB and M9 minimal medium supplemented with 1 mM LA. *E. coli* possesses only monounsaturated fatty acids and lacks the desaturases and elongases for PUFA biosynthesis (Cao *et al*., 2012). Though LA was easily incorporated in *E. coli* cell membrane lipids, no biotransformation of LA to ALA was detected (Figure 5.1).

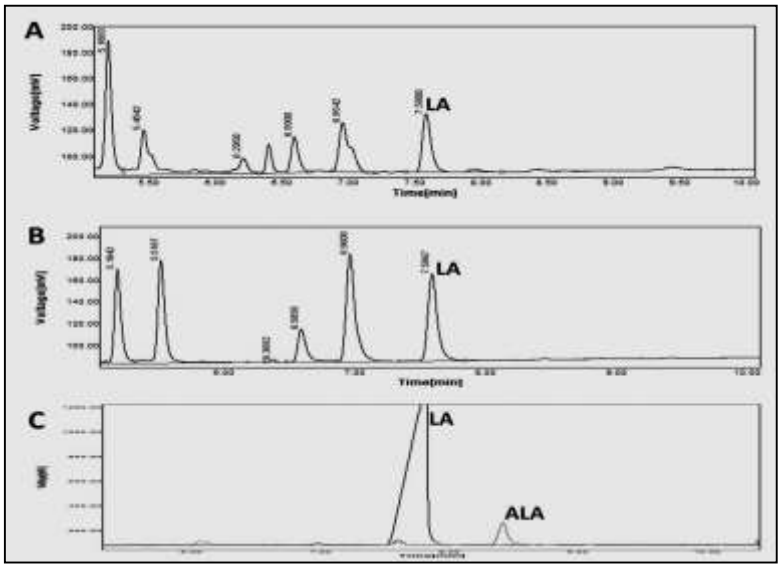

Figure 5.1 GC analysis of total fatty acids extracted from *E. coli* BL21(DE3) grown in LB medium supplemented with 1 mM LA. A: Recombinant construct pETSP28c, B: Control vector pET-28c(+), C: Standard fatty acids (LA and ALA) methyl esters. No peak of ALA was observed in test samples.

In the present study, overexpression of Ct-FAD-3 was achieved successfully in *E. coli* but no functional desaturase activity was seen both in vivo and in vitro. The absence of FAD-3 activity is whether due to the absence of proper membrane docking or need of essential post-translational modifications of the protein or need of co-factors (if any) is not clear.

During past few years, desaturases have been cloned from various organisms and have been attempted for their expression in *E. coli.* The characterization of these hydrophobic FAD-3 proteins was difficult in many cases due to problems associated with expression including formation of inclusion bodies due to hydrophobic regions (Demain and Vaishnav, 2009), toxic effect of overexpressed membrane proteins on the *E. coli* cells (Miroux and Walker, 1996; Dumon-Seignovert *et al*., 2004). In some cases expressed proteins were inactive in *E. coli* as has been observed here. However several soluble desaturases such as the palmitoyl (16:0)-ACP desaturase, stearoyl (18:0)-ACP-desaturase (Cahoon *et al*., 1996; Kiseleva *et al*., 2000; Cao *et al*., 2010) and few eukaryotic Δ-12 FAD (Apiradee *et al*., 2004; Li *et al*., 2006; Yin *et al*., 2007; Suresha *et al*., 2013) have been functional when expressed in *E. coli*. Heterologous production of EPA has been achieved in *E. coli* by expressing prokaryotic gene cluster from marine bacterium *Shewanella sp.* (Orikasa *et al*., 2006; Lee *et al*., 2009) but expression of eukaryotic FAD-3 in *E. coli* has not been successful (Chen *et al*., 2013).

In most of the cases heterologous *E. coli* systems are not feasible for production due to less productivity (total fatty acid content: 2% - 5% of dry cell mass) (Ratledge, 2004).

5.2 Confirmation of biotransformation product by GC-MS

Analysis of desaturase activity of the expressed protein was done by confirming the identity of ALA peak GC-MS analysis of the FAME samples prepared from biotransformation mixtures using *S. cerevisiae* W-9100 (pSP-*fad*-3). Mass spectra corresponding to the peaks of LA and ALA are shown in figure 5.2 A and 5.2 B respectively. The mass spectra of the putative FAME component showed characteristic peaks at m/z = 108 and molecular ion at m/z = 292 which is a characteristic identity of n-3 fatty acid ALA (5.2 B). No higher PUFAs were detected in total fatty acids extracted from the recombinant yeast culture. Biotransformation of LA to ALA confirmed omega-3

desaturation activity of the Ct-FAD-3 in the *S. cerevisiae* W-9100 (pSP-*fad*-3). These data supported the cell associated expression of recombinant desaturase in yeast, had the ability to catalyse the conversions from LA to ALA.

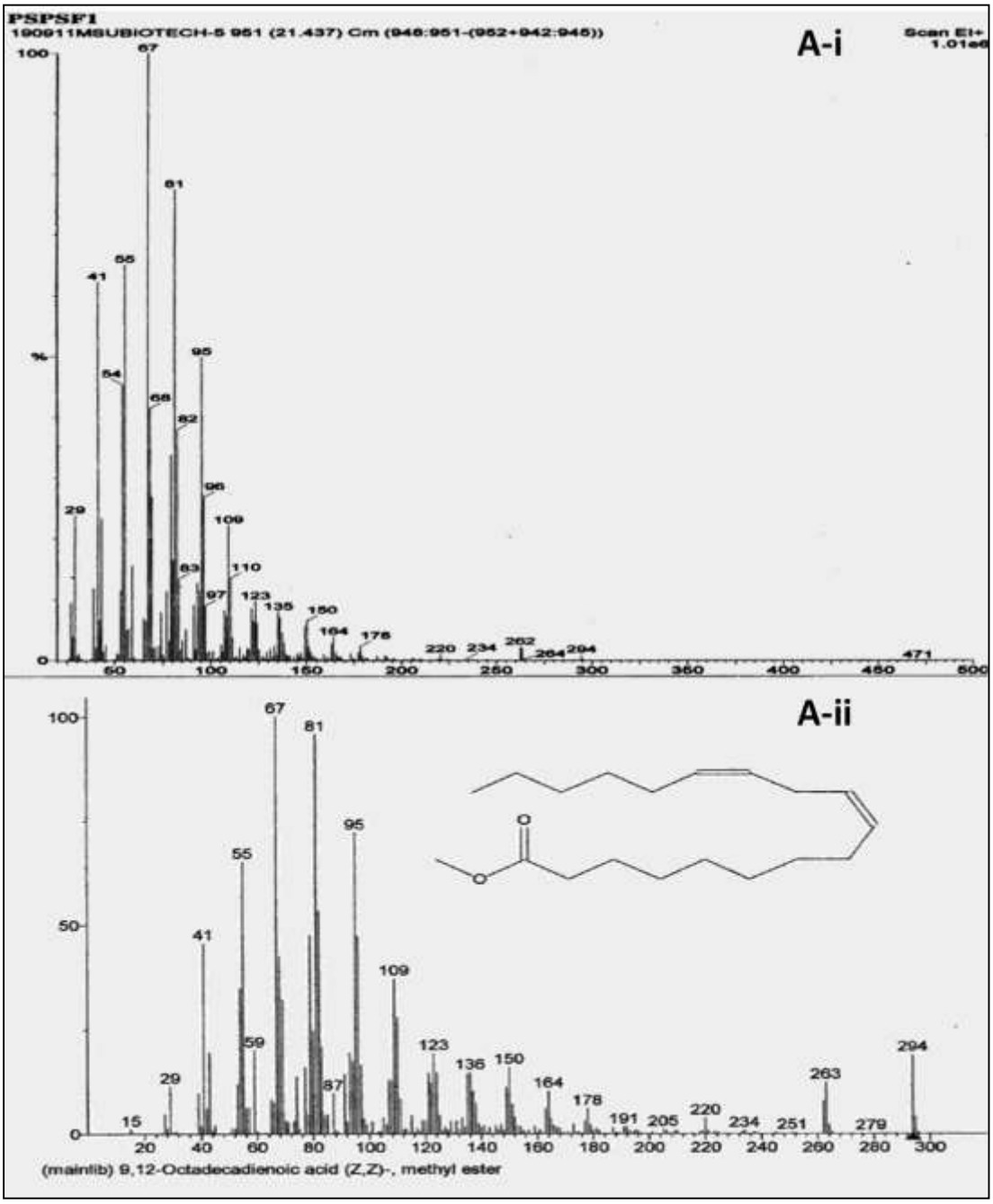

Figure 5.2 A: Mass spectra of LA (C18:2: n-6) peak. Characteristic peaks of LA at *m*/*z* = 150 and molecular ion at *m*/*z* = 294 were observed. A-i; Test mass spectra, A-ii; Standard mass spectra of LA from GC-MS library.

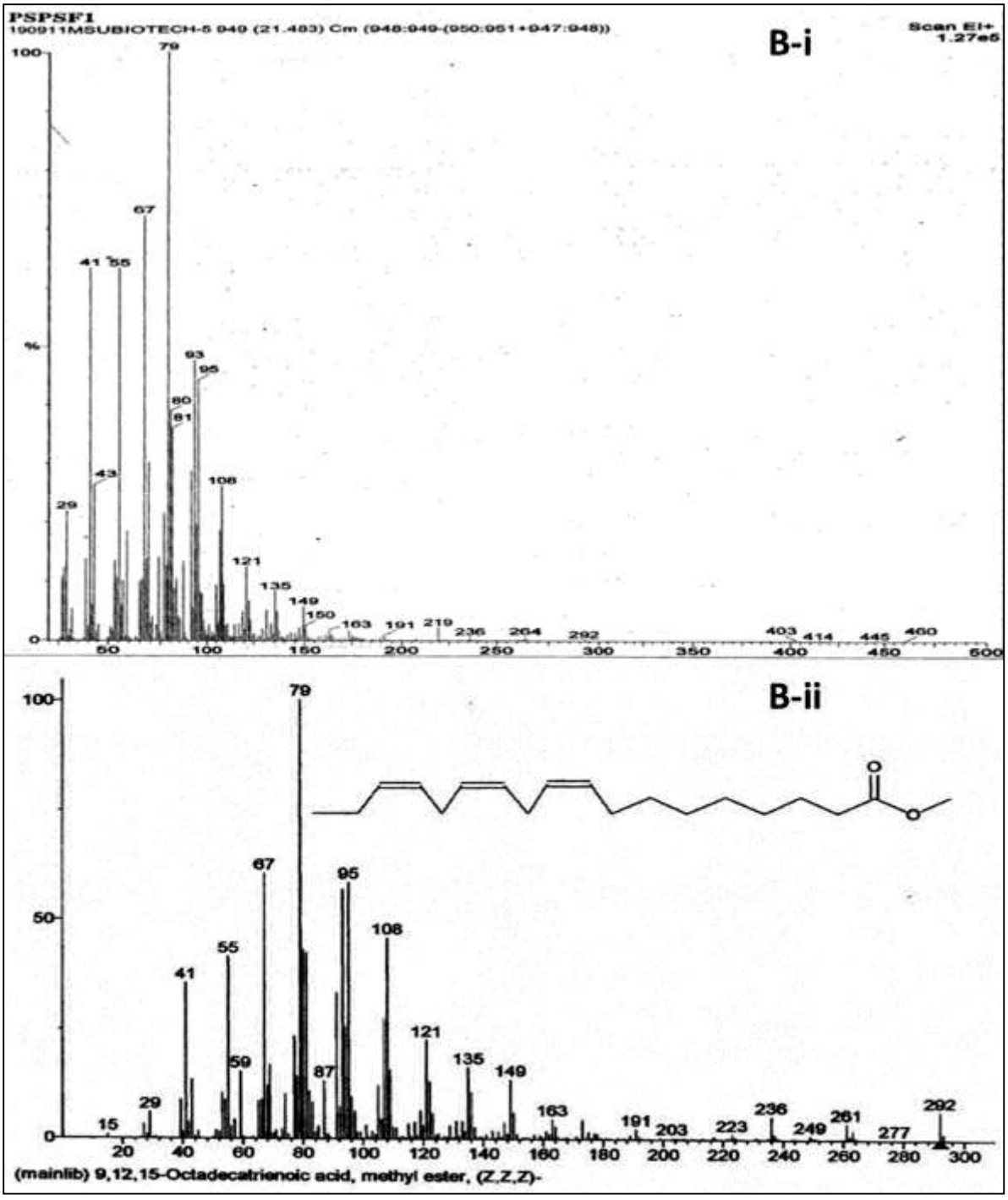

Figure 5.2 B: Mass spectra of ALA (C18:3: n-3) peak. Characteristic peaks of ALA at *m*/*z* = 108 and molecular ion at *m*/*z* = 292 were observed. B-i; Test mass spectra, B-ii; Standard mass spectra of ALA from GC-MS library.

5.3 Analysis of substrate specificity

The substrate specificity of the enzyme was determined by growing recombinant cells in the medium supplemented with known n-6 PUFAs (LA, C18; AA, C20) for their conversion to their corresponding n-3 derivatives, ALA, EPA respectively. No biotransformation of AA to EPA was observed, confirming the specificity of the protein towards C18 LA as a substrate and not to C20 (Figure 5.3).

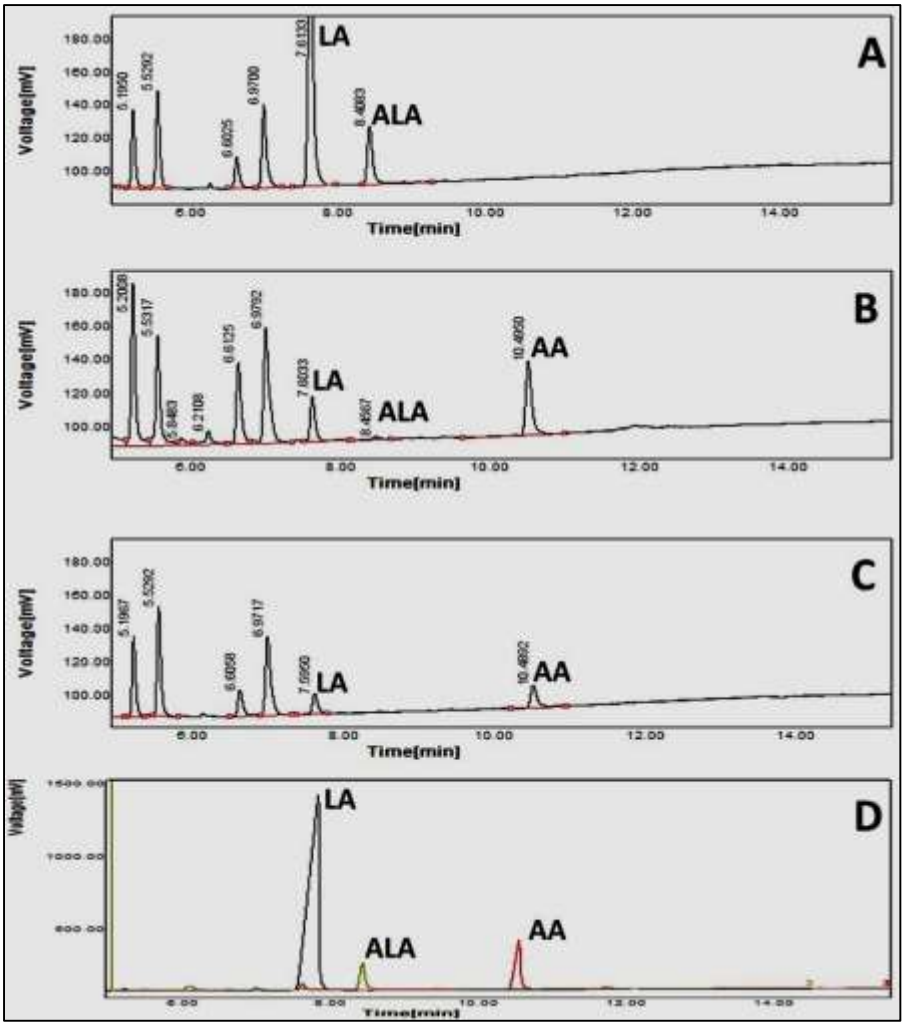

Figure 5.3 Substrate specificity of Ct-FAD-3 in *S. cerevisiae* W-9100 (pSP-*fad*-3). A: pSP-*fad*-3, supplemented with 1 mM LA, B: pSP-*fad*-3, 1 mM AA, C: Control vector, pGAL-MF; 1 mM AA, D: Standard fatty acids (LA, ALA and AA) methyl esters.

5.4 Bio-transformation of edible vegetable oil

The bio-transformation ability of the Recombinant construct was also analyzed by using commercially available sunflower oil.

Sunflower oil being routinely used edible oil with high content of LA (60% - 65%) was selected as a substrate for biotransformation. Culture grown up to early log phase was supplemented with pre-emulsified sunflower oil to a final concentration 1 mM equivalent of LA and grown overnight. Total fatty acids were extracted and analyzed by GC, recombinant yeast showed conversion of LA to ALA from supplied sunflower oil, hence capable of utilizing sunflower oil as a raw material for the bioconversion (Figure 5.4).

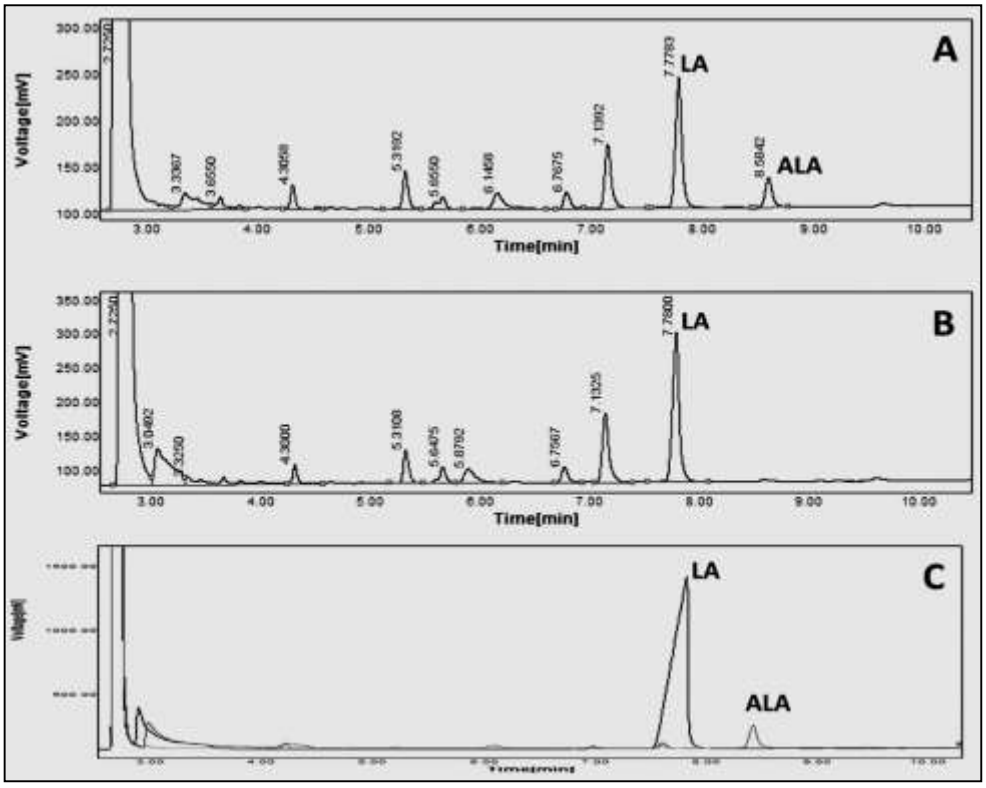

Figure 5.4 Biotransformation of sunflower oil in *S. cerevisiae* W-9100. A: pSP-*fad*-3, B: Control vector, pGAL-MF, C: Standard fatty acids (LA and ALA) methyl esters. The peak of ALA was observed in *S. cerevisiae* W-9100 pSP-*fad*-3 only.

5.5 Time course of enzyme activity

Time course of enzyme activity was done by using LA as a substrate. Here LA was supplemented in overnight grown cultures at OD_{600} 1.4, and allowed to metabolize the provided fatty acid substrate. Samples were collected for fatty acid extraction at regular intervals. Accumulation of LA within the cell and its conversion into ALA was found to be increased linearly until the overnight incubation at late log phase i.e. after 16 hrs (Figure 5.5) and the ALA: LA ratio obtained was 1: 3-4.

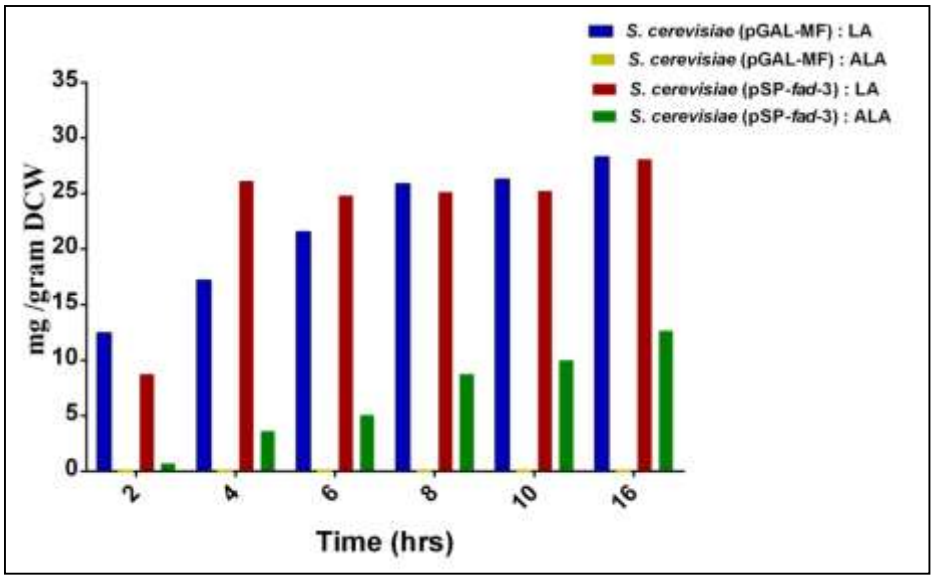

Figure 5.5 Time course analysis of the FAD-3 Activity. Yeast cultures were grown at 25 ºC in a SD Ura$^-$ medium containing 2% galactose supplemented with 1 mM LA.(DCW: dry cell weight)

The ALA: LA ratio after 16 hrs biotransformation was 1:3-4 wherein ALA was 12.2 ± 2 mg/g dry cell mass as detected by GC analysis. It was seen that even though FAD-3 was overexpressed in the yeast cells, the final ratio of ALA: LA was found to be remained constant (1:3-4) as that of resource yeast strain (PS-2) when supplemented with LA. This might be due to the involvement of genetic and various environmental factors which determine the fatty acid accumulation within the yeast cells (Xue *et al*., 2013). *S. cerevisiae* W-9100 can synthesize only monounsaturated fatty acids any other higher PUFAs are absent. This property of *S. cerevisiae* has been utilized in detection of expression and functional identification of number of desaturases; but since it is not oleaginous, relative fatty acid content and ALA remained within limits.