APPENDIX-I

I.A Polyacrylamide gel electrophoresis reagents

SDS-PAGE was performed using a 5% stacking gel and a 8-12% separating gel as described by Sambrook *et al*., 2001. Protein samples in SDS loading buffer were boiled for 4-5 min before being separated by SDS-PAGE. Afterwards, proteins were visualized by silver staining or by Coomassie Brilliant Blue R250 staining. The molecular weight standard used was from Bangalore genei and had the following size markers 205 kDa, 97.4 kDa, 66 kDa, 43 kDa and 29 kDa. About 2-20 µg of protein was applied to the gels.

30% Acrylamide stock solution: 23.2 g acrylamide + 0.8 g bisacrylamide (29:1) was dissolved in warm RO water and the volume made upto 80 ml. The solution was then filtered through Whatman filter paper and stored at 4 ˚C in the dark.

Tris buffers: Tris buffers for resolving (1.5 M Tris pH 8.8) and stacking gels (1 M Tris pH 6.8) were prepared by dissolving Tris base in RO water and adjusting the pH with conc. HCl. The solutions were then autoclaved at 15 lbs for 15 min and stored at 4 ˚C.

10% SDS (Sodium Dodecyl Sulphate): 2 g of electrophoretic grade SDS was dissolved in 20 ml autoclaved RO water and stored at RT.

10% APS (Ammonium Per Sulphate): 0.1 g of APS was dissolved in 1 ml of autoclaved RO water and prepared freshly each time.

TEMED (N,N,N’,N’-tetramethylethylenediamine): was a readymade solution stored in a dark bottle at 4 ˚C.

5 X Tris glycine tank buffer: 7.55 g Tris and 47 g glycine was dissolved in 500 ml RO water. The solution was autoclaved at 15 lbs for 15 min and stored at 4 ˚C.

2 X gel-loading buffer: 100 mM Tris-Cl (pH 6.8) + 20% (v/v) glycerol, 0.2% (w/v) bromophenol blue. SDS was added to 4% (v/v) for SDS-PAGE. β-mercaptoethanol was freshly added to 5% (v/v) at the time of sample preparation

I.B Dialysis buffer

80 ml of 1 M Tris pH 7 + 0.68 g of NaCl + 0.353 g of $CaCl_2.2H_2O$ were dissolved in RO water to make 1.6 L of solution which was autoclaved at 15 lbs for 15 min. The final concentration of each ingredient in the dialysis buffer was 50 mM Tris + 7.26 mM NaCl + 1.5 mM $CaCl_2.2H_2O$.

Preparation of dialysis tubing: Dialysis tubing (cut off 12 kDa) may contain significant amount of sulphur compounds and heavy metal compounds. These were removed by boiling the dialysis tubing in 2% sodium bicarbonate (w/v) + 0.05% EDTA (w/v) for about 15 min and then washed with autoclaved reverse osmosis (RO) water. This was followed by boiling again twice with RO water for 15 min periods. The prepared tubing was stored in water/dialysis buffer at 4 ˚C.

I.C Hydropathy index

The hydropathy index of an amino acid is a number representing the hydrophobic or hydrophilic properties of its side-chain. It was proposed in 1982 by Jack Kyte and Russell Doolittle. The larger the number is, the more hydrophobic the amino acid. The most hydrophobic amino acids are isoleucine (4.5) and valine (4.2). The most hydrophilic ones are arginine (-4.5) and lysine (-3.9). This is very important in determining the tertiary structure of a protein structure; hydrophobic amino acids tend to be internal; while hydrophilic amino acids are more commonly found towards the protein surface. Overall hydropathy plot of a polypeptide can give us an idea about the localization and topology of the protein.

Amino acids sorted by increasing hydropathy index

R	**K**	**N**	**D**	**Q**	**E**	**H**	**P**	**Y**	**W**	**S**	**T**	**G**	**A**	**M**	**C**	**F**	**L**	**V**	**I**
-4.5	-3.9	-3.5	-3.5	-3.5	-3.5	-3.2	-1.6	-1.3	-0.9	-0.8	-0.7	-0.4	1.8	1.9	2.5	2.8	3.8	4.2	4.5

Appendix II

A. Maps of vector plasmids used in present study

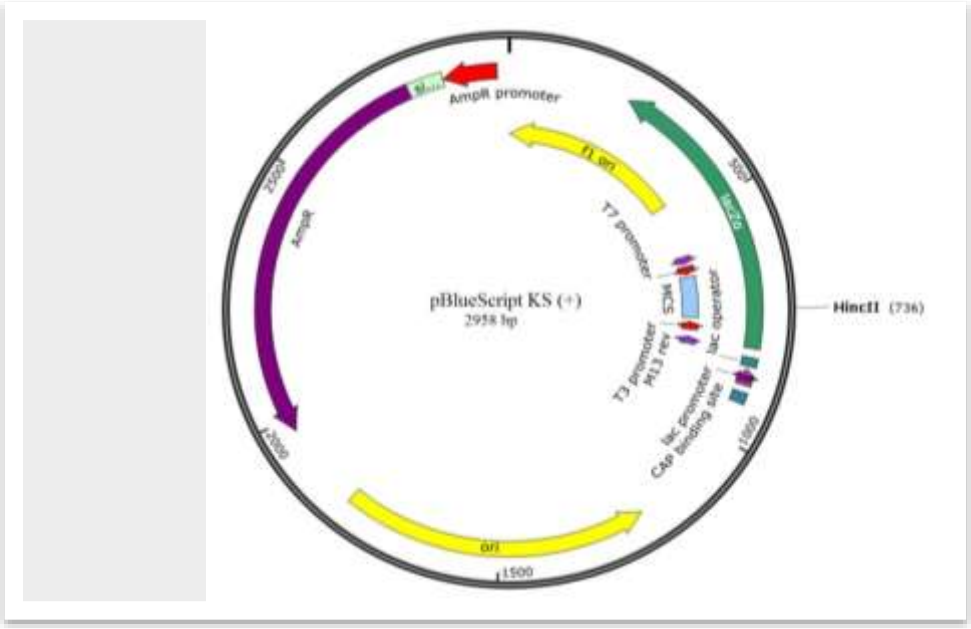


Figure II.A.1 pBlueScript KS (+)

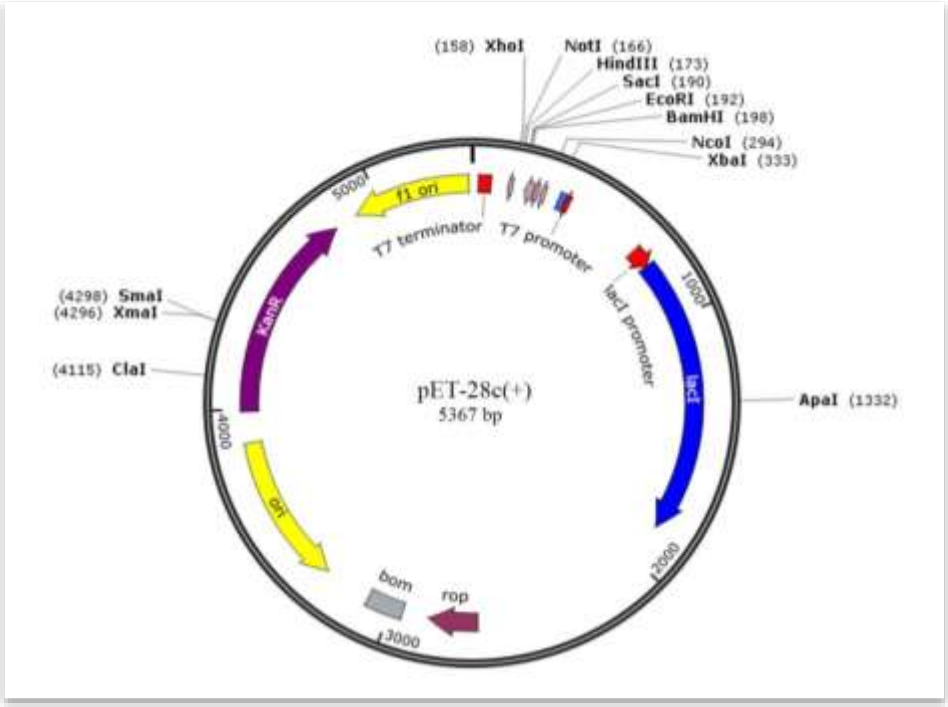


Figure II.A.2 pET-28c(+)

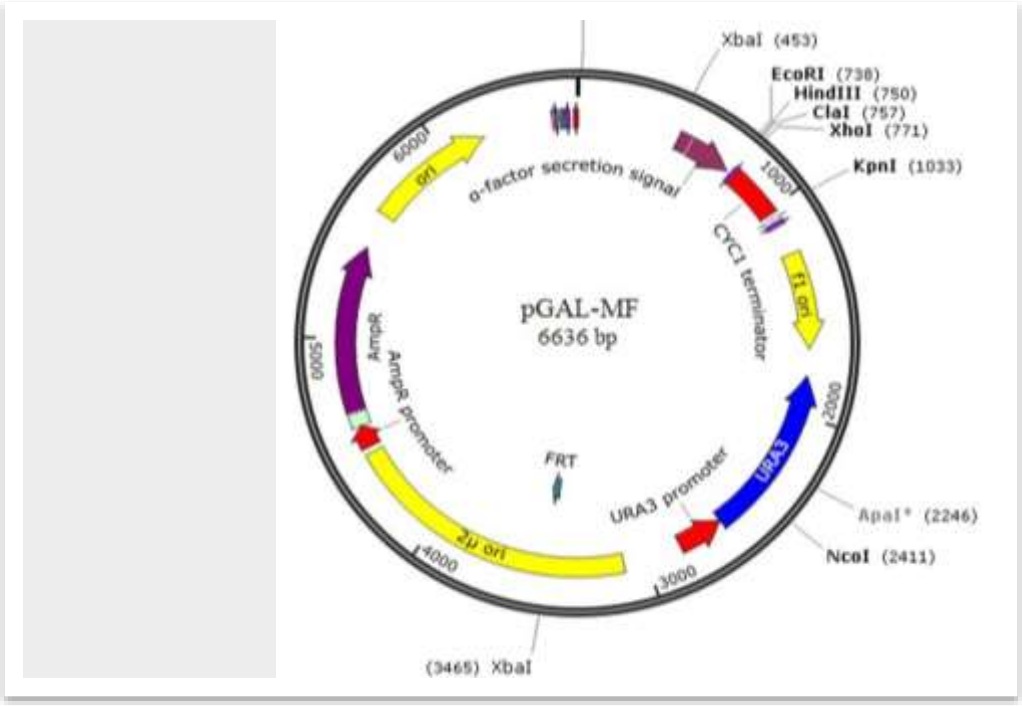
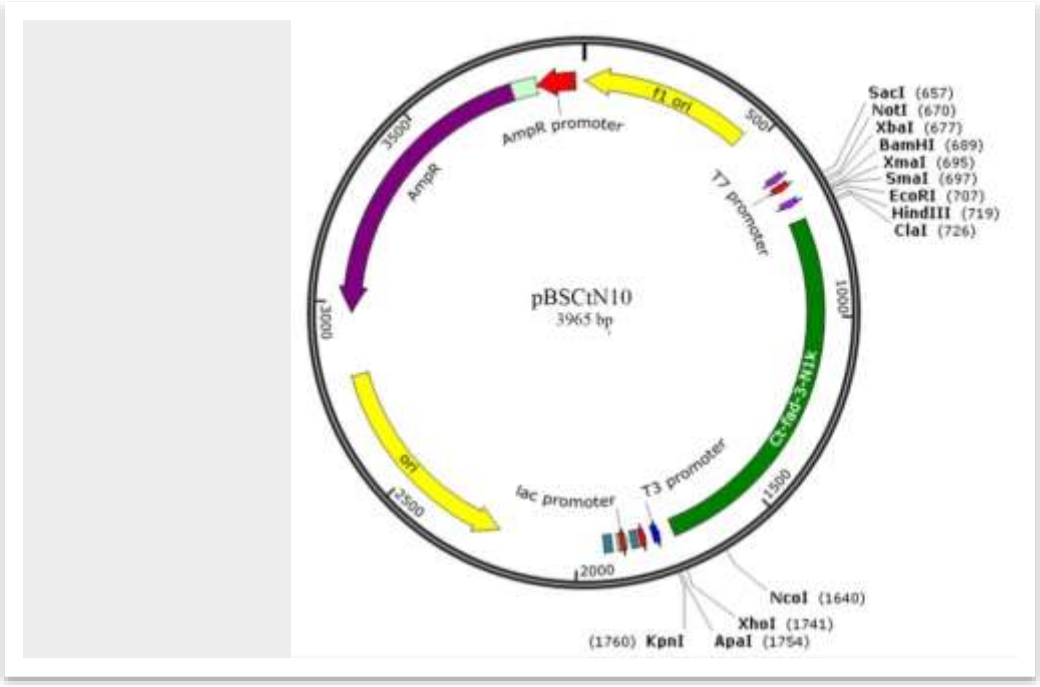
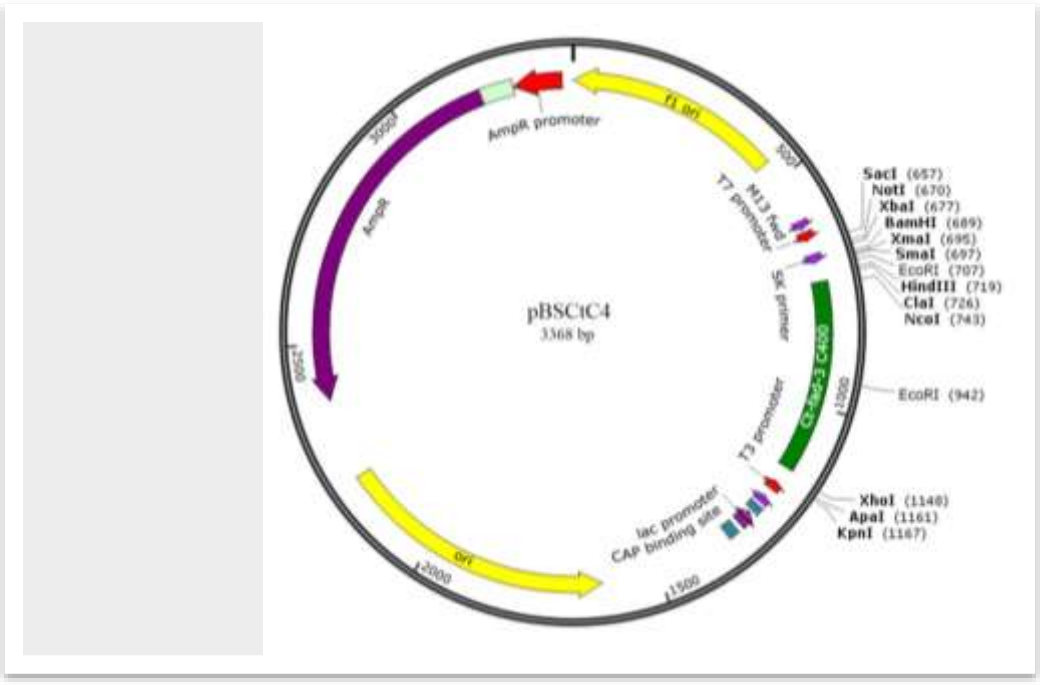


Figure II.A.3 pGAL-MF

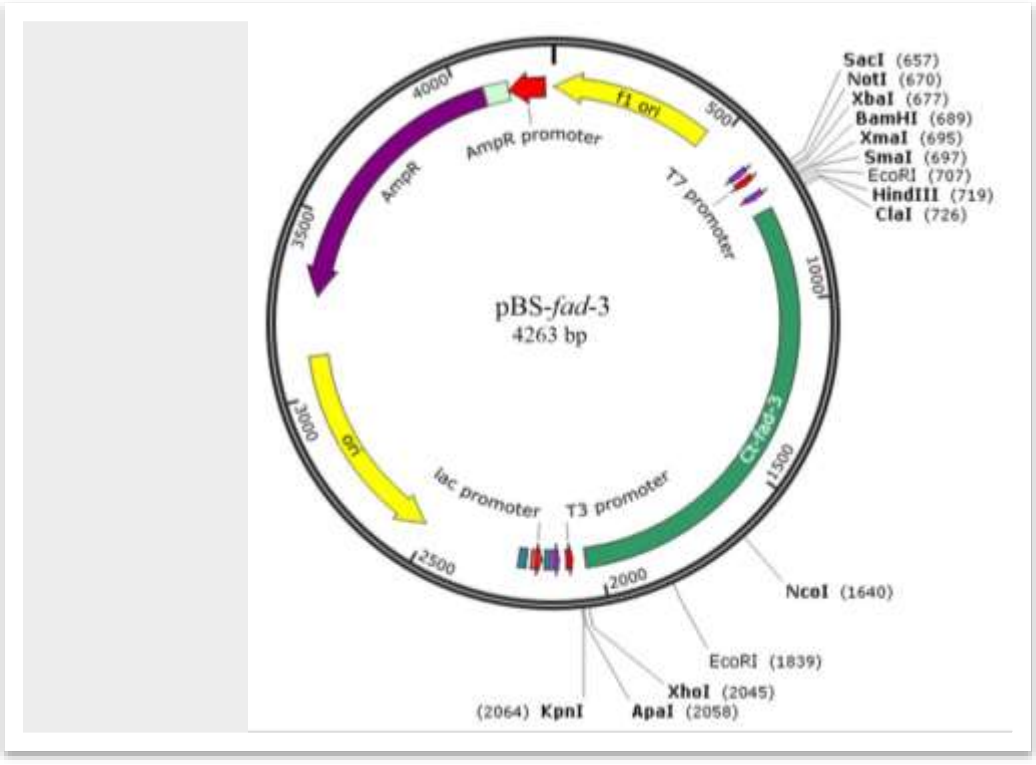
B. Maps of the recombinant construct generated in present study



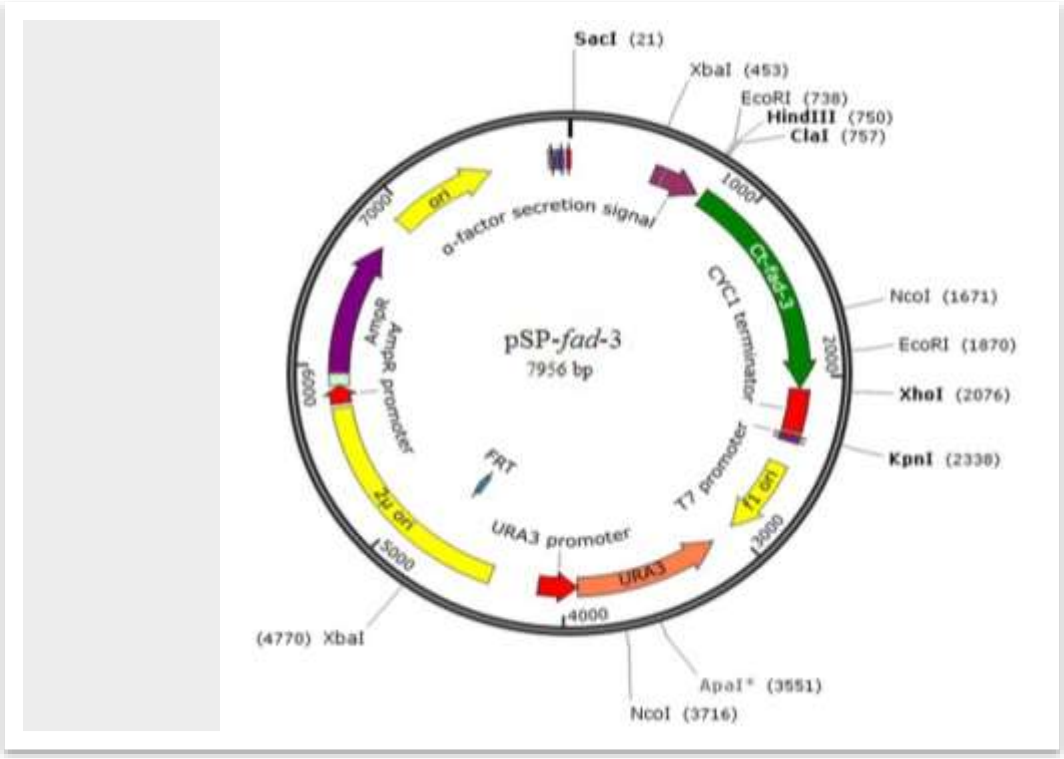
II.B.1 pBSCtN10 with 1007 bp N-terminal Ct-*fad*-3 fragment



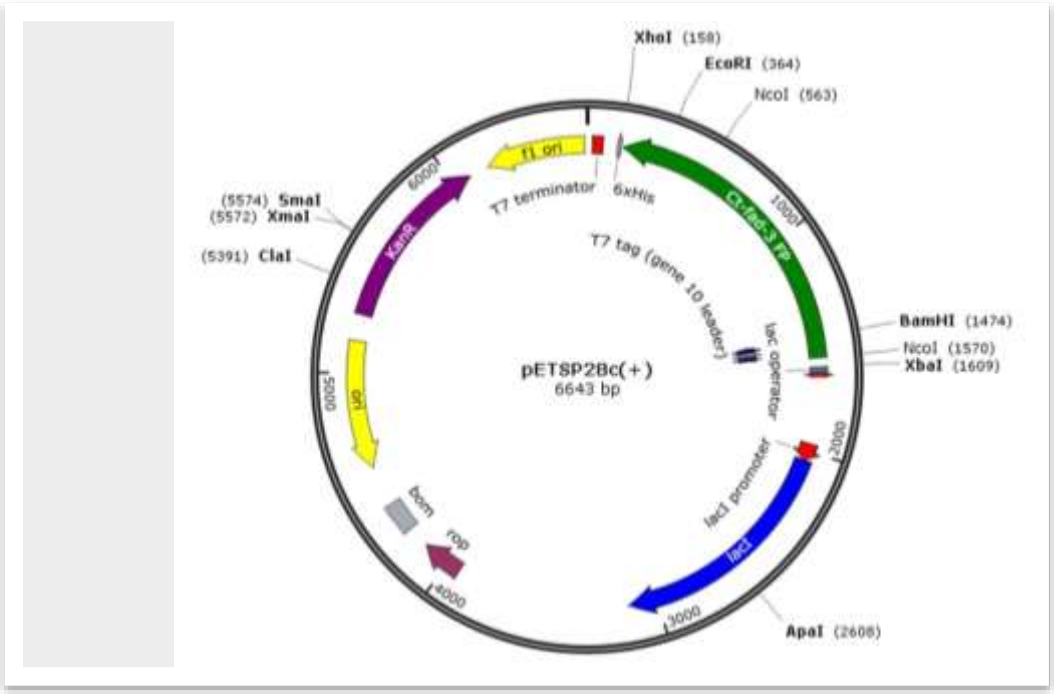
II.B.2 pBSCtC4 with 410 bp C-terminal Ct-*fad*-3 fragment



II.B.3 pBS-*fad*-3 with complete Ct-*fad*-3 gene



II.B.4 pSP-*fad*-3 with Complete Ct-*fad*-3 gene



II.B.5 pETSP28c(+) with complete Ct-*fad*-3 gene

C. Primer series used in this study

Primer	Sequence (5'⟶ 3')
OF1F	
OF1F1	GGTTTTTGGATTTTGGCTCAYGAITGYGG
OF1F2	GGTATATGGATTTTGGCTCAYGANTGYGGNCA
OF1F3	TGGATTTTGGSTCATGANTGYGGNCATTC
OF1F4	SACAYGAATGYGGICATTC
OF1F5K *Kodemia* bias	CCAYGAATGYGGYCACTC
OF1F5C *Candida* bias	GCYCAYGAATGYGGYCATGG
OF1F5S *S. klyuvery* bias	GCTCAYGAATGYGGYCACTC
OF2R	
OF2R1	ATAGTACAAGCAGCACCTTTTTGAWAIKTCC
OF2R2	AGTACAAGCTTCACCTTTTTACAWARWCCAYTC
OF2R3	TAGCAGCAGCACCTTTCRCAAARKTCCAYTC
OF2R4	CCTTTGGCAAARGTCCATTC
OF2R5KC	CCWYKRGCAAAAGTCCATTC
OF2R5SP	WYKRGCAAAAGTCCATTC
OF3R	
OF3R1	ATCTTTAGTCATATGACCAGTABYYTTRTGRTG
OF3R2	AAAWTAYCATATCTTTAGTCATATGACCAG
OF3R3	ATGGCCWGTWGCTTTATGRTGYTT

Primer	**Sequence (5'⟶ 3')**
Primers used in Amplification of Ct-*fad*-3 gene sequence	
OF5CtF	*ATG*AGYGTWGTTGARGCATCWTC
OF2CtR	CCWYKRGCAAAAGTCCATTC
OF6CtF	TGGTTTRTTCCATGGTTRTGG
OF7CtR	*CTA*ATCTYTWGGTTTAACWGGWCC
OF5CtFN	AGC*CATATG*AGTGTTGTTGAAGCATCT
OF7rCtX	CA*CTCGAGCTA*ATCCTTTGGTTTGACAGG
Primers used in 18S rDNA characterization	
P108	ACCTGGTTGATCCTGCCAGT
M2130	CAATAAATCCAAGAATTTCACC
P1190	CAATTGGAGGGCAAGTCTGG
M3490	TCAGTGTAGCGCGCGTGCGG
D) Primers used in -ITS1-5.8S- ITS2- region amplification	
ITS1	TCCGTAGGTGAACCTGCGG
ITS4	TCCTCCGCTTATTGATATGC

II.D.1 Ct-*fad*-3 ORF

```
   1 atgagtgttgttgaggcatcttcaagttctattgctaatgactct
     M  S  V  V  E  A  S  S  S  S  I  A  N  D  S
  46 actggtaacggtagtagtaacgttgttcaaagaggaaatatttct
     T  G  N  G  S  S  N  V  V  Q  R  G  N  I  S
  91 tcatttgcatcaactactgctactacaaatttaacaactattgat
     S  F  A  S  T  T  A  T  T  N  L  T  T  I  D
 136 acaaacggtaatgtttttaaagttccagattattccattaaagat
     T  N  G  N  V  F  K  V  P  D  Y  S  I  K  D
 181 attttacaagctattccaaaacattgttatgaaagatctttgatt
     I  L  Q  A  I  P  K  H  C  Y  E  R  S  L  I
 226 agatctttgggttatgttgttagagatatcaccatgatggtttta
     R  S  L  G  Y  V  V  R  D  I  T  M  M  V  L
 271 attagttatgttggacattcttttattccattggttgatattgaa
     I  S  Y  V  G  H  S  F  I  P  L  V  D  I  E
 316 aaccatgaaactttaagtactgttgttagaggttctttatggatg
     N  H  E  T  L  S  T  V  V  R  G  S  L  W  M
 361 gtccattcttacttaattgggttatttggttttggtttatggatt
     V  H  S  Y  L  I  G  L  F  G  F  G  L  W  I
 406 ttagctcatgaatgtggtcatggtgcattttcagattatcaaaat
     L  A  H  E  C  G  H  G  A  F  S  D  Y  Q  N
 451 ttaaatgatctaattggttgggttatacattcttatttgatggtt
     L  N  D  L  I  G  W  V  I  H  S  Y  L  M  V
 496 ccttacttttcatggaaattttctcatgctaaacatcataaagca
     P  Y  F  S  W  K  F  S  H  A  K  H  H  K  A
 541 actggtcatttaactaaagatatggttttcattccatatactaaa
     T  G  H  L  T  K  D  M  V  F  I  P  Y  T  K
 586 gaagaatatttagaaaagaataaagttgaaaaagtttctgaattg
     E  E  Y  L  E  K  N  K  V  E  K  V  S  E  L
 631 gttgaagaatctccaatttattccctttagttttaatttttcaa
     V  E  E  S  P  I  Y  S  L  L  V  L  I  F  Q
 676 caattgggtggtttacaattatatttagctaataatgcaactggt
     Q  L  G  G  L  Q  L  Y  L  A  N  N  A  T  G
 721 caagtttatcctggtgtttcatggtatgcaagatctcattattct
     Q  V  Y  P  G  V  S  W  Y  A  R  S  H  Y  S
 766 ccaatttctccagtttttgataaaaatcaatattggttcattgtt
     P  I  S  P  V  F  D  K  N  Q  Y  W  F  I  V
 811 ttatctgatattggtattatttcaactttaactgtggtttatcaa
     L  S  D  I  G  I  I  S  T  L  T  V  V  Y  Q
 856 tggtataaaaactttggtttatttaatatgatgatcaattggttt
     W  Y  K  N  F  G  L  F  N  M  M  I  N  W  F
 901 gttccatggttatgggttaatcattggttagttttgttacattt
     V  P  W  L  W  V  N  H  W  L  V  F  V  T  F
 946 ttacaacatactgatccaacaatgcctcattatgctgctaacgaa
     L  Q  H  T  D  P  T  M  P  H  Y  A  A  N  E
 991 tggacttttgctcgtggtgctgctgctacaattgatagaaatttt
     W  T  F  A  R  G  A  A  A  T  I  D  R  N  F
1036 ggatttgttggtcaacacatcttccatgatattattgaaactcat
     G  F  V  G  Q  H  I  F  H  D  I  I  E  T  H
1081 gttttacatcattatgtttcaagaattccattttataatgccaga
     V  L  H  H  Y  V  S  R  I  P  F  Y  N  A  R
1126 gaagctactgaagctattaaaaaagttatgggtgaacattataga
     E  A  T  E  A  I  K  K  V  M  G  E  H  Y  R
1171 tatgaaggtgaaaatatgtggttttctttatggaaatgtgttaga
```

```
    Y  E  G  E  N  M  W  F  S  L  W  K  C  V  R
1216 atgtgtcaatttgttgatgatgataaagaagatgccaaaggtgtt
    M  C  Q  F  V  D  D  D  K  E  D  A  K  G  V
1261 ttaatgtttagaaatgttaatggtcctgttaaaccaaaagattag 1305
    L  M  F  R  N  V  N  G  P  V  K  P  K  D  *
```

II.D.2 Ct-*fad*-3 gene sequence

ATGAGTGTTGTTGAGGCATCTTCAAGTTCTATTGCTAATGACTCTACTGGTAA
CGGTAGTAGTAACGTTGTTCAAAGAGGAAATATTTCTTCATTTGCATCAACTA
CTGCTACTACAAATTTAACAACTATTGATACAAACGGTAATGTTTTTAAAGTT
CCAGATTATTCCATTAAAGATATTTTACAAGCTATTCCAAAACATTGTTATGA
AAGATCTTTGATTAGATCTTTGGGTTATGTTGTTAGAGATATCACCATGATGG
TTTTAATTAGTTATGTTGGACATTCTTTTATTCCATTGGTTGATATTGAAAACC
ATGAAACTTTAAGTACTGTTGTTAGAGGTTCTTTATGGATGGTCCATTCTTAC
TTAATTGGGTTATTTGGTTTTGGTTTATGGATTTTAGCTCATGAATGTGGTCAT
GGTGCATTTTCAGATTATCAAAATTTAAATGATCTAATTGGTTGGGTTATACA
TTCTTATTTGATGGTTCCTTACTTTTCATGGAAATTTTCTCATGCTAAACATCA
TAAAGCAACTGGTCATTTAACTAAAGATATGGTTTTCATTCCATATACTAAAG
AAGAATATTTAGAAAAGAATAAAGTTGAAAAAGTTTCTGAATTGGTTGAAGA
ATCTCCAATTTATTCCCTTTTAGTTTTAATTTTTCAACAATTGGGTGGTTTACA
ATTATATTTAGCTAATAATGCAACTGGTCAAGTTTATCCTGGTGTTTCATGGT
ATGCAAGATCTCATTATTCTCCAATTTCTCCAGTTTTTGATAAAAATCAATATT
GGTTCATTGTTTTATCTGATATTGGTATTATTTCAACTTTAACTGTGGTTTATC
AATGGTATAAAAACTTTGGTTTATTTAATATGATGATCAATTGGTTTGTTCCA
TGGTTATGGGTTAATCATTGGTTAGTTTTTGTTACATTTTTACAACATACTGAT
CCAACAATGCCTCATTATGCTGCTAACGAATGGACTTTTGCTCGTGGTGCTGC
TGCTACAATTGATAGAAATTTTGGATTTGTTGGTCAACACATCTTCCATGATA
TTATTGAAACTCATGTTTTACATCATTATGTTTCAAGAATTCCATTTTATAATG
CCAGAGAAGCTACTGAAGCTATTAAAAAAGTTATGGGTGAACATTATAGATA
TGAAGGTGAAAATATGTGGTTTTCTTTATGGAAATGTGTTAGAATGTGTCAAT
TTGTTGATGATGATAAAGAAGATGCCAAAGGTGTTTTAATGTTTAGAAATGTT
AATGGTCCTGTTAAACCAAAAGATTAG

II.D.3Amino acid sequence of Ct-FAD-3

MSVVEASSSSIANDSTGNGSSNVVQRGNISSFASTTATTNLTTIDTNGNVFKVPDY
SIKDILQAIPKHCYERSLIRSLGYVVRDITMMVLISYVGHSFIPLVDIENHETLSTV
VRGSLWMVHSYLIGLFGFGLWILAHECGHGAFSDYQNLNDLIGWVIHSYLMVPY
FSWKFSHAKHHKATGHLTKDMVFIPYTKEEYLEKNKVEKVSELVEESPIYSLLVL
IFQQLGGLQLYLANNATGQVYPGVSWYARSHYSPISPVFDKNQYWFIVLSDIGIIS
TLTVVYQWYKNFGLFNMMINWFVPWLWVNHWLVFVTFLQHTDPTMPHYAAN
EWTFARGAAATIDRNFGFVGQHIFHDIIETHVLHHYVSRIPFYNAREATEAIKKV
MGEHYRYEGENMWFSLWKCVRMCQFVDDDKEDAKGVLMFRNVNGPVKPKD

434 amino acid polypeptide with 49850 Da molecular weight and pI 6.4

II.E CLUSTAL O(1.2.0) multiple sequence alignment

```
Pp_FAD_3      MSKVTVSGSEILE-G----STKTVRRSGNV-----ASFKQQKTAIDTFGNVFKVPDYTIK
Ct_FAD-3      MSVVEASSSS-IANDSTGNGSSNVVQRGNISSFASTTATTNLTTIDTNGNVFKVPDYSIK
Ca_FAD_3      MSVVEASSSS-VVEDST---ASNVVQRGNISSFASTTASSNLTTIDTNGKVFKVPDYSIK
Sk_FAD_3      MSIETVGSSSGVAINS------------KAVSSTATTVVQPKTAIDTNGNVFKVPDYTIK
Kl_FAD_3      -----MSKSTGVEHHISGVAT-------TETATETVTVPPAKTAIDTHGNIFKVPDYTIK
                    . *  :                .      .:     *:*** *::******:**

Pp_FAD_3      DILDAIPKHCYERSLVKSMSYVVRDIVAISAIAYVGLTYIPLLPN-------EFLRFAAW
Ct_FAD-3      DILQAIPKHCYERSLIRSLGYVVRDITMMVLISYVGHSFIPLVDIENHETLSTVVRGSLW
Ca_FAD_3      DILQAIPKHCYERSLIRSLGYVVRDITMMVIIGYVGHTFIPMVQIPEYPSLAYGLRGALW
Sk_FAD_3      DILSAIPKECYKRDTLWSLHYVVRDIAAILVIGYLGTNYIPVLFP-----NSALLRGIAY
Kl_FAD_3      DILGAIPKECYKRDTLWSLHYVVRDIIAICIIGYVGTNYIPVWFP-----NSGLLRFVAY
              *** ****.**:*. : *: ******  :  *.*:* .:**:           :*   :

Pp_FAD_3      SAYVFSISCFGFGIWILGHECGHSAFSNYGWVNDTVGWVLHSLVMVPYFSWKFSHAKHHK
Ct_FAD-3      MVHSYLIGLFGFGLWILAHECGHGAFSDYQNLNDLIGWVIHSYLMVPYFSWKFSHAKHHK
Ca_FAD_3      MVQSYCIGLFGFGLWILAHECGHGAFSDYQNINDFIGWVLHSYLIVPYFSWKFSHAKHHK
Sk_FAD_3      AIQSYLIGLFGFGLWILAHECGHSAFSESNAVNDTVGWVLHSWWMVPYFPWKFSHSKHHK
Kl_FAD_3      MVQSYLIGLFGFGLWILAHECGHGAFSDSRLINDTVGWVLHSWWMVPYFSWKFSHSKHHK
                  : *. ****:***.*****.***:   :** :***:**  :**** *****:****

Pp_FAD_3      ATGHMTRDMVFVPYTAEEFKEKHQVTSLHDIAEETPIYSVFALLFQQLGGLSLYLATNAT
Ct_FAD-3      ATGHLTKDMVFIPYTKEEYLEKNKVEKVSELVEESPIYSLLVLIFQQLGGLQLYLANNAT
Ca_FAD_3      ATGHLTKDMVFIPYTKEEYLEKNKVEKVADLMEESPIYSFLVLVFQQLGGLQLYLATNAT
Sk_FAD_3      ATGHMTRDMVFIPYTKDEFITMKKKSKFAEITEEAPVMTLFNLIAQQVGGLQLYLATNAT
Kl_FAD_3      ATGHLTRDMVFVPYTKKEYLEMKGKSKLREITEEAPIVTLLTLIGQQIGGLQLYLATNAT
              ****:*:****:*** .*:   .   .. :: **:*: :.: *: **:***.****.***

Pp_FAD_3      GQPYPGVSKFFRSHYWPSSPVFDKKDYWYIVLSDLGILATLTSVYTAYKVFGFWPTFITW
Ct_FAD-3      GQVYPGVSWYARSHYSPISPVFDKNQYWFIVLSDIGIISTLTVVYQWYKNFGLFNMMINW
Ca_FAD_3      GQVYPGYSKIAKSHYTPTSPVFDKHQYWYIVLSDIGIILAFTTVYQWYKNFGLFNMMINW
Sk_FAD_3      GQPYPGVKKFFKSHYWPTSPVFDAKDFWWIIMSDIGIVSTLLINYLWYRAYGAHVVLINW
Kl_FAD_3      GQSYPGVPKFFKSHYWPTSPVFDTKDFWYIILSDIGIISTLTINYLWAKTYGSHVMLINW
              ** ***     :*** * ***** .::*:*::**:**: ::   *   : :*    :*.*

Pp_FAD_3      FCPWILVNHWLVFVTFLQHTDSSMPHYDAQEWTFAKGAAATIDREFGILG-IIFHDIIET
Ct_FAD-3      FVPWLWVNHWLVFVTFLQHTDPTMPHYAANEWTFARGAAATIDRNFGFVGQHIFHDIIET
Ca_FAD_3      FVPWLWVNHWLVFVTFLQHTDPTMPHYTSKEWTFARGAAATIDRNFGFVGQHIFHDIIET
Sk_FAD_3      FIPWLWVNHWLVFVTFLQHTDPTMPHYDAEEWTFAKGAAATIDRNFGFVGQHIFHDIIET
Kl_FAD_3      FVPWLWVNHWLVFVTFLQHTDPTMPHYEASEWTFAKGAAATIDRNFGFVGQHIFHDIIET
              * **: ***************  :**** :.*****:********:**::*  ********

Pp_FAD_3      HVLHHYVSRIPFYHAREATECIKKVMGEHYRHTDENMWVSLWKTWRSCQFVENH----DG
Ct_FAD-3      HVLHHYVSRIPFYNAREATEAIKKVMGEHYRYEGENMWFSLWKCVRMCQFVDDDKEDAKG
Ca_FAD_3      HVLHHYVSRIPFYNAREATDAIRKVMGEHYRYEGESMWYSLWKCMRMCQFVDDDKEDAKG
Sk_FAD_3      HVLHHYCSRIPFYNARKATSAIKEVMGQHYRYEGENMWKSLWKVARSCQYVEGD----NG
Kl_FAD_3      HVLHHYCSRIPFYNARVATEAIKKVMGEHYRYEGENMWQSLWKVARSCQFVDGD----NG
              ****** ******.** **..*::***:***:  *.** ****  * **:*: .    .*

Pp_FAD_3      VYMFRNCNNVGVKPKDT---
Ct_FAD-3      VLMFRNVNG--PVKPKD---
Ca_FAD_3      VMMFRNVNGWGPVKPKD---
Sk_FAD_3      VRMFRNTNGVGVKPEDGSSQ
Kl_FAD_3      VLMFRNTNGVGAPCQE----
              * **** *       .
```

Figure 3.2 Comparison of the deduced amino-acid sequence of Ct-FAD-3 with previously reported FAD-3from several fungi. The amino-acid sequences of omega-3 desaturases from*Pichia pastoris* (Pp_FAD_3), *Candida tropicalis* (Ct_FAD_3), *Candida albicans* (Ca_FAD_3), *Saccharomyces kluyveri* (Sk_FAD_3), *Kluyveromyces lactis* (Kl_FAD_3) are used. Sequences were aligned using the **Clustal omega** algorithm. The three conserved 'Histidine' boxes are shaded with light grey.Candida specific conserved amino acid stretch is shaded with green color.

Identification, cloning, expression and characterization of omega-3 fatty acid desaturase from yeast as a novel source

Summary

Nutritional importance of omega-3 (n-3) polyunsaturated fatty acids (PUFAs) over n-6 PUFAs is well accepted. In our body, the essential fatty acid α-linolenic acid (ALA) (C-18:3 n-3) is metabolized to produce higher n-3 PUFAs viz. Eicosapentanoic acid (EPA) (C-20:5), Docosahexnoic acid (DHA) (C-22:6). Disruption in proper PUFA intake and metabolism (i.e. higher n-6: n-3 ratio in the diet) is associated with various health disorders.

Most edible plant seed oils are poor sources of essential n-3 PUFAs, while marine fishes and their oils are rich in n-3 PUFAs. However, dietary habits, cost, biomagnifications of toxic compounds, limit their use. Microbial oils have begun to be considered as possible alternative sources of n-3 PUFAs. Several microorganisms e.g. bacteria, microalgae, yeasts etc. have been characterized for the production of n-3 PUFAs and n-3 fatty acid desaturases (FAD-3) that converts n-6 fatty acids into n-3 fatty acids. Among these microorganisms yeast have better scope as they have relatively higher lipid content with similar fatty acids composition and energy value to plant oils thus can be utilized as an optimal and abundant source of PUFAs.

An intense molecular and biochemical screening was undertaken in this study, with the main objective of exploring yeast strains as a source of n-3 PUFAs and to investigate their applicability. Seventy yeast isolates from indigenous microflora from different sources were screened for presence of *fad*-3 gene by PCR, hybridization analysis and further for their ability to convert n-6 PUFAs to n-3 PUFAs mainly LA to ALA. Out of seventy screened yeast isolates, 25 isolates gave an amplicon of ~600 bp and 23 out of these 25, gave amplification (~145 bp) by subsequent semi-nested PCR. The PCR positive yeast strains were further screened by low stringency dot-blot hybridization and thereafter narrowed down by high stringency Southern hybridization using *fad*-3 (610 bp) sequence from *S. kluyveri* (NBRC 1893). Out of these 25 isolates, 22 isolates showed

positive results in dot blot analysis and 19 isolates gave positive signals in southern hybridization. The putative isolates were identified by -ITS1-5.8S-ITS2- region sequencing. Many of the selected isolates were identified as *Candida spp.* or closely related to *Candida genera* indicating a predominance of this genus among yeasts which contain functional FAD-3 activity. The identified isolates belong to the different genera *viz. Candida, Issatchenkia, Kodamaea, Meyerozyma, Pichia, Arxula* and *Rhodotorula*. Our efforts to find an evolutionary correlation to rationalise the distribution of *fad*-3 gene among these isolates gave somewhat imprecise interpretations due to lack of consensus in yeast taxonomy. Confinement of *fad*-3 to the identified similar or related organisms in different and distant ecological niches indicates high conservation and minimal horizontal transfer.

The LA to ALA biotransformation ability of yeast cultures was studied using pure LA as a substrate and the transformation products were analyzed by Silver ion TLC and further by quantification of ALA in their fatty acid profiles by gas chromatography (GC). Strains *C. parapsilosis* A-16, *C. tropicalis* PS-2, *I. orientalis* MI-1, and *P. kudriavzevii* MI-3 gave 2- to 4- fold high volumetric productivity of ALA (20-60 mg/L; approx. 4-7 g dry weight cells), compared to that of the standard strain *S. kluyveri* (10 mg/L). The LA content of cells were in the range of 35-120 mg/g dry cell weight and the ratio of n-3: n-6 PUFAs ranged in between 1:4-8, the values better than that of *S. kluyveri* (NBRC 1893) (1:10); higher PUFAs were not detected in these FAMEs.

In this work, *C. tropicalis* PS-2, isolated from '*Neera*' - a well-accepted beverage in India for its nutritional values, was selected for n-3-fatty acid desaturase (Ct-*fad*-3) gene isolation, cloning and characterization. The Ct-*fad*-3 gene was amplified as two separate fragments by PCR and recombined *in vitro* to produce the complete gene and sub-cloned in the yeast secretory expression vector pGAL-MF to produce a recombinant construct, pSP-*fad*-3. Ct-*fad*-3 sequence was submitted in Gene Bank; Accession No. ADN42964. The nucleotide sequence of Ct-*fad*-3 gene consisted of an open reading frame encoding for a protein with 434 amino acid residues containing three regions of conserved histidine cluster motifs *viz.* HXXXH, HXXHH, and HXXHH in dasaturase domain with a calculated molecular mass of 49.85 kDa and a pI of 6.4. Recombinant protein was

expressed in *S. cerevisiae* W9100 and expression of mRNA transcripts of *fad*-3 gene was confirmed by reverse transcriptase PCR. Overexpression of FAD-3 in yeast facilitated better growth at low temperature (20 ºC) as compared to the control yeast strain. Ct-*fad*-3 was expressed under the strong control of GAL L promoter, 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. The functional activity of the expressed FAD-3 was rather found to be cell associated and no transformation of LA was seen using protein content from culture supernatant. Hydropathy and topology analyses of Ct-FAD-3 also predicted 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 despite its fusion with secretory signal. In order to confirm the presence of FAD-3 protein, various procedures for solubilization of membrane proteins were employed, but no band corresponding to FAD-3 protein was detected on SDS-PAGE gel. For immunological detection of the expression of Ct-FAD-3 protein in yeast, cloning and heterologous expression of Ct-*fad*-3 in *E. coli* BL-21(DE3) was done using pET-28c(+) as an expression vector. Though Ct-FAD-3 protein was expressed in *E. coli*, no FAD-3 activity was seen. Polyclonal anti-FAD-3 IgG antibodies against purified FAD-3 protein were generated in rabbit and purified. Cell associated expression of FAD-3 protein in recombinant yeast was confirmed by ELISA and Western Blot analysis of total yeast cell proteins using anti-Ct-FAD-3 antibodies. The protein might have been overlapped by indigenous host cell protein hence was not seen in Coomassie or silver staining methods.

Biotransformation studies were done using whole cell / cell lysates of the recombinant yeasts as an enzyme source using pure LA and sunflower oil as substrates. Sunflower oil being routinely used edible oil with high content of LA (60-65%) was selected as a substrate for biotransformation. In both cases final ALA: LA ratio in recombinant cells was obtained within the range of 1: ~3-4. GC-MS analysis confirmed the presence of ALA in the FAMEs samples prepared from galactose induced cell mass of recombinant yeast after biotransformation. The expressed FAD-3 protein was found to be C-18 specific only with ability to transform LA to ALA, but no conversion of AA (C-20:4 n-6)

to EPA (C-20:5 n-3) was seen. It was seen that even though FAD-3 was overexpressed in the *S. cerevisiae* W9100, the final ratio of ALA: LA was found to be 1:3-4, as that of resource yeast strain (*C. tropicalis* PS-2) when supplemented with LA after 16 hrs biotransformation wherein ALA production amounted to 12.2 ± 2 mg / gm of dry wt. This might be due to the involvement of genetic and various environmental factors which determine the fatty acid accumulation within the yeast cells. The parent *S. cerevisiae* W9100 can synthesize only monounsaturated fatty acids. 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.

This research was aimed at exploring the potential yeast resources capable of producing n-3 PUFAs which may be further used as a source of essential PUFAs or proteins involved in PUFA biosynthesis. Our findings may give an insight regarding diversity in the yeasts containing PUFAs. In the present study, screening for n-3 PUFAs producers was limited to certain habitats that pose no health risks. Many of these ascomycetous yeasts associated with food and drink products could be explored as direct source of essential fatty acids. The concept of obtaining n-3 PUFAs from *S. cerevisiae* in sustainable quantities for human consumption can be of special attraction and may be explored further for probiotic preparations.

For future perspective, recombinant yeast strain expressing cloned *fad*-3 gene can be applied for production of n-3 PUFAs enriched neutraceuticals. Studies will be required to fully understand the structure/function relationship between different hydrophobic regions of Ct-FAD-3, their effect on overall topology, localization, regioselectivity and substrate specificity of the protein. Further studies regarding effect of recombinant protein expressed in *S. cerevisiae* or other probiotic organisms like *Lactobaccilus* spp. or *S. bolardii*, on animal model as n-3 PUFAs producers will be required to better understand its applicability. Experiments which replace certain amino acids so as to increase the substrate specificity of the protein would help to enlighten the new window to produce long chain PUFAs within the recombinant yeast by using single protein.