IDENTIFICATION, CLONING, EXPRESSION AND CHARACTERIZATION OF OMEGA-3 FATTY ACID DESATURASE FROM YEAST AS A NOVEL SOURCE

SUMMARY OF THESIS SUBMITTED TO

THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

MICROBIOLOGY

BY

SANDEEP G. PATIL

GUIDED BY

Dr. MRINALINI NAIR

DEPARTMENT OF MICROBIOLOGY & BIOTECHNOLOGY CENTRE
FACULTY OF SCIENCE
THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA
VADODARA – 390 002, GUJARAT
INDIA

2014

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 °C) as compared to the control yeast strain. Ct-*fad*-3 was expressed under the strong control of GAL 1 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.