Present Investigation

1.4 Present investigation

1.4.1 Definition of the problem

Lipids are the very important components of human diet. Study on beneficial role of n-3 PUFAs was triggered by the hypothesis on the healthy longevity of Eskimo population might be due to their traditional fish-rich diet containing DHA and EPA. The benefits of fish oil can be related with these long chain PUFAs which are not synthesized in plants.

In our body, biosynthesis of PUFAs is under strong feedback regulation and the n-3 and n-6 PUFAs are not interconvertable (Arterburn *et al.*, 2006). Ultimately the ratio of LA: ALA in our diet determines the ratio of n-6: n-3 PUFAs in the body, indicating the need of a balanced supply of essential fatty acids (Arterburn *et al.*, 2006; Sjogren *et al.*, 2008).

Although the recommended ratio of dietary n-6 and n-3 fatty acids is 5:1, it has shifted heavily toward n-6 fatty acids in the current diet up to 30-fold, attributable in part to the increased consumption of vegetable oils that contain abundant n-6 fatty acids, but less n-3 fatty acids (Damude and Kinney, 2008; Sayanova and Napier, 2011). To correct the imbalance, increased intake of n-3 PUFAs is advisable which is possible only with increased consumption of n-3 PUFAs-enriched/fortified foods or n-3 PUFA supplements.

Fishes are rich in DHA and EPA; which they derive from phytoplankton through bioaccumulation in food chain (Bell and Tocher, 2009). A variety of marine organisms and fish species such as cod, eels, herring, mackerel, salmon, sardines, sharks, tuna, trout etc. are the good sources of n-3 PUFAs (Bell and Tocher, 2009). For therapeutic use, generally capsules containing fish oils, especially fish liver oil (e.g. cod and halibut), have been used which are rich sources of EPA and DHA.

Global climate change and pollution of marine environments is directly responsible for their deterioration, resulting in decreased phytoplankton growth and reduced synthesis of n-3 fatty acids. Due to the many drawbacks of fish and fish-derived oil, including undesirable taste and odor, objections by vegetarians, chemical processing methods, presence of contaminants such as mercury, dioxins and polychlorinated biphenyls (Guallar *et al.*, 2002; Dorea, 2008). Variability in EPA and DHA content and declining world fish supply is also one of the causes for a need to develop non fish sources for n-3 PUFAs (Racine and Deckelbaum, 2007).

Dietary inclusion of ALA increases expression of PPARγ involved in its conversion to longer n-3 fatty acids in cattle and also raises n-3 fatty acid content, including DHA albeit at a lower rates in adult rats and nonhuman primates (reviewed in Barcelo-Coblijn and Murphy, 2009). The increased dietary supplements of DHA in porcine liver has been shown to down regulate ADD1 mRNA involved in the regulation of genes involved in synthesis of PUFAs (Hsu *et al.*, 2004). In human, the efficiencies of conversion of dietary ALA into EPA and DHA are in strong associations with SNP variants in the Fads1 and Fads2 genes, age, gender, and other factors (Schaeffer *et al.*, 2006; Malerba, 2008; Bokor *et al.*, 2010) thereby demanding dietary inclusion of EPA or DHA in individuals. Thus the production of ALA probably is a first step towards developing alternative to fish and other sources of EPA and DHA. Eventually such an expression system serve as a platform for production of EPA and DHA.

1.4.2 In search of alternative sources of n-3 PUFAs

For many years yeasts have been used as a tool for commercial production of variety of valuable metabolites. Yeasts and algae can produce oils and fatty acids (Subramaniam *et al.*, 2010); yeast have advantage over algae, bacteria and moulds, that the lipid content of yeasts is relatively higher in comparison with bacteria and microalgae (Meng *et al.*, 2009). The biomass of yeasts is devoid of endotoxins with similar fatty acids composition and energy value to plant oils thus can be utilized as an optimal and abundant source of PUFAs (Beopoulos *et al.*, 2011; Meng *et al.*, 2009). Microbial lipids have many advantages (short life cycle, less effects on production by climate, venue, season, easy scale-up *etc.*) that promise to overcome many limitations of plant oils, utilization of inexpensive substrates with yield and productivity is the major limiting factor (Subramaniam *et al.*, 2010).

A few yeasts including *S. kluyveri* (Oura and Kajiwara, 2004), *C. albicans* (Murayama *et al.*, 2006) express membrane bound Δ-15 desaturases. *S. cerevisiae* has been serving as a model organism for the development of metabolic engineering strategies to produce certain metabolites (Nevoigt, 2008; Hong and Nielsen, 2012). The concept of obtaining PUFAs from *S. cerevisiae* in sustainable quantities is particularly attractive but the quality as well as quantity of lipid content in *S. cerevisiae* is not high enough. Thus introduction of ability to produce essential n-3 PUFAs, not only ALA, but also EPA and DHA by metabolic

engineering will be beneficial for future applications. Biotransformation using recombinant yeast to increase percentage of desired n-3 PUFAs would provide an economical source of these important fatty acids (Uemura, 2012).

1.4.3 Objectives

- Identification of yeasts as a novel alternate source for n-3 fatty acid desaturases.
- Cloning and secretory expression of n-3 fatty acid desaturase.
- Purification and functional characterization of n-3 fatty acid desaturase.
- To assess the applicability of the recombinant yeast for transformation of LA (pure and from edible oils) to ALA.

1.4.4 This work

This research aims at exploiting the potential natural yeast resources for the production of PUFAs mainly ALA and developing techniques that can later be applied to the production of EPA and DHA in yeast. The primary aim is to screen and identify yeast strains containing 18-carbon PUFA-specific *fad*-3 genes, to characterize their enzyme activity and to isolate, clone and express the *fad*-3 gene from the prospective isolates in *S. cerevisiae*. More than seventy yeast strains, isolated from over-ripened fruits, fermented drinks and from soil in premises of edible oil mills in different tropical parts of India, were analyzed for the presence of *fad*-3 gene by PCRs and DNA hybridization which detected multiple clusters of 16-22 amino acids conserved among *fad*-3 genes of algae, fungi, and plants. Strains that produced n-3 PUFAs, α linolenic acid (ALA) were identified by 18s rDNA and ITS sequence analysis. The *fad*-3 gene was isolated from *Candida tropicalis* PS-2 (Ct-*fad*-3) and cloned into yeast expression vector pGAL-MF. Expression studies of the cloned Ct-*fad*-3 were carried out in *E. coli* BL21(DE3) and *S. cerevisiae* W 9100. Expression of Ct-FAD-3 protein was analyzed by Western blotting using polyclonal anti-FAD-3 antibodies raised in rabbit in this work. Final ALA content and LA: ALA ratio in the recombinant strains was assayed by biotransformation studies and detection of product by gas chromatography and mass spectrophotometry analysis. For future perspective, this recombinant yeast strain expressing cloned *fad*-3 gene can be applied for production of n-3 PUFAs enriched neutraceuticals.