Minor Research Project

Title: "Isolation, characterization of marine yeasts and selection of low cost cultivation media for production of Poly unsaturated fatty acids"

UGC Reference No: 47-502/12(WRO)

Completion Report

Submitted to

University Grants Commission,

Western Regional Office, Pune

By

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UGC Funded Minor Research Project

EXECUTIVE SUMMARY

Title: "Isolation, characterization of marine yeasts and selection of low cost cultivation media for production of PUFA"

(UGC Reference No: 47-502/12(WRO)dated 15 Feb.2013

Principal Investigator: Dr. Mrs. Anuradha A Jape, Yashwantrao Mohite College of Arts, Science and Commerce, Bharati Vidyapeeth Deemed University, Pune

Introduction

The Poly Unsaturated Fatty acids, PUFAs are essential nutrients as are required for normal development and metabolism. The main source of dietary PUFA is sea foods. The average intake of seafood varies with season, population, health status of the individuals. To fulfill the daily requirement of the growing population and considering the medicinal applications, techniques for better PUFA production is an area of intense research. Considering the limitations of vegetarian diet, depletion of marine fish, there is a need for alternative LC-PUFA sources. At present, commercially, the microbial PUFA and oils contribute a very small fraction but offer a sustainable option that can be potentially explored. In the marine ecosystem the lipids are accumulated by phytoplankton mainly algae and fungi and are transported to higher animals of the marine food chain. Thus the oleaginous marine yeasts and fungi appear as the potential alternative source for poly-unsaturated fatty acid production.

Brief objectives of the project

i. Isolation of marine yeasts

ii. Screening of marine yeasts for Poly Unsaturated Fatty Acid production

iii. Selection of low cost media for PUFA production

A brief summary of the work done

- Marine yeasts were studied for the potential to produce Poly Unsaturated Fatty Acids (PUFA). The marine water samples were obtained from Konkan regions and 54 yeasts were isolated. These yeasts were grown on the basal medium (BM containing yeast extract 0.3% glucose 1.0 % in sea water) and the fast growing 18 yeasts were selected for further studies. These 18 yeasts were studied for biochemical characters, screened for the

lipid accumulation property using primary screening techniques. The initial microscopy studies i.e. wet mount and modified Sudan Black B staining (Jape 2014) were useful in selecting the oil producing yeasts which were then identified using molecular technique.

- The analysis of yeast lipids using Nile red fluorescence microscopy (Figure1), FTIR spectroscopy and Gas Chromatography were useful in selecting the most promising PUFA producers among the studied yeasts of marine origin. The marine yeasts *Candida orthopsilosis, Candida tropicalis* and *Rhodotorula muciliginosa* were studied further. The selected yeasts were cultivated on whey based low cost liquid media for PUFA production. (Table I). (The study is published, Jape 2015).
- **In addition to the stated objectives**, experiments were further conducted to develop Solid substrate fermentation method to produce PUFA enriched cornmeal using selected marine yeasts. The yeasts were grown in solid state fermentation using corn+whey as substrate (Jape 2015) and biomeal (the fermented product) with high PUFA was produced (Figure 2). The toxicological analysis of the biomeal produced was conducted to check the safety. The animal toxicity studies (28 days repetitive dose study according to OECD guidelines) were conducted in wistar rat model. The clinical, haematological and serum parameters were monitored on weekly intervals. After giving the dose for 28 days, animals were ethunised and histopathology studies were conducted. The sub acute repetitive dose studies of biomeal showed no toxicological effects in wistar rat animal model.

Review of literature

The theme to "Bring back PUFA" in human food chain includes PUFA enriched feed for poultry and dairy animals. Thus production of PUFA rich food or feed supplement using PUFA producing oleaginous microbes is times need (Certik et al 2010). Marine yeasts offer the potential source of PUFA that can be easily scaled up on low value raw materials and could be employed as promising feed supplement. The demand, health benefits of PUFA and limitations in the current sources emphasizes the requirement for a simple system to mediate PUFA production. Therefore it is important to screen various environments to isolate and identify new oleaginous microorganisms which may produce high amount of the desired fatty acid. It is equally essential to optimize the fermentation parameters, to select low-cost substrates, and formulate a system for maximum production of oil or oil rich in high value fatty acids.

In view of this, the objectives of present work were set to study the potential of marine yeasts for PUFA production and select a low cost medium for PUFA production using marine yeasts.

Methodology

1. **Isolation of marine yeasts**
2. **Primary screening: Microscopy (Wet mount, Sudan Black B staining, Fluorescence staining using Nile Red)**
3. **Secondary screening :Analysis of PUFA using Gas chromatography**
4. **Biochemical characterization and molecular identification (18 S r RNA gene sequencing) of isolated marine yeasts (Performed at Microbial Culture Collection Centre,Pune)**
5. **Selection of Media and growth optimization**
6. **Solid substrate fermentation (SSF)**
7. **Toxicological study of biomeal prepared using SSF (28 Days repetitive dose subacute oral toxicity test in Wistar rat model according to OECD, Performed at National Institute of Toxicology, Pune).**

Results:

Isolation of marine yeasts:

Marine waters were sampled from Konkan coasts of India, 54 yeasts were isolated using Wickerham media. Characteristic yeast colonies were purified and maintained as 20.0 % Glycerol stocks.

1. **Primary screening :Microscopy**

The microscopic methods viz. wet mount and modified Sudan Black B staining of oil globules (Jape 2014) were used for the preliminary selection of oil accumulating yeasts. Out of 54 isolates, the fast growing 18 yeasts those could accumulate oil were selected and screened further. Also Nile red staining was performed to demonstrate oil accumulation

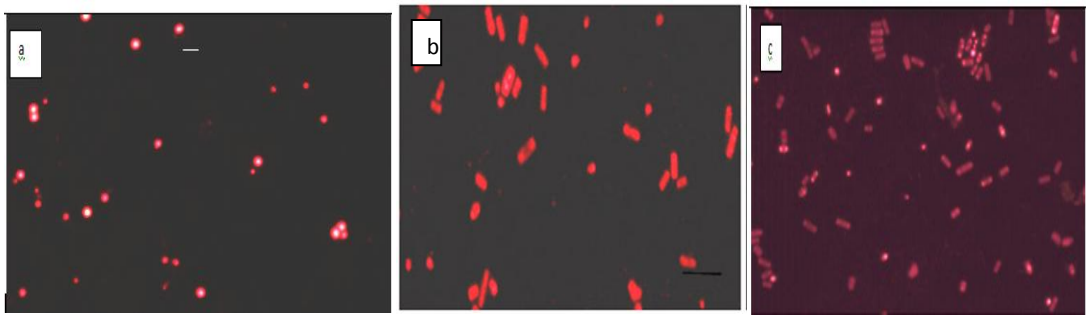

Figure1. Golden yellow fluorescence of **f**at globules in yeast cells stained with Nile red solution observed under Fluorescent Microscope. a: *Rhodotorula*, b, c: *Candida* spp.

2. **Secondary screening :Analysis of PUFA using Gas chromatography**

The oils extracted from the marine yeasts were derivatised to Fatty acyl methyl esters (FAMEs) and were analysed by gas chromatography- flame ionizing detector (GC-FID). The total PUFA content of the yeasts studied ranged between 10.44 - 38.82 (area %) in the yeast cells. The result showed that the omega-3: omega-6 ratio was 4:1 in the oil obtained from yeast cells and 10:1 in the oil extracted from the yeast cells, 1.4: 1. The ratio of omega-6: omega-3 is very important in using the yeasts as a PUFA source. The high ratio of 10:1 shows the potential of these oils and the marine yeasts studied as possible PUFA supplements for fortification or as food or feed additive.

3. **Characterisation and Identification of marine yeasts isolated**

The physiological characteristics of 18 yeast isolates were studied. (Fell 2001). The molecular identification of 11 marine yeasts isolates was performed on the basis of 18S rRNA gene sequence analysis (Molecular identification was done at Microbial Culture Collection, Pune). The similarity of 18 S rRNA gene sequences was more than 98.0% as compared to available sequences the reference for the BLAST analysis of sequences obtained. The isolates *C. orthopsilosis, C. parapsilosis, C. tropicalis, R. muciliginosa* were studied further.

4. **Selection of Media and growth optimization**

Various raw materials were screened for oil accumulation; whey was selected as promising raw material for oil production using marine yeasts (table1) (Jape2015). Growth parameters temperature, pH, salt concentration and rpm for shake flask culture were optimized.

Table1. PUFA content of selected yeasts cultivated in a basal medium(1.0g% Glucose +0.3g% YE in sea water) with various supplements (PUFA content expressed as area% of total lipids as detected by GC)

Yeast studied	**Glucose 10.0 g%**	**Malt powder 1.0 g%**	**Glycerol 4.0 g%**	**Whey 50% (v/v)**	**Flax oil 1.0g%**
R. muciliginosa	09.48	10.29	33.92	24.43	21.11
C. orthopsilosis	48.33	31.41	42.76	32.86	54.32
C .tropicalis	13.12	10.93	33.96	19.22	32.12

5. Solid substrate fermentation(SSF)

In further experiments, the yeasts were cultivated in SSF using corn meal substrate (corn+ whey) that was supplemented with various carbon sources, so as to increase C:N content that would lead to high lipid accumulation. The Solid state fermentation was carried out at 27^{o}C for 196 hrs. The supplement showed the increase in biomass, protein, lipid content, type and degree of unsaturation of fatty acids. The lipid content of the biomeal ranged between 3.0 to 10.0 % and was 1-3 times higher than in the substrate.

The oil content of the corn used was 2.56 area % and the major fatty acid component was Linoleic Acid 52.2 area % as detected by GC. The GC analysis showed that the LA content of the biomeal formed after the fermentation using marine yeasts was 42-47 area %. The higher PUFA including ALA, EPA and ARA (<1%), DHA (2-4%), and GLA (16%) were produced by the yeasts. The fatty acid produced by all the three marine yeasts studied also showed presence of saturated fats; Oleic and Palmitic acids were the major components. Also Eicosadienoic and Eicosatrienoic acid were noted in the oil from all the three marine yeasts and their concentration ranged between 5-8 area % of total lipids. The PUFA fraction contained LA as the major component, the major component of corn that was used as substrate in SSF.

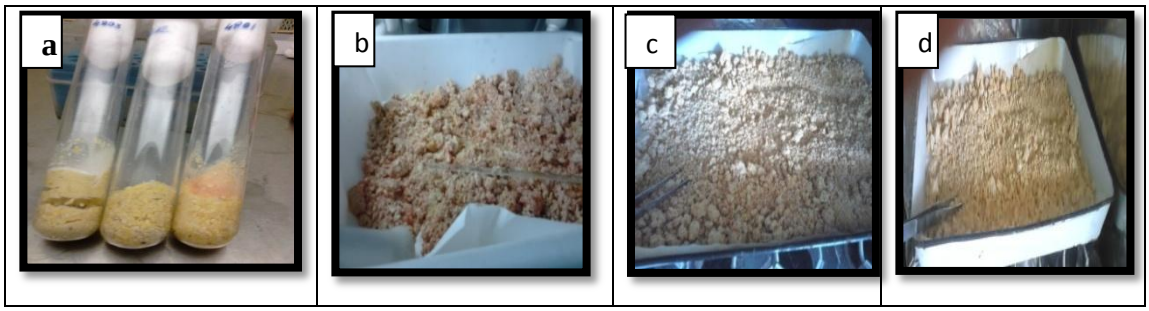

Figure 2 Biomeal preparation: Scale-up to 1.0kg.
(a: SSF in test tube, biomeal prepared using b *C.tropicalis*:, c: *C. orthopsilosis*, d*: R . muciliginosa*)

6. Toxicological study of biomeal prepared using SSF.

The cornmeal based substrate was fermented with these yeasts and biomeal generated was tested for its nutritional safety. In this study, 28 days oral toxicity test was done and at doses of 1000 mg/kg of biomeal with water as vehicle in female wistar rats (6 animals per group). Mortality, clinical signs and changes in body weight, haematology, serum nutrients, serum enzymes and serum wastes were monitored for 7, 14 and 28 days according to OECD Guidelines The recommended maximum dose for an unknown substance is 1000 mg/kg.

Throughout the 28 days of assay, the test group did not show any change in behavior, mortality, or clinical signs of toxicity compared to the control group. All animals survived to the end of the observation period and were subjected to terminal necropsy. Animals were euthanized by ether saturation after overnight fasting (approximately 18 h with water *ad libitum*).Target organs were observed, included the heart, lung, kidney, thymus, adrenal glands, brain, liver, ovary, pancreas, spleen. Results from this preliminary study show that the corn based fermented biomeal prepared using marine *C. orthosilopsis, C. tropicalis, R. muciliginosa* has no toxicity and are safe in wistar rats. **(The toxicity studies were conducted at NIT, Pune)**

Conclusion

Marine yeasts were isolated and 18 isolates were screened for PUFA production and three isolates were selected for further studies. Isolates were identified using biochemical and molecular methods as *Candida tropicalis, Candida orthosilopsis, Rhodotorula muciliginosa.*

Whey appears as a low cost, good medium for potential production of PUFA using marine yeasts. The study provides a simple method of SSF of corn powder using marine oleaginous yeasts. Results indicate that SSF offers favorable cultivation environment for simultaneous enrichment of corn powder with PUFA, pigments and proteins using marine oleaginous yeasts. This value added bioproduct may be used as low cost feed supplement and may create new perspectives for PUFA and protein rich cereal based balanced feed production

Outcome of study

- **Isolated Marine yeasts for PUFA production.**
- **Potential marine yeasts identified by molecular method; Gene sequences submitted to GeneBank**
- **Whey based medium formulated for production of PUFA using marine yeasts**
- **Optimized SSF method for production of PUFA enriched cornmeal**
- **Toxicological studies (28 days repetitive oral dose toxicity) conducted in Wistar rat model demonstrated safety of biomeal**

Future Research

- Studies on biomeal as feed supplement for poultry
- To develop a rural technology for biomeal preparation (SSF system)

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PUBLICATIONS AND PRESENTATIONS

Publications

- Jape Anuradha, *et al* (2015); 'Enrichment of nutritional value of cornmeal with protein and PUFA using oleaginous marine yeasts in solid state fermentation', Int J Pharm Bio Sci; 6(1): (B) 1237 – 1245 ISSN NO.6299-0975.
- Jape Anuradha**,** *et al*; (2014); 'Modified Sudan Black B staining method for rapid screening of oleaginous marine yeasts', Int. J. Curr. Microbiol. App. Sci.3 (9) 41-46. (ISSN: 2319-7692 (Print) ISSN 2319-7706.

- Jape A. A., *et al*; (2016); Marine yeasts from coastal waters of Kokan (India), as a promising source of polyunsaturated fatty acids' Asian Journal of Multidisciplinary Studies 4(3):122-3126.

Oral Presentations

- Jape A. A., "Prospecting marine yeasts for PUFA production", at DST sponsored National Conference on "Bio Engineering Sciences, Present Status and Future Perspectives (NCBES-13)",organized by College of Engineering Sciences, Pune. Date 15/03/2013 to 16/03/13.
- Jape A. A., "Poly Unsaturated Fatty Acid production using whey by marine *Candida tropicalis*" at "Third Global Sustainable Biotech Congress: An International Conference on innovations in Biotechnology and their applications, (GSBC 2014)", organized by CETYS University Mexico, and Global Biotech Forum, Nagpur with NMU, Jalgaon. Date 01/12/1014 to 05/12/ 2014

Poster Presentations

- Jape A. A., "Bring back poly unsaturated fatty acids to enrich human food chain by PUFA enriched feed for poultry and dairy animals' at "International Conference on Herbal and Synthetic Drug Studies (HSDS-2016)". The Kansas University Medical Center, Kansas City, USA. M.C.E. Society's Interdisciplinary Science and Technology Research Academy (ISTRA), Abeda lnamdar Senior College (Post Graduate Department of Chemistry and Research Center) & Allana College of Pharmacy, Azam Campus, Pune-411 001 Maharashtra (India). Date 07/01/2016 to 09/01/ 2016
- Jape A. A., "Molecular identification of marine *Rhodotorula* yeast and its spectroscopic analysis establishes unsaturated fatty acid accumulation"; at "ICAAM 2016: Asian Medicine- Global Health; organized by Society for Ethnopharmacology associated with International Society for Ethnopharmacology and Poona College of Pharamacy, BVDU, Pune. ", Date 3/01/2016 to 07/01/2016 (BEST POSTER AWARD)
- Jape A. A.; "A Pragmatic Approach 'Biotechnological method for production of novel functional cereals"; at International seminar on "Pharmaceuticals to Neutraceuticals", Organised by SPPU, Sinhagad College of Pharamacy, Pune. Date 15/01/2016-16/01/2016 (BEST POSTER AWARD)
- Jape A. A. "Marine yeasts from coastal waters of Kokan (India), as a promising source of polyunsaturated fatty acids" at National Seminar on Current trends in Biodiversity conservation and climate change, organized by Yashwantrao Mohite College of Arts, Science and Commerce, BVDU, Pune. Date 24/01/2016 to 25/01/2016