**Qualitative and Quantitative Analysis of Lipids Produced By Oleaginous Yeasts.**

**Introduction:** There always has been a high demand for lipid products for a variety of uses. Lipids have been of great importance biologically and otherwise. Lipids obtained from natural resources like fossil fuels, have been used since ancient times to satisfy man’s needs. Uses of naturally occurring oils which are to be used in the different aspects of biology are not always eco-friendly and contribute greatly towards chemical pollution. Lipids extracted from microbial sources, have proven to be as useful and functional as the conventional lipids. Usage of microbial lipid products for experimental purposes, in place of conventional organic lipids may contribute to the decrease in the demand for natural lipids. Microbial lipids are thus, fast gaining importance in recent markets with the increase in technology and development. Microbial lipids are far more easily extracted than their naturally obtained counterparts. Also, they exert less pressure on the environment for its digestion and degradation.

**Origin of the research problem:** Excessive use of naturally occurring lipids in turn causes a strain on the resources, thereby depleting them. To overcome this problem, scientists have proposed the use of microbial lipids. Studies have shown that, microbial sources such as bacteria, fungi and algae have proved to be an excellent source for lipids [15]. Out of these, oleaginous yeasts, are selected as model organisms for lipid production as they are said to accumulate triacylglycerol (TAGs) as cellular storage lipids, sometimes above 80% of their biomass [11]. A lot is known, about oleaginous yeasts and their lipid accumulating properties. But, successful extraction of these lipids and quantification of the same has not been worked upon. Qualitative confirmation and quantitative study of the produced lipids can in turn help us in optimizing the lipid production in yeasts by exploiting the different modes of action [2].

**Interdisciplinary Relevance:** A lot of work has been done in the past 30 years, on oleaginous yeasts, to maximize the lipid production. Every field of life sciences can be greatly benefitted, if microbial lipids are used in place of naturally occurring lipids. The process involved in the production of microbial lipids is way cheaper than compared to that of the conventional lipids. The final by products formed during this process, are not hazardous to the environment. These reasons prove that usage of microbial lipids from oleaginous organisms is an easy, eco-friendly and a cheaper method, which is applicable without creating any significant difference in the efficacy of the procedure involved [12].

**Rationale:** The experiment is designed in a particular order which will help us to methodically determine maximum extraction of oils produced by *Yarrowia lipolytica* NCIM 3590 and its estimation by colorimetric and gravimetric methods. Further experimentation can be carried out to reduce the scale of experiment to a microfluidic level.

* **Significance of the Study**: Large scale production of microbial lipids and other biologically important products can lead to successful replacement of the use of conventional lipids. Over use of conventional oils can thus be reduced and hence, be used sparingly.

Qualification and quantification of microbial oils produced by oleaginous yeasts will help us in selection of the optimum extraction and estimation procedure. This will in turn contribute in getting a better understanding of oleaginous yeasts and their oil production.

**Literature Review:** Oils produced by oleaginous yeasts have been in the limelight for a long period of time. Scientists had discovered the importance of these microbial oils way back in 1960's. The fact that yeasts can generate lipids from different carbon sources; have paved way for a broad range of research work [1]. The concept of use of oils produced by oleaginous micro-organisms as the supplementary sources of conventional oils and fats is relatively old. It has been attempted since the 1980s from bacteria, fungi and yeast and was also reported to be the convenient substitutes for conventional oils because of their ability to accumulate more than 60% lipids resembling the plant and animal oils. The exact biochemistry which determines the amount of lipids to be produced and stored in the cell structures of oleaginous yeasts is discussed by Ratledge C. The biochemistry of oleaginicity is discussed elaborately in the document [18]. These yeasts are also known for their ability of producing lipids in varying amounts in presence of different fatty acids and triglycerides which are present in the culture medium. The definitive pathway used by oleaginous yeasts has been traced, and the exact mechanism of oil production and lipid accumulation in them has been known [3].

One such oleaginous yeast, *Yarrowia lipolytica* is one of the most extensively studied non-pathogenic yeasts, being a strictly aerobic microorganism and capable of producing important metabolites along with an intense secretory activity. This study justifies the efforts to use it in the industrial field, in molecular biology and in genetics’ studies [5]. *Yarrowia lipolytica* has been in use, still is deployed in, or is considered for multiple industrial applications, like (i) as a high-quality protein source for livestock feeding, (ii) as a biotechnological production host for organic acids (citric acid) or hydrophobic substances such as polyunsaturated fatty acids (PUFAs) or carotenoids (iii) as a heterologous production host for pharmaceutical and industrial proteins and enzymes (iv) for the mass production of biofuels, as well as (v) for bioremediation purposes [10].

The fermentation process of *Yarrowia lipolytica* is opted more than other oleaginous organisms because of its economic feasibility. Here, the cost of the raw materials depends on the ratio of the lipids produced per amount of carbon source used, whereas the cost of the fermentation process is based on the ratio of produced lipids [2].  The total lipid content of biological samples is an important quantity used in many biochemical, physiological, and nutritional studies. Thus, reliable methods for the quantitative extraction of lipids from tissues are of critical importance. Isolation, or extraction, of lipid from tissues is performed with the use of various organic solvents. The alkane - assimilating *Yarowia lipolytica*, degrades hydrophobic substrates such as n-alkanes, fatty acids, fats and oils for which it has specific metabolic pathways. It utilizes these substrates for production of cellular lipids. A study has been done which includes the interaction of hydrophobic substrates with yeast cells, their uptake and transport, the primary alkane oxidation to the corresponding fatty alcohols and then by different enzymes to fatty acids, and the subsequent degradation in peroxisomal b-oxidation or storage into lipid bodies [11].

The techniques involved in the extraction and estimation of oils produced, are standardized by a variety of institutes who focus on this topic for research. The vanillin assay, which is widely used for the quantification of microbial lipids and is one of the simplest, precise and accurate spectrophotometric methods to quantitate lipids from oleaginous yeasts [4]. This has also been a topic for research, way back in 1972, which dealt with serum lipids [8]. Another colorimetric assay, which uses a more rapid and linear approach to quantification of lipids, dates back to 1963 [7]. For extraction of lipid bodies from the yeasts, methods like Bligh & Dyer’s method and Folch’s Method are used. The primary advantage of the Bligh and Dyer method is a reduction in the solvent/sample ratio The Folch method employs a ratio of 1 part sample to 20 parts 2:1 chloroform/methanol, followed by several washings of the crude extract. Despite this solvent reduction, the Bligh and Dyer method is nevertheless thought to yield recovery of ≥95% of total lipids. The samples of known high lipid content were greatly underestimated using the Bligh and Dyer method compared to the Folch method, although no difference is detected in the fatty acid composition under either method. The Bligh and Dyer method has undergone rigorous and favorable evaluations, and virtually all of these evaluations have been performed on samples containing less than 1.5% total lipid. This study concludes that Folch method and Bligh and Dyer's method gave results within a close range with some or no deviation [12].

Detection of intracellular lipid bodies in these yeasts, done by densitometric scanning of the dry cell biomass is discussed in the following study. *Yarrowia* is known to produce both intracellular and extracellular type of lipids, which makes it a model organism for qualitative and quantitative analysis [9]. A staining technique, commonly used for the detection of intracellular lipid bodies, is known to be a rapid, qualitative method. Fluorescent staining, by Nile Red stain, is widely used for detection purposes. However, it is only applicable to a limited number of organisms. Research has been done, to broaden the range of oleaginous organisms which can be subjected to fluorescence staining. A study has been performed, which work in this direction, which gives a high success rate [14]. The quantitative estimation of cellular lipids by fluorescent techniques, is fast gaining importance as more number of organisms are been subjected to this methodology. A popular research of lipid estimation, involving microalgae is done which can be standardized and applied to oleaginous yeasts [25].

Often *Saccharomyces cerevisiae*, commonly known as Baker’s Yeast, is taken up as a parallel in any experiment in opposition to oleaginous yeasts. It is said to show no cell surface hydrophobicity, no bioremediation properties and is not of oleaginous nature. It functions as a control in most of the oleaginous experiments. However, this study involves the lipid extraction from *S. cerevisiae*. It indicates that the presence of lipid particles in *S. cerevisiae* are equal amounts of triacylglycerols and sterol esters and the more unsaturated character of the fatty acids in the sterol esters may indicate that these particles serve as a store not only for energy production but also for membrane synthesis [6].

Release of hydrocarbons in water reservoirs, due to the activities held in petrochemical industries, is of major concern in the environment. Hydrocarbon components have been known to belong to the family of carcinogens and neurotoxic organic pollutants. Mechanical and chemical methods generally used to remove hydrocarbons from contaminated sites have limited effectiveness and can be expensive. Due to its ability to degrade, assimilate as well as produce lipid bodies for its growth and survival, *Yarrowia lipolytica* and its strains have been the prime focus of bioremediation studies and are being used as promising agents for treatment of contaminated areas [9].

This bioremediation technology is believed to be non-invasive and relatively cost-effective. Crude oil, one of the most important hydrocarbons, which is a biological pollutant, is degraded by *Yarrowia lipolytica* by biodegradation. The potential degradation of crude oil by *Yarrowia lipolytica* IMUFRJ 50682 has also been evaluated [13].

A number of oleaginous yeasts from different sources have been studied for production of Single Cell Oils (SCO) and Poly Unsaturated Fatty Acids (PUFA). The concept of single cell oil produced by oleaginous microorganisms as the supplementary sources of conventional oils and fats has attracted attention since the early 1980’s. Oleaginous yeasts are often considered for the production of single cell oil. The economics of these bioprocesses has become more favourable when zero or negative value waste substrates are utilized as carbon or nitrogen sources. *Yarrowia* is one such organism which is worked upon for production of Single Cell Oils. However, the medium required for the cultivation of these oils are costly and tend to produce a lot of by products. But efforts are taken to minimize the waste substrates produced and reduce the cost of the media involved [15].

To further, reduce the cost of cultivation media a study is done that shows oleaginous yeasts cultivated from lignocellulosic wastes. Lignocellulosic wastes from *Arundo donax* (AR) and *Sorghum bicolor* (SB) were used as a source of fermentable sugars for culturing the oleaginous yeasts *Lipomyces starkeyi*. The only growth condition modified was that a high ratio between the Carbon: Nitrogen ratio was maintained. This treatment made possible the growth of yeasts in the presence of raw hydrolysates, thus improving the production of microbial oils from oleaginous yeasts, potentially allowing a sustainable production of biodiesel. Several oleaginous yeasts can be grown in the presence of hydrolysates of lignocellulosic materials, as they are able to metabolize both hexose and pentose sugars [17].

It has been known that certain yeasts are known to produce higher concentrations of lipids when grown with an organic, rather than an inorganic, nitrogen source. A study was performed, which explains the effects of nitrogen sources on the lipid content of various yeasts. From such a study, a deeper understanding of the biochemical processes and control mechanisms operating in oleaginous yeasts can be sought and then can be used to advantage [16].

An interest in the production of SCO and PUFA from alternative sources for use in aquaculture feeds and human nutraceuticals has fuelled recent research into the molecular biology of PUFA production. The report also involves the screening of bacterial associates of marine sponges for SCO production. A key advantage of bacterial PUFA production is that only a single PUFA is produced, rather than the complex mixture yielded from fish or algal oils with convenience for the production of high-purity PUFA oils [18].

Another way of handling this problem is by modifying the genotype of the organism to increase the capacity of storage and production of lipids in the cell. *Y. lipolytica* can use fatty acids and alkanes as carbon sources. This yeast secretes emulsifiers i.e. liposan, modifies its cell surface hydrophobicity, stores lipids in specifically developed organelles, and uses a special set of enzymes to store and use alkanes or lipids as carbon sources. The β-oxidation pathway, which is heavily involved in the utilization of these substrates, is a common target for modifying the ability of *Y. lipolytica* to use lipids and alkanes as carbon sources. Genetic engineering can therefore be used to improve the ability of *Y. lipolytica* to store and utilize lipids. A modification of the genotype could be a useful alternative to the modification of growth conditions and medium composition that was previously used to improve intracellular lipid accumulation in *Y. lipolytica*. This study involves the strain MTLY36-2P of *Yarrowia lipolytica* which is found to be a good candidate for single-cell oil production [19].

Other species of *Yarrowia* are now being screened for the production of lipid production as the chances of limited phenotypic diversity are higher. It has been found that there are some species in the *Yarowia* clade, which show traits of being oleaginous [22].

Production of biodiesel from oleaginous yeasts is yet another novel research, which has emerged in the recent years. Biodiesel has received increasing attention as a result of globally rising crude oil prices, increasing carbon dioxide emissions, and growing expectations that biomass will be utilized instead of wasted. Biolipids, including triacylglycerol, produced by oleaginous yeasts have been confirmed to be among the most effective raw materials for biodiesel production. *Cryptococcus terricola*, an oleaginous yeast, which belongs to the basidiomycete family is frequently isolated from soils. Several reports have been published on lipid production by *C. terricola*. In this study, the oleaginous yeast *C. terricola* is said to directly assimilate soluble starch and thereby accumulate a considerable amount of lipids. The results provide a novel process for lipid production from starch biomass. More importantly, a system that does not require enzymatic hydrolysis would encourage further development in practical biodiesel production [24].

1. **International Status**: Efforts have been taken on a global scale, to know more about the oleaginous yeasts. These yeasts have been taken up as desired organisms to study intracellular and extracellular lipid production [3, 14, 19]. They have also been tested for the production bio-oils and bio-diesel which can be used as fuels, thus replacing the conventional fuels [13]. More and more research labs worldwide are taking interest in the lipid producing quality of yeasts. Scientists are coming up with novel ideas as to where these oils may have an application [5].
2. **National Status**: Indian research, to meet the ever – increasing demand for oils, have also tried to make use of microbial oils. The Regional Research Laboratory, India, has been working on Polyunsaturated fats **(PUFA)** produced by marine organisms [16]. Various techniques have been developed for the rapid screening of oleaginous yeasts. To tackle the environmental problems of hydrocarbon contamination in water bodies by the petrochemical industries, VIT Institute, Tamil Nadu. India also has been working on the microbial degradation of petroleum [5]. Nationally recognised institutes like Indian Institute of Technology and Indian Institute of Chemical Technologyhave been carrying out definitive projects in collaboration with renowned colleges, to encourage students to take up studies on oleaginous organisms [27].

**Aim**: To analyze the Lipids produced by Oleaginous Yeasts Qualitatively and Quantitatively.

**Objectives:**

a. To grow and maintain *Yarrowia lipolytica* NCIM 3590 on Maltose - Glucose - Yeast Extract - Peptone Media.

b. To extract the lipids produced by *Yarrowia lipolytica* NCIM 3590 by using various lipid extraction methods including gravimetric methods [11, 17, 19].

c. To confirm the production of lipids by the yeast by qualitative methods [6].

d. To analyse the extracted lipids by chromatographic techniques.

e. To quantify the amount of lipids produced by the yeast using techniques like vanillin assay and other colorimetric assays [4, 7].

f. To determine the effect of chemical emulsifiers like Tween 80 on *Yarrowia lipolytica* NCIM 3590.

g. To carry out colorimetric techniques on a microfluidic scale to minimise the scale of the experiments.

**Methodology and Plan of Work:**

1. **Plan of Work:**

|  |  |
| --- | --- |
| Work to be done: | Month wise distribution: |
| 1. To grow and maintain *Yarrowia lipolytica* NCIM 3590 on Maltose - Glucose - Yeast Extract - Peptone Media.  2. To determine the optimum method for the extraction of lipids from the yeast including the gravimetric method. | July – August |
| 3. To confirm the production of lipids by the yeast by qualitative methods.  4. To analyse the extracted lipids by chromatographic techniques.  5. To quantify the amount of lipids produced by oleaginous yeasts using techniques like vanillin assay and other colorimetric assays. | September – October |
| 6. To determine the effect of chemical emulsifiers like Tween 80 on *Yarrowia lipolytica* NCIM 3590.  7. To differentiate and compare the intracellular and extracellular lipids produced by the organism. | November |
| 8. To carry out colorimetric techniques on a microfluidic scale to minimize the scale of the experiments. | December |

**B. Methodology:**

1. The Standard strain of *Yarrowia lipolytica* will be grown on Malt Extract - Yeast - Glucose - Peptone media and maintained. [14].

2. Confirming the production of lipids by the organism *Yarrowia lipolytica* by Sudan Black Staining. Lipid bodies will be seen through microscopy [14].

3. Different lipid extraction methods like Folch’s method, Bligh and Dyer’s method, Soxhlet’s method and Ratledge’s method will be performed to determine the method giving maximum extraction of lipids. [1, 9, 17].

4. Gravimetric analysis of the extracted lipids will be performed for total extraction of the lipids [19].

5. Using techniques like HPLC and MS-GC for categorical analysis of the extracted lipids [12, 19].

6. Assays like Sulfo-phospho-Vanillin assay and colorimetric microdetermination for the qualitative and quantitative estimation of the extracted lipids will be performed [4, 6, 7].

7. Determination of the effect of chemical emulsifiers like Tween 80 on *Yarrowia lipolytica* NCIM 3590.

8. Colorimetric lipid estimation assays on a lab-on-paper scale to reduce the scale of the experiment.

**Expected Outcome**: After confirming the lipid production, extraction of lipids produced by *Yarrowia lipolytica* by using methods like Folch and Bligh and Dyer’s Method, will help to determine the optimum method of lipid extraction. The qualitative and quantitative analysis of microbial oils will provide a broader perspective in terms of the production of microbial oils under optimum conditions and will help in the standardisation of the extraction and estimation of the same. Using this data, further experimentation can be carried out, which will help in the better understanding of lipid production in *Yarrowia lipolytica*.

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* **Budget:**

1. **Consumables and Chemicals:**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Sr. No.** |  | **Media** | **Quantity** | **Price** |
|  |  | MGYP Media |  |  |
| 1 |  | Malt Extract | 100 | 338.00 |
| 2 |  | Glucose | 200 | 68.00 |
| 3 |  | Yeast Extract | 100 | 112.00 |
| 4 |  | Peptone | 150 | 357.00 |
| 5 |  | Agar | 300 | 510.00 |
|  |  | **Chemicals** | **Quantity** | **Price** |
| 6 |  | Sulphuric Acid | 150ml | 142.20 |
| 7 |  | Phosphoric Acid | 200ml | 300.00 |
| 8 |  | Chloroform | 100ml | 55.00 |
| 9 |  | Methanol | 50ml | 7.82 |
| 10 |  | Acetic Acid | 20ml | 97.60 |
| 11 |  | Copper Nitrate | 30mg | 158.10 |
|  |  | **Staining** |  |  |
| 12 |  | Crystal Violet | 50ml | 16.00 |
| 13 |  | Alcohol | 100ml | 25.00 |
| 14 |  | Sudan Black B | 50ml | 263.76 |
| 15 |  | Safranine | 50ml | 15.00 |
|  |  | Total |  | 2465.28 |

|  |  |  |  |
| --- | --- | --- | --- |
| **Sr. No.** | **Reagents** | **Quantity** | **Price** |
| 1. | Vanillin | 25gm | 393.60 |
| 2 | Olive Oil | 10ml | 115.00 |
| 3 | Palmitic Acid | 10ml | 55.00 |
| 4 | Oleic Acid | 20ml | 60.00 |
| 5 | Glycerol | 20ml | 50.00 |
| 6 | Triethanolamine | 50ml | 739.00 |
| 7 | Diethyldithiocarbamate | 50gm | 774.00 |
| 8 | Tween 80 | 5ml | 20.00 |
| 9 | Palm oil | 10ml | 75.80 |
| 10 | Castor Oil | 10ml | 20.00 |
| 11 | Butanol | 50ml | 408.00 |
|  | Total |  | 2710.40 |

1. **Glassware:**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Sr No.** | **Glassware** |  | **Capacity/size** | **Amount used** | **Price (Rs.)** |
| 1 | Tubes | Dilution tubes | 25 ml | 50 | 150.00 |
|  |  | Test tubes | 15 ml | 20 | 80.00 |
|  |  | Suspension tubes | 7 ml | 10 | 50.00 |
|  |  | Sugar tubes | 30 ml | 20 | 360.00 |
| 2 | Pipettes |  | 1 ml | 5 | 720.00 |
|  |  |  | 10 ml | 5 | 984.00 |
| 3 | Micropipettes |  | 2 - 20µl | 1 | 5750.00 |
|  |  |  | 100µl | 1 | 5750.00 |
|  |  |  | 1000µl | 1 | 5750.00 |
| 4 | Plastic measuring cylinder |  | 100 ml | 1 | 150.00 |
| 5 | Glass Conical Flask |  | 250 ml | 10 | 242.20 |
|  |  |  | 500 ml | 10 | 605.00 |
| 6 | Petri plates |  |  | 50 | 1000.00 |
| 7 | 96 well Microtiter Plate  (127mm x 86mm) |  |  | 1 | 90.00 |
| 8 | Side Arm Flasks |  | 250ml | 2 | 90.00 |
| 9 | Beaker |  | 250ml | 1 | 100.00 |
| Total |  |  |  |  | 21,871.20 |

1. **Others:**

|  |  |  |  |
| --- | --- | --- | --- |
| **Sr. No.** | **Other Products** | **Capacity** | **Price (Rs.)** |
| 1 | Cotton | - | 85.00 |
| 2 | Disinfectant | 100ml | 30.00 |
| 3 | Water Can | 5L | 20.00 |
| 4 | Distilled Water | 10L | 200.00 |
| 5 | Whatman Filter Paper No. 1 | 50cm x 50cm | 294.00 |
| 6 | pH Paper (2-8) | 10 | 145.00 |
| 7 | pH Paper (5-7.5) | 10 | 140.00 |
| 8 | Falkon Tubes | 25ml | 140.00 |
| 9 | Eppendorf’s Tubes | 1.5ml | 75.80 |
| 10 | Electricity | - | - |
| 11 | Space | 6.5 sq. feet | 1,62,500 |
| 12 | Computer Space + other stationary |  | 800.00 |
| 13 | Cartridges |  | 100.00 |
| 14 | Travelling |  | - |
| Total |  |  | 1,64,529.80 |

1. **Instruments:**

|  |  |  |  |
| --- | --- | --- | --- |
| Sr. No. | Name of the instrument | Manufacturer | Cost (Rs.) |
| 1 | Autoclave | Equitron | 48000 |
| 2 | Cyclomixer | Remi equipment | 6700 |
| 3 | Cooker | Butterfly | 1200 |
| 4 | Digital weighing balance | Contech | 28,000 |
| 5 | Hot air oven | Classic scientific | 25,000 |
| 6 | Microwave oven | Electrolux | 5500 |
| 7 | Refrigerator | Whirlpool classic | 22,200 |
| 8 | Incubator | Techno instrument | 35,000 |
| 9 | Shaker | Orbitek | 25,000 |
| 10 | Deep freeze | Krispcald | 48,000 |
| 11 | Small lab centrifuge | Remi equipment | 15,000 |
| 12 | Biosafety cabinet | Micro – filt (India) | 80,000 |
| 13 | Laminar air flow | Micro – filt (India) | 40,000 |
| 14 | Digital colorimeter | Systronics | 8500 |
| 15 | iMark Plate Reader | BioRad | 2,30,000 |
| 16 | Vortex | Remi | 2,000 |
| 17 | Sonicator | Probe | 8,000 |
| 18 | Compound / Light Microscope | Advance | 6,000 |
| 19 | Phase Contrast Microscope | Motic | 5,50,000 |
| Total |  |  | 11,92,100 |

1. **Infrastructure facilities**

|  |  |
| --- | --- |
| Lab space and furniture | 6.5 sq.ft working tables with gas connection |
| Water and electricity | Uninterrupted supply |
| Computer and documentation | 50 PC units with internet facility |
| Instrument room | No |
| Library periodicals | No |
| Telecommunication | No |
| Transportation | No |
| Animal house | No |

**Summary**:

|  |  |
| --- | --- |
| Instruments | 11,92,100 |
| Media | 1385.00 |
| Chemicals | 760.72 |
| Reagents | 2710.40 |
| Stains | 319.76 |
| Glassware | 21,871.20 |
| Miscellaneous | 1,64,529.80 |
| Total | 13,83,676.88 |