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PROBIOTIC *Rhodotorula mucilaginosa* ISOLATED FROM FERMENTED FOOD: INVESTIGATION OF PUFA PRODUCTION AND STRATEGY FOR HEALTH IMPROVEMENT

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Polyunsaturated fatty acids (PUFAs) are a vital component for human health. PUFA cannot be synthesized by human system and hence dependance on other sources has become inevitable.
Though porcine liver and fish oil were the dependable sources for ages past, yet cheaper microbial source was sought after and have gained importance as well. Such microbial oils normally used as biofuels can also be used for several therapeutic purposes. Hence, this study was designed to evaluate the quality and quantity of lipids produced by a probiotic yeast, *Rhodotorula mucilaginosa.* The lipid production potential of *Rhodotorula mucilaginosa* has indeed attracted a lot of attention. Isolation and characterization of the probiotic yeast with enhanced lipid production and determining the lipid components have become the aim of the work.

Keywords: *Rhodotorula mucilaginosa*; single cell oil; lipid; oleaginous.

1. INTRODUCTION

"Everybody requires fat for energy and other functions. Among fats, Polyunsaturated fats are a healthy choice. Eating healthier fats can lead to certain health benefits [1]. It is not enough to add foods high in unsaturated fats to a diet filled with unhealthy foods and fats". "Instead, replacement of saturated or trans fats with healthier fats is a good suggestion. Overall, eliminating saturated fats is twice as effective in lowering blood cholesterol levels as increasing polyunsaturated fats" [2]. "Polyunsaturated fatty acids (PUFAs) are fatty acids that contain more than one double bond in their backbone. This class includes many important compounds, such as essential fatty acids and those that give drying oils their characteristic property. In recent years it has been demonstrated that PUFA are regulators of lipid metabolism, and have anti-inflammatory and anticancer effects" [3].

"Microbes are also known to produce lipids and are called as microbial oils. Microbial oil accounts for the lipids produced by microorganisms including bacteria, yeasts, molds and algae that can produce and accumulate lipids upto 20-40% of the cell weight" [4]. Among the several microbes that have been explored for lipid production, the probiotic yeast, *Rhodotorula* sp. had also been explored to determine its lipid production ability [5,6]. Probiotics are live microorganisms that are intended to have health benefits when consumed or applied to the body. It is

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reported that microbial lipids have been preferred for its low toxicity and easy biodegradability. It is also highly preferred for its low SO2 emission when used as fuel in motor vehicles [7]. Yeast *Rhodotorula* is a wide spread saprophytic organism that can be isolated from several commonplaces such as, soil, fresh water, air and food samples.

"The efficiency of microbes to produce several metabolites as lipids (Single Cell Oil), proteins (Single Cell Protein) and pigments creates a demand for the use of such microbes in various industries" [8]. It is observed that lipid content and lipid profile differ between species and hence this study was undertaken in our college laboratory with an aim to evaluate the efficiency of lipid production by *Rhodotorula mucilaginosa* isolated from rice water. Owing to the above mentioned advantages, microbial oil is more superior to oil derived from other sources.

2. MATERIALS AND METHODS

2.1 Potato Dextrose Agar

Potato Dextrose agar- PDA contains dextrose as a carbohydrate source which serves as a growth stimulant and potato infusion that provides a nutrient base for luxuriant growth of most fungi. PDA- 3.9 g, Agar agar-0.8 g, 100ml of distilled water.

2.2 Rose Bengal Agar Medium

Rose Bengal agar- Is a selective medium to detect and enumerate yeasts and molds in food samples. RBA- 3.15 g, Agar- 0.8 g, 100ml of distilled water.

2.3 Liquid Media-Basal Broth

Basal Broth: 2g - Glucose, 0.4g -Yeast extract, 0.1g - KH_2Po_4, 0.05g - $MgSO_4$, 100ml distilled water.

2.4 Sample Collection

Rice water was obtained after draining the water from soaked rice for 30minutes. The probiotic yeast (*Rhodotorula mucilaginosa)* was isolated from the rice water sample by serially diluting (upto a dilution of $10^{-8)}$) and plating on PDA or RBA plates. The plates were incubated at 28±2ºC for 2 days and observed for yeast colonies [9]. Single colonies from the agar plate were selected and picked up and sub-cultured on to solid medium. Only colonies that exhibited orangish pink colour were chosen.

2.5 Identification of the Local Isolate

The primary identification of *Rhodotorula sp.* was done based on its pigmentation and morphology. Further confirmation was based on Gram staining and biochemical tests [10].

2.6 Maintenance of *Rhodotorula mucilaginosa* Strains

Purified isolated strains of *Rhodotorula mucilaginosa* were streaked on PDA/RBA medium. After a growth period of 2 days at room temperature, colonies of *Rhodotorula mucilaginosa* strains were stored at 4 ºC in a refrigerator. Subculturing of the strains was done every two months.

2.7 Preparation of Inoculum

A loopful of culture isolated from rice water was streaked on RBA plates and the colonies from RBA plate was introduced to 50 ml inoculation broth medium (Basal broth) in 250 ml flask. The culture was incubated at room temperature in shaker at 180 rpm for 48 hrs [11].

2.8 Molecular Characterization of the Isolate

Though biochemical tests were performed, 18srRNA sequencing was performed to confirm the genus and species.

2.9 Determination of Single Cell Oil Content

A rapid protocol to isolate lipids from yeast was followed according to Pan et al. [11] with slight modifications. The dry weight of the isolated lipids was determined.

2.10 Determination of Single Cell Oil (SCO) Productivity

Single cell oil productivity of the local isolate, *R. mucilaginosa* strain was determined by the following equation, [12].

SCO productivity = SCO weight (g/L) / cell dry weight (g/L) × 100.

2.11 Effects of Different pH Values on Growth of *R. mucilaginosa* Strains and Single Cell Oil Production

To evaluate the effects of pH on the growth and productivity of the isolate, various ranges of pH from 5.0 to 8.0 were applied on fermentation medium.

2.12 Screening for PUFA Production by H_2O_2 Plate Method

Rose Bengal agar medium with catalyse inhibitor (Sodium Azide) 0.006g added to the medium was used for the assay. Swabbed the isolate on to the plate, sterile discs soaked with the different concentrations of hydrogen peroxide (10ul to 30ul) were placed. Incubated the plates at room temperature for 12 to 72 hrs. to observe the zones of inhibition. Absence of zone formation confirms PUFA producer.

2.13 GC-MS Analysis of the Lipids Extracted from the Isolate

The lipids from *R. mucilaginosa* were subjected to the GC-MS analysis to determine the components of the lipids. Chromatography by Gas Mass Spectroscopy is an analytical technique for identifying various substances inside a test sample that incorporates the features of gas chromatography. Since it is used to conduct a 100 percent specific test that positively identifies the existence of a specific drug, GCMS has been recognized as the gold standard for forensic substance detection.

3. RESULTS

3.1 Isolation of Pigmented Yeast Strains

Among the several microbes isolated from rice water, only two colonies were pigmented (pink and yellow respectively). On microscopic examination, the pink colony demonstrated the typical morphology of yeast on Gram staining, which was in accordance to the report by Sakaki et al. [13]. Thus, the pigmented yeast was isolated from rice water.

3.2 Identification of the Pigmented Yeast Isolates

The isolated strain subjected to biochemical tests revealed the isolate to possess the characteristics of *R. mucilaginosa.* The cells under microscopic examination were ovoid to globule and grew rapidly after 24 hr. Though the optimum growth temperature was 30ºC, the isolates could grow well even at 37ºC.

3.3 Growth in Various Media

The yeast, *R. mucilaginosa* was grown in various media differing in carbon sources, and the growth was determined by the biomass obtained after 72 hrs of growth. The biomass of the yeast in various media as basal broth, carrot juice, potato juice and beetroot juice were 73g/l, 28.5g/l, 62.5g/l and 128.5g/l respectively.

3.4 Lipid Yield

Lipids from *R. mucilaginosa* were extracted using the protocol mentioned earlier and were quantified. It was observed that the efficiency of the isolate grown in basal broth was 57g/l.

SCO productivity = SCO weight (g/L) / cell dry weight (g/L) × 100.
= 57 / 73 X 100 = 78 %

3.5 GC-MS Analysis

To be aware of the components in the lipid sample from *Rhodotorula sp.,* GC MS analysis was performed. The presence of lipids in the chromatogram confirmed lipid production by *Rhodotorula sp*. The major fatty acid components identified were hexadecanoic acid, methyl ester, saturated lipid, 9-octadecenoic acid and methyl ester, omega -9- fatty acid (Oleic acid).

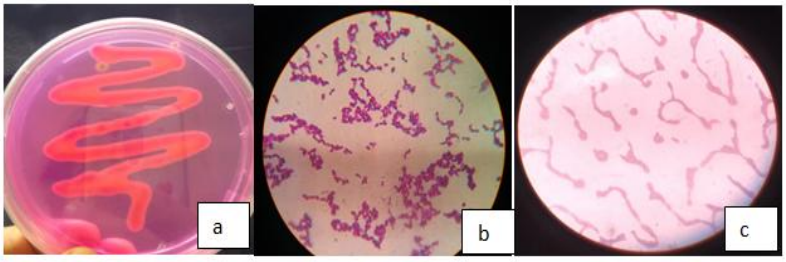

Fig. 1. a: *Rhodotorula sp.* isolated on RBA plate, b & c: Gram staining

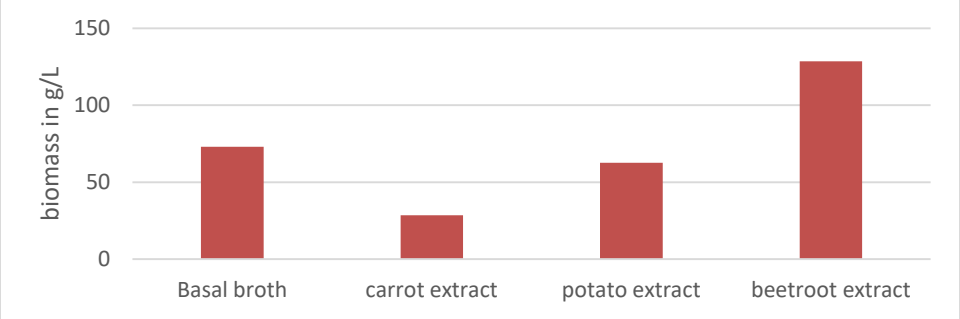

Fig. 2. Biomass yield of *Rhodotorula sp*. in different carbon source

50 ml of culture | 10 ml culture-centrifuged | Acid hydrolyzed | Lipid formation

Fig. 3. Lipid extraction from *Rhodotorula sp.*

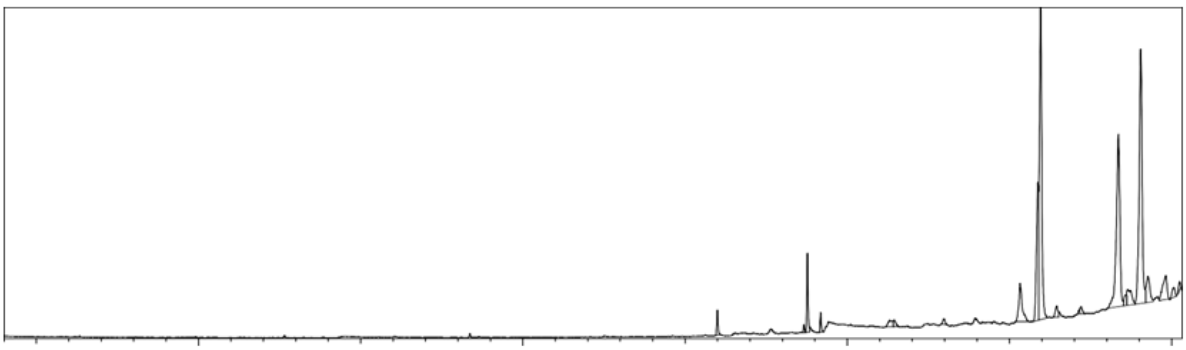

Fig .4. Chromatogram of the lipids isolated

Table 1. White Lipid profile of *Rhodotorula sp*. by GC MS analysis

Peak	Retention	Area	Area (%)	Name of compounds
1	26.037	376859	3.89	Hexadecanoic acid, methyl ester
2	28.82	782122	8.06	9-Octadecenoic acid, methylester, (E)-
3	28.913	2300369	23.72	stigmast-5-en-3-ol, (3. beta.,24s)-
4	33.595	1362288	14.05	4- Campestene-3-one
5	35.53	382265	3.94	24(S)- Ethyl-3.alpha.,-cyclocholest-22(E)-en-6-one
6	36.246	594537	6.13	silane, (9,19-cyclo-9.beta,-lanost-24-en-3.beta,-yloxy) trimethyl
7	38.235	178192	1.84	2-ethykidene-1,3—bis[(e)-2,4,6- trimethylphenyl methylene] cyclopentane
8	38.416	2826096	29.14	gamma,-sitostenone
9	38.48	666227	6.87	cholest-4-en-24-ol-3-one
10	39	230401	2.38	cyclononasiloxane, octadecamethyl

4. DISCUSSION

All microbes have the ability to produce lipids, which is an essential component of the cell membrane and organelles [14]. Though several microbes synthesize lipids for their survival and as part of their cellular component, yet very few of them produce lipids in excess that is accumulated as reserve storage material. Several genera such as *Cryptococcus, Cunninghamella*, and *Mortierella* are known to produce lipids with high amounts of PUFAs [15]. It is known that excess carbon triggers the production and accumulation of lipids and hence the use of cheap carbon source could enhance lipid production. This was also supported by increasing lipid production by employing use cheaper alternate raw materials, such as glycerol, sugars and plant residues [16,17,18,19,20,21], Zeng et al., 2013. Among the lipids, PUFA are of great significance, since they play a vital role in human health [22]. The supply of essential fatty acids in the diet is essential since mammals lack the ability to synthesize them [23]. The production of PUFAs in SCO comparable to plant oil makes them to be very valuable resources with additional merits of reduced time for culture and high purity of the lipids. Production of high content of lipids upto 40% using wheat straw and rice straw by *Cryptococcus curvatus* and *Tricosporon fermentens* was reported by Yu et al. [24] and Huang et al. [25] respectively. When compared with earlier reports, it is found that the *R. muciloginosa* strains from rice water used in this study produced higher biomass and lipid yield than those reported in literature, which suggests the need to identify the pathway and enzymes involved in enhanced lipid production. The enhanced lipid with high PUFA content could be commercialized in future as microbial oils [26-28]. Oils derived from microorganisms, alternatively known as single-cell oils (SCOs), are similar in composition to vegetable oils and animal fats [29-32]. However, single-cell oils are preferred to plant- and animal-derived oils because it is easy to scale up their production [33].

5. CONCLUSION

The work performed demonstrated the ability of *R. mucilaginosa* from rice water to yield maximum lipid content 57 g/L with a productivity of 78%. It is hence recommended that identifying the underlying mechanism may pave way for strain improvement for even a higher yield of microbial lipids, which could be used as microbial single cell oil.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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