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Lipid body formation by *Thraustochytrium aureum* (ATCC 34304) in response to cell age

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Abstract−The heterotrophic marine protist, *Thraustochytrium aureum* produces substantial amounts of polyunsaturated fatty acids (PUFAs). In the present investigation, changes in the lipid and fatty acid profiles of *T. aureum* were studied according to the culture age. *T. aureum* was grown in artificial sea water medium for 10 days at 25 °C in shake culture condition. One to 10 day old cell samples were analyzed for cell biomass production, total lipid content, fatty acid profile and lipid body formation. In all the samples tested, total lipid production was found to be directly proportional to the dry cell weight of *T. aureum*. In the early phase of cell growth, cell biomass production, lipid content and glucose consumption were found to be higher. Thin layer chromatographic analysis (TLC) of lipids showed the presence of triacylglycerol (TAG; 169 mg/g, 90%), phospholipids (PL; 83 mg/g, 66%) and sterol (ST; 6 mg/g, 5%), which were recorded at maximum levels in the early growth phase of the cells. The composition of PUFAs and saturated fatty acids (SFAs) of the cell biomass and lipid class components (TAG and PL) was identified by gas chromatographic analysis (GC). In the early phase of cell growth, production of PUFAs in the total fatty acids was found to have attained maximum levels (61.3%) in which docosahexaenoic acid alone showed higher content of occurrence (99.0 mg/g in total lipid; 65.2 mg/g in TAG and 41.0 mg/g in PL). In the middle phase of cell growth, palmitic acid production was found to be higher (36.7 mg/g in total lipid; 31.3 mg/g in TAG and 12.6 mg/g in PL). Transmission electron microscopic studies of the cells showed the presence of a membrane around the lipid bodies in the early phase of cell growth. TAG and PL were actively involved in the formation of lipid bodies in the cells of *T. aureum*. Large-sized lipid bodies accumulated in 3 day old cells which were then fragmented into smaller bodies in the late growth phase.

Key words: Lipid Synthesis, Lipid Body Formation, Culture Age, *Thraustochytrium aureum*, Triacylglycerol, Phospholipids, Fatty Acid Profile, Polyunsaturated Fatty Acids, Docosahexaenoic Acid

INTRODUCTION

Lipids and fats form a class of natural compounds that serve as sources of energy and are considered as important dietary constituents of our food. Lipids are indispensable for the growth and survival of all organisms. They are important structural components of membranes in many organisms and play a crucial role in energy storage. Fatty acids are widely known for their beneficial effects in the prevention and treatment of heart disease, high blood pressure, inflammation and certain cancers [1]. Natural sources of lipids include plants, animals and microorganisms. During the past three decades, an interest in long chain polyunsaturated fatty acids (PUFAs) obtained from fish oil has increased considerably, due to its beneficial health effects.

However, the production of PUFAs from fish oil has encountered many barriers such as low content of docosahexaenoic acid (DHA), undesirable fishy flavor, oxidation instability and long purification steps [1-3]. It is widely believed that the production of PUFAs from current sources will become inadequate for supplying the expanding market. In view of an increasing population and continued demand for industrial utilization, plant and animal sources alone cannot meet the total requirement. In order to meet the expected rise in demand and to circumvent the difficulties in producing PUFAs from fish oils, the discovery of alternate sources and production processes for PUFAs are very much essential. This will be achieved by the exploitation of microbial sources in the production of PUFAs [4], which may be offered as a future sustainable resource regarding industrial PUFA production.

Marine microorganisms belonging to achromatic stramenopiles such as thraustochytrids and labyrinthulids have been reported to produce significant quantities of PUFAs [5]. Recent studies have shown that some thraustochytrid strains can produce high biomass, containing substantial amounts of lipid rich PUFAs. It is also evident that cell yield and PUFA production by some *Thraustochytrium* spp. strains can be varied by manipulation of the physical and chemical parameters of the cultures [6]. Recent morphological studies have demonstrated that thraustochytrids contain distinct lipid bodies in which high levels of DHA are thought to exist [7]. Although the information on fatty acid production and its composition at different nutrient levels with reference to media optimization is available [8,9], only limited reports exist that address the production of different PUFAs and saturated fatty acids (SFAs) in respect to the age of the culture. Therefore, it is important to explore the relationship between lipid synthesis and lipid body formation in cell growth of *T. aureum*. The present investigation identifies the changes in lipid class, fatty acids production and lipid body formation in response to culture age.

EXPERIMENTAL

1. Culture Condition

In this study, the strain *Thraustochytrium aureum* (ATCC 34304)

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was used. Shaking culture (180 rpm) was performed in 500 ml Erlenmeyer flasks containing 200 ml of culture solution with 5% (v/v) inoculum at 25 °C for 10 days. The basal medium for growth optimization and seed culture contained (g/L) glucose, 10; yeast extract, 1; peptone, 1; NaCl, 24; $MgSO_4 \cdot 7H_2O$, 12; KCl, 0.75; $CaCl_2 \cdot 2H_2O$, 1; Tris, 1; Na_2EDTA, 0.012; $ZnSO_4 \cdot 7H_2O$, 0.002; $Na_2MoO_4 \cdot 2H_2O$, 0.001; $NaNO_3$, 0.04 and some components in tracers (g/L) which included thiamine-HCl, 0.01×10^{-3}; $NaHCO_3$, 0.1; vitamin B12, 1×10^{-6}; amino benzoate, 20×10^{-6}; calcium penthothenate, 10×10^{-6}; cyanocobalamin, 4×10^{-6}; $FeCl_3 \cdot 6H_2O$, 0.5×10^{-3}; $MnCl_2 \cdot 4H_2O$, 0.2×10^{-3}, $CoCl_2 \cdot 6H_2O$, 2×10^{-6} in 1 L artificial sea water with the pH adjusted to 6, which was essentially the same as medium described by Bajpai et al. [2] with some modification. Sampling was carried out every 24 h (from day 1 to day 10) to analyze the differences in dry cell weight, glucose consumption, lipid content, fatty acid profile and accumulation of lipids in cells of *Thraustochytrium aureum*. Culture age/Cell age was recorded for every 24 h from day 1 to 10 of the inoculated culture. It was a scale in order to mark the growth of cultured cells everyday to perform the above mentioned biochemical parameters.

2. Analytical Determination

Every 24 h the cultures were harvested and centrifuged at 3,000 rpm for 10 min in order to obtain the cell mass. The biomass (dry cell weight) was measured by weighing method. The glucose concentration was then determined by using a glucose analyzer (YSI 2700, Japan). Lipids were extracted from the dried cells [10] in order to identify the lipid class by TLC silica rod analysis [11,12]. Cell samples were esterified and different fatty acids from the total lipids and lipid class were characterized by gas chromatography [13,14].

3. Transmission Electron Microscopic Analysis (TEM)

Samples were prepared by centrifuging 1 ml of culture medium with cells at 3,000 rpm for 5 min. The pellets were suspended and washed thrice in 1 ml of 1 M phosphate buffer. Cells were first fixed in 3% glutaraldehyde for 4 h and then washed with 1 ml of 1 M phosphate buffer 3 times. The cells were again fixed with 1% osmium tetroxide for 30 min and washed 3 times in 1 ml of 1 M phosphate buffer [7]. Samples were then dehydrated with ethanol and embedded in spur resin (Epon-Poly/Bed 812, Polyscience, Inc.). Ultramicrotome cuts of 70 nm were selected and placed on a copper grid 3 mm in diameter. Specimens stained in uranyl acetate and lead acetate (metal staining) were viewed using TEM (Philips CM200).

RESULTS AND DISCUSSION

1. Biomass, Total Lipid Content and Glucose Consumption

In the present investigation, five day old cells of *T. aureum* exhibited higher amounts of cell biomass (3.9 g/L) and total lipids (0.8 g/L), with a rapid increase in both levels observed during the first five days after inoculation (Fig. 1). The glucose consumption rate also increased rapidly during the first five days of culture (8.8 g/L was utilized). The total content of lipid in the biomass also increased up to 24.4% in the early growth period of the cells. Cell growth of *T. aureum* achieved its maximum in the early growth phase, showing a rapid progressive increase in the production of total lipids and cell biomass. In an earlier report, maximum production of biomass in *T. aureum* was recorded to be 2 g/L on day 4 at 32 °C in 100 rpm shaking condition [11], whereas in the present study, the biomass

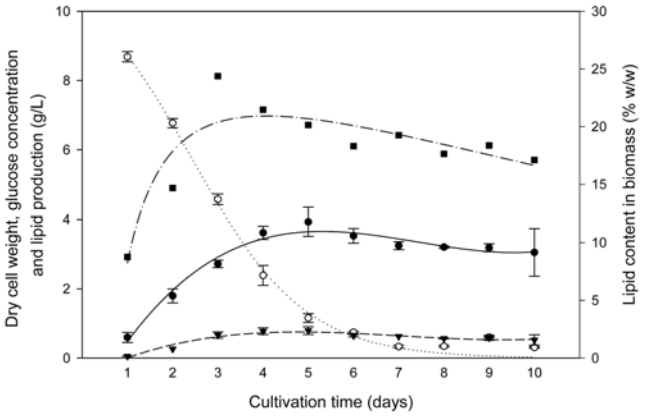

Fig. 1. Effect of culture age on glucose concentration of the medium (○), cell biomass (●), lipid content of the cells (▼) and Lipid content in biomass (■). Mean values (±SD) of three replicates are shown.

production was doubled in 5 day old culture at 25 °C in 180 rpm shaking condition. An intense occurrence of lipid synthesis was also encountered during the same period. This shows that the lipid synthesis is directly proportional to the cell growth at different ages. The growth of *T. aureum* in different concentrations of NaCl and $MgSo_4$ amended medium was also studied in which maximum cell biomass was recorded on 6 day old cells representing 3.2 g/L [15], whereas in the present investigation the production of biomass was found to be quantitatively high in the early phase of growth. Glucose-containing media with high NaCl led to the production of bulk lipid at the end of the exponential growth phase, which remained unaltered in the stationery phase. After day 5, lipid content and dry cell weight decreased gradually, whereas a steady rate was observed in the late growth period (7-10 day) with low glucose concentration (Fig. 1). With this glucose depletion, a decline phase was exhibited by showing a gradual decrease in the synthesis of cellular lipids which was directly proportional to the growth of the cell. This possible regulatory mechanism of growth-coupled lipid synthesis in the microorganisms was evidenced in the production of arachidonic acid in the fungus, *Mortierella alpine* [16]. The maximum amount of glucose and other nutrients of the media was utilized by the cells, and at the same time cell biomass and lipid content in biomass were also found to reach their maximum consumption in the early growth period of the cells. Furthermore, a maximum glucose consumption of 76% was achieved in the first four days by the cells in the culture medium. Initial glucose and other nutrient concentrations of the culture medium also played an important role in the higher production of biomass and lipids in the early period of cell growth, which is evidenced in *T. aureum* and *T. roseum* [1,2,9]. A regression analysis of the results of total lipids, dry cell weight and glucose concentration also shows statistical significance when compared with the data of day 1 to day 10 cell samples (Fig. 1).

2. Lipid Class and Fatty Acid Profile of TAG and PL

Changes in the ratio of lipid class composition were estimated by TLC silica rod analysis in order to find the difference in the lipid content in the biomass in relation to dry cell weight and total lipid production rate. A rapid progressive increase in the production of triacylglycerol (TAG) was observed at the beginning of the station-

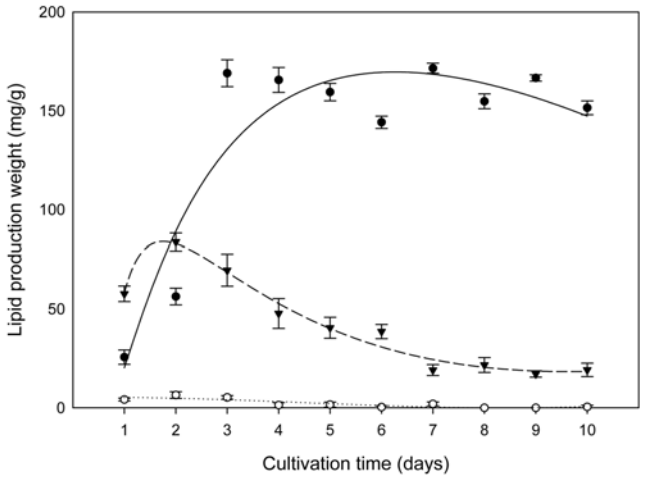

Fig. 2. Effects of cultivation time on lipid production weight in the cell (● TAG; ▼ PL; ○ ST). Mean values (±SD) of three replicates are shown.

ary phase of cell growth which reached a maximum of 156.7 mg/g in the first three days, whereas phospholipids (PL) and sterol (ST) conversely decreased in 2 day old cultures by obtaining enough glucose concentration (Fig. 2). Both PL (84.1 mg/g) and ST (6.5 mg/g) showed maximum production on day 2. Later in day 3, the TAG level showed a progressive decrease until day 6 followed by an unchanging rate from day 7 to 10. Changes in the composition of the TAG fatty acid profile including palmitic acid (PA; C16 : 0), stearic acid (SA; C18 : 0), docosapentanoic acid (DPA; C22 : 5) and docosahexaenoic acid (DHA; C22 : 6) showed a rapid increase in the first three days. Later the production level slightly decreased from day 4-8 followed by a uniform steady rate from day 8-10 (Table 1). Although the other fatty acids including oleic acid (OA, C18 : 1), linoleic acid (LA; C18 : 2), arachidonic acid (AA; C 20 : 4) and eicosapentanoic acid (EPA; C20 : 5) did not exhibit any significant changes in the production rate during the late growth phase, they did, however, show an increased rate in the early phase of growth (first 3 days). TAG showed a higher content of DHA (65.2 mg/g; 40%) in 3 day old cultures of *T. aureum*. In general, TAG is a non polar, neutral lipids found in abundance in the lipids of cell biomass. It is a major form of fatty acid, consisting of molecules of glycerol by which TAG can pass through the cell membranes freely. These are the essential components in cell lipids, playing an important role in metabolism as a primary energy source [17]. In the first five days, the maximum amount of glucose (carbon source) in the medium was utilized by the cells and produced maximum TAG on day 3 which was used as an energy source in the late growth phase of cells. TAG content was also confirmed with an electron micrograph, which showed maximum accumulation of lipid bodies in 3 day old cells. Later, the association and dissociation of lipid bodies led to decreased production in 6-10 day old cells. ST and PL showed a decrease in its level from day 3 to 7. In the late phase of cell growth (day 7 to 10), PL showed a steady level of production, whereas the presence of ST was undetected. The fatty acid profile of PL showed changes in the production of DHA, DPA, PA and SA as recorded in ten day old cultures (Table 2). Whereas the changes in the production of other fatty acids, which includes EPA, AA, LA, OA, and MA, have not been expressed in all 10 samples analyzed, they showed a higher content in the first three days. PA and SA showed a rapid increase in the first three days, and later from day 4 they showed a progressive decrease in their production level. The production levels of DHA and DPA in PL were found to be indirectly proportional to each other. The maximum production of DHA was recorded in the early growth phase (1-4 day), whereas in DPA, it was observed in the late growth phase of the cells (6-10 day). PL showed a higher content of DHA (41.0 mg/g) in 2 day old cells. The lipid content in the biomass increased rapidly in the first three days (244 mg/g) because of a rapid increase in the production of TAG in the early growth phase of the cells. PL is considered to be an important building block of all biomembranes which constitute phosphatidic acids. They are actively involved in cell signaling and lipid vesicle formation [17]. Electron micrographs showed clear membrane bound lipid bodies in 1-3 day old cells of *T. aureum,* which confirms the production of PL to be maximum and active in 3 day old cells where they are the building blocks of biomembranes. In the later phase of growth (9-10 day old cells), the membrane formation was not prominent

Table 1. Effect of culture age on production of fatty acids in TAG of *T. aureum* (SFAs; saturated fatty acids, PUFAs; polyunsaturated fatty acids, Mean values (±SD) of 3 replicates are shown)

FAs (mg/g)	Culture age (days)									
	1	2	3	4	5	6	7	8	9	10
C14 : 0	0.4±0.04	0.2±0.03	1.7±0.13	1.6±0.10	1.4±0.02	1.3±0.13	1.5±0.01	1.5±0.09	1.2±0.07	1.3±0.10
C16 : 0	4.6±0.27	8.6±0.45	31.3±1.34	28.4±0.13	31.5±1.04	34.7±0.40	34.7±0.39	31.2±0.79	29.6±0.82	28.7±0.71
C18 : 0	2.1±0.26	3.8±0.18	8.1±0.47	6.9±0.94	6.6±0.85	10.8±0.40	8.7±1.14	7.6±0.82	6.9±0.18	7.6±0.37
C18 : 1	2.3±0.19	6.2±0.23	25.9±0.73	24.9±0.39	25.3±0.66	27.2±0.90	27.0±0.96	25.2±0.81	22.1±0.79	24.1±1.27
C18 : 2	0.9±0.03	2.6±0.09	7.7±0.24	6.7±0.17	6.5±0.16	4.5±0.02	6.0±0.17	5.3±0.31	5.0±0.22	4.8±0.11
C20 : 4	1.2±0.02	2.2±0.09	3.8±0.23	3.7±0.10	3.4±0.03	2.0±0.07	3.6±0.04	2.1±0.03	2.7±0.15	2.2±0.05
C20 : 5	0.7±0.06	1.5±0.07	2.9±0.18	2.5±0.15	2.5±0.01	1.0±0.04	2.9±0.07	2.0±0.02	2.8±0.19	2.1±0.06
C22 : 5	1.3±0.23	4.0±0.15	10.1±0.18	9.6±0.09	9.6±0.51	8.7±0.33	12.0±0.17	10.2±0.27	13.9±0.37	12.1±0.16
C22 : 6	7.4±0.28	22.4±1.03	65.2±2.32	60.8±1.26	58.3±0.75	49.2±1.57	64.2±0.76	58.2±1.13	64.3±0.34	57.7±0.35
SFAs	7.1±0.19	12.6±0.22	41.1±0.65	36.9±0.39	39.5±1.97	46.8±0.31	44.9±0.51	40.3±0.57	37.7±0.36	37.6±0.39
PUFAs	13.8±0.13	38.9±0.28	115.6±0.65	108.2±0.36	105.6±0.52	92.6±0.49	115.7±0.61	103.0±0.43	110.8±0.34	103.0±0.33
Total	20.9±0.15	51.5±0.26	156.7±0.65	145.1±0.37	145.1±1.00	139.4±0.43	160.6±0.58	143.3±0.47	148.5±0.35	140.6±0.35

Table 2. Effect of culture age on the production of fatty acids in PL of *T. aureum* (SFAs; saturated fatty acids, PUFAs; polyunsaturated fatty acids, Mean values (±SD) of 3 replicates are shown)

FAs (mg/g)	Culture age (in days)									
	1	2	3	4	5	6	7	8	9	10
C14 : 0	0.2±0.09	0.6±0.04	0.3±0.06	0.1±0.03	0.1±0.01	0.2±0.03	0.1±0.01	0.0±0.00	0.1±0.01	0.2±0.01
C16 : 0	6.1±0.60	12.6±0.66	8.3±0.55	3.4±0.04	2.5±0.77	2.1±0.11	0.1±0.04	0.2±0.11	0.1±0.08	0.1±0.09
C18 : 0	0.5±0.58	2.1±0.26	1.0±0.19	0.6±0.27	0.4±0.72	0.5±0.11	0.2±0.13	0.2±0.11	0.1±0.02	0.1±0.05
C18 : 1	1.8±0.43	8.1±0.34	6.5±0.30	0.8±0.11	0.4±0.22	1.0±0.24	0.2±0.06	0.1±0.01	0.1±0.08	0.1±0.06
C18 : 2	1.0±0.06	2.1±0.13	1.9±0.10	0.1±0.05	0.1±0.04	0.7±0.01	0.1±0.02	0.1±0.04	0.1±0.02	0.1±0.01
C20 : 4	3.8±0.05	4.5±0.14	1.6±0.10	3.6±0.03	2.8±0.01	1.4±0.02	0.4±0.00	1.7±0.00	0.1±0.02	1.5±0.01
C20 : 5	2.4±0.12	3.9±0.11	2.2±0.08	2.8±0.04	2.6±0.00	2.2±0.01	2.1±0.01	2.6±0.00	1.2±0.02	2.1±0.01
C22 : 5	5.9±0.53	9.2±0.23	8.9±0.07	8.8±0.02	7.4±0.13	6.7±0.09	4.9±0.02	5.9±0.04	6.3±0.04	5.5±0.02
C22 : 6	30.6±0.62	41.0±0.54	33.8±0.95	25.6±0.36	22.9±0.19	16.8±0.42	10.5±0.20	10.3±0.16	8.6±0.03	8.8±0.04
SFAs	6.8±0.21	15.3±0.74	9.6±0.69	4.1±0.73	3.0±0.60	2.8±0.48	0.4±0.09	0.4±0.14	0.3±0.67	0.4±0.21
PUFAs	45.5±0.30	68.8±0.41	54.9±0.27	41.7±0.10	36.2±0.13	28.8±0.13	18.2±0.07	20.7±0.06	16.4±0.04	18.1±0.04
Total	52.3±0.34	84.1±0.39	64.5±0.27	45.8±0.11	39.2±0.24	31.6±0.12	18.6±0.07	21.1±0.07	16.7±0.04	18.5±0.04

Table 3. Effect of culture age on production of fatty acids in the cell of *T. aureum* (SFAs; saturated fatty acids, PUFAs; polyunsaturated fatty acids, Mean values (±SD) of 3 replicates are shown)

FAs (mg/g)	Culture age (in days)									
	1	2	3	4	5	6	7	8	9	10
C14 : 0	0.6±0.13	0.8±0.03	1.9±0.06	1.8±0.09	1.5±0.11	2.0±0.04	1.6±0.01	1.5±0.01	1.3±0.04	1.5±0.02
C16 : 0	10.6±0.00	21.2±0.99	36.7±1.07	34.7±0.91	32.0±0.95	36.8±1.00	34.8±0.08	31.4±0.12	29.7±0.84	28.8±0.31
C18 : 0	2.6±0.49	5.9±0.20	9.1±0.93	7.5±0.69	7.0±0.24	11.3±0.39	8.9±0.20	7.8±0.29	7.0±1.03	7.7±0.30
C18 : 1	4.0±0.22	12.7±1.08	34.0±1.07	25.7±0.27	25.7±0.52	28.2±0.99	27.2±0.49	25.3±0.94	22.2±1.01	24.1±1.11
C18 : 2	1.9±0.36	4.6±0.31	8.6±0.74	7.9±0.53	6.6±0.31	5.2±0.04	6.1±0.05	5.4±0.08	5.1±0.52	4.9±0.03
C20 : 4	5.0±0.89	6.7±0.39	7.2±0.81	5.4±0.26	6.3±0.43	3.5±0.11	4.1±0.08	3.7±0.16	2.8±0.76	3.6±0.21
C20 : 5	3.2±1.21	4.1±0.27	6.3±0.23	6.1±0.33	6.4±0.17	3.2±0.11	5.0±0.14	4.6±0.25	4.0±1.23	4.2±0.29
C22 : 5	7.2±0.59	13.2±0.56	19.1±1.23	18.3±0.28	17.0±0.95	15.4±0.49	16.9±0.35	15.9±0.70	20.1±0.43	17.6±0.98
C22 : 6	38.0±0.30	63.3±0.50	99.0±1.28	86.4±1.19	81.1±0.91	66.0±0.81	74.7±0.06	68.4±0.17	72.9±0.14	66.5±0.55
SFAs	13.8±0.43	27.9±0.32	47.7±0.27	44.0±0.11	40.5±0.50	50.1±0.08	45.3±0.06	40.7±0.08	38.0±0.04	38.0±0.05
PUFAs	59.3±0.59	104.6±0.68	174.2±0.83	149.8±0.56	143.1±0.97	121.5±0.42	134.0±0.19	123.3±0.38	127.1±0.98	120.9±0.53
Total	73.1±0.48	132.5±0.70	221.9±0.99	193.8±0.61	183.6±0.86	171.6±0.44	179.3±0.16	164.0±0.31	165.1±1.03	158.9±0.43

in the lipid bodies, which showed a progressive decrease in the level of PL. ST was found to be in low amounts and actively involved in the cellular membrane fluidity, taking part in the cell's developmental signaling as secondary messengers [17].

3. Fatty Acid Profile of Cell Biomass

GC analysis showed the changes of polyunsaturated and saturated fatty acids in the cell biomass in 10 days of growth (Table 3). In general, a rapid increase in the level of SFAs, unsaturated fatty acids, and PUFAs was observed in the first three days of cell growth. Later, a slight decrease in its level was noticed during days 4 to 6 followed by a steady rate from day 7 to 10. Of the nine different fatty acids analyzed, seven (MA, PA, SA, OA, LA, DPA, DHA), showed a rapid increase in their production in the first three days of early growth phase of cells, whereas AA and EPA showed a progressive increase with a maximum level on day 4. In the SFAs, total fatty acid expressed maximum levels on day 3 (47.7 mg/g) followed by PA (36.7 mg/g), SA (9.1 mg/g) and MA (1.9 mg/g). Later, they showed a progressive decrease in their levels between day 4 and 5, followed by a slight increase on day 6 and a progressive decrease from day 7 to 10. In the analyzed unsaturated fatty acid C18, total unsaturated fatty acid expressed a maximum level on day 3 (42.6 mg/g) followed by OA (34.0 mg/g) and LA (8.6 mg/g). Later they showed a progressive decrease in their level on day 4, followed by a slight increase on day 6 and 7 and a progressive decrease from day 8 and 9, whereas in the case of LA, a progressive decrease was observed between day 4 and 6 followed by a steady phase in its production level from day 7-10. A progressive increase in the level of PUFAs of C20 was analyzed in the early phase of cell growth and a maximum level was recorded on day 4 (13.5 mg/g), followed by AA (7.2 mg/g) and EPA (6.3 mg/g). Later, they showed a rapid decrease in their levels on day 6, followed by a slight increase on day 7 and a steady level from day 8 to 10. In the PUFAs of C22, a rapid increase in the level of fatty acids was observed on day 3 in which the total PUFAs expressed maximum level (174.2 mg/g) followed by DHA (99.0 mg/g) and DPA (19.1 mg/g), with DPA expression limited when compared with other C22 PUFAs. Later they showed a progressive decrease in its level from day 4 to 6, followed by a steady rate from day 7 to 10.

The fatty acid profiles of PUFAs (DHA, DPA, AA, EPA, LA, OA) and SFAs (PA, SA, MA) of cell biomass and lipid class (TAG, PL) showed rapid increase in production level within the first 3 days of *T. aureum* cultivation. Thus the early growth phase of the cells may be suitable for higher production of fatty acids since they show a higher content of cell biomass and lipid content within the first five days, which is evidenced in *Thraustochytrium* spp. [8,9,18]. Also the maximum amount of carbon source in the growth medium was utilized in the early phase of growth, which played a role in the synthesis of fatty acids during the first four days (1-3). Either the fatty acid content was decreased or the production rate was uniform (steady phase) in the late growth phase (4^{th} day onwards). This may focus nutritional depletion in the growth medium causing stress in the growth and developmental activities of the cells. Kang et al. have studied the effect of salt concentration (NaCl and $MgSo_4$) on the production of PUFAs in *T. aureum* in which the fatty acid profile was found to be at maximum synthesis level only during the late growth phase [15], whereas in the present study, it was found in the early phase of cell growth.

The maximum DHA production (389 mg/L; 99 mg/g; 44%) by *T. aureum* was higher and not comparable with other fatty acids in the overall analysis. Culture age and growth was found to play an important role in the production of DHA in the early growth period of the cells. The production of DHA from *T. aureum* was recorded as 49% (70 mg/g) and 51% (104 mg/g) in the earlier reports, but when the flask culture was subjected to 300 rpm shake culture for a period of 6 days, results showed 270 and 511 mg/L [2,3]. Iida et al. have also showed 40% DHA production in 200 rpm shake culture of *T. aureum* of 2.5 day old cells [8]. In the present investigation we obtained a maximum of 99 mg/g of DHA in the 3^{rd} day of cell growth with shaking at 180 rpm. Other species of *Thraustochytrium* studied earlier including *T. roseum* (ATCC 28210), *Thraustochytrium* sp. (ATCC 20892) and *Thraustochytrium* sp. (ATCC 26185) showed maximum production of DHA only during the late growth phase of culture [1,7,18]. Although production of DHA had been the main focus, it is evident in the present study that the strain, *T. aureum* also produces PUFAs including DPA, AA and OA in limited amounts. Fatty acid profiles of DHA producing thraustochytrids can be used to classify them into separate categories [19]. PUFAs profile of some thraustochytrid strains may produce 30% AA of the total fatty acids, with no other PUFA exceeding 10% of total fatty acids [20].

4. TEM Study of Lipid Bodies in the Cells

Ultrastructure of *T. aureum* contained numerous cytoplasmic bodies which have been confirmed as lipid bodies by TEM analysis with the help of uranyl acetate and lead acetate stain. The cells were heterogeneous in size and appearance ranging from 6-12 μm. Cytoplasm appeared as granular and filled with multiple oil globules or lipid bodies (0.2 to 3.2 μm in size) in an 1 day old to 10 day old cells (Fig. 3a-j). The size of the nucleus was larger and more prominent in 1 day and 2 day old cells (Fig. 3a, b), whereas it was smaller in other cells (3-10 day old cells). Small-sized lipid bodies were in limited number in 1 day old cells. In the 2 and 3 day old cells, accumulation of the lipid bodies became enriched and enlarged to form spherical shaped bodies (up to 3.2 μm diameter). Limitations of a membrane around the lipid bodies were found to be evident in the early growth phase of the cells (1-3 day old cells). The size of the

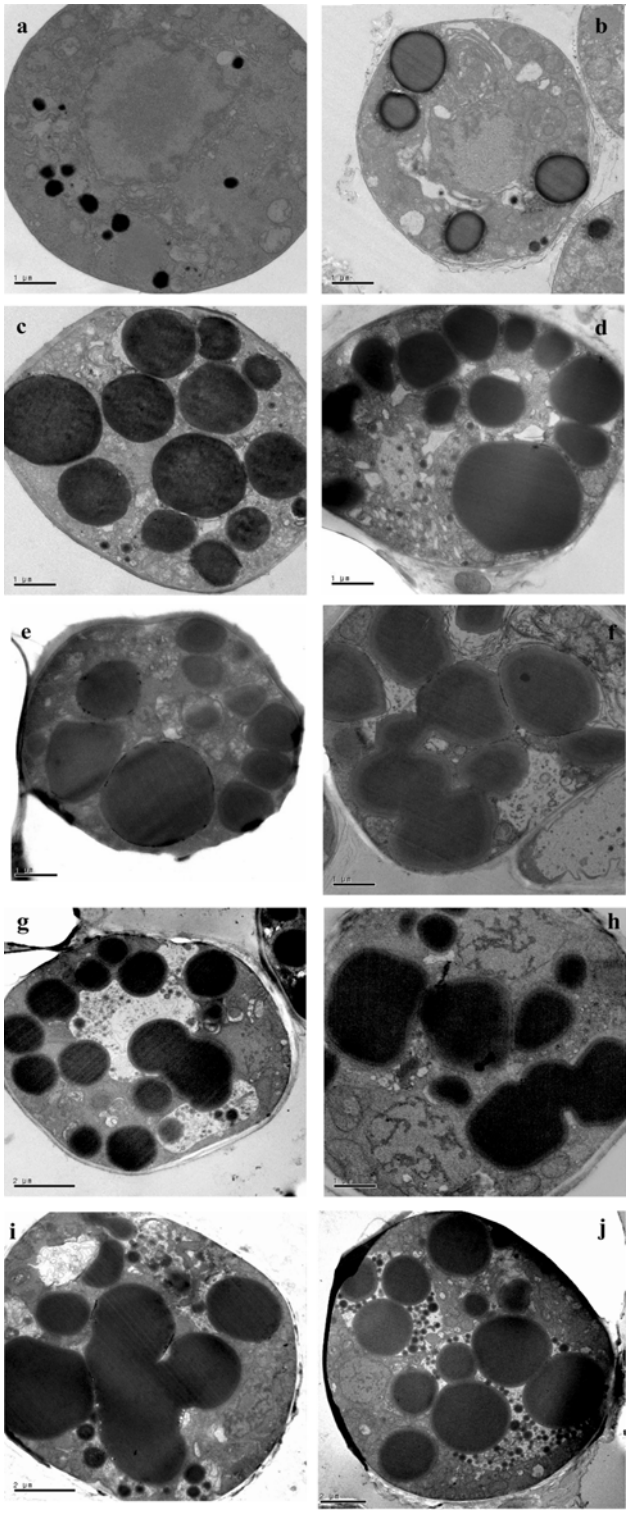

Fig. 3. Transmission electron micrograph of *Thraustochytrium aureum* cells showed the formation of lipid bodies in response to culture age. a. Origin of small lipid bodies (dark spot) near ER of the cytoplasm in 1 day old cell; b-e. 2 to 5 day old cells showing a progressive increase in size of the spherical bodies by the deposition of lipids; f. 6 day old cells showed the aggregation of lipid bodies arranged closer to each other; g-j. 7-10 day old cells showing aggregation of individual lipid bodies leading to the formation of small fragmented individual bodies. (a-f, h scale bar=1 μm; g, i-j, scale bar=2 μm).

lipids was found to be largest in the 3 day old cells, representing maximum accumulation of lipids (Fig. 3c) with lipid body content approximately 57% of the total area of the cell. Limiting membranes around the lipid body was not clear from 4-10 day old cells. In the 6 and 7 day old cells (Fig. 3f, g), the size of the lipid remained the same as in 4 and 5 day old cells. Lipid bodies present in the cells were associated closely by overlapping with each other. This association has led to the formation of a number of new small lipid bodies in the 7-10 days old cells (Fig. 3g-j). Also, the margins of the bodies were not distinct due to the fusion and dissociation of larger lipid bodies (Fig. 3j).

Lipid bodies in the cells were associated with hollow membrane-like structures that seemed to be associated with lipid synthesis and deposition. With continued lipid deposition, they became internalized within the lipid bodies. Sectional views of the TEM photographs indicate that the boundary layer of these structures was unit membrane that surrounds the lipid body in the early growth phase of cells (1-3 days). Here, maximum PL production was found to be actively involved in the synthesis of biomembranes [17] and involved in cell signaling which led to the formation of ER around the lipid body. As the structures become internalized during lipid body enlargement, their boundaries became indistinct, which seemed to be followed by loss of the internalized structures. This is evidenced in 4-6 day old cells of *Thraustochytrium* sp. [7] which were clearly observed in the present study. The presence of lipid bodies in *Thraustochytrium* and related species was observed by light [21] and transmission electron microscopy [7]. However, no report has been made on the membrane-like structures in species of this genus. Although it is generally accepted that lipid bodies arise from ER membranes [22], very little information on lipid body formation in fungi yeasts is available. However, a study on fluorescent staining of ER revealed a close association between ER and lipid bodies. This study is suggested in the fungi *Mortierella ramanniana* [23] and *Saccharomyces cerevisiae* [24] and also in a thraustochytrid, *Schizochytrium limacinum* [12]. In the present study during the of early phase of growth, the cells synthesizing lipids limited by ER membrane of the cytoplasm were clear in the early growth phase (1-3 days), where the lipid accumulation (TAG) and ST depletion were found to be maximum in size. This was evidenced by an earlier report on yeast, in which the yeast cells utilize TAG of lipid particles for membrane biosynthesis, especially during depletion of ST [25]. The maximum synthesis and continuous accumulation of lipids might be present in this period of cell growth. This may be due to the rapid and active growth of the cell and the utilization of a carbon source within the first five days. The same factors apply to production of TAG that was found to be at maximum levels during the early growth phase, which served as an energy source to perform cell metabolism [17]. All fatty acids obtained from cell biomass and lipid class components (TAG and PL) analyzed (C14 : 0, 16 : 0, C18 : 0, C18 : 1, C18 : 2, C20 : 4, 20 : 5, 22 : 5, 22 : 6) in the present investigation also showed a rapid increase in the production rate only during the early growth phase of the cells, which may induce the accumulation of lipid bodies. PUFAs in the total fatty acids of *T. aureum* showed a maximum level of 61.3% in which DHA production alone was represented by 44% in the early phase of cell growth, which was incomparable and appreciative when compared with the earlier reported *Thraustochytrium* species. The result of lipids was evidenced in *Thraustochytrium* sp. (ATCC 26185) in which the synthesis, deposition and association of lipids in cells were recorded on the 6^{th} day after inoculation [7]. Whereas in the present investigation, lipid bodies remained in the same size and tended to associate with the nearby bodies from day 5 onwards. Some of the natural organic compounds produced by the fungus may cause inhibition in the additional accumulation of lipid bodies after day 5, which was supported by an earlier report on yeast [26], in which the inhibitory effect of the compounds led to a 30-40% decrease in TAG accumulation without any additional accumulation of its intermediates. This suggests that suppression of the total carbon inflow into the TAG biosynthesis had taken place. During association, the lipids of 6-8 day old cells became fragmented to form numerous small bodies in the late growth phase (7-10 days). Some of the natural organic compounds produced by the fungus may cause inhibition in the additional accumulation of lipid bodies after day 5. In 9 and 10 day old cultures, most of the observed cells were broken up into a number of small fragmented lipids, which may be due to the active metabolism of the cell that utilizes its fragmented lipid bodies as an energy source to perform different functions [17]. Nutritional stress in the cells caused depletion of carbon and nitrogen sources in the medium during cell growth. In general, the size of lipid bodies increased because of the increased concentration of TAG, PL and fatty acids, cell biomass, glucose consumption and lipid content in the early period of cell growth, whereas in the late growth phase it decreased progressively due to the reduction or fragmentation of accumulated lipid bodies in the cells observed.

CONCLUSION

Overall, this study focused on a detailed understanding of the effects of growth periods on the changes in lipid and fatty acid composition and also lipid body accumulation of *T. aureum* for the first time. Based on the present work, the early growth phase of the *T. aureum* culture showed a higher production of biomass, lipid content (TAG, PL), PUFAs particularly DHA and other fatty acids in culture. Ultrastructure of the cells also revealed maximum accumulation of lipid bodies corresponding to the TAG quantity with the presence of a limiting membrane (ER) around lipid bodies especially during the depletion of ST in the early growth phase of the cells. It is evidenced that DHA is obtained directly from the cell biomass and TAG of *T. aureum* showing an equal and maximum synthesis. TAG plays an important role in metabolism as a primary energy source. PL found as a building block of all membranes is actively involved in cell signaling. This study provides a novel additional understanding of the relationship between culture age and fatty acid composition of the protist *T. aureum*. These results will also be helpful in future studies investigating the physiological and biochemical characteristics of *T. aureum* to further improve its biotechnological potential.

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