STUDIES ON OPTIMIZATION OF FERMENTATION PARAMETER FOR PUFAs PRODUCTION

Nakuleshwar D. Jasuja and Suresh C. Joshi*

Reproductive Toxicology Unit, Center for advanced studies,
Department of Zoology, University of Rajasthan, Jaipur – 302055

*e-mail: s_c_joshi2003@rediffmail.com

Summary

The effects of culture condition, pH, salinity and carbon sources on the production of docosahexaenoic acid (DHA) were investigated. Monosaccharides (glucose) and acetic acid supported good cell growth and DHA yield. A high content of DHA was obtained from culture on glucose. Thraustochytrids, in particular Schizochytrium spp., were used for the production of the valuable polyunsaturated fatty acid (PUFA), docosahexaenoic acid (DHA; 22:6, n-3). Growth of Schizochytrium spp., in a defined medium was initially made in shake-flask cultures to determine the optimum concentrations of carbon (glucose) and nitrogen ($NaCH_3COO$) that could be used by this microorganism. Fatty acid profile was affected by the different nutrient concentrations provided during fed batch culture. Consequently, lipid accumulation can be induced through the carbon and nitrogen source concentration in the medium to maximize the TFA (total fatty acid) and subsequently DHA productivity by this microorganism.

Key words: Docosahexaenoic acid (DHA), Monosaccharides, Polyunsaturated fatty acid (PUFA)

***Correspondence to:** Dr. Suresh C. Joshi, Associate Professor, 170, Vishwakarma Nagar-I, Maharani Farm, Durgapura, Jaipur (INDIA)-302018.

Phone: 91-141-2762525 **Fax:** 91-141-2740141 **e-mail:** s_c_joshi2003@rediffmail.com

Introduction

Omega-3 highly unsaturated fatty acids (HUFAs) particularly the very long chain ones of eicosapentaenoic acid (EPA; 22:5, n - 3) and docosahexaenoic acid (DHA; 22:6, n - 3), are of significant commercial interest as important dietary compounds for preventing abnormalities in immune system, inflammatory system, endocrine system, nervous system, arteriosclerosis and coronary heart disease, for alleviating inflammatory conditions and for retarding the growth of tumor cells (1,2,3). DHA is regarded to be essential for the proper visual and neurological development of infants, because of its role as a structural lipid component (4, 5, 6). DHA supplementation of juice at either 50 mg/day or 100 mg/day for 6 weeks was effective in increasing plasma phospholipid DHA contents of children (7). A high content of DHA in the brain and retina is important for proper nervous and visual function (8, 9). Long chain ω-3 polyunsaturated fatty acids can act as breast and colon cancer chemopreventive agents (10, 11).

Docosahexaenoic acid (DHA) and arachidonic acid (ARA) are produced exclusively from microorganisms Schizochytrium sp. is a marine thraustochytrids that synthesizes polyunsaturated fatty acids in which the valuable dietary docosahexaenoic acid (DHA; 22:6, n−3) represents 30–40% of the total fatty acids (12,13). In present investigation lipid and docosahexaenoic acid (DHA) accumulation into Schizochytrium spp. SC-I was studied under fed batch culture. Different incubation and nutrients were supplemented in a defined medium. The whole biomass of this organism may also be useful for the development of aquafeeds for farmed fish, which require a source of DHA for the development of fish fry (14, 15).

Materials and methods

The strain Schizochytrium sp. SC-I was obtained from Regional Research Laboratory, Jammu Tawi (CSIR Lab., RRL, J&K, INDIA). The strain was maintained on nutrient agar plates by streaking as tree like manner as mentioned in fig. 1.1 and subcultured after every five days. The isolation medium was modified (16) & consisted of glucose 20 g/L, KH_2PO_4 0.25 g/L, yeast extract 5 g/L, peptone 5 g/L, agar 2%, Seawater1000 ml, penicillin solution3 µg/ml, streptomycin solution 3 µg/ml, pH 7.0. All ingredients except antibiotic solutions were mixed and autoclaved. After autoclaving antibiotic solutions were added through micro filter. Above medium was poured on sterile petriplates, streaked and incubated for 24 hrs at 30°C.

Fig. 1.1. **Petriplates showing 24 hrs and 120 hrs streaked SC-I.**

Preparation of seed flask:

Seed flask was prepared for fermentation process using production medium (M_4) consisting glucose 20g/L, KH_2PO_4 0.25 g/L, yeast extract 10 g/L, peptone 10 g/L, sea salt 35 g/L., then autoclaved and inoculated with culture suspension harvested from agar petriplate. This flask was then kept on rotary shaker (210rpm) for 24 hrs at 30°C.After 24 hrs seed flasks were used for inoculation.

Biomass production and lipid extraction

After desired incubation period the culture broth from each flask was harvested separately by centrifugation at 8000 rpm for 10 min at 20^{0}C. Pellet and supernatant were separated. Weighing of the pellet was done for wet biomass estimation.

Ethyl acetate and methanol solvent system

Culture broth was centrifuged at 8000 rpm for 10 min at 20°C. 10ml methanol was added to pellet and maceration was done in pester motor. 20ml ethyl acetate was added in the separating funnel and shacked vigorously. After the separation of upper layer containing ethyl acetate, again 20ml ethyl acetate was added to lower layer and shacked vigorously. This process was repeated twice. Around 2-3 gm sodium sulphate was added to the polled upper layer. The above solution was filtered and distilled on rotor vapor at 45-65°C. The oil obtained was dissolved in 1ml of hexane and transfer to a pre weighed vial. The vial was kept open overnight for complete evaporation of solvent and weight of the lipid was taken.

Production of DHA on different fed-batches

a) Glucose Fed Batch

100ml of autoclaved media consisting glucose 20g/L, KH_2PO_4 0.25 g/L, yeast extract 5 g/L, peptone 5 g/L, inoculum's volume 10%, sea salt 35 g/L. was taken in 500ml of flask.10% inoculum's was added in each flask from seed flask. After inoculation all flasks were kept on rotary shaker and incubated at 30^0C. Glucose 10g/L was added after every 24 hrs and after 72, 96, 120 hrs experiment was terminated.

b) Acetic acid Fed Batch

Fed batch was done with acetic acid to production medium flask containing glucose as carbon source at concentration of 10 ml/l after each 24 hrs. Experiment was terminated on 48, 72, 96, 120 and 144 hrs and extraction of lipid was done as described above.

c) Sodium acetate Fed Batch

Fed batch was done with sodium acetate to production medium flask containing glucose as carbon source at concentration of 10g/l after each 24 hrs. i.e glucose was replaced with sodium acetate as a carbon source. Experiment was terminated on 96, 120 and 144 hrs and lipid was extracted and analyzed.

Effect of Sodium sulfate on production of DHA

100ml of autoclaved media containing glucose 20g/L, KH_2PO_4 0.25 g/L, yeast extract 10 g/L, peptone 10 g/L, sodium sulfate 5 g/L. was taken in 500ml of flask**.** Glucose fed batch was done to respective flask at concentration of 2% after every 24 hrs. Experiment was terminated on 72, 96, 120, 144 and 168 hrs and extracted as described above.

Effect of Incubation time on DHA production

Glucose Fed batch was done at concentration of 2% after every 24 hrs. Experiment was terminated on 120, 144, 172, 198 and 216 hrs and extracted out.

Optimization of pH for DHA production

Different initial medium pH used were 4, 5, 6, 7, 8, 9, and 10. M4 medium was prepared in phosphate buffer. pH above 7.7 and below 6.1 were adjusted using NaOH (1 N) and HCl (1 N) respectively. Glucose Fed batch was done to respective flask at concentration of 2% after each 24 hrs. Experiment was terminated on 144 hrs and lipid was extracted out as described earlier.

Results

Production of DHA by fed-batch glucose at shake flask level

Fig.1.2 shows that the production of DHA and biomass by fed batch glucose at shake level was higher at incubation of 120 and 144 hrs. Incubation at 72 and 96 hrs had given significant production of DHA. Carbon source is necessary for any organism to survive and have a great impact on the lipid production, when used in various concentrations and also mode of addition.

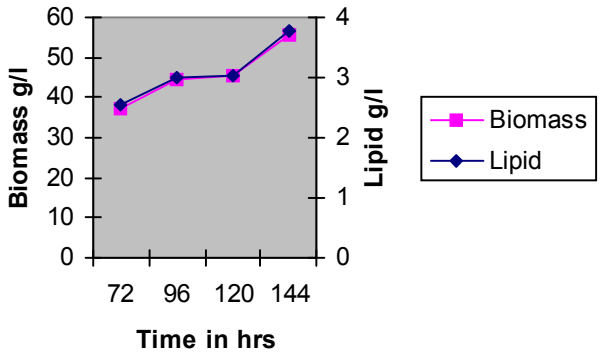

Fig. 1.2. Production of Lipid by SC-1 at different time intervals

Effect of acetic acid fed batch on biomass and lipid production

At the incubation of 144 hrs in presence of Acetic acid as carbon source the biomass increases but cell count was reduced. There was no tremendous change in lipid production between incubation periods of 96, 120 and 144 hrs. It was thought that instead of glucose, acetic acid may incorporate in the medium as a source of acetyl-CoA to increase the yield of PUFAs. But contrary to that when acetic acid was incorporated in medium both the biomass and lipid yield decreased markedly. This may be due to the pH effect that acetic acid decreased the pH of the medium to as low as 3.3 and hence created the unfriendly environment for the organism to grow effectively and hence lipid production was also low.

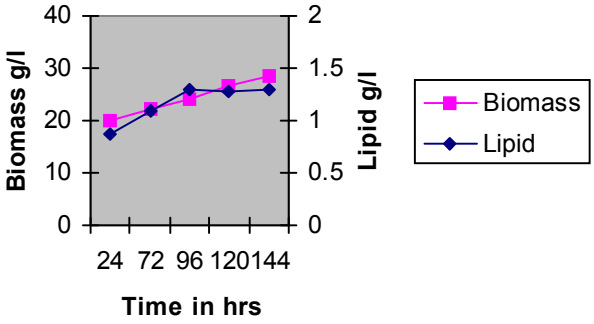

Fig.1.3 Graph showing effect of acetic acid.

Effect of sodium acetate on biomass and lipid production

In continuation to the above experiment sodium acetate was incorporated in the medium as a source of acetyl-CoA.

As the organism of interest was of marine origin, it was necessary that the medium contain sodium so that marine environment can be created for the proper growth. But sea salt, which was normally being used, was around 35 g/l and hence owing to its high concentration was not environmental friendly. So as an alternative to sea salt, sodium acetate (5 g/l) was used as a source for sodium ions.

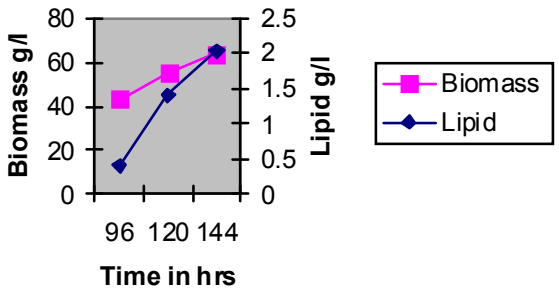

Fig.1.4. Graph showing effect of sodium acetate

Effect of sodium sulfate when used in place of sea salt on biomass and lipid production

The lipid production was very less from incubation period of 72 hrs to 144 hrs but after 144 hrs, the cell counts were increased double and lipid production was four fold at 168 hrs.

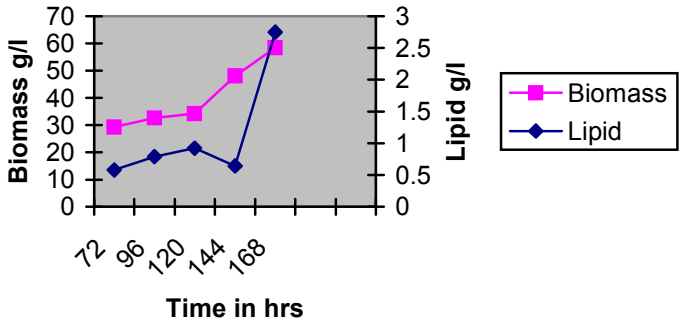

Fig. 1.5. Graph showing effect of sodium sulfate

Effect of Incubation time and glucose fed batch on biomass and lipid production

Effect of prolonged incubation period was studied by increasing the incubation period up to 216 hrs. Due to increase in the incubation period, the lipid accumulation was maximum than before reaching up to 6.7 g/L indicating that continuous glucose supply was utilized efficiently by the organism for lipid production.

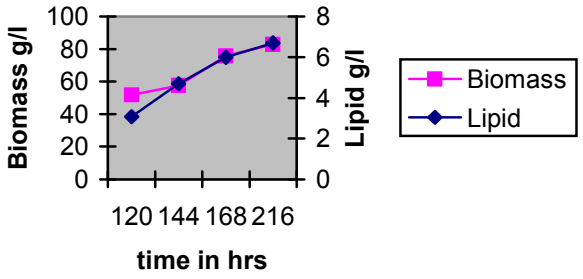

Fig 1.6. Graph showing effect of incubation time

Optimization of initial medium pH for the production of biomass and lipid

The more productive culture conditions for DHA production by SC-1 in this work was obtained at initial pH of 7.0 with 20 g L^{-1}glucose at 8-day incubation.

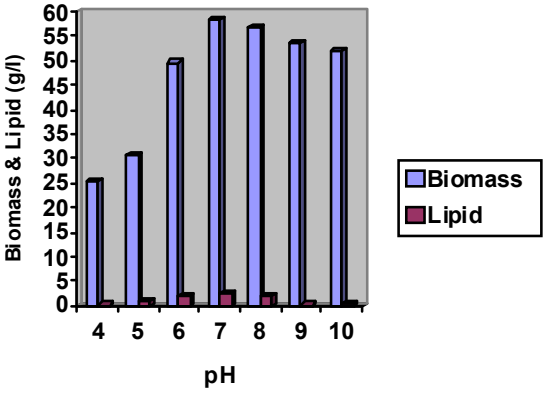

Fig. 1.7. Graph showing effect of different initial medium pH

Discussion

This research work tried to maximize DHA yield from oleaginous microorganism by optimization of glucose concentration, giving different carbon source, fed batch acetic acid and fed batch glucose in optimal conditions.

In this study it was found that instead of taking initial high concentration of glucose, addition of glucose in batches enhances the accumulation of lipid. As evident from the residual sugar estimation (17) that the supplied glucose is consumed within 24 hrs, fed batch of glucose enhanced the lipid production.

When supplying the acetic acid as carbon source in place of glucose, the biomass increases but lipid production remains constant if incubation period increases.

When as an alternative to sea salt, sodium acetate (5 g/l) was used as a source for sodium ions high biomass was obtained (60 g/l) but the lipid accumulated was not adequate when compared to biomass. When sodium acetate was replaced by sodium sulfate there was tremendous change in lipid production at 168hrs incubation. The lipid content increases to four fold as compare to sodium acetate. Incubation period is very important for any fermentation process. When studied the effects of incubation time with glucose fed batch on lipid production it was found that productivity increases as the incubation time increases.

The results presented in this study illustrated that the organism's growth, the lipid profile and the fatty-acid profile of SC-I can be enhanced by adjusting cultivation time, initial pH and medium composition. The dynamic interactions among the growth, fatty acid synthesis and nutrient limitation result in significant variation in the biomass and lipid production.

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