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Optimization of culture conditions and process parameter (pH)to enhance the production of DHA by using Schizochytrium sp. in submerged batch fermentation

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ABSTRACT: *Docosahexaenoic acid (DHA) is an important omega-3 fatty acid with many beneficial health effects on humans. Microalgae derived DHA has several advantages as compared to fish oil. Therefore, most of the commercial production is done now-a-days through fermentation using microalgae and Schizochytrium species are well studied in this regard. In the current study, Schizochytriumlimacinumwas used for optimization of culture conditions and process parameters for enhanced biomass and yield of DHA in fermentation. Our study highlights the use of initial culture growth attained after 28hours with pH and packed cell volume (PCV) values 5.8±0.2 and 05±1.0, respectively as lab inoculum for seed and production batch. Moreover, we studied the effect of varying pH(5.7±0.2, 6.2±0.2 and 6.8±0.2) on DHA production. Our results suggest that the optimal pH for maximum DHA production is 6.2±0.2 duringproduction fermentation batch.*
KEYWORDS*: Microalgae,DHA, fermentation, pH, extraction, HPLC*

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I. INTRODUCTION

The effectiveness of docosahexaenoic acid (DHA) in curing several diseases, such as cancer, depression, coronary heart disease, hypertension, depression, atherosclerosis, type-2 diabetes mellitus, and thrombosishaslong been discovered(Crawford et al., 1997; Nordøy et al., 2001; Das 2003). This makes DHA a widely used nutraceutical component in the food and feed market (Zeng et al., 2011). Most of the commercial source of DHA is fish oil derived from cold water fatty fish such as Tuna and Salmon. However, due to limited supply of fish oils and several drawbacksassociated with it alternative sources of DHA were explored (Wu et al., 2011; Ren et al., 2009). One of the major sources of DHA is microbial oil obtained from marine unicellular Thraustochytrids (Aurantiochytrium/ Schizochytrium and Crypthecodinium cohnii) produced under controlled conditions. This is considered to be an excellent source of DHA with various advantages likes fast heterotrophic growth, ability to produce toxin-free oils and suitability in commercial-scale fermentation (Gupta et al., 2012; Huang et al., 2012; Chang et al., 2013).

Improvement in DHA production by Thraustochytrids has been achieved in the last few decades with few genera likeSchizochytrium, Thraustochytrium andAurantiochytrium. Growth conditions and media components have been optimized for improved production of DHA in strains that belong to the above genera(Huang et al., 2012; Chang et al., 2013; Liu et al., 2014; Li et al., 2015).However, optimal culture conditions can vary significantly amonginterspecific and/or intraspecific strains.

The current study was carried out using Schizochytrium strain which is known for its enhanced capability to produce DHA when grown on glucose or fructose (Chatdumrong et al., 2007). Two parameters thatgreatly affect the yield of biomass and fatty acid composition of cultivated cells are physiological conditions and composition of medium (Luthra et al., 2014a; Patil and Gogate 2015).The amount of carbon and nitrogen in the medium determine growth and/or lipid accumulation.Yokochi, 1998have reported 32.5, 30.9 and 43.1% DHA in fatty acid composition using glucose, fructose and glycerol respectively, as carbon source in the medium.

Physiological conditions like pH of the medium may alter DHA yield and total lipid accumulation by affecting cell membrane function and the uptake of nutrients.A report suggests maximum DHA yield and biomass of S. limacinum at pH 7 (Wu et al. 2005).Other determinant is the salinity which regulates the cytoplasmic ion gradient and activity of enzymes (Kim et al. 2005).A study using different sea water concentrations revealed thatlowering the salinity from 28 to 18% resulted in higher DHA accumulation (Zhu et al. 2008).

In order to make it industrially feasible, several efforts have been made towards optimizing the culture conditions to enhance DHA production by most Thraustochytrids (Fan et al., 2007; Manikan et al., 2014). In optimizing the medium components, carbon and nitrogen content are the two most important components to be considered as their role in lipid accumulation has been extensively established.Moreover, DHA production also depends largely on process conditions (e.g., pH, temperature, aeration etc.), it is thus essential that for each thraustochytrid strain, culture conditions are screened individually in order to derive the optimal process parameters that maximize their DHA yield.Thus, this study intends to optimize the culture conditions and a process parameter (pH) to enhance the growthand DHA production of our Thraustochytrids isolate.

II. MATERIALS AND METHODS

Microorganism and initial growth medium

Schizochytriumlimacinum, used in the current studywas obtained from **American Type Culture Collection (ATCC-MYA-1381)**, stored as frozen cultures and cultivated on agar mediacontainingpotato dextrose agar (39.0 g/L), Sea water components with NaCl (12 g/L), $MgCl_2$ (5.5 g/l), $CaCl_2$ (1.0 g/l),KCl (0.35 g/l), Na_2SO_4 (2.0 g/l) and Agar (30 g/l), pH 5.8±0.2.and Agar (30 g/l), pH 5.8±0.2. The cultures were incubated at 28ºC for 96 hrs and the colonies were observed on plates. The cultures were further scaled up by sub culturing in inoculated lab inoculum medium containing of Dextrose (50 g/l), Yeast Extract (2.5 g/l), bacteriological peptone (2.5 g/l), potato dextrose agar (39.0 g/L), Sea water components (12 g/l NaCl,5.5 g/l $MgCl_2$,1.0 g/l $CaCl_2$, 0.35 g/l KCl, 2.0 g/l Na_2SO_4), trace salt solutions (0.1 g/l KBr, 0.03 g/l H_3BO_3, 0.03 NaF, 0.04 g/l $SrCl_2$, 0.02 g/l NH_4NO_3, 0.001 g/l $FeSO_4$ and 0.15 g/l Na_2SiO_3) in 2 L Erlenmeyerflask and incubated at 28ºC in an orbital shaker at 240rpm. After 24 hours, culture sample was collected every hour to evaluate the pH, packed cell volume (PCV) and microscopic analysis of culture growth

Culture conditions for seed batch

The mature culture obtainedin lab inoculum after 28 hours (as above) with pH 5.8±0.2 and PCV up to 05±1.0% was used as inoculum (transfer volume 10%) in seed media. Process pH was maintained around 6.0±0.2 throughout the batch with 25% liquor NH_3. Reducing sugar wasmaintained around 2.0 g/l by feeding50%sterile Dextrose solutionthroughout the batch.Tip speed (1.5 to 2.5 m/Sec) and airflow (0.5 to 1.0 vvm) were adjusted during the entire process to maintain dissolved oxygen (DO) above 10%. Back pressure (0.50-0.60 kg/cm^2) and temperature (28±1ºC) were maintained. The seed batch after 24 hours was transferred to fermenter to study the effect of changing pH range on the production of DHA (**Fig.1**).

Variation of pH in fermentation batch

The process parameters (speed and air flow) for production batch was 1.50 m/s and 0.50 to 1.00 vvm respectively and it was maintained throughout the cycle. A transfer volume of 10% was taken from seed batch for the production fermentation batch. Different pH (5.7±0.2, 6.2±0.2 and 6.8±0.2) were maintained in each production fermentation batch usingliquor NH_3. The level of reducing sugar was maintained 2.0 gm/l by feeding 50% sterile Dextrose solution, throughout the production batch.

Process Parameters for seed and production batch

Tip speed and airflow wereadjusted during the entire process to maintain dissolved oxygen (DO) above 10%. Back pressure (kg/cm^2)and temperature (ºC) were maintained at 0.50-0.60and 28±1 ºC respectively.

Estimation of Reducing Sugar

Reducing sugar estimationof the broth was done at every 04 hours interval using Dinitrosalicylic acid (DNSA) method.

HPLC Analysis for DHA

The amount of DHA in culture broth was analysed throughHPLC. Sample preparation for HPLC analysis was done using 2 ml of 10N sodium hydroxide solution and 5 ml ethanol and sonicating them for 2mins and 20 mins respectively with 2.50g of sample. Thereafter, 2ml of acetic acid was added to it and makeup the final volume 20 ml with ethanol. A portion of it was again filtered using 0.22µ disk filter and injected 20µl of sample solution in duplicate and chromatogram reading was recorded.This analysis was done after 48 hours of batch at every 12 hours interval. Hypersil BDS C-18, 100X4.6mm, 5µ column was used for the estimation of DHA. 0.1% ortho-phosphoric acid and acetonitrile was used as a mobile phase. The flow rate was set at 1.3 ml/min. The concentration of DHA was calculated by comparing the obtained peak area with standard area.

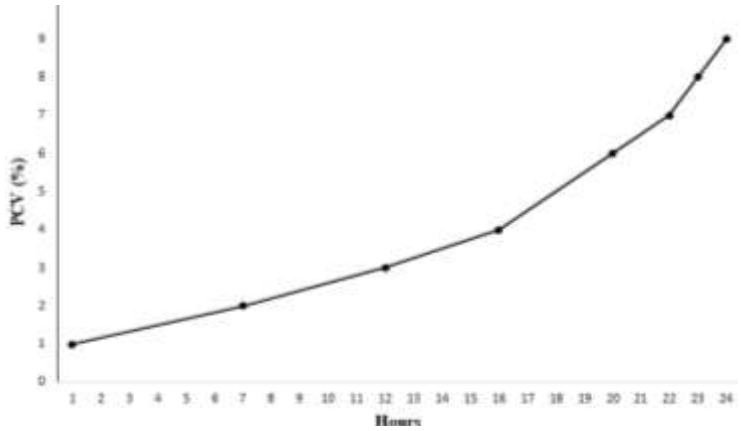

Figure 1. Packed cell volume (%) obtained after 24 hours

III. RESULTS AND DISCUSSION

Microscopic morphology at various stages of culture

The vegetative cells as observed under microscope were spherical and formed as cluster in broth. The cells possessed many spherical vacuoles as observed24 hrs after inoculating into fresh medium(**Fig. 2**). On agar plates, colonies consisted of spherical cells, but elongate amorphous cells were observed at the margins (**Fig. 3**). After inoculating into fresh medium, the size of vegetative cells increased.The morphology of the cells was also observed at production phase during the batch **(Fig. 4).**

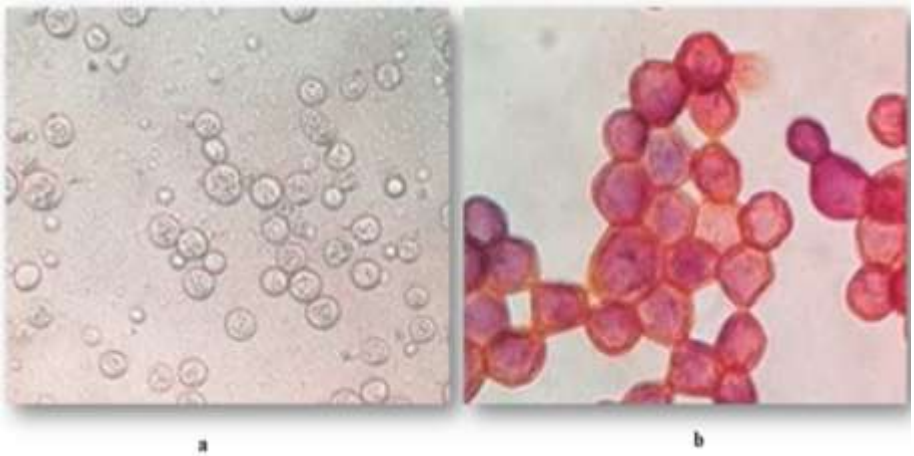

Figure 2: S. limacinum cells at initial stage as observed under microscope.

a. 24hrs after inoculation
b. Cells as observed after staining

Figure 3: Spherical colonies of S. limacinum on Agar plates

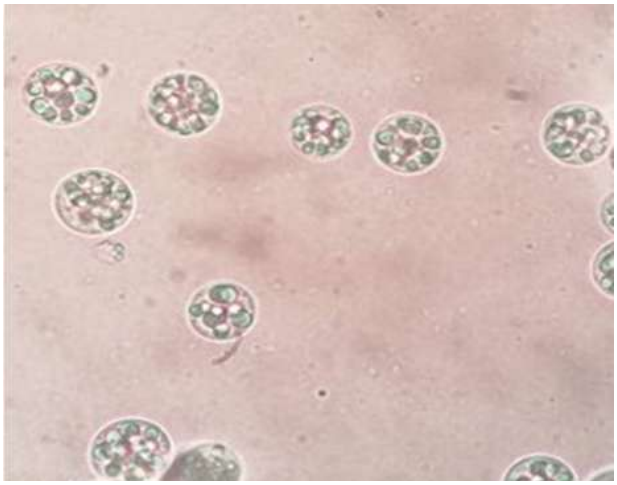

Figure 4:S. limacinum cells at production phase as observed under microscope

Effect of changing pH on DHA production

In order to study the effect of different pH on DHA production, we maintained 3 different pH conditions(5.7±0.2, 6.2±0.2 and 6.8±0.2) in each production fermentation batch. The PCV (%) and titre values of DHA obtained during production fermentation batch are shown in table 1. A maximum of 36% PCV with high titre value of DHA upto 5.5 g/l was obtained when pH value was maintained at 6.2±0.2as compared to other pH values maintained during fermentation batch (**Fig 5**).This increase in DHA titre value at 6.2±0.2 can be attributed to the optimal growth of the strain which is also well supported by the increase in PCV value. It is well established fact that the growth of microalgal strains is significantly influenced by changing pH (Fan et al., 2002). Our results are in close agreement with other reports that suggested pHrange affects DHA production from different strains of Thraustochytrids (Liu et al., 2014; Zhu et al., 2008; Arafiles et al., 2011).

S. No.	pH	PCV (%)	DHA (g/l)
1.	5.7±0.2	27	4.75
2.	6.2±0.2	36	5.56
3.	6.8±0.2	16	2.56

Table 1: DHA titre values under varying pH conditions in fermentation batch.

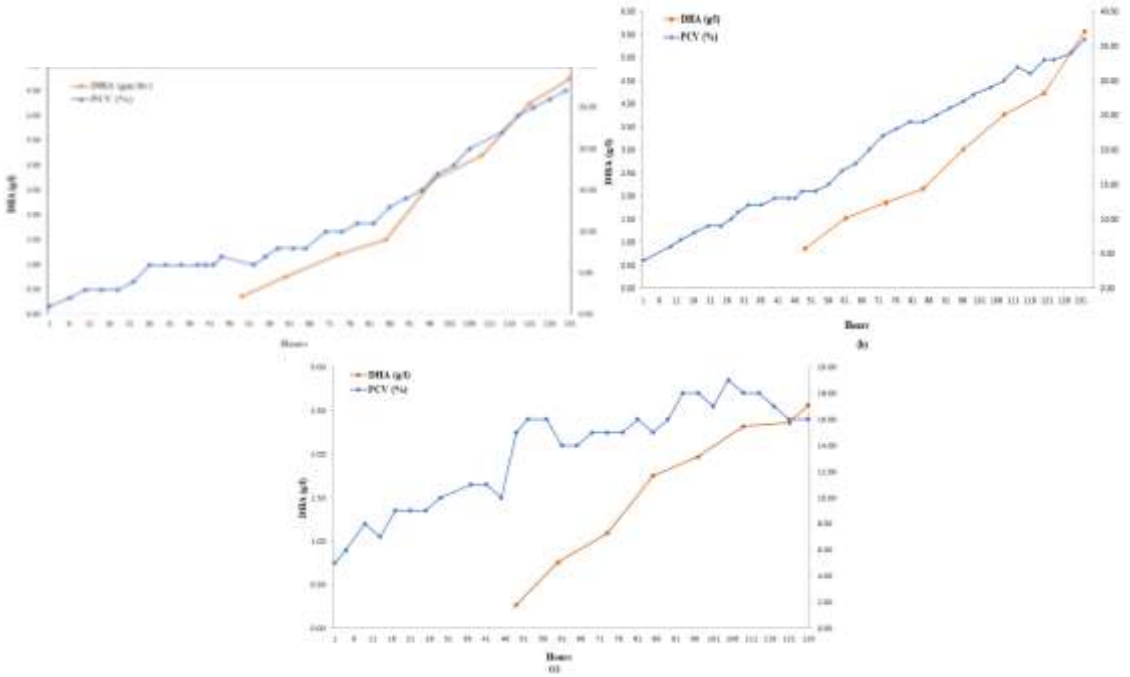

Figure 5. PCV (%) and DHA titre value (g/l) as obtained under (a) pH 5.7±0.2 (b) pH 6.2±0.2 (c) pH 6.8±0.2

IV. CONCLUSION

Since DHA production is highly influenced by growth phase, culture mode, process conditions (e.g., pH, temperature, aeration etc.), and nutrition (e.g., carbon, nitrogen, phosphorous, medium supplements, etc.), therefore it becomes essential to screen each thraustochytrid strain individually and optimize their culture conditions to derive the optimal process parameters that maximize their DHA yield. Hence, through this study we have modified and improvedfermentation processfor the industrial production of DHA from Schizochytriumlimacinum. Initial culture growth of Schizochytriumlimacinum and its optimization as lab inoculum was made for seed and production fermentation batch. Further, optimization of process parameter (pH) for enhancing the production of DHA has been achieved through this study. Through this study, we found an increase in DHA titre value up to 5.5 g/l at pH 6.2±0.2 with high PCV value up to 36%.

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