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Optimization of Docosahexaenoic Acid (DHA) Production and Improvement of Astaxanthin Content in a Mutant *Schizochytrium limacinum* Isolated from Mangrove Forest in Thailand

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ABSTRACT

Polyunsaturated fatty acids including DHA are essential dietary fatty acids. At present, fish oils are a major source, but an alternative supply is needed because of increasing demand and fish dwindling stocks. This need might be satisfied using a thraustochytrids found in mangrove forests of Thailand and identified by 18S rDNA sequencing as either *Schizochytrium limacinum* or *Thraustochytrium aggregatum*. *S. limacinum* was tested in various culture conditions to find the optimal yield of DHA. This culture medium contained 7.5% glucose, 0.5% peptone, 0.5% yeast extract (with either 0.25% soybean meal or 1% skimmed milk) and 0.75% sea salt at 20-30°C. The C:N ratio was about 15:1. The culture was mutated using NTG and one isolate showed high DHA content and also a red pigment identified as astaxanthin by TLC and HPLC. Astaxanthin synthesis peaked on day 6 - 10 of incubation in medium containing 2% glucose using shaking flasks at 180 rpm, 25°C, 2 kLux light intensity with a 18:6 h light:dark periods. Six days of incubation yielded the highest yields of both DHA (224.6 mg/l) and astaxanthin (8.9 µg/ml of medium). These results suggested that this microorganism could provide a commercial source of this valuable lipid and pigment.

Key words: astaxanthin, *Schizochytrium*, DHA, mutation, mangrove forest, Thailand

INTRODUCTION

Thraustochytrids such as *Schizochytrium* and *Thraustochytrium* are aquatic heterotrophic microorganisms commonly found in marine and estuarine environment (Barr, 1992). The capacity of thraustochytrids to accumulate large amounts of polyunsaturated fatty acids (PUFAs), especially omega-3 fatty acids including docosahexaenoic acid (C22:6, DHA), is well recognized (Lewis *et al*., 1999; Huang *et al*., 2001). They are important in preventing and treating pathologies such as coronary heart disease, stroke and rheumatoid arthritis (Kinsella, 1987), provide protection against asthma, dyslexia, depression and some forms of cancer (Simopoulos, 1989; Takahata *et al*., 1998). DHA is an essential fatty acid for neuronal development (Yongmanitchai and Ward, 1989). Demand of these fatty acids as a dietary supplement has increased and the major supply is presently derived from fish oil. But dwindling fish stocks and increasing demand has created a need

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for alternative sources of supply.

Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'dione) is a carotenoid found especially in marine crustaceans. It is added to food products (Vazquez *et al*., 1997) and use as a colorant for cultured fish, poultry (Johnson and An, 1991) and shrimp. It also acts as a scavenger of free oxygen radicals which damage DNA and oxidizes proteins (Schroeder and Johnson, 1993). Astaxanthin used as an animal feed is often produced commercially by chemical synthesis. However, the public have a preference for additives coming from natural source (Fang and Cheng, 1993) such as algae, fungi and small crustaceans. When in the food chain, they lead to pigmentation of larger animals including fish (especially salmon), lobsters, krill and small marine and freshwater organisms (Johnson and Lewis, 1979).

Recently, microbial production of astaxanthin pigment has been improved through isolated or combined strategies, *i.e.*, mutagenesis and media fermentation (Fontana *et al*., 1996). The thraustochytrids, *Schizochytrium aggregatum* (Valadon, 1976) and *Thraustochytrium* CHN-1 (Marvelisa *et al.*, 2003), have both been found to contain this pigment. This study aims to improve both the astaxanthin and DHA production by the creation of mutations of *Schizochytrium* sp. BR2.1.2 and also by optimizing the media and conditions in small scale cultures and then applying to larger vessels.

MATERIALS AND METHODS

Microorganisms

Wild type strain

The wild type of thraustochytrid selected strain BR2.1.2 was isolated from mangrove forest at Bang-rong area, Amphur Thalang in Phuket province, Southern Thailand. The isolation was carried out in GPY agar medium (Huang *et al*., 2001) by baiting technique. After a series of streaking on the agar medium, pure culture was obtained for this study.

Identification of microorganism by 18S rDNA sequencing

Morphological characteristics of thraustochytrid BR2.1.2 resembled *Schizochytrium*. Identification was further confirmed by 18S rDNA sequencing. Two primers of NS1 and NS8 were used for amplification of 18S rDNA by PCR technique using a thermal cycler (Perkin Elmer GeneAmp PCR system 2400). The amplification program was carried out following the protocol of Mo *et al*. (2001). Purified PCR product of 18S rDNA was analyzed by DNA autosequencer with NS1-8 primers set according to White *et al*. (1990)

Mutagenesis

Increased expression of astaxanthin was sought by mutagenesis of the wild type BR2.1.2 using N-methyl-N'-nitro-N-nitrosoguanidine (NTG) (Fluka Chem, AG) modified from Chaunpit (1993). The initial concentration of 1-9×10^6 cells/ml was treated with NTG (0.1 mg/ml) for 20 min with shaking. NTG was removed from suspension by centrifugation and cells pellet washed by 0.5 M phosphate buffer pH 7.0 and spreaded on GPY agar plate. The treated culture contained 0.05 – 0.1 % of the initial cells. Red colonies indicating accumulation of astaxanthin were collected for further assessment of growth, astaxanthin and DHA contents.

Optimization of growth and DHA production by wild type BR2.1.2

Culture conditions for thraustochytrid BR2.1.2 were optimized for growth and DHA production. The following conditions were applied throughout unless otherwise stated. Thirty milliliters of GPY medium (Huang *et al*., 2001) composed of 3% glucose, 1% peptone, 0.5% yeast extract and 50% of natural sea water was used as

the basal medium and placed in a 125 ml Erlenmeyer flask. All experiments were carried out in triplicate flasks. Cultivation was initiated by addition of 1 ml of inoculum (adjusted cells concentration to 1.0 at OD 600 nm). Incubation was done on a rotary shaker (Sac Science-ENG LTD, Part) at 140 rpm at room temperature for 4 days. To test different media, the basal media was modified in the following manner:

1. The carbon source replaced by either glucose, fructose, sucrose, glucose syrup and agricultural products i.e. molasses, and sugar cane juice (Sahakarnnamtan Co. Ltd., Chonburi, Thailand).

2. The nitrogen sources replaced by peptone, soybean meal, skimmed milk, ammonium sulfate, potassium nitrate, sodium nitrate, monosodium glutamate (MSG).

3. Sea salt concentration (salinity) 0-200% of sea water.

The effect of temperature on growth and DHA production were also determined by using temperature gradient incubator (Model TN-3, Toyo, Kagaku Sangyo Co., Ltd., Tokyo, Japan) set at 15, 20, 25, 30 and 35°C.

Optimization of growth and astaxanthin production by thraustochytrid mutant

The mutant was cultivated in a 125 ml Erlenmeyer flask containing 30 ml of GYC medium (Marvelisa *et al*., 2003) and kept in an incubator shaker at 180 rpm for 10 days at 25°C. Light was provided by fluorescent lamps at the intensity of 2 kLux with light:dark periods at 16:8 hrs. Effects of carbon sources such as sugar cane juice, molasses and maltose:glucose (1:1, w/w) contained the same carbon equivalent as 2% glucose were studied. Environmental conditions such as light intensity at 0, 5 and 10 kLux and temperature (as described above) were also determined.

Analytical procedures

Growth was determined as the dry weight of the cells (drying conditions).

Lipid was extracted by the modified method of Bligh and Dyer (1959), followed by transmethylation according to Holub and Skeaff (1987). Fatty acid methyl esters were analyzed in a gas-liquid chromatography (GC-14B; Shimadzu, Tokyo, Japan) equipped with flame ionization detector and a split injector at 1:40 ratio using capillary column in 30 m length, 0.25 mm internal diameter, 0.25 mm. film thickness (AT-WAX, Alltech Associates Inc, USA). Fatty acids were identified by comparing retention times with authentic standards from Sigma by using C-R6A Chromatopac Data Integrator (Shimadzu, Japan).

The astaxanthin content was determined by the method modified from Fontana *et al*. (1996). The concentration was quantified by using absorbance values at 479 nm calculated with the specific absorption coefficient $a_{(1cm,1\%)} = 1600$ as proposed by Anderwes *et al.* (1976). Isomers of astaxanthin were identified by thin layer chromatography according to Donkin (1976) compared with reference standards extracted from *Haematococcus pluvialis*. These determinations were confirmed by HPLC (model HP1100, Agilent Technology) following the procedure of Marvelisa *et al*. (2003).

RESULTS AND DISCUSSION

Identification of thraustochytrid BR2.1.2 by 18S rDNA sequence analysis

The corrected partial sequence of 18S rDNA of thraustochytrid BR2.1.2 was 912 bases in length after gaps, inserts and ambiguous positions had been removed and was deposited in DDBJ as accession number 794133. A phylogenetic tree was constructed from an alignment of the BR2.1.2 sequence with those from related species obtained from GenBank by the NJ method (Figure 1). It was clearly seen that

BR2.1.2 formed the same clade with *Thraustochytrium aggregatum* and *Schizochytrium limacinum* but with slight distance. Hence the strain BR2.1.2 was finally identified as *Schizochytrium limacinum.*

Effect of culture conditions on growth and DHA production by *S. limacinum* BR2.1.2

1. Carbon source

Among the various carbon sources tested, *S. limacinum* BR2.1.2 exhibited highest growth rates in 3% fructose and glucose with 14.3 and 13.4 g/l of CDW, respectively (Figure 2A). DHA yields were 392.5 and 362.1 mg/l with DHA contents at 49.1 and 49.7% of TFA, respectively. Although, relatively good growth rates were obtained in complex carbon sources, i.e. molasses (10.5 g/l) and sugar cane juice (11.5 g/l), DHA production was low. Sucrose and glucose syrup were poorer carbon source for this organism. The results coincided with those of Wu *et al*. (2005) as glucose syrup contained mainly oligosaccharides that could not support growth for many microorganisms. Although glucose was slightly inferior compared to fructose, it is considered to be the good carbon source, because it was ready available and substantially cheaper.

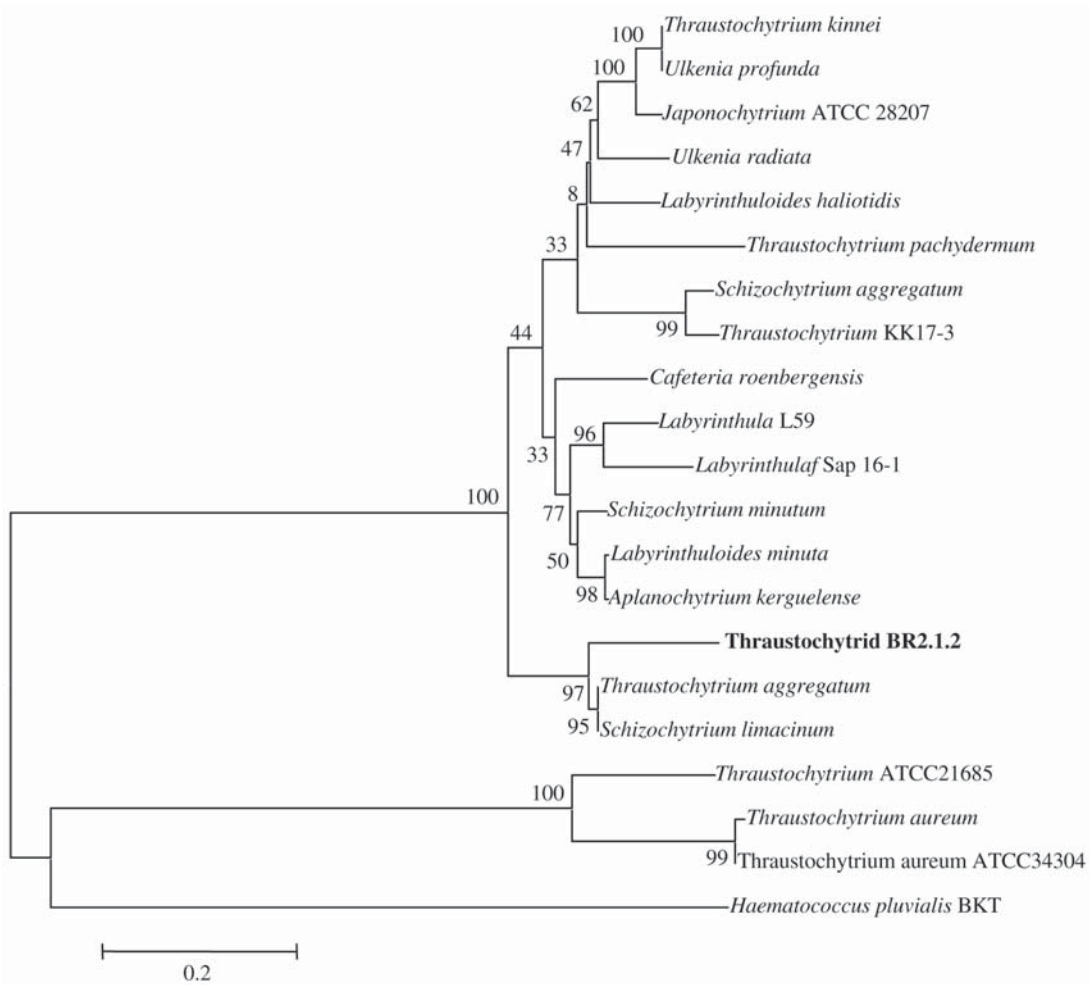

Figure 1 Phylogenetic tree reconstruction based on 18S rDNA sequence by neighbour-joining (NJ) method. The number at each branch shows bootstrap values 1000 replications.

Figure 2B demonstrates the effect of glucose concentration. Cell mass depended on glucose concentrations and was maximal with 7% (cell mass 28.3 g/l). However, the highest DHA production was obtained in 5% glucose (732 mg/l) making up 50.6% of TFA. When glucose concentration was increased to 7%, the proportion of DHA (641.1 mg/l) was 44.6% of TFA.

2. Nitrogen source

Further experiments used 5% glucose and 0.5% yeast extract to determine the effect of the nitrogen source in Figure 3A. Results revealed that among complex nitrogen sources (1% peptone) was the best in supporting growth for both CDW (20.9 g/l) and DHA (828.2 mg/l). Soybean meal and skimmed milk although relatively good nitrogen source for CDW but DHA production was considerably lower at 441.8 and 545.9 mg /l, respectively. In the medium containing 0.2% MSG, BR2.1.2 grew at 20.3 g/l and produced DHA 768.5 mg/l, almost the same levels as supported by 1% peptone. This result agrees with those using with *Thraustochytriun aureum* ATCC 34304 that grew well in medium containing glucose, peptone, yeast extract and

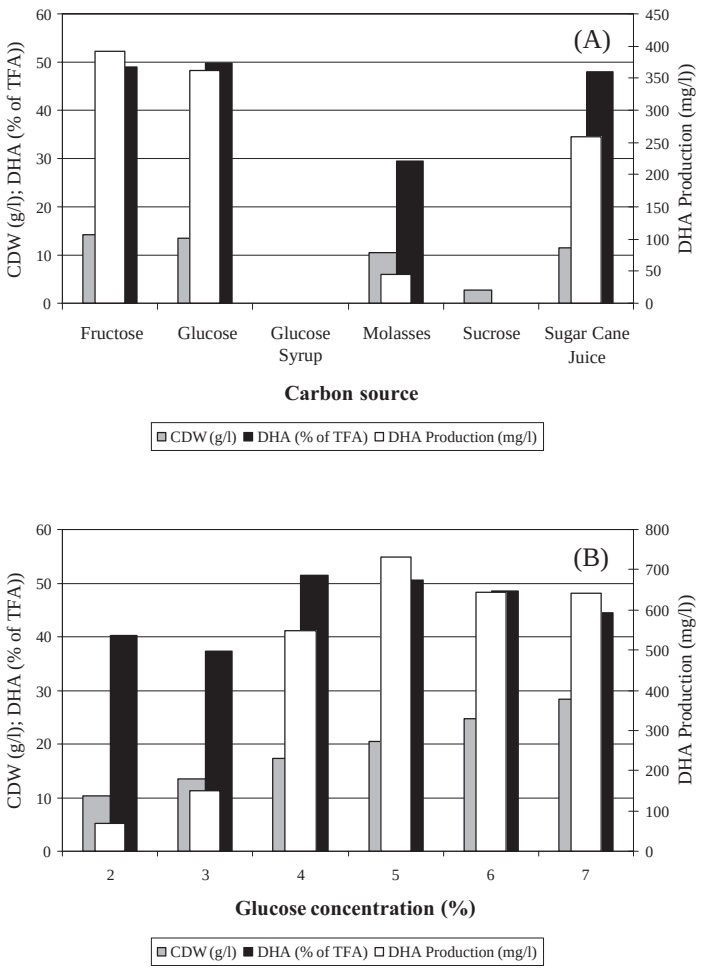

Figure 2 Effect of (A) carbon sources and (B) glucose concentration on growth and DHA production by *S. limacinum* BR2.1.2 cultivated at room temperature in shaker 140 rpm.

supplement with glutamate (Iida *et al*., 1996).

Although, soybean meal and skimmed milk were slightly inferior compared to peptone and probably not suitable as sole nitrogen source, they are agricultural products that are less expensive and readily available in Thailand. Moreover, soybean meal not only provided protein but also carbohydrate, fat, mineral and vitamins which was likely to support growth and DHA production of thraustochytrids (Fan *et al*., 2002). Therefore, they could partially replace peptone which was expensive and economically unsuitable for large scale production. Figure 3B shows the effect of various peptone and soybean meal mixtures on growth and DHA production by *S. limacinum* BR2.1.2. In this experiment, the base medium consisted of 5% glucose, 1% skimmed milk and 0.2% MSG and 0.5% yeast extract. Clearly, treatment with 0.5% peptone and 0.25% soybean meal produced highest DHA contents at 1,170.9 mg/l which was 45.3% of TFA.

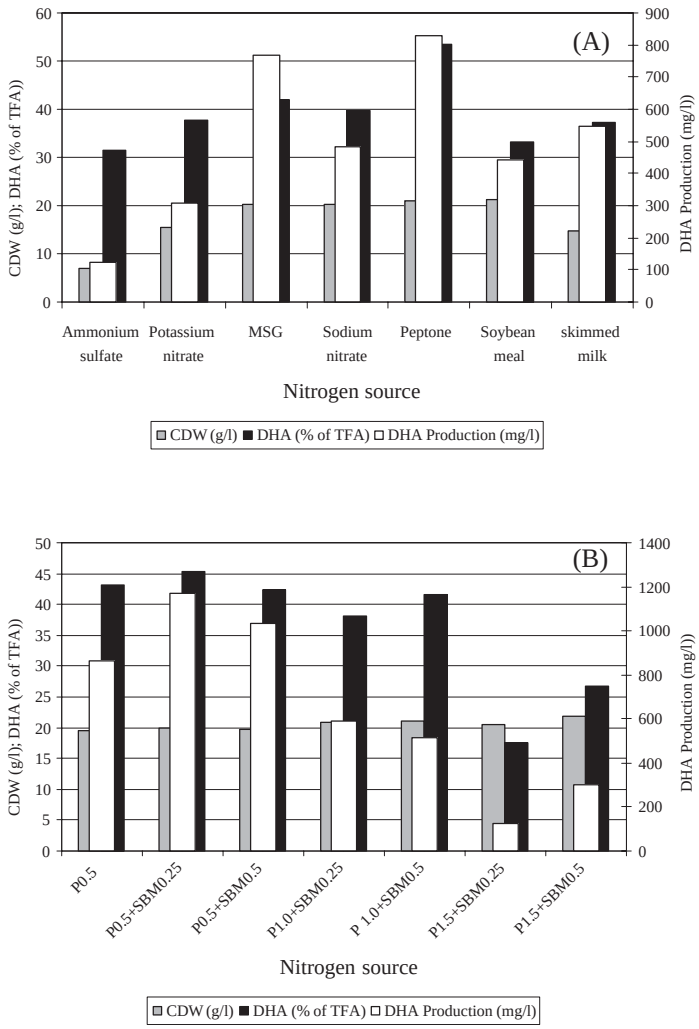

Figure 3 Effect of (A) single nitrogen source and (B) combined nitrogen source on growth and DHA production by *S. limacinum* BR2.1.2 cultivated at room temperature in shaker 140 rpm (P = peptone; SBM = soybean meal).

3. C/N ratio

Lipid accumulation in oleaginous microorganisms can be enhanced by providing excess carbon while limiting nitrogen (Ratledge, 2004). Figure 4 showed that optimum C/N ratio at 15:1 was suitable for *S. limacinum* BR2.1.2 in terms of growth and DHA production of 2,416.7 mg/l. Although, the cell concentration was improved (27.6 g/l) the highest biomass of 38.0 g/l was achieved in medium with C/N ratio of 20:1.

4. Salinity

Seawater was used as the source of salinity in this study. It should be noted that although *S. limacinum* BR2.1.2 was isolated from marine environment, it could grow and produced DHA at all levels of salinity (Figure 5). The results coincided with Yokochi *et al*. (1998) who reported that *S. limacinum* SR21 could grow in condition at zero salinity or without salt. However, a salinity equivalent to 25% of natural sea water appeared

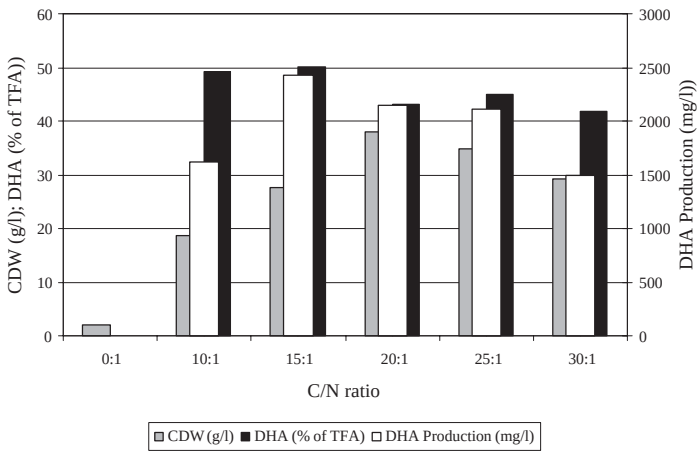

Figure 4 Effect of C:N ratio on growth and DHA production in *S. limacinum* BR2.1.2 cultivated at room temperature in shaker 140 rpm.

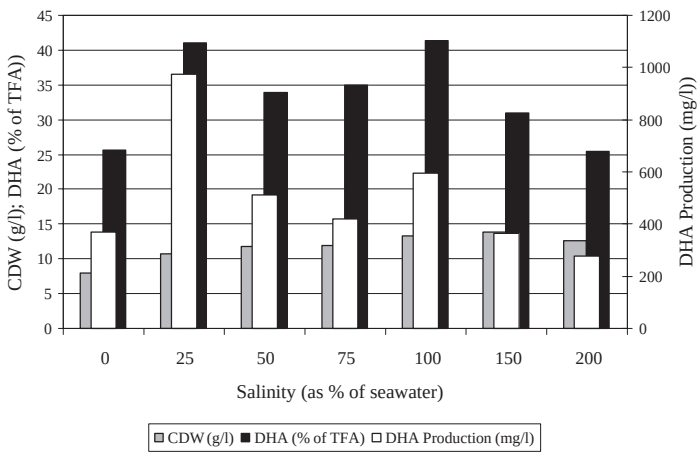

Figure 5 Effect of salinity (as % of seawater) on growth and DHA production in *S. limacinum* BR2.1.2 cultivated at room temperature in shaker 140 rpm.

optimal for *S. limacinum* BR2.1.2 for DHA production (975.4 mg/l , 41.1% of TFA). At the highest salinity (200%), the organism showed good growth but DHA production was lowest at 277.5 mg/l This contrasts to *T. aureum* which fails to grow at zero salinity and also completely inhibited at 200% salinity of sea water (Iida *et al*., 1996).

5. Effect of temperature

In this study cultures were grown in L-shaped tubes containing 10 ml medium and incubated in a temperature gradient incubator. *S. limacinum* BR2.1.2 grow well and produced fairly constant DHA levels at a wide range of temperature between 20-30°C. Growth of culture varied from 8.7-10.3 g/l, and DHA contents were 220-236 mg/l (Figure 6).

Improvement of astaxanthin content by mutation

Although, culture of *S. limacinum* BR2.1.2 in liquid GPY medium was creamy white color, it developed orange colonies on agar plate after several weeks of incubation. This might be explained by an accumulation of carotenoid pigments. Preliminary analysis of the pigments by TLC and HPLC confirmed that it was astaxanthin. Hence, it was considered to be appropriate to improve the content of this pigment in *S. limacinum* BR2.1.2 by mutation. If successful this organism would provide two important nutrients, *i.e.*, DHA and astaxanthin making it suitable for animal and human consumption

1. Isolation of *S. limacinum* BR2.1.2 mutants

From an initial *S. limacinum* BR2.1.2 concentration of 8.75×10^6 cells/ml, the culture was treated with NTG for 20 minutes which yielded a 0.05% cell survival rate. The treated culture was then plated on GYP medium but only one colony showed a distinctive red color. After sub-culturing for several times the deep red color persisted which showed that it was stably expressed. The mutant was then used for further investigation.

The mutant grew rapidly for the first 2 days with cell concentration of 7.8 g/l. Maximum cells mass was obtained on the 6^{th} days at 10.8 g/l and declined gradually (Figure 7). Astaxanthin contents in cell mass increased corresponding with growth and reached highest value at 8.9 µg/ml and remained relatively constant towards the end of fermentation. This result coincided with Marvelisa

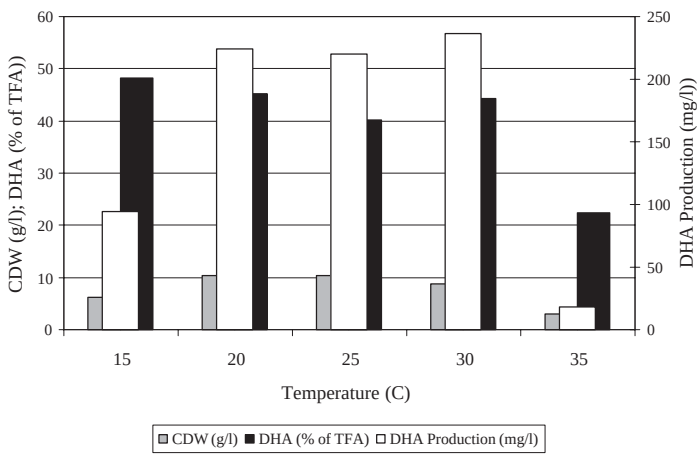

Figure 6 Effect of temperature on growth and DHA production in *S. limacinum* BR2.1.2 cultivated in L-shaped tubes.

et al. (2003) who reported that, carotenoid contents of *Thraustochytrium* CHN-1 paralleled the biomass and cell growth. The mutant *S. limacinum* BR2.1.2 could produce both DHA and astaxanthin at moderate amounts. However, DHA production decreased from 224.6 mg/l day 6 to only 29.8 mg/l at day 10. Hence it seemed that we have to sacrifice either DHA or astaxanthin production depending on the degree of necessity.

2. Effect of light intensity on astaxanthin accumulation by *S. limacinum* BR2.1.2 mutant

Under dark condition, the mutant accumulated 5.6 µg/ml of astaxanthin at 25°C after incubation for 8 days. However, when fluorescent light source of 5 kLux was provided, the culture produced higher pigment yield of 13.1 µg/ml. Further increase of light intensity to 10 kLux had adverse effect on astaxanthin production (10.7 µg/ml) (Figure 8). Therefore, moderate light was an important bioinduction for carotenogenesis as it was also shown by *Phycomyces blaksleeanus* and several species of *Rhodotolula* (Goodwin, 1984). Yamaoka *et al*. (2004) also demonstrated that *Thraustochytrium* sp. CHN-1 grown under

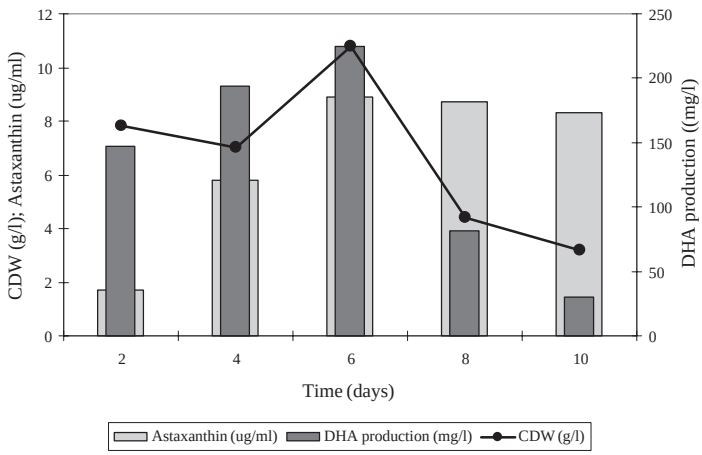

Figure 7 Growth, astaxanthin and DHA production by *S. limacinum* BR2.1.2 mutant strain in GYC broth at 25°C with 2 kLux light intensity and light:dark 16:8 hrs.

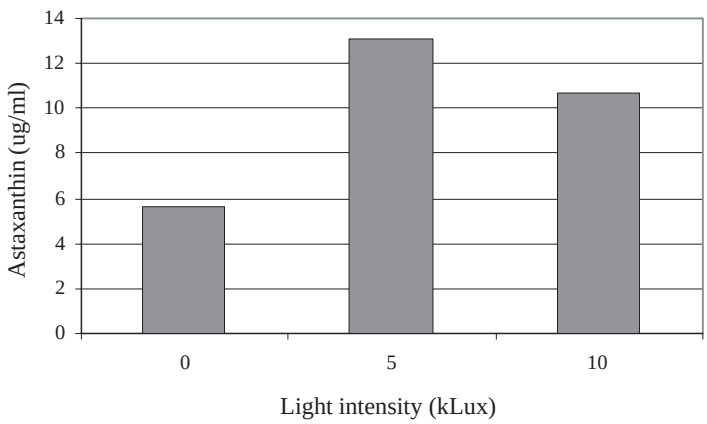

Figure 8 Effect of light intensity for astaxanthin production by *S. limacinum* BR2.1.2 mutant strain in GYC broth at 25°C, 180 rpm for 8 days.

fluorescent lamp at 1.5 kLux developed orange to red color.

CONCLUSIONS

A thraustochytrid strain BR2.1.2 was isolated from mangrove forest in Thailand. The strain showed an ability to grow rapidly while accumulating large amounts of DHA. Identification of the strain based on morphological characteristics and 18S rDNA sequence revealed that it belonged to *Schizochytrium limacinum* species. Under optimal culture conditions, *i.e*., 5% glucose, combined nitrogen source (0.5% peptone, 0.2% MSG, 0.25% soybean meal and 1% skimmed milk) and C/N ratio at 15:1, the DHA yield was 2,416.7 mg/l from a cell dry weight of 27.6 g/l. Furthermore *S. limacinum* BR2.1.2 had a unique feature of growing in media having a wide range of salinity equating to 0-200% seawater. When the strain was cultivated in liquid GPY medium the culture appeared creamy white color. But on agar medium with prolong incubation, color of the colony developed into typical orange color of carotenoid pigment which was identified as astaxanthin. Improvement of *S. limacinum* BR2.1.2 for astaxanthin content by mutation with NTG was carried out and resulted in a colony with intense red color. This mutant produced astaxanthin in liquid medium even without light. Optimization of culture conditions in liquid GYC medium, particularly high light intensity at 5 kLux at 25°C caused the mutant to accumulate the pigment at 13.1 µg/ml.

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