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Production of ω-3 Polyunsaturated Fatty Acids From Cull Potato Using an Algae Culture Process

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Abstract

Algal cultivation for converting cull potato to docosahexaenoic acid (DHA) was studied. *Schizochytrium limacinum* SR21 was selected as the better producing strain, compared with *Thraustochytrium aureum* because of higher cell density and DHA content. Used as both carbon and nitrogen source, an optimal ratio of hydrolyzed potato broth in the culture medium was determined as 50%, with which the highest production of 21.7 g/L dry algae biomass and 5.35 g/L DHA was obtained, with extra glucose supplemented. Repeat culture further improved the cell density but not fed batch culture, suggesting limited growth was most likely caused by metabolites inhibition.

Index Entries: Docosahexaenoic acid; microalgae; omega-3 fatty acid; *Schizochytrium*; cull potato; fish oil.

Introduction

ω-3 Polyunsaturated fatty acids (ω-3 PUFAs) are a group of fatty acids containing two or more double bonds, of which the last double bond is located at the third carbon atom from the methyl terminal. Docosahexaenoic acid (DHA, 22:6) is a particularly important ω-3 PUFA, with a 22-carbon chain and six double bonds. It has been reported that DHA is an essential nutrient during early human development *(1,2)*; it is supplied to the infant through the placenta during pregnancy and through human milk after birth. Being an important component of the photoreceptor cells of infants' retinas, DHA is also involved in the development of infants' brain tissues through incorporation in synaptic vesicles, myelin, and mitochondria. As a result of its important role in infant development,

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inclusion of supplementary DHA in infant formulas is recommended by the World Health Organization *(3)*.

The conventional source of ω-3 PUFAs is mostly from fish oil. Cod, salmon, sardine, mackerel, menhaden, anchovy, tuna, and seal are generally used for fish oil production. The quality of fish oil is variable, being dependent on fish species, season, and geographical location of the catch site. As marine fish oil is a complex mixture of fatty acids with varying chain lengths and degrees of saturation, DHA needs to be refined from fish oil for use in nutraceutical/pharmaceutical applications. The purification of DHA from low-grade fish oil can be difficult and costly *(4)*, and in addition, marine fish stocks are subject to seasonal and climatic variations, and might not be able to provide a steady supply for the increasing demands of DHA. In fact, fish are not capable of synthesizing PUFA *de novo* and much of their PUFA is derived from the primary producer in the oceanic environment: the microalgae or algae-like microorganisms *(5)*.

DHA produced from heterotrophic algal culture is taking on a more and more important role and could, with increased economic viability, gradually substitute fish oil, as it offers better taste, odor, and stability *(5)*. Although a large number of microalgae contain DHA, only a few species have demonstrated production potentials on an industrial scale *(6)*, in that they can accumulate high oil contents in their biomass, produce a high percentage of total lipids as DHA, and reach high biomass densities in a short time. Strains from the *Traustochytrid* marine protists and dinoflagellate *Crypthecodinium cohnii* have traditionally been considered to have the most potential in a commercial setting, especially the former *(5)*, which belongs to the genus *Thraustochytrium* and *Schizochytrim*. Initial research on *Thraustochytrium* produced relatively low cell densities around 5.0–20.0 g/L as well as low DHA content in the biomass. Lately, although, new *Schizochytrium* strains have been isolated *(7)*. In initial research with these strains, the highest DHA productivities were 2.0 g/L·d using optimized media *(8)*. The lipid extracted from the cell was about 50% of the dry cell weight and the DHA content of the lipid was 34% of total fatty acids. Optimal culture conditions for the specific strain *Schizochytrium limacinum* SR21 have also been investigated. A high total fatty acids content, up to more than 50%, was obtained with corn steep liquor as the nitrogen source and an increase in carbon source concentration led to a high-DHA yield *(9)*. In this work, a DHA yield of > 4.0 g/L was obtained in both glucose and glycerol media at 9% and 12% concentrations, respectively, which indicated that *S. limacinum* SR21 is a promising algae strain for DHA production.

High-yield production culture of *Schizochytrium* strains has also been investigated *(10)*. With increased carbon (glucose) and nitrogen (corn steep liquor and ammonium sulfate) sources in the medium, 48.1 g /L dry cell weight and 13.3 g/L DHA were produced in a 4-d culture with 12% glucose. The lipid content was 77.5% of dry cells, and the DHA content was 35.6% of total fatty acids. With this excellent performance, this algae

strain was eventually applied to the commercial sector. The new high cell density fed batch fermentation process developed by the commercial sector splits the overall fermentation process into a biomass density increasing stage and a DHA production stage; resulting in biomass densities of at least 100.0 g dry cell/L in the fermentation broth and at least 20% of their dry cell weight as lipids *(11)*.

Even with such yield and productivity improvements, fish oil is still a substantial competitive threat to the new commercialized DHA productions from algae fermentation *(12)*. Urea crystallization, supercritical fluid extraction, and high-performance liquid chromatography are used for extraction and purification of DHA from fish oil, which are difficult and costly. However, several companies have developed microencapsulated fish oil products that claim to have resolved much of the odor, stability, and taste issues associated with fish oil whereas simultaneously removing the need for costly purification. Thus, it is still indispensable for research to continue to decrease the cost of production from heterotrophic algal cell cultivation. Using under-utilized cull potato to replace glucose as a carbon source and to provide the nitrogen source might be a good choice. There are more than 10–15% of harvested potatoes that are classified as culls, and therefore unfit for market or processors. Development of new markets for the culls is essential to the profit margins of the potato producers because the cost to grow the culls is $70–120/t, whereas presently they can be sold as an animal feed for only about $10–20/t. Converting the culls to a value-added bioproduct is an excellent way to develop new markets and overcome the cost to produce the culls, as they can provide starch, protein, vitamins, and salt nutrients for the fermentation process. In fact, microbial production of lactic acid from potato starch has been widely reported *(13)*. Various bacteria such as *Lactobacillus*, *Lactococcus* and *Streptococcus*, and the fungi *Rhizopus* were used as the producers, with hydrolyzed potato starch as the carbon source *(14–16)*. Among them, some strains of *Rhizopus oryzae* can use potatoes as the sole nutrient supply in the culture medium. With one of these strains, Liu et al *(17)* optimized the culture condition to produce 33.3 g/L lactate. This indicates that cull potato can be an effective feedstock for the production of lactic acid and potentially other valued nutraceuticals.

Compared with lactate, DHA is more valuable. If these potatoes can be converted to DHA, it will not only benefit the DHA manufacturers, in that this will significantly reduce the medium cost of the production process, but also the farmers because the high value-added product will better offset the production costs. Preliminary research for the development of such a process was investigated in this article. The specific objectives of this study were:

1. Selecting a high yield DHA producer algae strain.
2. Determining the optimum ratio of HPB to the whole medium.
3. Developing a higher cell density culture process.

Methods

Algal Strains

The alga strain *Thraustochytrium aureum* (American Type Culture Collection [ATCC] 34304) and *S. limacinum* SR21, (ATCC MYA-1381) were used in the experiments. The seed cells were cultured in artificial seawater medium, as described by the University of Texas at Austin, Culture Collection of Algae (UTEX) (http://www.bio.utexas.edu/research/utex/), with 5.0 g/L glucose, as well as 1.0 g/L yeast extract, and 1.0 g/L peptone.

Culture Conditions

The culture medium for DHA production consisted of artificial sea water, 5.0 g/L corn steep solids (Sigma, St. Louis, MO), and 1.0 g/L ammonium acetate as nitrogen sources plus either pure glucose, or as described in the later experiments, a certain percentage of hydrolyzed potato broth (HPB). The initial medium pH was adjusted to 6.0 by addition of hydrochloric acid. The cells were cultured in 250-mL Erlenmeyer flasks at 20°C, with inoculums of 5 mL seed cells to 50 mL culture medium. After 6 d culture, the algae cells were harvested to conduct dry cell weight and fatty acid analysis.

To prepare HPB, cull potato was boiled and minced, mixed with certain volume of water and placed in a 5-L tank with agitation. Two enzymes, α-amylase and glucoamylase were used to hydrolyze the potato starch into glucose. The temperature used in the hydrolysis process was 55°C. After 4 d, the hydrolyzed broth was harvested and centrifuged to remove the solids. The glucose concentration in the liquid phase was determined and then diluted to 100.0 g/L with water.

Cell Growth and Fatty Acid Analysis

The algae biomass in the broth was harvested and centrifuged, removed from the medium, and washed twice with distilled water. The biomass was then dried at 105°C for 3 h and weighed.

One milliliter of the same biomass was distributed to screw top glass tubes; then placed in a freeze dryer overnight. The preparation of fatty acid methyl ester (FAME) directly from algae biomass was used, putting to use a method as described in Indarti *(18)*. After freeze-drying, a 4 mL mixture of methanol, concentrated sulfuric acid and chloroform (1.7 : 0.3 : 2.0 [v/v/v]) were added into the tube. Four milligrams arachidonic acid (C20 : 4) or heptadecanoic acid (C17 : 0) was added as the internal standard. Tubes were placed inside a heated water bath at 90°C for 40 min. On completion of the reaction, the tubes were cooled down to room temperature and weighed again to dismiss leaking samples. Then, 1 mL of distilled water was added into the mixture and thoroughly vortexed for 1 min. After the formation of two phases, the lower phase containing the FAME was transferred to a clean, 10-mL bottle and dried with anhydrous Na_2SO_4. Half milliliter-dried

Table 1
Comparing Cultures of the *S. limacinum* SR21 with *T. aureum*

Strain	Carbon source	Concentration (g/L)	Dry cell weight (g/L)	DHA yield (g/L)	DHA/ biomass (%)
T. aureum	Soluble starch	30	7.7	0.65	8.4
	Potato starch	30	ND[a]	0.40	ND[a]
S. limacinum	Pure glucose	30	12.6	2.30	18.2
SR21	Potato glucose	30	10.8	1.75	16.2
	Pure glucose	90	20.1	3.51	17.5
	Potato glucose	90	9.2	0.52	5.7

[a]Not determined, because residual potato starch was mixed with the algae biomass.

solutions were transferred into a vial and stored in a freezer (–20°C) for gas chromatography (GC) analysis. The method of FAME analysis using a GC was the same as reported by Wen and Chen *(19)*. Briefly, a GC machine from HP Inc. and a GC column from Restek (cat no: 12498) were used in this analysis. The parameters of this GC column are: 30 m × 0.32 mm ID × 0.25 μm.

Results

Alga Strain Selection

T. aureum can take starch directly as a carbon source to support its growth *(20)*, whereas *S. limacinum* SR21 grows poorly in starch *(9)*. The carbon in the cull potato mainly exists as starch. The cost of hydrolyzing the starch could be avoided if the starch could be utilized directly. In the preliminary work (data not shown) with *T. aureum*, the highest dry cell weight was obtained when the starch concentration was 30.0 g/L, thus, this concentration was used in this experiment. The performance of these two algal strains was compared, as shown in Table 1. *T. aureum* was cultured with both soluble starch (Sigma) and potato starch, whereas *S. limacinum* SR21 was cultured with two levels (30.0 g/L and 90.0 g/L) of pure glucose or a certain amount of HPB. Results of the study showed that *T. aureum* reached a maximum dry cell weight of 7.7 g/L, but with the same amount of carbon source, *S. limacinum* reached a maximum of 12.6 g/L dry biomass. Additionally, the DHA yield and DHA content in the biomass was much higher in *S. limacinum*. The fatty acids profile of *S. limacinum* SR 21 in the GC analysis was shown in Fig. 1.

Even higher dry cell weights and DHA yield were obtained with *S. limacinum* SR 21 when 90.0 g/L of glucose were used instead of 30.0 g/L. This indicated that *S. limacinum* SR 21 can grow well on a high carbon source concentration. Unfortunately, the culture result with HPB containing 90.0 g/L glucose was lower than its 30.0 g/L counterpart, which implies that inhibition occurred when a high percentage of HPB was used. These

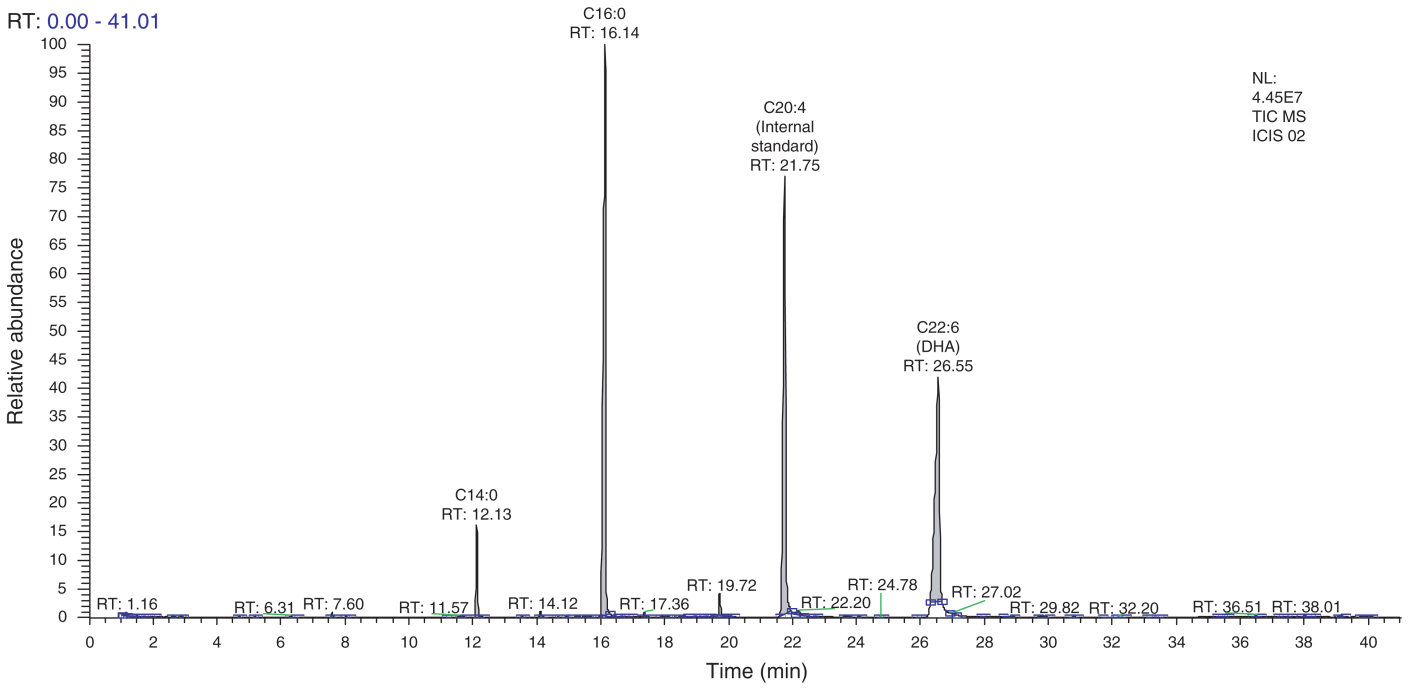

Fig 1. GC analysis of the FAME from *S. limacinum* SR 21.

Table 2
Culture Results With Various Concentration of HPB

Carbon source	Nitrogen source added	HPB/ medium (% [v/v])	Glucose concentration (g/L)	Nitrogen concentration (g/L)	Dry cell weight (g/L)	DHA yield (g/L)	DHA in biomass (%)
Group 1: HPB	5.0 g/L Corn steep solids	70	70	1.74	18.1	2.70	14.9
		50	50	1.40	18.6	2.70	14.5
		30	30	1.06	11.7	1.60	13.7
		70	70	1.19	17.3	2.79	16.2
Group 2: HPB	Without	50	50	0.85	17.0	2.81	16.5
		30	30	0.51	10.8	1.75	16.2
Group 3: Pure glucose	5.0 g/L Corn steep solids	–	70	0.55	21.2	3.76	17.7
		–	50	0.55	19.0	3.35	17.6
		–	30	0.55	12.6	2.30	18.2

results show that the performance of *S. limacinum* was much better than *T. aureum* and although the latter can directly utilize starch, the production efficiency was too low. Thus, *S. limacinum* was used as the DHA producing strain in the ensuing experiments.

HPB Percentage Optimization

As shown in Table 1, the growth of *S. limacinum* SR21 was inhibited with the use of a high HPB concentration, which has been inferred to be caused by the presence of a certain inhibitor in the HPB. One hypothesis was that a potential inhibitory source might be the high nitrogen source, in that the nitrogen content in the HPB was 1.7 g/L (total kjeldahl nitrogen [TKN]), which is equivalent to the nitrogen in 15.0 g/L of corn steep solids. It is well known that the C/N ratio strongly affects the lipids accumulation in the DHA producers, so determining the optimal HPB concentration and corresponding nitrogen concentration in the culture was necessary. In this experiment, the HPB culture was diluted to various concentrations, as shown in Table 2. The cultures with and without added nitrogen source (5.0 g/L corn steep solids) were also compared. The cultures with pure glucose were taken as the control.

Compared with the culture with 90.0 g/L pure glucose in which 20.1 g/L dry algae biomass was obtained, the biomass production in the culture with 90% HPB was only 9.2 g/L, as shown in Table 1. This indicates that there was severe inhibition in the culture with a high percentage of HPB. But in the range of 30–70%, the differences were not significant ($p > 0.05$, as shown in Table 3). This indicates that the inhibition were relatively moderate in the lower percentage of HPB, owing to the corresponding lower inhibitor

Table 3
The *p*-Value of Paired *t*-Test of Various Groups in Table 2

	Dry cell weight	DHA yield	DHA content in biomass
Group 2 vs Group 3	0.062	0.040	0.026
Group 1 vs Group 3	0.219	0.025	0.022
Group 1 vs Group 2	0.049	0.018	0.036

Table 4
Culture Results of HPB Medium With Glucose Added ($n = 3$)

Glucose added (g/L)	Dry cell weight (g/L)	DHA yield (g/L)	DHA content in biomass (%)
0	16.3 ± 0.1	2.69 ± 0.04	16.5 ± 0.3
20	21.7 ± 1.0	5.35 ± 0.04	24.7 ± 1.2
40	20.7 ± 0.4	4.83 ± 0.02	23.3 ± 0.5

concentration in the culture. Therefore, the results of this study showed that the original hypothesis of the high nitrogen concentration in the culture being the cause of the growth inhibition was incorrect, because a higher biomass production was obtained in the HPB culture with extra nitrogen as compared with the culture without any nitrogen addition.

The earlier data showed that it was not necessary to add an extra nitrogen source to the HPB culture, because the culture without any extra nitrogen supported good growth of the algae. Also, the extra nitrogen significantly decreased the DHA content in the biomass, compared with the culture without nitrogen added (Tables 2 and 3). In fact, 0.55 g/L nitrogen was enough to support 20.0 g/L algae biomass growth using pure glucose (Table 2). An equivalent nitrogen concentration (0.51 g/L) existed in the 30% HPB, suggesting that this would be the optimal HPB concentration, but taking into consideration that a higher biomass concentration may be reached in future work, which will need more nitrogen, the percentage of 50% HPB was taken as the basic medium in the ensuing work.

Culture With Glucose Added to HPB

Although the nitrogen concentration in the culture with 50% HPB is enough to support higher biomass density, the 50.0 g/L glucose in it may be a limiting factor, in that with the same concentration of pure glucose reported earlier, only 19.0 g/L dry biomass was obtained (Table 2). If this hypothesis is right, supplement of extra glucose in the culture should enhance the production. In this verification experiment, 20.0 g/L and 40.0 g/L pure glucose were added to the culture with 50% HPB with the results shown in Table 4.

Table 5
Repeat Culture Results (n = 3)

Culture day	Dry cell weight (g/L)	DHA yield (g/L)	DHA content in biomass (%)
6	20.1 ± 0.9	4.75 ± 0.05	23.7 ± 1.0
12	41.0 ± 1.0	9.14 ± 0.08	22.2 ± 0.7

Compared with the control, the added glucose significantly enhanced the cell density, to as much as 21.7 g/L dry cell biomass with 5.35 g/L DHA obtained in 6-d culture. However, there was no significant difference between the 20.0 and 40.0 g/L glucose added groups, which indicate that the glucose was no longer a limiting factor.

Feeding and Repeat Culture

To develop a higher cell density culture, a fed batch culture was conducted with a defined medium. In practice, each component of the medium, at half the amount of the initial culture, was added to the end of the previous batch culture. Unfortunately, there was no further increase in yield with respect to addition of all the supplements (data not shown). This indicated that the reduced growth in batch culture was not caused by depletion of certain nutrients and must therefore be a result of inhibition owing to accumulation of some metabolites. To verify this hypothesis, a repeat culture was conducted. In practice, at the 6th d of this culture, the fermentation broth was centrifuged and the spent medium was removed and replaced with fresh medium at which point the culturing process was allowed to proceed for another 6 d. The results of this repeat culture are shown in Table 5. The cell density climbed to 41.0 g/L in the repeat culture, and the DHA yield in these cultures reached 9.14 g/L. This verified that some metabolites in the culture inhibited the cells' growth. The analysis to determine what the inhibitory factors are and the method of how to avoid the inhibition are to be conducted in future work.

Discussion

Compared with *S. limacinum* SR 21, which has been proven to be a good DHA producer *(9)*, *T. aureum* obtained a much lower final cell density and DHA content, which led to very low production efficiency, even though *T. aureum* can take direct advantage of starch as a carbon source. Later experiments that used *S. limacinum* as the production strain within a HPB as both the carbon source and nitrogen source, attained similar results as that from the culture of pure glucose with added nitrogen source. This result along with the knowledge that the starch hydrolyzing process is widely used in the fermentation industry makes DHA production from cull potatoes a feasible and perhaps cost effective process. Also, this production efficiency may

be further increased if a simultaneous saccharification and fermentation process were to be used.

A higher ratio of carbon-to-nitrogen is preferred for this alga to accumulate fatty acids *(9)*, but the ratio of carbon-to-nitrogen in potato is not optimal for this process. To offset this, in this experiment, a HPB containing 100.0 g/L glucose was prepared, which correspondingly, contained 1.7 g/L nitrogen. The algae grew well in the culture without any extra nitrogen source having been added, which indicated that this amount of nitrogen was enough to support the algae's growth. This will significantly decrease the feedstock cost for DHA production, in that the cull potato provided both the carbon and nitrogen source. The low ratio of C/N in the original HPB caused a low DHA content in the biomass, but this was improved by reducing the percentage of HPB (reduction of the nitrogen source concentration) and adding extra glucose to the culture.

21.7 g/L dry cell biomass and 5.35 g/L DHA was obtained in the optimized culture. Compared with the results of the same algae strain by Yokochi et al *(9)* in which 4.0 g/L DHA and more than 30.0 g/L biomass was obtained with 90.0 g/L glucose in 50% concentration of seawater, we obtained less biomass production, but higher DHA yield. Although the productivity in these preliminary studies are still rather low, compared with the processes currently used commercially, they indicate cull-potato utilization is a feasible and promising way to produce DHA and in a more economically efficient manner because of the reduction in fermentation feedstock cost.

Conclusions

1. Although it cannot directly utilize potato starch, *S. limacinum* SR21 is a better DHA producer with cull potato as raw material, in that it accumulated a high content of DHA in the biomass, and reached a high culture cell density.
2. The ratio of carbon-to-nitrogen in the potato is not optimal for the DHA production process, but it can be improved by reducing the percentage of HPB in the medium and adding extra glucose in the culture.
3. The process of converting underutilized cull potato to DHA is promising and worth further study to reach a high cell density culture.

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