DAESANG Corporation

# DHA-rich Algal Oil from *Schizochytrium* sp.RT100

A submission to the UK Food Standards Agency requesting consideration of Substantial Equivalence in accordance with Regulation (EC) No 258/97 concerning novel foods and novel food ingredients

# I. Administrative data

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**Food ingredient:**

The food ingredient for which an opinion on Substantial Equivalence is requested is Daesang Corp.'s *Schizochytrium* sp.RT100 derived DHA-rich oil.

**Date of application**:

20 March, 2015.

## II. The Issue

In recent years, *Schizochytrium* sp.-derived docosahexaenoic (DHA)-rich oils have been the subject of several Novel Food Applications submitted under Regulation No 258/97 in the European Union (EU; EC, 1997). The first application for Novel Food authorization for *Schizochytrium* sp.-derived DHA-rich oil for general use as a nutritional ingredient in foods, was submitted by the United States (US)-based company OmegaTech Inc. to the Advisory Committee on Novel Foods and Processes (ACNFP) of the United Kingdom (UK) Food Standards Agency (UK FSA) in 2001.(Martek BioSciences Corporation, 2001)

After evaluation, the placing on the market of OmegaTech DHA-rich oil was authorized in 2003 following the issuing of the Commission Decision of 5 June 2003 (CD 2003/427/EC) authorizing the placing on the market of oil rich in DHA (docosahexaenoic acid) from the microalgae *Schizochytrium* sp. as a novel food ingredient (EC, 2003).

Following the acquisition of OmegaTech Inc. by Martek Biosciences Corp. in 2002, an application requesting an extension of use for the *Schizochytrium*-derived DHA-rich oil was filed with the UK FSA in 2008. The extension of use was authorized by Commission Decision 2009/778/EC (EC, 2009).

In 2011, the Canadian Company Ocean Nutrition Canada Ltd. submitted a request for an opinion on substantial equivalence of their *Schizchytrium* sp.ONC-T18 derived DHA-rich oil to that of Martek 's (formerly OmegaTech) DHA-rich oil already authorized (EC, 2003). In March 2012, The ACNFP concluded that the DHA rich algal oil produced by Ocean Nutrition Canada could be considered to be substantially equivalent to the existing DHA rich algal oil produced by Martek (ACNFP, 2012).

The current submission pertains to a similar request for an opinion on substantial equivalence of Daesang DHA-rich oil from *Schizochytrium* sp.RT100 to that of Martek Biosciences Corp., that was the initially authorized DHA-rich oil (EC, 2003), including the authorized extension of uses (EC, 2009). As per 14 July 2014, both Decisions 2003/427/EC and 2009/778/EC have been repealed following the issuing of Commission Implementing Decision of 14 July 2014 on authorizing the placing on the market of oil from the microalgae *Schizochytrium* sp. as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council (CD 2014/463/EU) and repealing Decisions 2003/427/EC and 2009/778/EC. Therefore, the current application for an opinion on substantial equivalence of *Schizochytrium* sp. DHA-rich oil therefore should be considered to relate to substantial equivalence to the Specification of oil from the microalgae *Schizochytrium* sp. as laid down in Annex 1 to this Commission Implementing Decision as well as the authorized uses of oil from the microalgae *Schizochytrium* sp. as laid down in Annex 2 of the Commission Implementing Decision 2014/463/EU (EC, 2014).

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# 1. Introduction

Docosahexaenoic acid (DHA; 22:6 n-3), a polyunsaturated fatty acid (PUFA), is linked to various health benefits in humans including cognitive and visual development of infants and reduced risk of cancer, cardiovascular diseases and mental illnesses of adults. Indeed, recently several health claims to be made on foods for DHA have been authorized in the EU. These authorized claims pertain to maintenance of normal brain function and vision and, in combination with eicosapentaenoic acid (EPA; 20:5 n-3) to contribute to the normal function of the heart.

Traditionally, long-chain polyunsaturated fatty acids (PUFAs), amongst which DHA, are obtained from marine fish such as salmon, mackerel, and tuna. Fish oil, with an annual production of over 1 million tons (2010) is at present the major source of DHA. However, heavy metal pollution and over-exploitation of the sea-fish resources jeopardize the sustainability of this source.

Some marine microalgae such as dinoflagellates and species in the Heterokonta phylum contain a high amount of DHA. However, the majority of those microalgae are photoautotrophic and as such being dependent on light as energy source and on weather conditions. Heterotrophic microalgae are able to take energy from simple organic substances without requiring light (Yokoyama & Honda, 2007). One of heterotrophic microalgae is *Schizochytrium* sp. which can be utilized as alternative to fish oils due to its rapid growth rate, its weather condition independency and its DHA content which reach to almost 49% of its total fat content (Renet al., 2010).

This DHA-rich algal oil is produced by the US-company Martek Biosciences Corp. that obtained marketing authorization for the product as a Novel Food in the European Union (EU) (EC, 2003). In 2004, the FDA did not object to the GRAS notification by Martek for its DHA algal oil derived from *Schizochytrium* sp.(FDA, 2004 - http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm153961.htm). DHA-rich algal oil is now available for use in foods and dietary supplements.

The current application pertains to a request for an opinion on substantial equivalence of Daesang's DHA-rich oil from *Schizochytrium* sp. with the originally authorized (EC, 2003) DHA-algal oil manufactured by Martek BioSciences Corp.

This submission will be compiled taking the ACNFP guidelines for the presentation of data to demonstrate substantial equivalence between a novel food or food ingredient and an existing counterpart into account (ACNFP, 2005). In the following sections it will be argued that based on the characterization of the source organism *Schizochytrium* sp.RT100, its method of production, the composition of the DHA-rich oil, its metabolism, its intended use and level of undesirable substances, we conclude that Daesang DHA-rich oil from *Schizochytrium* sp.RT100 is substantially equivalent to Martek's *Schizochytrium* sp.ATCC 20888-derived DHA algal oil.

# 2. Substantial equivalence of the source organism to DSM/Martek's *Schizochytrium* ATCC 20888

## 2.1 Taxonomy and Morphology of *Schizochytrium* sp.

*Schizochytrium* is a heterotrophic microalgae which belongs to the family of *Thraustochytriacaea* (Luyinget al., 2008). *Schizochytrium* is a spherical unicellular microorganism. According to Yokoyama et al. (2007),morphological characteristics of *Schizochytrium* under the microscope show ectoplasmic nets, formation of zoospores, aplanospores, and amoeboid cells of a size between 10-20 µm. The taxonomy details of *Schizochytrium* are as follows:

Kingdom : *Chromista* (*Stramenopila*)

Phylum : *Heterokonta*

Class : *Thraustochytridae*

Order : *Thraustochytriales*

Family : *Thraustochytriaceae*

Genus : *Schizochytrium*

Species : *Schizochytrium sp*.

*(Source: Leipe et al., 1994)*

*Schizochytrium* produces biflagellate zoospores and mature cells divide by repeated binary division to form diads, tetrads and clusters (Figure 1). Each *Schizochytrium* cell could develop into a sporangium that produces several zoospores (Kamlangdee & Fan, 2003).

The Daesang strain was originally isolated from a sea water sample obtained from the nearby sea of Iriomote island in the Okinawa Prefecture, Japan in 2005 by Prof. Daisuke Honda (Marine Biotechnology Institute Co., Ltd., Kamaishi, Iwate, Japan, and the National Institute of Bioscience and Human-Technology, Agency of Industrial and Technology, Tsukuba, Ibaraki, Japan), who is an expert in the field of marine biodiversity and systematics of marine algae.

Following selection of the strain on the basis of growth characteristics and high content of omega-3 polyunsaturated fatty acids, including docosahexaenoic acid (DHA), further characterization indicated that the strain belonged to the genus *Schizochytrium*. The strain has been purchased by Daesang Corp. and named RT100.

## 2.2 Taxonomic Classification

The *Labyrinthulomycota* (slime nets, net slime moulds) form a taxonomic phylum consisting of two families (Olive, 1975). One family (the *Thraustochytriaceae*) consists of several genera commonly referred to as “thraustochytrids”.

The traditional genera within the thraustochytrids are distinguishable based on the presence of certain morphological features, except for the genus *Thraustochytrium Sparrow*, 1936, which serves as a “catch-all” for the group and contains members that do not show the distinguishing characters of any other genus.

The other traditional genera include:

- (1) *Japanochytrium* (Kobayashi and Ookubo, 1953), whose species are distinguishable by the presence of a swelling (the supsoral apophysis) just below the sporulating structure,
- (2a) *Schizochytrium* (sensu stricto) (Goldstein and Belsky, 1964), whose members are characterized by the sporangia undergoing vegetative mitosis (successive bipartitioning) before the formation of spores,
- (3) *Ulkenia* (Gaertner, 1977), whose members are identified by the presence of an amoeboid protoplasm being released from the sporangium prior to cleavage into zoospores,
- (4) *Aplanochytrium* (Bahnweg and Sparrow, 1972) emend. Leander and Porter, 2000), the members of which display a unique gliding motility.

*(source: Yokoyama et al., 2007)*

For the original taxonomic classification, see also the World Register of Marine Species (http://www.marinespecies.org/aphia.php?p=taxdetails&id=22156).

Yokoyama and Honda (2007) conducted a taxonomic rearrangement of *Schizochytrium*, which resulted in the erection of two new genera, *Oblongichytrium* and *Aurantiochytrium* and an emended description of the genus *Schizochytrium*. These three genera can be distinguished by different fatty acid and pigment profiles, in addition to 18S rDNA sequence and morphological data.

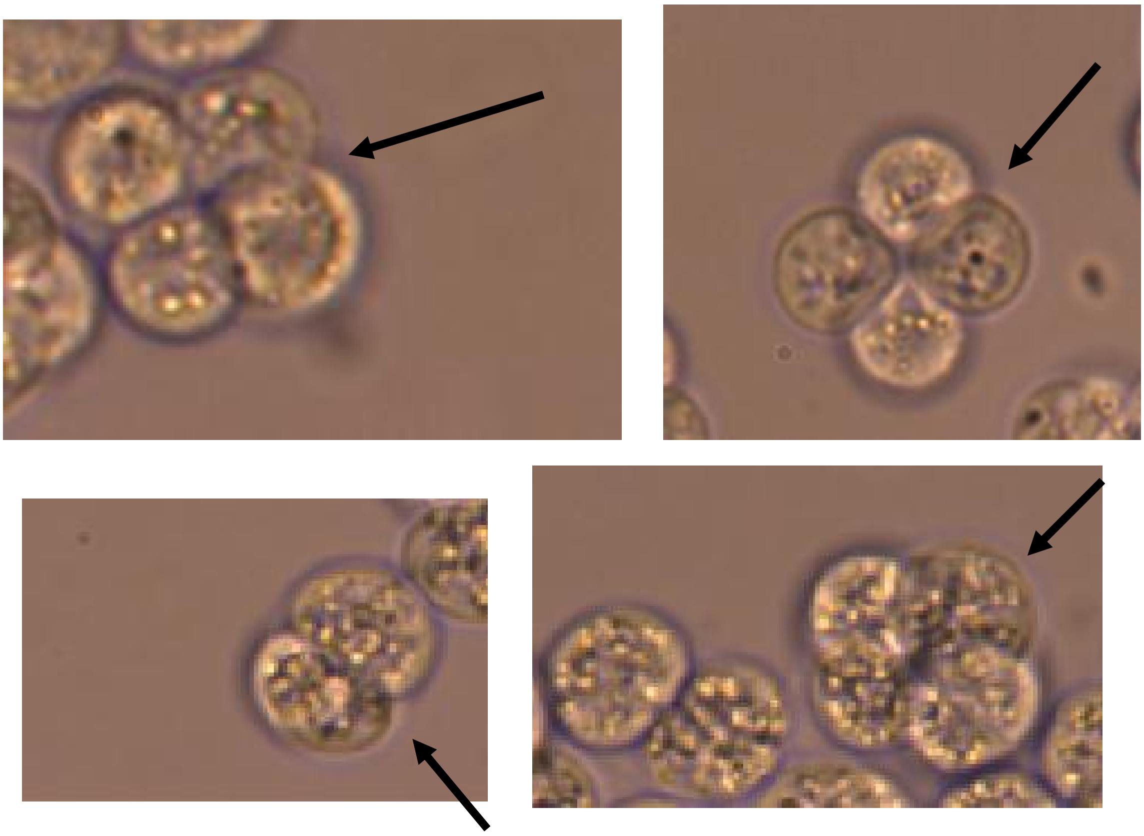
- (2b) *Schizochytrium* (emend. Yokoyama and Honda, 2007) members possess only beta-carotene as a carotenoid pigment and 20% arachidonic acid. Colonies are large because of successive bipartitioning.

- (5) *Oblongichytrium* (Yokoyama and Honda, 2007) members possess canthaxanthin, beta-carotene, abundant n-3 docosapentaenoic acid, and little n-6 docosapentaenoic acid. Colonies are also large due to successive bipartitioning.
- (6) *Aurantiochytrium* (Yokoyama and Honda, 2007) members possess astaxanthin, phoenicoxanthin, canthaxanthin, and beta-carotene as well as arachidonic acid and docosahexaenoic acid. Colonies are smaller, but sporangia still undergo successive bipartitioning.

## 2.3 Morphological Taxonomy

RT100, the mother strain of our industrial strain for producing oils rich in omega-3 fatty acids, was grown on solid agar media (GPY or equivalent) to evaluate gross morphological characters. Figure 1 shows its displaying successive bipartitioning.

**Figure 1: RT100 on solid agar, showing successive bipartitioning (arrows)**



Based on several images indicating successive bipartitioning in this strain, Dr. Honda, who initially isolated, cultured and characterized this strain, began to suspect that this strain was a close relative to (or a member of) the *Schizochytrium*, *Oblongichytrium*, or *Aurantiochytrium* genera. Moreover, RT100 did not display the amoeboid protoplast stage indicative of *Aurantiochytrium limacinum* or *Ulkenia*.

## 2.4 Phylogenetic Taxonomy -18S rRNA sequence comparison

Ribosomal RNA (rRNA) sequences have been aligned and compared in a number of living organisms, and this approach has provided a wealth of information about phylogenetic relationships. Studies of rRNA sequences have been used to infer phylogenetic history across a very broad spectrum, from studies among the basal lineages of life to relationships among closely related species and populations (Hillis and Dixon, 1991).

An analysis of aligned sequences of the four nuclear and two mitochondrial rRNA genes identified regions of these genes that are likely to be useful to address phylogenetic problems over a wide range of levels of divergence.

In general, the small subunit nuclear sequences appear to be best for elucidating Precambrian divergences, the large subunit nuclear sequences for Paleozoic and Mesozoic divergences, and the organellar sequences of both subunits for Cenozoic divergences (Figure 2). Thus, small subunit nuclear sequences (and hence RNA sequences) provide information on phylogenetic relationships that go furthest back in time (Hills & Dickson, 1991).

**Figure 2: Geological Time Periods**

*Mya: million years ago*

<table>
<tr><td rowspan="3">Phanerozoic Eon<br>(544 mya to present)</td><td>Cenozoic Era<br>(65 mya to today)</td><td>Quaternary (1.8 mya to today)<br>Holocene (11,000 years to today)<br>Pleistocene (1.8 mya to 11,000 yrs)<br>Tertiary (65 to 1.8 mya)<br>Pliocene (5 to 1.8 mya)<br>Miocene (23 to 5 mya)<br>Oligocene (38 to 23 mya)<br>Eocene (54 to 38 mya)<br>Paleocene (65 to 54 mya)</td></tr>
<tr><td>Mesozoic Era<br>(245 to 65 mya)</td><td>Cretaceous (146 to 65 mya)<br>Jurassic (208 to 146 mya)<br>Triassic (245 to 208 mya)</td></tr>
<tr><td>Paleozoic Era<br>(544 to 245 mya)</td><td>Permian (286 to 245 mya)<br>Carboniferous (360 to 286 mya)<br>Pennsylvanian (325 to 286 mya)<br>Mississippian (360 to 325 mya)<br>Devonian (410 to 360 mya)<br>Silurian (440 to 410 mya)<br>Ordovician (505 to 440 mya)<br>Cambrian (544 to 505 mya)<br>Tommotian (530 to 527 mya)</td></tr>
<tr><td rowspan="3">Precambrian Eon<br>(4,500 to 544 mya)</td><td>Proterozoic Era<br>(2500 to 544 mya)</td><td>Neoproterozoic (900 to 544 mya)<br>Vendian (650 to 544 mya)<br>Mesoproterozoic (1600 to 900 mya)<br>Paleoproterozoic (2500 to 1600 mya)</td></tr>
<tr><td colspan="2">Archaean<br>(3800 to 2500 mya)</td></tr>
<tr><td colspan="2">Hadean<br>(4500 to 3800 mya)</td></tr>
</table>

The small subunit (SSU) 18S rRNA gene is one of the most frequently used genes in phylogenetic studies and an important marker for random target polymerase chain reaction (PCR) in environmental biodiversity screening because rRNA gene sequences are easy to access due to highly conserved flanking regions allowing for the use of universal primers.

Honda et al. (1999) have performed a molecular phylogenetic analysis of *Labyrinthulids* and *Thraustochytrids* based on the sequencing of the 18S ribosomal RNA gene. A signature sequence region was identified as the 11 bases from positions 494-504 in the alignment (Figure 3) in helix 16 in region V3 on the secondary structure of small subunit ribosomal RNA molecule. The sequences of this region are relatively conserved among the sequences of stramenopiles (Figure 3). The sequences of this region in the labyrinthulid phylogeny group are relatively similar to those of the stramenopiles and alveolates as an outgroup (e.g. *Cafeteriaroenbergensis*, *Prorocentrum micans*), but the sequences of the thraustochytrids are quite different from those of other stramenopiles.

In addition, Honda et al. (1999) identified inserted signature sequences of 14-28 bases from positions 1,712-1,739 in the alignment in helix 46 in region V8on the secondary structure of small subunit ribosomal RNA molecule for only the thraustochytrid phylogenetic group (Figure 4). Both the labyrinthulid phylogenetic group and the other organisms did not possess this inserted signature (Figure 4).

**Figure 3: Shared signature sequences of the boxed region (positions 494-504) of secondary structure of 18S rRNA showing close relationships in the thraustochytrid phylogenetic group**

| | | 494 → 504 | | |
|---|---|---|---|---|
| *Japonochytrium* sp. | AAATTACT-CAAT | GTCAATTCGAC | GAAGTAGTGAC | Thraustochytrid phylogenetic group |
| *Labyrinthuloides haliotidis* | AAATTACT-CAAT | GTCAATTCGAC | GAAGTAGTGAC | |
| *Schizochytrium aggregatum* | AAATTACTGCAAG | TTCTACAGAAC | GAAGTAGTGAC | |
| *Schizochytrium limacinum* | AAATTACC-CACT | GTGGACTCCAC | GAGGTAGTGAC | |
| *Thraustochytrium aggregatum* | AAATTACC-CAAT | GGGGACTCCCC | GAGGTAGTGAC | |
| *Thraustochytrium aureum* | AAATTACT-CAAT | GTTGACTCGAC | GAAGTAGTGAC | |
| *Thraustochytrium kinnei* | AAATTACT-CTAT | GCCAACGCGGC | GAAGTAGTGAC | |
| *Thraustochytrium pachydermum* | AAATTACC-CAAT | GGTGGAATGCC | GAGGTAGTGAC | |
| *Thraustochytrium striatum* | AAATTACT-CAAT | GTTGACTCGAC | GAAGTAGTGAC | |
| *Ulkenia profunda #29* | AAATTACT-CAAT | GTCAATTCGAC | GAAGTAGTGAC | |
| *Ulkenia profunda* N 3077a | AAATTACT-CAAT | GTCAATTCGAC | GAAGTAGTGAC | |
| *Ulkenia radiata* | AAATTACT-CAAT | GTCAATTCGAC | GAAGTAGTGAC | |
| *Ulkenia visurgensis* | AAATTACT-CAAT | GTCAATTCGAC | GAAGTAGTGAC | |
| *Aplanochytrium kerguelense* | AAATTACC-CAAT | CCTGATACGGG | GAGGTAGTGAC | Labyrinthulid phylogenetic group |
| *Labyrinthula* sp. | AAATTACC-CAAT | CCTGACACGGG | GAGGTAGTGAC | |
| *Labyrinthuloides minuta* | AAATTACC-CAAT | CCTGATACGGG | GAGGTAGTGAC | |
| *Schizochytrium minuta* | AAATTACC-CAAT | CCTAATACGGG | GAGGTAGTGAC | |
| *Thraustochytrium multirudimentale* | AAATTACC-CAAT | CCTAACACGGG | GAGGTAGTGAC | |
| *Cafeteria roenbergensis* | AAATTACC-CAAT | CCTAAAGCAGG | GAGGTAGTGAC | uncertain stramenopile |
| *Prorocentrum micans* | AAATTACC-CAAT | CCTGACACAGG | GAGGTAGTGAC | alveolate |

*Source: Honda et al., 1999.*

**Figure 4: Shared signature sequences in 18S rRNA showing close relationships in the thraustochytrid phylogenetic group. The boxed region (positions 1712 - 1739) showing the large insertion present in all members of thraustochytrid phylogenetic group but absent from all other organisms**

| | | 1712 – 1739 | | |
|---|---|---|---|---|
| *Japonochytrium* sp. | TTCAACGAGTA | TGTGTTGT----TTGTTTAACGATGAAT | GACGGTCCTAGACA--GGAATG | Thraustochytrid phylogenetic group |
| *Labyrinthuloides haliotidis* | TTCAACGAGTT | TTTCAT-------CTTG-------ATGA | A-TA-TCCTTGGCC--GGAAGG | |
| *Schizochytrium aggregatum* | GGGAGCACGTT | GCTTTG---------TC--G---TACGA | CAACGTCCTGGGCC--GGAAGG | |
| *Schizochytrium limacinum* | TTCATCGGGTT | TTAATT--------C-A-TTTTATGGAA | TTGAGTGCTTGGTC--GGAAGG | |
| *Thraustochytrium aggregatum* | TTCAACAAGTC | CGGTAGTG-----G-GCTCTG-TGC-CT | ATTATTACTTTTCC--GAGAGG | |
| *Thraustochytrium aureum* | TTCAACGAGTA | TTTGTTTTTTTCTCATTTTGGGAGGGGG | CAGAGTCCTTGGCC--GGAAGG | |
| *Thraustochytrium kinnei* | TTCAACGAGTT | GTTTATGTTGCTGTTTTTGGCAA----- | TATT-TCCTTGTCC--GTTAGG | |
| *Thraustochytrium pachydermum* | TTCAACAAGTT | TTATTTAAAA----TTTATTTTATAAAT | TTTT-TCCTTGATC--GGAAGG | |
| *Thraustochytrium striatum* | TTCAACGAGTT | TTTTTG-------TTTCTTTGGAAATAA | AATG-TCCTTGATC--GGAAGG | |
| *Ulkenia profunda* #29 | TTCAACGAGTT | TTTCTTGT------TCTTTTAGGAATGA | GAAG-TCCTTGGCC--GGAAGG | |
| *Ulkenia profunda* N 3077a | TTCAACGAGTA | TTGTTCTATGCTTTTCGGAGTGTGGGAT | G----TCCTGGGCA--GGAATG | |
| *Ulkenia radiata* | TTCAACGAGTT | ATTCTTGT------TCTTTTAGGAATGA | GAAG-TCCTTGGCC--GGAAGG | |
| *Ulkenia visurgensis* | TTCAACGAGTA | TGTGTTGT---TTGTTTAACGATGAATG | ACGG-TCCTAGACA--GGAATG | |
| *Aplanochytrium kerguelense* | TTCAACGAGTT | ---------------------------- | TATA-ACCTTGGTT--GAAAAG | Labyrinthulid phylogenetic group |
| *Labyrinthula* sp. | GTCAGCGAGCT | C--------------------------- | -----TCCTGTATC--GAAAGG | |
| *Labyrinthuloides minuta* | TTCAACGAGTT | ---------------------------- | TATA-ACCTTGGTT--GAAAAG | |
| *Schizochytrium minuta* | TTCAACGAGTT | ---------------------------- | TATA-ACCTTGGCT--GAGAAG | |
| *Thraustochytrium multirudimentale* | TTCAACGAGTT | ---------------------------- | TATA-ACCTTGGCT--GAGAAG | |
| *Cafeteria roenbergensis* | TGCAACAAGTG | ---------------------------T | CTAC-ACGTTGGCCTCGAGAGG | uncertain stramenopile |
| *Prorocentrum micans* | TTCAACGAGTT | ---------------------------- | TATG-ACCTTGCCC--GATAGG | alveolate |

The data on 18S rRNA molecular phylogeny as presented above suggested that *Labyrinthula* sp. and Thraustochytrids separate into at least two major groups. Nevertheless, the three genera *Labyrinthuloides, Schizochytrium,* and *Thraustochytrium,* appeared in both groups and clearly did not reflect the generic (morphologic) circumscription of the thraustochytrids indicating that the examined strains of each genus do not form a monophylogenetic group (Honda et al., 1999).

Therefore, and also based on the images indicating successive bipartitioning described and shown above (Figure 1), it cannot be convincingly concluded at this stage that our strain RT100 belonged to the *Schizochytrium* genus on the basis of these morphological characteristics alone.

In order to get a more definitive answer on the taxonomic classification of our strain RT100 and, hence, on its relationship to Martek's *Schizochytrium* sp.ATCC 20888 , we first performed an analysis of the 18S rRNA signature sequences identified by Honda et al. (1999) and made a comparison with the simultaneously obtained 18S rRNA sequences of ATCC 20888 and of the more distantly related *Schizochytrium* sp. SR21 (Table 1). The latter strain had previously been described by Honda et al. (1998) who observed a morphology and life history similar to that of the *Ulkenia* and *Schizochytrium*. However, based on 18S rRNA analysis SR21 did not appear to cluster with any of the *Ulkenia* and *Schizochytrium* strains examined by Honda et al. (1999).

On the basis of 18s rRNA sequences (GenoTeck Co., Daejon, South Korea), the tested strains were classified to belong to *Thraustochytrid* Phylogenetic Group (TPG) I (sites 494-504) and/or TPG II (sites 1712-1739).

Comparisons of the results of TPG I and TPG II between the respective strains are presented in Table 1 and 2. Table 1(TPG I Comparison) shows a very high degree of homology between the strains.

However, the comparison of the sequences representing site 1712-1739 (Table 2) shows clear differences between the strains. On the basis of 18S rRNA comparison, it is suggested that our strain RT100 is highly likely to belong to the *Schizochytrium* genus.

**Table 1: 18S rRNA sequences of Thraustochytrid Phylogenetic Group I(sites 494-504 - shaded green) -Comparison by strains**

| | | | |
|---|---|---|---|
| *Schizochytriumsp*. SR21[1)] | CACT | GTGGACTCCAC | GAGGTAGTGA |
| *Schizochytriumsp.*ATCC 20888[2)] | CAAT | GTGGACTCCAC | GAGGTAGTGA |
| *Schizochytriumsp*. RT100[2)] | CAAT | GTGGACTCCAC | GAGGTAGTGA |

**Table 2: 18S rRNA sequences of Thraustochytrid Phylogenetic Group II (sites 1712-1739 – shaded green) - Comparison by strains**

| | | | |
|---|---|---|---|
| *Schizochytrium* sp. SR21[1)] | GGGTT CATC GGGTT | TTAATT-CATTTTAT---- GGAA | --TTGAG TGC- TTGGTC GGAAGG |
| *Schizochytrium* sp. ATCC 20888[2)] | GGGTT CAGC GGGTT | TTTGTT--GTGTTTT----- GCA | -CAGCGT TGCT TTGTC- GGAAGG |
| *Schizochytrium* sp. RT100[2)] | GGGTT CAGC GGGTC | TTTGTT--GTGTTT---- ACTCA | -CAGCGT TGCT TTGTC- GGAAGG |

1) Honda D. et al, 1999
2) Analysis result by Daesang laboratory

To further determine the phylogenetic relationship between our strain RT100 and Martek's ATCC 20888, 18s rRNA sequences were aligned (GenoTeck Co.) with other thraustochytrid sequences from Genbank (http://www.ncbi.nlm.nih.gov/genbank/) , and a bootstrap Maximum Likelihood (ML) phylogenetic tree (Figure 5) was generated using MEGA5.2 software with the settings as indicated in Figure 6.

The comparator18S rRNA sequences were selected from *Thraustochytrium, Schizochytrium, Aurantiochytrium, Japonochytrium, and Ulkenia*, genera within the family*Thraustochytriaceae*.

Figure 5 indicates that RT100 is closely related to *Schizochytrium* strain ATCC 20888. Both strains are grouped together in a monophyletic clade (100% of the bootstrap replications), indicating that both organisms are phylogenetically closely related.

Full 18S rRNA sequence alignment was performed at Daesang Laboratory applying BLAST program (available at NCBI(http://www.ncbi.nlm.nih.gov/). Results showed 98% homology between the two strains (see for full sequence alignment and comparison in Appendix A**).**

**Figure 5: ML trees showing relationships among selected thraustochytrid 18S rRNA sequences. Bootstrap values are indicated at the nodes. The scale bar at the bottom represents the % difference between distinct region sequences (e.g. 0.02 = 2%)**

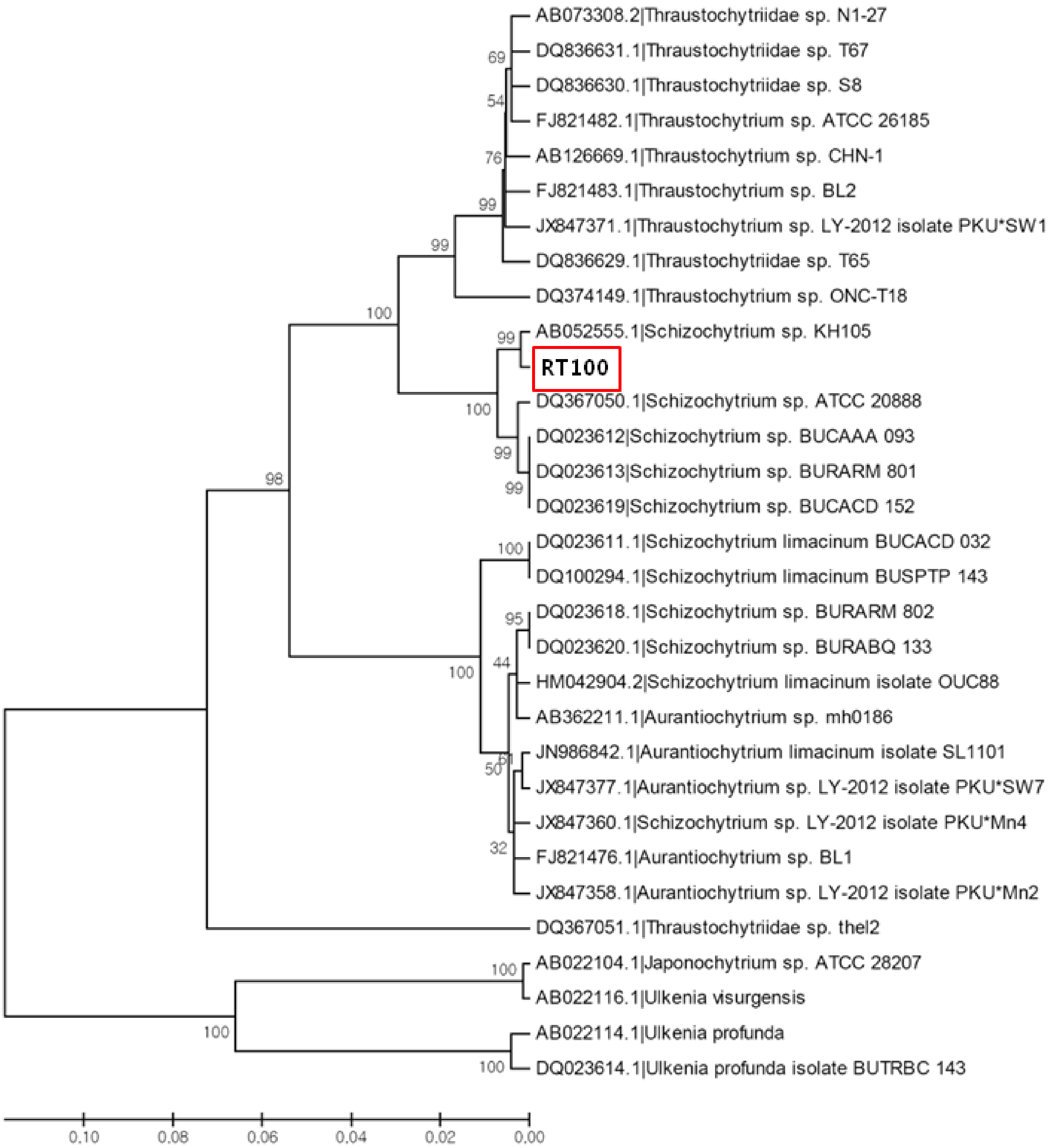


**Figure 6: Set conditions in MEGA5.2 for phylogenetic tree generation.**

Analysis
Analysis ------------------------ Phylogeny Reconstruction
Statistical Method -------------- Maximum Likelihood (ML)

Phylogeny Test
Test of Phylogeny --------------- Bootstrap method
No. of Bootstrap Replications --- 1000

Substitution Model
Substitutions Type -------------- Nucleotide
Model/Method -------------------- General Time Reversible model (GTR)

Rates and Patterns
Rates among Sites --------------- Gamma distributed with Invariant sites (G+I)
No of Discrete Gamma Categories - 5

Data Subset to Use
Gaps/Missing Data Treatment ----- Complete deletion

Tree Inference Options
ML Heuristic Method ------------- Nearest-Neighbor-Interchange (NNI)
Initial Tree for ML ------------- Make initial tree automatically (Default - NJ/BioNJ)
Branch Swap Filter -------------- Very Strong

*MEGA: Molecular Evolutionary Genetics Analysis*

## 2.5 Conclusion on substantial equivalence of source organisms

Given the morphological characteristic of successive bipartitioning observed in RT100 as well as its close 18S rRNA phylogenetic affinity with other *Schizochytrium* strains (notably ATCC 20888), it is the Dr. Daisuke Honda's expert opinion to classify this strain as belonging to the genus *Schizochytrium*.

Following its identification, *Schizochytrium* sp.RT100 has been registered in the Korean Collection of Type Cultures (KCTC) under number RT01000P1 (KCTC 10937BP).

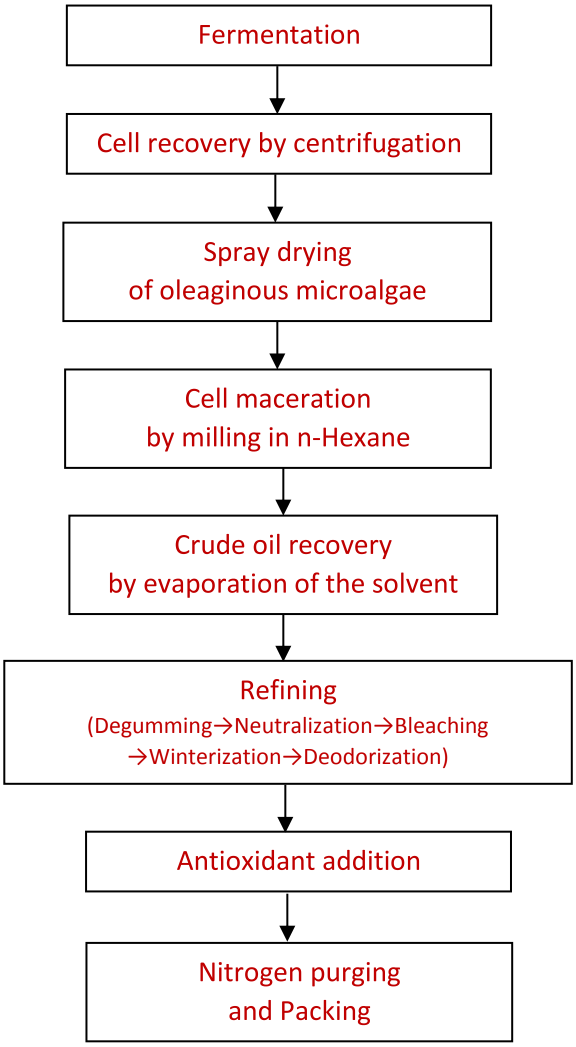
# 3. Substantial Equivalence of manufacturing process and characterization of Daesang DHA-rich oil from *Schizochytrium* RT100.

## 3.1 Culture conditions of Daesang's *Schizochytrium* sp.RT100

## 3.2 Manufacturing Process of Daesang DHA-rich oil from *Schizochytrium* sp.RT100

Recovery of DHA rich-oil from the algae biomass was subsequently performed applying processes commonly in use in the edible oil industry and following the steps as indicated in the flow diagram (Figure 7). Spray-dried microalgae were mixed with n-hexane at a fixed ratio and milled 2 times to macerate the cells and crude oil was extracted from the ruptured cells. The thus extracted oil was then separated from the cellular debris by gravitational precipitation and evaporated under vacuum to remove any residual solvent from the extracted oil. A concise description of the downstream processes is presented in Appendix B.

**Figure 7: Flow diagram of DHA-rich oil production from *Schizochytrium* sp.RT100**



# 4. Compositional Equivalence

## 4.1 Specification of Daesang DHA-rich oil from *Schizochytrium* sp.RT100

Applying the above described culture and manufacturing processes, the product specifications for the thus obtained DHA-rich oil are indicated in the Table 3. These are in compliance with the Product Specifications as per Annex 1 of Commission Implementing Decision 2014/463/EU, authorizing the placement on the market of Martek's (now DSM Nutritional Lipids) *Schizochytrium* sp. derived DHA-rich oil (Table 4 - below). Table 5 provides for the most recent analyses results of the three batches DHA-rich oil manufactured by Daesang and a comparator batch of Martek/DSMs' DHA-rich oil from *Schizochytrium* sp. (The analyses have been carried out by Intertek Food Services, Linden, Germany).

**Table 3: Daesang product specifications for DHA-rich oil produced by *Schizochytrium* sp.RT100 according to the above described manufacturing process**

| Test | Specification |
|---|---|
| Appearance | Yellowish liquid |
| Odor and taste | Characteristic |
| Docosahexaenoic Acid (mg/g) | NLT 400 |
| Acid Value (mg KOH/g) | NMT 0.5 |
| Peroxide Value (meq/kg) | NMT 5.0 |
| Residual Solvent (ppm as Hexane) | NMT 1.0 |
| Arsenic (ppm) | NMT 0.1 |
| Cadmium (ppm) | NMT 0.1 |
| Lead (ppm) | NMT 0.1 |
| Mercury (ppm) | NMT 0.04 |

*[$]: measured according to the Korean Foods Standards*
*[#] NLT: not less than*
** NMT: not more than*
*TFA: total fatty acids*

Commission Decision 2003/427/EC authorized the placing on the market of Martek's oil rich in DHA from the microalgae *Schizochytrium* sp. ATCC 20888 as a novel food ingredient as specified in Annex I to the Decision. Recently, Commission Decision 2003/427/EC and Commission Decision 2009/778/EC have been repealed following the issuing of Commission Implementing Decision of 14 July 2014 on authorizing the placing on the market of oil from the microalgae *Schizochytrium* sp. ATCC 20888 as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the

Council (CD 2014/463/EU). The unaltered product specifications are provided in Annex I to Commission Implementing Decision 2014/463/EU (Table 4).

**Table 4: Product Specifications for DHA-rich oil derived from *Schizochytrium* sp.**

**ANNEX I**

**SPECIFICATION OF OIL FROM THE MICRO-ALGAE *SCHIZOCHYTRIUM* SP.**

| Test | Specification |
|---|---|
| Acid Value | Not more than 0,5 mg KOH/g |
| Peroxide Value (PV) | Not more than 5,0 meq/kg |
| Moisture and volatiles | Not more than 0,05 % |
| Unsaponifiables | Not more than 4,5 % |
| Trans-fatty acids | Not more than 1,0 % |
| DHA content | Not less than 32,0% |

*Source: Annex I to the Commission Implementing Decision 2014/463/EU*

**Table 5: Compliance to EC specifications of Daesang DHA-rich oil from *Schizochytrium* sp.RT100 (three batches) compared with DSM/Martek from *Schizochytrium* sp.ATCC20888**

| Test | Specification per CD 2014/463/EU | Daesang | | | DSM/Martek | Compliance to EC Specifications |
|---|---|---|---|---|---|---|
| | | NMF2-2701140A1 | NMF2-2003140A1 | NMF2-0111130A1 | VY00081803 | |
| Acid value | NMT* 0.5 mg KOH/g | 0,22 | 0,22 | 0,20 | 0,35 | + |
| Peroxide Value | NMT 5.0 meq/kg oil | 4,22 | 2,66 | 3,12 | 0,196 | + |
| Moisture and volatiles | NMT 0.05% | 0,02 | 0,03 | 0,03 | 0,04 | + |
| Unsaponifiables | NMT 4.5% | 3,06 | 3,33 | 3,12 | 1,16 | + |
| Trans-fatty acids | NMT 1% | | | | | |
| C18:1 trans | | < 0.01 | < 0.01 | < 0.01 | < 0.01 | + |
| C18:2 trans (Sum of isomers) | | < 0.01 | < 0.01 | < 0.01 | < 0.01 | + |
| C18:3 trans (Sum of isomers) | | 0,1 | 0,1 | < 0.01 | < 0.01 | + |
| DHA content | NLT** 32% | 49.1 | 50.1 | 50.4 | 39.4 | + |

*NMT: Not More Than
**NLT: Not Less Than.

Table 5 indicates compliance of both Daesang's and Martek's DHA-rich oil from the respective *Schizochytrium* species with the product specifications as per Annex I of Regulation No. 2014/463/EU. Close equivalent identity of both oils is demonstrated. In addition, residual hexane was below detection level (< 1 mg/kg). (see Appendix C for test report).
The differences between the Daesang and DSM/Martek levels, especially with respect to peroxide value and the percentage of unsaponifiables are currently not known but may be attributed to the application of sunflower oil as carrier of adding antioxidants by DSM to stabilize the refined DHA oil. Again it is stressed that all samples are compliant with EU specifications.

# 5. Conclusion on substantial equivalence of manufacturing and characterization of product specifications

The manufacturing process and characterization of product specifications of Daesang DHA-rich oil from *Schizochyrium* sp. RT100 comply with the product specifications for Martek/DSM's DHA-rich oil from *Schizochytrium* sp. ATCC 20888 (Table 5) and those indicated in Annex I of Commission Implementing Decision 2014/463/EU (Figure 4) and can therefore be considered substantially equivalent to Martek/DSM's DHA-rich oil derived from *Schizochytrium* sp. ATCC 20888.

In conclusion, Daesang DHA-rich oil from *Schizochyrium* sp.RT100 (three batches) are in compliance with product specifications as per Annex I of Commission Decision 2014/463/EU and are therefore considered substantially equivalent to the product specifications of Marteks' DHA-rich oil from *Schizochytrium* sp.ATCC 20888.

## 5.1 Equivalence of proximate analysis

Proximate analysis has been performed for all four batches. In addition, it was shown that all Daesang DHA-rich oil from *Schizochytrium* sp.RT100, in addition to the reference oil from Martek/DSM did not contain protein and carbohydrates (detection limit. 0.1 %) (see Appendix C for test report). The results are indicated in Table 6 and show that the analysis results of Daesang DHA-rich oil from *Schizochytrium* sp.RT100 are very similar to those obtained for DSM/Martek from *Schizochytrium* sp.ATCC20888-derived oil. Taken together, with respect to the proximate analysis both strains are considered to be substantially equivalent.

**Table 6: Proximate analysis of Daesang DHA-rich oil from *Schizochytrium* sp. RT100 and DSM/Martek from *Schizochytrium* sp. ATCC20888**

| Test | Unit | Daesang | | | DSM/Martek |
|---|---|---|---|---|---|
| | | NMF2-2701140A1 | NMF2-2003140A1 | NMF2-0111130A1 | VY00081803 |
| Fat | % | 100 | 100 | 100 | 100 |
| Saturated fatty acids | % | 24,8 | 23,6 | 22,1 | 30,1 |
| Protein | % | < 0.1 | < 0.1 | < 0.1 | < 0.1 |
| Ash | % | < 0.1 | < 0.1 | < 0.1 | < 0.1 |
| Sodium | % | < 0.005 | < 0.005 | < 0.005 | < 0.005 |
| Carbohydrates | % | < 0.1 | < 0.1 | < 0.1 | < 0.1 |
| Energy | kJ/100g | 3.700 | 3.699 | 3.699 | 3.699 |
| Energy | kcal/100g | 899,9 | 899,9 | 899,9 | 899,8 |

## 5.2 Equivalence of fatty acid composition

The fatty acid composition of the 3 Daesang batches and two Martek/DSM batches is presented in Table 7. The last column indicates the average test values for Martek/OmegaTechs' original test batches as provided in the original Martek application. The column referring to DSM batch VY00081803 refers to the analyses results from a recently obtained (by us) DSM commercial batch. It is shown that the three Daesang batches have very similar compositions, with only minor differences in levels of individual fatty acids. Compared to the tested DSM batch, some more pronounced differences are noted, esp. for the levels of myristic acid, palmitic acid, oleic acid, docosapentaenoic acid (DPA; C22:5n6) and docosahexaenoic acid (DHA; C22:6n3).

As already indicated in the ACNFP COMMITTEE PAPER FOR DISCUSSION of February 2012(Document: ACNFP/105/3), related to the Substantial Equivalence notification as submitted by Ocean Nutrition Canada Ltd. (ONC), the committee accepted the view that the differences in composition due to differences in levels of oleic acid were likely to be due to the effect of blending the commercial product with vegetable oil (e.g. oleic acid) to obtain a consistent product that was within the published specification. In addition, the blending with the reported amount of oleic acid may also, in part, explain the differences in the levels of DPA and DHA between the Daesang batches and the batches produced by OmegaTech/Martek and DSM, as found in our analysis (see Table 7).

The level of myristic acid, which is consistent across the Daesang's batches (approx. 1.0 %), differs considerably from the levels found in the original Martek batch and the recently obtained DSM batch (6-10%). Although myristic acid production is intrinsic to *Schizochytrium* sp. we do not know the exact reasons for these differences, but the findings may amongst others be related to the difference in species type, differences in culture medium conditions, etc. Burdock & Carabin (2007) showed that myristic acid as a food ingredient has a very favorable safety profile. Taken together, we do not consider these lower levels of myristic acid to be of any safety concern.

It is noted that the DHA content of the Daesang oil is higher than that of the Martek/DSM oils. Apart from the issue of blending with oleic acid, as discussed above, we believe that specific strain characteristics of *Schizochytrium* sp.RT100 in combination with growth medium composition may (in part) explain these differences.

Also of note is that the higher level of DHA in the Daesang DHA-rich oil batches is not due to a chance finding caused by the use of the Intertek analysis method. Daesang DHA-rich oil product specifications (Table 3) established from the analysis of previous batches already indicated that the DHA-content of the oil should not be less than 40%, explaining the constant level of DHA across the various batches over time.

Taken together, given the fact that the DHA content of Daesang DHA-rich oil is in compliance with the authorized product specifications (CID 2014/463/EU) and given the relatively minor differences in percentage for some of the other fatty acids, we consider the fatty acid profiles of our DHA-rich oil to be substantially equivalent to DSM/Martek DHA-rich oil.

**Table 7: Fatty Acid Profiles of Daesang DHA-rich oil from *Schizochytrium* sp.RT100 and DSM/Martek from *Schizochytrium* sp.ATCC20888 with reference of OmegaTech**

| Fatty Acid Composition | | | | | | | |
|---|---|---|---|---|---|---|---|
| Fatty acid | Unit | Specification according to CD 2003/ | Daesang | | | DSM/Martek | OmegaTech Application (ATCC 20888) |
| | | | NMF2-2701140A1 | NMF2-2003140A1 | NMF2-0111130A1 | VY00081803 | |
| C12:0 (Lauric acid) | % | | - | - | - | 0,2 | 0,4 |
| C14:0 (Myristic acid) | % | | 1,2 | 1,1 | 0,9 | 6,3 | 10,11 |
| C16:0 (Palmitic acid) | % | | 16,4 | 15,5 | 14,2 | 17,3 | 23,68 |
| C16:1 (Palmitoleic acid and isomers) | % | | 0,4 | 0,3 | 0,2 | 0,2 | 1,76 |
| C17:0 (Heptadecanoic acid) | % | | 0,8 | 0,8 | 0,7 | 0,9 | - |
| C18:0 (Stearic acid) | % | | 0,8 | 0,7 | 0,6 | 0.9 | 0,45 |
| C18:1 (Oleic acid and isomers) | % | | 0,8 | 0,6 | 0,4 | 13,9 | 13,8 |
| C18:2 (Linoleic acid and isomers) | % | | 0,2 | 0,1 | 0,1 | 1,2 | 1,2 |
| C18:3n3 (α-Linolenic acid and isomers) | % | | 0,4 | 0,3 | 0,2 | 0,1 | - |
| C18:3n6 (γ-Linolenic acid) | % | | 0,3 | 0,3 | 0,3 | 0,2 | - |
| C20:0 (Arachidic acid) | % | | 0,1 | 0,2 | 0,1 | 0,1 | - |
| C20:1 (Eicosanoic acid and isomers) | % | | 0,1 | 0,1 | - | - | - |
| C20:3 (Eicosatrienoic acid and isomers) | % | | 0,7 | 0,7 | 0,8 | 0,4 | 0.87 |
| C20:4n6 (Arachidonic acid) | % | | 1,1 | 0,9 | 0,8 | 0,8 | 0,94 |
| C20:5n3 (Eicosapentaenoic acid) | % | | 3,0 | 2.4 | 2,2 | 1,0 | 2.63 |
| C22:0 (Behenic acid) | % | | - | - | - | 0,2 | - |
| C22:5n3 (Docosapentaenoic acid) | % | | 1,9 | 1,9 | 2,0 | 0,5 | - |
| C22:5n6 (Docosapentaenoic acid) | % | | 21,5 | 23.0 | 24,5 | 16,9 | 13.5 |
| C22:6n3 (Docosahexaenoic acid) | % | NLT 32.0 | 49,1 | 50.1 | 50,4 | 39,4 | 35.0 |
| C24:0 (Lignoceric acid) | % | | 0,1 | - | 0,2 | 0,2 | - |
| C18:1 trans | % | NMT 1.0 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | Max 2.0% |
| C18:2 trans (Sum of isomers) | % | | < 0.01 | < 0.01 | < 0.01 | < 0.01 | |
| C18:3 trans (Sum of isomers) | % | | 0,06 | 0,05 | < 0.01 | < 0.01 | |

'-' = not detected

The difference in fatty acid composition, although not investigated in depth, could be attributed to various culture conditions during growth as there are: pH control, dissolved oxygen (DO) level and the difference in nitrogen and carbohydrate sources. In the Daesang cultivation conditions pH level is not tightly controlled, whereas in the DSM/Martek methodology this is strictly kept at a level of

7.3. The same holds true for the DO level being not controlled under Daesang cultivation conditions in contrast to those by DSM/Martek. Finally beside ammonium sulfate monosodium glutamate is being applied as nitrogen source whereas ammonia in case of DSM/Martek. One important aspect in the DSM downstream protocol is the application of sunflower oil as carrier of adding antioxidants to stabilize the refined DHA oil. Oleic acid is one of the byproducts of sunflower oil in this process. Another important feature is the myristate concentration, the existence and non-existence of myristate cannot be the intrinsic characteristic of *Schizochytrium* sp. Of importance, the actual production strain of DSM shows similar DHA content of Daesang as compared to that in DSM/Martek (Barclay WR, U.S. Patent 5, 340, 742 (1994)).

## 5.3 Equivalence of nutritional value and metabolism

Because the new oil from Daesang Corp. does not substantially differ from Martek's DHA-rich oil that was initially authorized (Reg. 2003/427/EC) we consider that nutritional value (see also Table 6 for proximate analysis) as well as metabolism will not differ between the oils.

## 5.4 Substantial equivalence of Intended Use of Daesang DHA-rich oil from *Schizochytrium* sp.

The intended uses of Daesang DHA-rich oil from *Schizochytrium* sp. conform to the indicated applications and dosages as already authorized and listed in Annex II of Commission Implementing Decision 2014/463/EU (Table 8).
The applicant will advise customers of the DHA-rich oil about the authorized applications and maximum levels.

In addition, due to substantial equivalence of Daesang DHA-rich oil with DSM/Martek DHA-rich oil (established in this notification), future authorizations established by an application for the extension of uses will therefore also pertain to Daesang DHA-rich oil from *Schizochytrium* sp.RT100.

**Table 8: Authorized uses of oil from the microalgae *Schizochytrium* sp.**

**ANNEX II**

**AUTHORIZED USES OF OIL FROM THE MICRO-ALGAE *SCHIZOCHYTRIUM* SP.**

| Food category | Maximum use level of DHA |
|---|---|
| Dairy products except milk-based drinks | 200 mg/100 g or for cheese products 600 mg/100 g |
| Dairy analogues except drinks | 200 mg/100 g or for analogues to cheese products 600 mg/100 g |
| Spreadable fat and dressings | 600 mg/100 g |
| Breakfast cereals | 500 mg/100 g |
| Food supplements | 250 mg DHA per day as recommended by the manufacturer for normal population<br>450 mg DHA per day as recommended by the manufacturer for pregnant and lactating women |
| Foods intended for use in energy-restricted diets for weight reduction as defined in Directive 96/8/EC | 250 mg per meal replacement |
| Other foods for particular nutritional uses as defined in Directive 2009/39/EC excluding infant and follow on formulae | 200 mg/100 g |
| Dietary foods for special medical purposes | In accordance with the particular nutritional requirements of the persons for whom the products are intended |
| Bakery products (breads and rolls), sweet biscuits | 200 mg/100 g |
| Cereal bars | 500 mg/100 g |
| Cooking fats | 360 mg/100 g |
| Non-alcoholic beverages<br>(including dairy analogue and milk-based drinks) | 80 mg/100 g |

*Source: Annex II to the Commission Implementing Decision 2014/463/EU*

## 5.5 Substantial equivalence in levels of undesirable substances

### 5.5.1 Microbiological information

Microbiological information was determined for the microorganisms indicated in Table 9. The results are from three batches of RT100-oil and are compared to a recent DSM (formerly Martek) batch of the originally authorized *Schizochytrium* sp. derived DHA-rich oil (CD 2003/427/EC).

**Table 9: Microbiological analysis of Daesang DHA-rich oil from *Schizochytrium* sp.RT100 and DSM/Martek from *Schizochytrium* sp.ATCC20888**

| Parameter | Unit | Daesang | | | DSM/Martek |
|---|---|---|---|---|---|
| | | NMF2-2701140A1 | NMF2-2003140A1 | NMF2-0111130A1 | VY00081803 |
| Salmonella | in 25g | Negative | Negative | Negative | Negative |
| Coliform bacteria | cfu/g | < 10 | < 10 | < 10 | < 10 |
| E. coli | in 1g | Negative | Negative | Negative | Negative |
| Total aerobic count | cfu/g | < 100 | < 100 | < 100 | < 100 |
| Yeasts | cfu/g | < 100 | < 100 | < 100 | < 100 |
| Moulds | cfu/g | < 100 | < 100 | < 100 | < 100 |
| Staphylococcus aureus | cfu/g | < 10 | < 10 | < 10 | < 10 |

Values for all microorganisms were below detection limits indicating the absence of the microorganism (see Appendix C for test report).

### 5.5.2 Elemental analysis

Potential contamination with various metals, including the regulated heavy metals Lead and Mercury (Commission Regulation 1881/2006/EC) has been tested for the respective batches of Daesang DHA-rich oil *Schizochytrium* sp.RT100, along with a batch of the comparator DHA-rich oil from (now) DSM (Table 10). Analyzed metals in two Daesang batches as well as the DSM batch have levels below detection limits. Only for Daesang batch NMF2-0111130A1, levels for Lead and Copper slightly exceeded the detection limit (see Appendix C for test report; Table 10).
Regulation (EC) 1881/2006 does not specifically indicate maximum levels of lead for a product group comprised of amongst other marine oils of microbiological origin. Nevertheless, taking the most appropriate product group 'Fats and oils, including milk fat' (art. 3.1.14) into account, the maximum level for lead is 0.1 mg/kg, a level that all tested Daesang batches comply with.

For copper, no maximum levels for foods in the EU are regulated. Nevertheless, for the normal population an upper limit (UL) of 5 mg/day is derived, while for children up to 17 years of age, ULs have been set at 1-4 mg/day (Scientific Committee on Food, 2003).

We, therefore, consider the measurable levels of Lead and Copper in the one batch of Daesang DHA-rich oil (NMF2-0111130A1) to be very low and not of safety concern.

**Table 10: Elemental analysis of Daesang DHA-rich oil from *Schizochytrium* sp.RT100 and DSM/Martek from *Schizochytrium* sp.ATCC20888**

| Metal | Unit | Daesang | | | DSM/Martek |
|---|---|---|---|---|---|
| | | NMF2-2701140A1 | NMF2-2003140A1 | NMF2-0111130A1 | VY00081803 |
| Arsenic | mg/kg | < 0.02 | < 0.02 | < 0.02 | < 0.02 |
| Lead | mg/kg | < 0.02 | < 0.02 | 0.039 | < 0.02 |
| Mercury | mg/kg | < 0.02 | < 0.02 | < 0.02 | < 0.02 |
| Copper | mg/kg | < 0.05 | < 0.05 | 0.185 | < 0.05 |
| Iron | mg/kg | < 0.05 | < 0.05 | < 0.05 | < 0.05 |

### 5.5.3 Benzo(a)pyrene

Maximum permitted levels of benzo(a)pyrene are regulated by Commission Regulation EU 835/2011 (EC, 2011a), referring to *Section 6: Polycyclic aromatic hydrocarbons.* Under section 6.1.1 comprising 'Oils and fats (excluding cocoa butter and coconut oil) intended for direct human consumption or use as an ingredient in food' maximum levels for benzo(a)pyrene in foodstuffs are set at 2.0 µg/kg (Table 11) (see Appendix C for test report).

**Table 11: Levels of benzo(a)pyrene of Daesang DHA-rich oil from *Schizochytrium* sp.RT100 and DSM/Martek from *Schizochytrium* sp.ATCC20888**

| Substance | Unit | Daesang | | | DSM/Martek |
|---|---|---|---|---|---|
| | | NMF2-2701140A1 | NMF2-2003140A1 | NMF2-0111130A1 | VY00081803 |
| Benzo(a)pyren | µg/kg | < 0.5 | < 0.5 | < 0.5 | < 0.5 |

### 5.5.4 Dioxins, dioxin-like PCBs and non-dioxin-like PCBs

#### *5.5.4.1 Dioxins and dioxin like PCBs*

EC Maximum levels for dioxins, sum of dioxins and dioxin-like PCBs have been regulated by COMMISSION REGULATION (EU) No 1259/2011 amending Regulation (EC) No 1881/2006 as regards maximum levels for dioxins, dioxin-like PCBs and non-dioxin-like PCBs in foodstuffs (EC, 2011b).

The regulation provides for maximum levels of dioxins and dioxin-like PCBs allowed to be present in foodstuffs. In the Annex, under 5.7 (Marine oils (fish body oil, fish liver oil and oils of other marine organisms intended for human consumption)), the maximum levels are defined. Table 12 displays the measured levels of dioxins and dioxin-like PCBs in Daesang DHA-rich oil from *Schizochytrium* sp.RT100 referring to the maximum levels of dioxins and dioxin-like PCBs, as set by Reg. (EU) No. 1259/2011, levels are below the maximum permitted levels (see also Table 12 and Appendix C for test report).

**Table 12: Contents of dioxins and dioxin-like PCBs of Daesang DHA-rich oil from *Schizochytrium* sp.RT100 and DSM/Martek from *Schizochytrium* sp.ATCC20888**

| | Daesang | | | DSM/Martek | Max Level |
|---|---|---|---|---|---|
| | NMF2-2701140A1 | NMF2-2003140A1 | NMF2-0111130A1 | VY00081803 | |
| TE-WHO dioxins (quantification limits included) : ng/kg fat | 1.2 | 0.24 | 0.71 | 0.17 | dioxins* : 1.75 ng/kg fat |
| TE-WHO dioxin-like PCBs | 0.306 | 0.21 | 0.142 | 0.142 | dioxin-like PCBs: 4.25 ng/kg fat |
| TE-WHO total (quantification limits included) : ng/kg fat | 1.5 | 0.446 | 0.856 | 0.315 | Sum of dioxins + dioxin-like PCBs : 6.0 ng/kg fat |

** Maximum levels as per COMMISSION REGULATION (EU) No 1259/2011.*

#### *5.5.4.2 Non-dioxin-like PCBs*

Levels of non-dioxin-like PCBs for all tested batches were below the detection limit of 0.5 *µ*g/kg, therefore, do not pose a safety concern (see also Appendix C for test report).

### 5.5.5 Potential for contamination with Cyanobacteria

'Blue-green algae' or *Cyanobacteria* are a type of microscopic, algae-like bacteria which inhabit freshwater, coastal and marine waters. Due to the overlap in natural habitat of *Cyanobacteria* and *Schizochytrium* we considered the risk of potential contamination by *Cyanobacteria* during fermentation.

*Schizochytrium* sp. is a heterotrophic microorganism capable of growing in darkness. It derives its energy from at least one organic carbon substrate. Glucose is generally a preferred carbon source for the growth of most microorganisms. *Cyanobacteria*, on the other hand, photosynthesize like plants and have similar requirements for sunlight, nutrients and carbon dioxide to grow and produce oxygen.

Apart from the large differences in optimal growth conditions between *Schizochytrium* sp. and *Cyanobacteria* several additional measures are taken for the prevention of potential contamination of the *Schizochytrium* cultures by *Cyanobacteria* and other microorganisms. These are in part intrinsic to the culture requirements of *Schizochytrium* sp.

1) Only single isolated microalgae are inoculated implicating the absence of contaminating microorganisms.

2) Daesang's cultivation of microalgae is done in sterilized closed system fermenters which are completely isolated from light and ambient air. Supplied air for culturing is delivered through a filter.

3) Culture media and water are sterilized before use.

Considering the highly different optimal growth conditions of *Schizochytrium* sp. versus *Cyanobacteria* and by taking these additional measures, the culture conditions of *Schizochytrium* sp. provide for axenic growth conditions. It is therefore highly unlikely that the Daesang cultures of *Schizochytrium* would become contaminated with *Cyanobacteria*.

### 5.5.6 Potential for contamination with toxin-producing algae

As indicated in the section above, the culture conditions of *Schizochytrium* sp.RT100 are such that contamination with microorganisms, including microalgae capable of producing toxic substances, is highly unlikely to occur. Nevertheless, we have checked both *Schizochytrium* sp.RT100 algal biomass and the DHA-rich oil from the same batches for the presence of a series of algal toxins which are indicated in Table 13.
Analyses have been performed by the Dutch Reference Laboratory RIKILT, Wageningen, The Netherlands. The lipophilic toxins are determined applying one method (Standard Operating Procedure A1127_06) using LC-MS/MS. This broad spectrum analysis also includes Gymnodimine and cyclic imines like spirolides. Due to its hydrophilic nature, domoic acid was analyzed separately applying another SOP (A0935_02) (Table 13; see Appendix D for test reports).

The analyses indicated that for all algal toxins tested, levels were below detection level of the method used (see Appendix D for test reports). After consultation of RIKILT (wageningen, The Netherlands) it was agreed that safety limits in these DHA-rich oils cannot be established due to differences in consumptive behavior, variability of matrix in which the various toxins might be included and no toxicity testing performed as of yet in these oils. Moreover, all tests so far have been conducted in shellfish samples acknowledging the consumption of a certain quantity by a person with a known body weight. As stated clearly by RIKILT the concentrations of the various toxins as determined in the *Schizochytrium* sp.RT100 DHA-rich oil and listed in Table 13 are considered to be safe.

**Table 13: Algal toxins tested in *Schizochytrium* sp.RT100 DHA-rich oil, outcome of three replicates from the same source in oil.**

| Toxin | Reporting limit | Method |
|---|---|---|
| Domoic acid | < 1 mg/kg | IV_A0935_02 |
| Okadaic acid | < 40 µg/kg | IV-A1127_06 |
| Dinophysistoxin-1 | < 40 µg/kg | IV-A1127_06 |
| Dinophysistoxin -2 | < 40 µg/kg | IV-A1127_06 |
| Pectenotoxin-1 | Not detected | IV-A1127_06 |
| Pectenotoxin-2 | Not detected | IV-A1127_06 |
| **Total Okadaic Acid, Dinophysistoxins and Pectenotoxins** | **<160 OA TEQ*/kg** | IV-A1127_06 |
| Yessotoxin | < 125 µg/kg | IV-A1127_06 |
| 45-OH Yessotoxin | < 125 µg/kg | IV-A1127_06 |
| Homo Yessotoxin | < 125 µg/kg | IV-A1127_06 |
| 45-OH Homo Yessotoxin | < 125 µg/kg | IV-A1127_06 |
| **Total Yessotoxin** | **<3.75 mg YTX TEQ/kg** | IV-A1127_06 |
| Azaspiracid-1 | <40 µg/kg | IV-A1127_06 |
| Azaspiracid-2 | <40 µg/kg | IV-A1127_06 |
| Azaspiracid-3 | <40 µg/kg | IV-A1127_06 |
| **Total Azaspiracids** | **<160 µg AZA1 TEQ/kg** | IV-A1127_06 |
| 13-desmethyl spirolide C | <100 µg/kg | IV-A1127_06 |
| Gymnodimine | <50 µg/kg | IV-A1127_06 |

**TEQ: toxic equivalent. See Appendix D for test report*

### 5.5.7 Prymnesins

Prymnesins, produced by the very distantly related *Prymnesium parvum,* have not been determined. The actual number of different substances that comprise the "prymnesins" is presently not known, but their broad range of biological activities support the notion that extracts from cells and from cell free supernatants are composed of a complex and diverse mixture of toxic metabolites (Manning & La Claire, 2010).

Presently, there are no methods available for the specific and quantifiable detection of individual prymnesins. The structural elucidation of the complex prym1 and prym2 molecules has been a significant advancement in the study of this organism that has opened the door for the study of other toxic metabolites in *Prymnesium parvum*. Prym1 and prym2 were chemically characterized by positive-mode ESI-LC/MS and NMR.
While these and related analytical methods are highly-sensitive and capable of validating both mass and structural features, generating procedures for mass spectra can be very complex, expensive, and time-consuming. In addition, prymnesin-toxin-containing fractions must also be highly enriched and contain a few interfering substances for confident detection and quantification. Moreover, there are no standards presently available for prym1, prym2, or for crude extracts of prymnesins. Consequently, such spectroscopic methods are not practical for prymnesin identification in natural or cultured samples. The chemical isolation of individual prymnesins has proven especially challenging, and new methods will need to be developed for the specific *in vitro* detection of toxic metabolites from this alga (Manning& La Claire, 2010).

In a personal communication with prymnesin-expert Dr. Schonna Manning (University of Texas, Austin, Texas), she indicated that there are no known toxins produced by *Schizochytrium* sp. The policy for testing *Schizochytrium* sp. for the presence of prymnesin toxins has arisen from the belief that because *Schizochytrium* sp. is often referred to as "golden algae", even though this organism has no direct relationship to *Prymnesium parvum*, the toxin-producing golden alga, or any photosynthetic golden algae (diatoms, haptophytes, etc.) that contain the golden pigment, fucoxanthin.

*Schizochytrium* sp. is a heterotrophic (heterokont) alga cultivated for the production of omega fatty acids, EPA and DHA, and it would be highly unlikely that a heterotrophic growth platform would support the growth of *P. parvum*, as it is predominantly photoautotrophic (with some evidence of mixotrophy).

According to Dr. Manning, this nomenclature has led to many misunderstandings and, consequently, unnecessary requests for testing. Moreover, the polyketide prymensins in question are produced at such low levels in the cells (25 mg per 400 L of culture), which would require a very dense population of *P. parvum* for this organism to be a legitimate concern.

Taken together these arguments and the rigorous growth conditions of *Schizochytrum* sp.RT100, It is highly unlikely that *Schizochytrium* sp.RT100 cultures would be contaminated with *P. parvum* and its derived toxins.

Overall, there is no indication of algal toxin contamination of Daesang DHA-rich oil from *Schizochytrium* sp.RT100.

### 5.5.8 Hexane residue

Residual extraction solvent was measured applying Headspace-GC/MS. As shown in table 14, in all the batches tested, hexane levels were below the detection limit (< 1 mg/kg) indicating the virtual absence of hexane in the end product.

**Table 14: Hexane residue analysis of Daesang DHA-rich oil from *Schizochytrium* sp.RT100 and DSM/Martek from *Schizochytrium* sp.ATCC20888**

| Substance | Unit | Daesang | | | DSM/Martek |
|---|---|---|---|---|---|
| | | NMF2-2701140A1 | NMF2-2003140A1 | NMF2-0111130A1 | VY00081803 |
| Hexane | mg/kg | < 1 | < 1 | < 1 | < 1 |

Taken together, the data indicate that contamination with measurable concentrations of hexane in all batches tested is not present.

### 5.5.9 Conclusion on levels of undesirable substances

From the data above pertaining to the presence of undesirable substances in both the three Daesang batches as well as the Martek batch, we conclude that both oils are substantially equivalent with respect to the absence of potential contaminations, and that in the few cases where low levels were measurable, we argue that those do not to pose a safety concern.

# 6. Overall conclusion

Daesang's *Schizochytrium* sp.RT100 has been shown to display successive bipartitioning characteristic of *Schizochytrium* sp. and was shown by generation of a 18S RNA phylogenetic tree to be very closely related to Martek's (DSM's) *Schizochytrium* sp.ATCC20888.

With regard to composition we conclude that of Daesang DHA-rich oil from *Schizochytrium* sp.RT100 is substantially equivalent to the previously authorized DHA-rich oil of Martek BioSciences, (Commission Decision 2003/427/EC; Commission Decision 2009/778/EC).

Both oils do not differ with respect to nutritional value and metabolism. Also, there are no indications of significant differences in levels of undesired substances between Daesang Corp. RT100 oil and DSM/Martek ATCC20888 oil. In addition, the intended use of both products is similar and comply with the authorized uses by Commission Decision 2003/427/EC; Commission Decision 2009/778/EC which, for reasons of legal clarity, have recently been replaced by Commission Implementing Decision 2014/463/EU.

Taken together, we conclude that the Daesang DHA-rich oil from *Schizochytrium* sp.RT100 is substantially equivalent to the Martek BioSciences DHA-rich oil from *Schizochytrium* sp.ATCC20888 as authorizedby Commission Decision 2003/427/EC; Commission Decision 2009/778/EC in the context of Regulation 258/97 on Novel Foods.

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