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Comparison of Labyrinthulid Strains L4 and L75 by Fatty Acid Composition and Characteristics

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Labyrinthulids are unicellular heterotrophic marine microalgae. Two labyrinthulid strains, L4 and L75, which produce generous amounts of polyunsaturated fatty acids (PUFAs), were isolated from leaves floating in the coastal areas of Quang Ninh province, Vietnam, and Fukuoka Prefecture, Japan, respectively. Both strains had spindle-shaped cells surrounded by ectoplasmic networks. Numerous oil bodies were observed in each cell, mostly in the vicinity of cell membranes. When soybean oil or soybean lecithin was used as a carbon source with peptone as a nitrogen source, the proportion of PUFAs reached 25-30% and 50-56% for L4 and L75, respectively. After 14 days of growth at 25°C, L4 produced 0.3 mg PUFAs/g-agar in PYA-SBO medium and 0.6 mg PUFAs/g-agar in PYA-SBL medium. In comparison, L75 produced 0.2 mg PUFAs/g-agar in both types of media. The differences between the two strains included changes in cellular morphology and the capacity for attaching tightly to fibers when cultured in liquid PYA medium containing 2% SBL. In addition, when the strains were grown under the same conditions, L4 had a higher growth rate and produced more PUFAs than L75.

Key words: Heterotroph, Labyrinthulid, Polyunsaturated fatty acid, Marine microalgae, Fiber

Introduction

Labyrinthulids are unicellular heterotrophic marine microalgae. They were formerly considered to be fungi; however, they are presently recognized as stramenopiles of the kingdom Protista sensu lato (Honda et al., 1999). Cavalier-Smith (1994) and Cavalier-Smith et al. (1994) classified thraustochytrids and labyrinthulids under the kingdom Chromista, phylum Heterokonta, subphylum Labyrinthista, class Labyrinthulea, families Thrausto-chytridae and Labyrinthulidae, respectively. The distinguishing features of the species known as labyrinthulids and thraustochytrids were subsequently presented by Dick (2001). Members of the phylum Labyrinthulomycota, which are collectively known as marine slime molds, are parasitic or saprotrophic toward organic debris and marine organisms such as mollusks and aquatic plants. Inouye (2004) suggested that algae be defined as a polyphyletic assemblage of organisms including prokaryotic cyanobacteria and nine eukaryotic groups, which are distinguished from one another based on their cellular architecture and the composition of their photosynthetic antenna system. It is widely accepted that oxygenic photosynthesis originated in prokaryotes and eukaryotes as the result of a single endosymbiotic event called primary endosymbiosis, namely, the enslavement of a cyanobacterium by a heterotrophic eukaryotic protist (Archibald and Keeling, 2004). Although Heterokonphyta, Haptophyta, Dinophyta, Euglenophyta, and Apicomplexa lack nucleomorphs, these algae are believed to have arisen by secondary endosymbiosis (Archibald and Keeling, 2004). Cavalier-Smith (2004), Arisue et al. (2002), and Baldauf (2003) demonstrated that the ten to 12 super-groups of eukaryotes could be classified into two (unikonts and bikonts) or three (Amoebozoa, Opisthokonta, and bikonts) large assemblages. However, Cavalier-Smith (1989) and Yoon et al. (2002) proposed a single common origin for the kingdom Chromista, with an assemblage of algae comprised of cryptophytes, heterokontophytes, and haptophytes, based on trees of plastid-encoded genes. Other reports, such as those by Fast et al. (2001) and Cavalier-Smith (2004), have asserted that in the big trees of eukaryotes, stramenopiles and alveolates form a monophyletic clade, called chromalveolates.

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Apicomplexa, which once had plastids, lost their photosynthetic ability secondarily, but still retain plastids that they use for fatty acid and isoprenoid metabolism (Gardner et al., 2002). These facts suggest that plastids do not disappear, even when an organism loses its photosynthetic function. Heterotrophic members of the chromalveolate and other bikont lineages should be reexamined from this perspective (Inouye, 2004).

Labyrinthulids began to attract attention because of their ability to produce long-chain polyunsaturated fatty acids (PUFAs), such as docosahexaenoic (DHA, C22:6 n-3) and docosapentaenoic (DPA, C22:5 n-6). DHA is important due to its specific function in the brain (O'Brien and Sampson, 1965) and retina (Uauy et al., 1990). Although the physiological function of n-6 DPA has yet to be clarified, some compositional features have been reported. A relatively high level of n-6 DPA has been observed in the brain and retina (Homayoun et al., 1988; Bourre et al., 1989; Dyer and Carol, 1991) under conditions of n-3 essential fatty acid (EFA) deficiency. A deficiency in n-3 EFAs during lactation resulted in an increase in n-6 DPA and a decrease in the DHA level, even in the liver and serum lipids (Guesnet et al., 1988). The overall level of n-6 DPA in the biosphere is low, except in labyrinthulids and thraustochytrids, most of which contain DHA and a lesser amount of DPA. PUFA production has been improved in the genera *Crypthecodinium* (Jiang and Chen, 2000), *Mortierella* (Nakahara et al., 1992; Yamada et al., 1987), *Schizochytrium* (Nakahara et al., 1996), and *Thraustochytrium* (Iida et al., 1996). *Schizochytrium* and *Thraustochytrium* are used for the commercial production of omega-3 fatty acids. The ectoplasmic networks of labyrinthulids can digest bacteria, yeast, and other organisms (Porter, 1989). Isolation techniques for labyrinthulids have been proposed (Waltson and Ordal, 1957; Sakata and Iwamoto, 1995; Yokochi et al., 2001); in addition, PUFA production using labyrinthulids has been reported (Kumon et al., 2002; 2003). In this report, we present the results of a comparison between two labyrinthulid strains, L4 and L75, isolated from leaves floating in coastal areas of Southern Vietnam (Quang Ninh province) and Fukuoka Prefecture, Japan, respectively.

Materials and Methods

Culture of the labyrinthulids on solid medium

The base medium for the isolation of the labyrinthulids (GPY medium) contained glucose (2 g/L), peptone (1 g/L), and yeast extract (0.5 g/L) in artificial seawater containing 50% salt (50% ASW, approximately 1.5% NaCl; Tropic Marine Aquarientechnik, Wartenberg, Germany). PY medium consisted of polypeptone (1 g/L) and yeast extract (0.5 g/L) in 50% ASW. Solid agar medium (GPYA or PYA) was prepared by the addition of agar (15 g/L). As a food source for the labyrinthulids, a bacterial suspension of *Psychrobacter phenylpyruvicus* (100 µL containing 50 µg of cells, dry weight) grown in GPY medium was spread onto a PY agar plate (10 cm diameter). A square piece of inoculum (PYA medium, about 5×5 mm, on which labyrinthulids had already grown) was subsequently placed in the center. The culture was incubated in a stationary position at 25°C. For the improved medium, 1 L of PYA medium was supplemented with 5 g of soybean oil (SBO; Kumon et al., 2002) or soybean lecithin (SBL; Wako Pure Chemical Industries, Tokyo, Japan) as a carbon source plus 1.25 g of Tween-80 (Nacalai Tesque, Tokyo, Japan). The SBO or SBL and Tween-80 were autoclaved separately with the components of the medium and then mixed aseptically using disposable syringes to form an oil suspension (designated as PYA-SBO or PYA-SBL medium). In the oil-suspended medium, the labyrinthulids grew not only on the surface of the solid medium, but also inside of the medium (i.e., three-dimensional growth). A bacterial suspension (100 µL containing 50 µg of cells, dry weight) grown in GPY medium was spread onto a plate containing PYA-SBO medium. A square piece of inoculum (about 5×5 mm, on which labyrinthulids had already grown) was placed in the center of the PYA-SBO medium. The culture was incubated in a stationary position at 25°C.

Fibrous immobilization medium

Fibrous activated carbon (FMI; Unitika Co. Ltd.) with a six-bladed shape (8 mm in diameter and 10 mm in length) was used. A total of 5-15 FMIs plus 50-100 mL of PY-SBL medium (PY medium with 1-5% SBL in ASW with a 50% salt concentration) were placed in a 250-mL flask and incubated at 25°C with shaking at 120 rpm.

Isolation of labyrinthulids

Labyrinthulid strains L4 and L75 were isolated from floating leaves and seaweed collected from seawater in Southern Vietnam (Quang Ninh province in October of 2002) and Fukuoka Prefecture, Japan (in July of 2002), using the technique reported by Yokochi et al. (2001). The plant samples were cut into square pieces (-1 cm^2) and washed twice with sterilized 50% ASW. The cut samples were then placed aseptically onto GPYA medium on which *P.*

phenylpyruvicus had been grown. The plates were then sealed with sealing film and incubated at 25°C. The plates were observed daily for 7 days under an inverted microscope. The labyrinthulids were easily recognized due to the spindle shape and gliding movement of the cells. The isolated strains were kept on PYA-SBO medium as monoxenic cultures with *P. phenylpyruvicus*.

Morphological observation

Living cells in the colonies on the agar plates or in liquid medium were examined using an Olympus BX50 light microscope fitted with a Nomarski interference differential contrast objective. For the observation of oil bodies, one drop of cell suspension was mixed with one drop of Nile blue (10 μg/mL in acetone). The cells were then viewed with an epifluorescence microscope (Olympus BX60) under 520-550 nm excitation.

Lipid analysis

Square pieces of the cultivated agar media (about 2×2 cm^2) containing the labyrinthulids were removed, weighed, and dried at 105°C for 3 hr. The dried media were then directly methyl-esterified with 10% HCl in methanol. The resultant methyl esters were then applied to a gas chromatograph (GC-17-A Shimazu, Kyoto, Japan) equipped with a TC-70 capillary co-lumn (GL Science, Tokyo, Japan) under temperature programming (180 to 220°C at 4°C/min). The level of each fatty acid and the total fatty acids (TFAs) were quantified using arachidic acid (C20:0) and hepta-decanoic acid (C17:0) as internal standards. In this study, PUFA represents the amount of DHA and n-6 DPA, since no substantial amounts of eicosapen-taenoic acid or n-3 DPA were detected. The amount of PUFAs produced is expressed in terms of the g PUFAs/L medium, using the average specific gravity (0.96 g/L) of the cultivated media. PUFA content represents the amount of PUFAs over the TFAs in the cut agar culture (Kumon et al., 2003). For the liquid medium, 1 mL of medium containing labyrinthulid cells was used for the quantitative analysis of PUFA production. The data were analyzed using one-way analysis of variance. The significance of the differences among the means was established using Tukey's test ($P<0.05$) (Snedecor and Cochran, 1989).

Results

The isolated strains (L4 and L75) were cultivated on PYA-SBO medium with *P. phenylpyruvicus* for 7 days, and the PUFA content (%) of the cut agar culture was obtained by GLC analysis. The values were 25-30% and 50-56% per TFA in strains L4 and L75, respectively. Strains L4 and L75, which grew well on PYA-SBO medium, were used in each of the following experiments.

Differences between L4 and L75 cultivated on agar medium

We cultivated the labyrinthulid strains L4 and L75 on PYA agar medium containing 0.5% SBO or SBL. The growth rate and amount of PUFA produced by each strain are shown in Table 1. The growth rate for both strains was higher on PYA-SBL agar medium than on PYA-SBO. Strain L4 produced more PUFAs than strain L75; however, strain L75 exhibited a higher growth rate. Cultivation of labyrinthulids using 5 g/L SBL leads to higher PUFA production than cultivation using SBO (Table 1). This suggests that some component of SBL promotes labyrinthulid growth. Growth enhancement was achieved by the addition of SBL. The lipid composition of the paste from which SBL was prepared was 40.8% phospholipids, 40.0% triacylglycerol (TG), and others. The phospholipids included 16.6% phosphatidylcholine, 9.8% phosphatidylinositol, 8.1% phosphatidylethanolamine, and 6.3% phosphatidylacid. Tween-80 was used as a detergent, since SBL was insoluble in the PYA medium. The PYA-SBL medium was yellowish due to the pigments in the SBL. When our labyrinthulid strains were inoculated onto the center of a square of PYA-SBL agar medium with bacteria, three-dimensional growth was observed; that is, growth was observed not only on the surface of the medium but also inside of the medium. The same result was obtained using PYA-SBO medium after 7 and 14 days of cultivation at 25°C. The size and morphology of the cells were nearly identical on both media. PUFA production and the PUFA content were higher on PYA-SBL medium than on PYA-SBO medium (0.55 g/L and 29.2%, respectively, for L4). TG in the SBL may be an essential factor for the three-dimensional growth of labyrinthulids. SBL contains not only lipids but also many trace components and carotenoids. Some carotenoids function as antioxidants against the oxidation of lipids. These elements may also be responsible for the increased production of PUFAs in SBL versus SBO medium by our labyrinthulid strains. The droplets of SBL that were dispersed in the medium were smaller than those of SBO. This difference could conceivably have an effect on labyrinthulid growth and the PUFA yield. Fig. 1 shows the growth rates of labyrinthulid strains L4 and L75. Strain L4 grew preferentially on the side

Table 1. Growth rate and PUFAs production of Labyrinthulid strains L4 and L75 on PYA agar medium containing 0.5% SBO or SBL. Data are mean ± SD (n=6). Means in a column having different superscript letters are significantly different at $P<0.05$

Media	Strain	7 d-cultivation			14 d-cultivation			Note	
		Growth rate ($\times 10^6$ cells/mL)	PUFAs mg/g-agar (g/L)	PUFAs content (%)	Growth rate ($\times 10^6$ cells/mL)	PUFAs mg/g-agar (g/L)	PUFAs content (%)	Surface	3D
PYA-SBO (0.5%)	L4	5.71 ± 0.76^a	0.08 ± 0.02^a	25.80 ± 1.12^b	19.52 ± 0.32^a	0.30 ± 0.03^b	29.70 ± 1.96^b	-	++
	L75	11.47 ± 0.51^b	0.15 ± 0.03^a	52.60 ± 2.10	13.62 ± 0.62^b	0.17 ± 0.04^b	55.30 ± 2.94^b	+	+
PYA-SBL (0.5%)	L4	10.27 ± 0.32^a	0.11 ± 0.06^a	30.20 ± 1.56	27.85 ± 0.27^a	0.55 ± 0.02^b	29.20 ± 1.36^b	-	+++
	L75	14.32 ± 0.17^b	0.17 ± 0.04^b	53.90 ± 2.64^a	18.06 ± 0.11^b	0.22 ± 0.05^a	55.70 ± 2.56^b	+	++

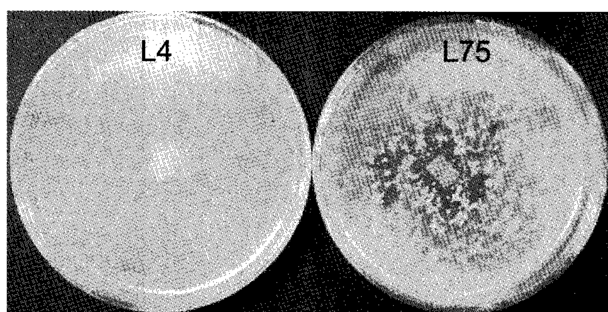

Fig. 1. Different growth rate of Labyrinthulid strains L4 and L75 on PYA medium containing 0.5% SBO.

of the agar medium, whereas strain L75 grew mostly on the surface in addition to showing three-dimensional growth. Following 14 days of culture at 25°C, L4 produced 0.30 mg/g-agar of n-6 DPA and DHA on PYA-SBO and 0.55 mg/g-agar on PYA-SBL. In comparison, L75 produced 0.17 mg/g-agar of n-6 DPA and DHA on PYA-SBO and 0.22 mg/g-agar on PYA-SBL.

Differences between L4 and L75 at various concentrations of SBL using fibrous immobilization medium

The effect of various concentrations of SBL on PUFA production in labyrinthulid strains L4 and L75 cultivated on fibrous immobilization medium is shown in Table 2. In both strains, a higher concentration of SBL resulted in increased PUFA production. When the concentration of SBL exceeded 2%, PUFA production in both strains reached its maximum value (1.29 g/L at 4% SBL for strain L4 and 0.57 g/L at 2% SBL for L75). At concentrations below 2%, PUFA production increased rapidly. It appears that at SBL concentrations above 2%, saturation of PUFA production occurred in both strains. The results in Table 2 indicate that the cells of strain L4 were present not only in the liquid portion of the culture but also in the fibrous portion. Thus, the L4 cells were able to adhere to the fibers, but were also easily released from the fibers and into the liquid medium. Contrary to L4, strain L75 was found mostly attached to the fibers and could not be easily removed by squeezing.

Differences between L4 and L75 cultivated in liquid medium containing 2% SBL

Based on our results, the presence of SBL at concentrations exceeding 2% enabled higher PUFA production compared to lower concentrations of SBL. To observe the labyrinthulid cells easily by microscopy, strains L4 and L75 were cultivated at 25°C in 100 mL of liquid medium containing 1 g/L polypeptone, 0.5 g/L yeast extract, 50% ASW, and 2% SBL with 15 fibers in a 250-mL flask with shaking at 120 rpm. Along with an analysis of PUFA production under these conditions, we recorded the morphological changes in the cells using a microscope. All changes in cellular morphology observed for strains L4 and L75 are shown in Fig. 2, whereas the PUFA production data are given in Table 3. Our results indicate that the L75 cells were largely attached to the fibers and were difficult to remove, while the cells of strain L4 were observed not only in the fibrous portion but also in the liquid. In addition, the L4 cells could be easily removed from the fibers. Thus, PUFA production in the mixed portion of strain L4 was higher than that in the liquid and fibrous parts, whereas PUFA production for strain L75 was higher in the fibrous portion than in the liquid or mixed portion. L4 produced more PUFAs than L75 during liquid culture using fibrous immobilization media; PUFA production after 14 days of cultivation reached 1.29 g/L in the mixed portion for L4 and 0.57 g/L in the fibrous portion for L75. As shown in Fig. 2B, after 7 days of cultivation under these conditions, the cells of L75 were larger and contained a higher number of oil bodies. The cells of strain L4 showed a similar morphology only after 14 days of cultivation (Fig. 2A).

Differences in the growth of L4 and L75 on fibers in PYA containing 2% SBL

We next examined the difference between strains

Table 2. Effect of different SBL concentrations on PUFAs production from two Labyrinthulid strains L4 and L75 by fiber cultivation. Data are mean±SD (n=6). Means in a column having different superscript letters are significantly different at $P<0.05$

Strain	Sample	Content of SBL concentration	After 7 d-cultivation		After 14 d-cultivation	
			PUFAs (g/L)	PUFAs content (%)	PUFAs (g/L)	PUFAs content (%)
L4	Liquid part	0.5%	0.06 ± 0.02^{a}	14.39 ± 1.38^{a}	0.04 ± 0.03^{b}	12.38 ± 1.46^{b}
		1%	0.35 ± 0.03	29.98 ± 2.25	0.26 ± 0.07	32.65 ± 2.17
		2%	0.79 ± 0.04^{a}	28.81 ± 2.12^{b}	0.89 ± 0.08^{b}	27.25 ± 2.55^{a}
		3%	0.69 ± 0.06	7.81 ± 1.28	0.72 ± 0.05	9.14 ± 1.67
		4%	0.70 ± 0.02^{a}	3.72 ± 1.67^{b}	0.77 ± 0.05^{a}	8.02 ± 1.55^{b}
		5%	0.31 ± 0.03^{b}	1.05 ± 0.34^{d}	0.44 ± 0.06^{a}	4.45 ± 1.06^{b}
	Fiber part	0.5%	0.11 ± 0.04^{a}	3.62 ± 1.02^{b}	0.15 ± 0.03^{a}	5.80 ± 0.55^{b}
		1%	0.79 ± 0.06	14.36 ± 2.23	0.42 ± 0.02	13.64 ± 1.89
		2%	0.82 ± 0.09^{b}	18.27 ± 1.11^{b}	0.60 ± 0.07^{b}	16.18 ± 2.02^{a}
		3%	0.80 ± 0.06	6.33 ± 1.62^{b}	0.72 ± 0.05^{a}	6.74 ± 0.76^{c}
		4%	0.89 ± 0.05^{a}	3.24 ± 0.87	0.75 ± 0.05	4.78 ± 0.79
		5%	0.58 ± 0.03	2.48 ± 0.59	0.64 ± 0.04	4.75 ± 0.45
	Mix	0.5%	0.06 ± 0.05^{a}	7.61 ±1.23^{b}	0.10 ± 0.04^{b}	15.63 ± 1.78^{b}
		1%	0.66 ± 0.04	25.05 ± 3.48	0.61 ± 0.03	29.28 ± 2.65
		2%	0.96 ± 0.06^{c}	23.23 ± 3.56^{b}	0.98 ± 0.05^{a}	24.67 ± 2.94^{b}
		3%	0.72 ± 0.08	7.77 ± 1.56	0.90 ± 0.04	7.93 ± 0.58
		4%	0.78 ± 0.03^{a}	3.72 ± 1.20^{b}	1.29 ± 0.07a	6.79 ± 0.94^{a}
		5%	0.70 ± 0.02	2.50 ± 0.54	0.61 ± 0.03	4.87 ± 0,40
L75	Liquid part	0.5%	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
		1%	0.15 ± 0.03	24.67 ± 2.96	0.12 ± 0.03	36.25 ± 2.47
		2%	0.17 ± 0.04^{a}	3.89 ± 0.45^{b}	0.17 ± 0.05^{b}	3.04 ± 0.93^{a}
		3%	0.20 ± 0.01	2.14 ± 1.23	0.18 ± 0.04	2.02 ± 0.10^{d}
		4%	0.26 ± 0.05^{a}	1.76 ± 0.22^{d}	0.19 ± 0.07^{a}	1.47 ± 0.10^{c}
		5%	0.20 ± 0.06	1.09 ± 0.26	0.23 ± 0.09	1.44 ± 0.09
	Fiber part	0.5%	0.17 ± 0.01^{a}	22.81 ± 2.15^{a}	0.17 ± 0.03^{b}	14.04 ± 1.04^{b}
		1%	0.24 ± 0.05	19.73 ± 1.93	0.39 ± 0.05	20.23 ± 2.12^{a}
		2%	0.42 ± 0.07^{a}	4.97 ± 0.79^{b}	0.57 ± 0.07^{b}	39.43 ± 2.86^{a}
		3%	0.27 ± 0.04^{c}	1.12 ± 0.36^{b}	0.26 ± 0.08^{a}	4.51 ± 0.22^{b}
		4%	0.26 ± 0.07^{b}	1.16 ± 0.19	0.27 ± 0.08^{c}	1.46 ± 0.62^{c}
		5%	0.39 ± 0.08^{c}	0.93 ± 0.06^{a}	0.34 ± 0.09^{d}	3.83 ± 0.56^{a}
	Mix	0.5%	0.02 ± 0.01^{a}	13.94 ± 1.45^{b}	0.00 ± 0.00	0.00 ± 0.00
		1%	0.26 ± 0.04	27.19 ± 2.29	0.18 ± 0.01	52.07 ± 3.98
		2%	0.28 ± 0.03^{a}	3.77 ± 0.46^{b}	0.45 ± 0.01^{b}	43.63 ± 2.87^{a}
		3%	0.30 ± 0.06	2.09 ± 0.16	0.30 ± 0.02	2.75 ± 0.13
		4%	0.36 ± 0.04^{b}	1.64 ± 0.67^{a}	0.29 ± 0.01^{c}	1.69 ± 0.10a
		5%	0.40 ± 0.08	1.35 ± 0.45	0.11 ± 0.03	1.65 ± 0.32

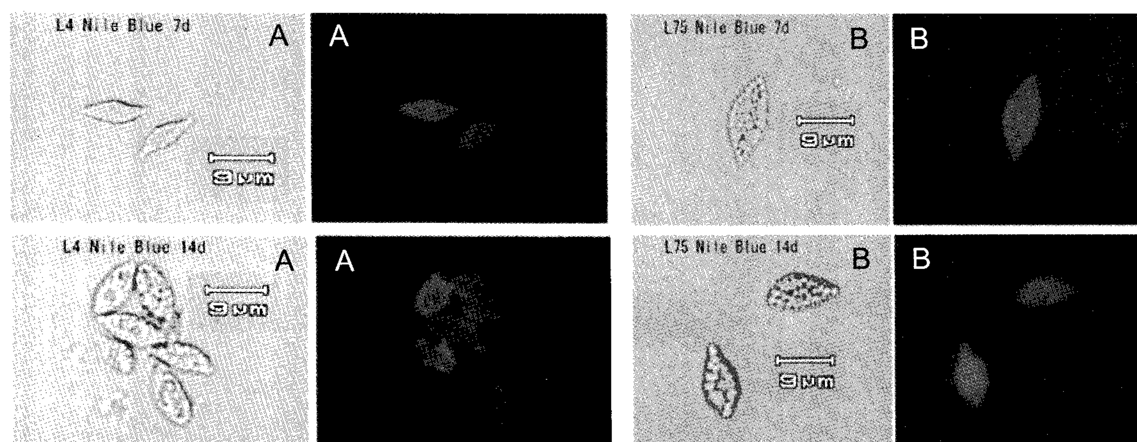

Fig. 2. Change in cell morphology of Labyrinthulid strain L4 (A) and L75 (B) cultivated in liquid medium containing 2% SBL after 7 and 14 days cultivation.

Table 3. Change of PUFAs production from two Labyrinthulid strains L4 and L75 in PYA medium containing 2% SBL. Data are mean±SD (n=6). Means in a column having different superscript letters are significantly different at $P<0.05$

Strain	Sample	7 d-cultivation		14 d-cultivation	
		PUFAs production (mg/g-agar or g/L)	PUFAs content (%)	PUFAs production (mg/g-agar or g/L)	PUFAs content (%)
L4	Liquide part	0.74 ± 0.09^{a}	28.81 ± 2.43^{b}	0.89 ± 0.10^{b}	27.25 ± 2.23^{b}
	Fibre part	0.82 ± 0.07^{b}	18.27 ± 2.17	0.60 ± 0.12^{b}	16.18 ± 1.76^{a}
	Mix	0.96 ± 0.06^{b}	23.23 ± 2.78^{a}	0.98 ± 0.19^{b}	24.67 ± 1.72^{a}
L75	Liquide part	0.17 ± 0.01^{a}	3.89 ± 0.53^{b}	0.17 ± 0.01^{b}	3.04 ± 0.65^{b}
	Fibre part	0.42 ± 0.01^{b}	4.59 ± 0.61^{a}	0.57 ± 0.19^{a}	43.43 ± 1.32^{b}
	Mix	0.27 ± 0.02^{b}	3.97 ± 0.29^{a}	0.45 ± 0.08^{a}	53.63 ± 1.53^{b}

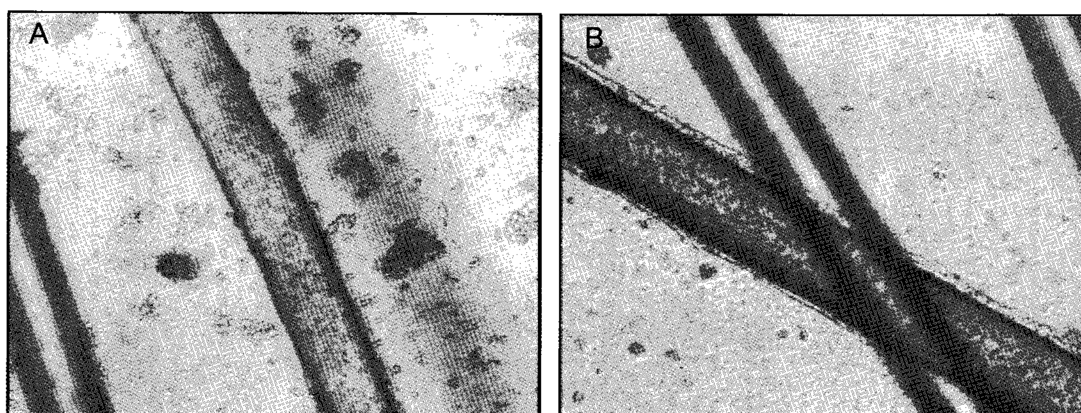

Fig. 3. Difference of two Labyrinthulid strains L4 (A) and L75 (B) in capacity of tight stick on fiber in PYA liqu id medium containing 2% SBL.

L4 and L75 in terms of their ability to adhere tightly to fibers in liquid PYA medium containing 2% SBL (Fig. 3). L75 could still be observed on the surface of the fibrous filaments after squeezing (Fig. 3B), whereas the same treatment removed most of the L4 cells from the fibers (Fig. 3A). The results in Fig. 3 also help explain why PUFA production in the mixed portion for strain L4 was higher than that in the liquid and fibrous portions. PUFA production by L75 in the fibrous portion was higher than that in the liquid and mixed portions (Tables 2 and 3).

Discussion

According to a review by Raghukumar (2002), labyrinthulids are prevalent on or in living marine algae (seaweeds) and seagrasses as parasites, commensals, or mutualists. In contrast, thraustochytrids are rarely found on living plants and appear to be inhibited by plant antimicrobial compounds. Porter (1989) suggested that there is only one genus (*Labyrinthula*) in the family Labyrinthulids, whereas there are several genera in the family Thraustochytrids. Eight species of *Labyrinthula* are currently recognized, which may be distinguished on the basis of cell size, color, and developmental stage: *L. macrocystis* Cienkowski 1867, *L. vitellina* Cienkowski 1867, *L. cienkowskii* Zopf 1892, *L. valkanovi* (Valk.) Karling 1944, *L. algeriensis* Hollande and Enjumet 1955, *L. roscoffensis* Chadefaud 1956, *L. coenocystis* Schmoller 1960, and *L. magnifica* (Valk.) Olive 1975. However, Raghukumar (2002) indicated that the labyrinthulids included the genera *Labyrinthula* Cienkowski and *Aplanochytrium* Bahnweg and Sparrow. Species of the genus *Labyrinthula* grow colonially, with numerous spindle-shaped cells linked together by means of an ectoplasmic net. Members of the genus *Aplanochytrium* Bahnweg and Sparrow multiply by means of spores that move in a gliding manner using ectoplasmic net elements. This genus, which was originally referred to as *Labyrinthuloides*, comprises a distinct lineage, the labyrinthuloids, among the *Labyrinthulomycetes*, alongside the thraustochytrids and labyrinthulids. There is a high level of morphological variability and overlap among the species of the *Labyrinthulomycetes*; thus, the taxonomy of these organisms should be carefully reconsidered using molecular methods (Honda et al.,

1999). Thraustochytrids and some labyrinthulids may be found in coastal and oceanic waters, pelagic and benthic regions, plant and animal substrata, and as saprobes and parasites (Raghukumar, 2002). *Labyrinthulomycetes* can cause disease in animals or live as commensals or mutualists within the guts and tissues of marine invertebrates (Raghukumar, 2002); moreover, they are saprobic toward animal materials such as feces and mollusk shells. However, labyrinthulids have attracted attention in recent years because of their ability to produce PUFAs. *Labyrinthulea* are important decomposers that degrade detritus, fallen leaves, and marine algae. They can also use dissolved organic nutrients released on the surface of aquatic plants and produce fatty acids. Thus, they may play an important role in the mineralization of organic matter in the sea and serve as food for grazers during certain seasons and in some locations. Their high omega-3 PUFA content suggests that they may be an important link in the food web. According to Raghukumar (2002), additional data regarding the ability of labyrinthulids to degrade various forms and concentrations of organic carbon in the sea, as well as their use of phagotrophy, will help resolve their exact ecological niche vis-à-vis bacteria in marine ecosystems.

The morphological features of strains L4 and L75 were similar to those of other labyrinthulids; the cells were approximately 8.23 ± 1.04 and 11 ± 1.34 μm in length and 3.23 ± 0.35 and 4.21 ± 0.54 μm in width, respectively (data not shown). *Heterokont biflagellate* zoospores were not observed on agar medium. Both strains formed spindle-shaped cells surrounded by ectoplasmic net elements, which is typical of labyrinthulids. Strains L4 and L75 also formed several oil bodies beneath the cell membrane (Fig. 2). We used optimal culture conditions for PUFA production (25°C, initial pH of 6-10, and ASW with a salt concentration of 50%) in our experiments. The lipid content in strains L4 and L75 was 25% and 18%, respectively. These values are lower than that reported for the labyrinthulid strain L59 by Kumon et al. (2003). EPA, n-6 DPA, and DHA were not included in the oil added to the media (the fatty acid composition of SBO and SBL is not shown here), nor were they present in the bacterium; they were produced only by the labyrinthulids. Therefore, PUFA (e.g., DHA and n-6DPA) production seems to be a suitable indicator of labyrinthulid growth, in addition to counting the cell number using the semi-quantitative Most Probable Number (MPN) technique or the acriflavine direct detection (AfDD) technique with epifluorescence microscopy, which have been used for labyrinthulids such as *Aplanochytrium* sp. (Raghukumar, 2002). Under our experimental conditions, the proportion of PUFAs in strains L4 and L75 reached 25-30% and 50-60%, respectively (Table 1). Labyrinthulid strain L4 produced 0.3 mg/g-agar PUFAs in PYA-SBO medium and 0.6 mg/g-agar in PYA-SBL medium after 14 days at 25°C. Strain L75 produced about 0.2 mg/g-agar PUFAs in both media (Table 1). Differences between the two strains were observed in terms of their cellular morphology and their ability to adhere tightly to fibers when cultured in liquid PYA medium containing 2% SBL (Figs. 1-3). The results shown in Tables 1-3 indicate that strain L4 had a higher growth rate and produced more PUFAs than strain L75 under the same conditions. Additional studies are needed to increase PUFA production in both strains, since the level of productivity obtained in the present study is insufficient for feeding experiments using rotifer, krill, and shrimp larvae in fibrous media. Microalgae can be mass-cultured if the conditions allow for the exclusion of all or most potential competitors, as in the case of the commercial production of *Spirulina* (Pulz and Gross, 2004), *Schizochytrium*, and *Thraustochytrium* (Sijtma and Swaff, 2004). Otherwise, a large amount of inoculum must be used for the production process. This requires algal strains that grow quickly under the conditions required for inoculum production while also being highly productive. This is a major issue for any large-scale algal production process, where the cost of inoculum production could easily dominate the overall economics, as it does with *Chlorella* and *Haematococcus* (Pulz and Gross, 2004; Boussiba, 2000). Microalgal PUFAs hold great promise on the biotechnological market as both food and feed; health-promoting purified PUFAs are added to infant milk formulas in Europe, while hens are fed with special microalgae-like heterotrophically grown *Schizochytrium* sp. or *Cryptheodinium* to produce Omega eggs (Pulz, 2004; De Swaff et al., 2003). Both applications have proven to be profitable. Labyrinthulea are important decomposers that degrade detritus, fallen leaves, and algae and produce fatty acids; thus, a diatom double-layer agar has been described for labyrinthulid isolation (Sakata and Iwamoto, 1995). Among the *Labyrinthula* spp., three types of PUFA profiles can be described: DHA/DPA/DTA (docosatetraenoic acid, C22:4 n-6), DPA, and DHA (Nakahara, 1996). PUFA production using labyrinthulids in liquid medium has not yet been reported. Since labyrinthulids require other microbes as their food, their culture must be monoxenic, which may lead to little growth in liquid

medium. However, PUFA production using labyrinthulids has been reported (Kumon et al., 2002; 2003). As intensive aquaculture continues to expand, so does the requirement for high-quality protein and oil sources. Fish meal and oil are major and increasingly expensive components of commercial aquaculture feeds, and their replacement has been the focus of considerable research for many species. The replacement of fish oils is viewed as a significant problem owing to the balance of EFAs in vegetable protein, particularly the absence of EPA (C20:5 n-3) and DHA (Lasen, 2000). Labyrinthulids may have greater potential for the industrial-scale production of EFAs than autotrophy because their culture is less complex. PUFA production by labyrinthulids for the aquaculture of rotifer, krill, and shrimp larvae in fibrous media has also become a topic of universal interest. Microalgal oils differ from most vegetable oils in being quite rich in PUFAs with four or more double bonds (Belarbi et al., 2000). Microalgae appear to be a source of renewable biodiesel, which is capable of meeting the global demand for transport fuels. The oil productivity of many microalgae greatly exceeds that of the best producing oil crops (Chisti, 2007). The oil content in microalgae such as *Schizochytrium* sp. can exceed 80% of the dry weight (Spolaore et al., 2006). Oil-producing heterotrophic microorganisms grown on a natural organic carbon source such as sugar can be used to make biodiesel (Ratledge and Wynn, 2002). On the other hand, heterotrophic production is not as efficient as using photosynthetic microalgae. The production of algal oils requires the ability to inexpensively produce large quantities of oil-rich microalgae. A microalgal biorefinery can simultaneously produce biodiesel, animal feed, biogas, and electrical power. The extraction of other high-value products may be feasible, depending on the specific microalga. Producing low-cost microalgal biodiesel requires improvements in algal biology through genetic and metabolic engineering (Chisti, 2007). The use of the biorefinery concept will further lower the cost of production.

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