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Raghukumar 2008). In spite of the numerous attempts to improve PUFA production in these organisms, only a few strains are currently available in culture collections, and isolation procedures have remained virtually unchanged since they were formulated more than 40 years ago (Vishniac 1956; Goldstein 1963a,b; Fuller et al. 1964). Porter (1990) summarized the two basic techniques applied for the isolation of thraustochytrids: (1) direct plating of appropriate sampled material onto a nutritive medium supplemented with antibiotics; or (2) placing a portion of the sample into baited (commonly pine pollen) seawater and the subsequent plating of colonized baits on the nutritive medium. Slight modifications of these procedures (the use of diverse baits and/or isolation media, variations on incubation temperature, etc.) have been adopted in different laboratories (Bahnweg and Sparrow 1974; Honda et al. 1998; Fan et al. 2002).

To study the thraustochytrids from temperate and cold environments of the southern region of South America (Argentina), pine pollen baiting and transference of the colonized baits to agarized glucose–peptone–yeast extract (GPY) medium have been used as the standard isolation method in our laboratories (Rosa et al. 2006). However, many of such microorganisms detected by microscopic examination in both the original samples and the colonized baits failed to be isolated with this methodology. As most of the previously studied strains came from tropical and subtropical regions, especially organic matter-rich mangroves areas (Raghukumar 2002), some of the features of the cultivation methodology should be adjusted to isolate local "elusive" thraustochytrids. The aim of this work is to determine suitable conditions for a more efficient isolation of representatives of this group of microorganisms from both cold and temperate environments. The adaptation of the classical methodology, including the use of two new culture media that allowed us the isolation of 35 new strains, is analyzed. The effects of the composition of these newly formulated and other already reported culture media on the relative growth of the strains were studied comparatively. Additionally, as agar content of culture media could limit thraustochytrids proliferation (Vishniac 1956), the effect of its concentration on isolates growth was also investigated. Based on previous reports from the literature, our expertise in this subject and the results reported herein, a standard methodology is proposed as a tool for improving the success of the isolation of thraustochytrids.

Materials and methods

Sampling

Samples were collected in 50-ml sterilized plastic containers from the following sites:

1. Two small saline environments (a saline pond outside Rada Tilly town, 45°55′ S–67°34′ W; and La Mata stream, 45°53′ S–67°33′ W) and two intertidal marine habitats (one near Comodoro Rivadavia city, 45°50′ S–67°28′ W; and the other next to Caleta Cordova crude oil port, 45°47′ S–67°24′ W); all these sites are in the vicinity of Comodoro Rivadavia city (CR samples), Chubut province; algal, plant, and animal debris; organic and inorganic sediments
2. Intertidal zones of (a) Puerto Madryn city (PM samples), 42°46′ S–65°01′ W; (b) Puerto Pirámides (PP samples), 42°34′ S–64°17′ W, Chubut province; and (c) Las Grutas beach (LG samples), 40°48′ S–65°05′ W, Río Negro province; algal fragments and inorganic sediments recently washed ashore
3. San Antonio Oeste salt marsh (SAO samples): 40°44′ S–64°56′ W, Chubut province; algal, plant, and animal debris; detritus and inorganic sediments
4. Ingeniero White industrial port: 38°47′ S–62°16′ W, Bahia Blanca city (BB samples), Buenos Aires province; sludge with bird feces and sediments
5. Intertidal environment of East Tunel Bay: 54°49′ S–68°07′ W, Tierra del Fuego province (TF samples); algal fragments
6. La Salada saline pond (LS samples): 39°27′ S–62°41′ W, Pedro Luro, Buenos Aires province; plant and algal debris and inorganic sediments

Sites 1–5 are coastal environments of the Argentinean continental shelf. Those waters are of subantarctic origin with a mean temperature ranging from 6.5° to 21°C (depending on the latitude and the season) and a regular salinity lower than 33.7 g/l (Guerrero and Piola 1997). Total organic carbon and total nitrogen present values from 0.25 to 2.00% (w/v) and from 0.06 to 0.20% (w/v), respectively (Premuzic et al. 1982); the abundance and nutritive quality of organic matter is related to the lower biological activity imposed by the lower temperature (Fernandez et al. 2007). Site 6 is a continental saline pond approximately 6 m deep lacking suspended sediments, with macrophytes and algae on the shorelines, mean temperature between 8° and 21°C, and salinity of 23 g/l (García 1993).

Isolation

A portion of each sample (water plus organic material) was placed into individual Petri dishes, adding a small amount of heat-sterilized pine and sweet gum (*Liquidambar* sp.) pollen grains and antibiotics (5×10^5 U/l penicillin G and 0.5 g/l streptomycin sulfate). Although only one kind of bait was used for simplicity, in those samples where they were not colonized, heat-autoclaved brine shrimp larvae were employed alternatively. Plates were incubated at 25°C and

Table 2 Collection data and characterization of the isolated thraustochytrids strains

Isolate (BAFC cult. #)	Sampling site[a]	Isolation medium	Colony morphology[b]	Taxon
3481	CR	SSA	wh, fl, dl, sc (pt)	*Ulkenia* aff. *visurgensis*
3482	CR	MC	pk-or, cv, dl, lc	*Thraustochytrium* sp. (nf)
3483	CR	MC	pk, cv, dl, vlc	*Thraustochytrium* sp. (nf)
3484	CR	MC-BHB	pk-or, cv, dl, ht	*Ulkenia* aff. *visurgensis*
3485	CR	MC	wh, pl, dl, sc	*Thraustochytrium* aff. *kinnei*
3486	CR	SSA	pk, cv, dl, mc	*Thraustochytrium* sp. (nf)
3487	CR	PSW	nd	*Schizochytrium* sp.
3488	CR	MC	cr, pl, dl, mc	*Thraustochytrium* sp. (nf)
3489	PM	MC-BHB	wh, pl, dl, sc	*Thraustochytrium* aff. *kinnei*
3490	PM	MC	wh, pl, dl, sc-mc	*Thraustochytrium* sp. (f)
3491	PP	SSA	pk-or, cv, dl, mc	*Thraustochytrium* sp. (f)
3492	PP	SSA	wh, fl, dl, sc (pt)	*Schizochytrium* sp.
3493	PP	SSA	hy, rs, gl, ht	*Schizochytrium* sp.
3494 and 3495	PP	MC-BHB	wh-cr, pl, dl, mc	*Thraustochytrium* aff. *kinnei*
3496	PP	MC	pk-or, rs, gl, mc	*Thraustochytrium* sp. (nf)
3497	PM	MC	wh-cr, pl, dl, mc	*Thraustochytrium* aff. *kinnei*
3498 and 3501	SAO	MC	pk-or, cv, dl, lc	*Thraustochytrium* sp. (nf)
3499	SAO	MC	pk, cv, dl, vlc	*Thraustochytrium* sp. (nf)
3500	SAO	MC	hy, rs, dl, sc	*Schizochytrium* sp.
3502	SAO	MC	cr-ye, pl, gl, mc (net)	*Schizochytrium* sp.
3503	LG	MC-BHB	wh, pl, dl, mc	*Thraustochytrium* aff. *kinnei*
3504	LG	SSA	hy, rs, gl, ht	*Schizochytrium* sp.
3505	LG	SSA	hy-wh, rs, gl, sc	*Schizochytrium* sp.
3506	BB	MC	pk-or, cv, dl, lc	*Thraustochytrium* sp. (nf)
3507	BB	MC-BHB	wh-cr, pl, dl, mc	*Thraustochytrium* aff. *kinnei*
3508	BB	PSW	nd	*Schizochytrium* sp.
3509	TF	MC-BHB	wh, cv-pl, dl, mc	*Thraustochytrium* aff. *kinnei*
3510, 3511, and 3514	LS	MC	cr-ye, pl, gl, mc (br)	*Schizochytrium* sp.
3512	LS	SSA	pk, cv, gl, ht	*Thraustochytrium* sp. (nf)
3513	LS	SSA	hy, fl, dl, sc	*Schizochytrium* sp.
3515	LS	MC	pk, cv, dl, lc	*Thraustochytrium* sp. (nf)

PSW pollen seawater, *n.d.* not determined, *f* and *nf* development of basal fundament or its absence, respectively

[a] *CR* Comodoro Rivadavia beach and saline ponds, *PM* Puerto Madryn beach, *PP* Puerto Pirámides beach, *LG* Las Grutas beach, *SAO* San Antonio Oeste salt marsh, *BB* Bahía Blanca industrial port, *TF* Tierra del Fuego beach, *LS* La Salada saline pond. For more details see "Materials and methods"

[b] The features observed were as follows, in order. Color: *wh* white, *ye* yellow, *pk* pink, *or* orange, *cr* cream, *hy* hyaline. Elevation: *f* flat, *rs* raised, *cv* convex, *pl* pulvinate. Surface: *gl* glistening, *dl* dull. Cell size: *sc* small cells, *mc* medium cells, *lc* large cells, *vlc* very large cells, *hs* heterogeneous cell size. Particular colony design: *pt* punctiform colony, *net* very prominent ectoplasmatic network, *br* brain-like appearance

Effect of agar concentration

Growth values for ten strains (Table 3) were compared in MC, MC-BHB, SSA, and H with 1, 2, and 3% (w/v) agar (USB Corporation, USA) after 10 days incubation. Water activity of each culture media before inoculation was measured in a convenient aliquot using an AquaLab Water Activity Meter (Decagon Devices) with a chilled mirror dewpoint sensor.

In all cases, cultures were incubated in darkness at 25°C.

Statistical analysis

Significant differences in GVs were evaluated by a one-way analysis of variance (ANOVA). Mean GVs for the factors "culture medium" (assay 1) and "agar percentage" (assay 2) were compared by a multiple range contrast (Fisher method). A regression analysis between water activity (dependent variable) and agar percentage (independent variable) of culture media was done, adjusting data to the linear model $Y = a + b \times X$. Statistical analysis

Table 3 Comparative growth of the isolated thraustochytrids in different culture media

Isolate (BAFCcult. #)	Growth Values (GVs)						Isolate GV mean
	GPY	**H**	**KMV**	**SSA**	**MC**	**MC-BHB**	
3481	1	1	2	2	1	1	1.33
3482	2	2	2	2	4	3	2.50
3483	1	2	2	2	3	3	2.17
3484	2	2	3	2	4	4	2.83
3485	4	3	2	2	3	4	3.00
3486	1	2	3	2	3	3	2.33
3488	4	3	3	2	3	3	3.00
3489	4	3	2	2	3	4	3.00
3490	2	3	2	2	4	4	2.83
3491	1	2	2	2	3	3	2.17
3492	0	1	2	2	1	0	1.00
3493	1	3	3	2	1	1	1.83
3495	3	2	2	2	3	4	2.67
3497	3	2	2	2	3	4	2.67
3499	1	3	2	2	3	2	2.17
3500	1	1	2	2	1	2	1.50
3501	1	3	2	2	3	2	2.17
3502	4	3	3	2	4	3	3.17
3503	3	2	2	2	3	4	2.67
3504	1	2	3	2	1	1	1.67
3505	0	1	2	2	0	1	1.00
3506	1	3	3	2	3	3	2.50
3509	1	2	3	2	3	4	2.50
3510	4	3	3	2	4	4	3.33
3512	1	3	3	2	3	1	2.17
3513	0	0	1	2	0	1	0.67
3515	1	2	3	2	2	2	2.00
SR21	4	4	4	2	4	4	3.67
GV mean of isolates on each medium	**1.86**	**2.25**	**2.43**	**2.00**	**2.61**	**2.68**	
Cultivation Efficiency (CE)	**42.8**	**82.1**	**96.4**	**100**	**75.0**	**75.0**	

Groups of isolates based in their growth on solid media: light grey, group 1 (GVs up to 2 in all media); white, group 2 (low GVs in GPY, intermediate GVs in KMV, H, and SSA, and high GVs in MC and MC-BHB); dark grey, group 3 (high GVs in all media). Cultivation efficiency (CE) for each medium was estimated as the percentage of strains attaining a GV $\geq$ 2 on it

seawater were employed to separate contaminating yeasts and protists. Finally, when bacterial proliferation could not be suppressed by penicillin G plus streptomycin, the use of a mixture of chloramphenicol, kanamycin, and tetracycline (0.1 g/l each) was effective.

A preliminary characterization of the isolates obtained is also presented in Table 2. Observations on morphology and development on pollen baits suggested that strains belong either to the genera *Schizochytrium* (sensu lato), *Ulkenia* (sensu lato), and *Thraustochytrium*. Only a few isolates could be identified at species level (*Thraustochytrium* aff. *kinnei* and *Ulkenia* aff. *visurgensis*), and they are presented under *affinis* status as the observations did not fit exactly with the original descriptions. Strains could be also distinguished by their colony morphology on isolation media: some of them presented a characteristic colony design, as BAFC cult. 3510, 3511, and 3514, that developed colonies with elevations and depressions that resembled the sulcus and gyrus pattern of the brain. Nineteen kinds of colonies could be differentiated among the 35 isolates, as the same colony morphology was shared by several strains from different samples (e.g., BAFC cult. 3482 and 3498).

Media composition and growth of the strains

To check that the newly formulated culture media (MC and MC-BHB) had been more suitable for the cultivation of most of the local strains than the "standard" culture media (GPY, KMV, H, and SSA), the effects of their composition on the growth of the isolates were compared. According to the results (Table 3), isolated thraustochytrids could be separated into three groups: group 1, which includes the isolates presenting GVs up to 2 in all culture media (21.4% over total); group 2, which includes the isolates that presented low GVs in GPY, intermediate GVs in KMV, H, and SSA, and high GVs in MC and MC-BHB (46.4%); and group 3, containing those strains presenting high GVs in all culture media (representing the 32.1% plus SR21).

Analysis of variance showed that the effect of the factors isolate and culture medium on GVs was statistically highly significant ($P < 0.0001$ and $P = 0.0003$, respectively).

According to our results, the use of a diversity of baits was the first aspect to be considered to improve success in the isolation of thraustochytrids. For instance, thraustochytrids in the samples dominated by animal detritus could colonize shrimp larvae, as proposed previously for *Ulkenia amoeboidea* (Bahnweg & Sparrow) A. Gaertner (Bahnweg and Sparrow 1974), but they failed to colonize pollen grains. This finding agrees with other reports on the growth of uncultured organisms by simulating the natural environment (Kaerberlein et al. 2002). Covering clean colonies with a drop of seawater was another procedure mimicking natural conditions. This approach has been widely applied with zoosporic fungi (Fuller and Jaworski 1987) and was very useful to stimulate sporulation, facilitate spreading, and improve growth of the isolates presented here. Another environmental factor that should be considered is the temperature of incubation. Although the isolation procedures were carried out at 25°C and thraustochytrids were observed in almost all the samples, materials from colder environments (such as those in the Antarctic) should be incubated at lower temperatures to allow the growth of cryophilic strains.

Incubation and observation of cultures over longer periods of time for what might be considered unimpressive signs of growth is one of the keys to cultivation success, particularly for such slow-growing strains that might never reach high yields (Leadbetter 2003). We found that several of local strains required long periods of incubation (at least 30 days after plating) to develop colonies, and in this sense they could be considered as elusive to previous standard cultivation techniques. Our results highlight the importance of a strict control of contaminants as a pivotal issue for the isolation of such slow-growing thraustochytrids. Different treatments were successfully applied according to the kind of contaminating microorganisms present. We showed that the fungicide benomyl and the alternative antibiotics chloramphenicol, kanamycin, and tetracycline suppressed mold and bacterial growth, respectively, without any negative impact on proliferation of thraustochytrids. When contaminating yeasts and other protists were present, their colonies could be separated from the thraustochytrid ones in solidified media only after lowering their concentration by successive transfers in baited-seawater cultures.

Formulation of new culture media was one of the effective strategies explored in this work to culture "elusive" thraustochytrids, particularly those that did not grow well in standard media reported in the literature, such as GPY. MC medium, designed in our laboratory, allowed the isolation of 17 strains. MC is almost a tenfold dilution of the main nitrogen and carbon sources of GPY plus the remaining nutrients of KMV, monosodium glutamate, and CSL (see Table 1). These two last nutrients were included in MC, taking into account the results of Iida et al. (1996) and Yokochi et al. (1998). They found, respectively, that addition of monosodium glutamate to GPY medium increased significantly the growth of a strain of *Thraustochytrium aureum* Goldstein, whereas CSL as a nitrogen source resulted in the highest dry cell weight in *Aurantiochytrium limacinum* SR21. For baited thraustochytrids with a preference for animal detritus (i.e., baited on autoclaved brine shrimp larvae) that could not be isolated in MC, MC-BHB medium (containing equal amounts of MC and brain–heart broth; see Table 1) was effective. Other components, not assayed herein, which could also be included in isolation media to improve thraustochytrids growth, are KH_2PO_4 and Tween 80 (Taoka et al. 2008).

Effectiveness of the newly formulated and other standard culture media to support growth of the isolates was compared as an attempt to establish more suitable conditions for the cultivation (and isolation) of new strains of thraustochytrids. The arbitrary scale based on the characteristics of colonies proposed in the present study showed that not every isolate could be grown successfully (i.e., attain a GV $\geq$ 2) on every media. Statistical analysis revealed that the tested culture media could be separated into three homogenous groups according to the mean GV of the isolates growing on them. Lower values were obtained in GPY and SSA for different reasons. GPY medium was suitable for culturing only a few strains (group 3), in spite of the fact that they showed high GVs on it. Although a slow mean GV was also obtained in SSA medium, it had the highest CE, as all isolates could grow on it, but not one could attain a GV higher than 2. Most of the local strains presented the highest GVs on MC and MC-BHB media (group 2). According to these results, the newly formulated MC medium is preferred for isolation among all the media tested. MC is preferred over MC-BHB, because the growth of the isolates was not significantly different on these and MC-BHB has a more complex composition. Although MC did not satisfy the requirement of all isolates (i.e., none of the strains belonging to group 1 attained a GV higher than 1 on it), it allows the rapid development of most of assayed organisms. Furthermore, SSA medium is recommended as a second option for those thraustochytrids observed in baits and samples that could not proliferate on MC. However, it must be pointed out that the poor growth and the soft, slimy consistence of SSA make microbiological work more difficult. Special care is needed when the streaking plate technique is carried out on this medium to obtain pure, single-cell colonies.

The effect of the concentration of the solidifying agent in the media on growth was also analyzed because some strains had seemed to be very sensitive to this factor during their isolation. Limited growth of thraustochytrids caused by higher agar content had been also reported previously by Vishniac (1956). Comparison of data showed that

Conclusion

Thirty-five new strains of thraustochytrids were isolated from temperate and cold environments in Southern Argentina by adjusting the classical procedures described in the literature. Modifications based on our results were integrated with the traditional procedures in the flow chart shown in Fig. 2. Alternative treatments are proposed according to the nature of the sample, the characteristics (mainly nutritional requirements) of thraustochytrids to be isolated, and the presence of contaminating microorganisms. The linear pathway from 1 to 6 (see solid lines in Fig. 2) should be followed first; adjustments based on our results to improve this procedure include the use of multiple types of baits and of isolation media, the observation of plates for at least 30 days, and covering the transferred colonies with a drop of seawater, among other methods. Observation of colony morphology is suggested as an easy method to identify purity of the isolates (branch 6), considering that this characteristic remained stable for each strain during the experiments described in this work. Branch 1′ was not applied in our own research, but it was suggested in the literature (Porter 1990; Fan et al. 2002). Successive subculture in baits in seawater is proposed as a strategy for isolating a strain when it grows poorly on solid media (pathway 3′–3″) and for decreasing the concentration of contaminants (pathway 3′–3 or branch 5′). The use of chloramphenicol, kanamycin or tetracycline, and benomyl (branch 5′) is also proposed to solve problems associated with contamination.

In selecting isolation media (see Fig. 2, branch 3), we propose the medium MC, formulated in the present research, as the most suitable one, and SSA (Porter 1990) as an alternative option, based on our results from comparative studies. More concentrated media (i.e., MC-BHB and GPY) did not improve the relative growth of isolates, suggesting that nutrient quality (and interactions) rather than quantity should be limiting for culturing thraustochytrids. Agar concentration for the isolation media should be no higher than 2% w/v, as this factor (or small molecules contained in it as impurities) was directly related to available water (A_w) and showed a significant effect on growth; this effect was more pronounced in more concentrated media. For practical purposes, formulation of new culture media for the isolation of thraustochytrids should take into account not only nutrient quality but also agar concentration, which should be high enough to allow microbiological work (e.g., separating and spreading cells and colonies with a dissecting needle), but low enough to let these microorganisms grow.

Cultivation of microorganisms with heterogeneous metabolic requirements demands culture media and isolation procedures that cover their range of ecophysiological necessities, as we considered in this work. The goal of this research was to expand the standard methodologies to gain access to baitable "elusive" thraustochytrid strains, improving the isolation and the study of the undocumented biodiversity of this group of microorganisms. It would be very interesting to integrate this kind of studies with metagenomic research in future studies.

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