

Lipid content and fatty acid composition of a marine-derived *Trichoderma longibrachiatum* strain cultured by agar surface and submerged fermentations

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Abstract:

A marine-related *Trichoderma longibrachiatum* strain isolated from mussels in a farming shellfish area was investigated for total lipid (TL) production, total lipid fatty acids (TLFA), and phospholipid fatty acids (PLFA). Fungal biomass was produced from two different marine-like culture ways, on agar surface and submerged fermentations (ASF and SmF, respectively), allowing useful comparisons. ASF produced a rather higher biomass amount than SmF with similar TL content. All fatty acids (FA), identified by gas chromatography–mass spectrometry (GC–MS), were ranged from 14 to 30 carbon atoms. Similar FA compositions were found in TL and phospholipids (PL) from biomasses obtained by both fermentation systems, including oleic (up to 15.3% of total FA mixture for SmF, and 33.9% for ASF), linoleic (46.1% for SmF, and 40.3% for ASF) and palmitic (28.1% for SmF, and 19.1% for ASF) acids as major components. Regarding the most common FA, lipid class and FA profiles observed did not show marked differences with those available for some *Trichoderma* terrestrial species. Some 2-hydroxylated FA and a rather unusual series of C18 unsaturated conjugated FA (CFA) were identified. In addition, fungal biomass production by ASF was found as an easy to operate process, especially for further screenings of marine-derived fungi.

Keywords: *Trichoderma longibrachiatum*; Agar surface fermentation; Submerged fermentation; Lipids; Fatty acids; Conjugated fatty acids

1. Introduction

Polyunsaturated fatty acids (PUFA), mainly from the (n-3) series, are now well known as essential lipids with major interest in human health and nutrition, and fish oils represent the main marine source of such fatty acids (FA) [1]. Nevertheless, given the worldwide decline of fish stocks, it seems very important to look for alternative sources including cultured microorganisms. Thus, new sources of interesting FA have been reported such as several algae, marine bacteria, protozoa and fungi [2]. Primitive fungi such as Oomycetes and Zygomycetes have been widely investigated and recognized as fungal sources of interesting PUFA such as arachidonic acid (ARA) or eicosapentaenoic acid (EPA) [3-7]. Various terrestrial species from the genus *Trichoderma* have been already studied for their lipid production [8-11]. Their major FA were 16:0, 9,12-18:2, 9-18:1 and 18:0 in concordance with those known to be accumulated in fungi [9]. *Trichoderma* species have been also widely studied for their enzyme production such as cellulases [12,13], chitinases [14], xylanases [15]. Moreover, *Trichoderma* species have been also studied for the production of flavour compounds such as 6-pentyl- α -pyrone [16-18]. In the other hand, strains from *Trichoderma* species deal with the production of small linear antimicrobial peptides, namely peptaibols [19]. In other studies, occurrence of marine-derived *Trichoderma* species has been reported in shellfish farming areas on the Atlantic coast [20]. Due to their ability to growth in this saline culture condition, it would be interesting to investigate lipids and FA profiles of those marine *Trichoderma* strains implanted on shellfish farming areas. Fungal lipids have been especially investigated in cultures performed in submerged fermentation (SmF). Our previous works showed that metabolites such as gliotoxin [21] and griseofulvin [22] had been produced from fungal culture in agar surface fermentation (ASF). Thus, this fermentation way could be evaluated for lipid production and FA composition. A comparative study on lipid accumulation, total lipid fatty acid (TLFA) and phospholipid fatty acid (PLFA) compositions of a marine strain of *T. longibrachiatum* through SmF and ASF has been carried out.

2. Material and methods

2.1. Fungal strain

The fungal strain was isolated from mussels (*Mytilus edulis*) collected in a shellfish-farming area from the estuary of the Loire River (Tharon, France). The strain was identified as *Trichoderma longibrachiatum* Rifai using metabolic profiles on Biolog FF MicroPlates™ and by sequencing the internal transcribed spacer (ITS) regions of the ribosomal RNA cluster. This strain was maintained on DCA tubes (dextrose 40 g, enzymatic digest of casein 10 g, agar 15 g, natural seawater 1 L), stored under a paraffin oil layer and conserved in our

fungal collection under the reference number MMS 151 (SMAB Marine Fungal Collection, University of Nantes).

2.2. Fungal cultures

Submerged liquid cultures were performed in 500 mL flasks containing 300 mL of GCY medium (glucose 20 g, casein pancreatic peptone 10 g, yeast extract 5 g, natural seawater 1 L). Spores were previously cultivated in ASF and incubated on DCA medium Roux flasks (235 mL) at 27°C for 10 days in order to produce sufficient quantity of spores for the suspension. Each Roux flask was inoculated with *T. longibrachiatum* conidia taken from stock cultures in Petri dishes (5 cm). Spore suspension was prepared with sterile seawater and after filtration, 160 ± 10^6 spores/mL were counted using Malassez cells. 1 mL of sterile suspension was inoculated in 500 mL flasks (final concentration of 0.5 ± 10^6 spores/mL) and incubated for 10 days according to a previous study of lipid accumulation by *Trichoderma* species cultivated in liquid cultures [10]. In the latter case, the maximum of the lipid content was observed globally between the 9th and 11th days. Incubation was performed at 27°C in natural light. Concerning solid cultures of *T. longibrachiatum*, ASF was performed in Petri dishes (20 cm) containing 125 mL of DCA medium. Each dish was incubated for 10 days (growth time required for total covering of dish surface by the strain) at 27°C in natural light similarly for SmF.

2.3. Lipid extraction

Concerning SmF, after incubation, the mycelium was filtered through Büchner and mycelial biomass was steeped twice in dichloromethane /methanol (1:2, then 2:1, v/v) for 2 h at room temperature. The combined extracts were filtered under vacuum (0.45 µm PTFE membrane filters, Sartorius, Göttingen, Germany), washed with water and evaporated to provide crude total lipids (TL). Cultures in ASF were stopped after scrapping off mycelium and conidia from agar surface. Lipid extraction was performed in the same way used for SmF. In both ways, the biomass amount considered is determined as the sum of delipidified matter and crude lipid extract.

2.4. Obtention of total lipid fatty acids (TLFA) and phospholipid fatty acids (PLFA)

The crude extract was splitted into two parts. The first part was saponified (KOH/EtOH, 2 mol/L) for 120 min under reflux. TLFA obtained were converted into fatty acid methyl esters (FAME) by reaction with 2% methanolic hydrogen chloride (30 min under reflux). *N*-acyl pyrrolidides (NAP) were prepared by treatment of FAME with pyrrolidine/ acetic acid (10:1, v/v) for 1 h under reflux. The second part of the crude extract was chromatographed on an open silica gel (60 Å 35-75 µm, SDS, Peypin, France) column to obtain the different lipid classes. Thus, neutral lipids were eluted by dichloromethane, glycolipids by acetone and finally phospholipids (PL) by methanol. This general method used for isolation and analysis of lipid mixtures has been

previously described [23]. PLFA were converted into methyl esters by transesterification with 5% methanolic hydrogen chloride (90 min under reflux). NAP were then prepared as previously described.

2.5. General experimental procedures

All samples were analysed using a Agilent model 6890 series II gas chromatograph linked to an Agilent 5973 series network mass selective detector (e.i. 70 eV) equipped with an Agilent model 5973N selective quadrupole mass detector. Separation was achieved with a CP-Sil 5 CB low bleed MS capillary column (60 m _ 0.25 mm i.d., 0.25 μ m phase thickness; Chrompack, Middelburg, The Netherlands). The carrier gas was helium at a constant flow rate of 1mL/min. The column temperature was programmed from 80°C to 170°C at 30°/min and 170 to 295°C at 3°C/min. Standard FAME mixtures were purchased from Sigma-Aldrich (St. Quentin Fallavier, France) including common FA mixture (ref.18917-1AMP) and conjugated linoleic acids (ref.O5632).

3. Results and discussion

3.1. Biomass, total lipid content and lipid classes

The amount of biomass produced per litre of culture medium by *T. longibrachiatum* in ASF was commonly around 10 times higher than those produced in SmF, showing that the use of an ASF could be more interesting in terms of biomass production (Table 1). In the latter case, only one fermentation step was actually required, while submerged culture needs two fermentation steps, an ASF for spore suspension and a SmF for lipid production. In both fermentation ways, TL production was almost identical, 9.9~10.0% of the biomass after extraction. These values are in agreement with previous studies concerning lipid synthesis by *T. viride* [8] and *T. reesei* [24]. Nevertheless, lipid accumulation in *Trichoderma* terrestrial strains were shown very dependent of different parameters as the culture media composition [10], the pH and the temperature [24] and total lipid contents in *Trichoderma* species could reach higher levels in optimum conditions.

Comparison of the lipid class composition of *T. longibrachiatum* obtained by SmF and ASF showed notable differences (Table 1). PL were the major lipid class in SmF (66.8%), whereas neutral lipids were predominant in ASF (58.1%). For both fermentations, glycolipids corresponded to the minor lipid class with similar proportions (12.2 in SmF, 12.3 in ASF). In our best knowledge, only few studies describe lipid class separation from *Trichoderma* species so far. Thus, lipid classes produced by *T. harzianum* and *T. viride* in glucose-ammonium-sulphate medium after a cultivation period of 8 days [10] showed the predominance of neutral lipids (72.9% and 63.2% respectively) as for *T. longibrachiatum* in ASF but not in SmF. However, in an early study on lipid synthesis by *T. viride*, Ballance and Crombie [8] showed that PL were less accumulated when spores were cultivated in glucose-agar slopes (21%) than mycelium cultivated in non-aerated glucose solutions (55%

respectively). Neutral lipid accumulations were also different according to the experimental conditions with predominance in glucose-agar slopes (64%) contrary to glucose solutions (36%). These observations, similar to the variation of lipid classes from cultures of *T. longibrachiatum* in SmF and ASF, emphasize the importance of experimental conditions (agar cultures or medium solutions) in the accumulation of neutral lipids or PL. Any detectable amount of free FA was observed in all samples studied. TLFA arising from saponification accounted for $32.0 \pm 5.4\%$ (w/w TL) in SmF, whereas they represented only $16.5 \pm 1.7\%$ in ASF.

Table 1

3.2. Fatty acid composition of Trichoderma longibrachiatum

General observations

About 40 FA were identified in *T. longibrachiatum* ranging from 14 to 30 carbon atoms. They are listed in the order of increasing retention time (Table 2). Each FA was identified as methyl ester and pyrrolidide, from its mass spectrum and its gas chromatography (GC) mobility (equivalent chain length ECL), with the help of usual key fragment ions. The use of NAP for gas chromatography /mass spectrometry (GC/MS) analysis allowed the location of unsaturations and methyl branches [25].

One of the most striking results of this study was the detection of several dienoic and trienoic C₁₈ conjugated fatty acids (CFA). Another interesting result was the identification of some 2-hydroxylated FA.

FA composition of T. longibrachiatum in SmF

Few variations of FA composition are observed between TL and PL. The same predominant major FA were present (9,12-18:2, 16:0, 9-18:1, 18:0), nevertheless content of palmitic acid was less important on TL (20.5%) than PL (28.1%) contrary to stearic acid, and more important in TL (10.4%) than in PL (4.6%). These variations of predominant FA with higher amounts in PL show that some FA (e.g. 9-18:1) could play a structural role, while others (e.g. 18:0) could represent storage lipids. It could be noted that such variation of stearic acid content was already reported [8,26]. In addition, palmitoleic acid was more present in TL than in PL. Smaller amounts of 14:0, 15:0, 17:0, 9-17:1 and 9,12-17:2 acids were identified in both TL and PL. Very long-chain FA were identified in TL as well in PL but a slight difference was observed for TL with the presence of 28:0 and 30:0.

In TL, fourteen CFA accounted for 9.0% including two major C_{18:2} CFA eluted just after stearic acid methyl ester (ECL=18.12 and 18.20, 4.6% and 1.8%, respectively). In PL, seven CFA were detected accounting for 1.2%. GC-MS Comparisons between the natural conjugated linoleic methyl esters with those from the standard mixture showed that the major C_{18:2} CFA produced by the fungal strain are likely isomeric 9,11-18:2 and 10,12-18:2. In our best knowledge, it is the first occurrence of C_{18:2} CFA from *Trichoderma* species. C_{18:2}

CFA have been especially described from animal sources as milk fat, cheese and ruminant meat and are formed by biohydrogenation and oxidation processes [27]. Efficient production of conjugated linoleic acid was obtained from various lactic acid bacteria [28]. Further studies should be devoted to the elucidation of the mechanism by which this fungal strain can produce such FA. These conjugated linoleic acid isomers have been attracted particular attention because of their remarkable biological activities: anti-carcinogenic, immune modulator, anti-diabetic, anti-obesity, anti-thrombotic and anti-atherogenic [29].

Up to ten conjugated linolenic acids have been detected in SmF and especially in TL. Five isomeric C_{18:3} CFA occur as major seed oils of several plants [30]. It seems to be possible that fungal strains could produce additional unusual C_{18:3} CFA. Although interesting beneficial effects in the field of health and nutrition are known for C_{18:3} CFA, there has been reported than they were stronger anti-carcinogens than conjugated linoleic acids [30]. Such C_{18:3} CFA do not seem to have attracted sufficient attention so far.

One other interesting result was the occurrence of two 2-hydroxylated FA namely 2-hydroxyoctadenoic (2-OH-18:0) and 2-hydroxytetracosanoic acids, readily identified as methyl esters from their GC mobilities and their mass spectra [31]. Such hydroxylated FA usually occur in fungal glycolipids [32], and sometimes in PL from various organisms such as marine sponges [31].

*FA composition of *T. longibrachiatum* in ASF*

Minor FA have been identified from PL, notably for some long-chain FA only presents in TL (20:2, 20:1, 20:0, 21:0, 23:0, 25:0 and 28:0) (Table 2). The usually observed decreasing order of the predominant FA in TL was the following: 9,12-18:2 (35.0%), 16:0 (19.1%), 9-18:1 (17.1%) and 18:0 (10.7%). In PL, two main differences were observed concerning oleic and stearic acids which were respectively present in higher amounts (33.9%) and smaller amounts (2.6%) than in TL. The higher proportion of oleic acid in PL suggests its potential structural role in cell membranes. Smaller amounts of 14:0, 15:0, 17:0, 9,12-17:2 and 9-17:1 acids were identified in both TL and PL.

CFA were also detected in cultures of *T. longibrachiatum* by ASF. In TL, eight CFA accounted for 4.5%. The same three major C_{18:2} CFA as observed in SmF were detected (2.1, 1.5 and 0.6%, respectively). In PL, only 3 CFA were detected and accounted for 0.5%. Two additional 2-hydroxylated FA, namely 2-hydroxypentacosanoic (2-OH-25:0) and 2-hydroxyhexacosanoic (2-OH-26:0) were identified.

*General comparison of FA composition of *T. longibrachiatum* in SmF and ASF*

The main difference of the FA composition between ASF and SmF is the notable variation of some predominant FA as oleic and palmitic acids. Although the oleic acid level was higher (33.9%) than the palmitic acid (17.2%) in PL from ASF, the reverse was observed in SmF for PL with higher content of palmitic acid (28.1%) followed by oleic acid (15.3%). In both culture ways, linoleic acid was the highest FA in TL as well as in PL. Similar amounts of linoleic acid has been reported in other *Trichoderma* species cultivated in liquid medium as *T. reseii* (47%) [11] and *T. harzianum* (48%) [10]. In both fermentations stearic acid was present in smaller levels in PL, showing that it likely does not play any structural role in the cell membrane. Similar observation was already reported [8,26].

The most striking difference between the both fermentation ways was the greatest number of CFA detected in SmF, especially in TL. The three major C_{18:2} CFA were detected in both fermentation ways and accounted respectively up to 6.9% in SmF and 4.2% in ASF. In order to improve the production of CFA and principally dienoic C₁₈, it seems better to use SmF than ASF for the culture of *T. longibrachiatum*. For both fermentations, the predominance of CFA in TL, while in PL suggests that these FA do not seem to have a structural role in cell membrane.

Table 2

3.4. General conclusion

Rather higher amount of biomass -around ten times- was produced in ASF than in SmF. Similar TL contents (10% of dried weight) were obtain in both fermentation ways. Nevertheless, TLFA were more important in SmF than in ASF. Regarding the common FA, FA profiles observed in this study for both fermentations did not show marked differences with those available for some *Trichoderma* terrestrial species. One of the most striking results is the first report of a series of CFA in a marine fungal strain occurring at relatively high levels compared with other natural sources. This study shows the interest to select SmF or ASF according to a particular purpose. Thus, in ASF fungal biomass production is an easy to operate process, requiring a direct step of cultivation, and could be especially interesting for further screenings of marine-derived fungi. On the other hand, it seems that lipid production using SmF could be more appropriate in terms of CFA production than ASF.

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Legends

Table 1

Biomass, total lipid content and lipid class composition of *Trichoderma longibrachiatum* in SmF (submerged fermentation) and in ASF (agar surface fermentation)

Table 2

Total lipid and phospholipid fatty acid composition^a from *Trichoderma longibrachiatum* in SmF and in ASF

Table 1

	Biomass ^a (g /L)	Lipids in biomass (%, w/w)	Lipid composition (% ,w/w)		
			Neutral lipids	Glycolipids	Phospholipids
SmF	0.8 ± 0.3	10.0 ± 0.7	21.0 ± 5.1	12.2 ± 3.5	66.8 ± 6.0
ASF	8.3 ± 1.5	9.9 ± 1.8	58.1 ± 7.3	12.3 ± 3.8	29.7 ± 5.5

^a as explained in Material and methods, expressed per litre of culture

Data are the mean of 3 replicates ± S.D.

Table 2

	ECL ^b	SmF		ASF	
		TLFA	PLFA	TLFA	PLFA
Tetradecanoic (14:0)	14.00	0.55 ± 0.26	0.33 ± 0.20	0.50 ± 0.04	0.40 ± 0.10
Pentadecanoic (15:0)	15.00	0.63 ± 0.20	0.84 ± 0.37	1.27 ± 0.03	0.63 ± 0.11
9-Hexadecanoic (16:1)	15.79	2.56 ± 0.15	1.25 ± 0.89	2.12 ± 0.02	0.73 ± 0.30
Hexadecanoic (16:0)	16.00	20.46 ± 0.93	28.13 ± 1.95	19.07 ± 0.73	17.21 ± 1.40
9,12-Heptadecadienoic (17:2)	16.64	0.57 ± 0.12	< 0.1	0.58 ± 0.04	0.41 ± 0.05
9-Heptadecenoic (17:1)	16.86	< 0.1	0.19 ± 0.09	0.71 ± 0.10	0.28 ± 0.12
Heptadecanoic (17:0)	17.00	0.64 ± 0.02	0.51 ± 0.24	1.07 ± 0.06	0.47 ± 0.10
9,12-Octadecadienoic (18:2)	17.66	38.16 ± 2.63	46.07 ± 5.26	34.96 ± 0.66	40.29 ± 1.43
9-Octadecenoic (18:1)	17.79	13.54 ± 2.69	15.27 ± 0.75	17.47 ± 0.48	33.91 ± 3.42
Octadecanoic (18:0)	18.00	10.43 ± 1.02	4.58 ± 1.95	10.65 ± 0.40	2.60 ± 0.17
Octadecatrienoic (cj-18:3)	18.05	0.46 ± 0.16	—	—	—
Octadecadienoic (cj-18:2)	18.12	4.60 ± 1.81	0.35 ± 0.05	2.13 ± 0.47	—
Octadecadienoic (cj-18:2)	18.20	1.77 ± 1.65	—	1.55 ± 0.29	0.11 ± 0.01
Octadecatrienoic (cj-18:3)	18.25	0.50 ± 0.21	—	—	—
Octadecadienoic (cj-18:2)	18.40	0.33 ± 0.16	0.25 ± 0.05	0.25 ± 0.08	—
Octadecadienoic (cj-18:2)	18.54	0.51 ± 0.23	0.15 ± 0.05	0.58 ± 0.20	0.37 ± 0.02
Nonadecanoic (19:0)	19.00	0.35 ± 0.04	—	0.52 ± 0.09	—
2-Hydroxyoctadecanoic (2-OH-18:0)	19.12	0.33 ± 0.02	< 0.1	0.59 ± 0.28	0.61 ± 0.03
Eicosanoic (20:0)	20.00	0.17 ± 0.06	0.36 ± 0.04	0.40 ± 0.05	—
Tetracosanoic (24:0)	24.00	1.30 ± 0.55	0.36 ± 0.15	1.00 ± 0.22	0.10 ± 0.01
2-Hydroxytetracosanoic (2-OH-24:0)	25.25	0.13 ± 0.00	0.14 ± 0.07	2.89 ± 0.14	0.99 ± 0.18
Hexacosanoic (26:0)	26.00	0.57 ± 0.06	0.14 ± 0.07	0.13 ± 0.04	0.13 ± 0.03

Also identified ($\leq 0.30\%$): cj-18:3 (18.33, 18.59, 18.79, 19.06, 19.08, 19.41, 19.49 and 19.55), 20:2 (19.65), 20:1 (19.79), 21:0 (21.00), 22:0 (22.00), 23:0 (23.00), 25:0 (25.00), 2-OH-25:0 (26.28), 2-OH-26:0 (27.28), 28:0 (28.00) and 30:0 (30.0)

cj for conjugated

^a % of total fatty acid content (mean of 3 replicates ± S.D.)

^b ECL (equivalent chain length)