HNUE JOURNAL OF SCIENCE DOI: 10.18173/2354-1059.2021-0050
Natural Sciences 2021, Volume 66, Issue 3, pp. 91-98
This paper is available online at http://stdb.hnue.edu.vn

UNUSUALLY LIPID PRODUCTION BY THE MARINE-DERIVED FUNGI *Penicillium restrictum*

Le Van Tuyen[1], Samuel Bertrand[2, 3], Nguyen Ngoc Linh[1], Tran Van Diep[1],
Phung Van Hoan[1], Vu Thanh Cong[1], Yves Francois Pouchus[2, 3] and Olivier Grovel[2, 3]
[1]Falculty of Pharmacy, Thanh Do University, [2]University of Nantes, France
[3]Corsaire-ThalassOMICS Metabolomics Facility, Biogenouest, University of Nantes, France

Abstract. Using marine-derived fungi as a source of lipid production holds promise as an alternative to industrial lipid production for health and nutrition in the future. In the present study, this strain showed a high production of lipid, about plus 80% of the total lipids in the host-derived medium. Gas chromatography analysis of fungal lipids revealed the presence of saturated (mainly palmitic acid C16: 0 and stearic acid C18: 0) and unsaturated fatty acid (mainly linoleic acid C18: 2, oleic acid C18: 1). These findings suggest this marine-derived fungus is a promising source for lipid production in various industrial applications. In particular, a fraction containing glycolipids of the crude extract exhibited potential cytotoxic activity on human oral epidermal carcinoma cell lines. This result is very interesting to further isolate and determine the molecular structure of bioactive glycolipids.

Keywords: *Penicillium restrictum*, lipids, MES-SSW, GC-MS.

1. Introduction

Lipids are the main component of the fungal cell membrane, it is considered as signaling molecules, and as an energy source [1]. Fungi are capable of accumulating intracellular lipids from substrates and carbohydrates. Oleaginous fungi were known for their ability to accumulate lipids such as triacylglycerols, sterols reaching more than 20% of biomass weight [1]. It can be increased up to 70% of their biomass under nitrogen limitation conditions. In particular, the single-cell oils accumulated by oleaginous fungi have received much attention as a potential feedstock for biodiesel production [2]. Several studies have shown that the fatty acid profile of fungal lipid is very similar to that of vegetable oils [3]. However, the application of fungal lipids was rare compared to vegetable lipids, although fungi have many advantages such as fast growth, short life cycle, easy cultivation on large scale.

Received June 17, 2021. Revised October 16, 2021. Accepted October 23, 2021.
Contact Le Van Tuyen, e-mail address: lvtuyen@thanhdo.edu.vn

Fungal lipids sources have been studied for many applications such as in pharmaceutical, food additives, and biodiesel. Several investigations have shown that fatty acid lithium or potassium salts from *Cunninghamella echinulata* containing the γ-linolenic acid (GLA, Δ6, 9, 12-C18:3), are potentially useful agents in the treatment of cancer [4, 5]. Some species of yeast have been proven capable of butter-like lipid production under limited culture conditions [6, 7]. The *Penicillium commune* NRC2016 isolated from soil was used for biodiesel production with the highest lipid accumulation of 99.1 mg/g in the presence of sweet sorghum [8]. A recent study has also used sugarcane bagasse hydrolysate to culture *Yarrowia lipolytica* for producing biodiesel [9]. Several steroids isolated from the fruit bodies of the inedible mushroom have shown potential cytotoxicity against four cancer cell lines KB (human epidermal carcinoma), MCF7 (human breast carcinoma), SK-LU-1 (human lung carcinoma), and Hep-G2 (hepatocellular carcinoma) [10].

Penicillium restrictum has a worldwide distribution and is grown on a wide variety of substrates [11]. This strain was employed for several biotechnological purposes such as enzyme technology, soil bioremediation [11]. But received little interest in the exploitation of lipid. Several previous studies showed the fatty acid composition in *Penicillium restrictum* [12, 13].

In-process research and development of new natural products of marine-derived fungi, the *Penicillium restrictum* has been found in the Loire estuary, France. Our previous study has shown that the mussel-derived strain of *P. restrictum* can produce a high chemical diversity of specific metabolites such as pyran-2-ones in host-derived media [14]. Our study introduces the observation of lipid and fatty acid production in this fermentation.

2. Content

2.1. Materials and methods

**** Fungal material***

The marine-derived strain, *Penicillium restrictum* MMS417, has been found from the blue mussel *Mytilus edulis* at Port Giraud in France. The strain has been previously identified [14]. The strain was conserved at the laboratory Mer-Molécule-Santé (MMS) EA2160, University of Nantes, France.

**** Culture of fungi***

Details for the preparation of mussel extract sucrose medium (MES-SSW) are presented in the previous studies [14]. The marine-derived strains were cultivated in 54 Erlenmeyer flasks containing 250 mL of MES-SSW medium and incubated at 27 ^{o}C for 14 days. The biomass obtained was extracted with the CH_2Cl_2/ethyl acetate mixture (1:1, *v/v*) resulting in the obtaining of 11.65 g of crude extract. This crude extract obtained was fractionated by thin-layer chromatography (TLC) on silica, by successive elution with hexane/ethyl acetate, and then with CH_2Cl_2/methanol with increasing polarity. The 11 fractions received include the following: 15.9 mg of F1, 3.2 mg of F2, 5214.5 mg of F3, 2223.1 mg of F4, 388.1mg of F5, 253.8 mg of F6, 53.3 mg of F7, 943.4 mg of F8, 230.5 mg of F9, 124.8 mg of F10, 27.4 mg of F11.

**** HPLC-HRESI-MS analyses of lipids***

High-performance liquid chromatography-high resolution electrospray ionization-mass spectrometry (HPLC-HRESI-MS) analyses were performed on fast liquid chromatography coupled with ion trap-time of flight mass spectrometry (UFLC-MS-IT-TOF) Shimadzu instrument, using a Kinetex C18 column (2.6 μm, 2.1 mm × 150 mm, Phenomenex) and following previously described conditions [15].

**** Protocol trans-esterification (preparation of fatty acid methyl esters - FAME - and N-acyl pyrrolidides - NAP)***

Crude extract fatty acid methyl esters (FAME) were prepared by protocol trans-esterification of fatty acids (5 hours at 80 °C under reflux with methanolic hydrogen chloride 3N /MeOH/$CHCl_3$ (5: 3: 1, *v/v/v*). *N*-acyl pyrrolidides (NAP) were prepared by direct treatment of the FAME with pyrrolidine/acetic acid (5: 1, *v/v*) for 60 minutes at 85 °C under reflux.

**** GC-MS analyses***

Fatty acid methyl esters (FAME) was analyzed using a gas chromatography-mass spectrometry (GC-MS) instrument (Hewlett Packard HP 6890-GC System, Agilent Technologies, Santa Clara, CA, USA) linked to a mass detector (HP 6890-E.I. 70 eV) equipped with an SLB-5^{TM} column (60 m × 0.25 mm × 0.25 µm). The carrier gas was helium at a flow rate of 1 mL/min. The temperature of the injector and detector were respectively set at 250 °C and 280 °C. One microliter was injected in splitless mode. The column temperature was held at 170 °C for 4 minutes and programmed to 300 °C at 3 °C/min. The solvent delay was 9 minutes.

**** Cytotoxicity assays***

Cytotoxicity assays were carried out using the MTT assay and following the procedure previously described [14].

2.2. Results and discussion

The crude extract of the MMS417 strain contained 4 very apolar fractions. Fractions F3 and F4, corresponding to a high proportion of the mass, formed a yellowish oil, solidifying in the cold. These thermal properties suggested that they might contain lipids. Lipids were organic molecules, which are divided into many different groups of properties. Among these groups, fatty acids, phospholipids, sphingolipids, glycerolipids, and sterols are common lipid molecules found in most cells [16]. Therefore, the fractions from F1 to F4 were analyzed by thin-layer chromatography (TLC) according to a protocol making it possible to easily identify the types of common lipid compounds contained (Figure 1).

The results showed that traces of sterols and a large amount of hydrocarbons consist of the F2 fraction. The F3 fraction is mainly made up of triglycerides, while the F4 fraction seems to contain mostly free fatty acids. Thus, this analysis confirmed the very high proportion of lipids in the crude extract, the sum of the four fractions F1 to F4 representing 7.4567 g, or 84.60% of the total crude extract. This phenomenon had not been observed previously with this fungal strain, which may suggest a change in its metabolism linked to a mutation that appeared during successive subcultures.

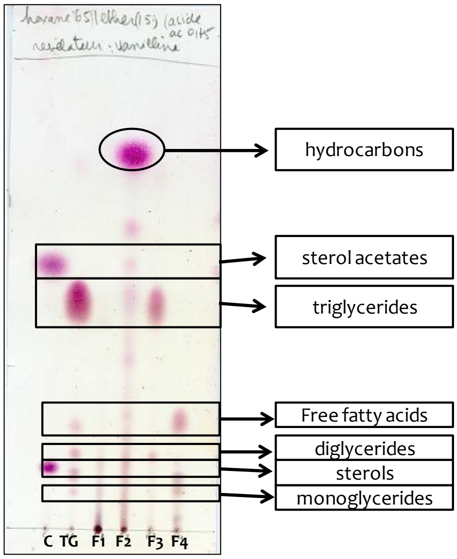

Figure 1. TLC plates of fractions F1 to F4 and control, triglycerides (silica, eluent: hexane/ether/acetic acid, 65 mL/15 mL/0.75 mL), vanillin sulphuric acid reagent

Liquid chromatography coupled to high-resolution mass spectrometry (HPLC-HRMS) is a technique used to analyze a total lipid profile in one run, from which different lipid classes can be separated [15-17]. The two fractions F3 and F4 were analyzed by HPLC-HRMS according to the lipid analysis method [15]. The lipid profiles of the fractions in this analysis make it possible to separate the compounds observed into 3 distinct classes: localization of free fatty acids (FA) between 2 minutes and 22 minutes (also specialized metabolites), phospholipids (PL), and glycolipids (GL) between 22 minutes and 35 minutes, and glycerides (TG) between 35 minutes and 43 minutes (Figure 2).

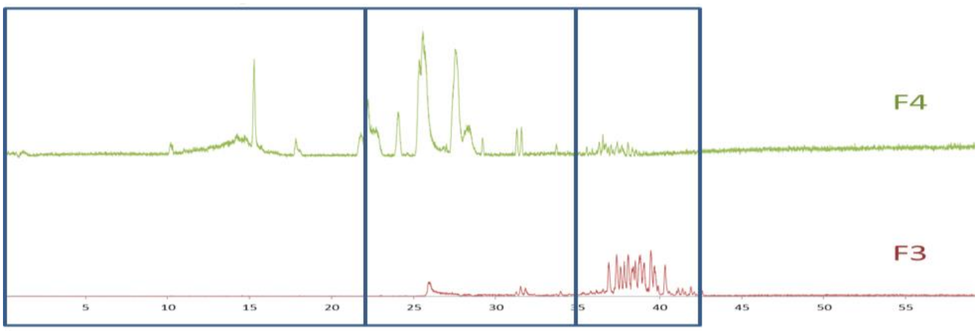

Figure 2. HPLC-MS chromatograms of the F3 and F4 fractions

Based on HPLC-HRMS analyzes, the F3 fraction contains only triglycerides and the F4 fraction is a fraction enriched in glycolipids and phospholipids. These complex lipids are containing fatty acids and the common type of membrane lipids. Fatty acids have a common basic structure with specific diversity determined by the chain length and the degree of unsaturation. For the detailed study of the triglycerides structure and other lipids in the F3 and F4 fractions, the fatty acid composition of the two fractions was investigated.

The analysis of the fatty acid composition of the F3 and F4 fractions was carried out by gas chromatography-mass spectrometry (GC-MS) analyses to make them volatile and to be able to identify them with certainty, they were chemically modified into methyl esters (FAME, allowing the identification of saturated fatty acid) or N-acyl pyrrolidines (NAP, for the identification of unsaturated fatty acid). The dominant components in F3 and F4 fractions were C16:0, C18:0, 9-C18:1, 9,12-C18:2 fatty acids (Figure 3). In general, both fractions have had the same qualitative pattern of FAs, but differed in the quantity of the FAs produced, except FAs for the following: 10-C19:1, C23:0, 9,12-C17:2 found in F3 fractions, not in F4 fractions; and similarly, C18:0, C18:3, 6,11-C20:2, 7-C20:1, 8,13-C22:2 found in F4 fractions, not in F3 fractions. This analysis showed that triglycerides species in the F3 fraction containing 17 fatty acids, with 14-24 carbon atom alkyl chain and 0-2 double bonds were found. The major molecular TG species in the F3 fraction contains the most abundant of FAs as follows: C16:0, C18:0, 9-C18:1, 9,12-C18:2.

A total of 19 fatty acids was identified in *P. restrictum* grown on a host-derived medium, having 14 to 24 carbons. Among these acids, the common acids as hexadecanoic acid (C16 : 0), 9-octadecenoic acid (9-C18: 1), octadecanoic acid (C18 : 0) present at high levels (Figure 3). In addition, a C18: 2 unsaturated fatty acid was found in high proportion. This result was consistent with previous studies [12]. The acids such as 9, 12-C17 : 2; 8-C18 : 1; C18 : 3; C19 : 0; 7-C20 : 1; 12-C20 : 1; 6, 11-C20 : 2; C22 : 0; 8, 13-C22 : 2; C23 : 0; C24 : 0 were detected with less than 2% of the total fatty acid content. These acids were not reported in previous studies of Stahl et al. [12]. Moreover, this phenomenon has also been observed when comparing our results with Oleinikova'study. Oleinikova *et al.* showed that the strain *Penicillium restrictum* grown on rice produces hexadecanoic acid (C16 : 0), octadecenoic acid (C18: 1), octadecanoic acid (C18 : 0) present in large quantities but they haven't reported the production of octadecadienoic acid (C18: 2) [13]. In our work, octadecadienoic acid (C18: 2) showed high levels in both F3 and F4 fractions (over 25% of total fatty acid). These results have confirmed that the culture medium affects the fatty acid composition produced and then, the change in fatty acid profiles was due to environmental changes in fatty acid metabolism. This represented an adaptation to the changing habitat of this strain.

As we know it, marine fungi have had to adapt to the changing environment to survive. Among the changes to which they have had to adapt, salinity was probably one of the environmental factors that affect the change in the lipid composition of the membrane of fungi [18]. These changes were necessary to maintain a fluid state of the cell membrane. Several lipids were directly responsible and indirectly influence the maintenance of membrane fluidity: the type of fatty acyl chains, the number of sterols, the nature of the head groups of polar phospholipids [19]. The majority of previous studies have been carried out on the effect of salt stress on the lipid composition and membrane fluidity of yeasts [18, 20, 21]. Our previous study has shown that the marine fungi *Penicillium restrictum* could produce specific metabolites in the host-derived medium. Further studies are needed on the environmental response of this fungal strain, especially in the marine environment; as well as on the effects of salinity, culture medium on lipid composition in this strain.

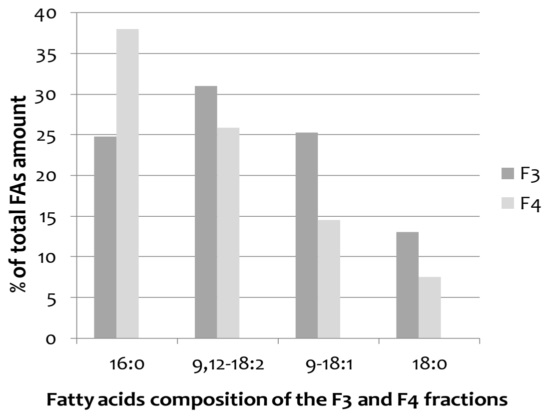

Figure 3. The proportion of fatty acids composition of the F3 and F4 fraction

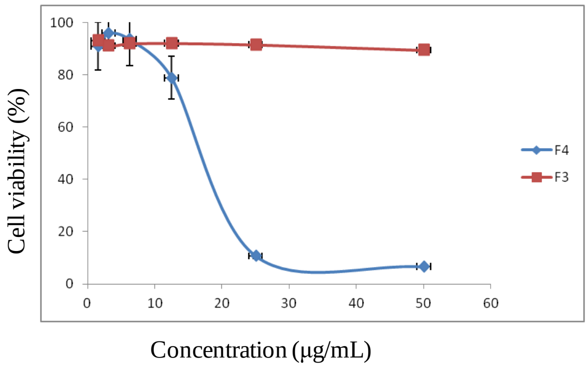

Figure 4. Evaluation of cytotoxicity on the KB cells of the F3 and F4 fractions

Glycolipids are amphiphiles compounds composed of a hydrophilic moiety, a sugar moiety, and a hydrophobic moiety, a fatty acid. They have also demonstrated their biological activity, as biosurfactants or modulators of membrane organization and they are potentially useful in health, with antimicrobial, immunomodulatory, or anticancer activities [22]. Therefore, F3 and F4 fractions were tested on the KB cell line to evaluate potential cytotoxic activity. Interestingly, the F4 fraction exhibits average activity (IC_{50} = 16.76 μg/mL) compared to the F3 fraction without significant cytotoxicity on the KB line (Figure 4). This result showed that no significant effect of the triglycerides in the F3 fraction was observed on the KB line. It is very interesting to further isolate and determine the molecular structure of bioactive glycolipid content in the F4 fraction.

3. Conclusions

The extract from MMS417 grown on a host-derived culture medium was largely composed of lipids (plus 80%), which were produced in an unusually high amount. Four main fatty acids were identified typical of fungal lipids. Thus, we obtained the following fours lipid fractions: hydrocarbons, triglycerides, phospholipids, glycolipids, sterols. Moreover, among them, the lipid fraction exhibits average activity on the KB line (IC_{50} = 16.76 μg/mL). This result is very interesting to further isolate and determine the molecular structure of bioactive compounds.

This observation is promising and further study of lipids is planned: alternative oil production processes, whether for biofuels or food, are a rapidly developing research avenue. The discovery of fungal strains capable of high lipid yields may represent an avenue to explore.

REFERENCES

[1] France T., Jean-Marc N., 2013. Microorganisms as sources of oils. *Oilseeds and fats, Crops and Lipids,* Vol. 20, Iss. 6, pp. D603.

[2] Mahesh K., Srijay K., Smita Z., Aditi P., Balu C., and Ameeta R. K., 2012. Single-cell oil of oleaginous fungi from the tropical mangrove wetlands as a potential feedstock for biodiesel. *Microbial Cell Factories,* Vol. 11, pp. 71.

[3] Gwendoline C., Vinod K., Régis N., Geneviève G., Pierre F., Ashok P., Carlos R. S., Christian L.., 2012. Recent developments in microbial oils production: a possible alternative to vegetable oils for biodiesel without competition with human food? *Brazilian Archives of Biology and Technology*, Vol. 55, Iss. 1, pp. 29-46.

[4] Raghda A., Stamatia B., Grammatiki F., Georgia S., Nikos A. D., Seraphim P., George A., 2015. Fatty acid lithium salts from *Cunninghamella echinulata* have cytotoxic and genotoxic effects on HL-60 human leukemia cells. *Engineering in Life Sciences,* Vol. 15, Iss. 2, pp. 243-253.

[5] Fotoon S., Ahmed E., Stamatia B., Anna M., Ayman I. E., Mohammed N. B. and George A., 2016. Production of polyunsaturated single cell oils possessing antimicrobial and anticancer properties. *Annals of Microbiology,* Vol. 66, pp. 937-948.

[6] Seraphim P., George A, 2010. *Arrowia lipolytica*: A model microorganism used for the production of tailor made lipids. *European Journal of Lipid Science and Technology,* Vol. 112, Iss. 6, pp. 639-654.

[7] Yongjun W., Verena S. and Jens N., 2017. Cocoa butter-like lipid production ability of non-oleaginous and oleaginous yeasts under nitrogen-limited culture conditions. *Applied Microbiology and Biotechnology,* Vol. 101, pp. 3577-3585.

[8] Sayeda A. A., Azhar A. H., Mohsen S. A., Osama H. El S. and Saher S. M, 2019. Optimization of culture conditions for biodiesel production from Egyptian isolate *Penicillium commune* NRC2016. *Bulletin of the National Research Centre,* Vol. 43, pp. 15.

[9] Ho Quoc Phong, Do Nguyen Tuong Vy, Huynh Lien Huong, Truong Thi Be Trinh, 2014. Research on synthesis of biodiesel from fat of Y. Lypolitica PO1G using subcritical method. *Can Tho University Journal of Science*. No. 33, pp. 22-28 (in Vietnamese).

[10] Onesy K., Vong A. K., Bounpong K., Sitha K., and Dang Ngoc Quang, 2016. Cytotoxic steroids from the mushroom *Ganoderma australe* were collected in Laos. *Vietnam Journal of Chemistry,* International Edition, Vol 54, Iss. 6, pp. 688-691.

[11] Rosario N. and Mario de S., 2012. *Penicillium restrictum* as an antagonist of plant pathogenic fungi. *Dynamic Biochemistry, process Biotechnology and Molecular Biology,* Vol. 6, Iss. 2, pp. 61-69.

[12] Peter D. S. and Michael J. K., 1996. Characterization and differentiation of filamentous fungi based on fatty acid composition. *Applied and Environmental Microbiology,* Vol. 62, Iss. 11, pp. 4136-4146.

[13] G. K. Oleinikova, O. F. Smetanina, Yu. V. Khudyakova, N. N. Kirichuk, and Sh. Sh. Afiyatullov, 2013. Non-polar compounds and free fatty acids from marine isolates of mycelial fungi. *Chemistry of Natural Compounds*, Vol. 49, Iss. 3, pp. 499-500.

[14] Le Van-Tuyen, Samuel B., Thibault R. d. P., F. Fleury, Nathalie C., S. Bourgeade-Delmas, E. Gentil, C. Logé, G. Genta-Jouve, and O. Grovel, 2021. Untargeted metabolomics approach for the discovery of environment-related Pyran-2-ones chemodiversity in a marine-sourced *Penicillium restrictum*. *Marine Drugs*, Vol. 19, Iss. 7, pp. 378.

[15] Dos S. D., A. C., Couzinet-Mossion, A., Ruiz, N., Bellec, M. L., Gentil, E., & Bertrand, G. W.-C. and S., 2017. Sugar induced modification in glycolipid production in *Acremonium* sp. revealed by LC-MS lipidomic approach. *Current Biotechnology,* Vol. 6, Iss. 3, pp. 227-237.

[16] Fahy Eoin, Subramaniam Shankar, Brown H Alex, Glass Christopher K, Merrill Alfred H, Murphy Robert C, Raetz Christian RH, Russell David W, Seyama Yousuke, Shaw Walter, 2005. A comprehensive classification system for lipids. *Journal of Lipid Research,* Vol. 46, Iss. 5, pp. 839-861.

[17] Oskar L. K.; Bernd P. W.; Thomas O. E., Sepp D. K., Gerald N. R., 2014. A versatile ultra-high performance LC-MS method for lipid profiling. *Journal of Chromatography B,* Vol. 951, Iss. 1, pp. 119-128.

[18] Turk M., Méjanelle, L., Šentjurc, M., Grimalt, J. O., Gunde-Cimerman, N., and Plemenitaš, A., 2004. Salt-induced changes in lipid composition and membrane fluidity of halophilic yeast-like melanized fungi. *Extremophiles,* Vol. *8,* Iss. 1, pp. 53-61.

[19] Russell N. J., 1989. Adaptive modifications in membranes of halotolerant and halophilic microorganisms. *Journal of Bioenergetics and Biomembranes,* Vol. 21, Iss. 1, pp. 93-113.

[20] Hosono K., 1992. Effect of salt stress on lipid composition and membrane fluidity of the salt-tolerant yeast *Zygosaccharomyces rouxii*. *Microbiology,* Vol. 138, Iss. 1, pp. 91-96.

[21] Andreishcheva E.N., Isakova E.P., Sidorov N.N., Abramova N.B., Ushakova N.A., Shaposhnikov G.L., Soares M.I.M., Zvyagilskaya R.A., 1999. Adaptation to salt stress in a salt-tolerant strain of the yeast *Yarrowia lipolytica*. *Biochemistry (Moscow),* Vol. 64, Iss. 9, pp. 1061-1067.

[22] Alejandro d. J. C.-S., Humberto H.-S., María E. J.-F.., 2013. Biological activity of glycolipids produced by microorganisms: New trends and possible therapeutic alternatives. *Microbiological Research,* Vol. 168, Iss. 1, pp. 22-32.