**RESEARCH ARTICLE**      **OPEN ACCESS**

# SELECTION OF FILAMENTOUS FUNGI FOR CONVERSION OF HIGH CONCENTRATIONS OF BIODIESEL-DERIVED GLYCERIN

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### ABSTRACT

Biodiesel-derived glycerin is a potential biotechnological substrate, but its bioconversion depends on finding resistant microbial strains, once this effluent can be inhibitory for growth. A total of 58 microbial isolates from 26 samples of soil, rotten leaves, and other fallen plant materials or contaminated foods, survived an enrichment selection performed at 25% of biodiesel-derived glycerin. *Aspergillus sp.* T7.1, *Penicillium sp.* T2.1 and T6.2 grew in submerged processes adjusted for citric acid production, at a final concentration of 100 g . $L^{-1}$ of glycerol derived from biodiesel as carbon source (1.1 M). Under these conditions, isolates T7.1 and T2.1 produced limited amounts of acid, but consumed 50% and 40% of the initial glycerol, at volumetric consumption rates of 0.3 g . $L^{-1}$ . $h^{-1}$ and 0.24 g . $L^{-1}$ . $h^{-1}$, respectively. Another isolate, *Penicillium sp.* T14.1 showed high index of acidogenesis in solid media and resisted to 3 M biodiesel-derived glycerol under submerged cultivation, producing 5 times more acid than the above strains. Results indicate the genus *Penicillium* as a good source of microbial agents for use of glycerin effluent.

## INTRODUCTION

The continuous search for sustainable and ecologically friendly processes has been one of the impellers of bioeconomy, a global tendency of using bio-based feed stocks (STAFFAS *et al.*, 2013, JOHNSON, 2017). Biofuels like biodiesel have achieved increased importance in this scenario, with a growing number of production facilities all around the world (HOOD, 2016). Biodiesel is chemically produced by the reaction of a vegetal oil or animal fat with an alcohol of low molecular weight, like ethanol or methanol, by transesterification (ALMEIDA *et al.*, 2012). As a consequence of the augment of biodiesel production, glycerol formed as an effluent is becoming easily available in large scale, and due to its low cost, glycerin has turned to be an interesting alternative substrate for industrial biotechnology (KALIA *et al.*, 2016). However, glycerin effluent of biodiesel can be contaminated with methanol, alkalis, fat acids and their salts, esters, sulfur compounds, proteins, and minerals (THOMPSON, 2006). Such contamination impacts the cost of glycerin purification and, thus, its commercial applications. Nevertheless, biodiesel derived glycerin need an adequate destination, once their discard results in environmental problems and requires

investment to control its potential as pollutant. Discarding glycerin also means loss of matter and energy. Conversion of crude glycerin is important to increase viability of biodiesel, and a variety of value added products can be formed from glycerol: 1,3-propanediol, hydrogen, ethanol, propanoic and citric acids, n-butanol, polymers, rhamnolipds, and others (GARLAPATI *et al.*, 2016, SALAZAR-BRYAM *et al.*, 2017). One main step for the biotechnological use of crude glycerin is to find microorganisms resistant to this toxic effluent, and which also be able to convert it to high-value biotechnological products (SZYMANOWSKA-POWALOWSKA, 2015). According to Samul *et al.* (2014) the number of studies reporting crude glycerol utilization by microorganisms as the sole carbon source is yet small, despite it is being more economically advantageous than the traditional use of pure glycerol. Miltrea *et al.* (2017) reviewed bioconversion of glycerol to 1,3-propanediol and citric acid, and reported a number of studies utilizing crude glycerol as substrate for yeast or bacteria. Most of the reports in the literature have utilized bacteria and yeasts for bioconversion of glycerol. Mixed cultures of bacteria were used to convert 100% of crude glycerol to 2,3-butanediol and 1,3-propanediol (PARATE *et al.*, 2018). Conversion of glycerol by filamentous fungi has been included in fewer studies, but some good results have

been published (CHATZIFRAGKOU *et al.*, 2011). Microbial ability to grow on glycerol varies greatly among species and strains, and is also influenced by culture conditions. Among the pathways for glycerol catabolism, the most known involves conversion to glycerol 3-phosphate, in a reaction catalyzed by glycerol-kinase. In the next reaction, glycerol 3-phosphate is converted to a glycolysis intermediate, dihydroxiacetone phosphate, by the action of glycerol 3-phosphate dehydrogenase (KLEIN *et al.*, 2017). The end product of glycolysis, piruvate, is precursor of several important biotechnological products, including organic acids. These have a wide range of applications in the pharmaceutical, food, and chemical industries. Citric acid, the main organic acid in the biotechnology industry (CIRIMINNA *et al.*, 2017), can be produced from glycerol by filamentous fungi (SHOW *et al.*, 2015) or yeast (RYWINSKA *et al.*, 2009, SABRA *et al.*, 2017). Several filamentous fungi, yeasts, and bacteria are able to grow on glycerol, but specific growth rates of strains, and their metabolic potential for biotechnological conversion can be quite different. The current state of biodiesel-derived glycerin conversion or remediation poses the need for more studies on microbial utilization of glycerol, including selection of microorganisms which tolerate high glycerol concentration and contamination. Once the necessary enzymes for glycerol metabolism are widely spread among microbial genera, the objective of this work was to search among the microbial diversity occurring in the campus of the Universidade Federal dos Vales do Jequitinhonha e Mucuri, in Diamantina-MG, and in indoor environments, strains able to grow on glycerin effluent of biodiesel. Isolates were screened for their ability to produce acid from glycerin in solid media. Those showing good acidogenesis were cultivated by submerged process, under conditions adjusted for citric acid production. Factorial planning was applied to determine effects of variables influencing growth of a high-potential-acidogenic strain cultivated in media supplemented with elevated concentrations of glycerin.

## MATERIAL AND METHODS

***Isolation of microbial strains:*** Samples of soil, rotten plant material and contaminated food were powdered when necessary and inoculated into 50 mL of enrichment solution contained in 500 mL Erlenmeyer flasks. Enrichment solution was prepared by diluting raw glycerin to 50% with distilled water and decreasing pH to 4.0 with sulfuric acid. Glycerin was obtained as effluent of biodiesel production from frying oil wastes, a process performed in the Laboratory of Biodiesel-UFVJM. An amount of sulfate precipitated was filtered off. After filtration, concentration of effluent was adjusted to 25% and used to select microorganisms able to survive these conditions. Samples were incubated at 30 ºC for 5 days under agitation (150 rpm). An aliquot of 0.1 mL was taken from each sample and spread onto the surface of potato dextrose agar slants. Cultures were incubated at 30 ºC and at room temperature, and the growing colonies were isolated. Each isolate was further cultivated onto potato dextrose agar (HiMedia®), Czapek agar (30 g . $L^{-1}$ sucrose; 20 g . $L^{-1}$ NaNO$_3$; 0.5 g . $L^{-1}$ KCl; 0.5 g . $L^{-1}$ Mg$_2$SO$_4$. 2 H$_2$O; 1 g . $L^{-1}$ K$_2$HPO$_4$; 0.01 g . $L^{-1}$ FeSO$_4$. 7 H$_2$O; 0.01 g . $L^{-1}$ ZnSO$_4$. 7 H$_2$O; 1 mL.$L^{-1}$ CuSO$_4$ 0.5%; 1.5% agar; pH 6.0) and/or yeast extract glucose agar (20 g . $L^{-1}$ glucose; 5 g . $L^{-1}$ yeast extract; 1.5% agar; pH 6.0). Isolates were classified as filamentous fungi, yeast or bacteria according to microscopic and macroscopic morphology. Filamentous fungi were grown on potato dextrose

agar by micro-cultivation. Grown samples were taken and transferred to a glass slide containing a drop of lactophenol blue. Analysis was performed at phase contrast with a microscope Olympus BX41. Whenever present, reproductive structures of fungal strains were also observed for identification at the genus level.

***Acidogenesis of isolates grown on glycerin:*** Isolates were evaluated to determine the potential to grow and produce acid on solid medium containing glycerin effluent of biodiesel as the sole carbon source. Disks of 5 mm of diameter were collected from colonies grown on potato dextrose agar, Czapek medium or yeast extract glucose agar at 30 ºC, and transferred to the center of medium for acidogenesis determination (20% glycerin treated as described above; 3 g . $L^{-1}$ (NH$_4$)$_2$SO$_4$; 1 g . $L^{-1}$ KH$_2$PO$_4$; 1 g . $L^{-1}$ K$_2$HPO$_4$; 0.04 g . $L^{-1}$ bromocresol green; 1.5% agar; pH 4.0). Incubation was at 30 ºC and at room temperature. Colony diameter and the acidic halo were measured at 24 h intervals. The index of acidogenesis was calculated as the ratio: diameter of the acidic halo/ colony diameter. Diameters were measured in two directions in at least three replicates, and expressed as mean ± standard deviation.

***Utilization of glycerin and growth of fungal isolates in submerged cultures:*** Filamentous fungi showing good index of acidogenesis, confluent growth and spore formation, were cultivated in 1 L fermentation flasks containing 200 mL of medium for citric acid production as modified from the literature (RUGSASEEL *et al.*, 1996): biodiesel-derived glycerin (10% glycerol); 3 g . $L^{-1}$ (NH$_4$)$_2$SO$_4$; 1 g . $L^{-1}$ KH$_2$PO$_4$; 1 g . $L^{-1}$ K$_2$HPO$_4$; 0.5 g . $L^{-1}$ MgSO$_4$. 7 H$_2$O; 0.0014 g . $L^{-1}$ MnSO$_4$; 0.001 g . $L^{-1}$ FeCl$_3$. 6 H$_2$O; pH 4.0. Final concentration of glycerol in media was adjusted for 10%, as measured by adaptation of an enzymatic commercial kit for triglycerides quantification (LabTest®). Cultures were inoculated with spores to a final concentration of $10^5$. mL$^{-1}$, and incubated at 30 ºC or at room temperature, according to the best temperature for acidogenesis previously determined. Submerged cultivations were conducted at 150 rpm of agitation. Biomass was determined as dry weight, and total acidity was determined by titration and expressed as citric acid (HU *et al.*, 2014).

***Factorial planning of biotechnological processes for glycerin utilization and conversion:*** A factorial planning $2^{6-1}$ was designed to evaluate variables influencing growth of a fungal strain selected for their high index of acidogenesis. Variables and their minimum and maximum levels were: glycerin concentration (20 to 60%); pH 2 to 7; K$_2$HPO$_4$ (0 to 5 g . $L^{-1}$); CaCl$_2$ (0 to 0.2 g . $L^{-1}$); sucrose (0 to 1 g . $L^{-1}$); time of incubation (3 to 9 days). A total of 32 bioprocesses combining the minimum and maximum levels of the variables analyzed, and 3 processes combining the central values of all variables were generated with the software *Statistica 7.0* (Statsoft Inc). Other medium constituents were adjusted as described above for submerged cultures. Planned processes were inoculated with pellets of mycelia produced after 72 h of cultivation at 30 ºC (150 rpm). The amount of pellets contained in 50 mL was used to inoculate each planned process.

## RESULTS AND DISCUSSION

***Isolation and selection of acid producers among strains resistant to biodiesel-derived glycerin:*** A total of 58 isolates from 26 samples of soil, rotten leaves, and other fallen plant

materials or contaminated foods were resistant to the enrichment cultivation. Among these, there were 39 filamentous fungi, 18 yeasts and 1 strain of bacterium. Isolates were coded and evaluated for their potential to produce acid from glycerin in solid media, as shown in Table 1. The number of surviving microorganisms after enrichment was small for most samples, even for materials usually highly colonized, like soil. Some samples yielded no surviving microorganisms, showing the stringency of such selective conditions and the toxicity of the effluent. Among 39 filamentous fungi, 16 produced reproductive structures that allowed identification at the genus level by analysis of micro and macroscopic morphologies. From these, 10 were classified as *Penicillium sp.*; 2 as *Talaromyces sp.*, 2 as *Aspergillus sp.*, 1 as *Chrysosporium sp.* and 1 as *Fusarium sp.*

All of the 4 isolates which produced an index of acidogenesis above 4.0 when cultivated at room temperature belonged to the genus *Penicillium* (Table 1). Similar results were verified when screening was performed at 30 °C: 6 isolates showing an index above 4.0 were also *Penicillium sp.* Thus, there was a prevalence of the genus *Penicillium* among survivors (10 of 16 identified fungi; 10 of 39 isolates of filamentous fungi), which also showed to have got potential for bioconversion of contaminated glycerin. The genus *Aspergillus*, of recognized biotechnological importance and ubiquitous occurrence, was under represented, with only 2 isolates surviving the selective enrichment. *Aspergilla* are known for their potential as acid producers, especially citric acid, whereas the main industrial producer is *Aspergillus niger*. Isolate *Aspergillus sp.* T7.1 showed reasonable index of acidogenesis, both at room

**Table 1. Microbial isolates resistant to biodiesel-derived glycerin and their potential for acidogenesis**

| Sample Code | Sample Type | Strain Code | Classification | IA Room Temperature | IA 30 ºC |
|---|---|---|---|---|---|
| 1 | Seed | T1.1 | *Penicillium* | 4.0 ± 0.5 | 4.6 ± 0.7 |
| | | T1.2 | *Penicillium* | 3.8 ± 0.6 | 4.5 ± 0.6 |
| 2 | Leaves | T2.1 | *Penicillium* | 4.4 ± 0.3 | 0.3 ± 0 |
| 3 | Rotten branches | T3.1 | *Penicillium* | 0 ± 0 | 0 ± 0 |
| 5 | Bread | T5.1 | Yeast | 0 ± 0 | 0 ± 0 |
| 6 | Cookie | T6.1 | *Talaromyces* | 0 ± 0 | 0 ± 0 |
| | | T6.2 | *Penicillium* | 4.4 ± 0.5 | 5.1 ± 0.4 |
| 7 | Pudding | T7.1 | *Aspergillus* | 3.5 ± 0.1 | 3.3 ± 0.2 |
| 8 | Rice | T8.1 | Yeast | 2.0 ± 0.5 | 3.3 ± 0.4 |
| | | T8.2 | Yeast | 1.5 ± 0.1 | 1.5 ± 0.2 |
| | | T8.3 | *Penicillium* | 3.5 ± 0.5 | 3.0 ± 0.8 |
| 9 | Leaves | T9.1 | *Penicillium* | 3.1 ± 0.2 | 4.9 ± 0.7 |
| | | T9.2 | *Penicillium* | 4.2 ± 0.2 | 4.1 ± 0.5 |
| 10 | Soil | T10.1 | Filamentous fungus | 0 ± 0 | 0 ± 0 |
| | | T10.3 | Yeast | 0.9 ± 0.6 | 2.1 ± 0.4 |
| | | T10.4 | Filamentous fungus | 0 ± 0 | 0 ± 0 |
| | | T10.5 | *Talaromyces* | 1.9 ± 0.2 | 1.6 ± 0.1 |
| 13 | Jatropha | T13.1 | Bacterium | 0 ± 0 | 0 ± 0 |
| 14 | Tamarindo pulp | T14.1 | *Penicillium* | 3.9 ± 0.3 | 6.2 ± 0.2 |
| | | T14.2 | Filamentous fungus | 3.2 ± 0.7 | 2.9 ± 0.2 |
| 16 | Passion fruit | T16.1 | Yeast | 0 ± 0 | 2.9 ± 1.2 |
| 17 | Lemongrass | T17.1 | Filamentous fungus | 0 ± 0 | 2.1 ± 0.3 |
| 18 | Leaves | T18.1 | Filamentous fungus | 0 ± 0 | 0 ± 0 |
| | | T18.2 | *Chrysosporium* | 1.4 ± 0.1 | 1.9 ± 0.3 |
| | | T18.3 | Yeast | 0 ± 0 | 0 ± 0 |
| | | T18.4 | Filamentous fungus | 0 ± 0 | 2.2 ± 0.2 |
| | | T18.5 | Filamentous fungus | 0 ± 0 | 0 ± 0 |
| | | T18.6 | Filamentous fungus | 0 ± 0 | 0 ± 0 |
| | | T18.7 | Yeast | 0.3 ± 0.5 | 1.9 ± 0.1 |
| | | T18.8 | Filamentous fungus | 0 ± 0 | 0 ± 0 |
| 19 | Shrub | T19.1 | Filamentous fungus | 0 ± 0 | 0 ± 0 |
| | | T19.2 | Filamentous fungus | 0 ± 0 | 0 ± 0 |
| | | T19.3 | Yeast | 0 ± 0 | 0 ± 0 |
| 20 | Branches | T20.1 | Yeast | 0 ± 0 | 0 ± 0 |
| 21 | Shrub | T21.1 | Yeast | 0 ± 0 | 0 ± 0 |
| | | T21.2 | Yeast | 0 ± 0 | 0 ± 0 |
| | | T21.3 | Yeast | 0 ± 0 | 0 ± 0 |
| | | T21.4 | Filamentous fungus | 0 ± 0 | 0 ± 0 |
| 22 | Branches | T22.1 | Filamentous fungus | 0 ± 0 | 0 ± 0 |
| | | T22.2 | *Fusarium* | 0 ± 0 | 3.2 ± 0.3 |
| | | T22.3 | *Penicillium* | 4.4 ± 0.1 | 4.0 ± 0.2 |
| | | T22.4 | Yeast | 0 ± 0 | 0 ± 0 |
| 23 | Branches | T23.1 | Yeast | 0 ± 0 | 0 ± 0 |
| | | T23.2 | Filamentous fungus | 0 ± 0 | 0 ± 0 |
| | | T23.3 | Filamentous fungus | 0.7 ± 0.5 | 0.3 ± 0.5 |
| 24 | Branches | T24.1 | Yeast | 0 ± 0 | 0 ± 0 |
| | | T24.2 | Filamentous fungus | 0 ± 0 | 0 ± 0 |
| 25 | Branches | T25.1 | Yeast | 0 ± 0 | 0 ± 0 |
| | | T25.2 | Filamentous fungus | 0 ± 0 | 0 ± 0 |
| | | T25.3 | Filamentous fungus | 0 ± 0 | 0 ± 0 |
| | | T25.4 | Filamentous fungus | 0 ± 0 | 3.6 ± 0.2 |
| | | T25.5 | Yeast | 0 ± 0 | 0 ± 0 |
| 26 | Branches | T26.1 | Yeast | 0 ± 0 | 0 ± 0 |
| | | T26.2 | Filamentous fungus | 0 ± 0 | 0 ± 0 |
| | | T26.3 | Filamentous fungus | 2.3 ± 0.2 | 1.5 ± 0.1 |
| | | T26.4 | *Aspergillus* | 3.1 ± 0.7 | 2.0 ± 0.1 |
| | | T26.5 | Filamentous fungus | 3.1 ± 0 | 1.8 ± 0.1 |
| | | T26.6 | Filamentous fungus | 3.3 ± 0.3 | 4.0 ± 0.4 |

temperature and at 30 °C. It is possible that acidogenesis of isolate T7.1 was not higher because it is a fast-growing fungi, once the index is calculated as the ratio of the acidic halo per colony diameter. On the opposite, some isolates of *Penicillium* did not grow well in the conditions studied. Isolates were coded according to their sample of origin (number before dot) and the sequential number of colonies growing in solid media (number after dot). IA, index of acidogenesis. Secretion of acid was evaluated in media supplemented with 20% of a 2X diluted biodiesel-derived glycerin adjusted to pH 4.0. Bromocresol green was used as indicator to evidence a yellow acidic halo against green medium. Each culture was started with a disk of mycelia (5 mm) transferred from colonies grown on Czapek agar. Index of acidogenesis was determined after 72 h of incubation as the ratio: diameter of the acidic halo/ colony diameter.

Microscopic morphology of isolates *Penicillium sp.* T6.2, *Penicillium sp.* T2.1, and *Aspergillus sp.* T7.1 are shown in Figure 1. Based on their acidogenesis, growth and spore formation they were selected for submerged processes to estimate their ability to grow and produce total acidity from glycerin in the conditions usually applied to citric acid fermentation. The best acidogenic isolate *Penicillium sp.* T14.1 was chosen for cultivation under conditions designed by factorial planning.

***Growth, glycerin utilization and acid production in submerged processes:*** Submerged bioprocesses containing 20% of biodiesel-derived glycerin were conducted at room temperature with isolate *Penicillium sp.*T2.1, and at 30ºC with *Aspergillus sp.* T7.1 and *Penicillium sp.* T6.2 (Figure 1). Besides their indexes of acidogenesis, good level of
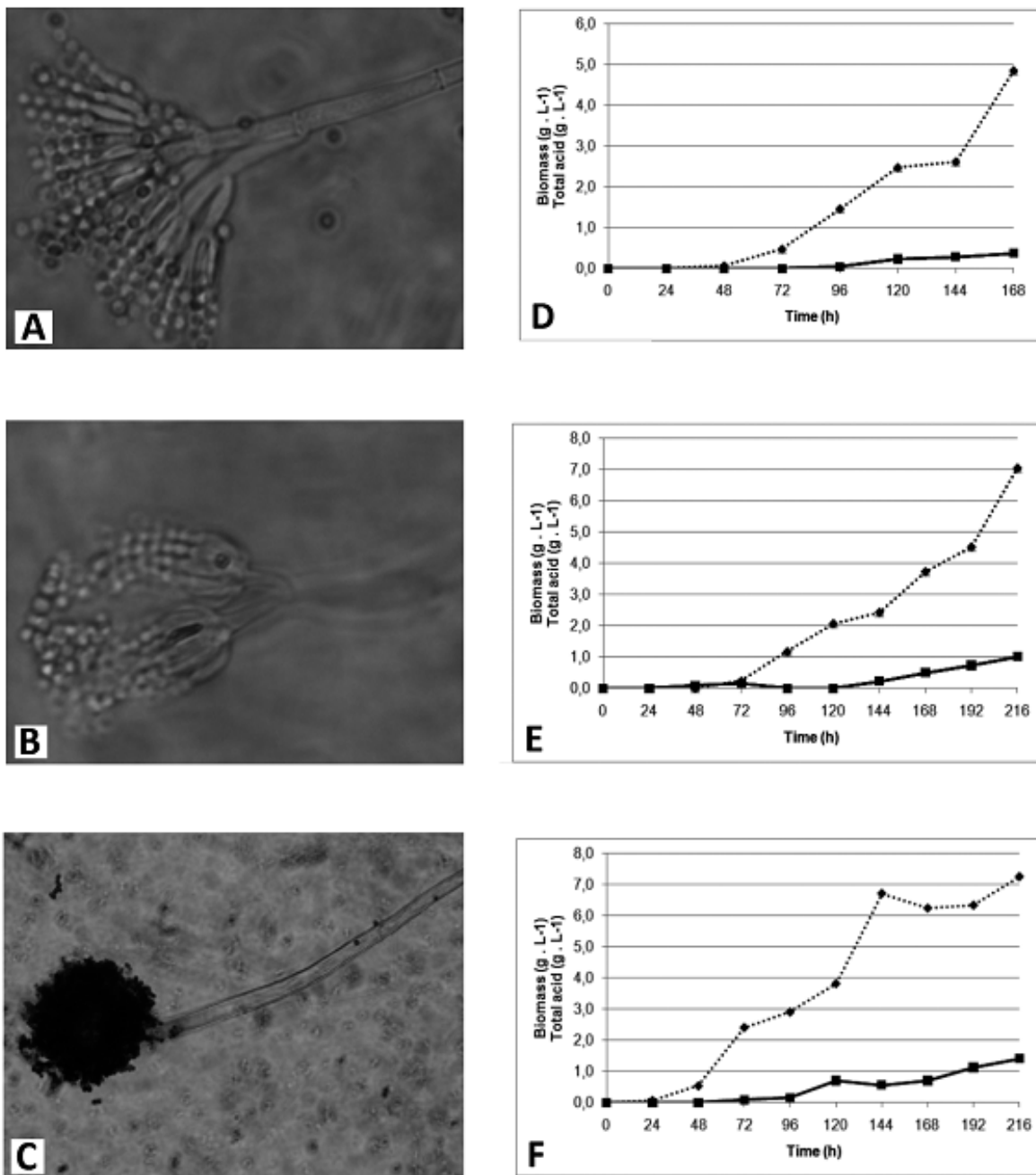


**Figure 1. Filamentous fungi utilizing glycerol for growth and acid secretion. A-C: microscopic morphologies showing conidiophores of *Penicillium sp.* T2.1 (A), *Penicillium sp.* T6.2 (B), and *Aspergillus sp.* T7.1 (C). D-E: Curves of growth (dotted lines) and total acidity (solid lines) of isolates T2.1 (D), T6.2 (E), and T7.1 (F) cultivated in media supplemented with biodiesel-derived glycerin – glycerol concentration: 100 g . L$^{-1}$ (1.1 M). Cultures were inoculated with $10^5$ conidia per mL, and incubated under 125 rpm of agitation and at 30º (E, F) or at room temperature (D) – according to the best condition for acid secretion determined during screening**

conidiation and growth were taken into account for selection of these isolates. It was observed that the isolate *Penicillium sp.* T2.1 presented detectable biomass formation after 48 h of cultivation, and reached a growth of 4.85 g . L$^{-1}$ after 168 h (Figure 1D). Although the total acidity remained low during the whole period of cultivation, experimental determination evidenced glycerol utilization with a decrease in the initial concentration (100 g . L$^{-1}$) to approximately 60 g . L$^{-1}$ after 168 h. This yields a rate of glycerol consumption of 0.24 g . L$^{-1}$ . h$^{-1}$, whereas 40% of the initial substrate were utilized. The volumetric biomass production was 0.05 g . L$^{-1}$ . h$^{-1}$ (72 to 168 h). Analysis of the growth curve of the isolate *Penicillium sp.* T6.2 showed that the exponential growth phase started after 72 h. Total acid production became detectable after 144 h (0.23 g . L$^{-1}$) and reached 1.02 g. L$^{-1}$ after 216 h (Figure 1), 5 times more than isolate T2.1. Biomass formation by isolate T6.2 reached 7 g . L$^{-1}$, with a volumetric biomass production from 72 to 216 h of 0.05 g . L$^{-1}$ . h$^{-1}$. Good biomass formation on glycerin as the sole carbon source and acidity production indicates that isolate T6.2 may have potential for bioconversion of glycerin effluent of biodiesel. Some strains of *Y. lipolytica* able to grow in media supplemented with biodiesel-derived glycerin were reported (ANDRÉ et al., 2009).

The best growing yeast strain formed 8.2 g . L$^{-1}$ of biomass and consumed 25.3 g . L$^{-1}$ of glycerol after 90 h of cultivation, whereas the initial concentration of glycerol in the cultivation medium was 30 g . L$^{-1}$ (ANDRÉ et al., 2009). In the present work, the initial concentration of glycerol in media was 100 g . L$^{-1}$. These results corroborate the potential of the fungal isolates T2.1 and T6.2 to bear high concentrations of biodiesel-derived glycerin and also for its utilization. Growth of *Aspergillus sp.* T7.1 became measurable after 24 h, showing it is a fast growing fungus when compared to *Penicillium sp.* T6.2 and *Penicillium sp.* T2.1. After 216 h, dry weight of biomass was 7.25 g . L$^{-1}$ (Figure 1). Accumulation of total acid in the culture filtrates started after 120 h, and the maximum was obtained after 9 days of cultivation (1.4 g . L$^{-1}$). Glycerol concentration decreased from 100 g . L$^{-1}$ to 50 g . L$^{-1}$ after 168 h of cultivation, yielding a rate of substrate consumption of 0.3 g . L$^{-1}$ . h$^{-1}$, slightly higher than that of isolate T2.1. Volumetric biomass formation was 0.06 g . L$^{-1}$ . h$^{-1}$ (48 to 144 h). Exponential growth ceased after 144 h. The specific growth rate presented by strain T7.1 from 48 to 144 h (0.01 h$^{-1}$) was lower than those of some yeast strains which showed high specific growth rates of 0.1 and 0.2 h$^{-1}$(KLEIN et al., 2017). However, *S. cerevisiae* usually grows faster than filamentous fungi, and the good reported growth rates of such yeasts strains were achieved in glycerol synthetic media, not in media supplemented with crude glycerin. A strain of the bacterium *Anaerobium acetethylicum* cultivated in 200 mM of glycerol showed a specific growth rate of 0.07 h$^{-1}$ during the exponential phase (PATIL et al., 2017).

This concentration represents 5 times less glycerol than 100 g . L$^{-1}$ which was added to media for cultivation of *Aspergillus sp.* T7.1, *Penicillium sp.* T2.1 and T6.2, corresponding to approximately 1.1 M. As reported by the same authors, growth of *A. acetethylicum* was completely inhibited at 1.5 M glycerol. The initial concentration of glycerol adjusted in the bioprocesses supplemented with glycerin in the present work, 100 g . L$^{-1}$, is considerably high if compared to the usual concentration of substrates in most media, which varies around 10 to 30 g . L$^{-1}$. The glycerol concentration in the submerged

bioprocesses conducted with strains T2.1, T6.2, and T7.1 is near to the high levels of carbon source that are needed for an efficient accumulation of product in citric acid fermentation, usually between 14 to 22% of sugar (MAX et al., 2010). Thus, the magnitude of growth and acidity produced by *Aspergillus sp.* T7.1 is taken as promising for further development of a bioconversion process for organic acid production, especially because media were not supplemented with pure glycerol, but with glycerin effluent of biodiesel production.

***Growth and acid production by* Penicillium sp. *T14.1 in planned bioprocesses:*** Another isolate showing high index of acidogenesis in solid media was cultivated in bioprocesses generated by factorial planning. The conditions generated for bioconversion of glycerin by *Penicillium sp.* T14.1 are shown in Table 2. As seen, the maximal acidity produced was 5.04 g . L$^{-1}$ after 9 days of cultivation in Bioprocess FC 22, which contained 60% glycerin, 5 g . L$^{-1}$ KH$_2$PO$_4$, no CaCl$_2$, 1 g . L$^{-1}$ Sucrose, pH 2.0. Similar production of total acid (4.62 g . L$^{-1}$) was verified after 3 days of cultivation in Bioprocess 30, supplemented with 60% glycerin, 5 g . L$^{-1}$ KH$_2$PO$_4$, 0.2 g . L$^{-1}$ CaCl2, 1 g . L$^{-1}$ Sucrose, pH 2.0. These are very low yields if compared to the high productivities reached with biotechnological strains cultivated under optimized conditions for citric acid production. However, strain T14.1 was able to secrete some acidity in a very high concentration of biodiesel-derived glycerin, what can be taken into account for further uses of this strain as a glycerol converter and for process optimization. The processes that yielded more accumulation of acid, FC 22, FC 30, and FC 29, have in common: pH 2.0, supplementation with KH$_2$PO$_4$ at 5.0 g . L$^{-1}$ and sucrose at 1 g . L$^{-1}$. On the opposite, there was no acid production in most of the processes in which pH was increased to 7.0, or 4.5, and in those with no supplementation of KH$_2$PO$_4$ or sucrose (Table 2). These data indicates that sucrose was beneficial for total acid accumulation. Results correlate with reports about the importance of sucrose for citric acid production. However, the usual concentration of this disaccharide for citric acid fermentation is between 14-20% (MAX et al., 2010).

As shown in Table 2, the 0.1% sucrose concentration in bioprocess FC 22 allowed production of 5.04 g . L$^{-1}$ of total acid. Assuming that the total amount of sucrose was converted to citric acid – an efficiency of 100% – the maximum amount to be produced would be 1 g . L$^{-1}$. Thus, if the acidity produced in bioprocess FC 22 be expressed as citric acid, most of the carbon source being converted must have been glycerol. In this sense, supplementation with sucrose may have stimulated acid production, but may not be taken alone as the single substrate being converted. Some of the glycerol present in the media may have been converted to acid too. Statistical effects of the independent variables on total acid production by isolate T14.1 and on its growth are shown by means of Pareto graphs (Figure 2). Sucrose presented the most significant effect for production of total acid (4.6) among the variables evaluated, but its interaction with pH was stronger and negative (-6.5), meaning that increases in both variables concomitantly decrease acid production by isolate T14.1. A similar result was observed for the interaction of glycerol and pH, which was also negative (-2.2). Glycerol concentration alone was not statistically significant for acid production by isolate T14.1, meaning that either 20% or 60% may induce acid formation, as seen for bioprocesses FC 22, FC 29, and FC 30 (Table 2). For biomass formation, glycerol and sucrose both presented negative effects of -2.2 and -3.4, respectively.

**Table 2. Factorial planning for bioprocess design, evaluation of growth, and total acid production by *Penicillium sp.* T14.1 cultivated in media supplemented with biodiesel-derived glycerin**

| Bioprocess | Glycerin (%) | pH | $KH_2PO_4$ (g.L$^{-1}$) | $CaCl_2$ (g.L$^{-1}$) | Sucrose (g.L$^{-1}$) | Time (days) | Total acid (g.L$^{-1}$) | Biomass (g.L$^{-1}$) | Bioprocess | Glycerin (%) | pH | $KH_2PO_4$ (g.L$^{-1}$) | $CaCl^2$ (g.L$^{-1}$) | Sucrose (g.L$^{-1}$) | Time (days) | Total acid (g.L$^{-1}$) | Biomass (g.L$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| FC1 | 20 | 2.0 | 0.0 | 0.0 | 0.0 | 3 | 0.14 | 0.086 | FC19 | 20 | 7.0 | 0.0 | 0.0 | 1.0 | 3 | 0.00 | 0.0396 |
| FC2 | 60 | 2.0 | 0.0 | 0.0 | 0.0 | 9 | 0.00 | 0.223 | FC20 | 60 | 7.0 | 0.0 | 0.0 | 1.0 | 9 | 0.14 | 0.0814 |
| FC3 | 20 | 7.0 | 0.0 | 0.0 | 0.0 | 9 | 0.98 | 1.1573 | FC21 | 20 | 2.0 | 5.0 | 0.0 | 1.0 | 3 | 2.10 | 0.0910 |
| FC4 | 60 | 7.0 | 0.0 | 0.0 | 0.0 | 3 | 0.14 | 0.1546 | FC22 | 60 | 2.0 | 5.0 | 0.0 | 1.0 | 9 | 5.04 | 0.1619 |
| FC5 | 20 | 2.0 | 5.0 | 0.0 | 0.0 | 9 | 0.00 | 0.0935 | FC23 | 20 | 7.0 | 5.0 | 0.0 | 1.0 | 9 | 1.40 | 0.3652 |
| FC6 | 60 | 2.0 | 5.0 | 0.0 | 0.0 | 3 | 0.00 | 0.1968 | FC24 | 60 | 7.0 | 5.0 | 0.0 | 1.0 | 3 | 0.70 | 0.0926 |
| FC7 | 20 | 7.0 | 5.0 | 0.0 | 0.0 | 3 | 1.12 | 1.4097 | FC25 | 20 | 2.0 | 0.0 | 0.2 | 1.0 | 3 | 1.12 | 0.0778 |
| FC8 | 60 | 7.0 | 5.0 | 0.0 | 0.0 | 9 | 1.40 | 1.1722 | FC26 | 60 | 2.0 | 0.0 | 0.2 | 1.0 | 9 | 2.66 | 0.2551 |
| FC9 | 20 | 2.0 | 0.0 | 0.2 | 0.0 | 9 | 0.00 | 0.0596 | FC27 | 20 | 7.0 | 0.0 | 0.2 | 1.0 | 9 | 0.00 | 0.4444 |
| FC10 | 60 | 2.0 | 0.0 | 0.2 | 0.0 | 3 | 0.00 | 0.2077 | FC28 | 60 | 7.0 | 0.0 | 0.2 | 1.0 | 9 | 0.00 | 0.1446 |
| FC11 | 20 | 7.0 | 0.0 | 0.2 | 0.0 | 3 | 0.70 | 1.1532 | FC29 | 20 | 2.0 | 5.0 | 0.2 | 1.0 | 9 | 3.22 | 0.1078 |
| FC12 | 60 | 7.0 | 0.0 | 0.2 | 0.0 | 9 | 0.42 | 0.2707 | FC30 | 60 | 2.0 | 5.0 | 0.2 | 1.0 | 3 | 4.62 | 0.2099 |
| FC13 | 20 | 2.0 | 5.0 | 0.2 | 0.0 | 3 | 0.00 | 0.0849 | FC31 | 20 | 7.0 | 5.0 | 0.2 | 1.0 | 3 | 0.98 | 0.4325 |
| FC14 | 60 | 2.0 | 5.0 | 0.2 | 0.0 | 9 | 0.00 | 0.1245 | FC32 | 60 | 7.0 | 5.0 | 0.2 | 1.0 | 9 | 0.42 | 0.3703 |
| FC15 | 20 | 7.0 | 5.0 | 0.2 | 0.0 | 9 | 1.96 | 3.6505 | FC33 (C) | 40 | 4.5 | 2.5 | 0.1 | 0.5 | 6 | 0.00 | 0.4267 |
| FC16 | 60 | 7.0 | 5.0 | 0.2 | 0.0 | 3 | 0.84 | 0.5496 | FC34 (C) | 40 | 4.5 | 2.5 | 0.1 | 0.5 | 6 | 0.00 | 0.2927 |
| FC17 | 20 | 2.0 | 0.0 | 0.0 | 1.0 | 9 | 1.96 | 0.0757 | FC35 (C) | 40 | 4.5 | 2.5 | 0.1 | 0.5 | 6 | 0.00 | 0.3318 |
| FC18 | 60 | 2.0 | 0.0 | 0.0 | 1.0 | 3 | 2.24 | 0.3012 | | | | | | | | | |

Bioprocesses FC1 to FC35 were performed in liquid media supplemented with 20% or 60% of a 2X diluted biodiesel-derived glycerin. Cultures were inoculated with an amount of mycelium pellets produced after 72 h of cultivation at 30 ºC (150 rpm) in media supplemented with glycerin to a 10% glycerol concentration. The amount of pellets contained in 50 mL was used to inoculate each planned process. Bioprocesses FC1 to FC35 were conducted at 30 ºC (150 rpm). Biomass was determined as dry weight. (C), central points

This was expected, once glycerin concentrations in media is very high and may be reaching prohibitive values for growth: 60% of glycerin yields 300 g . L$^{-1}$ glycerol (30%). The negative effects determined by statistical analysis of the factorial planning indicated that the lowest levels of these variables (0 for sucrose and 20% for glycerol) are better for fungal growth than their higher levels (Figure 2). Once the higher level of sucrose (1 g . L$^{-1}$) was added to a medium already containing glycerin (20% or 60%), its effect for growth would also be expected to be negative.

The variable pH presented a negative effect for acid production (-2.9). Several studies have reported that low pH values are better for citric acid production, being usually equal to 2.0, during the active phase of acid accumulation. Besides stimulating citric acid formation, low pH diminishes the risk of contamination by other microorganisms and inhibits formation of byproducts (PAPAGIANNI, 2007). Thus, the negative effect of pH on acid production by strain T14.1 is in accordance to the literature data on citric acid accumulation: increasing pH would decrease acid production. Ability of isolate T14.1 to tolerate high amounts of glycerin, and yet produce some acidity, indicates that further process of glycerin bioconversion may be adjusted with this strain. Increasing the pH level to 7.0 stimulates growth of *Penicillium sp.* T14.1 with an effect of 4.2, what is in agreement with the values usually utilized for cultivation of the genus. The interactions pH x sucrose, pH x $K_2HPO_4$, and pH x glycerol were negative for growth of the isolate T14.1 with effects of -3.6, -2.2,

and -3.1, respectively. At the values applied herein, $CaCl_2$ and $K_2HPO_4$ did not have significant effects for biomass formation (Figure 2). Additional supplementation of media with phosphate ($KH_2PO_4$) had a positive effect for acid accumulation (3.2). Thus, for acid formation by strain T14.1 the higher level of this compound (5.0 g . L$^{-1}$) is better. The best growth of isolate T14.1 occurred in Bioprocess FC 15, which was supplemented with 20% glycerin, no sucrose, 5 g . L$^{-1}$ $K_2HPO_4$, 0.2 g . L$^{-1}$ $CaCl_2$, pH 7.0 (Table 2). Total acid produced in this condition was almost 2 g . L$^{-1}$. Yet, biomass formation was 3.6 g . L$^{-1}$, which is almost 50% of the biomass produced by *Aspergillus sp.* T7.1 and *Penicillium sp.* T6.2 (Figure 1). Conditions of cultivation influenced oppositely growth and acid production by isolate T14.1, especially concerning pH and sucrose concentration. While low pH values are better for acid accumulation by isolate T14.1, higher values are better for its growth (Figure 2).

This poses a challenge for further adjustments of culture conditions, because a reasonable amount of biomass formation is important for bioconversion and product accumulation. Nonetheless, results herein showed the selective conditions and the screening procedures were efficient to obtain a number of isolates with potential to grow on and convert residual glycerin, and encourage further studies of glycerol bioconversion and of the metabolic routes utilized by strains isolated in this work.
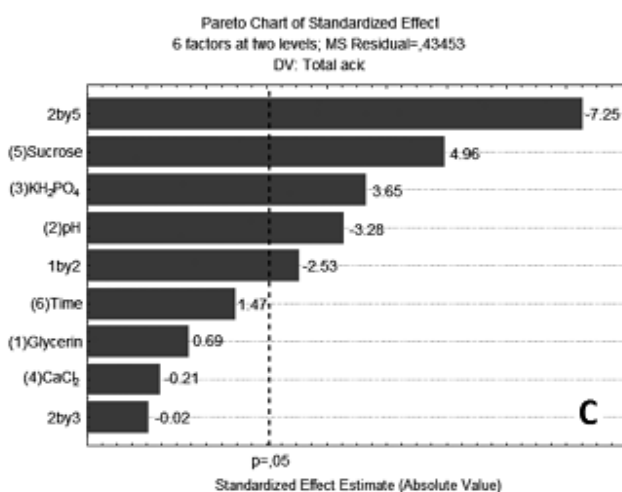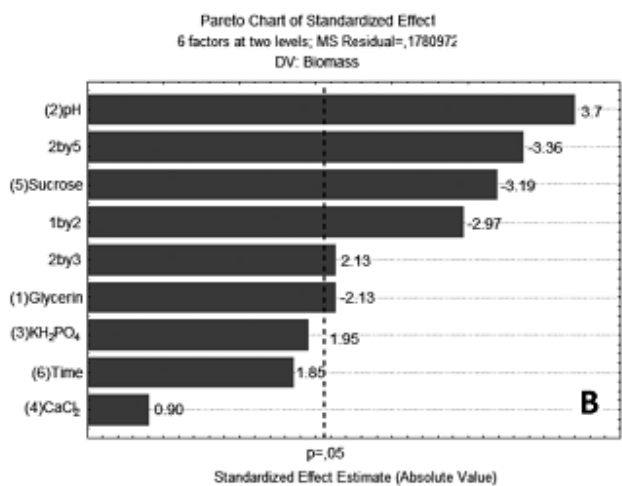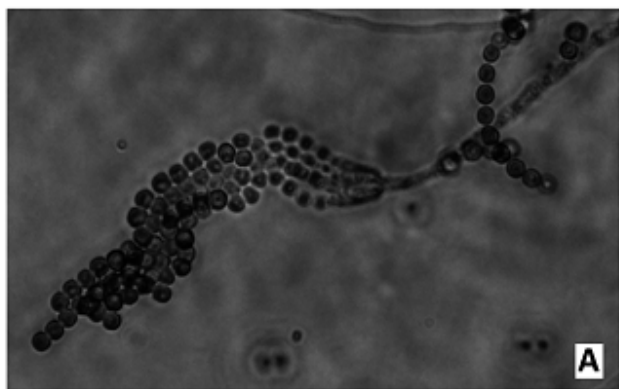
**Figure 2. Variables influencing growth and acid secretion by *Penicillium sp.* T14.1. A: conidiophore of isolate T14.1. B, C: Pareto chart showing effects and interaction of variables influencing biomass (B) and total acidity produced (C) by isolate T14.1 in submerged processes supplemented with 1.1 to 3 M biodiesel-derived glycerol**

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