Lipase and Surfactant Production by Fungi Isolated of Oily Residues of Environmental Sanitation in Liquid and Solid Phase Reactors

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Abstract

The use of lipolytic activity of fungal biomass as biocatalyst represent an attractive approach for oily waste treatment and or production of biodiesel from oils and greases wastes. Fifteen isolates of filamentous fungi and five of yeasts from oily residues were obtained and our lipolytic activity and biomass production were quantified with 120 h of growth in a minimal mineral media with 10 % of soybean oil. Lipolytic activity varied from 0.13 U.mg^{-1} protein for Rhizomucor sp. F18 to 18.06U.mg^{-1}protein for Penicilliumsp F2 and the biomass varied from 7.61 mg.mL^{-1} for Cladosporium sp. F28 to 12.68 mg.mL^{-1} for Rhizomucor sp. F18. Penicilliumsp F2 and Rhizomucor sp. F18were then evaluated in liquid phase reactor and solid phase reactor for lipolytic activity, surfactant activity, biomass production and removal of oils and greases of substrate, and qualified to future tests as whole-cell biocatalysts for hydrolysis and transesterification processes.

Keywords: Lipase-producing fungi, oily residues, growth substrate

1. Introduction

Lipases (triacylglycerol acyl hydrolase, E.C. 3.1.1.3) are enzymes that work on the aqueous- organic interface catalyzing hydrolytic reactions of triglycerides and performing esterification, transesterification or interesterification reactions in low water environment. The versatility of its properties, such as simplified mass production, high temperature stability and wide ranges of pH, high specificity and selectivity, makes them of great interest for industrial applications, with wide range of possible uses. (Reetz 2002; Carvalhoet al. 2005).

The commercial lipases produced from micro-organisms such as bacteria, yeasts and filamentous fungi. The latter are preferred producers of these enzymes, since it mostly present an extracellular activity, facilitating the extraction of proteins in supernatant media and fatty substrate as inductors in many species, such as *Geotrichumcandidum, Aspergillusniger, Aspergillusoryzae, Rhizopusdelemear* and *Penicilliumcyclopium*, among others, that present lipase production constitutively (Colen et al. 2006). However, use of lipases for hydrolysis of oily effluents (Mendeset al. 2005) and or transesterification of biodiesel still not competitive with other processes in use, mainly due to the cost of production of these enzymes, although advantageous under the environmental point of view (Bajajet al. 2010; Singh & Singh 2010).

Bacteria, yeasts and filamentous fungi that degrade water-insoluble substrates such as fat, oil and grease, among others, usually generate surfactant, which act as detergents and support the assimilation of oily compounds by production visible emulsions. Thus, the lipolytic activity is link with surfactant production, depending on both microbial strain and reaction factors involved.

Biosurfactants are amphipathic molecules, with low to medium molecular weight, surface-active, consisting of glycolipids, lypopeptides, phospholipids, polymeric compounds or fatty compounds acting extracellular or intracellularly. These compounds decrease the interfacial surface tension between liquid and nonpolar polymers, which are most effective in stabilizing oil-water emulsions (Desai & Banat1997; Martins et al. 2008). Concerning the main advantages of surfactants can be infer the low toxicity, biodegradability, reduction the surface tension, alkaline water solubility, thermal stability, resistance to high salt concentrations and stability at wide pH range. Although its broad use, including bioremediation of soil and oily wastewater treatment, its production cost is the main limiting factor (Banatet al. 2000; Nitschke & Pastore 2002; Castiglioni et al. 2009).

The production of biomolecules in solid phase reaction systems is a low-cost alternative, possibility to use environmental oily residues and agro industrials, as substrates for the production of lipases and or surfactant by these microorganisms. The low cost and abundance of residual materials contribute to lowering the cost of production (Damasoet al. 2008; Castiglioni et al. 2009). Additionally, solid phase reactors (SPR), characterized by microbial growth within solid particulate substrate, have proved to be an efficient way for the production of enzymes, especially by filamentous fungi, since provides the environmental conditions to micro-organisms similar to their natural habitat. However, due to the low moisture content of the solid phase reactor, the system tends to create heterogeneous environments and thus contributing to high variability observed in both experiments at full-scale process (Fernandes et al. 2007).

This work aimed isolate and evaluate the profile of lipase-producing fungi from oily residues of environmental sanitation, in liquid phase reactor (LPR) and solid phase reactor (SPR), targeting its towards application in hydrolysis in environmental sanitation and esterification and or transesterification in biodiesel production of residual oils and greases.

2. Materials and Methods

2.1. Isolationand Identification of Lipase-Producing Fungi

Isolation of lipase-producing filamentous fungi and yeasts was performed sampling 10 mL aliquots of each oily residues (sewage scum, grease-traps, septic tanks, sewage sludge and waste frying oil) added to 50 mL mineral minimal medium (MM) composed by($g.L^{-1}$): $(NH_4)_2 SO_4$, 5,0 g; KH_2PO_4, 0,9 g; NaCl,1,0 g; $MgSO_4$. $7H_2O$, 0,3; Na_2HPO_4, 6,2 g; soybean oil 10% and 1 mL micronutrients solution ($FeCl_3$. $6H_2O$, 2000 mg; $ZnCl_2$, 50 mg; $CuCl_2$. $2H_2O$, 30 mg; $MnCl_2$. $2H_2O$, 500 mg; $(NH_4)_6.Mo_7O_{24}$.$4H_2O$, 50 mg; $AlCl_3$, 50 mg; $CoCl_3$. $6H_2O$, 2000 mg). The inoculated medium incubated at 30°C during 96 h under shaking 120 rpm, for enrichment. After observed growth, the fungi were isolated in solid media composed by MM with 10 % of soybean oil, 0.1% Tween 80, 50 mg of streptomycin sulfate, 50 mg of chloramphenicol and 17 % of agar by water 1000 mL. Afterincubation at 30°C for 5days, isolated fungi were stored in tubes with potato dextrose agar (BDA)and maintained at 5^oC (Menezes &Assis 2004).

For identification of filamentous fungi isolated, were prepared microcultures in microscopic blades (Menezes & Assis 2004) and evaluated macroscopic and microscopic characteristics presented by fungi after 96 h of growth at 28° C in BDA culture media, were compared with the literature descriptions (Barnet & Hunter 1998). Was not carried the identification of yeast isolates.

2.2. Lipolytic Activity and Fungi Biomass Production in Liquid Medium

Aimed evaluate the 20 fungi isolates obtained in the previous step for lipolytic activity (LA) and fungal biomass production (FB), was utilized a liquid media composed by MM with 10 % of soybean oil and 0.1% Tween 80 (MMOS), using a 125 mL flask and 50 mL of liquid media. Each flask was inoculate with 1 mL suspension of filamentous fungi or yeast containing approximately 10^7spores or cells.mL^1. The flasks were incubate at 30 °C during 120 h under shaking of 150 rpm.

For evaluate the lipolytic activity, the contents of each flask was vacuum filtered and three aliquots of 1mL of the filtrate were transferred to sterile Eppendorf tubes and centrifuged at 15000 gand 4 °C by 20 min. The supernatant of each tube was collected and essayed for protein determination (Bradford 1976*)* and LA using p-NPP (p-nitrophenylpalmitate) with reaction to 37 °C for 15 min. evaluated by spectrophotometry (λ = 410 nm) and the lipolytic activity unit (U) defined as the amount of enzyme to hydrolyze1 µmol of ρ-nitrophenolmin^{-1}(Winkler & Stuckmann 1979).

For quantification by gravimetry the FB was separated from the supernatant by filtration of cultures with filter paper, Whatman No 1, followed by cleaning with acetone and successive washes with water qualitymilli-Q. Transferred to filter papers previously dried at 80° C, proceeded to drying to this temperature until constant weight and then weighing. The quantification of dry fungal biomass was by the difference between the measurements of the roles, with and without the presence of biomasses.

2.3. Liquid Phase Reactor (LPR) and Solid Phase Reactor (SPR) Assays

The *Penicillium* sp. F2 and *Rhizomucor* sp. F18selected based on their LA and FB in the previous step were evaluate in the sequence using liquid (LPR) and solid phase reactor (SPR) flasks systems.

InLPR 2L flask with 400 mL of MMOS was inoculate with each of these filamentous fungi in the concentration of10^7 spores.$mL^{-1}$of sterilized water and incubated under shaking of 150 rpm and 30°C for 120 h. Every 24 h up to 120 h, three 20 mL aliquots were taken from each flask and transferred to test tubes of 20 mL for pH measurements and biomass/supernatant separation by centrifugation (3000 g 15 min. 20°C) for LA, surfactant activity (SA) in supernatant, and FB quantification.

The LA of the fungi were measured in 24 h intervals, up of 120 h of incubation as previously described from the supernatant of three samples per treatment (Winkler &Stuckmann1979;Rowe & Howard 2002).

The SA determination was through the oil in water (O/W) emulsifying activities (Martins et al. 2008; Castiglione et al. 2009), using 3.5 mL of extract and 2 mL of soybean oil. The mixture was agitated in a vortex agitator at 700 rpmfor 1 min. After 60 min, the absorbance of the emulsified O/W mixture was read through a spectrophotometer at 610 nm. The O/W emulsifying activity was obtained by Eq. (1).

$$AE = [Abs.D]/[m\,(1-U)] \quad (1)$$

where: Abs = absorbance of the suspension of oil in water; D = dilution of the sample in water; m = mass of sample damp (g) and U = sample humidity from SPR. Two blanks were accomplished, the first using water instead of the sample and the second using not fermented culture media. The results were expressed in units emulsifiers defined as the mass needed of biosurfactantto increase the absorbance (Abs) in one (1) unit, when compared with the white.

In SPR 250 mL flasks containing a substrate composed of a mixture of sand and vermiculite 1:1(v/v), previously washed and autoclaved then filled with minimal medium (MM) up to 50% field capacity and 10% of soybean oil. The system inoculated with a suspension of fungal spores and incubated under static and dark conditions, at 30 °C for 120 h. Every 24 h, up 120 h, samples of 1 g taken from each reactor flask for pH, moisture, oil and grease removal (rO&G) of substrate and FB determination by ergosterol content. To quantify the LA and SA, two separate reactors employed by adding 120 mL sterile water at 25°C to the first flask and 120 mL sterile distilled water at 90°C to the second flask and maintained under continuous shaking 160 rpm 30 minutes at range temperature of 25 °C and 50 °C respectively. After, each flask content was vacuum filtered and LA and SA analyzed. The LA and SA determinations were as described in the previous step.

The quantification of oil and grease performed in SPR was conform the *Standard Methods for the Examination of Water and Wastewater*(APHA 1995), using the Soxhlet extraction technique, and the results expressed for weight of the substrate, sampled every 24 h up to 120 h and then transformed into removal percentage taking the initial time (To) as reference sampling.

In SPR the fungal biomass production was quantified byergosterol content present in the samples, via liquid chromatography (HPLC) using a C18 column, methanol as mobile phase and detection by PDA at 280 nm. The ergosterol peak was eluated and identified using ergosterol standard(Sigma) with 8 min elution time (Schnurer 1993, modified).

3. Results and Discussion

There are two basic types of microbial lipase, as to their form of production: the extracellular lipase, which secreted into the liquid medium and the intracellular lipase, also called lipase whole cell, which remains inside the cell or linked to its membrane or cell wall, which is less expensive to use in the first full-scale (Talukder et al. 2013).

Isolation and selection of good fungi lipases producers for future use in the hydrolysis of oily wastewater and domestic and industrial, in the synthesis of fatty acids esters from waste oils and fats (OGR), in the light of the high costs of these enzymes in pure market, mobilized to carry out this study. Filamentous fungi are preferred sources of lipase for commercial use because are easily extracted from fermentation processes and because are considered safe and easy handling, in addition to the growing potential of the same as the use for the said purposes, in the form of whole cell biocatalysts (Alberton et al. 2010; Andrade et al. 2014).

The various microbial colonies obtained from oily residues of environmental sanitation, enriched with the MM and 10% soybean oil, were obtained 20 pure cultures: 15 of filamentous fungi and 5 of the yeasts, after the successive passages through MMOS plus bacterial antibiotics. Our taxonomic identification was through of macroscopic structures presented by colonies in BDA in Petri dishes and microscopic structures observed in microscopic (Menezes &Assis 2004) and comparison with the specific literature descriptions (Barnett & Hunter 1998). The 20 isolates kept in BDA to 4° C, multiplied in the same and used to evaluate the lipolytic activity (LA) and fungal biomass production (BF), which occurred after 120 h in liquid phase reactor in MMOS at 30° C and 150 shake of 150 rpm (Table 1).

The LAof each fungi isolates evaluated on supernatant of liquid after growth ranged from 0.13 ± 0.03 $U.mg^{-1}$protein of *Rhizomucor* sp. F18 to 18.06 ± 0.36 $U.mg^{-1}$protein of *Penicillium* sp. F2. The biomass dry weight ranged from 7.82 ± 0.13$mg.mL^{-1}$of culture substrate for *Cladosporium* sp. F28 to 12.68 ± 0.15 $mg.mL^{-1}$ culture substrate of *Rhizomucor* sp. F18.Overall, statistically significant differences for LA and FB observed between the values obtained for various strains belonging to different taxonomic genera and between strains of the same genera (Table 1).Using similar methodology to quantify, however with greater time of incubation, Baron et al. (2005) observe LA of 11.82 ± 1.35 U. mg^{-1} protein for *P.coryophilum* IOC 4211, after 144 h of incubation at 29° C and 120 rpm. Using distinct methodology of quantification, with AL expressed volumetrically from cultivation in liquid medium composed of 1.0% of olive oil, 2.0%, 0.1% peptone yeast extract and 0.5% NaCl, Carvalho et al. (2005) obtained for *P.restrictum* AL of 13.0 $U.mL^{-1}$ with FB of 14.2 $mg.mL^{-1}$and for *P. solitum*LA of 10.5 $U.mL^{-1}$ and FB of 6.56 $mg.mL^{-1}$. Experimental results of the lipolytic activity and the growth of microbial biomass, in addition to the different constitutive potential for the production of lipase and growth between and within the same species are influenced by factors not constituting as the composition of the culture media, temperature, pH, agitation and forms of quantification as reviewed by Turky (2013).

Species *of Penicillium*: *P. citrinum, P. cyclopium, P. simplicissimum, P. caseicolum, P. restrictum, P. expansum, P. corylophilum, P. chrysogenum, P. roqueforti, P. camembertii, P. abeanum,* among others, are widely known forextracellularlipase production, measured in supernatant (Freire et al. 1997; Jesus et al. 1999; Reetz 2002; Baron et al. 2005;Carvalho et al. 2005;Azeredo et al., 2007).

The genus *Rhizomucor*, represented mainly by the specie R. *mihei*, stands out as a great producer of lipase, being the extracellular lipase produced by this species one of the most marketed today and the first whose structure was reported, having its activation interface very well elucidated and basis for enzyme modeling studies. Recently extensive review about the main uses of LRM (*Rhizomucormihei* lipase), and features some of the most relevant aspects of the same in the processing of oils and fats as hydrolysis of glycerides, transesterification, esterification, acidólise and interesterificação (Rodriguez& Fernando-Lafuente 2010).

Table 2 shows the mean results from liquid (LPR) and solid phase growth reactors (SPR) for fungi isolates F2 and F18 with 48 h growth and Figure 1 shows the observed values for solid phase reactor during 120 h growth for lipolytic activity, biomass and oils and greases consumption of the same fungi isolates.

From Table 2 and Figure 1 and 2 it can be seen that at 48 h the *Penicillium* sp. F2 has a lipolytic activity (LA) with values around 28 and 34 $U.mg^{-1}$protein on liquid and solid phase reactors respectively, corresponding to exo-lipolytic activity(measured in the supernatant) in contrast with *Rhizomucor*sp. F18 presenting a low lipolytic activity. In fact, the isolate F18 with lower exo-lipolytic activity showed some evidence of efficient lipolytic activity as seen by the biomass and oils and greases removal principally on solid phase reactor (SPR) with mean values of these variables as higher than isolate *Penicillium* sp F2 (Figure 4). This fact evidences the presence of endo-lipolytic system in *Rhizomucor* sp. F18 isolate maybe bound to cell wall or membranes and highly capable of transform the lipids present in the medium and converting them into biomass, as main carbon source.

Possibly this evidence is correlated with the results from some authors that did not find a straight correlation between biomass production and lipase activity (Carvalho et al. 2005), specially of *Penicillium*sp. F2 growing in liquid media.

The same trend observed in this work with results presented in Table 1, which did not match a relation of biomass and lipase activity maybe due to presence or interference of lipase production as exo-activity or endo-activity.
The biosurfactant production was higher in the *Rhizomucor* sp. F18 as compared with *Penicillium* sp. F2, and growing significantly more on liquid and solid phase reactors (Table2, Figure1 and 2). This may be due to endo-activity or cell bound enzyme of*Rhizomucor*F18 isolate in contrast with the activity found in *Penicillium* sp. F2. These results emphasizes that the presence of lipids in the medium and the surfactant activity presents a strong factor to increment the lipolytic activity as demonstrated for *Aspergillusfumigatus* and *Phialemonium*sp. (Martins et al. 2008).

The rOS of cultivation substrate, quantified through wide use methodology in the area of environmental sanitation for the determination of the content of oils and greases in wastewater, solid waste and sludge, consisted in this work methodological tool to estimate, indirectly, the hydrolytic capacity of two strains evaluated in SPR (Figure 4). Because the soybean oil was the onlyoily component of the substrate used and exclusive source of carbon for fungal growth inferred through its removal (consumption) and potential lipolytic activity profile, implicated in its transformation to biomass production by growing of fungi isolates. Oils and greases removal of79.30± 0.43% by *Penicillium* sp. F2 and of71.50 ± 0.32% by *Rhizomucor* sp.F18 allows similar interpretation to that which we gave to the results obtained in LPR which assigned the production of intracellular lipase, indirectly estimated, the significant responsibility for the lipolytic profile presented by *Rhizomucor* sp. F18 (Table 2 and Figure 4).

Conclusion

Fungi have a greater potential for production of extracellular lipases generally used as purified enzymes. Due to the instability of extracellular lipases and costly purification procedures, their application is limited by environmental and economic factors. Direct use of cells or biomass with endo-activity of lipases within a porous biomass support could represent a very attractive process for lipase production and applied to several processes as in case of bulk biodiesel production. The great advantage of such approach has correlated with the fact that it does not require enzyme purification step and the whole cell and solid substrates could act as immobilizing agent for enzyme support. Therefore, the fungi *Penicillium* sp. F2 and *Rhizomucor*sp. F18 showed potential for to be evaluate in different residual substrates on the optimization of parameters for extra and intracellular lipase production, aiming at its future use in processes of hydrolysis and transesterification of residual oils and greases of environmental sanitation.

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Table 1. Fungal biomass production (FB) and lipolytic activity (LA) of 15 filamentous fungi and 5 yeasts isolates on mineral minimal media and 10 % soybean oil (MMOS) in liquid phase reactor (LPR), with 120 h incubation at 30°C and 150 rpm agitation

Isolate	Taxonomic classification	LA* ($U.mg^{-1}$)		FB ($mg.mL^{-1}$ MMOS)	
F02	*Penicillium*sp.	18,06 ± 0,36	a	10,19 ± 0,13	cdef
F96	*Geotrichumcandidum*	14,25 ± 0,31	b	9,23 ± 0,18	h
F41	*Beauveriabassiana*	9,13 ± 0,23	c	9,62 ± 0,14	g
F28	*Cladosporium* sp.	6,10 ± 0,22	e	7,61 ± 0,15	j
F98	*Cladosporium* sp.	4,85 ± 0,16	f	7,82 ± 0,13	j
F64	*Colletotrichumgloeoesporioides*	3,79 ± 0,07	g	9,71± 0,16	g
F80	Yeast	2,74 ± 0,17	h	10,12 ± 0,16	ef
F102	*Penicillium* sp.	2,56 ± 0,05	h	8,77 ± 0,12	i
F72	*Colletotrichum* sp.	2,49 ± 0,25	h	9,05 ± 0,18	hi
F76	*Cladosporium* sp.	2,36 ± 0,39	h	9,02 ± 0,13	hi
F111	Yeast	1,53 ± 0,25	i	10,45 ± 0,11	cde
F104	*Botrytis* sp.	1,44 ± 0,11	i	11,89 ± 0,10	b
F107	Yeast	0,51± 0,10	j	10,25 ± 0, 80	cde
F35	*Beauveriabassiana*	0,47± 0,01	j	10,74 ± 0,19	c
F74	*Fusarium* sp.	0,39 ± 0,04	j	10,27 ± 0,12	cde
F108	Yeast	0,28 ± 0,08	j	9,07 ± 0,15	hi
F106	*Rhizomucor* sp.	0,25 ± 0,01	j	9,91 ± 0,12	fg
F07	*Verticillium* sp.	0,18± 0,01	j	10,50 ± 0,15	cd
F81	*Aspergillus* sp.	0,18 ± 0,05	j	9,87 ± 0,21	fg
F18	*Rhizomucor* sp.	0,13 ± 0,03	j	12,68 ± 0,15	a

* =Means (n = 4) followed by same letters, do not differ statistically significant by Tukey test (p = 0.05)

Table 2: Mean values (n=3) observed for lipolytic activity (LA), surfactant activity (SA) and fungal biomass production (FB) in 48 h growth, oils and greases removal (rOG) and hydrogen potential (pH)of growth substrate in 120 h growth, of *Penicillium* sp. F2 and *Rhizomucor* sp.F18 in liquid phase reactor (LPR) and solid phase reactor (SPR)

	LPR		SPR	
Parameters	**F2**	**F18**	**F2**	**F18**
LA ($U.mg^{-1}$)	28,09 ± 0,25	0,25 ± 0,02	34,11 ± 0,62	5,81± 0,25
SA ($EU.g^{-1}$)	4,23± 0,27	5,00 ± 0,21	8,58 ± 0,22	11,97 ± 0,95
FB ($mg.mL^{-1}$)	0,92 ± 0,14	2,12 ± 0,32	4,76 ± 0,26	5,87 ± 0,39
rOG (%)	NA	NA	79,30 ± 0,43	71,50 ± 0,32
pH	7,5 ± 0,08	7,0 ± 0,09	4,9 ± 0,05	4,4 ± 0,06

NA = not applicable.

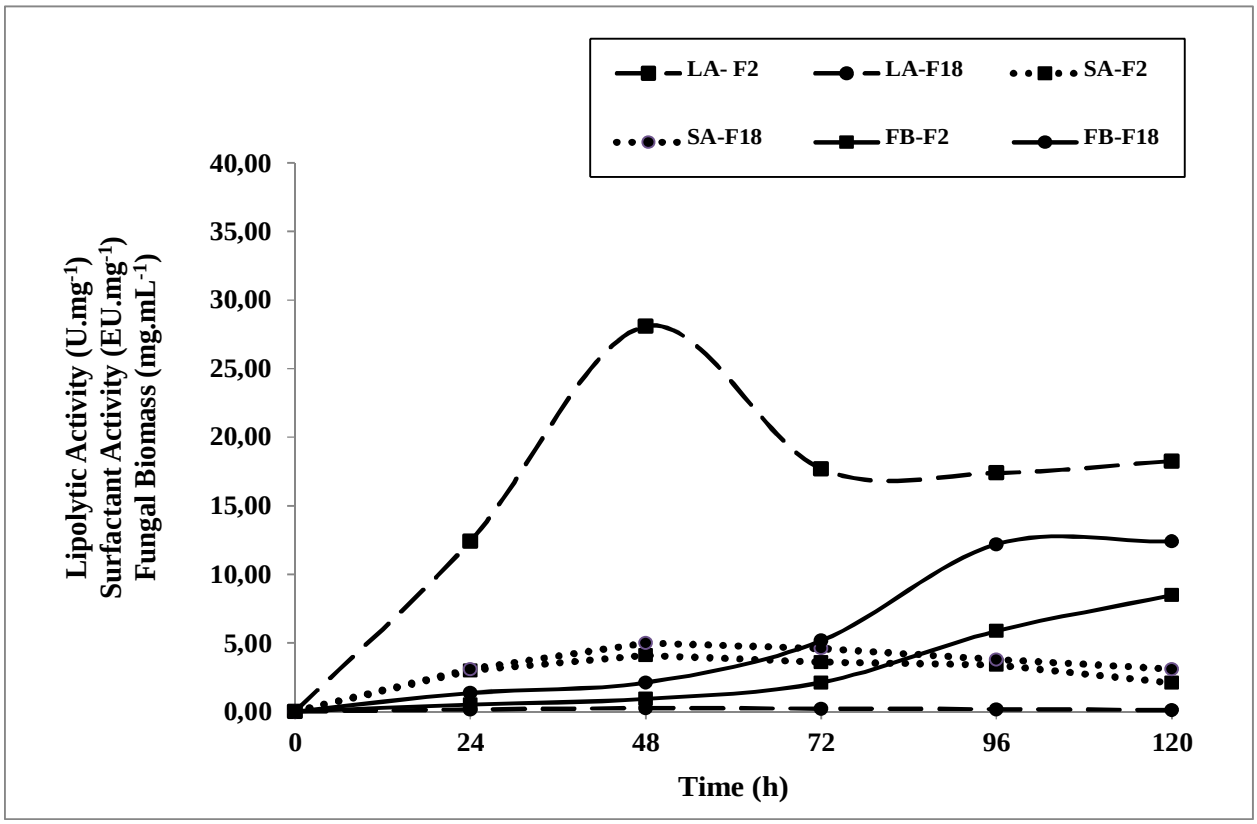

Figure 1. Lipolytic activity (LA), surfactant activity (SA) and fungal biomass production (FB) of *Penicillium* sp. F2 and *Rhizomucor* sp. F18 in liquid phase reactor (LPR) during incubation of 120 h at 30 °C and agitation of 150 rpm

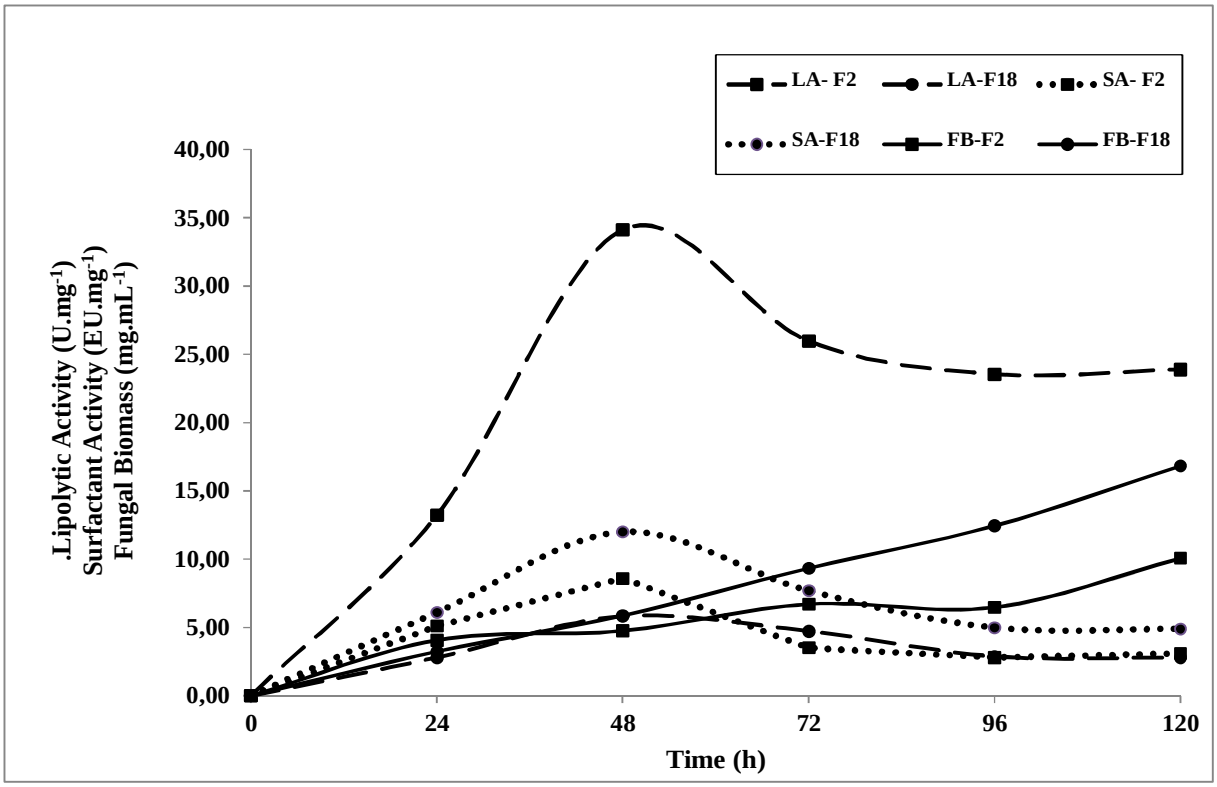

Figure 2: Lipolytic activity (LA), surfactant activity (SA) and fungal biomass production (FB) of *Penicillium* sp. F2 and *Rhizomucor* sp. F18 in solid phase reactor (SPR) during incubation of 120 h at 30 °C.

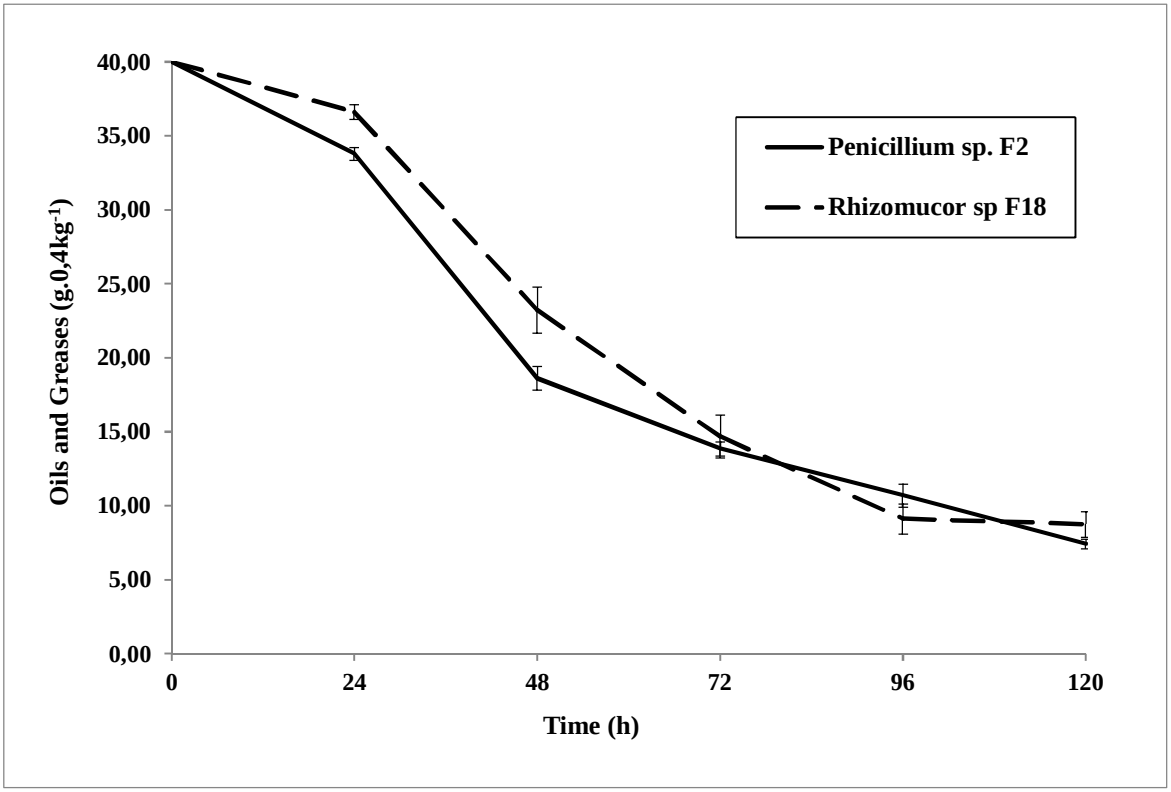

Figure 3: Removal of oil and greases (rOG) by *Penicillium*sp F2 and *Rhizomucor* sp. F18 on solid phase reactor (SPR) during 120 h of incubation at 30 °C.