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Full Length Research Paper

Production of glycolipids with antimicrobial activity by *Ustilago maydis* FBD12 in submerged culture

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***Ustilago maydis* FBD12 is a phytopathogenic fungus that grows in submerged environments and produces different metabolites, such as glycolipids (biosurfanctants), cyclic peptides, tryptophan derivatives, and enzymes. Biosurfactants are molecules with a high surface activity, as well as emulsifying properties. They are secondary metabolites whose main physiological role is to support the growth of microorganisms in water-immiscible substrates, through the reduction of the superficial tension of the interface. In the present study, the production of glycolipids by *U. maydis* FBD12 was investigated during its growth in fish and soy oils. The highest growth was observed at the 7th day of incubation with 16.8 and 7.25 g/L of biomass in fish and soy oils, respectively. An amount of 18.3 mg/100mL of glycolipid extract was obtained for fish oil, with an antioxidant capacity of 350 µMol ET/mL and a reduction in the minimum inhibitory concentration (MIC) towards *S. aureus* and *S. Typhimurium* of 25%. MIC decreased in 75% for *Staphylococcus aureus* when lipase was added to the fish oil culture medium. For soy oil, 9.6 mg/100mL of glycolipids were obtained with an antioxidant capacity of 232.5 µMol ET/mL and a MIC reduction of 25% in soy oil, which decreased 50% with the addition of lipase.**

Key words: Antimicrobials, biosurfactants, lipase, reductors sugars, secondary metabolites, *Ustilago*.

INTRODUCTION

Biosurfactants are amphiphatic compounds produced by organisms such as plants and animals, but mainly by microorganisms. They play an important role in the microorganism, therefore facilitating the mass transfer in the surface of the same (Desai and Banat, 1997; emulsification of insoluble substrates in water by extending the interface area between substrate and Kitamoto et al., 2002). They also intervene in microbial mobility, reducing the interfacial tension and facilitating movement in the interface when the microorganism is looking for new environments for growth, reproduction and colonization (Kearns and Losick, 2003). Biosurfactants are classified into 5 categories, based on their chemical structure: 1) Glycolipid, 2) Fatty acid/ phospholipid, 3) Lipopeptide/lipoprotein, 4) Polymeric, 5) Surfactant particles. Among these 5 groups, the glycolipid is the most widely studied since it has higher production yields as compared to other biosurfactants, as well as a higher potential in functions and applications (Desai and Banat, 1997).

Glycolipids have some additional advantages over chemical surfactants, such as their low toxicity, high biodegradability, emulsifying capacity, reduction of the superficial and interfacial tension, as well as their selective capacity and specificity at high temperatures, pH and extreme salinity (Desai and Banat, 1997; Colla et al., 2010). An additional advantage showed by some biosurfactants is antimicrobial activity, such as the one observed for mannosyl erythritol (Mel A and Mel B),

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Abbreviations: MIC, Minimum inhibitory concentration; **MCFCH,** culture media with hydrophobic carbon sources; **DNS,** dinitrosalicylic acid method; **TEAC,** antioxidant capacity in equivalents of trolox; **CCGL,** capillary gas-liquid chromatography; **AST,** soy trypticasein agar; **soy-L,** soy oil without addition lipase; **fish+L,** fish oil with added lipase; **soy+L,** soy oil with added lipase; **fish-L,** fish oil without addition lipase; **MEL,** mannosyl erythritol; **UA,** ustilagic acid; **TLC,** thin layer chromatography; **CCGL,** capillary gas-liquid chromatography; **FID,** flame ionization detector; **ABTS,** 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid); **AST,** soytrypticasein agar.

produced by *Candida antarctica,* which is specific against gram positive bacteria (Kitamoto et al.,1993), viscocinamide or viscoside that has antifungal properties and is produced by *Pseudomonas fluorescens* (Nielson et al.,1999), and rhamnolipids of *P. aeruginosa* AT10 with antimicrobial activity against *Escherichia coli, Alcaligenes faecalis* and *Staphylococcus epidermidis (*Abalos et al., 2001). Given the above, these substances may be an interesting alternative for different food, cosmetic and pharmaceutical industries (Berdnasky et al., 2004; Singh and Cameotra, 2004; Nitschke and Costa, 2007; Pornsunthorntawee et al., 2008).

The production of biosurfactants by microoorganisms may be affected by culture factors such as the nature of the carbon source. The production of glycolipids by *Torulopsis bombicola* is stimulated by the addition of vegetable oils during growth in a medium containing 10% glucose, which yielded 80 g/L (Asmer et al., 1988; Cooper et al., 1988). Stuwer et al. (1987) obtained yields of 90 g/L by using a medium with a mix of D-glucose and sunflower oil, showing evidence that the yield and type of surfactant is related to the carbon source and particularly to the type of carbohydrate. Morita et al. (2007) reported that the lipids in mannosyl erythritol (MEL) and the type of structural fatty acids of glycolipids produced by *Pseudozyma antarctica* depend on the type of carbon source (glucose or soy oil) used in the medium. Other factors, such as nitrogen sources, also affect the production of biosurfactants. Robert et al. (1989) pointed out that nitrate is the best source of nitrogen for the production of biosurfactants by *Pseudomonas* 44T1 and *Rhodococcus* ST-5 growing on paraffin and olive oil, respectively. Environmental and growth factors such as pH, temperature, agitation and concentration of salts are all external variables that affect the production of biosurfactants (Desai and Banat, 1997).

U. maydis is a dimorphic fungus, a basidiomycetous that belongs to the *Ustilaginomycetes* family. It is an infectious parasite of both maize (*Zea mays)* and its parent Teozintle (*Zea mexicana* subesp. *parviglumis* and subesp. *mexicana*), producing the disease known as “maize charcoal or huitlacoche”. This is characterized by an initial induction of chlorosis or pigmentation by anthocyanins, as well as for the formation of tumors in leaves, spikes, stems and cobs. It is widely spread and causes economic losses in many countries, nevertheless in Mexico it is considered a culinary delicacy since pre-Columbian times and it is becoming popular as an exotic, high-cuisine dish in other countries (Ruiz-Herrera and Martínez, 1998). *U. maydis* is additionally a producer of several secondary metabolites such as glycolipids or biosurfactants, compounds derived from tryptophan, cyclic peptides (Bolker et al., 2008), and proteases (Mercado Flores et al., 2004), among others.

U. maydis produces two types of extra-cellular biosurfactants, one is the cellobiose lipids or ustilagic acid (UA), where the disaccharide is joined to a ω-hydroxyl of 2,15,16-trihydroxy- or 15,16- dihydroxy hexadecanoic acid group through a glycosidic bond, while the cellobiose molecule is joined by esterification to the β-hydroxyhexanoic o β-hydroxyoctanoic acids. The second group is the mannosyl erythritol or ustilipids (MEL) 4-O-β-D-mannopyranosyl-D-erythritol, which is esterified to short chain (C_2 to C_8) and medium length (C_{10} to C_{18}) fatty acids, besides of acetyl groups. These compounds originate under limiting nitrogen conditions and hydrophobic carbon sources, where the production and structural nature of glycolipids depend on the nutritional conditions of the culture for the growth of the fungus (Spoeckner et al., 1999; Hewald et al., 2005; Bolker et al., 2008). The use of different carbon sources in the culture of *U. maydis* affects the quantity and type of the two main glycolipids produced. The culture of strains on hydrophobic substances containing unsaturated fatty acids produces 90% of mannosyl erythritol and 10% of cellobiose lipids (9/1). However, this proportion may be switched to 1/1 by using coconut oil or 1/9 by using glucose without the addition of substrate in the stationary phase. The addition of a carbon source during the same phase increase the yield of glycolipids from 2 to 30 g/L in limiting nitrogen conditions (Spoeckner et al.,1999). Given the above, the aim of the present work was to study the production of glycolipids by *U. maydis* FBD12 using different hydrophobic carbon sources, as well as to evaluate their antimicrobial activity.

MATERIALS AND METHODS

Hydrophobic substrates

Commercial soy and fish oils.

Standards of sugars and fatty acid methyl esters

Rhamnose and fatty acid methyl esters of sigma-aldrich were used in the present study as the reference in the TLC and CCGL runs.

Biological material

The diploid strain of *U. maydis* FBD12 was preserved in 50% glycerol (v/v) at -70°C. The strains of *Staphyloccocusaureus* ATCC 25923 and *Salmonella enterica Var. Typhimurium* ATCC 14028 were obtained from the medical bacteriology laboratory ENCB-IPN. The following were obtained from Sigma-Aldrich: lipase of *Candida rugosa* type VII, wheat germ lipase, and β-glucosidase from almonds.

Culture media

The culture medium YPD (1% yeast extract, 2% casein peptone and 2% dextrose) (Ausubel et al., 1994) was used. The composition of the culture media with hydrophobic carbon sources (MCFCH) for a volume of 500 mL was: 0.02 g citric acid, $MgSO_4$. $7H_2O$ 0.4 g, $FeSO_4$. $7H_2O$ 0.03 g, corn steep liquor 0.6 mL, $(NH_4)_2SO_4$ 1.3 g, $CaCO_3$ 1.5 g, hydrophobic substrate 20 mL, and KH_2PO_4 1 g (Spoeckner et al., 1999). The oils used for media preparation were previously bubbled with high-purity nitrogen and then sterilized in autoclave.

Maintenance and propagation of the fungus

The fungus was cultured in the YPD medium inside an incubator (New Brunswick Scientific Mod. G-25972420) at 30ºC/ 48 h/ 200 rpm. After 48 h, 0.5 mL of the culture were transferred into 1.5 mL vials and then added with 0.5 mL of 50% glycerol. The vials were stored at -70°C.

***U. maydis* FBD12 culture**

For the submerged culture, 125 mL baffled flasks were used at an operational volume of 35 mL of YPD and then incubated in a controlled temperature shaker (New Brunswick Scientific mod. G-25972420) at 30ºC/ 48 h/ 200 rpm. Afterwards the culture media were inoculated with hydrophobic carbon sources (MCFCH) with 3.55 mL (0.043 g/L) of the inoculum into 500 mL baffled beakers with an operational volume of 250 mL and then incubated in a controlled temperature shaker (Barnsted-Lab-line MaxQ 4000 Mod.SHKE-4000-7) at 100 rpm/30°C /9 days.

***U. maydis* FBD12 culture in hydrophobic carbon sources added with lipase**

The procedure described above was carried out and some samples were added with 2mg of lipase from *Candida rugosa* after 0, 3, 5, and 7 days (2 mg of *Candida rugosa* had an activity of 0.18U/mL as determined by the method described by Mark et al. (1992).

Determination of biomass, free fatty acids and lipase activity

Biomass (Monteiro et al., 2007), free fatty acids (NMX-F-101-1987; AOCS, 1998), and lipase activity (Mark et al., 1992) were determined after 3, 5, 7, and 9 days of culture.

Extraction and concentration of glycolipids

The culture so obtained was centrifuged at 12500 x g/30 min and the supernatant was adjusted at pH 2 with HCl and left to rest for 12 h at 4°C. Then it was centrifuged again at 12500 x g / 20 min and the precipitate was dissolved in HCl 0.4M. Afterwards it was extracted twice with $CHCl_3$:CH_3OH (2:1 v/v), the mix was vigorously agitated and left to rest to obtain a phase separation. The organic phase was recovered and evaporated until dry at 40°C to obtain a glycolipid dry extract (Monteiro et al., 2007).

Quantification of reducing sugars and lipids in the glycolipid extract

The sample was processed with hydrolytic enzymes in order to determine the content of reducing sugars in the following way: 0.5 mL of the raw glycolipid extract was dried with high-purity N_2, then it was immediately added with 1 mL of *C. rugosa* lipase (2 mg/mL) and 1 mL of a solution of ß-glucosidase (2mg/ml) in a sodium phosphate buffer 0.05 mM, pH 7.5. The sample was then incubated at 37°C/1h. Once this time had passed, 3 mL of petroleoum ether were added and then vigorously agitated, the aqueous phase was separated and the content of reducing sugars was determined by the dinitrosalicylic acid method (DNS) (Miller, 1959). Individual sugars were identified by thin layer chromatography (TLC) by comparison with standards using ethyl acetate: acetic acid: methanol: water (60:15:15:10) as mobile phase. The stationary phase was Silica gel 60 and the revealer α-naphthol-sulphuric acid (Jarusiewicz et al., 2006).

The organic phase containing the lipid fraction was esterified with CH_3OH/NaOH in BF_3 for a later capillary gas-liquid chromatography (CCGL) (AOAC, 2000). A gas chromatographer was used (Perkin Elmer Mod. Autosystem XL) equipped with a capillary injection system and flame ionization detector (FID). A PE-WAX column (Model N931-6413) with a length of 30 m, I.D. 0.32 mm, film of 0.5 µM, nitrogen flow of 10psi and temperature range of 0 to 275ºC was used. The gas chromatographer was calibrated according to the following parameters: point of injection 225ºC and detector 225ºC. The temperature profile was set as: initial temperature 120ºC, waiting time at the starting point 0 min, program range 5ºC/min, final temperature 190ºC, and final waiting time 10 min. Methyl esters were prepared from the samples as described above. 1µL of the reference methyl esters was injected into the gas chromatographer to obtain the reference chromatograph with the corresponding retention times. Then 1µL of the methyl esters of each problem sample were injected into the gas chromatographer and a comparative analysis of the results were performed.

Determination of the antioxidant capacity of the glycolipid extracts

A solution of the cation radical $ABTS^{\bullet+}$ was prepared by the reaction of the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) 7 mM and 2.5 mM $K_2S_2O_8$, which were mixed and the volume taken to 10 mL with distilled water. The mixture was allowed to rest in the dark for 12-16 h. The resulting solution of $ABTS^{\bullet+}$ was diluted in ethanol until reaching an absorbance of 0.7±0.02. Then 0.99 mL of this solution of $ABTS^{\bullet+}$ were added into the cell and finally 0.01 mL of the sample were mixed into it. The absorbance at 734 nm was registered at min 0 ($ABTS^{\bullet+}$) and the reading was recorded every min during the next 7 min. Data so obtained were interpolated into the standard curve (percentage of inhibition vs trolox concentration) in order to obtain the antioxidant capacity in µmol equivalents of trolox (TEAC) (Nenadis et al., 2004).

Analysis of the antimicrobial activity of the glycolipid extracts

The stock experimental cultures (*Staphyloccocusaureus* and *Salmonella enterica Var. Typhimurium)* were maintained at 4°C in soy trypticasein agar (AST) for its preservation until the microbial analysis. The preparation and standardization of the microbial inoculums consisted in transferring a loop of the stock culture into tubes filled with fresh soy trypticasein broth previously sterilized, and then incubated for 18-24 h/ 37ºC. Afterwards, serial dilutions were made into tubes with sterile soy trypticasein broth until reaching 12500 CFU/mL or a 0.5 turbidity in the McFarland scale. Then the dishes containing soy trypticasein agar were inoculated. The minimum inhibitory concentration (MIC) of the glycolipid extracts was determined (Miles and Misra, 1938). Glycolipid extracts for the analysis of microbial activity were bubbled with N_2 for 1 h. MIC was carried out by the method of dilution in broth using soy trypticasein broth as the growth medium. A stock solution of the sample antimicrobials was prepared by adding Tween 80 to a volume of 5 µL/ 200 µL glycolipid extract. Serial dilutions were made using soy trypticasein broth from this stock solution to obtain concentrations of 50, 25, 10, 5 and 2.5%. Each one was added with 10 µL of the corresponding standardized microbial inoculums. After the inoculation, 10 µL of each dilution were placed on soy trypticasein agar and incubated at 37ºC/24-18 h. Then a microbial count was performed and inoculated again in soy trypticasein agar for 18-24 h/ 37ºC before the count was made one more time. The MIC was the one with the lowest concentration of the antimicrobial in which bacteria do not show visible growth (Cheraif et al., 2007). All the experiments were made by triplicate and MS-Excel 97 and Sigma Plot version 10.0 were used to perform the statistical

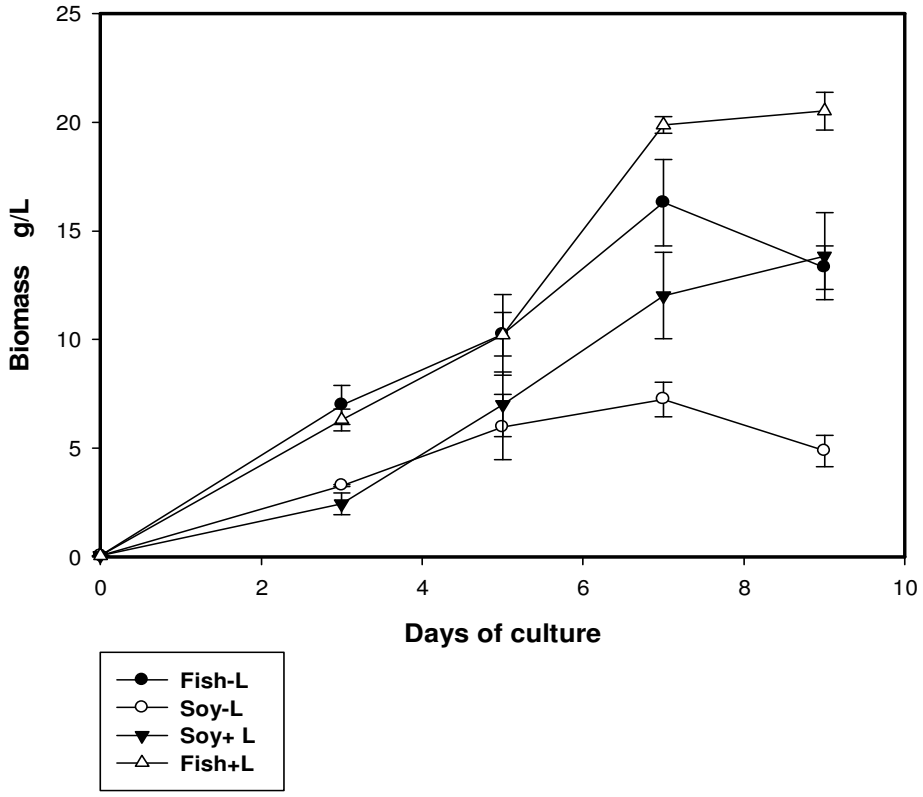

Figure 1. Biomass of *Ustilago maydis* FBD12 in soy oil and fish with addition of lipase (Soy+L)(Fish+L) and without addition of lipase (Soy-L)(Fish-L).

analysis.

RESULTS AND DISCUSSION

Growth of *Ustilago maydis* FBD12 in a culture medium with soy and fish oils

Figure 1 shows the biomass obtained by the culture in those media added with soy and fish oils with lipase (soy+L and fish+L) and without the addition of lipase (soy-L and fish-L). A higher biomass production was obtained with fish+L, followed by fish-L, soy+L and finally by soy-L.

In every case, a phase of constant growth was observed from day 3 through day 7, except for fish+L which had its higher value after 9 days of incubation (20.5 g/L), followed by fish-L at day 7 (16.3±2.0 g/L, decreasing at day 9 to 13.3±2 g/L). While the highest quantity of biomass was generated in the culture medium with soy+L at day 9 with 13.8 ± 2 g/L, in soy-L the highest quantity was observed at day 7, which later decreased until reaching 4.87 ±0.73 g/L on the 9th day (Figure 1).

The production of biomass increased significantly, as compared to the blank, when adding lipase to the medium formulation. The largest quantity was observed for fish+L after 9 days of incubation, with a 21% increase in the yield rate as compared to fish-L after 7 days.

Previous studies made on several microorganisms reported by Morita et al. (2008) mention that the growth of the yeast *Pseudozyma siamensis* CBS 9960 has a stationary phase after 9 days of incubation at 25°C, with a biomass production of approximately 20 g/L, using 4% safflower oil as carbon source in the production of mannosyl erythritol lipids. Spoeckner et al. (1999), on the other hand, observed a stationary phase after only 2 days of incubation with an approximate content of cellular protein of 1.25 g/mL for *U. maydis* DSM 4500 in a culture medium with sunflower fatty acids (85% oleic acid) as carbon source added batchwise after 2, 3, 4 and 5 days.

Yin et al. (2008) found a stationary phase after 12 h of incubation with approximately 5 g/L of biomass for *P. aeruginosa* S6 isolated from residual waters from the chemical oil industry, using petroleum hydrocarbons as sources of carbon for the production of rhamnolipids of low molecular weight. *Bacillus subtilis* ATCC 21332 showed a stationary phase in a submerged batch culture after 4 days of incubation and an approximate biomass of 2.4 g/L, using a hydrophobic diesel mix as a carbon source, as reported in a study of rhamnolipids degradation by Liang-Ming et al. (2008).

The above shows great differences in the quantities of biomass produced, from 2.4 to 20 g/L, obtained by different microorganisms and carbon sources, as well as

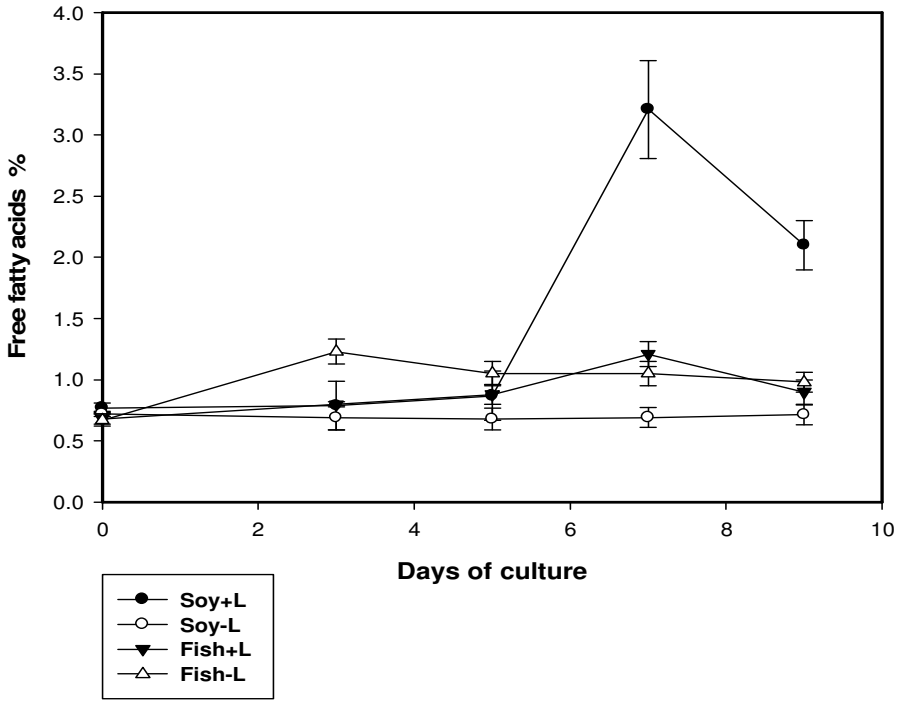

Figure 2. Percentage of free fatty acids in the culture médium for the growth of *Ustilago maydis* FBD12 using soy oil and fish with addition of lipase (Soy+L)(Fish+L) and without addition of lipase(Soy-L)(Fish-L).

type of culture.

Analysis of the percentage of free fatty acids in the culture médium

The percentage of free fatty acids originated from the oils used as carbon sources was determined. Soy oil had 0.23 ± 0.07% free fatty acids, while fish oil had 0.19 ± 0.02%.

Figure 2 shows the variation of the percentage of free fatty acids in culture media prepared with soy and fish oils and added with lipase (fish+L and soy+L) along the incubation period. Results show a constant increase with time, as compared to cultures that were not added with lipase (fish-L and soy-L). A higher percentage of free fatty acids was observed for these samples as compared to the enzyme-free cultures, with the highest values at day 7 for soy+L (3.2%). Fish+L showed its highest point at day 9 (1.2%), which may be due to a larger degree of hydrolysis of the triglycerides of the respective oils by the added enzyme, showing an additive effect when combined with the endogenous fungal enzyme.

In the cultures containing fish and soy oils, the addition of lipase increased the hydrolysis and the content of free fatty acids as compared to the cultures without the enzyme. The highest values were obtained for soy oil, but even when there was a higher degree of hydrolysis of the oil, the production of biomass was lower than the one obtained for fish. This indicates that in the soy culture, free fatty acids are used in a lower proportion for biomass production as compared to the fish culture. The changing behavior of the values of free fatty acids may be due to the degree of specificity of the enzyme towards the substrate, given the differences in the composition of fatty acids and chain lengths, which catalyzes the initial step in the metabolism of the hydrophobic substrate. The glycerol released as a product of the triglyceride metabolism is not a good substrate for *U. maydis*, since no growth has been observed when used as the only carbon source. It is possible that glycerol produced after triglyceride hydrolysis in the culture medium has an inhibitory effect in the metabolism of *U. maydis* affecting not only the growth, but also the production of glycolipids (Spoeckner et al., 1999). This may be the reason why the production of biomass is lower for soy-lipase even though this particular medium presents higher values of free fatty acids in the culture as compared to the ones obtained for fish+lipase.

Furthermore, there are microorganisms such as *Rhodotorula glutinis*, a type of yeast that is able to incorporate glycerol as a primary or secondary source of carbon when batch cultured with an initial concentration of 20 g/L glycerol, and has high biomass yields of up to 24.41 mg/L after 48 h of incubation. This is higher than values obtained by individual hydrophilic carbon sources,

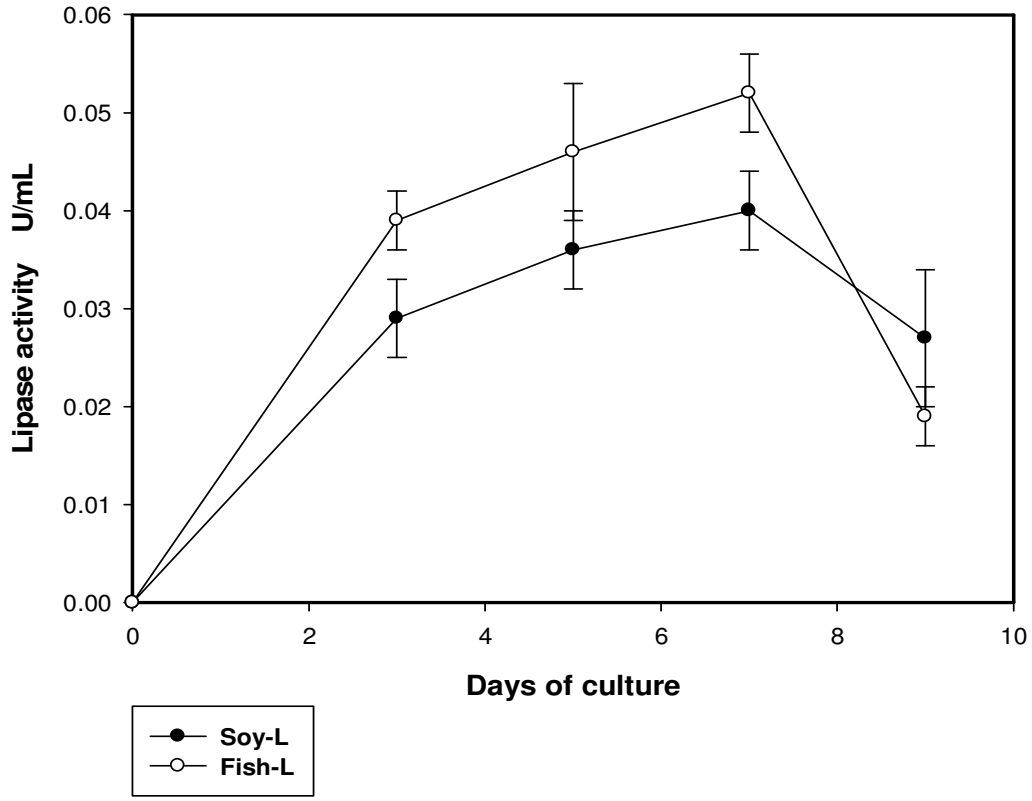

Figure 3. Lipase activity produced by *U. maydis* FBD12 during growth in fish and soy oil without addition of lipase (1U hydrolyses 1 microequivalents fatty acid triacetin in 1h/pH 7.4/37ºC).

such as sugars, producing and surviving at high concentrations of triacyl glycerols (up to 25%), destined for the production of biodiesel (Easterling et al., 2009).

Analysis of lipase activity from *U. maydis* FBD12 in culture media added with soy and fish oils

Figure 3 shows the activity of extracellular lipase produced by *U. maydis* FBD12. The highest values were observed at day 7 in fish-L with 0.05 U/mL and 0.3±0.02mg/mL of protein, and in soy-L with 0.04U/mL and 0.084±0.008 mg/mL. This correlates with the maximum growth phase, where high values of biomass were found for soy-L and fish-L.

The highest lipase activity for both oils was observed between days 5 and 7, when the highest proportion of glycolipids was obtained, as reported in the form of quantities of reducing sugars and biomass. The enzymetic activity of fish oil was the highest and yielded a higher production of glycolipids and biomass as compared to soy. This may be due to the initial catabolic step of the substrates, which is the action of the enzyme destined to the growth and production of glycolipids.

Morita et al. (2007) reported the presence of an extracelluar enzyme in a culture with intermittent feeding of glycerol of *P. antarctica* T-34, which takes part in the production of glycolipids of mannosyl erythritol with an activity of 9 U/mL after 10 days of incubation, using soy oil as the only carbon source.

Quantification of reducing sugars in glycolipid extracts

The analysis of reducing sugars in the glycolipid extracts (Figure 4) shows that the highest level was obtained after 9 days of incubation, being of 19.3 mg/100 mL for fish-L. In the case of soy-L, the highest production was observed at day 7 with 9.6 mg/100 mL and decreasing at day 9. The production of glycolipids of both oils is 2.29 times higher for fish-L as compared to soy-L. This relates to a better use of fish oil as carbon source in the production of glycolipids and biomass, as well as to a higher lipase activity.

It was observed that the sugar present in the glycolipid extracts after enzymatic hydrolysis as quantified by TLC was rhamnose, with a Rf of 0.7 for fish-L and fish+L, and a Rf of 0.69 for soy-L and soy+L. This was compared to a rhamnose standard, which has a Rf of 0.67.

Regarding the production of glycolipids, Morita et al. (2007) reported a maximum production from mannosyl

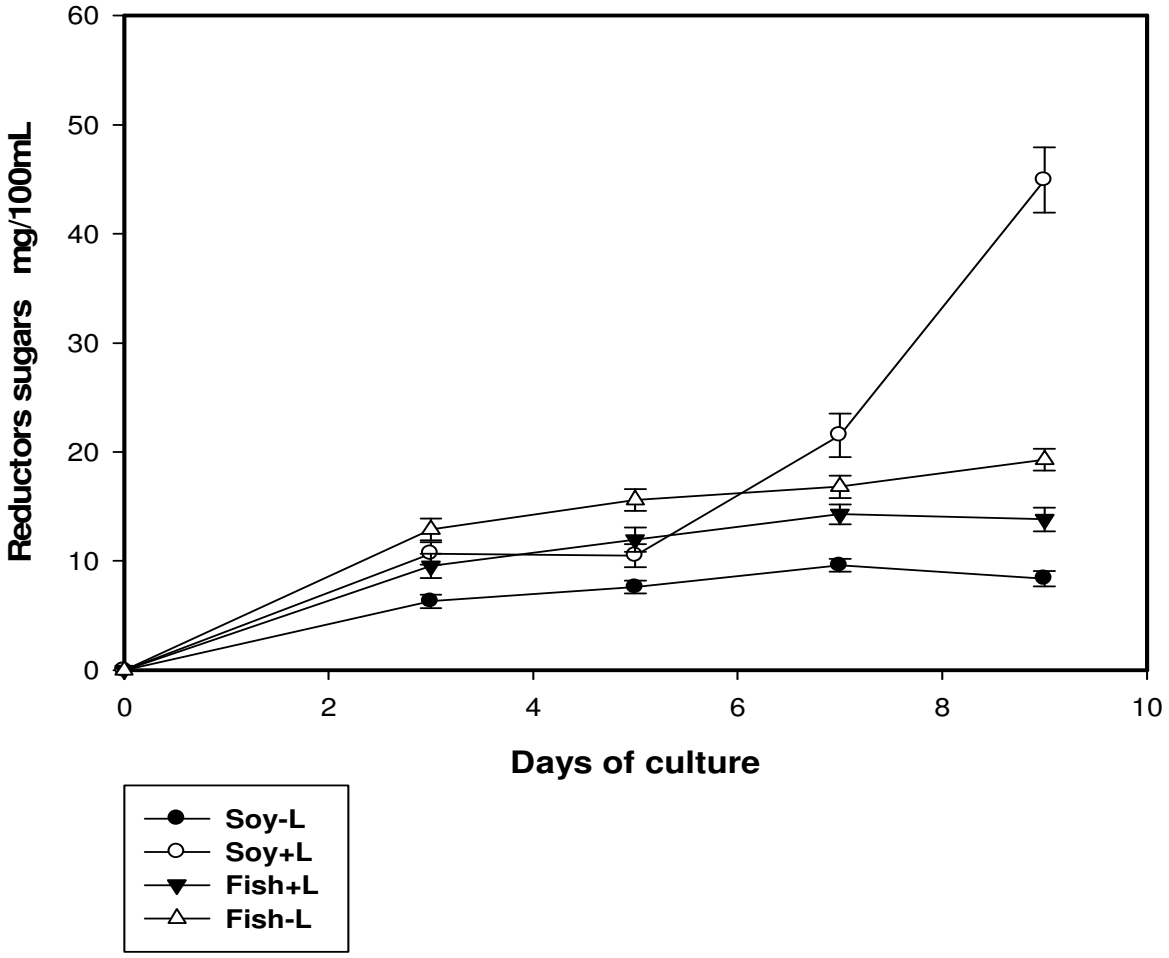

Figure 4. Reducing sugars content in the extract of glycolipids produced by *U. maydis* FBD12 in soy oil and fish with addition of lipase (Soy+L)(Fish+L) and without addition of lipase (Soy-L)(Fish-L).

Table 1. Major fatty acids found in soy and fish oils as a carbon source used in the study of the production of glycolipids.

Oil	C16	C16:1	C18	C18:1 mg/100 mL	C18:2	C18:3	Total
Soy	353.66±5	ND	123.33±5	790±10	1867±12	101.55±5	3235.54±7
Fish	215.68±10	898.3±5	523.9±5	1239.4±10	147.1±5	41.15±5	3065.53±6

ND:Undetected C10 Capric C16 Palmític C18:1 Oleic C16:1 Palmitoleic C18:2 Linoleic C18:3 Linolenic C18 Estearic.

erythritol of 400 mg/100 mL using *P. antarctica* JCM 10317 at 30ºC and 10% glycerol as carbon source, in a batch submerged culture after 7 days of incubation. Afterwards, these authors carried out a fed-batch culture added with a carbon source at 10% which yielded a production of mannosyl erythritol of 600 mg/100 mL after 14 days. They also mention that glycerol has negative effects on the cell growth and production at concentrations higher than 10%. Prieto et al. (2008) reported a maximum production of 142 mg/100 mL of rhamnose equivalent to rhamnolipids (a type of low-molecular weight glycolipids) in a batch culture of *P. aeruginosa* LBM10 after 4 days of incubation, using soy oil as carbon source and sodium nitrate as nitrogen source.

Lipid profile in glycolipid extracts

Table 1 shows the composition of the soy and fish oils used as carbon sources in the production on glycolipids. Both oils have a high content of unsaturated fatty acids of 85.25% in soy and 75.87% in fish. These results agree with the standards CODEX-STAN *210*; NMX-F-252-SCFI-2005 and with Gagliostro et al. (2006).

Regarding the fatty acid composition of the glycolipid extract from soy+L and soy-L, three different saturated

Table 2. Fatty acids found in extracts of glycolipids produced in soy oil with and without lipase.

DAY	C10	C12	C14	C16	C16:1	C16:2 mg/100 mL	C18	C18:1	C18:2	C18:3	Total
3-L	11.82	1.26	3.38	5.39	0.33	0.20	18.9	10.4	9.73	9.7	71.1
3+L	18.6	5.66	3.89	2.5	ND	0.47	5.31	3.06	8.14	1.03	48.6
5-L	11.88	3.16	10	35	0.25	0.20	0.7	10	2.9	0.024	74.1
5+L	8.0	12.49	24	1.51	0.11	0.78	1.14	0.33	0.20	0.25	48.8
7-L	6.00	5.07	50.6	4.93	1.13	0.38	2.19	3.13	1.03	0.67	75.1
7+L	20.8	10.8	7.60	1.5	0.11	0.35	0.43	0.31	0.08	1.09	43
9-L	5.30	5.16	49.26	4.91	4.06	0.52	2.49	0.256	0.67	0.99	73.6
9+L	49.7	2.21	47	9	0.68	0.22	0.23	0.91	0.11	1.21	111

+L with addition of lipase; -L without addition of lipase
ND:Undetected; C10 Capric; C12 Láuric; C14 Miristic; C16 Palmític; C18:1 Oléic; C16:1 Palmitoleic; C16:2 Palmitolenic; C18:2 Linoleic; C18:3 Linolenic; C18 Esteáric.

Table 3. Fatty acids found in extracts of glycolipids produced in fish oil with lipase (fish+L) and without lipase (Fish-L).

Day	C10	C12	C14	C14:1	C16	C16:1 mg/100mL	C18	C18:1	C18:2	C18:3	Total
3-L	8.41	5.97	4.9	1.42	31.6	0.49	4.78	7.3	5.9	0.37	71.4
3+L	12.9	6.20	20	3.6	4.54	ND	0.41	0.29	0.10	0.52	48.5
5-L	7.65	39.7	38.4	1.14	14.59	1.07	4.72	1.40	0.59	0.45	109.7
5+L	17.0	17.3	2.04	0.27	17.3	ND	0.37	0.21	0.09	0.38	54.9
7-L	12.9	42.5	9.39	ND	30.6	2.29	4.1	0.35	0.36	0.21	102.7
7+L	16.7	11.2	17.8	ND	67.8	ND	2	0.14	0.11	0.35	116.1
9-L	38.3	29.4	19.3	1.79	38	0.30	0.50	0.39	0.47	0.20	128.6
9+L	36.3	2.93	2.7	7.72	21.5	ND	1.9	0.61	0.30	2.16	76.12

+L with addition of lipase; -L without addition of lipase
ND: Undetected C10 Capric C12 Láuric C14 Miristic C16 Palmític C18:1 Oléic C16:1 Palmitoleic C18:2 Linoleic C18:3 Linolenic C18 Esteáric.

fatty acids were found (C10, C12, and C14) that were not detected in the initial analysis. These acids may be products of metabolism through β-oxidation and therefore incorporated into the structure of the glycolipid. Other two unsaturated acids C16:1 and C16:2 in the extract were not found in the original oils (Table 2).

The occurrence of fatty acids C10, C12 and C14 in soy+L extracts and C12 and C14 in soy-L showed a trend to increase with time. Their maximum values were found at day 5 for C10, at day 9 for C12, and at day 7 for C14. Regarding soy-L extracts, the maximum values for C10 were observed at day 5, C12 at day 9 and C14 at day 7. The total content of fatty acids in soy+L was highest after 9 days of incubation, as opposed to soy-L that had the highest content at day 7. In both cases, C10 and C14 fatty acids were majority. Furthermore, in the present study the higher proportion of reducing sugars and the biomass generated at the same incubation times were also reported. In extracts of soy+L and soy-L, it was observed that unsaturated fatty acids originally present in soy oil such as oleic, linoleic and linolenic tend to decrease. This happened as time increased and it is possibly due to degradation caused by the fungus metabolism during its growth. Simultaneously, shorter chain fatty acids appeared in the samples.

Table 3 shows the fatty acid composition in the glycolipid extracts of fish+L and fish-L. Three saturated fatty acids C10, C12 and C14 appeared in the medium and their concentration tended to increase with incubition time. Their maximum value for fish+L was observed at day 9 for C10, day 5 for C12 and day 3 for C14. In the case of fish-L, the maximum values were found at day 9 for C10, at day 7 for C12, and at day 5 for C14. It was observed that the total proportion of fatty acids for fish+L extracts was the highest after 7 days of incubation, while for fish-L it was at day 9. In both cases, the dominating fatty acids were C10, C12, C14 and C16 and the highest proportion of reducing sugars was found at the same incubation times, as well as quantity of biomass in fish-L and fish+L.

Regarding fish+L and fish-L, a tendency to decrease was observed in the proportion of unsaturated fatty acids such as oleic, linoleic and linolenic, which composed the majority of the original fish oil. This was observed after several days of incubation and may be due to the degradation of oil carried out by the fungus metabolism for its growth. Simultaneously, the content of shorter-length fatty acids tended to increase.

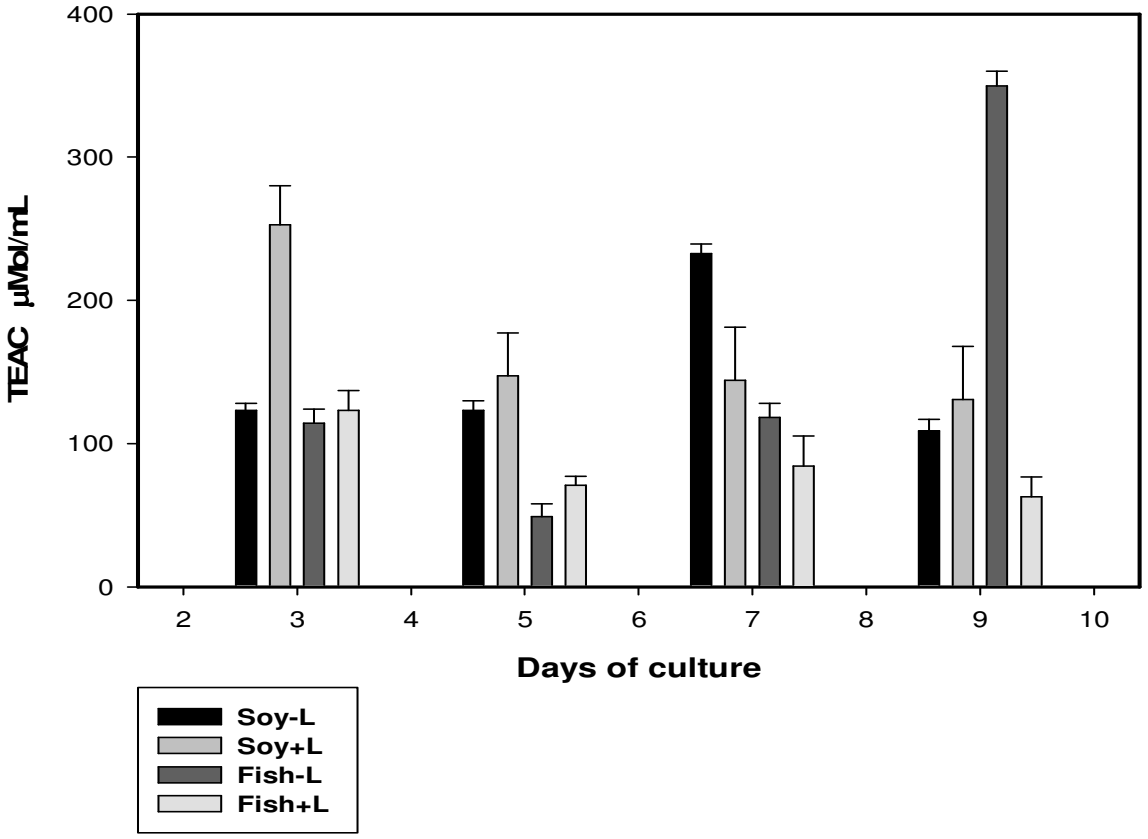

Figure 5. Antioxidant capacity in µMol equivalents of trolox (TEAC) of extracts of glycolipids produced by *U. maydis FBD12* in soy oil and fish with addition of lipase (Soy+L)(Fish+L) and without addition of lipase (Soy-L)(Fish-L).

The high proportion of saturated fatty acids in glycolipid extracts may be a result of the metabolism of the initial oils, which were the target of hydrolysis and β-oxidation during the growing of the fungus. They are also part of the structure of the hydrophobic portion of the synthesized glycolipids (Kitamoto et al., 1998; Kitamoto et al., 2002). Fukuoka et al. (2007) reported for yeast of the *Pseudozima* genre the synthesis of mannosyl erythritol glycolipids (MEL) in soy oil as the only carbon source; the products showed saturated fatty acids in their structure, mainly C8, C10, C12 and C16 after 8 days of incubation. Morita et al. (2007) reported the production of mannosyl erythritol glycolipids (MEL) by *P. antarctica* T-34 using soy oil as carbon source; these glycolipids showed mainly saturated fatty acids in their structure with chain lengths of C10, C8, C12, and C14, after 7 to 10 days of incubation.

Determination of antioxidant activity of glycolipid extracts

The antioxidant capacity of soy oil was of 1768.6 µMol TE/mL, while the one observed for fish oil was of 2249 µMol TE/mL, higher than the one showed by the different glycolipid extracts. This may be due to: 1) the high proportion of unsaturated fatty acids in the oil, and 2) the antioxidant TBHQ added to commercial oils, which is of 0.01% for soy and 0.005% for sunflower.

Figure 5 shows the antioxidant capacity of soy-L and soy+L glycolipid extracts, as well as of fish-L and fish+L. The highest antioxidant capacities were observed for soy-L at day 7 with 232 µMol TE/mL, and fish-L at day 9 with 350 µMol TE/mL.

The antioxidant capacity of glycolipid extracts obtained with lipase tended to decrease with incubation time, and they had a lower capacity as compared to the extracts obtained with oils without lipase addition. This decrease may be due to a higher triglyceride hydrolysis given by the enzyme, originating fatty acid units that are more readily oxidized by the fungus metabolism. The action of the enzyme may also cause a possible structural change in the composition of the glycolipids.

The difference between the antioxidant capacities of glycolipids obtained in fish-L and fish+L as compared to soy-L and soy+L may be due to the composition and proportion of mono- and polyunsaturated fatty acids present in different oils used as carbon sources, since the

Table 4. Minimum inhibitory concentration (MIC) of extracts of glycolipids produced in soy and fish oil with added lipase on *Salmonella enterica Var. Typhimurium* y *Staphylococcus aureus*

Source carbon	Glicolípids (%)	Log UFC/mL to 0 h	Log UFC/mL to 24 h	Log UFC/mL to 48 h
		Salmonella enterica Var. Typhimurium		
Soy Oil	5	4	0	0
	0	4	9	9
		Staphylococcus aureus		
	10	4	0	0
	0	4	9	9
		Salmonella enterica Var. Typhimurium		
Fish Oil	10	4	0	0
	0	4	9	9
		Staphylococcus aureus		
	10	4	0	0
	0	4	9	9

structure of the resulting glycolipids may be conditioned to the composition of the triglycerides present in the carbon source (Kitamoto et al., 2002; Syldatk and Wagner, 1987).

According to the standards CODEX-STAN 210 and NMX-F-252-SCFI-2005, soy oil has a composition of 80.5% unsaturated fatty acids (17% oleic acid, 59% linoleic acid and 4.5% linolenic acid). This may be the reason of the high antioxidant capacity of the oil and the glycolipids derived from it. The composition of fish oil agrees with Gagliostro et al. (2006), who report contents of unsaturated and saturated fatty acids of 71.16 and 26.92%, respectively.

In the present study, it was observed that glycolipids produced by *U. maydis* FBD12, due to their amphiphatic nature, may be used as antioxidants in systems formed by immiscible phases (emulsions), either alone or together with other antioxidants whose activity is weaker in these type of system, generating a possible synergy as antioxidant or antimicrobial and therefore contributing to the stability, protection against oxidation, and microbial degradation of the medium.

Effect of glycolipid extracts on the growth of *Staphylococcus aureus and Salmonella enterica Var. Typhimurium*

Glycolipid extracts produced in soy-L showed an antimicrobial effect at a MIC of 0.02 mg/mL after 24 h of exposure against *S. aureus,* while glycolipid extracts produced in fish-L showed a bactericide effect after 48 h at a MIC of 0.04 mg/mL.

When added with lipase, soy+L and fish+L extracts showed a better antimicrobial effect, decreasing MIC in 50% (0.01 mg/mL) for soy+L. In the case of fish+L, MIC was reduced in 75% (0.01 mg/mL) (Table 4).

Soy-L was tested on S. *enterica. Var. Typhimurium*, and it showed a bactericide effect at a MIC of 0.02 mg/mL after 24 h of exposure. On the other hand, fish-L extracts were less effective than soy-L with a bacteriostatic effect after 24 h of exposure and a bactericidal effect at a CMI of 0.04 mg/mL and exposure time of 48 h.

When adding lipase to the culture media, fish+L extracts tested against *S. Enterica Var. Typhimurium* showed a higher antimicrobial effect as compared to samples not added with the enzyme, reducing MIC in 75% (0.01 mg/mL) from a value of 0.04 mg/mL for fish-L. The extracts obtained for soy+L showed the same MIC of 0.02 mg/mL than the ones obtained for soy-L after 24 h of exposure (Table 4).

The results from the present study indicate that the addition of lipase into the culture medium of *U. maydis* FBD12 with soy and fish oils improves the antimicrobial activities of the resulting glycolipid extracts. Glycolipids derived from soy and fish obtained with and without the addition of lipase showed a higher antimicrobial activity against *S. aureus* as compared to *S. enterica Var. Typhimurium*, which may be explained by the structural difference of the cell wall observed in these micro-organisms. It is possible that the inhibition of microbial growth shown by the glycolipids produced by *U. maydis* FBD12 is related to chemical structures in the microorganisms with similar surface activity, therefore altering the permeability of the cytoplasmic membrane or otherwise damaging or inhibiting the synthesis of the cell wall (Pelczar et al., 1982).

When comparing to other reports related to the antimicrobial activity of glycolipids, it was observed that the MIC values of the extracts obtained in the present study are active at lower concentrations than other vege-table and glycolipid extracts, but higher when compared with some commonly used antibiotics. Kitamoto et al.

(2002) reported that the antimicrobial activity of some glycolipids such as mannosyl erythritol A and B had a MIC of 0.0125 and 0.025 mg/mL respectively, as well as a MIC of 0.128 mg/mL for rhamnolipids and a MIC higher than 0.80 mg/mL for sophorolipids in *S. aureus*. For *S. thypimurium* a MIC of 0.128 mg/mL was found in rhamnolipids, while Kikuc et al. (2008) reported for cardoon extracts (*Cynara cardunculus*) a MIC of 2 and 1.5 mg/mL for *S. aureus* and *Salmonella thyphimurium,* respectively. The antibiotic streptomycin had a MIC of 0.001 mg/mL for both bacterial strains.

Conclusions

The addition of lipase significantly increased the production of biomass of *U. maydis* FBD12 in culture media added with fish and soy oils. Among these, fish oil had the highest yield. The highest lipase activity of *U. maydis* FBD12 was observed after 5 and 7 days of incubation, which corresponded to the highest production of glycolipids and biomass. The production of glycolipids by *U. maydis* FBD12 in soy+L showed an increase as compared to soy-L, while the opposite happened for fish oil, observing that a lower amount of glycolipids was produced in fish+L as compared to fish-L. The highest antioxidant capacity of glycolipid extracts with and without the addition of lipase was found for soy-L at day 7 and fish-L at day 9. Glycolipid extracts from both sources with the addition of lipase showed a higher antimicrobial activity against *S. enterica Var. Typhimurium* and *S. aureus,* being more effective against the last microorganism.

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