Research Paper

Modulation of antimicrobial metabolites production by the fungus *Aspergillus parasiticus*

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Abstract

Biosynthesis of active secondary metabolites by fungi occurs as a specific response to the different growing environments. Changes in this environment alter the chemical and biological profiles leading to metabolites diversification and consequently to novel pharmacological applications. In this work, it was studied the influence of three parameters (fermentation length, medium composition and aeration) in the biosyntheses of antimicrobial metabolites by the fungus *Aspergillus parasiticus* in 10 distinct fermentation periods. Metabolism modulation in two culturing media, CYA and YES was evaluated by a 2^2 full factorial planning (ANOVA) and on a 2^3 factorial planning, role of aeration, medium composition and carbohydrate concentration were also evaluated. In overall, 120 different extracts were prepared, their HPLC profiles were obtained and the antimicrobial activity against *A. flavus*, *C. albicans*, *E. coli* and *S. aureus* of all extracts was evaluated by microdilution bioassay. Yield of kojic acid, a fine chemical produced by the fungus *A. parasiticus* was determined in all extracts. Statistical analyses pointed thirteen conditions able to modulate the production of bioactive metabolites by *A. parasiticus*. Effect of carbon source in metabolites diversification was significant as shown by the changes in the HPLC profiles of the extracts. Most of the extracts presented inhibition rates higher than that of kojic acid as for the extract obtained after 6 days of fermentation in YES medium under stirring. Kojic acid was not the only metabolite responsible for the activity since some highly active extracts showed to possess low amounts of this compound, as determined by HPLC.

Key words: *Aspergillus parasiticus*, factorial planning, metabolites diversification, kojic acid, antimicrobial activity.

Introduction

Throughout the centuries, many active metabolites were discovered from varied natural sources, such as superior plants, animals, insects and microorganisms. Amongst these sources, the microorganisms are probably the group that generates metabolites possessing the most readily industrial applications because they are chemo-organotrophic, present a high growing rate on a short cycle of life and generate large amounts of biomass in short time. Therefore, industrial production of fungal metabolites demands less complex operational control processes (Demain, 2000). By the other hand, the great number of existing microorganisms can make the initial chemo-biological prospection of extracts from natural sources very time-consuming, increasing the length of research in this area (Harvey, 2007). Therefore, the use of interactive chemo-biological tools to screen suitable conditions for bioactive metabolites production is very welcome (Lam, 2007). Natural products already isolated from fungi possess numerous pharmacological uses, in special as antibacterial and antifungal agents (Takahashi and Lucas, 2008a; Takahashi *et al.*, 2008b; Tanseer and Anjum, 2011; Newman and Cragg, 2012). Research in this area has been neglected in face of the profit perspective of some more fashioned diseases. However, the scope of antimicrobial activity has become increasingly important since fungal and bacterial infections have grown enormously in recent years, especially associated to individuals with immune system deficiency like those with acquired immunodeficiency syndrome (AIDS).

Life expectance increase, with the raise of a great number of elderly in the world population, the chronic use of corticosteroids and the boost of invasive medical procedures also contributed to the increase in the number of reported infections (Perfect, 2012). This new framework claims for the search of antimicrobial agents more effective and with less adverse effects. Production of antimicrobial metabolites by fungi is natural, since their survival in the natural habitats depends on their efficiency to stop the growth of other co-habitant microorganisms.

Fungi as *A. flavus* and *C. albicans* are related to important human infections that require close attention. *A. flavus* is responsible for huge losses in agricultural sanitary and nutritional quality in grains, through the production of secondary metabolites called aflatoxins, responsible for poisoning several species of animals, as well as being carcinogenic (Luo *et al.*, 2009; Roze *et al.*, 2011; Sajid *et al.*, 2011). This fungus is also responsible for causing pulmonary aspergillosis, an opportunistic disease that affects mostly people with an already weakened immune system (Mahmoud *et al.*, 2011; Mellon *et al.*, 2011). *C. albicans* can be normally found in the human body, but under certain conditions, this microorganism can cause infections known as candidiasis, which affect mainly immune deficient patients. Local *C. albicans* infections can quickly develop into serious systemic infections (Seneviratne *et al.*, 2008).

Several fungi from *Aspergillus* genus are also known for their ability to produce various bioactive metabolites for the pharmaceutical industry, such as terrecyclic acid A, metabolite with anticancer and antibacterial activity (Turbyville *et al.*, 2005; Bok *et al.*, 2006) and lovastatin, a very interesting metabolite used for cholesterol reduction (Bizukojc *et al.*, 2012), both produced by the fungus *Aspergillus terreus*. Other active compounds produced by *Aspergillus* species are asperlicin, metabolite of *Aspergillus alliaceus* employed to treat neurological disorders (Butler, 2008), echinocandin B produced by *A. nidulans*, with antifungal activity (Aly *et al.*, 2011) and fumagillin, metabolite of *A. fumigatus*, an angiogenesis inhibitor that has also antiparasitic activity (Sanchez, 2012).

A. parasiticus produces an interesting metabolite called kojic acid, a compound currently used in the pharmaceutical industry since it aggregates important biological activities, such as anti-inflammatory, antifungal, antibacterial, antitumor and insecticidal (Novontny *et al.*, 1999). Recently kojic acid also started to be employed in treatments for reduction of expression marks around the eyes area (Hyde *et al.*, 2010), broadening the scope of its industrial applications. In the present work, *A. parasiticus* was cultivated under 120 distinct conditions, and the changes in metabolites production were monitored by High Pressure Liquid Chromatography coupled to a diiodoarray detector (DAD-HPLC). The extracts obtained were submitted to a biological screening to monitor their antimicrobial activity against Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*), a yeast of relevant global clinical importance (*Candida albicans*) and a filamentous fungus (*Aspergillus flavus*) that causes not only human diseases but also agricultural losses. Kojic acid was quantified in all extracts by DAD-HPLC through the elaboration of a calibration curve using external standard methodology (Hu *et al.*, 2011) in order to understand kojic acid role in the extracts activity.

Materials and Methods

Microorganism and culturing conditions

The fungus *A. parasiticus* ATCC 15517 was obtained from the Microorganisms' Collection hold by Fundação Oswaldo Cruz (FIOCRUZ – RJ, Brazil). The fungus was stored on test tubes containing potato dextrose agar (PDA, Himedia, India) at the temperature of 11 °C. In the fermentations, *A. parasiticus* was grown in liquid media with the following compositions (in distilled water): CYA medium (35.0 g/L Czapek broth; 5.0 g/L yeast extract; 30.0 g/L sucrose), YES medium (150.0 g/L sucrose; 20.0 g/L malt extract; 0.5 g/L $MgSO_4.7H_2O$), LCG20 medium (20.0 g/L D-Glucose; 5.0 g/L bacteriological peptone; 1.0 g/L K_2HPO_4; 0.5 g/L $MgSO_4.7H_2O$; 5.0 g/L NaCl); LCG150 medium (150.0 g/L D-Glucose; 5.0 g/L bacteriological peptone; 1.0 g/L K_2HPO_4; 0.5 g/L $MgSO_4.7H_2O$; 5.0 g/L NaCl); medium LCS20 (20.0 g/L sucrose; 5.0 g/L bacteriological peptone; 1.0 g/L K_2HPO_4; 0.5 g/L $MgSO_4.7H_2O$; 5.0 g/L NaCl) and medium LCS150 (150.0 g/L sucrose; 5.0 g/L bacteriological peptone; 1.0 g/L K_2HPO_4; 0.5 g/L $MgSO_4.7H_2O$; 5.0 g/L NaCl).

Experimental planning

In order to optimize the production of active metabolites, three parameters (fermentation length, medium composition and aeration) were varied during the fermentation in 10 distinct fermentation periods (3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 days). For each period, the parameters were evaluated by a 2^2 full factorial planning (ANOVA), to screen two culturing media, CYA and YES. In this experiment, there were also evaluated the aeration and the medium composition. On a 2^3 factorial planning, there were studied the influence of the liquid media LCG and LCS and the role of the variables aeration, medium composition and carbohydrate concentration. Details on the experimental conditions can be found on Tables 1 and 2.

For the preparation of the spore suspension used in the experiments, *A. parasiticus* was incubated for 7 days at 28 °C in PDA. After this period, the spores were recovered, counted on a Neubauer Chamber and the appropriate dilution was prepared to achieve the final concentration of 5 x 10^6 spores/mL. The experiments took place upon the inoculation of a spore suspension in 500 mL Erlenmeyer flasks, containing 100 mL of culturing medium. There were used a

Table 1 - Levels of variables studied in the 2^2 factorial planning.

Variables	Levels	
	Inferior (-)	Superior (+)
Stirring (A), rpm	0	150
Culture medium	CYA	YES

Table 2 - Levels of variables studied in the 2^3 factorial planning.

Variables	Levels	
	Inferior (-)	Superior (+)
Stirring (A), rpm	0	150
Carbohydrate	Glucose	Sucrose
Carbohydrate concentration	20 mg L^{-1}	150 mg L^{-1}

total of 240 flasks, in which 120 different growing conditions were evaluated in duplicate.

Extracts preparation

The work up was carried on by vacuum filtration to separate the mycelium from the broth followed by extraction of the broth with ethyl acetate (3 x 50 mL/100 mL of broth) on a separating funnel. The organic layers were combined and the solvent was vacuum removed on a rota evaporator. The resulting extracts were transferred to clean vials and weighted.

High performance liquid chromatography analyses

Aliquots (10 mg) of the homogenised extracts were filtered with PTFE organic solvents modified membranes (MILLEX LCR 0.45 μm - Millipore) and analysed by High Performance Liquid Chromatography on a SHIMADZU LC10AD series Liquid Chromatograph coupled to an automatic sampler SIL-10AF SHIMADZU Auto Injector and a Diiodo Array Detector SPDM10avp. The separations were accomplished using a reversed phase Supelcosil ^{TM} LC18 column (250 x 4.6 mm; 5 μm). The volume of injected samples was 20 μL, at the concentration of 1 mg/mL. The system was operated by the CLASS LC10 Software. The mobile phase was constituted by a mixture of water containing 0.05% of formic acid and methanol. The linear elution gradient used was H$_2$0-Methanol 70:30 to 0:100 in 30 min, maintaining for additional 10 min in the last concentration. The gradient was brought back to the initial value in 5 min and maintained at this point for additional 5 min, to equilibrate the system, prior to the injection of the next sample. The eluent flux used was 0.5 mL/min.

External standardization of Kojic acid

To determine the amount of kojic acid produced in the experiments using different culture media, external standard methodology was used to build a productivity curve for this compound. A methanol solution of kojic acid (Acros Organics, Geel, Belgium) at the concentration of 1 mg/mL was prepared and, from it, 10 serial dilutions were prepared. Methanol solutions with known amounts of kojic acid were injected on the chromatograph. The calibration curve was generated by regression analysis (linear model) interposing the nominal concentrations of kojic acid solutions with the area of the peak obtained in the chromatogram. Statistical analysis of the data and return calculations were achieved using Microsoft Office Excel 2007. This experiment was carried out in quadruplicate.

Antimicrobial assays

Antibacterial and antifungal assays were carried out with the bacteria *S. aureus* ATCC 25923, *E. coli* ATCC 25723, the yeast *C. albicans* ATCC 18804 and the filamentous fungus *A. flavus* CCT 4952. The assays were conducted in microplates prepared with fixed concentrations of each extract (1000 μg/mL) and with kojic acid (250 μg/mL) in DMSO. Microorganisms were inoculated as suspensions. For the preparation of the spore suspension, *A. flavus* was incubated for 7 days at 28 °C in PDA. After this period, the spores were recovered, counted on a Neubauer Chamber and the appropriate dilution was prepared for the final concentration of 5 x 10^3 spores/mL. To prepare bacterial and *C. albicans* inocula, the respective strains were incubated for 24 h at 37 °C in Broth Heart Infusion medium (BHI). Turbidity was adjusted with aid of a spectrophotometer model SP-22, BIOSPECTRO, by adding sterile water to the cells until reaching 75-76% of transmittance at the fixed wavelength of 530 nm. Microorganisms growth was measured on a ELISA reader (Thermoplate, TP-READER), at 492 nm, after 24 and 48 h (antibacterial tests) and after 48 h for *C. albicans* and *A. flavus*. The assay was carried in duplicate.

Results

The HPLC profiles of the extracts prepared (120 in total) were obtained and the extracts were assayed to monitor the modulation of the metabolites antimicrobial profiles upon the growing conditions alterations. Afterwards, the results of the biological screening were analysed using a statistical tool to determine which interactions among the variables in study influenced the biological activity (Table 3). There were considered significant the variables that presented p ≤ 0.05, *i.e.*, a confidence interval of 95%.

Graphics referring to the response surface from the 2^2 (evaluation of CYA and YES media) and 2^3 (evaluation of factorial LCG and LCS media) complete factorial planning (ANOVA) were prepared in order to analyse the effect of the growing conditions on the production of bioactive metabolites by *A. parasiticus*. The analyses led into consideration the conditions in which the variables presented significant results. Figure 1 illustrates the 2^2 response graphic for

Table 3 - Culturing lengths, interactions between the variable that influenced the biological activity, microorganisms for which this influence was significant (p ≤ 0.05) and better conditions found for the culturing.

Experiment code	Fermentation lenght (days)	Interactions Studied	Microorganism	Better Growing Condition
C01	6	Stirring x Medium (CYA / YES)	*E. coli* and *S. aureus*	YES under stirring
C02	9	Stirring x carbohydrate concentration	*C. albicans*	LCG150 without stirring
C03				LCS150 without stirring
C04	12	Stirring x carbohydrate concentration	*E. coli*	LCG150 under stirring
C05				LCS150 under stirring
C06	15	Stirring x Medium (LCG / LCS)	*E. coli*	LCG20 without stirring
C07				LCG150 without stirring
C08	21	Stirring x carbohydrate concentration	*S. aureus*	LCG150 without stirring
C09				LCS150 without stirring
C10	24	Stirring x carbohydrate concentration x Medium (LCG / LCS)	*A. flavus*	LCG20 without stirring
C11			*E. coli* and *C. albicans*	LCG20 under stirring
C12	27	Stirring x Medium (LCG / LCS)	*A. flavus*, *C. albicans* and *S. aureus*	LCG20 under stirring
C13				LCG150 under stirring

antimicrobial activity against *E. coli* (Figure 1A) and *S. aureus* (Figure 1B) of extracts prepared after 6 days of *A. parasiticus* cultivation. It can be noticed that the interactions among the type of culturing medium employed in the fermentation and presence of stirring showed significant results. The optimal conditions found at this stage of the fermentative process comprised the use of both superior levels, *i.e.*, YES as the culture medium under stirring of 150 rpm (condition C01, Table 3).

Figure 2 shows a graphic for the response surface for the 2^3 planning for antimicrobial activity against *C. albicans* of extracts prepared after 24 days of *A. parasiticus* cultivation. It can be noticed that the interactions among the carbohydrate concentration, type of culturing medium employed in the fermentation and presence of stirring showed significant results. The optimal conditions found refer to the lower levels in relation to the type of carbohydrate and carbohydrate concentration and to the higher levels for stirring. Therefore, best condition, in this case, was found for LCG culture medium, with 20 g/L of glucose and under stirring of 150 rpm (condition C11, Table 3).

The yield of kojic acid in the extracts was determined using high-performance liquid chromatography with external calibration. Methanol solutions of kojic acid at known concentrations were injected in the HPLC chromatograph individually in order to trace a calibration curve. Kojic acid peak presented a wave length with maximum absorption in 270 nm and 100% of chromatographic pureness. The calibration curve, constructed through the intersection of the area of the chromatographic peaks and the nominal concentration, can be visualized in Figure 3. The procedure for adjustment of the straight line was carried through by the method of linear regression. Linear regression equation was $y = 139419507.07x - 2992550.64$ and the coefficient of correlation $r^2 = 0.99$. Kojic acid was screened by anti-

microbial assays against *A. flavus*, *C. albicans*, *E. coli* and *S. aureus* and its biological activity was compared to the activities detected for the crude extracts obtained from *A. parasiticus* in the diversified growing conditions utilized in this study. Figure 4 shows kojic acid antimicrobial profile as determined by the biological screening performed in this study.

Discussion

In this study, manipulation of growing conditions focused six different culture media: CYA, YES, LCG20, LCG150, LCS20 and LCS150. The CYA, YES and LCS possess sucrose as carbon source, varying only the composition of this component: CYA (30 g/L), LCS20 (20 g/L), YES and LCS150 (150 g/L). Culture medium LCG20 and LCG 150 present glucose as carbon source at concentrations of 20 and 150 g/L, respectively. As the nitrogen sources, LCG and LCS media have bacteriological peptone; medium YES has, in its composition, malt extract, and medium CYA, contains yeast extract. The production of metabolites by *A. parasiticus* was evaluated after 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 days and in presence or absence of stirring. The variation of these parameters has allowed to achieve a wide scope of biosynthetic responses, since, at the end, there were evaluated 120 different fermentation conditions. Many secondary metabolites of great economic importance are produced in low yields or even are not produced by some fungal species due to the use of unfavourable cultivation conditions (Bills *et al.*, 2008). By the other hand, production of bioactive metabolites as a result of manipulation of growing conditions has been shown to be important in the discovery of novel major bioactive metabolites, as exemplified by Bode *et al.* (2002), which
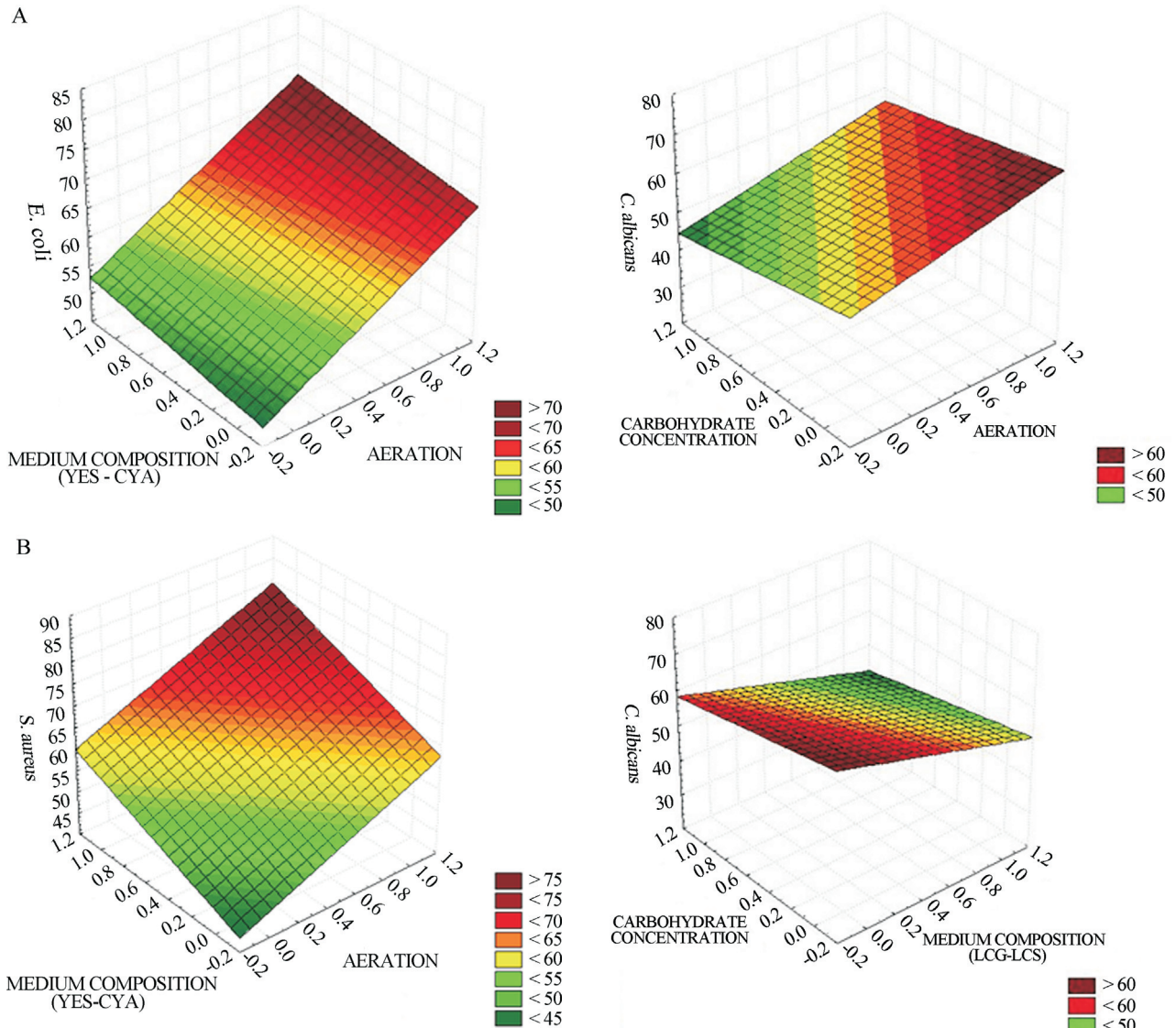
A

B

Figure 1 - Surface response graphical representation of the extracts activity against *E. coli* (A) and *S. aureus* (B) after six days of fungal growth.

isolated more than 20 different metabolites from the same organism, varying only the cultivation conditions.

Since *A. parasiticus* is known for producing large amounts of kojic acid, it was necessary to evaluate the influence of this compound in the results. Therefore, kojic acid activity was determined against all microorganisms targeted by this study and quantified in all extracts. Kojic acid presented moderate activity against all the tested microorganisms under the conditions utilized in this study, ranging from $45.63 \pm 0.09\%$ against *C. albicans* and up to $63.73 \pm 3.81\%$ against *S. aureus*. Therefore, if the extracts activity were only related to the presence of kojic acid, extracts presenting greater percent production of kojic acid should also present higher percentage of microbial inhibition when compared to extracts containing lower amounts of kojic acid. However, the bioassays with the crude ex-
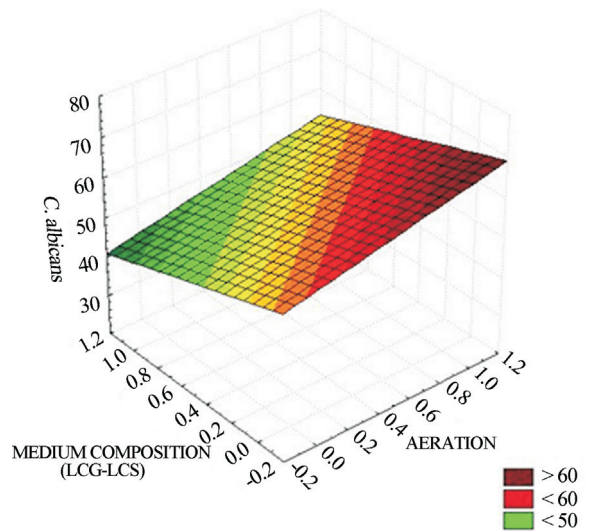

Figure 2 - Surface response graphics of the activity presented by *A. parasiticus* extracts prepared after 24 days of fungal culturing against *C. albicans*.
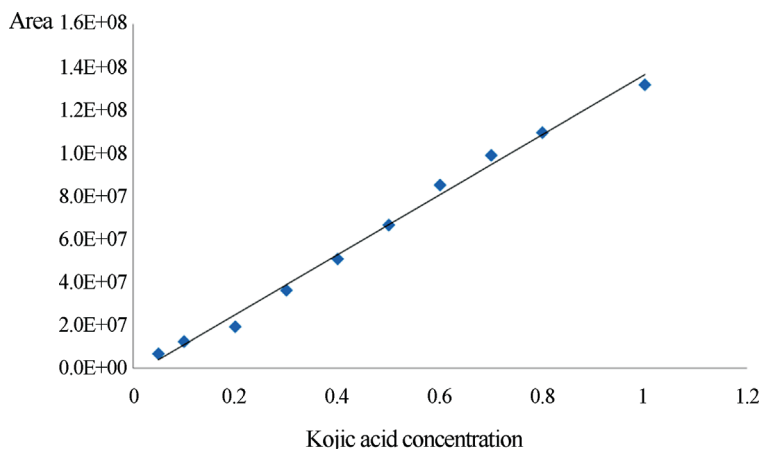
Figure 3 - Calibration curve for methanol solutions of kojic acid.
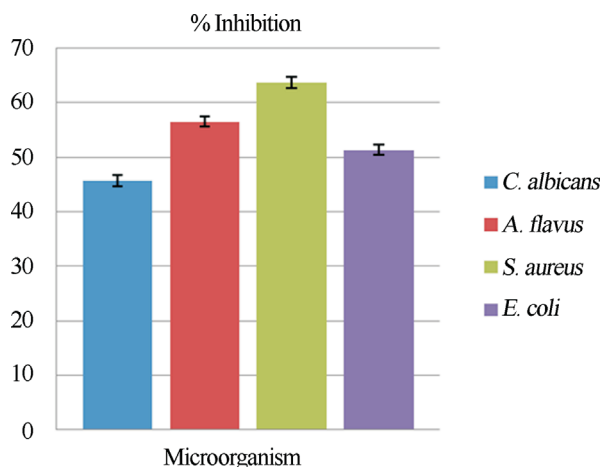

Figure 4 - Graphical representation of percentage inhibition of *A. flavus*, *C. albicans*, *E. coli* and *S. aureus* by kojic acid at 250 µg/mL.

tracts evidenced that most of them presented inhibition rates higher than that of kojic acid, as shown in Table 4, for some selected conditions.

This observation can be exemplified by the extract codified as C01, that showed activities against the microorganisms *E.coli* and *S. aureus* (50.42 ± 0.94% and 50.57 ± 4.71% respectively) at levels not consistent with their respective kojic acid contents (0.04%), evidencing the presence of other antimicrobial metabolites in the extracts. The presence of such novel bioactive compounds in the extracts was also observed in their HPLC-DAD profiles. Figure 5 (Chromatogram A) shows the chromatogram of extract C01.

Production of kojic acid by the extract obtained from the condition C09 reached 57.70%, where the carbon source used was sucrose, also used to prepare extract C01. In this case, nitrogen source, the culturing time (21 days) and the absence of agitation led to a different metabolites profile, as can be seen in Figure 5 (chromatograms A and

Table 4 - Percentages of *A. flavus*, *C. albicans*, *E. coli* and *S. aureus* inhibitions for different culture conditions *vs.* percentages of kojic acid determined in the respective extracts.

Code	Microorganism	Percentages of inhibition (%)	Kojic acid yield (%)
C01	*E. coli*	50.42 ± 0.94	0.04
	S. aureus	50.57 ± 4.71	
C02	*C. albicans*	53.04 ± 0.13	26.30
C03		54.66 ± 7.53	37.94
C04	*E. coli*	64.03 ± 0.82	35.38
C05		64.24 ± 6.57	34.55
C06	*E. coli*	69.78 ± 1.05	18.52
C07		54.62 ± 1.82	29.28
C08	*S. aureus*	52.21 ± 2.29	34.14
C09		49.15 ± 0.54	57.70
C10	*A. flavus*	41.09 ± 0.65	15.53
C11	*E. coli*	53.61 ± 4.91	10.23
	C. albicans	62.69 ± 1.39	
C12	*A. flavus*	49.75 ± 3.21	7.66
	C. albicans	60.23 ± 0.72	
	S. aureus	57.74 ± 0.99	
C13	*A. flavus*	49.74 ± 3.21	41.10
	C. albicans	58.44 ± 4.26	
	S. aureus	64.00 ± 1.66	

B). In the chromatogram B (Figure 5) the peak for kojic acid can be visualized with the retention time of 10.38 min.

The same occurred for fungal extracts produced under conditions C11 and C12 that, despite producing low amounts of kojic acid (10.23 and 7.66% respectively) presented high inhibition of the tested microorganisms (Table 4).When comparing the HPLC chromatogram of kojic acid with the chromatograms of the extract produced with con-
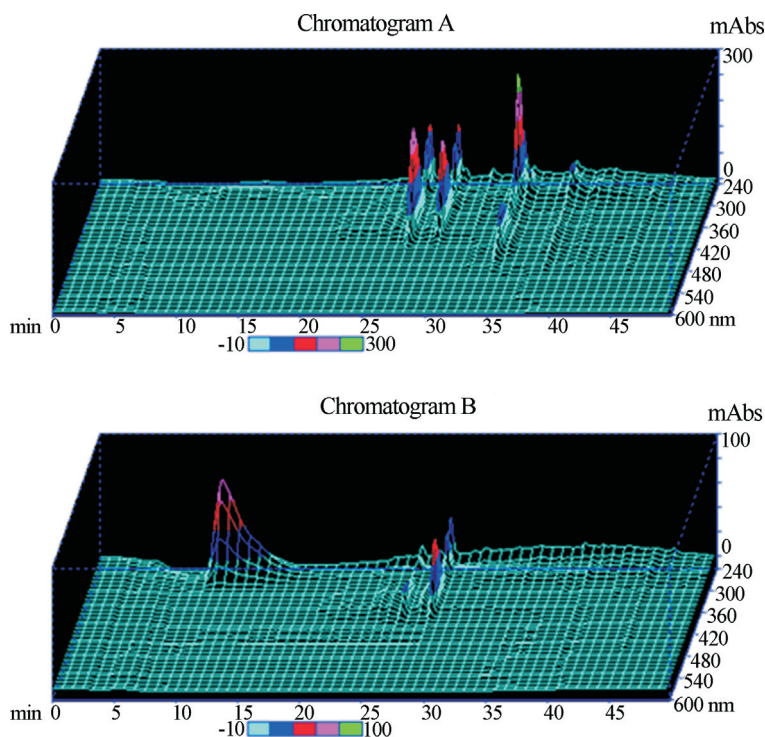
Figure 5 - Chromatograms (240 nm to 600 nm) of the extract obtained C01 (Chromatogram A) and C09 (Chromatogram B).

ditions C10 and C11 (without and with stirring respectively), represented in Figure 6, the presence of metabolites with retention times different of kojic acid was detected.

It is known that the use of glucose by microorganisms during the biosynthesis of kojic acid is essential (Bentley, 2006; El-Aasar, 2006), therefore higher yields ok kojic acid were observed in the culture media LCG that contained higher amounts of glucose (150 g/L). In the media LCS, where glucose was replaced by sucrose, production of kojic acid was also predominant since, in this case, glucose can be readily obtained upon sucrose hydrolysis. Interestingly, kojic acid production did not reach high yields when *A. parasiticus* was grown in the culture medium YES, which has also a high concentration of sucrose (150 g/L). In this case, other factors like cultivation time (6 days) and stirring (150 rpm) overcame glucose availability in the biosynthesis, leading to lower yield of kojic acid.

The diversity of metabolites produced by *A. parasiticus* in the different growing media utilized was accessed by HPLC-DAD, according to the number of peaks detected in the chromatograms with different retention indexes (RI) (Abreu *et al.*, 2012). The fermentation carried out employing condition C01, in addition to presenting the lowest production of kojic acid, offered the largest production of other secondary metabolites. As previously discussed, fungal growing using YES medium in such specific condition did not favour kojic acid biosynthesis. It is possible that the metabolites diversification, in this case, arose from an alternative biosynthetic route using fructose, since

sucrose enzymatic conversion to glucose also generates fructose, on an enzymatic reaction catalyzed by invertase.

Literature reports that alteration of the carbon source in the medium produces biological activity variation. For instance, extracts obtained using sucrose as a carbon source presented higher biological activity against microorganisms such as *E. coli* and *Fusarium oxysporium* when compared to extracts from culture media containing only glucose or fructose in their composition (El-Banna, 2006). Endophyte fungi *Apiospora montagnei* and *Arthrinium state* also produced extracts with high biological activity when cultured in media containing sucrose as carbon source (Ramos and Said, 2011). These data corroborate the results obtained, indicating that hydrolysis of sucrose into glucose and fructose allows the development of distinct biosynthetic routes for production of bioactive metabolites.

The biological tests results were very encouraging, especially those obtained for *A. parasiticus* extract produced in under culturing condition C01 that, in addition to present relevant activity against the bacteria *E. coli* and *S. aureus*, produced the highest metabolic diversity among all 120 different cultivation conditions evaluated in the biological and chromatographic screening. Use of medium LCG20 without stirring showed to be the most suitable condition (69.78 $\pm$ 1.05% inhibition) to produce metabolites active against *E. coli*. Among fungal conditions evaluated for production of bioactive metabolites, extract produced under condition C10 showed selectivity towards the fungus *A. flavus* (41.09 $\pm$ 0.65% activity). Conditions C02 C03 led
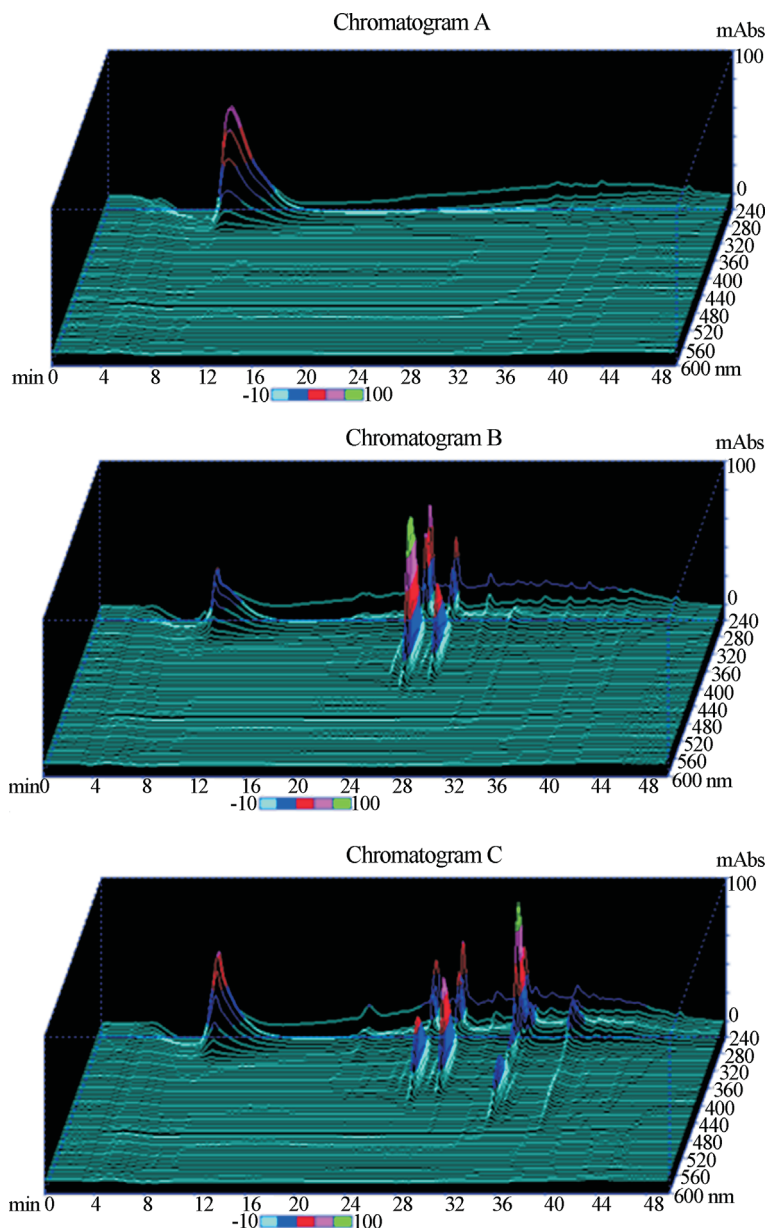
Figure 6 - 3D Chromatograms (240 nm to 600 nm) of kojic acid (Chromatogram A), extract C10 (Chromatogram B) and extract C11 (Chromatogram C).

to extracts selective towards the fungus *C. albicans* (53.04 ± 0.13 and 54.66 ± 7.53% activity respectively). These results demonstrate the great potential for production of bioactive metabolites under these specific conditions. Since the activity results are reported for crude extracts, antimicrobial activity in the range of 50% are extremely exciting, since major compounds in fungal extracts usually do not exceed 1% of the total extract.

Acknowledgments

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