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Enzymatic Potential of *Mucor inaequisporus* for Naringin Biotransformation, Accessed by Fractional Factorial Design and Mass Spectrometry Analysis

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Abstract

Whole cells of Mucor inaequisporus were used for biotransformation of the flavonoid naringin, as a green method for structural diversification of this class of natural product. The metabolism of the fungus was challenged against naringin under several culturing conditions, using statistical optimization. Eight parameters were evaluated comprising type and concentration of carbon source, substrate concentration, substrate addition time, shaking, luminosity, temperature and extraction time. Sixteen different culture conditions were tested in this screening. After LC-MS analysis, several biotransformation products were identified and naringenin was identified as the major product. Eleven biotransformation products were detected in Ft-HRMS analyses and identified as naringenin as major compound, 4'-methoxynaringenin, 4'-methoxynaringin, rhoifolin, apigenin, methoxyapigenin, acetylated naringin, and also two biflavonoids and two triflavonoids. Ft-HRMSn showed to be a powerful technique for structural elucidation of flavonoid biotransformation products. The fungus was capable to perform deglycosylation, reduction and O-methylation reactions at this substrate indicating good enzymatic potential, since these reactions weren't observed before for this fungus-genus.

Keywords: Biotransformation; Naringin; Statistical optimization; Mucor inaequisporus; Mass spectrometry; Flavonoid fragmentation; Ft-HRMS

Introduction

Flavonoids are an important and widespread group of plant natural products that possess many biological activities. These compounds are part of the wide range of substances called "polyphenols", which are widely known mainly by their antioxidant properties, and are present in human dietary sources showing great health benefits [1]. Therefore, researchers are constantly looking for new sources of flavonoids, new structures, and strategies for their interconversions. Flavonoid biotransformation is an important, usually "green", method for structural modification that may lead to high chemical diversity, which could improve bioavailability and biological properties. In some cases, the microorganisms can mimic mammalian and plant metabolism [2,3].

In recent studies, the biotransformation of naringin, which is a flavanone glycoside present in citrus fruits and grapefruit, and is responsible for the bitterness of citrus juices [4], was performed by several microorganisms using intact cells and isolated enzymes in order to improve some chemical and biological properties. *Bacillus stearothermophilus* maltogenic amylase, as an example, transglycosylated naringin to maltosylnaringin which is 250 times more soluble in water and 10 times less bitter than naringin [5], whereas a *Candida antarctica* lipase enhanced its liposolubility by sugar a4cylation [6]. Although several researchers are interested in naringin to naringenin bioconversion using naringinase [7-9] and to prunin [10,11], some studies showed great functionalization of naringin using microbial biotransformation. For instance, naringin metabolism by *Penicillium charlesii* produced two unknown compounds besides naringenin and prunin [12], whilst *Trichoderma harzianum* improved naringin antioxidative activity performing a B-ring mono- and dihydroxylation [13]. In other report, *Aspergillus saitoi* produced carthamidin and isocarthamidin, products of a ring hydroxylation at positions 6 and 8, respectively [14]. Also a C ring opening by *Butyrivibrio sp.* produced phloroglucinol and *p*-hydroxyphenylpropionic acid in addition to naringenin and neohesperidose when using intact cells for this microbial transformation [15].

Biotransformation reactions using factorial design approach are frequently used to optimize reactional parameters in some biochemical processes performed by microorganisms. The advantage is to test different conditions in reduced number of experiments, improving the process workout and observing synergic or antagonic effects among variables [16-19]. Our objectives are to study *via* statistical optimization a way to enhance naringin biotransformation by *Mucor inaequisporus* and show the capability of mass spectrometry to detect and identify biotransformation products. This is the first report about microbial transformation of naringin by a *Mucor* species. We found that this fungus was capable to perform deglycosylation, dehydrogenation and *O*-methylation in naringin, producing eleven biotransformation products.

Materials and Methods

General experimental procedures

The fungus *Mucor inaequisporus* was isolated from *Syzygium cumini* (L.) Skeels fruits and identified by sequencing two Internal

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Transcribed Space (ITS) rDNA regions [20]. The strain has been maintained on malt-agar slants with repeated subculturing. HPLC-Diode array detector analyses were performed in a SHIMADZU HPLC equipped with a PDA detector (Tokyo, Japan). HPLC-Electrospray ionization/High resolution mass spectrometry analyses were carried out on a LTQ-Orbitrap Thermo Fisher Scientific mass spectrometry system (Bremen, Germany). Naringin and naringenin were purchased from Aldrich. Fungus growth media were purchased from commercial suppliers and used as provided. All solvents were distilled before use.

Preparation of cultivation medium and culture conditions

The fungus was cultivated in a rich media composed of 50.0 $g.L^{-1}$ soluble starch or dextrose, 3.0 $g.L^{-1}$ sodium nitrate, 1.0 $g.L^{-1}$ potassium phosphate monobasic, 0.5 $g.L^{-1}$ magnesium sulphate heptahydrate, 0.5 $g.L^{-1}$ iron sulphate heptahydrate, 0.5 $g.L^{-1}$ potassium chloride and 20.0 $g.L^{-1}$ yeast extract. The culture conditions are designed by a 2^{8-4} fractional factorial design experiment described in Table 1. The eight factors (variables) analyzed were (1) type and (2) concentration of carbon source, (3) substrate concentration, (4) substrate addition day, (5) shaking, (6) luminosity, (7) temperature and (8) extraction day. These variables were tested in two levels named -1 (lower level) and +1 (higher level) and more details can be observed at Table 1. The spore suspension of *M. inaequisporus* was used to inoculate into culture medium using 100 µL of 10^6 $CFU.mL^{-1}$ spore suspension. Cultures were grown in 125 mL capacity Erlenmeyer flasks in triplicate and the substrate was prepared in dimethyl sulfoxide.

Extraction and LC-DAD analysis

The mycelium extraction was done by harvesting fungal mycelia by filtration on paper filter no. 11 and extracting it with 50 mL of ethanol. The aqueous phase was extracted in triplicate with 50 mL of ethyl acetate. The extracts were dried under reduced pressure, re-dissolved in 3 mL of methanol and analyzed by reversed-phase LC-DAD.

The samples were analyzed by a gradient HPLC method for the detection of the naringin metabolites by injecting 10 µL of sample. The column used was a PHENOMENEX Luna Phenyl-hexyl 5 µ 250 × 4.6 mm. The mobile-phase composition consisted of water with 0.1% of formic acid (A) and methanol with 0.1% of formic acid (B) pumped at 1.0 $mL.min^{-1}$. The mobile phase varied from 20% B in a linear gradient of 20 to 50% B over 10 minutes, followed by a linear gradient of 50% to 100% B in 20 minutes. After 10 minutes in 100% B, the gradient shifts to 20%B and stay in 20% B until next analysis. Naringin and its metabolites were detected at the wavelength of 289 nm and the data analysis was performed by CLASS-VP software.

LC-MS/MS analyses

HPLC-ESI/HRMS analyses were carried out on a LTQ-Orbitrap Thermo Fisher Scientific mass spectrometry system (Bremen, Germany) with the resolution set at 60K and operating in negative scan mode from 100-1500 Da. HPLC was fitted with a PHENOMENEX Luna Phenyl-Hexyl 5 µ 250 × 4.6 mm and samples were eluted using a linear gradient elution with acetonitrile and water from 20 to 100% with a 1.0 $mL.min^{-1}$ flow.

Results and Discussion

Compounds identification

After extraction, the extracts were analyzed by LC-DAD and several chromatographic peaks were detected and exhibiting similar UV profile of naringin (**1**). The analyses and comparison with standard compounds indicated that part of **1** remained in all extracts and that the major biotransformation product was naringenin (**2**) by comparison with standards, while several candidate products were identified, as shown in Figure 1. HRMS analyses were performed with tandem mass spectrometry experiments in order to identify their chemical structures.

The peaks next to **1** and **2** showed similar UV pattern indicating them as possible biotransformation products. Their identification was achieved by HRMS analyses and comparison with literature information, according the discussions below. All identified compounds, which are shown in Figure 2, were not detected in control experiments, with fungus growing in the absence of substrate, and also in the medium with substrate but without fungus.

Naringin (**1**) fragmentation pattern is very characteristic due to

	1 Carbon	2 [Carbon]	3 [Substr]	4 Substr. add. time	5 = 123 Shak.	6 = 134 Lum.	7 = 234 Temp.	8 = 1234 Extr. time
f1	-1	-1	-1	-1	-1	-1	-1	1
f2	1	-1	-1	-1	1	1	-1	-1
f3	-1	1	-1	-1	1	-1	1	-1
f4	1	1	-1	-1	-1	1	1	1
f5	-1	-1	1	-1	1	1	1	-1
f6	1	-1	1	-1	-1	-1	1	1
f7	-1	1	1	-1	-1	1	-1	1
f8	1	1	1	-1	1	-1	-1	-1
f9	-1	-1	-1	1	-1	1	1	-1
f10	1	-1	-1	1	1	-1	1	1
f11	-1	1	-1	1	1	1	-1	1
f12	1	1	-1	1	-1	-1	-1	-1
f13	-1	-1	1	1	1	-1	-1	1
f14	1	-1	1	1	-1	1	-1	-1
f15	-1	1	1	1	-1	-1	1	-1
f16	1	1	1	1	1	1	1	1
Levels (-1 and +1) description								
-1	Soluble starch	10 $g.L^{-1}$	5 mg	1 day	Static	Dark	25°C	12 days
1	Dextrose	50 $g.L^{-1}$	40 mg	7 days	200 rpm	Light	35°C	20 days

Table 1: 2^{8-4} fractional factorial design for biotransformation of naringin by *M. inaequisporus* with factors and its levels (-1 and 1) in different flasks (f1-f16).

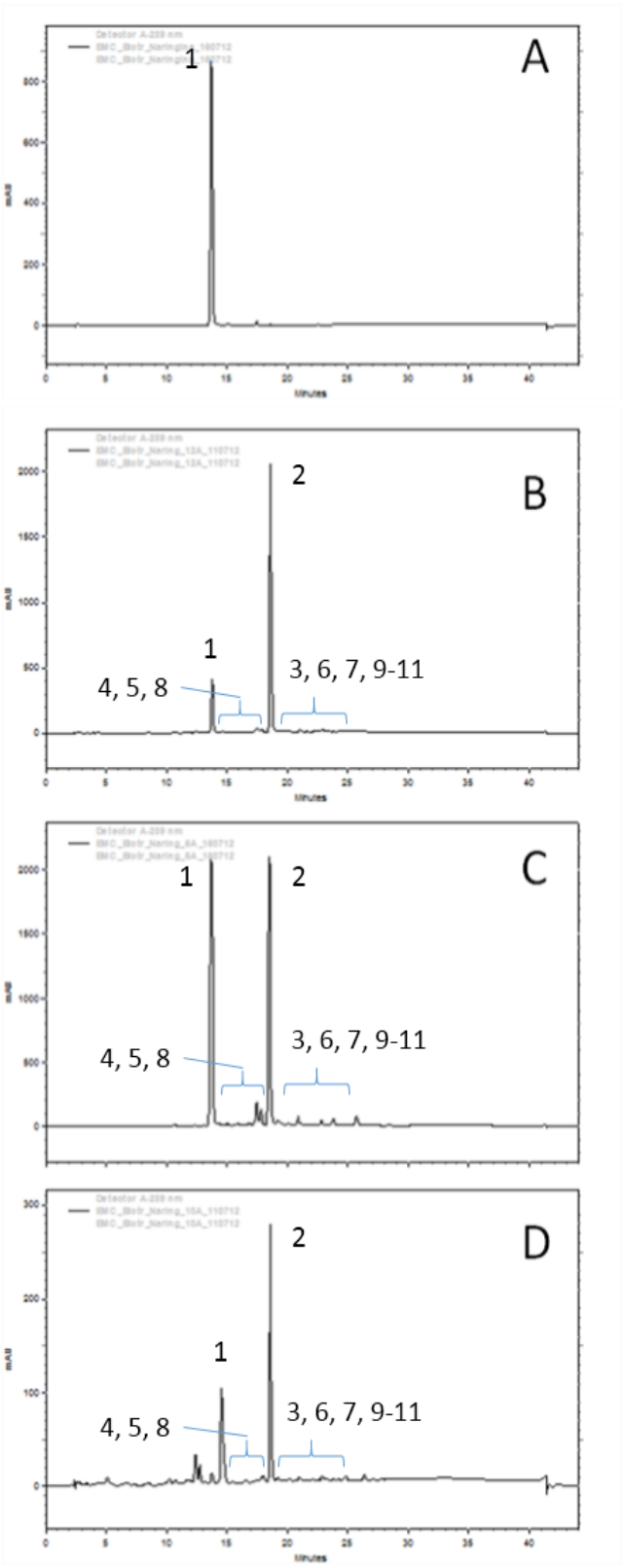

Figure 1: LC-DAD chromatograms at 289 nm. A) naringin standard (1); B) flask number 12 – ethyl acetate extract (f12); C) flask number 6 – ethyl acetate extract (f6); D) flask number 10 – ethyl acetate extract (f10). The numbers of identified compounds were also shown.

C-ring retro Diels-Alder cleavage and sugar loss. The ethyl acetate extract from flask 6 of the designed experiment was utilized for the mass spectrometry analyses interpretation and identification of compounds; since it contain all biotransformation products detected at others extracts.

The HRMS ESI mass spectrum of **1** in negative mode shows a deprotonated molecule at *m/z* 579.1715 ($[M-H]^-$) corresponding to molecular formula $C_{27}H_{31}O_{14}^-$. MS^2 experiment led to *m/z* 271.0593 which corresponds to Y_0^- fragment, the same as deprotonated naringenin (**2**), according flavonoid fragmentation nomenclature states [21,22]. This was confirmed by MS^3 experiments of *m/z* 271.0593, which led to *m/z* 151.0038 ($C_7H_3O_4^-$) and *m/z* 119.0498 ($C_8H_7O^-$), $^{1,3}A^-$ and $^{1,3}B^-$ fragments from C-ring retro Diels-Alder. It was also detected the ion at *m/z* 165.0194 ($C_8H_5O_4^-$) correspondent to a retrocyclization ($^{0,4}B^-$ ion) and *m/z* 459.1117 ($C_{19}H_{23}O_{13}^-$) that is a $^{0,2}X_1^-$ fragment from sugar fragmentation. Table 2 summarizes the HRMS data for the detected compounds and the proposed fragmentation pathway for all the discussed structures is shown at Figure 3.

The spectrum of **2**, previously identified with comparison to standard as naringenin in LC-DAD, exhibited a deprotonated molecule at *m/z* 271.0606 correspondent to $C_{15}H_{11}O_5^-$. The MS^2 spectrum produced ions at *m/z* 165.0189 ($C_8H_5O_4^-$), *m/z* 151.0033 ($C_7H_3O_4^-$), *m/z* 119.0498 ($C_8H_7O^-$) and *m/z* 107.0136 ($C_6H_3O_2^-$) corresponding to $^{0,4}B^-$, $^{1,3}A^-$, $^{1,3}B^-$ and $^{0,4}A^-$, respectively.

The mass spectrum of **3** showed a deprotonated molecule at *m/z* 285.0767 ($C_{16}H_{13}O_5^-$), an addition of 14 Da to **2**, indicating a methylation. The fragment at *m/z* 270.0519 ($C_{15}H_{10}O_5^-$) mean a loss of methyl radical, confirming the methylation and fragments at *m/z* 243.0650 ($C_{14}H_{11}O_4^-$) indicate ketene loss (42 Da) from deprotonated molecule, previously reported for naringin fragmentation [23]. Ions with *m/z* 151.0030 and 107.0134 corresponding to $^{1,3}A^-$ and $^{0,4}A^-$ part of molecule confirms that the methyl moiety is located at 4´ position at B ring, since *O*-methoxylated fragment produced is neutral. There is one fragment at *m/z* 164.0108 ($C_8H_4O_4^-$) corresponding to ketene loss radical ion $^{0,4}B^{\cdot-}$. Thus, **3** is identified as 4'-methoxynaringenin.

The mass spectrum of **4** indicates a 14 Da addition, methylation, to naringin, since deprotonated ion at *m/z* 593.1873 correspond to $C_{28}H_{33}O_{14}$. This modification was confirmed to be in position 4´ because the MS^2 showed a fragment at *m/z* 285.0750 similar to **3** and MS^3 of this ion showed the same fragmentation pattern, confirming **4** as 4'-methoxynaringin.

A loss of 2 Da in comparison to **1** was detected in the full scan spectrum of **5**, indicating a loss of two hydrogens. The *m/z* 577.1560 was correlated to the molecular formula $C_{27}H_{29}O_{14}^-$ and the fragmentation produced the *m/z* 269.0435 ion ($C_{15}H_9O_5^-$) which corresponds to a dehydrogenation at C ring in **2**. In that way, **5** could be identified as rhoifolin.

The spectrum for the next compound (**6**) shows a deprotonated molecule at *m/z* 269.0453 ($C_{15}H_9O_5^-$), which suggest to be apigenin, the rhoifolin aglycone. The MS^2 confirmed the proposal, since it produced the $^{1,3}B^-$ fragment at 117.0341 ($C_8H_5O^-$). The addition of a methyl (14 Da) to **6** gave the **7** mass spectrum with a deprotonated molecule at *m/z* 283.0606 ($C_{16}H_{11}O_5^-$), which corresponds to methoxyapigenin, but no fragment was detected in MS^2 experiments. Low resolution mass spectrometry collision-induced dissociation experiments evidences

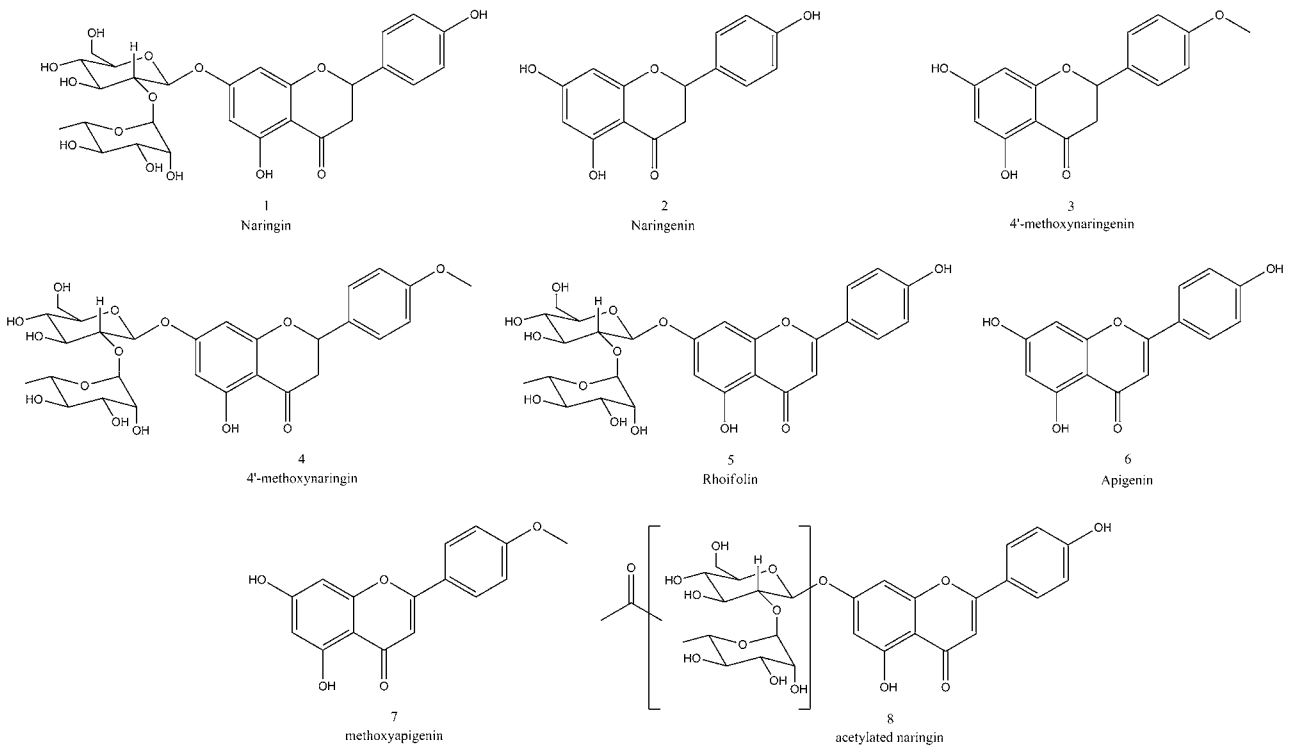

Figure 2: Naringin (**1**) and the biotransformation products chemical structures (**2-8**) identified by HRMS analyses

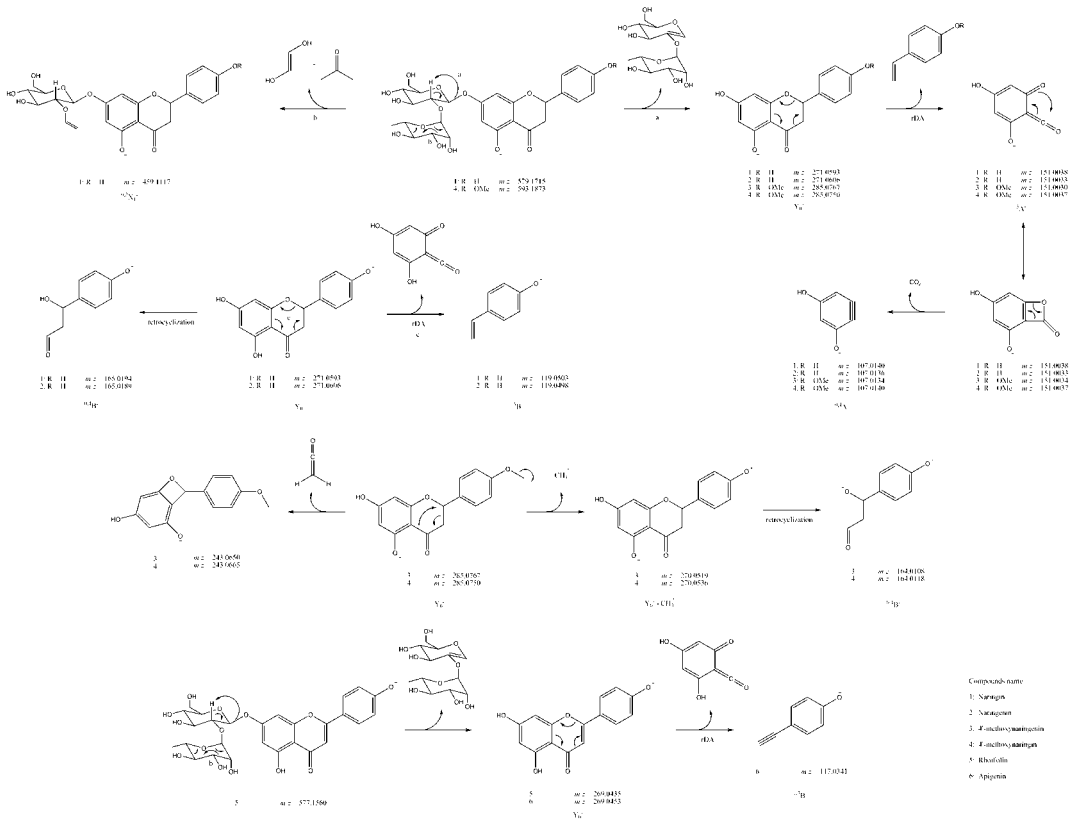

Figure 3: Main fragmentations pathway for naringin biotransformation products based on their MS^2 and MS^3 spectra

methyl position in the molecule at A ring (at position 4 or 6) as *m/z* 119 ($^{1,3}B^-$) and *m/z* 163 ($^{1,3}A^-$) ions were observed.

One acylated biotransformation product of **1** was detected (**8**) with *m/z* 621.1818 ($C_{29}H_{33}O_{15}^-$), corresponding to an addition of 42 Da. The MS^2 experiments exhibit the ions at *m/z* 579.1689 ($C_{27}H_{31}O_{14}^-$), *m/z* 561.1585 ($C_{27}H_{29}O_{13}^-$), *m/z* 501.1225 ($C_{25}H_{25}O_{11}^-$) and *m/z* 271.0596 ($C_{15}H_{11}O_5^-$), corresponding to loss of ketene, water from deprotonated naringin, acetic acid and sugar, respectively. Due to 42 and 60 Da loss from parent ion, the acylation is located at saccharide moiety, but the exact position of acetylation in the saccharide could not be determined using only mass spectrometry.

Some unexpected deprotonated molecules with *m/z* 553.1143 ($C_{31}H_{21}O_{10}^-$), *m/z* 555.1291 ($C_{31}H_{23}O_{10}^-$), *m/z* 839.1973 ($C_{47}H_{35}O_{15}^-$) and *m/z* 863.2397 ($C_{43}H_{43}O_{19}^-$) were detected (**9**, **10**, **11** and **12**, respectively). They exhibited a similar fragmentation pattern, suggesting, for example, **9** and **10** to be a pair of biflavonoid composed of **2** and **7** due to MS^2 ions at *m/z* 271.0595 ($C_{15}H_{11}O_5^-$) and *m/z* 283.0595 ($C_{16}H_{11}O_5^-$). These fragments were confirmed by MS^3 data at *m/z* 151.0040 ($C_7H_3O_4^-$) and *m/z* 163.0040 ($C_8H_3O_4^-$), respectively. The mass spectrum of **11** present *m/z* 555.1256 ($C_{31}H_{23}O_{10}^-$) and *m/z* 283.0589 ($C_{16}H_{11}O_5^-$) as MS^2 fragments, indicating a coupling of **7** and **9** molecules, as MS^3 experiment for *m/z* 555.1256 ($C_{31}H_{23}O_{10}^-$) confirmed this as the same ion as **9** due to *m/z* 283.0616 ($C_{16}H_{11}O_5^-$) and *m/z* 271.0616 ($C_{15}H_{11}O_5^-$). Therefore this compound was suggested to be a triflavonoid. Accordingly, 12 was suggested to be another triflavonoid as result of coupling 7 to 1, due its MS^2 spectrum exhibits an ion at *m/z* 579.1675 ($C_{27}H_{31}O_{14}^-$). This is a very interesting result, as biflavonoids and triflavonoids were not reported as biotransformation products in the literature, being the first report of this event.

Fractional Factorial design

It is possible to verify that different parameters for culture conditions affect directly biotransformation of naringin by *M. inaequisporus*, similar as proposed by the OSMAC methodology for secondary metabolism [24]. During the experimental part 11 compounds were observed in the total ion chromatogram of extracts. The relative area of each compound was recorded (as symbols "-" for undetected, "+", "++" and "+++" for relative areas of increasing relative concentration) and our intention was to identify a condition that is able to produce more compounds as shown in Table 3. In addition, our main interest is also to identify an experiment that produces more rhoifolin, apigenin and methoxyapigenin. The biflavonoids and triflavonoids were not accounted for this calculation.

In this case, weights were attributed for each response: naringin, for example, received a weight 1, naringenin was 2, 4-methoxynaringenin and 4- methoxynaringin were 3 and the three most important compounds mentioned before received a weight 4. After that, all 8 responses for each experiment were combined using geometric mean. This response was used to calculate the effects and to identify the most important variables. The fractional factorial design permits to identify the factors that enhance the biotransformation capability of the microorganism. The most influent factors are in this order, luminosity (variable 6), carbon source (variable 1) and shaking (variable 5) as seen for flasks 6 and 12. The best responses were obtained when the flasks were maintained in the dark (level -1 for variable 6), dextrose was used (level +1 for variable 1) and static (level -1 for variable 5). The other variables (2, 3, 4, 7 and 8) did not present remarkable effects in the studied levels and can be fixed in the most suitable condition.

Mucor inaequisporus biotransformation capability

The *Mucor* is the biggest genus from Mucorales order and comprises in the major part of saprophytic microorganisms that causes food ripening and grain contamination. Some few species are mammalian and plant pathogens [25]. One of the main biotechnology applications for *Mucor* genera is to biotransform natural products, especially steroidal like compounds and terpenoids [26,27]. On the other hand, there are few reports about biotransformation of flavonoids by *Mucor* genera. *Mucor ramannianus* can perform A- and B-ring hydroxylation, demethylation, sulfation and glycosylation in hesperetin [28], hydroxylation, sulfation and glycosylation in cannflavin A and B [29], glycosylation in kaempferol [30], B-ring hydroxylation, glycosylation, and carbonyl reduction in 4'-hydroxyflavanone [31]. Furthermore, *Mucor hiemalis* glycosylated 8-prenylnaringenin [32], *Mucor spinosus* performed the same reaction in cardamonin [33] and a *Mucor miehei* lipase was used to prepare 3-*O*-acylcatechins [34], demonstrating the enzymatic potential for flavonoid modification of this genera.

The LC-MS/MS analyses at high resolution allowed the identification of several microbial products as naringenin (**2**), 4'-methoxynaringenin (**3**), 4'-methoxynaringin (**4**), rhoifolin (**5**), apigenin (**6**), 4- or 6-methoxyapigenin (**7**) and a sugar acetylated naringin (**8**). Biflavonoids and triflavonoids were also detected in some extracts. There are no literature report for biflavonoids (**9** and **10**) and triflavonoids (**11** and **12**) as metabolites obtained from microbial transformation of flavonoids. The microorganism was able to perform deglycosylation, dehydrogenation and *O*-methylation whereas the common hydroxylation [28,29,31] and glycosylation [28-33] reaction for *Mucor* species was not observed. The proposed biotransformation pathway is represented at Figure 4. Naringin, the identified naringin metabolites and its qualitatives correlation in each flask are summarized in Table 3.

Apigenin was once reported as biotransformation product from naringin [35] as well as acylated naringin, produced by Lipozyme catalysis from *Thermomyces lanuginosus*, so this is the first time a whole cell microorganism perform these reactions in naringin [36]. In contrast, 4'-methoxynaringin, 4'-methoxynaringenin, methoxyapigenin and rhoifolin were not previously reported as naringin biotransformation product in literature, so these novel results are very interesting for assessing the *Mucor inaequisporus* enzymatic potential.

Conclusion

The fungus *Mucor inaequisporus* was capable to perform the biotransformation of naringin to eleven different products which were identified through $HRMS^n$ analysis and extensive fragmentation studies. The great number and great structural diversity created by the fungus indicates the vast enzymatic machinery and therefore it inspires the use of this microorganism as a living catalyst that could be used as source for important structural modifications in other substrates and source of valuable enzymes. The mass spectrometry has showed to be a powerful technique for elucidation of flavonoid biotransformation products.

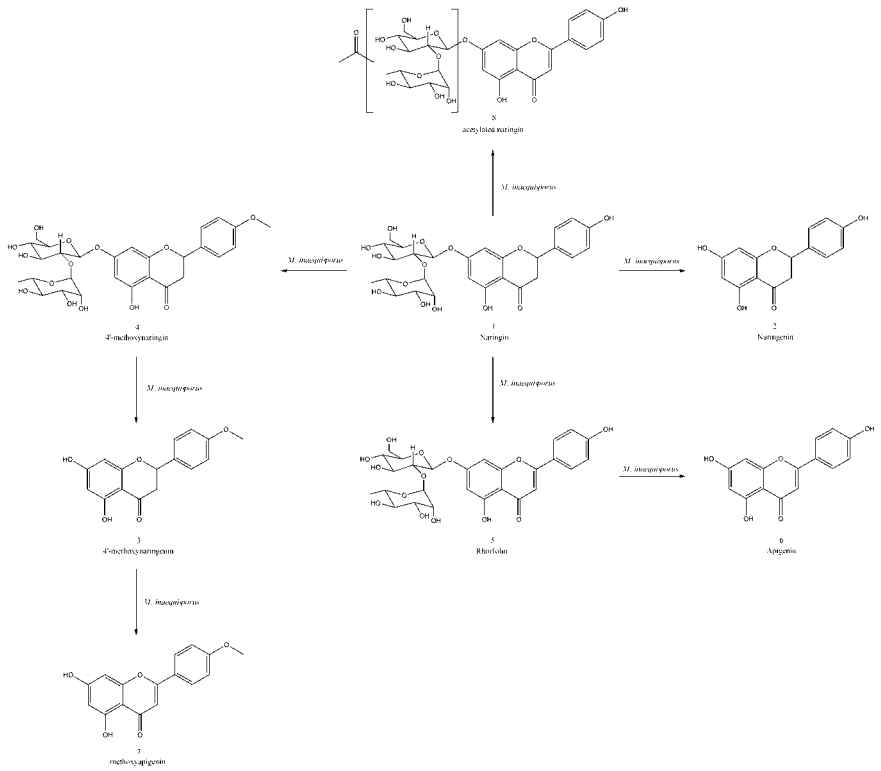

Figure 4: Proposed biotransformation pathway of naringin (**1**) by *Mucor inaequisporus*

Entry	Name	Deprotonated molecule	HRMS		MS^n fragments
			Exp.	Δppm	
1	Naringin	$C_{27}H_{31}O_{14}^-$	579.1715	-1.1	459.1117 ($C_{19}H_{23}O_{13}^-$), 271.0593 ($C_{15}H_{11}O_5^-$), MS^3: 165.0194 ($C_8H_5O_4^-$), 151.0038 ($C_7H_3O_4^-$),119.0503 ($C_8H_7O^-$), 107.0140 ($C_6H_3O_2^-$)
2	Naringenin	$C_{15}H_{11}O_5^-$	271.0606	-1.9	165.0189 ($C_8H_5O_4^-$), 151.0033 ($C_7H_3O_4^-$), 119.0498 ($C_8H_7O^-$), 107.0136 ($C_6H_3O_2^-$)
3	4'-methoxynaringenin	$C_{16}H_{13}O_5^-$	285.0767	-0.7	270.0519 ($C_{15}H_{10}O_5^-$) / 243.0650 ($C_{14}H_{11}O_4^-$) / 164.0108 ($C_8H_4O_4^-$) / 151.0030 ($C_7H_3O_4^-$) / 107.0134 ($C_6H_3O_2^-$)
4	4'-methoxynaringin	$C_{28}H_{33}O_{14}^-$	593.1873	-0.5	285.0750 ($C_{16}H_{13}O_5^-$), MS^3: 270.0536 ($C_{15}H_{10}O_5^-$) / 243.0665 ($C_{14}H_{11}O_4^-$) / 164.0118 ($C_8H_4O_4^-$) / 151.0037 ($C_7H_3O_4^-$) / 107.0140 ($C_6H_3O_2^-$)
5	Rhoifolin	$C_{27}H_{29}O_{14}^-$	577.1560	-0.5	269.0435 ($C_{15}H_9O_5^-$)
6	Apigenin	$C_{15}H_9O_5^-$	269.0453	-0.7	117.0341 ($C_8H_5O^-$)
7	Methoxyapigenin	$C_{16}H_{11}O_5^-$	283.0606	-2.1	HRMS fragments not detected
8	Acetylated naringin	$C_{29}H_{33}O_{15}^-$	621.1818	0.2	579.1689 ($C_{27}H_{31}O_{14}^-$) / 561.1585 ($C_{27}H_{29}O_{13}^-$) / 501.1225 ($C_{25}H_{25}O_{11}^-$) / 271.0596 ($C_{15}H_{11}O_5^-$)

Table 2: HRMS Full Scan and MS^n data for naringin (1) and biotransformation products (2-8)

Compound	f1	f2	f3	f4	f5	f6	f7	f8	f9	f10	f11	f12	f13	f14	f15	f16
Naringin (substrate)	+++	+++	+++	++	+++	++	+++	+++	+++	+	+++	++	+++	+++	+++	+++
Naringenin	+	+	+	+	+	+++	+	++	+	+++	+	+++	+	+	++	+
4'-methoxynaringenin	-	-	+	-	+	++	-	+	-	+	-	++	-	+	+	-
4'-methoxynaringin	++	++	++	++	++	+	++	++	++	+	++	++	++	++	++	++
Rhoifolin	++	++	++	+	++	++	++	++	++	-	++	+	++	++	++	++
Apigenin	+	-	+	-	-	++	+	+	-	+	-	++	-	+	++	-
Methoxyapigenin	-	-	-	-	-	++	-	+	-	+	-	++	-	-	+	+
Acetylated naringin	++	++	++	++	++	+	++	++	++	+	++	++	++	++	++	++

Table 3: Qualitative correlation between naringin and identified biotransformation products in all experiment flasks where "+" means detected by MS, "++" means formation of a chromatographic band, "+++" means majority and "-" means undetected

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