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Potential of yeast antagonists on *invitro* biodegradation of ochratoxin A

S. Patharajan [a,*], K.R.N. Reddy [a], V. Karthikeyan [a], D. Spadaro [b], A. Lore [a], M.L. Gullino [a], A. Garibaldi [a]

[a] Centre of Competence for the Innovation in the Agro-environmental Sector, Università degli Studi di Torino, via L. da Vinci 44, I-10095 Grugliasco (TO), Italy
[b] DiVaPRA-Plant Pathology, Università degli Studi di Torino, via L. da Vinci 44, I-10095 Grugliasco (TO), Italy

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ABSTRACT

Six antagonistic yeast strains were screened initially for their ability to degrade Ochratoxin A (OTA) in liquid medium amended with OTA (7.5 μg ml^{-1}) and different concentrations of yeast cells at 30 °C. Highest OTA degradation was observed when the yeast cell concentration used at 1×10^8 cells/ml ranging from 7.8 to 84%. Out of six yeast strains, three strains [*Metschnikowia pulcherrima* (MACH1), *Pichia guilliemonodii* (M8) and *Rhodococcus erythropolis* (AR14)] were selected for further studies. In preliminary studies yeast strains were evaluated for their ability to degrade OTA at different temperature regimes (10, 15, 20, 25, 30 and 35 °C) and found that 30 °C was the optimum temperature for yeast growth and highest OTA degradation ranging from 10.4 to 83.5%. These three strains were further evaluated to test their potential on OTA degradation using different concentrations of OTA (5 and 10 μg ml^{-1}) only at 30 °C. Three strains showed more or less same results with that of 7.5 μg ml^{-1} concentration ranging from 7.5 to 81%. The three yeast antagonists were further tested to confirm either degradation or cell wall adsorption of OTA at different time intervals. Among the strains, MACH1 effectively degraded the OTA (>80%) at 30 °C after 15 days incubation compared to other strains tested and few amounts of OTA adsorption was observed in the yeast cell wall. LC–MS studies revealed that no by-product like α-OT or Phenylalanine was found during the degradation process. Therefore, further studies are needed to understand the mechanism of action of these yeast strains during OTA degradation.

1. Introduction

Ochratoxin A (OTA) is the most important mycotoxin produced by several species of *Aspergillus* and *Penicillium*. It has been reported that *Aspergillus* section *Nigri* (black aspergilli) are the dominant fungi in the grape fields before harvest and some of its members had shown to produce OTA on grapes (Battilani et al., 2003; Belli, Ramos, Sanchis, & Marin, 2004). In particular *Aspergillus carbonarius* seems to be as a target pathogen, because high percentages (75–100%) of its isolates are able to produce OTA (Belli, Ramos, Coronas, Sanchis, & Marn, 2005) isolated from grapes. The occurrence of OTA was first reported by Zimmerli and Dick (1996) in grape juice and wine. OTA was also found in several food commodities such as grapes, grape juices, cereals, beverages, spices and dried fruits (JEFCA, 2001; O'Brien & Dietrich, 2005). OTA is a nephrotoxic, hepatotoxic, genotoxic, teratogenic and immunotoxic to animals, humans and its carcinogenicity is well-established (Castegnaro et al., 1998). It has been linked to Balkan Endemic Nephropathy and the development of tumors in the urinary tract in humans (Radic, Fuchs, Peraica, & Lucis, 1997). The International Agency for Research on Cancer classified OTA as a possible human carcinogen (group 2B) (IARC, 1993). The contamination of grapes and their by-products by OTA has been emerged as a big problem for the health risk related due to the consumption of such products by human beings (Otteneder & Majerus, 2000). Several strategies are available for the detoxification of mycotoxins. These can be classified as physical, chemical, physicochemical and (micro) biological approaches (Varga & Toth, 2004). However, physical and chemical methods met with varying degrees of success. Microbes or their enzymes could be applied for mycotoxin detoxification; such a biological approaches are now being widely studied (Fuchs et al., 2008; Schatzmayr et al., 2006). Such studies led to the identification of several microbes and their enzymes capable of detoxifying OTA, including bacteria (Silva, Galli, & Grazioli, 2003), yeasts (Angioni et al., 2007; Molnar, Scahtzmayr, Fuchs, & Prillinger, 2004; Schatzmayr et al., 2006), filamentous ascomycetes (Varga, Rigo, & Teren, 2000; Varga, Peteri, Tabori, Teren, & Vagvolgyi, 2005) and basidiomycetes (Engelhardt, 2002). However, in the present study, we carried out *invitro* experiments to evaluate the six antagonistic yeast strains for their ability to degrade OTA in liquid medium.

* Corresponding author. Tel.: +39 011 6708942; fax: +39 011 6709307.
E-mail address: spatharajan@yahoo.co.in (S. Patharajan).

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2. Materials and methods

2.1. Yeast strains and culture conditions

Six strains of yeast *viz*., *Kloeckera lindneri* (GAL5), *Metschnikowia pulcherrima* (MACH1), *M. pulcherrima* (M320), *Pichia guilliermondii* (M8), *P. guilliermondii* (M29) and *Rhodococcus erythropolis* (AR14) were obtained from Agroinnova culture collection centre, University of Torino, Torino, Italy and maintained on malt extract agar slants at 4 °C for further studies. For the preparation of cell suspension, these strains were grown in PM broth (yeast extract 0.5%, sucrose 1%, peptone 0.5% and malt extract 0.2%) (Peteri, Teren, Vagvolgyi, & Varga, 2007) for 2 days at 30 °C and brought to final concentration of 1×10^6, 1×10^7 and 1×10^8 cells/ml.

2.2. OTA degradation by yeast strains using different concentrations of cell suspension

Five ml aliquots of PM broth containing 7.5 μg ml^{-1} OTA (Sigma–Aldrich Chemical Co., O1877, USA) were inoculated with 100 μl cell suspension of six yeast strains individually containing 1×10^6, 1×10^7 and 1×10^8 cells/ml and incubated at 30 °C. Then the cultures were harvested at 24 h, 3, 5, 7, 11, 13 and 15 day intervals by centrifuging the culture at 3500 rpm for 10 min to get cell free culture filtrate. Then the OTA was extracted from culture filtrate using Ochra Test™ column according to the manufacturer instructions. Briefly, 1 ml of culture filtrate was diluted ten times by adding deionised water and the samples were passed through Ochra Test™ immunoaffinity column. After that the columns were washed with 5 ml of water solution containing 2.5% NaCl and 0.5% $NaHCO_3$ followed by 7 ml of deionised water. Then OTA was eluted by adding 0.75 ml of methanol to the columns and collected in amber colour vials. The eluate was dried using N_2 gas through micro plate evaporator. The dried OTA was re-dissolved in 0.5 ml of HPLC grade methanol and stored at 4 °C for further analysis. Controls were included with only media with OTA and media inoculated with yeast strains (without OTA) to find the any metabolite production by yeast strains. Five replications were maintained for each treatment and the experiment was repeated twice.

2.3. OTA degradation by yeast strains at different temperature regimes

For these studies we have selected three yeast strains (MACH1, M8 and AR14) based on the results obtained from previous experiment. To examine the effect of temperature on OTA degradation by yeast, 100 μl cell suspension of three strains containing 1×10^8 cells/ml were inoculated into 5 ml aliquots of PM broth containing 7.5 μg ml^{-1} of OTA. The cultures were cultivated at different temperatures (10, 15, 20, 25, 30, 35 °C). After incubation, the cultures were harvested at different intervals and extracted the OTA as described above. These three strains were further evaluated to test OTA degradation using different concentrations of OTA (5 and 10 μg ml^{-1}) only at 30 °C and harvested at different intervals (after 3, 7, 11 and 15 days) and further processed as described above. Five replications were maintained for each treatment and the experiment was repeated twice.

2.4. Studies on confirmation of either degradation or cell wall adsorption of OTA

After standardizing the yeast cell concentration and optimum temperature, studies were carried out to confirm whether OTA degradation or adsorption by yeast cell wall. For this experiment, 100 μl yeast cell suspension of three strains containing 1×10^8 cells/ml was inoculated in to 5 ml of PM broth containing 7.5 μg ml^{-1} of OTA. The liquid cultures were incubated at 30 °C and the cultures were harvested at 24 h, 3, 5, 7, 11, 13 and 15 days after incubation. Then the cultures were centrifuged at 3500 rpm for 10 min to separate the cells. The OTA was extracted from culture filtrates as described above. OTA was also extracted from yeast cell wall to confirm whether degradation or cell wall adsorption. Briefly, the pellet was sonicated with saline solution (0.85% NaCl w/v) for 30 min and washed twice with saline solution by centrifugation at 4000 rpm for 10 min at 4 °C. The pellet was suspended in 3 ml of saline solution and 3 ml of ethyl acetate. Then they were thoroughly mixed and centrifuged at 4000 rpm for 10 min at 4 °C. The organic phase was separated and evaporated by flash evaporator (system Juan model RC60) and re-dissolved in 1 ml of methanol and analysed through HPLC. Five replications were maintained for each treatment and the experiment was repeated twice.

2.5. Influence of OTA on colony forming units of yeast strains

The colony forming unit (CFUs) was carried out after 1, 3, 5, 7, 11, 13 and 15 days of incubation in PM medium with OTA (7.5 μg ml^{-1}) and without OTA. Yeast cultures were spread on a surface of PM solid medium plate and incubated at 30 °C and the number of colonies formed was counted before and after the incubation periods. The investigation was carried out in triplicate for each treatment.

2.6. Quantification of OTA by HPLC

The OTA was estimated by the method of Bragulat, Abarca, and Cabanes (2001). Samples were analysed in an HPLC Agilent series 1100 formed by a degasser, an autosampler, a quaternary pump, a thermostated column and a fluorimeter. An analytical column RP-18 (150 mm × 4.6 mm i.d., 5 μm) with a pre-column was used. The mobile phase, eluting at 1 ml min^{-1}, consisted of an isocratic mixture of acetonitrile:water:acetic acid (49.5:49.5:1.0) for 18 min. 100 μl of sample were injected onto the HPLC column and OTA was detected through a fluorimeter (λ_{exc} 330 nm; λ_{em} 460 nm) after a retention time of 6.15 min. The amount of OTA in the final solution was determined by using a calibration graph of concentration versus peak area and expressed as ng ml^{-1}, achieved by injection onto the HPLC column of 100 μl of standard solutions of OTA (Sigma–Aldrich chemical Co, 01877). The standard solutions had concentrations of 0.5, 1.0, 5.0, 10.0, 25.0, 50.0 and 100.0 ng ml^{-1}.

2.7. Recovery assay

PM broth was spiked at three concentrations of OTA (0.1, 0.2 and 10.0 ng ml^{-1}). After 1 h, samples were processed according to the above procedure for extraction and estimation of OTA. Recovery experiments were done in four replicates. Recoveries ranged from 90.8 to 92.1%. The repeatability ranged from 2.64 to 2.71% for replicate analysis. The detection limit of the analysis was 0.01 ng OTA ml^{-1} of PM medium.

2.8. LC–MS analysis for detection of OTA byproducts

After estimating the OTA from the samples using HPLC, same samples were used to test the detection of byproducts of OTA by LC–MS analysis. An HPLC system equipped with an SPD11 Avp DAD detector, an SIL 11 AD vp autoinjector, and an LC 10 AD binary pump coupled on line with an MS2010 mass spectrometer (Shimadzu). UV and MS data were acquired and processed using Shimadzu "LC–MS solution" software. Isocratic elution was preformed with H_2O (0.1% TFA): CH_3CN (0.1% TFA, 99% purity) (50:50 v/v) for 20 min. Waters Symmetry C_{18} column was used and its dimension

Table 1
OTA (initial concentration 7.5 μg ml^{-1}) degradation by different antagonistic yeast strains with different cell concentrations at 30 °C.

Cell concentrations	Yeast strains	OTA content and percent degradation						
		24 h	3rd day	5th day	7th day	11th day	13th day	15th day
1×10^6	MACH1	6.16(17.8%) ± 0.06	5.8(22.6%) ± 0.26	4.8(36%) ± 0.22	4.21(43.8%) ± 0.18	3.91(47.8%) ± 0.40	3.61(51.8%) ± 0.29	2.05(72.6%) ± 0.20
	M8	6.2(17.3%) ± 0.18	6.1(18.6%) ± 0.31	5.54(26.1%) ± 0.43	4.72(37%) ± 0.17	4.34(42.1%) ± 0.17	3.21(57.2%) ± 0.40	3(60%) ± 0.21
	M29	6.03(19.6%) ± 0.60	5.95(20.6%) ± 0.69	5.68(24.2%) ± 0.34	5.11(31.8%) ± 0.27	4.94(34.1%) ± 0.25	4.8(36%) ± 0.25	3.95(47.3%) ± 0.27
	M320	6.13(18.2%) ± 0.23	6.1(18.6%) ± 0.68	5.71(23.8%) ± 0.31	5.1(32%) ± 0.61	5.02(33%) ± 0.28	4.9(34.6%) ± 0.22	4.01(46.5%) ± 0.32
	GAL5	6.1(18.6%) ± 0.19	6(20%) ± 0.54	5.48(26.9%) ± 0.22	5.01(33.2%) ± 0.15	4.9(34.6%) ± 0.25	4.78(36.2%) ± 0.38	4.13(44.9%) ± 0.16
	AR14	6.19(17.4%) ± 0.17	6.04(19.4%) ± 0.25	6.26(16.5%) ± 0.31	6.15(18%) ± 0.21	6.09(18.8%) ± 0.17	5.82(22.4%) ± 0.20	5.75(23.3%) ± 0.47
	Control	7.45(0.6%) ± 0.13	7.45(0.6%) ± 0.14	7.42(1.0%) ± 0.44	7.25(3.3%) ± 0.11	7.32(2.4%) ± 0.55	7.3(2.6%) ± 0.32	7.3(2.6%) ± 0.31
1×10^7	MACH1	6.16(17.8%) ± 0.06	5.63(24.9%) ± 0.40	4.61(38.5%) ± 0.43	3.96(47.2%) ± 0.18	3.54(52.8%) ± 0.33	3.15(58%) ± 0.12	1.41(81.2%) ± 0.37
	M8	6.12(18.4%) ± 0.11	5.82(22.4%) ± 0.33	5.04(32.8%) ± 0.25	4.34(42.1%) ± 0.26	4.09(45.4%) ± 0.18	2.91(61.2%) ± 0.24	2.89(61.4%) ± 0.28
	M29	6.02(19.7%) ± 0.19	5.96(20.5%) ± 0.64	5.44(27.4%) ± 0.37	4.83(35.6%) ± 0.35	4.88(35%) ± 0.39	4.70(37.3%) ± 0.36	3.81(49.2%) ± 0.26
	M320	6.06(19.2%) ± 0.62	6.04(19.4%) ± 0.69	5.58(25.6%) ± 0.42	4.93(34.2%) ± 0.38	4.95(34%) ± 0.29	4.81(35.8%) ± 0.42	3.88(48.2%) ± 0.43
	GAL5	6.09(18.8%) ± 0.29	5.89(21.4%) ± 0.76	5.26(30.6%) ± 0.39	4.82(35.7%) ± 0.42	4.77(36.4%) ± 0.28	4.61(38.5%) ± 0.40	3.96(47.2%) ± 0.45
	AR14	6.16(17.8%) ± 0.17	6.03(19.6%) ± 0.22	6.14(18.1%) ± 0.43	6.05(19.3%) ± 0.24	6.03(19.6%) ± 0.24	5.80(22.6%) ± 0.25	5.71(23.8%) ± 0.26
	Control	7.45(0.6%) ± 0.13	7.45(0.6%) ± 0.14	7.42(1.0%) ± 0.44	7.25(3.3%) ± 0.11	7.32(2.4%) ± 0.55	7.3(2.6%) ± 0.32	7.3(2.6%) ± 0.31
1×10^8	MACH1	6.14(18.1%) ± 0.04	5.6(25.3%) ± 0.44	4.59(38.8%) ± 0.34	3.91(47.8%) ± 0.29	3.5(53.3%) ± 0.28	3.11(58.5%) ± 0.13	1.2(84%) ± 0.40
	M8	6.03(19.6%) ± 0.17	5.71(23.8%) ± 0.29	4.92(34.4%) ± 0.29	4.27(43%) ± 0.27	4.06(45.8%) ± 0.16	2.84(62.1%) ± 0.29	2.5(66.6%) ± 0.31
	M29	6.05(19.3%) ± 0.64	5.87(21.7%) ± 0.66	5.38(28.2%) ± 0.36	4.79(36.1%) ± 0.35	4.83(35.6%) ± 0.28	4.63(38.2%) ± 0.37	3.25(56%) ± 0.41
	M320	6.01(19.8%) ± 0.36	5.9(21.3%) ± 0.60	5.45(27.3%) ± 0.37	4.88(34.9%) ± 0.37	4.89(34.8%) ± 0.29	4.73(36.9%) ± 0.46	3.26(56.5%) ± 0.38
	GAL5	6.08(18.9%) ± 0.64	5.77(23%) ± 0.64	5.11(31.8%) ± 0.19	4.82(35.7%) ± 0.19	4.65(38%) ± 0.42	4.5(40%) ± 0.32	3.68(51%) ± 0.37
	AR14	6.91(7.8%) ± 0.14	6.8(9.3%) ± 0.21	6.8(9.3%) ± 0.42	6.55(12.6%) ± 0.42	6.1(18.6%) ± 0.42	5.85(22%) ± 0.20	5.56(25.8%) ± 0.86
	Control	7.45(0.6%) ± 0.13	7.45(0.6%) ± 0.14	7.42(1.0%) ± 0.44	7.25(3.3%) ± 0.11	7.32(2.4%) ± 0.55	7.3(2.6%) ± 0.32	7.3(2.6%) ± 0.31

Table 2
OTA (initial concentration 7.5 μg ml^{-1}) degradation by selected antagonistic yeast strains (10^8 cells/ml) at different temperature regimes.

Yeast strains	Temp (°C)	OTA content and percent degradation						
		24 h	3rd day	5th day	7th day	11th day	13th day	15th day
MACH1	10	6.25(16.6%) ± 0.22	6.12(18.4%) ± 0.47	5.9(21.3%) ± 0.26	5.88(21.6%) ± 0.26	5.72(23.7%) ± 0.52	5.54(26.1%) ± 0.53	5.14(31.4%) ± 0.71
	15	6.25(16.6%) ± 0.21	6.12(18.4%) ± 0.46	5.9(21.3%) ± 0.45	5.88(21.6%) ± 0.26	5.72(23.7%) ± 0.39	5.54(26.1%) ± 0.49	5.14(31.4%) ± 0.77
	20	6.07(19%) ± 0.14	5.8(22.6%) ± 0.29	5.3(29.3%) ± 0.64	5.0(33.3%) ± 0.64	4.76(37.3%) ± 0.81	4.4(41.3%) ± 0.49	3.9(48%) ± 0.47
	25	6.07(19%) ± 0.14	5.66(24.5%) ± 0.22	5.04(32.8%) ± 0.63	4.52(43.3%) ± 0.45	4.12(45%) ± 0.39	3.8(49.3%) ± 0.52	2.56(65.8%) ± 0.59
	30	6.16(17.8%) ± 0.06	5.63(25%) ± 0.38	4.61(38.5%) ± 0.39	3.96(47.2%) ± 0.17	3.54(52.8%) ± 0.31	3.15(58%) ± 0.12	1.2(83.5%) ± 0.35
	35	6.2(17.3%) ± 0.12	5.7(24%) ± 0.26	4.4(41.3%) ± 0.39	4.0(46.6%) ± 0.16	3.6(52%) ± 0.26	3.62(51.7%) ± 0.18	2.85(62%) ± 0.33
M8	10	6.44(14.1%) ± 0.24	6.41(14.5%) ± 0.22	6.38(14.9%) ± 0.53	6.14(18.1%) ± 0.39	6.0(20%) ± 0.33	5.92(21%) ± 0.40	5.74(23.4%) ± 0.36
	15	6.46(13.8%) ± 0.22	6.37(15%) ± 0.27	6.48(13.6%) ± 0.36	6.36(15.2%) ± 0.29	6.0(20%) ± 0.33	5.92(21%) ± 0.49	5.8(22.6%) ± 0.51
	20	6.18(17.6%) ± 0.17	6.05(19.3%) ± 0.17	5.8(22.6%) ± 0.54	5.56(25.8%) ± 0.55	5.0(33.3%) ± 0.36	4.74(36.8%) ± 0.87	4.56(39.2%) ± 0.96
	25	6.16(17.8%) ± 0.19	5.82(22.4%) ± 0.36	5.36(28.5%) ± 0.65	4.82(35.7%) ± 0.77	4.56(39.2%) ± 0.70	4.26(43.2%) ± 0.41	3.8(49.3%) ± 0.53
	30	6.12(18.4%) ± 0.10	5.82(22.4%) ± 0.32	5.04(32.8%) ± 0.24	4.34(42.1%) ± 0.25	4.09(45.4%) ± 0.17	2.91(61.2%) ± 0.22	2.65(65.3%) ± 0.26
	35	6.3(16%) ± 0.36	5.9(21.3%) ± 0.38	5.2(30.6%) ± 0.43	4.5(40%) ± 0.24	4.3(42.6%) ± 0.14	3.52(53%) ± 0.18	2.9(61.3%) ± 0.21
AR14	10	6.6(12%) ± 0.27	6.66(11.2%) ± 0.14	6.4(14.6%) ± 0.55	6.46(13.8%) ± 0.43	6.26(16.5%) ± 0.31	6.08(18.9%) ± 0.38	6.02(19.7%) ± 0.36
	15	6.9(8%) ± 0.35	6.84(8.8%) ± 0.11	6.82(9%) ± 0.34	6.66(11.2%) ± 0.42	6.4(14.6%) ± 0.20	6.32(15.7%) ± 0.19	6.12(18.4%) ± 0.24
	20	6.66(10.4%) ± 0.25	6.58(12.2%) ± 0.15	6.34(15.4%) ± 0.51	6.3(16%) ± 0.27	6.26(16.5%) ± 0.44	5.96(20.5%) ± 0.44	5.78(22.9%) ± 0.42
	25	6.72(10.4%) ± 0.24	6.6(12%) ± 0.19	6.64(11.4%) ± 0.20	6.2(17.3%) ± 0.18	6.32(15.7%) ± 0.32	6.0(20%) ± 0.26	5.92(21%) ± 0.54
	30	6.72(10.4%) ± 0.34	6.68(10.9%) ± 0.32	6.55(12.6%) ± 0.20	6.52(13%) ± 0.48	6.42(14.4%) ± 0.23	6.46(13.8%) ± 0.38	5.68(24.2%) ± 0.24
	35	6.7(10.6%) ± 0.36	6.7(10.6%) ± 0.28	6.6(12%) ± 0.20	6.5(13.3%) ± 0.48	6.4(14.6%) ± 0.34	6.6(12%) ± 0.26	6.0(20%) ± 0.18

Table 3
Effect of yeast antagonists on degradation of OTA used at different concentrations at 30 °C.

OTA concentration	Intervals	Yeast strains and OTA content and percent degradation		
		MACH1	M8	AR14
5 μg ml^{-1}	3 days	3.78(24.4%) ± 0.23	3.85(23%) ± 0.26	4.45(11%) ± 0.52
	7 days	2.46(51%) ± 0.45	2.95(41%) ± 0.31	4.22(15.6%) ± 0.48
	11 days	2.15(57%) ± 0.26	2.52(49.6%) ± 0.35	4.20(16%) ± 0.32
	15 days	0.95(81%) ± 0.35	2.12(58%) ± 0.52	3.98(20.4%) ± 0.21
	Control	4.78(4.4%) ± 0.15	4.75(5%) ± 0.47	4.85(3%) ± 0.38
10 μg ml^{-1}	3 days	7.45(25.5%) ± 0.62	8.01(20%) ± 0.25	9.25(7.5%) ± 0.45
	7 days	5.36(46.4%) ± 0.42	6.11(39%) ± 0.64	8.55(14.5%) ± 0.32
	11 days	5.12(49%) ± 0.28	5.16(48.4%) ± 0.35	8.26(17.4%) ± 0.61
	15 days	1.95(80.5%) ± 0.32	3.45(65.5%) ± 0.32	7.95(20.5%) ± 0.42
	Control	9.45(5.5) ± 0.54	9.74(2.6%) ± 0.42	9.82(1.8%) ± 0.39

was 150 × 2.1 mm i.d., 3.5 μm particle size. The injection volume was 20 μl and the flow rate was set at 0.2 ml min^{-1}. According to the λ_{max} of the UV spectra of OTA, UV-DAD detection was carried out at 215 nm for the detection of phenylalanine. In this chromatographic condition, the retention time for OTA was 9.58 min. The ESI-MS interface was operated in the positive mode: ESI CDL, 200 °C; block at 200 °C; flow gas (N2) at 4.5 ml min^{-1}; probe voltage, 3 kV; scan mode, 100–600 amu; selected ion monitoring, 405; 427 amu for OTA; and 257 amu for α-OTA (the analysis for the determination of α-OTA residues was done in the flow injection mode), respectively.

3. Results

3.1. OTA degrading ability of yeast strains

In a preliminary study six yeast isolates were tested for their ability to degrade OTA under *invitro* conditions using three different cell concentrations (1×10^6, 1×10^7 and 1×10^8 cells/ml). All the strains were able to degrade OTA in a liquid medium (PM). The degradation of OTA was started at 24 h and it was continued until day 15. Among the six strains with three different cell concentrations, five strains at 1×10^8 cells/ml were effectively degraded OTA more than 50% after 15 days incubation at 30 °C. Of which, MACH1 able to degrade OTA at maximum level (84%) followed by other strains M8 (66.6%), M29 (56%), M320 (56.5%) and GAL5 (51%) at 30 °C after 15 days interval. Strain AR14 was showed very low degradation (25.8%) even after 15 days incubation at 30 °C (Table 1). In control also we observed some degradation (0.6–2.6%) of OTA. This is may be due to some other environmental factors.

3.2. OTA degradation by yeast strains at different temperature regimes

After the preliminary screening, two effective strains, MACH1 and M8 and one ineffective strain (AR14) were further selected for their degradation ability of OTA at different temperatures using at 1×10^8 cells/ml. Six different temperatures were studied to optimize the OTA degradation and found that highest degradation of OTA at 30 °C compared to other temperature regimes by both yeast strains. The degradation was started at 24 h and it was continued until 15 days incubation. Strain MACH1 effectively degraded the OTA (83.5%) followed by M8 (65.3%) at 30 °C after 15 days incubation (Table 2). Strain AR14 showed less degradation (24.2%). We observed some degradation in control (0.6–3%). These three strains were further tested using different concentrations of OTA (5 and 10 μg ml^{-1}). All strains were degraded the OTA more or less same with that of 7.5 μg ml^{-1} ranging from 7.5 to 81% (Table 3).

3.3. Confirmation studies for either degradation or cell wall adsorption of OTA

In this study we confirmed that either degradation or cell wall adsorption of OTA by yeast strains. In this experiment we extracted the OTA from cell wall and culture filtrate separately after the

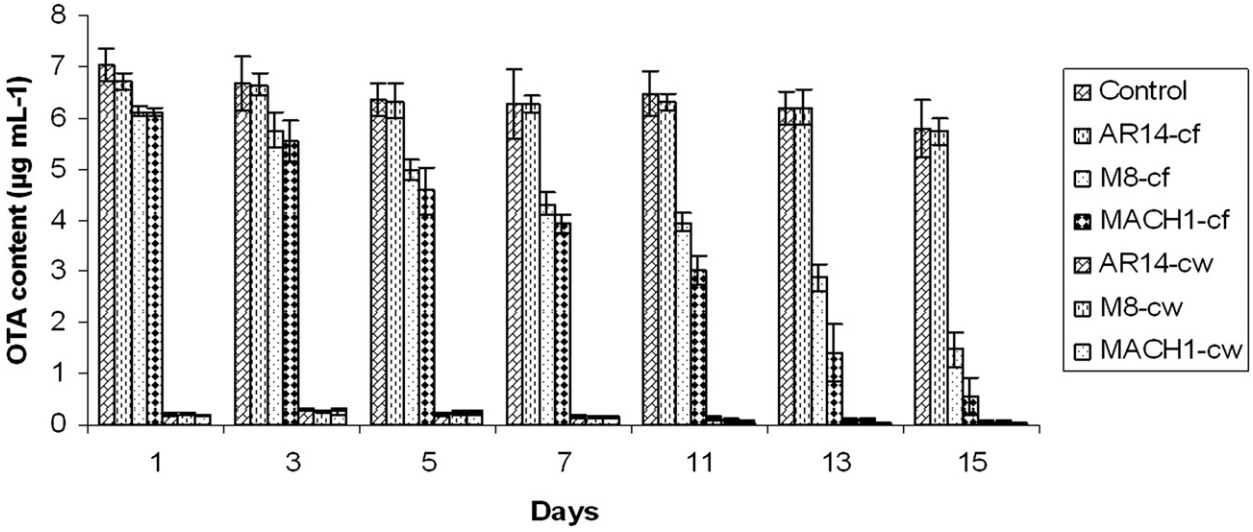

Fig. 1. OTA degradation and or adsorption by the selected antagonistic yeast strain at different intervals. MACH1– *Metschnikowia pulcherrima*; M8– *Pichia guillermonodii*; AR14– *Rhodococcus erythropolis*. cf – Culture filtrate; cw – Cell wall.

Table 4
CFU of the selected yeast antagonistic strains at different intervals (30 °C).

Treatment	CFU/ml							
	Initial	24 h	Day 3	Day 5	Day 7	Day 11	Day 13	Day 15
OTA + MACH1	1.0×10^8	1.2×10^9	1.7×10^9	1.0×10^9	2.0×10^8	1.8×10^7	1.5×10^7	1.0×10^7
OTA + M8	1.0×10^8	1.0×10^9	1.4×10^9	1.0×10^9	1.5×10^8	1.5×10^7	1.0×10^7	1.0×10^7
OTA + AR14	1.0×10^8	1.0×10^8	1.0×10^9	1.2×10^8	0.5×10^8	2.0×10^7	1.5×10^6	1.0×10^6
MACH1 alone	1.0×10^8	1.5×10^9	1.0×10^{10}	2.0×10^9	1.0×10^9	2.5×10^7	2.0×10^7	1.5×10^7
M8 alone	1.0×10^8	1.0×10^9	1.8×10^9	2.0×10^9	1.3×10^9	2.0×10^7	1.8×10^7	1.0×10^7
AR14 alone	1.0×10^8	1.0×10^8	1.0×10^9	3.0×10^8	1.0×10^8	8.6×10^7	1.5×10^7	1.0×10^7

incubation period at 30 °C. MACH1 and M8 started to degrade OTA from day 1 to day 15 of the intervals. The cell wall adsorption of OTA is very less by yeast strains ranging from 4 to 5.5% with MACH1 and 2.2–3.4% with M8 after 15 days interval (Fig. 1). In order to assess bio absorption and adsorption effects by yeast strains, further experiment was conducted with heat killed inactivated yeast strains in the same experimental condition and found no diminution of the initial OTA concentration occurred during 90 h (data not shown), so that both bio absorption and adsorption effects could be excluded.

3.4. Influence of OTA on yeast colony forming units

In order to study the effect of OTA toxicity on the yeast strains, the colony forming units (CFU) for yeast concentrations were recorded at different day intervals. *Invitro* inhibitory effects were determined by distinguishing between CFU without and with OTA. As shown in Table 4 addition of OTA, had minor effect on the growth of yeast strains used for this study. We have not observed any major variations in yeast cell concentration after adding different concentrations of OTA (5 and 10 μg ml^{-1}) in liquid medium.

3.5. Detection of OTA by-products by LC–MS

The main by-products of OTA during fungi mediated degradation were α-OTA and phenylalanine. LC–MS was the only method for compound identifications matching t_R values and ESI fragmentation patterns with the authentic standard. In the present study, our sample analysis performed in the positive ESI mode gave the following fragments and present abundances fragmented ions at: *m/z* 405 (100%) $[M + H]^+$, 427 (25%) $[M + Na]^+$. Confirmation of the OTA was assessed in selective ion monitoring (SIM), recording the ions 405 and 427 (Fig. 2). We have not found any peaks related to α-OTA and phenylalanine.

4. Discussion

Mycotoxin contamination of agricultural products and fruit juices is a serious health hazard throughout the world. The present study demonstrated that the antagonistic yeast strains were able to degrade OTA during their growth in nutrient medium. In a preliminary study, four antagonistic yeast strains were able to degrade OTA (>50%) in a liquid medium and one strain (MACH1) more than 80% within 15 days at 30 °C. There is no influence of OTA on yeast cell concentration even at higher concentrations. OTA degradation mediated by microorganisms has been studied by many authors. For example, Peteri et al. (2007) reported that more than 90% of OTA degradation by yeast strains on 7th day incubation at 20 °C. In another study, Bejaoui, Mathieu, Taillandier, and Lebrihi (2004) reported about 90% of OTA reduction by *Saccharomyces* strains at 30 °C on 72 h. Our yeast strains also started to degrade OTA from

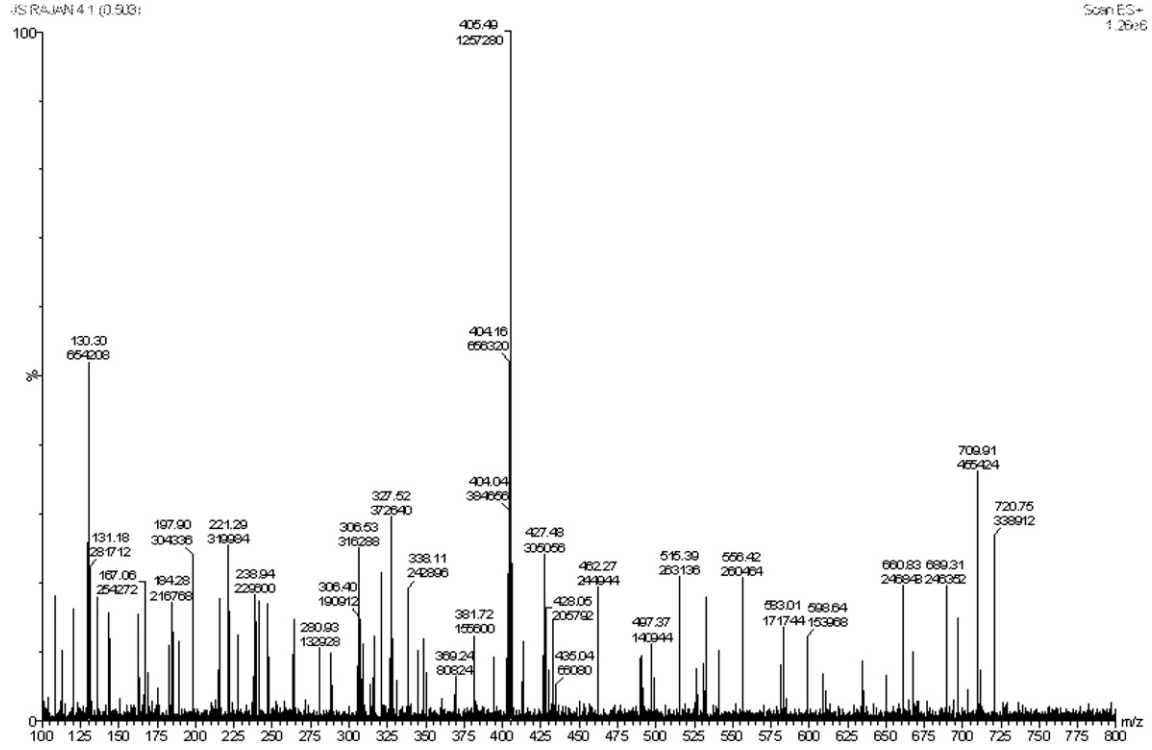

Fig. 2. ESI-MS mass spectra of OTA; *m/z* 405 $[M + H]^+$ molecular ion, *m/z* 427 $[M + Na]^+$ ion.

24 h and it was continued until 15 days incubation (>80%). Valero, Sanchis, Ramos, and Marin (2008) observed the significant OTA degradation by *Botrytis cinerea* on OTA spiked medium under *invitro* conditions. Varga et al. (2000) and Abrunhosa, Serra, and Venancio (2002) studied the OTA degradation activity in liquid medium by *Aspergillus* spp. and they found that *A. fumigatus* and black *Aspergillus* strains were able to detoxify OTA in culture media.

Kinetic study on the confirmation of OTA degradation or cell wall adsorption by selected yeast strains revealed that MACH1 and M8 have the ability to degrade highest OTA in a liquid medium. The yeast cell walls were adsorbed very less amount of OTA during the incubation period. These results are in agreement with Angioni et al. (2007) who had reported cell walls of *Saccharomyces cerevisiae* and *Kloeckera apiculata* do not adsorb the OTA under *invitro* interaction between OTA and the yeast strains. In a study, Bejaoui et al. (2004) had found that 90% of OTA rapidly bound to yeast cell wall in synthetic medium and natural grape juice medium. Our results also showed that yeast cell walls used in this study have the ability to absorb OTA (<6%) during degradation in liquid medium. Cecchini, Morassut, Garcia Moruno, and Di Stefano (2006) reported that up to 70% of OTA removal by yeast strains during fermentation of white and red musts by adsorption rather than biodegradation. In our study we found that the OTA biodegradation or cell wall absorption was strain dependent and one strain (AR14) was found sensitive to OTA. These results are in agreement with Piotrowska and Zakowska (2005) who had reported some strains of lactic acid bacteria was sensitive to OTA and others were capable of reducing OTA at different levels and they concluded that the ability of lactic acid bacteria in reducing OTA levels was strain dependent.

LC–MS studies revealed that OTA by-products like Phenylalanine or α-OTA was not found either in culture filtrate or yeast cell wall during OTA degradation. The absence of α-OTA and phyenlalaine in the media and in the pellet was ensured with the finding of Angioni et al. (2007). But this result did not ensure that the common degradation pathway suggested by Abrunhosa et al. (2002), because phenylalanine and α-OTA could also react with other compounds in the media. Many authors reported that the OTA adsorption phenomena. When the degradation product searched by these authors, they did not find any specified product, which allowed them to conclude that OTA was adsorbed by the yeast (Angioni et al., 2007). Bejaoui et al. (2004) reported that the ability to remove OTA residues from conidia of *Aspergillus* species. This experiment carried out on both SGM and natural red wine led to a decrease of OTA content and in some cases to the occurrence of α-OTA, but the removing effects were ascribed to adsorption on the pellet. On the other hand, no analysis was carried out on the yeast cell walls, and the degradation products may potentially be chemicals that are not detectable, neither with the UV nor with the fluorescence detector. Nevertheless, the decrease of OTA levels found by Bejaoui et al. (2004) is also comparable with the present study. Ringot et al. (2005) reported *invitro* experiments of the spontaneous nature of OTA to adsorb on vinaccia with 16% of yeast, dry yeast cell walls, and β-glucan. In the wine, at pH 3.5, OTA is partially dissociate, showing a positive charge on the amino group ($NH4+$); for these reasons, the OTA phenolic groups and carboxyl groups could be involved in different mechanisms of absorption by phenolic substances present in the lees. Leong, Hocking, Varelis, Giannikopoulos, and Scott (2006) reported that the fate of OTA during vinification, after inoculation with two different strains of *A.carbonarius*. No OTA reduction was reported during vinification. But, during the solid-liquid separation stage, they observed a higher repartition of OTA in the solid phase. However, they concluded that further research was needed on the nature of OTA binding precipitated by grape constituents and yeasts.

5. Conclusion

The results of this study conclude that antagonistic yeast strains were able to degrade OTA more than 80% under *invitro* conditions. These yeast strains can be effectively used to reduce OTA up to acceptable levels in beverages and other food products. The absence of OTA in yeast cell wall excluded an adsorbing effect by different antagonistic yeast strains studied, and the absence of OTA by-products like α-OTA and phenylalanine suggested that other degradation pathways of OTA. The use of OTA isotopically labelled could help us to understand the various degradation pathways of this mycotoxin. Further studies on mechanism of yeasts during OTA degradation is under progress.

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