DEGRADATION of AFLATOXIN B_1 in VARIOUS FOODS by *Nocardia corynebacteriodes* (*Flavobacterium aurantiacum*) NRRL B-184 *

Çeşitli Gıda Maddelerinden *Nocardia corynebacteriodes* (*Flavobacterium aurantiacum*) NRRL B-184 ile Aflatoksin B_1'in Degradasyonu

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ABSTRACT

In this study, the ability of *Nocardia corynebacteriodes* NRRL B-184 strain to remove aflatoxin B_1 in PB solution and dry red pepper, corn, black olive, soy bean, dry fig and also hazelnut, was investigated. The activated *N. corynebacteriodes* strain was incubated in TSB at 30°C for 95 h and growing curve was obtained. According to non-linear regression analysis, Modified Gompertz model was fitted best with experimental data. The μ_{max} and λ were found as 0.073 h^{-1} and 5.244 h^{-1}, respectively. It was observed that *N. corynebacteriodes* strain increased by 2.7 log and reached to the stationary phase within 45 h. The bacteria were still in that phase at 95 h. First order reaction kinetics was fitted best with the degradation kinetics in PB and food mediums. In PB medium, the "k value" was found higher and followed by milled dry fig and whole black olive. At the end of incubation, the reduction of aflatoxin B_1 content were resulted in the range of 84.28% and 98.84% at soy bean and hazelnut, respectively. After incubation, aflatoxin content of milled red pepper, dry fig and hazelnut that contain 500 ng g^{-1} aflatoxin B_1 and whole hazelnut (1000 ng g^{-1} aflatoxin B_1) was decreased to permitted level of this toxin in Turkish Food Codex.

Key Words: *F. aurantiacum*, Aflatoxin B1, Detoxification, Kinetic

ÖZET

Bu çalışmada, *Flavobacterium aurantiacum* NRRL B-184 suşunun potasyum fosfat tamponu (PFT) ortamında ve sıklıkla aflatoksin sorunu yaşanan kırmızı biber, mısır, zeytin, soya fasulyesi, kuru incir ve fındıkta aflatoksin B_1 (AFB_1)'i ortamdan uzaklaştırma yeteneği araştırılmıştır. Aktifleştirilmiş *F. aurantiacum* NRRL B-184 suşunun triptik soy broth (TSB) besiyerinde ve 30°C inkübasyon sıcaklığındaki gelişimine ait gelişme eğrisi elde edilmiş ve doğrusal olmayan regresyon analizi sonuçlarına göre gelişme eğrisini tanımlamada Modifiye Gompertz modelinin daha uygun olduğu sonucuna varılmıştır. Modelden elde edilen verilere göre, *F. aurantiacum* NRRL B-184 suşunun μ_{max} 0.073 $saat^{-1}$, λ ise 5.244 saat olduğu hesaplanmıştır. *F. aurantiacum* NRRL B-184 suşunun 45 saatte 2.7 log'luk bir artışla durgun faza geçtiği ve 95. saat sonunda hala durgun fazda olduğu belirlenmiştir. PFT ve gıda ortamlarındaki azalmanın "Birinci Dereceden Reaksiyon Kinetiğine" uygun olduğu tespit edilmiştir. Bu modele göre, "k" değeri en yüksek PFT ortamlarında bulunmuş ve bunu öğütülmüş ürünlerde genel itibariyle incir, bütün ürünlerde ise zeytin izlemiştir. İnkübasyon süresi sonunda ürünlerin AFB_1

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içeriğindeki azalma 1000 ng g^{-1} AFB_1 içeren bütün haldeki soya fasulyesinde %84.28 ile en düşük ve 500 ng g^{-1} AFB_1 içeren öğütülmüş fındıkta %99.84 ile en fazla olarak gerçekleşmiştir. 72 saatlik inkübasyon sonunda 500 ng g^{-1} AFB_1 içeren öğütülmüş kırmızı biber, 500 ng g^{-1} AFB_1 içeren öğütülmüş kuru incir ile 500 ve 1000 ng g^{-1} AFB_1 içeren öğütülmüş fındık örnekleri Türk Gıda Kodeksinde bu ürünlerin insan gıdası olarak kullanılması durumunda izin verilen AFB_1 düzeylerine inebilmiştir.
Anahtar Kelimeler: *F. aurantiacum*, Aflatoksin B_1,Detoksifikasyon, Kinetik

INTRODUCTION

Mycotoxins are extremely toxic chemical substances produced by certain filamentous fungi growing naturally in many agricultural commodities especially in corn, various nuts, oilseeds, dry red pepper and dry fig in the field and also during harvest, storage, transportation and processing into food or animal feed (D'souza and Brackett, 2000; Smith, 2001). Aflatoxins, a group of secondary metabolites produced by *A. flavus* and *A. parasiticus*, are among the most potent of all mycotoxins (Hao and Brackett, 1989). These compounds are highly toxic, carcinogenic, mutagenic, teratogenic and immunosupressor (Line and Brakett, 1995; Stark, 2001). Aflatoxin B_1 is considered the most potent carcinogen of all the aflatoxins. Due to high incidence of aflatoxin in agricultural commodities, it poses major health and economical problems. Aflatoxin B_1 is carcinogenic to many organs and primary liver cancer is one of the most prevalent human cancers in developing countries. Very strong correlation exists between the daily dietary intake of aflatoxin B_1 and the incidence of primary liver cancer in humans. Although, it is believed that there are combined actions of aflatoxins and hepatitis B virus infection leading to hepatocellular carcinoma (Van Genderen, 1997; Stark, 2001). Physical segregation, solvent extraction and inactivation by physical, chemical or biological methods are presently used to reduce aflatoxin content in food and feedstuffs (Hao and Brackett, 1988; D'souza and Brackett, 2000). The use of many available physical and chemical methods for the detoxification is restricted due to limited efficacy, losses of nutritional value and high cost. Therefore, detoxification by biodegradation should be the best solution for removal of mycotoxins under mild conditions without using harmful chemicals and significant nutritional losses and also retaining the palatability of decontaminated food and feed (Bata and Lasztity, 1999). Ciegler et al. (1966) screened approximately 1000 microorganisms for their ability to degrade aflatoxins. They concluded that *Flavobacterium aurantiacum* NRRL B-184 was the only bacterium capable of removing aflatoxins irreversibly from test substrates. Except of *F. aurantiacum*, some of the bacteria, yeasts and molds have an absorption or degradation ability of aflatoxin B_1 (Doyle et al., 1982; Teniola et al., 2005). In this study, growth characteristics of *N. corynebacteriodes* NRRL B-184 (formerly erroneously classified as *Flavobacterium aurantiacum*) in tryptic soy broth were determined and the ability of this bacterium to degrade aflatoxin B_1 from PB and some of the agricultural commodities was investigated.

MATERIALS and METHODS

Preparation of food products

Corn, black olive, soybean, dry fig and hazelnut were purchased from local markets in Adana and dry red pepper in Kahramanmaraş, Turkey. Milled and whole forms of the products were sterilized in autoclaved at 121°C for 15 min. and then used. The products were prepared just before using.

Reagents

All solvents (HPLC grade), all reagents (analytical grade), tryptic soy broth (TSB) and tryptic soy agar (TSA) were purchased from Merck (Germany). Standard of aflatoxin B_1 from Sigma (Germany) and immunoaffinity columns (IAC) Aflaprep (R-Biopharm Rhóne, Glasgow, Scotland) were used.

Cell Culture

Nocardia corynebacteriodes NRRL B-184 was obtained from D.P. Labeda (USDA, National Center for Agricultural Utilization Research, Microbial Genomics & Bioprocessing Research Unit, Peoria, Illinois) in lyophilized form. The cells were activated in TSB at 30°C for 48 h and stock culture was maintained on TSA. Cells were activated by three successive transfers in TSB and incubated at 30°C for 48 h with no agitation.

Growth of *N. corynebacteriodes* NRRL B-184 in TSB

Activated *N. corynebacteriodes* culture about 10^7 (CFU mL^{-1}) cells was transferred into a flask containing 100 ml of culture medium. The inoculated flasks were incubated at 30°C in a water bath with a shaker and continuously agitated at 50 rpm for 95 h. Populations of *N. corynebacteriodes* were determined periodically during 95 h incubation. Microbial growth was monitored by measuring the optical density (OD) at 540 nm of the culture suspensions (Lillehoj et al. 1967). In order to convert OD values to CFU mL^{-1}, serial dilutions were made from stock culture solution. The OD values for the dilutions were measured with UV-Vis spectrophotometer (Shimadzu) and also bacterial enumeration by surface spreading on TSA was performed. Calibration curve was drawn by plotting OD versus number of *N. corynebacteriodes* (log CFU mL^{-1}).

Harvest of stationary phase cells of *N. corynebacteriodes*

Approximately 10^7 (CFU mL^{-1}) cells *N. corynebacteriodes* were inoculated into flask containing 50 ml of TSB and incubated at 30°C for 72 h in a water bath with a shaker and continuously agitated at 50 rpm. After incubation, TSB containing *N. corynebacteriodes* was centrifuged at 3500 rpm for 20 min. and the supernatant was discarded. The pellet washed with 0.067 M PB (pH 6.7) and recentrifuged. The bacterial cell pellet was resuspended in buffer and used in the experiments.

Determination of AFB_1 degradation in PB and food mediums

Different concentrations (500, 1000 and 2000 ng mL^{-1}) of AFB_1 were added into flasks and the solvent was evaporated by nitrogen (N_2) gas at 30°C. Sterilized PB (50 mL)

and then stationary cells (10^7 CFU mL^{-1}) of *N. corynebacteriodes* was supplemented. After the addition of sterilized food samples (50 g) into flask different concentrations (500, 1000 and 2000 ng mL^{-1}) of AFB_1 were added. According to Das and Mishra (2000) test samples were waited for about 2h in a cool and dark place to penetration of the toxin. Stationary cells (10^7 CFU mL^{-1}) of *N. corynebacteriodes* were inoculated and sterilized PB (50 mL) was added later. Controls containing AFB_1 but no cells were also prepared. All samples were incubated at 30°C in a water bath with shaker and continuously agitated at 50 rpm for 72 h. AFB_1 contents were determined by taking duplicate samples after 0, 6, 12, 24, 36, 48 and 72 h. Experiment were replicated triple. Triple replication was made in all trials.

Determination of AFB_1

Preparation of AFB_1 standard and phosphate buffered saline solutions and also extraction and clean up procedure were determined by AOAC (2000). AFB_1 was determined by AOAC Official Method 999.07. 100 µL of the sample was injected into HPLC. Determinations of AFB_1 levels were carried out by HPLC using the following equipment: A Hewlett Packard HPLC system (Hewlett Packard, Agilent 1100, Palo Alto, USA) equipped with an auto sampler Agilent 1100 Series and a HP Agilent 1100 fluorescence detector; excitation and emission wavelengths were 360 and 440 nm, respectively. The HPLC column was Ace 5 C18 (25 cm-4.6 mm i.d.) (Advanced Chromatography Technologies, Aberdeen, Scotland). The mobile phase was the mixture of acetonitrile–methanol–water (2:3:6, v/v/v) with the addition of 120 mg L^{-1} potassium bromide and 350 µ L^{-1} nitric acid. The flow rate was 1 mL min^{-1}. For the post column derivatization a Kobra cell (Rhone Diagnostics, Glasgow, UK) was used. The determination coefficient of the standard curve was 0.9998. Samples were artificially contaminated with AFB_1 at two levels (10-50 ng mL^{-1}). Based on results for spiked samples, the mean recoveries (n=12) were found to be 87±4.33 % for dry red pepper, 92±3.52 % for corn, 93±3.66 % for black olive, 93±4.95 % for soy bean, 90±3.26 % for dry fig and 95±4.12 % for hazelnut.

Model equations fitting

For describing the bacterial growth curve, Gompertz and Logistic models were used. The growth parameters were calculated according to the modified Gompertz and modified Logistic function indicated by Zwietering et al., 1990. All the models were fit to the data using SigmaPlot (10.0) non-linear regression procedure. The goodness of fit was evaluated by means of the residual sum of squares (RSS), mean square error (MSE) and determination coefficient (R^2).

Statistical Analysis

Three repetitions were made in all experiments and each data was a mean of six analyses. The data was evaluated by analysis of variance using SPSS 10.0. Duncan's multiple range test was used at a significance level of 0.05.

RESULT AND DISCUSSION

Predictive modeling is a promising field of food microbiology. In describing the behavior of microorganisms under different conditions, the use of mathematical models is receiving great attention (Zwietering et. al, 1990; Giannuzzi et al., 1997). Therefore, there are several sigmoid functions describing a bacterial growth curve such as Gompertz and Logistic models. In this study, the activated *N. corynebacteriodes* NRRL B-184 strain was incubated in TSB at 30°C for 95 h and growing curve was obtained. The modified Gompertz and modified Logistic equations were fitted to microbial counts of *N. corynebacteriodes* strain. The values for the model parameters and results of the statistical analysis are summarized in Table 1. In both models, a good agreement between experimental data and predicted values was obtained. In both models the value of A and μ_{max} were found similar in both models. However, λ was lower in modified Gompertz model. Modified Gompertz model gave slightly better descriptions (low RSS, MSE and high R^2 values) of the growth curves than the modified logistic model. It was observed that *N. corynebacteriodes* strain increased by 2.7 log and reached to the stationary phase within 45 h. The bacteria were still in that phase at 95 h. Hao ve Brackett (1989) were indicated that the stationary phase of growth was reached after 36 h incubation at similar conditions. In another study, μ_{max} was 0.085 h^{-1} and λ was found unreliable (Özkaya, 2001).

Table 1. The Values for the Model Parameters and Results of the Statistical Analysis

Model	A	μ_m (hour^{-1})	λ (hour)	SEE	RSS	MSE	R^2
Modified Gompertz	2.693	0.073	5.244	0.120	0.201	0.012	0.987
Modified Logistic	2.651	0.072	6.393	0.125	0.218	0.013	0.985

In PB

Aflatoxin B_1 content of the PB mediums were shown in Fig.1. During incubation, aflatoxin B_1 contents of control samples were not changed significantly. However in PB mediums, the level of aflatoxin B_1 was decreased continuously. This situation was indicated that, the reduction of aflatoxin B_1 content was relevant to the activity of *N. corynebacteriodes* cells. Except for controls, aflatoxin B_1 content of the samples was reduced approximately 52, 94 and 99 % at the end of the 8, 36 and 72 hours, respectively. The effect of toxin concentration and incubation time on reduction of aflatoxin B_1 were found statistically important in PB medium and all food products ($p<0.05$). Degradation rate constants were obtained by fitting experimental data to zero and first order reaction. In PB, first order reaction kinetics was fitted best with the reduction kinetics. The determination coefficients were changed in the range of 0.990-0.995. Line and Brackett (1995a) and Özkaya (2001) were indicated that reduction kinetics of aflatoxin B_1 were fitted well with first order kinetics. The reduction rates of aflatoxin B_1 in the PB solutions (500, 1000 and 2000 ng ml^{-1} aflatoxin B_1) were 0.093, 0.085 and 0.091 h^{-1}, respectively.

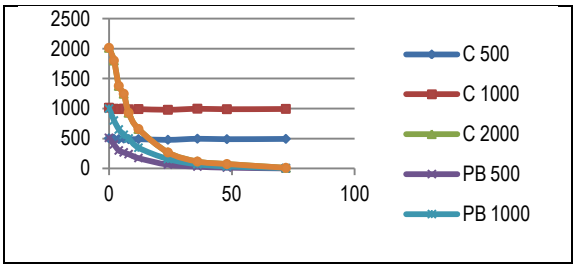

Fig 1. PB Reduction of Aflatoxin B_1 Content of the PB Mediums (C:Control).

Smiley and Draughon (2000) observed that about 74.5% AFB_1 degradation by cell-free extract of *F. aurantiacum* was obtained at 24 h. Teniola et al. (2005) indicated that crude cell-free extracts of *Nocardia corynebacteriodes D*SM 12676 were showed 60% degradation of AFB_1 after 24 h.

Dry red pepper

The dry red peppers that contain 500, 1000 and 2000 ng g^{-1} aflatoxin B_1 were incubated with stationary cells of *N. corynebacteriodes* and alteration of aflatoxin B_1 contents was shown in Fig.2. Reductions of 99.17, 98.65 and 96.52 % in aflatoxin B_1 level of milled samples were observed at the end of the incubation. In whole form of dry red peppers, the reduction ratios were lower than the milled ones. However, this diversity was statistically insignificant. In a study, red peppers that contain approximately 300, 500 and 1500 ng g^{-1} natural aflatoxin B_1 were degreased by *F. aurantiacum* cells at a ratio of 92.8, 99.9 and 98.3 %, respectively (Özkaya, 2001). It could be thought that the differences between reduction ratios were the result from the counts of the cells. The determination coefficients of the milled and whole samples that contain 500, 1000 and 2000 ng mL^{-1} aflatoxin B_1 were changed in the range of 0.973-0.984. As is observed in other food samples, the degradation kinetics were fitted well with first order kinetics in red peppers. The determination coefficients of the milled and whole samples that contain 500, 1000 and 2000 ng mL^{-1} aflatoxin B_1 were changed in the range of 0.973-0.984. The reduction rates of aflatoxin B_1 in milled dry red peppers (500, 1000 and 2000 ng mL^{-1} aflatoxin B_1) were 0.062, 0.051 and 0.044 h^{-1} and 0.044, 0.043 and 0.038 h^{-1} in whole samples, respectively. After incubation (72 h), aflatoxin content of milled dry red pepper that contain 500 ng g^{-1} aflatoxin B_1 was decreased to permitted level of this toxin in Turkish Food Codex.

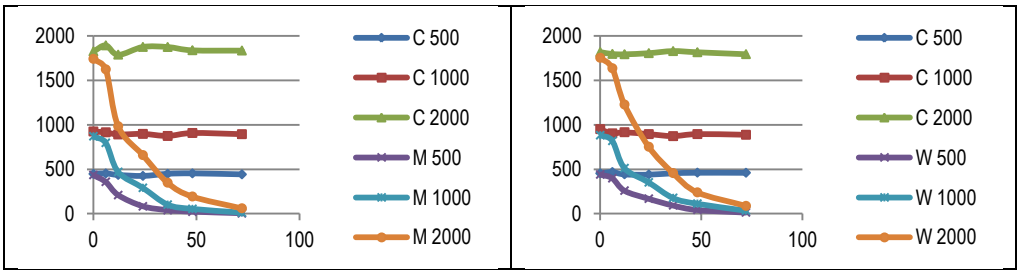

Fig 2. The Reduction of Aflatoxin B_1 in Red Pepers by *N. corynebacteriodes* (C:Control; M:Milled; W:Whole)

Corn

The reduction of aflatoxin B_1 in corn samples was shown in Fig.3. After 72 hours, a reduction of 97.44, 96.09 and 95.96 % in milled and 95.25, 94.01 and 94.15 % in whole corns was observed at 500, 1000 and 2000 ng g^{-1} aflatoxin B_1 concentration, respectively. Ciegler et al. (1966) notified that viable cells of *F. aurantiacum* (10^{13} CFU mL^{-1}) were added to aflatoxin contaminated corn and after 72 h at 28°C, all of the aflatoxin B_1 (16 ng g^{-1}) was removed. The determination coefficients of the milled and whole corns that contain 500, 1000 and 2000 ng mL^{-1} aflatoxin B_1 were changed in the range of 0.986-0.997. The reduction rates of aflatoxin B_1 in milled corns (500, 1000 and 2000 ng mL^{-1} aflatoxin B_1) were 0.050, 0.051 and 0.046 h^{-1} and 0.043, 0.044 and 0.040 h^{-1} in whole samples, respectively. After incubation, none of the samples were decreased to permitted level of this toxin in Turkish Food Codex.

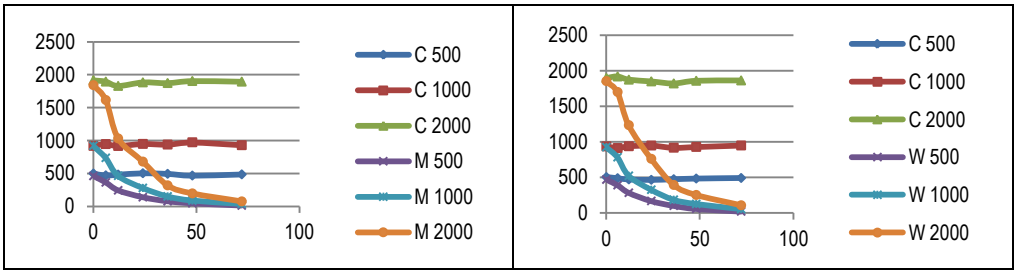

Fig 3. The Reduction of Aflatoxin B_1 in Corn by *N. corynebacteriodes*

Black Olive

After incubation (72 h), reductions of 98.69, 98.14 and 99.14 % in aflatoxin B_1 level of milled black olive were observed. Degradation of aflatoxin B_1 in black olives by *N. corynebacteriodes* was shown in Fig.4. The reduction ratios of whole form samples were lower than the milled ones. However, this diversity was statistically insignificant. The determination coefficients of all samples were changed in the range of 0.983-0.988 inmilled and 0.979-0.985 in whole olives. In milled black olives (500, 1000 and 2000 ng mL^{-1} aflatoxin B_1) the reduction rates of aflatoxin B_1 were 0.055, 0.052 and 0.051 h^{-1} and also 0.053, 0.047 and 0.045 h^{-1} in whole samples, respectively. End of the incubation,

none of the samples were decreased to permitted level of this toxin for human consumption in Turkish Food Codex.

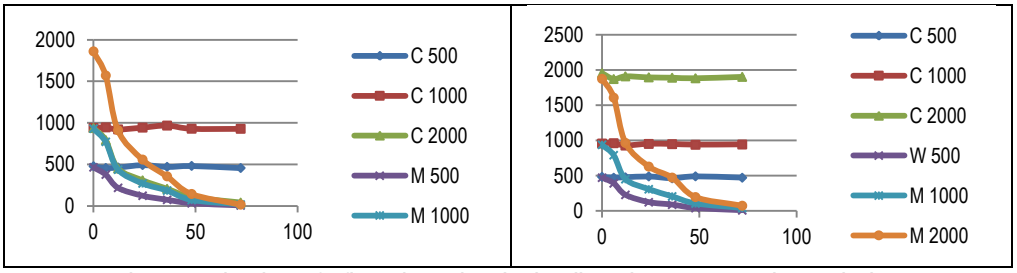

Fig 4. Reduction of aflatoxin B_1 in Black Olives by *N. corynebacteriodes*.

Soybean

After incubation process, approximately 12 % and 15 % of the initial concentration of aflatoxin B_1 were remained in milled and whole soybean samples, respectively (Fig.5). Ciegler et al. (1966) indicated that 8 mg g^{-1} aflatoxin B_1 was decreaded to 2 mg g^{-1} in 12 h at 28°C by *F. aurantiacum* NRRL B-184. The remained toxin was also completely removed at the end of the incubation time. In milled and whole samples, the determination coefficients were changed between 0.995-0.996 and 0.994-0.999, respectively. The reduction rates of aflatoxin B_1 (500, 1000 and 2000 ng mL^{-1}) were 0.055, 0.052 and 0.051h^{-1} and 0.053, 0.047 ve 0.045 h^{-1} in milled and whole soybeans, respectively. The lowest degradation rate constant was found in soy bean samples. Whereas the highest rate constant was in hazelnuts. None of the samples were decreased to permitted level of aflatoxin B_1 for human consumption in Turkish Food Codex after incubation (72 h).

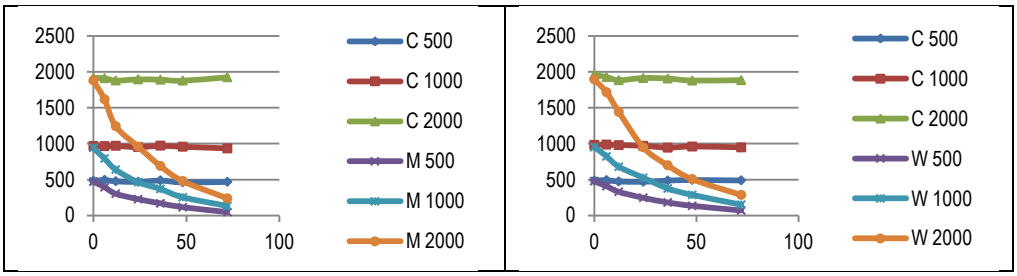

Fig 5 The Reduction of Aflatoxin B_1 in Soy Bean by *N. corynebacteriodes*

Dry Fig

The reduction of aflatoxin B_1 concentrations in dry figs was shown in Fig.6. After 72 hours, a reduction of 99.34, 98.30 and 97.83 % in milled and 98.17, 95.80 and 95.25% in whole dry figs was observed at 500, 1000 and 2000 ng g^{-1} aflatoxin B_1 concentration, respectively. The differences between reduction ratios of milled and whole samples were statistically insignificant. The determination coefficients of all samples were

changed in the range of 0.990-0.997 in milled and 0.988-0.996 in whole samples. The reduction rates of AFB_1 in milled dry figs were 0.054 h^{-1} and same in all toxin concentrations. In whole samples, degradation rate of AFB_1 were 0.048, 0.046 and 0.042 h^{-1} at the concentrations of 500, 1000 and 2000 ng mL^{-1}, respectively. After incubation (72 h), aflatoxin content of milled dry figs that contain aflatoxin B_1 (500 ng g^{-1}) was decreased to permitted level of this toxin for human consumption in Turkish Food Codex.

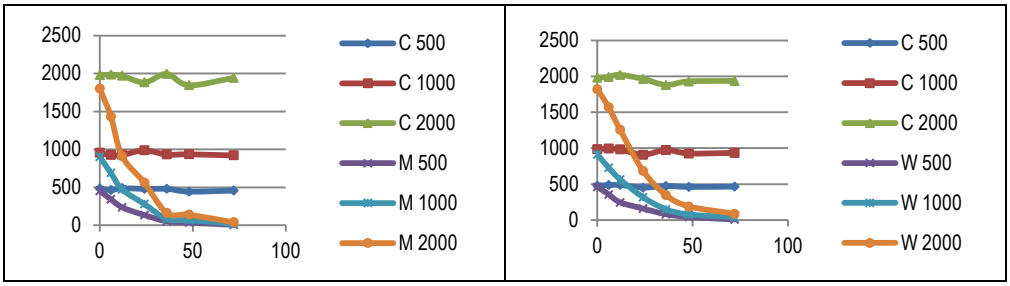

Fig 6. The Reduction of Aflatoxin B_1 in Dry Fig by *N. corynebacteriodes*

Hazelnut

After 72 hours, a reduction of 99.84, 99.47 and 99.04 % in milled and 97.69, 98.11 and 96.48 % in whole hazelnuts was observed at 500, 1000 and 2000 ng g^{-1} aflatoxin B_1 concentration, respectively. The determination coefficients of the milled and whole samples that contain 500, 1000 and 2000 ng mL^{-1} aflatoxin B_1 were changed in the range of 0.986-0.997. The highest rate constant was found in hazelnuts. The reduction rates of aflatoxin B_1 in milled hazelnuts (500, 1000 and 2000 ng mL^{-1} aflatoxin B_1) were 0.067, 0.053 and 0.048 h^{-1} and 0.052, 0.044 and 0.040 h^{-1} in whole samples, respectively. After incubation, aflatoxin contents of milled hazelnut that contain 500 and 1000 ng g^{-1} aflatoxin B_1 were decreased to permitted level for human consumption in Turkish Food Codex.

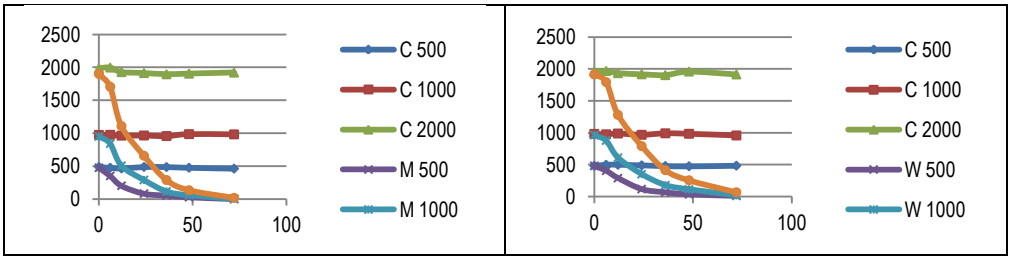

Fig 7. The Reduction of Aflatoxin B_1 in Hazel Nut by *N. corynebacteriodes*

CONCLUSION

Whenever an agricultural commodity has been contaminated with aflatoxins there are only two approaches that can be used. The contaminated product can be discarded or the toxin can be degradated into less toxic or non-toxic products. Especially

in developing countries, the contaminated products are usually consumed by humans and animals. The consumption of these crops could be caused major health and economical problems. Therefore, the development of safer, rapid and cost-effective degradation methods is essential. The ability of *N. corynebacteriodes* to detoxify aflatoxin without the need for exogenous sources could be important in the usage of this organism at various crops. Prevention of nutritional value in detoxified commodities was quite significant. A better understanding of the AFB_1 degradative pathway is necessary for the practical application of this biological method. In this research, identification of AFB_1 degradation products and toxicity analysis were not examined. Degradation products of AFB_1 should be determined and identified with advanced analysis techniques. However, the absence of toxicity would need to be confirmed for the detoxification method to be acceptable.

Researches should be continued to detect the degradation mechanism of AFB_1 and optimise this method to detoxify contaminated agricultural crops to produce safe products.

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