DEGRADATION OF SELECTED MICROBIAL AFLATOXINS FROM *ASPERGILLUS PARASITICUS* BY PARTIAL PURIFIED LACCASE FROM *CORIOLUS HIRSUTUS*

by

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This thesis is dedicated to Jean-Charles and my parents, with love.

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CONTRIBUTION OF THE AUTHORS AND CO-AUTHORS

The present author, Sabrina Borgomano, was responsible for the concepts, the designs and the fulfillment of the experimental work and the preparation of the manuscripts for their submission.

Dr. Monique Lacroix, the thesis supervisor, and Dr. Selim Kermasha, the thesis co-supervisor, supervised the research work, provided valuable input and advices, monitored the progress of the work and critically reviewed and edited the manuscripts, prior to their submission.

Dr. Khanh D. Vu contributed to the statistical analysis using response-surface methodology and reviewed this part in the manuscript.

Dr. Richard St-Louis carried out the LC/MS structural analysis of aflatoxins and their enzymatic degradation products and contributed to the analysis of data.

Dr. Varoujan Yaylayan contributed to the FTIR analysis.

ABSTRACT

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The production of the major aflatoxin isorforms by selected fungal strains, including *Aspergillus flavus* and *Aspergillus parasiticus* as well as their degradation by a laccase from *Coriolus hirstutus* were investigated. *A. parasiticus* was found to be the most appropriate one in term of its capacity to produce the highest aflatoxins level, when potato dextrose agar was used as the initial inoculation medium. Using a fungal biomass concentration of 150 g/L obtained after 168 h of incubation provided the highest yield of 73.603 mg aflatoxins/L culture medium. The effect of cryoprotectants on the dry aflatoxins recovery was also investigated, where 1.5% of mannitol was found the most appropriate for the recovery of high yield and improved quality of aflatoxins extract. A reverse-phase/high-performance liquid chromatography, at 365 nm, was used to separate and characterize the aflatoxins, where AFG2, AFG1, AFB2 and AFB1, were eluted at 3.70, 4.20, 4.80 and 5.08 min, respectively. The biocatalysis of a partial purified laccase (PPL), from *Coriolus hirsutus*, using the major aflatoxin isoforms, AFB1, AFB2, AFG1 and AFG2, as substrates, was investigated. The PPL exhibited the highest specific activity of 0.133, 0.124, 0.155 and 0.138 μmol of degradation product per mg protein per min, for AFB1, AFB2, AFG1 and AFG2, respectively, at 57.5ºC and pH 6.0. The kinetic studies indicated that the K_m value was 0.646, 0.128, 0.237 and 0.284 μM and a V_{max} value was 10.40, 9.68, 13.00 and 10.80 for AFB1, AFB2, AFG1 and AFG2, respectively. In addition, the presence of 0.4 mM of dithiothreitol and diethyldithiocarbamic acid strongly inhibited the laccase activity, whereas 10 mM of kojik acid and 0.01 mM of *p*-coumaric acid greatly promoted its activity. An experimental strategy for seeking the optimum conditions of the degradation of the major aflatoxin isoforms was investigated. A five-level by three factors central composite design (CCD) was used to evaluate the effect of the enzyme concentration X1, the aflatoxins concentration X2 and the incubation time X3, on the percentage of degradation for each aflatoxin, including AFB1, AFB2, AFG1 and AFG2. The experimental findings demonstrated that the predicted values of aflatoxins degradation were in good agreement with the experimental results, where the R^2 value of the polynomial model for the prediction of AFB1, AFB2, AFG1 and AFG2 degradation percentage was 0.89, 0.94, 0.92 and 0.89, respectively. In addition, the

results also indicated that the incubation time and the enzyme level, with higher coefficient values, greatly influenced aflatoxin AFB1 degradation more than the substrate concentration. For AFB2 degradation, enzyme level, with higher coefficient, had higher effect than incubation time and substrate concentration. For AFG1 and AFG2 degradation, the substrate and enzyme concentrations, with higher coefficient values, had higher effects than incubation time. The optimal predicted conditions for a 38.2, 30.1, 76.4 and 100% of AFB1, AFB2, AFG1 and AFG2 degradation, respectively, were 31.5 U/nmol aflatoxins for enzyme, 96.2, 191.0, 32.7 and 42.2 nmol, respectively, for AFB1, AFB2, AFG1 and AFG2 and 55.2 min for time. The characterization of the laccase-catalyzed end products was obtained by Fourier-transform infrared spectroscopy (FTIR), high-performance liquid chromatography (HPLC), and liquid chromatography/mass spectrometry (LC/MS). The structural analyses showed that the laccase-catalyzed degradation of major aflatoxin isoforms resulted in the production of several degradation products, with a wide range of structures and molecular weights. The most abundant molecular ion peak was at m/z 327.254, 205.069, 205.069 and 196.656 Da for AFB1, AFB2, AFG1 and AFG2, respectively. The FTIR and MS analyses also showed that the major mechanisms of aflatoxins degradation by laccase were epoxydation, hydroxylation, *O*-demethylation, deshydrogenation, dehydratation, reduction of the double bond and keto-reduction a well as the loss of ketone, oxygen, carbon and methyl molecules, leading to the modification either of the furofuran moiety, the coumarin or the lactone ring as well as to the formation of nontoxic products.

RÉSUMÉ

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La production des principales formes des aflatoxines par des souches fongiques sélectionnées, comprenant *Aspergillus flavus* et *Aspergillus parasiticus*, ainsi que leur dégradation par la laccase de *Coriolus hirstutus* ont été étudiés. *A. parasiticus* s'est révélée être la souche la plus appropriée en termes de sa capacité à produire les taux plus élevés d'aflatoxines, lorsque la gélose dextrosée à la pomme de terre était utilisée comme milieu initial d'inoculation. L'utilisant d'une concentration de 150 g/L d'une biomasse fongique, obtenue après 168 h d'incubation, permettait d'obtenir la concentration la plus élevée de 73,603 mg d'aflatoxines/L de milieu de culture. L'effet de cryoprotectants sur la récupération des extraits secs d'aflatoxines a également été étudié, où 1,5% de mannitol a été trouvé comme étant le plus adéquate pour obtenir à la fois une plus grande quantité et une meilleure qualité d'extraits d'aflatoxines. Une chromatographie liquide à haute performance en phase inverse, à une longueur d'onde de 365 nm, a été utilisée pour séparer et caractériser les aflatoxines, où les aflatoxines AFG2, AFG1, AFB2 et AFB1, étaient éluées à 3,70, 4,20, 4,80 et 5,08 min, respectivement. La biocatalyse par la laccase partiellement purifiée, provenant de *Coriolus hirsutus*, en utilisant les principales aflatoxines, AFB1, AFB2, AFG1 et AFG2, comme substrats a été étudiée. L'extrait enzymatique présentait une plus forte activité spécifique de 0,133, 0,124, 0,155 et 0,138 µmol de produit dégradé par mg de protéine par minute, pour les aflatoxines AFB1, AFB2, AFG1 et AFG2, respectivement, à une température de 57.5ºC et un pH de 6,0. Les études cinétiques ont indiqué que la valeur de k_m était de 0,646, 0,128, 0,237 et 0,284 µM et celle de V_{max} était de 10,40, 9,68, 13,00 et 10,80 pour les aflatoxines AFB1, AFB2, AFG1 et AFG2, respectivement. En outre, la présence de 0,4 mM de dithiothréitol et d'acide diéthyldithiocarbamique inhibait fortement l'activité de la laccase, alors que 10 mM d'acide kojique et 0,01 mM d''acide *p*-coumarique favorisait grandement son activité. Une stratégie expérimentale pour rechercher les conditions optimales de la dégradation des principales aflatoxines a été étudiée. Un plan de composites centrés (CCD) à cinq-niveaux-par-trois-facteurs a été utilisé pour évaluer l'effet de la concentration de l'enzyme X1, la concentration des aflatoxines X2, et le temps d'incubation X3, sur le pourcentage de dégradation de chaque aflatoxine, dont les aflatoxines AFB1, AFB2, AFG1 et AFG2. Les résultats

expérimentaux ont montré que les valeurs prédites pour la dégradation des aflatoxines étaient en adéquation avec les résultats expérimentaux, où la valeur R^2 du modèle polynômial pour la prédiction du pourcentage de dégradation des aflatoxines AFB1, AFB2, AFG1 et AFG2 était de 0,89, 0,94, 0,92 et 0,89, respectivement. En outre, les résultats indiquaient également que le temps d'incubation et la concentration de l'enzyme, à des valeurs élevées, influençaient plus fortement la dégradation de l'aflatoxine AFB1 que la concentration du substrat. Pour la dégradation de l'aflatoxine AFB2, la concentration de l'enzyme avec un coefficient plus élevé avait un effet plus important que le temps d'incubation et la concentration du substrat. Pour la dégradation des aflatoxines AFG1 et AFG2, les concentrations d'enzyme et de substrats, à des valeurs élevées, engendraient des effets plus importants que le temps d'incubation. Les conditions optimales prédites pour une dégradation respective de 38,2, 30,1, 76,4 et 100% des aflatoxines AFB1, AFB2, AFG1 et AFG2, étaient 31,5 U/nmol d'aflatoxines pour l'enzyme, 96,2, 191,0, 32,7 et 42,2 nmol, respectivement, pour les aflatoxines AFB1, AFB2, AFG1 et AFG2 et 55,2 min pour le temps. La caractérisation des produits finaux issus de la dégradation par la laccase a été obtenue par spectroscopie à transformée de Fourier (FTIR), par chromatographie liquide à haute performance (HPLC), ainsi que par une chromatographie liquide couplait à un spectromètre de masse (LC/MS). Les analyses structurales ont montré que la dégradation des principales aflatoxines par la laccase a mené à la production de plusieurs produits de dégradation, de structures et de masses moléculaires variées. Le pic de l'ion moléculaire m/z le plus abondant était de 327,254, 205,069, 205,069 et 196,656 Da, pour les aflatoxines AFB1, AFB2, AFG1 et AFG2, respectivement. Les analyses FTIR et MS ont suggéré que les principaux mécanismes de dégradation des aflatoxines par laccase pouvait être l'époxydation, l'hydroxylation, l'*O*-déméthylation, la déshydrogénation, la déshydratation, la réduction de la double liaison et celle des cétones ainsi que la perte de cétone, d'oxygène, de carbone et de méthyle, conduisant à la modification soit du groupe furofurane, de la coumarine ou du cycle lactone, ainsi qu'à la formation de produits non toxiques.

CLAIMS OF ORIGINAL RESEARCH

(1) It is the first time where the development of a new method for the recovery of aflatoxins from a liquid medium culture of *Aspergillus parasiticus* was carried out, without the use of solvents and in presence of cryoprotectant.

(2) This is the first study, in which the enzymatic extract preparation of laccase, from *Coriolus hirsutus*, was investigated in terms of its optimum reaction temperature, optimum pH, kinetic studies and effects of selected chemical agents, using the major aflatoxins isoforms, AFB1, AFB2, AFG1 and AFG2, as substrates.

(3) A developed process for the degradation of the major aflatoxin isoforms, using the microbial laccase, was optimized by central composite design.

(4) In order to propose an enzymatic degradation mechanism of aflatoxins by laccase, this is the first study in which the end products resulted from the laccase enzymatic reaction were characterized in terms of their chemical properties.

SOMMAIRE

Les mycotoxines sont des métabolites secondaires toxiques produits par diverses moisissures sur plusieurs produits agricoles et aliments pour animaux, sous certaines conditions environnementales, et peuvent avoir des effets toxiques chez les organismes supérieurs qui consomment des produits contaminés. Par conséquent, la contamination des aliments et aliments pour animaux par les mycotoxines est une question de sécurité alimentaire grave qui affecte à la fois la compétitivité de l'agriculture, le domaine de l'exportation et la santé humaine et animale. Le terme mycotoxine vient du grec *«mycos*» qui signifie champignon et du latin «*toxicum*» qui signifie poison. Ce terme commence à apparaitre au milieu du 20^{e} siècle durant plusieurs épidémies majeures dont celle de 1960 en Angleterre connue sous le nom de "Turkey X disease", ou maladie de la dinde, qui a causée la mort d'une grande partie de la population. Trois cents mycotoxines ont été identifiées et caractérisées chimiquement, parmi celles-ci, de nombreuses ont été trouvées comme contaminants d'aliments. En 1985, l'Organisation des Nations Unies pour l'alimentation et l'agriculture estimait que 25% des récoltes de céréales dans le monde étaient affectées par des mycotoxines, ce qui a pour effet de réduire la qualité des aliments, tant végétale qu'animale, disponible au niveau mondial. De plus, les mycotoxines sont responsables d'intoxications aiguës parfois mortelles, notamment chez les animaux d'élevage, et d'intoxications chroniques. Parmi ces mycotoxines, les aflatoxines ont été largement étudiées en raison de leur présence fréquente dans les aliments, en particulier dans les pays en voie de développement, et de leurs effets mutagènes et cancérigènes. Les aflatoxines sont des dérivés difurano-coumarines produites sous forme de métabolites secondaires par des espèces d'*Aspergillus*, principalement *A. flavus A. parasiticus, A. nomius, et A. Tamarii*. Parmi les plus courantes, on trouve les aflatoxines AFB1, AFB2, AFG1, AFG2, AFM1 et AFM2. Ces aflatoxines, toxines les plus étudiées, sont nuisibles à la fois chez l'homme et chez l'animal, de par leur pouvoir cancérigène élevé. Plus de 50 pays ont instauré ou proposé des règlementations de contrôle pour les aflatoxines dans les aliments et aliments pour animaux. Ces toxines continuent non seulement d'imposer des problèmes d'ordre de santé publique mais aussi d'ordre économique car les aliments et cultures contaminés ne peuvent pas être vendus. Ces contaminations sont principalement observées lors de mauvaises gestions de récoltes des cultures

et de stockage des aliments. Actuellement, Il existe de nombreux procédés à la fois physiques et chimiques de détoxification de l'aflatoxine, qui consistent en l'élimination ou en l'inactivation de la toxine dans les produits contaminés. Néanmoins, aucune de ces méthodes conventionnelles n'est idéale ou applicable car elles sont responsables de la formation de sous-produits toxiques ainsi que de la perte de la qualité nutritionnelle et de la valeur économique. De ce fait, l'emploi d'une méthode biologique notamment celle d'enzymes serait une approche éventuelle pouvant conduire à l'amélioration des procédés agro-industriels, et à la réduction des coûts énergétiques associés au traitement ainsi qu'à l'amélioration de la qualité nutritionnelle des aliments. L'approche actuelle de la dégradation biologique des aflatoxines est basée sur les processus microbiens impliqués dans la dégradation des composés organiques aromatiques complexes tels que la lignine. En effet, parmi les composés poly-phénoliques présents dans la nature, la lignine est sans doute le composé le plus abondant ainsi que le plus hétérogène et récalcitrant pouvant être dégradé par voie microbienne. Cependant, les microorganismes ont développé des moyens qui peuvent dégrader ces composés complexes récalcitrants, comme l'ouverture de la structure de la lignine, la dépolymérisation, et enfin de la minéralisation. Les champignons sont considérés comme étant le principal groupe responsable de la dégradation de polyphénoliques xénobiotiques en raison de leur grand répertoire d'enzymes extracellulaires. Le groupe des basidiomycètes est connu comme étant activement impliqué dans la dégradation de la lignine. Comme ces champignons ont le potentiel de dégrader la lignine, ainsi qu'une large gamme d'hydrocarbures aromatiques polycycliques, leur rôle dans la dégradation d'autres substances cancérigènes, telles que les aflatoxines est possible. Parmi les enzymes des basidiomycètes, les peroxydases ont montré une grande efficacité dans la détoxification des aflatoxines. Cependant, la production d'hydroperoxydes lors de ce procédé, qui à leur tour se décomposent en radicaux libres pouvant réagir avec la toxine peut poser problème, d'où la nécessité de trouver un autre système de détoxification enzymatique de l'aflatoxine. La laccase, une autre enzyme appartenant au groupe des basidiomycètes notamment synthétisé à partir de l'espèce de *Coriolus hirsutus* (champignon à la pourriture blanche), posséderait la capacité de dégrader et/ou détoxifier les aflatoxines. Les laccases (hydroquinone: oxygène oxydoréductase, EC 1.10.3.2) sont des polyphénols oxydases qui appartiennent à la famille des protéines bleues multi-cuivres. En plus d'être des enzymes industriellement pertinentes utilisées dans diverses applications, les laccases ont un rôle important dans le cycle du carbone et pourraient contribuer à dégrader un large éventail de

xenoaromatiques. En outre, elles jouent un rôle essentiel dans de nombreuses activités cellulaires comme la biosynthèse de la paroi cellulaire des plantes, la pigmentation des spores bactériennes et fongiques, la dégradation de la lignine, et l'oxydation des composés phénoliques. En raison de leurs larges spécificités de substrat, leurs grands pouvoirs catalyseurs et leurs capacités à utiliser l'oxygène de l'environnement en tant que cofacteur, les laccases fongiques sont utilisées dans une large gamme d'applications biotechnologiques et industrielles. De nombreux chercheurs ont montré que la détoxification des aflatoxines par les laccases est à la fois efficace et rapide et contrairement aux peroxydases, le mécanisme de détoxification n'engendre pas la formation de radicaux libres, ce qui rendrait l'ensemble du processus plus sûr.

L'objectif général de la recherche proposée est d'étudier le mécanisme de dégradation des aflatoxines selectionnées par la laccase partiellement purifiée de *Corolius hirsutus*.

Au cours de cette étude, la production de la biomasse de *Coriolus hirsutus* et la récupération de sa laccase partiellement purifiée ont été réalisées grâce à l'utilisation de plusieurs procédés comme l'ultrafiltration, la précipitation des protéines au sulfate d'ammonium (60-80%), une dialyse et enfin par l'utilisation d'un système de lyophilisation dans le but de récupérer l'enzyme sous forme de poudre.

De plus, la production de la biomasse d'*Aspergillus parasiticus* et la récupération des quatre principales aflatoxines AFB1, AFB2, AFG1 et AFG2 ont été réalisées. En outre, afin d'éviter des risques supplémentaires que peut causer une extraction par solvant (chloroforme), une nouvelle méthode d'extraction des aflatoxines sans l'emploi de solvants a été développée grâce à l'utilisation d'un système d'ultrafiltration. D'autre part, un procédé de lyophilisation en utilisant une concentration de mannitol de 1,5% a été utilisé afin d'obtenir un extrait sec.

L'étude de la cinétique de dégradation de la laccase en utilisant les aflatoxines comme substrats a été effectuée dans le but de déterminer le temps, la température et le pH optimum de la réaction. De plus, les paramètres cinétiques de la réaction de dégradation des aflatoxines par la laccase, incluant l'activité spécifique et totale ainsi que les valeurs du k_m, V_{max} et k_{cat} ont été déterminés afin de connaitre les aspects de la catalyse des aflatoxines par la laccase. Par ailleurs, l'effet

d'activateurs et d'inhibiteurs sur l'activité de dégradation de la laccase partiellement purifiée a été étudié, ainsi que le type d'inhibition exercé sur la réaction enzymatique.

Une analyse statistique, en utilisant la méthodologie de réponse de surface couplée à un plan de composites centrés, a était réalisée dans le but de déterminer la dégradation maximale des aflatoxines par la laccase, au moyen de trois paramètres sélectionnés, la concentration d'enzyme, la concentration des aflatoxines utilisées comme substrats et le temps d'incubation. L'analyse chromatographique liquide à haute performance des aflatoxines résiduelles après réaction enzymatique a été effectuée afin de déterminer le taux de dégradation des quatre principales formes des aflatoxines par la laccase.

La caractérisation structurale des aflatoxines résiduelles et des produits finaux issus de la dégradation enzymatique a été réalisée afin de déterminer le mécanisme de dégradation des aflatoxines par la laccase. Les analyses à spectroscopie infrarouge à transformée de Fourier (FTIR) et à spectroscopie de masse (SM) ont été entreprises dans le but de confirmer la présence et d'identifier les produits de dégradations afin de proposer un ou des mécanisme/s de dégradation des aflatoxines AFB1, AFB2, AFG1 et AFG2 par la laccase.

Discussion sur la Production et la Caractérisation des Aflatoxines d'Aspergillus parasiticus

La sélection de la souche fongique d'*Aspergillus* est essentielle dans la production des toxines. Les deux souches fongiques, *Aspergillus parasiticus* et *A. flavus,* ont été reportées pour produire les principales formes des aflatoxines, dont AFB1, AFB2, AFG1 et AFG2 (Fig. 3.1). Cependant *A. parasiticus* a été capable de produire le taux le plus élevé d'aflatoxines à deux différents temps d'incubation, 24 et 168 h.

La présence de certains nutriments dans les milieux de culture, Czapek's agar (CA) et gélose dextrosée à la pomme de terre (PDA), joue un rôle important sur la production des aflatoxines. Les résultats (Tableau 3.1) ont indiqué qu'*A. parasiticus* produisait 38% plus d'aflatoxines sur la gélose dextrosée à la pomme de terre que sur Czapek's agar. Cette augmentation du taux de toxines peut être due à la présence de glucose dans PDA au lieu de sucrose dans CA. De plus, le pH des milieux de culture peut également être un élément jouant sur la production des toxines puisque ce denier est acide dans PDA alors qu'il est plutôt neutre dans CA.

D'autres paramètres, tels que la concentration fongique et le temps d'incubation, peuvent favoriser la synthèse d'aflatoxines. Les résultats (Tableau 3.2) ont montré qu'une biomasse de 150 g/L de milieu de culture permettait d'obtenir une plus grande concentration d'aflatoxines de 56,2 µg aflatoxines/g d'extrait sec comparé à des biomasses de 50 et 100 g. En outre, les résultats (Tableaux 3.3 et 3.4) ont démontré qu'un temps d'incubation de 48 h et 168 h dans les milieux de pré-culture et culture, respectivement, permettaient d'obtenir les taux les plus élevés d'aflatoxines de 353,8, 331,6, 433,6, 388,4 et 694,7, 646,4, 845,5, 757,5 µg aflatoxines/g d'extrait sec pour les aflatoxines AFB1, AFB2, AFG1 et AFG2, respectivement. Cependant au delà de ces temps de fermentation, une diminution du taux de toxines était observée, probablement due à la synthèse d'enzymes par le mycélium plus âgé, capablent de dégrader les aflatoxines. On peut en conclure que la concentration des aflatoxines est dépendante de la souche fongique, de la concentration de la biomasse et des conditions de culture.

La caractérisation des aflatoxines a été réalisée par chromatographie liquide à haute performance en phase inverse à 365 nm, où les aflatoxines AFG2, AFG1, AFB2 et AFB1 étaient éluées à 3,70, 4,20, 4,80 and 5,08 min, respectivement.

Discussion sur la Cinétique de Dégradation des Aflatoxines Sélectionnées par la Laccase de Coriolus Hirsutus

Il est important d'optimiser les paramètres de l'enzyme afin d'obtenir un taux d'oxydation maximal pour un substrat spécifique. De ce fait, les effets de la température, du pH, de la charge protéique ont été étudiés sur l'activité de la laccase en utilisant les aflatoxines AFB1, AFB2, AFG1 et AFG2, comme substrats. Les résultats expérimentaux (Fig. 4.2, 4.3 et 4.4) ont montré qu'une température de 57,5ºC, un pH de 6,0 et une charge protéique de 16 µg de protéines/mL réaction permettaient d'obtenir la plus grande activité enzymatique de 127, 118, 148 et 132 nmol produit par mg de protéine par min, pour les aflatoxines AFB1, AFB2, AFG1 and AFG2, respectivement.

De plus, les paramètres cinétiques de l'activité de dégradation de la laccase dont l'activité spécifique, l'activité totale ainsi que les valeurs du K_m, V_{max} et K_{cat}, en utilisant les aflatoxines AFB1, AFB2, AFG1 et AFG2, comme substrats ont également été déterminés. Les résultats (Tableau 4.1) ont montré que l'activité spécifique et l'activité totale de l'enzyme étaient

respectivement de 0,133, 0,124, 0,155, 0,138 µmol de produit par mg de protéine par min et de 2,527, 2,356, 2,945, 2,622 µmol par min pour les aflatoxines AFB1, AFB2, AFG1 and AFG2, respectivement. De plus, la valeur de K_m était de 0,646, 0,128, 0,237 et 0,284 µM et celle de V_{max} était de 10,40, 9,68, 13,00 et 10,80 µM par mg de protéine par min pour les aflatoxines AFB1, AFB2, AFG1 et AFG2, respectivement. De surcroît, la laccase a montré une efficacité catalytique selon l'ordre suivant AFB2 > AFG1 > AFG2 > AFB1, avec une valeur K_{cat} de 75, 55, 38 et 16 molécule/min, respectivement.

Un autre facteur important pour connaitre le mécanisme d'action d'une enzyme est l'effet de molécules activatrices et inhibitrices sur l'activité de cette dernière. De ce fait, l'effet d'agents chimiques, incluant l'acide citrique, l'imidazole, le *N*-hydroxyphthalimide (HPI), l'acide diethyldithiocarbamique (DDC), dithiothréitol (DTT), le sel diammonium 2,2'-azino-bis (acide 3-ethylbenzothiazoline-6-sulfonique) (ABTS), l'acide kojique et l'acide *p*-coumaric ainsi que des ions métalliques, tels que le chlorure de cuivre ($CuCl_2$) et le sulfate de cuivre ($CuSO_4$), ont été étudiés sur l'activité de la laccase. (Fig. 4.5 et 4.6).

Les résultats expérimentaux (Fig. 4.5) ont démontré que les molécules suivantes, l'HPI, l'ABTS, l'acide kojique, l'acide *p*-coumarique et l'imidazole, avaient un effet activateur sur l'activité de la laccase. On peut en conclure que la conjugaison de la laccase avec de molécules de faible poids moléculaire, tel que l'ABTS ou avec des médiateurs synthétiques de type NOH^- tel que HPI pourrait à la fois augmenter le taux de rendement de la transformation des substrats mais également élargir le répertoire de l'enzyme pour des substrats non phénoliques pour lesquels l'enzyme aurait normalement peu ou pas d'activité. De plus, les résultats suggèrent que les composés dérivés des phénols, tels que l'acide *p*-coumarique, constituaient des médiateurs hautement efficaces pour l'activité enzymatique de la lacccase.

Les résultats expérimentaux (Fig. 4.6) ont démontré que les molécules suivantes, DTT, DDC, L-cysteine, l'acide citrique, $CuSO_4$ et $CuCl_2$, avaient un effet inhibiteur sur l'activité de la laccase. On peut en conclure que les composés avec des groupements sulfhydryles (-S-H), tels que L-cysteine, DTT et DDC sont des chélateurs spécifiques des ions métalliques qui inhibent l'activité de la laccase par la formation de complexes avec les ions cuivres de son site actif, conduisant à la modification de ce dernier. L'acide citrique quant à lui pourrait former un complexe avec les ions

cuivre de la laccase conduisant également à la modification du site actif de la laccase. Concernant les ions métalliques $CuSO_4$ and $CuCl_2$, leurs effets inhibiteurs pourrait être du à un excès de cuivre qui conduirait à une modification de la structure de l'enzyme et ainsi à l'annihilation de son activité.

De plus, la comparaison des valeurs de K_m et V_{max} en absence et en présence des molécules inhibitrices a permis de déterminer le type d'inhibition que ces dernières exercent sur l'activité de la laccase. Les résultats expérimentaux (Tableau 4.2) ont permis de montrer que l'acide citrique, $CuSO_4$ et DCC exerçaient une inhibition incompétitive sur l'activité de la laccase. Quant à $CuCl_2$, L-cysteine et DTT, ils exerçaient des inhibitions non compétitives mixtes, non compétitives et compétitives, respectivement, sur l'activité de la laccase.

Discussion sur l'Étude Statistique de la Dégradation des Aflatoxines par la Laccase

La méthodologie de réponse de surface (RSM) est un ensemble de techniques mathématiques et statistiques utiles pour modéliser et analyser des problèmes où une réponse est affectée par différents facteurs, plans d'expériences raffinés, notions d'analyse mathématique et calcul différentiel, dont l'objectif est d'optimiser la réponse. Un plan de composites centrés (CCD) à cinq-niveaux-par-trois-facteurs a été utilisé pour évaluer l'effet de la concentration de l'enzyme X1, la concentration des aflatoxines X2, et le temps d'incubation X3, sur le pourcentage de dégradation de chaque aflatoxine, dont les aflatoxines AFB1, AFB2, AFG1 et AFG2.

Les résultats des analyses ANOVA et de régression (Tableau 5.3) ont montré que les valeurs du coefficient de déterminstion R^2 du modèle polynômial pour la prédiction du pourcentage de dégradation des aflatoxines AFB1, AFB2, AFG1 et AFG2 étaient de 0,89, 0,94, 0,92 et 0,89, respectivement, ce qui impliquait que 89, 94, 92 et 89% de variations dans la réponse pouvaient être expliquées par le modèle.

Pour l'aflatoxine AFB1, les résultats ont démontré que les variables linéaires et quadratiques ainsi que l'interaction entre l'enzyme et la concentration de l'AFB1 étaient statistiquement significatives ($P \leq 0,05$) ou marginalement significatives ($P \leq 0,1$), alors que l'interaction entre la concentration de l'enzyme et le temps d'incubation n'était pas significative ($P > 0,1$).

Pour l'aflatoxine AFB2, les résultats ont démontré que toutes les variables linéaires et quadratiques ainsi que l'interaction entre l'enzyme et la concentration de l'AFB2 étaient statistiquement significatives ($P \leq 0,05$) ou marginalement significatives ($P \leq 0,1$). Cependant l'interaction entre la concentration de l'AFB2 et le temps d'incubation n'était pas significative ($P > 0,1$).

Pour l'aflatoxine AFG1, les résultats ont démontré que les variables linéaires et quadratiques ainsi que les interactions étaient statistiquement significatives ($P \leq 0,05$) ou marginalement significatives ($P \leq 0,1$), à l'exception de la variable linéaire du temps d'incubation ($P > 0,1$).

Pour l'aflatoxine AFG2, les résultats ont démontré que toutes les variables linéaires ainsi que la variable quadratique des concentrations de l'enzyme et du substrat étaient statistiquement significatives ($P \leq 0,05$) ou marginalement significatives ($P \leq 0,1$), alors que la variable quadratique du temps d'incubation et celles des interractions ne l'étaient pas ($P > 0,1$).

En outre, les résultats indiquaient également que le temps d'incubation et la concentration de l'enzyme, à des valeurs élevées, influençaient plus fortement la dégradation de l'aflatoxine AFB1 que la concentration du substrat. Pour la dégradation de l'aflatoxine AFB2, la concentration de l'enzyme avec un coefficient plus élevé avait un effet plus important que le temps d'incubation et que la concentration du substrat. Pour la dégradation des aflatoxines AFG1 et AFG2, les concentrations d'enzyme et de substrats, à des valeurs élevées, engendraient des effets plus importants que le temps d'incubation.

Les résultats ont indiqué que les valeurs prédites pour la dégradation des aflatoxines étaient en accords avec les résultats expérimentaux, où la dégradation maximale des aflatoxines, soit de 38,2, 30,1, 76,4 et 100% pour l'AFB1, AFB2, AFG1 et AFG2 respectivement, était obtenue avec une concentration enzymatique de 31,5 U/nmol d'aflatoxines, une concentration de substrat de 96,2, 191,0, 32,7 et 42,2 nmol, respectivement, pour les aflatoxines AFB1, AFB2, AFG1 et AFG2 et un temps d'incubation de 55,2 min.

De cette étude on peut on conclure que la laccase de *C. hirsutus* peut être efficacement utilisée comme système biologique pour dégrader les principales formes d'aflatoxines d'*A. parasiticus.*

De plus, un plan de composites centrés couplé avec la méthodologie de réponse de surface peut être utilisé avec succès pour optimiser la dégradation enzymatique des aflatoxines.

Discussion sur la Caractérisation Structurale et les Mécanismes Proposés pour la Dégradation des Aflatoxines par la Laccase

Afin de déterminer le mécanisme de dégradation des aflatoxines par la laccase, la caractérisation structurale des aflatoxines résiduelles ainsi que des produits finaux issus de la dégradation enzymatique a été réalisée.

Les résultats de l'analyse chromatographique des aflatoxines et de leurs produits de dégradation finaux (Fig. 6.2) ont montré qu'après 55 min de traitement avec la laccase, l'aire du pic de chaque aflatoxine diminuait significativement et que de nouveaux pics apparaissaient, preuve de la formation de nouveaux produits issus de la dégradation enzymatique.

Les analyses de la spectroscopie infrarouge à transformée de Fourier (FTIR) ont été utilisées comme outil pour l'élucidation de la structure des produits finaux issus de la catalyse des aflatoxines par la laccase. Les résultats (Fig. 6.4) ont indiqué que les spectres IR des produits de dégradation enzymatiques finaux des aflatoxines montraient plusieurs différences dans les pics d'absorption par rapport à ceux des substrats. Les différences majeures des spectres après traitement enzymatique se situaient au niveau des pics d'absorption à 3100-2900, entre 1770 et 1650, et entre 1300 et 1000 cm^{-1}, ainsi qu'avec l'apparition d'un nouveau pic à 1375 cm^{-1}. Ces changements seraient probablement dus à des modifications de la structure des aflatoxines, notamment au niveau de la coumarine et des autres cycles aromatiques tels que les lactones et les furanes. De plus, le pic apparaissant à 1375 cm^{-1} pourrait suggérer la conversion du groupe méthoxyle du cycle aromatique en groupe hydroxyle. Pour le spectre de l'aflatoxine AFB1 transformée, un nouveau pic asymétrique apparaissait à 825 cm^{-1}, ce qui pourrait correspondre à la vibration d'élongation d'un groupe époxyde positionné sur le cycle furane de la toxine.

Les analyses de la spectrométrie de masse (Figs. 6.5.et 6.6) ont montré que la dégradation par la laccase des principales formes des aflatoxines a mené à la production de plusieurs produits de dégradation, ayant des structures et des masses moléculaires variées, dont le pic de l'ion moléculaire m/z le plus abondant était de 327,254, 205,069, 205,069 et 196,656 Da, pour les

aflatoxines AFB1, AFB2, AFG1 et AFG2, respectivement. Les résultats expérimentaux ont suggéré que les principaux mécanismes de dégradation des aflatoxines par laccase pouvait être l'époxydation, l'hydroxylation, l'*O*-déméthylation, la déshydrogénation, la déshydratation, la réduction de la double liaison et celle des cétones ainsi que la perte de cétone, d'oxygène, de carbone et de méthyle, conduisant à la modification soit du groupe furofurane, de la coumarine ou du cycle lactone, ainsi qu'à la formation de produits non toxiques.

Conclusion

Les données expérimentales obtenues tout au long de cette étude ont montré que parmi les souches fongiques étudiées, *Aspergillus parasiticus* sur PDA était la plus appropriée pour produire un rendement plus élevé des principales formes des aflatoxines, AFB1, AFB2, AFG1 et AFG2. Au cours de la fermentation liquide de la souche fongique, divers facteurs peuvent influencer la production des aflatoxines, tels que l'activité de l'eau, la température et le temps d'incubation. De plus, la composition en nutriments du milieu de culture affecte également la production des aflatoxines. L'analyse HPLC en phase inverse à 365 nm de l'extrait sec des aflatoxines a montré la présence des principales formes des aflatoxines.

Les résultats recueillis dans cette étude ont montré que l'activité enzymatique est influencée à la fois par des propriétés spécifiques, notamment la concentration du substrat, par la présence d'activateurs et d'inhibiteurs et par des effets non spécifiques tels que tampon, le pH et la température. Les résultats expérimentaux ont indiqué qu'une température de 57,5°C et un pH de 6,0, étaient les conditions optimales pour améliorer l'activité de dégradation de la laccase pour les aflatoxines, AFB1, AFB2, AFG1 et AFG2. En outre, les effets des ions métalliques, des sels et des agents chimiques sélectionnés sur l'activité de dégradation de la laccase sont fortement tributaires de leurs natures et de leurs concentrations.

La laccase de *C. hirsutus* peut être utilisée de manière efficace pour dégrader les principales formes des aflatoxines d'*A. parasiticus* avec un maximum de dégradation de 38,2, 30,1, 76,4 et 100% des aflatoxines AFB1, AFB2, AFG1 et AFG2, respectivement, avec des conditions optimisées.

Les données expérimentales obtenues tout au long de cette étude ont suggéré que la laccase peut dégrader les aflatoxines, qui sont dérivés de difurocoumarin, en plusieurs produits de dégradation, avec un large éventail de structures et de poids moléculaires, où les plus importants étaient identifiés comme des époxydes, des diols et des dérivés coumarines. Les analyses structurales des produits finaux ont suggéré que la laccase peut soit modifier la structure des aflatoxines soit conduire à la production de précurseurs, qui seront à leurs tours convertis en des dérivés d'aflatoxines avec des propriétés potentiellement non toxiques.

En résumé, la laccase partiellement purifiée produite à partir de *C. hirsutus* peut éventuellement être appliquée pour éliminer les aflatoxines dans les aliments contaminés afin de diminuer les risques de ces toxines à la fois sur santé humaine et animale.

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LIST OF ABBREVIATION

ABTS	2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
ADTZ	Aflatoxin detoxifizyme
AFB1	Aflatoxin B1
AFB2	Aflatoxin B2
AFB2a	Aflatoxin B2a
AFG1	Aflatoxin G1
AFG2	Aflatoxin G2
AFH1	Aflatoxin H1
AFL	Aflatoxicol
AFM1	Aflatoxin M1
AFM2	Aflatoxin M2
AFO	Aflatoxin-oxydase
AFP1	Aflatoxin P1
AFP2	Aflatoxin P2
AFQ1	Aflatoxin Q1
AFQ2	Aflatoxin Q2
ATCC	American Type Culture Collection
a_w	Water activity
BT	Benzotriazole
CA	Czapek's agar
Ca^{2+}	Calcium (II)

C–C	Carbon to carbon coupling bond
C=C	Carbon to carbon coupling double bond
CCD	Complex Composite Design
C–H	Carbon to hydrogen bond
=CH	Alken
CH_2	Carbon to two hydrogen bonds
Cl^-	Chloride ion
Co^{2+}	Cobalt (II)
C=O	Ketone group
COO	Carbon to two oxygen bonds
CPZ	Chlorpromazine
Cu^{2+}	Cupper (II)
$CuCl_2$	Copper chloride molecule
$CuSO_4$	Copper sulfate molecule
CYP 1A2	Cytochrome 1A2
CYP 3A4	Cytochrome 3A4
CYP 3A5	Cytochrome 3A5
CYP 450	Cytochrome P450
DAD	Diode-array detector
DCC	Diethyldithiocarbamic acid
DMP	2,6- dimethoxyphenol
DTGS	Detracted triglycine sulfate detector
DTT	Dithiothreitol

E°	Redox potential
ELISA	Enzyme linked immuno-sorbent assay
EPR	Electron-paramagnetic resonance
F^{-}	Fluoride ion
FAO	Food and Agriculture Organization
$Fe_2(SO_4)_3$	Ferric sulfate
FTIR	Fourier-transform infrared spectroscopy
γ	Gamma rays
GSH	Glutathione
GST	Glutathione-S-transferase
H_2SO_4	Sulfuric acid
HBT	1-Hydroxybenzotriazole
HCl	Hydrochloric acid
HNNS	4-Hydroxy-3-nitroso-1-naphthalenesulfonic acid
HPI	N-hydroxyphtalimide
HPLC	High-Performance Liquid Chromatography
HPLC-MS	High-Performance Liquid Chromatography-Mass Spectroscopy
HXH	Helix-X-Helix
HIC	Hydrophobic interaction chromatography
K_{cat}	Catalytic efficiency determined as V_{max}/K_m
KCN	Potassium cyanide
*k*Da	Kilo dalton
KH_2PO_4	Monopotassium phosphate

K_m	Michaelis constant
LC-MS	Liquid Chromatography-Mass Spectrometry
LC-MS-MS	Liquid Chromatography-tandem Mass Spectrometry
LOX	Lipoxygenase
mARN	Messenger ARN
Mg^{2+}	Magnesium (II)
$MgSO_4.7H_2O$	Magnesium sulfate
Mn^{2+}	Manganese (II)
$MnSO_4$	Manganese sulfate
MS	Mass spectrometry
MW	Molecular weight
$Na_2B_4O_7$	Sodium tetraborate decahydrate
$(NH_4)_6Mo_7O_{24}$	Ammonium molybdate tetrahydrate
$(NH_4)_2SO_4$	Ammonium sulfate
NMR	Nuclear magnetic resonance
NNDS	1-Nitroso-2-naphthol-3,6-disulfonic acid
NOH	*N*-hydroxy moiety
o-	Ortho
O_3	Ozone
O–CH3	Methoxy group
OH	Hydroxide group
p-	Para
P	Probability

PDA	Potato dextrose agar
PHS	Prostaglandin H synthase
PPL	Partial purified laccase
PZ	Promazine
R^2	Coefficient of determination
RBB	Remazol brilliant blue
RP-HPLC	Reversed-Phase High-Performance Liquid Chromatography
RSD	Relative standard deviation
RSM	Response surface methodology
SD	Standard deviation
SEC	Size-exclusion chromatography
SYG	Syringaldazine
TFA	Trifluoroacetic acid
Tg	Glass transition temperature
TLC	Thin-layer chromatography
T	Type
UV	Ultraviolet
VIS	Visible
V_{max}	Maximum reaction rate
Zn^{2+}	Zinc (II)
$ZnSO_4$	Zinc sulfate

CHAPTER I

INTRODUCTION

Mycotoxins are secondary metabolites, produced under certain environmental conditions by the growth of various molds on several agricultural products and animal feed, and can have toxic effects (Rustom, 1997). Contaminations are mainly resulted from improper harvest as well as pro-and post-harvest management of the crops in poor environmental control and conditions during processing, packaging, storage and distribution (Mishra and Das, 2003). The food mycotoxins and feed contamination are serious food safety issues that could have a major health and economical impacts. Several hundreds of mycotoxins have been identified and chemically characterized, of which many of them were found as food contaminants (Richard *et al*., 2003). In addition, to their responsibility for acute poisoning (Hussein and Brasel, 2001), mycotoxins are also involved in the deterioration of food quality (Bhat *et al*., 2010).

Because of their frequent presence in food and feedstuffs and their potential toxic, mutagenic, teratogenic and carcinogenic effects, aflatoxins have been widely studied among mycotoxins (Reddy *et al*., 2010). Aflatoxins are difurano-coumarin derivatives produced as secondary metabolites by *Aspergillus* species, including *A. flavus*, *A. parasiticus, A. nomius* and *A. Tamarii* (Bhat *et al.*, 2010). The most common aflatoxins in food and feed products are AFB1, AFB2, AFG1, AFG2, AFM1 and AFM2 (Pietri *et al.*, 2012). These aflatoxins were identified on the basis of their blue or green fluorescence under ultraviolet light and on their relative chromatographic mobility in thin-layer chromatography of silica gel (Keller *et al*., 2005). The tolerable level of aflatoxins for human is ranged from 2 to 20 µg/kg (Rustom, 1997).

Although there are many physical and chemical detoxification processes for aflatoxins which consist of the removal or inactivation of the toxins in the contaminated products, none of these conventional methods are applicable for all different types of food and food products. In addition, these methods require a high cost; hence, the use of a biological strategy and more especially the use of enzymes could be a potential approach that could improve the agri-food industrial processes and to preserve the nutritional quality of food and food products (Kabak *et al*., 2006). Although peroxidases showed high efficiency in aflatoxins degradation, the presence of hydroperoxides during this process resulted by the production of free radicals that can react with the toxins (Mishra and Das, 2003; Tripathi and Mishra, 2011). These free radicals, produced through the substrate oxidation by peroxidases, may initiate lipid peroxidation and hence could bind to cellular proteins as well as DNA. The reactive molecules can epoxidize many substrates,

including polycyclic aromatic hydrocarbons, generally resulting in increasing toxicity of the respective substrates (Levi, 2004). Hence the aflatoxins peroxidases degradation causes problems, the need to find another enzyme system is essential, where laccase could be the appropriate one (Alberts *et al*., 2009).

Laccases are oxygen oxidoreductases, as part of multi-blue copper proteins. They are ubiquitous in the nature and are produced by a wide variety of plants, fungi and bacteria. In fungi, laccases can be found in ascomycetes, deuteromycetes and most white-rot basidiomycetes (Baldrian, 2006). White-rot fungi are well known and have long been investigated as one of the most abundant producers of fungal laccases (Marques de Souza and Peralta, 2003). Laccases play an essential role in many cellular activities, such as the biosynthesis of the cell wall of plants, the pigmentation of fungal and bacterial spores, the lignin degradation and the oxidation of phenolic compounds as well as in the carbon cycle, and may contribute to the degradation of a wide range of xenoaromatics (Madhavi and Lele, 2009).

Due to their broad substrate specificity, their great power of catalysis and their ability to use oxygen from the environment as a cofactor, fungal laccases are used in a wide range of biotechnological and industrial applications (Thurston, 1994). These applications include lignocellulosic degradation, pulp delignification, textile dye bleaching, polycyclic aromatic hydrocarbon degradation, bioremediation, biosensors, detergent manufacturing, wine clarification and fruit juice stabilization as well as olive oil mist wastewater removal and organic synthesis (Octavio *et al*., 2006). Alberts *et al*. (2009) reported the ability of pure laccase, from *Trametes versicolor*, to detoxify aflatoxin AFB1; this detoxification is fast and effective and unlike peroxidases, the mechanism of detoxification does not cause the formation of free radicals, which makes the whole process safer.

The possible mechanism for the detoxification of aflatoxins by laccase could involve two different pathways, the removal of the double bond positioned at the terminal furan ring and the opening of the lactone ring (Liu *et al*., 1998; Mishra and Das, 2003). Wang *et al*. (2011) reported that the oxidation of the 8,9-vinil bond of AFB1, by peroxidase groups, including laccase, resulted by the formation of the AFB1-8,9-epoxide, followed by its hydrolysis to generate the AFB1-8,9-dihydrodiol. In addition, Taylor *et al*. (2010), showed that the two dependent

reductases FDR-A and -B, from *Mycobacteria*, catalyzed the reduction of AFB1, AFB2, AFG1 and AFG2; this reduction wasn't due to that of the furan moiety by these enzymes but possibly due to the reduction of the double bond of the *α, β*-unsaturated ester moiety between the lactone ring in AFG1 and AFG2 and the lactone and cyclopentenone rings in AFB1 and AFB2.

The literature indicated the presence of wide range of methods, used for the characterization of aflatoxins and their enzyme-catalyzed end products. Among them, high-performance liquid chromatography (HPLC) was the most common method for the separation, quantification and identification of aflatoxins and their degradation products (Turner *et al*., 2009). Although the aflatoxins separation can be achieved with both normal (NP) and reversed phases (RP) columns, RP ones are the more commonly used (Medina and Magan, 2012). In addition, other methods called rapid methods for aflatoxins analysis, such as enzyme-linked immunosorbent assay (ELISA), have been developed to determine and to quantify aflatoxins (Zheng *et al*., 2006). Fourier-transform infrared (FTIR) is a method used to confirm the presence of aflatoxins and their characteristics, where the analysis is rapid and requires only minimal sample size and chemicals (Mirghani *et al*., 2001). Mass spectrometry (MS) is also widely used for the characterization of aflatoxins and their degradation products (Wang *et al*., 2011; Samuel *et al*., 2014). Nuclear magnetic resonance (NMR) spectroscopy was also used to determine the structure of aflatoxin AFB1 metabolite (Wang *et al*., 2011).

The present thesis contains seven chapters. Chapter I provides a general introduction, whereas chapter II covers the literature review of the most recent and relevant informations related to the undertaken research work. Chapter III describes the production and the recovery of the major aflatoxin isoforms, obtained from selected fungal strains, using selected cryoprotectants as well as their structural characterizations. Chapter IV describes the characterization of the enzymatic activity in terms of its optimum pH, optimum reaction temperature as well as its kinetic characterizations, using the major aflatoxin isoforms as substrates. The effects of selected chemicals and metal ions on the laccase activity are also express. Chapter V deals with the modeling and the optimization of laccase enzymatic degradation of selected microbial aflatoxins using response surface methodology, through three selected parameters, enzyme concentration, aflatoxin concentration and incubation time. Chapter VI covers the purification and the characterization of the laccase-catalyzed end products in terms of their chemical structure.

Chapter VII provides a general conclusion on the overall work and observations made during this study.

CHAPTER II

LITERATURE REVIEW

DETOXIFICATION OF AFLATOXINS BY LACCASE: A REVIEW

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2.1. Contribution of the Authors

The present author, Sabrina Borgomano, was responsible for this manuscript preparation of the submitted papers.

Dr. Monique Lacroix, the thesis supervisor, and Dr. Selim Kermasha, the thesis co-supervisor, supervised the research work, critically reviewed and edited the manuscript prior to its submission.

2.2. Aflatoxins

2.2.1. Sources, Structures and Properties of Aflatoxins

Aflatoxins are highly toxic compounds, mutagenic, teratogenic, and carcinogenic called mycotoxins. They are produced as secondary metabolites by fungi belonging to several species of *Aspergillus*, especially *A. flavus*, *A. parasiticus*, *A. nomius*, and *A. tamarii* (Brown *et al.*, 1999; Bhat *et al.*, 2010). They were discovered in the early 1960s when thousands of turkey poults mysteriously died in hatcheries in and around London. All of the dead turkeys had been fed with the same Brazilian peanut meal. The meal was heavily contaminated with a common species of mould (Keller *et al*., 2005). The aflatoxin name comes from the combination of "a" for the genus *Aspergillus* and the "fla" for *flavus* species, which means poison toxin (Bhat *et al*., 2010). From the agricultural point of view, the two most important sources are the fungi *Aspergillus flavus* and *A. parasiticus*. These two parasitic fungi can successfully colonize and produce aflatoxins, although *A. flavus* is known as the most aggressive and the most predominant one on corn, peanuts, cotton and nuts (Payne, 1998; Brown *et al.*, 1999). The genus *Aspergillus* belongs to a class of fungi known as the Hyphomycetes, belonging to the subdivision of the Deuteromycotina fungi. The Hyphomycetes consist of organisms including conidia states which they produce directly on mycelia or conidiophores (Ellis *et al*., 1991). Overall, aflatoxins contaminate a wide range of foods such as maize, rice, groundnuts, oilseeds, vegetable oils, spices, soybean, cotton and nuts (Brown *et al*., 1999; Bhat *et al.*, 2010). Three other sources of contamination are eggs, dairy products and natural medicines (Bhat *et al*., 2010).

The aflatoxin-producing fungi show large variations in their growth needs. For example, the range of minimum temperature for the growth of *A. parasiticus* is between 6 and 8°C and the maximum is between 44 and 66°C, with an optimum from 25 to 35°C (Dienner *et al*., 1982), while *A. flavus* may grow between 12 and 42°C, with an optimum around 30°C (Brackett, 1989). Thus, aflatoxins have a strong presence in the tropical and subtropical regions where temperature and humidity are optimal for toxins production.

Aflatoxins are an assembly of a coumarin and 3-furan. Currently, 18 different types of aflatoxins have been identified; whom AFB1, AFB2, AFG1, AFG2, AFM1 and AFM2 are the most common in food products (Fig. 2.1). Among these, the aflatoxins AFB1 and AFG1 are the most frequently produced and have a greater toxic potential (Mishra and Das, 2003).

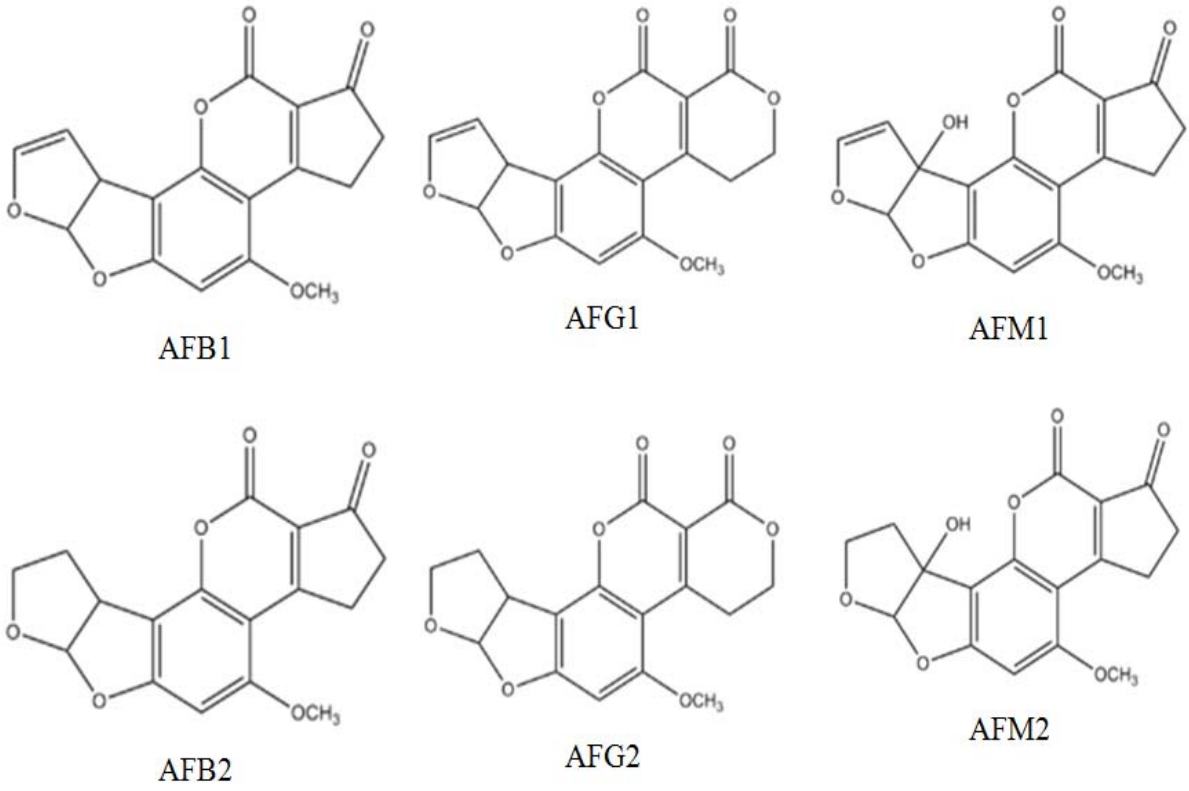

Figure 2.1. Chemical structure of the most common aflatoxins.

The structural difference design may be determined by fluorescence. Indeed, when the chromatograms of aflatoxin extracts were observed under ultraviolet light, a complex set of fluorescent compounds is usually present. Two emit visible blue light and were named AFB1 and AFB2 and two emit a yellow-green light called AFG1 and AFG2. In addition, the M-form is produced in small quantities and it is derived from the metabolism of aflatoxin AFB1 and AFB2 in animals (Bhat *et al.,* 2010). Some physical properties of aflatoxins are summarized in Table 2.1.

Table 2.1. Summary of the physical characteristics of aflatoxins.

Aflatoxin	Molecular formula	Molecular weight	Melting point
AFB1	$C_{17}H_{12}O_6$	312	268-269
AFB2	$C_{17}H_{14}O_6$	314	286-289
AFG1	$C_{17}H_{12}O_7$	328	244-246
AFG2	$C_{17}H_{14}O_7$	330	237-240
AFM1	$C_{17}H_{12}O_7$	326	299
AFM2	$C_{17}H_{14}O_7$	330	293

The spectral characteristics of aflatoxins have been determined by several studies (Asao *et al*., 1965; Wogan, 1966). Wogan (1966) reported that ultraviolet absorption spectra of AFB1, AFB2, AFG1 and AFG2 are very similar, showing two maximal absorptions at 265 and 363 nm. The molar extinction coefficients show that AFB1 and AFG2 absorb more intensely than AFB2 and AFG1 at these two wavelengths. Regarding AFM1 and AFM2, their absorption maxima are between 264 and 357 nm. Due to the close structural similarities, the infrared absorption spectra of these compounds are also very similar. The maximum fluorescence emission for AFB1, AFB2 and AFM1 has been reported at 425 nm and it is at 450 nm for AFG1 and AFG2 (Wogan, 1966; Bhat *et al*., 2010). Despite the various forms found, all the aflatoxins share a common structure that is double cyclic coumarin structure (Guan *et al.*, 2008).

2.2.2. Biochemical Mode of Action of Aflatoxins

Aflatoxins are highly liposoluble metabolites and are readily absorbed through the gastrointestinal and respiratory tracts into blood stream (Bbosa *et al*., 2013). Human and animals are exposed to aflatoxins by two major ways, the direct ingestion of aflatoxin-contaminated foods or from milk and milk products like cheese and powdered milk as well as other animal tissues contamined with mainly AFM1 or by inhalation of dust particles of aflatoxins especially

AFB1 in contaminated foods in industries and factories (Bbosa *et al*., 2013). After ingestion of the aflatoxins in the body, they are absorbed across the cell membranes, where they reach the blood circulation. They are distributed from blood to different tissues and to the liver, the main organ of metabolism of xenobiotics. Aflatoxins are mainly metabolized by the liver into a reactive epoxide intermediate or hydroxylated into the less harmful aflatoxin AFM1 (Wu *et al*., 2009). The physico-chemical and biochemical characteristics of the AFB1 molecule reveal two important sites for its toxic activity (Mishra and Das, 2003). The first site is a double bond in position 8,9 of the furan ring. The interaction of aflatoxin with DNA and proteins, which occurs on this site alters the biochemical functions of these macromolecules and leads to undesirable effects at the cellular level. The second reactive group is the lactone ring in the coumarin (Mishra and Das, 2003). This cycle is easily hydrolyzed and it is therefore vulnerable to the degradation. The toxic effects of AFB1 occur after metabolic activation of the molecule by the oxidase function of microsomal system. This metabolic activation of AFB1 may lead either to the pathway of the toxicity or that of the detoxification or both or lead to the formation of less toxic metabolites. AFB1 are metabolized by the cytochrome P450 (CYP450) microsomal enzymes to aflatoxin-8,9-epoxide, a reactive oxygen species that binds to DNA and to albumin in the blood serum, forming adducts and hence causing DNA damage and induce liver cancer or may bind to proteins and cause acute toxicity (aflatoxicosis). The predominant human CYP450 isoforms involved in human metabolism of AFB1 are CYP3A4 and CYP1A2. Both enzymes catalyze the biotransformation of AFB1 into the highly reactive exo-8,9-epoxide (Guengerich *et al*., 1998). CYP1A2 is also able to catalyze both the epoxidation of AFB1 to form an endo-epoxide and the hydroxylation of AFB1 to form aflatoxin AFM1, which is less potent than AFB1 (Wild *et al.,* 2002). These reactions are considered as the major detoxification metabolic pathway for the aflatoxins. The CYP3A4 is the major CYP450 enzyme responsible for activation of AFB1 into the epoxide form as well as into AFQ1 form, a less toxic metabolite. The CYP3A5 may also metabolize AFB1 mainly into the exo-epoxide and some into AFQ1 (Wang *et al*., 1998).

The CYP3A7 is a major CYP450 enzyme isoform in human fetal liver and metabolizes AFB1 into the 8,9-epoxide that may cause fetal defects to the developing fetus (Kitada *et al*., 1989). The binding of AFB1 to DNA and DNA adduction by AFB1-8,9-exo-epoxide have been reported to cause a functional changes of DNA conformation and mutation leading to incorrect

protein synthesis (Mishra and Das, 2003). The epoxide is highly unstable and binds with high affinity to guanine bases in DNA to form aflatoxin-N7-guanine (Guengerich, 2001). The aflatoxin-N7-guanine has been shown to be able to transform guanine (purine) to thymine (pyrimidine) nucleobases, a transversion mutation in DNA, and hence affecting the p53 suppressor gene in the cell cycle (Bbosa *et al*., 2013). The p53 gene is important in preventing cell cycle progression when there are DNA mutations, or signaling apoptosis (Li *et al*., 1993). Raney *et al*. (1993) studied the interaction of AFB1 with DNA and DNA adduction by AFB1-8,9-epoxide. Their result showed that the duplex structure promotes the formation of adducts. The yields of adduct formation were compared for forms A, B and Z DNA helices. Around, 12 times less adducts were produced from the A helix shape compared to the B form, whereas no adduct was produced from the Z form. They concluded that the reaction of AFB1-8,9 epoxide with DNA occurs via a transition state complex intercalated with the B form of the double helix. Aflatoxin binds with lysine of serum albumin resulting in the formation of AFB1-lysine complex (Mishra and Das, 2003). Ch'ih *et al*. (1993) studied the nuclear translocation of AFB1-protein complex. They showed the *in vitro* binding of AFB1-3 [H] to various proteins by dialysis at 23°C, where the binding capacity of AFB1-3 [H] (mmol/mol) changed according to nature of the protein; pyruvate kinase had the highest affinity, following by albumin-NLS and albumin then carbonic anhydrase, after that RNase and finally the histones.

Aflatoxins can also interfere with DNA replication or with the transcription of messenger RNA (mRNA) into protein by interacting with the DNA-dependent RNA polymerase activity and thus causes degranulation of endoplasmic reticulum (Sharma *et al*., 2011). Clifford and Rees (1967) showed that the administration of a single dose of 7 mg/kg of AFB1 in rats resulted in slow development of periportal necrosis. Moreover, liver enzymes have been released into the serum after 48h of intoxication, followed by an increase in the activity of phosphatase and bilirubin. Clifford *et al*. (1967) demonstrated that the underlying biochemical changes, in the development of liver necrosis in rats after administration of AFB1, were initiated by the interaction of the toxin with DNA. This interaction prevented the RNA polymerase to transcribe the DNA and inhibits the formation of mRNA. The failure of the formation of mRNA leads to an inhibition of protein synthesis, which would cause the necrosis of the liver. The reduction in protein content in

body tissues like in skeletal muscle, heart, liver and kidney resulted by the increase of liver and kidney necrosis (Sharma *et al*., 2011).

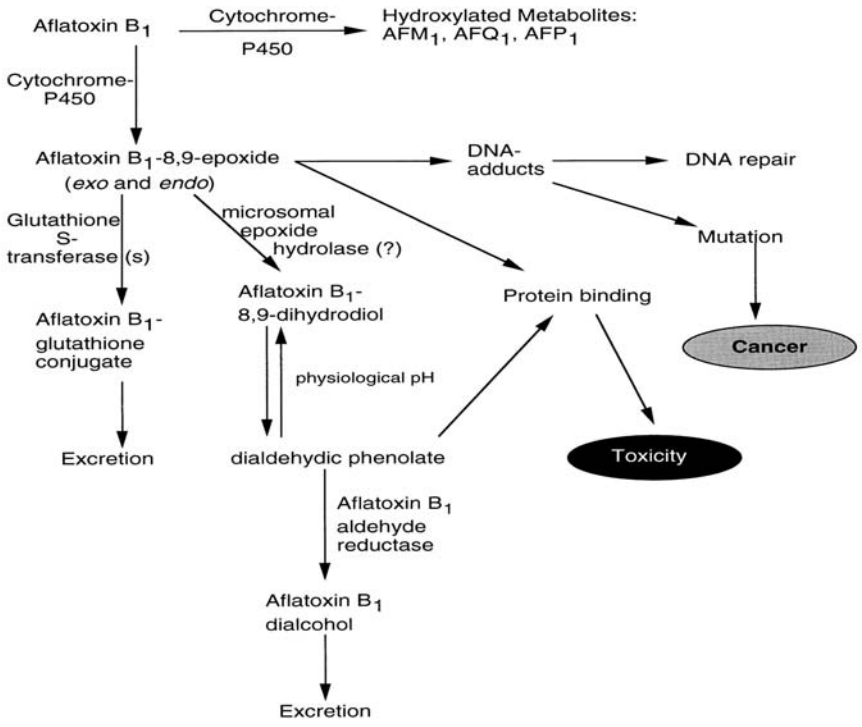

Figure 2.2. Biotransformation pathway of aflatoxin AFB1 (Bammler *et al*., 2000).

AFB1-8,9-epoxide can also be combined with proteins and glutathione (GSH) leading to its excretion by the body. Glutathione pathway has an essential role in the detoxification of AFB1. The catalyze of the conjugation of glutathione to AFB1-8,9-epoxides by the glutathione-S-transferase (GST) is the most important reaction to prevent the epoxide binding to DNA and also to various cell proteins (Sherratt *et al*., 2001). The AFB1-8,9-exo and -endo-epoxides are conjugated with the glutathione to form AFB-mercapturate by the catalyze by GST. The glutathione-aflatoxin conjugate is transported from the cells with an ATP-dependent multidrug resistance protein through an accelerated process (Farombi and Nwaokeafor, 2005). Sarasin *et al*. (1977) revealed that a mechanism for DNA repair occurs in human cells after treatment with AFB1. Aflatoxins, which are potent inhibitors of protein synthesis, affect the differentiation in primordial cells (Mishra and Das, 2003) and lead to a teratogenic effect in some animals.

The reactive aflatoxin-8,9-epoxide also binds to mitochondrial DNA (mitDNA) during hepatocarcinogenesis that avoid the ATP production and FAD/NAD-linked enzymatic functions

leading to the disruption of mitochondrial functions in the various parts of the body that require production of energy in the form of ATP (Mishra and Das, 2003). The damages of aflatoxin in mitochondria may lead to mitochondrial diseases and may be responsible for aging mechanisms (WHO, 2008). Wogan *et al*. (1971) studied the structure-activity relationship and carcinogenicity of aflatoxins. Large doses of aflatoxins cause complete inhibition of biochemical processes and lower doses affect different metabolic pathways. They inhibit the absorption of O_2 throughout the tissues by acting on the adenosine triphosphatase enzyme of electron transport chain resulting in a lower production of ATP (Mishra and Das, 2003). Aflatoxins also reduce the level of liver glycogen, probably by inhibiting the glycogenesis or depression of glucose transport into liver cells or by accelerating glycogenolysis (Mishra and Das, 2003).

In the hepatocytes of the liver, AFB1 are converted into aflatoxicol by cytoplasmic reductase and by microsomal mixed-function oxidase system to form AFB1-8,9-epoxide (highly toxic, mutagenic, and carcinogenic), AFM1 (toxic) and AFQ1, AFP1, $AFB2_a$ (all relatively nontoxic), that can be deposited in various body tissues as well as in edible animal products (Wu *et al*., 2009). These metabolites can undergo a detoxification by several molecules and excreted from the body, such as aflatoxin AFQ1, which is excreted in the urine.

2.2.3. Toxicity and Its Effects

Aflatoxins have been reported to affect the various body organs, including the liver, kidneys, lungs, brain, testes and many endocrine and exocrine organs, such as the heart, skeletal muscles and the different body systems (Bbosa *et al*., 2013). Repeated consumption of foods/feeds contaminated with high levels of aflatoxins may lead to acute aflatoxicosis and regular intake, even at low levels (ppb), is responsible for the production of bile, hepatic necrosis, the osteosclerosis of bone, childhood cirrhosis, the immune system suppression, and the veno-occlusive lesions. Biological effects of aflatoxins ingestion can be categorized as toxic, carcinogenic, mutagenic, and teratogenic (Rutsom *et al*., 1997). These effects are influenced by several factors, including the variation of species, sex, age, diet as well as chemical interaction between aflatoxins and other compounds in the biological system (Mishra and Das, 2003). In addition, the dose level ingested and the period of exposure of the toxin on the body plays a very important role.

Aflatoxins are also linked to kwashiorkor, a disease related to the protein-energy malnutrition (Bhat *et al*., 2010). Studies reported that AFB1 and aflatoxicol were detected more frequently in the serum, liver, urine and feces of children suffering from kwashiorkor (Bhat *et al*., 2010). The role of aflatoxins in the development of Reye's syndrome (encephalopathy with severe lesion in the liver and kidney following influenza or varicella disease) has never been proved, regardless of the frequent detection of aflatoxins in the liver of children dead from this disease (Casteels-van Daele and Eggermont, 1994). Aflatoxins may be transferred into AFM1 form, from mother to infant by breast milk (Rastogi *et al.*, 2004). Ghiasian and Maghsood (2012) reported that children exposed to AFM1 through milk or it's by products may become prone to infectious diseases, underweight, and stunted during infancy for the rest of their life. Egal *et al*. (2005) reported that 90% of children in West Africa, especially in Benin and in Togo, are exposed to aflatoxins due to the consumption of contaminated maize and groundnuts, which leads to a measurable deterioration of the child growth. Severe liver damages among the malnourished adults during the 1970s, with a fatal outcome have been also reported after severe cases of acute aflatoxicosis in parts of Asia and Africa (Krishnamachari *et al*, 1975; Bhat and Krishnamachari, 1977).

The ingestion of Aflatoxin AFB1 during pregnancy may cause risk to the intrauterine life. AFB1 has been reported to have prenatal effects on certain animals. This aflatoxin is a potent inhibitor of protein synthesis in eukaryotic cells, thus it impairs differentiation in sensitive primordial cells (Ellis *et al*., 1991). The prenatal effect of the AFB1 varies greatly during the period of the gestation; nevertheless it is known that the embryo is most susceptible during the early stages of morphological differentiation causing a high proportion of malformed and dead or reabsorbed fetuses (Ellis *et al*., 1991). Wangikar *et al*. (2005) reported that New Zealand white rabbits, between 6 and 18 days of gestation, intubated with corn oil contamined with aflatoxins presented pathomorphological lesions observed in various fetal tissues.

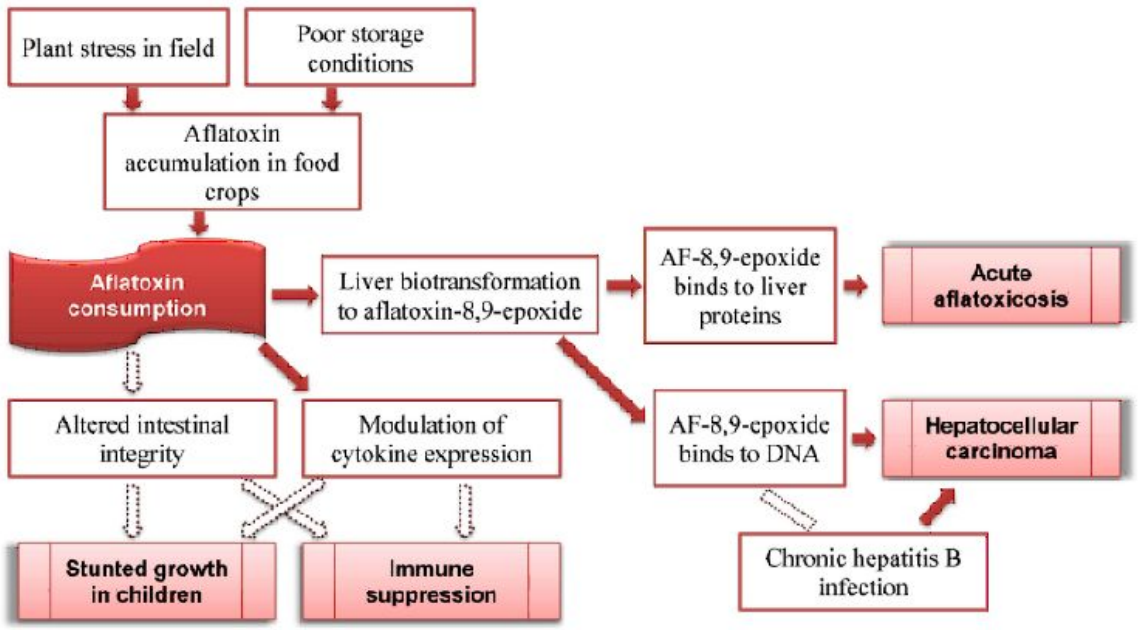

Figure 2.3. Aflatoxin disease pathways (Bbosa *et al*., 2013).

Aflatoxins are considered as co-factors in liver cancer (hepatocellular carcinoma) with hepatitis-B virus (HBV) in tropical Africa (FAO, 1997). HBV interferes with the ability of hepatocytes to metabolize aflatoxins, therefore, an aflatoxin AFM1-DNA conjugate exists for a longer period of time in the liver, which increases the probability of damage to the suppressor genes tumors. Among animals, monogastric farm animals, such as poultry and pigs, are at high risk, since a large part of their staple diet consists of cereals. In addition, these animals do not have the digestive system of ruminants. The rumen tank is composed of a multitude of microorganisms which may degrade toxins before being absorbed by the intestine. The susceptibility of the animals to the contamination of aflatoxins depends on the species, the age, and the diet. Bonomi *et al*. (1994) reported that ingestion of contaminated livestock feed can lead to substantial losses in productivity and the quality of the meat. The main symptoms of acute aflatoxicosis in mammals include lethargy, ataxia, rough hair coat, and increased liver size. With chronic exposure, the first symptoms of aflatoxin poisoning include the reduction of milk production and the decrease of appetite (Bhat *et al*., 2010). To fight against the potential danger of aflatoxins and to ensure the safety of human health, the U.S. Food and Drug Administration has established an acceptable range of the consumption level from zero tolerance to a maximum of 20 ppb for most foods (Mishra and Das, 2003; Sandosskumar *et al*., 2007). However, the European Committee

defined the total tolerable intake at a lower level, 4 ppb (Bhat *et al*., 2010). According to the FAO/WHO (World Health Organization) in 1990, the human tolerable limit for AFB1 is 5 µg/kg in food products and for the total aflatoxins, AFB1+AFG1+AFB2+AFG2, it is 15 µg/kg in foods. For AFM1 a strict limitation has been established at 0.05 µg/kg (Rustom, 1997). Overall, consumption of aflatoxins suppresses the immune system of the animal and human having impacts on the individual's daily activity and health conditions as well as causes lung injury and birth defects (Bbosa *et al*., 2013).

2.2.4. Toxins Preventive Methods

2.2.4.1. Control Strategies at Pre-Harvest Stage

According to the Food and Agriculture Organization (FAO), 25% of agricultural products in the world are contaminated with mycotoxins, leading to significant economic losses (Kabak *et al*., 2006). The mycotoxicity occurs much more in developing countries than in developed countries. This is only due to the lack of adequate laws and strict regulations imposed in the practices of agriculture and food production. The level of aflatoxin concentration authorized in food is continually declined; however, it is difficult to produce food with such small amounts of aflatoxins. It is estimated that more than 1.5 billion crop losses that occur each year are due to aflatoxins contamination (Cleveland *et al*., 2003). Several codes of practice have been developed by the Codex Alimentarius for the prevention and the reduction of mycotoxins in cereals, peanuts and raw materials. The development and acceptance of the code in general use by the Codex provides uniform guidelines for all countries to try to control and manage contamination by various mycotoxins. For this practice to be effective, it is necessary that the producers of each country respect the general principles of the code, taking into consideration their own cultural practices, cultures and climatic factors. It is well known that mycotoxins contamination of agricultural products may occur at harvest and during storage (Kabak *et al*., 2006). However, Lisker and Lillehoj (1991) showed that the seeds are contaminated with mycotoxins, essentially at the pre-harvest stage. Thus, it is suggested that a good management of conditions during storage and especially during periods of pre-harvest is essential in reducing or, better yet, in the elimination of toxins contamination (Brown *et al*., 1999, Mishra and Das, 2003).

2.2.4.2. Prevention of Aflatoxins Contamination

Prevention of fungal attack is possible using various means such as the use of good seeds, good irrigation method, the application of crop rotation, proper harvesting and drying technicals and the storage conditions management (Mishra and Das, 2003). However, other management practices, including the composition of the land, the soil cultivation, the irrigation and the use of fertilizers are known to affect mycotoxins formation in the field (Kabak *et al*., 2006). Crop rotation is important to break the chain of toxins production. Numerous studies reported the effectiveness of crop rotation to control toxins contamination (Jouany, 2007) such as the rotation of wheat and legume material (Kabak *et al*., 2006). Dill-Macky and Jones (2000) and Schaafsma *et al*. (2001) reported that the wheat crop grown after soybeans or maize reduced the incidence of toxins contamination in wheat grains. In addition, some crops such as potato, clover, and alfalfa are recommended in crop rotation to reduce the contamination of the field (Codex Alimentarius Commission, 2002). However, other studies have shown the ineffectiveness of this method; in semi-arid areas, the growth of *Aspergillus* may be very high, and crop rotation may have little influence on the fungal activity (Kabak *et al*., 2006). The soil composition may also play an important role in the contaminations by molds. For example, light and sandy soils promote rapid growth of fungi, especially under dry conditions. Heavier soils have a higher level of water retention which reduces the onset of a drought, which may be partly responsible for the lower aflatoxins contamination in peanuts grown on such soils. In Mediterranean climates, it is common to leave the plowed fields exposed in the autumn sun in order to destroy the fungal material that may contaminate the next crop (Nicholson *et al*., 2003). Irrigation is also a convenient way of reducing plant stress in some situations. It is first necessary that all plants in the field have an adequate supply of water if irrigation is used. It is known that rainfall excess during anthesis (flowering) creates favorable conditions for contaminations, hence irrigation during anthesis and maturation of crops, especially wheat, barley and rye, should be avoided (Codex Alimentarius Commission, 2002). It is important to note that soil analyses should be conducted to determine its need or no in fertilizers and/or in conditioners to ensure adequate soil pH, plant nutrition and to avoid physiological stress, especially during seeds development that can make them more susceptible to fungal contaminations (Kabak *et al*., 2006). The application of fertilizer can affect crops by altering the rate of residues decompositions, acting on the rate of plant growth, and changing the soil structure and microbial activity (Jouany, 2007).

Environmental conditions such as relative humidity and temperature are also factors known to have a significant effect on the growth of molds (Ciegler *et al*., 1966a).

There is evidence that plants damaged by drought are more susceptible to contaminations, therefore the planting of crops should be scheduled to avoid both high temperatures and drought stress during seed germination and full maturity can be an important point of control in the prevention of contamination by mycotoxins (Kabak *et al*., 2006). Another factor which is known to increase the sensitivity of agricultural products to the invasion of toxigenic molds is an injury caused by insects, birds, or damage due to rodents (Kabak *et al*., 2006). Insects, fungal infections and damages from the animals must be controlled by a proper use of registered insecticides, pesticides, fungicides, and other appropriate control practices in pest management (Codex Alimentarius Commission, 2002). In France, the Ministry of Agriculture has allowed the use of many fungicides, including tebuconazole and metconazole. In the European Union, fungicides must first be shown to be safe for both the environment and human beings before being allowed. Pesticides and fungicides may be useful for monitoring the production of mycotoxins under certain conditions, while other researchers have found that pesticides are ineffective in the control of mycotoxins production by *Fusarium* and *Aspergillus* species (Kabak *et al*., 2006). The main effect of pesticides in plant growth in the field is the control of insect damage, thus reducing the risk of mycotoxigenic molds invasion and the synthesis of toxins (report of the European Commission, 1999).

A number of biological and chemical control agents have been reported to inhibit aflatoxigenic molds growth and subsequent aflatoxins biosynthesis (Kabak *et al*., 2006). The application of competitive nonaflatoxigenic strains of *Aspergillus flavus* and *A. parasiticus* has clearly been shown to reduce the aflatoxins contamination of agricultural commodities, including peanuts, rice, maize, and cottonseed, mainly through a competition for the substrates and through the production of inhibitory metabolites (Kabak *et al*., 2006). Dorner *et al*. (1992) reported that the presence of *A parasiticus*, nonaflatoxigenic strains, in peanut crops resulted by a decrease of aflatoxins level from 531,96 and 241 $g.kg^{-1}$ to 11,1 and 40 $\mu g.kg^{-1}$, respectively.

Kabak *et al*. (2006) reported that many phenolic compounds, which are secondary metabolites synthesized by the phenylpropanoid biosynthetic pathway plants, may exert metabolic effects on

the biosynthetic pathway of aflatoxins. The acetosyringone, syringaldehyde, and sinapinic acid at concentration of 4 $mmol^{-1}$ have been reported to reduce the biosynthesis of aflatoxin by 96, 74 and 32%, respectively. Lee *et al*. (2001) reported that plant-derived compounds, such as antharaquinones, coumarins and flavonoids, can act as potent inhibitors in the biotransformation of aflatoxin AFB1 into AFB1-8,9-epoxide.

2.2.4.3. The Selection of Resistant Varieties

The method of artificial intelligence such as the use of resistant species to fungi is another option in the prevention of toxins contamination in food crops (Mishra and Das, 2003). The differences between plant species seem to differ between countries; this is probably due to the differences in the gene pool in the breeding program of each country and the various environmental and agronomic conditions in which seed varieties have grown. A number of researchers have worked on genetic resistance to *Aspergillus* infection and subsequent aflatoxins production. Since the early 1970s, much work has been done to identify genotypes of genetically resistant crops both in the laboratory and in the field in order to control of aflatoxigenic molds growth and aflatoxins biosynthesis (Kabak et *al.*, 2006; Cleveland *et al*., 2003). This led to the identification of a number of sources well characterized both resistant to infection by *Aspergillus flavus* and to the aflatoxin production. These include kernel proteins such as a 14-*k*Da trypsin-inhibiting protein as well as globulin 1 and 2 and a 22-*k*Da zeamatin protein (Chen *et al*., 2001). In addition, kernel proteins of maize genotypes GT-MAS and Mp-420 have also been identified to be involved in the resistance to *A. flavus* infection and aflatoxins contamination due to greater distribution on kernel surfaces of proteins to GT-MAS and Mp-420 genotypes which can inhibit and/or restrict entry of the aflatoxigenic moulds (Guo *et al*., 1998). The choice of variety of seeds can also be important and therefore before planting, farmers should consult appropriate services for plant breeding and agricultural services to determine if their cultivars are suitable for their region, and the availability of varieties that are resistant to various factors, such as insect as well as microbial and fungal attacks, that can affect the safety and quality of the seeds (Kabak et *al.*, 2006). However, resistance to the invasion of toxigenic fungi has been attributed to several biochemical, environmental and physical factors. Uncontrollable factors which could lead to failure of the use of certain selected varieties resistant to fungi (Mishra and Das, 2003).

2.2.4.4. The Use of Genetic Engineering

Genetic engineering is another way to reduce the risk from mycotoxins. Control of aflatoxins is possible by targeting the mechanisms governing the biosynthesis of aflatoxins. It can be achieved with the identification of genes and enzymes responsible for the synthesis of aflatoxins and the transfer of genes that encode resistant factors inhibiting toxins synthesis (Mishra and Das, 2003). This can lead to the formation of resistant varieties to moulds attacks or inhibit toxins production. Cleveland *et al*. (2004) showed that the resistance improved in some volatile compounds of plants, including n-decyl aldehyde, hexanol and octanol, has a negative impact on the growth of *Aspergillu*s, thereby controlling its toxin production. The use of genetic engineering is very useful in preventing the occurrence of food risks. However, it does not guarantee the development of toxins during improper storage and during transport conditions that promote the growth of fungi (Mishra and Das, 2003). The genetically modified crops are a similar technique that may be applied in the culture itself (Halasz *et al.*, 2009; Khlangwiset and Wu, 2010). This method can reduce aflatoxins contamination by protecting crops from wounds and damages by discouraging the fungi to penetrate and colonize the interior of the food product. This approach is most appropriate to prevent the contamination of food products by aflatoxigenic fungi. However, prevention is not always possible under certain conditions and agronomic practices, especially when environmental conditions create an atmosphere favorable to fungal growth and toxins production. In this context, detoxification would be an option for products already contaminated by toxic metabolites of fungi.

2.2.5. Degradation of Aflatoxins

The prevention of mycotoxins contamination before harvest or during post-harvest and storage is not always possible; therefore detoxification processes play an important role to limit the presence of mycotoxins. The detoxification of mycotoxins is obtained by removing or inactivating the toxins in contaminated products by chemical, physical, or biological methods. Kabak and *al.* (2006) and Mishra and Das (2003) reported that decontamination processes have to respond to certain standards in order to reduce the toxicity and the economic impact of mycotoxins.

- They must destroy, remove or inactivate mycotoxins;

- They must not produce or leave toxic and/or carcinogenic/mutagenic residues in the final products or food products from animals fed with food decontaminated;

- They should not affect the physico-chemical, nutritional and sensory properties of the food products;

- They must be able to destroy fungal spores and mycelium to prevent mycotoxins formation under favorable conditions;

- They must be technically and economically feasible.

2.2.5.1. Physical Methods

Many physical strategies have been applied for the decontamination of mycotoxins. These include the thermal inactivation and the inactivation by radiation. Radiations are classified into two categories, ionizing and non-ionizing. The ionizing radiations, including X-rays, gamma rays (γ), UV rays, lead to the changes in molecules of the irradiated food products. These molecular changes might be quite harmful to living organisms exposed to large doses of ionizing radiation. The non-ionizing radiations, including radio waves, microwaves, infrared waves, visible light, lead to an increase in the temperature, usually accompanied by molecular changes that are no hazardous to human health (Kabak *et al*., 2006). The use of ionizing radiation to free foods from pathogenic microorganisms is among the methods applicable in food preservation (Kyzlink, 1990). Most mycotoxins are relatively heat stable in a range of temperatures corresponding to the food processing (Kabak *et al*., 2006). For the aflatoxins, the degradation by temperature occures between 237 and 306°C, therefore, little or no destruction occurs during normal cooking conditions such as boiling, frying and pasteurization. The degradation of aflatoxins by heat treatment is also related to several factors, including the moisture content, as well as the pH and the ionic strength of the food (Rustom, 1997). The toxins degradation by heat treatment also depends on the type of mycotoxins, their concentrations, the extent of binding between the toxin and the feed, the degree of heat penetration, as well as the heating temperature and the time processing (Kabak *et al*., 2006).

Heathcote and Hibbert (1978) reported that the increase of the moisture content of food product resulted by the increase of aflatoxins degradation. These authors demonstrated that when flour

and cotton seed contaminated with aflatoxins AFB1 and AFB2 were heated at 100ºC during 1 hour with a moisture content of 30%, the aflatoxins were degraded by 74.8%. Whereas, in the same conditions but with a moisture content of 6.6% only 2.7% of the aflatoxins degradation occurred. It has been suggested that the presence of moisture in the food contributes to the opening of the lactone ring of AFB1 to form a carboxylic acid, followed by its decarboxylation induced by the heat treatment (Kabak *et al*, 2006).

Pluyer *et al*. (1987) showed that roasting peanuts at 150°C during 30 min resulted by a reduction of 30% in aflatoxins level, while a 60% reduction of aflatoxins level were reported after roasting at 150°C during 90 min. Kabak *et al*. (2006) reported that aflatoxin AFB1 was degraded by 38.0, 41.5 and 47.6% in unsalted peanuts, and by 2 and 5% in salted peanuts, when a traditional roasting method at 150°C during 30 min was used. While the use of high temperatures can be use to degrade the aflatoxins, it can also alter the nutritional and organoleptic properties of the food products.

Radiations involving X and γ rays have been widely studied as a method for mycotoxins degradation (Scott, 1998). The inactivation of mycotoxins by radiation depends on the degree of the radiation dose, the type of food and mycotoxins (Samarajeewa *et al*., 1990). Irradiation is a non-thermal treatment and is increasingly called "cold pasteurization" because it eliminates food borne pathogens without increasing the temperature of the product; moreover, the average doses of 10 kGy have no toxicological hazard (Kabak *et al*., 2006).

Gamma irradiation has been shown to destroy the conidia of *Aspergillus flavus* and inhibit the aflatoxins production in foods products (Chang and Markakis, 1982). Chang and Markakis (1982) reported that different γ irradiations doses, from 0 to 4 Gy reduced aflatoxins production in barley contaminated with *Aspergillus parasiticus* strain. Aziz and Youssef (2002) also showed that a dose of 20 kGy was sufficient for complete destruction of aflatoxin AFB1 in peanut, yellow corn, wheat and cotton seed meal. In addition, Jalili *et al*. (2010) demonstrated that a gamma-ray dose of 60 kGy was able to degrade 43, 24, 40 and 36% of aflatoxins AFB1, AFB2, AFG1 and AFG2, respectively. On the other hand, Frank and Grunewald (1970) reported that the dosage required to totally eliminating aflatoxin AFB1 from food and feed products was too high and it could cause a significant deterioration of the quality and the organoleptic properties of the

irradiated food products. In addition, the effectiveness of this method depends on the type of food and the moisture content. Water content has an important role in the destruction of mycotoxins by gamma rays, since the radiolysis of water leads to the formation of highly reactive free radicals that can attack the aflatoxin AFB1 at the terminal furan ring and produce molecules with lower toxicity and biological activities. Ghanem *et al*. (2008) reported that a gamma-ray dose of 10 kGy was able to degrade 58.6, 68.8, 84.6, 81.1 and 87.8% of aflatoxin AFB1 of in peanuts, peeled pistachios, unpeeled pistachios, corn and rice samples, respectively. Whereas, in the samples containing oil, the aflatoxin AFB1 degradation by a gamma-ray dose of 10 kGy was not effective.

Samarajeewa *et al*. (1990) reported that the aflatoxins were also sensitive to the UV rays at 222, 265 and 362 nm, which may lead to the formation of several photodegradation products. The aflatoxins AFBl and AFGl underwent photochemically degradation reactions when exposed to UV light at 365 nm during 1 h on silica gel TLC plates (Rustom, 1997). In addition, peanut and oil treated with UV light during 2 h resulted by the destruction of 40 to 45% of aflatoxins (Kabak *et al*., 2006). Yousef and Marth (1986) demonstrated that the aflatoxin AFM1 in milk was degraded by 56.2% with the use of UV radiation at 365 nm during 30 min. In addition, solar energy can also be used for the destruction of mycotoxins in food products, since UV rays are present in sunlight (Rustom, 1997). Kabak *et al*. (2006) reported that approximately 70% of AFB1 present in coconut oil artificially contaminated was destroyed by solar energy. It was also showed that aflatoxin AFB1 was completely degraded from peanut oil in transparent glass and translucent plastic containers after 18-24 h of sunlight exposure (Kabak *et al*., 2006).

2.2.5.2. Chemical Methods

A wide range of chemical compounds can reduce, destroy or inactivate mycotoxins. These chemical compounds include acids (ex: hydrochloric, sulfuric, salicylic, sulfamic and sulfosalicylic acids), bases (ex: ammonia, sodium hydroxide), oxidizing agents (ex: hydrogen peroxide, ozone), reducing agents (ex: sodium bisulfite, sodium sorbate), chlorinating agents (ex: sodium hypochlorite, chlorine dioxide and chlorine gas), and other reagents such as formaldehyde. Strong acids convert aflatoxins to form less toxic compounds, such as hemiacetal forms, through the hydration mechanism (Pons *et al*., 1972). Tabata *et al*. (1994) showed that 1%

of hydrochloric (HCl) or sulfuric (H_2SO_4) acid solutions completely degraded the aflatoxins AFB1 and AFG1 and reduced by 13 and 18% the aflatoxins AFB2 and AFG2, respectively.

Hasan (1996) reported that a concentration of 3*N* of salicylic, sulfamic, and sulfosalicylic acids, with 25% of moisture during 72 h at 25°C, reduced 90% of 1 ppm of aflatoxin AFB1 in sorghum grains. Inorganic and organic bases are effective and relatively inexpensive chemical compounds to destroy aflatoxins and other important mycotoxins in a wide range of contaminated agricultural products. Among these chemical methods, ammoniation is an approved procedure for detoxification of contaminated agricultural and food products by the aflatoxins (Kabak *et al*., 2006). The process involves the use of ammonium hydroxide or ammonia gases, which are both also effective for the detoxification of aflatoxins in peanuts, cotton and corn (Samarajeewa *et al*., 1990; Piva *et al.*, 1995). The success of this degradation process depends on the amount of ammonia, the reaction time, as well as the ranges of the temperature and the pressure used (Samarajeewa *et al*., 1990). Treatments under high pressure are more effective in the detoxification of contaminated food products than treatments under atmospheric pressure, with the advantage that the ammoniation at high pressure requires both lower ammonia concentrations and less processing time. The level of aflatoxins destruction by high pressure ammoniation may be increased with that in temperature. Kabak *et al*. (2006) reported that aflatoxin AFB1 in grain was degraded by 88% with the use of 2% of ammonia under atmospheric pressure and room temperature during 24 h, while it was completely degraded with the same concentration of ammonia but under high temperature and pressure (121°C/2 bar). Aflatoxin AFB1 degradation by ammonia is a result of the hydrolysis of the lactone ring followed by a decarboxylation which leads to the formation of a non-toxic compound, namely aflatoxin AFD1, and the loss of the cyclopentenone ring (Kabak *et al*., 2006).

The effect of alkaline agents, such as sodium, potassium or calcium hydroxide, on the destruction of mycotoxins is slightly lower than the effect of ammonia treatment (Samarajeewa *et al*., 1990). Kabak *et al*. (2006) reported that the treatment of peanut flour contaminated by AFB1, containing 30% moisture, with 2% of sodium hydroxide at 100°C during 120 min reduced the the toxin in trace state. The aflatoxin AFB1 dstruction in solutions at 110°C by alkaline agents is as the following order, potassium hydroxide> sodium hydroxide> potassium

carbonate> sodium carbonate> potassium bicarbonate> ammonium hydroxide> sodium bicarbonate> ammonium carbonate (Samarajeewa *et al*., 1990).

Several oxidizing agents were successfully used to detoxify mycotoxins from contaminated agricultural products. Ozone or triatomic oxygen (O_3) is a strong oxidant and reacts through many chemical groups, although it is particularly effective with olefinic double bonds (Kabak *et al*., 2006). Beuchat *et al*. (1999) demonstrated that aflatoxins were destroyed when cotton seed and peanuts were treated with aqueous O_3. The C=C double bond of the unsaturated terminal furan ring of aflatoxins AFB1, AFM1 and AFG1 is sensitive to O_3 and other oxydants. However, aflatoxins AFB2, AFM2 and AFG2 are resistant to oxidation by ozone because of the lack of the double bond in the terminal furan ring (Kabak *et al*. 2006).

The use of reducing agent is another way to degrade the aflatoxins. Sodium bisulfite is commonly used as a food additive because of its properties to inhibit enzymatic degradation and acts as an antioxidant and bacteriostatic agent (Kabak *et al*. 2006). Sodium bisulfite reacts with the double bond on position 8,9 of the terminal furan ring of aflatoxin AFB1 to form a sulfonate, called S-AFB1, (Samarajeewa *et al*. 1990; Piva *et al*, 1995; Scott, 1998). This reaction reduces the mutagenic potential of the aflatoxin AFB1 molecule. However, since aflatoxin AFB2 doesn't have this double bond, the degradation by bisulfite is not possible (King *et al*., 2005). Doyle *et al*. (1982) showed that concentrations of 0.5 and 2% of sodium bisulfite degraded by 80 and 90%, respectively, the level of aflatoxin AFB1 in corn. Kabak *et al*. (2006) also reported that a treatment with 200 ppm of sodium sorbate degraded by 68% the major aflatoxin isoforms, AFB1, AFB2, AFG1 and AFG2, in the dried figs.

Other agents such as aqueous chlorine are commonly used in the food industry to sanitize food processing equipments and to wash a wide variety of raw materials such as fruits, nuts, meat, and fish (Samarajeewa *et al*., 1991). Chlorinating agents, including chlorine and sodium hypochlorite, have been reported to destroy mycotoxins. Kabak *et al*. (2006) reported that the application of gaseous chlorine at 10% degraded more than 90% of aflatoxins in peanuts.

2.2.5.3. The Physical Separation

Since the detoxification of mycotoxins by chemicals is not always an acceptable practice in some areas due to adverse effects, the physical separation of contaminated crops is another important option for producers. Such means include sorting, segregation density, washing, milling, solvent extraction and adsorption.

Minimizing or preventing food contamination by fungal spores by cleaning the external surface of the grains and the removal of damaged kernels is a complementary way to reduce the infection of healthy grains by the contaminated ones. Sorting discolored or damaged crops, containing visible molds, may lead to the elimination of large quantities of mycotoxins from the cultures (Kabak *et al*., 2006). Various studies indicate that sorting removes large quantity of aflatoxins present at the harvest period (Schatzki, 1995, 1998). Manual, mechanical and electronic methods can be used for the separation of cultures. Manual selection is based on the fact that the damaged products vary in size, shape, color, and often visible mold present on the affected foods may be seen (Kabak *et al*., 2006). For example, sorting which removes visibly mouldy apples reduces the level of mould to an acceptable level (FAO, 2001). Although the manual selection is the easiest way to remove contaminated food, it remains a tedious process because of the time consuming. Partial removal of aflatoxins can be achieved by fluorescence sorting of maize, cottonseeds, and dried figs, where a positive correlation has been reported between the observation of a bright light greenish-yellow under UV light at 365 nm and the presence of aflatoxins in these commodities (Kabak *et al*. 2006).

Another effective method to reduce the level of mycotoxins is the use of flotation devices and segregation density that have been reported to significantly reduce the content of mycotoxins in crops, particularly aflatoxin levels in toxic corn grain. Seeds contaminated with mycotoxins show different physical properties from those undamaged and can be separated by segregation density in some liquid, or by the fractionation based on specific gravity tables.

A simple method to reduce mycotoxins in cereals and grains is to wash them with a solution based on water and sodium carbonate. Moreover, washing food with tap water pressure significantly reduces the level of mycotoxins in the contaminated products (Wilson *et al*., 2004).

The removal of some components on the grains during milling can reduce toxins level in contaminated grains. Milling has no direct effect on the mycotoxins content on the seeds; however, it changes the distribution of the toxin in the different fractions (Kabak *et al*., 2006).

Extraction with solvents is a technique capable to extract mycotoxins from contaminated food products such as oilseeds and cottonseed. The most commonly solvents used are 100 and 95% ethanol, 90% aqueous acetone, 100% methanol, aqueous isopropanol, 80% isopropanol, hexane-methanol 73:27, methanol-water 80:20, acetonitrile-water 90:10, hexane-ethanol-water 85:12:3 and acetone-hexane-water 54:44:2 propanol-water 80:20, 100% benzene and chloroform (Scott, 1998; Blesa *et al.,* 2003). Solvents extraction can effectively remove aflatoxins from oilseed meal without either the formation of toxic or any reduction in nutritional properties or qualities. However, the large-scale application of this technique is limited by the high cost and problems related to the disposal of toxic extract (Kabak *et al*., 2006).

Another method which can reduce the quantity of mycotoxins in food is adsorption. Some adsorbents may bind and eliminate mycotoxins from aqueous solutions (Kabak *et al*., 2006). Charcoal and bentonite are two binders' agents active on mycotoxins. The particle size of the adsorbent and heat treatment affect the level of mycotoxins degradation. Bentonite removes between 65 and 79% of aflatoxin AFM1 in the milk, and nearly 100% of aflatoxins in liquid solutions (Kabak *et al*., 2006).

Although many physical and chemical treatments can destroy mycotoxins present in many foods, they can still reduce the nutritional value of food products or produce toxic compounds and harmful residues with side effects that can potentially affect public health. In addition, these methods are generally expensive and can pollute the environment.

2.2.6. Aflatoxins Biodegradation Using Microoganisms

The use of many physical and chemical methods for the detoxification of agricultural products contaminated with mycotoxins is limited due to problems with security issues, the possible loss of the nutritional quality of processed products, coupled with their limited effectiveness and their high costs. This led to the search of alternative strategies such as biological agents. Progress in this area has been supported by recent advances in molecular biology, genetic engineering and

microbial genomics associated with the discovery of a wide range of catabolic in the microbial world. Many micro-organisms, including bacteria, actinomycetes, yeasts, fungi and algae, may degrade aflatoxins. Ciegler *et al*. (1966a,b) have identified about 1,000 micro-organisms with the ability to destroy or transform aflatoxins AFB1 and AFG1.

2.2.6.1. Degradation of Aflatoxins by Bacteria

Temperature and pH influence the absorption of the toxins by the cells. Mishra and Das (2003) reported that a large population of cells (10^{11} cells/mL) permanently removed a high percentage of aflatoxins in solution without being affected. *Flavobacterium aurantiacum* can significantly remove aflatoxin AFB1, from a liquid medium and from a wide variety of food products, including milk, peanuts and corn, without forming toxic products (Mishra and Das, 2003). Hao and Brackett (1988) reported the effectiveness of this organism since it eliminated aflatoxin AFB1 in milk and peanuts. Moreover, Kabak *et al*. (2006) reported that *Flavobacterium aurantiacum* NRRL-B-184 strain removed from 79.9 to 98.9, 92.6 to 99.8, and 88.7 to 100% of AFB1 in peanuts, phosphored- salin buffer and red pepper, respectively, in 48 h. These studies led to investigate the mechanisms of degradation, mainly, if this bacterium degrades aflatoxins or if the disappearance of the toxin resulted from the cells adsorption. Line *et al*. (1994) used ^{14}C labeled aflatoxin AFB1, treated with *F. aurantiacum,* to trace and detect the labeled product with a scintillation detector. The CO_2, the aflatoxin AFB1 degradation products and the water-soluble toxins adsorbed by the cells were measured. It has been shown that the elimination of aflatoxin AFB1 levels by living cells was significantly higher compared to that of dead cells, but these two types of cells adsorbed a certain amount of aflatoxin AFB1. In addition, the release of marked CO_2 only by living cells showed that some amount of aflatoxin AFB1 was metabolized by *Flavobacterium* cells. D'Souza and Brackett (1998) studied the role of metal ions (Cu^{2+}, $Mn^{2+,}$ Zn^{2+} and Co^{2+}) to understand the enzyme system involved in the degradation of aflatoxin AFB1 by *F. aurantiacum*. The effects of divalent chelators and the inhibitors groups, including seryl and sulfhydryl, were also studied. It is shown that the presence of copper and zinc ions inhibits the degradation of aflatoxin. However, the divalent cations Ca^{2+} and Mg^{2+} stimulate the degradation of aflatoxin AFB1 by *F.aurantiacum*. Crude protein extracts (800g of total/mL protein) from *F. aurantiacum* were tested for their ability to degrade aflatoxin AFB1 in aqueous solution. They were able to remove 74.1% of aflatoxins. Karunaratne *et al*. (1990) showed that

Lactobacillus acidophilus, *L. bulgaricus* and *L. planatarum* could be used either to prevent mold growth or to degrade aflatoxins. Kankaanpää *et al*. (2000) studied the adhesion of aflatoxin AFB1 by *Lactobacillus rhamnosus* GG strain, using an adhesion Caco-2 as model. They reported that the removal of aflatoxin AFB1 by the strain reduced adhesion capacity from 30 to 5%. Therefore, the aflatoxins may affect the adhesion of probiotic bacteria capable to sequester them, and subsequently it may reduce the accumulation of aflatoxin AFB1 in the intestine via increased aflatoxin-bacteria complex. Mishra and Das (2003) reported that among several strains, including *Bacillus megaterium*, *B. laterosporus*, *Cellulomonas cartae*, *Flavobacterium odoratum*, *Phyllobacterium rubiacearum*, *Pseudomonas aurofaciens* and *Xanthomonas maltophila* in dual culture with *Aspergillus flavus,* only *F. odoratum* inhibited the biosynthesis of aflatoxins.

2.2.6.2. Degradation of Aflatoxins by Fungi

A number of different fungal cultures have the ability to detoxify aflatoxins. Fungal strains, such as *Trichoderma* sp. 639, *Phoma* sp., *Rhizopus* sp. 668, *Rhizopus* sp. 720, *Sporotrichum* sp. ADA, *Sporotrichum* sp. SF and *Altemaria* sp, may degrade between 65 and 99% of aflatoxin AFB1 in 5 days at 28°C (Kabak *et al*., 2006). Ciegler *et al*. (1966b) studied the production of aflatoxins AFB1 and AFG1 and their degradation by several strains of *A. flavus*, including NRRL 2999, 3000, A-13570 and A-13367, also known as M001, in liquid medium. The aflatoxin production was observed during the first 72 h of growth, before its decrease. Hamid and Smith (1987) showed that molds able to produce aflatoxins could also degrade them, where the production of aflatoxins by *Aspergillus flavus* and *A. parasiticus* reached a maximum after 12 days and then decreased due to the aflatoxin-degradative activity in the mycelium and the cell-free extracts of these fungi. Kabak *et al*. (2006) reported that the addition of cycloheximide, hydrochloride SKF 525-A or metyrapone in the cultures of *A. flavus* prevents the aflatoxins degradation by the mycelium. In the cell extracts, the aflatoxins degradation was inhibited by the presence of SKF 525-A, metyrapone, and cytochrome-C, but not by potassium cyanide (KCN). In all free cell extracts, the aflatoxins degradation was enhanced by the addition of NADPH and $NaIO_4$. Hamid and Smith (1987) suggested the probable involvement of cytochrome P-450 mono-oxygenases in the aflatoxin-degradation activity of *A. flavus*. Cole *et al*. (1972) studied the conversion of aflatoxin AFB1 into isomeric hydroxy compounds by *Rhizopus* species. Two new fluorescent

compounds have been characterized by the use of AFB1 ^{14}C as stereoisomers of hydroxylated AFB1. Nakazato *et al*. (1990) studied the inter-conversion of AFB1 into aflatoxicol (AFL) by several fungi. They found that four fungal strains, including *Aspergillus niger*, *Eurotium herbariorum*, *Rhizopus* sp and nonaflatoxigenic *A. flavus,* could convert aflatoxin AFB1 in aflatoxicol. They suggested that the inter-conversion occurs through intracellular enzymes of *A. flavus* and *Rhizopus sp*. Chaurasia and Sinha (1994) showed the potential of biological control of aflatoxins in developing peanut by nonatoxigenic strains of *A. flavus* in the greenhouse experiment. They inoculated strains in the root of peanut plants aged from 1 to 2 weeks at the pre-harvest stage; on the seven non-toxigenic strains tested, six showed a reduction in aflatoxins contamination by *A. flavus*. Kusumaningtyas *et al*. (2006) reported that *Rhizopus oligosporus* which grew with an aflatoxigenic *A. flavus* strain, during 5 days of fermentation, was also able to inhibit the synthesis and to degrade the toxins.

2.2.6.3. The Use of Enzymes

Specific enzymes capable to degrade aflatoxins were purified from microbial systems. Detoxification by specific enzymes avoids the problems associated with the use of micro-organisms such as the modification of the the flavor and the nutritional value of the food products as well as avoiding the formation of toxic compounds from the degradation process. Motomura *et al*. (2003) isolated and purified a novel enzyme, from *Pleurotus osteatus*, able to degrade the aflatoxin AFB1. In addition, this new enzyme showed greater activity at 25°C with a pH between 4.0 and 5.0. Fluorescence measurements suggested that this enzyme cleaved the lactone ring of aflatoxin, although the degradation products of aflatoxin were not clearly studied. Liu *et al*. (2001) purified and isolated an enzyme called aflatoxin detoxifizyme (ADTZ), from *Armillariella tabescens*, able to detoxify the aflatoxin AFB1 by undergoing an opening in its difurane cycle, making it less toxic. The optimum activity of this enzyme was obtained at 35°C with a pH of 6.8. These authors (1998) isolated a multienzyme complex, from *A. tabescens,* which was able to degrade aflatoxin AFB1; they proposed a degradation pathway of AFB1 by this enzyme extract, where aflatoxin AFB1 is first transformed into AFB1-epoxide, followed by its hydrolysis to form a dihydrodiol and then difurane ring would open in the subsequent hydrolysis step.

2.2.7. Metabolism of aflatoxin in Human and Animals

Generally, there is a great diversity in the metabolism of aflatoxins among the different animal species. There are four maetabolic pathways of AFB1, the *O*-demethylation into AFPl, keto-reduction into aflatoxicol (AFL), the epoxidation into AFB-8,9-epoxide (highly toxic, mutagenic and carcinogenic) and the hydroxylation into AFM1 (toxic), AFH1, AFQ1 or $AFB2_a$ (Wu *et al*., 2009). There are also three metabolic pathways for AFB1-8,9-epoxide, the hydrolysis to form AFB1-8,9-dihydrodiol, the conjugation with guanine bases in DNA to form AFB1-8,9-dihydro-8-(N'-gnanyl)-3-hydroxy (AFB-N'-Gua), and the conjugation with soluble nucleophilic molecules such as glutathione (Wu *et al*., 2009). Various forms of CYP-450 are used for different biotransformations in the species. The AFB1 is converted into AFM1, AFQ1 and AFB1-8,9-epoxide by a mono-oxygenase from rat liver. In addition, Yoshizawa *et al*. (1982) found that AFM1 was only catalyzed by CYP-448, while AFQ1 was catalyzed by both CYP-450 and CYP-448 in rat liver. CYP3A4 and CYPIA2, belonging to mono-oxygenase CYP-450, are majors isoforms involved in human liver. However, lipoxygenase (LOX) and prostaglandin H synthase (PHS) are the key enzymes of the aflatoxins biotransformation in human lung, whereas P-450 plays a relatively minor role (Wu *et al*., 2009). The metabolism of aflatoxins *in vitro* and *in vivo* has been largely studied. Roebuck and Wogan (1977) reported that the aflatoxicol (AFL) was found to be the major metabolite of the aflatoxin AFB1 in the duck liver, whereas the aflatoxin AFB1 seems be converted both into aflatoxins AFQ1 and AFP1 in the monkey and human liver (Wu *et al*., 2009). In addition, the aflatoxin AFB1 could be converted into aflatoxins AFM1 and AFM2, in many species, including ducks, rats, mice and monkeys, except humans. Roebuck *et al*. (1978) studied the *in vitro* metabolism of the aflatoxin AFB2 in humans and animals. The duck liver had higher activity than tissues from other species. The aflatoxicol (AFL) as well as the aflatoxins AFB1, AFM1 and AFM2 have been found in the duck liver. The aflatoxin AFB1 was also transformed into aflatoxins AFQ2 and AFP2 by the enzymes of rat, mouse and human livers. In chicken liver, a peptide (or amino acid) conjugated to AFB2 and a glucuronide conjugated to AFM1 were the major metabolites of aflatoxin AFB1 *in vivo*. Salhab and Edwards (1977) studied the *in vitro* metabolism of AFL in animals and humans livers. They reported that AFL was metabolized into aflatoxins AFB1, AFQ1, AFH1, AFP1, AFM1 and AFM2 by the enzymes of S12 liver fractions of monkey, dog, rat, mouse and human. Liver tissus

from human and hamster had the most active pathway to convert AFL into aflatoxin AFB1 while those of rabbit and trout had a greater ability to convert the aflatoxin AFB1 into AFL.

2.3. Detection and Characterization Methods for the Aflatoxins and Their Degradation Products

The fact that most aflatoxins are toxic at very low concentrations requires sensitive and reliable methods for their detection (Turner *et al*., 2009). There are a diversity of well established methodologies reported to analyze aflatoxins and their degradation products in foods and solutions such as thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), Fourier-transform infrared (FTIR) spectroscopy, mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. The enzyme-catalyzed end products of aflatoxins obtained by laccases might show unpredictable molecular structures with various structures and other properties. The selection of the proper analytical strategies may depend subsequently on the purpose of the study as well as the nature of the sample (Naczk and Shahidi, 2006). The detection methods used for aflatoxins and their degradation products should be robust, sensitive and have a high degree of flexibility (Turner *et al*., 2009).

2.3.1. Thin Layer Chromatography

Thin-layer chromatography (TLC) is a method widely used for mycotoxins quantification and semi-quantification, using UV detection and silica gel for coating the plates. This method is commonly used due to its high throughput of samples, low operating cost and ease of identification of target compounds (Espinosa-Calderón *et al*., 2011). TLC involves the coating of a glass plate with silica gel and applying a concentrated sample of aflatoxins on a baseline. Separation occurs by solvent migration via capillary action followed by drying leading to the characterization of the resultant spots (Elis *et al*., 1991). The quantitation of the aflatoxins may be achieved by several methods. One of these methods is the visual estimation, using comparison of aflatoxins with standards known as R_f values. However, this techique presents inaccurate and imprecise quantification (Elis *et al*., 1991). New quantification method has been developed based on a fluorodensitometric detection, where the TLC plates are examined under UV light and scanned with a photometer. This technique allows both the exact location of the position of

fluorescent spots and a precise measurement of the intensity of the aflatoxins fluorescence (Elis *et al*., 1991).

2.3.2. High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is the most common method used for the separation, quantification and identification of aflatoxins and their degradation products. The aflatoxins separation can be achieved with both normal (NP) and reversed phases (RP) columns; however, RP ones are the more commonly used (Medina and Magan, 2012). HPLC is coupled with UV, fluorescence, amperometric and diode array detectors for the aflatoxins detection, which is based on the presence of a chromophore in the molecules (Turner *et al*., 2009). HPLC involves the separation of sample constituents, followed by their detection and their quantification. The separation is achieved by a competitive distribution of the sample between the mobile liquid phase and the stationary phase that is supported in a column (Elis *et al*., 1991). UV detection at 365 nm affords to detect all the peaks of the major aflatoxin isoforms, AFB1, AFB2, AFG1 and AFG2. On the other hand, although the fluorescence detection, 365 nm excitation, 415 nm emission, has greater sensitivity for AFB2 and AFG2 than for AFB1 and AFG1 (Joshua, 1993). The current approach to improve the fluorescence detectability of the aflatoxins AFB1 and AFG1 is the use of pre- and post-columns derivatization. The pre-column approach uses the formation of the corresponding aflatoxin hemiacetals using trifluoroacetic acid (TFA), while for the post-column one, either bromide or pyridinium hydrobromide perbromide are used as agents for the bromination of the aflatoxins (Espinosa-Calderón *et al*., 2011).

2.3.3. Enzyme-Linked Immunosorbent Assay

The use of enzyme-linked immunosorbent assay (ELISA) methods for aflatoxins detection became very popular recently due to their relatively low cost, their avaibility, their sensitivity, their specificity as well as their easy application (Zheng *et al*., 2006). The detection of aflatoxins by ELISA is based on a competitive reaction that uses either a primary antibody specific for the target molecule or a conjugate between an enzyme and the required target. The complex formed will then interact with a chromogenic substrate to give a measurable result (Turner *et al*., 2009). The intensity of the solution is measured optically using an ELISA reader. ELISA has been employed to detect aflatoxins in maize (Turner *et al*., 2009). Kolosova *et al*. (2006) reported the effectiveness of the aflatoxin AFB1 detection in grain samples by direct competitive ELISA

based on a monoclonal antibody, where the detectable level was between 0.1 and 10 μg.L^{-1}. Rastogi *et al*. (2004) also used competitive ELISA technique to detect the occurrence of aflatoxin AFM1 in Indian infant milk products and liquid milk samples. Although ELISA detection is highly specific and sensitive, the fact that the target compounds are mycotoxins and not antigens, the compounds with similar chemical groups can also interact with the antibodies resulting in non-specific signal.

2.3.4. Fourier-Transform Infrared Spectroscopy

Fourier transform infrared spectroscopy (FTIR) has been underutilized for the detection of aflatoxins (Tripathi and Mishra, 2009). This technique presents several advantages such as fast, easy equipment operation, good accuracy, performing nondestructive analyzes as well as it requires only minimal sample size and chemicals. This technique is used to identify the types of chemical bonds in a molecule. One of the strengths of FTIR spectroscopy is its ability to obtain spectra from a very wide range of different compounds to discern small differences that would otherwise be missed in the raw spectrum (Mirghani *et al*., 2001). It can be applied to the analysis of solid, liquid and gasse products. FTIR is based on the absorption of the infrared light by several molecules in a sample. The chemical structure and three-dimensional orientation of the molecules are responsible for generating different IR absorption. In addition, when the chemical bond absorbs the infrared light, it vibrates in varying ways depending on its own nature (Santos *et al*., 2010). Mirghani *et al*. (2001) reported the effectiveness of FTIR to determine aflatoxin contents in groundnut and groundnut cake.

2.3.5. Liquid Chromatography/Mass spectrometry

Liquid chromatography (LC) coupled with a mass spectrometry (MS) detection have been developed and applied in residual analysis of foods. The high selectivity and sensitivity of MS detection methods associated with the resolution of LC provide decisive advantages to perform qualitative as well as quantitative analysis of a wide range of molecules at trace levels. The specificity of the liquid chromatography coupled with MS is permitted by the unique nature of the mass spectrum and the sensitivity of instrumentation. For chromatographic resolution and sensitivity, different solvents and columns were optimized. It was found that a simple solvent system using water, methanol, ammonium acetate, and a C18 column worked very well (Ventura *et al*., 2004). The multi-aflatoxin method by liquid chromatography-tandem mass spectrometry

(LC-MS-MS) was a good combination for the determination of the aflatoxins, AFB1, AFB2, AFG1 and AFG2, in cereal and corn extract due to its high sensitivity and high selectivity. Another advantage of this method is that ion suppression was not observed for all food samples studied (Vahl and Jorgensen, 1998). Hurst *et al*. (1991) used HPLC coupled with a thermospray MS for the detection and the characterization of the aflatoxins, AFB1, AFB2, AFG1 and AFG2, in peanuts. Cappiello *et al*. (1995) reported the use of LC-MS to analyse the aflatoxins in peanut meals. In addition, Wang *et al*. (2011) used LC-MS method for the identification and the characterization of aflatoxin AFB1 and its degradation products obtained by gamma ray radiation. Velazhahan *et al*. (2010) also demonstrated the efficiency of mass spectrometry method to characterize the degradation products of aflatoxins AFG1 obtained with the use of seed extracts of the medicinal plant, *Trachyspermum ammi.*

2.3.6. Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) spectroscopy is demonstrably one of the principal methods for obtaining detailed structural information of compounds. It is considered the most informative method to analyze compounds due to its ability to differentiate between most structural, conformational and optical isomers. However, the sensitivity of the NMR spectroscopy to a wide range of compounds in a multi-component mixtures lead to ambiguous or incomplete assignments, thus delaying detection even with the use of multidimensional NMR (Es-Safi *et al*., 2002). Rice *et al*. (2004) used LC-MS and NMR to identify major aflatoxin isoforms, AFB1, AFB2, AFG1 and AFG2, in mixtures based on their fingerprint spectral regions.

2.4. Optimization of the Bioprocess using Response Surface Methodology

Response surface methodology (RSM) is a powerful statistical analysis technique which is well suited to modeling complex multivariate processes, where a response is influenced by several variables (Cowpe *et al*., 2007). The objective of this method is to optimize the levels of the variables in order to obtain the best system performance (Bezerra *et al*., 2008). RSM consists of a group of mathematical and statistical techniques that are based on the fit of empirical models to the experimental data obtained in relation to experimental design, useful for developing, improving and optimizing processes (Kong *et al.,* 2012). This method is widely used in the chemical (Song *et al*., 2011) and biological fields (Tabandeh *et al*., 2008), food science (Gan and Latiff, 2011), microbiology and enzyme applications (Levin *et al*., 2008; *Li et al*., 2009). The

main advantages of RSM are the reduced number of experimental runs needed to provide sufficient information for statistically acceptable data, which results in lower reagent consumption and considerably less laboratory work (Ferreira *et al*., 2007). It is a faster and a less expensive method for gathering research results than classical one-variable-at-a-time or full-factorial experimentation, and allows the interactive effects among the variables studied (Myers *et al*., 2009).

The application of RSM as an optimization technique needs six different stages. The selection of independent variables of major effects on the system through screening studies and the delimitation of the experimental region, according to the objective of the study and the experience of the researcher; the choice of the experimental design and carrying out the experiments according to the selected experimental matrix; the mathematic-statistical treatment of the obtained experimental data through the fit of a polynomial function; the evaluation of the model's fitness; the verification of the necessity and possibility of performing a displacement in direction to the optimal region; and obtaining the optimum values for each studied variable (Bezerra *et al*., 2008).

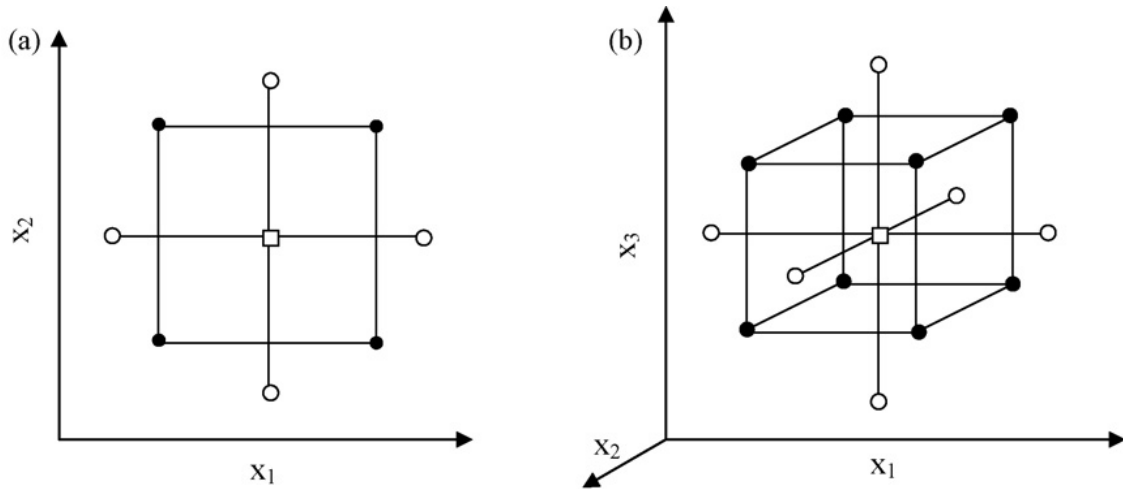

Figure 2.4. The representations of the central composite designs for (a) two- and (b) three-variables optimization (Bezerra *et al*., 2008).

The central composite design (CCD) is composed of three parts, a full factorial or fractional factorial design; an additional design, forming a star pattern, in which experimental points are at

an α distance from its center; and a central point (Bezerra *et al*., 2008). One of the most important characteristic of the CCD is that all the factors are studied in five levels, including -α, -1, 0, +1, +α.

RSM has been successfully applied to optimize the degradation process of aflatoxins. Tripathi and Mishra (2011) used RSM to optimize the peroxidase-catalyzed enzymatic degradation of aflatoxin AFB1 from red chili powder. Under the optimal conditions, the yield of AFB1 degradation was 70%. Arzandeh and Jinap (2011) reported that RSM was appropriate to study the degradation of the aflatoxins AFB1, AFB2, AFG1 and AFG2 by different heating methods. Under the optimal conditions, aflatoxins AFB1, AFB2, AFG1 and AFG2 were reduced by 78.4, 57.3, 73.9 and 75.2%, repectively. Jalili *et al*. (2010) also indicated that RSM was useful to evaluate the degradation of the major aflatoxin isoforms by a wild range of gamma radiation. Under the optimal conditions, aflatoxins AFB1, AFB2, AFG1 and AFG2 were degraded by 43, 24, 40 and 36%, repectively.

2.5. Laccase

The use of enzymes in the food industry is well known. Enzymes are specific biological catalysts able to react under mild conditions of temperature and pH. They are produced in plants, animals or microorganisms, and therefore perceived as natural. They are non-toxic food components and they are preferred to chemicals in food bio-transformation processing (Minussi *et al*., 2002). The use of enzymes may lead to the improvement of agro-industrial processes in order to reduce the energy costs associated with processing, in improving the nutritional quality of food, and in the creation of a new generation of products and alternative applications for several agricultural products.

Laccases (EC 1.10.3.2, p-biphenol oxygen oxidoreductase) are multi-copper proteins among ascorbate oxidase, ceruloplasmin, and bilirubin oxidase (Claus, 2004). Laccase was first described by Yoshida (1883), and has been characterized as an oxidase containing metal ions by Bertrand (1986). They use molecular oxygen to catalyze the oxidation of a wide variety of organic and inorganic substrates, including mono-, di-, polyphenols, aminophenols, methoxy phenols, aromatic amines and ascorbate (Thurston, 1994). Laccases are involved in the

pathogenesis, immunity, in the morphogenesis of bodies and the renewal of the metabolism of complex organic substances such as lignin and humic substances (Rogalski *et al*., 2011).

2.5.1. Structure and Functions

The molecular mass of the laccases ranges in size from 50 to 110 *k*Da. Laccases are a glycosylated monomer or homodimer protein made of 500-580 amino acids having more or less carbohydrate compounds (10-45%) in fungi, bacteria and plants. These carbohydrate compounds contain monosaccharides, including hexoamines, glucose, fructose and arabinose (Madhavi and Lele, 2009). Among saccharides, one of the most important for maintaining the high stability and normal functions of the enzyme is mannose, and 10 to 50% of the laccase's weight belongs to saccharides (Madhavi and Lele, 2009). The active sites of laccases contain three copper centers, including type 1, type 2 and type 3, that can be identified on the basis of their spectroscopic properties (Nakamura and Go, 2005). Type 1 has a blue copper paramagnetic ion, with a strong absorbance at 610 nm. Type 2 has a paramagnetic non-blue copper ion and exhibits a weak absorption in the visible region. Type 3 has two diamagnetic antiferromagnetic copper ions forming a binuclear copper center coupled; wich absorbs at 330 nm.

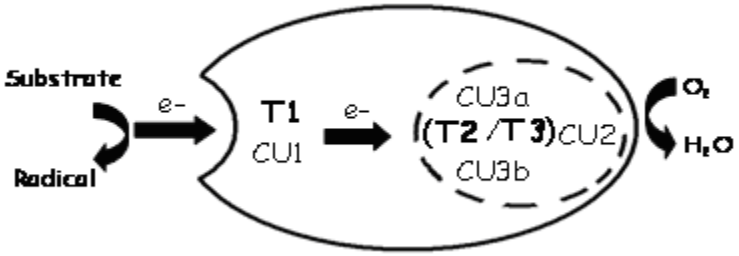

Figure 2.5. Schematic mechanism of the action of laccases (modified from Mougin *et al*., 2003).

Type 1 copper has a trigonal coordination, with two histidine and cysteine as fixed equatorial ligands and generally one variable axial position. The axial ligand is a methionine in bacteria and it's a leucine or a phenylalanine in fungal laccases (Claus, 2004). The ligand in the axial position strongly affects the oxidation potential of enzyme, and eventually may provide a mechanism for regulating its activity. A mutation of phenylalanine or methionine significantly reduced the oxidation potential of the fungal laccase from *Trametes villosa* (Kumar *et al*., 2003). Type 1 copper confers the typical blue color to the multi-copper proteins, which results from the intense

electronic absorption caused by the covalent copper-cysteine bond. Because of its high redox potential +790 mV, type 1 copper is the site where substrate oxidation takes place and mediates the transfer of the extracted electron to the T2/T3 site. Type 2 copper does not show absorption in the visible spectrum and discloses paramagnetic properties. It is positioned close to the type 3 copper, a binuclear center spectroscopically characterized by an electron adsorption at 330 nm and the absence of an electron paramagnetic resonance (EPR) signal which is the result of the anti-ferromagnetic coupling of the pair of copper. Type 2 and type 3 copper form a trinuclear cluster, where the reduction of molecular oxygen and the release of water takes place. Kumar *et al*. (2003) reported that multiple sequence alignments of more than 100 laccases allowed the identification of four regions, L1-L4. The 12 amino acid residues in the enzymes known as copper ligands are located inside these regions. The amino acid ligands of the tricyclic pole are eight histidines of four helix-X-helix (HXH) pattern. In this pattern, X is the cysteine binding T1 copper while each of histidines is bounded to one of the two type 3 coppers (Claus, 2004).

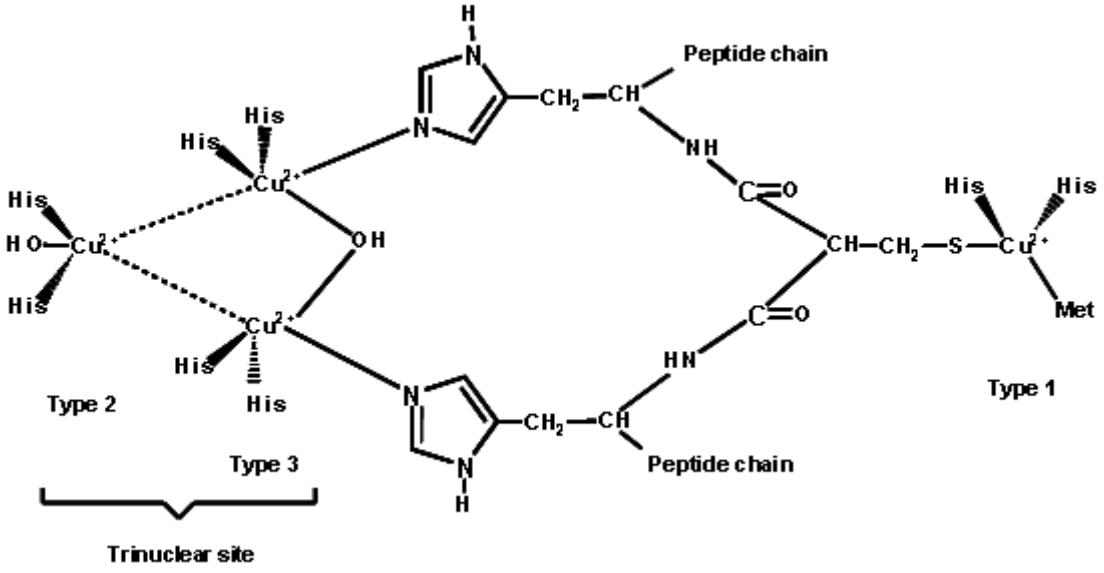

Figure 2.6. The composition of the active site of laccases (Octavio *et al*, 2006).

In addition, laccases play several important roles in bacteria, plants and fungi. The Laccases are responsible for the formation of pigment in the mycelium and fruiting bodies, improve the adhesion between cells, contribute to the formation of rhizomorphs and are also responsible for the formation of polyphenolic glue that binds together the hyphae. The activity of laccases may

contribute to the recovery of the cell wall regeneration in protoplasts of higher plants. De Marco and Roubelakis-Angelakis (1997) measured the laccases activity in regenerating and non-regenerating protoplasts isolated from leaves of tobacco (*Nicotiana tabacum*). They found that the laccases activity decreased shortly after isolation. The activity increased in the protoplasts regenerated during a culture period of six days, but it was undetectable in non-regenerated protoplasts. Leaves with an injury showed an immediate increase of laccases activity, while peroxidases activity increased slowly and reached a peak after only 4 days. They concluded that the laccases were the only effective polymerizing enzymes during the first days of protoplast culture and could be involved in the early stages of healing of injured leaves, substituting the peroxidases activity in the reconstitution of the cell walls before that the hydrogen peroxide becomes available.

Various plant pathogens also produce extracellular laccases that allow them to overcome the immune response of the host (Thurston, 1994). Laccases are also used in various medical applications and in the detoxification process. Indeed, they facilitate the detoxification of plant tissue through the oxidation of antifungal phenols or in disabling the phytoalexins (Madhavi and Lele, 2009). It was established that the laccases are involved in various cellular and microbial activities. Recent studies on the physiological function of the laccases have been investigated, including the biosynthesis of plant cell wall, the phytopathogenicity, wood degradation, humification, the sclerotization of insect and bacterial melanization (Madhavi and Lele, 2009). Moreover, the laccases are widely used in many industrial fields.

2.5.2. Sources of Laccases

The laccases are more widely distributed in higher plants, fungi and bacteria. Laccases of the plants have been identified in the trees, cabbages, turnips, beets, apples, asparagus, potatoes, pears and various other fruits and vegetables (Madhavi and Lele, 2009). Five distinct laccases were found to be present in the xylem of *Populus euramericana* (Mayer and Staples, 2002). Most laccases described in the literature have been isolated from higher fungi. They were isolated from ascomycetes, deuteromycetes and basidiomycetes fungi. The laccases from *Monocillium indicum* were the first to be characterized showing peroxidase activity (Madhavi and Lele, 2009). The most common laccases producer are white rot fungi such as *Trametes versicolor*, *Trametes hirsuta*, *Trametes ochracea*, *Trametes villosa*, *Trametes gallica*, *Cerena*

maxima, *Coriolposis polyzona*, *Lentinus tigrinus*, *Pleurotus eryngii*, etc. (Madhavi and Lele, 2009). Although laccases are widely distributed in plants and fungi, laccases activity have been reported only in few bacteria, including *Azospirillum lipoferum*, *Marinomonas mediterranea*, *Streptomyces griseus*, and *Bacillus subtilis* (Madhavi and Lele, 2009). Laccases are also present in a dozen kinds of insects, including *Bombyx*, *Calliphora*, *Diploptera*, *Drosophila*, *Lucilia*, *Manduca*, *Musca*, *Oryctes Papilio Phormia*, *Rhodnius*, *Sarcophaga*, *Schistocerca* and *Tenebrio* (Xu, 1999). Laccases are ubiquitous, exceptionally versatile enzymes (Mayer and Staples, 2002), and distributed in all areas of life.

2.5.3. Properties

Most studied laccases are extracellular proteins, although intracellular laccases have been detected in several fungi and insects. Fungal laccases have isoelectric points (pI) from 3 to 7, while plants laccases have pI around 9 (Madhavi and Lele, 2009). The main difference between the two enzymes is that fungal enzymes have their optimum pH between 3.6 and 5.2, whereas the laccases from plants had an optimum pH between 6.8 and 7.4. This difference in pH may be due to the adaptation of fungal enzymes to acidic conditions unlike those from intracellular plants that have an optimum pH close to the physiological range (Madhavi and Lele, 2009). In addition, the pH of laccases is highly dependent on the substrate. The optimal properties of these enzymes vary depending on their source, their adaptation to grow in acidic environment and their physiological functions (Gianfreda *et al*, 1998; Madhavi and Lele, 2009).

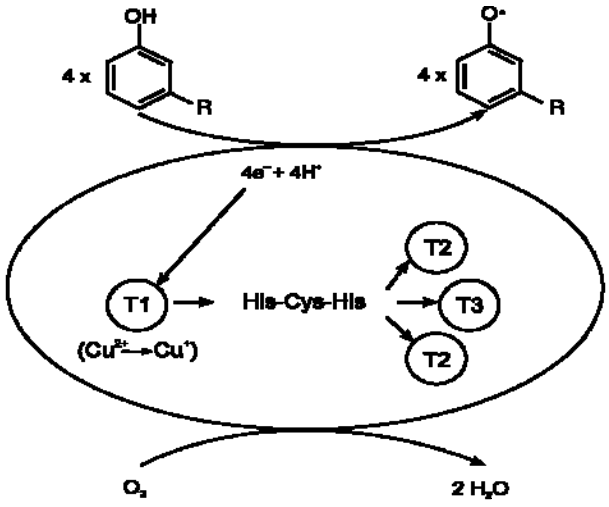

Figure 2.7. Catalytic cycle of laccases (Baldrian, 2006).

Laccases catalyse the reduction of O_2 to H_2O using a wide range of phenolic and non phenolic compounds (though not tyrosine) as hydrogen donors (Baldrian, 2006). The catalysis mechanism via laccases is based on the activity of the copper centers present in the enzyme molecule (Claus, 2004). Laccases catalyse occurs in three steps: (1) The substrates are oxidized by the T1 copper, (2) the extracted electrons are transferred, probably through a strongly conserved His-Cys-His tripeptide motif, to the T2/T3 site, (3) molecular oxygen is reduced to water (Baldrian, 2006) (Fig. 2.7).

Laccases are known to be highly oxidizing, with high redox potential (E^0) ranges (Baldrian, 2006). The reactivity of laccases has been directly related to their standard redox potential E˚ of the T1 site which is thought to play a major role in the performance of the enzyme and their substrate-specificity (Shleev *et al*., 2004; Alcalde, 2007). The redox potential of T1 centers can vary widely between laccases from different sources, from 450–480 mV in *Myceliophthora thermophila* to 760–790mV in *Polyporus pinsitus* (Xu *et al*., 2000) and the presence of four cupric ions, each co-ordinated to a single polypeptide chain, is an absolute requirement for an optimal activity (Baldrian, 2006). Laccases are also able to oxidize substrates of lower or higher redox potential, when coupled with mediators resulting in a higher rate of substrate oxidation and broader substrate specificity (Alcalde, 2007). Laccases coupled with mediators may thus be used to extend their substrate range to non phenolic compounds (Gianfreda *et al*., 1999). Some mediators are 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 1-hydroxybenzotriazole (HBT), benzotriazole (BT), remazol brilliant blue (RBB), chlorpromazine (CPZ), promazine (PZ), 1-nitroso-2-naphthol-3,6-disulfonic acid (NNDS) and 4-hydroxy-3-nitroso-1-naphthalenesulfonic acid (HNNS) (Octavio *et al*., 2006). The laccases-catalyzed reaction becomes a two-step process, where the mediator first is oxidized by the enzymes to form the oxidized mediators and these later can successfully oxydized the substrate, with the ionization potentials exceeding that of the laccases (Fig. 2.8).

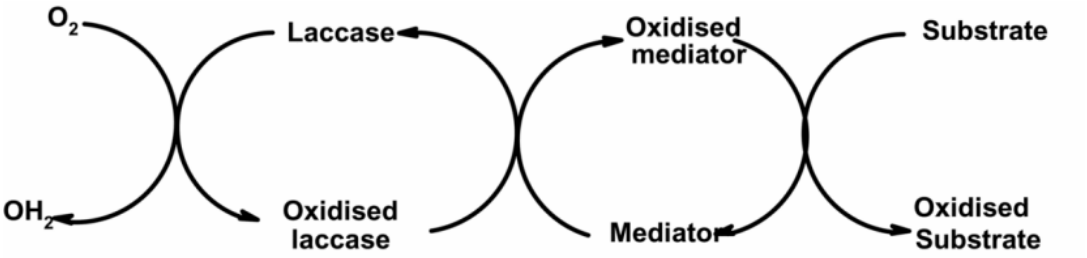

Figure 2.8. Laccases mediator redox cycles (Morozova *et al*., 2007).

2.5.4. Inducers of Fungal Laccases Production

It has been recently shown that culture conditions affect fungal physiology and the expression of enzymes; therefore it is important to study the effects of inducers on enzymes production. In basidiomycetes fungi, extracellular laccases are produced in small amounts, however, their production can be considerably improved by the presence of inducers, mainly aromatic or phenolic compounds related to lignin or lignin derivatives (Octavio *et al*., 2006). Octavio *et al*. (2006) reported that veratryl alcohol enhanced laccase production by 10-fold. The ascomycete, *Botryosphaeria* sp, produced two extracellular constitutive laccases. Dekker and Barbosa (2001) also showed that the presence of 40 mM of veratryl alcohol in the growth culture media stimulated the production of the two extracellular laccases, from ascomycete *Botryosphaeria* sp, by 100 and 25 fold, respectively. Octavio *et al*. (2006) reported that laccases production from *Coriolus hirsutus* was also increased in presence of syringaldazine and guaiacol.

Koroljova-Skorobogat'ko *et al.* (1998) suggested that the most effective inducer of extracellular laccases synthesis in the culture of *Coriolus hirsutus* was syringaldazine, which with a concentration of 0.11 μM increased the enzymes yield by 1000%.

Non-lignin related compounds, such as gallic and ferulic acid, can also play the role of inducers, improving the production of laccases from *Botrytis cinerea* (Fortina *et al*., 1996) and *Pleurotus sajor-caju* (Rescigno *et al*., 1993), respectively. In addition, ethanol can also stimulated laccase gene expression and consequently improve laccases production. According to Octavio *et al*. (2006), the presence of ethanol 40 g ethanol/L in medium increased by over 20-fold the laccases production as compared without ethanol. This stimulatory effect was also reported for others white rot fungi such as *Coriolus hirsutus* and *Grifola frondosa*, (In-Young *et al.,* 1999). Lee *et*

al. (1999) also reported that the addition of 50 mL/L ethanol in a culture of *Tinea versicolor* increased laccases activity by 24-fold, whereas the addition of 25 mL/L ethanol in a culture of *C. hirsutus* enhanced the enzymes activity by 4-fold (Zouari-Mechichi *et al*., 2006). However, an excessive concentration of ethanol may severely inhibit laccases production (Lee *et al*., 1999).

Copper is a metal ion which is considered as one of the most efficient substrate to induce laccases production, this inducer effect may be due to the presence of this metal ion in the enzymes structure (Octavio *et al.,* 2006). Octavio *et al*. (2006) reported that the production of extracellular laccases from *Trametes pubescens* MB 89, a white-rot fungus, can be considerably stimulated by the addition of Cu (II) in the millimolar range. Zouari-Mechichi *et al*. (2006) demonstrated that 300 µM of copper in the basal medium of *T. trogii* enhanced the production of its laccases by 100-fold; however, higher concentrations of copper resulted by a decrease in the fungal biomass and laccases activity, which is probably due to a toxic effect on the fungal culture. Gnannamani *et al*. (2006) suggested that the sensitivity or resistance of fungal species to copper may vary, since it may act as an inducer for some species and as an inhibitor for others.

Octavio *et al*. (2006) reported that in presence of glucose, as the main carbon source, significant increase in laccases production, from *T. pubescens*, was observed only after it was completely consumed from the culture medium. Hao *et al*. (2007) studied the effect of different concentrations of glucose as a carbon source on laccases activity. They reported that increasing the glucose concentration from 5 to 20 g/L resulted in a more than 5-fold increase in laccases activity, whereas increasing the concentration to 40 g/L resulted in a lower activity. In addition, the presence of nitrogen source has an important effect on laccase synthesis. Hao *et al*. (2007) also studied the effect of different nitrogen sources on the laccases activity, from *Pestalotiopsis* sp., in presence of 20 g/L glucose in the growth culture media. Their result showed that among the nitrogen sources, 10 g/L ammonium tartrate resulted in the highest laccases activity. Moreover, a further increase in the concentration of this nutrient to 15 g/L did not result in an increase in laccases activity.

Zouari-Mechichi *et al*. (2006) suggested that laccases production could be affected by the fungal species, the strain and the culture conditions. In addition, the concentration and the time of the addition of each inducer could have considerable effects (Cavallazzi *et al*., 2005). Galhaup *et al*.

(2002) reported that an optimized medium containing 40 g/L of glucose, 10 g/L of peptone from meat, 1 g/L of $MgSO_4.7H_2O$ and in presence of 2.0 mM Cu^{2+}, provided the highest laccases activity of 330 U/mL.

2.5.5. Substrate Specificity

One of the unique features of the laccases is their broad specificity for substrates. Accordingly, laccases have been shown to oxidize many compounds such as monophenols, *o*- and *p*-diphenols, polyphenol and syringaldazine (common laccases substrate). They all act as electron donors in the reaction and are oxidized (Flurkey, 2003). The catalysis of lignin degradation by laccases is also based on oxidation; degradation begins with the oxidation of phenolic compounds and the production of phenoxy radicals (Marjasvaara *et al*. 2006). It was found that the function of laccases in plants is different from that found in fungi. Indeed, in the plants, they owe a system of lignin synthesizing while in fungi they are a system to degrade lignin. During depolymerization, highly reactive and toxic species are formed. The mycelia of fungi must be protected against these harmful substances. It could be that one of the functions of the laccases is to trap these compounds in promoting the polymerization before they can enter in the hyphae. There is still no information available for the role of laccases in lignification and delignification process (Alcalde, 2007). Unlike peroxidases that require the presence of hydrogen peroxide as an electron acceptor, molecular oxygen can be used as an electron acceptor by laccases, which makes these enzymes an attractive biocatalyst for many applications (Michizoe *et al*., 2005). Compared with other enzymes, laccases are reported to be non-specific and a wide range of substances are suitable substrates. Although most substrates of laccases are a variety of phenolic compounds, the reactivity of these enzymes could be extended by using mediators. In combination with the so-called mediator substances, such as ABTS, the spectrum of the substrate becomes even wider including non-phenolic aromatic molecules (Rittstieg *et al*., 2002). It is known that the range of substrates oxidized by laccases varies from one laccase to another complicating the precise role of laccases activity (Alcalde, 2007). Moreover, their specificity depends mainly on the structure of the laccases and the redox potential of the T1 copper (Gorbacheva *et al*., 2009).

2.5.6. The Effect of pH on Laccases Activity

Laccases have an optimum pH in the acidic range (pH 3.0 to 6.0) (Flurkey, 2003). The pH value is mainly depending on the type of substrate chosen to the assay of laccases (Flurkey, 2003). At high pH values, the enzymes activity may decrease by the binding of a hydroxide anion to the T2/T3 copper of the laccases that may interrupt the electron transfer from T1 to T2/T3 sites (Baldrian, 2006). The rate of oxidation and reaction products may vary depending on the pH (Baldrian, 2006). The optimum pH is often dependent on the nature of substrate used for laccases assay and the type of the products formed also depend on the pH and the substrate as well (Flurkey, 2003). In presence of phenols, the optimum pH may range from 3 to 7 for fungal laccases and up to 9 for plant laccases (Madhavi and Lele, 2009). When ABTS is used as substrate, the optimum pH are more acidic and are found in the range between 3 and 5 (Gianfreda *et al.*, 1998; Heinzkill *et al*. 1998). In general, laccases activity has a bell-shaped pH profile with an optimum pH that varies considerably, depending on the substrate, oxygen, or the enzyme itself (Xu, 1997).

2.5.7. The Effect of Temperature on Laccases Activity

Laccases are heat stable with a certain thermal stability properties associated with each laccase isoform (Flurkey, 2003). The laccases activity is best when temperature is between 20 to 70˚C. Indeed, their activities gradually increased when temperature increased from 20 to 70°C (Gianfreda *et al.*, 1998). Few have been found to have an optimum temperature below 35°C (Baldrian, 2006). The optimum temperature of laccase activity depends on the sources of the laccases, their adaptation to grow at acidic conditions and their physiological functions (Gianfreda *et al.*, 1998; Madhavi and Lele, 2009). Wang *et al*. (2010) reported that the optimum reaction temperature of laccase isoenzyme I (LacI) was 65, 70, 45 and 70°C when ABTS, DMP, SYR and guaiacol were used as substrates, respectively. However, for the isoenzyme II (LacII), using the same substrates, the optimum reaction temperature was 70, 75, 50 and 50°C, respectively. Nevertheless, the optimum temperature of laccases activity is between 50 and 70°C (Baldrian, 2006) such as this one from *Chaetomium thermophilium* which has an optimum temperature around 70°C.

2.5.8. The Effect of Activators

Enzyme activators are molecules that bind enzymes in order to enhance their activity. Many compounds are found to enhance laccase activity, mainly aromatic or phenolic compounds, such as veratryl alcohol, 2,5-xylidine, guaiacol, gallic acid, ferulic acid, and metal ions (Octavio *et al*., 2006). Cupper is considered to be the most efficient one maybe because this metal is part of the enzymes structure (Tychanowicz *et al*., 2006; Neifar *et al*., 2010). Rosconi *et al*. (2005) studied the effect of selected salts, ammonium sulfate and potassium sulfate, on laccase activity. The addition of these salts increased laccase activity up to 4.0 and 3.5-fold, respectively.

2.5.9. The Effect of Inhibitors

Inhibitor compounds are substances which inactivate the desired enzyme (Johannes and Majcherczyk, 2000). Laccases are liable to undergo inactivation. Many compounds are found to inhibit laccases. Small anions, such as azide, halides, cyanide, thiocyanide, fluoride and hydroxide, bind to the type 2 and 3 copper, which result in the interruption of internal electron transfer and inhibit laccases activity (Nitheranont *et al*., 2011; Lorenzo *et al*., 2005; Solomon, *et al*., 1996.). Other inhibitors include metal ions, fatty acids, sulfhydryl reagents, hydroxyglycine, kojic acid, desferal and cationic quaternary ammonium detergents (Ivanka *et al*., 2010). They may involve in amino acid residue modifications, conformational changes or copper chelation.

2.5.10. Extraction and Purification of Laccases

The purification and enrichment of enzymes such as laccases is of great interest for biotechnology. Laccase have been usually purified from extracellular fungal fluids. The first steps of laccases purification consist to the cells removal (filtration, centrifugation) (Flurkey, 2003), followed by the concentration of the extracellular fluid using ultrafiltration, precipitation methods (organic solvent, salting-out) and dialysis (Gerken *et al.,* 2006).

2.5.10.1. The Ultrafiltration

The ultrafiltration is a filtration where the liquid passes through a semi-permeable membrane due to a difference in pressure (transmembrane pressure or TMP) (Li, 2009). The filter retains all particles larger than its porosity (Cheryan, 1998). The particles suspended in solution or high molecular weight are retained, while water and low molecular weight molecules pass through the membrane (Maurel, 1993). This separation process is used in industry for purifying and/or

concentrating solutions of macromolecules (10^3-10^6 Da) (Maurel, 1993), especially proteins (Susanto *et al*., 2008). Ultrafiltration operates using mild conditions of low temperature and pressure (Rajeeva and Lele, 2010).

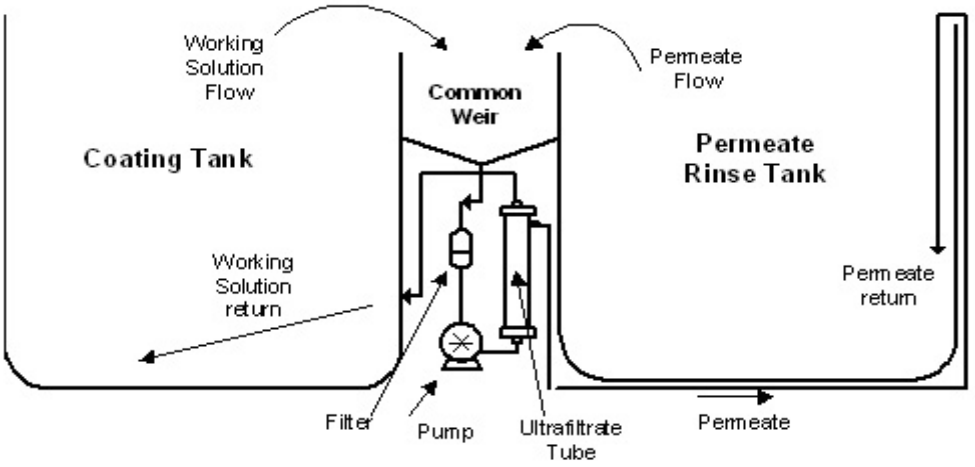

Figure 2.9. Schematic of the ultrafiltration system.

2.5.10.2. Purification of Proteins

Precipitation of proteins using salting out process is based on precipitation of the proteins, without denaturation, by the increase of the salt concentration at a high level into the solution. The proteins in a buffer solution are highly hydrated, in other words, the ionic groups on the surface of the proteins are attracted and binded to water molecules very closely. When a lot of salt, such as ammonium sulfate, is added to the proteins solution, the salt ions attract water molecules away from the proteins. This is partly because the salt ions have a greater density than proteins. While the salt is added and that these ions bind to water molecules, the protein molecules are forced to interact with one another and they begin gathering. Thus, when enough salt was added, the proteins will begin to precipitate. After, proteins can be collected by centrifugation, and then redissolved in a solution using a buffer with a low salt content.

These methods are usually followed by a chromatographic purification process (Gerken *et al*., 2006). The concentrated extract will be subjected to ion-exchange chromatography (IEC) that could be followed by size exclusion chromatography (SEC). The enzymes are considered to be highly purified after IEC and SEC, but higher purification methods could be done by

hydrophobic interaction chromatography (HIC) (Flurkey, 2003). Purified laccases are usually stable for several years when stored frozen and show a typically blue color when concentrated (Flurkey, 2003).

2.5.11. Freeze-Drying Treatment and Use of Cryo-and Lyoprotectants

Freeze drying involves the removal of water or other solvent from a frozen product by a process called sublimation. It occurs when a frozen liquid goes directly to the gaseous state without passing through the liquid phase. Elimination of water using freeze-drying system is the most common stabilization method used for preservation in food industry. Moreover, it's one of the main process used to produce stable protein and polypeptides which are physically and/or chemically unstable in aqueous solution (Passot *et al.*, 2005). Lyophilisation is commonly used in pharmaceutical and biotechnological industries to improve stability of the product. The goal of freeze-drying is to remove water and dry the sample. The resulting highly porous cake has low moisture and can be stored at extended period of time at low temperature - 80ºC (Tsinontides *et al*., 2004). The disadvantages of this method are the freezing and drying stresses of the samples during the process. Stresses, such as solute concentration, formation of ice crystals, pH changes, increase of the ionic strength and the removal of the protein hydration shell, may lead to the denaturation of proteins to various degrees (Wang, 2000; Bhatnagar *et al.*, 2007). In addition, even after a successful lyophilisation, the stability of protein is limited during long-term storage at -80ºC. It can appear that after long-term storage, the protein stability in solid state was equal or even worse than that in liquid (Wang, 2000).

Freeze-drying process is divided in two major steps: (1) the freezing of a protein solution, (2) the drying of the frozen solid under vacuum. The drying step is also composed of two phases: primary and secondary drying. The primary drying removes the frozen water and the secondary drying removes the non-frozen 'bound' water (Wang, 2000). In order to protect proteins integrity and to reduce the degradation of active component during freezing and drying processes as well as storage, stabilizers are often required in a protein formulation (Wang, 2000; Tang and Pikal, 2005). Many stabilizers such as sugars (sucrose), polyols (mannitol, glycerol) and polymers (dextrane) are both as effective cryoprotectants and remarkable lyoprotectants. Stabilizers can protect the proteins by direct interaction with the proteins themselves to form a monomolecular layer on the proteins surface (Tang and Pikal, 2005).

The mechanism of cryoprotection involves mainly the preferential interaction. Preferential interaction means that in aqueous solution the proteins prefer to interact with either water or an excipient. In the presence of a stabilizing excipient, the proteins prefer to interact with water (preferential hydration) than the excipient which is preferentially excluded from the domain of the proteins (preferential exclusion). In this case, the excipient is usually associated with an increase in the surface tension of water. Others mechanisms than preferential interaction can be involved in the proteins protection. Protein stabilizers, which are excluded from proteins surface in solution, can also stabilize proteins during freezing by reduction of the surface tension. In addition, cryoprotectants can increase the viscosity of a solution and thus avoid the limiting protein structural changes (Pikal, 1999) and can also modify the size of ice crystals (Wang, 2000). Stabilization of proteins by polymers can be due to their properties such as preferential exclusion, surface activity, steric hindrance of protein–protein interactions as well as to their capacity to increase the solution viscosity limiting protein structural movement. In addition, polymers, such as dextran, have been reported to stabilize proteins by raising the glass transition temperature (Tg) of a protein formulation significantly (Skrabanja *et al*., 1994) without affecting the capacity to inhibit protein unfolding during lyophilisation and resulted in improved storage stability (Wang, 2000).

Since during lyophilization, the preferential interaction mechanism is no longer applicable because the hydration shell of proteins is removed, the major mechanism of proteins stabilization by lyoprotectants is the formation of an amorphous glass (Wang, 2000). Amorphous materials are structurally more similar to a liquid than crystalline materials. The formation of a glass increases the viscosity and this high viscosity increases protein stability by slowing down the interconversion of the conformational relaxation of the proteins, thus the lyoprotectants preserve the folded form of the proteins (Wang, 2000). In addition to glass formation, many excipients, especially polymers, can stabilize proteins by increasing Tg of protein formulations, since higher Tgs generally result in more stable protein formulations during lyophilisation (Wang, 2000). Another mechanism involved during lyophilisation is the water replacement. This mechanism involves the formation of hydrogen bonds between proteins and excipients at the end of the drying process (Carpenter *et al*., 1990). These excipients preserve the native structures of proteins by serving as water substitutes.

2.5.12. Assay Methods for Laccases Activity

To measure laccases activity, several methods are used such as measurement of oxygen consumption (Gigi *et al*., 1981), spectrofluorimetric (Zuyun *et al*., 1998) and spectrophotometric methods (Flurkey, 2003). The determination of laccases activity by spectrophotometric method, based on the detection of colored end products generated from quinines is the most used since this method is simple and rapid. In the colorimetric assay, laccases react with the substrate by oxidation generating a color which varies depending on the nature of the latter. Several substrates that give uniquely colored products have been used in the determination of laccases activity such as 2,6-Dimethoxyphenol (DMP) (brown), ABTS (blue-green) and syringaldazine (SYR) (pink). SYR is the most common substrate used for laccases activity (Thurston, 1994). Harkin *et al*. (1974) reported that when laccases are assayed with SYR, a pink through red to purple color appeared. These authors also reported that using laccases having high activity in presence of SYR, a purple color appeared immediately.

2.5.13. Application of Laccases

Due to their broad substrate specificity, their high catalytic rate and their ability to use oxygen from the environment as co-factor, fungal laccases are used in many biotechnological and industrial applications. Laccases could be used in the degradation of lignin, bioremediation, biosensors as well as in cosmetics (Octavio *et al*., 2006; Minussi *et al*., 2002). Laccases are secreted by most basidiomycetes and play an essential role in the ligninolytic activity of these fungi. A detailed understanding of lignin degradation is necessary to improve the process of pulping and bleaching in the pulp and paper (Youn *et al*., 1995). In the paper industry, the laccases have been used to remove lignin from the pulp (Madhavi and Lele, 2009). Although, chlorine and conventional chemical oxidants are very effective, they can cause serious problems in the disposal of by-products or a loss of strength of the cellulose fibers. This could even lead to the release of toxic chemicals into the environment (Couto and Herrera, 2006). Laccases could be applied in the oxidative degradation of lignin and reduce the use of chemical oxidants (Camarero *et al*. 2004). Traditional methods of pulping and bleaching of wood pulp for paper production are energy intensive and produce toxic wastes. Natural methods, such as those used by fungi, can be effective, economically viable and environmentally friendly (Couto and Herrera, 2006).

Many researchers have studied both the degradation and the elimination of environmental pollutants using oxidoreductases, including laccases (Gianfreda and Bollag, 2002), because of their high ability to catalyze the oxidation of aromatic compounds (Bourbonnais and Praice, 1990; Gianfreda *et al*., 1999). In contrast to peroxidases that require hydrogen peroxide as electrons acceptor, the molecular oxygen may be used as electrons acceptor by laccases, which makes these enzymes an attractive biocatalyst for bioremediation (Michizoe *et al*., 2005). Most phenolic compounds are toxic and cause coloration of the receiving waters, it is essential to decontaminate effluents containing such compounds (Madhavi and Lele, 2009). The effluents contaminated with phenolic compounds are conventionally treated by various methods including solvent extraction, distillation, adsorption and chemical oxidation. Although these methods are useful, they have some drawbacks such as high cost, incomplete purification, formation of other hazardous by-products or restricted applicability as regards which pollutants they can effectively remove (Shin-ya *et al*., 2005). Microbial or enzyme-based treatments for the removal of phenolic compounds have offered some distinct advantages over physical and chemical removal methods. The catalyze of phenolic compounds by laccases leads to the production of higher oligomers and polymers less toxics of low solubility, which then precipitate and can be readily removed by sedimentation or filtration (Madhavi and Lele, 2009).

Phenolic compound effluents have been found to exhibit different toxicity and to be persistency, therefore, the determination of phenolic compounds is very important (Freire *et al*., 2002; Jegan Roy *et al*., 2005). Spectrophotometric, gas chromatography, liquid chromatography and capillary electrophoresis are the most commonly methods used for the determination of phenolic compounds. However, these methods are time consuming including complicate sample pre-treatment. In addition, the equipment is expensive and is not generally portable. Therefore, there is an interest in developing simple, sensitive, specific, accurate and portable system, such as biosensor for determination of these compounds (Abdullah *et al*., 2007). Many biosensors containing laccases have been developed for phenolic compounds determination (Minussi *et al*., 2002), since these enzymes catalyze the oxidation of polyphenols by molecular oxygen. The biosensors act as biological reconnaissance and interact with the analyte being tested; the biological response is converted into an electrical signal by a transducer (Gomes and Rebelo, 2003).

Bleaching and/or dying of hair usually involve the use of harsh chemicals which can damage the hair. It has been found that dye precursors can be oxidized to the effective coloring agent using laccases instead of the chemical agents (Madhavi and Lele, 2009). Laccases could be used with hydroxystilbenes as hair bleach. Pigments used in cosmetic application could be produced using laccase catalysed oxidation reactions (Alcalde, 2007).

Enzymes (amylases, proteases, cellulases, etc.) have been used as dough and/or bread improving agents (Madhavi and Lele, 2009; Minussi *et al*., 2002) due to their ability to cross-link biopolymers (Osma *et al.,* 2010). The use of laccases results in an increased volume, an improved crumb structure, and softness of the baked product, as well as increased strength, stability and reduced stickiness, thereby improving the machinability of the dough (Osma *et al.,* 2010). Their effects on the dough have been found to be particularly interesting when poor quality of flour has been used (Minussi *et al*., 2002). Si (1994) suggested that when laccases were added to dough used for producing baked products, they may exert an oxidizing effect on the dough constituents and thereby serve to improve the strength of gluten structures in dough and/or baked products.

2.5.14. Degradation of Aflatoxins Using Laccases

Toxins in contact with food products pose a great health concern to the general public. Thus, it is very important to eliminate, or better yet, avoid at all costs any food contamination by toxic substances. Three possible techniques have been used to detoxify products contaminated with toxins. Conventional methods, both chemical and physical, to degrade the toxins are more or less effective on certain foods and still poses too much problems concerning security issues. They can reduce the nutritional value of food products or produce toxic compounds and harmful residues that can potentially affect public health as well as they are generally expensive and can pollute the environment. Therefore, the use of biological methods is a way which may lead to the improvement of agro-industrial processes and to reduce energy costs associated with the processing for improving the safety and the nutritional quality (Kabak *et al*., 2006). According to Food and Agricultural Organization, the mycotoxins subjected to the enzymes should be completely destroyed or modified into a toxic free compound (Mishra and Das, 2003; Kabak *et al.*, 2006).

The degradation of toxins by biological approach is possible through the studies on the functions and characteristics of microorganisms (Karlovsky, 1999). The most recent biological method is the use of enzymes. The current approach of the biological degradation of aflatoxins is based on the degradation of complex organic aromatic compounds such as lignin, which is probably the most abundant polyphenolic xenobiotics (Alberts *et al*., 2009). The fungus known as basidiomycetes has the potential to degrade lignin and a wide range of polycyclic aromatic hydrocarbons because of their large diversity of extracellular enzymes production, and their role in the degradation of other carcinogens such as aflatoxins is probably possible (Christian *et al*., 2005). Among the enzymes produced by basidiomycetes, laccases could have the ability to detoxify aflatoxins (Alberts *et al*., 2009) Karlovsky (1999) reported that the aflatoxins can be converted into other non-toxic compouns in plants, suggesting the presence of natural laccases and enzymes able to degrade the toxins present in the plant.

2.5.15. Degradation Mechanism

The possible mechanisms for the detoxification of aflatoxins by laccases could involve two different pathways, the removal of the double bond positioned at the terminal furan ring and the opening of the lactone ring (Liu *et al*., 1998; Mishra and Das, 2003). Once the lactone ring is opened, further reactions may occur to alter the binding properties of terminal furan ring to DNA and proteins. Wang *et al*. (2011) reported that the degradation of aflatoxin AFB1 involve the oxidation of the 8,9-vinil bond of the toxin to form the aflatoxin AFB1-8,9-epoxide, followed by its hydrolysis to generate the AFB1-8,9-dihydrodiol. This is an important step to reduce affinity of the epoxide with DNA and other enzymes which can lead to undesirable mutations (Mishra and Das, 2003). Treatment with laccases can alter aflatoxin's structure and can form reduced toxical compounds and at a greater extent to non toxic products (Mishra and Das, 2003).

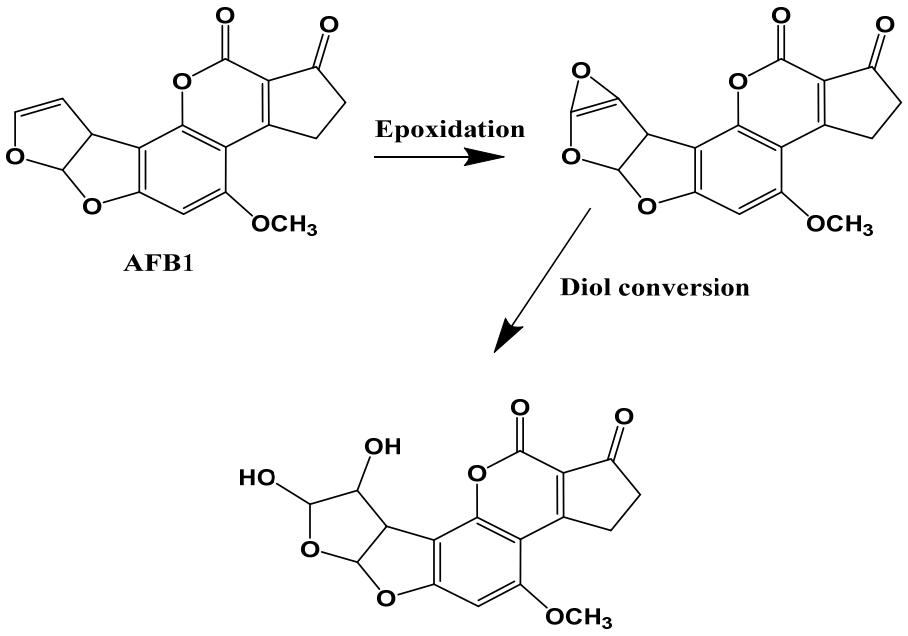

Figure 2.10. Proposed pathway of degradation of AFB1 by laccases (Wu *et al*., 2009).

Mishra and Das (2003) reported that the extent of aflatoxin AFB1 degradation is dependent on the quantitative level of peroxidases groups, including laccases. They also reported that the effectiveness of peroxidases of fungi in the aflatoxins degradation requires the production of hydroperoxides. Peroxidases can produce hypochlorite and singlet oxygen in the presence of hydrogen peroxide and chloride ion (Allen, 1975). During the catalytic oxidation of the substrate by these enzymes, free radicals are produced that react with the toxins. These reactive molecules can epoxidize many substrates, including polycyclic aromatic hydrocarbons, resulting in increased toxicity of the respective substrates (Levi, 2004). They also may initiate lipid peroxidation, bind to cellular proteins or DNA. Another activation pathway involves the formation of a peroxyl radical from subsequent metabolism of prostaglandin G2. In comparison with laccase whose mechanism skips the formation of free radicals, which makes the whole process safer while achieving similar results (Alberts *et al*., 2009).

2.6. Aim, Hypothesis and Specific Objectives

2.6.1. Aim of the Research

The overall objective of the research work was to investigate the degradation of selected aflatoxins, including AFB1, AFB2, AFG1 and AFG2, by selected microbial laccase.

2.6.2. Hypothesis

1. Aflatoxins, a group of mycotoxins, are highly toxic, mutagenic, teratogenic, and carcinogenic. The presence of aflatoxins in foods and feedstuffs is a serious concern for human and animal health as well as for an economical impact. Although many physical and chemical treatments have reported to reduce or degrade aflatoxins in contaminated food and animal feed, they can still reduce the nutritional value of food or produce toxic compounds and harmful residues that can potentially affect public health. The use of biological methods in particular enzymes could be a potential appropriate approach for agro-industrial processes, the safety as well as the nutritional quality and economic value. This approach is based on the degradation of complex organic aromatic compounds, such as lignin, which is probably the most abundant polyphenolic xenobiotics, by the enzymes from basidiomycetes. Among the enzymes produced by basidiomycetes, laccases could have the ability to degrade the aflatoxins. Due to their broad substrate specificity and great power catalyst as well as to their ability to use oxygen in the environment as a cofactor, fungal laccases are used in a wide range of biotechnological and industrial applications.

2. It could be possible to study the potential degradation of selected microbial aflatoxins by partial purified laccase (PPL) obtained from liquid cuture media of *Coriolus hirsutus* and optimize its parameters in order to achieve the maximum enzymatic degradation of the major aflatoxin isoforms.

3. It could be possible to study the residual aflatoxins and their degradation products obtained after laccase catalyse in order to propose a degradation mechanism.

2.6.3. Specific Objectives of the Research

(i) To investigate the production, recovery and characterization of major aflatoxin isoforms, AFB1, AFB2, AFG1 and AFG2, by selected fungal strains, including *Aspergillus parasiticus* and *Aspergillus flavus*.

(ii) To optimize the enzymatic reaction of laccase, obtained from *Coriolus hirsutus*, in terms of its optimum pH and reaction temperature as well as its kinetic characteristics, using the major aflatoxin isoforms as substrates. The effects of selected chemicals and metal ions on the laccase activity were also investigated.

(iii) To determine the maximum laccase enzymatic degradation rate of the selected microbial aflatoxins, through three selected parameters, enzyme concentration, aflatoxin concentration and incubation time, using response surface methodology based on central composite design.

(iv) To characterize the chemical structure of laccase-catalyzed end products and to propose a potential mechanism for the aflatoxins degradation.

2.6.4. Means to achieve the objectives

(i) The recovery of the major aflatoxin isoforms, AFB1, AFB2, AFG1 and AFG2, from the culture medium of *Aspergillus parasiticus* (Hsieh and Mateles, 1971) was performed using an ultra-filtration system, without the use of solvents. In addition, a lyophilization method using 1.5 % of mannitol was used in order to obtain an aflatoxins dry extract. The separation and the characterization of aflatoxins were performed by reversed-phase/high-performance liquid chromatography (RP-HPLC) (Joshua, 1993).

(ii) The recovery of the partially purified laccase (PPL), from the culture medium of *Coriolus hirsutus* was performed with the use of a wide range of methods, including ultrafiltration, protein precipitation with ammonium sulfate by 80% of saturation, dialysis and a freeze-drying system in order to obtain an enzymatic dry extract. The kinetics parameters of the PPL, with the use of aflatoxins as substrates, were performed using a wide range of temperatures and pH. The effect of activators and inhibitors on laccase activity was performed usind selected chemicals agents, including citric acid, L-cysteine, imidazole,

N-hydroxyphthalimide (HPI), dithiothreitol (DTT), diethyldithiocarbamic acid (DDC), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), kojic acid, and *p*-coumaric acid as well as metal ions, including cupric chloride and copper sulfate.

(iii) An experimental strategy for seeking the optimum conditions of the degradation of the major aflatoxin isoforms was performed using central composite design (CCD) with three independent variables, including initial aflatoxins concentration (nmol; X1), enzyme amount (units/nmol aflatoxins; X2) and incubation time (min; X3) at five levels (Tripathi and Mishra, 2011).

(iv) The structural characterization of microbial aflatoxins and their enzymatic degradation products was performed using RP-HPLC (Joshua, 1993), Fourier-transformed infrared (FTIR) (Mirghaniand *et al*., 2001; Samuel *et al*., 2014) and liquid chromatography/mass spectrometry (LC/MS) analyses (Wang *et al*., 2011; Farzaneh *et al*., 2012).

METHODOLOGY

Coriolus Hirsutus and his Laccase

Coriolus hirsustus belongs to the class of the Basidiomycetes of the order of the Polyporales from *Coriolaceae* family and from *Trametes* gender. His cap is usually semicircular and between 4 and 12 cm in diameter when fully grown. Initially white or cream with the upper surface covered in silvery hairs and tends to develop an ochre or brownish region near the margin and are concentrically zoned with narrow yellow-ochre or brown regions and are visibly ridged at maturity. Adjacent caps sometimes merge and fuse together, and the fruitbodies gradually turn greyer before decaying. The tubes are white and up to 6 mm deep, terminating in mainly roundish pores often varying randomly in size, with medium width from 2 to 3 mm and are visible to the naked eye, and sometimes merging to produce a few angular/elongated pores; typically 3 to 4 pores per mm. Initially white, the pore surface turns cream and later ochre or pale brown. The foot is non-existent or insignificant.

The laccase purification from *Coriolus hirsutus* is 14.5-fold with an overall yield of 32.3%. The enzyme is a monomeric glycoprotein with 11% carbohydrate content, an isoelectric point of 7.4, and a molecular mass of 73 kDa. The N-terminal amino acid sequence showed low homology to those of the laccases of other white-rot basidiomycetes. Spectroscopic analyses revealed a typical laccase active site with three Cu centers. The absorption spectrum showed a type 1 signal at around 600 nm and a type 3 signal near to 330 nm. Type 3 Cu showed fluorescence emission near 418 nm and an excitation maximum at 332 nm. The highest rate of 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonate) oxidation for the enzyme was reached at 45°C, and the pH optima of the enzyme varied and was substrate dependent in the range of 2.5 to 4.0. The enzyme oxidized a variety of the usual laccase substrates, including lignin- related phenols and had highest affinity toward guaiacol.

Ultra-filtration

Tangential flow ultra-filtration is sufficient for concentrating large volumes of protein solution (from several liters to thousands). This technique applies pressure to the solution to cause the flow of water and dissolved low molecular weight solutes through the membrane, while high molecular weight solutes are retained Since the fluid flow is rapidly directed across the surface

of the membrane rather than perpendicular to its surface, tangential flow ultra-filtration maintains a clean membrane surface by sweeping away the particles that might otherwise block the pores. The volume of the concentrate slowly drops as low molecular weight solutes and the water in the filtrate pass through the membrane. Ultra-filtration operates using mild conditions of low temperature and pressure (Fig.2.9).

Ammonium Sulfate Precipitation of Proteins

Precipitation techniques are used most commonly during early stages of a purification sequence. For many years, ammonium sulfate fractionation has been commonly used to partially purify laccases from the crude filtrate of their sources. Ammonium sulfate is the most common salt used in enzyme purification because it combines many useful features, such as salting out effectiveness, high solubility and low price. Increasing the salt concentration to a very high level will cause proteins to precipitate from solution without denaturation if done in a gentle manner. First, we want to understand why the protein precipitates. A protein in a buffer solution is very highly hydrated, in other words, the ionic groups on the surface of the protein attract and bind many water molecules very tightly.

When a lot of salt, such as ammonium sulfate, is added to the protein solution, the salt ions attract the water molecules away from the protein. This is partly since the salt ions have a much greater charge density than the proteins. So as the salt is added and these small ions bind water molecules, the protein molecules are forced to interact with themselves and begin to aggregate
So when enough salt has been added, the proteins will be begin to precipitate. If this is carried out at a cold temperature like in ice, the proteins will precipitate without denaturation. Thus, the proteins can be collected by centrifugation and then redissolved in solution using a buffer with low salt content. This process is called "Salting Out" and works best with divalent anions like sulfate, especially ammonium sulfate which is highly soluble at ice temperatures. Ammonium sulfate precipitation is useful for concentrating dilute solutions of proteins. It is also useful for fractionating a mixture of proteins. Since large proteins tend to precipitate first, smaller ones will stay in solution. Through this precipitation, you are also able to increase the purity of your protein of interest.

Dialysis of Proteins

After a protein has been ammonium sulfate precipitate and taken back up in buffer at a much greater protein concentration than before precipitation, the solution will contain a lot of residual ammonium sulfate which was bound to the protein. One way to remove this excess salt is to dialyze the protein against a buffer low in salt concentration. First, the concentrated protein solution is placed in dialysis bag with small holes which allow water and salt to pass out of the bag while protein is retained. Next the dialysis bag is placed in a large volume of buffer and stirred for many hours (16 to 24 hours), which allows the solution inside the bag to equilibrate with the solution outside the bag with respect to salt concentration. When this process of equilibration is repeated several times (replacing the external solution with low salt solution each time), the protein solution in the bag will reach a low salt concentration

This process should be done in buffer to prevent the protein from denaturing due to the fact that distilled or deionized water is too low in salt and may have an undesirable pH for your protein, which may cause it to denature. The dialysis is a good way to exchange the buffer the protein is in at the same time you get rid of excess salt

Aspergillus Parasiticus and his Aflatoxins

This species is characterized by is greenish-yellow to olive surface and may have a white border. His texture is often floccose, especially near to the center and overall can be velvety to woolly. Colony diameters on Czapek's Agar are from 1.3 to 1.5 cm in 14 days at 25°C, dense, raised. Conidial heads radiate to loosely columnar, white to cartidge buff. The mycelium is white to pale capucine buff. Exudate is abundant, light yellow. Soluble pigment is buff-yellow to amber brown. The stipes (140 - 1510 × 2.8 - 11.1 µm) is colorless or light yellow to pale brown, smooth to slightly roughen. The vesicles are obovoid, subglobose or pyriform and about 6.0 - 29.4 µm wide. Metulae cover 1/2 to 4/5 of the vesicle from which the phialides (4.0 - 9.4 × 2.5 - 5.7 µm) form. The metulae support the phialides and together form the biseriate structure phialides. Conidia are globose to subglobose with 2.0 - 4.0 µm in diameter, smooth walled. Colony diameters on Malt Extract Agar are from 3.0 to 3.5 cm in 14 days at 25°C, plane to velutinous. The conidial heads are radiate to columnar, white to light buff. The mycelium is white to pinkish buff. Soluble pigment is red brown to mikado brown.

Aspergillus flavus produces mainly aflatoxin AFB1 and AFB2 while *A. parasiticus* produces aflatoxins AFB1, AFB2, AFG1 and AFG2. Several factors affect the growth of *A. parasiticus* and aflatoxins biosynthesis. Among these factors, temperature and relative humidity are the most important ones, where the optimum temperature is around 30ºC and relative humidity is between 0.95 and 0.99. In addition, the nutrient composition of the culture medium also plays a role in the aflatoxins. Simple sugars such as glucose, fructose and sucrose are preferred sources of carbon. The nitrogen source such as peptone can promote the production of toxins. Moreover, nitrogen may also be an important factor in the production of toxins. The presence of ammonium favored the production, whereas the presence of nitrate would tend to inhibit it. The presence of metals such as zinc, magnesium, manganese, molybdenum, aluminum and iron are also important for the fungal growth. Zinc is particularly important both for fungal growth and the aflatoxins biosynthesis.

Although pH has a secondary effect on the toxins biosynthesis of toxins, an acidic pH, between 4 and 6, would seem to favor the aflatoxins production. In addition, the presence of light is essential for vegetative growth and toxins production in both liquid and solid growth media by having a photochemical effect on the medium.

Aflatoxins-producing fungi are widespread around the world, in countries with temperate, subtropical and tropical climates and can contaminate a wide range of agricultural commodities, including cereal grains (corn, rice, barley, wheat), oilseeds (sunflower, soybeans), nuts (pistachios, almonds, coconut) and spices (pepper, paprika, ginger) or in milk and derivatives.

High-Performance Liquid Chromatography (HPLC)

It is one of the most common methods used to separate, identify, and quantify each component in a mixture. Its high precision allows the search of traces and it is also possible to couple it with a mass spectrometer. Chromatography can be described as a mass transfer process involving adsorption. HPLC relies on pumps to pass a pressurized liquid and a sample mixture through a column filled with a sorbent, leading to the separation of the sample components. The active component of the column, the sorbent, is typically a granular material made of solid particles (e.g. silica, polymers, etc.), 2-50 micrometers in size. The components of the sample mixture are separated from each other due to their different degrees of interaction with the sorbent particles.

The pressurized liquid is typically a mixture of solvents (e.g. water, acetonitrile and/or methanol) and is referred to as a "mobile phase". Its composition and temperature play a major role in the separation process by influencing the interactions taking place between sample components and sorbent. These interactions are physical in nature, such as hydrophobic (dispersive), dipole–dipole and ionic, most often a combination. The sample mixture to be separated and analyzed is introduced, in a discrete small volume (typically microliters), into the stream of mobile phase percolating through the column. The components of the sample move through the column at different velocities, which are function of specific physical interactions with the sorbent (also called stationary phase). The velocity of each component depends on its chemical nature, on the nature of the stationary phase (column) and on the composition of the mobile phase. The time at which a specific analyte elutes (emerges from the column) is called its retention time. The retention time measured under particular conditions is considered an identifying characteristic of a given analyte. A separation in which the mobile phase composition remains constant throughout the procedure is termed isocratic (meaning constant composition). On the other hand, a separation in which the mobile phase composition is changed during the separation process is described as a gradient elution.

Fourier Transform Infrared Spectroscopy (FTIR)

It is based on the absorption of infrared radiation by the analyzed sample. It allows detection of characteristics via chemical bonds vibration, performing the analysis of chemical functional groups present in the sample. When the wavelength (energy) supplied by the light beam is close to the vibrational energy of the molecule, the latter will absorb the radiation and record a decrease in the reflected or transmitted intensity. The infrared region between 4000 cm^{-1} and 400 cm^{-1} corresponds to the energy range of vibration of the molecules.

Mass Spectrometry

It is a powerful analytical technique used to quantify known materials, to identify unknown compounds within a sample, and to elucidate the structure and chemical properties of different molecules. The complete process involves the conversion of the sample into gaseous ions, with or without fragmentation, which are then characterized by their mass to charge ratios (m/z) and relative abundances. This technique basically studies the effect of ionizing energy on molecules. It depends upon chemical reactions in the gas phase in which sample molecules are consumed

during the formation of ionic and neutral species. A mass spectrometer generates multiple ions from the sample under investigation; it then separates them according to their specific mass-to-charge ratio (m/z), and then records the relative abundance of each ion type. The first step in the mass spectrometric analysis of compounds is the production of gas phase ions of the compound, basically by electron ionization. This molecular ion undergoes fragmentation. Each primary product ion derived from the molecular ion, in turn, undergoes fragmentation, and so on. The ions are separated in the mass spectrometer according to their mass-to-charge ratio, and are detected in proportion to their abundance. A mass spectrum of the molecule is thus produced. It displays the result in the form of a plot of ion abundance versus mass-to-charge ratio. Ions provide information concerning the nature and the structure of their precursor molecule. In the spectrum of a pure compound, the molecular ion, if present, appears at the highest value of m/z (followed by ions containing heavier isotopes) and gives the molecular mass of the compound. The devices can be used either with a direct injection system (analysis of pure substances) or coupled with a chromatography system (TLC, GLC, HPLC, SFC). Some more sophisticated devices, such as MS-MS system used to analyze mixtures without chromatography. The first MS stage is used to select an ion, and the second analyze the ions from the fragmentation thereof. A mass spectrum is a real identity card of molecules.

CHAPTER III

ARTICLE II

PRODUCTION, RECOVERY AND CHARACTERIZATION OF AFLATOXINS, OBTAINED FROM ASPERGILLUS PARASITICUS

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3.1. Contribution of the Authors

The present author, Sabrina Borgomano, was responsible for the concepts, the designs and the fulfillment of this experimental work and the preparation of this manuscript for its submission.

Dr. Monique Lacroix, the thesis supervisor, and Dr. Selim Kermasha, the thesis supervisor, supervised this research work, provided valuable input and advices, monitored the progress of this work and critically reviewed and edited this manuscript, prior to its submission.

3.2. Résumé

La production d'exo-aflatoxines par des souches fongiques sélectionnées, dont *Aspergillus flavus* et *Aspergillus parasiticus*, a été étudiée, en utilisant un milieu de fermentation liquide. Les effets de la composition du milieu initial d'inoculation (MII), de la concentration de la biomasse microbienne et de la durée d'incubation ainsi que l'utilisation de cryoprotectants selectionnés, conprenant le mannitol et le dextrane, ont été déterminés sur le niveau des aflatoxines. *A. parasiticus* a montré une plus grande capacité à produire les aflatoxines lorsque la gélose dextrosée à la pomme de terre était utilisée comme milieu initial d'inoculation. En outre, le rendement le plus élevé de 73,603 mg aflatoxines/L de milieu de culture a été obtenu avec une concentration de biomasse fongique de 150 g/L après 168 h d'incubation. Les résultats indiquent que la récupération la plus élevée et la meilleure qualité de l'extrait sec d'aflatoxines ont été obtenues avec 1,5% de mannitol, utilisé comme cryoprotectant, où l'extrait sec d'aflatoxines était complètement soluble dans une solution à 40% de methanol. La séparation et la caractérisation des aflatoxines ont été effectuées par chromatographie liquide à haute performance en phase inverse à une longueur d'onde spécifique de 365 nm.

3.3. Abstract

The production of exo-aflatoxins by selected fungal strains, including *Aspergillus flavus* and *Aspergillus parasiticus* was investigated, using liquid-state fermentation. The effects of the initial inoculation medium (IIM) composition, the microbial biomass concentration and the incubation time as well as the use of selected cryoprotectants, including mannitol and dextran, on the aflatoxins yield were determined. *A. parasiticus* showed the highest aflatoxin-production ability, when potato dextrose agar was used as the IIM. In addition, the highest yield of 73.603 mg aflatoxins/L culture medium was obtained with a fungal biomass concentration of 150 g/L after 168 h of incubation. The results indicated that the highest recovery and the better quality of dried aflatoxins extract were obtained with 1.5% of mannitol, used as cryoprotectant, where the dry aflatoxins extract solubility was complete in 40% methanol solution. The separation and characterization of aflatoxins were carried out with reverse-phase/high-performance liquid chromatography at a specific wavelength of 365 nm.

Keywords: *Aspergillus parasiticus*, aflatoxins production, incubation time, cryoprotectant, RP-HPLC

3.4. Introduction

Aflatoxins, which are highly toxic, mutagenic, teratogenic and carcinogenic compounds, are produced as secondary metabolites by fungi belonging to several *Aspergillus* species (Bhat *et al.*, 2010). Aflatoxins are an assembly of a coumarin and 3-furan, where aflatoxins AFB1, AFB2, AFG1 and AFG2 are the most common and potent ones in contaminated food products and feed; however, aflatoxin AFB1 is the most toxic one (Pietri *et al*, 2012). The tolerable level of aflatoxins is pending on the nature of food and on the regulations that ranged between 2 and 20 μg/kg (Rustom, 1997).

The nature of the toxin is pending on the type of the microorganism; *Aspergillus flavus* produces mainly aflatoxins AFB1 and AFB2, whereas *Aspergillus parasiticus* produces aflatoxins AFB1, AFB2, AFG1 and AFG2 (Bhat *et al.*, 2010). Several fermentation parameters, including temperature and water activity (a_w), could play an important role in the aflatoxins production (Bhat *et al.*, 2010). In addition, the aflatoxins yield is also pending on the nature of carbon source, used for the microbial biomass production, where glucose, fructose and sucrose are the most preferred ones (Klich, 2007).

The recovery of aflatoxins from solutions and contaminated products could be obtained by a variety of slightly polar solvents (Blesa *et al.,* 2003). The most common technique used to analyze aflatoxins is high-performance liquid chromatography (HPLC). Although, the separation and characterization of aflatoxins can be achieved with both normal-phase (NP) and reversed-phase (RP), RP is the more appropriate one. In addition, RP-HPLC can be coupled with UV absorption, fluorescence and mass spectroscopy (MS), to achieve the characterization of the different forms of aflatoxins (Afsah-Hejri *et al*., 2011).

The overall aim of this research work was the optimization of biomass production of *A. parasiticus* and the recovery of aflatoxins. The specific objectives were to study the effects of selected solid media cultures, incubation time and selected cryoprotectants on the aflatoxins production and their recovery.

3.5. Materials and Methods

3.5.1. Micoorganism and Culture Conditions

Aspergillus parasiticus (26864) and *Aspergillus flavus* (26771) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). *A. parasiticus* was maintained through periodic transfer onto potato dextrose agar medium (PDA) plates at pH 5.6 and Czapek's agar medium (CA) plates at pH 7.3 and incubated at 24ºC. *A. flavus* was maintained on Czapek's agar medium plates and incubated at 24ºC. For aflatoxins production, a pre-culture and a culture liquid media were prepared using the method previously described by Hsieh and Mateles (1971).

3.5.2. Media Preparation and Inoculation

The fermentation process of *A. parasiticus* and *A. flavus* was carried out according to Hsieh and Mateles (1971). The pre-culture liquid medium contained 50.0 g glucose, 3.0 g $(NH4)_2SO_4$, 10.0 g KH_2PO_4, 2.0 g $MgSO_4.7H_2O$, 0.7 mg $Na_2B_4O_7.10H_2O$, 0.5 mg $(NH_4)_6Mo_7O_{24}.4H_2O$, 10.0 mg $Fe_2(SO4)_3.6H_2O$, 0.3 mg $CuSO_4.5H_2O$, 0.11 mg $MnSO_4.H_2O$ and 17.6 mg $ZnSO_4.7H_2O$ per liter. Culture liquid medium contained 15.0 g glucose; 5.0 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KC1 and the same trace metal elements used in the pre-culture medium.

The aflatoxins production was initiated by the inoculation of two cylinders of 7 mm diameter of the fungi, picked from the potato dextrose agar (PDA) or Czapek's agar (CA) plate cultures into 200 mL of pre-culture medium in a 500 mL-Erlenmeyer flask. This primary culture was incubated for two days at 30°C under agitation using a rotary shaker incubator (New Brunswick Scientific Co., Inc.; Edison, N.J.) at 100 rpm for the first day and at 200 rpm for the second one. The pre-cultures were used to inoculate 2 L-Erlenmeyer flask containing 200 mL of liquid culture medium, before its incubation for one day at 30°C under agitation using the rotary shaker incubator at 200 rpm. The mycelium pellets were removed throughout a cheese cloth filtration.

3.5.3. Recovery of Aflatoxins

The extracellular toxins were recovered from the culture medium by ultra-filtration, using Prep/Scale TFF Cartridge (2.5 ft^2) of polyethylene polypropylene low protein-binding membranes (1 *k*Da cut-off filter) under a pressure of 10 psi (Millipore, Milford, MA). The cryoprotectant, mannitol or dextran, was added to the resulted ultrafiltred fraction containing the

aflatoxins, before its lyophilization, using a freeze dryer (Labconco Co., Kansas City, MO). The lyophilized aflatoxins fraction was stored at -80ºC and subjected for further investigation.

3.5.4. Spectrometric Determination of Aflatoxins

The aflatoxins were determined spectrophotometrically at 365 nm, using a Beckman DU 650 spectrophotometer (San Raman, CA). The lyophilized aflatoxins extract was solubilized in 40% methanol (1:10; w/v).

3.5.5. Statistical Analyses

Data were expressed as means of triplicate trials and their respective standard deviations (SD). The percent relative standard deviation (RSD) was calculated as the SD divided by the mean multiplied by 100. Statistical analyses were performed, using STATISTICA 10.0 (StatSoft, Tulsa, OK). A post hoc comparison was made, using Tukey's test. The results were considered to be statistically different at $P \leq 0.05$.

3.5.6. HPLC Analyses

The separation and the tentative characterization of aflatoxins, AFB1, AFB2, AFG1 and AFG2, was performed by reversed-phase/high-performance liquid chromatography (RP-HPLC), using a Beckman Gold system 126 (Beckman Instrument Inc., San Raman, CA), equipped with a UV diode-array detector (DAD), set at 365 nm (Beckman, model 168). A 20 µL sample was injected into an allsphere C18 column (4.6 mm x 250 mm, 5 µm; Alltech, Deerfield, IL), thermostated at 50°C. The isocratic solvent elution was a mixture of water/methanol (40:60, v/v), at a flow rate of 1 mL/min.

3.6. Results and Discussion

3.6.1. Selection of Aspergillus Strain

The production of the major isoforms of aflatoxins, AFB1, AFB2, AFG1 and AFG2, by *A. parasiticus* and *A. flavus* was investigated using PDA and CA as the initial inoculation medium, respectively. Figure 3.1 shows that both strains were able to produce the major isoforms of aflatoxins at two stages of incubation time, 24 and 168 h. For *A. parasiticus* at 24 h of fermentation, 259.0, 241.8, 314.9 and 283.8 µg aflatoxins/g dry extract of AFB1, AFB2, AFG1 and AFG2 were, respectively, recovered, whereas, at 168 h, 720.7, 675.1, 882.3 and 788.7 µg aflatoxins/g dry extract of AFB1, AFB2, AFG1 and AFG2 were, respectively, recovered. For *A.*

flavus at 24 h of fermentation, 220.9, 207.2, 270.6 and 246.6 µg aflatoxins/g dry extract of AFB1, AFB2, AFG1 and AFG2 were, respectively, recovered, whereas, at 168 h, 414.3, 387.8, 507.1 and 451.1 µg aflatoxins/g dry extract of AFB1, AFB2, AFG1 and AFG2 were, respectively, recovered. The overall results indicated that *A. parasiticus* produced the highest aflatoxins yield as compared to that of *A. flavus*.

The experimental findings (Fig. 3.1) are in agreement with those of Wei and Jong (1986), in which *A. parasiticus* 26864 showed the highest ability for the production of the major aflatoxin isoforms as compared to *A. flavus* 26771; these authors reported that the use of *A. parasiticus*, resulted by the production of 1255.2, 16.0, 510.0 and 8.3 µg/mL of AFB1, AFB2, AFG1 and AFG2, respectively, whereas, the use of *A. flavus* resulted by the production of 224.4, 5.2, 340.0 and 5.5 µg/mL of AFB1, AFB2, AFG1 and AFG2, respectively. In addition, Fente *et al*. (2001) showed that among different strains of *A. flavus* and *A. parasiticus,* only *A. parasiticus* NR2999 was able to produce the four mainly isoforms of aflatoxins. Codner *et al*. (1963) also obtained the highest aflatoxins yield after fermentation of *A. parasiticus* IMI 15957 as compared to that obtained by *A. flavus*.

3.6.2. Effect of the Initial Inoculation Medium on Aflatoxins Yield

The effect of different nutriments on aflatoxins yield, using CA and PDA as the initial inoculation media, was investigated. The results (Table 3.1) indicate that the aflatoxins yield, obtained with *A. parasiticus* on PDA, was 108.3, 101.4, 132.8 and 118.8 µg aflatoxins/g dry extract for AFB1, AFB2, AFG1 and AFG2, respectively, whereas that for *A. parasiticus* on CA, it was 78.0, 72.8, 95.4 and 85.8 µg aflatoxins/g dry extract for AFB1, AFB2, AFG1 and AFG2, respectively. The experimental findings suggest that there was a significant ($P \leq 0.05$) increase of 38% in aflatoxins yield by *A. parasiticus* with PDA compared to that with CA. These results could be due to the presence of glucose as the carbon source in PDA as compared to the sucrose in CA.

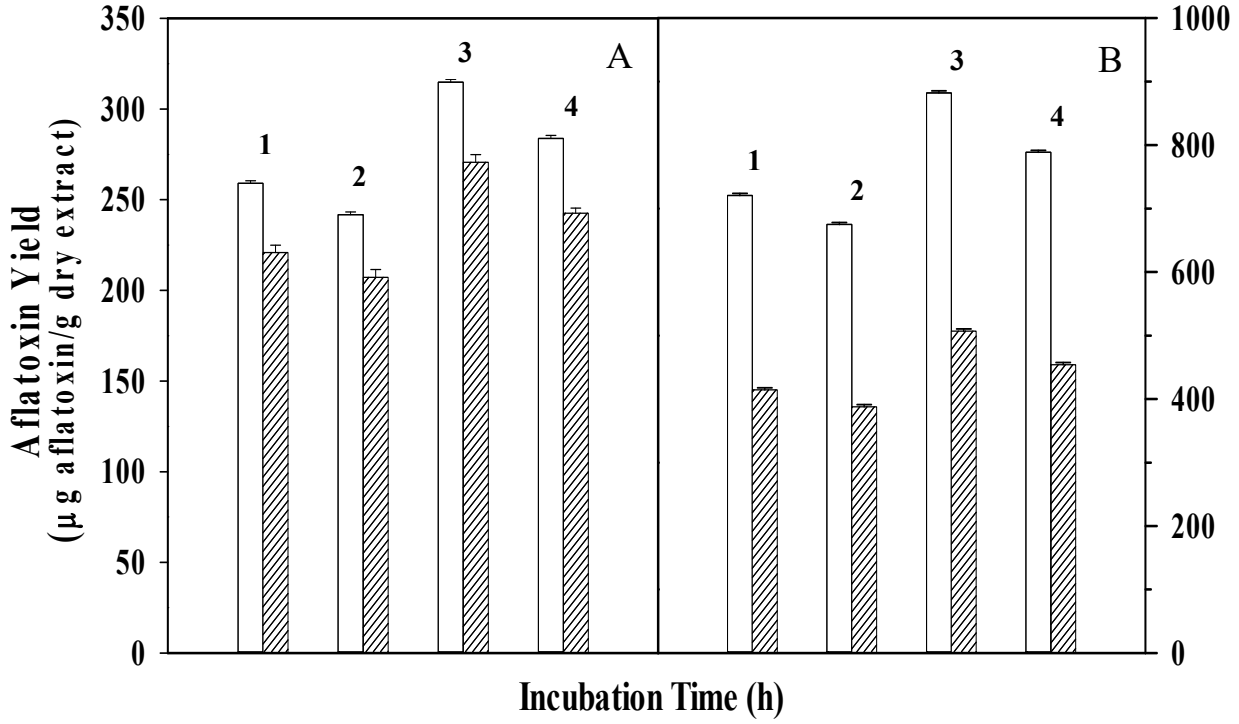

Figure 3.1. Aflatoxins obtained from the culture of *Aspergillus parasiticus* ☐ and *Aspergillus flavus* ▨ after (A) 24 and (B) 168 h of incubation in culture media, (1) AFB1, (2) AFB2, (3) AFG1 and (4) AFG2.

These findings are in agreement with those of Buchanan *et al*. (1984), who reported that glucose is the most appropriate carbon source compared to sucrose for the production of aflatoxins. In addition, the increase in aflatoxins yield with the use of PDA, could be also attributed to the initial pH of the medium which was at 5.6 for PDA, whereas it was at 7.3 for CA. Klich (2006) indicated that the highest aflatoxins yield was obtained with that having acidic pH, at 4 and 6.

The decrease in aflatoxins yield could also be attributed to the degradation of aflatoxins in CA, by fungal enzyme synthesized by mycelia (Huynh *et al*., 1984). Callaghan *et al*. (2003) reported that CA is a permissive medium that allows the growth of the fungi as well as the production of the toxins on the plates, whereas PDA is a restrictive medium which only allows the growth of the mold on the plates without the toxins formation. The overall results indicate that the highest aflatoxins yield was obtained with PDA as the solid medium.

3.6.3. Effect of the Biomass Concentration on Aflatoxins Yield

The effect of the biomass of *A. parasiticus* on the aflatoxins yield was investigated. Table 3.2 shows that the increase in aflatoxin yield was obtained in concomitant with that of the wet microbial biomass, with 13.6, 31.5 and 56.2 µg aflatoxins/g dry extract per 50, 100 and 150 g biomass/L culture medium, respectively. These experimental results are similar to those obtained by Hsieh and Mateles (1971), who reported that the optimum of aflatoxins yield was reached with a cell concentration of 150 g/L of culture medium. Tzanidi *et al*. (2012) showed that when the mycelium concentration increased from 0 to 21 g/L, the aflatoxins yield increased from 0 to 753.1 µg/L, respectively. In addition, Detroy *et al*. (1971) reported that the synthesis of aflatoxins was accelerated with the biomass increase. However, Schindler *et al*. (1967) reported that the maximal fungi growth was not correlated with the aflatoxins production.

Table 3.1. Effect of nutriments of PDA and CA used as solid media on aflatoxin yield.

Aflatoxin yield (µg aflatoxin/g dry extract)[a]							
PDA[b]				CA[b]			
AFB1[c]	AFB2[c]	AFG1[c]	AFG2[c]	AFB1[c]	AFB2[c]	AFG1[c]	AFG2[c]
108.3 (0.6)[d]	101.4 (0.6)[d]	132.8 (0.8)[d]	118.8 (0.8)[d]	78.0 (0.6)[d]	72.8 (0.6)[d]	95.4 (0.6)[d]	85.8 (0.6)[d]

[a]Products issued from fraction IIb obtained by ultrafiltration (1 *k*Da) of the crude exo-culture media of *A. parasiticus* after 24 h of incubation.

[b]Solid media used.

[c]Aflatoxins AFB1, AFB2, AFG1 and AFG2, with molecular mass of 312, 314, 328 and 330 $g.mol^{-1}$, respectively, characterized spectrophotometrically at 365 nm, by their extension coefficient of 21,800, 14,700, 16,100 and 19,300, respectively, produced from exo-culture media of *A. parasiticus.*

[d]Relative standard deviation (RSD) was calculated as the standard deviation of triplicate samples divided by their mean multiplied by 100.

Table 3.2. Effect of the *Aspergillus parasiticus* biomass on aflatoxin yield.

Weight (g)[b]	Aflatoxin yield (µg aflatoxin/g dry extract)[a]			
	AFB1[c]	AFB2[c]	AFG1[c]	AFG2
0	0 (0.0)[d]	0 (0.0)[d]	0 (0.0)[d]	0 (0.0)[d]
50	12.9 (12.9)	11.9 (12.8)	15.6 (12.9)	14.0 (12.9)
100	29.6 (4.5)	27.6 (4.5)	36.4 (4.5)	32.7 (4.5)
150	52.7 (16.7)	49.6 (16.6)	64.6 (16.5)	58.1 (16.5)

[a]Products issued from fraction IIb obtained by ultrafiltration (1 *k*Da) of the crude exo-culture media of *A. parasiticus* after 24 h of incubation.

[b]Concentration of wet fungi (g/L) filtered from the pre-culture medium and inoculated in the culture medium.

[c]Aflatoxins AFB1, AFB2, AFG1 and AFG2, with molecular mass of 312, 314, 328 and 330 $g.mol^{-1}$, respectively, characterized spectrophotometrically at 365 nm, by their extension coefficient of 21,800, 14,700, 16,100 and 19,300, respectively, produced from exo-culture media of *A. parasiticus.*

[d]Relative standard deviation (RSD) was calculated as the standard deviation of triplicate samples divided by their mean multiplied by 100.

3.6.4. Effect of Incubation Time on Aflatoxins Yield

The effect of incubation time on the aflatoxins yield in the pre-culture and in the culture media was investigated. Table 3.3 shows that 48 h of fermentation of *A. parasiticus*, in pre-culture medium, resulted by the highest yield of 353.8, 331.6, 433.6 and 388.4 µg aflatoxins/g dry extract for AFB1, AFB2, AFG1 and AFG2, respectively, however, beyond 48 h of fermentation, the yield dropped to 331.7, 310.9, 406.1 and 364.0 µg aflatoxins/g dry extract for AFB1, AFB2, AFG1 and AFG2, respectively. The overall results indicate that the aflatoxins yield increased in concomitant with the increase in fermentation time in pre-culture medium, up to 48 h and before its decrease. These experimental results are in agreement with those of Hsieh and Mateles (1971), where the highest aflatoxins production was obtained after 48 h of incubation in pre-culture, before it was dropped drastically.

Table 3.4 shows that 168 h of fermentation of *A. parasiticus*, in culture medium, resulted by the highest yield of 694.7, 646.4, 845.5 and 757.5 µg aflatoxins/g dry extract for AFB1, AFB2, AFG1 and AFG2, respectively, however, the results also shows that beyond 168 h of incubation, the yield dropped to 480.1, 453.9, 593.3 and 531.5 µg aflatoxins/g dry extract for AFB1, AFB2, AFG1 and AFG2, respectively. The decrease in aflatoxins yield may be due to the presence of peroxidase in the medium, synthesized by aged mycelia of *A. Parasiticus* (Doyle and Marth, 1979), whereas, in the presence of young ones, there was little or absence of such activity (Huynh and Lloyd, 1984). The results (Table 3.4) are in agreement with those obtained by Tzanidi *et al*. (2012), who reported that aflatoxins production was related with the mycelium growth and the incubation time. However, the present results differ from those of Hsieh and Mateles (1971), who indicated that after 48 h of incubation in the pre-culture medium, a linear aflatoxins production was obtained only during the first 20 h of fermentation. Kheiralla *et al*. (1992) also reported that the highest aflatoxins production was obtained after 14 days of fermentation before its decrease. Ciegler *et al*. (1966b) indicated that the highest aflatoxins production, from *A. parasiticus* strain NRRL 3000, was obtained after 72 h of incubation.

Table 3.3. Effect of pre-culture incubation time on aflatoxins yield obtained from the culture of *Aspergillus parasiticus*.

Time (h)[b]	Aflatoxin yield (µg aflatoxin/g dry extract)[a]			
	AFB1[b]	AFB2[b]	AFG1[b]	AFG2[b]
0	0 (0.0)[d]	0 (0.0)[d]	0 (0.0)[d]	0 (0.0)[d]
24	130.7 (1.7)	147.0 (1.7)	140.1 (1.7)	117.5 (1.8)
48	353.8 (7.8)	331.6 (7.8)	433.6 (7.8)	388.4 (7.9)
72	331.7 (2.7)	310.9 (2.7)	406.1 (2.7)	364.0 (2.8)

[a]Products issued from fraction IIb obtained by ultrafiltration (1 *k*Da) of the crude exo-culture media of *A. parasiticus.*

[b]Incubation time *of A. parasiticus* in the pre-culture medium before enrichment with the culture medium for 24h.

[c]Aflatoxins AFB1, AFB2, AFG1 and AFG2, with molecular mass of 312, 314, 328 and 330 $g.mol^{-1}$, respectively, characterized spectrophotometrically at 365 nm, by their extension coefficient of 21,800, 14,700, 16,100 and 19,300, respectively, produced from exo-culture media of *A. parasiticus.*

[d]Relative standard deviation (RSD) was calculated as the standard deviation of triplicate samples divided by their mean multiplied by 100.

Table 3.4. Effect of incubation time in culture medium on aflatoxins yield obtained from the culture of *Aspergillus parasiticus.*

Time (h)[b]	Aflatoxin yield (µg aflatoxin/g dry extract)[a]			
	AFB1[c]	AFB2[c]	AFG1[c]	AFG2[c]
0	0	0	0	0
6	111.4 (1.6)[d]	104.6 (1.4)[d]	136.8 (1.5)[d]	122.8 (1.5)[d]
9	117.0 (1.2)	109.6 (1.2)	143.3 (1.5)	128.4 (1.3)
12	127.3 (1.7)	119.3 (1.7)	156.1 (1.5)	139.9 (1.4)
15	236.2 (4.4)	221.4 (4.5)	289.3 (4.3)	259.1 (4.4)
18	241.8 (3.4)	226.7 (3.4)	289.3 (3.4)	265.7 (3.4)
24	259.0 (1.5)	241.8 (1.5)	314.9 (1.5)	283.8 (1.6)
36	285.5 (2.3)	267.5 (2.3)	349.6 (2.2)	313.5 (2.3)
48	368.8 (6.2)	345.7 (6.2)	451.7 (6.2)	404.9 (5.6)
90	412.5 (3.0)	386.5 (3.0)	505.4 (3.0)	452.8 (3.0)
120	469.3 (13.1)	440.3 (13.1)	575.3 (13.3)	515.5 (13.3)
168	694.7 (12.5)	646.4 (12.4)	845.5 (12.8)	757.5 (12.5)
200	480.1 (2.4)	453.9 (2.4)	593.3 (2.4)	531.5 (2.4)

[a]Products issued from fraction IIb obtained by ultrafiltration (1 *k*Da) of the crude exo-culture media of *A. parasiticus.*

[b]Different incubation time of *A. parasiticus* in the culture liquid medium after 48 h in pre-culture medium.

[c]Aflatoxins AFB1, AFB2, AFG1 and AFG2, with molecular mass of 312, 314, 328 and 330 $g.mol^{-1}$, respectively, characterized spectrophotometrically at 365 nm, by their extension coefficient of 21,800, 14,700, 16,100 and 19,300, respectively, produced from exo-culture media of *A. parasiticus.*

[d]Relative standard deviation (RSD) was calculated as the standard deviation of triplicate samples divided by their mean multiplied by 100.

The overall results suggest that the aflatoxins yield significantly ($P \leq 0.05$) increased with the concomitant increase in incubation time, until a certain critical point where, there was a possible degradation of aflatoxins. In addition, the incubation time at which the cultures reached its maximum aflatoxins yield was dependent on strain and culture conditions (Klich, 2007).

3.6.5. Effect of Cryoprotectants on the Dry Aflatoxins Recovery and Quality

The effect of selected cyoprotectant additives, including mannitol and dextran, in terms of their efficiency on the recovery and the quality of the aflatoxins dry extract, was investigated. Table 3.5 shows that the lyophilization of aflatoxins extract, without the use of any cryoprotectant, resulted by a sticky structure. However, the use of the selected cryoprotectants, mannitol or dextran, provided a better recovery for the dry extract. The results suggest that the quality of the dry aflatoxins, obtained with the use of dextran and mannitol, was concentration dependant, where 3.5 and 3% of mannitol and dextran, respectively, were the most appropriate concentrations of cryoprotectants to obtain a better quality of dry extract. These findings are in agreement with those of Wang (2000), who indicated that both sugars and polyols were effective cryoprotectants and remarkable lyoprotectants, where their level of stabilization was concentration pending. These results are in agreement with those of Carpenter *et al.* (1997), who reported that the use of mannitol during lyophilisation resulted by a mechanically stronger dry powder with higher structural quality than dextran. In addition, Prestrelski *et al*. (1993) indicated that an appropriate choice of cryoprotectant was needed to protect the sample from denaturation during lyophilization as well as to provide a glassy matrix required for long-term storage stability in the dried solid.

Figure 3.2 shows that the aflatoxins yield decreased with the concomitant increased in cryoprotectant concentration. There was a significant ($P \leq 0.05$) decrease of 164.0 and 125.5% in aflatoxins yield in presence of 3.5 and 3% (w/v) of mannitol and dextran, respectively, as compared to that obtained with 1%. The experimental results indicate that 1.5% (w/v) of cryoprotectant, mannitol or dextran, ensured an appropriate quality of the dry extract and resulted by a high recovery in aflatoxins yield.

Table 3.5. Effect of cryoprotectants concentrations on the quality of the dry extract from the culture of *Aspergillus parasiticus* using potato dextrose agar as selected solid medium.

Mannitol		Dextran	
(%)[a]	Quality[b]	(%)[a]	Quality[b]
0	NR[c]	0	NR[c]
1.00	-	1.00	+/-
1.25	+/-	1.25	+
1.50	++	1.50	++
1.75	++	1.75	++
2.00	++	2.00	+++
2.50	+++	2.50	+++
3.00	+++	3.00	+++
3.50	+++	_	_

[a]Cryoprotectant concentration in percentage (w/v) used in the fraction IIb.

[b]Quality of powder was defined as the final texture of lyophilised aflatoxins from the crude exo-culture media of *A. Parasiticus,* and expressed with qualitative evaluation: -: Bad powder quality, +/-: Average powder quality, +: Good powder quality, ++: Very good powder quality, +++: Excellent powder quality.

[c]No recovery for any extract

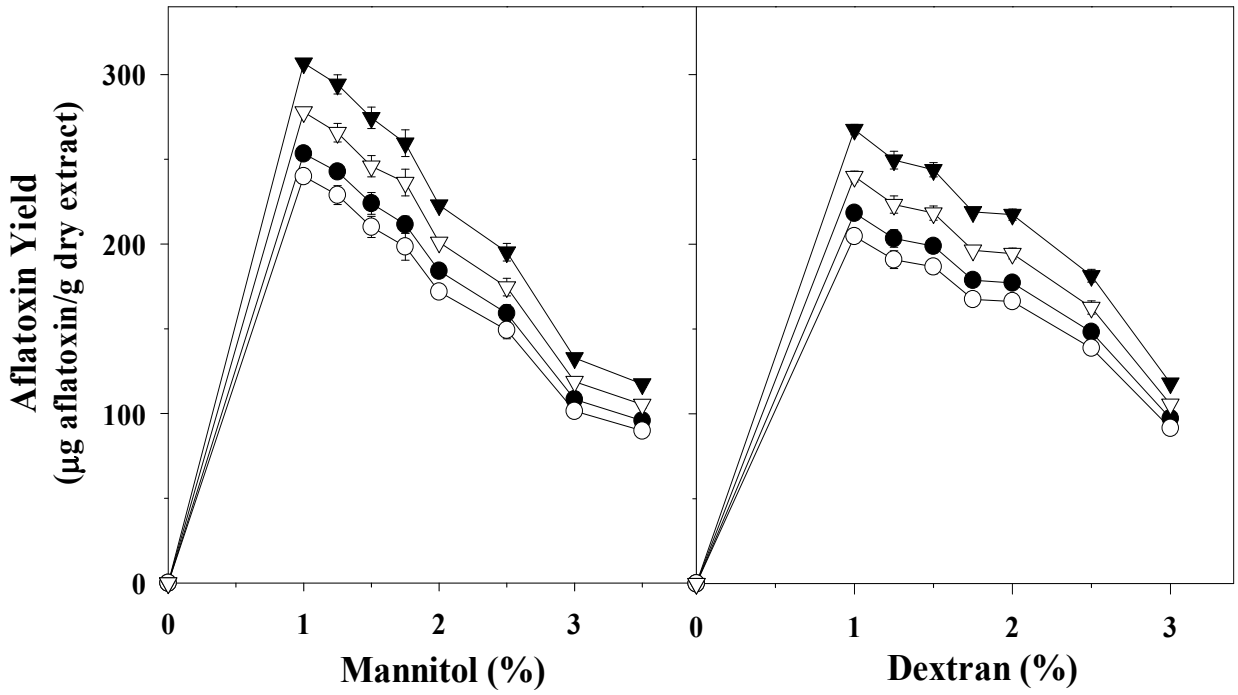

Figure 3.2. Effect of cryoprotectant concentrations on the aflatoxins yield obtained by culture of *Aspergillus parasiticus* in culture media during 24 h using potato dextrose agar as solid media. AFB1 (●), AFB2 (○), AFG1 (▼), and AFG2 (▽).

On the other hand, the presence of 1.5% mannitol in the extract resulted by the recovery of 224.0, 210.1, 274.5 and 245.9 µg aflatoxins/g dry extract of AFB1, AFB2, AFG1 and AFG2, respectively, whereas with that of 1.5% dextran, resulted by 198.9, 186.7, 243.9 and 218.4 µg aflatoxins/g dry extract of AFB1, AFB2, AFG1 and AFG2, respectively. The experimental findings suggest that use of 1.5% mannitol resulted by a significant ($P \leq 0.05$) increase of 11% in aflatoxins yield as compared to 1.5% for dextran.

3.6.6. Effect of the Selected Cryoprotectants on the Dry Extract Solubility

The effect of 1.5% of cryoprotectants, on the solubility of the dry extract in different methanol gradient solutions, was investigated (data not shown). In presence of 1.5% mannitol, a 40% of methanol solution was suitable for a complete dissolution of the dry aflatoxins extract. However, in presence of 1.5% dextran, a 100% of aqueous solution was needed for a complete dissolution of the dry extract. Diaz and Cepeda (2012) reported that the aflatoxins, solubilized in aqueous solution, were instable and underwent degradation because of the presence of the double bonds in their chemicals structures which are vulnerable to hydrolysis. However, the instability of aflatoxins in aqueous solution could be counteracted by the presence of at least a 20% polar organic solvent. These results are in agreement with those of Carpenter *et al*. (1997) who reported that mannitol is preferred to dextran for its dissolution properties.

3.6.7. HPLC Analysis of Aflatoxins

The RP-HPLC analysis was used to separate and to characterize at 365 nm, the different forms of aflatoxins in the dry extract. Figure 3.3 shows the chromatogram of the major aflatoxins of the dry extract. Table 3.6 shows that aflatoxins standards AFG2, AFG1, AFB2 and AFB1 were eluted at 3.70, 4.20, 4.80 and 5.08 min, respectively. On the other hand, peaks # 3, 4, 5 and 6 of the dry extract were, respectively, eluted at 2.80, 3.20, 4.80 and 5.08 min. The experimental findings suggest that the peaks # 5 and 6 of the dry extract could be characterize as the aflatoxins AFB2 and AFB1. Peaks # 3 and 4 could be tentatively characterized as the aflatoxins AFG2 and AFG1, respectively; these two fractions were also spectrophotrometically characterized.

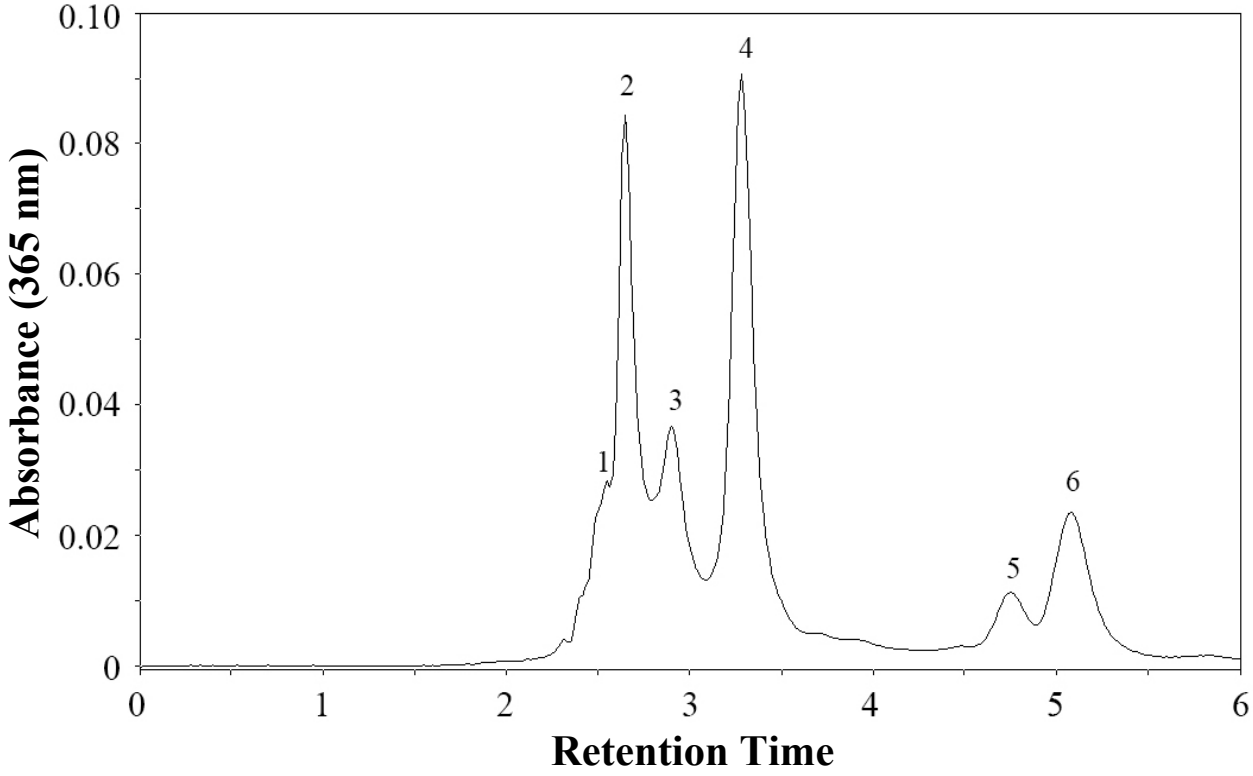

Figure 3.3. HPLC chromatogram of the aflatoxins from *Aspergillus parasiticus* at 365 nm, using an allsphere column C18 thermostated at 50°C, with a mix of methanol/water (60/40; v/v) as mobile phase, and a flow rate of 1 mL/min.

Table 3.6. Comparison of the retention times and peak areas between the aflatoxins from *Aspergillus parasiticus* and the aflatoxin standards AFB1, AFB2, AFG1 and AFG2 at 365 nm.

Peak	RT (min)[a]	Peak area[b]
Product peak # 1[c]	2.30 (0.00)[d]	338954 (2.71)[b]
Product peak # 2	2.50 (0.00)	142761 (6.30)
Product peak # 3	2.80 (0.00)	266624 (3.66)
Product peak # 4	3.20 (0.00)	859064 (5.40)
Product peak # 5	4.80 (0.00)	101287 (7.49)
Product peak # 6	5.08 (0.00)	401019 (9.93)
Standard AFB1[e]	5.08 (0.18)	6614017 (7.50)
Standard AFB2	4.80 (0.19)	814200 (19.35)
Standard AFG1	4.20 (2.38)	1355070 (4.80)
Standard AFG2	3.70 (2.56)	1391858 (8.65)

[a]Mean of retention time in minute, calculated from the values of triplicate samples. Results obtained from reversed-phase high performance liquid chromatography (RP-HPLC) using an allsphere column C18 thermostated at 50°C. The mobile phase used was a mix of methanol/water (60/40; v/v), using a flow rate of 1 mL/min.

[b]Mean of peak area, calculated from the values of triplicate HPLC analysis.

[c]Injection of 20 µL of a stock solution of 100 mg/mL of the product issued from fraction IIb obtained by ultrafiltration (1 *k*Da) of the crude exo-culture media of *A. parasiticus.*

[d]Relative standard deviation (RSD) was calculated as the standard deviation of triplicate samples divided by their mean multiplied by 100.

[e]Injection of 20 µL of each standards obtained from Sigma (Sigma-Aldrich, Co).

The experimental results are in agreement with those of Joshua (1993), where the aflatoxins were eluted at 8.08, 9.84, 10.74 and 13.24 min for AFG2, AFG1, AFB2 and AFB1, respectively, with a UV detection at 360 nm. In addition, Chiavaro *et al*. (2001), also reported that aflatoxins from food and feed were also eluted in the same order, with 2.8, 3.4, 5.0 and 6.9 min, for AFG2, AFG1, AFB2 and AFB1, respectively, with a UV detection at 365 nm.

3.7. Conclusion

The experimental data obtained throughout this study showed that among the fungal strains investigated, *Aspergillus parasiticus* on PDA was the most appropriate one for the production of high aflatoxins yield. In addition, the high recovery of good matrix aflatoxins dry extract was obtained by the use of 1.5% (w/v) of the lyoprotectant mannitol.

CHAPTER IV

ARTICLE III

KINETIC DEGRADATION OF SELECTED AFLATOXINS BY THE LACCASE FROM *CORIOLUS HIRSUTUS*

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4.1. Contribution of the Authors

The present author, Sabrina Borgomano, was responsible for the concepts, the designs and the fulfillment of the experimental work and the preparation of this manuscript for its submission.

Dr. Monique Lacroix, the thesis supervisor, and Dr. Selim Kermasha, the thesis supervisor, supervised the research work, provided valuable input and advices, monitored the progress of the work and critically reviewed and edited this manuscript, prior to its submission.

4.2. Résumé

La biocatalyse de la laccase partiellement purifiée (LPP) de *Coriolus hirsutus* a été optimisée en termes de température et de pH, en utilisant comme substrat les principales formes des aflatoxines, AFB1, AFB2, AFG1 et AFG2. La cinétique de la dégradation enzymatique par la laccase microbienne des substrats sélectionnés a également été étudiée. L'exo-laccase de *C. hirsutus* a été soumise à une purification partielle par précipitation au sulfate d'ammonium à 60-80% de saturation. L'extrait enzymatique de la laccase partiellement purifiée présentait la plus forte activité spécifique de 0,133, 0,124, 0,155 et 0,138 umol de produit par mg de protéine par minute, pour les aflatoxines AFB1, AFB2, AFG1 et AFG2, respectivement, à une température de 57,5ºC et un pH de 6,0. En outre, les études cinétiques ont montré une valeur K_m de 0,646, 0,128, 0,237 et 0,284 uM et une valeur V_{max} de 10,40, 9,68, 13,00 et 10,80 pour les aflatoxines AFB1, AFB2, AFG1 et AFG2, respectivement. Les effets de métaux lourds ainsi que ceux de sels et d'agents chimiques sélectionnés sur l'activité de la laccase ont également été étudiés. La présence de 0,4 mM de dithiothréitol et d'acide diéthyldithiocarbamique inhibait fortement l'activité de laccase, alors que 10 mM d'acide kojik et 0,01 mM d'acide *p*-coumarique favorisaient grandement son activité.

4.3. Abstract

The biocatalysis of partial purified laccase (PPL) from *Coriolus hirsutus* was optimised in terms of temperature and pH, using major aflatoxin isoforms, AFB1, AFB2, AFG1 and AFG2, as substrates. The kinetic of enzymatic degradation by this microbial laccase of the selected substrates was also investigated. The exo-crude laccase from *C. hirsutus* was subjected to a partial purification by ammonium sulfate precipitation 60-80% of saturation. The partial purified enzymatic extract laccase exhibited the highest specific activity of 0.133, 0.124, 0.155 and 0.138 µmol of product per mg protein per min, for AFB1, AFB2, AFG1 and AFG2, respectively, at 57.5ºC and pH 6.0. In addition, the kinetic studies showed a K_m value of 0.646, 0.128, 0.237 and 0.284 µM and a V_{max} value of 10.40, 9.68, 13.00 and 10.80 for AFB1, AFB2, AFG1 and AFG2, respectively. The effects of heavy metals as well as selected salts and chemical agents on the laccase activity were also investigated. The presence of 0.4 mM of dithiothreitol and diethyldithiocarbamic acid strongly inhibited the laccase activity, whereas 10 mM kojik and 0.01 mM *p*-coumaric acid greatly promoted its activity.

Keywords: Aflatoxins, partial purified laccase, degradation, kinetic parameters, activators, inhibitors

4.4. Introduction

Aflatoxins are an assembly of a coumarin and 3 furans (difurano-coumarin derivatives), where AFB1, AFB2, AFG1 and AFG2 are the most common and potent toxins in food products (Medina and Magan, 2012). Aflatoxins, mainly produced by *Aspergillus flavus* and *Aspergillus Parasiticus* (Bhat *et al.*, 2010), have been widely study because of their toxic, mutagenic, teratogenic and carcinogenic properties for humans and animals health. A wide range of strategies, including physical, chemical and biological, are employed to detoxify the contaminated products with aflatoxins. Although, the use of chemical and physical strategies to degrade the aflatoxins is effective to a certain degree on foods, their degradations still pose many problems concerning security issues. They cause loss in the nutritional quality, they are responsible for the formation of dangerous toxic unwanted compounds and their effectiveness is still limited because of the high cost (Kabak *et al*., 2006). Biological treatments, with the use of enzymes, have been proposed, which is more effective, fast and less expensive that chemical and physical degradations (Alberts *et al.*, 2009).

Laccases (EC 1.10.3.2) are polyphenol oxidases that belong to the family of blue multi-copper proteins, which contain copper atoms at their catalytic center (Baldrian, 2006). As oxidoreductive enzymes, laccases can oxidize a substrate with a reduction of the oxygen molecule to water; laccases can act on a very broad variety of substrates, including mono-, di- and polyphenols, aminophenols and methoxyphenols (Xiao *et al*., 2004). Laccases are widely distributed in eukaryotes, such as fungi, higher plants, and insects as well as in prokaryotes, such as bacteria. Among fungal species, laccases can be found in ascomycetes, deuteromycetes and in most white-rot basidiomycetes, where the white-rot fungi are well known as one of the most abundant producers of laccases (Baldrian, 2006). Laccases are also involved in the pathogenesis, immunity, in the morphogenesis of bodies and the renewal of the metabolism of complex organic substances such as lignin and humic substances.

Due to their stability, low substrate specificity, higher redox potential and their ability to use environmental oxygen as co-factor as well as the ease of their use, fungal laccases have the potential to be used in many environmental and biotechnological applications (Myasoedova *et al*., 2008). In addition, the low costs of laccases production as well as their stability at high temperatures and different pH conditions are parameters which made their use very attractive for

biotechnological applications (Patrick *et al*., 2009). There is an increased interest in laccases for the degradation of toxin, in pulp delignification, bioremediation, biosensors, detergent manufacturing, wine clarification and fruit juice stabilization as well as in degradation of polyphenolic xenobiotics (Octavio *et al*., 2006).

The aim of this work was to study the potential degradation of aflatoxins by the partially purified laccase, obtained from *Coriolus hirsutus* (basidiomycetes). The specific objectives were to determine the optimal conditions for the laccase activity as well as its kinetic characteristics, including K_m, V_{max} and K_{cat}. The effects of selected chemicals and metal ions on the laccase activity were also investigated.

4.5. Materials and Methods

4.5.1. Organism and Culture Conditions of Coriolus hirsutus and Aspergillus Parasiticus

Coriolus hirsutus (MYA-828) was obtained from the American Type Culture Collection (ATCC, Manassas, VA.). *C. hirsutus* was maintained onto malt agar media plates and incubated at 20ºC. For laccase production a basal liquid medium was prepared, using the method previously described by Taqi (2012).

Aspergillus parasiticus (26864) was obtained from the American Type Culture Collection (ATCC; Manassas, VA). *A. parasiticus* was maintained through periodic transfer onto potatoes dextrose agar media plates and incubated at 24ºC; for aflatoxins production, a basal liquid medium was prepared using the method previously described by Hsieh and Mateles (1971).

4.5.2. Preparation of Laccase Extract

The fermentation process was carried out according to Taqi (2012). The mycelium pellets from culture liquid medium were removed throughout the use of a cheese-cloth. The culture medium was ultra-filtered using Prep/Scale TFF Cartridge (2.5 ft^2) with polyethersulfone low protein-binding membranes (10 *k*Da cut-off filter) and a pressure of 10 psi (MILLIPORE, Mississauga, ON, CA). According to a method of Taqi (2012), the recovered concentrate from ultra-filtration was partially purified by using ammonium sulphate precipitation. Solid ammonium sulfate was added to give 60% saturation and the precipitated proteins were removed by centrifugation at 10,000 xg for 30 min. The supernatant was adjusted to 80% saturation with solid ammonium

sulfate. The precipitated proteins were collected by centrifugation at 10,000 xg for 45 min and redissolved in a minimal volume of 0.01 M sodium acetate buffer, pH 5.0 and subjected to a dialysis. The resulting dialyzed enzymatic fractions were lyophilized using a freeze dryer (Labconco Corporation, Kansas City, MO). The lyophilized enzymatic fraction was stored at -80ºC and subjected for its enzymatic characterizations.

4.5.3. Laccase Assay

Laccase activity was assayed spectrophotometrically, using 4 mM syringaldazine (SYG) in ethanol (ε_{525}=65000 $M^{-1}cm^{-1}$). The reaction mixture contained 15 µL substrate and 200 µL enzyme dilutions. The reaction medium was adjusted with sodium acetate buffer (0.1 M, pH 5.0) to a total volume of 700 µL. The enzymatic reactions were carried out at room temperature (25°C) and one unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmol of product of syringaldazine per min.

4.5.4. Protein Determination

The protein content of the samples was determined according to a modification of the Lowry method (Hartree, 1972) using bovine serum albumin as a standard for the calibration curve.

4.5.5. Aflatoxins Preparation

The fermentation process of *A. parasiticus* was carried out according to a modification of the method of Hsieh and Mateles (1971) developed in our laboratory and previously described in chapter III. The mycelium pellets from culture liquid medium were removed throughout a cheese cloth filtration. The extracellular toxins were recovered from the culture medium by ultra-filtration, using Prep/Scale TFF Cartridge (2.5 ft^2) of polyethylene polypropylene low protein-binding membranes (1 *k*Da cut-off filter) under a pressure of 10 psi (Millipore, Milford, MA). The resulted ultrafiltred fraction, containing the aflatoxins, was lyophilized using a freeze dryer (Labconco Co.). The lyophilized aflatoxins fraction was stored at -80ºC and subjected for further investigation.

4.5.6. Degradation Activity Using Aflatoxins as Substrates

Laccase activity was assayed spectrophotometrically at 365 nm, using a Beckman DU-650 spectrophotometer (Beckman Instruments Inc.; San Ramon, CA). Stock solution of substrate, 0.1 g/mL in 40% methanol and enzyme suspension of PPL, 0.04 g/mL (0.32 mg protein/mL), were prepared. The reaction mixture contained 150 µL substrate and 200 µL diluted enzyme. The reaction medium was adjusted with sodium acetate buffer (0.1 M, pH 5.0) to a total volume of 700 µL. The reaction was initiated by the enzyme addition. The enzymatic reaction was carried out at room temperature (25ºC) and one unit of enzyme activity was defined as the amount of enzyme producing 1 µmol of product per min.

4.5.7. Determination of the Optimum Temperature

The effect of the reaction temperature on laccase activity was assayed as described previously, using a wide range of temperatures, from 25 to 65°C and aflatoxins as substrates.

4.5.8. Determination of the Optimum pH

The effect of pH on laccase activity was assayed as described previously, using a wide range of pH values, from 4.0 to 8.0, using citrate phosphate buffer (0.1 M, pH 4.0 to 6.0) and sodium phosphate buffer (0.1 M, pH 6.0 to 8.0) and aflatoxins as substrates.

4.5.9. Effect of Protein Load on Laccase Activity

The effect of enzymatic protein content on laccase activity in citrate phosphate buffer (0.1 M, pH 6.0) was determined as described previously, by varying the amount of protein from 0 to 40 µg/mL. The enzymatic reactions were carried out at 57.5ºC, and one unit of the specific activity of laccase was defined as the amount of enzyme producing 1 µmol of product per min.

4.5.10. Kinetic Parameters of Partially Purified Laccase, Using Aflatoxins as Substrates

The effect of substrate concentration on the activity of laccase was investigated, using a 0 to 128.2 nM for AFB1, 0 to 254.8 nM for AFB2, 0 to 43.6 nM for AFG1 and 0 to 56.3 nM for AFG2. The enzymatic assay was carried out using the optimal reaction temperature and pH. K_m and V_{max} values were calculated from the Lineweaver-Burk plot, whereas the catalytic efficiency (K_{cat}) was determined as V_{max}/K_m.

4.5.11. Effect of Selected Chemicals on Laccase Activity

The effects of selected chemical compounds, including citric acid, N-hydroxyphthalimide (HPI), imidazole, *p*-coumaric acid, L-cysteine, dithiothreitol (DTT), diethyldithiocarbamic acid (DDC), kojic acid and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) as well as selected heavy metals, including copper sulfate ($CuSO_4$) and copper chloride ($CuCl_2$), were investigated on laccase activity, using a wide range from 0 to 26 mM.

4.6. Results and Discussion

4.6.1. The Catalyse of Aflatoxins by Laccase

The formation of the products obtain by laccase activity, using major aflatoxin isoforms as substrates, has been investigated. Figure 4.1 shows that there was an increase in the product formation with that of the time reaction up to 90 min. The experimental results are in agreement with those of Liu *et al*. (1998) and Wang *et al*. (2011), who proposed that during enzymatic reaction, AFB1 was firstly oxidized in the 8,9-vinyl bond position to generate AFB1-8,9-epoxide and later hydrolyzed in AFB1-8,9-dihydrodiol using a multienzyme from *Armillaria tabescens* and a manganese peroxidase from *Phanerochaete sordida* YK-624, respectively. The overall findings suggest that the aflatoxins AFB1, AFB2, AFG1 and AFG2 could be oxidized by laccase.

4.6.2. Effect of the Temperature on Laccase Degradation Activity

The effect of the reaction temperature on laccase activity was investigated. Figure 4.2 shows that the highest specific activity of laccase was obtained at 57.5ºC, when the aflatoxins AFB1, AFB2, AFG1 and AFG2 were used as substrates. The results also indicated that further increases or decreases in temperature resulted by a gradual inhibition in laccase activity. Baldrian (2006) reported that most fungal laccases have an optimum reaction temperature ranging from 35 to 70ºC. Koroleva *et al*. (2001) indicated that the enzymatic activity of laccase from *C. hirsutus* and *Coriolus zonatus* increases when the temperature rises up to 55°C, after which thermo-inactivation occurs. The sudden decrease of activity above 55°C can hardly be explained by protein unfolding, but it could be caused by conformational changes in both type 1 and type 2

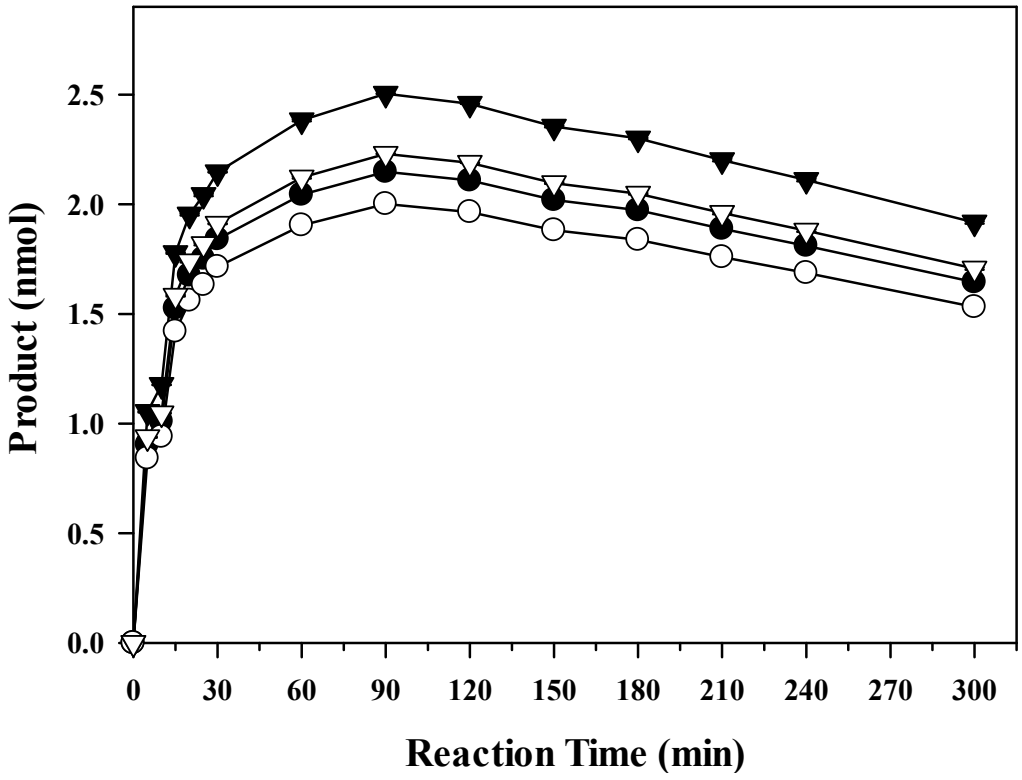

Figure 4.1. Formation of the products during the catalysis of the partially purified laccase (PPL), from *Coriolus hirsutus*, using the aflatoxins, (●) AFB1, (○) AFB2, (▼) AFG1 and (▽) AFG2 from *Aspergillus parasiticus* as substrates. Laccase activity was measured under standard assay conditions (25°C) using sodium acetate buffer (0.1M, pH 5.0). The error bars in the figure indicate the standard deviation.

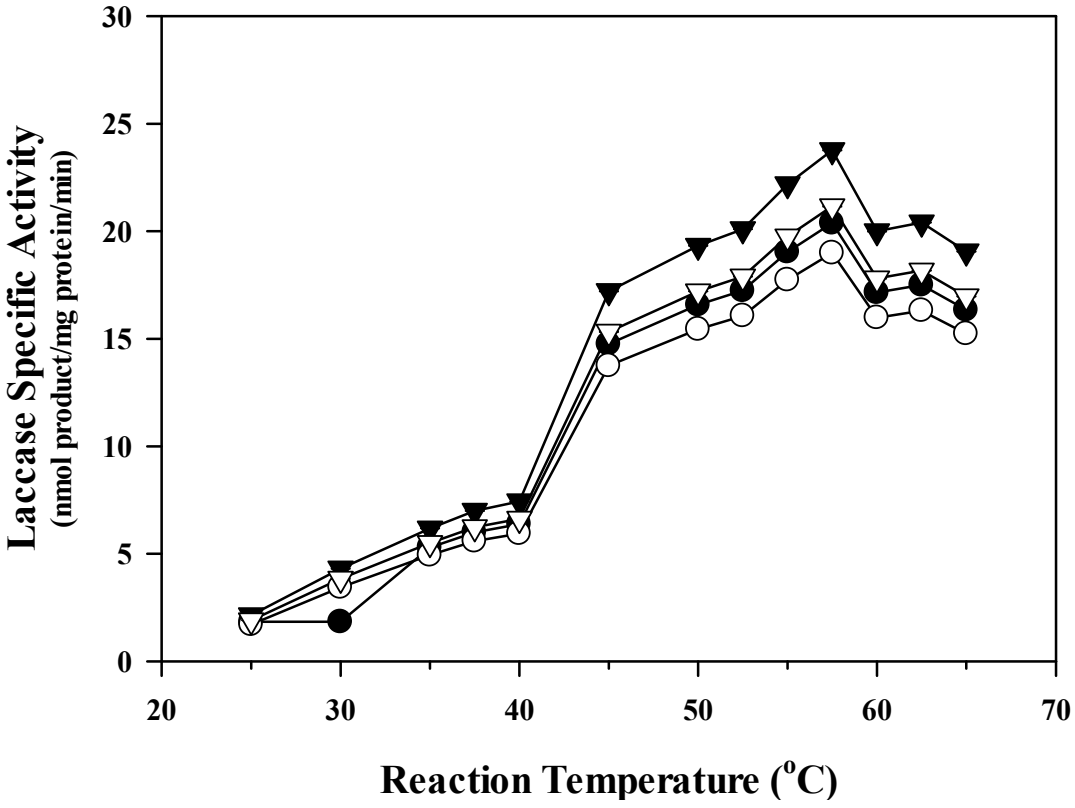

Figure 4.2. Effect of reaction temperature on the partial purified laccase (PPL) activity from *Coriolus hirsustus*, where the specific activity (nmol product/mg protein/min) for aflatoxin AFB1 was (●), AFB2 was (○), AFG1 was (▼) and AFG2 was (▽). Laccase activity was measured under standard assay conditions using sodium acetate buffer (0.1M, pH 5.0) during 30 min. The error bars in the figure indicate the standard deviation.

copper centers. Higher temperature might lead to a release of the type 2 copper ion, which is completely absent at 70°C. At temperatures higher than 70°C, the type 1 and type 3 copper sites are completely disintegrated and the protein unfolding could occur. The experimental findings are similar to those of Chefetz *et al*. (1998) who indicated that the optimum reaction temperature for the purified laccase from *Chaetomium thermophilium* was ranged between 50 to 60ºC, when SYR was used as a substrate.

4.6.3. Effect of the pH on Laccase Degradation Activity

The effect of pH, from 4.0 to 8.0, on laccase activity using AFB1, AFB2, AFG1 and AFG2 as substrates, was investigated. Figure 4.3 indicates that the optimum pH for the laccase catalysed reaction was 6.0; however there was a drastic decrease above this pH, with a strong enzyme inhibition at pH 8.0. Xu (1997) reported that the laccase activity has a bell-shaped pH profile with an optimum pH that varies considerably. Balbrian (2006) and Xu (1997) reported that the low laccase activity at neutral and alkaline pH values could be attributed to the inhibition obtained by the bonding of the hydroxide anion to the T2/T3 copper of the active site of the enzyme. On the other hand, at higher pH the redox potential of the phenolic substrate decreases, which makes the substrate more susceptible to the oxidation by laccase. The experimental findings are in agreement with those obtained by Hublik and Schinner (2000), who reported that the highest activity of laccase from *Pleurotus ostreatus* was obtained at pH 5.8, using SYR as substrate. Ullrich *et al*. (2005) also reported that the laccase from the medicinal mushroom, *Agaricus blazei*, had a maximum activity for the oxidation of 2,6-dimetoxyphenol (DMP) and SYR at pH 5.5. In addition, Liu *et al*. (2001) indicated that the highest enzyme activity of *Armillariella tabascens* was obtained at pH 6.5, when aflatoxin AFB1 was used as substrate. Zhao *et al*. (2010) also reported that an enzyme from *Myxococcus fulvus* ANSM068 (MADE) had a maximum activity at pH 6.0, using aflatoxins AFB1, AFG1 and AFM1 as substrates. On the other hand, Linke *et al*. (2005) reported that the highest laccase activity of the extracellular crude laccase from *Pleurotus sapidus* was obtained at pH 3.5, using ABTS as substrate. In general fungal laccases are active at low pH values and at higher ones (Baldrian, 2006). Moreover, their pH optima are substrate dependent (Shin and Lee, 2000).

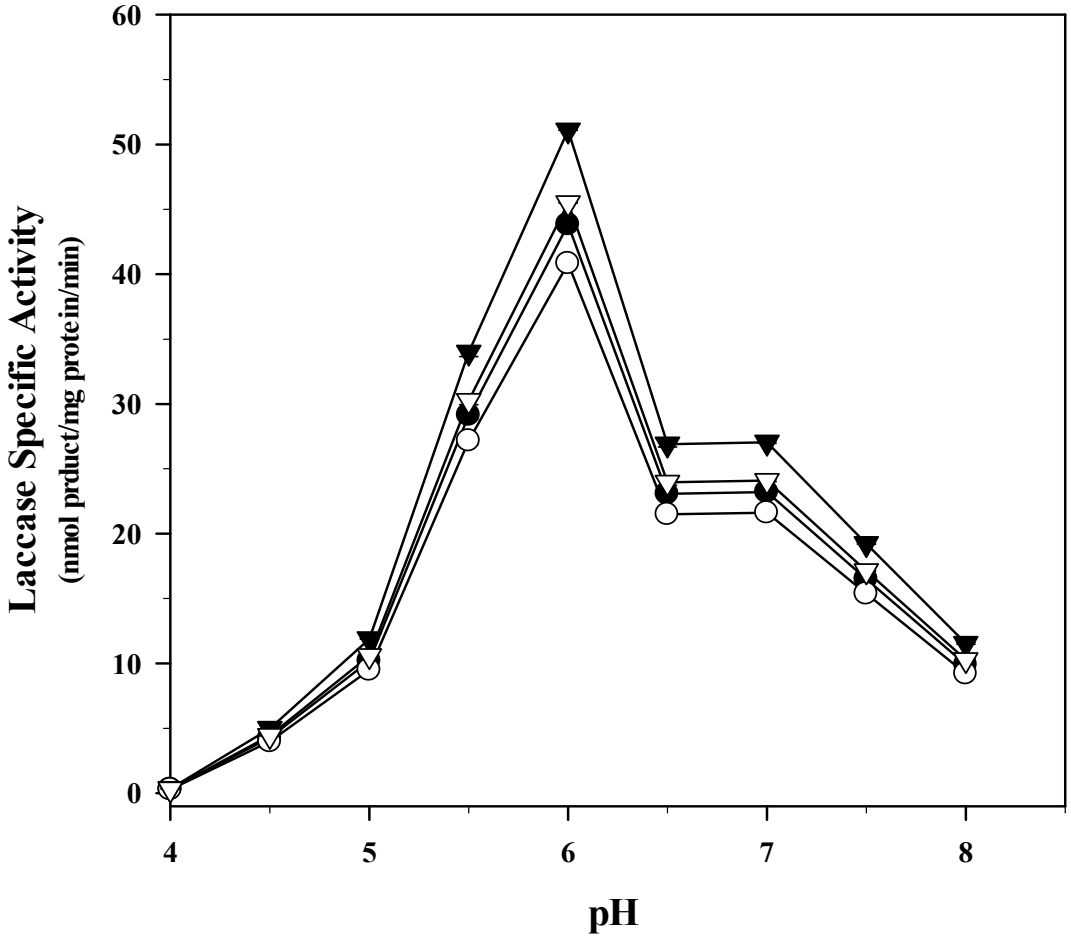

Figure 4.3. Effect of pH on the partially purified laccase (PPL) activity from *Coriolus hirsutus*, using the aflatoxins, (●) AFB1, (○) AFB2, (▼) AFG1 and (▽) AFG2 from *Aspergillus parasiticus* as substrates. Laccase activity was measured under optimized conditions (57.5°C during 30 min) using citrate phosphate buffer (0.1 M, pH 4.0 – 6.0) and sodium phosphate buffer (0.1 M, pH 6.0 – 8.0). The error bars in the figure indicate the standard deviation.

4.6.4. Effect of Enzymatic Protein Load on Laccase Activity

Figure 4.4 shows that the laccase activity increased with that in protein load, up to 127, 118, 148 and 132 nmol product per mg protein per min with 16 µg protein/mL reaction for AFB1, AFB2, AFG1 and AFG2, respectively. However, further increase in the protein load, up to 40 µg protein/mL reaction resulted by 59.9% decrease in the enzymatic activity. Hong-Mei and Nicell (2008) reported that an optimum enzyme concentration should be considered to maximize the rate of oxidation of a specific substrate.

4.6.5. Kinetic Parameters of Partially Purified Laccase, Using Aflatoxins as Substrates

The kinetic parameters of laccase degradation activity, including specific activity, total activity, K_m, V_{max} and K_{cat}, using aflatoxins, AFB1, AFB2, AFG1 and AFG2, as substrates were investigated. Table 4.1 indicates that the specific activity for PPL using the aflatoxins, AFB1, AFB2, AFG1 and AFG2, as a substrates were 0.133, 0.124, 0.155 and 0.138 µmol of product per mg protein per min, respectively, with a pH of 6.0 and at a temperature of 57.5°C. The total activity was calculated as 2,527, 2,356, 2,945 and 2,622 µmol per min for the AFB1, AFB2, AFG1 and AFG2, respectively.

The K_m value for laccase was 0.646, 0.128, 0.237 and 0.284 µM for AFB1, AFB2, AFG1 and AFG2, respectively. The PPL showed a substrate affinity in the following order, AFB2, AFG1, AFG2 and AFB1. These results suggest a same behavior of the enzyme for these substrates, which may be correlated to their structural homology (Zouari-Mechichi *et al*., 2006). The V_{max} value for laccase was 10.40, 9.68, 13.00 and 10.80 µM per mg protein per min for AFB1, AFB2, AFG1 and AFG2, respectively. In addition, the highest catalytic efficiency (K_{cat}) which translates the number of aflatoxin molecules bonded on the catalytic site of the enzyme per minute, calculated as V_{max}/K_m, was obtained with AFB2, 75 molecules/min, following by AFG1, 55 molecules/min, AFG2, 38 molecules/min and AFB1, 16 molecules/min. The overall findings suggest that the laccase reacts at a high rate with the molecules of aflatoxins AFB1, AFB2, AFG1 and AFG2. The possible molecular changes that follow the treatment of aflatoxins by PPL under described conditions are not known, but from the literature reports, it is known that conversion of aflatoxins in epoxide and aflatoxicol is regarded to be the detoxification steps. In addition, the toxicity of these metabolites are lower than those of the AFB1, AFB2, AFG1 and AFG2, themselves (Bintvihok and Kositcharoenkul 2006; Wang *et al*., 2011).

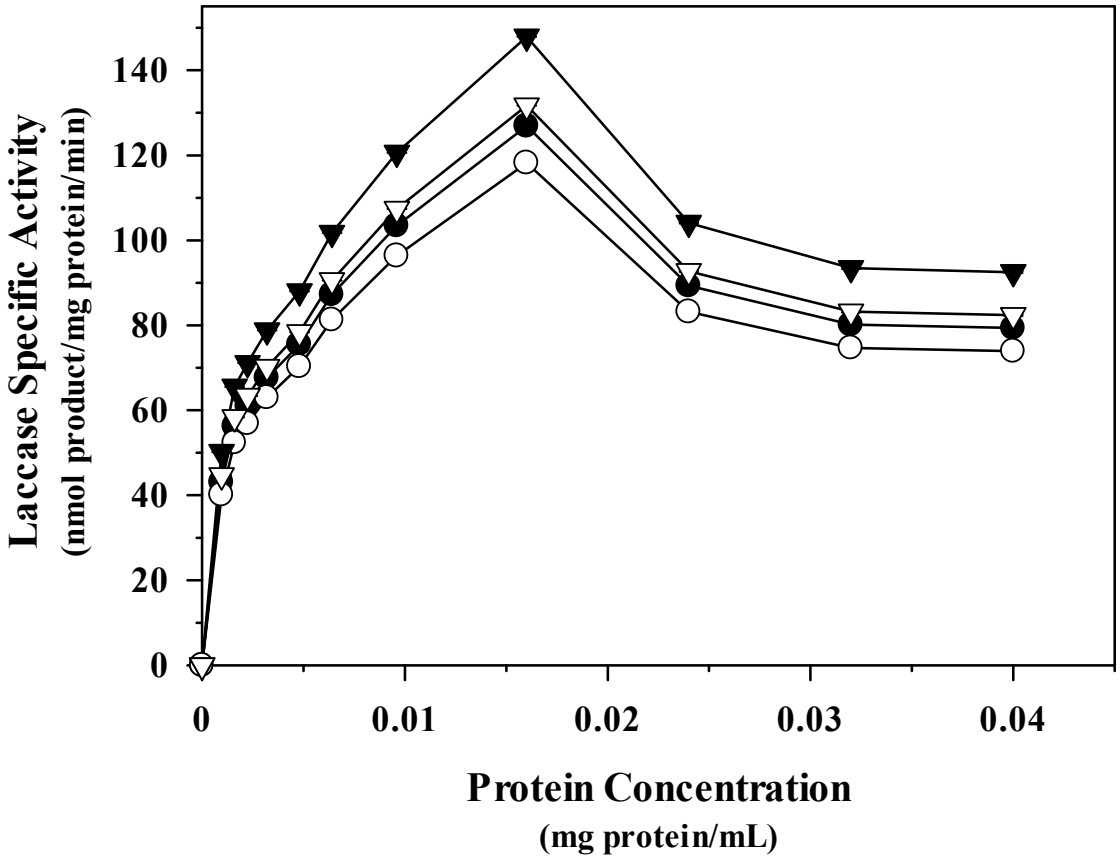

Figure 4.4. Effect of protein load on the laccase (PPL) activity from *Coriolus hirsutus*, using the aflatoxins, (●) AFB1, (○) AFB2, (▼) AFG1 and (▽) AFG2 from *Aspergillus parasiticus* as substrates. Laccase activity was measured under optimized conditions (57.5°C during 30 min) using citrate phosphate buffer (0.1 M, pH 6.0) The error bars in the figure indicate the standard deviation.

Table 4.1. Kinetic parameters of the partial purified laccase using aflatoxins AFB1, AFB2, AFG1 and AFG2 from *Aspergillus parasiticus*.

Substrate	Specific activity[a]	Total activity[b]	K_m[c]	V_{max}[d]	K_{cat}[e]
AFB1	0.133	2,527	0.646	10.40	16.1
AFB2	0.124	2,356	0.128	9.68	75.6
AFG1	0.155	2,945	0.237	13.00	54.9
AFG2	0.138	2,622	0.284	10.80	38.0

[a]The specific activity of laccase was defined as μmol of produced product per mg protein per min (0.32 mg protein/mL).

[b]Total activity was defined by multiplying the total protein by the specific activity.

[c]Michaelis-Menten constant (K_m) was defined as μM of substrate.

[d]Maximum reaction velocity (V_{max}) was defined as uM of products formed per mg protein per min.

[e]The catalytic efficiency was defined as the ratio of V_{max} to $K_{m..}$

Laccases are considered to be non-specific to their substrates, being able to oxidize a wide range of aromatic compounds (Madhavi and Lele, 2009), such as phenols and leads to formation of dehydrogenation products (Alcalde, 2007). Aflatoxins (chemically furocoumarins) a type of aromatic compounds, can also be used as a suitable substrates by laccase and oxidized to some less toxic residues.

4.6.6. Effects of Selected Chemicals on Laccase Activity

The Effects of chemical agents, including citric acid, L-cysteine, imidazole, *N*-hydroxyphtalimide (HPI), dithiothreitol (DTT), diethyldithiocarbamic acid (DDC), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), kojic acid, and *p*-coumaric acid as well as metal ions, including copper chloride and copper sulfate, on laccase activity were investigated (Figs. 4.5 and 4.6).

4.6.6.1. Effects of Activators

Figure 4.5A shows that 5 mM of HPI, a synthetic mediator of the type -NOH^-, enhanced laccase activity by 88.2%, when the aflatoxins were used as substrates. The experimental findings are in agreement with those of Fabrini *et al*. (2002), where 6.0 mM of HPI strongly activated laccase (76%), when 4-methoxybenzyl alcohol was used as substrate. Sealey *et al*. (1998) reported that HPI was a mediator for laccase with improved delignification properties for chemical pulps. The experimental findings suggest that HPI is one of the most efficient mediators for the oxidation of non-phenolic substrates, where *N*-oxyl radical generated from the *N*-hydroxy moiety by laccase could be an active specie in the oxidation cycle (Baiocco *et al.,* 2002)

Figure 4.5B indicates that ABTS, enhanced the enzyme activity from 0 to 80.5% at all the selected concentrations from 0 to 0.3 mM, respectively, using the aflatoxins as substrates. The experimental results are in agreement with those of Hou *et al*. (2004), who showed that using 0.16% of ABTS allowed 90% of the decolorization of anthraquinone dye. In addition, Fabbrini *et al*. (2002) reported that 6 mM of ABTS increased laccase activity by 22% using 4-methoxybenzyl alcohol as substrate. In addition, Bourbonnais and Paice (1992) reported that ABTS improved pulp delignification using laccase as enzyme. The experimental findings suggest that ABTS is as an efficient mediator of laccase activity towards non-phenolic substrates (Xu *et al*, 2000).

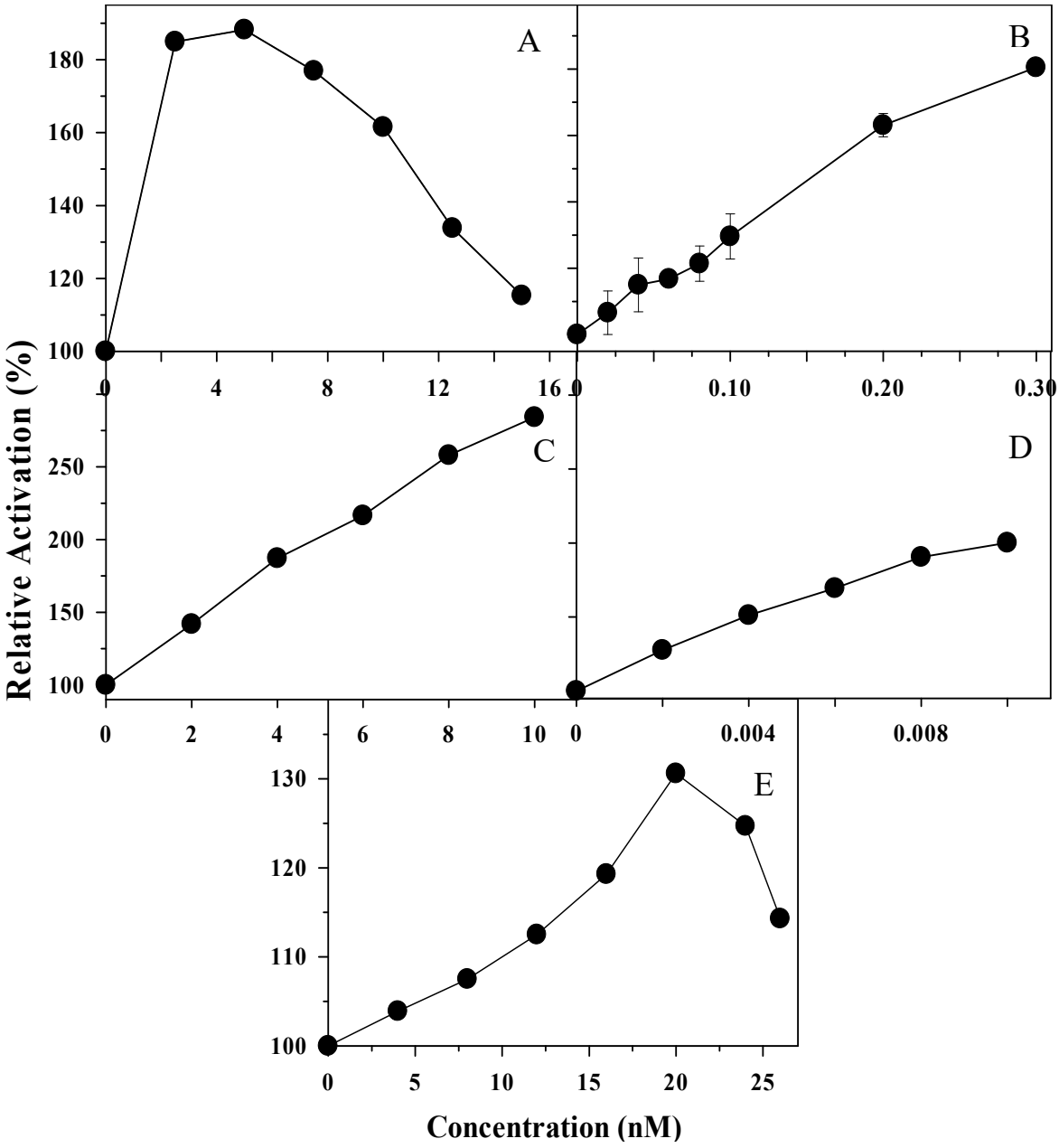

Figure 4.5. Effect of chemical agents, (A) N-hydroxyphtalimide, (B) 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), (C) kojic acid, (D) *p*-coumaric acid and (E) imidazole on the partially purified laccase (PPL) activity, using aflatoxins AFB1, AFB2, AFG1 and AFG2 from *Aspergillus parasiticus* as substrates. Laccase activity was measured using citrate phosphate buffer (0.1 M, pH 6.0) at 57.5°C, during 30 min. The error bars in the figure indicate the standard deviation.

The overall results suggest that the combination of the laccase with low molecular weight molecules such as ABTS or synthetic mediators of the type -NOH^{-} such as HPI, not only lead to higher rates and yields in the transformation of laccase substrates but also add new oxidative reactions to the laccase repertory towards substrates in which the enzyme alone had no or only marginal activity (Kunamneni *et al*., 2008).

Figure 4.5C shows that kojik acid increased laccase activity at all the selected concentrations, using aflatoxins as substrates. The experimental findings differ from those of Saito *et al*. (2003) in which laccase activity was strongly inhibited by 96% in presence of 1 mM of kojic acid using syringaldazine as substrate. In addition, Nagai *et al*. (2002) also reported that 0.1 and 1 mM of kojic acid caused an inhibition of 45.6 and 70% of laccase activity, respectively, when ABTS was used as substrate.

Figure 4.5D demonstrates that *p*-coumaric acid improved laccase activity from 27.7 to 100.1% at very low concentrations from 0.002 to 0.01 mM, respectively, when the aflatoxins were used as substrates. Camarero *et al*. (2005) reported that 50 μM of *p*-coumaric acid allowed up to 20% decolorization of the recalcitrant dye Azure B by laccase. On the other hand, the experimental findings are in disagreement with those of Saito *et al*. (2003) and Nagai *et al*. (2002) in which 1 mM of *p*-coumaric acid strongly inhibited by 98 and 91.5% laccase activity, using SYR and ABTS, respectively, as substates. The experimental findings suggest that phenolic compounds derived from lignin degradation, such as *p*-coumaric acid, are highly-efficient laccase mediators of natural origin (Kunamneni *et al.*, 2008).

Figure 4.5E demonstrates that imidazole enhanced the enzyme activity from 0 to 30.6% at all the selected concentrations from 0 to 20 mM, respectively, before its decrease, using aflatoxins as substrates. The experimental findings are in agreement with those of Taqi *et al*. (2012), in which 0.8 mM of imidazole activated by 30% laccase activity when DMP was used as substrate. In addition, Rosconi *et al*. (2005) showed that 20 mM of imidazole strongly activated laccase activity by 83%, using SYR as substrate.

4.6.6.2. Effects of Inhibitors

Figure 4.6A demonstrates that DTT strongly inhibited laccase activity at all the selected concentrations from 0 to 1 mM, where the inhibition increased with that of DTT concentration. Laccase activity was inhibited by 78.2% in presence of 1 mM of DTT when aflatoxins were used as substrates. Lu *et al*. (2007) reported that 1.0 mM of DTT strongly inhibited laccase activity by 90%, when ABTS was used as substrate. Saito *et al*. (2003) and Nagai *et al*. (2002) showed that 0.1 and 1 mM of DTT led to a complete inhibition of laccase activity using SYR and ABTS, respectively, as substrates. The sensitivity of PPL for dithiothreitol was very similar to that seen with the laccase from *C. hirsutus* of Shin and Lee (2000), which results in a strong inhibition of the activity. The experimental findings suggest that the inhibition by this compound, which is a metal ions chelator, may be due to the presence of copper in the laccase catalytic center.

The effect of metal ions such as $CuSO_4$ and $CuCl_2$, was also investigated on laccase activity. Figures 4.6B and 4.6D show that copper chloride and copper sulfate inhibited laccase activity at all the selected concentrations, when the aflatoxins were used as substrates. Laccase activity was inhibited by 91.3 and 70% in presence of 10 mM of $CuCl_2$ and 12 Mm of $CuSO_4$, respectively. The experimental findings are agree with those of Lorenzo *et al*. (2005), who reported an inhibition of 40% of laccase activity in presence of 20 mM of Cu^{2+} and a complete inhibition in presence of 80 mM of Cu^{2+}, when SYR was used as substrate. Abadulla *et al.* (2000) and Galhaup *et al*. (2002) indicated that the halides F^- and Cl^- were the strongest halides inhibitors for laccases, and the degree of inhibition of laccases by halides seemed to be linked to the availability of copper atoms (type 2 and type 3) in the active site. Farnet *et al*. (2008) demonstrated that chloride ions act as competitive inhibitors with the electron donor in laccase. On the other hand, the experimental results differ from those of Lu *et al*. (2013), who showed an increase of laccase activity by 45.7% in presence of 10 mM Cu^{2+}. In addition, Murugesan *et al*. (2009) reported that 10 mM of $CuSO_4$ enhanced laccase activity by 72%, using ABTS as substrate. Guo *et al*. (2011) also reported that copper increases laccase activity at low concentrations, however, excess concentrations inhibit laccase activity. The overall findings suggest that although the laccase is a copper-containing protein (Guo *et al*., 2011), the inhibition of laccase activity by an excess of copper can be due to change in the enzyme structure, leading to a loss of its activity (Nitheranont *et al*., 2011).

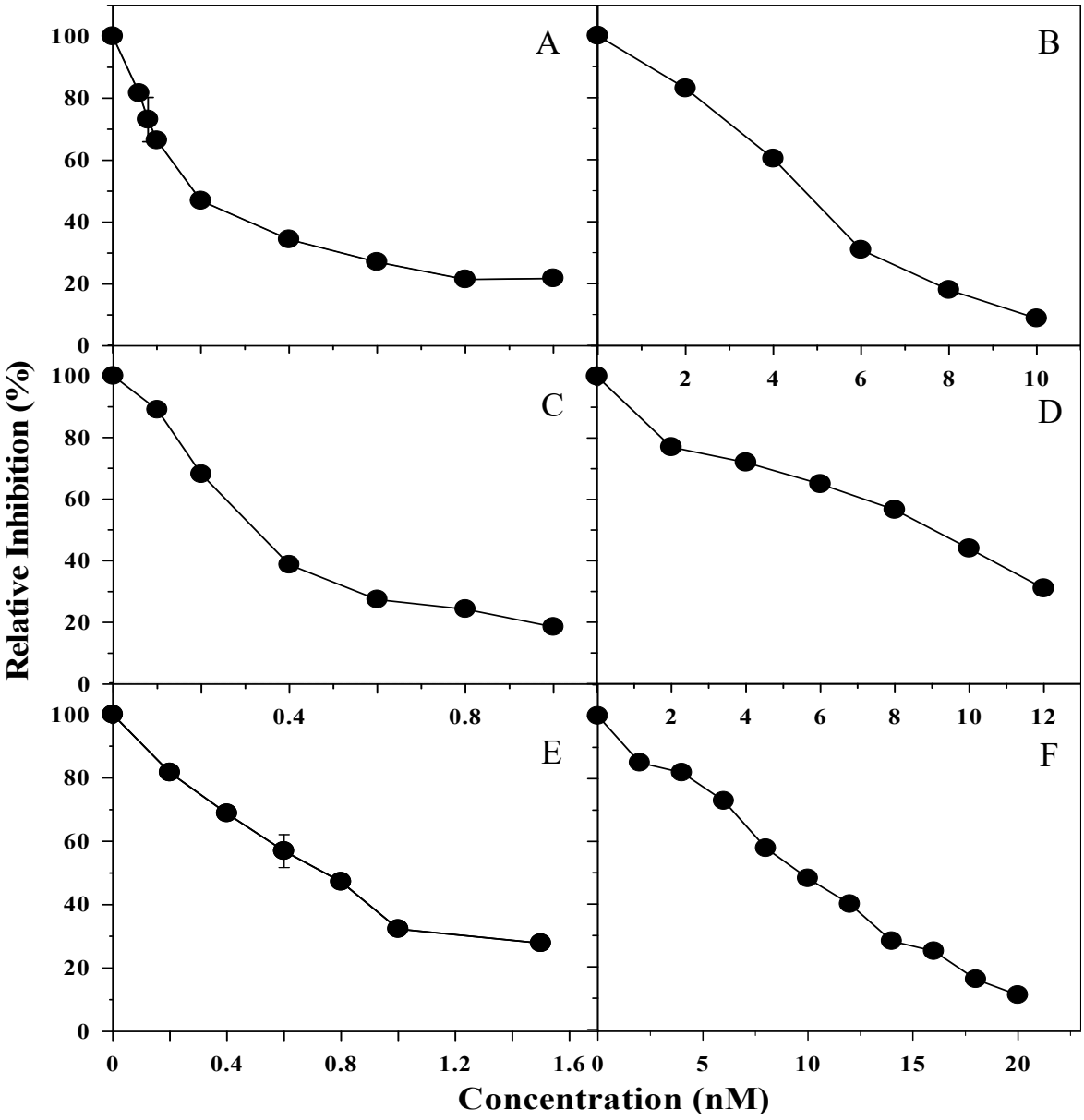

Figure 4.6. Effect of chemical agents and metal ions, (A) dithiothreitol, (B) copper chloride, (C) diethyldithiocarbamic acid, (D) copper sulfate, (E) L-cysteine and (F) citric acid on the partially purified laccase (PPL) activity, using the aflatoxins AFB1, AFB2, AFG1 and AFG2 from *Aspergillus parasiticus* as substrates. Laccase activity was measured using citrate phosphate buffer (0.1 M, pH 6.0) at 57.5°C, during 30 min. The error bars in the figure indicate the standard deviation.

Figure 4.6C shows that DDC inhibited laccase activity when the aflatoxins were used as substrates. The inhibition goes along with the increase in the concentration of this compound, with 81.5% of inhibition in presence of 1 mM of DDC. The experimental results are in agreement with those of Lu *et al*. (2013), who reported that 1 mM of DDC completely inhibited laccase activity with ABTS used as substrate. The experimental findings suggest that DDC, which is a copper chelator, inhibits laccase activity by modification of the active site of the enzyme (Sadhasivam *et al*., 2008).

Figure 4.6E shows that L-cysteine inhibited laccase activity, where the inhibition increased with the concomitant increase of L-cysteine concentration up to 1.5 mM, with an inhibition rate of 72.2%. The experimental findings are in agreement with those of Saito *et al*. (2003), who showed that laccase activity was completely inhibited in presence of 1mM of L-cysteine. Nagai *et al*. (2002) also found that laccase activity was completely inhibited in presence 1 mM of L-cysteine using ABTS as substrat. The experimental results suggest that L-cysteine, an essential amino acid of the active site of fungal laccases (Thurston 1994), is a classical inhibitor of phenol oxidase-type activities where the presence or an excess of this one, can inhibit laccases activity (Lu *et al*., 2007).

Figure 4.6F indicates that citric acid inhibited laccase activity, where the inhibition increased with that of the citric acid concentration and reached an inhibition rate of 90% in presence of 20 mM of citric acid. The results (Fig 6F) are in agreement with those of Lorenzo *et al*. (2005) in which 17 mM of citric acid strongly inhibited, more than 90%, laccase activity, with DMP and SYR used as substrates. The experimental findings suggest that citric acid can form a complex compound with laccases copper ions, leading to a modification in the laccases active site (Zavarzina *et al*., 2004).

The overall results indicate that the sulfhydryl organic compounds such as L-cysteine, DTT and DDC are metal specific chelators which inhibit laccase activity through the formation of complexes with copper ions, modifying its active site (Khlifi *et al*., 2010).

Table 4.2 and figure 4.7 indicate that the K_m and V_{max} values for the partial purified laccase with the use of aflatoxins, AFB1, AFB2, AFG1 and AFG2, as substrates were 0.646, 0.128, 0.237, 0.284 µM of substrate and 10.4, 9.7, 13.0, 10.8 µM of products per mg protein per min, respectively, at temperature of 57.5°C and pH of 6.0. $CuSO_4$ exhibited an uncompetitive inhibitory effect towards laccase activity as indicated by the corresponding K_m and V_{max} values of 0.113, 0.224, 0.041, 0.050 µM and 1.19, 1.11, 1.46, 1.24 µM/mg protein/min, for AFB1, AFB, AFG1 and AFG2, respectively. On the other hand, $CuCl_2$ shows a noncompetitive mixte inhibitory effect towards PPL activity as indicate by the corresponding K_m and V_{max} values of 0.470, 0.934, 0.170, 0.206 µM and 2.79, 2.60, 3.45, 2.89 µM/mg protein/min, respectively.

The experimental results (Table 4.2 and Fig. 4.8) show that citric acid also exhibited an uncompetitive inhibitory effect towards PPL activity as indicated by the corresponding K_m and V_{max} values of 0.028, 0.111, 0.0191, 0.022 µM, and 0.17, 0.20, 0.25, 0.20 µM/mg protein/min, respectively.

As regards the metal specific chelators i.e., L-cysteine, DTT and DDC, the results indicate that L-cysteine had a noncompetitive inhibitory effect towards PPL actvity, when the aflatoxins, AFB1, AFB2, AFG1 and AFG2, were used as substrates, as demonstrated by the values of the corresponding K_m and V_{max} of 0.675, 0.134, 0.238, 0.296 and 3.51, 3.27, 4.26, 3.65 µM/mg protein/min, respectively.

The experimental findings (Table 4.2 and Fig. 4.8) also show that DTT had a competitive inhibitory effect towards PPL activity, when AFB1, AFB2, AFG1 and AFG2 were used as substrate, as demonstrated by the values of the corresponding K_m and V_{max} of 2.040, 0.421, 0.545, 0.894 µM and 9.25, 8.62, 8.67, 9.60 µM/mg protein/min, respectively.

On the other hand, the present results demonstrate that DDC exhibited an uncompetitive inhibitory effect towards PPL activity as indicate by the corresponding K_{max} and V_{max} values of 0.082, 0.176, 0.025, 0.035 µM and 1.0, 1.0, 1.04, 1.0 µM/mg protein/min, for AFB1, AFB2, AFG1 and AFG2, respectively.

Table 4.2. Inhibitory effects of selected chemicals on partial purified laccase.

Inhibitor	K_m[a]				V_{max}[b]				K_{cat}[c]				
	AFB1	AFB2	AFG1	AFG2	AFB1	AFB2	AFG1	AFG2	AFB1	AFB2	AFG1	AFG2	Type of inhibition
Blank	0.646	0.128	0.237	0.284	10.4	9.70	13.00	10.80	16	75.8	55	38	No inhibition
Citric acid	0.028	0.111	0.0191	0.022	0.17	0.20	0.25	0.20	6	2.0	1	9	Uncompetitive
$CuSO_4$	0.113	0.224	0.041	0.050	1.19	1.11	1.46	1.24	11	5.0	36	25	Uncompetitive
$CuCl_2$	0.470	0.934	0.170	0.206	2.79	2.60	3.45	2.89	6	3.0	20	14	Mixte
L-cysteine	0.675	0.134	0.238	0.296	3.51	3.27	4.26	3.65	5	2.0	18	12	Non-competitive
DTT[d]	2,040	0.421	0.545	0.894	9.25	8.62	8.67	9.60	5	21.0	2	11	Competitive
DDC[e]	0.082	0.176	0.025	0.035	1.00	1.00	1.04	1.00	12	6.0	42	29	Uncompetitive

[a]Michaelis-Menten constant (K_m) was defined as μM of substrate. The reaction was carried at 57.5ºC, using citrate phosphate buffer (0.1 M, pH 6.0).

[b]Maximum reaction velocity (V_{max}) was defined as μM of products formed per mg protein per min.

[c]The catalytic efficiency was defined as the ratio of V_{max} to K_m.

[d]Dithiothreitol.

[e]Diethyldithiocarbamic acid.

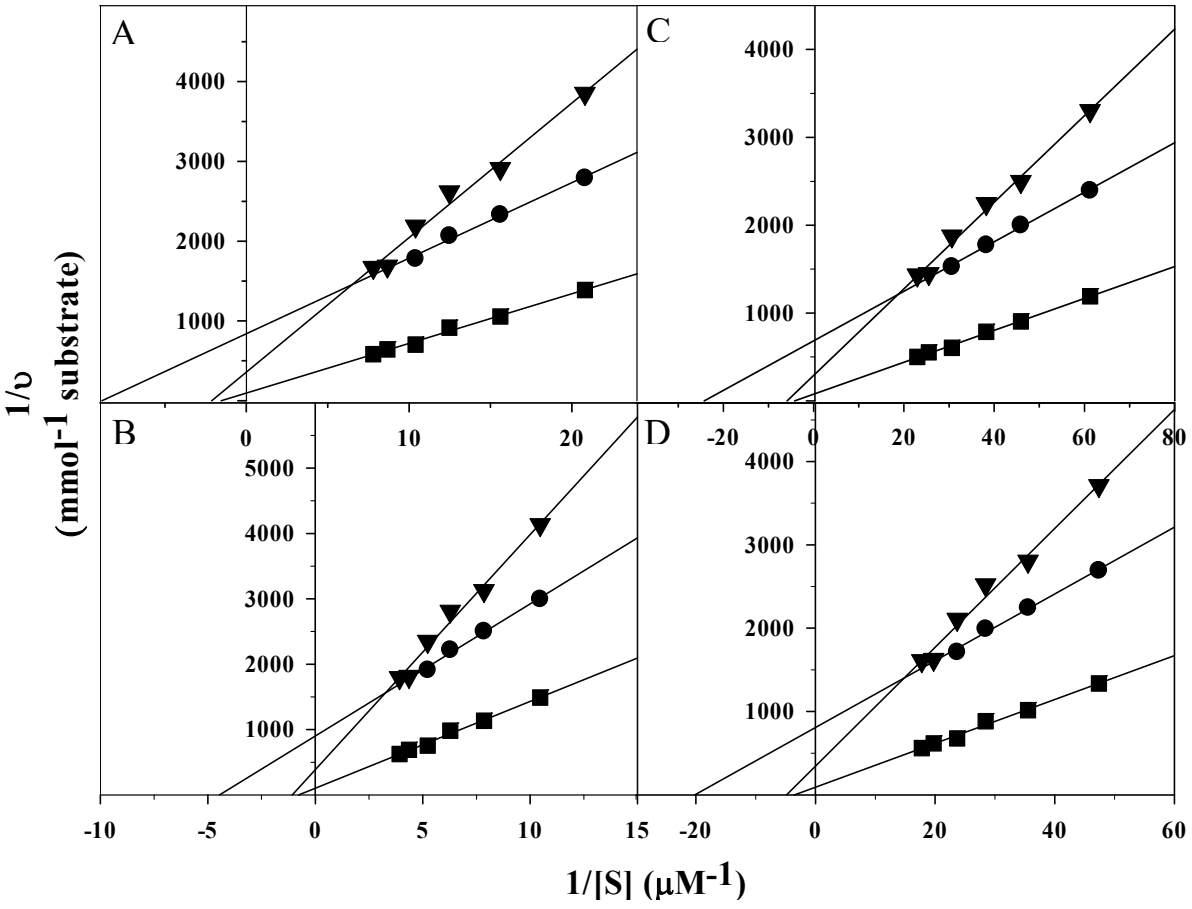

Figure 4.7. Lineweaver-Burk plots of 1/υ versus 1/[S] with (A) AFB1, (B) AFB2, (C) AFG1, (D) AFG2 and (●) 10 mM copper sulfate and (▼) 8.0 mM copper chloride as inhibitors. The enzymatic assay was performed with the partial purified laccase alone (■) and in the presence of inhibitors.

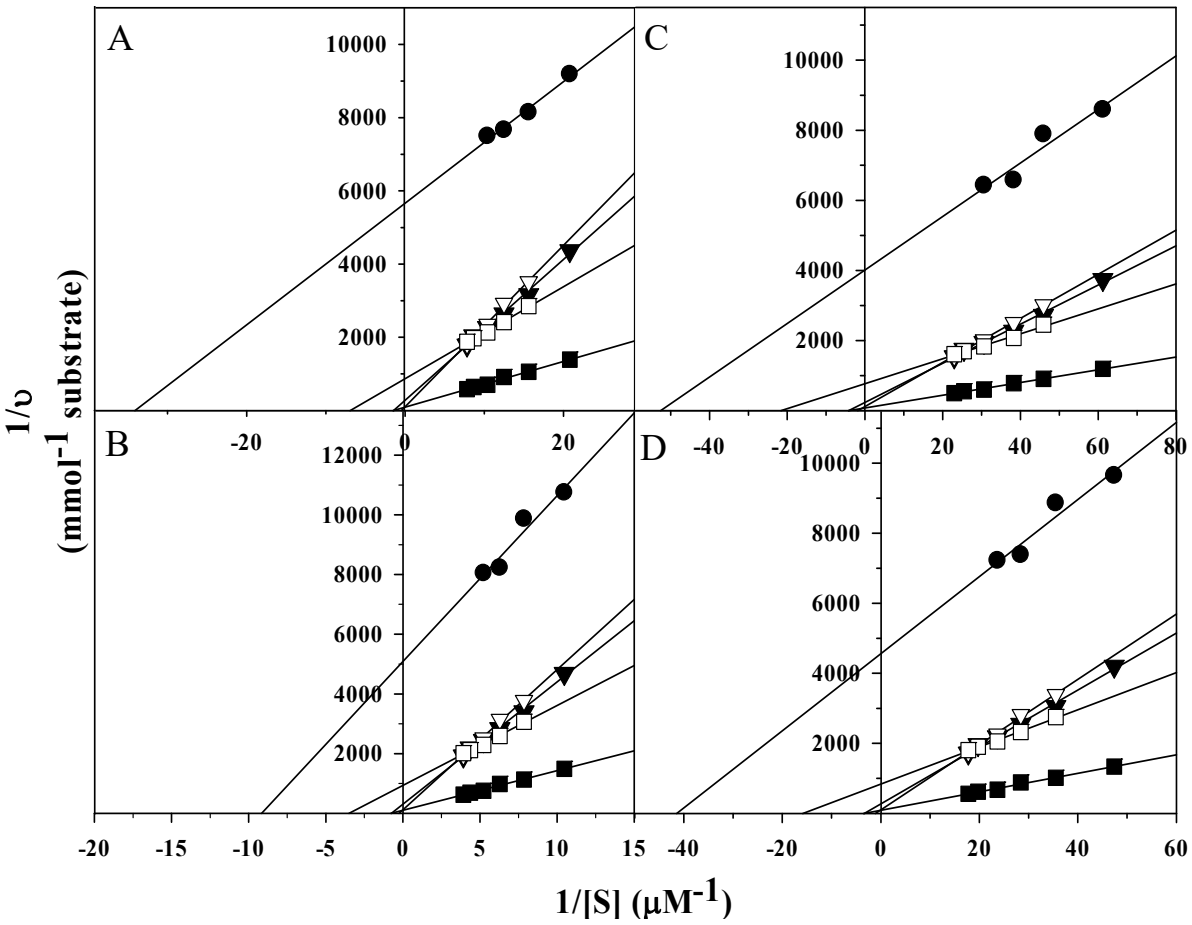

Figure 4.8. Lineweaver-Burk plots of 1/υ versus 1/[S] with (A) AFB1, (B) AFB2, (C) AFG1, (D) AFG2 and (●) 20 Mm citric acid, (▼) 1.0 mm L-cysteine, (▽) 0.4 mm dithiothreitol, (□) 0.4 mm diethyldithiocarbamic acid as inhibitors. The enzymatic assay was performed with the partial purified laccase alone (■) and in the presence of inhibitor.

4.7. Conclusion

The results gathered in this study showed that the enzymatic activity is influenced both by specific properties, including substrate concentration, activators and inhibitors and by non specific effects such as buffer, pH and temperature. The experimental findings suggest that a temperature of 57.5°C and a pH of 6.0, are the optimale conditions to improve laccase degradation activity for the aflatoxins, AFB1, AFB2, AFG1 and AFG2. In addition, the effects of metal ions, salts and some selected chemical agents on the laccase degradation activity were highly dependent on the agents' nature and concentrations. The results suggest that the partial purified laccase produced from *C. hirsutus* can possibly be applied to eliminate aflatoxins in contaminated food and feed in order to decrease the hazards of aflatoxins for both humans and animals health.

CHAPTER V

ARTICLE IV

MODELING AND OPTIMIZATION OF LACCASE ENZYMATIC DEGRADATION OF SELECTED MICROBIAL AFLATOXINS, USING RESPONSE SURFACE METHODOLOGY

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5.1. Contribution of the Authors

The present author, Sabrina Borgomano, was responsible for the concepts, the designs and the fulfillment of this experimental work and the preparation of this manuscript for its submission.

Dr. Monique Lacroix, the thesis supervisor, and Dr. Selim Kermasha, the thesis supervisor, supervised this research work, provided valuable input and advices, monitored the progress of this work and critically reviewed and edited this manuscript, prior to its submission.

Dr. Khanh D. Vu contributed to the statistical analysis using response-surface methodology and reviewed this part in this manuscript.

5.2. Résumé

La méthodologie de réponse de surface (RSM) a été utilisée pour optimiser la catalyse de dégradation de la laccase pour les principales formes des aflatoxines, dont les aflatoxines AFB1, AFB2, AFG1 et AFG2, obtenues à partir d'*Aspergillus parasiticus*. Une série de 19 expériences a été réalisée, en utilisant un plan de composites centrés (CCD) avec trois variables indépendantes, dont la concentration en enzyme, la concentration des aflatoxines, et le temps d'incubation, ainsi que quatre réponses, le pourcentage de dégradation pour chaque aflatoxine. Les analyses CCD et RSM ont été utilisées pour étudier à la fois les interactions entre les trois variables indépendantes et pour optimiser la dégradation des aflatoxines par l'extrait enzymatique de la laccase de *Coriolus hirsutus*. Les équations polynomiales ont été générées à partir de l'analyse de régression des variables. Les résultats ont démontré que les valeurs prédites pour la dégradation des aflatoxines sont en accord avec les résultats expérimentaux, où le pourcentage maximum de la dégradation des aflatoxines, 38,2, 30,1, 76,4 et 100% pour les aflatoxines AFB1, AFB2, AFG1 et AFG2, respectivement, ont été obtenus avec des concentrations de substrat de 96,2, 191,0, 32,7 et 42,2 nmol d'AFB1, AFB2, AFG1 et AFG2, respectivement, ainsi qu'une concentration d'enzyme de 31,5 U/nmol aflatoxine et un temps d'incubation de 55,2 min.

5.3. Abstact

Response surface methodology (RSM) was used to optimize the laccase-catalyzed degradation for the major aflatoxin isoforms, including aflatoxins AFB1, AFB2, AFG1 and AFG2, obtained from *Aspergillus parasiticus*. A series of 19 experiments were carried out, using central composite design (CCD) with three independent variables, including enzyme concentration, aflatoxins concentration and incubation time as well as four responses, the percentage of degradation for each aflatoxin. CCD and RSM analyses were used to investigate both the interactions between the three independent variables and to optimize the degradation of aflatoxins by a laccase enzymatic extract from *Coriolus hirsutus*. Polynomial equations were generated based on the regression analysis of the variables. The results demonstrated that the predicted values of aflatoxins degradation were in good agreement with the experimental results, where the maximum percentage of aflatoxins degradation, 38.2, 30.1, 76.4 and 100% for AFB1, AFB2, AFG1 and AFG2, respectively, were obtained with 96.2, 191.0, 32.7 and 42.2 nmol of AFB1, AFB2, AFG1 and AFG2, respectively, as substrate concentrations as well as an enzyme concentration of 31.5 U/nmol aflatoxin and an incubation time of 55.2 min.

Keywords: Aflatoxins, degradation, laccase, optimization, response surface methodology, central composite design

5.4. Introduction

Aflatoxins are the most potent natural mycotoxins produced by fungi, mainly *Aspergillus flavus* and *A. Parasiticus* (Bhat *et al.*, 2010) and have been widely investigated because of their mutagenic, teratogenic, carcinogenic and hepatogenic properties for both humans and animals health. Aflatoxins are an assembly of a coumarin and 3-furan, where aflatoxins AFB1, AFB2, AFG1 and AFG2 are the most common and potent in food products. Aflatoxins contaminate a wide range of food commodities and agricultural food and feed (Pietri *et al.*, 2012), with a limited tolerance for human food and animal feed of 20 ppb for total aflatoxins (Park and Liang 1993).

Although several physico-chemical methods have been reported to reduce or to degrade aflatoxins in contaminated food and animal feed, any of these processes are widely accepted. These methods are responsible for the formation of toxic by-products as well as for the loss in nutritional quality and economic value (Kabak *et al*., 2006). Hence, it was important to develop a novel approach, using biological methods in particular enzymes, which could be cost effective, non toxic and environmentally friendly for the aflatoxins degradation (Wu *et al*., 2009). The degradation of aflatoxins by enzymes, especially peroxidases group, from different sources has been reported (Mishra and Das, 2003).

Laccases (EC 1.10.3.2) are oxidoreductases that catalyze the oxidation of a broad variety of aromatic compounds, in particular phenolic compounds, by oxidizing the substrate (Marjasvaara *et al*., 2006). The advantages of laccases are that they do not require hydrogen peroxide for the substrate oxidation and instead, they use the oxygen as a non-limiting electron acceptor as well as their broad specificity for substrates. Laccases are widelly used in pulp delignification, bioremediation, biosensors and degradation of polyphenolic xenobiotics as well as detergent manufacturing, wine clarification and fruit juice stabilization (Octavio *et al*., 2006). The cost of laccases production as well as their stability makes them suitable for diverse biotechnological applications (Flurkey, 2003; Baldrian, 2006).

Response surface methodology (RSM), as a collection of mathematical and statistical technique, defines the effect of the independent variables, alone or in combination, on the process (Kong *et*

al., 2012). It has been successfully applied to optimize the enzymatic degradation of AFB1 in red chili powder (Tripathi and Mishra, 2011).

The overall aim of the present work was to determine the degradation of major aflatoxin isoforms, AFB1, AFB2, AFG1 and AFG2, by a laccase enzyme extract from *Corilus hirsutus*. The specific objectives were to optimize three selected parameters, including enzyme concentration, aflatoxins concentration and incubation time, as well as to study their interactions to achieve maximum enzymatic degradation of each aflatoxin isoform using RSM based on central composite design (CCD).

5.5. Materials and Methods

5.5.1. Organism and Culture Conditions of Coriolus Hirsutus and Aspergillus Parasiticus

Coriolus hirsutus (MYA-828), used for the production of laccase, was obtained from the American Type Culture Collection (ATCC, Manassas, VA.). *C. hirsutus* was maintained onto malt agar media plates and incubated at 20ºC. For laccase production a basal liquid medium was prepared, using the method previously described by Taqi (2012).

Aspergillus parasiticus (26864), used for the production of aflatoxins, was obtained from the American Type Culture Collection (ATCC; Manassas, VA). *A. parasiticus* was maintained through periodic transfer onto potatoes dextrose agar media plates and incubated at 24ºC; for aflatoxins production, a basal liquid medium was prepared using the method previously described by Hsieh and Mateles (1971).

5.5.2. Preparation of Laccase Extract

Laccase enzymatic extract was obtained according to the procedure described by Taqi (2012). The mycelium pellets from culture liquid medium were removed throughout the use of a cheese-cloth filtration. The culture medium was ultra-filtered and partially purified by 80% saturation of ammonium sulphate precipitation.

5.5.3. Aflatoxins Preparation

The fermentation process of *A. parasiticus* was carried out according to a modification of the method of Hsieh and Mateles (1971) developed in our laboratory and previously described in chapter III. The mycelium pellets from culture liquid medium were removed throughout a

cheese-cloth filtration. The extracellular toxins were recovered from the culture medium by ultra-filtration, according to the method of chapter III.

5.5.4. Aflatoxins Degradation by Partial Purified Laccase

A stock solution of afltoxins, 1 g/10 mL, was prepared in 40% methanol and diluted to get the different concentration range from 28.8 to 163.64 nM for AFB1, 57.2 to 325.0 nM for AFB2, 9.74 to 55.6 nM for AFG1 and 12.6 to 71.8 nM for AFG2. Measured amount of partial purified laccase, 0.04 g/mL (0.32 mg protein/mL), was dissolved in citrate phosphate buffer (0.1 M, pH 6.0) and used to detoxify the different levels of aflatoxins. The enzyme concentration varied from 5.012 to 58.0 U/nM aflatoxin. The reaction medium was adjusted with citrate phosphate buffer (0.1 M, pH 6.0) to a total volume of 700 µL. The reaction was initiated by the enzyme addition and was carried out at 57.5ºC. The reaction mixture was stirred at 100 rpm and incubated up to 55.2 min. The reaction was stopped by adding 700 µL of chloroform and the degradation rate of aflatoxins was determined at the end of every incubation period.

5.5.5. Extraction and Quantification of Aflatoxins and Their End Products by RP-HPLC

At the end of every incubation time at 57.5ºC, aflatoxins were extracted three successive times with chloroform (1:1, v⁄v) as described by Teniola *et al*. (2005). The three chloroform extracts were combined and evaporated under nitrogen, and samples were dissolved in methanol 99%, filtered and analysed by reversed-phase/high-performance liquid chromatography (RP-HPLC). RP-HPLC analysis was performed to characterize and quantify the major aflatoxin isoforms, AFB1, AFB2, AFG1 and AFG2, using a Beckman Gold system 126 (Beckman Instrument Inc., San Raman, CA), equipped with a UV diode-array detector (DAD), set at 365 nm (Beckman, model 168). A 20 µL sample was injected into an allsphere C18 column (4.6 mm x 250 mm, 5 µm; Alltech, Deerfield, IL), thermostated at 50°C. The isocratic solvent elution was a mixture of water/methanol (40:60, v/v), at a flow rate of 1 mL/min. The percentage of degraded aflatoxins was calculated, using the equation (5-1).

(1 - Area peak of treated aflatoxins/ Area peak of untreated aflatoxins)/100 ..... (5-1)

5.5.6. Response Surface Methodology Study, Experimental Design

A central composite design with three independent variables, including enzyme amount (units/nmol aflatoxins; X1), initial aflatoxins concentration (nmol; X2) and incubation time (min;

X3) at five levels were used to study their effects on responses, which is the aflatoxins degradation percentage (Y). The coded values of different levels, +1.682, +1, 0, -1 and -1.682 (Table 5.1) were selected according to the limiting values of the variables and converted into their real form as X1, X2 and X3, respectively. A total of 19 experiments were conducted according to the central composite design (CCD) in random order (Table 5.2). RSM was applied to the experimental data using Statistica (version 10.0, StatSoft, Tulsa, OK). The experimental results were fitted via a regression procedure and expressed by the following second order polynomial equation.

$$y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \quad \text{..... (5-2)}$$

Y is the predicted response (the degradation percentage of aflatoxins), X_i X_j are the independent variables, β_0 is the intercept, β_i is the linear coefficient of each independent factor, β_{ii} is the quadratic coefficient of each independent factor, and β_{ij} is the interaction coefficient between two independent factors. The goodness of fit of the developed nonlinear equations was tested by F value and the coefficient of determination (R^2). Response surfaces were obtained using the fitted model by keeping one independent variable at a constant value while changing the other two variables for visualizing the effect of process variables on the responses and to find optimum parameter combinations using the same software.

5.6. Results and Discussion

5.6.1. Modeling and Optimization

The aflatoxins degradation by PPL under different combinations, as defined in the CCD (Tables 5.1 and 5.2) was analyzed by ANOVA using Statistica (version 10.0, StatSoft, Tulsa, OK), and the results are presented in Table 5.3. The ANOVA and regression analysis were used to determine the lack of fit and the significant effect of the independent variables on aflatoxins degradation as well as to determine the regression coefficient of the model. The surface response of the polynomial model (Figures 5.1 to 5.4) showed the effect of the independent variables on aflatoxins reduction.

5.6.1.1. Regression Analyses of the Designs

The ANOVA analysis for aflatoxin AFB1 degradation (Table 5.3) shows that the *P* value for the *lack of fit* of the model was *s*ignificant. The lack of fit measures the failure of the model to represent the data in the experimental domain at points which are not included in the regression. The coefficient of determination (R^2) is of prime importance in a predictive modeling system involving optimization of several factors (Khan *et al*., 2014). A high R^2 value implies that the polynomial model is accurate in predicting the responses of the system. The R^2 value of the polynomial model for the prediction of AFB1 degradation percentage was 0.89, which implies that 89% of the variation in the response could be explained by the model. It suggests a good fit of the data in the reduced quadratic model and a good correlation between the experimental results and the theoretical values predicted by the model equation. The regression table also determines the significance of the linear, quadratic and interaction constant coefficients, where those with *P* value ≤ 0.05 were considered as significant. Negative coefficient values indicate that individual or interactions factors negatively affect the aflatoxin AFB1 degradation, whereas positive coefficient values mean that these factors increase the aflatoxins AFB1 degradation in the tested range. The results (Table 5.3) also show that the linear and the quadratic variables as well as the interaction effects between enzyme and AFB1 concentration were statistically significant ($P \leq 0.05$) or marginally significant ($P \leq 0.1$), whereas the interaction effect of enzyme concentration and incubation time was not significant ($P > 0.1$). All the linear coefficients of the variables as well as this of interaction of enzyme concentration and incubation time had a positive effect on the percentage of AFB1 degradation. Whereas, the quadratic coefficient of the variables and this of the interaction between the enzyme and substrate concentrations produced negative effects on the response. The regression table indicates that incubation time and enzyme concentration with higher coefficient values greatly influenced aflatoxin AFB1 degradation, more than substrate concentration.

Table 5.1. Variables and their levels for central composite design.

Independent variables	Aflatoxins	Level of factors				
		Level -2	Level -1	Level 0	Level +1	Level +2
Enzyme concentration[a]		5.012	15.75	31.5	47.25	58.0
Substrate concentration (nM)[b]	AFB1	28.8	64.1	96.2	114.3	163.6
	AFB2	57.2	127.3	191.0	286.5	325.0
	AFG1	9.8	21.8	32.7	49.1	55.6
	AFG2	12.6	28.1	42.2	63.3	71.8
Incubation time (min)[c]		4.7	15	30	45	55.2

[a]Partial purified laccase from *C. hirsutus* in U/nmol Aflatoxin (nmol.min^{-1}.nmol Aflatoxin).

[b]Major aflatoxin isoforms, AFB1, AFB2, AFG1 and AFG2, from *A. parasiticus* in nM, used as substrates for PPL reaction.

[c]Time reaction in minute of partial purified laccase activity using aflatoxins from *A. parasiticus* as substrates.

Table 5.2. Aflatoxins degradation by partial purified laccase.

	Independent variables						Response (aflatoxins reduction %)			
Run	X_1[a]	X_2[b1]	X_2[b2]	X_2[b3]	X_2[b4]	X_3[c]	AFB1	AFB2	AFG1	AFG2
1	15.75	64.1	127.3	21.8	28.1	15.0	20.2	13.5	34.6	35.0
2	15.75	64.1	127.3	21.8	28.1	45.0	26.2	24.4	51.5	75.0
3	15.75	144.3	286.5	49.1	63.3	15.0	18.0	15.1	39.5	30.4
4	15.75	144.3	286.5	49.1	63.3	45.0	26.9	22.3	54.4	72.3
5	47.25	64.1	127.3	21.8	28.1	15.0	19.7	20.2	55.0	62.9
6	47.25	64.1	127.3	21.8	28.1	45.0	28.8	28.9	62.0	85.9
7	47.25	144.3	286.5	49.1	63.3	15.0	3.8	8.8	20.6	46.4
8	47.25	144.3	286.5	49.1	63.3	45.0	25.2	14.5	67.2	72.7
9	5.01	96.2	191.0	32.7	42.2	30.0	19.8	9.6	48.4	45.2
10	58.00	96.2	191.0	32.7	42.2	30.0	26.2	20.3	68.5	82.2
11	31.50	28.8	57.2	9.8	12.6	30.0	25.8	22.7	31.1	37.9
12	31.50	163.6	325.0	55.6	71.8	30.0	24.1	19.4	36.4	61.4
13	31.50	96.2	191.0	32.7	42.2	4.8	4.2	9.8	49.5	100.0
14	31.50	96.2	191.0	32.7	42.2	55.2	38.2	30.1	76.4	100.0
15	31.50	96.2	191.0	32.7	42.2	30.0	30.0	24.6	68.8	100.0
16	31.50	96.2	191.0	32.7	42.2	30.9	29.6	25.2	66.3	100.0
17	31.50	96.2	191.0	32.7	42.2	30.0	31.5	24.2	65.8	100.0
18	31.50	96.2	191.0	32.7	42.2	30.0	30.6	24.5	67.5	100.0
19	31.50	96.2	191.0	32.7	42.2	30.0	28.9	24.8	67.8	100.0

[a]Enzyme concentration (U/nM aflatoxin).

[b]Aflatoxins concentration (nM), with (1) AFB1, (2) AFB2, (3) AFG1 and (4) AFG2.

[c]Different incubation times in minutes of reaction between enzyme and aflatoxins.

Since the interaction coefficient between enzyme concentration and AFB1 concentration was not statically significant (P > 0.05), it wasn't included in the polynomial equation. Similarly, the interaction coefficient of enzyme concentration and incubation time was also excluded from the polynomial equation, as it was not statistically significant (P > 0.05). Based on the regression analysis, the final polynomial equation for prediction of aflatoxin AFB1 degradation is given in the equation (5-3).

$$AFB1\% = -23.53 + 0.85 \times X_1 - 0.01 \times X_1^2 + 0.36 \times X_2 - 0.0014 \times X_2^2 + 1.15 \times X_3 - 0.02 \times X_3^2 \quad (5\text{-}3)$$

The ANOVA analysis for aflatoxin AFB2 degradation shows that the *P* value for the *lack of fit* of the model was *s*ignificant (table 5.3). The R^2 value for prediction model on AFB2 degradation was 0.94, which implies that 94% of variation response could be explained by the model, suggesting that the polynomial equation is accurate in predicting the responses of the model. The results (Table 5.3) demonstrate that all the linear and the quadratic variables were statistically significant ($P \leq 0.05$) or marginally significant ($P \leq 0.1$), as well as the interaction effects between enzyme and AFB2 concentration. However, the interaction effect between substrate concentration and incubation time was not significant ($P > 0.1$). All the linear coefficients of the variables imparted positive effects on the response. On the other hand, the quadratic coefficient of the variables and the interactions produced negative effects on the response. Table 5.3 also shows that enzyme concentration with higher coefficient values greatly influenced aflatoxin AFB2 degradation, more than incubation time and substrate concentration. Although the coefficient of interaction between substrate concentration and incubation time was not statistically significant ($P > 0.1$), it was included in the polynomial equation, in order to negate an underestimation of AFB2 degradation percentage values. Based on the regression analysis, the final polynomial equation for the prediction of aflatoxin AFB2 degradation is given in the equation (5-4).

$$AFB2\% = -30.01 + 1.48 \times X_1 - 0.014 \times X_1^2 + 0.17 \times X_2 - 0.0002 \times X_2^2 + 0.8 \times X_3 - 0.005 \times X_3^2 - 0.003 \times X_1 \times X_2 - 0.0008 X_2 \times X_3 \quad (5\text{-}4)$$

Table 5.3. Response surface regression analyses of aflatoxins degradation by the partial purified laccase.

	Regression analysis							
	AFB1(%)[a]		AFB2(%)[a]		AFG1(%)[a]		AFG2(%)[a]	
Factors[b]	Coefficient	*P* value[c]	Coefficient	*P* value[c]	Coefficient	*P* value[c]	Coefficient	*P* value[c]
Constant	-23.53	0.10	-30.01	<0.01	-56.90	<0.05	-161.67	<0.01
X1 (L)[d]	0.85	0.05	148	<0.001	1.90	<0.01	5.334	<0.001
X1 (Q)[e]	-0.01	<0.05	-0.014	<0.001	-0.01	<0.10	-0.06	<0.001
X2 (L)[d]	0.3561	<0.05	0.17	<0.01	4.35	<0.001	6.10	<0.001
X2 (Q)[e]	-0.0014	0.06	-0.0002	<0.05	-0.06	<0.001	-0.07	<0.001
X3 (L)[d]	1.15	<0.01	0.80	<0.01	0.51	0.40	1.73	0.10
X3 (Q)[e]	-0.02	<0.01	-0.005	0.06	-0.013	0.10	-0.01	0.50
X1xX2	-0.004	<0.10	-0.003	<0.001	-0.02	0.05	-0.011	0.45
X1xX3	0.008	0.18	N/A[f]	N/A[f]	N/A[f]	N/A[f]	-0.02	0.33
X2xX3	N/A[f]	N/A[f]	-0.0008	0.24	0.024	<0.05	N/A[f]	N/A[f]
Lack of fit		≤0.05		≤0.05		0.07		≤0.05

[a]Degradation of aflatoxins, AFB1, AFB2, AFG1 and AFG2, in percent.

[b]Factors that influenced the aflatoxins degradation: (X1) enzyme concentration (U/nM aflatoxins), (X2) aflatoxins concentration (nmol), (X3) incubation times (min).

[c]*P* value is the probability of error ($P \leq 0.05$ means that the effect of a factor is significant).

[d]L represents linear coefficient.

[e]Q represents quadratic coefficient.

[f]N/A not applicable for the model.

The ANOVA analysis for aflatoxin AFG1 degradation shows that the *P* value for the *lack of fit* of the responses investigated in this study was not significant (table 5.3), indicating that the model equation is adequate for predicting the percentage of AFG1 reduction under any combination of values of the variables. The R^2 value for the prediction model of aflatoxin AFG1 degradation was 0.92, which implied that 92% of the variation in the response could be explained by the model. It suggests that the polynomial equation is accurate in predicting the responses of the model. The results (Table 5.3) demonstrate that all the linear, quadratic and interaction variables were statistically significant ($P \leq 0.05$) or marginally significant ($P \leq 0.1$), except for the linear incubation time variable ($P > 0.1$). All the linear coefficients of the variables imparted positive effects on the response as well as this of interaction between substrate and incubation time. Whereas, the quadratic coefficient of the variables and the interactions between enzyme and substrate concentrations produced negative effects on the response. Table 5.3 also show that substrate and enzyme concentrations, with higher coefficient values, greatly influenced aflatoxin AFG1 degradation, more than incubation time and the interaction of substrate concentration and incubation time. Although the linear positive incubation time effect was not statistically significant ($P > 0.1$), it was included in the polynomial equation because it's main effect. Based on the regression analysis, the final polynomial equation obtained for prediction of aflatoxin AFG1 degradation is given in the equation (5-5).

$$\text{AFG1\%} = -56.90 + 1.90 \times X_1 - 0.01 \times X_1^2 + 4.35 \times X_2 - 0.06 \times X_2^2 + 0.51 \times X_3 - 0.013 \times X_3^2 - 0.02 \times X_1 \times X_2 + 0.024 \times X_2 \times X_3 \quad (5\text{-}5)$$

The ANOVA analysis for aflatoxin AFG2 degradation shows that the *P* value for the *lack of fit* of the model was *s*ignificant (Table 5.3). The R^2 value for the prediction model on aflatoxin AFG2 degradation was 0.89, which implied that 89% of variation could be explained by the model, suggesting that the polynomial equation can be used to predict the responses. The results (Table 5.3) demonstrate that all the linear variables and those of the quadratic variables of enzyme and substrate concentrations were statistically significant ($P \leq 0.05$) or marginally significant ($P \leq 0.1$), whereas, the quadratic coefficient of the incubation time and the interaction coefficient were not significant ($P > 0.1$). All the linear coefficients of the variables imparted positive effects on the response. On the other hand, all the quadratic coefficients of the variables and the interactions produced negative effects on the response. Table 3 also shows that enzyme

and substrates concentrations with higher coefficient values influenced aflatoxin AFG2 degradation, more than incubation time. Although, the quadratic coefficient of incubation time was not statistically significant ($P > 0.1$), it was included in the polynomial equation, because it's main effect. Similarly, the interactions among three independent factors were also included in the polynomial equation, in order to negate an underestimation of AFG2 degradation percentage values, although they were not statistically significant ($P > 0.1$). Based on the regression analysis, the final polynomial equation obtained for aflatoxin AFG2 degradation is given in the equation (5-6).

$$AFG2\% = -161.67 + 5.334 \times X_1 - 0.060 \times X_1^2 + 6.10 \times X_2 - 0.07 \times X_2^2 + 1.73 \times X_3 - 0.01 \times X_3^2 - 0.011 \times X_1 \times X_2 - 0.02 \times X_1 \times X_3 \quad (5\text{-}6)$$

Response surface methodology has been successfully used to optimize the degradation of aflatoxins by biological, physical and chemical methods. Tripathi and Mishra (2011) used RSM to optimize the peroxidase-catalyzed enzymatic degradation of aflatoxin AFB1 from red chili powder. The second-order polynomial equation showed a high R^2value of 0.92, suggesting the fit of the model. Aroyeun *et al.* (2013) effectively used RSM to study the effect of the essential oil of *Aframomum. Danielli* on the reduction of aflatoxin AFB1 in cocoa beans contaminated with *Aspergillus flavus*. They demonstrated that this method was adequate to predict the effect of *A. Danielli* oil on the growth of *A. flavus* and the aflatoxin AFB1 production, with R^2 values of 0.90 and 0.83, respectively. Arzandeh and Jinap (2011) reported that RSM was appropriate to predict the degradation rate of aflatoxins AFB1, AFB2, AFG1 and AFG2 by different heating methods, with R^2 values of 0.999, for each one. Jalili *et al*. (2010) also indicated that RSM was applied to evaluate the effect of gamma ray dose ranging from 0 to 60 kGy on major aflatoxin isoforms degradation. High R^2 values were obtained for aflatoxins degradation with 0.95, 0.93, 0.88 and 0.89 for AFB1, AFB2, AFG1 and AFG2, respectively.

5.6.1.2. Response Surface Plot

5.6.1.2.1. Aflatoxin AFB1 Degradation

The combined effects of enzyme concentration (X1) and AFB1 concentration (X2) on the AFB1 degradation, at fixed incubation time (30 min), were investigated. Figure 5.1A shows that the AFB1 degradation increased when enzyme and AFB1 concentrations increased up to 40 U/nM aflatoxin and 99.6 nM, respectively. However, further increases in the concentration of both the components decreased the AFB1 degradation. The results suggest that initially when substrate concentration is low, all the active sites of the enzyme are free to bind the substrate, and thus, there is high rate of reaction and better percent degradation, but at certain level, the substrate concentration becomes the limiting factor, and all the active sites of the enzyme are already occupied (Tripathi and Mishra, 2011). The region corresponding for maximum AFB1 degradation had optimum values near to 31.5 U/nM aflatoxin and 96.2 nM for variables X1 and X2, respectively, with response value of 38.2%.

The combined effects of enzyme concentration (X1) and incubation time (X3), at fixed AFB1 concentration (96.2 nM), on the response were investigated. Figure 5.1B shows that the increase in incubation time had more significant effect on the AFB1 degradation than enzyme concentration, where it increased with the concomitant increase in incubation time. The AFB1 degradation increased from 4.2 to 38.2% when incubation time increased from 4.8 to 55.2 min. It also increased with that in enzyme concentration up to 40 U/nM aflatoxin. The initial percent of AFB1 degradation of 19.8% at 5.01 U/nM AFB1 increased to around 38.9% at 31.5 U/nM aflatoxin. However, further increase in enzyme concentration, at fixed substrate (AFB1) concentration, didn't increase the percentage of degradation. The results suggest that all the enzyme/substrate complexes have been formed and no substrate was left to bind the added enzyme, resulting in no products formation (Tripathi and Mishra, 2011). The optimum levels for X1 and X3 obtained at fixed value of X2 were near to 31.5 U/nM aflatoxins and 55.2 min, respectively. The overall results suggest that the maximum of AFB1 degradation (38.2 %) can be obtained by optimized conditions, including enzyme concentration of 31.5 U/nM aflatoxin, substrate concentration of 96.2 nM and incubation time of 55.2 min.

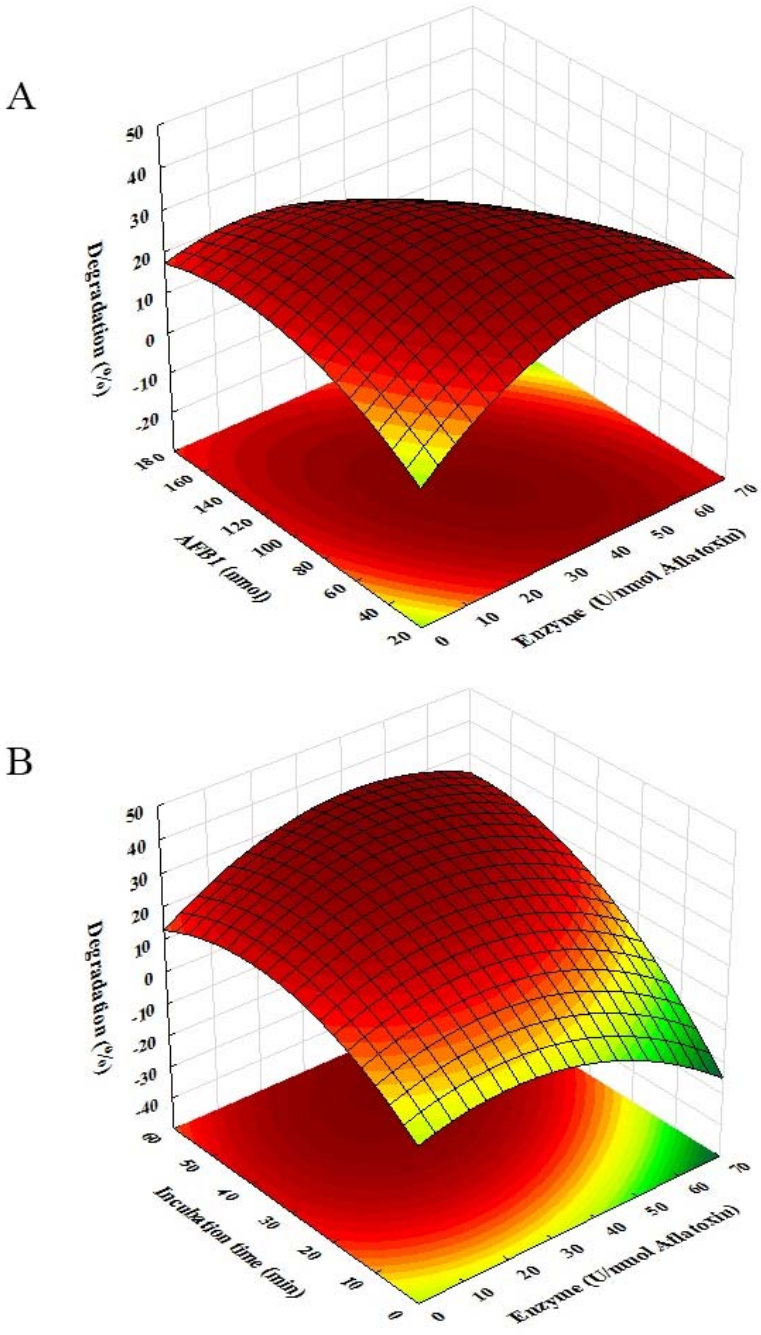

Figure 5.1. Response surface 3-D plot showing the effects of the independent variables on AFB1 degradation. (A) Effect of enzyme concentration (U/nM Aflatoxin) and AFB1 concentration (nM) while keeping incubation time (X_3) constant and (B) Effect of enzyme concentration (U/nM Aflatoxin) and incubation time (min) while keeping substrate concentration (X_2) constant.

5.6.1.2.2. Aflatoxin AFB2 Degradation

The combined effects of enzyme concentration (X1) and AFB2 concentration (X2) on the AFB2 degradation, at fixed incubation time (30 min), were investigated. Figure 5.2A shows that the AFB2 degradation increased when enzyme and AFB2 concentrations increased up to 50 U/nM aflatoxin and 150 nM, respectively. However, further increases in the concentration of both the components didn't increase the AFB2 degradation. The optimum values for maximum AFB2 degradation were near to 31.5 U/nM aflatoxin and 191 nM for variables X1 and X2, respectively, with response value of 30.1%.

The combined effects of enzyme concentration (X1) at different incubation time (X3), at fixed AFB2 concentration (191 nM), on the response were investigated. Figure 5.2B shows that the AFB2 degradation increased with that in enzyme concentration up to 35 U/nM aflatoxin. In addition, it increased with the concomitant increase in incubation time. The AFB2 degradation increased from 12.5 to 30.1%, when incubation time increased from 4.8 to 55.2 min. The optimum levels for X1 and X3 obtained at fixed value of X2 were near to 31.5 U/nM aflatoxin and 55.2 min, respectively. The overall results suggest that maximum aflatoxin AFB2 degradation (30.1%) can be obtained by optimized conditions, including enzyme concentration of 31.5 U/nM aflatoxin, substrate concentration of 191 nM and incubation time of 55.2 min.

5.6.1.2.3. Aflatoxin AFG1 Degradation

The combined effects of enzyme concentration (X1) and AFG1 concentration (X2) on the AFG1 degradation, at fixed incubation time (30 min), were investigated. Figure 5.3A shows that the AFG1 degradation increased when enzyme and AFB1 concentrations increased up to 50 U/nM aflatoxin and 35 nM, respectively. However, further increases in the concentration of both the components decreased the AFG1 degradation. The optimum values for maximum AFG1 degradation were near to 31.5 U/nM aflatoxin and 32.7 nM for variables X1 and X2, respectively, with response value of 76.4%.

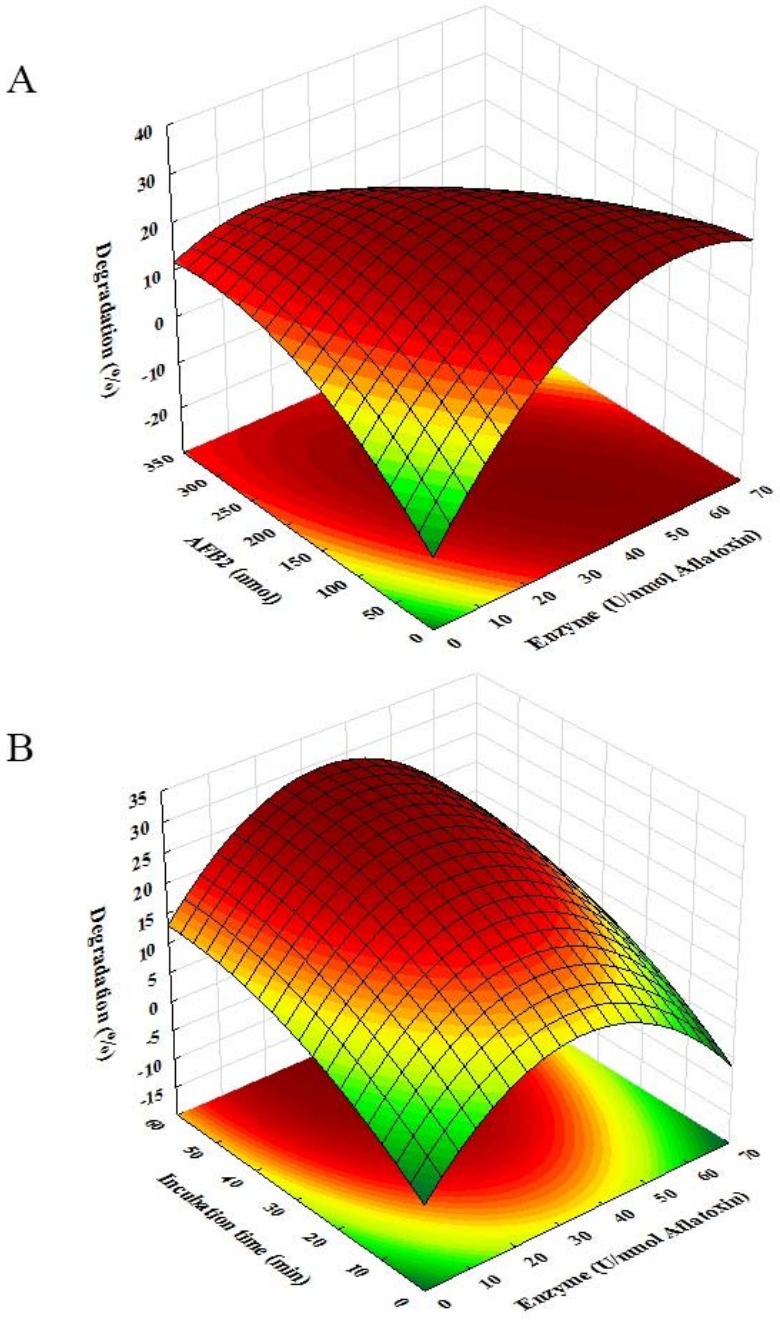

Figure 5.2. Response surface 3-D plot showing the effects of the independent variables on AFB2 degradation. (A) Effect of enzyme concentration (U/nM Aflatoxin) and AFB2 concentration (nM) while keeping incubation time (X_3) constant and (B) Effect of enzyme concentration (U/nM Aflatoxin) and incubation time (min) while keeping substrate concentration (X_2) constant.

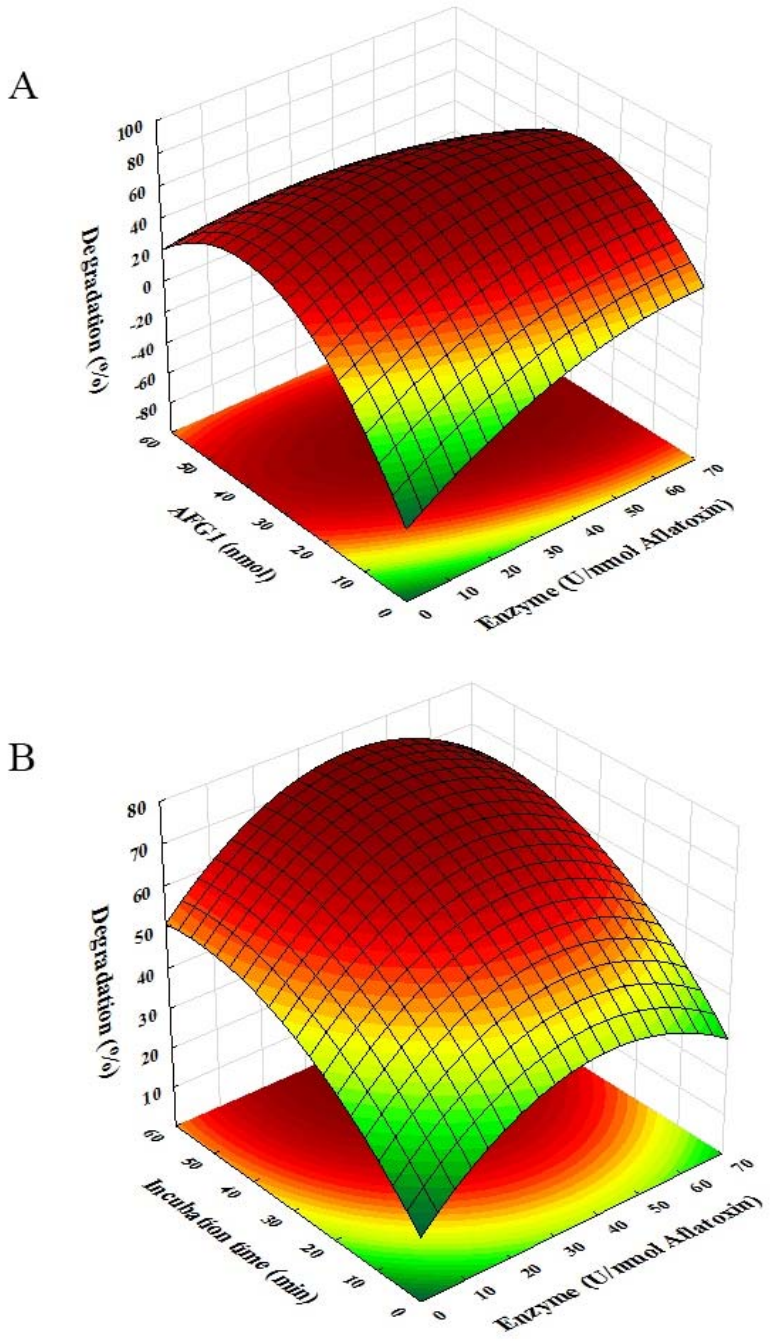

Figure 5.3. Response surface 3-D plot showing the effects of the independent variables on AFG1 degradation. (A) Effect of enzyme concentration (U/nM Aflatoxin) and AFG1 concentration (nM) while keeping incubation time (X_3) constant and (B) Effect of enzyme concentration (U/nM Aflatoxin) and incubation time (min) while keeping substrate concentration (X_2) constant.

The combined effects of enzyme concentration (X1) and incubation time (X3), at fixed AFG1 concentration (32.7 nM), on the response were investigated. Figure 5.3B shows that the AFG1 degradation increased when enzyme concentration increased up to 50 U/nM aflatoxin. In addition, AFG1 degradation increased with the concomitant increase in incubation time, where it increased from 49.5 to 76.4% when incubation time increased from 4.8 to 55.2 min. However, further increases in enzyme concentration didn't increase the percentage of degradation. The optimum levels for X1 and X3 obtained at fixed value of X2 were near to 31.5 U/nM aflatoxins and 55.2 min, respectively. The overall results suggest that maximum aflatoxin AFG1 degradation (76.4%) can be obtained by optimized conditions, including enzyme concentration of 31.5 U/nM aflatoxin, substrate concentration of 32.7 nM and incubation time of 55.2 min.

5.6.1.2.4. Aflatoxin AFG2 Degradation

The combined effects of enzyme concentration (X1) and AFG2 concentration (X2) on the AFG2 degradation, at fixed incubation time (30 min), were investigated. Figure 5.4A shows that the highest AFG2 degradation was obtained with an enzyme and AFG2 concentrations around to 40 U/nM aflatoxin and 45 nM, respectively. However, further increases or decreases of these two variables resulted in lower AFG2 degradation. The optimum values for maximum AFG2 degradation were near to 31.5 U/nM AFG2 and 42.2 nM for variables X1 and X2, respectively, with response values of 100%.

The combined effects of enzyme concentration (X1) and incubation time (X3), at fixed AFG2 concentration (42.2 nM), on the response were investigated. Figure 5.4B shows that the AFG2 degradation increased with that in enzyme concentration up to 40 U/nM aflatoxin. In addition, the degradation increased with the concomitant increase in incubation time. However, further increases in enzyme concentration decreased the percentage of degradation. The optimum levels for X1 and X3 obtained at fixed value of X2 were near to 31.5 U/nM aflatoxin and 55.2 min, respectively. The overall results suggest that the complete aflatoxin AFG1 degradation (100%) can be obtained by optimized conditions, including enzyme concentration of 31.5 U/nM aflatoxin, substrate concentration of 42.2 nM and incubation time of 55.2 min.

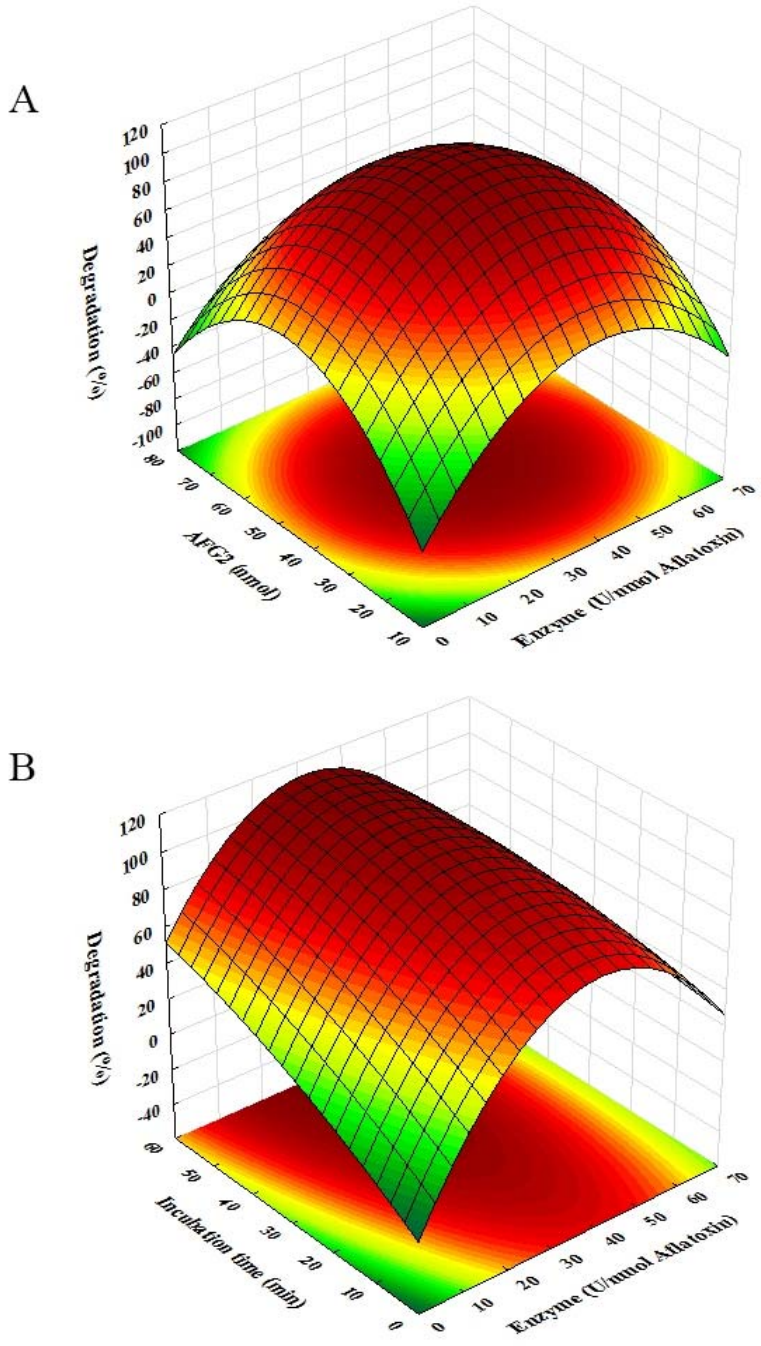

Figure 5.4. Response surface 3-D plot showing the effects of the independent variables on AFG2 degradation. (A) Effect of enzyme concentration (U/nM Aflatoxin) and AFG2 concentration (nM) while keeping incubation time (X_3) constant and (B) Effect of enzyme concentration (U/nM Aflatoxin) and incubation time (min) while keeping substrate concentration (X_2) constant.

Kong *et al*, (2012) reported that RSM was an effective method to optimize the degradation conditions of AFB1 by *Rhodococcus erythropolis* in liquid culture. They show that selected parameters, including temperature, pH, liquid volume and incubation time, had significant effects on the degradation of AFB1. An increase in liquid volume, suggesting an increase in bacteria growth, increased the degradation of AFB1. In addition, the degradation of AFB1 increased with the concomitant increase in incubation time. Tripathi and Mishra (2011) reported that peroxidase and aflatoxin AFB1 concentrations had significant effects on AFB1 degradation; the increase in both enzyme and substrate concentrations resulted by the increase of AFB1 degradation up to certain level, before its decrease. They also showed that AFB1 degradation increased with incubation time up to 24 h. Das and Mishra (2000) reported that pure commercial horse radish peroxidase degraded AFB1 up to 60%. In addition, partially purified peroxidase enzymes, from freshly harvested radish root, at two different concentrations 20 and 30 U/mg of protein, decreased AFB1 by approximately 30 and 38%, respectively. They studied the effect of enzyme and aflatoxin AFB1 concentrations as well as the incubation time. These authors showed that an increase in AFB1 concentration, up 1 mM, increased AFB1 conversion. Beyond this substrate concentration, no significant reaction was observed. They also demonstrated that AFB1 degradation increased with that in enzyme concentration, where an enzyme concentration of 2 U/mM of substrate was considered as the optimum. In addition, the degradation of AFB1 increased with the concomitant increase in incubation time, up to 60 min. Arzandeh and Jinap (2011) reported that time and aflatoxins concentration were the key factors affecting the aflatoxins reduction, where an increase in both time and substrates concentration was accompanied by an increase in aflatoxins AFB1 and AFB2 reduction. They also indicated that aflatoxins concentration had positive effects on AFG1 and AFG2 degradation. However, the time was not shown to be significant. Several enzymatic extracts, purified from microbial systems, have been used to degrade the aflatoxins (Wu *et al*., 2009). Alberts *et al*. (2009) reported that AFB1 was degraded by 87.34% when pure laccase, from *Trametes versicolor,* was used for 72h at 30ºC. Wang *et al*. (2011) also reported that a manganese peroxidase, from *Phanerochaete sordid* YK-624, was able to degrade 86% of AFB1 in 48 h. Motomura *et al*. (2003) showed that an enzyme, obtained from *Pleurotus ostreatus,* reduced AFB1 by 50% at 25ºC. Liu *et al*. (2001) reported that a purified enzyme, from *Armillariella tabescens*, called Aflatoxin Detoxifizyme (ADTZ) had the capacity to degrade and detoxify AFB1.

5.7. Conclusion

The overall experimental findings suggested that laccase from *C. hirsutus* can be efficiently used to degrade the major aflatoxin isoforms from *A. parasiticus* up to 38.2, 30.1, 76.4 and 100% of degradation, under the optimized conditions, for AFB1, AFB2, AFG1 and AFG2, respectively. Using RSM analysis, the three variables, enzyme concentration, aflatoxins concentration and incubation time, had linear and quadratic effects on aflatoxins degradation at significant level. In addition, the model equations are adequate for predicting the degradation percentage of aflatoxins under certain combination of values of the variables. Experimental design coupled with RSM can be successfully used to optimize the enzymatic degradation of aflatoxins and have great potential for practical applications.

CHAPTER VI

ARTICLE V

STRUCTURAL CHARACTERIZATION AND PROPOSED MECHANISMS FOR THE AFLATOXINS DEGRADATION BY A SELECTED MICROBIAL LACCASE

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6.1. Contribution of the Authors

The present author, Sabrina Borgomano, was responsible for the concepts, the designs and the fulfillment of the experimental work and the preparation of this manuscript for its submission.

Dr. Monique Lacroix, the thesis supervisor, and Dr. Selim Kermasha, the thesis supervisor, supervised the research work, provided valuable input and advices, monitored the progress of the work and critically reviewed and edited the manuscripts, prior to its submission.

Dr. Richard St-Louis carried out the LC/MS structural analysis of aflatoxins and their enzymatic degradation products and contributed to the analysis of data.

Dr. Varoujan Yaylayan contributed to the FTIR analysis.

6.2. Résumé

La dégradation des aflatoxines par une laccase partiellement purifiée (LPP), du champignon à la pourriture blanche *Coriolus hirsutus*, a été étudiée. En utilisant les principales formes des aflatoxines, AFB1, AFB2, AFG1 et AFG2, comme substrats pour la LPP, les aflatoxines résiduelles ainsi que les produits finaux de dégradation ont été analysés par chromatographie liquide à haute performance en phase inverse (RP-HPLC). Les résultats ont montré un taux de dégradation de 38,2, 30,1, 76,4 et 100% pour les aflatoxines AFB1, AFB2, AFG1 et AFG2, respectivement, après 55 minutes de réaction. La caractérisation structurale des aflatoxines purifiées provenant d'*Aspergillus parasiticus* et leurs produits finaux issus de la dégradation enzymatique a été réalisée, en utilisant la spectroscopie infrarouge à transformée de Fourier (IRTF) et la spectrométrie de masse (SM). Les résultats ont montré que la dégradation des aflatoxines a entraîné la formation de plusieurs produits finaux, dont le plus abondant était le pic de l'ion moléculaire m/z à 327,254, 205,069, 205,069 et 196,656 pour les aflatoxines AFB1, AFB2, AFG1 et AFG2, respectivement. Les analyses IRTF et SM ont suggéré que les mécanismes de la dégradation enzymatique des aflatoxines pouvait être l'époxydation, l'hydroxylation, l'*O*-déméthylation, la déshydrogénation, la déshydratation, la réduction de la double liaison et la céto-réduction ainsi que la perte de la cétone, de l'oxygène, du carbone et du méthyle qui pourraient conduire à la modification soit du cycle furofuran, de la coumarine ou du cycle lactone, ainsi qu'à la formation de produits non toxiques.

6.3. Abstract

The aflatoxins degradation by a partial purified laccase (PPL), from white-rot fungi *Coriolus hirsutus,* was investigated. Using the major aflatoxin isoforms, AFB1, AFB2, AFG1 and AFG2, as substrates for the PPL, the residual aflatoxins as well as the degradation end products were analyzed by reversed-phase/high-performance liquid chromatography (RP-HPLC). The results showed a degradation rate of 38.2, 30.1, 76.4 and 100% for AFB1, AFB2, AFG1 and AFG2, respectively, after 55 min of reaction. The structural characterization of the purified aflatoxins from *Aspergillus parasiticus* and their enzymatic degradation end products was investigated, using of Fourier-transform infrared spectroscopy (FTIR) and mass spectrometry (MS). The results showed that the degradation of the aflatoxins resulted in the formation of a wide range of several end products, where the most abundant one was the molecular ion peak at m/z 327.254, 205.069, 205.069 and 196.656 for AFB1, AFB2, AFG1 and AFG2, respectively. The FTIR and MS analyses suggested that the mechanisms of aflatoxins enzymatic degradation could be the epoxydation, hydroxylation, *O*-demethylation, dehydrogenation, dehydratation, reduction of the double bond and keto-reduction a well as the loss of ketone, oxygen, carbon and methyl molecules which could lead to the modification of either furofuran moiety, coumarin or the lactone ring as well as to the formation of nontoxic products.

Keywords: Aflatoxins, laccase, degradation, structural analyses, mechanism of degradation

6.4. Introduction

Aflatoxins, a group of mycotoxins, are highly toxic, mutagenic, teratogenic, and carcinogenic. They are produced as secondary metabolites via the polyketide pathway by fungi belonging to several species of *Aspergillus*, especially *A. flavus*, *A. parasiticus*, and are the most potent in food and feed products (Bhat *et al*, 2010). Aflatoxins are an assembly of a coumarin and 3-furan (difurano-coumarin derivatives), where aflatoxins AFB1, AFB2, AFG1 and AFG2 are the major isoforms in food and feed products. The presence of aflatoxins in foods and feedstuffs is a serious concern for human and animal health as well as for an economical impact (Van Egmont *et al.,* 2007).

Although many physical and chemical treatments can destroy aflatoxins in contaminated food products by the removal or the inactivation of the toxin, they can still reduce the nutritional value of food or produce toxic compounds and harmful residues that can potentially affect public health. The use of biological methods could be a potential appropriate approach for agro-industrial processes and the safety as well as the nutritional quality (Kabak *et al*., 2006). This approach is based on the degradation of complex organic aromatic compounds, such as lignin, which is probably the most abundant polyphenolic xenobiotics (Alberts *et al*., 2009). The fungus known as basidiomycetes has the potential to degrade lignin and a wide range of polycyclic aromatic hydrocarbons as well as other carcinogens such as aflatoxins (Christian *et al*., 2005). Among the enzymes produced by basidiomycetes, laccase could have the ability to detoxify aflatoxins (Alberts *et al*., 2009). Laccase (hydroquinone: oxygen oxidoreductase, EC 1.10.3.2) a polyphenol oxidase, which belongs to the family of multi-blue copper protein (Baldrian, 2006), is an industrially relevant enzyme where it plays an important role in the carbon cycle degradation and may contribute to the degradation of a wide range of compounds, including xenoaromatics and lignin as well as the oxidation of phenolics compounds (Madhavi and Lele, 2009). Due to their broad substrate specificity and great power catalyst as well as to their ability to use oxygen in the environment as a cofactor, fungal laccases are used in a wide range of biotechnological and industrial applications (Myasoedova *et al*., 2008).

The aim of the present study was to investigate the degradation of selected aflatoxins by partial purified laccase (PPL) from *Coriolus hirsutus* and their structural characterization. In addition, the structural characterization of the degradation end products of aflatoxins AFB1, AFB2, AFG1

and AFG2, from *Aspergillus parasiticus,* was also carried out using Fourier transform infrared spectroscopy (FTIR) and masse spectroscopy (MS).

6.5. Materials and Methods

6.5.1. Organism and Culture conditions of Coriolus Hirsutus and Aspergillus Parasiticus

Coriolus hirsutus (MYA-828), used for the production of laccase, was obtained from the American Type Culture Collection (ATCC, Manassas, VA.). *C. hirsutus* was maintained onto malt agar media plates and incubated at 20ºC. For laccase production a basal liquid medium was prepared, using the method previously described by Taqi (2012).

Aspergillus parasiticus (26864), used for the production of aflatoxins, was obtained from the American Type Culture Collection (ATCC; Manassas, VA). *A. parasiticus* was maintained through periodic transfer onto potatoes dextrose agar media plates and incubated at 24ºC; for aflatoxins production, a basal liquid medium for strain was prepared using the method previously described by Hsieh and Mateles (1971).

6.5.2. Preparation of Laccase Extract

Laccase enzymatic extract was obtained according to the procedure described by Taqi (2012). The mycelium pellets from culture liquid medium were removed throughout the use of a cheese-cloth filtration. The culture medium was ultra-filtered and partially purified by 80% saturation of ammonium sulphate precipitation.

6.5.3. Aflatoxins Preparation

The fermentation process of *A. parasiticus* was carried out according to a modification of the method of Hsieh and Mateles (1971) developed in our laboratory and previously described in chapter III. The mycelium pellets from culture liquid medium were removed throughout a cheese-cloth filtration. The extracellular toxins were recovered from the culture medium by ultra-filtration, according to the method of chapter III.

6.5.4. Aflatoxins Degradation by Partial Purified Laccase

Laccase reaction was performed using 7 mL of reaction mixture containing 1.5 mL of aflatoxins or 96.2 nM of AFB1, 191 nM of AFB2, 32.7 nM of AFG1 and 42.2 nM of AFG2, and 0.5 mL of stock solution of partial purified laccase containing 0.04 g/mL (0.32 mg/mL of protein). The reaction medium was adjusted with citrate phosphate buffer (0.1 M, pH 6.0) to a total volume of

7 mL. The reaction was initiated by the enzyme addition and was carried out at 57.5ºC. The reaction mixture was stirred at 100 rpm and incubated up to 55 min. The reaction was stopped by adding 7 mL of chloroform and the degradation rate of aflatoxins was determined at the end of every incubation period.

6.5.5. Aflatoxins Standards Degradation by Partial Purified Laccase

Laccase reactions using aflatoxin standards were performed. Stock solutions of 1 µM of each aflatoxin were prepared by their solubilisation in methanol 99%. The enzymatic reactions containing, 67.3, 133.7, 22.9 and 29.6 µL of AFB1, AFB2, AFG1 and AFG2, respectively, were initiated by the addition of 50 µL enzyme suspension (0.32 mg/mL of protein). The reaction media were adjusted with citrate phosphate buffer (0.1 M, pH 6.0) to a total volume of 700 µL. The enzymatic reactions were carried out at 57.5ºC, during 55 min under agitation at 100 rpm.

6.5.6. Extraction and Quantification of Aflatoxins and Their Enzymatic Degradation End Products by RP-HPLC

Following incubation at 57.5ºC, aflatoxins and their end products were extracted three successive times from the samples with chloroform (1:1, v/v) as described by Teniola *et al*. (2005). The three chloroform extracts were combined and evaporated under nitrogen. The samples were dissolved in methanol 99%, filtered and analyzed by reversed-phase/high-performance liquid chromatography (RP-HPLC). RP-HPLC analysis was performed to characterize and to quantify the major aflatoxin isoforms, AFB1, AFB2, AFG1 and AFG2 as well as their degradation end products, using a Beckman Gold system 126 (Beckman Instrument Inc., San Raman, CA), equipped with a UV diode-array detector (DAD), set at 365 nm (Beckman, model 168). A 20 µL sample was injected into an allsphere C18 column (4.6 mm x 250 mm, 5 µm; Alltech, Deerfield, IL), thermostated at 50°C. The isocratic solvent elution was a mixture of water/methanol (40:60, v/v), at a flow rate of 1 mL/min. The percentage of degraded aflatoxins was calculated using the equation (6-1).

(1 - Area peak of treated aflatoxins/Area peak of untreated aflatoxins)/100 …..…... (6-1)

6.5.7. Purification of Aflatoxins and Their Enzymatic Degradation End Products by HPLC

At the end of 55 min of reaction, the aflatoxins and their end-products were extracted three successive times from the samples with chloroform (1:1, v⁄v) as described by Teniola *et al*. (2005). The three chloroform extracts were combined and evaporated under nitrogen, and the samples were dissolved in methanol, filtered and each compound was purified by high-performance liquid chromatography (HPLC). HPLC analysis was performed to purify the major aflatoxin isoforms, AFB1, AFB2, AFG1 and AFG2 as well as their degradation end products using a Beckman Gold system 126 (Beckman Instrument Inc., San Raman, CA), equipped with a UV diode-array detector (DAD), set at 365 nm (Beckman, Model 168). A 500 µL sample was analyzed and purified on a Zorbax SB-C18 column (9.4 mm x 250 mm, 5 µm) thermostated at 50°C. The isocratic solvent elution was a mixture of water/methanol (40:60, v/v), at a flow rate of 2 mL/min.

6.5.8. Structural Characterization of Aflatoxins and Their Enzymatic Degradation Products

6.5.8.1. Fourier-Transform Infrared Analyses

Fourier-transform infrared (FTIR) analysis was carried out as a tool for the elucidation of the structure of the laccase-catalyzed end products. The recovered purified end products were placed on the ATR crystal and immediately scanned, at room temperature, and their infrared spectra were acquired, using a Bruker Alpha-P spectrometer (Bruker Optic GmbH; Ettlingen, GE), equipped with a detracted triglycine sulfate (DTGS) detector, a temperature-controlled, single-bounce diamond ATR crystal and a pressure application device for solid samples. A total of 32 scans at 4 cm-1 resolution were co-added. Processing of the FTIR data was performed with Bruker OPUS software.

6.5.8.2. Mass-spectroscopy Analysis

The samples were prepared in methanol and the compounds were separated by liquid chromatography (Accela system, ThermoFisher) and detected by UV/VIS and high resolution mass spectrometry (LTQ Orbitrap Discovery, ThermoFisher) after ionization of the column effluent in an ESI source. The LC column was a Zorbax SB-C18 (2.1 mm x 75 mm, 3.5 µm) with an Orbit C18 pre-column. Isocratic elution was performed with a mixture of 30% methanol containing 0.1% acid formic and 70% of a mixture of 90% isopropanol and 10% acetonitrile, containing 20 mM of NH_4COOH. The elution was carried out with 0.2 mL/min.

6.5.9. Statistical analysis

Data were expressed as means of triplicate trials and their respective standard deviations (SD). The percent relative standard deviation (RSD) was calculated as the SD divided by the mean multiplied by 100. Statistical analyses were performed, using STATISTICA 10.0 (StatSoft, Tulsa, OK). A post hoc comparison was made, using Tukey's test. The results were considered to be statistically different at $P \leq 0.05$.

6.6. Results and Discussion

6.6.1. Degradation of aflatoxins by PPL

The biological degradation of the most common and potent isoforms of aflatoxins, AFB1, AFB2, AFG1 and AFG2, by PPL, over 55 min of treatment, was investigated. Figure 6.1 shows that after 55 min of reaction in presence of 16 μg of protein, the aflatoxins AFB1, AFB2, AFG1 and AFG2, were significantly ($P \leq 0.05$) reduced by 38.2, 30.1, 76.4 and 100%, respectively. Table 6.1 also indicates that the peak area of each aflatoxin was significantly ($P \leq 0.05$) reduced.

These experimental data are in agreement with those of Alberts *et al*. (2009), who reported that AFB1 was degraded by 87.34%, when pure laccase, from *Trametes versicolor*, was used for 72 h at 30ºC. Cao *et al*. (2011) demonstrated that aflatoxin-oxydase (AFO), from *Armillariella tabescence*, degraded AFB1 by the cleavage of bis-furan ring. Tripathi and Mishra (2011) reported that a peroxidase, from garlic bulb of *Allium sativum*, degraded AFB1 by 70% in 26 h. Wang *et al*. (2011) also reported that a manganese peroxidase, from *Phanerochaete sordid* YK-624, was able to degrade 86% of AFB1 in 48 h. Motomura *et al*. (2003) showed that an enzyme, obtained from *Pleurotus ostreatus*, reduced AFB1 rate by 50% at 25ºC.

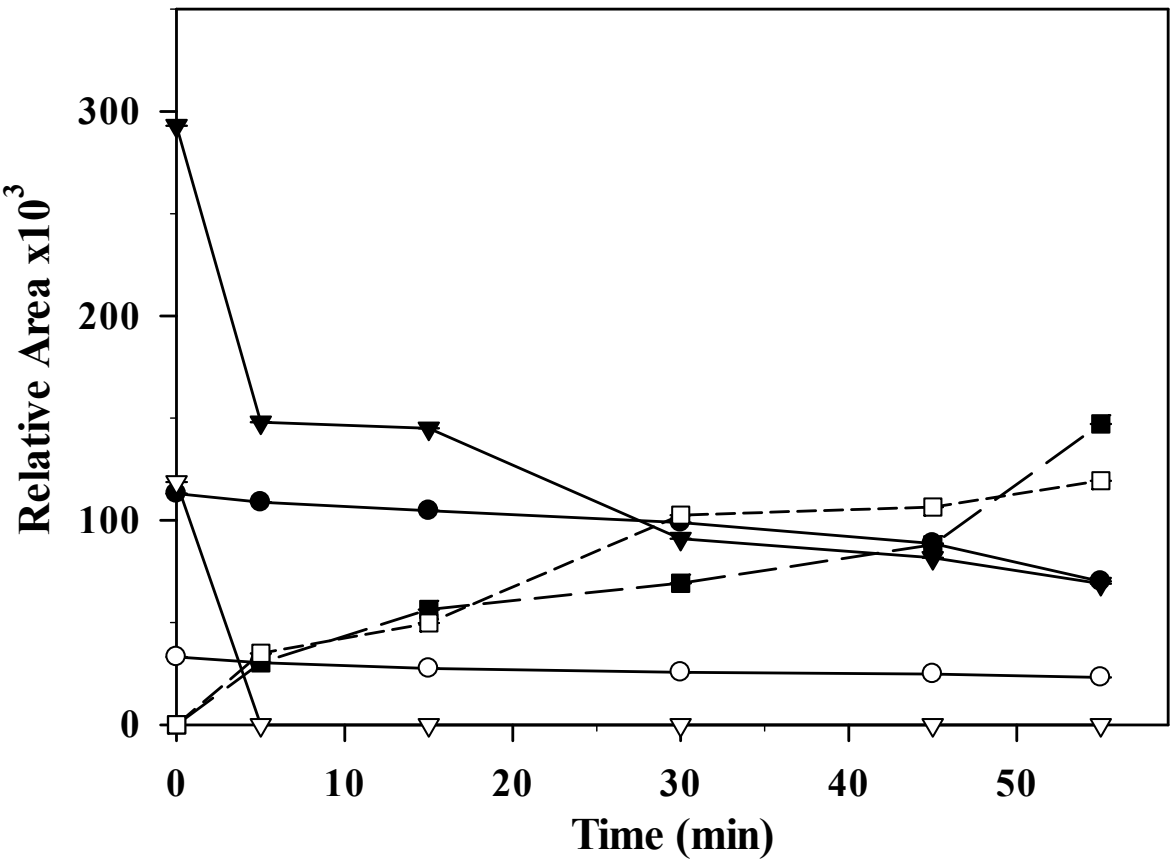

Figure 6.1. Degradation of aflatoxins, AFB1 (●), AFB2 (○), AFG1 (▼) and AFG2 (▽), from *Aspergillus parasiticus,* with the partial purified laccase from *Coriolus hirsutus* over 55 min. The symbols ■ & □ correspond to the formation of the enzymatic degradation products during laccase treatment.

Table 6.1. HPLC analysis of the degradation of aflatoxins at 365 nm obtained by liquid culture media of *Aspergillus parasiticus*, using the partial purified laccase from C*oriolus hirsutus*.

Time (min)[b]	Relative Peak Area[a]						
	AFB1[c]	AFB2[c]	AFG1[c]	AFG2[c]	U_1[d]	U_2[d]	U_3[d]
0	113,07 (0.4)[f]	33,13 (2.8)[f]	293,07 (0.2)[f]	118,70 (0.2)[f]	146,78 (0.6)[f]	Nd[e] (0.0)[f]	Nd[e] (0.0)[f]
5	108,82 (7.2)	30,33 (6.3)	147,96 (1.5)	Nd[e] (0.0)	165,76 (1.8)	30,33 (0.2)	34,90 (4.5)
15	104,65 (0.4)	27,50 (0.3)	144,97 (1.2)	Nd[e] (0.0)	15,75 (1.5)	56,31 (1.1)	49,71 (1.2)
30	98,95 (2.5)	25,64 (0.1)	91,06 (0.1)	Nd[e] (0.0)	4,36 (6.8)	69,19 (2.9)	102,46 (2.3)
45	88,68 (1.5)	24,88 (3.6)	81.62 (1.6)	Nd[e] (0.0)	20,89 (3.2)	88,00 (2.1)	106, 143 (.7)
55	70,14 (0.6)	23,14 (0.1)	69,04 (2.6)	Nd[e] (0.0)	14,43 (1.4)	147,09 (2.6)	119,32 (1.2)

[a]Mean of peak area, calculated from the values of triplicate samples. Results obtained from reversed-phase high performance liquid chromatography (RP-HPLC) using an allsphere column C18 thermostated at 50°C. The mobile phase used was a mix of methanol/water (60/40; v/v), using a flow rate of 1 mL/min.

[b]Time reaction in minute of partial purified laccase activity using the aflatoxins from *A. parasiticus* as substrates.

[c]Aflatoxins issued from fraction IIb obtained by ultrafiltration (1 *k*Da) of the crude exo-culture media of *A.parasiticus.*

[d]Unknown products, the enzymatic degradation end products of aflatoxins AFB1, AFB2, AFG1 and AFG2.

[e]Not determined, because the peak area was below the detection limit or not repeatable.

[f]Standard deviation (SD) was calculated as the standard deviation of triplicate samples.

Liu *et al*. (2001) reported that a purified enzyme, from *Armillariella tabescens*, called aflatoxin detoxifizyme (ADTZ), had the capacity to degrade and detoxify AFB1 by undergoing an opening in its difurane cycle, making it less toxic; these authors (1998) proposed a pathway of the degradation of AFB1 by multienzyme complex from *A. Tabescens*, where the first step is the transformation of AFB1 to AFB1-epoxide, followed by the hydrolysis of the epoxide to give the dihydrodiol, and then the difuran ring could open in the subsequent hydrolysis step. Das and Mishra (2000) reported that an enzymatic treatment of AFB1 with a peroxidase, from horseradish, resulted by its decrease to 53 and 60%, in groundnut meal and liquid culture, respectively. Guengerich *et al*. (1998) indicated that several cytochrome P450 enzymes, such as P450 3A4, oxidize AFB1 into less toxic products. In addition, Singh (1997) demonstrated that microsomal peroxidases temperature-dependent were able to degrade aflatoxins. Doyle and Marth (1979) indicated a direct correlation between the amount of peroxidases produced by *Aspergillus parasiticus* and the AFB1 degradation. Laccase is nonspecific and non-stereoselective enzyme that could not only oxidizes phenolic compounds and degrades lignin but also acts on a very broad variety of substrates (Wang *et al*., 2011). The overall results suggest that laccase from *C. hirsutus* could be applied to the degradation of aflatoxins AFB1, AFB2, AFG1 and AFG2, which are difurocoumarin derivates and non-polyphenolic compounds, and thus avoiding the problems associated with the use of micro-organisms who can change the flavor, the nutritional value of the food products and the acceptability of the products without the production of toxic compounds (Wu *et al*., 2009).

6.6.2. HPLC Characterisation of Microbial Aflatoxins and Their Enzymatic Degradation Products

The residual aflatoxins from AFB1, AFB2, AFG1, AFG2, and their enzymatic degradation products, were analyzed by reversed-phase/high-performance liquid chromatography (RP-HPLC). Figure 6.2 shows the HPLC profile of each sample over the reached time. At time 0 min, the HPLC chromatogram shows that peaks 2, 3, 4 and 5, eluted respectively at 2.3, 3.2, 5.0 and 5.5 min, corresponded to the aflatoxins AFG2, AFG1, AFB2 and AFB1, respectively. After 55 min of PPL treatment, the peak area of each aflatoxin significantly ($P \leq 0.05$) decreased. The results also indicate that new peaks 1' and 2', eluted respectively at 2.6 and 2.8 min, corresponded to the enzymatic degradation products.

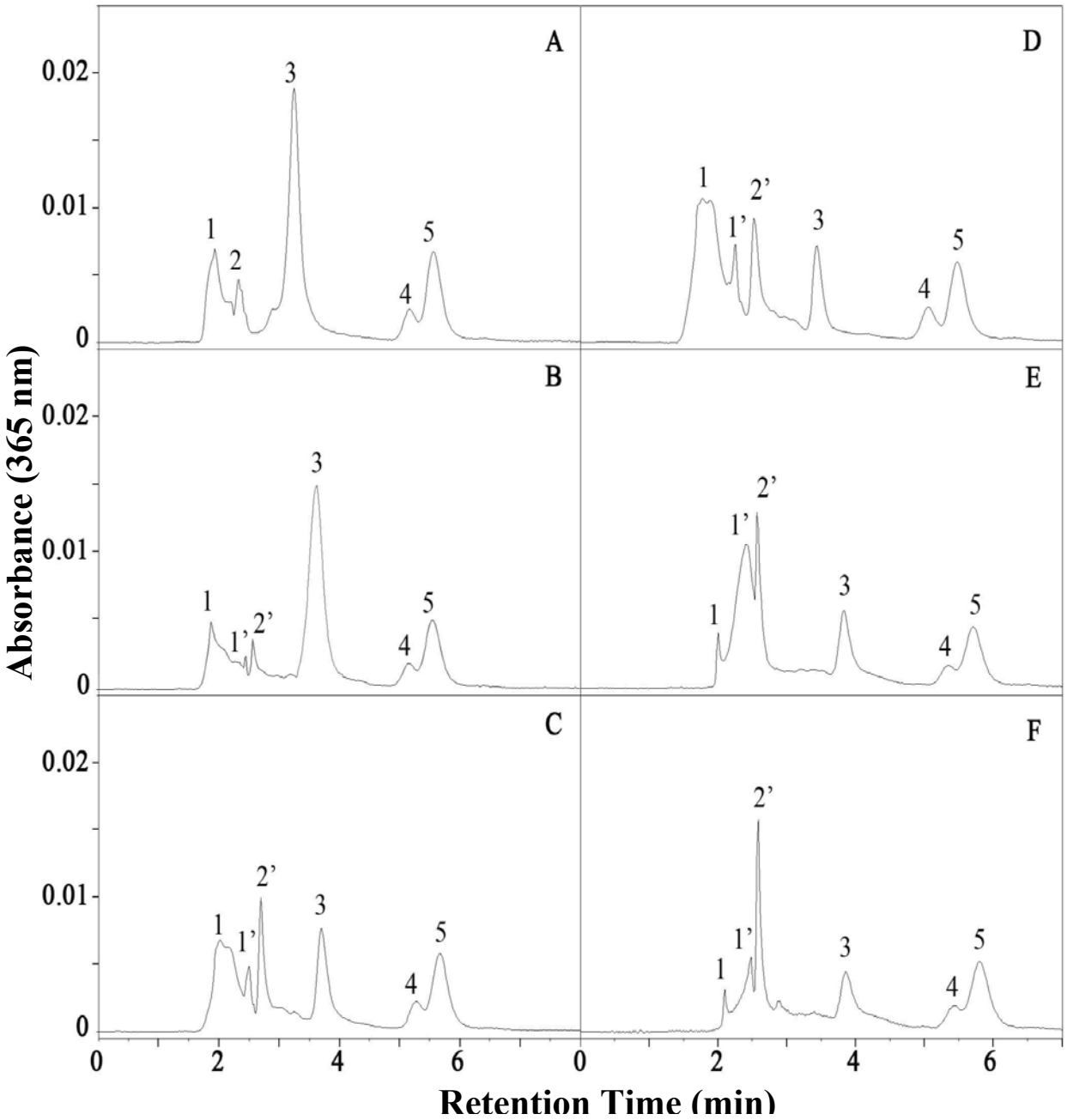

Figure 6.2. Chromatograms of HPLC analysis at 365 nm of the laccase reaction mixture containing aflatoxins AFB1 peak #5, AFB2 peak #4, AFG1 peak #3 & AFG2 peak #2 and their degradation products peaks #1, 1' and 2'. The laccase reaction was the incubation of the aflatoxins, AFB1, AFB2, AFG1 and AFG2, from *A. parasiticus,* with the partial purified laccase as well as the citrate phosphate buffer (0.1M, pH 6.0 at 57.5°C) over 55 min, (A) 0 min, (B) 5 min, (C) 15 min, (D) 30 min, (E) 45 min, (F) 55 min.

Figure 6.3 shows the elution profiles of the initial aflatoxin standards at time 0 and 55 min. The experimental results demonstrated that the peaks of standard aflatoxins, AFB1, AFB2, AFG1 and AFG2, were eluted respectively at 5.6, 5.3, 4.6 and 4.3, and significantly ($P \leq 0.05$) decreased after enzymatic treatment. The peaks b and c, eluted respectively at 2.5 and 3.2 min, corresponded to the enzymatic degradation products of the standard aflatoxins.

The experimental findings are in agreement with those of Samuel *et al*. (2014), who reported the reduction of AFB1 and the detection of its degradation products after treatment with *Pseudomonas putida*. Farzaneh *et al*. (2012) also indicated that after treatment of AFB1 by *Bacillus subtilis*, a different product that the parent one was detected. In addition, Wang *et al.* (2011) reported the presence of new metabolite generated by the action of the manganese peroxidase, from *Phanerochaete sordida* YK-624, on aflatoxin AFB1. Velazhahan *et al*. (2010) also reported the presence of new peaks after AFG1 degradation by seed extracts, from *Trachyspermum ammi*. The experimental results suggest the degradation of the major aflatoxin isoforms, AFB1, AFB2, AFG1 and AFG2, by PPL and their conversion into other products having different chemical properties that the parent ones (Albert *et al*., 2006). In addition, there may be more than one compound formed during degradation (Samuel *et al*., 2014).

6.6.3. Fourier-Transform Infrared Analysis

Fourier-transform infrared (FTIR) analysis was carried out as a tool for the elucidation of the structure of the laccase-catalyzed end products. Figure 6.4 shows that the aflatoxins, AFB1, AFB2, AFG1 and AFG2, exhibit the same characteristic absorption bands, since they have structural similarity designs, except for the bands corresponding to C=O (ketone) and C–O of the coumarin and furan rings, which show higher intensity in G aflatoxins. Diaz *et al*. (2012) indicated that there are two major differences between B and G aflatoxins. First, the presences of a six-membered lactone ring in the G-type aflatoxins instead of a pentenone ring in the B-type aflatoxins. Second, aflatoxins AFB1 and AFG1 have an additional unsaturated bond between carbons 8 and 9.

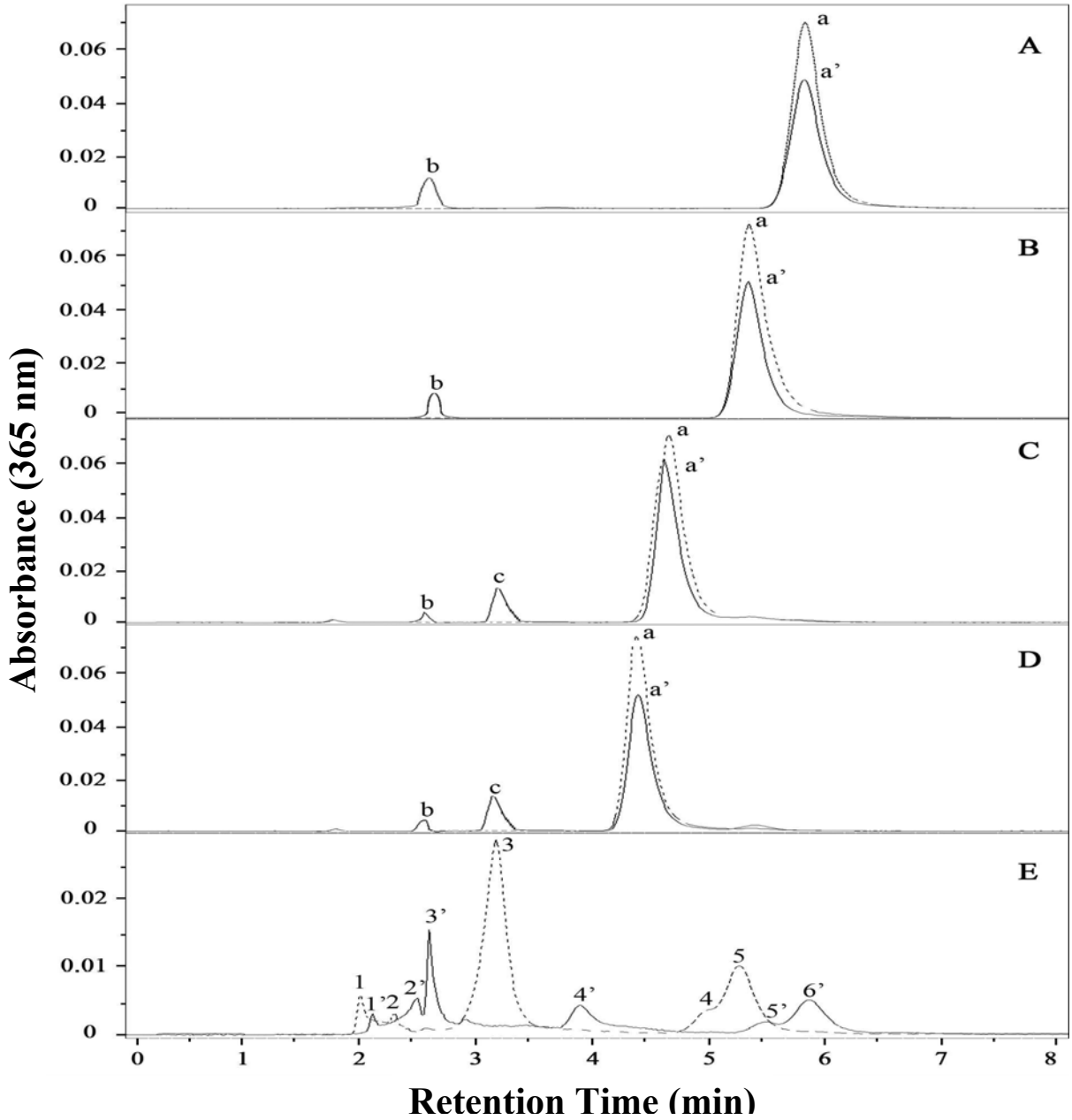

Figure 6.3. Chromatograms of HPLC analysis of aflatoxins standards and from *A. parasiticus* subjected to the degradation by laccase from *C. hirsutus.* (A) Aflatoxin B1 "AFB1", (B) Aflatoxin B2 "AFB2", (C) Aflatoxin G1 "AFG1", (D) Aflatoxin G2 "AFG2". Initial aflatoxins standards (a), residual ones (a') and products of aflatoxins degradation (b) & (c). (E) Aflatoxins from *A. parasiticus,* (-----) initial products from *A. parasiticus* with peaks # 1, 2, 3, 4 and 5 and (——) products from *A. parasiticus* after degradation with peaks 1', 2', 3', 4', 5' and 6'.

The aflatoxins IR spectra (Fig. 6.4) show weak intensity bands at wavelengths 3100–2850 cm^{-1} assigned to the stretching of CH_2, aromatic =CH, –C–H, C=C, –C–H of methoxy group and phenyls. The characteristic overtone bands from 2000 to 1770 were specific of aromatic meta-substituted ring. The strong peaks in the region between 1770 and 1650 cm^{-1} were attributed to the C=O stretching of the coumarin moiety (Arvide *et al*., 2008). The two peaks at 1610 and 1590 cm^{-1} were assigned to the aromatic C=C stretching. The peaks which appear at 1582 and 1425 cm^{-1} corresponded to the stretching bands of C bonded with two oxygens (COO) of the coumarin ring (Arvide *et al*., 2008). The two peaks in the in the region between 1485 and 1450 were specific of the skeletal vibration of C–C ring stretching. The region between 1400 and 1300 cm^{-1} was attributed to the C-H bending within the ring structure as well as that of the methyl group (O–CH3). Several infrared strong bands in the region between 1300 and 1000 cm^{-1} were assigned to the C-O stretching vibration bonds. These infrared bands indicated the presence of different compounds, including a carboxylate, a cyclopentanone and an aromatic structure. The region between 950 and 690 cm^{-1} was attributed to the C-H bending out of plane which can support the presence of an aromatic structure.

Figure 6.4 indicates that the IR spectra of the enzymatic degradation end products of the aflatoxins showed several differences in absorption peaks as compared to that of the substrate. On the other hand, the IR spectra of the four transformed aflatoxins show the same profil (Figs. 4A' to 4D'). A broad absorption band around 3400 cm^{-1} indicated the presence of hydroxyl functions (Van Cauwenberge *et al*., 2012; Samuel *et al*., 2014). Cole *et al*. (1972) reported the probable ketone hydroxylation of the coumarin moiety during biological treatment with *Rhizopus* spp. The increase of the absorption peak at 3100-2900 cm^{-1} as well as the reduction of the overtone bands absorption may suggested a modification of methyl group and aromatic rings (Guan *et al*., 2010). The two peaks in the area between 1770 and 1650 cm^{-1} in the aflatoxins spectra changed into one peak after treatment and the absorption peaks at 1610 and 1590 cm^{-1} were reduced. In addition, the absorption peak at 1582 cm^{-1} was also reduced and that at 1425 cm^{-1} disappeared after enzymatic treatment. Guan *et al*. (2010) indicated that these changes could be attributed to the modification of either coumarin, five-membered pentanone or the six-membered lactone rings. A new absorption peak appeared at 1375 cm^{-1}, characteristic of OH bending, suggesting the conversion of the methoxy group into hydroxyl group (Coates, 2000).

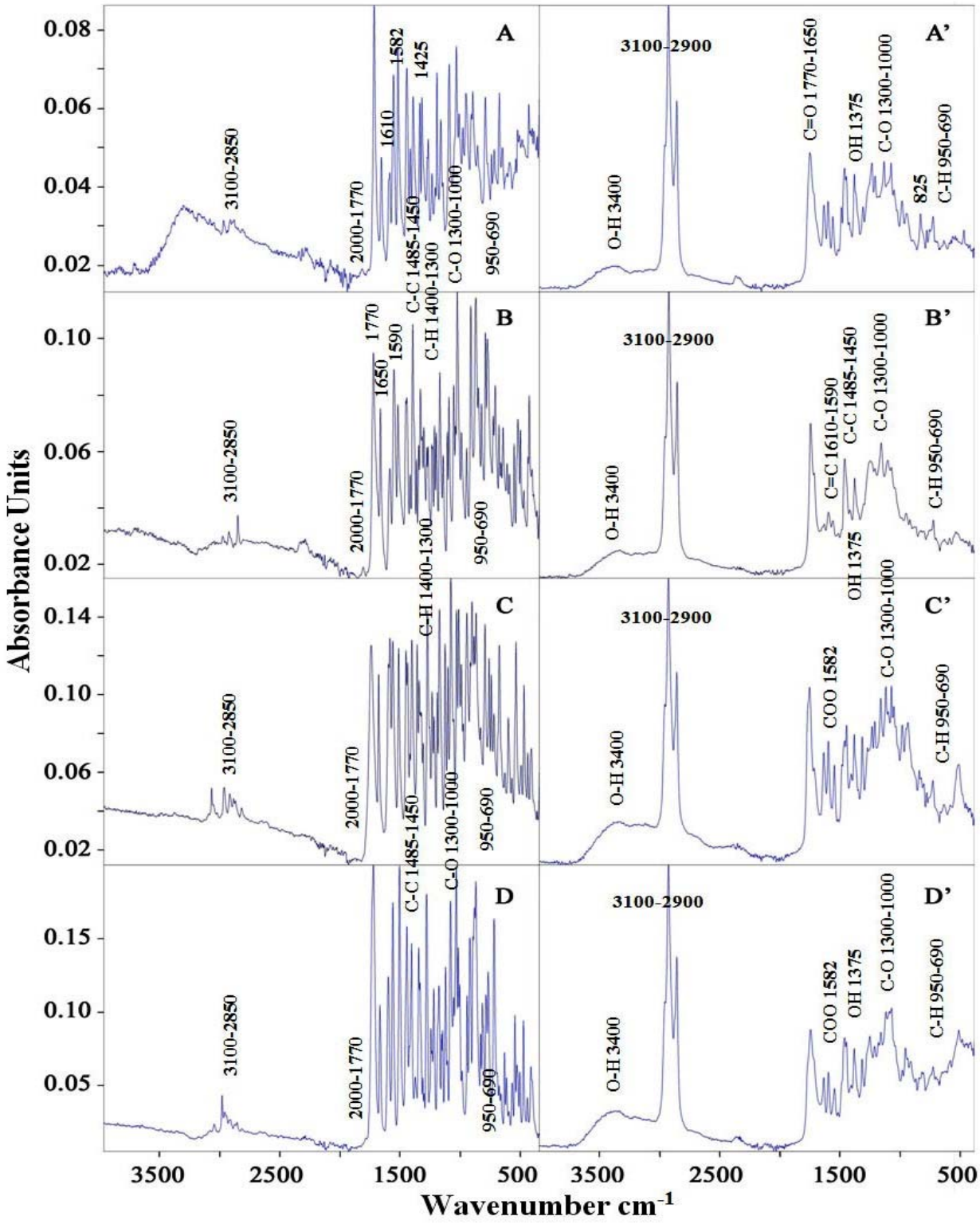

Figure 6.4. Fourier transform infrared spectroscopy (FTIR) spectra of the aflatoxins AFB1 (A), AFB2 (B), AFG1 (C), AFG2 (D) and the purified laccase-catalyzed end products of aflatoxins AFB1 (A'), AFB2 (B'), AFG1 (C'), AFG2 (D').

A modification of the absorption involving the C-O stretching bonds between 1300 and 1000 cm^{-1} was observed, where the reduction of the intensity and absence of bands may suggested an alteration of the furan rings (furofuran moiety) as well as that of the methoxy group after enzymatic treatment (Liu *et al*., 1998; Samuel *et al*., 2014). In addition, many bands referring to the C-H deformation vibration were observed in the region between 950 and 650 cm^{-1}, which suggests a modification of the aromatic rings (Al-Kadhemy and Rasheed, 2013). For the transformed aflatoxin AFB1, a new asymmetric peak appeared at 825 cm^{-1}, which could correspond to the stretching vibration of an epoxy group positioned on the furan ring.

Samuel *et al*. (2014) proposed that the lactone ring of AFB1 opened in the presence of *Pseudomonas putida,* which resulted in decarbonylation of AFB1. Mishra and Das (2003) indicated that fluorescence intensity of AFB1 treated with purified enzyme decreased, suggesting the enzymatic cleavage of the lactone ring. Wang *et al*. (2011) reported the oxidation of the 8,9-vinil bond of aflatoxin AFB1 by the manganese peroxidase, from *Phanerochaete sordida* YK-624, to form the AFB1-8,9-epoxide. Diaz *et al*. (2012) indicated that double bonds and functional groups of aflatoxins can undergo oxidation/reduction reactions. They also suggested that the six-membered lactone ring of G aflatoxins is more susceptible to the oxidation and the degradation than the five-membered pentanone ring of B aflatoxins. Wogan *et al*. (1971) reported that the furofuran moiety and the presence of the double bond in the terminal furan ring are responsible of the toxic and carcinogenic activities of the aflatoxins. Hence the modification of these functions could reduce the toxicity of toxins (Motomura *et al*., 2003; Wang *et al*., 2011). The overall findings suggest the transformation of the major aflatoxin isoforms by the laccase into less toxic compounds.

6.6.4. Mass Spectrometry Analysis

The characterization of laccase-catalyzed end products was investigated by liquid chromatography/mass spectrometry (LC/MS) analyses in order to identify their structure as well as to propose a mechanism for their degradation. As the author is aware there is little information on the enzymatic degradation of the major aflatoxin isoforms. The results (Table 6.2 and Fig. 6.5) show the emergence of several compounds, formed by laccase degradation of the major aflatoxin isoforms, used as substrates. The fragment products in the mass spectrum of aflatoxins, before degradation reaction corresponded to the mass fragments of the toxins obtained during their ionization in the MS.

Table 6.2. Mass of the major aflatotoxin isoforms and their degradation end products obtained from laccase catalysis using liquid chromatography-UV linear trap quadropole (LTQ) mass spectrometry.

Compound	Retention time[a]	Mass (m/z)[b]	Formula[c]
AFB1[d]	0.95	313.071	$C_{17}H_{13}O_6$
Transformed[e]	1.33	327.254	$C_{17}H_{11}O_7$
		285.076	$C_{16}H_{12}O_5$
		255.185	$C_{15}H_{11}O_4$
		196.863	$C_{10}H_{13}O_4$
		195.194	$C_{10}H_{11}O_4$
AFB2[a]	1.03	315.088	$C_{17}H_{15}O_6$
Transformed[e]	0.92	288.862	$C_{15}H_{13}O_6$
		254.900	$C_{15}H_{11}O_4$
		205.069	$C_{12}H_{13}O_3$
		186.913	$C_{13}H_{13}O_2$
AFG1[a]	0.94	329.066	$C_{17}H_{13}O_7$
Transformed[e]	1.05	288.862	$C_{15}H_{13}O_6$
		254.900	$C_{15}H_{11}O_4$
		205.069	$C_{12}H_{13}O_3$
		186.913	$C_{13}H_{13}O_2$
AFG2[a]	1.08	331.080	$C_{17}H_{15}O_7$
Transformed[e]	0.68	362.240	$C_{17}H_{14}O_9$
		340.258	$C_{16}H_{20}O_8$
		322.247	$C_{16}H_{18}O_7$
		227.174	$C_{14}H_{11}O_3$
		196.656	$C_{10}H_{13}O_4$
		195.028	$C_{10}H_{11}O_4$

[a]Retention time in minute. Results obtained from liquid chromatography (LC) using a Zorbax SB-C18 colum coupled with an Orbit C18 pre-column. The mobile phase used was a mix of 30% methanol containing 0.1% acid formic and 70% of a mixture of 90% isopropanol and 10% acetonitrile, containing 20 mM of NH4COOH. The elution was carried out with 0.2 mL/min.

[b]m/z of $[M+H]^+$ (ionized mass from the protonation of neutral mass M+H).

[c]Elemental composition of afltoxins and their degradation end products.

[d]Major aflatoxin isoforms, AFB1, AFB2, AFG1 and AFG2, from *A. parasiticus*, used as substrates for laccase reaction.

[e]Degradation end products of aflatoxins obtained from laccase catalysis.

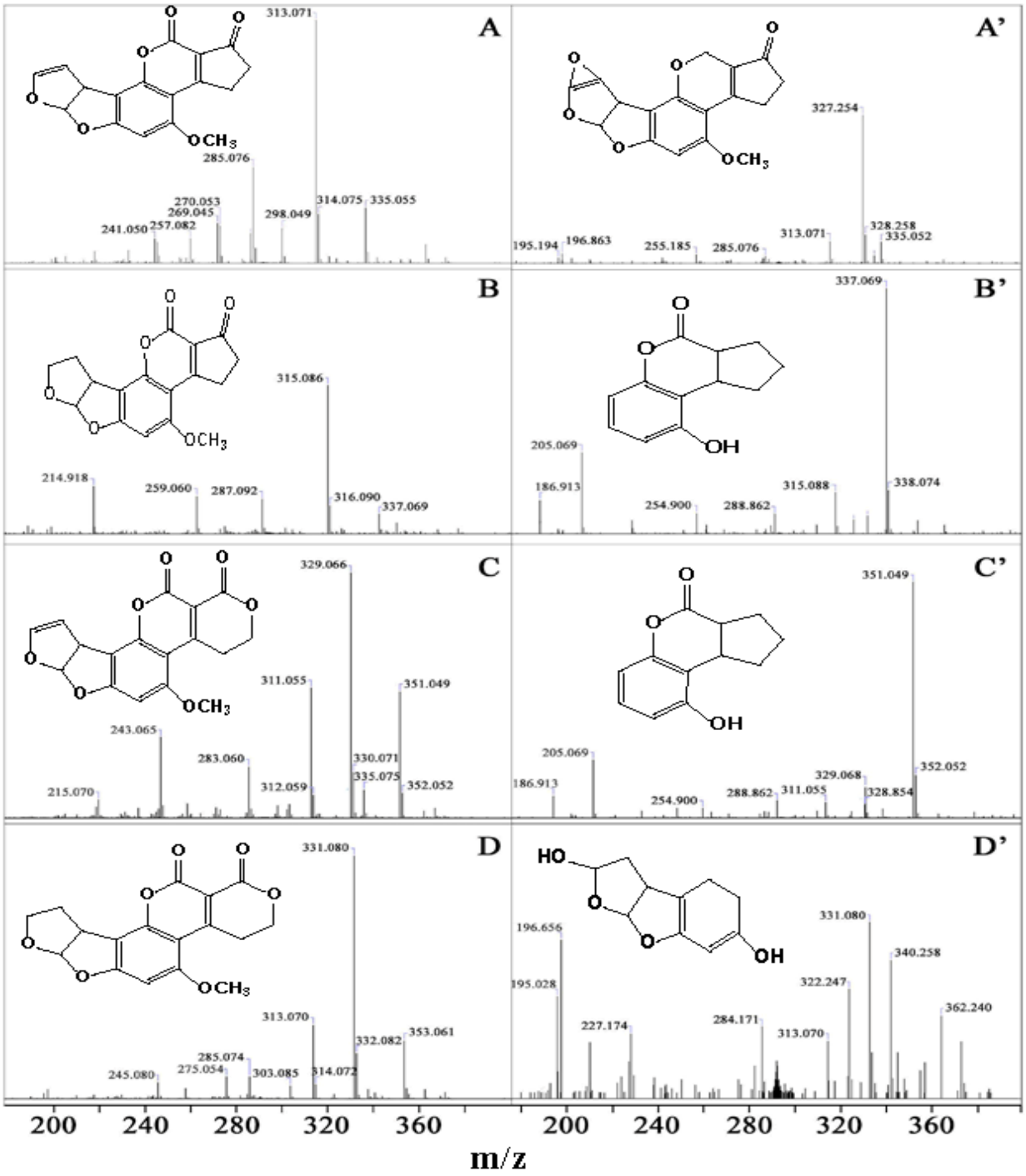

Figure 6.5. Liquid chromatography/mass spectrometry (LC/MS) chromatograms of the aflatoxins, AFB1 (A), AFB2 (B), AFG1 (C), AFG2 (D) and the purified laccase-catalyzed end product of aflatoxins AFB1 (A'), AFB2 (B'), AFG1 (C'), AFG2 (D').

The compounds with a mass pair in the spectrum may resulted from a rearrangement (α cleavage) or by the loss of small molecules, occurred during the electronic impact in the MS.

6.6.4.1. Aflatoxin AFB1 Degradation Pathways

The mass spectrum (Fig. 6.5A') of the laccase reaction, using aflatoxin AFB1 as substrate, shows intense peak values at m/z 327.254, 285.076, 255.185, 196.863 and 195.194; these ions were not present in the mass spectrum (Fig. 5A) before reaction, suggesting that they may correspond to the enzymatic end products. The mass spectrum also shows that the m/z 313.07 $[M+H]^+$ and 335.052 $[M+Na]^+$ values could be the residual AFB1.

Table 6.2 and Figure 6.6A indicate that the compound $C_{17}H_{11}O_7$, with a mass of 327. 254 m/z, had one more oxygen molecule than that in AFB1; this product, which is the most abundant one, may resulted by the epoxidation of the furan double bond of AFB1 or from the diol conversion of the epoxide, followed by the loss of one H_2O molecule and the dehydrogenation reaction. The results also show that the compound $C_{16}H_{12}O_5$, with a mass of 285.076, identified as 2,3,3a,6a-tetrahydrofuro[2,3-b]furan 8-methoxy-1,2-dihydro-3H-cyclopenta[b]benzofuran-3-one, had one less CO molecule than that in AFB1. The experimental findings indicate that the compound $C_{15}H_{11}O_4$, with a mass of 255.185 m/z, identified as 1,2,5a,8a-tetrahydro-10H-cyclobuta[c]furo[3',2':4,5]furo[2,3-h]chromen-10-one, could have a diol as the precursor. The loss of two H_2O molecules in the diol may led to the modification of metoxy group as well as to the loss of two carbon monoxide (CO) molecules. Another fragmentation pathway could be proposed, where the precursor of this compound resulted from the hydroxylation of AFB1 into $AFB2_a$. When one H_2O molecule in the $AFB2_a$ was lost, successive fragmentation may occurred, that led to the loss of the methoxy and CO groups. A third fragmentation pathway could be suggested for the production of $C_{15}H_{11}O_4$, identified as 2,3,3a,6a-tetrahydrofuro[2,3-b]furan-1,2-dihydrocyclobuta[b]benzofuran-7-carbaldehyde; in this case, the precursor may resulted from the hydroxylation of AFB1 into $AFB2_a$, followed by its loss of two CO and H_2O molecules. In addition, the compounds $C_{10}H_{13}O_4$ and $C_{10}H_{11}O_4$, with masses of 196.863 and 195.194 m/z, respectively, identified as 2,3,3a,4,5,8a-hexahydrofuro[2,3-b]benzofuran-2,6-diol and 3a,4,5,8a-tetrahydrofuro[2,3-b]benzofuran-2,6-diol, respectively, could have the aflatoxin AFP1 as the precursor, which may resulted from the *O*-demethylation and the hydroxylation of AFB1. The masses of the 196.863 and 195.194 m/z products suggest that the loss of CO and C_2H_2 group was the main fragmentation pathway.

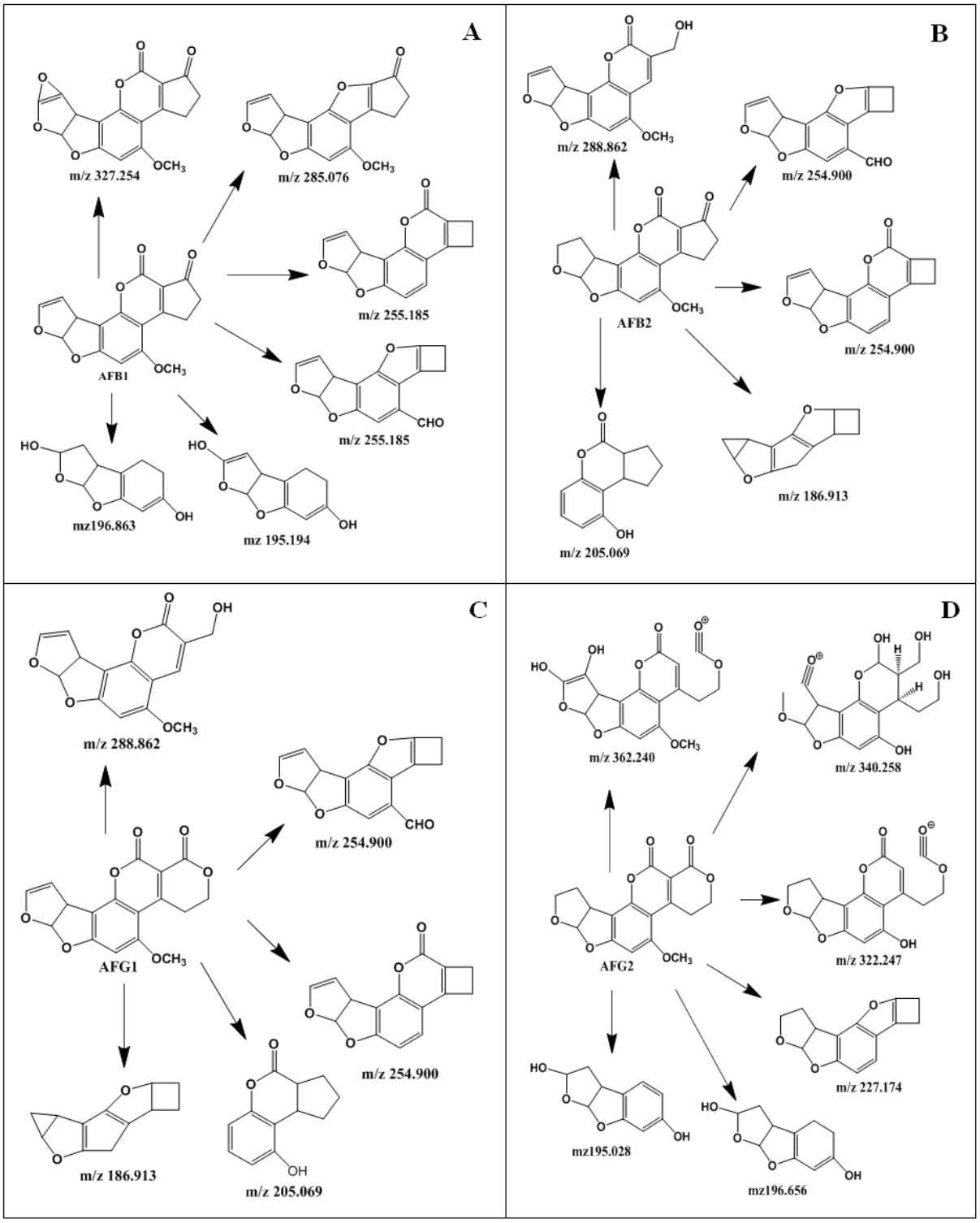

Figure 6.6. Degradation products from aflatoxins, (A) AFB1, (B) AFB2, (G) AFG1, and (D) AFG2.

6.6.4.2. Aflatoxin AFB2 Fragmentation Pathways

The mass spectrum (Fig. 6.5B') of the laccase reaction, using aflatoxin AFB2 as substrate, shows intense peak values at m/z 288.900, 254.900, 205.069 and 186.913, these ions were not present in the mass spectrum (Fig. 6.5B) before reaction, suggesting that they may correspond to the enzymatic end products. The mass spectrum also shows that the m/z 315.088 $[M+H]^+$ and 337.069 $[M+Na]^+$ values could be the residual AFB2.

Table 6.2 and Figure 6.6B indicate that the compound $C_{15}H_{13}O_6$, 288.900 m/z, identified as 3-(hydroxymethyl)-5-methoxy-7a,10a-dihydro-2H-furo[3',2':4,5]furo[2,3-h]chromen-2-one, may resulted from the keto-reduction of the AFB2 lactone ring, followed by its opening with a loss of C_2H_2 molecule as well as the dehydrogenation of furofuran moiety double bond. A second fragmentation pathway of this compound was possible; it consisted of the conversion of AFB2 into AFB1, followed by its hydroxylation to form the aflatoxicol as well as the opening of the lactone ring, leading hence to the loss of C_2H_2 molecule. The experimental data suggest that the precursor of the compound $C_{15}H_{11}O_4$, 254.90 m/z, identified as 1,2,5a,8a-tetrahydro-10H-cyclobuta[c]furo[3',2':4,5]furo[2,3-h]chromen-10-one, may resulted from the hydroxylation of AFB2 into AFM2. When one H_2O molecule of the hyroxyled compound was lost, successive fragmentation may occurred, leading hence to the loss of two CO groups. The results suggest that the precursor of the most abundant compound $C_{12}H_{13}O_3$, 205.069 m/z, identified as 9-hydroxy-2,3,3a,9b-tetrahydrocyclopenta[c]chromen-4(1H)-one, may resulted from the *O*-demethylation of AFB2. A successive fragmentation of the precursor may led to the loss of the furofuran moiety as well as to that of the oxygen molecule on the five-membered pentanone ring. In addition, the compound $C_{13}H_{13}O_2$, 186.913 m/z, identified as 1,1a,3,5b-tetrahydrocyclopenta [b]cyclopropa [d]furan-(1S,5S)-2-oxabicyclo[3.2.0]heptanes, may resulted from the modification and the fragmentation of AFB2. The conversion of the methoxy group of AFB2 into ketone may allowed a successive fragmentation of the toxin. The mass of the 186.913 m/z compound suggests that the loss of CO was the main fragmentation pathway.

6.6.4.3. Aflatoxin AFG1 Fragmentation Pathways

The mass spectrum (Fig. 6.5C') of the laccase reaction, using aflatoxin AFG1 as substrate, shows intense peak values at m/z 288.286, 254.900, 205.069 and 186.913; these ions were not present in the mass spectrum (Fig. 6.5C) before reaction, suggesting hence that they were the

products of the laccase reaction. The mass spectrum also shows that the m/z 329.07 $[M+H]^+$ and 351.05 $[M+Na]^+$ values could be the residual AFG1.

Table 6.2 and Figure 6.6C suggest that the compound $C_{15}H_{13}O_6$, 288.862 m/z, identified as 3-(hydroxymethyl)-5-methoxy-7a, 10a-dihydro-2H-furo[3',2':4,5]furo[2,3-h]chromen-2-one, may resulted from the keto-reduction of the lactone ring, followed by its opening with a loss of C_2H_2O molecule. The experimental findings suggest that the precursor of the compound $C_{15}H_{11}O_4$, 254.900 m/z, identified as 2,3,3a,6a-tetrahydrofuro[2,3-b]furan 1,2-dihydrocyclobuta [b]benzofuran-7-carbaldehyde, may resulted from the hydroxylation of AFG1; successive fragmentation of the precursor, mainly the loss of CO group, may led to the formation of this product. Another fragmentation pathway to obtain the compound $C_{15}H_{11}O_4$, 254.900 m/z, identified as 1,2,5a,8a-tetrahydro-10H-cyclobuta[c]furo[3',2':4,5]furo[2,3-h]chromen-10-one, was possible; the precursor of this compound may resulted from the conversion of AFG1 into diol. When two H_2O molecules of diol were lost, successive fragmentation may occurred, that could led to the loss of two CO groups as well as to that of oxygen molecule. In addition, the most predominant compound $C_{12}H_{13}O_3$, 205.069 m/z, identified as 9-hydroxy-2,3,3a,9b-tetrahydrocyclopenta [c]chromen-4(1H)-one, may resulted from the fragmentation of AFG1 and the modification of the methoxy group. A successive fragmentation of AFG1 may led to the loss of furofuran moiety as well as to that of the oxygen molecules of the six-membered lactone ring; this compound may also resulted by another fragmentation pathway, which consisted of the *O*-demethylation of the AFG1, followed by its successive fragmentation, leading to the loss of the furofuran moiety as well as to that of the oxygen molecules of the six-membered lactone ring. The compound $C_{13}H_{13}O_2$, 186.913 m/z, identified as 1,1a,3,5b-tetrahydrocyclopenta[b] cyclopropa[d]furan-(1S,5S)-2-oxabicyclo[3.2.0]heptanes, may resulted from the fragmentation of AFG1. The mass of 186.913 m/z suggests a modification of the metoxy group into ketone of AFG1, where the loss of the CO molecules was the main fragmentation pathway.

6.6.4.4. Aflatoxin AFG2 Fragmentation Pathways

The mass spectrum (Fig.6.5D') of the laccase reaction, using aflatoxin AFG2 as substrate, shows intense peak values at m/z 362.240, 340.258, 313.070, 227.174, 196.656 and 195.028; these ions were not present in the mass spectrum (Fig. 6.5D) before reaction, suggesting hence that they were the products of the enzyme reaction. The mass spectrum also shows that the m/z 331.08 $[M+H]^+$ and 353.061 $[M+Na]^+$ values could be the residual AFG2.

Table 6.2 and Figure 6.6D show that the compound $C_{17}H_{14}O_9$, 362.240 m/z, identified as ((2-(9,10-dihydroxy-5-methoxy-2-oxo-7a,10a-dihydro-2H-furo[3',2':4,5]furo[2,3-h]chromen-4-yl) ethoxy) methylidyne) oxonium, had two more hydroxyl groups than that in AFG2, which may suggest that it resulted from the hydroxylation of AFG2, followed by a rearrangement in order to acquire this pair mass. The results indicate that the compound $C_{16}H_{10}O_8$, 340.258, identified as (((3R,4R)-2,5-dihydroxy-4-(2-hydroxyethyl)-3-(hydroxymethyl)-8-methoxy-3,4,8,9-tetrahydro-2Hfuro[2,3-h] chromen-9-yl) methylidyne) oxonium, had five more hydrogen and one more oxygen molecules as well as one less carbon molecule in the structure than that in AFG2; the mass of this compound may suggest the *O*-demethylation and the hydroxylation of AFG2 as well as the reduction of the ketone and the oxygen in the six-membered lactone ring and the keto-reduction in the second lactone ring. The reduction of the ketone and the oxygen in the six-membered lactone ring may led to the opening of the ring. In order to obtain the pair mass of this fragment, a possible rearrangement may occurred. The compound $C_{16}H_{18}O_{7,}$ 322.247 m/z, identified as ((2-(5-hydroxy-2-oxo-7a,9,10,10a-tetrahydro-2H-furo[3',2':4,5]furo[2,3-h]chromen-4-yl) ethoxy) methylidyne) oxonium, with one less carbon group may resulted from the *O*-demethylation of AFG2; the pair mass of this fragment may also resulted from a rearrangement of the compound structure. The experimental data suggest that the precursor of the compound $C_{14}H_{11}O_3$, 227.174 m/z, identified as hexahydrofuro [2,3-b]furan-1,2-dihydrocyclobuta[b]benzofuran, may resulted from the hydroxylation of AFG2; a successive fragmentation of this precursor may led to the loss of the two ketones and one oxygen in the lactone rings. The precursor of the compounds $C_{10}H_{13}O_4$ and $C_{10}H_{11}O_{4,}$ with a mass of 196.656 (the most predominant one) and 195.028 m/z, respectively, identified as 2,3,3a,4,5,8a-hexahydrofuro[2,3-b]benzofuran-2,6-diol and 3a,4,5,8a-tetrahydrofuro[2,3-b]benzofuran-2,6-diol, respectively, may resulted from the *O*-demethylation and the hydroxylation of AFG2; a successive fragmentation of the precursor may led to the loss of the six membered-lactone rings and the reduction of the furofuran moiety double bond as well as to that of the six membered ring.

Luo *et al*. (2013) identified six degradation products from AFB1, treated with ozone, with ion masses m/z of 371.1342, 347.0767, 327.0505, 319.0818, 317.0661 and 305.1025, where the degradation reaction occurred on the double bond in the terminal furan ring of the toxin.

Farzaneh *et al*. (2012) showed that there were three abundant fragments corresponding to the degradation of aflatoxin AFB1, with ion masses of m/z 285, 269 and 241; these authors suggested that the product ions of m/z 285 and 269 may be obtained by the neutral loss of (CO) and ($COCH_3$), respectively, from the coumarin moiety of AFB1. Velazhahan *et al*. (2010) reported that the most abundant degradation product of aflatoxin AFG1 had a molecular ion peak at m/z 288.29, where the MS/MS analysis of the precursor ion at m/z 288.29 showed a fragment transition at m/z 270.16 corresponding to the loss of 18 Da, which suggested that the degradation of aflatoxin AFG1 occurred through the modification of the lactone ring structure. Wang *et al*. (2011) reported the oxidation of the 8,9-vinil bond of AFB1 by the manganese peroxidase, from *Phanerochaete sordida* YK-624, to form the AFB1-8,9-epoxide, followed by the hydrolysis of the epoxide to generate the AFB1-8,9-dihydrodiol. Wu *et al*. (2009) indicated the presence of AFB1-8,9-dihydrodiol in some animals, contaminated with AFB1, that may be obtained by the hydrolysis of AFB1-8,9-epoxide, which was formed when the 8,9-vinyl bond was oxidized by the microsomal cytochrome P450 system. Mishra and Das (2003) reported that the degradation of AFB1 and AFG1 involved the oxidation of the unsaturated carbons of the furan ring by mammalian liver cytochrome P450. In addition, Guengerich *et al*. (1998) showed that the generated epoxide was spontaneously hydrolyzed into AFB1-8,9-dihydrodiol. Sabbioni *et al*. (1987) demonstrated that the AFB1-8,9-dihydrodiol, generated from AFB1 by albumin, was less toxic than AFB1, since it can be rearranged and hence formed a reactive dialdehyde that can react with the primary amine groups in proteins by Schiff base reactions; this prevents the formation of DNA adducts, which can cause mutation. Another degradation process has been described by Taylor *et al*. (2010), who showed that the two dependent reductases FDR-A and –B, from *Mycobacteria*, catalyzed the reduction of AFB1, AFB2, AFG1 and AFG2; this reduction wasn't due to that of the furan moiety by these enzymes but possibly due to the reduction of the double bond of the *α, β*-unsaturated ester moiety between the lactone ring in AFG1 and AFG2 and the lactone and cyclopentenone ring in AFB1 and AFB2. Wu e*t al*. (2009) reported that fungi could convert AFB1 into aflatoxicol-A (AFL-A), which was in its turn converted into aflatoxicol-B (AFL-B). Fungi are also able to convert AFL into AFB1, which can be further converted into aflatoxin $AFB2_a$. Megalla and Mohran (1984) showed that yeast could also degraded AFB1 into $AFB2_a$, during the fermentation of dairy products.

The overall findings suggest that the major enzymatic degradation pathways of aflatoxins by laccase are either the additional or the fragmentation reactions, including the epoxydation, hydroxylation, *O*-demethylation, dehydrogenation, dehydratation, reduction of the double bond and the keto-reduction as well as the loss of ketone, oxygen, carbon and methyl molecules.

6.7. Conclusion

The experimental data, obtained throughout this study, suggested that the PPL can degrade the aflatoxins AFB1, AFB2, AFG1 and AFG2, which are difurocoumarin derivates, into several degradation products, with a wide range of structures and molecular weights, where the major ones were identified as epoxide, diol and coumarin derivates. The structural analyses of the enzymatic end products suggested that PPL can either alter aflatoxins structure or lead to the production of precursors, which were in their turn converted into aflatoxins derivates, with potentially nontoxic properties.

CHAPTER VII

GENERAL CONCLUSION AND FUTURE DEVELOPMENTS

The present study was aimed at the investigation of the major aflatoxin isoforms degradation, including AFB1, AFB2, AFG1 and AFG2, by a selected microbial laccase and proposed a potential mechanism for such pathway. The experimental data showed that among the fungal strains investigated, *Aspergillus parasiticus* was the most appropriate one for the production of aflatoxins. Mannitol appeared to be the most suitable cryoprotectant in terms of its recovery capacity and to obtain better quality of aflatoxins dry extract.

The biocatalysis of laccase, from *Coriolus hirsutus*, showed that high temperature and low pH were appropriate to improve the laccase activity for the aflatoxins degradation. In addition, the effects of metal ions, salts and some selected chemical agents on the laccase degradation activity were highly dependent on the agents' nature and concentrations.

Central composite design (CCD), with response surface methodology (RSM), was successfully used to optimize the enzymatic degradation of the major aflatoxin isoforms by the laccase. The experimental findings indicated that enzyme and substrates concentrations had major effects on the degradation process. In addition, the statistical analyses showed that in order to achieve a higher degree of aflatoxins degradation, it was essential to maintain the enzyme and substrates concentrations at their (0) average, while the incubation time should be maintained to its higher level (+2).

Using the major aflatoxin isoforms as substrate models, the characterization of laccase-catalyzed end products was investigated, using Fourier-transform infrared spectroscopy (FTIR), high-performance liquid chromatography (HPLC), as well as liquid chromatography/mass spectrometry (LC/MS). The overall results showed that the laccase-catalyzed degradation of aflatoxins resulted in the production of several degradation products, with a wide range of structures and molecular weights. The most abundant characteristic molecular ions of the degradation products were epoxide, diol and coumarin derivates. In addition, the structural analyses showed that the characterized degradation end products resulted either from the synthesis or from the fragmentation of precursors or from the residual aflatoxins. The major mechanisms of the aflatoxins degradation by laccase could be epoxydation, hydroxylation, *O*-demethylation, deshydrogenation, dehydratation, reduction of the double bond and keto-reduction a well as the loss of ketone, oxygen, carbon and methyl molecules. This degradation

may led to the modification of either furofuran moiety, coumarin or the lactone ring as well as to the formation of nontoxic products.

The overall experimental results obtained through the present study suggest that the investigated microbial laccase could be possibly applied to degrade the aflatoxins in the contaminated food and feed products. This approach could be a valid alternative to the physical and chemical aflatoxins degradation, since it is a biotechnological one and economically viable.

The fisrt objective was the production, the recovery and the characterization of the major aflatoxins from selected fungal strains, including *A. parasiticus* and *A. flavus*. In this study, the experimental findings indicated that the aflatoxins production was dependant of several fermentation factors. The first one was the source of the strains, where *A. parasiticus* produced the highest aflatoxins concentration than *A. flavus*. Secondly, the nutriment in solid culture medium of PDA was the most appropriate one for the production of aflatoxins. This could be explained by the presence of glucose as preferred source of carbon as compared to the sucrose in CA. In addition, it was showed that pH can also have effect of the production, where acidic one seemed to be more suitable than the neutral one to obtain the highest aflatoxins yield. Finally, the results showed that the toxins production was also related to the biomass concentration and to the incubation time, where the highest aflatoxins yield was obtained with a cell concentration of 150 g/L of culture medium and incubation time of 48 and 168 h in pre-culture and culture media, respectively. The literature suggested that aged mycelium of *A. parasiticus,* produced proxidases that was able to degrade afltoxins in liquid medium. In this study, a new process implying the use of ultra-filtration system was developed for the recovey of aflatoxins. In order to obtain aflatoxins dry extract with high recovery and good structural quality, the use of cryoprotectants during lyophilization, was needed. The results suggested that 1.5% of mannitol was the most appropriate concentration to ensure the highest rheological quality of dry extract as well as the highest recovery of aflatoxins.

The potential degradation of aflatoxins by the partial purified laccase from *C. hirsutus* was studied. The kinetic degradation study indicated that the optimisation of different parameters, including temperature, pH, protein load, were essential to obtain the highest enzyme activity for the aflatoxins degradation. It was found that a temperature of 57.5C, a pH of 6.0 as well as a

potein load of 16 µg/mL reaction medium resulted by the highest laccase activity. It was suggested that laccases are active at high temperature and low pH, where below or above of these optimal conditions laccases can undergo conformational change in copper centers as well as to an inhibition of the active site of enzymes. The effect of different activators and inhibitors on laccase activity in order to identify the mechanism of action of the enzyme was also investigated. It was found that HPI, ABTS, kojic acid, *p*-coumaric acid and imidazole had activator effects on laccase activity. It was suggested that the interaction of laccase with low molecular weight molecules such as ABTS or synthetic mediators of the type -NOH^- such as HPI, not only resulted by the higher rates and yields in the transformation of laccase substrates but also added new oxidative reactions to the laccase repertory towards substrates in which the enzyme alone had no or only marginal activity. In addition, phenolic compounds derived from lignin degradation seemed to be highly-efficient laccase mediators of natural origin. On the other hand, DTT, DDC, L-cystein, citric acid as well as $CuSO_4$ and $CuCl_2$ had inhibitory effects on laccase activity. It was suggested that the sulfhydryl organic compounds and citric acid are metal specific chelators which inhibit laccase activity through the formation of complexes with copper ions, modifying its active site. In addition, although the laccase is a copper-containing protein, the presence of metal ions in excess concentrations inhibits its activity.

In order to optimize the maximum laccase enzymatic degradation of each microbial aflatoxin, the effects of three selected reaction parameters, including enzyme and aflatoxin concentrations as well as incubation times, at five levels and the relationships between them have been investigated using response surface methodology (RSM), which is based on Central composite design (CCD). ANOVA and regression analyses showed that the R^2 values of the polynomial model for prediction of aflatoxins AFB1, AFB2, AFG1 and AFG2 degradation were 0.89, 0.94, 0.92 and 0.89, respectively, which implies that 89, 94, 92 and 89% of variation response could be explained by the model, suggesting that the polynomial equation is accurate in predicting the responses of the model. In addition it was demonstrated that the incubation time and the enzyme level, with higher coefficient values, greatly influenced aflatoxin AFB1 degradation more than the substrate concentration. For AFB2 degradation, enzyme level, with higher coefficient, had higher effect than incubation time and substrate concentration. For AFG1 and AFG2 degradation, the substrate and enzyme concentrations, with higher coefficient values, had higher

effects than incubation time. The optimal condition for 38.2, 30.1, 76.4 and 100% degradation of aflatoxins AFB1, AFB2, AFG1 and AFG2, was obtained with an enzyme concentration of 31.5 U/nmol aflatoxins and substrate concentration of 96.2, 191.0, 32.7 and 42.2 nmol of AFB1, AFB2, AFG1 and AFG2, respectively. In addition, it could conclude that RSM and sequential experiments could be used to overcome a saddle point in order to meet the objective of maximization of the bioprocess.

Finally, the last objective of the research work was to characterize the degradation end products of aflatoxins by the partial purified laccase in order to propose a potential mechanism for such pathway. The FTIR analysis suggested that the IR spectra of the enzymatic degradation end products of the aflatoxins showed several differences in absorption peaks as compared to that of the substrates. The major differences lie around absorption peak area at 3100-2900, between 1770 and 1650 and between 1300 and 1000 cm^{-1} as well as with the apparition of new absorption peak at 1375 cm^{-1}. It was suggested that these changes could be attributed to the modification of either coumarin, five-membered pentanone or the six-membered lactone rings as well as to the conversion of the methoxy group into hydroxyl group of the aflatoxins. For the transformed aflatoxin AFB1, a new asymmetric peak appeared at 825 cm^{-1}, which could correspond to the stretching vibration of an epoxy group positioned on the furan ring. The MS analysis showed that the degradation of the aflatoxins by laccase resulted in the formation of a wide range of several end products, where the most abundant one was the molecular ion peak at m/z 327.254, 205.069, 205.069 and 196.656 for AFB1, AFB2, AFG1 and AFG2, respectively. The structural characterization study of the degradation end products of aflatoxins by the partial purified laccase confirmed their bioconversion into potentially less toxic products.

In perspective, it would be interesting to investigate *in situ* the degradation of an induced aflatoxin food product. The food product will be then treated with laccase in order to demonstrate the degradation of aflatoxins as well as to characterize the enzymatic degradation end products. The overall of the experimental findings of the research work could lay the ground for a potential industrial application of the enzymatic approach for the decontamination of food and food products.

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