ANTIFUNGAL ACTIVITY OF PYRAZINE COMPOUNDS

ARCHANA BUKKA

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ANTIFUNGAL ACTIVITY OF PYRAZINE COMPOUNDS

A Thesis

Presented to the

Graduate Council of

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in Biology

by

Archana Bukka

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ABSTRACT

Pyrazine and its derivatives represent an important group of nitrogen containing heterocyclic aromatic compounds, some of which occur naturally and some are xenobiotic. Pyrazine compounds enter into the environment as constituents of pesticides, insecticides, dyes and waste waters from pharmaceutical industries. Pyrazines have a wide range of antibacterial activity.

This research was aimed at studying the antifungal activity of pyrazine compounds. Antifungal susceptibility tests showed that among pyrazine compounds, 2-amino pyrazine had inhibited the growth of most of the fungi tested. Pyrazine had inhibitory effect on a few fungi tested, whereas 2-pyrazine carboxylic acid had no inhibitory effect on all fungi tested. Studies on biodegradation of pyrazine involving *A. fumigatus* revealed that pyrazine was not degraded when supplied as sole carbon and nitrogen source, sole carbon source, and sole nitrogen source. ***Pencillium notatum***, ***P. chrysogenum***, and ***Phanerochaete chrysosporium*** were also unable to degrade pyrazine when served as sole carbon source.

It was concluded that high concentrations of 2-amino pyrazine and pyrazine may result in potential problems in nature. This study also demonstrated that pyrazine is a recalcitrant compound and was not degraded by the most efficient biodegrading fungi.

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CHAPTER I

INTRODUCTION

A large variety of aromatic substances participate in life processes, and their degradation forms an important part of the natural carbon cycle. Human activities are an additional source of synthetic organic chemicals in the environment. Two-thirds of known chemicals that contain heterocyclic aromatic compounds are xenobiotic and are relatively recalcitrant to biodegradation (Kaiser et al., 1996).

Heterocyclic aromatic compounds as environmental pollutants

Nitrogen containing heterocyclic aromatic compounds are widely used in the preparation of various drugs, as dyes in textile industries, and as flavors in the food industry. Wastewaters of a number of pharmaceutical, textile and food industries contain considerable amounts of heterocyclic aromatic compounds. When these compounds are disposed of they cause environmental pollution in soils and bodies of water due to run-off and through seepage. Because most microorganisms cannot metabolize them, heterocyclic aromatic compounds tend to accumulate in the environment, and cause environmental contamination (Kaiser et al., 1996).

Pyrazines

Pyrazines and their derivatives are an important group of nitrogen containing heterocyclic aromatic compounds, some of which occur naturally and some are xenobiotic. Pyrazine is chemically similar to benzene ring except the 1st and 4th carbon positions are occupied by nitrogen atoms. Many pyrazine derivatives contribute to the flavor of roasted and cooked foods. Pyrazines formed in heated food result from condensation reactions between sugars and amino acids (Table 1). One of the most common naturally occurring pyrazine compounds is aspergillic acid, an antimicrobial agent produced by the fungal genus *Aspergillus*.

Man made pyrazines

A large variety of man made pyrazine compounds is widely used in pesticides, textiles, food, and pharmaceutical industries. Pyrazines are used in food industry as dyes, aromas and flavoring agents. Methyl pyrrolo [1,2-a] pyrazine is a major constituent of the odor of roasted meat. 2-methoxy 3-methyl pyrazine is used as a flavoring agent of coffee and cocoa (Mega and Sizes, 1973).

Pyrazines are indispensable to the dye industry. Dyes such as mauveine (red color) are widely used. Thionazin [O,O- Diethyl-O-(2-pyrazinyl) phosphorothioate is a soil insecticide and nematocide that is produced commercially. Thionazin enters into the environment as emission during its manufacture, formulation and application as a pesticide. It is highly mobile in soil and also contaminates bodies of water. Thionazin has been found in acidic soil up to one year after its application.

Antibacterial activity of pyrazine compounds

A multidrug therapy including pyrazinamide has been used to treat tuberculosis. 5-Hydroxy pyrazine 2-carboxylic acid is a versatile building block for the synthesis of various 2,5 disubstituted pyrazines which have been more active against tuberculosis bacilli (*Mycobacterium*) than pyrazinamide (Cynamon et al., 1995; Bergmanm et al., 1996). Derivatives of pyrazinedicarboxaimido have inhibitory effects on bacterial species like *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Zahid et al., 1999).

Role of Fungi in nature

Fungi can be harmful as well as beneficial to mankind. Many of the fungi found in soil are responsible for causing diseases in economically important plants. Some fungi are also responsible for causing disease in humans and other animals. Certain fungi are also involved in property damage. Materials such as clothing, tentage, optical equipment, leather goods, plastic objects, photographic film, paper goods, and electronic equipment are deteriorated by fungus growth. Fungi (and bacteria) found in natural waters and soil have a broad ability to utilize (catabolize) organic matter through their various digestive and respiratory processes, thus recycling the fixed organic carbon back into harmless biomass and carbon dioxide. Decay of organic matter by fungi benefits man in three important ways: (1) organic debris is continuously being removed from environment; (2) large quantities of carbon dioxide are released into the atomsphere and made available for green plants to synthesize sugar; and (3) formation of humus. Materials like starch, cellulose, hemicellulose, lignin, suberin, cutin, pectin and sugar are degraded by fungi. Some fungi have the ability to degrade environmental pollutants and xenbiotic

compounds (Table 2). If the fungi and bacteria should suddenly lose their capacity for bringing about the decay of organic debris, life would become exceedingly burdensome and disagreeable and, conceivably, might cease altogether.

Table 1: PYRAZINES IN FOODS

PYRAZINE DERIVATIVES	FOODS
Unsubstituted	Barley (roasted), Casein, Coffee, Peanuts (roasted), Soybean.
2-Methyl pyrazine	Beef, Casein, Coffee and Non fat dry milk.
2,5-Dimethyl pyrazine	Cocoa products, Beef fat (heated) Casein.
2-Ethyl-5-methyl pyrazine	Barley (roasted), Beef (fried), Popcorn, Potato chips, whey.
2,5-Dimethyl- 3-ethyl pyrazine	Potato chips, Barley, Beef, Soya protein.
2-Vinyl pyrazine	Coffee, Peanuts.
3-Iso propyl –2methoxy pyrazine	Peas (green)
5-Acetyl 2-methyl pyrazine	Cocoa products

Source: Mega and Sizes, 1973

Table 2. Environmental pollutants degraded by fungi

Name of the oragnism	Compounds degraded
Phanerochaete chrysosporium	Polyaromatic hydrocarbons, dioxins, polychlorinated biphenyls and other halogenated aromatics, some dyes, trinitro toluene and other nitro explosives, cyanides, azide, carbon tetrachloride, pentachlorophenol, toxaphene, creosote, and coal tars
Aspergillus niger	2-Aminobenzoate, Benzoate, Phenanthrene
Neurospora crassa	2-Aminobenzoate
Fusarium sp.	Propanil, cyanide

Source: The University of Minnesota Biocatalysis/Biodegradation Database
http://umbbd.ahc.umn.edu/

OBJECTIVES

Since pyrazine compounds have antibacterial properties and some are xenobiotic there is a possibility that they may interfere with the natural microbial flora when discharged into the environment. Despite the possible potentially serious consequences of the release of pyrazine compounds, no research has been conducted to determine their fate in the environment. Since many of the pyrazine compounds have antibacterial properties there is a possibility that these compounds might have antifungal properties. The goal of this study was to examine the antifungal activity of selected pyrazine compounds (pyrazine, 2-amino pyrazine, and 2- pyrazine carboxylic acid) on fungi that are found in natural environment (soil) which play an important role in biodegradation processes. Then based on the results of antifungal studies, biodegradation studies on pyrazine were conducted because there is a possibility that resistant organisms might have the ability to degrade pyrazine compounds. The following fungi were used as test organisms for antifungal studies:

Aspergillus fumigatus

Aspergillus niger

Aspergillus oryzae

Aspergillus flavus

Chytridium confervae

Cladosporum carrionii

Fusarium oxysporum

Neurospora crassa

Pencillium notatum

Pencillium chrysogenum

Phanerochaete chrysosporium

Pythium sp.

Saprolegnia sp.

Sordoria firmicola

SIGNIFICANCE OF THE STUDY

The results of this study will be used as a baseline to determine the role of pyrazine compounds (inhibitory or non-inhibitory effect) on natural fungal flora. This information may then be used for evaluating the effect that pyrazine compounds have on the biodegradation of other compounds by natural fungal flora in the environment. The results might also be used as a screening method to identify microorganisms capable of degrading pyrazine and its derivatives.

CHAPTER II

LITERATURE REVIEW

ANTIFUNGAL STUDIES

No information is available on antifungal activity of pyrazine compounds. Few studies have demonstrated the antibacterial properties of pyrazine compounds. Studies by Cynamon et al., (1995) and Bergman et al., (1996) demonstrated the in-vitro antimycobacterial activity of pyrazionic acid esters. Studies by Zahid et al., (1999) provided additional information on antibacterial activity of pyrazine derivatives. Mutagenic studies of pyrazine compounds have been conducted on *Salmonella typhimurium* (information obtained from Chemical Carcinogenesis Research Information System). All these studies focused on the effects of pyrazine compounds from a medical point of view. Studies demonstrating the environmental fate of pyrazine compounds are not available. Information is not available about the effects of pyrazine compounds on NATURAL MICROBIAL FLORA.

BIODEGRADATION STUDIES

An enormous amount of literature is available on biodegradation of nitrogen containing heterocyclic aromatic compounds. Microbial degradation of various aromatic hydrocarbons involves two initial steps: dioxygenase mediated hydroxylation, followed by dehydrogenation. These reactions yield catechol, which is subsequently oxidized either by *ortho* cleavage or *meta* cleavage pathways. The final products of both pathways

are molecules that can enter the tricarboxylic acid cycle. A nitrogen-containing heterocyclic aromatic ring differs from a benzene ring in having a lone pair of electrons on the nitrogen, making the aromatic ring less susceptible to electrophilic attack (Shukla, 1986). This phenomenon is evident in the microbial degradation of quinoline compounds wherein the benzene ring of the quinoline molecule is preferably degraded over a pyridine ring (Taniuchi and Hayaishi, 1963). Studies by Kaiser et al., (1991) and Korosteleva et al., (1981) deal with degradation of pyridine and its derivatives. These studies indicated that mechanism of cleavage of the pyridine ring is uncertain but demonstrated that metabolism of hydroxylated and carboxylated pyridines is initiated by hydroxylation. In degradation of these pyridine derivatives, di- and trihydroxypyridine derivatives are formed prior to ring cleavage. Studies by Gauthier and Rittenberg (1971) showed that in nicotine transformation, the pyrrolidine ring is attacked and converted to trihydroxypyridine. Studies by Aislabie et al., (1990) and Shukla (1986) showed that quinoline metabolism by *Pseudomonas* sp. is initiated by hydroxylation(s) on the molecule followed by cleavage of pyridine ring to yield 8-hyroxy coumarin, which is further metabolized via 2,3- dihydroxyphenyl propionic acid. Since degradation of most of the nitrogen containing aromatic compounds is initiated by hydroxylation reaction, the same process might be applicable to degradation of pyrazine compounds. All these studies basically used the same assay procedure, whereby the medium containing the compound to be degraded is monitored by scanning the broth for absorption spectra of the compound being analyzed. No information is available on biodegradation studies involving pyrazine or its derivatives. Hence, the procedure employed for biodegradation of quinoline compounds was used in this study (Shukla, 1986).

CHAPTER III

MATERIALS AND METHODS

ANTIFUNGAL SUSCEPTIBILITY STUDIES

Antifungal activity of pyrazine compounds (2-pyrazine carboxylic acid, 2-amino pyrazine and 2-cyano pyrazine) was evaluated using the Broth microdilution method for the determination of minimum inhibitory concentration (MIC) against selected fungal organisms (Denning et al., 1997).

Preparation of compound serial dilutions

Stock solutions of 2-amino pyrazine, pyrazine and 2-pyrazine carboxylic acid (160mM) were prepared using sterile ethanol as solvent. The start solution (80mM) was prepared by diluting stock solution in sterile ethanol by 1/ 2. One hundred microliters of sterile RPMI medium was added to wells in columns 2- 11 of a microtitre plate. Two hundred microliters of start solution was added to the well in column 1. Serial dilution of 100µl volumes from columns 1-11 was performed, discarding the extra 100µl from the well in column 11.This resulted a final range of serial dilutions from 40mM –0.03mM.

Inoculum preparation

A loopful of test organism spores was transferred into 5ml of PBS (phosphate buffer saline) /Tween. The suspension was vortexed. The number of spores present per ml of PBS/Tween was counted using a haemocytometer, and the spore concentration was adjusted to 1x 10^6 spores/ml using RPMI medium.

Inoculation of assay plates

One hundred microliters of the inoculum was added to all wells in the appropriate row. The concentration of the final inoculum was $5x10^5$ spores/ml. A growth control (compound free) well was included for each assay. A sterility control well (inoculum free) was also included to validate the sterility of the assay medium. A solvent control that contained the concentration of solvent used to dissolve the compounds was also included. Microtitre plates were incubated aerobically at 37° C. The incubation period varied with the organism being tested. Growth was indicated by turbidity and was best determined by comparison with sterility control. The minimum inhibitory concentration (MIC) was read visually as the well containing the lowest concentration of compound exhibiting no growth.

Viability tests

Viability tests were performed on all test organisms. Aliquots were taken from the wells were no growth was observed and streaked on agar slants. Agar slants were aerobically incubated at 37^0C. The incubation period (48 to 168 hours) varied depending upon the organism tested.

BIODEGRADATION STUDIES

Based on the results from antifungal susceptibility studies (discussed in detail here in chapter IV) *A. fumigatus*, *P. chyrsosporium*, *P. notatum*, and *P. chrysogenum* were selected for biodegradation studies. Because *A. fumigatus* was resistant to all three pyrazine compounds tested at all concentrations, it was selected for biodegradation studies wherein pyrazine served as sole carbon and nitrogen source, sole carbon source and sole nitrogen source. Nonspecific activity of the ligninase enzyme system of

P. chrysosporium enables it to degrade a wide range of heterocyclic aromatic compounds, hence there is a possibility that it can degrade pyrazine. Even though growth of the *P. notatum* was inhibited at 40mM of pyrazine it was selected for biodegradation studies because of its broad range of enzymatic activity. *P. chrysogenum* was resistant to pyrazine at all concentrations tested, and because of its broad range of enzymatic activity, it was selected for biodegradation studies. In biodegradation studies using *P. chyrsosporium*, *P. notatum*, and *P. chrysogenum,* pyrazine served as sole carbon source.

Media

The medium used for the growth of *A. fumigatus, P. chyrsosporium*, *P. notatum*, and *P. chrysogenum* contained (in grams per liter) K_2HPO_4, 12.5; KH_2PO_4, 3.8; $MgSO_4$. $7H_2O$, 0.1; yeast extract, 0.1; peptone, 0.1; sucrose, 2.0. Sucrose solution was prepared separately, autoclaved and added to media before use. One ml of trace element solution was added to the media. The trace element solution contained (in milligrams per liter) boric acid, 0.001; Copper Sulfate, 0.01; Ferrous Sulfate, 0.02; Zinc Sulfate, 0.2. The salts were dissolved separately (at a concentration of 100 times that required in the medium), the pH was adjusted to 7 with 1N HCl or 1N NaOH, and solutions were autoclaved and added to media before use. The media were sterilized at a 15-lb/in^2 pressure of steam for 20 minutes. Media used for biodegradation studies had the above-mentioned composition except yeast extract, peptone, and sucrose were omitted from the media.

Biodegradation of pyrazine

A pure culture of *A. fumigatus* was subjected to membrane filtration. Filtrate containing cells was washed with 1% saline three times and then inoculated into replicate flasks of biodegradation medium containing 3mM of pyrazine. To test the ability of *A.*

fumigatus to utilize pyrazine as its sole carbon source, the medium was supplemented with 0.1% ammonium sulfate. Ammonium sulfate was omitted from the medium to determine the ability of *A. fumigatus* to utilize pyrazine as sole source of carbon and nitrogen. To test the ability of *A. fumigatus* to utilize pyrazine as sole nitrogen source, the medium was supplemented with 1% sucrose and ammonium sulfate was omitted from the assay medium. The cultures were incubated for 7 days at 28°C. Sterility controls were also included. Samples from the cultures, as well as from sterility controls were collected every 24 hours. Broth samples were subjected to membrane filtration and broth was analyzed by using a UV spectrophotometer to detect the pyrazine and biotransformation products. The above-mentioned procedure was employed in biodegradtion studies by *P. notatum*, *P.chrysogenum*, and *P. chrysosporium* where pyrazine served as sole carbon source and cultures were monitored for 5 days.

Viability tests

To check the viability of the organism, cultures from biodegradation medium were streaked on agar slants after the test period for biodegradation studies was completed.

Analytical methods

Pyrazine has an absorption peak at 280nm (Fig. 1). Broth samples were scanned from 200 to 300 nm by using a Spectronic Genesys Spectrophotometer. Degradation of pyrazine is detected by qualitative or quantitative changes in absorption spectra when compared with uninoculated controls.

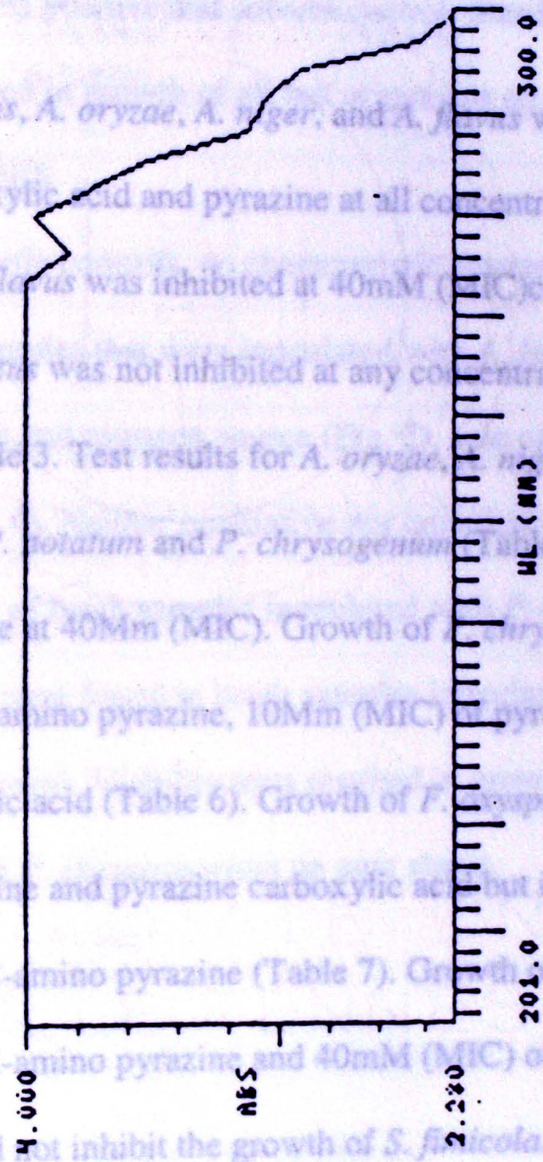


Fig 1. UV absorption spectra of pyrazine

CHAPTER IV

RESULTS

ANTIFUNGAL STUDIES

Growth of *A. fumigatus*, *A. oryzae*, *A. niger*, and *A. flavus* was not inhibited in the presence of 2-pyrazine carboxylic acid and pyrazine at all concentrations tested. Growth of *A. oryzae*, *A.niger*, and *A. flavus* was inhibited at 40mM (MIC)concentration of 2-amino pyrazine but *A. fumigatus* was not inhibited at any concentration. Test results for *A. fumigatus* are shown in table 3. Test results for *A. oryzae*, *A. niger*, and *A. flavus* are shown in table 4. Growth of *P. notatum* and *P. chrysogenum* (Table 5) was inhibited by 2-amino pyrazine and pyrazine at 40Mm (MIC). Growth of *P. chrysosporium* was inhibited at 5mM (MIC) of 2-amino pyrazine, 10Mm (MIC) of pyrazine and 10mM (MIC) of 2-pyrazine carboxylic acid (Table 6). Growth of *F. oxysporum* was not inhibited in presence of pyrazine and pyrazine carboxylic acid but its growth was inhibited at 20mM (MIC) of 2-amino pyrazine (Table 7). Growth of *S. firmicola* was inhibited at 10mM (MIC) of 2-amino pyrazine and 40mM (MIC) of pyrazine (Table 8). 2-pyrazine carboxylic acid did not inhibit the growth of *S. fimicola*. *C. carrionii* was growing in presence of pyrazine carboxylic acid and pyrazine at all concentrations tested but its growth was inhibited at 40mM (MIC) of 2-amino pyrazine (Table 9). MIC of 2-amino pyrazine, pyrazine, and 2-pyrazine carboxylic acid against *Pythium* sp. was 20mM (Table 10). Growth of *C. confervae* was inhibited at 20mM (MIC) of 2-amino pyrazine,

and 40 mM (MIC) of pyrazine. Pyrazine carboxylic acid failed to inhibit the growth of *C. confervae* (Table 11). Growth of *N. crassa* was inhibited at 40mM (MIC) of both 2-amino pyrazine and pyrazine but its growth was not inhibited by pyrazine carboxylic acid (Table 12). Growth of *Saprolegnia* was inhibited at 20mM (MIC) of all three pyrazine compounds tested (Table 13). In all the susceptibility tests carried out, growth of the test organisms was observed in both positive and solvent control. Sterility controls were negative. Viability tests resulted in growth of all test organisms on agar slants.

BIODEGRADATION STUDIES

When compared to sterile controls, no characteristic changes were observed in absorption spectra of broth samples that were inoculated with ***A. fumigatus*** where pyrazine served as sole carbon and nitrogen source (Fig. 2), sole carbon source (Fig. 3), and sole nitrogen source (Fig. 4). Neither qualitative nor quantitative changes were detected in absorption spectra of broth samples inoculated with ***P. notatum*** and ***P. chrysogenum***. Similar results were found in broth samples inoculated with ***P. chrysosporium*** (results not shown). Viability tests resulted in growth of ***A. fumigatus***, ***P. notatum***, ***P. chrysogenum***, and ***P. chrysosporium*** on agar slants.

Table 3. Results of broth microdilution method for the determination of minimum inhibitory concentration (MIC) of pyrazine compounds against *Aspergillus fumigatus.*

Concentrations (mM)	Compounds		
	2- Amino pyrazine	Pyrazine	2-Pyrazine carboxylic acid
40.0	+	+	+
20.0	+	+	+
10.0	+	+	+
5.00	+	+	+
2.50	+	+	+
1.25	+	+	+
0.62	+	+	+
0.31	+	+	+
0.12	+	+	+
0.06	+	+	+
0.03	+	+	+

\+ Growth present

\- Growth absent

MIC of pyrazine compunds is > 40mM.

Results are expressed after 48 hours of incubation period.

Table 4. Results of broth microdilution method for the determination of minimum inhibitory concentration (MIC) of pyrazine compounds against *Aspergillus oryzae*, *A. niger*, and *A. flavus*.

Concentrations (mM)	Compounds		
	2- Amino	Pyrazine	2-Pyrazine
40.0	-	+	+
20.0	+	+	+
10.0	+	+	+
5.00	+	+	+
2.50	+	+	+
1.25	+	+	+
0.62	+	+	+
0.31	+	+	+
0.12	+	+	+
0.06	+	+	+
0.03	+	+	+

\+ Growth present

\- Growth absent

MIC of 2-amino pyrazine is 40mM.

MIC of pyrazine and 2-pyrazine carboxylic acid is > 40mM.

Results are expressed after 48 hours of incubation period.

Table 5. Results of broth microdilution method for the determination of minimum inhibitory concentration (MIC) of pyrazine compounds against *Pencillium notatum* and *P. chrysogenum.*

Concentrations (mM)	Compounds		
	2- Amino	Pyrazine	2-Pyrazine
40.0	-	-	+
20.0	+	+	+
10.0	+	+	+
5.00	+	+	+
2.50	+	+	+
1.25	+	+	+
0.62	+	+	+
0.31	+	+	+
0.12	+	+	+
0.06	+	+	+
0.03	+	+	+

\+ Growth present

\- Growth absent

MIC of 2-amino pyrazine and pyrazine is 40mM.

MIC of 2-pyrazine carboxylic acid is > 40mM.

Results are expressed after 48 hours of incubation period.

Table 6. Results of broth microdilution method for the determination of minimum inhibitory concentration (MIC) of pyrazine compounds against *Phanerochaete chrysosporium*.

Concentrations (mM)	Compounds		
	2- Amino	Pyrazine	2-Pyrazine
40.0	-	-	-
20.0	-	-	-
10.0	-	-	-
5.00	-	+	+
2.50	+	+	+
1.25	+	+	+
0.62	+	+	+
0.31	+	+	+
0.12	+	+	+
0.06	+	+	+
0.03	+	+	+

\+ Growth present

\- Growth absent

MIC of 2-amino pyrazine is 40mM.

MIC of pyrazine and 2-pyrazine carboxylic acid is > 40mM.

Results are expressed after 120 hours of incubation period.

Table 7. Results of broth microdilution method for the determination of minimum inhibitory concentration (MIC) of pyrazine compounds against *Fusarium oxysporum.*

Concentrations (mM)	Compounds		
	2- Amino	Pyrazine	2-Pyrazine
40.0	-	+	+
20.0	-	+	+
10.0	+	+	+
5.00	+	+	+
2.50	+	+	+
1.25	+	+	+
0.62	+	+	+
0.31	+	+	+
0.12	+	+	+
0.06	+	+	+
0.03	+	+	+

\+ Growth present

\- Growth absent

MIC of 2-amino pyrazine is 20mM.

MIC of pyrazine and 2-pyrazine carboxylic acid is > 40mM.

Results are expressed after 48 hours of incubation period.

Table 8. Results of broth microdilution method for the determination of minimum inhibitory concentration (MIC) of pyrazine compounds against *Sordoria firmicola.*

Concentrations (mM)	Compounds		
	2- Amino	Pyrazine	2-Pyrazine
40.0	-	-	+
20.0	-	+	+
10.0	-	+	+
5.00	+	+	+
2.50	+	+	+
1.25	+	+	+
0.62	+	+	+
0.31	+	+	+
0.12	+	+	+
0.06	+	+	+
0.03	+	+	+

\+ Growth present

\- Growth absent

MIC of 2-amino pyrazine and pyrazine is 10mM, 40mM respectively.

MIC of 2-pyrazine carboxylic acid is > 40mM.

Results are expressed after 120 hours of incubation period.

Table 9. Results of broth microdilution method for the determination of minimum inhibitory concentration (MIC) of pyrazine compounds against *Cladosporum carrionii.*

Concentrations (mM)	Compounds		
	2- Amino	Pyrazine	2-Pyrazine
40.0	-	+	+
20.0	+	+	+
10.0	+	+	+
5.00	+	+	+
2.50	+	+	+
1.25	+	+	+
0.62	+	+	+
0.31	+	+	+
0.12	+	+	+
0.06	+	+	+
0.03	+	+	+

\+ Growth present

\- Growth absent

MIC of 2-amino pyrazine is 40mM respectively.

MIC of pyrazine and 2-pyrazine carboxylic acid is > 40mM.

Results are expressed after 72 hours of incubation period.

Table 10. Results of broth microdilution method for the determination of minimum inhibitory concentration (MIC) of pyrazine compounds against *Pythium* sp.

Concentrations (mM)	Compounds		
	2- Amino	Pyrazine	2-Pyrazine
40.0	-	-	-
20.0	-	-	-
10.0	+	+	+
5.00	+	+	+
2.50	+	+	+
1.25	+	+	+
0.62	+	+	+
0.31	+	+	+
0.12	+	+	+
0.06	+	+	+
0.03	+	+	+

\+ Growth present

\- Growth absent

MIC of pyrazine compounds is 20mM.

Results are expressed after 48 hours of incubation period.

Table 11. Results of broth microdilution method for the determination of minimum inhibitory concentration (MIC) of pyrazine compounds against *Chytridium confervae.*

Concentrations (mM)	Compounds		
	2- Amino	Pyrazine	2-Pyrazine
40.0	-	-	+
20.0	-	+	+
10.0	+	+	+
5.00	+	+	+
2.50	+	+	+
1.25	+	+	+
0.62	+	+	+
0.31	+	+	+
0.12	+	+	+
0.06	+	+	+
0.03	+	+	+

\+ Growth present

\- Growth absent

MIC of 2-amino pyrazine and pyrazine is 20mM, 40mM respectively.

MIC of 2-pyrazine carboxylic acid is > 40mM.

Results are expressed after 72 hours of incubation period.

Table 12. Results of broth microdilution method for the determination of minimum inhibitory concentration (MIC) of pyrazine compounds against *Neurospora crassa.*

Concentrations (mM)	Compounds		
	2- Amino	Pyrazine	2-Pyrazine
40.0	-	-	+
20.0	+	+	+
10.0	+	+	+
5.00	+	+	+
2.50	+	+	+
1.25	+	+	+
0.62	+	+	+
0.31	+	+	+
0.12	+	+	+
0.06	+	+	+
0.03	+	+	+

\+ Growth present

\- Growth absent

MIC of 2-amino pyrazine and pyrazine is 40mM.

MIC of 2-pyrazine carboxylic acid is > 40mM.

Results are expressed after 48 hours of incubation period.

Table 13. Results of broth microdilution method for the determination of minimum inhibitory concentration (MIC) of pyrazine compounds against *Saprolegni* sp.

Concentrations (mM)	Compounds		
	2- Amino	Pyrazine	2-Pyrazine
40.0	-	-	-
20.0	-	-	-
10.0	+	+	+
5.00	+	+	+
2.50	+	+	+
1.25	+	+	+
0.62	+	+	+
0.31	+	+	+
0.12	+	+	+
0.06	+	+	+
0.03	+	+	+

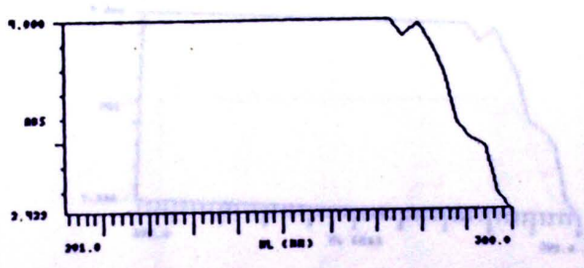
\+ Growth present

\- Growth absent

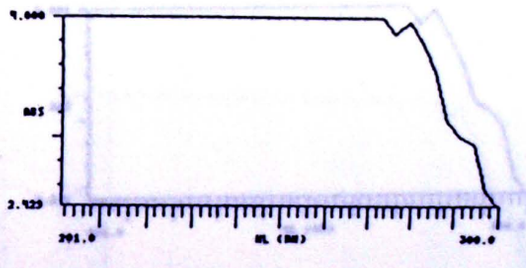
MIC of pyrazine compounds is 20mM.

Results are expressed after 168 hours of incubation period.

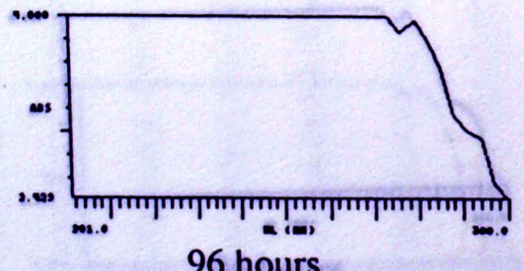
O hour



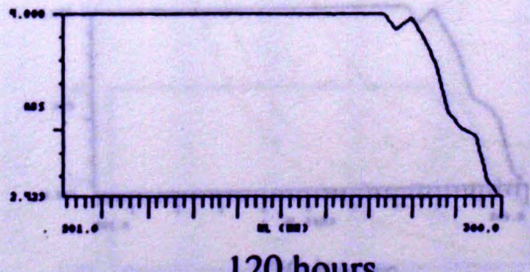
48 hours



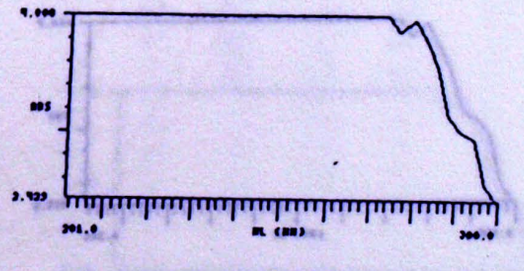
72 hours



96 hours



120 hours



144 hours

Fig. 2. UV absorption spectra of cell-free fermentation broths at different periods of incubation of *A. fumigatus* in pyrazine (as sole carbon and nitrogen source) medium.

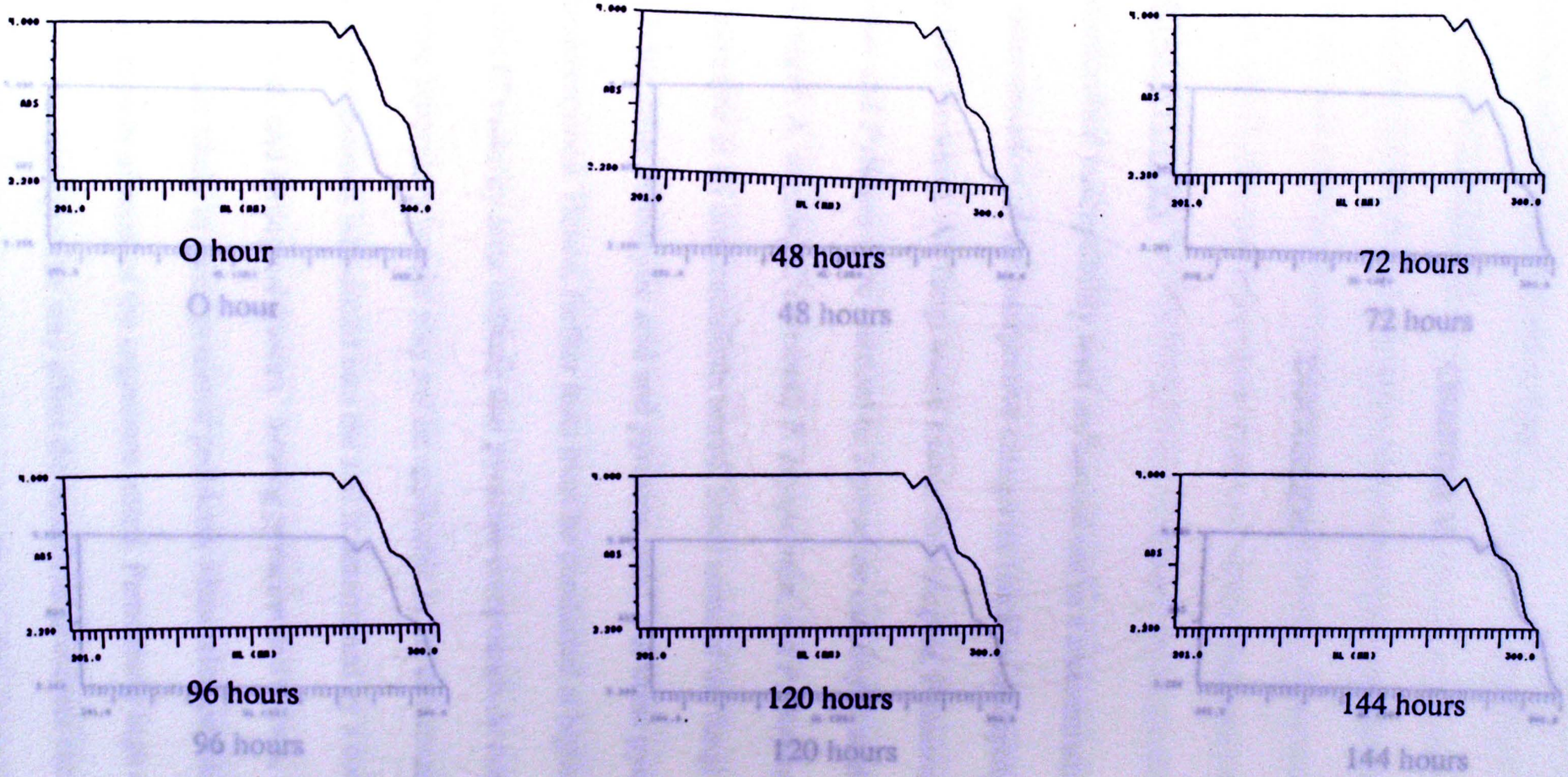


Fig. 3. UV absorption spectra of cell-free fermentation broths at different periods of incubation of *A. fumigatus* in pyrazine (as sole carbon source) medium.

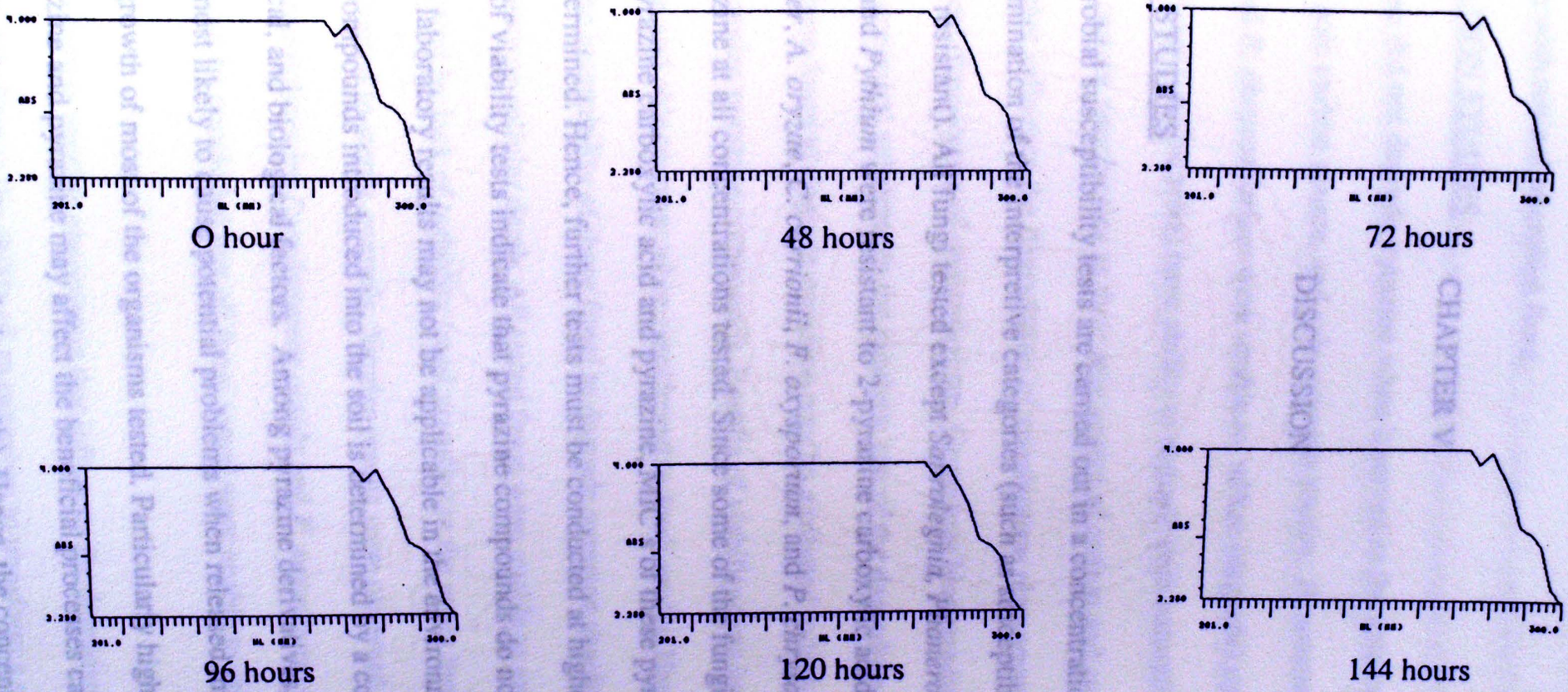


Fig. 4. UV absorption spectra of cell-free fermentation broths at different periods of incubation of *A. fumigatus* in pyrazine (as sole nitrogen source) medium.

CHAPTER V

DISCUSSION

ANTIFUNGAL STUDIES

Antimicrobial susceptibility tests are carried out in a concentration range that allows for determination of the interpretive categories (such as susceptible, moderately susceptible, and resistant). All fungi tested except *Saprolegnia, Phanerochaete chrysosporium* and *Pythium* were resistant to 2-pyrazine carboxylic acid. ***A. fumigatus***, ***A. flavus***, ***A. niger***, ***A. oryzae***, ***C. carrionii***, ***F. oxysporium***, and ***P. chrysogenum*** were resistant to pyrazine at all concentrations tested. Since some of the fungi tested were resistant to 2- pyrazine carboxylic acid and pyrazine, MIC's of these pyrazine compounds could not be determined. Hence, further tests must be conducted at higher concentration ranges. Results of viability tests indicate that pyrazine compounds do not affect fungal viability. These laboratory results may not be applicable in the environment because the fate of organic compounds introduced into the soil is determined by a combination of physical, chemical, and biological factors. Among pyrazine derivatives, 2-amino pyrazine is the most likely to cause potential problems when released into nature because it inhibited the growth of most of the organisms tested. Particularly high concentrations of 2-amino pyrazine and pyrazine may affect the beneficial processes carried out by these fungi in nature (indirectly by affecting their growth). Hence, the concentration of

pyrazine compounds must be reduced before they are discharged into nature so that they will not interfere with natural microbial flora.

BIODEGRADATION STUDIES

A. fumigatus did not degrade pyrazine when it served as the sole carbon and nitrogen source, sole carbon source, and sole nitrogen source. *P. notatum*, *P. chrysogenum*, and *P. chrysosporium* were unable to utilize (degrade) pyrazine as sole carbon source. Fungi (and bacteria) have ability to degrade both naturally occurring compounds and certain xenobiotics as their sole source of carbon and energy. There are two key factors that influence the degradation of xenobiotics- phenomena of gratuitous biodegradation and phenomena of cometabolism. Gratuitous biodegradation requires adherence of an unnatural substrate to the catalytic site of the degrading enzyme. Incapability of these fungi to degrade pyrazine may be attributed to lack of enzyme systems that recognize pyrazine compounds. Results of viability tests indicate that these fungi were viable and able to grow when transferred to fresh media. Hence, it can be concluded that the incapability of these fungi to degrade pyrazine should not be linked to an inactive cell state or cellular death. All tested fungi were unable to grow in presence of pyrazine as indicated by lack of turbidity (read visually) in the assay medium. Turbidity was same as compared to sterile controls. Similar results were observed in biodegradation studies of methylquinolines by *Pseudomonas putida* and *P. aeruginosa* (Aislabie et al., 1990). Hence, it can be concluded that all fungi tested were unable to utilize pyrazine as growth substrate.

Cometabolism is another phenomena that influence the degradation of xenobiotics. Cometabolism is the ability of an organism to transform a non-growth

substrate as long as a growth substrate or other transformable compound is also present. Racke et al (1990) showed that degradation of 3,5trichloro-2-pyridinol occurs in soil through a cometabolic process. Hence, it is possible that biodegradation of pyrazine could be achieved if biodegradation assay medium were supplied with growth substrates. Many enzymes of the pathway involved in the degradation of benzoate are induced when non-growing *Pseudomonas* cells "adapt" to the presence of benzoate in the medium (Meer et al., 1992). There is a possibility that these fungi could degrade pyrazine if they were allowed to adapt in the pyrazine medium. Adaptation might be achieved by extending the incubation period.

In conclusion, the present study demonstrated that high concentrations of 2-amino pyrazine and pyrazine have inhibitory effects on most of the fungi studied but 2- pyrazine carboxylic acid does not. Hence, high concentrations of pyrazine compounds in the environment may result in potential problems in nature. This study also demonstrated that pyrazine is a recalcitrant compound and was not degraded by the most efficient biodegrading fungi. Further studies should be conducted to gain an understanding of the antifungal activity of pyrazine compounds. Also further investigations must be done to isolate organisms capable of degrading pyrazines.

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VITA

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