Preliminary Characterization of IDCase in *Neurospora crassa* and other fungi organisms

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Abstract

Preliminary characterization of isoorotate decarboxylase (IDCase) in *Neurospora crassa* and other fungi organisms was determined by a radioactivity-based assay using the radioactive isotope carbon 14 to label the substrate (isoorotate, IOA) at C-7. A crude enzyme preparation was obtained from *Neurospora crassa* which stoichiometrically converts IOA to uracil and carbon dioxide (CO_2). The decarboxylation assay measures the amount of IDCase activity within a protein preparation by detecting the amount of $^{14}CO_2$ released from IOA in a single reaction at a given time point. Wild-type (74A) *N. crassa* had a specific enzyme activity of 0.10 nmol/min/mg which was not significantly different when the organism was grown in the presence of uracil. Mutant strains of *N. crassa* that lack certain thymidine salvage enzymes were obtained and tested to observe if whether IDCase levels can be altered with changes of pyrimidine and nitrogen sources in the growth medium. The mutant strain 2203 showed a 3-fold increase in specific enzyme activity when it was grown in the presence of uracil and about a 15-fold increase in the presence of thymine. The labeled strain 2204 showed more than a 2-fold increase in the presence of uracil. When compared to wildtype *Aspergillus nidulans* showed more than a 1-fold increase in specific enzyme activity. The other organisms assayed for IDCase activity did not have a significant amount of specific enzyme activity. Chemically similar compounds were tested as inhibitors of IDCase. 5-HU and 5-FU inhibits about 40 % of the enzyme activity. Although 5-NO_2U inhibits almost all of the enzyme activity. The results of the assay indicate that this organism and others (where noted) utilize the thymidine salvage pathway to salvage and synthesize pyrimidines needed for ribonucleic acid (RNA), hence deoxyribonucleic acid (DNA) synthesis.

Acknowledgments

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The author would like to dedicate this thesis to her parents, John Cannon (In Loving Memory) and Liddie Cannon, whose love, aspiration, encouragement and continuous support by my mother made completing this study more desirable.

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* None of the figures included in this thesis are drawn to scale.

List of Symbols

Symbol	Definition
α	alpha
ml	milliliter
µl	microliter
nm	nanometer
g	gram
mg	milligram
M	Molar
mM	millimolar
µM	micromolar
^{o}C	degree Celsius

Chapter One

Introduction

Nucleotides are biologically ubiquitous [1] substances that participate in almost all biochemical processes. They are made up of a nitrogenous base (purine or pyrimidine), a 5-carbon sugar (carbohydrate), usually a ribose or deoxyribose, and a phosphate group. The activated form of nucleotides are called nucleoside triphosphates (NTPs) which are high energy compounds whose present amount regulates many metabolic pathways. NTPs are used in the synthesis of nucleic acids. The major pyrimidine components of nucleic acids are cytosine and uracil (which are incorporated in RNA), and thymine which is mainly found in DNA; the main purine residues are adenine and guanine. [1] Other important purines and pyrimidines are inosine and orotidine respectively. This discussion will focus on pyrimidine metabolism in Fungi.

Uridine Monophosphate (UMP) or uridylic acid is a pyrimidine ribonucleotide that has important biological functions including acting as a precursor for pyrimidine nucleotide biosynthesis. The phosphorylated form of UMP (UTP) is used in glycogen synthesis and other metabolic pathways that may require high energy-rich compounds. Glutamine donates an amine group to UTP to make the pyrimidine ribonucleotide CTP by the enzyme CTP synthetase. The deoxynucleotide dUMP can be methylated by the enzyme thymidylate synthase with N^5, N^{10} - methylenetetrahydrofolate (MTHF) acting as the methyl donor to produce dTMP. [1] Two of the four NTPs, dTTP and dCTP, are required for DNA synthesis. The required amounts can be regulated through feedback control with the other dNTPs, dGTP and dATP.

The biosynthesis of UMP can occur in two distinct metabolic pathways, the *de novo* pathway and the more unique thymidine salvage pathway. All organisms contain the *de novo* pathway. Purine nucleotides derive heavily from the synthesis or degradation of nucleic acids and are present in every body cell. The *de novo* purine pathway shows the biosynthesis of the purine nucleotide Inosine Monophosphate (IMP) from α D-Ribose-5-Phosphate [1] by eleven enzymatic catalyzed reactions. The high energy compound Adenosine Triphosphate (ATP) is used to assist the reaction.

More importantly, pyrimidine nucleotide synthesis also occurs by a *de novo* pathway in six enzymatic steps beginning with the amino acid glutamine which provides NH_3, the bicarbonate ion (HCO_3^-), and two moles of ATP [1] (see Fig.-1a). In this pathway enzyme two add the reactants together, enzyme three causes a ring closure, enzyme four catalyzes the formation of a double bond between C-5 and C-6, enzyme five adds a sugar phosphate to give the substrate OMP of the well studied enzyme orotidine monophosphate (ODCase) and this sixth enzyme decarboxylates at C-6 to give the pyrimidine ribonucleotide UMP. This is one way to form this pyrimidine.

Other pathways use preformed pyrimidines as substrates. An example of such a pathway is the thymidine salvage pathway which catalyzes the synthesis of the nucleic acid base uracil (Neuhard, 1983 [2]) from which UMP can be easily synthesized, see Fig.-1b. In this pathway uracil is synthesized in four enzymatic reactions. First, the pyrimidine deoxynucleoside thymidine is broken down in two steps. The first is done by an unknown enzyme encoded by the gene *uc2* to produce the thymine ribonucleoside. Thymine is then enzymatically converted to uracil 5-carboxylic acid (IOA) by the enzyme thymine 7-hydroxylase (THase) which is encoded by the gene *uc3*. This happens in three steps producing an alcohol (5-hydroxymethyluracil, 5-HMU) and an aldehyde (5-

formyluracil) as intermediates. In the last step IOA is decarboxylated by IDCase to uracil which can be converted to UMP by the ezyme uracil phosphoribosyltransferase (UPRTase). UMP can also be easily synthesized from deoxyuridine.

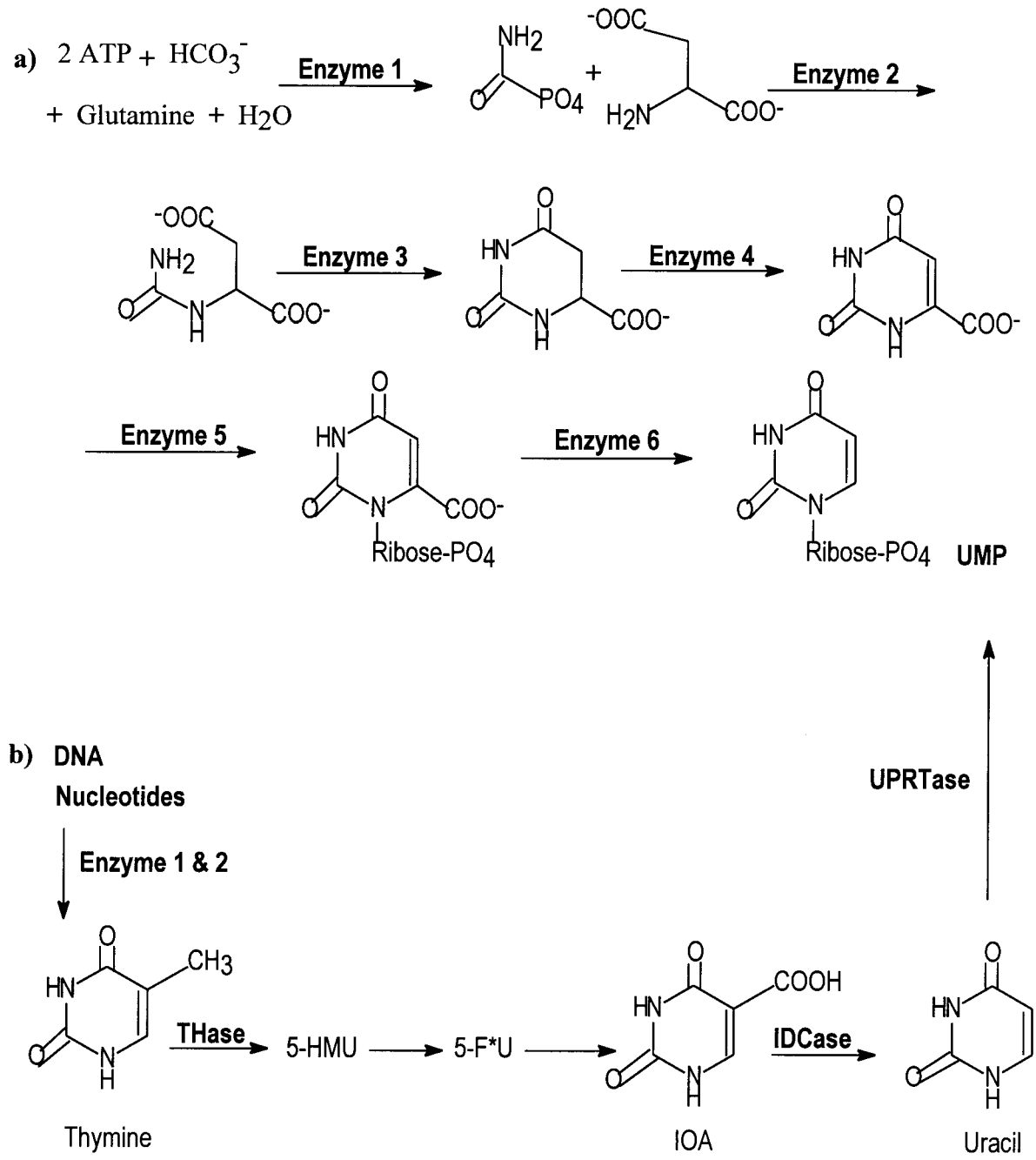


Fig.-1a. The *de novo* pyrimidine biosynthetic pathway.
1b. Thymidine salvage pathway

The thymidine salvage pathway was first identified in the fungus *Neurospora crassa* (which was discovered by Shear and Dodge [3]) from which only the enzyme THase has been purified to an appreciable degree, but both THase and IDCase show substrate specificity. This enzyme has been purified 1300-fold from *Neurospora* crassa. [4] The substrates that are produced and used during the oxidation of thymine to IOA by THase are an alkane, an alcohol, and an aldehyde. Pyrimidines chemically related to thymine have been tested positive to serve as substrates for THase. [4] These are 1-methyluracil, 1-ethyluracil and 6-azathymine. IDCase has only been detected at a slightly measurable amount that allowed for it to be partially purified. The stoichiometry of the reaction of IOA to uracil has been shown from a crude cell-free extract that orotic acid from the *de novo* pathway cannot substitute IOA as substrate for IDCase. [4] Other chemically related pyrimidines have not yet been found to serve as substrates.

The focus of this project was to determine under certain growth conditions if the pyrimidine producing enzyme IDCase could be detected at a measurable amount in lower eukaryotic organisms that utilize the thymidine salvage pathway for nucleic acid metabolism. IDCase is a key enzyme and is important for the completion of the pathway which could be effected by the enzyme inhibitors, 5-hydroxyuracil (5-HU), 5-fluorouracil (5-FU), and 5-nitrouracil (5-NO_2U). If the pyrimidine nucleobase is not synthesized then other nucleotide production could be stopped. This would mean that the synthesis of RNA and DNA could be blocked or terminated. Therefore, organisms that do not contain the *de novo* pathway but utilize this pathway for pyrimidine biosynthesis would eventually lose all of its activity and die. *Neurospora crassa* is the main organism in which IDCase was readily observable and was used as a control for comparison while assaying other organisms that contain thymidine salvage enzymes. Positive activity of IDCase allows the product of the pathway, uracil, to be produced in a certain quantity

that can be measured by correlating the amount of CO_2 released in a reaction mixture, indicating how active the enzyme was.

Chapter Two

Assays for Iso-orotate Decarboxylase

A decarboxylase assay has been developed to detect the levels of IDCase in the organisms by measuring the amount of CO_2 discharged. All decarboxylase enzymes cause the release of CO_2 from their substrates. Each decarboxylase reaction can be assayed so that the amount of CO_2 liberated can be measured by different methods and correlated to the amount of product formed.

Ultraviolet spectroscopy was used to enhance the accuracy in measurement of enzyme activity by providing exact values of concentration for the reactants in the incubation mixture. IOA and all of the protease and reaction inhibitors used in the incubation mixture were first measured on the spectrometer. This was done in order to obtain correct concentrations of the dilutions so that quantitative and qualitative errors are minimized and measurements read from the completed reaction will be more accurate.

A spectrophotometric assay using ultraviolet spectroscopy was developed to observe the simulated conversion of IOA to uracil by measuring the absorbance at a particular wavelength, Fig.- 4. This assay was done in a similar manner as that developed for the well studied enzyme ODCase (Lieberman et al., 1954[5]). When taking a UV spectrum it is a good idea to use quartz cuvettes instead of plastic because plastic cuvettes may absorb UV light and interfere with the spectrum. The enzyme activity can be detected by utilizing the difference in the ability of the substrate and the product to absorb ultraviolet light. The difference in absorbance patterns of IOA and uracil is greatest at a wavelength of 280 nm.The transitional metal zinc (II) was added to the mix to observe the effects it has on the absorbance pattern of the reaction. Some benefits with this assay is that it is inexpensive, easy to perform and is not hazardous to your health. Also, measurements can

be taken at different time points in the same reaction mixture. However, major drawbacks are that the detection is not extremely sensitive and the assay is limited to using a narrow concentration range. This means that spectrophotometric assays are usually unable to detect anything less than 5 % conversion of reactants to products.

The substrate IOA enriched with ^{14}C at C-7 can be easily synthesized from the reaction of radioactive formaldehyde with uracil. The reaction was completed in two steps with the intermediate being radioactive 5-HMU. The first step was done under basic conditions to ensure nucleophilic attack by electrons at C-5 of uracil on the carbonyl ^{14}C of formaldehyde. The second step was done by a platinum-catalyzed oxidation reaction of the 5-HMU with O_2. This is a simple reaction and was carried out over a longer period of time than normal. This was done to ensure that 100% of the radioactive ^{14}C was converted to the product, see Fig-5.

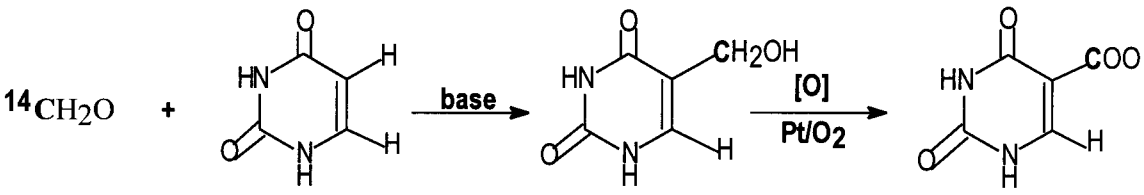


Fig. 5. The synthesis of ^{14}C labeled IOA from formaldehyde and uracil.

A radioactivity-based assay was developed by using the radioactively labeled substrate. This technique is based on the original assay of the enzymatic conversion of IOA to uracil and CO_2 (Palmatier et al., 1970 [6]). Each was measured for radioactivity by reading the radioactive counts on the filter paper of each mix in a scintillation counter. A cup suspended in an assay vial contained a small strip of paper that had been dampened in base, to absorb the $^{14}CO_2$ as it is formed by the activity of the enzyme that is present in the incubation mixture of protein lysate and [7-^{14} C] IOA. Using a syringe, hydrochloric acid (HCl) was injected through the rubber septum into the mixture so as to stop the reaction and drive the $^{14}CO_2$ out of the reaction mix. The vials were incubated for about an hour to allow all of the $^{14}CO_2$ to absorb. After this, the strip was oven dried and placed in a scintillation counter to measure the content of ^{14}C. This measurement correlates to the amount of IDCase activity present in a reaction mixture. See Fig.-6. for calculations. The problems with this assay is that it is more hazardous to our health because the radioactive isotope contain low energy beta particals which could be damaging to the surface of skin. This assay requires cautious and necessary safety procedures, and is more expensive. However, the assay allows a broader concentration range to be used if necessary and is more sensitive in its measurements. Less than 5 % conversion can be easily detected. Different time points can also be taken but, they have to be taken of different reaction mixtures. For example, to measure activity at 5 and 10 minutes, two separate reaction mixtures are needed. Whereas in the spectrophotometric assay the same reaction mixture can be used to measure different time points.

Counts of $^{14}CO_2$ (cpm) / Counting efficiency (cpm/dpm) =
Radioactivity of $^{14}CO_2$ (dpm)

Radioactivity of $^{14}CO_2$ (dpm) / Specific activity of $^{14}CO_2$ (dpm/nm) = nm of product

nm of product / Time (min) = Enzyme activity (nm/min)

Enzyme activity (nm/min) / Total Protein (μg) = Specific activity (nm/min/μg)

Fig.-6. Calculation of specific IDCase activity from radioactive counts per vial.

IDCase was originally assayed by a radioactivity-based assay using the radioactive isotope carbon 14 (^{14}C). IOA was radioactively labeled with ^{14}C at first C-2 and then C-7 but the set up of the standard incubation mixture was a bit different. A test tube containing the incubation mixture was enclosed in a centrifuge tube but held above the bottom of the tube with a glass rod. Beneath the test-tube was the filter paper that had been saturated not with NaOH but nuclear Chicago solubilizer reagent. [6] The centrifuge tube was then stoppered with a rubber cap. The addition of trichloracetic acid (which was used for the same purpose as the HCl), the incubation period, and the reading of the filter paper in the scintillation vials were done in the same manner as previously discussed.

A Bovine Serum Albumin (BSA) standard protein curve produced by Bradford assays was used to determine the protein concentration of the lysates which in turn was used to calculate the specific activity of the enzyme. The BSA standard was made with Glutamate Dehydrogenase (GDH) buffer instead of water because the protein lysate was in buffer, although the dilutions were made with water.

Preparation of Protein Samples Necessary for Assays

Protein lysates from the organisms containing the thymidine salvage pathway were all prepared in a similar manner, although the growth conditions varied. Before inoculation all medias were autoclaved to kill any growth of bacteria in the media or in the non-sterile flask. Cultures were harvested using vacuum filtration immediately after their growth period or after they have grown to a dense enough culture to be assayed so that they would not overgrow. During vacuum filtration the tissue should not be left to over dry because the enzyme may not be stable enough for over drying. The length of time for harvesting is not an important factor as long as the tissue is kept frozen at about -70 $^{\circ}C$.

When preparing the lysate the tube was vortexed to break up all the tissue cells and allow the protease inhibitors to do their job. A tissue tearor was later used for the same purpose. The longer the vortex time the more concentrated the protein becomes. Although, too long of a time doesn't do much good.

Proteases are protein enzymes that are synthesized and transported in the cell as pre-proenzymes which function as degradative enzymes of specific cellular protein. They acquire additional functional properties because they may be post-translationally modified by glycosylation and carboxylation of clotting factors. [7] These proteases are used in biological reactions or released to their sites of physiological function after being stored in special cellular compartments. When the proteases are stored they are stored in active, inactive or in undeveloped form [7] which is as an enzyme inhibitor bound to the protein. The amount of proteases in a biochemical procedure should be kept at a mininum so that measurements for the enzyme being detected would be more accurate.

Protease inhibitors are small molecules that block the active form of proteases from breaking down the native and cellular membrane protein. Protease inhibitors have been isolated from or detected in blood plasma, interstitial fluid, mucous secretions, and intracellular materials. [7] The capacity of these protease inhibitors in an organism is particularly high towards digestive enzymes because they break up the food so that it can be absorbed. Therefore, the inhibitor's main function within the organism is protective, to immediately inhibit potentially digestive proteases whenever they may escape from their natural target compartments. [7] The protease inhibitors used in the experiments were pepstatin A, leupeptin, and phenylmethylsulfonylfluoride (PMSF).

To concentrate the enzyme activity and remove some unwanted proteins, measured amounts of ammonium sulfate ($AmSO_4$) were rapidly stirred into the protein lysate. This was done until the desired percent of saturation was obtained and the resultant precipitate

was then immediately removed by centrifugation. The precipitate was resuspended in a volume of GDH buffer and pipetted into a dialysis bag, which was then placed in a cold beaker of buffer and dialyzed for an hour. The cold buffer help to stabilize the protein so that during dialysis any unwanted molecules will be transfered out of the bag into the beaker of Tris-EDTA buffer. The solution was kept cold so that the proteases would stay less active and the desired enzyme could maintain its activity. An hour is a long enough time for metabolites other than IDCase to dialyze out. In a simple way this process can be used to help purify samples by removing some of the impurities and leaving behind most of the protein.

Chapter Three

Decarboxylation reactions result in the production of carbon dioxide as it is mechanistically cleaved from the substrate of the involved enzymes. Decarboxylation mechanisms occur in many biosynthetic pathways to produce different biological molecules. There are decarboxylation mechanisms using metal ion-dependent enzymes. Metal ions are important to vital functioning of metabolic processes. The metabolism of nucleic acids and the action of the enzymes involved are closely interwoven with the properties of certain metal ions [8] as catalyst. For example, nucleic acid polymerases and nucleases require metal ions for activity.

One common feature of some decarboxylation reactions and other biochemical reactions, as well as many mechanistically similar reactions of amino acids, is the requirement for pyridoxal, also known as coenzyme B_6. [9] The term coenzyme refers to biochemical function and vitamin refers to dietary requirement. The elimination of CO_2 from appropriate amino acids depend on the ability of a Schiff base with pyridoxal to stabilize a negative charge on the α carbon of the substrate. [9] Thus, pyridoxal enzymes are used in the metabolism of amino acids which are the building blocks of proteins.

Other enzymes used in decarboxylations are thiamine-dependent, and miscellaneous enzymes as ODCase. Thiamine is a vitamin that has a competitiveness to bind to proteins. It is a coenzyme that participates in reactions forming and breaking carbon-carbon bonds. [9] Thiamine acts as a cofactor to enzymes that catalyze the decarboxylation of α-keto acids, including pyruvic acid which occurs in the citric acid cycle. The decarboxylate enzyme ODCase is also used in the metabolism of nucleotides. It occurs in the last reaction of the *de novo* pyrimidine biosynthetic pathway.

The possible catalytic mechanism of IDCase is similar to that of Thymidylate Synthase (TS) in higher organisms. Both substrates would undergo nucleophilic attack. See Fig.-7. for the mechanism of TS (Voet, 1995 [1]). In the TS mechanism, the dUMP undergoes nucleophilic attack by the enzyme at C-6. N^5,N^{10}-Methylene THF is exposed to acid to produce DHF. In the second reaction the electrons at C-5 of the dUMP-enzyme complex attacks the nitrile carbon of DHF to give a bound complex. In the third reaction the electrons on the dUMP-enzyme bound base deprotonates the hydrogen on C-5 and breaks away the DHF. The bound enzyme comes off and dTMP is produced which is required for DNA synthesis.

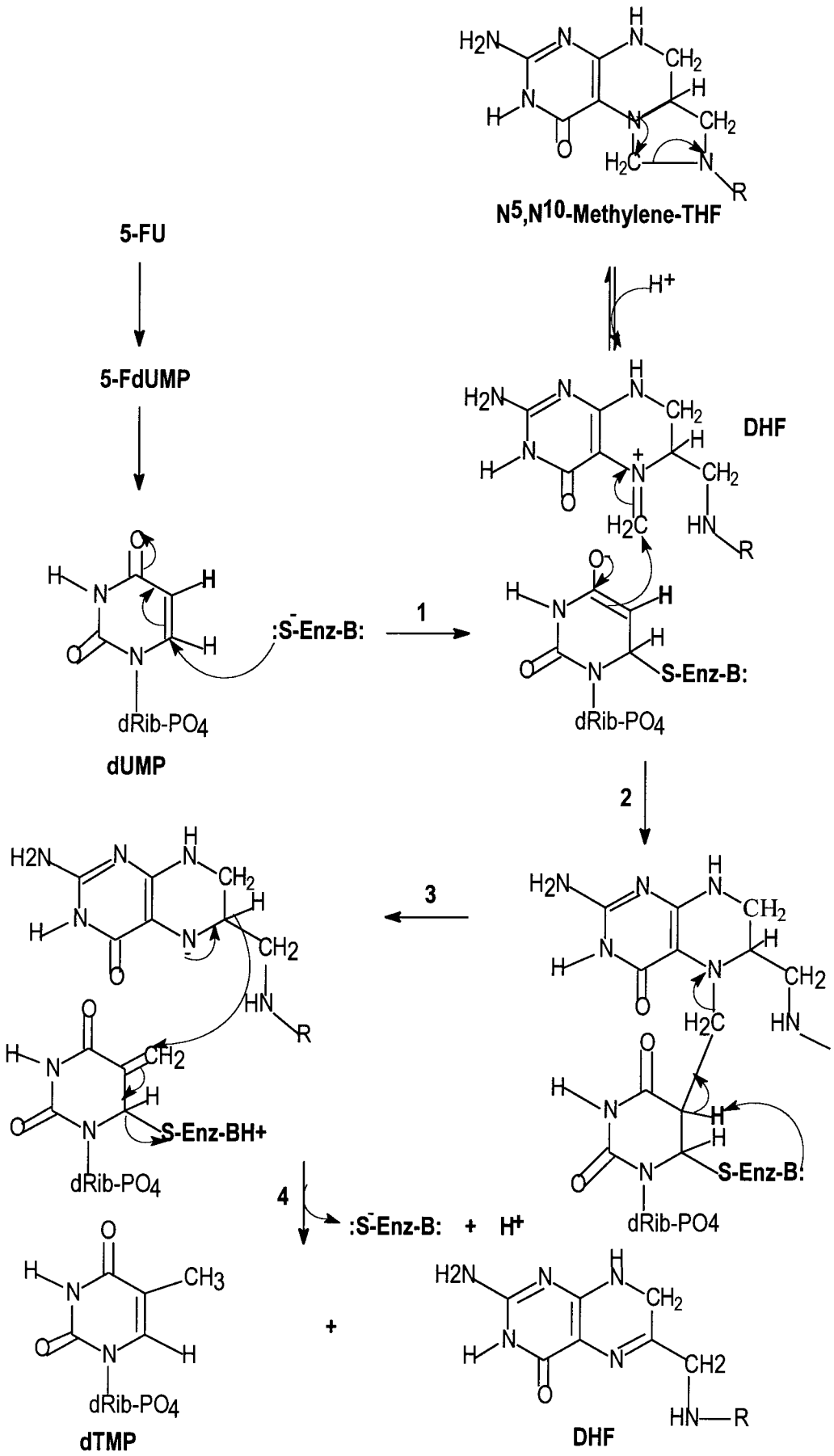


Fig.-7. The catalytic mechanism of thymidylate synthase.Inhibition is caused when 5-FdUMP enters the pathway in place of dUMP.

It is possible that the chemical mechanism of IDCase occurs in a similar manner (see Fig.-8). (a) IOA could be attacked at C-6 by an enzyme-nucleophile complex breaking the double bond between C-5 and C-6. (b) a pair of electrons between the carboxylate and C-5 are moved onto the ring, displacing the CO_2 and leaving a negative charge on the oxygen (enolate). The double bond on the oxygen reforms by the lone pair of electrons on the oxygen, causing a transfer of the bond between C-4 and C-5 to C-5 and C-6 which causes the nucleophile-enzyme to be released. This produces the nucleobase uracil and a free enzyme.

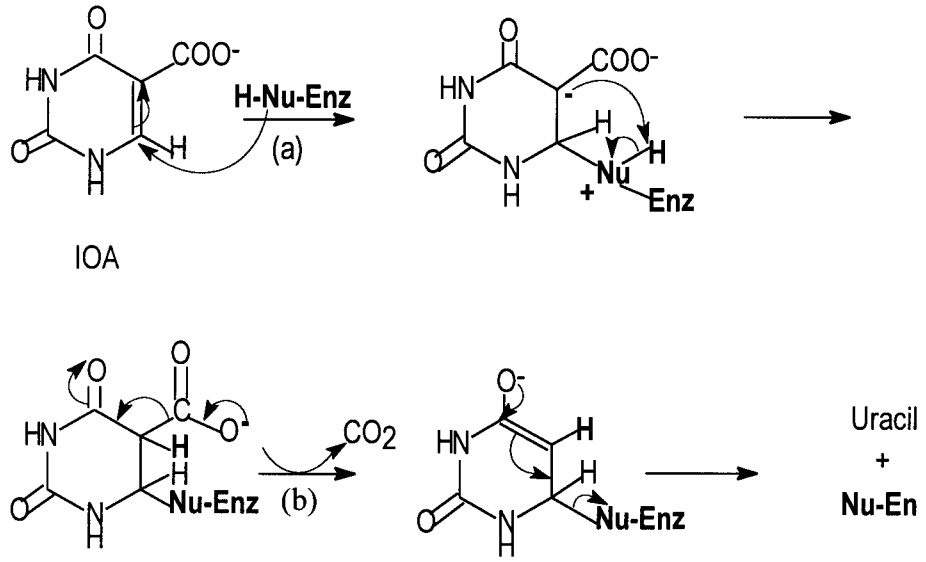


Fig.-8. Proposed Catalytic Mechanism for the enzyme IDCase.
Nu represents a nucleophile and Enz represents the enzyme IDCase.

There were three compounds tested as possible inhibitors on the action of IDCase. A similar structure of the original substrate IOA with a strong electron withdrawing group at C-5 could work as an inhibitor of IDCase if the proposed mechanism is actually operating. 5-HU, 5-FU, and 5-NO_2U were predicted inhibitors and chosen to be tested. A hydroxy group is a weaker electron withdrawing group and less electronegative than a nitro and fluoro group. Therefore, 5-HU would not be expected to have much inhibition on the enzyme in the pathway. Because of the fluorine's strong electronegativity it could be expected to have greater inhibition than 5-NO_2U, though 5-NO_2U is a better electron withdrawing group.

The inhibition of IDCase by 5-FU could represent an additional site of metabolic interference in the mode of action of this compound. There are three possible metabolic pathways where the inhibition by 5-FU has been observed. 1) The first and best described is the inhibition on the conversion of dUMP to dTMP by TS (Fig.-7). In fungi 5-FC is converted to 5-FU by cytosine deaminase and then to 5-F-dUMP which is the inhibitor of TS. However, in higher organisms as eukaryotes 5-FU has to be intravenously injected then it is converted to 5-F-dUMP. This compound enters the TS pathway as dUMP normally would and proceed through the mechanism until reaction three. The mechanism gets inhibited here because the enzyme-bound base cannot remove the fluorine that has replaced the hydrogen at C-5. Therefore, the reaction stops and DNA synthesis is blocked because dTMP is unable to be produced. 2) Another metabolic pathway is the synthesis of RNA. Since RNA incorporates uracil into its growing chain its synthesis can always be blocked by 5-F-UTP producing fluoronated RNA instead. 3) The third metabolic pathway affected by 5-FU is that of thymine to uracil (Fig.- 9). 5-FU is an artificial compound that is added to the protein lysate to compete with IOA for IDCase. Because of the structural similarity 5-FU would undergo IDCase mechanism and cause inhibition at the

second step. IDCase cannot decarboxylate a fluorine. The reaction stops at this step and uracil, hence UMP, cannot be synthesized by this pathway. Therefore, microorganisms or mutant strains lacking the *de novo* pathway would not be able to synthesize required pyrimidine nucleotides, hence RNA and DNA. Also, the protein would have no activity. The same inhibition results when 5-NO_2U or any other strong inhibitor is used.

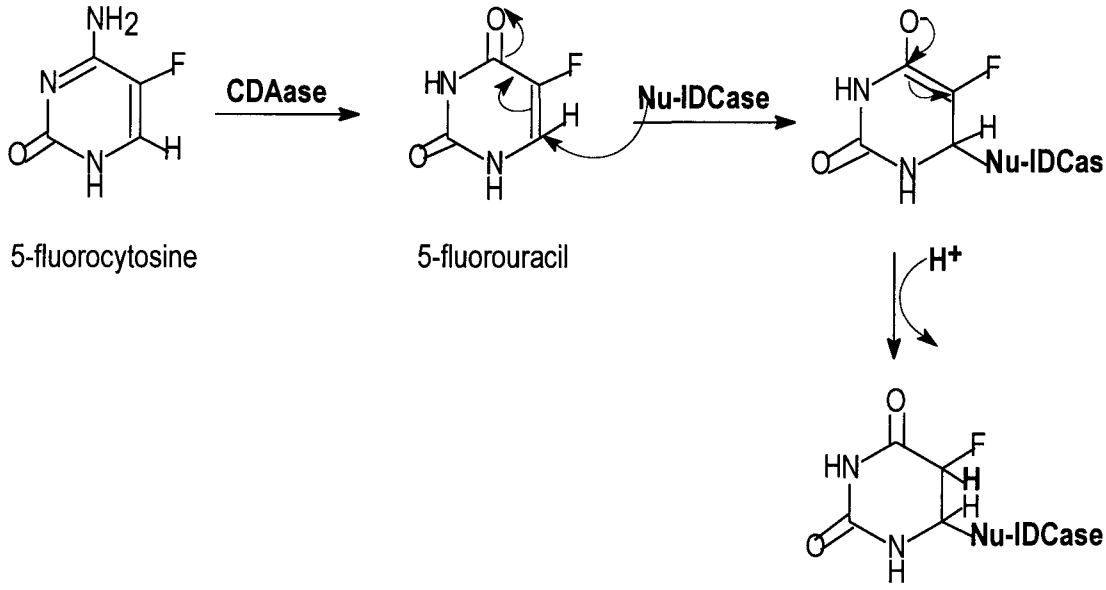


Fig.-9. Proposed Mechanism of Inhibition of IDCase by 5-fluoro-pyrimidines

Chapter Four

Filamentous Fungi and Yeast

Fungi are an immense group of organisms that are characterized and divided on the basis of sexual reproduction. They may be unicellular or multicellular. Some fungi may grow as unicellular for a period of time or in a certain environment, and become multicellular later on or under alternate conditions. Such an organism would be dimorphic. Many of the fungi parasitic to man or animal have this dimorphism, growing in one form in the body and in another form on artificial culture media. [10] The multicellular fungi are composed of cells arranged end-toend to form filaments which branch, rebranch and interwine to form a structure called mycelium. In fungi it is difficult to determine whether the observed cellular changes are temporary or permanent, spontaneous or induced, mutant or hybrid. In the laboratory experiments these variations can be troublesome because they lead to a loss or alteration of specific systems that are being studied. However, these variations are important in explaining the origin and evolution of new races and species.

Yeast are phylogenetically a heterogenous group [10] and are considered to be true Fungi whose usual and dominant growth form is unicellular, though a few cells may be temporarily attached in irregular clusters when actively growing. Yeast are microscopic organisms which sometimes multiply by an asexual process called budding. They can exist in three somewhat different states: (1) the resting state; (2) the growing state; (3) and the spore bearing state [11] (where the spore produced is kind of a reproductive body). If yeast cells have been grown without sufficient nourishment then they would not grow by the normal method of budding, instead the cells would break up into several spores. Economically, yeast are the most important among the natural agents that bring about

fermentation. The rising of bread by yeast is just as truly a fermentation as is the manufacturing of beer. Yeast have extreme use in scientific research as host organisms. It can be obtained at a low price and easily grown over a short period of time.

The filamentous fungi have also been a popular experimental model. These fungi include the ascomyceteous *Neurospora* crassa, Aspergillus *nidulans* (heterothallic mating) and *Neurospora africana* (homothallic mating). In ascomycetes the sexual ascospores are endogenous. Another group of fungi, the imperfect fungi, includes *Candida albicans* and *Candida stellatoidea* (yeasts with clinical significance). The fungi imperfecti have no known sexual phase. All of the fungi can be considered filamentous. *Candida* forms both mycelium and single budding cells, but fail to form ascospores. [10] However, because of its yeast-like form and dimorphism it is sometimes filamentous. Some of these microorganisms probably contain some type of regulatory gene that control the production of THase. For example, the regulatory gene *areA300* in *Aspergillus nidulans* considerably elevate the levels of THase by at least partly alleviating the nitrogen metabolite repression and oxygen repression on the synthesis of THase. [12] However, none of these fungi have been measured for the production of IDCase, the enzyme whose substrate IOA is the product of the THase catalyzed reaction in the salvage pathway. The amounts of IDCase in these organisms were calculated in the same manner as the levels of THase. Value comparisons of specifc enzyme activity are in Table-20. However, THase activity was measured from both a crude and pure sample, but IDCase activity was detected from a crude extract because it has not been purified to any appreciable degree.

The ascomyceteous fungi, *Neurospora crassa* (heterothallic mating), is a type of red bread mold that utilize both the *de novo* and thymidine pathway to synthesize and salvage pyrimidines. Mutant strains of *Neurospora crassa* (Williams and Mitchell, 1969 [4]) that

were deficient in a complete thymidine salvage and *de novo* pathway were obtained. The mutations were given the stock center numbers 2203 and 2204 Strain 2203 lacks the genes *pyr4*, *uc1*, and *uc2* where *pyr4* encodes the enzyme ODCase, *uc1* is a regulatory gene of a unknown mechanism and *uc2* encodes the enzyme that converts thymidine to thymine and deoxyuridine to uridine. Strain 2204 also lacks the genes *pyr4* and *uc1* but also *uc3* which encodes the thymidine salvage enzyme THase. Growth conditions for these strains of *Neurospora* includes a nitrogen and pyrimidine source since they lack pathways that would enable them to produce their own. Wild type *Neurospora crassa* was grown in media containing NH_3 (Vogels [13] media) and NO_3^{2-} (Westergaard and Mitchell [14] media) to find out which works better as a nitrogen source, see Fig.-2, and then the muatant strains were grown on the most suitable media. Since both strains do not contain the *pyr4* gene then they can not produce the enzyme that catalyze the last step in the *de novo* pathway, ODCase. This means that they are unable to synthesize pyrimidines by this pathway and would therefore need some type of nucleic acid base as uracil or thymine in their growth medium to use as a pyrimidine source and obtain normal growth. This makes them unusual. However, mutants not containing the de novo pathway are desired so that the only decarboxylate enzyme being detected will be IDCase and not ODCase. Growth medias of these pyrimidine mutants were prepared to observe if *Neurospora* will grow on the substrate IOA and consequently make more of the enzyme IDCase. If so, its gene would likely be over-expressed and more uracil will be produced, therefore more UMP. Over-expression of this gene would make attempts at gene isolation and cloning easier.

There were four growth conditions made for pyrimidine metabolism-deficient *Neurospora crassa* strain 2203. One consisted of only the minimal medium and was expected to have negative growth. Another consisted of minimal plus uracil and was

expected to have positive growth. The third consisted of the minimal plus thymine and was also expected to have positive growth. The last growth condidtion consisted of IOA and was predicted to have positive growth. For the second strain labeled 2204, there were only two growth conditions. One consists of minimal medium with thymine but of course would have negative growth since it lacks the enzyme *uc3*. The other consists of the minimal with IOA and the results were unexpected because of the lack of *uc3*. Once the culture grew, cell preparations were made in order to analyze the activity of IDCase.

The gram-negative bacterium *E.coli* was used as a host to screen a cDNA library constructed with all genes showing IDCase activity. *E.coli* is a haploid organism with a single circular chromosome which has enough DNA to account for approximately 3000 genes. [15] The *E.coli* strain used requires uracil to grow. An attempt to isolate the IDCase gene was designed based on the possibility that a uracil-requiring strain might be able to grow using IOA as sole pyrimidine source, if it acquired the IDCase gene from a library. If the wrong gene or one lacking IDCase activity gets cloned into the *E.coli* plasmid then the strain would be disrupted and the cells would not be able to make the uracil needed for growth because the pathway would be incomplete. This means that the cells would die if the strain was grown in media lacking a pyrimidine. If the *E.coli* plasmid does pick up the IDCase gene then the cells would grow on media lacking uracil because they would be able to make their own. Agarose gel electrophoresis was used to select for the plasmid DNAs that showed some type of gene insert. Then those plasmids were assayed for IDCase activity.

Chapter Five

(Experimental)

Materials

All chemicals and biological materials were obtained from commercial sources except where specified. *Candida albicans* was provided by St. Elizabeth's Hospital and the rich Sabouraud's media used for its growth was prepared by the Health and Safety Department (HSD) at Youngstown State University (YSU). The *Candida stellatoidea* was provided by Dr. John Yemma's research lab at YSU. The radioactive isotope ^{14}C was obtain from Dupont-NEN.

The radioactively labeled substrate, [7-^{14}C] iso-orotate (IOA), was synthesized from uracil and [1-^{14}C] formaldehyde in two steps (Cline and Fink, 1959 [16]). The first step is done under basic conditions to produce [7-^{14}C]HMU which takes about three days. The second step is oxidation and involves the addition of platinum oxide (PtO_2) to complete the synthesis of [5-^{14}C] iso-orotate by oxidizing HMU. IOA was pre-acidified to drive off excess CO_2 out of the solution so that the radioactive counts measured would only be that which comes from the conversion of IOA to uracil and CO_2. To acidify IOA 2 ml was dispensed in a assay vial covered with a rubber septum and micro amounts of 2 M HCl was injected into the liquid until the color indicated pH paper read about 4.0. Then the vial was incubated at room temperature for an hour to let the reaction take place.

Methods

Tissue Preparations:

Growth conditions for species of the genus *Neurospora* The type of minimal media used was by Westergaard and Mitchell, Table-1. A 10X stock concentration was made and diluted 50 ml in 450 ml of dH_2O to give 500 ml of 1X solution. Sucrose was added 10 g / 500 ml as the sugar source. Fifty mililiter aliquots of the 1X Westergaard solution were poured into ten 250 ml Erlenmeyer flask. When growing the mutant strains of *Neurospora crassa*, 2203 and 2204, 560 µl of 20 mg/ml uracil was added to some flasks and 625 µl of 20 mg/ml thymine was added to others. Both pyrimidines were added to give a final concentration of 2 mM. When testing the growth of the mutants on IOA, 526 µl of 20 mg/ml IOA was added. The opening of each flask was covered with aluminum or stuffed with cotton and sterilized by autoclaving for about 20 min. on slow exhaust. This was done for each media preparation before inoculation. After the flasks cooled to room temperature about a loop full of the *Neurospora crassa* culture was added to each flask. The metal loop used was sterilized with a flame and sterile water. The flasks were then incubated at 30 °C for about 3 $^1/_2$ days. Each 50 ml media flask provided about 1g of *N. crassa* tissue. *N. africana* took about 5 days to grow and give about 1 g of tissue per 50 ml of media.

Table-1a. Westergaard and Mitchell Crossing Medium	
To make 1 liter of a 1X stock solution add the following;	
Compounds	**Amount**
KNO_3	1.0 g
KH_2PO_4	1.0 g
$MgSO_4$ 7 H_2O	0.5 g
NaCl	0.1 g
$CaCl_2$	0.1 g
Trace Elements	0.2 ml
Biotin (50 X)	0.2 ml
dH_2O	1000 ml

Table-1b Trace Element Solution	
* In 95 ml of dH_2O completely dissolve the following;	
Compound	**Amount**
Citric acid $\cdot 1H_2O$	5.0 g
$ZnSO_4 \cdot 7H_2O$	5.0 g
$Fe(NH_4)(SO_4)_2 \cdot 6H_2O$	1.0 g
$CuSO_4 \cdot 5H_2O$	0.25 g
$MnSO_4 \cdot 1H_2O$	0.25 g
H_3BO_3 anhydrous	0.05 g
$Na_2MoO_4 \cdot 2H_2O$	0.05 g
$CDCl_3$	1.0 ml
dH_2O to make 100 ml	

Growth Conditions for *Aspergillus* *Aspergillus nidulans* was grown in Hunter's [17] minimal media, 50 ml / flask, refer Table-2. After all of the salt reagents were added and dissolved in dH_2O then the pH was adjusted to 6.5 with 2 M HCL. Glucose and Hunter's trace elements were then added. The dH_2O was added to complete the volume. *Aspergillus* took about 5 days to grow to a dense culture giving about 1 g of tissue per 50 ml of media. The protein lysate was made in the same manner as *Neurospora*.

Table-2a Hunter's Minimal Media	
50 ml / flask	**Total Stock Volume 500 ml**
Reagents	**Amount**
$NaNO_3$	3.0 g
KCl	0.26 g
$MgSO_4 \cdot 7H_2O$	0.26 g
KH_2PO_4	0.76 g
Glucose	5.0 g
Trace elements	1.0 ml

Table-2b Hunter's Trace Elements	
2 ml / liter	**Total Stock Volume 100 ml**
Reagents	**Amount**
dH_2O	100 ml
$ZnSO_4 \cdot 7H_2O$	2.20 g
H_3BO_3	1.10 g
$MnCl_2 \cdot 4H_2O$	0.50 g
$FeSO_4 \cdot 7H_2O$	0.50 g
$CoCl_2 \cdot 6H_2O$	0.16 g
$CuSO_4 \cdot 5H_2O$	0.16 g
$(NH_4)Mo_7O_4 \cdot 4H_2O$	0.11 g
EDTA*	5.00 g

Growth Conditions for species of *Candida* *Candida albicans* was grown in 150 ml of Sabouraud's + dextrose media (Table-21; dissolve ingredients, adjust pH to 5.7, dispense into tubes and autoclave at 121 °C for 10 min.) and grew at 30 °C to a dense enough culture in 24 hrs to give approximately 2 g of tissue per 150 ml of media. Although, when this species was grown in Sabouraud's + lactose it took twice as long to grow and only provided about 1.5 g of tissue per 150 ml flask. *Candida stellatoidea* was also grown in Sabouraud's but uses lactose better than dextrose as a sugar source. This culture was slow growing at 30 °C in broth and took about 72 hrs to give precisely 1.3 g of tissue per 150 ml of media. *Candida* will grow at temperatures between 25°C - 37°C. Dimorphic fungi are filamentous when cultured at 25 °C; yeast-like on culture at 37 °C. These cells were harvested by centrifugation.

Harvesting Tissue Cultures Each flask of *N. crassa*, *N. africana* or *A. nidulans* tissue were filtered by vacuum filtration, not allowing the tissue to dry completely. The filter paper was changed and the buchner funnel was rinsed with dH_2O every three flask. The tissue was weighed in a plastic boat on a Mettler PM 100 scale and in some cases frozen in a VWR Scientific freezer set at -70 °C until it was ready to be lysed.

Protein Lysate In a sterile, 50 ml Fisher brand conical tube approximately 2 g of protein tissue was mixed with 20 ml of GDH-pH 8 lyses buffer minus ethylenediaminetetra-acetic acid (EDTA) while maintained on ice. EDTA was added to the buffer in one experiment to see if it would have an effect on the enzyme activity (See Fig.-17 for results). The amount of tissue added differs between fungi organisms. *Aspergillus nidulans* and *Neurospora africana* requires about 4 g of tissue because the density of their tissue was less than that of *Neurospora crassa*. Approximately 5 g of glass beads were added to the 50 ml tube with three protease inhibitors all having a concentration of 1 mg / ml, 13.3 µl of pepstatin A (3.3 µl / 5 ml), 6.8 µl of leupeptin
(1.7 µl / 5 ml) and 40 µl of PMSF (10 µl / 5 ml). Once these inhibitors were prepared they were used for about six weeks before fresh inhibitors were made, see Table-3. The glass beads were used to

cut the protein tissue and the inhibitors block the enzymatic activity of the proteases which degrade the protein. The 50 ml tube was then vortexed on a junior mixer for 15 min. to break up the cells. An experiment was done to determine if vortexing time would be a factor in making the protein solution more concentrated. The results are shown in Fig.-17. After vortexing the solution was spun down by a Beckman GPR table top Centrifuge at about 3500 rpm for 10 min. The supernatant was transferred to a new sterile tube which was then sometimes frozen for a short period of time or assayed.

Table-3 Solutions Required for Making a Protein Lysate	
GDH Extract - EDTA (Stock Volume 100 ml)	
Reagent	**Amount**
dH_2O	96.3 ml
1 M Tris, pH 8.0	3.0 ml
Beta-mercaptoethanol	7.0 µl
Protease Inhibitors (Stock Solution)	
Pepstatin A	3 mg in 3 ml of EtOH
Leupeptin	3 mg in 3 ml of EtOH
PMSF	3 mg in 3 ml of dH_2O

* The PMSF is usually much more concentrated: 100 mM (174 mg/10 ml)

Protein Lysate for Candida The method of making a protein lysate for *Candida* was a little different from the others. After the cultures had grown instead of vacuum filtering, each flask containing 150 ml of broth were dispensed into 50 ml conical tubes and spun down in a Beckman GPR table-top centrifuge. The supernatant was discarded. The tubes were weighed before adding the culture and after discarding the supernatant in order to obtain an approximate weight of the pellets (tissue). The pellets were resuspended in a small volume of GDH buffer. The tubes were vortexed to assure the pellets were resuspended. The resuspensions were all dispensed into one tube and GDH was added to complete the desired volume. The protease inhibitors were added to this tube in an amount that was twice as much as that used for the other organisms because *Candida* is expected to have a lot more proteases. The rest of the procedure was done as previously discussed.

Ammoniun Sulfate Precipitation $AmSO_4$ was ground with a mortar and pestle into a thin powder. About 4 ml of protein lysate was saved to run as a control in the radioactivity assay. The rest of the lysate was poured into a 50 ml beaker while sitting in a glass bowl of ice on a Corning Stirrer/Hot Plate. The solution was kept at 4 ^{o}C . A stirring bar was added to mix the solution. The addition of $AmSO_4$ corresponds to the volume of the protein solution (See Table-4 for values of percent saturation [18]). The given amount of $AmSO_4$ was added to the beaker and allowed to stir for about 20 min. The solution was poured into a clean centrifuge tube and spun down in a Beckman L7-35 Ultracentrifuge for 12 min. at 15000 rpm to obtain a pellet. The supernatant was then poured back into the 50 ml beaker for the next addition. The $AmSO_4$ pellet from the second addition was resuspended in about 2 ml of GDH buffer (pH 8.0). This suspension was poured into a dialysis bag which had been soaking in a cold beaker of Tris-glycerol buffer (10 % glycerol in 50 mM tris), pH 8.0. The mix was then dialyzed for an hour in another beaker of cold buffer while sitting in a refrigerator. The 0 % saturation is the protein lysate without any $AmSO_4$ addition and the unprecipitated lysate is that lysate after the last $AmSO_4$ % saturation addition has been spun to a pellet. The unprecipitated solution was also dialyzed. After dialysis the solution was pipetted from the bag into a sterile 25 ml conical tube

and maintained on ice until used in the assay mix. See Fig.-18 for results. Only 30 minute time point was taken for the $AmSO_4$ % saturations to allow time for reaction.

Table-4 Ammonium Sulfate Precipitation	
The Addition of $AmSO_4$ Per 20 ml of Lysate Solution	
Amount	**Percent Saturation**
0.00 g	0%
2.12 g	20%
1.10 g	30%
1.12 g	40%
1.16 g	50%
1.20 g	60%
1.24 g	70%
1.30 g	80%

Quantitating the IDCase and Protease Inhibitors This is done by UltraViolet Spectroscopy. The actual concentration of each inhibitor was determined from the absorbance reading by using the mathematical equation representative of Beer's Law, $A = \in bc$. A is the dimensionless absorbance, $\in$ is the molar absorptivity or commonly called the extinction coefficient and has the units M^{-1} cm^{-1} because the product $\in bc$ must be dimensionless, b is the path length which is always equal to 1 cm and c is the concentration given in units of mol/l (M). [19] The $\in$ is the characteristic of a substance that tells how much light is absorbed at a certain wavelength. Each inhibitor has an $\in$ and A that was used to help calculate the exact c, see Table-5. The application of Beer's law is limited to a narrow concentration range in which absorbances are measured at less than or equal to 1. This means that the mixtures have to be diluted to fit the range. For example, a 10 mM solution of 5-FU was diluted 1/10 to give 1 mM and then diluted 1/10 again to give a final concentration of 0.1 mM which was measured on the spectrophotometer. The absorbance of a diluted solution is used to calculate the concentration of the concentrated solution from which the dilution was prepared.

Table-5 UV Spectra Data Results for Determining Actual Concentrations					Stock conc.
Inhibitor	**Absorbance**	**Wavelength**	**Extinction Coeff.**	**Expected Conc.**	**Actual Conc.**
5-FU	0.7	266 nm	7070	.100 mM	.070 mM
5-NO_2U	0.2287	342 nm	16,000	0.785 mM	0.0143 mM
5-HU	0.1099	305 nm	5,000	0.640 mM	0.0220 mM

UV Spectrophotometric Assay Absorbances were measured on a Hewlett-Packard 8452A Diode Array Spectrophotometer. Solutions used for this assay were 100 mM Tris pH 7.4, 500 μM IOA, 20 mg / ml of uracil, 1 mM zinc (Zn^{2+}) and dH_2O. Contents were added together and mixed in a 1.5 ml microfuge tube and 1.5 ml quartz cuvettes were used to read the wavelengths. Each microfuge tube contained 250 μl of 1 mM Zn^{2+} and the tube without Zn^{2+} contained more dH_2O. The volume addition of Tris was consistent, 20 μl of 100 mM Tris. Different concentrations of IOA and uracil were added to each tube, see Table-6. Distilled water was added last to complete a final volume of 1000 μl. The spectrophotometer was blanked with 980 μl of distilled water and 20 μl of 100 mM Tris. The absorbances were measured at a wavelength range of 220 - 320 nm in order to see the pattern of the entire reaction.

Table-6 UV Spectrophotometric Assay: Volume and Concentration of major components					
[IOA] initial	Volume of IOA added	[IOA] final	[uracil] initial	Volume of uracil added	[uracil] final
500 μM	100 μl	50 μM	500 μM	0 μl	0 μM
	80 μl	40 μM		20 μl	10 μM
	60 μl	30 μM		40 μl	20 μM
	40 μl	20 μM		60 μl	30 μM
	20 μl	10 μM		80 μl	40 μM
	0 μl	0 μM		100 μl	50 μM

Radioactivity Assay Each assay vial contains 450 µl of protein lysate, 50µl of acidified substrate, IOA, and approximately 0.2 ml of 2 M HCl which was added at the end of the reaction time to stop the reaction. The total IOA concentration of each vial was 50 µM. When assaying with the enzyme inhibitors, 5 µl of the desired concentration of inhibitor was added to the assay vials of choice. This small amount is not enough to significantly affect the total volume. First the protein lysate was added to the vials and then the substrate was added to each vial in 1 minute increments. Assays were carried out at 30°C. When used, the enzyme inhibitors were added right before the substrate. The vials were quenched with the 2 M HCl using a 1 ml syringe at different times during the assay depending on the desired time point. See Table-7 for guidelines. The assay vials were placed in a Dubnoff metabolic shaking incubator for about an hour. After this the wicks were taken out of the vials with tweezers, placed in a square glass dish containing numbered wells and put into a Napco E. series vacuum oven until the they dried or hardened. The wicks were then taken out and placed in scintillation vials containing enough Fisher ScintiSafe Econo 1 scintillation fluid to cover the wicks. The scintillation vials were placed in a Packard Tri-Carb 1900CA Liquid Scintillation Analyzer where the radioactivity of each vial was measured for 5 minutes in units of Cpm. Duplicate assays of a given enzyme preparation were taken at each time point and usually agreed within $^{+}$/- 11 % of the mean.

Table-7 Guidelines for the Radioactivity-Based Assay (Duplicate Assays)			
Assay Vial	**Time Point (min.)**	**Start Time (min.)**	**Quench Time (min.)**
1 , 2	0	0 , 1	0 , 1
3 , 4	10	2 , 3	12 , 13
5 , 6	20	4 , 5	24 , 25
7 , 8	30	6 , 7	36 , 37

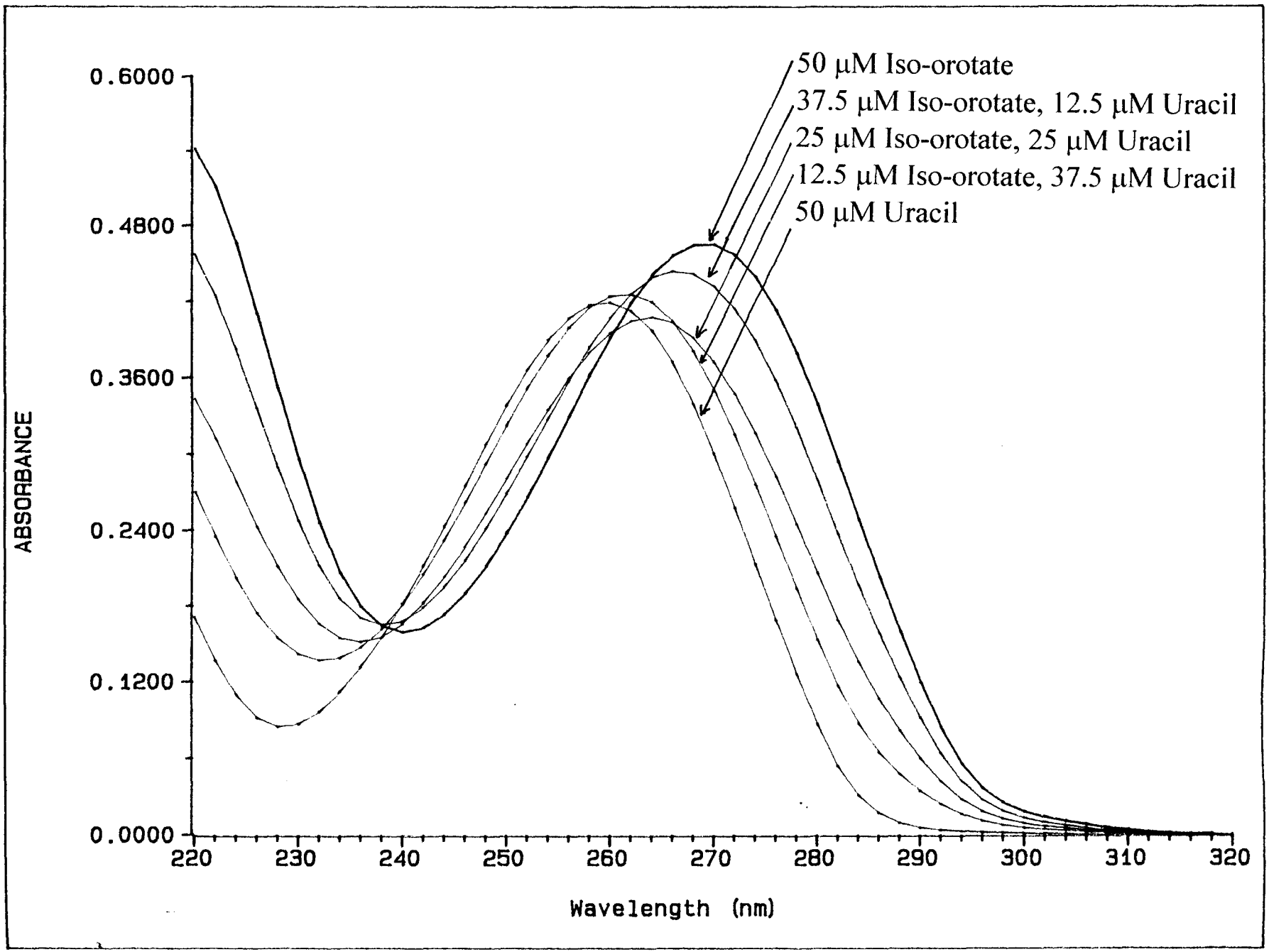
BSA Standard Protein Assay The BSA standard concentration used was 1 mg / ml of GDH buffer. Dilutions with water in 1.5 ml microfuge tubes were made to obtain the following BSA concentrations; 0, 0.10, 0.25, 0.50, 0.75, and 1.00 mg / ml. The protein lysate was normally diluted to give absorbance (optical density, OD) readings that fit inside the BSA concentration range. The dilutions were usually 12.5-100 % of the protein lysate. In 1.5 ml microfuge tubes 790 µl of dH_2O, 200 µl of Coomassie Blue (G-250) reagent, and 10 µl of BSA concentrations were measured out to give a final volume of 1 ml. The same was done for 10 µl of each lysate dilution. The tubes were vortexed to mix the contents and then set at room temperature for 5-10 min. to allow the reaction to take place. The content of each microfuge tube was dispensed into the 1.5 ml quartz cuvette one at a time, thoroughly rinsing with dH_2O after each use. Single absorbance measurements were taken of each tube at the wavelengths 596 nm. Once these measurements were obtained they were used in the calculation for specific enzyme activity (refer to Fig.-6.).

Chapter Six

Results and Discussion

Fig.-4 shows a spectrum of the simulated conversion of 50 μM IOA to 50 μM uracil giving two different isosbestic points at about 238 nm and 260 nm. The isosbestic points provides good evidence that only two species exist in the spectrum. The absorbances at the wavelength of the isosbestic points are constant because the sum of the extinction coefficients and concentrations of the reactant and product are constant. The maximum absorbance between the 50, 37.5 and 25 μM IOA were approximately 0.03 abs apart. The 12.5 and 0 μM IOA was expected and should have had a lower maximum absorbance than the 25 μM concentration. As the concentrations of the reactant decreased the wavelength of the maximum absorbances slightly shifted about 2 nm to the left beginning around 268 nm and ending at 260 nm. This spectrum was taken in the absence of Zn^{2+} because in its presences the maximum absorbances decreased, the spectrum shifted to the right and the lines that defined the isosbestic points were scattered out, putting them around 242 nm and 165 nm (data not included). The amount of enzyme activity in the mixtures can be calculated by measuring at the wavelength that shows the greatest difference between IOA and uracil to absorb UV light. This difference is at 280 nm. See Table-5 for concentrations and volume additions. Because of the problems, this assay was not relied on to detect IDCase activity in the microbiological cultures. The radioactivity assay was used instead.

Fig.-4. UV Spectrophotometric assay of the similated conversion of IOA to uracil


50 μM Iso-orotate
37.5 μM Iso-orotate, 12.5 μM Uracil
25 μM Iso-orotate, 25 μM Uracil
12.5 μM Iso-orotate, 37.5 μM Uracil
50 μM Uracil
ABSORBANCE
0.6000
0.4800
0.3600
0.2400
0.1200
0.0000
220
230
240
250
260
270
280
290
300
310
320
Wavelength (nm)

Results from the radioactivity-based assay show that IDCase activity in a fresh protein lysate of wild-type *N. crassa* (74A) was about 2-fold greater when the organism was grown on Westergaard's (NO_3) than Vogel's (NH_3). See Fig.-2 for the graph of radioactive counts. Therefore, NO_3 works better for inducing IDCase as the sole nitrogen source in the growth media than NH_3. However, if the purpose is to study the effects that heavy metals and enzyme inhibitors have on the protein or enzyme activity then Vogel's media may be the ideal to use because it provides more tissue to work with. Most assays show a steady increase in activity from the 0 to the 30 minute time point. After 30 minutes the activity of the enzyme begins to level off. This can be due to protease digestion of IDCase or because the protein starts to run out of substrate. Radioactive counts that were significantly higher than the background were taken as real activity, see Table-8. By observing Fig.-2, the protein activity of frozen Westergaard's lysate appears to be only about 10 % more than frozen Vogels lysate. Fresh Westergaard's appears to have 35 % more protein activity than Westergaard's tissue that had been frozen for a period of time. Fresh Vogel's show 30 % more protein activity than frozen Vogel's. The protein concentration in Vogels was 1 $^1/_2$-fold greater than Westergaard's, but the specific enzyme activity in Westergaard's was about 2-fold greater. See Table-22 for values of specific enzyme activity in the $AmSO_4$ precipitation and all of the microorganisms studied in this thesis.

Table- 8 Radioactive counts of IDCase activity in wild-type *N. crassa* grown on Westergaards and Vogels media

		avg. CPM-BKG		
Time Points (min)	Westergaards (fresh)	Westergaards (frozen)	Vogels (fresh)	Vogels (frozen)
0	0	0	0	0
10	699	1032	484	221
20	1128	644.5	832	570.5
30	1367	974	1038	748
40	1374	947.5	1036	894.5

All radioactive values are in CPMs

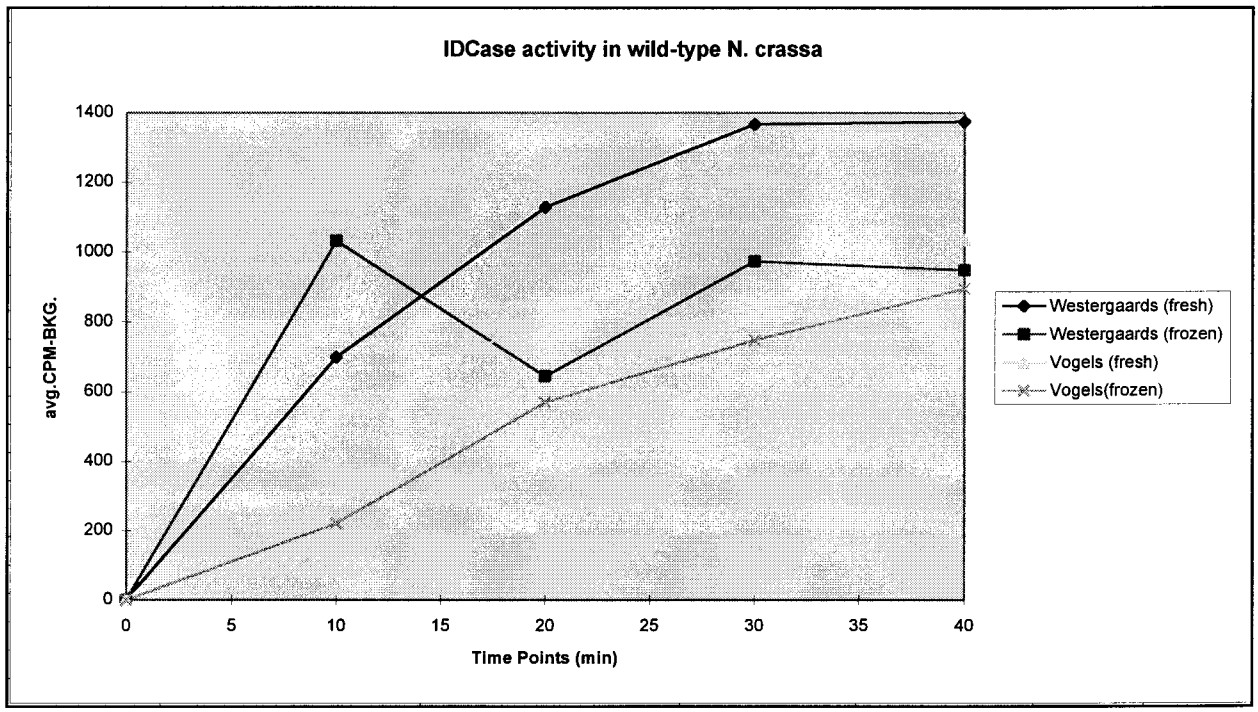


Fig.-2 IDCase activity in wild-type *N. crassa* grown on Westergaards and Vogels media.

An experiment was done where 74A was grown on Westergaard's plus thymine and uracil to observe which base would cause the protein to have more enzyme activity. There were four flask of 74A + thy and three of 74A + ura and 74A alone. The 74A + thy produced a total of 1.422 g of tissue, 74A + ura produced 0.653 g, and 74A produced 0.494 g. Proportional amounts of buffer were used in the lysates. The BSA standard protein assay provided absorbance readings that were used to determine the protein concentration of each sample. Fig.-11 shows an example of how the protein concentration was determined using a standard BSA plot (see Table-10 for values). There was not a significant difference between the protein concentration of the tissues. 74A + ura was a little more than 1-fold greater than 74A + thy and 74 A was a little more than 1-fold greater than 74A + ura. The difference between the specific enzyme activity of 74A and 74A + ura was also not that significant. However, the specific enzyme activity in 74A + thy was about 2 ½ -fold greater than the others. The graph is shown in Fig.-10 and the data is in Table-9.

Table-9 Radoiactive counts of IDCase in wild-type *N. crassa*			
Time Points (min)	**74A**	**74A + thy**	**74A + ura**
0	0	0	0
10	37	72.5	37
20	121	183	55.5
30	61.5	245	106.5

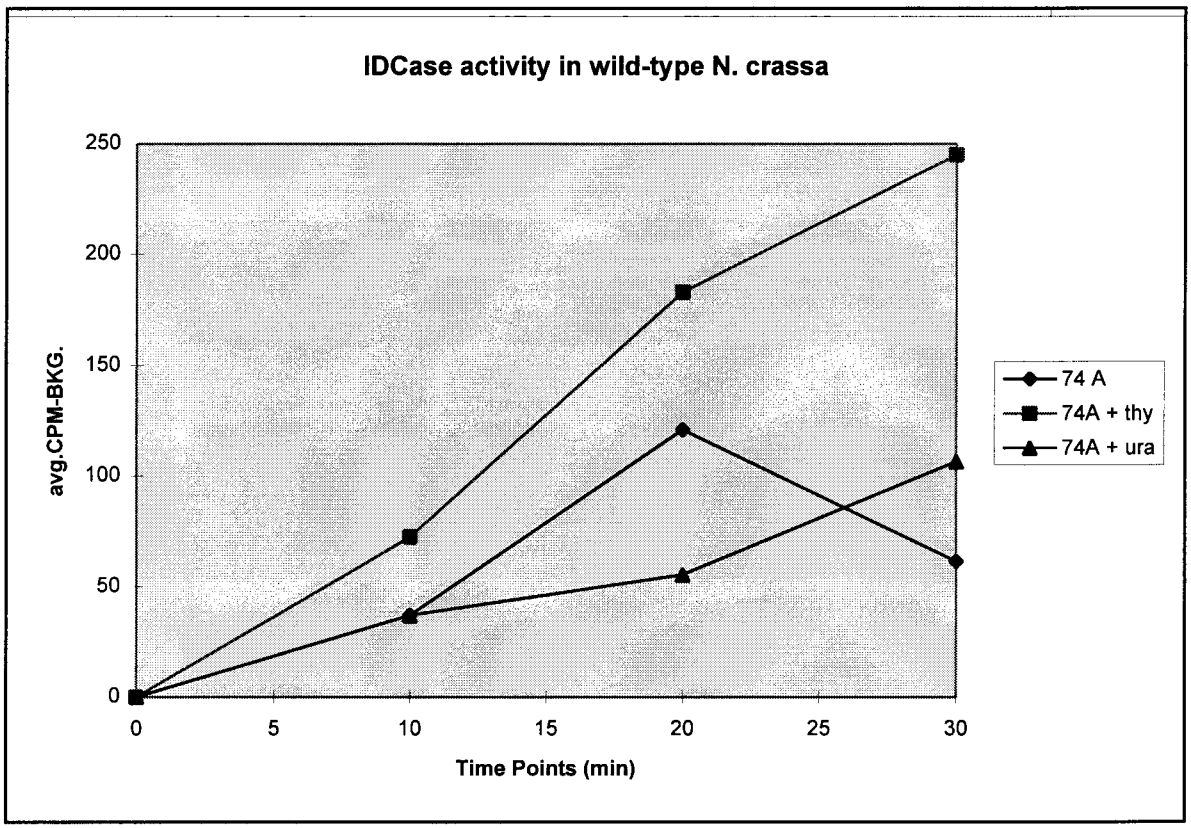


Fig.-10 IDCase activity in wild-type *N. crassa* grown in the presence of thymine and uracil.

Table-10 BSA Standard Protein Assay		
Sample	**Conc.**	**596 nm**
[BSA]	0.1	0.007
mg/ml	0.25	0.056
	0.5	0.145
74A	12.50%	0.12
74A + ura	12.50%	0.105
74A + thy	25%	0.079

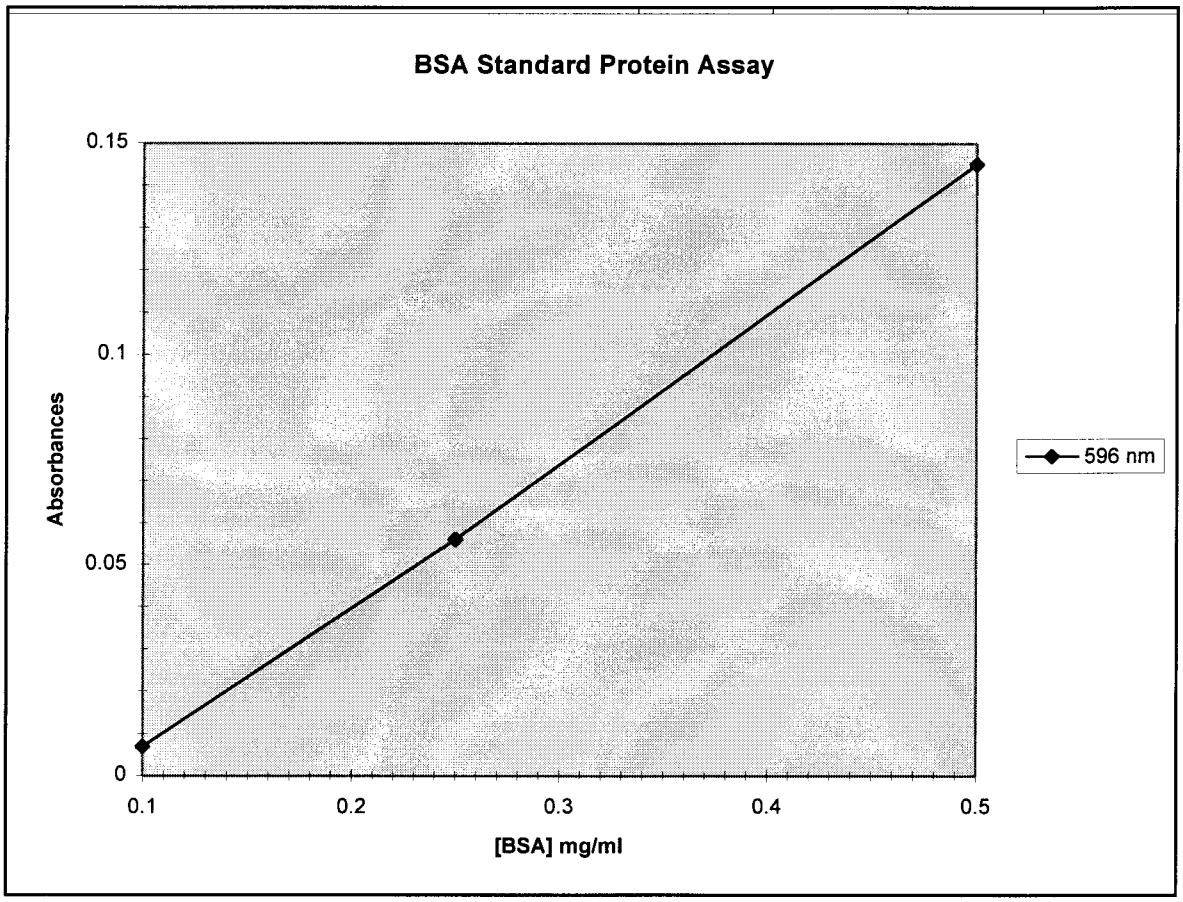


Fig.-11 BSA standard protein determination curve. The concentrations of the *Neurospora* protein samples are obtained by using the absorbances to trace across and down the standard plot to the standard concentration.

If protease inhibitors are included in the lysate then there will be more protein activity, thus possibly more enzyme activity. Vogel's media was used to test the effects that these protease inhibitors have on protein activity, see Fig.-12 and Table-11. The protein concentration in the lysate with proteases inhibitor was 1 $^{1}/_{2}$-fold less than that without. However, there was about

35 % more protein activity and the specific enzyme activity was more than 2-fold greater in the lysate containing protease inhibitors All of the microbiological cultures contained protease inhibitors in their lysate except in the experiment of Westergaards vs. Vogels as a better growth media.

Table-11 Radioactive counts of IDCase activity affected by the Protease inhibitors		
Time Points (min)	(+) Protease Inhibitor	(-) Protease Inhibitor.
0	0	0
10	625.6	1186.4
20	992.8	1708.8
30	1336	1900.8
40	1568	1982.4

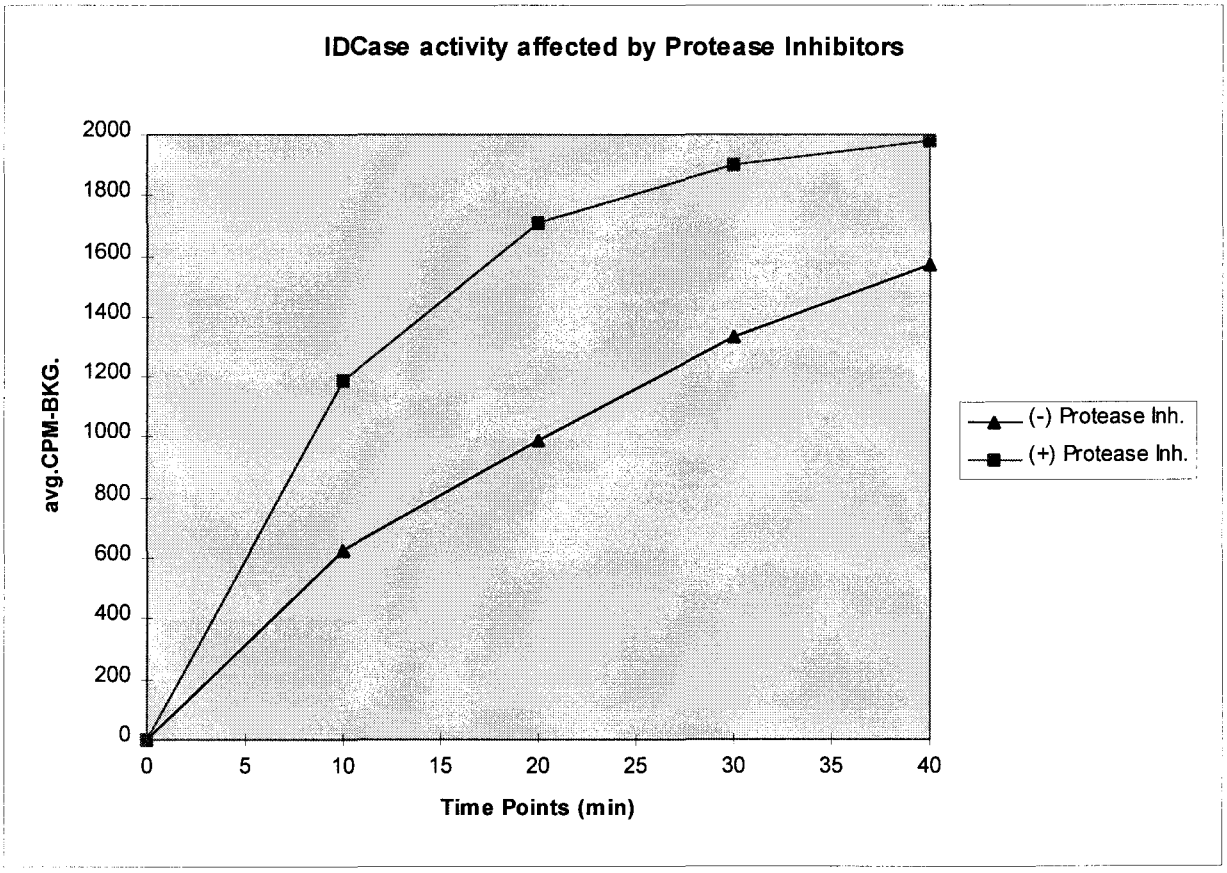


Fig.-12 IDCase activity in wild-type *Neurospora crass* grown on Westergaards vs. that on Vogels media.

Vogel's media was also used to study the effects that EDTA may have had on protein and enzyme activity. EDTA can act as a chelating agent and bind to transition metals or as a protease inhibitor. Since the structure of IDCase is not known, it is also not known if it contains any transition metals in its complex. For this reason EDTA was tested before its trial use as a protease inhibitor. The EDTA concentrations in the protein lysate were 0, 1, and 10 mM. Results from the radioactivity assay indicate that the protein and enzyme activity between the 0 and 1 mM are relatively similar. Although, the 10 mM concentration showed 40 % more activity. Therefore, the 1mM didn't have much of an effect on the activity but the 10 mM help increase it and appears to be useful as a protease inhibitor, see Fig.-13 and Table-12.

The BSA standard protein assay was used to analyze the effects that vortexing time has on protein concnetration. *E.coli* tissue cultures were used for this purpose. The more protein tissue present in a sample the more the absorbance seems to increase. There was not much difference between the solutions that were vortexed for 5 and 10 minutes. The absorbances were only about 0.006 abs apart, although the 10 minute lysate was more. However, the solution that was vortexed for 15 minutes was about 0.025 abs more than the 10 minute solution. The absorbances for these solutions were not high enough to fit into the BSA standard protein range and consequently the exact concentration cannot be determined (so the graph of protein concentration determination is not included). Though the data obtained is good eneough to conclude that the longer the vortexing time the more concentrated the protein lysate becomes. The data is reliable enough to have sufficient use in any microbiological culture.

Table-12 Radioactive counts of IDCase activity affected by EDTA			
Time Points (min)	0 mM	1 mM	10 mM
0	0	0	0
10	278	294.5	367
20	546	488	904.5
30	548	710.5	1201

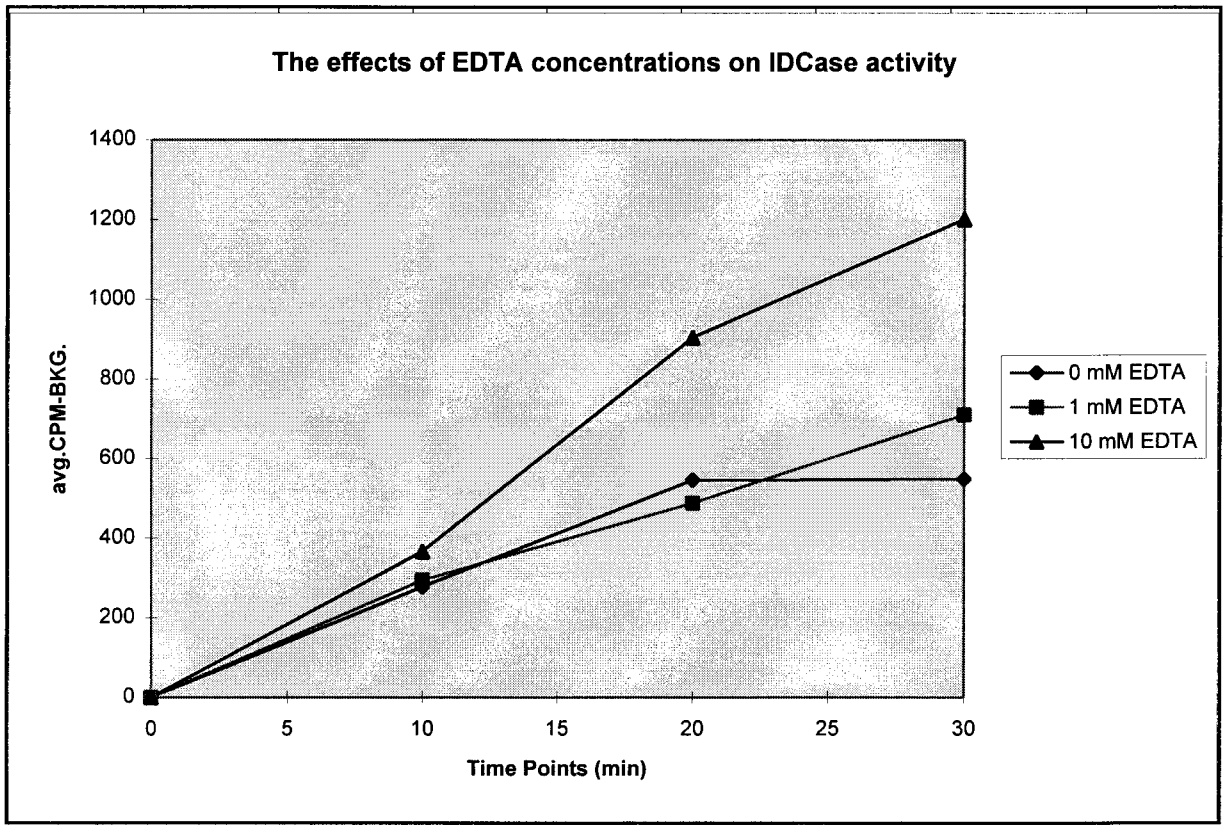


Fig.-13 The effects of different EDTA concentrations on IDCase activity in wild-type *N. crassa.*

Since Westergaards seem to show more enzyme activity the mutant strains of *N. crassa*, 2203 and 2204, were grown on Westergaards. Uracil or thymine have to be used as the sole nucleobase because the cell culture did not seem to utilize IOA well enough to obtain average or less than average growth when IOA was used as the nucleic acid base. When the cells were first grown on uracil or thymine then transferred to IOA after they had begun to grow, they were still able to continue normal growth.

In this experiment each strain was grown in the presence of uracil and thymine to see if the same results would occur with the mutants as they did in the wild-type. There were eleven flask of Westergaards media prepared for each strain, five consisting of thymine and six consisting of uracil. After inoculation of each strain the cultures grew for four days producing 0.810 g of tissue for 2203 + ura, 0.543 g of tissue for 2203 + thy, 0.912 g of tissue for 2204 + ura, and 0.758 g of tissue for 2204 + thy. Proportional volumes of GDH buffer was added. The latter part of the results was baffling considering that strain 2204 lacks the gene encoding the enzyme THase that makes the IOA, and so 2204 was expected to have negative growth on thymine because it would not be able to utilize it. The protein concentration of each strain varied. Strains 2203 + ura and 2204 + thy had about four times as much protein as 2203 + thy and 2204 + ura and had relatively the same level of protein activity. Although, 2203 + thy had about 25 % more protein activity by the 30 minute time point than 2203 + ura and 2204 + thy. Strain 2204 was suppose to have positive growth on uracil but appeared to have 70 % less protein activity than 2204 + thy. However, there was not a significant difference in the specific enzyme activity of 2204 + ura and 2204 + thy. Though there was a significant difference between the two 2203's. 2203 + thy had about $4\ ^{1}/_{2}$ -fold more specific enzyme activity than 2203 + ura. This result along with that of 74A + thy indicates that thymine works better

than uracil to induce IDCase activity. Fig.-3 shows a graph of the enzyme activity in these mutant strains, see Table-13 for values.

Table-13 Radioactive Counts of IDCase Activity in Mutant Strains of *N. crassa*				
Time Points (min)	**2203 + ura**	**2203 + thy**	**2204 + ura**	**2204 + thy**
0	0	0	0	0
10	314.5	312.5	86.5	315.5
20	431.5	486	115	347.5
30	521	691	186.5	516.5

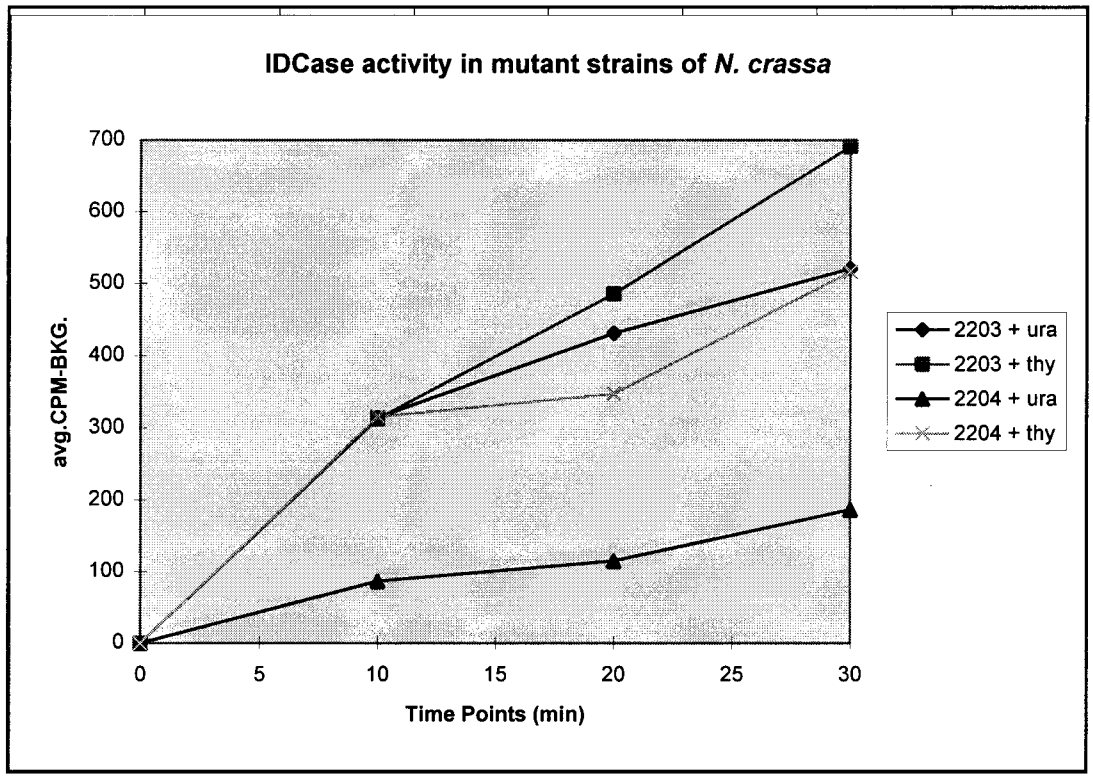


Fig.-3 IDCase activity in mutant strains of *N. crassa* grown on thymine and uracil

The *africana* species of *Neurospora* seem to have very little enzyme activity early in the reaction (before 10 minutes), but the activity begins to decrease as the reaction proceeds (Fig.-14 and Table-14). The radioactive counts at the 10 minute time point was only 35.5 cpm above background (The 0 time point was read and substracted out as background). It then descended to 16 cpm and 4 cpm above background at the 20 and 30 minute time points respectively. The amount of protein in this species was not much different from that of wild-type *crassa.* The specific enzyme activity was almost nothing. The amount that showed probably came from background or something else.

Aspergillus nidulans had IDCase activity at an amount that was a bit less than what was first detected (literature value) in wildtype *N. crassa.* The 10 minute time point was only 59.5 cpm above background, but a steady increase to the 20 (cpm almost twice as much) and 30 minute time points was expected. However, the activity started to bear off and descend before the 30 minute time point (Fig.-15 and Table-15). In order to know if what had happened was real, the protein concentration and specifc enzyme activity was calculated. The protein concentration ended up being higher than in all the other microorganisms except the 74A + thy. The specific activity at the 20 minute time point was relatively the same as that of 74A + ura. The 10 minute time point of 74A + ura was a little less than $1\ ^{1}/_{2}$-fold of the specific activity of this organism.

Fig.-16 shows that both species of *Candida* had relatively no IDCase activity. For these species measurements were taken at 5 minute time points as well in order to make sure that the first assumption (that *Candida* doesn't use the TSP) was real. There was a very small amount of cpm over background at the 5 minute time point of *C. albicans* and a steady decrease through to 30 minutes (Table-16). The *stellatoidea* species had about the same specific enzyme activity as *N. africana* at the 10 minute time point. The protein

concentration in the *C. albicans* was high as that in *Aspergillus nidulans*. The protein concentration of *C. stellatoidea* was about the same as the 74A. The specific enzyme activity of *C. albicans* was about twice as less than that in *C. stellatoidea* and *N. africana*.

Table-14 Radioactive counts od IDCase in *Neurospora africana.*	
Time Points (min)	avg.CPM-BKG.
0	0
10	35.5
20	16
30	4

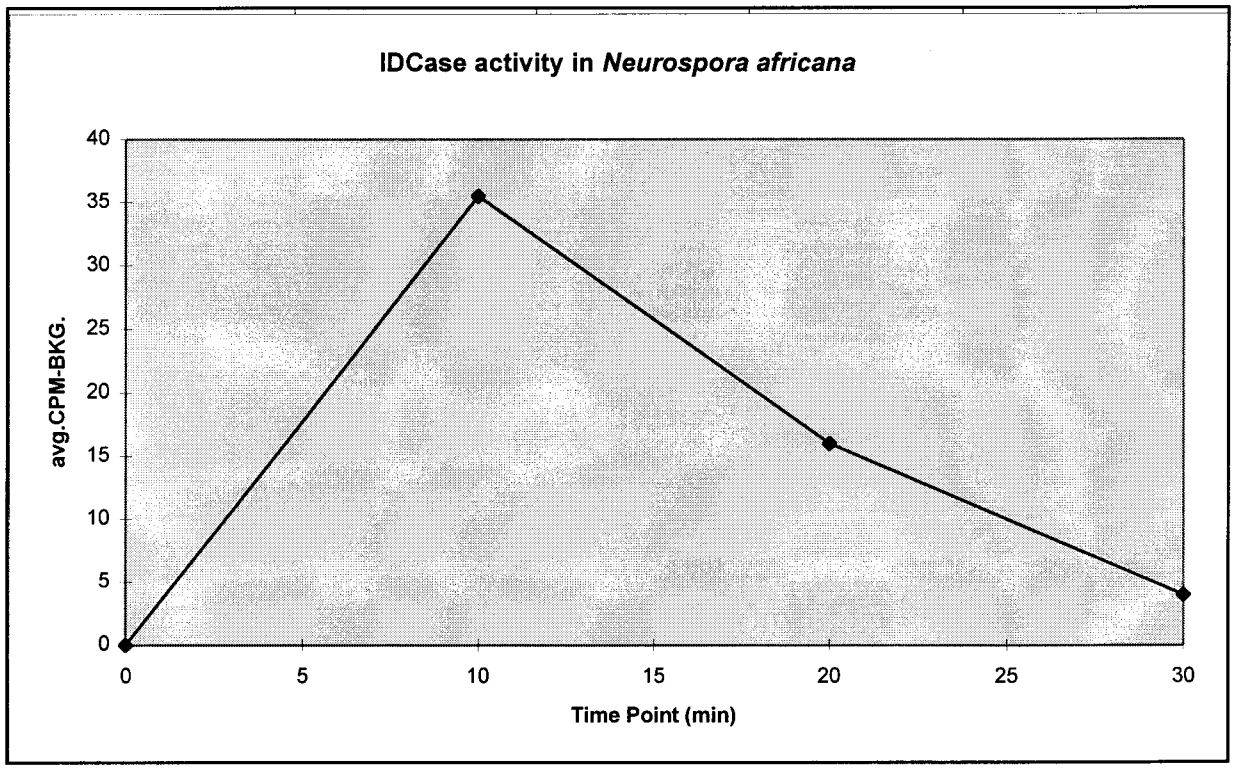


Fig.-14 The amount of IDCase activity in *Neurospora africana.*

Table-15 Radioactive counts of IDCase in *Aspergillus nidulans*	
Time Points	**avg.CPM-BKG**
0	0
10	59.5
20	103
30	96

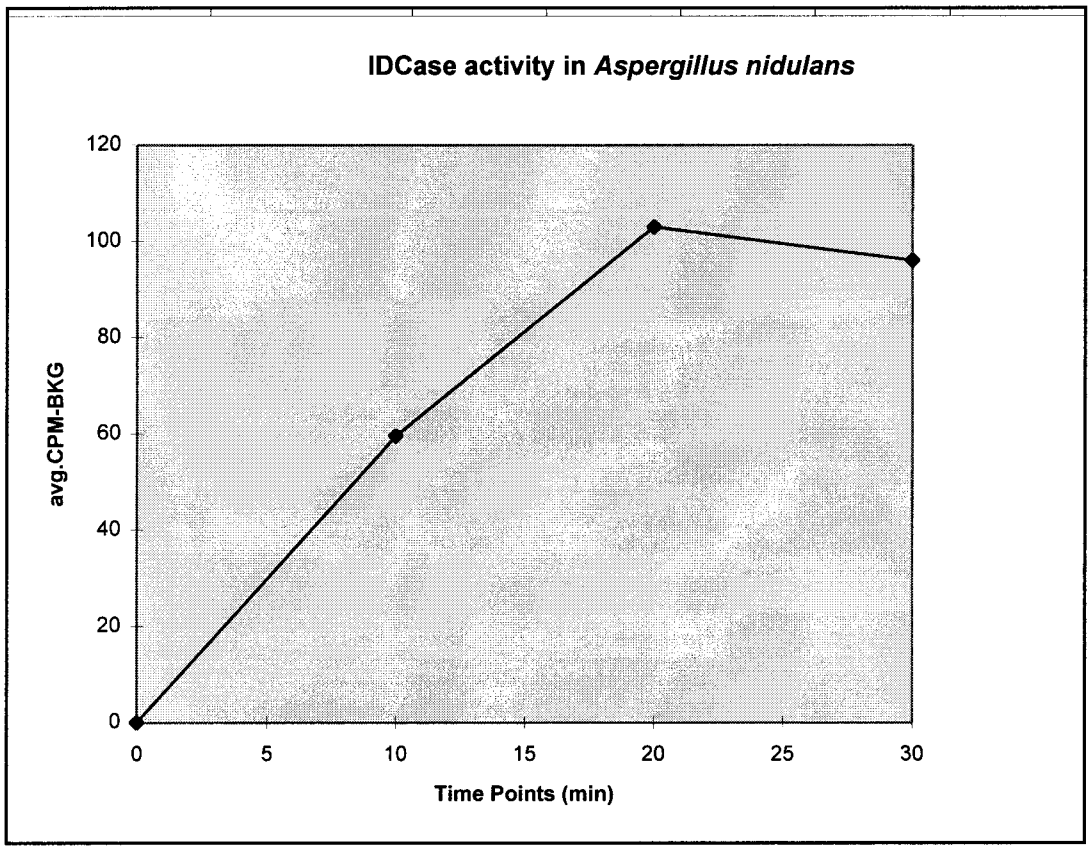


Fig.-15 The amount of IDCase activity in *Aspergillus nidulans*.

Table-16 Radioactive counts of IDCase in species of *Candida*		
Species	**Time Points (min)**	**avg.CPM-BKG.**
albicans	0	0
	5	9.5
	10	-1.5
	20	-42.5
	30	-61
stellatoidea	0	0
	5	-1
	10	19.5
	20	11.5

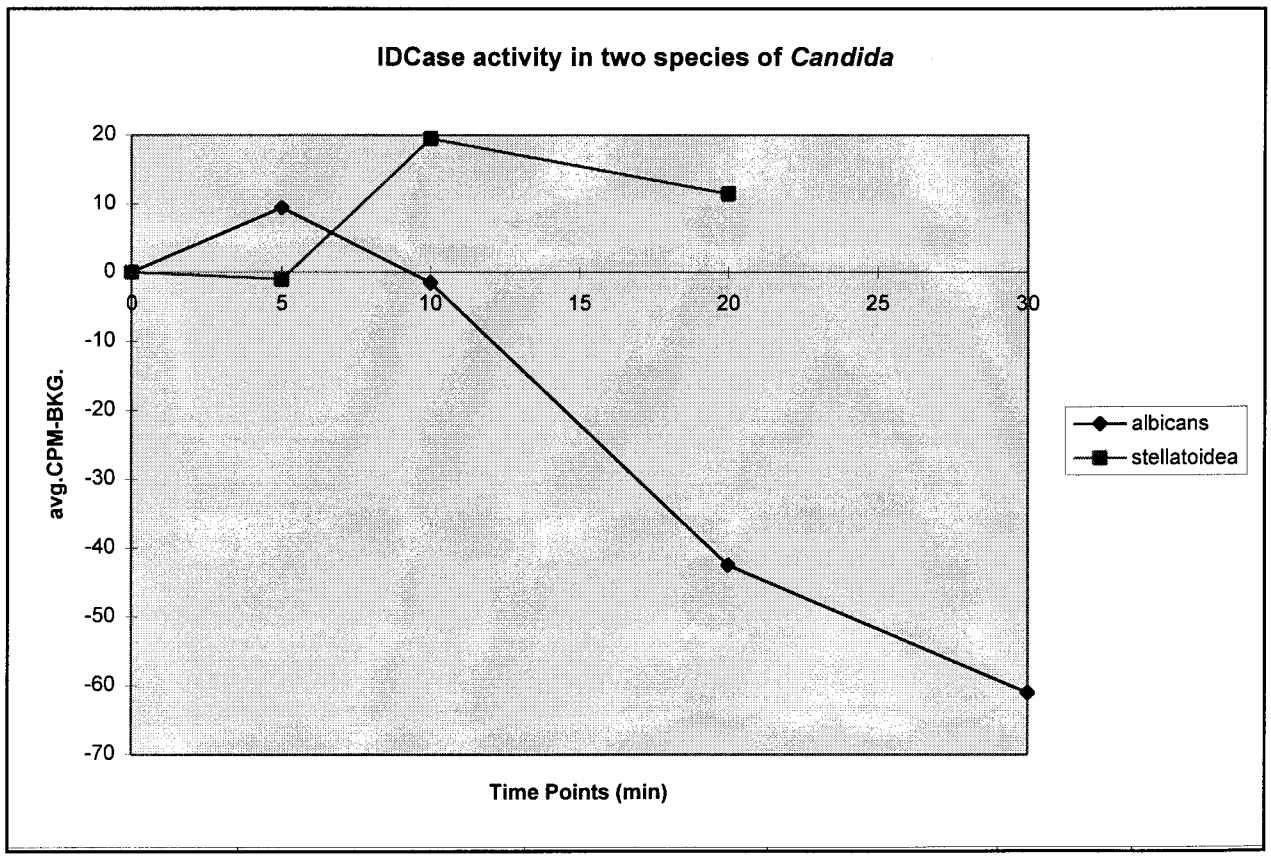


Fig.-16 The amount of IDCase activity in *Candida albicans* and *Candida stellatoidea.*

Wild-type *N. crassa* was used to test the inhibtion of IDCase activity by 5-HU, 5-FU, and5-NO_2U. These inhibitors were allowed to mix with the protein lysate before adding the substrate to the assay vial. Fig.-17 shows that 22 μM 5-HU seem to inhibit about 40 % of the enzyme activity. The 0 minute time point was relatively the same as the original protein lysate (without the inhibitor, $^-$5-FHU). There was a steady increase in $^+$5-HU following below the $^-$5-HU (Table-17). The $^-$5-FU and 10 μM $^+$5-FU at the 10 minute time point starts off about the same, but separates right after. Both increase in enzyme activity but the $^+$5-FU increases at a level about 40 % below the $^-$5-FU at the 20 and 30 minute time point, see Fig.-18 and Table-18. After 30 minutes the enzyme activity begins to increase again to about the same amount as the $^-$5-FU. A reasonable answer for this happening could be that the inhibitor ran out and the ezyme regained its activity. Fig.-19 shows that 1.43 μM 5-NO_2U inhibits just about all the enzyme activity. The activity at the 10 minute time point only begins at 13.5 cpm which when compared to the original (347 cpm) is only about 4 % activity. The activity increase to 56 cpm then decrease to 31.5 cpm, 8.5 % and 2.5 % activity respectively (Table- 19). A BSA standard assay was not done on the inhibitors. Therefore, the enzyme activity was estimated from the cpm values and not specific.

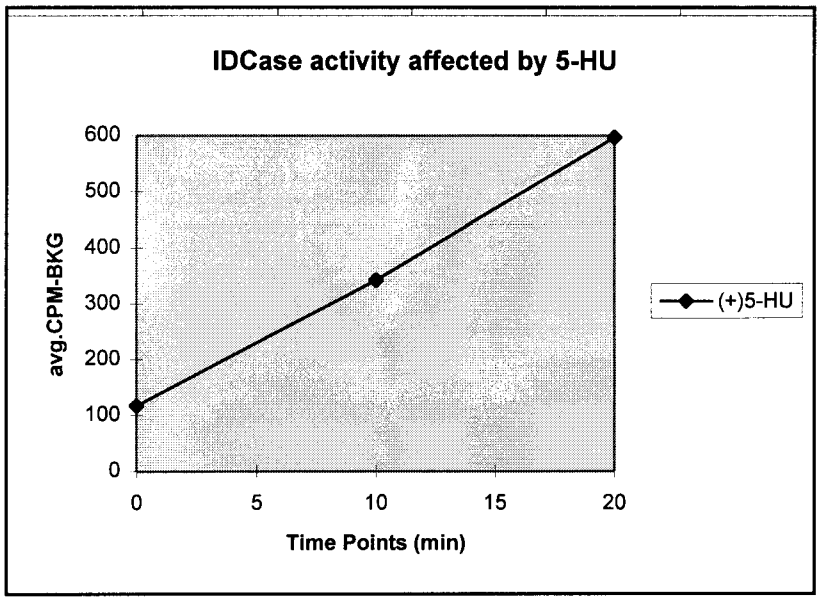
Out of the different $AmSO_4$ % saturations, the results of radioactivity-based and BSA standard assay indicated that the 60 % and 80 % saturations when dialyzed precipitated more protein and had a higher amount of specific enzyme activity, see Table-20 for radioactive counts (figure is not included). Since the first experiment showed that most of the enzyme was precipitated somewhere between the 60 % and 80 % saturated solutions, the first two time points were taken for the 80 % solution. The protein concentration for

the 60 % solution was about 2-fold greater than the unprecipitated solution which was a little more than 1-fold higher than wild-type. The 60 % solution also had 1.5-fold more protein than the 80 5 solution. Though the specific enzyme activity of the 60 % solution was nearly the same as that of *N. africana* and *C. stellatoidea*. Both the 10 and 20 minute time points of the 80 % saturated solution showed more than a 6-fold increase in specifc enzyme activity than wild-type.

Table-17 Radioactive counts of IDCase inhibition by 5-HU		
Time Points (min)	(a). (+) 5-HU	(b). (-) 5-HU
0	113.5	117
10	?	343
20	292.5	596.5
30	481	?

? means values were not obtained

(a).



(b).

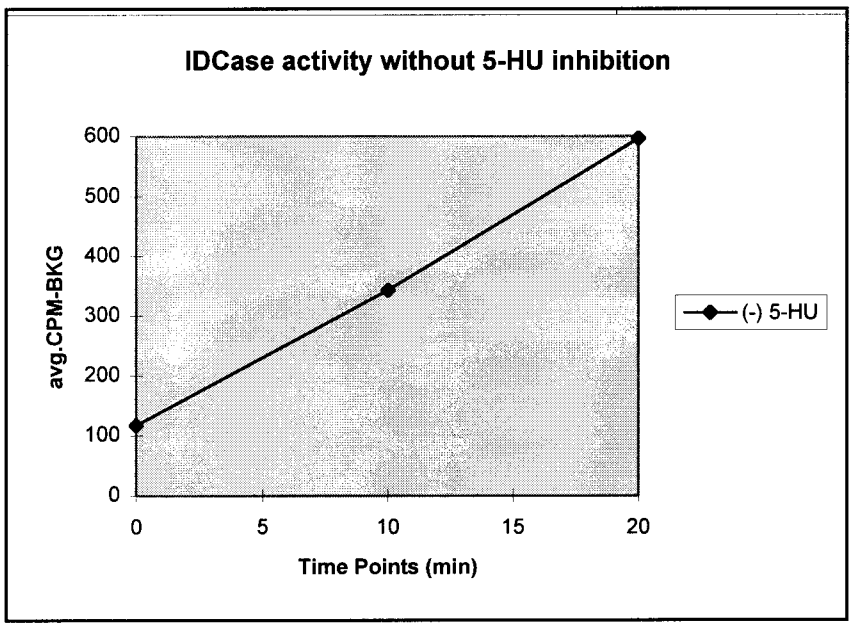


Fig.-17 (a). 22 μM 5-HU inhibiting enzyme, thus protein activity.

(b). Enzyme and protein activity without 5-HU.

Table-18 Radioactive counts of IDCase inhibition by 5-FU		
Time Points (min)	(+) 5-FU	(-) 5-FU
0	215	226.5
10	290	473.5
20	437.5	614
30	740.5	690.5

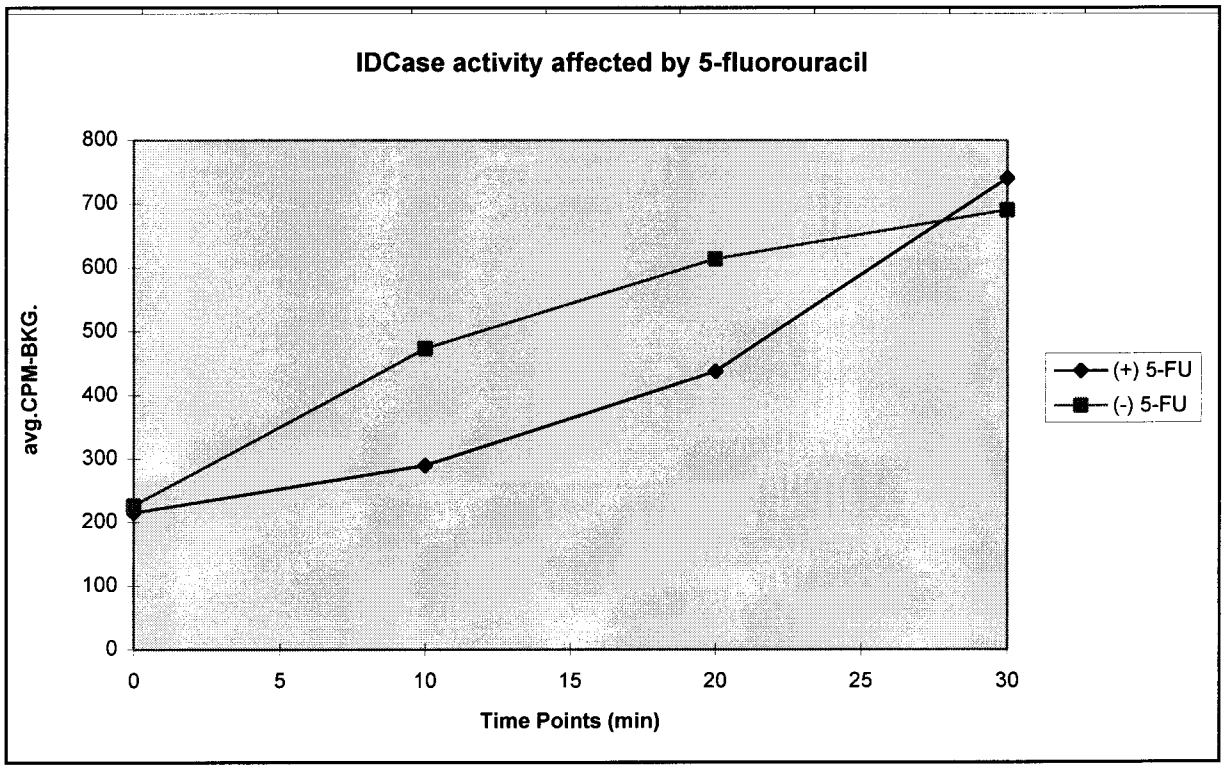


Fig.-18 The amount of IDCase activity affected by 10 μM 5-fluorouracil

Table-19 Radioactive counts of IDCase inhibition by 5-NO_2		
Time Points (min)	(+) 5-NO_2	(-) 5-NO_2
0	0	45
10	13.5	347
20	56	659
30	31.5	1213

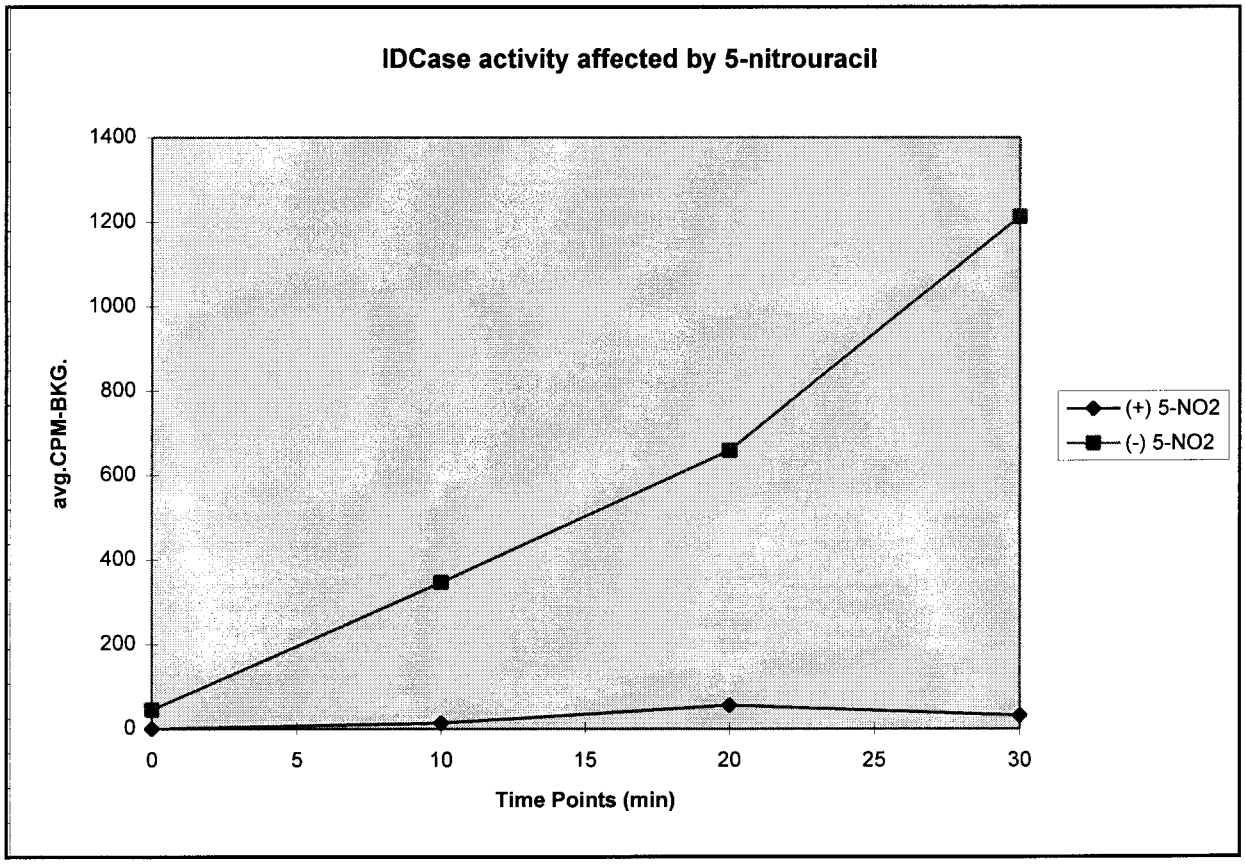


Fig.-19 The amount of IDCase activity affected by 1.43 μM 5-NO_2uracil

Table-20 Radioactive counts of IDCase activity after $AmSO_4$ Precipitation		
% Saturations	**Time Point (min)**	**averageCPM-BKG.**
Unprecipitated	0	20
	30	18.5
0%	30	2363
60%	30	414.5
80%	10	1748.5
	20	3362

Table-21 Sabouraud's Media (1 liter)	
Compound	**Amount**
Dextrose	20 g
Peptone	10 g
dH_2O	1000 ml

Table-22	Specific Enzyme Activity in Microorganisms							*Specific activity*
Organism	Time(min)	CPM	DPM	nCi	nmol	nmol/min	Protein(mg)	nmol/min/mg
74 A	10	37	74	0.185	0.185	0.0185	0.1935	0.0956
	30	61.5	123	0.3075	0.3075	0.0103	0.1935	0.053
74 A + ura	10	37	74	0.185	0.185	0.0185	0.1734	0.1067
	30	106.5	213	0.5325	0.5325	0.0178	0.1734	0.1024
74 A + thy	10	72.5	145	0.3625	0.3625	0.0363	0.1403	0.2584
	30	245	490	1.225	1.225	0.0408	0.1403	0.2911
2203 + ura	10	314.5	629	1.5753	1.5753	0.1575	0.489	0.3221
	20	431.5	863	2.1575	2.1575	0.1079	0.489	0.2206
	30	521	1024	2.605	2.605	0.0868	0.489	0.1776
2203 + thy	10	321.5	643	1.6075	1.6075	0.1068	0.1107	1.4519
	20	486	972	2.43	2.43	0.1215	0.1107	1.0974
	30	691	1382	3.455	3.455	0.1152	0.1107	1.0402
2204 + ura	20	115	230	0.575	0.575	0.0288	0.1174	0.245
	30	186.5	373	0.9325	0.9325	0.0311	0.1174	0.2648
2204 + thy	10	315.5	631	1.5775	1.5775	0.1578	0.391	0.0403
	30	516.5	1033	2.5825	2.5825	0.0861	0.391	0.2202
N.africana	10	90.5	181	0.0823	0.0823	0.0082	0.1724	0.0477
	20	71	142	0.0646	0.0646	0.0032	0.1724	0.0187
A. nidulans	10	125	250	0.625	0.625	0.0625	0.417	0.1499
	20	168.5	337	0.8425	0.8425	0.0421	0.417	0.101
C. stellatoidea	10	19.5	39	0.0975	0.0975	0.0098	0.1958	0.0498
	20	11.5	23	0.0575	0.0575	0.0029	0.1958	0.0147
C. albicans	10	14.5	29	0.0725	0.0725	0.0073	0.4437	0.0163
[a] **60 %**	30	495.5	991	0.45	0.45	0.015	0.352	0.043
[b] **80 %**	10	1819.5	3639	1.65	1.65	0.165	0.225	0.73
	20	3418	6836	3.1	3.1	0.155	0.225	0.689
[c] **80 %**	10	1819.5	3639	1.65	1.65	0.165	0.087	1.9
	20	3418	6836	3.1	3.1	0.155	0.087	1.78
unprecipitated	30	87	174	0.079	0.079	0.0026	0.191	0.0136
	30	87	174	0.079	0.079	0.0026	0.068	0.0387

[a] is a 50/50 dilution of the 60 % saturated solution

[b] is the pure 80 % saturated solution

[c] is a 50/50 dilution of the 80 % saturated solution

Chapter Seven

Conclusion

Based on the specific activity of the enzyme, it can be confidently concluded that the heterothallic *N. crassa* and *A. nidulans* utilize the thymine salvage pathway to synthesize and salvage pyrimidines more than the homothallic *N. africana* and dimorphic *Candida*. The *Candida* was somewhat used as a negative control and expected not to have much specific enzyme activity because it does not use the thymine salvage pathway. *N. africana* has very little (almost none) specific enzyme activity, but is more in agreement with *Candida*. The growth and protein concentration of the *africana* species of *Neurospora* indicates that it probably relies on other biosynthetic pathways for pyrimidine metabolism. *A. nidulans* has specific enzyme activity compatible to that of wild-type *N. crassa* which is known to use the thymine salvage pathway and expected to have an amount of specific enzyme activity congruent to that THase in wildtype.

Only four of the *E.coli* plasmids showed some type of insert. Although one plasmid showed radioactive counts above background, the counts was not significantly high and there was relatively no specific enzyme activity (so results are not shown). Attempts to isolate, thus clone, a gene for IDCase have recently been unsuccessful. Research in this area is being continued.

All of the results on the mutant strains of *N. crassa* provided evidence that IDCase is an inducible enzyme, giving the organism an advantage that can be eliminated by enzyme inhibition. The mutants were able to grow under certain environmental conditions, producing higher specific enzyme activity under some of these conditions more than others. The NH_3 worked better as a nitrogen source to give more protein tissue, but NO_3

worked better to give give a higher specific enzyme activity. Thymine works better than uracil as a pyrimidine source to induce IDCase. The ability of the enzyme to be induced under certain conditions gives the microorganism the advanatge of using the thymine salvage pathway to produce high amounts of uracil which gets incorporated into RNA. If the pathway is producing high amounts of uracil then more cellular RNA is being synthesized, thus DNA. Because of the fluorine's strong electronegativity it could be expected to have greater inhibition on IDCase than 5-NO_2U, but it does not because 5-NO_2U is a stronger electron withdrawing group. The 5-NO_2U prove to work better as an enzyme inhibitor. A small amount of this enzyme inhibitor could eliminate the advantage of high cellular DNA production by blocking the production of uracil and inhibiting the synthesis of RNA. The 5-HU and 5-FU works as average inhibitors on IDCase, but if knocking out the enzyme activity completely is desired then 5-NO_2 would be the best to use.

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