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ERGOTHIONEINE DEGRADATION AND PROPERTIES OF ERGOTHIONASE FROM *AGROBACTERIUM RADIOBACTER*

JIKU MANAMTHARAYIL JOSE

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Reviewed and approved* by the following:

Allen T. Phillips, PhD.
Professor Emeritus of Biochemistry
Thesis Supervisor

Chen-Pei David Tu, PhD.
Professor of Biochemistry
Honors Adviser

Wendy Hanna-Rose
Associate Department Head for Undergraduate studies
Department of Biochemistry and Molecular Biology

* Signatures are on file in the Schreyer Honors College.

ABSTRACT

Ergothioneine is a naturally occurring sulfur containing amino acid that is obtained through the diet and is assimilated by several tissues in millimolar concentrations, where it functions as a natural antioxidant and part of the thiol defense system against oxidative stress. With the emerging role of oxidative stress in the etiology of various cardiovascular and neurological diseases, the study of ergothioneine biodegradation pathway is very crucial.

In this study, we investigated the ability of a bacterial strain, *Agrobacterium radiobacter,* to degrade ergothioneine and use it as a sole nitrogen source for growth. The characteristics of the enzyme ergothionase, which is responsible for the breakdown of ergothioneine, were examined. We made an effort to extract ergothioneine from the ergothioneine synthesizing King Oyster mushrooms. The protective role of ergothioneine against oxidative stress in *Agrobacterium* was also studied.

Our data suggest that the *Agrobacterium radiobacter* is capable of utilizing ergothioneine as a sole nitrogen source, where ergothionase degrades ergothioneine into its first intermediate product thiolurocanate and the byproduct trimethylamine. We found that *A. radiobacter* uses only two of the three nitrogen atoms present in ergothioneine for its growth. Ergothioneine also had a protective role against oxidative stress induced by H_2O_2 in *Agrobacterium.* Ergothioneine was successfully extracted from King Oyster mushroom powder through a three step purification procedure.

TABLE OF CONTENTS

LIST OF FIGURES

.

LIST OF TABLES

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Section-1

INTRODUCTION

Ergothioneine, the betaine of 2-thiolhistidine, was discovered in the beginning of the twentieth century. Humans not do synthesize ergothioneine (ERT) but derive it exclusively from diet, primarily through the consumption of edible mushrooms. The widespread occurrence of ERT in animal blood and tissue, the avidity of ERT assimilation within cells, its highly specific transport system and its evolutionary conservation within mammalian cells suggest an important physiological role for this molecule. A lot of ERT research to understand its physiological role has been conducted in the early to mid-20th century. However even after a century of research its exact physiological function and degradation pathway still remain a mystery.

Recent studies have shown that ERT has antioxidant properties which enable it to scavenge hydroxyl and peroxynitrite radicals as well as activated oxygen species such as singlet oxygen. It has also been able to mitigate the progression of chronic neurodegenerative diseases such as Alzheimer's where oxidative stress is a big concern. The recent discovery of ERT as a key biogenic substrate for the organic cation/carnitine trasporter 1 (OCNT1), now also known as the ergothioneine transporter (ERTT), has caused additional interest in the study of ERT. OCNT1 has been associated with autoimmune and inflammatory disorders such as rheumatoid arthritis and Crohn's disease (Grundemann, 2005). The evolutionary conservation of this highly specific transport system indicates a specific role for ERT in human physiology; however an exact molecular mechanism for such a function is yet to be elucidated.

With the current restriction on many synthetic antioxidants due to their possible carcinogenicity (Chen *et al*., 1992), many researchers and food scientists are interested in alternative natural antioxidants such as ERT. The antioxidant properties of ERT and its nutritional benefits have heightened interest in ERT research. Thus it is crucial to understand how ERT functions, what determines its accumulation within cells, and how it is eventually broken down. Presently only a few bacterial species are known to have the enzymes necessary for its utilization.

The ability to degrade ERT is not a universal property and only few strains of bacteria have been reported to grow on ERT as sole source of carbon or nitrogen. A bacterial strain, identified as *Agrobacterium radiobacter,* that had previously been isolated from soil by enrichment culture techniques, was used for studies reported herein. This strain of *Agrobacterium* is capable of utilizing ERT as its sole nitrogen source for growth. A cell-free extract of *Agrobacterium* was found to contain the enzyme ergothionase which is capable of degrading ERT into thiolurocanic acid and trimethylamine (Fig 3).

Our primary goal in this research was to study the properties the enzyme ergothionase from *Agrobacterium* and how it is involved in the biodegradation of ERT. Our secondary goal was to investigate the ability of *Agrobacterium* to use ERT as its sole nitrogen source for growth. We also wanted to develop a method for purification of ERT from King Oyster mushroom to compensate for the low commercial availability and thus high cost of ERT.

Section-2

LITERATURE REVIEW

Ergothioneine, a natural sulfur containing amino acid, has captivated the attention of biochemists for more than a century now because of its unique biochemical and physiological functions. The presence of ERT in the mammalian tissue and erythrocytes had intrigued the interest of several biochemists in the early nineteenth century. Its chemistry, distribution and biosynthesis have been extensively studied. ERT have been shown to form stable complexes with several transition metal ions such as iron and copper ion which are very unique for a thiol compound. Although various speculations about the biological function of ergothioneine have been made, a complete definitive idea of its function and degradation pathway still remains a mystery. The recent advances in the ERT research during the last couple of decades have suggested that ERT is a natural antioxidant which protects against gamma and UV radiations.

Ergothioneine is a α-N,N,N-trimethyl-2-thiolhistidine betaine (Fig 1). ERT was first isolated from ergot fungus and later shown in the red blood cells of mammals and birds (Hunter and Eagles, 1925; Gulland and Peters, 1930), in boar seminal fluid (Mann and Leone, 1953), and in various tissues of mammals for up to 1-2 mM (Melville et.al., 1953b). It exists as a thione-thiol tautomer with thione form predominating in the aqueous solution of physiological pH. However, ERT is exclusively biosynthesized in fungi and some mycobacteria (Melville *et al*., 1956b). In humans, ERT is absorbed only through consumption of plant materials, primarily various edible mushrooms (Melville *et al*., 1956a). Since animals cannot synthesize ERT, there must exist certain mechanisms

for its uptake and retention within the cells. Studies have shown that only cells which contain the ERT transporter (ERTT) receptor, also known as the OCTN1 receptor, such as the human erythrocytes, can uptake and maintain intracellular ERT against a concentration gradient (Grundemann, 2005).

The biosynthesis of ERT has been already studied, and it is suggested that ERT is derived from histidine (Melville *et al*., 1957). However even less is known about its biodegradation pathway. Studies on the biodegradation of ERT in several bacteria have found that the ERT is broken down into its first intermediate thiolurocanic acid (TUA) and the byproduct trimethylamine (TMA) by the enzyme ergothionase (Wolff, 1962)

I. History:

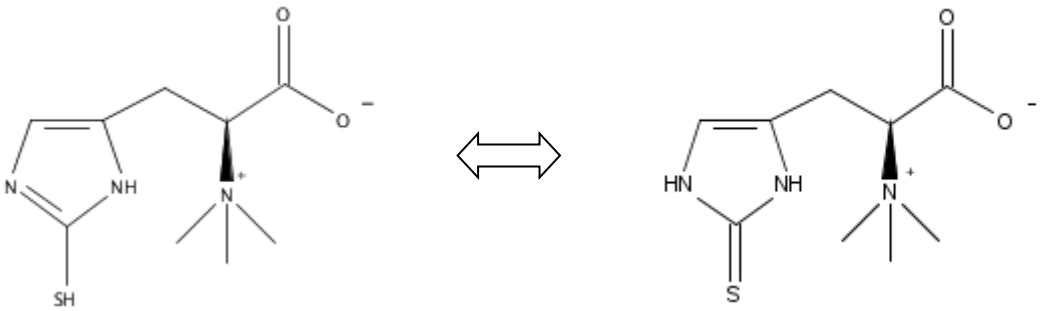
Research on ERT had started as early as 1909. Ergothioneine was first isolated as a crystalline sulfur-containing compound by Tanret (1909), during his studies on *Claviceps purpurea* (the ergot fungus that causes infection in rye grain). The empirical formula was deduced to be $C_9H_{15}O_2N_3S$. Later Barger and Ewins (1911) proved ERT as the betaine of thiolhistidine and proposed its current chemical structure.

The discovery of ERT in blood (Hunter and Eagles, 1925; Benedict *et al*., 1926) stimulated a lot of interest in this compound in the 1920's and led to many publications about its physical and chemical characteristics. One of the most important contributions was made by G. Hunter (1928), when he developed a quantitative assay for ERT based on Pauly's diazo test where ERT was coupled with diazotized sulfanilic acid to give a bright red colored solution (Hunter diazo test).

Soon thereafter, ERT was reported to be found in tissue and red blood cells (RBC) of various animal species. The blood ERT level of these animals was influenced by the diet. Mushrooms such as the King Bolete mushroom and Oyster mushroom, oats, corn and wheat were found to contain high levels of ERT (Eagles and Vars, 1928), although it is likely that the ERT content in cereal grains resulted from association with or contamination by, ERT-producing microorganisms. Ever since the discovery of ERT in 1901, numerous attempts have been made to isolate ERT from various sources and to understand its physiological and biochemical importance.

II. Chemical Properties of Ergothioneine:

Ergothioneine is the only known naturally occurring compound with an imidazole-2-thione moiety. (Skellern, 1989). It is a very hydrophilic amino acid whose sulfhydryl group is under tautomeric thiol/thione equilibrium due to the presence of a sulfur atom bonded to the 2-position of the imidazole ring (Fig 1).



Thiol Thione

Figure-1: Ergothioneine thiol-thione equilibrium.

At physiologic pH, ERT exists mostly in thione form and is stable towards oxidative dimerization in aerated aqueous solutions. (Xu and Yadan, 1995). It is interesting that ERT differs from other biologically occurring thiols such as cysteine or glutathione because of its predominant thione form in the physiological conditions. At physiological pH of 7.4, ERT does not form ERT disulfide (non-autoxidizable). ERT disulfide (Fig-2) is formed only in artificial experimental conditions of low pH (Ey *et al*., 2007). It might seem like the absence of the aliphatic thiol group would limit the antioxidant activity and potential efficacy of ERT compared to the other natural antioxidant. However studies have shown that ERT has a higher antioxidant activity than other natural antioxidants like glutathione and trolox (Franzoni *et al*., 2006).

L-Ergothioneine was synthesized in the laboratory using the procedures of Ashley and Harington (1930) and Xu and Yadan (1995). Both methods of synthesis are quite complex and involve many steps. In the Harington and Ashley method (1930), the attempt to synthesize ERT was done by the benzoylation of ethyl α-chloro-β-glyoxaline-4 (or 5)-propionate (I) followed by conversion of the product into ethyl α-chloro-γ-keto-δ-benzamidovalerate (II); the product II was hydrolysed to the aminoketone and aminoketone was converted into α-chloro-β-2-thiolglyoxaline-4(or 5)-propionic acid which with trimethylamine yielded ERT (Harington and Overhoff, 1933). This method often had a very poor yield of ERT. The Xu and Yadan (1995) method of ERT synthesis consist of a series of sequential cleavage and reformation of the imidazole ring of L-histidine with phenyl chlorothionoformate via a Bamberger-type intermediate. This

method, although complex, gave much better yield (34%), and enabled the preparation of ERT on a large scale.

Ergothioneine behaves as a weak monoacidic base and is essentially neutral to litmus. The presence of the sulfur atom on ERT affects the basicity of the normal imidazole ring and makes the ring system somewhat acidic (Melville, 1959b). The presence of the quaternary ammonium group in ERT is responsible for the formation of various ERT salts, such as chloroplatinate and phosphotungstate. The two most characteristic chemical reactions of ERT are the ready oxidation of the sulfur atom and the lability of the trimethylammonium radical towards alkali (Melville, 1959b).

Under severe alkaline conditions which readily split the sulfur from cysteine as inorganic sulfide, ERT loses trimethylamine but the thiolimidazole portion of the molecule remains essentially intact and thiolurocanic acid can be recovered in good yield (Barger and Ewins, 1911). The sulfur atom is very sensitive to oxidizing agents and readily forms sulfuric acid when ERT is treated with bromine water (Barger and Ewins, 1911).

Ergothioneine can be rapidly oxidized to its disulphide form (Fig 2) by treating it with equivalent amount of H_2O_2 in strong acid solution. It can also be oxidized slowly to the disulphide form by oxygen in the presence of copper and a strong acid solution (Heath and Toennies, 1958)

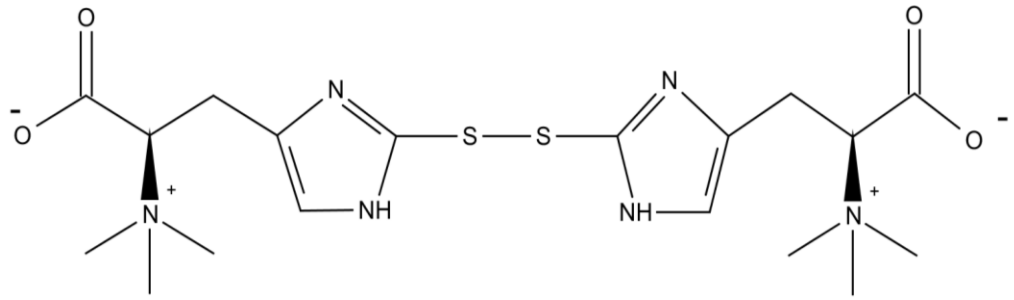


Figure-2: Structure of ergothioneine-disulphide.

The ERT disulfide also gives a positive Hunter diazo test. However ERT disulfide, in contrast to glutathione disulfide, cannot accumulate to any significant extent in tissues as it would be immediately reduced to ERT (the more stable form) by cysteine or reduced glutathione (Heath and Toennies, 1958).

III. Physical Properties of Ergothioneine:

Ergothioneine is a colorless, odorless substance which is moderately soluble in cold water, very soluble in hot water and slightly soluble in ethanol and insoluble in nonpolar solvents. The anhydrous material melts with decomposition at 256-257^{o}C in the capillary tube but does not melt up to 300^{o}C on the micro stage (Melville *et al*., 1959b) The configuration of the α-carbon atom is similar to the L-series of amino acids. The ultraviolet absorption spectrum in water displays a single peak at 257-258 nm (Holiday, 1930; Heath *et al*., 1951) while in 95% ethanol the peak shifts to 264 nm (Melville *et al*., 1955)

IV. Ergothioneine Biosynthesis:

Ergothioneine is synthesized by many common fungi, such as the ergot fungus, *Claviceps purpurea* and *Neurospora crassa* (Melville *et al*., 1957). Early studies on *Neurospora crassa,* have proved that ERT is derived from histidine, by the formation of C^{14}-ERT from L-histidine-2-C^{14} without dilution of radioactivity (Melville *et al*., 1957). This accounts for the structural similarity between histidine and ERT. The amino acid cysteine provides the sulfhydryl group and the methionine provides the methyl groups through transmethylation reaction (Melville *et al*., 1957; Melville *et al*., 1959a). It is also suggested that the imidazole ring of the histidine remains intact in the conversion to ERT and hercynine is the most likely intermediate (Melville *et al*., 1957).

Current evidence suggest that ERT is naturally synthesized not only in fungi but also in soil Actinomycetales bacteria, including *Streptomyces, Mycobacterium* and *Nocardia* (Genghof, 1970). However, none of the common bacteria such as *Staphylococcus aureus* or *Escherichia coli* showed evidence of ERT synthesis. (Melville *et al*., 1956b). Bacteria, just like mammals, were able to incorporate and concentrate the exogenous ERT. However, even among bacteria certain genera such as *Staphylococcus aureus* have impressive ability to incorporate exogenous ERT. On the other hand, bacteria like *Pseudomonas fluorescens* completely lack the ability to assimilate exogenous ERT (Melville *et al*., 1956b). This difference in the assimilation capacity might be attributed to the expression of enzymes such as ergothionase, available to degrade ERT and the presence of ERTT.

ERT biosynthesis has not been detected in any higher plants or animal species. It has been suggested that plants absorb ERT synthesized by the fungi or Actinomycetales bacteria through their root system (Melville *et al*., 1956a). Thus the ERT enters and accumulates in the food chain through these ERT absorbing plants. Since no evidence has been found for ERT synthesis besides fungi (mushrooms) and some mycobacteria, it is likely that any significant incorporation of ERT within the non-fungal food chain requires the ERTT-mediated intracellular uptake (Ey *et al*., 2007).

V. Ergothionase and Biodegradation of Ergothioneine:

The biodegradation of the ERT accumulated within the cells is an important concept that is far less known compared to the biosynthesis of ERT. As described above, the biosynthesis of ERT is exclusive to few fungi and mycobacteria. However since a significant number of organisms including humans incorporate ERT, through their ERTT expressing cells; it is pivotal to understand the steps of ERT degradation.

Several studies have shown that bacteria such as *Alcaligenes faecalis and Escherichia coli* can grow on ERT as a sole source of carbon or nitrogen (Wolff, 1962). These organisms were found to have an enzyme called ergothionase, which breaks down ERT into thiolurocanic acid (TUA) and trimethylamine (TMA). A cell cell-free extract of *Escherichia coli* W was found to be capable of rapidly converting ERT into TUA and TMA (Wolff, 1962). The following reaction was reported:

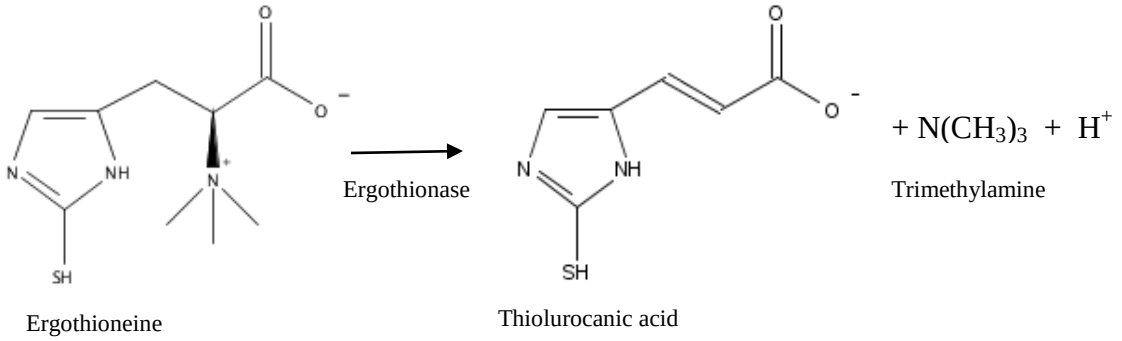


Figure-3: Degradation of ergothioneine by ergothionase enzyme

The formation of TUA was measured spectrophotometrically at 311nm. A pH of 7.4 to 7.5 was found to be optimal for growth and ergothionase induction in *E. coli* W (Wolff, 1962). The enzyme was reported to have a Km value of 5.6 X 10^{-5} M for ERT.

The reaction in Fig-3 is essentially the first step of ERT biodegradation, producing the first intermediate TUA and the byproduct TMA. This reaction appears to be similar to the first step of histidine biodegradation by the enzyme histidase. TMA was not further metabolized in *Alcaligenes faecalis*, whereas TUA slowly disappeared from the incubation mixture (Appleman and Kelly, 1960). The disappearance of TUA suggests that it is converted to a second, but still unknown, intermediate in the pathway, possibly similar to the disappearance of urocanate in histidine biodegradation. However the breakdown of ERT proceeds through a different pathway than histidine. Organisms grown on ERT were not induced for the enzymes needed to attack histidine or urocanic acid, and conversely, those grown on histidine were unable to form TUA from ERT (Wolff, 1962).

Appleman and Booth (1963) have identified glutamate, ammonia and hydrogen sulfide as the end product of ERT degradation by cell-free extracts of *Alcaligenes faecalis*. They have also suggested that the cleavage of the imidazole ring during degradation appeared to be hydrolytic rather than oxidative. But the intermediate steps of ERT biodegradation still remain to be demonstrated.

VI. Distribution of Ergothioneine in Animals:

Several investigations have confirmed the widespread nature of ERT in animals. The work of Hunter (1928) demonstrated its presence in erythrocytes. It also occurs in birds, marine crab, rat and pig (Gulland and Peters, 1930; Lutwak-Mann, 1952). High concentrations of ERT have been found in the seminal vesicular secretion of pig (Mann and Leone, 1953). It is also reported in the human bone marrow (Fraser, 1951) and urine (Sullivan and Hess, 1933). Levels of ERT vary in the human body with highest concentration in erythrocytes, liver, eye-lens, semen and skin (Melville, 1955).

There are several factors that control the blood ERT level. Some of them include diet, geography (Eagles and Vars, 1928), age, race and sex (Touster and Yarbro, 1952). The variations of ERT level with the diet further suggest that the ERT is not synthesized in animals but is purely exogenous. Once incorporated, the cells are able to concentrate ERT because of its long half-life. Studies have shown that the half-life of ingested ERT in rat and pig is about 1 month, although seminal ERT could be detected in pig as long as 83 days after ingestion (Sakrak *et al*., 2008).

Another factor which might be responsible for the distribution of ERT within mammalian tissue is the highly specific transporter of ERT called the Ergothioneine transporter (ERTT). The ERTT is in fact the organic cation/carnitine transporter 1 (OCNT1). Since OCNT1 transports ERT 100 times more efficiently that carnitine, it is rightfully named (functionally) as the ERTT (Grundemann, 2005). Cells such as the hematopoietic cells and liver cells which express ERTT have a high accumulation and retention level of ERT. By contrast, cells which lack ERTT do not contain ERT since ERT is impermeable to the plasma membrane (Grundemann, 2005).

In humans ERT is found in all ERTT-expressing tissues or cell lineages which are derived from the OCTN1-expressing progenitors (Grundemann, 2005). This primarily includes cells of the myeloid lineage such as monocytes and erythrocytes where concentration of ERT can range from 10-15 μmole/L whereas no ERT was found in the extracellular fluid (Taubert *et al*., 2005). However a much higher concentration of ERT was reported in patients with rheumatoid arthritis, where oxidative stress is a big concern (Taubert *et al*., 2006).

VII. Distribution of Ergothioneine in Plants and Mushrooms:

The presence of ERT was found in many cereal grains such as oats and corn. The highest concentration of ERT was found in specialty mushrooms such as *Boletus edulis* (King bolete mushroom) and *Pleurotus ostreatus* (Oyster mushroom) in which ERT concentration ranged from 400-2000 mg/kg dry weight of mushroom (Ey *et al*., 2007). The high concentration of ERT in mushrooms is not surprising since it is exclusively

synthesized by fungi and some mycobacteria. There is a significant variation in the ERT content among the same variety of mushroom itself depending on different cultural factors, harvestation of mushroom at different maturation stages of fungal cycle and whether the mushroom has spore-bearing or spore-falling fruitbodies, since only the fungal spores have the ability to synthesize ERT and not fungal mycelium (Ey *et al*., 2007).

Also certain plant products such as black bean (13.9mg/kg), kidney bean (4.52mg/kg), oat bran (4.41mg/kg) and garlic (3.11mg/kg) contained much higher ERT compared to other plant products (Ey *et al*., 2007). Since no evidence had been found for ERT synthesis in higher plants, the higher concentration of ERT in certain plants can be assumed to its close association with the ERT-synthesizing fungi or mycobacteria in their roots (Melville *et al*., 1956a). Another assumption is that the ERT content in complex plants (also animals) is dependent on the expression of the ERTT (OCTN1), the highly specific transporter of ERT (Grundemann, 2005), within the cells. Only cells which express ERTT in the membrane are capable of incorporating the ERT within the cells. Thus plants which have a higher expression of ERTT in cells would have a higher ERT content. Studies have shown that there is no correlation between ERT content and other natural antioxidant content like glutathione within the cells, since glutathione uptake is completely independent of the expression of ERTT (Demirkol *et al*., 2004).

VIII. Biological Activity of Ergothioneine:

A lot of effort has been made to explore the biological function of ERT. A unified concept of ERT function has not been elucidated yet; however some interesting aspects of ERT function have been recorded in the literature. Current research points to the protective function of ERT against oxidative stress and its role in disease pathophysiology and prevention. Some of the current studies on ERT which highlight its biological importance are as follows:

1. As an Antioxidant:

ERT is found mainly in the thione form (Fig-1) and therefore does not dimerize under oxidation like glutathione. When acting as an antioxidant, its -(SH) group is oxidized but very rapidly reduced back to its thione form non-enzymatically because of its unique tautomerization (Paul and Snyder, 2009). In contrast, other antioxidants such as glutathione are totally depleted (oxidized) when confronted with oxidative stress and need special enzymes such as glutathione reductase to convert back into the reduced state. Also most natural antioxidants are selective to the class of molecules that are reduced. The hydrophilic antioxidants such as glutathione and ascorbate protect water soluble protein whereas the hydrophobic antioxidant bilirubin protects against lipid peroxidation. However ERT, a water soluble molecule, appears to prevent both carbonylation of water soluble proteins, resembling hydrophilic antioxidants, and lipid peroxidation, similar to hydrophobic antioxidants (Paul and Snyder, 2009). Therefore

ERT might serve as a better antioxidant than glutathione and other classic antioxidants. In fact an *in vitro* study conducted by Franzoni *et al*. (2006) to assess the antioxidant activity of ERT, found that the ERT was the most active scavenger of free radicals as compared to classic antioxidants such as glutathione, uric acid and trolox. However contrasting to this result another study conducted by Ey *et al*. (2007) analyzing the antioxidant effect of ERT found that ERT has a low radical scavenging capacity compared to glutathione due to its extremely low reducing potency (Eo=-60mV) and the thiol/thione tautomerism. These differences could be attributed to the variations in the experimental model used in the studies. ERT possesses anti-hydroxyl, anti-peroxyl as well as anti-peroxynitrite radicals antioxidant activity. It also protects tissues against the toxic effects of HOCl (Akanmu *et al*., 1991).

It is suggested that ERT inhibits the generation of singlet oxygen by quenching excited states of photosensitisers due to the protonated imidazole ring (Hartman, 1990). The sulfhydryl (thiol) group (SH) of ERT can serve as a proton donor similar to glutathione and might be responsible for the biological activity of ERT. The ability of ERT to inhibit peroxidation suggests that ERT could have a protective role *in vivo* against the peroxidation induced by haem proteins plus H_2O_2 in heart and muscle (Akanmu *et al*., 1991). Indeed a study by Arduini et.al (1990) has shown that ERT can protect against reperfusion injury in isolated rat hearts. This demonstrates the power of ERT as an antioxidant and may find applications in pharmaceutical industry.

2. In Crustaceans and Sea Food Industry:

The development of melanosis or blackspot formation in crustaceans such as shrimp is a very common phenomenon. The melanosis is caused by the polymerization of phenolic compounds into an insoluble black pigment called melanin. This polymerization is initiated by the action of an enzyme complex called polyphenoloxidase (PPO). The melanosis as well as the lipid oxidation significantly affects the flavor and odor of these sea foods. This causes a huge loss to the sea food industry.

A recent study by Encarnacion *et al*. (2010) confirmed that the application of L-ERT from edible mushroom *Flammulina velutipes* significantly inhibited the PPO activity and delayed melanosis in shrimp. The ERT also restored the fresh color of the meat and prevented browning of the apple. The mixing of mushroom extract containing ERT in beef and fish meat also suppressed lipid oxidation. This study accentuates the inhibitory effect and usefulness of ERT.

3. As a Physiological Cytoprotectant:

A recent study conducted by Paul BD and Snyder SH in the John Hopkins department of Neuroscience (2009) about the highly specific transporter of ERT called Ergothioneine Transporter (ERTT) in the mammalian tissue suggest significant physiological roles of ERT. In this study, the ERTT was depleted from the cells using RNA interference techniques. The cells lacking ERTT were shown to be more susceptible to oxidative stress, resulting in increased mitochondrial DNA damage, protein

oxidation and lipid peroxidation resulting from the superoxide generated in the course of electron transport cycle. This signifies the importance of ERT as a natural antioxidant and its role can be paralleled to the more common antioxidants such as glutathione. The protective role of ERT was limited to the ERTT-expressing cells such as the erythrocytes and liver whereas glutathione was able to protect all stress-related cells irrespective of the ERTT expression (Ey *et al*., 2007). However, ERT may provide a more stable mode of cytoprotection because of its elongated half-life (about 1 month) and non-enzymatic tautomerization. The exact molecular mechanism of the cytoprotective effect of ERT still remains a mystery.

3. As a Chelater of Metal Ion:

ERT forms very stable complex with various transition metals such as iron and copper. The ERT-copper ion complex is very stable and does not decompose to generate oxygen radical. In contrast, other antioxidants such as glutathione are rapidly oxidized by copper ions with the production of toxic free radicals. The chelation of copper ions in the non-redox active form in hemoglobin and myoglobin might be a major function of ERT in erythrocytes and other tissue of the body (Akanmu *et al*., 1991). A recent study had found that the ERT prevents copper-induced oxidative damage to DNA and protein by forming a redox-inactive ergothioneine-copper complex. (Zhu et at., 2011)

4. As an Antimutagen:

ERT protects the cell from single stranded DNA breaks caused by UV radiation. In a study conducted by Botta *et al*. (2008) to evaluate the role of ERT in preventing ssDNA break, a maximal protection rate of about 60% was obtained at a concentration of 0.5mM. It has been suggested that the UV radiation causes oxidative lesions which could lead to endogenous photosensitizers. These excited states of photosensitizers are quenched by the ERT (Botta *et al*., 2008).

ERT also inhibits the PARP-1 cleavage (Markova *et al*., 2009). PARP-1 is a critical enzyme in the DNA damage network in the epidermal cells. The basal epidermal keratinocyte cells of the skin synthesize ERTT (ERT transporter), enabling them to absorb ERT from the blood and protect the skin against UV radiations (Markova *et al*., 2009). Thus ERT is becoming a part of many effective sunscreens.

5. As a Neuron protector:

The existence of ERT in different regions of the bovine central nervous system, especially in cerebellum, indicates that ERT might have a role in the central nervous system (Briggs, 1972). It has also been found to have an excitatory effect on the neurons (Avanzino *et al*., 1966). ERT has been reported to have neuroprotective function because it protects rat pheochromocytoma (PC12) cells from oxidative and nitrosative cell death caused by β-amyloid peptide (Jung *et al*., 2003). β-Amyloid peptide is the major

component of senile plaques (which is toxic to neurons) and is thought to have a major role in the development and progression of Alzheimer's disease.

In many of these neurodegenerative disorders such as Alzheimer, injury to neuron can be caused due to overstimulation of the receptors by excitatory amino acids such as glutamate and aspartate. (Lipton, 1994). The N-methyl-D-aspartate (NMDA) is a glutamate receptors involved in many brain functions such as memory and neuronal development (Lipton, 1994). The overstimulation of NMDA receptors causes production of free radical and neuronal death. In vivo studies have also shown that ERT protects retinal neurons from NMDA-induced excitatoxicity (Moncaster *et al*., 2002)

Thus ERT could be used as an effective neuroprotective antioxidant supplements preventing chronic neurodegenerative disorders such as Alzheimer's where pathogenesis might be linked to oxidative stress. (Jung *et al*., 2003).

6. In Human Health Condition:

A H-NMR study conducted by Turner *et al*. (2009) to identify biomarkers present in erythrocytes that would distinguish between normal pregnant woman and those who are suffering from preeclampsia (pregnancy complication which is characterized by high blood pressure and protein in urine) found significant higher concentration of ERT in the erythrocytes of preclamptic patients. This result directly attributes to the oxidative stress and reactive oxygen species (ROS)-scavenging properties of ERT since preeclampsia is thought to be caused by oxidative stress.

Another study indicates that ERT plays a protective role against cardiovascular disease by interfering with events that contribute to atherogenesis like reducing adhesion molecule expression and monocyte adhesion to aortic endothelial cells (Martin, 2010). The results of this study suggest that a consumption of fruits and vegetables, especially rich in ERT would be important to prevent cardiovascular disease.

A single nucleotide polymorphism in the OCTN1 gene, the ERT transporter, has shown to increase the risk for Crohn's disease (Petermann *et al*., 2009). This mutation in the ERTT causes the cell to absorb ERT at a much higher rate leading to high levels of ERT accumulation in Crohn's patients. The unusually high level of ERT could lead to an antioxidant overload in erythrocytes or epithelium, leading to a pro-oxidant effects or immune imbalance (Petermann *et al*., 2009). Thus many Crohn's patient are mushroom intolerant since mushrooms have a high concentration of ERT.

Thus research is continuing to provide evidence for the positive role of ERT in the disease pathophysiology of human, especially in the cases of oxidative stress. Defects and polymorphism in the ERTT gene has been linked to various inflammatory and autoimmune disease which are related to oxidative stress (Petermann *et al*., 2009). Therefore it is also necessary to evaluate the safety of the dietary intake of ERT as an antioxidant as ERT may cause aerobic nitrosation or divalent metal chelation (Petermann *et al*., 2009). Preliminary research has confirmed that ERT has no mutagenic activity on bacterial test strains (Schauss *et al*., 2010). However more research must be done to define the specific role of ERT in the disease pathophysiology.

Section-3

MATERIALS AND METHODS

I. Estimation of Ergothioneine (Modified Hunter Diazo Test):

The quantitative estimation of ERT in solution is an important step for all of our procedures. ERT content in a solution was estimated using a modified version of the Hunter diazo test. This test, based on the Pauly reaction, is a colorimetric method in which ERT is coupled with diazotized sulfanilic acid and a strong alkali to give a typical reddish-magenta colored product (Hunter, 1928). No other naturally occurring compounds are known to give a product with the same color characteristics. Histidine gives a yellow colored product with the Hunter diazo test.

The modification was necessary to accommodate the presence of ethanol in most of our assays. The regular Hunter diazo test did not give consistent results with ethanol since it causes instability of the dye and leads to bleaching of color. The modified procedures were done according to the methods based on Macpherson's (1941) colorimetric estimation of histidine.

In this method, 1 ml of sample containing ERT was mixed with 100ul of 1% sulfanilic acid in 10% HCl followed by the addition of 100ul of 5% sodium nitrite. The solution was mixed thoroughly and kept at room temperature. After 10-15 min, 300ul of 20% NaOH was added to develop the characteristic reddish-magenta color. The absorbance of the colored product (1.5ml) was measured in a Gilford spectrophotometer

at 445nm (the maximum absorbance wavelength for the modified Hunter product of ERT with NaOH) and compared to the standard curve to determine the µg of ERT.

The whole reaction was carried out in the room temperature, in contrast to the Hunter diazo test, where reactions were carried out at 0^{o}C. Also in contrast to the methods suggested by Macpherson (1941), we used NaOH to make the solution alkaline and thereby inducing the color formation, instead of using Na_2CO_3, because Na_2CO_3 caused the formation of bubbles (probably because of the formation of CO_2 gas) in the cuvette, which could interfere with the spectrophotometric reading. The color of the product remained stable for 1-2 hour.

II. Extraction of Ergothioneine from Mushroom:

A purification procedure was carried out to extract ERT quantitatively from mushroom. The dried King Oyster mushroom powder, provided by Dr. Beelman's lab, was used since King Oyster mushroom was known to contain high concentrations of ERT. A three step extraction procedure was used which included hot water extraction (Step 1), Dowex-1-acetate anion exchange (Step-2) and alumina adsorption chromatography (Step 3).

1. Hot Water Extraction (Step 1):

The first step of hot water extraction was used to separate the water soluble, low molecular weight substances (which include ERT) from the high molecular weight, non-

water soluble substances (which include cell debris, membranes and lipids). In this method 5 g of King Oyster mushroom powder was blended with 50mL of boiling water in a Waring commercial blender for 5 minutes. The blended solution was centrifuged for 15 min at 3000 rpm and the supernatant-1 was collected.

The pellet was again resuspended in 50 mL of boiling water and centrifuged for 15min in 3000rpm. The supernatant-2 was collected and mixed with supernatant-1. The resulting pellet was resuspended one more time in 50 mL of boiling water and centrifuged to collect supernatant-3. The combined supernatants were then centrifuged at a high speed of 15000 rpm for 15 min to remove turbidity. The resulting supernatant was vacuum evaporated to 5 mL, in order to perform step-2 of extraction.

2. Dowex-1-acetate Anion Exchange (Step-2):

In the second step of extraction, an anion exchange was performed to remove all negative charged species (at pH 7) from the step-1 solution. Dowex-1-acetate was obtained from Bio Rad with a cross-linking capacity of 1.2 milli-equivalents/mL of the resin bed and a mesh size of approximately 100. A 10 g quantity of Dowex-1-acetate was mixed with 15 mL of water to form a slurry. The slurry was poured in a 1.5 cm X 30 cm sized column to form a 20 mL resin bed. A 3 ml of Step-1 solution, adjusted to pH 7, was passed through the column. The column was washed with 40 ml of water (eluent). The flow rate was adjusted to be 1ml/minute.

The eluate was collected as 5ml portions in a test tube. The collected elutes were tested for the presence of ERT by modified Hunter diazo test. The tubes with positive Hunter diazo test were combined and vacuum evaporated to 5 ml.

3. Alumina Adsorption Chromatography (Step-3):

In the final step of ERT purification, an alumina adsorption chromatography was performed to separate ERT from histidine, both of which give a positive Hunter diazo test.

Alumina—Alumina of the type F-20 with 80-200 mesh, obtained from Sigma Chemicals, was used for the chromatography. A 25 g portion of alumina was washed by decantation several times with distilled water to remove the finest material.

Solvent—A 75% ethanol in water with 1% formic acid was used as the solvent (75:25:1). The addition of an organic acid (formic acid) aids in maintaining high recoveries. Citric acid could also be used instead of formic acid, but better results were obtained with formic acid. The column solvent was made freshly by adding 1 volume of formic acid (88%) to 100 volumes of aqueous alcohol (75 volume of 190 proof pure ethanol and 25 volume of water)

The 25g alumina was resuspended in the solvent and placed in a 1.5cm X 30cm sized column partially filled with the solvent to form a 30 ml resin bed. 1ml of the sample containing histidine and ERT was loaded to the top of the column slowly. The column was eluted with the same solvent (150ml) at a flow rate of 1ml/5min (12ml/hour). The slow flow rate was necessary to achieve sharp distinct peaks for histidine and ERT.

At a higher flow rate, both peaks fused together to give a broad non-distinguishable peak. 5ml fractions were collected using a fraction collector and the presence of Pauly positive material (histidine or ERT) was confirmed by performing the modified Hunter diazo test on each fraction. The fractions within the ERT peak were combined and evaporated to dryness, and the ERT content was determined.

III. Biochemical Test for *Agrobacterium:*

The identity of the *Agrobacterium* species was confirmed through the biochemical test described by Bernaerts and De Ley (1963). This test is based on the fact that the only *Agrobacterium* produces 3-ketoglycoside from the corresponding disaccharides and bionic acids through a unique mechanism of oxidation at carbon-3 of the glycosyl moiety in disaccharides and bionic acids. In our case we used the disaccharide lactose as a medium to detect 3-ketoglycoside.

The unknown bacterial species as well as the *E. coli* species (control) were grown for 1-2 days in a glucose medium (1% yeast extract, 2 % glucose and 2 % Bacto-agar) at 25-30^{o}C. After 2 days, a loopful of both bacteria was deposited on the lactose medium plates (1% lactose, 0.1% yeast extract and 2% Bacto-agar), as a small heap about 0.5 cm or less in diameter at the center of the plate. The lactose plates were incubated at room temperature for 1-2 days until bacterial colonies could be easily seen. The newly grown colonies on the lactose plate were flooded with a shallow layer of Benedict reagent and left at room temperature. If 3-ketoglycoside (in this case 3-ketolactose) were formed in

the lactose medium, then a yellow ring of Cu_2O should be visible around the cell mass in the presence of Benedict reagent.

IV. Growth of *Agrobacterium* in Ergothioneine:

The bacterium *Agrobacterium radiobacter* was isolated from soil through enrichment culture techniques by Dr. Dennis Hernandez; identification was accomplished by a commercial service (Midland Corp.). The *Agrobacterium* cultures were grown aerobically at 30^{o}C in non-nitrogen containing 1X-M9 minimal medium for 24 hours. The medium contained 10mM of glucose (carbon source), 10^{-2}mM of $CaCl_2$, 2mM of $MgSO_4$ and 10mM of ERT (the sole nitrogen source). The medium was incubated for 24 hours in a water bath shaker at 30^{o}C.

V. Preparation of Crude Extract:

Cells grown overnight in the M9 minimal medium with ERT as nitrogen source were spun down and washed 2 times with 1% NaCl. The washed cells (pellet) were resuspended in 1 mL of 0.2 M CHES buffer and sonicated 6 times, each lasting for 30 seconds. The cell suspension was kept in ice during sonication to prevent denaturation. After sonication, the cell debris were removed by high speed centrifugation at 4^{o}C for 6 minutes. The resulting supernatant (Crude Extract) was collected and stored at -20^{o}C.

The protein concentration of the crude extract was determined using the Bradford assay (Bradford, 1976). The reagents and the Coomassie blue dye was obtained from the

Bio Rad Laboratories, Hercules, CA. The assay was done according to the Bio Rad macro-method for protein assay. Bovine serum albumin (BSA) was used as the standard protein to produce the standard curve of protein concentration versus absorbance at 595nm.

VI. Ergothionase Assay:

The ergothionase assay was based on the enzymatic conversion of ERT into TUA and TMA, following TUA formation by its specific UV absorbance at 311nm. The assay was performed in aerobic conditions at 30^{o}C in a Gilford spectrophotometer with cooling system.

The typical reaction assay (1ml) contained 20mM of CHES buffer, 1mM of the substrate ERT, varying amounts of crude extract and water. The substrate concentration was kept constant while the amount of crude extract was varied in each assay (10ul-40ul). The final volume was made to 1ml with water; assays were conducted in quartz cuvettes. The substrate ERT was added last and the cuvette contents were mixed immediately before measuring absorbance in the spectrophotometer at 311 nm. The temperature inside the spectrophotometer was maintained at 30^{o}C using the cooling system. All solutions were kept in ice prior to use in the assay.

The change in absorbance due to the formation of TUA was measured at 311nm and recorded using the Gilford chart recorder. The rate was calculated from the slope of the optical density versus time graph.

VII. Estimation of Trimethylamine (TMA):

The TMA content in the bacterial culture medium was estimated using the micro-estimation method described by Cromwell (1950). In this method bacterial culture samples were centrifuged to separate the supernatant. The supernatant (0.05 mL) was diluted to 1.0 mL with water. Then toluene (5 mL) along with 1.5 mL of saturated potassium carbonate was added to the solution in a glass stoppered tube. The tube was vigorously shaken for 10 seconds, and the layers were allowed to separate. The bottom layer (aqueous) was removed with a Pasteur pipette, and the toluene/TMA component was dried by adding 0.25 g of anhydrous sodium sulfate for 10 minutes. After vigorous shaking, the sodium sulfate was allowed to settle and 4 mL of the toluene solution was removed. Following this, 0.1 mL of cis-aconitate solution (2.5% aconitic acid in glacial acetic acid) and 2 mL of acetic anhydride were added. The solution was then heated in a water bath for 10 min at 65^{o}C. Twenty-five minutes after the solution had cooled, the OD was measured at 540nm.

VIII. Paper Chromatography:

Paper chromatography was performed to identify ERT and distinguish it from histidine and TUA. Chromatograms were developed on Whatman no. 1 paper by ascending technique in 1-butanol-acetic acid-water (4:1:5 by volume) using the upper phase of the solvent.

Developed chromatograms were examined using Pauly spray (Hunter diazo reagent) in which the chromatograms were sprayed with 1% sulfanilic acid in 1M HCl, followed by 0.7% sodium nitrite. The chromatogram was allowed to air dry and then re-sprayed with 10% sodium carbonate solution to develop the bright spots. The paper chromatogram was also examined under ultraviolet light before the Pauly spray to further detect the ERT and TUA.

Unless otherwise specified, routine biochemicals and buffers used in the assays such as CHES (2-[N-Cyclohexylamino] ethane-sulfonic acid), CAPS (3-[Cyclohexylamino]-1-propane-sulfonic acid) and HEPES (4-[2-Hydroxyethyl]-1-piperazineethane-sulfonic acid) were obtained from Sigma Chemical Co., St. Louis. The commercial ERT obtained from Oxis International Inc., was used for all growth and enzyme experiments.

Section-4

RESULTS

I. Estimation of Ergothioneine:

A standard method for the estimation of ERT was developed through the modified Hunter diazo test described above. The spectrophotometric properties of the diazotized ERT product developed through the modified Hunter diazo test were studied. The alkaline medium (Na_2CO_3 or NaOH), used to induce the color formations seemed to make a difference in the spectrophotometric properties of the product. Therefore we used NaOH as the alkalinizing medium for all further studies.

1. Absorbance Spectrum for the Diazotized Ergothioneine Product:

A 1ml solution containing 80 µg of ERT was prepared and the ERT content was estimated using the modified Hunter diazo method described. The absorbance of the reddish-magenta product was measured in the Gilford spectrophotometer with wavelengths ranging from 400-550 nm (Fig 4).

The results from the Fig-4, suggests that the maximum absorbance for the modified Hunter diazo product for ERT is 445 nm. At 445 nm, 80 µg of ERT gave a maximum absorbance of 1.41. This value is significantly different from the results obtained in the original Hunter diazo product of ERT, in which the maximum absorbance occurred at 510nm (Hunter, 1928). This difference can be attributed to the alterations made in the estimation of ERT due to the presence of ethanol. The extinction coefficient

for ERT in this assay was found to be 8.5 X 10^3 $M^{-1}cm^{-1}$ at 445 nm, compared to the extinction coefficient of 5.4 X 10^3 $M^{-1}cm^{-1}$ at 540 nm. Thus all the further studies involving estimation of ERT, were made at 445nm

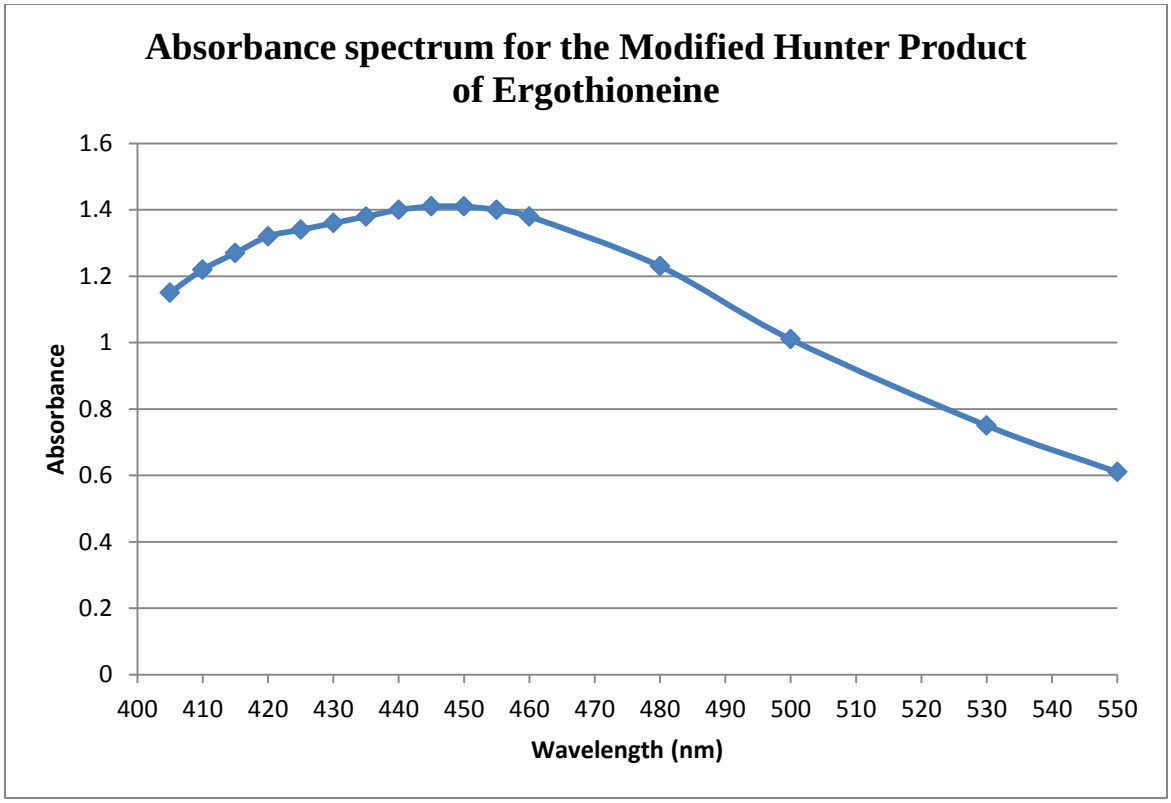


Figure-4: Absorbance spectrum for the diazotized ergothioneine product formed through the modified Hunter diazo test with NaOH as the alkaline medium.

2. Standard Curve of Ergothioneine:

A standard curve for ERT color in the modified Hunter diazo test as a function of ERT concentration was prepared spectrophotometrically. A 1 mL solution contained ERT in the range of 5-40 µg. The optical densities were measured in a Gilford

spectrophotometer at 445nm, where the Hunter product of ERT had a maximum absorbance.

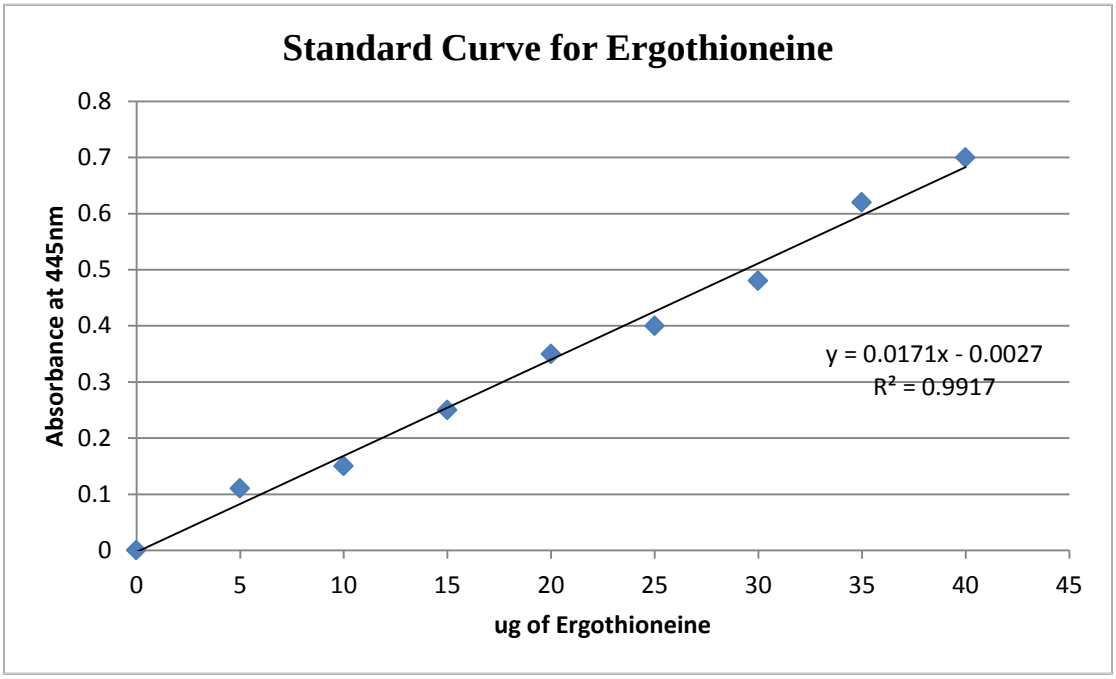


Figure-5: Comparison of ERT concentration (µg) with optical density (at 445 nm)

The relationship of optical density to ERT concentration was found to be a straight line function (Fig 5). Approximately 40 µg of ERT produces the maximum absorbance readable with fair accuracy. The sensitivity of this test is appreciably greater than the sensitivity of the original Hunter test performed using a Duboscq colorimeter (Hunter, 1928). The above graph (Fig-5) is used to estimate the ERT content in an unknown solution.

II. Extraction of Ergothioneine from Mushroom

ERT is found in several varieties of mushrooms. The ERT content in several mushrooms has been estimated through HPLC techniques (Ey *et al*., 2007). In this study we wanted to purify ERT from mushroom and use this ERT as a substrate for inducing the expression of ergothionase. The ERT was extracted from the King Oyster dried mushroom powder. We developed a 3-step purification strategy to extract ERT from the dried King Oyster mushroom power, which included hot water extraction (Step-1), Dowex-1-acetate anion exchange (Step-2) and alumina adsorption chromatography (Step-3).

1. Hot Water Extraction (Step-1):

In the first step of purification, 5 g of dried King Oyster mushroom powder were extracted three times using 50ml of boiling water per extraction. This process enabled us to separate the water soluble, polar and low molecular weight molecules (like ERT) from the non-water soluble, non-polar and high molecular weight substances. The resulting hot water extract (supernatant) containing ERT, was vacuum evaporated to 5ml and the ERT content in the solution was determined. The results of this purification are summarized in Table-1.

Results from the Step-1 purification allowed us to recover about 22.9 mg of Pauly positive material from 5 grams of mushroom powder. This recovery is based on the assumption that all the Pauly units (absorbance from the modified Hunter diazo product)

are due to the ERT and histidine. Histidine may also be present in this fraction since both histidine and ERT have similar properties and give a positive Hunter diazo test. They could be separated only in Step-3. Thus the original recovery of ERT would be much less than reported here.

Table-1: Step-1 (hot water extraction) purification chart

Hot water extraction	
Grams of Mushroom powder used	5 g
Volume used for 1st, 2nd and 3rd extraction	50 ml each
Total Volume of the Extract recovered	136 ml
Volume of recovered extract after vacuum evaporation	5 ml
ERT estimation from recovered extract	
Volume Analyzed	10 µl
Pauly positive material from the ERT-standard curve	46 µg
Total Pauly positive material present in the recovered extract	22900 µg (22.9 mg)

2. Dowex-1-acetate Anion Exchange (Step-2)

The Step-2 anion exchange was done using Dowex 1-acetate as the resin to separate all the positively charged species from the negative charged species. At pH 7, ERT (as well as histidine) has a positive charge. This allowed us to separate ERT from the negatively charged species in the extract. 3ml of the Step-1 extract was passed through the Dowex-1-acetate column and eluted using water as described in the procedure. Results of this purification are summarized in Table-2.

Results from the Step-2 purification allowed us to recover 12.2 mg (53.34 %) of the Pauly positive material from the Step-1 extract. This amount still accounts for both

ERT and histidine, both positive at pH 7, in the Step-2 extract. However histidine is only slightly positive at pH 7 compared to the ERT, which has a strong positive charge. This could account for the loss of Pauly positive material in Step-2.

Table-2: Step-2 (anion exchange) purification chart

Dowex-1-acetate anion exchange	
Volume of Step-1 extract used for Step-2	3 ml
Volume of Eluent (water used) used	40 ml
Total Volume of the Eluate recovered	35 ml
Volume of solution after vacuum evaporation	5 ml
ERT estimation from recovered solution	
Volume Analyzed	100 µl
Pauly positive material from the ERT standard curve	174 µg
Total Pauly positive material present in the recovered extract	8700 µg (8.7 mg)
Percentage recovery of material from Step-2 treatment	63.04 %

3. Alumina Adsorption Chromatography (Step-3):

The third step of alumina adsorption chromatography was done to separate histidine from ERT. Both histidine and ERT are very similar in structure and give a positive Hunter diazo test due to the presence of the imidazole group. These similarities made it harder to separate them quantitatively using common separation techniques. However we knew that histidine and ERT could be separated in an alumina column where ERT elutes much earlier than the histidine in an ethanol solvent (Melville *et al*., 1953).

Two milligrams of pure ERT and histidine were mixed together in the solvent (75 ethanol: 25 water: 2 formic acid) to form a 1ml sample. This sample was loaded to the alumina column and 30 fractions were collected according to the procedures described above. Each fraction (5ml) was analyzed using the modified Hunter diazo method and the absorbance values are noted (Fig-6).

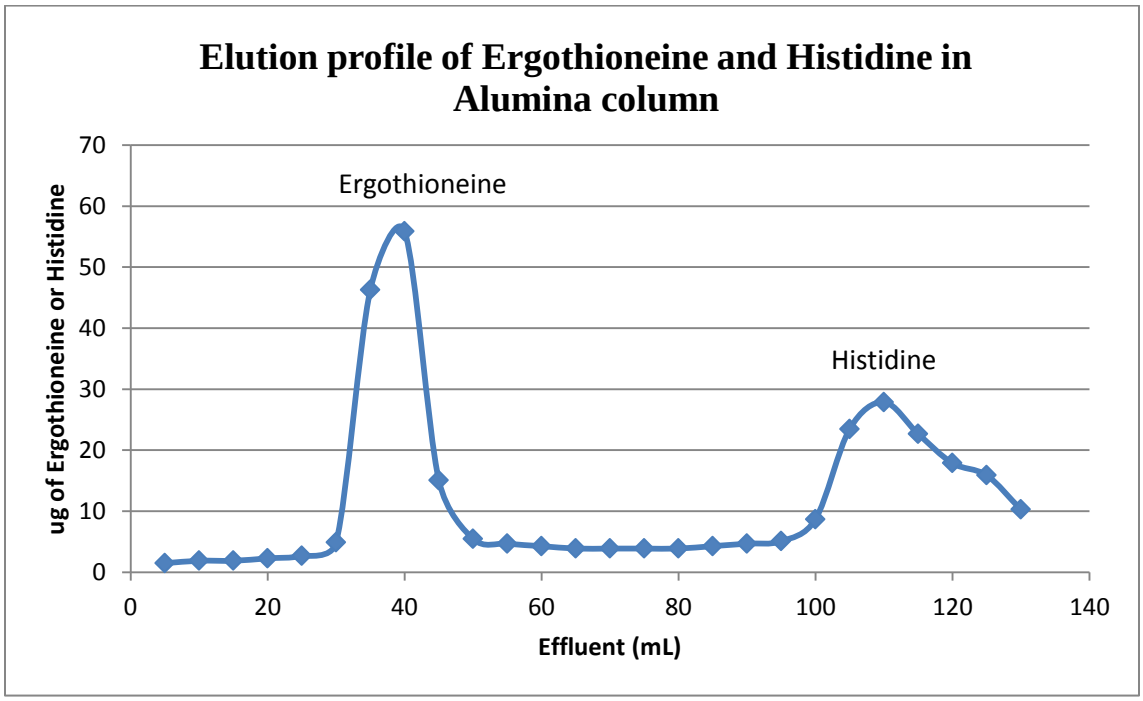


Figure-6: Elution profile of ergothioneine and histidine in alumina column.

We saw two distinct peaks (Fig-6), from the known samples of ERT and histidine on the alumina column. We know from the literature that ERT elutes first compared to the histidine in a alumina column (Melville *et al*., 1953). Also fractions 7-9 (35-45ml) showed a distinct red color (characteristic of the ERT) in the modified Hunter diazo test, while fractions 21-24 (105-120 ml) showed a distinct yellow color (characteristic of

histidine). These features allowed us to designate the first peak as ERT and the second peak as histidine.

Now, 1ml of the final Step-2 extract was loaded on the alumina column and 30 fractions were again collected. Each fraction (5ml) was analyzed using the modified Hunter diazo method and the absorbance values are plotted in Fig-7.

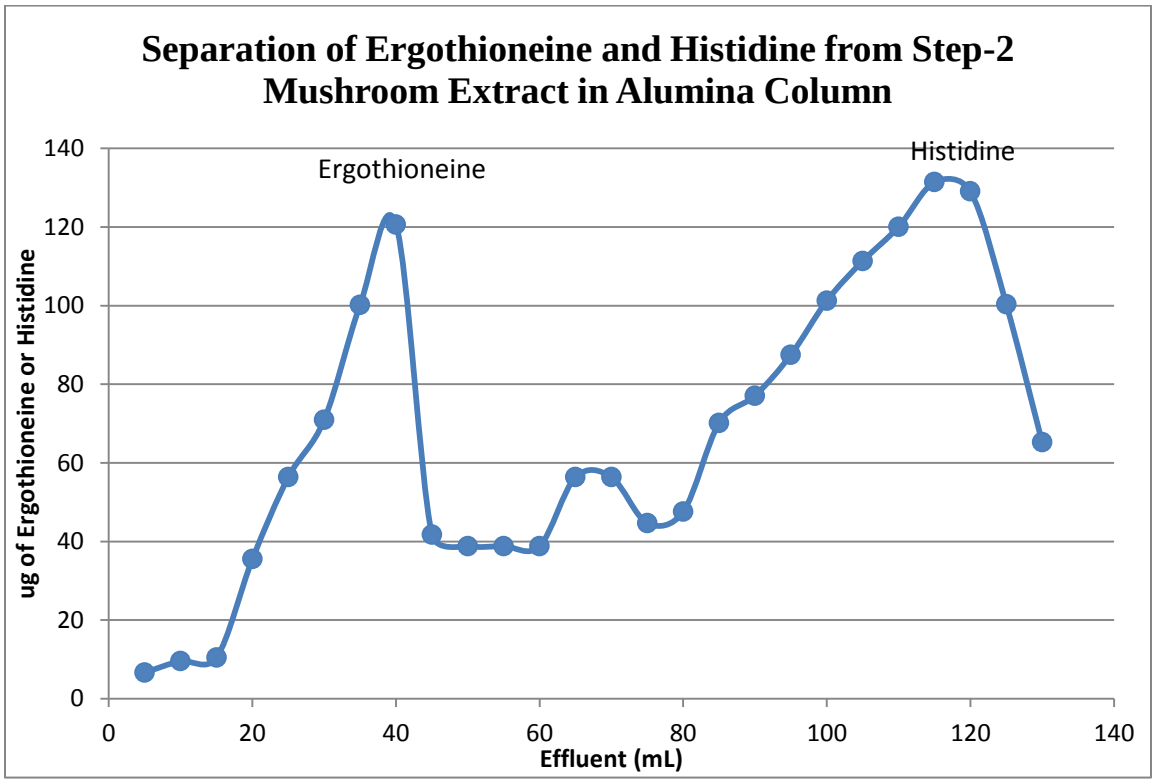


Figure-7: Separation of histidine and ergothioneine from Step-2 mushroom extract in alumina adsorption column.

Once again we saw two distinct peaks (Fig 7), similar to the standard ERT and histidine elution profile (Fig-6). The ERT peak had its maximum value at the 8th fraction (40ml) and the second (histidine) peak had the highest value at the 22nd fraction (110ml).

These results suggest that both ERT and histidine were present in the Step-2 extract and they were separated distinctly in the alumina column.

In order to further analyze, fractions 6, 7 and 8 (25-40ml) were combined and vacuum evaporated to 5ml. The ERT content was determined using the modified Hunter diazo test, which gave the distinctive red color. The results are summarized in Table-3.

Table-3: Step-3 (alumina column) purification chart

Alumina adsorption chromatography	
Volume of Step-2 extract used for Step-3	1ml
Fractions combined for pooling ERT	6, 7 and 8 (25-40ml)
Total volume of the combined ERT material	15 ml
Volume of solution after vacuum evaporation	5 ml
ERT estimation from combined fractions	
Volume Analyzed	100 µl
ERT from the standard curve	10.1 µg
Total ERT present in the recovered extract	503 µg (0.5 mg)
Percentage recovery of ERT from Step-3 treatment	28.9 %
Percentage Overall ERT recovery (from initial extract)	10.9 %

Thus we purified 0.5 mg (28.9 % recovery) of the ERT from the Step-2 extract, which contained 1.7 mg of Pauly positive material per ml. This recovery is based on a more conservative and stringent effort where only three fractions containing the highest ERT have been combined to avoid any histidine contamination. We would have gotten a higher recovery if we included fractions 5 and 9. The combined vacuum evaporated Step-3 solution was confirmed to contain ERT by doing a paper chromatography which showed no evidence of histidine presence.

III. Ergothioneine as a Nitrogen Source for *Agrobacterium radiobacter*:

Several strains of bacteria such as *E .coli* W were reported to utilize ERT as their sole nitrogen source for growth (Wolff, 1962). We wanted to determine if *Agrobacterium radiobacter* can utilize ERT as its sole source of nitrogen by breaking down ERT into thiolurocanic acid and trimethylamine.

1. Biochemical Test for *Agrobacterium* species:

We confirmed the identity our species through the biochemical test described in the materials and methods. Our bacterial species showed a yellow ring of Cu_2O around the cell mass, confirming that it is an *Agrobacterium* species (Fig-8)

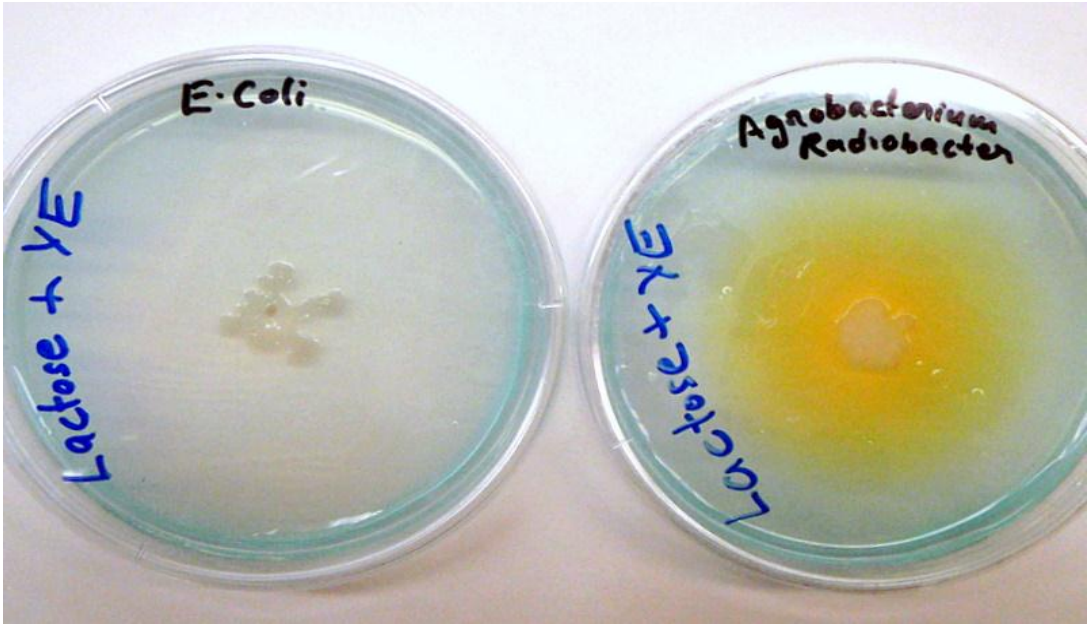


Figure-8: Biochemical test for *Agrobacterium*

The characteristic yellow color in the lactose medium remained stable for about 4 hours. Our control organism, *E .coli* did not show any yellow colored ring in the lactose medium. Thus, this *Agrobacterium* species has been used for all further studies.

2. Growth of *Agrobacterium* on Ergothioneine as Sole Nitrogen Source:

The *Agrobacterium* was grown in 1mM of ERT and histidine as described in the procedures above. The growth was monitored by taking absorbance readings (540nm) at regular intervals in a Gilford spectrophotometer. The results are plotted in Fig-9.

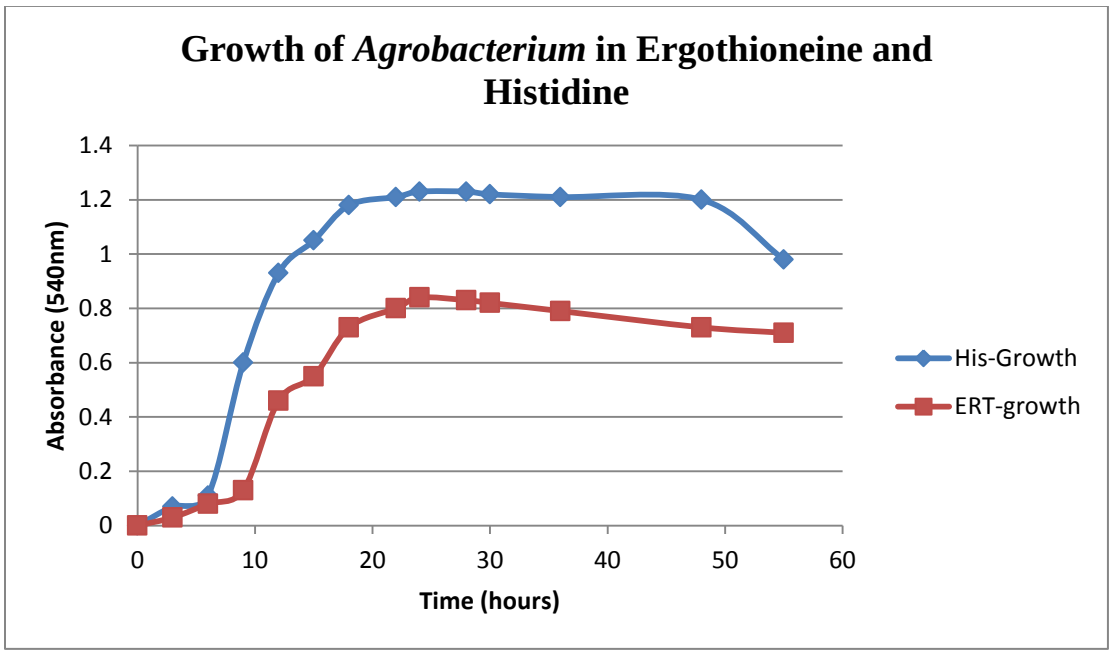


Figure-9: Growth of *Agrobacterium* in ERT and histidine as nitrogen source

The results from Fig-9 suggest that our species *Agrobacterium radiobacter* can utilize ERT as its sole source of nitrogen. The growth in ERT reaches a plateau in about 20 hours with a highest growth (0.84) recorded in the 24th hour. After 30th hour, the

growth curve starts to decline due to cell death. The growth in histidine is also very similar to the growth in ERT, with a highest growth in the 24th hour. However, at the 24th hour the growth in histidine is about one-third higher (1.23) than the growth in ERT (0.84) when both were present initially at the same concentration. This variation can be attributed to the difference in the nitrogen utilization pattern of *Agrobacterium.* The details of this pattern have been investigated in the following experiment.

3. Ergothioneine Degradation by *Agrobacterium*:

The above experiment showed that *Agrobacterium* can utilize ERT as a nitrogen source for growth. We wanted to confirm this conclusion by monitoring the disappearance of ERT from the medium. Various bacterial species have been shown to rapidly consume ERT and convert it to TUA and TMA (Wolff, 1962).

In this experiment we grew a culture of *Agrobacterium* in 2mM of ERT. A 100 µl of the growing culture were centrifuged at different times. The supernatant was separated and the ERT content remaining was estimated (Fig-10).

The results from Fig-10 suggest that *Agrobacterium radiobacter* degrades ERT and utilizes it as its nitrogen source. In the beginning (0th hour), the ERT concentration of the supernatant is about 2mM, which is same as the ERT concentration of the medium. As time progresses, the *Agrobacterium* transports ERT from the medium into its cells. Towards the end of the 20th hour, almost none of the ERT is remaining in the supernatant because it has all been absorbed by the *Agrobacterium* cells.

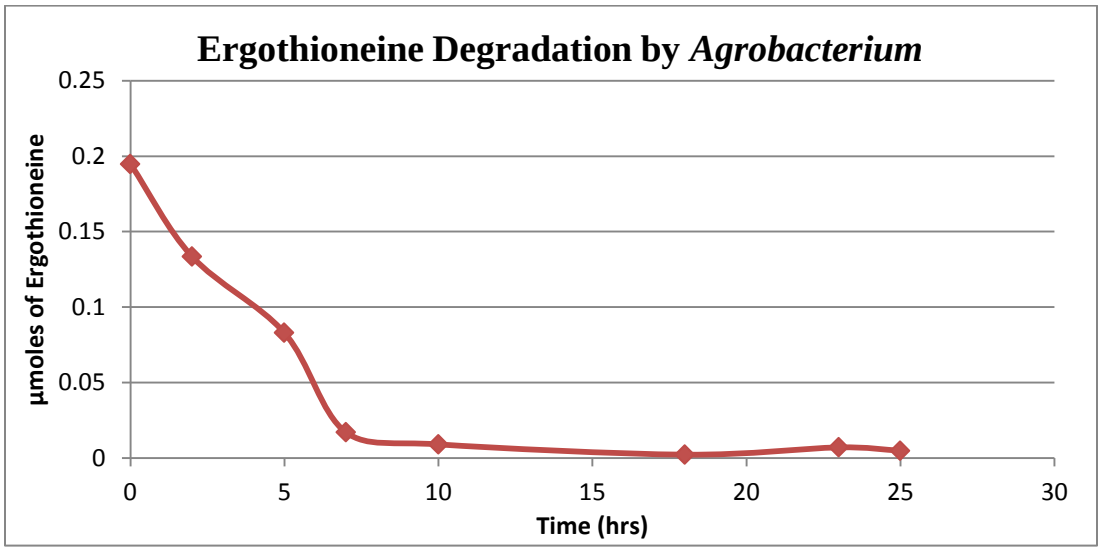


Figure-10: Ergothioneine degradation by *Agrobacterium*

As a negative control *Agrobacterium radiobacter Tn5*, a mutant version of *Agrobacterium radiobacter*, did not grow on the ERT medium, even after 120 hours of incubation, suggesting that *Agrobacterium Tn5* cannot absorb ERT from the medium.

IV. Nitrogen Utilization Pattern in *Agrobacterium*:

The variation in the growth pattern observed in Fig-9 intrigued our interest to further study the nitrogen utilization pattern in *Agrobacterium*. According to the literature, organisms when confronted with ERT as the sole nitrogen source induce the expression of ERT degradation enzymes (first one being ergothionase), which breaks down ERT to extract the nitrogens. This mechanism might be very similar to the action of histidine degradation enzymes, starting with the irreversible nonoxidative deamination of histidine into urocanic acid by the first enzyme histidase. However both mechanisms must be different since we get a higher growth in 1mM of histidine compared to 1mM of

ERT. In fact the results from Fig-9 suggests that the growth in ERT is only two-thirds the growth in histidine, even though the number of nitrogens in both compounds is same. This suggests that *Agrobacterium* can only use two out of the three nitrogens present in ERT.

1. Nitrogen Growth Experiment:

In this experiment a standard nitrogen utilization pattern for *Agrobacterium* growth was created by growing *Agrobacterium* in different concentrations of ammonium sulfate as the sole nitrogen source for 24 hours; this time is sufficient to allow complete utilization of the available nitrogens (Fig-11). The final concentration of nitrogen in the growth medium was calculated by multiplying the concentration of ammonium sulfate by two.

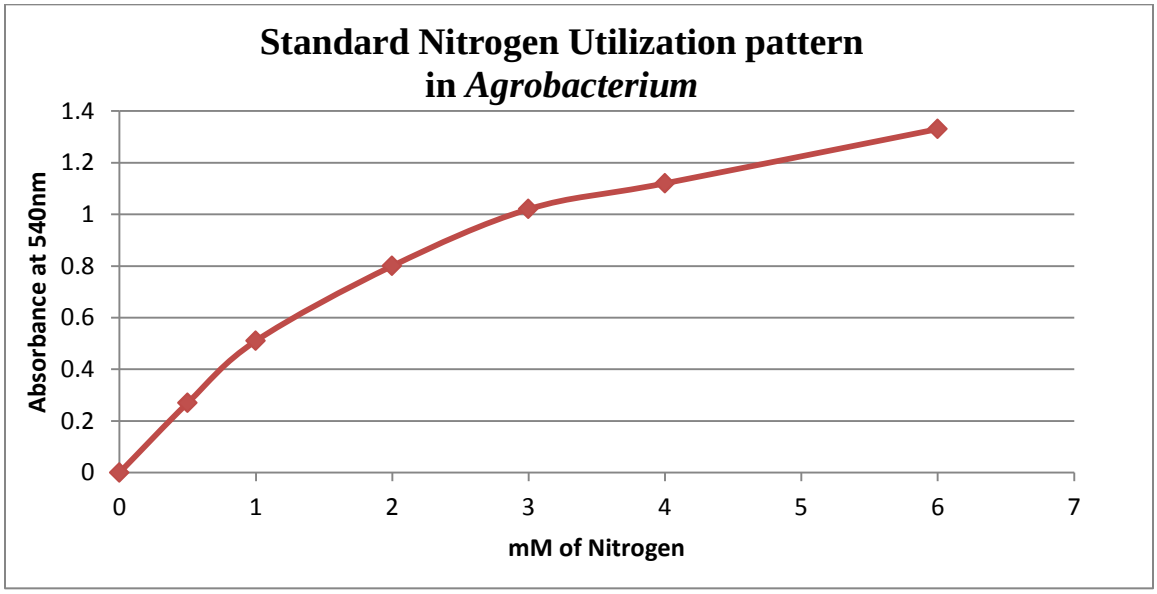


Figure-11: Standard nitrogen utilization pattern in *Agrobacterium* growth

After creating the Standard nitrogen curve, *Agrobacterium* were grown in 0.5, 0.75 and 1 mM of ERT as the sole nitrogen source. The conditions were kept similar to the growth in ammonium sulfate as the sole nitrogen source. After 24 hours, the growth was measured as the absorbance in Gilford spectrophotometer at 540nm (Table-4)

Since this experiment requires our ERT to be 100% pure, we determined the purity of our commercially available ERT through its extinction coefficient ($14000\ M^{-1}cm^{-1}$) given by Carlsson *et al*., (1974) at pH 7 and 257 nm. We found that our sample ERT is only about 80% pure.

Table-4: Nitrogen utilization data for *Agrobacterium* growth

ERT (mM)	ERT corrected (80% pure) (mM)	Theoretical nitrogen utilization (mM)	OD (540nm)	Observed nitrogen utilization (mM) (From Fig-11)	Fraction of nitrogen used
0.5	0.4	1.2	0.42	0.8	0.66
0.75	0.6	1.8	0.56	1.2	0.66
1	0.8	2.4	0.70	1.6	0.68

The data from Table-4 suggests that *Agrobacterium* only uses two-thirds of the nitrogen present in the ERT. This may account for the difference of growth pattern in ERT and histidine in Fig-9. In histidine the *Agrobacterium* probably utilizes all three of the nitrogens which results in the higher growth (one-third higher) than ERT.

2. Growth in Trimethylamine:

Having confirmed that *Agrobacterium* utilizes only two out of the three nitrogens present in the ERT, we concluded that it would be useful to know the identity of the nitrogens which are used by *Agrobacterium.* It is possible that these are the nitrogens present in the imidazole ring (Fig-1). In order to confirm this identity, we looked at the degradation pathway of ERT. ERT is biodegraded to TUA and TMA. The two nitrogens present in the imidazole group are retained within the TUA, while the tertiary nitrogen is released as TMA. So, we hypothesized that TMA is not used further by the organism but simply accumulates in the medium.

We used the method described by Cromwell (1950), to micro-estimate trimethylamine contained in a solution. A standard curve was created for comparing the absorbance given by solutions of varying trimethylamine concentrations (Fig-12). We found a linear relationship between the optical density and trimethylamine concentration.

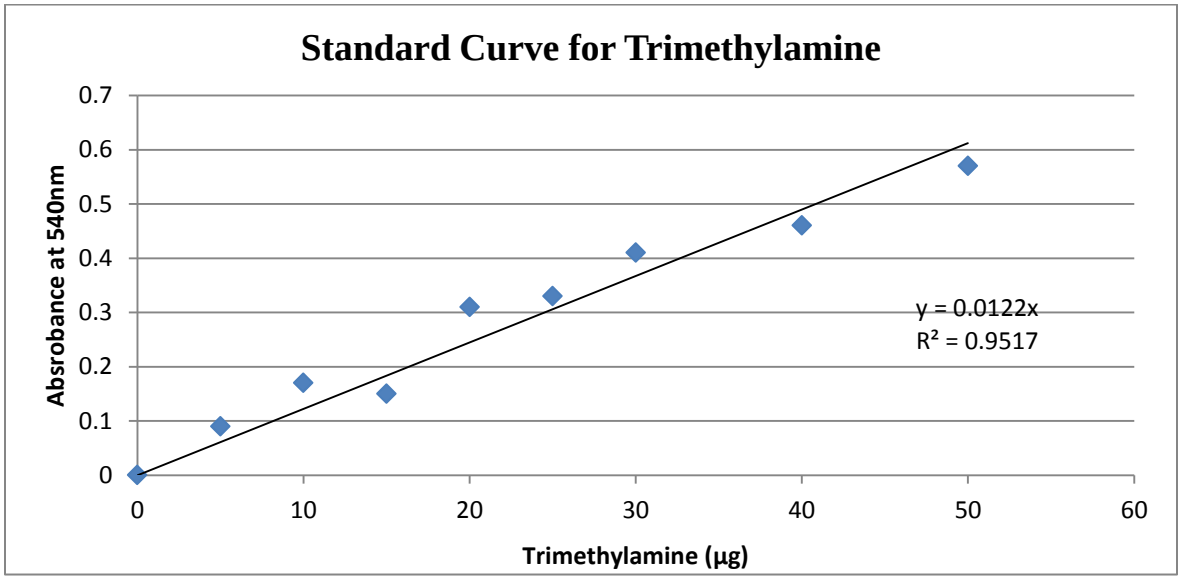


Figure-12: Standard curve for trimethylamine estimation

We grew an *Agrobacterium* culture with 2 mM of ERT. After 24 hours, the cells were centrifuged to form a pellet. The supernatant was separated and its TMA content was estimated using the Cromwell (1950) method (Table-5). The theoretical TMA estimations were done based on the assumption that 2 mM of ERT degrades to give 2 mM of TMA in a 1:1 ratio (Fig-3). The molecular weight of TMA is 59 g/mole.

Table-5: Estimation of trimethylamine (TMA) from the *Agrobacterium* culture.

Amount of supernatant (µl)	Theoretical estimation of TMA (µg)	OD (540nm)	TMA from the standard curve (µg)	% recovery
100	11.8	0.13	10.5	88.9
200	23.6	0.27	22.4	94.9

Results from Table-5 suggest that the TMA is not utilized by *Agrobacterium* for growth, but is retained in the medium. Thus *Agrobacterium* only uses the nitrogens present in the imidazole group and the nitrogen in TMA is not utilized. However in histidine degradation, histidine breaks down into urocanate and ammonia, both of which can be utilized as a nitrogen source for *Agrobacterium.* Thus we see a higher growth of *Agrobacterium* in histidine compared to growth on ERT.

V. Ergothionase Gene Expression and Assay:

The ergothionase gene is found in the ERT metabolizing organisms such as *Agrobacterium radiobacter* and *E. coli* W. In case of the ERT metabolizing *Agrobacterium,* we see a gradual decrease of ERT with a temporary accumulation of

TUA, since ergothionase breaks down ERT into TUA and TMA. The accumulation of TUA is temporary because TUA is broken down into the 2nd intermediate of ERT breakdown pathway by some enzyme, possibly a thiolurocanase analogous to urocanase that participate in histidine degradation.

1. Assay for Ergothionase.

A convenient method for following the ergothionase reaction (Fig-3) in *Agrobacterium* was based on the property that the accumulation of TUA is characterized by an increase in the absorbance at 311nm. An *Agrobacterium* culture was grown in 10 mM ERT as sole nitrogen source, and the crude extract (CE) was prepared according to the procedures described above. The ergothionase assay was performed (as described in Materials and Methods section) and the change in absorbance at 311nm was recorded.

Different concentrations of ergothionase were used by adding 10, 20, 40, 60 and 80 µl of CE with a fixed 1mM of ERT in the final assay. The rate was calculated from the slope of the line on the chart (ΔOD/min). We found that the assay was not accurate for estimating enzyme activity when the rate exceeded 0.1 ΔOD/min.

Thus only rates below 0.1 ΔOD/min with a fixed 1mM of ERT was used for the actual spectrophotometric ergothionase assay. No reactions were observed in the absence of the extract. The absorbance values were recorded by the Gilford chart recorder and the values are plotted in Fig-13.

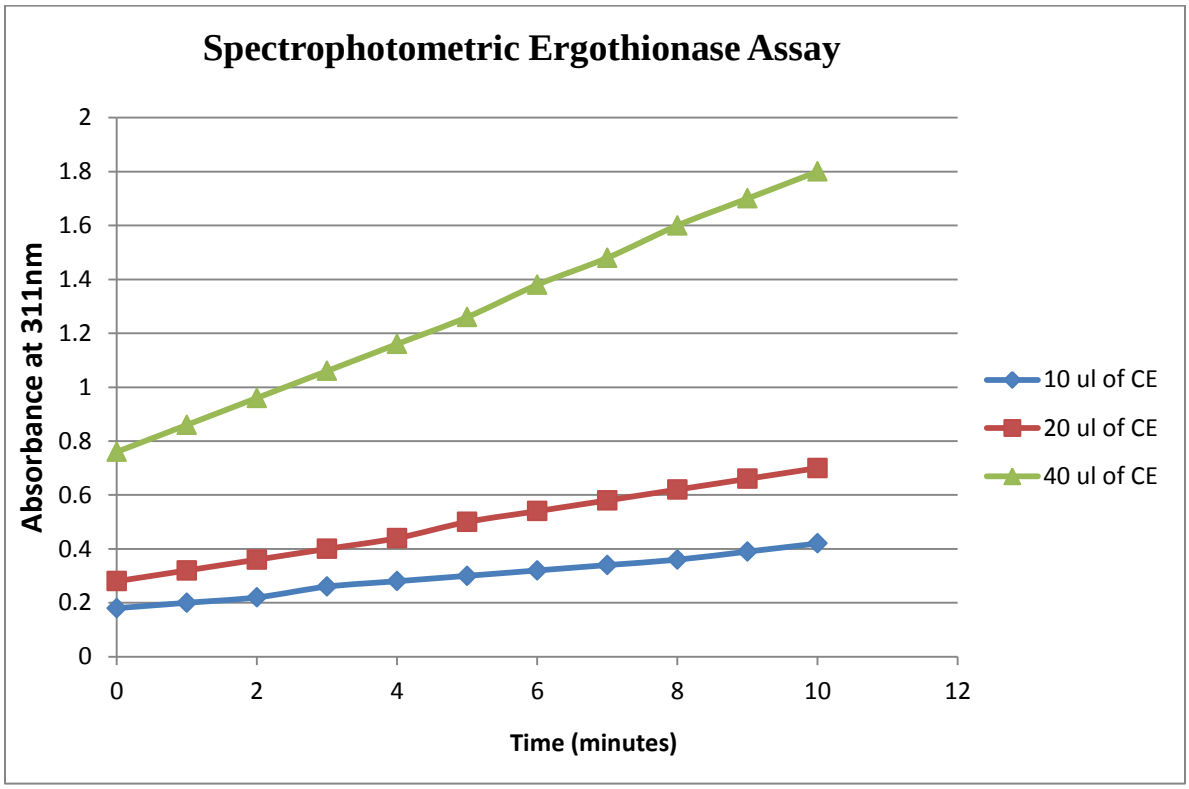


Figure-13: Spectrophotometric ergothionase assay measuring the accumulation of thiolurocanate.

2. Total Activity of Ergothionase:

The reaction rates for ergothionase were calculated from the slopes of plots shown in Fig-13. The extinction coefficient (ε) for TU was 1.9 X 10^4 L.moles^{-1}cm^{-1} at 311nm (Wolff, 1962). The total assay volume was 1ml (10^{-3} L). The total activity of ergothionase was measured in Units. A Unit of ergothionase activity was defined as the amount of enzyme which, in the standard assay (1ml) at 30^{o}C, catalyzed the conversion of 1 µmoles of ERT per minute. The average total activity in this sample of extract was calculated to be 0.13 Units/ml (Table-6).

Table-6: Total activity of ergothionase in crude extract:

Amount of CE (µl)	Rate (ΔOD/min)	Amount of product formed per min (Activity) $C=(Rate/\varepsilon) \times (10^{-3} L/ 10^{-6})$ (µmoles/min=Units)	Total activity in the CE (Units/ ml of CE)	Average total activity of Ergothionase (µmoles/min/ml) (Units/ml)
10	0.026	0.00136	0.13	0.13
20	0.047	0.00247	0.12	
40	0.105	0.00549	0.13	

3. Total Protein Content in Crude Extract:

The total protein content in the crude extract was determined through Bradford assay using the bovine serum albumin (BSA) as the standard. A standard curve for the BSA protein was made using different concentrations of BSA and measuring their absorbance at 595nm (Fig-15).

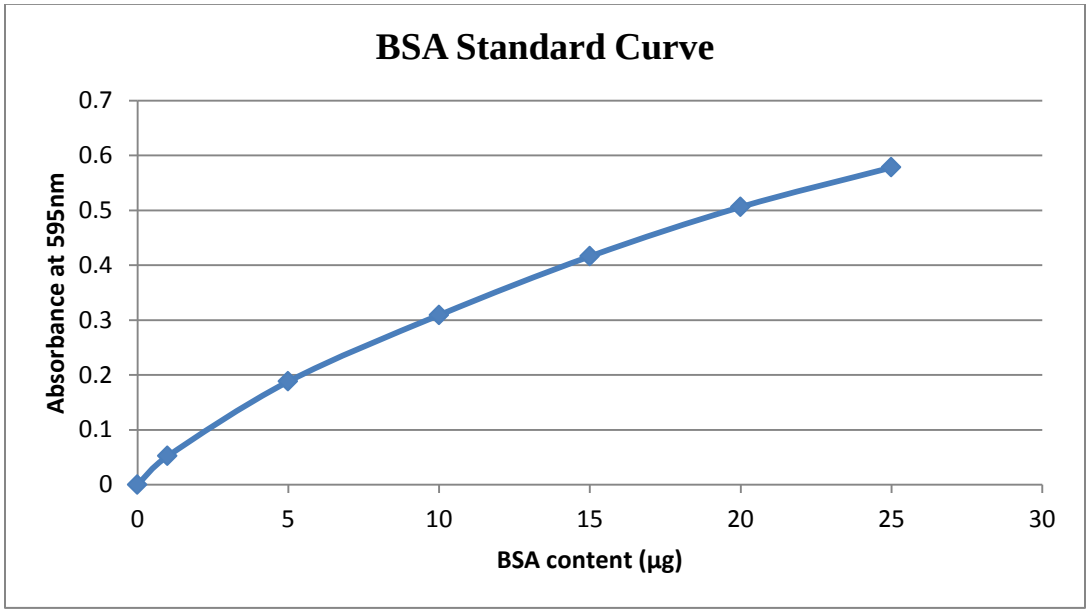


Figure-14: BSA standard curve for protein concentration determination

The total protein content of the crude extract was then determined by performing a Bradford assay on 100 µl of CE and measuring the absorbance at 595 nm. Comparing the absorbance value (0.112) to Fig-14, we found that the total protein content of the crude extract was 25 µg (0.025 mg) in the total 1mL of CE.

4. Specific Activity of Ergothionase in Crude Extract:

The specific activity is the activity of ergothionase per milligram of total protein. It is found by dividing ergothionase activity per mL by protein concentration per mL. In our case the specific activity of ergothionase in the CE was found to be:
(0.13 Units/ml) / (0.025 mg/ml) = 5.2 Units/mg.

5. Fate of Trimethylamine (TMA):

Results from Table-5 suggest that the TMA formed from the degradation of ERT is not further utilized. However in Experiment-IV, ERT was used in the medium as the sole nitrogen source. To confirm that TMA cannot provide nitrogen for growth, we attempted to grow *Agrobacterium* cultures in 1 mM TMA as the sole source of nitrogen. A positive control culture was also grown in 1mM of ERT (Fig-15).

The results from Fig-15 illustrate that *Agrobacterium radiobacter* cannot utilize TMA for growth and is just a byproduct of the 1st step of ERT degradation. The actual pathway intermediate is TUA, which disappears from the medium to form the 2nd intermediate of ERT degradation.

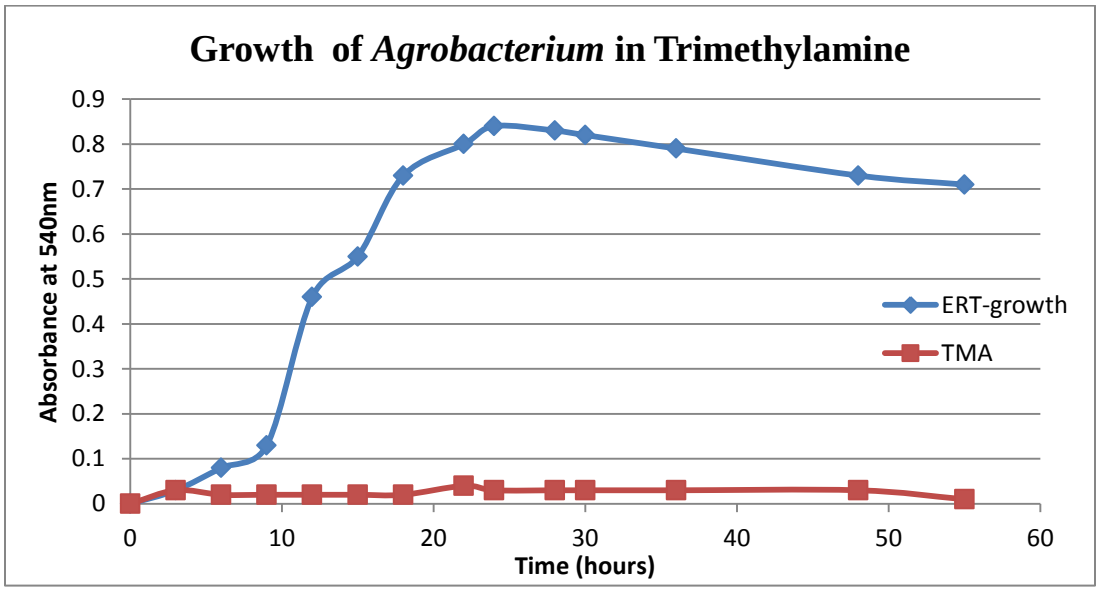


Figure-15: Growth of *Agrobacterium* in trimethylamine

VI. Properties of Ergothionase from *Agrobacterium*:

Several properties of the enzyme ergothionase were investigated. The effects of pH and buffer on the activity of ergothionase, as well as the kinetic properties of ergothionase were studied using the spectrophotometric ergothionase assay.

1. Effect of pH and Buffer:

The variation of ergothionase activity with pH, using several buffers, is shown in Fig-16 below. An ergothionase assay was performed with 5 mM of ERT, 50 µl of crude extract and 200 mM of various buffers in the assay. CHES buffer with a pKa of 9.3,

CAPS buffer with a pKa of 10.4 and HEPES buffer with a pKa of 7.5 were used in their useful pH range to evaluate their effect on the activity of ergothionase. The activity was measured as normally done and the relative activity was plotted against various pH values (Fig-16).

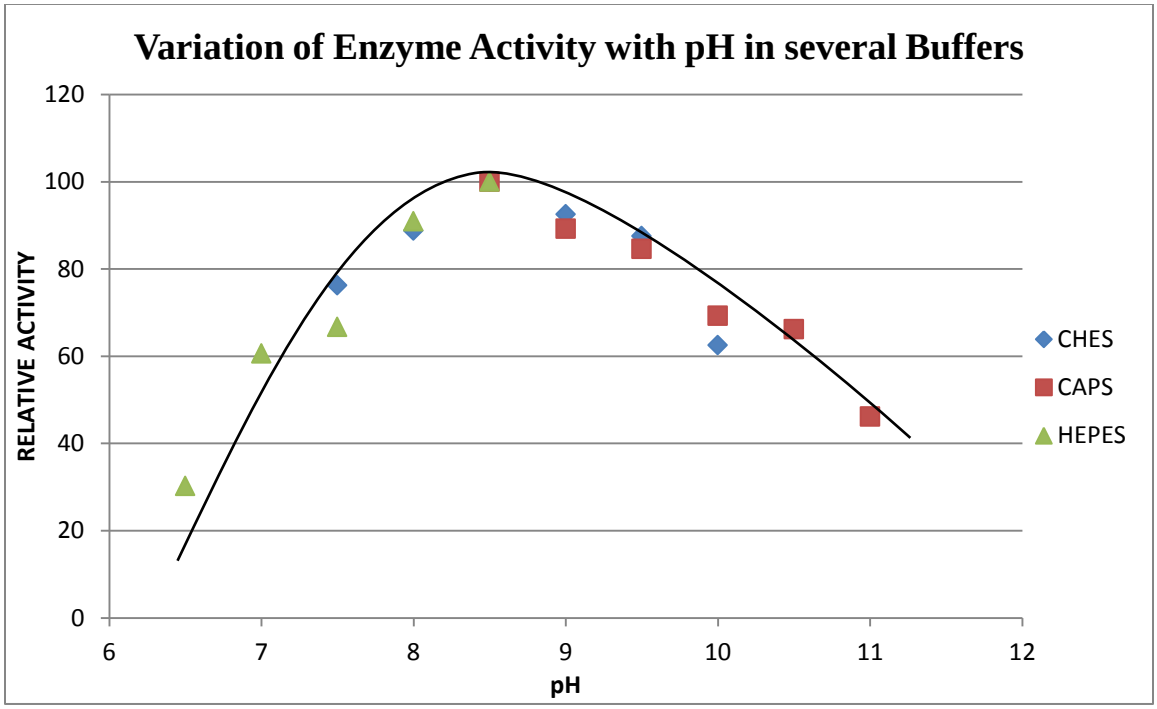


Figure-16: Variation of enzyme activity with pH in several buffers.

The optimum pH for the ergothionase activity lies between 8.0 and 9.0, with the highest activity at pH 8.5. The rate decreased rapidly as the solution became more acidic or alkaline. The rate decreased to less than 64% of the maximum when the pH was made below 7 or above 10. At pH 4, no activity could be detected, suggesting the complete destruction of ergothionase. Also the CHES buffer seemed to contribute the highest activity in the range near optimum pH.

2. Kinetic Properties of Ergothionase:

The kinetic properties of Km and Vmax for the enzyme ergothionase were determined to measure the affinity with which the enzyme binds to its substrate ERT. For studying the enzyme kinetics, the reaction rate was measured while varying the concentration of substrate (ERT).

An appropriate enzyme concentration was necessary for the subsequent kinetic analysis. So, we needed to adjust the concentration of ergothionase (from CE) in the assay to give a rate of approximately 0.1 ΔOD/min, which previous studies (see Experiment-V.1) had shown was the limit at which linearity between product formation rate and enzyme level could be observed. The ergothionase concentration was varied with a fixed 1 mM of ERT in the final assay and a suitable enzyme concentration was determined (Table-7). The rate was measured from the slope and the activity (µmoles/min) was calculated by multiplying rate with 0.0523 (see Table-6 for this conversion factor).

Table-7: Determining an appropriate enzyme concentration for kinetic studies.

Amount of CE added to assay (µl)	ERT concentration in the assay (mM)	Rate (Abs/min)	Activity (Rate X 0.0523) µmoles/min
10	1	0.03	0.0016
20	1	0.07	0.0037
40	1	0.12	0.0063
60	1	0.13	0.0068
80	1	0.15	0.0076

From Table-7, we can see that the extract volume to be added to the assay to get a 0.1 ΔOD/min rate was 40 µl. Thus, this quantity was used for further kinetic studies in this experiment.

Kinetic studies were performed using the standard ergothionase assay (30^{o}C, pH 8.5 and 1ml final volume) by varying the concentration of substrate ERT from 0.05 mM to 1 mM with a fixed volume of extract (Table-8). We observed an increase in the activity of ergothionase with the increase in ERT concentration.

Table-8: Enzyme activity in different substrate (ERT) concentrations.

Amount of CE added to assay (µl)	ERT concentration in the assay (mM)	Rate (ΔOD/min)	Activity (Rate X 0.0523) µmoles/min
40	0.05	0.052	0.0027
40	0.07	0.061	0.0032
40	0.1	0.069	0.0036
40	0.2	0.083	0.0043
40	0.5	0.103	0.0054
40	1	0.110	0.0059

The affinity of ergothionase for its substrate ERT was estimated by the kinetic constant Km. This constant was determined from the saturation curve of velocity as a function of ERT in the presence of a constant amount of extract. This relationship was shown according to the Lineweaver-Burk double reciprocal method (Fig-17).

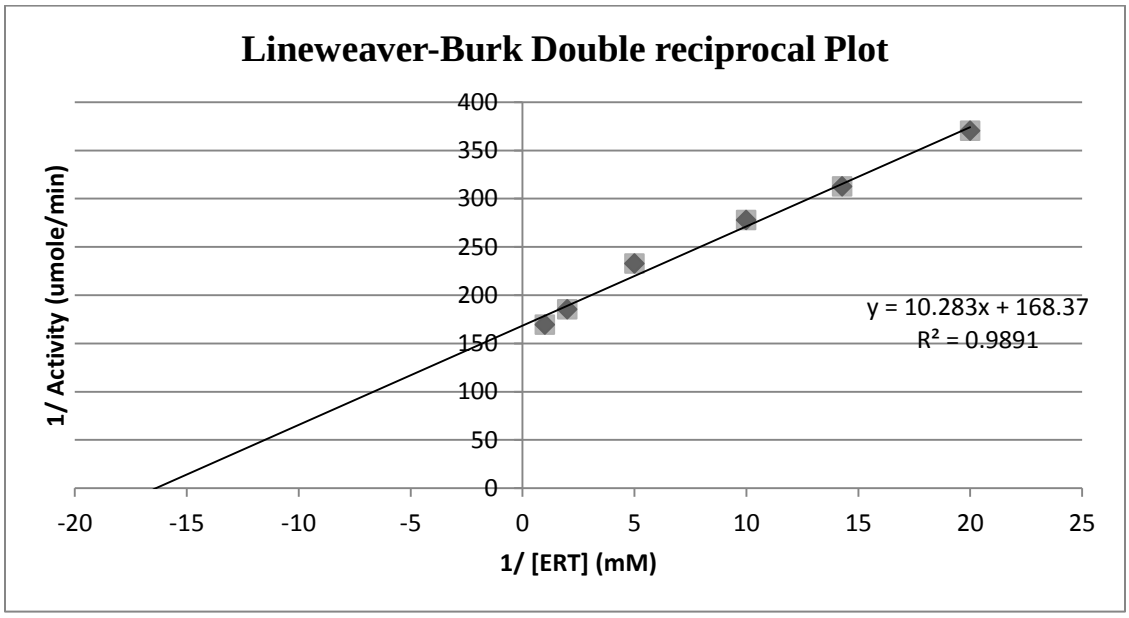


Figure-17: Lineweaver-Burk plot for the reciprocal of reaction velocity versus reciprocal of substrate concentration.

In Figure-17, the y-intercept defines the reciprocal of Vmax, while the x-intercept reveals the reciprocal of the negative Km. The slope of the line represents Km/Vmax value. Thus from Fig-17, the Km for ergothionase with ERT as substrate is about 0.061 mM or 6.1 X 10^{-5} M. However, since our commercial ERT is only 80% pure, the corrected Km value would be 0.049 mM. Similarly the Vmax for the quantity of enzyme used is in the range of 0.0059 Units (µmoles/min). The Km value suggests a high affinity between ERT and the enzyme ergothionase. Moreover, it also confirms that our standard assay conditions in which 1 mM ERT was used are such that the measured velocities are close to Vmax values, meaning such standard assays are conducted under "zero order" reaction conditions where velocity is primarily a direct function of the amount of enzyme added up to some limit.

VII. Ergothioneine's Protective Role against Oxidative Stress:

From our discussion in the Literature Review, we understand that ERT has a very specific protective role against oxidative stress. In this experiment, we wanted to evaluate whether ERT has any protective effect on the growth of *Agrobacterium* in an oxidative stress induced situation. Several *Agrobacterium* cultures were grown (as done previously), in 0.26 M H_2O_2 with different nitrogen sources. All the nitrogen sources, 1 mM histidine, 1.5 mM ERT and 1.5 mM ammonium sulfate, provided 3 mM of nitrogen available for growth. The growth of the culture was measured spectrophotometrically at 540nm. The results are plotted in Fig-18.

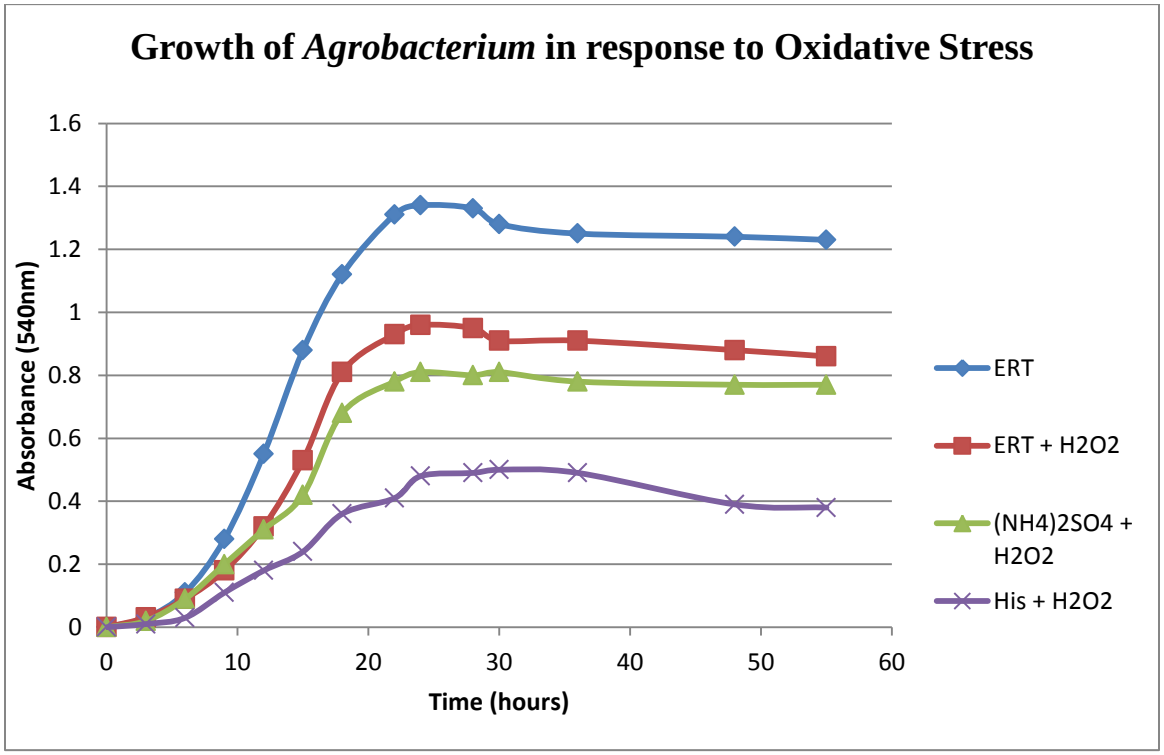


Figure-18: Growth of *Agrobacterium* in response to oxidative stress induced by hydrogen peroxide.

The results from Fig-18 indicate that ERT has a positive protective effect in the growth of *Agrobacterium* against oxidative stress induced by H_2O_2. We can clearly see that the growth in ERT without the H_2O_2 is much higher. In fact the growth in ERT without H_2O_2 is about 30% higher than the growth in ERT with H_2O_2. This shows that the H_2O_2 has a marked effect on the growth on *Agrobacterium.* Comparing the growths in H_2O_2 with ERT, histidine and ammonium sulfate, we can see that the growth in ERT is almost 50% higher than the growth in histidine and almost 15% higher than the growth in ammonium sulfate.

Also *Agrobacterium* cells grown in excess ammonium sulfate with 0.5 mM of ERT had a higher growth (11%) than the cells grown without ERT in a medium containing H_2O_2. This signifies the protective role of ERT against oxidative stress.

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Section-5

DISCUSSION

In this study we have investigated the role of ERT as a nitrogen source in supporting the growth of *Agrobacterium radiobacter*. Ergothionase, the first enzyme involved in the biodegradation of ERT, breaks down ERT into thiolurocanic acid and trimethylamine. We also characterized the ergothionase from *Agrobacterium* in terms of its activity and kinetic properties.

ERT is structurally very similar to histidine and both give a positive Hunter diazo test. It was necessary to establish a critical method to estimate ERT quantitatively in a solution. We used a modified Hunter diazo method, based on the methods of Macpherson's (1941). This method allowed us to accurately estimate ERT, even if ethanol was present in the solution. Also the sensitivity of this method was much higher (about 25 times) than the original Hunter diazo method, which was very critical since a lot of assays in our experiments require accurate estimation of very low concentrations of ERT. It must be noted that the alkaline medium (NaOH or Na_2CO_3) used to induce the color formation at the end of the reaction, makes a difference in the intensity of the color. So, it is necessary to use the same alkaline medium for all experiments to obtain consistent results. In our case, we used NaOH instead of Na_2CO_3 since Na_2CO_3 can cause the formation of CO_2 gas in the cuvette, which can compromise the spectrophotometric measurements.

The colored diazotized ERT product formed through the modified Hunter diazo test had a maximum absorbance of 445 nm (Fig-4), in contrast to the traditional 510 nm

in the original Hunter diazo assay. It must be noted from Fig-4 that the sensitivity of the colored product does not appreciably change in the absorbance range of 420-450 nm. However we wanted to avoid the lower values since the colored diazotized histidine product has a maximum absorbance at 400 nm. Thus all ERT measurements were made at 445 nm.

Due to complexity of the ERT synthesis and its limited supply, we decided to purify ERT from the mushrooms. King Oyster mushroom, which was already established to contain a high concentration of ERT (about 2 mg per gram of mushroom powder according to the studies from Dr. Beelman's lab), was used for this purpose. We employed a three step purification procedure consisting of hot water extraction, anion exchange and alumina adsorption chromatography. The first step of hot water extraction, done to separate the non-polar and high molecular weight debris, allowed us to extract about 23 mg of Pauly positive material. The second step of Dowex-1-acetate anion exchange, done to separate the negatively charged species, allowed us to recover about 63 % of Pauly positive material from Step-1 extract. It must be noted that the total recovery in these two steps represents a combination of ERT and histidine, both of which give a positive Hunter diazo test.

The step of alumina adsorption chromatography (Step-3) was the most important and critical step because it separated histidine from ERT, two structurally similar compounds and therefore must be done according to the conditions described in Materials and Methods section. An elution profile for ERT and histidine mixture in alumina column was done to confirm the ability of the system to separate the compounds in two

distinct peaks. The Step-2 extract also formed two distinct peaks in the alumina column (Fig-7) when done under the same conditions as the standard profile, with a high ERT peak surrounding fraction-8 (40 ml effluent) similar to the standard profile. The histidine peak was much broader compared to the ERT peak and had a poor resolution. We also obtained a small middle peak, whose identity could not be confirmed. It might be a mixture of both ERT and histidine and so was avoided. A conservative method was adopted to collect and combine only 3 fractions in the ERT peak with the highest absorbance to avoid any contamination from histidine. The combined solutions were vacuum evaporated to concentrate ERT and used as nitrogen source for *Agrobacterium* growth. A positive ergothionase assay from the growth in Step-3 extract as a sole nitrogen source confirmed the presence of ERT. Thus we successfully recovered 11% ERT (0.5 mg) from the initial extract (Step-1) containing 4.6 mg/mL of Pauly positive material (Table-3). This represents a 25% recovery of ERT from the estimated 2 mg ERT per gram of King Oyster mushroom. The recovery was low because of the conservative approach used in Step-3 and loss of material during many vacuum evaporation processes.

We have found that *Agrobacterium* can use ERT as a sole nitrogen source for its growth. The concentration of ERT in the medium kept decreasing with time as the *Agrobacterium* grew. After 20 hours of incubation, none of the ERT was found in the medium (Fig-10). The organism must have broken the imidazole ring of ERT to extract the nitrogens contained in it during the biodegradation of ERT. A mutant version of *Agrobacterium* genetically transformed by transposon (*Tn5*) mutagenesis was unable to

grow on ERT as the nitrogen source. This indicates that the *Tn5* might have caused a mutation in the ERT biodegradation genes or in the gene encoding ERT transporter. Many other bacterial species were not able to grow on ERT as the sole nitrogen source because they lack the ERT biodegradation genes, however most of them (especially mammals) can use ERT as an antioxidant by absorbing ERT through their high specific ERT transporter.

The *Agrobacterium* growth in histidine was one-third higher compared to the growth in ERT, even though the concentrations of ERT and histidine were same (1 mM). We found that that *Agrobacterium* uses only 2 out of the 3 nitrogens present in ERT, while it uses all 3 nitrogens from histidine. This happens because in the biodegradation pathway of ERT, the first byproduct is TMA, a compound which cannot provide nitrogen for the growth of *Agrobacterium* (Fig-15). On the other hand, in the biodegradation pathway of histidine, the first byproduct is ammonia, a product which definitely supports the growth of *Agrobacterium.*

The strong absorption of thiolurocanic acid (1^{st} intermediate) at 311nm and pH 9 provided a convenient method for determining the activity of ergothionase. Neither ERT nor urocanic acid has a significant absorption at 311nm. However the possibility that an unknown product of the ERT biodegradation pathway with similar absorption characteristics may occur in the reaction mixture cannot be eliminated until the complete series of biodegradative products from ERT is known. The TMA formed in the first step of ERT degradation was not used by the *Agrobacterium* and has been recovered to 95% from the medium (Table-5). *Agrobacterium* cells grown in histidine or ammonia showed

no ergothionase activity. Also no activity was detected when histidine or TMA was provided as the substrate in the ergothionase assay. This shows that the ergothionase is very specific for its substrate ERT.

Several properties of the enzyme ergothionase were investigated including optimum pH, buffer and kinetic properties. The highest activity was attained with the CHES buffer at pH 8.5. The rate decreased rapidly with more acidic or alkaline pH. Protonation or deprotonation seems to deactivate the active sites causing the enzyme to lose its activity. This result is very similar to the property of the ergothionase from *E. coli* W, where the optimum pH was 9 (Wolff, 1962).

Kinetic properties such as Km and Vmax were studied to evaluate the affinity of ergothionase for its substrate ERT. We found that the activity increased linearly with increasing concentration of enzyme up to a certain point (40ul). After 40ul of CE, the linearity kept decreasing and the curve eventually flattened out. In order to find the Km we increased the concentration of substrate while keeping the concentration of enzyme constant (40ul of CE). In this case we see a linear increase in the activity of the enzyme in the lower ERT concentration range. However in the highest concentrations, the activity remains relatively constant (Table-9). This is because when the velocity approaches Vmax, the velocity curve flattens out. Near Vmax all the enzymes active site is fully occupied by the substrate and increasing substrate concentration would not increase the velocity. The corrected Km value from Fig-17 was found to be 0.049 mM. This value was similar to the Km reported from ergothionase of *E.coli,* in which the Km

was about 0.056 mM (Wolff, 1962). The small value of the Km represents that the ergothionase has a tight binding to its substrate ERT in the active site.

The protective role of ERT against oxidative stress induced by H_2O_2 in *Agrobacterium* was studied by growing cells in excess of ammonium sulfate (nitrogen source) and H_2O_2 with and without ERT. We found that the cell cultures containing H_2O_2 with ERT had a much higher growth compared to the cultures without ERT. This illustrates the protective effect of ERT. In Fig-18, we saw that the cells grown in $(NH_4)_2SO_4 + H_2O_2$ had a growth almost similar to the growth in ERT + H_2O_2. But this increase in growth in the ammonium sulfate medium might be related to fact that the nitrogens in ammonium sulfate are more accessible than the nitrogens present in histidine and ERT. A more comparative scenario can be deduced from looking at the growth in ERT + H_2O_2 and histidine + H_2O_2. Here both ERT and histidine have their nitrogens enclosed within the imidazole ring and *Agrobacterium* would have to put some effort in breaking that ring. In this case we can clearly see that the growth in ERT + H_2O_2 is much higher than the growth in histidine + H_2O_2, again suggesting the protective effect of ERT against oxidative stress. These effects may not be too evident in the growth of single celled *Agrobacterium* since the rapid growth in bacteria prevents the accumulation of oxidative stress-impaired macromolecules unlike plant and animal cells where severe oxidative stress induces a specific programmed death pathway-apoptosis (Sigler *et al*., 1999). Thus the benefits of ERT could have a profound effect in more complex animals.

Thus we have studied various aspects and properties of ERT and how it could act as a nitrogen source for *Agrobacterium.* We have also investigated the first step in the

biodegradation pathway of ERT, and analyzed the characteristics of the enzyme ergothionase. Although ERT is not made in our body, its accumulation within a wide variety of cells and highly specific transporter makes it a very important physiological molecule. It is one of few dietary antioxidants with a well-known specific transport system in humans. Its protective role against oxidative stress and inflammatory response is just the beginning of understanding the true potential of this molecule. Thus more research needs to be done in terms of deducing all the intermediate products of ERT biodegradation and the enzymes involved it.

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Section-6

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Academic Vita of Jiku M. Jose

Jiku M. Jose
10227 Dedaker St.
Philadelphia, PA 19116
jikumjose@yahoo.com

Education:

Bachelors of Science in Biochemistry and Molecular Biology, Penn State 2011
Minor in Biology
Honors in Biochemistry and Molecular Biology
Thesis Title: Ergothioneine Degradation and Properties of Ergothionase from *Agrobacterium Radiobacter*
Thesis Supervisor: Dr. Allen T. Phillips, PhD

Employment:

Computer Lab Consultant, IST Dept. Penn State, University Park, PA (2007-2011)
Library Assistant, Penn State Abington, PA (2007-2009)
Teachers Assistant, Penn State Abington, PA (Spring 2008)

Honors and Awards:

Lapinski award from BMB department, Penn State
Berg award from BMB department, Penn State
Outstanding Freshman Chemistry award, Penn State Abington
George and Elizabeth Smollett award
Tsui Honors scholarship
Penn State Abington Honors Program
Dean's List

Extracurricular Activities:

Phi-Kappa-Phi honors society
International Affairs President for the Student Government Association, Penn State
Science and Environment club, Penn State Abington
Student volunteer at Mount Nittany medical center, State college, PA
Volunteer at Abington Neurological associates.