PURIFICATION AND CHARACTERIZATION OF NOVEL NUCLEASES FROM A THERMOPHILIC FUNGUS

A Thesis Presented

By

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ABSTRACT

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A thermophilic fungus was isolated from composted horse manure. The organism was as a Chaetomium sp. by sequencing the highly conserved ITS region of the fungus and comparing to known regions in a genomic database and was referred to as TM-417. TM-417 was found to have an optimal growth temperature of 45 ^{o}C and an optimal pH of 7.0. An extracellular DNase and RNase was found to be produced by the isolate and were purified 145.58-fold and 127.6-fold respectively using a combination of size exclusion chromatography and a novel affinity membrane purification system. The extent of purification was determined electrophoretically using 4-15% gradient polyacrylamide gels.

Both DNase and RNase were dependent on metal co-factors for activity. The metal ion Mg^{2+} was the preferred ion for the DNase, whereas for the RNase, Zn^{2+} and Mn^{2+} yielded an increase in enzyme activity over that with Mg^{2+}. The purified DNase demonstrated maximum activity at pH 6.0 with no activity at pH 2.0 or 10.0. The RNase exhibited two peaks of maximum activity, on at pH 3.0 and the other at pH 7.0 with no activity at pH 2.0 or 10.0. The optimal temperature for the purified DNase was 65^{o}C. The optimal temperature for the RNase was 70^{o}C. The molecular of the DNase and RNase were determined to be 56 kDa and 69kDa respectively

using a Sephadex G-75 column. A standard curve was generated using several standard proteins of known molecular weight.

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ABBREVIATIONS

Media:

YpSs = yeast protein soluble starch

DYpSs = DNase test yeast protein soluble starch

Amino Acids:

Ser= serine

Tyr = tyrosine

His = histidine

Nucleic Enzymes:

DNase = deoxyribonuclease

RNase = ribonuclease

Nucleotides:

A = adenosine

C = cytidine

G = guanosine

T = thymidine

U = uridine

Mononucleotides:

d-pA, d-pT,…, etc…. = deoxyadenosine-5'-phosphate, deoxythymidine-5'-phosphate, etc…….

d-Ap, d-At, ….,etc… = deoxyadenosine-3'-phosphate, deoxyadenosine-3'-phosphate, etc…….

Oligonucleotides:

d-pCpC, d-pApT, ……, etc…. = 5'-deoxycytidine (3' → 5') deoxycytidine, etc……

Polynucleotides:

DNA = deoxyribonucleic acid

RNA = ribonucleic acid

Reagents:

EDTA = Ethylene Diamine Tetra Acetic acid

EGTA = Ethylene Glycol Tetra Acetic acid

Other:

UV = ultraviolet

RPM = revolution per minuet

INTRODUCTION

Introduction

Composting is an economical and environmentally sound method for the breakdown of organic matter usually resulting in the production of usable end products (i.e. fertilizers, various substrates, and biogas) (Anastasi *et al.,*2005). One fundamental stage of composting is thermogenesis which results from organismal activity within the compost (Kane & Mullins, 1973). Originally the component believed to be responsible for the breakdown and conversion of decaying matter was the naturally occurring bacterial flora; however, it has been found that fungi play an intricate part with an average fungal to prokaryote ratio of 2:1 (Sparling, 1982). Temperatures exceeding 60^{o}C are quite common for compost. These high temperatures support and promote the growth of thermophiles that thrive in temperatures around that of active compost, between 45-62^{o}C (Cooney & Emerson, 1964; Anastasi *et al.* , 2005). Thermophilic bacteria and fungi are commonly found within active compost heaps (Kane & Mullins, 1973; Salar & Aneja, 2007).

Thermophilic fungi aid in thermogenesis, the destruction of pathogens, and the breakdown of various organic materials (Hart *et al.*, 2002; Norman, 1932; Kane & Mullins, 1973). They use the decaying matter as a source of nutrients and are mainly responsible for compost maturation (Anastasi *et al.*, 2005). The accessibility of the nutrients is reliant on the various types of extracellular enzymes produced by the organism (Johri & Satyanarayana, 1999; Fraser & Low, 1993). Therefore to fully understand the breakdown of compost associated with thermophilic fungi the enzymes of these organisms must be well understood.

Extracellular nucleases (DNA and RNA degrading enzyme) of fungal origin may aid in the composting process. As organic matter is broken down, nucleic acids are released into the surrounding environment. Free DNA and RNA are vulnerable to hydrolysis by extracellular nucleases secreted by thermophilic fungi. With many mesophilic fungi, nucleases are often excreted out into the environment for the sole purpose of providing the purine and pyrimidine requirements of the organism. The same can be said for thermophiles (Johri & Satyanarayana, 1999; Maheshwari *et al.*, 2000).

The purpose of this research is to improve on the isolation, purification, and characterization of extracellular nucleases produced by thermophilic fungi. The objectives are as follows:

1) Isolation of a thermophilic fungus that produces thermophilic nucleases
2) Identification of the isolated fungus
3) Purification of the thermophilic nucleases
4) Characterization of the thermophilic nucleases

Hopefully this work will shed some light on the enzymatic digestion of DNA occurring during the composting process.

CHAPTER 1
LITERATURE REVIEW

Thermophilic Fungi

History

Thermophilic fungi were first officially defined by Cooney and Emerson (1964) as fungi having a maximum temperature at or above 50°C and a minimum of 20°C necessary for growth. Thermophilic fungi constitute a small group of eukarya that exhibit the ability to thrive at temperatures normally detrimental to the majority of fungal species. Of the 100,000 recorded fungal species roughly 50 species have been described that have ability to successfully grow at these elevated temperatures (Salar & Aneja, 2007) with probably many more yet to be described.

The first study regarding thermophilic fungi was published by the German scientist Lindt (1886). He described the characteristics of the first true thermophilic fungus, *Mucor pusillusaI.* Cohn (1889) demonstrated that molding barley seedlings when held in an insulated container, would reach temperatures as high as 60^{o}C. He suggested that this heating was due to the metabolic activity of molds. Tsiklinskaya (1899) classified and named the thermophilic fungus *Thermomyces lanuginosus,* which was found growing on a potato that had been inoculated with garden soil. Up to the late 19th century, thermophilic fungi were accidently being discovered as contaminates of plated substratum (Cooney & Emerson, 1964).

It was not until 1907 that most of the early information regarding thermophilic fungi was published primarily in part by Miehe (Cooney & Emerson, 1964). Miehe showed that damp, packed hay or leaves self-heated to 60^{o}C within one to several days (Miehe, 1907). He also

showed that steamed hay did not heat and that steamed hay inoculated with soil suspensions did (Miehe, 1907). Miehe (1907) further investigated the micro-flora associated with self-heating hay and compost systems along with comparing the heating capacities of mesophilic and thermophilic organisms. Miehi was the first person to work extensively with thermophilic organisms and ultimately established the first collection of thermophilic fungi along with numerous thermophilic bacteria and actinomycetes (Cooney & Emerson, 1964).

The Second World War brought an unexpected interest to the world of thermophilic fungi. During the war the need for finding alternate sources of rubber led to the study of the rubber-producing guayule shrub, *Parthenium argentatum* (Cooney & Emerson, 1964). Through a series of trials, Allen and Emerson (1949) found that the extractability and physical properties of the rubber improved when the shrub was put through a process involving the chopping and storage of the shrubs in rets. Rets are used for the aerobic biological decomposition of the guayule shrub, a process known as retting (Shekhar Sharma, 1989). They further demonstrated that the improvements resulted from the utilization and reduction of resin in the crude rubber by a thermophilic micro-flora (Allen & Emerson, 1949). From this micro-flora several species of thermophilic fungi were isolated with temperature maxima up to 60^{o}C (Allen & Emerson, 1949). With the announcement for the potential commercial utilization of thermophilic fungi, the search for new species and other potentially commercially important enzymes was initiated.

Ecology

Allen and Emerson's (1949) assessment of the thermophilic flora associated with the decomposition of guayule shrubs suggested that moisture and nutrient content were crucial for the observed improvements. Thermophilic fungi thrive at thermogenic conditions (Johri & Satyanarayana, 1999). Examples include various types of soils and places where ample plant

decomposition occurs such as: compost heaps, piles of hay, stored grains, wood chip piles, nesting material of birds and animals (Salar & Aneja, 2007). In sum, decaying organic matter in warm, humid, and aerobic environments provide the basic conditions for development and growth of thermophiles (Salar & Aneja, 2007). In fact a 5 cm pile of leaves is adequate for the development of thermophilic fungi (Noack, 1912).

Depending on the availability of nutrients and environmental conditions, thermopiles may populate the substrata as either resting propagules (spores) or as active mycelia (Salar & Aneja, 2007). According to Maheswari et al. (1987) soils in tropical regions do not appear to have a higher population of thermophilic fungi than more temperate environments. It was suggested the widespread occurrence was due to the dissemination of propagules from decomposing organic masses (Maheshwari *et al.*, 1987).

Aquatic environments, though initially thought of as an unusual environment for such organisms, have been studied for the presence of thermophilic fungi. Natural geothermal sites, such as hot springs and hot spring effluent channels were considered the most favorable locations (Ellis D. H., 1980). Tansey and Borck (1971) successfully isolated thermophilic fungi from hot spring effluent channels at Yellowstone National Park. Hedger (1974) also demonstrated that thermophilic fungi populated hot springs in Indonesia, but the total flora was limited to a few species. Man-made heated aquatic habitats such as steam-line discharge sites and heated effluents of nuclear reactors were found to be another rich source for thermophilic fungi (Tansey & Brock, 1972; 1978).

The existence of thermophilic fungi in the sediment of lakes was first reported by Tubaki *et al* (1974). Several species were isolated from mud on the bottom of cold lakes in Japan. Unlike the previously heated natural environments, the ambient temperature at the bottom of

the lakes was 6-7^{o}C (Tubaki, 1974). The culturing of these organisms unfortunately did not prove they were growing and actively involved in the decomposition actions taken place in the mud (Ellis D. H., 1980). The highly unfavorable conditions within the lake, such as the low temperatures (on average 6-7^{o}C) and low oxygen levels (average 10 ppm, <1.0 ppm at a depth of 31 meters) disproved any speculations (Ellis D. H., 1980; Salar & Aneja, 2007). Their presence in the sedimentary mud was explained by the washing of the surrounding terrestrial habitats into the lakes (Tubaki, 1974).

In 1971, thermophilic fungi were isolated from another surprising environment, Antarctic and Sub-Antarctic soils (Cameron, 1971). Again there was no evidence suggesting growth, but the observations do show that propagules of thermophilic fungi populate Antarctic soils (Ellis D. H., 1980). The idea of thermophilic fungal spores being transferred from a habitable environment to a non-habitable one can help explain the occurrence of these organisms in such uninviting environments.

Unfortunately for many of the early papers dealing with the isolation of thermophilic fungi from extreme environments such as lake beds, artic soil, and hot springs the organisms were not identified.

Nutritional Requirements

The substrates suitable for thermophilic fungi encompass an array of organic matter which is difficult to reproduce in a laboratory setting. It was originally thought that thermophilic fungi had complex or unusual nutritional requirements (Cooney & Emerson, 1964). Miller *et al.* (1974) stated that "no defined medium could be produced in which the thermophilic fungi would grow….". Rosenberg (1975) reported that thermophilic fungi required 0.01% yeast extract and Wali *et al.* (1978) showed that growth in a liquid media containing glucose and

ammonium sulfate required a supplementation of succinic acid. The observation by Wali *et al.* was later disproved by Gupta and Maheshwari (1985) who demonstrated the low phosphate concentration in the culture medium lowered the pH to a level unfavorable for growth. Furthermore, they determined that only one tri-carboxylic acid stimulated growth, which was due to its buffering capacity rather than any nutritional benefit.

It is now known that thermophilic fungi grow in simple media containing carbon, nitrogen and a few mineral salts (Salar & Aneja, 2007). Nitrogen sources that support good growth include sodium nitrate, potassium nitrate, asparagine, and yeast extracts (Satyanarayana & Johri, 1984). *Humicola lanuginose* utilizes ammonium as a nitrogen source and glucose, sucrose, maltose, mannose, galactose, xylose, starch and lignin as carbon sources (Johri & Satyanarayana, 1999). Several other thermophilic fungi were found to utilize cellulose, hemicellulose, lignocellulose, and pectin as carbon sources as well (Basu, 1980; Deploey, 1976).

Thermophilic fungi can be isolated and grown on a variety of mycological media, yet according to Cooney and Emerson (1964), most are unsatisfactory. For example, Cooney and Emerson (1964) noted that nutrient agar was found to favor bacterial growth and was subject to excessive dehydration at high temperature. Through trial and error they concluded that yeast starch agar and yeast glucose agar were the most suitable for thermophilic fungi, and are still the most commonly used media today (Salar & Aneja, 2007).

Isolation

Man-made and/or natural areas of organic decay are the most promising for the growth of thermophilic fungi. Natural substrata may already contain visible fungal growth which can be sub-cultured directly onto laboratory media. However, in most cases a natural substratum does not show any evidence of fungal growth prior to incubation (Cooney & Emerson, 1964). To

greatly increase the yield of growth, Cooney and Emerson (1964) suggested that the substratum be chopped into small, well moistened pieces and incubated in dishes lined with several thicknesses of moist paper toweling. For the initial growth Cooney and Emerson (1964) suggested that incubation temperatures ranging from 45^{o}C and up be implemented. For initial isolation from natural substratum, it was found that temperatures 40^{0}C and lower allow many ubiquitous fungi to grow inhibiting their thermophilic counterparts (Cooney & Emerson, 1964). After substantial growth, spores or active mycelia should be transferred to various laboratory media and incubated at various temperatures to determine the optimal conditions for growth (Cooney & Emerson, 1964).

Taxonomic Identification of Thermophilic Fungi

In 1964 the first comprehensive collection of information regarding the morphology of thermophilic fungi was published by Cooney and Emerson. Here they provided diagrams and pictures of key features used for the identification of thermophilic fungi. Up to that time only 13 species had been identified. As of today there are 50 or so identified fungal thermophiles, 30 of which were discovered in this past decade (Mouchacca, 2000). Many species have been redefined, resulting in changes to much of the original classification implemented by early taxonomic findings (Maheshwari *et al.*, 2000; Mouchacca, 2000). Zygomycetes, Ascomycetes, and Deuteromycetes make up the population of known thermophiles with no reports of true thermophiles from either Myxomycetes or Basidiomycetes (Salar & Aneja, 2007).

The classification of fungi was and is still to this day largely based on morphological features. Vegetative hyphae, sexual and asexual reproduction and color, shape or septation of spores are all characters used for the determination of fungal species (Hall, 1969). The morphological approach of classifying fungal species is a sound and traditional method yet it is

limited (Parmeter, 1965). The presence of suitable diagnostic features are the key to morphological identification, however there have been cases where these are lacking. As described by Barron (1968) the classification of polymorphic fungi is quite difficult since these fungi have the ability to produce one or several spore forms. In some situations suitable morphological features are rare, sporadic, or lacking which places impeding limitations on such methods of classification (Hall, 1969). An unreliable taxonomy of a species creates unnecessary obstacles making a reliable taxonomy ever more important for the successful study of the species (Göker *et al.*, 2009).

With thermophilic fungi that are culturally and morphologically similar, ecological traits such as cardinal temperatures for growth and the ability to degrade particular substrates are essential for proper classification (Salar & Aneja, 2007). The nomenclature for the taxonomy of thermophilic fungi is often confusing and contradicting, as explained by Salar and Aneja (2007), the confusion is caused by scattered taxonomic literature, the description of the same species under different names, and numerous misidentifications.

Due to such challenges, molecular analysis based on DNA sequences is the most reliable method for the determination of various isolated samples (Pan, et al., 2010). However the typing of all known thermophiles is incomplete, therefore the ability to identify samples is often limited to the size of the fungal database. Also, the number of known thermophiles is extremely low and one would expect the possibility of finding new species to be high.

Thermophilic Fungi in Biotechnology

Thermophilic fungi play a necessary role in the degradation of agro-residues, mushroom production, solid waste management, and in the breakdown of plant material (Johri &

Satyanarayana, 1999). Thermophilic fungi have been shown to produce antibacterial and antifungal compounds and have the ability to release volatile molecules which regulate *in situ* growth behavior via fungistatic and auto-inhibition mechanisms (Rode *et al.*,1947; Bai MP, 1966; Prakash, 1984)

The ultimate biotechnological potential of thermophilic fungi lies in their secretory enzymes. These enzymes have the potential to exhibit desirable properties such as high temperature optima and long shelf life (Baerlocher, 2006). Enzymes from thermophilic fungi have been studied for their suitability in bioprocesses and to understand the similarities and differences between enzymes from thermophilic and mesophilic organisms (Maheshwari *et al*., 2000).

In the dairy industry, proteases such as chymosin are used in the industrial preparation of cheese. Traditionally, chymosin was collected from the stomach contents of milk-feeding calves, but this task was difficult and there was an effort to find satisfactory substitutes (Kumar *et al.*, 2010). Arima *et al*. (1968; 1967) found a soil isolate of *Mucor pusillus* that produced a protease with a high ratio of milk clotting to proteolytic activity. *Mucor miehei* was also shown to produce a strong milk clotting protease similar to that of *Mucor pusillus* (Ottesen & Rickert, 1970). Today, there are a variety of thermophilic fungal rennets available for the dairy industry (Garg & Johri, 1999). The discovery of these proteases resulted in an increased effort to isolate other thermophilic fungi for their potential industrial applications (Maheshwari *et al.*, 2000).

Besides proteases, a whole host of enzymes have been isolated from thermophilic fungi. An extracellular lipase was purified from *Humicola lanuginose* and later cloned and expressed in *Aspergillus oryzae* (Arima *et al.*, 1972; Huge-Jensen. B, 1989). The enzyme α-amylase is produced by all species of thermophilic fungi, yet only *Thermomyces lanuginosus* α-amylase has

been characterized (Maheshwari *et al.*, 2000; Arnesen, 1998). Glucoamylases and cellulases have also been reported (Basaveswara *et al.*, 1981; Tosi L., 1993).

The production of xylanases from thermophilic origins is an increasing area of interest in the research community (Maheshwari *et al.*, 2000). Besides cellulose, xylan is the most abundant polysaccharide in nature. The complete digestion of xylan is a complex process requiring the cooperation of multiple hydrolytic enzymes (Cuyversa *et al.* 2011). Thermophilic xylanases are receiving considerable attention due to their ability to aid in the bio-bleaching of paper pulp and for improving the overall digestibility of animal feed (Maheshwari*et al.*, 2000). Laccases, invertases, phytases and a multitude of other enzymes are produced either intra- or extracellularly by thermophilic fungi (Johri & Satyanarayana, 1999; Maheshwari *et al.*, 2000). In view of their potential production of commercially significant enzymes, thermophilic fungi will continue to be examined for more heat and storage stable enzymes.

General Characteristics of Nucleases

Nucleases are enzymes which catalyze the cleavage of the inter-nucleotide bridges, without the release of inorganic phosphate (Privat de Garilhe M. , 1967). These inter-nucleotide bridges are extremely resistant to hydrolysis (Gerlt, 1993). In fact, the halftime for the hydrolysis of the simplest phosphodiester in 1 M NaOH was determined to be roughly 35 years at 35^{o}C (Chin *et al.*, 1989). This stability is necessary for the geometric integrity of molecules such as DNA and RNA, but poses a challenge for enzymes involved in the synthesis, repair and destruction/recycling of such molecules (Gerlt, 1993).

Mechanism

If any reaction involving phosphodiester nucleotides is to occur in living systems, a more efficient process of cleavage must be utilized (Gerlt, 1993). Nucleases provide such a process;

Serpersu *et al.* (1987) found that the hydrolysis of the phosphodiester bonds in DNA was accelerated by a factor of $\geq 10^6$ relative to the rate of spontaneous hydrolysis.

Nucleases cleave phosphodiester bonds via a S_N2 associative mechanism using acid-base catalysis (Gerlt *et al.*, 1983). A base activates the nucleophile by deprotonation; the departing group is then protonated by the acid producing the end products (YangI *et al.*, 1990). The most common nucleophiles used by nucleases are water molecules (Yang *et al.*, 1990). As stated above, a base removes a proton from the nucleophile. In the case of water a reactive hydroxyl group is left , which goes on and attacks one of the two bridging phosphodiester bonds, either 3' or 5' (Gerlt, 1993). Besides water, the hydroxyl groups of both DNA and RNA have been shown to be nucleophiles (Doudna & Cech, 2002; van Gent, Mizuuchi, & Gellert, 1996) along with side chains of Ser, Tyr and His for DNA cleavage (Grindley *et al.*, 2006) and 2' hydroxyls of RNA and free ribonucleotides for RNA cleavage (Raines R. T., 1998).

All nucleases do not conform to the phosphodiester bond cleavage via hydrolysis. A variety of nucleases attack the C-O bond (Gerlt, 1993). These nucleases are responsible for the repair of damaged DNA and dissemble DNA through β-elimination reactions (Bailly & Verly, 1987; Mazumder *et al.*, 1990). DNA strand cleavage occurs on the 3' side of aldehydic abasic sites, which are present in damaged DNA bases (Gerlt, 1993). The abasic sites form when the hydrolysis of the N-glycosylic bond occurs between a base and its associated deoxyribose group (Gerlt, 1993). The damaged bases that are typically targeted by repair nucleases include cyclobutane pyrimidine, oxidized pyrimidines, and oxidized purines (Linn *et al.* 1993).

As stated before, nucleases may attack either from the 3' end or the 5' end of nucleic acid molecules, and some attack from both (Mishra N. C., 1995). The resulting products of this reaction are always 3' or 5' nucleotides, and currently there is no known individual nuclease that

produces both forms of nucleotides. This restriction may be due in part to the difference in the topography of the region surrounding the 3'and 5' carbon of the sugar molecules (Mishra N. C., 1995) .

Site Specificity

A variety of nucleases exhibit site specificity and have the ability to recognize certain sequences of nucleotides (Grindley *et al.*, 2006). These sequences direct the nuclease to make a cut either within or outside these sequences (Mishra N. C., 1995) . For example, a micrococcal nuclease attacks at A/T pairs and cleaves exclusively at NpA and NpT diester bonds (Horz & Altenburger, 1981). Holley (1966) showed that site specific cleavage is a characteristic property of multiple RNases. Some RNases showed specificity, not towards specific sequences, but towards specific size, stero-specificty, and/or secondary structure of the RNA molecule (Penswick & Holley, 1965).

Sugar Specificity

There are three possibilities for the pentose sugar specificity of nucleases; DNA specific (DNase), RNA specific (RNase) and quite a number of nucleases are sugar non-specific and can cleave both RNA and DNA. Non-specific nucleases show no specificity towards the nature of the pentose sugar moiety. Therefore DNA, RNA and other various poly-nucleotides are hydrolyzed, resulting in the formation of oligonucleotides terminated by either a 3' or 5' group (Mishra N. C., 1995). Non-specific nucleases have been shown to have some specificity towards the nature of the linkages, specifically the purine or pyrimidine linkages (Privat de Garilhe M. , 1967). Non-specific nucleases have varying degrees of "blindness to sugar" according to Wechter and Mikulski (1968). They demonstrated that some nucleases like venom exonucleases and mung bean nucleases are totally unspecific towards varying sugar groups; whereas micrococcal

nuclease was unspecific to DNA or RNA but was incapable of attacking arabinose (Wechter & Mikulski, 1968).

Kunitz (1940; 1948) described two nucleases based on sugar specificity, resulting in two different classifications, deoxyribonucleases (DNase) and ribonucleases (RNase). RNases and DNases are sugar specific and therefore have substrate preference, RNA for RNase and DNA for DNase. This substrate preference inhibits their ability to attack other poly-nucleotides lacking the preferable sugar structure.

Strand Specificity

Nucleases may show some specificity towards either double or single strand nucleic acids (Mishra N. C., 1995). With double strand nucleic acids, nucleases can only approach from the outside where external nucleophiles are limited (Yang W. , 2011). Single stranded nucleic acids are much easier for nucleases to attack because bases are not stacked allowing access to the nucleophiles (Yang W. , 2011). Nucleases that cleave double stranded nucleic acids tend to attack at the minor groove (Yang W. , 2011). Sequence specific nucleases tend to attack the major groove (Lee *et al*. , 2005).

Metal Ion Dependent and Independent Nucleases

There are three major classes of nucleases that are divided based on their metal-ion dependence. The groups consist of two-metal-ion catalysis, one-metal-ion catalysis, and metal-ion-independent catalysis nucleases (Yang W. , 2011). Two-metal-ion catalysis nucleases require two metal ions at the active site (Beese & Steitz, 1991). This two-metal-ion catalysis mechanism has been proven to be used by all DNA and RNA polymerases and many nucleases (Steitz, 1998). In all cases, the products are 5'-phosphate and 3'-hydroxyl groups (Yang W. , 2011). Two-metal-

ion nucleases can attack and hydrolyze native DNA with high substrate specificity and efficient product release (Yang W. , 2011; 2006). The ions of choice are Mg^{2+} and Ca^{2+} but other ions such as Mn^{2+} may take the place of Mg^{2+} (Yang, Lee, & Nowotny, 2006; Hopfner *et al.*, 2001).

One-metal-ion catalysis nucleases require only one metal ion and like the two-metal-ion catalysis nuclease can hydrolyze native DNA and produce 5'-phosphate and 3'-hydroxy products. (Yang W. , 2008; 2011). These nucleases show less substrate specificity and discrimination when compared to the two-metal-ion nucleases (Yang W. , 2008). A number of one-metal-ion nucleases are sugar non-specific and demonstrate little sequence specificity (Hsia *et al.,*2005).

Metal-ion-independent nucleases do not require metal ions to catalysis the hydrolysis of nucleic acids, instead they require and form intermediate compounds that aid in this process (Yang W. , 2011). Unlike their metal-ion using relatives, these nucleases do not use water molecules as nucleophiles. This forces metal-ion-independent nucleases to use a multi-step cleavage process for the hydrolysis of phosphodiester bonds (Yang W. , 2011). RNases not requiring metal ions use a 2'-hydroxyl group adjacent to the scissile phosphate as the nucleophile for the formation of their 2'-3' cyclic phosphate intermediates (Yang W. , 2011). DNases use positively charged side chains to align themselves with the scissile phosphate and to stabilize penta-covalent intermediate. The phospho-enzyme intermediates are formed by the hydroxyl groups on the Tyr, Ser or His side chains (Champoux, 2001; Grindley *et al.*, 2006; Sasnauskas *et al.*, 2007).

Mode of Attack

Endonucleases

Nucleases themselves are subdivided into two specific classes, endonucleases and exonucleases. Both cleave phosphodiester bonds, but substrate recognition and product

formation determines if they are endo- or exonucleases (Garilhe, 1967). Endonucleases attack phosphodiester bonds situated in the middle of nucleic chains, forming oligonucleotides and some mononucleotide fragments. One of the most classically researched endonucleases is bovine pancreatic ribonucleases (Privat de Garilhe M. , 1967). This small molecular weight protein was first isolated and studied by Kunitz (1940). Pure pancreatic RNase was found not only to break down RNA, but also polyadenylic acid and polyribose phosphate (Privat de Garilhe M. , 1967).

Georgatsos and Laskowski (1962) purified and studied nucleases found in snake venom, specifically the venom from *Bothrops atrox*. The nucleases demonstrated non-specific nuclease characteristic, attacking both DNA and RNA at the same rate and producing 3'-phosphate terminated nucleotides (Georgatsos & Laskowski, 1962). The preferred bond linkage was determined to be d-Gp-Gp. Following the initial hydrolysis of the desired bonds, the specificity with respect to adjacent bases decreased and the chain length of the substrate became a limiting factor (Georgatsos & Laskowski, 1962).

Endonucleases play an important role in the removal and repair of damaged DNA lesions that occur during our lifetime (Gratchev, 2008). UV radiation is involved in the physical damage of DNA, creating damaged sections of DNA known as lesions (Uchida *et al.*, 2002). It has been estimated that under strong sunlight exposed epidermis cells develop about 40000 damaged sites in one hour (Uchida *et al.*, 2002). The human body has a series of proteins which remove and replaces these damaged sections of DNA. Human XPG protein and ERCC1-XPF are structure specific, sugar selective endonucleases that make necessary cuts around DNA lesions during repair process (O'Donovan *et al.*, 1994). XPG is a 135 kDa protein necessary for one of the most important DNA repair mechanisms known as nucleotide excision repair (NER)

(Gratchev, 2008). NER can eliminate large areas of damaged DNA by removing damaged regions that contain lesions (Gratchev, 2008). XPG makes an incision in the DNA backbone 3'to the lesion near the double and single strand junction and promotes the incision on the 5' side by ERCC1-XPF (Gratchev, 2008). These endonucleases allow for the dissociation of the damaged fragment and the synthesis and ligation of the new DNA strand (Gratchev, 2008).

Exonucleases

Exonucleases also break the phosphodiester bonds holding nucleic acids together but only attack from one end sequentially cleaving mono and/or dinucleotides.

Escherichia coli has 14 DNA exonucleases, all of which play diverse and important roles in DNA metabolism. Exonuclease I has a molecular weight of 70 Kdal (Bachmann & Brooks Low, 1980) and preference for single stranded DNA, attacking from the 3' hydroxyl end and producing 5' mononucleotides (Weiss, 1981). Exonuclease I and Exonuclease II have roles in DNA recombination, with the latter being associated with the proofreading 3'-5' exonucleolytic function of DNA polymerase I (Mishra N. C., 1995).

Exonuclease III is a small monomeric protein (MW 28Kdal) that demonstrates four types of catalytic activity, which include double strand specific 3'-5' exo-deoxyribonuclease activity (Weiss, 1981; Richardson & Kornberg, 1964), RNase activity (Weiss, 1981), 3' phosphatase and AP-endonuclease activity (Weiss, 1981). The versatility of Exonuclease III has allowed for its utilization in generation of primers for DNA synthesis, the identification of AP sites in DNA and distinction from accidentally incorporated ribonucleotides in DNA, and for the mapping of specific protein binding sites on DNA (Mishra N. C., 1995). Viswanathan and Lovett (1999)discovered and characterized a new exonuclease present in *E. coli n*amed exonucleases X.

This nuclease was found to degrade both single and double stranded DNA with 3'-5' specificity. If deleted, the UV sensitivity of the bacterium was found to greatly increase and if over expressed, was capable of repairing UV damaged DNA (Viswanathan & Lovett, 1999).

Fungal Nucleases

Fungal nucleases adhere to the classical definition of nucleases and are either intra- or extracellular in nature. In 1975, Hanking and Anagnostakis examined 10 species of thermophilic fungi for the production of extracellular enzymes. They found that 8 out of the 10 species examined produced extracellular DNase activity and 5 out of 7 produced extracellular RNase activity (Hankin & Anagnostakis, 1975). Two distinct but related fungal nucleases are the secreted single-strand specific endonucleases and the intracellular endo-exonucleases (Fraser & Low, 1993). The extracellular nucleases main function is to scavenge phosphate and nucleosides for cell growth, whereas the endo-exonucleases likely play a role in DNA repair, recombination, and possible DNA replication (Fraser & Low, 1993).

One extensively studied fungal extracellular nuclease is nuclease S1. Nuclease S1 is a heat stable, 32 kDa, endo-exonucleolytic glycoprotein originally isolated from a digestive enzyme preparation from *Aspergilus oryaze* (Shishido & Ando, 1982). The enzyme attacks both single-stranded DNA and RNA, yielding 5'-monophophates and small amounts of dinucleotides (Fraser & Low, 1993; Shishido & Ando, 1982). Double stranded DNA is not hydrolyzed (Shishido & Ando, 1982). The nuclease is inhibited 50% by 2mM phosphate and shows Zn^{2+} dependent activity with optimal activity at 1 mM (Shishido & Ando, 1982; Fraser & Low, 1993). Nuclease S1 has thermal stability with strong nucleolytic activity at 65^0C (Ando, 1966). S1 is inhibited by EDTA, with no activity reported at a 0.1*m*M concentration (Fraser & Low, 1993). Yet, S1 can be

70% reactivated with Zn^{++} and Co^{++} ions but not at all with Mg^{++} and Ca^{++} (Shishido & Ando, 1982).

The secretion of nucleases like S1 sheds light on the main biological role of these enzymes. These enzymes scavenge nucleosides and phosphate necessary for growth. Ultimately, S1 is like nuclease I of many plant species, all of which have similar dependences, pH optima, molecular weights and biological function (Fraser & Low, 1993).

Classification of Ribonucleases

Ribonucleases (RNase) are found in all organisms including animals, plants and microorganisms and were the first nucleases to be described (Mishra N. C., 2002). Ribonuclease was first purified and crystallized from beef pancreas by Kunitz (1940). Ribonucleases can hydrolyze single stranded or double stranded RNA as well as RNA present as a DNA-RNA hybrid (Mishra N. C., 1995). Bovine pancreatic ribonuclease is the most characterized of all nucleases and was the first enzyme to be subjected to sequencing, protein crystallographic, and protein folding studies (Raines R. T., 1998). Currently there are hundreds of known RNases that have been fully or partially characterized, however the most well-known and studied RNase is bovine pancreatic RNase.

Bovine Pancreatic RNase

In 1940, bovine pancreatic RNase (RNase A) was successfully purified and crystallized by Kunitz (Kunitz M. , 1940). When the purified enzyme was subjected to ICR-50 chromatography, five different RNases were found (Plummer & Hirs, 1963). However, all of the RNases were derived from RNase A. It was later found by Salnikow *et al.* (1970) that all five forms of bovine

pancreatic RNase had the same amino acid sequence and differences between them were due to post-translational modifications.

Purified RNase was the first enzymatic tool that was used to distinguish between DNA and RNA (Laskowski, 1982). The discovery that RNase A cleaved RNA after pyrimidine residues further increased the usefulness of the enzyme (Schmidt, et al., 1951). RNase A was also the first chemically synthesized enzyme using solid phase synthesis and solution methods (Gutte & Merrifield, 1971; Yagima & Fujii, 1980). RNase A is small (15 kDa), heat stable nuclease exhibiting endonuclease activity, cleaving single stranded RNA at the 3' end producing 3'-phosphorylated mono- or oligonucleotides (Privat de Garilhe M. , 1967; Mishra N. C., 2002). The optimal temperature for RNase A was determined to be 65 ^{o}C. RNase A activity occurs in the pH range 7 – 8.2, with optimal activity at pH 7.7 (Privat de Garilhe M. , 1967). RNase A exists as a dimer consisting of 124 amino acids with four disulfide bonds (Mishra N. C., 2002). Disruption of these disulfide bonds causes disorientation of the secondary structure as well as rupture of the tertiary configuration, making the four disulfide bonds necessary for the stability of the enzyme.

Currently, RNase A is commonly used to degrade contaminating RNA molecules during recombinant plasmid synthesis and for mapping single nucleotide mismatches in DNA: RNA hybrids (Mishra N. C., 2002).

Classification of Deoxyribonuclease

Deoxyribonucleases (DNases) were first isolated and studied from calf thymus. (Mishra N. C., 1995). DNase was the second enzyme whose amino acid sequence was fully determined (Liao *et al.*, 1973). DNases are nucleases with specificity towards deoxypentose sugar moieties, therefore hydrolyzing phosphodiester bonds in deoxyribonucleic acid. Like all nucleases, DNases

are divided into two groups based on their nucleolytic properties. It was shown by Frankel and Richardson that exonucleases required a free terminus for nucleolytic attack on the phosphodiester bonds in a nucleic acid molecule (Frenkel & Richardson, 1971). Pritchard et al. (1977) showed that a venom exonuclease had the ability to hydrolyze a highly super coiled circular DNA but not relaxed circular DNA. An assortment of DNases show specific specificity towards nucleotide sequence, topology, and damaged segments of DNA (Mishra N. C., 1995). DNases with exonucleolytic properties hydrolyze DNA in either a 5'→3' or 3'→5' direction. DNases with double strand specificity on average tend to only hydrolyze half of the DNA (Linn, 1981). DNases are ubiquitous in nature; bacteria produce a whole host and are the subject of current study, yet the most well characterized deoxyribonucleases are DNase I and DNase II (Linn, 1981).

Deoxyribonuclease I

Bovine pancreatic DNase is a glycoprotein made of 260 amino acids which was first crystallized by Kunitz (1948) and fully characterized by Moore (1981) (Liao *et al.*, 1973). It was also the first deoxyribonuclease to be purified without any ribonuclease contamination (Linn, 1981). DNase I class enzymes have endonucleolytic properties yielding primarily 5'-phosphodi- and oligonucleotide end products (Moore, 1981). DNase I is more widespread than DNase II and is found mostly in the pancreas and digestive tissues (Mishra N. C., 2002). DNase I is prevalent in mammals and bacteria, but not in low eukaryotes or plants (Yang W. , 2011). With native DNA, the initial mode of attack is directed by the enzymes specificity for nucleotide sequences leading to a single stranded break (Mishra N. C., 1995). Following this attack the enzymes losses its specificity and suffers from auto-retardation (Mishra N. C., 2002). DNase I is a two metal ion catalysis dependent nuclease required both Mg^{2+} and Ca^{2+} for optimal activity (Yang W. , 2011).

The hydrolysis products of DNase I were examined by Garilhe and Laskowski (1955) via ion exchange chromatography with an increasing ammonium formate gradient. Chromatography of DNase I enzymatically digested DNA reveled a mixture or mono-, di, tri-, and tetranucleotides. The first peaks eluted by a 0.1 M formate buffer included d-pC and d-pT mononucleotides; they were the only mononucleotides present and made up less than 1% of all nucleotide products (Privat de Garilhe & Laskowski, 1955). Dinucleotides d-pCpC, d-pCpT, and d-pCpA followed in the sequential fractions using a 0.25 M formate buffer (Privat de Garilhe & Laskowski, 1955). The remaining fractions eluted with a 1.0 M formate buffer yielded mixtures of di-, tri-, and tetranucleotides (Privat de Garilhe & Laskowski, 1955). The hydrolysis of the trinucleotide d-ApApTp by DNase I results in the formation of d-ApA and d-pTp (Potter *et al.*, 1958). The observation that the hydrolysis of oligonucleotides by DNase I is more difficult than that of macromolecular DNA was first noted by Fredericq (1961) and later confirmed by Vanecko and Laskowski (1961). In general it can be said that the shorter the fragments of DNA the more difficult it is hydrolyze (Privat de Garilhe M. , 1967).

The mycelia of *Neurospora crassa* and *Aspergillus nidulans* both secrete an Mg^{++}, Mn^{++}, or Ca^{++} dependent DNase I-like endonuclease (Fraser & Low, 1993). DNase A, nuclease from *Neurospora,* shows no strand specificity and is depressed 200-fold when grown on a phosphate free medium (Fraser M. , 1979; Kafer & Witchell, 1984). This enzyme degrades native DNA, helical RNA, and ordered synthetic polynucleotides producing 5'-phospho-terminated products (Shishido & Ando, 1982). The nuclease from *Aspergillus*, known as DNase 4, is a double strand specific endonuclease (Campbell & Winder, 1983). Both DNase A and DNase 4 have molecular masses between 60-65 kDa and generate the same 5'-phospho-terminated products (Fraser & Low, 1993).

Deoxyribonuclease II

DNase II, also known as acid DNase, is a 38 kDa glycoprotein that has been purified to homogeneity and is very well characterized (Mishra N. C., 2002; Laskowski, 1967). Optimum enzymatic activity is situated around a pH of 4.5 (Privat de Garilhe M. , 1967). DNase II, unlike DNase I, is a metal-ion-independent nuclease, which can catalyze cleavage reaction in the absence of metal ions (Yang W. , 2011). High concentrations of divalent cations retard DNase II activity, with optimum Mg^{2+} concentrations centered on 0.002 – 0.0025 M (Oth *et al.*, 1958). DNase II attacks in two endonucleolytic phases with a rapid initial phase followed by a very slow second phase (Mishra N. C., 1995). First, the enzyme rapidly makes a double strand break in one hit (Mishra N. C., 2002). The second phase however requires two hits to cause a double strand break (Mishra N. C., 2002). Like DNaseI, DNase II is subject to auto-retardation. The cleavage products of DNase II are 5'-phosphate and 3'-hydroxyl (Yang W. , 2011). Like other metal-ion-independent DNases the positively charges side chains are used to guide and align the enzyme to the scissile phosphate along with stabilizing the intermediate (Yang W. , 2011). DNase II found in the lysosomes of macrophages plays an important role in the breakdown of DNA following the engulfment of apoptotic cells (Nagata, 2007). Without DNase II, the residual DNA fragments would eventually lead to an autoimmune disease (Yang W. , 2011).

Catalytic Properties

Divalent Metal Ions

The conformational activation and activity of DNase I and variety of other DNases are dependent upon the presence of certain divalent metal ions which may have more than one function (Price, 1975). It was shown by Kunitz (1950) and later confirmed by Wiberg (1958) and Shack and Byum (1964) that the concentration of Mg^{2+} needed for activity is proportional to the

DNA concentration. DNase I requires the presence of divalent cations such as Mg^{2+} or Mn^{2+} which are crucial to the structural integrity of the enzyme (Dupureur, 2008). Calcium binding to the DNase I is required to reform the disulfide bonds in reduced DNase I and for the protection for proteolytic enzymes such as chymotrypsin and trypsin (Price *et al.*, 1969; Hugli T. E., 1973).

Depending on the presence of certain ions, DNase I either makes a double stranded break or a single stranded nick (Mishra N. C., 2002). Double stranded scission can be expected in the presence of Mg^{2+} and Ca^{2+} which is when the affinity for the substrate is at its maximum (Moore, 1981). Single strand scissions and changes in specificity occur when the Ca^{2+} concentration is subpar (Moore, 1981). The same enzyme is further inhibited by anions such as fluoride citrate, arsenate, and borate (Privat de Garilhe M. , 1967). Becking and Hurst (1963) demonstrated that the hydrolysis of DNA by DNase I in the presence of Mg^{2+} resulted in a 5% and 49% increase of mononucleotide and dinucleotide formation respectively. Activity of the enzyme is highest with a combination of Mg^{2+} and Ca^{2+} (Price, 1975; Pan & Lazarus, 1999), while combinations of Mg^{2+} with Ba^{2+}, or Sr^{2+} ions show slightly lower activity (Shack & Bynum, 1964).

Jones (1996) suggested that the metal ions are essential for the phosphodiester cleavage mechanism. The bivalent metal ions serve two roles; first Ca^{2+} must be bound to the enzyme and second, Mg^{2+} must be bound to the substrate (Moore, 1981). It was found by Price (1972) that two Ca^{2+} bound with an average K_d of 1.4×10^{-5} and with Mg^{2+} the K_d for two sites was 2.3×10^{-4}. Through further observation Price (1975) noticed that with reagents purified to reduce Ca^{2+} levels DNase in the presence of Mg^{2+} is about 99.5% inactive. A Ca^{2+} concentration of 0.1 mM yields the highest activity where concentrations as high as 1.0 mM show inhibitory affects (Moore, 1981). The concentration of Ca^{2+} in bovine pancreatic juice is 4×10^{-4} to 3×10^{-3}

M which is in range that can keep DNase functional in its physiological environment (Frouin & Gerard, 1912).

Inhibition of DNase

DNase I is a nucleolytic enzyme that requires divalent metal ions for activation, therefore the removal of such ions will inhibit enzyme function. It was shown by Price (1975) that purified enzyme reagents lacking Ca^{2+} resulted in very low levels of activity. Therefore the addition of any chelating agent such as EGTA and/or EDTA would greatly inhibit enzyme activity. In the presence of a very low concentration of EGTA (0.1mM) DNase I activity was undetectable (Price, 1975). Anions also inhibit DNase I activity based on their reactions with required ions (Garilhe, 1967). Festy et al (1965) confirmed that a 10^{-4} M concentration of beryllium ions could inhibit DNase I activity up to 50%. DNase I is inactivated by iodoacetate at pH 7.2 in the presence of either Mn^{2+} or Cu^{2+} ions (Catley *et al.*, 1969). Nitration and the addition of methanesulfonyl chloride at pH 5 both inactivate DNase I (Hugli & Stein, 1971; Poulos & Price, 1974). Eron and McAuslan (1966) demonstrated that both actinomycin D and ethidium bromide inhibited activity. DNase I is strongly inhibited by actin (Lazarides & Lindberg, 1974). The inhibitory effect results from DNase I depolymerizing the filamentous actin and forming a stable complex of 1 mole of DNase I with 1 mole of globular actin (Hitchcock *et al.*, 1976; Mannherz, Leigh *et al.*, 1975).

DNase II is strongly inhibited by Mg^{2+} at concentration higher than 1m*M* (Mishra N. C., 1995). Purified DNase II is inhibited by RNA (Mishra N. C., 1995). Like many other biological proteins, both types of DNase are susceptible to proteolytic enzymes such as trypsin, chymotrypsin, ficin, and papain (Maxwell *et al.*, 1963).

Assay and Purification Techniques

Challenge of DNase Studies

There are numerous assays for DNase activity. The substrate for these assays is native DNA, since there is no uniform synthetic substrate available (Laskowski, 1966). A major problem associated with the study of DNases is the assay. With all assay techniques problems arise in obtaining the specific substrate in sufficient amounts, identifying the proper products, and quantitating the product with sufficient specificity and sensitivity (Linn, 1981). Numerous assays for DNase have been suggested and utilized to try to limit these problems. One problem in particular is the phenomenon of auto-retardation, but since the substrate is native DNA this problem cannot be avoided (Laskowski, 1966). All methods, not only suffer from this persevering phenomena but also from their own limitations. Assay procedures are the major limiting factor in studying DNases (Linn, 1981).

With all DNase assays the effect measured is not constant with each enzymatic attack (Linn, 1981). This inconstancy is most obvious with older assay techniques such as the viscosimetric and spectrophotometric methods (Laskowski, 1966). With the viscosimetric method, only double strand breaks matter and with the spectrophotometric methods, the location of the cleaved bond and the nature of the adjacent bases all have a strong influence on the measured effect (Laskowski, 1966).

Nonspecific, highly active DNases are easily detected and studied by the degradation of large polynucleotides monitored by radioactivity, UV absorbance and/or viscosity (Linn, 1981). However, these methods did not cater to the more sugar and sequence specific DNases. With the advent of utilization of circular DNA with DNase studies, endonucleases could be monitored

without interference by exonucleases, but more importantly as little as one phosphodiester bond cleavage per molecule could be detected (Linn, 1981).

The study of DNases involved in the repair and recombination are often limited in regards to the use of an appropriate substrate (Linn *et al.*, 1993). The limited or unavailability of substrates with one particular type of well characterized damaged DNA limits analysis (Linn, 1981). For example, any damaged DNA present in the substrate forms too many potential substrates and the use of single strand specific assays limit the credibility of the enzyme function (Linn, 1981). DNases involved with recombination often require substrates that are currently unknown or are so rare that it must be quantitated by techniques that are often too cumbersome to perform on a routine basis (Linn, 1981).

Various Assay Techniques

As stated before, there are a variety of techniques available for the analysis of DNase activity all of which can be broken down into seven groups: 1) the measure of the release of protons, 2) physicochemical methods, 3) changes in affinity for dyes, 4) products of depolymerization, 5) biological activity, 6) histochemical methods, and 7) radioactive substrates (Kurnick N. B., 1962; Laskowski, 1966). The simplest method for detection of DNase activity involves the diffusion of DNase throughout agar gel. DNA suspended within an agar gel matrix will be hydrolyzed *in situ* during the diffusion of the enzyme. Zones of clearing are then measured; the diameter of the diffusion zone is directly proportional to the logarithm of the enzyme concentration, therefore allowing a semi-quantitative study of the activity of the DNase (Privat de Garilhe M. , 1967). A dye, usually Methyl Green, is used to visualize the extent of hydrolysis. Methyl green intercalates between the stacked bases of native DNA with approximately one dye molecule binding per 5 base pairs resulting in an enhanced green

coloration (Kurnick & Mirsky, 1950; Kurozumi, Kurihara, Hachimori, & Shibata, 1963). Once the DNA is hydrolyzed the overall absorbance decreases. Methyl green is freed from DNA upon hydrolysis and spontaneously decolorizes due to the tautomerization of its structure (Sinicropi *et al.,* 1994).

Two classical methods often used for the study of DNases are the viscosimetric and spectrophotometric methods. The viscosimetric method is based on the decrease in the viscosity of a nucleic acid solution upon its digestion with nucleases (Privat de Garilhe M. , 1967). The spectrophotometric methods measure the changes in hyperchromacity of digested nucleic acids (Mishra N. C., 1995). The viscosimetric method first described by Laskowski and Seidel (1945), is a sensitive method and has the advantage of being able to examine solutions that are strongly absorbing in the ultraviolet. However, this method can only be used for the initial stages of the reaction and is for mostly crude sample systems (Laskowski, 1966).

A typical protocol involves the enzyme sample to be added to an Oswald viscometer already containing a certain volume of substrate. The time it takes for the enzyme-substrate mixture to flow between two designated points is recorded at sequential time intervals. This method is at best a semi quantitative one for the study of purified enzyme, or the study of levels of DNase activity in different tissues or microbial cultures (Laskowski, 1966). However, the use of this method for the rapid characterization of a nuclease is helpful since endonucleases show high viscosimetric values, whereas exonucleases can hardly be detected (Williams *et al.*, 1961).

First proposed by Kunitz (1950), the spectrophotometric analysis of DNase is one of the most commonly used methods. This assay is based on the rise in optical density at 260 nm resulting from the hydrolysis of DNA (Privat de Garilhe M. , 1967). The actual measured affect results from the modification of the DNA spatial conformation, known as the hyperchromic

effect (Privat de Garilhe M. , 1967). This method is recommended for the evaluation of purified samples however it is almost useless for rigorous kinetic studies since the effect measured varies with the substrate, the stage of the reaction and the type of bond cleavage (Laskowski, 1966).

Purification Techniques

Kunitz (1950) described the initial isolation of DNase I from beef pancreas. Preliminary purification was carried out by separating a protein fraction from fresh beef pancreas by means of an acid extraction. Fractionation by the use of ammonium sulfate and ethyl alcohol further removed inert proteins resulting in a purified crystalline DNase.

As with any purification system, the method of purification is dependent on the characteristics of the protein. Variables such as temperature, pH, and buffer ionic strength all have to be taken into consideration when designing a purification scheme. DNases are isolated and purified using classical techniques (Linn, 1981). Preliminary purification, especially if from a biological source such as beef pancrease, is necessary for the removal of undesirable contaminants (Kunitz M. , 1950). Such purification includes acid wash membrane filtration and crude 0.2 saturation ammonium sulfate precipitation (Kunitz M. , 1950). Dialysis is a technique often used to lower the ionic strength of a sample. This in turn helps retard the precipitation of protein during concentration and prepares samples for more advance separation methods such as ion exchange and affinity chromatography (Cooper, The Tools of Biochemistry, 1977).

Affinity, size exclusion, and ion exchange are types of chromatography regularly used in the purification of nucleases. In 1994 Ito *et al.* purified a fungal nuclease using a combination of ion exchange and size exclusion chromatography. A DNase contaminant of *Aspergillus oryzae* prepared α-amylase was purified 1550-fold using a combination of acetone precipitation and ion

exchange chromatography (Rushizky & Whitlock, 1977). An alkaline DNase was purified from testes of the crab *Neptunus astatus* by ammonium sulfate precipitation and size exclusion chromatography (Georgatosos, 1965).

The purification of an enzyme allows for the study of its catalytic activities and its response to regulatory molecules that raise or lower its overall activity (Kornberg, 1990). It is necessary to achieve the highest level of purity possible to ensure accurate and reliable enzyme characterization.

CHAPTER 2
MATERIALS AND METHODS

Fungal Methods

Culture Isolation

Cultures were isolated from local farms (Amherst, MA), compost and horse manure piles. Care was taken to ensure that samples were collected from the center or as close to the center of the piles. Compost was allowed to incubate at 55^{o}C for three days. Following incubation, the compost was placed on yeast protein soluble starch (YpSs) agar plates and allowed to incubate at 45^{o}C and 55^{o}C. To limit bacterial contamination, the initial plates used for isolation contained 10 ppm of each following antibiotics: polymyxin B, penicillin G, ampicillin, and streptomycin. Each day after the initial inoculation, the plates were examined for fungal growth under a dissecting microscope. Any visible growth was transferred to YpSs plates and incubated at 45^{o}C and 55^{o}C until substantial visible growth was observed. Each fungal sample was continually plated until isolation was achieved. Isolated cultures were stored on YpSs slants.

Initial DNase Screening

Isolated cultures were plated on yeast protein soluble starch DNase test agar (DYpSs) and incubated at 45^{o}C and 55^{o}C for seven days. Following the incubation period, each plate was examined for a zone of clearing around the fungal colony. Fungi which exhibited DNase activity were surrounded by a clear zone due to the release and de-coloration of the indicator dye. Samples with zones were transferred to DYpSs plates and incubated at 32^{o}C, 37^{o}C, 40^{o}C, 45^{o}C, and 55^{o}C to determine optimal growth and enzymatic temperature. Cultures not displaying any activity were placed in storage.

Preparation of Sample for Enzymatic Study

Isolated cultures were transferred to Fernbach flasks containing 500 mL of YpSs broth. Each flask was placed on a shaker and incubated at 55^{o}C and 115 RPMs for three days. After three days the flask was allowed to incubate statically for another 4 days. Cell mass was removed from the sample by vacuum filtration through coarse filter paper (Fisherbrand Filter Paper P8). All collected cell mass was washed with 250 ml of 0.1M ammonium acetate to remove any trace nutrients and dried for two days at 55^{o}C. The filtrate was filtered under vacuum through medium (Fisherbrand Filter Paper P5), and fine (Fisherbrand Filter Paper P2) filter paper. The filtered sample represented the crude enzyme. To inhibit any bacterial and/or fungal growth 0.02% sodium azide was added to the crude enzyme sample. Dried cell mass was weighed. All filtered samples were kept at ambient temperature.

Enzyme Assays

Acid Soluble Assay for Enzyme Activity

DNase and RNase activity was determined by measuring acid soluble nucleic acids. The method used in this study was a modified version of Eaves and Jeffries (1963). Enzyme sample (0.75 mL) was added to 0.75 mL of substrate (Fish sperm DNA, USB Lot #126531, or yeast RNA; 1mg/ml (Sigma R6625 Lot# 110M1168V), 10 µmol $MgSO_4$ in 0.1 M imidazole, pH 7) and incubated in a water bath set to the desired temperature. The reaction was stopped by adding 0.5 mL of uranylacetate-perchloric acid reagent (0.25% uranylacetate in 10% perchloric acid). Reaction tubes were cooled in an ice bath for 15 min. The mixture was diluted with 2.0 mL of deionized water and the precipitate removed by centrifugation at 13,400 rpm for 5 min at ambient temperature. The supernatant was diluted as required and the absorption at 260nm was measured against a reagent blank prepared by adding the uranylacetate-perchloric acid

reagent to the substrate prior to the addition of the enzyme. One unit of enzyme activity is defined as an increase in absorbance of 0.05 units in a cuvette of 1 cm light path at 260nm.

Spectrophotometric Assay of DNase Activity

The method used for this study was a modified version of the one used by Wang and Levin (2010). Approximately 2.25mL of substrate (40 µg/mL fish sperm DNA (USB Lot #126531), 10 µmol $MgSO_4$ in 0.1 M imidazole, pH 7) was added to a cuvette (1 cm path length) and placed into a temperature controlled cuvette holder equilibrated to the desired temperature. Partially purified enzyme (0.75mL) was added and mixed with a cuvette mixing device, for a total volume of 3 mL. The increase in absorbance at 260nm was recorded. The blank consisted of 2.25 mL of substrate solution and 0.75 mL of deionized water.

Spectrophotometric Assay of RNase Activity

The method used for this study was a modified version of the one used by Kunitz (1946). Approximately 2.25 mL of substrate (yeast RNA; 1mg/ml (Sigma R6625 Lot# 110M1168V), 10 µmol $MgSO_4$ in 0.1 M imidazole, pH 7) was added to a cuvette (1 cm path length) and placed into a temperature controlled cuvette holder and equilibrated to the desired temperature. Partially purified enzyme (0.75mL) was added and mixed with a cuvette mixing device, for a total volume of 3 mL. The decrease in absorbance at 300nm was recorded. The blank consisted of 2.25 mL of substrate solution and 0.75 mL of deionized water. The recorded absorbance was then converted to $\log(E - E_f) + 10$ where E = A_{300} and E_f = A_{300} after 30 min of digestion.

Enzyme Destruction Assay

Two water baths were used for this assay, one set to 55° C and another set to the desired temperature (65 and 68 ^{o}C for DNase and 90 and 95 ^{o}C for RNase). Enzyme sample was placed in the water bath set to the desired temperature and allowed to equilibrate. Once the

enzyme preparation reached the desired temperature, enzyme (0.75 mL) was removed and added to 0.75 mL of substrate (Fish sperm DNA, USB Lot #126531, or yeast RNA; 1mg/ml (Sigma R6625 Lot# 110M1168V), 10 µmol $MgSO_4$ in 0.1 M imidazole, pH 7) equilibrated at 55° C. In the case of DNase, the sample was allowed to incubate for 1 hour; for RNase the incubation time was 10 min. Enzyme sample was tested at various time intervals during the incubation at the desired temperature.

Protein Determination

Protein was determined by a modified version of the Lowry method and by measuring absorbance at 280nm (Lowry *et al.*, 1951). For the modified Lowry method 0.5 mL of 1% cupric sulfate•5 H_2O was added to 0.5 mL of 2% sodium tartrate and mixed with 50 mL of 2% sodium carbonate (reagent A). To determine the protein content 0.5 mL of sample was added to 5 mL of reagent A and allowed to incubate for 15 min at 45^{o}C. Following incubation, 0.5 mL of 1 N Folin-Ciocalteu's reagent (Sigma Lot# 21K36) was added. Care was taken to ensure that the sample was being mixed when the Folin-Ciocalteu's reagent was added. The sample was allowed to sit for 30 min. The absorbance was measured at 750 nm. The blank was generated by substituting the sample with deionized water. The standard curve can be found in the appendix as Figure27.

Concentration Methods

Dialysis

Dialysis of the crude sample was performed against deionized water for 12 hrs. Dialysis tubing (Fisherbrand #21-152-5 Lot#9403) with a flat with of 40mm and a molecular weight cutoff of 6-8 kDa was used.

Ultrafiltration Membrane Concentration

Crude enzyme sample was concentrated using a pressure cell (Amicon© 500mL pressure cell) and 10 kDa regenerated cellulose membrane (Millipore© Utrafiltration YM10 Dia. 76mm, Lot # COSA17530). Prior to sample filtration, 200 mL of distilled water followed by 100mL of 1% (w/v) bovine serum albumin solution was passed through the pressure cell/membrane apparatus. This was to ensure that no protein from the sample would bind to the membrane. The resulting sample was designated the concentrated enzyme sample.

Purification Methods

Sephadex G-50 Column Chromatography

Sephadex G-50 (Pharmacia Fine Chemicals Lot# 2638) was hydrated in deionized water for 3 hours at 100^{o}C prior to loading the column. A 45 cm x 2.5cm glass column was used. A 34 cm long column of Sephadex G-50 was equilibrated for 24 hours with a pH 7, 0.5 M imidazole buffer containing 0.02% sodium azide. A portion (1 mL) of concentrated sample was loaded on the bottom of the gel bed and eluted ascendingly at a rate of 2 mL/min (120 mL/hr) in a 2 - 5°C chromatography refrigerator. Fractions (6.6 mL) were collected using a Gilson FC 203B fraction collector.

Ceramic Hydroxyapatite Chromatography

Ceramic hydroxyapatite (Bio-Rad) was suspended in deionized water. A 45 cm x 2.5cm glass column was used and loaded forming a 9 cm long column. The column was equilibrated for 24 hours with buffer containing 0.5 M imidazole, 5 mM sodium phosphate and 0.02% sodium azide at pH 7. Fractions (6.6 mL each) with the highest specific activity from ten Sephadex G-50 runs were pooled and concentrated down to 10 mL by ultrafiltration membrane concentration. The concentrated sample was applied to the bottom of the column and eluted ascendingly with

a sodium phosphate gradient (0 to 0.5M) in a pH 7, 0.5 M imidazole buffer containing 0.02% sodium azide with a flow rate of 0.8 mL/min (48 mL/hr) in a 2 - 5°C chromatography refrigerator. Fractions (6.6mL) were collected using a Gilson FC 203B fraction collector. The column was regenerated by washing with 3-5 column volumes of buffer containing 0.5 M imidazole and 0.5M sodium phosphate at pH 7. The rate of the elution gradient was determined using crystal violet and measuring the increase in absorbance at 600 nm. The concentration gradient can be found in the appendix under Figure 28.

Membrane Purification

A 47 mm, 0.2 µm FP-Vericel membrane (Pall Life Sciences P/N 66477 Lot# T11779) was rinsed through a Millipore© membrane filtration unit, under vacuum with 200 mL of deionized water. 1.0 g of DNA (Origin: salmon sperm, USB Lot #126531) was dissolved in 50 mL of 2x SSC buffer (1.75% sodium chloride, 0.88% sodium citrate, adjusted to pH 7) and boiled for one minute. The boiled solution was placed in ice and cooled to 10^0 C. Once at the desired temperature the rinsed membrane was added to the solution and placed in an ice bucket. The ice bucket was placed on a shaker at 75 RPMs for 60 min at ambient temperature. After 60 min the membrane was removed, rinsed with deionized water and dried using a stream of hot air.

To purify the nucleases, 10 mL of sample from four Sephadex G-50 column runs was chilled to 5^0 C and place in an ice bucket on a shaker set to 50 RPMs in a 2° C refrigerator. The DNA coated membrane was added to the chilled sample and removed after 3 min. The membrane was then removed, rinsed quickly with deionized water and transferred to another beaker on ice containing a 0.5% NaCl and 0.5 M imidazole (pH 7) buffer in a 2° C refrigerator. The buffer was also chilled to 5°C prior to elution.

Molecular Weight Determination via Sephadex G-75 Chromatography

The molecular weights of the purified nucleases were determined using a column of Sephadex G-75 (Pharmacia Fine Chemicals Lot# 8591) with a gel bed of 2.5 x 30 cm and a pH 7, 0.5 M imidazole buffer containing 0.02% sodium azide. The flow rate for the column was 0.8 mL /min (48 mL/hr). The K_{av} values of enzymes with known molecular weights were used to determine the molecular weight of the purified nucleases from a standard curve. Highly purified ribonuclease A (13,700 Da), α-chymotrypsinogen (25,000 Da), α-amylase (51,000 Da), and enolase (82,000 Da) as well as Blue Dextran 2,000 were used to generate the standard curve.

Electrophoresis

Sample Purity

Electrophoresis was carried out in a vertical Bio-Rad Mini-Protean® system using a Tris/Glycine/SDS buffer (Bio-Rad). The sample was diluted 1:1 with a Lamemmli buffer (Bio-Rad) /β-mercaptoethanol (Fisher Scientific Lot# 940723) solution. The solution was 950 µL Lamemmli buffer and 50 µL β-mercaptoethanol. The mixture was heated for 5 min at 99^{o}C in a thermal cycler (Techne Model FTGENE5D). Protein was separated using 7.5% and 4-15% gradient Mini-Protean® TGX^{TM} 30µL well precast gels (Bio-Rad). The gels were loaded with 30 µL of sample and run at 155 V for 45 min. Gels were placed on a shaker set to low speed and stained using 50 mL of Acqua Stain (Bulldog Bio Lot# 009C) for 60 min. The bands were photographed with a PowerShot G10 Digital Canon Camera equipped with an orange filter lens.

Determining Mode of Attack

The method in which the nucleases attacked the substrate was examined using agarose gel electrophoresis. Partially purified enzyme (0.75 mL) was added to 0.75 mL of the appropriate substrate (Fish sperm DNA, USB Lot #126531, or yeast RNA; 1mg/ml (Sigma R6625 Lot#

110M1168V), 10 µmol $MgSO_4$ in 0.1 M imidazole, pH 7) and incubated in a water bath set to 55° C. For DNase activity the time intervals were 0, 30, 60, and 90 min and 0, 10, 20, 30 min for RNase activity. The reactions were stopped by adding 50 mM EDTA to the reaction tubes followed by placing them in an ice bath.

The degradation products were resolved on 0.3% agarose gels (DNA grade agarose, (cat no. BP164-100, Fisher Scientific) in TAE buffer (40 mM Tris base, 40 mM acetic acid, 1 mM EDTA) at 100 V for 45 min. 20 µL of digested substrate was added to the agarose gel wells. Following electrophoresis the gels were stained with ethidium bromide (0.2 µg/mL) for 30 min. The bands were visualized at 302 nm with a transilluminator (Spectronics Corp., NY, USA) and photographed with a PowerShot G10 Digital Canon Camera equipped with a green filter lens.

Relative fluorescence was determined using the NIH photo analyzing program Image J. Average values were generated from triplicate gels.

CHAPTER 3
RESULTS AND DISCUSSION

Development of a Novel Affinity Membrane Purification System for Deoxyribonuclease

Development

The concept for a membrane based affinity purification system stemmed from the idea of affinity chromatography and its simple yet powerful purification method. Using traditional non-affinity systems, such as size exclusion of ion/cation exchange chromatography, multiple step processes are needed to purify the target enzyme. This is due to the way in which the enzymes are separated by those specific techniques. Size and ion/cation exchange chromatography are based on small differences between proteins that often demonstrate low specificity towards the desired protein (Cooper, 1977). Affinity chromatography does not suffer from this; in fact affinity chromatography is successful because it exploits the biological function of the enzyme by using the enzymes nature affinity for a specific substrate.

In regards to the purification of deoxyribonuclease (DNase), affinity chromatography systems have been used with great success. For example, DNA covalently bound to agarose and carboxymethyl cellulose has been used for the purification of DNase and other DNA binding enzymes (Bautz & Dunn, 1971; Tanaka, et al., 1980; Chockalingam, Jurado, & Jarrett, 2001). The main drawback to these methods is the preparing, packing, and equilibration of columns which in some cases may take multiple days to complete. This is why the idea of using a relatively simple, column-free affinity purification system is so attractive.

Dubitsky and Perreault (2010) demonstrated that single stranded DNA can hydrophobic bind to unmodified polyvinylidene fluoride (PVDF) membranes. When a nucleic acid makes contact with PVDF membranes interactions between the hydrophobic regions of the membrane and the molecule occur (Dubitsky & Perreault, 2010). The group was able to bind over 1000 ng of DNA per cm of membrane. The bond strengths between the nucleic acids and membrane are very high and are a direct result of interactions between the hydrophobic secondary structures of the nucleic acids and the hydrophobic membrane (Dubitsky & Perreault, 2010). The affinity between deoxyribonuclease and DNA alludes to the possibility of a DNA coated membrane purification system.

To purify a deoxyribonuclease, a DNA coated membrane would be placed into a partially purified sample under refrigerated conditions. In addition to refrigeration, the process would occur on ice with EDTA to help minimize the breakdown of bound DNA. After a certain period of time, the membrane would be removed from the sample and placed into an elution buffer containing NaCl, effectively separating the deoxyribonuclease from the miscellaneous protein found in the original sample. A visual representation of the process can be seen in Diagram 1. To test this proposed method, the purification of commercial deoxyribonuclease I from a solution of bovine serum albumin was attempted.

Separation of DNase I from bovine serum albumin

Commercial grade DNase I (Sigma Analytical, Lot# 070M7032V) and bovine serum albumin were used to test the effectiveness of the proposed affinity membrane purification system. To produce the single stranded DNA, 1.0 g of DNA (Origin: fish sperm, USB Lot #126531) was dissolved in 50 mL of 2x SSC buffer and boiled for one minute. The complete process of coating the membranes with the single stranded DNA can be found in the materials and methods

section. 2 mg of BSA and 0.5 mg of DNase I were dissolved in 10 ml of buffer (0.05 M imidazole, 0.1 M EDTA, adjusted to pH 7) and placed on ice in refrigerated conditions (2-5^{o}C). The test solution was allowed to equilibrate for 15 min. An elution buffer containing 0.05M imidazole and 0.5% NaCl (adjusted to pH 7) was also placed on ice in refrigerated conditions (2-5^{o} C) and allowed to equilibrate for 15 min. Both the sample and elution buffer were on a shaker set to 75 RPMs.

Once the sample and elution buffer were equilibrated a DNA coated PVDF membrane was allowed to soak in the sample solution for 3 min while shaking at 75 RPMs. After the allotted time, the membrane was removed, rinsed with deionized water and placed into the elution buffer for three min. Following the elution process the membrane was rinsed with deionized water and discarded. Ten DNA coated PVDF membranes were used to purify the test sample. The purified sample was dialyzed prior to analysis.

The results of the purification can be seen in Table 1. The DNase I was successfully separated from the BSA resulting in a 6-fold increase in specific activity with 80% enzyme recovery. After membrane purification, the final protein content was 0.31 mg, which was an 81% decrease when compared to the original BSA/DNase I sample.

Table 1

Overall purification of the commercial DNase I using affinity membrane purification [a]

Fraction	Vol (mL)	Protein Content (mg)	Total activity (U)	Sp act (U/mg)	Recovery (%)	Increase in sp act
BSA/DNase I	10.0	2.35	43.6	18.6	100	1
Membrane Purification	10.0	0.31	34.83	112.3	80	6

[a] One unit of enzyme activity is defined as an increase in absorbance of 0.05 units in a cuvette of 1 cm path length at 260nm

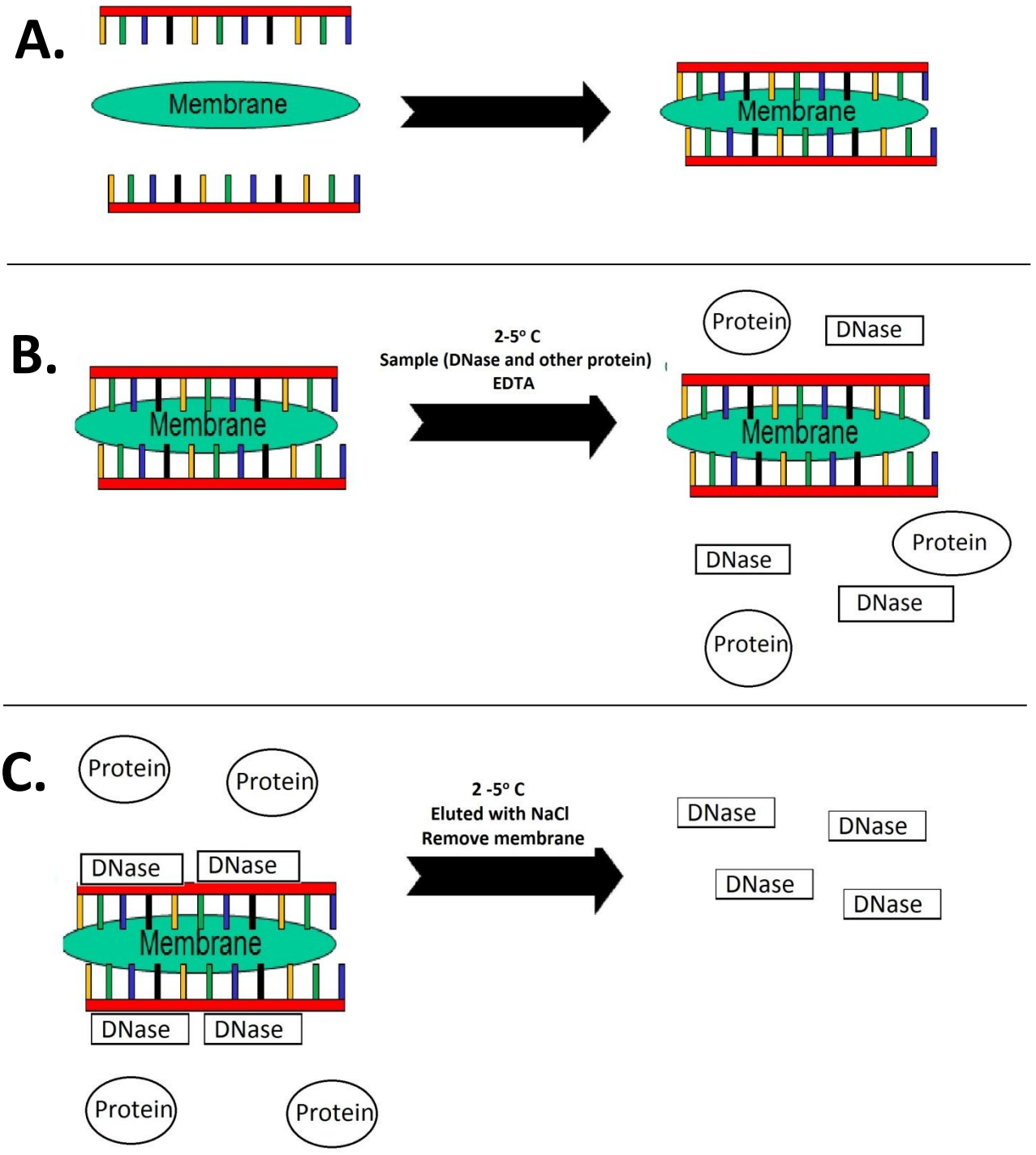


Diagram 1 – A diagrammatic representation of the affinity membrane purification process. A). Illustrates the binding of single stranded DNA to the PVDF membrane. B). Shows the addition of the membrane to the partially purified system under the indicated conditions. C). Illustrates the binding of the DNase to the DNA coated membrane followed by the elution process and separation of the DNase from extraneous protein.

Enzyme activity was measured using the acid soluble assay with a 10 minute digestion time at 25° C. The time allowed for enzyme-membrane binding was 3 min which was determined to be sufficient for the purification process. Other time intervals were tried (5, 10, and 15 min); however there was no significant difference in purification. Various NaCl concentrations were also tested (0.5%, 1%, 2%, 3% and 5%) but all enzyme activity was successfully removed from the membrane with only 0.5% (Figure 1.1).

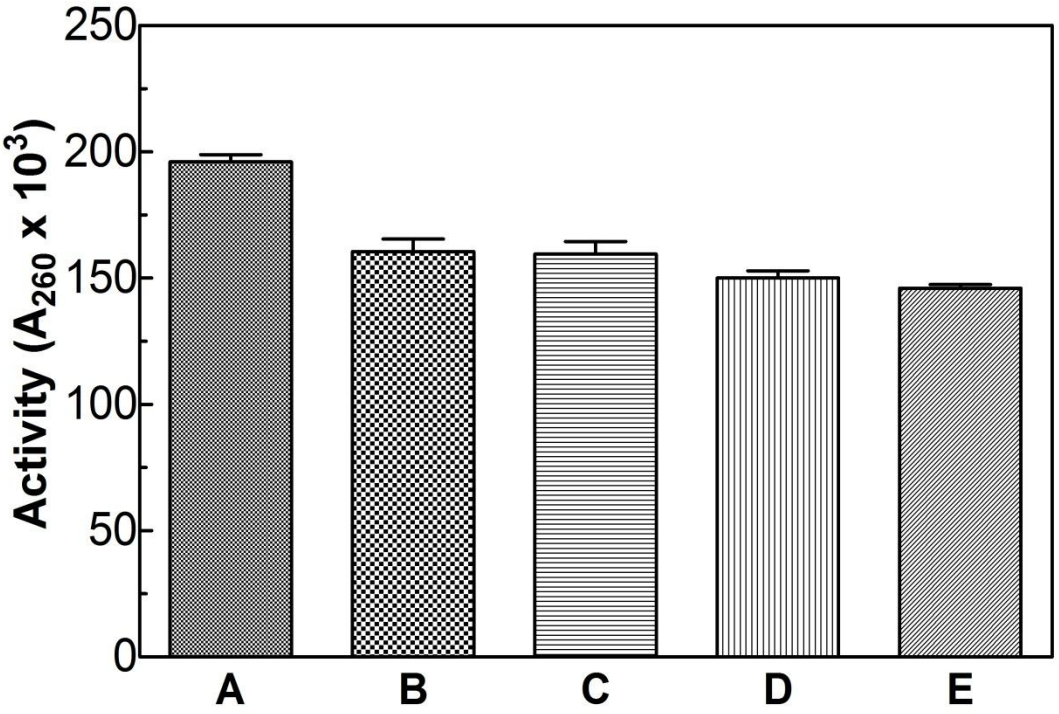


Fig. 1.1 – Effect of salt concentration on elution of bound DNase I from the DNA coated membrane; A) DNaseI/BSA sample, B) purified sample (0.5% NaCl), C) purified sample (1.0% NaCl), D) purified sample (3.0% NaCl), and E) purified sample (5.0% NaCl). There was no significant difference between the four salt concentrations tested. Using this method about 80% of the original activity was recovered for both 0.5% and 1.0%. A slight, but not significant decrease in activity was noted at both 3.0% (P_{valie} = 0.1212) and 5.0% (P_{value} = 0.0576) when compared to both 0.5% and 1.0% concentrations using an unpaired t-test with a 95% confidence interval. Enzyme activity was assessed using the acid soluble assay with a digestion time of 10 min at 25° C. All tests were performed in triplicate.

A slight, but not significant decrease in enzyme activity was noted at both of the high salt concentrations (3.0 and 5.0%). Total enzyme recovery for 3.0% and 5.0% NaCl was only 76% and 74% respectively. The decrease in activity may have been due to partial denaturation of DNase I as a result of the elevated salt content.

Enzyme purity was assessed using 4-15% gradient polyacrylamide gels. The purification of the DNase I using the affinity membrane purification system can be seen in Figure 1.2. The four lanes in Figure 1.2 are: 1) 250 kD – 10 kD protein ladder, 2) BSA (2 mg), 3) DNase I (0.5 mg), 4) BSA + DNase I (2.5 mg), 5) eluted membrane sample. The effectiveness of this method can be clearly seen with the separation of the DNase I from the BSA with only a small amount of BSA carry over.

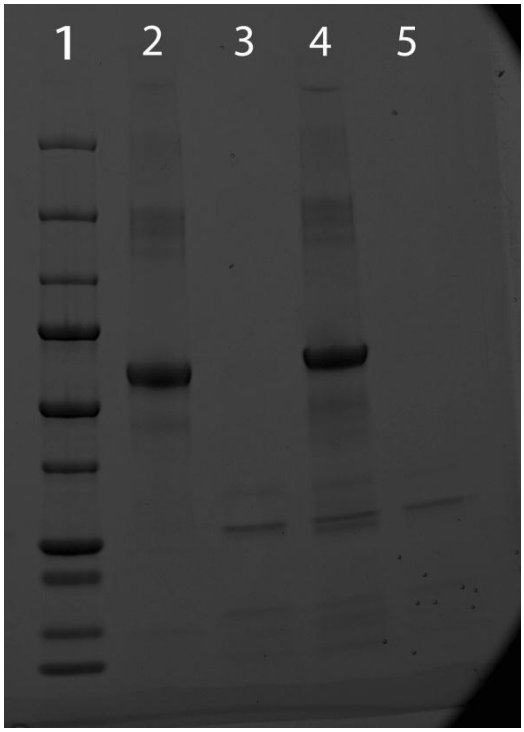


Fig 1.2– DNase I purification was determined using 4 -15% gradient polyacrylamide gel electrophoresis. The lanes are as follows: 1) 250 kD – 10 kD protein ladder, 2) BSA, 3) DNase I, 4) BSA + DNase I, and 5) A, eluted membrane sample.

Isolation of a DNase Producing Fungus

Isolation

Samples of compost were taken from the University of Massachusetts Amherst and various farms from the Amherst, MA area and incubated for three days at 55° C. Small amounts of incubated compost were spread onto yeast protein soluble starch agar plates (YpSs) and allowed to incubate at 45 and 55° C. For five days following the initial inoculation, the plates were examined for fungal growth under a dissecting microscope. Any visible growth was transferred to YpSs plates and incubated at 45 and 55^{o}C until substantial visible growth was observed. Attempts at initial isolation were foiled by bacterial growth which dominated the plate. To prevent future bacterial growth, an antibacterial cocktail was added to the YpSs plates used for future isolation. The cocktail included 10 ppm of each of the following: polymyxin B, penicillin G, ampicillin, and streptomycin. No bacterial growth was seen following the addition of this cocktail.

Seven fungal isolates were collected and tested for DNase activity. Each isolate was plated on yeast protein soluble starch DNase test agar (DYpSs) and incubated at 45 and 55^{o}C for seven days. Three of the initial seven isolates had DNase activity, which was indicated by a zone of clearing under and around the growth. However the zones of clearing for these isolates were very weak. To encourage growth of DNase producing fungi a DNA enrichment broth (0.25% Fish sperm DNA, USB Lot #126531, 10 ppm polymyxin B, 10 ppm penicillin-G, 10 ppm Ampicillin, 10 ppm streptomycin, 0.005% methyl green) was inoculated with pieces of compost and incubated at 55^{o} C on a shaker set at 115 RPM. The flask was checked every day for signs of de-coloration, and after five days the blue/green color was gone. The liquid culture was plated on YpSs and incubated at 45 and 55°C. Five isolates were collected and transferred to DYpSs plates and

incubated again at 45 and 55° C for 5 days. Two of the five isolates were positive for DNase activity, one at 45°C and another at 55° C.

The isolate that demonstrated activity at 45° C was plated again on DYpsS and incubated at 55°C for 5 days. The organism did not grow. The isolate that demonstrated activity at 55° C was plated on DYpSs and incubated for 5 days at 45° C. The organism grew much larger at 45° C then it did at 55° C; however there was minimal DNase activity. The organism with the highest amount of DNase activated at 55^{o} C was used for this study.

Identification

Initial attempts to identify the organism were based on observed morphology using conventional microscopy. A wet mount slide was made using a liquid culture of the isolated fungus. The hyphae were septate with an average diameter 2 – 3 µm. No clamp connections were present on the hyphae; however there were swollen cells. No fruiting bodies or spores were seen suggesting that the organism was either in its imperfect stage or was a sterile organism.

The organism was grown on different media (oatmeal, water, yeast glucose, cellulose, and czapek's agar), grown at different pH, incubated for different time periods, and grown in different lighting conditions to try to encourage spore formation. All attempts failed. Because of the size of the hyphae, it was quite difficult to examine the organism. Therefore scanning electron microscopy was used to get a more detailed picture. Scanning electron microscopy was performed on a JEOL JSM-5400 scanning electron microscope. The results can be seen in Figure 2. Again no significant fruiting bodies or spores were seen.

Since no identifying structures were observed, the highly conserved ITS region was amplified using the polymerase chain reaction and sequenced using an automated Sanger method. The results were compared to a reference databank using the NCBI Blast program. The organism was identified with a 91% match as a Chaetomium sp. and is now referred as strain TM-417.

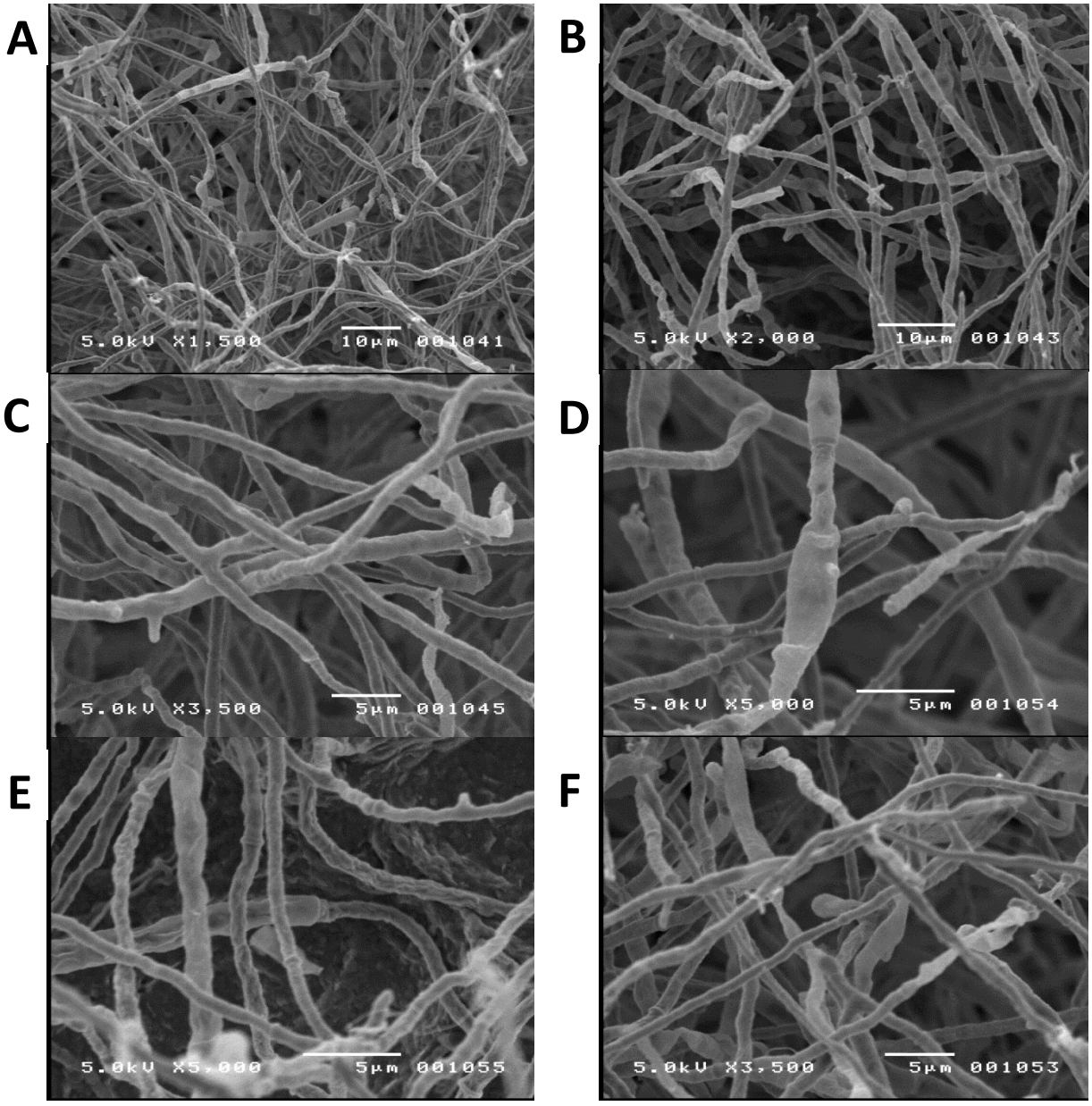


Fig. 2- Scanning electron microscopy images of TM-417 when performed on a JEOL JSM-5400 scanning electron microscope. No identifying structures were noticed. However, swollen cells can be seen in D and E.

Characterization of isolate TM-417

Optimal Temperature

The optimal growth temperature of the isolated fungus was assessed two ways; 1) measurable growth on agar plates, and 2) dry cell mass after growth in liquid culture. For the first method, YpSs agar plates were inoculated with a 10 mm plug of the isolate taken from a stock culture plate. The plates were partially sealed with parafilm and incubated at various temperatures (20, 32, 37, 45, 55, and 60° C) for five days. The diameter of the fungal growth was measured in four different directions (Figure 3.1) and the average size was determined. A chart containing the average growth size in regards to temperature can be seen in Table 2.

It can be noted from both Table 2 and Figure 3.2 that the optimal and maximum growth temperature for this organism was 45 and 60° C respectively. The morphology of the isolate was visibly different at 55° C when compared to 45°C. The hyphae at 55°C seem to have collapsed with only the perimeter having vertical hyphal growth (Figure 3.2 F). The thickness of the fungal growth (vertical hyphae) at 45° C was observed to be between 1 and 5 mm. At 55° C hyphae in the collapse region were immeasurable and the growth around the perimeter was 0.5 – 1 mm.

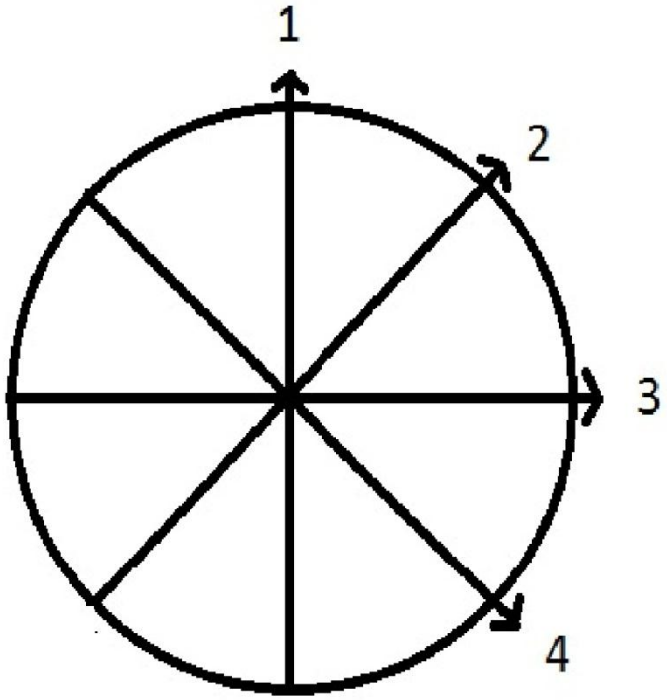


Fig. 3.1– Fungal growth on agar plates was measured in four directions and the average taken. The measurements taken were used to represent the average fungal growth observed on agar plates incubated at various temperatures.

Results from the liquid culture study can be seen in Figure 3.2 and Table 2. Since sporulation could not be induced, a blender was used to produce a semi-uniform inoculum. First, a culture was grown in YpSs broth for 5 days at 45° C on a shaker set to 115 RPM. The culture was loaded into a sterile blender bottle and pulsed five times using an Oster Osterizer Classic© blender. Blended culture (1 ml) was transferred to a sterilized 250 ml baffled flask containing 100 ml of YpSs broth. Inoculated flasks were incubated at various temperatures (20, 32, 37, 45, 55, and 60° C) on a rotary shaker set to 115 RPM for five days.

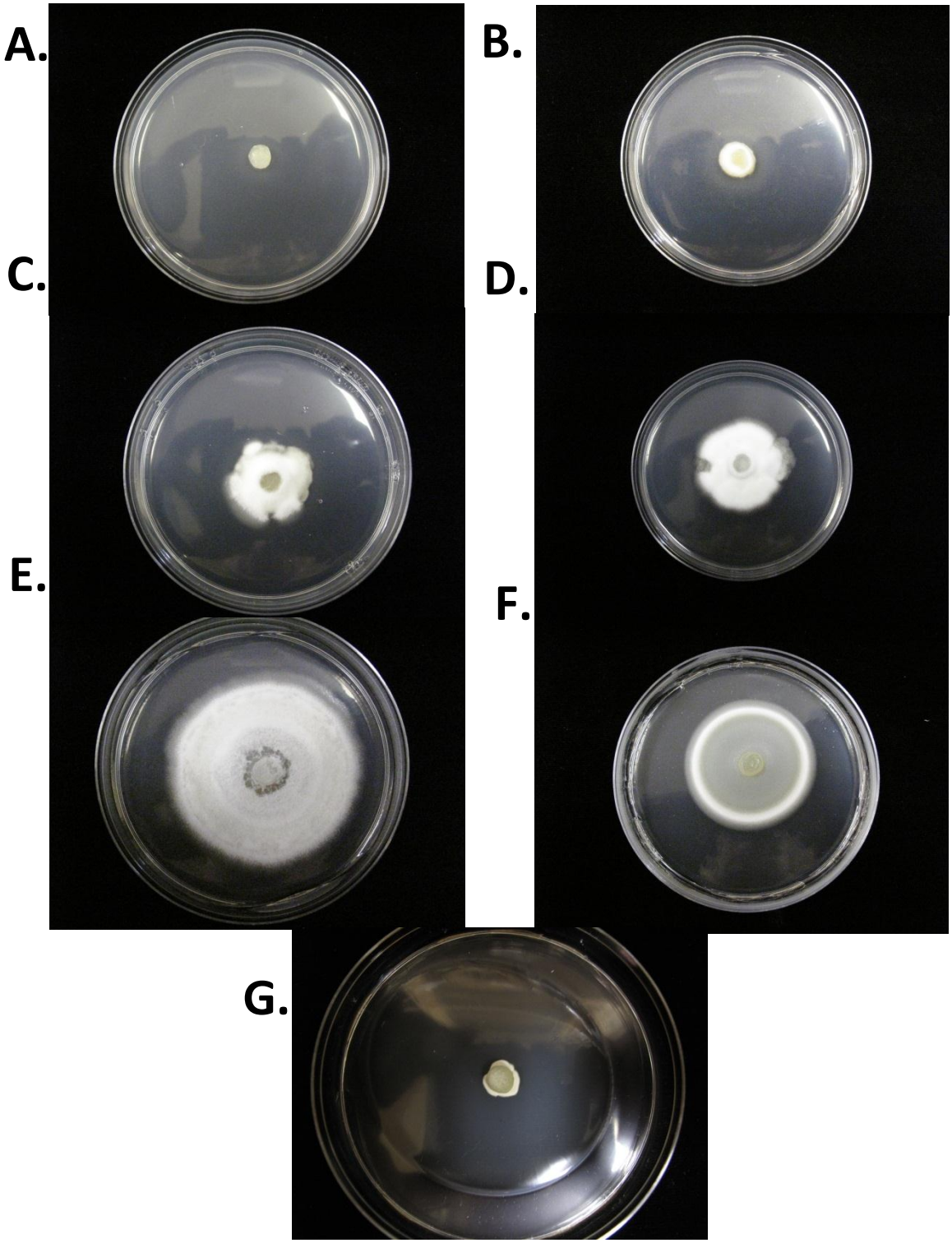


Fig. 3.2– Fungal growth on YpSs agar plates incubated at various temperatures for five days. The pictures are as follows: (A). 20°C, (B). 32° C, (C). 37° C, (D). 40° C, (E).45° C, (F). 55° C and (G). 60° C.

After the incubation period, a Buchner funnel lined with medium filter paper was used to filter out mycelia. The mycelia were washed with 250 mL of 0.1M ammonium to remove any trace nutrients. The mycelial mass were dried at 55° C overnight and weighed the next day. Again, the optimal temperature was determined to be 45° C with an overall dry cell weight of 241 g. No growth occurred at 20 °C for either method. This organism can be considered a true thermophile since the definition, as provided by Cooney and Emerson (1964), states that a fungal thermophile must not grow at or below 20° C and have a maximum temperature at or above 50° C.

Table 2

Diameter of fungal growth at various temperatures [a]

Temperature (° C)	Avg. Diameter Size (mm)
20	0 ± 0
32	14 ± 0
37	26 ± 0.75
40	41 ± 1.5
45	63 ± 1.4
55	47 ± 1.61
60	9 ± 1.22

[a] Fungal growth on agar plates was measured in four directions and the average taken. All temperatures were assessed in triplicate.

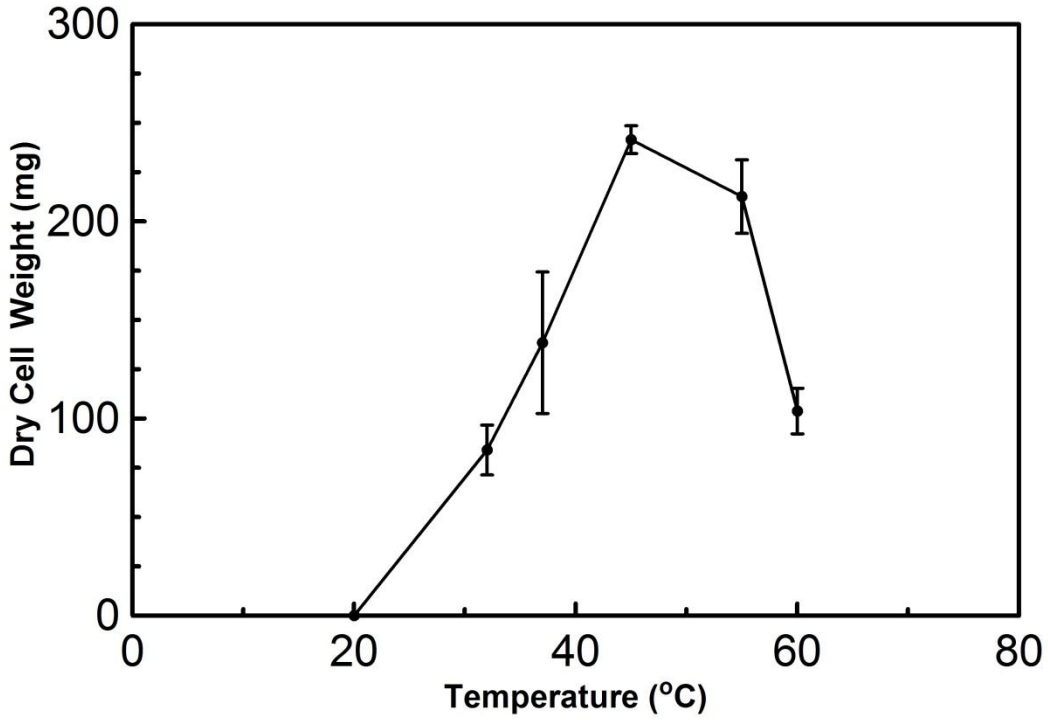


Fig. 3.3– Dry cell mass of fungal growth in a liquid culture at various temperatures. Samples were filtered, rinsed (0.1 M ammonium acetate) and dried at 55° C overnight prior to weighing. All temperatures were assessed in triplicate.

Optimal pH

The isolated organism's optimal pH for growth was determined by measuring dry weight after incubating for five days at 55° C. A modified formulation of the standard YpSs broth was developed for this experiment (0.05 M K_2HPO_4, 0.05 M tartaric acid, 0.4% yeast extract, 0.05% $MgSO_4$, 1.5% soluble starch, adjusted to desired pH) . Instead of just using 0.05 M potassium phosphate, 0.05 M tartaric acid was also added to cover the lower pH range. As seen in Figure 4, the optimal pH for this organism was 7 with no growth below pH 5.

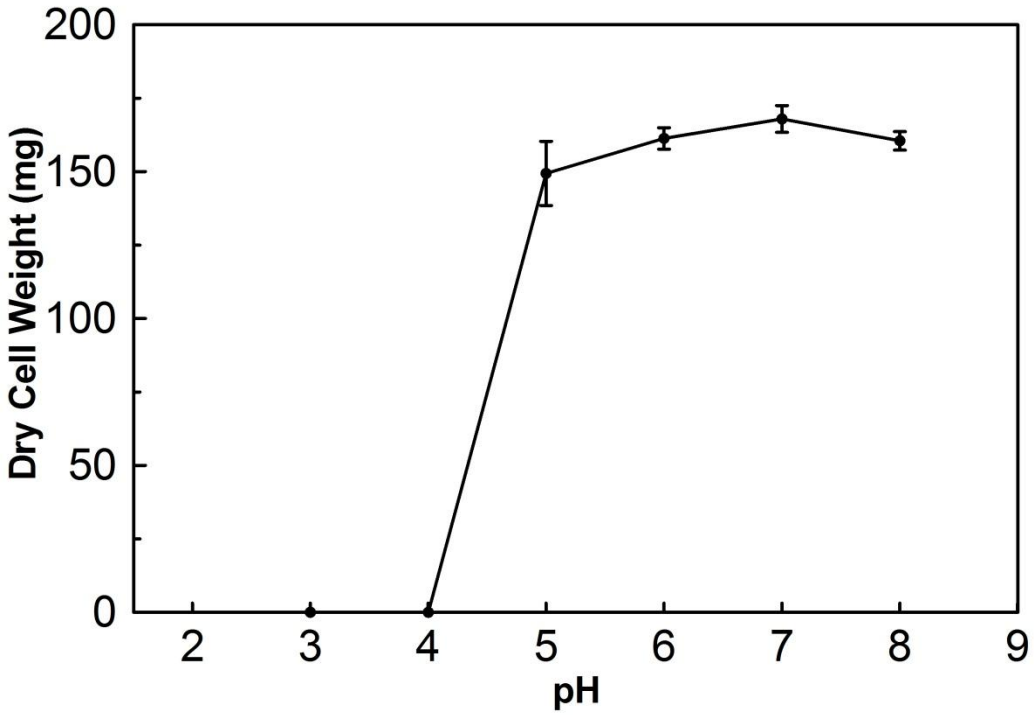


Fig. 4 – Dry cell weight of fungal growth in respect to different pH values. Growth occurred between pH 5 and 8 with no growth below pH 5. All tests were performed in triplicate.

Nutritional Characteristics

The nutritional characteristics of the organism, in regards to carbon and nitrogen requirements were examined. The original medium (YpSs) used to grow the organism was made up of four basic ingredients; sodium phosphate, magnesium sulfate, yeast extract and soluble starch. Yeast extract can be considered the main nitrogen source and the soluble starch the main carbon source. The utilization of different carbon sources was tested by substituting the soluble starch for other carbon sources while keeping the rest of the formulation the same (0.4% yeast extract, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 1.5% carbon source, adjusted to pH 7). 50 ml of modified broth was added to a 250 ml baffled flask and sterilized. Inoculation and dry cell

weight determination were performed in the same manner as the liquid culture temperature growth studies. The carbon sources tested were: xylose, glucose, lactose, sucrose, fructose, soluble starch, maltose, sorbose, galactose, sorbitol, and manitol. A control flask was used to determine the weight of the initial inoculum and any growth that may have resulted from nutrient carry over. The control medium was the same as the modified carbon medium except no carbon source was added. The results from the carbon study can be seen in Figure 5.1.

Statistically significant levels of growth occurred with all carbon sources when compared to the control using an unpaired t-test with a 95% confidence level. The sugar alcohols, sorbitol and manitol, demonstrated the highest amount of growth followed by galactose and glucose. The degree of utilization of the sugar alcohols is quite interesting. The utilization of sugar in fungi is dependent on membrane specific carrier proteins which have been shown to demonstrate a degree of substrate specificity. Fungi also produce a carrier specifically designed for glucose (Deacon, 1997). But it has been shown that when glucose levels are low, this carrier can also transport other sugars, including sugar alcohols. However the affinity for the other sugars is less than that for the originally intended substrate (Deacon, 1997). Since there was more growth with the sugar alcohols than glucose, a sugar alcohol specific membrane carrier may be actively expressed in the presence of these compounds.

The utilization of lactose was compared to both glucose and galactose. Lactose is a disaccharide comprised of glucose and galactose. Thus, in theory, the amount of cell growth from both glucose and galactose should equal that of lactose. However the molecular weights of glucose and galactose are both about 180. Lactose only has a molecular weight of 342 which is less than glucose and galactose combined (molecular weight about 360). To compensate for this discrepancy, the total combined dry cell weight for glucose and galactose was multiplied by the

ratio: $\frac{342}{360}$. When adjusted there was no significant difference between the dry cell weight of lactose and the combined weight of glucose and galactose (Figure 5.2).

The carbon source information also provides some insight on other extracellular enzymes produced by the fungus. For example since soluble starch and sucrose are utilized amylase and invertase must be produced and excreted by the isolate.

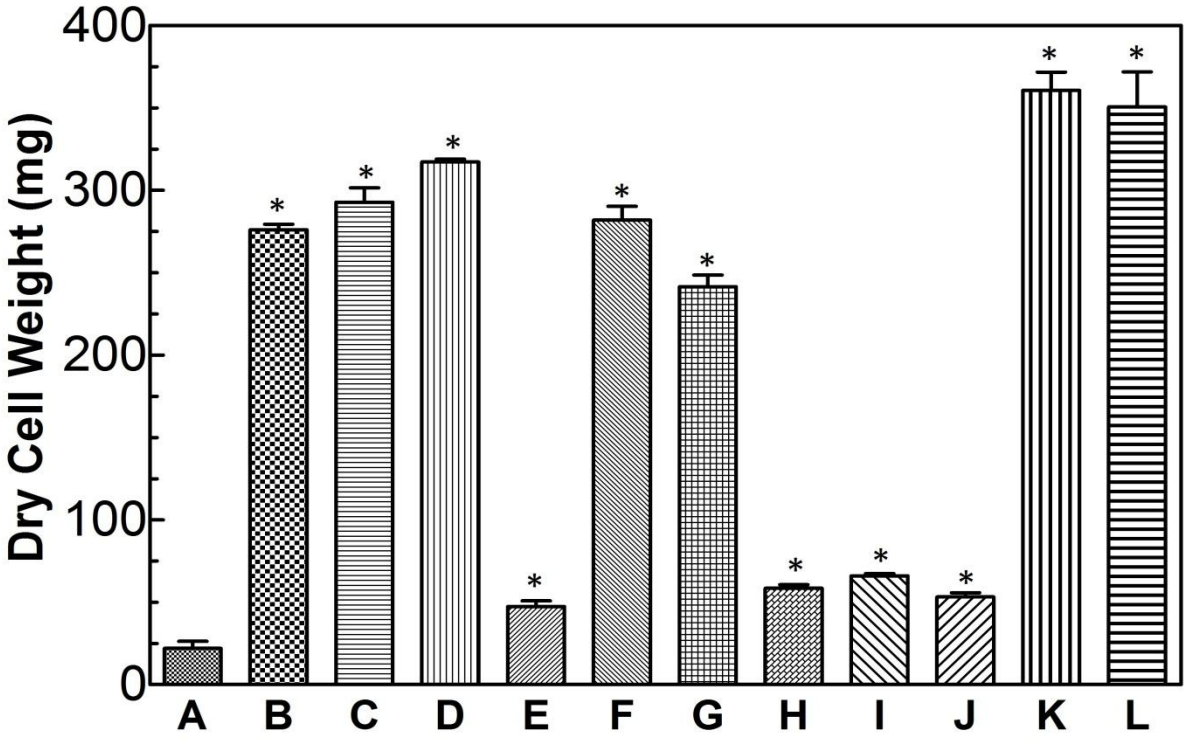


Fig. 5.1– Dry cell weight of fungal growth as a result of different carbon sources; (A) control, (B) xylose, (C) glucose, (D) lactose, (E) sucrose, (F) fructose, (G) soluble starch, (H) maltose,(I) sorbose, (J) galactose, (K) sorbitol, and (L) manitol. All tested carbon sources showed statistically significant growth (*) when compared to the control flask using an unpaired t-test with a 95% confidence interval. All tests were performed in triplicate.

Fig. 5.2– Comparison of dry cell weight of fungal growth in regards to lactose and glucose + galactose when multiplied by the ratio: $\frac{342}{360}$. Dry cell weight differences between sources were not statistically significant (P_{value} = 0.1201) when tested using an unpaired t-test with a 95% confidence interval. All tests were performed in triplicate.

The utilization of different nitrogen sources was tested by substituting the yeast extract for other nitrogen sources while keeping the rest of the original nutrient formula the same. In these studies soluble starch was used as the carbon source. The nitrogen sources tested were: "Vita Free" casein, L-glutamin acid, glycine, L-alanine, peptone, ammonium sulfate, and yeast extract. A control flask was used to determine the weight of the initial inoculum and any growth that may have resulted from nutrient carry over. The control media was the same as the

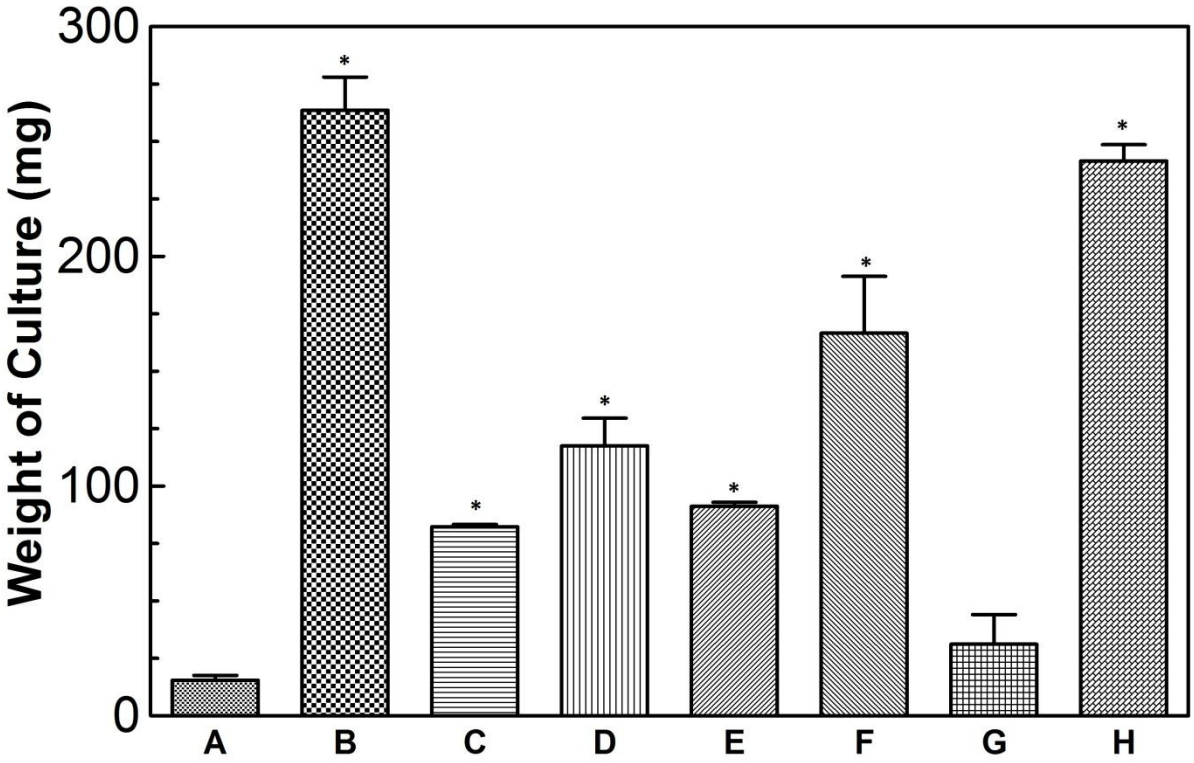


Fig. 5.3 - Dry cell weight of fungal growth with different nitrogen sources; A) control, B) "Vita Free" casein, C) L-glutamin acid, D) glycine, E) L-alanine, F) peptone, G) ammonium sulfate, and H) yeast extract. Six out of seven nitrogen sources showed statistically significant growth when compared to the control flask using an unpaired t-test with a 95% confidence interval. All tests were performed in triplicate.

modified nitrogen media except no nitrogen source was added. The results from the nitrogen study can be seen in Figure 5.3.

Among the seven nitrogen sources tested, six demonstrated a statistically significant increase in growth when compared to the control flask using an unpaired t-test with a 95% confident interval. The nitrogen source, ammonium sulfate did not yield significant growth. The nitrogen source that provided the greatest amount of growth was the "Vita Free" casein (United States Biochemical Corp., Lot# 29307). This form of casein is specifically designed and used to grow vitamin deficient cultures. From the data seen in Figure 5.3, it is clear that this organism does not require vitamin supplementation to grow. Also, the breakdown and utilization of

casein indicates that an extracellular protease is produced. Yeast extract also supported a large amount of growth, second only to the casein. Even with the excess nutrients and vitamins provided by the yeast extract, there was no significant difference (P_{value} = 0.1929) between the "Vita Free" casein and yeast extract indicating that under these conditions the same amount of maximum growth can be achieved without the presence of vitamins (Figure 5.4).

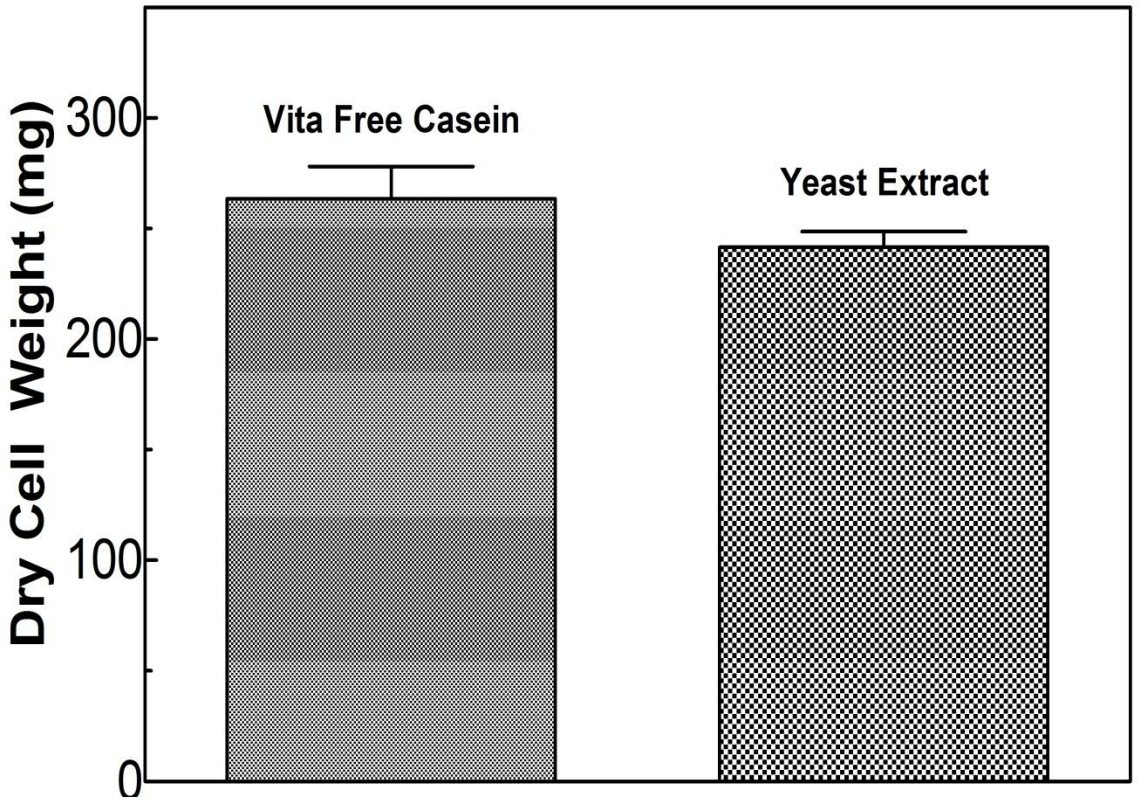


Fig. 5.4 - Comparison of dry cell weight of fungal growth in regards to Vita Free casein and yeast extract as nitrogen sources. Dry cell weight differences between sources were not statistically significant (P_{value} = 0.1929) when tested using an unpaired t-test with a 95% confidence interval. All tests were performed in triplicate.

The ability of the organism to utilize aromatic/ ring structured amino acids in the presence of ammonium sulfate was also examined. The same modified broth from the initial nitrogen source study was used except 0.2% ammonium sulfate was added (0.2% ammonium sulfate, 0.2% aromatic amino acid, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 1.5% soluble starch, adjusted to pH 7). Combinations of the following aromatic amino acids were used: proline, tryptophan, phenylalanine, and tyrosine. Figure 5.5 shows the difference in dry cell weight between cultures grown with just ammonium sulfate and cultures grown with ammonium sulfate plus individual or a combination of aromatic amino acids.

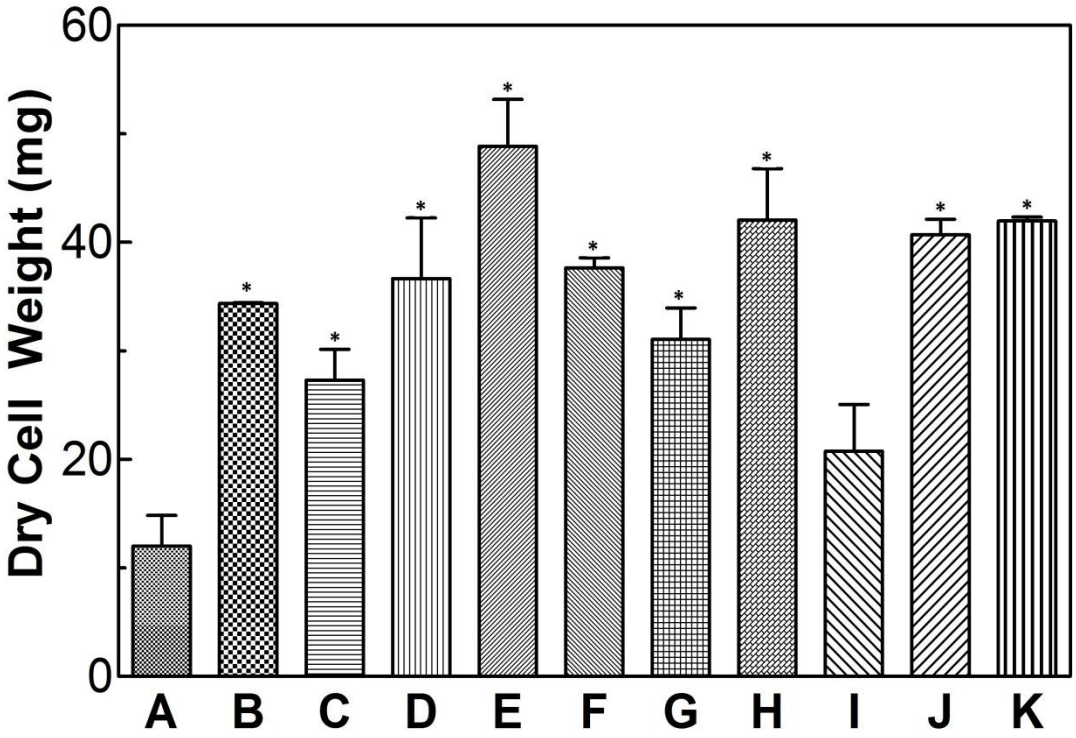


Fig. 5.5– Dry cell weight of fungal growth when using different aromatic amino acids as nitrogen sources; (A) ammonium sulfate, (B) proline, (C) tryptophan, (D) phenylalanine, (E) tyrosine, (F) proline + tryptophan, (G) proline + phenylalanine, (H) proline + tyrosine,(I) tryptophan + phenylalanine, (J) tryptophan + tyrosine, and (K) phenylalanine + tyrosine. Nine out of ten aromatic amino acid combinations showed statistically significant growth (*) when compared to the measured growth achieved with just ammonium sulfate. Data was analyzed using an unpaired t-test with a 95% confidence interval and all tests were performed in triplicate.

Nine out of the ten aromatic amino acid and ammonium sulfate combinations resulted in statistically significant growth when compared to growth from just ammonium sulfate. Only the sample with tryptophan, phenylalanine, and ammonium sulfate was not significant. It should be noted that the amount of growth between single amino acids and paired amino acids was not additive. For example, the dry cell weight of proline and tryptophan combined is not equal to the total dry cell weight of samples with just proline or tryptophan.

One possible explanation for this is the uptake inhibition of certain amino acids as a result of the presence of other amino acids. It has been shown that amino acids may act as competitive inhibitors for the uptake of other amino acids (Deacon, 1997; Kelly, 1969; EcCleston & Kelly, 1972).This phenomenon may explain the insignificant growth experienced with the tryptophan, phenylalanine, and ammonium sulfate. Kelly (1969) demonstrated that tryptophan inhibited phenylalanine incorporation into phenylalanine deficient *Thiobacillus neapolitanus* by 66%. Kelly later demonstrated that tryptophan significantly decreased phenylalanine uptake into *Pseudomonas aeruginosa* and *Methylococcus capsulatus* (Kelly, 1969; EcCleston & Kelly, 1972). Though these studies were performed on bacteria, there is no evidence dismissing the possibility of this phenomenon in the fungal world.

Effect of Dye Concentration on Fungal Growth

The effect of different dyes on the growth of the organism was assessed. The dye methyl green was frequently incorporated into both agar plates and broth and therefore any possible inhibition of growth due to the dye should be noted. The dyes selected were methyl green, methyl blue, and crystal violet. Each dye was added to YpSs agar plates prior to sterilization in the following amounts: 0 mM, 2 mM, 4 mM, 6 mM, 8 mM, and 10 mM respectively. The results of this study can be found in Figure 5.6. Methyl blue resulted in the

least amount of inhibition, with all of the concentrations decreasing the overall diameter by only 16%. 2 ml of methyl green inhibited growth by 52% and crystal violet inhibited growth at all concentrations. However, the amount of methyl green used for the visualization of DNase activity is in the order of millimoles, therefore inhibition of growth by methyl green was not considered to be an issue.

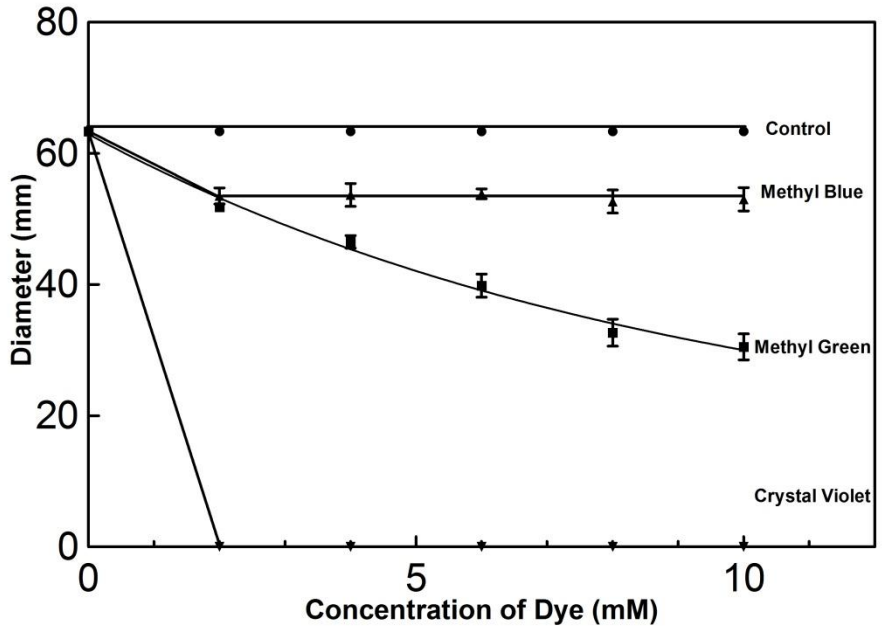


Fig. 5.6– The effect of dyes on the growth of the fungus. Various amounts of 1 M dyes were added prior to pouring the YpSs agar plates. Plates were inoculated with 10 mm plugs of the isolate taken from a stock culture plate. The plates were allowed to incubate at 55° C for five days and growth was measured in four directions as explained in Figure 1. The concentration used in the culture media was close to that of 0 on the x-axis. All tests were performed in triplicate.

Initial DNase Studies

To generate a sample with a substantial amount of extracellular DNase activity, the isolated organism was grown in a 2 L Fernbach flask with 500 ml YpSs liquid broth containing

both DNA and methyl green. The broth was inoculated with 5 ml of a liquid culture that was pulsed five times using an Oster Osterizer Classic© blender. Once inoculated the culture was placed on a shaker set at 115 RPM and incubated until the blue/green color was gone. With this method, the color in the flask was gone after five days.

To try to decrease the incubation period and increase DNase production, another flask was inoculated and incubated for 3 days at 55° C on a shaker set to 115 RPM. After the 3 days, the shaker was stopped and the culture was allowed to incubate statically until the color was gone. With static growth, the color in the flask was gone after an additional two days of incubation. The overall incubation time did not decrease with static growth; however the overall amount of DNase activity was significantly higher in the static culture than the agitated culture (Figure 6.1). The enzyme activity was measured using the acid soluble assay with a 60 minute digestion time at 55° C. To prepare the sample, 4 ml was removed from the incubated flask and centrifuged at 13,400 RPM for 10 min. The supernatant was used to determine enzyme activity.

Fungi grown in a static liquid broth develop into a dense fungal mat, whereas agitated fungi develop into individual spheres. It is known that fungi grown statically results in higher amounts of enzyme activity then the activity achieved with agitated growth. As seen in Figure 6.1, static growth resulted in a 269% increase in enzyme activity when compared to the agitated culture.

Effect of Temperature on Methyl Green Stability

Due to the high incubation temperatures it was necessary to make sure the decoloration was in fact a direct result of DNase activity and not the oxidation of the dye itself. To test this, two flasks containing DYpSs broth were incubated at 55° C for five days. One flask was inoculated with 5 ml of blended culture and the other was not. The stability of methyl green was

measured by recording the absorbance of each flask at 645 nm (abs. max for methyl green). The enzyme activity of the inoculated flask was also tested every day using the acid soluble assay with a 60 minute digestion time at 55^{o} C. The samples were prepared in the same manner as the agitated and static growth supernatants prior to analysis.

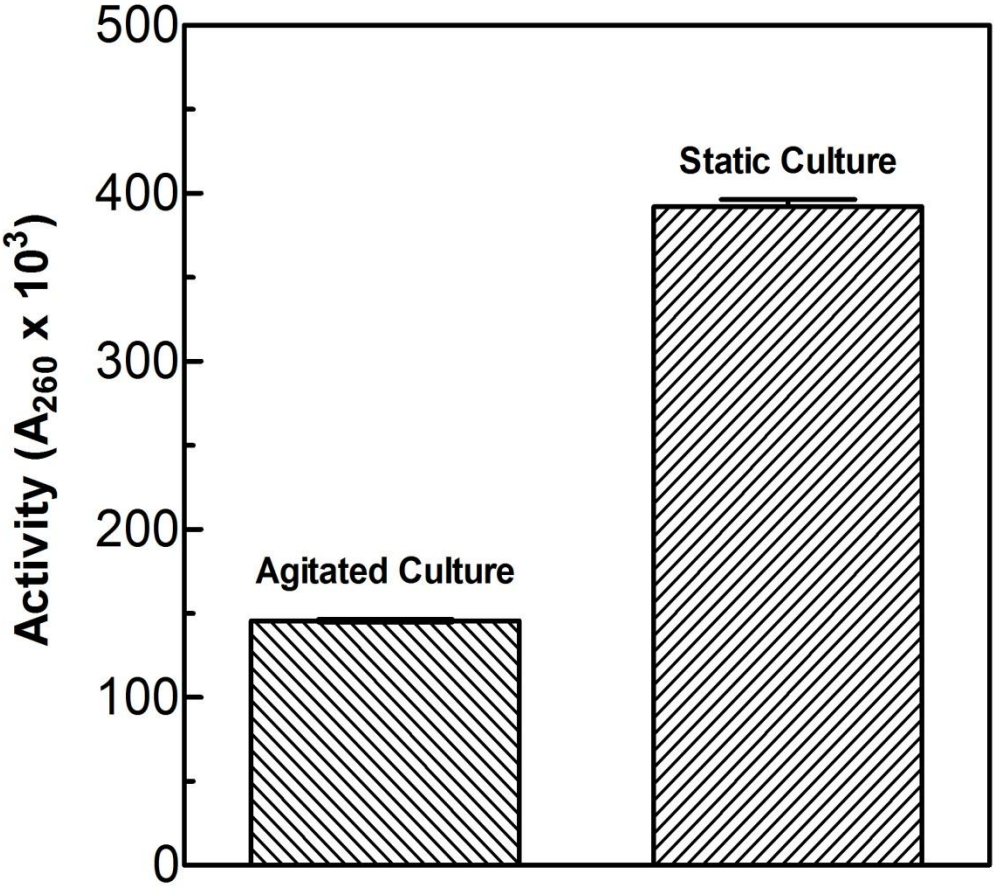


Fig. 6.1– The difference in DNase activity between crude samples prepared from static and agitated cultures. There was a significant increase in DNase activity when the fungal culture was grown statically compared to agitated incubation. The supernatant from 4 ml of centrifuged (13,400 RPM for10 min) culture was used to determine enzyme activity. Activity was measured using the acid soluble assay with a 60 min digestion time at 55^{o} C. All assays were performed in triplicate.

There was no significant decoloration in the control sample indicating that the incubation temperature was not responsible for the change in color. The graph clearly depicts the correlation between an increase in DNase activity and a decrease in A_{645} readings. Based on the information provided by Figure 6.2, optimal DNase activity was achieved after 5 days (3 days agitated and 2 days static) of incubation at 55° C. As a result of these findings, crude enzyme sample was harvested after incubating (3 days agitated and 2 days static) at 55° C for five days.

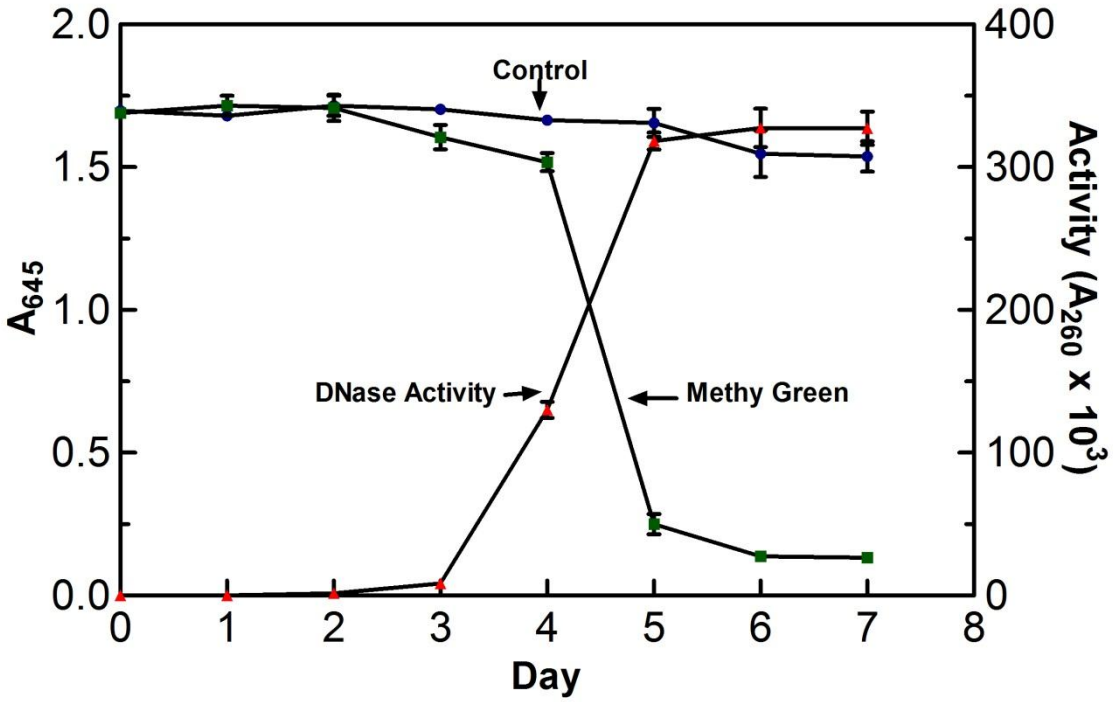


Fig. 6.2– The effect of temperature and DNase activity on the decoloration of the DYpSs liquid media. Based on the results from the control flask, incubation at 55° C did not have any significant effects on the color intensity of the media. However a direct relationship between an increase in DNase activity and the decrease in A_{645} can be seen. Color intensity was measured at 645 nm, which is methyl greens optimal absorption wavelength. Enzyme activity was measured using the acid soluble assay with a 60 min digestion time at 55° C. All assays were performed in triplicate.

Effect of Temperature on DNase and potential RNase production

A correlation between temperature and DNase production was established by inoculating DYpSs agar plates with a 10 mm plug taken from a stock culture plate. The plates were incubated at various temperatures (2-5, 20, 32, 37, 45, 55, and 60° C) for five days. The zones of clearing were measured in four different directions from the edge of visible growth to the furthest spot of clearing (Figure 7). The organism was also inoculated on YpSs RNase test agar plates (RYpSs) to test for the presence of an extracellular ribonuclease (RNase).

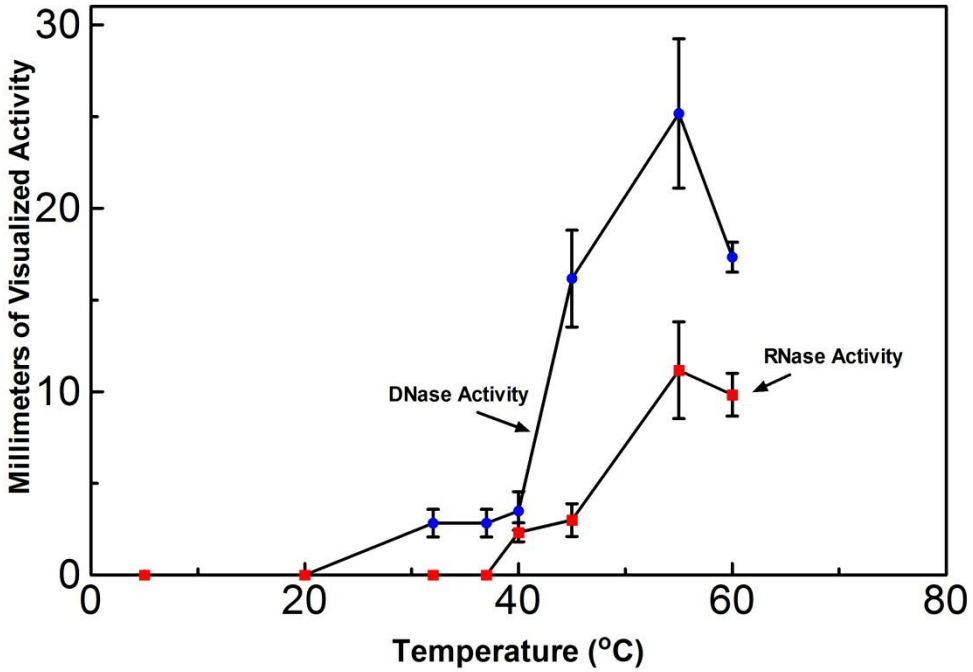


Fig. 7– The effect of temperature on DNase and RNase production by the isolated organism. DYpSs agar plates were inoculated with a 10 mm plug taken from a stock culture plate. The plates were incubated at various temperatures (2-5, 32, 37, 45, 55, and 60° C) for five days. The zones of clearing were measured in four different directions from the edge of visible growth to the furthest spot of clearing. Test was performed in triplicate.

The RNase test media uses the dye thionine to visualize RNase activity. Thionine forms a blue complex with RNA and when the RNA is digested by an RNase produced by an organism a red or pink halo forms around the growth. RYpSs plates were inoculated with 10 mm plugs taken from a stock culture plate and incubated at various temperatures (2-5, 20, 32, 37, 45, 55, and 60° C) for five days. The pink or red halos were measured in a similar manner as the DYpSs agar plates were. The results for both DNase and RNase activity at the various temperatures can be seen in Figure 7.

The organism tested positive for RNase activity at 40, 45, 55, and 60^{o} C, with the highest amount of activity present at 55^{o} C. DNase activity was detected from 32 to 60^{o}C and the highest amount of activity was also at 55^{o} C.

DNase Induction Studies

A series of experiments were designed to see if production of the DNase was induced by the presence of DNA. Blended culture (1.0 ml) was transferred to a sterilized 250 ml baffled flask containing 100 ml of YpSs broth. The culture was allowed to incubate for 5 days at 55^{o} C on a shaker set to 100 RPM. After the incubation period DNase activity was measured using the acid soluble assay with a digestion time of 60 min at 55^{o} C. DNA (1.0 g) was dissolved in 10 ml of deionized water, filter sterilized, and added to the 5 day old flasks. The flasks were incubated for another 4 days under the same conditions. DNase activity was measured every day following the addition of the sterilized DNA. In order to analyze the enzyme activity in each flask, 4 ml was removed from the each flask and centrifuged at 13,400 RPM for 10 min. The resulting supernatant was used to measure enzyme activity.

After growing for five days in the absence of DNA, the overall amount of DNase produced by the culture was significantly lower than cultures grown in the presence of DNA

(Figure 8.1). Flasks in which DNA was added after the five day incubation period showed a spike in DNase activity overnight. Figure 8.2 represents a typical increase in DNase activity observed after 1 g of DNA was added and activity was assessed every 12 hours.

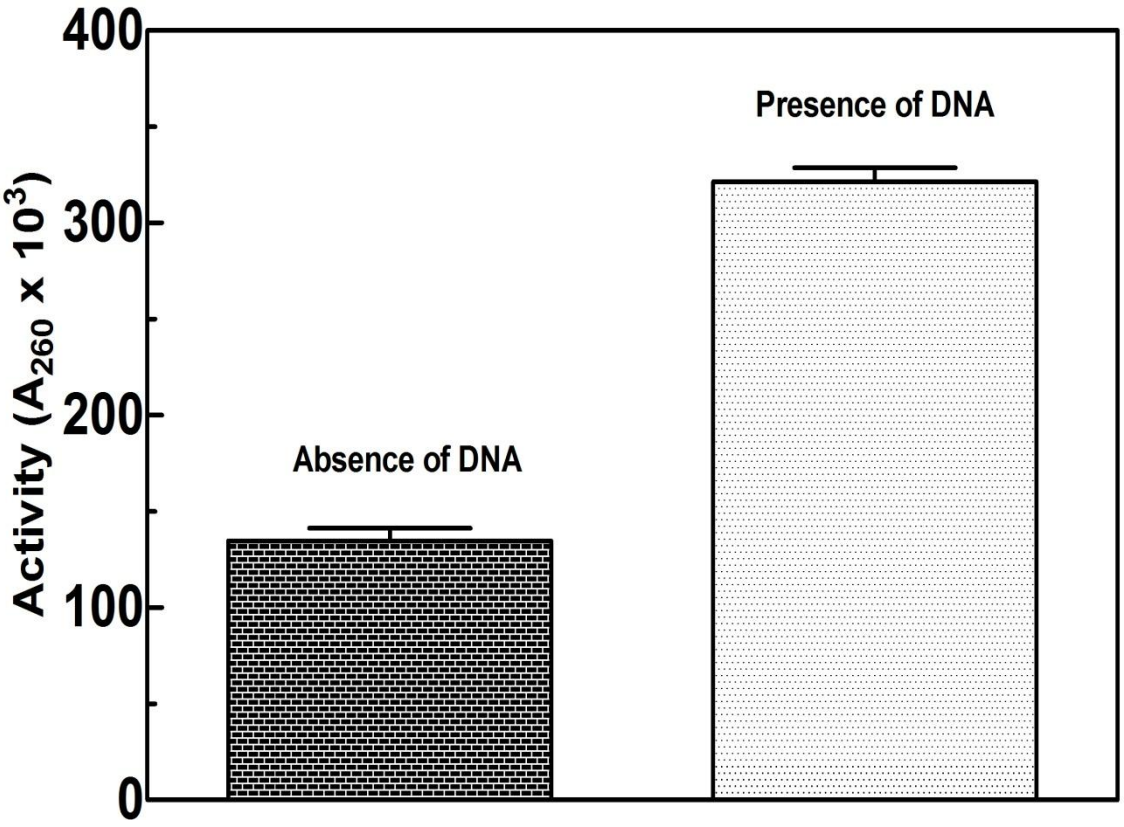


Fig. 8.1– Difference in DNase activity when a culture was grown with and without DNA present in the culture medium after incubating for five days at 55°C on a shaker set to 100 RPM. Culture grown in the presence of DNA demonstrated a 239% increase in enzyme activity compared to a culture grown in the absence DNA. Activity was measured using the acid soluble assay with a 60 minute digestion time at 55° C. All measurements were performed in triplicate. The results from 3 flasks were used to calculate DNase activity.

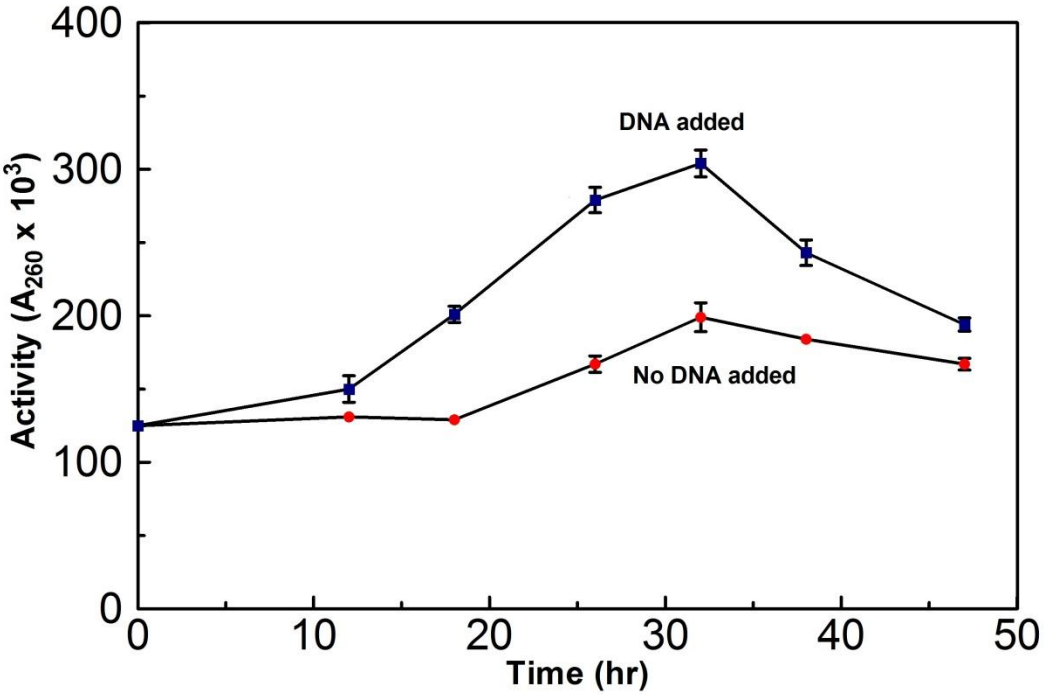


Fig. 8.2– Observed increase in DNase activity after 1 g of filter sterilized DNA was added to a culture flask that was previously growing in the absence of DNA for five days at 55° C on a shaker set to 100 RPM (0 time on graph). Cultures showed a significant increase in activity following the addition of DNA at all assay intervals when compared to the culture with no DNA added. Activity was measured using the acid soluble assay with a 60 minute digestion time at 55° C. All tests were performed in triplicate.

The sugar deoxyribose was investigated as a possible inducing factor for expression of the DNase. Deoxyribose is only found in DNA and makes up the structural backbone of the molecule. To test this theory, flasks grown in the absence of DNA were spiked with 1 g of filter sterilized deoxyribose sugar after five days of incubation at 55° C. The spiked flask was incubated for another 3 days under the same conditions and the increase in DNase activity was recorded daily. The deoxyribose sugar did create a rise in DNase activity. However, it was not as dramatic as the stimulation by DNA (Figure 8.3). As seen in Figure 8.3, the maximum DNase activity was achieved on the second day following the addition of the sugar. In comparison, the DNase activity reached its maximum after 24 hr.; however the activity on the second day was

lower than the activity initiated from the deoxyribose. On the third day both activity from the DNA and deoxyribose was almost comparable to that of the control. This decrease in activity may be the result of protease activity that may have leaked out of the aging cells. Overall, DNA did induce the most amount of DNase activity in a shorter amount of time then deoxyribose. However DNase activity is induced in the presence of deoxyribose suggesting that deoxyribose may play a role in the induction of the DNase.

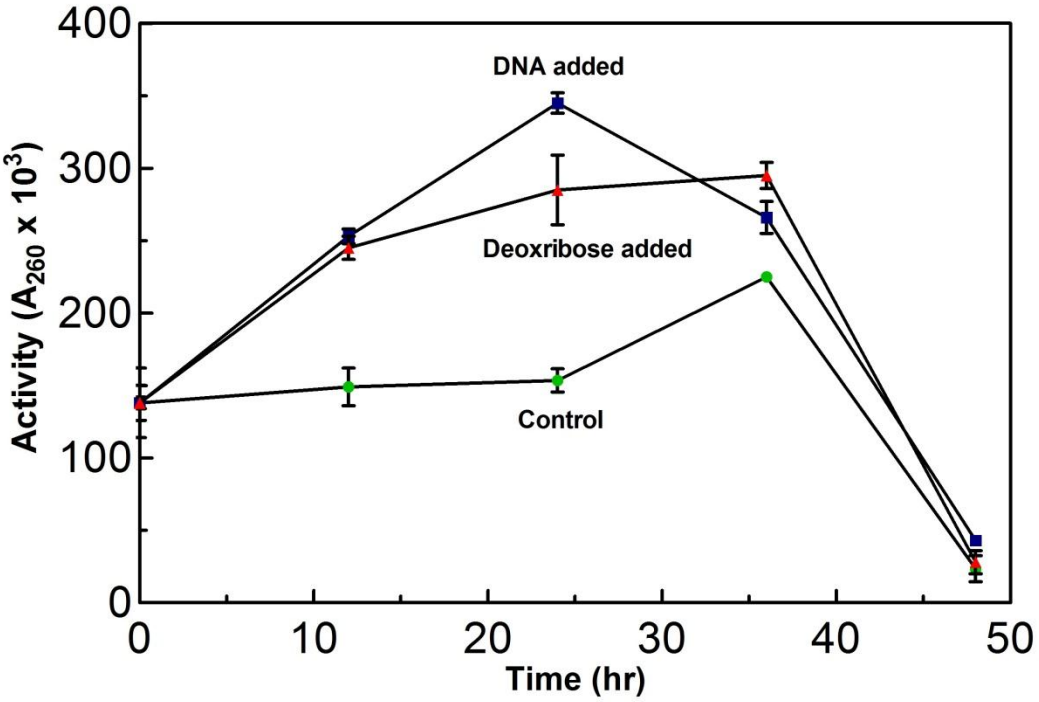


Fig. 8.3– Comparison of increase in DNase activity achieved when either 1g of DNA or 1 g of deoxyribose was added to a culture flask that was previously growing in the absence of DNA for five days at 55° C on a shaker set to 100 RPM. Cultures showed a significant increase in activity at all assayed intervals following the addition of DNA or deoxyribose when compared to the culture with no DNA added. Activity was measured using the acid soluble assay with a 60 minute digestion time at 55° C. All tests were performed in triplicate.

Purification of Extracellular Nucleases

Concentration of Sample

Following filtration and dialysis of the culture, the enzyme activity of the crude sample was analyzed for both DNase and RNase activity using the acid soluble assay at 55° C. Crude sample (250 ml) was concentrated down at room temperature to 39 ml using an ultra-filtration apparatus with a membrane cut off of 10 kDa, resulting in a concentration factor of 6.41.

The enzyme activity of the concentrated sample was tested for both nucleases. Results from both the crude and concentrate assays were plotted and their slopes analyzed. If no enzyme activity was lost during the concentration then the rate or slope of the concentrated sample when divided by the concentration factor would equal that of the crude.

Figure 9 shows the observed DNase and RNase activity, respectively. Both of the concentrated samples, when divided by the concentration factor were 98% of the original crude slopes indicating that only 2% of the total enzyme activity was lost during the concentration process, see Table 3. Overall there was a 9.07-fold increase in specific activity when compared to the original crude sample. In regards to overall protein, the crude sample had 320 mg of protein. After concentration, the overall protein content was reduced by 89% to 35 mg.

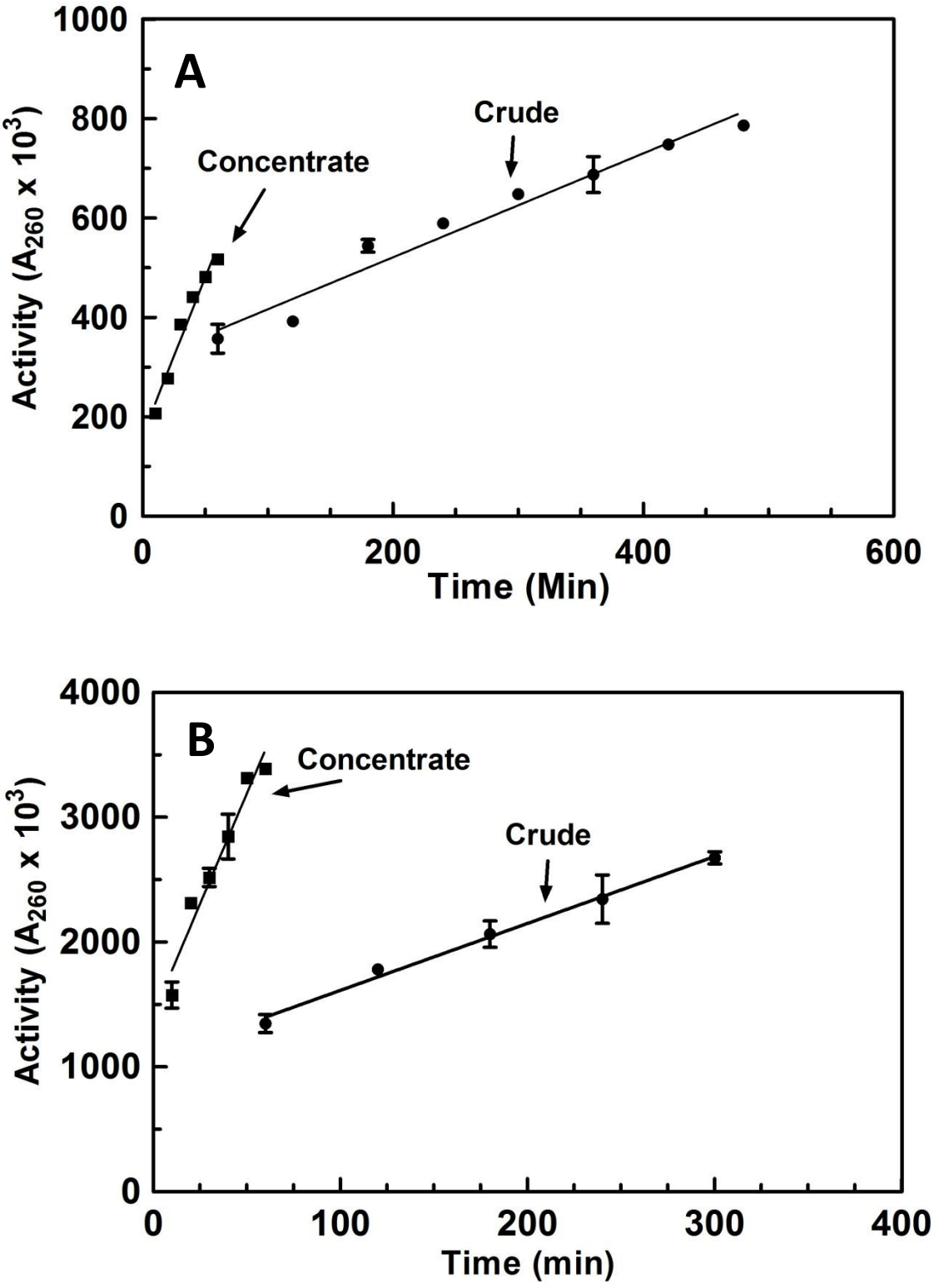


Fig. 9–Comparison of the rates or slopes of the crude and concentrated samples in regards to enzyme activity. A, represents DNase activity and B, represents RNase activity. The assay used for these experiments was the acid soluble assay which was performed in triplicate at 55° C.

Table 3

Numerical values for crude and concentrated sample slopes and adjusted slopes [a]

	Sample	Slope x 10^3 (S)	Concentration Factor (CF)	Adjusted Slope	Concentrate Slope x 10^3	Crude Slope x 10^3	Difference
DNase Activity	Crude	1	6.41	6.41	6.3		2%
	Concentrate	6.3	6.41	0.98		1	2%
RNase Activity	Crude	0.7	6.41	4.5	4.4		2%
	Concentrate	4.4	6.41	0.68		0.7	3%

[a] **The crude sample was adjusted by multiplying the slope by the concentration factor. The slope for the concentrated sample was adjusted by dividing by the concentration factor. The averaged percent difference for both nucleases is considered to be ~ 2%.**

Sephadex G-50 Purification

The next stage of purification involved passing the concentrated sample through a Sephadex G-50 column. The void volume for the column was determined to be 52.8 ml using Blue Dextran 2000 (Pharmacia Fine Chemicals, Lot# 8050). The first fraction demonstrating a measureable absorbance reading at 280nm was fraction 9 with additional readings extending to fraction 50. Fractions 9 through 15 had DNase activity and fractions 9 through 16 had RNase activity (Figure 10). Tubes with DNase activity were pooled together and the same for the tubes with RNase activity. The remaining fractions demonstrated no activity.

Initially, gel chromatography was performed at room temperature with the idea that the nucleases would be stable due to their thermophilic nature. However, when purification was performed in a 2 - 5° C refrigerator the percent recovery increased, from 42.79% to 64.41% for the DNase. The refrigerated sample also had a 109% increase in specific activity compared to the non-refrigerated sample (Table 4). The increase in specific activity for the non-refrigerated sample was 38.03 fold compared to a 73.59 fold increase for the refrigerated sample.

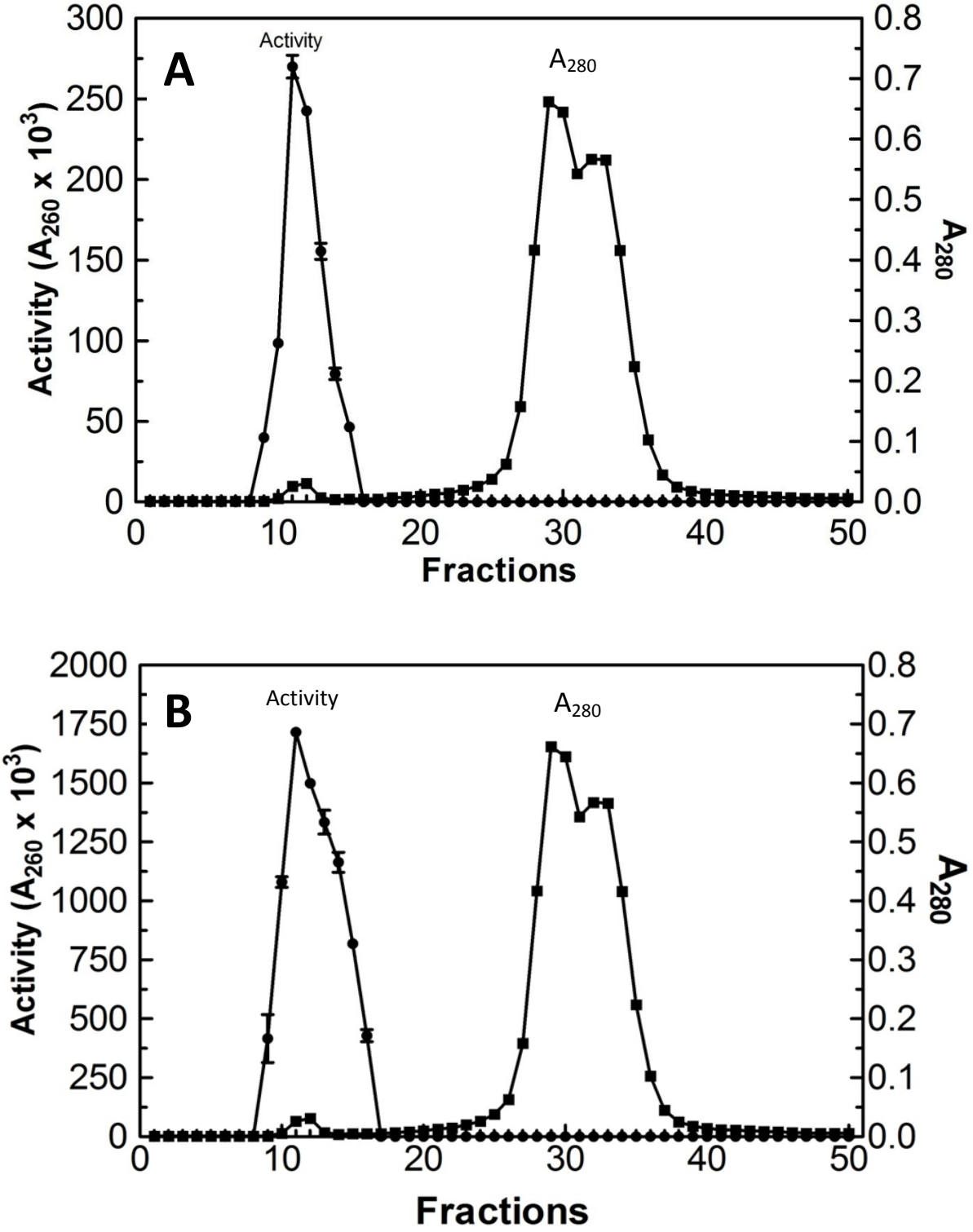


Fig. 10 –Typical plots of protein and enzyme activity when 1 mL of concentrated sample was passed through a Sephadex G-50 column at 2 - 5°C. A, represents DNase activity and B, represents RNase activity. The activity was measured using the acid soluble assay at 55° C and protein was estimated by determining the absorbance at 280nm.

The RNase was only purified under refrigerated conditions due to the improvements seen in the percent recovery of the DNase (Table 4). Purification via Sephadex G-50 resulted in a 91.5-fold increase in specific activity. A typical plot of protein and activity for RNase and DNase can be seen in Figure 10.

Ceramic Hydroxyapatite Purification

Samples from ten Sephadex G-50 runs were pooled together and concentrated down from 462 ml to 45 ml. 10 ml of this concentrated, pooled Sephadex G-50 sample were loaded onto the ceramic hydroxyapatite column. Like the Sephadex G-50, ceramic hydroxyapatite column chromatography was performed at ambient temperature followed by trials in a 2 - 5°C refrigerator. Non-refrigerated chromatography resulted in only a 7.6% recovery of DNase activity along with a 52.55-fold increase in specific activity. Refrigerated chromatography recovered 10.9% of the original activity with a 166.37-fold increase in specific activity, a 316% increase over the non-refrigerated sample. Typical plots of protein and DNase activity can be seen in Figure 11.1. The protein for both the non-refrigerated and refrigerated samples was reduced to 0.21 mg and 0.22 mg respectively from 2.8 mg per Sephadex G-50 column run.

Ceramic hydroxyapatite purification did not result in sufficient activity to allow for kinetic studies. The overall amount of activity was low even though the specific activity had undergone a 166.37-fold increase. Also RNase activity was not successfully separated from the DNase activity. Upon inspection of Figure 11.2, two peaks representing RNase activity are present with the first peak being coincident with the DNase peak. This could be due to: 1) The presence of two nucleases, one being a non-specific nuclease degrading both RNA and DNA and the other having specificity towards RNA only, 2) The presence of three enzymes, two different RNases and one DNase, or 3) The presence of only two enzymes, one DNase and one RNase with

the latter of the two not uniformly eluting off the column. The overall purification of the DNase, in both refrigerated and non-refrigerated conditions, can be seen in Table 4.

Table 4

Comparison of the overall purification of the DNase performed using ceramic hydroxyapatite

Temperature	Fraction	Vol (mL)	Protein Content (mg)	Total activity (U)	Sp act (U/mg)	Recovery (%)	Increase in sp act
Ambient (~20°C)	Crude	250.0	320.0	1807.5	5.65	100	1
	Concentrated	39.0	35.0	1793.26	51.24	98	9.07
	Sephadex G-50	56	3.6	773.53	214.87	42.79	38.03
	Hydroxyapatite	70	0.22	138.13	627.87	7.6	52.55
Refrigerated (2 - 5°C)	Crude	250.0	320.0	1807.5	5.65	100	1
	Concentrated	39.0	35.0	1793.26	51.24	98	9.07
	Sephadex G-50	46.2	2.8	1164.24	415.8	64.41	73.59
	Hydroxyapatite	52.8	0.21	197.4	940	10.9	166.37

[a] One unit of enzyme activity is defined as an increase in absorbance of 0.05 units in a cuvette of 1 cm path length at 260nm

Membrane Purification

Affinity membrane purification was used as an alternative to the ceramic hydroxyapatite method with the idea that the DNase would be separated from the RNase based on its substrate specificity. This approach embodied the mechanism that when the DNA was bound to the membrane the DNase would in turn selectively bind to the DNA leaving the RNase in the supernatant. However protein may also bind hydrophobically to these membranes, some small transfer of RNase and other miscellaneous protein was expected.

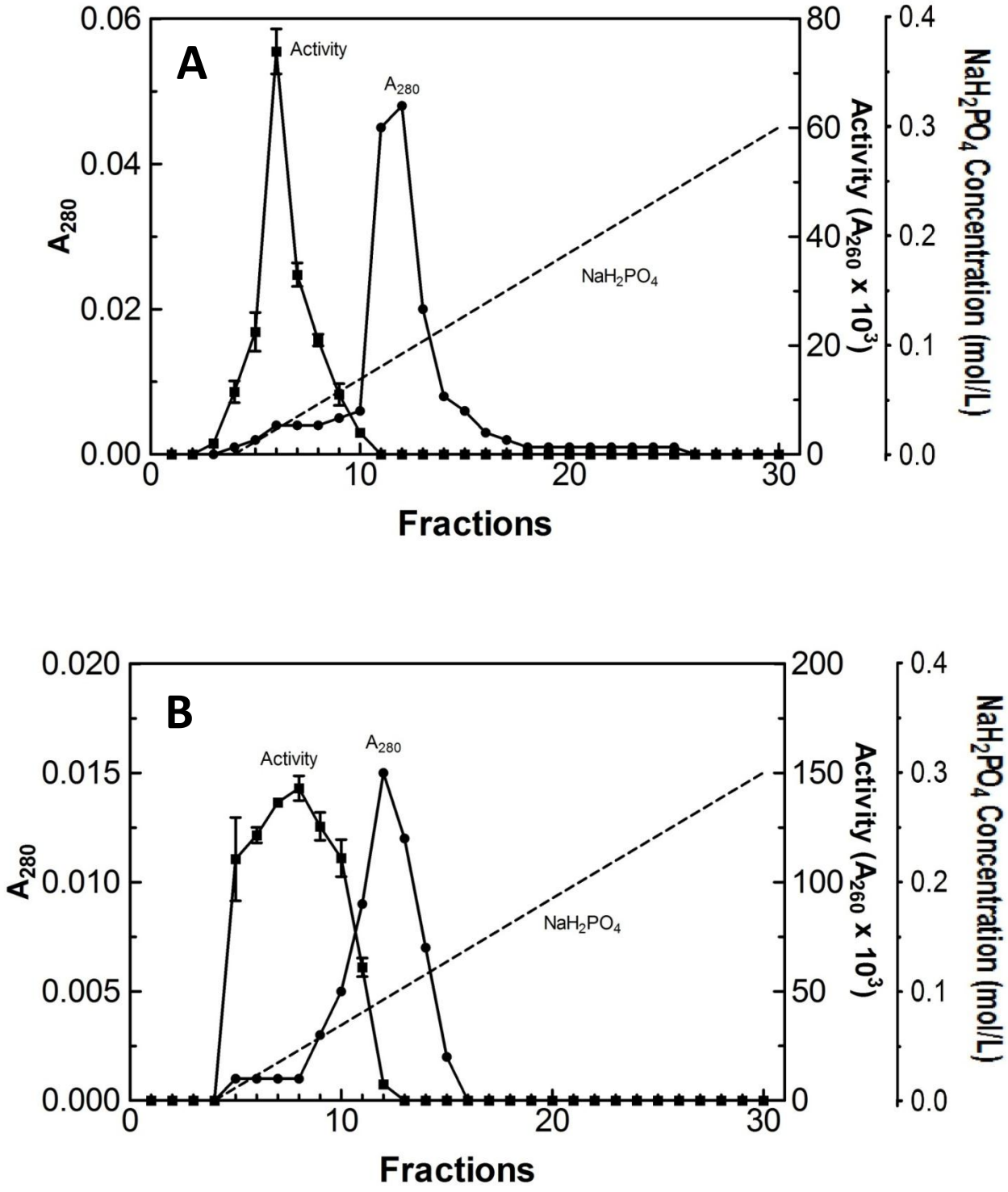


Fig. 11.1–Typical plots of protein and enzyme activity when 10 ml of Sephadex G-50 sample is eluted from a ceramic hydroxyapatite column using a sodium phosphate gradient. A, represents purification at ambient temperature and B, represents purification at 2 - 5° C. The activity was measured using the acid soluble assay at 55° C and protein was estimated by determining the absorbance at 280nm. Sodium phosphate gradient was from 0 to 0.3 mol.

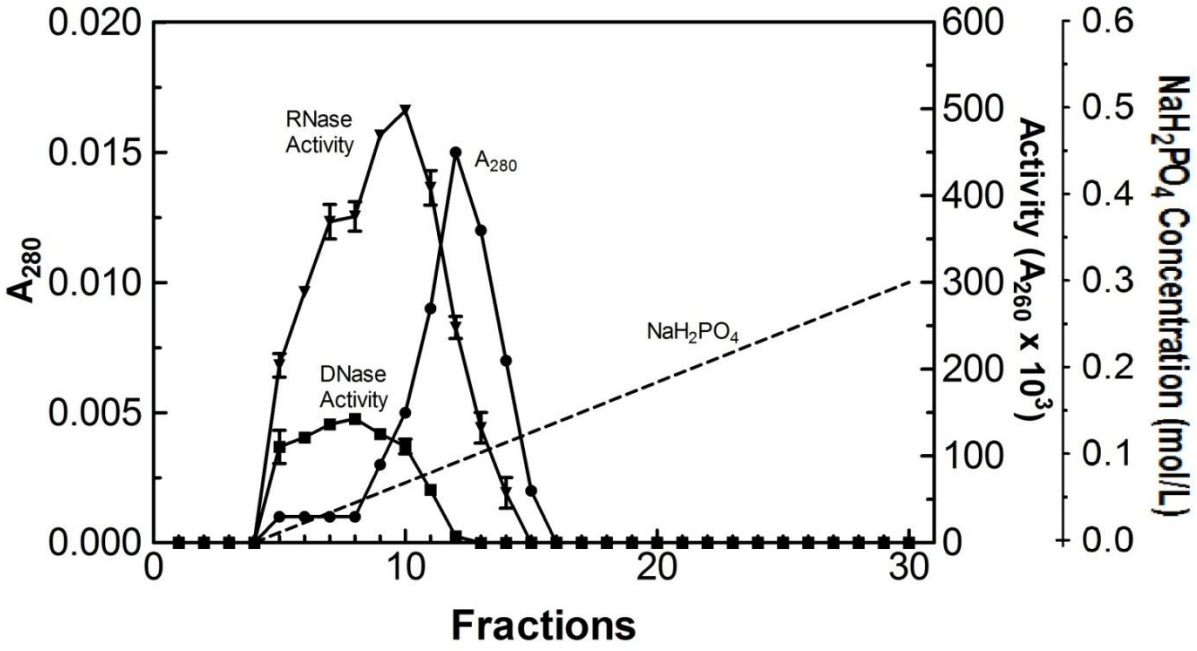


Fig. 11.2 –Protein and enzyme activity when 10 ml of Sephadex G-50 sample is eluted from a ceramic hydroxyapatite column using a sodium phosphate gradient. The peak for DNase activity is clearly positioned within the peaks representing RNase activity. Activity was measured using the acid soluble assay and protein was estimated by determining the absorbance at 280nm. Sodium phosphate gradient was from 0 to 0.3 mol.

Four pooled Sephadex G-50 refrigerated column runs were concentrated to 45ml using the pressurized ultrafiltration cell as described earlier. From this, 10 ml were purified using 10 DNA coated membranes. This was done for the remaining volume as well. Following the elution process, the final sample was dialyzed prior to analysis. The elution sample was checked for DNase activity and purity. Using this method 25% of the DNase was recovered with a 145-fold increase in specific activity when compared to the crude sample (Table 5). This purification procedure resulted in a final reduction in protein by over 550-fold, with a final protein content of 0.57mg (Table 5). It was from this sample that all of the characterization and kinetic data was determined for the DNase.

For the RNase, the original 10 ml following membrane purification was checked for activity and purity. Membrane purification resulted in the retention of 76% of the initial activity

with a 121-fold increase in specific activity (Table 5). The overall protein content was 168 times less than that of the original crude sample, with the final concentration being just under 2 mg (Table 5). It was from this sample that all of the characterization and kinetic data was determined for the RNase. The final overall purification data for the DNase and RNase can be seen in Table 5.

Enzyme purity was assessed using 4-15% gradient polyacrylamide gels. The purification of both the DNase and RNase can be seen in Figure 12.

Table 5
Comparison of the overall purification of both DNase and RNase activity using affinity membrane purification

Nuclease	Fraction	Vol (mL)	Protein Content (mg)	Total activity (U)	Sp act (U/mg)	Recovery (%)	Increase in sp act
DNase [a]	Crude	250.0	320.0	1408.5	4.40	100	1
	Concentrated	39.0	35.0	1379.26	39.41	97.9	8.96
	Sephadex G-50	46.2	2.8	854.98	305.35	60.70	69.40
	Membrane	45	0.57	365.12	640.56	25.22	145.58
RNase [b]	Crude	250.0	320.0	10476	33	100	1
	Concentrated	39.0	35.0	10257	293	98	8.9
	Sephadex G-50	46.2	2.8	8456	3020	81	91.5
	Membrane	45	1.9	8006	4214	76	127.6

[a] One unit of enzyme activity is defined as an increase in absorbance of 0.05 units in a cuvette of 1 cm path length at 260nm
[b] One unit of enzyme activity is defined as an increase in absorbance of 0.1 units in a cuvette of 1 cm path length at 260nm

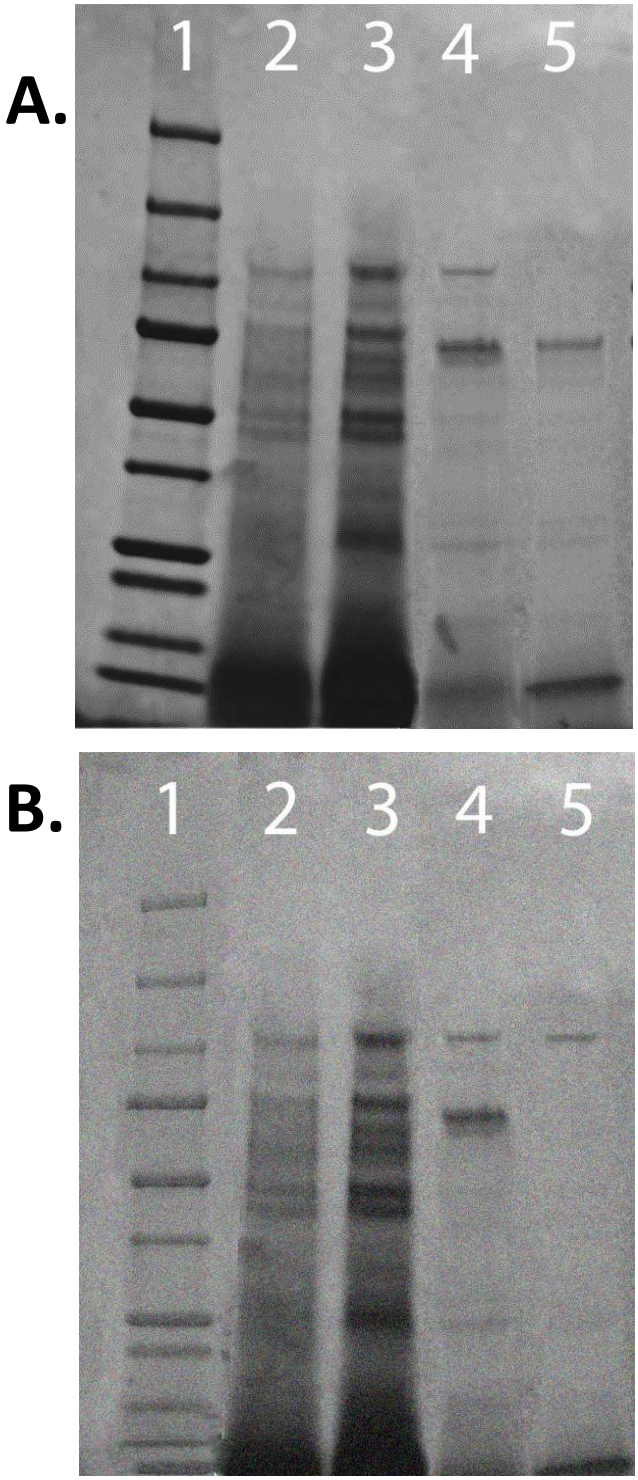


Fig.12– Enzyme purification was determined using 4 -15% gradient polyacrylamide gel electrophoresis. The lanes are as follows: 1) 250 kD – 10 kD protein ladder, 2) crude sample, 3) concentrated sample, 4) Sephadex G-50 sample, and 5) A, eluted membrane sample (DNase) and B, Sephadex G-50 sample after membrane purification (RNase).

Characterization of the DNase

Effect of Temperature on Enzymatic Activity

The temperature profile of the purified DNase can be seen in Figure 13.1. The enzymatic activity was analyzed using the real-time spectrophotometric assay at various temperatures. 0.75 ml of purified DNase was added to 2.25 ml of equilibrated substrate (40 µg/mL fish sperm DNA (USB Lot #126531), 10 µmolMgSO4 in 0.1 M imidazole, pH 7) and the absorbance at 260 nm was recorded over time. The optimal temperature was found to be 65° C with little activity at 68° C and no measurable activity at 70° C (data not shown). The activity at the optimal temperature was 121% greater than at 40° C.

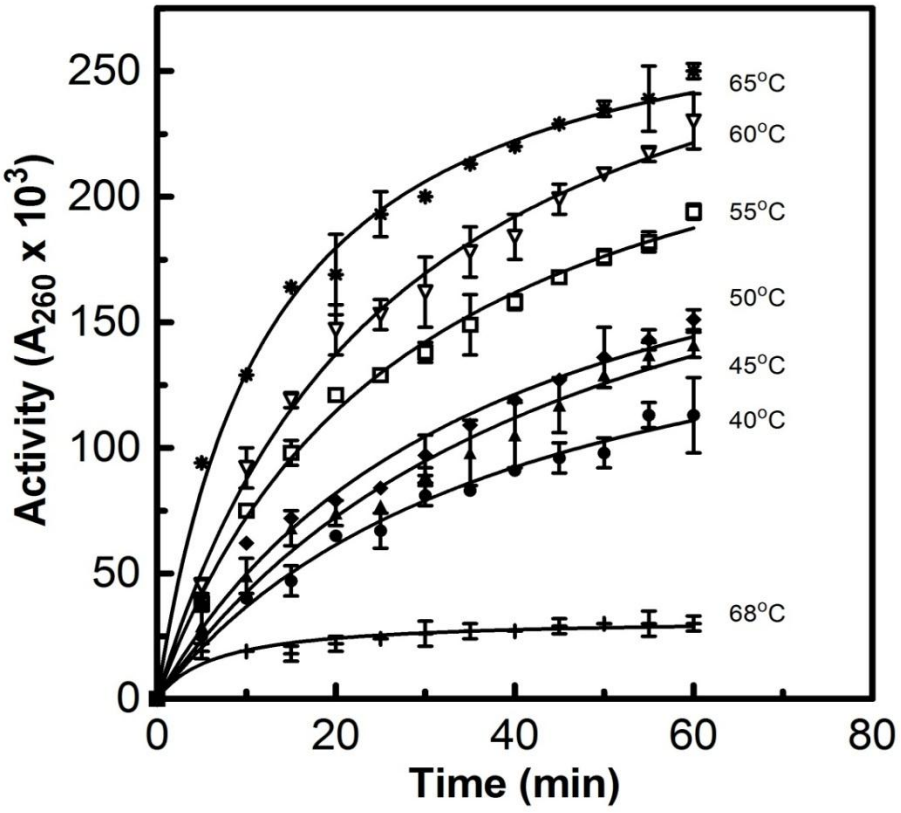


Fig. 13.1– The effect of temperature on purified DNase activity. The assay used for this experiment was the real-time spectrophotometric assay which was performed in triplicate.

The rate of enzyme destruction, as seen in Figure 13.2, was established at both 68° C and 65°C. No enzyme destruction was detected at 65° C during 30 min; which was expected since the optimum temperature for the DNase was determined to be 65° C. However, at 68° C a bi-phasic destruction plot was observed, which may elude to the presence of enzymatic subunits. The bi-phasic nature of the plot may suggest that one subunit or a particular part of the enzyme may be more susceptible to higher temperatures subsequently resulting in irreversible denaturation.

Irreversible denaturation is dependent on both time and temperature. As an enzyme is exposed to high temperatures for extended periods of time, it transforms to inactive conformational states. If the enzyme is not completely denatured, it may transform back or at least partially back to its active conformational state with a lowering in temperature. However, in most cases a majority of the activity is lost. This might explain the change in activity seen at 68° C.

Effect of pH on Enzymatic Activity

The pH profile for the purified DNase was determined using the acid soluble assay along with measuring the zone of clearing on pH adjusted DTest agar plates (recipe found in appendix), all of which were performed in triplicate. The digestion time for the acid soluble assay was 60 min throughout the pH range. A buffer system comprised of imidazole, malic acid and glycine was used to provide a substrate that could buffer throughout the desired pH range. With this buffering system, a continual plot was generated alleviating the need for duplicate plots or breaks in the graph which are generally seen when separate buffer systems are used.

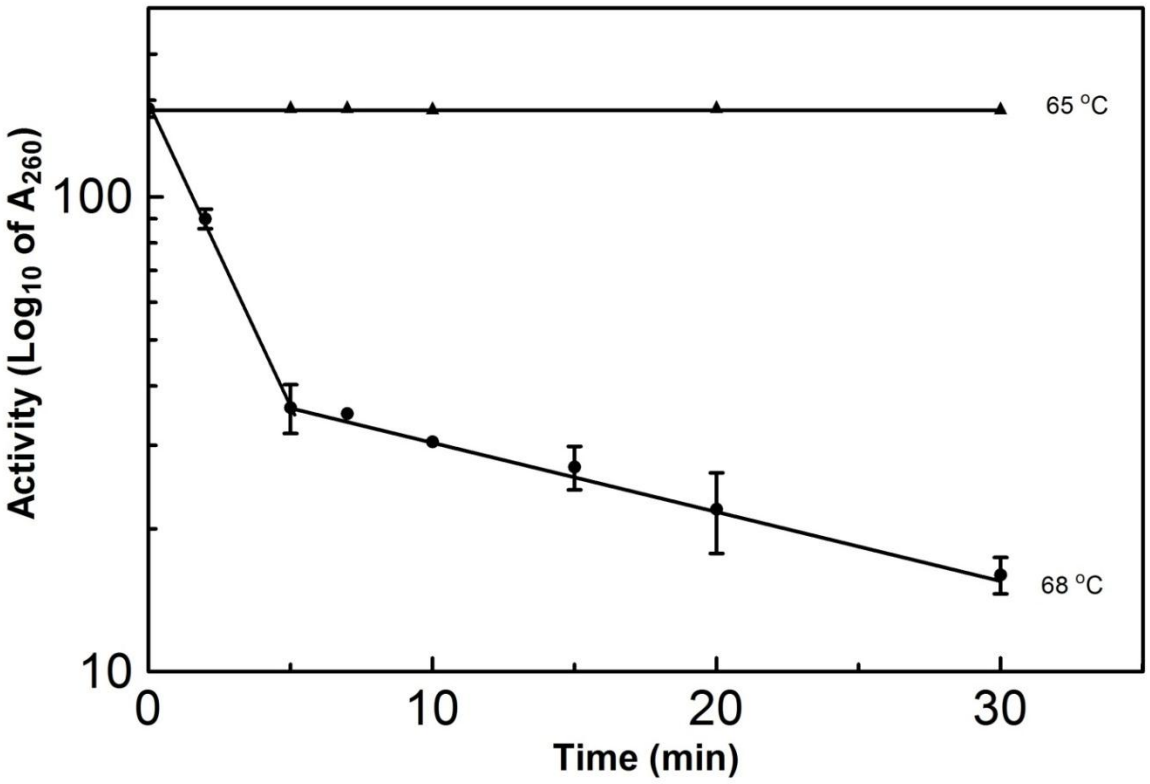


Figure 13.2– Destruction plot of the purified DNase at 68° C. The assay used for this experiment was a modified version of the acid soluble assay which is explained in detail in the materials and methods section.

For the clearing study, 10 mm diameter wells were made in the center of the agar plates and loaded with 200 µL of purified DNase. The loaded plates were then incubated at 55° C and the zones of clearing were measured after 12 hours of incubation. Both studies showed the highest amount of activity at pH 6 (Figure 14).

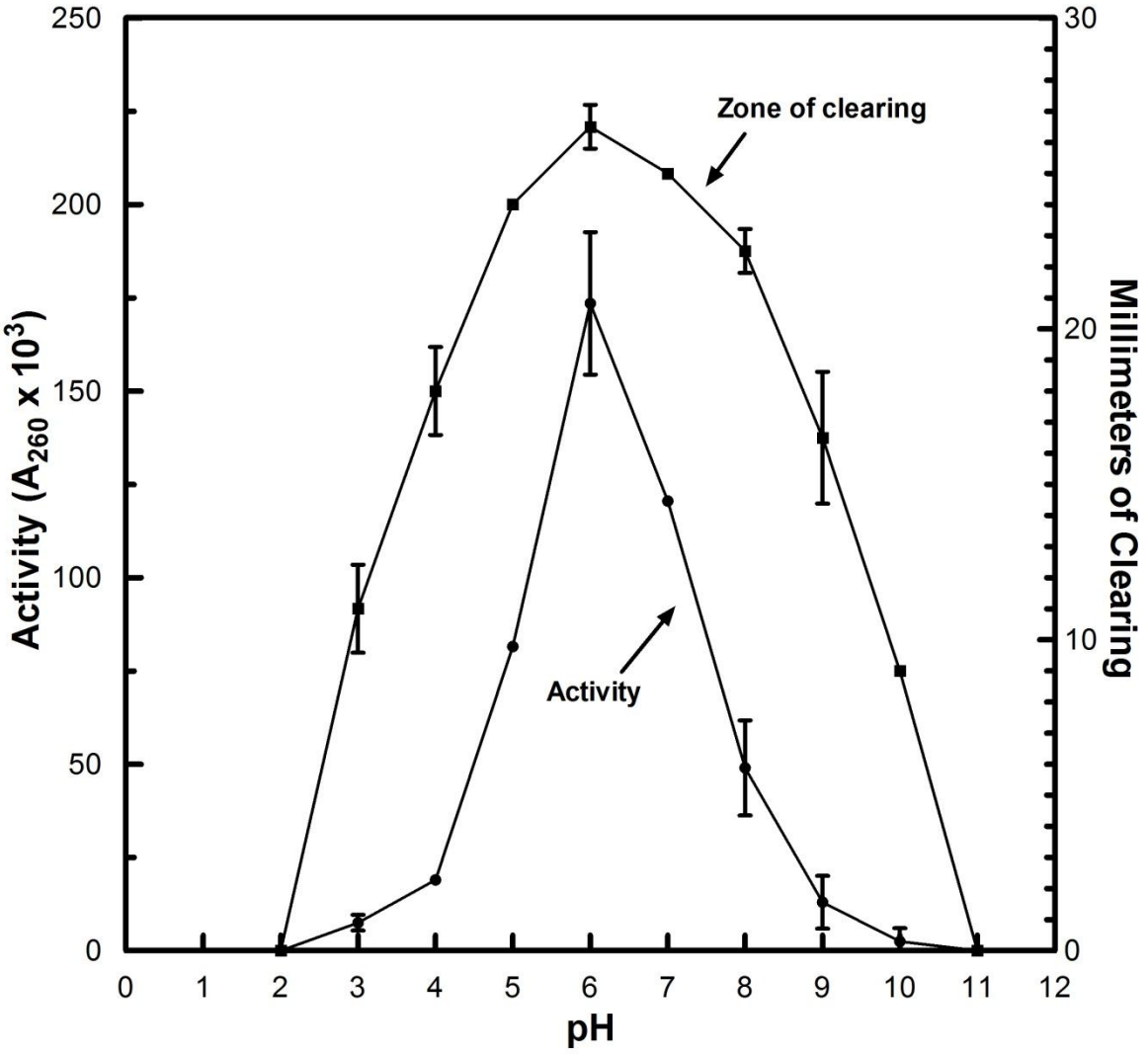


Fig. 14– Plot of the purified DNase pH profile in regards to activity and millimeters of clearing. The activity was measured using the acid soluble assay at 55° C with varying pH. The zone of clearing was determined by measuring the diameter of the visible zone which also included the well.

Effect of EDTA on Enzymatic Activity

EDTA was added to purified DNase containing 2 mM/mL $MgSO_4$ to determine the inhibitory effect of removing the co-factor. Figure 15 shows that 1 mM/ml EDTA and 2 mM EDTA/ml reduced activity by 51% and 98% respectively.

Substrate Specificity of the DNase

The specificity of the DNase towards single stranded (ssDNA) or native DNA (dsDNA) was assessed using the acid soluble assay with a 60 minute digestion time at 55° C. To make ssDNA, the substrate (Fish sperm DNA, USB Lot #126531, 10 µmol $MgSO_4$ in 0.1 M imidazole, pH 7) was boiled in a microwave for 30 seconds, rapidly cooled in an ice bath to 55° C and then placed in a water bath set to 55° C. The DNase demonstrated a significant difference in substrate preference, with a 121% higher specificity for ssDNA than dsDNA (Figure 16).

Estimated Molecular Weight of the DNase

The molecular weight of the purified DNase was estimated using a column of Sephadex G-75 and by determining the K_{av} values for the purified DNase and protein references (Figure 17). This value is a constant for a compound when passed through a specific type of Sephadex gel. The K_{av} values of enzymes with known molecular weights were used to calculate the log of the molecular weight of the DNase from a standard curve.

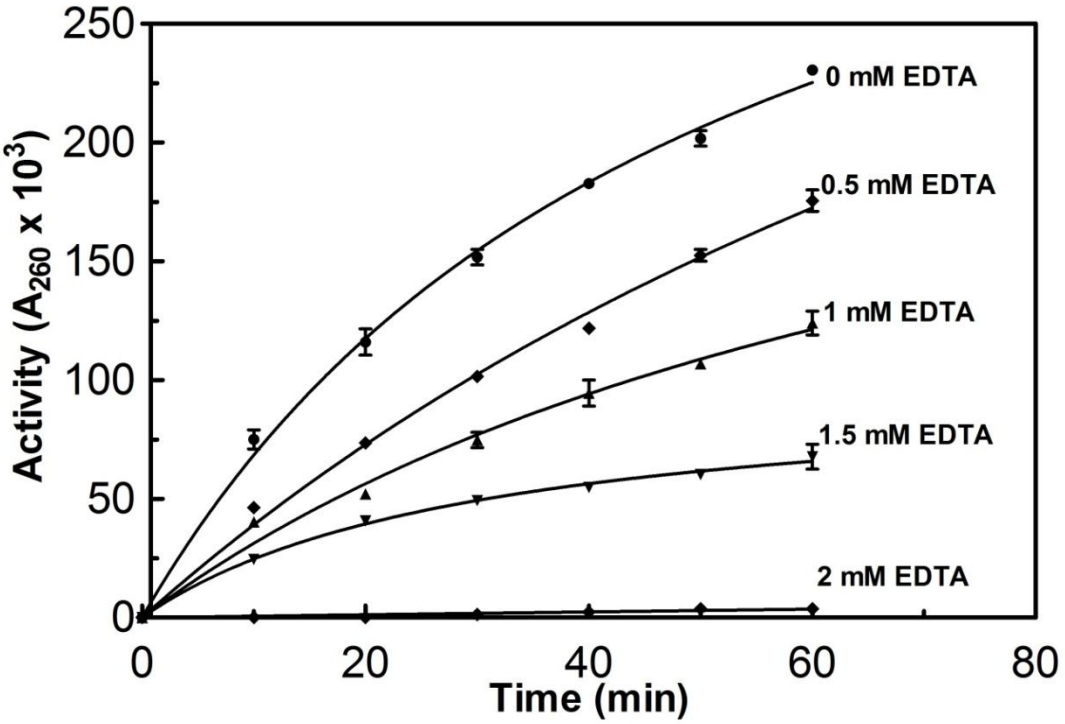


Fig. 15– Effect of EDTA on DNase activity. Results were obtained using the real-time spectrophotometric assay at 55° C with the designated amount of EDTA added to the substrate.

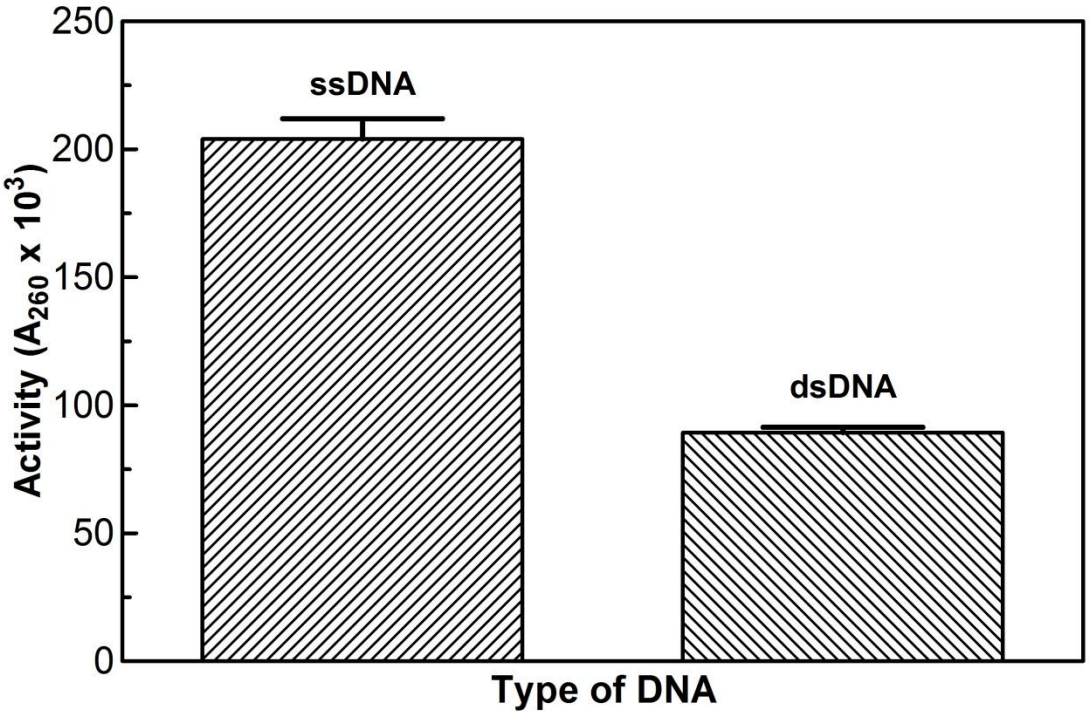


Fig. 16– Difference in DNase specificity towards ssDNA and dsDNA. The activity was determined using the acid soluble acid at 55° C with a 60 minute digestion time. There was significant difference in activity between the substrates when compared using an unpaired t-test with a 95% confidence interval.

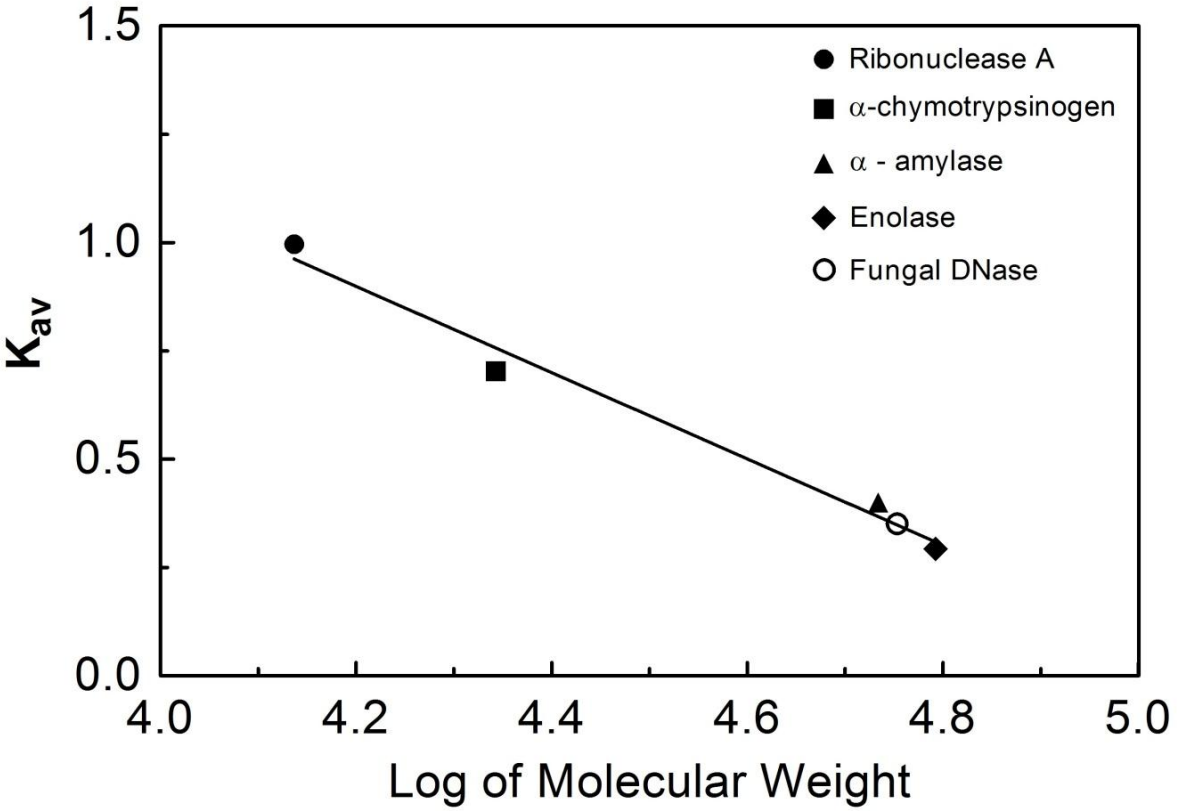


Fig. 17– Molecular weight determination of the purified DNase. Molecular weights of the protein standards were: ribonuclease A, 13,700; α-chymotypsinogen, 25,000; α-amylase, 51,000; and enolase, 82,000. The log molecular weight of the DNase was determined to be 4.753. This is roughly equal to a molecular weight of 56 kDa.

The K_{av} values were determined for all standard proteins and the purified DNase using the equation:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

$V_e = elution\ volume\ for\ the\ protein$

$V_o = elution\ volume\ for\ Blue\ Dextran\ 2{,}000$

$V_t = total\ bed\ volume$

The calculated K_{av} values were plotted against the log molecular weight of the standard proteins(Figure 17). Plotting the K_{av} value for the purified DNase resulted in a log molecular weight of 4.753 which is roughly equal to a molecular weight of 56,000 Da.

Mode of Attack for DNase

To determine the method in which the DNase attacked DNA, purified DNase was allowed to digest a DNA substrate for 30, 60, and 90 min. 20 µL of digested substrate electrophoresed through a 0.3% (w/v) agarose gel, stained (EZ-Vision Three DNA Dye, Amresco, Lot# 1091C159) and visualized under UV light. The NIH photo analyzing program Image J was used to measure relative fluorescence of the visualized bands.

The decrease in relative fluorescence over the course of digestion can be seen in Figure 8 and Table 6. Following 90 min of digestion, the intensity in relative fluorescence decreased a total of 85%. Since single nucleotides cannot be visualized, only the decrease in molecular weight and relative fluorescence can be detected.

The DNase exhibited exonuclease activity based on the single point progression of the digested substrate through the gel (Figure 19). An endonuclease would have given rise to the visualization of multiple DNA bands of varying size. Exonucleases cleave off one nucleotide at a time, slowly decreasing the molecular weight of the substrate. In respect to the purified DNase, the digested substrate progressed as a single point, traveling further down the gel with increased digestion times indicating a decrease in molecular weight which is characteristic of exonuclease activity.

DNase K_m and V_{max}

The K_m and V_{max} for the purified DNase was estimated by plotting the velocities against the substrate concentration (Figure 18). The velocities for each concentration were generated using the real-time spectrophotometric assay at 55° C. The DNase was estimated to have a K_m value of 149 and a V_{max} value of 0.008.

Table 6

Decrease in relative fluorescence over time

Digestion Time (min)	Relative Fluorescence	Decrease (%)
0	111807	0
30	65641	41
60	30823	72
90	16932	85

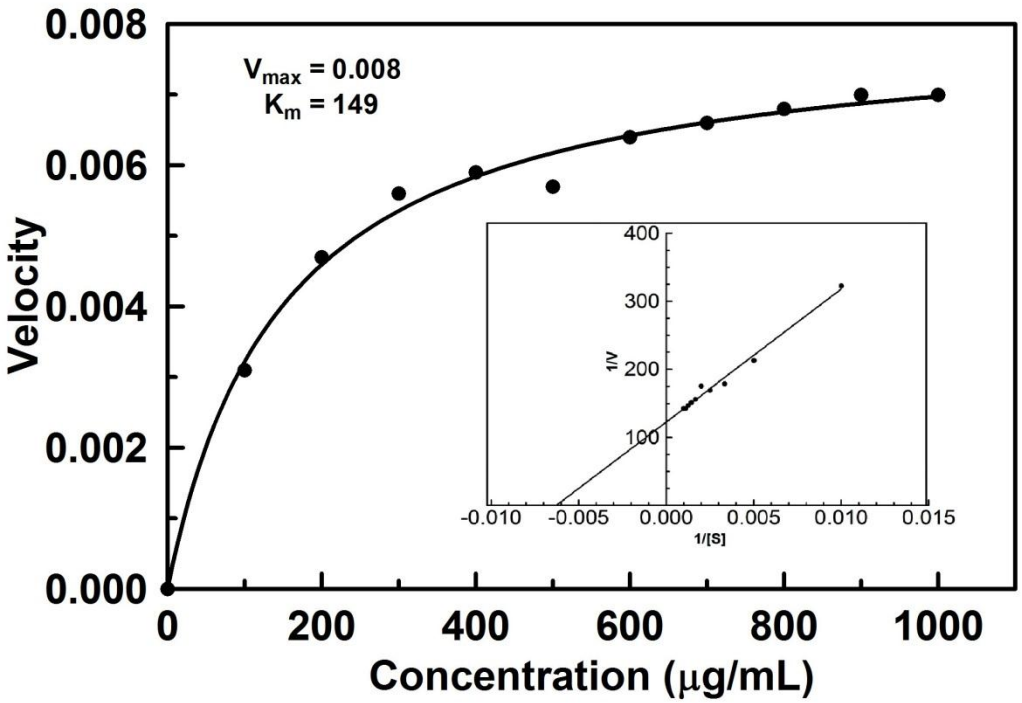


Fig. 18– Michaelis-Menten and Lineweaver-Burk plots of DNase activity. The K_m value was estimated to be 149 with a V_{max} of 0.008. The real-time spectrophotometric assay at 55° C with varying DNA substrate concentrations was used to determine the velocities. The data was then converted to make the Lineweaver-Burk plot.

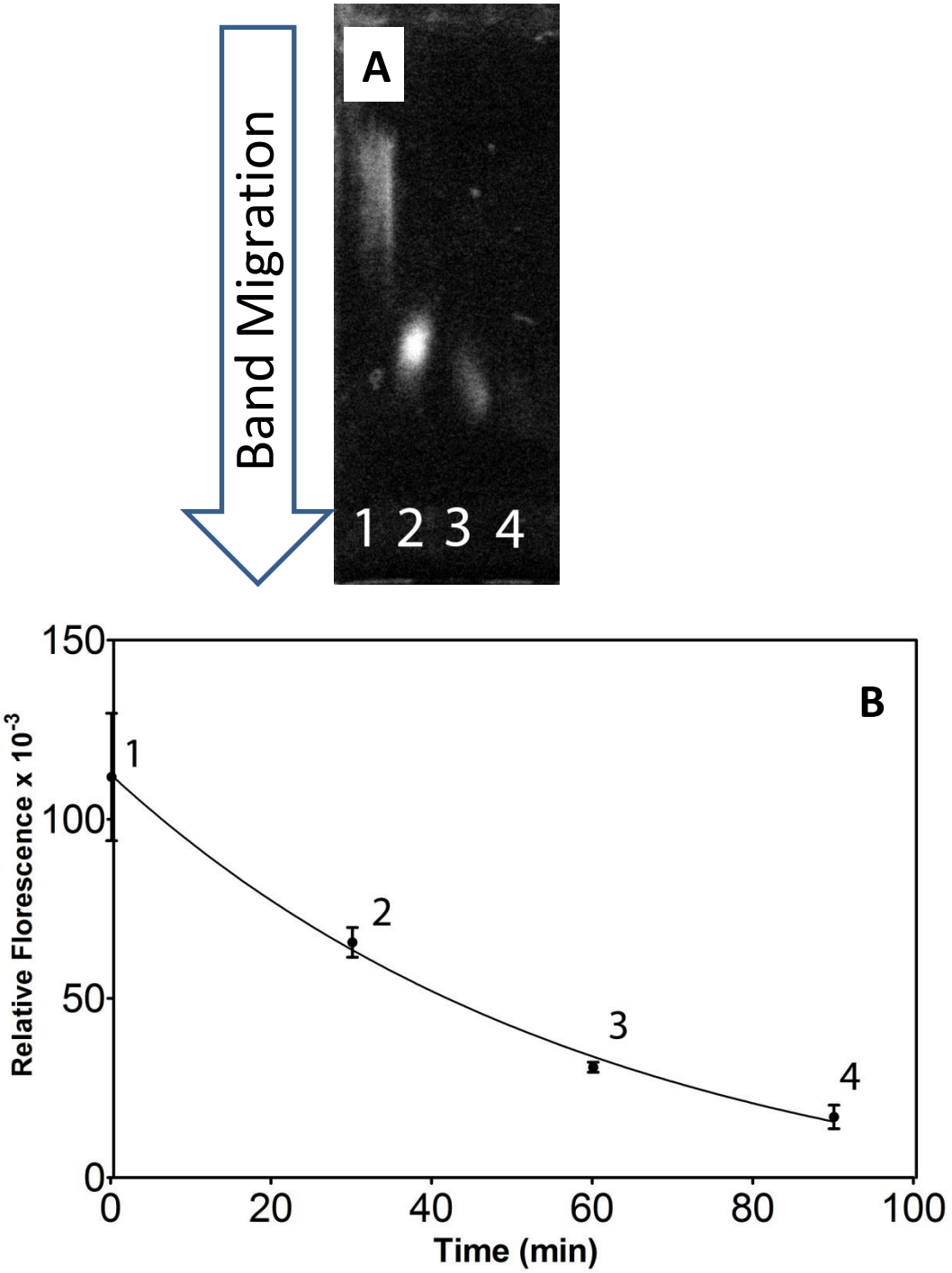


Fig. 19– Determining the method in which the DNase attacked the substrate; A, a typical agarose gel image of digested substrate with lanes: 1) undigested substrate, 2) 30 minute digestion, 3) 60 minute digestion, 4) 90 minute digestion; B, Plot of the decrease in relative fluorescence over time. Each point can be referenced to the corresponding numbered lane in A. Relative florescence from 3 agarose gels were used to generate the plot.

Characterization of the Partially Purified RNase

Effect of Temperature on Enzyme Activity

The effect of temperature on enzyme activity for the partially purified RNase was assessed two ways. The first method involved using the acid soluble assay at various temperatures. Using this method the optimal and maximum temperature for enzyme activity was determined to be 70° C and 95° C with no measurable activity above the maximal temperature (Figure 20.1). The activity at the optimal temperature was 73% greater than at 50° C.

The second method used a modified version of the real-time spectrophotometric assay developed by Kunitz (1946). 1.0 ml of partially purified RNase was added to 2.0 ml of substrate and the decrease in absorbance at 300 nm was recorded as seen in Figure 20.2. Prior to analysis both the sample and buffer were equilibrated to the desirable temperature. Using this method the optimal temperature was again found to be 70° C however the maximum temperature was not assessed using this method.

The rate of partially purified RNase denaturation was assessed in a similar manner to that of the DNase. Based on the results from the acid soluble assay, 90 and 95° C were the temperatures selected. The denaturation plot can be seen in Figure 20.3. An enzyme sample exposed to 90° C for 10 min resulted in a 92% decrease in activity when compared to unexposed enzyme. After 7 min at 95° C, 100% of the activity was lost while enzyme exposed to 95° C for 6 min only lost 95% of the activity.

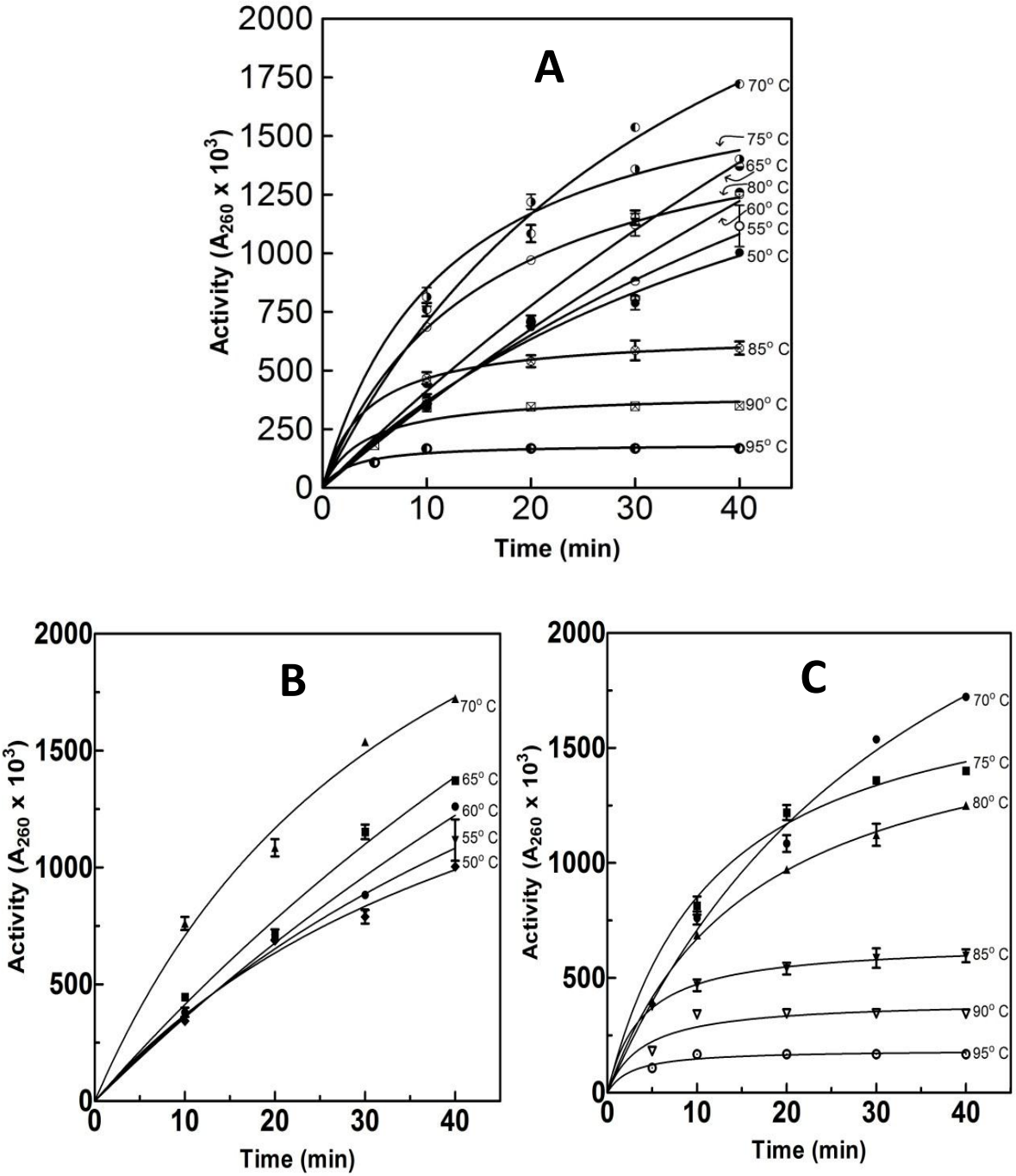


Fig. 20.1– The effect of temperature on the activity of the partially purified RNase. The plots are as follows: A, plot of all of the temperatures assayed for activity; B, plot of the temperatures 50 - 70° C; and C, plot of the temperatures 70 - 95° C. Activity was measured using the acid soluble assay which was performed in triplicate.

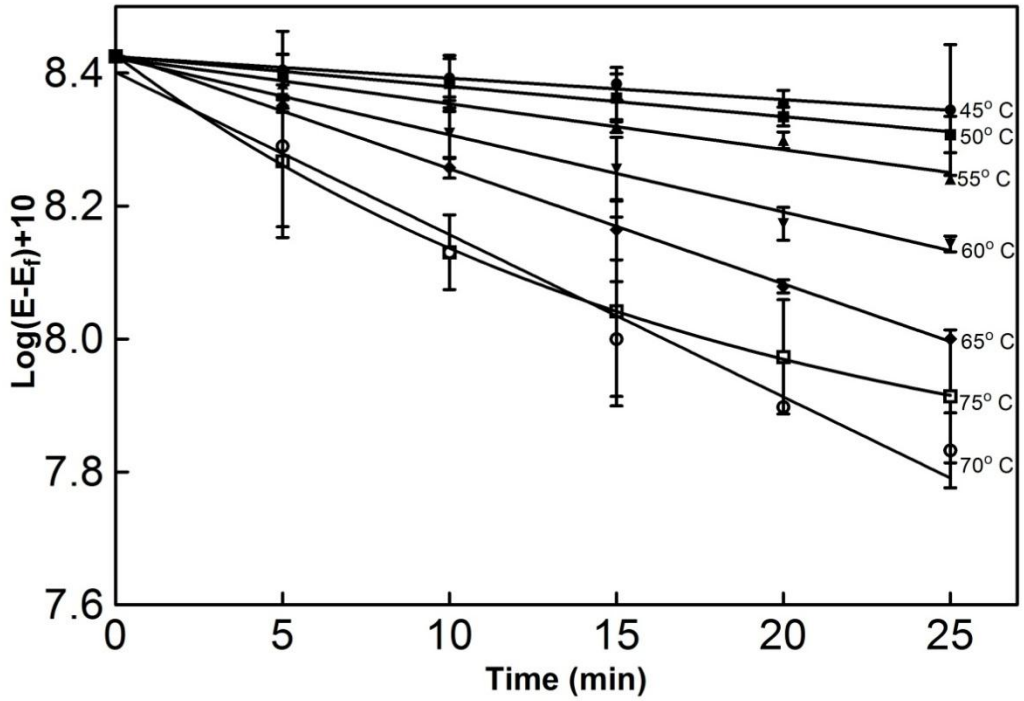


Fig. 20.2– Effect of temperature on partially purified RNase activity. Measurements were in triplicate using a modified version of the real-time assay developed by Kunitz (1946).

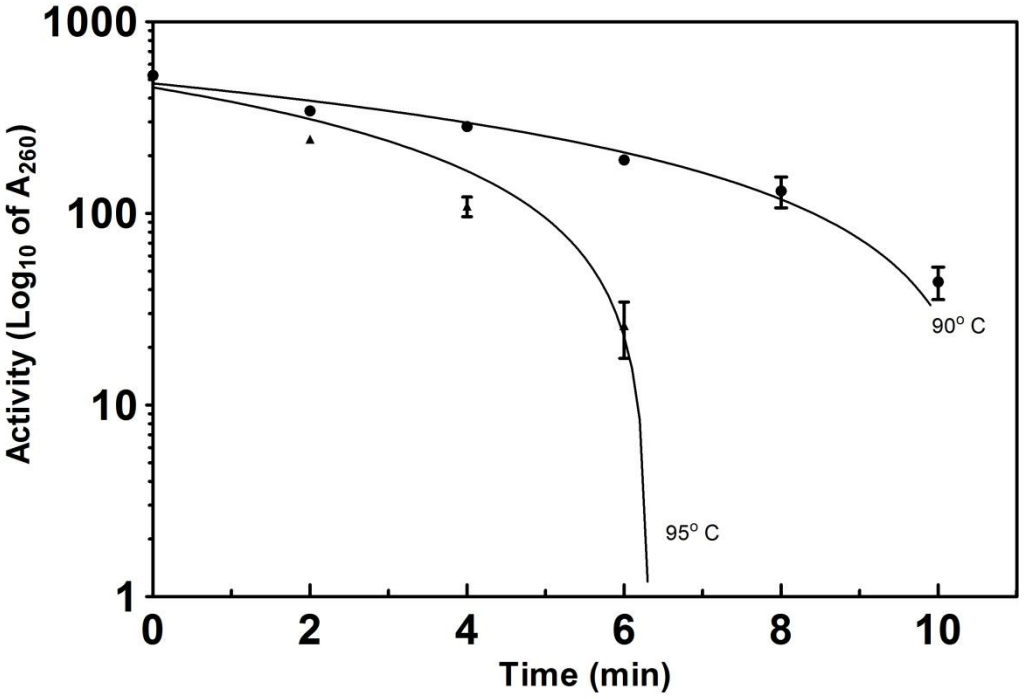


Fig. 20.3– Plots of the denaturation of partially purified RNase at 90 and 95° C. The assay used for this experiment was a modified version of the acid soluble assay which is explained in detail in the materials and methods section.

Effect of pH on Enzymatic Activity

The activity of the partially purified RNase was determined at various pH values between pH 2 and 10. A modified version of the acid soluble assay was used which involved adding 0.75 ml of partially purified RNase to 0.75 ml of substrate (yeast RNA; 1mg/ml (Sigma R6625 Lot# 110M1168V), 10 µmol $MgSO_4$ in a 0.1M imidazole, malic acid, and glycine buffer, at various pH) and allowed to incubate at 55° C for 10 min. The reaction was stopped by adding 0.5mL of uranylacetate-perchloric acid reagent (0.25% uranylacetate in 10% perchloric acid). The rest of the procedure was carried out as described in the materials and methods section. The buffering system used for this assay was the same as the one used for the purified DNase.

The partially purified RNase demonstrated activity throughout the pH range of 3 – 9 and had optimal activity at both pH 3 and 7 (Figure 21). No activity was detected at pH 2 or 10. The presence of two optimal pH peaks may indicate the presence of two different RNases. Upon recollection of Figure 11.2, two peaks are present for RNase activity. However Figure 4 shows a single prominent band which raises the question; if there is only one band how can two different nucleases be present?

It could be possible that the enzymes are of similar molecular weight and do not separate under electrophoresis or by size exclusion chromatography. Yet when the partially purified sample was passed through the ceramic hydroxyapatite column the enzymes partially separated generating the two peaks see in Figure 11.2. Ceramic hydroxyapatite separates protein based on charge and is often used to separate proteins that show as a single band after electrophoresis (CHT™ Ceramic Hydroxyapatite Instruction Manual). Therefore to obtain a better understanding of the system, partially purified RNase would have to be purified using ceramic hydroxyapatite chromatography.

Effect of Dialysis and EDTA on Enzymatic Activity

The effect of dialysis and EDTA on the activity of partially purified RNase was assessed. Partially purified RNase was dialyzed by filtering the sample with 500 ml of deionized water using a pressure cell (Amicon© 500mL pressure cell) equipped with a 10 kDa regenerated cellulose membrane (Millipore© Utrafiltration YM10 Dia. 76mm, Lot # COSA17530) ensuring that the final sample volume was the same as the initial sample volume prior to dialysis. It was found that there was no significant difference between dialyzed and non-dialyzed sample, indicating that Mg^{2+} may not be the co-factor for the partially purified RNase (Figure 22.1).

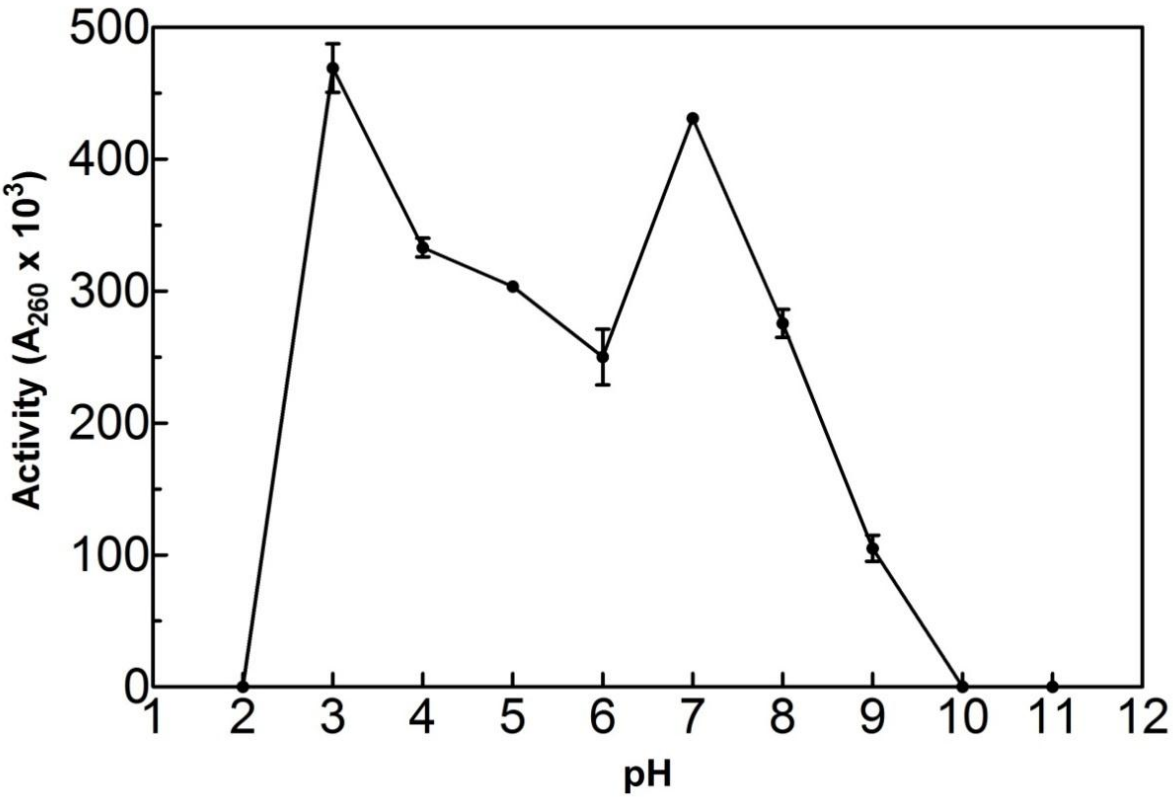


Fig. 21– Plot of the partially purified RNase pH profile. The activity was measured using a modified version of the acid soluble assay at 55° C with varying pH.

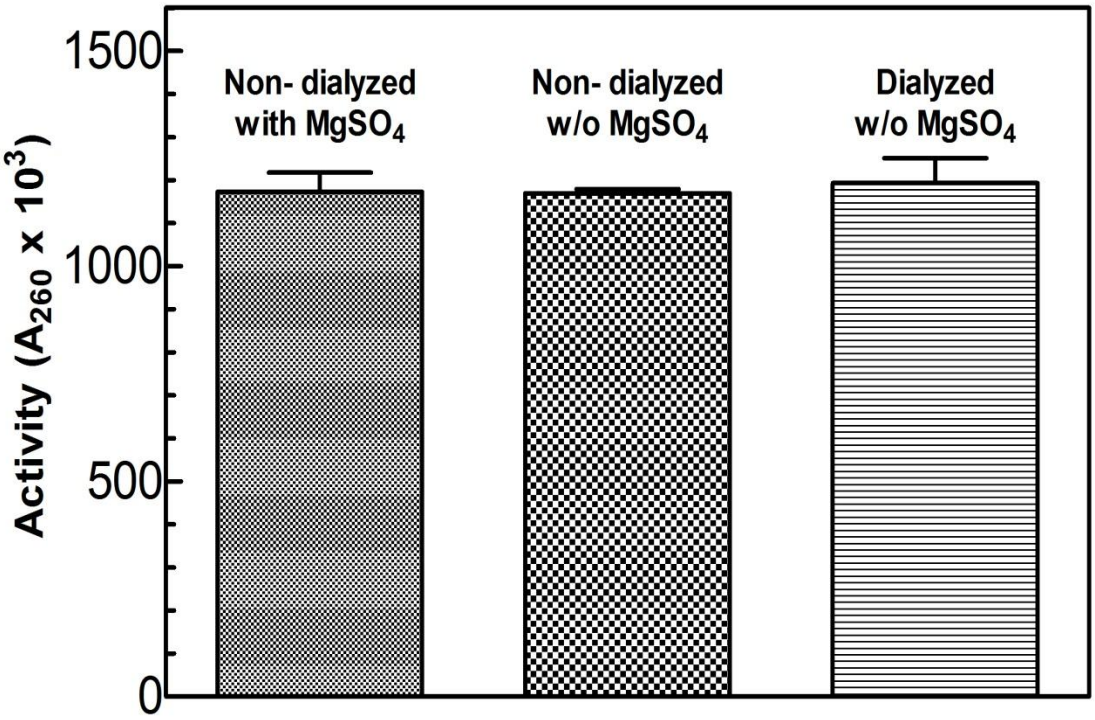


Fig. 22.1–Effect of dialysis and the addition of $MgSO_4$ on enzyme activity is displayed in this figure. Dialyzed and non-dialyzed samples assayed for activity without the addition of $MgSO_4$ had no significant difference in activity when compared to a non-dialyzed sampled assayed with $MgSO_4$. The acid soluble assay was used for this experiment following a 40 min digestion time and was performed in triplicate.

The effect of EDTA on enzyme activity was also examined. 0.75 ml of partially purified RNase was added to 0.75 ml of substrate (yeast RNA; 1mg/ml (Sigma R6625 Lot# 110M1168V), 2 mM $MgSO_4$, with 0 mM, 0.5 mM, 1.0 mM, 1.5 mM, or 2.0 mM EDTA in 0.1 M imidazole, pH 7)) and incubated in a water bath set to 55° C. The reaction was stopped by adding 0.5mL of uranylacetate-perchloric acid reagent (0.25% uranylacetate in 10% perchloric acid). The addition of 2.0 mM of EDTA resulted in only a 35% decrease in enzyme activity. All activity was stopped when 20 mM of EDTA was added (Figure 22.2).

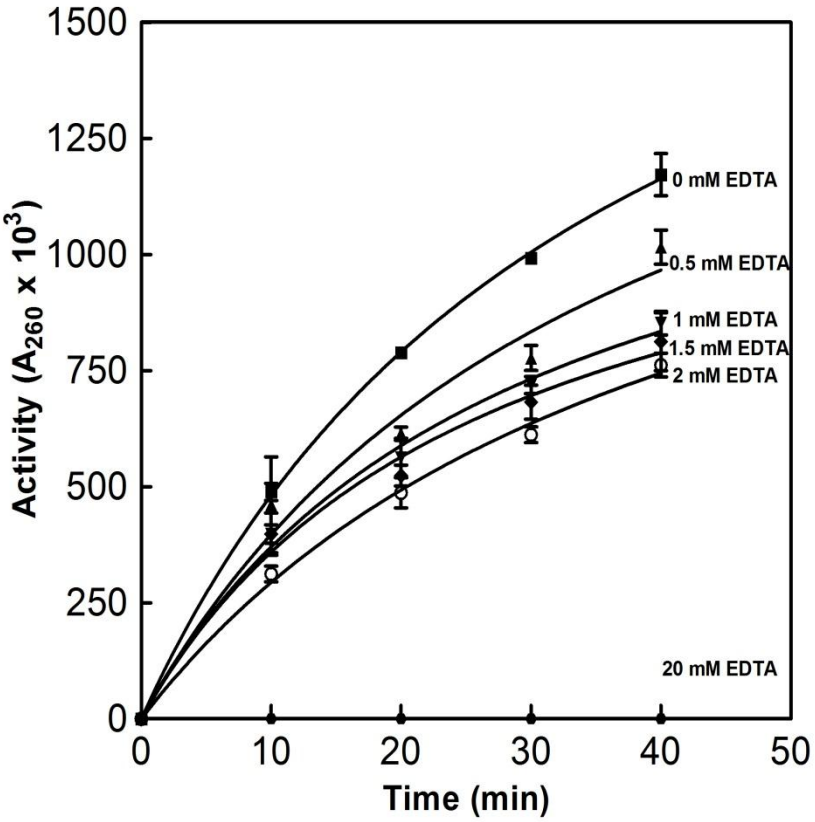


Fig. 22.2–The effect of EDTA on partially purified RNase activity. The results were obtained using the acid soluble assay at 55° C with the designated amount of EDTA added to the substrate.

Effect of Metal Ions on Enzymatic Activity

Partially purified RNase stripped of its metal co-factor from the addition of 20 mM EDTA/ml was used to determine the effect of different metal ions on enzyme activity. The ions tested were zinc (as zinc acetate, Fisher Scientific, Lot# 771994), magnesium (as magnesium sulfate, Fisher Scientific, Lot# 901737), and manganese (as manganese chloride, Fisher Scientific, Lot# 764882); 50 mM of the desired metal ion was added to the substrate to ensure that the metal ions would not be sequestered by unbound EDTA. The acid soluble assay at 55° C was used to measure enzyme activity for all metal ions (Figure 23). The metal ions zinc and manganese both demonstrated the highest amount of activity with only a 3% difference in

activity between them. The magnesium ion resulted in about 50% of the activity achieved with the zinc and manganese ions suggesting that the metal ion zinc or manganese results in higher enzyme activity than magnesium.

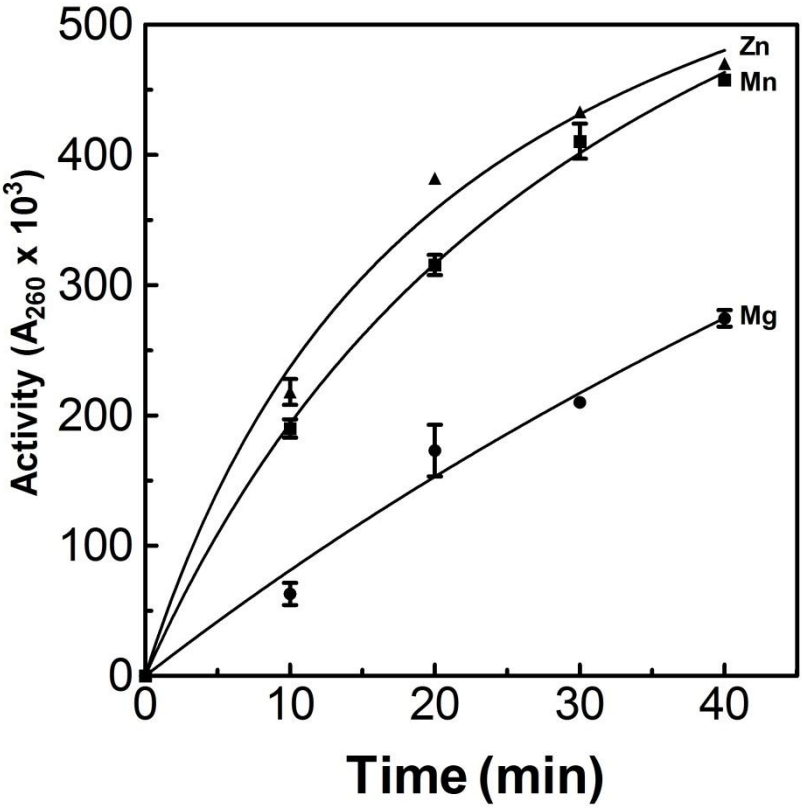


Fig. 23–The effect of various metal ions on partially purified RNase activity. The results were obtained using the acid soluble assay at 55° C with 50 mM/per ml of the designated metal ions added to the substrate. Prior to the experiment 20mM/per ml of EDTA was added to the partially purified RNase.

Estimated Molecular Weight of the Partially Purified RNase

The molecular weight of the partially purified RNase was estimated using a column of Sephadex G-75. K_{av} values of enzymes with known molecular weights were used to calculate the log of the molecular weight of the RNase from a standard curve. The method used was

performed in the same manner as the estimation of the purified DNase. Chromatography of the RNase yielded a log molecular weight of 4.84 and therefore the partially purified RNase has an estimated molecular weight of 69,000 (Figure 24).

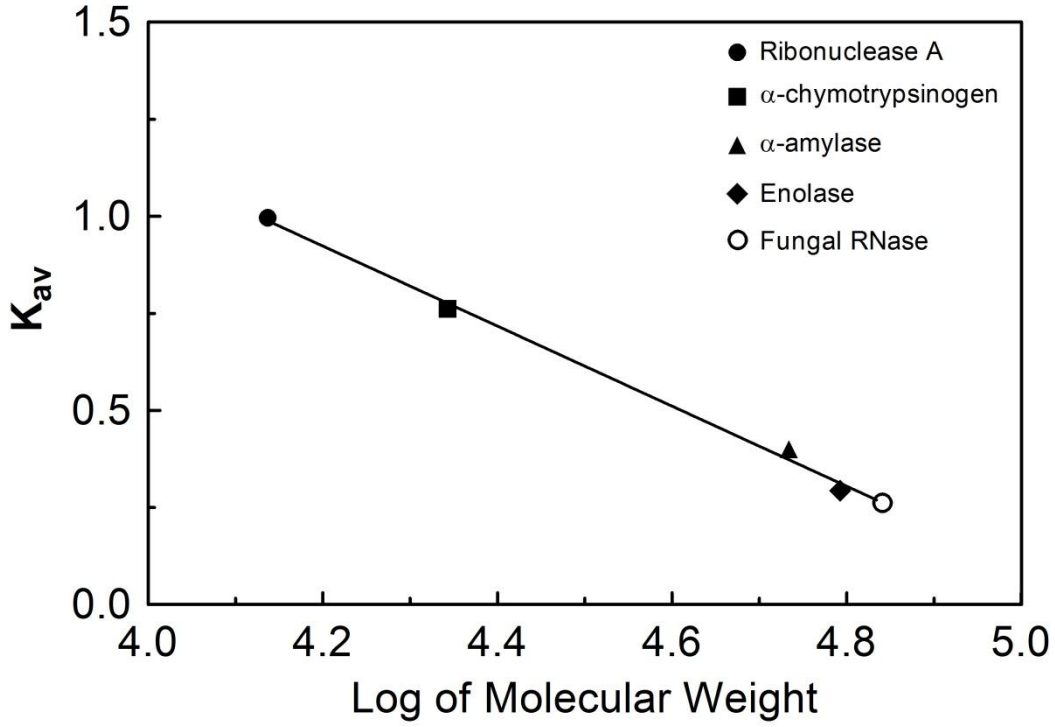


Fig. 24– Molecular weight determination of the partially purified RNase. Molecular weights of protein standards were: ribonuclease A, 13,700; α-chymotypsinogen, 25,000; α-amylase, 51,000; and enolase, 82,000. The log molecular weight of the DNase was determined to be 4.841 which is roughly equal to a molecular weight of 69 kDa.

RNase K_m and V_{max}

The K_m and V_{max} for the partially purified RNase was estimated by plotting the velocities against the substrate concentration (Figure 25). The velocities for each concentration were generated using the acid soluble assay at 55° C with varying concentrations of RNA. The RNase was estimated to have a K_m value of 169 and a V_{max} value of 0.019.

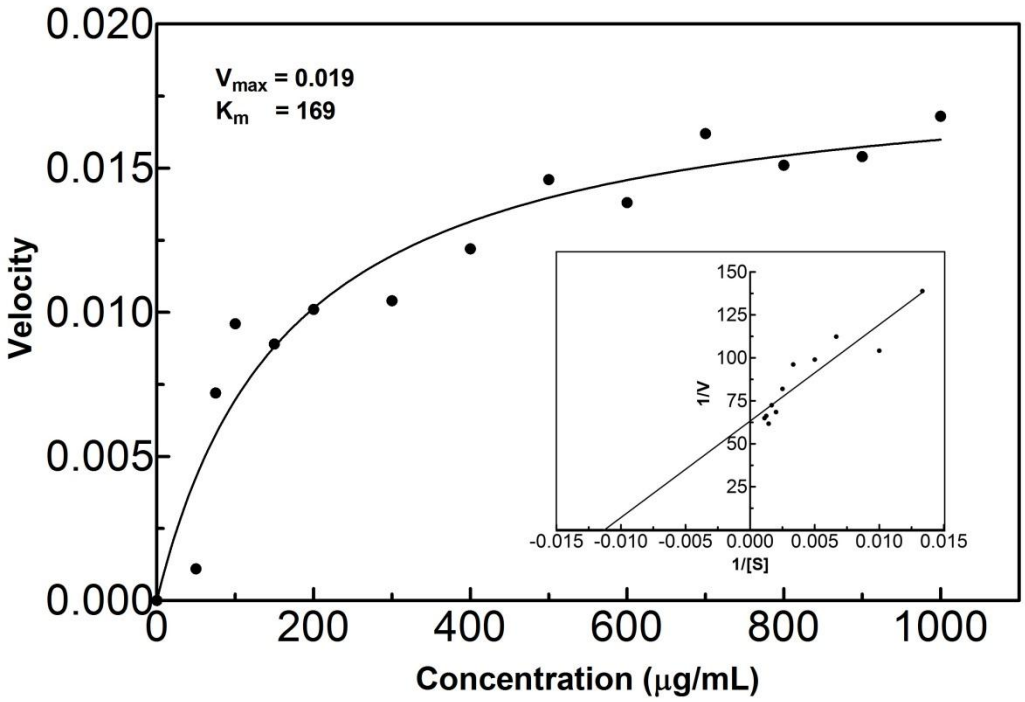


Fig. 25– Michaelis-Menten and Lineweaver-Burk plots of RNase activity. The K_m value was estimated to be 169 with a V_{max} of 0.019. The acid soluble assay at 55° C with varying RNA substrate concentrations was used to determine the velocities. The data was then converted to generate the Lineweaver-Burk plot.

Mode of Attack for the Partially Purified RNase

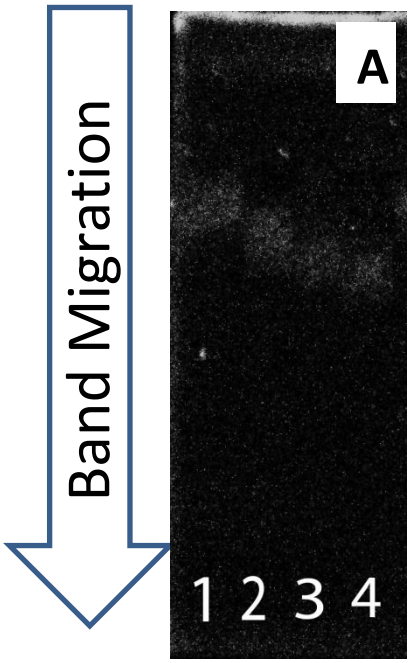
To determine the mode in which the RNase attacked the substrate, partially purified RNase was allowed to digest RNA substrate for 10, 20, and 30 min. The process of loading and running the 0.3% agarose gel was identical to the DNase method; however the bands were visualized using 0.2 μg/ml ethidum bromide. The decrease in relative fluorescence can be seen in Figure 25 and Table 7. A 30 min digestion of the RNA substrate resulted in a 51% decrease in relative fluorescence. Like the DNase, the partially purified RNase also demonstrated exonuclease activity since only one band was visualized during the digestion. The migration

pattern of the bands also suggests a uniform decrease in the molecular weight of the substrate with an increase in digestion time.

Table 7

Decrease in relative fluorescence of RNA substrate over time

Digestion Time (min)	Relative Fluorescence	Decrease (%)
0	**35333**	**0**
10	**27624**	**22**
20	**22014**	**38**
30	**17295**	**51**



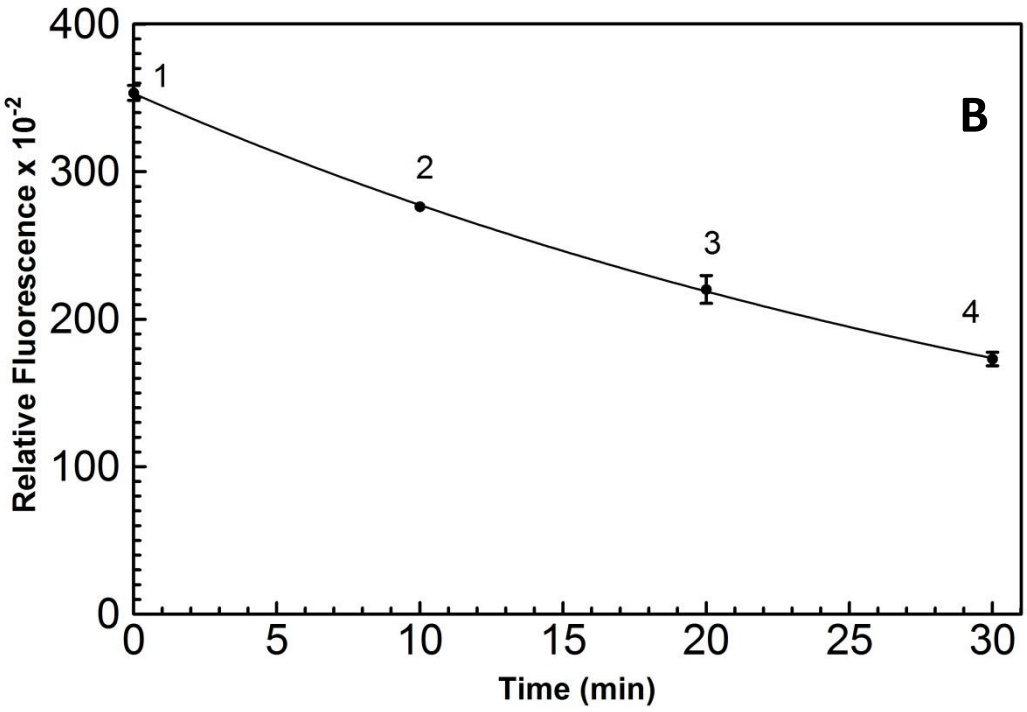


Fig. 26– Determining the method in which the partially purified RNase attacked the RNA substrate; A, a typical agarose gel image of digested substrate with lanes: (1) undigested substrate, (2) 10 minute digestion, (3) 20 minute digestion, (4) 30 minute digestion; B, Plot of the decrease in relative fluorescence over time. Each point can be referenced to the corresponding numbered lane in A. Relative florescence from 3 agarose gels were used to generate the plot.

Summary and Conclusion

Seven thermophilic fungi were isolated from the compost collected around the Amherst, MA area, but only one demonstrated DNase activity at 55^{o}C. The organism was identified with a 91% match as a Chaetomium sp. by sequencing the highly conserved ITS region of the fungus and comparing to known regions in a genomic database. The strain was referred to as TM-417. A culture medium containing 0.4% yeast extract, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 1.5% Soluble Starch, 0.005% methyl green, and 0.2% DNA adjust to pH7 was found to be optimal for DNase production by TM-417. Maximum DNase activity was achieved after five days of growth (3 days agitated at 115 RPM and 2 days static) at 55^{o} C. Complete decoloration of the indicator dye methyl green also occurred after five days of incubation.

The plots of crude and concentrated nucleases were compared by analyzing the generated slopes. These slopes were directly related to the rate of enzyme activity. When the slopes of the crude and concentrated samples were analyzed, 98% of enzyme activity was recovered for both DNase and RNase, with an 8.96-fold and 8.9-fold increase in specific activity. The concentrated sample was subjected to gel filtration on Sephadex G-50. The active fractions from Sephadex G-50 gave a 73.59-fold and 91.5-fold increase in specific activity for the DNase and RNase respectively when compared to the crude sample. For the final stage of purification, the partially purified sample was subjected to affinity membrane purification. The increase in specific activity for the DNase and RNase following this purification step was 145.58-fold and 127.6-fold when compared to the crude sample.

Electrophoresis of the purified DNase on a 4-15% polyacrylamide gradient gel yielded a single band indicating DNase homogeneity. In terms of the RNase, electrophoresis on a 4-15% polyacrylamide gradient gel also yielded a single band, however based on the previous

purification attempts using ceramic hydroxyapatite the level of purification could not be determined. Initial purification via ceramic hydroxyapatite resulted in two peaks of RNase activity. However when the RNase was partially purified using the affinity membrane purification system, it was assumed that if the sample was subjected to ceramic hydroxyapatite chromatography two peaks of activity would be present. Even though the partially purified RNase sample was visualized as one band under electrophoresis, the two peaks from ceramic hydroxyapatite purification indicates the possibility that multiple RNases are present.

Both DNase and RNase were dependent on metal co-factors for activity. The metal ion Mg^{2+} was the preferred ion for the DNase, whereas for the RNase, Zn^{2+} and Mn^{2+} yielded an increase in enzyme activity over that with Mg^{2+}. Both nucleases were inhibited by EDTA.

The purified DNase demonstrated maximum activity at pH 6.0 with no activity at pH 2.0 or 10.0. The RNase exhibited two peaks of maximum activity, on at pH 3.0 and the other at pH 7.0 with no activity at pH 2.0 or 10.0.

Both nucleases were notably stable to elevated temperatures. The optimal temperature for the purified DNase was 65^{o}C. However, over 88% of the enzyme activity was lost after heating the purified sample at 68^{o} C for 30 min. The optimal temperature for the partially purified RNase was found to be 70^{o}C. All activity was lost after heating the RNase at 95^{o} C for 7 min.

The molecular weight of both nucleases was determined using a Sephadex G-75 column. A standard curve was generated using several standard proteins of known molecular weight. The molecular of the DNase and RNase were determined to be 56 kDa and 69kDa respectively.

A membrane based affinity purification system was developed for the purification of the DNA specific nuclease, DNase I. Single stranded DNA was bound to unmodified polyvinylidene fluoride (PVDF) membranes which were used to purify DNase I from a solution of bovine serum albumin. Using 10 coated membranes a 6-fold increase in specific activity was achieved with 80% enzyme recovery.

This novel method was then applied to a biological sample with great success. Affinity membrane purification is a simple, yet powerful tool that is comparable to the results of traditional chromatography. Overall, this method is very practical since separation was achieved with minimal loss of activity.

Appendix

APPENDIX
SUPPLEMENTARY MATERIALS

Media:

1) Yeast protein soluble starch medium (YpSs) (Cooney & Emerson, 1964)

   0.4% Yeast Extract
   0.1% K_2HPO_4
   0.05% $MgSO_4$
   1.5% Soluble Starch
   Adjust to pH7

2) Yeast protein soluble starch DNase medium (DYpSs)

   0.4% Yeast Extract
   0.1% K_2HPO_4
   0.05% $MgSO_4$
   1.5% Soluble Starch
   0.005% Methyl Green
   0.2% DNA
   Adjust to pH 7

3) DNase test medium (DTest)

   0.1% K_2HPO_4
   0.05% $MgSO_4$
   0.005% Methyl Green
   0.2% DNA
   0.02% Sodium Azide
   Adjust to pH 7

4) Yeast-glucose medium (YG) (Cooney & Emerson, 1964)

   0.5% Yeast Extract
   1% Glucose

5) Oatmeal medium (OA) (Cooney & Emerson, 1964)

   5% Oatmeal
      (In 700 ml tap water, steamed for one hour and strained through cheesecloth)

6) Czapek's medium (C3) (Cooney & Emerson, 1964)

0.3% $NaHO_3$
1% K_2HPO_4
0.5% $MgSO_4 \bullet 7H_20$
0.5% KCL
0.01% $FeSO_4 \bullet 7H_2O$
3% Sucrose

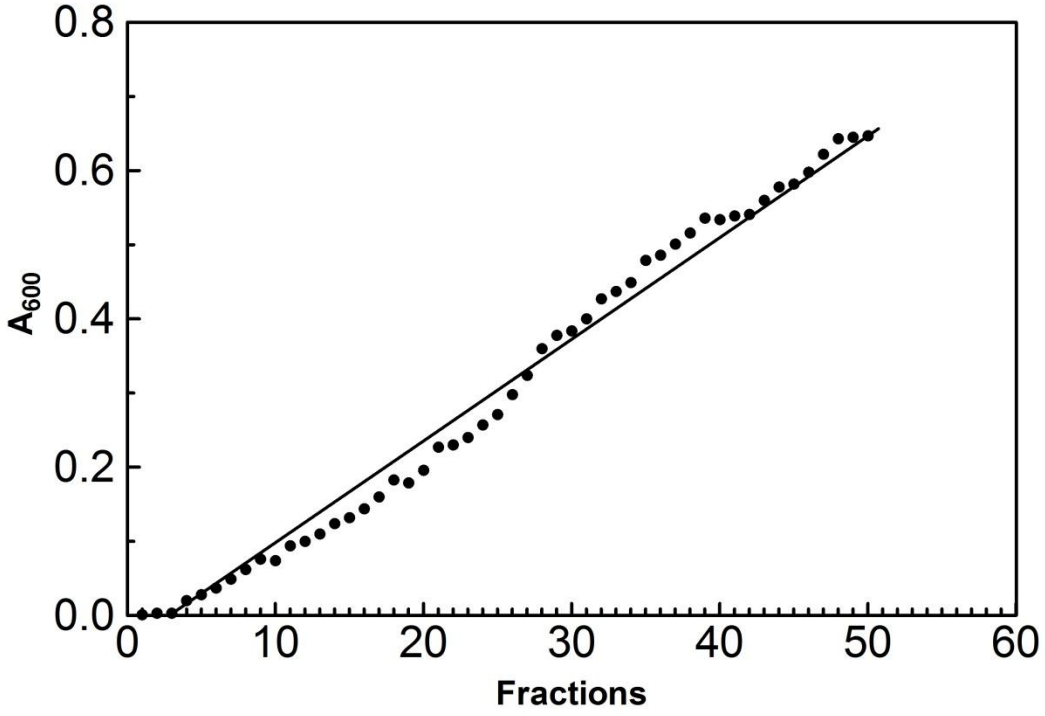


Fig. 27– The rate of the elution gradient for ceramic hydroxyapatite chromatography determined using crystal violet and recording the absorbance at A_{600} for each fraction.

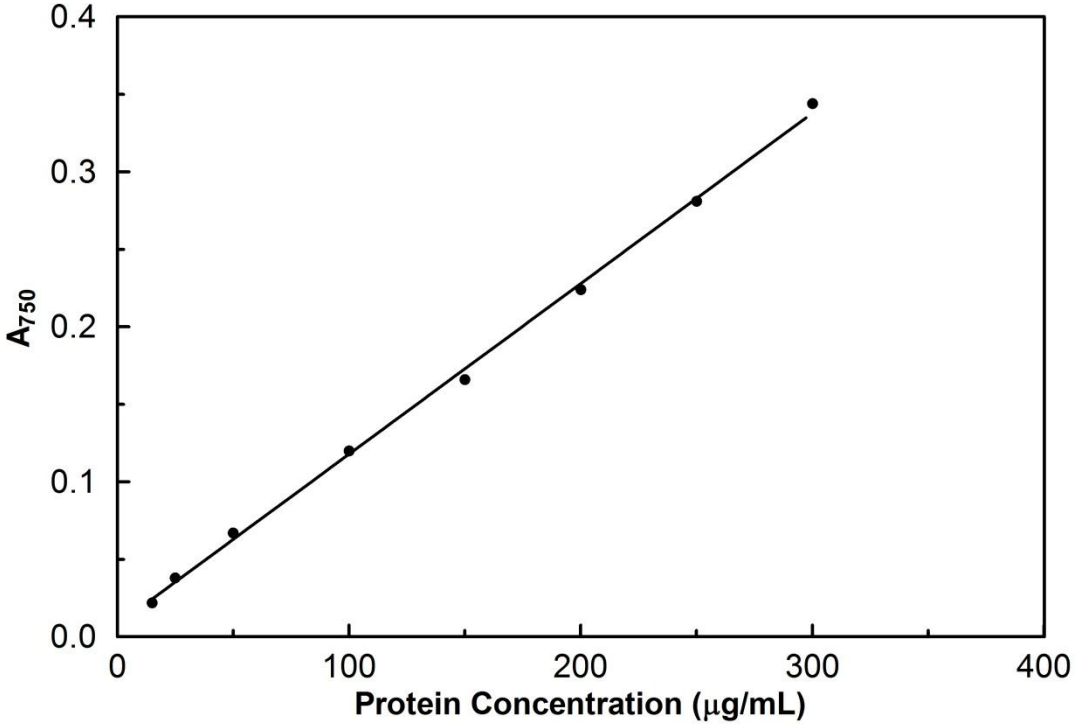


Fig. 28– Standard curve for the Lowry assay which was determined using various concentrations of bovine serum albumin.

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