Purification and characterization of a novel protease from *Burkholderia* strain 2.2 N

By

Sally Nicole Jewell

Thesis submitted to the faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of

Master of Science

In

Microbiology

Joseph O. Falkinham, III., Ph.D., Co-Chair
Malcolm Potts, Ph.D., Co-Chair
Charles Hagedorn, Ph.D.

September 6, 2000
Blacksburg, Virginia

Keywords: protease, protein purification, *Burkholderia, Pseudomonas*

Purification and characterization of a novel protease from *Burkholderia* strain 2.2 N

Sally Nicole Jewell

ABSTRACT

The bacterium *Burkholderia* strain 2.2 N is a soil isolate and a member of a group of non-obligative predator bacteria that can prey on other microorganisms or grow saprophytically. The bacterium has anti-bacterial, anti-fungal, anti-yeast and anti-protozoan activities. *Burkholderia* strain 2.2 N culture shows hydrolysis on Milk Casein Agar, indicating the bacterium also produces a protease.

Azocoll hydrolysis was used to detect and measure protease activity. Protease activity was two-fold higher at pH of 7.5 than pH 9.0 and 25-fold at pH 4.0. Cultures grown in media containing 1.0 % yeast extract (YE), tryptic soy, tryptone or beef extract had protease activity, whereas activity was absent in cultures grown in media containing peptone, soytone, casitone, or tryptone as sole protein source. Addition of 1.0 % sucrose or glucose to 1.0 % YE medium increased protease activity 1.8-fold and 1.4-fold, respectively. Protease activity was 2-fold higher in cultures grown in media containing 1.0 % YE and 10 mM $MgCl_2$ or $FeCl_2$, than in 1.0 % YE medium lacking metals or containing 10 mM $MnCl_2$ or $CaCl_2$. The 1.0 % YE medium containing either $ZnCl_2$ or $CuCl_2$ lacked protease activity (< 5.0 %). In cultures grown in 1.0 % YE at 30° C with rotation at 120 rpm, protease activity was higher in stationary phase (0.38 units /mg protein) than in exponential phase (0.04 units/mg protein). The *Burkholderia* strain 2.2 N

protease is evidently exported from cells because 86 % of the total proteolytic activity of cells was found in the cell-free culture medium.

The cell free filtered culture supernatant medium assayed at 4 °C had protease activity, however at a three-fold lower specific activity compared to the same supernatant assayed at 37 °C. Protease activity was lower in filtered culture supernatants stored at 4 °C, room temperature, and 30 °C. Protease activity in samples stored at 4 °C was only 40 % (24 hours) and 15 % (48 hours) of activity at time zero. Protease activity in samples stored at room temperature was only 45 % (24 hours) and 35 % (48 hours) of activity at time zero. Protease activity in samples stored at 30 °C was only 78 % (24 hours) and 9 % (48 hours) of activity at time zero.

Purification of the protease from filtered culture supernatant medium by ammonium sulfate precipitation, increased the protease activity 20-fold. An eluted protein fraction from DEAE-Sepharose column chromatography had 50-fold higher protease activity. Protease activity was inhibited by 10 mM 1-10-phenathroline, EDTA and EGTA, all metalloprotease inhibitors. Purified protease activity inactivated with 10 mM 1-10-phenanthroline or 10 mM EGTA was regained through the addition of Ca^{2+} or Mg^{2+}. Protease activity was reduced by exposure to dithiothreitol (29 % with 1 mM and 84 % with 10 mM), a disulfide bond inhibitor. Protease activity was not inhibited by leupeptin or phenylmethylsulphonyl flouride.

Casein polyacrylamide zymography revealed a band of hydrolysis at approximately 60,000 Da. SDS-PAGE resolved a doublet band present at 60,000 Da present in both the filtered culture supernatant sample and the ammonium sulfate / DEAE-Shepharose column chromatography purified protease sample.

Burkholderia strain 2.2 N protease is a metalloprotease with a broad temperature range of activity. It has a molecular weight of approximately 60,000 Da.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Burkholderia strain 2.2 N

Burkholderia strain 2.2 N (ATCC 55961) is a novel bacterium isolated from a Hagerstown silty clay loam sample (pH 6.2). The soil sample was collected on the Pennsylvania State University campus under the supervision of Lester E. Casida, Jr., Ph.D., in 1995. The bacterium was isolated from one individual soil sample. Since the first soil isolation, isolates that share the same rRNA gene sequence and cultural and biochemical characteristics have been isolated from a sandy loam sample in South Hampton County, Virginia (unpublished data).

Burkholderia strain 2.2 N is a Gram-negative, aerobic, motile bacterium. The cells are approximately 0.5 - 1.0 μm in width by 1.5 - 3.0 μm long. The cells form 1 - 2 mm diameter amber-colored (AM-type) beehive shaped colonies on Tryptic Soy Agar medium containing 0.2 % sucrose, after 48 hours of incubation at 30° C. Strain 2.2 N grows between 25° C – 37° C, but poorly at 45° C. A transparent, flat, stable colonial variant (TP-type), approximately 2 - 3 mm in diameter, appears at a frequency of 1 - 4 % after growth in Tryptic Soy and Sucrose broth (TSB + S). The predominant colony type is the amber-colored, which gives rise to both amber and transparent types. The transparent colony type gives rise to only other transparent types.

Classification of *Burkholderia*

Species within what is now called the genus *Burkholderia* were originally classified as members of the genus *Pseudomonas*. However, recent studies have shown species of the genus *Burkholderia*, while sharing characteristics in common with members of the genus

Pseudomonas, are distinct and separate based on rRNA sequence and fatty acid composition (Kersters et al., 1996).

The genus *Pseudomonas* was created in 1894, however numerous incompletely described strains of aerobic, polarly flagellated, Gram-negative, rod-like bacteria were placed in this large group (De Ley, 1992). In the late 1960's work began on re-classification of the pseudomonads. Based on phenotypic similarities and deoxyribonucleic acid (DNA)-DNA hybridization experiments, pseudomonads were separated into five different "species groups" (Stanier et al., 1966). However some of the species were quite heterogeneous with respect to DNA homology (Palleroni et al., 1973). The Stanier group continued their classification work, showed there were clear distinctions between species of the pseudomonads based on rRNA:DNA hybridization results, and confirmed five genus groups (Palleroni et al., 1973). Additionally, on a wider variety of *Proteobacteria* species (which includes the pseudomonads), phylogenetic distance between each of the rRNA groups was experimentally determined using the rRNA:DNA hybridization experiments (De Ley, 1992; Gillis et al., 1995). Finally, phenotypic characterization, cellular lipid and fatty acid analysis, and 16S rDNA nucleotide sequencing confirmed the differences and led to discrimination of additional species (Kersters et al., 1996).

The most significant result of this re-classification, was the placement of a number of *Pseudomonas* species within the α-, β-, γ-subclasses of the Proteobacteria. Indicating a number of the *Pseudomonas* species were closely related to other genera, such as; *Xanthamonas, Vibrio, Enterobacteriaceae, Bordetella,* etc. (Kersters et al., 1996). Some of the *Pseudomonas* species that were renamed, are now members of the *Burkholderia* and the *Ralstonia* genus.

Genetic and phenotypic characteristics that distinguish *Burkholderia* from *Pseudomonas* and *Ralstonia* are listed in the table below.

Table 1: Distinguishing characteristics between the *Burkholderia, Pseudomonas* and *Ralstonia* genome's.

Characteristics	*Burkholderia*	*Pseudomonas*	*Ralstonia*
16s rRNA Similarity (%):			
Burkholderia	100	83	90
Pseudomonas	83	100	80
Ralstonia	90	80	100
Mol % G+C Content	66-68	60-68	64-67
Fusaric acid resistance	Yes	No	No
Cellular Fatty Acids:			
C19CPA	Yes	Yes	No
C16:1	Yes	Yes	Yes
Cellular Lipids:			
OL-1 and OL-2	Yes	Yes	No
AL-X	Yes	Yes	No
Hydrolysis of Esculin	Yes	No	No
Lysine and Ornithine Decarboxylase	Yes	No	No

(Yabuuchi et al., 1992, Xiang et al., 1993, Yabuuchi et al., 1995)

Although a number of genetic and biochemical characteristics distinguish *Burkholderia* from *Pseudomonas* and *Ralstonia*, there is one characteristic that these three species groups share; the production of an extracellular protease.

Biochemical Characteristics of *Burkholderia* strain 2.2 N

Cultural, enzymatic, biochemical characteristics, and 16s rRNA sequence comparison's of *Burkholderia* strain 2.2 N distinguish it from other members of *Burkholderia*. The bacterium

is oxidase- and indol-negative and reduces nitrates to nitrites. The enzyme activities exhibited by Strain 2.2 N are as follows; acid phosphatase, alkaline phosphatase, cysteine arylamidase, esterase (C4), esterase lipase, gelatinase, leucine arylamidase, N-acetyl-β-glucosaminidase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, β-glucosidase, and valine acrylamidase. Although the cells are capable of utilizing glucose for growth, Strain 2.2 N fails to ferment glucose or other carbohydrates. Compared to *Pseudomonas, Ralstonia,* or other *Burkholderia,* Strain 2.2 N utilizes a narrower range of substrates as carbon or energy sources. For instance, it is unable to utilize or form acids from the following; glycerol, inositol, mannitol, or sorbitol. It can, however, utilize the following substrates; adipic acid, D-arabinose, L-arabinose, capric acid, cellobiose, citric acid, esculin, D-fucose, galactose, gluconate, D-glucose, lactose, D-lyxose, malic acid, maltose, and mannose.

One characteristic that places this bacterium in the genus *Burkholderia,* is its resistance to a variety of antibiotics (e.g. tetracycline, cefotaxime, trimethoprim, amikacin, gentamycin, etc.) as well as a wide range of antimicrobial activity and the production of proteases.

Characteristics of Predator Bacteria

Strain 2.2 N shares characteristics in common with a group of non-obligate predator bacteria that can prey on other microorganisms or can grow saprophytically (Casida, 1988). These bacteria survive by utilizing soluble nutrients in the soil environment, or by utilizing nutrients obtained from prey cells that they have killed. Soluble nutrients are their first choice, but if unavailable, they will destroy prey cells (Casida, 1988).

Bacteria account for the largest portion of the microbial population of soil, in diversity and in numbers (Pelzcar et al., 1993). Those bacteria that prey, can coexist in the same soil

sample. Evidently, they do not attack one another, but if nutrient conditions are depleted these bacteria can attack both non-predator bacteria as well as other predator bacteria, playing an important and major role in controlling bacterial processes in soil (Casida, 1992). These processes include the maintenance of the microbial population as well as providing nutrients, such as cellulose and proteins, as well as other organic molecules. For less versatile biochemical species, bacteria are able to break down complex molecules into smaller, more readily metabolized molecules (Pelzcar et al., 1993).

Predator bacteria can inhibit the growth of a wide variety of organisms, for example; bacteria, fungi, yeast, protozoa, and other predators (Casida, 1988). This fact places them at the top of the hierarchy of non-obligate bacterial predators in soil (Casida, 1988 and Zeph et al., 1986).

Antimicrobial Activities

Common to many bacteria is the production of antimicrobial compounds or small molecules. *Burkholderia* strain 2.2 N has a broad range of extracellular antimicrobial activities. For example, these antimicrobial activities include, but are not limited to the following; anti-bacterial (e.g., *Micrococcus luteus* and *Escherichia coli*), anti-yeast (e.g., *Saccharomyces cerevisiae, Candida albicans* and *Cryptococcus neoformans*), anti-fungal (e.g., *Aspergillus niger* and *Botrytis cineria*), and anti-protozoan (e.g., *Tetrahymena pyriformis* and *Acanthamoebae polyphaga*) activities.

In addition to the production of two extracellular low molecular weight antimicrobial compounds (Falkinham, unpublished data), *Burkholderia* strain 2.2 N produces a protease. This was reflected by its ability to produce clear zones on Milk Casein Agar medium. Also, an

acetone precipitate of culture medium also exhibited cleared zones on Milk Casein Agar (Cain, unplublished). It is believed the protease is used in conjunction with its extracellular compounds for predatorial use within the environmental setting.

Proteases

Proteases form a large group of enzymes, ubiquitous in nature and found in a wide variety of microorganisms. They are molecules of relatively small size and are compact, spherical structures that catalyze the peptide bond cleavage in proteins (Polgar, 1989). These enzymes are important in a number of diverse and crucial biological processes; for example, they are involved in the regulation of metabolism and gene expression, enzyme modification, pathogenicity, and the hydrolysis of large proteins to smaller molecules for transport and metabolism (Rao et al., 1998).

Proteases are difficult to characterize because of their diversity of action and structure. Originally proteases were classified based on molecular size, charge or substrate specificity. However, with the advent of molecular biology, proteases are now grouped into families based on the following; chemical nature of the catalytic or active sites, mechanism(s) of action, and the evolutionary relationship of their three-dimensional structure (Beynon et al., 1989 and Rao et al., 1998).

Proteases are broadly divided into either exopeptidases or endopeptidases depending on their site of action. If the enzyme cleaves the peptide bond proximal to the amino or carboxy terminus of the substrate, they are classified as exopeptidases. If the enzyme cleave peptide bonds distant from the termini of a substrate, they are classified as endopeptidases.

Based on the functional group present at the active site and their catalytic mechanism, proteases are then categorized into four groups; serine proteases, aspartic proteases, cysteine /thiol proteases, or metalloproteases. Four classes of endoproteases have been identified in living organisms and three of the four classes of endoproteases have been isolated and purified in bacteria; serine, cysteine, and metalloproteases (Liao et al., 1998).

Proteases of *Pseudomonas* and *Burkholderia*

Proteases from a number of different *Pseudomonas* species and one from *Burkholderia cepacia* have been purified and characterized. All of the proteases that have been purified to date are extracellular metalloproteases isolated from culture supernatants. These enzymes are all between approximately 20 – 60 kDa (Liao et al., 1998, McKevitt et al., 1989, and Sexton et al., 1994). Although they share the fact that they are extracellular metalloproteases, the enzymes are quite diverse.

Pseudomonas aeruginosa has a number of diverse proteases that have been purified and characterized from multiple strains; strain PST-01 (Ogino et al., 1999), strain PAO1 and strain DG1 (Olson et al., 1992), strain PA103-29 (Engel et al., 1998) and strain FRD2 (Kessler et al., 1998). These proteases are all extracellular and directly associated with this species virulence (Kessler et al., 1998). *Pseudomonas aeruginosa* secretes an elastase as a preproenzyme, with a weight of 53 kDa, which is then processed into its mature form, with a molecular weight of 33 kDa (Olson et al., 1992 and Kessler el al, 1998). It is a neutral metalloprotease, requiring zinc for enzymatic reactivity and calcium for stability (Olson et al., 1998). Strain FRD2 secretes the LasA metalloendoprotease as a proenzyme with a molecular weight of 42 kDa. The protease is then processed extracellularly into its mature form with a molecular weight of 20 kDa (Kessler et

al., 1998). Both elastase and LasA protease can degrade elastin, some collagens, and the LasA protease also has high stapholytic activity (Olson et al., 1998 and Kessler et al., 1998). Another extracellular protease, Protease IV, is as a serine protease. Produced by strain PA103-29, it has a molecular weight of 26 kDa, an isoelectric point of 8.7 and optimum enzymatic activity at pH 10 and 45° C (Engel et al., 1998). *P. aeruginosa* strain PST-01 produces another type of protease, an organic solvent-stable protease with a molecular weight of 38 kDa, an optimum temperature of 55° C and a pH of 8.5. This protease is stable over a pH range of 8 – 12 and at temperatures below 50° C. Inhibitor assay experiments concluded the enzyme was a metalloprotease, inhibited by EDTA, 1,10-phenanthroline, and phosphoramidon (Ogino et al., 1998).

Burkholderia pseudomallei, produces an extracellular alkaline metalloprotease involved in the bacteria's pathogenicity and development of melioidosis in humans and animals (Sexton et al., 1994). The enzyme has a molecular weight of 36 kDa, is optimally active at 60° C, a pH of 8 and requires iron for maximal activity. Interestingly, antibodies directed against an alkaline protease produced by *P. aeruginosa* cross-reacted with the *P. pseudomallei* protease, indicating the protease belongs to the family of alkaline proteases sensitive to metal chelators. This was confirmed by its sensitivity to 1,10-phenanthroline, 8-hydroxyquinolone, dithiothreithol, 2,2'-dipyridyl, and Zincov, all metalloprotease inhibitors (Sexton et al., 1994). N-terminal sequences of the protease purified showed homology existed between members of the alkaline protease group (Sexton et al., 1994).

Pseudomonas fluorescens strains M3/6, CYO91, and AFT 36 all produce metalloproteases (Liao et al., 1997, Kohlman et al., 1991, and Stepaniak, 1982). These proteases are associated with and cause spoilage of milk and dairy products within the food industry, subsequently, the enzymes have broad temperature ranges for stability and activity. *P.*

fluorescens strain CY091 produces a protease with a high temperature stability that has an estimated molecular weight of 50 kDa (Liao et al., 1997). *P. fluorescens* strain M3/6 produces an alkaline endometalloprotease with a molecular weight of 45 kDa, exhibiting a high temperature stability and a broad pH range for activity (Kohlmann, et al., 1991). Finally *P. fluorescens* strain AFT 36 produces a metalloprotease of 46 kDa (Stepaniak et al., 1982). The protease is active at pH 6.5 and 45° C. The protease also retained 30 % of maximal activity at 7° C and 16 % at 4° C, indicating a cold sensitive but active protease (Stepaniak et al., 1928).

Burkholderia cepacia produces an extracellular protease, believed to be one of the bacterium's many virulence factors (McKevitt et al., 1988). The protease has a molecular weight of 34 kDa, a pH optimum of 6 and a temperature optimum of 45° C. Protease activity decreased when incubated with EDTA and O-phenathroline, indicating it was a metalloprotease. It was also inhibited by the disulfide bond reducing agent, dithiothreitol (McKevitt et al., 1989).

Bacterial Proteases in the Industry Setting

One objective of purifying, characterizing, then cloning a bacterial protease has been the production of enzymes for commercial purposes. Bacterial proteases have come to represent one of the largest classes of industrial enzymes, accounting for 40 % of the total worldwide sale of enzymes (Rao et al., 1998).

Microbial proteases represent a good source of enzymes due to a number of characteristics. For instance; their broad biochemical diversity, the rapid growth of microorganisms, the limited space required for cell cultivation, as well as the ease at which the enzymes can be genetically manipulated to generate new enzymes for various applications (Rao et al., 1998).

HYPOTHESIS

Burkholderia strain 2.2 N produces a protease that has antimicrobial activity.

SPECIFIC OBJECTIVES

1. Identify an assay for measuring protease activity.
2. Identify medium and conditions required for high protease production.
3. Identify the timing of protease production during growth.
4. Determine the location of the protease activity.
5. Purify the protease.
6. Measure the effect of chelators, metal ions and protease inhibitors on the activity of the protease.
7. Measure the antimicrobial activity of the purified protease.

RATIONALE of the EXPERIMENTAL APPROACH

Previous articles describe the purification and characterization of extracellular proteases from *Pseudomonas* and *Burkholderia* species, sequentially utilizing ammonium sulfate precipitation, ion-exchange chromatography, and gel electrophoresis (Liao et al., 1998, McKevitt et al., 1989, and Sexton et al., 1994). These methods were used to purify and characterize the novel protease of *Burkholderia* strain 2.2 N.

MATERIALS AND METHODS

Bacterial Strains

Burkholderia strain 2.2 N (ATCC 55961) is a Gram negative bacterium, that forms 1mm diameter amber-colored, beehive-shaped colonies on one quarter strength Tryptic Soy Broth (Difco, Detroit, MI) + 0.2 % Sucrose agar medium incubated at 30° C for 48 hours (Casida, 1992). A transparent, flat colonial variant (TP-type) that is 2 mm in diameter and appears at a frequency of 1 - 4 %. The colony morphology of the TP-type is stable.

Growth Conditions

Burkholderia strain 2.2 N was grown in one quarter strength Tryptic Soy Broth (Difco, Detroit, MI) medium containing 0.2 % sucrose (TSB +S) at 30° C in baffled 250 ml Erlenmeyer flasks with aeration (120 rpm). To determine the influence of protein, carbon, and nitrogen sources on protease production, Strain 2.2 N was grown under the same conditions in a minimal medium (MS) containing 1.0 % yeast extract (Difco, Detroit, MI), 0.3 % $MgSO_4 \cdot 7H_20$, 1.0 % $NaHPO_4$, and 1.0 % K_2HPO_4 (Liao et al., 1998). For protease isolation, Strain 2.2 N was grown under the same conditions in an optimized protease producing medium that contained 1.0 % yeast extract, 1.0 % NaH_2PO_4, and 1.0 % K_2HPO_4 and 10 mM $MgCl_2$.

Detection of Protease Activity by Clearing on Milk Casein Agar

Cultures (10 µl) were spotted on the surface of Milk Agar [i.e., skim milk powder 0.1 % (Difco, Detroit, MI) and 0.2 % agar]. Plates were incubated at 30° C for 48 hours and examined for zones of clearing in positions where the filtrate was spotted.

Measurement of Protease Activity by p-Nitroanilide Hydrolysis

The following protease substrates were used; N-succinyl-ala-ala-pro-phe-p-nitroanilide substrate for chymotrypsin and peptidyl prolyl isomerase, N-succinyl-ala-ala-pro-leu-p-nitroanilide substrate for elastase, N-succinyl-gly-gly-phe-p-nitroanilide substrate for chymotrypsin, and N-benzoyl-L-arginine p-nitroanilide (BAPNA) a chromogenic trypsin substrate. All were obtained from Sigma Chemical Co., St. Louis, MO).

To clean 16 x 125 mm screw-capped tubes, 5 mg pNA substrate and 5 ml 0.1 M Tris-HCl buffer (pH 8.1) containing 0.02 M $CaCl_2$ was added. For background hydrolysis, (negative control), 0.1 ml dH_20 was added to the tube, for positive hydrolysis control, 0.1 ml of Protease VIII (0.2 mg / ml) (Sigma Chemical Co., St. Louis, MO) was added. The test sample was 0.1 ml of a cell-free culture filtrate of Strain 2.2 N.

Tubes were incubated at 37° C for 2, 4, 6, 8 and 10 minutes and absorbance at 405 nm was measured against the Tris-HCl buffer. Hydrolysis due to enzyme activity was determined as, the auto-hydrolysis absorbance subtracted from the absorbance of each tube and activities.

Measurement of Protease Activity by Azocoll Hydrolysis

To clean 16 x 125 mm screw-capped tubes, 10 mg Azocoll (Sigma Chemical Co., St. Louis, MO) was added. To each tube, 4.5 ml of either 10 mM potassium phosphate buffer (pH 7.5), 10 mM Tris-HCl buffer (pH 9), or 10 mM sodium acetate buffer (pH 4.5). To the same tube, 0.5 ml of test material was added. For a negative control (auto-hydrolysis), 0.5 ml of buffer was added. For the positive control, 0.5 ml Protease VIII solution (0.2 mg / ml) was added. Tubes were vortexed on high for 30 seconds using a Vortex Genie-2 (Scientific Industries, Springfield, MA). Following incubation for 25 minutes at 37º C with agitation, tubes were placed in an ice-water slurry. Suspensions were filtered through Whatman No. 4 filter paper

cones in glass funnels into tubes in an ice-water bath to prevent continued Azocoll hydrolysis. The absorbance of the released azo-dye was measured at 520 nm against a reagent control blank. For calculation of specific protease activity, absorbance at 520 nm was multiplied by 2.965 to obtain mg Azocoll hydrolyzed in 25 minutes at 37° C per volume of test sample (Chavira et al., 1984). One unit of protease activity was defined as the amount of enzyme that yielded an increase in absorbance (at 520 nm) in 25 minutes at 37° C. Protease activity was expressed in U / mg.

Measurement of Protein Concentration

Protein concentrations were measured by the method by Lowry et al. (1952).

Effect of pH on Enzyme Activity

Cells were grown in 25 ml one quarter strength TSB + S broth medium in a 250 ml baffled Erlenmeyer flask at 30° C for 16 hours with rotation at 120 rpm. These variables were selected on the basis of convenience. Cells were pelleted by centrifugation (10,000 x g for 30 minutes) at 25º C and the supernatant was collected and filtered through 0.2 µm pore size filter. To measure the effect of pH on enzyme activity, Azocoll assays were performed using filtered supernatant and 10 mM potassium phosphate buffer at pH 4.5, 7.5, and 9.

Effect of Temperature on Protease Stability and Enzyme Activity

Cells were grown in 25 ml one quarter strength TSB + S broth medium in a 250 ml baffled Erlenmeyer flask at 30° C for 16 hours with rotation at 120 rpm. Cells were pelleted by centrifugation (10,000 x g for 30 minutes) at 25º C and the supernatant was collected and filtered

through 0.2 µm pore size filter. Culture supernatants were incubated at 4°, 25°, and 30° C for 48 hours. At two hour time points the protease activity and protein concentrations were measured and specific activity calculated. The protease activity (Azocoll) was also measured at 4° C (buffer and supernatant cooled before assays begun) and 30° C. The protease activity and protein concentration was measured and specific activity calculated.

Effect of Different Protein Sources on Production of Protease Activity

Cells were grown in 25 ml one quarter strength TSB + S broth medium in a 250 ml Erlenmeyer flask at 30° C for 16 hours with aeration (120 rpm). Cells were also grown in MS broth medium at the same conditions, substituting the following for 1.0 % Tryptic Soy: 1.0 % yeast extract, 1.0 % casitone, 1.0 % peptone, 1.0 % tryptone, and 1.0 % beef extract. Cultures were incubated 16 hours (i.e. onset of protease activity) and cultures were harvested and centrifuged at 10,000 x g for 30 minutes at 25º C. The culture supernatant was collected and filtered through a 0.2 µm pore size filter. The protease activity and protein concentration of each was measured and specific activity calculated.

Effect of Different Carbohydrates on Enzyme Production

Cells were grown in 25 ml MS broth medium containing 1.0 % yeast extract in a 250 ml baffled Erlenmeyer flask at 30° C for 16 hours with rotation at 120 rpm. Cells were also grown in the same medium under the same conditions, containing either 1.0 % sucrose or 1.0 % glucose. These two sugars were selected because they are the few utilized by Strain 2.2 N (Cain et al., 2000). Cultures were incubated 16 hours (i.e. onset of protease activity) and cultures were harvested and centrifuged at 10,000 x g for 30 minutes at 25º C. The culture supernatant was

collected and filtered through a 0.2 µm pore size filter. The protease activity and protein concentration of each was measured and specific activity calculated.

Effect of Divalent Cations on Production of Protease Activity of Cultures

Cells were grown in 25 ml MS broth medium with 1.0 % yeast extract in a 250 ml baffled flask at 30° C for 16 hours with rotation at 120 rpm. Cells were also grown in the same medium under the same conditions, containing either 1 mM or 10 mM concentrations of $CaCl_2$, $MgCl_2$, $FeCl_2$, $ZnCl_2$, $CuCl_2$, and $MnCl_2$ (Sexton et al., 1994). Cultures were incubated 16 hours (i.e. onset of protease activity) and cultures were harvested and centrifuged at 10,000 x g for 30 minutes at 25º C. The culture supernatant was collected and filtered through a 0.2 µm pore size filter. The protease activity and protein concentration of each was measured and specific activity calculated.

Growth Curve and Timing of Protease Activity in Cultures

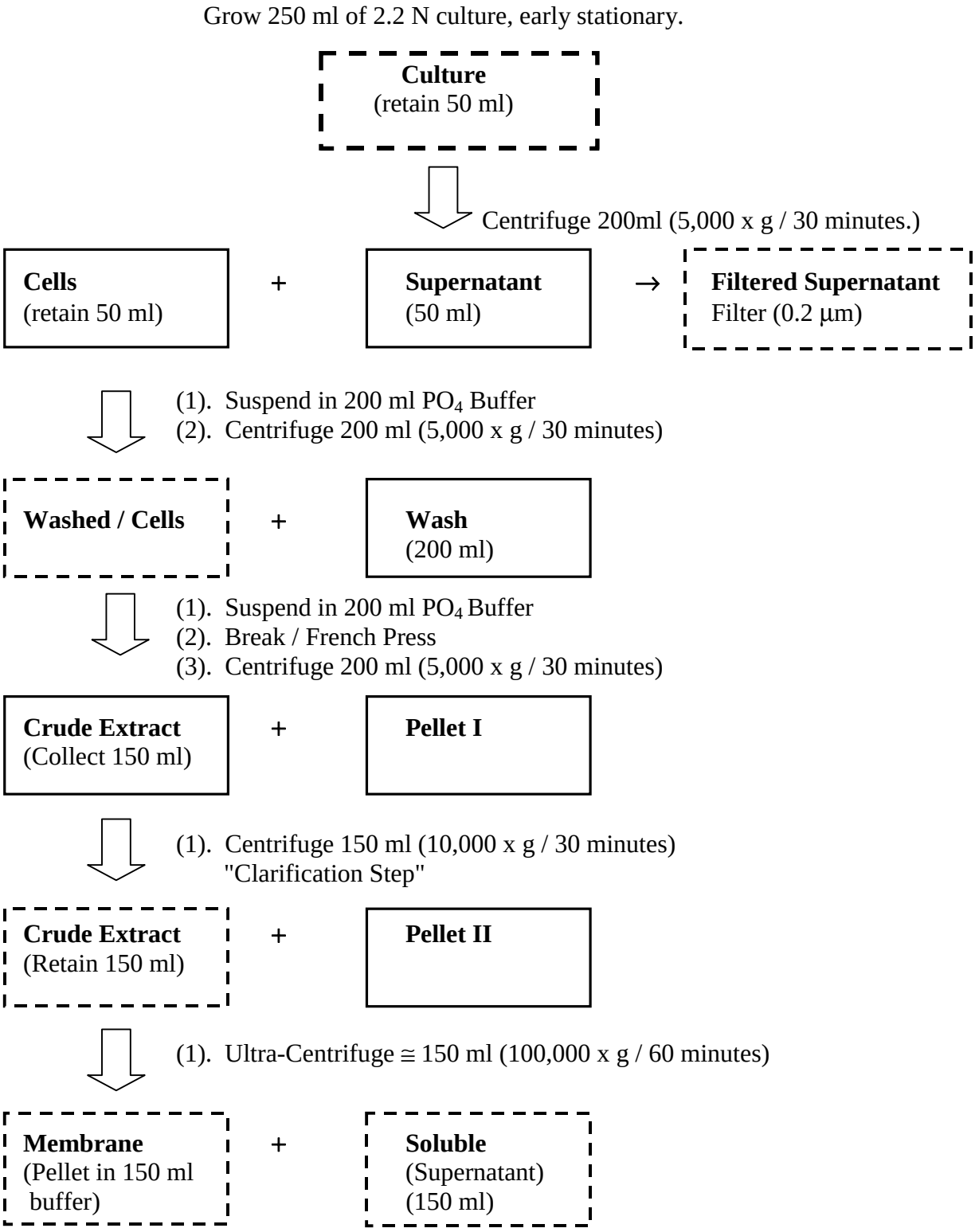
The growth of *Burkholderia* strain 2.2 N was characterized in one quarter strength TSB + S broth at 30° C to 48 hours with rotation at 120 rpm and in the medium that showed the highest protease production, 1.0 % YE, 1.0 % K_2HPO_4, 1.0 % NaH_2PO_4 with 10 mM $MgCl_2$ broth, at 30° C to 60 hours with rotation at 120 rpm. The culture samples were collected hourly and culture turbidity (i.e. absorbance 580 nm) measured. The protease activity of the filtered culture supernatant was measured on samples collected every 2 hours testing using Azocoll hydrolysis assays. Protease activity of the supernatant was also tested, by the clearing on Milk Casein Agar. Samples were collected every 6 hours and serially diluted in broth medium and 0.1 ml spread on one quarter strength TSB + S medium to measure the number of colony forming units (CFU).

Determination of the Cellular Location of Protease Activity

Cells were grown in 125 ml of 1.0 % yeast extract, 1.0 % K_2HPO_4, 1.0 % NaH_2PO_4 with 10 mM $MgCl_2$ broth in 500 ml baffled flasks at 30° C and 120 rpm. Cultures were harvested at early stationary phase (approximately 16 hours). A sample (50 ml) of the whole culture was collected and stored in a ice-water slurry (Culture Sample). The remaining culture was centrifuged (10,000 x g for 30 minutes at 4º C) and the culture supernatant collected and filter-sterilized by passage through a 0.2 um pore size filter and stored in an ice-water slurry (Supernatant Sample). The cell pellet was resuspended in an equal volume of 10 mM phosphate buffer (pH 7.5) and a portion collected and stored in an ice-water slurry (Cell Sample). The cells were centrifuged (10,000 x g for 30 minutes at 4° C) and the wash retained in an ice-water slurry (Wash Sample). The cell pellet was resuspended in an equal volume of buffer (repeat of step 4). A sample of the cells in the remaining suspension was broken using a French Press. The unbroken cells and cell debris were separated from the crude extract by centrifugation (15,000 x g for 5 minutes at 4º C). The cells and cell debris were suspended in the original volume of 10 mM phosphate buffer (pH 7.0) and stored in an ice-water slurry (Debris Sample). A portion of the crude extract was stored in a ice-water slurry (Crude Extract Sample). The remaining portion of the crude extract was centrifuged (100,000 x g for 60 minutes) at 4º C to pellet the membrane fraction. The supernatant fraction was collected and stored in an ice-water slurry (Soluble Sample) and the pellet suspended in an equal volume of 10 mM phosphate buffer (pH 7.0) and stored in an ice-water slurry (Membrane Sample). The protease activity and protein concentration was measured and specific activity determined for the following fractions; filtered supernatant, washed cells, crude extract, membrane and soluble (Figure 1). As markers, β-

galactosidase (soluble) and alkaline phosphatase (periplasmic) activities were measured on the same fractions.

Figure 1. Fractionation Flow-Chart



Ammonium Sulfate Precipitation

Burkholderia strain 2.2 N cells were grown in 1.0 % yeast extract, 1.0 % NaH_2PO_4, 1.0 % K_2HPO_4 and 10 mM $MgCl^{2+}$ medium to 45 hours. Cells were harvested and centrifuged (10,000 x g for 30 minutes at 25º C). The supernatant was then filtered using 0.45 μm pore size filters. Ammonium sulfate was poured slowly into the supernatant over a period of ten minutes, allowing the salt to slowly dissolve. The supernatant was continually stirred at room temperature for an additional 25 minutes. Precipitates were recovered by centrifugation (10,000 x g for 10 minutes at 25º C), and dissolved in 10mM Tris-HCl, 10mM NaCl buffer (pH 8.0). Each $(NH_4)_2SO_4$ fraction was dialyzed overnight against the same buffer (1:50 volume). The protease activity and protein concentration was measured and specific activity calculated.

To determine the precipitation fraction that contained the largest percent of protease activity, twenty percent fractions (0 – 100 %) were retained. Protease activity and protein concentration was measured and specific activity calculated. The 20 % fractions with the highest protease activity were determined.

Ion-Exchange Chromatography

The dialyzed ammonium sulfate fractions were concentrated using an ultrafiltration cell (Amicon, Danvers, MA) through a membrane pore size > 5,000 MW (Diaflo, Danvers, MA). The resulting concentrated samples (4-5 ml, 2.0 - 2.5 mg) were applied to a column (16 cm length, 2 cm diameter) of DEAE-Sepharose™ Fast Flow beads (Amersham Pharmacia, Piscataway, NJ). The column was equilibrated with 150 mL 1M NaCl buffer (pH 8.0) and washed with 150 mL of 10mM Tris. Using a Fast Performance Liquid Chromatography System (Amersham Pharmacia, Piscataway, NJ) proteins were eluted with 10 mM Tris and 0 to 1.0 M

NaCl gradient. Eluted fractions (4 mL) were collected and absorbance was measured at 280 nm. Protein peaks were pooled then dialyzed overnight against the Tris, NaCl buffer. The protease activity and protein concentration of fractions was measured and specific activity calculated.

SDS-Polyacrylamide Electrophoresis and Blotting

SDS-PAGE of *Burkholderia* strain 2.2 N growth medium, filtered culture supernatant, ammonium sulfate, and DEAE fractions containing protease activity were carried out as described by Laemmli (1970) using 5.0 % stacking and 12.0 % polyacrylamide resolving gels (1.5 mm thickness). Gels were run using a Mini-Protean II 2-D Cell apparatus (Bio-Rad Laboratories, Hercules, CA). Low weight molecular weight standards (Bio-Rad Laboratories, Hercules, CA) and pre-stained molecular weight markers (Bio-Rad Laboratories, Hercules, CA) were used to estimate the molecular weight of separated proteins. Samples of protein preparations and molecular weight standards were dissolved in a solution containing 60 mM Tris-HCl (pH 6.8), 14.4 mM ß-mercaptoethanol, 2.0 % SDS, 25.0 % (vol / vol) glycerol, and 0.01% (wt / sol) Bromophenol Blue and immediately heated at 100º C for 10 minutes. Electrophoresis was carried out at a constant current of 100 V / 40 mA per gel until the tracking dye (bromophenol blue) reached the bottom of the gel (approximately 1 hour total running time).

SDS-PAGE gels were stained for protein using either a Coomassie Brilliant Blue Stain (Bollag et al.,1991) or a silver stain using the Silver Stain Plus Kit and methods from Bio-Rad Laboratories, Hercules, CA (Gottlieb et al., 1987).

Zymography

Premade, commercially available 12.0 % casein and 10 % gelatin zymography gels (Bio-Rad Laboratories, Hercules, CA). Gels were inserted into a Mini-Protean II 2-D Cell apparatus (Bio-Rad Laboratories, Hercules, CA). The chamber was filled with 25 mM Tris, 192 mM glycine and 0.1 % SDS Buffer. Samples to test were diluted 1 part sample with 2 parts zymography sample buffer; 62.5 mM Tris-HCl, pH 6.8, 4.0 % SDS, 25.0 % glycerol and 0.01% (wt / sol) Bromophenol Blue. Samples were loaded and the gels run at 100 V constant for approximately 1 hour. Pre-stained molecular weight markers (Bio-Rad Laboratories, Hercules, CA) were used to estimate the molecular weight of separated proteins. The gels were removed from the apparatus and incubated for 30 minutes at room temperature in 100 ml of 2.5% Triton X-100 renaturation buffer on an orbital shaker. The renaturation buffer was removed and 100 ml of 50 mM Tris-Cl, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij-35 development buffer was added. Gels were incubated on the orbital shaker at 37º C overnight. The development buffer was poured off and the gels were washed with water. Coomassie Brilliant Blue was added and the gels were incubated on an orbital shaker for 1 hour at room temperature. The gels were de-stained over night with 40 % methanol, 10 % acetic acid until clear bands appeared against the blue background.

Inhibitor and Metal Chelator Assay's

Purified *Burkholderia* strain 2.2 N protease was assayed in the presence and absence of various chelators and potential inhibitors used at 0.1 mM, 1.0 mM, and 10.0 mM concentrations (Sexton et al., 1994). In sterile test tubes, 2.2 N protease sample, inhibitor, and Azocoll buffer was incubated for 30 minutes at 37° C. Each sample was added immediately added to a 15 x 125

mm test tube each containing 10 mg Azocoll. Azocoll assays were performed at the same conditions as described above to determine protease activity.

The following inhibitors were tested for their ability to inhibit protease activity; Ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(ß-aminoethyl ether)-*N,N,N'N'*-tetra acetic acid (EGTA), 1-10-phenanthroline (1-10-PA), phenylmethylsulphyonyl (PMSF), Leupeptin, and dithiothreitol (DTT), all were obtained from Sigma Chemical Co., St. Louis, MO.

The following metal salts were tested for their ability to stimulate or inhibit protease activity; $FeCl_2$, $CaCl_2$, $ZnCl_2$, $CuCl_2$, $MgCl_2$, $MnCl_2$. In metal requirement studies, divalent cations were added to assay tubes to a final concentration of 1 mM. Protease assays were carried out in Azocoll buffer that had been depleted of divalent cations by overnight incubation in 5 g / 100 mL Chelex® 100 (Bio-Rad Laboratories, Hercules, CA), or in the presence of chelators. In sterile test tubes, 2.2 N protease sample, inhibitor, divalent cation, and chelexed Azocoll buffer was incubated for 30 minutes at 37° C. Each sample was added immediately added to a 15 x 125 mm test tube each containing 10 mg Azocoll. Azocoll assays were performed at the same conditions as described above to determine protease activity.

Antimicrobial Activity

To determine if the purified protease from *Burkholderia* strain 2.2 N had antimicrobial activity, samples were tested against the microorganisms that Strain 2.2 N inhibits (Cain et al., 2000): *Aspergillus niger, Sacchromyces cerevisiae, Micrococcus luteus, Candida albicans,* and *Cryptococcus neoformans.* Samples of purified protease (10 µl) were spotted onto agar medium containing lawns of the different organisms (Appendix I). Zones of inhibition of growth were determined.

RESULTS

Determination of Colony Morphology

Burkholderia strain 2.2 N produced a transparent, flat, stable colony variant that appeared at a frequency of 1 – 4 %. That the colony variant has no protease activity, therefore, an increase of transparent cells in cultures decreases the specific protease activity (Cain et al., 2000). The colony morphology of all *Burkholderia* strain 2.2 N cultures grown was determined to insure the colony variant was present at < 1.0 %.

All culture's grown were streaked onto ¼ tryptic soy broth with 0.2 % sucrose agar and plates were incubated 30 °C for 48 hours. All cultures showed colony morphology consisting of amber colonies with < 1.0 % of the transparent colony variants present.

Determination of the Presence of Protease Activity

Burkholderia strain 2.2 N culture samples showed clearing zones on the milk casein agar indicating the hydrolysis of the proteins within the agar and thus the presence of a protease(s). Zones measuring 13 - 15 mm of complete hydrolysis of the milk casein agar occurred after plates were spotted with 10 µl culture and incubated for 48 hours at 30° C.

Selection of a Protease Assay

To determine the best assay for measurement of protease activity, three assays were tested and compared. Filtered *Burkholderia* strain 2.2 N supernatant was used to determine the optimum conditions for hydrolysis of four different substrate types; p-Nitroanilide's (pNAs), Azocoll and Milk Casein Agar.

Assay tubes containing culture supernatants of *Burkholderia* strain 2.2 N were incubated with the four different p-nitroanilide protease substrates exhibited hydrolysis (i.e. protease activity), however the formation of precipitates in solution also occurred. The negative controls containing the four substrates but no culture supernatant lacked the precipitate. Additional negative controls containing the buffer and culture supernatant but no substrates showed no precipitation. The positive control containing Protease VIII showed hydrolysis of the substrates, as indicated by a color change within the assay tubes, with no precipitate forming. It is likely that the buffer composition or concentration caused the precipitation between the pNA substrates and either the protease or other proteins within the culture supernatant. Although there was hydrolysis of the p-nitoanilide protease substrates, precipitation prevented accurate measurement of reaction rates This problem interfered with the spectrophotometer reading, therefore this assay system could not be used.

The Azocoll assay tubes containing both the *Burkholderia* strain 2.2 N supernatant as well as the positive control, showed hydrolysis of the dye-linked insoluble collagen. Hydrolysis caused a release of a purple dye, which allowed tubes to be immediately read spectrophotomically or by eyesight. The optimal pH for maximum protease activity was determined using three different pHs of the buffer.

Effect of pH on Protease Activity

Protease activity was present at all three pHs (4.0, 7.5 and 9.0) in both the tubes containing the filtrated culture supernatant as well as the positive control. Assays at a pH 7.5 showed the highest protease activity compared to a pH 9.0, which was 27 % lower (Table 2).

Protease assays performed at a pH of 4.5 showed little to no protease activity, 92 % lower than assays conducted at pH 7.5 (Table 2).

Table 2: Azocoll Assay Activity at Different Buffer pHs.

(mg Azocoll hydrolyzed / mL of filtered culture supernatant)

pH 4.5	pH 7.5	pH 9.0
0.02	0.26	0.19
8 %	100 %	73 %

(Results are means of three experiments.)

Azocoll substrate hydrolysis at a pH of 7.5 provided a rapid and simple method that could be used for measurement of activity of a large number of samples. Using Azocoll as a substrate proved to be the most effective, replicative assay to measure the presence and specific activity of protease.

Effect of Temperature on Enzyme Activity

Experimental conditions, such as temperature and time, can affect the activity of enzymes. The protease activity of *Burkholderia* strain 2.2 N filtered culture supernatant samples were measured at different temperature's to determine its stability during purification. Strain 2.2 N protease activity was measured at 4º C (i.e., storage temperature for protease samples) and 37º C.

Buffers and test tubes were brought to temperature before the protease sample was added to assay tubes. Strain 2.2 N filtered culture supernatant assayed at 4º C, showed a three-fold decrease in specific protease activity compared to the assays conducted at a temperature of 37º C

(Table 2). Although there was a decrease in activity at 4º C, there was still enzyme activity present at the low temperature.

Table 3: Effect of Temperature on Enzyme Activity

Temperature	Specific Protease Activity		
	Experiment # 1	Experiment # 2	Average
4 °C	0.0330	0.0643	0.0487
37 °C	0.1434	0.1133	0.1284

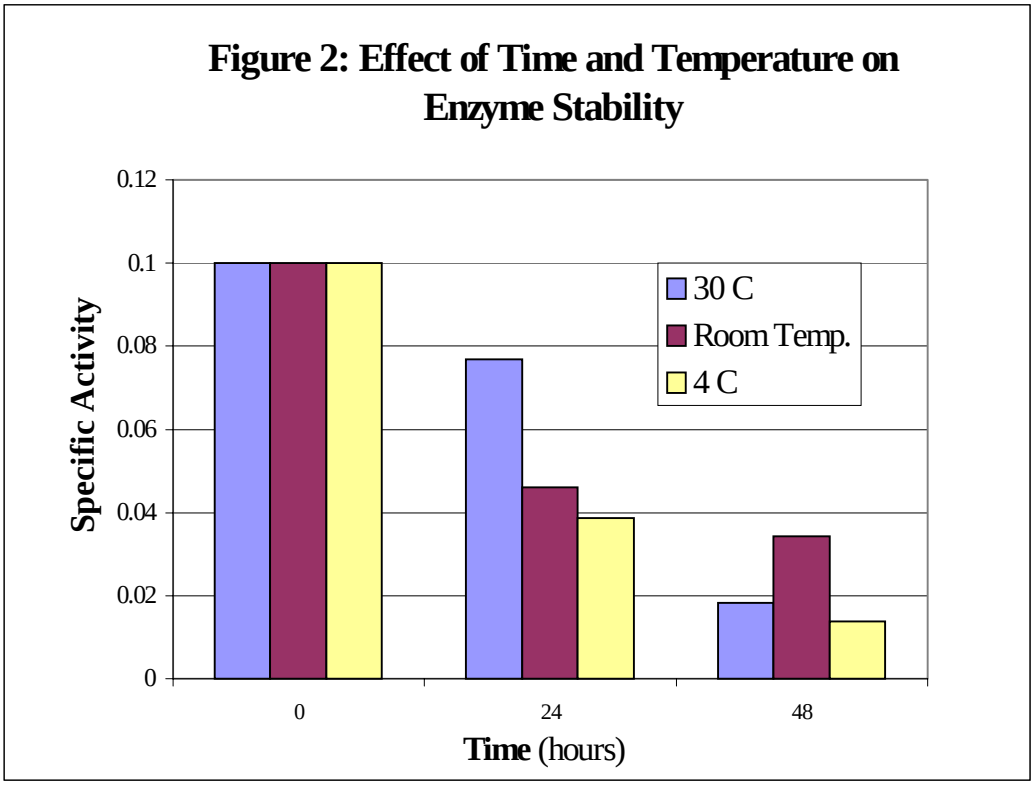
Effect of Time on Enzyme Activity

Another important experimental condition when working with enzymes is time. Proteases are subject to auto-hydrolysis (Beynon and Bond, 1989). To measure *Burkholderia* strain 2.2 N protease stability and auto-hydrolysis, a filtered culture supernatant was stored at three different temperatures (4°, 25°, and 30° C) and protease activity measured initially and after 24, and 48 hours.

Burkholderia strain 2.2 N filtered culture supernatant showed a loss of activity after storage at all three temperatures (Figure 2). The loss in activity was evident at 24 hours and then again at 48 hours an additional loss in activity was also seen. The greatest loss of activity was observed in supernatant stored at 4° C, decreasing two-fold in specific activity within a 24 hour time point (Figure 2). Protease activity in samples stored at 4 °C dropped 60 % after 24 hours, and 85 % after 48 hours. Although there was a decrease in enzyme activity at 4° C, the enzyme was active at the lower temperature.

Filtered culture supernatant samples kept at room temperature maintained a higher specific activity compared to samples stored at 4° and 30° C. Protease activity dropped 55 % after 24 hours, and 65 % after 48 hours. The lowest decrease in specific activity was measured in supernatant samples stored at 30° C. Protease activity dropped 22 % after 24 hours, and 81 % after 48 hours. All samples showed no cellular growth to provide for the additional protease activity.

Loss in activity in supernatant samples stored at all three temperatures indicated the enzyme is susceptible to auto-hydrolysis. Purification was conducted at room temperature as rapidly as possible, with no long time storage of protease samples to maintain high levels of activity.



Effect of Different Protein and Carbohydrates on Protease Activity

To identify the components for a defined medium yielding a high level of protease activity, cells were grown in different broth media containing different protein and carbon sources. The first medium component that was tested was the protein source.

Burkholderia strain 2.2 N cells were grown in ¼ strength tryptic soy broth + 0.2 % sucrose, for a comparison. Cells were grown in a minimal salt medium with either of the following; 1.0 % tryptic soy, 1.0 % $(NH_4)_2SO_4$, 1.0% yeast extract, 1.0 % peptone, 1.0 % soytone, 1.0 % casitone, 1.0 % tryptone, and 1.0 % beef extract.

Filtered culture supernatant from cells grown in the minimal medium containing 1.0 % $(NH_4)_2SO_4$, 1.0 % peptone, 1.0 % soytone and 1.0 % casitone showed little to no protease activity. Filtered culture supernatant of cultures grown in 1.0 % yeast extract, 1.0 % tryptone, and 1.0 % beef extract all showed protease activity (Table 4). Both tryptone and beef extract (Amersham Pharmacia, Piscataway, NJ) showed less protease activity as those cells grown in medium containing yeast extract.

Culture supernatant of cells grown in ¼ strength tryptic soy broth + 0.2 % sucrose had four times the protease activity compared to cells grown in a minimal salt medium containing yeast extract (Table 4). However, the ¼ strength tryptic soy broth + 0.2 % sucrose, is a complex medium and there was a requirement for a minimal medium.

A minimal medium was best to look at different carbon sources, metal ion requirements, and the protease production by Strain 2.2 N in each medium.

Table 4: Effect of Different Protein Sources on Protease Activity

Media	Specific Activity (mg Azocoll hydrolyzed / mg protein)	Protease Activity
¼ Tryptic Soy Broth + Sucrose	0.43	+
1.0 % $(NH_4)_2SO_4$	≤ 0.02	-
1.0 % Yeast Extract	0.14	+
1.0 % Peptone	≤ 0.008	-
1.0 % Soytone	≤ 0.03	-
1.0 % Casitone	≤ 0.003	-
1.0 % Tryptone	0.09	+
1.0 % Beef Extract	0.07	+

(Results are means of three experiments.)

The effect of addition of sucrose and glucose to the minimal medium, 1.0 % yeast extract, 1.0 % K_2HPO_4, 1.0 % NaH_2PO_4 with 10 mM Mg^{2+} on protease production was measured. The cells were grown under the same conditions in parallel with a 1.0 % yeast extract medium containing no carbon source and a ¼ strength tryptic soy broth + 0.2 % sucrose medium.

The addition of either a 1.0 % sucrose or a 1.0 % glucose to the 1.0 % yeast extract broth minimal medium, increased the protease production (Table 5). Addition of sucrose increased the protease production by 1.8-fold, and addition of the glucose increased the protease by 1.4-fold compared to the medium lacking carbohydrate. Although the addition of a carbon source to the

medium increased the overall protease production of the cells, it resulted in a viscous culture supernatant. This caused problems upon filtering and the methods of enzyme purification.

The specific activity of protease production was higher in the medium containing 1.0 % yeast extract, than the medium containing ¼ tryptic soy broth + 0.2 % sucrose (Table 5). Previously protease production was higher in the ¼ tryptic soy broth + 0.2 % sucrose (Table 4). Variability was a problem in protease production when repeating the media studies. All medium and inoculum preparations were identical, as well as growth of *Burkholderia* strain 2.2 N, the protease production varied within the 1.0 % yeast extract and the ¼ tryptic soy broth + 0.2 % sucrose cultures. Although there was variability, protease production within these two media was consistently higher than other media tested.

Table 5: Effect of Different Carbohydrates on Protease Activity

Media	**Specific Activity** (mg Azocoll hydrolyzed / mg protein)
¼ Tryptic Soy Broth + 0.2 % Sucrose	0.230
1.0 % Yeast Extract	0.358
1.0 % Yeast Extract + 1.0 % Glucose	0.510
1.0 % Yeast Extract + 1.0 % Sucrose	0.653

(Results are means of three experiments.)

Divalent Cation Required for Protease Production and Activity

Cells may require divalent cations for the production of proteases (Olson et al., 1992) and may require divalent cations for their activity (Beynon et al., 1989). To determine whether divalent cations were required for the production of *Burkholderia* strain 2.2 N protease, cells were grown in the 1.0 % yeast extract medium. To the medium, 10 mM concentrations of six different divalent cations were added.

Cells grown in 1.0 % yeast extract supplemented with 10 mM $MgCl_2$ and 10 mM $FeCl_2$ showed a two-fold higher increase in protease activity (Table 6), than in 1.0 % yeast extract lacking metals or containing 10 mM $CaCl_2$ or 10 mM $MnCl_2$. Cells grown in 1.0 % yeast extract medium containing either $ZnCl_2$ or $CuCl_2$ showed little to no protease activity (< 0.5 %).

Table 6: Effect of Different Divalent Cations on Protease Activity

Media	**Specific Activity** (mg Azocoll hydrolyzed / mg protein)
1.0 % Yeast Extract (YE)	1.14
1.0 % YE + 10mM Ca^{2+}	1.36
1.0 % YE + 10mM Mg^{2+}	2.92
1.0 % YE + 10mM Fe^{2+}	2.41
1.0 % YE + 10mM Zn^{2+}	≤ 0.04
1.0 % YE + 10mM Cu^{2+}	0.08
1.0 % YE + 10mM Mn^{2+}	1.41

(Results are means of three experiments.)

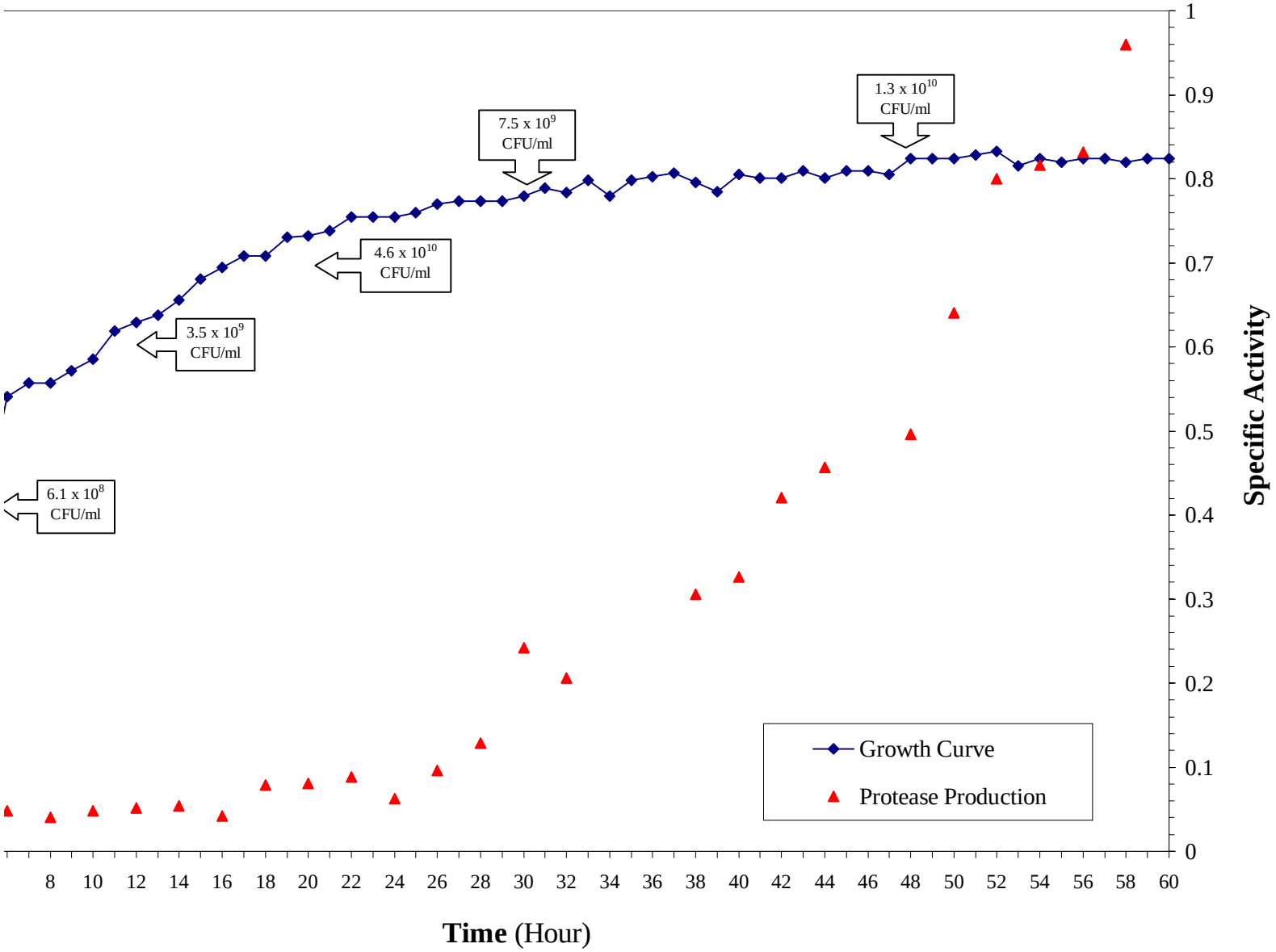
A medium consisting of 1.0 % yeast extract, 1.0 % K_2HPO_4, 1.0 % NaH_2PO_4 with 10 mM $MgCl_2$ broth provided the best and most simplest medium for the purification of a possible extracellular enzyme.

Growth and Protease Production *Burkholderia* strain 2.2 N in 1.0 % Yeast Extract Minimal Medium Containing 10 mM $MgCl_2$

To determine the growth characteristics and protease production for *Burkholderia* strain 2.2 N cells were grown at 30 ° C, with rotation at 120 RPM, in the best minimal medium as determined from previous experiments; 1.0 % yeast extract, 1.0 % K_2HPO_4, 1.0 % NaH_2PO_4 with 10 mM $MgCl_2$. Incubation was carried out in duplicate, samples collected every 2 hours and protease activity measured. Absorbance measurements were made hourly and samples removed to measure CFU every 6 hours.

Burkholderia strain 2.2 N cells begin exponential growth approximately 2 hours after inoculation and enter stationary phase at approximately 16 hours (Figure 3). This exponential phase showed possible biphasic growth with a shift at 4- 6 hours (Figure 3). At 60 hours the cells appeared to be still in stationary phase, with no indication of a death phase. Protease appeared at low levels immediately after inoculation. There was a noticeable rise in the protease which began at 18 hours during early stationary growth (Figure 3). Protease activity continually increased after 18 hours through the entire growth curve measured. For the purification of protease, it was decided that culture samples should be taken at a time point later than 18 hours (early stationary) of growth. For maximal protease production and better enzyme isolation, it was determined that cultures should be grown to a late stage in stationary growth.

Figure 3: Growth Curve and Protease Production of *rkholderia* strain 2.2 N in 1.0 % Yeast Extract with 10mM $MgCl_2$



Determination of the Cellular Location of the Protease

To identify the cellular location of the protease, different fractions from *Burkholderia* strain 2.2N cells grown in 1.0 % yeast extract with 10 mM $MgCl_2$ were isolated and assayed for protease activity. Activity of alkaline phosphatase (a periplasmic membrane enzyme) was measured to identify whether the protease was a periplasmic enzyme, and β-galactosidase (a soluble enzyme) as markers to ensure fractions isolated were representative. Tables 7 and 8 show fractionation activity assay results and overall percentages.

Protease activity was present in all fractions tested. The culture fraction showed a specific activity of 0.64, the filtered culture supernatant had a specific activity of 1.10, and the cells in buffer measured 0.98. The filtered culture supernatant had the highest overall specific protease activity and contained 86 % of the overall protease percentage of the culture.

Reduced protease activity was found in the culture fraction compared to the filtered culture supernatant. Possibly, the whole cells or cellular debris within the culture may have interfered with the protease assay. The whole cell fraction accounted for 108 % of the protease activity, higher than the culture fraction (100 %). This high percentage could be due to the fact that the assay was conducted at 37° C for 25 minutes, which allowed for cells to produce more protease.

The crude extract fraction had a specific protease activity of 0.009 units and the soluble fraction measured a specific protease activity of 0.0012 units. The membrane fraction had a higher specific protease activity of 0.17, and contained 319 % percent activity of the crude extract. This high percentage could be due to the fact that the membrane was homogenized before assays were conducted, releasing protease that was present within the membrane.

Alkaline phosphatase and β-galactosidase activities were present in all fractions. Alkaline phosphatase present in the culture supernatant fraction contained only 3 % of the total enzyme present in the culture. The cells contained 177 % of the culture activity. This data indicated that the periplasmic enzyme was associated with the cells and not the supernatant culture medium. β-galactosidase activity was present in low percentages in the supernatant (11 %) and in the cell fraction (20 %) compared to the culture fraction.

Both marker enzyme activities were present in the crude extract, membrane and soluble fractions. Alkaline phosphatase activity was present in the membrane fraction at 81 % of the crude extract fraction and in the soluble fraction at 53 % of the crude extract fraction. This indicated that the periplasmic enzyme was associated with the membrane fraction as well as the soluble fraction.

β-galactosidase activity was present in the membrane fraction at 4 % of the crude extract fraction and in the soluble fraction at 92 % of the crude extract fraction. This indicated that there was a high level of the soluble enzyme in the soluble fraction and low in the membrane fractions.

Table 7: Fractionation Assay Activities

Fraction	Specific Protease Activity (mg / mg protein)	Specific Activity Alkaline Phosphatase (activity / mg protein)	Specific Activity β-Galactosidase (activity / mg protein)
Culture	0.64	0.00036	0.00073
Supernatant	1.10	0.000022	0.00030
Cells / Buffer	0.98	0.0009	0.00011
Crude Extract	0.009	0.0004	0.0011
Membrane	0.17	0.0021	0.0025
Soluble	0.0012	0.00025	0.0011

(Results are means of three experiments.)

Table 8: Fractionation Recovery Activities

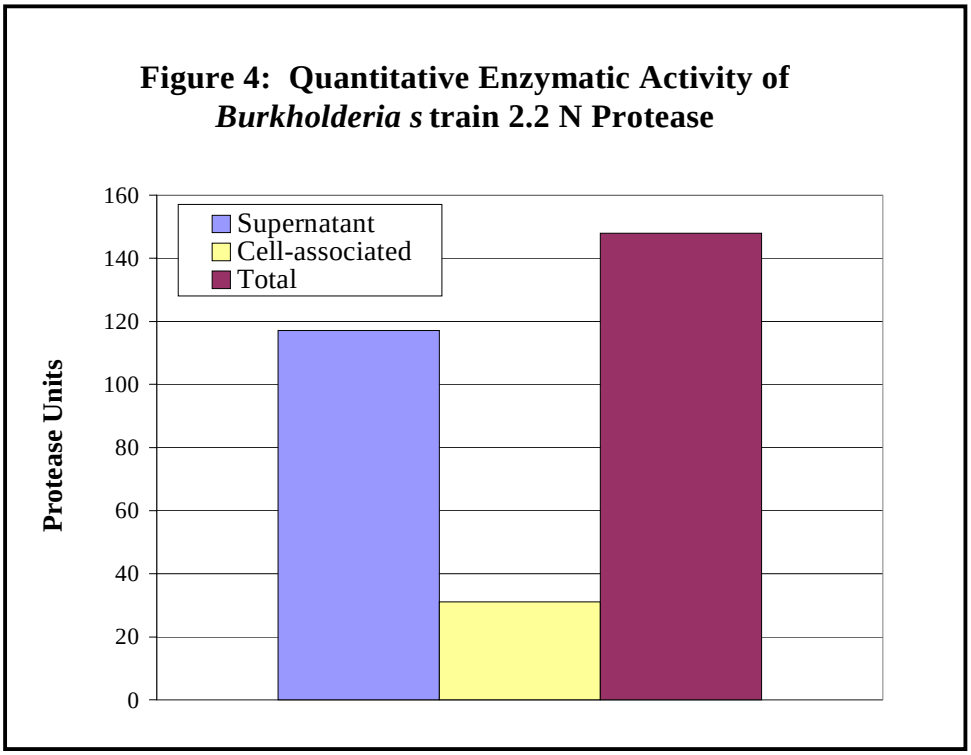
Fraction Recovery	Protein (mg / ml)	Protease	Alkaline Phosphatase	β-galactosidase
Culture	2.2	100 %	100 %	100 %
Supernatant	1.1	86 %	3 %	11 %
Cells / Buffer	1.6	108 %	177 %	20 %
Crude Extract	1.2	100 %	100 %	100 %
Membrane	0.2	319 %	81 %	4 %
Soluble	1.1	12 %	53 %	92 %

Note: Percentages reported are of either the culture or crude extract fractions (treated as 100 % activity).

(Results are means of three experiments.)

The high percentage of protease within the cellular membrane fraction (319 %), as well as in the supernatant fraction (86 %) indicated the protease was exported from the cells (Figure 4). This was supported by the low percentage of protease in the soluble cell fraction (12 %).

Figure 4: Quantitative Enzymatic Activity of *Burkholderia s* train 2.2 N Protease



Ammonium Sulfate Precipitation

Ammonium sulfate precipitation was performed as the first step of protease purification. Ammonium sulfate fractions collected at levels of saturation of 20 % (w/v) were collected and assayed for specific protease activity after dialysis. Fractions collected at 40 – 60 % and 60 – 80 % ammonium sulfate contained the highest percent of specific protease activity, with a total of 66.4 %, and accounted for 27 % of the total protein concentration (Table 9).

To more precisely define the ammonium sulfate fraction containing the highest amount of specific protease activity, smaller ammonium sulfate fractions were collected over the range of 40 – 80 %. It was determined that the 40 – 70 % ammonium sulfate fraction contained 53 % of

the protease activity whereas the 70 – 80 % fraction contained < 1 % of the protease activity (Table 9). The 10 % precipitation between 70 – 80 % and allowed for removal of 1 % of the supernatant proteins.

An ammonium sulfate precipitation between 40 – 70 % was used for purifying the protease and resulted in a 20-fold increase in specific activity compared to the unconcentrated supernatant (Table 9).

Table 9: Ammonium Sulfate Precipitation

Experiment #1

Salt Added	Percent of Enzyme Specific Activity	Percent of Total Protein Concentration
0 - 20 %	4.6 %	19 %
20 - 40 %	17.3 %	14 %
40 - 60 %	**41.1 %**	**14 %**
60 - 80 %	**25.3 %**	**13 %**
80 - 100 %	4.1 %	16 %
100 % +	7.6 %	24 %

Experiment #2

40 - 70 %	**53 %**	**17 %**
70 - 80 %	< 1 %	1 %

(Results are means of multiple experiments.)

Ion Exchange Chromatography

Dialyzed ammonium sulfate precipitates (40 – 70 %) were loaded onto a DEAE-Sepharose column. Proteins were eluted using a 10 mM Tris buffer (pH 8.0) and 0 – 1.0 M NaCl at a pH of 8.0 and eluted proteins were collected. Fractions were pooled, absorbance read, and tested for specific protease activity.

Four absorption peaks at 280 nm were observed (Figure 5). Peak 1 contained the greatest protease activity (4.0 specific protease activity) found in the buffer fractions containing 0.0 - 0.16 M NaCl. The column purification resulted in a 44-fold increase in specific activity compared to the unconcentrated supernatant, and a 2.2-fold increase compared to ammonium sulfate precipitation (Table 10).

The first pooled column fraction was dialyzed overnight and then re-loaded onto the same DEAE-cellulose column that had been washed and re-calibrated. Eight or greater absorption peaks at 280 nm were observed as illustrated in Figure 6. The protease activity was again associated with the pooled fractions at the beginning of the NaCl gradient (0.0 - 0.25 M NaCl), within the first 2 – 3 protein peaks. The second column purification resulted in a 49-fold increase in specific activity compared to the first column purification, increasing the first column purification by 10 % of the total activity (Table 10).

Figure 5: DEAE-Sepaharose Column Run of 40 - 70% Ammonium Sulfate Fraction from *Burkholderia* strain 2.2 N Filtered Supernatant

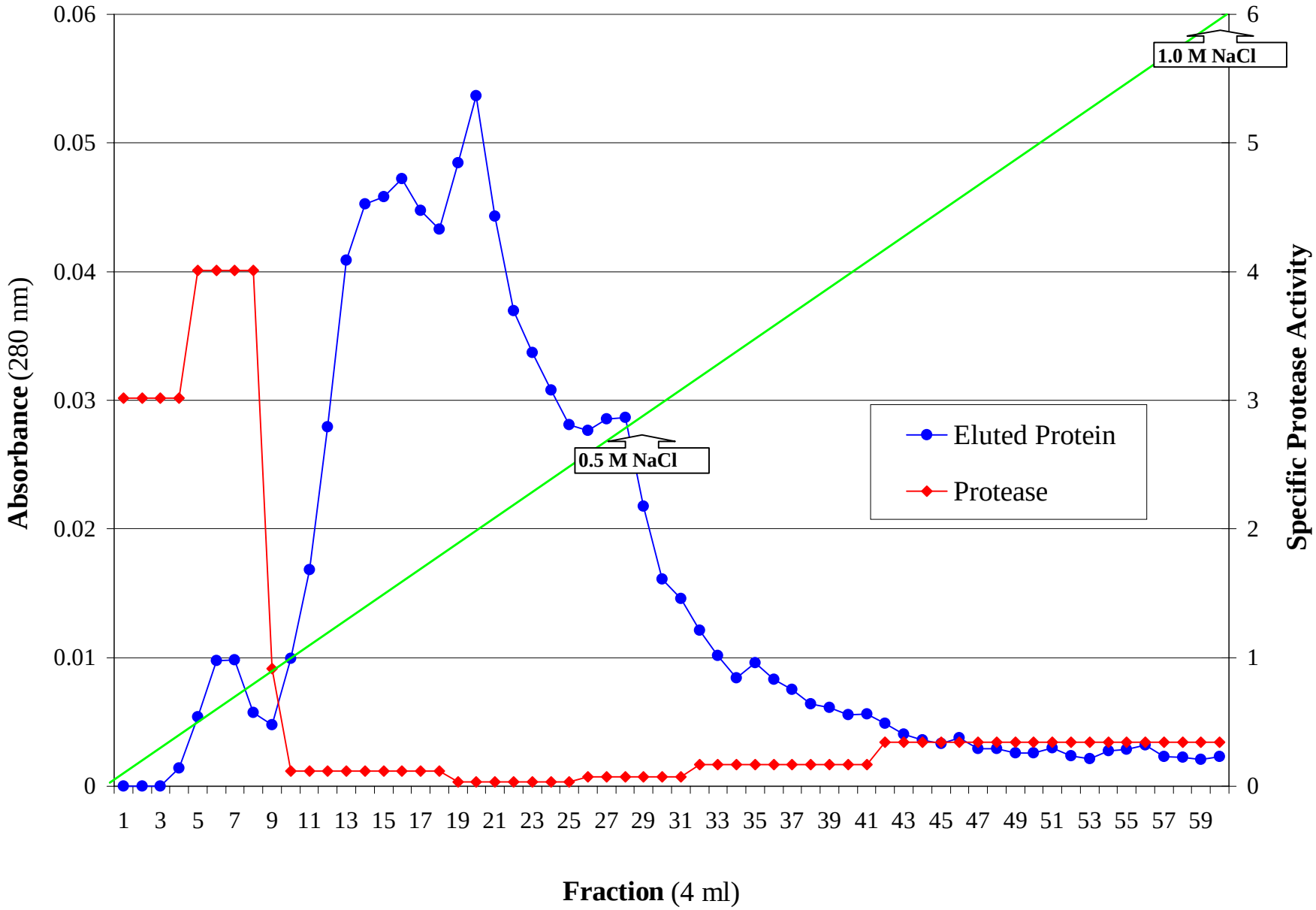


Figure 6: DEAE-Sepharose Column Run with Peak # 1 from First DEAE Column

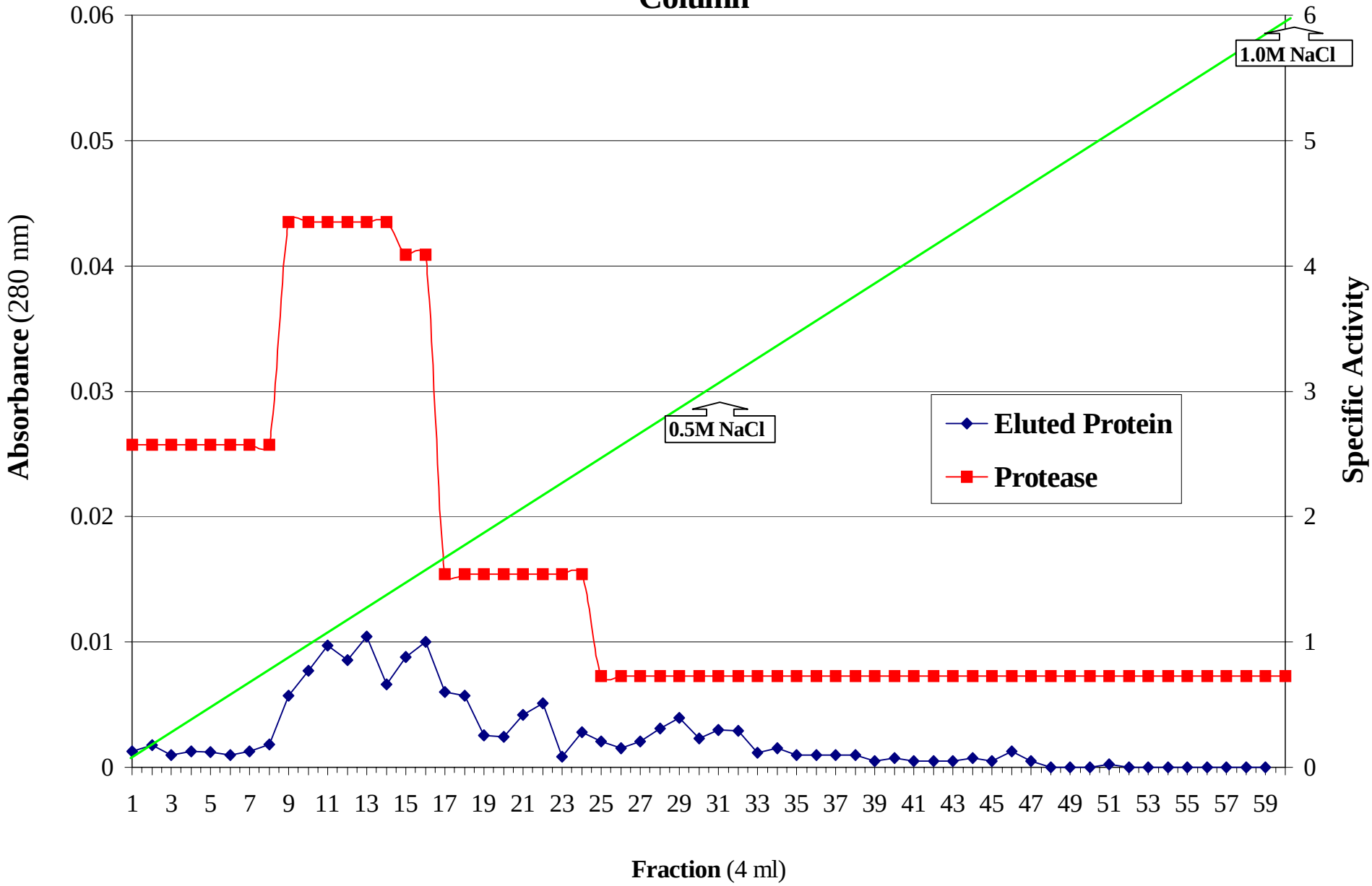


Table 10: Purification of *Burkholderia* strain 2.2 N Protease

Purification Stage	Volume	Protein	Total Protein	Activity	Total Activity	Specific Activity	Purification (Fold)
	(ml)	(mg / ml)	(mg)	(U / ml)	(U)	(U / mg of protein)	
Culture Supernatant	4,100	1.60	6560	0.1360	558	0.09	
Ammonium sulfate Precipitation	7	0.50	3.50	0.9200	6.4	1.8	20
DEAE-Sephacel # 1	28	0.08	2.24	0.3030	8.5	4.0	44
DEAE-Sephacel # 2	39	0.065	3.12	0.2760	8.2	4.4	49

Effect of Inhibitors and Metal Chelators on Protease Activity

Inhibitors and metal chelators can reduce the hydrolysis of a substrate by proteolytic enzymes. Due to their specificity, inhibitors and chelators can also add in the characterization of novel proteases by examining hydrolysis rates of a protease in their presence.

Proteolytic activity, measured by the Azocoll assay, decreased after incubation of purified protease with the inhibitor 1-10-phenanthroline(1-10-PA) (Table 11). Activity decreased 86 % when incubated with 10 mM 1-10-PA, and decreased 80 % when incubated with 1 mM 1-10-PA. Activity was also inhibited 70 % when incubated with 10 mM EDTA and by 69 % when incubated with 10 mM EGTA. Both EDTA and EGTA reduced the protease activity at 1 mM concentrations, but not as significant as when incubated with 1 mM 1-10-PA.

Activity of the protease was not inhibited or significantly decreased when incubated with phenylmethylsulphyonly flouride (PMSF) or leupeptin. When incubated with dithiothreitol (DTT), a reducer of disulfide bonds, protease activity was decreased by 29 % when incubated with a 1 mM concentration and 84 % with a 10 mM concentration.

Protease samples that had been chelexed regained partial activity (66 % and 51 %) by adding back $CaCl_2$ or $MgCl_2$ (Table 13). The addition of $ZnCl_2$ or $MnCl_2$ partially restored some protease activity. Addition of either $CaCl_2$ or $ZnCl_2$ to inhibited protease samples (EGTA and 1-10-phenanthroline) restored protease activity back to 79 - 100 % of control levels (Table 13).

Table 11: Effect of Chelators and Inhibitors on Protease Activity

Chelator or Inhibitor[a]	Conc. (mM)	% Activity Remaining[b]	Class – Enzyme Inhibitor
EDTA	0.1	>100	Metalloprotease
	1	>100	
	10	30	
EGTA	0.1	>100	Metalloprotease
	1.0	78	
	10	31	
1-10-PA	0.1	>100	Metalloprotease, Zn^{2+}
	1	20	
	10	14	
PMSF	0.1	>100	Serine
	1	>100	
	10	18	
Leupeptin	0.1	>100	Serine / cysteine
	1	>100	
	10	64	
DTT	0.1	>100	Disulfide bonds
	1	71	
	10	16	

[a] Azocoll assays were carried out under standard conditions in the presence of the chelators listed. EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N'N'*-tetra acetic acid; 1-10-PA., 1,10-Phenanthroline; PMSF, phenylmethylsulphyonyl fluoride; DTT, dithiothreitol.

[b] The activity in the reaction mixture containing no inhibitor was considered 100 %.

Table 12: Effects of Chelators and Metal Ions on Protease Activity

Addition	**Activity Remaining** (Percent)
None	100
Chelex	25
1 mM Ca^{2+}	66
1 mM Zn^{2+}	35
1 mM Mn^{2+}	10
1 mM Mg^{2+}	51

Note: Azocoll assays were carried out under standard conditions in the standard Azocoll buffer or chelexed Azocoll buffer, to which metals were added back.

Table 13: Effects of Chelators and Metal Ions on Protease Activity

Addition	**Activity Remaining (Percent)**
None	100
EGTA	64
1-10-PA	5
EGTA + Ca^{2+}	79
EGTA + Zn^{2+}	> 100
1-10-PA + Ca^{2+}	> 100
1-10-PA + Zn^{2+}	> 100

Note: Azocoll assays were carried out under standard conditions in the standard Azocoll buffer with metal chelators, to which metals were added back.

SDS-Polyacrylamide Gel Electrophoresis and Zymography

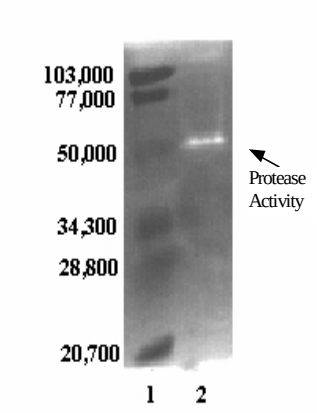
The 5,000 Da membrane ultrafiltrated and concentrated *Burkholderia* strain 2.2 N filtered culture supernatant was run on both 12 % casein and 10 % gelatin polyacrylamide zymogram gels under denaturing conditions. Following renaturation the gel was incubated overnight (30 °C) to allow for hydrolysis of the casein and gelatin, the gels were stained with Coomassie blue. A clear zone was observed in the 12 % casein zymogram. The zone of clearing corresponded to a migration distance consistent with an approximate molecular weight of 60,000 Da, by comparison to real weight standards (Figure 7). There was no cleared band present in the 10 % gelatin zymogram. It is possible that additional proteases are produced by Strain 2.2 N. They're absence in the zymogram could have been due to the denaturation without adequate renaturation.

Samples from the protease purification steps (100 ug concentrated filtered culture supernatant and 100 ug eluted protein from DEAE-column chromatography) were run under denaturing conditions on a 12 % SDS-polyacrylamide gel stained with silver (Figure 8). A doublet present in both the concentrated filtered culture supernatant lane and the eluted protein sample from the DEAE-columns at a migration consistent with a 60,000 Da real weight was observed. This corresponded with the molecular weight (60,000 Da) as the protease activity band seen in the 12 % casein Zymogram (Figure 7).

Antimicrobial Activity of the Purified Protease

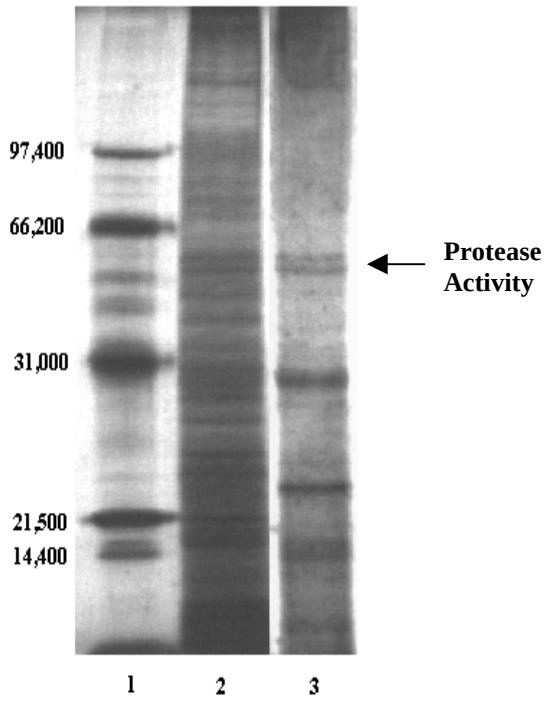
Burkholderia strain 2.2 N filtered culture supernatant exhibited antimicrobial activity against all tested microorganisms. The purified protease samples did not show any zone of inhibitions on any of the following lawns; *Aspergillus niger, Sacchromyces cerevisiae, Micrococcus luteus, Candida albicans,* or *Cryptococcus neoformans*. The purified protease did not exhibit antimicrobial activity against these five organisms.

Figure 7: 12 % Casein Polyacrylamide Zymogram



Lane 1, molecular weight markers: phosphorylase B (103,000), bovine serium albumin (79,000), ovalbumin (50,100), carbonic anhydrase (28,400), soybean trypsin inhibitor (28,400) and lysozyme (20,800). Lane 2, 1.4 mg concentrated *Burkholderia* strain 2.2 N filtered culture supernatant.

Figure 8: 12 % SDS-PAGE (silver stained)



Lane 1, molecular weight markers (Figure 7). Lane 2, 100 ug concentrated *Burkholderia* strain 2.2 N filtered culture supernatant. Lane 3, 100 ug DEAE column eluted fraction.

DISCUSSION

Protease Activity of *Burkholderia* strain 2.2 N

Protease activity of *Burkholderia* strain 2.2 N cultures or culture fractions could be detected by spotting 10 µl samples on Milk Casein Agar. Zones of clearing were indicative of hydrolysis of the milk casein. Using Azocoll as a protease substrate, the highest specific protease activity of the *Burkholderia* strain 2.2 N filtered culture supernatant was exhibited at pH 7.5. Lower protease activities were exhibited in assays conducted at pH 9.0 (27 %) and 4.5 (92 %). These results indicated that *Burkholderia* strain 2.2 N protease was more active at a neutral pH. Neutral proteases have been reported in both *Pseudomonas* and *Burkholderia*. *B. cepacia* produces a protease with an optimal pH of 6.0 (McKevitt et al., 1989), and a number of *P. fluorescens* strains produce proteases active at neutral pHs (Stepaniak et al., 1982; Mitchell et al., 1989).

Effects of Temperature and Time on Protease Activity

Temperature influenced the activity of *Burkholderia* strain 2.2 N protease. Filtered culture supernatant assayed at 4° C showed protease activity, however at a three-fold lower specific activity than at 37° C. Protease activity was lost in filtered culture supernatant samples stored at 4° C, room temperature, and 30° C. Specifically, protease activity of samples stored at 4° C exhibited the greatest loss of activity over time. Samples stored at 4° C retained only 40 % specific activity after 24 hours, and 15 % after 48 hours. Protease activity of samples stored at room temperature fell 55 % after 24 hours, and 65 % total after 48 hours. Finally, protease activity of samples stored at 30° C lost 22 % after 24 hours, and 81 % total after 48 hours.

Burkholderia strain 2.2 N protease is capable of auto-hydrolysis over a temperature range of 4° C to 30° C. Protease activity at temperatures higher than 30° C was not measured. Therefore the optimal temperature for activity can not be determined. The temperature range of activity of proteases of *Burkholderia* and *Pseudomonas* can range from as low as 4° C to as high as 60° C (Mitchell et al., 1989; Sexton et al., 1994; Stepaniak et al., 1982; Ogino et al., 1999). Filtered culture supernatants of *Burkholderia* strain 2.2 N exhibited proteolytic activity at 4 °C, indicating the enzyme is cold active and therefore auto-hydrolytic at low temperatures. Proteases active at low temperatures are not common, however like Strain 2.2 N, a few proteases have been isolated that exhibit this type of activity. *P. flourescens* strains P26, 32A, P1 and P27 isolated from pasteurized milk, produces a protease which has activity at 5° C (Mitchell et al., 1989; Stepaniak et al., 1982) and *P. pseudomallei* produces an extracellular protease which is active at 4° C (Sexton et al., 1994).

Effect of Medium Composition on Production of *Burkholderia* strain 2.2 N Protease

Burkholderia strain 2.2 N cells grown in 1.0 % yeast extract and one-quarter-strength Tryptic Soy Broth containing 0.2 % sucrose showed the highest specific protease activity in filtered culture supernatants. There was substantial variation in the protease activity of different cultures. This could have been due to the use of different Tryptic Soy and Yeast Extracts. These contain a low percentage of ash that includes metals. Metals may increase or decrease the production of proteases as well as their stability (Kohlman et al., 1991, Olson et al., 1992, Sexton et al., 1994 and Stepaniak et al., 1982).

The addition of 10 mM $MgCl_2$ to a minimal salt medium containing 1.0 % yeast extract, 1.0 % K_2HPO_4, 1.0 % NaH_2PO_4 increased the specific protease activity two-fold. A medium

consisting of 1.0 % yeast extract, 1.0 % K_2HPO_4, 1.0 % NaH_2PO_4 with 10 mM $MgCl_2$ provided the simplest and cheapest medium for the growth of *Burkholderia* strain 2.2 N and purification of a large amount of protease. Addition of metal ions to growth media has been shown to not only increase protease activity, but may also be required for the production and stability of some proteases (Liao et al., 1998; Kohlman et al., 1991; Olson et al., 1992).

Timing of Production of *Burkholderia* strain 2.2 N Protease

Burkholderia strain 2.2 N cells began exponential growth 2 hours after inoculation at one (rapid) rate and shifted to a lower rate of growth at 4 – 6 hours (Figure 3). At 16 hours the cells entered stationary phase and remained in stationary phase for up to 60 hours, the end point of the growth curve measured. There was no evident death or lytic phase. Protease activity was present at low levels in the cell-free culture supernatant immediately after inoculation. The protease activity rose at 18 hours, as cells began stationary phase. Protease activity continually increased after 18 hours through 60 hours (Figure 3). This pattern is indicative of a secondary metabolite.

Location of *Burkholderia* strain 2.2 N Protease Activity

Filtered culture supernatants had 86 % of the protease activity of *Burkholderia* strain 2.2 N cultures. Further, when crude extracts were separated into soluble and membrane fractions, all activity was present in the membrane fraction. Low percentages of alkaline phosphatase (3 %) and ß-galactosidase (11 %) in the cell-free culture supernatant fraction suggested that Strain 2.2 N cells did not leak enzymes. On the basis of that data I propose that *Burkholderia* strain 2.2 N protease is an extracellular enzyme, consistent with published *Pseudomonas* and *Burkholderia*

proteases. Localization experiments with *P. fluorescens* showed more extracellular proteolytic than cell-associated activity (Kohlmann et al., 1991; Liao et al., 1998). Further, it was shown that *P. aeruginosa* strain PA103-29 protease activity was secreted into the medium during growth (Engel et al., 1998).

It is not known if the enzyme is transported as a pre / pro-enzyme, then cleaved to its mature form extracellularly. Generally, *Pseudomonas* and *Burkholderia* strains secrete proteases as pre / pro-enzymes which are cleaved to an active form extracellularly (Kessler et al., 1998; Olson et al., 1992).

Purification of *Burkholderia* strain 2.2 N Protease

Ammonium sulfate purification increased the protease activity 20-fold compared to the activity present in the unconcentrated culture supernatant. This precipitation step also decreased the overall protein concentration (70 - 90 %) in the precipitation fraction containing the highest protease activity. Two DEAE-Sepharose column purification steps followed the ammonium sulfate purification. The protease activity was present in the 0 - 0.25 M NaCl gradient in both column runs. Ammonium sulfate precipitation followed by DEAE-Sepharose column chromatography of *Burkholderia* strain 2.2 N supernatant increased specific activity 50-fold. The purification process took approximately 72 – 96 hours. Although the purification steps were conducted at room temperature and specific activity increased, because the protease was subject to auto-hydrolysis, it is likely higher yields and specific activity can be attained by preventing proteolytic activity.

Increases of the Strain 2.2 N protease activity using ammonium precipitation (20-fold increase) are consistent with published literature, which shows a purification (fold) range from

1.9 – 96 (Liao et al., 1998; McKevitt et al., 1989; Sexton et al., 1994). Subsequent increases in protease activity (50-fold increase) using DEAE-column chromatography were also consistent with purification ranges from 7.8 – 555 (Liao et al., 1998; McKevitt et al., 1989; Sexton et al., 1994).

SDS-Polyacrylamide Gel Electrophoresis and Zymography

The cell-free, filtered culture supernatant of *Burkholderia* strain 2.2 N exhibited a band of hydrolysis at a molecular weight of 60,000 Da on a 12 % casein zymogram (Figure 7), indicating there was one protease within the extracellular supernatant. This band of hydrolysis at 60,000 Da corresponded to a doublet band present in both the filtered culture supernatant and purified protease sample on a 12 % SDS-polyacrylamide gel (Figure 8). No corresponding band of activity was observed in the ammonium sulfate purified fraction. The same bands were not seen in lanes containing the ammonium sulfate fraction. This might have been due to the fact the samples were not fully dialyzed or not enough protein was loaded onto the gels for the bands to be present. It can be concluded that the protease is approximately 60,000 Da.

Because protease activity was present at increasing specific activities during the steps of purification, it is likely that the differences in protein band patters seen in the SDS-PAGE gels were due to proteolytic activity.

Characterization of *Burkholderia* strain 2.2 N Protease

Purified protease samples were assayed in the presence of inhibitors and metal chelators. The failure of either serine and cysteine proteinase inhibitors (i.e., leupeptin and PMSF) and sensitivity to metal chelating inhibitors suggest that Strain 2.2 N protease is a metalloproteinase

(Powers et al., 1986). A decrease in activity was measured in the presence of two metal chelators, EGTA and EDTA. However, inhibition by EDTA is not a good indicator of a metalloprotease because a large number of other enzymes require calcium for activity and EDTA is an excellent calcium chelator (Powers et al., 1986). The strongest inhibitor of protease activity was 1-10-phenanthroline, which is the preferred metalloproteinase inhibitor, as it will not remove calcium (Powers et al., 1986).

Protease samples were incubated with Chelex® 100 and regained partial activity (66 % and 51 %) upon addition of $CaCl_2$ or $MgCl_2$. Addition of either $CaCl_2$ or $ZnCl_2$ to inhibited protease samples (i.e., EGTA and 1-10-phenanthroline) restored protease activity to 79 - 100 % of control levels.

Burkholderia strain 2.2 N protease exhibited specificity of an extracellular metalloprotease with possible divalent metal requirements of Mg^{2+}, Ca^{2+}, or Zn^{2+}. Requirements of divalent metals are common to other *Pseudomonas* and *Burkholderia* proteases. *Pseudomonas pseudomallei* strain 319 metalloprotease requires Fe^{2+} for activity (Sexton et al., 1994) and *P. aeruginosa* strain PAO1 produces both an elastase with Zn^{2+} requirements and a protease that requires both Ca^{2+} and Zn^{2+} (Olson et al., 1992).

Antimicrobial Activity of the Purified Protease

Burkholderia strain 2.2 N filtered culture supernatant exhibited broad spectrum antimicrobial activity against all target microorganisms. However, purified protease samples from ammonium sulfate precipitation and DEAE-Sepharose column chromatography did not show any zones of inhibition against *A. niger, S. cerevisiae, M. luteus, C. albicans or C. neoformans*. The purified protease did not exhibit antimicrobial activity.

REFERENCES

Abe, M. and T. Nakazawa. 1996. The *dsbB* gene product is required for protease production by *Burkholderia cepacia*. Infect. Immun. **64:**4378-4380.

Beynon, J. and J.S. Bond. 1989. Proteolytic enzymes, a practical approach. IRL Press, New York, NY.

Bollag, D.M and S.J. Edelstein. 1991. Protein Methods. Wiley-Liss, Inc., New York, NY.

Cain, C.C., A. T. Henry, R.H Waldo, III., L.J. Casida, Jr., and J.O. Falkinham, III. 2000. Identification and characteristics of a nonobligate bacterial predator of the genus *Burkholderia* with broad spectrum antimicrobial activity. Appl. Environ. Microbiol. Vol. 66.

Casida, L.E., Jr. 1988. Minireview: nonobligate bacterial predation of bacteria in soil. Microb. Ecol. **15:**1-8

Casida, L.E., Jr. 1992. Competitive ability and survival in soil of *Pseudomonas* strain 679-2, a dominant, nonobligate bacterial predator of bacteria. Appl. Environ. Microbiol. **58:**32-37.

Casida, L.E., Jr. 1993. Predatory *Pseudomonas* strain as a control of bacterial and fungal plant pathogens. United States Patent Number 5,232,850.

Chavira, R., Jr., J. Thomas, and J.H. Hageman. 1984. Assaying proteinases with azocoll. Anal. Biochem. **136:**446-450.

De Ley, J. 1992. The Proteobacteria. Ribosomal RNA cistron similarities and bacterial taxonomy, pp. 2111-2140. In: The prokaryotes, a handbook on the biology of bacteria, ecophysiology, isolation, identification and applications (A. Balows, H.G.Truper, M. Dworkin, W. Harder and K.H Schleifer, eds.) 2nd Ed., Vol. II, Springer Verlag.

Engel, L.S., J.M. Hill, A.R. Caballero, L.C. Green, and R.J. O'Callaghan. 1998. Protease IV, a unique extracellular protease and virulence factor from *Pseudomonas aeruginosa*. J. Biol. Chem. **273:**16792-16797.

Gillis, M., T.V. Van, R. Bardin, M. Goor, P. Hebbar, A. Willems, P. Segers, K. Kersters, T. Heulin, and M.P. Fernandez. 1995. Polyphasic taxonomy in the genus Burkholderia leading to an emended description of the genus and proposition of *Burkholderia vietnamiensis* sp. Nov. for N2-fixing isolates from rice in Vietnam. Int. J. Syst. Bacteriol. **45:** 274-289.

Gottlieb, M. and Chavko, M. 1987. Silver staining of native and denatured eucaryotic DNA in agarose gels. Anal. Biochem. **165:**33-37.

Kersters, K., W. Ludwig, M. Vancanneyt, P.D. Vos, M. Gillis, and K-H. Schleifer. 1996. Recent changes in the classification of the *Pseudomonads*: an overview. System. Appl. Microbiol. **19:**465-477.

Kessler, E., M. Safrin, J.K. Gustin, and D.E. Ohman. 1998. Elastase and the LasA protease of *Pseudomonas aeruginosa* are secreted with their propeptides. J. Biol. Chem. 273:30225-30231.

Kohlmann, K.L., S.S. Nielson, and M.R. Ladisch. 1991. Purification and characterization of an extracellular protease produced by *Pseudomonas florescens* M3/6. J. Dairy Sci. **74:**4125-4136.

Krieg, N.R. and J.G. Holt. 1984. Bergey's manual of systematic bacteriology. Volume 1. Williams and Wilkins, Baltimore, MD. Pgs. 142-201.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London). **227:**680-685.

Liao, C.H. and D.E. McCallus. 1997. Biochemical and genetic characterization of an extracellular protease from *Pseudomonas fluorescens* CY091. Appl. Environ. Microbiol. **64:**914-921.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265-275.

McKevitt, A., S. Bajaksouzian, J.D. Klinger, D.E. Woods. 1989. Purification and characterization of an extracellular protease from *Pseudomonas cepacia.* Appl. Environ. Microbiol. **57:**771-778.

Mitchell, S.L., and R.T. Marshall. 1989. Properties of heat-stable proteases of *Pseudomonas flurescens*: characterization and hydrolysis of milk proteins. J. Dairy Sci. **1987:**864-874.

Nelson, J.W., S.L. Butler, D. Krieg, and J.R.W. Cowan. 1994. Virulence factors of *Burkholderia cepacia*. FEMS Immunol. Med. Microbiol. **8:**89-98.

Ogino, H., F. Watanabe, M. Yamada, S. Nakagawa, T. Hirose, A. Noguchi, M. Yasuda, and H. Ishikawa. 1999. Purification and characterization of organic solvent-stable protease from organic solvent-tolerant *Pseudomonas aeruginosa* PST-01. J. Biosci. Bioeng. **87:**61-68.

Olson, J.C., and D.E. Ohman. 1992. Efficient production and processing of elastase and LasA by *Pseudomonas aeruginosa* require zinc and calcium ions. J. Bacteriol. **174:**4140-4147.

Oppert, B., K.J. Kramer, W.H. McGaughey. 1997. Rapid microplate assay for substrates and inhibitors of proteinase mixtures. BioTechnology **23:**70-72.

Palleroni, N.J., R. Kunisawa, R. Contopoulou, and M. Doudoroff. 1973. Nucleic acid homologies in the genus *Pseudomonas.* Int. J. Syst. Bacteriol. **23**:333-339.

Pelzcar, Jr., M.J., E.C.S. Chan, and N.R. Krieg. 1993. Microbiology Concepts and Applications. McGraw-Hill, Inc., New York, N.Y. Pgs. 772-778.

Polgar, Laszlo. 1989. Mechanisms of protease action. CRC Press, Inc., Boca Raton, Florida. Pgs. 43-76.

Powers, J.C. and J.W. Harper. 1986. "Inhibitors of metalloproteases". Protease Inhibitors. Elsevier Science Publishers, New York, NY. Pgs. 219-298.

Rao, M.B., A.M. Tanksale, M.S. Ghatge, and V.V Deshpande. 1998. Molecular and biotechnological aspects of microbial proteases. Microbiol. Mol. Biol. Rev. **62:**597-635.

Sambrook. J., E.F. Fritsch, and T. Maniatis. Molecular Cloning. Second Edition. 1989. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY.

Sexton, M.M., A.L. Jones, W. Chaowagul, and D.E. Woods. 1994. Purification and characterization of a protease from *Pseudomonas pseudomallei*. Can. J. Microbiol. **40:**903-910.

Stanier, R.Y., N.J. Palleroni and M. Doudoroff. 1966. The aerobic Pseudomonads: a taxonomic study. J. Gen. Microbiol. **43:**159-271.

Stepaniak, L., P.F. Fox, and C. Daly. 1982. Isolation and general characterization of a heat-stable proteinase from *Pseudomonas fluorescens* AFT 36. Biochim. Biophys. Acta. **717:**376-383.

Tyler, S.D., C.A. Strathdee, K.R. Rozee, and W.M. Johnson. 1995. Oligonucleotide primers designed to differentiate pathogenic *Pseudomonads* on the basis of the sequencing genes coding for 16S-23S rRNA internal transcribed spacers. Clin. Diagn. Lab. Immunol. **2:**448-453.

Urakami, T., C. Ito-Yoshida, H. Araki, T. Kijima, K. Suzuki, and K. Komagata. 1994. Transfer of *Pseudomonas plantarii* and *Pseudomonas glumae* to *Burkholderia* as *Burkholderia* spp. and description of *Burkholderia vandii* sp. nov. Int. J. Syst. Bacteriol. **44**:235-245.

Xiang, L., M. Dorsch, T. Del Dot, L.I. Sly, E. Stackebrandt and A.C. Hayward. 1993. Phylogenetic studies of the rRNA group II pseudomonads based on 16s rRNA gene sequences. J. Appl. Environ. Bacteriol. **74:**324-329.

Yabuuchi, E., Y. Kosako, H. Oyaizu, I. Yano, H. Hotta, Y. Hashimoto, T. Ezaki and M. Arakawa. 1993. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. Microbiol. Immunol. **36:**1251-1275.

Yabuuchi, E., Y. Kosako, I. Yano, H. Hotta, and Y. Nishiuchi. 1995. Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov.: proposal of *Ralstonia picketti* comb. nov., *Ralstonia solanacearum* com. nov. and *Ralstonia eutropha* com. nov. Microbiol. Immunol. **39**:897-904.

Young, L.W. 1980. The role of exotoxins in the pathogenesis of *Pseudomonas aeruginosa.* J. Infect. Dis. **142:**626-630.

Zeph, L.R. and L.E. Casida, Jr. 1986. Gram-negative versus gram-positive (Actinomycete) nonobligate bacterial predators of bacteria in soil. App. Environ. Microbiol. **54:**819-823.

APPENDIX I.

Standard Operating Procedure (SOP)

Zone of Inhibition Assay for Antimicrobial Activity of Cell-Free Culture-Fractions or Products.

Objective

Measure the antimicrobial activities of cell-free culture, culture-fractions or products by zone-of-inhibiton assay.

Starting Materials

(1) Cell-free culture fractions or products.
(2) Standard, reference spray dried *Burkholderia* strain 2.2N material suspended at 1% (wt/vol) in sterile distilled water (1gm aliquots of powder stored at -70˚C).
(3) Butanol extracts of cultures or culture fractions of *Burkholderia* strain 2.2N.
(4) Cultures of susceptible, target microorganisms: *Saccharomyces cerevisiae*, *Aspergillus niger*, and *Micrococcus luteus*.

Equipment

Autoclave
30° C Incubator
30° C Shaking Water Bath
45° C Water Bath
Centrifuge
Heating block (45° C)
13 x 100 mm capped test tubes
Micropipettors

Media

Brain Heart Infusion Broth with Sucrose (BHIB+S)

3.7 gm Brain Heart Infusion Broth (Difco 0037-17-8) (one-tenth strength)
2.0 gm sucrose (Fisher BP220-212)
1 liter distilled water

For solid media: add 15 gm agar (BBL 11853) / Liter

Tryptic Soy Broth with Sucrose (TSB+S)

7.5 gm Tryptic Soy Broth (BBL 4399071)
2.0 gm sucrose (Fisher BP220-212)
1 liter distilled water

For solid media add 15 g agar (BBL 11853) / Liter

Potato Dextrose Agar (PDA)

39 gm Potato Dextrose Agar (Difco 0013-17-6)
1 liter distilled water

BHIB+S Top Aagar

1.85 gm Brain Heart Infusion Broth (Difco 0037-17-8) (one-tenth strength)
1.0 gm sucrose
6.0 gm agar (BBL 11853)
500 ml distilled water

Dissolve agar by heating and stirring.
Aliquot 100 ml into 5 milk-dilution bottles.

Buffered Saline Gelatin (BSG)

0.1 gm gelatin (Difco 0143-01)
8.5 gm NaCl
0.3 gm KH_2PO_4
0.6 gm Na_2HPO_4
1 liter distilled water

Preparation of Susceptible Target Microorganisms

***Saccharomyces cerevisiae* and *Micrococcus luteus*:**

(1) Inoculate single colony into 20 ml of BHIB+S broth in a 125 ml Erlenmeyer flask
(2) Incubate for 48 hrs at 30°C at 60 rpm.
(3) Transfer culture into a 16 x 125 mm sterile screw capped test tube and store at 4°C.
(4) Good for two weeks.

***Aspergillus niger*:**

(1) Streak 100 µl of spore suspension on the surface of PDA agar.
(2) Incubate at 30° with the medium side on the bottom (lid up). Do not seal plate with Parafilm.
(3) Incubate medium until the surface is completely black (2-3 days) with spores.
(4) Harvest spores by pipetting 10 ml sterile BSG onto culture surface. Suspend spores in the BSG by scraping the surface with a glass rod with a circular spreading motion.
(5) Recover spore suspension by collecting the liquid using a sterile 10 ml serological pipette.
(6) Transfer liquid through sterilie glass wool contained in a funnel into a 16 x 125 mm screw cap centrifuge tube.
(7) Collect spores by centrifugation (5000 x g for 10 minutes at room temperature).
(8) Discard supernatant liquid and suspend spores in BSG to equal a 0.5 MacFarland Standard.
(9) Store at 4° C.

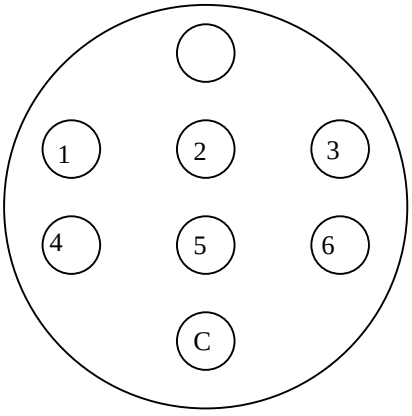
Measurement of Antimicrobial Activity

Measure Activity of 5 Separate Cell-Free Suspensions or Liquid Products

1. Suspend dry product at 1% (wt/vol) or use liquid product directly.
2. Prepare a two-fold dultuion series to 1:4096 for 5 separate cell-free suspensions or liquids of each product to be tested.

3. Prepare a two-fold dilution series to 1:4096 for the standard reference spray dried material suspended at 1% (wt/vol) concentration in sterile distilled water (i.e., for quality assurance and quality control, QA/QC).
4. Melt top agar by autoclaving for 7 min. Cool to 45° C and transfer 3.5 ml each to 13 x 100 mm test tubes. Place in 45° C heating block.
5. Label medium-containing portion of BHIB + S plates.
6. Add the following volumes (each) of culture or suspension of target susceptible microorganisms to 3.5 ml top agar: (i.e., 75 µl *S. cerevisiae*., 100 µl *M. luteus*, or 100 µl *A. niger*).
7. Mix suspension by vortexing without creating bubbles and immediately distribute suspension evenly onto the surface of the agar medium
8. Allow agar to solidify 5 minutes before spotting materials to be tested.
9. Using the pattern (below) spot 10 µl of all undiluted samples and dilutions, in duplicate on the lawn(s). Include the following controls: growth medium, BSG or diluent (negative control) and undiluted standard, reference spray dried material suspended at 1% (wt/vol) concentration in sterile water (positive control).
10. Allow spotted samples to air-dry completely, with lid off, before transferring plate to incubator.
11. Incubate plates at 30˚ C for 24 hours under the following conditions: Plates containing *S.cerevisiae* and *M.luteus* incubate inverted. Plates containing *A.niger* , parafilm and incubate with the agar side down, lid up.
12. Measure diameter of zone of inhibition for each dilution and identify the highest dilution yielding a zone of inhibition . Compute the highest average dilution with a zone of inhibition for each of the 5 identical samples of a single cell-free products or liquid suspensions tested.
13. Identify the highest dilution yielding a zone of inhibition for the standard reference spray dried material. Recordthe highest dilution with a zone of inhibition and compare to the recorded values for the other assays. Value should be within one standard deviation (one dilution) of mean for previous recorded values.

Example



M = Growth medium , BSG and diluent (Negative Control)
1-6 = Samples and dilutions to be tested
C = Reference Spray Dried Material (Positive Control)

APPENDIX II.

Sally Nicole Jewell

4619 N. Dittmar Road • Arlington, Virginia 22207 • (703) 534-4619 • jewell@vt.edu

EDUCATION

Master of Science, Microbiology, 2000.
GPA; 3.70 (4.0 scale)
Department of Biology, Fralin Biotechnology Center
Virginia Polytechnic Institute and State University, Blacksburg, VA.

Thesis:
Purification and Characterization of a Novel Protease from *Burkholderia s*train 2.2 N.

Bachelor of Science, Biology, 1997.
Minor: Chemistry Option: Microbiology / Immunology
Virginia Polytechnic Institute and State University, Blacksburg, VA.

PROFESSIONAL ACCOMPLISHMENTS

Research Consultant Dominion BioSciences, Inc.
Blacksburg, VA. August, 1999 – present.

Purifying and characterizing a novel protease from a new species of bacteria
- Protein characterization using chelator, metal ion and inhibitor assays
- Use of column chromatography, and gel electrophoresis for protein purification
- Determination of location of protease using enzyme specific assays
- Optimization of media and growth conditions for protease overproduction

Purifying novel antimicrobial biological compounds
- Preparing antimicrobial samples for chemical identification
- Preparing Standard Operating Procedures and written reports
- Transfer of biological assays and techniques to chemical collaborators

Laboratory Specialist Senior Department of Microbiology, Fralin Biotechnology Center
Virginia Tech, June, 1997 - August, 1999.

Identification and Characterization of predator bacteria
- Nested 16S rRNA PCR identified new species of ubiquitous bacteria
- Sequence analysis, multiple alignments and primer design
- Biochemical and 16s rRNA identification of bacterial species
- Bacterial and DNA isolation from soil samples

Scale up antibiotic production to 30L fermentation
- Monitored and supervised Quality Control / Quality Assurance program
- Developed antimicrobial and antifungal activity assays
- Determined optimum medium and transfer of techniques for scale-up
- Maintained culture collection and transfer of strains

Managerial / Supervisory Responsibilities
- Technical training and supervision of lab support personnel and undergraduate students
- Maintained laboratory data, records and formatted research reports
- Monitored laboratory equipment and inventory purchases

Teaching Assistant Department of Biology,
Virginia Tech, August, 1999 – May, 2000

- Teaching evaluations, 3.70 (4.0 scale)
- Lecturing general microbiology and principles of biology topics
- Teaching undergraduate students laboratory skills

PUBLICATIONS:

Jewell, S.N., R.H. Waldo, C.C. Cain, and J.O. Falkinham, III. Rapid Detection of Lytic Antimicrobial Activity Against Yeast and Filamentous Fungi. (submitting)

PRESENTATIONS:

Jewell, S.N., and J.O. Falkinham, III. 2000. Purification and Characterization of a novel protease from *Burkholderia casidae* Strain 2.2N. 100th General Meeting of the American Society for Microbiology, Los Angeles, CA.

Jewell, S.N., and J.O. Falkinham, III. 1999. Purification of a novel protease from *Burkholderia casidae* Strain 2.2N. The ASM Virginia Branch Annual State Meeting, Blacksburg, VA.

Hall, D.J., **S.N. Jewell**, J.O. Falkinham, III. 1999. Isolation and Characterization of Predator Bacteria from Soil. Virginia Tech Research Symposium, Blacksburg, VA.

Jewell, S.N., R.H. Waldo, C.C. Cain, J.O. Falkinham, III. 1998. Rapid Detection of Lytic Antimicrobial Activity Against Yeast and Filamentous Fungi. 98th General Meeting of the American Society for Microbiology, Atlanta, GA.

GRANTS & AWARDS:

1999 Graduate Research Award, The ASM VA Branch Annual State Meeting
1999 Graduate Student Association, Research Development Grant, Virginia Tech
1999 Department of Biology, Research Development Grant, Virginia Tech

AFFILIATIONS:

1996 – present American Society for Microbiology