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ACQUISITION OF HEAT STABLE ENZYMES FROM THERMOPHILIC MICROORGANISMS: PEROXIDASES, UREASES, AND GLUCOSE OXIDASES

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13. ABSTRACT (Maximum 200 words)

Thermophilic microorganisms producing heat stable peroxidases, ureases and glucose oxidase have been collected and isolated. Optimization methodology for the production and purification of these enzymes has been investigated in detail. The 16S rRNA and fatty acid analyses suggest the three organisms submitted to US Army Chemical Research, Development and Engineering Center (CRDEC) have not been previously isolated and identified.

The most noteworthy observation was a heat stable urease with a molecular weight (MW) between 120,000 and 130,000. Considering the poor heat stability of Jack Bean urease and MW of 550,000, this heat stable urease is a particularly valuable enzyme for conjugation reactions used for a host of diagnostic assays.

The bacterium producing a peroxidase with slightly better stability than a recent Japanese isolate, was observed to have a distinctive microscopic appearance and may later be classified in a new genus. A total of 4 thermophiles were submitted to CRDEC: #197 for peroxidase; #408 and 429 for urease; and #370 for glucose oxidase. Detailed methodology for culturing, fermenting, maintaining and assaying the enzymes are described.

Fermentation evaluation in reactors to 15 L was accomplished and detailed instructions for these organisms for enzyme production and purification were developed. Fermentations will require close monitoring of several parameters to achieve optimum production. Further investigation on the purification of all three enzymes is essential to achieve efficient removal of protease and other non specific proteins without losing major portions of the enzymes of interest.

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General Commercialization

Commercial potential for heat stable enzymes and other bioactive compounds produced by thermophilic microorganisms is substantial and growing rapidly. Although specific enzymes examined in this project have not yet been commercialized, this SBIR grant has led directly to a number of contracts for J. K. Research.

The investigation of peroxidase producing microorganism has transitioned to a contract with a major biotechnology company to examine related protective mechanisms. That contract provides for one year of research and exclusive rights and sales of those products to the biotechnology company, insuring commercialization of all products developed. Since the peroxidase from this SBIR project was so stable compared with others presently available, we anticipate that current distributed samples to various companies will undoubtedly lead to commercialization.

Numerous inquires have been received on all three enzymes investigated in this SBIR project and other heat stable enzymes. Interest has been sufficient for us to reach agreement with a marketing firm, Omega Biologicals, to assist us with further marketing and licensing of associated technologies.

A proposal, partially based on the urease findings, has been written and submitted to the Department of the Navy. We have recently been notified of its recommendation for funding and anticipate project initiation within the next 2-3 months. Heat stable enzymes are of interest to a number of Chemical Companies. Preliminary discussions indicate the urease to be the most intriguing. Results at the very end of the project revealed that this enzyme is a very low molecular weight urease, which will be an important marketing point for many potential users, so again we do anticipate eventually being able to commercialize this enzyme.

The glucose oxidase generated the least interest among potential users, largely because the product already on the market is relatively heat stable. However, one pharmaceutical company is interested and has already provided us with two small contracts for additional work on this enzyme.

J. K. Research is currently negotiating with five biotechnology/ pharmaceutical firms for additional research contracts. At least three would be in the form of direct contracts with large firms and one would be a cooperative effort on an SBIR proposal.

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Summary

Microorganisms producing heat stable peroxidase, urease and glucose oxidase have been collected and isolated. Optimization methodology for the production and purification of these enzymes has been investigated in detail. 16S rRNA and fatty acid analyses suggest the three organisms submitted to CRDEC have not been previously isolated and identified and thus are considered novel microorganisms.

The most noteworthy observation was a heat stable urease with a molecular weight between 120,000 and 130,000. Considering the poor heat stability of Jack Bean urease and MW of 550,000, this heat stable urease is a particularly valuable enzyme for conjugation reactions used for a host of diagnostic assays.

The bacterium producing a peroxidase with slightly better stability than a recent Japanese isolate, was observed to have a distinctive microscopic appearance and may later be classified in a new genus. A total of 4 thermophiles were submitted to CRDEC: #197 for peroxidase; #408 and 429 for urease; and #370 for glucose oxidase. Detailed methodology for culturing, fermenting, maintaining and assaying the enzymes of interest are described.

Fermentation evaluation in reactors to 15 L was accomplished and detailed instructions for these organisms for enzyme production and purification were developed. Fermentations will require close monitoring of several parameters to achieve optimum production. Further investigation on the purification of all three enzymes is essential to achieve efficient removal of protease and other non specific proteins without loosing major portions of the enzymes of interest.

This SBIR project has led to a contract with a biotechnology company and an additional SBIR contract. An agreement with a marketing firm will help further commercialization of these heat stable enzymes:

- A peroxidase with slightly better stability than recent Japanese isolate
- A urease with molecular weight ~125,000 (Jack Bean urease MW 550,000) of particular value for labeling

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PREFACE

The work described in this report was authorized under Contract No. DAAA15-89-C-0501. The work was started in August 1989 and completed in August 1991.

The use of trade names or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

This report has been approved for release to the public.

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We also wish to thank the National Park Service for permitting us to collect samples from Yellowstone Park. The co-operation of all Park Service personnel we dealt with contributed to the success of our research. We would like to specifically mention the assistance provided by Rick Hutchinson, District Rangers John Lounsbury, Joe Evans, Jerry Mernin, and Steve Frye and Administrator John Varley.

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Peroxidase

I. Introduction

An environment considered to be extreme for many organisms is optimal for a few others. Certain microorganisms actually thrive under temperature extremes and in the presence of high concentrations of acids, heavy metals, sulfur, radioactivity and UV radiation. One reason these organisms can withstand extreme conditions is they produce enzymes which are inherently more stable. Changes in the amino acid sequence resulting in increased intramolecular bonding add up to a significantly more stable enzyme. Most research has examined effects of these bonding changes on thermostability but these changes also increase enzyme stability in the presence of other disruptive factors such as detergents and organic solvents.

Peroxidase, one enzyme of the cellular antioxidant defense system, is frequently used as a label in ELISA type assays. Poor temperature stability of horseradish peroxidase, the most widely available peroxidase, led to a search for a more thermal tolerant source.

Origin

The study of peroxidase in thermophiles is not without precedent. Five well characterized thermophiles are known to produce peroxidase along with a number of unidentified thermophilic isolates. Allgood and Perry compared peroxidase levels (units per mg protein) in *Thermus aquaticus* (18), *Thermomicrobium roseum* (11), three strains of *Thermoleophilum album* (42-93) and *E. coli* (471) in aerated cultures. The stability of these peroxidases seems questionable since when they were assayed at 45°C or above, only the peroxidase from *T. aquaticus* and *E. coli* could be detected (Allgood C). *Thermus thermophilus* was also identified as a thermophile producing peroxidase (MacMichael). More recently, the Japanese have raised *Bacillus stearothermophilus* to produce a heat stable peroxidase, with optimal activity at 70°C (Loprasert).

Geothermal activity is responsible for the creation of numerous extreme microenvironments. The thermal features in Yellowstone National Park make up the largest and most varied array of microbial habitats on earth. These relatively undisturbed, extreme environments which have permitted the evolution of unusual microorganisms, were the source of all microorganisms described in this investigation.

During Phase I research, 200 samples were collected from a range of thermal waters (Combie and Runnion). A number of peroxidase producing organisms were isolated. These isolates fell into two groups, organisms producing peroxidase that was most active near neutral pH and acidophiles producing a peroxidase that was most active between pH 2 and 3. The acidophiles were likely several strains of one organism. Although the acidophiles produced high levels of peroxidase and after submitting samples to CRDEC, the enzymes were determined unsuitable for CRDEC needs. Accordingly, this report will focus on the peroxidases that proved to be most active and stable under neutral pH conditions. Data on the acidophile is summarized near the end of this report.

Enzyme Assay

Chromogen Selection.. The peroxidase reaction consists of the transfer of hydrogen or electrons from an oxidative chromogen to the highly reactive catabolized radical products of the substrate, hydrogen peroxide. The criteria for a chromogen to be useful in the peroxidase reaction is the ability to change color upon oxidation. A large number of compounds can serve as chromogens. Several chromogens were tested over the course of this project for two reasons: (1) Peroxidases from various sources differ in regard to their apparent reaction rates with different chromogens and (2) As a result of

the ability to penetrate intact cells, a select few chromogens can be used to detect peroxidases inside the cells. The latter avoids the necessity of cell disruption, a useful alternative when screening large numbers of organisms. Following is a list of chromogens tested:

Table 1. Oxidative Chromogens Used With Peroxidases

ABTS	2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)
Aminoantipyrine	Phenol-aminoantipyrine
DMP	N,N-dimethyl-p-phenylenediamine
Isoproterenol	3-4-dihydroxyphenyl-β-ethanol-isopropylamine
Pinacyanol Cl	2,2'-Trimethinequinocyanine chloride
TMBZ	3,3'5,5'-Tetramethylbenzidine

One of the more successful chromogens was TMBZ (Bos, Parham), for it was readily adapted to both screening and later quantitative assays. TMBZ is not soluble in water but could be readily dissolved in dimethylsulfoxide (DMSO). Since cells were permeabilized by the DMSO-methanol, total peroxidase concentrations could be determined without the necessity of breaking open the cells. TMBZ has been reported to be non-mutagenic (Bos) although this point has been disputed (Giunta). TMBZ also yields a color superior to that obtained with commonly used diamines. During screening, TMBZ dissolved in DMSO and methanol was added to slants of the test organism. Following an initial incubation, a dilute solution of hydrogen peroxide was added and the slants incubated for a second but shorter time period generating a green color produced by the oxidation of the TMBZ. Most assays were conducted at 20°C for conventional use and convenience. However, assays were also performed at 37°C and 50°C to demonstrate the stability of the enzymes under stressful conditions and improved signals with these enzymes in assays at elevated temperatures. The following quantitative assays are described for incubation at 20°C, however, temperatures may be increased and incubation times decreased as desired:

Peroxidase Assay - *TMBZ*

1 mL Diluted Peroxidase
2 mL 0.2 M Sodium phosphate buffer, pH 6
1 mL .01% TMBZ in 1:9 DMSO: methanol
1 mL Dilute H_2O_2 (30% H_2O_2 - 25 µL / 100 mL water)
Incubate 10 minutes at 20°C
Read green color at 400 nm

Isoproterenol, a sympathomimetic amine used in medical practice, has been successfully used for whole cell microbial peroxidase tests. Catalase does not interfere with this assay and unlike most other chromogens is definitely not toxic or carcinogenic. This adrenergic compound was used extensively during initial screening of water samples and on later occasion when it was convenient to not disrupt the cells.

Peroxidase Assay - *Isoproterenol*

0.5 mL Dilute Peroxidase
3.6 mL Isoproterenol (.5% in acidified water - allow to stand 1 hour before use)
0.2 mL Dilute H_2O_2 (30% H_2O_2 - 0.5 mL / 37 mL water)
Incubate 10 minutes at 20°C
Read at 480 nm

Aminoantipyrine was another chromogen evaluated (Saunders, Shinmen). This assay produced better color at pH 7 to 7.5 than at the pH 5-6 usually employed with many of the other peroxidase assays. Loprasert et al. substituted dichlorophenol for the phenol and used this assay for the peroxidase obtained from thermophilic *B. stearothermophilus*.

Peroxidase Assay - *Aminoantipyrine*	
Dilute peroxidase	1 mL
Sodium phosphate buffer (0.2 M, pH 7.4)	2 mL
Aminoantipyrine (0.04 gm/100 mL water)	1 mL
Phenol (0.60 gm/10 mL water)	1 mL
Dilute H_2O_2 (25 µL 30% H_2O_2/100 mL water)	1 mL
Incubate 30 minutes at 20°C	
Read at 500 nm	

ABTS is perhaps the most widely used chromogen for peroxidase assays. It was in use at CRDEC at the time this work was initiated and was used in the majority of the assays conducted during this investigation and the quantitative assays of the original organism screening.

Some spontaneous color development will be observed for ABTS, particularly as the pH decreases below 6. Therefore a reagent blank must be included in the assay. ABTS has also been reported to be mutagenic by the Ames test (Bos) and therefore caution should be exercised when handling.

Peroxidase Assay - *ABTS*	
Dilute peroxidase	1 mL
Sodium phosphate (0.2 M, pH 6)	2 mL
ABTS (0.01 gm/1 mL)	0.1 mL
Dilute H_2O_2 (100 µL 30% H_2O_2/100 mL)	1 mL
Incubate 30 minutes at 20°C	
Read at 420 nm	

The differential signal sensitivities using several chromogens with various organisms is illustrated in table 2. ABTS and TMBZ assays were performed at pH 6 while phenol-aminoantipyrine assays were performed at pH 7.4. The relative ease of oxidation of the individual chromogens and the differences in assay pH presumably account for some of the observed variations.

Table 2. Signal Sensitivity for Various Chromogens

Chromogen	*JK Research Isolate #*		
	54	135	197
ABTS	0.199*	0.180	0.212
TMBZ	0.152	0.310	0.131
Aminoantipyrine	0.118	0.525	0.107

* Values represent a change in optical density or absorbance occuring from the oxidation of the respective chromogens.

Another substrate used during early qualitative screening was N,N-dimethyl-p- phenylenediamine.

Peroxidase Assay - *N,N-dimethyl-p-phenylenediamine*	
Dilute Peroxidase	0.5 mL
Buffer (pH 6)	3 mL
DMP (0.25 gm/100 mL water)	0.2 mL
H_2O_2 (30% H_2O_2 - 0.1 mL / 100 mL water)	0.2 mL
Incubate 30 minutes at 20°C	
Read at 485 nm	

This assay often resulted in a hazy solution, necessitating centrifugation prior to reading and was therefore abandoned.

Pinacyanol chloride was also evaluated but observed not to be useful despite the fact that others found this to give dramatically increased color changes with peroxidases from certain fungi (Glenn).

Vilter assayed peroxidase in the presence of vanadium and noted a dramatic increase in color change (Vilter). Although the addition of vanadium to the assay solution can produce inceased color, we observed more color in the reagent blank as compared with the same assay medium in the presence of peroxidase. Other elements including both manganese and iron salts also produced more color in the reagent blank than was observed in the presence of peroxidase. When EDTA was added to the assay, the color of both the experimental and the control tubes was decreased although in this case the control was less than when enzyme was present.

pH Optimum . The optimum assay pH depends on both the reagents selected and the source of the peroxidase. Optimal pH for most peroxidases in the literature has been reported in the range of 5 to 6.5, although optima at pH extremes have been documented. Peroxidase produced by isolate #130 had an optimum of 2.5 using the ABTS assay. Yumoto et al reported on an alkalophilic *Bacillus* YN-2000 with an optimal pH at 9 (Yumoto).

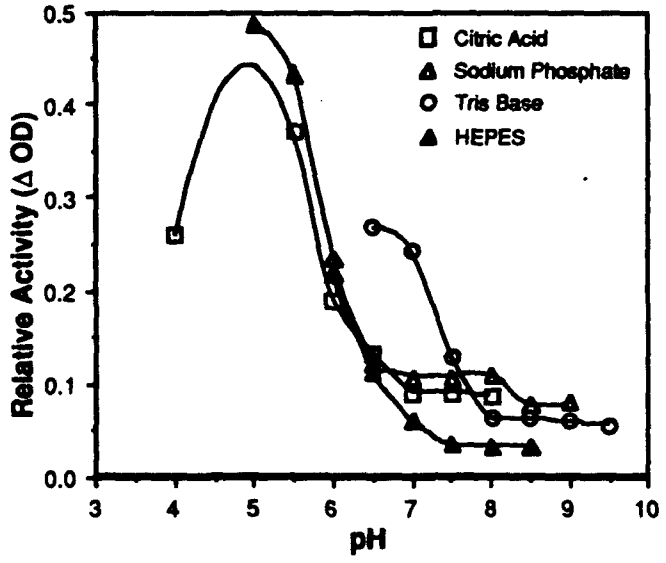


Figure 1: pH profiles for isolate #197. Color development in ABTS assay. Aliquots (0.5 mL) of samples added to four 0.2 M buffers adjusted to the appropriate pHs. Reagent blank was high at pH 5, so pH 6 was routinely used in assays.

Optimal pH for isolate #197 was determined using the ABTS procedure (Figure 1). Four 0.2 M buffers were prepared to span a pH range from 4 to 9.5. The buffers were used in the range in which they have some buffering capacity, although it should be noted that at either end of the pH curves, the buffering capacity is not optimal. The buffers chosen were citric acid (adjusted with sodium hydroxide), sodium phosphate, Trizma (Tris [hydroxymethyl]aminomethane) and HEPES (N-[2-hydroxyethyl]piperazine-N'-[2 ethanesulfonic acid]). Under these conditions, #197 peroxidase showed optimum activity at pH 5 in sodium phosphate buffer (figure 1). However, the color obtained in the reagent blank at this pH was unacceptably high so pH 6 was routinely used in assays. Tris base and HEPES do not buffer well at the lower pHs. It should be noted that there was little difference observed in #197 peroxidase activity among the four buffers in the pH range where all could be effectively utilized.

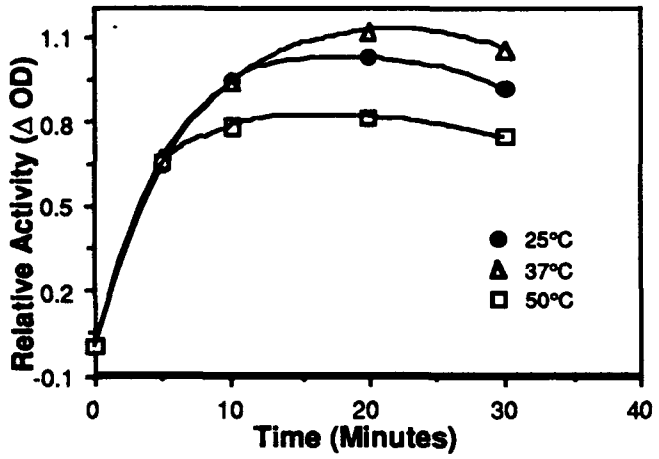


Figure 2: Optimal assay temperature for isolate #197 in sodium phosphate buffer containing the chromogen, ABTS. The decrease in color development at 50°C may be a resultant instability of hydrogen peroxide at this temperature.

Temperature Optimum . The objective of this project, to locate organisms producing heat stable enzymes, although an influencing factor, does not dictate the optimum functioning temperature of the enzyme. Enzymes functioning at room temperature as well as elevated temperatures possibly encountered in field conditions are highly desirable and achievable outcomes of such a project. During this investigation, assays were routinely performed at room temperature, however, the same organisms were known to produce peroxidases capable of withstanding and functioning at elevated temperatures. The optimal assay condition for #197 was observed to be 20 min at 37°C, although an assay time ranging from 5 to 30 mins at 25-50°C resulted in satisfactory color development (figure 2).

Substantial increases in activity with increasing temperature are common among thermophilically derived enzymes. The Japanese found *B. stearothermophilus* had optimal activity at 70°C (Loprasert). Allgood and Perry had observed a four-fold increase in *T. aquaticus* peroxidase activity when the assay temperature was raised from 25 to 65°C. However, for two other thermophiles, *T. roseum* and *T. album*, the same investigators were able to detect activity only at 25°C, not at 45 or 65°C (Allgood C).

II. Organism Culturing and Isolation

Original Habitat

Habitat temperature and pH are critical data for laboratory culturing, isolation and process engineering scale-up. The original habitat temperature and pH for a few organism isolates were as follows:

Organism	*Temperature (°C)*	*pH*
54	59	5.5
72	59	9.1
135	47	6.4
197	51	6.5

Water samples collected from natural sites were analyzed for calcium, iron, phosphorus, sulfur, vanadium, cobalt, magnesium, potassium, zinc, copper, manganese, sodium, chloride and various forms of nitrogen. In addition to pH and temperature observations, these data provided valuable initial information for media optimization. This data collection was only done for two organisms, isolates #130 and 135, but proved to be of substantial value. However, there are two problems with this type of information: (1) The microorganisms are not necessarily growing under optimal conditions; in fact, often

they have simply found a niche uninhabited by competitors; and (2) The optimal conditions for growth may not coincide with the optimal conditions for production of the desired enzyme.

For isolate #135, the sulfate, magnesium, potassium and sodium concentrations observed in the original habitat closely corresponded to the experimentally determined optimal growth concentrations. Several trace metals not detected in the original habitat were evaluated but not observed to enhance peroxidase production by this organism. Where differences did occur, in most cases, the optimized medium contained orders of magnitude more of the element than had been observed in the original location. This was true for phosphorus, manganese, iron, calcium and nitrogen. Chloride, the one exception, was detected at 0.9 gm/L in the original habitat and was added only incidentally as a contaminant in the 1 gm/L of peptone in the optimized media.

Organism Isolation

Starting Media. Organisms producing heat stable peroxidases were originally isolated on either dilute YM or an initial JK Research derived (autotrophic) media (JK1), adjusted to the approximate pH of the original habitat using sulfuric acid or sodium hydroxide (table 3).

Table 3. Original Isolation Medium

Dilute YM Media

Component	*Concentration (g/ L)*
yeast extract	1.0
malt extract	0.5
peptone	1.0
dextrose	1.0

JK1 Media

Component	*Concentration (g/ L)*
Na_2HPO_4	1.16
KH_2PO_4	0.54
KNO_3	0.15
$MgSO_2 \cdot 7H_2O$	0.10
$CaCl_2 \cdot 6H_2O$	0.10
trace elements	*
dextrose	1.00
peptone	0.20
yeast extract	0.20

* An aliquot of 2 mL of a liter of micronutrients containing 0.29 gm $FeCl_3 \cdot H_2O$, 2.28 gm $MnSO_4 \cdot H_2O$, 0.4 gm $ZnSO_4 \cdot 7H_2O$, 1.5 gm H_3BO_3, 0.05 gm $CuSO_4 \cdot 5H_2O$, 0.2 gm Na_2MoO_4 and 0.05 gm $CoCl_2 \cdot 6H_2O$ was added.

When preparing the media described in this report, the best results are obtained by separately autoclaving the defined salts, sugars and complex components such as yeast extract or peptone. Extremes of pH were avoided for dextrose, yeast extract and peptone. Dextrose will easily caramelize when heated at low or high pHs and yeast extract and peptone may hydrolyze. If pH adjustment of the final medium was necessary, a pH adjustment of the defined salts or an addition of sterile acid or base after autoclaving was preferable. By adding 0.40 gm calcium chloride and 10 gm Gel-e™ to any of the formulas, a gelled medium can be obtained for use in storage slants (tubes) or plates.

Optimized Media. Media formulations were optimized for the four most closely investigated peroxidase producing organisms (isolate #'s 54, 72, 135 and 197) (table 4). The first three had originally been isolated on dilute YM medium while #197 appeared to be autotrophic, growing best on medium JK1. The process for arriving at these formulas is discussed in the following sections.

Table 4. Optimized Media Formulations for 4 Peroxidase Producing Isolates

Isolate #54	
Component	*g/L*
Peptone	1.0
Yeast Extract	1.0
$MnSO_4 \cdot H_2O$	0.01
$MgSO_4 \cdot 7H_2O$	0.10
KNO_3	5.0
KH_2PO_4	0.7
$K_2HPO_4 \cdot 3H_2O$	0.3
$CaCO_3$	2.0
$CaCl_2 \cdot 2H_2O$	0.5

Isolate #72(B)	
Component	*g/L*
Peptone	1.0
Yeast Extract	0.5
Dextrose	1.0
$MnSO_4 \cdot H_2O$	0.25
$MgSO_4 \cdot 7H_2O$	0.0001
$ZnSO_4 \cdot 7H_2O$	0.0001
$VdSO_4 \cdot H_2O$	0.00001
Fe citrate	0.0001
$K_2HPO_4 \cdot 3H_2O$	1.5
NH_4SO_4	10

Isolate #135(D)	
Component	*g/L*
Peptone	1.0
Dextrose	0.25
$MgSO_4 \cdot 7H_2O$	0.01
$MnSO_4 \cdot H_2O$	0.05
Fe citrate	0.0001
$CaCO_3$	1.0
$K_2HPO_4 \cdot 3H_2O$	0.1
KH_2PO_4	0.1
NH_4SO_4	0.2
$NaNO_3$	0.1

Isolate #197	
Component	*g/L*
Dextrose	0.5
$MgSO_4 \cdot 7H_2O$	0.25
$MnSO_4 \cdot H_2O$	0.01
NH_4Cl	0.10
KNO_3	4.0
NH_4SO_4	1.0
KH_2PO_4	1.1
$K_2HPO_4 \cdot 3H_2O$	0.5
$CaCO_3$	0.5

Media Optimization Process

Starting with the medium formulation on which the organism had been originally isolated, each element was added or substituted, usually at 4 to 5 different concentrations including a zero concentration. Some elements were evaluated in multiple forms, ie. calcium was added as sulfate, chloride and carbonate. Several nitrogen sources were evaluated including yeast extract, peptone, ammonium salts, and nitrates. Carbon source was evaluated as dextrose, sucrose and molasses. Nitrogen and/or carbon limitation have been documented to stimulate lignin peroxidase production (Tonon). Similarily, isolate #197 produced less peroxidase in the presence of 1 g/L dextrose as compared with 0.5 g/L dextrose. Dextrose in relatively low concentrations was also superior for isolates #72 and 135 while no sugar was added to media for isolate #54. Apparently isolate #54 obtains sufficient carbon from the yeast extract and peptone. However, the literature does support increased protease production on carbon or nitrogen limitation (Allison, Dosoretz). Therefore, attempts to increase dextrose concentration was performed in an effort to reduce protease production albeit without success.

Carbon. Isolate #197 was submitted to Microbe Inotech Laboratories, Inc. (MIL) in St Louis, MO for characterization of carbon utilization. The assays, performed in commercially prepared 96 well microplates containing various organic compounds, can be particularly beneficial in biodegradation and selective media development. Unfortunately, the assays were not initiated until near the end of the project. Based on the results of this relatively inexpensive analysis, we would suggest acquiring this information in the earliest stages of a similar project.

In the analysis, the organisms are streaked onto nutrient medium supporting vigorous growth and incubated at 50°C. Colonies are lifted from the culture plates using a saline moistened cotton swab and a suspension of uniform turbidity is prepared in 0.85% saline. The microplate wells containing the carbon source to be tested and the reductive indicator, tetrazolium, are inoculated with the bacterial suspension at a rate of 150 µL per well. The plate is covered with the microplate lid and incubated at the standard assay intervals of 50°C for 4 and 24 hours. As a result of little response, the plates were further incubated at 23°C for a total of 72 hours prior to a third reading being taken. The degree of tetrazolium reduction producing various shades of purple was monitored at 590 nm. The color of each well was referenced against the negative control so that any purple color recorded above the control level was considered as a positive utilization of the given carbon source. The data is reported as the percent color change as compared to the control (Table 5). In the standard 4 and 24 hour assays, as illustrated, isolate #197 utilized only α-D-glucose. On further incubation to 72 hours, the organism used an additional 23 carbon sources. However, all values were quite low in comparison with other organism isolates.

Calcium. Calcium carbonate exhibited an unexpected requirement in peroxidase production by organism isolates #135, 72, 197 and 54. Isolate #197 was isolated from a location actually saturated with calcium carbonate, although increasing it10-fold proved slightly inhibitory. However, for organism isolates 135 and 54, calcium carbonate enhanced the production of peroxidase, a phenomenon not previously documented. The insolubility of calcium carbonate complicated the purification process by clogging the Minitan membranes. Therefore, attempts to either eliminate calcium carbonate or to substitute a more soluble form of calcium was undertaken. The elimination of calcium carbonate in the medium could only be accomplished for isolate #72 and soluble salts of calcium could not be substituted. Interestingly, manipulation of aeration parameters had a significant effect on the calcium carbonate requirement. When producing inoculum, initial organisms were lifted from a gelled plate and presumably carried calcium impurities from the Gelrite. However, when calcium carbonate is used, it should be autoclaved separately from other components to avoid the formation of calcium carbonate aggregates.

Trace Elements. Once the macronutrient concentrations were established, other trace elements were evaluated. The most important mechanism for biochemical dioxygen activation involves oxygen complexation and/or reduction by transition metal (Aust). For this reason, special attention was given to

Table 5. Carbon Utilization Pattern Recognition Data (MIL, Inc.)

Peroxidase producing isolate #197

Carbon Source	4 hrs.	24 hrs.	72 hrs.	Carbon Source	4 hrs.	24 hrs.	72 hrs.
α-cyclodextrin	N	N	30	itaconic acid	N	N	N
dextrin	N	N	51	α-keto butyric acid	N	N	20
glycogen	N	N	50	α-keto glutaric acid	N	N	N
tween 40	N	N	70	α-keto valeric acid	N	N	N
tween 80	N	N	N	D,L-lactic acid	N	N	N
N-acetyl-D-galactosamine	N	N	N	malonic acid	N	N	N
N-acetyl-D-glucosamine	N	N	22	propionic acid	N	N	N
adonitol	N	N	N	quinic acid	N	N	N
L-arabinose	N	N	N	D-saccharic acid	N	N	N
D-arabitol	N	N	N	sebacic acid	N	N	N
cellobiose	N	N	27	succinic acid	N	N	N
i-erythritol	N	N	N	bromo succinic acid	N	N	20
D-fructose	N	N	32	succinamic acid	N	N	N
L-fucose	N	N	N	glucuronamide	N	N	N
D-galactose	N	N	N	alaninamide	N	N	N
gentiobiose	N	N	23	D-alanine	N	N	N
α-D-glucose	N	54	65	L-alanine	N	N	N
m-inositol	N	N	N	L-alanyl-glycine	N	N	N
α-lactose	N	N	48	L-asparagine	N	N	N
lactulose	N	N	26	L-aspartic acid	N	N	N
maltose	N	N	54	L-glutamic acid	N	N	N
D-mannitol	N	N	N	glycyl-L-aspartic acid	N	N	N
D-mannose	N	N	42	glycyl-L-glutamic acid	N	N	N
D-melibiose	N	N	N	L-histidine	N	N	N
β-methyl-glucoside	N	N	26	hydroxy L-proline	N	N	N
psicose	N	N	26	L-leucine	N	N	N
D-raffinose	N	N	N	L-ornithine	N	N	N
L-rhamnose	N	N	N	L-phenylalanine	N	N	N
D-sorbitol	N	N	22	L-proline	N	N	N
sucrose	N	N	53	L-pyroglutamic	N	N	N
D-trehalose	N	N	53	D-serine	N	N	N
turanose	N	N	33	L-serine	N	N	N
xylitol	N	N	N	L-threonine	N	N	N
methyl pyruvate	N	N	32	D,L-carnitine	N	N	N
mono-methyl-succinate	N	N	N	γ-aminobutyric acid	N	N	N
acetic acid	N	N	N	urocanic acid	N	N	N
cis-aconitic acid	N	N	N	inosine	N	N	N
citric acid	N	N	N	uridine	N	N	N
formic acid	N	N	N	thymidine	N	N	N
D-galactonic acid lactone	N	N	N	phenylethylamine	N	N	N
D-galacturonic acid	N	N	9	putrescine	N	N	N
D-gluconic acid	N	N	N	2-amino ethanol	N	N	N
D-glucosaminic acid	N	N	N	2,3-butanediol	N	N	27
D-glucuronic acid	N	N	N	glycerol	N	N	N
α-hydroxy-butyric acid	N	N	N	D,L-α-glycerol phosphate	N	N	N
β-hydroxy-butyric acid	N	N	N	glucose-1-phosphate	N	N	N
γ-hydroxy-butyric acid	N	N	N	glucose-6-phosphate	N	N	N
P-hydroxy-phenylacetic acid	N	N	N				

* Values are percentage color change on microtiter wells (MIL) over the control and represent a relative proportional quantity of utilization (N = Negative Utilization).

closely examining the effects of iron and copper on peroxidase production. Copper was not observed to have any detectable effect on the four organisms investigated. Iron did have an effect, although the concentration required varied significantly for various cultures even of the same organism Apparently this was in response to other parameters including the type and size of hardware used. Although optimizations involving small volumes were performed in all glass containers, scale up engineering was conducted in 5 and 14 L fermentors with stainless steel cooling coils and agitators. The requirements for certain trace elements that were exhibited in glass containers, were often observed unnecessary in the larger containers that included metal parts. Although heme peroxidases contain iron, the addition of 0.001 gm ferrous sulfate/L to the medium of isolate #197 was distinctly inhibitory in the metal containing fermentors. On the other hand, ferrous sulfate can spontaneously catabolyze hydrogen peroxide and so might actually act as a peroxidase competitor if present in sufficient quantities. Although yeast extract and peptone would serve as sources of iron and other trace elements, two optimized formulas did include an iron salt.

A dramatic enhancement of peroxidase activity from *Ascophyllum nodosum* has been observed on the addition of vanadium salts to the assay solution (Vilter). In our experience, vanadium appeared to result in color development in the ABTS assay whether or not peroxidase was also present. However, vanadyl sulfate was added to the media to determine the effect on peroxidase production. Under these conditions, some color was produced in the ABTS assay and as expected, less color development was exhibited in controls containing no organisms than in active cultures except at very high levels of vanadium. The conclusion was reached that vanadium did have some positive effect either on the production or on the activity of the peroxidase. The problem of significant color development in the controls needs further investigation before the addition of this rather expensive element to the medium can be justified.

Other trace elements including cobalt, zinc, molybdenum, boron, silicon dioxide, selenium, aluminum, nickel and strontium have been evaluated. Cobalt and copper do stimulate lipid peroxidation and so might be expected to increase peroxidase production. Copper, zinc and perhaps manganese are required by superoxide dismutase, resulting in the production of hydrogen peroxide. On evaluation of these trace elements, there appeared little effect on peroxidase production. Some peroxidases produced by *Phanerochaete chrysosporium* are manganese dependent (Leisola). All four optimized media do include manganese, generally at concentrations higher than would be typical for microbial media. Glutathione peroxidase contains selenium, but the addition of selenium to the media had no effect on these organisms' ability to produce peroxidase.

Complex components such as yeast extract, not only provide organic carbon and nitrogen, but also trace elements and vitamins. Because many vitamins are not particularly stable at elevated temperatures, it is fairly unusual to find a thermophile requiring vitamins. *T. thermophilus* has been reported to require biotin for peroxidase production. However, both biotin and thiamin were evaluated on #197 and observed to have no effect.

Oxygen. Comparing effects of oxygen on mesophilic and thermophilic bacteria, mesophiles are often observed to respond to an increase in oxygen tension by synthesizing increased amounts of one or more of the enzymes of the oxygen defense system, ie. superoxide dismutase, catalase and peroxidase. Since oxygen is less soluble in water at elevated temperatures, thermophiles have evolved under lower oxygen concentrations. Allgood and Perry investigated how thermophiles tolerated increased oxygen tensions. Variations were observed among the organisms studied and media components also had some effect. From their small survey, they concluded that thermophiles exhibited a limited response to increased aeration, perhaps because they do not normally encounter high concentrations of oxygen in their environment. In fact, some organisms actually produced more peroxidase in static cultures, although differences were not dramatic (Allgood C). Using some of the same thermophiles, MacMichael observed a general increase in superoxide dismutase and catalase but not for peroxidase when aeration levels were increased (MacMichael). Although concentrations of superoxide dismutase and catalase in thermophiles in these studies were comparable to those concentrations observed in *Escherichia coli*, peroxidase concentrations are generally much lower in the thermophiles (Allgood C).

High oxygen partial pressures have been documented to stimulate lignin (Tonon) and certain mesophile (Hansson) peroxidase production but had little effect on our thermophile peroxidase production. In fact, as observed with isolate #197, excess aeration was distinctly inhibitory for peroxidase production. This organism, however, is aerobic and some oxygen must be present. As will be illustrated later in the fermentation section, peroxidase production only increased following biomass production and on subsequent reduction of oxygen concentrations. Obviously other parameters were involved, but limitation of available oxygen was a major factor.

Inducers. Perry and co-workers have experimented numerously on the effect of certain inducers on peroxidase production. The addition of methyl viologen (Paraquat) at micromolar levels generated a stress that resulted in an increased cellular concentration of toxic by-products caused by the univalent reduction of oxygen. This in turn led to an induction of the oxygen defense enzymes, most notably catalase and peroxidase. Similarily, *T. album* increased peroxidase levels 9-fold in the presence of 2.5 micromoles of methyl viologen (Allgood A). However, superoxide dismutase, at least in thermophiles, was generally unaffected by methyl viologen (Allgood B). When evaluated on our thermophiles, methyl viologen had no significant effect on peroxidase production.

The addition of hydrogen peroxide is also known to increase peroxidase concentrations under certain conditions. Hyslop investigated this phenomenon intensely and determined that low concentrations of hydrogen peroxide dramatically increased peroxidase concentrations as a result of an increase in protein synthesis as if preparing the cell for an onslaught of even more hydrogen peroxide. However, high concentrations of hydrogen peroxide did, as expected, simply kill the cells (Hyslop). Several experiments were performed on the addition of hydrogen peroxide directly to the medium or placing it in a separate container in the center of the fermentor. On occasion, an increase in peroxidase production was observed, but the results were inconsistent. This may have been due to the difficulty of maintaining a given concentration of hydrogen peroxide at elevated temperatures.

Peroxidase production is also known to be enhanced by certain stress conditions. This was particularly dramatic for isolate #197. In the early stages of fermentation, organisms appeared as medium sized rods, some with a curve such is typically observed with the genus *Vibrio*. However, following the diminishing of dextrose below detectable levels and the subsequent minimization of oxygen concentrations, there was a dramatic change in the appearance of the cells. The formation of chains, coils, spirals and even complete loops was initiated and the cells also became noticeably smaller. Under these stress conditions, peroxidase production flourished. Initiating a fermentation with higher concentrations of dextrose or oxygen, always resulted in less production of peroxidase. Conditions of extreme stress were also observed. Initiating a fermentation with no added air, produced so few cells that a later addition of small amounts of oxygen or sugar would result in peroxidase production. This approach seemed to be more difficult to control, however, and was abandoned in favor of an initial quantity of 0.5 gm dextrose per liter and 0.1 mL air/mL media/min. Adjusting glucose or air during the fermentation, generally yielded no significant improvement.

Antioxidant mechanisms also have a role in radiation injury and radioprotection (Chow). Various radionuclides are quite common in thermal waters. Isolate #197 originated from an area known to possess high levels of radon. Radium has also been observed in the same thermal basin.

III. Fermentation

Initial aeration experiments were performed on isolates #72 and 135. Peroxidase production from cultures raised on magnetic stirrers was compared against those raised in mini-fermentors using 0.25, 1.25 or 2.5 mL air/mL medium/min . For isolate #72, only the stirred culture and the one with 0.25 mL air produced detectable levels of peroxidase. Progressively decreasing concentrations of peroxidase was evident from isolate #135 as the aeration rate was increased through 1.25 mL air, with none produced at 2.5 mL air.

Monitorization of Fermentation Parameters

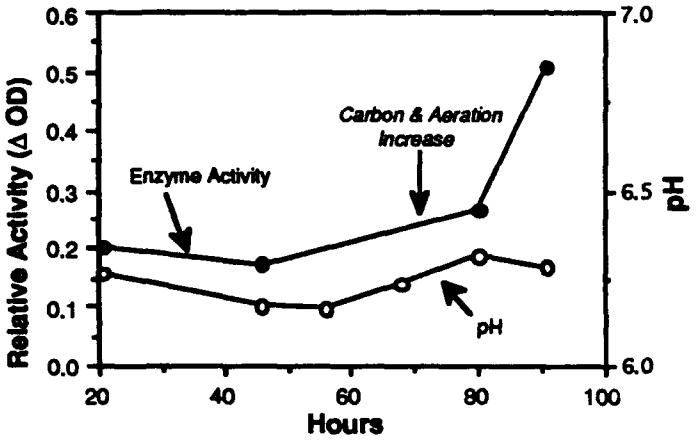


Figure 3: Enzyme production during fermentation. The pH varied only slightly during fermentation even when parameters were manipulated. The addition of dextrose and air have minimal effect on pH although peroxidase levels increased significantly.

A control of pH did not appear to be a problem. In either mini-fermentors or the 5 and 14 liter fermentors, the pH was seldom observed out of the range 5.8-6.8. However, the pH generally decreased slightly during the early stages of fermentation followed by a slight increase near the end . As long as the pH remained weakly acidic to neutral, no correlation between pH and peroxidase production was readily apparent.

On numerous occasions, fermentations were allowed to proceed beyond 48 hours. Inevitably, there was a decline in peroxidase concentration, regardless of attempts to manipulate various parameters. The addition of dextrose or nitrogen, an aeration increase or decrease or a manipulation of pH could not arrest the steady decline of peroxidase concentration. The one exception was fermentations with no air added until late in the fermentation (figure 3). At this point, the addition of air and/or dextrose did increase the very low concentrations of peroxidase.

As with any fermentation, various parameters must be closely monitored. Although operator intervention was generally not necessary, monitoring the parameters was required for proper determination of the optimal harvesting time and signaling the end of fermentation. Variations in fermentations performed in the same vessel can be sufficient to shift the peak of peroxidase production by many hours. Even fermentations set up side by side using the same inoculum will not produce comparable concentrations of peroxidase at exactly the same time.

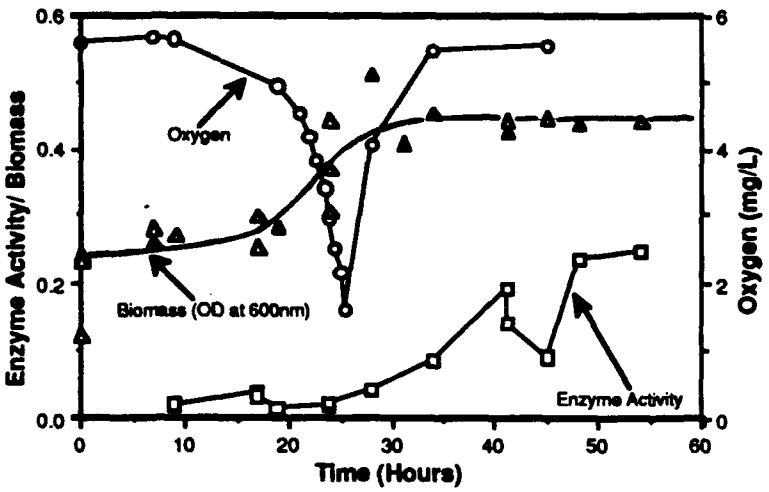


Figure 4: Monitorization of enzyme activity, biomass, and oxygen during fermentation of isolate #197. Following a decrease in dextrose below 0.1 g/L and a minimal concentration of oxygen , peroxidase levels increased. The ABTS assay was used for monitoring peroxidase activity.

Four monitorizations crucial to successful fermentations are:

1. **Oxygen** Concentration
2. **Dextrose** Concentration
3. **Enzyme** Concentration
4. **Microscopic Appearance**

Further optimization of fermentation includied increasing the inoculum, maintaining oxygen input, slight alterations in the media (decreased potassium nitrate by 20%, decreased manganese by 20%) and decreasing agitation rate by 25% (figure 5). The fermentation illustrated in figure 5 yielded the best peroxidase concentration in the shortest period of time. Notice that peroxidase was increased an order of magnitude over the results in figure 4. Optimization was an ongoing process, with small improvements obtained with many fermentations.

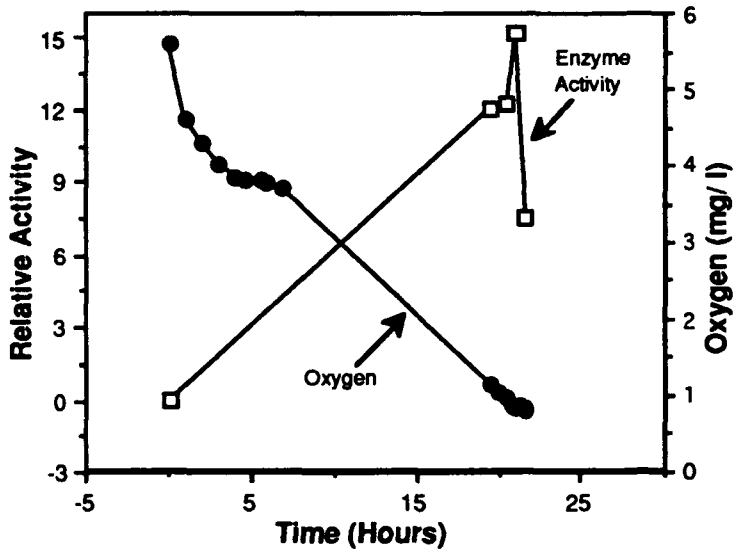


Figure 5: Monitorization of enzyme activity and oxygen during the fermentation of isolate #197. One mL of fermentation broth was withdrawn at intervals, assayed using the ABTS assay and absorbance monitored at 420 nm. Optimized medium, 0.1 mL air/ mL medium/ min and a 350 rpm agitation rate were used. Fermentors were inoculated with a 24 hr culture at a rate of 50 mL/L medium.

Oxygen. The decrease in oxygen concentration from that at saturation is quite dramatic as temperature increases (ie. air saturated distilled water at 37°C is 7.04 mg oxygen/L while at 60°C that value diminishes to 4.48 mg oxygen/L) (Allgood B). Since oxygen has a key role in the regulation of peroxidase production, a temperature change of only a degree or two can shift the timing of the enzyme production peak. At 50°C and saturation, the medium contains approximately 5.5 mg oxygen / L. The addition of 0.1 mL air/ mL media/min and an agitation rate of 350 rpm allowed for a steady concentration of oxygen to be maintained during the initial stages of fermentation. As biomass approaches a point where oxygen is being consumed more rapidly than it is being added, a very sharp decline in oxygen concentration is observed. Upon striking a minimum of 1-2 mg oxygen/ L, peroxidase production begins to increase rapidly. As fermentation progresses, the peroxidase concentration eventually decreases. Late in fermentation, a sulfurous odor is often detected.

Dextrose. Dextrose concentrations were monitored semi-quantitatively using a dipstick approach. Dextrose concentrations appear to be equally as important as oxygen for regulating peroxidase production. Peroxidase production has been well established to be initiated by stress conditions. Various stresses have been investigated in different organisms. Hyslop experimented with hydrogen peroxide (Hyslop), Perry's lab focused on the toxicant methyl viologen (Allgood A), while others investigated the effects of excess oxygen or radiation (Chow). For isolate #197, we experimented with a combination of minimal dextrose and oxygen concentrations and consistently observed induced peroxidase production. Adding more dextrose initially, delayed the time of minimal dextrose concentration and subsequently resulted in reduced peroxidase production, even though the fermentation was prolonged. Peroxidase production began to increase only once the dextrose concentration diminished below the detection limit of 0.1 gm/L.

Several experiments were performed on various agitation rates and air additions. Reduced peroxidase production was observed when agitation was increased to 450 rpm and no air added. However, peroxidase production began to increase on the addition of either 1 mL air/ mL medium/ min or 0.5 gm dextrose/ L of medium. However, this approach was more difficult to control and the results were inconsistent.

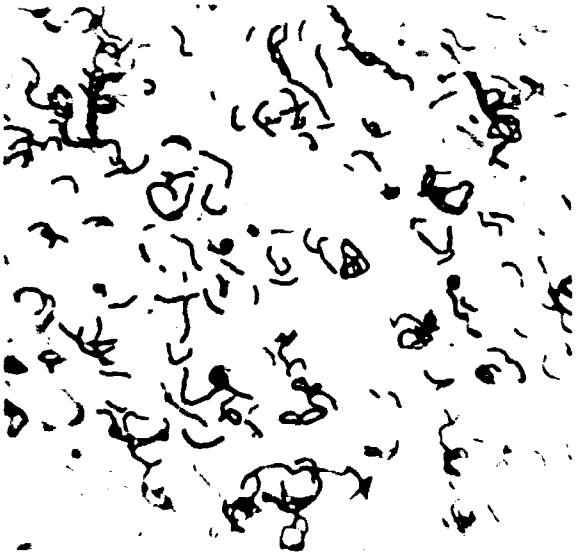
The best combination of stress inducers for isolate #197 was restricted oxygen and dextrose. Research focusing on the timing of additions and minimizations and interrelationships between these two parameters is necessary for further optimization of peroxidase production.

Enzyme Concentration. Enzyme concentrations must be monitored at frequent intervals during the second half of fermentation. The fraction of the peroxidase excreted from the cells and recovered in the supernatant varied as will be discussed later. Therefore, sonication of the cells to release peroxidase followed by assaying for total peroxidase concentrations (ie. the peroxidase in the supernatant plus the peroxidase released from cells) is necessary. Peroxidase concentrations can decrease rapidly necessitating frequent assaying for peroxidase once the dextrose is no longer detectable and the oxygen concentrations are minimal.

Microscopic Appearance. Seldom do microbiologists place much emphasis on the microscopic appearance of the organisms being investigated. However, microscopic examination of isolate #197 provided a wealth of information. In enriched media with an ample supply of oxygen, this organism appeared as a medium sized rod, sometimes slightly curved (photo 1). As fermentation proceeded, and the organisms encountered a more stressful environment, they began to form chains, spirals and tight coils (photo 2). Late in fermentation at a time of maximum peroxidase production, individual cells often form a complete circle. The cell size decreases and crystal violet stain uptake becomes poor, if at all (Photo 3).

Subjection of the organisms to proper stress at the proper time is critical in achieving high peroxidase output. In photos 1-3, the changes are dramatic. Observing cells microscopically during fermentation may provide more information than any other single test. Under excessive stress, the cells become very small and the most extreme tight coils are observed. If signs of extreme stress are observed early in fermentation, microscopic appearance serves as an early warning that the organisms are being subjected to such stress. Late in fermentation, however, the appearance of large, slightly curved rods indicate peroxidase production will be low due to insufficient stress being placed on the organisms. If an oxygen probe should malfunction, semi-quantitative dextrose measurements cannot detect the minute concentrations of dextrose required to maintain the fermentation and the appearance of a thermophilic contaminant, although unlikely, is possible. Simple microscopic observations can reassure the microbiologist that the fermentation is proceeding normally, or provide an early warning that something is wrong.

Photograph1: Organisms raised in stirred flasks exhibiting slightly curved rods and an occasional spore. (Hiph Powered Field (HPF) (1000x magnification), Oil Immersion).



Photograph 2: Approximately 10-12 hours into fermentation, the rods become more curved as the effects of stress are first observed. (Hiph Powered Field (HPF) (1000x magnification), Oil Immersion).

Other parameters. An estimate of biomass can be obtained by observing the absorbance or optical density of the whole media at 600 nm. To insure that the insolubility of calcium carbonate does not interfere, readings must be taken immediately after mixing so particles will be uniformly dispersed. As a result of the small size of these organisms, the change in shape and the tendency for some organisms (ie. Isolate # 197) to form clumps, absorbance is not a good indication of cell numbers and an accurate estimate cannot be achieved for any given OD. However, as is illustrated in figure 4, absorbance does

Photograph 3: At the time of maximum peroxidase production, the organisms exhibit effects of extreme stress. Organism #197 forms tight coils that are observed in clumps. The cells are small and stain poorly. (Hiph Powered Field (HPF) (1000x magnification), Oil Immersion).

increase at a fairly steady rate through most of the fermentation. For isolate #197, a culture suspension with an OD at 600nm of 0.35 was serially diluted and spread over pour plates. The plate count indicated the original culture contained only 2.2 x 10^3 organisms/ mL. However, direct microscopic examination suggested a much higher titer. Apparently single colonies are being formed from clumps of microorganisms.

The pH is another parameter that should be monitored during fermentation. Normally, only minor changes will be observed in pH, however, a deviation in pH may indicate contamination or an error in media preparation. There appears to be little relationship between peroxidase production and pH for #197. The pH of the optimized medium for this organism is approximately 6.3-6.4. The final pH of the best fermentation was 5.9.

IV. Organism Lysis and Enzyme Secretion

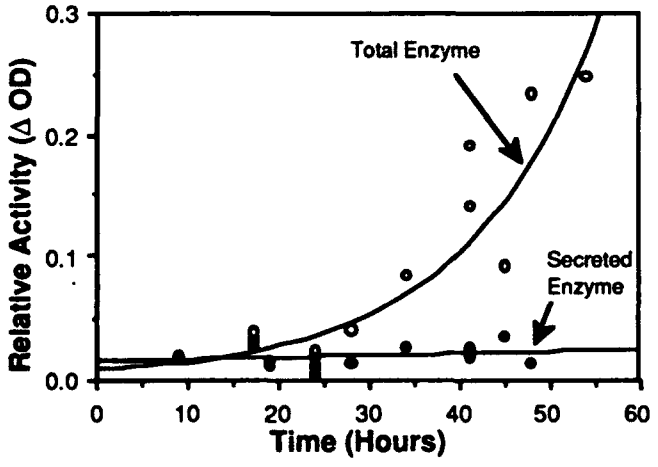


Figure 6: Enzyme production by isolate #197 during fermentation using 0.1 mL air/ mL medium/ min and an agitation rate of 450 rpm in a 5 liter fermentor. Minimal levels of enzyme were passively excreted from the cells.

Passive Cellular Secretion

Comparing the peroxidase concentration in the supernatant with the total cellular peroxidase, *ie. the amount released from the cells upon* sonication plus the amount found in the supernatant, (figure 6), the concentrations in the supernatant remain relatively low throughout fermentation when the aeration rate is tightly controlled at 0.1 mL air/ mL medium/ min.

During fermention with no added air and a constant agitation of 450 rpm, a different profile of the fraction of supernatant to total peroxidase was observed (figure 7). Because concentrations were so low initially, the small amount of peroxidase in the supernatant was above that found in the cells, perhaps simply reflecting peroxidase that was available in the inoculum. However, concentrations in the

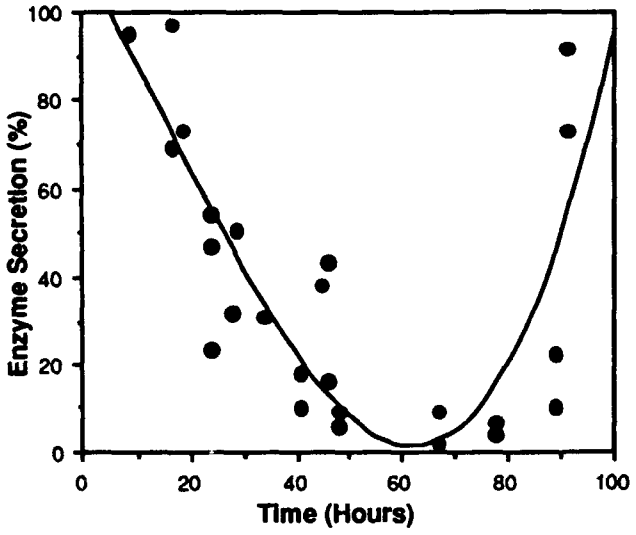


Figure 7: Secretion of enzyme from isolate #197 cells during fermentation. No air was added to this fermentation but agitation was maintained at 450 rpm.

supernatant increased sharply near the end of this very long fermentation.

In mini-fermentors with air added at a rate of 1 mL/ mL medium/ min and no agitation, the fraction of supernatant peroxidase averaged 77-91% by the end of fermentation. Unfortunately the total peroxidase levels were generally lower than what was obtained in the larger, agitated fermentors. These total peroxidase concentrations, obtained during the manipulation of media components, varied considerably. In conclusion, the fraction of supernatant peroxidase versus the cell bound fraction varied with culture conditions. Therefore, assaying peroxidase activity in the supernatant as well as total peroxidase activity is essential. On a small scale, the fraction containing less peroxidase may be discarded. However, on a production scale, limits must be set on levels below which it is uneconomical to attempt to recover additional enzyme. A low level of peroxidase in10,000 gallons of biomass may add up to a substantial amount of finished product. Economic questions remain to be addressed for these processes.

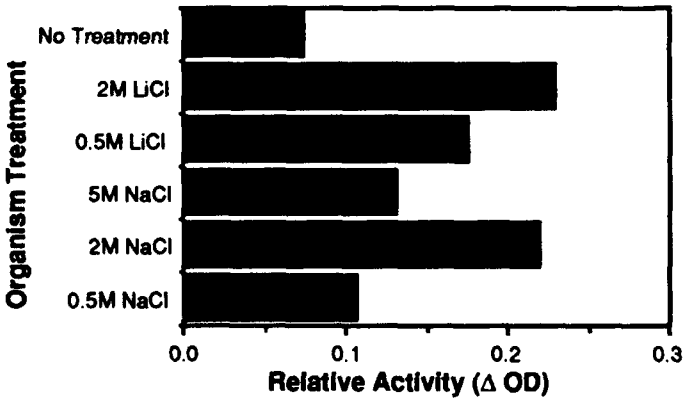


Figure 8: Passive secretion of enzyme from Isolate #135 cells using ionic strength variation. Cells were allowed to stand overnight in various salt solutions and the supernatant was assayed for peroxidase.

Ionic Strength Variation

Earlier in this investigation, peroxidase was observed to spontaneously be released from the cells by allowing them to stand overnight at room temperature in various salt solutions. The largest fraction of the enzyme being released from isolate #135 cells was observed to occur in 2 M solutions of either sodium or lithium chloride (figure 8). Lysozyme, also evaluated as a cell disrupter, was inefficient at releasing enzyme. As a result of later sonication procedures proving much better at releasing enzyme than chemical methods such methods were abandoned.

V. Enzyme Processing/ Purification

Organism Concentration

Traditionally, enzyme purification protocols incorporate a centrifugation step followed by some method of cell disruption, ammonium sulfate precipitation of the enzyme and finally removal of the salt by dialysis or column chromatography. With recent advances in membrane technology many of the problems associated with membranes plugging with cellular debris have been solved. As a result, some form of filtration system is replacing the centrifuge, ammonium sulfate and dialysis procedures.

The filtration system used in this investigation was a Minitan Ultrafiltration System from Millipore Corp. The system allowed both tangential flow microporous and ultrafiltration processing more rapidly

than traditional methods. This versatile system allowed processing of varying volumes of material dependent on system configuration. Eight hydrophilic Durapore (PVDF) membrane plates with a total area of 480 sq cm (0.65 μ pore size) were stacked in a series configuration between acrylic manifold plates. Silicone retentate separators provided a gasket seal between plates and created a sweeping flow across the upstream filter surfaces (Millipore). Torque pressure was adjusted to 80 in lbs. Although the PVDF was supposed to be low protein binding, a loss of a significant portion of the enzyme was observed. Coating the membranes with a solution of 5% nonfat dry milk which covered available binding sites helped prevent enzyme loss. The milk was rinsed away using 0.2 M sodium phosphate buffer, pH 6, prior to processing the fermentation broth. Because the rather insoluble calcium carbonate tended to clog the pores, allowing the fermentation media to settle and pumping from near the top of the vessel into the Minitan was preferable. Transmembrane pressure was maintained below 1-2 psi. Clarified media was collected from the filtrate line, while the cells became more and more concentrated in the retentate container. The process was considered complete when the cellular material was concentrated to 250 ml.

Sonication

The cellular concentrate was placed in a 400 mL beaker in an ice bath over a magnetic stirrer, and a stir bar added. The one inch horn of a 600 W ultrasonicator was placed at the edge of the vortex created by the stirrer, approximately one inch into the solution. The sonicator was turned to full power for 30 minutes to achieve cell disruption. As a consequence of the cells releasing proteases during disruption, the sonicated material must be kept cold to avoid proteolytic digestion of the peroxidases. PMSF at 1 mM was observed to inhibit much of the proteolytic activity, however, this chemical is carcinogenic, and its use could be avoided by adequate icing of the sample. Furthermore, sonication experts have determined that superior cell breakage is achieved at lower temperatures.

Cellular debris was separated from the supernatant containing the peroxidase by processing the sonicated solution through the Minitan in exactly the same configuration as previously described. Approximately 220 mL of the clarified enzyme solution was collected on ice from the filtrate line. As the volume of cellular debris approached 50 mL (hold up volume of the instrument is 30 mL, leaving approximately 20 mL in the retentate container) the cells were washed several times with 50 ml portions of buffer. This resulted in a final filtrate volume of approximately 500 mL. In the calculation of enzyme yield (Table 5), washing of the cellular material was not employed. For processing small volumes, washing the cellular debris did not recover significantly more enzyme. However, for larger volumes, significant quantities of enzyme were recovered. Also in the calculation of enzyme yield, the filtrate from the first Minitan filtration was not routinely saved. Again, with small volumes, the amount of enzyme in this fraction was not usually significant, although with production size volumes this fraction must be assayed for peroxidase prior to being discarded. Excessive variation of yield was encountered in each fermentation to assume there was insufficient enzyme in any fraction. Our recommendation is that assays for peroxidase be conducted on each fraction throughout the entire process.

The fully assembled Minitan plates were washed with 8 mL bleach/ L according to the manufacturers instructions and allowed to soak at least overnight in a 0.1% solution of sodium dodecyl sulfate (SDS) at 50°C.

Enzyme Concentration

At this point, there were one or more containers of clarified solutions containing peroxidase. The Minitan was assembled with 10,000 molecular weight cut off filters of regenerated cellulose (designated PLGC). The system was operated at 10 psi, concentrating the clarified material to 30 ml. As a precaution, both the collection vessel and the clarified sample were maintained over ice to avoid degradation of the peroxidases by proteases. Proteases produced by #197 do have fairly low activity at room temperature; even at 37°C, the protease assay was held several hours before substantial activity on Azocoll was observed. However, once the peroxidase was produced, allowing proteases to destroy even a small fraction was not sensible.

Again, the waste filtrate was evaluated to determine the quantity of peroxidase passing through the filter. In actual practice and regardless of the membrane nominal molecular weight cutoff of 10,000 and the substantially higher molecular weight for this peroxidase, roughly one quarter to one third of the peroxidase was observed in the waste filtrate. This material, filtered through the system a second time, usually resulted in much reduced levels of peroxidase in the filtrate. The two fractions were pooled, yielding a 30 mL concentrate for subsequent column chromatography.

Chromatography

Anion Exchange. Most column purification procedures reported in the literature initially use DEAE (diethylaminoethyl) on various polymeric supports. At the time this task was initiated, we obtained samples of an experimental anion exchanger from BioRad Corp. This material, later named Macro-Prep 50 Q was a strongly basic anion exchanger with quaternary ammonium groups interacting with negatively charged biomolecules, much the same as DEAE exchangers.

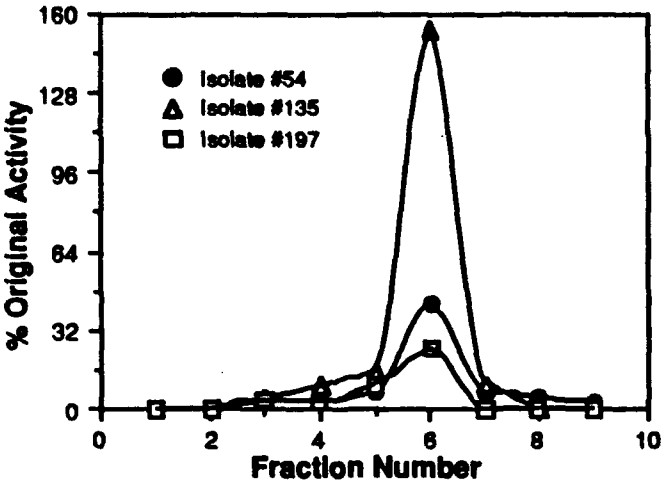


Figure 9: Anion exchange chromatography of peroxidase from various organism isolates. Elutions were achieved using successive additions of 0.2M sodium phosphate buffer (pH 7) containing 0.1-0.6M NaCl at 0.1M concentration intervals.

Macro-prep 50 Q and DEAE cellulose were evaluated on peroxidase preparations from isolates #54, 72 and 197 (figure 9) at both 4 and 20°C. No significant difference was observed between the two temperatures for either packing material. However, the Macro-Prep 50 Q did allow greater recovery of peroxidase and, unlike the DEAE cellulose, did not present any flow problems. The peroxidase sample was applied to the column in a 0.2 M sodium phosphate buffer (pH 6). Columns were eluted with sodium chloride in an analogous buffer adjusted to pH 7 at 0.1M intervals in the range of 0.1 to 0.6 M. All three isolates were initially eluted in the 0.4 M NaCl fraction regardless that they were three distinctly different microorganisms as will be discussed later. The increase in activity of the eluate for isolate #135 over that of the crude concentrate is encouraging and suggests that some sort of inhibitor had been removed by the chromatography. This increase in activity might not be associated with usual specific activity increases with increased purity as will be discussed later.

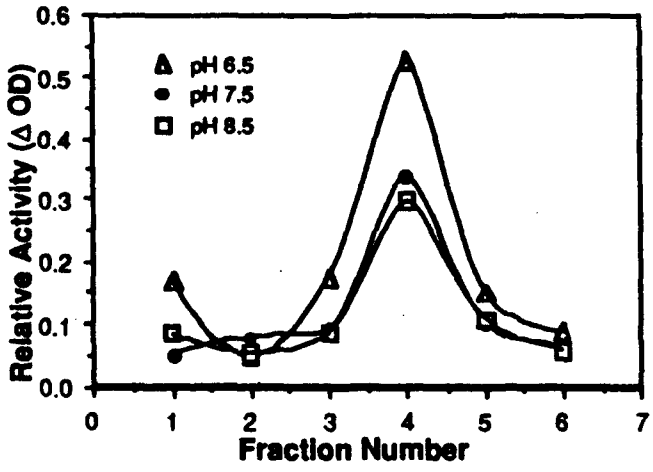


Figure 10: Effect of pH on enzyme chromatography of isolate #197. Elution of peroxidase from Macro-Prep 50 Q using 50mM sodium phosphate buffer at pH 6.5, 7.5 and 8.5.

On changing the pH of the elution buffer there was no difference in the elution pattern at the three pH's evaluated (figure 10). However, there was a slight decrease in the amount of peroxidase recovered as the pH was increased. As development of chromatographic purifications progressed, peroxidase began to elute in the 0.2 to 0.3 M NaCl fractions. In light of later work, insufficient volumes of eluants applied to the earlier columns apparently resulted in differences in the ionic strength elution patterns. Still later experiments exhibited more peroxidase being eluted predominantly in the 0.2 M NaCl

fraction.

Prior to proceeding to other chromatographic procedures, the removal of accumulated salt from the anion exchange column was necessary. Columns are available especially designed for this purpose. However, the Minitan was a more rapid approach. The instrument was assembled with the concentration membranes (10,000 MW cutoff) and the sample to be desalted was concentrated to 30 mL as previously described. The sample was then diluted one to one with phosphate buffer (0.2M at pH 7) and the concentration step similarily repeated. A sufficient number of dilutions (ie. 5 dilutions for 0.2 M salt) and concentrations were repeated to reduce the salt concentration below 0.01 M. The small volumes allowed this step to be completed in a few minutes.

Protease Removal. A common reason for instability of enzyme preparations is the presence of proteases which degrade enzymes of interest. Proteases are a secondary line of defense against oxygen radicals as they remove damaged material. Produced late in fermentation, they may partially explain the decrease in peroxidase levels observed after 48 hours.

All peroxidase preparations were assayed and observed to contain proteases. The separation and removal of proteases from the peroxidase containing fraction is an important criterion used in determining the effectiveness of purification techniques. Azocasein was used initially for protease detection but the results were not definitive. An assay using Azocoll was developed and used to monitor results of later column chromatographic procedures:

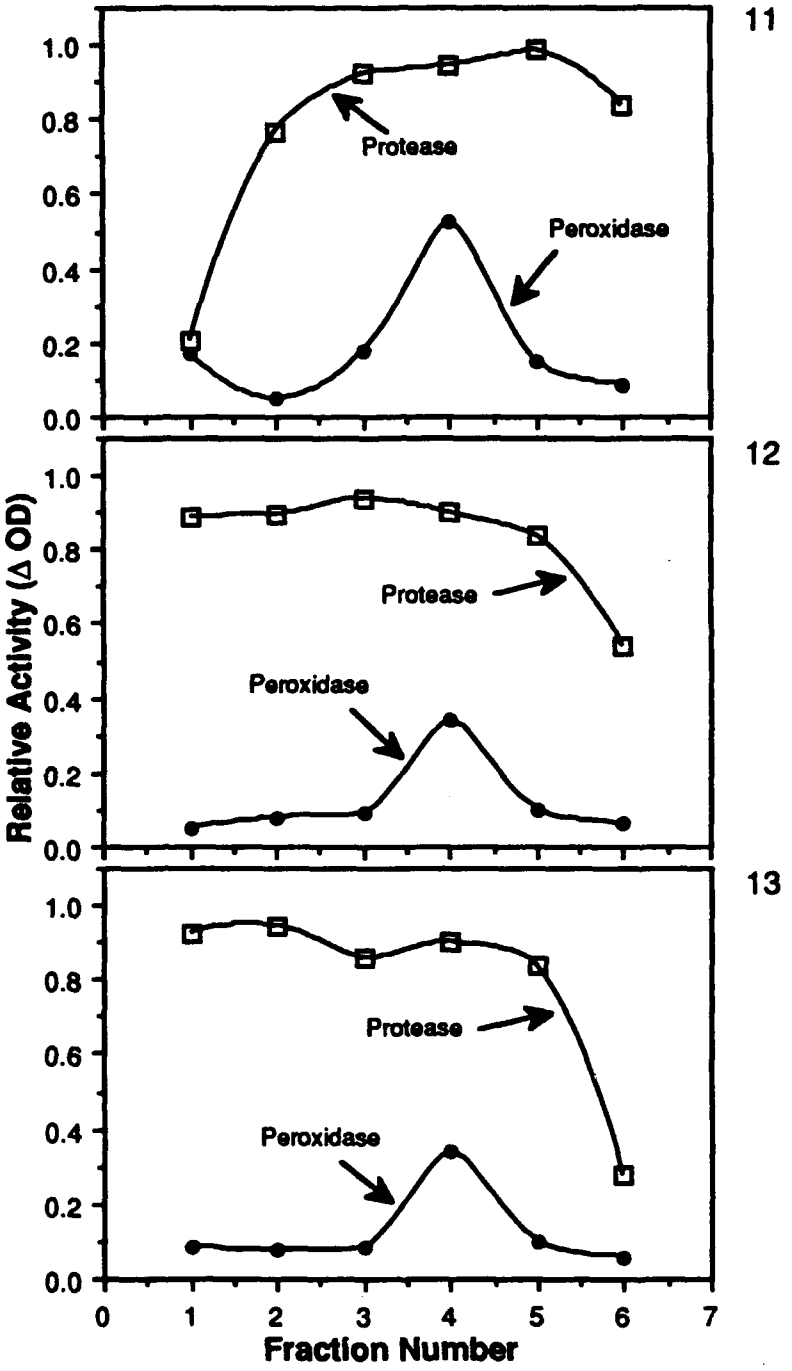
Protease Assay

Assay buffer: 0.05 M Tris base containing 1mM $CaCl_2$ adjusted to pH 7.8 with 1M HCl
Azocoll: 0.25 gm Azocoll into 50 mL rapidly stirring protease assay buffer

---

Assay: 0.5 mL eluate or concentrate
Blank: 0.5 mL 0.2 M Sodium phosphate buffer (pH 6) in duplicate
Add 1 mL Azocoll
Incubate 37°C for 12 hours
Centrifuge
Add 2 mL of water to the supernatant
Read at 520 nm against blank

The separation of protease from peroxidase during the chromatographic process was of the next highest priority. If this could be achieved in a single step, the purification process would be significantly simplified and accelerated. As illustrated in figures 11-13, protease was eluted in all fractions, including the peroxidase fractions and regardless of the pH.



Figures 11, 12 and 13: Separation of peroxidase from protease on strong anion column. Elution of both enzymes from Macro-Prep 50 Q matrices was accomplished using 50 mM sodium phosphate buffers at pH 6.5 (11), 7.5 (12) and 8.5 (13).

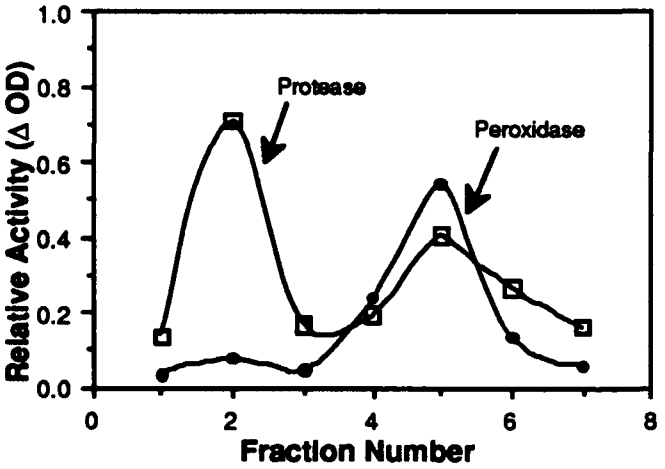


Figure 14: Step elution of peroxidase from Macro-Prep 50 Q using 50mM sodium phosphate buffer, pH 8.5. The fraction number represents arbitrarily chosen pooled volumes of the following elution sequence: (1) Buffer eluted sample, (2) Buffer, (3) Buffer, (4) 0.1 M NaCl in buffer, (5) 0.2 M NaCl in buffer, (6) 0.3 M NaCl in buffer, (7) 0.4 M NaCl in buffer.

Since buffer was eluting the protease, an attempt to flush the protease before elution of the peroxidase was begun. The effect of this second wash is illustrated in figure 14. Although this technique proved beneficial, a substantial amount of protease was still being eluted in the peroxidase fraction.

In light of these encouraging results, the procedure was altered to include four buffer washes and three washes with 0.08 M NaCl in buffer. This low level of salt was insufficient to elute the peroxidase but was added in an attempt to remove additional contaminants. The removal of even a larger portion of the proteases by this approach is illustrated in figure 15. The anion exchanger was regenerated between successive chromatography procedures with 1 N HCl and more thoroughly cleaned in 20% ethanol following every 4 to 5 passages.

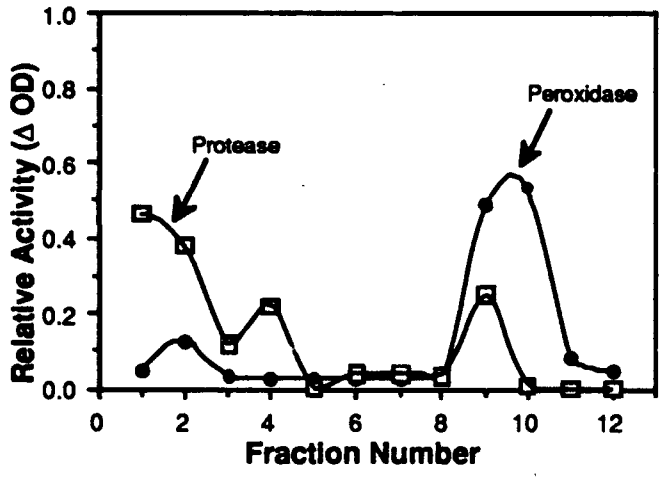


Figure 15: Separation of peroxidase from protease on Macro-Prep 50 Q using 50 mM sodium phosphate buffer. Results are the average of three runs. Fraction number represents elution of: (1) Sample diluted 1:2 with buffer, (2-5) Buffer, (6-8) 0.1 M NaCl in buffer, (9-12) 0.2 M NaCl in buffer.

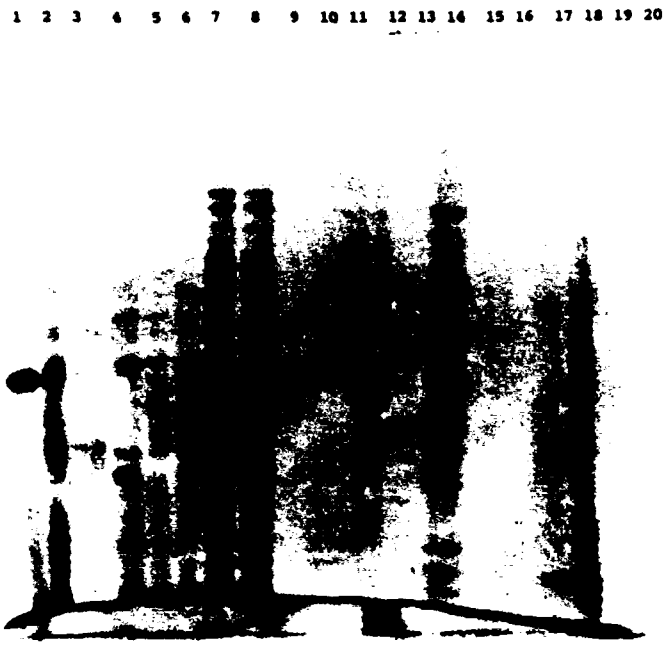
At this point, the decision was adopted to use extensive column washing to remove as much protease as possible followed by the use of a second column to remove the remainder of the protease. Numerous columns were evaluated for this purpose. A strong cation exchanger not only was an inefficient binder of peroxidase, but allowed proteases to simultaneously elute with the peroxidase. Several dye ligand columns were also investigated. Peroxidase #197 did not adhere to Matrex gel red A or Matrex gel green A from Amicon. A CM Affi-Gel Blue gel column used to separate out proteases from serum samples did not bind either the peroxidase or protease of #197. An affinity Affi-Gel Heparin binding column has been demonstrated for several protein classes including proteases. A theory was developed that if protease but not peroxidase bound to the column, a successful protocol for removal of protease would be obtained. However, the Affi-gel heparin affinity gel from Bio-Rad did not bind either protease or peroxidase of isolate #197.

Phenyl Boronate. Phenyl boronate has been used successfully in the purification of both horseradish peroxidase and serine proteases. Phenyl boronate, unlike most ligands used in affinity chromatography, interacts predominantly by forming a temporary covalent bond with a particular chemical moiety - the 1,2-cis-diol. The boronate ligand binds any molecule with the proper chemical composition, regardless of size. One important exception is the specific, non-covalent interactions with serine proteases (Amicon). A boronate column was regenerated with 6 M guanidine HCl. The column was washed extensively with 50 mM HEPES (pH 7) containing 10 mM magnesium chloride and the sample, diluted 1:3 in the same HEPES buffer, was applied to the column. The column was then washed with the analogous buffer without magnesium chloride. Peroxidase did not adhere to the column and was eluted in this

wash. The manufacturers's instructions were followed to remove proteases which included an additional wash with 10 mM EDTA. A further improvement was later developed by adding 0.5 M glycerol (Akparov), which results in tighter binding of the proteases to the boronate column, and thus better separation from the peroxidase.

Electrophoresis

Two SDS-PAGE gels were electrophoresed under non-reducing conditions, heated at 37°C in buffer. One was stained with Coomassie brilliant blue R250 to allow observation of all proteins in each sample (Photo 4). Dye column concentrates and samples eluted from anion exchange columns exhibited



Photograph 4: SDS PAGE non-reduced gel stained for protein detection. The lane identification is identical to photograph 5 with the exception that lane 20 is missing.

numerous protein bands. As expected, the concentrates from both the green 5 and boronate columns exhibited substantially fewer bands as a result of extended purification. Lanes 11 and 12 exhibited substantial streaking and were later repeated (Photo 5). A similar number of reduced bands is observed , but the preparations are by no means purified. No protein bands were observed in lane 15, suggesting the molecular weight of isolate #130 to be less than 10,000.

The second gel was processed to allow specific detection of peroxidase activity by the method of Graham (Graham)with the same samples in the same lanes (Photo 5). Five mg 3,3'-diaminobenzidine tetrahydrochloride was dissolved in 10 mL 0.1 M Tris buffer, pH 7.6. To this solution was added 0.1 mL of a 3% aqueous solution of hydrogen peroxide and the final solution added to the gel. The gel was incubated for 5-10 min at room temperature and the reaction stopped by rinsing thoroughly with distilled water. Peroxidase activity was detected in the second concentrate (lane 2), very weakly in eluates from the anion columns (lanes 3-6), stronger in concentrates from the anion columns (lanes 7 and 8), none from the first eluate of a green 5 column (lane 9) and approximately the same level in the first boronate column and the second green 5 column as the concentrates from the anion columns (lanes 10 and 11). Markedly more activity was observed in the eluate from the second boronate column (lane 12) although the fewest bands were exhibited on the protein gel. Lanes 13 and 14 of isolate #130 showed peroxidase activity at a lower molecular weight. The two very strong reactions are of horseradish peroxidase.

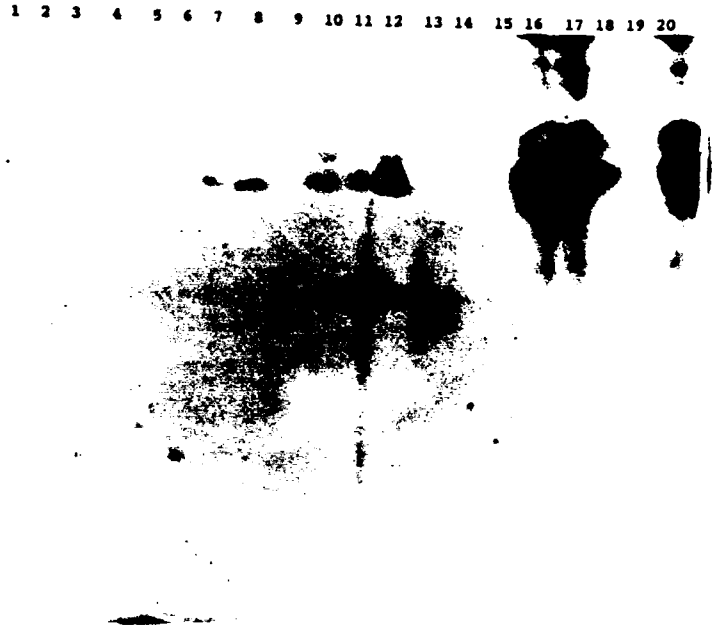


Photo 5: SDS PAGE non-reduced gel stained for peroxidase enzyme activity. Electrophoretic separation of samples from various purification stages for peroxidase #197 (Lanes 1-12), peroxidase #130 (Lanes 13-15) and Horseradish Peroxidase (Lanes 16, 17 and 20)

Lane #	*Preparation*
1 - 2	Crude Concentrate
3 - 6	Eluate from anion column under various pHs
7 - 8	Concentrate from anion columns
9, 11	Concentrate from green 5 column
10, 12	Concentrate from boronate column
13 - 14	Crude extract of #130
15	Dialyzed #130 supernatant from10,000 MW cutoff

Horseradish peroxidase has a molecular weight of 40,000 (Glenn). Most other peroxidases from plants, fungi, and bacteria have been observed in the same range. Peroxidase produced by *Phanerochaete chrysosporium* has a molecular weight of 46,000 (Glenn), *Mucor hiemalis*, 45,000 (Aisaka), *Euphorbia characias*, 48,000 (Floris), *Arthromyces ramosus*, 41,000 (Shinmen). Only human salivary peroxidases have been found to be substantially larger with MW ranging from 78,000 to 280,000 (Mansson-Rahemtulla). On the substrate gel, #197 peroxidase and horseradish peroxidase migrate to about 100-110,000 MW level. The non-reducing conditions apparently allow the enzyme to form some sort of dimer. Since the horseradish peroxidase band appears at the same location, the peroxidase of isolate #197 is suggested to be of approximately the same size as most other peroxidases. The peroxidase from #130 was of much lower molecular weight, perhaps not forming the dimer.

VI. Enzyme Characterization

Specific Activity

As the desired enzyme is progressively more highly purified, increasing amounts of peroxidase are discarded with contaminants. The process of discarding protein not contributing to the peroxidase activity increases the specific activity of the product at the expense of losing substantial quantities of specific enzyme. Although enzyme yields vary greatly in the literature, a 10% recovery is not unusual (Aisaka, Loprasert).

Table 6. Percent Recovery Of Peroxidase From Successive Processing

Whole fermentation broth	100
Clarified and concentrated on Minitan	38
Anion exchanger	14
Phenyl boronate	18

The average final recovery from the best three processings in this investigation was 18%, although significant differences were evident (table 6). Over half of the original enzyme activity in the crude extract was lost following processing through the Minitan. Because of the relatively small size of the peroxidase molecule, a substantial portion of the enzyme ends up in the waste. For small volumes in developmental investigation, this was not a problem, but would be unacceptable on a commercial scale. Millipore does manufacture a 5000 MW cutoff membrane, that was not evaluated. As the pore size is decreased, the processing time is increased. One possibility would be to do a preliminary concentration using the 10,000 MW cutoff membranes, then concentrate the waste through 5,000 MW cutoff membranes and pool the two fractions.

Over half the activity in the clarified concentrate was lost on the anion exchanger. Part of the problem was that the peroxidase was being discarded with the protease containing frations. Research targeted at the nature of the proteases contaminating these preparations is necessary.

Enzyme Stability

The main objective of this project was to locate microorganisms producing heat stable peroxidases. The peroxidase from the thermophilic bacterium #197 was far more stable than the widely used horseradish peroxidase and was slightly more stable than a thermophilic peroxidase from *Bacillus stearothermophilus* isolated by the Japanese (Loprasert).

Purified peroxidase produced by isolate #197 is compared in table 7 with the most widely used horseradish peroxidase and with the peroxidase from *B. stearothermophilus* as described by Loprasert (Loprasert). Although the enzyme is unlikely to be exposed to 70°C, stability at elevated temperatures translates to increased stabilities at lower temperatures. Thus storage would be feasible at ambient temperatures rather than the necessity of refrigeration.

Table 7. Stability of Various Peroxidases at 70°C
(% Activity Remaining)

Time	JKR 197	*Bacillus stearothermophilus (Loprasert et al.)*	Horseradish
10 minutes	104	80	3
30 minutes	86	--	2
3 hours	53	--	2
7 hours	17	--	--

In initial stability analysis, several peroxidase producing isolates were incubated at 50, 60 and 70°C and pH 6 for 18 hours (table 8). The most important finding is the high percent of activity remaining following 18 hours, regardless of significant quantities of protease present. These preparations also contained cellular material to which peroxidase could bind. This suggests that immobilization of peroxidase from these thermophiles could further improve their heat stability.

Table 8. 18 Hour Stability at pH 6

% Activity Remaining

Isolate	*50°C*	*60°C*	*70°C*
24 B	57	71	45
25 A	83	70	54
31 B	81	78	63
31 C	76	79	43
41 A	78	72	46
69 B	58	75	52
72 B	78	71	59
122 A	81	78	55
135 D	76	73	49
195	76	74	56
246 A	78	79	41
246 B	69	57	45
251 A	84	81	57

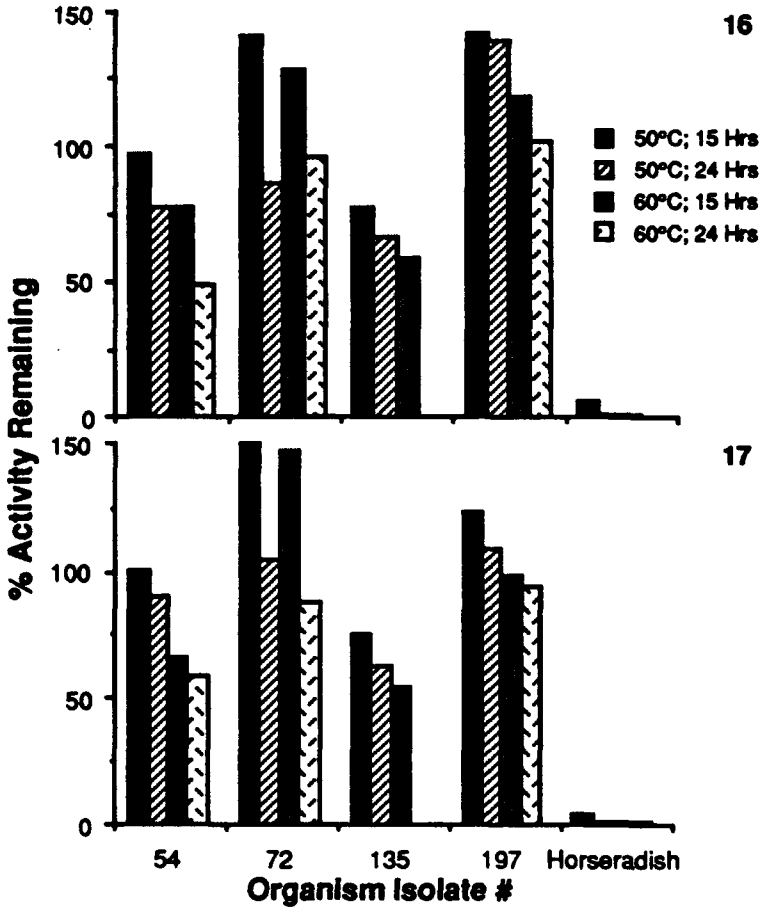


Figure 16 and 17. Crude peroxidase stability in sodium phosphate (16) and KCl (1M) (17) following the incubation at 50 and 60°C for 15 and 24 hours as compared with horseradish peroxidase incubated under analogous conditions. KCl has no apparent stabilizing advantage over phosphate buffer.

Crude extracts of isolates #54, 72, 135 and 197 were incubated in phosphate buffer at varying temperatures and the fraction of activity remaining at given intervals was compared with the activity observed at time zero and to Horseradish Peroxidase (figure 16).

Numerous chemical compounds have been employed in an attempt to increase the stability of enzymes. Some of these stabilizers were evaluated to determine their effect on our thermophilic peroxidases. One approach was to decrease the free water by increasing the salt concentration. Potassium chloride (figure 17) had a very small effect on both the microbial and horseradish peroxidases. In fact some preparations actually lost more activity in KCl than in buffer. However, an initial increase in enzyme activity was observed in the presence of KCl. Heat labile inhibitors or tertiary structure rearrangement may explain this activity increase.

Sodium phosphate, Tris and HEPES buffers were evaluated, but enzyme stability was unaffected in these buffers. Glycerol has been widely used to stabilize enzymes as has addition of calcium ions and polyethylene glycol (Anawis). Interestingly, none of these additives contributed significantly to the stability of #197 peroxidase perhaps because the stability of #197 peroxidase was already substantially greater than other peroxidases on which these stabilizers had been found useful.

As discussed earlier, the primary reason for instability of the enzymes is protease activity. Even preparations that have been fairly well purified may have sufficient protease activity to adversely affect stability of the desired enzyme. Kelley has suggested that most of the proteases in thermophiles can be classified as serine proteases (Kelley) and indeed, when the protease inhibitor, PMSF, was added to a crude preparation of #197, the sample did exhibit a greater retention of peroxidase activity at 75°C. The addition of other protease inhibitors, ie. aprotinin and antipain, had no effect. The addition of EDTA at a rate of either 0.05 or 1.0 mM did increase the peroxidase activity, indicating the potential presence of metalloproteases.

Stability studies of peroxidase at 25, 37 and 50°C and purified through anion exchange and boronate columns are illustrated in figure 18. Following 5 days of incubation, more than 50% of enzyme activity was retained at all three temperatures.

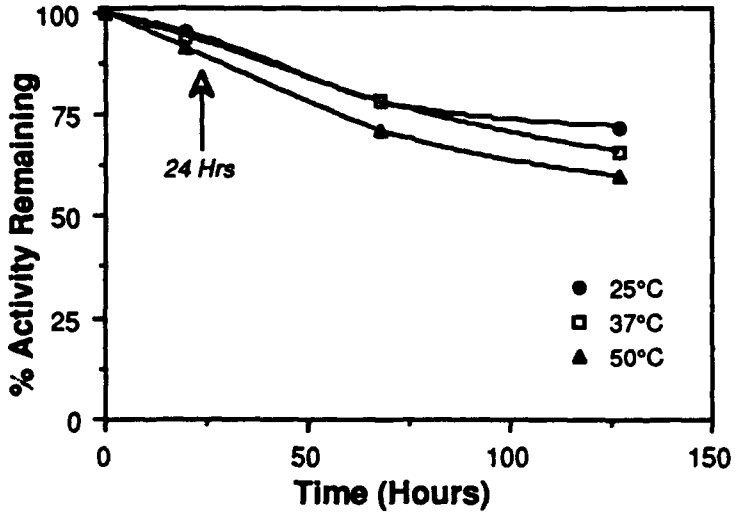


Figure 18: Stability of peroxidase #197 purified through anion exchange and phenylboronate columns. Assays were conducted in buffer at pH 6 containing the indicator, ABTS.

For comparison, stability of other peroxidases reported in the literature follows:

Table 9. Stability Comparison of Other Peroxidase Producing Organisms

Source	Half Life		Reference
	Temperature	*Time*	
Mucor hiemalis	60°C	15 min	Aisaka et al., 1983
Lignin	60°C	0.2 hours	
	50°C	13 hours	Barclay et al., 1990
Bacillus stearothermophilus			
..... .in 10% glycerol	30°C	64% retention after 34 days	
.....in buffer	70°C	80% retention after 10 min	Loprasert et al., 1989

For comparison with other thermophiles, a culture of *Thermus aquaticus* (ATCC #27634) was obtained from the American Type Culture Collection (ATCC). The concentrations of peroxidase produced were low compared with isolates #54, 72, 135 and 197. Also, the assay color completely faded after remaining at room temperature for a few minutes. In contrast, the absorbances of the assay solutions for the JK Research peroxidases increased over comparable time periods at ambient temperatures. Whether an inhibitor was present, the enzyme was unstable under assay conditions or other reasons is unknown. When incubated at higher temperatures, the *T. aquaticus* peroxidase assays did retain their absorbances suggesting the possibility of an inhibitor that is particularly active at 20°C or that is heat labile.

VII. Organism Identification

Fatty Acid Analysis

To ensure the four neutral peroxidases selected for in depth investigation weren't just strains of the same organism or even different isolates of the same organism, the isolates were submitted to Five Star Laboratories in Branford, CT for fatty acid analyses. As illustrated in table 10, the isolates were distinctly different organisms. Note particularly the differences in the 15:0 iso and anteiso fractions.

Although this information assured us that these organisms were four distinctly different organisms, fatty acid analyses are only available for a select few thermophiles. As a result, this information did not aid in identification. Over the course of this investigation, various investigators were contacted for assistance with identification. Dr. Karl Stetter, a German scientist who has published extensively on thermophiles, was unable to take on the additional work. However, several other institutes including ATCC, Dr. Don Crawford at the University of Idaho and Dr. Karl Woese at the University of Illinois did agree to examine the peroxidase producing organisms. After receiving the data, all felt that identification was going to be more involved than they had anticipated. Isolate #197 was a particular puzzle because of its distinctive appearance, which was not familiar to any of these people. By this time, #197 had been selected as the organism for the focus of the remainder of the project. Several people at Montana State University, accustomed to looking at microorganisms from Yellowstone, were questioned about the unique appearance of #197, but no one was able to recognize it.

As observed in other thermophilic bacteria, iso and ante iso fatty acid chains predominate. For moderate thermophiles of the genus *Bacillus*, 34-64% of cell lipids are of the 15-iso and 17-iso fatty acids. In the more extreme thermophiles of this genus, around 80% of the total fatty acids are iso forms of 15, 16 and 17 carbon chains (Edwards). Of the 4 organisms submitted to CRDEC, three closely resemble the extreme thermophiles. The percentage of fatty acids occurring as iso forms of 15, 16 and 17 carbons

Table 10. Fatty Acid Profile
(% Fatty Acid)

Fatty Acid *	Isolate #			
	197	*135*	*72*	*54*
SOLVENT PEAK				
9:0			1.09	
10:0			0.53	
11:0 ISO	0.80	0.53	1.23	0.89
14:0 ISO	1.12	0.45	1.63	2.20
14:0			0.43	0.59
15:0 ISO	16.29	53.22	40.07	24.62
15:0 ANTE ISO	1.12	13.41	21.93	36.43
15:0			0.88	0.84
¥ 16:1 ISO I				
14:0 3 OH	3.67			
16:0 ISO	44.19	3.55	7.76	5.33
¥ 16:1 TRANS 9				
15:0 20H	3.19			
16:0	3.02	1.55	3.26	2.39
17:1 ISO H	5.47			
17:1 ANTE ISO A	0.98			
17:0 ISO	11.28	19.67	14.09	16.47
17:0 ANTE ISO	5.92	7.63	6.73	10.24
18:0 ISO	2.14			
18:0	0.82		0.37	

* Conventional Fatty Acid Nomenclature denoting carbon chain length and arranged by retention times within a Gas Chromatograph - Microbial Lipids, Vol 1 (1988) Ratledge, C. and Wilkinson, S.J., Eds.
¥ Two combined (double) peaks were observed. Substituted OH fatty acids are eluted later than non-OH substituted.

are: peroxidase #197 = 72%; urease #408 = 49%; urease #429 = 76% and glucose oxidase #370 = 74%. The unusual pattern for #197 where the 16 iso form predominates is especially noted.

16S rRNA Sequencing

Since these bacteria were isolated from the extreme environments of thermal waters and the original collection focused on the maximum diversity of microhabitats rather than the few pools that have been extensively sampled by others, it is not surprising that none of the organisms, including #197 had been previously identified and details added to any of the data bases searched.

Isolate #197 was selected for 16S rRNA sequencing. Samples were submitted to PhyloGen Corp. in Boston, MA. The sequencing and data analysis were performed by Dr. Daniel Distel of PhyloGen and Harvard. The resulting sequence was aligned with *E. coli* and compared with other organisms. Since there was no match with any other organism in the data bank, a similarity analysis was performed, comparing #197 with the currently available sequences in the Woese/Olsen collection at the Argonne

National Laboratory. Similarity was computed as the fraction of nucleotide positions which are identical in a given pair of aligned sequences. Data was weighted by applying a mask to the sequence, instructing the computer only to compare sequence positions which have been determined unambiguously in all sequences being compared and which can be aligned with homologous nucleotide positions in all sequences being compared. This was necessary since 16S rRNAs vary considerably in length. One can only compare positions that occur in all sequences. #197 shared the highest similarity values with gram positive bacteria. The highest similarity value was 89.5% for *Lactobacillus thermophilus* and *B. subtilis* for organisms already in the data bank. As will be seen later, this is actually a fairly low similarity value, suggesting #197 may not belong in either genus. Since much of the data base consists of sequences determined by Dr. Woese, thermophiles are well represented. Although #197 could not be identified, this data does tell us that the approach used for collection did not result in re-isolating the same organisms that many others have already isolated, but rather turned up novel organisms. The acquired sequence data is a permanent, phylogenetically consistent and unambiguous descriptor of the specific strain submitted to CRDEC. As new sequences on thermophiles becomes available, the sequence for #197 will become more valuable for classification.

Unfortunately there is no consensus on the level of 16S rRNA sequence similarity that constitutes strain, species or generic differences. This is largely due to the fact that well studied bacterial groups have been frequently split while in general less well known bacterial groups have been lumped. The result is that the currently used binomial classification system is not phylogenetically consistent. Examples may be found where two organisms with a 98.9% similarity value have been classified in two different genera. Yet two other organisms with lower similarity values may be found in the same genus.

Molecular taxonomic studies of the genus *Bacillus* have shown that it is a heterogeneous group of organisms that can hardly be considered closely related. All members of this genus form spores. This characteristic is so distinctive that taxonomists have been reluctant to create separate genera (Brock). Consequently this is a large group of rather diverse organisms. Identification of an organism in this genus really provided very little useful information. *L. thermophilus* is not a "typical" *lactobacillus* and the organisms lumped within this genus are fairly diverse phylogenetically.

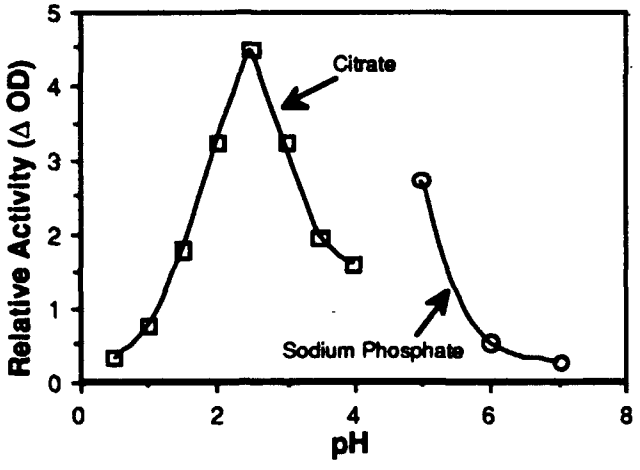


Figure 19: Optimum pH for peroxidase #130. Assays were conducted in 0.2 M citric acid and sodium phosphate buffers at varying pH's.

Specific (*Acid*) Peroxidase

Organism isolate #130 was isolated from a habitat of pH 2.4 and 47°C. Not surprisingly, the resulting peroxidase produced was most active and most stable under strongly acid conditions. The optimum pH for this peroxidase was determined to be 2.5 in citrate buffer (figure 19).

This organism produced extremely high concentrations of peroxidase, ie. roughly 3 orders of magnitude more than the neutral peroxidase producers. Therefore, assaying for the enzyme could be conducted under the same conditions of pH and temperature as used for neutral peroxidases to yield a detectable signal within a reasonable time period. At very low pH, the background color produced in the ABTS assay becomes quite substantial, making subtraction of the reagent control value essential.

Optimized Medium. Following is the formula for the optimized medium:

Optimized medium for #130

Component	*g/ L*
Yeast Extract	0.5
Peptone	2.0
Dextrose	0.5
NaCl	0.30
$MnSO_4 \cdot H_2O$	0.01
$MgSO_4 \cdot 7H_2O$	0.25
$NaHCO_3$	1.00
Ferrous Sulfate	0.001
NH_4SO_4	10
SiO_2	0.1

Although the organism would grow in the dark, the organism is a facultative phototroph and the degree of peroxidase production appeared to be influenced by light. Once green growth became visible and light was constantly supplied, maximum peroxidase production resulted. During the first days of the growth period, fermentors were subjected to a light/dark cycle. The organism required substantial amounts of oxygen, provided by bubbling in air at a rate of 1 mL/ mL medium/ min and maintaining the agitation rate at 150 to 200 rpm, dependent on which agitators were being used. For other organisms, aerated mini- fermentors could produce enzyme at approximately the same concentration as in the 5 and 14 liter New Brunswick fermentors. Nevertheless, peroxidase #130 was produced at a concentration 10-fold greater in the larger fermentors.

Processing. Although the enzyme was readily and passively secreted from the cell, it tended to adhere to everything. The cells even adhered to one another. Several salts and other chemicals were added at varying concentrations in an attempt to prevent this adherence (table 11). Enabling the cells to remain in processing buffer for several days in the refrigerator was observed to release far more enzyme than any other approach. Preparations were stable for many months under these conditions so subsequently, cells were constantly maintained in the refrigerator with buffer being replaced at intervals.

Table 11. Peroxidase #130 Harvesting

Organism Treatment	*Relative Activity (ΔOD)*
Supernatant from Cells Sitting in Citric Acid Buffer (0.2M, pH 2.5) for One Month	7.120
25 % Tween 80	0.676
10 % Tween 80	1.150
1 % Tween 80	2.340
0.1 % Tween 80	4.000
Na_2HPO_4	1.850
NaH_2PO_4	0.875
Distilled Water	1.580
Citric Acid Buffer 0.2 M	1.330
2 M $CaCl_2$	0.304
1 M $CaCl_2$	0.742
0.5 M $CaCl_2$	0.800
0.1 M $CaCl_2$	1.090
2 M $LiCl_2$	0.494
1 M $LiCl_2$	0.770
0.5 M $LiCl_2$	0.930
0.1 M $LiCl_2$	0.626
2 M NaCl	0.468
1 M NaCl	0.698
0.5 M NaCl	0.860
0.1 M NaCl	0.935
2 M KCl	0.502
1 M KCl	0.596
0.5 M KCl	0.670
0.1 M KCl	0.690
2 M $MgCl_2$	0.242
1 M $MgCl_2$	0.532
0.5 M $MgCl_2$	0.820
0.1 M $MgCl_2$	1.025

The peroxidase also adhered to the low protein binding membranes used in processing. After some experimentation, suitable filters were located, ie. Millex GV (table 12).

Table 12. Filter Retention of Peroxidase # 130

Filter Description			Activity Recovered
Manufacturer	*Type*	*Pore Size (μ)*	*(%)*
	Centrifuged Control		100
Millipore	Millex GV - Durapore in Minitan	0.22	107
S & S	Blue - 190 - 2020	0.2	91
MSI	N025P02500 70 Anotop	0.2	68
Nuclepore	Polycarbonate	0.22	28
Nalgene	Nylon195 - 2020		27
Gelman	Supor 450 Polysulfone	0.45	17
Millipore	Millex HA	0.45	15

The large cell mass also tended to clog the Minitan membranes of which were difficult to clean. Nevertheless, enzyme recovery was excellent from the Minitan (table 13).

Table 13. Activity Recovery of Peroxidase #130 thru Minitan™ at pH 6

Crude Biomass	0.135
Theoretical Total	0.135 X 15* = 2.025
Cell Concentrate	2.000
Supernatant Concentrate	0.196
Cell + Supernatant Concentrate	2.196
Percent Recovered	> 100%

*15 Fold Concentrate

As a result of the extremely low pH requirements, this enzyme was not suitable as a label in ELISA type assays. Intense interest in this preparation, however, was generated for industrial processes. For such applications, a very inexpensive, not highly purified enzyme would be preferred. In fact, using either whole fermentation medium or a cell concentrate would be most practical. Therefore, minimum investigation on purification was required. This peroxidase was observed to adhere to green and blue A dye ligand columns from Amicon in a pH 2 citric acid buffer and was eluted in 0.1 M NaCl in citric acid buffer.

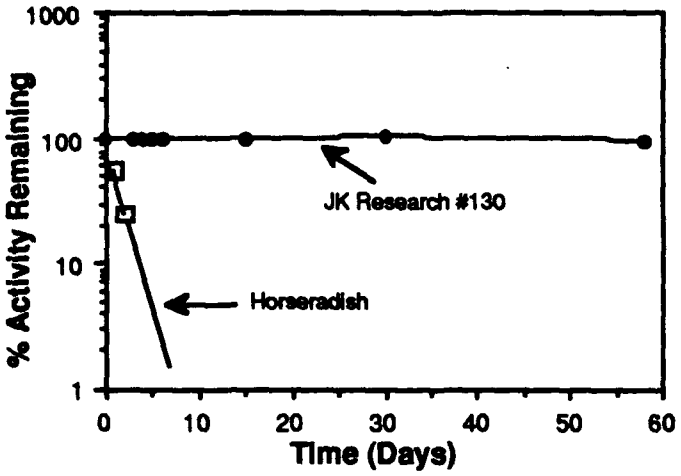


Figure 20: Stability of peroxidase #130 in citric acid buffer at pH 2 and 45°C.

Stability. The stability of peroxidase from isolate #130 was rather interesting. Poor stability at pH 7 and limited stability at 50°C or above was exhibited. However, nearly full activity was retained for 2 months at 45°C and a pH of 2-3 (figure 20). At 50°C, the half life was approximately one week while at 70°C only 8% of the activity remained following 24 hours.

VIII. Commercialization

Neutral Peroxidase

Peroxidase is used for signal generation in numerous research methods and laboratory assays, as evidenced by the long list of companies marketing peroxidase conjugated products.

Interest in this enzyme has been expressed by numerous diagnostic companies. Specifically the peroxidase from isolate #130 has been one of the first enzymes to be distributed for evaluation. Although impressed by the acid stability of the enzyme, the product possessed limited use.

A sample of the purified peroxidase produced by #197 has been submitted to international laboratories in Germany for evaluation. Discussions with domestic chemical companies indicate intense

interest in all heat stable enzymes, although the strongest interest appears to be in ureases.

Acid Peroxidase

Industrial application of peroxidases has been limited predominantly because of their relative instability, high isolation and purification costs, and the difficulty in recovering active enzyme after completion of the catalytic process (Kadima).

Considerable interest has been generated by the peroxidase produced by isolate #130. A pulp and paper company has evaluated a small sample and felt the enzyme compared favorably with other peroxidases. A requested 10,000 units of the enzyme has recently been shipped.

Another Pulp and Paper company has also indicated interest in these peroxidases. Because isolate #130 releases peroxidase into the medium and produces the enzyme at comparably high levels, this isolate is of more interest to companies dealing with large quantities of enzyme. Minimal processing would minimize the cost of the enzyme. Initial samples have been prepared and recently submitted and larger quantities are being produced for scale-up testing.

Immobilization of leachable toxic soil pollutants has been accomplished with oxidative enzymes (Shannon). Lignin peroxidases have been used to degrade xenobiotics including DDT, Lindane and certain PCB congeners. Fieldable sensors such as a cyanide detecting sensor based on peroxidase have been developed (Smit). All of these applications address a low cost, stable peroxidase. J. K. Research plans to submit samples of #130 peroxidase to companies interested in these areas of bioremediation.

Halogenation is a key step in the production of many industrial chemicals. Horseradish peroxidase is an iodoperoxidase; assays have not been performed to determine which halogens #130 might be able to utilize. Since hypohalous acid has known antibacterial properties, haloperoxidases have been used as in situ antibacterial generating agents. In fact a patent has been issued in which a haloperoxidase, halide ion and hydrogen peroxide are added to an industrial effluent as a broad spectrum biocide (Neidleman). J. K. Research will submit samples to companies with interest in these applications using peroxidase.

IX. Future Investigations

Perhaps one of the most promising areas for further research would be to optimize use of the phenyl boronate column to remove protease. In one run, two-thirds of the peroxidase applied to the boronate column was lost; in another run, measured activity actually doubled. Although these were different preparations and changes had been made in preceding steps, the same procedure was followed in both cases on the boronate column. More needs to be learned about factors influencing the performance of the phenylboronate. The manufacturer's instructions for use of the phenylboronate column to recover protease were followed, but proteases from this thermophile could be expected to vary substantially from the proteases used by the manufacturer.

The marketplace is currently being flooded with new purification products. One example is the numerous dye ligands now available. A green dye ligand produced by Lexton reportedly binds peroxidase. Green dye ligands from Amicon and Sigma would not bind peroxidase from #197, although the peroxidase of #130 would adhere to the Amicon column packing. The dye ligands offer a powerful yet inexpensive form of affinity chromatography. Further experimentation with conditions aimed at improving the adherence of #197 peroxidase to green dye ligand columns from various manufacturers has the potential for significantly improving peroxidase separation from protease.

If the molecular weights of the proteases differ enough from the peroxidase, all the peroxidase containing fractions from the anion exchanger could be saved, regardless of how much protease was present. Perhaps protease could be more efficiently removed by size exclusion chromatography.

Unfortunately, literature on other organisms suggests this may not be a viable alternative.

Other forms of affinity chromatography were not tested but also offer great potential. Epoxy-activated media has been successfully used for horseradish peroxidase (Alden). American International Chemical, Inc. now offers anion exchangers with varying porosities. Hydrophobic interaction and gel permeation chromatography have been used for purification of numerous enzymes with excellent recovery.

Although pH, salt concentrations and buffers were varied and some early work was done on temperature effects, numerous other parameters affecting the efficiency of any one chromatographic technique were not tested at all. Buffer strength, use of alternative salts, addition of certain trace elements and column dimensions remain to be tested and optimized.

Temperature stability of the #197 peroxidase offers some interesting variations on the standard purification procedures. During stability studies, peroxidase activity was noticed to slightly increase especially for the first one or two readings after incubation at an elevated temperature was initiated. This was a consistent observation and applied to both the neutral and acid peroxidases. Informal conversations with other scientists revealed that this phenomenon had also been observed with certain other enzymes produced by thermophiles. The explanation is unknown. However, a rearrangement of the tertiary structure occuring on a change in temperature has been suggested. Another possibility is that certain contaminants are being destroyed at the higher temperature. Experiments are necessary to determine if a preliminary incubation at an elevated temperature has any effect on subsequent purification. If the genetic information, ie. DNA, for the production of peroxidase #197 were inserted into a mesophile, ie. E. coli, inactivation of inhibitors might readily be achieved by temperature elevation, although that technique would not remove the excess, unreactive protein.

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Urease

I. Introduction

Development of colorimetric immunoassays in the early 1980's, using the enzyme urease as a label, (Chandler, Lo, Myerhoff) has stimulated the more recent development of novel urease based immunoassays using potentiometry with a silicon sensor (Olson) and fluorescence with fiber optic technology (Rhines). Urease catalyzes the hydrolysis of urea to ultimately form two molecules of ammonia. At near neutral pH, ammonia molecules become protonated, resulting in a net pH increase, a reaction visually followed by pH sensitive dyes. Although not optimal for all assays, the distinct color change in the colorimetric immunoassay produced by urease using bromocresol purple as an indicator (yellow to purple), gives this nonradiometric label an advantage over other enzymes currently used. The substrate, urea, is safe, inexpensive and stable in solution (Mobley). The absence of urease in most mammalian tissues reduces background noise resulting in increased sensitivity in assays involving mammalian cells. (Mobley)

Commercially available urease comes largely from Jack beans. Manufacturers suggest storage at 4°C or below for stability. Such low temperatures are not practical for home health care testing kits, diagnostic assays in physician offices and health clinics and fieldable kits for monitoring ecotoxicity and pollution. Greater enzyme stability under a wider range of conditions is required. Extensive screening of thermophiles during this project resulted in selection of two bacteria producing thermally stable urease.

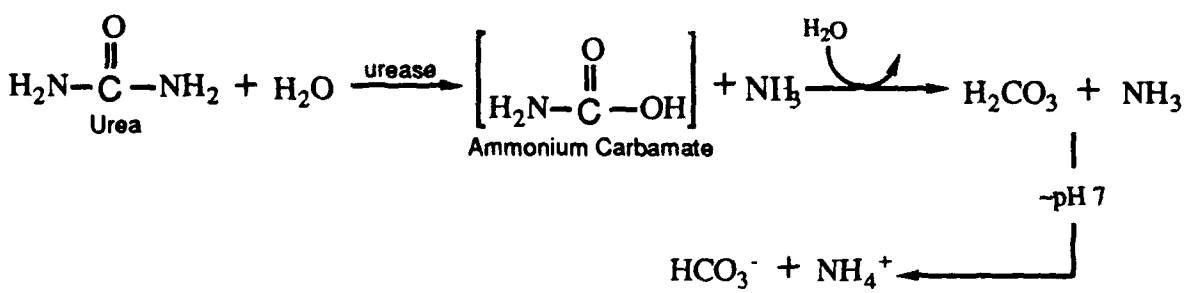
Origin

Urease is found in many plants, bacteria, fungi, yeast and algae. Among soil bacteria, up to 30% produce urease (Mobley) but little work has been published on its occurrence in thermophiles. Bhatnagar et. al. did speculate on the existence of urease in *Methanobacterium thermoautotrophicum* (Bhatnagar) but the enzyme was not identified or further characterized. The only definitive reference in the literature to a urease produced by a thermophile is in a Japanese patent on *Bacillus* sp. TB-90 (Takashio).

Many thermal waters are low in nitrogen, particularly organic forms. Unlike many moderate temperature habitats, high temperature waters seldom have any urea, and other forms of fixed nitrogen are not abundant. Interestingly, out of 10,000 thermal features in Yellowstone, only one small thermal basin has high levels of nitrogen in the water. For the thermophiles screened in this study, urease production was not common.

Enzyme Assays

Urease converts urea to ammonia and carbamate. Carbamate spontaneously breaks down to a second ammonia and carbonic acid. At near neutral pH, carbonic acid dissociates and the ammonia becomes protonated, thus resulting in a net pH increase. This reaction was the basis for both the qualitative screening and quantitative assays. This reaction is frequently applied in assays incorporating a pH indicator; the results are read by eye or quantitated spectrophotometrically. More specifically, urea



and bromocresol purple (Chandler) or phenol red (Mobley) is incorporated into the media or added to a buffered enzyme solution. A color change caused by a pH increase is indicative of urease activity.

Since our media had several starting pHs (pH approximated that of the original habitat) the process was simplified by using a micro pH electrode and recording the actual pH change at intervals. Cells grown in liquid culture could be assayed directly. All isolates studied here either transported urea across intact membranes or exported the urease. Although the disruption of cells prior to testing was observed unnecessary, the process did initially accelerate the reaction.

Protocol for Assaying Urease Enzyme Activity Throughout the Investigation

1 mL dilute urease
2 mL 50 mM citric acid buffer, pH 7
1 mL urea (25 gm urea /L)
Determine pH at time zero
Incubate 30 min at 50°C or 1 hr at 20°C
Determine difference between initial and final pH

For the initial screening assay, a 200 mM phosphate buffer was used. Following the determination of the optimum pH using four different buffers, a change was made to a 50 mM citrate buffer. This alteration increased assay sensitivity by reducing the buffering effect and enabling a change in pH to be exhibited sooner.

All the ureases included here with the exception of #429 were observed to possess increased activities as the temperature progressed to 70°C. However, since the most useful enzyme should be active over a wide temperature range, much of the investigation was conducted at ambient temperature. Since the enzyme reaction produced by isolate #429 was often too slow to detect activity in thirty minutes at ambient temperature, the assays were conducted at 50°C.

Discriminatory Analysis and Validation of Urease Presence The degradation of urea by microorganisms involves either urease or ATP-urea amidolyase (UALase). Generally UALase has been found in yeast and green algae, while urease is usually found in blue-green algae, higher plants, bacteria and fungi. As a result of the lack of information on urea degrading thermophiles, an experiment was performed to determine if the associated activities originated from urease or UALase. The strong inhibition of urease but not UALase by hydroxyurea has been well established (Mackeraas, Rai). As observed in table 1, all the isolates evaluated in this investigation were producing urease and not UALase. However, it is noted that #429 was not inhibited as much at any level of hydroxyurea as were ureases produced by other organisms. This isolate, raised at 70°C (20°C above other isolates), was also the most heat stable urease and originated from among the hottest habitats.

Table 1. Urease Inhibition By Hydroxyurea (%)

Culture	Hydroxyurea Concentration (g/ mL)		
	0.00009	*0.00076*	*0.02*
197	ND*	ND	100.0
211	ND	ND	100.0
288	39.0	80.2	96.7
339	ND	ND	100.0
404	52.3	85.0	98.1
408	39.2	78.5	96.2
413	51.1	85.6	96.7
429	30.1	57.5	82.2

Mobley observed 50.0% inhibition using *Campylobacter pylori* and 0.00009 gm hydroxyurea/mL. Mackeraas observed 73.0% inhibition using *Anabaena cylindrica* and 0.00076 gm hydroxyurea/mL.
The absence of UALase is indicated by the high percentage of inhibition.
* ND = Not Determined.

Optimum pH. The pH optimum for ureases has been well established to be strongly buffer dependent. Ureases have been observed to be inhibited by sodium, potassium and ammonium ions and phosphate especially at pHs less than 7. Nakano observed optimal pH's of 6.2 , 7.4 and 7.3 for maleate, Tris-HCl and phosphate buffers, respectively (Nakano). Additionally, Jespersen and Taylor observed optimal pHs ranging from 6.5 to 8.0 for phosphate, maleate, citrate and Tris buffers (Jespersen, Taylor). The relative amount of activity also varied significantly with the buffer even at the optimal pH. Only 42% of the urease activity in maleate buffer was observed at the optimal pH in phosphate buffer (Nakano). Similar results can be observed in figures 2 - 4 which depict pH activity profiles for 3 organisms. These profiles were performed with an excess of urea substrate to prevent interferences from alterations in Km. Rather high buffer concentrations were also used to impede major pH changes which would otherwise result in artifactual phase shifts of the profiles. For all three isolates, citrate buffer proved to be superior, yielding as much as 3-fold greater activities. In contrast, isolates #197 and 429 exhibited no activity in phosphate buffer at pH's less than 7. These curves must be interpreted with some caution. As the assay proceeds, the pH increases. Therefore, the pH at which the enzyme is actually optimal may be above the initial pH shown. Urease may also be inhibited by the presence of generated ammonia as the reaction progresses toward increased pH's. As noted earlier, the substrate concentration is very high. In the presence of lower levels of urea, and thus lower levels of generated ammonia, less urease inhibition may occur. Less concentrated buffers would also permit greater pH changes to occur more quickly thereby increasing assay sensitivity.

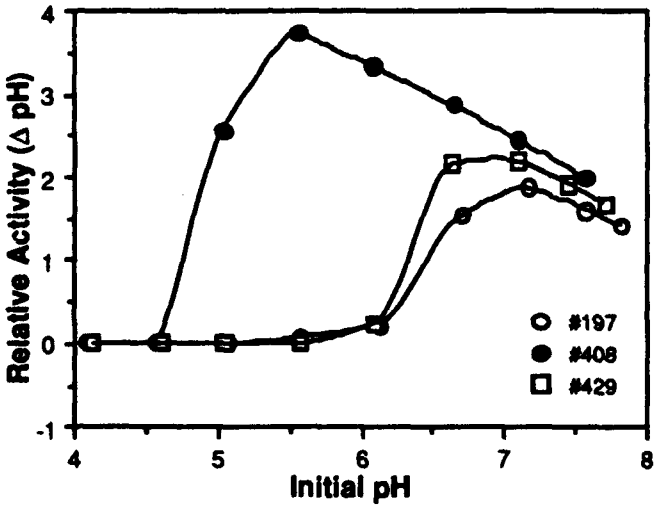
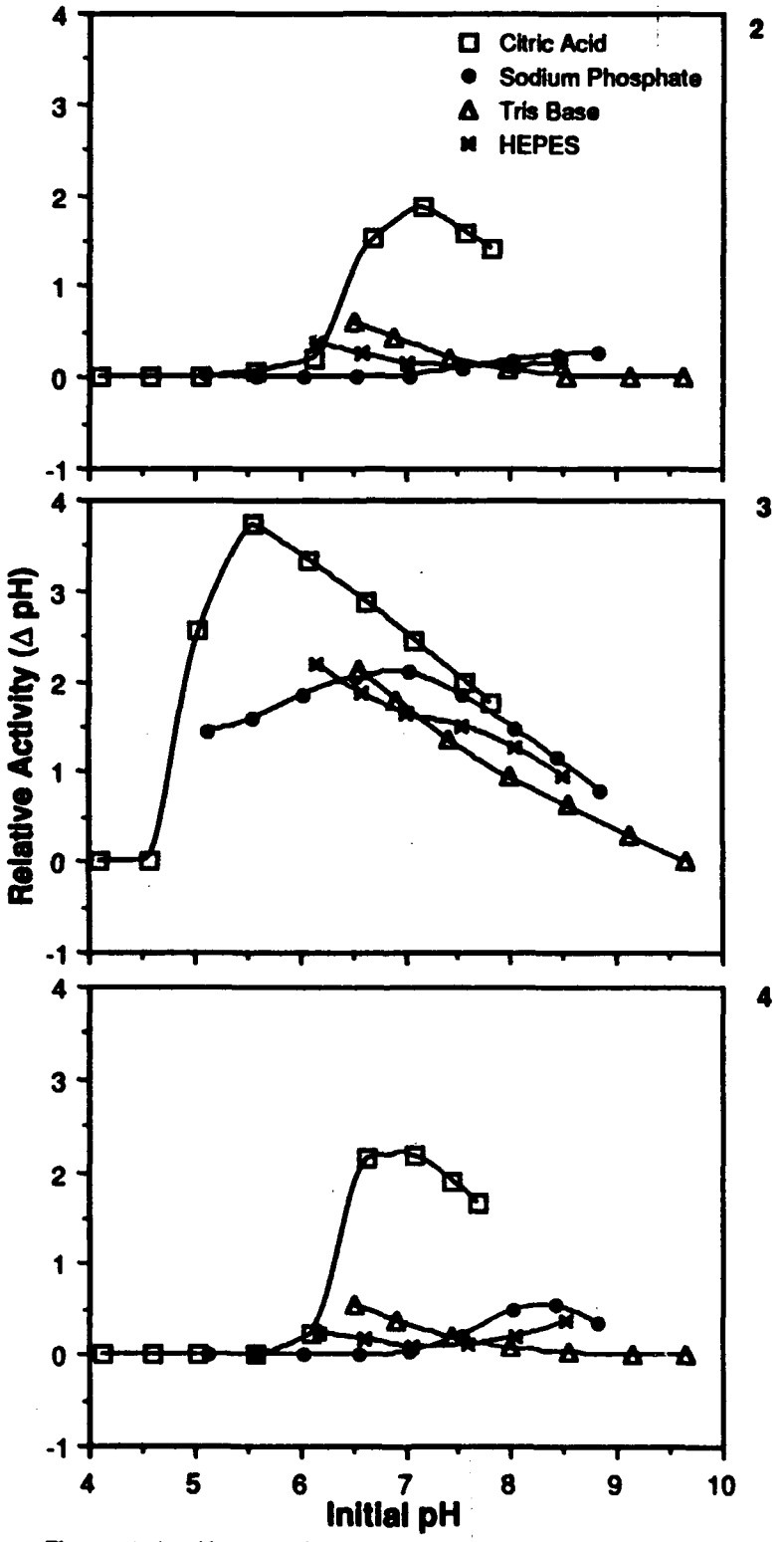


Figure 1: Comparison of pH profiles for ureases from organism isolates #197, 408 and 429 in optimum citrate buffer (200 mM) containing 1 mM EDTA.



Figures 2-4: pH profiles for ureases from organism isolates #197U (2), 408 (3) and 429 (4) in 4 common buffers at 200 mM containing 1 mM EDTA.

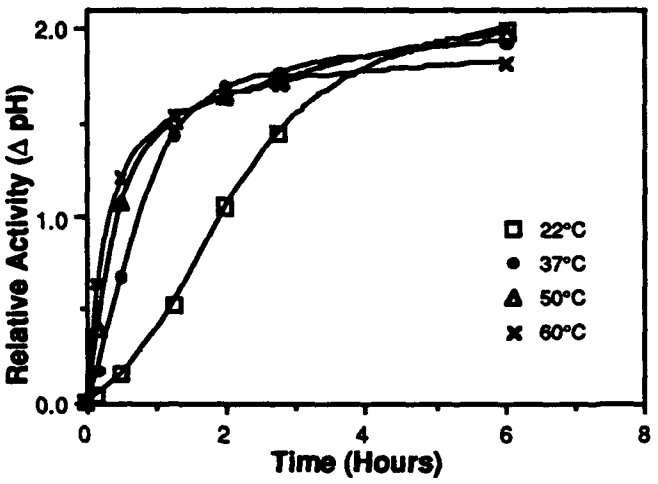


Figure 5: Temperature effect on the assay of urease from organism isolate #413 in 50mM HEPES containing 1mM EDTA.

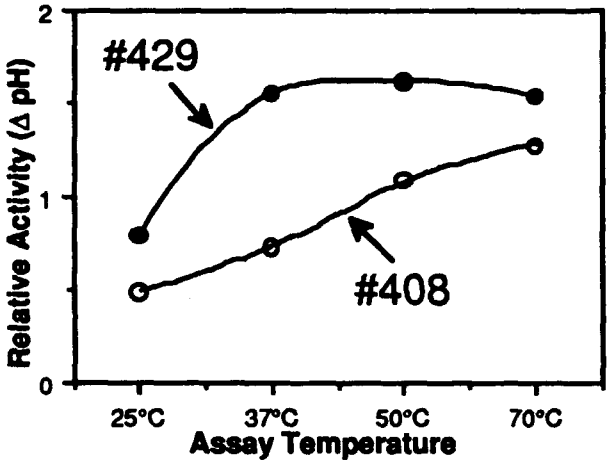


Figure 6: Comparison of assay tempertaures for ureases from isolates #429 and 408 in 50 mM citrate buffer (pH 7) illustrates anticipated relative thermophilities. Ureases were partially purified.

Optimum Temperature. Two factors must be considered when determining the optimum assay temperature : (1) temperature and (2) time. As illustrated in figure 5, prolonging the assay results in the convergence of data points among incubation temperatures as the buffer pH is driven toward alkalinity due to the consumption of hydrogen ions in the formation of ammonium ions. The enhanced and near linear activities observed with increasing temperatures, particularly for short incubation periods, is presumably due to the expulsion of inhibitory ammonium ions as observed by Magana-Plaza (Magana-Plaza). Ammonia may be more toxic for urease at higher temperatures or ammonia may be driven off as a gas faster than ammonium ion is formed and a pH change produced. A substrate limiting condition, similar to the pH stabilization observed with prolonged incubation at individual temperatures, can also be observed as the temperature of the enzyme is increased. Given sufficient time, even assays conducted at 22°C will show nearly as large a pH change as assays performed at 60°C.

The optimum assay temperature for two isolates is illustrated in figure 6. This work was done on partially purified enzyme so the loss of activity at higher temperatures, may indicate inhibition by the large amount of ammonia being produced or may simply reflect activity of small amounts of proteases not removed during the purification process.

II. Organism Collection, Culturing and Isolation

Water samples containing microorganisms were collected from the nearby Yellowstone thermal basins. Sites were selected based on temperature, pH and known nitrogen analyses. Efforts centered on near neutral pH waters between 50 and 70°C, including waters of both high and low nitrogen levels. An attempt to increase the chances of recovering ureolytic organisms was accomplished using autosampler vials containing varying nitrogen sources. The septa in the caps were replaced with porous membranes. Vials were left in thermal pools for 24 hours. No microbes were recovered from the vials with urea, but a number of organisms were recovered from vials containing either ammonium sulfate or potassium nitrate. Additional organisms from J. K. Research's thermophile collection were also screened for urease activity.

Samples were returned to the laboratory the same day they were collected and plated on the following medium: 0.25 g/ L peptone, 0.25 g/ L yeast extract, 2.0 g/ L dextrose, 0.05 g/ L magnesium

sulfate, 0.20 g/ L calcium chloride, 0.5 g/ L sodium chloride, 0.5 g/ L urea (filter sterilized, not used in controls), 1.0 g/ L sodium phosphate (monobasic or dibasic according to the desired pH), 8.0 g/ L Gelrite, 1.0 mg/ L manganese sulfate, 0.005 mg/ L zinc sulfate, and 0.01 mg/ L nickel chloride. Incubators were kept constant at 50, 60 and 70°C and cultures were incubated at the temperature most closely approximating that of the original habitat.

Two hundred thirty cultures were evaluated for stable enzyme production. Twenty seven of these organisms exhibited a positive reaction in the qualitative screening, i.e. a pH increase in the presence of urea compared with comparable media without urea (figure 7).

Significant Urease Positive Cultures Collected

Culture ID	*Habitat pH*	*Habitat Temp (°C)*
26(C)X	8.2	53
30(A)X	8.8	49
30(B)X	8.8	49
56(A)X	8.8	47
70(B)X	8.8	50
88X	6.6	55
135(C)F	6.4	47
153(A)F	5.8	52
189(A)F	6.7	53
197X	6.5	51
208(B)	5.0	56
208(A)X	5.0	56
211(A)	6.9	57
237	7.1	54
256(B)X	5.2	54
256(C)X	5.2	54
288B	6.0	50
301	8.1	58
312	7.0	50
316	7.0	50
339	6.0	75
341	2.6	49
345	7.7	49
369	7.5	47
408	5.4	50
413	6.7	56
429	8.5	70

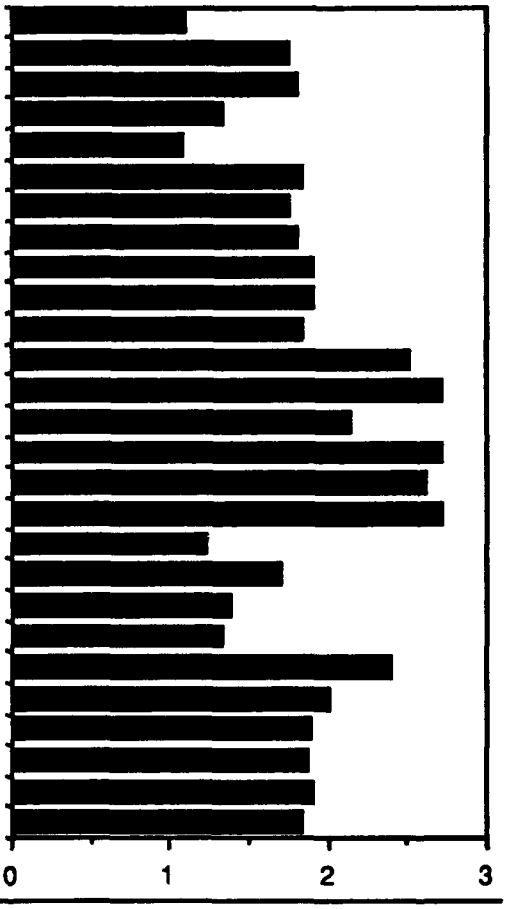


Figure 7: pH of cultures were compared on media with and without urea. Those producing a pH difference greater than one were considered positive in this screening assay.

Since a simple pH difference observed between media with and without urea was only a presumptive determination, organisms were subjected to a series of additional evaluations to ensure the production of specific urease. Organisms cultured on media with 10 gm urea/ L and monitoring for an odor of ammonia was one such experiment (figure 8).

Later observations revealed the odor of ammonia was either very weak or generally not detected when media containing only 1 gm urea/ L was used in the assay. However, enzyme activity in quantitative determinations were much higher in media with 1 gm rather than 10 gm urea/ L. This may

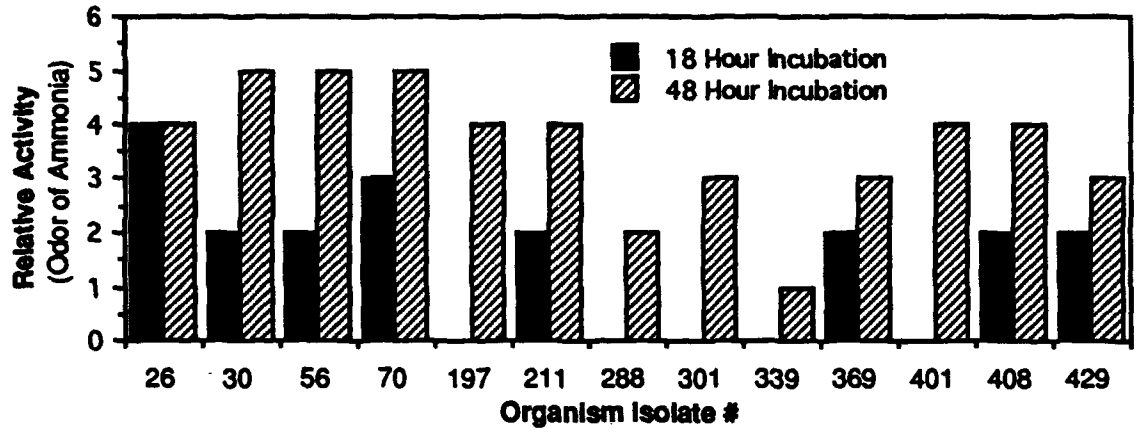


Figure 8: Qualitative screening of organism isolates by the odor of ammonia liberated on solid media containing 10 gm urea/L. The plates were incubated at 18 and 48 hrs at appropriate growth temperatures for individual organisms (n=2 by two people).

indicate an insensitivity of the olfactory response rather than lack of enzyme activity.

Microorganisms were raised in analogous media prepared as a broth rather than gelled plates. Ammonium ion levels were semi-quantitated using Merck's EM Quant test strips. Six cultures produced undetectable levels of ammonium ion. The two cultures selected for submission to CRDEC, #408 and 429, were among the best producers of ammonium, as seen in table 2.

Table 2. EM QUANT. NH_4^+ Concentrations
(10 gm urea/ L media)

Culture	*Ammonium Ion (mg/ L)*
26	0
30	0
56	0
70	0
197	60
208	0
211	100
288	200
301	10
339	0
369	30
408	**100**
429	**400**

Preliminary advanced investigation was initiated while screening continued. Only ten isolates were deemed to possess sufficient urease activity to warrant further investigation. These organisms came from habitats ranging in temperature from 45 to 75°C and pH 5.4 to 8.5, as listed in table 3. Only one of

these would produce urease at 70°C, at readily detectable levels.

Table 3. Isolates Selected for Further Investigation

	Habitat of Origin	
Number	*°C*	*pH*
197X	51	6.5
211(A)	57	6.9
288(B)	45	
301	58	8.1
339	75	
369	47	7.5
401	66	7.4
408	50	5.4
413	56	6.7
429	70	8.5

The two organisms, isolates #408 and 429, were determined to produce the highest level and most stable urease. The following collection results are provided.

Urease Collection History

Screened 230 Cultures

27 Isolates Yielded a pH Change >1 pH unit

10 Isolates Selected for Further Investigation

2 Isolates Submitted to CRDEC (#408 & 429)

Media Optimization

Ureolytic organisms were isolated on several different media. Some of these organisms were previously collected as potential producers of peroxidase and glucose oxidase. Other urease producing organisms were collected in membrane enrichment vials. A media similar to that described early in the collection section without the trace elements was used as an initial starting point.

Carbon Source. Dextrose is frequently used as a sole carbon source in minimal media for laboratory culturing of numerous microorganisms. Although others have observed repression by dextrose (Yuodval'kita) we observed a significant increase in urease activity as dextrose levels were raised to 25 gm/L. This increase is presumably due to increased biomass accumulation which is consistent with other investigators who observed increases in urease activity during the early stationary phase or late log phase (Bast). Similar or perhaps better urease production was also observed using molasses as a carbon source, however it was found to be rather messy to work with. Such increases are expected considering the complexity of composition and the presence of considerable micronutrients. An attempt was then made to determine the equivalent concentration of dextrose required for comparable production

of urease using molasses.

Table 4. Effect of Dextrose Concentration on Urease Production from Several Organism Isolates

Organism Isolate #	*Dextrose Concentration*	
	2 g/ L	*25 g/ L*
197	1.16[a]	2.73
211	1.84	2.67
288	1.19	2.45
301	0.85	2.13
339	1.26	2.26
369	0.57	1.52

[a] Urease activity expressed as a change in pH units

Use of 25 g/ L dextrose was compared with 25, 50 or 100 g/ L of molasses for isolate #408 (figures 9 and 10). Although the higher levels of molasses did significantly improve urease production, the large

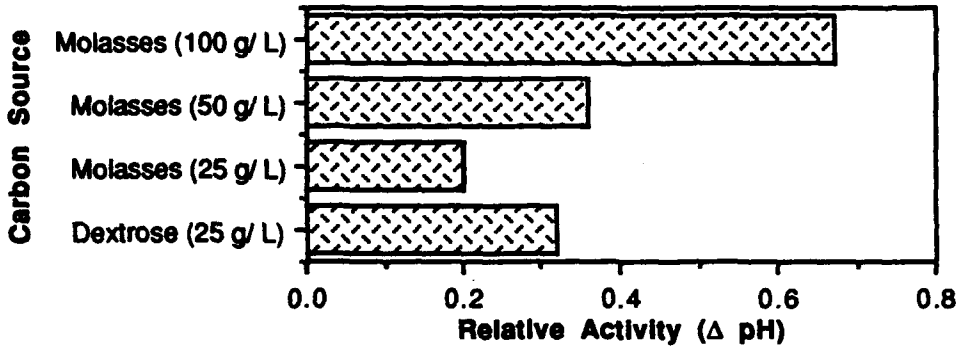


Figure 9: Effect of molasses and dextrose as carbon sources on urease production from organism isolate #408. The equivalent concentration of dextrose required for comparable production of urease using 50g/ L molasses was observed at approximately 25g/ L. Further increasing dextrose to 50 gm/ L did not result in urease production equivalent to that obtained with 100 gm molasses/ L (Data not shown).

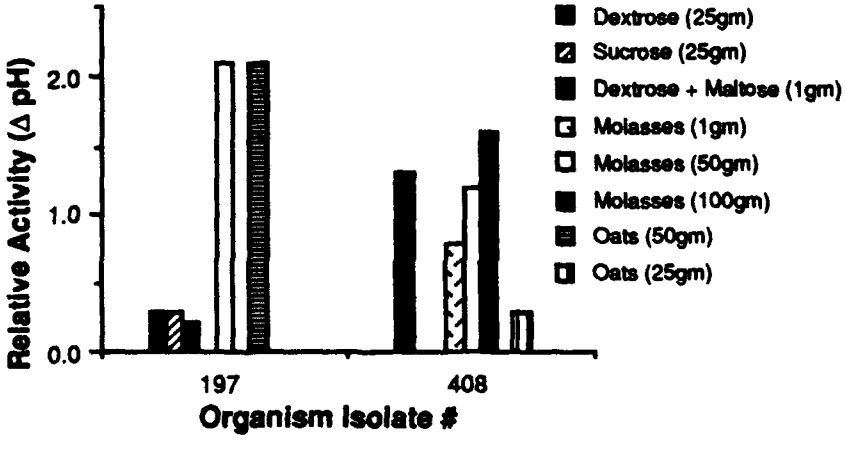


Figure 10: The effect of carbon sources on urease production. Although carbon utilization tests (table 5) demonstrated the use of sucrose by #408, substitution of dextrose always resulted in reduced or no urease production.

quantities of molasses in the medium caused problems in the chromatographic purification process due to the chemical complexity of the molasses. The addition of oats to the medium dramatically increased the viscosity and prevented adequate aeration. On occasion, however, excellent urease production was obtained on oats, despite the poor aeration.

Isolates #408 and 429 were submitted to MIL, Inc. for characterization of their utilization of various carbon

Table 5. Carbon Utilization Pattern Recognition Data (MIL, Inc.)

Urease producing isolates #408 and 429

Carbon Source	#408			#429		
	4hrs	*24 hrs*	*72 hrs*	*4 hrs*	*24 hrs*	*72 hrs*
α-cyclodextrin	N	N	27	N	N	N
dextrin	83	133	214	N	N	N
glycogen	51	104	192	N	N	49
tween 40	N	N	58	N	N	104
tween 80	N	N	N	N	N	N
N-acetyl-D-galactosamine	N	N	N	N	N	N
N-acetyl-D-glucosamine	33	N	22	N	N	N
adonitol	N	N	N	N	N	N
L-arabinose	50	N	50	N	N	N
D-arabitol	N	N	N	N	N	N
cellobiose	49	N	61	N	N	N
i-erythritol	N	N	16	N	N	N
D-fructose	34	N	67	N	N	N
L-fucose	N	N	14	N	N	N
D-galactose	N	32	44	N	N	N
gentiobiose	N	N	50	N	N	N
α-D-glucose	43	59	90	N	63	52
m-inositol	N	34	41	N	N	N
α-lactose	52	57	80	N	N	N
lactulose	38	N	52	N	N	N
maltose	47	N	78	N	68	61
D-mannitol	N	N	N	N	N	N
D-mannose	N	32	65	N	N	N
D-melibiose	N	N	N	N	N	N
β-methyl-glucoside	N	N	19	N	N	N
psicose	63	98	148	N	N	51
D-raffinose	N	N	25	N	N	N
L-rhamnose	N	N	N	N	N	N
D-sorbitol	N	N	N	N	N	N
sucrose	38	65	75	N	N	N
D-trehalose	39	52	76	N	N	N
turanose	34	40	61	N	N	N
xylitol	N	N	N	N	N	N
methyl pyruvate	N	N	34	111	192	214
mono-methyl-succinate	N	N	N	59	237	303
acetic acid	47	40	53	N	N	N
cis-aconitic acid	N	N	N	N	N	N
citric acid	N	N	N	N	N	N
formic acid	N	N	N	N	N	N
D-galactonic acid lactone	N	N	N	N	N	N
D-galacturonic acid	N	N	N	N	N	N
D-gluconic acid	N	N	N	N	N	N
D-glucosaminic acid	N	N	N	N	N	N
D-glucuronic acid	N	N	N	N	N	N
α-hydroxy-butyric acid	N	N	N	N	N	N

* Values are percentage color change on microtiter wells (MIL) over the control and represent a relative proportional quantity of utilization. N = Negative.

Carbon Source	#408			#429		
	4hrs	24 hrs	72 hrs	4 hrs	24 hrs	72 hrs
β-hydroxy-butyric acid	N	N	N	N	N	N
γ–hydroxy-butyric acid	N	N	N	N	N	N
P-hydroxy-phenylacetic acid	N	N	N	N	N	N
itaconic acid	N	N	N	N	N	N
α-keto butyric acid	N	N	30	N	123	120
α-keto glutaric acid	N	N	N	N	N	N
α-keto valeric acid	N	N	27	49	208	240
D,L-lactic acid	N	N	N	N	74	55
malonic acid	N	N	N	N	N	N
propionic acid	N	N	N	N	N	N
quinic acid	N	N	N	N	N	N
D-saccharic acid	N	N	N	N	N	N
sebacic acid	N	N	N	N	N	N
succinic acid	N	N	N	N	N	N
bromo succinic acid	N	N	16	N	N	N
succinamic acid	N	N	N	N	N	N
glucuronamide	N	N	N	N	N	N
alaninamide	N	N	N	38	142	113
D-alanine	N	N	N	N	N	N
L-alanine	N	N	N	N	65	N
L-alanyl-glycine	N	N	N	N	N	N
L-asparagine	N	N	N	N	N	N
L-aspartic acid	N	N	N	N	N	N
L-glutamic acid	N	N	N	N	N	N
glycyl-L-aspartic acid	N	N	N	N	N	N
glycyl-L-glutamic acid	N	N	N	N	57	48
L-histidine	N	N	N	N	N	N
hydroxy L-proline	N	N	N	N	N	N
L-leucine	32	N	22	N	78	69
L-ornithine	N	N	23	N	N	N
L-phenylalanine	N	N	N	N	N	N
L-proline	N	N	N	36	199	216
L-pyroglutamic	N	N	N	N	N	N
D-serine	N	N	N	N	N	N
L-serine	N	N	N	N	N	N
L-threonine	N	N	N	N	N	N
D,L-carnitine	N	N	N	N	N	N
γ–aminobutyric acid	N	N	N	N	N	N
urocanic acid	N	N	N	N	N	79
inosine	N	N	N	N	N	N
uridine	45	N	55	N	N	N
thymidine	37	34	58	N	N	N
phenylethylamine	N	N	N	N	N	N
putrescine	N	N	N	N	N	N
2-amino ethanol	N	N	N	N	N	N
2,3-butanediol	N	N	29	N	N	N
glycerol	N	N	N	N	N	N
D,L-α-glycerol phosphate	N	N	N	N	N	N
glucose-1-phosphate	N	N	N	N	N	N
glucose-6-phosphate	N	N	N	N	N	N

sources. These assays, performed in 96 well microplates, can be particularly useful in biodegradation and selective media development. Unfortunately, these assays were not performed until near the end of the project. Based on the results of this inexpensive test, we would suggest acquiring this information in the earliest stages of a similar project.

The protocol used by MIL, Inc. initially involved streaking the organisms on a nutrient medium that would support vigorous growth and incubating at 50°C. Colonies were removed from the culture plate using a saline moistened cotton swab. A suspension of uniform turbidity was prepared in 0.85% saline. The microtiter wells containing the carbon source to be tested and the reductive indicator, tetrazolium, were then inoculated with the bacterial suspension at a rate of 150 µL per well and covered with a microplate lid. Covered plates were incubated at 50°C for the standard intervals of 4 and 24 hours. As a result of the unusually low responses of other thermophiles being evaluated simultaneously, plates were further incubated at 23°C for a total of 72 hours. The degree of tetrazolium reduction producing shades of purple was monitored at 590 nm and referenced against a negative control. Any purple color recorded above the control was taken as a positive utilization of the given carbon source. The data were reported as the percent color change as compared to the control (table 5).

Isolate #408 appeared to use a number of carbon sources much more readily than glucose. These sources included dextrin, glycogen and β-methyl-glucoside. An inexpensive source of dextrin has the potential for increasing enzyme production (provided this carbon source doesn't result in negative feedback) and decreasing production costs. Perhaps more interesting are the carbon sources favored by #429. This organism made rather marginal use of α-D-glucose and did not ferment many of the more common sugars such as lactose, fructose, mannose, galactose and sucrose. However, reactions in wells with methyl pyruvate, mono-methyl- succinate, α-keto valeric acid and L-proline were very high.

Nitrogen Source. Regulation of urease production may be repressible, inducible or constitutive (Bast). In many bacterial species, urease production is regulated in conjunction with the nitrogen regulatory system. Synthesis may be de-repressed under nitrogen limiting conditions or induced by the presence of urea. For organisms in which urease production is constitutive, enzyme levels are unaffected by addition or limitation of nitrogenous compounds (Mobley).

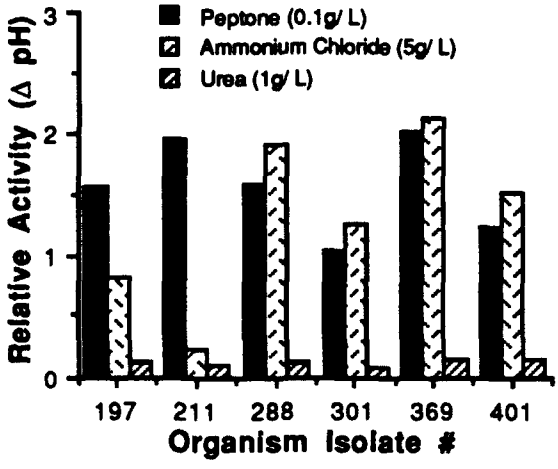


Figure 11: The effect of nitrogen sources on urease production in several organism isolates. Media containing urea resulted in significant decreases in urease production as compared with low levels of peptone or high concentrations of ammonium chloride.

In this investigation, urease production was significantly inhibited in the presence of urea for all isolates tested. In fact, membrane enrichment vials used for some of the collections described previously are recalled not to have yielded any organisms when vials contained urea, while numerous bacteria were recovered from the same locations when vials contained either ammonia or nitrate. Bast observed similar correlation of urease production with ammonia concentration where urease biosynthesis oscillated with varying levels of exogenous ammonia (Bast). The induction of urease by urea was also suggested to be secondary, a less effective mechanism of urease formation. This is further illustrated in figure 11 where 6 organisms were evaluated for urease activity in the presence of 3 compounds functioning as nitrogen sources. The presence of urea as a sole nitrogen source resulted in significantly less activity in all 6 isolates. Other urea derivatives, uric acid and hydroxyurea were also found to inhibit urease production.

Work on isolate 429, the only organism raised at 70 °C, compared the effects of casein, tryptone,

urea and ammonium sulfate. The following table shows that neither urea nor ammonium sulfate were satisfactory nitrogen sources.

Table 6. Effect Of Nitrogen Sources On Urease #429 Production

Nitrogen Source	g/ L	Activity *(ΔpH)*
Casein	1.0	1.09
Tryptone	0.5	0.79
Urea	0.5	0.17
Caseamino acids*	0.5	0.02
Ammonium Sulfate	0.5	0.15

*Caseamino acid s aggregate in the medium. Initial trials yielded poor results, but better dispersal was obtained later resulting in only slightly better activities.

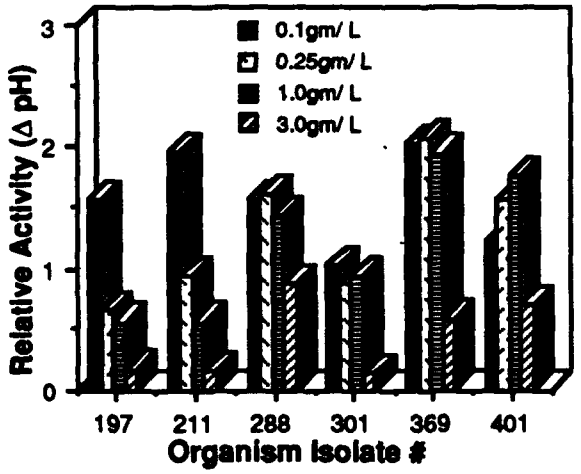


Figure 12: The effect of peptone concentration as a sole nitrogen source on urease production in several organism isolates. High concentrations were inhibitory while concentrations of 0.10 to 1.0 gm peptone/ L were optimal. The exact concentration was specific for selected organisms.

Replacement of the peptone with either casein or tryptone were satisfactory substitutes, but uric acid was not. Casein at 1 g/ L was optimal; raising casein to 5 g/ L or lowering it to 0.1 g/ L caused a 80-90% reduction in urease levels for isolate #408. Isolate #429 did equally well on casein, caseamino acids or tryptone in the range of 0.5-2.0 g/ L. Casein was not particularly water soluble and tended to become gummy after autoclaving, and seemed to make foaming problems worse than other nitrogen sources. However, by the end of a fermentation, all the gummy material had disappeared. Final media formulas included tryptone, simply because it was the easiest to handle.

From the carbon utilization data, the fact that neither organism could use histidine should be noted. In an investigation with *Klebsiella pneumoniae*, Jahns observed a 10-fold increase in urease production when 20 mM histidine was substituted for either urea or ammonium salts (Jahns).

Table 7. Effect of Nitrogen Source on Urease Production

Organism Isolate #	*Nitrogen Source/ Concentration (grams/ Liter)*					
	Peptone .1	*Peptone .25*	*Peptone 1*	*Peptone 3*	*NH_4Cl 5*	*Urea 1*
197	1.57[a]	0.67	0.57	0.17	0.82	0.12
211	1.97	0.94	0.56	0.15	0.24	0.10
288	1.58	1.61	1.44	0.88	1.91	0.10
301	1.03	0.88	0.93	0.14	1.26	0.07
369	2.02	2.07	1.94	0.59	2.13	0.13
401	1.23	1.57	1.78	0.71	1.53	0.14

[a] Urease activities expressed as a change in pH units

[b] Data has been graphically represented in figures 11 and 12

Oxygen. The effect of air on urease production was examined by comparing the concentration of urease produced at 3 days among cultures magnetically stirred at 400 rpm (minimal aeration), shaken in a waterbath at 80 rpm (intermediate aeration) and sparged with air at a rate of 1 mL air/mL medium/min (maximal aeration).

The significant increase in urease production observed at higher aeration rates might simply represent the organism's response to an increased nitrogen availability. However, a similar increase in biomass with subsequent increased urease yields as observed in the presence of added dextrose is also possible.

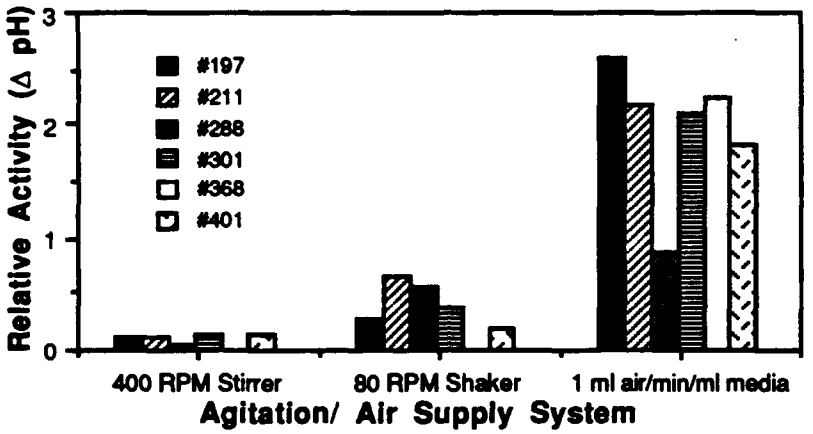


Figure 13: Effect of aeration on urease production for several organism isolates in 3 day cultures subjected to magnetic stirring (minimal aeration), shaker (intermediate aeration) and constant bubbling of 1 mL air/mL medium/min (maximum aeration).

Other Nutrients. Media optimization included the investigation of various trace metals. Although urease is a nickel containing enzyme, the addition of nickel was observed unnecessary. The concentration of trace metals, including nickel, required by the organisms are likely to be readily obtained from complex nitrogen sources. However, the stainless steel agitators and heat exchangers in the large fermentors undoubtedly contribute to the available nickel. Zinc sulfate at 0.01 g/ L was observed to be inhibitory, however at one tenth the concentration, the benefits varied among organisms. Zinc sulfate was observed not required for either isolates #408 or 429. Magnesium sulfate had no detrimental effect to a maximum of 0.25 g/ L, however at 0.5 g/L, some decrease in urease production was generally observed. Evaluations were also performed on molybdenum, manganese and cobalt salts. A micronutrient mixture normally used for other thermophiles, gave superior results on additions of single trace elements or combinations of only two or three.

Both isolates #408 and 429 could thrive in either sodium or potassium phosphate buffer, however, tris buffer resulted in substantially reduced urease cncentrations.

Vitamins are not often required by organisms inhabiting high temperatures. Similarily, biotin and thiamin were evaluated and observed unnecessary for urease production.

Table 8. Final Optimized Medium Formula

Component	*(g/L)*
Yeast Extract	0.5
Dextrose	25.0
Magnesium Sulfate Heptahydrate	0.1
Casein	1.0
Micronutrients	*
Sodium Phosphate Dibasic	3.0

Components must be autoclaved separately.
* A 1.0mL aliquot of micronutrients consisting of 0.29g/ L ferric chloride hexahydrate, 2.28g/ L manganese sulfate monohydrate, 0.40g/ L zinc sulfate heptahydrate, 1.50 g/ L boric acid, 0.05 g/ L copper sulfate pentahydrate, 0.20 g/ L molybdic acid dihydrate sodium salt and 0.05 g/ L cobalt chloride hexahydrate is added to 1 L of medium.

III. Fermentation

Temperature

Fatty acid analyses on 7 isolates, #197U, 211, 288, 301, 408U, 413 and 429, suggested that all belonged to the genus *Bacillus*. Although they were by no means all from the same species, similar organisms were eliminated as discussed under organism identification, allowing more effort to be concentrated on distinctly different species. Because the very large genus *Bacillus* includes many well known mesophiles and thermophiles, comparative data is available on stabilities of certain enzymes. For example, some thermophilic bacteria have been demonstrated to adapt to growth at both high and low temperatures. Haberstich and Zuber showed a thermoadaptation (reversible conversion) for certain bacilli if they were cultivated at an intermediate temperature of 44-52°C. These cells were then cultivated at 5°C intervals from 30° to 70°C. Crude extracts of cells raised at the higher temperatures contained enzymes that were more thermostable than the corresponding enzyme from cells raised at the lower temperatures (Haberstich). Since several of the urease producing organisms would grow at 60-65°C, and #429 would grow at 70°C, a series of experiments were performed on raising temperatures either initially or during fermentation to determine the effect on enzyme thermal stability. Although biomass was produced at most temperatures, urease concentrations in all except #429 diminished significantly or were not detectable at the higher temperatures. Although isolate #429 did produce urease at 70°C, the concentrations were initially much lower than what could be obtained with other organisms at lower temperatures. However, by the end of the project, urease production by #429 was improved to the point that levels approached those of the lower temperature organisms.

As the temperature rises, oxygen solubility in a liquid decreases. Many extreme thermophiles isolated to date are anaerobic. Isolate #429 is aerobic, but growth rates and subsequently urease production are reduced as dissolved oxygen becomes limiting. At 70°C, the growth medium for #429 had to be supersaturated with dissolved oxygen for optimum urease production.

Controls

The following parameters were monitored during fermentations: enzyme activity, dissolved oxygen, microscopic appearance, biomass and pH. Dextrose and ammonium ion concentrations, monitored at the end of fermentations, may be useful in future fermentations. Although many variables such as

quantity and age of the inoculum and media components will affect fermentation, frequent monitoring of enzyme activity and oxygen are the most critical.

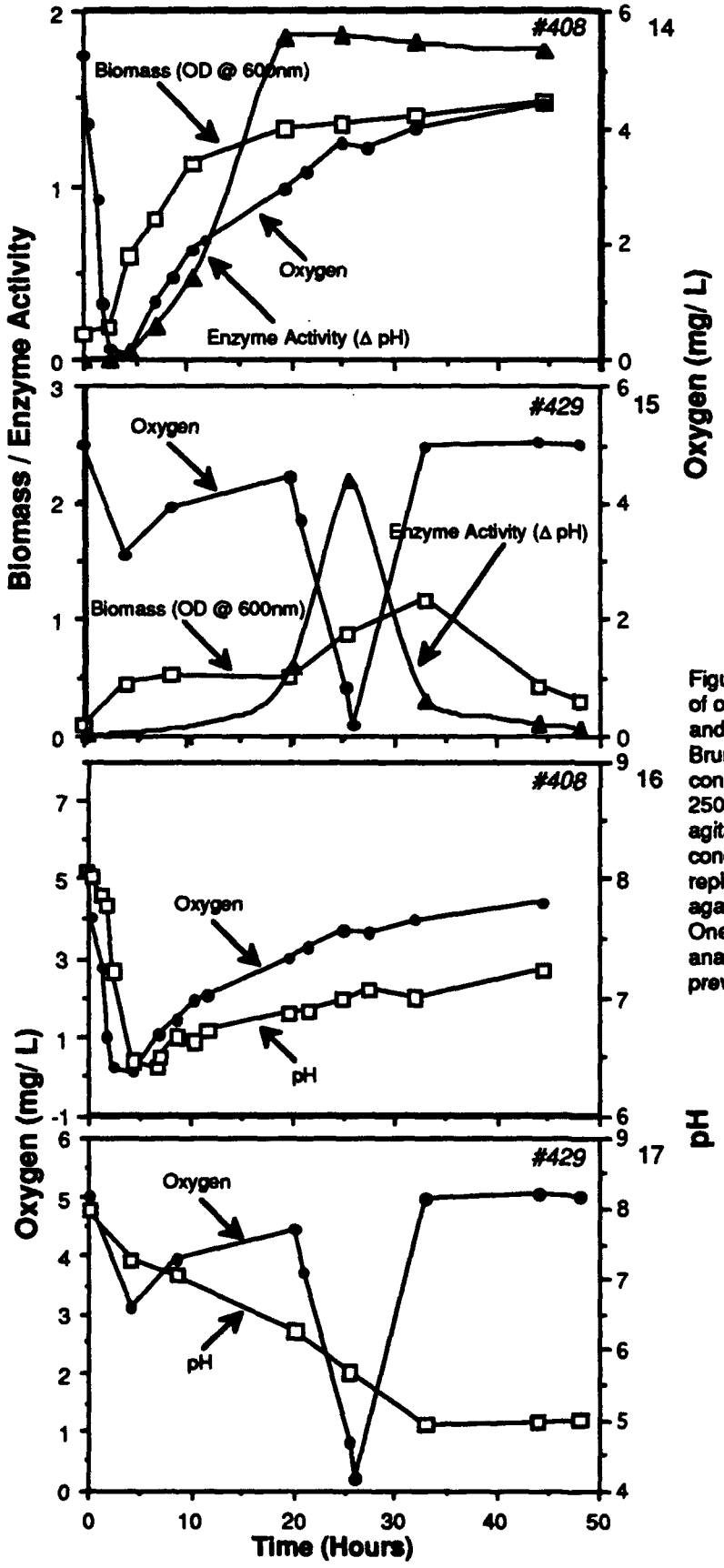
Enzyme Activity. Urease cellular and supernatant activity must be monitored at frequent intervals. Cell disruption was observed to slightly shorten the length of time required for assay incubation.

Protocol for Monitoring Enzyme Activity During Fermentation

1 mL dilute urease
2 mL 50 mM citric acid buffer, pH 7
1 mL urea (25 gm urea/ L)
Determine pH at time zero
Incubate 30 min at 50°C or 1 hr at 20°C
Determine difference between initial and final pH

Dissolved Oxygen . As temperature increases, the amount of oxygen required for saturation of a liquid decreasesquite dramatically. For example, air saturated distilled water at 37°C contains 7.04 mg/ L of oxygen while at 60°C it decreases to 4.48 mg/ L (Allgood). At 50°C and at saturation, a medium will contain only approximately 5.5 mg oxygen per liter. In an effort to increase the amount of oxygen, the medium of #429 at 70°C was supersaturated by increasing the agitation rate to 500 rpm. However, this agitation created a problem with foaming. Foaming could be controlled by adding the nitrogen source in increments at 0, 8.5, 20, 21 and 25.5 hrs. Rather than a sharp decline in oxygen and peak enzyme concentrations by 18 hours as for #408 (figure 14), the fermentations with #429 were initiated more slowly and oxygen was not minimal until 24 hrs, nearly coinciding with maximum urease concentrations. This illustrates that further investigation on the fermentation procedure of #429 is required. The possibility that a slower fermentation is optimal for #429 exists. The substitution of tryptone for casein was also observed to be beneficial in decreasing the foaming problem, although did not eliminate it.

Oxygen concentrations decreased at varying rates depending on the organism, condition of the inoculum and manipulation of nitrogen additions. As oxygen concentration decreased, however, urease production accelerated and on return to saturation, urease concentrations declined. Since oxygen was added at a constant rate, changes in oxygen concentration reflect use by the bacteria. In other words, urease production was observed during the time of maximum cell growth and/or maximum metabolic rate.



Figures 14 - 17: Fermentation monitorization of organism isolates #408 at 50°C (14 and 16) and #429 at 70°C (15 and 17) in 5 L New Brunswick fermentors. Air was introduced at a constant rate of 1 mL air/ mL medium/ min and 250 rpm agitation for #408 (14) and 500 rpm agitation for #429 (15). Oxygen concentrations from figures 14 and 15 were replotted on figures 16 and 17, respectively, against pH alterations during fermentation. One mL samples were withdrawn and analyzed for enzyme activity using the previously described protocol.

Microscopic Appearance. Like all members of the genus Bacillus, isolates #408 and 429 form spores that are generally observed following the exponential phase and well into the log phase of the microbial sigmoid growth curve. Isolate #408 cells were enumerated on slides prepared at intervals during the log phase of the fermentation. Generation times were estimated to be between 20 and 60 minutes.

Photograph 1: Urease producing isolate #429. Rod shaped bacteria 10 hours into fermentation. Isolates #408 and exhibited similar microscopic appearance. High powered field (HPF) 1000X magnification, oil immersion.

Biomass . An estimate of cell density was determined by monitoring optical density at an absorbance of 600 nm. A comparison between optical density and plate counts for both isolates #408 and 429 revealed that an OD of 0.6 corresponded to a plate count of 1 x 10^{12} bacteria/ mL. Urease activity was observed to approximately parallel cell density, increasing to a maximum and plateaing or actually diminishing if fermentations were prolonged.

pH. The pH of the medium prior to inoculation was approximately 8. The medium containing isolate #408 tended to have a higher pH during fermentation than the broth of #429. The pH of the broth of #408 declined to a minimum of 6.41 at 6.75 hours, followed by a slow return to pH 7.24 by the end of the fermentation and nearly paralleled oxygen levels (figures 14 and 16). In contrast, the pH of the broth containing isolate #429, declined to 4.94 at 33 hours and had only increased slightly to 5.00 by the end of the fermentation. Initiating the fermentation of #408 at pH 8.0 rather than 7 - 7.5 was observed to yield increased urease production, however, attempts to maintain a higher pH through the addition of buffer during fermentation resulted in failure. Review of the final data for #429 illustrates that fermentations ending between 5.5 and 6.7 consistently produced more urease than those ending from 5.0-5.9 (note overlap). Again, the addition of buffer during a fermentation only served to destroy the fermentation. More investigation is necessary, perhaps initiating at a higher pH or using a stronger buffering system. Too high of a pH must also be avoided; in experiments where the complex nitrogen source was added incrementally, the pH remained between 7 and 8 but little urease was produced.

Other Parameters. Dextrose was not observed below 10 g/ L as would be expected when starting with such high levels. Ammonium ion levels were measured in the range of 5-20 mg/ L at the end of

fermentation but there appeared to be no correlation between urease production and final ammonium levels.

IV. Organism Lysis and Enzyme Secretion

A certain portion of the urease was retained inside the cells, although that portion varied significantly with culture conditions. When harvesting urease, it was essential to break the cells open. All fractions had to be assayed for enzyme activity during processing. A number of cell disruption methods were attempted as illustrated in table 9. Sonication resulted in the best recovery of urease from the cells.

Table 9. Cell Disruption Methods
Enzyme Activity of Supernatant

Control - No treatment	0.73*
Nitrogen Cavitation - 1 hr	1.32
5 Minute Sonication	1.98
Multiple Freeze Thaw (3 times)	0.23
Lysozyme + 5 min Sonication	1.19

*Enzyme activity is reported as a change in pH from a 1 mL sample size.

The duration of sonication required for complete cell disruption depended on the organism, temperature and sample size. As illustrated in figure 18, organism isolate #211 was more readily disrupted than #197U. Further investigation on #197U revealed better than 90% recovery from 1 liter of medium on 45 minutes of sonication, while analogous results were obtained in 20 minutes when the volume was decreased to 200 mL.

V. Enzyme Purification

Organism Concentration

The filtration system used for this project was a Minitan Ultrafiltration System from Millipore. The system allowed both tangential flow microporous and ultrafiltration processing more rapidly than traditional methods. Eight hydrophilic Durapore (PVDF) membrane plates with a total area of 480 sq cm were stacked in a series configuration between acrylic manifold plates. Silicone retentate separators provided a gasket seal between plates and created a sweeping flow across the upstream filter surfaces (Millipore). Torque pressure was adjusted to 80 in lbs. Although the PVDF was supposed to be low protein binding, a portion of the enzyme was observed being lost during this step. Coating the membranes with a solution of 5% nonfat dry milk, covered active sites and prevented enzyme loss. The milk was rinsed with 50 mM HEPES, pH 7, before the fermentation broth was processed. Transmembrane pressure was kept below 1-2 psi. Clarified media was collected from the filtrate line, while the cells became more and more concentrated in the retentate container. The process was considered complete when the cellular material was concentrated to 250 mL.

Sonication

The cellular concentrate (250 mL) was placed in a 400 ml beaker in an ice bath over a magnetic stirrer. A stir bar was added and the one inch horn of a 600 W ultrasonicator was placed at the edge of the vortex created by the stirrer, approximately one inch into the solution. The sonicator was turned to full power for 30 minutes to achieve cell disruption. This protocol was used for both extracts from isolates #408 and 429. It was important that the sonicated material be kept cold. As the cells are disrupted,

proteases are released. Their activity could be substantially reduced by proper icing of the sample. Furthermore, sonication experts have determined that superior cell breakage is achieved at lower temperatures.

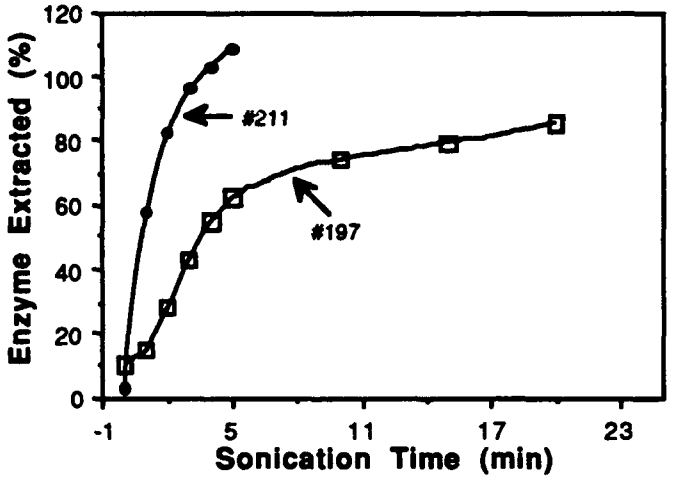


Figure 18: The effect of sonication on enzyme secretion from organism isolates #197U and 211. Sonication of 100 mL fermentation broth was achieved using a 600W sonicator with a 1 inch horn.

The separation of cellular debris from soluble urease was performed by processing the sonicated solution through the Minitan in exactly the same configuration as previously described during organism concentration. Approximately 220 mL of clarified enzyme solution was collected on ice from the filtrate line. As the volume of cellular debris approached 50 mL (hold up volume of the instrument is 30 mL, leaving approximately 20 mL in the retentate container) the cells were washed several times with 50 mL portions of buffer. This resulted in a final filtrate volume of approximately 500 mL. Urease analyses were performed on each fraction throughout the process.

The assembled Minitan plates were washed with 8 mL bleach/ L according to the manufacturers instructions and allowed to soak at least overnight in a 0.1% solution of sodium dodecyl sulfate (SDS) at 50°C.

Enzyme Concentration

At this point, the clarified urease solution was ready for concentration. The Minitan was assembled with 10,000 molecular weight cut off filters of regenerated cellulose (designated PLGC). The system was operated at 20 psi, concentrating the clarified material to 30 ml. Since problems were not encountered with urease penetrating the membrane, this higher pressure could be used to enhance the processing rate. Both the collection vessel and the clarified sample were kept on ice to avoid protease degradation of urease. The waste filtrate was assayed to determine the quantity urease passing through the filter. Since the molecular weight of the urease was sufficiently larger than the membrane cutoff, little urease was usually observed in the waste. The assays also assured that the membranes had not developed holes during processing.

Table 10 illustrates relative amounts of urease in each fraction obtained during processing through the Minitan. Later, as further improvements were made, a maximum of 91% and 93% of activity in the fermentation broths of organism isolates #408 and #429, respectively, was recovered in the concentrates. A higher processing temperature for #408 resulted in a larger portion of excreted urease from the cell, but a decrease in total recoverable urease. The use of molasses as a carbon source also resulted in a reduced excretion of urease into the medium compared with the use of dextrose under comparable conditions. In the larger fermentors, a larger portion of the urease was observed in the cells when compared to the mini-fermentors. Again, this data re-enforces the fact that all fractions must be assayed for urease activity, prior to the assumption that a given portion can be discarded.

Preparation of Clarified Concentrate

(1) Fermentation medium was clarified on the Minitan, concentrating the cells down to 250 mL.
(2) Cells were stirred in an ice bath during a 30 minute sonication with a 600 W instrument and one inch horn.
(3) Cells and clarified media were concentrated on the Minitan and the concentrates combined.

Table 10. Processing Yield of Urease Enzyme #413 Through Minitan
(Sample Not Sonicated) §

Treatment	TOTAL UNITS* *(ΔpH)*
Original	737
Original centrifuged	348
Filtrate	227
Retentate	197
Retentate centrifuged	57
Washed cells	172
Washed cells centrifuged	30
Concentrate	143
Discard	0

(Sample Sonicated)

Original	530
Original centrifuged	470
Filtrate	324
Retentate	141
Retentate centrifuged	100
Washed cells	128
Washed cells centrifuged	82
Concentrate	256
Discard	0

Units are expressed as the pH change ocurring in 1 mL aliquots multiplied by the volume being processed.
§ When the sample was not sonicated, 19% of the original activity was recovered in the concentrate.
When cells were sonicated, 48% of the original activity was recovered in the concentrate.

The literature is surprisingly consistent in reporting urease buffers containing 1 mM EDTA and 1 mM mercaptoethanol to sequester inhibitory trace metals and prevent oxidation and the formation of disulfide bonds. However, in this investigation, mercaptoethanol was observed to have a slight inhibitory effect on urease activity. Although mercaptoethanol does oxidize with time resulting in gradual full recovery of enzyme activity, only EDTA was added to our buffers.

Chromatography

Affinity. Affinity chromatography is a powerful purification technique for enhancing specificity of purified biologicals. The technique capitalizes on the specificity of the active site for binding substrates or substrate analogs. Numerous investigators have used either urea or hydroxyurea immobilized to a solid surface, although not all had found it to be a viable approach (Creaser, Mendes, Wong). Similarily in this investigation, urea or hydroxyurea was attached to a column. In theory, the urease attaches to these substrates or substrate analogs while all impurities are washed from the column. Attempts to affinity purify heat stable ureases in this investigation were not successful.

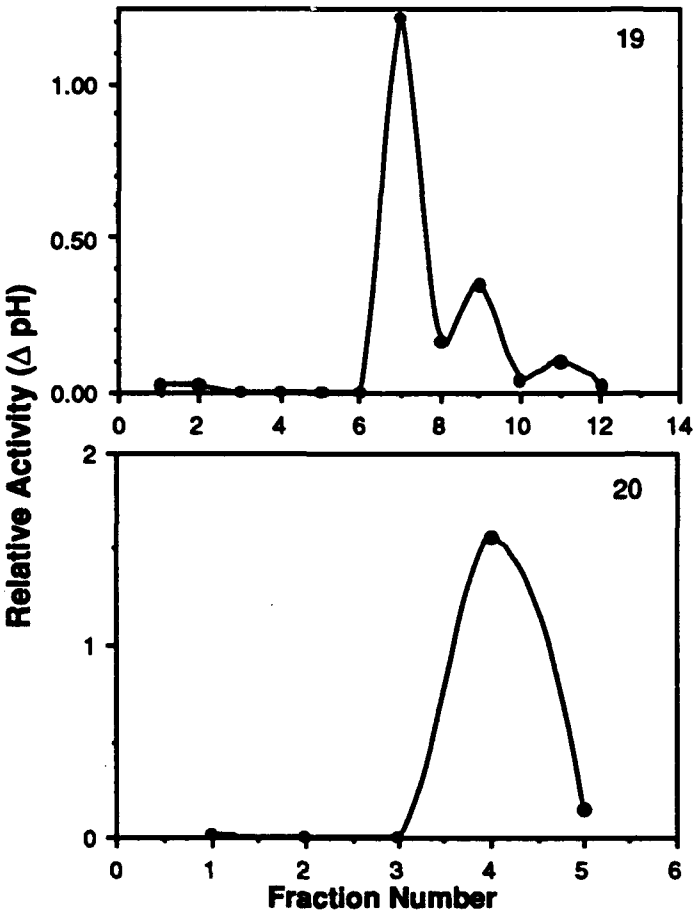


Figure 19 and 20. Purification of urease enzyme #413 (19) and #429 (20) via strong anionic chromatography using Macro-Prep 50 Q column material from Bio-Rad. Elution was achieved using a manual .1 - 2M NaCl gradient in sodium phosphate buffer (pH 7.1) for #413 (19) and a .01 - .5M NaCl gradient in 50mM HEPES buffer (pH 7) containing 1mM EDTA for #429 (20). A ..... mL sample in sodium phosphate buffer (pH 7.1) (19) and HEPES buffer (pH 7) was applied to the column. Column size = 1x5 cm; Flow rate = 0.5 mL/ min.

Anion Exchanger. Most published purification procedures incorporate an anion exchanger such as DEAE (Breitenbach, Dunn, Hu, Nakano). Similarily, a strong anion exchanger, Macro-Prep 50 Q, from Bio-Rad was observed to yield good results. Urease from isolates #288, 408, 413 and 429 was evaluated and observed to behave similarily on this exchanger. Initially, a 20 mM sodium phosphate buffer, pH 7.1, was used. Following sample application, the column was washed with buffer and buffer containing 0.1 M NaCl. Urease was eluted with 0.2 and 0.3 M NaCl in the same buffer (figure 19).

Later the buffer was changed to 50 mM HEPES, pH 7, containing 1 mM EDTA. The purification of #429 through the Macro- Prep 50 Q exchanger shows the elution of urease in 0.5 M NaCl (Figure 20). Urease could also be eluted with 0.1 M calcium chloride in buffer, but recovery was slightly less than with the higher concentrations of sodium chloride. Similar results were also observed for #408. Columns were regenerated with 10 mL of 1N HCl. The eluate was desalted and concentrated prior to application to the next column.

Protease Removal. All of the preparations were observed to contain protease. One important criterion used to judge the effectiveness of purification techniques is the separation and removal of proteases from the urease fraction. An assay incorporating Azocoll was used to monitor the presence of protease in the eluates of later column chromatography procedures. This procedure follows:

Protease Assay

Assay buffer: 0.05 M Tris base with 1 mM $CaCl_2$ bring to pH 7.8 with 1 M HCl
Azocoll: 0.25 gm Azocoll into 50 mL rapidly stirring assay buffer

Sample: 0.5 mL eluate
Blank: 0.5 mL 50 mM HEPES, pH 7 (in duplicate)
Add 1 mL Azocoll
Incubate 37°C for 12 hours
Centrifuge
Dilute supernatant to 2 mL with water
Read at 520 nm against blank

The separation of protease from urease during anionic chromatography was investigated by assaying for both enzymes in all fractions during elution. As illustrated in figure 21, protease was eluted in the same fraction as urease. The use of a phenyl boronate column previously used to remove protease from peroxidase fractions was of no use in this case.

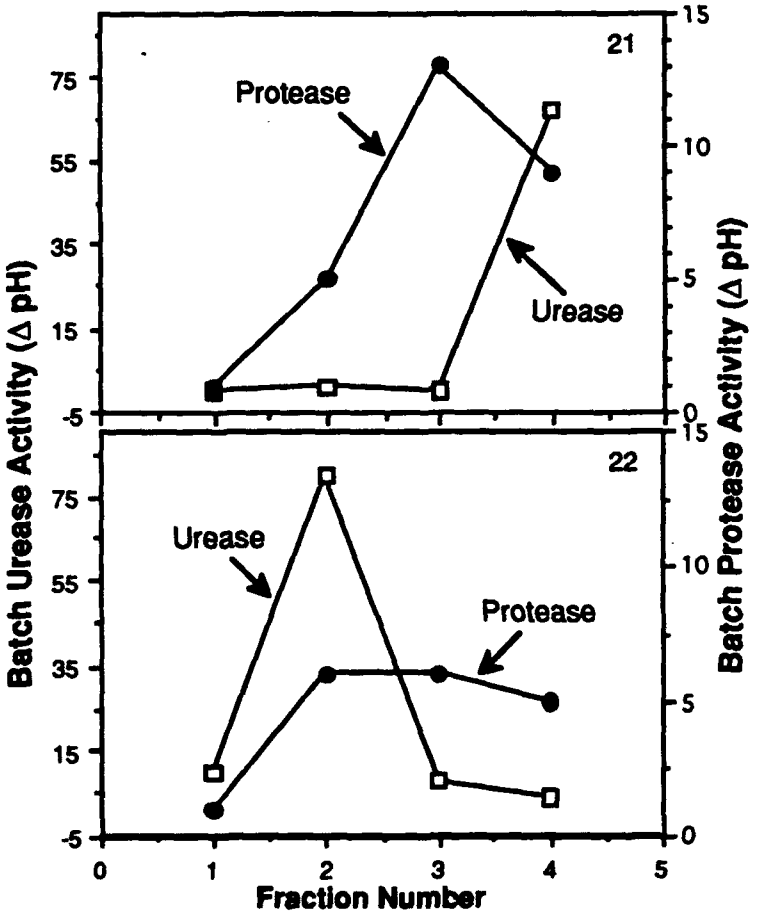


Figure 21 and 22: Separation of proteases from ureases of isolate #429 on a strong anionic exchanger (21) followed by desalting, reconcentration and affinity chromatography on a green 5 dye ligand from Sigma (22). A 1 mL sample diluted 1:1 with 50 mM HEPES (pH 7) containing 1mM EDTA was applied to the column and eluted using a manual 0.1- 0.5M NaCl gradient in the analogous buffer. The y-axis activities represent relative total enzyme units based on a 1 mL assay volume and a 3500 mL batch volume.

Dye Ligands. Other column matrices such as dye ligands were evaluated for their efficiency at removing proteases. The urease did not adhere to Amicon dyes such as red A, green, orange, blue A, and blue B using four different buffers at pH's ranging from 5.5 to 7.8. Up to 100% of the activity was recovered from all dye columns. In addition, the stability of urease from the green dye column appeared

more stable at room temperature than did analogous urease preparations from other dye ligand columns. The possibility that some inhibitor or proteolytic enzyme was being removed by the green dye ligand is suggested despite the fact that the urease did not adhere at all.

Another dye ligand from Sigma Chemical Co., denoted green 5, was also evaluated. The sample was applied to the column diluted 1:1 in 50mM citric acid, pH 7, containing 0.25 mM EDTA. Urease was removed in the same buffer wash. Contaminants were eluted with 0.1 M NaCl. The column was regenerated with 8 M urea in 0.5 M NaOH.

Although much of the protease was removed with the dye ligands, a small amount remained (figure 22). Until all protease is removed from the preparations, stability will be adversely affected. Further purification investigation is crucial and necessary for these urease preparations.

Table 11. Purification and Recovery Of Urease Enzymes #408 and 429

	Total Volume (ml)	Enzyme Activity (Δ pH) 5 min Assay	Total Units*	% recovery
Organism Isolate #408				
Whole Media	2500	0.14	350	100
Clarified	2300	0.02	46	13
Whole Cells	200	1.38	276	79
Clarified + Sonicated With Clarified Cells	30	10.60	318	91
Anion Exchange, Desalted, then Concentrated	30	4.00	120	34
Divide In Thirds:				
Partial 1: Boronate column and concentrated	30	0.40	12	3
Partial 2: Anion column and concentrated	30	0.40	12	3
Partial 3: Green 5 dye column, then concentrated	530	0.60	18	5
Organism Isolate #429				
Whole Media	7000	0.07	490	100
Filtrate + Sonicated and Clarified Cells	6500	0.07	455	93
Concentrate (Negative for Protease)	30	4.60	138	28
Waste (Positive for Protease)	6500	0.05	325	66
Anion Exchange, Desalted, Concentrated	30	3.40	102	21
Green 5 Dye Column, Concentrated	30	2.80	84	17

*Units - 1 unit is relative and defined as the number of pH units changed per ml of diluted enzyme

Assays were read at 5 mins. This was possible once the enzyme had been partially purified and concentrated. Only in the dilute fermentation medium were urease concentrations so low as to require prolonged incubations.

No attempt was made to wash the cells to recover more enzyme for these recovery studies. There was excellent recovery from the clarification and concentration steps through the Minitan. Most of the enzyme was lost during the column chromatography due to high level protease contamination of the fractions which were discarded. On a small scale this was an acceptable alternative to running another column. However, more work on improving the efficiency of protease removal is required prior to scaling for production.

Electrophoresis

Urease samples were electrophoresed on two non-denaturing 7.5% polyacrylamide (PA) gels. For protein detection, one gel was stained with Coomassie brilliant blue R250 (Gel 1) while the other was processed to allow specific detection of urease activity (Gel 2).

On gel 1, the heavily streaked lanes corresponding to samples of #429 that had been raised at 70°C were highly noticeable. Lane 18, after partial purification through the anion and green 5 columns, did exhibit some more clearly defined bands, but the heavy streaking remained. Interestingly, when raised at 60°C, #429 did not exhibit the heavy streaking as seen in lanes 8 and 12. Although the green 5, boronate or two runs through the anion exchange columns was adequate in purifying #408 preparations, huge amounts of contaminating material remained in the #429 preparations using analogous procedures.

For the urease activity gel (gel 2), the PA gel was soaked in 20 volumes of 50 mM citrate buffer, pH 6. Buffer was changed three times, at 1 hr, 4 hr and again after standing overnight. This thorough changing of the buffer was critical in aiding the removal of non-specific proteins; insufficient washing also resulted in a pH that caused problems with the substrate/staining solution. Substrate/staining solution of 1 mL 8 M urea, 20 mg Nitro Blue tetrazolium, 1 mL mercaptoethanol and 20 mL 50 mM citrate buffer, pH 6 was poured onto the gel (Creaser). The color was allowed to develop 10-15 minutes. The gels were closely monitored to prevent excessive color development that resulted in a

Gel 1: PAGE gel of urease samples at varying stages of purification stained for protein detection.

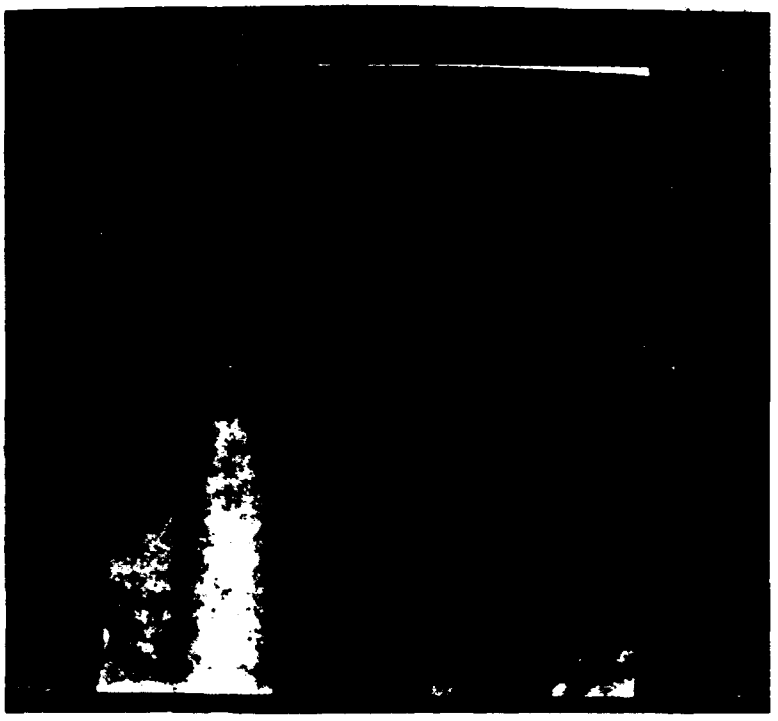
Lanes 1-4	Eluate from various dye ligand columns, #408
Lane 5	Eluate from anion exchanger, pH 8.2, #408
Lane 6	Eluate from anion exchanger (pH 7) #408 raised at 50°C
Lane 7	Eluate from anion exchanger (pH 7) #429 raised at 70°C
Lane 8	Eluate from anion exchanger (pH 7) #429 raised at 60°C
Lanes 9-10	Fermentation media, no purification
Lane 11	Unpurified #429 raised at 70°C
Lane 12	Same as lane 8
Lane 13	Same as lane 6
Lane 15	Eluate from anion and green columns, #408
Lane 16	Eluate from anion and boronate, #408
Lane 17	Eluate from twice through anion column, #408
Lane 18	Eluate from anion and green columns, #429

dark purple precipitate covering the gel.

The substrate gel with #429 shows urease with a molecular weight of 120,000-130,000 in lanes 3 and 5. Urease produced by #408 and Jack Bean urease remained near the top of the 7.5% T gel, indicating molecular weights over 200,000. The badly streaked lane of #429 raised at 70°C and eluted from the anion column, showed no urease activity. It is possible that this was an artifact caused by the heavy streaking.

The low molecular weight of the #429 urease will be of particular value for labelling. Most ureases are quite large molecules causing problems with steric hindrance. Most bacterial ureases studied to date have a molecular weight in the range of 200,000 to 380,000. One unidentified bacterium has been listed in the literature as having a Mr of 125,000 and there is one report of *Ureaplasma urealyticum* with a molecular weight of 150,000 although others have reported up to 380,000 (Mobley). Active subunits must be considered, but the substrate gel clearly shows no activity at the top of the #429 lanes; #408 and Jack Bean urease on the same gel show no activity except at the top of the lanes.

The most significant observation of both protein gels (#408 and 429) is the large number of distinct bands still present even after purification through both the anion and green 5 columns and knowing that a low level of protease activity could still be detected. For labeling purposes however, much more purification will be necessary. As non-specific or proteolytic proteins are removed, the specific activity will increase.



Gel 2: PAGE gel of urease samples at varying stages of purification stained for urease activity.

Lane 1	#408 eluate from anion column
Lane 2	#429 raised at 70°C, eluate from anion column
Lane 3	#429 raised at 60°C, eluate from anion column
Lane 4	#408 eluate from anion and green columns
Lane 5	#429 raised at 70°C, eluate from anion and green columns
Lane 6	Jack Bean urease

VI. Enzyme Characterization

Specific Activity

Once the enzyme preparations had been partially purified, samples were submitted to Michael Williamson at CRDEC. As part of his evaluation of the urease preparations, he performed all assays reported in this section on specific activity. Specific activities of enzymes, frequently reported as units of activity per mg of protein, are used as comparative indicators of enzyme preparation quality and quantity. Increases in purity are reflected by subsequent increases in specific activity. The range of specific activities are extremely varied among ureases from various sources and are highly dependant on the choice of buffer, pH, solvent components and assay temperature. Highly purified microbial ureases with specific activities of 9 to 5500 µmoles of urea/min/mg protein have been documented (Mobley). Others have reported specific activities in the range of 0.6 -15 µmolar units/mg protein (Breitenbach, Creaser, Dunn, Magana- Plaza, Nakano, Todd). In contrast, urease from Jack Beans has been reported to exhibit a specific activity of 3500 µmolar units/mg protein at 37°C and pH 7. Sigma Chemical Co. distributes a Jack Bean urease with approximately 780 µmolar units/mg protein at 25°C and pH 7. Among the bacterial sources, only *Ureaplasma urealyticum* exhibits a specific activity (Saada) comparable to Jack Bean urease (Wong).

In this investigation, a preliminary attempt to obtain specific activities was initiated using freshly reconstituted commercial Jack Bean urease from Sigma Chemical Co. (Type VII containing 6000 µmolar units per 8.5 mg solid where 1 µmolar unit is equivalent to 1 µmole of ammonia liberated per minute at 25°C). A standard curve was constructed using a serially diluted preparation and a previously determined optimal Jack Bean urease. Samples of Jack Bean urease were assayed in 5 mM phosphate buffer adjusted to a final pH of 8 containing 1 mM EDTA, 100 mM urea, 0.05% BSA and 0.001% phenolphthalein. For ureases from the thermophiles under investigation, 50 mM citrate buffer containing 1 mM EDTA, 100 mM urea and 0.001% phenol red adjusted to pH 7 was used to obtain catalytic rates at 25-70°C. The specific activities in table 12 are expressed as Jack Bean (JB) µmolar units. Although the specific activities of the semi-purified, cell-free extracts are comparable to other reported bacterial ureases, considerably enhanced activities are likely to be achieved with further investigation and refined purification strategies.

Table 12. Specific Activities For Thermostable Ureases

Isolate	*25°C*	*37°C*	*50°C*	*70°C*
197	3.44	4.93	7.90	10.63
408	8.70	16.53	25.85	39.94
429	0.30	0.478	1.22	16.73

Activities represent Jack Bean µmolar units extrapolated from a standard curve.

For optimum performance and catalytic efficiency, information on kinetic parameters such as the Michaelis Menten constant (Km) and maximum velocity (Vmax) are often required for enzymes used in analytical procedures. Kinetic estimates were obtained using a similar procedure where reaction rate was monitored as a function of substrate concentration between 0.25 mM and 333 mM in 50 mM citrate, 1 mM EDTA and 0.001% phenol red adjusted to pH 7. The suitability of the procedure was previously verified using Jack Bean urease in which Km's of 3.5-4.0 were achieved and closely approximate the 2.9 value reported by Blakeley (Blakeley, Mobley). The Km constants for thermostable ureases ranged from 5-15 mM for organisms #197 and 408 and 0.2-0.6 mM for #429, dependent on the temperature for assay.

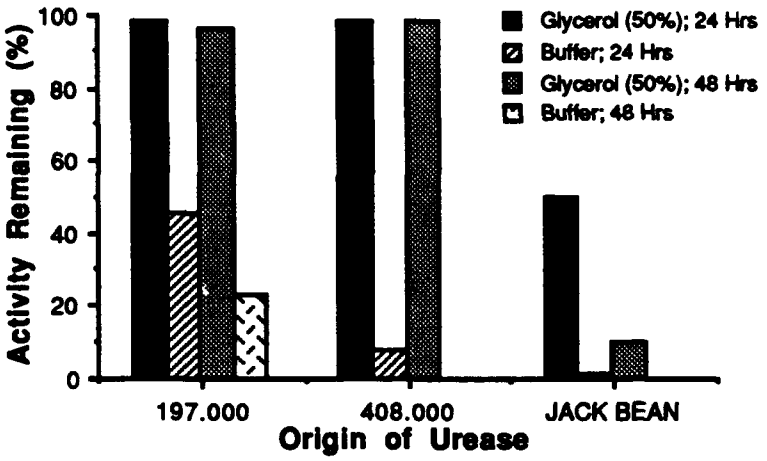


Figure 23: Stabilizing effect of 50% glycerol on crude urease preparations from isolates #197 and 408 as compared with a commercial preparation of Jack Bean urease. Stability evaluations were conducted at 50°C.

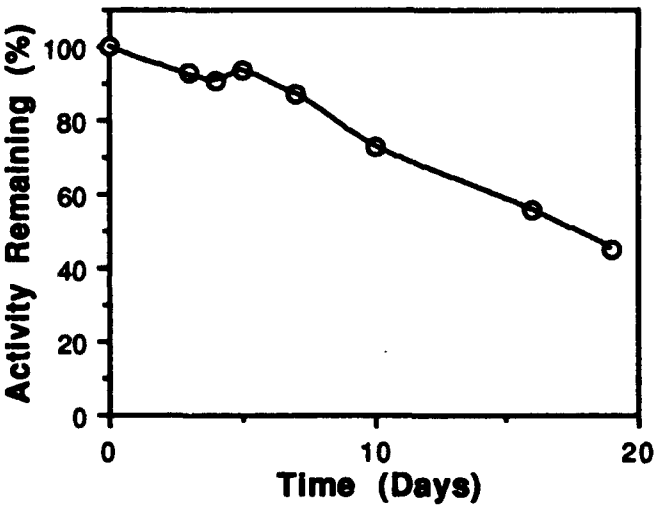


Figure 24: Stability of a crude preparation of urease from isolate #408 at room temperature (20°C). A half life of 18 days with proteases present is illustrated.

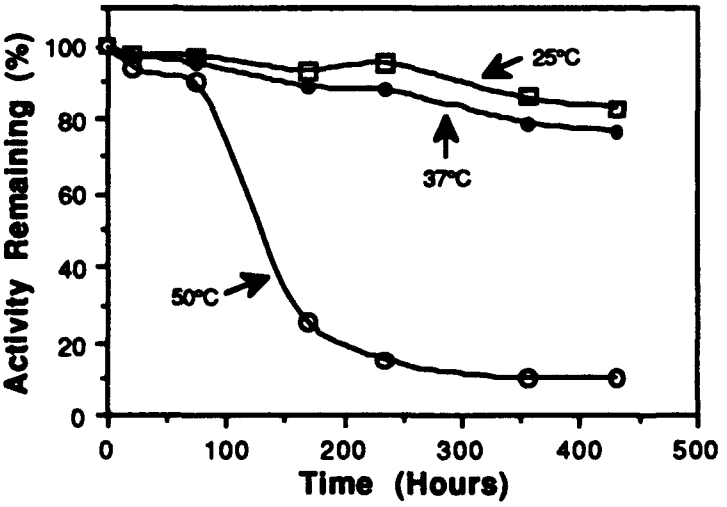


Figure 25: Stability of partially purified urease #429. A 10 mL sample was diluted with 20 mL of 50mM sodium phosphate, pH 6.5, and applied to a column (2.5 x 30 cm). The column was washed with 150 mL of the same buffer followed by 150 mL of 0.08M NaCl in buffer. The urease was eluted with 150 mL of 0.2M NaCl in buffer.

The increase in Km at elevated temperatures is consistent with that observed by Magana- Plaza who correlated the increase with an inhibition by ammonia. The inhibition was further observed to coincide with a change from Michaelis Menten kinetics to a sigmoidal type profile. This effect was particularly evident with isolate #429 and was greater as the substrate concentration was progressively increased above 10 mM. At substrate concentrations less the 10 mM, the kinetic profile exhibited a linear Michaelis Menten mechanism. For bacterial ureases, Km values are generally reported in the range of 10-50 mM and seldom as low as 0.5 or as high as 100 mM. Considering the extreme thermophilicity of isolate #429 (activity optimum above 60°C) and the limited data on other thermostable ureases, uncommonly low reported Km's may not be so unusual.

Enzyme Stability

A number of experiments looked at the effects of stabilizers used by others for urease, among them EDTA, nickel, glycerol and diethanolamine. Only the glycerol plus EDTA combination was of significant benefit (Table 13). The data are listed as the % of activity remaining after nearly 9 days at either of the two incubation temperatures. Extracts still contained some protease activity at this time.

Table 13. Stability Of Crude Urease
(Sonicated Organism from #197U)

	% Activity		
	*Buffer **	*KCl **	*Glycerol**
0 hours	100.0	100.0	100.0
24 hours			
4°C	89.4	89.3	94.9
20°C	80.9	88.4	94.9
50°C	30.3	81.3	77.8
60°C	7.9	45.5	50.5
48 hours			
4°C	105.6	104.5	114.1
20°C	92.1	105.4	118.2
50°C	11.2	74.1	80.8
60°C	0.0	23.2	30.3

*A 0.05M HEPES buffer containing 1mM EDTA was used as a control and compared with 1M KCl and 50% glycerol prepared in the same buffer.

(Comparison of #408, 429 and Jack Bean in 50% Glycerol Containing 1mM EDTA)

	37°C	50°C
209 Hours		
#408	103	90
#429	98	94
Jack bean	46	7

VII. Enzyme Application

Immobilization

The stability of enzymes is well established to be enhanced by immobilization. Immobilization of urease from #197 on polysaccharide spheres was accomplished following desalting after elution from the anion exchanger. The soluble enzyme was mixed with fresh anion exchange medium, allowed to stand at room temperature for two hours, washed and dried overnight at room temperature. Following incubation for 64 hours at 50°C, 69% of the activity at time zero, remained. Although not as good as when incubated in glycerol, it did demonstrate the value of immobilization. The treatment with 2.5% glutaraldehyde, however, resulted in a loss of activity as did incubating the exchanger wet, rather than dried. Neither urease from #197 or 408 could be attached to aminopropyl glass beads, although #408 was successfully immobilized on arylamine glass beads. Arylamine glass beads and a partially purified extract of urease #408 were mixed and allowed to stand 24 hours at room temperature. The beads were washed twice in buffer and dried, again at room temperature. Following incubation for 48 hrs at 50°C, 100% of the urease activity was retained relative to the activity at time zero.

Antibody Conjugation. Urease from #429 that had been partially purified through the anion exchanger was used in the following experiment by Dr. Don Burgess of BioSciences. It must be rernembered that some protease would still be present after this partial purification scheme. One mL urease extract (protein approx. 5.12 mg/ mL) was conjugated to 116 µL goat anti-*T. foetus* antibody (approx. 43.8 mg/ mL) (Nakane). After conjugation, unconjugated antibody and urease were separated from the IgG-Urease conjugate by gel filtration chromatography on Sepacryl 300 HR. The void volume containing the IgG-Urease conjugate was collected.

Bromocresol purple/urea was used in an enzyme assay performed on the IgG-Urease conjugate revealing approximately 25% of the total activity in purified IgG-Urease conjugate compared to the unpurified conjugate mixture.

Table 14. Validation Analysis of IgG-Urease Conjugation Reaction

Fraction	Volume *(µl)*	Relative Enzyme Activity *(Absorbance @ 590nm)*
Column Purified IgG-Urease	10	0.240
Column Purified IgG-Urease	20	0.508
Unpurified IgG-Urease	10	1.022

Enzyme Linked Immunosorbent Assay (ELISA)

A simplified, simulated ELISA was performed to illustrate practical utility. Insoluble bacteria, known not to possess urease activity, were incubated with an anti-bacteria-urease conjugate followed by washing and incubation in an enzyme substrate solution to elicit a colorimetric signal. This analysis, although crude and preliminary, verified the existence of urease activity linked to the specific antibody. A more refined experiment using fixed *T. foetus* organisms were incubated in 10 mM citrate buffer, pH 7, as a control or IgG-Urease in the same citrate buffer for 15 min. Organisms were washed in citrate buffer twice and resuspended in bromocresol purple/urea substrate solution and incubated at 40°C for 15 min (table 15).

Table 15. T. foetus - Anti T. foetus Model Immunoassay

Reaction	*Relative Urease Activity (Absorbances @590 nm)*
T. foetus + Buffer Control	0.501
IgG-Urease + T. foetus	0.773

VIII. Organism Identification

Fatty Acid Analyses

Fatty acid analyses were performed on 7 isolates. Values were compared to determine which organisms might be duplicates and which were distinctly different organisms. Isolates #288 and 301 appear to be identical, as do #211 and 413. There is some degree of relatedness among all 6 organisms raised at 50°C. The two isolates submitted to CRDEC, #408 and 429 are distinctly different bacteria.

Table 16. Fatty Acid Profiles

*Fatty Acid**	JKR Media at 50°C						TSB at 60°C
	#197U 11/4/90	*#211 10/31/90*	*#288 10/31/90*	*#301 10/31/90*	*#408 U 10/31/90*	*#413 10/31/90*	*#429 5/23/91*
SOLVENT PEAK							
12:0				0.25			
14:0 Iso	3.76	1.09	1.48	1.24	2.58	1.34	0.55
14:0	0.68		0.19	0.27			1.19
15:0 Iso	31.48	39.91	42.5	41.86	28.12	39.54	50.80
15:0 Anteiso	23.58	15.32	12.05	12.55	16.69	14.05	3.74
15:0	0.57						0.69
16:0 Iso	13.71	10.22	12.2	10.53	19.36	12.13	3.27
¥ 16:1 Trans9 15i 20H							0.84
16:0	3.66	2.76	2.41	2.32	3.06	2.61	8.44
¥ 17:1 Iso I Anteiso B					0.51		
17:1 Iso H							0.55
17:0 Iso	13.67	21.08	21.54	22.30	17.76	21.15	22.20
17:0 Anteiso	8.35	9.62	6.82	7.80	9.80	8.66	7.74
18:0 Iso			0.34	0.30	1.01		
18:0	0.54		0.26	0.27	0.67	0.51	
19:0 Iso			0.23	0.31	0.44		

* Conventional Fatty Acid Nomenclature denoting carbon chain length and arranged by retention times within a Gas Chromatograph - Microbial Lipids, Vol 1 (1988) Ratledge, C. and Wilkinson, S.J., Eds.

¥ Two combined (double) peaks were observed. Substituted OH fatty acids are eluted later than non-OH substituted.

16S rRNA Sequences

As discussed earlier, several attempts were made to identify the microorganisms submitted to CRDEC. Since these bacteria were isolated from extreme environments of thermal waters and the original collection focused on the maximum diversity of microhabitats rather than the few pools that have been extensively sampled by others, it is not surprising that none of the organisms, including #408 and 429, had not been previously identified and details added to any of the data bases searched.

Isolate #408 was selected for 16S rRNA sequencing and and the resultant sequence aligned with *E. coli*. This sequence is a permanent, phylogenetically consistent and unambiguous descriptor of the specific strain submitted to CRDEC. As new sequences on thermophiles becomes available, the sequence for #408 will become more valuable for classification and identification.

Samples were submitted to PhyloGen where sequencing and data analysis was performed by Dr. Daniel Distel, of PhyloGen and Harvard University. Since there was no match with any other organism in the data bank, a similarity analysis was performed, comparing #408 with the currently available sequences in the Woese/Olsen collection, Argonne National Laboratory. Similarity was computed as the fraction of nucleotide positions which are identical in a given pair of aligned sequences. Data was weighted by applying a mask to the sequence, instructing the computer only to compare sequence positions which have been determined unambiguously in all sequences being compared and which can be aligned with homologous nucleotide positions in all sequences being compared. This was necessary since 16S rRNAs vary considerably in length. One can only compare positions that occur in all sequences. Isolate #408 shared the highest similarity values with gram positive bacteria. The highest similarity value was 98.2% with *Bacillus subtilis*.

Unfortunately there is no consensus on the level of 16S rRNA sequence similarity that constitutes strain, species or generic differences. This is largely due to the fact that well studied bacterial groups have been frequently split while in general less well known bacterial groups have been lumped. The result is that the currently used binomial classification system is not phylogenetically consistent. Examples may be found where two organisms with a 98.9% similarity value have been classified in two different genera. Yet two other organisms with lower similarity values may be found in the same genus.

The conclusion by Dr. Distel was that #408 was a thermophilic *Bacillus* closely allied with *B. subtilis*.

Molecular taxonomic studies of the genus *Bacillus* have shown that it is a heterogeneous group of organisms that can hardly be considered closely related. All members of this genus form spores. This characteristic is so distinctive that taxonomists have been reluctant to create separate genera (Brock). Consequently this is a large group of rather diverse organisms.

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Glucose Oxidase

I. Introduction

Commercially available glucose oxidase is almost entirely isolated from the fungus *Aspergillus niger*. With rare exceptions, the literature has reported the acquisition of the enzyme from fungi. No reports have been located on the production of glucose oxidase by thermophilic microorganisms. In an earlier investigation, a number of microorganisms producing glucose oxidase were isolated from the thermal waters of Yellowstone National Park, but at extremely low levels. Furthermore, most of the isolates originated from lower temperature locations. As temperature increases, oxygen solubility in a liquid decreases. Consequently, many thermophiles are anaerobic; even those that are aerobic are adapted to lower oxygen levels than would be common for mesophiles. Furthermore, glucose has not been observed at high levels in the thermal waters of Yellowstone. Even if it were, at higher temperatures and particularly at pH extremes, the sugar would tend to caramelize and degrade. Therefore, thermophiles producing an enzyme requiring oxygen and acting on glucose would not be common in this location. Despite a diligent search, exceptions to this generalization were not observed.

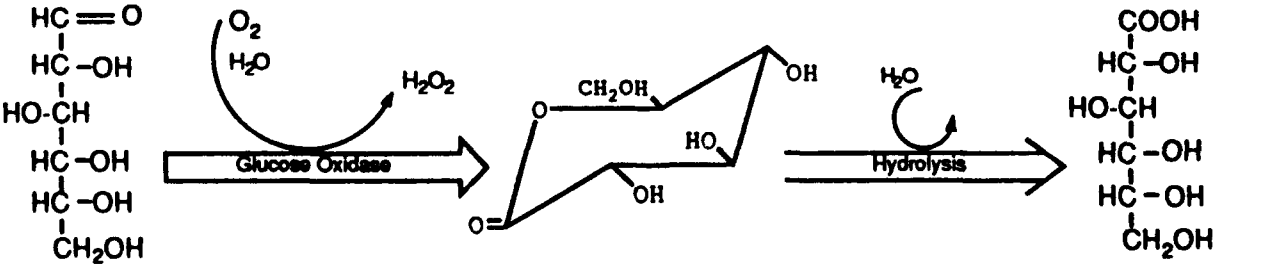
Therefore, between the earlier and present investigations, alternative high glucose habitats at elevated temperatures were sampled. Included were a honey processing plant, a sorghum processing plant, a raw sugar importer who had already identified thermophiles in sugar samples, a sugar beet processing plant and a fruit flavored syrup packager. Microorganisms isolated from these locations formed the bulk of the investigation on glucose oxidase, although 15 isolates from earlier studies were also included.

Seventeen isolates were recovered that produced glucose oxidase. There did not appear to be a great diversity of organisms. As habitats become more extreme there is a tendency for the number of different microorganisms to decrease. Although there is some evidence that novel isolation techniques might help refute this generalized statement, this does tend to be true with use of currently known techniques.

Four isolates were selected for the majority of this investigation. Isolates # 305, 370, 246 and 408G were collected and isolated from the fruit flavored syrup packager, sorghum processing plant and two locations in Yellowstone, respectively. The fatty acid analyses indicated that all 4 organisms were closely related. This was later reinforced for the two organisms selected for carbon utilization tests (#305 and 370), where use of any given carbon source was not matched with use of that same source by the second organism in only 18 times out of the 95 tests.

II. Enzyme Assays

Glucose oxidase, a flavoprotein, converts glucose to a lactone and offers several approaches to measuring the enzyme activity: (1) oxygen consumption, (2) hydrogen peroxide production and (3) gluconic acid production.



α-D-Glucose D-Glucono-δ-Lactone α-Gluconic Acid

The lactone spontaneously hydrolyzes to gluconic acid

Oxygen Consumption Many of the earliest procedures for measurement of glucose oxidase activity used a Warburg manometer to measure the oxygen uptake (Pazur). Methods for glucose concentration were also determined enzymatically by a procedure in which the amount of oxygen depleted from a solution in the presence of glucose oxidase was observed. The difference between the initial and final oxygen levels, as detected by a polarographic oxygen analyzer, was taken as a measure of the glucose concentration (Kadish). For much of this investigation, this method was adapted for measuring glucose oxidase activity, rather than glucose levels. In this case, a known amount of glucose was added to a buffered solution of the glucose oxidase preparation. The decrease in oxygen concentration compared with a reagent blank was taken as a measure of glucose oxidase activity (Doppner, Tsuge).

Activity Measurement - *Polarographic oxygen probe*

1 mL dilute glucose oxidase
2 mL 0.2 M sodium phosphate buffer, pH 6
1 mL glucose (100 gm/ L)
Incubate 1 hr at 20°C.
Measure oxygen concentration and compare with oxygen concentration of reagent blank.
A lower oxygen level corresponds with a higher level of glucose oxidase activity.

The oxidation of α-D-glucose is 150 times slower than β-D-glucose. The β-D-glucose is far more expensive, so solutions were made with α-D-glucose at least one day before it was needed to insure mutarotation. After standing for a period of time, glucose solutions end up as 36% α-D-glucose and 64% β-D-glucose.

Hydrogen Peroxidase Most colorimetric methods rely on a coupled enzyme system with the ultimate oxidation of a substrate such as o-dianisidine or ABTS to a colored product (Bergmeyer, Kelley, Schephartz, Ye). A known excess amount of peroxidase is added, so the color development will be proportional to the amount of hydrogen peroxide produced.

In the initial investigation, the following assay was consistently used. As will be discussed later, there are numerous variables that may or may not interfere with assay results. Although this assay was almost always used successfully, on occasion and for unknown reasons, no glucose oxidase was detected, controls were positive or results were difficult to interpret. Therefore, this method was abandoned in the later part of the investigation for the oxygen depletion method, which is a more direct quantitation of glucose oxidase activity.

Glucose oxidase - *ABTS Method*

1.0 mL dilute glucose oxidase
3.0 mL 0.2 M Na phosphate buffer, pH 6
0.2 mL glucose (10 g/ L)
0.2 mL 20 mM ABTS (0.22 gm/ 20 mL water)
1.0 mL peroxidase (10 mg/ 20 mL water)
Incubate 30 min at 20°C
Read at 420 nm against reagent blank

A similar assay using o-dianisidine was evaluated with no significant difference in results from those obtained with the ABTS assay. A reducible quinone such as benzoquinone has been used, reading the reduced form in the UV region (Ciucu). Glucose oxidase produced by isolate #370 and peroxidase from

isolate #197 were used successfully in the ABTS coupled enzyme assay, following partial purification of both enzymes.

One report exist on the use of a platinum electrode to measure the amount of hydrogen peroxide produced. However, the association of hydrogen peroxide generation with flavoprotein oxidation must be remembered and so the source of hydrogen peroxide must be taken into account. Accumulated hydrogen peroxide will inhibit glucose oxidase activity, particularly the reduced form of the enzyme. The effect is more noticeable with the immobilized enzyme (Greenfield).

Gluconic Acid Production Methods have been published on the measurement of gluconic acid production by a titrimetric assay (Finnsugar).

For some screening assays, organisms were streaked on media containing high concentrations of glucose and the subsequent resulting changes in pH were monitored using micro pH probes. Although this technique was designed to monitor the production of gluconic acid, other acidic metabolic products would also cause a similar decrease in pH. Interestingly, one organism produced a pH reduction of approximately 3 pH units and 5 organisms reduced the pH by 1 to 2 pH units. Those organisms producing marked pH reductions, later tested positive for glucose oxidase activity by the colorimetric method.

A method for screening large numbers of organisms for glucose oxidase production included methyl red in the media with the appearance of a red zone around a colony taken as presumptive evidence of gluconic acid production (Markwell). This approach was considered, but since media had several different initial pH's to correspond with the habitat pH, direct measurement of the pH change was selected for ease of experimentation and to eliminate the necessity for preparing several media.

Interferences Citrate will cause autooxidation of glucose (Schepartz). Addition of sulfhydryl reagents such as mercaptoethanol will interfere with the glucose oxidase assay (Kilburn). Many procedures call for saturating the assay solution with oxygen prior to analysis, while others use sealed containers or open tubes. One report claims sodium is required for the reaction (Schepartz). Chloride ion has been observed to be a competitive inhibitor of glucose oxidase from *Aspergillus niger* (Rogers).

Hydrogen peroxide can inhibit glucose oxidase activity. Catalase is often added to the assay mixture to catalyze the presence of excess hydrogen peroxide. However, excessive catalase will break down all of the hydrogen peroxide as it's formed and the color development in assays using ABTS, o- dianisidine or aminoantipyrine will be prevented. This dilemma has produced numerous approaches to modifying the basic glucose oxidase assay. As compared with 4- aminoantipyrine, one group observed inhibition of catalase activity by o-dianisidine enough so that catalase inhibition was not a problem (vanPee). However, the potential carcinogenicity of o-dianisidine made others uneasy with this substrate (Bergmeyer). Kunz suggested the use of sodium azide to suppress catalase activity (Kunz) while others added potassium cyanide to inhibit the catalase (Kelley, Kleppe). Apparently the reaction with cyanide varies with the source of the glucose oxidase, for Schepartz observed cyanide to be strongly inhibitory (Schepartz). Glucose oxidase from #246, 305, 370 and 408G were strongly inhibited by sodium cyanide, however, the presence or absence of sodium azide had little effect on oxygen depletion.

Optimum pH As for the colorimetric assays, the optimum pH will partially depend on the reagents used. Since the oxygen depletion method obviated this problem, pH curves for glucose oxidase were determined using the oxygen probe (figure 1). The pH optimum for #370 was observed to be in the range 6.0 and 6.5 when assaying was performed in citric acid, sodium phosphate or Tris buffers. The activity in HEPES buffer was quite different and exhibited a very broad optimum from pH 5.5 to 8.5. Activity above pH 8.5 was not assayed using HEPES since the buffering capacity is very poor above that pH. The very sharp decrease in activity between pH 5.0 and 5.5 was also noteworthy and activity was particularly low at any pH in Tris buffer. Since sodium phosphate buffer was used for the colorimetric method, and maximum activity approximated that in citric acid buffer, sodium phosphate buffer was selected for most of the subsequent assays.

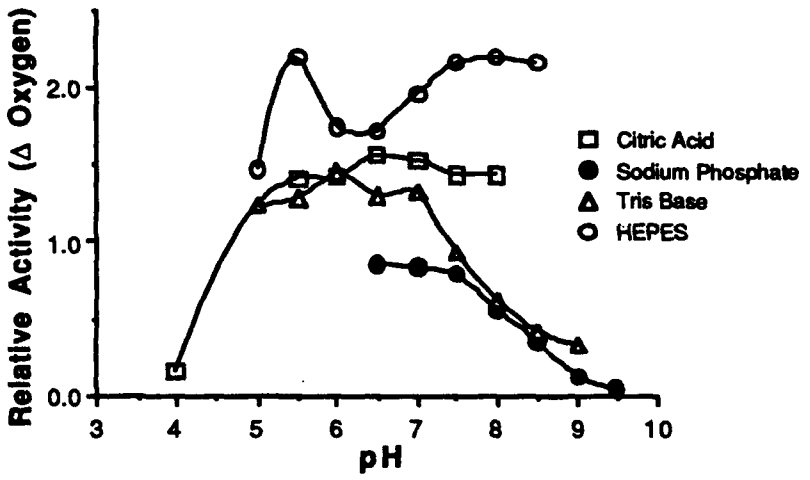


Figure 1: pH profiles for isolate #370 in 200 mM buffers containing 1 mM EDTA. Oxygen depletion was monitored using a polarographic oxygen probe. n=3.

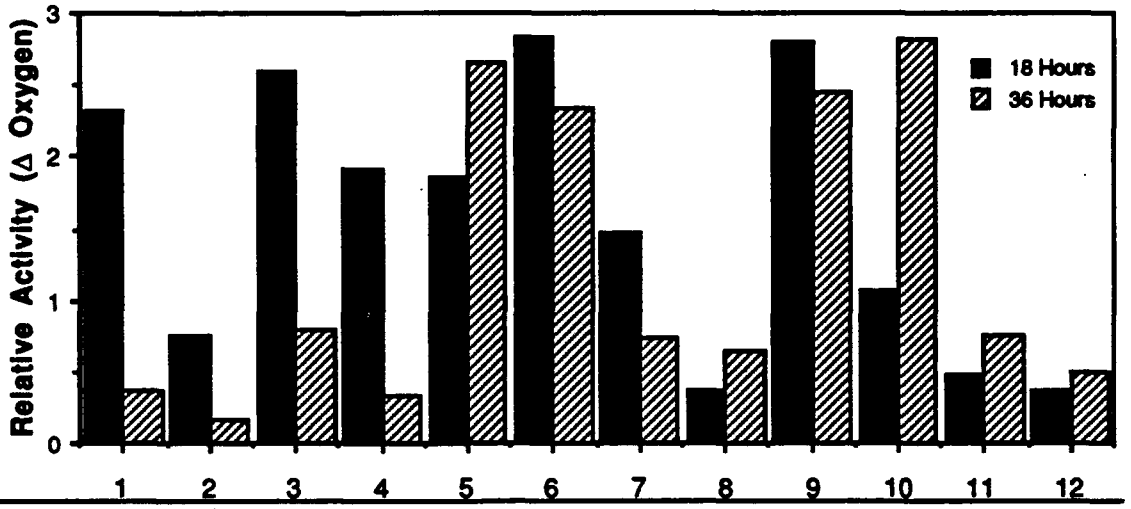
Optimum Temperature Measurement of glucose oxidase activity using the polarographic oxygen probe at different temperatures, raises problems. Since oxygen becomes less soluble in solution as the temperature is increased, a significant drop in dissolved oxygen will be observed, even in the absence of any enzyme. For this reason, blanks must be run with the assay to enable correction. In experiments to determine the optimum temperature, test samples appeared to reflect an increase in glucose oxidase activity as the temperature was raised from 25 to 37°C. However, a substantial increase in the blank reading caused the corrected test value to actually change very little. The effect was even more dramatic at 50°C, where the corrected test value was actually below that at 37°C. A second problem was that the enzyme was never completely free of proteases which further decreased glucose oxidase activity as the temperature increased and the proteases became stimulated. Further investigation is required to characterize the optimum temperature for glucose oxidase. All proteases must be removed and an alternative assay used to monitor the glucose oxidase activity.

III. Media Optimization

Glucose oxidase was one of the first enzymes to be used commercially. Consequently, there is a substantial body of literature on the optimization of media for enzyme production. Unfortunately most of these procedures were performed on fungi, and transferring this information to thermophilic bacteria didn't always work. However, it did serve as a starting point. Rather than starting with one medium and making additions and subtractions, as was performed for urease and peroxidase, several media were evaluated and changes made to each. Even after one isolate was selected, several basic formulas were still being evaluated. Figure 2 and table 1 illustrates the results for 12 media evaluated for the growth of isolate #370.

Table 1. Media Compositions Evaluated

Figure 2. Growth of isolate #370 in mini-fermentors containing 12 different media and a 1 mL air/ mL medium/ min aeration. Glucose oxidase was monitored at 18, 36 and 48 hours (data for 48 hours not shown).



Molasses						20						
Malt Extract	1								3			
Yeast Extract	7		0.2	0.2	0.2	0.2	0.2	0.05	3	3		
Peptone		0.1	0.1	0.1	0.1	0.1	0.1		5	3		
Dextrose	10	25	1	25	5			18	50	40	50	
Sucrose							5					50
Glycerol		5						20				
Citric acid		0.1	0.1	0.1	0.1	0.1	0.1	0.1			1	
$(NH_4)_2SO_4$		0.1	0.1	0.1	0.1	0.1	0.1	0.4				
$NaNO_3$		1	1	1	1	1	1	2			6	
$NaCO_3$										10		
$MgSO_4 \cdot 7H_2O$	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.25			0.25	1.1
$MnSO_4 \cdot H_2O$	0.02											
$K_2HPO_4 \cdot 3H_2O$	0.3	1	1	1	2	2	2	1				
KH_2PO_4											0.25	0.15
$FeSO_4 \cdot 7H_2O$	0.001							0.005				
$FeCl_3 \cdot 6H_2O$											0.01	
Na_2HPO_4	0.3											
$CaSO_4 \cdot 2H_2O$										3		
$CaCl_2 \cdot 2H_2O$											2	0.55
KCl								0.5			0.25	
NaCl												0.15
$ZnSO_4 \cdot 7H_2O$												0.0015
Trace Elements		1	1	1	1	1	1	0.5				

* Values are reported as g/ L.

Optimized Medium

Medium #9 was closest to that eventually optimized. The final formulation used the same ingredients, with some components at slightly lower concentrations. In general, activities at 36 and 48 hours decreased. At 36 hrs, the two exceptions were Media #5 and 10. Medium #5 was identical to #4 except that it was better buffered and had a lower initial dextrose concentration.

Optimized Media for Growth of Isolate #370

Component	*g/L*
Malt Extract	2.0
Yeast Extract	3.0
Peptone	3.0
Dextrose	40.0

Calcium Medium #10 includes an insoluble form of calcium. The only formulas which used soluble calcium chloride were Media #11 and 12 which proved to be very poor producing substrates for glucose oxidase. Rogalski et al observed that calcium carbonate increased enzyme production by A. niger (Rogalski) although Pitt et al. observed that soluble forms of calcium inhibited enzyme production in another fungus (Pitt). Numerous analysis were perfoprmed on the effects of calcium. Although calcium in a soluble form was observed to be generally inhibitory for glucose oxidase production by our thermophiles, much as had been also observed for some fungi, the inclusion of calcium in microbiological media is not uncommon. In fungi as expected due to the synthesis of the calcium chelator, dipicolinic acid, the presence of calcium increased sporulation, however, the same correlation could not be made for our thermophiles.

Carbon Since the carbon source and its concentration would be expected to have a significant impact on glucose oxidase production, a major emphasis was directed to carbon. The evaluation of 12 media in figure 2 illustrates some effects of carbon source on glucose oxidase production by #370, that proved to be generally true for all isolates analysed. Media #6 and 9 gave similar results; the difference in the two formulas was that one contained molasses at approximately twice the carbon rate as the other which contained dextrose. Molasses had given problems earlier with urease in the column chromatography so its use was not pursued.

Media #2 and 8 gave very poor results. A common ingredient of both was glycerol. Glycerol is included in several formulas for fungal glucose oxidase production. However, as confirmed by carbon utilization analysis, #370 made little use of glycerol and #305 did not use it at all. Both Media #2 and 8 also included ample dextrose, so in this case, the glycerol may be exerting an inhibitory effect, in addition to not being used by the thermophiles.

Sucrose was also evaluated as a carbon source. Sucrose has often been substituted for dextrose in fungal media for glucose oxidase production. But, as again later confirmed by the carbon utilization tests, #305 and 370 made fairly poor use of sucrose at 50°C although better use at 23°C can be observed (table 2). Isolate #370 did produce glucose oxidase on media containing sucrose, but at a much lower rate than was observed in the presence of dextrose.

Isolates #305 and 370 were submitted to Microbe Inotech Laboratories, Inc. (MIL) in St Louis, MO for characterization of carbon utilization. The assays, performed in commercially prepared 96 well microplates containing various organic compounds, can be particularly beneficial in biodegradation and selective media development. Unfortunately, the assays were not initiated until near the end of the

Table 2. Carbon Utilization Pattern Recognition Data (MIL, Inc.)

Glucose Oxidase Producing Isolates # 370 and 305

	#370			#305		
Carbon Source	*4*	*24*	*72*	*4*	*24*	*72*
α-cyclodextrin	N	47	78	N	N	90
dextrin	38	57	117	59	N	186
glycogen	N	54	121	42	N	141
tween 40	N	N	194	N	N	72
tween 80	N	N	N	N	N	N
N-acetyl-D-galactosamine	N	N	N	N	N	N
N-acetyl-D-glucosamine	N	N	50	N	N	50
adonitol	N	N	N	N	N	N
L-arabinose	N	N	59	N	N	N
D-arabitol	N	N	N	N	N	N
cellobiose	N	N	65	44	N	91
i-erythritol	N	N	N	N	N	N
D-fructose	N	54	91	N	N	84
L-fucose	N	N	N	N	N	N
D-galactose	N	N	N	N	N	N
gentiobiose	N	N	47	N	N	57
α-D-glucose	24	70	116	60	N	183
m-inositol	N	N	N	10	N	N
α-lactose	N	46	80	39	N	80
lactulose	N	N	73	39	N	104
maltose	32	54	116	68	N	131
D-mannitol	N	N	N	N	N	N
D-mannose	N	41	91	57	N	121
D-melibiose	N	N	N	N	N	N
β-methyl-glucoside	N	N	49	39	N	94
psicose	N	59	90	45	N	116
D-raffinose	N	N	N	N	N	N
L-rhamnose	N	N	N	N	N	N
D-sorbitol	N	N	49	N	N	52
Sucrose	N	52	72	52	N	132
D-trehalose	31	54	99	53	N	126
turanose	35	54	101	44	N	118
xylitol	N	N	N	N	N	N
methyl pyruvate	N	N	50	N	N	98
mono-methyl-succinate	N	N	N	N	N	N
acetic acid	N	N	N	N	N	N
cis-aconitic acid	N	N	N	N	N	N
citric acid	N	N	N	N	N	N
formic acid	N	N	N	N	N	N
D-galacturonic acid	N	N	N	N	N	N
D-galacturonic acid	N	N	42	N	N	N
D-gluconic acid	N	N	N	N	N	N
D-glucosaminic acid	N	N	N	N	N	N
D-glucuronic acid	N	N	N	N	N	N
α-hydroxy-butyric acid	N	N	N	N	N	N
β-hydroxy-butyric acid	N	N	N	N	N	N
γ-hydroxy-butyric acid	N	N	N	N	N	N
P-hydroxy-phenylacetic acid	N	N	N	N	N	N

* Values are percentage color change on microtiter wells (MIL) over the control and represent a relative proportional quantity of utilization. N = No Reaction.

Carbon Utilization Pattern Recognition Data (MIL, Inc.)

Glucose Oxidase Producing Isolates # 370 and 305

	#370			#305		
Carbon Source	*4*	*24*	*72*	*4*	*24*	*72*
Itaconic acid	N	N	N	N	N	N
α-keto butyric acid	N	41	52	N	N	62
α-keto glutaric acid	N	N	N	N	N	N
α-keto valeric acid	N	N	34	N	N	N
D,L-lactic acid	N	N	N	N	N	N
malonic acid	N	N	N	N	N	N
propionic acid	N	N	N	N	N	N
quinic acid	N	N	N	N	N	N
D-saccharic acid	N	N	N	N	N	N
sebacic acid	N	N	N	N	N	N
succinic acid	N	N	N	N	N	N
bromo succinic acid	N	N	N	N	N	N
succinamic acid	N	N	N	N	33	N
glucuronamide	N	N	N	N	N	N
alaninamide	N	N	N	N	47	N
D-alanine	N	N	38	N	53	N
L-alanine	N	N	N	N	51	N
L-alanyl-glycine	N	N	N	N	N	N
L-asparagine	N	N	N	N	N	N
L-aspartic acid	N	N	N	N	N	N
L-glutamic acid	N	N	N	N	52	N
glycyl-L-aspartic acid	N	N	N	N	30	N
glycyl-L-glutamic acid	N	N	36	N	N	N
L-histidine	N	N	N	N	48	N
hydroxy L-proline	N	N	N	N	N	N
L-leucine	N	N	N	27	50	N
L-ornithine	N	N	N	N	35	N
L-phenylalanine	N	N	N	N	29	N
L-proline	N	N	N	N	N	N
L-pyroglutamic	N	N	N	N	77	N
D-serine	N	N	N	N	N	N
L-serine	N	N	N	N	N	N
L-threonine	N	N	N	N	N	N
D,L-carnitine	N	N	N	N	23	N
γ-aminobutyric acid	N	N	N	N	N	N
urocanic acid	N	N	N	N	N	N
inosine	N	N	N	N	N	N
uridine	N	N	41	N	N	53
thymidine	N	N	53	N	N	59
phenylethylamine	N	N	N	N	N	N
putrescine	N	N	N	N	N	N
2-amino ethanol	N	N	N	N	N	N
2,3-butanediol	N	N	35	39	N	52
glycerol	N	N	N	N	41	N
D,L-α-glycerol phosphate	N	N	N	N	69	N
glucose-1-phosphate	N	N	38	N	N	N
glucose-6-phosphate	N	N	75	N	N	55

* Values are percentage color change on microtiter wells (MIL) over the control and represent a relative proportional quantity of utilization. N = No Reaction.

project. Based on the results of this relatively inexpensive analysis, we would suggest acquiring this information in the earliest stages of a similar project.

In the analysis, the organisms are streaked onto nutrient medium supporting vigorous growth and incubated at 50°C. Colonies are lifted from the culture plates using a saline moistened cotton swab and a suspension of uniform turbidity is prepared in 0.85% saline. The microplate wells containing the reductive indicator, tetrazolium, are inoculated with the bacterial suspension at a rate of 150 µL per well. The plate is covered with the microplate lid and incubated at the standard assay intervals of 50°C for 4 and 24 hours. As a result of little response, the plates were further incubated at 23°C for a total of 72 hours prior to a third reading being taken. The degree of tetrazolium reduction producing various shades of purple was monitored at 590 nm. The color of each well was referenced against the negative control so that any purple color recorded above the control level was considered as a positive utilization of the given carbon source. The data is reported as the percent color change as compared to the control (table 2). Both #305 and 370 utilized many of the same carbon sources. Both readily fermented dextrin, glycogen, tween 40, and many of the more common sugars including glucose, fructose, arabinose, maltose, sucrose and mannose. In total, #305 utilized 31 different carbon sources while #370 was able to use 39.

Of all the media formulas in the literature only two were for bacteria, and these were for anaerobes. No analysis on thermophiles for glucose oxidase production was located. One medium used for bacteria included Tween. Although tween was not evaluated, after reviewing the carbon utilization tests and observing that both #305 and 370 utilized Tween 40, it appears that this carbon source should have been included.

Initiating fermentation with lower concentrations of dextrose followed by subsequent additions of more dextrose was attempted several times, but never increased glucose oxidase production.

Inducers And Inhibitors Figure 2 reinforces the generally lower glucose oxidase production on inclusion of defined salts media with or without low levels of complex components such as yeast extract or peptone, as opposed to media prepared solely of the more complex ingredients. Medium #5 seemed to be the best compromise between these two types of media.

Iron was included in all media, either as an added salt or as part of a complex component similar to yeast extract. The literature indicates that this element is required for glucose oxidase production.

Most experimentation with other trace metals has revolved around secretion of glucose oxidase into the media (Pitt). For fungi, this is particularly important, in that many fungi are difficult to break up sufficiently to release the enzyme.

Another trace element that appears to have a role in glucose oxidase production is manganese. Manganese has been claimed to inhibit glucose oxidase production (Mischak), however, after closer examination of the reports, manganese appears to be actually required as minimizing its concentrations promoted better excretion of the glucose oxidase into the media. There also may be variation among organisms evaluated. Manganese, in addition to that present in yeast extract or peptone, did not improve glucose oxidase production among our thermophiles.

IV. Fermentation

The medium formulation used for much of the work in the larger fermentors was identical to the originally determined optimized media:

Optimized Media for Fermentation of Glucose Oxidase

Component	*g/ L*
Malt Extract	2.0
Yeast Extract	3.0
Peptone	3.0
Dextrose	40.0

Enzyme Activity

The relationships of glucose oxidase, oxygen, biomass and pH are illustrated in figure 3. For glucose oxidase production, the 2 most important parameters to be monitored were enzyme activity and oxygen.

Glucose oxidase fermentations were quite straight forward. As the biomass increased, the high air input could no longer maintain an aerobic system and oxygen concentrations decreased very rapidly and glucose oxidase concentrations increased. Approaching oxygen concentrations below 2 mg/ L, the fermentation slowed and glucose oxidase activities leveled off.

Under the conditions evaluated, glucose oxidase was largely intracellular with only low concentrations observed outside the cells. However, media manipulation on fungi has been observed to force the excretion of glucose oxidase (Mischak, Pitt). There is little reason to believe, the same phenomenon could not be induced in these thermophiles. Therefore, both supernatant and disrupted cells should be evaluated.

Oxygen

Oxygen was probably the most critical parameter to monitor during enzyme production. Air was bubbled into the New Brunswick fermentors at a rate of 0.2 mL air/ mL medium/ min and an agitation rate of 250 rpm. Once the oxygen began to decrease rapidly, glucose oxidase activities increased. A reversal of the oxygen decrease signaled a slow down in the metabolic activity. The fermentation must be halted before enzyme concentrations begin to decrease. For mini-fermentors, air was bubbled in at a rate of 1 mL air/ mL medium/ min but without agitation which resulted in oxygen limitation as was observed by the much slower fermentations.

Biomass

A rough measure of biomass was obtained by measuring absorbance at 600 nm. Comparison between absorbance and culture plates revealed that an absorbance of 7.6 corresponded to 7×10^{12} colony forming units (CFU) per mL. The high concentrations of malt extract, yeast extract and peptone make the medium a fairly dark amber color, adding to the observed high absorbance.

pH

The pH appeared to vary little over the course of a short fermentation. On prolongation of fermentation for several days, the final pH might be as low as 4.9, however most often, final pHs ended up between 5 and 6.5. Interestingly, out of several media evaluated, the media always turned yellow

when glucose oxidase reached high levels and the fermentation slowed due to oxygen limitation.

Prior investigations of pH on *A. niger* cultures have concluded that citric acid was the predominant acid formed when the pH was below 2 while gluconic acid dominated when the pH was above 5. Glucose oxidase, responsible for gluconic acid accumulation, has been theorized to become inactivated below pH 5. Further, the formation of glucose oxidase has been obsereved to be induced by a pH change from under 2 to over 5 during a fermentation (Mischak). The pH optimization graphs for isolate #370 also tend to support this theory (figure 1).

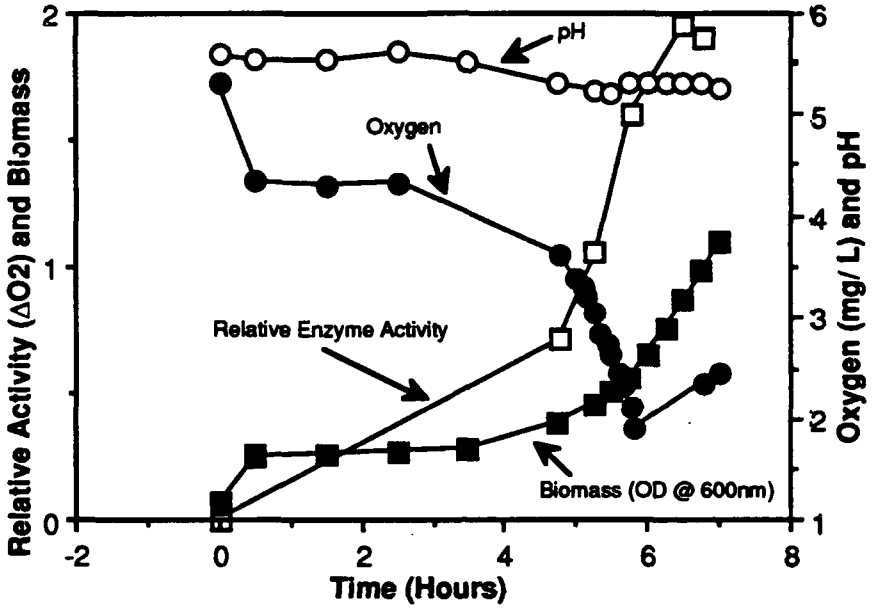
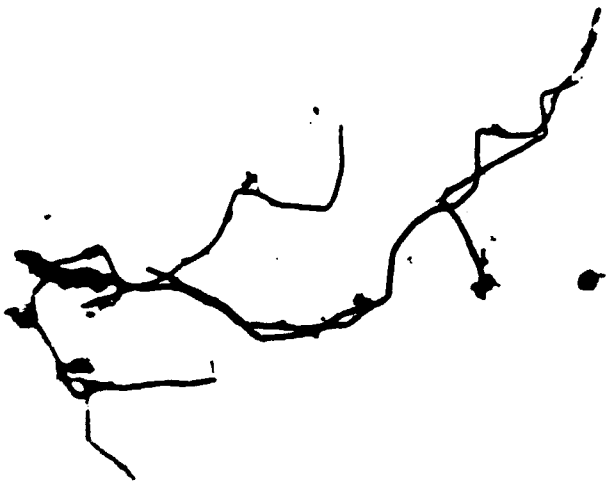


Figure 3. Optimized enzyme production from isolate #370 during fermentation.

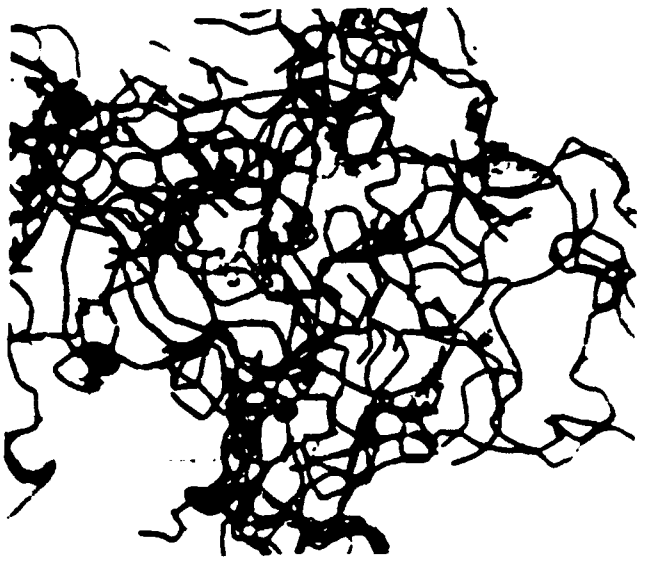
Other Parameters

Microscopic appearance simply showed the increase in numbers of organisms, reflected in the absorbance readings. Initially rods were observed singly, but as cell division rate increased, more chains of rods were observed (Photo A, B and C).

Photograph A. Isolate #370 at the initiation of fermentation.



Photograph B. Five hours into fermentation, chains of rods are becoming visible.



Photograph C. Seven and a half hours into fermentation.

Because the initial concentration of dextrose was so high, glucose enzymatic test strips from Eli Lilly Co. could not detect changes in dextrose levels because the concentrations did not diminish below 20 gm per liter which was the detection limit of the strips. One literature report where dextrose concentrations were monitored in *A. niger* fermentation, did indicate dextrose concentration decreasing to near zero by 60 hours after initiating at 35 g/ L (Rogalski).

V. Enzyme Purification

Organism Concentration

The filtration system used for this project was a Minitan Ultrafiltration System from Millipore. The system allowed both tangential flow microporous and ultrafiltration processing more rapidly than traditional methods. Eight hydrophilic Durapore (PVDF) membrane plates with a total area of 480 sq cm were stacked in a series configuration between acrylic manifold plates. Silicone retentate separators provided a gasket seal between plates and created a sweeping flow across the upstream filter surfaces (Millipore). Torque pressure was adjusted to 80 in lbs. Although the PVDF was supposed to be low protein binding, some enzyme was apparently being lost during this step. Coating the membranes with a solution of 5% nonfat dry milk, covered active sites and helped prevent enzyme loss. The milk was rinsed with 0.2 M sodium phosphate buffer, pH 6, prior to processing the fermentation broth. Transmembrane pressure was maintained below 1-2 psi. Clarified media was collected from the filtrate line, while the cells became more and more concentrated in the retentate container. The process was considered complete when the cellular material was concentrated down to 250 ml.

Sonication and Clarification

The cellular concentrate was placed in a 400 ml beaker in an ice bath over a magnetic stirrer, and a stir bar added. The one inch horn of a 600 W ultrasonicator was placed approximately one inch into the solution and at the edge of the vortex created by the stirrer. The sonicator was turned to full power for 30 minutes to achieve cell disruption. It was important that the sonicated material be kept cold to decrease protease activity.

Cellular debris was separated from the fluid now containing the glucose oxidase by processing the sonicated solution through the Minitan in exactly the same configuration as previously described. Approximately 220 mL clarified enzyme solution was collected on ice from the filtrate line. As the volume

of cellular debris approached 50 mL (hold up volume of the instrument is 30 mL, leaving approximately 20 mL in the retentate container) the cells were washed several times with 50 mL portions of buffer. This resulted in a final filtrate volume of approximately 500 mL. It should also be noted that the filtrate from the first step, equivalent to the supernatant, was not routinely saved. Little glucose oxidase was excreted from the cells. However, because of the very large volume of filtrate, it was found that barely detectable levels of glucose oxidase at this step, did become significant when the filtrate was concentrated. Five thousand mL of filtrate still had less activity than 250 mL of cells. On a large scale, the economics of recovery from supernatant must be determined.

The Minitan plates were washed in place with 8 mL bleach per liter according to the manufacturers instructions and allowed to soak at least overnight in a 0.1% solution of sodium dodecyl sulfate (SDS) at 50°C.

Enzyme Concentration

The Minitan was then set up with 10,000 molecular weight cut off filters of regenerated cellulose (designated PLGC). The system was run at 10 psi, concentrating the clarified material down to 30 mL. Both the collection vessel and the clarified sample were maintained on ice to avoid protease degradation of the glucose oxidase. Because the molecular weight was substantially higher than for peroxidase, there were no problems with glucose oxidase penetrating the membranes and ending up in the waste.

Chromatography

Anion Exchange Glucose oxidase was applied to a strong anion exchanger, Bio- Rad's Macro-Prep 50 Q. Following washing in 1 mM sodium phosphate buffer, pH 6, the enzyme was eluted in 25 mM sodium phosphate. Additional washing with 100 and 200 mM sodium phosphate recovered little additional glucose oxidase activity. As noted earlier, chloride ion has been shown to inhibit glucose oxidase from some sources (Rogers), so this method was attempted first in order to avoid the salt.

As discussed later under dye ligands, protease was almost completely separated from the glucose oxidase fraction, although approximately 80% of the enzyme was lost. More thorough washing of the column with 25 mM sodium phosphate, did recover more enzyme but higher concentrations of proteases resulted.

Protease Removal One common reason for the instability of enzyme preparations is the presence of proteases which may destroy enzymes of interest.

These glucose oxidase preparations were evaluated and observed to contain protease. The separation and removal of proteases from the glucose oxidase fraction is an important criterion used to determine the effectiveness of purification techniques. The procedure used for protease detection follows:

Protease Assay

Assay Buffer: 0.05 M Tris base with 1 mM $CaCl_2$ bring to pH 7.8 with 1 M HCl
Azocoll: 0.25 gm Azocoll into 50 mL rapidly stirring protease assay buffer

Assay: 0.5 mL eluate or concentrate
Blank: 0.5 mL 0.2 M Na phosphate buffer, pH 6 (run 2 blanks)
Add 1 mL Azocoll
Incubate 37°C for 12 hours
Centrifuge
Dilute supernatant with 2 mL of water
Read at 520 nm against blank

Dye Ligands The adherence of glucose oxidase to a number of dye ligands has been documented (Lexton). Purification appeared to be excellent. The duplication of those results with glucose oxidase from isolate #370 was attempted. Eight dye ligands were selected; Red A was from Amicon and the remaining 7 dye ligands were from Sigma Chemical Co. To each 2 mL column, 1 mL dilute glucose oxidase was applied. Columns were washed with 10 mL 20 mM sodium phosphate buffer, pH 6. Columns were eluted with 0.05 M NaCl and 0.5 M NaCl in the same buffer. Assays were performed by the oxygen depletion method and protease was checked in all samples to determine how much protease was in the same fraction as the glucose oxidase. Most of the columns were removing a portion of the protease. The green 5 column did the poorest job of separating protease from the buffer fraction containing the bulk of the glucose oxidase activity. The yellow 3 dye ligand removed the most protease from the eluate fraction containing the most glucose oxidase. When the same sample was applied to the Macro-Prep 50 Q column and eluted with increasing strengths of sodium phosphate buffer, protease in the eluate containing the highest level of glucose oxidase was only 0.007 mg/ L oxygen, less than one-half that observed with the best dye ligand, yellow 3. Unfortunately, the glucose oxidase read only 1.17 mg/ L oxygen compared with the 4.82-5.80 mg/ L oxygen exhibited by the samples from the dye columns. It is possible that the aforementioned inhibition by chloride ion was giving falsely low readings for glucose oxidase activity in the two salt eluates. However, readings in the buffer were as high as expected based on activity of the original samples.

Table 3. Purification Of #370 Glucose Oxidase On Dye Ligands

Dye	*Sample*	*Buffer*	*.05 M NaCl*	*.5 M NaCl*	*Protease*
Red A	0.45*	5.44	1.22	0.61	.041
Blue 3GA	0.00	5.06	0.51	0.43	.027
Brown	0.72	4.95	0.83	0.55	.036
Yellow 3	0.18	5.41	0.65	0.61	.015
Yellow 86	0.01	5.32	0.45	0.69	.021
Blue 4	0.52	4.82	0.46	0.59	.036
Green 5	0.63	4.91	0.49	0.58	.101
Blue 72	1.42	5.80	0.29	0.64	.035

* Values represent relative enzyme activities as a decrease in oxygen concentration (mg/ L)

Red A was selected for preparation of larger quantities of glucose oxidase. Although much of the protease was removed, enough was left to severely limit stability of the glucose oxidase. This was particularly noticeable at 50°C or higher. One explanation is that the protease was less active at 37°C where the protease was assayed and more active at 50°C where the stability analyses were performed. Although the anion exchanger removed more protease than any single dye ligand, significant loss of glucose oxidase also resulted. A suggested alternative would be to use 2 or even 3 dye ligands and to eliminate the anion exchanger.

Other Chromatographic Techniques A small but active amount of protease remained in glucose oxidase fractions after being applied to both the anion exchanger and Red A dye ligand column. This undoubtedly contributed to the relatively low stability of the glucose oxidase. There are two possible approaches to correcting this problem: (1) Further purification or (2) Manipulation of media to encourage expulsion of the glucose oxidase into the media, therefore obviating the necessity to break open the cells, releasing proteases along with the glucose oxidase.

Eriksson et al utilized hydrophobic interaction chromatography on a pentylagarose column for glucose oxidase purification (Eriksson). There are several examples of purification on various dye ligand columns; additional work changing buffers, pH and eluants might improve separation of protease from

the glucose oxidase. As discussed in the assay section, high catalase activity can interfere with detection of glucose oxidase activity. Use of increasing concentrations of sodium acetate rather than sodium phosphate buffer on an anion exchanger has been successful at separating out the catalase, although the effect on protease was not tested (Swoboda).

Electrophoresis

Samples of #370 after concentration on the Minitan and elution from the anion and Red A columns were applied to an SDS-PAGE gel. Electrophoresis was performed under non-reducing conditions and stained with Coomassie brilliant blue R250. Additional purification is required as was apparent by the many protein bands remaining in all samples. Molecular weights of most glucose oxidases range between 150,000 and 180,000 (Kelley, Schepartz). Substrate gels were not performed and since many bands remained on the protein gels, the molecular weight of this preparation was difficult to determine.

VI. Enzyme Characterization

Recovery from the clarification and concentration steps of the Minitan was poor. Following sonication, the microscopic appearance of cells indicated good cell disruption. However, on separation of cellular debris, enzyme activity was very low suggesting the glucose oxidase was adhering to the cells. Cellular debris was washed with buffer during the processing but was insufficient at recovering enzyme. Activity did increase after elution from the anion exchanger suggesting that inhibitors were being removed. Although a 2% recovery is not uncommon for highly purified enzymes, this preparation still retained a large amount of inactive protein, including protease.

Table 4. Recovery Of Glucose Oxidase

	Sample Activity (ΔO_2)	Total Volume (mL)	Total Units (ΔO_2 X mL)	Recovery (%)
Fermentation broth	1.90	5000	9500	100
Minitan concentrate	12.40	30	372	4
Anion exchanger	3.44	400	1376	14
Red A dye ligand	5.20	30	156	2

* Values represent relative enzyme activities as a decrease in oxygen concentration (mg/ L).

Enzyme Stability

The presence of small amounts of protease in the final samples was a major cause for glucose oxidase instability. Numerous stabilizers commonly used for enhancing the stability of glucose oxidase are easily located in the literature. Some of these stabilizers and compounds to inhibit interfering substances such as catalase (sodium azide and cyanide - see assay section) were evaluated. Various samples concentrated from the Macro-Prep 50 Q and Red A columns were incubated 24 hours at 50°C in the presence of additives listed in table 5. Results were reported as the increase in activity over that observed in an untreated sample.

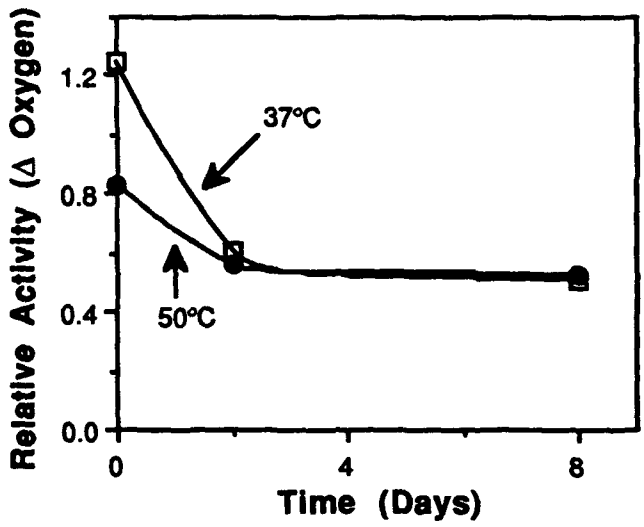


Figure 4. Stability of Glucose Oxidase stability from isolate #370.

Table 5. Effects of Additives on the Stability of Isolate #370

Treatment	Relative Activity *(% increase Over Control)*
PMSF	Decrease
Sodium cyanide	Decrease
4 M xylitol	Decrease
Ferrous sulfate	16
Polyethylene glycol	43
Citric acid buffer, pH 7	57
HEPES buffer (pH 7) containing $MgCl_2$ (10 mM) & glycerol (0.5 M)	78
Trace metals	75
20% Glycerol	88
Sodium azide	78

As observed during assay optimization, sodium cyanide was strongly inhibitory. Sodium azide may have been aiding the inhibition of catalase. PMSF, intended to inhibit serine proteases, was strongly inhibitory for glucose oxidase. Although Ye et al. had observed xylitol to be beneficial for stabilizing glucose oxidase from *Aspergillus niger*, glucose oxidase (#370) was actually slightly less stable in 4 M xylitol. Rather than having any effect on glucose oxidase, the citric acid may have caused oxidation of the substrate, glucose. As noted earlier during the addition of ferrous sulfate and a trace metal solution, iron was observed to be required for glucose oxidase activity. As noted earlier, iron is required for glucose oxidase as has been illustrated by the addition of ferrous sulfate and a trace metal solution. When iron was added alone, an addition rate of 1 mg/ mL was observed to be optimal while in the trace metals, an optimal rate was observed at 0.029 mg/ mL. Other elements within the trace metal solution including manganese, zinc, boron, copper, molybdenum and cobalt, may have contributed to the stability. Glycerol was also observed to significantly enhanced the enzyme stability.

Since isolate #370 was raised at 50°C, the glucose oxidase enzyme would be expected to be much more stable at that temperature in the absence of additives. One possible exception would be the

requirement for ferrous ions. The improvement of stability on removal of inhibitors is possible considering that a high degree of purification was not achieved.

VII. Organism Identification

Fatty Acid Analysis

Four isolates, selected for fatty acid analysis, proved to be closely related (table 6). Moreover, isolates #370 and 408U may be identical. All 4 isolates are likely from the same genus, with #305 and 360B belonging to different subspecies.

Although the organisms were collected from several locations around the United States, all of the original habitats were high in sugar concentration and, likewise, the isolation media for these four isolates were similar.

Table 6. Fatty Acid Profile of Organisms Raised at 50°C for 24 Hrs in TSB Media

	Isolate #			
*Fatty Acid**	*305*	*360B*	*370*	*408U*
SOLVENT PEAK				
14:0 ISO	0.50	0.44	0.43	0.39
14:0		0.29		
15:0 ISO	38.39	40.95	45.93	44.00
15:0 ANTEISO	14.51	11.17	12.41	12.12
16:0 ISO	4.50	4.11	3.54	4.01
16:0	3.28	5.53	3.42	3.58
15:0 ISO 30H	0.57		0.36	
17:1 ISO E		0.36		
17:0 ISO	25.89	22.67	24.84	26.36
17:0 ANTEISO	11.22	8.24	8.32	8.78
18:0	0.57	0.84	0.36	0.43
19:0	0.57	0.40	0.39	0.34

* Conventional Fatty Acid Nomenclature denoting carbon chain length and arranged by retention times from a Gas Chromatograph - Microbial Lipids, Vol 1 (1988) Ratledge, C. and Wilkinson, S.J., Eds.
¥ Two combined (double) peaks were observed. Substituted OH fatty acids are eluted later than non-OH substituted.

16S rRNA Sequence Analysis

Isolate #370 was selected for 16S rRNA sequencing and submitted to PhyloGen. Sequencing and data analysis was performed by Dr. Daniel Distel, of PhyloGen and Harvard University. The sequence was aligned with *E. coli*. Since there was no match with any other organism in the data bank, a similarity analysis was performed with the current available sequences in the Woese/Olsen collection, Argonne National Laboratory. Similarity was computed as the fraction of nucleotide positions which are identical in a given pair of aligned sequences. Data was weighted by applying a mask to the sequence, instructing the computer only to compare sequence positions which have been determined unambiguously in all

sequences being compared and which can be aligned with homologous nucleotide positions in all sequences being compared. This was necessary since 16S rRNAs vary considerably in length. One can only compare positions that occur in all sequences. Isolate #370 shared the highest similarity values with gram positive bacteria. Considering how different the fatty acid analyses were for #370 and the urease producing organism #408U, and the completely different collection locations, the nearly identical sequences of the 16S fragment was surprising. Unfortunately, the only two differences occurred in the single stranded portion which could not be verified. Two considerations are: (1) Since all three sequences were performed at the same time, there is concern of sample switching, and (2) Distinctly different organisms will have similar sequences if they are closely related. Assuming correct sequences, the conclusions reached for isolate #408 would also apply for isolate #370. More specifically, this glucose oxidase producing organism also belongs to the genus *Bacillus*. The sequence data is a permanent, phylogenetically consistent and unambiguous descriptor of the specific strain submitted to CRDEC. As new sequences on thermophiles becomes available, the sequence for #370 will become more valuable for classification.

Comparing the major peaks in the fatty acid analyses, distinct differences are readily observed between organisms producing urease and glucose oxidase (table 7).

Table 7. Fatty Acid Analysis Comparison

Fatty Acid	Glucose oxidase *#370*	Urease *#408U*
15:0 ISO	45.93	28.12
16:0 ISO	3.54	19.36
17:0 ISO	24.84	17.76

Unfortunately there is no consensus on the level of 16S rRNA sequence similarity that constitutes strain, species or generic differences. This is largely due to the fact that well studied bacterial groups have been frequently split while in general less well known bacterial groups have been lumped. The result is that the currently used binomial classification system is not phylogenetically consistent. Examples may be located where two organisms with a 98.9% similarity value have been classified in two different genuses. Yet two other organisms with lower similarity values may be found in the same genus.

Molecular taxonomic studies of the genus *Bacillus* have indicated the group to be heterogeneous and can hardly be considered closely related. All members of this genus form spores. This characteristic is so distinctive that taxonomists have been reluctant to create separate genera (Brock). Consequently this is a large group of rather diverse organisms.

VIII. Commercialization

The reaction catalyzed by glucose oxidase has been adapted to many practical applications. Most commonly, it has been used to detect and estimate glucose in bodily fluids or in a coupled system in conjunction with peroxidase, in various assay procedures. Glucose oxidase will remove glucose in the presence of an excess of oxygen or hydrogen peroxide or it can be used to deoxygenate systems if an excess of glucose is present (Ward). In the food industry, glucose oxidase has been used to desugar egg whites and prevent off flavors in whole eggs. Glucose oxidase is used to deoxygenate beer, carbonated soft drinks and to remove the oxygen from sealed containers (Ward). Glucose oxidase is also used to prevent oxidation of salad dressings, mayonnaise and fresh, frozen or canned fruits and fish (Finnsugar). Less well known applications include applications where the hydrogen peroxide produced by the reaction of glucose oxidase on glucose has been applied to killing certain pathogens. Attachment of glucose oxidase to magnetotactic bacteria offer further refinement of medical applications (Matsunaga).

Glucose oxidase from *A. niger* is relatively stable, certainly when compared with the poor stability of peroxidase from horseradish or urease from Jack Beans. For most applications in the food industry, it is desirable for the enzyme to react for a short period of time. There is no need for the activity to continue once glucose or oxygen have been removed. Understandably, when contacting users of glucose oxidase in the food industry, we observed little interest in a more stable form. One company, with a pharmaceutical application did indicate an interest in a glucose oxidase that was markedly more stable than what is currently available. In fact, J. K. Research is currently performing two specific research projects in this area.

The organism producing glucose oxidase submitted to CRDEC does grow at least to 60°C. Therefore it would be expected that removal of proteolytic activity, interference from catalase and other unknown factors, would result in a preparation with long term stability to at least 50°C.

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Abbreviated Procedures

Heat Stable Peroxidase #197

Culturing Criteria and Characteristics

Organism Characteristics

Gram stain negative
Short rods, curved under stress
Spore former
Aerobic
50°C

Inoculum

Transfer a loopful of colonies from gelled medium to 200 mL liquid medium in 500 mL stirred flask.
Add 0.0001 gm ferric chloride/ L
Use magnetic stirrer at 400 rpm.
Incubate at 50°C for 24 hours.
Add 50 mL inoculum per liter of fermentation medium.

Growth Media

Component	*g/ L*
dextrose	0.5
$MgSO_4 \cdot 7H_2O$	0.25
$MnSO_4 \cdot H_2O$	0.01
NH_4Cl	0.10
KNO_3	4.0
NH_4SO_4	1.0
KH_2PO_4	1.1
$K_2HPO4 \cdot 3H_2O$	0.5
$CaCO_3$	0.5

For gelled medium:
add 0.4 gm $CaCl_2$ and 10 gm Gelrite.
Incubate gelled plates or tubes at 50°C for 24 hrs.

Fermentation Monitor: (1) Dextrose, (2) Dissolved oxygen, (3) Microscopic appearance, (4) Peroxidase (total and supernatant), (5) Biomass, (6) pH.

Note: The initial objective is to produce as much biomass as possible. When both dextrose and oxygen become limiting factors, effects on microscopic appearance will be observed as more curved, twisted, coiled and deformed cells. Peroxidase production is enhanced under these adverse conditions. However, do not allow the fermentation to become completely anaerobic or the carbon to be limiting too early in the fermentation.

(1) *DEXTROSE* Dextrose should fall steadily from 0.5 gm/L to less than 0.1 gm/L.

(2) *DISSOLVED OXYGEN* 0.1 mL air/ mL medium/ minute and 350 rpm agitation. Adjust so that oxygen level falls steadily to minimal value. Do not allow to go below 0.5 mg O_2/L. As dextrose reaches zero, dissolved O_2 levels will stop falling.

(3) *MICROSCOPIC APPEARANCE* Initially 0 - 1 rods, often slightly curved will be observed in each HPF (1000x magnification). By the end of the fermentation, there will be several hundred cells/ HPF and they will be markedly more curved, twisted, coiled and deformed.

(4) *Enzyme Activity* Assay peroxidase in supernatant from whole culture and supernatant from disrupted (sonicated) cells.

1 mL Dilute Peroxidase
2 mL 0.2 M Sodium Phosphate, pH 6
0.1 mL Dilute ABTS (0.01 gm ABTS/ mL water)
1 mL Dilute H_2O_2 (0.1 mL 30% H_2O_2/100 mL water)
Incubate 20 minutes, 37°C
Read at 420 nm against reagent blank

(5) *BIOMASS* Read absorbance at 600 nm to use as a measure of cell density. Read immediately after mixing since calcium carbonate particulates settle rapidly and will cause variability in readings.

(6) *pH* pH will fall only slightly from about 6.3 - 6.5 at zero hours, reaching a minimum near pH 6 as peroxidase production begins.

Processing

(1) Organism Concentration (separation of cells from medium): Minitan™ (Millipore) with 8 PVDF, 0.65 μ plates, run at 1 psi. Coat plate with 5% nonfat dry milk. Rinse with 0.2 M sodium phosphate, pH 6.

Stop processing when only 250 ml cells remain.

Assay for peroxidase content of clarified portion and cellular portion. (Cells must be disrupted or whole cell assay used.) Often, clarified portion will have low level of peroxidase that can be discarded.

(2) Cell disruption (to break open cells and release peroxidase): Place 250 ml cells in 400 ml beaker in ice bath with magnetic stirrer. Place 1" horn of sonicator at edge of vortex. Sonicate 30 minutes with 600 W at full power. Since protease will also be released, remaining processing steps must be conducted in a cold room or ice baths.

(3) Clarification (repeat clarification step to separate cellular debris from released enzyme): Repeat step one except clarify all material, leaving only the hold up volume.

(4) Enzyme Concentration: Minitan with 8 PLGC plates, 10,000 MW cutoff. At 10 psi, concentrate clarified enzyme until only hold up volume remains (30 ml).

Purification

Strong anion exchanger

(1) Pour 50 mL Macro-Prep 50 Q (Bio-Rad) into 1" X 12" column. Dilute 10 mL concentrate from step 4 above with 20 mL 50 mM sodium phosphate buffer, pH 6.5 and apply to column.
(2) Wash with 150 mL of same buffer.
(3) Wash with 150 mL of 0.08 M NaCl in same buffer.
(4) Elute enzyme with 150 mL of 0.2 M NaCl in same buffer. Collect in several fractions.
(5) Regenerate column with 1 N HCl, followed by extensive washing in buffer. (Occasionally the column must also be washed with 20% ethanol.)

Assays. Assay fractions for peroxidase and protease, pooling those positive for peroxidase:

Peroxidase Assay

1 mL eluate
2 mL 0.2 M Sodium phosphate, pH 6
0.1 mL ABTS (0.01 gm ABTS/1 mL water)
1 mL dilute H_2O_2 (100 μL 30% H_2O_2/100 mL)
Incubate 20 min, 37°C
Read at 420 nm against reagent blank

Protease Assay

Assay buffer: 0.05 M Tris base with 1 mM $CaCl_2$; bring to pH 7.8 with 1 M HCl
Azocoll: 0.25 gm Azocoll into 50 mL rapidly stirring protease assay buffer

Assay: 0.5 mL eluate
Blank: 0.5 mL 0.2 M Na phosphate buffer, pH 6
Add 1 mL Azocoll
Incubate 37°C for 12 hours
Centrifuge
Dilute supernatant with 2 mL of water
Read at 520 nm against reagent blank

Purification (cont.)

Desalt

Desalt using concentration membranes on Minitan™. Concentrate pooled eluate to 30 mL, dilute with buffer and re-concentrate. Repeat until calculated salt concentration is below 0.01 M.

Boronate

(1) Pour 50 mL Matrex Gel PBA-30 (Amicon) into 1" X 12" column. Dilute 10 mL desalted concentrate with 20 mL 50 mM HEPES, pH 7 containing 10 mM $MgCl_2$ and 0.5 M glycerol. Apply sample to column.

(2) Wash with 150 mL 50 mM HEPES, pH 7 with 0.5 M glycerol (No $MgCl_2$). Peroxidase does not bind and will be recovered in this wash.

(3) Remaining steps are to remove proteases and other contaminants from the column. Wash with 150 mL 50 mM HEPES, pH 7 containing 20 mM EDTA. Regenerate using 50 ml 6 M guanidine. Wash extensively with HEPES buffer until wash returns to pH 7.

pH adjustment

This step is performed in a manner analogous to the desalting step using the Minitan concentration membranes. In this case, 0.2 M Na phosphate, pH 6 buffer is used for the repeated dilutions, resulting in a concentrated sample of heat stable peroxidase in pH 6 buffer. Repeat peroxidase and protease assays. If protease is still present, run sample through boronate column again. Final sample must not contain any protease.

Heat Stable Urease #408 And 429

Organism Characteristics
Rods
Spore former
Aerobic
50°C for #408, 70°C for #429

Growth Medium

	g/L
Dextrose	25
Yeast Extract	0.5
Casein or Tryptone	1.0
$MgSO4 \cdot 7H_2O$	0.10
Na_2HPO_4	3.0
Micronutrients	*see page 59*

For gelled medium, add 0.4 gm calcium chloride and 10 gm Gelrite. Incubate gelled plates or tubes, 24 hrs at 50°C for #408 and 70°C for #429

Inoculum Transfer loopful of colonies from gelled medium to 200 mL liquid medium in 500 mL mini-fermentor. Aerate at 1 mL air/mL medium/min. Incubate at 50°C for #408 or 70°C for #429 for 24 hrs. Add 50 mL inoculum per L of fermentation medium.

Fermentation Monitor:
(1) Urease Activity
(2) Dissolved Oxygen
(3) Microscopic Appearance
(4) Biomass
(5) pH

Note: **#408:** Within the first 12 hrs, most of biomass should be produced causing a dramatic fall in oxygen levels. During the following 12 hrs, much of the urease production will take place, accompanied by slow increases in biomass, oxygen and pH.

#429: This organism is raised at 70°C. At this temperature, oxygen is poorly soluble. The key is to supersaturate the medium with oxygen, without increasing foaming. To date, this has been accomplished by reducing fermentation (organism growth) and adding nitrogen slowly.

(1) *Enzyme Activity* Monitor urease in supernatant from whole culture and supernatant from disrupted (sonicated) cells.

Urease assay

1 mL dilute urease
2 mL 50 mM citric acid buffer, pH 7
1 mL urea (25 gm urea/L)
Determine pH at time zero
Incubate 30 min at 50°C or 1 hr at 20°C
Determine difference between initial and final pH

(2) *Dissolved Oxygen* 1 mL air/ mL medium/minute and 250 rpm for #408 or 500 rpm for #429. Adjust so that oxygen concentration decreases steadily to minimal value. Do not allow to go below 1 mg O_2/L.

(3) *Microscopic Appearance* Initially, 0 - 1 rods, will be observed in each High Power Field (HPF) (1000x Magnification, Oil Immersion). By the end of the fermentation, there will be several hundred cells/HPF and spores will be observed.

(4) *Biomass* Monitor absorbance at 600 nm to use as an estimation of cell density.

(5) *pH* Initial pH for this medium is approx. 8. pH for #408 will closely parallel oxygen levels, falling to 6.0-6.5 within the first few hrs, then rising slowly to end the fermentation above pH 7. For #429, if nitrogen is added at several times over the first 24 hrs, pH will drift downward slowly, beginning to rise only after oxygen levels begin to rise.

Processing

(1) Organism Concentration: Minitan (Millipore) with 8 PVDF, 0.65μ plates, run at 1 psi. Coat plate with 5% nonfat dry milk. Rinse with 50 mM HEPES, pH 7.

Stop processing when only 250 mL of cells remain.

Assay for urease content of clarified portion and cellular portion.

(2) Cell disruption (to break open cells and release urease): Place 250 mL cells in 400 mL beaker in ice bath with magnetic stirrer. Place 1" horn of sonicator at edge of vortex. Sonicate 30 minutes with 600 W at full power. Since protease will also be released, remaining processing steps must be conducted in a cold room or ice baths.

(3) Clarification (repeat clarification step to separate cellular debris from released enzyme): Repeat step one except clarify all material, leaving only the hold up volume.

(4) Enzyme Concentration: Minitan with 8 PLGC plates, 10,000 MW cutoff. At 20 psi, concentrate clarified enzyme until only hold up volume remains (30 ml).

Purification

Strong anion exchanger

(1) Pour 50 mL Macro-Prep 50 Q (Bio-Rad) into a 1" X 12" column. Dilute 10 mL of enzyme concentrate with 20 mL 50 mM HEPES buffer, pH 7 and apply to column.
(2) Wash with 150 mL of same buffer.
(3) Wash with 150 mL of 0.1 M NaCl in same buffer.
(4) Elute urease with 150 mL of 0.2 M NaCl in same buffer. Collect in several fractions.
(5) Regenerate column with 1 N HCl, followed by extensive washing in buffer (Occasionally the column must also be washed with 20% ethanol)

Assays Assay fractions for urease and protease, pooling those positive for urease.

Urease Assay

1 mL eluate
2 mL 50 mM citric acid buffer, pH 7
1 mL urea (25 gm urea/L)
Determine pH at time zero
Incubate 30 min at 50°C or 1 hr at 20°C
Determine difference between initial and final pH

Protease Assay

Assay buffer: 0.05 M Tris base with 1 mM $CaCl_2$; bring to pH 7.8 with 1 M HCl
Azocoll: 0.25 gm Azocoll into 50 ml rapidly stirring protease assay buffer

Test: 0.5 mL eluate
Blank: 0.5 mL of 50 mM HEPES (pH 7)
Add 1 mL Azocoll
Incubate 37°C for 12 hours
Centrifuge
Dilute supernatant with 2 mL of water
Read at 520 nm against reagent blank

Desalt

Desalt using concentration membranes (10000 MWCO) on Minitan. Concentrate pooled eluate to 30 mL, dilute with buffer and re-concentrate. Repeat until calculated salt concentration is below 0.01 M.

Green 5 Chromatography

(1) Pour 50 mL Green 5 dye-ligand (Sigma) into 1" X 12" column. Dilute 10 mL desalted concentrate with 10 mL 50 mM HEPES, pH 7. Apply sample to column.

(2) Wash with 150 mL 50 mM HEPES, pH 7. Urease does not bind and will be recovered in this wash. Reconcentrate on Minitan.

(3) Remove column bound contaminants with 0.5 M NaCl. Regenerate column with 8 M urea in 0.5 M NaOH.

(4) Assay for urease and protease. All protease must be removed to avoid adversely affecting urease stability.

Heat Stable Glucose Oxidase # 370

Organism Characteristics	
	Rods
	Spore former
	Aerobic
	50°C

Growth Medium

	g/L
Malt Extract	2.0
Yeast Extract	3.0
Peptone	3.0
Dextrose	40.0

Gelled medium, add 0.4 gm calcium chloride and10 gm Gelrite.
Incubate gelled plates or tubes, 24 hrs at 50°C.

Inoculum Transfer loopful of colonies from gelled medium to 200 mL liquid medium in 500 mL mini-fermentor.
Aerate at 1 mL air/mL medium/min.
Incubate at 50°C for 24 hrs.
Add 50 mL inoculum per L of fermentation medium.

Fermentation Monitor:
(1) Enzyme Activity
(2) Dissolved Oxygen
(3) Microscopic Appearance
(4) Biomass
(5) pH

(1) *Enzyme Activity* Assay glucose oxidase of supernatant and disrupted (sonicated) cells.

Enzyme Activity - Polarographic oxygen probe

1 mL Diluted Sample
2 mL 0.2 M sodium phosphate buffer, pH 6
1 mL Glucose (100 g/L)

Incubate 1 hr at 20°C.

Measure oxygen concentration and compare with oxygen concentration of reagent blank. A lower oxygen level corresponds with a higher level of glucose oxidase activity. Report as a change in oxygen concentration.

(2) *Dissolved Oxygen* 0.2 mL air/mL medium/minute and 250 rpm. Adjust so that oxygen level falls steadily to minimal value. Do not allow to go below 1 mg O_2/L.

(3) *Microscopic Appearance* Initially 0 - 1 rods, will be observed in each HPF (1000x Magnification, Oil immersion). As the biomass begins to increase rapidly, chains of rods become more common.

(4) *Biomass* Monitor optical density at 600 nm to use as a measure of cell density.

(5) *pH* pH will vary little, generally staying between 5 and 6.5.

Processing

(1) Organism Concentration (separation of cells from medium): Minitan (Millipore) with 8 PVDF, 0.65 μ plates, run at 1 psi. Coat plate with 5% nonfat dry milk. Rinse with 0.2 M sodium phosphate buffer, pH 6.

Stop processing when only 250 mL cells remain.
Assay for glucose oxidase content of clarified portion and cellular portion.

(2) Cell disruption (to break open cells and release urease): Place 250 mL cells in 400 mL beaker in ice bath with magnetic stirrer. Place 1" horn of sonicator at edge of vortex. Sonicate 30 minutes with 600 W at full power. Since protease will also be released, remaining processing steps must be conducted in a cold room or ice baths.

(3) Clarification (repeat clarification step to separate cellular debris from released enzyme): Repeat step one except clarify all material, leaving only the hold up volume.

(4) Enzyme Concentration: Minitan with 8 PLGC plates, 10,000 MW cutoff. At 10 psi, concentrate clarified enzyme until only hold up volume remains (30 mL).

Purification

Strong anion exchanger

(1) Pour 50 mL Macro-Prep 50 Q (Bio-Rad) into 1" X 12" column. Dilute 10 mL concentrate from step 4 above with 20 mL 1 mM sodium phosphate buffer, pH 6 and apply to column.

(2) Wash with 50 mL of same buffer.

(3) Elute glucose oxidase with 300 mL of 25 mM sodium phosphate buffer, collecting in several fractions.

(4) Wash with 300 mL 200 mM sodium phosphate buffer.

(5) Regenerate column with 1 N HCl, followed by extensive washing in buffer. (Occasionally the column must also be washed with 20% ethanol.)

Enzyme Assays. Assay fractions for glucose oxidase and protease, pooling those positive for glucose oxidase.

Glucose oxidase - Polarographic oxygen probe

1 mL Eluate
2 mL 0.2 M sodium phosphate buffer, pH 6
1 mL glucose (100 gm/L)

Incubate 1 hr at 20°C.
Measure oxygen concentration and compare with oxygen concentration of reagent blank.

Protease Assay

Protease assay buffer: 0.05 M Tris base with 1 mM $CaCl_2$; bring to pH 7.8 with 1 M HCl
Azocoll: 0.25 gm Azocoll into 50 mL rapidly stirring protease assay buffer

Test: 0.5 mL eluate
Blank: 0.5 mL sodium phosphate, pH 6
Add 1 mL Azocoll
Incubate 37oC for 12 hours
Centrifuge
Dilute supernatant with 2 mL of water
Read at 520 nm against reagent blank

Red A Chromatography

(1) Pour 50 mL Red A dye ligand (Amicon) into 1" X 12" column. Dilute 10 mL concentrate with 10 mL 50 mM Sodium phosphate, pH 6. Apply sample to column.

(2) Elute glucose oxidase with 150 mL 50 mM sodium phosphate, pH 6. Glucose oxidase does not bind and will be recovered in this wash. Reconcentrate on Minitan.

(3) Remove column bound contaminants with 0.5 M NaCl. Regenerate column with 8 M urea in 0.5 M NaOH.

(4) Assay for glucose oxidase and protease. All protease must be removed to avoid adversely affecting glucose oxidase stability.

Safety Considerations

Chemicals and Reagents

Under certain circumstances nearly all chemicals are potentially hazardous. General safety procedures, applicable to all chemicals, should be in place. Safety awareness is the most important rule in any laboratory. For all laboratory chemicals normal precautions such as avoiding inhaling dust, preventing chemicals from coming in contact with the skin or eyes, never ingesting any chemicals and general fire safety procedures must be followed.

Many of the chemicals described in this report are used in the culture media as nutrients for the microorganisms. Media are made up in very dilute solutions, not over a few grams of various compounds per liter of media. At the end of a fermentation, many of the compounds have been completely metabolized, thus decreasing various hazards. Material Safety Data Sheets (MSDSs) for all chemicals have been previously submitted to CRDEC and should be consulted for specific precautions.

Media used for chromatographic purification is generally considered to be of low toxicity. In fact some manufacturers have not yet prepared MSDSs for some of these products. Those available have been filed with CRDEC.

Chemicals for assays are used in extremely small quantities. Probably the most questionable compounds are those used as chromogens in various peroxidase and glucose oxidase assays. ABTS is mutagenic by the Ames test (Bos). Questions have been raised about some of the alternative chromogens as discussed under "Enzyme assays." Since ABTS was already in use at CRDEC at the start of this project, and it is one of the most widely used chromogens for this type of assay, it was selected for inclusion in the final procedures. Again, the MSDSs should be consulted for details of precautions.

OSHA has published detailed procedures (29 CFR 1910; compare also 45 Fed. Reg. 5002-5296) for working with substances they have classified as carcinogens. Although no chemicals recommended for use in the final procedures occur on the OSHA list (Prudent Practices for Handling Hazardous Chemicals in Laboratories), these procedures should be followed when handling compounds for which carcinogenicity has been questioned, namely ABTS (or certain alternative peroxidase and glucose oxidase chromogens). PMSF was used in experimental procedures during the conduct of this project, but was not recommended for use in the final procedures. PMSF breaks down rapidly in aqueous solution, particularly at an alkaline pH, so can be readily inactivated before disposal.

Chemicals must be disposed of in such a way that people and the environment are subjected to minimal harm. Again, the MSDSs should be consulted for specific handling requirements and the institutional safety plan for safe and regular disposal of chemicals should be followed.

Microorganisms

The microorganisms submitted to CRDEC are thermophiles isolated from thermal waters of Yellowstone Park. As a result of their temperature requirements, thermophilic bacteria have not been implicated in disease states of either warm or cold blooded animals. The attached letter from Dr Karl Woese, a recognized leader in thermophile research, confirms the current opinion. A search of two computerized data bases, BIOSIS and AGRICOLA, did not turn up any references to disease caused by thermophilic members of the genera Bacillus or Lactobacillus. Since the four organisms submitted to CRDEC were not identified, some uncertainty remains. 16S rRNA analyses were conducted on three of the isolates and fatty acid analyses on all four. None matched the profile of any organism in any of the computerized data banks that were searched. Although risks are perceived as minimal, the same general safety precautions that would be applicable in any routine microbiology laboratory should be followed when handling these organisms. Any direct contact with a culture should be avoided. Care must be taken not to allow the organisms to come in contact with the skin or eyes, production of aerosols

an inhalation of bacteria is to be avoided and solutions of these organisms should not be pipetted by mouth. All of the investigations with all of the organisms were conducted on the open benchtop in our laboratory according to general microbiological practices outlined in the CDC/ NIH Guideline Booklet. According to these guidelines and in light of the discussions within this report, we perceive all of the organisms delivered to CRDEC to be classified as BioLevel Safety 1 as described in the same aforementioned text.

Organism Maintenance and Preservation

The organisms were cultured on slants or plates from standard microbiological isolation techniques. For storage up to 6 months, cultures were maintained on gelled slants. Since thermophiles do not grow at temperatures significantly below 50°C, they were stored at room temperature in tightly capped tubes. Attempts to store the tubes at 4°C resulted in significant loss of many cultures as the culture media became dehydrated and cracked.

Thermophiles can be stored frozen for many years without significant loss of viability. Although other procedures are available, the following procedure was selected (Daggett): Cells were harvested from the late log or early stationary growth phase using standard microbiological practices and scraping several loopfuls of biomass off a gelled plate (Glass or polymethylpentene (PMP) plates must be used for culturing thermophiles as the high temperature requirement for growth solubilizes conventional plastic). The biomass was suspended in 0.75 mL double strength medium (to allow single strength on later 1:1 dilution with glycerol) in 2 mL storage vials. An equal volume of sterile 25% glycerol was added and the vial shaken gently to break up clumps of biomass and ensure mixing of the glycerol with the medium. The 25% glycerol concentration was experimentally determined as the minimal glycerol necessay to insure freezing prevention at -30 to 40°. Vials were allowed to stand for 30 min at 20°C before transferring them to a minus 40°C freezer. Programmable cooling units which control the rate of freezing at 1°C per min have been demonstrated to increase viability, but were not used for these cultures (Dagget and Simione, *Cryopreservation Manual*, 1987). Ultra-low freezers at -70 to -90°C would allow longer storage than can be expected at -40°C.

Conclusions/ Recommendations

This investigation has resulted in significant information that will be beneficial to the improved development of consumer products to aid the detection and diagnosis of health and environmental disorders in addition to aiding the military in achieving a stable and reproducible chemical and biological detection device. More specifically, significant findings include:

- Heat stable ureases with molecular weights ranging from 120,000 to 130,000 which is of particular value for protein labeling and considering the 550,000 MW of the conventionally used Jack Bean urease.

- A heat stable peroxidase with slightly better stability than a recent Japanese isolate.

- The 16S rRNA analysis indicates that the three organisms submitted to the US Army, CRDEC had not been previously isolated and identified and supports the theory that the thermophile s are novel in origin.

- Further investigation on the purification of all three enzymes described is required to more efficiently remove proteases and other inactive proteins.

- The subsequent fermentations require close monitoring of enzyme concentrations and dissolved oxygen for all three enzymes. Other parameters vary in importance with the organism involved.

- This SBIR project has directly led to both commercial and research opportunities.

Two of these conclusions lend themselves to the basis for recommendations : 1) Further purification and 2) Monitoring fermentations.

Further Purification

Further purification is required for all three enzymes. This process is critical to the commercial success of the peroxidase, urease and glucose oxidase enzymes. The importance of this step for any enzyme may be judged by the current flood of new purification products being brought onto the market. Numerous column packings are being introduced to deal with the removal of proteases. Phenylboronate was the most successful of those evaluated in this project but its use must either be further optimized or more alternative products tested. Various forms of affinity, hydrophobic interaction (HIC) and size-exclusion (SEC) chromatographies have great potential for more efficient recovery of each enzyme. Although a substantial amount of testing was conducted during this project, further optimization is necessary. The prediction of how a particular enzyme will behave on a given column packing is impossible. Additionally, these heat stable enzymes often exhibited quite different behaviors compared with commonly available forms of these enzymes from plants, eucaryotic sources and mesophiles. An expanded testing regime is needed for all three enzymes.

Monitor Fermentations

The need to closely monitor each fermentation during production cannot be overemphasized. Enzyme and oxygen concentrations are critical parameters for all three enzymes. For peroxidase, the unusual microscopic changes and narrow range of dextrose concentrations are two additional values to be closely monitored. The biomass (cell growth), pH and microscopic examination of urease and glucose oxidase fermentations will provide the advanced warning of problems.

Biological systems involve numerous variables; small changes in one parameter can have a significant effect on the whole fermentation. Although the fermentations described in this project are not unusually difficult, minor variations in the inoculum, temperature, water source, pH or source of media components can change the optimum time for harvesting the culture. Hence the need to monitor every run carefully.

Commercial Materials and Addresses

Enzyme Purification Filters And Chromatographic Media

General Classification	Type	Source	Catalogue #
Clarification membranes	Durapore membranes	Millipore	DVLP OMP 04
Concentration membranes	regenerated cellulose	Millipore	PLGC OMP 04
Strong anion exchanger	Macro-Prep 50 Q	Bio-Rad	156-0050
Boronate	Matrex Gel PBA-30	Amicon	19605
Red dye ligand	Matrex Gel Red A	Amicon	19111
Green dye ligand	Reactive green 5 agarose	Sigma	R2257

Company	Address	Order Phone	Technical Phone
Schweizerhal	South Plainfield, NJ	800-243-6564	201-753-5000
DIFCO Laboratories	P.O. Box 331058, Detroit, MI 48232-7058	313-462-8500	
Aldrich Chemical Co., Inc.	1001 West Saint Paul Ave., Milwaukee, WI 53233	800-558-9160	800-231-8327
Fisher Scientific	8030 S. 228th Street, P.O. Box 1148, Kent, WA 98032	800-342-3002	
Fisher Scientific (Corp. Hdqts)	711 Forbes Ave., Pittsburgh, PA 15219	412-562-8300	
Millipore Corporation	448 Grandview Dr., So. San Francisco, CA 94080	800-632-2708	800-632-2708
Millipore Corporation	Bedford, MA 01730	800-225-1380	800-225-1380
BIO-RAD (Div. Hdqts.)	3300 Regatta Blvd., Richmond, CA 94804	800-227-5589	
BIO-RAD (E. Reg. Office)	P.O. Box 1229, 85A Marcus Dr., Melville, N.Y. 11747	800-645-3227	
Amicon Division (W.R. Grace & Co.)	24 Cherry Hill Dr., Danvers, MA 01923	800-3443-0696	800-3434-1397
SIGMA Chemical Co.	P.O. Bos 14508, St. Louis, MO 63178-9916	800-325-3010	800-325-5832

List of Chemical Abbreviations

ABTS	2,2'-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic Acid)
EDTA	Ethylenediaminetetraacetic Acid
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic Acid)
Pinacyanol Cl	2,2'-Trimethinequinocyanine chloride
PMSF	Phenylmethanesulfonyl Fluoride, α-toluenesulfonyl Fluoride
TMBZ	3,3',5,5'-Tetramethylbenzidine
TRIZMA BASE	Tris(Hydroxymethyl)aminomethane
TRIZMA HCl	Tris(Hydroxymethyl)aminomethane Hydrochloride