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Devulcanization of Rubber Crumb Using Sulfur Oxidizing *Archaea*

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Abstract

Waste tires pose an ever-growing disposal and environmental problem. In the United States alone it is estimated that there are 2.5 billion waste tires sitting in landfills and this number is growing by 200 million every year. In order to recycle the rubber in these tires the rubber must be effectively devulcanized by breaking the carbon sulfur cross-links that are present. The need for a more cost-effective devulcanization process that produces rubber of higher quality has driven research in this field. It was the goal of my project to develop rubber recycling methods involving sulfur oxidizing *Archaea* and to determine the usefulness of these methods. In order to accomplish this goal two major methods were investigated: one using whole cells from the species *Sulfolobus solfataricus* P2, another using an enzyme, the sulfur oxygenase/reductase (Sor) from *Acidianus ambivalens*. My results have shown that both the whole cell method and the enzyme method oxidize the sulfur found in rubber crumb, thereby, causing some degree of devulcanization.

An attempt was made to locate homologues to *sor* found in a number of species related to *Acidianus ambivalens*. Interestingly, after exhausting a number of methods, this search resulted in no homologue being found. The possibility of a novel form of sulfur metabolism is discussed.

Résumé

Les pneus en caoutchouc usagés posent plusieurs problèmes environnementaux. Il est estimé qu'aux États-Unis il y a 2,5 milliards de pneus en caoutchouc usagés qui remplissent les réservoirs de déchets et ce chiffre augmente de 200 millions par année. Afin de recycler ce caoutchouc il faut le dévulcaniser en cassant les liens entre le carbone et le souffre. La recherche dans ce domaine est poussé par le besoin de développer un processus de dévulcanisation qui est moins dispendieux et qui produit quand même du caoutchouc de haute qualité. L'objectif de cette recherche était de développer des moyens de recycler le caoutchouc en utilisant les Archaea qui peuvent oxider le souffre. Deux méthodes ont été étudiées afin d'accomplir cet objectif. La première utilise des cellules intactes de l'espèce *Sulfolobus solfataricus* P2 et la deuxième utilise l'enzyme oxygenase/réductase du souffre isolé de l'espèce *Acidianus ambivalens*. Mes résultats démontrent que ces deux méthodes induisent un certain niveau de dévulcanisation en oxidant le souffre retrouvé dans des miettes de caoutchouc.

Plusieurs méthodes ont été utilisées afin de localiser des homologues au gène *sor* qui se retrouvent chez plusieurs espèces voisines de *Acidianus ambivalens*. Malgré ces efforts aucun gène homologue a été trouvé. La possibilité d'un nouveau métabolisme du souffre est discuté.

Acknowledgements

I would like to thank Dr. Robert L. Charlebois, for not only giving the opportunity to work on this exciting project, but also for his help, encouragement and guidance. During this research I have grown both as a scientist and as a person and his influence has been significant in both areas.

I would also like to thank all of the people who worked beside me. During my time in this lab I have made friends who made all of the hard work a lot of fun. Anick De Moors and Ghislaine Allard in particular made sure there was never a day without a good laugh (usually something about some ones dreams). I would also like to thank Julie Chapados for never failing to have good Monday morning gossip.

I would also like to acknowledge the hard work of Dr. Kris Kostanski in performing the UV Chromatography analysis and Dr. Arnulf Kletzin for his kind gift of pSOR-1/17.

I would also like to thank my parents for their support, both financial and moral, throughout my years of education.

And finally, I would like to thank my wife Debbie for her patience, encouragement and love.

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INTRODUCTION

The goal of my research was to investigate the possible uses of members of the *Sulfolobales* in the devulcanization of rubber crumb. Two major areas were the focus of this investigation. The first area of interest was to study the ability of *Sulfolobus solfataricus* P2 to oxidize sulfur when grown in the presence of rubber crumb and to optimize this process. The second area was the use of the sulfur oxygenase (Sor) enzyme from *Acidianus ambivalens* to devulcanize rubber. To accomplish this the *sor* gene was expressed in *Escherichia coli* and the protein partially purified. In both of these areas the main objective was to determine if there were any possible industrial applications for either of these treatments in the recycling of vulcanized rubber. The recycling of rubber is a major industry with potential economic and environmental benefit (for more information please see these websites: www.tirerecycling.com/index.html, www.link-pl.com.au and www.tnrcc.state.tx.us/waste/msw/tires/index.html). With the current technology this industry is expanding every year and rubber crumb is quickly becoming a valuable commodity. What follows in this introduction is a description of the *Sulfolobales*, sulfur metabolism, and an explanation of why given the nature of these organisms and the structure of vulcanized rubber this study was initiated.

I) *Sulfolobales*

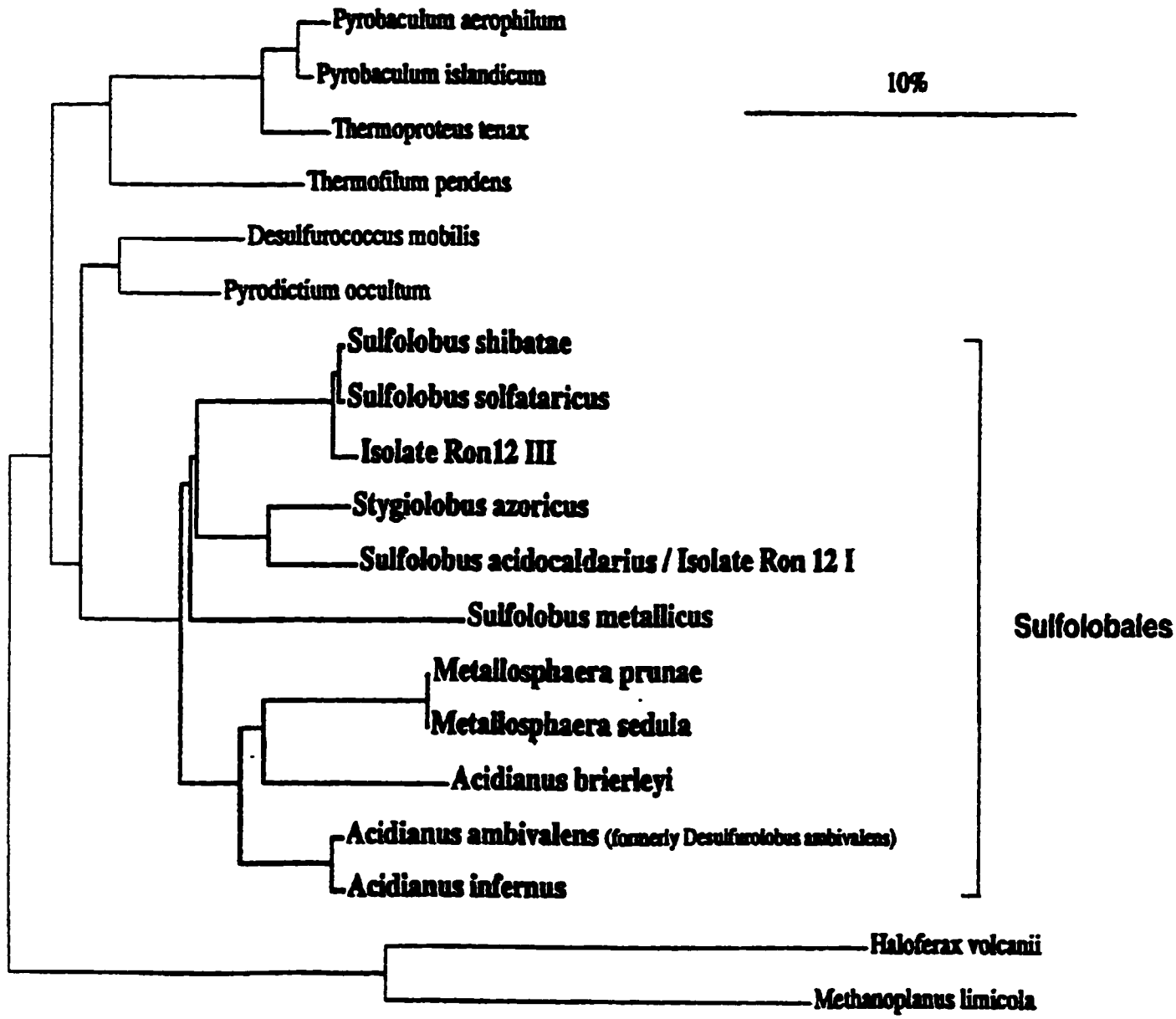
I.A. Phylogenetic Position

Based on ribosomal RNA sequences all life on this planet has been divided into three domains: *Archaea, Bacteria* and *Eucarya* (Woese and Fox 1977; Woese *et. al.*, 1990). Of these three domains, the *Archaea* and the *Bacteria* are grouped broadly

together as procaryotes based on a general similarity in morphology and mode of life; both groups are made up of single-celled organisms. More recently however, similarities between the *Archaea* and the *Eucarya* in areas of gene structure and expression have been discovered in contrast to the metabolic and morphological similarities found between the *Archaea* and the *Bacteria* (for review see Brown and Doolittle 1997). These conflicting similarities have led to a great deal of debate over the relationships between the three major domains and how they have diverged (Keeling *et. al.* 1994, Keeling and Doolittle 1995, Brinkmann and Philippe 1999 and Zillig 1991). Very recent studies, involving the sequence of t-RNA synthetases and other genes, have done little to clarify the situation (Gribaldo and Cammarano 1998, Siatecka *et. al.* 1998, Taupin and Leberman 1999, Doolittle 1999 and Koch 1998). However, as the *Sulfolobales* are an order in the domain *Archaea* it will be this domain that the remainder of this section will focus on.

The *Archaea* are split into three major divisions: the *Euryarchaeota*, the *Korarchaeota* and the *Crenarchaeota* (Woese *et. al.* 1990). The *Euryarchaeota* are composed primarily of the methanogens and the extreme halophiles along with one genus of sulfate reducing species and two genera of extreme thermophiles. The recent discovery of low temperature marine *Archaea* has given rise to the *Korarchaeota* (Barns *et. al.* 1996). The *Sulfolobales*, along with the sulfur reducing extreme thermophiles, make up the *Crenarchaeota*. Within the *Sulfolobales* there have been four genera described: *Sulfolobus, Acidianus, Metallosphaera* and *Stygiolobus*. The relationships between these genera are shown in figure #1 (Sanz *et. al.* 1994, Kurosawa *et. al.* 1995, Trevisanato *et. al.* 1996 and Fuchs *et. al.* 1996).

Figure #1: Phylogentic tree depicting the relationships between members of the *Sulfolobales*. The tree is based on 16S rDNA and was reproduced from Fuchs *et. al.* 1996.


Pyrobaculum aerophilum
Pyrobaculum islandicum
10%
Thermoproteus tenax
Thermofilum pendens
Desulfurococcus mobilis
Pyrodictium occultum
Sulfolobus shibatae
Sulfolobus solfataricus
Isolate Ron12 III
Stygiolobus azoricus
Sulfolobus acidocaldarius / Isolate Ron 12 I
Sulfolobus metallicus
Sulfolobales
Metallosphaera prunae
Metallosphaera sedula
Acidianus brierleyi
Acidianus ambivalens (formerly Desulfurolobus ambivalens)
Acidianus infernus
Haloferax volcanii
Methanoplanus limicola

I.B. General Metabolism

In nature members of the *Sulfolobales* inhabit continental sulfur springs known as solfataras. In these solfataras the *Sulfolobales* grow under what we consider extreme conditions for the existence of life. The range of temperature optima for these genera is from 65°C to 90°C and the pH optimum from 2-4 (Brock *et. al.* 1972, Segerer *et. al.* 1986, Sergerer *et. al.* 1991 and Huber *et. al.* 1989). Essentially these species grow in what amounts to very hot sulfuric acid. Another interesting feature of the *Sulfolobales* is the ability, and some cases the dependency, to survive by either the oxidation or the reduction of sulfur. Unlike their close relatives, the truly sulfur dependent organisms in the *Crenarchaeota* (*Pyrobaculum* and *Pyrodictum*), which are found near deep sea vents and grow at high temperatures by reducing sulfur, many of the *Sulfolobales* are capable of heterotrophic growth. During heterotrophic growth carbon sources such as glucose or sucrose are used to produce energy (De Rosa *et. al.* 1984). When growing lithotrophically energy is produced by oxidizing either sulfur or ferrous iron and carbon is fixed from CO_2 (Shivvers and Brock 1973 and Ishii *et. al.* 1997). However, all species capable of growth on carbon sources grow much faster and to higher cell densities when in a carbon source medium as opposed to a sulfur medium (Mosser *et. al.* 1974). In this study, sulfur metabolism is of primary importance and will be discussed in a later section in extensive detail.

I.C. Industrial uses of *Sulfolobales*

Due to the thermal stability of their enzymes and their unique forms of metabolism, members of the *Sulfolobales* have been considered for a number of biotechnological processes. One group of enzymes that has been studied for this purpose

are enzymes involved in trehalose biosynthesis and hydrolysis (Kobayashi *et. al.* 1996 and Kato *et. al.* 1996). These enzymes have been used in industrial scale production of trehalose, a disaccharide with potential uses as a food additive (Nakada *et. al.* 1996). There has also been a large amount of work on a number of dehydrogenases including glutamate, succinate and alcohol dehydrogenase (Facchiano *et. al.* 1995, Moll and Schafer 1991 and Raia *et. al.* 1996). By far however, the vast majority of the published work linking *Sulfolobales* to an industrial use deals with possible utilization of sulfur and iron oxidation. Most of these studies focus on two major uses: the desulfurization of fossil fuels and the bioleaching of minerals.

Fossil fuels such as coal and petroleum products are major sources of energy for our society. One of the problems with the burning of fossil fuels is the relatively high sulfur content in the fuel. The sulfur concentration ranges from 0.5 to 6% (Wheelock 1977). When these fuels are burned the sulfur is released into the atmosphere as sulfur dioxide. Sulfur dioxide is a major cause of acid rain which is harmful to both animal and plant life (Skinner and Porter 1992). This is a problem whether the fuel being burned is coal to produce electricity or gasoline in the cars we drive. For this reason a number of methods have been investigated to reduce the amount of sulfur in the fuels we burn. Chemical and physical methods that have been developed are expensive, use a large amount of energy and are hard on equipment due to the corrosive nature of the by-products (Wheelock 1977 and Meyers 1977). In many cases the energy producing characteristics of the fuel are also reduced by these methods. More recently microbial methods have been investigated, as they are less expensive, require less energy and the characteristics of the fuel are not affected. The first microbes investigated for the

desulfurization of fossil fuels were species of *Thiobacillus* and *Rhodococcus* (Dugan and Apel 1978 and Denome 1994). The results of these studies showed moderate success. Due to the thermostable ability to oxidize the sulfur in these fuels, members of the *Sulfolobales* were used to increase the effectiveness of the process. A number of studies have shown that *Sulfolobus* cells are capable of oxidizing sulfur in coal (Kargi and Robinson 1982) and in dibenzothiophene (DBT), a material commonly used to mimic sulfur compounds in gasoline (Kargi and Robinson 1983 and Kargi 1987). Kargi and Robinson published the first of these studies in 1982. They showed that with little optimization *Sulfolobus acidocaldarius* could oxidize up to 50 % of the sulfur found in coal. Since their original work there have been a number of studies optimizing factors such as pH, particle size, and sulfur content in the coal (Kargi and Robinson 1984, Chen and Skidmore 1987 and Vitaya and Toda 1991). All of these studies have led to a better understanding of the factors involved and also to studies involved in scaling up the process (Nixon and Norris 1992).

The use of members of the *Sulfolobales* for bioleaching has been primarily focused on the dissolution of pyrite. This process occurs due to the oxidation of the iron and sulfur in the pyrite and releases gold as well as other valuable metals (Ngubane and Baecker 1990). Again, as with the desulfurization of fossil fuels, this microbial process was first studied using species of *Thiobacillus* (Silverman 1967, Torma and Sakaguchi 1978, Chan and Myerson 1982 and Konishi *et. al.* 1990). The use of members of the *Sulfolobales* was thought to have more potential than the mesophilic *Thiobacillus* for two reasons: 1) the fermentation would not have to be cooled, thus saving money and 2) the oxidation rates would be higher for both the biological and chemical reactions at the

higher growth temperatures. Ngubane and Becker performed the first study in 1990. They found that the rate of oxidation was some 3-5 times higher using *S. acidocaldarius* than with *Thiobacillus* species. Further studies have been done optimizing the fermentation conditions (Lindstrom et al 1993, Han and Kelly 1997 and Konishi *et al* 1997). These studies have looked at factors such as pH, particle size, temperature and the presence of yeast extract. The kinetics of the process have also been studied and the relative contributions of the biological and chemical reactions has been studied. This study showed that the ratio of biological oxidation to chemical oxidation was 2:1 (Vitaya *et. al.* 1994). There has also been one study attempting to adapt this fermentation to a continuous leaching apparatus similar to what would be used in an industrial setting (Lindstrom and Gunneriusson 1990).

All of these studies into bioleaching and desulfurization provide excellent justification for this study, as rubber shares many similarities with coal, which make the processes similar. In fact, there have been recent reports of groups working on developing a cell based method for devulcanizing rubber crumb using *S. acidocaldarius* (Coghlan 1995 and Siuru 1997).

II) Sulfur Oxidation in Procaryotes

Among procaryotes elemental sulfur or related reduced sulfur compounds such as hydrogen sulfide (H_2S), thiosulfate ($S\text{-}SO_3^-$) and polythionates ($^-O_3S - S_N - SO_3^-$) are important electron donors in energy producing reactions (for reviews see Wood *et. al.* 1987, Friedrich 1998 and Kelly *et. al.* 1997). The oxidation of these sulfur compounds can be directly energy producing as in chemolithotrophic *Bacteria* (ie. *Thiobacillus*, *Rhodococcus* and *Paracoccus*) and *Archaea* (ie. *Sulfolobus* and *Acidianus*) or can be

indirect as in anaerobic sulfur phototrophs (ie. *Rhodobacter*). In the lithotrophic case, over 100 years of investigation have revealed that there are at least two major pathways involved. One pathway involving a polythionate intermediate (S4I pathway), is thought to be found only in obligate chemolithotrophs such as species of *Thiobacillus* (Lu *et. al.* 1985 and Kelly 1988). The second pathway (PSO pathway) does not involve polythionates and is thought to occur in facultative heterotrophs such as species of *Paracoccus* (Kelly and Syrett 1966). It is important to note before I go into detail explaining these two pathways that they both deal primarily with the oxidation of thiosulfate and not with the oxidation of elemental sulfur. In fact, the process of oxidizing elemental sulfur is still a major question that has not been answered. The oxidation of sulfur compounds by phototrophs is also well understood but this usually deals with sulfur in the form of H_2S. All of the data leading to the development of these three pathways has been obtained from *Bacteria*. There is very little known about sulfur oxidation in *Archaea*. The pathway may or may not resemble either of the pathways I am about to describe. However, a brief description of what is known about lithotrophic sulfur oxidation pathways is important given the focus of this investigation.

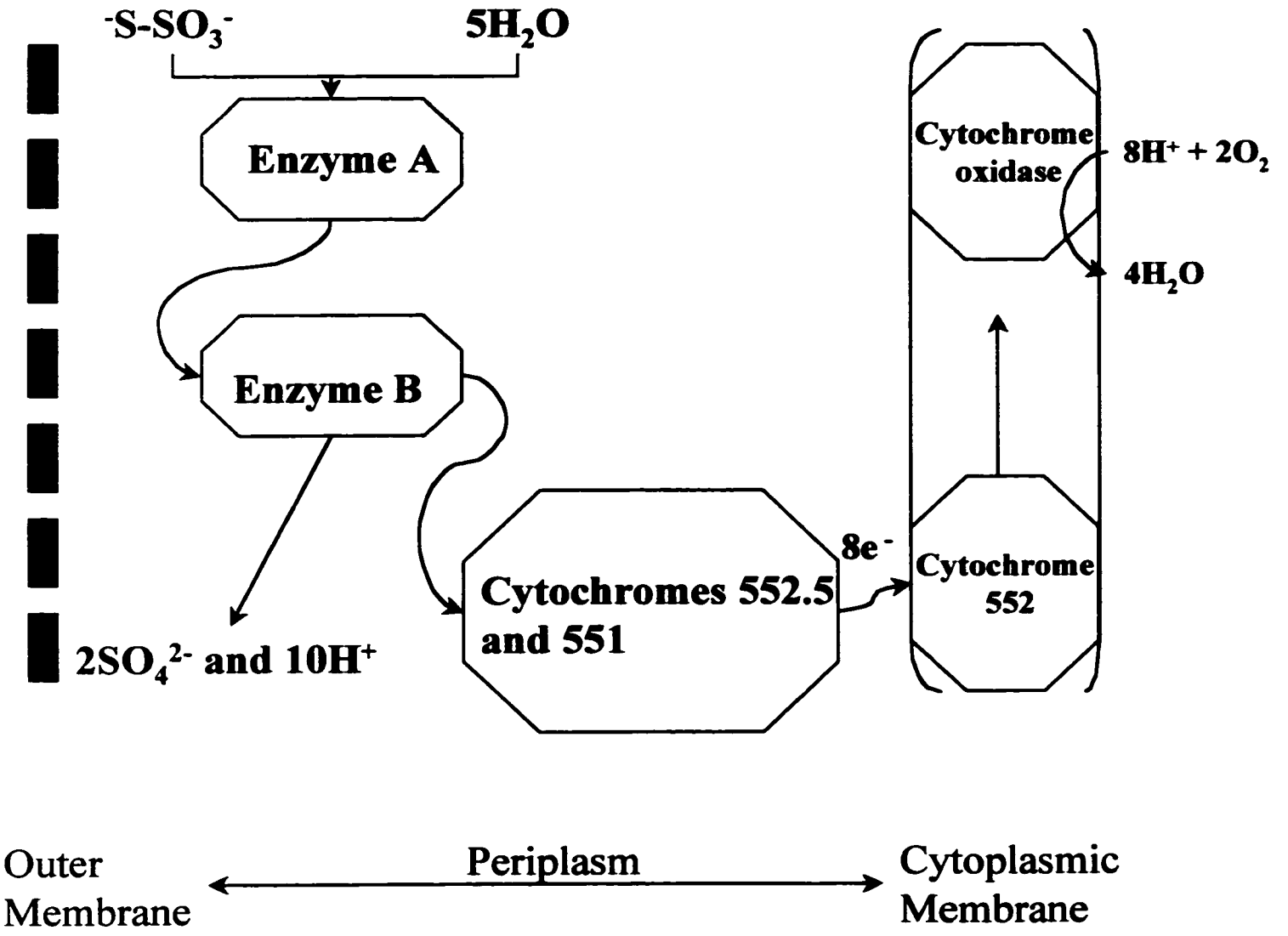
II.A. The *Paracoccus* Sulfur Oxidizing Pathway (PSO)

As I mentioned previously this pathway is believed to occur in facultative heterotrophs capable of growth on carbon sources as well as on reduced sulfur compounds. These organisms are capable of the same modes of metabolism as members of the *Sulfolobales* but do so at mesophilic temperatures. The PSO pathway is composed of two enzymes and two cytochromes. Together these four constituents effect the conversion of thiosulfate to sulfate while transferring electrons to more conventional

c-type cytochromes (Lu *et. al.* 1983 and 1984). The first step of this pathway is the binding of thiosulfate by enzyme A (thiosulfate-binding enzyme) which is a 16 kDa protein (Lu *et. al.* 1984, 1985 and 1988). This enzyme binds thiosulfate in a stoichiometric fashion with one mole of enzyme binding one mole of thiosulfate. Binding of thiosulfate occurs in the periplasm, as does most of the pathway (see figure #2). The $A\text{-}O_3S\text{-}S^-$ complex then binds to enzyme B of the system. Enzyme B is a 61 kDa protein and is thought to be responsible for the oxidation of the SO_3 and perhaps the S moieties to sulfate (Lu and Kelly 1983 and Wodara *et. al.* 1994). The enzyme has a manganese cluster near the reaction center of the enzyme and it has been proposed that this would allow for the binding of the water molecules needed for the oxidation of sulfur compounds (Cammack *et. al.* 1989). It has also been shown that there may be a molybdenum cofactor involved in the action of this enzyme, as mutants in thiosulfate metabolism have been shown to be defective in the synthesis of molybdenum cofactors (Lu and Kelly 1984). It is interesting to note that there have been studies showing that sulfur oxidation in species of *Sulfolobus* can be improved by adding molybdenum to the medium (Buckingham *et. al.* 1989). This similarity may suggest that the pathways share similar components.

The function of the cytochromes in this system is to shuttle electrons to the more conventional cytochromes (Lu and Kelly 1984b and 1988). In this system no free intermediates have been detected and all four components are necessary for the efficient oxidation of thiosulfate. In studies where different enzymes of the system have been left out, little or no activity has been seen (Lu *et. al.* 1985). The actual chemical mechanism

Figure #2: The PSO pathway for the oxidation of thiosulfate. Shown are the enzymes thought to be involved and their corresponding substrates and products. The compartment within the cell where the reaction occurs is also indicated. Modified from Kelly *et. al.* 1998


-S-SO3-
5H2O
Enzyme A
Enzyme B
2SO4 2- and 10H+
Cytochromes 552.5 and 551
8e -
Cytochrome 552
Cytochrome oxidase
8H+ + 2O2
4H2O
Outer Membrane
Periplasm
Cytoplasmic Membrane

behind the binding of the thiosulfate molecule and its subsequent oxidation is not known but there has been one model that has been supported by studies using purified enzymes and horse heart cytochrome c. These studies indicate that the reaction has two steps; one of these steps very rapid and one slower (Fersnt 1985). The model that has been proposed is shown in figure #3. Simply put, enzyme A binds the sulfonate group of thiosulfate ($A\text{-}O_3S\text{-}S^-$). The subsequent binding of enzyme B to the sulfur atom of thiosulfate causes the rapid release of the SO_4^{-2}. This is then followed by the slower cytochrome reducing steps. In figure #3 one can see that enzyme B is responsible for the conversion of elemental sulfur to sulfate through sequential hydration and dehydrogenation steps. The exact mechanism of this reaction is speculative; in fact there have been some studies indicating that there may be another enzyme involved (Kelly *et. al.* 1997).

II.B. The Polythionate Intermediate Pathway (S4I)

Unlike the PSO pathway, little of the enzymology of the S4I pathway is known. Most of what is known about this pathway has been found out using ^{35}S labelled thiosulfate and following the movements of this radiolabelled sulfur over a time course (Kelly and Syrett 1966). The results of these studies have led to the proposed pathway shown in figure #4. In this pathway thiosulfate enters the cell and in the periplasm is converted to tetrathionate, which is then moved across the cytoplasmic membrane (Prank *et. al* 1990 and Meutenberg 1993). Inside the cytoplasm tetrathionate is converted into sulfate which is then exported out of the cell (Kelly 1988). The reaction produces H^+ and electrons, which can then be used to produce a proton motive force. However, as I

Figure #3: The exact chemical changes which are thought to occur during the oxidation of thiosulfate by the PSO system. It is interesting to note the enzyme B is thought to convert sulfur to sulfate by successive hydrations and dehydrogenations (steps 3 - 6). Modified from Kelly *et. al.* 1998.

1) $\mathbf{EnzymeA + [\,^{-}O_3S\text{-}S^{-}] \rightarrow A\text{-}O_3S\text{-}S^{-}}$

2) $\mathbf{A\text{-}O_3S\text{-}S^{-} + EnzymeB\text{-}S^{-} + H_2O \rightarrow B\text{-}S\text{-}S^{-} + SO_4^{-2} + 2H^{+} + 2e^{-} + A}$

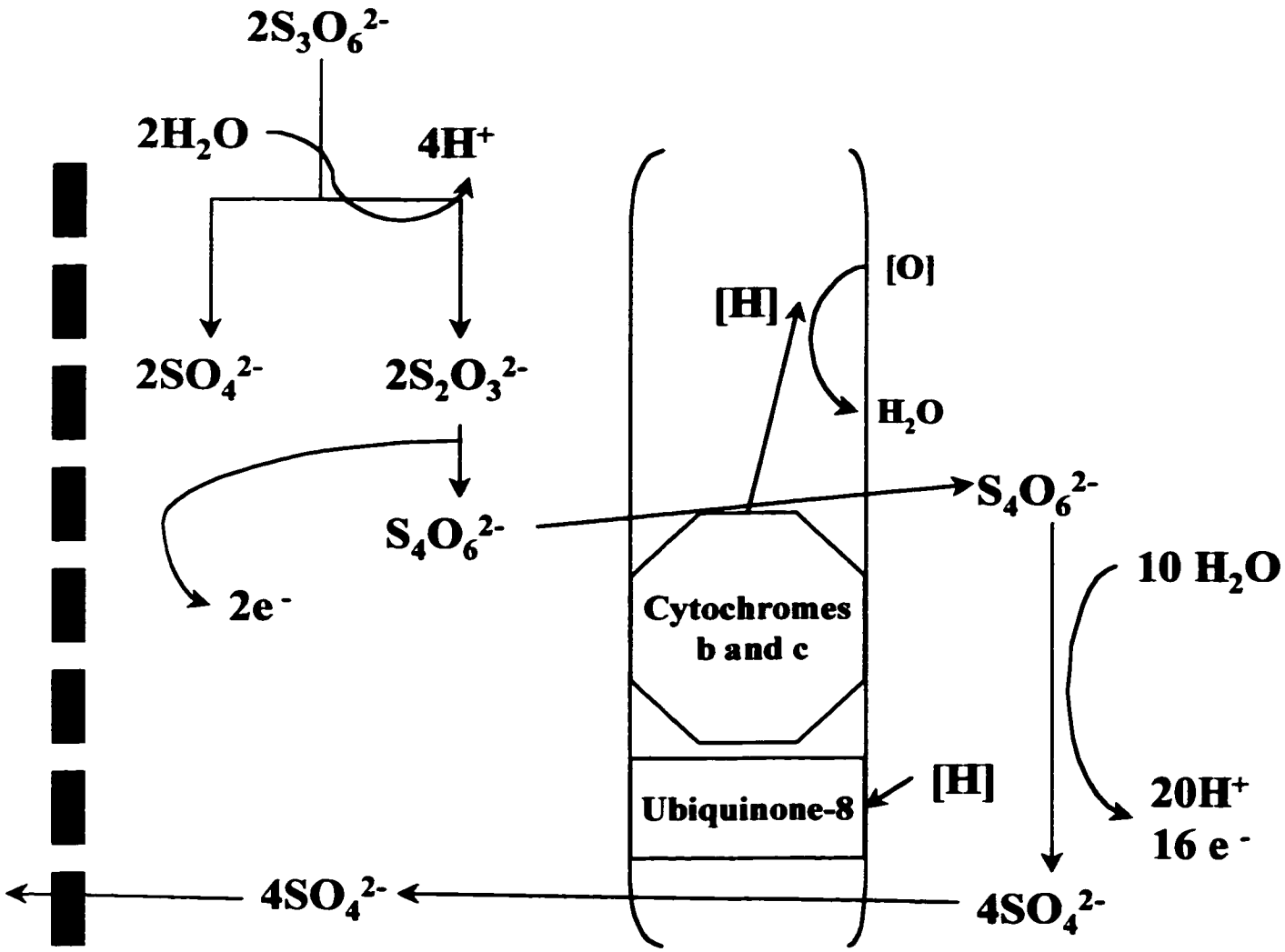
3) $\mathbf{B\text{-}S\text{-}S^{-} + H_2O \rightarrow B\text{-}S\text{-}SO^{-} + 2H^{+} + 2e^{-}}$

4) $\mathbf{B\text{-}S\text{-}SO^{-} + H_2O \rightarrow B\text{-}S\text{-}SO_2^{-} + 2H^{+} + 2e^{-}}$

5) $\mathbf{B\text{-}S\text{-}SO_2^{-} + H_2O \rightarrow B\text{-}S\text{-}SO_3^{-} + 2H^{+} + 2e^{-}}$

6) $\mathbf{B\text{-}S\text{-}SO_3^{-} + H_2O \rightarrow B\text{-}S^{-} + SO_4^{-2} + 2H^{+}}$

Figure #4: The S4I intermediate pathway for the oxidation of thiosulfate. Shown are the chemical changes which have been noted and the cellular compartment in which they are located. It is important to remember that none of the enzymology for these reactions is known. Modified from Kelly *et. al.* 1997.



Outer Membrane

Cytoplasmic Membrane

previously mentioned, very little enzymology is known for this pathway and until that biochemistry is done the exact mechanism is speculative.

II.C. Sulfur Oxidation in the *Sulfolobales*

There has been only one enzyme described which is likely to be involved in sulfur metabolism in the *Sulfolobales*. This enzyme is the sulfur oxygenase from two species of *Acidianus*. The gene for this enzyme has been sequenced and its activity studied. One study by Emmel *et. al.* found that the enzyme from *A. brierleyi* oxidized sulfur to form sulfite and small amounts of thiosulfate (Emmel *et.al.* 1986). Another study by Kletzin found that the enzyme from *A. ambivalens* was able to produce hydrogen sulfide in addition to producing sulfite and thiosulfate (Kletzin 1989). The reaction is proposed to occur by the following:

$$5[S] + O_2 + 4OH^- \rightarrow HSO_3^- + S_2O_3^{2-} + 2HS^- + H^+$$

It is believed that the thiosulfate produced in this reaction is a result of chemical interaction between sulfite and sulfur. This would lead to the products of the sulfur oxygenase being sulfite and hydrogen sulfide in a 1:1 ratio. This reaction is very different from the hydration-dehydrogenation reaction of enzyme B in the PSO pathway. The sulfur oxygenase is a 550 kDa enzyme consisting of identical 40 kDa subunits (Kletzin 1992). The metabolic significance of this enzyme is still vague however, as there has been no link to the electron transport chain found. For the purpose of this study it has a number of attractive characteristics: one characteristic being that this enzyme is not thought to require a cofactor; another being that it has already been shown to have *in vitro* activity. Both of these are important when attempting to express functional proteins in *E.*

coli and when attempting to devise a simple enzyme incubation to accomplish a chemical change.

III) Expression of Heterologous Proteins in *Escherichia coli*

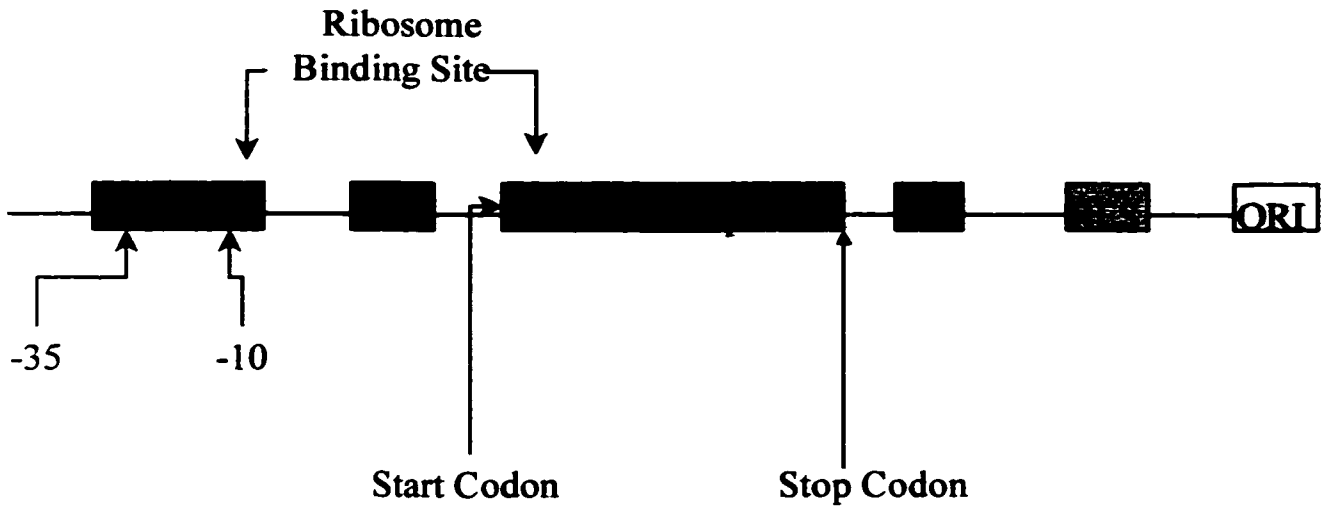
Escherichia coli is the most frequently used prokaryotic host for the expression of foreign proteins. The main contributing factor in this extensive use is the vast knowledge of the genetics and physiology of this organism. However, even with this understanding of the host organism the expression of heterologous proteins is still an unpredictable experience. Characteristics of the foreign gene, its corresponding mRNA and of the protein itself often make the outcome of expression work very much uncertain. In this section I will briefly describe some of the basic characteristics of an *E. coli* expression system. I will then review some of the major problems encountered while expressing proteins in *E. coli* and the strategies that are currently being used to solve these problems.

III. A. General Characteristics of an *Escherichia coli* Expression System

A crucial component of a heterologous expression system is the vector that the gene of interest will be cloned into. The choice of vector can influence how expression is induced, how high the expression will be, whether the protein is exported out of the cell and whether the protein is fused to another molecule for easier purification (for reviews see Hannig and Makrides 1998 and Hockney 1994). In this section I will state the major components of a prokaryotic expression vector (see figure # 5) and briefly describe the function of each of these components.

The promoter is the element that has the most direct effect on the cloned gene. In general terms a good expression promoter will be a strong promoter when induced

Figure # 5: Schematic of a typical expression vector. Major features include the promoter (P), the Shine-Dalgarno sequence (SD), the transcription terminator (TT), the antibiotic resistance gene (RES) and the origin of replication (ORI).
The presence of some or of all of these factors is needed for the expression of proteins in a prokaryotic host. Drawing is not to scale.


Ribosome
Binding Site
ORI
-35
-10
Start Codon
Stop Codon

(express protein at 10-20% of total cellular protein) and show low levels of uninduced expression. This second point is extremely important when expressing proteins that may be toxic to *E. coli*. The most widely used promoters in research are *tac*, *lac* and *T7*. However, there are more than 30 that have been used and more are always being developed (Hannig and Makrides 1998). All three of these commonly used promoters are induced with the addition of IPTG and show various levels of expression. For industrial uses these promoters are not ideal, as the inducer, IPTG is very expensive when used on an industrial scale. For this reason, industrial processes use promoters that can be induced more cheaply such as by thermal induction (*P7* promoter) or by starvation of compounds like phosphate or tryptophan (*pho* and *trp* promoters).

For there to be proper transcription of the cloned gene and this gene only, transcription terminators are placed upstream of the promoter as well as downstream of the cloned gene. This prevents transcription of regions upstream of the cloned gene, which may affect the promoter, and also prevents transcription through the cloned gene into the origin of replication, which could affect the copy number of the plasmid. To allow translation of the gene a ribosome-binding site and Shine-Dalgarno sequence can be placed downstream of the promoter and upstream of the multiple cloning site. In order to enhance translation further the region around the ribosome binding site and sometimes even the 5' end of the gene are made as A/T rich as possible. It is thought that this prevents the formation of secondary structure that would inhibit ribosome binding. In order to halt translation at the proper location a modified stop codon (UAAU) and/or all three normal stop codons are placed at the end of the cloned gene. As with most vectors, expression vectors also possess an origin of replication that controls the copy number of

the plasmid and an antibiotic resistance gene for selection of the plasmid. In most cases it is ideal to keep the copy number rather low to prevent toxic effects of uninduced expression.

These elements represent the basic constituents of a prokaryotic expression vector. There are other features that are often added to deal with specific problems which may arise during the expression. I will go into detail about these features in the next section.

III.B. Obstacles to Proper Expression and Strategies to Overcome these Obstacles

The expression of a single protein at very high levels, especially when this protein is a foreign protein, puts *E. coli* under an enormous stress (for review see Betton *et. al.* 1998). In most cases the protein is being expressed at such a high level that the cell can not keep up and a large fraction of the proteins are folded incorrectly. This causes two problems when expressing foreign proteins. One problem is the formation of inclusion bodies; the other is the degradation of the improperly folded proteins.

When *E. coli* is presented with an unusually large amount of incorrectly folded protein in its cytoplasm it aggregates these proteins into large insoluble masses called inclusion bodies (for review see Schein 1989). It is very difficult, in most cases, to recover functional proteins from inclusion bodies. However, one benefit to having an expressed protein found in inclusion bodies is that it is protected from degradation. *E. coli* possess a large number of proteases including Lon, OmpT and Clp (for review see Gottesman 1996) which are used under normal growth conditions to control gene expression and monitor protein levels in the cell. When faced with large amounts of misfolded protein *E. coli* uses these proteases to degrade them. This degradation causes the protein to have a modified structure and/or function depending on the severity of the

degradation. To limit these problems as much as possible, a number of strategies have been developed.

To increase folding and decrease inclusion body formation one of the most widely used methods is the fusion of the protein of interest to other proteins. Some examples of fusion proteins that are commonly used are maltose binding protein, thioredoxin and DsbA (Zhang *et. al.* 1998, LaVallie *et. al.* 1993, LaVallie and McCoy 1995). It is thought that these fused proteins aid in the folding of the foreign protein because they are recognized by the folding machinery found in *E. coli*. Fused proteins are then removed after purification by site-specific proteases, which recognize a site in the linker between the two proteins. Another method, which is commonly used to increase proper folding, is to express molecular chaperones at high levels along with the protein of interest (for reviews see Georgiou and Valax 1996 and Wall and Pluckthum 1995). The first study to show that this strategy could work used the chaperones GroEL and GroES (Goloubinoof *et. al.* 1989). Since this original work was done, there have been a number of successful studies using this combination of chaperones as well as DnaK/J (Lee and Olins 1992). Yet another strategy involves slowing the growth rate of *E. coli* by lowering the growth temperature. If *E. coli* is growing slower and producing proteins slower, it is thought that there will be more time for the heterologous proteins to fold correctly. This type of thinking has led to lowering the incubation temperature to 30° or 25°, from the optimal 37° (Schein and Noteborn 1988). In most cases it has been found that this helps to some extent but usually lowers the total amount of heterologous protein which is finally recovered as the cells do not grow as densely as they do at higher temperatures. A more drastic measure, which is sometimes taken with proteins that do not fold properly, is to

attach a secretion leader such as OmpA or PelB (Abrahmsen *et. al.* 1986). The addition of this sequence causes the expressed protein to be exported to the periplasm where conditions for folding and aggregation into inclusion bodies are very different from that in the cytoplasm. All of the strategies I have outlined to increase the proper folding of heterologous proteins have been shown to work in some instances and not in others. The bottom line is, there is no accepted protocol for expressing correctly folded proteins. In the end the characteristics of the expressed protein itself and how it interacts with the cytoplasmic environment are what determines whether one of these strategies will work or not. A better understanding of protein folding and of chaperones in *E. coli* is needed if a truly universal method for correctly folding heterologous proteins is to be developed.

Reducing the degradation of expressed proteins has been attempted using many of the same strategies designed to reduce inclusion bodies (for reviews see Enfors 1992 and Murby *et. al.* 1996)). One strategy that is being shown to be particularly useful, is the use of protease-minus strains that are still capable of growth (Meerman and Georgiou 1994). The major problem has been that a great number of these strains are not particularly healthy; they grow poorly and often lyse during growth. Nonetheless, progress has been made in this area and strains such as BL21 (Lon and OmpT minus) have become commonly used (Studier *et. al.* 1990). Other strains lacking many more proteases have been developed but have not been adopted for use on a large scale as problems with their growth are still prevalent (Meerman and Georgiou 1994).

In the previous paragraphs I have outlined the problems and solutions for a heterologous protein to be expressed in *E. coli*. However, there is also often a problem of getting very low or no expression of a foreign gene in *E. coli*. The problem in some

cases where expression is very low is due to the codon usage bias found in *E. coli* (Sharp *et. al.* 1988). *E. coli* tends to use certain codons more than others which code for the same amino acid. This is either a result of or causes the corresponding tRNA pool to be biased. If a foreign gene contains many codons which are found in low frequency in *E. coli* then it is likely that the ribosome will stall along the mRNA as the tRNA for that codon is not available. This in turn causes the expression of the gene to be low. The solution to this problem is, to change the first 10 codons to *E. coli* preferred codons, or to simultaneously express rare tRNA genes (Andrews *et. al.* 1996 and Brinkmann *et. al.* 1989). In more drastic cases a synthetic gene using all *E. coli* preferred codons can be constructed. Expression of foreign genes may also be low if the protein produced is toxic to *E. coli*. Often when this is the case the solution is to export the protein either into the periplasm or the medium by fusing secretion leaders to the gene. This however, brings on other problems such as purifying the protein. Once the protein is exported to the medium it is found in very low concentrations (Hannig and Makrides 1998).

All of these problems with expressing proteins are found when expressing bacterial, archaeal and eucaryotic proteins. They are however more prevalent when expressing eucaryotic proteins. In fact, there have been a large number of *Sulfolobus* proteins expressed in *E. coli,* which have been soluble and functional. These include proteins such as: carboxypeptidase, fumarase, phosphoglyceraldehyde-3-phosphate dehydrogenase, β-glycosidase and β-galactosidase to name a few (Colombo *et. al.* 1994 and 1995, Jones *et. al.* 1995 and Moracci *et. al.* 1992 and 1995). This success is a good indicator that a wide variety of *Sulfolobus* proteins express well in *E. coli*.

IV) Rubber Structure and Recycling

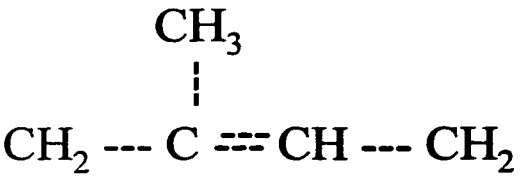
Currently, in the United States alone, waste tires are accumulating at a rate of 200 million per year. This is in addition to the estimated 2.5 billion which are already in tire mounds across the United States (Coghlan 1995). These waste tires represent not only an enormous environmental problem but also a significant resource to be utilized. In this section I will describe what is currently being done to recycle these tires, as well as how the knowledge derived from this research may aid in this industrial process. However, in order to completely comprehend aspects of the recycling process an understanding of the structure of rubber is required.

IV.A. Rubber Structure and Vulcanization

Rubber, and elastomers in general, are broadly divided into two categories: natural and synthetic. Natural rubber is purified from latex, which is the sap of *Hevea brasiliensis*; while synthetic rubber is derived from petrochemicals. In either case the rubber is composed of long polymers based on simple hydrocarbon subunits (see figure #6) (Morton 1981). These polymer chains interact through molecular chain entanglements and weak interactions, such as hydrogen bounds, to give virgin rubber very plastic like physical properties.

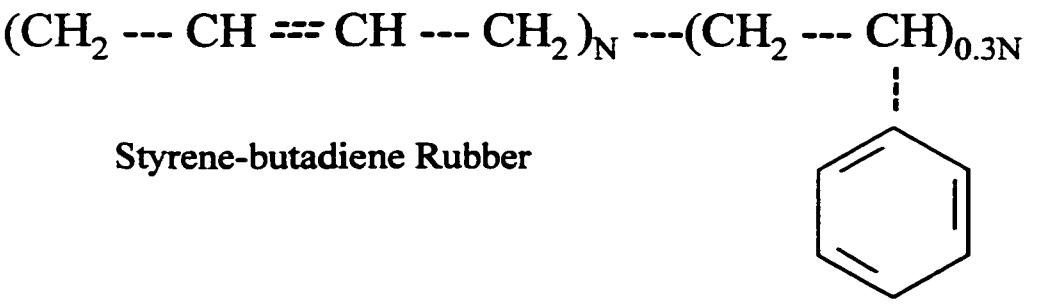
In order to increase the durability and therefore the usefulness of rubber a process known as vulcanization is used. Vulcanization is defined as an intermolecular reaction that increases elasticity while decreasing plasticity in an elastomeric compound (Long 1985). Vulcanization reduces the amount of permanent deformation in a rubber compound when it is exposed to a deforming force. In general terms vulcanization shifts

Figure # 6: Representative types of rubber compounds used to make tires and other rubber products. In each case the basic unit of the hydrocarbon polymer is shown.

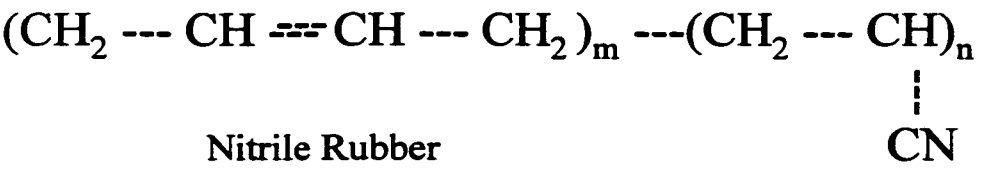


Natural Rubber

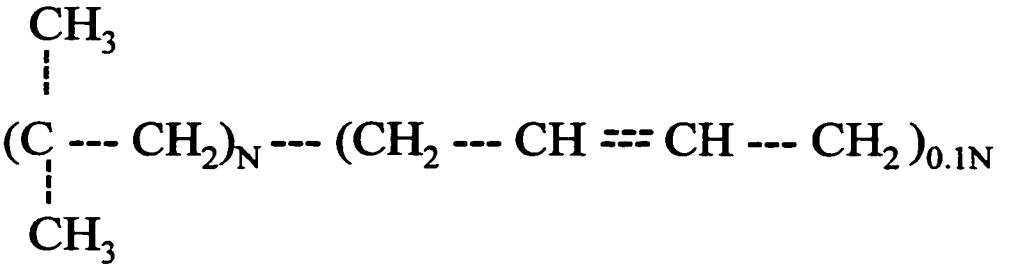
Polybutadiene Rubber



Styrene-butadiene Rubber



Nitrile Rubber



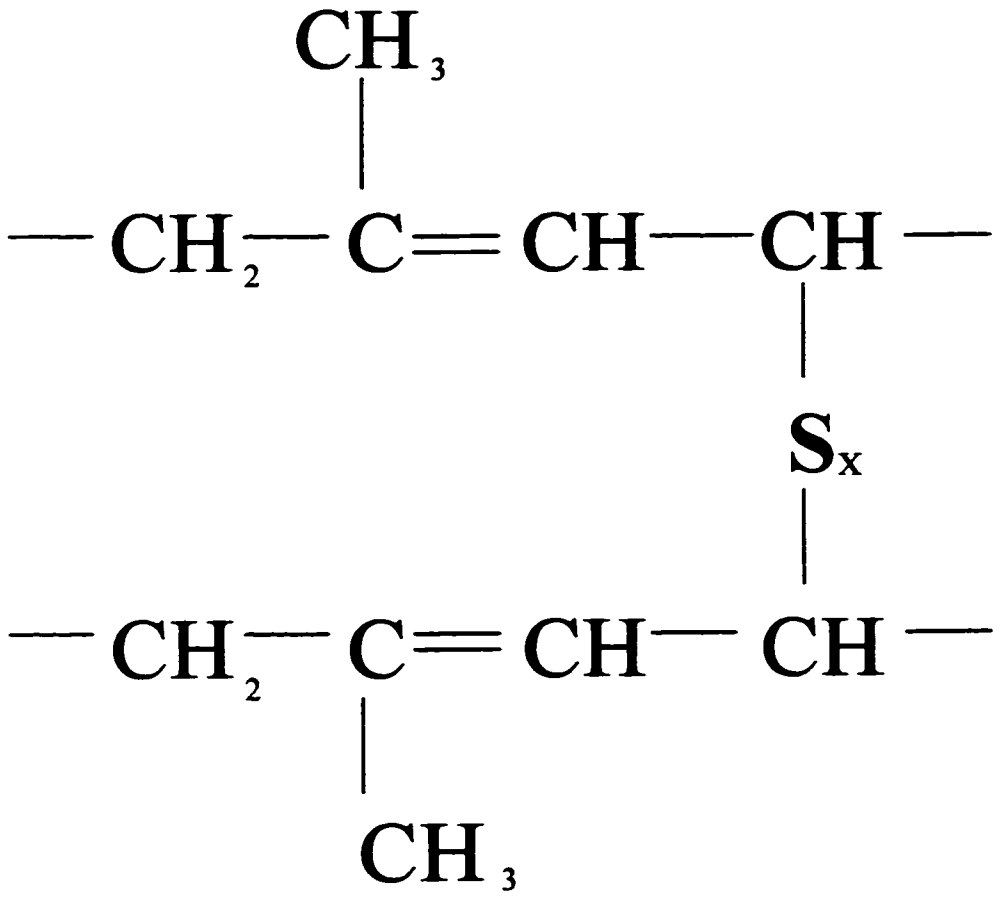
Butyl Rubber

the properties of virgin rubber from viscous plastic behavior to strength and elasticity. The process is based on the formation of cross-links between the hydrocarbon chains found in rubber. In its simplest form vulcanization is the heating of rubber, under pressure and in the presence of 3-8% sulfur. Over time however, the process has been developed to include chemical accelerators (ie. sources of organic radicals such as mercaptobenzothiazole) and anti-degradants (ie. antioxidants, antiozonants, anti flex- cracking agents, heat stabilizers and metal poison inhibitors) (Waddell 1990). These compounds decrease the amount of sulfur used in the process, reduce the vulcanization time and enhance the durability of the resulting rubber compound (Waddell 1990). The key to vulcanization is the formation of sulfur cross-links between the hydrocarbon chains (see figure #7). In accelerated vulcanization, these cross-links are formed exclusively at the allylic carbon and are usually either mono or disulfidic (Long 1985). It is the presence of these sulfur cross-links that make the interaction between the hydrocarbon chains durable enough to be used in the manufacturing of products like tires.

IV. B. Rubber Recycling

Traditionally, rubber has been recycled by grinding it into a fine powder, and then using this material as a filler in newly manufactured rubber products (Burgoyne *et. al.* 1976). However, it has been found that when this rubber crumb is added at higher than 5% (w/w) the physical properties of the rubber begin to degrade (Coghlan 1995). It is thought that this degradation of physical properties is due to the low number of sites, in the rubber crumb, which are available to form new sulfur cross-links (Siuru 1998). Most of the cross-link sites in the rubber crumb had been previously occupied when the rubber

Figure # 7: Chemical changes which occur during vulcanization cause the formation of sulfur cross-links between the hydrocarbon chains. Shown is a monomer of vulcanized natural rubber. The sulfur bonds may consist of single sulfur atoms or polysulfidic chains.



crumb was initially vulcanized. If rubber crumb could be devulcanized it may be possible to increase the percentage used as filler in new rubber products. The improved quality of the rubber with increased amounts of rubber crumb would only be possible if the removal of sulfur from the rubber crumb allowed new cross-links to be formed.

Currently, industry uses thermomechanical processes to break the sulfur cross-links (Mike Burgoyne personal communication). These processes are costly in terms of energy and equipment, much the same as the thermomechanical processes involved in the desulfurization of coal. The goal of this project was to investigate the usefulness of sulfur-oxidizing *Archaea* in removing the sulfur cross-links found in rubber crumb, in the hope of applying this procedure to the devulcanization and eventual recycling of rubber.

[1]

Materials and Methods

I) Whole Cell Methods

The most direct method of devulcanizing rubber crumb using sulfur oxidizing *Archaea* is to simply incubate the cells with rubber and allow them to oxidize the sulfur present. This major group of experiments was focused on investigating a number of factors that affect the amount of sulfur oxidation during this incubation.

I.A. Growing *Sulfolobus solfataricus* P2

i) Rich medium Growth

Sulfolobus solfataricus P2 (DSM 1617) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM, Braunschweig, Germany). *S. solfataricus* P2 was grown in Allen's medium (1959) as modified by Brock *et. al.* (1972). This medium is composed of a basal salts solution supplemented with 0.2% (w/v) sucrose and 0.1% (w/v) yeast extract. The basal salts portion of the medium contained (per litre): 1.3 g $(NH_4)_2SO_4$, 280 mg KH_2PO_4, 250 mg $MgSO_4 \cdot 7H_2O$, 70 mg $Ca_2Cl_2 \cdot 2H_2O$, 20 mg $FeCl_3 \cdot 6H_2O$, 4.5 mg $Na_2B_4O_7 \cdot 10H_2O$, 1.8 mg $MnCl_2 \cdot 4H_2O$, 220 ng $ZnSO_4 \cdot 7H_2O$, 50 ng $CuCl_2 \cdot 2H_2O$, 30 ng $VOSO_4 \cdot 2H_2O$, 30 ng $Na_2MoO_4 \cdot 2H_2O$ and 10 ng $CoSO_4$. The pH of this medium was adjusted to 3 with diluted H_2SO_4 (1:50 (v/v) in water). The 0.2% sucrose and the 0.1% yeast extract were added prior to pH adjustment by adding 500 µL of 20% sucrose and 500 µL of 10% yeast extract to 50 mL of medium.

[1] Unless otherwise stated refer to appendix one for recipes to media and solutions

Cultures were grown in glass hybridization tubes by inoculating 50 mL of medium with an aliquot from a frozen stock or with 500 μL of a liquid culture. Liquid cultures remained viable at room temperature for up to two weeks. After inoculation the cultures were then incubated aerobically for 3-4 days, until the medium became cloudy (OD_{600} ~1), at 78° C in a Tekstar hybridization incubator with moderate agitation. These rich medium grown cells were then harvested by centrifugation in a Baxter Scientific Megafuge (rotor 2101) at 3700 rpm for 7 minutes and used for growth experiments involving rubber and sulfur.

ii) Sulfur and Rubber Growth

Pellets from cells grown in rich medium were resuspended in the rich medium described above with the following changes for all experiments, unless otherwise stated: there was no yeast extract or sucrose added, 10g/L of sulfur or 200 g/L rubber crumb (mesh 80) were added to the medium and 5 mM sodium bicarbonate was added. The sulfur content of the rubber crumb was ~ 2.5%. Characteristics of the medium unique to each group of experiments are described below.

a) pH experiments

In this group of experiments the pH of the medium was varied to include values of 1, 2 and 3. This was done by adding the appropriate volume of diluted H_2SO_4 (1:50 v/v in water). The amount of sulfur oxidized under each condition was then monitored.

b) Carbon Source Experiments

This group of experiments focused on the type of carbon source added to the medium and how this would affect sulfur oxidation. To accomplish this a number of possible sources were used. These included gaseous CO_2, sucrose and sodium

bicarbonate. In the gassing experiments the medium was gassed with CO_2 for 30 seconds at a number of frequencies: once per experiment, every other day throughout the experiment, every day throughout the experiment, twice a day or not at all. For the sucrose experiments 0.16%, 0.12%, 0.08% and 0.04% sucrose were added. In the sodium bicarbonate experiments 5, 10, 20, 30 and 40 mM concentrations were added to the medium.

c) Particle Size Experiments

The goal of these experiments was to elucidate the effect particle size would have on the rate of sulfur oxidation. To accomplish this two different mesh sizes of rubber crumb were used, mesh 20 and mesh 80. These correspond to sizes in microns of 125 and 30 respectively.

I.B. Monitoring of Sulfur Oxidation in Rubber and Sulfur Growth

i) Sulfate Readings

When sulfur is oxidized by *Sulfolobus* sulfate is formed. I therefore decided to monitor the oxidation of sulfur by measuring the concentration of sulfate in the surrounding medium during growth on sulfur and rubber. Under acidic conditions sulfate ions precipitate with the addition of barium chloride (Hanson 1973). Using this as the basis for an assay the amount of sulfate in a solution was determined. This was done by measuring the optical density of the solution after the addition of barium chloride and comparing this to a standard curve derived from solutions of known sulfate concentration. In brief the method is as follows: 1 mL of sample to be tested was mixed with 90 mL of water and 5 mL of conditioning reagent (75 g $NaCl_2$, 30 mL concentrated HCl, 100 mL isopropanol, 50 mL of glycerol and 300 mL of distilled water). Then 0.5 g of barium

chloride was added and the mixture stirred gently for 1 minute. Finally, the absorbance at 420 nm was measured, after zeroing the spectrophotometer with water. This value could then be converted to a concentration (g/L) using the formula $[SO_4^{-2}]=OD_{420nm} / 0.1033$ derived from the standard curve (see appendix II). For statistical analysis the rate of sulfate production in grams per litre per day was calculated using linear regression. These rates were then compared by either ANOVA or by t-tests depending on the number of treatments being compared. This analysis was performed using the computer software package GraphPad Prism version 2.01.

II) Enzyme Based Methods

In these enzyme-based methods I was attempting to clone and express the sulfur oxygenase/reductase gene from *Acidianus ambivalens* (Kletzin 1992) in *E. coli*. The product of this gene had previously been shown to have the ability to oxidize sulfur (Kletzin 1989) and it was my intention to investigate whether it would be applicable to the devulcanization of rubber crumb.

II.A. Cloning of the Sulfur Oxygenase (*sor*)

i) Description of Original *sor* Clone

Plasmid pSOR-1/17 (Kletzin 1992) was kindly provided by Dr. Arnulf Kletzin (Max-Planck-Institut für Biochemie, Martinsried, Germany). This plasmid was constructed by inserting a 5.8 kbp Hind III fragment of *Acidianus ambivalens* (previously known as *Desulfurolobus ambivalens*) genomic DNA into pUC18. This 5.8 kbp insert contained 3 kbp of DNA which had been sequenced. This DNA contained 4 open reading frames (ORFs) one of which was found to code for the sulfur oxygenase/reductase or *sor*.

ii) PCR Amplification of *sor* from pSOR-1/17

In order to clone the entire *sor* gene, and only the *sor* gene, it was necessary to separate the *sor* gene from the other ORFs on plasmid pSOR-1/17. To do this primers for the 5` and 3` end of the *sor* gene were made, sorF (5` **ATGCCGAAACCATACGTT GCTATAAAC** 3`) and sorR (5` **TTATTGTTCGTTTAAATATTCTCTC** 3`) respectively. The gene was then amplified using the polymerase chain reaction (PCR) (Saiki et. al. 1988). PCR was performed on a Gene Amp 9600 PCR system using 10 ng of plasmid pSOR-1/17 as template, 30 pmol of each primer, PCR buffer with $MgCl_2$ and 1 unit of Taq polymerase from Boehringer Mannheim. The thermal cycling conditions were as follows: 95° for 5 minutes, 30 cycles of: (94° for 1 minute, 55° for 2 minutes and 72° for 2 minutes), 74° for 10 minutes and finishing with lowering the temperature to 4°. To check for the presence and size of this PCR product 5 μL were run on a 1% agarose gel.

iii) Cloning of the *sor* gene into pUC18

To clone the PCR product I needed to repair the 5` and 3` ends and then clone by blunt-end ligating it into pUC18.

a) End repair of PCR amplified *sor*

One microlitre of PCR-amplified *sor* was added to 11.7 μL of H_2O, 2 μL of 10X Klenow buffer, 1.5 μL of dNTP (1mM), 0.3 μL T7 DNA pol form II (3 units) and 2.5 μL of sequencing grade Klenow (2.5 units). This mixture was incubated at 12° C for 30 minutes to repair the ends and then at 65 ° C for 5 minutes to end the reaction. To further clean the DNA it was precipitated by adding 10 μL of H_20, 3 μL of 3 M sodium acetate

pH 5.5 and 70 μL of 95% ethanol. The DNA was pelleted by centrifugation at 10 000 rpm for 10 minutes in a IEC Micromax microcentrifuge and then washed with 70 % ethanol, air dried and resuspended in 13.6 μL of water by incubation at 37° for 1 hour with occasional vortexing.

b) Phosphorylation of end-repaired *sor* gene

Phosphorylation of the end-repaired *sor* gene was carried out by adding 2 μL of 10 mM ATP, 2 μL of 10 X PNK buffer (New England Biolabs) and 2.4 μL (24 units) of polynucleotide kinase (New England Biolabs) to the product of end repair. This mixture was then incubated at 37° for 30 minutes and the reaction was halted by incubation at 65° for 10 minutes.

c) Ligation of PCR-amplified *sor* into pUC18

One microlitre of 10X ligase buffer without ATP, 1 μL (50 ng) of *SmaI* cut-dephosphorylated pUC18 (Pharmacia), 0.3 μL of 10 mM ATP and 1.2 μL of H_2O were added to 10 μL of end-repaired and phosphorylated PCR product. The ingredients were mixed well and then 1.5 μL of T4 DNA ligase (1.5 U) was added. The mixture was then incubated at 12° for 18 hours.

d) Transformation of pUC18/*sor*

Epicurian coli XL1-Blue MRF` supercompetent cells (Stratagene) were thawed on ice. Sixty microlitres of these cells were then transferred into a 14 mL polypropylene tube and incubated with 1.0 μL of 1:10 β-mercaptoethanol for 10 minutes on ice. 5 μL of ligation were then added and the mixture was incubated for 30 minutes on ice. After the 30 minute incubation the cells were heat shocked at 42° for 45 seconds and then incubated on ice for 2 minutes. After the two minute incubation 0.9 mL of SOC broth

was added and the cells were allowed to recover for one hour at 37 ° with gentle shaking. Following recovery 200 μL, 100 μL and 50 μL volumes of the cells were plated onto YT-amp-Xgal-IPTG plates and incubated for 18 hours at 37°.

e) Storage of pUC18/*sor* clones

Ninety-six white colonies were picked using autoclaved wooden toothpicks and grown in 100 μL of YT-amp broth in 96 well microtiter dishes at 37° for 18 hours. After growth 30 μL of sterile 75% glycerol was added with thorough mixing and the cultures were frozen at -80°.

iv) Screening of pUC/*sor* clones for positives

a) Preparation of blots for hybridization

Prior to the freezing of the pUC18/*sor* clones each clone was stamped onto GeneScreen Plus membranes (Dupont / NEN) using a 96 pronged metal stamp. The membranes were placed onto YT-amp plates and the clones allowed to grow for 22 hours at 37 °. Before the membranes could be hybridized they had to be treated to bind and denature the DNA and to remove the cellular debris. To accomplish this the membranes were washed for 5 minutes in each of the following solutions: 1) 10 % SDS (to lyse the bacteria), 2) 0.5 M NaOH (denaturing solution), 3) 0.5 M Tris-Cl pH 7.4 and 1.5 M NaCl (neutralizing solution), 4) 2X SSC (washing solution). After this washing the blots were air dried and then autoclaved for 1 minute on the dry cycle in order to bind the DNA. Immediately before hybridization the blots were soaked in prewash solution (0.5% SDS, 1mM EDTA and 5X SSC) for 1 hour at 55°. Following this the cell debris was removed manually with a Kimwipe.

b) Hybridization

Blots prepared as above were placed into glass hybridization tubes and prehybridized in 15 mL of hybridization solution (1M NaCl, 50mM Tris-Cl pH 7.6, 5% SDS and 50 % v/v formamide) and 375 μL of 3 mg/mL denatured and sheared herring sperm DNA at 38° for 2 hours.

To make the probe 1 μL of random hexamer primers (2.5 μg/μL) was added to 500 ng of PCR amplified *sor* and brought up to a final volume of 8.5 μL with H_2O. The mixture was then boiled for 5 minutes and quick chilled on ice. Next 1.25 μL of 10X RP-C buffer, 1.5 μL of BSA, 1 μL of [α - ^{32}] dCTP (10 μCi/μL) and 0.5 μL (1U) of labeling grade Klenow enzyme were added, in order, and the labeling reaction was performed at 37° for 45 minutes. The probe was then precipitated by adding two volumes of 0.5 M ammonium acetate in 95 % ethanol, centrifuged for 10 minutes at 10 000 rpm on a Sarstedt model MH2 microcentrifuge and resuspended in 100 μL of H_2O. Before adding the probe to the hybridization solution it was boiled for 3 minutes and quick chilled on ice. Once the probe was added the blots were incubated overnight at 38°.

The next day the hybridization solution was drained and the blots rinsed twice with 2 X SSC. The hybridization tube was then half filled (75 mL) with prewarmed 2 X SSC / 1% SDS and incubated for 1 hour at 65°. Following this wash the blots were washed twice in 2 X SSC and placed in an autoradiography cassette between sheets of plastic wrap and allowed to expose Kodak X-OMAT film with an intensifying screen at -80°.

c) Preparation of plasmid DNA of positive clones for sequencing

Clones, which were shown to be positive for the presence of the *sor* gene, were sequenced in order to determine that the insert was indeed the *sor* gene and that there were no errors introduced during the PCR amplification of the gene. Plasmid DNA was extracted from 5 mL cultures (YT-amp medium) grown at 37° for 18 hours using the Nucleobond AX PC-20 kit. Cells were pelleted by centrifuging for 7 minutes at 3700 rpm in a Beckman Scientific Megafuge (rotor 2101) and resuspended in 500 μL of buffer S1 (50 mM Tris-Cl, 10 mM EDTA and 400 μg RNase A /mL; pH 8.0). Cell lysis was accomplished by adding 500 μL of buffer S2 (200mM NaOH and 1 % SDS) and mixing gently so as not to sheer the genomic DNA. To neutralize this solution and to precipitate the cellular debris 500 μL of buffer S3 (2.8M potassium acetate pH 5.2) was added. Following this addition, the cellular debris was pelleted by centrifuging for 10 minutes at 13 000, in a IEC Micromax microcentrifuge, and the supernatant was transferred to a Nucleobond AX column that had been washed with 1 mL of buffer N2 (100 mM Tris/H_3PO_4, 15 % Ethanol and 900 mM KCl pH 6.3). After the sample had passed through the column three 1 mL washes of buffer N3 (100 mM Tris/H_3PO_4, 15 % ethanol and 1.15 M KCl pH 6.3) were added to the column. Plasmid DNA was then eluted from the column with 800 μL of buffer N5 (100mM Tris/H_3PO_4, 15 % ethanol and 1 M KCl pH 8.5) and finally precipitated with 600 μL isopropanol, washed with 70 % ethanol and resuspended in 20 μL of H_2O.

d) Sequencing of pUC/*sor* clones

Sequencing was performed using the Applied Biosystems Prism Dye terminator cycle sequencing kit. In each reaction 500 ng of plasmid DNA, 3.2 pmol of either M13

universal forward, reverse, sorF or sorR primers and 7 μL of reaction terminator mix in a final volume of 20 μL were placed in a PCR tube. The sequencing reaction went through the following cycles: 96° for 1.5 minutes; 25 cycles of 96° seconds, 50° for 5 seconds and 60° for 4 minutes.

Sequencing reactions were cleaned by adding 2 μL of 3 M sodium acetate pH 4.6 and 50 μL of 95 % ethanol followed by centrifuging at 10 000 rpm for 15 minutes. Pellets were washed with 70 % ethanol and vacuum dried for 1 hour. Dried pellets were stored at -20° until run on a sequencing gel. The sequencing gel was run by André Bergeron on an Applied Biosystems ABI373 sequencer.

e) Assembly and Analysis of Sequences

Assembly of the sequences into a contig was performed using the Staden package version 1995a (Dear and Staden 1991). Once this contig was assembled the FASTA format sequence for the contig was used to perform a BLASTX search using the NCBI web site: http://www.ncbi.nlm.nih.gov/. The identification of the PCR product as the *sor* gene could then be made. This in turn led to the cloning of this PCR product into the expression vector pKK223-3 (Pharmacia).

v) Cloning of PCR amplified *sor* into expression vector pKK223-3

a) Preparation of pKK223-3

Two point five micrograms of pKK223-3 (Pharmacia) was digested with SmaI (10U) for 3 hours at 37° using the supplied buffer (Sure Cut A from Boehringer Mannheim). This digested DNA was purified by adding 1 volume of phenol/chloroform (1:1 v/v) and vortexing vigorously. The DNA was precipitated by adding 1:10 volume of sodium acetate and 2 volumes of ethanol. After centrifugation the DNA was washed in

70% ethanol and resuspended in 50 µL of TE (10 mM Tris pH 8, 1 mM EDTA). This linearized vector DNA was dephosphorylated by adding 1 µL (1U) of alkaline phosphatase (Boehringer Mannheim) and incubating at 37° for 1 hour. The enzyme was deactivated by heat denaturation at 65° for 15 minutes. The DNA was then ethanol precipitated as stated above and resuspended in 50 µL of TE.

b) Making competent *E. coli* JM105

Escherichia coli JM105 (*thi, rpsL(Str^r), endA, sbcN15, hsdR4, supE, Δ (lac-proAB), F' [traD36, proAB$^+$, lacI^q, lacZΔM15]*) was grown in 4 mL of YT at 37° for 18 hours with shaking in a 14 mL polypropylene tube. One millilitre of this overnight culture was used to inoculate 50 mL of YT in a 500 mL flask. This culture was then incubated at 37° with shaking until the OD_{600} was 0.5-0.7. The cells were chilled on ice and pelleted by centrifuging at 3700 rpm in a Beckman Scientific Megafuge (rotor 2101) for 10 minutes. It was important that from this point on the cells remained chilled. The cell pellet was resuspended in 25 mL of 100 mM $CaCl_2$ by gently pipetting up and down with a 25 mL sterile glass pipette. The cells were incubated on ice for 1-2 hours. Following incubation the cells were centrifuged at 3700 rpm in a Beckman Scientific Megafuge (rotor 2101) for 10 minutes and resuspended in 5 mL of 100 mM $CaCl_2$ by gentle pipetting. One point five millilitres of sterile glycerol was added and the solution mixed gently by rocking. The cells were then frozen at -80° in 200µL aliquots.

c) Ligation of PCR-amplified *sor* into pKK223-3

PCR-amplified *sor* was end repaired and phosphorylated as mentioned previously. The ligation was setup by adding 1.5 µL, *Sma*I digested dephosphorylated pKK223-3, 1 µL of 10X ligase buffer without ATP, 0.3 µL of 10 mM ATP, 0.7 µL H_2O and 1.5 µL T4

DNA ligase (1.5 U) to 10 μL of end-repaired, phosphorylated *sor* and incubated at 12^0 for 16 hours.

d) Transformation of *E. coli* JM105 with pKK223-3/*sor*

The 15 μL ligation mixture was added to 200 μL of competent *E. coli* JM105. The cells and the DNA were incubated on ice for 30 minutes and then heat-shocked at 42° for 45 seconds. After chilling on ice 750 μL of SOC broth was added and the cells were incubated at 37° for 1 hour with gentle shaking. Following this incubation 50, 150 and 200 μL amounts were spread onto YT-amp-Xgal-IPTG plates and incubated for 18 hours at 37°.

e) Storage of pKK223-3/*sor* clones

Ninety-six white colonies were picked, using autoclaved wooden toothpicks, and grown in 100 μL of YT-amp broth in 96 well microtiter dishes at 37^0 for 18 hours. After growth, 30 μL of sterile 75% glycerol was added with thorough mixing and the cultures were frozen at -80°.

f) Screening for positive clones

The screening for positives was performed as described for the cloning of *sor* into pUC18.

g) Sequencing of positive pKK223-3/*sor* clones

Sequencing was performed as previously described with the pUC18/*sor* clones except the primers used were pKKF (5` **TGAGCGGATAACAATTTCACAC** 3`), sorF and sorR.

h) Assembly and Analysis of sequences

The assembly and analysis performed on the raw sequence data was the same as for the pUC18/*sor* clones with special attention being paid to the orientation of the gene within the vector. This was necessary because the ligation was blunt-ended and therefore the insert could enter in either orientation. To analyze this, the sequence from the pKKF primer was BLAST searched to determine whether this sequence matched the 5` or the 3` end of the *sor* gene. If it matched the 5` end of the gene then the insert was in the correct orientation. When a clone with the correct orientation was found it was chosen to work with in expression studies. To determine if the entire sequence of the cloned gene was correct its sequence was aligned with the known sequence for the *sor* gene using ClustalW version 1.60 (Thompson *et al* 1994).

II.B. Expression of *sor* in *Escherichia coli JM105*

i) Growth Conditions and Induction of Expression of *sor*

As a starter culture 10 mL of YT-amp was inoculated from a frozen stock using a sterilized wooden stick and incubated at 37° for 18 hours. This starter culture was then used to inoculate a 1 L culture of YT-amp and it was incubated for 12 hours until the OD_{600}= 0.8 - 1. Once the culture had reached an OD_{600}= 0.8 - 1 the expression of *sor* was induced by adding 1 mL of 1 M IPTG to give a final concentration of 1 mM. The culture was then allowed to grow for another 24 hours before the extraction of the proteins was done.

ii) Extraction and Purification of Sor

After growing under induced conditions for 24 hours the cells were collected by centrifugation at 3700 rpm in a Beckman Scientific Megafuge (rotor 2101) for 10 minutes

in a chilled rotor. The cell pellet was then washed in 30 mL of ice cold 50 mM sodium phosphate buffer to remove traces of medium from the pellet. The pellet was resuspended in extraction buffer (50 mM sodium phosphate buffer with 0.7 mM phenylmethanesulfonyl fluoride) by pipeting and vortexing. At this stage it was very important to thoroughly resuspend the cells. Two millilitres of 10 mg/mL lysozyme solution was then added to the cells and incubated for 20 minutes at room temperature. After the cell wall had been digested by the lysozyme 1 mL of 120 mM sodium deoxycholate and 1 mL of 0.5 mM DNase1 (150 U) were added to further degrade the cell envelope and to digest the DNA. After a 30 minute incubation at room temperature 50 mg of protamine sulfate was added and the mixture was vortexed thoroughly. The protamine sulfate was used to precipitate the nucleic acids. This precipitation was aided by centrifuging the mixture at 4300 rpm in a Beckman Scientific Megafuge (rotor 2101) for 15 minutes. The supernatant from this centrifugation was then subjected to two rounds of incubation at 80° for 20 minutes, chilling on ice and centrifugation at 4300 rpm in a Beckman Scientific Megafuge (rotor 2101) for 15 minutes. After each centrifugation the supernatant was kept. The purpose of this step was to heat denature the native *E. coli* proteins and therefore somewhat purify the heat stable Sor. The protein sample was now ready to be analyzed by SDS-PAGE.

iii) SDS-PAGE of protein extracted from *E. coli* pKK223-3/*sor*

a) Pouring of acrylamide gels for SDS-PAGE

To ensure the glass plates were sufficiently clean they were washed thoroughly with detergent and water and then rinsed with 95% ethanol and allowed to dry. Finally the plates were scrubbed with a Kimwipe wetted with 95 % ethanol and allowed to dry.

The plates were then clamped together and the sides of the plates sealed with 1% agarose to prevent leakage. Once this had been done the resolving gel could be made and poured. The resolving gel consisted of 10.65 mL H_2O, 7.5 mL of 1.5 M Tris-Cl pH 8.8, 300 μL of 10% SDS, 11.4 mL of 40 % acrylamide:bisacrylamide (19:1) from Biorad, 150 μL of 10% ammonium persulfate and 30 μL of TEMED. The above ingredients were added in the order listed and mixed well. The resolving gel was poured using a 10 mL pipette until it was approximately 1.5 centimetres from the bottom of the comb. The resolving gel was overlaid with H_2O and allowed to polymerize. Once the resolving gel had polymerized the overlaying water was poured off and any excess removed using filter paper. Now the stacking gel could be poured. The stacking gel consisted of 6.4 mL H_2O, 2.5 mL of 0.5 M Tris-Cl pH 6.8, 100 μL 10% SDS, 1.3 mL of 40% acrylamide:bisacrylamide (19:1) from Biorad, 50 μL of ammonium persulfate and 20 μL of TEMED. The ingredients were again added in order and mixed well. The stacking gel was poured using a Pasteur pipette until it reached the very top of the glass plate. The comb was then added and the stacking gel allowed to polymerize. Once the stacking gel had polymerized the comb was removed and the wells were rinsed well with H_2O to remove any unpolymerized acrylamide.

b) Preparation of samples for SDS-PAGE

In preparation for running of a gel the protein extracts were mixed with sample buffer and boiled for three minutes. Ten microlitres of 5 X sample buffer (4 mL H_2O, 1 mL 0.5 M Tris pH 8.8, 1.8 mL of 10 % SDS, 800 μL of glycerol, 400 μL of β-mercaptoethanol and 200 μL of 0.1% bromophenol blue) was mixed with 40 μL of

sample. After three minutes of boiling the sample was quick chilled on ice and was then ready to be loaded.

c) Electrophoresis of Proteins using Polyacrylamide Gels

The gel was clamped into the electrophoresis chamber and the upper reservoir was sealed with agarose to prevent leakage. 10 X running buffer (15 g Tris, 72 g glycine and 5 g SDS per litre) was then diluted 1:10 with H_2O and added to both the upper and lower buffer tanks and the samples were loaded. The gel was then run for 18 hours at 12 V/cm.

d) Visualization of Proteins Using Coomassie Blue

Protein gels were stained using Coomassie Blue R-250 dye (Biorad). The gel was soaked in staining solution (0.2% Coomassie Blue R-250, 10% acetic acid and 40% methanol) for 3 hours at room temperature with gentle shaking. Destaining was performed in a 10% acetic acid and 10 % methanol solution at room temperature with gentle shaking for approximately 48 hours or until the background was clear. For destaining, small pieces of Kimwipe were added to the solution to bind the dye as it was removed form the gel.

iv) In vitro Assay Using Recombinant Sor

a) Enzyme Incubation

Total protein extracted as described above was added to 1 litre of prewarmed (78°) assay buffer (70 mM Tris-acetate, 0.005 % Tween 20 and 2% wt/vol powdered sulfur or 20% wt/vol rubber crumb) and incubated at 78°. During the incubation samples were taken at appropriate time points to be analyzed for changes in sulfate, thiosulfate and sulfite concentrations.

b) Determination of Sulfate, Sulfite and Thiosulfate Concentrations

The three possible products from the enzyme reaction with sulfur were sulfate, hydrogen sulfide, thiosulfate and/or sulfite. Therefore to determine the presence or absence of activity it was necessary to test for any changes in concentrations of all four of these substances.

The method for determining sulfate concentration in this case was the same as previously mentioned in the whole cell section. The method was as follows: 1 mL of sample to be tested was mixed with 90 mL of water and 5 mL of conditioning reagent (75 g $NaCl_2$, 30 mL concentrated HCl, 100 mL isopropanol, 50 mL of glycerol and 300 mL of distilled water). Point five grams of barium chloride was added and mixing was continued for 1 minute. Finally, the absorbance at 420 nm was measured after zeroing the spectrophotometer with water. This value could then be converted to a concentration in grams/litre. For statistical analysis a rate of sulfate production in grams per litre per day was calculated using linear regression. These rates were then compared by either ANOVA or by t-tests depending on the number of treatments being compared. This analysis was performed using the software package GraphPad Prism version 2.01.

The basis for the method I used for measuring sulfite was this compound's ability to reduce Fuchsin, thereby causing a change in the spectrometric characteristics of a solution (Kletzin 1989). In this method an acidic solution of Fuchsin was made by adding 40 mg of Fuchsin to 87.5 mL of H_2O and 12.5 mL of concentrated H_2SO_4. Next 50 µL of this solution was added to 195 µL of H_2O and 250 µL of the sample to be tested. After a ten minute incubation at room temperature 5 µL of formalin was added and the reaction was incubated for another 90 minutes at room temperature, after which the absorbance at

570 nm was taken. The concentration of sulfite was then determined by comparison to a standard curve (see appendix II).

The measurement of thiosulfate concentration was based on the titration of this chemical with iodine (Hanson 1973). The first step was to prepare the sample by adding the sample to H_2O to give a final volume of 200 mL (in most cases 100 mL of sample was tested) and adding 50 mL of diluted H_2SO_4 (1:3 v/v in H_2O). To this mixture 25 mL of 0.0125 N iodine was added and the mixture immediately titrated with 0.0125 N thiosulfate until the iodine was consumed. Next a few drops of 2% starch indicator solution (VWR) was added to the sample and the titration with 0.0125 N thiosulfate was continued until the solution turned blue. The final volume of thiosulfate used was recorded. The same procedure was then carried out on 200 mL of water as a blank determination. The concentration of thiosulfate in the sample was then calculated using the formula:

$$[S_2O_3^{2-}]\text{mg/L} = 1400\,(V_2 - V_1)\,/V$$

where V_2= the volume of thiosulfate used with the sample (mL)

V_1= the volume of thiosulfate used in the blank (mL)

V = the volume of sample (mL)

III) Locating Homologues to the Sulfur Oxygenase

The goal of this group of experiments was to locate homologues to the *sor* gene in a number of species related to *A. ambivalens*. To accomplish this goal cosmid libraries and genomic DNA were screened by hybridizations using the *A. ambivalens* copy of *sor* as a probe. Genomic DNA was also screened by PCR using degenerate primers designed

using the *A. ambivalens* and the *Aquifex aeolicus* Sor sequences (forward 5` GGICCIAARGTITGYAT 3`; reverse 5` RTGCATYTCYTCRTGRTC 3`, see figure # 8).

III.A. Screening Cosmid Libraries for *sor*

Cosmid libraries for 8 species of sulfur oxidizing *Archaea* were kindly prepared and provided by Anick De Moors. These libraries were provided on colony blots, which were prepared using the same method previously described in the section on screening for pUC/*sor* clones. The eight species screened using this method were *Sulfolobus solfataricus* P2, *Sulfolobus solfataricus* P1, *Sulfolobus solfataricus* MT4, *Sulfolobus shibatae*, *Sulfolobus acidocaldarius*, *Sulfolobus hakonensis*, *Sulfolobus tokodaii* and *Metallosphaera sedula*. The relationship between these organisms and *A. ambivalens* is shown in figure #1.

Colony blots for these species were screened with the PCR-amplified *sor* gene from *A. ambivalens* using the same hybridization procedure as outlined in the section on screening for pUC/*sor* clones.

III.B. Screening Genomic DNA for *sor*

i) Growth of Organisms

The growth of *S. solfataricus* P2 was previously described in the section on growth on rich medium. The other seven species were grown in the same Allen's medium and using the same method described in that section with the following changes. *S. solfataricus* P1 was supplemented with 0.2% maltose instead of 0.2% sucrose and *M. sedula* was given only 0.1% yeast extract with no sugar. Each organism was grown at the specific temperature which was optimal for its growth: *M. sedula* 65°C; *S. acidocaldarius* 70°C ; *S. solfataricus* MT4 85°C and the remaining species at 78°C. Stocks of each

Figure #8: Alignment of protein-coding sequences for the *sor* gene from *Acidianus ambivalens* and *Aquifex aeolicus*. The alignment was performed using ClustalW version 1.60. Regions shown in red were used to construct degenerate primers.

Forward

```
Aquifex aeolicus       VAINMAELKNEPKTFEMFASVGPKVCMVTARHPGFVGFQNHIQIGILPFGNRYGGAKM--
                       VAIN A++ N P+  E+   VGPKVC+ TA H GF+GF   IQ+G  P G R+G AK+
Acidianus ambivalens   VAINKAKVNNTPRFQELMYQVGPKVCITTAMHEGFLGFHAFIQVGAHPLGGRFGAAKVIN
```

Reverse

```
Aquifex aeolicus       -----DMTKESST------VRVLQYTFWKDWKDHEEMHRQNWSYLFRLCYSCASQMIWGP
                           + +E  T      + + QYT W+ W+DHEEMH Q +  +F LC  C ++++ GP
Acidianus ambivalens   ADSLEAIQREGLTAIKYNPLELWQYTVWEKWEDHEEMHYQQFDRIFELCVGCFTEVVEGP


Aquifex aeolicus       WEPIYEIIYANMPINTEMTDFTAVVGKKF---AEGKPLDIPVISQPYGKRVVAFAEHSVI
                        EPIY +  ANMP   + TD   V+G+      +GK   IP + +   +R+     H V
Acidianus ambivalens   NEPIYVVREANMPPVIDFTDVPKVLGESMMQAMQGKG-GIPKV-RLLTQRIAVVGIHKVK


Aquifex aeolicus       PGKEKQFEDAIVRTLEMLKK-APGFLGAMVLKEIGVSGIGSMQFGAKGFHQVLENPGSLE
                        G E++F +  V TLE+L K + G +G M+L++IG S  G+ Q     F +V+ + G++
Acidianus ambivalens   EGMEEKFIEGAVETLELLNKYSAGMIGWMILEKIGESPYGTFQIKPPEFWEVVASRGAVP


Aquifex aeolicus       PDPNNVM---YSVPEAK----NTPQQYIVHVEWANTDALMFGMGRVLLYPELRQVHDE-V
                       P     +   Y  PE +      P+++IVH+EW+  ++ MFG     + P++++VHDE V
Acidianus ambivalens   PKTRETIWGEYGKPEFQLEPAGHPKEFIVHMEWSRPESAMFGPALTAVNPKIKKVHDEKV


Aquifex aeolicus       LDTLVY-GPYIRILNPMME
                       + TL +  PY ++  P+ME
Acidianus ambivalens   MTTLAHIPPYYKVFMPLME
```

species were stored at -80° C using a protocol suggested by the supplier (DSM Braunschwieg, Germany). Ten millilitres of a grown culture were brought to room temperature and then the pH was equilibrated with an excess of $CaCl_2$ (~200 mg). The culture was then left at room temperature for 30 minutes to allow the precipitation of the $CaSO_4$ that was formed. The supernatant was then removed and centrifuged for 10 minutes at 3700 rpm in a Beckman Scientific Megafuge (rotor 2101). The resulting pellet was resuspended in 1 mL of fresh medium equilibrated with $CaCl_2$. Finally glycerol was added to a concentration of 10% and the cells frozen at -80°C.

ii) Extraction of Genomic DNA

Genomic DNA from each of the eight species was extracted from a 50 mL culture which had reached early to mid exponential phase (OD~0.5-0.8). The phase of growth of the cells was very important. If the cells were in late exponential or stationary phase they would not lyse well and if they had not grown enough the yield of DNA was very low. After growth the cells were pelleted by centrifugation at 3700 rpm in a Beckman Scientific Megafuge (rotor 2101) for 10 minutes and resuspended in 200 µL of TE (10 mM Tris-Cl and 1mM EDTA). Cell lysis was accomplished by adding 20 mL of STE (1% lauroyl sarcosine, 50mM Tris-Cl pH 8 and 50 mM EDTA). The cells were incubated in STE for 30 minutes with periodic gentle mixing. Next 3 phenol/chloroform (1:1 v/v) extractions were performed by adding 20 mL phenol/chloroform (1:1 v/v) and mixing gently for 5 minutes followed by centrifugation at 3700 rpm in a Beckman Scientific Megafuge (rotor 2101) for 10 minutes. Genomic DNA was then precipitated by adding 1/10 volume of 3 M sodium acetate and 2 volumes of 95% ethanol. The DNA

was washed with 70% ethanol, dried and finally resuspended in 400 μL of TE by incubating at 4°C for 7 days.

iii) Construction of Genomic Blots

To screen genomic DNA for the presence of *sor* I first had to digest the DNA with a restriction enzyme. To do this 3 μg of genomic DNA was digested with 5 units of enzyme and the digests were run for 18 hours at 2.5 V/cm on a 0.6% agarose gel. The DNA was then transferred to GeneScreen membrane (Dupont/NEN) using a Tyler VT-20 vacuum transfer apparatus. Prior to the transfer being set up the membrane was soaked in H_2O for one minute. To transfer the DNA, the gel was submerged in a number of solutions while the vacuum was applied. The first treatment was with depurination solution (0.25 M HCl) for 3 minutes. The gel was then neutralized by a 3 minute wash with neutralizing solution (1.5 M NaCl and 0.5 M NaOH) and transferred for 30 minutes using transfer solution (0.4 M NaOH). After the transfer was complete the membrane was rinsed in 2X SSC and the DNA bound by UV irradiation for 5 minutes.

iv) Hybridization of Genomic Blots with PCR amplified *sor*

Genomic blots were screened with the PCR-amplified *sor* gene from *A. ambivalens* using the same hybrdization procedure as outlined in the section on screening for pUC/*sor* clones with the following changes. Due to the heterologous nature of this hybridization a number of factors affecting the hybridization were changed. Trials using different concentrations of formamide (0, 10, 20, 30 and 50 %) in the hydridization solution were performed, as well as trials in which the hybridization and wash temperatures were lowered (as low as 30° and 55° respectively) in order to find the optimal conditions.

v) Screening Genomic DNA using PCR

Degenerate primers designed on the sequence alignment of the *A. ambivalens* and the *Aquifex aeolicus* (Deckert *et. al.* 1998) *sor* sequences were used in an attempt to PCR amplify the *sor* gene from all of the species previously screened using hybridization (see figure #8). The PCR cycle used was the same as previously used and the Mg^{++} concentration was varied from 1-5 mM. As a positive control plasmid pSOR-1/17 was used.

IV) Analysis of Treated Rubber

From monitoring the production of sulfate either when rubber was incubated with whole cells or with enzyme I knew that sulfur had been oxidized. However, I did not know what, changes if any, this had made to the structure of the rubber. Furthermore, it would be these changes in the structure of the rubber that would be of particular interest if either of these treatments were to be used to recycle rubber. To determine whether there had been any structural change in the rubber I extracted the soluble phase of the rubber. The thought behind this was that if sulfur cross-links in the rubber were broken there would be larger molecular weight molecules in the soluble fraction as they would no longer be cross-linked. The soluble phase was then analyzed using UV chromatography.

IV.A. Extraction of Soluble Phase

To extract the soluble phase of the rubber a continuous solvent extraction apparatus was used. This apparatus consisted of a 500 mL round bottom flask, a Soxhlet extraction tube, a coarse glass extraction thimble and a Allihn condenser. This apparatus works by boiling the solvent in the flask. The vapours from this bypass the extraction tube and are condensed by the condenser. The hot solvent then runs down into the

thimble which is inside the extraction tube. When the extraction tube is full it empties back into the flask. The cycle then repeats over and over again with the soluble fraction being left in the flask after each round.

To purify the soluble fraction from the treated rubber 10 g of rubber was first extracted with acetone for 24 hours. This extraction removes all of the very low molecular weight molecules such as oil. After this extraction the rubber was dried under vacuum to remove all of the acetone. Once the rubber was dried it was extracted again for 24 hours using chloroform as the solvent. This extraction removed all of the larger molecular weight molecules which could be extracted from the rubber. This was the soluble phase that I was interested in. After the chloroform extraction the soluble phase was dried with heat and vacuum. The dried samples were then ready to be analyzed.

IV.B. UV Chromatography of the soluble phase

Soluble phases extracted from rubber samples were sent to Dr. Kris Kostanski for UV chromatography. This apparatus separates the molecules in the sample by size and then the relative amount of each is determined by the absorbance at 220 nm. In this case the samples were run on a 4XStyrAGel column, in THF (5β-Pregnan-3α,11β,17α,21-tetrol-20-one) buffer and at room temperature. The molecular weight of the samples was determined by comparison to standards which were run simultaneously (see appendix 5 for examples). From page 121 of this appendix the absorbance is read on the Y axis. Also on this page the position of each of the molecular weight markers with respect to the sample is shown. Page 123 shows the molecular weights of each of the standards. These weights were then used to determine the molecular weight of the major peak.

Results

This research involved developing and assessing the applicability of several approaches for the devulcanization of rubber crumb. More specifically, my focus was the devulcanization of rubber crumb involving the use of sulfur oxidizing *Archaea* and their enzymes. *Sulfolobus solfataricus* P2 was used in the whole cell method, which involved the growth of cells using rubber crumb as the sole source of sulfur and therefore energy. The desired effect being the oxidation of sulfur in the rubber crumb. Another method, using an enzyme, the sulfur oxygenase/reductase from *Acidianus ambivalens*, also attempted to devulcanize rubber crumb by oxidizing the sulfur present.

In the development of the whole cell method a number of factors that were likely to affect the oxidation of sulfur by the cells were studied in order to optimize the process. These factors included the type of carbon source, the pH of the medium and particle size of the rubber crumb. To test the applicability of the sulfur oxygenase in the devulcanization of rubber it was necessary to clone this enzyme and express it in *Escherichia coli*. The enzyme was then partially purified and incubated with the rubber crumb in an appropriate buffer. The activity of the enzyme was then assayed.

Once these methods had been developed the evaluation of their usefulness was undertaken. The first evaluation was based on the monitoring of sulfur oxidation. From this monitoring, rates of sulfur oxidation were calculated. These rates were then compared between different treatments in the whole cell methods, and between the whole cell and the enzyme methods using statistical analysis. Evaluation of the treatments was also done by testing the rubber crumb that had been treated by each of the methods. This testing attempted to discern the chemical changes which had occurred in the rubber crumb as a result of the treatments.

I) Whole Cell Methods

S. solfataricus P2 was incubated with rubber crumb and a number of factors were studied. One factor, which I thought would have a significant impact on the oxidation of sulfur, was the type of carbon source utilized by the cells. When growing on sulfur, species of *Sulfolobus* gain energy from the oxidation of sulfur but they still need a source of carbon. The accessibility of this carbon and the efficiency with which the cells are able to use the carbon will affect the growth and health of the cell. This in turn will impact the amount of sulfur the cells oxidize. To determine which types of carbon source would better suit this application a number were tested and their affect on sulfur oxidation monitored. Figure # 9 shows the production of sulfate by *S. solfataricus* when grown on rubber of mesh size 20 and in a medium of pH1. Sulfate production was monitored in all whole cell experiments as it is the end product of sulfur metabolism. The factor that was varied with each trial, was the frequency of gassing with carbon dioxide. From this figure one can see that the frequency of gassing has a dramatic effect on the oxidation of sulfur. It appears as though gassing with carbon dioxide gives the highest rate of sulfur oxidation when it is done once every 24 hours. If the frequency is increased from this a dramatic decrease in the rate of sulfur oxidation is noted. When the rates of sulfur oxidation between these treatments were compared using ANOVA it was confirmed that the rates are significantly different. The p-value was < 0.0001, see table #1. The results of the statistical analysis are also shown in a more comprehensive manner in appendix III. Another carbon source, which was tested, was the addition of sodium bicarbonate to the medium. To study the effect of the addition of this compound to the medium, various concentrations were added and the oxidation of sulfur was monitored. The size of the rubber particles and the pH of the medium were maintained at the same values as in the previous gassing experiments. The results of the sodium bicarbonate experiments are shown in figure # 10. It appears, from this figure, as though a sodium bicarbonate concentration of 5 mM gave the highest rate of sulfur oxidation. When the ANOVA

Figure # 9 : The production of sulfate during several trials of *Sulfolobus* growth on rubber crumb. The variable which was changed in these trials is the frequency of gassing with carbon dioxide. The duration of the gassing was 30 seconds. Error bars represent the standard error.

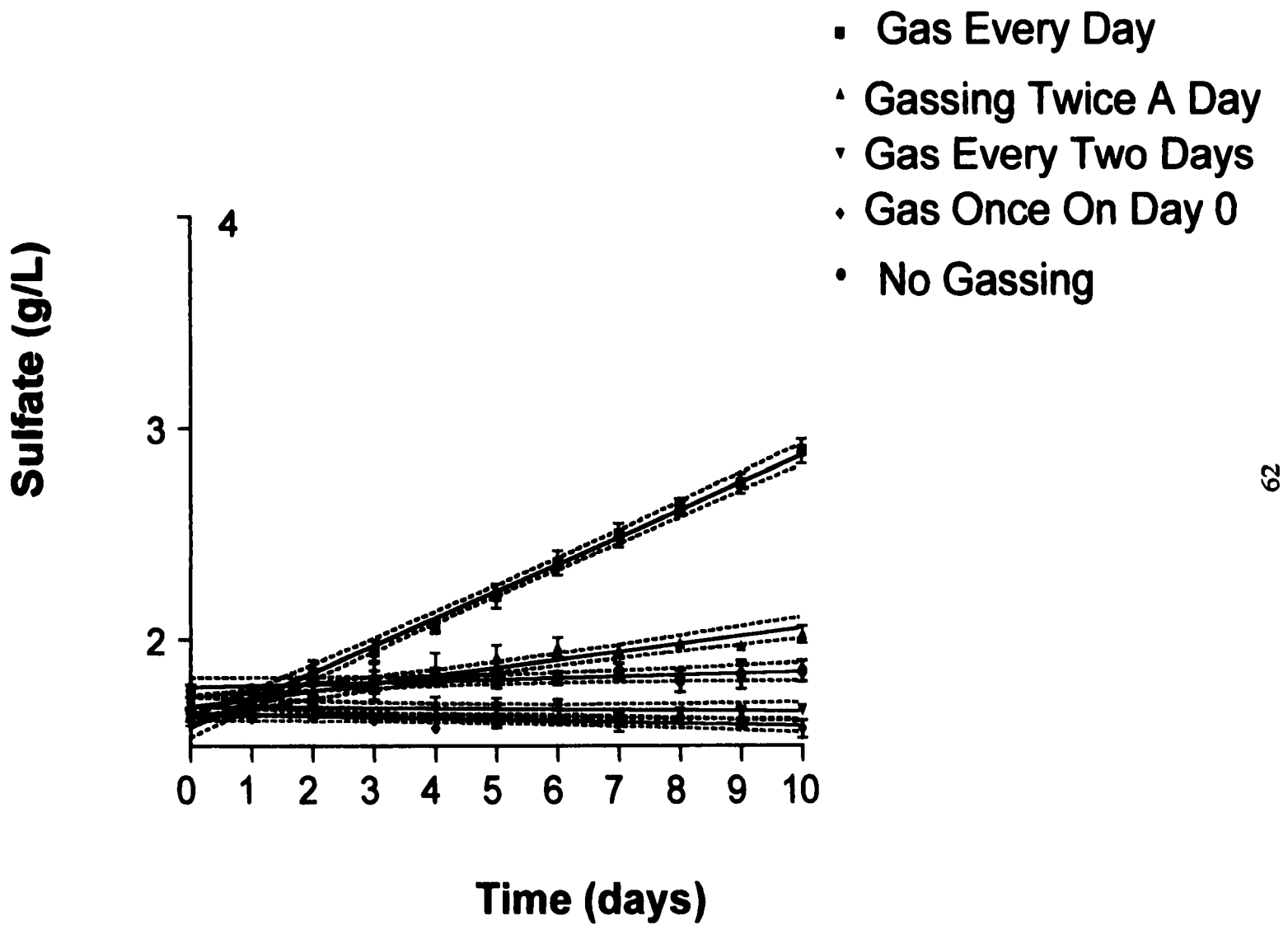
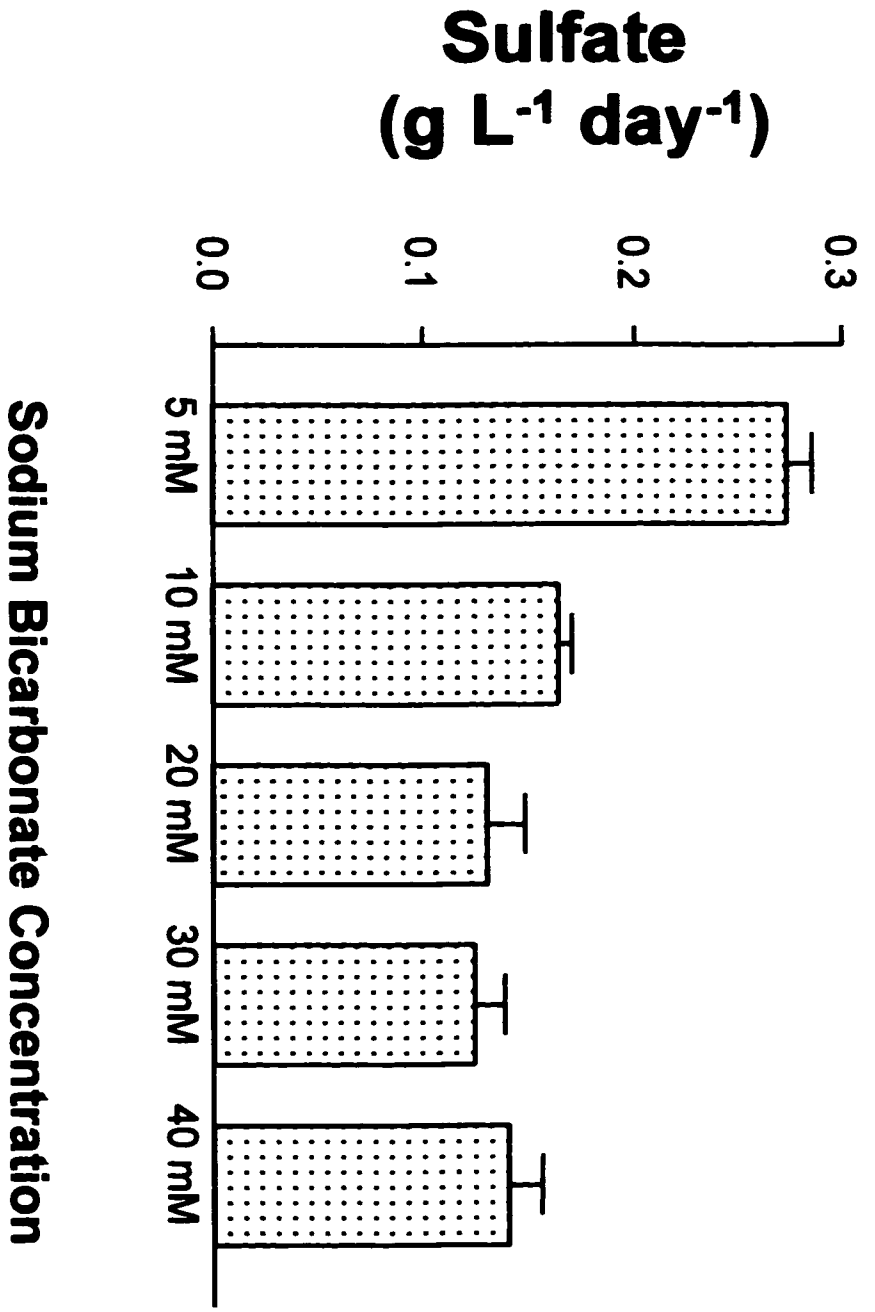

Gas Every Day
Gassing Twice A Day
Gas Every Two Days
Gas Once On Day 0
No Gassing
Sulfate (g/L)
4
3
2
0 1 2 3 4 5 6 7 8 9 10
Time (days)

Table # 1: Statistical analysis of the rates of sulfate production for both the enzyme and the whole cell experiments. In cases when more than two rates were being compared ANOVA analysis was performed; in other instances t-tests were performed. In all analyses it was confirmed that there was a significant difference between the rates. Analysis was performed using the GraphPad Prism version 2.10.

	CO_2 gassing conditions	Bicarbonate concentration	5 mM bicarbonate vs. gassing every day	pH of medium	Size of rubber crumb	Cells vs. enzyme	Preincubated vs. normal
Type of test	One-way ANOVA	One-way ANOVA	Unpaired t-test	One-way ANOVA	Unpaired t-test	Unpaired t-test	Unpaired t-test
P-value	< 0.0001	< 0.0001	0.0003	< 0.0001	< 0.0001	< 0.0001	0.5992

Figure # 10 : The effect of sodium bicarbonate on the production of sulfate during the growth of *Sulfolobus* on rubber crumb is shown in this graph. The error bars represent the standard error after three trials of each treatment had been performed.


Sulfate
(g L-1 day-1)
0.0
0.1
0.2
0.3
5 mM
10 mM
20 mM
30 mM
40 mM
Sodium Bicarbonate Concentration

analysis was performed it was confirmed that this rate was significantly different from that of the other concentrations. The p-value for this test was < 0.0001 and is shown in table # 1. The rate of sulfur oxidation using 5 mM sodium bicarbonate was next compared to the rate for gassing with carbon dioxide every day, the highest for the gassing experiments. The t-test for this comparison suggests that the rate was significantly higher using 5 mM sodium bicarbonate. The p-value for this test was 0.0003 (table #1). The other carbon source studied was sucrose. I found that with even very low concentrations of sucrose there was no oxidation of sulfur. The presence of even 0.04 % sucrose inhibited sulfur metabolism.

The next factor, which I studied, was the pH of the medium. In previous studies it had been shown that this factor has an influence on the ability of the cells to attach to coal particles (Chen and Skidmore 1987). In the previous carbon source experiments the pH was kept at 1. For these experiments the pH was varied to include values of 2 and 3 as well. The results from these trials are shown in figure #11. This bar graph shows the rate of sulfur oxidation over the course of the experiment. After comparison of the rates using ANOVA it was found that the rates were significantly different with the rate at pH 1 being the highest. Again the p-value of < 0.0001 is shown in table #1.

The final factor, whose effect I was interested in, was the size of the rubber particles used. In both the previous studies the size used was 20 mesh which is approximately 125 microns. In this part of the study an additional size was added, which was 80 mesh or 30 microns. This size is the smallest size, which is currently available. It is more expensive to grind the rubber to this size as compared to the mesh 20 but my thinking was that this decrease in size would increase the amount of sulfur oxidation. From figure #12 one can see that this was indeed the case. The decrease in size from 20 to 80 mesh did increase the rate of sulfur oxidation. This difference which is seen in the graph was found to be statistically significant when the rates were compared using a t-test. The p-value for this test was <0.0001 (see table # 1).

Figure # 11 : The effect of pH on the production of sulfate during the growth of *Sulfolobus* on rubber crumb. The error bars represent the standard error after three trials of each treatment had been performed.

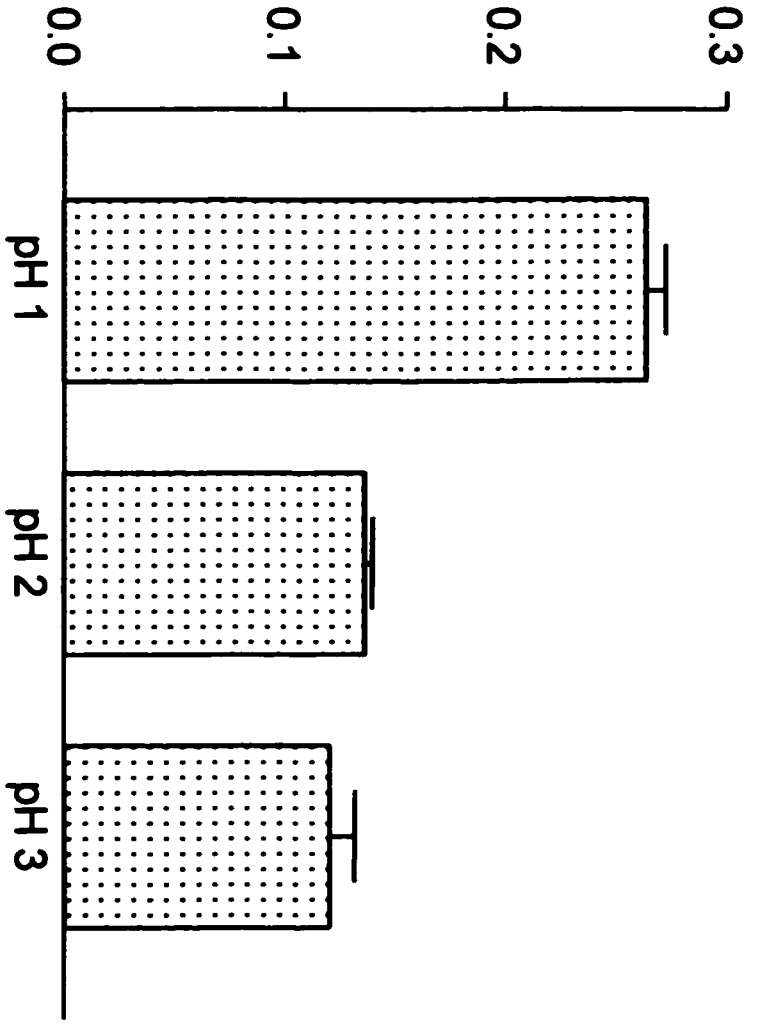
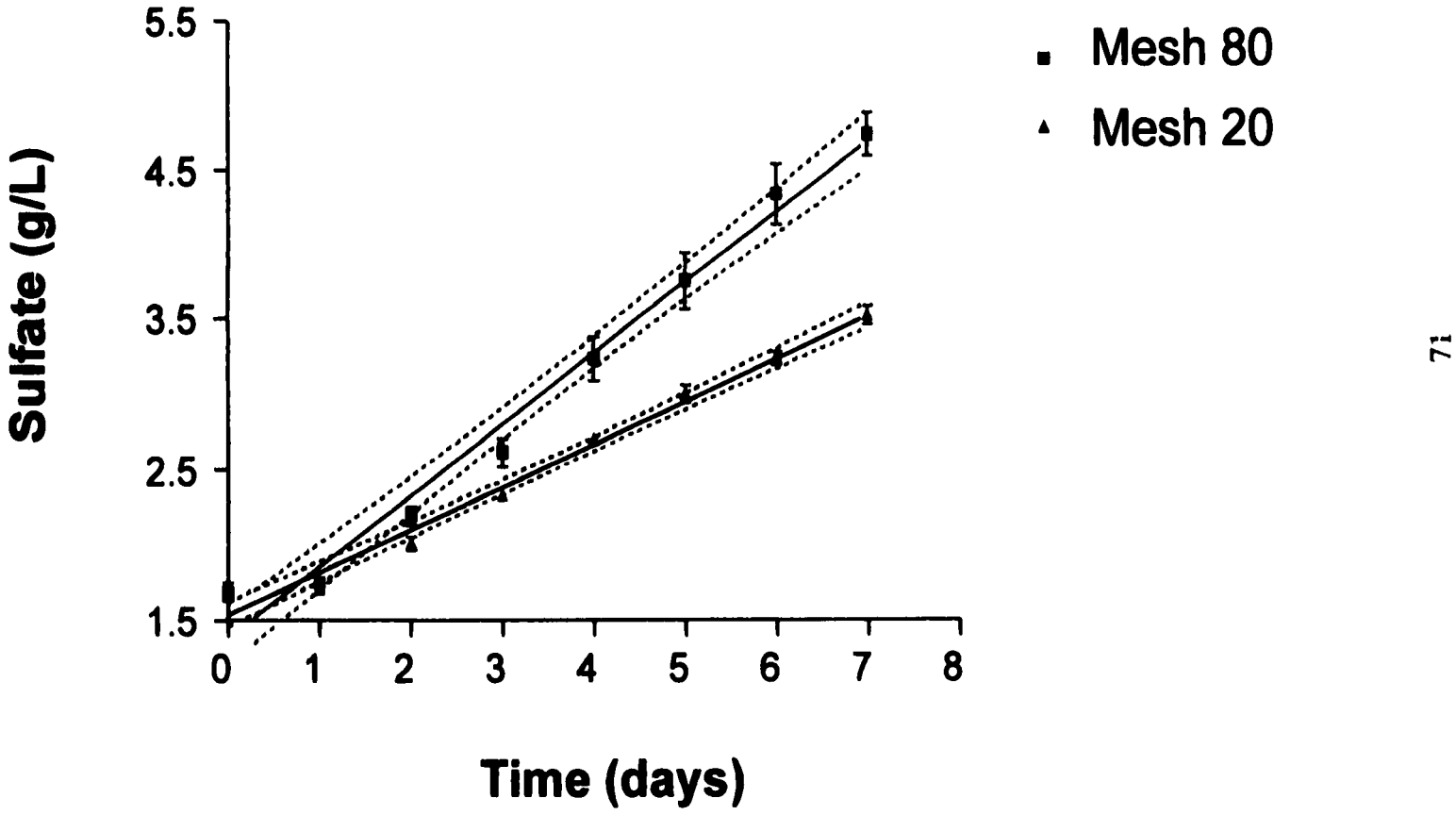

Sulfate
(g L-1 day-1)
0.0
0.1
0.2
0.3
pH 1
pH 2
pH 3

Figure # 12 : During the growth of *Sulfolobus* on rubber crumb the particle size was either 20 or 80 mesh. The effect of this change is shown here. The error bars represent the standard error which was calculated after three trials.


Sulfate (g/L)
5.5
4.5
3.5
2.5
1.5
0
1
2
3
4
5
6
7
8
Time (days)
Mesh 80
Mesh 20

In this whole cell section of my research I was attempting to develop a method for growing *S. solfataricus* on ground rubber. Once this was done it was my goal to optimize a number of key factors involved in this process. I found that the highest rate of sulfur oxidation occurred at pH 1, using 5 mM sodium bicarbonate as the carbon source and using mesh 80 rubber particles. The actual rate of sulfate production for these conditions was 0.47 g/L/day. It was now my intention to determine if an enzyme system could be applied to the devulcanization of rubber. If this were possible, I would then compare the rate of sulfur oxidation using the enzyme system to the rate obtained in the whole cell experiments.

II) Cloning and Expression of *sor* in *Escherichia coli*

The goal of this second part of my research was to clone and express the sulfur oxygenase from *Acidianus ambivalens*. To do this, I obtained a clone from Dr. Arnulf Kletzin, which contained the *sor* gene as part of a 5.3 kbp insert. The *sor* gene is ~ 1 kbp in length. The first step in cloning this gene was to design primers for the ends of the gene and to amplify the gene using PCR. The amplified product was then blunt-end ligated into both pUC18 and pKK223-3. The resulting clones were screened by hybridization. From this hybridization 24 of the 96 clones screened positive for the presence of the *sor* gene. Two of the positive clones were chosen to be sequenced. When these sequences were searched against Genbank it was confirmed that it was indeed the *sor* gene which had been amplified and cloned. It was also determined that one of these clones was in the wrong orientation while the other was in the correct orientation. Through further sequencing it was confirmed that the entire gene was cloned and that there were no errors introduced during the PCR. In appendix IV, the sequence of the clone I created is aligned with the known sequence of the *sor* gene. This alignment was performed using Clustal W.

Once it was confirmed that an error-free copy of the intact *sor* gene had been cloned into the expression vector it was time to express this gene in *E. coli* and determine the level of expression by SDS-PAGE. The results of this are shown in figure # 13. The *sor* gene product is ~40 kDa in size. From this gel it is clear that there is a band of approximately this size which appears in the induced cultures, but not in either of the negative controls. Also from this figure, it is clear that the heat purification step substantially reduced the amount of *E. coli* protein in the sample (compare lanes 3-6 to lane 2). Now that I was convinced that the sulfur oxygenase was being expressed and that I was able to somewhat purify this protein, it was my intent to assay for the function of this protein.

III) *In vitro* testing of recombinant Sor

The goal of expressing the sulfur oxygenase in *E. coli* was to attempt to use this recombinant protein to devulcanize rubber crumb. In order for this to work the protein would not only have to be expressed but would have to be expressed in a functional form. To test for function the enzyme was incubated with rubber and a number of possible products were tested for. These products, which had been indicated from previous studies with the native sulfur oxygenase, were sulfite, thiosulfate and sulfate. The results of this testing showed that there was no thiosulfate or sulfite produced when the enzyme was incubated with the rubber crumb but there was sulfate produced. The results of this incubation are shown in figure #14. From this curve it is clear that there is significant sulfate production when the recombinant sulfur oxygenase is incubated with rubber crumb. This production of sulfate indicates that there was oxidation of the sulfur in the rubber crumb and therefore some degree of devulcanization. This curve also suggests that for some reason the enzyme does seem to lose its activity after ~ 4 days. The next step was to compare the rate of sulfur oxidation for the enzyme incubation over the first 4 days to the best rate obtained for the whole cell incubation. The rate of sulfur oxidation

Figure # 13 : Gel demonstrating that the induction and expression of *sor* in *E. coli* did occur. Lanes 1 and 7 are the marker lanes, the size of each band in kDa is shown to the right of the figure. Lane two is a protein extract which has not been heat treated to remove the *E. coli* proteins. Lanes 3 and 4 are negative controls in which there was no insert in pKK223-3 or the culture was not induced respectively. Lanes 5 and 6 are cultures which possess the pKK223-3/*sor* plasmid and have been induced with 1 and 5 mM IPTG respectively.

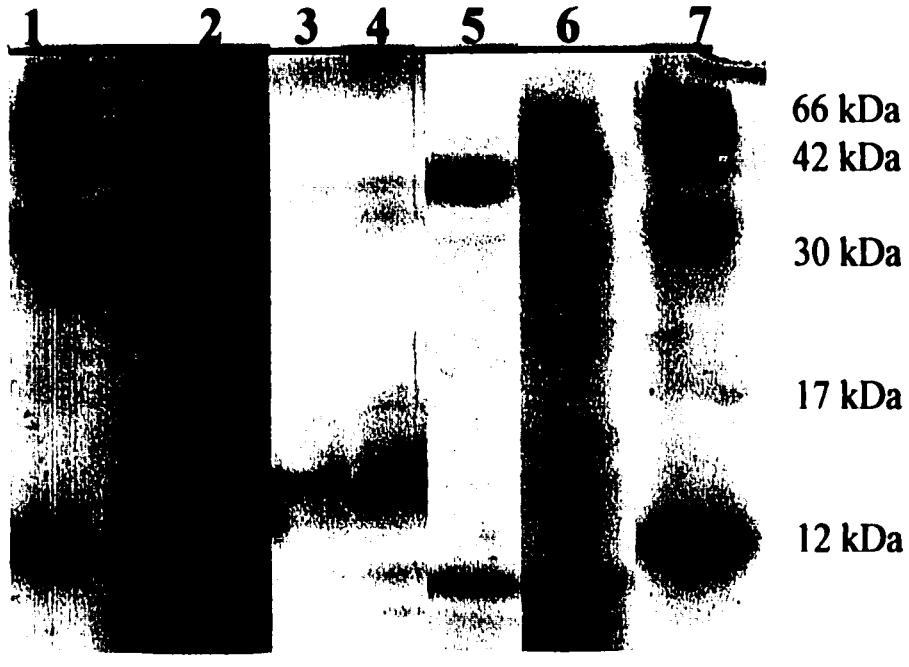
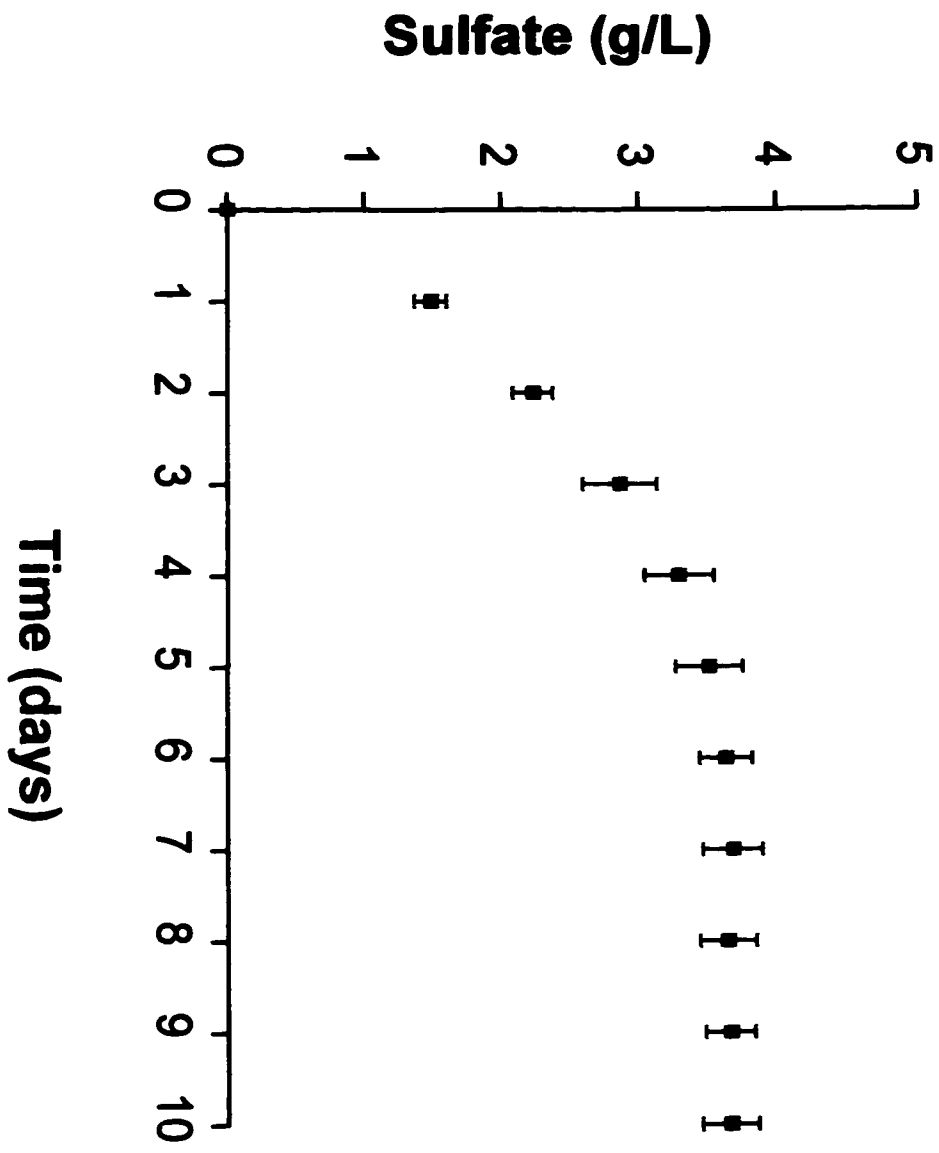

1
2
3
4
5
6
7
66 kDa
42 kDa
30 kDa
17 kDa
12 kDa

Figure # 14 : The production of sulfate when the recombinant sulfur oxygenase was incubated with rubber of mesh size 80. There appears to be an initial rate of sulfur oxidation which is higher than that observed using whole cells but this rate quickly drops off after ~ 4 days of incubation. Error bars represent the standard error.


Sulfate (g/L)
0
1
2
3
4
5
Time (days)
0
1
2
3
4
5
6
7
8
9
10

in the two methods are shown in figure # 15. The rates were compared by performing a t-test. The findings of this test suggest that rates are significantly different. The p-value for this test was <0.0001 and is shown in table # 1. This confirmed that the enzyme method oxidized sulfur in rubber crumb at a higher rate than the whole cell method.

In addition to simply testing for enzyme activity, an experiment was performed to test for the binding of the enzyme to the rubber. If during the incubation this type of binding did occur it would greatly affect the future industrial process, as a batch of enzyme would only be useful for one treatment. In this experiment recombinant Sor was incubated with rubber overnight followed by the removal of the rubber by centrifugation. The supernatant was then reincubated with rubber and sulfate production monitored. This preincubation did not titrate out the enzyme as the rate of sulfate production was found to be similar to that for other *in vitro* assays (see table #1).

IV) Testing of the end products

At this point of my research I was confident that there had been sulfur oxidized in the rubber crumb in both the enzyme and the whole cell incubations. However, I was not sure if the oxidation of this sulfur had made any meaningful changes to the cross-linking levels in the rubber crumb. To test for this, the rubber company involved in the funding of this research suggested a procedure. This procedure involved extracting the soluble portion of the rubber crumb using solvents and then analyzing this soluble phase using UV chromatography. The idea behind this method is that as cross-links are broken larger molecular weight molecules will be present in the soluble phase. The relative proportion of these larger molecular weight portions can then be detected using UV chromatography.

The method as described above, was performed on a number of samples including rubber that had been incubated with whole cells and enzymes. Negative controls for this analysis included rubber that had been treated with medium, with buffer and rubber that had not been treated at all. A summary of the results of this work is shown in figure # 16

Figure # 15 : Comparison of the rate of sulfate production during cell growth on rubber and during enzyme incubation with rubber. The rate for the enzyme incubation was calculated from the first four days as shown in figure # 14. It is clear that sulfate is produced at a higher rate by the enzyme. Error bars represent the standard error.

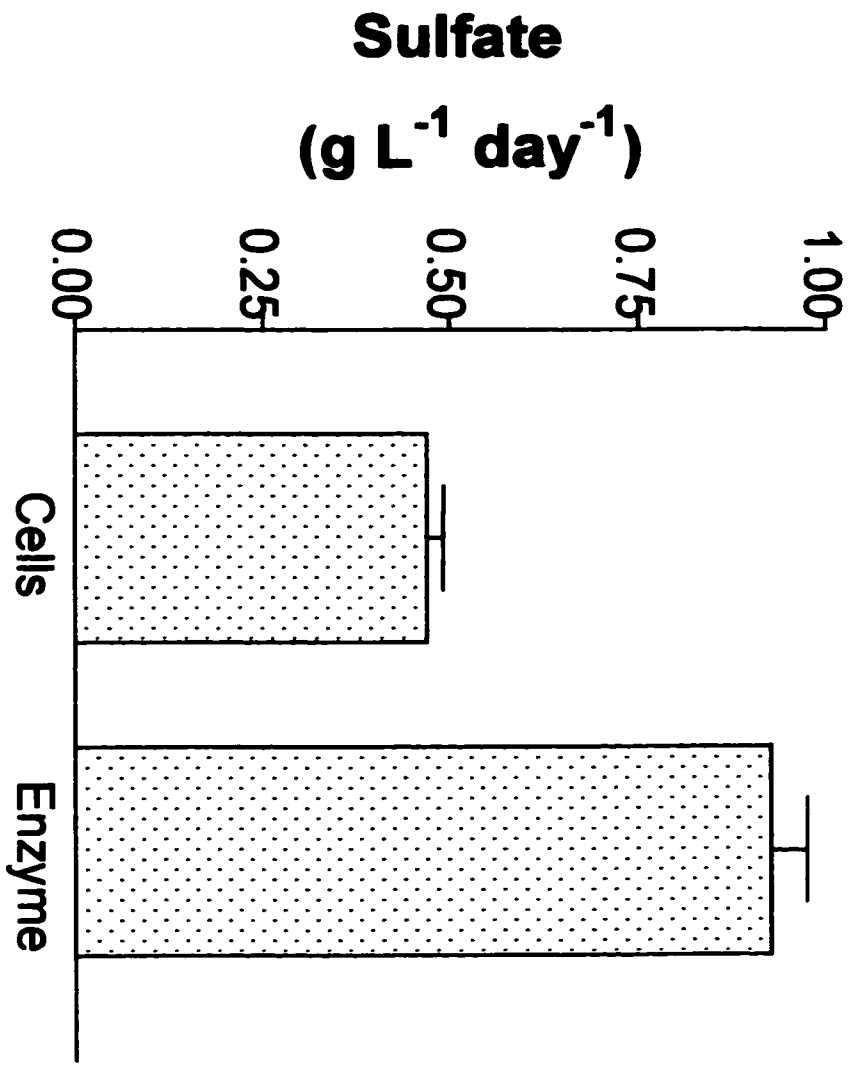
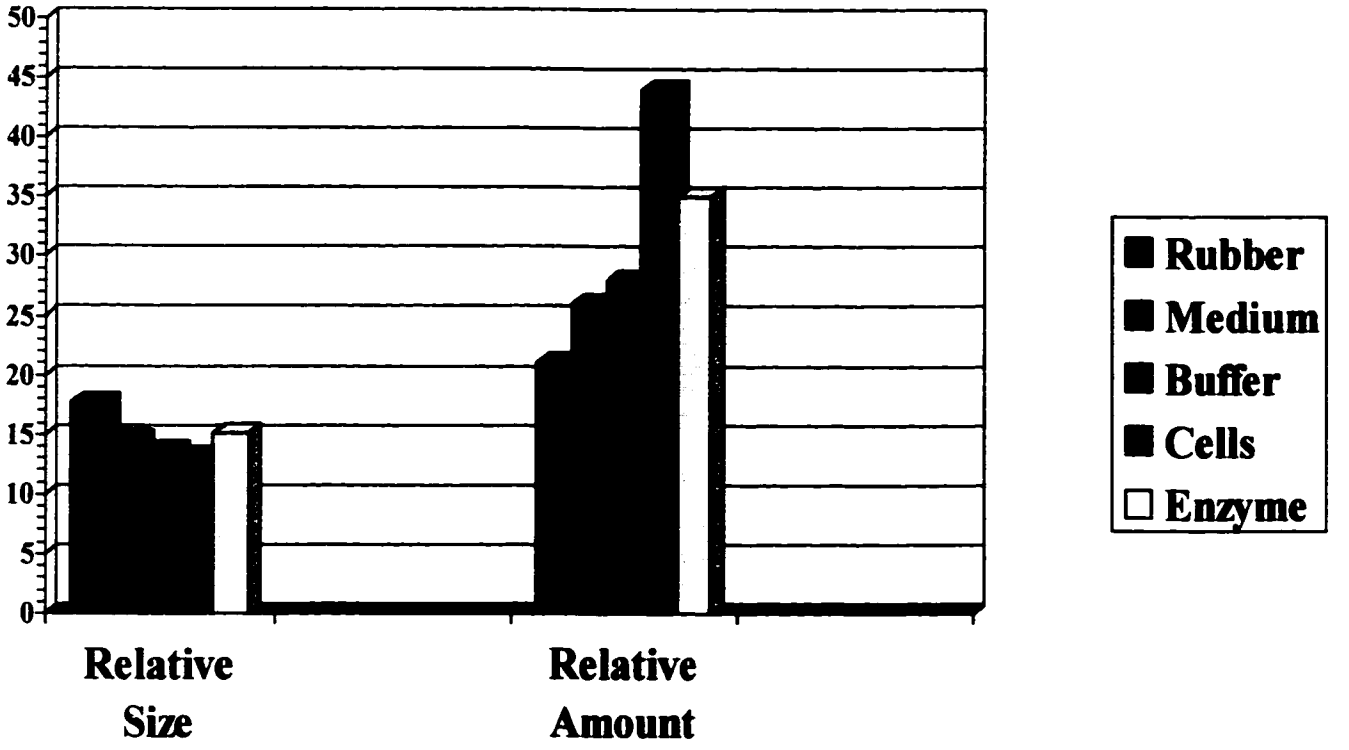

Sulfate
(g L-1 day-1)
0.00
0.25
0.50
0.75
1.00
Cells
Enzyme

Figure # 16: Results of the rubber testing using UV chromatography. Shown are the results for non-treated rubber, medium, buffer, whole cell and enzyme treated rubber. The size of the hydrocarbon which makes up the major peak is approximately the same for each of the samples and no trend is visible. However, the relative amount of hydrocarbon in the major peak increases when the rubber is treated with either the whole cell or the enzyme. The size is shown in kilodaltons while the amount is measured by absorbance at 220 nm.


50
45
40
35
30
25
20
15
10
5
0
Relative Size
Relative Amount
Rubber
Medium
Buffer
Cells
Enzyme

and the raw data is shown in appendix V. Basically, what was found, was that there was no increase in the size of the hydrocarbon chains found in the soluble phase of the treated rubber. This was the case for the rubber crumb treated with enzyme and with whole cells. What was indicated by this analysis, was that the relative amount of hydrocarbon material found in the soluble phase increased when the rubber crumb was treated with either the whole cells or the enzyme.

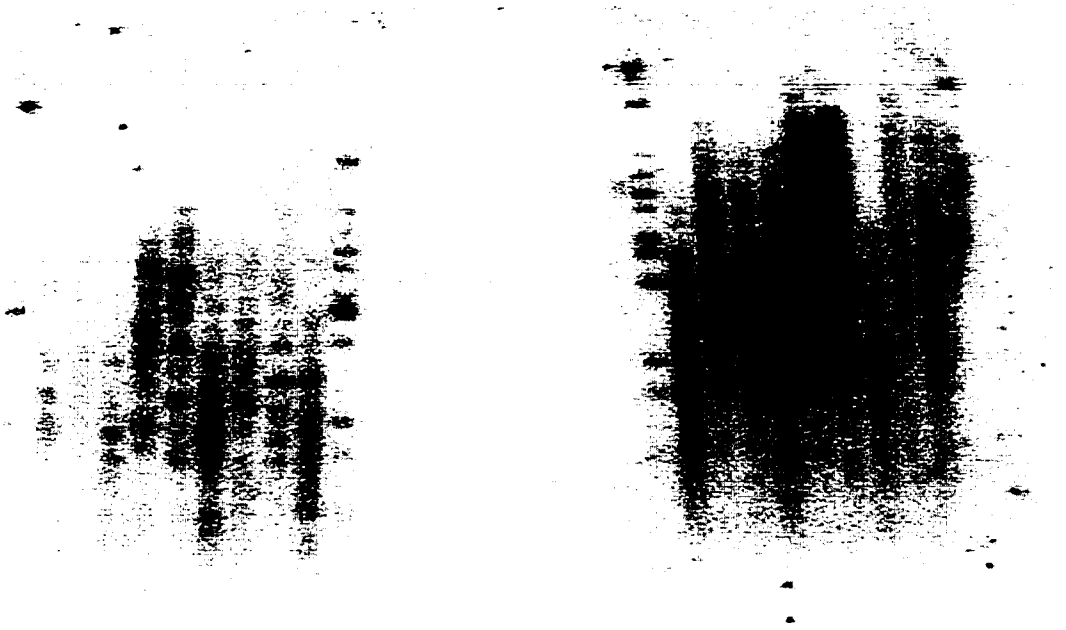
V) The Search for *sor* homologues

Another aspect to my research was the search to identify sulfur oxygenase homologues in a number of species related to *A. ambivalens*. The purpose of this search was to possibly locate an enzyme that was better suited to the rubber devulcanization process than the *A. ambivalens* enzyme. Two methods were used to accomplish this goal.

One of these methods was to use the sulfur oxygenase from *A. ambivalens* as a probe and screen, using hybridization, a number of libraries and genomic DNA from related species. The first type of library that was screened was a cosmid library. These libraries had been constructed for the following species: *Sulfolobus solfataricus* P2, *Sulfolobus solfataricus* P1, *Sulfolobus solfataricus* MT4, *Sulfolobus shibatae*, *Sulfolobus acidocaldarius*, *Sulfolobus hakonensis*, *Sulfolobus tokodaii* and *Metallosphaera sedula*. Screening of these libraries led to the discovery of no positive clones. As no positives had been found through the library screenings, I next attempted to locate a homologue using genomic DNA from the same representative species, again screening by hybridization using the *A. ambivalens sor* gene as a probe. The results of a number of trials are shown in figure # 17. In these trials genomic DNA from the previously mentioned species was screened. The concentration of formamide was varied in order to adjust the stringency of the hybridization. In figure # 17 the results of the trials with 0 and 10 percent formamide are shown. The results clearly indicate that under these conditions there is a large amount of non-specific binding. When the concentration is

Figure #17: Representative blots containing digested genomic DNA from various species of *Sulfolobales*. From left to right each blot contains *S. solfataricus* P2, *S. acidocaldarius* and *S. shibatae* genomic DNA cut with HindIII, EcoRI and a double digest with both enzymes. In each blot the outermost left and right lanes are size markers. Under conditions where the hybridization solution contained either 0 or 10 % formamide there is extensive background when the blots were screened for the presence of the sulfur oxygenase gene. If the amount of formamide was raised to 20, 30 or 50% no binding was seen (data not shown).

10% formamide **0% formamide**



raised from this level to 20%, 30% or 50% there is no binding of the probe. Hybridization and washing temperatures were also varied to adjust the strigency, but similar results were obtained. Having exhausted possible hybridization methods for locating a homologue, I next attempted to locate a homologue using PCR. To do this degenerate primers were designed based on the sequence for Sor found in *A. ambivalens* and *Aquifex aeolicus* (figure # 8). These primers were used in an attempt to amplify *sor* homologues from genomic DNA from the same species previously mentioned. Unfortunately, under a number of different PCR conditions no product was observed for any of the species tested. In each case however, the positive control did show a product of the expected size.

Discussion

I) Summary of Major Findings

This research project was concerned with the devulcanization of rubber crumb using sulfur oxidizing *Archaea* and their enzymes. To accomplish this, *Sulfolobus solfataricus* P2 was grown on rubber crumb and a number of factors that influence this growth were optimized. The optimization of this process was statistically tested using ANOVA and comparative t-tests. The maximum rate of sulfur oxidation occurred when the pH of the medium was 1, when rubber of size 80 mesh was used and when the carbon source was 5 mM sodium bicarbonate. The rate of sulfur oxidation was monitored by determining the increase in sulfate, the end product of sulfur oxidation. Under optimized conditions the rate of sulfate production was 0.47 g/L/day. In addition to the whole cell approach an enzyme-based method was also undertaken. In this enzyme-based portion of my research the sulfur oxygenase/reductase from *Acidianus ambivalens* was cloned and expressed in *Escherichia coli*. Recombinant Sor was then incubated with rubber crumb and the end products assayed for. It was found that oxidation of sulfur did occur and the rate of sulfate production was found to be 0.92 g/L/day. Statistical analysis showed that this rate was significantly higher than the rate of 0.47 g/L/day found in the whole cell experiments.

Another part of this study was an attempt to identify homologues to the *sor* gene in a number of species related to *A. ambivalens*. In this attempt, hybridization and PCR based screening methods were used. The results of this screening suggest that the species which were screened, do not have a sulfur oxygenase/reductase gene.

II) Whole Cell Investigations

Before beginning this study it was not known whether *Sulfolobus* could be grown using the sulfur found in rubber crumb as the sole energy source. From this study, along with others which have recently been reported (Romine and Romine 1998), it is now clear that this is indeed possible. During my investigation of this process I wanted to optimize a number of factors including pH, carbon source and particle size of the rubber crumb.

The results for the particle size and the pH experiments showed that a smaller particle size and a pH of 1 were the best. These findings agree with the results of other studies involving growth of *Sulfolobus* on sulfur, coal and pyrite. In these previous studies it had been shown that the attachment of the cells to the insoluble coal particles was facilitated at a pH of 1 (Chen and Skidmore 1987 and Vitaya and Toda 1991). The particle size of sulfur sources like rubber, pyrite, coal and powdered sulfur is important because all of these sources are insoluble in the growth medium. Due to this insolubility, the cells must attach to the particles and slowly dissolve them (Kargi *et.al.* 1982). When the particles are smaller there is a greater surface area available to the cells. For this reason when the particle size is reduced the cells have greater access to the sulfur and the rate of sulfur oxidation is increased.

As I mentioned previously, when *Sulfolobus* is growing on sulfur they oxidize this sulfur to produce energy. However, the cells still need a source of carbon in order to survive. Sodium bicarbonate has previously been used as a carbon source in studies involving the growth of *Sulfolobus acidocaldarius* on tetrathionate (Buckingham *et.al.* 1989). However, sodium bicarbonate is not the most widely used carbon source in

Sulfolobus medium. The most commonly used method of providing carbon is to bubble the medium continuously with a mixture of 10% carbon dioxide in air. This method, however, requires an incubator that has the ability to reach temperatures up to 80° C and be able to allow the gassing of the culture. I did not have an incubator with these capabilities, so I could not test this type of carbon source. However, I did use a method of gassing using 100 % carbon dioxide, at a number of different frequencies, but this method gave rates of sulfur oxidation that were significantly lower than those found when 5 mM sodium bicarbonate was used. It is possible, that if I had access to an incubator as described above, that the rate of sulfur oxidation would have been even higher than that which I obtained using sodium bicarbonate.

An important consideration when deciding if this whole cell method was useful for the devulcanization of rubber crumb was the exact amount of sulfur that had been removed from the rubber. It is quite likely that the effectiveness of the cells is limited to the surface of the rubber crumb. This is simply because this is the only part of the rubber crumb exposed to the cells. A very recent study by Robert and Margaret Romine investigated the use of *Sulfolobus acidocaldarius* to oxidize the sulfur found in the rubber crumb. They found that after 7 days incubation ~14% of the sulfur found in the rubber had been oxidized (Romine and Romine 1998). During my study, after optimization, I found that ~ 20% of the sulfur in the rubber had been oxidized. This number was calculated by comparing the number of moles of sulfur present in the rubber crumb to the number of moles of sulfate produced. In this calculation the amount of sulfur in the rubber was approximated to be 2.5% w/w (Mike Burgoyne personal communication). The increase in the amount of sulfur oxidized in my study indicates that the optimization

had an effect on the amount of sulfur oxidized. In the study by the Romines, they were not interested in studying the actual growth of *Sulfolobus*, but were more focussed on the structural changes which occurred in the rubber. They, therefore, did not attempt to change any of the culture conditions to obtain more sulfur oxidation.

Overall, the whole cell method proved to be very effective. The cells were able to survive in the presence of the rubber crumb and were indeed able to oxidize the sulfur that is found in the rubber. Comparison of the amounts of sulfur removed in this study, to the one other study with a similar focus, suggests that the optimization of culture conditions did have an impact on the amount of sulfur oxidized. The major limitation in this section of my research was the type of incubator used. Ideally, a fermentation apparatus with a large (15 litre) capacity, an ability to gas the culture on a continuous basis and a vigorous stirring ability would have been used. Having this type of equipment may have led to an increase in the rate and/or the amount of sulfur oxidized. However, given this limitation, there was still significant amounts of sulfur oxidized from the rubber crumb, ~ 20%.

III) Enzyme Method

The sulfur oxygenase/reductase from *A. ambivalens* has been the subject of a number of studies by Dr. Arnulf Kletzin. In these studies the sulfur oxidizing activities of the native enzyme were ascertained. The enzyme was found to react with sulfur to form sulfite and hydrogen sulfide (Kletzin 1989). The sulfite was then chemically converted to thiosulfate, due to the presence of sulfur. I felt that this activity would be ideal for the purpose of devulcanizing rubber crumb. To obtain large amounts of this

enzyme it was cloned and expressed in *E. coli*. From numerous other studies it was quite evident to me that archaeal proteins express well in this host and in a large number of cases retain their function (Colombo *et. al.* 1994 and 1995, Jones *et. al.* 1995 and Moracci *et. al.* 1992 and 1995). I found that indeed the *sor* gene was expressed. However, when the *sor* gene was expressed in *E. coli* the function of the protein was modified. Instead of producing sulfite and thiosulfate when incubated with sulfur, the recombinant Sor produced sulfate. One possible reason for this is that the analytical tests I performed allowed the spontaneous conversion of the sulfite and thiosulfate to sulfate. This would occur through oxidation of sulfite during the handling of the samples. I, however, do not feel this is a likely explanation. For each of the analytical methods I performed positive controls using known quantities of either sulfite, thiosulfate or sulfate and in each case was able to detect the presence of the expected amount. If there were an error, inherent to the handling of the samples, this would not have been possible. A second reason for this modified function could be a change in structure of the protein. This could be changes at any level of structure, from primary to tertiary. I again do not feel this is the most probable explanation. The cloned *sor* gene was sequenced and it was confirmed that there were no errors. Also, there have been literally dozens of archaeal genes cloned and expressed in *E. coli* and none have had their function modified. There have, however, been two reported cases where *Sulfolobus* proteins, expressed in *E. coli*, showed modified structure. In one instance there was an amino acid substitution which caused the recombinant protein to become less thermostable (Fusi *et. al.* 1997). In another instance, the protein was produced without the usual N-methyl lysines (Moracci *et. al.* 1995). This change did not affect the function of the protein. I therefore, do not

completely discount the possibility that there was, somehow, a change in the structure of the protein, which caused the change in function. I feel the most probable reason for the difference in function, is the presence of a cofactor in the studies involving the native protein. The native Sor is a 550 kDa complex made up of identical 40 kDa subunits. Given the large size of this complex it is possible that a small tightly bound cofactor would not have been noticed during the purification.

The production of sulfate when the recombinant Sor was exposed to rubber crumb was found to be more rapid than when exposed to whole cells. The rate for the enzyme was 0.92 g/L/day opposed to 0.47g/L/day for the whole cells. This result is very exciting as any time which is saved in the processing of the rubber makes the process more viable from an economic standpoint. It is important to note that even though the rate of sulfur oxidation is higher in the enzyme-based method the total amount of sulfur oxidized is approximately the same (see figures #12 and 14).

What is not clear from my study is how much enzyme is needed to obtain these results. It is possible that using less enzyme, would allow for the same amount of activity, if the enzyme was not limiting at the concentration I used. The main reason I did not attempt to investigate this question, is the purity of the recombinant protein and the inconsistency of the amount of expression. When the protein was expressed and purified through heat treatment there was still a large amount of *E. coli* protein present. Also, there was significant variability in the relative proportions of Sor and *E. coli* proteins found in each trial. These two factors made it impossible to know exactly how much Sor was being added in each experiment. This problem could be responsible for the greater variation seen in the enzyme experiments (see figure # 15). In order to

investigate the question of enzyme amount, one would need to further purify the enzyme. One would then be able to know exactly how much enzyme was being added and be able to characterize this relationship.

IV) Testing of Treated Rubber

Although I was confident that sulfur in the rubber crumb had been oxidized it was more important to know whether or not the characteristics of the rubber had been changed. The eventual use of the treated rubber is to mix it with virgin rubber. This mixture would then be revulcanized producing a new rubber product with similar characteristics to one made entirely of virgin rubber. Currently 5% rubber crumb can be mixed with virgin rubber without affecting the characteristics of the end product. If the rubber is treated to form new sites for revulcanization it is likely that this amount could be increased to 20% (Coghlan 1995 and Siuru 1997). To determine whether there had been changes in the rubber structure the soluble phase of the ground rubber was analysed, the thought being, that if sulfur cross-links had been broken, there would be a change in the soluble phase to larger hydrocarbon chains. The analysis did not show this. Instead the trend was more material in the soluble phase, with no change in the size of the hydrocarbons. The most likely reason for this difference in the results, from what was expected, is the sensitivity of this test. The analysis that was performed was designed by the company funding this research to test their treated rubber crumb. The method that they currently use affects not only the surface of the rubber crumb but the entire particle. This is in contrast to either of the methods I used, which only affected the surface of the particles. Because of this, the amount of material released into the soluble phase would

be much less in the rubber treated by either the whole cell or the enzyme methods. I believe that because of this, the change in the hydrocarbon size was not seen.

In the study by Robert and Margaret Romine, they were extremely interested in changes that occurred to the rubber crumb when it was incubated with *S. acidocaldarius*. They tested the rubber using a number of methods including: Fourier transform infrared spectroscopy, X-ray analysis of near-edge surfaces, as well as strength tests on revulcanized rubber. They found that up to 15 % rubber crumb could be added to virgin rubber, without affecting the strength of the revulcanized rubber. Interestingly, they also found that the rubber crumb that had been treated for the full seven days, until complete oxidation, did not give revulcanization results as good as those for rubber that had been treated for only 2-3 days. They found the difference between day 3 and day 7 treated rubber, was the presence of sulfoxide and sulfone compounds on the surface of the rubber at day 3. By day 7 of the incubation, these compounds had been oxidized by the cells and were no longer present. They speculated that these sulfoxide and sulfone compounds interact with the virgin rubber making the resulting rubber easier to revulcanize. This finding is very important to the enzyme results that I received. If with the cells a 2-3 day incubation is sufficient, then, given the shorter time frame of the enzyme method, perhaps a treatment of hours would be sufficient. When looking at the process from an economic standpoint this difference in incubation time is extremely important. It is also possible that given the specificity of the sulfur oxygenase, for sulfur, that the enzyme method would not oxidize the sulfoxide and sulfone compounds, therefore, removing the need for tight regulation of the process.

V) Does *Sulfolobus* have *sor*?

The final part of my research was an attempt to locate homologues of the *A. ambivalens sor* gene. The reason this search was undertaken was to find a version of the *sor* gene that may have been better suited to the devulcanization of rubber. Both hybridization and PCR using degenerate primers were used to screen a number of species, which are related to *Acidianus* (see figure #1). Unfortunately, the results of both of these screenings proved to be negative. This result was somewhat surprising as a homologue to *sor*, found in a bacterium (*Aquifex aeolicus*), shows a fairly high degree of similarity (see figure #8). One thing that *Acidianus* and *Aquifex* have in common, that differs from species of *Sulfolobus*, is the ability to grow anaerobically. Another piece of evidence for the lack of a *sor* gene in species of *Sulfolobus* was obtained by searching the database provided by the *Sulfolobus* genome project. The most recent search was of approximately 75% of the genome, which has been finished to date. I searched for any sulfur metabolism genes which had been identified in *Thiobacillus*, *Rhodococcus* and *Acidianus*, and found none. I did however, find the two ORF's that are found before and after the *sor* gene in *Acidianus*. In previous studies it was found that these ORF's were not transcriptionally related to *sor* (Kletzin 1992). These findings suggest that perhaps *Sulfolobus* uses a novel form of sulfur metabolism. I eagerly anticipate the completion of the genome sequencing project, as this will truly answer the question of the presence of *sor* in *Sulfolobus*.

VI) Conclusions and Future Studies

This study was designed to investigate the usefulness of sulfur oxidizing *Archaea* and their enzymes in the devulcanization of rubber crumb. I have found that both a whole cell system, based on *Sulfolobus solfataricus* P2, and an enzyme system, utilizing recombinant sulfur oxygenase/reductase from *Acidianus ambivalens*, are capable of oxidizing the sulfur found in the cross-links of vulcanized rubber. Furthermore, my results are comparable to the only other study on this subject. What remains to be answered is the financial viability of these processes as well as a better characterization of the treated rubber. Both of these questions are best answered by experts in the appropriate fields. Some interesting scientific questions have also come out of this study. The modified activity of the recombinant Sor provides the basis for a number of studies that could look at the structure of the recombinant enzyme as well as investigating the possibility of a cofactor associated with the native enzyme. Another question that remains to be answered is the type of sulfur oxidizing enzymes that are found in species of *Sulfolobus*. Do these species possess a highly divergent copy of the *sor* gene or do they have an entirely different set of enzymes?

Overall, I believe this study to be successful; it provided the answer to the key question that it was designed to answer, while providing the knowledge to ask new questions.

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Appendix IA: Recipes for solutions

Denaturing solution: 0.5 M NaOH, 1.5 M NaCl

20 g NaOH
87.6 g NaCl
bring volume to one litre with water

Depurination solution: 0.25 M HCl

10.78 ml concentrated HCl
489.2 ml water

Hybridization solution: 1 M NaCl, 50 mM, Tris-Cl pH 7.6, 5% SDS, 50% formamide

29.2 g NaCl
25 ml 1 M Tris-Cl pH 7.6
25 g SDS
250 ml formamide
bring to 500 ml with water

10X Klenow buffer: 0.5 M Tris-Cl pH 9.5, 0.1 M $MgCl_2$, 50 mM dithiothreitol, 1 mM spermidine

10X ligase buffer without ATP: 660 mM Tris-Cl pH 7.6, 50 mM $MgCl_2$, 10 mM dithiothreitol

Loading buffer (protein gels):

4 ml water
1 ml 0.5 M Tris-Cl pH 8.8
1.8 ml 10% SDS
800 µl glycerol
400 µl betamercaptoethanol
200 µl 0.1% bromphenol blue

Neutralizing solution: 0.5 M Tris-Cl pH 7.4, 1.5 M NaCl

500 ml of 1 M Tris-Cl pH 7.4
87.6 g NaCl
bring volume to one litre with water

10X RE buffer:

500 mM Tris-Cl pH8
500 mM KCl
100 mM $MgCl_2$
100 mM dithithreitol

10X RP-C:

200mM Tris-Cl pH 8
100 mM $MgCl_2$
50 mM dithiothreitol
660 µM each of dG, dA and dT
25% glycerol

10 X SDS Page Running Buffer:

15 g Tris-Cl pH 7.8
72 g glycine
5 g SDS
bring volume to one litre with water

Size Marker (λ - *BstE*II-*Xho*I-*Xba*I):

λ DNA digested with *BstE*II: 120 µg λ DNA
80 µl of 10X RE buffer
20 µl of 4 M NaCl
10 µl *BstE*II (100U)
Bring volume to 800 µl with water
incubate at 65° for 3 hours

λ DNA digested with *Xho*I: 40 µg of λ DNA
80 µl of 10 X RE buffer
5 µl of *Xho*I (50 U)
bring volume to 800 µl with water
incubate at 37° for 3 hours

λ DNA digested with *Xba* I: 40 µg of λ DNA
80 µl of 10X RE buffer
8 µl of *Xba*I (80U)
bring volume to 800 µl with water
incubate at 37° for 3 hours

Mix together each of the digests: 100 µl λ DNA digested with *BstE*II
100 µl λ DNA digested with *Xho*I
100 µl λ DNA digested with *Xba* I

100 µl TE
100 µl 0.25 M EDTA
150 µl loading buffer

20X SSC: 3 M NaCl, 0.3 M sodium citrate

350.6 g NaCl
176.4 g sodium citrate
bring volume to two litres with water

STE: 1% N-lauroyl sarcosine, 50 mM Tris-Cl, 50 mM EDTA

0.2 g N-lauroyl sarcosine
1 ml Tris-Cl pH 8
2 ml 0.5 M EDTA pH 8

40X TAE: 2 M Tris, 80 mM EDTA, 0.8 M sodium acetate

484.5 Tris-Cl pH 8
59.9 g EDTA
217.8 g sodium acetate
add 1857.6 ml of water
adjust pH to 7.2 by adding 142.4 ml of acetic acid

TE: 10 mM Tris-Cl pH8, 1 mM EDTA pH8

10 ml of 1 M Tris-Cl pH8
2 ml of 500 mM EDTA
bring volume to one litre with water and autoclave

Transfer solution: 0.4 M NaOH

16 g NaOH
bring volume to one litre of water

Prewash solution: 5 X SSC, 0.5% SDS, 1 mM EDTA

500 ml of 20X SSC
10 g SDS
0.74 g EDTA
bring volume to 2 litres with water

Appendix IB: Recipes for Media

***Sulfolobus* media:**

Broth: 500 µl 100X Salt solution
250 µl 200X Salt solution
50 µl of 1000X Salt solution
500 µl 20% sucrose
500 µl 10% yeast extract
100-250 µl 1:50 H_2SO_4 in water
48 ml water

100X Salts: 260 g $(NH_4)_2SO_4$
50 g $MgSO_4 \cdot 7H_2O$
autoclave
add
4 g $FeCl_3 \cdot 6H_2O$
3 ml 50% H_2SO_4

200X Salts: 90 ml of 10 mg/ml $Na_2B_4O_7 \cdot 10H_2O$
36 ml of 10 mg/ml $MnCl_2 \cdot 4H_2O$
4.4 ml of 10 mg/ml $ZnSO_4 \cdot 7H_2O$
1 ml of 10 mg/ml $CuCl_2 \cdot 2H_2O$
0.6 ml of 10 mg/ml $VOSO_4 \cdot 2H_2O$
0.6 ml of 10 mg/ml $Na_2MoO_4 \cdot 2H_2O$
0.2 ml of 10 mg/ml $CoSO_4$
56 g KH_2PO_4
autoclave
add 5 ml 50% H_2SO_4

1000X Salts: 14 g $Ca_2Cl_2 \cdot 2H_2O$

YT Broth:

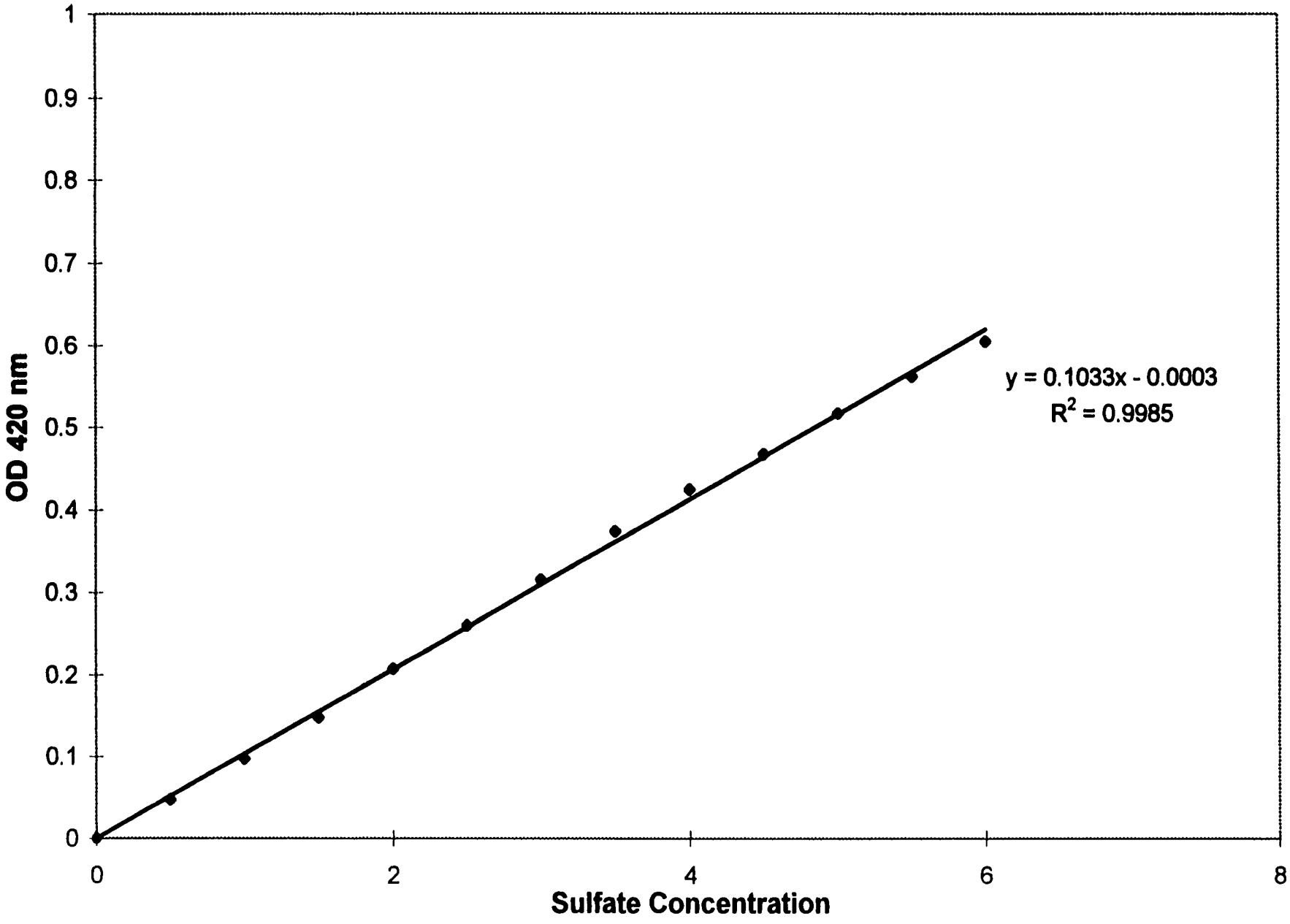
8 g tryptone
5 g yeast extract
5 g NaCl
bring volume to one litre with water and autoclave
for ampicillin broth add 70 mg filter-sterilized ampicillin

YT-Ampicillin-Xgal-IPTG plates:

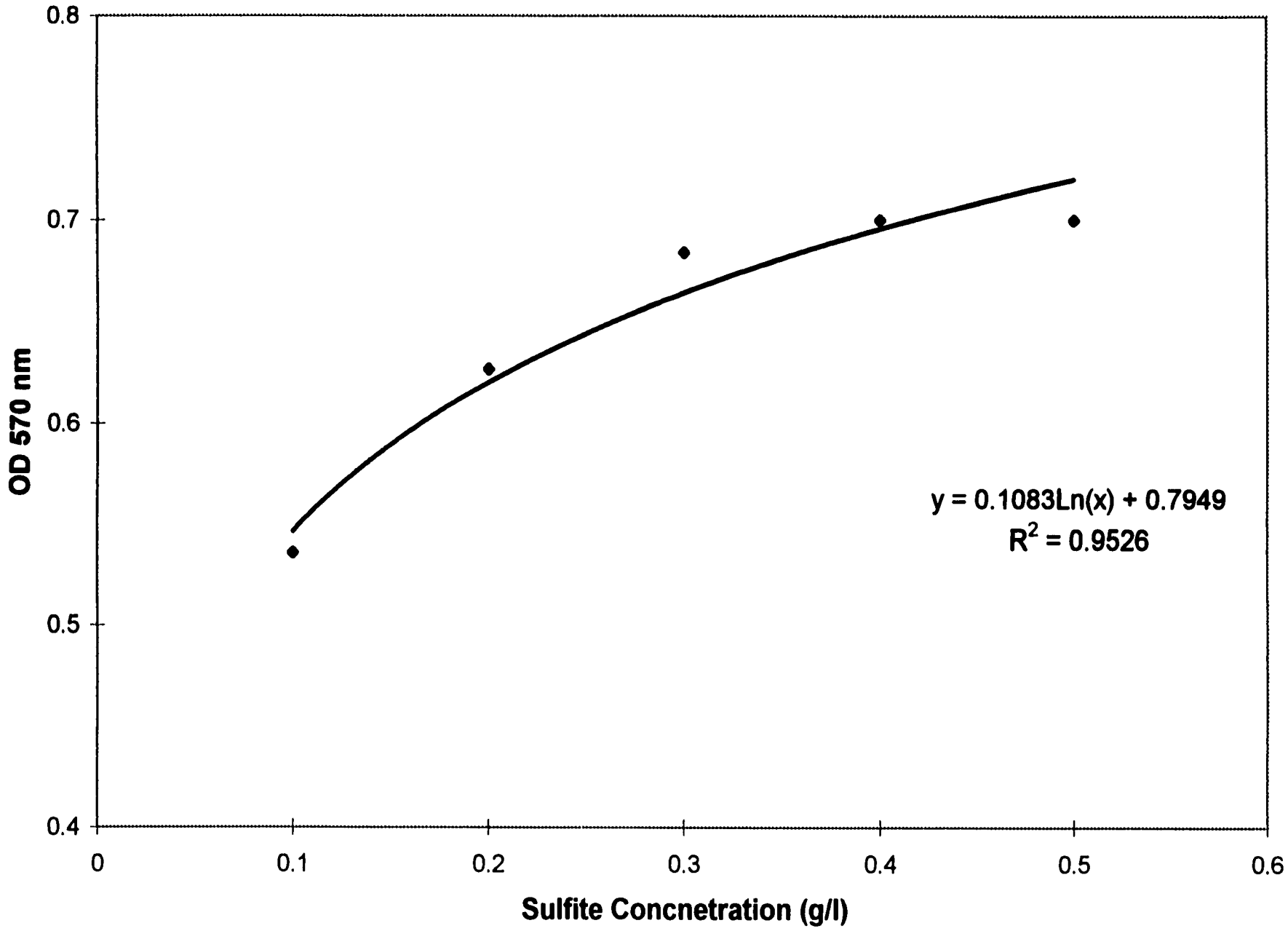
8 g tryptone
5 g yeast extract
5 g NaCl
15 g agar
bring volume to one litre with water and autoclave
cool to 55°
add 70 mg filter-sterilized ampicillin, 2 ml 2% xgal and 0.4 ml 1 M IPTG

Appendix II: Standard Curves for Sulfite and Sulfate Assays

Sulfate Standard Curve



Sulfite Standard Curve



Appendix III: Statistical Analysis

Sodium Bicarbonate ANOVA Results

One-way analysis of variance
  P value P<0.0001
  P value summary ***
  Are means signif. different? (P < 0.05) Yes
  Number of groups 5
  F 20.52
  R squared 0.8914

ANOVA Table	SS	df	MS
Treatment (between columns)	0.04596	4	0.01149
Residual (within columns)	0.005600	10	0.00056
Total	0.05156	14	

Gassing Conditions ANOVA Results

One-way analysis of variance
  P value P<0.0001
  P value summary ***
  Are means signif. different? (P < 0.05) Yes
  Number of groups 5
  F 15.48
  R squared 0.5533

Bartlett's test for equal variances
  Bartlett's statistic (corrected) 96.63
  P value P<0.0001
  P value summary ***
  Do the variances differ signif. (P < 0.05) Yes

ANOVA Table	SS	df	MS
Treatment (between columns)	2.513	4	0.6281
Residual (within columns)	2.029	50	0.04057
Total	4.541	54	

5 mM Sodium Bicarbonate and Gassing Everyday t-test

Unpaired t test
  P value 0.0004
  P value summary ***
  Are means signif. different? (P < 0.05) Yes
  One- or two-tailed P value? Two-tailed
  t, df t=10.67 df=4

How big is the difference?
  Mean ± SEM of column A 0.2733 ± 0.01202 N=3
  Mean ± SEM of column F 0.1267 ± 0.006667 N=3
  Difference between means 0.1467 ± 0.01374
  95% confidence interval -0.1848 to -0.1085

R squared 0.9661

F test to compare variances
F, DFn, Dfd 3.250, 2, 2
P value 0.2353
P value summary ns
Are variances significantly different? No

pH ANOVA Results

One-way analysis of variance
P value P<0.0001
P value summary ***
Are means signif. different? (P < 0.05) Yes
Number of groups 3
F 82.95
R squared 0.9651

ANOVA Table	SS	df	MS
Treatment (between columns)	0.03687	2	0.01843
Residual (within columns)	0.001333	6	0.0002222
Total	0.03820	8	

Rubber Crumb Size t-test Results

Unpaired t test
P value P<0.0001
P value summary ***
Are means signif. different? (P < 0.05) Yes
One- or two-tailed P value? Two-tailed
t, df t=7.977 df=64

How big is the difference?
Mean ± SEM of column A 0.4706 ± 0.02172 N=33
Mean ± SEM of column B 0.2812 ± 0.009594 N=33
Difference between means 0.1894 ± 0.02374
95% confidence interval -0.2369 to -0.1419
R squared 0.4985

F test to compare variances
F, DFn, Dfd 5.125, 32, 32
P value P<0.0001
P value summary ***
Are variances significantly different? Yes

CELLS compared to Enzyme t-test

Unpaired t test
P value P<0.0001
P value summary ***
Are means signif. different? (P < 0.05) Yes
One- or two-tailed P value? Two-tailed
t, df t=8.553 df=64

How big is the difference?
Mean ± SEM of column A 0.4706 ± 0.02172 N=33
Mean ± SEM of column B 0.9256 ± 0.04856 N=33

Difference between means -0.4550 ± 0.05320
95% confidence interval 0.3487 to 0.5613
R squared 0.5334

F test to compare variances
F,DFn, Dfd 4.998, 32, 32
P value P<0.0001
P value summary ***
Are variances significantly different? Yes

Preincubated compared to normal enzyme t-test

Unpaired t test
P value 0.5852
P value summary ns
Are means signif. different? (P < 0.05) No
One- or two-tailed P value? Two-tailed
t, df t=0.5929 df=4

How big is the difference?
Mean ± SEM of column A 0.6667 ± 0.08819 N=3
Mean ± SEM of column B 0.7300 ± 0.06028 N=3
Difference between means -0.06333 ± 0.1068
95% confidence interval -0.2332 to 0.3599
R squared 0.08078

F test to compare variances
F,DFn, Dfd 2.141, 2, 2
P value 0.3184
P value summary ns
Are variances significantly different? No

Appendix IV:
Sequence Alignment for *sor* gene and pKK223-3/*sor*

```
sor gene        ----------------------------------------ATGCCGAAACCATACG
pKK223-3/sor    CTAGCATGCCTGCAGGTCNNNTCTNNNNNNNNNNNNCNAATTCAAATGCCGAAACCATACG
                                                        ****************

sor gene        TTGCTATAAACATGGCAGAATTAAAGAATGAACCTAAAACTTTTGAAATGTTTGCCTCAG
pKK223-3/sor    TTGCTATAAACATGGCAGAATTAAAGAATGAACCTAAAACTTTTGAAATGTTTGCCTCAG
                ************************************************************

sor gene        TAGGACCGAAGGTCTGCATGGTAACAGCAAGGCATCCGGGCTTTGTTGGTTTTCAAAACC
pKK223-3/sor    TAGGACCGAAGGTCTGCATGGTAACAGCAAGGCATCCGGGCTTTGTTGGTTTTCAAAACC
                ************************************************************

sor gene        ATATACAAATAGGAATTTTGCCATTCGGAAACAGATACGGCGGAGCTAAAATGGACATGA
pKK223-3/sor    ATATACAAATAGGAATTTTGCCATTCGGAAACAGATACGGCGGAGCTAAAATGGACATGA
                ************************************************************

sor gene        CTAAGGAAAGTAGTACTGTCAGAGTTTTACAGTACACCTTCTGGAAAGATTGGAAAGACC
pKK223-3/sor    CTAAGGAAAGTAGTACTGTCAGAGTTTTACAGTACACCTTCTGGAAAGATTGGAAAGACC
                ************************************************************

sor gene        ATGAAGAAATGCACAGGCAAAACTGGAGTTACTTATTCAGGCTATGCTATTCATGCGCTT
pKK223-3/sor    ATGAAGAAATGCACAGGCAAAACTGGAGTTACTTATTCAGGCTATGCTATTCATGCGCTT
                ************************************************************

sor gene        CACAAATGATATGGGGACCCTGGGAGCCAATTTATGAAATAATCTACGCAAACATGCCTA
pKK223-3/sor    CACAAATGATATGGGGACCCTGGGAGCCAATTTATGAAATAATCTACGCAAACATGCCTA
                ************************************************************

sor gene        TAAACACTGAAATGACCGACTTCACTGCAGTTGTAGGAAAGAAGTTCGCAGAAGGAAAGC
pKK223-3/sor    TAAACACTGAAATGACCGACTTCACTGCAGTTGTAGGAAAGAAGTTCGCAGAAGGAAAGC
                ************************************************************

sor gene        CTTTAGATATTCCAGTTATTTCACAACCATATGGAAAGAGAGTTGTTGCCTTTGCAGAGC
pkk223-3/sor    CTTTAGATATTCCAGTTATTTCACAACCATATGGAAAGAGAGTTGTTGCCTTTGCAGAGC
                ************************************************************

sor gene        ACTCAGTAATTCCAGGCAAAGAGAAGCAATTTGAGGACGCAATAGTTAGGACTTTAGAAA
pKK223-3/sor    ACTCAGTAATTCCAGGCAAAGAGAAGCAATTTGAGGACGCAATAGTTAGGACTTTAGAAA
                ************************************************************

sor gene        TGTTAAAGAAAGCTCCTGGCTTCTTAGGTGCAATGGTATTAAAGGAAATAGGAGTTTCCG
pKK223-3/sor    TGTTAAAGAAAGCTCCTGGCTTCTTAGGTGCAATGGTATTAAAGGAAATAGGAGTTTCCG
                ************************************************************

sor gene        GAATTGGAAGCATGCAATTCGGTGCCAAGGGATTCCATCAAGTCTTAGAGAACCCTGGAT
pKK223-3/sor    GAATTGGAAGCATGCAATTCGGTGCCAAGGGATTCCATCAAGTCTTAGAGAACCCTGGAT
                ************************************************************

sor gene        CACTTGAGCCAGATCCAAATAATGTAATGTATTCAGTCCCAGAAGCAAAGAATACTCCAC
pKK223-3/sor    CACTTGAGCCAGATCCAAATAATGTAATGTATTCAGTCCCAGAAGCAAAGAATACTCCAC
                ************************************************************
```

```
sor gene          AACAATACATAGTTCATGTAGAATGGGCAAATACTGATGCTTTAATGTTTGGAATGGGTA
pKK223-3/sor      AACAATACATAGTTCATGTAGAATGGGCAAATACTGATGCTTTAATGTTTGGAATGGGTA
                  ************************************************************


sor gene          GAGTACTATTATATCCTGAGCTAAGACAAGTACACGACGAAGTTTTAGACACATTAGTAT
pKK223-3/sor      GAGTACTATTATATCCTGAGCTAAGACAAGTACACGACGAAGTTTTAGACACATTAGTAT
                  ************************************************************

sor gene          ACGGACCTTACATTAGAATATTAAATCCAATGATGGAAGGCACATTCTGGAGAGAATATT
pKK223-3/sor      ACGGACCTTACATTAGAATATTAAATCCAATGATGGAAGGCACATTCTGGAGAGAATATT
                  ************************************************************

sor gene          TAAACGAACAATAA----------------
pKK223-3/sor      TAAACGAACAATAATCGAATTGGNANNNNG
                  **************
```

Appendix V: Raw Data From Rubber Testing

Polymer Laboratories
PL GPC Software

11:32 Mon Jun 15 1998

Unknown 8MY29.014
R 1

Acquired : 11:31 Mon Jun 15 1998
Operator L.K.KOSTANSKI

Concentration :
Injection Volume :
Solvent : THF
Column Set : 4XSTYRAGEL

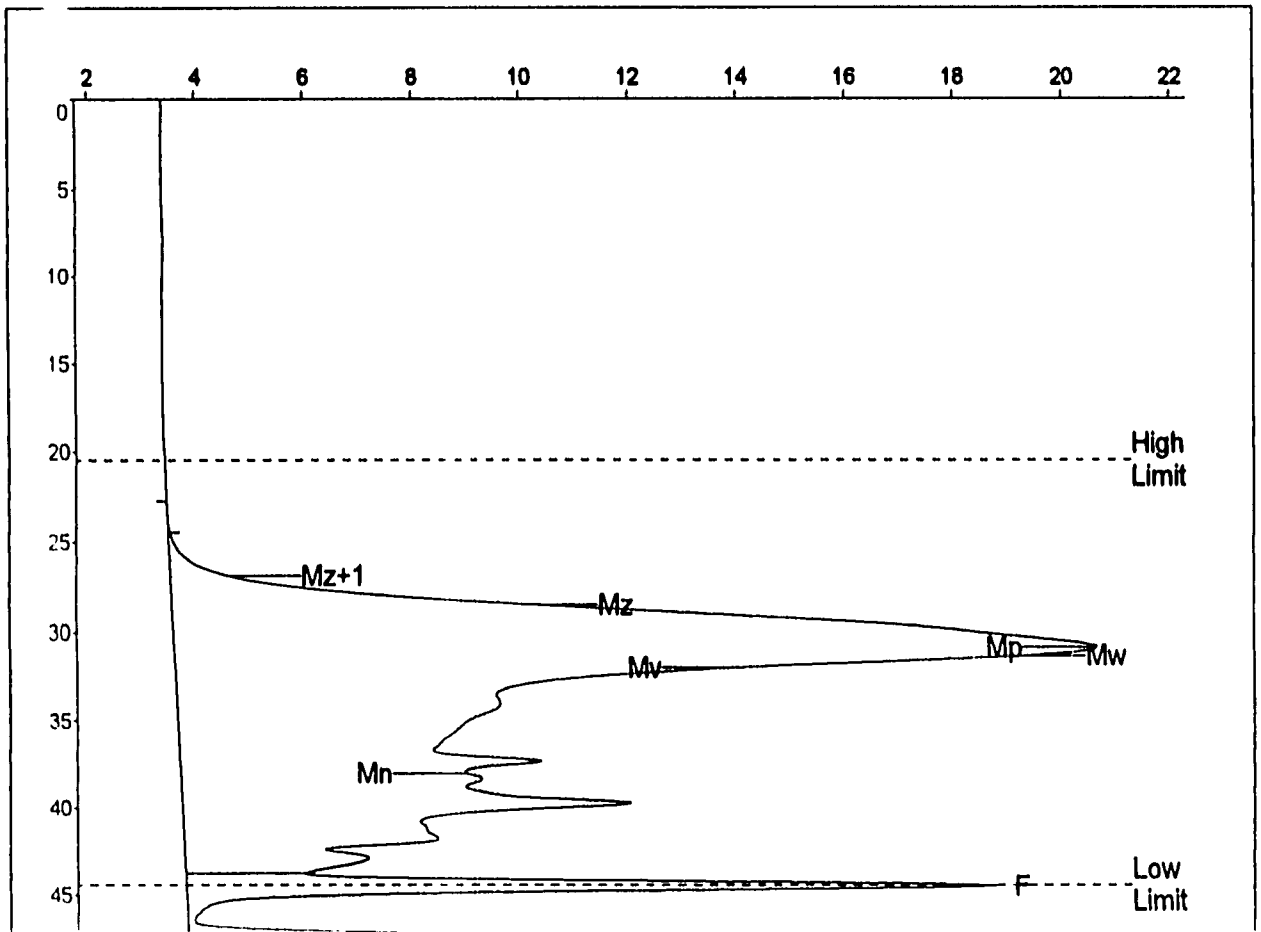
Detector : RI_DAD
Temperature : RT
Flow Rate : 1.000
Standards : PS

Method 11
Comments :

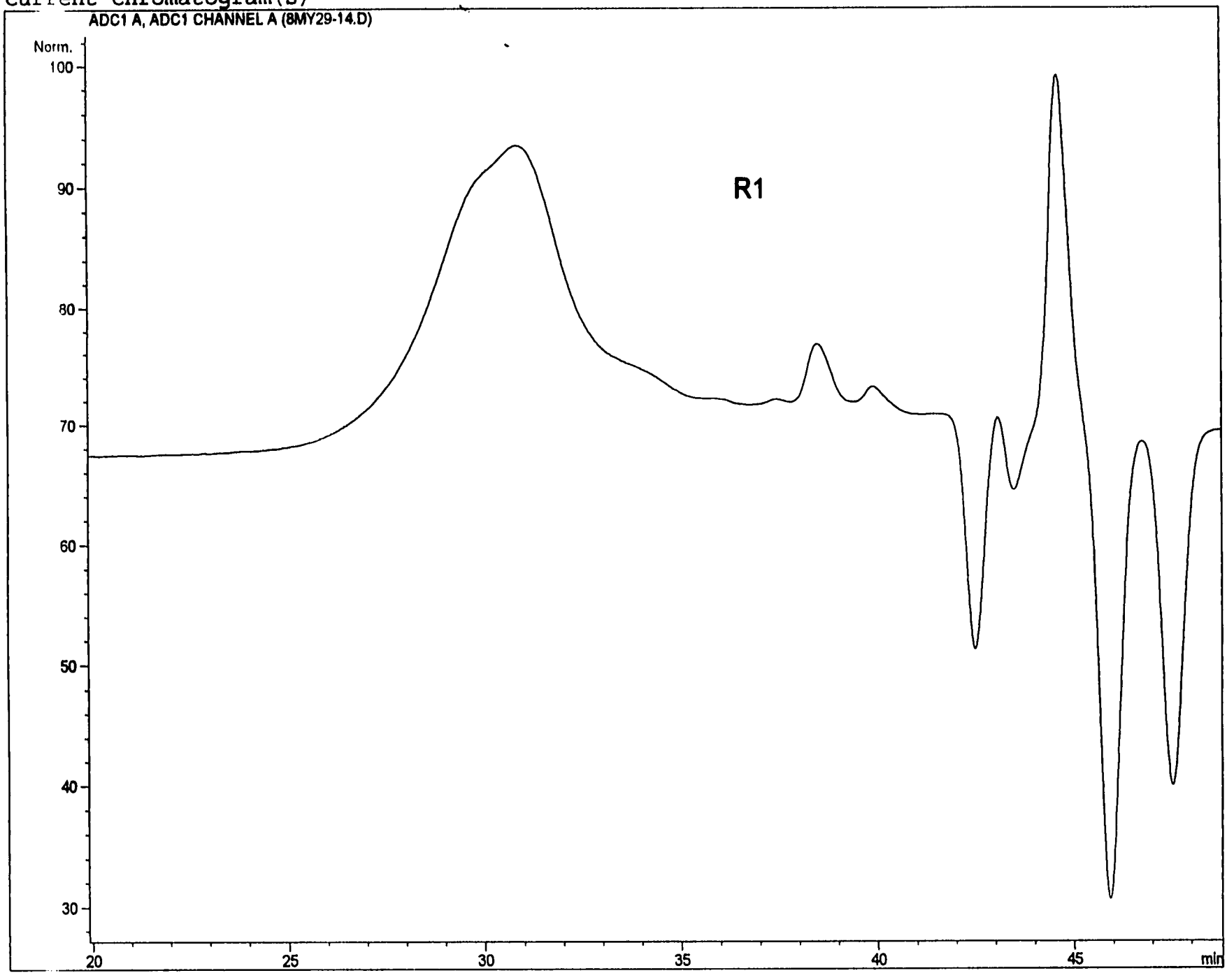
Calibration Using : Narrow Standards
Calibration Limits : 20.46 to 44.43 Mins
Flow Rate Marker : found at : 44.45

Curve Used : 3rd Order Polynomial
Last Calibrated : Mon Jun 15 11:10:40 1998
In Standards at : 44.50 Mins

Broad Peak Start : 24.48 End : 43.73 Mins



Current Chromatogram(s)



122

Polymer Laboratories PL GPC Software

Filename : 8MY29.014
Sample Name : R 1
Run Type : Unknown
Method : 11

Date : 11:32 Mon Jun 15 1998
Date Acquired : 11:31 Mon Jun 15 1998
Operator L.K.KOSTANSKI

CALIBRATION PARAMETERS :

Type : Narrow Standards
Curve Used : 3rd Order Polynomial
Coefficients : Log(M) = A + BT + CT2 + DT3
A=12.932137, B=-0.305157, C=-0.000455, D=0.000040

Limits of Referenced Calibration: 20.46 Mins to 44.43 Mins

Calibrated on : Mon Jun 15 11:10:40 1998

Standards :
K Value: 14.1000 * 10e-5 Alpha Value : 0.700

Sample:
K Value 14.1000 * 10e-5 Alpha Value : 0.700

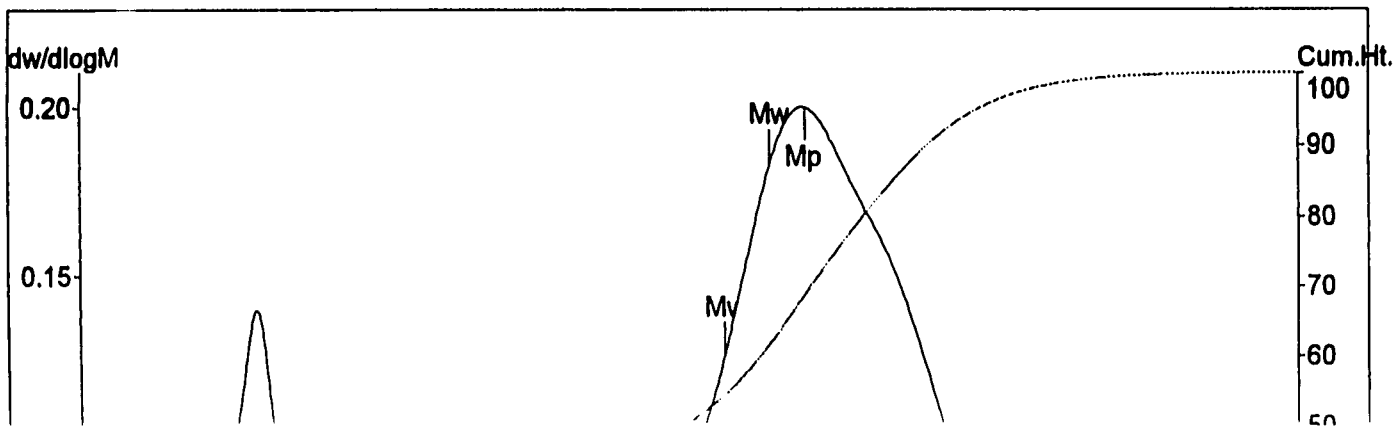
Flow Rate Marker:
Found in Standards at 44.50 Mins
Found in Sample at 44.45 Mins

Broad Peak Markers: Start 24.48 Mins End 43.73 Mins

MOLECULAR WEIGHT PARAMETERS

Mp =	17691	Mz =	59134
Mn =	684	Mz+1 =	144713
Mw =	13767	Mv =	9981

Poly dispersity (Mw/Mn) = 20.114
Peak Area 2414791

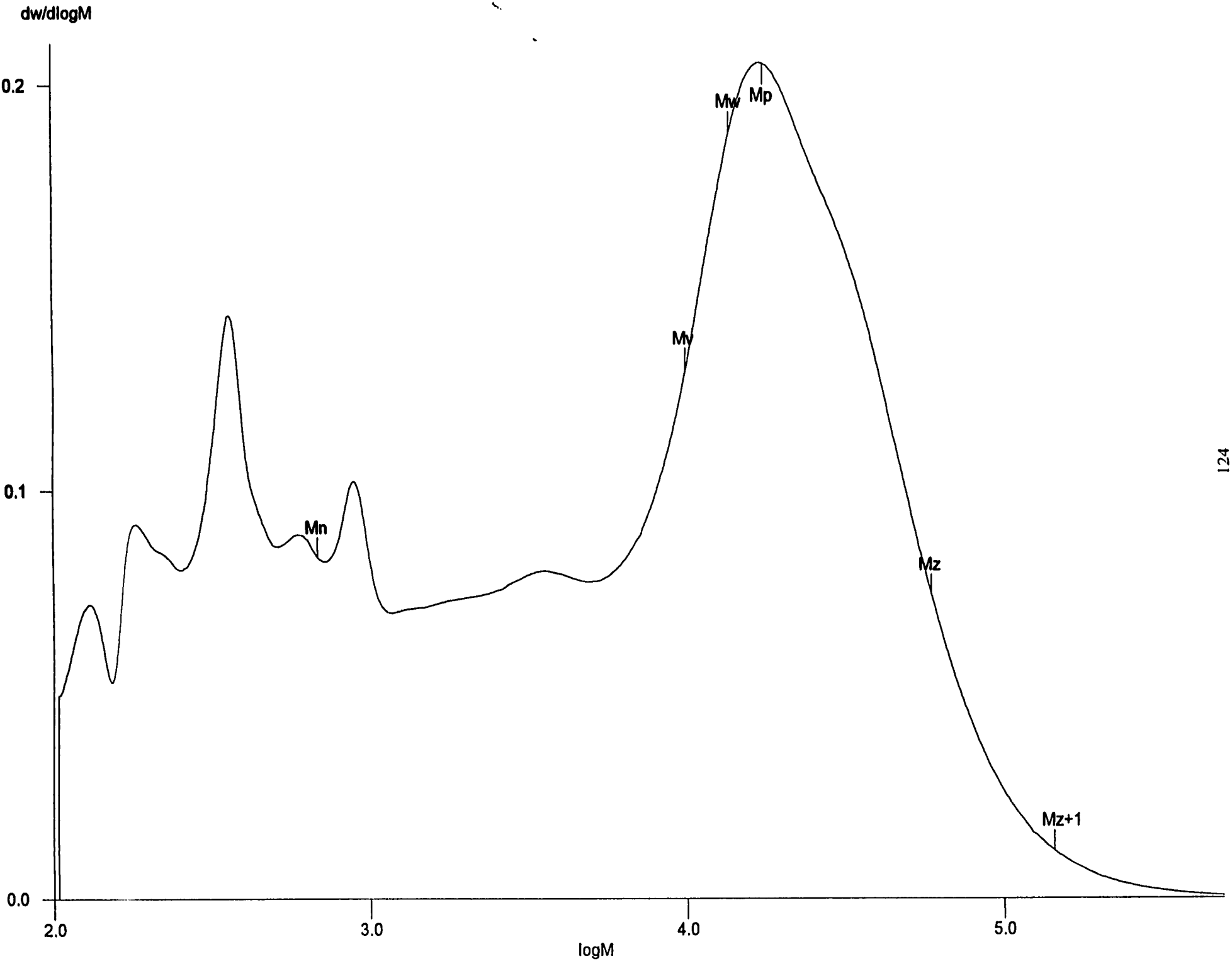


GPC Single Channel Online 1 11:32 Mon Jun 15 1998

Raw Data: Reanalysed from 8MY29.014

Differential Molecular Weight Graph for Sample 14

Sample Name : R 1



Polymer Laboratories
PL GPC Software

11:34 Mon Jun 15 1998

Unknown 8MY29.015
M1

Acquired : 11:32 Mon Jun 15 1998
Operator L.K.KOSTANSKI

Concentration :
Injection Volume :
Solvent : THF
Column Set : 4XSTYRAGEL

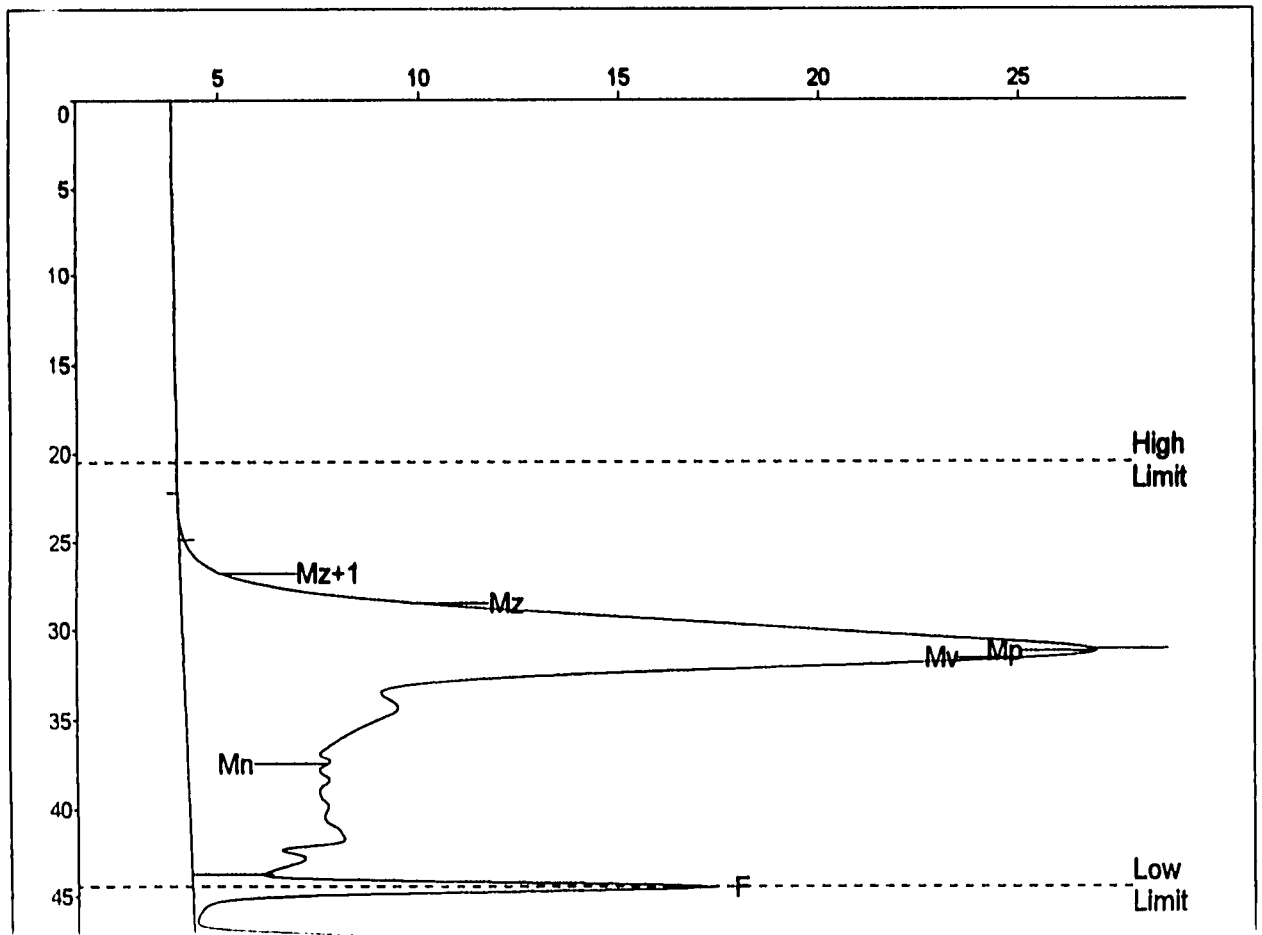
Detector : RI_DAD
Temperature : RT
Flow Rate : 1.000
Standards : PS

Method 11
Comments :

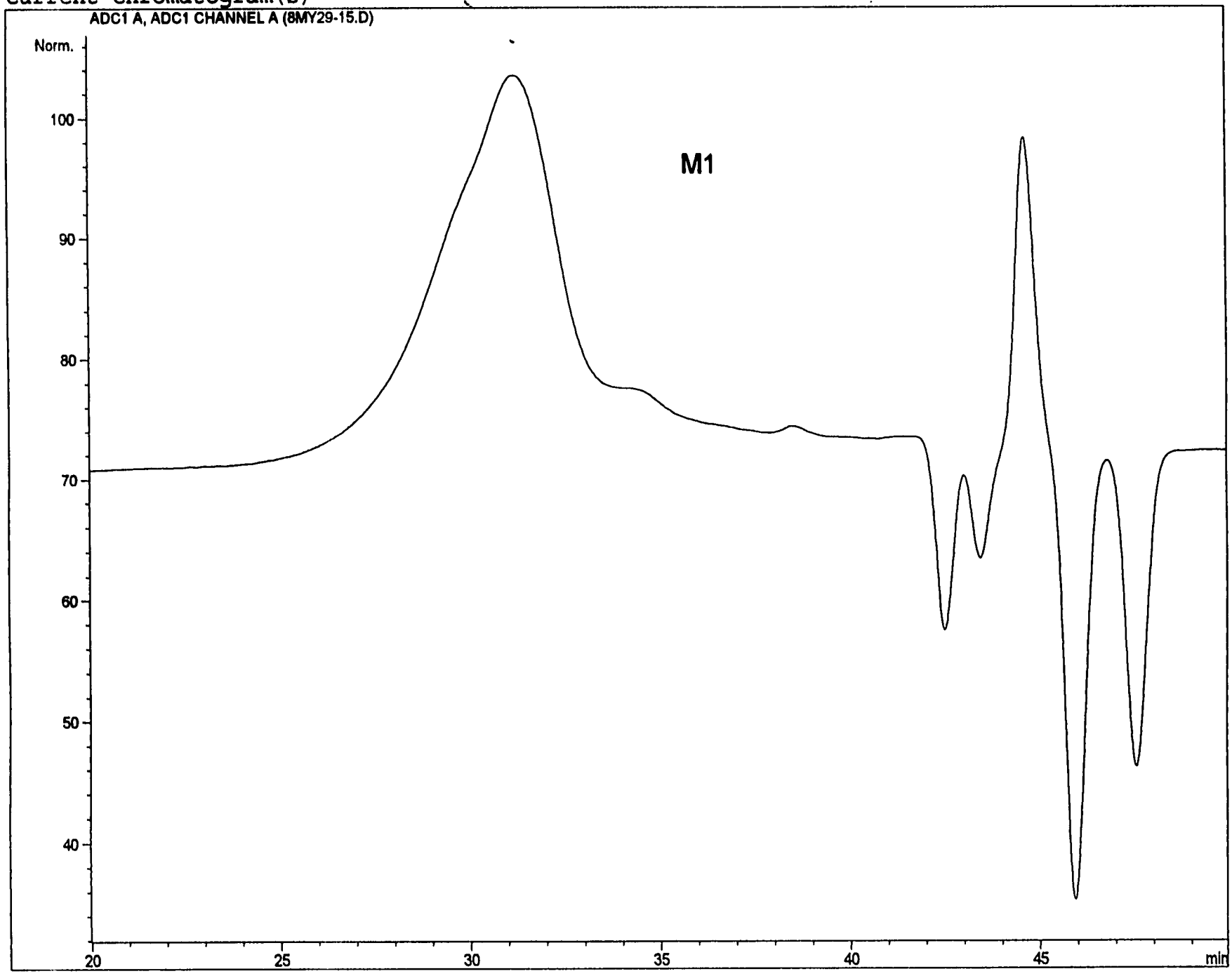
Calibration Using : Narrow Standards
Calibration Limits : 20.46 to 44.43 Mins
Flow Rate Marker : found at : 44.45

Curve Used : 3rd Order Polynomial
Last Calibrated : Mon Jun 15 11:10:40 1998
In Standards at : 44.50 Mins

Broad Peak Start : 24.86 End : 43.73 Mins



Current Chromatogram(s)



126

Polymer Laboratories PL GPC Software

Filename : 8MY29.015
Sample Name : M1
Run Type : Unknown
Method : 11

Date : 11:34 Mon Jun 15 1998
Date Acquired : 11:32 Mon Jun 15 1998
Operator L.K.KOSTANSKI

CALIBRATION PARAMETERS :

Type : Narrow Standards
Curve Used : 3rd Order Polynomial
Coefficients : Log(M) = A + BT + CT2 + DT3
A=12.932137, B=-0.305157, C=-0.000455, D=0.000040

Limits of Referenced Calibration: 20.46 Mins to 44.43 Mins
Calibrated on : Mon Jun 15 11:10:40 1998

Standards :
K Value: 14.1000 * 10e-5 Alpha Value : 0.700
Flow Rate Marker:
Broad Peak Markers: Start 24.86 Mins End 43.73 Mins

Sample:
K Value 14.1000 * 10e-5 Alpha Value : 0.700
Found in Standards at 44.50 Mins
Found in Sample at 44.45 Mins

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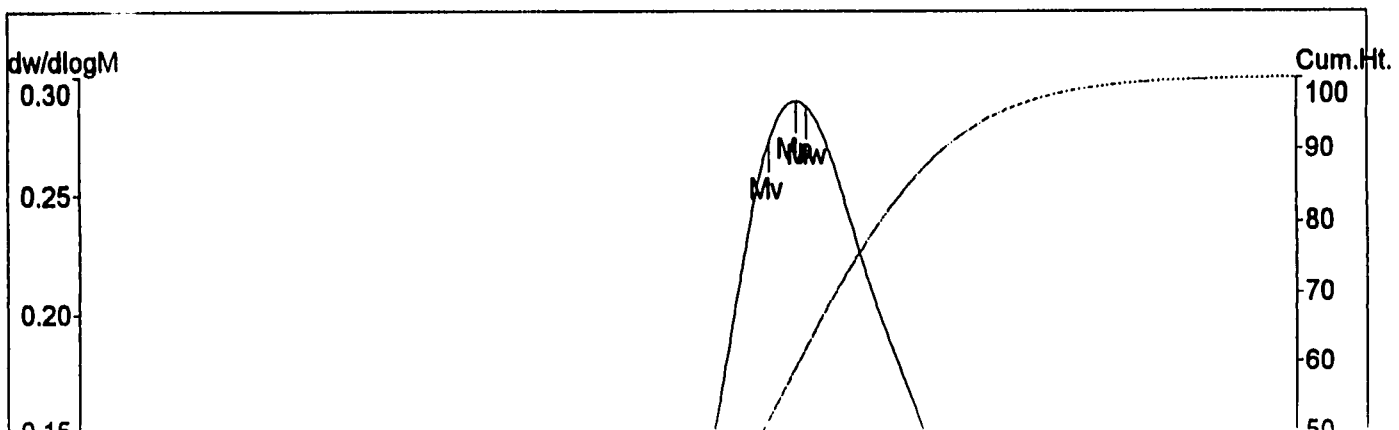
MOLECULAR WEIGHT PARAMETERS

Mp =	14687	Mz =	58865
Mn =	859	Mz+1 =	149326
Mw =	15723	Mv =	12153

Poly dispersity (Mw/Mn) = 18.297
Peak Area 2345267



127

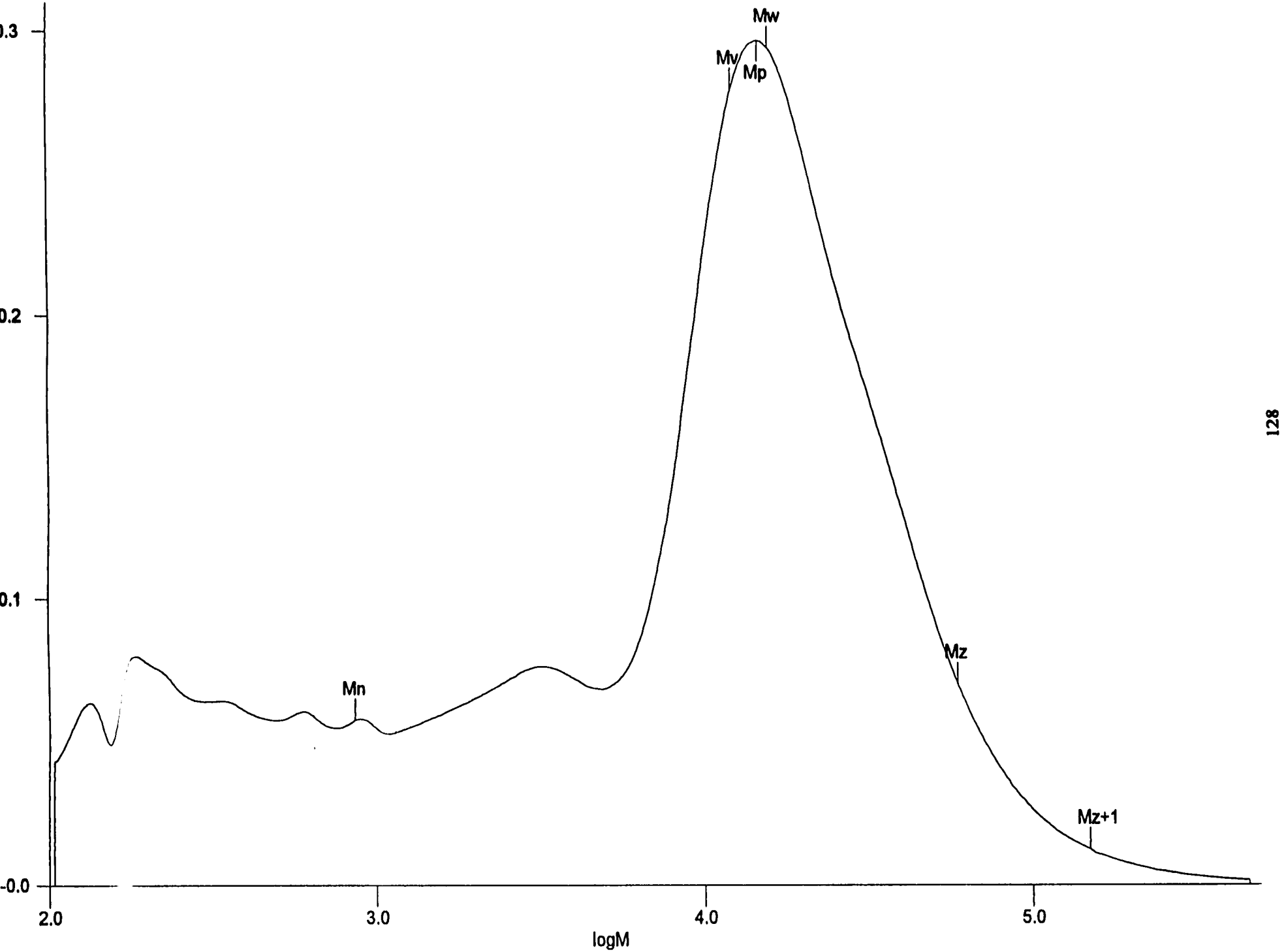
GPC Single Channel Online 1 11:35 Mon Jun 15 1998

Raw Data: Reanalysed from 8MY29.015

Differential Molecular Weight Graph for Sample 15

Sample Name : M1

dw/dlogM



128

Polymer Laboratories
PL GPC Software

11:27 Mon Jun 15 1998

Unknown 8MY29.012
B 1

Acquired : 11:25 Mon Jun 15 1998
Operator L.K.KOSTANSKI

Concentration :
Injection Volume :
Solvent : THF
Column Set : 4XSTYRAGEL

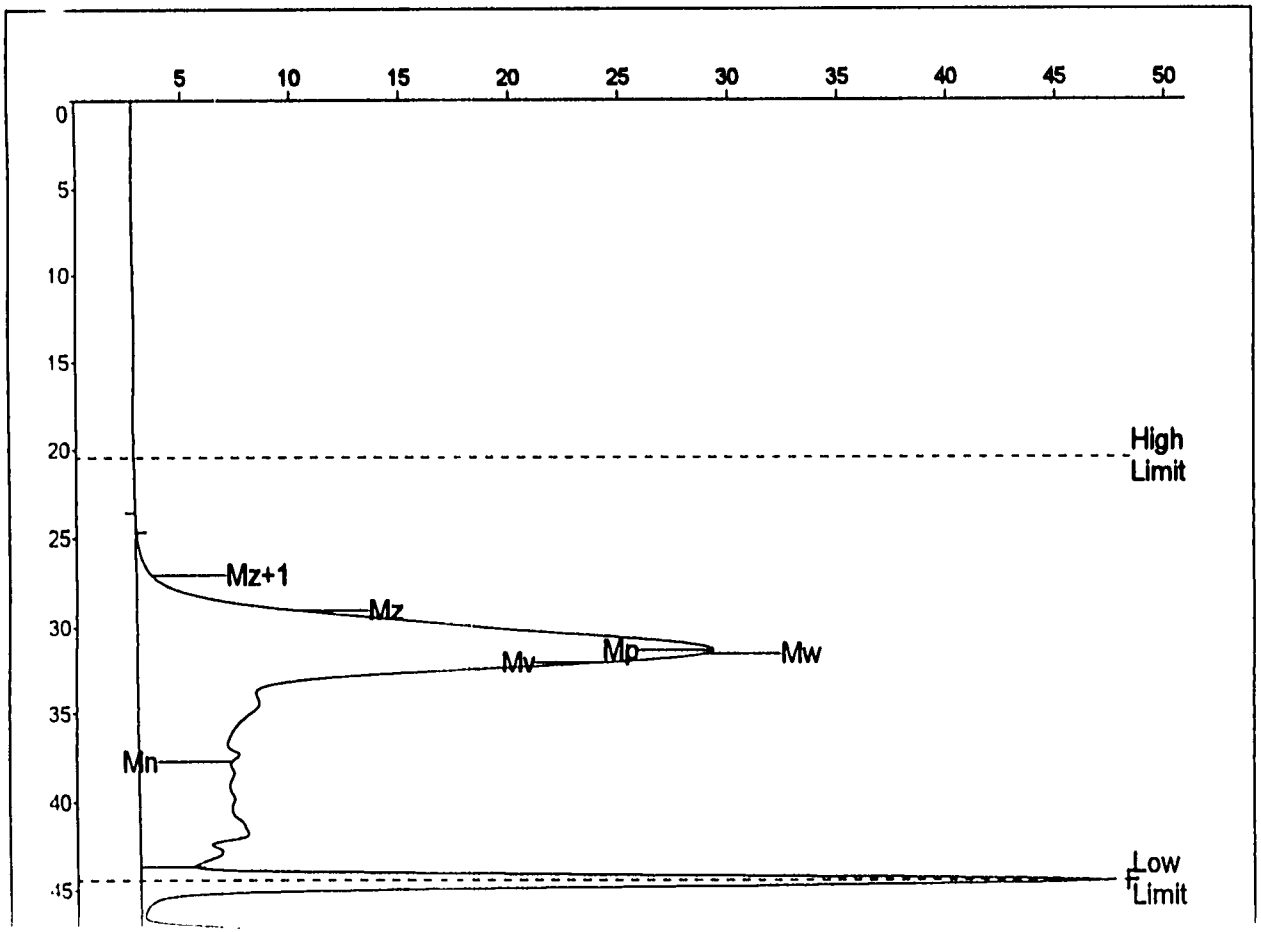
Detector : RI_DAD
Temperature : RT
Flow Rate : 1.000
Standards : PS

Method 11
Comments :

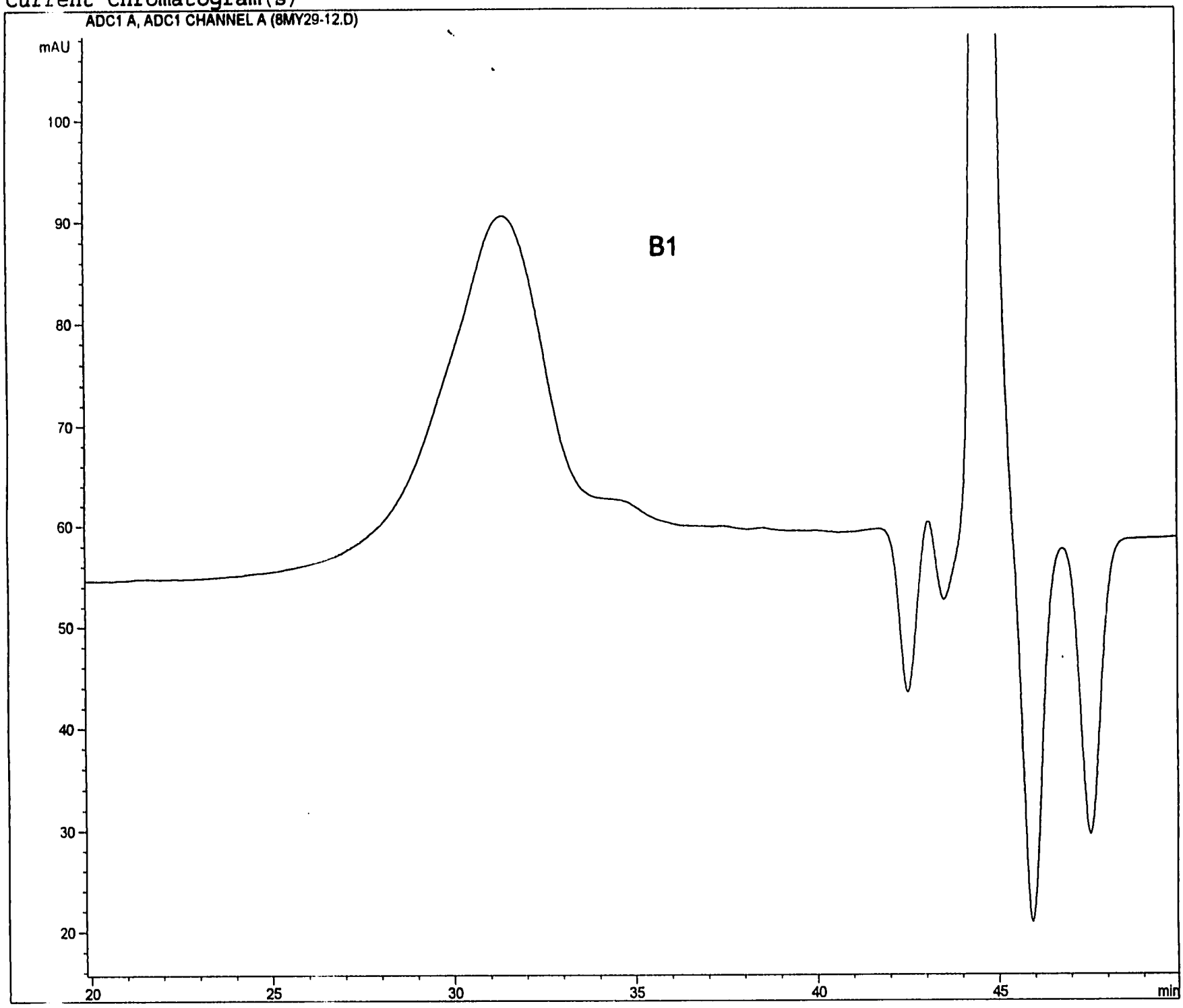
Calibration Using : Narrow Standards
Calibration Limits : 20.46 to 44.43 Mins
Flow Rate Marker : found at : 44.45

Curve Used : 3rd Order Polynomial
Last Calibrated : Mon Jun 15 11:10:40 1998
in Standards at : 44.50 Mins

Broad Peak Start : 24.70 End : 43.63 Mins



Current Chromatogram(s)



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Polymer Laboratories PL GPC Software

Filename : 8MY29.012
Sample Name : B 1
Run Type : Unknown
Method : 11

Date : 11:27 Mon Jun 15 1998
Date Acquired : 11:25 Mon Jun 15 1998
Operator L.K.KOSTANSKI

CALIBRATION PARAMETERS :

Type : Narrow Standards
Curve Used : 3rd Order Polynomial
Coefficients : Log(M) = A + BT + CT2 + DT3

A=12.932137, B=-0.305157, C=-0.000455, D=0.000040

Limits of Referenced Calibration: 20.46 Mins to 44.43 Mins

Calibrated on : Mon Jun 15 11:10:40 1998

Standards :

K Value: 14.1000 * 10e-5 Alpha Value : 0.700

Flow Rate Marker:

Broad Peak Markers: Start 24.70 Mins End 43.63 Mins

Sample:

K Value 14.1000 * 10e-5 Alpha Value : 0.700

Found in Standards at 44.50 Mins
Found in Sample at 44.45 Mins

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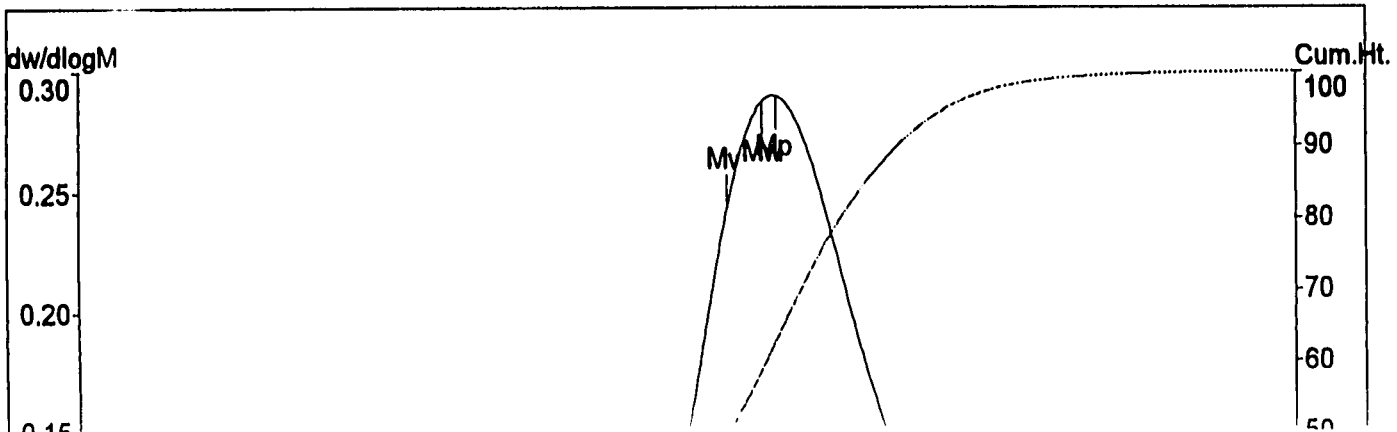
MOLECULAR WEIGHT PARAMETERS

Mp =	13663	Mz =	44628
Mn =	775	Mz+1 =	132412
Mw =	12389	Mv =	9672

Poly dispersity (Mw/Mn) = 15.967
Peak Area 2640601

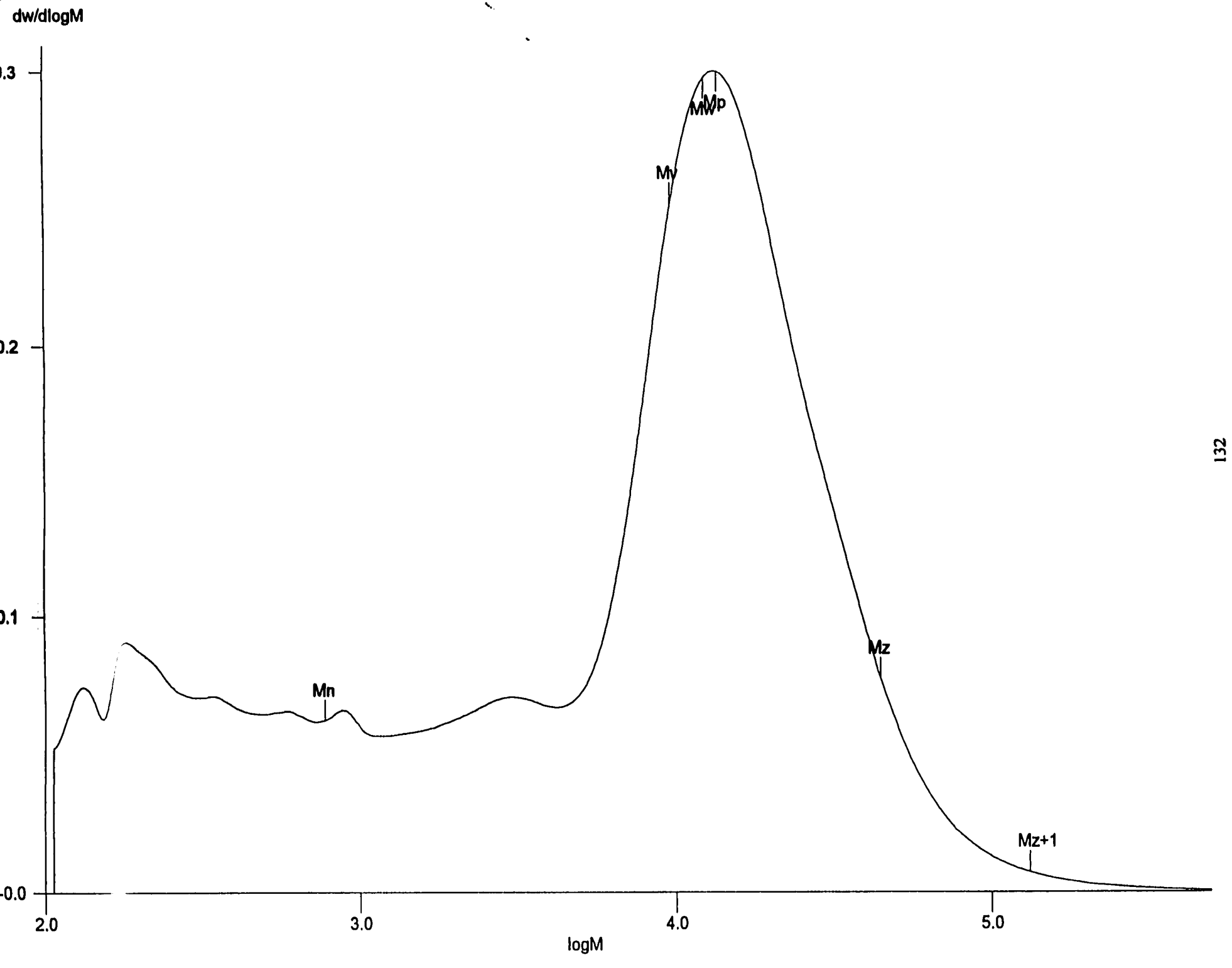


GPC Single Channel Online 1 11:28 Mon Jun 15 1998

Raw Data: Reanalysed from 8MY29.012

Differential Molecular Weight Graph for Sample 12

Sample Name : B 1



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Polymer Laboratories
PL GPC Software

11:30 Mon Jun 15 1998

Unknown 8MY29.013
C 1

Acquired : 11:28 Mon Jun 15 1998
Operator L.K.KOSTANSKI

Concentration :
Injection Volume :
Solvent : THF
Column Set : 4XSTYRAGEL

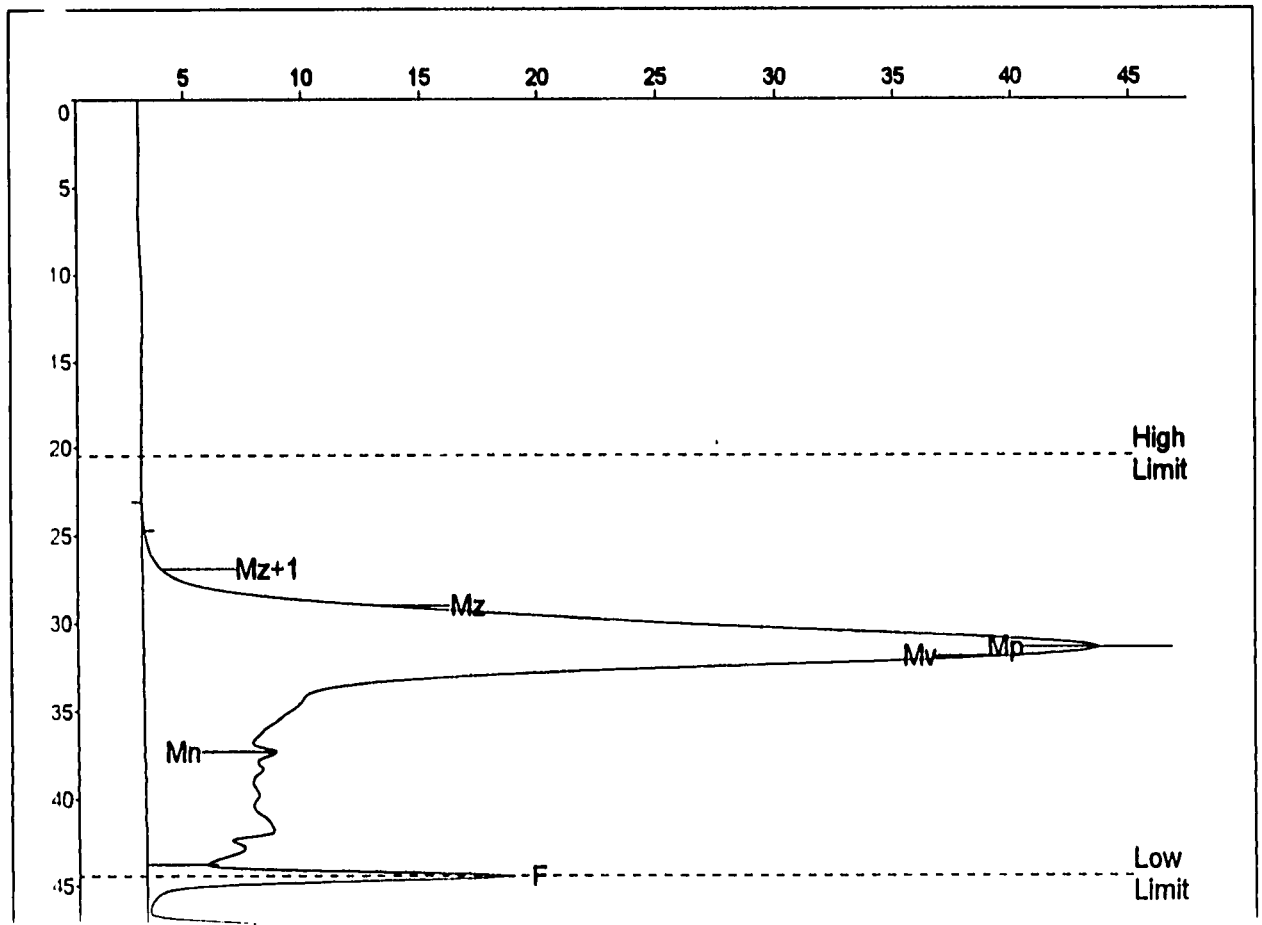
Detector : RI_DAD
Temperature : RT
Flow Rate : 1.000
Standards : PS

Method 11
Comments :

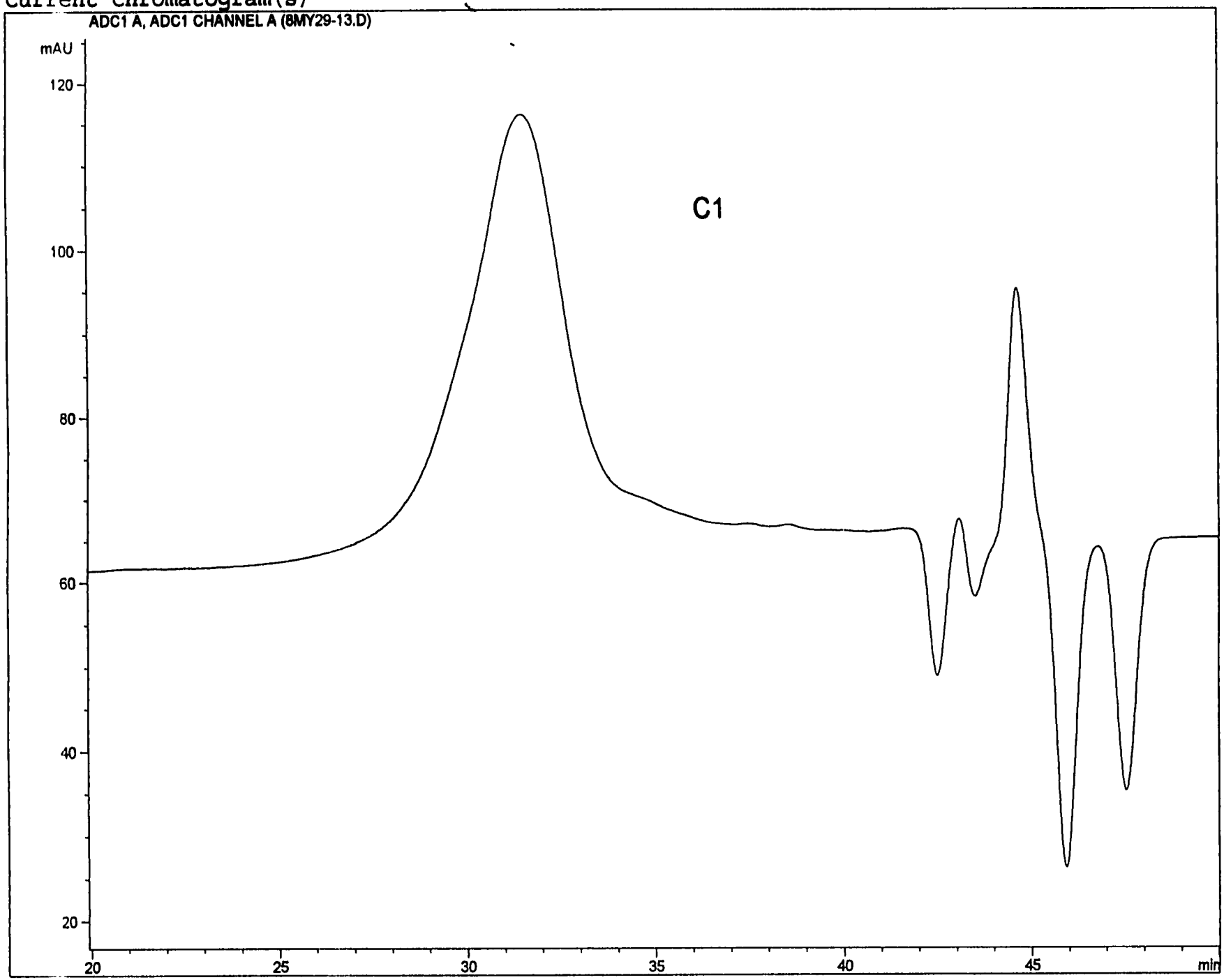
Calibration Using : Narrow Standards
Calibration Limits : 20.46 to 44.43 Mins
Flow Rate Marker : found at : 44.45

Curve Used : 3rd Order Polynomial
Last Calibrated : Mon Jun 15 11:10:40 1998
In Standards at : 44.50 Mins

Broad Peak Start : 24.69 End : 43.74 Mins



Current Chromatogram(s)



134

Polymer Laboratories PL GPC Software

Filename : 8MY29.013
Sample Name : C 1
Run Type : Unknown
Method : 11

Date : 11:30 Mon Jun 15 1998
Date Acquired : 11:28 Mon Jun 15 1998
Operator L.K.KOSTANSKI

CALIBRATION PARAMETERS :

Type : Narrow Standards
Curve Used : 3rd Order Polynomial
Coefficients : Log(M) = A + BT + CT2 + DT3

A=12.932137, B=-0.305157, C=-0.000455, D=0.000040

Limits of Referenced Calibration: 20.46 Mins to 44.43 Mins

Calibrated on : Mon Jun 15 11:10:40 1998

Standards :
K Value: 14.1000 * 10e-5 Alpha Value : 0.700
Flow Rate Marker:
Broad Peak Markers: Start 24.69 Mins End 43.74 Mins

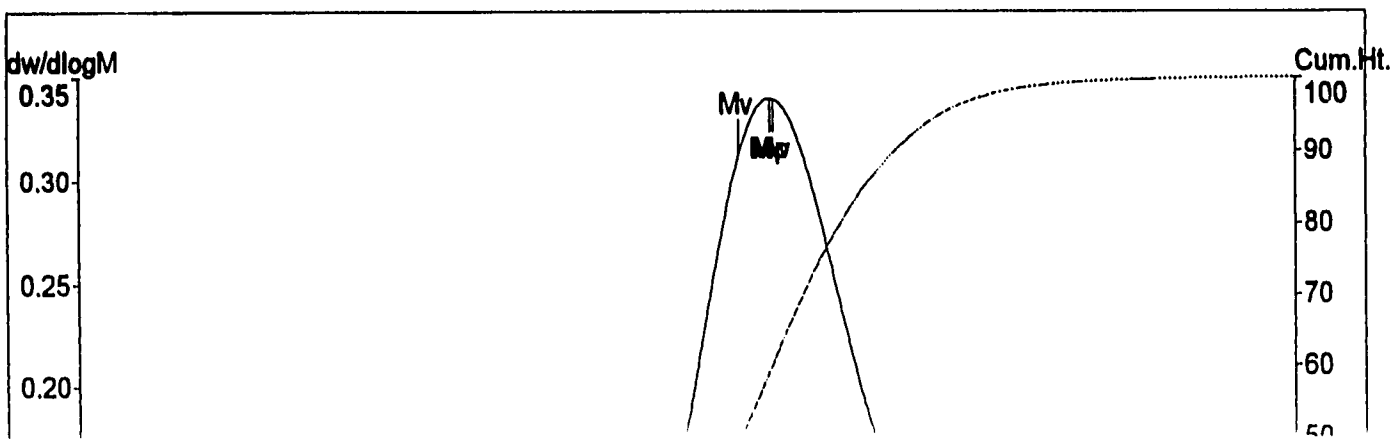
Sample:
K Value 14.1000 * 10e-5 Alpha Value : 0.700
Found in Standards at 44.50 Mins
Found in Sample at 44.45 Mins

135

MOLECULAR WEIGHT PARAMETERS

Mp =	13232	Mz =	44050
Mn =	907	Mz+1 =	141173
Mw =	12979	Mv =	10441

Poly dispersity (Mw/Mn) = 14.303
Peak Area 3622408

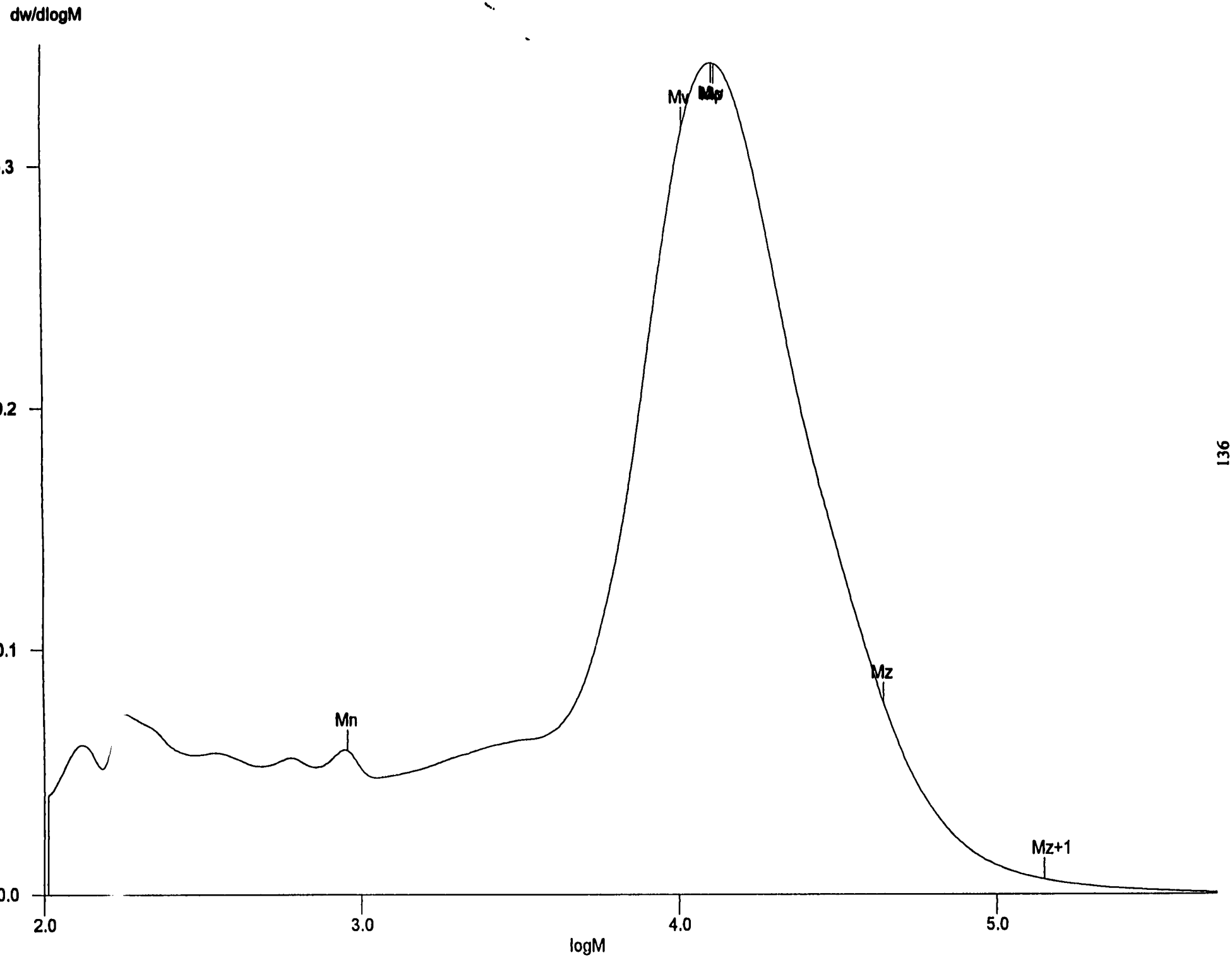


GPC Single Channel Online 1 11:30 Mon Jun 15 1998

Raw Data: Reanalysed from 8MY29.013

Differential Molecular Weight Graph for Sample 13

Sample Name : C 1



Polymer Laboratories
PL GPC Software

11:37 Mon Jun 15 1998

Unknown 8MY29.016
E 1

Acquired : 11:35 Mon Jun 15 1998
Operator L.K.KOSTANSKI

Concentration :
Injection Volume :
Solvent : THF
Column Set : 4XSTYRAGEL

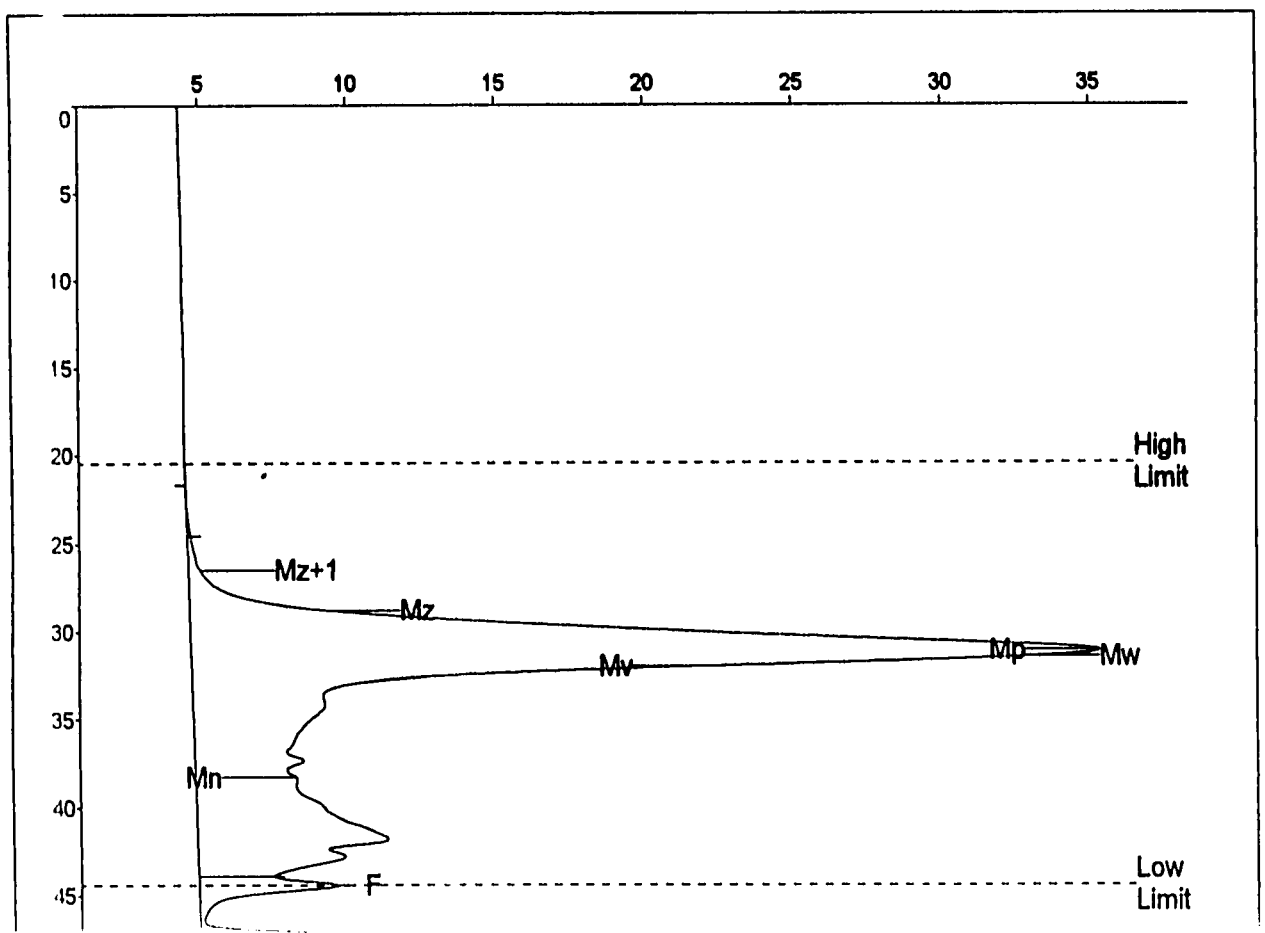
Detector : RI_DAD
Temperature : RT
Flow Rate : 1.000
Standards : PS

Method 11
Comments :

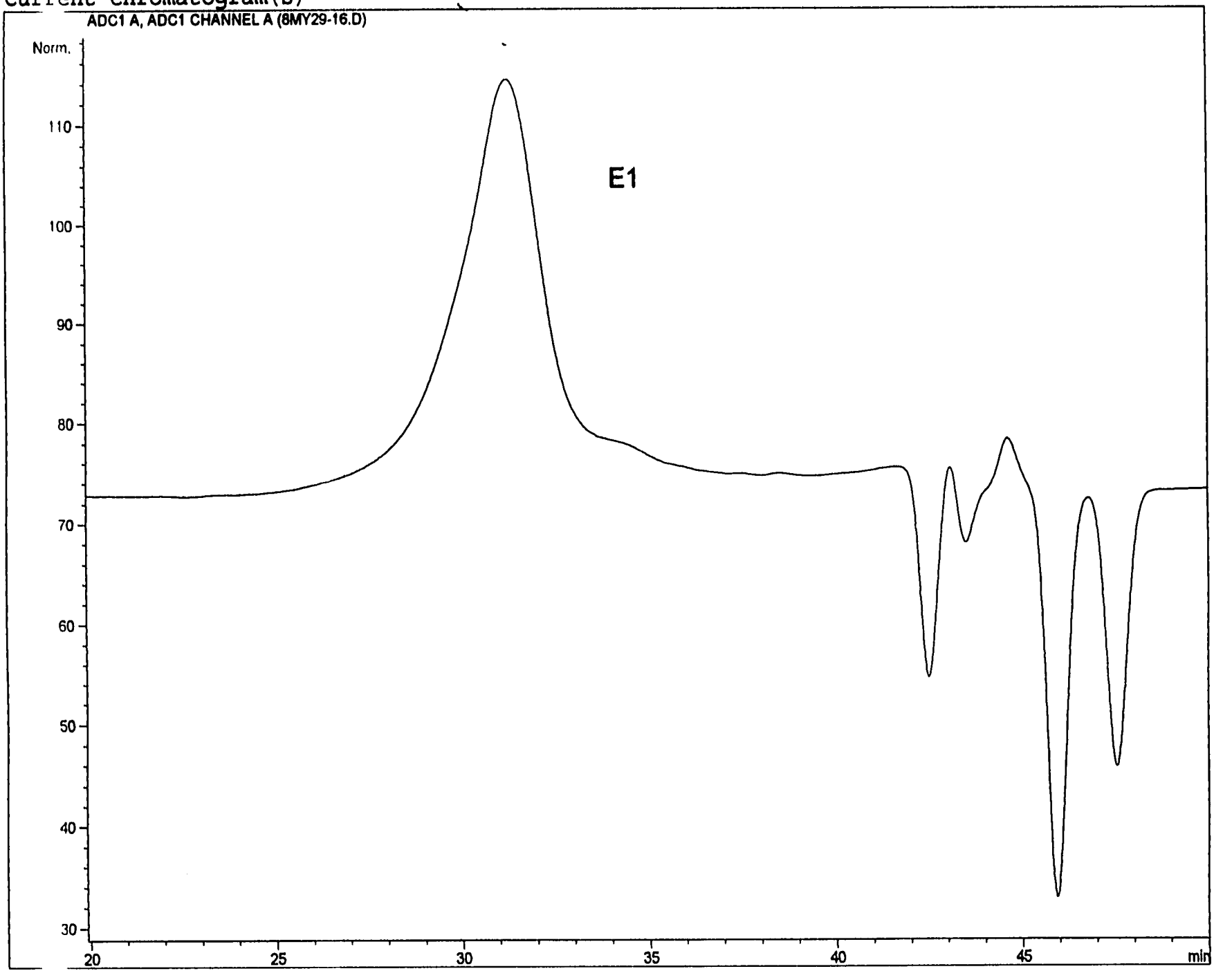
Calibration Using : Narrow Standards
Calibration Limits : 20.46 to 44.42 Mins
Flow Rate Marker : found at : 44.43

Curve Used : 3rd Order Polynomial
Last Calibrated : Mon Jun 15 11:10:40 1998
in Standards at : 44.50 Mins

Broad Peak Start : 24.54 End : 43.92 Mins



Current Chromatogram(s)



138

Polymer Laboratories PL GPC Software

Filename : 8MY29.016
Sample Name : E 1
Run Type : Unknown
Method : 11

Date : 11:37 Mon Jun 15 1998
Date Acquired : 11:35 Mon Jun 15 1998
Operator L.K.KOSTANSKI

CALIBRATION PARAMETERS :

Type : Narrow Standards
Curve Used : 3rd Order Polynomial
Coefficients : Log(M) = A + BT + CT2 + DT3
A=12.932137, B=-0.305157, C=-0.000455, D=0.000040

Limits of Referenced Calibration: 20.46 Mins to 44.42 Mins

Calibrated on : Mon Jun 15 11:10:40 1998

Standards :
K Value: 14.1000 * 10e-5 Alpha Value : 0.700

Sample:
K Value 14.1000 * 10e-5 Alpha Value : 0.700

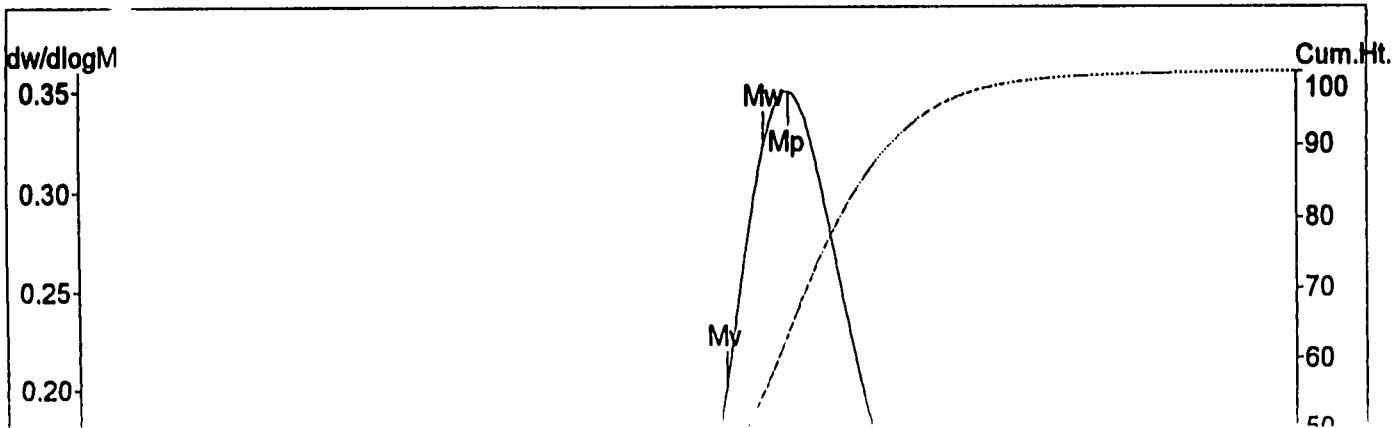
Flow Rate Marker: Found in Standards at 44.50 Mins
Found in Sample at 44.43 Mins

Broad Peak Markers: Start 24.54 Mins End 43.92 Mins

MOLECULAR WEIGHT PARAMETERS

Mp =	15082	Mz =	51547
Mn =	620	Mz+1 =	184201
Mw =	12614	Mv =	9716

Poly dispersity (Mw/Mn) = 20.332
Peak Area 2554359



GPC Single Channel Online 1 11:37 Mon Jun 15 1998

Raw Data: Reanalysed from 8MY29.016

Differential Molecular Weight Graph for Sample 16

Sample Name : E 1