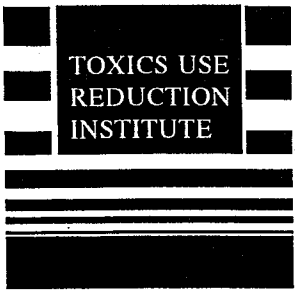

THE MASSACHUSETTS
TOXICS USE REDUCTION INSTITUTE

TOXICS USE REDUCTION FROM PRODUCT INCEPTION:

NATURALLY DERIVED γ-POLY (GLUTAMIC ACID)

Technical Report No. 1 **1992**

University of Massachusetts Lowell

TOXICS USE REDUCTION FROM PRODUCT INCEPTION:

NATURALLY DERIVED γ-POLY(GLUTAMIC ACID) AS A SUBSTITUTE MATERIAL FOR ACRYLIC WATER SOLUBLE IONIC POLYMERS

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The Toxics Use Reduction Institute Research Fellowship Program

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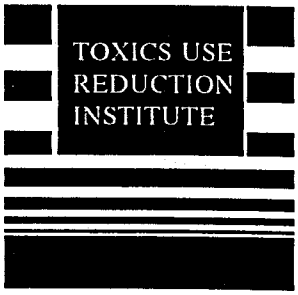


The Toxics Use Reduction Institute is a multi-disciplinary research, education, and policy center established by the Massachusetts Toxics Use Reduction Act of 1989. The Institute sponsors and conducts research, organizes education and training programs, and provides technical support to governments to promote the reduction in the use of toxic chemicals or the generation of toxic chemical byproducts in industry and commerce. Further information can be obtained by writing the Toxics Use Reduction Institute, University of Massachusetts Lowell, One University Avenue, Lowell, Massachusetts 01854.

PREFACE

In 1991 the Toxics Use Reduction Institute established the Research Fellows Program at the University of Massachusetts Lowell (UML). The Research Fellows Program funds toxics use reduction research projects performed by a graduate student and their advisor. The goals of the Research Fellows Program are:

- to develop technologies, materials, processes, and methods for implementing toxics use reduction techniques,
- to develop an understanding of toxics use reduction among UML graduate students and faculty,
- to facilitate the integration of the concept of toxics use reduction into UML research projects,
- to provide UML faculty with "incubator" funding for toxics use reduction related research, and
- to act as a liaison between Massachusetts industries and UML faculty.

The types of projects funded through the Research Fellows Program are technology, methods, and policy research projects. Each final project report is published by the Institute. The opinions and conclusions expressed in this Research Fellow report are those of the authors and not necessarily those of the Toxics Use Reduction Institute.

INTRODUCTION

Naturally occurring polymers have attracted considerable interest from polymer scientists in recent years. This interest is due in part to an increased awareness in the environment and the desire to produce environmentally safe materials. These polymers not only occur from natural resources and are biodegradable, but in lieu of the limitations of our oil supplies, they offer a possible alternative to petroleum based polymers. The use of natural compounds and a biocatalyst in an aqueous solvent for synthesis of a polymer is clearly an intelligent approach to reducing the corresponding toxic chemicals used and formed during a production process. Therefore, the research conducted during the past year has explored the exciting potential of the biological synthetic route to produce the water soluble nylon γ-poly(glutamic acid), PGA.

PGA is a capsular polyamide formed by a number of Bacillus species including *Bacillus licheniformis* 9945a, *Bacillus subtilis* (natto), *Bacillus megaterium* and *Bacillus anthracis*. It has been shown that the activated glutamate monomers are polymerized in a ribosome-independent manner via γ linkages between the α amino and the γ carboxylic acid functional groups [1]. PGA produced by *B. anthracis* has been shown to be a homopolymer of predominantly D-glutamic acid residues, tightly bound to the cells as a capsule [2]. PGA produced by *B. megaterium* was shown to be a copolymer of both L and D isomers of glutamic acid [3]. Evidence exists that the PGA formed by B. *licheniformis* 9945a, which is secreted extracellularly, is a mixture of homopolymers containing either L- or D- stereoisomers [4]. Leonard et al. [4] reported that the stereochemistry of *B. licheniformis* PGA could be controlled by the composition of the production medium. Specifically, they found the concentration of manganese varied inversely with the percentage of L-glutamate units in the polymer. Troy reported, however, that they were unable to confirm these observations [1,2].

We are currently investigating the biosynthesis of PGA by *B. licheniformis* 9945a, looking at the utilization of carbon sources and effects of the divalent cations in

the medium on the resulting polymer. By understanding the physiology of PGA production, we hope to manipulate the fermentation conditions in order to create new polymers with various functional properties.

EXPERIMENTAL

Since Leonard et al. had reported that the stereochemistry of γ-PGA could be modulated by varying the manganese concentration in the medium [4], but Troy had reported that he could not confirm this result [1,2], we wanted to resolve this extremely important and fascinating conflict in the literature. We were interested to see if the stereochemistry could indeed be altered by variation in the media manganese concentration. In addition we wanted to investigate whether the γ-PGA stereochemistry varies as a function of time since it is not known whether one stereoisomer is produced preferentially over the other at various times in the growth cycle. There are a number of practical reasons for interest in obtaining stereochemical control. For one, it would be expected that significant differences in the mechanical and thermal properties of materials would be found for γ-PGA with different polymer stereochemistries. Furthermore, since γ-PGA has considerable interest for medical applications, important differences in the body's tolerance and metabolism for the various γ-PGA stereochemical forms would be anticipated. Indeed, it was found that γ-[D]-PGA has been found to be immunogenic, whereas γ-[L]-PGA is not [5]. Therefore, we have initiated studies to gain a better understanding of how *B. licheniformis* is capable of variation in the polymer product stereochemistry. This process is indeed a unique phenomena for a whole cell biocatalyst system and, as stated above, has important practical applications. An experiment was designed to investigate five different $MnSO_4 \cdot H2O$ concentrations: 6.15×10^{-4}, 6.15×10^{-5}, 6.15×10^{-6}, 6.16×10^{-7} and 0 molar. The cell viability, polymer yield, molecular weight, and stereochemistry were monitored over a period of six days.

METHODS

Organism and Strain Maintenance - *Bacillus licheniformis* ATCC 9945a was obtained from the American Type Culture Collection. Before experiments on PGA production were undertaken, highly mucoid (PGA producing) colonies of *B. licheniformis* 9945a were selected by repeatedly streaking on modified Sauton's solid medium [6]. When homogeneously mucoid colonies were obtained by the above, a broth culture of modified Sauton's medium (100mL) was inoculated with a young mucoid colony and incubated at 37°C with shaking (250RPM) for 48h. The culture was then mixed with 100mL 20% sterile glycerol, transferred to 1mL cryogenic vials and frozen in a dry ice ethanol bath before being immersed in liquid nitrogen in a storage dewar.

Culture Conditions and Polymer Production - PGA was produced in 100mL cultures of Medium E [6] (using 500mL Erlenmeyer flask) inoculated with 1mL of a thawed B. Licheniformis 9945a cell suspension. The formulation of Medium E, in grams per liter (g/L) is as follows: L-Glutamic acid, 20.0; Citric acid, 12.0; Glycerol, 80.0; NH_4Cl, 7.0; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeCl_3 \cdot 6H_2O$, 0.04; $CaCl_2 \cdot 2H_2O$, 0.15; $MnSO_4 \cdot H_2O$, 0.104. The culture was incubated at 37°C and shaken at 250 RPM. γ-PGA production was normally monitored as a function of time over a period of six days.

Polymer isolation: The contents of the flasks are blended with 50.0g of ice for 1 minute and then centrifuged at 10,000 RPM for 10 minutes. The supernatant (approximately 100mL) is slowly poured into ice cold 95% ethanol (300mL) and the polymer was collected by wrapping onto a glass rod as it was precipitated from solution. The precipitated PGA is placed in a vial, washed once with acetone, dried under vacuum and weighed to determine the crude polymer yield.

Purification of Crude PGA: The crude PGA is dissolved in approximately 100mL of distilled deionized H_2O and filtered through a 0.45υm filter to remove cells using a Minitan^{TM} tangential flow filtration apparatus (Millipore Corp. Waltham, MA). Subsequent to the removal of cells, the polymer solution was dialyzed using the Minitan^{TM} equipped with a 30,000 cutoff membrane. The completion of the removal of salts is verified by the absence of a precipitation when the filtrate is added to a $AgNO_3$ solution. The polymer solution was then concentrated to about half its volume using the Minitan^{TM} apparatus and this solution was placed in an ice bath and brought to a pH of 1.5 using 10 N HCl. The acidified polymer is precipitated in 350mL (7x volume) of a 1:1 ether:n-propanol mixture to yield the free acid, H-γ-PGA. It should be noted that this purification procedure can also be performed directly on the culture without first isolating the polymer as the so called crude produce (see above).. The only additional step is to centrifuge the culture prior to filtering through the 0.45υm filter in order to remove the majority of the cells.

Molecular Weight Determination: Molecular weight determinations of γ-PGA were carried out by gel permeation system (GPC) using Waters 600E system controller, equipped with Shodex KB800 series columns (two of KB80M, and one of KB802.5) and an RI detector. Pullulan standards of narrow polydispersirty obtained from Polysciences were used to calibrate the system. The mobile phase was 0.3M Na_2SO_4 that was brought to a pH to 4.0 using glacial acetic acid. The molecular weight of γ-PGA was routinely measured by removing a small aliquot from the culture which was then filtered through a 0.45υm cellulose acetate membrane (to remove the cells) and directly injected into the GPC to perform the analysis.

Quantitation of Poly(glutamic acid): Two approaches have been used in our laboratory to quantitate the γ-PGA formed under specific culture conditions. One approach used

was simply to weight the purified γ-PGA isolated from the fermentation broth taking care to carry out all steps in a quantitative manner. The method used for polymer purification has been described above. In addition, an analytical method was developed such that the yield was determined using gel permeation chromatography (GPC). A standard curve of γ-PGA concentration vs. the area under the GPC peak was constructed using purified γ-PGA standard samples. The conditions used for this GPC analysis was identical to the described above for the determination of γ-PGA molecular weight. The polymer yield was then routinely measured by removing a small aliquot from the culture after a specific culture time, filtering it through a 0.45 μm cellulose acetate membrane to remove the cells, and injecting this filtrate directly into the GPC. Using the standard curve described above, the concentration of γ-PGA was then calculated.

Stereochemical Analysis of γ-PGA: The repeat unit stereochemistry of the γ-PGA formed after various cultivation times and media conditions was measured by reverse phase HPLC. Samples were prepared by filtering an aliquot of the crude culture through a 0.45 μm cellulose acetate membrane to remove the cells. Salts and other low molecular weight impurities were them removed by filtration using Amicon 30,00 molecular weight cutoff Semi-Prep^{TM} filters. The retentate remaining above the Amicon filter membranes was washed, and subsequently concentrated, by performing repetitive centrifugation and addition of fresh glass distilled deionized water. The polymer was then hydrolyzed by first adding concentrated HCl such that the final concentration of HCl was 2N and, subsequently, maintaining this solution at 110^{o}C for three hours. The hydrolyzed samples were derivatized using Marfey's reagent [7]. The resulting diasteriomer were analyzed by HPLC using a Pierce RP-18 column attached to a Waters 600E system with UV detection (Waters Model 474).

RESULTS AND DISCUSSION:

One aim of this work was to improve the methods used to culture *B. licheniformis* 9945a and produce γ-PGA Initially, we used a 2-step batch culture method in which colonies from Sauton's agar plates were used as inocula for primary batch cultures in Sauton's broth. After 48 h incubation, aliquots were used as inocula for γ-PGA production cultures using Medium E broth [6]. This method proved problematic because *B. licheniformis* 9945a often degenerates to a non-PGA producing (non-mucoid, or rough colony type) variant. Therefore, inconsistent growth and polymer yield resulted from this 2-step batch culture approach. Although the molecular nature underlying the degeneration to a non-γ-PGA producing *B. licheniformis* variant is not presently understood, it has been repeatedly reported in the literature [1,2,3,4,5].

Another approach was investigated to obtain an inoculum for γ-PGA fermentations that proved quite valuable in improving the culture to culture polymer formation reproducibility. This was accomplished by adding glycerol as a cryoprotectant at a final concentration of 10% v/v to a culture of *B. licheniformis* 9945a grown in Sauton's broth and freezing this mixture in multiple 1 mL aliquots. When the aliquots were subsequently thawed and used to inoculate Medium E batch cultures, levels of crude PGA of >20g/L were consistently obtained. Since, following this method, each culture is inoculated with a subsample of the original culture, the serious problems which were previously encountered in obtaining reproducible results from γ-PGA polymer formation cultures have been eliminated. In addition, by eliminating routine plating and the need for primary Sauton's broth cultures, both time and materials are also reduced.

An additional objective of this study has been to develop new analytical approaches for γ-PGA cultures so that more detailed information may be obtained using small culture aliquots that are withdrawn at various time intervals during the fermentation process. Previously, information such as γ-PGA yield was obtained by the

laborious process of precipitating the polymer and subsequent purification (see *Experimental section*). In this work, we investigated whether GPC cold serve as a convenient approach to quantitate the γ-PGA yield. Although GPC is typically used for measuring molecular weight of polymeric materials, a standard curve was constructed of γ-PGA concentration vs. the area under the GPC peak using purified γ-PGA standard sample. The graph resulted in a line with a correlation coefficient, determined by linear regression, of 0.998. Analysis of the γ-PGA concentrations which were present in the media at a given time were then performed by withdrawing a small aliquot from the cultures, removal of the cells by filtration and injection of the filtrate into the GPC system (see *Experimental section for details*). Simultaneously, the GPC traces obtained were used to determine the number and molecular weight averages of the product formed (see *Experimental section*).

Previous studies by Leonard et al. [4] reported that the stereochemistry of γ-PGA formed by *B. licheniformis* could be controlled by changing the concentration of manganese in the cultivation medium. In contradiction to this report by Leonard et al. Troy [1,2] claimed for studies carried out on the same bacterial strain that the stereochemistry of γ-PGA was not effected by the medium manganese concentration. Of course, this disagreement in the literature is of great concern since the stereochemical modulation of a polymer formed in aqueous media by a biocatalyst system is a feature which, to our knowledge, has only been documented for the γ-PGA formation process. Therefore, we carried our investigations to determine whether stereochemical modulation, as described by Leonard et al., was possible with the identical *B. licheniformis* strain. In addition, the analytical methods described above in combination with standard procedures (see *Experimental section*) allowed us to study in great detail the effects of the initial medium $mans_4$ concentration on cell viability and γ-PGA yield, molecular weight and stereochemistry. The experiment was performed twice to

determine whether the trends observed were indeed reproducible. The results of this work is described below.

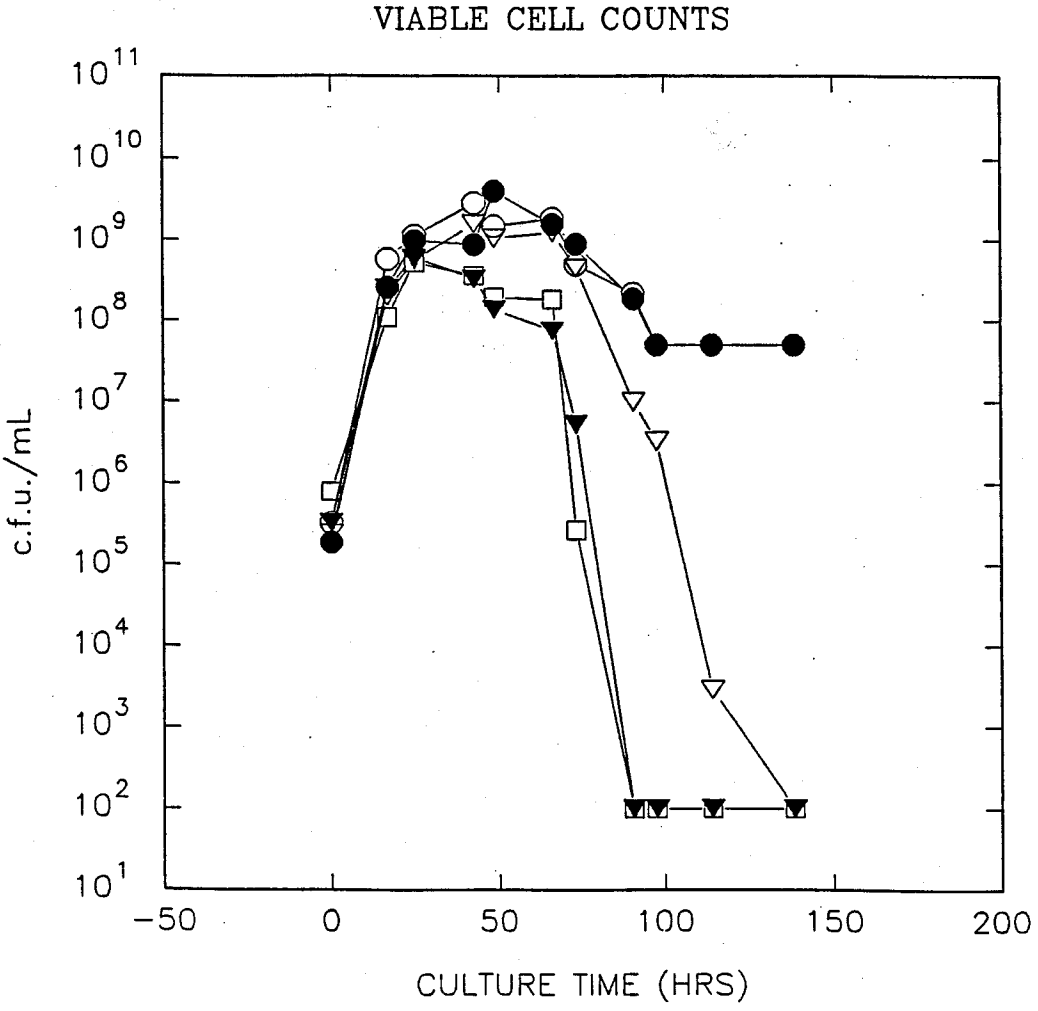
It was determined that the cell viability begins to decrease after a relatively shorter period for cultures which contained relatively lower concentrations of manganese sulfate (Figure 1). Furthermore, the γ-PGA yield appears to be higher in the cultures containing higher concentrations of manganese sulfate (Figure 2). Also, the number average molecular weights and the weight average molecular weights decreased in magnitude over the six day period with all concentrations of manganese sulfate; however, it appears that the final molecular weights of the relatively higher manganese sulfate containing cultures are relatively lower. It should be noted that these results were shown to be reproducible in two experimental trials.

An analysis was carried out to determine whether the stereochemistry of the γ-PGA formed changes as a function of the culture time. Results are reported from two experimental trials (Figures 3 and 4). When relatively higher concentrations of manganese sulfate (6.15×10^{-4} and 6.15×10^{-5}) were added to the cultivation media, the percentage of [L]-glutamate repeat units in the hydrolyzed polymer remained at approximately 10% throughout the culture period. The results of this experiment were found to be in close agreement for the two experimental trials which were carried out The γ-PGA repeat unit stereochemistry was dramatically altered when relatively lower concentrations of manganese sulfate (6.15×10^{-7} and 0 M) were added to the cultivation media. At these manganese sulfate concentrations, the percentage of [L]-glutamate repeat units in the hydrolyzed polymer was approximately 50% for both experimental trials. Similar to that described above, the polymer stereochemistry did not change appreciably as a function of the culture time. Interestingly, the results obtained at the intermediate manganese sulfate concentration of 6.15x10-6 M were rather different for both experimental trials that were performed. In one case, the percentage of [L]-glutamate repeat units in γ-PGA was approximately 10 % [L]-glutamate at early culture

time periods and then increased to about 65 %, and subsequently decreased to approximately 40 % as the culture time progressed. When this experiment was repeated, surprisingly the stereochemistry of the γ-PGA formed for the intermediate manganese sulfate concentration of 6.15×10^{-6} M did not change dramatically as a function of the culture time. The γ-PGA stereochemical composition was found to remain at approximately 40 % [L]-glutamate repeat units throughout the cultivation period. The difference between the experimental trials indicates that the 6.15x10-6 M manganese sulfate concentration is at a critical value where depletion of the manganese from the media at some culture time due to, for example, slightly variable growth and carbon source metabolism, can result in a dramatic switch in the product stereochemistry.

The control exhibited in γ-PGA stereochemistry which has been documented by the work described above demonstrates conclusively that this fascinating and unique feature of γ-poly(glutamic acid) biosynthesis does indeed take place. This result has great importance since it provides the basis for the development of methods where the product stereochemistry can be carefully controlled. Therefore, γ-PGA can now be produced in a stereochemical form that provides the desired biological and material properties.

FIGURE 1

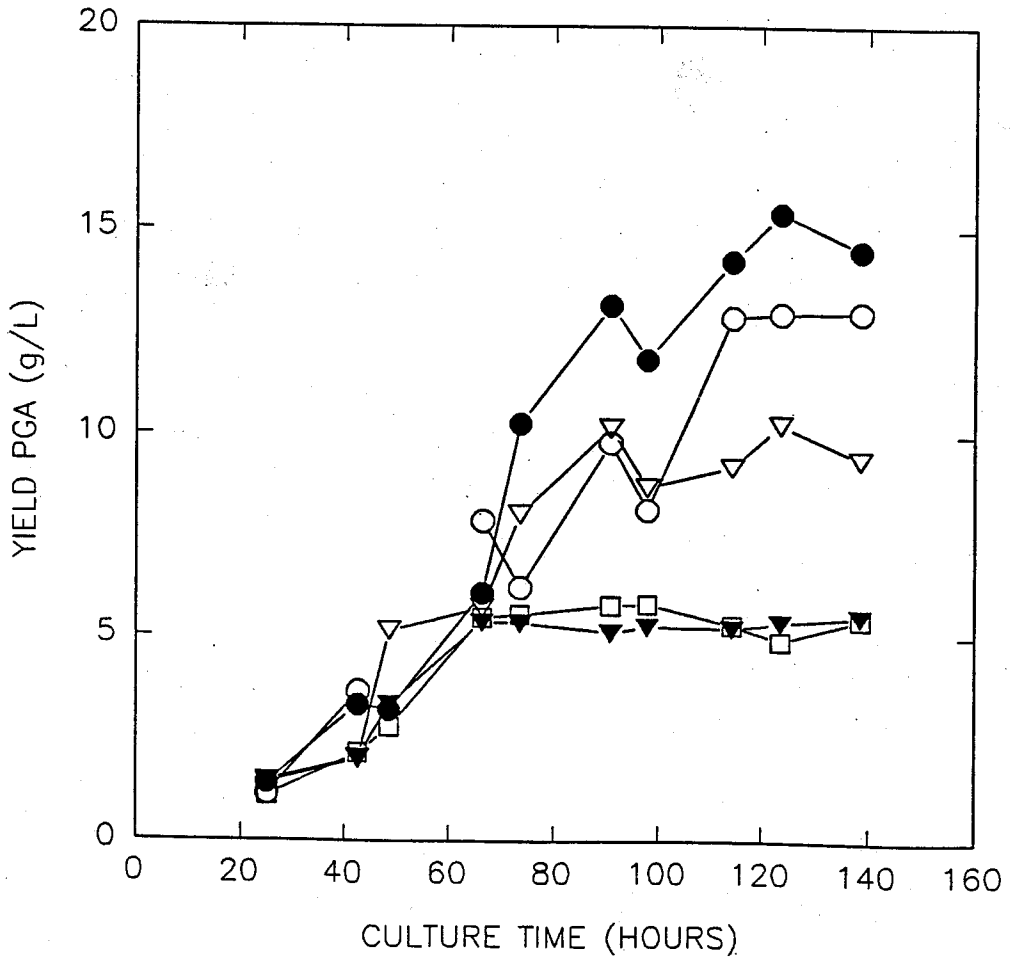


KEY

Symbol	[$MnSO_4 \cdot H_2O$], Molar
○	6.15×10^{-4}
●	6.15×10^{-5}
▽	6.15×10^{-6}
▼	6.15×10^{-7}
□	0

FIGURE 2

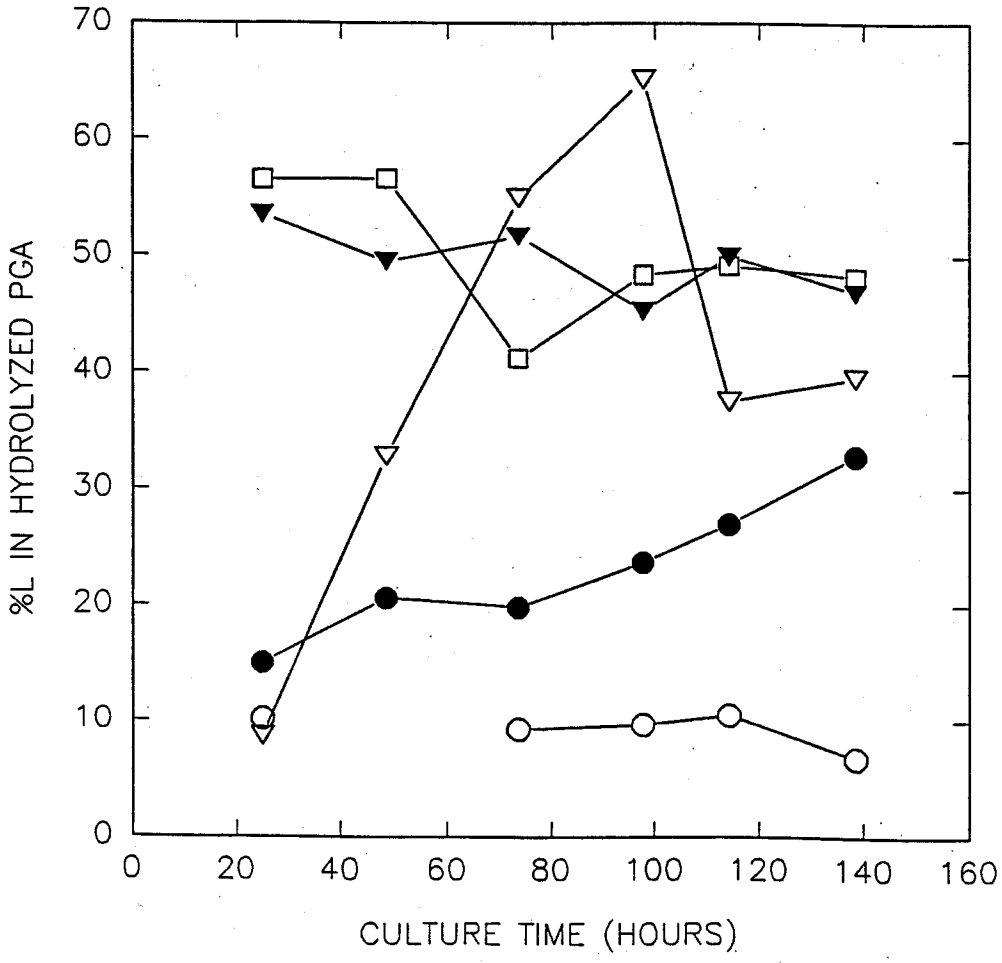
PGA YIELD vs. CULTURE TIME



KEY

Symbol	[$MnSO_4 \cdot H_2O$], Molar
○	6.15×10^{-4}
●	6.15×10^{-5}
▽	6.15×10^{-6}
▼	6.15×10^{-7}
□	0

FIGURE 3
%L in HYDROLYZED PGA vs. CULTURE TIME



KEY

Symbol	[$MnSO_4 \cdot H_2O$], Molar
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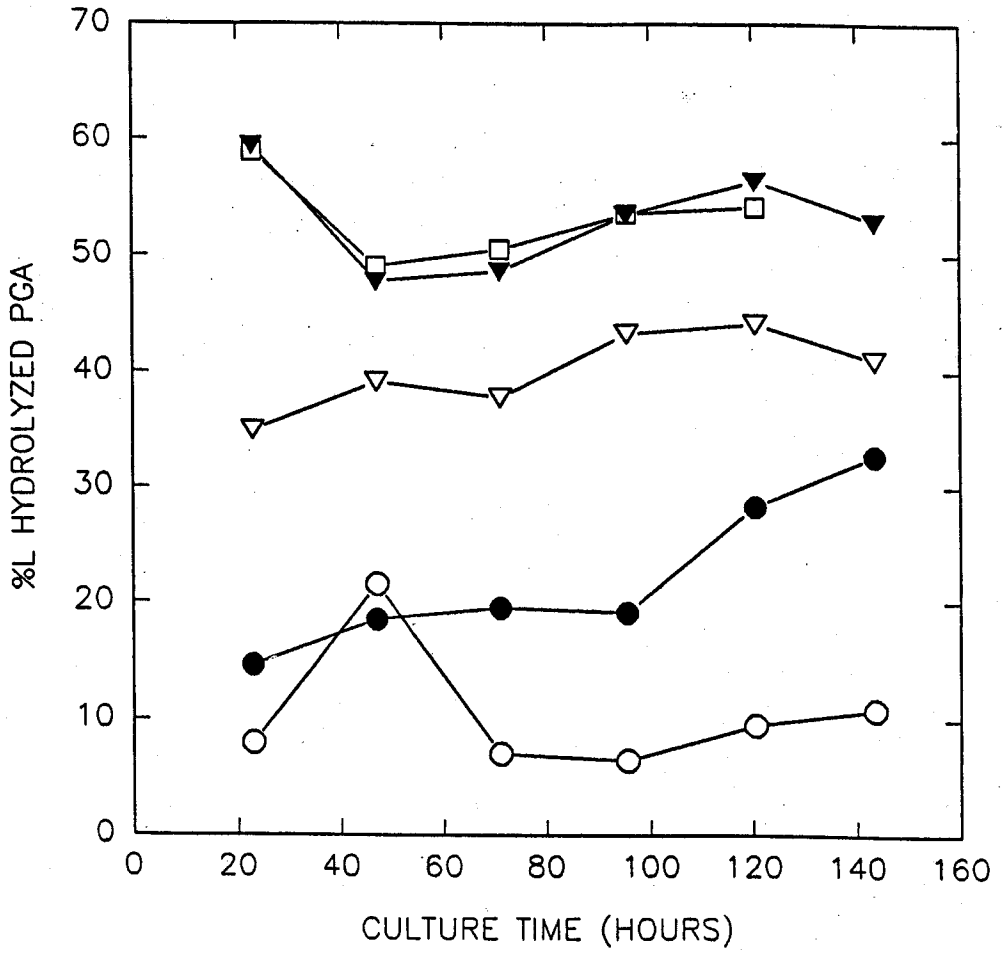
●	6.15×10^{-5}
▽	6.15×10^{-6}
▼	6.15×10^{-7}
□	0

FIGURE 4

%L in Hydrolyzed PGA vs. CULTURE TIME



KEY

Symbol	[$MnSO_4 \cdot H_2O$], Molar
○	6.15×10^{-4}
●	6.15×10^{-5}
▽	6.15×10^{-6}
▼	6.15×10^{-7}
□	0

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