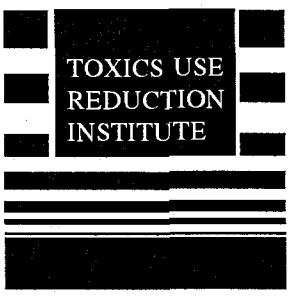

THE MASSACHUSETTS
TOXICS USE REDUCTION INSTITUTE

A BIOLOGICAL PROCESS TO MAKE WATER SOLUBLE IONIC POLYMERS: γ-POLY(GLUTAMIC ACID)

Production and Isolation

Technical Report No. 14 1993

University of Massachusetts Lowell

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Production and Isolation

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The Toxics Use Reduction Institute Research Fellows Program

June 1993

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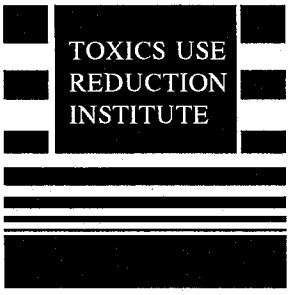

The Toxics Use Reduction Institute is a multi-disciplinary research, education, and policy center established by the Massachusetts Toxics Use Reduction Act of 1989. The Institute sponsors and conducts research, organizes education and training programs, and provides technical support to governments to 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.

Toxics Use Reduction Institute
Research Fellows Program

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 graduate students and their advisors. The goals of the 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, and
- to provide UML faculty with "incubator" funding for toxics use reduction related research.

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

INTRODUCTION:

In the *1992 Final Report* on γ-PGA, the modulation of γ-PGA stereochemistry by changing the concentration of manganese sulfate in the fermentation media was discussed. Results on the stereochemical analysis of the polymers formed as a function of culture time were presented. In first performing this experiment, it was found that at higher media concentrations of manganese sulfate ($6.15x10^{-4}$ and $6.15x10^{-5}$ M), the [L]-glutamate ([L]-glu) isomeric content in the hydrolyzed γ-PGA was approximately 10%. In contrast, media which contained lower concentrations of manganese sulfate ($6.15x10^{-7}$ and 0 M) resulted in γ-PGA with an [L] isomer content of approximately 50% . Interestingly, at the intermediate manganese sulfate concentration of $6.15x10^{-6}$ M, the [L]-glu isomeric content in the hydrolyzed γ-PGA formed started at around 10%, increased during the culture to aproximately 65%, then decreased to about 40%. This experiment was repeated during this report period and the values of % [L]-glu as a function of culture time were almost identical to those reported earlier at the higher and lower manganese sulfate media concentrations. However, the results obtained at the intermediate manganese sulfate concentration of $6.15x10^{-6}$ M were quite different from those reported earlier (see *Final Report, 1992*). Specifically, the percentage of [L]-glu in the product changed little remaining at approximately 40% throughout the course of the cultivation time period (see the two dissimilar curves at $MnSO_4$ concentrations of 6.15 x 10^{-6} M in Figure 1). It appears from these results that the [L]-glu stereochemical composition at this manganese sulfate concentration was approaching a critical value where the % [L]-glu changes from appromately 10 to 50 %. Therefore, an experiment was specifically designed to obtain additional information on a narrowed range of manganese sulfate concentrations near to 6.15 x 10^{-6} M. Two other manganese sulfate concentrations which are slightly higher and lower than $6.15x10^{-6}$ M, specifically $3.38x10^{-5}$ and $3.38x10^{-6}$ M, were chosen for investigation.

Furthermore, it became apparent from analysis of the results reported on stereochemical modulation as a function of manganese sulfate media concentration (see *results herein and the Final Report, 1992)* that the effects of sulfate ion concentration changes had not been considered. Changes in γ-PGA stereochemistry had been interpreted as being due to modulation of the media manganese concentration making the assumption that the sulfate ion concentration is not playing a role in stereochemical modulation. The effects of sulfate ion concentration must then be evaluated as a single variable parameter so that one may properly interpret the results obtained for the two parameter manganese sulfate

concentration experiments. Therefore, an experiment was designed where manganese sulfate in Medium E (see *Experimental Section* for composition) was substituted with manganese chloride to evaluate the effects of sulfate ion concentration as a single variable. In addition, sodium sulfate was used in place of manganese sulfate so that the mangenese II concentration could be investigated as a single variable. It is assumed in these experiments that the corresponding small changes in media sodium and chloride concentrations will have negligible effects on the results obtained.

Differences in the utilization of carbon sources (see *Results and Discussion Section*) between cultures containing high ($6.15x10^{-4}$M $MnSO_4$) and no manganese sulfate generated questions regarding the incorporation of carbon sources other than glutamic acid into γ-PGA. The possibility of sources other than glutamic acid is of particular interest since in the future it is hoped to be able to use analogs of these carbon sources to produce new polymers via biosynthesis. An experiment was designed enriching with ^{13}C labelled 1,5,-citric acid and 1,2 L-glutamic acid. This was performed as a 2^3 factorial experiment in which there were three variables, each at two levels, for a total of eight combinations. The three variables and the two levels were: (1) $6.15x10^{-4}$M $MnSO_4$/ No $MnSO_4$, (2) 7% ^{13}C labelled 1,2 L-GLU/ non-labelled GLU, and (3) 7% ^{13}C labelled 1,5-citric acid/ non-labelled citric acid. Analysis by ^{13}C NMR will show both direct incorporation of glutamic acid, or citric acid, as well as provide information if scrambling of the label occurs.

EXPERIMENTAL:

VARIATION OF [$MnSO_4$] IN MEDIUM E:

Bacillus licheniformis 9945a was grown in Medium E (Medium E in g/L consists of: L-Glutamic acid, 20.0; Citric acid, 12.0; Glycerol, 80.0; NH_4Cl, 7.0; K_2HPO_4, 0.5; $MgSO_4$ $7H_2O$, 0.5; $FeCl_3$ $6H_2O$, 0.04; $CaCl_2$ $2H_2O$, 0.15; $MnSO_4$ H_2O, 6.15 x 10^{-4}M) where the concentration of $MnSO_4$ was varied. The cell viability, yield of γ-PGA, molecular weight and stereochemistry were monitored over a six day cultivation time period.

CARBON SOURCE UTILIZATION

The utilization of glutamic acid, citric acid, and glycerol for cultures of *B. licheniformis* 9945a grown in Medium E with $6.15x10^{-4}$M $MnSO_4$ and no $MnSO_4$ was determined using the Mannheim boehringer Enzyme Analysis Kits, catalog numbers: L-Glu, 139092; citiric acid, 139076; and glycerol, 148270.

INVESTIGATIONS OF Mn^{++} AND SO_4^- AS INDEPENDENT VARIABLES

B. licheniformis 9945a was grown in Media in which the manganese sulfate component of Medium E was substituted with $6.15x10^{-4}$ M $MnCl_2$ and $NaSO_4$, respectively. The cell viability, yield of γ-PGA, molecular weight and stereochemistry were monitored throughout the six day cultivation time period.

INVESTIGATION OF THE INCORPORATION OF ^{13}C LABELLED 1,5,-CITRATE AND 1,2 L-GLUTAMIC ACID IN γ-PGA

B. licheniformis 9945a was grown in media containing various combinations of enriched ^{13}C labelled carbon sources, and high and low concentrations of manganese sulfate. This was performed as a 2^3 factorial experiment in which there were three variables, each at two levels, for a total of eight combinations. The three variables and the two levels were: (1) $6.15x10^{-4}$M $MnSO_4$/ No $MnSO_4$, (2) 7% ^{13}C labelled 1,2 L-GLU/ non-labelled GLU, and (3) 7% ^{13}C labelled 1,5-citric acid/ non-labelled citric acid. The cell viability, yield of γ-PGA, molecular weight, were monitored throughout the four day period.

RESULTS AND DISCUSSION:

VARIATION OF [$MnSO_4$] IN MEDIUM E :

An experiment was carried out to determine the effects of manganese sulfate media concentrations which varied slightly from that of 6.15×10^{-6} M. This study was initiated since it appears that this manganese sulfate concentration is approaching a critical value where the % [L]-glu in the γ-PGA formed changes from appromately 10 to 50 %. It was observed that within the relatively narrow window of manganese sulfate concentrations chosen for investigation (3.38×10^{-5} to 3.38×10^{-6} M), dramatic changes in cell viability were observed. Specifically, a relatively faster decline in cell viability after a 50 h cultivation time period for lower media manganese sulfate concentrations was observed (see Figure 2). This was previously noted in an experiment where the manganese sulfate media concentration was varied over a relatively broader concentration range (from 6.15×10^{-4} to 0 M, see *Final Report, 1992*). From the results in Figure 2 it can be concluded that for fermentation conditions leading to 10^9 cfu/mL viable cells in the early stationary phase, media manganese concentrations $\leq 3.38 \times 10^{-5}$ and $> 6.15 \times 10^{-6}$ M are required to maintain cell viability at high levels for culture times > 50 hours. The maintanence of relatively higher cell viability at culture time periods of > 50 hours for the manganese sulfate concentration of 3.38×10^{-5} lead to sustained polymer formation from 50 to 120

hour culture times (see Figure 3) so that up to 18 g/L γ-PGA was formed. In contrast, for media manganese sulfate concentrations of 6.15 and 3.38 x 10^{-6} M, little additional γ-PGA was formed for cultivation periods beyond 65 hours (see Figure 3). A correlation between higher cell viability and greater γ-PGA formation is not surprising. However, when the maximum volumetric production of γ-PGA were compared for an experiment where the manganese sulfate concentrations ranged from 0 to 6.15 x 10^{-4}, the optimal γ-PGA formation was found at the intermediate manganese sulfate concentration of 6.15 x 10^{-5}. Furthermore, when the results from Figure 3 are superimposed on that of the previous experiment (see Figure 4, results from Figure 3 are presented as the bold lines on the graph) it appears that the manganese sulfate concentration of 3.38 x 10^{-5} M which is intermediate between 6.15 x 10^{-5} and 6.15 x 10^{-6} M shows relatively higher γ-PGA yields. Excellent reproducibility between the two experiments is indicated by comparision of the γ-PGA volumetric production as a function of the culture time for the 6.15 x 10^{-6} M manganese sulfate concentration (see Figure 4, bold and dashed lines are used to differentiate the two different experiments). This gives a higher degree of confidence that a direct comparison of the results from the two respective experiments (see Figure 4) is exceptable. Therefore, this work shows that an optimal manganese sulfate concentration for γ-PGA formation exists at a manganese sulfate concentration which is > 6.15 x 10^{-6} and < 6.15 x 10^{-5} for cell growth reaching 10^9 cfu/mL in the early stationary phase. Furthermore, it is clear that beyond considerations of stereochemical modulation, the manganese sulfate available in the culture media is a critical parameter for optimizing polymer productivity in the system.

CARBON SOURCE UTILIZATION

The results in Figures 5, 6, and 7 show the utilzation of glutamic acid, citric acid, and glycerol, respectively for cultures of *B. licheniformis* grown in medium E with 6.15x10^{-4}M manganese sulfate, and no mangese sulfate. Interestingly, the carbon source utilization profile differs between the high and low manganese cultures, indicative of different motabolisms. Perhaps most interesting is the marked difference in the uptake of citric acid as shown in Figure 6; the high manganese containing culture completely depletes citric acid, whereas the citric acid is virtually untouched in the culture containg no manganese sulfate. These differences in the utilization of carbon sources introduces the question of what role do they play as the "building: blocks" for γ-PGA. One should not simply assume that the resulting γ-PGA arises strictly from the glutamic acid. One of the reasons for wanting to better understand the incroporation of carbon sources into the polymer is the hope to make analogs of theses carbon sources in attempts to create new

polymers via biosynthesis. A ^{13}C labelled experiment was designed to help answer some of the questions regarding the incorporation of glutamic acid and citric acid to form γ-PGA (see section entitled INVESTIGATION OF THE INCORPORATION OF ^{13}C LABELLED 1,5,-CITRATE AND 1,2 L-GLUTAMIC ACID IN γ-PGA).

INVESTIGATION OF Mn++ vs. SO4--:

Results in Figure 8 show that in the absence of manganese but with a sulfate ion concentration of 6.15×10^{-4}, cell viability declines rapidly after appoximately a 40 hour cultivation time. In contrast, when the culture contains manganese chloride in place of sodium sulfate at identical concentrations, the cell viability is maintained at approximately 10^9 cfu/mL up to approximately 65 hours before showing a similar rapid decline (see Figure 8). This indicates that the loss in cell viability previously noted for relatively lower manganese sulfate concentrations is due in large part to the deficiency in manganese II ions in the cultivation media. However, it should be noted that the decrease in media sulfate concentration resulting from the use of manganese chloride in place of manganese sulfate also resulted in an enhanced loss in cell viability. Therefore, a component of the loss in cell viability associated with a decrease in manganese sulfate concentration can be attributed to the decreased availability of sulfate ions in the media. Interestingly, it appears that the decreased sulfate ion media concentration resulting from substitution of manganese sulfate by manganese chloride (at 6.15×10^{-4} concentrations) appears to have resulted in increased volumetric γ-PGA production from approximately 13 to 19 g/L (see Figures 4 and 9 respectively). The reason for this apparent increase in γ-PGA yield at relatively lower sodium sulfate ion concentrations is currently unknown. The stereochemistry results shown in Figure 10 indicate that the modulation in stereochemistry is due to the manganese ion, and not the sulfate. The culture in which the medium had been substituted with manganese chloride in place of manganese sulfate, had a fairly low value of the L-isomer of around 15% which is consistent with a culture containing 6.15×10^{-4}M manganese sulfate. The culture grown in the medium substituted with sodium sulfate, however, had approximately 50% of the L-isomer. A 50% formation of the L-isomer is typical for cultures grown in medium E lacking in manganese sulfate.

INVESTIGATION OF THE INCORPORATION OF ^{13}C LABELLED 1,5,-CITRATE AND 1,2 L-GLUTAMIC ACID IN γ-PGA

Differences in the carbon source utilization profiles between cultures containing 6.15×10^{-4}M $MnSO_4$ and no $MnSO_4$ (see Figures 5, 6, and 7), resulted in questions regarding the

incorporation of carbon sources other than glutamic acid to form γ-PGA. An experiment was designed enriching with ^{13}C labelled carbon sources, 1,5-citric acid and 1,2 L-glutamic acid, to provide information on their incorporation into the polymer. Analysis by ^{13}C NMR will show both direct incorporation of glutamic acid, or citric acid, as well as provide information if scrambling of the label occurs. This experiment has been carried out. The NMR results have not been obtained at this time.

FIGURE 1 %L IN HYDROLYZED PGA AS A FUNCTION OF MnSO4

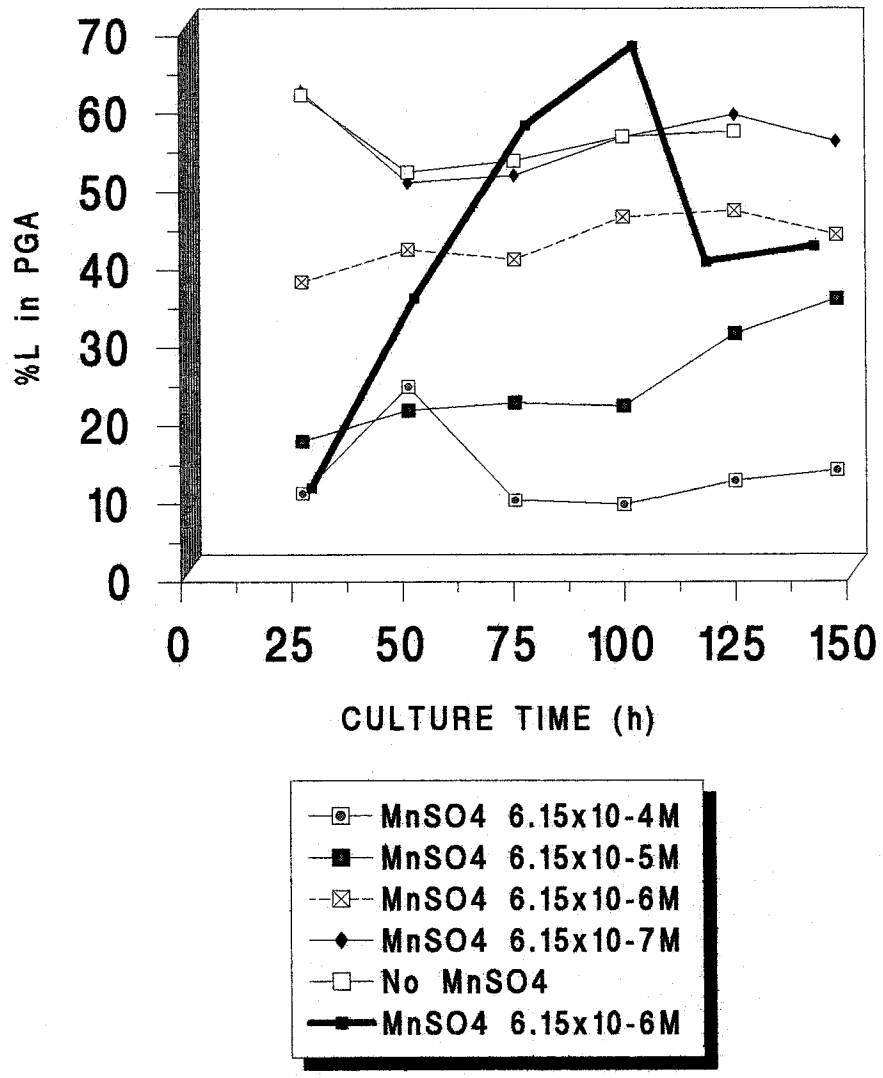

FIGURE 2 NUMBER OF VIABLE CELLS AS A FUNCTION OF [MnSO4]

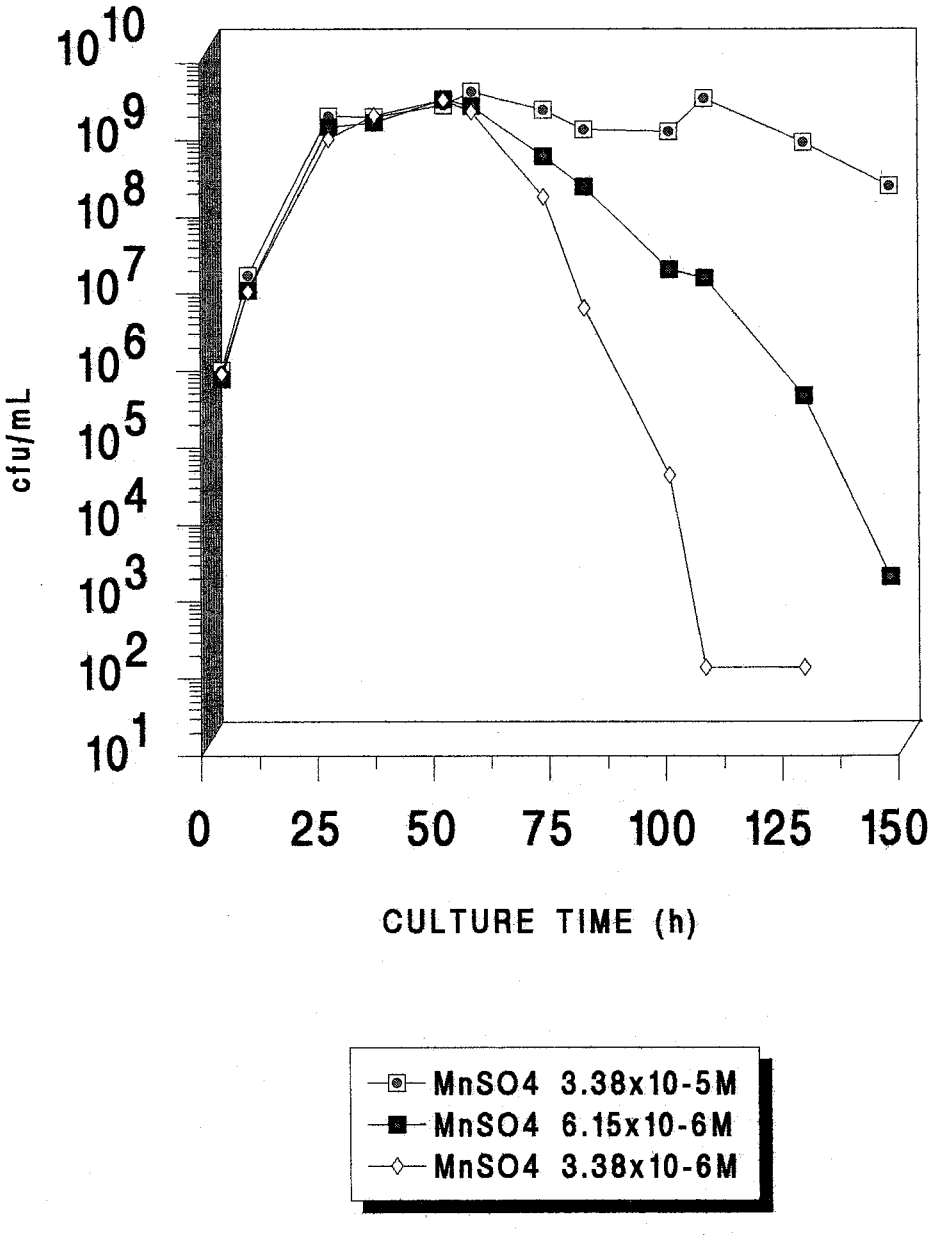

FIGURE 3 YIELD OF PGA AS A FUNCTION OF [MnSO4]

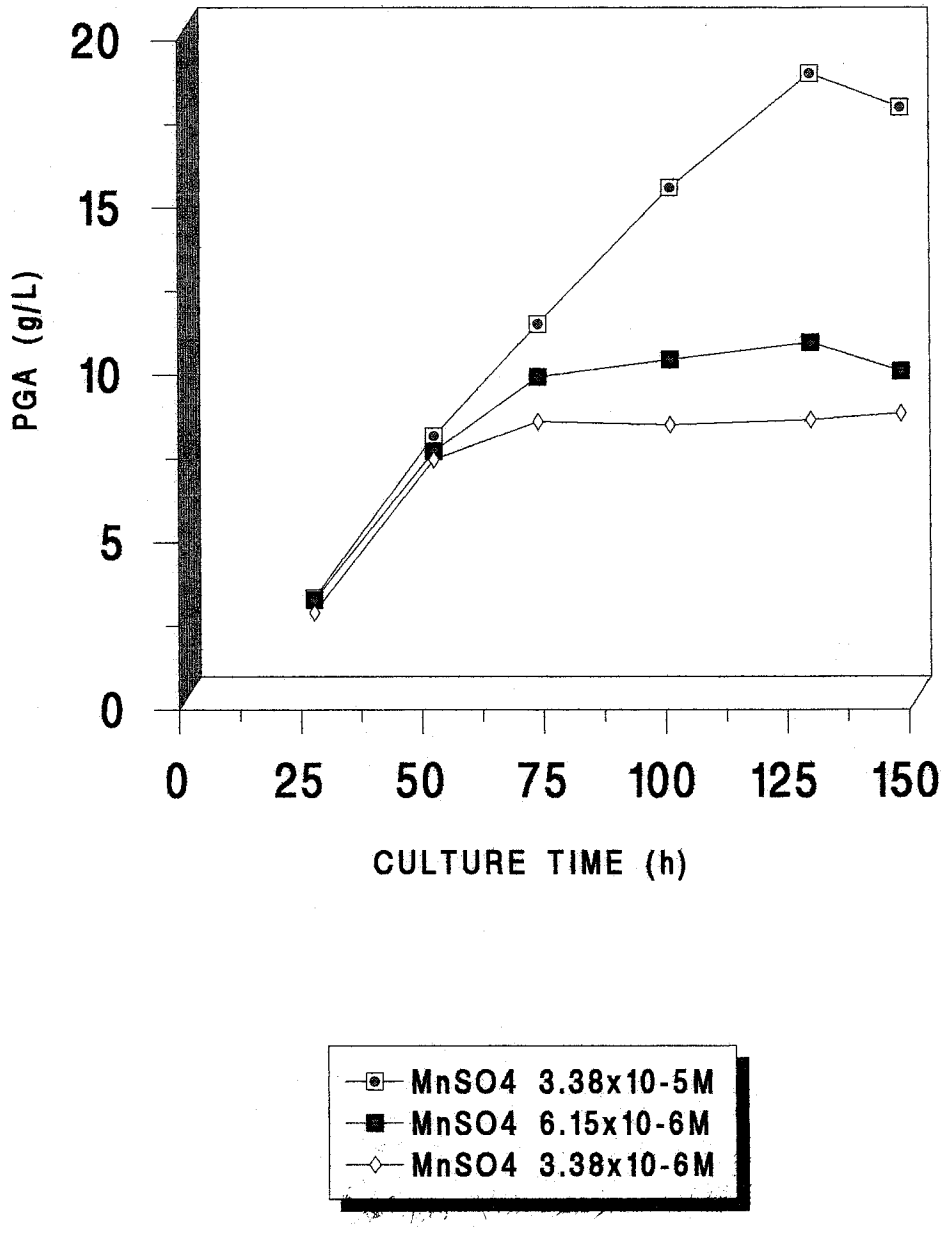

FIGURE 4 Yield of PGA isolated from cultures of B. licheniformis grown in Medium E with various concentratrations of manganese sulfate.

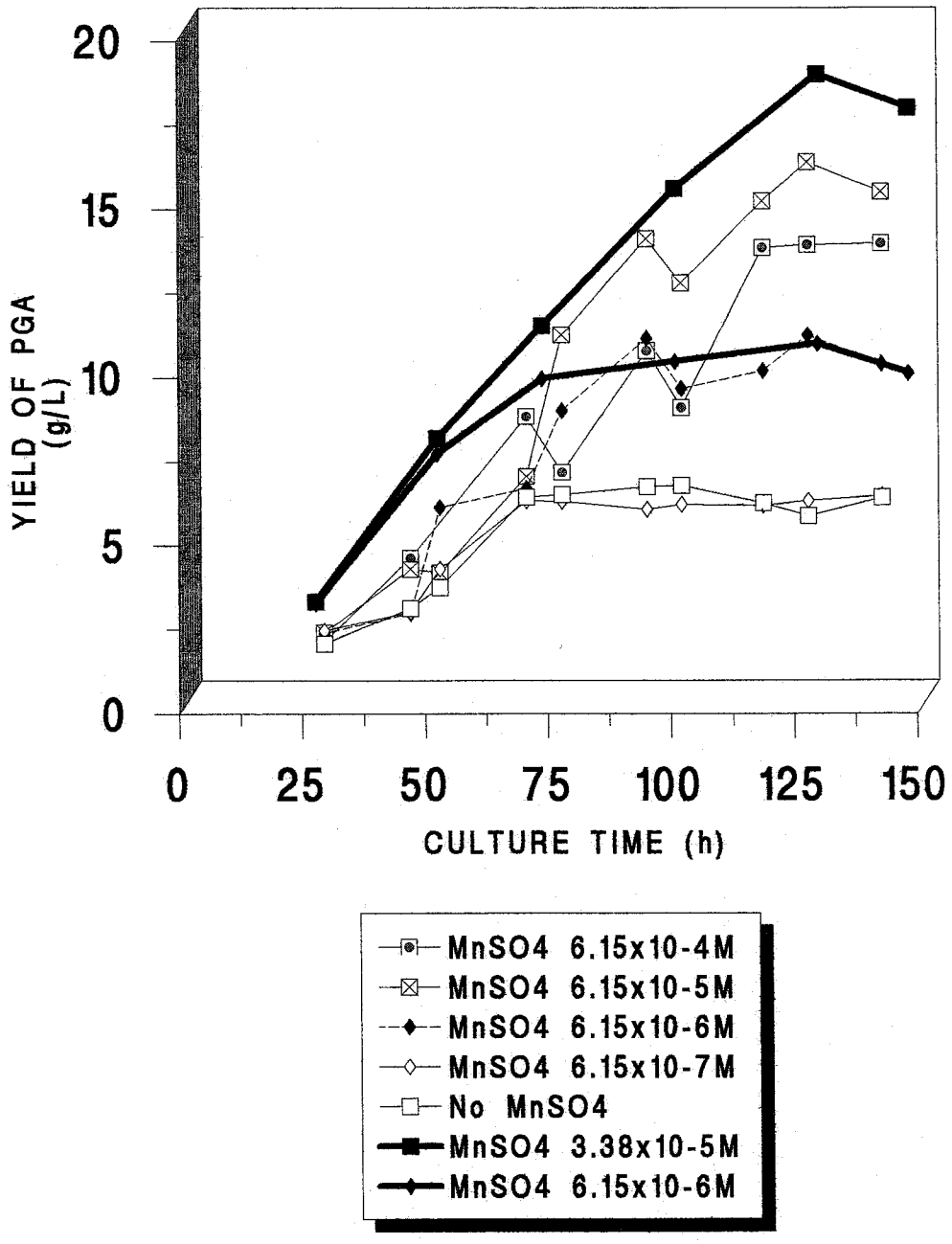

FIGURE 5 Glutamic acid use in culutures of B. licheniformis grown in Medium E with high (6.15x10-4M) and low (0.0M) manganese sulfate concentrations.

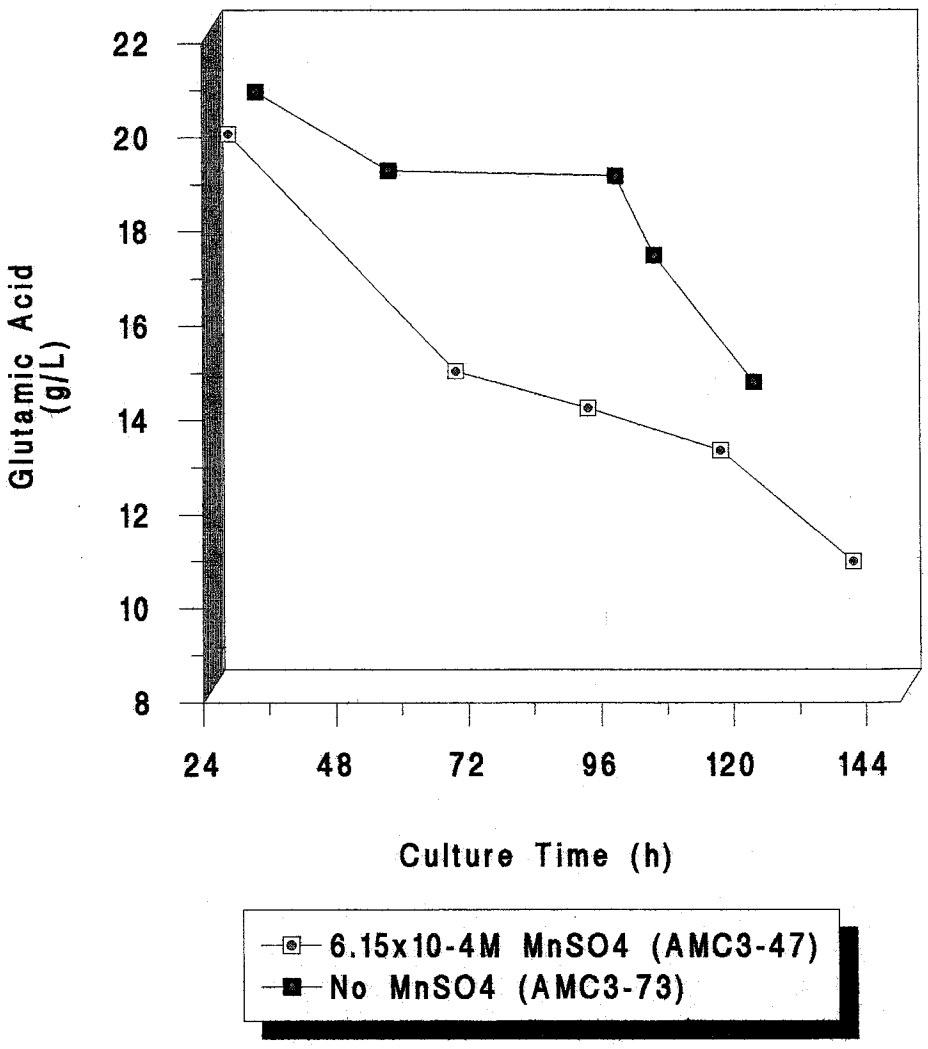

FIGURE 6 Citrate use in cultures of B. licheniformis grown in Medium E with high (6.15x10-4M) and low (0.0M) concentrations of manganese sulfate.

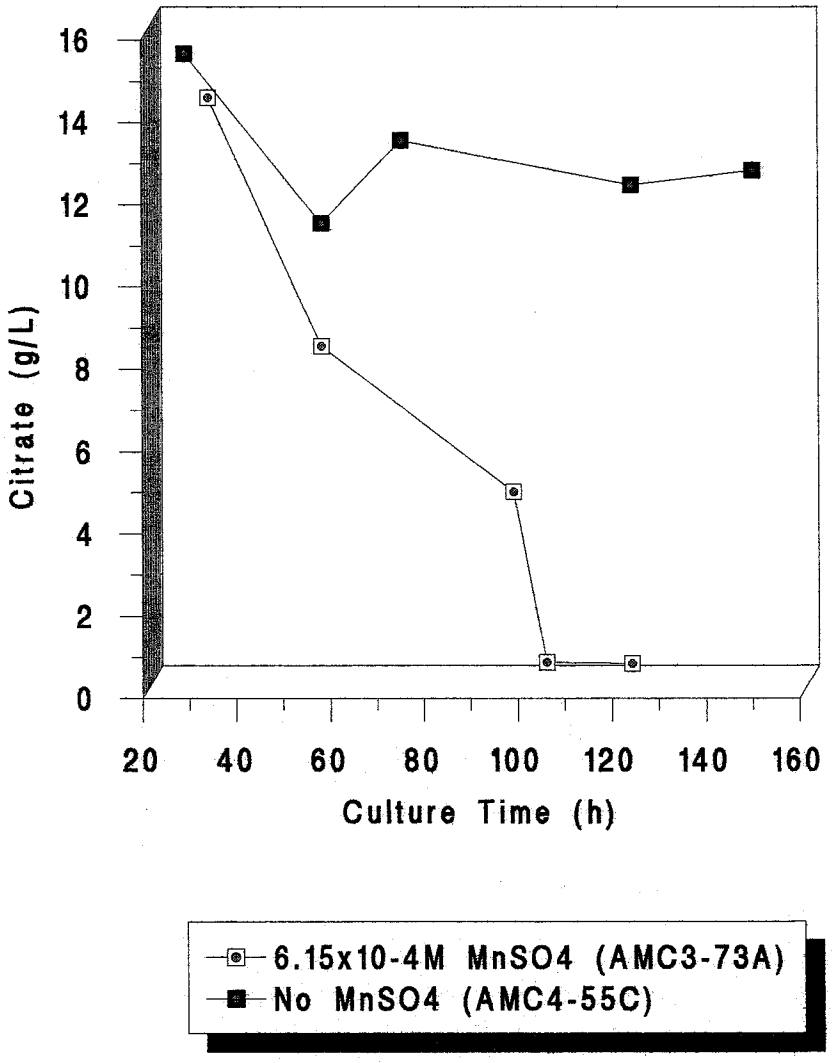

FIGURE 7 Glycerol use in culutures of B. licheniformis grown in Medium E with high (6.15x10-4M) and low (0.0M) manganese sulfate concentrations.

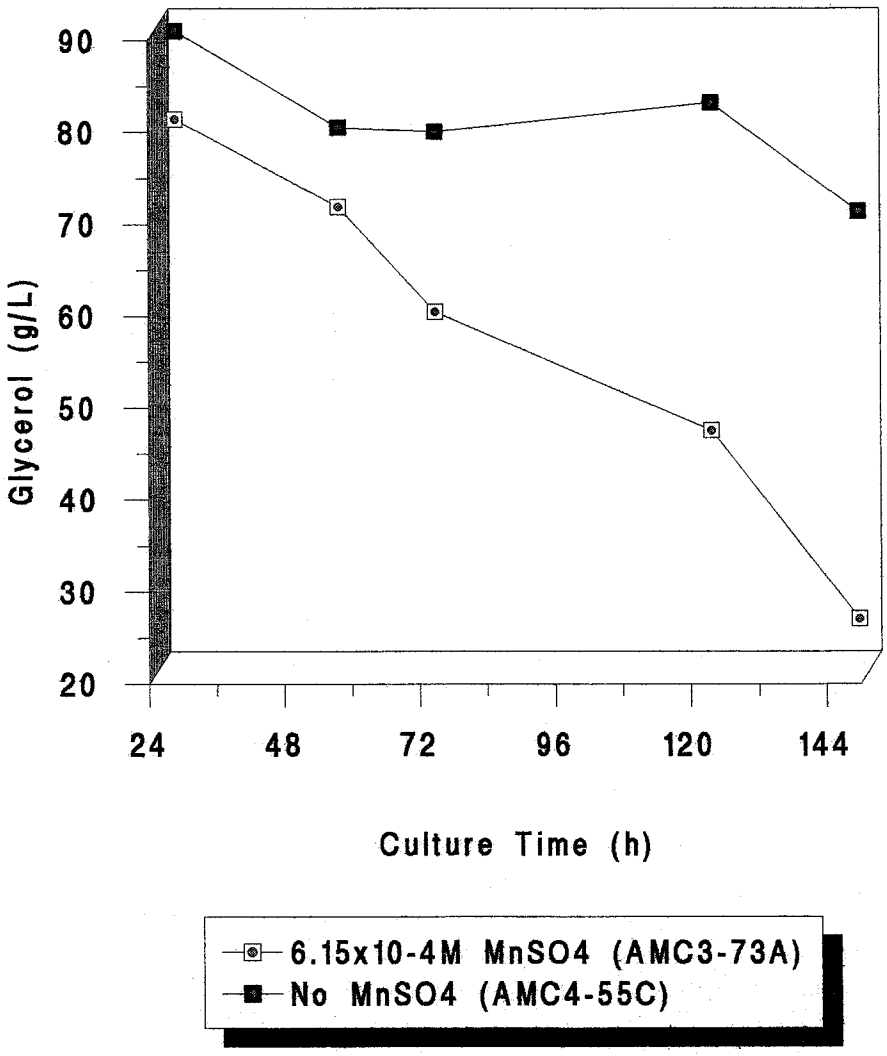

FIGURE 8 Investigation of Mn++ vs. SO4: Yield of PGA

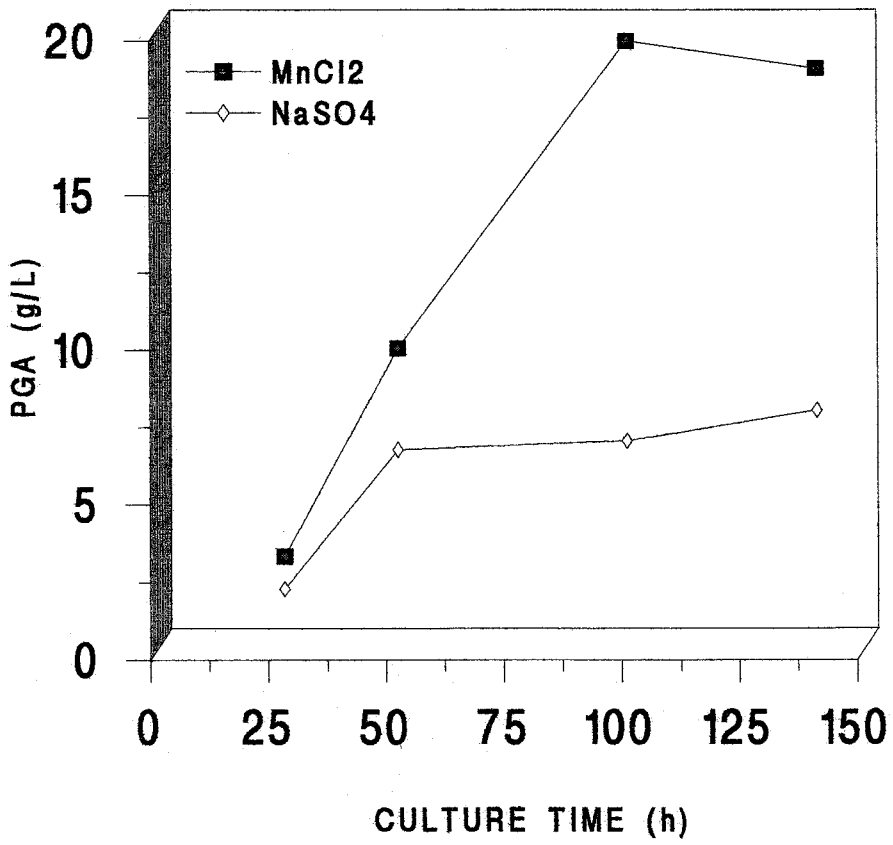

FIGURE 9 INVESTIGATION OF Mn++ vs. S04-- NUMBER OF VIABLE CELLS

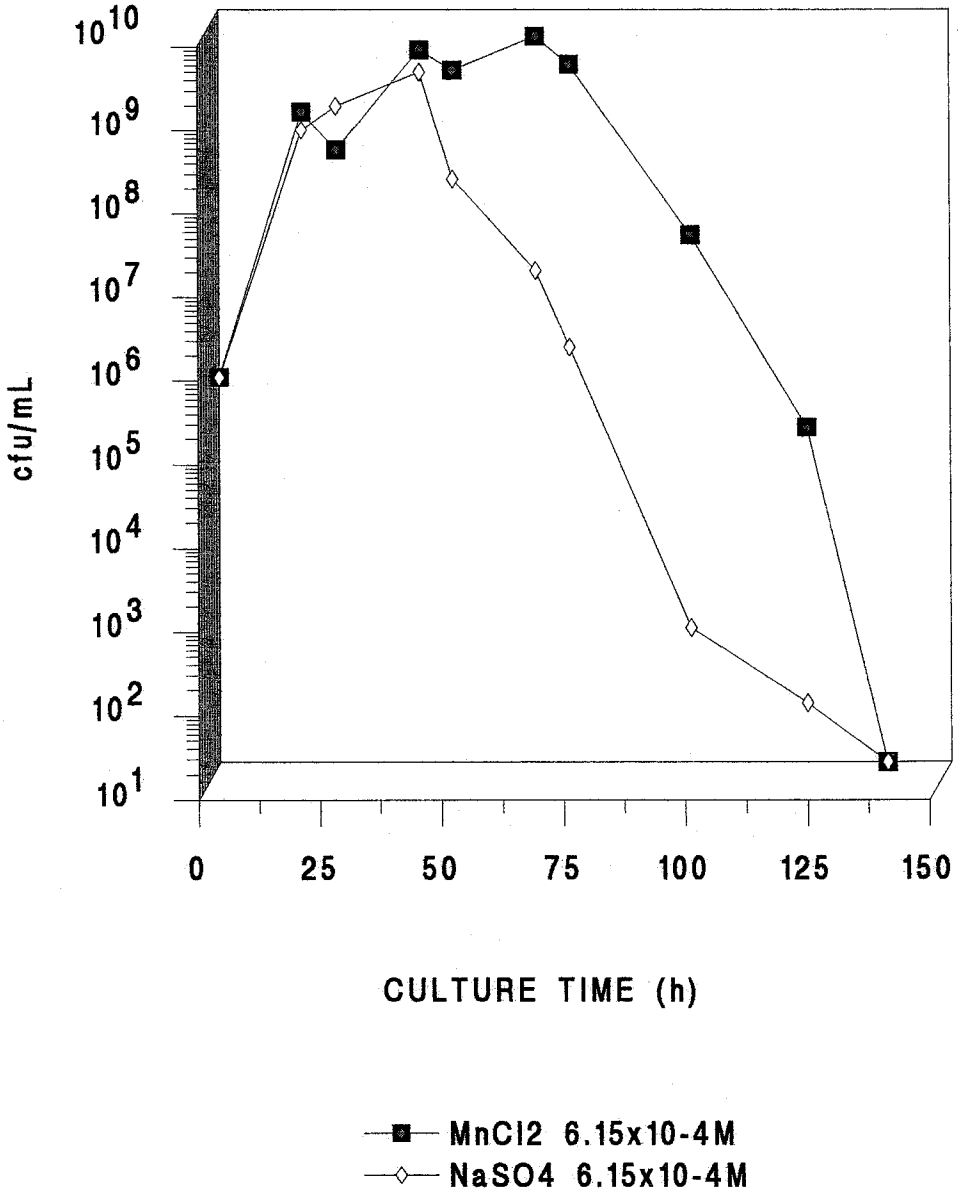

FIGURE 10 The percentage of L-glutamic acid in hydrolyzed PGA obtained from cultures of B. licheniformis grown in Medium E in which the MnSO4 was substituted with 6.15x10-4M MnCl2 and NaSO4, respectively.