Effects of Glucose and Glycerol on γ-Poly(glutamic acid) Formation by *Bacillus licheniformis* ATCC 9945a

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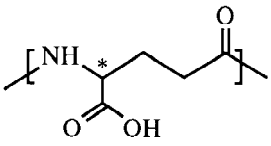
Abstract: *Bacillus licheniformis* ATCC 9945a is one of the bacterial strains that produce γ-poly(glutamic acid) (γ-PGA). The use of carbohydrate medium components for γ-PGA production was explored. Cells were grown in shake flasks or in controlled pH fermentors using medium formulations that contain different carbon sources. During the cultivations, aliquots were removed to monitor cell growth, carbon utilization, polymer production, and polymer molecular weight. Glucose was a better carbon source than glycerol for cell growth. Furthermore, glucose was utilized at a faster rate than glycerol, citrate, or glutamate. However, by using mixtures of glucose and glycerol in medium formulations, the efficiency of γ-PGA production increased. For example, by increasing the glycerol in medium formulations from 0 to 40 g/L, the γ-PGA broth concentration after 96 h increased from 5.7 to 20.5 g/L. Considering that glycerol utilization was low for the glucose/glycerol mixtures studied, it was unclear as to the mechanism by which glycerol leads to enhanced product formation. Cell growth and concomitant γ-PGA production (12 g/L) at pH 6.5 was possible using glucose as a carbon source if trace amounts (0.5 g/L each) of citrate and glutamate were present in the medium. We suggested that citrate and glutamate were useful in preventing salt precipitation from the medium. In addition, glutamate may be preferred relative to ammonium chloride as a nitrogen source. The conversion of glucose to γ-PGA by the strain ATCC 9945a was believed to occur by glycolysis of glucose to acetyl-CoA and tricarboxylic acid (TCA) cycle intermediates that were then metabolized via the TCA cycle to form α-ketoglutarate, which is a direct glutamate precursor. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng* **57:** 430–437, 1998.
Keywords: glucose; glycerol; γ-poly(glutamic acid); *Bacillus licheniformis* ATCC 9945a

INTRODUCTION

Natural macromolecules produced by microorganisms and that are useful for industry consist mostly of polysaccharides. Xanthan gum by *Xanthomonas campestris*, dextran by *Leuconostoc mesenteroides*, curdlan by *Agrobacterium tumefaciens* and *Alcaligenes faecalis*, pullulan by *Aureobasidium pullulans*, gellan by *Pseudomonas elodea*, and alginate by *Azotobacter vinelandii* are important representative examples of microbial polysaccharides (Linton et al., 1991). Based on increased incentives to produce polymers from sustainable resources that are also biodegradable, there has been renewed interest in the development of γ-poly(glutamic acid) (γ-PGA) (Birrer et al., 1994; Cheng et al., 1989; Cromwick and Gross, 1995a,b; Cromwick et al., 1996; Goto and Kunioka, 1992; Hara et al., 1982, 1986; Kubota et al., 1993; Mclean et al., 1990). Attractive properties of γ-PGA are that it is water soluble, anionic, and edible. These and other features make it of interest for applications in the fields of medicine, foods, plastics, and oil recovery.

γ-PGA is an extracellular polymer produced by certain bacillus species (Cheng et al., 1989; Goto and Kunioka, 1992; Hara et al., 1982; Housewright, 1962; Kubota et al., 1993), including *Bacillus licheniformis* ATCC 9945a (Birrer et al., 1994; Leonard et al., 1958; Mclean et al., 1990; Troy, 1973, 1985). Its structure is unusual because it is a homopolymer of glutamic acid that has amide linkages between glutamate γ-carboxyl and α-amino groups (Troy, 1973).



Production of γ-PGA was most extensively studied in *B. anthracis* (Roelants and Goodman, 1968; Thorne, 1956) and *B. licheniformis* ATCC 9945a. Work has been carried out on the nutritional requirements for cell growth, improving conditions for γ-PGA production (Kubota et al., 1993; Leonard et al., 1958; Ward et al., 1963) and variation in chain [d]/[l]-repeat unit composition (Cromwick and Gross, 1995a; Hara et al., 1986; Thorne et al., 1954). For example, it is known that variation in the medium Mn(II) concentra-

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tion can be used to regulate the relative concentration of [d]- and [l]-glutamate repeat units in γ-PGA produced by *B. licheniformis* ATCC 9945a (Cromwick and Gross, 1995a).

Often *B. licheniformis* 9945A and other strains were said to require [l]-glutamic acid for high levels of polymer formation (Housewright, 1962; Thorne et al., 1954). However, Murao et al. (1969) reported that *B. subtilis* 5E did not require glutamate in the medium. Furthermore, of 17 amino acids tested, l-proline was preferred by *B. subtilis* 5E for polymer production (Murao et al., 1971). Ward et al. (1963) reported that culturing of *B. subtilis* NRRL B-2612 on wheat gluten (the insoluble protein component of wheat) resulted in γ-PGA production. Kubota et al. (1993) isolated *B. subtilis* F-2-01 (FERM P-9082) from soil for investigations of γ-PGA production. The bacterium was cultivated on a medium containing 2.0% veal infusion broth, 0.1% glucose, and supplements of different amino acids. Of the amino acids investigated, l-glutamic acid resulted in the highest enhancement in γ-PGA formation. Considering the biosynthetic pathway of γ-PGA, which likely involves the TCA cycle (Cromwick and Gross, 1995b; Goto and Kunioka, 1992), the unrelated carbon source glucose could, in principle, be used as a primary source of carbon for both cell growth and γ-PGA production.

According to Leonard et al. (1958), γ-PGA was obtained from a shake-flask culture of *B. licheniformis* ATCC 9945a at a maximum concentration of 19.6 g/L when the cells were grown in medium E at 37°C with shaking at 250 ppm for 96 h. Medium E had an extraordinarily high concentration of carbon sources. Among them, the glycerol concentration was the highest (80 g/L). The mechanism and biosynthetic pathway for γ-PGA synthesis have not been clearly resolved, although the involvement of the tricarboxylic acid (TCA) cycle has not been disputed (Cromwick and Gross, 1995b; Goto and Kunioka, 1992).

In this study work was carried out to determine whether medium formulations that contained glucose as a carbon source were suitable for *B. licheniformis* ATCC 9945a growth and γ-PGA production. Experiments were also performed to investigate how the glycerol concentration in the medium affected cell growth and γ-PGA production. The polymers formed were analyzed by size exclusion chromatography (SEC) to determine whether the medium formulation resulted in γ-PGAs of variable molecular weight. Starting with medium E that contains glycerol, glutamate, and citrate as carbon sources (Leonard et al., 1958), we reduced and replaced carbon sources necessary for γ-PGA production. In addition to shake-flask studies, fermentations were also carried out with controlled pH so that effects of medium pH were eliminated. Experimental data presented herein strongly argue that glucose is a good carbon source for cell growth and γ-PGA production by this bacterium.

MATERIALS AND METHODS

Strain Information

B. licheniformis ATCC 9945a was obtained from the American Type Culture Collection (ATCC). Highly mucoid colonies capable of high γ-PGA production were selected, grown in liquid broth, transferred to cryovials, and then cryogenically frozen in liquid nitrogen. For storage of bacterial cells, the strain was grown in medium E (Hanby and Rydon, 1946) and 20% sterile glycerol was added to the culture prior to freezing (Birrer et al., 1994). After thawing these cryogenically frozen cells were used for inoculation of cultures in all experiments described below. Details of the method used were described by us elsewhere (Birrer et al., 1994).

Optical Density Determination

To monitor cell growth, aliquots of samples were withdrawn at predetermined time intervals from the shake-flask and fermentor cultures and their optical densities were determined with a UV-VIS spectrophotometer (ULTROSPEC 4050, LKB) at 660 nm. To count viable cells in the shake-flask cultures, samples were diluted with sterile distilled water, plated on principal component analysis (PCA, Difco) medium, and incubated at 37°C overnight.

γ-PGA Quantitation and Molecular Weight Analysis

Aliquots from shake-flask and fermentor cultures were first passed through a 0.45-μm filter and then injected into an HPLC that was fitted with columns for SEC. Conditions for HPLC operation, the method used for determining the concentration of γ-PGA in culture broth, and the method for measurement of γ-PGA molecular weight averages followed the procedures described elsewhere exactly (Birrer et al.. 1994).

Determination of Carbon Source Concentration

The concentrations of glucose, glycerol, citrate, and [l]-glutamate were determined enzymatically using analysis kits purchased from Boehringer Mannheim Biochemicals. Samples removed from shake-flask cultures were passed through 0.45-μm filters and then treated according to detailed procedures described in product information from the supplier (Boehringer Mannheim Biochemicals).

Shake-Flask Cultures

Shake-flask cultures were carried in 1-L Erlenmeyer flasks. Prior to their use, these flasks were acid washed with 10% nitric acid and then thoroughly rinsed with deionized water. The shake flasks were started by rapidly thawing a cryovial of frozen cells in a 37°C bath and transferring 0.5 mL from the cryovial into 200 mL of a modified medium E (Leonard and Housewright, 1963) formulation (see below) that had been autoclaved for 20 min for sterilization. Culture flasks were incubated at 37°C in an orbital shaker at 250 rpm for 4 days. All experiments in shake flasks were carried out in duplicate and data from the two cultures were reported as the mean.

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; $MgSO_4 \cdot 7H_2O$, 0.5; $FeCl_3 \cdot 6H_2O$, 0.04; $CaCl_2 \cdot 2H_2O$, 0.15; and $MnSO_4 \cdot H_2O$, 0.104 (Leonard and Housewright, 1963). The $MnSO_4$ concentration used in this study was 615 μM and glycerol was either completely or partially replaced by glucose (see Table I). Each modified medium had the same total concentration (% w/v) of carbon sources (Table I). The initial pH for each batch culture was adjusted to 7.4 with NaOH but decreased to 6.9 after autoclaving. All chemicals, unless otherwise specified, were obtained from J. T. Baker Inc. (Phillipsburg, NJ, Baker reagent grade).

Fermentor Cultures

To maintain cultures at constant pH, a 2.0-L fermentor (Marubishi, Japan) was used for bacterial growth and γ-PGA production. Medium E composition was modified as follows: glucose was added as a carbon source (see Table II); glycerol, glutamate, and/or citrate were omitted (see Table II); the NH_4Cl concentration was increased from 7.0 to 10 g/L; K_2HPO_4 was omitted; and KH_2PO_4 (0.340 g/L) and $Na_2HPO_4 \cdot 12H_2O$ (0.895 g/L) were added. To prevent the precipitation of various medium components, glucose and the medium salts $MgSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$, and $MnSO_4 \cdot H_2O$ were added separately after sterilization. One liter of modified medium E was added to the fermentor, adjusted to pH 6.5, autoclaved, and inoculated by rapidly thawing a cryovial of frozen cells in a 37°C water bath and transferring 1.0 mL from the cryovial into the fermentor. The fermentor stirring speed and aeration rate was increased gradually from 50 rpm and 0.5 L/min to 500 rpm and 2.0 L/min as the culture viscosity and cell density increased. The incubation temperature was 32°C and the pH was continuously monitored and automatically maintained at 6.5 by the mechanical addition of 2*N* HCl or 2*N* NaOH.

RESULTS AND DISCUSSION

Shake-Flask Cultures: Variation of Glucose to Glycerol Ratio

Shake-flask cultures containing different concentrations of glucose and glycerol were incubated for 96 h and the turbidity was measured at specific time intervals to monitor cell growth (see Fig. 1). In these experiments, an equivalent concentration (w/v) of glucose was substituted in place of glycerol in media formulations (see Table I). Relative to medium E (0/80, ratio of glucose/glycerol), cultures on media L (40/40), M (50/30), N (60/20), O (70/10), and P (80/0) all resulted in higher cell growth. However, differences in glucose concentrations between media L (40/40) and P (80/0) did not result in substantial changes in the growth of *B. licheniformis* ATCC 9945a. These results were further substantiated by viable cell concentrations (cfu/mL) measured at 0- (2.5×10^5) and 32-h cultivation times. At 32 h, the culture on medium E had a viable cell concentration of 5.0×10^8. In contrast, the viable cell concentrations for cultures on media L (40/40) and P (80/0) were all about 2 times higher (between 9.1 and 9.9×10^8).

The relationship between the utilization of carbon sources

Table I. Medium carbon source compositions[a] for shake flask experiments to determine the effect of the glucose/glycerol ratio on *B. licheniformis* 9945a growth, carbon source utilization and γ-PGA production.

	Carbon source components and concentrations (g/L)			
Medium	Glucose	Glycerol	[L]-glutamic acid	Citric acid
E(0/80)[b]	0.0	80.0	20.0	12.0
L(40/40)	40.0	40.0	20.0	12.0
M(50/30)	50.0	30.0	20.0	12.0
N(60/20)	60.0	20.0	20.0	12.0
O(70/10)	70.0	10.0	20.0	12.0
P(80/0)	80.0	0.0	20.0	12.0

[a]Other non-carbon medium components were identical for all of these media and are given in the Materials and Methods section.

[b]The values in parenthesis are the medium concentrations in g/L of glucose/glycerol.

Table II. Medium carbon source compositions[a] for fermentations of *B. licheniformis* 9945a growth performed with controlled pH (6.5).

	Carbon source components and concentrations (g/L)			
Medium	Glucose	[L]-glutamic acid	Citric acid	Glycerol
W(80/0/30)[b]	80.0	0.0	0.0	30.0
X(68/0/12/30)	68.0	0.0	12.0	30.0
Y(68/12/0/30)	68.0	12.0	0.0	30.0
Z(50/0.5/32/30)	50.0	0.5	32.0	30.0
A(50/0.5/0.5/50)	50.0	0.5	0.5	50.0
C(100/0.5/0.5/0)	100.0	0.5	0.5	0.0

[a]Other non-carbon medium components were identical for all of these media and are given in the Materials and Methods section.

[b]The values in parenthesis are medium concentrations in g/L of glucose/glutamate/citric acid/glycerol.

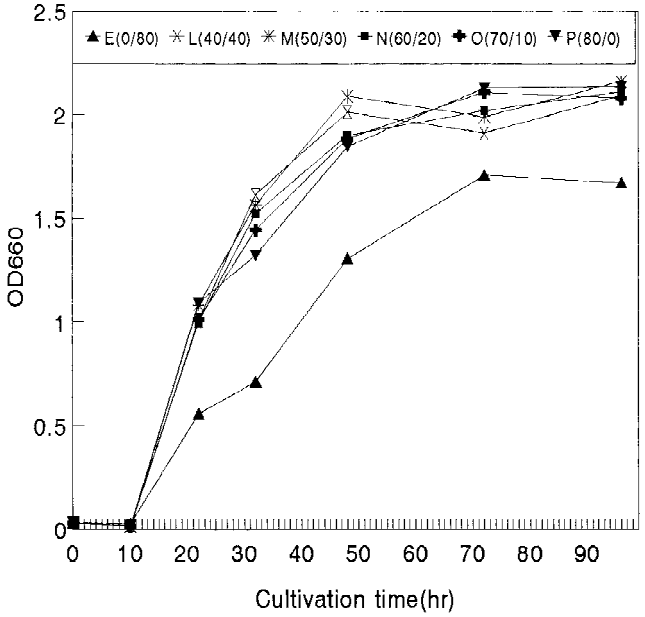

Figure 1. Changes in optical density at 660 nm for shake-flask cultivations of *B. licheniformis* ATCC 9945a. See Table I for medium compositions.

in the medium and the glucose/glycerol ratio was studied (see Figs. 2, 3). The rate of glucose utilization was relatively unchanged, regardless of the initial glucose and glycerol concentrations (Fig. 2). Glycerol was not utilized for cultures on N (60/20) and O (70/10). In cultures on L (40/40) and M (50/30), low levels of glycerol utilization were observed that corresponded with cultivation times where glucose was exhausted. Furthermore, comparison of cultures on E (0/80) and P (80/0) showed that glucose was utilized to much higher extents than glycerol (80 and 30 g/L, respectively). Thus, strain 9945a more readily metabolizes glucose than glycerol. This is consistent with the better growth of this organism on medium P (80/0) than medium E (0/80) (see Fig. 1 and above). Glucose was also utilized at a faster rate than either citrate or glutamate for cultures on L (40/40) through P (80/0). Interestingly, by increasing the content of glycerol in medium formulations from 0 to 30 g/L, the glutamate utilization increased from 6 to 14 g/L for 96-h incubations (Fig. 3). For the series of cultures on E (0/80) to P (80/0), medium P (80/0), which does not contain glycerol, showed the lowest glutamate utilization. However, medium M (50/30) and L (40/40) showed the highest glutamate utilization. The culture on E (0/80) showed an intermediate level of glutamate utilization (8 g/L). In comparison to glutamate, citrate utilization was similarly effected by the content of glycerol in medium formulations. Cultures on P (80/0), L (40/40), and E (0/80) utilized 0.0, 11.5, and 9.0 g/L of citrate over the 96-h incubation period. Thus, substitution of ~40 g/L of glucose in place of glycerol in medium formulations resulted in enhanced metabolism of both glutamate and citrate.

Because glutamate and citrate are known precursors of γ-PGA (Cromwick and Gross, 1995b), it was anticipated that increased glutamate and citrate metabolism would result in higher γ-PGA production. As suspected, the γ-PGA broth concentration during the 96-h incubation period showed a strong dependence on the initial glucose to glycerol ratio. Figure 4 shows that low levels (<1 g/L) of γ-PGA were produced by 22 h, which correlates with little glutamate and citrate consumption over a similar time period (Fig. 3). In all cases, γ-PGA concentration increased for incubation times from 22 to 48 h. Extending the culture time beyond 48 h resulted in increased γ-PGA concentrations for incubations on media E (0/80), L (40/40), N (60/20), and M (50/30) (see Fig. 4). As the glycerol concentration in medium formulations was increased from 0 to 40 g/L, the γ-PGA concentration after 96-h incubations increased from 5.7 to 20.5 g/L. A further increase in the glycerol concentration from 40 to 80 g/L resulted in decreased γ-PGA concentration (to 13.0 g/L). Therefore, our results on γ-PGA concentrations are consistent with the general trend that

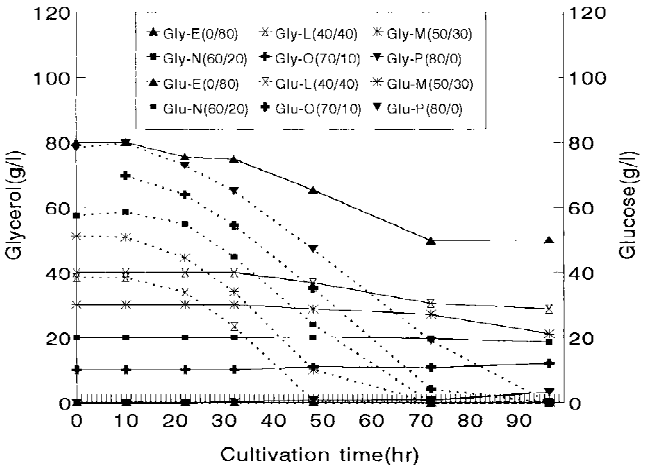

Figure 2. Changes in glycerol (solid lines) and glucose (dotted lines) concentrations for shake-flask cultures of *B. licheniformis* ATCC 9945a. See Table I for medium compositions.

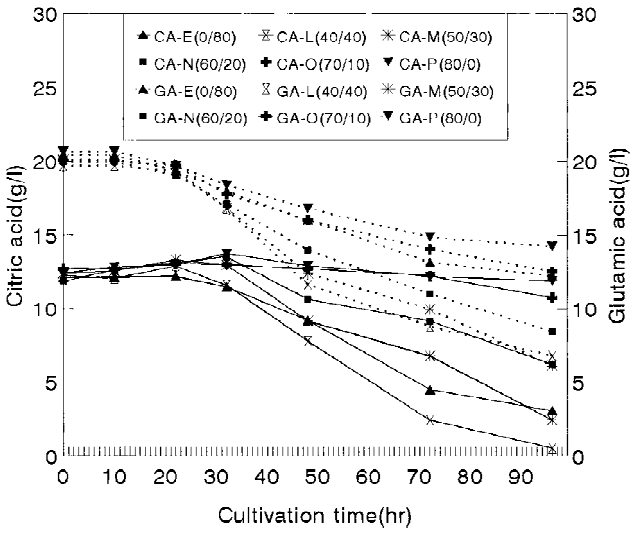

Figure 3. Changes in citric (solid lines) and glutamic acid (dotted lines) concentrations for shake-lask cultures of *B. licheniformis* ATCC 9945a. See Table I for medium compositions.

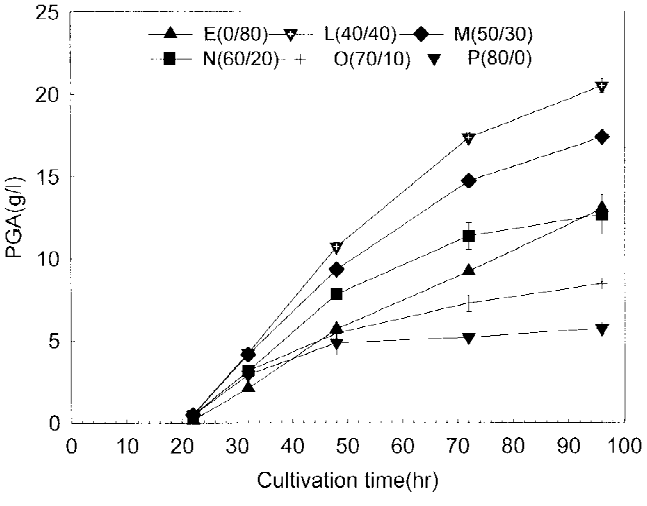

Figure 4. γ-PGA concentration as function of culture time and medium formulation for shake-flask incubations of *B. licheniformis* ATCC 9945a. See Table I for medium compositions. Error bars are given showing standard deviations for $n = 3$.

citrate and glutamate utilization increased as the glycerol medium concentration was increased from 0 to 40 g/L (see Fig. 3). However, because glycerol utilization was low for the glucose/glycerol mixtures studied (see Fig. 2) and, moreover, increasing the glycerol concentration from 0 to 40 g/L did not substantially alter cell growth (see Fig. 1), it was unclear as to why glycerol addition to the medium resulted in such large increases in γ-PGA formation.

Troy found that a polyglutamyl synthetase complex from *B. licheniformis* 9945a catalyzes a sequence of membrane-associated enzymatic reactions in which [l]-glutamic acid is activated, racemized, and polymerized to form predominantly [d]-γ-PGA (Troy, 1973). Furthermore, he showed a nearly twofold stimulation of polyglutamyl synthetase activity by the addition of 0.1*M* glycerol (Troy, 1973). Thus, considering the cell-free studies by Troy and the results of this work, it appears that glycerol may function to increase the activity of enzymes that are involved in monomer and/or polymer synthesis. However, the mechanism by which glycerol functions to stimulate carbon source metabolism and γ-PGA formation remains unknown.

The molecular weight of γ-PGA products will need to be ''tailored'' to meet various application specifications. For example, low and high molecular weight products will be more suitable for uses in detergency and superadsorbents, respectively. Therefore, it was of interest to determine whether changing the glucose/glycerol ratio would alter γ-PGA molecular weight. Weight average molecular weight (M_w) values as a function of the incubation time were plotted for cultures on E (0/80) through P (80/0) (Fig. 5). At 22 h when culture broth γ-PGA concentrations were low, M_w values ranged from about 4.9 to 5.4 million g/mol. For all of these cultures, M_w decreased as the incubation time increased and culture broth γ-PGA concentrations increased or remained unchanged. This was similarly observed by us elsewhere for fermentations on medium E where the Mn(II) concentration in the medium was varied (Cromwick and Gross, 1995a). By 96 h, the M_w values for these γ-PGA products ranged from about 900,000 to 2 million g/mol, which would still be considered rather high. Furthermore, the product molecular weight distributions (M_w/number average molecular weight) were between 2.5 and 3.0. Therefore, regardless of the glucose/glycerol ratio, high molecular weight products resulted.

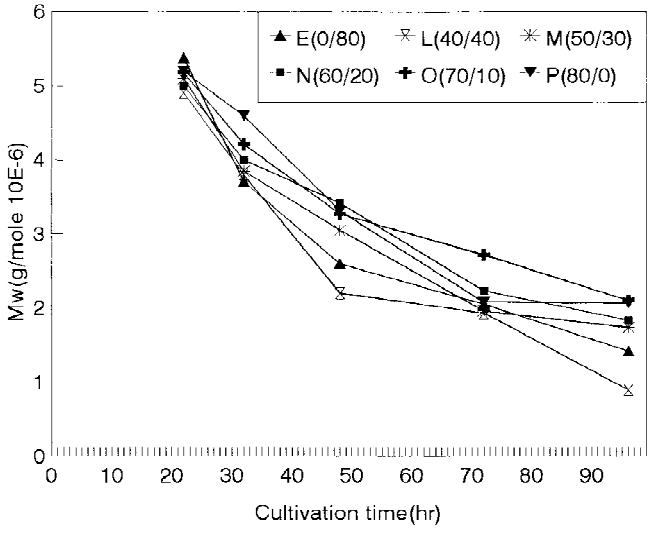

Figure 5. γ-PGA weight average molecular weights (M_w) as a function of culture time for shake-flask cultures of *B. licheniformis* ATCC 9945a. See Table I for medium compositions.

The initial pH of the shake-flask experiments was 6.9. After 48 h, the pH values of cultures on E (0/80), L (40/40), M (50/30), N (60/20), O (70/10), and P (80/0) were 6.0, 5.7, 5.6, 5.7, 5.6, and 5.5, respectively. When these fermentations were harvested at 96 h, the pH values were 5.3, 5.4, 6.0, 6.3, 6.4, and 5.7, respectively. These variations in supernatent pH would not be expected to affect polymer production (Cromwick et al., 1996).

Experiments in Fermentors with Controlled pH

In a previous article we reported on how removing either citrate, glutamate, or glycerol from the medium E formulation effects γ-PGA formation by *B. licheniformis* 9945a (Birrer et al., 1994). When citrate was omitted, the pH fell to a value of about 5.0 within the first 24 h and subsequently decreased to pH 4.5 by 58 h. When glycerol was omitted, the pH remained at about 7.0 for 28 h and then increased until a value >9.0 was reached. Both of these cultures showed low γ-PGA concentrations in the medium that were attributed to either cell death or low productivity at high pH. Furthermore, in another study, *B. licheniformis* 9945a was grown on medium E in batch fermentations where the pH was maintained at 5.5, 6.5, 7.4, and 8.25 (Cromwick et al., 1996). Fermentations at pH 6.5 resulted in relatively higher γ-PGA broth concentration and specific γ-PGA production rate. Therefore, the studies described below on the substitution of glucose for medium E carbon sources (see Table II) were carried out by controlling the pH at 6.5. In addition, many of the medium formulations selected for investigation contained mixtures of glucose and glycerol to exploit the results found in the shake-flask studies described above.

Initially, we considered the growth of *B. licheniformis* ATCC 9945a during fermentations on W (80/0/0/30), X (68/0/12/30), Y (68/12/0/30), and Z (50/0.5/32/30), which all contain 30 g/L of glycerol (see Table II, Fig. 6). *B. licheniformis* ATCC 9945a did not grow in medium W where glutamate and citrate were omitted. Therefore, the glucose-glycerol medium was not sufficient to support bacterial growth even though the medium pH was maintained at 6.5. However, when 12 g/L citrate was added to this fermentation at 52 h, the optical density began to increase after a lag of 40 h. A similar lag time (50 h) was observed for the fermentation on X (68/0/12/30), which contained citrate. Interestingly, the addition of a trace amount of glutamate (0.5 g/L) to the glucose-citrate-glycerol medium Z (50/0.5/

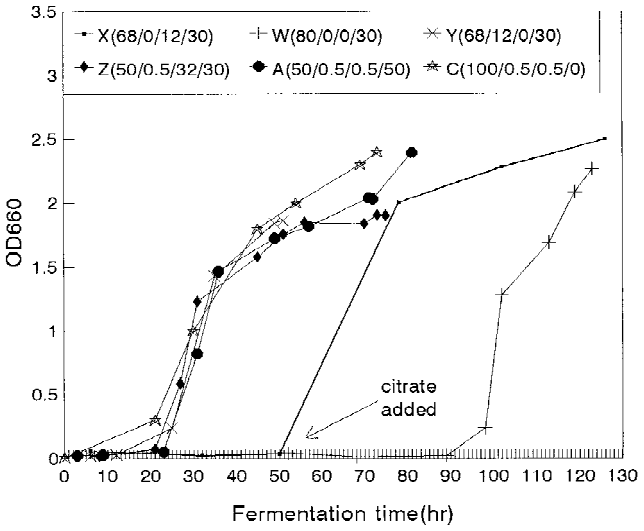

Figure 6. Changes in optical density at 660 nm for cultivations of *B. licheniformis* ATCC 9945a carried out at pH 6.5 in fermentors. See Table II for medium compositions.

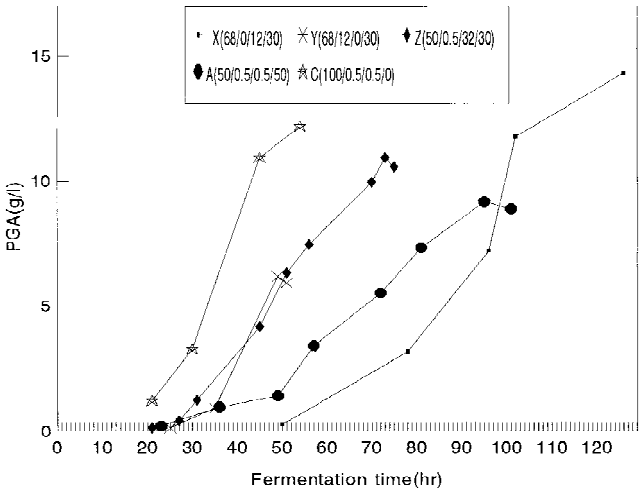

Figure 7. γ-PGA concentrations in culture broths for cultivations of *B. licheniformis* ATCC 9945a carried out at pH 6.5 in fermentors. See Table II for medium compositions.

32/30) reduced the growth lag time to 20 h. Furthermore, the omission of citrate from the medium formulation that contained glucose-glutamate-glycerol [Y (68/12/0/30)] resulted in a relatively short growth lag period (12 h). Interestingly, it was noticed that a red precipitate was formed in the glucose-glycerol medium W (80/0/0/30) immediately after the addition of sterile $FeCl_3 \cdot 6H_2O$ to complete the medium prior to inoculation. This precipitate disappeared when citrate was added to the medium. Therefore, one explanation for no cell growth on medium W (80/0/0/30) was the precipitation of an essential nutrient such as an iron complex that was made available to cells by solubilization when either citrate or glutamate was included in the medium formulation. Another possible factor is that glutamate might function as a better nitrogen source than NH_4Cl. This would be consistent with the fact that the fermentation on medium X (68/0/12/30) had a longer lag period than the fermentation on medium Y (68/12/0/30). When cells were incubated in glucose-glycerol medium that contained only low levels of glutamate and citrate [A (50/0.5/0.5/50)], the cells grew after only a ~20-h lag period. Even in the absence of glycerol, that is, in the medium that contained glucose and trace amounts (0.5 g/L) of glutamate and citrate as carbon sources [C (100/0.5/0.5/0)], the cells grew similarly to fermentations containing relatively large amounts of glutamate and citrate (20 and 12 g/L, respectively) (Birrer et al., 1994; Cromwick and Gross, 1995a). These results suggest that cell growth occurs in glucose minimal medium provided that critical medium nutrients are solubilized.

Figure 7 shows the γ-PGA broth concentration or volumetric yield as a function of culture time for the controlled pH fermentation experiments (see Table II). High concentrations of γ-PGA (~12 g/L in 50 h) were produced during the fermentation on the glucose minimal medium C (100/0.5/0.5/0). Also, the fermentation on medium A (50/0.5/0.5/50) gave ~9 g/L γ-PGA by ~95 h. Because the concentration of γ-PGA was well above the sum of glutamate and citrate added to these medium formulations, we concluded that the carbon for γ-PGA synthesis was in large part supplied by glucose for the fermentation on medium C (100/0.5/0.5/0) and by glucose and/or glycerol for the fermentation on medium A (50/0.5/0.5/50). γ-PGA formation on medium C (100/0.5/0.5/0) occurred concurrently with cell growth. Similarly, Goto and Kunioka (1992), working with another bacillus strain (*B. subtilis* IFO3335), showed that γ-PGA formation occurred with a maximum volumetric yield (10.4 g/L) by the early stationary phase (40 h). However, earlier work by us with *B. licheniformis* 9945a in modified medium E formulations containing glutamate, glycerol, and citrate (20, 80, and 12 g/L, respectively) with differing Mn(II) concentrations (0–615 μ*M*) showed that polymer formation occurred primarily in the early and later stationary phases (Cromwick and Gross, 1995a). Interestingly, for the fermentations on medium formulations other than C (100/0.5/0.5/0), γ-PGA was mainly formed in the early and late stationary phase (see Fig. 7).

Figure 8 provides the results of γ-PGA M_w as a function of culture time. The culture time at which γ-PGA began to increase in concentration in the medium corresponded to the formation of relatively higher molecular weight product. As the concentration of γ-PGA in the culture broth increased the M_w decreased. Such behavior is consistent with that observed in the shake-flask experiments (Fig. 5) as well as in previous work by us (Birrer et al., 1994; Cromwick and Gross, 1995a) and others (Leonard and Housewright, 1963; Thorne et al., 1954; Troy, 1973). It is likely that product degradation that occurs concurrently with product accumulation is due to the presence of γ-glutamyl depolymerase activity in the extracellular medium of *B. licheniformis* 9945a (Leonard and Housewright, 1963; Thorne et al.,

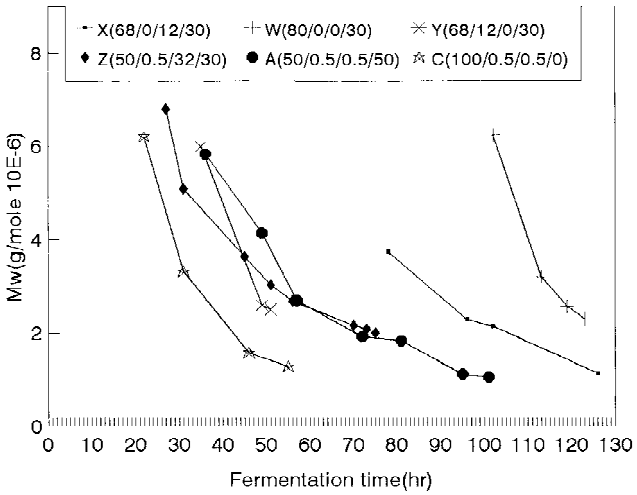

Figure 8. γ-PGA weight average molecular weights (M_w) as a function of culture time for cultivations of *B. licheniformis* ATCC 9945a carried out at pH 6.5 in fermentors. See Table II for medium compositions.

1954; Troy, 1973) or to the action of a depolymerase that is intracellularly located or cell bound (Birrer et al., 1994).

SUMMARY

Shake-flask incubation experiments showed that glucose was utilized to a much greater extent than glycerol. However, by adding controlled amounts of glycerol to medium formulations, γ-PGA production by *B. licheniformis* ATCC 9945a was increased. Furthermore, by using mixtures of glucose and glycerol in the medium, enhanced metabolism of both glutamate and citrate resulted. The increased utilization of glutamate and citrate corresponded with higher γ-PGA production as was anticipated based on the fact that glutamate and citrate are known precursors of γ-PGA. Considering that glycerol utilization was low for the glucose-glycerol mixtures studied, it was unclear as to the mechanism by which glycerol leads to enhanced product formation.

Batch fermentation experiments conducted at pH 6.5 demonstrated that *B. licheniformis* 9945a can metabolize carbon from glucose to form γ-PGA. It appeared that low levels of citrate and glutamate added to glucose minimal medium was useful in decreasing the cell growth lag time. Possibly citrate and glutamate functioned by solubilizing critical medium nutrients such as Fe^{+++}.

The conversion of glucose to γ-PGA suggests that γ-PGA synthesis by the strain ATCC 9945a can occur by the glycolysis of glucose to acetyl-CoA and TCA cycle intermediates that are then metabolized via the TCA cycle to form α-ketoglutarate, which is a direct glutamate precursor. Indeed, in a previous study ^{13}C-labeled glutamate was used to show that a large fraction of γ-PGA repeat units can be formed from provided glutamate with retention of the glutamate carbon skeleton (Cromwick and Gross, 1995a).

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