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PENICILLIN ACYLASE OF *ESCHERICHIA COLI*

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Abstract

A new strain of Escherichia coli *was isolated locally and possessed high penicillin acylase (EC 3.5.1.11) activity. The production and kinetic studies were done with the cell bound enzyme of this strain and compared to that of the reference strain,* E. coli *ATCC 9637. They were quite similar, in general, except that at 28°C and with 0.064% phenylacetic acid in the growth medium the new isolate possessed higher enzyme activity per mg cell dry weight. Moreover, a mutant of this new isolate was found to possess even higher penicillin acylase activity under the same conditions. A genetic manipulation program is underway to develop this mutant for industrial use.*

Introduction

Bacterial penicillin acylase (EC 3.5.1.11) is now being used on an industrial scale to catalyse the conversion of benzylpenicillin to 6-aminopenicillanic acid (6-APA) and phenylacetic acid (PAA)[1-3]. The product, 6-APA, is the important key intermediate for preparation of semisynthetic penicillins[4]. This has stimulated investigators to search for microbial species producing a more suitable penicillin acylase. Among various bacteria capable of producing pencillin acylase, *E. coli* was most widely studies, especially strain ATCC 9637[5-8].

The production of penicillin acylase in *E. coli* is induced by phenylacetic acid[5,7] and better production is obtained with growth conditions at low temperature (26° C) and with low dissolved oxygen in the medium[5,7,9]. Also, enzyme production is repressed by a high level of carbohydrate such as glucose or polyalcohols such as glycerol and this repressive effect can be overcome by cAMP[7,10,11]. *E. coli* penicillin acylase has also been subjected to substrate and product inhibitions[12-15]. Inhibition by 6-APA was shown to be noncompetitive while that by phenylacetic acid was competitive[3,12].

It is the purpose of this study to obtain local isolates of *E. coli* that would be suitable for production of penicillin acylase in Thailand. The criteria used for strain selection would be high enzyme yield at high temperature (37° C - 40° C) and in the absence of phenylacetic acid (the usual inducer) if possible. Other useful characteristics would be the ability to maintain high activity under high concentrations of substrate and product and stability under variable conditions.

Materials and Methods

Reagents. All reagents used were of analytical grades. Penicillin-G potassium salt, penicillin-V potassium salt, ampicillin, 6-aminopenicillanic acid, phenylacetic acid, and p-dimethylaminobenzaldehyde were obtained from Sigma Chemical Company, Saint Louis, Missouri. Ready made silica gel TLC aluminum sheets (Art. 5554) were obtained from E. Merck, Darmstadt. All media used were from Difco Laboratories, Detroit, Michigan.

Organisms. The organisms used in this study were *E. coli* ATCC 9637, *E. coli* strain 194 and its mutant *E. coli* strain 194-1 from our stock culture. The cultures were maintained on nutrient agar slants and subcultured every two weeks. The stock cultures were kept at 4° C.

Growth conditions. The cultures were grown in 500 ml Erlenmyer flasks containing 125 ml of liquid medium. They were shaken at approximately 200 rpm on a rotary shaker (Gyrotory shaker, New Brunswick, New Jersy) for 18-24 hours at either 28° C or 37° C as specified. Unless otherwise indicated, the liquid medium was composed of Bacto peptone (0.5%), Bacto beef extract (0.3%) and phenylacetic acid (0.064%). The pH of the medium was adjusted to 7.0 before sterilization.

Mutation experiment. E. coli cells grown in nutrient broth at 28°C for 18 hours were centrifuged, washed once, resuspended in phosphate buffer (0.05 M, pH 7.5) and adjusted to approximately 1×10^8 cells/ml. The bacterial suspension was subjected to UV irradiation[15]. Then 0.1 ml of each dilution (10^{-5} and 10^{-6}) was spread on each of two agar plates [bacto-peptone (0.5%), bacto-beef extract (0.3%), bacto-agar (1.2%) adjusted to pH 7.0 before sterilization] which were then incubated overnight at 37°C. The next day they were overlayered with 5 ml of nutrient agar containing 25,000 µg penicillin-G and 0.01 ml of an overnight culture broth [peptone (1%) and NaCl (0.5%)] of *S. marcescens.* The overlayered plates were incubated at 28°C, overnight and then examined for clear inhibition zones around the *E. coli* colonies. These clear zones resulted from the failure of growth of *Serratia marcescens* to grow and they indicated the ability of the relevant *E. coli* to produce penicillin acylase.

Determination of penicillin acylase activity. The penicillin acylase assay employed bacterial cells as a source of enzyme in the reaction mixture. Bacterial cells were washed once and resuspended in phosphate buffer (0.05 M, pH 7.5). The bacterial suspension was adjusted to 30 mg cell dry weight per ml. In general, unless noted otherwise, five ml of reaction mixture contained one ml of cell suspension and 25,000 µg of penicillin-G potassium salt in phosphate buffer. The reaction mixture was shaken at

40 °C for 20 min. after which 0.5 ml of the reaction mixture was assayed employing the method described by Balasingham, et al.[12] One unit of enzyme activity was defined as the amount of cell bound enzyme required to produce 1 umole of 6-APA per mg of cell dry weight per min.

Thin layer chromatography. Ready made silica gel plates were employed in this study. Samples along with standard solutions of 6-APA, penicillin-G and penicilloic acid were applied to the plates. The identity of standards was verified by bioassay and by Rf values in the literature. The penicilloic acid used as a standard was obtained as a cell-free supernatant from a mixture of 50,000 g penicillin-G, many loopfuls of *E. coli* colonies known to produce penicillinase, and phosphate buffer (0.05 M, pH 7.5) that had been incubated at 37 °C for 30 min. After application of samples and standards, the chromatogram was developed with n-butylacetate: n-butanol : acetic acid : water (80 : 15 : 40 : 24) for 1.5 hours. Spots developed were detected with ultraviolet light, iodine vapor, or sulfuric acid : methanol (50 : 50) with heat. They were also subjected to bioautography with *Serratia marcescens* which is sensitive to 6-APA but resistant to penicillin-G[16].

Results

Bacteria capable of producing penicillin acylase. The *E. coli* strains used in this study were selected from a screening program for penicillin acylase producing bacteria by our groups. Strain 194 was first selected because it gave higher penicillin acylase activity than *E. coli* ATCC 9637 under our culture conditions. Percent conversions to 6-APA in 60 min at 28 °C and 0.064% PAA were 73.8 in *E. coli* 194 and 58.3 in *E. coli* ATCC 9637 (see Table 1). In more recent experiments *E. coli* 194-1, a mutant of *E. coli* 194 was selected on the basis of even more enzyme activity as shown in Table 1. None of these bacteria produced penicillinase under the conditions studies.

Enzyme production required phenylacetic acid at 0.064% final concentration as an inducer in the culture broth. At higher concentrations of phenylacetic acid e.g., (0.15%) growth at 28 °C was suppressed so that there were too few cells for enzyme assays especially for *E. coli* 9637 and isolate 194. Enzyme production was also affected by temperature i.e., it was produced only during growth at 28 °C but not during growth at 37 °C, though growth at 37 °C was better.

Activities on different substrates. Only penicillin-G, penicillin-V, and ampicillin were tested. It was found that the cell-bound *E. coli* 194 was most active on penicillin-G and less active on ampicillin and penicillin-V (70% and 10% of that on penicillin-G, respectively).

TABLE 1. EFFECTS OF GROWTH CONDITIONS ON CELL MASS AND PENICILLIN ACYLASE PRODUCTION BY *E. COLI.*

Isolate No.	Growth Conditions		Cell Mass	% 6-APA conversion
	Temp. °C	/PAA[a]		
194	28	0.064	3.48	73.8
194-1	28	0.064	4.08	91.0
ATCC 9637	28	0.064	4.56	58.3
194	28	0.15	very low	ND[b]
194-1	28	0.15	5.04	99.2
ATCC 9637	28	0.15	very low	ND
194	37	0.064	5.52	4.6
194-1	37	0.064	4.56	4.9
ATCC 9637	37	0.064	4.56	4.6
194	37	0.15	7.8	5.0
194-1	37	0.15	7.0	4.3
ATCC 9637	37	0.15	6.7	7.6

[a] PAA stands for phenylacetic acid.

[b] ND = Not done.

Effects of pH on the deacylation of penicillin-G. The enzyme assays at various pH's were performed similarly to the penicillin acylase assay described in the Materials and Methods. Buffers at pH's 4.0, 5.0 and 5.6 were 0.05 M sodium acetate buffer, at 6.0, 7.0, 7.5 and 8.0 were 0.05 M phosphate buffer and at 9.2 and 10.1 were 0.05 M sodium carbonate buffer. The assays were run for 20 min at 40°C. The optimum pH was found to be broad, ranging from 6.0 to 8.1 with pH 8.1 giving the highest activity see Figure la.

Effects of temperature on the deacylation of penicillin-G. The test performed using *E. coli* 194 as in the usual penicillin acylase assay except that the incubation temperatures were varied from 30°C to 67°C. The results showed that within 20 min of incubation the temperature of 60°C gave the highest percentage yield of 6-APA (Figure 1b).

Effects of pH and temperature on the stability of cell-bound enzyme. E. coli 194 was employed in these experiments. For the effect of pH on stability of the enzyme, bacterial cells equivalent to 30 mg cell dry weight were suspended in 5 ml of the appropriate buffers at pH's 4.0, 6.0, 7.1, 7.5, 8.1, 9.2 and 10.1 for 1 hour. Then, the cells were centrifuged and resuspended in 5 ml of 0.05 M phosphate buffer pH 7.5 and assayed for enzyme activity. For the effect of temperature on stability of the enzyme, bacterial cell suspensions (30 mg cell dry weight in 0.05 M phosphate buffer, pH 7.5), were treated at various temperatures(4°C, 30°C, 40°C, 50°C and 60°C)for 24 hours before being assayed for penicillin acylase activity at 40°C, for 20 min. The results showed that the cell-bound enzyme was quite stable at acid, neutral and slightly alkaline pH's but severely damaged at pH 10.1 (Figure 1c). It was also stable at low temperatures and was destroyed at high temperatures as expected (Figure 1d). At 30°C and 40°C the activity was the same as that at 4°C while at 50°C the activity was reduced to 10% of that at 4°C. At 60°C there was no activity.

Determination of K_m and V_{max}. These two parameters were determined for the cell bound enzyme of *E. coli* 194 using penicillin-G potassium salt. The experiments were done in duplicate with concentrations of substrate employed at 5, 10, 15, 20, 30 and 40 mmoles/1. The mean values of the duplicate was used for determination of Km and V_{max}. K_m and V_{max} at pH 7.5, 60°C were determined from Lineweaver Burk plots to be 6.25 mM and 0.189 umoles 6-APA/mg cell dry weight/min, respectively (as in Figure 2).

Determination of K_i of phenylacetic acid. The reaction mixture consisted, in addition to the usual, of phenylacetic acid at 5, 10, 15, and 20 mM final concentrations. The results showed that K_i for phenylacetic acid was 20 mM.

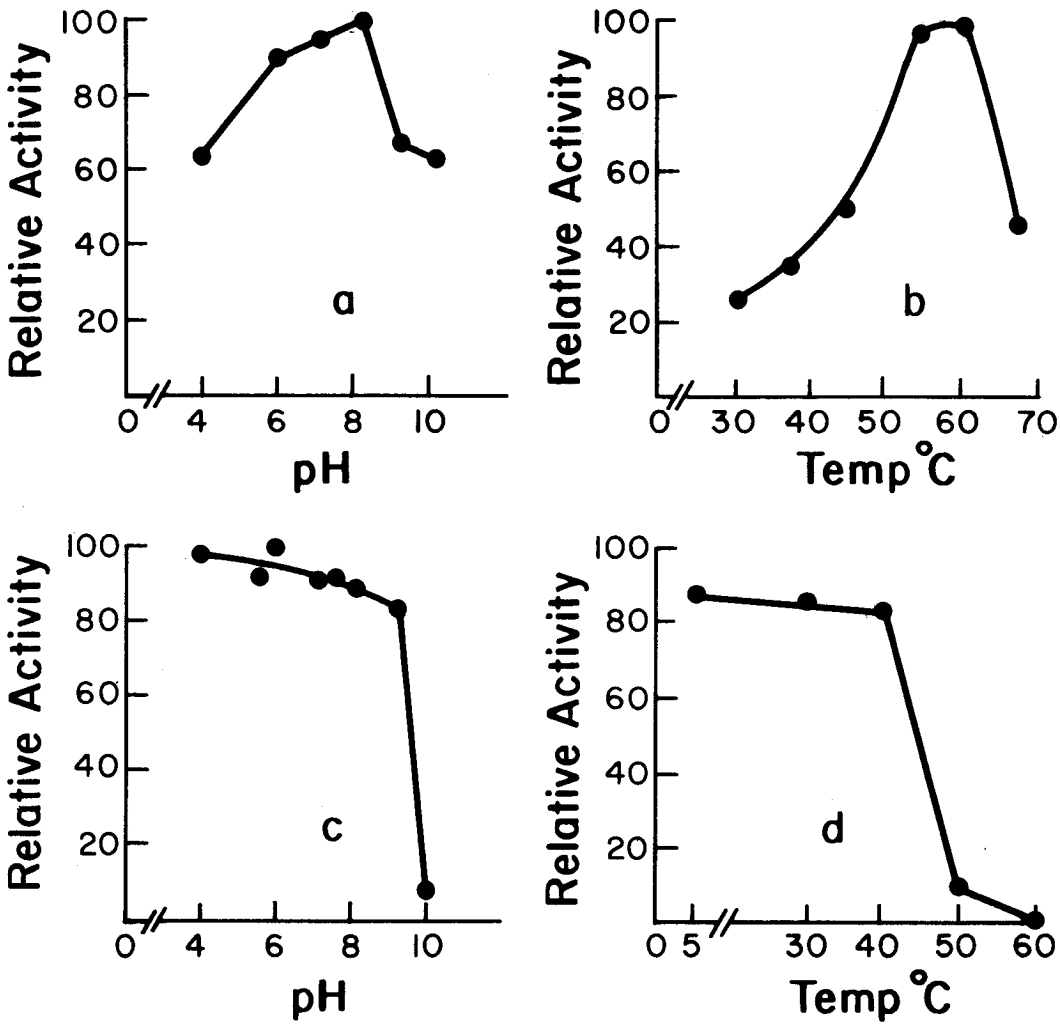

Figure 1. Characteristics of penicillin acylase from *E. coli* strain 194: Effects of (a) pH on enzyme activity; (b) temperature on enzyme activity; (c) pH on enzyme stability; and (d) temperature on enzyme stability.

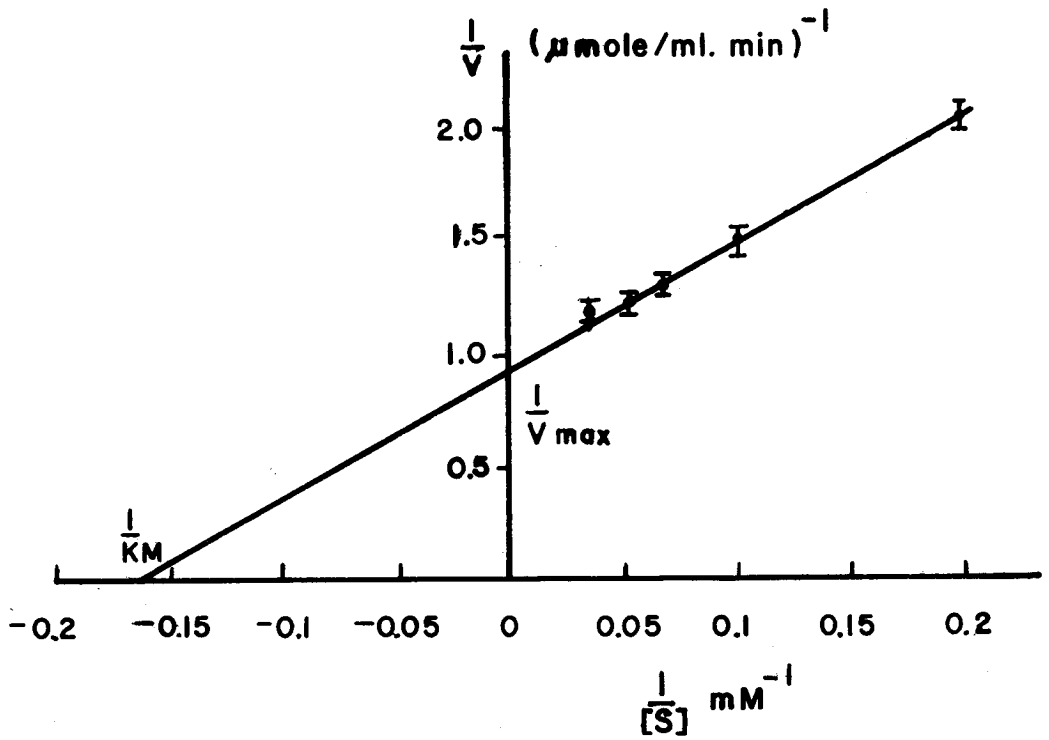

Figure 2. Lineweaver Burk plot for determination of K_m and V_{max} of enzyme activity for *E. coli* strain 194.

Physical and biological determinations of 6-APA after enzymatic hydrolysis. Thin layer chromatography of the cell-free supernate from the enzyme assay mixture showed the presence of 6-APA with an R_f value of 0.25 as compared to the R_f values of 0.70 and 0.35 for penicillin G and penicilloic acid, respectively. The spot with an Rf value 0.25 was biologically confirmed to be 6-APA by bioautography using *Serratia marcescens*-ATCC 27,117 which is inhibited by 6-APA but not by penicillin-G or penicilloic acid.

Discussion

The production of penicillin acylase during growth of *E. coli* was shown to be affected by temperature and by the presence of phenylacetic acid as an inducer (Table 1). Our results were similar to those previously reported[7,9,17]. However, the final concentration of phenylacetic acid used in our study was only 0.064% while others have used 0.15-0.2%. With 0.15% phenylacetic acid in our growth conditions at 28 °C growth of the reference strain and isolate 194 was suppressed but growth for the mutant was not. Whether phenylacetic acid from Sigma contained impurities that were toxic to the bacteria we do not know. No other source of phenylacetic acid was used in this study.

In this study, with growth conditions at 28 °C and 0.064% phenylacetic acid, *E. coli* isolate 194 was shown to possess higher penicillin acylase activity per mg cell dry weight when compared to the reference strain *E. coli* ATCC 9637. Moreover, a recent isolate designated *E. coli* 194-1 derived by mutagenesis possessed even higher enzyme activity. The percent conversion of penicillin-G to 6-APA with strain 194-1 was shown to be 91% in one hour at 40 °C, pH 7.5. This was much higher than the value of 58% for *E. coli* ATCC 9637. Previous works by other investigators showed 62-85% conversion in four hours with *E. coli* ATCC 9637 at 30 °C, pH 7.5[17] and 92% in one hour with *E. coli* NCIB 8743A at 50 °C, pH 8.2[2]. The considerable lower values for the first could be because the enzyme assay was done at a lower temperature or the growth conditions were different. It also seems that the isolate 194-1 was better than the second (*E. coli* NCIB 8743A) since the same percent conversion of 6-APA could be achieved at a lower assay temperature. We selected 40 °C for our penicillin acylase assay since the enzyme was quite active and stable at this temperature, less active at 30 °C and more active but less stable at 50 °C.

The optimum pH and temperature for the penicillin acylase activity of isolate 194 was shown to be similar to those reported for this enzyme by other investigators[2,17] i.e., at pH's ranged from 6.0 - 8.1 at 60 °C.

Isolate 194 was shown to have penicillin acylase of type II as classified by Hamilton-Miller[18]. It does not possessed penicillinase activity as the filtrate from the assay mixture showed no penicilloic acid on TLC plates and the organism was negative for the penicillinase test by the iodometric method[19].

The Km and V_{max} values obtained with isolate 194 were in the range of those previously reported by others. For example, our K_m value was 6.25 mM while others have reported 4.7 mM[8], 17.5 mM[17] and 30.9 mM[2]. Our V_{max} value in umoles 6-APA/mg cell dry weight/min. was 0.189 while others have reported 0.03[17] and 0.614[2]. The differences in K_m or V_{max} values obtained by us and by others could be attributed to differences in the strains of bacteria used and/or to the conditions of assay. Also, the inhibition constant K_i for phenylacetic acid obtained by us was similar to that obtained by Park, *et al*[8]. (i.e., 20 mM vs 28.9 mM, respectively).

Although the kinetic and stability work have yet to be done with penicillin acylase of *E. coli* 194-1, we expected similar results but higher activity to be obtained with this mutant. However, assays of cells grown at 37 °C in liquid media with or without phenylacetic acid were negative. Tests are underway to determine the reason for this discrepancy. We have attempted to isolated mutants that produce penicillin acylase resistant to high concentrations of phenylacetic acid and this has also been unsuccessful. Work is continuing with isolate 194-1 to obtain further mutants with some or all of the desired properties mentioned above.

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บทคัดย่อ

ผู้วิจัยได้คัดเลือก เอชเชอริเชีย โคไล *(Escherichia coli)* สายพันธุ์ใหม่ซึ่งสร้างเอ็นไซม์ เพ็นนิซิลลินเอซีเลส สูง เมื่อเปรียบเทียบตัวควบคุมการสร้างและคุณสมบัติของเอ็นไซม์นี้ ใน เอชเชอริเชียโคไล สายพันธุ์ใหม่ กับสายพันธุ์ที่เคยรายงานไว้คือ (*E. coli* ATCC 9637) พบว่าคุณสมบัติทั่ว ๆ ไปคล้ายกัน นอกเสียจากว่าเมื่อทำการเลี้ยงที่ 28°C ในอาหารที่มี 0.064% กรดฟีนิลอะซีติคด้วย สายพันธุ์ใหม่นี้มีความสามารถสูงกว่าเมื่อเปรียบเทียบต่อน้ำหนักของเซลล์ที่เท่ากัน นอกจากนี้ผู้วิจัยยังได้ทำการศึกษาการผ่าเหล่าของสายพันธุ์ใหม่ และได้สายพันธุ์ใหม่ ซึ่งให้เอ็นไซม์สูงขึ้นไปอีก ขณะนี้ได้ใช้วิธีการทางพันธุกรรมเพื่อทำให้ได้สายพันธุ์ที่มีคุณสมบัติเหมาะกับงานอุตสาหกรรมต่อไป