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Comparative Production of 6-Aminopenicillanic Acid by Different *E. coli* Strains and Their Acridine Orange (AO) Induced Mutants

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Abstract: The present study was conducted to see the difference in production of 6-APA I) between wild strains of *E. coli* collected from local environment and their acridine orange (AO) induced mutants and ii) between mutants and *E. coli* strains (ATCC 11105 and ATCC 9637) of American Type Culture Collection (ATCC) used commercially for enzymatic production of 6-APA. The optimum conditions for bioconversion were standardized and 6-APA was obtained in crystalline form. Relative PGA activity of local and foreign *E. coli* strains varied significantly with the highest being 12.7 in mutant strain (BDCS-N-M36) and the lowest 4.3 mg 6-APA $h^{-1}mg^{-1}$ wet cells in foreign strain (ATCC 11105). The enzyme activity exhibited by mutant strain (BDCS-N-M36) was also two folds higher compared to that in wild parent BDCS-N-W50 (6.3 mg 6-APA h^{-1} mg^{-1} wet cells). The overall production of 6-APA and conversion ratios ranged between 0.25-0.41 g of 6-APA per 0.5 g of penicillin G and 51-83%, respectively. Maximum conversion ratio (83%) was achieved by using crude cells of mutant strain (BDCS-N-M36) which is the highest value ever reported by crude cells on a shake-flask scale whereas reported 6-APA production by immobilized cells is 60-90% in batch and continuous systems. Results are being discussed with reference to importance of local bacterial strains and their significance for industrially important enzymes.

Key words: *E. coli*, penicillin G acylase, 6-aminopenicillanic acid, bioconversion

INTRODUCTION

The 6-aminopenicillanic acid (6-APA) is a starting compound for the synthesis of β-lactam antibiotics such as ampicillin and amoxicillin (Shewale and SivaRaman, 1989; Shewale *et al*., 1990). Penicillin G Acylase (PGA) is a microbial enzyme which is widely used as biocatalyst (Ma *et al*., 1992) for the production of 6-APA. Several approaches have been used to improve *E. coli* PGA production, particularly by using mutagenesis (Chou *et al*., 1999; Liu *et al*., 2000; Parekh *et al*., 2000) and genetic and protein engineering techniques (Chou *et al*., 2000). Biocatalytic conversion has many advantages as it involves fewer steps (Sheldon, 2000) and can lead to five-fold reduction in waste volume compared to the conventional chemical procedures (Egorov *et al*., 2000; Van de Sandt and de Vroom, 2000).

Immobilized preparations of both enzymes and cells have been successfully used in the pharmaceutical industry to produce 6-APA of which over 10,000 tonnes 6-APA is being produced annually using immobilized enzyme penicillin acylase (Ishimura and Suga, 1992; Liao *et al*., 1999; Scaramozzino *et al*., 2005). Immobilized penicillin G acylase is also being used for enzymatic conversion of penicillin G to phenylacetic acid (PAA) and 6-APA (Nabais and Cardoso, 2000). A composite system of immobilized enzyme reactor combined with ion exchange column has been used for hydrolysis of penicillin G to 6-APA (Jian *et al*., 1995). *E. coli* strains with intracellular penicillin acylase can also be used for the production of 6-APA in aqueous two-phase system (Cao *et al*., 2004). The purpose of present study is therefore to use crude cells of various *E. coli* strains and their acridine orange induced mutants possessing enhanced PGA activity for production of 6-APA. The objectives were to detect I) quantitative variations in production of 6-APA using crude cells of different strains, ii) differences in 6-APA production by wild type and mutant strains and iii) differences in production of 6-APA by crude cells and the reported production of 6-APA by

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immobilized cells. Success in this will help identification of bacterial strains used for cost-effective production of 6-APA.

MATERIALS AND METHODS

Bacterial strains: Three locally collected PGA producing *E. coli* strains BDCS-N-FMu10, BDCS-N-S21 and BDCS-N-W50 used in this study were isolated from different habitats (Arshad *et al*., 2006a) whereas BDCS-N-M1, BDCS-N-M23 and BDCS-N-M36 were obtained through mutation induced by acridine orange (AO) in the wild strains (Arshad *et al*., 2006b). Foreign *E. coli* strains (ATCC 9637 and ATCC 11105) were acquired from American Type Culture Collection (ATCC).

Culture conditions: Selected PGA producing strains were cultivated into 250 mL Erlenmeyer flasks containing MII medium [1.25 g of Corn steep liquor (CSL), 0.1 g of ammonium sulphate and 0.15 g of phenylacetic acid (PAA) per 100 mL]; pH of medium was adjusted to 6.5 with I M NaOH. Fermentation was carried out on a rotary shaker (150 rpm) at 28°C for 18 h on a shake-flask scale. After overnight incubation, the cells were separated by centrifugation at 5000 x g at 4°C for 10 min and washed with 0.05 M potassium phosphate buffer, pH 7.5.

Determination of PGA activity: One gram wet cells were re-suspended in 20 mL of potassium phosphate buffer, pH 7.5. Enzyme assay was performed on cell suspension and enzymatic reaction proceeded in a 250 mL Erlenmeyer flask. Penicillin G solution was prepared by dissolving 0.5 g of penicillin G into 100 mL of phosphate buffer, pH 7.5 This solution was added to cell suspension and the reaction mixture was kept shaking (50 rpm) at 40°C for 4 h. The amount of 6-APA produced using intact bacterial cells containing PGA was determined by p-dimethylaminobenzaldehyde (PDAB) method at 415 nm (Saba *et al*., 1998). One unit of enzyme activity was defined as the amount of 6-APA produced per h per milligram wet cells (mg 6-APA h^{-1} mg^{-1} wet cells) under assay conditions.

Bioconversion of penicillin G to 6-APA: Isolation of 6-APA from the reaction mixture was carried out after enzyme fermentation. Reaction conditions were standardized for conversion of penicillin G into 6-APA and recovery of 6-APA in crystalline form using intact cells. Reaction mixture was centrifuged, cells were discarded and supernatant was processed for 6-APA isolation. The pH of supernatant was adjusted to 2.0 with 6 M H_2SO_4. PAA and residual penicillin G were extracted from the reaction mixture at pH 2.0 with 120 mL of n-butylacetate. After phase separation the pH of aqueous phase was adjusted to 7.0 with $Ba(OH)_2$ and centrifuged. After centrifugation, supernatant was concentrated to 20 mL by evaporation under vacuum (<30°C), 40 mL of methanol was added to it before it was kept in refrigerator overnight and then filtered. Filtrate was re-concentrated under vacuum and precipitated by adjusting the pH to 4.3 with 6 M HCl. Crystals of 6-APA were washed with cold water and dried under vacuum. The conversion ratio (C) of penicillin G to 6-APA was calculated as:

$$C = \frac{\text{Amount of 6 – APA produced} \times 100}{\text{Amount of penicillin G used}}$$

Statistical analysis: Differences in PGA activity and 6-APA production of different strains were expressed as mean for three independent experiments and were evaluated by one-way Analysis Of Variance (ANOVA) followed by Duncan's Multiple Range (DMR) test. Data were analyzed by computer using Microsoft Excel software.

RESULTS

PGA activity: Relative PGA activity varied significantly amongst the local wild strains, mutants and the foreign *E. coli* strains (Table 1), with the highest (12.7 mg 6-APA h^{-1} mg^{-1} wet cells) being observed in BDCS-N-M36 (mutant) and the lowest (4.3 mg 6-APA h^{-1} mg^{-1} wet cells) in ATCC 11105. The enzyme activity exhibited by mutant strain (BDCS-N-M36) was two fold higher compared to that in wild parent (BDCS-N-W50) as well as foreign *E. coli* strains. Mutant BDCS-N-M23 showed a slight increase (7.1 mg 6-APA h^{-1} mg^{-1}

Table 1: Relative Penicillin G Acylase (PGA) activity and amount of 6-APA produced by locally collected (parent/mutant) and foreign *E. coli* strains

	PGA activity	Amount of 6-APA produced
Strain	mg 6-APA h^{-1} mg^{-1} wet cells	g 6-APA/0.5 g penicillin G
Local		
Parent		
BDCS-N-FMu10	6.6c	0.340c
BDCS-N-S21	6.5c	0.337c
BDCS-N-W50	6.3c	0.327cd
Mutant		
BDCS-N-M1	4.9d	0.310d
BDCS-N-M23	7.1b	0.377b
BDCS-N-M36	12.7a	0.413a
Foreign		
ATCC 9637	4.7d	0.273e
ATCC 11105	4.3e	0.253e
One-way analysis of variance (ANOVA)	F = 695.63*** df = 7,16 p<0.05	F = 71*** df = 7,16 p<0.05

6-APA: 6-aminopenicillanic acid; ATCC: American Type Culture Collection. Values are mean; numbers with different letters are significantly different with Duncan's Multiple Range (DMR) at p<0.05; *** highly significant

wet cells) in PGA activity compared to that (6.5 mg 6-APA h^{-1} mg^{-1} wet cells) in parent (BDCS-N-S21) however, compared to the parent strain BDCS-N-FMu10, PGA activity decreased in mutant BDCS-N-M1. One-way Analysis Of Variance (ANOVA) test revealed a highly significant difference among the mutants, parents and foreign strains ($F = 695.63^{***}$; df = 7,16; $p<0.05$). Duncan's Multiple Range (DMR) test indicated that mean enzyme activity was significantly ($p<0.05$) higher in two mutants (BDCS-N-M23 and BDCS-N-M36) followed by wild parental strains (BDCS-N-FMu10, BDCS-N-S21 and BDCS-N-W50). There were considerable variations among the wild strains and mutants whereas mutant *E. coli* strains expressed more PGA activity than the foreign strains (ATCC 9637 and ATCC 11105) acquired from American Type Culture Collection.

6-APA production: Considerable variations were observed in the conversion ratio of locally collected wild/mutant and foreign strains. The overall production of 6-APA ranged between 0.253 and 0.413 g of 6-APA per 0.5 g of penicillin G (Table 1) and bioconversion ranged between 51 to 83% (Table 2). The maximum conversion of penicillin G into 6-APA (0.413 g) was achieved using mutant strain BDCS-N-M36 with maximum intracellular PGA used as biocatalyst. In BDCS-N-M36 and BDCS-N-M23, one gram of wet cells converted 0.5 g of penicillin G into 0.413 and 0.377 g of 6-APA which led to 83 and 75% conversion, respectively. Recovery percentage of 6-APA was significantly less in foreign *E. coli* strain 55% in ATCC 9637 and 51% in ATCC 11105, which indicated that not only the mutant, but the wild parental strains (BDCS-N-FMu10, BDCS-N-S21 and BDCS-N-W50) with bioconversion of 68, 67 and 65%, respectively were better for bioconversion of penicillin G into 6-APA. One-way Analysis Of Variance (ANOVA) test for 6-APA conversion ratios (Table 1) showed a highly significant difference among mutant, parent and foreign strains ($F = 71^{***}$; df = 7,16; $p<0.05$). Duncan's Multiple Range (DMR) test indicated that 6-APA yield was significantly higher ($p<0.05$) in two mutants (BDCS-N-M36 and BDCS-N-M23) followed by wild strains (BDCS-N-FMu10, BDCS-N-S21 and BDCS-N-W50). The *E. coli* mutant strains with high intracellular PGA activity showed excellent potential for bioconversion of penicillin G into 6-APA than foreign *E. coli* strains (ATCC 9637 and ATCC 11105).

Table 2: Enzymatic conversion of penicillin G into 6-APA by PGA producing *E. coli* strains

Strain	6-APA conversion %
Local	
Parent	
BDCS-N-FMu10	68
BDCS-N-S21	67
BDCS-N-W50	65
Mutant	
BDCS-N-M1	62
BDCS-N-M23	75
BDCS-N-M36	83
Foreign	
ATCC 9637	55
ATCC 11105	51

6-APA: 6-aminopenicillanic acid

DISCUSSION

The enzymatic synthesis of 6-APA reduce/eliminate the production of waste and the dependence on organic solvents (Wegman *et al*., 2001). In the present study, 6-APA synthesis was achieved by using whole cells of PGA producing wild type and mutant *E. coli* strains on a shake-flask scale. The traditional chemical procedure for 6-APA production has gradually been replaced by biotransformation and improvements have been made in purification, stability and immobilization of penicillin acylase used for these transformations (Parmar *et al*., 2000). We have reported a relatively simple approach for improvement in the production of 6-APA. Optimum conditions were standardized for bioconversion and enzyme activity was induced by supplementing the fermentation medium with 0.15% PAA (Arshad and Ahmad, 2000).

The amount of 6-APA obtained through PGA activity of mutant BDCS-N-M36 was much higher than wild strains and was possibly achieved due to higher PGA activity and negligible β-lactamase expression in AO induced mutants (Arshad *et al*., 2006b). Penicillin G acylase activity in wild strains was enhanced by strain improvement through genetic manipulation. Mutagenesis of bacterial strains is one of the most frequently employed methods of improvement (Chou *et al*., 1999; Liu *et al*., 2000), but most often *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) has been used for this purpose (Chou *et al*., 1999). We have used AO-induced mutants (Arshad *et al*., 2006b) for the first time to enhance the production of 6-APA. Therefore, the present study reports the potential efficacy of PGA activity of mutant strains for the production of 6-APA.The process of enzyme fermentation and 6-APA crystallization was optimized by controlling reaction conditions and removing coexistence of PGA with β-lactamase (Arshad and Saba, 2001); A key enzyme which cleaves the β-lactam ring in penicillin and hampers the conversion of penicillin into 6-APA (Livermore, 1995).

The results provides experimental evidence that mutant *E. coli* strains, with enhanced PGA and negligible β-lactamase activity, are the potential biocatalyst for production of 6-APA. The pre-requisites for an efficient

6-APA biosynthesis using intact cells as enzyme source are: (i) high production of PGA and (ii) hydrolysis of penicillin. It is evident from the present study that more the PGA activity expressed by *E. coli* strains, the more will be conversion of penicillin G into 6-APA. Since our wild *E. coli* strains BDCS-N-FMul 0, BDCS-N-S21 and BDCS-N-W50 were good producers of PGA which resulted into 6-APA recovery of 68, 67 and 65%, respectively. This conversion ratio was obtained using crude cell suspension of wild strains having β-lactamase. However, 6-APA recovery in wild strains was higher than foreign strains ATCC 9637 (55%) and ATCC 11105 (51%) which reached to 83% and 77% in mutants BDCS-N-M36 and BDCS-N-M23 after reduction and/or elimination of β-lactamase activity by AO mutagenesis. In the present study, 83% conversion rate was achieved by using crude cells of mutant (BDCS-N-M36) that was obtained by strain improvement through genetic manipulation. The reported 6-APA production by immobilized cells is 60-90% in batch and continuous systems (Norouzian *et al*., 2002) whereas we are reporting for the first time here 83% production of 6-APA by crude cells on a shake-flask scale.

E. coli ATCC 11105 penicillin acylase has been used for the synthesis of β-lactam antibiotics (Bruggink *et al*., 1998), but due to its kinetic characteristics, penicillin acylase produced by this strain shows low potential for the synthesis of 6-APA. This however, can be improved by mutagenesis. Previously, studies were carried out to modify wild *E. coli* strains for hyper-production of PGA and thus mutants with enhanced PGA and reduced/negligible β-lactamase activity were developed (Arshad *et al*., 2006b). In the present study, we describe the potential o f AO induced mutants for production of 6-APA. However, such report is not available on the enhanced production of PGA and enzymatic synthesis of 6-APA. Our mutants revealed higher PGA activity and more 6-APA yield as compared to enzyme activity expressed by locally collected wild type *E. coli* and foreign strains specifically *E. coli* ATCC 11105, which is the best source for PGA production (Alkema *et al*., 2000; Arroyo *et al*., 2003). The enhanced PGA activity improved the capacity of mutants which led to high 6-APA conversion.

More than 80% of 6-APA is produced enzymatically (Bruggink *et al*., 1998) and substantial improvements such as new enzymes, better enzyme derivatives and improved reaction designs have continesously been made to improve the enzymatic approaches. Hyper-producing mutant strains and enzymatic process used in the present study are economically viable and environmental friendly. Because of significant economic investments, the development of industrial processes for 6-APA and other compounds will only be successful if the hyper-producing strains with broadened specificity, increased specific activity and overall stability are locally available. The availability of strains (wild and mutants) with potentiality of producing high levels of penicillin acylases is thus a key factor in the development of biocatalytic process for the synthesis of 6-APA.

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