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# *Beijerinckia indica* var. *penicillanicum* penicillin V acylase: enhanced enzyme production by catabolite repression-resistant mutant and effect of solvents on enzyme activity

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## SUMMARY

*Beijerinckia indica* var. *penicillanicum* mutant UREMS-5, producing 168% more penicillin V acylase, was obtained by successive treatment with UV, γ-irradiation and ethylmethane sulfonate. Penicillin V acylase production by the mutant strain was resistant to catabolite repression by glucose. Incorporation of glucose, sodium glutamate and vegetable oils in the medium enhanced enzyme production. The maximum specific production of penicillin V acylase was 244 IU/g dry weight of cells. Effect of solvents on hydrolysis of penicillin V by soluble penicillin V acylase and whole cells was studied. Methylene chloride, chloroform and carbon tetrachloride significantly stimulated the rate of penicillin V hydrolysis by whole cells.

## INTRODUCTION

Penicillin V acylase (PVA) catalyses hydrolysis of the penicillin V molecule to generate the β-lactam nucleus, 6-aminopenicillanic acid (6-APA), and side chain phenoxyacetic acid [2,13,20]. PVA is used in pharmaceutical industries for the production of 6-APA, which is further converted chemically into semisynthetic penicillins of commercial importance [4,11,20]. About 12.5% of the total 6-APA produced enzymatically involves using PVA [20]. PVA is produced mainly by fungi and actinomycetes [17]. However, certain bacterial isolates such as *Pseudomonas acidovorans* have a good potential for commercial production of PVA [7].

We have reported earlier that *Beijerinckia indica* var. *penicillanicum* SKB 3133 produces levels of PVA comparable to those produced by *Pleurotus ostreatus* (previously described as *Bovista plumbea*) [18]. The bacterium produces PVA constitutively and intracellularly and the enzyme production is repressed by sugars. The present paper describes the isolation of a catabolite repression-resistant mutant of *B. indica* var. *penicillanicum* that produces 168% more PVA than the parent strain. Incorporation of certain solvents in the reaction mixture increases the activity of the enzyme present in the cells.

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## MATERIALS AND METHODS

*Organism.* *Beijerinckia indica* var. *penicillanicum* SKB 3133 was used in this study [18]. The parent culture and mutants isolated were maintained on nutrient agar slants and preserved at 5 °C after lyophilization.

*Fermentation.* The culture was grown under submerged conditions as described previously [18]. The basal medium for PVA production (Medium III) contained: yeast extract, 0.5%; potassium dihydrogen phosphate, 0.1%; casamino acids, 2.0%; and corn steep liquor, 3.0%; pH 6.0–6.5 [18]. Glucose supplements were sterilized separately. Supplements of seed oils were sonicated with distilled water (16 ml/ml of oil) and sterilized separately, prior to addition to the medium. Fermentations were carried out on a rotary shaker (240 rpm) at 25 °C in 500-ml Erlenmeyer flasks containing 50 ml medium.

Cells were harvested by centrifugation at 8000 rpm for 10 min (Sorval RC-5 centrifuge), washed thrice with distilled water, and then recentrifuged.

### *Mutation studies*

*UV-treatment.* The cells from an agar slant of *B. indica* var. *penicillanicum* SKB 3133 were suspended in 20 ml of

sterile distilled water and transferred to a sterile petri dish (90 mm diameter). The suspension was exposed to UV radiation using a UV tube of 30 W as source. The cell suspension was kept stirring at a distance of 30 cm from the source for 5, 10 and 15 min. Samples, drawn at different time intervals, were serially diluted before plating on nutrient agar plates. The agar plates were incubated at 24 °C for 24 h. Single colonies were picked and tested for enzyme production. The colonies were designated as U and numbered sequentially.

*γ-Irradiation.* The cells from an agar slant of *B. indica* var. *penicillanicum* U-10 were suspended in 5 ml of sterile distilled water. 1 ml of this suspension was further diluted with 5 ml of sterile distilled water and aseptically distributed into three different sterilized tubes. The tubes were exposed to γ-radiation for 5, 10 and 15 min, respectively, using $Co^{60}$ as the source in a Gamma Chamber (Isotope Division, BARC, Bombay). The γ-irradiated cell suspensions were serially diluted with sterile distilled water and plated on nutrient agar plates. The plates were incubated at 24 °C for 24 h. Single colonies were picked and tested for the production of PVA. The colonies were designated as UR and numbered sequentially.

*Ethylmethane sulfonate (EMS) treatment.* A cell suspension was prepared by using 5 ml of sterile distilled water and an agar slant of *B. indica* var. *penicillanicum* UR-51. 1 ml of this suspension was distributed into four sterile tubes and to each of these, 4 ml of sterile distilled water, containing different quantities of EMS, was added so that the final concentrations of EMS were 0.05, 0.25 and 0.5%. The tubes were shaken for 30 min at room temperature. The treated cells were aseptically washed thrice with sterile distilled water. Treated cell suspensions were serially diluted and plated on nutrient agar plates. The plates were incubated at 24 °C for 24 h. Single colonies were isolated and screened for the production of PVA. The colonies were designated as UREMS and numbered sequentially.

*Enzyme assay.* The cells collected from 5 ml of broth were resuspended in 5 ml of 0.1 M phosphate buffer (pH 6.5). 0.5 ml of this suspension was mixed with 0.5 ml of 0.1 M phosphate buffer (pH 6.5) and 1.0 ml of potassium penicillin V solution (40 mg/ml) in 0.1 M phosphate buffer (pH 6.5). The reaction mixture was incubated for 30 min at 40 °C under gentle shaking so that the cells remained in suspension. At the end of the incubation, the reaction was terminated by addition of 1.0 ml of 2.0 M acetate buffer (pH 3.5). The reaction mixture was centrifuged and an aliquot (0.5 ml) was processed for estimation of 6-APA by *p*-dimethylaminobenzaldehyde method [14].

For studying the effect of solvents, the harvested cells were suspended in 0.1 M phosphate buffer (pH 6.5) so that the absorbance of the suspension at 625 mm was 3.0 and 0.5 ml of this suspension was used for the assay.

One unit of enzyme activity is the amount of enzyme required for production of 1 μmol of 6-APA per min.

*PVA assay in presence of solvents.* The solvents were incorporated into the reaction mixture prior to the addition of penicillin V solution. The volume of buffer was corrected for the added solvent so that the final volume of the reaction mixture remained 2.0 ml. The tubes were stoppered during incubation.

## RESULTS AND DISCUSSION

The objectives of a strain improvement program of penicillin acylase-producing cultures, in general, are to isolate: β-lactamase negative mutants, constitutive producers, mutants resistant to catabolite repression and strains producing greater quantities of penicillin acylase

TABLE 1

Effect of glucose and sodium glutamate on PVA production by *B. indica* var. *penicillanicum* SKB 3133 and mutant UREMS-5

| Medium | SKB 3133 | | | | UREMS-5 | | | |
|---|---|---|---|---|---|---|---|---|
| | Final pH | Growth (g/l) | Activity (IU/ml) | Specific production (IU/g DCW) | Final pH | Growth (g/l) | Activity (IU/ml) | Specific production (IU/g DCW) |
| Medium III | 9.0 | 4.5 | 0.41 | 91 | 9.1 | 5.1 | 0.75 | 146 |
| + Glucose (0.3%) | 8.3 | 4.2 | 0.36 | 85 | 8.7 | 5.8 | 1.25 | 215 |
| + Glucose (1.0%) | 8.0 | 3.4 | 0.28 | 82 | 8.6 | 6.0 | 1.08 | 180 |
| + Na Glutamate (1.0%) | 9.1 | 4.8 | 0.40 | 83 | 8.9 | 5.5 | 1.00 | 182 |
| + Glucose (0.3%) + Na Glutamate (1.0%) | 9.1 | 5.2 | 0.47 | 90 | 9.0 | 7.6 | 1.50 | 197 |

The experiments were repeated thrice with similar results.

[3]. *B. indica* var. *penicillanicum* produces PVA constitutively and is a non-producer of β-lactamase [18]. We, therefore, have attempted to isolate a hyper-producing mutant that is resistant to catabolite repression. Since our purpose was to obtain mutants of a specific type and not to determine mutation frequency in relation to dosage, wave length or other variables, we did not make exact measurement of dosage or mutation rate.

*Isolation of mutants*

The parent strain *B. indica* var. *penicillanicum* 3133 producing 91 IU/g dry cell weight was mutated using physical mutagenic treatments such as UV treatment, γ-irradiation and chemical mutagenesis using EMS in series to isolate hyper-producing mutants. Of the 123 colonies isolated after UV treatment, U-10 (isolate from 10 min exposure) showed specific production of 113 IU/g dry cell weight and was selected for further mutagenesis by γ-radiation. Three-hundred-and-seventy-five colonies were isolated after radiation with γ-rays for 5, 10 and 15 min. The mutant UR-51 (isolate from 15 min exposure) showed specific production of 129 IU/g dry cell weight. The mutant UR-51 was further treated with EMS at 0.05, 0.25 and 0.5% concentrations. Two-hundred-and-fifty colonies were isolated and the mutant UREMS-5 (isolated from 0.05% EMS treatment) showed maximum specific production of 146 IU/g dry cell weight. Thus, a 60% increase in specific production of PVA was achieved by the mutation program.

*Properties of the UREMS-5 mutant and parent strain*

Penicillin acylases, in general, are catabolically repressed by glucose [13]. We have compared the synthesis of PVA by SKB-3133 and mutant UREMS-5 as a function of glucose concentration (Table 1). PVA production by parent strain SKB-3133 was repressed by incorporation of glucose in the medium. Addition of sodium glutamate reversed the effect. On the other hand, production of PVA by UREMS-5 was increased by 66 and 44% with the addition of glucose at 0.3 and 1.0%, respectively. Thus, the mutant UREMS-5 was resistant to catabolite repression by glucose. Incorporation of sodium glutamate enhanced the enzyme production further to 1.5 IU/ml (Table 1). However, the specific production decreased from 215 to 197 IU/g dry cell weight due to higher growth.

*Effect of vegetable oils on PVA production by mutant UREMS-5*

During our studies with *Kluyvera citrophila*, it was observed that certain vegetable oils increased the penicillin G acylase production (unpublished results). Incorporation of groundnut, soyabean and olive oil at 0.6% stimulated the PVA production by 6.6, 40 and 46.6%, respectively (Table 2). Highest specific production of 244 IU/g dry cell weight which is 168% more than the parent strain was achieved with olive oil. The stimulatory effect is probably due to the fatty acids present in the oil since 9.64% increase in PVA production was observed by incorporation of oleic acid.

TABLE 2

Effect of vegetable oils on the PVA production by mutant UREMS-5

| Vegetable oil (0.6%) | Final pH | Growth (g/l) | Activity (IU/ml) | Specific production (IU/g DCW) |
|---|---|---|---|---|
| Nil[a] | 9.0 | 7.6 | 1.5 | 197 |
| Groundnut oil | 8.9 | 7.7 | 1.6 | 208 |
| Soyabean oil | 8.9 | 9.1 | 2.1 | 231 |
| Olive oil | 9.0 | 9.0 | 2.2 | 244 |
| Oleic acid | 8.8 | 8.8 | 1.9 | 216 |

[a] Medium III + glucose, 0.3% + Na glutamate, 1.0%.
The experiments were repeated thrice with similar results.

Production of PVA by various organisms reported in the literature is summarized in Table 3. It is evident that the *B. indica* var. *penicillanicum* UREMS-5 is an excellent source of PVA. It produces 103% higher PVA than does *P. ostreatus* NRRL 3824 [16]. We are not aware of any

TABLE 3

Comparison of PVA production by various organisms

| Organism | PVA (IU/ml) | Specific production (IU/g DCW) | Reference |
|---|---|---|---|
| *Beijerinckia indica* var. *penicillanicum* | | | |
| UREMS-5 | 2.2 | 244 | |
| *Bacillus sphaericus* | | | |
| NCTC 10338 | 0.09 | NA[a] | 1 |
| *Fusarium* | | | |
| *avenaceum* | NA | 9.8 | 21 |
| *Pleurotus ostreatus* | | | |
| *(Bovista plumbea)* | | | |
| NRRL 3501 | NA | 24.67 | 12 |
| NRRL 3824 | NA | 70 | 15 |
| NRRL 3824 | NA | 120 | 16 |
| *Pseudomonas acidovorans* | | | |
| isolate E | 0.5–0.71 | NA | 7 |

[a] Not available.

report in the published literature of cultures producing such large amounts of PVA. The mutant UREMS-5 has retained its increased productivity and has not reverted for over 1 year with frequent subculturing on nutrient agar.

*Effect of organic solvents on hydrolysis of penicillin V*

Microbial cells containing PVA have been used for conversion of penicillin V to 6-APA [19]. One of the major factors that limits the rate of reaction when whole cells are used catalytically is the permeability of substrate and products. It has been demonstrated that the treatment of *Gluconobacter melanogenus* cells with certain solvents stimulated the conversion of L-sorbose to L-sorbosone [8]. The effect has been attributed to the increased permeability of cell membrane. Addition of small volumes of toluene to *Escherichia coli* K 12 cells leads to faster equilibrium of intracellular contents of small molecules with any given extracellular concentration [3]. Aspects of biocatalytic systems in organic media were recently reviewed [6]. Prerequisites for the utilization of any solvent system is that the solvent should not alter the enzyme conformation nor should it hinder the enzyme-substrate interactions. We have studied the effect of a wide range of solvents on the rate of penicillin V hydrolysis by *B. indica* var. *penicillanicum* UREMS-5. The solvents were incorporated so that the final concentration of solvent in the reaction mixture was 1.0%. The results are summarized in Table 4. Methanol, propanol, isopropyl alcohol, methylene chloride, chloroform, carbon tetrachloride, acetone, ethyl acetate, benzene, toluene, hexane, methyl cellosolve, acetonitrile and acrylonitrile stimulated the rate of penicillin V hydrolysis to varying degrees. All other solvents reduced the rate of penicillin V hydrolysis. In particular *o*-oxylene, dioxane, formaldehyde and diethyl sulphate reduced the rate of hydrolysis by more than 90%. A stimulation of penicillin V hydrolysis greater than 30% was observed with methylene chloride, chloroform and carbon tetrachloride. The stimulatory effect of these solvents was studied as a function of their concentration in the reaction mixture (Fig. 1). It should be noted that chloroform at 0.5% concentration stimulated the rate of hydrolysis by 84% and at 2.0% and above, a toxic effect, decreasing the rate of reaction, was observed. Maximum stimulation with methylene chloride and carbon tetrachloride at 1.0% concentration was 79 and 92%, respectively. At higher concentration the stimulatory effect of both the solvents was reduced.

The stimulatory effect of these solvents can be attributed to one or more of the following factors: (i) increased cell wall permeability for penicillin V, 6-APA and phenoxyacetic acid thereby decreasing the diffusional limitations; (ii) increased rate of catalysis by PVA itself; (iii) increase in the rate of Schiff's base formation of 6-APA and *p*-dimethylaminobenzaldehyde during estimation of 6-APA [14] thereby reflecting a higher rate of reaction.

Methylene chloride, chloroform and carbon tetrachloride when incorporated at 1.0% concentration to the reaction mixture for 6-APA estimation [14] did not alter the color development i.e. the rate of Schiff's base formation. The effect of these solvents on soluble PVA (prepared by disruption of cells of *B. indica* var. *penicillanicum*

TABLE 4

Effect of solvents on the hydrolysis of penicillin V by whole cells of *B. indica* var. *penicillanicum* UREMS-5

| Solvent (1%) | Relative hydrolysis (%) |
|---|---|
| Nil | 100 |
| Methanol | 124 |
| Ethanol | 81 |
| Propanol | 116 |
| *n*-Butanol | 89 |
| *n*-Heptanol | 34 |
| Isopropyl alcohol | 125 |
| Benzyl alcohol | 25 |
| Amyl alcohol | 70 |
| Isoamyl alcohol | 42 |
| Ethylene glycol | 98 |
| Polyethylene glycol | 91 |
| Methylene chloride | 179 |
| Chloroform | 136 |
| Carbon tetrachloride | 192 |
| Acetone | 123 |
| Ethyl acetate | 110 |
| Ethyl acetoacetate | 71 |
| Diethyl ether | 80 |
| Butyl acetate | 87 |
| Benzene | 123 |
| Toluene | 119 |
| Hexane | 114 |
| Cyclohexinon | 12 |
| Pyridine | 55 |
| *o*-Xylene | 7 |
| Glycerine | 90 |
| *o*-Dichlorobenzene | 38 |
| Methyl cellosolve | 121 |
| Dimethylsulfoxide | 94 |
| Dioxane | 4 |
| Formaldehyde | 6 |
| Formamide | 96 |
| Acetonitrile | 108 |
| Acrylonitrile | 123 |
| Diethyl sulphate | 1 |

The experiments were repeated thrice with similar results.

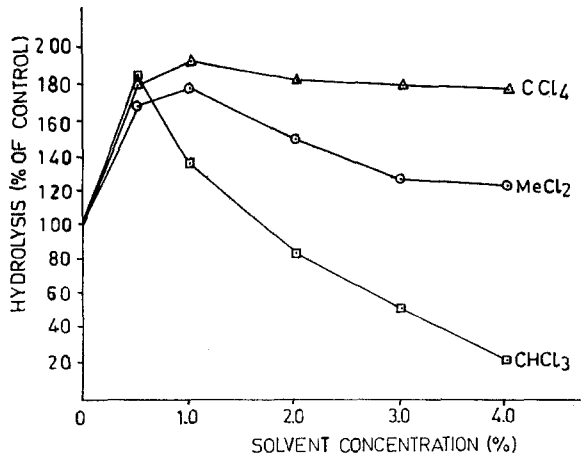


Fig. 1. Effect of solvent concentration on the hydrolysis of Pen V by mutant UREMS-5. Symbols: ○, methylene chloride; □, chloroform; △, carbon tetrachloride.

UREMS-5) and whole cells is compared in Table 5. Methylene chloride and carbon tetrachloride increase the activity of soluble PVA by 25 and 40% whereas chloroform inhibited the soluble PVA activity by 40%. However, when whole cells were used, the effect of all these solvents was amplified and the PVA activity was increased by 79, 36 and 92% with methylene chloride, chloroform and carbon tetrachloride, respectively. Increase in the rate of acylation by penicillin acylases with solvents like polyethylene glycol, methanol, ethanol, 2-propanol and acetone has been documented [5,9,10]. The enhanced effect of methylene chloride, chloroform and carbon tetrachloride in increasing the PVA activity of whole cells of *B. indica* var. *penicillanicum* UREMS-5 may be attributed to increased permeability of cells towards penicillin V, 6-APA and phenoxyacetic acid and altered enzyme catalysis.

TABLE 5

Effect of solvent on the hydrolysis of penicillin V by soluble PVA and whole cells of mutant UREMS-5

| Solvent | PVA activity (%) | |
|---|---|---|
| (1.0%) | Soluble PVA | Whole cells |
| Nil | 100 | 100 |
| Methylene chloride | 125 | 179 |
| Chloroform | 60 | 136 |
| Carbon tetrachloride | 140 | 192 |

The experiments were repeated thrice with similar results.

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