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Effect of organic solvents on cell-bound penicillin V acylase activity of *Erwinia aroideae* (DSMZ 30186): A permeabilization effect

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ABSTRACT

Erwinia aroideae (DSMZ 30186) is a potential microbial culture to produce intracellular penicillin V acylase (PVA). The whole cell PVA activity was improved by permeabilization with various organic solvents. The cell-bound PVA activity showed an eightfold increase upon treatment with chloroform (5 $\mu L/mg_{dry\ biomass}$) for 10 min and diethyl ether (10 $\mu L/mg_{dry\ biomass}$) for 45 min. Hexane, toluene, ethyl acetate and dichloromethane enhanced the enzyme activity up to two-, six-, four- and two-fold, respectively; whereas, PVA activity declined drastically on permeabilization with acetone, pyridine and alcohols. The physicochemical properties of the organic solvents used for permeabilization were correlated with the change in activity. It was found that solvents with high hydrophobicity ($\log P > 0.68$) and lower dielectric constant (<9) were relatively effective in increasing PVA activity. These results allow systematic selection of suitable solvent for best performance.

1. Introduction

Gram-negative bacteria possess an outer membrane (OM) in addition to the plasma membrane. It functions mainly as a protective layer to prevent entry of toxic substances into the cell. The OM forms a proficient barrier against hydrophilic macromolecules and hydrophobic substances due to a lipopolysaccharide layer on the membrane surface. However, many detergents such as triton X-100 [1], tweens [2], *N-cetyl-N,N,N*-trimethylammoniumbromide (CTAB) [3] and organic solvents [4] are capable of disrupting the integrity of the OM. OM resists the movement of substrate and product by imposing limits on diffusion, however the drawback can be circumvented by permeabilizing the cells. Permeability issues in gram-negative bacteria have been recently discussed by Chen [5]. Intracellular enzyme activity of wild-type microorganisms as well as recombinant strains can be enhanced, and used, by cell permeabilization. It is an easy method to improve cell-bound activity keeping the viability of the cell intact. Toluene, chloroform and other organic solvents have been used successfully for permeabilization leading to enhancement in the enzyme activity in bacteria and yeast cells [4,6,7]. Water-in-hexane *macro*- and microemulsions stabilized by sodium *bis*-(2-ethylhexyl) sulfosuccinate (AOT) were used for selective permeabilization of *Escherichia coli* cells to extract and purify penicillin acylase [8]. Cell permeabilization with organic solvents is a straightforward method to accelerate *in vivo* hydrolysis of substrate. However, little information is available on the effects of physicochemical properties of organic solvents on the cell permeabilization process. Most of the attempts involve selection of suitable solvent by trial and error method. Here we have explored the factors that affect the performance of organic solvents in permeabilizing gram-negative cells.

Penicillin acylases (E.C. 3.5.1.11) are used to produce 6-aminopenicillanic acid (6-APA), which is the starting molecule for the synthesis of clinically useful penicillins [9,10]. About 85% of 6-APA is produced enzymatically using penicillin G acylases (PGA), and only 15% by penicillin V acylases (PVA) [11]. Although media standardization, optimization of cultural conditions and genetic engineering methods have been used to improve PVA production, higher PVA activity is still required to produce commercially striking biocatalysts [12]. Periplasmic penicillin G acylase activity from *E. coli* was correlated with physicochemical properties of solvents recently [6], similar studies conducted for penicillin V acylase, as both belong to the same group of enzymes, to support the previous PGA report.

In the present work, we are reporting the effect of various organic solvents on whole cell PVA activity of *Erwinia aroideaea*; and enhancement in activity, due to permeabilization, also correlated with physicochemical properties of the solvents, such as dielectric constant and hydrophobicity ($\log P$). Effect of solvents to cell

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biomass ratio and kinetics of permeabilization were also studied to develop a commercially attractive biocatalyst.

2. Materials and methods

2.1. Materials

Penicillin V potassium salt was a kind gift from Hindustan Antibiotics Pune, India. *Para*-Dimethylaminobenzaldehyde (Himedia, India) and organic solvents (Qualigens, India) were of analytic grade.

2.2. Microorganism and growth conditions

Standardization of fermentation media and cultural conditions such as incubation period and temperature, initial pH of media as well as dispensing volume of medium for the production of penicillin V acylase from *E. aroideae* (DSMZ 30186) was carried out. *E. aroideae* cells were grown in nutrient broth (NB) with sodium glutamate (g/L, peptone 10, beef extract 10, NaCl 5 and sodium glutamate 10, pH 7.0) and incubated at 28 °C for 36 h with shaking (200 rpm) in 250 mL Erlenmeyer flasks containing 50 mL of medium. Cells were harvested by centrifugation at 10,000 rpm for 10 min, washed twice with 0.1 M sodium phosphate buffer pH 7.0 and resuspended in the same buffer and used for permeabilization. All the experiments were repeated three times with the freshly grown cells for the reproducibility of results.

2.3. Permeabilization with solvents

The amount of cell suspension required to obtain 10–20 mg biomass was centrifuged at 10,000 rpm for 3 min; the pellet was resuspended in required amount of (between 5 and 100 $\mu L/mg_{dry\ biomass}$) organic solvent and thoroughly mixed by vortexing. The suspension was incubated at room temperature for 15 min. Cells were harvested by centrifugation for 5 min at 10,000 rpm and 4 °C, and resuspended in 0.1 M sodium phosphate buffer pH 7.0. Treated cells were analyzed for PVA activity by standard enzyme assay; untreated cells served as control.

2.4. Analytical methods

Cell-bound penicillin V acylase activity was determined by the method of Bomstein and Evans [13], as modified by Shewale et al. [14], measuring the amount of 6-APA formed at 40 °C, employing 2% (w/v) solution of penicillin V, potassium salt, in 0.1 M sodium phosphate buffer pH 6.0. The 6-APA formed was estimated using 6% (w/v) *p*-dimethylaminobenzaldehyde (PDAB) in methanol. One unit (IU) of PVA activity is defined as the amount of enzyme that produces 1 μmol 6-APA per minute under the conditions defined. The cells were incubated for 20 min, under the assay conditions, to measure the enzyme activity, and the product–time relationship was linear up to the mentioned period of time.

Biomass concentration was determined from optical density measurements at 600 nm and converted to dry weight with a standard curve. The biomass represented here is dry biomass of cells and enzyme activity represented here in IU/g dry biomass. All the experiments were repeated three times with the freshly grown culture and the values reported here in tables and figures represent their mean value.

3. Results and discussion

3.1. Permeabilization with organic solvents

Results obtained from the treatment of *E. aroideae* cells with organic solvents are depicted in Table 1. Eleven different organic solvents were used to permeabilize *E. aroideae* cells. PVA activity was positively influenced by xylene, hexane, toluene, chloroform, diethyl ether, ethyl acetate and dichloromethane when the cells were incubated with the solvent at 20 $\mu l/mg_{dry\ biomass}$ concentration for 15 min. In contrast, solvents such as pyridine, acetone and alcohols (ethanol and methanol) drastically decreased the PVA activity. Various amounts and time intervals were used to screen the enhancement pattern of solvents and finally 20 $\mu l/mg_{dry\ biomass}$ and 15 min time interval was selected for data interpretation.

Enzyme activity was increased by xylene on incubation for 2 min only; further incubation led to inhibition of enzyme activity. Hexane, toluene, chloroform, diethyl ether, dichloromethane and ethyl acetate continued to enhance activity constantly even after 2 min incubation (Table 1). Further standardization of permeabilization conditions was carried out with those solvents, which enhanced cell-bound activity.

No activity was detected in the supernatant of the reaction mixture indicating that there was no leakage of enzyme from the cells into the external medium during permeabilization. Effect of temperature, on permeabilization of cells, was studied in the range of 4–37 °C, however there was no distinct effect of temperature on permeabilization of *E. aroideae* cells to enhance whole cell PVA activity. Consequently, all subsequent experiments were carried out at room temperature (25 °C).

There are many reports in which physicochemical properties of solvents are related to effects on the activity and stability of free enzymes [15–18]. However, little information is available on the effect of solvents on enzyme activity during cell permeabilization processes. De Leon et al. [6] reported permeabilization of recombinant *E. coli* cells expressing PGA using organic solvents and showed 380% increase in enzyme activity. Krishnan et al. [7] reported a threefold increase in lactate dehydrogenase activity of *Lactobacillus plantarum* cells permeabilized with 1% (v/v) diethyl ether (0.1 $\mu l/mg_{dry\ biomass}$ approximately); toluene and toluene–ethanol methods produced lesser improvements.

3.2. Effect of dielectric constant and hydrophobicity of solvent

It has been reported that electrostatic forces affect protein structures and their functionality [15,18,19]. Correlation between dielectric constant values of solvents and change in PVA activity is shown in Fig. 1. Permeabilization of *E. aroideae* cells with solvents of dielectric constant lower than 9 significantly increased PVA activity. Dielectric constant represents the dipole moment of the solvent molecules and directly affects the flexibility of proteins [20]. The whole cell PVA activity of *E. aroideae* was enhanced more than fourfolds, by the solvents exhibiting dielectric constant in between 2.0 and 6.0; and the highest enhancement (728%) was observed in case of chloroform, dielectric constant 4.8. PVA activity was dropped sharply by the solvents exhibiting dielectric constant more than 9.1. Penicillin G acylase activity from *E. coli* was enhanced by the solvents with dielectric constant <5 [6]. In general, enzymes are known to have a hydration shell. In reaction mixtures containing water-miscible organic solvents, distortion of the hydration shell caused by introduction of organic solvent into the enzyme solution upsets the system of interactions supporting the native conformation, which results in the loss of catalytic activity [21]. Therefore, inhibition observed in case of pyridine, acetone and alcohols was probably due to denaturation of the enzyme. Affleck et al. [15] have