Chapter 6

Application of immobilized penicillin V acylase system for 6-APA production
Chapter 6: Immobilization of PaPVA

6.1. Introduction:

Penicillin acylases have been used in the pharmaceutical industry since their discovery for the production of 6-APA and semi-synthetic antibiotics. Beta-lactam antibiotics (penicillins and cephalosporins) make up over 65% of the world market for antibiotics (Elander 2003). Significant advances have occurred in the last two decades, in the use of enzymes as a greener and inexpensive approach (Chandel et al. 2008) to meet the requirements for bulk antibiotic production. PGA produced by *E. coli* has been employed extensively to produce 6-APA (Maresova et al. 2014) and semi-synthetic antibiotics including ampicillin, amoxicillin, cephalothin and so on. Strain improvement, media optimization techniques and the use of thermostable PGAs (*Alcaligenes faecalis*, Verhaert et al. 1997) have helped enhance the efficiency of application of penicillin acylases in the pharmaceutical industry. In addition, PGAs have also been applied in peptide synthesis and resolution of racemic mixtures (Arroyo et al. 2003).

On the other hand the use of PVAs in the production of 6-APA has been very limited, probably owing to the slightly higher cost of Pen V substrate and the non-availability of highly active enzymes so far. Nevertheless, some authors have emphasized the advantages of using a Pen V-PVA system for 6-APA production (Shewale and Sudhakaran 1997). Pen V has greater stability in aqueous solutions, especially at lower pH required for extraction. PVAs also possess higher activity in acidic pH range and show better conversion efficiency at higher substrate concentrations. These characteristics emphasize the need for increased application of PVAs in the industry. Presently, most of the 6-APA is produced using PGAs, and the Pen V – PVA system only accounts for 10-15% of the total 6-APA production.

For industrial applications, free enzymes or whole cells are usually immobilized on various supports. Enzymes and cells can be immobilized through a variety of techniques (Matiasson 1983) including adsorption, encapsulation and covalent cross-linking. Different supports such as alginate, chitosan, polyvinyl alcohol, mesoporous silica, ion-exchange resins and so on, have been applied for immobilizing cells or enzymes. The alginate – CaCl$_2$ system has been one of the most widely used and mild, inexpensive technique for the immobilization of living cells and enzymes (Zhou et al. 2010). Immobilization protects the enzymes from changes in process...
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conditions such as pH, temperature and mechanical shear, and enhances their stability (Sheldon 2007). Immobilized enzymes and cells can also be reused a number of times with minimal damage to their conversion efficiency. Other techniques such as cell permeabilization and cross-linking with glutaraldehyde or polyethylene imine (PEI) serve to further enhance the activity and stability of the enzymes (Felix 1982, Prabhune et al. 1992).

While a plethora of immobilized PGA systems and better downstream processing steps have been developed over the years, not much advances have been associated with the application of PVA-Pen V system in the industry. This chapter details the attempts to immobilize the recombinant E. coli cells producing PaPVA and develop this system for maximum conversion of Pen V to 6-APA.

6.2. Materials and methods:

6.2.1. Materials:

Sodium alginate and media components were procured from HiMedia, India. Penicillin V (potassium salt) was a gift from Sparsh Biotech, Ahmedabad (India). Cetyltrimethyl ammonium bromide (CTAB) was obtained from Qualigens, India and all other chemicals were of analytical or HPLC grade.

6.2.2. Cloning and expression of PaPVA enzyme:

The pva gene from P. atrosepticum was cloned into a pET28b plasmid vector between NcoI and XhoI restriction sites. The PVA enzyme was expressed in E. coli BL21 star cells with a C-terminal His-tag as described in Chapter 2.

6.2.3. Cultivation of E. coli – PaPVA cells:

For maximum production of biomass, the cells were cultivated in Studier’s (2005) auto-induction medium ZYM-5052. After 4 h incubation at 37°C (OD600 ~ 1.0), the culture was transferred to 27°C for 18 h. The resultant biomass was harvested by centrifugation and washed with phosphate buffered saline (PBS) pH 7.2.
6.2.4. PVA activity assay and biotransformation:

Pen V hydrolysis activity was estimated by studying the formation of Schiff’s conjugate with the product 6-APA and p-dimethyl amino benzaldehyde (Shewale et al. 1987). One unit (IU) of enzyme activity was defined as the amount of enzyme producing 1 μmol 6-APA in 1 min under standard conditions (pH 5, 45°C). The biotransformation was carried out in a lab-scale bioreactor, with a double-jacketed vessel connected to a water bath, with 20mg free cells or corresponding amount of beads in 50 ml of Pen V (2% w/v).

6.2.5. Permeabilization of E. coli – PaPVA cells:

E. coli – PaPVA cells were permeabilized using 0.1% (w/v) cetyl triethyl ammonium bromide (CTAB) detergent (20 mg wet cells/ml) with a treatment time of 15 min at 25°C. The cells were then washed twice with distilled water to remove excess CTAB.

6.2.6. Immobilization of E. coli – PaPVA cells:

Freshly harvested cells from 50 ml overnight culture were permeabilized with CTAB, and added (550 mg wet weight) to appropriate volume of 2% (w/v) sodium alginate. The suspension was extruded dropwise through a thin needle into ice-cold solution of 0.2 M CaCl₂·2H₂O while stirring gently. The calcium alginate beads formed (1.5-2 mm diameter) were allowed to harden in the same solution for 1h and stored in CaCl₂ solution at 4°C for 18h. The beads were washed with distilled water and treated with 0.2% (w/v) glutaraldehyde for 30 min, giving a cell loading of 10% w/w (wet cell mass/ weight of beads). They were then washed thoroughly with distilled water and stored in 50mM CaCl₂ at 4°C, for use in the biotransformation reaction.

6.2.7. Optimization of reaction parameters:

In separate experiments, the biotransformation of penicillin V to 6-APA was carried out using different specified temperatures, pH values, biocatalyst concentration, and the initial substrate concentration to optimize the reaction parameters to achieve maximum conversion efficiency. Biotransformation was carried out using 5 g alginate beads (with a cell loading of 100 mg/g beads) in 50 ml of Pen V with
constant stirring at 200 rpm. Aliquots (0.5 ml) were withdrawn at regular time intervals and amount of 6-APA was determined.

6.2.8. Stability and Recyclability of the immobilized system:

The storage stability of alginate beads with immobilized cells was estimated by determining the conversion of Pen V to 6-APA after different time intervals. Beads (5g, with 0.5 g wet cell weight) were either stored wet in 50 mM CaCl\(_2\) solution at 4°C or dried to constant weight at room temperature and stored dry. The reusability of the immobilized system was assessed by carrying out the hydrolysis of 4 % (w/v) Pen V (in 0.1 M acetate buffer pH 5) at 35°C. After each cycle of hydrolysis (1 h), the solution with the reaction products was removed; the beads were taken out and re-hardened in 0.2M CaCl\(_2\) for 1 h. The next cycle was then started with fresh substrate. 6-APA yield was calculated for each cycle.

6.3. Results and Discussion:

6.3.1. Cultivation of E. coli – PaPVA:

As described in Chapter 2, PaPVA has been expressed in E. coli with high protein yields over 250 mg/l on LB supplemented with kanamycin. To further enhance the cell mass and enzyme productivity, E. coli – PaPVA was grown in Studier’s (2005) auto-induction media (AIM, ZYM-5052).

| Table 6.1. Enzyme productivity of E. coli – PaPVA on different media. |
|-------------------|-----------------|---------|-------------------|-------------------|
| Medium            | Kanamycin (µg/ml) | IPTG (mM) | Incubation time (h) | PVA Activity (IU/g) | Cell mass (g/L) | Productivity (IU/L) |
| LB                | 35              | 0.2       | 24                 | 1959              | 9.2             | 18023               |
|                   |                 |           | 48                 | 1393              | 8.6             | 11910               |
| Terrific broth    | 100             | 0.2       | 24                 | 1879              | 17.0            | 31849               |
|                   |                 |           | 48                 | 2015              | 17.4            | 35061               |
| AIM               | 100             | -         | 24                 | 2511              | 20.7            | 51852               |
|                   |                 |           | 48                 | 2697              | 19.2            | 51782               |
AIM provides a better supply of nutrients and microelements for the bacteria to grow, resulting in higher cell densities. In addition, the presence of lactose negates the need for use of IPTG to induce recombinant protein production. In the case of PaPVA, growth in AIM provided a cell mass of 20.7 g/l with 51852 IU/L enzyme productivity within 24 h (Table 6.1). This could be easily scaled up using a fermentor and high aeration rates for application of recombinant PaPVA enzyme in the pharmaceutical industry.

6.3.2. Permeabilization of E. coli – PaPVA cells:

The use of whole cells for biotransformation circumvents the problem of purification and provides a more favourable environment for the enzymes to function (Babu and Panda 1991). Additionally, immobilization of cells could facilitate product separation and also make the cells recyclable, thus simplifying the process and lowering the cost of production. However, it also suffers from the disadvantage of relatively lower activity compared to free enzymes, as a result of reduced diffusion of reactants or products through the cell membrane (Wang et al. 2012). Permeabilization involves the use of chemical agents to slightly weaken the membrane, thereby alleviating this problem (Felix 1982).

![Cell-bound PVA activity of E. coli – PaPVA cells permeabilized with different detergents (0.1% w/v) or organic solvents (1ml/20 mg cells) for 15 min.](image)

**Fig. 6.1.** Cell-bound PVA activity of *E. coli* – PaPVA cells permeabilized with different detergents (0.1% w/v) or organic solvents (1ml/20 mg cells) for 15 min.
In this study, a variety of detergents and solvents were explored for the permeabilization of *E. coli* cells expressing *PaPVA*. Cationic detergent CTAB significantly increased in the cell bound PVA activity by 14.4 fold, while certain solvents like dichloromethane and ethylacetate showed a moderate increase in PVA activity (Fig. 6.1). Kumar et al. (2008) have reported the enhancement of cell-bound PVA activity in yeast *Rhodotorula aurantiaca* using CTAB. Many other studies (Prabhune et al. 1992, Nagalakshmi and Pai 1994, Norouzian et al. 2002, Cheng et al. 2006) have identified CTAB as a mild but potent permeabilizing agent for enhancing PGA activity in *E. coli*. The free permeabilized *E.coli – PaPVA* cells exhibited 36,700 IU/g cell bound activity on treatment with 0.1% (w/v) CTAB for 15 min.

**6.3.3. Encapsulation of *E. coli – PaPVA* cells in alginate:**

While there has been a perennial interest in the immobilization of PGA as pure enzyme or cells (Cheng et al. 2006), there are very few reports of PVA based immobilized enzyme systems. Initial commercial preparations that have been employed in the industry include Novozyme 217 (Gestrelius 1982, Karlsen and Villadsen 1984) and Semacylase (Mollgaard 1987, Mollgaard and Karlsen 1988) by Novo Industri, Denmark. Sudhakaran and Shewale (1993) have immobilized the partially purified PVA enzyme from *Fusarium* sp. SKF 235 on a cation exchange resin (Amberlite CG-50) with a specific activity of 250-280 IU/g beads. Another study (Torres-Bacete et al. 2000) details the covalent immobilization of PVA from *Streptomyces lavendulae* on Eupergit C epoxy-activated acrylic beads.

However, the application of immobilized PVA is scarce probably owing to the higher substrate costs and non-availability of high activity enzyme preparations. The development of a commercial PVA/Pen V based enzyme process for 6-APA production has been stagnant over the last decade. This study was an attempt to develop an immobilized system using *E. coli – PaPVA* cells as a preliminary step for industrial application of this enzyme. The elevated specific activity of *PaPVA* and high recombinant enzyme yields make it an attractive proposition for its industrial use. Initial trials were made to immobilize the extracted and purified enzyme by covalent cross-linking with glutaraldehyde to form cross-linked enzyme aggregates, or with epoxy polymer beads (DILbeads, Fermenta Biotech, Thane, India). Such
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Attempts suffered from low recovery of enzyme activity (<10%) and reduction in enzyme stability. Hence, the encapsulation of whole cells was preferred. *E. coli – PaPVA* whole cells were permeabilized by treatment with CTAB and encapsulated in 2% calcium alginate hydrogel for biotransformation (Fig. 6.2). Further, the cells were cross-linked with 0.2% (w/v) glutaraldehyde for 30 min. Glutaraldehyde treatment has been reported to enhance the stability of the immobilized cell system by preventing enzyme leakage (Prabhune et al. 1992, Cheng et al. 2006).

![Calcium alginate beads with encapsulated E. coli – PaPVA cells.](image)

Comparative biotransformation studies with 2% (w/v) Pen V showed that permeabilized free cells gave a faster initial reaction rate and consequently a higher yield of 6-APA after reaction for 40 min as against immobilized preparation (Fig. 6.3). This might be attributed to (i) lower transfer rate of substrate and product inside and out of the immobilized cells through the alginate matrix, or (ii) hydrophilic nature of alginate leading to a lower concentration of hydrophobic Pen V surrounding the immobilized cells. However, both free and immobilized cells achieved full conversion at the end of 60 min. There was a gradual decline in 6-APA yield after 60 min in the case of free cells and 90 min with immobilized cells (data not shown), possibly due to decomposition of the product.

In spite of lower initial reaction rate and slightly longer reaction time, immobilized cells could also achieve maximum yield of 6-APA equal to that of free cells. In view of their potential for reuse and better stability, immobilized cells can be generally used efficiently for the biotransformation process (Prabhune et al. 1992; Cheng et al. 2006).
Fig. 6.3. Conversion of Pen V (2% w/v) to 6-APA by permeabilized free cells (E. coli – PaPVA) and cells immobilized in alginate beads.

6.3.4. Effect of reaction parameters:

Both free and immobilized cells showed an optimum pH of 4 and optimum temperature of 45°C for maximum initial activity (Fig. 6.4). Immobilized cells showed better activity over a wider pH and temperature range compared to free cells. However, biotransformation reaction for a longer period of time under the determined optimal conditions showed lower conversion and reduction in 6-APA yield, possibly as a result of weakening of beads or inactivation of cells. Therefore, milder conditions of pH 5 and temperature 35°C were selected for the biotransformation reaction.

Fig. 6.4. Relative initial activity of free and immobilized PaPVA – E. coli cells at increasing pH and temperature. Initial enzyme activity was measured after 5 min reaction.
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The cell loading was optimum at 10% (w/w), with 5g of beads containing 0.5 g wet cell weight used for each reaction. Higher cell concentrations did not hasten the conversion, presumably due to mass transfer limitations.

The initial Pen V concentration was varied from 2-8% (w/v). The fresh weight concentration of the immobilized cells was set at 10% (w/w), and the reaction was carried out at 35°C and pH 5 for a period of 90 min. Substrate conversion profiles (Fig. 6.5) showed that almost complete conversion to 6-APA (97-100%) could be achieved till a concentration of 4% Pen V in 60 min. Further increase in initial substrate concentration progressively slowed the reaction, leading to a reduction in conversion. This could possibly be caused by the occurrence of substrate inhibition in PaPVA enzyme (explored in Chapter 2) or inhibition by the product 6-APA (Gestrelius et al. 1983). Substrate and product inhibition has been documented even in the case of immobilized penicillin G acylases (Prabhune et al. 2002; Cheng et al. 2006). Sudhakaran and Shewale (1993) have also reported an optimum concentration of 4% (w/v) Pen V for the production of 6-APA. Regardless of inhibition, it is significant that the immobilized cells were able to achieve full conversion of 4% (w/v) Pen V within 60 min, which was better than earlier reports on PVA (Sudhakaran and Shewale 1993; Gestrelius et al. 1983; Singh et al. 1988). In addition, the enhanced activity of PaPVA and high expression yields could help reduce the amount of biocatalyst required to scale up the process.

![Conversion of Pen V to 6-APA by immobilized PaPVA – E. coli cells at increasing initial concentrations (% w/v) of Pen V.](image)

Fig. 6.5. Conversion of Pen V to 6-APA by immobilized PaPVA – E. coli cells at increasing initial concentrations (% w/v) of Pen V.
6.3.5. **Storage and recyclability of immobilized system:**

The immobilized cells showed good storage stability when stored in 50 mM CaCl$_2$ at 4°C, effecting 80% conversion to 6-APA in 60 min even after 28 days (Fig. 6.6). When the beads were dried, their weight decreased to 0.2g from 5g initial weight; however, they re-swelled to 2g in water. Although drying reduced the enzyme activity to 50% conversion in 60 min, the dried beads could be stored at room temperature for longer time periods without further loss of activity (Fig. 6.6).

The recyclability of the immobilized cell system was rather low, as a set of beads could only be used for three cycles (1h each) before they became weak and started to disintegrate. This probably happens due to the action of the monovalent cations (Na, K) from the acetate buffer and Pen V (potassium salt), that are known to compete with calcium ions for guluronic acid binding sites and gradually weaken the alginate gel structure (LeRoux et al. 1999). Re-hardening of the beads in 0.2M CaCl$_2$ solution for 1h between cycles helped to an extent in retaining the activity and integrity of the immobilized cell system for at least 10 cycles (75% conversion to 6-APA).

**Fig. 6.6.** (a) Storage stability of wet beads (4°C) and dry beads (room temperature, 25 °C) containing PaPVA – *E. coli* cells. (b) Recyclability of immobilized PaPVA – *E. coli* cells without hardening, and hardening in 0.2M CaCl$_2$ for 1h after each cycle.

Torres-Bacete et al. (2000) have reported the use of immobilized S/PVVA for 50 consecutive batch reactions, while the immobilized PVA from *Fusarium* sp. could be used for 68 consecutive cycles with average 85% conversion (Sudhakaran and Shewale 1993). Although low recyclability of *E. coli* – PaPVA immobilized on
alginate compared to other PVA-based systems is a concern, it is highly probable that the use of stronger supports based on synthetic polymers like the polyvinyl alcohol-based Lentikats® (Cardenas-Fernandez et al. 2012) could improve the potential for reuse. Nevertheless, it should be emphasized that PaPVA boasts of the maximum specific activity among reported PVA enzymes, and the use of recombinant *E. coli* gives high enzyme productivity. Further, protein engineering strategies could pave a way to understand and circumvent the substrate and product inhibition mechanisms slowing down the reaction. These factors, along with the results presented in this study, make a convincing argument for the industrial applicability of the *PaPVA* based immobilized system as a viable economical alternative for the production of the active pharmaceutical intermediate 6-APA from penicillin V.