Journal of Molecular Catalysis B: Enzymatic 57 (2009) 67–71

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

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

Atul Kumar, Archana Pundle*

Division of Biochemical Sciences, National Chemical Laboratory, Dr. Homi Bhabha Road, Pune 411008, India

ARTICLE INFO

Article history:
Received 1 April 2008
Received in revised form 18 June 2008
Accepted 23 June 2008
Available online 1 July 2008

Keywords:
Penicillin V acylase
Erwinia aroideae
Permeabilization
Dielectric constant
Hydrophobicity
Organic solvents

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

* Corresponding author. Tel.: +91 20 25902239; fax: +91 20 25902648.
E-mail address: av.pundle@ncl.res.in (A. Pundle).

1381-1177/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2008.06.018

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

Table 1
PVA activity of *E. aroideae* (DSMZ 30186) cells on permeabilization with different organic solvents

Solvents	Incubation time (min)	Dielectric constants ($C^2/N\,m^2$)	Log *P* (–)	Density (g/mL)	PVA activity (% of control)
Hexane	15	1.89	3.5	0.659	221
Toluene	15	2.38	2.5	0.865	426
Xylene[a]	2	2.4	3.1	0.86	466
Diethyl ether	15	4.34	0.85	0.715	569
Chloroform	15	4.8	2	1.492	728
Ethyl acetate	15	6.0	0.68	0.894	407
Dichloromethane	15	9.1	1.2	1.326	199
Pyridine	15	12.5	0.71	0.983	56
Acetone	15	20.7	−0.23	0.791	43
Ethanol	15	24.3	−0.24	0.798	39
Methanol	15	32.6	−0.76	0.791	38
Control (water)	15	78.5		1	100

Cell-bound enzyme was assayed at 40 °C for 20 min; activity of untreated cells considered as 100% (22.5 IU/g). All experiments were repeated three times with the fresh culture and the values reported here represent their mean value. Cells were permeabilized with 20 μl/mg biomass solvents for 15 min. Densities are taken from Merck Index. Dielectric constants and log *P* values are taken from http://www.asiinstr.com/dcl.html [24] and Ref. [25], respectively: *P* is the partition coefficient for the solvent between 1-octanol and water.

[a] In case of xylene, permeabilization period was restricted to 2 min as further exposure reduced PVA activity.

demonstrated dramatically decreased motions in the vicinity of two spin-labeled amino acids (Met-192 and Ser-195), with decreasing solvent dielectric constant, a trend consistent with changes in the electrostatic force between charged residues of the protein, might be cause of inactivation of *E. aroideae* PVA by the solvents with higher dielectric constant.

Whole cell PVA activity was also correlated with the hydrophobicity of the solvents (Fig. 2). Hydrophobicity is commonly measured as log *P*, where *P* is the partition coefficient of the solvent between 1-octanol and water. Solvents with higher polarity (i.e. those with low log *P* values) drastically reduced PVA activity. However, solvents with higher hydrophobicity (log *P* > 0.68) such as chloroform, diethyl ether, toluene, xylene, hexane, dichloromethane and ethyl acetate, enhanced PVA activity of whole cells. Short chain alcohols and acetone have been shown to improve the activity of PVA from *Streptomyces lavendulae* [22]; however alcohols and acetone were found to be inhibitory in the present case. The cell-bound PVA activity of *E. aroideae* was enhanced by solvents with intermediate hydrophobicity (0.68–3.5); however the maximum enhancement of the enzyme activity observed in case of chloroform, log *P* = 2.0. Cell-bound PGA activity of *E. coli* was enhanced by the solvents with log *P* value of 0.85–3.5 [6]. Flores et al. [4] analyzed the effect of solvent on permeabilization of *Kluyveromyces lactis* cells with chloroform, toluene and ethanol on β-galactosidase activity and found that, chloroform and toluene were more efficient than ethanol.

It is interesting to note that solvents with, dielectric constant 2.0–6.0, and hydrophobicity 0.68–2.5, have shown optima for enhancing cell-bound PVA activity from *E. aroideae*; amongst all the solvents used, chloroform, dielectric constant 2.0 and log *P* = 4.8, was the best solvents to enhance whole-cell enzyme activity. Dichloromethane exhibiting higher dielectric constant and log *P* values, 9.1 and 1.2 respectively, was effective in permeabilizing and enhancing PVA activity up to 199% of control, where as it was reported to be inhibitory in case of *S. lavendulae* PVA [22].

The densities of organic solvents are usually less than 1 g/mL (Table 1), except chloroform (1.492 g/mL) and dichloromethane (1.326 g/mL), and in water-immiscible solvents organic phase remains at the top. In case of chloroform and dichloromethane the evaporation of the solvents was not observed at permeabilization conditions due to its high density, which reduces the risk due to inflammable nature of solvent and favors to scale up the process.

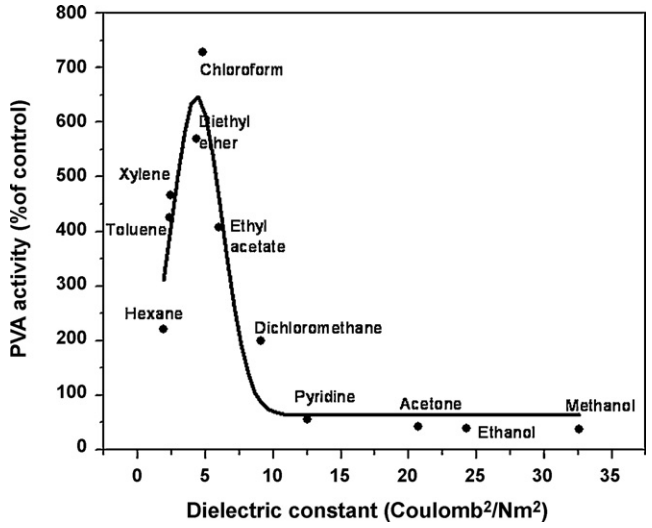

Fig. 1. Correlation between dielectric constant of organic solvents and PVA activity (% of control) of *E. aroideae*. Cells were treated with 20 μl/$mg_{biomass}$ concentration of solvent for 15 min except xylene (2 min). All experiments were repeated three times with the fresh culture and the values reported here represent their mean value.

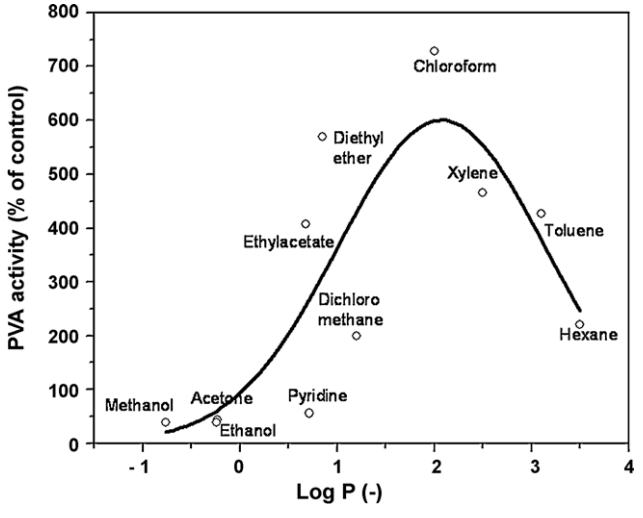

Fig. 2. Correlation between hydrophobicity (log *P*) values of organic solvents and PVA activity (% of control) of *E. aroideae*. Cells were treated with 20 μl/$mg_{biomass}$ concentration of solvent for 15 min except xylene (2 min). All experiments were repeated three times with the fresh culture and the values reported here represent their mean value.

3.3. Effect of solvent concentration

The effect of organic solvents to cell mass ratio on the enhancement of PVA activity is depicted in Fig. 3. Different concentrations (0–100 μL/$mg_{dry\ biomass}$) of selected solvents were tested to observe the effect on cell permeabilization. Different optima were obtained for each solvent, might be due to the characteristic of the solvents. PVA activity increased steeply upto 5 μL/$mg_{dry\ biomass}$ of chloroform however it decreased drastically on further addition of the solvent. Chloroform enhanced penicillin V hydrolysis at 10% concentration, the most, by *S. lavendulae* PVA [22]. A sixfold increase in PVA activity was observed when cells were incubated with diethyl ether at 5 μL/$mg_{dry\ biomass}$ concentration; PVA activity slightly decreased with the further increase in concentration. Toluene enhanced enzyme activity, sixfold, at concentration of 20 μL/$mg_{dry\ biomass}$, but further addition of solvent led to decrease in activity. In case of hexane, the maximum, 166% PVA activity, of control, was achieved at 20 μL/$mg_{dry\ biomass}$ concentration; further addition of solvent did not accelerate cell-bound PVA activity. De Leon et al. [6] have reported permeabilization of *E. coli* and demonstrated increase of PGA activity upto 380% using chloroform 30 μL/$mg_{dry\ biomass}$ while acetone and pyridine reduced and alcohols inactivated the enzyme activity. Similar results were obtained in the present study, however chloroform enhanced PVA activity at exceedingly lower concentration (5 μL/$mg_{dry\ biomass}$) than reported by De Leon et al. [6]. Diethyl ether failed to improve PGA activity in the latter case while it enhanced PVA activity up to sixfold in case of *E. aroideae* cells when incubated at 5 μL/$mg_{dry\ biomass}$ concentration. 1% ethyl ether was optimal concentration to increase lactate dehydrogenase activity in *L. plantarum* [7]. Cell-bound PVA activity was enhanced upto 421%, of control, when cells were incubated at 5 μL/$mg_{dry\ biomass}$ with ethyl acetate; however further increase in proportion of the solvent led to drastically decrease in the activity. In monophasic water–organic solvent system, enzymatic activity is normally lost at high solvent by replacement of water in the protein's hydration surface layer by organic solvent [23] that might be reason for decrease in PVA activity at higher concentration of organic solvents.

3.4. Effect of time of permeabilization

The effect of period of permeabilization for various solvents is depicted in Fig. 4. A tremendous increase in whole cell PVA activity

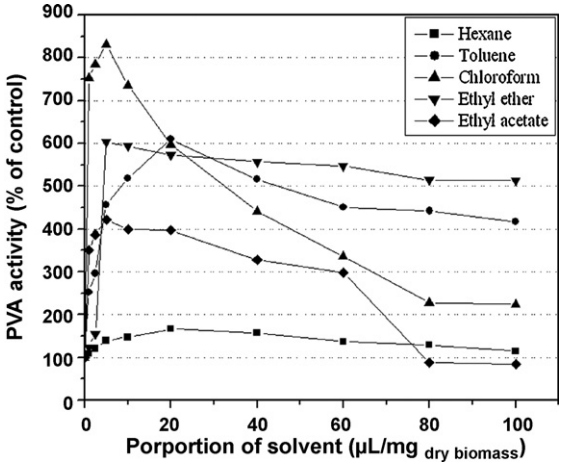

Fig. 3. Effect of solvent to cell mass ratio on PVA activity of *E. aroideae* cells. Cells were permeabilized for 15 min in a range of solvent concentrations. The activity of untreated cells considered as 100% (24.0 IU/g). All experiments were repeated three times with the fresh culture and the values reported here represent their mean value.

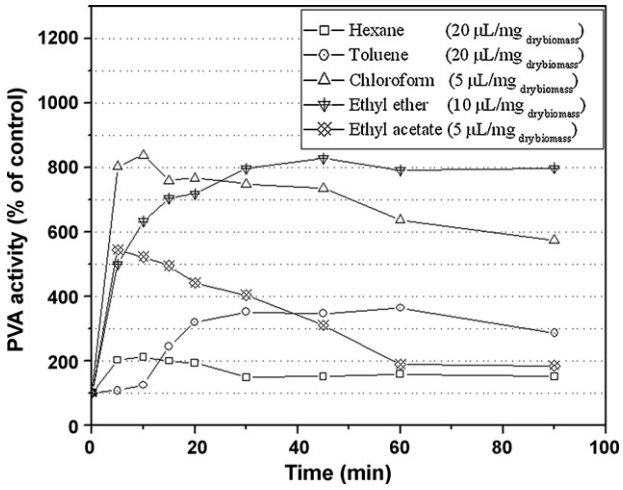

Fig. 4. Effect of permeabilization time on PVA activity of *E. aroideae* cells. Cells were incubated with the solvent for various time intervals and sampled for PVA activity under standard assay conditions. Activity of untreated cells considered as 100% (26.3 IU/g). All experiments were repeated three times with the fresh culture and the values reported here represent their mean value.

was observed when cells were incubated with the various selected solvents, for more than 30 min. The optimum concentration of each solvent, which showed maxima at the particular concentration, was used to achieve maximum possible, permeabilization effect and, enhancement in the enzyme activity. An 839% increase in PVA activity was observed when cells were exposed to chloroform for just 10 min at 5 μL/$mg_{dry\ biomass}$ concentration; and then a saturated behavior with the time was observed at further incubation, which led to decrease in the activity. Diethyl ether showed 829% PVA activity, of control, when incubated for 45 min but a slight decrease, 798% PVA activity, of control, was observed on further incubation. Similar results were obtained with hexane and toluene with 210% and 364% PVA activity, of control, respectively. Ethyl acetate was found to be effective for permeabilization of *E. aroideae*; it enhanced PVA activity up to 544%, of control, when the cells were incubated just for 5 min; the enzyme activity decreased drastically on further incubation. Hydrolysis of penicillin V was increased by *S. lavendulae* PVA, using chloroform in the medium [22]; similar results were observed in the present case of whole cell PVA activity from *E. aroideae*. In the view of this, the direct binding of the solvent on specific binding sites of enzyme might be the contributing factor that determines PVA activity [17]. The prolonged exposure of cells to the solvent led to distortion of the hydration shell of enzyme, resulted in loss of the activity. Penicillin G acylase activity of *E. coli* increased upto 334% when cells were incubated for 10 min in chloroform [6] where as, in the present study, 839% increase in PVA activity was observed at the same incubation time. This is the first time that eightfold increase in PVA activity has been achieved using chloroform and diethyl ether. Krishnan et al. [7] reported that 1 min was optimum to increase the lactate dehydrogenase activity of *L. plantarum* cells with ethyl ether at 28 °C.

4. Conclusions

Penicillin V acylase is a pharmaceutically important enzyme therefore it is relevant to enhance cell-bound activity to use cells as biocatalysts on commercial scale. Solvents with low dielectric constant and high hydrophobicity are effective in improving cell-bound PVA activity of *E. aroideae*. Correlation between physicochemical properties of solvents and change in PVA activity provides clues for selection of a suitable solvent to improve cell-bound activity in

gram-negative bacteria. In this study, for the first time, we report eightfold increase in whole cell PVA activity by employing chloroform and diethyl ether as permeabilizing agents. Solvents such as hexane and toluene were also effective at permeabilizing *E. aroideae* cells and enhancing PVA activity. Chloroform and diethyl ether are cheap reagents, and their volatile nature facilitates removal from the reaction mixture. These two reagents are effective at permeabilizing *E. aroideae* cells and to achieve maximum PVA activity within short period of treatment. These features make the process convenient to scale up.

Acknowledgements

Atul Kumar thanks the Council of Scientific and Industrial Research (CSIR, New Delhi) for the award of senior research fellowship. Authors thank Dr. Tanuja Gupte, Hindustan Antibiotics Pune, India, for providing penicillin V potassium salt. Help of Mr Sharath Balakrishnan is acknowledged for manuscript preparation.

References

[1] D. Galabova, B. Tuleva, D. Spasova, Enzyme Microbiol. Technol. 18 (1996) 18–22.
[2] E. Galindo, G. Salcedo, Enzyme Microbiol. Technol. 19 (1996) 145–149.
[3] A.A. Prabhune, B.S. Rao, A.V. Pundle, H. SivaRaman, Enzyme Microbiol. Technol. 14 (1992) 161–163.
[4] M.V. Flores, C.E. Voget, R.J.J. Ertola, Enzyme Microbiol. Technol. 16 (1994) 340–346.
[5] R.R. Chen, Appl. Microbiol. Biotechnol. 74 (2007) 730–738.
[6] A. De Leon, B. Garcia, A.P. Barda de la Rosa, F. Villasenor, A. Estrada, R. Lopez-Revilla, Process Biochem. 39 (2003) 301–305.
[7] S. Krishnan, L. Gowda, N.G. Karanth, Process Biochem. 35 (2000) 1191–1198.
[8] R.B. Mutalik, V.G. Gaikar, Enzyme Microbiol. Technol. 32 (2003) 14–26.
[9] S. Ospina, E. Merino, O.T. Ramierez, A. Lopez-Mungia, Biotechnol. Lett. 17 (1995) 615–620.
[10] V.K. Sudhakaran, J.G. Shewale, World J. Microbiol. Biotechnol. 9 (1993) 630–634.
[11] M. Arroyo, I. de la Mata, C. Acebal, M.P. Castillon, Appl. Microbiol. Biotechnol. 60 (2003) 507–514.
[12] J.G. Shewale, V.K. Sudhakaran, Enzyme Microbiol. Technol. 20 (1997) 402–410.
[13] J. Bomstein, W.G. Evans, Anal. Chem. 37 (1965) 576–578.
[14] J.G. Shewale, K.K. Kumar, G.R. Ambekar, Biotechnol. Tech. 1 (1987) 69–72.
[15] R. Affleck, C.A. Haynes, D.S. Clark, Proc. Natl. Acad. Sci. U.S.A 89 (1992) 5167–5170.
[16] P.J. Halling, Biotech. Adv. 5 (1987) 47–84.
[17] E. Liepinsh, G. Otting, Nat. Biotechnol. 15 (1997) 264–268.
[18] S. Yoshida, T. Watanabe, Y. Honda, M. Kunawhara, J. Mol. Catal. B: Enzymetic 2 (1997) 243–251.
[19] M.F. Perutz, Science 201 (1976) 1187–1191.
[20] R.M. Guinn, P.S. Skerker, P. Kavanaugh, D.S. Clark, Biotechnol. Bioeng. 37 (1991) 303–308.
[21] L.M. Simon, M. Kotormán, A. Szabó, J. Nemcsók, I. Laczko, Process Biochem. 42 (2007) 909–912.
[22] M. Arroyo, R. Torres, I. de la Mata, M.P. Castillon, C. Acebal, Enzyme Microbiol. Technol. 25 (1999) 378–383.
[23] L.A.S. Gorman, J.S. Dordick, Biotechnol. Bioeng. 39 (1992) 392–397.
[24] Dielectric Constants References Guide 2001. ASI Instruments, Inc., http://www.asiinstr.com/dcl.html.
[25] C. Laane, S. Boeren, K. Vos, C. Veeger, Biotechnol. Bioeng. 30 (1987) 81–87.