Appl Biochem Biotechnol (2009) 157:463–472
DOI 10.1007/s12010-008-8294-0

Effect of Cultural Conditions and Media Constituents on Production of Penicillin V Acylase and CTAB Treatment to Enhance Whole-Cell Enzyme Activity of *Rhodotorula aurantiaca* (NCIM 3425)

Atul Kumar · Sanjay Singh · Pankaj Poddar · Asmita Prabhune · Archana Pundle

Received: 13 March 2008 / Accepted: 27 May 2008 /
Published online: 15 July 2008

Abstract Penicillin V acylase (PVA) is a pharmaceutically important enzyme as it plays a vital role in the manufacture of semi-synthetic β-lactam antibiotics. *Rhodotorula aurantiaca* (NCIM 3425) produced high levels of intracellular penicillin V acylase after 18 h at pH 8.0 and temperature 27 °C. Fructose was the best carbon source for PVA production, whereas tryptone was the best nitrogen source to produce the enzyme up to 170 and 1,088 IU/l of culture, respectively. Additionally, the cell-bound PVA activity was enhanced on treatment with cationic detergent. Whole-cell activity was found to be doubled (204%) on treatment of 0.01 g dry weight of cells with 50 μg/ml solution of *N*-cetyl-*N*,*N*,*N*-trimethylammoniumbromide at pH 8.0 for 1 h at room temperature. Atomic force microscopy images of permeabilized cells show perturbation in the cell wall and offer first-ever visual illustration of surface structure modifications that occur during permeabilization of *R. aurantiaca* cells leading to enhancement in activity of intracellular enzyme.

Keywords Penicillin V acylase · *Rhodotorula aurantiaca* · Intracellular enzyme · Permeabilization · Atomic force microscopy

Introduction

Penicillin acylases (penicillin amidohydrolases, EC 3.5.1.11) hydrolyze the acyl side chain of penicillins to yield 6-aminopenicillanic acid (6-APA) and the corresponding organic acids. 6-APA is a key intermediate required in the manufacture of semi-synthetic penicillins such as ampicillin, amoxicillin, etc. Penicillin acylase selectively hydrolyzes the amide

A. Kumar · S. Singh · A. Prabhune · A. Pundle (✉)
Division of Biochemical Sciences, National Chemical Laboratory, Pune 411 008, India
e-mail: av.pundle@ncl.res.in

S. Singh · P. Poddar
Nanoscience group, Physical/Material Chemistry Division, National Chemical Laboratory, Pune 411 008, India

bond at the side chain of the penicillin moiety, keeping β-lactam amide bond intact [1, 2, 3]. Microbial producers of penicillin V acylase (PVA) occur widely in nature [4]. Shewale and Shudhakaran [5] have described the potential applications of PVA in the production of 6-APA.

The catalytic activities of intracellular enzymes may be low in whole-cell biocatalysts due to the impermeability of the cell to substrate and/or product as they diffuse to and from the reaction medium. Perforating the natural barrier by efficient permeabilization process produces whole-cell biocatalysts with high and stable enzyme activity [6]. Various methods of permeabilization of microbial cells have been reported by treating the cells with organic solvents [7–9], polyethylenimine (PEI) [10], lactic acid [11], and detergents such as *N*-cetyl-*N*,*N*,*N*-trimethylammoniumbromide (CTAB) [12–14] and Tween [15]. Permeability issues in gram-negative bacteria have been recently discussed by Chen [16]. Apart from that, a number of chemical and physical treatment methods have been described to permeabilize yeast cells [17].

Atomic force microscopy (AFM) is a powerful tool in microbiology which allows high-resolution imaging of cell structure in the conditions close to the native state. The technique provides three-dimensional images of surface ultrastructures with molecular resolution under physiological conditions with minimal sample preparation [18].

Penicillin V acylase is not only important to the pharmaceutical industry, but it is important also because it belongs to Ntn-hydrolases, the newly evolved super family. The proteins that belong to this family characteristically possess N-terminal residue as a nucleophile participating in the catalysis, exposed by autocatalytic processing of the precursor [19]. Penicillin V acylase from *Bacillus sphaericus* has been purified, and the three-dimensional structure of the same has been reported by our group [20, 21]. Keeping in mind the potential applications of PVA, the present study was undertaken to explore the PVA production ability of the much less-studied yeast. *Rhodotorula aurantiaca*, psychrophilic yeast, is a new eukaryotic source of PVA. Optimization of cultural conditions and effect of carbon and nitrogen sources on enzyme production were studied and reported here. Also, we have used cationic detergent, *N*-cetyl-*N*,*N*,*N*-trimethylammoniumbromide, to permeabilize *R. aurantiaca* cells at extremely low concentration to enhance cell-bound PVA activity; and surface changes on microbial cell wall were studied using AFM technique.

Materials and Methods

Materials

Penicillin V potassium salt was a kind gift from Hindustan Antibiotics, Pune, India. Peptone, yeast extract, sodium glutamate, tryptone, urea, soybean casein digest, $NH_4H_2PO_4$, $(NH_4)_2SO_4$, ammonium chloride, ammonium nitrate, glucose, mannitol, inulin, sorbitol, glycerol, and digitonin were procured from Himedia, India. *N*-cetyl-*N*,*N*,*N*-trimethylammoniumbromide and sodium dodecyl sulphate, triton X-100, ethanol, and toluene were obtained from Merck (I). All other reagents and chemicals used were of high purity and analytical grade.

Microorganisms

For isolation and screening of penicillin V acylase producers, soil samples, drainage, and waste material were collected from the vicinity of Hindustan Antibiotics, Pune, and other standard-type cultures were obtained from National Collection of Industrial Microorganism

(NCIM), National Chemical Laboratory (NCL), Pune, India. Screening for penicillin-acylase-producing microorganisms was carried out by plate assay method [22]. *R. aurantiaca* NCIM 3425, NCYC 138 was obtained from NCIM, NCL, Pune, India.

Culture Media

Batch fermentation was carried out in Erlenmeyer flasks (250 ml) containing 50 ml of medium inoculated with 10% *v/v* of seed culture at 28 °C for 24 h at 180 rpm. The minimal medium used for enzyme production contained (g/l): Na_2HPO_4, 12.8; KH_2PO_4, 3.1; NaCl, 1.0; $MgSO_4{\cdot}7H_2O$, 0.2; glucose, 4.0; and $NaNO_3$, 3.0, and the pH was adjusted to 7.0. Minimal medium was used as a basal medium for the fermentation study.

Optimization of Cultural Conditions and Effect of Media Supplements for Maximum PVA Production

All experiments for optimization of incubation period, optimum temperature, initial pH of media, and inoculum size, etc. were carried out under shake-flask conditions in 250 ml Erlenmeyer flasks containing 50 ml of minimal medium. Cells were grown at different temperatures (25, 27, 28, 30, 40, and 50 °C) to standardize the optimum temperature for PVA production. The pH of medium was adjusted to 4–10 to standardize the initial pH of medium. To standardize optimum incubation period for the production of enzyme, samples were removed every 6 h interval. All the experiments were followed by determination of cell biomass and cell-bound enzyme activity. All the optimal conditions were kept constant in all other experiments performed to determine the maximum PVA productivity.

Glucose of the minimal medium was replaced by various carbon sources (2.0% *w/v*), and $NaNO_3$ of minimal medium was replaced by various nitrogen sources (0.3% *w/v*) to study the effect of carbon and nitrogen sources, respectively, on the production of penicillin V acylase. All the experiments for optimization of cultural conditions and media constituents were carried out in triplicate with the freshly grown culture, and the values reported in tables and figures represent their mean value.

Permeabilization Procedure

R. aurantiaca cells were washed with 0.1 M phosphate buffer pH 6.0. Thirty milligrams wet cells were suspended in 0.1 M Tris–HCl buffer pH 8.0 containing CTAB to final concentration of 0.005% (*w/v*). The reaction mixture was then incubated for 1 h at room temperature (25 °C) with intermittent gentle mixing. The treated cells were recovered by centrifugation at 10,000 rpm and 4 °C for 5 min and washed with 0.1 M Tris–HCl buffer pH 8.0. Cell-bound PVA activity was determined by standard enzyme assay. The cells treated simultaneously in absence of CTAB served as control.

Enzyme Assay

Cell-bound penicillin V acylase activity was determined by the method of Bomstein and Evans [23], 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 citrate buffer pH 5.5. The 6-APA formed was estimated using 6% (*w/v*) *p*-dimethylaminobenzaldehyde 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.

Biomass concentration was determined from optical density measurements at 600 nm and converted to dry weight of cells (DW) with a standard curve. The biomass reported here is dry weight of cells; and enzyme activity represents IU/g DW. All the experiments were carried out in triplicate with the freshly grown culture, and the values reported in tables and figures represent their mean value.

Sample Preparation and Atomic Force Microscopy Analysis

For this purpose, samples were prepared by washing the CTAB-treated (611 cfu/ml) as well as untreated *R. aurantiaca* cells (645 cfu/ml) with 0.1 M Tris–HCl buffer, pH 8.0. These cells were immobilized on the freshly cleaved surface of mica substrate by drop-coating them followed by the air-drying of the samples at room temperature for 3 h. After fixation, the unbound cells were rinsed away with the same buffer. The mica substrate was mounted on a 6399e-piezoscanner (10 μm) for AFM imaging. The images were recorded in height mode for quantitative information on sample surface topography.

The cell imaging was carried out by an atomic force microscopy operating in contact mode using a Multimode™ scanning probe microscope by VEECO Instruments, USA, equipped with Nano-Scope IV™ controller. Standard silicon nitrate AFM cantilevers, T= 0.4–0.7 μm, (Model: NP-20), with spring constant of 0.12 N/m, and a nominal tip radius of <20 nm, by VEECO Instruments, USA, were used to image both the control and CTAB-treated cells. The samples were imaged in air using the contact mode with settings of 512 pixels/line and 2.98 Hz scan rate. Some of the images were first-order-flattened and contrast-enhanced using Nanoscope 5.30r2 for better demonstration on details.

Results and Discussion

Effect of Cultural Conditions on Production of PVA

While exploring the natural microflora and the available standard cultures from national culture collection, we identified a new source of penicillin V acylase, yeast *R. aurantiaca* (NCIM 3425). To achieve maximum production of enzyme from the organism, optimization studies of cultural conditions were carried out, and the results are depicted in Fig. 1 and summarized in Table 1. An incubation period of 15–18 h was found to be optimum for the production of PVA from *R. aurantiaca*; further incubation led to drastically decreased PVA production. The *R. aurantiaca* cell mass increased constantly up to 36 h and slightly decreased thereafter; however, PVA activity reached maximum at 18 h and drastically decreased at further incubation (Fig. 1a); we concluded that the enzyme was produced in the exponential phase of the yeast cell growth. Optimum parameters for production of penicillin V acylase from *B. sphaericus*, *Streptomyces lavendulae* and *R. aurantiaca* are compared in Table 2. The optimum incubation period for the production of penicillin V acylase from *B. sphaericus* and *S. lavendulae* was 20 and 278 h, respectively (Table 2). *R. aurantiaca* PVA production was very much influenced by fermentation temperature. Maximum PVA was produced, 12 IU/g DW, at 27 °C; the enzyme production lowers remarkably above and below 27 °C (Fig. 1b). PVA production was dropped with the drop of cell biomass above 28 °C. Production of PVA from *B. sphaericus* was optimum at 25 °C [24], whereas in the present case, 27 °C was the optimum temperature for enzyme production; PVA was optimally produced by *S. lavendulae* [25], an actinomyces, at 28 °C (Table 2).

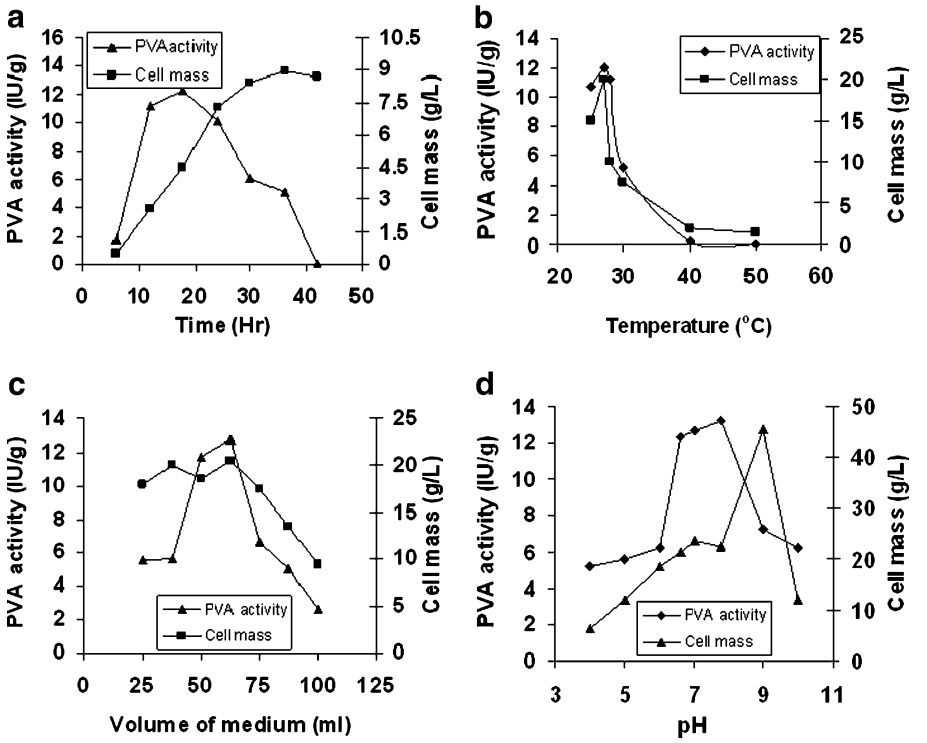

Fig. 1 **a** Optimum incubation period for the production of penicillin V acylases from *R. aurantiaca*. **b** Temperature optimization for the production of PVA. **c** Optimum dispensing volume of media for the production of PVA. **d** Optimum initial pH of medium for the enzyme production

Various volumes of media (25–100 ml) were dispensed in 250 ml Erlenmeyer flask to study the effect of aeration, and the maximum enzyme production was found at dispensing volume of 62.5 ml medium at 180 rpm (Fig. 1c). The initial pH of medium, 8.0, was found to be the best for enzyme production, 13.25 IU/g DW (Fig. 1d). The cell growth of *R. aurantiaca* increased constantly and reached highest (45.5 g cells/l), at pH 9.0; however, further increase in media pH drastically decreased the cell biomass; *Rhodotorula glutinis*, a yeast, has been reported to grow near pH 9.0 [26, 27]; it is true even in the case of *R. aurantiaca.* PVA production from *B. sphaericus* was optimum at pH 7.5, whereas production of PVA from *S. lavendulae* was optimum at pH 6.8 (Table 2).

Table 1 Optimum conditions for the production of penicillin V acylase from *R. aurantiaca* (NCIM 3425).

Parameters	Vales
Incubation period (h)	18
Incubation temperature (°C)	27
Volume of medium (milliliter per 250 ml flask)	62.5
Initial pH of media	8.0

Table 2 Comparison of optimum parameters for PVA production from different microorganisms.

Microorganism	Parameters			
	Incubation period (h)	Incubation temperature (°C)	pH of media	References
R. aurantiaca	18	27	8.0	Present studies
B. sphaericus	20	25	7.5	[24]
S. lavendulae	278	28	6.8	[25]

Effect of Carbon and Nitrogen Sources on Production of PVA

Various carbon sources were used to study the effect on PVA production from *R. aurantiaca*, and the results are depicted in Table 3. Mannitol, fructose, sorbitol, and galactose produced PVA up to 103, 68.3, 60.4, and 58.8 IU/g DW, respectively; however, overall PVA productivity was maximum (170 IU/l of culture broth) when fructose was used in minimal medium. Mannitol, fructose, and sucrose improved the penicillin G acylase (PGA) production by a 10% higher level from *Bacillus* sp. [28]. Similarly, Senthilvel and Pai [29] reported that the production of penicillin G acylase by *Bacillus megaterium* was increased by sucrose and repressed by glucose and fructose. In *Escherichia coli* also, the production of penicillin G acylase was repressed by glucose, fructose, maltose, and glycerol [30]. In the present studies, the addition of glucose, fructose, or sucrose did not repress but enhanced the PVA production from *R. aurantiaca.* Presence of inulin in minimal medium did not increase PVA activity, whereas it enhanced the cell mass and subsequently the productivity. *S. lavendulae* produced high levels of penicillin V acylase (178 IU/l of culture broth) when grown in skim milk [25].

Various nitrogen sources were used to study the effect on PVA production from *R. aurantiaca*, and the results are depicted in Table 4. Tryptone, sodium glutamate, peptone, and ammonium phosphate produced PVA up to 89.9, 85.3, 70.9, and 58.9 IU/g DW; however, the specific PVA productivity was maximum (1,088 IU/l) while using tryptone in minimal medium. Tryptone and peptone increased PGA productivity without increasing the cell growth in *Bacillus* sp. [28]. Similarly, in the present study, tryptone and peptone

Table 3 Effect of carbon sources on production of penicillin V acylase from *R. aurantiaca* (NCIM 3425).

Carbon sources	PVA activity (IU/g DW)	Cell weight (g DW/l)	Productivity (IU/l)
None	12.3	5.3	66
Glucose	44.1	3.3	148
Mannitol	103	1.5	154
Sorbitol	60.4	2.5	153
Glycerol	50.2	2.7	136
Fructose	68.3	2.5	170
Galactose	58.8	2.2	131
Maltose	42.2	3.4	146
Sucrose	52.2	3.1	162
Lactose	46.6	2.8	134
Mannose	45.0	3.6	162
Inulin	26.0	5.9	154

Table 4 Effect of nitrogen sources on production of penicillin V acylase from *R. aurantiaca* (NCIM 3425).

Nitrogen source	PVA activity (IU/g DW)	Cell weight (g DW/l)	Productivity (IU/l)
None	26.0	18.7	488
Sodium glutamate	85.3	12.1	1,033
Urea	59.3	7.9	468
Yeast extract	67.9	14.9	1,012
Tryptone	89.9	12.1	1,088
Soybean casein digest	46.5	21.3	990
Peptone	70.9	14.9	1,061
$NH_4H_2PO_4$	58.9	19.9	1,172
$(NH_4)_2SO_4$	46.6	11.5	538
NH_4Cl	64.9	11.9	775
KNO_3	52.9	9.4	498
NH_4NO_3	55.6	8.8	492

enhanced PVA production without increasing the cell growth. Gentina et al. [31] have also reported, after studying various complex nitrogen sources, that casein hyrdrolysate is the best nitrogen source for PGA production by *B. megaterium* ATTC 14945. Penicillin V acylase production was 1.3-fold more when the cornsteep liquor was replaced with

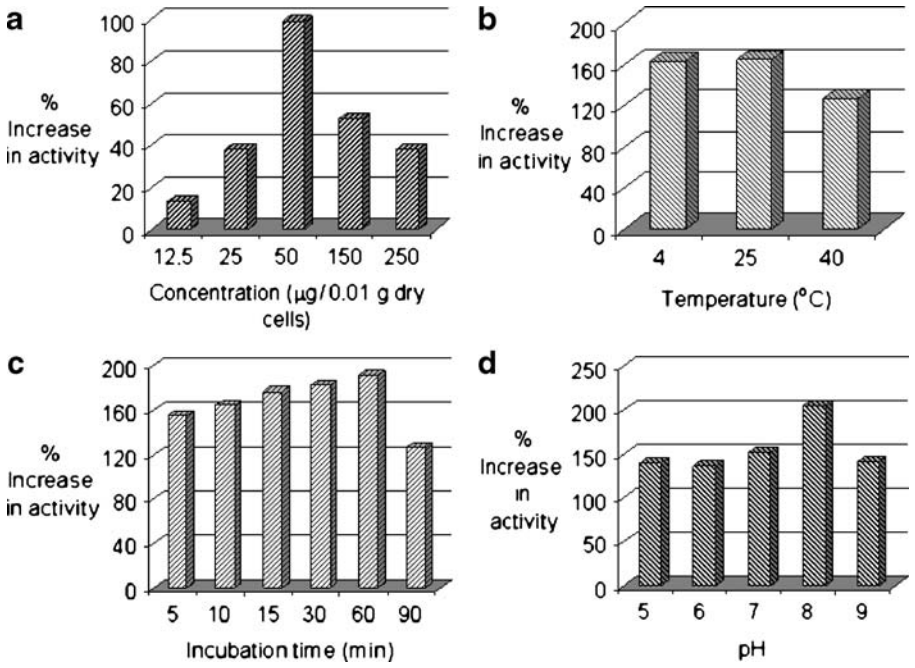

Fig. 2 **a** Effect of concentration of CTAB for the permeabilization of *R. aurantiaca* cells. The activity of untreated cells served as 100% (80.2 IU/g DW). **b** Effect of temperature on *R. aurantiaca* cells for permeabilization. The activity of untreated cells served as 100% (72.10 IU/g DW). **c** Effect of incubation period for permeabilization treatment of *R. aurantiaca* cells. The activity of untreated cells served as 100% (79.2 IU/g DW). **d** Effect of pH of CTAB solution for permeabilization of *R. aurantiaca* cells. The activity of untreated cells served as 100% (85.5 IU/g DW)

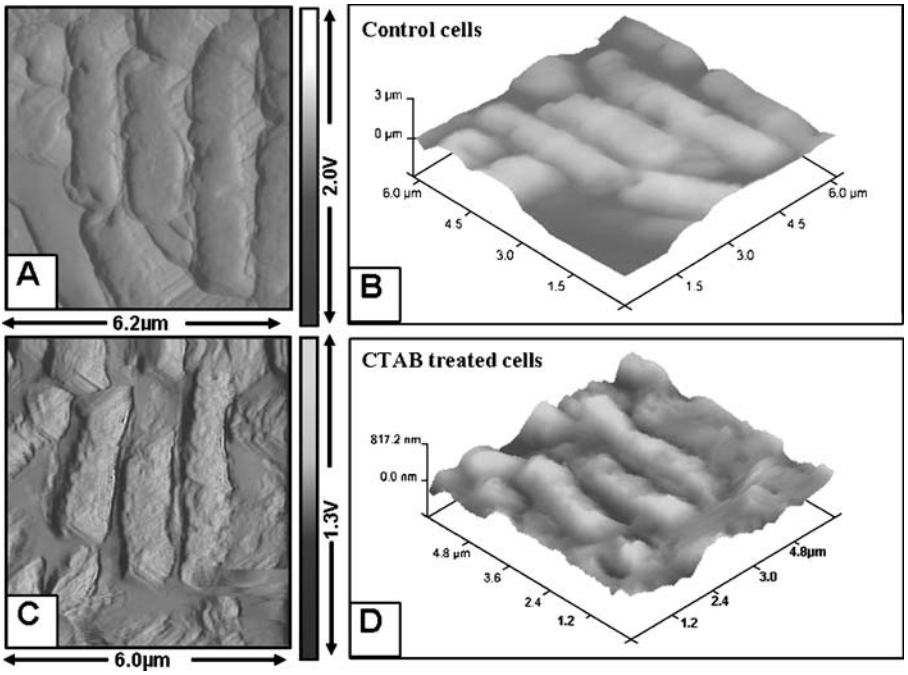

Fig. 3 Atomic force microscopy images: **a** deflection mode image; **b** three-dimensional image of height mode of control (untreated) of *R. aurantiaca* cells (scan size 6.2×6.2 µm); **c** deflection mode image; **d** three-dimensional image of height mode of CTAB-treated *R. aurantiaca* cells (scan size 6.0×6.0µm)

neopeptone [32]. Production of penicillin V acylase from *B. sphaericus* was achieved up to 100 IU/g DW [24]. This is the first time we are reporting PVA production up to 1,088 IU/l from *R. aurantiaca* culture.

Permeabilization of *R. aurantiaca* Cells

Various methods were attempted to enhance cell-bound PVA activity by permeabilizing *R. aurantiaca* cells. Treatment of cells with enzymes and antibiotics had no remarkable effect under the experimental conditions; the solvent treatment showed loss of PVA activity (data not shown), while the detergents showed interesting results. Digitonin (0.1%) showed 15% increase in PVA activity with respect to control (100%, 75.1 IU/g DW). Gowda et al. [33] used digitonin, a mild detergent, to permeabilize *Kluyveromyces fragilis* cells to obtain increased activity of intracellular enzymes like alcohol dehydrogenase, β-galactosidase, etc. Triton X-100 (0.02%) inhibited whole-cell *R. aurantiaca* PVA activity (87% residual activity) with respect to control (100%, 78.5 IU/g DW); whereas it was used to permeabilize yeast, *Yarrowia lipolytica* [34]. CTAB showed maximum escalation, 100% increase in activity with respect to control (80.2 IU/g DW), at very low concentration (50 µg/0.01 g DW; Fig. 2a). Hence, further experiments were carried out to optimize the conditions for permeabilization to obtain maximum enhancement in activity at lowest detergent concentration. PVA activity was increased up to 165% with respect to control (100%, 72.10 IU/g DW), when cells were permeabilized at room temperature, 25 °C (Fig. 2b). An increase of 192% in PVA activity was obtained, with respect to control

(100%, 79.2 IU/g DW), for 60 min incubation; however, further incubation led to inhibition of enzyme activity (Fig. 2c). There was no leakage of enzyme in the medium, which was traceable during the experimental period. Cell-bound PVA activity was increased up to 204% when the cells were treated with CTAB, suspended in Tris–HCl buffer pH 8.0 (Fig. 2d). Cell-bound PVA activity was enhanced up to 204% when all the optimized parameters were used. Earlier, we have reported enhanced production of 6-APA from benzylpenicillin by *E. coli* cells, which were sequentially treated with CTAB and glutaraldehyde then immobilized in open-pore polyacrylamide beads for continuous operation [12].

Atomic Force Microscopy

AFM images of control (untreated) and CTAB-treated yeast cells showed the changes that occurred on the cell surface due to permeabilization. The topography of the control as well as CTAB-treated cells is depicted in Fig. 3. The untreated cells had smooth surface where the length of yeast cells was around 3.6–4.0 μm and width around 1.5–1.8 μm (Fig. 3a, b). The damage to the cell surface, in case of CTAB-treated cells, was clearly noticed (Fig. 3c, d). However, it is assumed that the damaged surface accelerated the transport of the substrate, penicillin V, and the product, 6-APA, across the cell wall resulting in enhanced rate of hydrolysis.

AFM studies of *R. aurantiaca*, a newly identified PVA producer, reveals that controlled permeabilization treatment brings about subtle structural changes in the cell wall resulting in enhancement of intracellular enzyme activity. We are reporting here for the first time the visual illustration of alterations occurring on *R. aurantiaca* cell surface during permeabilization process.

Conclusion

This is the first time we are reporting very high production of penicillin V acylase from a yeast source, *R. aurantiaca* (NCIM 3425). Optimization of cultural conditions and studies of various carbon and nitrogen sources gave specific conditions for maximum production of PVA from the newly identified source. Furthermore, whole-cell penicillin V acylase activity was enhanced up to 204% by treating the cells with a cationic detergent, *N*-cetyl-*N*,*N*,*N*-trimethylammoniumbromide, so that the whole cells can be used as biocatalysts. Furthermore, atomic force microscopy study gave first-ever visual evidence of change in the surface topography of *R. aurantiaca* cells due to permeabilization process that leads to the enhancement of cell-bound PVA activity.

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.

References

1. Shewale, J. G., Deshpande, B. S., Shudhakarn, V., & Amberkar, S. S. (1990). *Process Biochemistry*, *25*, 97–103.
2. Valle, F., Balbas, P., Merino, E., & Bolivar, F. (1991). *Trends in Biochemical Sciences*, *16*, 36–40. doi:10.1016/0968-0004(91)90014-M.

3. Bruggink, A., Ross, E. C., & de Vroom, E. (1998). *Organic Process Research & Development*, *2*, 128–133. doi:10.1021/op9700643.
4. Sudhakaran, V. K., & Borkar, P. S. (1985). *Hindustan Antibiotics Bulletin*, *27*, 44–62.
5. Shewale, J. G., & Sudhakaran, V. K. (1997). *Enzyme and Microbial Technology*, *20*, 402–410. doi:10.1016/S0141-0229(96)00176-7.
6. Siso, M. I. G., Cerdan, E., Picos, M. A. F., Ramil, E., Belmonte, E. R., & Torres, A. R. (1992). *Biotechnology Techniques*, *6*, 289–393. doi:10.1007/BF02439313.
7. Flores, M. V., Voget, C. E., & Ertola, R. J. J. (1994). *Enzyme and Microbial Technology*, *16*, 340–346. doi:10.1016/0141-0229(94)90177-5.
8. De Leon, A., Garcia, B., Barda de la Rosa, A. P., Villasenor, F., Estrada, A., & Lopez-Revilla, R. (2003). *Process Biochemistry*, *39*, 301–305. doi:10.1016/S0032-9592(03)00079-7.
9. Krishnan, S., Gowda, L. R., & Karanth, N. G. (2000). *Process Biochemistry*, *35*, 1191–1198. doi:10.1016/S0032-9592(00)00162-X.
10. Helander, I. M., Alakomi, H., Latva-Kala, K., & Koski, P. (1997). *Microbiology*, *143*, 3193–3199.
11. Alakomi, H. L., Skytta, A., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., & Helander, I. M. (2000). *Applied and Environmental Microbiology*, *66*, 2001–2005. doi:10.1128/AEM.66.5.2001-2005.2000.
12. Prabhune, A. A., Rao, B. S., Pundle, A. V., & SivaRaman, H. (1992). *Enzyme and Microbial Technology*, *14*, 161–163. doi:10.1016/0141-0229(92)90176-O.
13. Canovas, M., Torroglosa, T., & Iborra, J. L. (2004). *Enzyme and Microbial Technology*, *37*, 300–308. doi:10.1016/j.enzmictec.2004.07.023.
14. Cheng, S., Wei, D., Song, Q., & Zhao, X. (2006). *Biotechnology Letters*, *28*, 1129–1133. doi:10.1007/s10529-006-9067-x.
15. Galindo, E., & Salcedo, G. (1996). *Enzyme and Microbial Technology*, *19*, 145–149. doi:10.1016/0141-0229(95)00215-4.
16. Chen, R. R. (2007). *Applied Microbiology and Biotechnology*, *74*, 730–738. doi:10.1007/s00253-006-0811-x.
17. Felix, H., Nuesch, J., & Wehrli, W. (1980). *Analytical Biochemistry*, *103*, 81–86. doi:10.1016/0003-2697(80)90240-7.
18. Dufrene, Y. F. (2002). *Journal of Bacteriology*, *184*, 5205–5213. doi:10.1128/JB.184.19.5205-5213.2002.
19. Brannigan, J. A., Dodson, G., Duggleby, H. J., Moody, P. C., Smith, J. L., Tomchick, D. R., et al. (1995). *Nature*, *378*, 416–419. doi:10.1038/378416a0.
20. Pundle, A. V., & SivaRaman, H. (1997). *Current Microbiology*, *34*, 144–148. doi:10.1007/s002849900159.
21. Suresh, C. G., Pundle, A. V., SivaRaman, H., Rao, K. N., Brannigan, J. A., McVey, C. E., et al. (1999). *Nature Structural Biology*, *6*, 14–416. doi:10.1038/8213.
22. Meevootisom, V., Somsuk, P., Prachaktam, R., & Flegel, T. W. (1983). *Applied and Environmental Microbiology*, *46*, 1227–1229.
23. Bomstein, J., & Evans, W. G. (1965). *Analytical Chemistry*, *37*, 576–578. doi:10.1021/ac60223a034.
24. Pundle, A. V., & SivaRaman, H. (1994). *Biotechnology Letters*, *16*, 1041–1046. doi:10.1007/BF01022400.
25. Torres, R., Roman, F., de la Mata, I., Acebal, C., & Castillon, M. P. (1999). *Applied Microbiology and Biotechnology*, *53*, 81–84. doi:10.1007/s002530051618.
26. Iizuka, H., & Goto, S. (1973). *Methods of classification and identifcation for yeast* (2nd ed.). Tokyo: University of Tokyo Press.
27. Kurita, O., & Yamazaji, E. (2002). *Current Microbiology*, *45*, 277–280. doi:10.1007/s00284-002-3735-4.
28. Rajendaran, J., Krishnakumar, V., & Gunasekaran, P. (2003). *World Journal of Microbiology & Biotechnology*, *19*, 07–110.
29. Senthilvel, S. G., & Pai, J. S. (1998). *Journal of Scientific and Industrial Research*, *57*, 781–784.
30. Shewale, J. G., & SivaRaman, H. (1989). *Process Biochemistry*, *24*, 146–154.
31. Gentina, J. C., Acevedo, F., & Villagra, M. P. (1997). *World Journal of Microbiology & Biotechnology*, *13*, 127–128. doi:10.1007/BF02770818.
32. Sudhakan, V. K., & Shewale, J. C. (1993). *World Journal of Microbiology & Biotechnology*, *9*, 233–239. doi:10.1007/BF00327845.
33. Gowda, L. R., Joshi, M. S., & Bhat, S. G. (1988). *Analytical Biochemistry*, *175*, 531–536. doi:10.1016/0003-2697(88)90579-9.
34. Galabova, D., Tuleva, B., & Spasova, D. (1996). *Enzyme and Microbial Technology*, *18*, 18–22. doi:10.1016/0141-0229(96)00063-4.