Ann Microbiol
DOI 10.1007/s13213-011-0374-7

ORIGINAL ARTICLE

Characterization of a new *Bacillus cereus* ATUAVP1846 strain producing penicillin V acylase, and optimization of fermentation parameters

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Received: 27 January 2011 / Accepted: 11 October 2011

Abstract Penicillin acylases are involved chiefly in the industrial production of semisynthetic penicillins, which remain the most widely used group of antibiotics. We have isolated a new bacterial strain ATUAVP1846 that produces penicillin V acylase (PVA). Phylogenetic analysis using 16S rRNA sequencing showed 99.37% homology with *Bacillus cereus*. Maximum PVA production was observed with *B. cereus* ATUAVP1846 at 30°C, pH 7 after 24 h fermentation time under submerged conditions. Highest enzyme productivity was achieved using sucrose as carbon source, and tryptone and ammonium hydrogen phosphate as nitrogen sources. Minimal medium containing 0.4% glucose and 0.3% ammonium hydrogen phosphate was found to be optimal for maximum PVA production from *B. cereus* ATUAVP1846. The crude enzyme from *B. cereus* ATUAVP1846 was partially purified using ammonium sulfate fractionation and showed highest enzymatic activity in the hydrolysis of penicillin V at 40°C and pH 6. The crude enzyme preparation also showed unique substrate specificity, preferring ampicillin and cephalexin over penicillin V.

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Keywords Penicillin acylase · *Bacillus cereus* · 16S rRNA · Isolation · Ntn hydrolase · Optimization

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 bond at the side chain of the penicillin moiety, keeping the β-lactam amide bond intact (Shewale et al. 1990).

Screening of penicillin acylase-producing organisms has shown that these enzymes are produced by bacteria, actinomycetes, yeasts and fungi (Arroyo et al. 2005). Penicillin V acylase (PVA) is produced intracellularly by several bacteria like *Beijerinckia indica* var. *penicillanicum*, *Bacillus sphaericus*, *Bacillus subtilis*, *Cryptococcus* sp., *Erwinia aroideae*, *Micrococcus ureae* and *Pseudomonas acidovorans*; by yeast *Rhodotorula glutinis* and by fungi *Penicillium* sp. and *Fusarium* sp. It is produced extracellularly by *Streptomyces lavendulae*, *Streptoverticillium* sp., *Fusarium* sp. SKF 235 and *Pleurotus ostreatus* (Shewale and Sudhakaran 1997). Penicillin acylases belong to the N-terminal nucleophile (Ntn)-hydrolase superfamily, which is constituted of enzymes that share a common structural fold and possess a catalytic serine or cysteine or threonine residue at the N-terminal end (Brannigan et al. 1995).

In addition to their application in the production of semisynthetic antibiotics, penicillin acylases are useful

Published online: 03 November 2011

as biocatalysts in many potentially valuable reactions such as protection of amino and hydroxyl groups in peptide synthesis, as well as in the resolution of racemic mixtures of chiral compounds (Fuganti et al. 1986; Arroyo et al. 2003).

Penicillin V acylase from *B. sphaericus* (NCIM 2478) has been purified and characterized in our laboratory (Pundle and Sivaraman 1997), and the three-dimensional (3D) structure was reported subsequently (Suresh et al. 1999). We have demonstrated the post-translational auto-proteolytic processing of PVA from *B. sphaericus* by crystallization of three catalytically inactive mutants of the enzyme in precursor and processed forms (Chandra et al. 2005). Cloning, purification, crystallization and preliminary structural studies of PVA from *B. subtilis* strain IG-20 (NCIMB 11621) (Rathinaswamy et al. 2005), and the evolutionary relationship between a conjugated bile salt hydrolase from *Bifidobacterium longum* and PVA have also been described by our group (Kumar et al. 2006).

Bacteria from various samples screened to find new microbial sources of PVA. In this paper, we report the isolation of a new penicillin acylase producer strain ATUAVP1846, which was isolated from a soil sample, and its subsequent identification as *Bacillus cereus*. Intracellular PVA is produced constitutively by *B. cereus*.

Materials and methods

Materials

Penicillin V potassium salt and different β-lactam substrates were kind gifts from Hindustan Antibiotics (Pune, India) and KDL Biotech (Khopoli, India). Peptone, yeast extract, beef extract, tryptone and skim milk were procured from Himedia (India). Corn steep liquor was procured from Hindustan Antibiotics (Pune, India). 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 (18°37′8″N 73°48′35″E); and other standard type cultures were obtained from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune, India. Screening for penicillin acylase-producing microorganisms was carried out by plate assay method with *Serratia marcescens* (ATCC 27117) according to Meevootisom et al. (1983).

Identification of PVA-producing strains

The phylogenetic profile of the PVA-producing isolate ATUAVP1846 was performed at Disha Institute of Biotechnology (Nagpur, India) using 16S rRNA sequencing and fatty acid methyl ester (FAME) analysis. The 16S rRNA gene of the strain ATUAVP 1846 was amplified by polymerase chain reaction (PCR) from genomic DNA. Related sequences retrieved from the GenBank database were aligned by using CLUSTAL_X (Thompson et al. 1997). A neighbour-joining phylogenetic tree (Saitou and Nei 1987) was constructed using PHYLIP (Felsenstein 1993) based on evolutionary distances determined with Kimura's two-parameter model (Kimura 1980). Bootstrap analysis (Felsenstein 1985) was performed for 1,000 replications. The 16S rRNA sequence analyzed in this study was deposited in NCBI GenBank database under the accession number JN183063.

Fermentation studies

Batch fermentation was carried out in Erlenmeyer flasks (250 mL) containing 50 mL medium, inoculated with 10% v/v of seed culture in nutrient broth and incubated 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. Nutrient broth and minimal medium were used as basal media for the fermentation study.

Optimization of cultural conditions and effect of media supplements for maximum PVA production

All initial fermentation experiments for optimization of incubation time, temperature, initial pH of media, and inoculum size, etc., were carried out under shake-flask conditions in 250 mL Erlenmeyer flasks containing 50 mL nutrient broth. To estimate the optimum incubation period for the production of enzyme, samples were withdrawn at intervals of 6 h. Varying volumes of media in 250 mL flasks were used to study the effect of aeration on enzyme production. Cell mass and PVA activity were determined for all the experiments, and these optimum conditions were kept constant in further studies performed to determine the maximum PVA productivity.

Glucose in the minimal medium was replaced by various carbon sources (2.0% w/v), and $NaNO_3$ in 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 PVA.

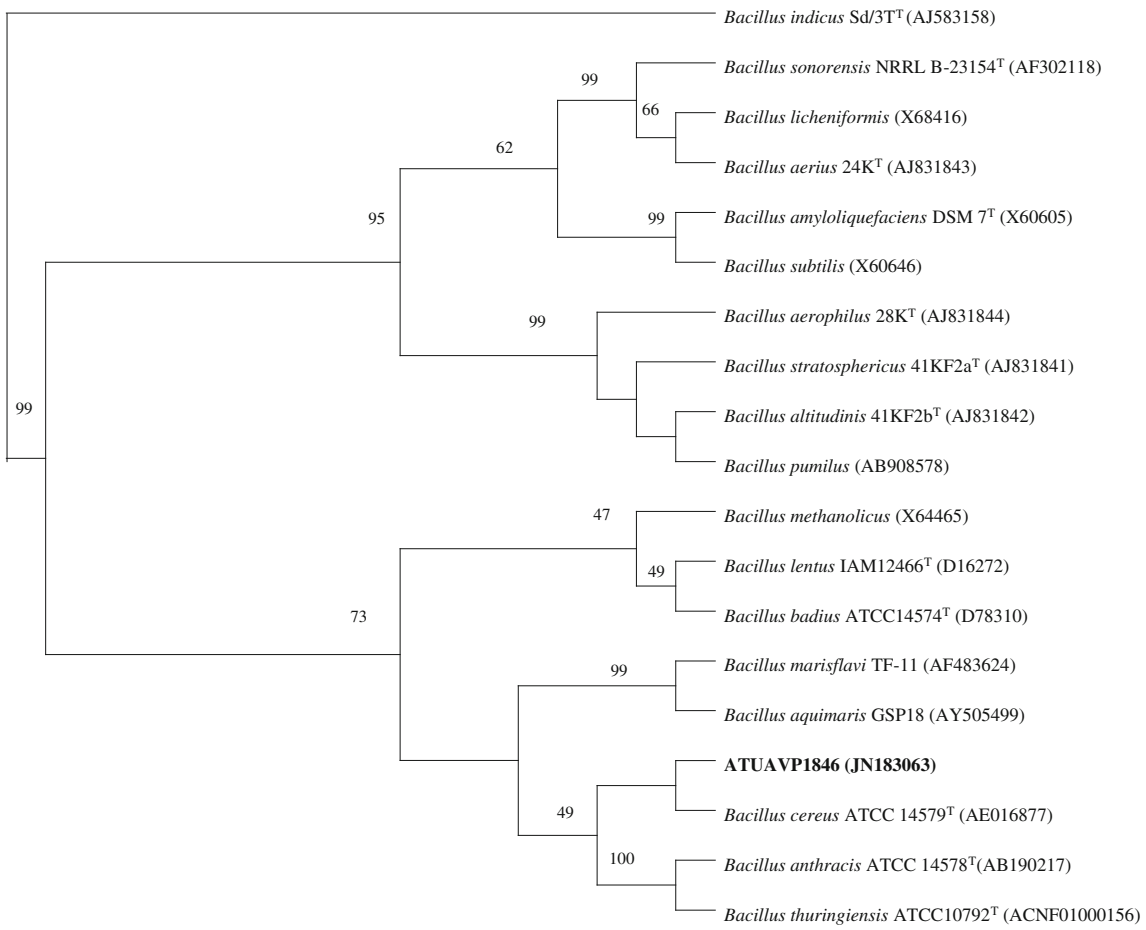

Fig. 1 Neighbour-joining tree based on 16S rRNA gene showing the positions of strain ATUAVP1846 and related taxa. Numbers indicate percentage bootstrap values based on 1,000 replications

Various concentrations of corn steep liquor and skim milk were also added to the minimal medium as supplements to check their effect on PVA production. The culture was grown at 30°C for 24 h before experimental use, and the values reported are averages of at least three repetitions.

Enzyme assay

Activity of cell-bound and crude penicillin V acylase was determined by the method of Bomstein and Evans (1965), modified by Shewale et al. (1987), measuring the amount of 6-APA formed at 40°C, employing 2% w/v solution of penicillin V (potassium salt) in 0.1 M phosphate buffer pH 7.0. The 6-APA formed was estimated using 6% (w/v) *p*-dimethyl amino benzaldehyde (DAB) in methanol. One unit (IU) 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. Productivity (IU/L) was calculated as the product of cell mass and enzyme activity.

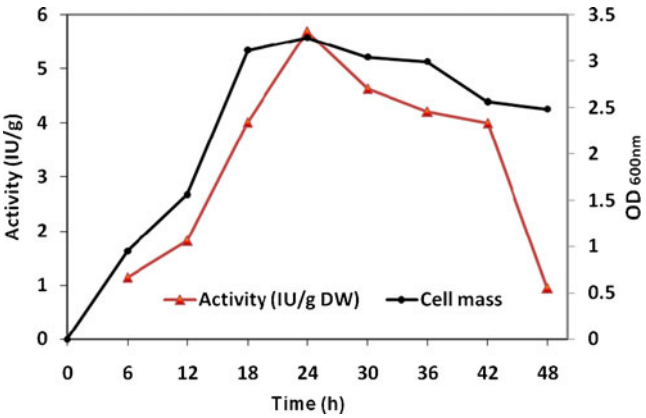

Fig. 2 Effect of incubation period on the growth and penicillin V acylase (PVA) production of *Bacillus cereus*. OD at 600 nm was used as indication of cell mass

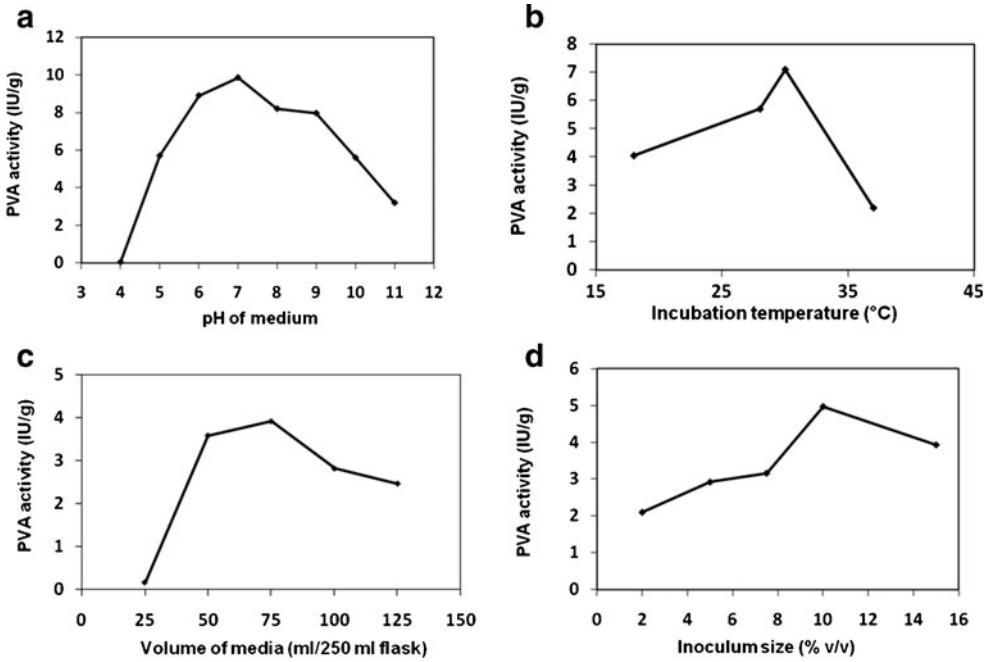

Fig. 3 Effect of **a** initial pH of medium, **b** temperature, **c** media volume and **d** inoculum size on production of PVA from *Bacillus cereus* ATUAVP1846

Preparation of crude enzyme

The enzyme was produced by growing *B. cereus* ATUAVP1846 in minimal medium containing 0.4% glucose and 0.3% $(NH_4)_2HPO_4$ under optimal culture conditions. The cells were collected by centrifugation at 5,000 rpm for 30 min and processed further. For preparation of crude enzyme, the cells were suspended in 50 mM phosphate buffer pH 7 (containing 1 mM EDTA and 1 mM DTT) and disrupted by sonication in an ice bath using a Branson Digital sonifier. Cell debris was removed by centrifugation at 10,000 rpm for 30 min, and the clear supernatant was subjected to fractionation by the addition of fine powder of ammonium sulfate while stirring. The fraction from 30–60% saturation was dialyzed against 10 mM phosphate buffer pH 7 and used as the source of crude or partially purified enzyme for further characterization studies. Enzyme assay was carried out as detailed above with 50 μL crude enzyme to determine the activity, and protein concentration measurements were performed using standard Folin-Lowry method (Lowry et al. 1951).

Effect of temperature and pH on the activity of PVA from *B. cereus* ATUAVP1846 and substrate specificity

The influence of temperature on the catalytic activity of partially purified PVA was determined by measuring the

Table 1 Effect of carbon sources on the production of penicillin V acylase (PVA) from *Bacillus cereus* ATUAVP1846

Carbon source	PVA activity (IU/g)	Cell weight (g/L)	Productivity (IU/L)
D-Mannose	2.61	1.70	4.45
D-Galactose	2.48	0.80	1.98
D-Sorbitol	1.09	1.05	1.15
D-Fructose	3.42	1.35	4.62
Lactose	3.29	2.95	9.73
Mannitol	1.72	2.00	3.45
Sucrose	4.47	7.50	33.55
Glucose	4.89	5.55	27.16
Glycerol	5.13	1.07	8.73
Inulin	4.02	10.15	40.83

Table 2 Effect of nitrogen sources on the production of PVA from *B. cereus* ATUAVP1846

Nitrogen source	PVA activity (IU/g)	Cell weight (g/L)	Productivity (IU/L)
KNO_3	5.98	6.00	35.88
NH_4NO_3	6.16	15.65	96.45
NH_4Cl	4.74	17.85	84.60
$(NH_4)_2SO_4$	5.72	17.75	101.56
Yeast extract	5.75	18.20	104.68
Tryptone	7.50	17.40	130.53
Soyabean casein digest	7.27	13.35	97.11
Peptone	5.35	16.40	87.89
$(NH_4)_2HPO_4$	8.92	14.90	132.96
Glutamate	4.47	25.70	114.89
Urea	4.32	17.40	75.22
Control ($NaNO_3$)	5.26	17.45	91.91

Table 3 Effect of corn steep liquor and skim milk on the production of PVA from *B. cereus* ATUAVP1846

Concentration (% w/v)	PVA activity (IU/g)	Cell weight (g/L)	Productivity (IU/L)
Corn steep liquor			
1	2.76	4.95	13.66
2	2.81	11.65	32.75
3	2.81	13.5	38.02
4	3.42	20.2	69.13
5	3.79	23.45	89.05
6	3.63	27.35	99.42
7	2.93	29.65	87.07
8	2.55	30.4	77.53
Skim milk			
0.1	1.43	2.3	3.29
0.2	3.33	3.85	12.83
0.5	4.27	7.7	32.92
1.0	6.94	12.7	88.23
2.0	2.96	13.65	40.49
3.0	1.87	20	37.53

enzyme activity at temperatures ranging from 20°C to 50°C, at pH 7.0. The influence of pH on the enzyme activity was determined by measuring the enzyme activity at varying pH values ranging from 4 to 9 using different suitable buffers at 0.1 M: sodium acetate (pH 4.0 and 5.0), sodium phosphate (pH 6.0 and 7.0), and Tris HCl buffer (pH 8.0, and 9.0), respectively. The partially purified enzyme fraction was also checked for substrate specificity using different β-lactam substrates, with appropriate compounds for calibration (6-APA and 7-ADCA).

Results and discussion

Isolation and identification of *B. cereus* ATUAVP1846

While exploring the natural micro flora and the available standard cultures from NCIM, we isolated a new source of PVA, which was identified using 16S rDNA sequencing analysis as *Bacillus cereus* (Fig. 1). The bacterium was isolated from soil samples taken from the vicinity of Hindustan Antibiotics, Pune. Production of PVA was confirmed by the formation of a clear zone in the plate assay with *Serratia marcescens* (zone diameter 4.1 cm). Phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that strain ATUAVP1846 (accession number in GenBank: JN183063) fell within the branch encompassing members of the genus *Bacillus* (Fig. 1) and was related most closely to *Bacillus cereus* (99.37% 16S rRNA gene sequence similarity). FAME analysis of ATUAVP1846 also showed a similarity index of 0.320 for *B. cereus* subgroup B. Based on these data, the PVA-producing isolate was designated as *Bacillus cereus* strain ATUAVP1846.

Effect of culture conditions on production of PVA

To achieve maximum production of enzyme from the organism, fermentation studies were carried out to determine the optimum culture conditions, and the results are depicted in Figs. 2 and 3. An incubation period of 24 h was found to be optimum for production of PVA from *B. cereus*; production decreased thereafter. Cell mass increased up to 18 h, then remained almost constant until 48 h, and decreased on further incubation. We concluded that PVA is produced constitutively, with maximum production in the stationary phase of bacterial cell growth. PVA production was also influenced to a great extent by change in temperature and pH of medium. Maximum enzyme production was observed at 30°C and pH 7.0, with 10% inoculum. PVA production decreased drastically at higher temperatures and at pH values beyond 6–9. Similar conditions have been reported in the case of *B. sphaericus* (Pundle and Sivaraman 1994), *S. lavendulae* (Torres et al. 1998) and *R. aurantiaca* (Kumar et al. 2008).

Various volumes of media (25–100 mL) were dispensed in 250 mL Erlenmeyer flasks to study the effect of aeration, and the maximum enzyme production was found at dispensing volume of 75 mL medium at 180 rpm orbital shaking.

Effect of media components on production of PVA

Various carbon and nitrogen sources were used to study the effect on PVA production from *B. cereus* in minimal medium. The effect of carbon sources on PVA production is indicated in Table 1. Maximum enzyme production was observed with glycerol (5.13 IU/g cell dry weight) and

Table 4 Purification table for partial purification of *B. cereus* PVA

Purification step	PVA activity (IU)	Protein (mg)	Specific activity (IU/mg)	Yield (%)	Purification fold
Sonication	18.37	856.70	0.0214	100	1
$(NH_4)_2SO_4$ precipitate (30-60%)	13.89	334.65	0.0415	75.61	1.94

Table 5 Substrate specificity of partially purified PVA from *B. cereus* ATUAVP1846

Substrate (5 mg/mL)	Activity (IU/mL)
Penicillin V	0.636
Penicillin G	0.290
Ampicillin (Na-salt)	1.648
Amoxicillin	0.444
Cloxacillin	0.352
Dicloxacillin	0.358
Cephalexin	9.425 (7-ADCA)

glucose (4.89 IU/g) as carbon sources, while inulin and sucrose increased the cell mass, in turn resulting in the highest productivity (40.8 and 33.5 IU/L, respectively). Catabolite repression of PVA production by glucose has been reported in *S. lavendulae* (Torres et al. 1999) and *Chainia* (Chauhan et al. 1998), whereas glucose did not repress enzyme production in certain other organisms (Carlsen and Emborg 1981; Vandamme and Voets 1975). In the case of *B. cereus*, the use of glucose as carbon source enhanced PVA production.

The use of $(NH_4)_2HPO_4$ (8.92 IU/g) and tryptone (7.50 IU/mL) as nitrogen sources resulted in maximum PVA production (Table 2). Ammonium hydrogen phosphate also gave the highest productivity (133 IU/L). As indicated in Table 3, various concentrations of corn steep liquor and skim milk were tested as media supplements. Carlsen and Emborg (1981) have reported enhanced PVA production from *B. sphaericus* on a medium containing 4% corn steep liquor, while *S. lavendulae* has been observed to produce high levels of PVA (178 IU/L of culture broth) when grown using skim milk as sole carbon source. In the case of *B. cereus*, 6% corn steep liquor and 1% skim milk were observed to be optimum for PVA production. However, minimal medium with 0.4% glucose and 0.3% $(NH_4)_2HPO_4$ without any other supplements was devised as the most favourable medium for maximum PVA production from *B. cereus* (133 IU/L).

Partial purification and properties of crude enzyme

Bacillus cereus cells were sonicated to get the crude extract, and PVA was partially purified using ammonium sulfate precipitation as given in Tables 4. The fraction from 30% to 60% saturation was dialyzed against 10 mM phosphate buffer pH 6.5 and the enzyme preparation (specific activity 0.042 IU/mg) was used for further studies.

Maximum activity of *B. cereus* PVA was observed at pH 6 and 40°C (Fig. 4). The substrate specificity of the enzyme was observed as given in Table 5. Curiously, ampicillin and cephalexin were hydrolyzed to a much greater extent than Penicillin V, which had been assumed to be the primary substrate. Skrob et al. (2003) have characterized a novel penicillin G acylase that hydrolyzes ampicillin, amoxicillin and cephalexin at almost twice the rate of hydrolysis of Penicillin G. PVA from *Streptomyces lavendulae* has also been reported to have a greater specificity for Penicillin K and other aliphatic penicillins over Penicillin V (Torres-Guzman et al. 2002). Further purification of *B. cereus* PVA to homogeneity could help unravel the novel characteristics of the enzyme.

Conclusion

This study reports the isolation of a new PVA-producing strain ATUAVP1846 that was identified as *Bacillus cereus* using 16SrRNA and FAME analysis. The fermentation parameters for production of the enzyme were optimized and the effect of media components and supplements was also studied. *Bacillus cereus* ATUAVP1846 produced maximum PVA at 30°C, pH 7 after 24 h. Highest enzyme production was achieved using an optimal minimal medium containing 0.4% glucose and 0.3% $(NH_4)_2HPO_4$. The crude enzyme from *B. cereus* ATUAVP1846 was partially purified using ammonium sulfate fractionation and found to be most active at 40°C and pH 6. In addition, the enzyme preparation also showed unusual substrate specificity, having preference for ampicillin and cephalexin over penicillin V.

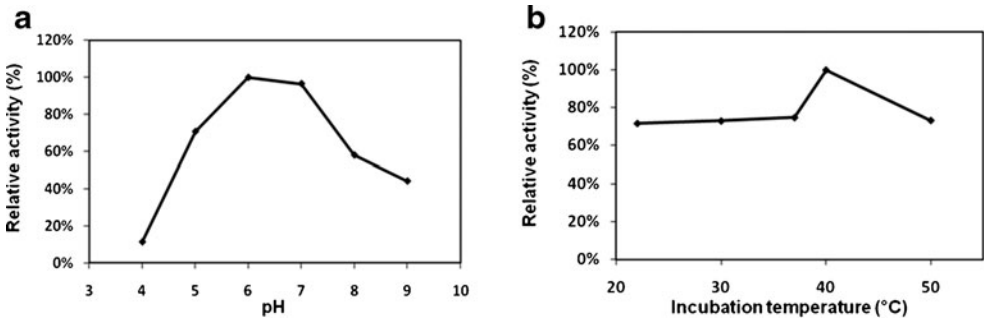

Fig. 4 Effect of **a** pH and **b** temperature on the activity of partially purified PVA from *B. cereus* ATUAVP1846. Enzyme activity values at pH 6 (6.97 IU/mL) and at 40°C (3.924 IU/mL) were considered 100%, respectively

Acknowledgments Avinash V.S. thanks the Council of Scientific and Industrial Research (CSIR), India for the award of Junior Research Fellowship (JRF). The authors thank Dr. Tanuja Gupte, Hindustan Antibiotics, Pune, India for providing Penicillin V potassium salt.

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