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COMPLETE SPECIFICATION

(See Section 10 and Rule 13)

A RECOMBINANT PENICILLIN V ACYLASE AND PROCESS FOR THE PREPARTION THEREOF

COUNCIL OF SCIENTIFIC & INDUSTRIAL RESEARCH,

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The following specification particularly describes the invention and the manner in which it is to be performed:

FIELD OF THE INVENTION

The present invention relates to a novel recombinant penicillin V acylase (PVA) enzyme and a process for the cloning and expression thereof. Further, the present invention relates to enhanced activity and increased specificity of the instant recombinant pen V acylase towards the synthesis of 6-amino penicillanic acid (6-APA).

BACKGROUND AND PRIOR ART OF THE INVENTION

Penicillin acylases cleave the acyl side chain of penicillins, and are mainly used in the production of semisynthetic beta-lactam antibiotics. Penicillin V acylases preferentially hydrolyze phenoxymethyl penicillin (penV). They belong to the Ntn-hydrolase protein family, with cysteine as N-terminal nucleophile. In addition, they display several more properties and therefore, are the subject matter of several studies.

Penicillin acylases (penicillin amidohydrolases/penicillin amidases), catalyze the selective hydrolysis of relatively stable amide bond in penicillins and some cephalosporins while leaving the labile β-lactam ring intact. Penicillin acylases are important in pharmaceutical industry for the production of semi-synthetic β-lactam antibiotics via the key intermediates 6-aminopenicillanic acid (6-APA) and 7-amino-3-deacetoxycephalosporanic acid (7-ADCA) (Shewale and Sivaraman, *Process Biochemistry* 24:146-154, 1989). Based on their substrate specificity, penicillin acylases have been classified into three groups: penicillin G acylases, penicillin V acylases and ampicillin acylases. Penicillin acylase is produced intracellularly and extracellularly in many kinds of organisms; however, the penicillin G acylase from *E.coli* has been prominently studied and used for industrial applications. Penicillin acylases can also be employed in other useful biotransformations, such as peptide synthesis (protection and deprotection of the amino groups of amino acids by direct enzymatic synthesis and acyl group transfer reactions) and the resolution of racemic mixtures of chiral compounds (amino acids, amines, β-amino esters and secondary alcohols) (Arroyo *et al*, Applied Microbiology and Biotechnology, 60:507-514, 2003).

Shewale and Sudharakaran in *Enzyme and Microbial Technology 20:402-410, 1997* state that pen V acylase is a better option than G-acylase for reasons including economics advantages as well its improved stability and better conversion rate to 6-amino penicillanic acid (6-APA). Penicillin V acylase shows higher stability in aqueous solutions at low pH during extraction from the fermented broth, which could lead to a higher yield of 6-APA. Penicillin V acylase also achieve higher conversion at higher substrate concentration as compared to Penicillin G acylases and its broader optimal pH range reduces buffering requirements during hydrolysis. Sudhakaran and Shewale *(World Journal of Microbiology and Biotechnology 9:630-634, 1993)* have used immobilized PVA from *Fusarium* sp. for the production of 6-APA.

The following table provides a comparison on the activity of the enzyme PVA from different sources.

Citation	Organism	Specific activity of enzyme
Current Work	*P. atrosepticum*	434 IU/mg(pure enzyme) 758 IU/g cells (cell bound activity)
D1	*Bacillus sphaericus*	20.2 IU/mg(pure enzyme)
D2	*Bacillus subtilis*	203.68 IU/g cells (cell-bound activity) 5.06 IU/mg pure protein (Rathinaswamy et al. 2012)
D3	*P. atrosepticum*	16.6 IU/mg(enzyme purified from wild type, not recombinant)
D4	*P. atrosepticum*	Only sequence mentioned, no functional/activity studies
D5	*P. atrosepticum*	Only sequence mentioned, no functional/activity studies

WO86/0929 by *Gatenbeck Sten et al* discloses a recombinant DNA molecule comprising at least one DNA sequence coding for penicillin V amidase or a derivative thereof, and to a process for its preparation. The inventors of WO'29 suggest that to produce PVA enzyme in a commcercial scale it would be highly desirable to use a microorganism which exhibits an increased production of penicillin V amidase. Penicillin V acylases from Gram-negative bacteria are enzymes secreted in the periplasmic region of cells; however this region of the cells is susceptible to activity of hydrolytic enzymes and cell wall leakage, and not conducive for production of large amounts of protein.

US Patent Publication No. 2005142652 relates to a recombinant plasmid, wherein pET-26b(+) cloning/expression region with SEQ ID No. 1 is cloned between BamH I site 198 and Nde I site 288. Also, it relates to a recombinant *E. coli* strain PTA 2456. Further, it relates to a process for the production of large amount of Penicillin V acylase using recombinant *E. Coli* strain PTA 2456. The amount of Penicillin V acylase obtained in the recombinant stain is about 57 to 65 times more than in the ordinary conditions. However, the drawbacks associated with this prior art are that the enzyme is less stable at acidic pH and the purified enzyme shows less activity than the present invention.

Reference may be made to a research study titled, "Structural modelling of substrate binding and inhibition in penicillin V acylase from *Pectobacterium atrosepticum*" authored by Avinash VS, et al published in *Biochem Biophys Res Commun. 2013 Aug 9;437(4):538-43*, which discloses considerable sequence and structural similarity between Penicillin V acylases (PVAs) and bile salt hydrolases (BSHs); however, they vary significantly in their substrate specificity. The inventors of D3 have identified PVA from Gram-negative bacteria, *Pectobacterium atrosepticum* (PaPVA) that turned out to be a remote homolog of the PVAs and BSHs that are already a part of the art. Even though the active site residues were conserved in *Pa*PVA it showed high specificity towards penV. The penV acylase activity was inhibited by bile salts. Comparative modelling and docking studies were performed to understand the structural differences of the binding site that confer this characteristic property. The study indicated that *Pa*PVA exhibited significant

differences in structure, which are contrary to those of known PVAs. Therefore, indicating that enzymes from Gram-negative bacteria require further investigation.

Despite the advantages of using PVAs, only 15% of all manufactured 6-APA worldwide is produced from Penicillin V acylases while the Pen G acylase from *E.coli* is more widely used. This is due to the slightly higher cost of the substrate (Pen V) and the non-availability of microbial strains with large enzyme production capability and PVAs with enhanced activity.
Therefore, there is a need in the art to provide alternate processes for synthesis of Penicillin V acylase and an enzyme with high specificity and conversion rate of Pen V to 6-APA. But the alternate provisions made should not take away the advantages pen V acylase enjoys over pen G acylase such as acid stability and improved conversion to 6-APA, along with economic advantages.

Therefore, keeping in view the aforesaid, the inventors of the present invention realized that there exists a dire need to provide recombinant penicillin V acylase which is secreted in the cytoplasmic region of the recombinant host strain thereby exhibiting enhanced specificity, increased activity, high stability at acidic pH and better conversion rate of penV to 6-APA, and as a consequence having great potential in the development of newer antibiotics especially semisynthetic beta-lactam antibiotics.

OBJECTS OF THE INVENTION

The main object of the present invention is thus to provide a novel recombinant penicillin V acylase (PVA) having enhanced enzymatic activity and increased specificity towards the synthesis of 6-amino penicillanic acid.
Another object of the present invention is to provide a recombinant PVA enzyme wherein the expression of the protein has been translocated to the cytoplasmic region of the cell.
Still another object of the present invention is to provide a process for the synthesis of the said recombinant penicillin V acylase.
Yet another object of the invention is to immobilize the instant recombinant pen V acylase enzyme on different supports so as to further improve its stability and reusability.

SUMMARY OF THE INVENTION

The present invention relates to a recombinant penicillin V acylase enzyme and a process for the synthesis thereof, wherein the said penicillin V acylase has enhanced specificity for the substrate i.e. phenoxymethyl penicillin (penV) and increased activity resulting in the synthesis of 6-aminopenicillanic acid.

Penicillin V acylase (PVA) is an enzyme employed in the removal of phenoxyacetic acid group of phenoxymethyl penicillin (Pen V) by hydrolysis to yield 6-aminopenicillanic acid (6-APA) which is used as a precursor in the commercial production of semi-synthetic penicillin.

Accordingly, the nucleotide sequence encoding the said penicillin V acylase enzyme lacking the periplasmic signal sequence is cloned into a plasmid cloning vector followed by expression of the gene in *Escherichia coli* to produce the protein i.e. penicillin V acylase in the cytoplasm in increased concentrations.

The nucleotide sequence encoding PVA is isolated from *Pectobacterium atrosepticum* (DSM 30186) and *Agrobacterium tumefaciens* (ATCC 33970). More particularly, the nucleotide sequence without that part of the gene sequence that encodes the periplasmic signal sequence, i.e. the 29 amino acid periplasmic signal sequence of penicillin V acylase is cloned into a cloning vector i.e. pET28b (*P. atrosepticum)* or pET22b (*A. tumefaciens)* followed by expression of the protein in E.coli. The expressed protein has a methionine residue added before the N-terminal cysteine, which is later processed by simple removal of methionine in the host *E. coli*. The protein is expressed in *E.coli* with a C-terminal 6X His-tag, in the cytoplasmic soluble fraction.
The enzyme synthesized by the instant process may be immobilized on solid support such as alginate, polyvinyl alcohol, acrylamide, Eupergit C and such like to improve the stability and reusability for industrial applications.
Accordingly, cloned *Escherichia coli* comprising the gene coding for pen V acylase from organisms selected form *Pectobacterium atrosepticum Pa*PVA and *Agrobacterium tumefaciens Atp*VA expressed in the *E.coli* is provided.

In an aspect, the present invention provides recombinant penicillin V acylase having at least 30% identity to Seq Id No. 1, wherein penicillin V acylase is devoid of the periplasmic signal sequence and encoded by nucleotide sequence having Seq ID No. 2.
In another aspect, the present invention provides a process for production of pen V acylase from cloned *E.coli* comprising cloning the gene encoding pen V acylase from organisms selected form *Pectobacterium atrosepticum* (PaPVA) and *Agrobacterium tumefaciens* (AtpVA) into a suitable vector and expressing the plasmid containing gene in *E.coli* BL 21 cells. The invention also provides a recombinant E.coli strain designated as MCC0018.
In yet another aspect, the present invention provides a process for the preparation of penicillin V acylase wherein the yield of penicillin V acylase is > 200 mg/l. Furthermore, the instant pen V acylase synthesized by the said process is stable at acidic pH.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

Figure 1 depicts SDS-PAGE of purified *Pa*PVA on 12% gel under denaturing conditions. Lane 1 –marker (individual molecular weight in kDa), 2 – *Pa*PVA in Fig 1(a) and MALDI spectrum showing subunit (monomer) molecular weight of *Pa*PVA in Fig 1(b).

Figure 2 depicts optimum pH in Fig 2(a) and temperature in Fig 2(b) for the instant recombinant *Pa*PVA enzyme activity. Activity at pH 5 and 45°C (360 IU/mg) taken as 100%.

Figure 3 depicts the effect of pH on PaPVA stability, wherein (a) depicts residual activity after 4h at pH 1to11, (b) Far UV CD spectra and (c) Near UV CD spectra of PaPVA at different pH.

Figure 4 depicts the effect of temperature on *Pa*PVA stability, wherein (a) Residual activity after 30 min incubation at temperatures 30-90°C and (b) Far UV CD spectra of PaPVA enzyme at different temperatures.

Figure 6 depicts the effect of solvents on *Pa*PVA activity. Enzyme was incubated with respective solvents for 30 min at 25°C and assayed. Activity with water was taken as control (100%).

Figure 7 depicts Kinetic *v* vs [S] curve for (a) Penicillin V and (b) NIPOAB substrates. $K_{0.5}$, V_{max} and *h* values are given in inset.

Figure 8 depicts (a) Activation and Inhibition of *Pa*PVA by bile salts at fixed Pen V concentration (50 mM); (b) *v*/[S] curves of increasing Pen V concentrations in the presence of different concentrations of GDCA.

Figure 9 depicts the multiple sequence alignment similar to *Pa*PVA using Clustal W. *Bsu*PVA (*B. subtilis),* *Bsp*PVA (*B. sphaericus*), *Bl*BSH (*Bifidobacterium longum*), *Pa*PVA (*Pectobacterium atrosepticum*), *Vc*PVA (*Vibrio cholerae*), *Bt*BSH (*Bacteroides thetaiotamicron)* and *Cp*BSH (*Clostridium perfringens)*. * Residues conserved in active sites of all choloylglycine hydrolases are highlighted.

DETAILED DESCRIPTION OF THE INVENTION

Deposition Details of the biological material employed in the instant invention

E. coli: Standard molecular biology cloning hosts, DH5alpha and BL21star cells were obtained from Life Technologies (Invitrogen™) vide catalog no.: DH5alpha - 18258-012, BL21 star cells - C6010-03 and plysS cell lines – C6060-03.

Pectobacterium atrosepticum was procured from DSMZ Germany vide No. DSM30186.

Agrobacterium tumefaciens was obtained from ATCC vide No. ATCC 33970.

The recombinant E.coli BL21 cells harbouring the cloned gene represented by SEQ ID No. 2 were deposited with NCCS, Pune, India on 07/April/2014 vide deposition No. MCC0018.

The present invention discloses a recombinant penicillin V acylase having an amino acid sequence depicted in SEQ ID No. 1 which is devoid of the periplasmic signal sequence and is the amino acid sequence of *Pectobacterium atrosepticum*.

In the most preferred embodiment, the present invention provides a recombinant penicillin V acylase having the amino acid sequence as represented by SEQ ID No. 1 and wherein the said penicillin V acylase is devoid of the periplasmic signal sequence and is encoded by nucleotide sequence represented by SEQ ID No. 2.

In accordance with this preferred embodiment, the instant recombinant PVA enzyme is devoid of the 29 amino acid periplasmic signal sequence that would otherwise direct the enzyme to the periplasmic region of the cell after protein translation. However, in the present invention the instant enzyme is expressed in the

cytoplasmic fraction of the host cell. Expression of foreign proteins in the inner or outer membrane of host cells may interfere with the normal cellular functioning of the cell and hence may be lethal to its regulation. Expression of protein in the cytoplasm would help in increased protein production, and would not interfere with protein folding, since the enzyme in the present invention doesn't contain any disulphide bonds.The instant recombinant penicillin V acylase secreted in the cytoplasm does not precipitate in inclusion bodies post incubation at 27°C. Yield of the instant recombinant protein is achieved in the range of 250 – 300 mg per litre of culture which is very high compared to the reported model recombinant proteins in *E.coli*.

Further, the present invention provides that directing the expression of the recombinant penicillin V acylase in the cytoplasm of the cell facilitates the retention of the enzyme with enhanced enzymatic activity. In group of enzymes belonging to the penicillin V acylase family, the catalytic residues including Cys1, Arg 19, Asp 22, Trp 87 and Arg 175 are well conserved. The penicillin V acylase isolated from *P. artosepticum* exhibits significant insertions in regions constituting the active site loops, restricting the size of the active site and making it highly specific for penicillin V. Therefore, the removal of the periplasmic signal sequence in penicillin V acylase protein across the spectra of Gram negative bacteria can be employed in synthesis of penicillin V acylase having at least 30% identity to SEQ ID No. 1 of the instant invention.

In an embodiment, the nucleotide sequence encoding penicillin V acylase is from Gram-negative bacteria, selected from *Pectobacterium atrosepticum* (PaPVA) and *Agrobacterium tumefaciens* (AtPVA).

Nucleotide sequence encoding penicillin V acylase annotated as a choloyglycine hydrolase sequence was retrieved from the NCBI database (PaPVA – Eca3205, AtPVA – Atu4586).
Accordingly, the gene sequences encoding penicillin V acylase [of the respective micro-organisms] were retrieved from GenBank and the genes were amplified in PCR using sequence-specific primers, wherein PaFP and PaRP represent the forward primer and reverse primer respectively for amplification of PaPVA and AtFP and AtRP represent the forward primer [FP] and reverse primer [RP] respectively for amplification of AtPVA.

*Pa*FP: GGC TAG CAT ATG TGT ACG CGG TTC GTT TAT CTG GAT CC (Seq Id No: 3)
*Pa*RP: GAT ACT CTC GAG GAG CCC CGC GAA TTC AAA CGG TTG (Seq Id No: 4)

*At*FP: GCT TGA CAT ATG TGC ACG CGT TTC GTT TAT ATA G (Seq Id No: 5)
*At*RP: CTG AAT CTC GAG AAG CCC GAG AAA CTT GAA AG (Seq Id No: 6)

The amplified DNA was cut at specific sites using restriction enzymes and ligated into an appropriate pET expression vector as given below. The recombinant vector was then transformed into *E.coli* BL21 star and plysS cell lines for protein expression. The respective restriction enzymes and the vectors used for the cloning of the amplified DNA are provided in the following Table 1.

Table 1: Restriction enzymes and the vectors employed in cloning

Source	Restriction enzymes	Vector	Antibiotic selection
Pectobacterium atrosepticum	NcoI/XhoI*	pET28b	Kanamycin (35 μg/ml)
Agrobacterium tumefaciens	NdeI/XhoI	pET22b	Ampicillin (100 μg/ml)

In another embodiment, the nucleotide sequence encoding the claimed recombinant penicillin V acylase [PaPVA] devoid of the periplasmic signal sequence is represented by SEQ ID No. 2.

The recombinant *E.coli* (BL21 star) cells were grown at 37° C in LB medium supplemented with respective antibiotic (Table 1) and induced after 3 h (A_{600nm} = 0.6) with 0.2 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside). After induction, they were transferred to 27° C and grown with shaking (200 rpm) overnight (12-14 h). The cells were harvested by centrifugation and sonicated to release the enzyme. The enzymes were purified in a single step using Ni^{2+} affinity chromatography since they contained a 6X C-terminal His-tag. After purification, the enzymes were dialyzed into suitable buffers to maintain their activity and stability. (PaPVA – 20 mM acetate pH 5.2, 100 mM NaCl and 1mM DTT; AtPVA – 10 mM Tris Cl pH 7.5, 100mM NaCl and 1mM DTT) *DTT - dithiothreitol

In another embodiment, the present invention provides synthesis of 6-APA using the recombinant *E.coli* as the suitable host cell for expression of recombinant *Pa*PVA yielding 60 mg/ml protein from 1 g recombinant cells, thus amounting to 250-300 mg protein/L culture.

In a characteristic embodiment, the instant recombinant penicillin V acylase is characterized by positive co-operativity and substrate inhibition.

Additionally, *Pa*PVA and *At*PVA also possess high specific activity (434 and 205 mol/min/mg respectively) and enhanced catalytic efficiency than other PVAs reported as yet, thus showing a high conversion rate (penV to 6-APA).

Table2: Comparison of specific activity values for reported PVA with PaPVA

PVA source	Specific activity (umol/min/mg)	References
Fusarium sp.	38-41	Sudhakaran and Shewale (World Journal of Microbiology and Biotechnology 9:630-634, 1993)
Streptomyces lavendulae	0.47	Torres *et al* (Progress in Biotechnology 15:719-724, 1998)
Bacillus sphaericus	29.2	Pundle and Sivaraman (Current Microbiology 34:144-148, 1997)
P. atrosepticum	434	Instant invention
A. tumefaciens	203	Instant invention

In a preferred embodiment, *Pa*PVA is stable for >6h till 50° C, in the pH range 3-6; while *At*PVA is stable at pH 4-7. These parameters like improved activity and stability provide a convincing argument for the potential of these enzymes in the pharmaceutical industry.

In another preferred embodiment, the instant recombinant penicillin V acylase is immobilized on solid substrates which may be selected from the group consisting of alginate, acrylamide, macroporous beads and such like to improve the stability and reusability for industrial applications.

In yet another preferred embodiment, the present invention provides compositions comprising the instant recombinant penicillin V acylase to catalyse the conversion of Penicillin V to provide 6-amino penicillanic acid in higher concentrations.

In another preferred embodiment, the present invention provides a process for the preparation of the recombinant penicillin V acylase represented by SEQ ID No. 1, wherein the steps comprising:

(a) cloning the nucleotide sequence having SEQ ID No: 2, wherein SEQ ID No: 2 is devoid of the part of the sequence encoding the periplasmic signal sequence of penicillin V acylase, into a vector;

(b) introducing the cloning vector of step (a) into a suitable host cell to express penicillin V acylase in high yield, and

(c) purifying the expressed PVA by affinity column chromatography,

wherein, penicillin V acylase is synthesized in the cytoplasmic fraction of the host cell.

Advantageously, the instant recombinant enzyme (3 mg/ml) stored at 4°C retained its activity for more than 30 days with a specific activity of 434 IU/mg.

Thus, the recombinant strain MCC0018 allows for large scale production of the enzyme with minimal equipment. The enzyme can be easily purified and has a high conversion rate of penV to 6-APA. It is also stable at pH 3-6 at 20-50° C, making it a highly suitable candidate for production of 6-APA and subsequent development of newer antibiotics. The enzyme can be immobilized on a suitable matrix to improve its stability and reusability.

EXAMPLES

Following examples are given by way of illustration and therefore should not be construed to limit the scope of the present invention.

Example 1

Cloning of nucleotide sequence encoding penicillin V acylase:

The nucleotide sequence encoding penicillin V acylase in *Pectobacterium atrosepticum* was retrieved from GenBank database (choloyl glycine hydrolase gene, ECA3205 – Accession no NC_004547.2). That part of

the nucleotide sequence encoding the periplasmic signal of penicillin V acylase was eliminated and the resultant *pva* gene was cloned in pET28b vector and expressed in *E. coli* BL 21 star cells.

Prior to cloning and expression of the gene sequence encoding penicillin V acylase, the nucleotide sequence devoid of that part of the sequence encoding the 29 amino acid periplasmic signal sequence before N-terminal cysteine was amplified using polymerase chain reaction (PCR) from the genomic DNA of the bacterium, with a sequence-specific pair of forward and reverse primers represented by SEQ ID No. 3 and 4 respectively.

These primers carried restriction sites for PciI and XhoI respectively (shown in bold). Amplification was carried out using genomic DNA (100ng) as template and 10 pmol of primer. After an initial denaturation of DNA at 95° C for 5 min, 30 cycles of denaturation (95°C for 30 s), annealing (54°C for 30 s) and extension (68°C for 60 s) were executed, with a final extension at 68°C for 10 min. The amplified gene (1.1 kb) was digested using the restriction enzymes PciI and Xho I for 4 h at 37°C to generate sticky ends compatible with the digested expression vector. The vector was prepared by restriction digestion of the pET28b plasmid with enzymes NcoI and XhoI. After digestion, the DNA samples were eluted from agarose gel. The digested PCR product and vector were ligated using T4 DNA ligase at 16° C overnight in a 10µl reaction. The ligation mixture was transformed into $CaCl_2$-competent *E.coli* DH5α cells by heat-shock carried at 42°C for 1 min and selected on Luria-Bertani medium (LB agar) containing 35 µg/ml kanamycin. The clones containing the ligated plasmid were confirmed by colony PCR with vector specific and sequence specific primers, and the insert was sequenced to confirm the absence of any mutations in the recombinant gene

Example 2

Expression of recombinant PVA

The plasmid vector pET28b comprising *Pa*PVA which was extracted from $CaCl_2$-competent *E.coli* DH5α cells was further transformed in *E. coli* BL21 star cells for protein expression. The resultant *Pa*PVA enzyme was found to be stably expressed in *E.coli* in soluble form, with a C-terminal 6X His tag. This was confirmed by electrophoresis (SDS-PAGE) and enzyme activity assays. The enzyme was expressed in the cytoplasm, since the nucleotide sequence coding for periplasmic signal was removed while cloning, thereby generating an amino acid sequence devoid of the periplasmic signal sequence.

Example 3

Process of production of PVA

The cloned cells of *E.coli-Pa*PVA [MCC0018] were maintained on LB agar containing 35µg/ml kanamycin. For production of the *Pa*PVA enzyme, the cells were cultivated in LB broth. A 1% inoculum was used and the cells were initially grown at 37° C for 3 h, with orbital shaking at 200 rpm. After reaching an OD (600nm) of 0.6, the protein production was induced by the addition of 0.2mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and the culture was transferred to 27° C at 200 rpm for overnight incubation. The cells were then harvested by centrifugation and stored frozen at -20° C. The protein did not precipitate in

inclusion bodies, even after overnight incubation at 27°C. Additionally, protein yields comfortably reached 250 – 300 mg per litre of culture, which is very high compared to model recombinant proteins in *E.coli*.

Enzyme Purification

About 1g cells were thawed and resuspended in lysis buffer [25mM TrisCl pH 7.0, 0.3M NaCl, 10mM $MgCl_2$) and sonicated for cell lysis. The sonicate obtained was subjected to clarification by centrifugation, and the supernatant was directly loaded on a HIS-Select Ni^{2+} affinity chromatographic column (Sigma) equilibrated with the lysis buffer in the presence of 2mM β-mercaptoethanol. The flow through was discarded, and the column was washed with the lysis buffer to remove unbound protein. The protein was then eluted from the column by passing the elution buffer [lysis buffer + 250 mM imidazole, final pH = 7.0] and the eluted protein fractions were dialyzed against 20mM acetate buffer pH 5 containing 1mM DTT. The purity of the protein was determined by SDS-PAGE and stored at 4°C.

A small amount of precipitate was formed on dialysis, which was clarified by high speed centrifugation. The enzyme (3 mg/ml) stored at 4°C retained its activity for more than 30 days. The final yield of protein was 62 mg/ g wet cells, with a specific activity of 434 IU/mg. *Pa*PVA exhibits many fold greater specific activity for Pen V than other PVAs from Gram-positive bacteria, actinomycetes, fungi and yeast. Such high activity and high protein yields make *Pa*PVA a valuable enzyme for use in the pharmaceutical industry.

Example 4

CHARACTERIZATION OF RECOMBINANT PENICILLIN V ACYLASE

Sequence Analysis

The nucleotide sequence of *Pa*PVA ECA3205 retrieved from the full genome sequence of *Pectobacterium atrosepticum* (NCBI Gene ID: 2881437) is annotated in the GenBank database as choloylgycine hydrolase, which also includes bile salt hydrolases. Phylogenetic clustering and biochemical assays have confirmed the enzyme as a penicillin V acylase.

Multiple sequence alignment with other reported choloylglycine hydrolase sequences was carried out using Clustal W.

The *pva* enzyme contains a 29-amino acid signal sequence that directs the enzyme to the periplasm. This signal peptide is present in most Gram-negative choloylglycine homologs; however, the length and sequence is dependent on the bacterial species.

Figure 9 depicts the sequence alignment results of *Pa*PVa with penicillin V acylase produced by other Gram negative and Gram positive bacteria. It is observed therein that in *Pa*PVA all the catalytic residues including Cys1, Arg 19, Asp 22, Trp 87 and Arg 175 are well conserved, The PaPVA enzyme also exhibits significant insertions in regions constituting the active site loops, compared to PVAs from other bacteria. All Gram-negative PVA sequences including *Pa*PVA lack the 20-amino acid sequence responsible for tetrameric subunit association. *Pa*PVA has only 25% sequence similarity with already characterized PVAs from

Bacillus and BSHs from Gram-positive bacteria, which furthers the argument for the uniqueness of the enzyme discussed in the present invention.

Determination of molecular weight

The subunit molecular weight was ascertained using Matrix-associated laser desorption ionization-mass spectrometry (MALDI, Perkin Elmer) using a sinapinic acid matrix. To determine the native molecular weight of *Pa*PVA, 200 µl of protein (7 mg/ml) was run on size exclusion chromatography column (ENrich^{TM} 650, 10 x 300 mm, BioRad) using a BioRad NGC^{TM}10 medium pressure chromatography system.

MALDI analysis exhibited a single peak of 39, 191 Da, corresponding to the subunit molecular weight of *Pa*PVA enzyme (Fig. 1). The native molecular weight was estimated to be 154 kDa using size exclusion chromatography, which confirms a tetrameric association. The isoelectric point of the enzyme was 8.4.

Determination of the effect of pH, temperature on Pa PVA activity and stability

The PVA activity was assayed at different pH values from 4 – 9 and temperatures varying from 20 – 70°C to ascertain the optimum conditions for enzyme activity. *Pa*PVA stability was studied using protein incubated in 20 mM acetate buffer pH 5.0 for 30 min at temperatures from 30 to 90°C. Effect of pH was studied by incubating the protein in 100 mM buffers of different pH (1-11) for 4 h and assaying the residual activity.

The instant recombinant *Pa*PVA enzyme exhibited maximum activity at an optimum pH of 5 (acetate buffer) in the assay (Peak obtained around pH 5 in Fig. 2). The enzyme showed a narrow pH spectrum (4-6) of activity and was most active in the acidic pH range. The optimum temperature for activity was 45°C; the enzyme showed very little activity when temperatures reached 60°C.

Although the enzyme was fairly stable in a wide range of pH (3-10), protein precipitation occurred at high concentrations (>0.5mg/ml) in alkaline pH, due to proximity to the isoelectric point. The far UV CD spectrum showed a significant change in the minima when incubated at pH 1; and there was a loss of ellipticity in the near UV region corresponding to a collapse in tertiary structure (Fig. 3).

The enzyme showed no significant changes in secondary structure till 70°C (Fig. 4). *Pa*PVA showed significant loss of activity only at 80°C; this is an improvement in stability over previously reported PVA/BSH enzymes reported from Gram-positive bacteria that lose their tertiary structure at 60°C. This is an additional favourable feature for industrial applicability of *Pa*PVA, besides high yield and specific activity..

Analysis of protein conformational stability and unfolding

The conformational stability of *Pa*PVA was studied using fluorescence and circular dichroism (CD) measurements to determine the changes in the secondary structure content and tertiary structure of the protein at different pH (1-11), temperature (30- 90°C). Samples (1ml, 25 µg *Pa*PVA) in 20 mM acetate buffer pH 5.0 were used to study the protein stability. Far UV CD spectra were recorded on a Jasco-J815

spectropolarimeter (Jasco, MD, USA) at 25 °C using a cell of 0.1 cm path length. Each spectrum was the average of 3 scans (with correction of buffer signal) from 250 to 190 nm with 1 nm bandwidth and a scan speed of 100 nm/min. Near UV spectra were recorded from 350 to 310 nm using a 0.5 cm cell. The raw CD signal was converted to mean residue ellipticity, and linear fits and graphs were generated using MicroCal Origin software.

Kinetic parameters and inhibition

Kinetic constants (Km and Vmax) were measured by assaying the instant recombinant enzyme activity with increasing concentrations of penicillin V (potassium salt, Sigma) as substrate, 5-240mM under the optimum conditions. GraphPad Prism version 5.01 (GraphPad software, La Jolla California USA, www.graphpad.com) was used to fit the kinetic data to an allosteric sigmoidal plot using non-linear regression. In case of the synthetic substrate NIPOAB, the hydrolysis of the substrate was followed at 405 nm in a spectrophotometer with increasing substrate concentrations (0.5-12mM). The effect on PVA activity by the presence of bile salts or POAA was estimated using a range of concentrations of the compound (5µM to 40mM) and 50mM Pen V. Plots of v/[S] were prepared at 0.1, 1 and 10mM glycodeoxy cholic acid (GDCA) concentration with pen V as substrate.

Kinetics of substrate binding

PVAs reported so far have followed normal Michaelis-Menten (MM) kinetics. However, *Pa*PVA kinetics was more complex with distinct deviation from MM curve, showing cooperative behaviour and substrate inhibition (Fig. 7). The kinetic parameters for the enzyme were computed using the initial part of the curve before the onset of substrate inhibition. The Hill's equation was used for computing the parameters $K_{0.5}$, h and Vmax. *Pa*PVA exhibits a high Vmax value (602 IU/g), much greater than any other acylase active on Pen V reported so far. Apart from longer loops surrounding the active site, the enzyme also contains two tryptophans (W23, 87) that are probably involved in aromatic stacking interactions to stabilize the substrate binding and thus increase the catalytic rate. *Pa*PVA also showed a Hill's coefficient of 2.105, indicating apparent positive cooperativity. Cooperative behaviour of bile salt hydrolase has been reported in *Lactobacillus salivarius* [23], while the enzyme showed curves similar to MM kinetics when DTT was present in the assay mixture. However, in the case of *Pa*PVA, the presence or absence of DTT did not cause any significant change in the allosteric nature of the enzyme. Cooperative behaviour was also seen with the synthetic substrate NIPOAB, although with a lower Hill's coefficient (h=1.768), and higher $K_{0.5}$ (= 11.532 mM) compared to other PVAs (Fig. 7).

Another interesting aspect of *Pa*PVA was the occurrence of apparent substrate inhibition at concentrations higher than 80 mM Pen V. Substrate inhibition has not been reported in any choloylglycine hydrolase so far Previously characterized PVAs and BSHs have been reported to follow classical MM kinetics, with no substrate inhibition even at high concentrations of Pen V or GDCA.

Using a homology model of *Pa*PVA and enzyme purified from native *P. atrosepticum,* the inhibitory action of bile salts on *Pa*PVA by binding to the active site in reverse orientation was shown. On extending the range of concentrations of bile salts tested, low concentrations of bile salts (5 to 500 μM) activated the enzyme to a maximum of 140%, while concentrations from 1 mM inhibit the enzyme at fixed substrate concentration ([Pen V] = 50 mM). Kinetics curves at three different GDCA concentrations (0.1 mM, 1 mM and 5mM) indicate changes in both Vmax and Km, signifying a competitive inhibition component (Fig. 8). The enzyme was also not inhibited by POAA till very high concentrations (>50mM).

Example 5

Enzyme activity assay

The enzyme activity of *Pa*PVA was determined by estimating the concentration of 6-APA formed from Penicillin V using p-dimethyl amino benzaldehyde *(Shewale et al, Biotechnology Techniques, 1:69-72, 1987).* The instant enzyme (1ug) was incubated with 50mM Pen V in 0.1 M acetate buffer pH 5 for 5 mins; then an equal volume of CPB (citrate-phosphate buffer pH 2.5) was added to quench the reaction. The supernatant was diluted 4x with CPB and treated with 0.6% w/v pDAB to form a yellow-coloured product (read at 415 nm).

The reaction was carried out for 5 min with 1.2 μM enzyme and 50 mM Pen V. One unit (IU) of PVA activity was defined as the amount of enzyme required to liberate 1 μmol of 6-APA per min under the mentioned assay conditions. In the case of NIPOAB used as substrate, the enzyme was added to 1ml of 2 mM substrate (2% DMSO effective concentration).

The *Pa*PVA enzyme showed a very high specific activity around 430 IU/mg for Pen V, which is many fold higher than reported values for penicillin V acylases. It did not hydrolyze any other beta-lactam substrate to a significant extent; however, it was inhibited by bile salts (Avinash *et al*, Biochemical and Biophysical Research Communications, 437:538-543, 2013). PaPVA showed optimum activity at pH 5 and 45° C.

Example 6

Stability of *Pa*PVA

Stability of *Pa*PVA was determined by incubating the instant recombinant enzyme (100ug) in 0.1M buffer having varying pH in the range 1-11, temperatures in the range of 30-50°C and assaying for residual activity. The enzyme was stable in acidic pH range 3-6 and till 40°C, showing <25% loss of activity after 48h. Loss of enzyme activity was significant after 6h in the alkaline pH range (7-10). The enzyme also showed aggregation and drastic loss of activity at temperatures above 60°C within 1 h.

Example 7

Effect of protein modifiers on enzyme activity

*Pa*PVA was extensively dialyzed to remove additives and was incubated with reducing agent DTT and metal-chelating agent ethylene diamine tetraacetic acid (EDTA) in a 100μl reaction mixture for 30 min at

25ºC. Enzyme activity was assayed after incubation; untreated enzyme served as control. Employing similar experimental conditions the effect of divalent metal ions, detergents and solvents was also studied. All experiments were performed independently in triplicates and results expressed as averages with <5% standard deviation.

Results indicated loss of enzyme activity when DTT was removed; there was moderate improvement with the re-addition of DTT, although the enzyme did not regain its original activity. The enzyme was stored in a buffer containing 100 mM NaCl and 1mM DTT; and DTT was not included in the assay buffer. There was no significant change in *Pa*PVA activity in the presence of EDTA. In the presence of metal ions that bind to sulfhydryl groups (Hg, Ag), the enzyme was completely inactivated within 15 min. The activity of *Pa*PVA was significantly enhanced by treatment with detergents for 30 min at room temperature. Cationic detergent CTAB increased the enzyme activity by over 250%, while non-ionic detergents (Tween 80, Triton) enhanced the activity by 150% at concentrations higher than their CMCs. Detergents are known to reverse the aggregation of proteins and stabilize them, which could be a reason for the enhanced activity. The anionic detergent SDS, however, rapidly deactivated the enzyme at 0.1% concentration (Table 1).

Certain solvents like isopropanol, 2-butanone, acetonitrile and ethyl acetate were also found enhance *Pa*PVA activity (150-200%) at 5% (v/v) concentration in the assay mixture (Fig. 6). There was a gradual increase in enzyme activity till 15% ethylene glycol. The protein was stable for 4 h in 10% isopropanol, losing <20% of its original activity; higher concentrations deactivated the enzyme rapidly. Other alcohols and glycols caused a moderate reduction in PVA activity. Aprotic solvents (DMSO, DMF, dioxan and tetrahydrofuran), and non-polar hydrocarbons (chloroform, dichloromethane) inhibited or deactivated the enzyme. The increase in activity in the presence of isopropanol could be a result of its nucleophilic effect helping in the deacylation of the intermediate-acyl enzyme, thereby releasing in the synthesis of the product faster.

Table 3

Additives	**Concentration**	**Relative activity (%)**
DTT	1 mM	114.5 ± 0.4
	10 mM	123.2 ± 6.1
βME	1 mM	119.0 ±5.8
	10 mM	105.3 ± 3.2
EDTA	1 mM	101.1 ± 4.5
	10 mM	101.1 ±6.3
CTAB	0.1 %	250.1±4.7
	0.5 %	270.6±0.6
Triton X-100	0.1 %	180.4±1.3
	0.5 %	185.3±0.5
Tween 20	0.1 %	175.1±4.3
	0.5 %	153.3±2.9
SDS	0.1 %	8.9±1.7
	0.5 %	0

Example 8

Immobilization of recombinant PVA

The instant recombinant penicillin V acylase was immobilized on compatible substrates thereby facilitating the use of the enzyme repeatedly, thus indicating the biodegradable nature of the enzyme. The enzyme was immobilized on substrates selected from the group consisting of alginate, acrylamide, and macroporous beads.

Method and composition of the immobilized enzyme

E. coli BL 21 containing the recombinant PaPVA enzyme was grown as in example 3 and cells were harvested using centrifugation. The cells (10 mg) were suspended in 0.5 ml distilled water, and mixed with equal volume of a solution of 4% sodium alginate (Himedia, India). The alginate-cell mixture was taken in a syringe and extruded through a 0.5mm needle into a solution of 0.2M $CaCl_2$ while stirring. The calcium alginate beads formed were hardened in the calcium chloride solution for 30 min and then washed with deionized water and stored at 4°C. Activity of the *Pa*PVA enzyme in immobilized cells was assayed using the calcium alginate beads as detailed in example 5.

Immobilization matrix	Cell-bound activity (IU/g cells)	Activity recovery (%)
Free cells	758.02	100
1% Ca-alginate	178.77	23.6
2% Ca-alginate	211.95	28

ADVANTAGES OF THE INVENTION

- The claimed recombinant penicillin V acylase exhibits high specificity, enhanced activity towards Pen V and yield of enzyme.
- It is free from inclusion bodies.
- The recombinant enzyme is easy to purify by a single step process.
- Secretion of the protein is in the cytoplasm, rather than the periplasmic region, which facilitates better production and recovery of the instant recombinant *Pa*PVA, since it evades the reactions with proteolytic enzymes present in the periplasm,
- The stability of *Pa*PVA and optimum activity in acidic pH augurs well for industrial catalysis of Pen V, since the substrate is also stable in the same pH range.

SEQUENCES USED IN THE INVENTION

SEQ ID No. 1: Amino acid sequence of recombinant penicillin V acylase devoid of the periplasmic signal sequence

SEQ ID No. 2: Nucleotide sequence encoding the recombinant penicillin V acylase of SEQ ID No.1.

SEQ ID No. 3: FORWARD PRIMER FOR THE AMPLIFICATION OF PaPVA.

SEQ ID No. 4: REVERSE PRIMER FOR THE AMPLIFICATION OF PaPVA.

SEQ ID No. 5: FORWARD PRIMER FOR THE AMPLIFICATION OF AtPVA.

SEQ ID No. 6: REVERSE PRIMER FOR THE AMPLIFICATION OF AtPVA.

SEQ ID No. 7: Amino acid sequence of the recombinant AtPVA (*Agrobacterium tumefaciens)* devoid of periplasmic signal sequence

SEQ ID No. 8: Nucleotide sequence encoding the recombinant penicillin V acylase of SEQ ID No.7

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We claim:

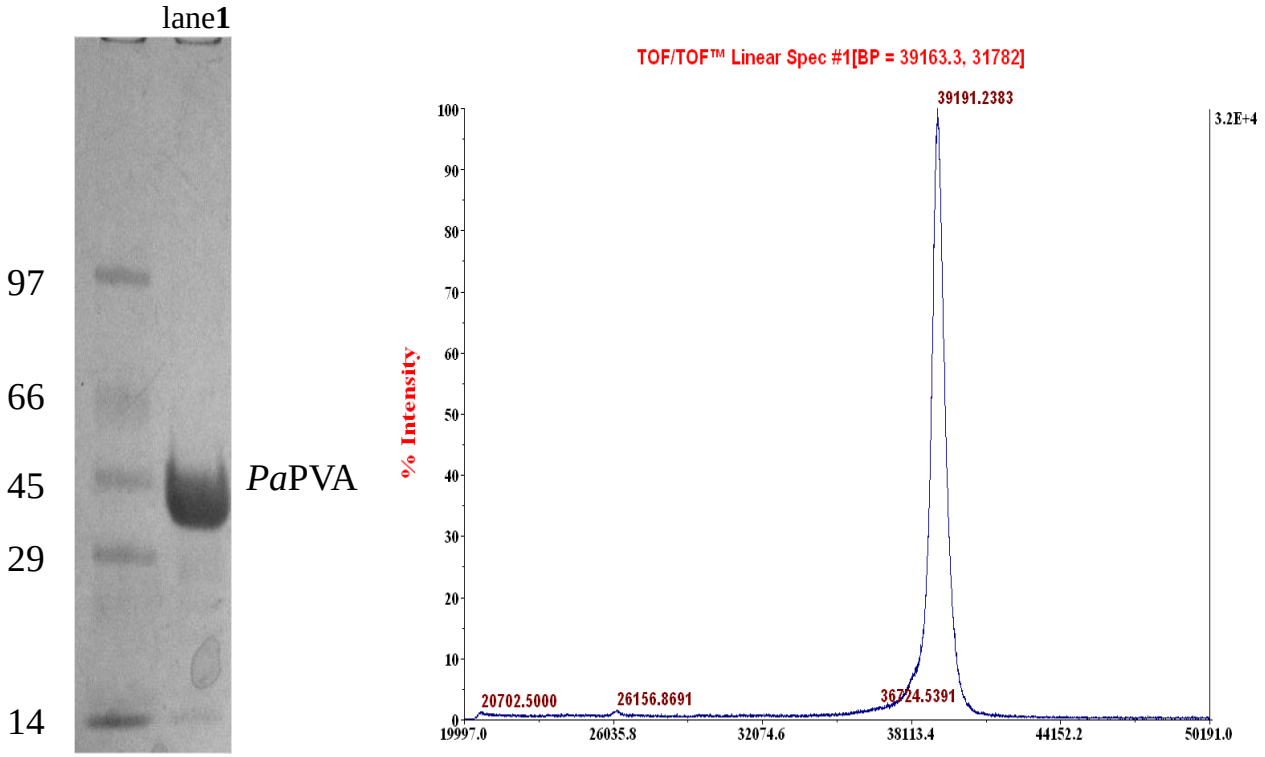
1. A recombinant penicillin V acylase enzyme represented by SEQ ID No. 1.
2. The enzyme as claimed in claim 1, wherein it is encoded by the nucleotide sequence represented by SEQ ID No. 2, which is devoid of the part of the sequence encoding the periplasmic signal sequence.
3. The enzyme as claimed in claim 1, wherein SEQ ID No. 2 is cloned in a vector and expressed in a host cell.
4. The enzyme as claimed in claim 1, wherein the vector is selected from the group consisting of pET28b and pET22b.
5. The enzyme as claimed in claim 1, wherein the host cell is *E. coli* BL 21.
6. The enzyme as claimed in claim 1, wherein it is stable at pH ranging from 3 to 6 and at temperature ranging from 20 to 50 degree C.
7. The enzyme as claimed in claim 1, wherein it is immobilized on solid substrates selected from the group consisting of alginate, acrylamide, and macroporous beads.
8. A process for the preparation of the recombinant penicillin V acylase represented by SEQ ID No. 1 and as claimed in claim 1, wherein the steps comprising:
   (a) cloning the nucleotide sequence represented by SEQ ID No. 2 into a vector;
   (b) introducing the cloning vector of step (a) into a suitable host cell to express penicillin V acylase in high yield, and
   (c) purifying the expressed PVA enzyme by affinity column chromatography,

   wherein, the prepared recombinant penicillin V acylase enzyme is secreted in the cytoplasmic fraction of the host cell.
9. The process as claimed in claim 8, wherein the yield of penicillin V acylase is in the range of 250 to 300 mg/l of culture.
10. A recombinant bacterial strain of MCC0018.

ABSTRACT

A RECOMBINANT PENICILLIN V ACYLASE AND PROCESS FOR THE PREPARTION THEREOF

The present invention provides recombinant penicillin V acylase represented by SEQ ID No.1 having enhanced activity and increased specificity towards the synthesis of 6-amino-penicillanic acid. The nucleotide sequence encoding the said penicillin V acylase enzyme lacking the periplasmic signal sequence is cloned into a plasmid cloning vector followed by expression of the gene in *Escherichia coli* to produce the desired recombinant protein i.e. penicillin V acylase in the cytoplasm in increased concentrations.



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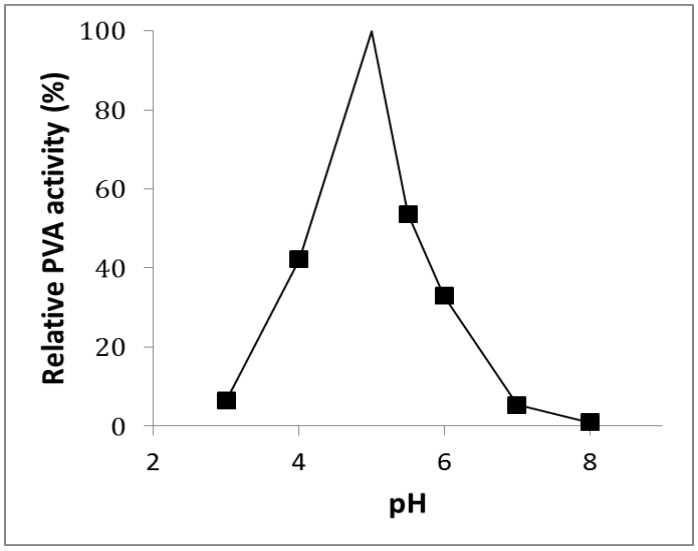

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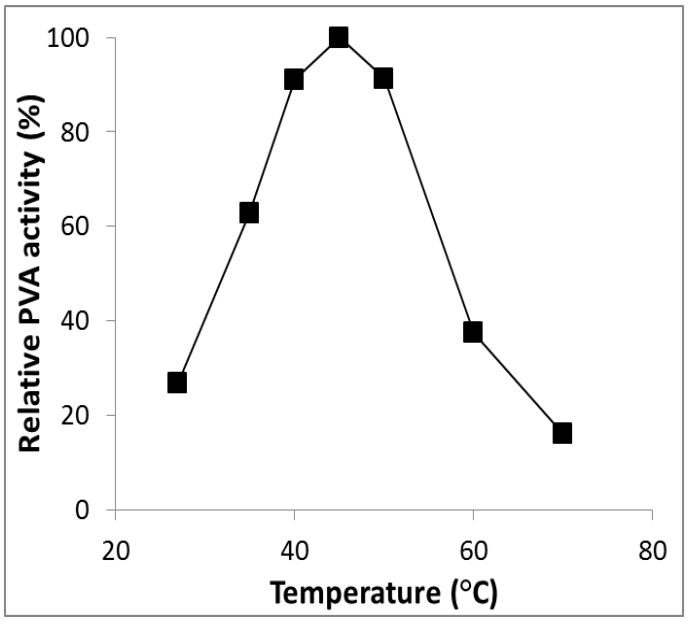

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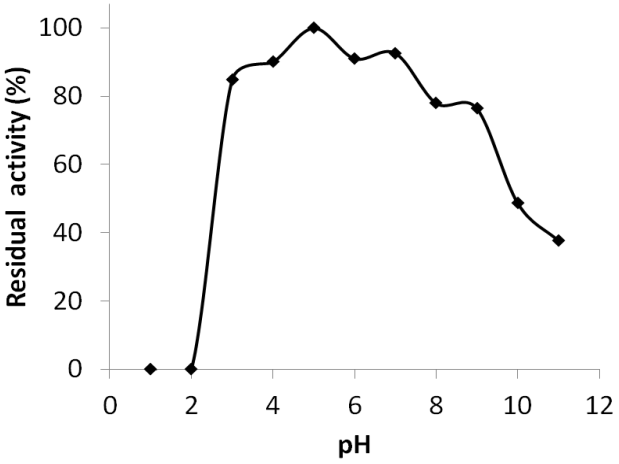

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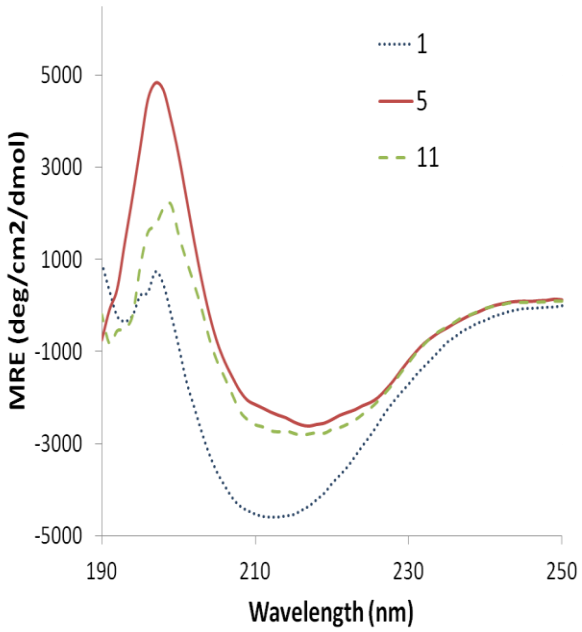

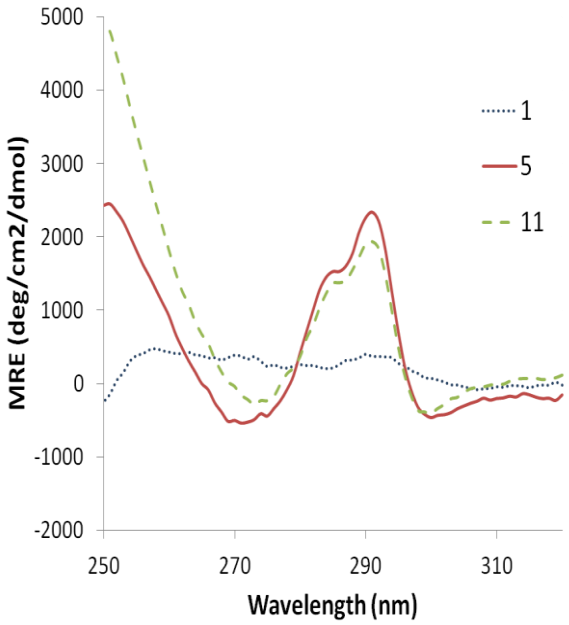

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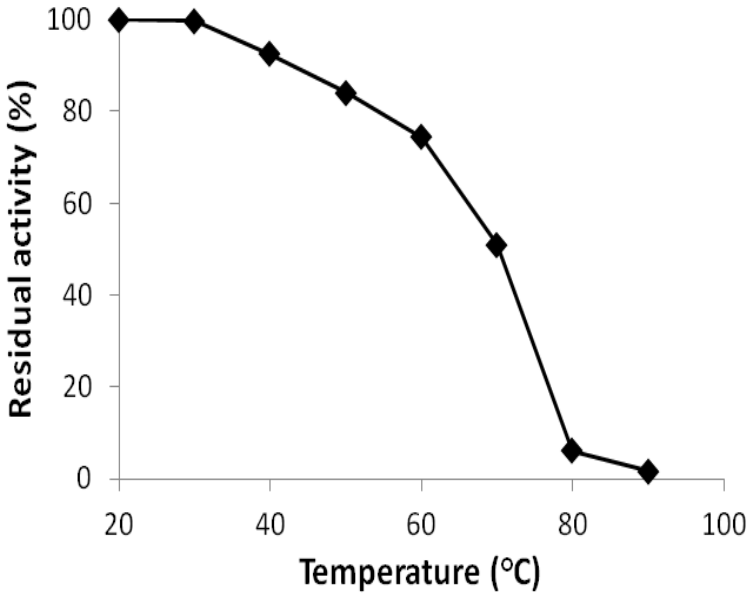

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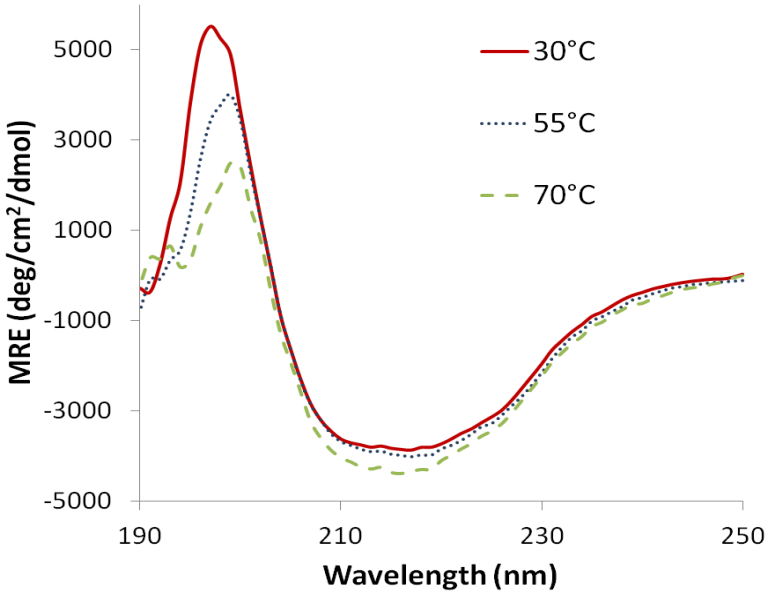

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Figure 4

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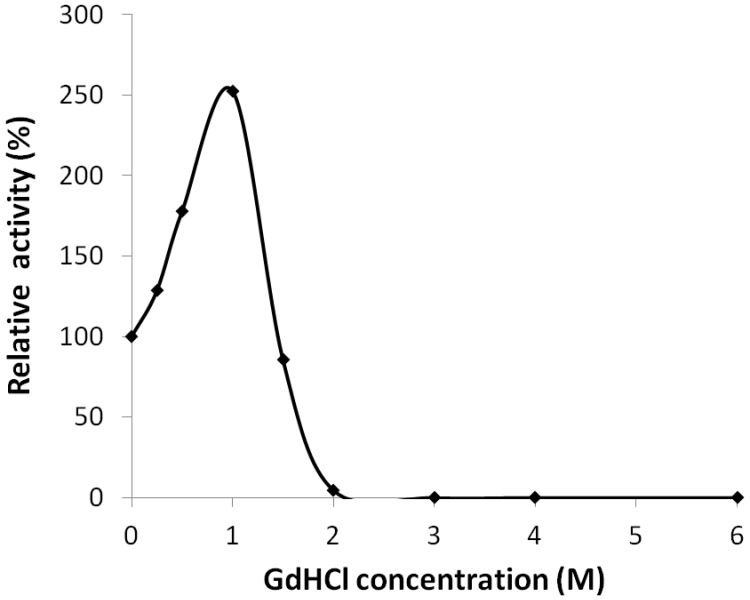

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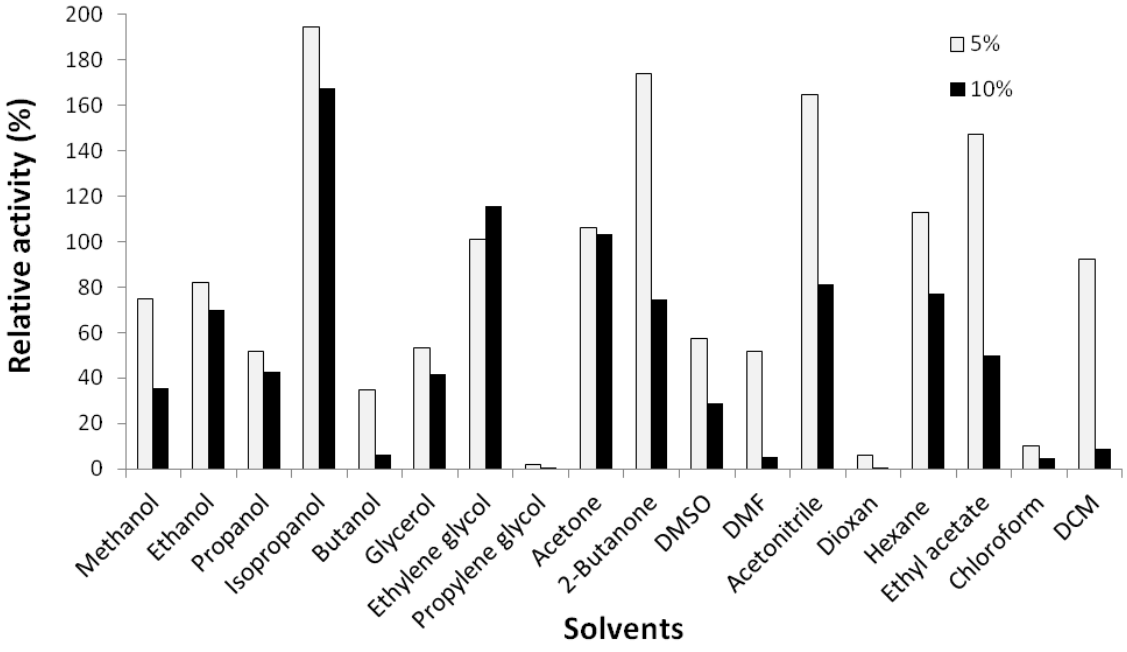

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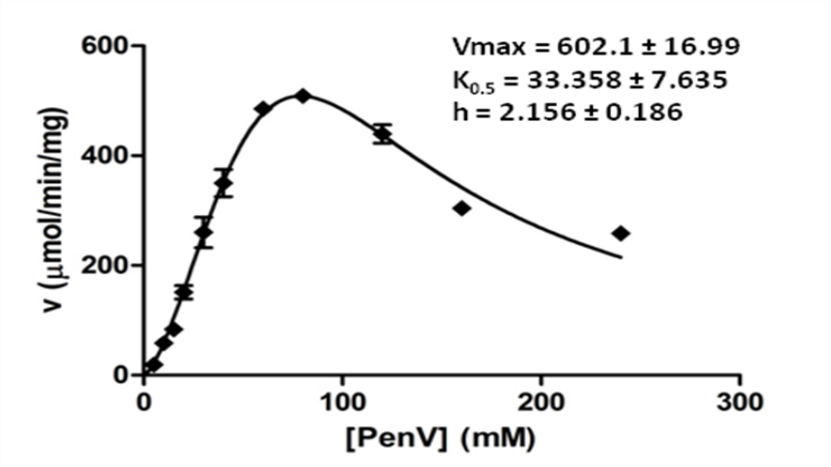

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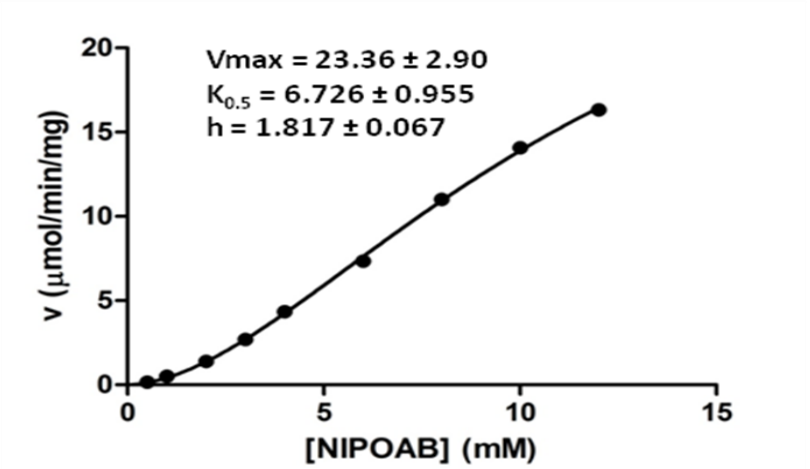

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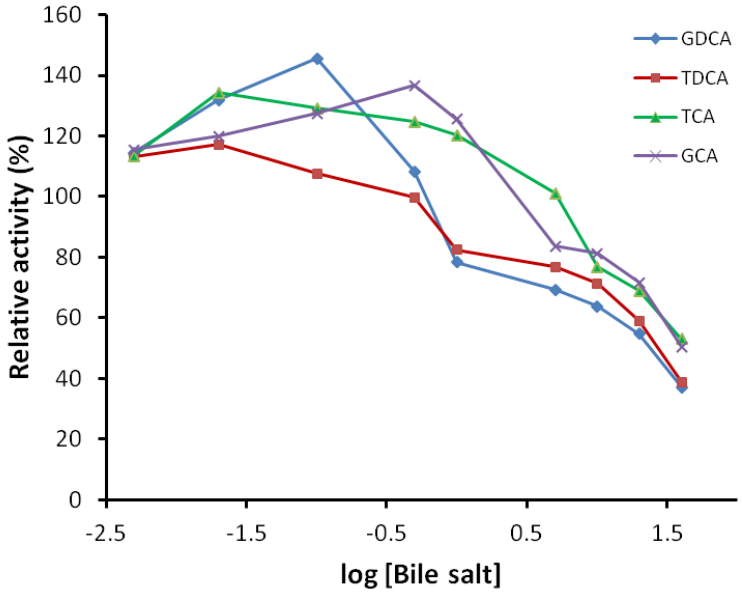

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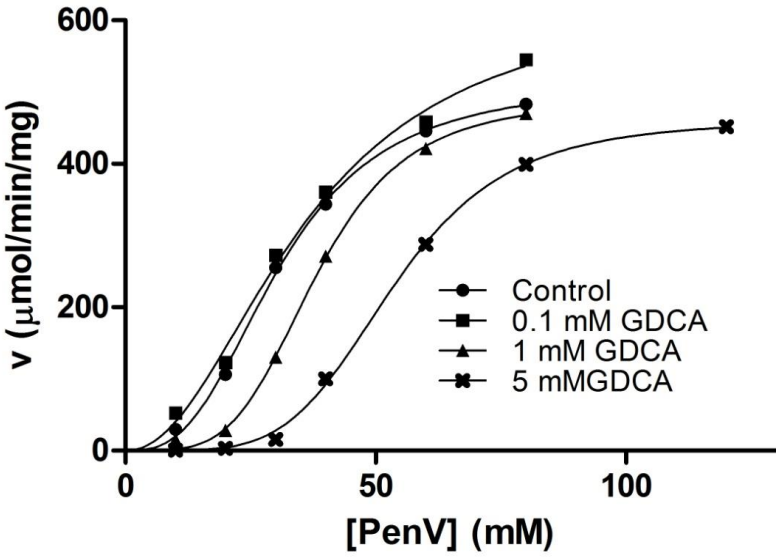

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Figure 8

APPLICANT

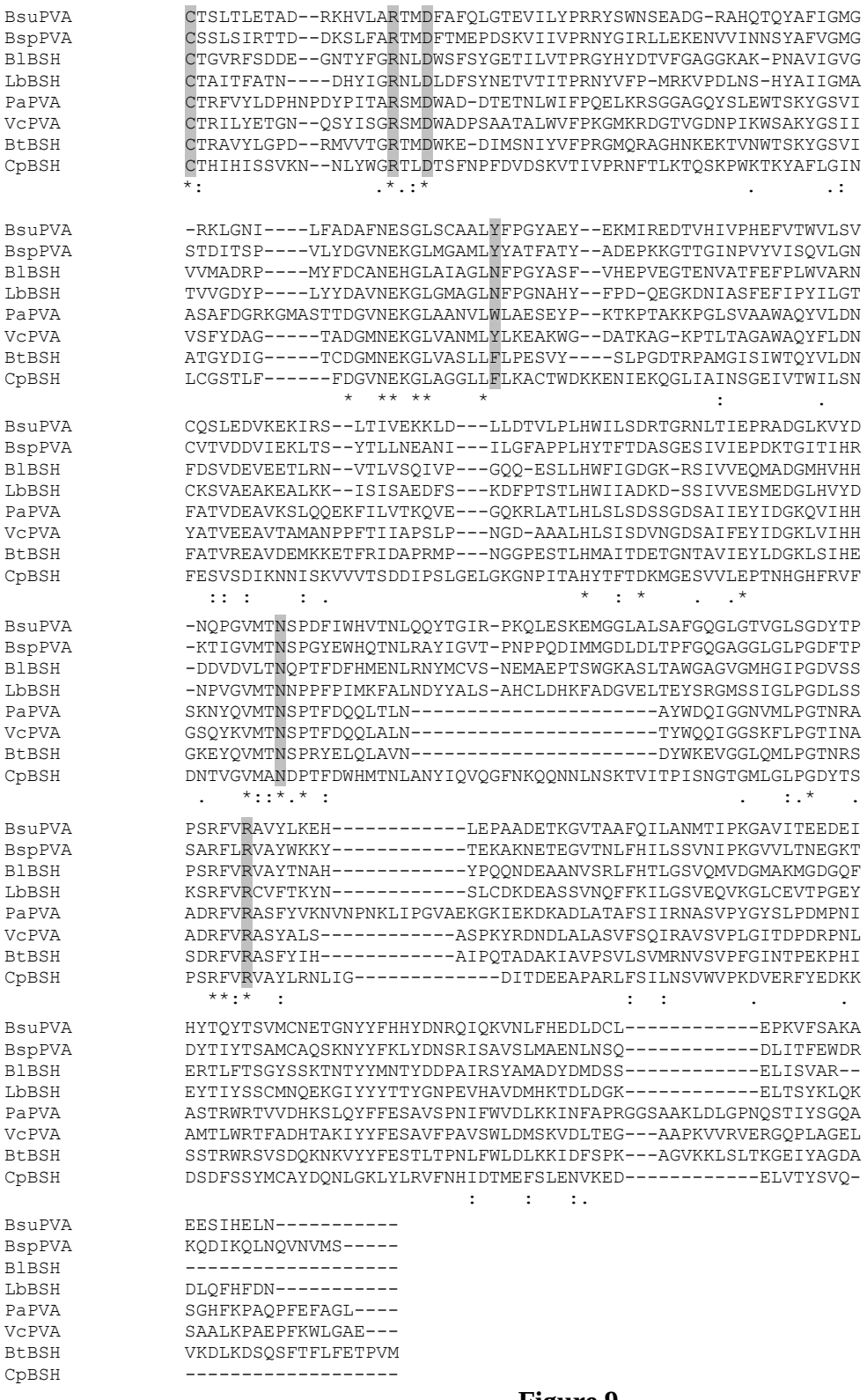

Figure 9

APPLICANT