ENZYMES FROM SOLVENT TOLERANT *PSEUDOMONAS AERUGINOSA* PseA FOR BIOTECHNOLOGICAL APPLICATIONS

by

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DEPARTMENT OF CHEMISTRY

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Enzymes, Formation - Biotechnological application

Dedicated to my Parents

CERTIFICATE

This is to certify that the thesis entitled "Enzymes from Solvent Tolerant *Pseudomonas aeruginosa* PseA for Biotechnological Applications" being submitted by **Ms. RUCHI GAUR** to the Indian Institute of Technology, Delhi for the award of the degree of *Doctor of Philosophy* in Chemistry is a record of bonafide research work carried out by her. Ms. Gaur has worked under my guidance and supervision, and has fulfilled the requirements for the submission of the thesis which, to my knowledge, has reached the requisite standard.

The results contained in this dissertation have not been submitted in part or full to any other University or Institute for the award of any degree or diploma.

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Ruchi

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Abstract

The thesis concerns the studies on enzymes from solvent tolerant strain of *Pseudomonas aeruginosa* PseA. The main part of the work deals with the studies on adaptive features imparting solvent stability to the bacteria, purification and characterization of solvent stable lipase and aminopeptidase, molecular basis of adaptation and their biotechnological implications.

Solvent tolerant bacteria are relatively new group of extremophilic microbes that thrive in the presence of high concentration of solvents. There is an increasing interest in their enzymes for use in non-aqueous enzymology. *P. aeruginosa* PseA, an organic solvent tolerant strain previously isolated from soil by solvent enrichment was considered for its growth in presence of solvents of varying hydrophobicities. The effects of solvents on PseA cells and their adaptation strategies were studied by Transmission Electron Microscopy, Atomic Force Microscopy, UV-VIS spectrophotometry and other techniques.

P. aeruginosa PseA was found to be a lipase and aminopeptidase producer indicated by substrate agar plate assays. It secreted novel lipase, which exhibited remarkable stability in solvents with log *P* ranging from 2.5-7.0, non-ionic/ anionic surfactants, oxidizing agents and native/ commercial proteases.

To enhance the production level of this potential lipase, the media optimization was carried out by the Response Surface Methodology (RSM). Optimization enhanced the lipase production by 6.06 fold over that of un-optimized media. Glucose as carbon source (1.0 g/L), tryptone, (10.0 g/L) and yeast extract (0.2 g/L) as nitrogen and vitamin sources, gum arabic (0.2 g/L), $MgSO_4$ (1.0 g/L) and $NaNO_3$ (0.2 g/L) as metal ion and salt in culture media (pH 6.5), inoculated with 2% of 12 h grown mother culture and incubation for 48 h at 25°C with shaking at 120 rpm were found to be the best culture condition leading to maximum lipase production of 4970 IU/ml. This level of lipase is comparable to other strains of *P. aeruginosa*.

P. aeruginosa PseA lipase was purified 8.6 fold with 51.6% recovery by ultrafiltration and Sephadex® G-100 gel filtration chromatography. The relative molecular mass of enzyme was estimated to be ~60 kDa by Sodium dodecylsulphate-polyacrylamide gel electrophoresis. It was recovered in the form of aggregates. The purified PseA lipase exhibited *K*m: 70.4 mM and *V*max: 2.24 µmol/ min/ mg towards *p*-nitrophenyl palmitate (*p*NPP) as substrate. The lipase was most active at 40°C. It preferred long chain fatty acid ester (C16) as substrate. Cysteine was found to be important for PseA lipase activity. The PseA lipase was remarkably stable in both hydrophilic and hydrophobic solvent. The stability in hydrophilic solvents was unique feature of *P. aeruginosa* PseA lipase. It was found to be detergent compatible being stable in surfactants and bleach oxidants.

To explore the biotechnological potential of PseA lipase, its application in oil hydrolysis was investigated. It hydrolyzed palm oil most efficiently among olive,

coconut, palm, mustard, linseed and castor oils. Conditions for palm oil hydrolysis were optimized by RSM. The optimized conditions were: PseA lipase, 317 IU in 230 µl water, pH 6.0, palm oil (0.1g in 2 ml isooctane containing 0.02%, v/v Tween-80) with continuous shaking at 150 rpm and 30°C. 89% palm oil was hydrolysis within 2 h under these conditions. Palm oil hydrolysis by whole cells of *P. aeruginosa* PseA was also undertaken. The conditions were optimized by RSM as, minimal medium 'M' supplemented with palm oil (2%, w/v) and Tween-80 (0.2%, v/v); temperature, 30°C and pH, 6.5. PseA whole cells hydrolyzed 80% palm oil in 48 h under optimized conditions. The oil hydrolysis correlated well with lipase secretion by PseA.

P. aeruginosa PseA, aminopeptidase, was also studied. It was purified to homogeneity by Q-Sepharose® anion-exchange chromatography attaining 11 fold purification with 21% yield. The Mr of PseA aminopeptidase was estimated to be ~56 kDa. Partial N-terminal sequence of purified enzyme was found to be "T-P-G-K-P-N-P-S-I-C-K-S-P-L-L". PseA aminopeptidase exhibited *K*m: 3.02 mM, *V*max 6.71 µmol/ mg/ min, pH optimum: 8.0, temperature optimum: 60°C towards $_{L}$-leu-*p*-nitroanilide as substrate. The enzyme was stable in the pH range of 6.0-8.0 and temperature 60 and 70°C. The enzyme belonged to the class of zinc metallo-peptidases. PseA aminopeptidase was remarkably stable in both hydrophilic and hydrophobic solvents. Here again, the stability in hydrophilic solvents is a unique feature of PseA aminopeptidase not reported in other *P. aeruginosa* aminopeptidases.

To establish the structural features responsible for imparting solvent tolerance, the primary structure, conserved motif and hydropathy profile of PseA aminopeptidase were

investigated and compared with other known metallo-aminopeptidases. The N-terminal amino acid sequence of the purified PseA aminopeptidase was used to search NCBI database for its homologues. An exact match with "probable aminopeptidase" having alternate gene name *pepB* from *P. aeruginosa* PAO1, chromosome no PA2939, was found. The c-DNA of PAO1 was used to design the polymerase chain reaction primers in order to clone the corresponding gene of PseA. The clone DNA fragment contained a 1,611 base pair open reading frame encoding a 536 amino acid polypeptide (57.5 kDa).

The deduced protein contains a segment of first 24 residues as a signal peptide (2.57 kDa) followed by a 1.28 kDa propeptide (12 residues) and the 53.6 kDa mature enzyme (500 residues). The alignment of full length amino acid sequence showed that the zinc binding residues His-296, Asp-308, Glu-341, Asp-369 and His-467 in PseA aminopeptidase were highly conserved in the aminopeptidases belonging to family M28 (clan MH). This indicated PseA aminopeptidase to be a new member of family M28 of the metallo-peptidases.

P. aeruginosa PseA lipase Protein Coated Microcrystals (PCMCs) were successfully prepared using *n*-propanol as the precipitating solvent. Transmission Electron Microscopy confirmed the formation of microcrystals. PCMCs were thermally more stable showing 4.65, 2.56 and 1.24 folds higher half-lives ($T_{1/2}$) at 45, 55 and 60°C as compared to free lipase. PseA lipase PCMCs were more efficient catalyst under non-aqueous conditions.

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