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Isolation and Characterization of *Pseudomonas aeruginosa* Strain-VA8 that produces Heme Oxygenase (PigA(HO))

Kalieswaran Vidhya, Balraj Janani, Jairaman Karunyadevi, Sunder Sunganya, Jayaraman Angayarkanni*

Department of Microbial Biotechnology, Bharathiar University, Coimbatore, Tamil Nadu, India.

***Corresponding author:* Jayaraman Angayarkanni, Department of Microbial biotechnology, Bharathiar University, Coimbatore 641046, India.**

Abstract:

Heme oxygenase (HO) is a microsomal enzyme which catalyses the first and rate limiting step in the degradation of heme. Human heme oxygenase-1 (HO-1) was reported to have cytoprotective, anti-apoptotic and anti-inflammatory activity. The *HMOX* genes were ubiquitously expressed in most living organisms like bacteria, algae, plants, insects and mammals. *Pseudomonas aeruginosa,* an opportunistic pathogen which is a major cause of nosocomial infections worldwide, was found to have heme degradation activity. Under pathogenic iron limiting conditions *P.aeruginosa* will internalise host heme and degrade it by producing an enzyme called heme oxygenase (PigA(HO)). The PigA(HO) is a product of *PigA* gene and it degrades heme to Fe^{2+}, biliverdin and carbon monoxide (CO). Since both human HO-1 and PigA(HO) catalyses heme degradation, the present study was aimed at the isolation and characterization of *P.aeruginosa* that produces PigA(HO). The organism was isolated from hospital waste dump site soil and was identified as *P.aeruginosa* Strain-VA8 by 16S rRNA gene sequencing (GenBank Id: KJ419347.1) and phylogenetic analysis. The organism was confirmed to produce PigA(HO) by heme oxygenase enzyme activity assay. The molecular weight of PigA(HO) was determined to be 23kDa by SDS-PAGE analysis and enzyme activity was confirmed by zymography.

Keywords: Heme oxygenase, *P.aeruginosa,* cytoprotective, anti-apoptotic, anti-inflammatory.

Introduction:

Heme oxygenase (HO, EC 1.14.99.3) was identified by Tenhunen and his colleagues in 1968 when they tried to describe the mechanism for heme catabolism. HO is a microsomal enzyme which catalyses the first and rate limiting step in the degradation of heme (Tenhunen et al., 1968). There are three known isoforms of HO, of which HO-1 is an inducible and HO-2 and HO-3 are constitutively expressed (Morse and Choi, 2005).

The substrate for HO activity i.e., heme exists essentially as the prosthetic group of heme proteins. Under oxidative stress, some hemeproteins can release their prosthetic heme group, which catalyses the production of free radicals in an unfettered manner. Cells can avoid this pro-oxidant effect of free heme by rapid induction of HO-1, which increases the rate of free heme catabolism and protects the cell from oxidative stress (Seixas et al., 2009).

HO-1 expression will increase in response to various physical stresses like hypoxia, UVA irradiation and chemical stress like and heme, Sodium arsenite, NO, CoPPIX, ZnPPIX, Cobalt chloride respectively (Ryter et al., 2006). Upregulation of HO-1 is responsible for cytoprotection against various physical and chemical stresses. Studies on HO-1 deficient mice proved that this system is indispensable for survival and protection from oxidant stress (Poss and Tonegawa, 1997). The first human case of HO-1 deficiency was reported in a 6-year-old boy, who suffered similar symptoms as like HO-1 deficient mice. The symptoms include stunted growth, anemia, iron deposition, leukocytosis, persistent proteinuria and hematuria, coagulation defects, hyperlipidemia, and hypobilirubinemia. Microscopic examination of renal biopsy samples revealed mesangial cell proliferation, lymphocyte infiltration, and detachment of the glomerular capillary endothelium (Yachie et al., 1999). These findings suggested the importance of HO-1 as a cytoprotective agent and a cheaper and easily available source of HO should be found to use this enzyme as a therapeutic agent. *Pseudomonas aeruginosa* was reported to have heme degradation activity and *pigA* gene of *P.aeruginosa* was shown to encode heme oxygenase protein (PigA(HO)) (Ratliff et al., 2001).

P.aeruginosa is a gram-negative rod shaped opportunistic pathogen and is carried by ~10% of healthy human population. It has the potential to severely infect immuno-compromised patients, who are infected with cystic fibrosis, emphysema, cancer or serious burn victims and is becoming a leading

cause of nosocomial infections in hospitals and community settings (Ochsner et al., 2000). Pathogenic bacteria require iron for its infection, colonization and survival inside the host but the availability of free iron in the human host is very low (10^{-9}M) because iron is sequestered into iron containing compounds and iron binding proteins (Wang et al., 2007). Since *P.aeruginosa* prefers an aerobic metabolism it requires respiratory enzymes that needs iron for their function hence it has relatively high demand for iron. Iron restriction plays a central role in the stress response of *P.aeruginosa* which has thus evolved numerous iron acquisition systems. These include the release of siderophores, production of extracellular proteases and secretion of cytotoxic exotoxin A (Ochsner et al., 2000). *P.aeruginosa* also has sophisticated mechanisms to utilize iron from heme and other iron-binding proteins through two operons regulated by *Fur* (Fe uptake regulator). The two operons were *phu* (*Pseudomonas* heme uptake) and *has* (Heme acquisition system). After internalization of heme into cytoplasm, heme will be delivered to PigA(HO) for its degradation to biliverdin, Fe^{2+} and CO. Biliverdin and CO will be released out and Fe^{2+} will be utilized by *P.aeruginosa* for their metabolic use (Wang et al., 2007). Since *P.aeruginosa* was reported to have heme degradation activity through production of PigA(HO) the present study was aimed at the isolation and characterization of *P.aeruginosa* from hospital waste dump site and to confirm the ability of organism to produce PigA(HO).

Materials and methods:

Chemical and media components:

Hemin, NADPH, solvents, media component, all other chemicals and rat liver cytosol were purchased from Himedia and Thermo Fisher Scientific respetively.

Sample collection:

The soil samples were collected in pre-sterilized bags from hospital waste dumping areas near Vadavalli, Coimbatore, India, after digging the ground for about 10 cm.

Enumeration of bacteria from soil sample:

Soil samples collected were sieved and weighed accurately. The weighed samples were serially diluted up to 10^{-8} dilutions and spread plated on nutrient agar plates. The plates were incubated at 37˚C

for 24 h. The bacterial colonies grown in 10^{-4} to 10^{-6} dilutions were taken for further tests (Dubey and Maheshwari, 2002).

Screening for *P.aeruginosa*:

The colony morphology, pigmentation and other colony characters similar to *P.aeruginosa* were observed on the plates. The bacterial colonies (Strain-VA1 to VA25) were taken for gram staining. The colonies which had gram negative rod-shaped organisms (Strain-VA2, 5, 8, 10, 12, 15, 16, 17 and 23) were taken for catalase test. The colonies which showed catalase positive (Strain-VA-5, 8, 15 and 17) were taken for oxidase test. The oxidase positive colonies (Strain-VA8 and 17) were picked and quadrant streaked on King's B medium (*Pseudomonas* selective medium) and then on Cetrimide agar medium (selective medium for isolation of *P.aeruginosa*) and incubated overnight at 37˚C. The organism grown in King's B medium was observed under UV light for fluorescence. The pure culture (Strain-VA8) grown in Cetrimide agar plate was sub cultured and refrigerated for further study (Dubey and Maheshwari, 2002).

Biochemical and molecular characterisation:

The biochemical characterization of the isolated Strain-VA8 was done by series of biochemical tests including Carbohydrate fermentation, IMViC, TSI, Urease, Hemolysis, Starch and Gelatin hydrolysis (Dubey and Maheshwari, 2002). Further for molecular characterization, the amplification of 16S rRNA gene was done in PCR using Universal 16S Forward and 16S Reverse primer (initial denaturation at 95˚C (5 min), denaturation at 94ºC (30 sec), annealing at 54ºC (30 sec), primer extension at 72ºC (45 sec) and primer elongation at 72˚C (10 min)). The PCR product was column purified and sequenced using AB instrument model 3730XL. The obtained sequence was subjected to the closest possible species search using BLAST programme of NCBI and phylogenetic analysis was done using MEGA software.

Extraction of intracellular enzyme (PigA(HO)):

The isolated Strain-VA8 was cultured in nutrient broth and the cells were pelleted at 10000 rpm for 15 min at 4˚C. The pellets were washed with sterile cold double distilled water by centrifuging at 10000 rpm for 10 min at 4°C. The washed pellets were suspended in 50mM Tris-HCl (pH 7.8) containing 1mM EDTA (pH 8.0) and 1mM PMSF. The suspended cells were sonicated for 2 min then the intracellular

enzymes were extracted by centrifuging at 10000 rpm for 20 min at 4°C. The supernatant collected was stored at -20˚C and used as crude enzyme (Maines et al., 1986). The protein concentration of the crude enzyme was estimated by Lowry's method using BSA as a standard (5-50mg) (Lowry et al., 1951). Then the crude enzyme was partially purified by Ethanol precipitation. The crude enzyme and pre-chilled ethanol (3:1) were mixed and incubated overnight at 4˚C. Then the precipitate was collected by centrifuging at 10000 rpm for 20 min at 4˚C. The pellets were dissolved in 50mM Tris-HCl and stored at -20˚C for further analysis.

Enzyme activity assay for PigA(HO):

To 500µL of enzyme, 200µl of 100µM Hemin, 50µl of 0.8µM NADPH, 10µl of Rat liver cytosol (20mg/ml) and 240µL of 50mM Tris-HCl (pH 7.0) were added and incubated at 37°C in dark for 1 h. After incubation period 1mL of chloroform was added and vortexed vigorously. The chloroform layer was separated by centrifuging at 10000 for 5 min. The bilirubin extracted in chloroform layer was measured by taking the difference in absorbance at 464 and 530nm (e = 40 mM $^{-1}$cm^{-1}) (Motterlini et al., 2000). Then the enzyme activity was expressed in terms of µM of bilirubin formed per mL of enzyme and the specific activity was expressed in terms of µM of bilirubin formed per mg of protein.

SDS-PAGE analysis

The molecular weight of PigA(HO) was determined by SDS-PAGE analysis using 15% gel with standard protein marker in the first lane. Then the gel was placed in CBB-Staining solution overnight and de-stained until the band appeared (Walker, 2002)

Zymography:

The enzyme activity of PigA(HO) in partially purified enzyme was confirmed by Zymography (an electrophoretic technique based on SDS-PAGE, which included the substrate copolymerization with separating gel. The Zymogram analysis was performed by modifying the method of Tracz. The sample and Zymogram loading dye (1:1) without SDS was kept in shaking condition for 20 min. Then the sample was loaded in Separating gel (12%) which contains 50µL of Hemin (1mg/mL of DMSO) and Stacking gel (5%). After electrophoresing for 3 h under cooling condition, the gel was washed with 2.5 % Triton-X-100

for 2 hr and incubated overnight in Activation buffer which mimics the PigA(HO) enzyme assay condition. Then the gel was stained with CBB-R-250 Staining solution for 3 hr and de-stained (Tracz et al., 2008).

Results:

Screening for *Pseudomonas aeruginosa*

P.aeruginosa is a gram-negative rod shaped bacterium, which grows as flat elevated colonies with irregular borders and it shows positive for both catalase and oxidase test. Based on this, about twenty-five strains (Strain-VA1 to VA25) were isolated from soil sample collected from hospital waste dump site. Since hospital settings had more number of nosocomial infection causing pathogens, soil sample from the hospital waste dump site was chosen for the present study. Among 25 strains isolated, nine strains (Strain-VA2, 5, 8, 10, 12, 15, 16, 17 and 23) which showed gram negative rod-shaped organisms on gram staining were taken for catalase test. The Catalase positive strains (Strain-VA5, 8, 15 and 17) were taken for oxidase test and oxidase positive strains (Strain-VA8 and 17) were grown in King's B medium and then on Cetrimide agar medium. The Strain-VA8 showed green fluorescence in King's B medium, which showed that the strain could produce fluorescein and it belongs to *Pseudomonas* species **(Fig. 1 a)**. The Strain-VA8 also had the capacity to grow in cetrimide agar plates, which is a selective medium for isolation of *P.aeruginosa* **(Fig. 1 b)**.

a **b**

Fig. 1 Isolated Strain-VA8 grown in selective media [a) Cetrimide agar; b) King's B medium]

Biochemical and molecular characterization:

The macro and microscopic characteristics of the selected strain-VA8 were shown in **Table 1**. The strain-VA8 showed flat, elevated colonies with irregular borders and grape-like odour which is a characteristic odour of *P.aeruginosa*. The organism produced green pigment and showed purple colour rods (Gram negative) under microscopic examination on Gram staining.

Table 1 Macro and Microscopic characters of the selected Strain-VA8

S.No	Character	Observation
1.	Colony morphology	**Flat elevated colonies with irregular borders**
2.	Pigmentation	**Green pigmentation**
3.	Odour	**Grape-like odour**
4.	Gram staining	**Gram negative rods**

Table 2 Biochemical characterization of the selected Strain-VA8

S.No	Biochemical test	Observation
1.	Catalase, Oxidase, Citrate	**Positive**
2.	Indole, MRVP, Starch and Gelatin hydrolysis, Urease	**Negative**
3.	TSI	**Alkaline slant-Alkaline butt**
4.	Hemolysis	**β-hemolysis**

The biochemical characteristics of the selected Strain-VA8 were shown in **Table 2**. The selected Strain-VA8 showed positive for catalase, oxidase and citrate test. On TSI and hemolysis test the selected Strain-VA8 showed alkaline slant-alkaline butt and β-hemolysis respectively. The selected Strain-VA8

showed negative for indole, MRVP, Starch hydrolysis, Gelatin hydrolysis and Urease test. On carbohydrate fermentation test, the selected Strain-VA8 showed positive for glucose, fructose, dextrose, Galactose and negative for sucrose, maltose, lactose, sorbose, xylose and inulin (**Table 3**).

Table 3 Carbohydrate fermentation profile of selected Strain-VA8

S.No	Sugar	Observation
1.	Glucose, Fructose, Dextrose, Galactose,	**Positive**
2.	Sucrose, Maltose, Lactose, Sorbose, Xylose, Inulin	**Negative**

From the macro and microscopic examination and biochemical characterization it was confirmed that the isolated Strain-VA8 belonged to ***Pseudomonas*** species and the strain was able to grow in *P.aeruginosa* selective medium. Further molecular characterization was done to confirm the selected Strain-VA8 to be *P.aeruginosa* at Genus level.

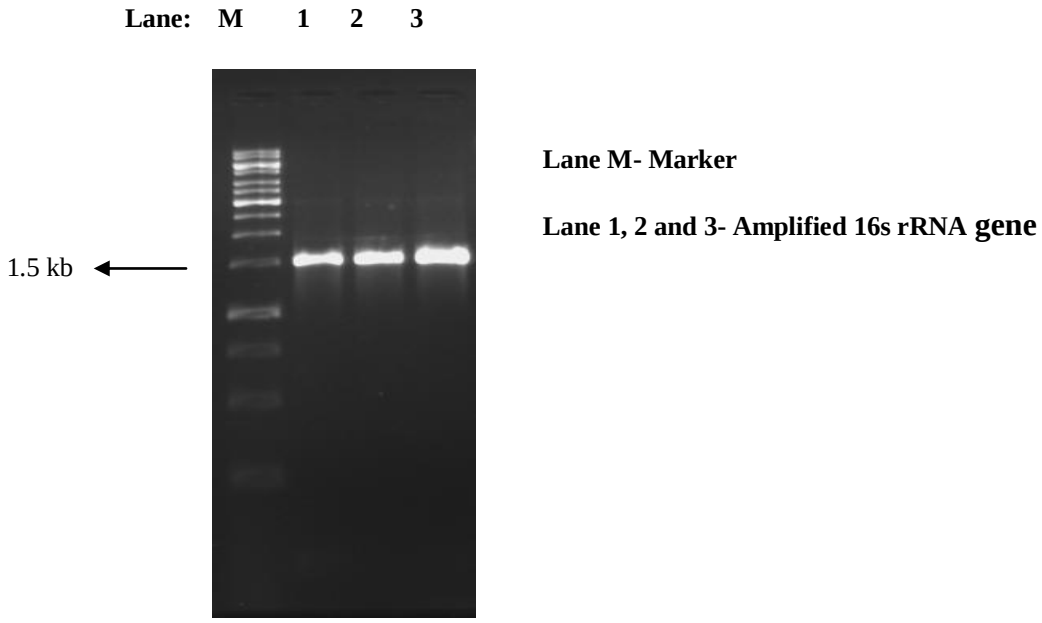

Fig. 2 Amplification of 16s rRNA gene

The amplified PCR products showed a band near 1.5 Kb, which is the length of 16S rRNA gene (**Fig. 2**). The PCR product was sequenced, aligned and submitted to NCBI (**GenBank Id: KJ419347.1**). The Phylogenetic tree was constructed which showed that the isolated Strain-VA8 was closely related to type strain NR 026078 *P.aeruginosa*^{T} and the isolated Strain-VA8 showed 99 % relatedness to the type strain (**Fig. 3**). From the above results, it was confirmed that the isolated Strain-VA8 was *P.aeruginosa*.

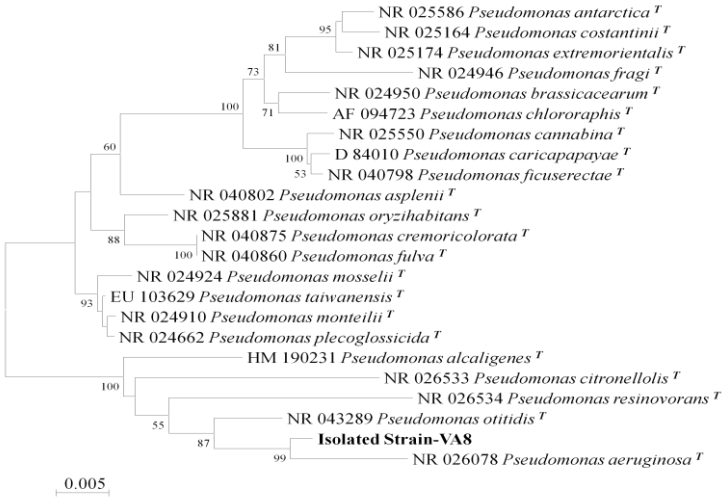

Fig. 3 Phylogenetic tree of isolated Strain-VA8

Enzyme assay for PigA(HO):

The PigA(HO) enzyme activity assay was performed and the results were shown in **Table 4**. The enzyme activity of crude enzyme and ethanol precipitate was found to be 3.4 and 1.65 U/mL respectively. Whereas the specific activity of crude enzyme and ethanol precipitate was found to be 3.569 and 4.125 U/mg. From the results, it was clear that the isolated Strain-VA8 had PigA(HO) enzyme activity and the enzyme activity was not lost but increased during ethanol precipitation.

Table 4 Enzyme assay for PigA(HO)

Enzyme	Protein content (mg/mL)	Enzyme activity (U/mL)	Specific activity (U/mg)
Crude	0.98	3.4	3.569
Ethanol precipitate	0.4	1.65	4.125

SDS-PAGE analysis:

On SDS-PAGE analysis a clear band behind 20 kDa marker was observed both in crude enzyme and ethanol precipitate which could be attributed as PigA(HO) (23KDa) (**Fig. 4**).

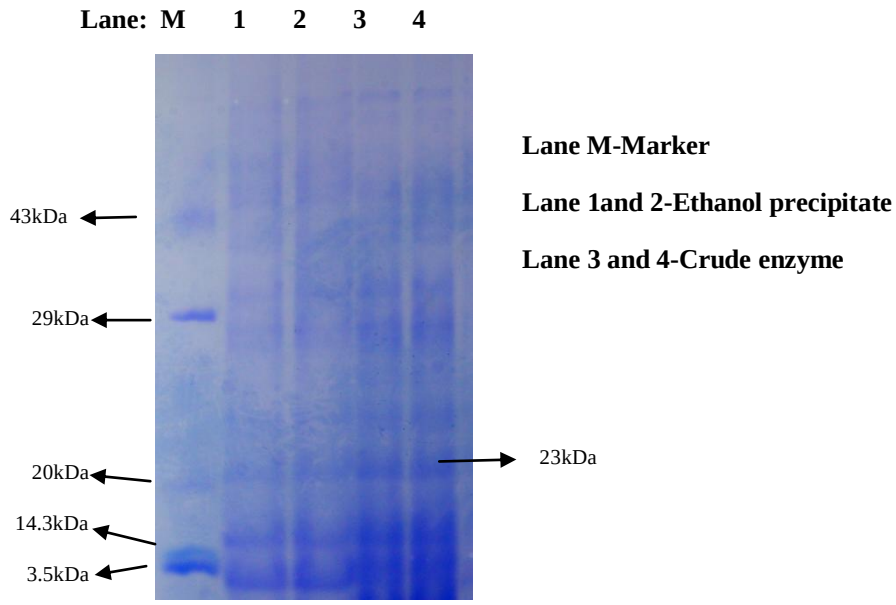

Fig.4 SDS-PAGE analysis of PigA(HO) from isolated Strain-VA8

Zymography:

The zone of lysis was observed in separating gel copolymerised with substrate hemin (**Fig. 5**). The clear zone was appeared surrounding the lane, which might be due to the migration of PigA(HO) through the lane which eventually caused lysis of hemin surrounding the lane.

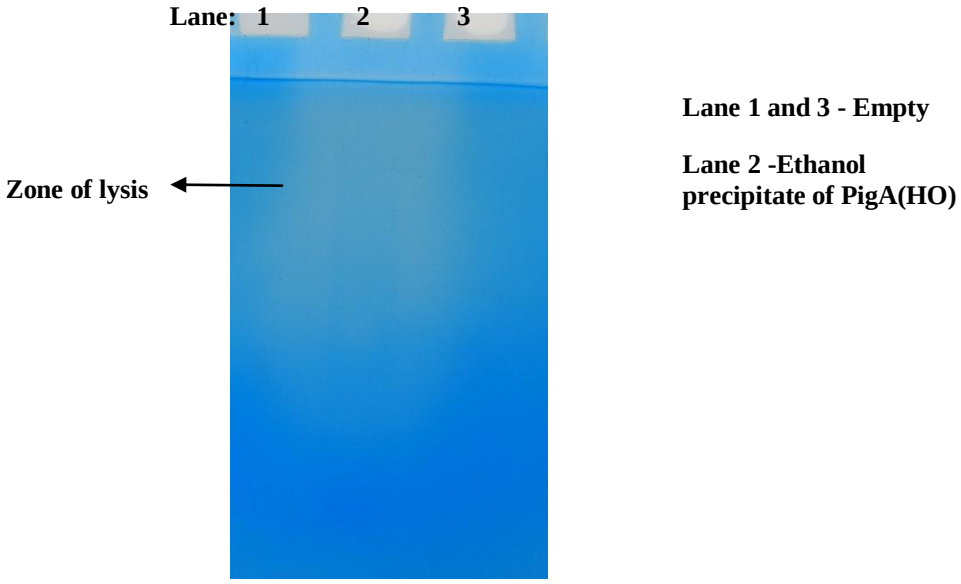

Fig.5 Zymogram analysis of PigA(HO) from isolated Strain-VA8

Discussion:

HO along with its by-products i.e., bilirubin, Fe^{2+} and CO had anti-inflammatory, anti-apoptotic, anti-proliferative, vasodilatory and antioxidant properties (Kirkby and Adin, 2006). The anti-proliferative effect of HO-1 was reported in rat and human breast cancer cell line (Hill et al., 2005). Since HO was reported to have therapeutic importance a cheap and alternative form of HO is required. Microorganisms are well-known, easy and cheap source of many therapeutically important enzymes and compounds. Microorganisms do not possess any circulating system but it requires iron for its survival hence under pathogenic iron limiting conditions microorganisms like *P.aeruginosa* will utilize host heme by production of heme degrading enzyme PigA(HO). Under iron limiting conditions about 118 genes was found to be upregulated of which *PigA* gene was found to be upregulated by 138-fold (Wang et al.,

2007). Since HO had therapeutic importance and *P.aeruginosa* was reported to have upregulated PigA(HO) activity, the present study was aimed at the isolation and characterization of *P.aeruginosa*, which has 37% genome sequence homology with *HemO* of *Neisseria meningitides,* which is a gram-negative organism that shares considerable homology with known human HOs and their phenotypic studies revealed the role of *HemO* gene product in the assimilation of heme and protection against heme toxicity (Zhu et al., 2000). In the present study *P.aeruginosa* was isolated from soil samples collected from the hospital waste dump site, which are prone to exposure with nosocomial infection causing pathogens and *P.aeruginosa* was an important nosocomial infection causing pathogen. The isolated Strain-VA8 was confirmed to be *P.aeruginosa* by biochemical and molecular characterization. The intracellular enzyme PigA(HO) was extracted from isolated Strain-VA8 and the enzyme activity of PigA(HO) was found to be 3.4 and 1.65 U/mL (Crude enzyme and ethanol precipitate respectively), which was more than the enzyme activity reported for Red algae *Cyanidium caldarium* (1.2 U/mL) (Cornejo and Beale, 1988) and it was less than the enzyme activity reported for Chick liver (66.7 U/mL) (Bonkovsky et al., 1990). The chick is an eukaryote with circulating RBC's in which the heme protein turn over would be more hence the enzyme activity of HO from chick liver was more than PigA(HO) from *P.aeruginosa*. On SDS-PAGE analysis a band near 20kDa was observed and it was already reported that the wild type PigA(HO) of *P.aeruginosa* had a molecular weight of 23kDa (Ratliff et al., 2001) hence the band near 20kDa could be attributed as PigA(HO) which is 23 kDa. But the molecular weight of *HemO*(HO) of six commensal *Neisserial* isolates was reported to be 26kDa which was more than molecular weight of PigA(HO) in the present study yet expresses the same function (Zhu et al., 2000). On zymography, initially the zone of lysis was not observed in samples which contained SDS in loading dye later when the denaturing agents were removed the zone of lysis was observed. This may be because of the denaturation of enzyme by SDS, which led to loss of enzyme activity (Tracz et al., 2008). In SDS-PAGE analysis the negatively charged sulfonic group of CBB-R-250 binds to the positive charge of basic amino acids like histidine, lysine and arginine and about 1.5 to 3 molecules of CBB-R-250 binds to each positive charge (Tal et al. 1985). Likewise, here in zymography CBB-R-250 binds to the positively charged Ferric (Fe^{2+}) centre of hemin to stain the gel and in places where the hemin was lysed by PigA(HO) a clear zone was observed. This confirmed the HO enzyme activity of PigA(HO) extracted from isolated *P.aeruginosa* Strain-VA8. The specific activity of PigA(HO) of isolated strain-VA8 was found to be 3.569 U/mg and even

after ethanol precipitation the specific activity was increased only to 4.125 U/mg. The isolated strain *P.aeruginosa* strain-VA8 showed a considerable amount of enzyme activity but is very less when we want to test it as a therapeutic protein. Hence further cloning and expression of *PigA* gene of *P.aeruginosa* Strain-VA8 is required to get high yield of protein and to test the ability of enzyme to be used as therapeutic protein.

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