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ISOLATION, SCREENING, AND PARTIAL CHARACTERIZATION OF OXALATE OXIDASE ENZYME FROM *PSEUDOMONAS* STRAIN UNDER INDUCED OXALATE STRESS CONDITION

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ABSTRACT

Oxalate oxidase (OxO), a manganese dependent enzyme, is involved in the catalysis of oxalate oxidation to carbon dioxide with the formation of hydrogen peroxide. OxO is present in the cell wall of plants. It increases the resistivity against diseases and external stress. Oxalate is found as waste metabolites in mammals, excess accumulation of oxalate results in hyperoxaluria and urolithiasis in humans. These disorders can be diagnosed by the help of OxO in many analytical methods. The present study is aimed on isolation of OxO enzyme from *Pseudomonas* strain under high salt stress. The OxO enzyme was produced in bulk under selected salt stress. It was partially purified by ammonium sulphate precipitation method, dialysis and ion-exchange chromatography. The OxO enzyme on enzymatic analysis showed potent activity concerning oxalic acid substrate. The microtiter plate analysis confirmed the ability of OxO enzyme purified from *Pseudomonas strain* in the diagnosis of oxalate-related disorders and in the future could be used to prepare the diagnostic biosensors.

Keywords: Oxalate oxidase, Salt stress, *Pseudomonas* strain, Peroxidase

INTRODUCTION

The daily consumption of a large amount of oxalate causes Oxalosis or the formation of calcium oxalate deposits in vital tissues or organs of the body, which could be fatal to humans (**Sanz & Reig, 1992**). Oxalate is observed as a crucial part of stones found in kidney disorders. Thus, the determination of oxalate content in urine is essential to patients with kidney stone problems. The oxalate determination can be done through clinical analysis of blood and urine using the OxO enzyme. Thus, the OxO became an essential candidate for researchers for the diagnosis of kidney stones (**Pundir *et al.*, 1993**). The OxO enzyme mechanism involves the oxidation of oxalate to carbon dioxide along with hydrogen peroxide. The OxO is dependent on manganese ions. Researchers isolated the OxO enzyme from fungi, bacteria, and plants (**Carrillo *et al.*, 2008; Zhou *et al.*, 1998**). This enzyme produces two moles of carbon dioxide and one mole of hydrogen peroxide from one mole of oxalate and oxygen. Oxalate oxidase from wheat and barley is known as germin-like oxalate oxidases found in the cell walls of embryos during germination (**Grąz *et al.*, 2016; Lane, 1994**). The Basidiomycete *Abortiporus biennis* strain possesses oxalate oxidase in the mycelium (**Koyama, 1988**). Researchers proposed the purification of membrane-bound oxalate oxidase from *Pseudomonas* sp. OX-53. Microbes are known for their potential contribution as a useful source of enzymes (**Vázquez *et al.*, 2019**). Microorganisms with high yield, easy culturing, and isolation strategies proves low-cost source in many industrial applications (**Chiriboga, 1966; Datta & Meeuse, 1955; Dumas *et al.*, 1993; Dunwell, 1998; Hu *et al.*, 2015**). Researchers used the OxO enzyme isolated from these sources in many diagnostic and clinical treatments for Urolithiasis (**Chauhan *et al.*, 2012**). Conventional analytical techniques such as Gas and liquid chromatography were studied for OxO analysis, but they have chances of sample degradation (**Fiorito & Córdoba de Torresi, 2004; Harris *et al.*, 2004**). In the era of rapid and robust screening of samples in clinical laboratories, sensitivity is most important. Thus, it is the need of the hour to prepare the biosensors based on OxO for reliable detection of oxalate associated disorders (**Yriberri & Posen, 1980**). The present study is focused on the screening and production of oxalate oxidase enzymes from *Pseudomonas* species isolated from soil under high-stress conditions.

MATERIAL AND METHODS

Pseudomonas Agar for fluorescein production (PAF), *Pseudomonas* Agar (PMP) for the detection of pyocyanin production, Normal saline solution, NaCl, Potassium oxalate (PO), Cetrimide agar, Simmons Citrate, Methyl Red medium, Voges-Proskauer medium, Glycerol, Yeast extract, Peptone, Dipotassium phosphate, Magnesium sulphate, Oxalic acid, Potassium oxalate, Ammonium sulphate, Tris-Cl buffer, Protein ladder, 3-methyl-2- benzothiazolinone hydrazone (MBTH), N, N-dimethylaniline (DMA), Ethylenediaminetetraacetic acid (EDTA). All chemicals were of analytical grade and purchased from Himedia, India, and standard oxalate oxidase enzyme from BioVision.

Isolation of *Pseudomonas* species from soil

The soil samples were collected from different locations of Sant Gadge Baba Amravati, University campus, Amravati Maharashtra. The isolation of *Pseudomonas* species was done on two specific enrichment media, *Pseudomonas* Agar **(PAF)** for the detection of fluorescein production (**King *et al.*, 1954**) and *Pseudomonas* Agar **(PMP)** for the detection of pyocyanin production by *Pseudomonas* species were used for screening and isolation of bacteria. Flask of PAP and PAF medium broth were prepared and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. 1 gm of soil sample was added in normal saline (0.9%) and mixed it entirely, and from that, 100 µl of supernatant was transferred to each PAF and PMP in 250 ml flask with 100 ml media 4 flasks for each sample. It was incubated at 37ºC for 24 hrs, and results were observed. Further, the bacterial growth was further were used for the spread plate culture of the sample using PMP and PAF media.. Next, plates were incubated at 37ºC for 24 hours, and colonies those fluoresced under UV light (λ= 356 nm) were selected and were purified on the same media by repetitive streaking.

Bacterial Strain Identification

Biochemical characterization

The isolated culture was screened for *Pseudomonas* bacterial culture identification biochemical test. The bacterial isolate was identified by performing biochemical tests such as Cetrimide agar, Cytochrome Oxidase, Catalase, Citrate utilization test, Methyl-red, and Voges–Proskauer test by standard methods (**Islam & Roy, 2018**).

Identification of *Pseudomonas* culture by VITEK 2 system (software version VT2-08.01)

Isolated bacterial culture was inoculated on a nutrient medium and incubated at 37ºC for 24 hrs. Suspensions were prepared by emulsifying bacterial isolates in 0.45% saline to the equivalent of a 0.5 McFarland turbidity standard, 1.5×10^7 CFU/ml. The same suspension was used for identification on the VITEK 2 system. The 0.5 McFarland bacterial suspension was diluted; cards were automatically filled, sealed, and loaded into the VITEK 2 instrument for incubation and reading (**Garcia-Garrote *et al.*, 2000**).

Screening of medium for salt stress oxalic acid and potassium oxalate for isolated *Pseudomonas* strain

The growth medium was prepared in 250 ml flask composed of 1% glycerol, 1% yeast extract, 1% peptone, 0.1% dipotassium phosphate, 0.05% magnesium sulphate with 1% oxalic acid, and 2% potassium oxalate as a variant for the screening of suitable substrate for bacterial growth to produce oxalate enzyme under stress condition.

Process optimization for maximum bacterial biomass production by a various dose of potassium oxalate salt

The bacterial broth (PAF) was prepared in which content increase the concentration of potassium oxalate ranges from 1 to 12% were added, sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The isolated culture from 1.0% PO (Potassium oxalate) medium was inoculated in each flask and kept for observation for 24 hr at 37ºC. 200 µl sample was added in 96 well plates, and the absorbance was noted at 625 nm using Biotek® H1-SYNERGY ELISA spectrophotometer.

Bulk production in suspension medium and harvesting of cells

Pseudomonas aeruginosa was grown in PAF medium broth (500 ml) with 2% Potassium oxalate supplemented in 1000 ml flask for 3-4 days at 37ºC.
The heat cold shock method was used on obtained bacterial cell suspension. The sample was extracted in liquid nitrogen and resuspended in 20mM Tris-Cl pH (8) with 10mM Phenyl methyl sulphonyl fluoride (PMSF) (**Alt *et al.*, 1975; MacGregor, 1977**).The 200 ml of obtained sample sonicated under the ice-cold condition operated for 1 hr cycle at 30% frequency with the pulse rate 5 seconds in ice-cold condition. Then sonicated mat samples centrifuged at 7000 rpm for 30 min and enzyme extracted (**Anjum *et al.*, 2015; Kanauchi *et al*., 2009**).

Characterization of Oxalate oxidase (OxO) enzyme

Ammonium sulphate fractionation and dialysis

Ammonium sulphate fractionation was performed in two stages, at an initial concentration of 0-60% of ammonium sulphate was added slowly until it completely dissolved in cold condition at 4ºC for 5 hrs and later it was kept on stirring overnight a 4°C. After the precipitation process was completed, it was centrifuged at 4000 rpm, and the pellet was resuspended in buffer (Tris-Cl pH 8), and the supernatant was collected and used for the second stage of the 80% ammonium sulphate fractionation process. The 60% pellet was resuspended in buffer (Tris-Cl pH 8) subjected to dialysis using a dialysis sack mwco (110) Hi-Media dialysis membrane. The dialysis sack was attached to a float at one end and immersed in 100 times the volume of the sample and dialyzed against appropriate buffer (PBS) for 24 hr at 4ºC with continuous stirring, resuspended in buffer and stored at 4ºC. The appropriate dialysis buffer was determined by the subsequent purification procedure to be undertaken. After completion of 60% and 80%, dialysis samples were stored for further analysis and assay purposes **(Hu & Guo, 2009**).

Protein estimation and SDS gel electrophoresis after partial purification of oxalate oxidase from oxalate degrading bacteria

The unknown protein quantification was done by Biuret method, and SDS-PAGE was performed on 12% separating and 5% stacking polyacrylamide gels at 25°C using TARSON Mini Vertical Electrophoresis Assembly (7080). The gels loaded with the sample were run at 25/50/100 V till the dye front reached the end of separating gel. The protein bands were visualized by staining with Coomassie brilliant blue (CBB) G-250 (**Laemmli, 1970)**.

Ion Exchange Column Chromatography (Fractionations) of Partial Purified Enzyme

Column chromatography with DEAE-cellulose chromatography Macro-Prep ®DEAE medium was used, which is an anion exchange medium. It was purchased from BioRad laboratories. The glass chromatography column was packed up to 15 cm height 50% slurry of the Macro-Prep ®DEAE medium. It was equilibrated with 20 mM tris CL buffer of pH 8 for the column chromatography process. Gradients of 50 ml were prepared to range between 200 to 800 mM NaCl in 20 mM tris-Cl buffer of pH 8. Further, 50 ml of 20 mM tris Cl buffer column was used to wash the column. Next, a 60% Ammonium sulphate precipitated sample (partially purified enzyme) was dialyzed, and it was loaded on the column. The column was washed with the same buffer to remove the unbound proteins, and the enzyme was eluted by applying a linear gradient of NaCl from 200 to 800 mM, and fractions of 2 ml were collected. Active fractions were pooled and stored for further analysis (**Kotsira & Clonis, 1997**).

Bacterial oxalate oxidase enzyme activity

MBTH (3-methyl benzathoizaline hydrazine) used to detect release of hydrogen peroxide in the enzymatic reaction of Barley oxidase enzyme upto 1mM. In the assay of oxalate oxidase, hydrogen peroxide is produced which is coupled with HRP which catalyses the MBTH-DMA (**Kanauchi *et al.*, 2009 ; Goodwin *et al.*, 2017**).

Oxalate + $O_2 \rightarrow 2CO_2 + H_2O_2$ (catalyzed by oxalate oxidase)
MBTH + H_2O_2 + DMA → indamine dye (purple color) + $2H_2O$ (catalyzed by added peroxidases)

Substrate concentrations were prepared as 10-50 µL of 200 mM oxalic acid in 20 mM tris Cl buffer pH 8. About 200 µL of crude bacterial enzyme, 60%, and 80% fractionated dialysis protein sample, and 50 µL substrate was mixed in 24 well plate and kept in incubation for 37ºC for 30 min. After completion of incubation time, 300 µL of MBTH solution (MBTH solution was prepared in 0.2 M sodium acetate buffer (pH 4.0) with 0.79 mM DMA and 0.11 mM MBTH) was added along with 100 µL of 100 mM EDTA solution and incubated for 5 min. After that, 20 µL of horseradish peroxidase enzyme solution (HRP) was added (1 mg/mL, Himedia, TC487). HRP enzyme solution (Immediately before use, prepare a solution containing 1 mg/ml of Peroxidase, in cold deionized water). The plate was incubated for 1hr in dark condition. The reading was noted on SYNERGY H1 microplate reader Biotek® at 600 nm (**Kanauchi *et al.*, 2009 ; Goodwin *et al.*, 2017**).

RESULTS AND DISCUSSION

Biochemical characterization

Biochemical characterization tests were performed for isolated bacterial samples for the identification of *Pseudomonas* species. The test results are shown in **Table 1. Figure 1A**. Showed that no fluorescence on plates as compared to **Figure 1B** and **Figure 1C**. Yellow pigment like colonies on PAF plates in visible light conditions was observed, and under UV transilluminator (**Figure 1B**), it showed greenish fluorescence (**Figure 1C**). The Oxidase test was found to be positive for the isolated sample, as shown in **Figure 2B** (bacterial sample without PO) and **Figure 2C** (bacterial sample with PO) compared to **Figure 2A** which was a negative control broth**.** Catalase test was also found to be positive, as a bacterial broth sample reacted to H_2O_2 to produce effervescence shown in **Figure 3B** with reference to negative control **Figure 3A.**

Table 1 Biochemical characterization of isolated bacterial samples

Sr. no	Biochemical test	Observation	Inference
1	Cetrimide agar test	Fluorescence in UV light	+ve
2	Citrate test	Green to blue colour change	+ve
3	Oxidase test	Dark blue-purple colour change within 10-30 sec.	+ve
4	Catalase test	Bubble formation	+ve
5	Pigment formation	Yellow-green colour	+ve
6	Methyl Red test	No colour change	-ve
7	Voges Proskauer test	No colour change	-ve

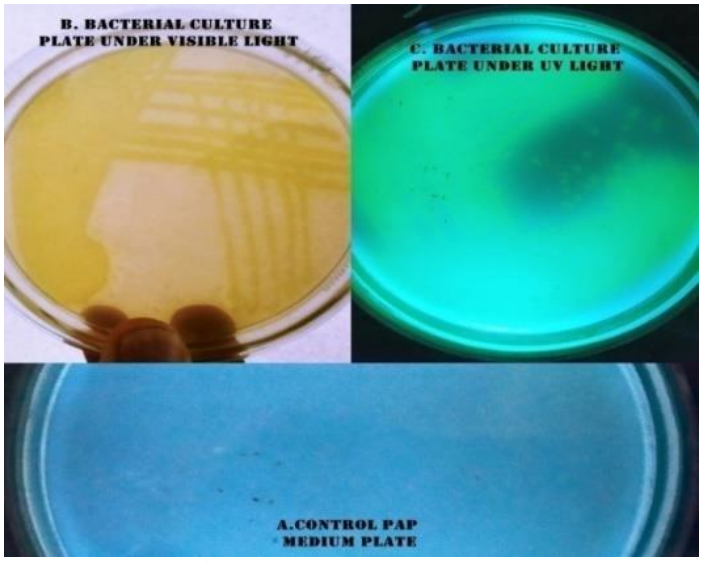

Figure 1 Screening of isolated *Pseudomonas species* in selective media, A: Control plate (PAP), B: Bacteria under visible light (PAF), C: Bacteria showing green fluorescence under UV light (PAF).

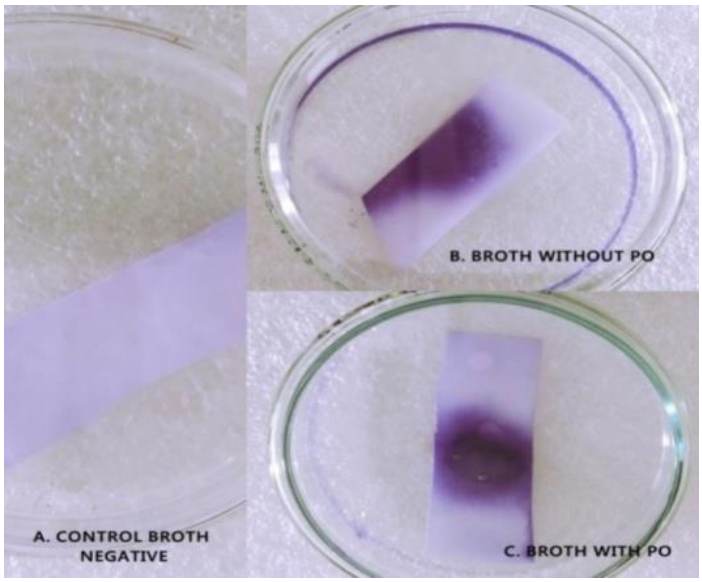

Figure 2 Comparative oxidase test of bacteria A. control broth: colourless, B: Broth without PO- oxidase-positive, C. Broth with PO: oxidase-positive

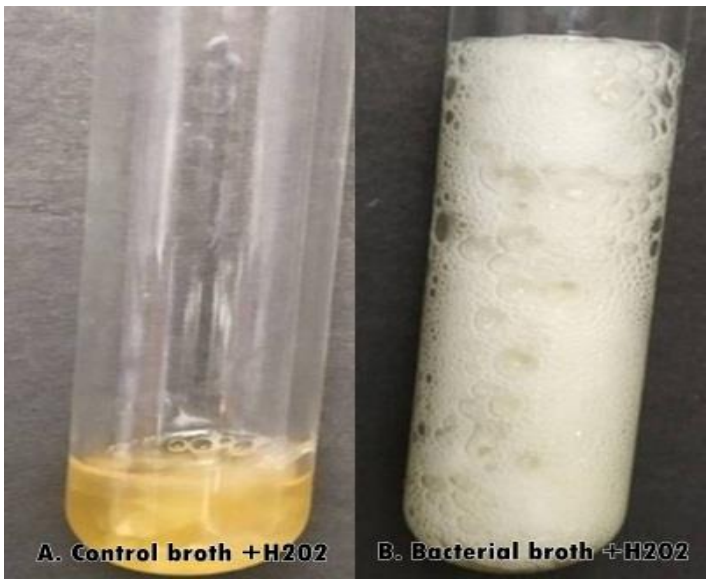

Figure 3 Screening of bacteria for the catalase test, A. Control broth sample: negative and B: Bacterial broth showing bubble formation ie, positive

Identification of oxidase producing Bacterial culture by VITEK 2 system (software version VT2-08.01)

The isolated colony was observed used for preparing a bacterial suspension of 0.5 McFarland. That sample was used for identification, and results were obtained after 6.78 hrs. The given Gram-negative bacteria *Pseudomonas aeruginosa* was identified using the VITEK 2 system.

Screening of medium for salt stress oxalic acid and potassium oxalate for isolated *Pseudomonas* strain

The increasing bacterial growth was observed in 3% Potassium oxalate medium compared to 2% oxalic acid medium. The Potassium oxalate supplemented medium showed increased growth shown in **Table 2** and **Figure4.**

Table 2 Effect on bacterial growth supplemented with oxalic acid and potassium oxalate in the growth medium

Sr. No	Type of sample	Mean Absorbance (O.D.)
1	Control	0.062
2	Oxalic acid 2%	0.258
3	Potassium oxalate 3%	2.200

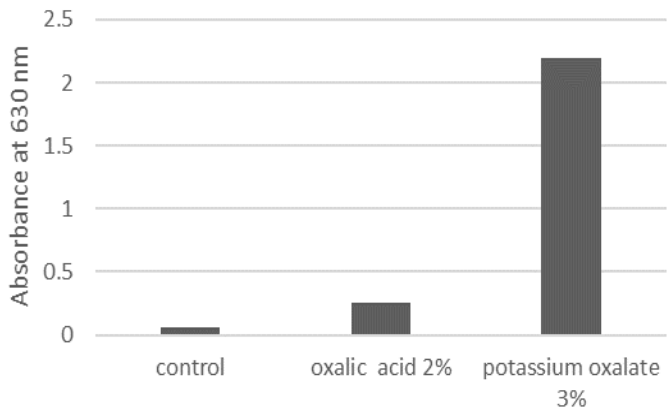

Figure 4 Comparative bacterial growth under oxalic acid and Potassium oxalate supplemented medium

Process Optimization for Maximum Bacterial Biomass Production by different Concentration of Potassium Oxalate Salt

The cultured bacteria showed growth in salt stress range between 1 to 12% PO containing medium. The flask also showed bubbles formation, which are the indicators for the presnce of oxidase enzyme. The results are shown in **Table 3** and **Figure5**. It is clearn that 3% of PO concentration have maximum bacterial growth. Hence 3% concentration of PO is selected in growth medium or is found to be more suitable for bulk production of oxalate enzyme.

Table 3 Effect on the growth of bacteria supplemented with different concentration of potassium oxalate (salt stress)

Concentration of Potassium oxalate (%)	Mean absorbance	Bubble formation
Control	0.062	-
1	0.22	-
2	0.464	++++
3	**0.934**	+++++++
4	0.492	+++
5	0.298	++
6	0.251	++
7	0.248	++
8	0.131	++
9	0.128	+
10	0.125	+
11	0.125	+
12	0.120	+

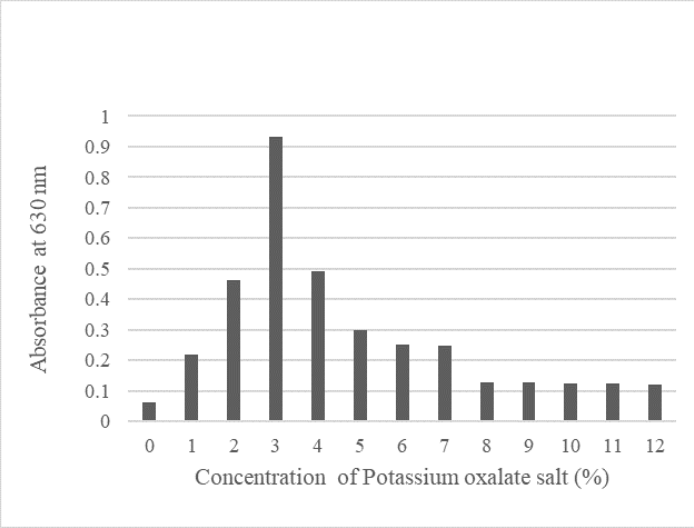

Figure 5 Effect on bacterial growth supplemented with increasing concentration of potassium oxalate salt (Induced stress condition)

Bulk production in suspension medium and harvesting of cells

Pseudomonas aeruginosa isolated and identified by **VITEK 2 System, version 08.01** was further grown in PAF medium broth with 2% Potassium oxalate and incubated for 3-4 days at 37ºC. A thick slimy mat – like biomass was observed at the end of incubation. The matt was pale yellow, very viscous in texture like egg yolk, and the bubble formation observed in culture. The mat was transferred and extracted in liquid nitrogen, the resultant extract was kept in a buffer having PMSF solution to avoid degradation of enzyme. The extracted sample was subjected to centrifugation. The collected supernatants of extract then proceeded for sonication under the cold condition for better recovery of enzyme and sample was stored for further analysis (**Dashek, 2018**).

Characterization of oxalate oxidase enzyme

Ammonium sulphate fractionation and dialysis:

The partially purified samples were collected and subjected to protein estimation (**Dashek, 2018; Neut *et al.*, 2005**).

Table 4 Protein estimation of ammonium precipitated samples

Sr. No	Concentration (BSA 5mg/ml)	Mean Absorbance y = 0.000x + 0.152 R² = 0.979
1	100 µl	0.173
2	200 µl	0.217
3	400 µl	0.279
4	600 µl	0.36
5	800 µl	0.424
6	1000 µl	0.447
7	Crude extract	0.238
8	60%	0.368
9	80%	0.157

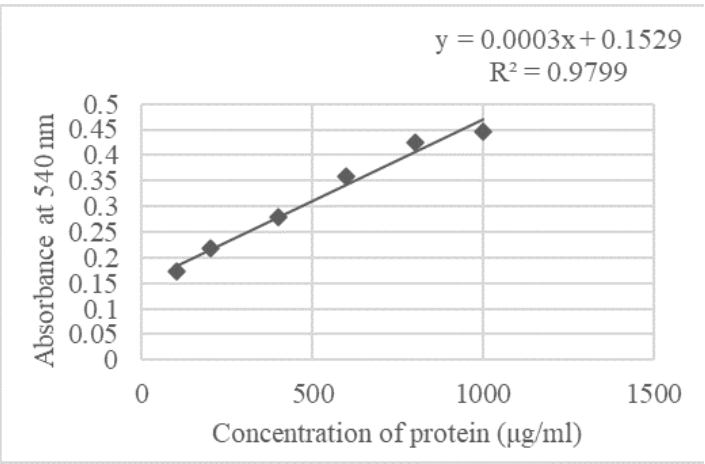

Figure 6 Standard calibration graph for protein estimation using standard BSA (5 mg/ml)

Protein estimation and SDS gel electrophoresis after partial purification of oxalate oxidase from oxalate degrading bacteria

Bacterial oxidase enzyme was subjected to total protein content evaluation. It showed that protein content of crude bacterial oxidase enzyme is 0.086 mg/gm, 60% fractionated dialysis sample was 0.216 mg/gm and 80% fractionated dialysis sample was found to be 0.005 mg/gm. It was deduced by the BSA calibration graph (**Figure 6 and Table 4)**. The SDS gel electrophoresis was performed for the crude enzyme, 60% and 80% dialyzed sample. The 60% dialyzed sample showed more prominent bands than crude and 80% dialyzed sample (**Figure 4**). The enzyme oxalate oxidase from *Pseudomonas aeruginosa* is observed to be the molecular weight between 45 Da to 95 Da in all the three samples (**Sambrook & Russell, 2001; Steinberg, 2009**).

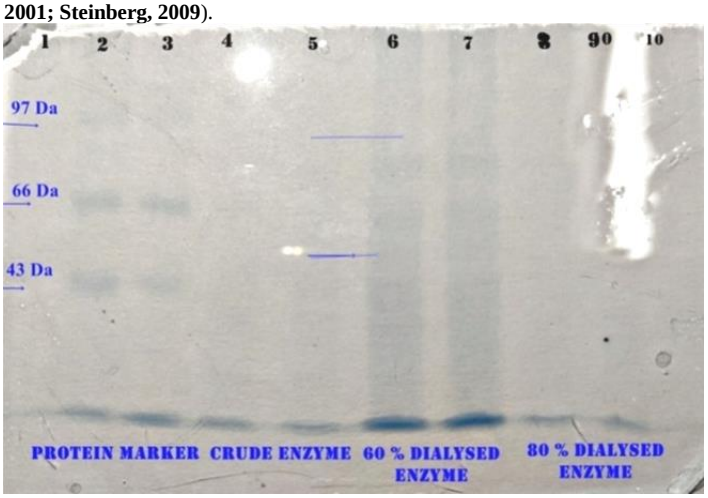

Figure 7 SDS page electrophoresis of protein marker lane-2, 3; crude enzyme lane 4,5; 60% dialyzed enzyme lane 6,7; 80% dialyzed enzyme lane 8,9. SDS-Polyacrylamide gel electrophoresis of oxalate oxidase. To the left is the gel, with the left hand representing the molecular weight standards and the oxalate oxidase band in different lanes indicated by the arrow.

Ion Exchange Column Chromatography (Fractionations) of Partial Purified Enzyme

The ion exchange Column chromatography was done using DEAE-cellulose chromatography Macro-Prep®DEAE medium, and fractions were collected for further enzyme activity (**Palanivelu, 2018; Zhang *et al.*, 1996**).

Bacterial oxalate oxidase enzyme activity

In the reaction, oxalate oxidase catalyzes the production of hydrogen peroxide from oxalate; the hydrogen peroxide further reacts with MBTH and DMA in the presence of peroxidase enzyme to form a purple color product (Indamine dye). The color of indamine dye was read at 600 nm using SYNERGY H1 microtiter plate reader from Biotek®. The results indicate the increase in absorbance as the indamine concentration increases, which can be correlated with the activity of the oxalate oxidase enzyme. The standard linear curve was prepared using standard hydrogen peroxide. The activity can be defined as the amount of oxalate unit required to produce the 1 µmol of hydrogen peroxide at 55°C per minute. The H_2O_2 detection was performed using MBTH indamines dye measured using Result layout colorimetric scheme of MBTH based standard H_2O_2 detection using HRP of reaction samples shown in **Figure 8**. The activity was shown in **Figure 9,** it shows that a 60% dialyzed sample has more significant H_2O_2 release in the reaction compared to the standard OxO enzyme, 80% dialyzed sample, and crude enzyme **(Goodwin *et al.*, 2017; Goyal *et al.*, 1999; Zhang *et al.*, 1996**).Some of the difficuties faced during study include the detection of H_2O_2 becomes unstable at higher temperature, thus the wstudy should be done at lower tempratures.

Figure 8 Result layout colorimetric scheme of MBTH based standard H_2O_2 detection using HRP

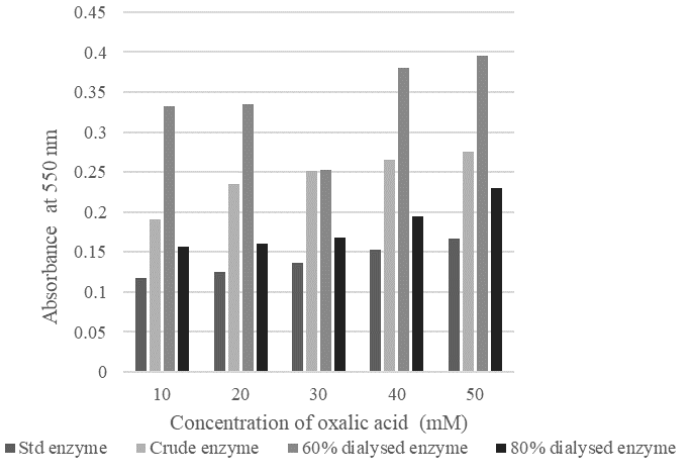

Figure 9 Comparative activity of the crude enzyme, purified 60 and 80% dialyzed sample with standard OxO enzyme (BioVision)

CONCLUSION

Findings provide new directions for research in the study of Oxalate oxidase enzyme. Isolation and production of an oxalate degrading enzyme strains under salt stress conditions. Further research focused on the enzyme activity of isolated bacterial enzyme and optimization of methods for the determination of oxalate concentration in the sample. Hence the microbial oxalate enzyme can be used for widespread application in the enzyme industry and diagnostics.

Author contributions: This work was carried out in collboration between all authors. All authors have accepted the responsibility for the entire content of this submitted manuscript and approved the submission. Authors AP, RS and LD conceptulized and performed the experiments. AP and HP performed the formal analysis and investigation. AP, RS and HP also performed the writing, editing and reviewing of the article. All authors read and approved the manuscript.

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Competing intrest: The authors declare that they have no competing intrest.

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