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CLONING, EXPRESSION AND PURIFICATION OF THE MANGANESE SUPEROXIDE DISMUTASE (MNSOD) GENE FROM BACILLUS ARYABHATTAI AB211

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ABSTRACT

The organism Bacillus aryabhattai AB211,is a plant growth promoting rhizobacteria. It has robust stress responsive genes, which enable them to withstand the oxidative stress condition, generated in the plant rhizosphere. SOD (superoxide dismutase) is basically an enzyme which catalyses dismutation i.e partitioning of the O2-(superoxide radical) into molecular oxygen and hydrogen peroxide. There are mainly three types of SOD genes in Bacillus aryabhattai AB211: FeSOD (which binds Fe), MnSOD(which binds Mn) and Cu/ZnSOD(which binds Cu/Zn). This project mainly deals with the MnSOD genes(located at the cytoplasm). These genes were cloned, to produce the protein and the expression of these proteins were checked at different temperature and at different concentrations of IPTG induction. After expression, purification of the MnSOD protein was done using Ni-NTA Affinity Chromatography, followed by Dialysis and Ion-Exchange Chromatography.

KEYWORDS: SOD, Cloning, expression, IPTG induction, Purification.

INTRODUCTION

Bacillus aryabhattai

Bacillus are a group of rod-shaped, gram positive bacteria, aerobic (under some conditions) or anaerobic and widely found in soil and water. Bacillus aryabhattai AB211, which is a plant growth promoting, Gram-positive bacterium,was isolated from the rhizoshpere of Tea. The genome sequencing of AB211 revealed the presence of 5,403,026 bp chromosome. Functional annotation or by determining the biological activity, some genes are revealed which are mainly responsible for flagella biosynthesis, bioflim formation of acetic acid production, acetoin production etc. Gene analysis revealed that AB211 has an active carbohydrate metabolism which implied that these bacterium can utilize the root exudates and other organic materials as a source of energy. They have genes for oxidative stress tolerance, resistance to heavy metals, heat shock tolerance and are highly potential as a PGPR

Superoxide Dismutase

SOD is an enzyme that catalyses the dismutation i.e partitioning of the Superoxide (O2-) radical into either ordinary molecular oxygen O2 and hydrogen peroxide. It is a byproduct of oxygen metabolism and if not regulated properly, causes cell damage. These SOD acts as an anti-oxidant and thus helps in defense in nearly all living cell exposed to oxygen. Now oxidative stress can be caused due to various biotic and abiotic factors. Reactive oxygen species (ROS) can be formed as a result of drought, injury, herbicides or pesticides, ozone or plant metabolic activity. To be specific molecular oxygen is reduced to O2-(ROS). Superoxide is known to denature enzymes, oxidize lipids etc. SOD, on the other hand catalyses the production of molecular oxygen and hydrogen peroxide from superoxide radical, which results in less harmful activity. With the increased levels of oxidative stress, SOD concentration also increases. Thus they are in the first line of defense for ROS.

SOD and its types

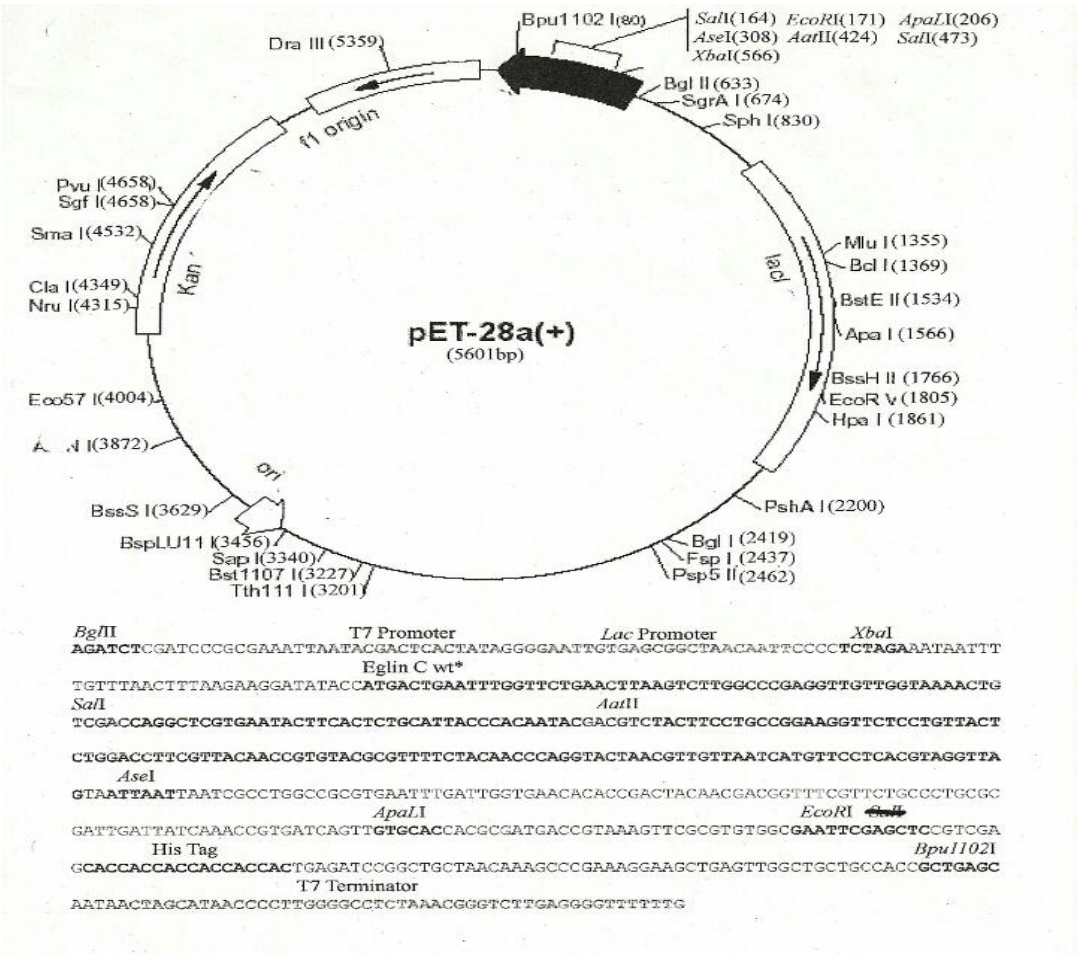
SOD genes in Bacillus aryabhattai are mainly of three types:

1- FeSOD
This binds Fe and is mainly located at the cytoplasm
2- Cu/ZnSOD
This mainly binds Cu/Zn and is located at the periplasm
And the one that we are dealing with
3- MnSOD
This binds Mn and is located at the cytoplasm.

Pet28a Vector

The features of the Pet28a vector are as follows

- Bacterial expression vector with T7 lac promoter.
- It adds N-terminal His-tag
- It has thrombin cleavage site
- C-terminal His-tag
- Kanamycin resistant



Fig, 1: Shows Pet 28a Vector.

MATERIALS AND METHODS

Strain and Cell Culture

Bacillus aryabhattai AB211 was isolated from the rhizosphere of tea from the Darjeeling district. Strain AB211 was grown in M9 minimal medium supplemented with glucose and incubated at 37 degree celcius.

Cloning

Procedure

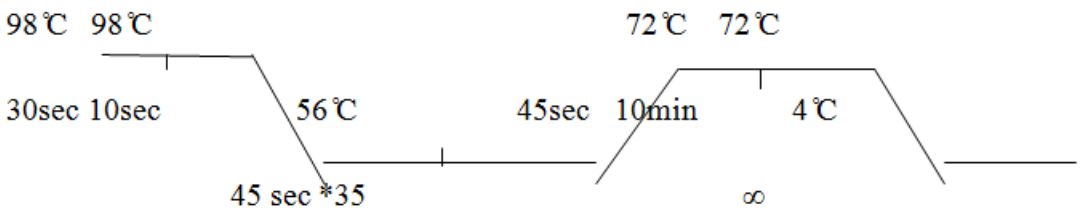
- **Genomic DNA isolation**

The genomic DNA was isolated by the CTAB method

- **PCR**

The targeted gene was amplified by using the PCR method

PCR condition



- **Restriction digestion and vector digestion**

After PCR, the amplified gene of interest needs to be inserted into the plasmid which contains all regulatory sequences. Thus both sides of the plasmid and either end of the genes are cut with the same enzymes called the restriction enzymes. Restriction enzymes NcoI and XhoI are used.

- **Ligation**

After gel electrophoresis, the plasmid and the purified gene of interest are mixed together and they join via complementary base pairing. But this force is very weak, so to create a strong phosphodiester bond, the enzyme ligase is added.

- **Transformation**

After ligation there are some plasmids which contain the insert and there are some plasmids which have not been able to ligate successfully. In this case bacteria are used. Each bacterial cell takes up one plasmid molecule at a time from the ligation mixture. Now a plasmid has a antibiotic resistant gene and a replication of origin. As the bacteria grows, the replication ori duplicates the plasmid independently of the bacterial chromosome in the host cell, so that the plasmid can replicate and the antibiotic resistant gene is expressed. For this project MnSOD was transformed in XL1B competant cells to increase the number of cloned plasmid. The key steps we have followed in bacterial transformation are competant cell preparation, transformation of cells, cell recovery and cell plating. As we have transformed, MnSOD in C43(a strain of E.coli for protein expression), the natural competency of E.coli being very low, the cells were made competant by heat shock method. So in this process, the chemically competant cells and the ligated mixture is mixed and kept in ice. Thus they were incubated in ice for about 30 mins. Heat shock was given to the mixture at 42 ℃ for 30 sec and they were again transferred back to ice. The cells were then kept in recovery for about 1 hour. After that cells were plated in LB agar plate with suitable antibiotics.

- **Colony PCR**

As colonies appeared on the Kan plate, Colony PCR was done to find out which of the colonies contains the required insert.

- **Isolation of plasmid**

The required protocol of alkaline lysis was followed and the plasmid was isolated.

- **Double Digestion**

The plasmid was cut with the same restriction enzymes (Nco1 and Xho1) to check for the presence of the required clone.

- Clone was confirmed & Sequencing was done.

Clonning Strategy

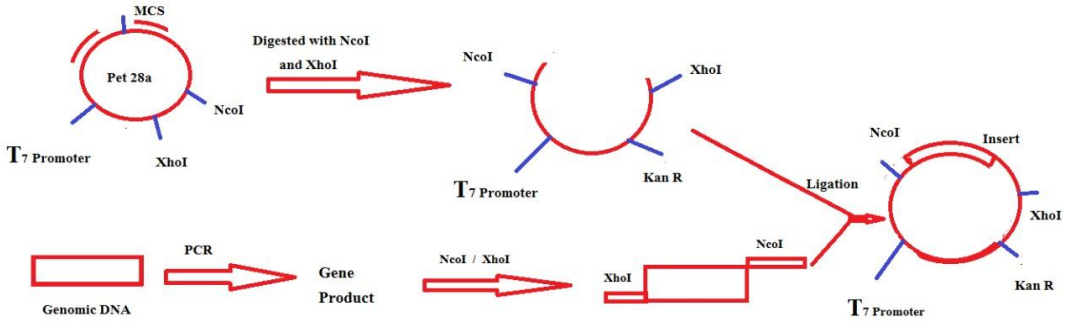


Fig. 2: Shows cloning strategy.

Expression of the MnSOD proteins at 37℃

Method

- For expression, 5ml of culture was given from MnSOD transformed in C43 plate with 5µl Kanamycin and 5µl Chloramphenicol. They were then kept in induction at 37℃ for 3hours till it reached 0.6 OD.
- Out of this, 1ml uninduced was kept aside and the rest 4ml was given 500µm IPTG induction for about 3 hours.
- 1ml induced and 1ml uninduced were resuspended in 100µl Lysis buffer (50mM Tris + 150mM NaCl). After that 20µl dye was added and they were heated for about 30 mins at 95C.
- 3ml induced samples were resuspended in 300µl lysis buffer.
- 3ml induced samples were sonicated at 90% amplitude, 10 secs each for 3 times.
- Centrifuged at 13,000 rpm for 30 min at 4℃.
- For the supernatant,60µl dye was added, heated at 95℃ for 15 mins and then loaded on the gel.
- For pellet, it was resuspended in 300µl lysis buffer, vortexed and 60µl dye was added. It was heated at 95℃ for 15 mins and then loaded in the gel.

MnSOD protein expression at 16℃ and 37℃

To check the expression of proteins at different temperatures, two temperatures, 16℃ and 37℃ were taken.

- The same process was repeated i.e induction was given at 37℃ for 3 hours and at 16℃ overnight.
- They were then ran in SDS PAGE with uninduced, induced, sup, pellet for 16℃ and uninduced, induced, sup, pellet for 37℃.

Expression of MnSOD proteins at different concentrations (300µM,400µM,500µM and 600µM)

- 5ml culture was given from MnSOD transformed in C43 with 5µl chloramphenicol and 5µl kanamycin. They were then incubated at 37 ℃ for about 3 hours till they reached 0.6OD
- 1ml uninduced was kept aside.
- The rest 4ml was given IPTG induction. As we go on adding IPTG, we gradually increase the concentrations. 1.5µl was given to 300µM, 2.0µL was given to 400µM and so on.
- 1ml induced and 1 ml uninduced was resuspended in 100µl lysis buffer.20µl dye was added and heated for 30mins at 95 ℃
- 3ml induced samples were resuspended in 300µl lysis buffer
- 3ml induced samples of 300µM, 400µM,500µM and 600µM were sonicated at 90%A ,for 10 secs each,3times
- Centrifuged at 13,000rpm for 30min at 4 ℃
- For sup,60µl dye was added, heated at 95 ℃ for 15min and was then loaded in gel
- For pellet, it was resuspended in 300µl lysis buffer. 60µl dye was added, heated at 95℃ for 15 min and then loaded in gel.

Purification of the Mn SOD protein using Ni-NTA Affinity chromatography

Affinity chromatography is mainly used for purification and separation of biological samples. In this type of chromatography, a ligand molecule, which is bounded to the protein to be purified is immobilized on a matrix (generally glass bead/agarose).

Ni-NTA is a nickel charged affinity resin that is used to purify recombinant proteins containing the His-tag.

Method

- For purification of the protein, column equilibration was done.300µl agarose was added to the column. The column was then washed with 10 CV water and 10 CV lysis buffer.
- On the other hand, the pellet was suspended in 5ml of lysis buffer, 50µl PMSF and a pinch of lysozyme was added.
- It was then sonicated at 10%A for 15 secs, 3 times.
- Then they were taken in the oakridge tube centrifuged at 9,500 rpm, 30 min at 4℃.
- 1 ml of the sup was kept aside.
- Rest of the 4ml sup was mixed with the Ni-NTA bead and kept in a rocker, in ice for about 1 hour.
- After 1 hour the sample was loaded into the column in the cold room.
- After that, flow through 1 and flow through 2 was collected for 2ml of the sample. These are those proteins that do not have any affinity for the His-tag and they flow straight through.
- For 2ml sample, wash buffer 1 and wash buffer 2(10mM and 20mM Imidazole+lysis buffer) was added. These wash buffers are mainly added to remove any non-specific binding of protein.
- 700µl E1(100µM Imidazole+lysis buffer) was added and E1 was collected
- 700µl E2(200mM Imidazole+lysis buffer) was added and E2 was collected
- 700µl E3(300mM Imidazole+lysis buffer) was added and E3 was collected
- 700µl E4(400mM Imidazole+lysis buffer) was added and E4 was collected
- 700µl E5(500mM Imidazole+lysis buffer) was added and E5 was collected
- 700µl E6(1M Imidazole+lysis buffer) was added and E6 was collected
- Column was washed with 10 CV 1M Imidazole
- Column was washed with 10 CV autoclaved water
- Finally 20% Ethanol was added to the coloumn
- SDS PAGE was then operated successfully

DIALYSIS

Since the protein was purified with contaminants, dialysis followed by Ion-Exchange chromatography was opted.

Dialysis uses the difference in their diffusion rates through a semi-permeable membrane. As a result, the large molecules cannot pass through and the small molecules will pass efficiently when equilibrium is reached.

METHOD

- 1.5ml eppendorf caps were cut.
- Add 200µl of the sample E1 containing protein into the cap.
- Wrap it with the dialysis membrane.
- Then put in a sterile beaker containing 200ml buffer.
- It was kept in stirring condition at 4℃ overnight.

Ion-Exchange Chromatography

In this case, proteins are separated due to difference in their affinity. Charged molecules bind to oppositely charged groups. Anions bind to cationic groups and cations bind to anionic groups. For the cationic exchange we have used CM and for anionic exchange we have used Q-Sepharose. The isoelectric point of our MnSOD protein is 5.21 and the pKa of Tris is 8. So the pH is greater than the pI and thus the H+ concentration becomes less which makes them deprotonated. Thus they become negatively charged. But as we increase the

concentration of NaCL, the Cl will bind with the bead and thus our protein will be free.

METHOD

- At first buffers were prepared
- Then the beads Q-Sepharose and CM (50µl) were washed with water twice.Little amount of sup was kept so that the beads do not dry up.
- After that the binding buffer was added (50mMTris+150mM NaCl), loaded in spin and the sup was discarded. Then binding buffer was added (wash2) and sup was discarded. Protein was then added to the tube.
- The tubes were kept in rocker in ice.
- The samples were subjected to spin
- Sup was collected for CM and Q and kept in the respective tubes.
- 500µl of wash1 was added in CM and Q tubes and they were loaded in the spin
- This process was repeated for the rest of the tubes.
- At the end a little amount of sup was kept and ethanol was added to it, loaded on the spin and sup was discarded.
- Stored at 4C

RESULTS AND DISCUSSION

Fig. 3: PCR of the three SOD genes.

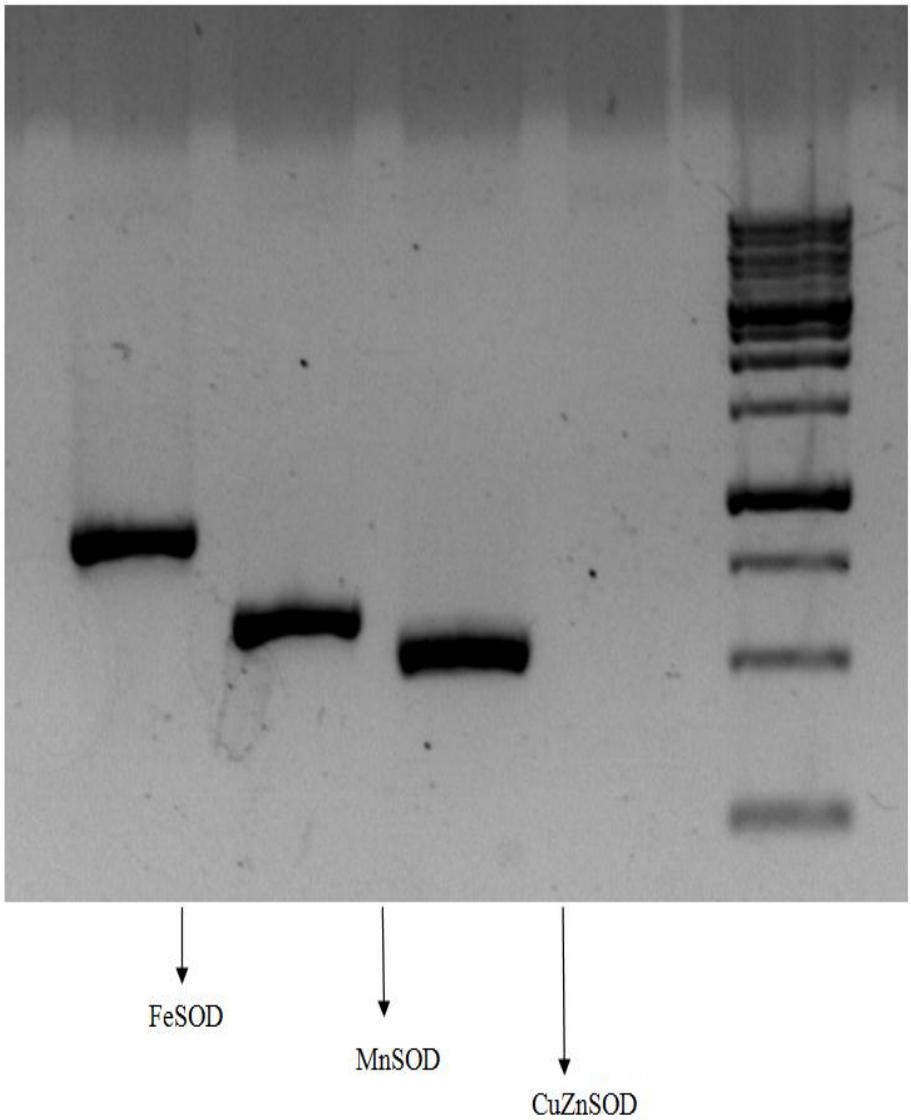


Fig. 3: Shows the PCR products of the three superoxide dismutase genes. The middle band is that of MnSOD, the one that we are concerned with.

Fig. 4: Colony PCR of MnSOD.

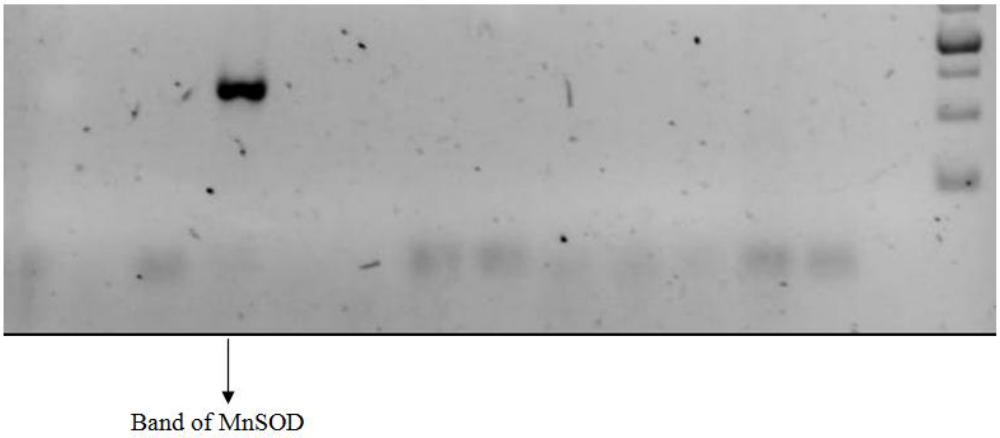


Fig. 4: Shows the colony PCR of the manganese superoxide dismutase genes with respect to the ladder.

Fig. 5: Double digestion product of MnSOD.

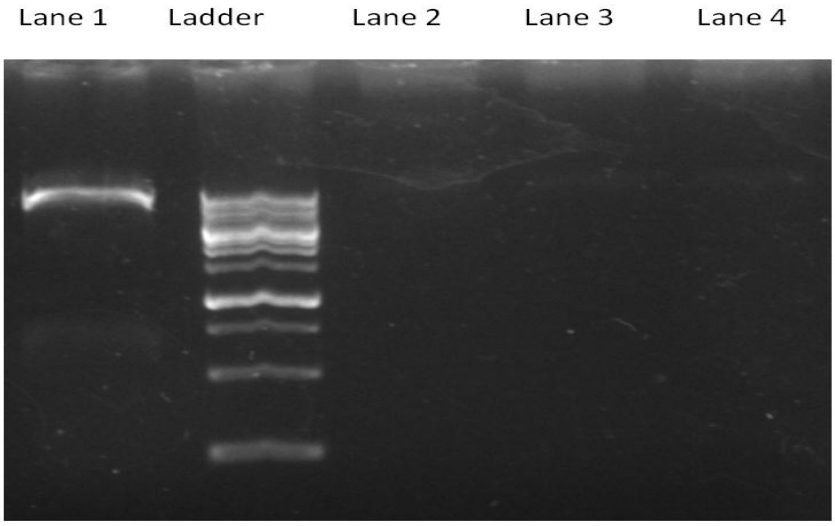


Fig. 5: shows the double digested product of MnSOD genes. Fig3 confirms successful cloning of the MnSOD proteins into the planned vectors. Lane 1 shows the insert and the plasmid.

Fig. 6: Protein expression of MnSOD at 37°C.

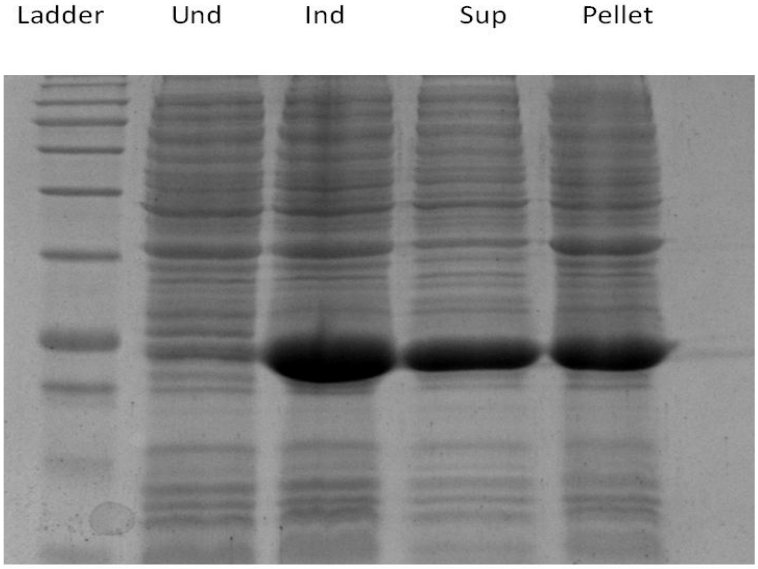


Fig. 6: Shows the expression of MnSOD proteins in C43, at 37℃ with IPTG induction.

Fig. 7: MnSOD protein expression at different temperatures (16°C and 37°C).

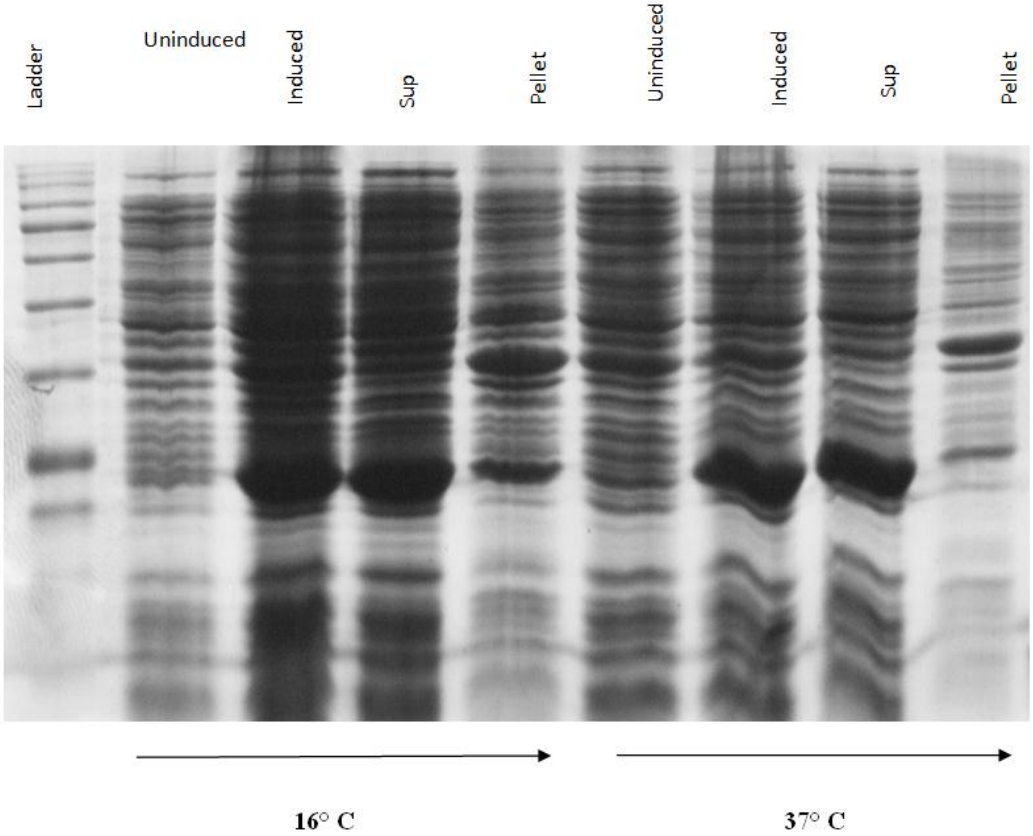


Fig. 7: Shows the expression of MnSOD proteins at two different temperatures with IPTG induction, transformed in C43. The proteins were expressed better at 16℃ since we got thicker bands as compared to the bands at 37℃ which is also very much evident from the picture.

Fig. 8: MnSoD Protein Expression at different concentration.

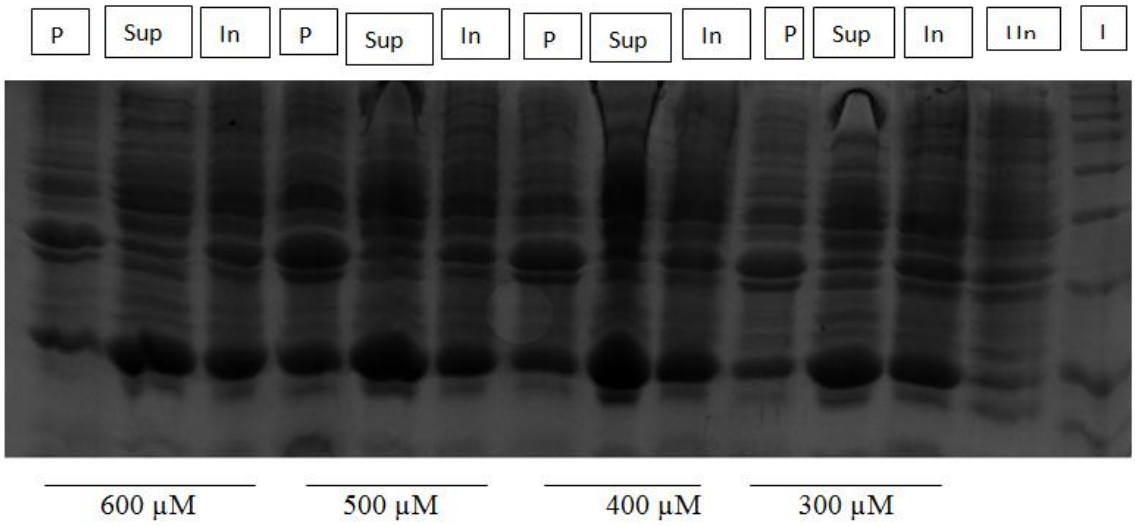


Fig. 8: demonstrates expression of MnSOD Proteins at different concentrations ie, 300 µM, 400µM, 500µM & 600µM. The proteins express better at 600µM concentration because the amount present in pellet is insignificant.

Index/Key:
Un – uninduced
In – Induced
Sup – Supernatant
P – Pellet

Fig. 9: MnSOD protein purification using NiNTA affinity chromatography.

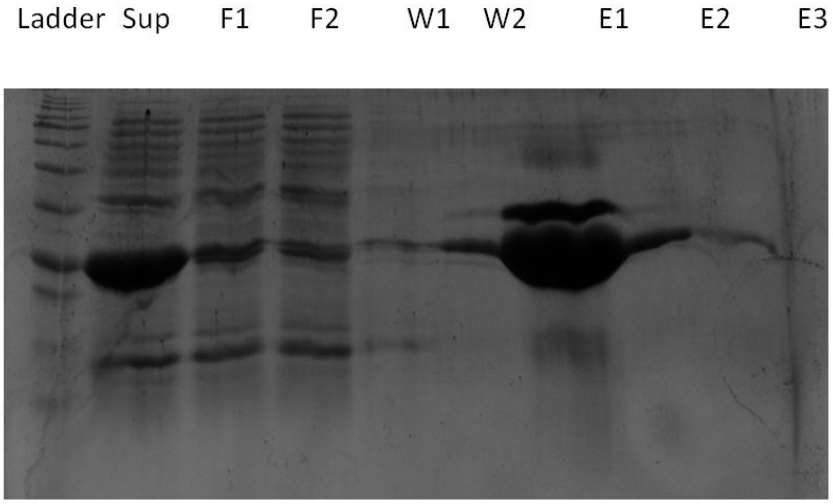


Fig. 9: Shows the result after purification was done with the Ni-NTA agarose bead using the principle of affinity chromatography. The protein of interest was eluted in Elution1 (E1) but with impurities, which is further subjected to dialysis and ion exchange chromatography.

Fig. 10: Protein purification using Ion-Exchange chromatography.

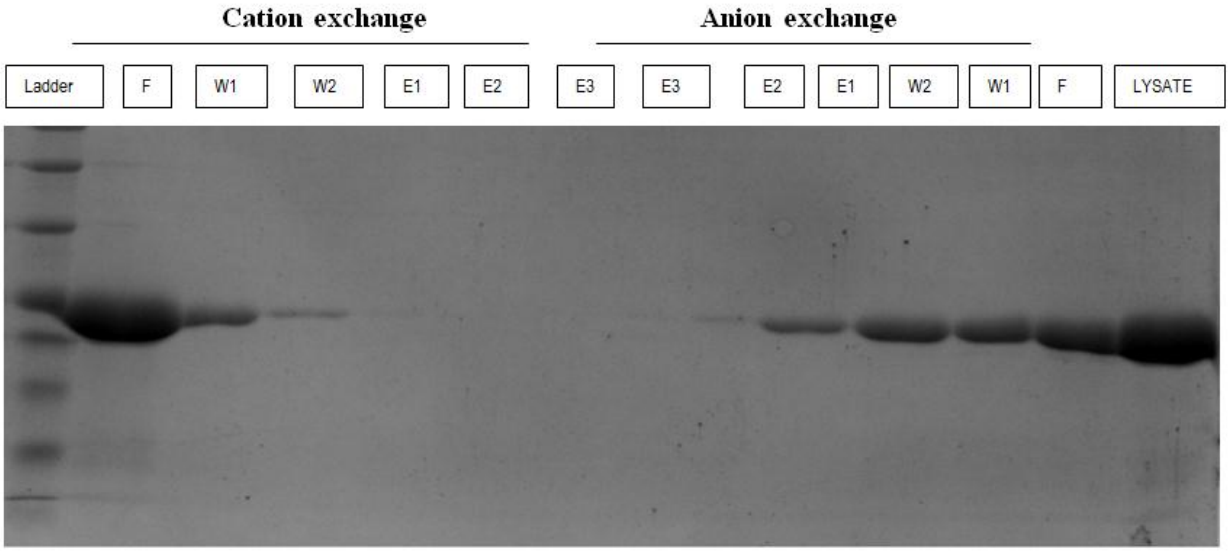


Fig. 10: Shows the purification of the MnSOD proteins after cap dialysis and Ion-Exchange Chromatography was performed. The amount of protein in cation exchange is almost insignificant. Anion exchange has significant amount of protein. The proteins are present in W1 and W2 mainly along with E1 which shows the residual proteins.

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