CHAPTER 6

SUMMARY AND CONCLUSIONS

1. *Mycobacterium tuberculosis* H37Rv cells were grown on modified Youmans medium at 37°C. Log phase cells were harvested.
2. Genomic DNA was extracted from these cells by CTAB method.
3. PCR primers were designed for the amplification of structural *asd* gene using DNA Stryder software. The primers were successfully used for the amplification of *asd* gene. The PCR reaction was optimized. A hot start (94°C/5 min) was followed by 35 cycles of amplification comprising each of 94°C, 63°C and 72°C (denaturation, annealing and extension, respectively) for 1 min each. An end filling step of 72°C/10 min was given in the end.
4. The PCR amplified product was of the expected size (around 1.0 kb). Reported size of *M. tb asd* gene is 1038 bp.
5. The PCR product was purified from agarose gel and ligated into pGEM-T Easy vector at an insert to vector molar ratio of 3:1.
6. An aliquot of ligated mix was transformed into $CaCl_2$ treated competent cells of *E. coli* JM109 and subsequently plated on LB agar plates supplemented with ampicillin, IPTG and X-gal.
7. White colonies (i.e. transformants) were picked and checked for the presence of *asd* insert by PCR. Recombinant *asd*$^+$ pGEM-T Easy plasmid was extracted from one of the positive clones. This plasmid was used as a template in a fresh PCR reaction using a new set of primers F2 and R2 incorporating *Kpn*I and *Hin*dIII restriction sites respectively.
8. The PCR product was gel purified, ligated into pGEM-T Easy vector and transformed into *E. coli* JM109 cells as described above.
9. Recombinant colonies were selected and checked for the presence of insert as mentioned above. Recombinant *asd*$^+$ pGEM-T Easy vector (with sites) was extracted and double digested using *kpn*I and *Hin*dIII restriction enzymes.
10. pQE-30 expression vector was extracted from *E. coli* DH5α cells and digested with *kpn*I and *Hin*dIII restriction enzymes as well, thus generating

cohesive ends for the purpose of ligation of the digestion product (*asd* gene) into the linearized expression vector.

11. The *asd* insert and linearized pQE-30 vector were then purified from agarose gel. The insert was ligated into the pQE-30 expression vector at an insert to vector molar ratio of 2:1. In this way we achieved directional and in-frame sub-cloning of the *asd* gene into the expression vector. The ligation product was then transformed into competent *E. coli* M15 cells and selected on LB + amp + km plates.
12. Recombinant colonies were screened for the presence of insert by digestion and PCR.
13. Recombinant colonies were grown in LB broth containing ampicillin and kanamycin and the expression of ASD enzyme was induced by IPTG after a culture $O.D._{600}$ of 0.8 was achieved. Induction conditions were 1 mM IPTG/ 37°C/ 4 hrs.
14. The induced cells were harvested and lysed in the loading buffer. The cell lysate was loaded onto 10% SDS-PAGE and resolved using a current of 20 mA. A thick band corresponding to *M. tb* ASD (approx. 38-40 KDa) appeared on the gel. Densitometric analysis showed the recombinant protein to be overexpressed at a level of 40% of total cell proteins.
15. Western blotting of the induced sample was successfully carried out using primary anti-6xHis antibodies. Clear bands appeared on nitrocellulose membrane, therefore suggesting that the 6xHis tag and the *asd* gene were in-frame. No truncated product was formed, ruling out the possibility of PCR induced errors.
16. Three clones showing high level of expression of the recombinant protein were subjected to forward and reverse DNA sequencing. The result of one clone shared 100% identity with *asd* gene as reported in the complete genome sequence of *M. tb* H37Rv. The sequence was submitted to GenBank and appears with accession number AY372113.

17. Preliminary studies on the large scale production of recombinant ASD by induction studies (37°C, 1 mM IPTG , 4 h) revealed the formation of inclusion bodies in the cytoplasm and hence little functionally active ASD.
18. To achieve the expression of soluble and active enzyme, induction conditions were altered. Induction temperature and IPTG concentration were reduced to check the increase in soluble enzyme production. It was found that the maximum soluble enzyme was achieved at 23°C/ 0.15 mM/ 5 h. Alteration of induction conditions beyond this did not result in additional enzyme activity. Hence, these conditions were adopted for further experiments.
19. The recombinant ASD was purified using Ni-NTA affinity chromatography. The enzyme containing sample was passed through the matrix to facilitate the binding of the 6xHistidine tag to the nickel moiety. An imidazole gradient was used to elute the desired protein. Recombinant ASD was eluted at 150-160 mM imidazole concentration. Approximately 8-10 mg of the enzyme could be purified from a 1 L culture.
20. The samples containing purified ASD were pooled and passed through a G-25 Sephadex desalting column to get rid of imidazole. Using this technique, recombinant ASD enzyme was purified to near homogeneity (96% purity).
21. The enzyme was found to possess a specific activity of 13.4 μmol min^{-1} μg^{-1} and turnover number (K_{cat}) of 8.49 sec^{-1}.

In conclusion, the functional ASD enzyme of *M. tb* H37Rv was obtained by gene cloning, intracellular expression and protein purification using affinity chromatography and the enzyme characterization was carried out successfully. This enzyme is a validated drug target and the future work in the laboratory would focus on deducing the three-dimensional structure of the enzyme and design of inhibitors, which could be used as drugs against TB.