CHAPTER 5

SUMMARY AND CONCLUSIONS

A total of 450 bacterial cultures from In-house culture center were screened for nitrilase production by employing bromothymol blue (BTB) agar plate method as primary screening method and 17 strains were short-listed. Further, these isolates were screened for specific nitrilases towards bioconversion of acrylonitrile and iminodiacetonitrile (IDAN) using HPLC (high performance liquid chromatography) and UPLC (ultra-performance liquid chromatography), respectively and short-listed to 8. Out of 8 isolates, 6 strains were exhibited activity towards bioconversion of both the nitriles acrylonitrile and IDAN. Remaining two strains (IICT-A38 & IICT-akl387) have shown activity exclusively for bioconversion of acrylonitrile. From both the above methods, it was observed that only 8 strains out of 17 BTB plate positives exhibited nitrilase activity. The false positives (9 isolates) obtained in BTB agar plate method might be due to other acidic metabolites. From the analysis of above screening methods it can be concluded that the direct screening methods like HPLC and UPLC asaays are more reliable than indirect methods like BTB agar plate method because of the probability of getting more false positives in BTB agar plate method due to interference of other substances along with nitrilase produced by microbes. Though HPLC and UPLC assays are final confirmation to assess nitrilase activity, inconvenient to use for screening of a large number of isolates as time consuming and expensive.

The six strains which were positive for acrylonitrile and IDAN hydrolysis identified up to species level by morphological, physiological, biochemical characteristics and 16S rDNA sequence analysis. The isolated microorganisms were identified as *Alcaligenes faecalis, Bacillus subtilis, Lysinibacillus boronitolerans and Bacillus* sp. Among these isolates, a new microorganism, namely, *Lysinibacillus boronitolerans* was identified as new additions to the list of nitrilase producing microorganisms. Except *Alcaligenes faecalis*, remaining isolates were identified as new addendum to the list of IDAN hydrolyzing microorganisms. However, 4 strains were known for hydrolysis of nitriles other than IDAN. IICT-A3, identified as *Alcaligenes faecalis*, was promising with highest conversion rate for acrylonitrile while IICT-akl252

(identified as *Lysinibacillus boronitolerans)* exhibited highest conversion rate for IDAN hydrolysis. Hence, furtehr studies were carried out with these isolates.

To develop a feasible industrial process, growth medium composition and reaction parameters are critical and need to be optimized for higher bioconversion. Thus, we have conducted studies towards the optimization of reaction and media parameters that play important role in production of acrylic acid and IDA by A. faecalis IICT-A3 and *Lysinibacillus boronitolerans* akl-252, respectively. Both the strains assimilated all the carbon sources tested, however, highest nitrilase activity for *A. faecalis* IICT-A3 was recorded when glucose used as carbon source at a concentration of 10 g/L where as in case of *L. boronitolerans* IICT-akl252 the highest nitrilase activity was observed in presence of sucrose at a concentration of 20 g/L. Nitrilases are generally inducible in nature; when a panel of inducers was tested acrylonitrile acted as the most suitable inducer for *A. faecalis* culture at 0.1% while highest production of nitrilase achieved with 0.2 % IDAN from *L. boronitolerans* IICT-akl252.

Further, optimization studies with respect to reaction conditions were conducted including different concentrations of substrate, biomass, temperature and pH. The conversion of acrylonitrile to acrylic acid was optimum between 3 to 4 mM of acrylonitrile substrate for *A. faecalis* IICT-A3 while highest activity was achieved with 100 mM of IDAN substrate concentration with *L. boronitolerans* IICT-akl252 culture. The optimum pH and temperature for the highest nitrilase activity with A. *faecalis* IICT-A3 were at pH 8 and temperatures of 35°C, respectively. In case of *L. boronitolerans* IICT-akl252 isolate, nitrilase activity was higher at 30°C while optimum pH recorded at 6.5. Amount of biomass in the reaction mixture also affects the degree of bioconversion as well as the reaction rate. The highest nitrilase activity was observed when 100 mg of wet cell weight of *A. faecalis* IICT-A3 was used whereas maximum activity attained with 150 mg of wet cell weight of *L. boronitolerans* IICT-akl252.

After optimization of medium and reaction conditions the nitrilase activity of both the strains, *A. faecalis* IICT-A3 (8.5U increase) and *L. boronitolerans* IICT-akl252 (37U increase) was significantly improved. These results indicate that, these strains might be suitable candidates for production of acrylic acid and IDA at the industrial scale.

Thermostability of the nitrilase from *A. faecalis* IICT-A3 and *L. boronitolerans* IICT-akl252 investigated by pre-incubating the reaction mixtures at various temperatures ranging from 10 to 70°C for 1h. The nitrilase activity from *A. faecalis* IICT-A3 was intact up to 60°C while nitrilase from *L. boronitolerans* shown stability up to 50°C for indicating these both enzymes are fairly thermostable which is useful in industrial applications. Both the enzymes exhibited broad substrate specificity i.e. for aliphatic, aromatic and heteroaromatic substrates, thus these strains further can be extended for bioconversion of other nitriles substrates as well as.

Further, we aimed to purify the nitrilase from *A. faecalis* IICT-A3 and to this regard the nitrilase specific primers were adopted from the previous that was publised by Lie et al., 2011. Initially, a 1.1 kb amplicon corresponding nitrilase gene was fished out from genomic DNA of *A. faecalis* IICT-A3 and cloned successfully into pTNOT vector using TA cloning strategy. Further, to express the desired gene encoding nitrilase was subcloned into an expression vector pET28a(+).The positive transformant containing recombinant plasmid was identified by double digestion. The production of enzyme from recombinant cell was achieved by inducing the cells at 0.6 OD (600 nm) with IPTG for 3 h. The nitrilase protein containing His-tag was purified using Ni-NTA affinity chromatography. The purified enzyme exhibited an apparent molecular weight of 44 kDa. This purified nitrilase shares higher similarity with the nitrilase protein of *Alcaligenes faecalis* ZJUTB10. Further studies are required to determine important properties like thermostability, substrate specifity, kinetic properties etc.