Nitriles are used as feedstock material in chemical and pharmaceutical industries for the synthesis of a wide range of compounds, drug intermediates, pesticides (such as bromoxynil and dichlobenil), polymers and even solvents, such as acetonitrile (Jallageas et al., 1980; Cowan et al., 1998; Prasad et al., 2007; DeSantis and DiCosimo, 2009). Mostly mesophilic bacterial species having nitrilase activity has been isolated from environmental samples (Layh et al., 1997).

The present study deals with hyper induction responsible for microbial growth, optimization of reaction conditions in order to enhance nitrilase activity, immobilization of whole cells and their optimization to increase thermo stability, amplification, cloning and in silico studies on nitrilase gene of *R. pyridinivorans* NIT-36. Further, statistical optimization using OVAT approach and RSM was carried out for the improvement of nitrilase activity.

Bacterial sample was procured from our lab at Shoolini University. This strain was previously identified and characterized as *Rhodococcus pyridinivorans* NIT-36 on the basis of biochemical tests and 16S rRNA sequencing. Since nitrilase is an inducible enzyme so the medium was induced with isobutyronitrile. Hyper induced cells were used for optimization of reaction conditions and immobilization. The major findings of the experiments carried out in the present investigation have been discussed in the following sections.

5.1 Hyper induction of nitrilase in *R. pyridinivorans* NIT-36

Nitriles are also a major potential source of environmental hazards. Nitrilases hydrolyse nitriles under mild conditions and in a stereo- or regioselective way. The nitrilase expression in *R. pyridinivorans* NIT-36 declined after 36 h incubation. The reduction of inducer in the growth medium was prevailing through multiple feeding of inducer (isobutyronitrile) in the medium to improve the nitrilase expression (Bhatia et al., 2013). The expression of nitrilase increases due to multiple feeding of isobutyronitrile after fixed interval of time. The expression of nitrilase in *R. rhodochrous* J1 and *R. rhodochrous* K22 was carried out by multiple feeding of isovaleronitrile in the growth medium (Nagasawa et al., 1988 and Kobayashi et al. 1990 a, b). Inducer feeding was used to increase the bacterial biomass and enzyme production; higher inducer concentration and frequent feedings inhibited the growth.
and enzyme production, therefore feeding after intervals was preferred as nitriles are toxic so bacteria takes time to consume them. Isovaleronitrile was very toxic so the incubation time was increased to 96 h and 120 h for *R. rhodochrous* J1 and *R. rhodochrous* K22 respectively for maximum growth and nitrilase expression. Bhalla *et al.*, 1992 reported expression of nitrilase for *R. rhodochrous* PA-34 in short incubation of 30 h at 28°C.

### 5.2 Substrate specificity of the nitrilase of *R. pyridinivorans* NIT-36

The nitrilase activity of *R. pyridinivorans* NIT-36 was tested for nine different nitriles. These were acetonitrile, acrylonitrile, adiponitrile, benzonitrile, butyronitrile, isobutyronitrile, mandelonitrile, propionitrile and valeronitrile. The nitrilase of *R. pyridinivorans* NIT-36 showed higher specific activity towards benzonitrile and no activity towards mandelonitrile. Previously, available findings on different nitrilases isolated from different microorganisms propose that different nitrilase have specific substrate affinity. It was noticed that *Rhodococcus* sp. and *Rhodococcus facians* both exhibited specificity towards benzonitrile (Gupta *et al.*, 2010).

### 5.3 Optimization of reaction parameters by one variable at a time approach

The nitrilase activity in the free cells of *R. pyridinivorans* NIT-36 was analyzed for various buffer systems as acetate buffer, sodium citrate, sodium phosphate, potassium phosphate and borax NaOH. Most species of *Rhodococcus* growth at neutral pH (7.0) like *Rhodococcus* species growing at pH 7.0 are *Rhodococcus erythropolis* MTCC 1526 (Kamble *et al.*, 2010), *Rhodococcus ruber* strain AKSH-84 (Kamal *et al.*, 2010) and *Rhodococcus* sp. strain YH3-3 (Kato *et al.*, 1999). Gupta *et al.*, 2010 optimized pH of *Rhodococcus* sp. and *Rhodococcus facians* was 8.0 and potassium phosphate buffer (0.1 M) showing its slightly alkaline nature.

The resting cells of the *R. pyridinivorans* NIT-36 showed increase in nitrilase activity up to 40°C after which a decline in activity was observed. This confirms that nitrilase of *R. pyridinivorans* NIT-36 was active under mesophilic conditions and not able to tolerate high temperature. The enzyme was most stable at 20°C and decrease in thermo stability was observed with increase in temperature for benzonitrile as substrates. Nitrilases from other mesophilic *rhodococcus* like *Rhodococcus* sp. and *Rhodococcus facians* (Gupta *et al.*, 2010), *Rhodococcus erythropolis* MTCC 1526
(Kamble et al., 2010), Rhodococcus ruber strain AKSH-84 (Ahmed et al., 2010) and Rhodococcus sp. strain YH3-3 (Kato et al., 1999) recorded optimum growth and enzyme production at 25 to 45°C.

The cells of Rhodococcus pyridinivorans NIT-36 showed best activity for 150 mM substrate (benzonitrile) after this concentration substrate start inhibiting the enzyme activity. With an increase in substrate concentration the activity declined. According to Gupta et al., (2010) benzonitrile in concentration 40 mM showed best results for Rhodococcus sp. and R. facians. Rhodococcus sp. strain YH3-3 showed maximum growth at 50 mM of E-pyridine-3-aldoxime as substrate (Kato et al., 1999). As per Kamal et al., (2010) Rhodococcus ruber strain AKSH-84 the yield of bioconversion using whole resting cells was recorded to be 63% (acrylic acid concentration was 126 mM) after 120 min, which is plausibly due to the slower mass transfer of the substrates and products into and out of the cells. The yield of bioconversion using purified nitrilase (50 U/mg) was observed to be 92% (acrylic acid concentration was 183 mM) after 30 min.

Along with substrate concentration cell volume is also important factor for enzyme activity. The nitrilase activity was observed maximum when cell volume was 10 µl. There was steady decrease with increase in cell volume.

Nitrilases do not need any metal ions as co-factor for their activity. The resting cells of Rhodococcus pyridinivorans NIT-36 showed 74% reduction in activity in favor of Cu²⁺ and 31% reduction for K⁺. In previous studies inhibition of specific activity of nitrilase was reported due to metal ions like Co²⁺, Hg²⁺ and Ag⁺ as they hinder the cell growth of Rhodococcus rhodochrous J1 (Nagasawa et al., 1988). According to Kato et al., (1999) there is no effect of metal ions on activity and heavy metals inhibit the activity.

Response surface methodology (RSM) and central composite design (CCD) was a helpful tool for investigating and exploring the reaction conditions for maximum nitrilase production. Four variables i.e. temperature, pH, substrate concentration and cell volume were taken in consideration for optimization. Total of 30 runs were performed and optimum pH 7.5, temperature 40°C, substrate concentration 150 mM and cell volume 10 µl was recorded. The R² value (0.85%)
and model F-value (11.19) implies that the model is significant. According to Kamble et al., (2010), total 22 experiments were carried out and the optimal values were reaction pH 6.85, temperature of 24.8°C, cell concentration of 190.98 mg/ml, and substrate concentration of 21.98 mM. The $R^2$ value (0.85%) and model F-value (11.19) implies that the model is significant.

5.4 Immobilization of whole cells nitrilase of *Rhodococcus pyridinivorans* NIT-36

In order to enhance the maximum catalytic potential of nitrilase of *Rhodococcus pyridinivorans* NIT-36, the immobilization of enzyme was carried out. Chitosan was used as matrix for immobilization of *Rhodococcus pyridinivorans* NIT-36 cells for conversion of nitriles to corresponding amino acids. The reaction parameters were optimized with immobilized cells similar to free cells. The resultant microspheres exhibited cell immobilization capacity of 450mg/g.

Immobilization is the simple way for detachment of the enzyme from product during the reaction which consists of two-phases, one phase containing the enzyme and the other phase containing the product. Immobilization is achieved by fixing the enzyme to some other material called matrix (Engasser et al., 1976). A wide range of insoluble materials used to immobilize the enzymes they may be inert polymeric or inorganic matrices (Besombes et al., 1997). Advantages of immobilized enzymes are: it minimizes the enzyme loss in the product as it is easy to separate reaction mixture; it reduces the production cost by repetitive use of enzyme; stability and activity control to prevent denaturation of enzyme in the reaction medium.

Chitosan is a random mix of deacetylated (D-glucosamine) and acetylated (N-acetyl-D-glucosamine) units acquired from the shells of crabs, lobsters, shrimps and other crustaceans. Alkali treatment of shells makes it a natural polymer as well as water-soluble (though mostly at acidic pH) and beside it this treatment made it attracted to negative charges and excellent biocompatibility (Gavhane et al., 2013). Chitosan is produced by enzymatic N-deacetylation under relatively mild conditions (Harish et al., 2005). The commercially available chitosan is mostly derived from chitin by alkaline N-deacetylation of crustaceans because it is easily accessible (Amorim et al., 2003 and Cervera et al., 2004).
Chitosan chemically bind with negatively charged fats, lipids and bile acids as it possesses a positive ionic charge (Sandford, 1992). It is a non-toxic, biodegradable and biocompatible polymer. Over the last several years chitosan is used as promising renewable polymeric materials because of vast applications in the biotechnology for enzyme immobilization, purification, pharmaceutical, wastewater treatment, food industries for food formulations (Knorr, 1984). The cross-linking reaction is mostly effected by the size and type of cross-linker agent and the functional groups of chitosan as diffusion is easy when the molecular size of crosslinker is small. The interaction between crosslinker and chitosan forms covalent or ionic bonds (Simon & Murphy, 1994).

Advantage of chitosan is it’s compatibility with the enzyme, that’s why it is extensively used (Singh et al., 2011). Chitosan is easily dissolved in diluted acetic acid and have free amine groups which are active site in many chemical reactions (Knaul et al., 1999). Above pH 7.0 chitosan solubility is poor. At higher pH, precipitation or gelation may occur and the chitosan solution result in formation of gel as a result of binding of poly-ion complex with anionic hydrocolloid (Kurita et al., 1998). It is a biopolymer having high molecular weight.

A number of matrices have been used for whole cell immobilization of nitrilase enzyme. This include sol-gel silica hybrids used for thermophilic Bacillus sp. UG-5B (Kabaivanova et al., 2008), alginate beads used for Pseudomonas putida MTCC 5110 (Banerejee et al., 2006), Alcaligenes facealis MTCC 126 (Kaul et al., 2006), polyethyleneimine/glutaraldehyde for Arthrobacter nitroguajacolius ZJUTB 06-99 (Shen et al., 2009), agar beads for Nocardia globerula NHB-2 (Raj et al., 2007) and polyacrylamide gel disc for Rhodococcus rhodochrous (Chand et al., 2008). Chitosan linked immobilization has been carried out for a number of enzymes viz. lipase (Huang et al., 2007), β-glucosidase (Changa et al., 2007), tyrosinase (Wang et al., 2002), amylase (Rana et al., 2014), β-glactosidase (Chen et al., 2013) and cellulase (Mao et al., 2006). The majority of these enzymes are extracellular enzymes.

Immobilization of nitrilase was previously reported using calcium alginate, sodium alginate and agar. Raj et al., (2007) immobilized the cells of Nocardia globerula NHB-2 having nitrilase activity that were used to catalyse the transformation of benzonitrile to benzoic acid. Bhalla et al., (2009) observed
maximum nitrilase activity of the immobilized cells when beads were prepared with
1% agar. Nigam et al., (2009) investigated that thermophilic nitrilase produced from a
new isolate Streptomyces sp. MTCC 7546 in both the free and immobilized state.

Characterization of immobilized beads was studied by FTIR, XRD and SEM. FTIR showed characteristic peaks for chitosan as well as for nitrilase of Rhodococcus
pyridinivorans NIT-36 and they are due to presence of alcohols, phenols, carboxylic
acids, esters, alkynes, amines or ether. There is slight modification in peaks of
chitosan and immobilized nitrilase. FTIR suggest that some interaction is occurring
between the glutaraldehyde or glyoxal and the amine group on the chitosan backbone
(Knaul et al., 1999). Rana et al., (2014) also observed modification of characteristic
peaks of reference chitosan-cl-poly(AAm) and amylase immobilized spectrum. XRD
analysis showed structural change in chitosan due to immobilization. For chitosan
characteristic peak was observed at 2θ=19-21º and 26-32º and for immobilized
nitrilase 2θ=21-24º and 33-39º. It confirms the crystalline nature of chitosan after
immobilization. According to Rana et al., (2014) modification in the 2θ values
supports the effective and uniform crosslinking that result in increased crystalinity.
The rigid crystalline structure of pure chitosan is stabilized by intra and
intermolecular hydrogen bonds. SEM was also executed to analyze enzyme
adsorption on matrix. SEM images showed outgrowth due to glutaraldehyde cross-
linking. It also showed covalent interaction occurred between immobilized cells and
matrix. According to Rana et al., SEM results supported the porous microsphere
structure for chitosan-cl-poly(AAm) with non-aggregated amylase immobilization,
which accounts for comparable activity of immobilized amylase (3.28 µmol/ml/min)
in contrast to free amylase (3.46 µmol/ml/min).

Substrate affinity for immobilized cells showed maximum activity for
acrylonitrile. Other substrates except isobutyronitrile showed no activity. This
variation in substrate affinity is due to the differential nature of enzyme-substrate
interactions. Acrylonitrile due to its low molecular weight displayed easier diffusion
potential across the immobilization matrix while benzonitrile owing to its high
molecular weight must have encountered greater hindrance in mass transfer leading to
reduction in activity of immobilized cells.
Optimization by one variable at a time approach (OVAT) for temperature, buffer system with pH range, buffer molarity, substrate concentration and incubation time was performed. The buffer system with varying pH range was optimized and maximum activity was observed at 7.5 for potassium phosphate buffer (0.1 M). Bandopadhyay et al., (1986) and Dhillon et al., (1999) observed maximum nitrilase activity for agar entrapped cell at pH 8.0 in Arthobacter sp strain J1 and Pseudomonas species respectively. However, pH 9.2 was observed to be best for immobilized cells reported in Klebsiella pneumoniae sp. (Stalker et al., 1987) and Fusarium solani IMI196840 (Harper et al., 1977).

Optimum temperature for nitrilase activity of immobilized cells was 60°C. The temperature was varied from 10-70°C. Nitrilase activity of immobilized cells decreased with increase in temperature after 60°C. The increase in temperature after immobilization contributes to its thermo stability. Raj et al., (2007) observed maximum nitrilase activity for agar immobilized cells at 40°C in Nocardia globerula NHB-2. Nigam et al., (2009) immobilized whole cells of Streptomyces sp. MTCC 7546 in agar-agar and observed biotransformation of acrylonitrile at a high temperature (50°C) in a batch mode of operation.

Optimization of incubation time and substrate concentration was carried out. Maximum activity was observed after incubation of 15 minutes for 200 mM substrate (acrylonitrile). However the nitrilase of Nocardia globerula NHB-2 (Raj et al., 2007) exhibited optimum substrate concentration was 4% (v/v). According to Li et al., (2015) immobilized cells showed excellent substrate tolerance when substrate (3-cyanopyridine) concentration was 700 mM.

Optimization of immobilized cells by RSM and CCD (central composite design) was analyzed. The activity obtained with immobilized cells was considerably less than with free cells. The maximum activity was observed at 60°C with 7.0 pH potassium phosphate buffer (0.1 M) and 200 mM substrate concentration. The R² value (0.96%) and model F-value (28.95) implies that the model is significant.

Reusability of Immobilized cell as analyzed up to 10 cycles and 50% activity was retained after seven cycles. Mustacchi et al., (2005) immobilize cells of
Rhodococcus rhodochrous within alginate beads with a 26% reduction in the biotransformation rate.

A 2.38 fold enhancement in nitrilase activity for non-immobilized cells and a 4.39 fold improvement in the nitrilase activity of immobilized cells underline the utility of employing a statistical optimization approach.

5.5 PCR amplification, sequencing and cloning

Primers for amplification of nitrilase gene of R. pyridinivorans NIT-36 was 5’-AAAAGAATTCCGGAGGTCGGGGGAGC-3’ and 5’-AATACATATGGGTCAGGGCGAGG-3’ designed by Primer3 online software. Restriction sites inserted were EcoRI and NdeI. Previously reported primers for amplification are 5’-TTCATATGGTCGAATACACAAAC-3’ and 5’-TTAAGCTTTCAGATGGGAGGCTG-3’ with restriction sites NdeI and HindIII of R. rhodochrous ATCC 33278 (Yeom et al., 2008). 5’-AGGTACGCATATGGTCGAATACACAAAC-3’ and 5’-TACAAGCTTCGAGTCAGATGGGAGGCG-3’ with restriction sites NdeI and HindIII of R. rhodochrous tg1-A6 (Luo et al., 2010).

A segment of 1351 bp was obtained, followed by nucleotide BLAST showing 100% similarity with Rhodococcus sp. 2G, complete genome, R. pyridinivorans SB3094, complete genome and Comamonas kerstersii strain 8943, complete genome and 99% similarity with Rhodococcus sp. p52, complete genome and 82% with Micromonospora siamensis strain DSM 45097 genome assembly. Thereafter, protein BLAST showed 92% similarity with R. rhodochrous tg1-A6, R. rhodochrous PA-34 and R. ruber.

Purified PCR product was cloned into pUC19 vector (2686bp). Multiple sequence alignment was done for the identification of active sites. As per Heinemann et al., (2003) gene encoding a putative nitrilase was identified in the genome sequence of the photosynthetic cyanobacterium Synechocystis sp. strain PCC6803. The gene was amplified by PCR and cloned into an expression vector. According to Luo et al., (2010) nitrilase gene of R. rhodochrous tg1-A6 was amplified with using PCR and sequenced. The open reading frame of the nitrilase gene contains 1,101 base pairs, which encodes a putative polypeptide of 366 amino acid residues. The nitrilase gene
was cloned into an expression vector pET-28a and expressed in an *E. coli* strain BL21 (DE3).

Kaplan *et al.*, (2011) amplified nitrilase gene using degenerate forward primer from N-terminal fragment sequence and reverse primer from internal peptide fragment sequence of *Aspergillus fumigatus* further followed by cloning partial cDNA of the nitrilase gene. The pOK101 and pOK102 resulting vectors were transformed into *E. coli*.

According to Yeom *et al.*, (2008) nitrilase from *R. rhodochrous* ATCC 33278 hydrolyses both aliphatic and aromatic nitriles. Replacing Tyr-142 in the wild-type enzyme with the phenylalanine did not alter specificity for any of the substrate. However, the mutants containing non-polar aliphatic amino acids (alanine, valine, and leucine) at position 142 were specific only for aromatic substrates such as benzonitrile, *m*-tolunitrile and 2-cyanopyridine, and not for aliphatic substrates.

### 5.6 Homology modeling and docking studies of nitrilase

The nitrilase superfamily is comprised of various thiol enzymes that participate in natural product biosynthesis and post-translational modification in plants, animals, fungi and certain prokaryotes. The superfamily can be classified into 13 branches according to sequence similarity and the presence of additional domains out of which nine conclude specificity for specific nitrile- or amide-hydrolysis or amide-condensation reactions (Pace and Brenner, 2001). According to Kiziak *et al.*, (2009) cysteine residue in the catalytic center was in close proximity to Ala-165 and Cys-163 residues of the *P. fluorescens* EBC191 nitrilase, proved to be accountable for the enantioselectivity and product formation.

Liu *et al.*, (2011) reported *in silico* studies of nitrilase from *Alcaligenes faecalis* ZJUTB10, considering Glu-47 as a general base and Cys-163 attacks the cyano group of nitriles where as Lys-147 supported stabilization of the tetrahedral intermediate structure. Chen *et al.*, (2015) analyzed the binding of dinitrile as substrate to catalytic triad by hydrogen linkage. Cys and Glu bound to two cyano groups of dinitrile which resulted in stable transition state. They reported the importance of distance between cyano group and sulphur in catalytic reaction. Low or
no activity was most likely due to extended distance connecting the cyano and sulphur group.

Three-dimensional structure of nitrilase from *R. pyridinivorans NIT-36* has been modeled. The conserved catalytic triad is Glu62, Lys53 and Cys89. Three-dimensional structure of *Rhodococcus* sp. BX2, *R. ruber*, *R. rhodochrous* tg1-A6 and *Nocardia globerula* were also modeled and their conserved catalytic triad is Glu48, Lys 131 and Cys 165. Nitrilase from *R. pyridinivorans NIT-36*, *Rhodococcus* sp. BX2, *R. ruber*, *R. rhodochrous* tg1-A6 and *Nocardia globerula* were docked with nine ligands (acetonitrile, acrylonitrile, adiponitrile, benzonitrile, butyronitrile, isobutyronitrile, mandelonitrile, propionitrile and valeronitrile). *R. pyridinivorans NIT-36 and Rhodococcus* sp. BX2 showed hydrogen bonding with adiponitrile among other nitriles.

Many forces like hydrophobic, dispersion, or van der Waals, hydrogen bonding and electrostatic are involved in docking. In *R. pyridinivorans NIT-36* Valine and Leucine amino acid are interacting with most of the substrates, thereby signifying their importance in the hydrophobic interaction with ligand. For *Rhodococcus* sp. BX2 and *Nocardia globerula* Valine amino acid is involved in most of the substrate interactions, but in case of *R. ruber* and *R. rhodochrous* tg1-A6 Glycine amino acid seems to be playing a crucial role in enzymatic activity which is evident by its maximum repetition with majority of substrates.

The conserved catalytic triad for nitrilase is Glu-48, Lys-131 and Cys-165 (Thuku, 2006). In the present case, the nitrilase of *R. pyridinivorans NIT-36* seems to share the same catalytic triad with most of the nitrilases of other bacteria, but the only variation encountered is in the relative positions of the three amino acids, i.e., Glu-62, Lys-53 and Cys-89. This fact underlines the variation that is present in the nitrilase of *R. pyridinivorans NIT-36*.

From docking studies of nitrilase of different bacterial isolates with aliphatic substrate (adiponitrile) it may be concluded that residues of catalytic triad showed direct interaction with substrate for *R. rhodochrous* tg1-A6 where as there was indirect interaction of substrate with catalytic triad through nearby amino acids for the other nitrilases. All the five nitrilases from various bacteria tend to form hydrophobic
bond with adiponitrile though the formation of hydrogen bond interaction is present only for nitrilase of *R. pyridinivorans* NIT-36 and *Rhodococcus* sp. BX2.

Docking studies with aromatic substrate (benzonitrile) reveals that there was no direct interaction of catalytic triad with the substrate and a variety of amino acids lying in close proximity with the active site are playing a critical role in enzyme-substrate interaction. This holds true for the nitrilases of *R. pyridinivorans* NIT-36, *R. rhodochrous* tg1-A6, *Rhodococcus* sp. BX2 and *Nocardia globerula*, on the other hand the amino acids of nitrilase enzyme of *R. ruber* displaying interaction with the substrate are distantly located from the active site. This *in silico* analysis of the aromatic substrate further states that there is absence of hydrogen bond formation and only hydrophobic interactions are facilitating the attachment of the substrate (benzonitrile) with the respective nitrilases.

*In silico* homology modeling and interaction of the ligand and the target protein provides a better understanding of the mechanism by which nitrilase enzyme works and further helps in identifying the residues that are involved in the interactions. It may therefore be concluded that besides the catalytic triad, a number of other amino acids of the enzyme are also playing a substantial role in effective enzymatic degradation of various nitrile compounds. These variations that exist in the nitrilases of various microbes further illustrate the enzymatic diversity that exists in the nitrilase superfamily.