Chapter 2 REVIEW OF LITERATURE

2.1 History of Daptomycin and related work

Daptomycin is a modern day cyclic lipopeptide antibiotic with a unique antibacterial mechanism against gram-positive bacteria like methicillin-susceptible *Staphylococcus aureus,* methicillin-resistant *S. aureus,* vancomycin-resistant *S. aureus,* penicillin-resistant *Streptococcus Pneumonia,* also; ampicillin and vancomycin-resistant Enterococci. Keeping in view, the potential importance of this antibiotic for the humans, these studies were focused with the objective of enhancement of microbial production of Daptomycin using different processing strategy and the utilization of Daptomycin against drug resistant MRSA strain. For the better understanding of the studies related with this work, chronologically arranged literature of this subject has been presented below:

1987-1990:

This period marks the advent of Daptomycin. Daptomycin was discovered in early 1980s. It is a natural product of soil actinomycetes, *Streptomyces roseosporus,* was isolated by scientists at the famous Eli Lilly Company from a soil sample taken from Mount Ararat (Turkey). This sporulating actinomycetes produced a complete family of lipopeptide antibiotics designated A21987C [Debono *et al*., 1987].

The literature reports during this period tell us about the potential activity of Daptomycin against methicillin-resistant staphylococci and bactericidal activity against *Streptococcus*

faecalis. Its mechanism of action involves bactericidal mechanism of action that is far different from those of Vancomycin and the popular β-lactam antibiotics [Allen *et al*., 1987].

Initially, Daptomycin was produced using *Streptomyces roseosporus* NRRL 11379 by several resin processes [Debono *et al*., 1988]. Literatures of this period also depict the structure of Daptomycin and its biosynthesis. The peptide portion of the compound contained a 13 amino acid chain linked by an ester bond between the carboxyl group of L-kynurenine13 (Kyn) and the hydroxyl group of L-Thr4 to form a 10 amino acid ring with a three amino acid tail. The three major components, A21978C1–3, have 11, 12 or 13-carbon branched-chain fatty acids, respectively, attached to the terminal amino group of L-Trp [Debono *et al*., 1987]. Its direct utility was impeded by rising toxicity led by various acyl groups attached to the exocyclic amino acids, but paved the way to alter the bioactivity through acyl group exchange[Debono *et al*., 1988]. The fatty acid side chains of A21978C are readily removed by incubation with Actinoplanes utahensis [Boeck *et al*., 1988], and the cyclic peptide can be reacylated with n-decanoyl fatty acid to produce daptomycin. Eli Lilly scientists also isolated a strain of *Actinoplanes utahensis* that produced a secreted deacylase that could cleave the natural longchain lipid side chains from the A21978C factors[Boeck *et al*.,1988]enabling the production of the core cyclic peptide for reacylation with different lipid side chain[Debono *et al*.,1988].

The research article by Debono *et al*., focuses on the structural background of Daptomycin.Daptomycin is composed of decanoyl side chain connected to the N-terminal tryptophan of a 13 amino acid peptide chain. The C-terminal amino acid,kynurenine,is

connected via an ester bond to the hydroxyl side chain of threonine. Daptomycin contains two dissociable side chain amines (primary amine at ornithine and an aromatic amine at kynurenine) and four side chain carboxylic acids (three aspartic acid and one methyl glutamic acid) [Debono *et al*.,1988]. The fermentative production of Daptomycin required n-decanoic acid which is the precursor and is added to the fermentation of *S. roseosporus* [Huber *et al*., 1987]. Lee *et al*., 1989 studied the aspartyl transpeptidation of Daptomycin in aqueous solutions. Daptomycin helped in the treatment of skin and skin structure infections caused by Gram-positive pathogens [Canepari *et al*., 1990].

1991-2000:

Daptomycin has a novel mechanism of action. Scientists at Eli Lilly developed and implemented clinical studies of intravenous (IV) Daptomycin during the late 1980s and early 1990s [Alborn *et al*., 1991; Allen *et al.,* 1991]. The most important literature regarding the discovery and development of Daptomycin belong to the following era. There were several research papers which presented various perspectives, speculations, interpretations regarding Daptomycin in this era. Non-ribosomal machinery involving several enzymes is engaged in Daptomycin synthesis. (NRPS) [McHenney *et al*., 1998; Wessels *et al*., 1996]. In this system, very large enzyme complexes composed of several subunits, with catalytic domains recognize specific amino acids and join them together in the proper order [Marahiel *et al*., 1997] and finally Daptomycin is developed and put to function. The genetic makeup for the functioning of NRPS system were discovered later where transposon mutagenesis, partial DNA sequence analysis of cosmid clones and gene-disruption experiments were used to identify the genes for the daptomycin (dpt) synthetase[McHenney *et al*., 1998). The

suitability of genetic engineering for manipulation of structure and hence function of this important drug were also discussed well in several reports on Daptomycin[Hosted & Baltz, 1996, 1997; McHenney & Baltz, 1996] .

Daptomycin was chosen for clinical development because of its in vivo efficacy and low toxicity in animals[Baltz,1997]. Earlier enzymatic deacylation, coupled with chemical reacylation was used which was a costly affair and therefore, scientists at Eli Lilly developed a process that fed decanoic acid as precursor element during fermentation to produce Daptomycin [Huber *et al*., 1998].Scientists at Eli Lilly gave birth to this wonderful anti-MRSA antibiotic and nurtured it to give its best potency in the early 1990s [Wooworth *et al*., 1992]. In early 1992, Richard Baltz and colleagues at Eli Lilly started to develop combinatorial biosynthetic approaches for modifications of the nonribosomal peptide synthetase to alter peptide cores for improvement of the therapeutic value of the antibiotic [Mc Henney and Baltz,1996; Baltz *et al*.,1997;Mc Henney *et al*.,1998].

In the initial trials, Daptomycin was well tolerated in healthy volunteers at up to 6 mg/kg IV in 2 divided doses per day[Baltz,1997]. At a higher dosage, such as 8 mg/kg per day, 2 of 5 volunteers developed the unacceptable adverse effects involving the musculoskeletal system with accompanying increases in creatine phosphokinase (CPK) levels (a marker of potential adverse effects involving the musculoskeletal system) [Tally *et al*.,1999]. Eli Lilly suspended subsequent trials to prevent side effects.

Years later, Baltz got associated with Tally at Cubist and their fruitful scientific relation eventually brought Daptomycin to Cubist in 2001. They together utilized their knowledge to

develop combinatorial biosynthesis mechanism to generate derivatives of Daptomycin that were not attainable through medicinal chemistry. The Cubist Daptomycin in-licensing agreement was finalized on 7 November 1997. Tally and Cubist team focused their efforts on developing Daptomycin formulations and treatment approaches for oral and topical clinical indications. Oral or dermal administration could minimize risks for the adverse effects involving the musculoskeletal system that resulted in the termination of the IV Daptomycin clinical programs at Eli Lilly [Tally,1999].

The Cubist research team was sure that Daptomycin had good attributes (e.g., high bactericidal activity, and high potency against gram-positive pathogens, including drug-resistant and drug-susceptible *S. aureus* and vancomycin-resistant Enterococci) and their further efforts were worthy enough to tackle the side effects of the drug that had troubled the Eli Lilly scientists. The adverse effects on musculoskeletal system could be checked by measuring the level of the CPK enzyme. The phase 2 trial results were satisfactory as they suggested that Daptomycin could be could combat complicated SSSI and bacteremia, though at higher doses [Oleson, 2000].

From medical point of view, Phase III and Phase IV trials proved that Daptomycin could now provide serious and life-threatening hospital-acquired infections.

Cubist then started to develop Daptomycin antibiotic for broad range of medical usage including the treatment of serious gram-positive infections, complicated SSSI and bacteremia with suspected infective endocarditis. In March 1998, a pre-investigational new drug

document was submitted to the US Food and Drug Administration (FDA) to inform the agency of the Cubist plans to take Daptomycin for the IV treatment of serious gram-positive infections and to seek its advice on this program.

In 1998, Cubist filed a patent application for administration of Daptomycin, Oleson and Tally being the key inventors [Oleson *et al*., 2000]. Several pharmacokinetic studies were conducted to show the efficacy of Daptomycin in a number of in vivo animal models of the soft tissues, bloodstream, kideney, heart, bone and especially those caused by gram positive resistant bacteria [Safdar *et al*., 1999].

2000-2017:

The biosynthetic gene clusters for Daptomycin and several other lipopeptide family members like Calcium dependent antibiotics (CDA) from *Streptomyces coelicolor* A(3)2 [Hojati *et al*., 2002], A54145 from *Streptomyces fradiae* A54145 [Miao *et al*., 2006], and friulimicin from *Actinoplanes friuliensis* [Muller *et al*., 2007] have been isolated and characterized for enhanced antibacterial activity . In this era, biosynthesis, combinatorial synthesis, route of mechanism of action and application of Daptomycin have been extensively covered [Baltz, 2008, Baltz, 2009]. On the basis of the literary reports, Daptomycin dosages of 4 and 6 mg/ kg per day were found to be safe and effectual by the FDA for complicated SSSI and *S. aureus* bacteremia (including right-sided endocarditis) [Cubicin,2007].In early 2003, Daptomycin for injection (Cubicin), a first-in-class acidic lipopeptide IV antibiotic, was approved by the FDA at a dosage of 4 mg/kg given once daily for the treatment of complicated SSSI caused by specific gram-positive bacteria. Since November 2003, this drug had the most financial success as anti-MRSA antibiotic in US history. In 2006, it was finally

approved for curing of *S. aureus* bacteremia, including right-sided infective endocarditis, at a dosage of 6 mg/kg given once daily [Rolston, 2007; Sauermann *et al*., 2008]. Still, Daptomycin could not meet non-inferiority criteria for the therapy of community acquired pneumonia. This low efficacy was attributed to inhibition by pulmonary surfactants [Silverman *et al*.,2005]. After Daptomycin approval and market launch, there was a continuous strive for its improvement through genetic engineering and metabolic engineering. The structural diversity and the bioactivity of Daptomycin was improved through strategic biosynthesis of the lead compound [Baltz, 2009; Kopp *et al*.,2006]. Nonribosomal peptide, Daptomycin is synthesized through the nonribosomal peptide-synthetase (NRPS) mechanism which is independent of messenger RNA. Each module involves several enzymes responsible for the specific recognition, activation, covalent binding and incorporation of a building block into the oligopeptide chain and can be furthermore divided into catalytic domains [Fischbach, 2006]. In the acylation of the *N*-terminal amino acid, the initiation mechanism consists of an acyl-CoA ligase, that activates the fatty acid, and an acyl-carrier-protein (ACP) to which the FA is covalently bound [Miao *et al*., 2005]. The *N*-terminal C-domain (type CIII) of the initiation module subsequently catalyzes the condensation between the FA and Trp1 and chain elongation is commenced [Rausch *et al*., 2007]. Initiation of daptomycin biosynthesis is mediated by the action of the two distinct enzymes DptE and DptF, encoded upstream of *dptD* [Wittmann *et al*., 2008]. DptE, which shares a high degree of homology to the acyl-CoA ligase superfamily, was shown to activate the fatty acid moiety attached to the *N*-terminus of daptomycin in an ATP-dependent manner. The activated FA is subsequently transferred onto the 4'-Ppan group of DptF, the cognate acyl-carrier-protein (ACP). The *N*-terminal C-domain of the DptA

initiation module is predicted to catalyze the condensation of the ACP-bound FA and tryptophan. The broad substrate tolerance of DptE towards the length and type of fatty acids is believed to reflect in the composition of the A21978C factors [Silverman *et al*., 2003] After the initiation, chain elongation is mediated by the linearly operating NRPSs DptA, DptBC and DptD. The three epimerization domains in the synthetases are associated with D-configured amino acids D-Asn2, D-Ala8 and D-Ser11, respectively [Miao *et al*.,2005]. The nonproteinogenic amino acids Orn6 and Kyn13 and the methylated glutamate residue (MeGlu12) are located within the ten-membered macrolactone ring. Deacylation was achieved by a highly efficient deacylase derived from *Actinoplanes utahensis*. Reacylation was performed chemically with activated acyl esters after protection of side-chain nucleophiles. The generated derivatives varied not only in the acyl functionality but also in the number of exocyclic amino acids (e.g. _-N-acylated-Phe) [Nguyen *et al*.,2006; Liao *et al*.,2012]. But,enzymatic method was tedious and costly.

Therefore, the precursor-directed fermentation helped high-quantity production of Daptomycin and substituted the time-consuming process of deacylation and reacylation. Further, removal of the *N*-terminal two exocyclic amino acids and coupling of *N*-decanoylated dipeptides to the Orn-protected undecapeptide took place. Preferring this method, Miao *et al.* evaluated the role of Asn2-sterochemistry on biological activity. Here, the L-Asn isomer revealed a 10-fold decrease in antibacterial activity [Miao *et al*., 2005]. The modification of side-chains has been applied for the acylation of the Orn6 -amino group [Hill *et al*., 2003]. In a row of acylations and consequetive evaluation of antibacterial properties, it was depicted that inclusion of long chain alkyls was not accepted, whereas

coupling of Trp preserved bioactivity. The conversion of the free Orn6 amine to an amide enhanced the MIC, also side chain modification was considered [Silverman,2001]. A reductive alkylation of the Orn6 amine to cause numerous benzylic substitutions like amide or sulfonamide groups as polar functionalities was explored. SAR studies done with these derivatives conclude that an increased electron deficiency of the aryl moiety as well as heterocyclic spacers and polar groups lead to maintaining antimicrobial activities [Hill *et al*.,2003].

Chemoenzymatic methodology for the biosynthesis of Daptomycin analogues was also attempted and investigated by uniting organic synthesis and enzymatic processes. The catalytic portion used is the TE-domain located in the *C*-terminal module of the final NRPS syatem. Again, the excised TE-domains lead to the macrocyclization of activated thioester substrates, similar to the native PCP-bound oligopeptide, the entire enzymatic machinery can be substituted likewise [Kopp *et al*.,2006,Grunewald *et al.*,2004; Sieber *et al*.,2003;Sieber *et al*.,2004]. Utilization of recombinant TE from the CDA biosynthetic machinery was also done for the production of daptomycin analogues [Grunewald *et al*.,2004]. In this, the excised domain catalyzed the macrolactonization of linear daptomycin analogues. In the course of this project seven positions within daptomycin were varied, including the acidic residues of the Ca2+-binding motif DXDG and SAR studies were carried out against *Bacillus subtilis*. The substitution of MeGlu12 with Glu led to a sevenfold increase of the MIC compared to the native compound and proved to be in full support of Nguyen *et al*[Nguyen *et al*.,2006]. Also, the replacement of Kyn13 with Trp13 enhanced the MIC. Modification in Ca2+-binding motif by single replacement of Asp7 and Asp9 with Asn eliminated

bactericidal activity, proving the significance of the acidic residues. The substitution of the exocyclic Asp3 with Asn did not diminish bactericidal activity drastically. This confirmed that only Asp7 and Asp9, at the conserved EF-hand motif DXDG, are important for cation binding [Yazawa *et al*., 1980]. An important study focused on the generation of Daptomycin through enzymatic cyclization of the linear thioesters by the TE-domains in Daptomycin NRPSs [Kopp *et al*., 2006]. SAR studies of the lipopeptide variants included an alternative macrolactam. The significance of intact DXDG-motif and the MeGlu12-residue was confirmed due to elimination of antibacterial activity in the case of Asp7 and Asp9 substitutions, and a sixfold increase in MIC when removal of MeGlu12. Structural hybrid molecules consisting of an exocyclic daptomycin peptide and endocyclic A54145 peptide cores displayed similar MICs. In totality, it can be stated that chemoenzymatic approaches offer the possibility to fasten up the construct of Daptomycin analogues that can be investigated towards SAR. Taking into the cons of this method, the *in vitro* is the low quantity of derivative generated, scientists in collaboration with the pharmaceutical industry set out to exploit the known biosynthetic machinery *in vivo* [Robbel and Marheil,2010].

After the chemoenzymatic method, the much valued approach was combinatorial approach of biosynthesis of Daptomycin which considered entire reprogramming of genes responsible for the enzymatic machinery of Daptomycin [Walsch *et al*.,2003]. *S. roseosporus* can habituate genetic manipulations and the responsible gene cluster has been sequenced, cloned and heterologously expressed [Penn *et al*.,2006] . Extensive and exhaustive research was done in combinatorial biosynthesis of daptomycin and hybrid molecules in *S. roseosporus* [Baltz *et al*.,2006;Einstein *et al*.,2010;Miao *et al*.,2006]. The modular NRPS assembly line

recommended substitutions and manipulations through tailoring from a single module to multi modules. Initially, *dptA* and *dptD* were removed from the original point [Coeffet *et al*.,2006]. These genes were subsequently hosted into *S. roseosporus* to trans-complement deletions of *dptA* and *dptD* through construction of plasmid cloning vectors leading to conjugal transfer of genetic information from *Escherichia coli* to the target strain [Mc Henney *et al*., 2006].

Linezolid shows antibacterial activity through disruption of translation of messenger RNA (mRNA) into proteins in the ribosome. It prevents the initiation complexes as it binds to the 23S portion of the 50S subunit [Ament *et al*., 2002]. The drug resistance of most Gram-negative bacteria towards linezolid is because of efflux pumps, which excrete out linezolid from cell faster than it can act. Vancomycin inhibits cell wall formation in gram-positive bacteria [Rathe *et al*., 2007]. Vancomycin is not ineffective against gram-negative bacteria. The hydrophilic molecule is capable to form hydrogen bond with the terminal D-alanyl-D-alanine moieties of the NAM/NAG-peptides. This binding of vancomycin to the D-Ala-D-Ala impedes cell wall synthesis of the long polymers of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) which prevents the backbone polymers. In resistant bacteria, the last D-ala residue has been substituted by a D-lactate, so vancomycin cannot engage. In the nonresistant bacteria, the vancomycin bound to the peptide chains does not allow them from interacting properly with the cell wall cross-linking enzyme. But, in the resistant bacteria, stable cross links get formed .The unique mode of action Daptomycin in antibacterial activities can be clarified by its mode of action [Rybak, 2000].

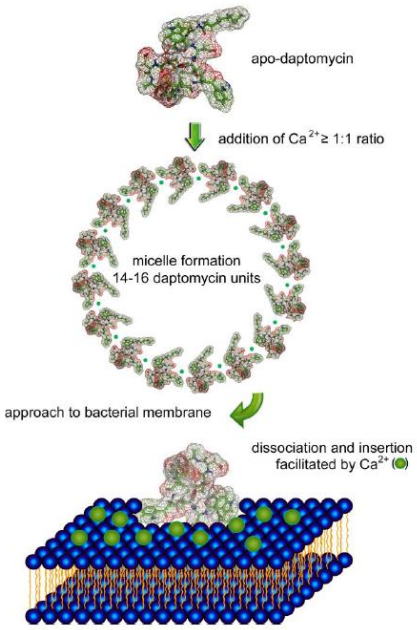

Figure 2.1 Mechanism of action of Daptomycin [Robbel and Marheil, 2010]

The present mode of action of Daptomycin helps to understand the discrete role of calcium ions in the antimicrobial activities. The initial studies by Jung *et al*. proposed a two-step mechanism of action derived from structural changes observed in NMRexperiments, CD-measurements and fluorescence spectroscopy. In the first step Ca2+ binds to daptomycin in solution and induces a conformational change, increasing amphipathicity and decreasing its charge [Jung *et al*.,2004]. This process facilitates oligomerization and leads to micelle formation which allows daptomycin to interact with neutral or acidic membranes. In a second step, Ca2+ bridges the gap between daptomycin and the acidic phospholipids. As indicated by CD measurements, daptomycin undergoes a second structural transition allowing a deeper insertion into the membrane bilayer. In contrast to the study conducted by Silverman *et al*., it was proposed that cytoplasmic membrane depolarization is not the main cause of cell death as it occurred subsequently [Silverman *et al*., 2003]. The recent studies cancel this theory based on NMR-structure of Daptomycin in the presence of Mg2+. It was found that Mg2+

also promotes micelle formation, but no conformational change is induced. Daptomycin led to oligomerization consisting of 14-16 monomers in presence of Ca2+ in a 1:1-ratio. The divalent cations cover the negatively charged residues and led to micelle formation either by stacking interactions between aromatic residues or by layout of the lipid tails towards the inner side of the micelle. Scott *et al.* [Scott *et al.*, 2007] confirmed that Daptomycin experiences only a small conformational rearrangement upon tie to DHPC in the presence of Ca2+.

Table 2.1 Microbial strains producing Daptomycin [Penn *et al*.,2006; I-Son *et al*.,2013;Yu *et al*.,2011; Lu *et al*.,2011]

Strains	**Daptomycin**	**Working Volume(L)**	**Culture time (h)**
Streptomyces roseosporus NRRL 11379	812	3.6	282
Streptomyces roseosporus LC-51	632	7.5	132
Streptomyces roseosporus NRRL 11379	296	7.0	144

The challenge to produce sufficient Daptomycin to fulfill the demand in the healthcare sector has led to various processing strategies to enhance its production. The wild type strain of

Streptomyces roseosporus NRRL11379 has been preferably used for Daptomycin production [I-Son *et al*., 2013]. Daptomycin is produced by fermentation and the characterizations of impurities are done based on detailed investigation of Daptomycin impurity profile. The main impurities include Anhydro-Daptomycin, ß-aspartyl Isomer and Lactone. Changes are made to the purification steps and to the final packaging of Daptomycin. The analytical outcomes reveal that Daptomycin used in medical applications are in accordance with the current and the previous processes are physicochemically equivalent [Linli *et al*.,2013].

Many mutant strains are being worked upon for improved Daptomycin production. For instance, *S.roseosporus* LC-51, a mutant of *S. roseosporus* NRRL11379 has shown better ability for Daptomycin production. Rational screening based on metabolic engineering is being considered for improvement of the selection process. Low power laser irradiation technology is being used for high yielding strains, nowadays [Lu *et al*., 2011]. Chemical mutation by N-methyl- N-nitro-N nitrosoguanidine (NTG) has been reported as a successful method for mutation and screening of highyield strains, which can be adapted by any in Pharmaceutical Industry for Antibiotic Production [Yu *et al*.,2011]. Various strategies, such as medium optimization, fermentative strategy, genetic engineering modification and metabolic flux analysis have been established to achieve higher production of Daptomycin.

Enzymatic reactions within biosynthetic cycle are controlled by various cofactors which play a vital role in the fermentation process. Thus, the management of cofactors distribution in the fermentation process could be important to increase the yield of the antibiotics. The outcomes of several cofactors of enzymes on Daptomycin production were evaluated, which included Tetrahydrofolic acid (THF) ,Nicotinic acid (VPP), Cyanocobalamin (VB12),

Riboflavin (VB2), Heme, Thiamine (VB1), Biotin (VH), and Pyridoxal 5-phosphate (VB6). Daptomycin yield intensified upto 632 mg/l, which is over 4.5-fold higher than that of the control (without cofactors), at 132 h in a 7.5 Litre fermenter, by addition all of the eight cofactors at optimized concentrations (VPP 4 mg/l, VB2 0.5 mg/l, Heme 9 mg/l, VB1 0.4 mg/l, VH 0.1 mg/l, VB12 0.04 mg/l, THF 6 mg/l and VB6 0.4 mg/l). This strategy used for increasing the Daptomycin production in *Streptomyces roseosporus* LC-51 by optimization of cofactors concentration in the fermentation broth may provide a suitable option to enhance the production of metabolites in other *Streptomyces* [Yu *et al*., 2011; Muller *et al*.,2013].

Downstream processing is very important aspect in biochemical industries, especially in pharmaceutical companies. Some significant solvent extraction methods are carried out in the industry for Daptomycin production [Yu *et al*., 2011]. Product analysis and purification is a very important task in antibiotic fermentation. The finished product specification includes various tests for appearance, identification (UV and FTIR), pH, assay (HPLC), powder fill weight, degradation products, uniformity of content, water content, bacterial endotoxins, particulate contamination, sterility, container closure integrity and reconstitution time. X-ray diffraction studies indicated that Daptomycin powder is amorphous. It is highly soluble in water. Stress stability studies showed that it degrades when exposed to direct light, heat, oxygen and to extreme pH in solution [Huang *et al*., 2011].

The efficacy of Daptomycin was tested in various models. Safety pharmacology was investigated in rodents, dogs, and in vitro models. No significant side effects on the cardiovascular, respiratory, renal, gastrointestinal or immune systems were observed. Daptomycin’s effects on the central nervous system as well as the neuromuscular system

were investigated through in vivo and in vitro studies. Effects on the nervous and/or muscular system were evident at high dose intravenous (IV) levels in rodents (≥ 50 mg/kg). Marked effects on the central nervous system in animals were observed only at IV dose levels of ≥ 150 mg/kg. At doses ≥ 200 mg/kg in mice, tremors and clonic convulsions were observed; with death occurring at ≥ 1000 mg/kg. No corresponding effects were observed in rats at 150 mg/kg [Dvorchik *et al*.,2003]. In vitro, at concentrations up to 4 mg/kg/day, no side effects were seen in cardiac or smooth muscles. The effect was mainly seen rat uterus. But, these symptoms were seen at a very high concentrations i.e. about 30-fold greater than the intended dose of 4 mg/kg/day [Liang *et al*., 2009]. Daptomycin is the active ingredient in Cubicin with sodium hydroxide as inactive ingredient. Cubicin is supplied in a single-use 10 mL vial, containing 500mg/vial as a sterile, lyophilized powder of Daptomycin for IV with 0.9 % sodium chloride injection. Daptomycin has a high aqueous solubility (> 1 g/mL) [Vihena *et al*., 2012].

Daptomycin shows poor oral bioavailability and can only be administered parenterally [Rice *et al*., 2009]. Dosing is based on the body weight of the patient. Daptomycin was poorly absorbed orally and exhibited slow excretion through the gastrointestinal tract with > 90% excreted in the faeces. Limited data indicated a relatively high bioavailability after SC or IP administration. Daptomycin plasma profile following IV injection was consistent with a 2-compartement model with a rapid distribution phase and slower elimination phase. Daptomycin exhibited linear kinetics in case of intravenous injection for varied dose ranges of 1 to 50 mg/kg in the rat, 1 to 200 mg/kg in the dog, and 1 to 25 mg/kg in the monkey. For all species, plasma clearance, volume of distribution, and terminal half-life were dose-

independent over the linear range. The terminal half-life of Daptomycin was 1 – 3 hours in rodents and 2 – 4 hours in non-rodents. The pharmacokinetic profile of Daptomycin was similar between strain and gender and was not altered upon repeated daily administration for up to 6 months [San *et al*., 2002].

The reason for the low volume of distribution is the drug's high plasma protein binding that has been reported to be 90 %. This high plasma protein binding always needs to be considered when comparing Daptomycin plasma concentrations with their respective MIC values. Microdialysis is an appropriate technique to monitor the unbound, active drug concentrations in tissues.

In the rat, the only species in which tissue distribution was studied, where Daptomycin distributes rapidly from the plasma to the tissues with a distribution phase half-life ($t_{½}$ α) of approximately 7 minutes. The volume of distribution was essentially the same in animal and human models. Daptomycin appears to distribute preferentially to the kidneys, reflecting the vascularisation of the tissue as well as renal concentration of the drug during excretion. In general, the half-life in tissues was slightly greater than that in plasma and tissue levels were higher after repeated dosing than after a single dose. No accumulation was observed in plasma upon repeated administration in rats, dogs, or monkeys. There is no data on the excretion of Daptomycin into milk. Published data indicated that the extent of protein binding was the same in mice, rabbits and humans [Vihena *et al*.,2012].

The half-life of Daptomycin is approximately 8 hours. The long half-life is the restricted glomerular filtration due to the high protein binding. It facilitates the possibility of a once-

daily dosing regimen [Rice *et al*., 2009]. In mice, rats, dogs, monkeys most of the compound (≥ 70 %) was recovered in the urinary within 48 hours post-dose. Faecal excretion accounted for approximately 3 to 10 % of the administered radioactivity in these species. This is comparable to the human data. As expected, patients with impaired renal function show longer half-lives and require longer dosing intervals [Kerry *et al*.,2010].

In rats less than 2 % of the administered radioactivity was recovered in the expired air. A study in juvenile dogs showed that total systemic clearance appears to be faster in juvenile dogs as compared to adults, resulting in shorter terminal half-life, at the same dose level. In rats with renal impairment, the systemic clearance was reduced by ~70 % compared to that of normal rats. This resulted in a ~1.5 to 2-fold increase in peak plasma concentration (Tmax), and increased half-life. Volume of distribution was decreased by 53 %.

Daptomycin is a new generation lipopeptide antibiotic approved by the Food and Drug Administration in 2003 for the treatment of skin and skin structure infections caused by Gram-positive pathogens and for curing bacteraemia and right-sided endocarditis caused by *S. aureus* strains and MRSA in 2006. The clinical approval of Daptomycin was on the basis of randomized, investigator-blinded trials comparing Daptomycin to either Vancomycin or conventional antibiotic Penicillin in the treatment of complicated skin and skin structure infections. Clinical trials are on-going examining Daptomycin's efficacy in the treatment of complicated urinary tract infections, bacteraemia, and endocarditis. Trials in endocarditis and bacteraemia are utilizing a higher dose (6 mg/kg) than the currently approved dose. Merck obtained rights to Cubicin through its $8.4-billion acquisition of Cubist Pharmaceuticals in

December 2014.Cubicin and Cubicin RF are the two products being manufactured and marketed by Merck at present [Gonzalez *et al*.,2017].

Daptomycin is a bactericidal antibiotic with excellent activity against Gram-positive organisms. The market prospective of Daptomycin is very promising and the demand of Daptomycin in the health sector is increasing. More importantly, doctors now have a reliable treatment for life-threatening infections, especially the drug-resistant which cause high mortality. However, the yield of Daptomycin needs to be further improved to reduce the cost of industrial production which is now $152 per 500mg vial. The cost reduction of this high-value secondary metabolite can be achieved through higher production; therefore, various disciplines of Biotechnology, Genetic Engineering, Metabolic Engineering and Biochemical engineering should combine to enhance the production of the antibiotics.

Decanoic acid is the crucial precursor for Daptomycin production. The antibiotic production is not initiated from *S.roseosporus* without decanoic acid addition. Decanoic acid, sodium decanoate and Cuphea oil [Penn *et al*., 2006] have been used to enhance the Daptomycin fermentation. The problems of precursor inhibition, substrate inhibition and product inhibition of *S.roseosporus* are addressed by several researchers in their literature indicating that the growth of cells would be inhibited by the toxicity of n-decanoic acid without optimization of n-decanoic acid during fermentation [Yu *et al*.,2011]. Decanoic acid, Sodium decanoate and Cuphea oil were compared for Daptomycin production.

Table 2.2 Precursor directed production of Daptomycin [I-Son *et al*.,2013; Lu *et al*.,2011; Gianluca Bertetti *et al*.,2010]

Precursors	**Daptomycin Production(mg/L)**	**Culture time(h)**	**Working Volume(L)**
Without any precursor	NIL	282	3.6
Decanoic acid	296	144	7
Sodium decanoate	812	282	3.6
Cuphea Oil	600	186	20

Sodium decanoate as the precursor favored Daptomycin production as compared to aliphatic fatty acids which were examined for improvement of several other lipopeptide antibiotics. Significant amount of Daptomycin production took place when sodium decanoate was added at the second day after inoculation. Delaying the addition time of the precursor also impeded cell growth [I-Son *et al*.,2013].

Substrate inhibition and product inhibition are two common problems that obstruct antibiotic production. In order to scale up the production of antibiotic without losing much product to substrate inhibition, improved fed batch strategy is established as a feedback control. Studies have shown that Daptomycin was produced in shake-flasks, batch, and fed-batch fermentation. However, the production of Daptomycin was effectively increased in by intermittent feeding of dextrin up to 812.0 mg/L from 217.5 mg/L in batch fermentation.

Fed-batch strategy developed in fermentation is often used to tackle substrate inhibition [Mueller *et al*., 2013].

A comprehensive metabolic flux analysis model was set up and used to evaluate Daptomycin metabolism, amino acid utilization, and simultaneous consumption of nutrient sources. Stoichiometric model were used to demonstrate the intracellular carbon flux distribution. This was an innovative approach which compared the flux changes for different fermentation conditions and helped to interpret of the dependency of Daptomycin yield on environmental perturbations (e.g. pH) and principal pathways. Experimental and calculated values for both the specific growth rate of *Streptomyces roseosporus* LC-54-20 (mutated strain) and Daptomycin production rate indicated that the in silico model proved a powerful tool to analyze the metabolic behaviors. Flux distribution pattern revealed that the antibiotic production could be majorly influenced by the branch nodes of glucose 6-phosphate, 3-phosphoglycerate, phosphoenolpyruvate, pyruvate, and oxaloacetate. Unlike the studies conducted by scientists earlier , where decanoic acid was the sole precursor which controlled Daptomycin production, five precursors enhanced the yield of Daptomycin. The strategy was different from conventional medium design. There is a critical role of metabolic engineering of the genes involved in biosynthetic pathway of Daptomycin in betterment of carbon uptake and precursor tolerance [Huang *et al*.,2011].

The total chemical method of synthesis of Daptomycin was discussed wherein instead of biosynthesis, a complex strategy was developed using a combination of solution-phase synthesis and SPPS to successfully assemble the linear Daptomycin peptide precursor. An efficient and scalable ozonolysis strategy was introduced to synthesize Kyn containing peptide fragments from the corresponding Trp-containing peptides. The key

macrocyclization step in the synthesis of Daptomycin was achieved via a chemoselective serine ligation, which enabled the large-scale synthesis of daptomycin since the cyclization could be accomplished at concentrations of up to 50 mM. Two important conclusions were drawn from this methodology -serine/threonine ligation could be a general tool for peptide cyclization; [Wong *et al*., 2013] and ozonolysis of a Trp-containing peptide fragment can lead to the formation of the corresponding Kyn-containing peptide fragment in a highly efficient manner if the Trp moiety is protected. This success suggests that a Kyn-containing cyclic peptide could be readily synthesized via serine/threonine ligation and ozonolysis from a suitable linear Trp-containing peptide precursor [Lam *et al*., 2014; Baltz *et al*., 2014]. The chemical synthesis of key intermediates involves multiple steps and hazardous reaction conditions. To develop high-valued antibiotics at large-scale like Daptomycin, various strategies should be taken up to improve mass transfer characteristics. The fermentation process is highly aerobic and involves actinomycetes. Free and immobilized microbial cells can be cultivated using various cultivation modes of batch and continuous strategy using different bioreactors (stirred tank bioreactor, air lift bioreactor and packed bed column) [Mahapatra *et al*., 2002 ; Gaurav *et al*.,2012].

2.2 Research Gaps and Perspectives

The health care sector is severely intimidated about multiple drug failure due to drug resistance [Fluit *et al*. 2001]. Researchers around the world are focused mainly with metabolic engineering and genetic improvisation of several crucial life-saving drugs that can tackle this issue. But, there is also an urge to enhance their production to reduce the cost of their industrial applicability. Hence, benefitting the society. As a biochemical engineer, the

main concern was to improve Daptomycin production through useful processing strategies. Daptomycin is an important cyclic lipopeptide antibiotic produced by *Streptomyces roseosporus*, which can effectively combat methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococci* due to its unique mechanism of action [Eisenstein *et al*. 2010] .The filamentous nature of *S. roseosporus*, influences the rheology of the fermentation broth. This hampers its production to a great extent. As the literature portrays, researchers have attempted to improve the yield via genetic engineering and metabolic flux analysis [Huang *et al*., 2012]. But, there have been very few attempts towards the process strategies for improved production of Daptomycin and strategies to strengthen its anti-drug resistance activity. Instances of the development of Daptomycin resistance in *Enterococcus faecium* and *S. aureus* raise concerns about decreasing clinical effectiveness of Daptomycin will also have to be addressed with the passage of time [Montero *et al*., 2008 and Friedman *et al*., 2006]. The morphology of *S.roseosporus* is defined by prominent features like spore chains, germinating spores, vegetative mycelium and aerial hyphae [Huber *et al*., 1987].The structural characteristics depicted that mycelium of cells were elongated and thick at 24hours without Daptomycin being synthesized. It then transformed to a slender and apical form after fermentation of Daptomycin at 192 hours. The addition of decanoic acid leads to the accumulation of Daptomycin and cell mass is reduced after stationary phase. Daptomycin being an idiolyte needs sufficient biomass for better production. The cell growth is significantly affected by stress conditions in the broth. The study of rheological properties influenced by morphological changes in the fermentation broth is of industrial importance and is indispensable to enhance the yield of the desired product as it depends on the biomass cultured [Olsvik *et al*.,1992]. The filamentous nature of actinomycetes leads to viscous

fermentation broths with shear thinning nature [Riley *et al*., 2000]. The structural changes of cell wall may be closely linked with the resistance to shear [Petersen *et al*., 2008; Olsvik *et al*., 1994]. So, to enhance Daptomycin production, there is a need to know the flow behavior of culture broths. There is a strong need to establish the relationship between fermentation broth properties and productivity of Daptomycin. This is the first aspect that can be strategically worked upon.

The second concern is to retain the cells of *S.roseosporus* to improve Daptomycin production through reusability. Cell immobilization has many operational and commercial benefits such as improved biosynthetic activities, cell reusability and aversion of cell washout at higher fermentation dilution rates [Hasan *et al*., 2009].The crucial factors associated with cell immobilization are mass transfer limitation and dilution rate optimization [Westman *et.al*., 2015]. Previous reports have explored how the phenomenon of immobilization instantiates the potential of bioprocesses for secondary metabolite production [Kundu *et al*. 1992; Mahapatra *et al*. 2002]. Whole cell immobilization in a packed bed bioreactor has been explored for Cephalosporins fermentation in the past [Kundu *et al*., 1992, Kundu *et al*., 2003]. Higher cell concentration can be maintained by immobilization methods which enhance the productivity of the secondary metabolite as it is a non-growth associated product. Prolonged reusability is also an advantage of whole-cell immobilization as explored in previous works using airlift bioreactors and stirred tank bioreactors [Srivastava and Kundu 1998; Mishra *et al*. 2005]. Also, the physiological state and morphological differentiation of filamentous microorganisms can be related to changes in growth conditions during submerged cultivations [Kundu *et al*. 2000].Morphological features have been correlated

with the production of secondary metabolites at several instances [O'Cleirigh *et al*. 2005; Papagianni and Mattey 2006]. Improved antimicrobials' production was observed through pellet formation [Choi *et al*. 2000]. Therefore, yield of crucial drugs like Daptomycin can be improved through morphological changes of producer microorganism and immobilization strategies.

Airlift reactors have been widely applied in chemical, biochemical as well as for the water treatment due to their simple construction, applicability for shear sensitive microorganisms, low power consumption, improved mixing and high mass transfer efficiency [Chisti *et al*.,1989;Luo and Dahhan ,2008;Jin *et al*.,2006]. Low volume and high value products can be synthesized in this type of bioreactor efficiently. As we know that the airlift bioreactors are classified into two categories: external loop airlift bioreactor and internal loop airlift bioreactor. The former is composed of two conduits connected at the top and the bottom, while the latter consists of two concentric cylinders [Baten *et al*., 2003]. A typical internal loop airlift reactor consists of important parts like riser, downcomer as well as gas disengagement sections. The density difference induced by the gas holdup difference between riser and downcomer is the main driving force to form the liquid circulation in airlift reactors [Xu *et al*., 2012]. Gas holdup, liquid and mass transfer coefficient are the crucial hydrodynamic parameters for airlift bioreactors. The consideration of superficial gas velocity, reactor geometry and sparger structure is essential for the design of airlift bioreactors. Extensive studies on the hydrodynamics in airlift bioreactors have been done in the past. Kilonzo *et al*. [Kilonzo *et al*.,2010] investigated the mass transfer characteristics in an inverse internal loop airlift-driven fibrous-bed bioreactor. Blazej *et al*. [Blazej *et al*., 2004] studied the hydrodynamics of an internal loop airlift bioreactor. Lu *et al*. [Lu *et al*., 2000]

compared the hydrodynamics and mass transfer characteristics between a modified square airlift reactor and traditional airlift reactors. Some researchers investigated the local hydrodynamic characteristics of airlift reactors [Olivieri *et al*.,2007;Young *et al*.,1991]. There are also some important studies regarding the influence of the structure of gas sparger in airlift reactors [Merchuk *et al*.,1998;Mc Manamey *et al*.,1984]. The influence of the sparger structure on gas holdup, liquid velocity and volumetric mass transfer coefficient in an annulus sparged internal loop airlift reactor holds great significance [Bello *et al*., 1985]. Mass transfer of Oxygen plays an important role in the bioreactor performance especially for aerobic fermentation processes. The volumetric mass transfer coefficient, K_La, is applied to measure the oxygen transfer in the bioreactor. Since the value of K_La depends on the interfacial area of gas bubbles and the physical properties of fluid, bubble size and bubble number in the reactor have substantial effect on the mass transfer. Many investigations have been done on determining the bubble size distribution [Miyahara *et al*., 1986; Evans *et al*., 1992). Tung *et al*. [Tung *et al*., 1997] proposed an airlift reactor with double net draft tubes in a laboratory scale and applied it to cultivation of baker's yeast. The two strategies i.e. influence of sparger structure and bubble size distribution can be taken into consideration to improve the production of pharmaceuticals by improving hydrodynamics inside the bioreactor.

Modern day antibiotics are continuously playing catch-up with multiple drug resistance bacteria species [Costerton *et al*., 1999;Rai *et al*.,2009;Stewart *et al*.,2001]. The rate of new antimicrobial drug development is pretty slow and the day is not far when the world may face an epidemic of untreatable infections in catastrophic number [Mah *et al*.,2001;Taubes,2008;Neu,1992;Cohen ,1992]. This fear inspires the strategies to circumvent

antibiotic resistance through efficient paradigms of applicability like combinatorial drug therapy or synergistic therapy [Hancock *et al*.,2006;Lemire *et al*.,2013]. Applying this knowledge of combination drugs, drug resistance can be impeded to a certain extent. Daptomycin is one of the lasting antimicrobials with therapeutic efficacy in fighting the multidrug resistance Gram-positive bacteria [Steenbergen *et al*.,2005]. Resistance against Daptomycin was recorded upto 0.2% for clinical trial cases [Muangsiri *et al*.,2006].Nevertheless, this statistics is not permanent. Also, due to unfavorable pharmacokinetics, Daptomycin could reach the infection site at a suboptimal dose over a therapy time of 20 days [Muangsiri *et al*., 2006]. Daptomycin exerts its bactericidal effect through lipophilic tail based bacterial membrane damage. Suboptimal dose over an extended therapy period coupled with a one unique bactericidal mechanism have been recognized to be the common cause of antibiotic drug resistance phenomenon [Vilhena *et al*.,2012].Thus, it is predictable that Daptomycin will follow the ultimate course of several conventional antibiotics and be rendered obsolete due to drug resistance. Thus, it is imperative to develop efficient strategies to protect and extend the efficacy of the drug against the multidrug-resistant bacteria. Combinatorial therapy of antibiotics or antibiotic with nanoparticles synergistically improve the performance of each component of the combination [Levy *et al*., 2004; Steenbergen,2009]. The two noble metal nanoparticles i.e. Silver nanoparticles and Gold nanoparticles and their amalgamation as bimetallic nanoparticles have been known to exert a wide-spectrum antimicrobial property through various killing mechanisms (e.g., membrane damage, DNA damage, and perturbation of cell metabolism triggered by a high reactive oxygen species (ROS) production) [Rizello *et al*.,2014;Chernousova *et al*.,2013].Such characteristics make metal nanoparticles potential candidate for the

combinatorial therapy with Daptomycin. In order to channelize the synergism, both nanoparticles and Daptomycin can be delivered in a single package for effective topical antimicrobial activity against MRSA strain(s) which are mainly responsible for skin infections and sepsis. Evidences of modest side-effects described for Daptomycin intravenous administration, topical use of Daptomycin needs attention [Gonzalez-Ruiz A *et al.,* 2016].

2.3 OBJECTIVES

The research work was undertaken with the aim to produce Daptomycin, a potential cyclic-lipopeptide antibiotic produced by *Streptomyces roseosporus* that has progressed as a significant anti-MRSA (methicillin-resistant *Staphylococcus aureus*) antibiotic. The work has been initiated to explore the strategies for the improvement of production of Daptomycin and its application against MRSA. The entire work was divided into the following phases:

The **first** phase of the work involved the procurement of the producer microorganism and optimization of several parameters, *viz*. inoculum development, medium composition, growth parameters, different physical parameters like pH, temperature, incubation time and various carbon sources, etc. and validation of optimized parameters using both one factor at a time and statistical methodology and further evaluation of the kinetic parameters from the shake flask studies using the optimized media.

The **second** phase of the work involved the evaluation of rheological properties of the fermentation broth which was a serious concern in process intensification of Daptomycin.

The **third** phase of the work dealt with the production of Daptomycin using different modes of bioreactors using various cell processing strategies.

The **fourth** phase of the work was concerned with the mass transfer studies of the fermentation broth using different spargers.

The **last phase** of the work dealt with the application of Daptomycin against MRSA using its synergistic effect with noble metal nanoparticles in the form of topical gel.