REVIEW OF LITERATURE

2.1 Rapamycin

In 1964, a Canadian travelled to Vai Atare region of Easter island, which was locally known as Rapa Nui island in the middle of Pacific. The soil samples collected from this island were taken to Ayerst's research laboratories. There a microbiologist team which included an Indian Suren Sehgal [Kahan, 2003], isolated an actinomycetes *Streptomyces hygroscopicus*. Study of the fermentation broth of this actinomycetes led to the discovery of Rapamycin [Venzina *et al.*, 1975] (name kept after the place of origin). Structural studies of rapamycin showed that it is similar to antibiotics and chemically falls under the group of macrocyclic polyketide with 31-membered cyclic structure.

Rapamycin is also known as sirolimus (generic name) and exhibits antifungal activities. Discoverers of rapamycin also found out that it shows antifungal activities (against *Candida albicans* with minimum inhibitory concentration < 0.02-0.2 µg/mL) but is not active against bacteria. Another milestone was achieved when immunosuppressant activity of this compound was discovered. Later on September 15, 1999, rapamycin was approved as a drug by the US- FDA for renal transplant. Then, it was sent to the National Cancer Institute (NCI) where the anti-tumour property of rapamycin was verified against some tumor cell lines of humans [Seto, 2012]. This screening as anti-tumor agent was carried out against 60 cell lines in which rapamycin inhibited the growth of colon 26, mammary, EM ependymoblastoma and B16 43 malanocarcinoma [Douros *et al.*, 1981]. This led to a series of investigation of antibiotic activity of rapamycin against *Saccharomyces cerevisiae, Caenorhabditis elegans,* Drosophila, fungus and mammals [Arndt *et al.*, 1999, Seto, 2012]. When molecular mechanism responsible for inhibitory activities shown by rapamycin was investigated, it was found that it targets a protein which was named as Target of Rapamycin (TOR). This protein

was found to be evolutionary conserved and among mammals, 95% homology was found and is known as mammalian Target of Rapamycin (mTOR) [Seto, 2012].

2.2 Chemical Structure and Nature of Rapamycin

Rapamycin is a triene macrolide which is colourless when crystallized from ether. The melting point is about 183°C-185°C and molecular weight 914.187 g/mol with molecular formula $C_{51}H_{79}NO_{13}$. LD_{50} value for toxicity i.p. mice was found to be 600 mg/Kg [Venzina *et al.*, 1975].

The IUPAC name of Rapamycin is (3*S*,6*R*,7*E*,9*R*,10*R*,12*R*,14*S*,15*E*,17*E*,19*E*,21*S*,23*S*,26*R*,27*R*,34a*S*)9,10,12,13,14,21,22,23,24,25,26,27,32,33,34,34a-hexadecahydro-9,27-dihydroxy-3-[(1*R*)-2-[(1*S*,3*R*,4*R*)-4-hydroxy-3 methoxycyclohexyl]-1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-23,27-epoxy-3*H* pyrido[2,1-c] [1,4]-oxaazacyclohentriacontine-1,5,11,28,29-(4*H*,6*H*,31*H*)-pentone) [Gandhi *et al.*, 2010].

Swindells *et al.* studied the structure of X-ray crystal of rapamycin as shown in Fig 2.1. Their study determined that the structure of rapamycin contains a macrocycle ring of 35 atoms. They reported the presence of an amide between C-16 and N-17 and an oxygen bridge between C-9 and C-14. An α-carbonyl piperidine unit was found to be planar [Swindells *et al.*, 1978]. The structure of rapamycin-based on Nuclear Magnetic Resonance (NMR) Spectroscopy was described by Findlay *et al.* They studied ^{13}C and ^{1}H NMR spectra of rapamycin. The structure exhibited two conformational isomeric forms which were found to exist due to the cis-trans rotation. This amidic bond rotation was found within the macrolide ring [Findlay *et al.*, 1980].

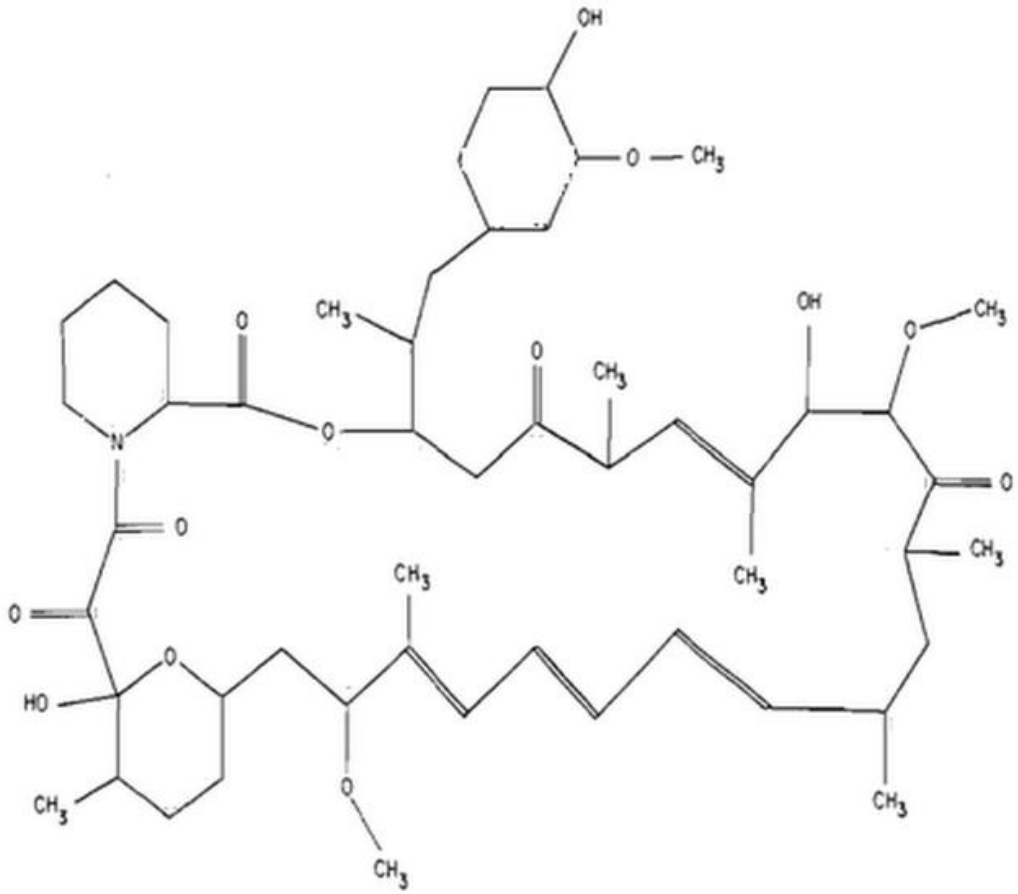

Fig 2.1 Structure of Rapamycin [Swindells *et al.*, 1978]

Rapamycin is not soluble in water and soluble in chloroform, ether, methanol, acetone and N,N dimethylformamide while it is sparingly soluble in petroleum ether and hexane [https://pubchem.ncbi.nlm.nih.gov/compound/rapamycin#section=GHS-Classification, accessed on October 15, 2017]. Gandhi *et al.* measured the rapamycin solubility in various solvents as methanol, chloroform, acetone, dichloromethane, ethanol at different temperatures ranging from 245 to 295 K. The analysis was done using the gravimetric method. They also determined the dependency of crystal size of rapamycin on its solubility. The stability of crystals based on their size was also evaluated [Gandhi *et al.*, 2010]. Simamora *et al.* studied the use of water-miscible co-solvents for enhancing the solubility of rapamycin in water. This strategy led to more than 100 times enhancement in the solubility of rapamycin in water [Simamora *et al.*, 2001].

As the immunosuppressive action of rapamycin was established, different clinical trials were performed to examine the efficacy of the compound as drug. These trials include phase 1 which evaluated the tolerability, safety and pharmacokinetics. Phase 2 investigations included dose finding and drug combination studies. Phase 3 incorporated clinical studies on large randomized population. In September 1999, US- FDA approved the use of rapamycin with cyclosporine and steroids. The European agency approved the use of rapamycin in the year 2000 [Kahan *et al.*, 2001].

2.3 Immunosuppressive action of rapamycin

Mechanism of action of rapamycin as an immunosuppressive agent was studied by Dupont *et al*. Action initiates when T cells identify the complex formed between Major Histocompatibility Complex Class II (MHC II) molecules and Antigen Presenting Cells (APCs). This results in down signaling by T cell receptors to calcineurins. Calcineurins then cause dephosphorylation of Nuclear Factor of activated T cells (NFAT). NFAT

then get transferred to the nucleus. Inside the nucleus, NFAT acts as a transcription factor and controls the production of interleukin-2 (IL-2). IL-2 interacts with its receptor IL-2 receptor (IL-2R) and facilitates further T cell proliferation and signaling during graft rejection. Rapamycin prevents graft rejection by binding to FK506 binding protein (FKBP). This rapamycin-FKBP complex then attaches to mammalian target of rapamycin (mTOR) which prevents IL-2R signaling. Rapamycin also interferes with the co-stimulatory signal of CD-28 which further aids in tolerance induction [Dupont *et al.*, 2003].

2.4 Producer microorganism

Rapamycin was first noted to be produced by *Streptomyces hygroscopicus* AY B-994 [Sehgal *et al.*, 1975]. *S.hygroscopicus* belongs to kingdom bacteria and phylum actinobacteria. Vezina *et al.* first reported about the morphological characteristics of the isolated microorganism. They found that aerial mycelium of the isolated strain is monopodially branched. The color of the spores was grey and turned to grey-brown after sometime during prolonged incubation. The characteristic appearance of vegetative and aerial mycelium in different culture media has been listed in their report [Vezina *et al.*, 1975]. They also described the physiological characteristics of the strain. It was found to grow in pH range of 6-8 while the range of temperature for growth was 20-37 °C.

2.4.1 Biosynthetic gene cluster of *S.hygroscopicus* for production of rapamycin

Schwecke *et al.* reported about the three polyketide synthase gene clusters known as rapA, rapB and rapC which are responsible for rapamycin synthesis by *S.hygroscopicus*. These polyketide synthase genes code for three multi-enzyme subunits known as Raps1,

Raps2 and Raps3 respectively. These subunits contain total 14 enzymatic modules such that chain elongation is carried out on each of these modules. In the end, a pipecolate incorporating enzyme coded by rapP gene catalyzes the transfer of pipecolate moiety on the extended chain and facilitates the closure of the macrocyclic ring of rapamycin. The biosynthetic gene cluster for rapamycin was classified to belong to polyketide synthase type I [Schwecke *et al.,* 1995]. Schwecke *et al.* also reported that the DNA for rapamycin biosynthesis was 107.3 kbp in size and had 24 open reading frames. Other studies were also conducted by researchers to determine the major biosynthetic pathways and enzymes involved in rapamycin production [Andexer *et al.,* 2011, Aparicio *et al.,* 1996] Park *et al.* reviewed the different work done by researchers to construct rapamycin analogs using mutagenesis and mutasynthesis. They proposed that application of metabolic engineering to regulate the carbon flux can contribute towards the enhancement of production of rapamycin and its derivative [Park *et al.,* 2010].

2.4.2 Precursors for rapamycin biosynthesis

Paiva *et al.* reported that a shikimic acid derivative is a precursor for rapamycin biosynthesis and act as a starter unit. They used ^{13}C labeled shikimic acid and found that it was incorporated into the cyclohexane moiety. Though they also found that addition of exogenous shikimic acid did not enhance the rapamycin concentration in a complex medium [Paiva *et al.,* 1993a]. Another study was carried out by the same group which found that rapamycin production was significantly enhanced by the addition of 57 mmol/L of shikimic acid in a chemically defined medium [Fang *et al.,* 1995]. Thus, it was found that 4,5-dihydroxycyclohex-1-enecarboxylic acid acts as a starter unit of rapamycin biosynthesis which initiates the linear chain of biosynthesis by ATP dependant CoA ligase present at the loading domain of Raps1.

Acetate and propionate were also found to act as a precursor of rapamycin by Paiva *et al.* They used ^{13}C labeled carbon atoms to identify the precursors responsible for rapamycin biosynthesis [Paiva *et al.*, 1991]. They also found that radio-labeled methionine was incorporated into the methoxy group of rapamycin. On the contrary, the addition of exogenous methionine was found to be inhibitory for rapamycin biosynthesis.

L-lysine was also found to act as a precursor for rapamycin production [Paiva *et al.*, 1993b]. They found that L-^{14}C-lysine and D,L- ^{3}H pipecolate were integrated into the nitrogenous ring of rapamycin. Thus, L-lysine derived pipecolate was found to act as a precursor for rapamycin. König *et al.* analyzed the nucleotide sequence and heterologous expression of RapP which facilitates the incorporation of pipecolate into the structure of rapamycin [König *et al.*, 1997]. Gatto *et al.* observed the bioconversion of L-lysine to pipecolate by lysine cyclodeaminase present in rapamycin biosynthetic gene cluster [Gatto *et al.*, 2006].

2.4.3 Overall biosynthetic pathway of rapamycin

Rapamycin is a polyketide compound which is synthesized by a large biosynthetic gene cluster. The overall pathway for biosynthesis of a polyketide antibiotic has been shown in Fig 2.2, glucose is used as the substrate. Carbon sources other than glucose can also be utilized as a substrate for rapamycin production. Fig 2.3 shows the biosynthetic route of different precursors of rapamycin when glucose, fructose or mannose are used as carbon source.

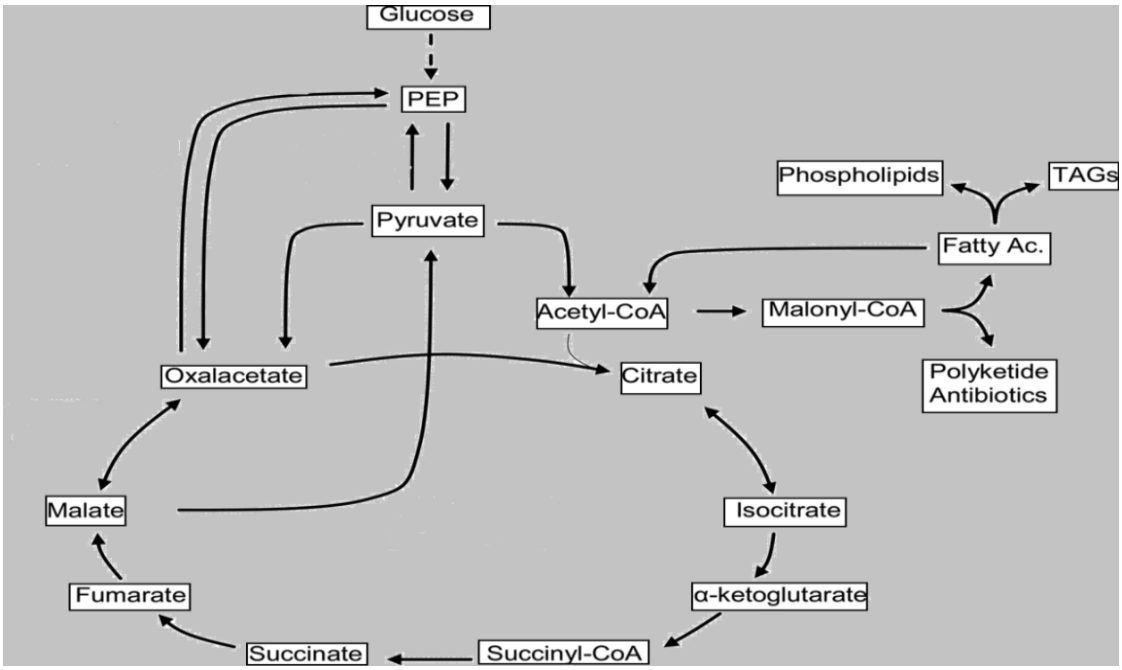

Fig 2.2 Biosynthetic pathway of polyketide antibiotics [Rodriguez *et al.*, 2012]

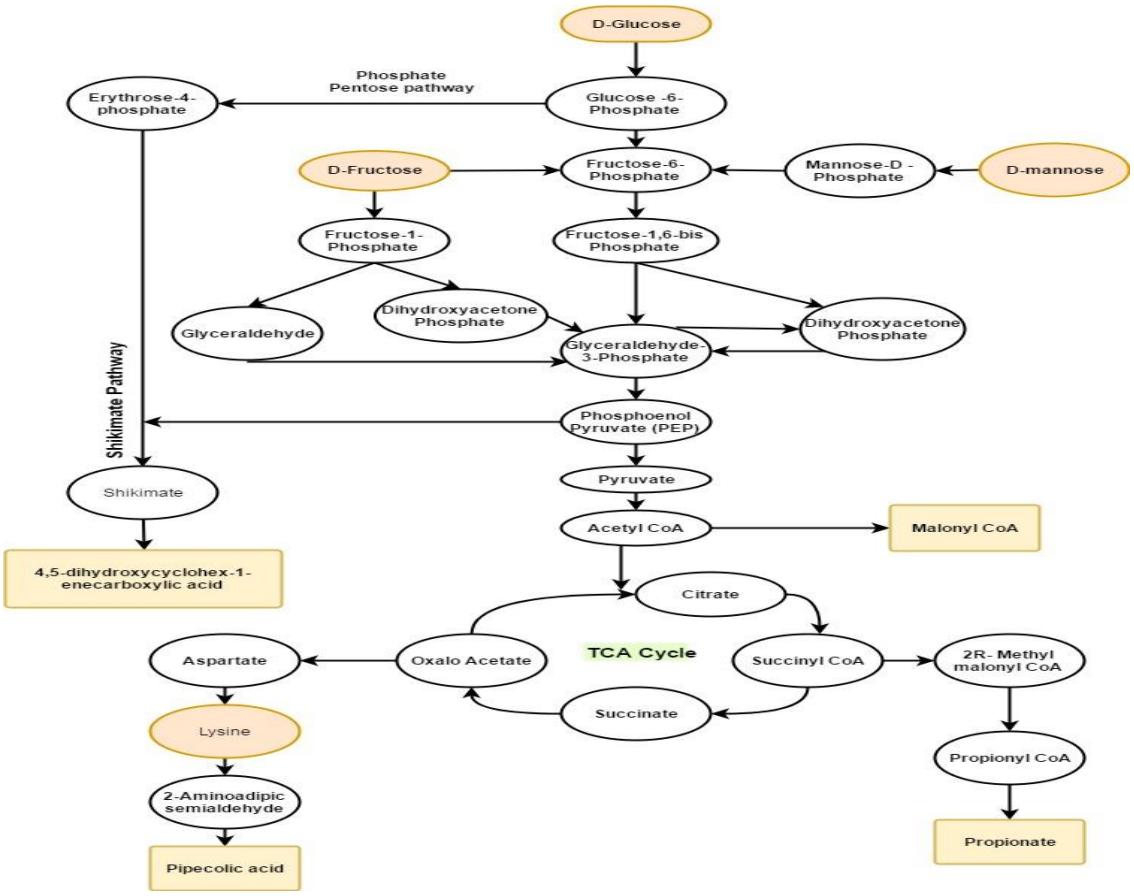

Fig 2.3 Route of precursors' biosynthesis for rapamycin production

Schekwe *et al.* proposed that the starter unit, 4,5-dihydroxycyclohex-1-enecarboxylic acid loads onto the loading domain. This precursor unit is then extended by the addition of propionate and acetate units by module 1 to module 14. Then, a pipecolate moiety is added by RapP which closes the macrocycle ring of rapamycin [Schwecke *et al.*, 1995].

2.5 Production of rapamycin

The production of rapamycin can be facilitated by both chemical as well as biological means.

2.5.1 Chemical production of rapamycin

Many workers have studied the chemical route of rapamycin synthesis [Ley *et al.*, 2009, Maddess *et al.*, 2007, Nicolaou *et al.*, 1993]. They explored the chemical production process of rapamycin which is a very large polyketide compound consisting of plethora of functional groups and geometrical centers. Nicolaou *et al.* attempted to chemically synthesize rapamycin by using acyclic precursors and adopted a "stitching – cyclisation" process. The strategy was designed so as to prevent the instability problems. While Ley *et al.* adopted a "catechol-templating/ macro-etherification" methodology for preparing the cyclic core of the compound. Though the synthesis of rapamycin was accomplished through the chemical route but it was a quite cumbersome method as some functional groups like cyclic acetals, pipecolinate moieties created hurdles. The stereochemistry of the compound was also a hindrance in the process.

2.5.2 Microbial production of rapamycin

Rapamycin production from microbial sources has received much attention by the scientific community as it is easy and environmental friendly approach. Many strategies

have been adopted to improve the titer of rapamycin production including strain improvement of *Streptomyces hygroscopicus* which covers genetic manipulation, random mutagenesis and precursor engineering. Another strategy is optimization of rapamycin production process which includes medium composition, precursor supplementation as well as modulation of operational parameters.

2.5.3 Strain Improvement

High throughput sequencing has been employed for improvisation of product yield by many researchers. Xu *et al.* carried out chemical mutagenesis using nitrosoguanidine (NTG) and screened 7000 isolated out of which 10 were found high yielding. The process was carried out on 96- well plate [Xu *et al.*, 2005]. Others have employed protoplast fusion strategy for improving the yield of rapamycin. Cheng *et al.* have evaluated the effect of treating the protoplast of a rapamycin producing strain, *Streptomyces hygroscopicus* FC904 which was obtained from soil samples of Fuzhou, China. They treated the protoplast of the parent strain with different chemicals including erythromycin, gentamycin, NTG and mitomycin C. Best result was obtained with gentamycin treated protoplasts, and the mutant C^{14} was obtained. The spores of mutant C^{14} was then treated with gentamycin and NTG to obtain a high yielding strain [Cheng *et al.*, 2001]. Chen *et al.* have evaluated the protoplast based techniques for this purpose. They tried protoplast mutation and fusion techniques. Both intraspecies and interspecies protoplasts were fused and their effects on rapamycin production were evaluated. High production was achieved when interspecies protoplast fusion was carried out along with genome shuffling [Chen *et al.*, 2009]. Zhu *et al.* designed a metabolic pathway based mutational approach to enhance the production of rapamycin. Their strategy incorporated reduction of glucose repression and development of

tryptophan and phenylalanine auxotrophs using UV mutagenesis. Using step-wise mutagenesis methodology they developed a strain known as *Streptomyces hygroscopicus* HD-04 [Zhu *et al.*, 2010]. Another study was carried out by Jung *et al.*, in which they employed UV mutagenesis and propionate supplementation for increased production of rapamycin by 5.5 fold and developed a mutant strain *Streptomyces hygroscopicus* UV2-2 [Jung *et al.*, 2011]. Zou *et al.* adopted a precursor engineering approach for enhancement of rapamycin production [Zou *et al.*, 2013]. Rapamycin concentration obtained during production process using different strains has been shown in Table 2.1.

Table 2.1 Rapamycin production using different strains of *Streptomyces hygroscopicus*

Producer strain	**Rapamycin production (mg/L)**	**Reference**
S. hygroscopicus FC904	139	*[Cheng et al., 2001]*
S. hygroscopicus N5632	420	*[Xu et al., 2005]*
S. hygroscopicus R060107	500	*[Chen et al., 2008]*
S. hygroscopicus GS-1437	445	*[Chen et al., 2009]*
S. hygroscopicus HD-04-S	450	*[Zhu et al., 2010]*
S. hygroscopicusU1-6E7	307	*[Zou et al., 2013]*

Though rapamycin titer with mutant strains was high but their reversion to wild strain with reduced yield with subsequent sub-culturing may question their industrial applicability and integrity as high- performance strain [Lee *et al.*, 2015].

2.5.4 Optimization of production parameters

Another strategy for enhancement of rapamycin production involves optimization of nutritional supply and process parameters. Different strategies have been suggested in the literature which includes medium optimization and controlling process parameters. Statistical optimization technique has evolved as a rapid method of medium optimization compared to the classical method or the one factor at a time methodology. Following sections will describe the feasibility of different strategies for optimization of rapamycin production.

2.5.4.1 Classical approach

This strategy includes varying the concentration or value of one factor at a time (OFAT) for every important parameter and finding their values to achieve maximum production. OFAT method of optimization includes removal supplementation or removal of the medium components [Singh *et al.,* 2017]. Singh *et.al.,* have earlier reported that removal of soyabean meal, NaCl or glycerol cause 20-40% reduction in an antifungal compound produced by *Streptomyces capoamus* [Singh *et al.*, 2008]. The most significant components of any medium are the carbon and nitrogen sources as they provide the structural backbone of cells as well as the desired compounds. Carbon source plays a very important role in providing energy to the cells and production of primary and secondary metabolites. The utilization rate of carbon substrate had been linked to the rate of primary and secondary metabolite formation in the literature [Singh *et al.*, 2017]. Study has been carried out to illustrate that production of antibiotics is enhanced when the carbon source is less readily utilized [Marwick *et al.*, 1999]. *Bacillus lichenformis* has shown to produce antibiotic bacitracin during growth phase when the slowly metabolized growth medium was used [Haavik, 1974]. Similarly,

nitrogen sources also influence the production process of many metabolites. The microbes utilize both organic as well as inorganic sources, but they affect the production of the desired compound. Certain amino acids have caused enhancement in secondary metabolites production while others inhibit them [Marwick *et al.*, 1999]. For rapamycin biosynthesis it has been studied that where L-lysine causes enhancement, L-phenylalanine and L-methionine cause reduction in production [Cheng *et al.*, 1995a]. Cheng *et al.* also optimized the salt concentrations of phosphate, magnesium, ammonium and iron of a defined medium for rapamycin production using OFAT methodology. They found that very low concentration of ammonium and magnesium salts are required for rapamycin production [Cheng *et al.*, 1995b]. Others have optimized seed medium composition as well as inoculum size and age for rapamycin production using OFAT strategy [Hamdy *et al.*, 2011]. Thus, OFAT methodology is still under use for optimization of physical as well as chemical process parameters including temperature, pH, rate of agitation and aeration as it is simple to execute. In another study, they reported that 5 g/L of potassium dihydrogen phosphate (KH_2PO_4) yielded maximum production of rapamycin when added in a complex medium [Sallam *et al.*, 2010]. On the other hand, the major roadblock in efficiency of OFAT is difficulty in prediction of interactive effect of the parameters. The time required for performing experiments using this technique is also large [Gupte *et al.*, 2003, Vaidya *et al.*, 2003].

2.5.4.2 Statistical optimization

To overcome the disadvantages associated with OFAT methodology, statistical optimization has been adopted in many studies. According to Fisher, the efficiency of optimization process increases as changing one factor at a time is replaced with

changing more than one parameter at a time [Fisher, 1992]. The statistical methodology is used for simultaneous comparison of many process variables with reduced number of experiments as compared to the OFAT method [Elibol, 2004].

Plackett Burman Design [PBD] has been employed by many researchers for optimization of significant parameters for production of many biochemicals [Banga *et al.*, 2008, Castro *et al.*, 1992, Ghanem *et al.*, 2000]. The negative side of using PBD is that it does not involve the interactive effect of the parameters therefore other designs also need consideration.

Box *et al.* were the first to use central composite design (CCD)[Box *et al.*, 1992]. CCD is a tool of response surface methodology (RSM) and used to construct a quadratic relation of second order. The experiments are designed such that there are three sets of experiments. One set includes the maximum and minimum level denoted as +1 and -1 of some of the parameters, the other set has a median value of every factor called as center points and the last set has star points having value of one factor both above and below the factorial levels. CCD was used to optimize the production parameters for antibiotics xenocoumacins and nematophin production from *Xenorhabdus nematophila* [Wang *et al.*, 2008]. Similarly, others have employed this tool for optimization of cellulase production using agricultural waste by *Trichoderma reesei* Rut C30 [Muthuvelayudham *et al.*, 2010]. Though CCD has found much use in statistical designing of medium components, but it lacks in predictions as the second order polynomial equation provides an inadequate prediction of optimal values and does not adequately considers the metabolic complexity of the microbial system [Singh *et al.*, 2017].

Another computational tool available for statistical optimization is the artificial neural network (ANN). ANN is designed so as to mimic the biological neural networks in

terms of architecture. ANN works in a way which is similar to the learning process by brain and consists of neurons which are weighted and then evaluated using different functions. ANN is based on adaptive learning and feedback control mechanism [Calise *et al.*, 2001]. ANN is described to work as "black box" model in which the relation between the key variables is not described, but believed to occur [Franco-Lara *et al.*, 2006]. ANN- based training of the input variables is carried out to adapt them for function approximation. After training, the model is validated and tested. Peng *et al.* have utilized ANN based strategy to optimize the production of an antifungal antibiotic iturin A produced by *Bacillus subtilis* [Peng *et al.*, 2014].

Genetic algorithm (GA) is another approach for optimization of production process variables and uses the theory of "survival of the fittest". GA adopts a process of natural selection to find the best individual which can give maximum output [Houck *et al.*, 1995]. GA first randomly selects an individual to act as a parent which is then used to produce offspring using any one of the two operations. These operations are mutation and cross-over. While the mutation involves one of the parents, the cross -over is implemented using both the parents. Then the GA selects the best individuals to act as a parent for next generation. GA finds its application in cases where optimization problem is complex due to non-differentiable, highly non-linear, stochastic or discontinuous objective functions. Many workers have studied the application of ANN-GA coupled optimization technique [Baishan *et al.*, 2003, Desai *et al.*, 2006]. GA was used for optimization of rifamycin B production [Bapat *et al.*, 2004]. Singh *et al.*, optimized the medium constituents for actinomycin V production by *Streptomyces triostinicus* using ANN-GA based strategy and reported four times higher production [Singh *et al.*, 2009].

2.6 Strategies for modulation of the production process

Effects of various strategies of the production of antibiotics have been evaluated which include immobilization of microorganisms, production in different non-conventional reactors and the effect of stress conditions and variation in the feeding regime of the substrate.

2.6.1 Immobilization of producer organism

Different workers have studied the effect of immobilization of cell mass on antibiotic production. *Penicillium chrysogenum* was immobilized on celite particles [Kim *et al.*, 1986] and urethane foam [Kobayashi *et al.*, 1990]. In another study, different support materials were evaluated for their efficacy in immobilizing *Cephalosporium acreminium* NCIM 1069 for Cephalosporin C production [Kundu *et al.*, 1992]. They found that among calcium alginate, silk sachets and bagasse, the second carrier was best in facilitating reduced cell growth as well as specific antibiotic production rate. Cephalosporin production was also evaluated using immobilized cells of *Streptomyces clavuligerus* NRRL 3585. A significant increase in specific antibiotic production was noted in this study [Freeman *et al.*, 1981].

Streptomyces T 59-235 (produces tylosin) and *Streptomyces tendae* Tu 901(produces nikkomycin) were immobilized by using different support materials and the fermentation was studied for 300 hours [Veelken *et al.*, 1982]. In another study, the production of rifamycin was evaluated through repeated batch fermentations using immobilized *Amycolatopsis mediterranei* CBS 42575 which caused reduction of fermentation time without reducing the product concentration [Trück *et al.*, 1990]. Erythromycin production was studied using cell entrapment strategy by *Streptomyces*

erythraeus. It was found that entrapped cells showed higher total antibiotic production as compared to free cells [Bandyopadhyay *et al.*, 1993].

Streptomyces rimosus TM-55 was immobilized in calcium alginate and the effect of immobilization on oxytetracycline production was evaluated. It was found that the fermentation could be continued for 28 days by repeated batch with significant production rate [Yang *et al.*, 2001].

In another study, the production of acetylisovaleryltylosin significantly increased when cells of *Streptomyces thermotolerans*11432 were immobilized on polyurethane foam in a semi-continuous mode. The carriers were pretreated and immobilized to evaluate the production in repeated batch mode [Zhu *et al.*, 2015].

2.6.2 Production in Air Lift Reactor

Aerobic fermentations require adequate oxygen supply for the microorganisms, as oxygen is very less soluble and commonly act as a limiting nutrient [Chisti *et al.*, 1989]. The fast rate of oxygen utilization must be complemented with the oxygen supply rate else the fermentation environment will become anoxic. Thus, the volumetric oxygen transfer coefficient (k_La) becomes the determining parameter for evaluation of the performance of a bioreactor for an aerobic fermentation. Oxygen serves many roles for cellular growth and oxygen transfer. Oxygen acts as terminal electron acceptor of the Electron Transport System (ETS) of aerobic respiration. Moreover, oxygen is also required for biosynthesis of oxygen heterocycles [Hemmerling *et al.*, 2016]. The oxygen supply in stirred tank reactor sometimes become limiting due to impeller flooding and the high mechanical energy requirement. Therefore, non-conventional bioreactors are used to overcome such drawbacks. Airlift bioreactor is one among them. Table 2.2 lists the advantages associated with airlift bioreactor and stirred tank

bioreactor. Airlift reactors exhibit improved performance ratio in terms of oxygen transfer rate when compared to agitated tanks as shown in Fig 2.4.

Sikula *et al.,* carried out mathematical modeling of gluconic acid fermentation by *Aspergillus niger* in an internal loop airlift reactor [Sikula *et al.,* 2007]. Modeling was carried out by dividing the reactors into different zones. The parameters for modeling such as gas hold up, velocity of circulation and volumetric oxygen transfer rates were calculated.

Table 2.2 Advantages of airlift bioreactor and stirred tank bioreactor

Airlift Bioreactor	**Stirred Tank Bioreactor**
Low shear fields	Homogenization
Good mixing	Suspension of solids
Better defined flow	Dispersion of gas-liquid mixtures
Low power input	Exchange of heat

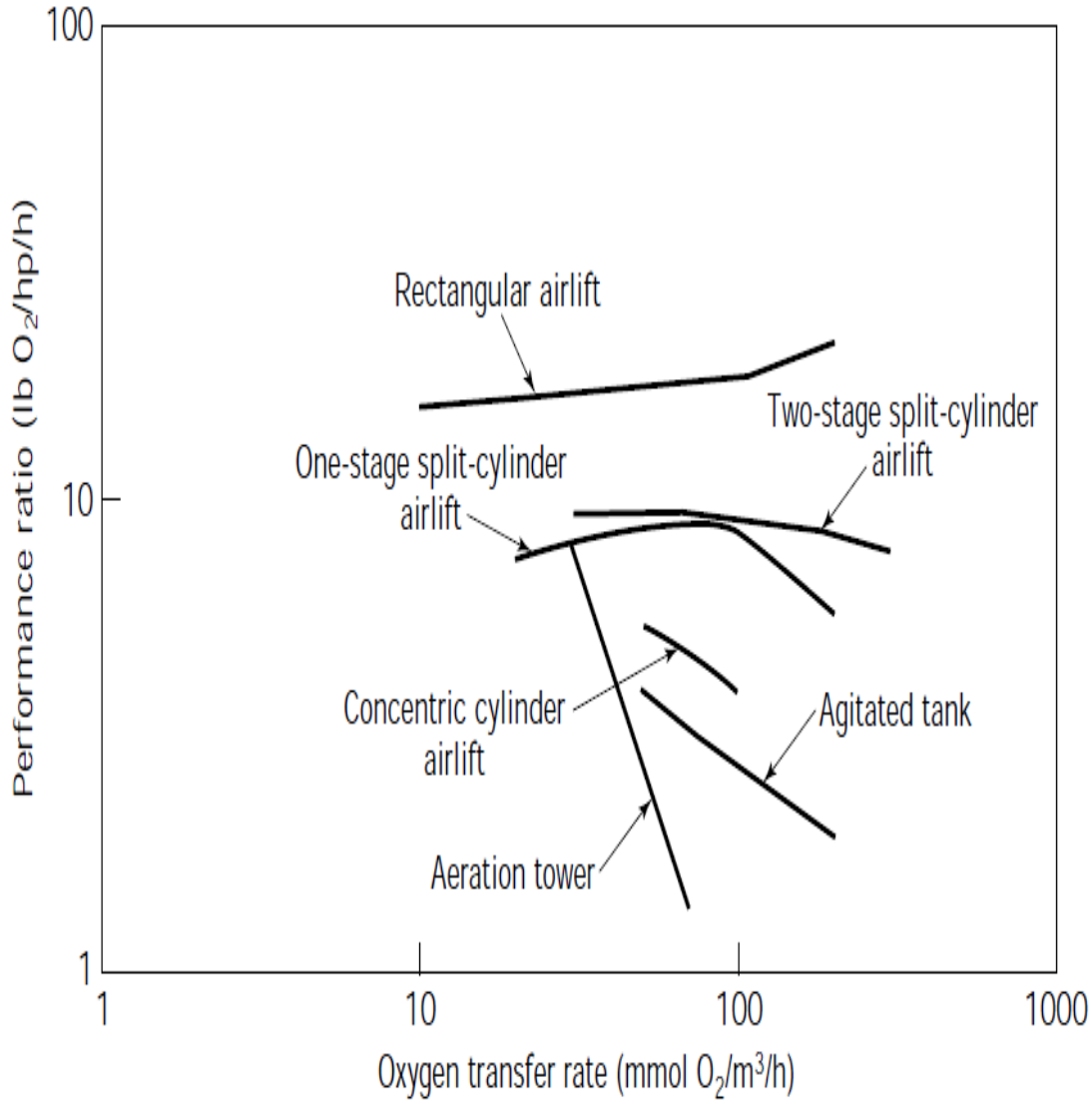

Fig 2.4 Variation of performance ratio with oxygen-transfer rate in different types of ALRs versus an agitation tank (adapted from [Orazem *et al.*, 1979])

2.6.2.1 Use of airlift reactors for antibiotic production

Effect of agitation on the production of antibiotics by *Streptomyces* species has been studied well [Olmos *et al.*, 2013]. No clear correlation has been proposed in this regard though some have noted an increase in antibiotic production at higher agitation speed. Rosa *et al.*, suggested that clavulanic acid production by *S. clavuligerus* enhanced with an increase in stirrer speed [Rosa *et al.*, 2005]. While in another study, clavulanic acid produced by *Streptomyces clavuligerus* ATCC 27064 did not show any direct relation with stirrer speed [Belmar-Beiny *et al.*, 1991]. Thus, use of airlift bioreactor without provision of agitation can be an alternate reactor system to study the antibiotic production profile.

Production of many antibiotics has been carried out using airlift bioreactors. Erythromycin production was evaluated in a 0.25 m^3 airlift bioreactor and the effect of two sparger design was studied using *Saccharopolyspora erythraea* [Pollard *et al*., 1998]. The study showed that erythromycin production rate is correlated to the energy dissipation rate. The specific productivity enhanced when the dissolved oxygen heterogeneity was reduced. In another study, production of neomycin in airlift bioreactor was evaluated using *S. marinensis* [Srinivasulu *et al.*, 2002] and *S. fradiae* [Park *et al.*, 1994] by immobilizing the cell systems. Srivastava *et al.*, studied the effect of Cephalosporin C production in an airlift bioreactor and the volumetric oxygen transfer coefficient was evaluated. The results showed that the specific antibiotic productivity enhanced comparatively [Srivastava *et al.*, 1999]. Gavrilescu *et al.*, evaluated the effect of bacitracin production by *Bacillus licheniformis* in an external loop airlift reactor. The production of the antibiotic was studied at different aeration rates. The results obtained with ALR were compared with STR. It was found that the reduction in power consumption was about 17-64% in ALR. Volumetric mass transfer

coefficient in the riser was found to be higher as compared to STR. They also proposed that ALR provides a low shear environment for the growth of cells [Gavrilescu *et al.*, 1993]. Schügerl compared the production of different secondary metabolites in STR and ALR. The study incorporated the production of antibiotics viz. cephalosporin C, penicillin V and tetracycline produced by *Cephalosporium acremonium, Penicillium chrysogenum a*nd *Streptomyces aureofaciens* respectively. The comparison was made in terms of oxygen transfer rate, substrate consumption, specific productivities and product yields [Schügerl, 1990]. Theobald *et al.*, studied the simocyclinones D8 antibiotics produced by *Streptomyces antibioticus* Tü 6040 in an airlift bioreactor. The kinetics of production, the growth of cells and the substrate utilization were evaluated using an airlift bioreactor. Low shear conditions in the airlift bioreactor led to the formation of pellets of size 1-2 mm diameter [Theobald *et al.*, 2000]. Gavrilescu *et al.*, studied the effect of production of antibiotics in internal and external loop bioreactors. The antibiotics studied were Cephalosporin C, nystatin and bacitracin by *C.acremonium, S. noursei* and *B.licheniformis* respectively and compared the parameters such as oxygen transfer ability, production of cell mass, product concentration, substrate utilization rate and energy efficiency. The production process in ALR was found to be 30-40% more energy efficient as compared to STR though the specific productivity of Cephalosporin C was comparable in the two cases [Gavrilescu *et al.*, 1998].

2.6.3 Co-culture of competitor species

Another strategy which could affect the production of antibiotics by the microorganism is co-culture of different species which exhibit competition under natural conditions.

Streptomyces species have three stages of life cycle viz. vegetative hyphae, aerial hyphae and sporulation. The spores then germinate again for vegetative hyphae

synthesis. As the nutrients deplete, the vegetative mycelium undergoes autocatalytic degradation for aerial mycelium development followed by sporulation [Chater *et al.*, 2011]. The initiation of sporulation is accompanied by the onset of secondary metabolite production [Barka *et al.*, 2016]. Recent works have explored the effect of mimicking the natural ecosystem of co-existence of antibiotic producers with other species and investigated their effect on the production of antibiotics [Van der Meij *et al.*, 2017]. According to another review, *Streptomyces* species undergo exploratory growth during fungal interaction and limited nutrient supply which lead to secretion of a volatile organic compound (VOC) by *Streptomyces* which is known as trimethyl amines and modulates the antibiotic secretion by them [Jones *et al.*, 2017]. According to Shank and Kolter, during co-culture interactions, small molecules act as signal molecules and affect developmental phenomena as sporulation and secondary metabolite production [Shank *et al.*, 2009].

2.6.3.1 Co-culture for antibiotic production

The microbial community tends to exhibit a physiological and metabolic response to interspecies interactions. This complex behavior can be studied with reference to antibiotic production by them.

Slattery *et al.* investigated the effect of competition between *Streptomyces tenjimariensis* and other marine microbial species on istamycin production. It was found that 12 bacterial species induced the antibiotic production. They found a considerable increase in istamycin production in case of co-culture as compared to the single culture system [Slattery *et al.*, 2001].

Fukuda *et al.* studied the effect of co-culture of *Bacillus subtilis* IFO 3335 and *Rhizopus peka* P8. They found that antibiotic activity increased. A similar result was obtained

when *B. subtilis* and *Rhizopus oligosporus* P12 were co-cultured in submerged fermentation. They optimized various parameters affecting the co-cultivation of *B.subtilis* and *R. peka.* Solid state fermentation of the co- culture was also analyzed. Antibiotic activity was measured against *Escherichia coli* IFO 3792 [Fukuda *et al.,* 2008].

Luti *et al.,* noted that the production of undecylprodigiosin enhanced by three times when the producer microorganism *Streptomyces coelicolor* was co-cultured with *Bacillus subtilis*. On the other hand, when *S.coelicolor* was co-cultivated with *Staphylococcus aureus,* the product concentration was enhanced by five times. They studied the effect of interaction on antibiotic production both with live and dead cells [Luti *et al.,* 2011]. Pérez *et al.* discovered that the interaction of *S. coelicolor* with *Myxococcus xanthus* leads to enhanced production of actinorhodin. The co-culture interaction triggered the production by 20 times. A similar result was also found with interaction of *S. coelicolor* with other bacteria with lower enhancement [Pérez *et al.,* 2011].

In another study based on finding the solution to the vascular wilt of many plants caused by *Verticillium dahlia,* an antibiotic undecylprodigiosin was found to be effective. Meschke *et al.,* noted that the production of undecylprodigiosin was enhanced when the producer organism *Streptomyces lividans* was co-cultured with *Verticillium dahlia* [Meschke et al., 2012]. Similarly, Schäberle *et al.* suggested that as *Streptomyces* and myxobacteria exist together in their natural habitat, therefore, their interspecies interaction can be analyzed for production of antibiotic. Co-cultivation of *Streptomyces coelicolor* with *Corallococcus coralloides* was found to stimulate the production of undecylprodigiosin (Red). They also found that the signal for enhancement was

triggered by a water-soluble compound which was extracted from *C. coralloides* [Schäberle et al., 2014].

Sung *et al.* examined the effect of co-culture of a marine-based *Streptomyces* sp. strain PTY087I2 with three human pathogens. These pathogens were *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis*. It was observed that the co-culture with each of these microbes led to enhanced production of antibiotics namely granatomycin D, granaticin and dihydrogranaticin B [Sung *et al.*, 2017].

Marmann *et al.* reviewed co-cultivation as an impactful tool for exploring the potential of biochemical production by different microbial interactions. They proposed that antagonism or competition can be harnessed as a tool for enhancement of constitutively expressed gene products or discovery of cryptic metabolites [Marmann *et al.*, 2014]. Similarly, Abdelmohsen *et al.* compiled different studies carried out which resulted in enhanced production of secondary metabolites in actinomycetes sp. In this review, they presented the effect of co-cultivation of actinomycetes with other actinomycetes, bacteria and fungi. They have also included the molecular and chemical elicitors which are involved in the process [Abdelmohsen *et al.*, 2015].

2.6.4 Fed - batch strategy

Batch fermentation is often considered as the simplest mode for production. It is characterized by the addition of all the medium components at the beginning of the batch and harvesting is accomplished towards the end of it. However, certain shortcomings are associated with batch fermentation such as it has long downtime and does not facilitate control of growth and product formation rates. Moreover, many times batch fermentation is associated with carbon catabolite repression and substrate inhibition which reduce the yield of the product. To overcome such problems fed-batch

strategy is adopted. Industrial application of fed-batch fermentation in stirred tank reactor is increasing. The feeding strategy is designed based on product and process parameters. The control of dissolved oxygen is one such process parameter which affects the production. Bodizs *et al.* have detailed the application of fed - batch fermentation for *Aspergillus oryzae* fermentation for amylase production in 2500 L stirred tank reactor at Novozymes pilot plant located at Bagsvaerd, Denmark. This production strategy has incorporated the dissolved oxygen based setpoint which considers the other process parameters such rheological and morphological variations during the process. The process was divided into three stages. Early batch phase was followed by a linear and constant feeding regime. A mechanistic model was proposed to determine the set point based on a rheological study which did not match the experimental results as it did not consider the effect of pH variation [Bodizs *et al.*, 2007].

Another important factor which controls the fermentation process is the carbon source. The nature and concentration of the carbon source regulate both the cellular growth as well as product formation.

Antibiotics production is favored with a reduced rate of utilization of carbon source. Both limited substrate supply and decreased growth rate are responsible for generating signals which control the production of secondary metabolites. It has been earlier studied that growth rate signals activate a master gene which controls chemical differentiation as well as morphogenesis [Demain *et al.*, 1995]. They also listed the secondary metabolites and the carbon sources which interfere with their production. Rapid assimilation of carbon source leads to repression of secondary metabolite synthesis [Uguru *et al.*, 2005]. Moreover, rapamycin and other polyketides are tightly regulated by gene clusters which code for type 1 polyketide syntheses [Lal *et al.*, 2000].

Actinorhodin production by *Streptomyces lividans* is inhibited by glucose as it represses the biosynthesis of *afs*R2 mRNA which codes for a protein responsible for the enhancement of secondary metabolites [Kim *et al.*, 2001]. Another example is the production of anthracycline by *Streptomyces peucetius* which is repressed by glucose at an initial concentration above 100 mM. This repression was not evident when glucose was added during stationary phase [Escalante *et al.*, 1999]. Inoue *et al.* observed the effect of carbon catabolite repression of retamycin production using *Streptomyces olindensis* ICB20. They found that highest retamycin specific production rate when 10g/L of glucose was used [Inoue *et al.*, 2007].

Design of fed - batch fermentation can vary from linear or exponential to pulse addition which can be based on the calculation of maintenance energy requirement. Vicik *et al.* carried out fed- batch fermentation for cephalosporin C production using *Cephalosporium acremonium* by regulating the specific growth rate at a lower level as compared to maximum specific growth rate until the desired cell mass was achieved. This study was done by simultaneous feeding of methionine and sucrose in an exponential manner [Vicik *et al.*, 1990]. El-Sedawy *et al.* studied the fed - batch rifamycin B production by *Amycolatopsis mediterranei* . They studied the effect of intermittent feeding of optimized media in a lab scale fermentor. Glucose, glucose syrup and uracil were fed at different time intervals. This feeding strategy led to an increase in rifamycin B production by two times [El-Sedawy *et al.*, 2007].

In another study, Ng *et al.* used a fed - batch fermentation for daptomycin production by *Streptomyces roseosporus* NRRL11379 with precursor addition at an optimal flow rate and the supply of glycerol as secondary carbon source. Sodium decanoate was added as a precursor for daptomycin production. The precursor supplementation was initiated after 48 hours of fermentation at an optimal flow rate of 600 mg/L day. The fed- batch

fermentation strategy led to enhancement of daptomycin production up to 812 mg/L [Ng *et al.*, 2014]. Elsayed *et al.*, studied the batch and fed-batch production strategy for oxytetracycline fermentation by *Streptomyces rimosus.* The fermentation was carried out at semi-industrial scale using a 15 L bioreactor. Based on batch data, fed - batch fermentation strategy was designed. Linear feeding strategy was adopted and feeding rate was kept as 0.33 g/L/h. They evaluated mono-glucose feeding as well as complete medium feeding and found that the second strategy led to 26% less oxytetracycline production [Elsayed *et al.*, 2015].

Thus, fed - batch strategy has been successfully implemented for enhancement in the production of many secondary metabolites both as lab and industrial scale.

2.7 Purification of rapamycin

Macrolide antibiotics have lactone rings substituted with deoxy sugars. This class of antibiotics includes tacrolimus, pimecrolimus, sirolimus or rapamycin, erythromycin, clarithromycin and azithromycin. Macrolides are commonly produced by fermentation involving complex medium composition, cellular metabolites and salts. After production, purification is an essential step for stepwise removal of unwanted components from the fermentation broth. Fermentation broth consists of a complex mixture of cells, intracellular and extracellular product, converted and unconverted substrates which include the desired product at a very low concentration [Demain *et al.*, 1999]. Thus, the desired compound needs to be purified as the impurities in pharmaceutical components are unwanted. The purity of any compound to act as a pharmaceutical ingredient must match the purity standards given in the guidelines of regulating authorities such as the US- FDA. The presence of impurities can be detected by several means such as High - performance liquid chromatography (HPLC).

Separation techniques employed for extraction of the product depends on the product location (intracellular or extracellular) as well as on the chemical nature of the product which includes its charge, size, and solubility in various solvents. The information about the other impurities found in the liquid may also help in their selective removal. Thus, purification increases the concentration of the desired product. Chromatography is a common technique employed for purification of fermentation broth which includes high-performance liquid chromatography (HPLC), elution chromatography such as silica gel chromatography.

Many workers have proposed different purification techniques for macrolide purification. Wilson *et al.* obtained a US patent as they proposed the method for separation of a macrolide antibiotic isolated from *Streptomyces avermitilis*. According to them, the purification of the macrolide was brought about by dissolving the extract in a hydrocarbon solvent. It was then extracted with solvents like ethylene glycol. The extract was then recovered using a solvent more polar than hydrocarbon solvent [Cole *et al.*, 1979]. Keri *et al,* suggested that purification of macrolides can be realized by providing charge to the macrolides which are then loaded onto sorption resins. Sorption resins exhibit porous structure which can be used for absorption and desorption of different chemical compounds. Subsequently, the bed of sorption resins was eluted with acetonitrile or other similar solvents. Then the fraction of eluent containing macrolide was collected to obtain macrolide with reduced impurities [Keri *et al.*, 2006]. The same group of inventors suggested a method of treating the macrolides with a polar solvent, a hydrocarbon solvent and water. The pH was kept around 7 and temperature range between -2°C to 35°C for minimum one hour. This method was suggested for purification and isolation of macrolide crystals [Keri *et al.*, 2007].

Similarly, some researchers have worked for purification of rapamycin from the crude fermentation broth. A method (as shown in Fig 2.5) has been devised by Patil *et al.* for purification of rapamycin using stepwise purification technique in which the broth was first extracted using different solvents and concentrated. The acidic and basic impurities were removed by washing with $NaHCO_3$ and HCl respectively. The sample was then purified using silica gel column chromatography and stirred with diethyl ether at low temperature to recover rapamycin crystals of high purity [Patil *et al.*, 2010].

Rani *et al.* studied the recovery of rapamycin produced by *S.hygroscopicus* MTCC 5681 in which they employed decanter centrifuge for extraction of biomass from the fermentation broth. 1600 L of broth was purified using sequential steps of biomass separation, extraction, concentration, aqueous sodium bicarbonate washing, extraction with toluene, silica gel chromatographic purification and crystallization to obtain 97% pure rapamycin. Crystallized product was further purified using flash chromatography to recover rapamycin with >99% purity [Rani *et al.*, 2013]. The purity of rapamycin can be analyzed by HPLC. Different protocols for determination of rapamycin have been reported as shown in Table 2.3. Thus, HPLC can be used as a robust and reliable method for quantitative estimation of rapamycin.

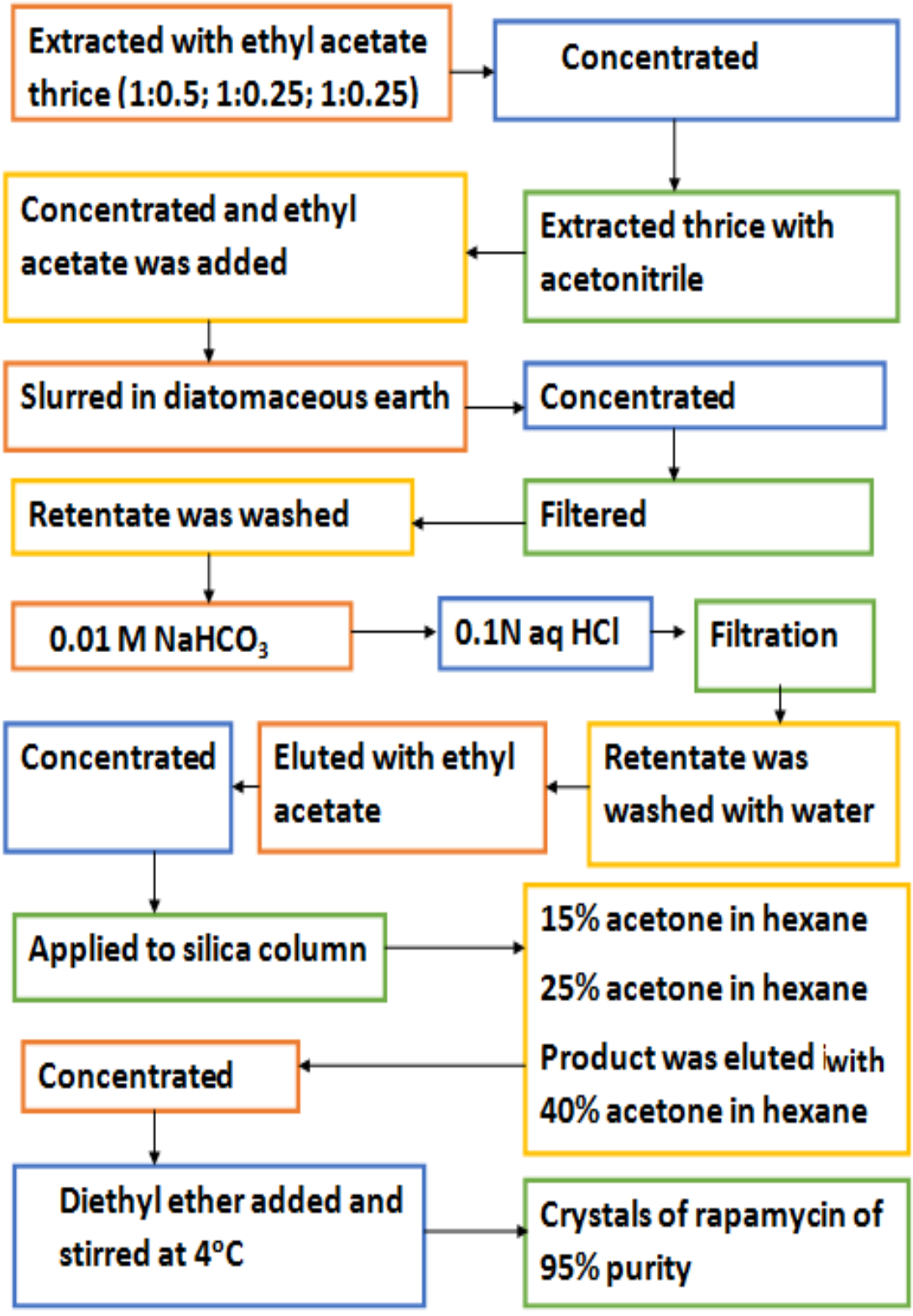

Fig 2.5 Steps of purification of rapamycin [Patil *et al.*, 2010]

Table 2.3 HPLC methods for detection of rapamycin from the fermentation broth

S.No.	Column	Solvent system (Ratio)	Isocratic/ Gradient	Flow Rate	Wave-length	References
1	Agilent Eclipse XDB-C8, 3.5µm, diameter 4.6 mm length 150 mm	A:Acetonitrile B: 2mM KH_2PO_4 in water	Gradient	1.5 mL/ min	287 nm	[Patil *et al.*, 2010]
2	Waters C18 reverse phase column	A: Acetonitrile B: water (80:20)	Isocratic	1 mL /min	277 nm	[Kim *et al.*, 2014]
3	C18 reverse phase (Novapak 3.9x 150 mm Millipore)	A:1,4 dioxane B: water (55:45)	Isocratic	1 mL /min	287 nm	[Kojima *et al.*, 1998]
4	Hitachi HPLC Veropak C18	A: Methanol B: water C:Acetonitrile (80:20:0.1)	Isocratic	2 mL / min	254 nm	[Yen *et al.*, 2013]
5	Hewlett Packard model 1100 (4.6 x 150 mm, 5µm, 120 Å)	A: 0.025% formic acid in water B: 0.025% formic acid in Acetonitrile	Gradient	0.8 mL /min	PDA detection	[Ritacco *et al.*, 2005]
6	Phenomennex Hyperclone (4.6 mm x 150 mm) 3 µm	A: 0.01 M Ammonium acetate (10% (v/v) Acetonitrile and 0.01% (v/v) trifluoro acetic acid) B: 0.01 M Ammonium acetate (90% Acetonitrile and 0.01% (v/v) trifluoro acetic acid)	Gradient	1mL /min	280 nm	[Kuščer *et al.*, 2007]
7	Waters C18 Hypersil column (4.6 x 250 mm and 5 mm particle size)	A: Methanol B: Acetonitrile (80:20)	Isocratic	1mL /min	272 nm	[Dutta *et al.*, 2014]

2.8 Stability of rapamycin

Rapamycin chemical stability in several organic solvents was examined by Ricciutelli *et al.* They used many organic solvents such as dichloromethane, chloroform, hexane, ethyl alcohol, methyl alcohol, acetone, pentane, ethyl acetate and tetrahydrofuran. The analysis was done using reverse phase HPLC and diode array detector (DAD) along with mass spectrometry (MS) [Ricciutelli *et al.*, 2006].

OBJECTIVES

Based on the review of the literature, the strategies for enhanced production of rapamycin were designed and evaluated.

The scheme of work was as follows:

1) Statistical optimization using RSM, ANN and GA hybrid methodology
    a. Study of fermentation kinetics of rapamycin production using optimized medium in Stirred Tank Reactor
2) Strategies for modification of rapamycin production process
    a. Effect of immobilization of *Streptomyces hygroscopicus*
    b. Effect of enhanced dissolved oxygen supply by carrying out production in an Airlift reactor
    c. Effect of stress conditions on rapamycin production by co-culture of *Streptomyces hygroscopicus* with a competitive microorganism
    d. Effect of extended stationary phase on the production of rapamycin using fed - batch fermentation
3) Purification and structural validation of rapamycin