CONCLUSION

Strain *Streptomyces hygroscopicus* NRRL 5491 procured from NRRL, USA was chosen for fermentation of rapamycin using complex media with growing conditions of 200 rpm, 6.0 pH and 28°C temperature in shake flask.

Fermentation medium was optimized using hybrid statistical analysis where data set was generated using Central composite Design (CCD). This led to the design of 20 experiments which were carried out in shake flasks. The coefficient of determination was found to be 98.41%.

The results of these 20 experiments were then used to train Artificial Neural Network (ANN). This application divided the input vectors and target vectors into three sets. One set for training, second to validate and third for test. Three input variables were used which are mannose (14.95-25.04 g/L), soyabean meal (11.59-28.408 g/L) and lysine concentration (1.59-18.40 g/L) and were normalized. ANN model provided accurate predictions as the data were trained in 1077 epoch with R value 0.99 and mean sq error value 0.305.

The trained data's fitness was evaluated using the Genetic Algorithm (GA) tool. The parameters taken for GA optimization were population size of 10, mutation rate of 0.1 and uniform cross overrate of 0.8. Five experiments were carried out under predicted conditions. From these experiments, the observed experimental production is 320 mg/L which was very close to the software predicted result of 323 mg/L. The optimal concentrations for the three medium components were 25.47, 15.39 and 17.48 mg/L for mannose, soyabean meal and L-lysine respectively.

Rapamycin production was then carried out in 3L Stirred tank reactor with two impellers. Kinetic analysis was done at different initial substrate concentrations. As the culture was active, the growth phase was initiated almost immediately after inoculation and reached stationary phase in 72 hours where the maximum rate of production of rapamycin was attained. Maximum production of rapamycin was obtained when initial mannose concentration was kept at 25 g/L. Modeling of growth, rapamycin production and mannose utilization was carried out.

Then rheological analysis of the culture obtained from 3L STR was carried out which showed that the relation between shear stress and shear rate can best described by Power Law model from 24 hours to 120 hours (R^2 value 0.97-0.95). The variation of apparent viscosity, flow behavior index and consistency index with respect to time was also calculated.

Immobilization of *Streptomyces hygroscopicus* was carried out using four different support materials i.e, polyurethane foam (PUF), sintered beads, glass beads and foam peanuts. These carriers were pretreated based on previous reports. The cells of *Streptomyces hygroscopicus* were immobilized using these carriers. Maximum immobilization of cells was observed in case of PUF as it has high porosity. SEM images show that uniform distribution of film of *Streptomyces* mycelia in case of PUF. The immobilized cells were then transferred to the previously optimized production medium where the cell release study was carried out. Maximum cell release was found in case of glass beads relating to its low porosity. It was found that highest production was 291 mg rapamycin/g of PUF and minimum in case of glass beads. The cell recycle study shows that immobilization

sustained well for three cycles when cells were immobilized on PUF as the cell release was about 15%.

Another strategy was to culture *S.hygroscopicus* in an internal loop Air Lift Reactor (ALR) using optimized media for rapamycin production. 3L of production medium was inoculated with 300 mL of seed culture medium and production was carried out for 120 hours under different flow rates in batch mode. Variation of gas hold-up with superficial gas velocity was estimated. It was found that maximum production of rapamycin was obtained at 1.5 vvm of flow rate.

It was also observed that comparable production of rapamycin took place in STR at 1 vvm and ALR at 1.5 vvm. The ALR batch had higher biomass production probably due to reduced shear stress. Kinetic analysis of the rapamycin production in the two reactors shows that $\mu_{max,}$ $Y_{X/S,}$ q_p and $Y_{P/x}$ was higher in case of ALR.

Another study was carried out to mimic the natural conditions for rapamycin production by creating the stress conditions and employ it to induce enhanced production. Culture of *Streptomyces hygroscopicus* was first grown on MGYP solid media under co-culture conditions with *C.albicans* for three generations. The colony of *S.hygroscopicus* was then isolated from every generation which was growing in vicinity to the competitor strain and was transferred to the successive generation. The colony selected from the third generation was finally grown in liquid medium to assess the production of rapamycin under different inoculating conditions of competitive strain of *C.albicans*.

It was found that maximum production of rapamycin was attained when 3% of *C.albicans* was inoculated with 10% of *S.hygroscopicus*. Also the production increased up to 368

mg/L when 3% of *C.albicans* was inoculated after 24 hours of inoculation of *S.hygroscopicus*.

Then the effect of extended stationary phase on rapamycin production was analyzed. As found during kinetic analysis that maximum production of rapamycin took place when 25 g/L of mannose was used and beyond this, the concentration of the product decreased. Thus, another strategy was fed-batch fermentation which was carried out with 25 g/L of mannose as initial concentration. Two precursors were fed at the initiation of stationary phase while maintenance energy was supplied as the substrate concentration decreased to extend the stationary phase. This led to increase in rapamycin production up to 481 mg/L at 144 h with higher productivity as compared to the batch culture.

The broth obtained from fed- batch fermentation was then purified. Crude broth was first extracted thrice with ethyl acetate followed by removal of colored impurities using diatomaceous earth. HPTLC of the extract was also carried out which showed that it contained rapamycin as one of the bands had same retention time as the standard. The extract was then subjected to basic and acidic wash followed by neutralization of the pH and washing of residue with distilled water. The extract was subjected to silica gel column. Initially, polar impurities were removed followed by elution of non-polar rapamycin.

The fractions showing presence of rapamycin were pooled and concentrated. Finally HPLC and FTIR were carried out to estimate the purity of the sample.

HPLC was carried out which showed that the sample was 45% pure after solvent extraction while it was 88% pure after stirring in diethyl ether. When FTIR was carried out for sample and standard rapamycin, it showed similar peaks in terms of transmittance. The overlay diagram clearly depicts the purity of the sample.

Thus, these strategies for enhanced production of rapamycin could be employed for scale – up of the process.

Future scope of this work includes carrying out immobilization and fed- batch studies in Airlift reactor. The effect of other microorganism grown in co-culture conditions with *Streptomyces hygroscopicus* could also be analyzed. Moreover, metabolic flux analysis could provide an insight for tailoring the biosynthetic pathway so as to enhance the production of rapamycin.