MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and Reagents

The chemicals and other consumables used in the experiments were mostly purchased from companies such as: Merck (India); S.D. Fine Chemicals (India); Himedia (Mumbai, India); Qualigens Fine Chemicals Ltd. (India) and Sisco Research Laboratories (Mumbai, India). Pure rapamycin was purchased from ApexBio LLC (USA).

3.1.2 Microorganisms

Microorganisms were procured from National Collection of Industrial Microorganism (NCIM at National Chemical Laboratory, Pune, India) and Northern Regional Research Laboratory (NRRL, USA) as listed in Table 3.1. The agar plate culture of *S.hygroscopicus* and *C.albicans* has been shown in Fig 3.1.

Table 3.1 List of microorganisms obtained from different culture collections

Sl. No.	Microorganisms procured	Culture Collections
1.	*Streptomyces hygroscopicus*	NRRL 5491
2.	*Candida albicans*	NCIM 3471

3.1.3 Instruments and Equipments used

Table 3.2 provides the list of equipments and instruments mainly used for the experiments and analysis.

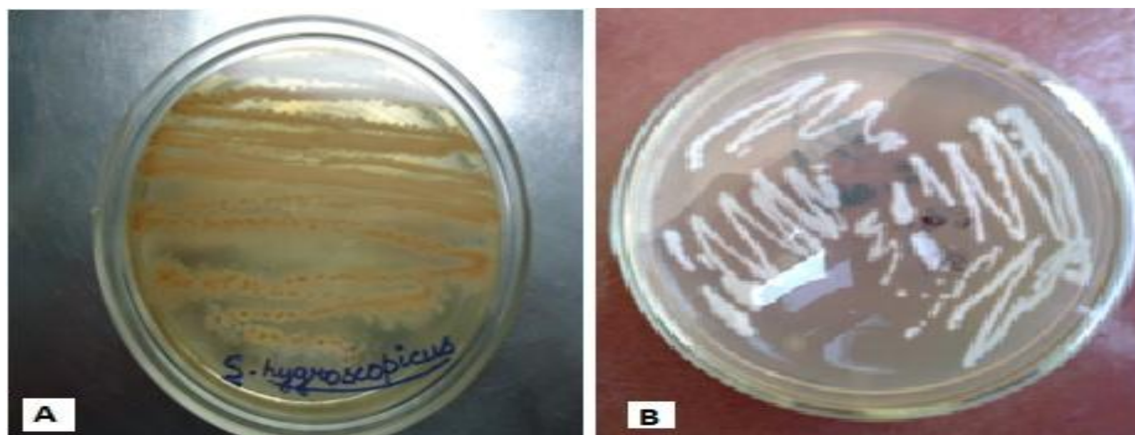


Fig 3.1 Microorganisms grown on agar plates A) *S. hygroscopicus* NRRL 5491 B) *Candida albicans* NCIM 3471

Table 3.2 List of equipments used for experiments

Sl. No.	Equipments/Instruments	Manufacturing Company
1.	Laminar Air Flow	Ikon Instruments
2.	UV/Vis Spectrophometer	Shimadzu UV-1800
3.	Hot Air Oven	Ikon Instruments
4.	Centrifuge	Remi R-24
5.	Shaking Incubator	Remi CIS -24 Plus
6.	pH meter	Toshcon
7.	HPLC system	Waters
8.	3L Fermentor	Scigenics India
9.	Air Lift Reactor	Indigenously designed
10.	Lyophilizer	Labconco
11.	FTIR analyzer	Shimadzu IR Affinity IS
12.	HPTLC	Camag
13.	Viscometer	Brookfield
14.	Light Microscope	Olympus CKX53
15.	Scanning Electron Microscope	Evo 18 Research, Zeiss SEM
16.	Rotary Vacuum Evaporator	Cyberlab

3.1.4 Media

Media sterilization was carried out by autoclaving for 15 min at 15 psig unless otherwise specified. Carbon and nitrogen sources were autoclaved separately. Heat labile medium components were filter sterilized using sterile nylon filters (0.2 µm pore size).

3.1.4.1 Maintenance media

Oat meal medium was used as maintenance medium for *Streptomyces hygroscopicus* NRRL 5491 with following medium composition:

Components	**Composition**
Oat Meal	20.0 g
Agar	20.0 g
Distilled water	1000 mL
	pH =7.0

YEPD medium was used for routine sub-culturing of *Candida albicans* NCIM 3471 at 25°C containing (g/L):

Components	**Composition**
Glucose	20.0 g
Yeast Extract	10.0 g
Bacto-Peptone	20.0 g
Agar	20.0 g
Distilled water	1000 mL
	pH =5.6

3.1.4.2 Seed Culture medium

MGYP medium which was used as seed culture medium for rapamycin production contained following components (g/L):

Components	**Composition**
Malt Extract	3.0 g
Yeast Extract	3.0 g
Peptone	5.0 g
Glucose	10 g
Agar	20.0 g
Distilled water	1000 mL
	pH =6.0

3.1.4.3 Production medium (modified Sallam *et al.* 2011)

Rapamycin production medium contained following components (g/L):

Components	**Composition**
Soyabean Meal	20.0 g
Mannose	20.0 g
L-Lysine	10.0 g
Potassium dihydrogen phosphate	5 g
Distilled water	1000 mL
	pH =6.0

3.1.5 Staining Reagents

3.1.5.1 Gram Stain

Gram staining was used for confirmation of presence of *Streptomyces hygroscopicus* as they are Gram positive

i) Crystal violet

a) *Solution A*

Crystal violet (90% dye content)	2.0 g
Ethyl alcohol (95%)	20 mL

b) *Solution B*

Ammonium oxalate	0.8 g
Distilled water	80 mL

Solution A and B were mixed completely

ii) Gram's iodine

Iodine	1.0 g
Distilled water	300 mL
Iodine	1.0 g

iii) Safranin solution

Safranin	0.25 mL
Ethyl alcohol (95%)	10 mL
Distilled water	100 mL

3.1.6 Software used

The software packages which were used for processing and analysis of experimental data are shown in Table 3.3.

Table 3.3 Software packages used for data processing and analysis

S.No.	Software Packages
1	MS Excel 2010
2	Matlab version 7.0 Mathworks Inc.
3	Minitab Version 15.1.0.0, USA
4	Origin Pro- 8.0, OriginLab Corp., MA, USA
5	Empower2 (HPLC)
6	winCATS Planar Chromatography Manager (HPTLC)
7	Lab Solutions IR (FTIR)

3.2 Methods

3.2.1 Maintenance of cultures

The culture of *S.hygroscopicus* was obtained in lyophilized form which was revived on oat meal agar plates. The active culture was routinely sub-cultured in 3-4 weeks and was stored as slants at 4±1 °C. To store for longer period, the spores were collected and stored in 20% glycerol solution.

The culture of *C.albicans* was regularly sub-cultured on YEPD agar medium and was stored as slants at 4±1 °C.

3.2.2 Visualization under microscope

3.2.2.1 Light microscopy

The morphology of *S.hygroscopicus* NRRL 5491 was visualized in different samples collected at different time intervals to verify that samples were contamination free. The

samples were stained with Grams reagent and then visualized under the microscope (Olympus).

3.2.2.2 Scanning Electron Microscopy

The immobilized cells were visualized using scanning electron microscope (Evo 18 Research, Zeiss SEM) which was carried out at an acceleration voltage of 20.0 kV after coating with gold particles by Q 150R ES Quorum. Before carrying out SEM, the cells were fixed by using 0.25% glutaraldehyde solution (prepared in sodium phosphate buffer – pH 7.2) for 30 h. Then the samples were dehydrated using varying ethanol concentrations ranging from 30% to 90% and incubated for 10 minutes. Final dehydration step used absolute ethanol and the samples were incubated for 1h.

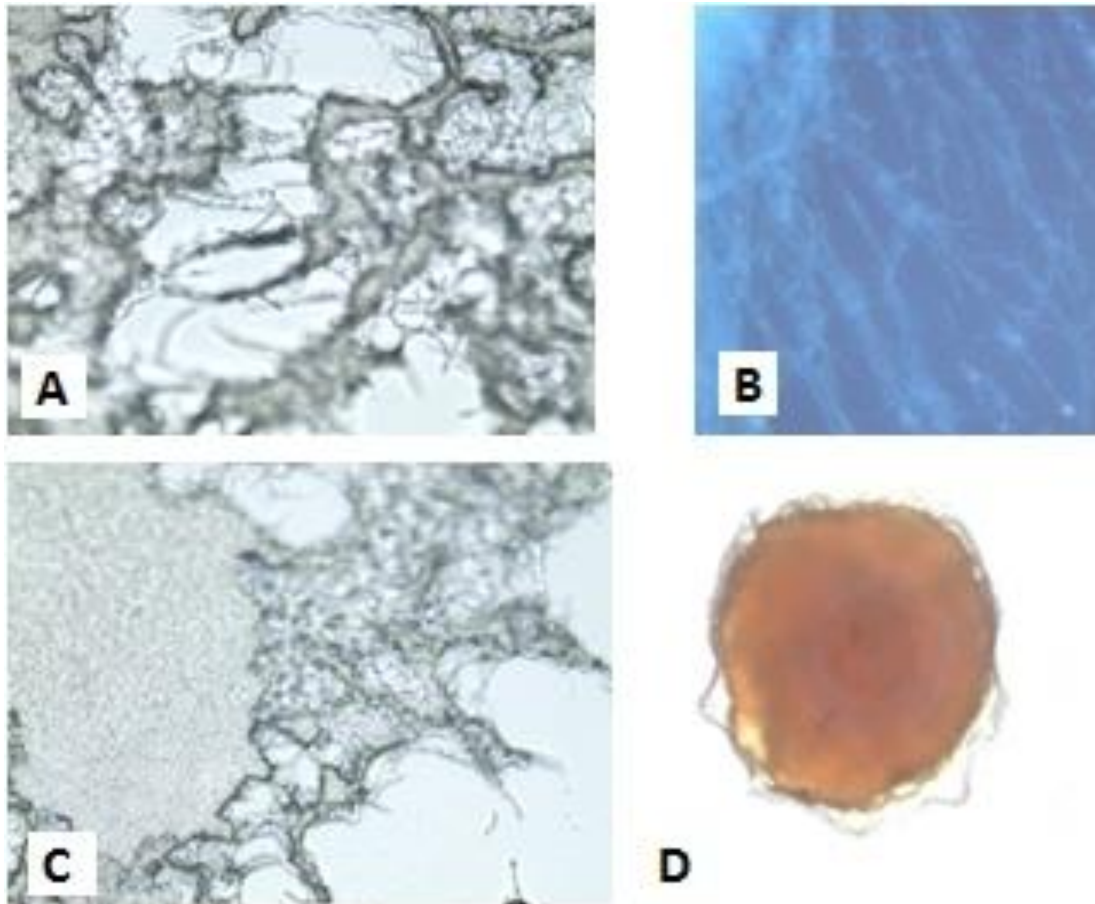


Fig 3.2 Microscopic image of *S.hygroscopicus*

Fig A and B: Mycelia of *S.hygroscopicus (under microscope 400 X)*

Fig Cand D: Pellet of *S.hygroscopicus (under microscope 400 X and 40 X respectively)*

3.2.3 Production of Rapamycin

Rapamycin production was carried out in following steps:

i. **Shake Flask Culture:** Initially, the production of rapamycin was studied in 250 mL Erlenmeyer flask.

ii. **Rapamycin production studies in stirred tank reactor:** Subsequently, rapamycin production was studied in 3L in-situ sterilizable jacketed stirred tank bioreactor (Scigenics, India Pvt. Ltd) using the statistically optimised medium.

iii. **Rapamycin production studies in airlift bioreactor:** Rapamycin production was also evaluated in 3L air lift reactor made of borosilicate glass.

3.2.3.1 Inoculum Preparation

A loopful of culture grown on slants was transferred to 10 mL of liquid MGYP medium for 24 h at 28 °C. 5 mL of this culture was then transferred to 50 mL of seed medium in 250 mL Erlenmeyer Flask and was allowed to grow for 2 days at 28°C and 200 rpm.

3.2.3.2 Shake Flask Studies

The production of rapamycin was studied in 250 mL Erlenmeyer/ shake flask containing 50 mL of production media (modified Hamdy *et al.,* 2010) containing: Soyabean Meal 20 g/L, KH_2PO_4 5 g/L, Mannose 20 g/L and L-lysine 10 g/L. The flask was inoculated with inoculum (10% v/v) from 2 days old seed culture and was cultivated at 28°C by shaking at 200 rpm. All the experiments were conducted in triplicates and the average value was measured. The samples were collected at 24 h intervals and were analysed for biomass, reducing sugar and rapamycin concentration.

3.2.3.4 Production in Stirred tank Bioreactor

Rapamycin production in 3L stirred tank bioreactor (Scigenics India Pvt. Ltd.) was studied which had 2L as working volume. The fermentor was sterilized using in-situ sterilization method. Media was sterilized along with pH and Dissolved Oxygen (DO) probe and during sterilization the inlet pressure was maintained at 1.5 bar while the exhaust pressure was kept 1.2 bar. pH was controlled automatically using After cooling, when the temperature reached 28°C, 10% inoculum was added aseptically using peristaltic pump. During fermentation temperature was maintained at 28°C, inlet pressure 1 bar and exhaust pressure 0.5 bar. The culture was agitated at 400 rpm using 2 ruston turbine impellers each having 6 blades and diameter of impellers were 50 mm. Aeration at 1 vvm was provided using a porous sparger with 12 holes each of diameter 1 mm. pH was maintained automatically using 0.1 M HCl and 0.1 M NaOH while the foam was controlled using 0.5% silicone oil.

3.2.3.5 Production of rapamycin in 3L airlift bioreactor

Rapamycin production in 3L airlift bioreactor was studied. Fig 3.3 shows the internal loop airlift bioreactor which was indigenously designed and fabricated to evaluate the production of rapamycin. The dimensions were suitable for autoclaving the fermentor. The airlift reactor was designed so as to minimize the pressure drop. The height to diameter ratio was kept high (H/D = 10), which was suitable for effective mixing. The liquid circulation velocity and gas hold up was also sufficient to support aerobic fermentation process with high mycelia content. This fermentor was provided with a glass jacket within which water was circulated and appropriate temperature for fermentation was maintained

by connecting it with a automated-chiller. A sterilizable pH and DO probe was connected to the air lift reactor from the top of the reactor.

Aeration was facilitated in the ALR by means of a perforated sparger which was connected to an air filter. Fig 3.4 illustrates the schematic diagram of set up of the internal loop airlift reactor. The supply of air in the fermentor is regulated with the help of rotameter (Bioengineering AG) and the aeration rate is calculated in terms of volume of air per volume of media per minute (vvm). Aseptic sealing of all the ports was done during the fermentation. 1 mL of silicone oil was added as an antifoam agent.

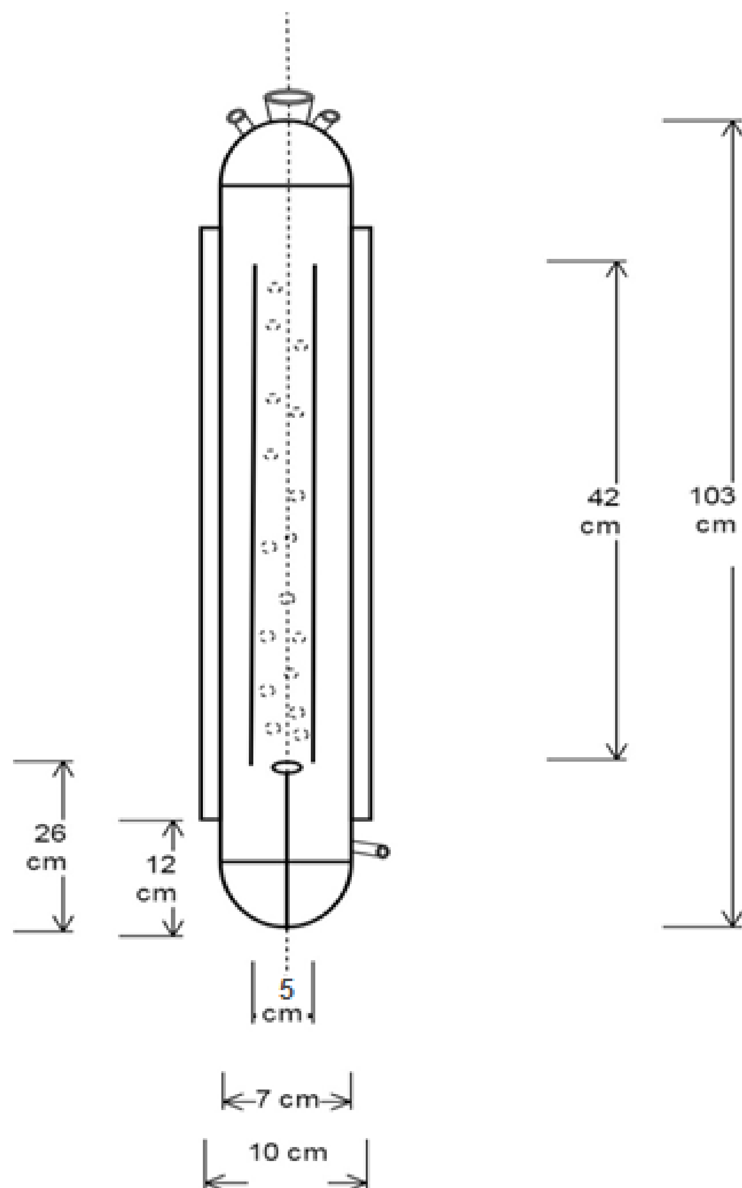
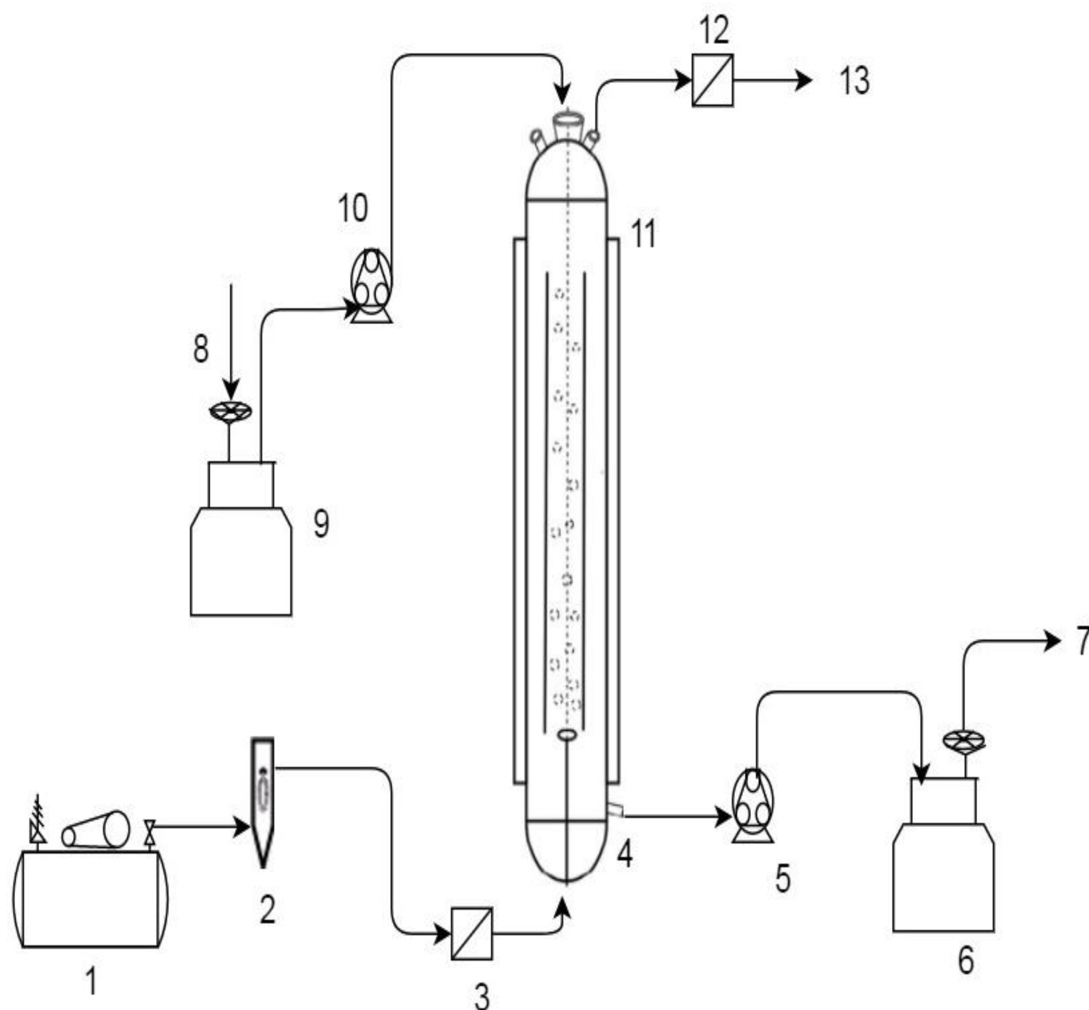


Fig 3.3 Schematic diagram of internal loop airlift reactor (dimensions not to scale)



1: Air Compressor
2: Rotameter
3 & 12: Air Filter
4: Sampling Port
5 & 10: Peristaltic Pump
6 & 9: Medium and Sample Reservoirs
7 & 8 : Reservoir Air Filters
11: Internal Loop Air Lift Reactor
13: Effluent Air

Fig 3.4 Schematic diagram of set up of Internal Loop Air lift Reactor

3.2.4 Optimization of media components

3.2.4.1 Study of interactive effect of parameters with Central Composite Design (CCD)

The effects of three independent process parameters (mannose, soyabean meal, L-lysine) on rapamycin production were analyzed using a Central Composite design (CCD). It was performed at five experimental levels: -α, −1, 0, +1, +α. For a three-factor design, comprising of six central points, a total of 20 experiments were performed. The various combinations used in these 20 experiments were used as input data set for training using ANN code written in MATLAB.

Out of these 20 experimental data we have taken 12 as training set, 4 as validation set to validate that the network is generalizing and to stop training before overfitting and remaining 4 as completely independent test of network generalization. This was done using default function for random data division.

Each experiment was performed in triplicate and the mean value reported. The regression analysis was done using software (Minitab 15) with the numerical data obtained after performing the experiments. The individual as well as the interactive effect of each parameter were determined and the process was modeled using the analysis of variance (ANOVA).

3.2.4.2 Artificial neural network (ANN)

The neural networks are adjusted, or trained, so that a particular input leads to a specific target output. The ANN works on error- back-propagation (EBP) method in which input vectors and the corresponding target vectors are used to train a network until it can

approximate a function. The basic artificial neural network architecture has three layers, input layer, hidden layer and output layer. Each of these layers consists of a number of nodes which are linked to subsequent nodes of subsequent layers with weighted connections. Before training the weights are initialized to random values. The reason to initialize weights with small values is to prevent saturation. The newff command automatically initializes the weights. This function takes a network object as input and returns a network object with all weights and biases initialized.

The normalization process for the raw inputs has great effect on preparing the data to be suitable for the training. Without this normalization, training the neural networks would have been very slow. Generally, the normalization step is applied to both the input vectors and the target vectors in the data set. In this way, the network output always falls into a normalized range. The network output can then be reverse transformed back into the units of the original target data when the network is put to use in the field. The 'mapminmax' function maps the range of input values to the range [0 1], or normalizes the input values. Training is often faster when values are normalized. Training is carried out for minimization of the errors which is done through multiple iterations using the parallel processing unit. Training process includes calculation of the mean square error between the experimental and the predicted values. This error value was then propagated backward through Levenberg-Marquardt back propagation algorithm. This process is repeated till the error value reaches the certain level which minimizes the difference between the two. Training is followed by validation which is carried out using data set which has not been used for training. A feed forward neural network (FFNN) has been used for modeling

which consists of input, hidden and output layers [Zafar *et al.*, 2012]. This FFNN is also known as multi layer perceptron [Gardner *et al.*, 1998].

The application randomly divides the input vectors and target vectors into three sets. One set for training, second to validate that the network is generalizing and to stop training before over fitting and last as a completely independent test of network generalization. This involves randomly extracting test set, developing a neural network model based on validation set and test set. This is followed by repeating the process with several random divisions of data. The reported results are based on the average performance of all randomly extracted test set [Smith *et al.*, 2002]. After training, ANN can predict the output when any input similar to the pattern that it has learnt is fed. ANN tends to inherently match the input vector (i.e., medium composition) to the output vector (rapamycin production).

For training of experimental data generated by CCD with artificial neural network, we used feed forward neural network and for training the network back propagation Levenberg-Marquardt algorithm was applied. Tansig and purelin transfer functions have been used for hidden and output layer respectively shown as below:

For hidden layer [Xu *et al.*, 2006]:

$$a_1 = \text{tansig}(IW_{1,1} + b_1) \qquad - (3.2.1)$$

For output layer [Xu *et al.*, 2006]

$$a_2 = \text{purelin}(LW_{2,1} + b_2) \qquad - (3.2.2)$$

where a_1 and a_2 were the output of the hidden layer, and output layer respectively. $IW_{1,1}$ and $LW_{2,1}$ were the input weight matrix and output weight matrix whereas b_1 and b_2 were the bias of hidden layer and output layer.

The input data were previously normalized and then fed into ANN for training. After training these data were fed to the fitness evaluation function for getting the best solution of all three input variables through GA.

3.2.4.3 Genetic algorithm

The genetic algorithm (GA) is a method for solving both constrained and unconstrained optimization problems that is based on natural selection, the process that drives biological evolution. Once a generation capable ANN-based process model with good prediction accuracy is developed, a genetic algorithm can be used to optimize its input space representing process variables, with a view of maximizing the process performance.

Genetic algorithm is based on theory of natural selection. This process comprises of three steps: selection, genetic operations and substitution or replacement. Initial population in form of group of chromosomes is randomly generated. GA repeatedly changes a population of individual solutions. At every step, the genetic algorithm chooses the individuals randomly from the current population to act as parents and employs them to generate the children for the next generation. This is carried out by a main loop of operations consisting of: (i) selection of more fit parent chromosomes to develop a mating pool (ii) crossover, which is generation of offspring solutions by pair-wise crossing-over of the constituents between pairs of better parent chromosomes and (iii) mutation i.e., random changes in the elements of the offspring strings, is done [Baishan *et al.*, 2003, Freyer, 1992]. With successive generations, the population advances toward an optimal solution. The offsprings are then examined based on the fitness function and then they are selected as parents for next generation. In this manner with successive iterations the best individual is selected which becomes the solution to the problem [Tang *et al.*, 1996].

Process optimization by GA requires several iterations to declare the global optimum solutions. These iterations were carried out by changing the input space parameters. These repetitions ensured that the entire searching space has been utilized to attain a global optimal solution. These multiple iterations lead to a state when the same optimal solution for most of the input conditions is acquired. This state then assures that the global optimal solution has been attained.

3.2.5 Study of immobilization of *Streptomyces hygroscopicus*

Streptomyces hygroscopicus were immobilized on different carriers and production of rapamycin was evaluated in shake flask culture. Fig 3.5 shows different carriers/ support materials used for immobilization. Table 3.4 describes the composition of support materials.

Table 3.4 Composition of different carriers used for immobilization

S.No.	Different carriers used for immobilization	Composition
1	Polyurethane foam	polyol, isocyante and water
2	Glass Beads	sodium aluminium silicate
3	Siran beads	borosilicate based
4	Foam Peanuts	corn starch

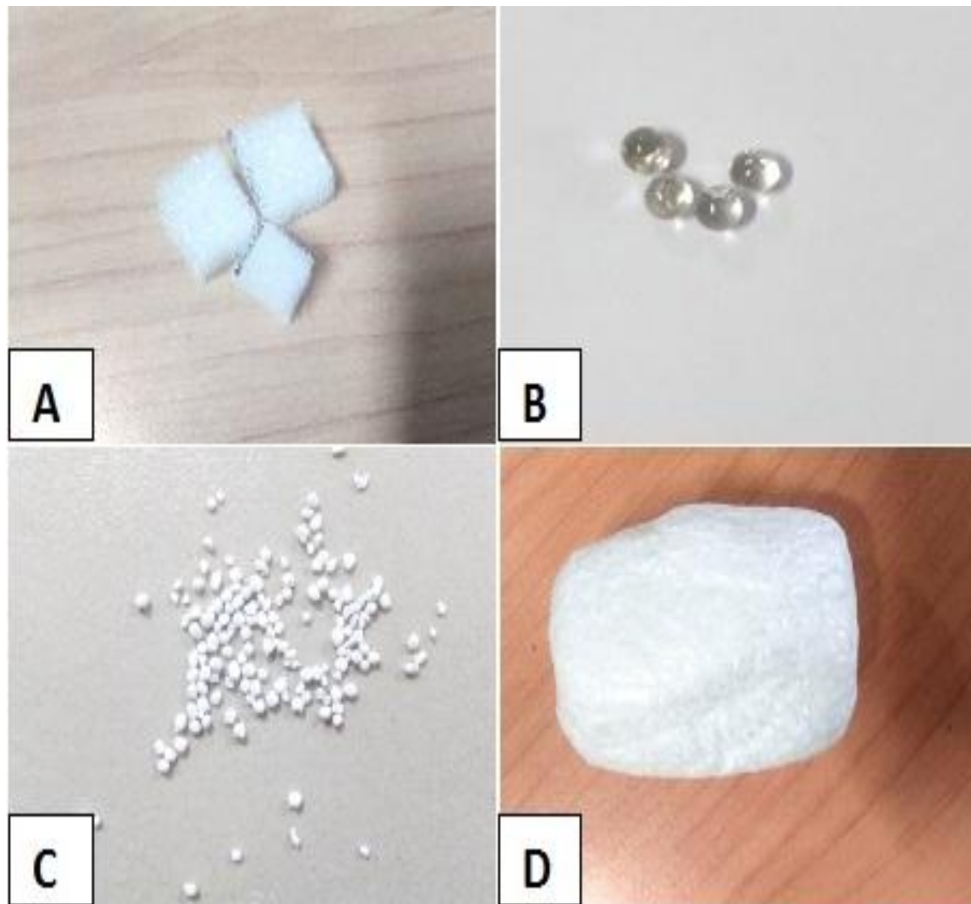


Fig 3.5 Different carriers used for immobilization

A. Polyurethane foam B. Glass Beads C. Siran beads D. Foam Peanuts

3.2.5.1 Measurement of porosity of carriers

Porosity of different carriers was determined by measuring the weight of the carriers (w1). Their volume was calculated as v1. The carriers were soaked in distilled water and placed in vacuum oven for 2 minutes. The saturated carriers were rolled over tissue paper to remove excess water. The weight of the carriers saturated with water was calculated as w2. The porosity of the carriers was calculated by the following equation:

$$Porosity = v2/v1 \qquad - (3.2.3)$$

Where, $v2 = w2 - w1$

3.2.5.2 Pre-treatment of carriers

The PUF and foam peanuts were cut into 1 cm x 1cm x 1 cm cubes and were washed with autoclaved distilled water.

Cubes of PUF were submerged in glutaraldehyde (4% w/v) as described by Zhu et. al. [4] . Then, they were rinsed with distilled water and soaked in 4% HCl for 10h. Subsequently, all the cubes were washed several timed with distilled water and dried at 50 °C. PUF cubes were pre-treated based on study carried out by Zhu *et. al.* [Zhu *et al.*, 2015].

Glass beads diameter was approximately 0.5 cm. Glass beads were submerged in concentrated Nitric acid for 3 hours and later with distilled water. Siran beads (particle size ranging from 600-1000 µm) were washed twice with distilled water and were then heated in muffle furnace at 600°C for 16 hours. Foam peanuts were washed with distilled water and then dried in oven at 50°C.

3.2.5.3 Cell Immobilization Study

Following pre-treatment all carriers were immobilized by *Streptomyces hygroscopicus* NRRL 5491. This was done by adding inoculum from freshly prepared seed culture to autoclaved carriers containing 50 mL of seed culture media, under sterile conditions. It was cultured for 48 hours at 200 rpm, 28°C and immobilized carriers were regularly withdrawn from the culture. These were rinsed twice with sterile water and were lyophilized for 10 h. The weight of immobilized cells was determined by calculating the difference in weight of lyophilized support and pre-determined weight of support material.

3.2.5.4 Cell Release Study

Cell release study was carried out by withdrawing 2 mL of fermentation broth at regular intervals and concentrations of dry cell mass in the samples were determined. Samples were then filtered through pre-weighed filters and were washed with distilled water. Filters were then dried by deep freezing and subsequent lyophilization. The weight of released cells was then determined by calculating the difference in weight of lyophilized filters and their initial weight.

3.2.6 Co-culture Technique

Co-culture strategy was adopted in which the producer strain *Streptomyces hygroscopicus* NRRL 5491 and its competitor strain *Candida albicans* NCIM 3471 were grown together in agar as well as liquid media.

3.2.6.1 Co-culture on agar media

S.hygroscopicus was first adapted to grow with *C.albicans* on MGYP agar medium by streaking the culture of *C.albicans* at the centre while *S.hygroscopicus* was streaked along the periphery. The culture of *S.hygroscopicus* growing adjacent to the competitor strain was carefully collected using sterile loop and was streaked on the next plate in similar manner. In this manner, the culture of *S.hygroscopicus* was grown for three generations. The plates were incubated at 28°C for 72 hours.

3.2.6.2 Co-culture in liquid media

A loopful of culture of *S.hygroscopicus* was collected and transferred to MGYP broth and was grown for three days at 28°C and pH 6.0. This culture was then transferred to the production media such that the inoculum size was 10% (v/v). Then *C.albicans* was introduced into the production media and different studies were conducted to evaluate the effect of co-culture on rapamycin production.

3.2.7 Analytical Techniques

Different techniques were employed to analyze the samples obtained during fermentation of rapamycin which were as follows:

3.2.7.1 Estimation of reducing sugar concentration

Reducing sugar concentration was determined using dinitrosalicylic acid (DNS) method which was based on earlier report [Miller, 1959]. DNS reagent was prepared by adding 5g of DNS to 250 mL distilled water and heated at 80°C. On cooling, 100 mL of 2N NaOH

was added alongwith 150 g of Potassium sodium tartrate tetrahydrate (Rochelle Salt). The final volume of the reagent was made upto 500 mL.

To prepare the standard curve stock solution of 10 mg/mL of mannose was made. Different dilutions were prepared such that final concentration ranges from 0.2 mg/mL to 1 mg/mL.

To estimate the reducing sugar in the sample, the broth samples were centrifuged and the supernatent was collected and appropriately diluted with distilled water. 2 mL of distilled water, sample and standards were taken in standards test tubes. The test tube containing distilled water was marked as blank. To each of the test tube, 3 mL of DNS reagent was added and placed in water bath at 95°C for 5 minutes. After removing from water bath 5mL of distilled water was added immediately to each of the test tubes and allowed to reach room temperature. Absorbance for standards and samples were noted using UV/Vis spectrophotometer against blank at 540 nm. Standard curve was plotted as shown in Fig 3.6 and the value of coefficient of linear regression was 0.991. The standard graph was then used for calculating the concentration of the reducing sugar in the samples.

The standard graph was used for calculating the concentration of the reducing sugar in the samples.

3.2.7.2 Estimation of dry cell mass

Microbial cell mass was determined by centrifugation of a fixed volume of broth samples. The pellet was collected in pre-weighed container and dried until the cell mass reaches constant weight in a hot air oven at 80°C. The weight of the container was subtracted from the final weight to get the dry cell mass concentration.

3.2.7.3 Estimation of rapamycin concentration

Estimation of rapamycin concentration was carried out using HPLC. For HPLC analysis samples were filtered through 0.2 micron syringe filters (nylon microfilters, Axiva). Analysis was carried out by Waters HPLC and 20 µL samples were passed through C18 column (SunFire™ C18, 5µm) and analysed by PDA detector (Waters 2998 Photodiode Array Detector) at 272 nm and computed using Empower Pro software. Solvent system comprised of methanol and acetonitrile in ratio 80:20 and flow rate was kept 1 mL/min [Rao *et al.*, 2011].

Standard plot (Fig 3.7) shows that linear correlation between peak area/height and rapamycin concentration was obtained.

3.2.7.3 Estimation of k_La

Estimation of k_La of the fermentation broth was done by using dynamic gassing out technique using a polarographic DO electrode (Mettler Toledo) based on earlier study [Bandyopadhyay *et al.*, 1967].

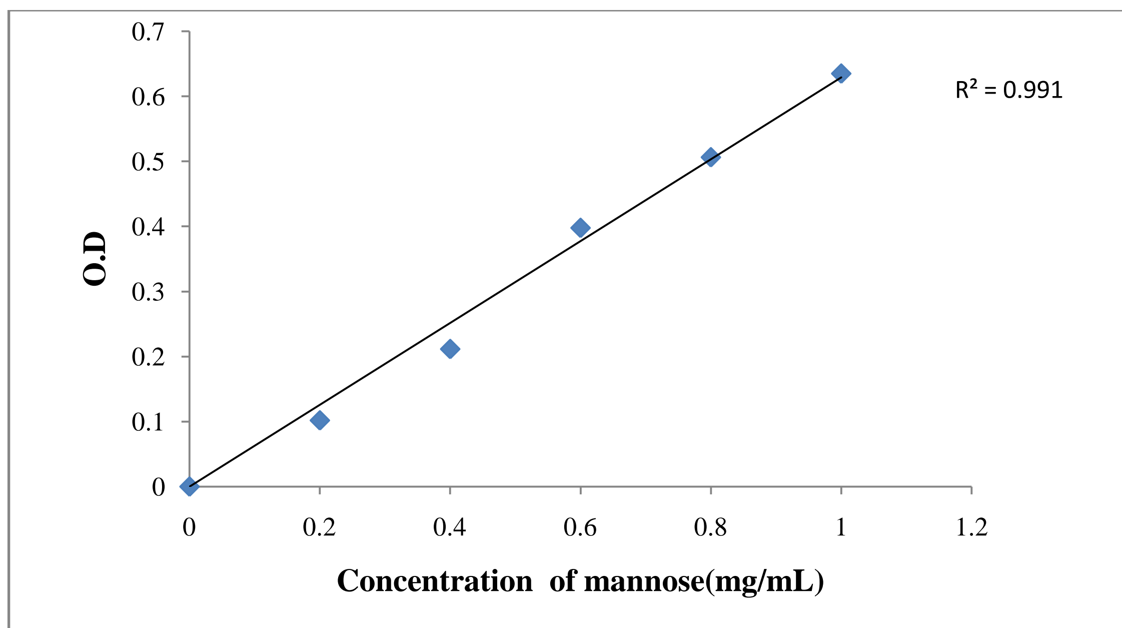


Fig 3.6 Standard curve for reducing sugar estimation using DNS method

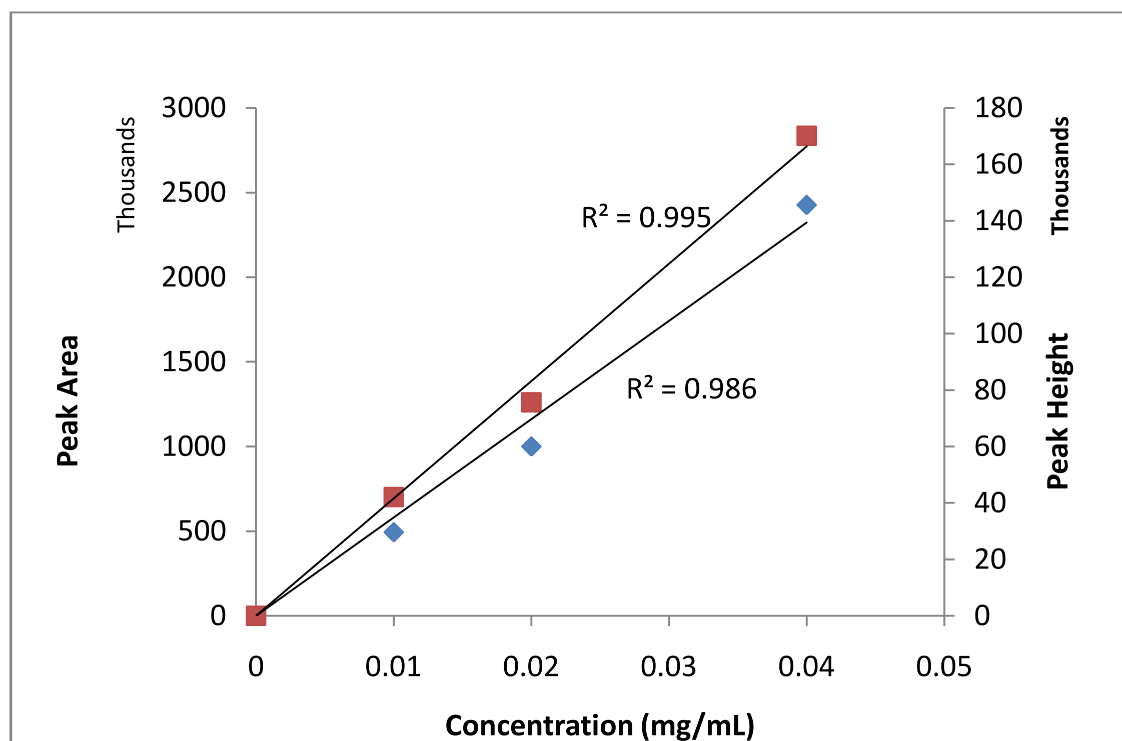


Fig 3.7 Standard HPLC plot based on peak area and height for determination of rapamycin concentration (Peak area: ◊ and Peak height: □)

3.2.7.4 Estimation of broth viscosity

Rheological analysis of the broth was done using Brookfield DVE cylindrical viscometer installed at Department of Pharmaceutical Engineering and Technology, IIT (BHU) Varanasi. Bubbles were removed from the samples and rheological analysis was carried out. Shear rate ($\dot{\gamma}$) and shear stress (τ) were calculated using the following formula (http://www.viscometers.org/PDF/Downloads/More%20Solutions.pdf):

$$\dot{\gamma} = \frac{2\omega\ R_c^2\ R_b^2}{x^2(R_C^2 - R_b^2)} \qquad \text{- (3.2.4)}$$

Where $\dot{\gamma}$ is shear rate, ω is angular velocity of the sample, R_c is radius of the container, R_b is radius of the spindle, x is the radius at which shear rate is determined.

$$\tau = \frac{M}{2\pi R_b^2 L} \qquad \text{- (3.2.5)}$$

Where τ is shear stress, M is input torque and L is effective length of the spindle.

Different rheological models as shown in Table 3.5, were assessed and the fitness of experimental data into these models was determined.

Table 3.5 Different models used for rheological studies

S.No.	Rheological Model	Equation
1	Newtonian	$\tau = \eta * \gamma$
2	Power Law	$\tau = k\gamma^n$
3	Bingham Plastic	$\tau = \tau_o + K_p\gamma$
4	Casson Plastic	$\sqrt{\tau} = \sqrt{\tau_c} + K_c \sqrt{\gamma}$

(η : Newtonian viscosity; k: consistency index; n: flow behavior index; τ_o: Bingham Yield stress; K_p: Bingham viscosity; τ_c: Casson yield; K_c: Casson viscosity)

3.2.7.5 HPTLC analysis

High Performance Thin Layer Chromatography was carried out with Camag Linomat 5 HPTLC system installed at Department of Pharmaceutical Engineering and Technology, IIT (BHU) Varanasi. The software used for data processing was winCATS Planar Chromatography Manager. Sample was applied on 20.0 x 10.0 cm HPTLC plates silica gel 60 F 254 manufactured by E.Merck KGaA. Both standard and samples were run on TLC plate using Methanol: Acetonitrile in ratio 80: 20. Plate was developed in oven at 60°C. Plates were visualised at 254 nm by Camag TLC scanner.

3.2.7.6 Silica column chromatography

Silica gel column chromatography was carried out using silica gel 60-200 mesh size. The column used for chromatography was 45 cm x 3 cm by dimension. The column was washed using 15% acetone in hexane and then 25% acetone in hexane. While the product was eluted using 40% acetone in hexane as mobile phase [Patil *et al.*, 2010].

3.2.7.7 FTIR analysis

FTIR analysis of purified sample and standard rapamycin was carried out using Shimadzu FTIR system and the spectra was obtained which showed the variation of transmittance (%T) with wavenumber (cm^{-1}). Presence of the characteristic peak at a particular wavenumber was correlated to the functional group found in rapamycin.