3. Materials and Methods

3.1 Materials

The materials (microorganisms, equipments, chemicals and media) used in the present study has been discussed in this part.

3.1.1 Microorganism

The Microfungus ***Penicillium brevicompactum*** MTCC 549 used in this work was purchased from Institute of Microbial Technology (IMTECH) Chandigarh, India. The stock culture of *Penicillium brevicompactum* MTCC 549 was grown aseptically on Czapek Dox agar slants and stored at 4 °C. The inoculum was prepared by transferring spores with a sterile loop in Czapek Dox broth and incubated at 28 °C for 3-5 days. The spore suspension was used as an inoculum for shake flask and bioreactor experiments.

3.1.2 Equipments

Autoclave (Khera Instruments, India): Used for sterilization of the media before inoculation and prior to disposal of the fermented /contaminated material.

Distilled Water plant (Infusil, India): Used as water source for media preparation and other experimental procedures.

Digital weighing balance (Danwer Scales): For weighing chemicals (mg level).

Cold centrifuge (Remi C 24 Plus, India): Used for separating the insoluble particles in solution.

Fourier Transform Infrared Spectrometer (Shimadzu, Japan): Used to identify the functional groups present in the mycophenolic acid sample and standard.

Fridge (Whirlpool, India), Deep fridge (Blue star): For storing the heat sensitive chemicals and preservation of culture.

Heater (Bajaj, India): Used for regular heating purpose

High Performance Liquid Chromatography (Waters Alliance, USA): Used for quantitative analysis of mycophenolic acid.

Hot air oven (Ikon instruments): Used for drying glass wares.

Orbital Rotary Shaker Incubator (Remi CIS 24 plus, India): Used for growth of microorganisms at constant temperature.

Laminar air flow cabinet (Ikon instruments): For inoculation under aseptic condition.

Magnetic stirrer (Remi, India): Used for mixing of chemicals.

pH meter (Toshcon industries): Used for pH measurement.

Viscometer (Brookfield, India): For measuring broth viscosity.

UV-Vis Spectrophotometer (Shimadzu, Japan): For routine optical measurements.

Water bath (National Scientific): Used for carrying out experiments at constant temperature.

Bioreactor (BioEngineering KLF, India): Used for production of mycophenolic acid.

3.1.3 Chemicals and Reagents

All chemicals used for different experimental studies, procured from Merck, SRL, HiMedia, India and Sigma Co., USA are listed below:

Acetone, acetonitrile, activated charcoal, agar, alumina, copper sulphate, 3,5-dinitro salicylic acid (DNS), dipotassium hydrogen phosphate, enzymatically hydrolyzed casein, ethanol, ethyl acetate, ferric chloride, ferrous sulphate, formaldehyde, glycerol, glucose, glycine, glycerol, hydrochloric acid, iodine, isopropyl alcohol, kieselguhr (diatomaceous earth), lactophenol, lactose, magnesium sulphate, malt extract, manganese sulphate, methanol, methionine, mycophenolic acid (standard), peptone, potassium chloride, potassium dihydrogen phosphate, potassium ferricyanide, potassium molebdate, silica gel, silicon oil, sodium hydroxide, sodium nitrate, sucrose, sulphuric acid, soybean meal, Trypan blue, Tween 80, yeast extract, zinc sulphate.

3.2 Media

3.2.1 Seed Culture Medium Composition for *Penicillium brevicompactum*

Seed culture was used to inoculate production media. The composition of growth medium are as follows

Constituents		**Quantities**
Sucrose	-	30.0 g/L
$NaNO_3$	-	2.0 g/L
KCl	-	0.5 g/L

$MgSO_4.7H_2O$	-	0.5 g/L
K_2HPO_4	-	1.0 g/L
$FeSO_4.7H_2O$	-	0.01 g/L
Agar	-	15.0 g/L
pH	-	7.3 - 7.5

All chemicals were dissolved in distilled water. The pH of the media was adjusted to 7.3-7.5 using 2 M HCl or 2 M NaOH before sterilization. The fermentation was carried out on a orbital rotary shaker (REMI, INDIA) at 200 rpm and 28 °C for 3-5 days.

3.2.2 Media for Mycophenolic Acid Production

The fermentation culture medium for the production of Mycophenolic Acid had the following composition

Constituents		**Quantities**
Glucose	-	60.0 g/L
Enzymatically Hydrolyzed Casein	-	15.0 g/L
Glycine	-	9.0 g/L
Methionine	-	0.5 g/L
KH_2PO_4	-	5.0 g/L

$MgSO_4.7H_2O$	-	1.0 g/L
Trace element	-	1.0 mL/L
pH	-	5.5 - 6.0

The trace element solution contains

$FeSO_4.7H_2O$	-	2.2 g/L
$CuSO_4.5H_2O$	-	0.3 g/L
$ZnSO_4.7H_2O$	-	2.4 g/L
$MnSO_4.4H_2O$	-	0.16 g/L
$KMoO_4$	-	0.2 g/L

All chemicals were dissolved in distilled water. The pH of the media was maintained at 5.5-6.0 with 2 M HCl or 2 M NaOH. The components of the media except glycine, methionine, and trace elements mixture were separately autoclaved at 121 °C for 15 min. The trace element solution, methionine, and glycine were sterilized by using a sterile 0.2 µm membrane filter (Axiva). The flasks containing production medium were incubated on a orbital rotary shaker at 200 rpm and 28 °C for ten days.

3.2.3 Method of Sterilization

Media sterilization was done by using autoclave at 121 ℃ or 15 psi for 20 min.

3.3 Experimental Set-up for Mycophenolic Acid Production

Mycophenolic Acid production was carried out in a 3.7 L bench-scale bioreactor (Bioengineering) having 2.5 L of working volume. The main specifications of the bench scale bioreactor (Bioengineering) system are as follows:

Impeller used in 3.7 L fermentor for proper mixing of nutrients was bottom driven type, have turbine of 2 mm thickness. The number of impellers used in the fermentor was 2, having 6 numbers of blades in each impeller. A ring-type sparger with 12 holes of 78 mm diameter was used for proper aeration. 4 numbers of baffles of 14 mm width were used in the fermentor. The working volume of the fermentor was 2.5 L.

The controllers for all important parameters such as agitation speed, pH, dissolved oxygen, and temperature were equipped in a bioreactor. A pre-installed software program for fermentation (BioSCADA, flexible software that is adaptable to user requirements) in the system was used for online monitoring and control of the fermentation process. To adjust the dissolved oxygen concentration level of the fermentation broth before the inoculation at 100%, the air was used as the inlet gas at the fermentation temperature. A polarographic type dissolved oxygen probe (Mettler-Toledo, Switzerland) with the replaceable membrane was used to analyze the dissolved oxygen concentration. It is connected to a dissolved oxygen analyzer of the computer-controlled fermentation system. A sterilizable pH electrode (Mettler-Toledo, Switzerland) was used to measure the culture broth's pH value. For the 3.7 L fermentor system, the supplied air, exhaust pipes, and other parts were sterilized by autoclave. A steam-heated jacket sterilized the culture vessel and broth. The photograph of continuous stirred tank bioreactor and its experimental setup is shown in Figure 3.1.

Batch fermentation was carried out in a 3.7 L bioreactor (Bioengineering) having 2.5 L of working volume. The 5% (v/v) of four-day-old culture was used as inoculum. The initial pH of the media was set at 5.5, incubation temperature of bioreactor kept at 28 °C for ten days, and the agitation speed and aeration rate of the bioreactor was 200 rpm, and 2 vvm, respectively. Intermittently, samples were collected aseptically and analyzed for biomass, product, and substrate concentration.

The Fed-batch procedure was carried out using media with an initial glucose concentration of 36 g/L in 3.7 L of continuous stirred tank bioreactor, followed by additions of glucose while maintaining the other variables at a constant level. The medium pH was initially set to 5.5, the incubation temperature of the bioreactor was 28 °C, the agitation rate of the bioreactor was 200 rpm, the aeration rate was 2 vvm, and the fermentation process continued for 12 days. Samples were taken intermittently and examined for cell mass, glucose concentration, and product concentration.

Continuous fermentation process was carried out to study the productivity of mycophenolic acid in the 3.7 L stirred tank bioreactor at a temperature of 28 °C. A two channelled peristaltic pump was equipped with the bioreactor for feeding the substrate and for effluent withdrawal. The substrate feeding was continuously provided with a constant flow rate, and the effluent was removed with the same flow rate. Samples were examined for dry cell weight, substrate concentration, and product concentration.

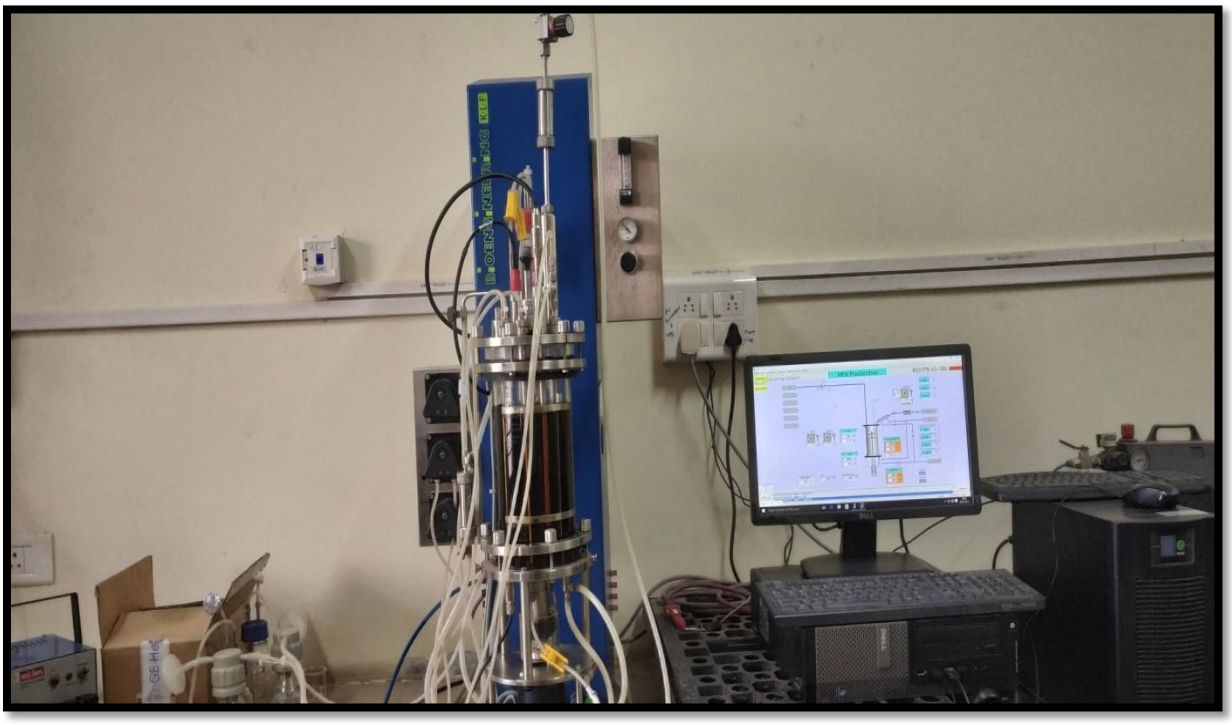

Figure 3.1. Photograph of stirred tank bioreactor and its experimental setup

3.4 Volumetric oxygen transfer coefficient (k_La) determination

The dynamic gassing-out technique was used to determine the k_La according to the method of Garćia Ochoa et al. [Garcia-Ochoa and Gomez, 2009]. A quick responding dissolved oxygen sensor was used to measure the dissolved oxygen. The dissolved oxygen was measured with a membrane covered polarographic oxygen electrode that was heat sterilizable.

Using mass balance the equation can be defined as:

$$\frac{dC_L}{dt} = k_La\ (C^* - C_L)$$

Where C is the concentration of dissolved oxygen at particular time and C^* is the saturated concentration of dissolved oxygen.

The equation for changes in dissolved oxygen concentration could be established:

$$\frac{dC_L}{dt} = k_L a\,(C^* - C_L) - q_{o2}X$$

Where dC_L/dt is the difference between dissolved oxygen concentrations at different times. K_La is the volumetric oxygen transfer coefficient, and C^* and C_L are the saturated dissolved oxygen concentration and dissolved oxygen at different times, respectively. $Q_{o2}X$ is the rate of oxygen consumption. X is cell mass concentration. The k_La values were calculated from the graph (DO vs. Time). Figure 3.2 represents the dissolved oxygen concentration during the dynamic gassing-out technique for the determination of k_La.

When oxygen supply id off, then

$$\frac{dC_L}{dt} = -\,q_{o2}X$$

The term - $q_{o2}X$ in the equation was determined from the decrease of C with time, when the air supply was turned off. Volumetric mass transfer coefficient (K_La) was determined at different aeration rates. The oxygen transfer rate from the gas phase into the broth was calculated according to the following equation:

$OTR = k_La\,(C^* - C)$

Where, OTR is the oxygen transfer rate from gas phase to liquid phase.

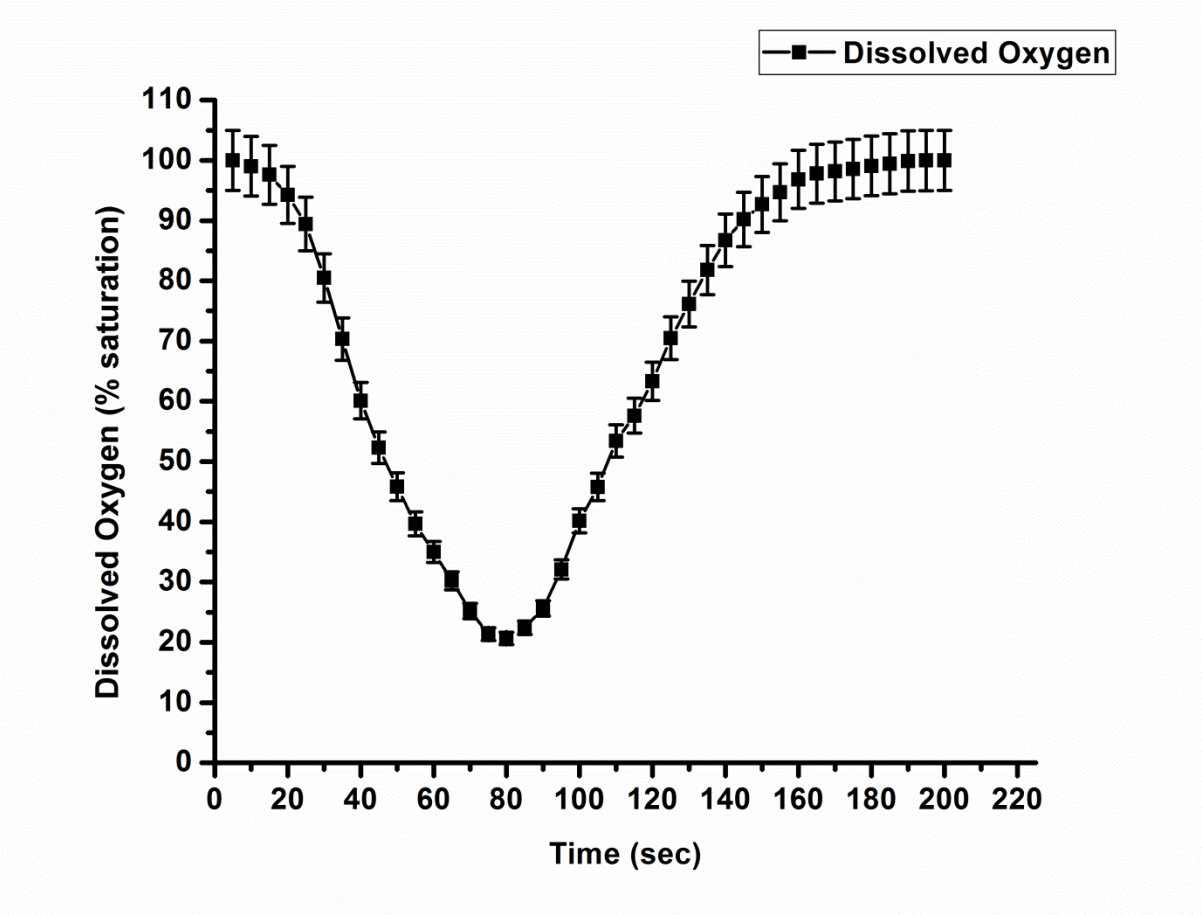

Figure 3.2. Experimental graph obtained during the dynamic gassing-out method for k_La determination

3.5 Microscopic Image Analysis for Morphological Changes

The morphological variations of the collected samples were evaluated using microscopy (Olympus CKX53) through a software program (Magvision) installed in the system. The magnification of the microscope was 40 times. The diluted samples were stained with a small amount (0.8 mL) of lactophenol Trypan Blue (0.1 -0.5% of Trypan Blue dye in Lactophenol), and a drop of about 0.5 mL sample was spread evenly onto a slide.

3.6 Estimation of Broth Viscosity

Viscosity measurements were used to study the physical properties of fermented broth during mycophenolic acid production and to obtain values of consistency index (*K*) and flow behaviour index (*n*). Bubble free samples were collected at regular intervals to perform rheological measurements.

The rheological properties of the samples were measured using Brookfield viscometer. The non-Newtonian behaviour of the microbial cell mass was analyses as per the power-law model. The power-law model described the shear thinning effect of the fermented broth. This model has defined mainly two parameters. The power-law model was expressed in the equation:

$$\tau = K\gamma^n$$

Where τ denotes shear stress, γ is the shear rate, *K* is the consistency index, and *n* is the flow behaviour index. The *K* and *n* characterized the rheology of the power-law model. The *n* value was used to determine the flow characteristic of shear-thinning ($n < 1$) and shear thickening ($n > 1$) behaviour of the fermented broth.

3.7 Analytical Methods

3.7.1 Estimation of Biomass

Dry cell weight was measured by taking 10 mL of fermented broth sample and centrifuged it at 10,000 rpm for 10 minutes. The supernatant was discarded and pellets were washed twice with distilled water.

The centrifuged biomass was transferred to a pre weighed aluminium foil cups and kept it in hot air oven at 90 ℃ for 24 h or until a constant weight was achieved.

The dry cell weight was determined by subtracting the weight of aluminium foil cups form the weight of dried pellets.

3.7.2 Determining of Reducing Sugar by DNS Method

The estimation of glucose concentration was done using the dinitrosalicylic acid (DNS) method described by Miller *et al.* [Miller, 1959]. A sample volume of 1 mL of properly diluted reducing sugar solution was taken in a test tube followed by addition of 3 mL of DNS solution. Then, the tubes were kept in boiling water bath for 5 min at 95 ℃. Immediately after boiling 1 mL of 40% rochelle salt solution was added to the reaction mixture and cooled under ice cold water. Absorbance of each was taken at 540 nm wavelength against the blank prepared by adding 3 mL of DNS solution in 2 mL of distilled water. Amount of reducing sugar was calculated from the standard curve of glucose (1 mg/mL) stock solution.

A standard curve was plotted between optical density at 540 nm versus amount of glucose (mg) in each sample. The standard plot is shown in Figure 3.3. The same procedure was follow to measure glucose in each unknown sample. Amount of glucose in each unknown sample were correspondingly read from the standard plot.

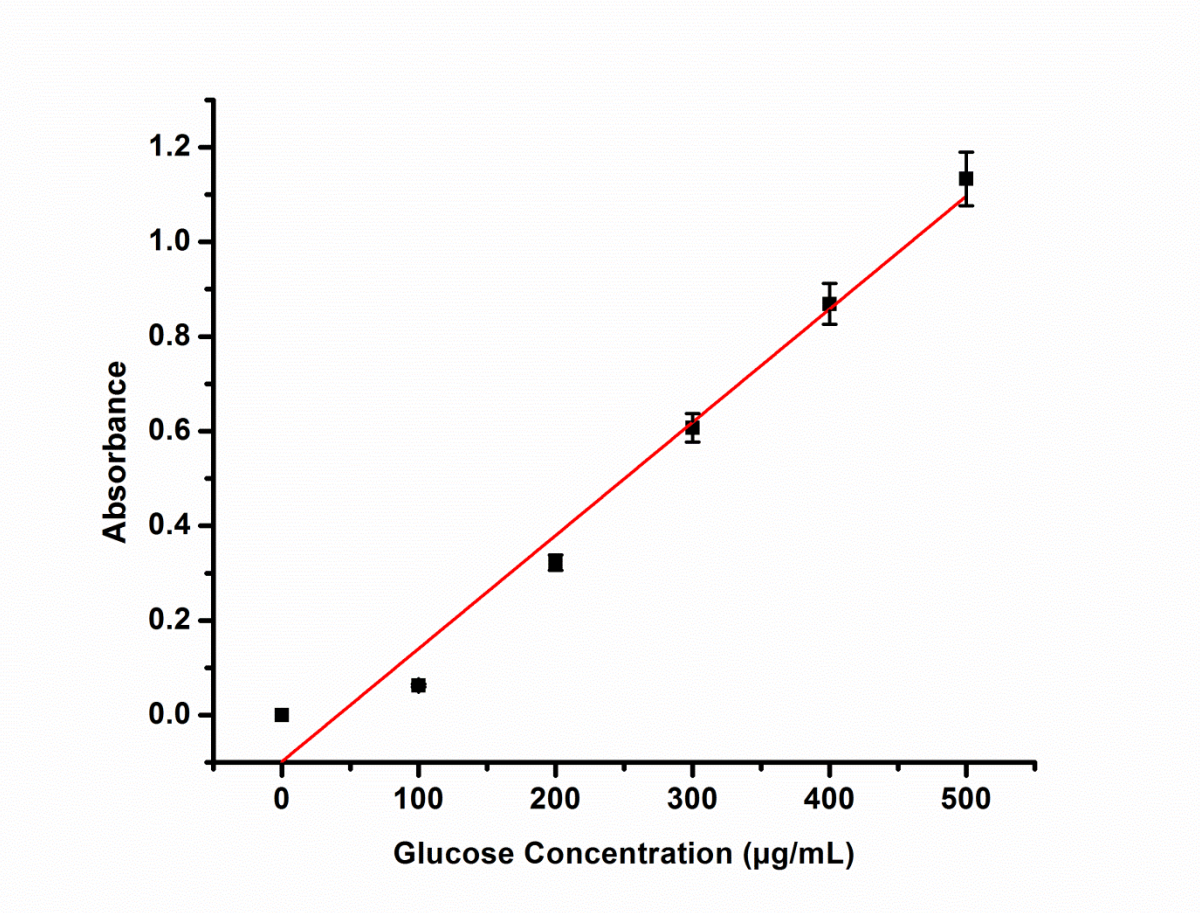

Figure 3.3. Standard plot for reducing sugar estimation by DNS method

3.7.3 Estimation of Mycophenolic Acid Concentration

The standard curve used in this experiment was prepared by HPLC grade authentic MPA (HIMEDIA). The stock solution of MPA (1 mg/mL) was prepared in methanol and stored at -20 °C. The working solution of MPA concentration in the range of 10-100 µg/mL was prepared by serial dilution of the stock solution with methanol. The MPA concentration of samples was measured and evaluated.

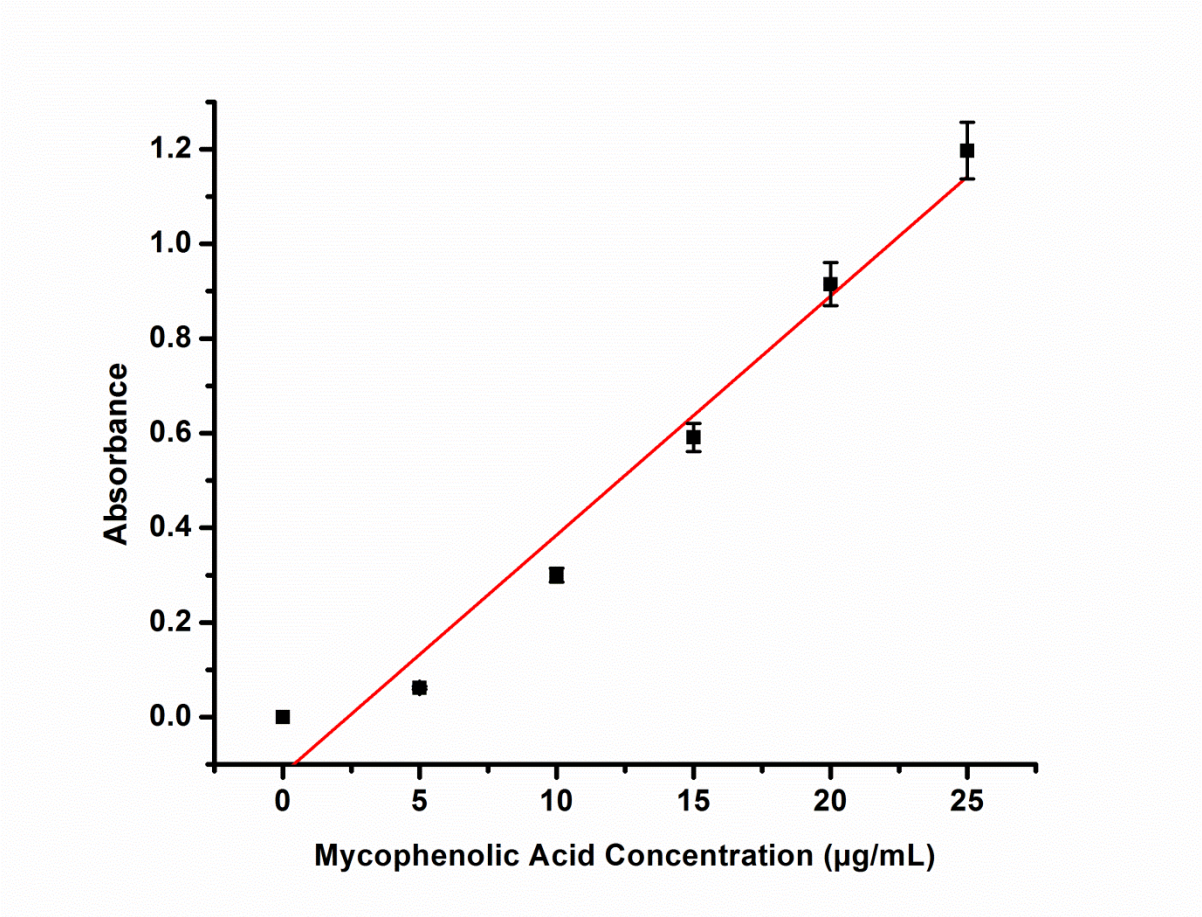

Figure 3.4. Standard plot for estimation of mycophenolic acid.

3.8 Extraction of Mycophenolic Acid

The submerged fermentation was carried out for ten days, following which the crude fermented broth was filtered using a sintered glass vacuum filtration unit and the pH was adjusted to 2 with 2 M H_2SO_4. The mycelia were washed with the organic solvent to recover the mycophenolic acid. The filtrate of every round of extraction was collected and added to the previous liquid.

The crude fermentation broth was taken for solvent extraction processes. The extraction was carried out using different organic solvents to compare the extraction

efficiency for mycophenolic acid. The selection of various solvents was done on the basis of their polarity.

3.9 Column Chromatography

The purification of mycophenolic acid from the fermented broth after solvent extraction was done by column chromatography method. To enhance the amount of purified mycophenolic acid, different columns were used on the basis of polarity. Semi-processed sample of mycophenolic acid was passed through column of the diameter of 3 cm and a length of 50 cm using a peristaltic pump at a speed of 2 mL/min.

3.10 HPLC Study for Mycophenolic Acid Quantification

Estimation and quantification of mycophenolic acid was done by HPLC method. The mycophenolic acid, produced by the fermentation process, was purified and further analyzed using high-performance liquid chromatography (HPLC). The sample was collected and dissolved into 1 mL of methanol, filtered through a 0.45 µm filter and analyze via HPLC (Waters Alliance, USA) with a C_{18} column (5 µm, 4.6×250 mm) at 40 °C column temperature. A photodiode array detector was used at a wavelength of 220 nm for the analysis of mycophenolic acid present in the sample. The method consisted of an isocratic elution mobile phase of water and acetonitrile (50:50 by volume) at pH 3 and a flow rate of 0.5 mL/min. The sample injection volume was 20 µL with run time of 15 min.

The standard curve used in this experiment was prepared by HPLC grade authentic MPA (HIMEDIA). The stock solution of MPA (1 mg/mL) was prepared in methanol and stored at -20 °C. The working solution of MPA concentration in the range of 10-100 µg/mL

was prepared by serial dilution of the stock solution with methanol. The MPA concentration of samples was measured and evaluated.

3.11 FTIR Study for Qualitative Analysis of Mycophenolic Acid

Fourier transform infrared spectroscopy (FTIR) was performed to determine the functional groups and the chemical bonds present in the sample. Samples were prepared for infrared analysis by mixing 1 mg of standard and purified mycophenolic acid with 100 mg of KBr and pressing the mixture into the form of a pellet at 134 MPa for 2-3 min to obtain pellet. The FTIR spectrum of the pellet was collected from 500 cm^{-1} to 4000 cm^{-1} wavenumber by using a FTIR spectrometer with sample dispersed in the pellets of KBr.

3.12 Response surface methodology

Response Surface Methodology (RSM) was developed by Box and collaborators [Gilmour, 2006]. It is a compilation of statistical and mathematical tools extensively used for mounting, improving and optimizing processes based on the fit of a polynomial equation to the experimental data. The most widespread application of RSM is to study the combined results of several input variables and to search best possible setting for a multivariable system. The main goal of response surface is to efficiently hunt for the optimum values of the variables such that the response is maximized. The input variables are called independent variables, i.e. they are subject to the control of the researchers. A combination of factors generating a certain optimum response can be recognized through factorial design as well as RSM.

3.13 Central Composite Designs

In the present study, Central Composite Design (CCD) based on RSM was used to analyze the effects of several parameters on elution of mycophenolic acid. It has been widely used statistical method based on multivariate nonlinear model for the optimization of process variables and also used to determine the regression model equations and operating conditions from the appropriate experiments. All the experiments were performed in triplicates. In coded terms the lowest, central and the highest levels for the variables were -1, 0 and +1 respectively. A total 20 different combinations were chosen in random order according to a CCD configuration for three variables. The variables of the experiments were coded according to the following equation:

$$\text{Coded value} = \frac{\text{Actual Level} - (\text{Higher Level} + \text{Lower Level})/2}{(\text{Higher Level} + \text{Lower Level})/2}$$

The experimental data were analyzed by the response surface regression method to fit the following second order polynomial equation:

$$Y = \beta_o + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \ldots..$$

Where, Y is the predicted response; β_o is the offset term; β_i is the linear effect; β_{ii} is the square effect; β_{ij} is the interaction effect. X_i is *i*th independent variable. The second order polynomial coefficients were calculated using the Minitab software version 16.

The statistical analysis of the model was performed in the form of analysis of variance (ANOVA). This analysis included Fisher's F-test (Overall model significance), its associated probability p(F), correlation coefficient R, determination coefficient R^2 which

measures the goodness of fit of regression model. It also includes Student's t-value for the estimated coefficients and the associated probabilities p(t). For each variable, the quadratic models were represented as surface plots. The optimal combinations were determined from the surface plots.

3.14 Software

The design experiments and date analysis were carried out using Minitab 16 software. The statistical analysis was performed using multiple regressions and ANOVA with the software Minitab 16.