4. Results and Discussion

All the results obtained through experimental studies on mycophenolic acid production were explained in this part of thesis. This section comprises the laboratory and bioreactor studies as well as describes the broth hydrodynamics. It also includes the microbiological aspects of mycophenolic acid production. This part of thesis includes studies on process parameter optimization, kinetic parameters, fungus morphology, broth rheology, hydrodynamics and mycophenolic acid production using different modes of fermentation. The optimization of purification process of mycophenolic acid using different columns and solvent extraction using different solvents also includes in this part. Figure 4.1 represents the schematic diagram of experimental setup.

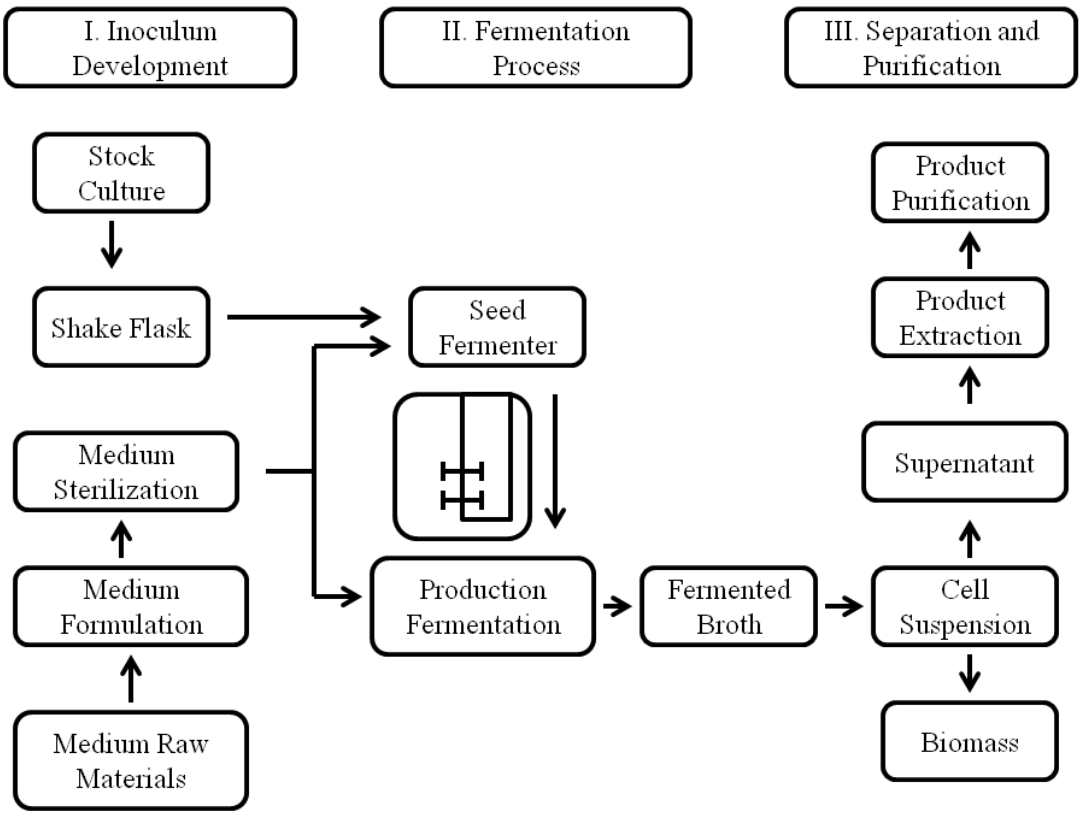

Figure 4.1. Schematic diagram of the experimental setup

4.1 Media Optimization Studies for Mycophenolic Acid Production

Secondary metabolite biosynthesis has been reliant on carbon and nitrogen sources; however the outcomes are uneven and vary depending on the strain and growth conditions.

The biosynthetic production process must be optimized since it provides a summary of the key parameters that must be considered during the growth of organisms for biosynthesis. Various batch runs in Erlenmeyer shake flasks have been performed to optimize the production process of mycophenolic acid. The optimized parameters have been further used for biosynthesis of mycophenolic acid in stirred tank bioreactor.

Different carbon, nitrogen sources and precursors were used for mycophenolic acid production studies. Because these nutrients are closely related with the formation of biomass and metabolites, it is assumed that the culture nutrients carbon and nitrogen sources have a major influence in fermentation productivity. Through catabolic suppression, the concentration of carbon source can control secondary metabolism. Carbon and nitrogen sources are essential for secondary metabolite fermentation; not only must they ensure growth and production, but the economy also influences their selection.

4.1.1 Studies on Carbon Sources

The efficiency of various carbon sources in increasing the mycophenolic acid titre was investigated. Batch fermentations employing diverse carbon sources such as glucose, lactose, sucrose, and glycerol were carried out in triplicate in shake flasks.

Studies found that glucose was rapidly processed among the various carbon sources studied. It was found that glucose was metabolized very speedily under high oxygen consumption and was fully depleted before the biomass could be built up [Patel,

et al., 2016; Sadhukhan, et al., 1999]. The yield of mycophenolic acid with glucose was appreciably high. The yield of mycophenolic acid using other carbon sources was observed lower than glucose. With a higher level of biomass and the maximum cell yield on sugar, glucose appears to be the best choice for cell growth and mycophenolic acid production. Figure 4.2 showed the effect of various carbon sources on mycophenolic acid concentration.

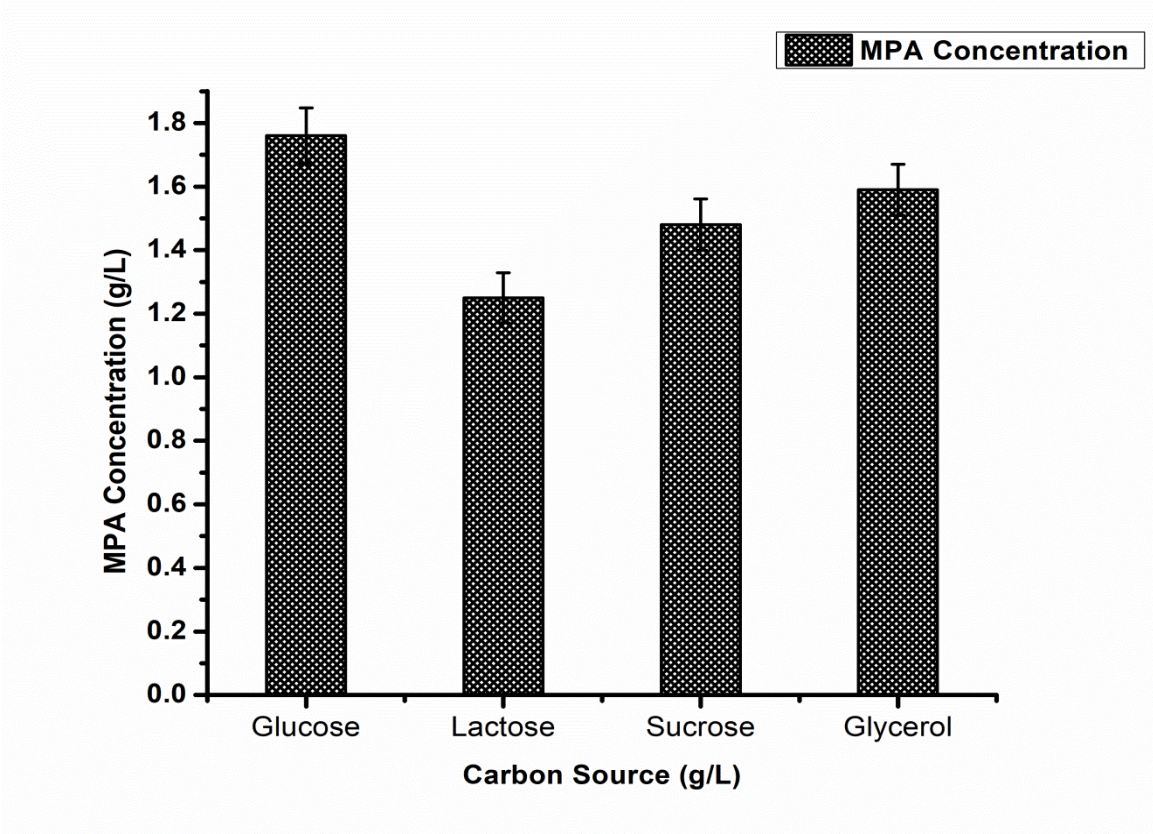

Figure 4.2. Effect of various carbon sources on MPA concentration

4.1.2 Studies on Nitrogen Sources

Another important limiting factor in the regulation of mycophenolic acid production and development is nitrogen availability. As a result, glucose was chosen as the sole carbon source, and a variety of complex nitrogen sources, such as yeast extract,

peptone, malt extract, and soybean meal, were investigated for their impact on fungal growth and mycophenolic acid production.

Various fermentation batches were carried out using various complicated nitrogen sources. Peptone was found to be the most efficient nitrogen source for the biosynthesis of mycophenolic acid, followed by yeast extract and malt extract. The maximal mycophenolic acid titre and a maximal productivity were reached with peptone as nitrogen source [Ismaiel, et al., 2014; Wu, et al., 2022]. Figure 4.3 showed the effect of different nitrogen sources on mycophenolic acid concentration.

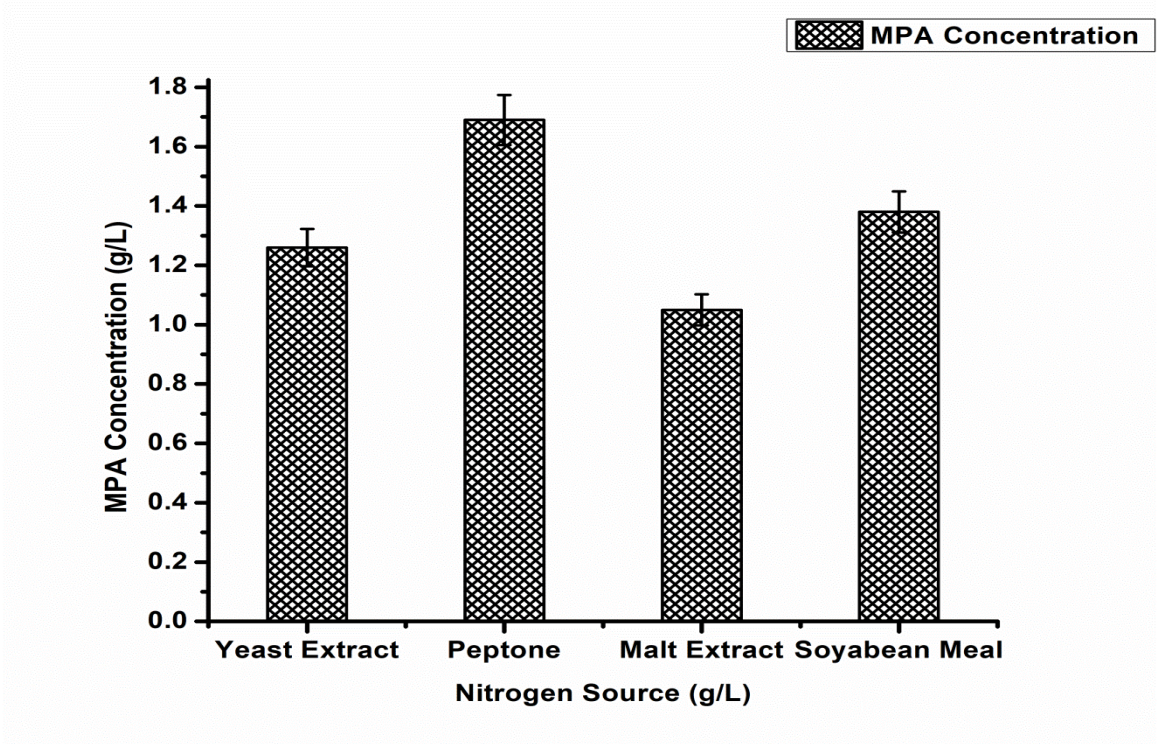

Figure 4.3. Effect of different nitrogen sources on MPA concentration

4.1.3 Studies on Methionine Addition

The impact of methionine supplementation in cultivation media on production of mycophenolic acid was investigated. Methionine was used as a precursor for the production of mycophenolic acid in this work, which was done in triplicate in a shake

flask. The medium was supplemented with methionine at various concentrations ranging from 0.5 to 2.0 g/L.

It was found that as the concentration of methionine increases, the concentration of mycophenolic acid drops. Mycophenolic acid production drops to 0.87 g/L at 2.0 g/L of methionine, which could be related to the inhibitory impact of methionine at higher concentrations. The fact that methionine functions as a precursor for the methylation step in mycophenolic acid biosynthesis may explain the rise in mycophenolic acid production. However, it is unclear whether the increase in mycophenolic acid titres was due to the methyl donor role of methionine or the activation of biosynthetic enzymes that dominated the polyketide pathway in the strain [Patel, et al., 2017; Wu, Li, 2022]. Figure 4.4 shows the effect of different concentrations of methionine on mycophenolic acid production.

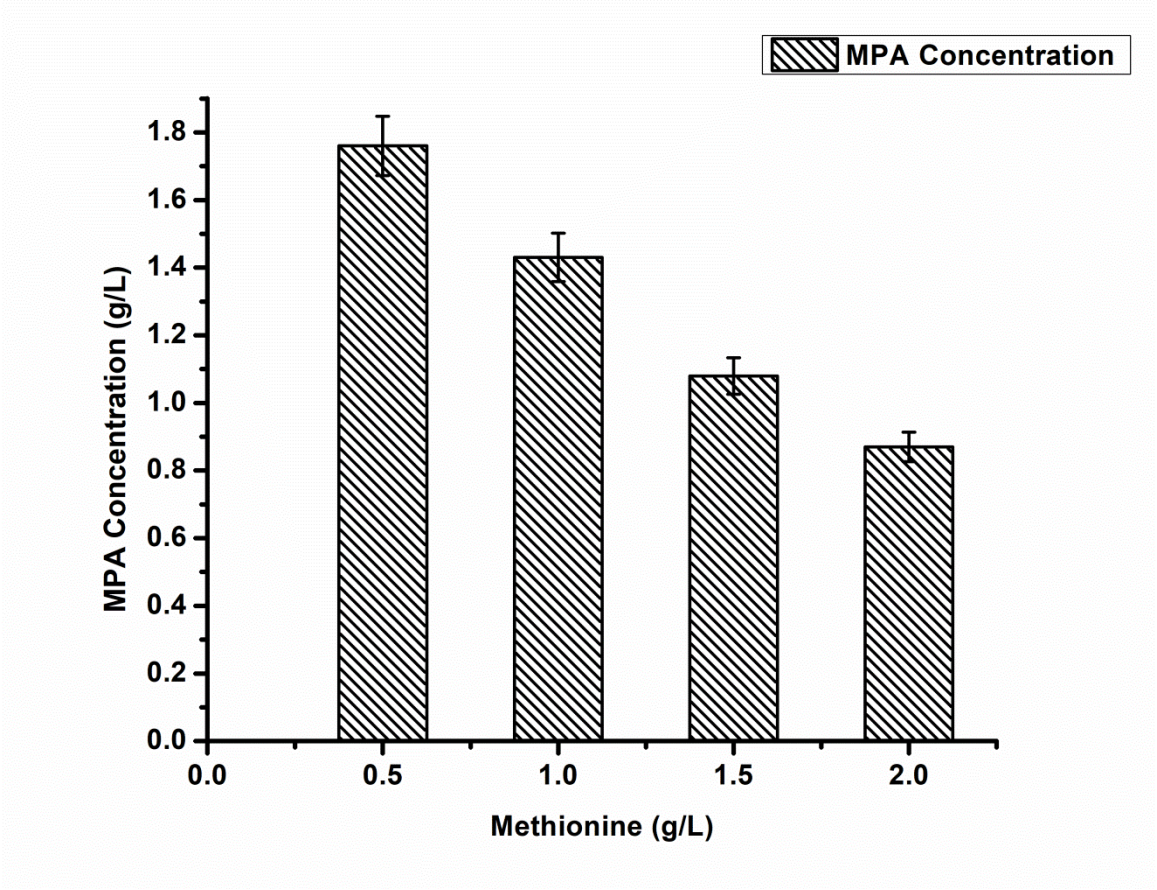

Figure 4.4. Effect of different concentrations of methionine on MPA production

4.1.4 Studies on Glycine Addition

The effect of glycine as a precursor in cultivation media on production of mycophenolic acid was investigated. The medium was supplemented with glycine at various concentrations ranging from 3.0 to 12.0 g/L. It was observed that as the concentration of glycine increases, the concentration of mycophenolic acid also increases. Higher concentration of glycine (12 g/L) caused a drop in mycophenolic acid concentration, which could be showed the inhibitory effect of glycine at higher concentrations [Patel, Patil, 2016; Wu, Li, 2022]. Figure 4.5 represents the effect of different glycine concentrations on mycophenolic acid production.

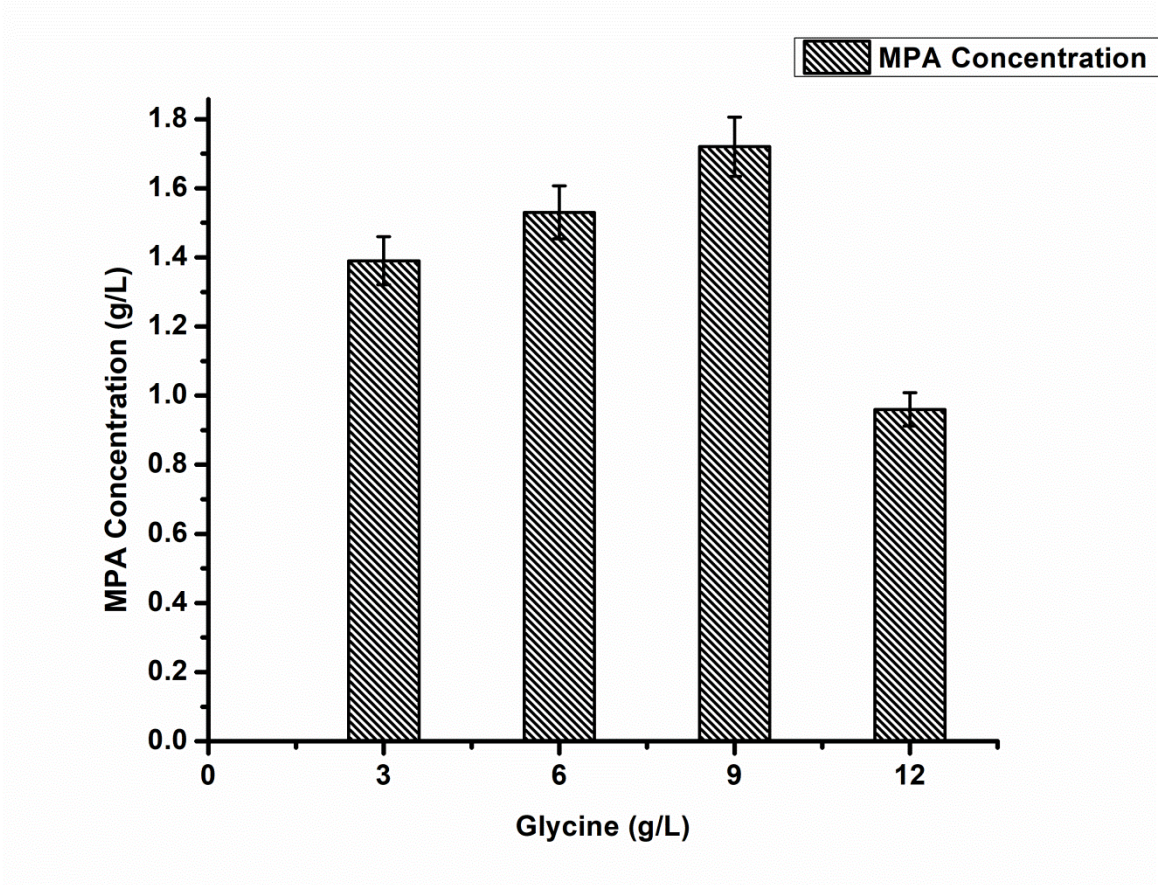

Figure 4.5. Effect of different concentration of glycine on MPA production

4.2 Optimization Studies of Process Parameters for Mycophenolic Acid

Fermentation is a complex bioprocess. Biosynthesis of secondary metabolite has been dependent on the medium components such as carbon, nitrogen, pH and temperature as well as process parameters like agitation rate aeration rate and dissolved oxygen concentration. The results are quite inconsistent and vary with the used strain and growth conditions.

The optimization of biosynthetic production process required to give an overview of important parameters during the cultivation of organism for biosynthesis. In the view of mycophenolic acid production process, various batch runs in Erlenmeyer shake flasks and bioreactor have been done to optimize the mycophenolic acid production process.

It is often difficult to monitor, identify or predict the process behaviour. Based on the available process databases, mathematical equations are used to build powerful models [Pilat, et al., 1976]. These models are useful in the study of microbial growth kinetics [Humphrey and AE, 1979], optimization of process parameters, downstream processing [McNeil and Harvey, 1993] etc. Models have proved their potential use in industry to predict subsequent events. Oxygen transfer is the most important parameter in fungal fermentations. Mathematical models of oxygen transfer during mycophenolic acid production by *Penicillium brevicompactum* while using continuous stirred tank bioreactor and shake flask have been developed [Van Suijdam, et al., 1978; Van Suijdam, et al., 1980].

Mycophenolic Acid production studies were carried out in shake flask and bioreactor using different process parameters.

4.2.1 Effect of Agitation Rate on Mycophenolic Acid Production

Mycophenolic acid production and cell growth studies were investigated at various agitation speeds 100, 150, 200 and 250 rpm on stirred tank bioreactor. The significant changes in fungal morphology were observed with the change in agitation rate (Figure 4.6 and Figure 4.7). The morphological changes exhibit primary effect on product formation in this fungal fermentation process. The rheological behaviour of fermentation broth also changed quite significantly during mycophenolic acid production at different agitation rates. In the fermentation process, the agitation could mainly cause mixing and shear. Agitation in the fermentation process makes oxygen, heat, and mix nutrients thoroughly and be transferred efficiently in the broth. The contact area between gas and liquid was also improved by dispersing the air into small bubbles with the help of agitation [Bandaiphet and Prasertsan, 2006; Feng, et al., 2003].

It was observed that at lower agitation speed of 100 rpm (Figure 4.6), the mycelia grow in the form of clump resulting in increase of viscosity of the fermented broth. Limitations of oxygen occurred when the agitation speed is too slow this causes deficiency of oxygen during fermentation process. It showed that an efficient supply of oxygen was required for mycophenolic acid production. At 100 rpm, where oxygen deficit condition occurred, 21.89 g/L biomass was formed which gave 1.45 g/L mycophenolic acid (Figure 4.7).

At 150 rpm, it was observed that mycelia grow in small hyphae form. The maximum biomass was formed at 150 rpm was 22.99 g/L (Figure 4.6) which gave 1.57 g/L of mycophenolic acid concentration. Figure 4.6 represent the biomass and mycophenolic acid production at 150 rpm.

The highest mycophenolic acid concentration was observed at 200 rpm, which was about 1.73 g/L. The biomass was formed approximately 25.09 g/L (Figure 4.7) at this rpm. During this fermentation process at 200 rpm, morphological characteristic of fungus was closely relatable to the compact and round pellets. Figure 4.6 showed the result at 200 rpm.

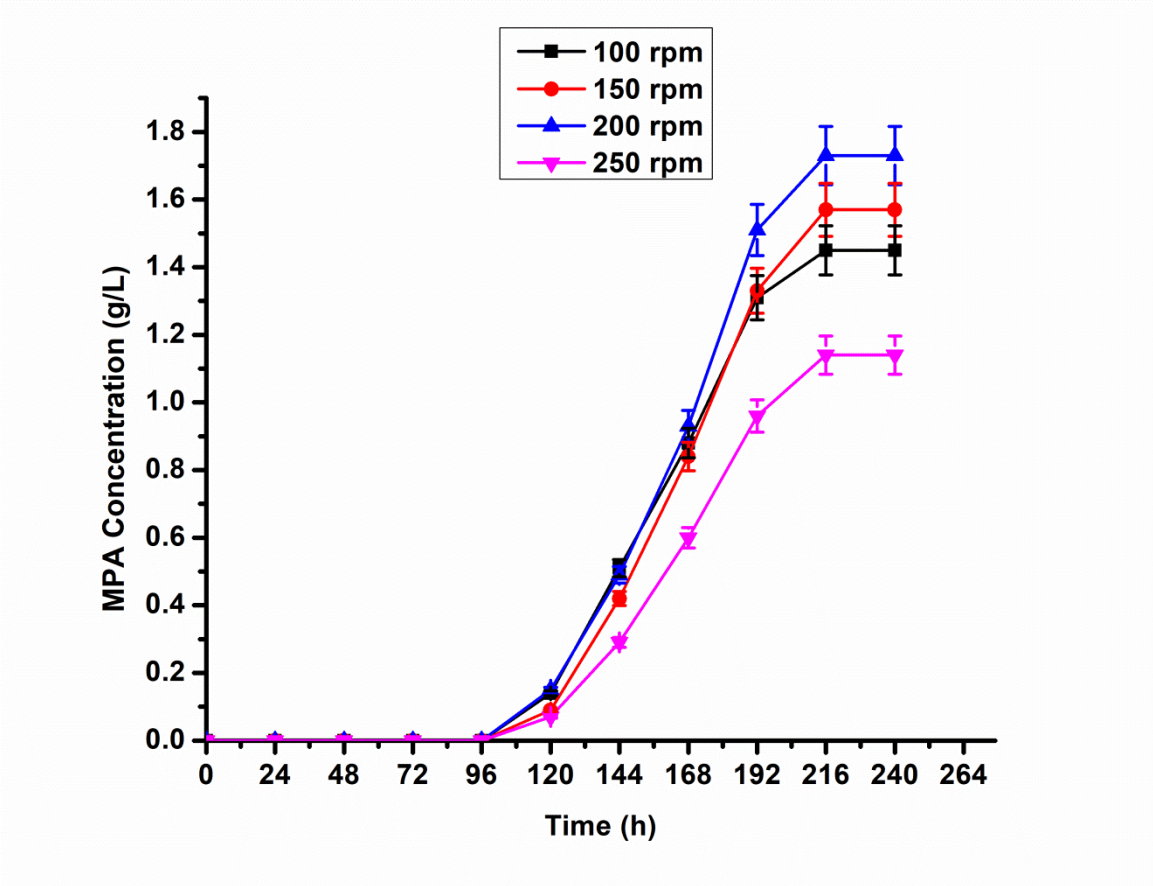

Figure 4.6. Effect of agitation rates (100 rpm - 250 rpm) on mycophenolic acid concentration

When the agitation speed was maintain about 250 rpm, it was observed that dissolved oxygen transfer rate was high but due to high shear rate rupturing of cells occurred during the growth phase. Pellets formed in this condition were rather weak and fluffy, but intrinsic strength of the pellets was high. During this fermentation process at

250 rpm, biomass was formed about 19.75 g/L which produce approximately 1.14 g/L of mycophenolic acid concentration (Figure 4.6 and Figure 4.7).

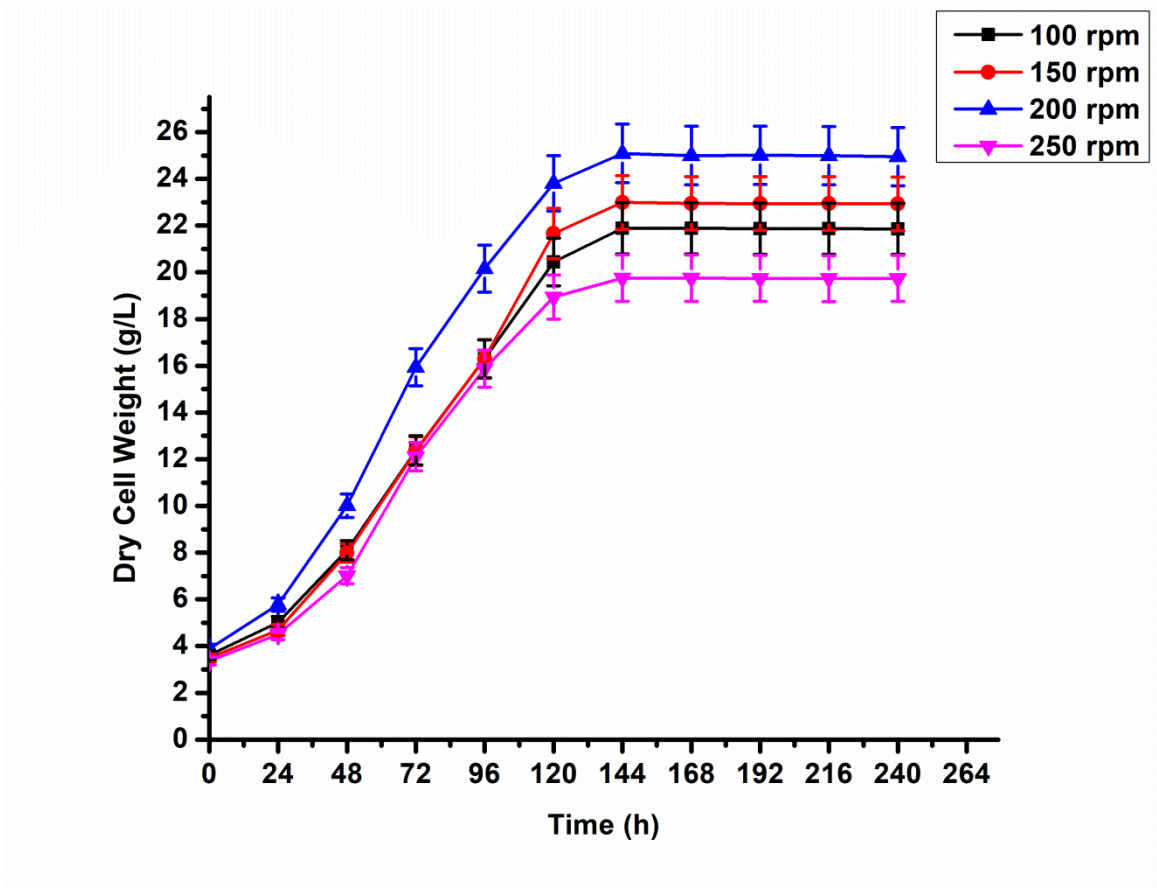

Figure 4.7. Effect of agitation rate (100 rpm - 250 rpm) on biomass concentration

Almost complete consumption of sugar was observed for the culture grown at 250 rpm. Also, it was observed that the number of pellets was accompanied by reduced pellet size. This leads to formation of secondary pellets. This presumably due to the increased oxygen transfer rate, which is a limiting nutrient for fungal growth in pellets form.

These results stated that there is an optimum between morphology and agitation for the fungal fermentation process, where agitation might also be internally regulated with the availability of dissolved oxygen to the cells. It also prevents the mycelia from clustering to favour of oxygen absorption. The high agitation speed increases the power

consumption and creates heterogeneous mixing of nutrients. When the agitation rate is too high it increases shear forces that can damage fragile microorganisms and affect product formation. On the other side, when the agitation speed is too slow, the viscosity of the fermented broth will increase, leading to a reduction in mass transfer efficiency. Agitation in fermentation process interacts with the culturing environments, which affect the product formation [Cui, et al., 1997]. The results represent that a low dissolved oxygen concentration in the fermentation broth strongly inhibits formation of product.

4.2.2 Effect of Aeration Rate on Mycophenolic Acid Production

The effect of aeration rate on the production of mycophenolic acid and cell growth was examined during fermentation process. Aeration rate of the fermentation system was adjusted to 0.5, 1.0, 1.5, and 2.0 vvm using rotameter. Experiments were carried out using *Penicillium brevicompactum* in stirred tank bioreactor. In aerobic fermentation process, oxygen transfer majorly affects the product formation by influencing metabolic activities and pathways. Dissolved oxygen concentration was observed for all aeration rates. It was found that all dissolved oxygen values gradually decreases to a minimum level within the 120 h of fermentation, and then kept constant afterward at 10-40 %. The dissolved oxygen values increased in the stationary phase with an increase in aeration rates but did not depends on the agitation rate. Therefore, oxygen transfer into microbial cells in a fermentor depends on physiology of microorganism and efficiency of bioreactor.

It was observed that at 0.5 vvm aeration speed, proper growth of mycelia was not occurred due to deficiency of oxygen transfer. Autolysis of pellets occurred due to reduction of dissolved oxygen and it forms loose mycelia clump. The maximum biomass was formed about 20.27 g/L which was produced approximately 1.16 g/L of

mycophenolic acid concentration. Figure 4.8 showed the results of fermentation process during production of mycophenolic acid.

In aerobic fermentation process, oxygen transfer plays a vital role in product formation. At 1.0 vvm aeration rate showed in Figure 4.8, 21.25 g/L biomass was formed which was produced about 1.39 g/L of mycophenolic acid.

At 1.5 vvm aeration speed, maximum dry cell weight was formed about 25.99 g/L and 1.69 g/L of mycophenolic acid was produced. Figure 4.8 and figure 4.9 represents biomass and mycophenolic acid concentration.

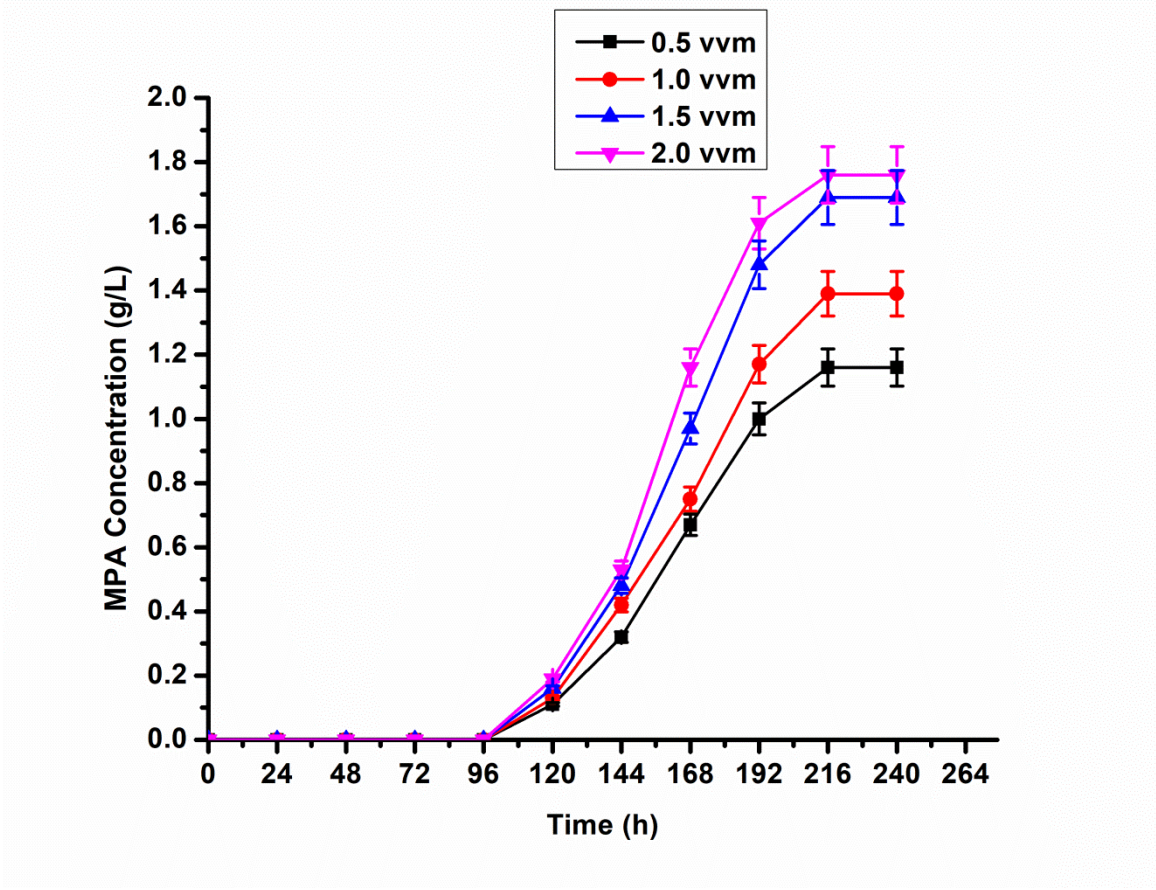

Figure 4.8. Effect of aeration rates (0.5 vvm – 2.0 vvm) on mycophenolic acid concentration

At higher aeration rate 2.0 vvm, rapid mycelia growth occurred due to complete consumption of substrate. During growth phase, free mycelia population was very low, which indicated that pellets were more productive morphological form. Denser pellets were formed due to high dissolved oxygen tension, whereas under very low dissolved oxygen tension weak and fluffy pellets were formed. The maximum biomass was formed about 27.33 g/L which produced approximately 1.76 g/L of mycophenolic acid (Figure 4.9).

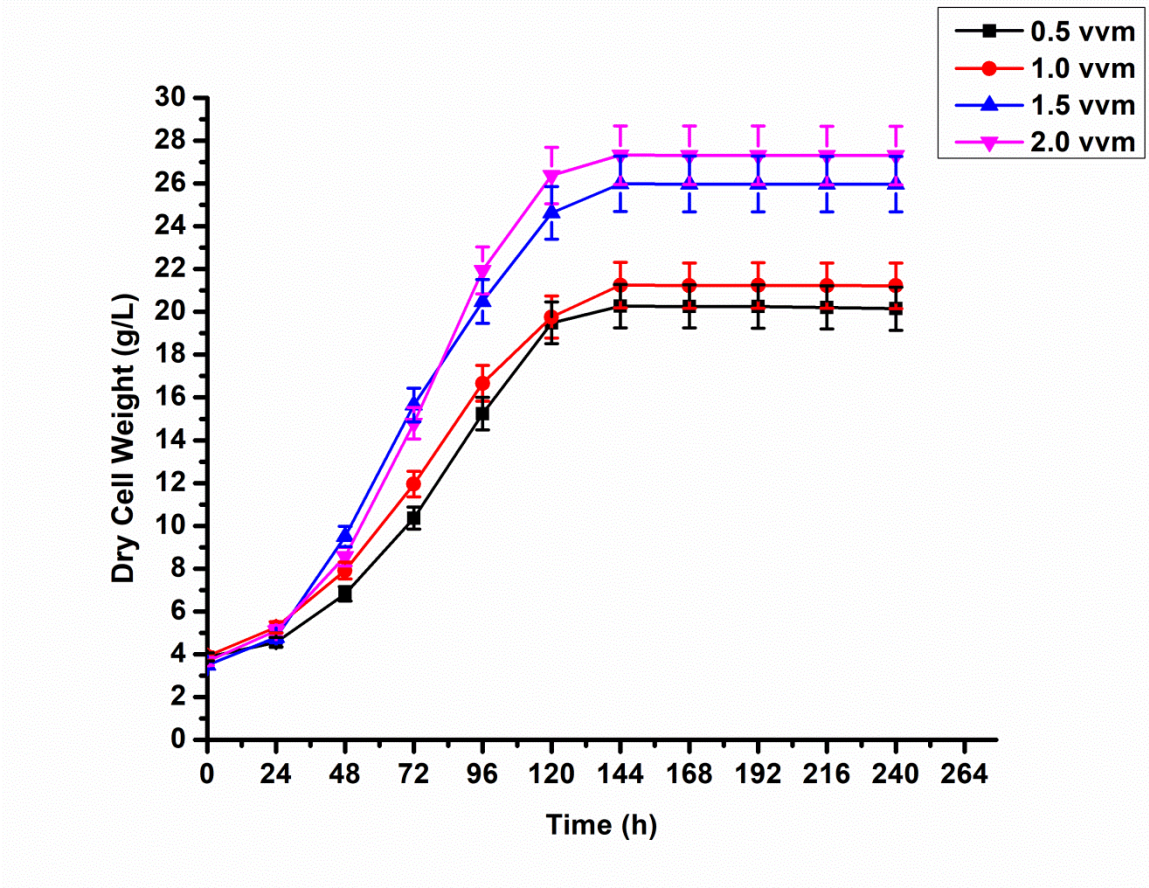

Figure 4.9. Effect of aeration rates (0.5 vvm - 2.0 vvm) on biomass concentration

Aeration in bioreactors supplies the necessary oxygen for cell growth, and it also eliminates the exhaust gases generated during the fermentation process. However, a higher aeration rate results in a reduction in the volume of fermentation broth. In the

aerobic fermentation process, oxygen supply is essential for microorganisms for their growth, but excessive oxygen concentration may toxic for some microbes [Kim, et al., 2003]. The oxygen toxicity situation did not occur in our study since the maximum production of mycophenolic acid 1.76 g/L was achieved at a higher aeration rate of 2.0 vvm.

4.3 Production Kinetics Studies of Mycophenolic Acid using the Batch Fermentation Process

The batch fermentation process was performed to produce mycophenolic acid in a 3.7 L stirred tank bioreactor for 10 days. In batch fermentation, the complete medium was used, as stated before. Figure 4.10 indicates the variation in product formation, dry cell weight, and concentration of the substrate. It was observed that initial growth of *Penicillium brevicompactum* occurs till 144 h wherein glucose is completely exhausted. The maximum dry cell weight 25.94 g/L was obtained at 144 h of fermentation corresponding to biomass yield ($Y_{x/s}$) on glucose of 0.379 g cell mass/g substrate. The maximum consumption of glucose was observed from 24 h to 144 h of the fermentation process, and the growth of the microbial cells increased and reached its maximum at the same time. As the biomass increased, glucose was consumed rapidly. The dissolved oxygen concentration dropped to almost below the critical point about 96 h due to rapid growth and glucose intake, despite the increased agitation speed in attempts to boost the supply of oxygen. After the biomass growth had ceased, MPA concentration started to rise. This confirms that the MPA is a secondary metabolite. The biosynthesis of MPA was mainly initiated from 120 h onwards. Nearly all glucose was consumed at about 240 hours and, thus, oxygen demand decreased, and the dissolved oxygen concentration in the bioreactor started to increase. At the end of fermentation, the maximum MPA concentration of 1.84 g/L was reached.

Mycophenolic acid production rate increases sharply after 144 h and extends till 216 h. the yield of mycophenolic acid on biomass ($Y_{p/s}$) is 0.0321 g mycophenolic acid/ g biomass. The kinetic analysis shows the specific growth rate (μ) was 0.013 h^{-1}. The maintenance energy (m) was calculated about 0.009 g substrate/g biomass/h. The batch fermentation process of MPA production was evaluated. Kinetic parameters were determined by using experimental data for glucose and biomass concentration during the growth phase of *P. brevicompactum* in the bioreactor. The specific growth rate, the specific substrate uptake rate, and specific product formation rate were observed to be 0.013 h^{-1}, 0.057 h^{-1}, and 0.004 h^{-1}, respectively. The yield of MPA on substrate and biomass were found 0.02 g/g substrate and 0.06 g/g biomass, respectively. During the stationary phase of the fermentation process, higher MPA production was obtained when the specific growth rate was constant at a given fermentation time [Krull, et al., 2013]. Table 4.1 showed the obtained value of kinetic parameters.

Table 4.1 Comparison of different kinetic parameters for mycophenolic acid production

S. No.	Parameters	Value
1.	**Maximum Dry Cell Weight (X_m)**	25.94 g/L
2.	**Maximum Production (P_m)**	1.84 g/L
3.	**Specific Growth Rate (μ)**	0.013 h^{-1}
4.	**Maintenance Energy (m)**	0.009 g substrate/g biomass/h
5.	**Yx/s**	0.379 g biomass/g substrate
6.	**Yp/s**	0.0321 g mycophenolic acid/g substrate

7.	**Yp/x**	0.067 g mycophenolic acid/g biomass
8.	**Product formation rate (q_p)**	0.004 h^{-1}
9.	**Substrate uptake rate (q_s)**	0.057 h^{-1}

Batch fermentation was carried out for the production of mycophenolic acid using the fungal strain *P. brevicompactum* MTCC 549. A 3.7 L stirred tank bioreactor containing 2.5 L of production media was inoculated with 5% (v/v) of the freshly prepared seed culture of the above-referred strain which was subcultured several times to give active growing cells. The initial pH of the media was adjusted to 5.5, temperature to 28 °C, agitation speed of the bioreactor at 200 rpm, and the fermentation continued for ten days. Samples were intermittently collected and analyzed for cell biomass, product yield, and residual substrate concentration. The variation in product formation, cell mass, and substrate concentration are shown in Figure 4.10. The concentration of the crude mycophenolic acid product was observed to be 1.84 g/L, which was confirmed using high-performance liquid chromatography (HPLC). The cell growth was observed to increase from 24 h to 144 h during which the consumption of glucose was also observed to be at its maximum. After a period of 144 h, the log phase subsided which marked the beginning of the stationary phase.

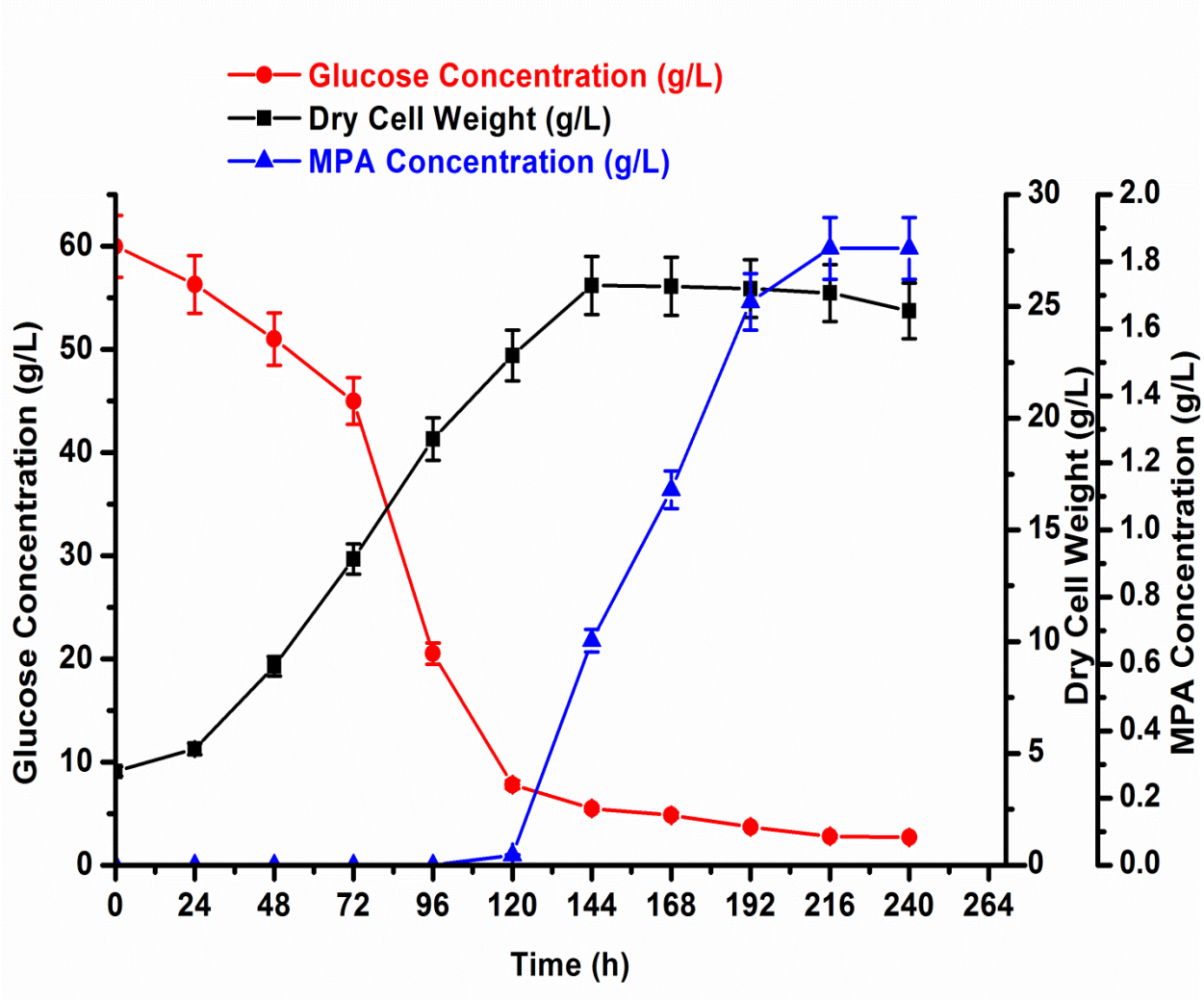

Figure 4.10. Dry cell weight, glucose concentration, and mycophenolic acid production profiles during the growth of *P. brevicompactum* MTCC 549.

The maximum production of MPA was observed after 216 h, as shown in Figure 4.10 MPA production started after 120 h when the culture was in its late log phase. As is well known, most of the secondary metabolites are synthesized when the active growth ceases, and the culture starts entering the stationary phase. The same phenomenon was found for synthesis of MPA in *P. brevicompactum* MTCC 549.

4.4 Study of Morphological Variations during Mycophenolic Acid Production

The present work was performed to study the morphological changes in fungal cells during mycophenolic acid production. Batch fermentation was conducted for mycophenolic acid production in 3.7 L stirred tank bioreactor. Fermentation broth was sampled and morphological variations were measured during 10 days of fermentation period for mycophenolic acid production. At high cell mass concentration, microorganisms grow as long, thin, branched threads of mycelium. The mycelial suspensions constitute viscous non-Newtonian fluid. A cluster of entangled hyphae constitute mycelia that are dispersed discretely in the broth.

4.4.1 Morphological Characterization

Batch fermentation was conducted for 10 days in 3.7 L stirred tank bioreactor. Samples were collected intermittently and analysed during the growth phase of *P. brevicompactum* for cell morphological characteristic changes (Figure 4.11). The early growth phase (trophophase) was followed by a late production phase of mycophenolic acid (Idiophase), which begins after 120 h and lasts until fermentation lasts 240 h. The growth of the fungus ceases in the later fermentation stages.

The morphological changes were examined by microscopy during the growth of *P.brevicompactum*. Typical morphological changes were found to occur in the fermentation broth over ten days. In the early fermentation stage, the filamentous hyphae exist as the growth continues to grow thicker, stouter, and exhibit branching patterns of the 2^{nd}-day hyphae. As well as 3^{rd} day onwards, the thick hyphae formed into swollen hyphal fragments, rapidly increasing the number of tips, shortening the hyphal length, and increasing the thickness of cells. In the fermentation broth, some spherical and swollen arthrospores were also found, and their number began to increase from the 5^{th} day on.

Significant numbers of arthrospores were detected from the 6^{th} day until the 9^{th} day. The remaining hyphae, however, became slim and split into smaller fragments. *P.brevicompactum's* filamentous growth results in extremely viscous fermented broth with non-Newtonian, pseudoplastic flow behaviour [Olsvik and Kristiansen, 1994].

It has been observed that there is an increase in production of mycophenolic acid with the formation of arthrospores, which lasts until the 9^{th} day. It is believed that the productivity of mycelium increases with the rise in the fermentation age of the branching frequency. It was observed that when sufficient substrate is available, an enhancement of branching frequency occurs.

It was found that the early stage of growth, the mean length of freely dispersed mycelia appeared to increase from 24 h to 72 h, indicating that the breakage rate was lower than the growth rate. The breakage rate was higher than the growth rate during the deceleration phase (96 h to 144 h), leading to a decrease in mycelia length. It was shown that the breakage was smaller during the decline phase in comparison to that occurs in the deceleration phase [Makagiansar, et al., 1993].

It was observed that during deceleration phase the breakdown of larger mycelia occurs gradually. Growth was negligible compared with breakage in this period. It was seen that the mycelia are likely to have a distribution of mechanical strength and some of them that survive the imposed shear forces, are physically strong and continue to grow at the end of the breakage period.

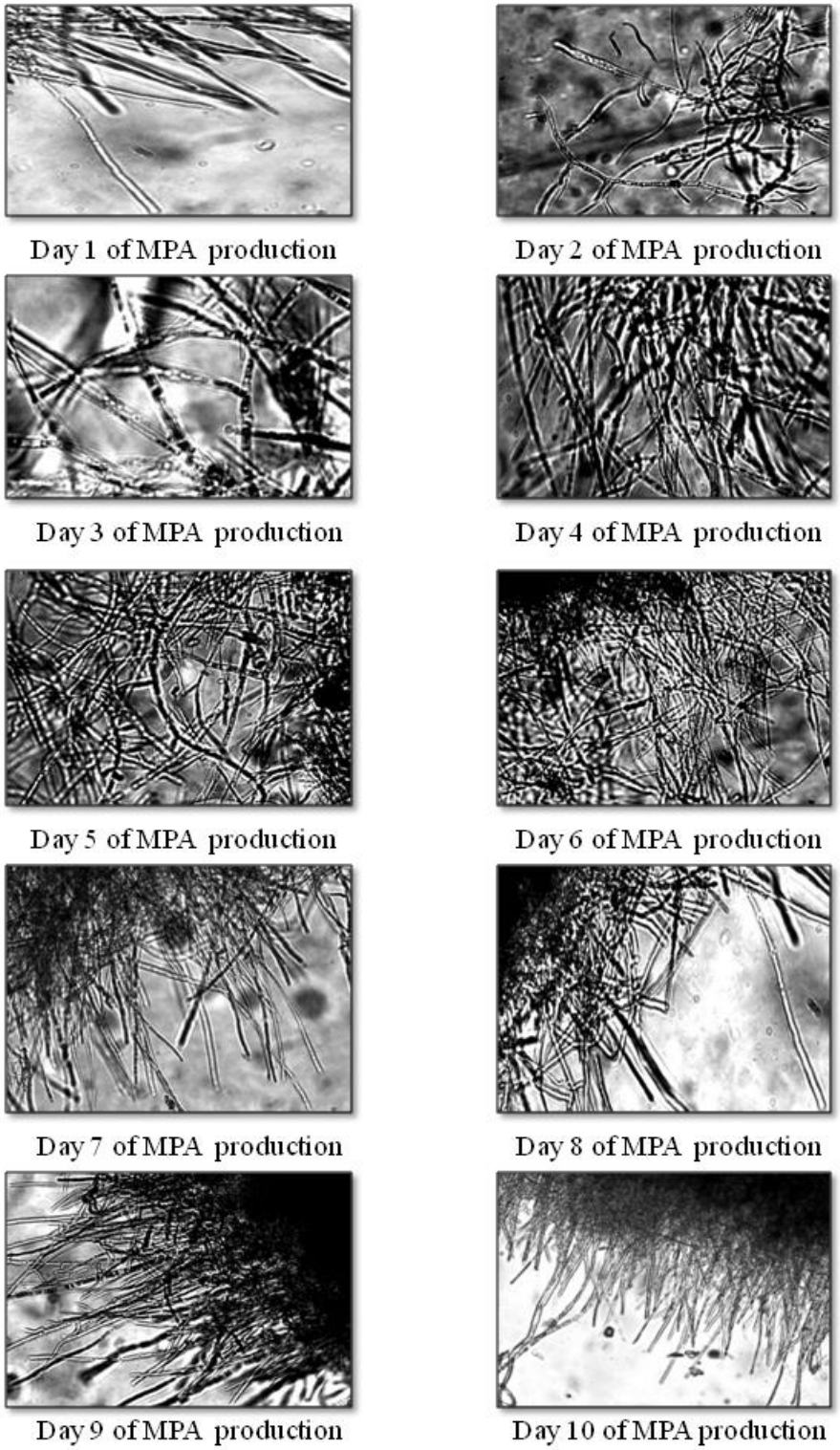

Figure 4.11. Morphological changes during ten days fermentation process for Mycophenolic acid

4.5 Rheological Properties of Fermented Broth during Mycophenolic Acid Production

Batch fermentation of MPA production was carried out, and samples were collected in triplicate for evaluating the cell mass and viscosity of fermented broth. It was observed that fermented broth exhibits a typical pseudoplastic non-Newtonian behaviour and follows the power-law model. The viscosity of the fermented broth increased in the early hours of the fermentation process and reached a maximum at about 144 h. A maximum dry cell weight of approximately 25.99 g/L was found at 144 h. As seen in microscopy, the mycelium changed into a compact structure. The change in the viscosity of the fermented broth with biomass concentration depicts the enhancement of viscosity with morphological differentiation of *P. brevicompactum*. In the later fermentation stages, after 144 h viscosity of the broth was reduced. It may be attributed to the variation of morphological characteristics of the mycelia.

Viscosity variation can be correlated to the change in biomass of the fermented broth. Figure 4.12 shows that the viscosity increased with the increasing biomass content, reaches a maximum of approximately 55.12 cp at 144 h, and then declined. The decrease in viscosity may be attributed to the differentiation of swollen hyphae fragments into arthrospores. The increase in hyphae thickness led to clump formation. Figure 4.12 showed the viscosity and cell mass variation during the production of mycophenolic acid.

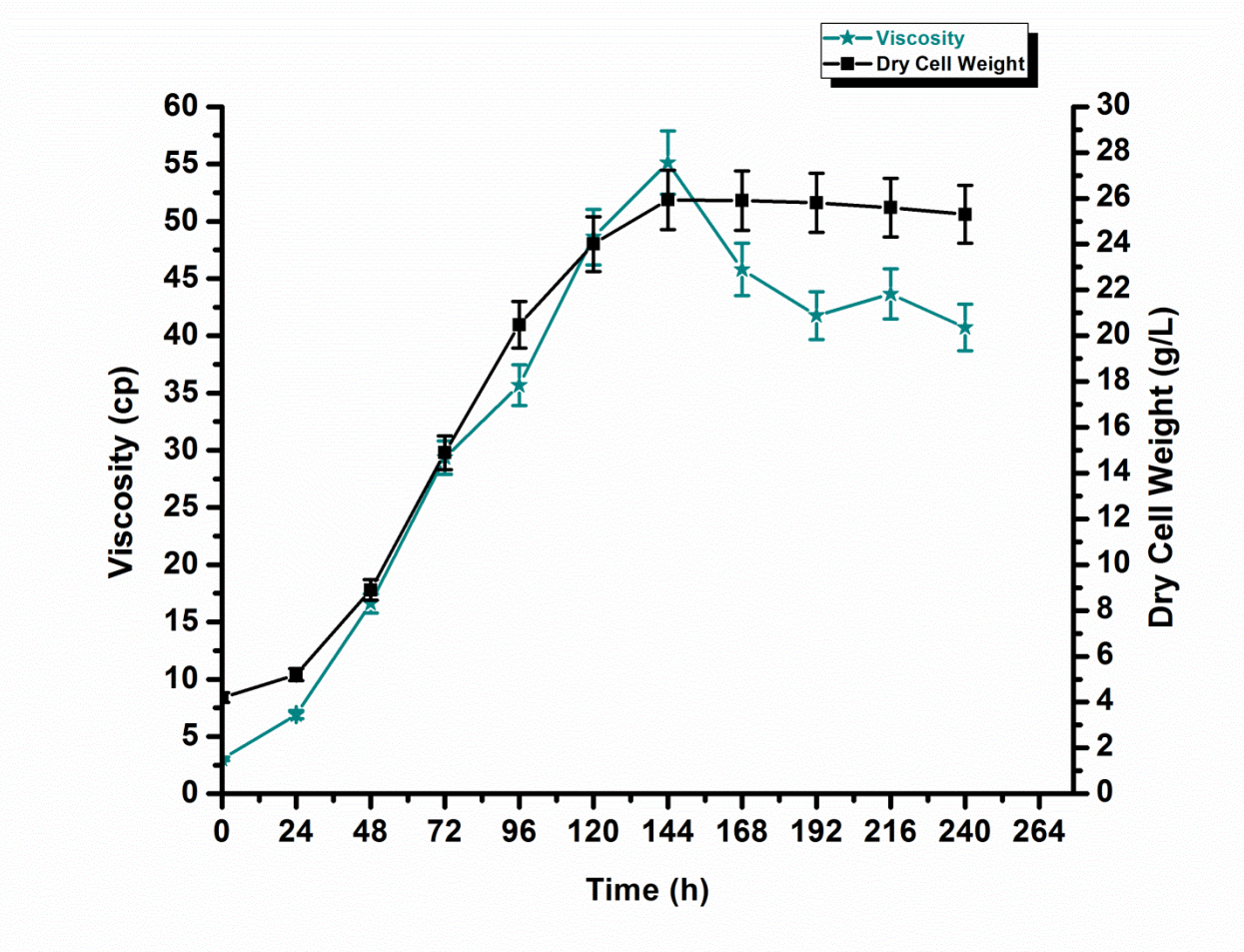

Figure 4.12. Variation of dry cell weight and viscosity with fermentation time

4.5.1 Consistency index (k) and Flow behaviour index (n) of the fermented broth

The rheological patterns of the fermented broth of *P. brevicompactum* exhibit the non-Newtonian behaviour and follow the power-law model. The consistency index (*k*) and flow behaviour index (*n*) of the power-law model was also evaluated. It was observed that *k* initially increases rapidly with the cell mass from 0.023 $Pa.s^n$ at 48 h to 0.067 $Pa.s^n$ at 144 h at 25.99 g/L biomass concentration and the values of *n* were observed to decrease from 0.71 to 0.45. The observed *n* values were less than one ($n < 1$), which depicts that the fermented broth exhibits a typical pseudoplastic non-Newtonian behaviour. The consistency index and flow behaviour index demonstrate that the

fermented broth becomes more shears thinning with time [Patel, et al., 2021]. The consistency index and flow behaviour index of the power-law model were taken into consideration to show the shear-thinning nature of the fermented broth during the fermentation of *Penicillium brevicompactum* (Figure 4.13).

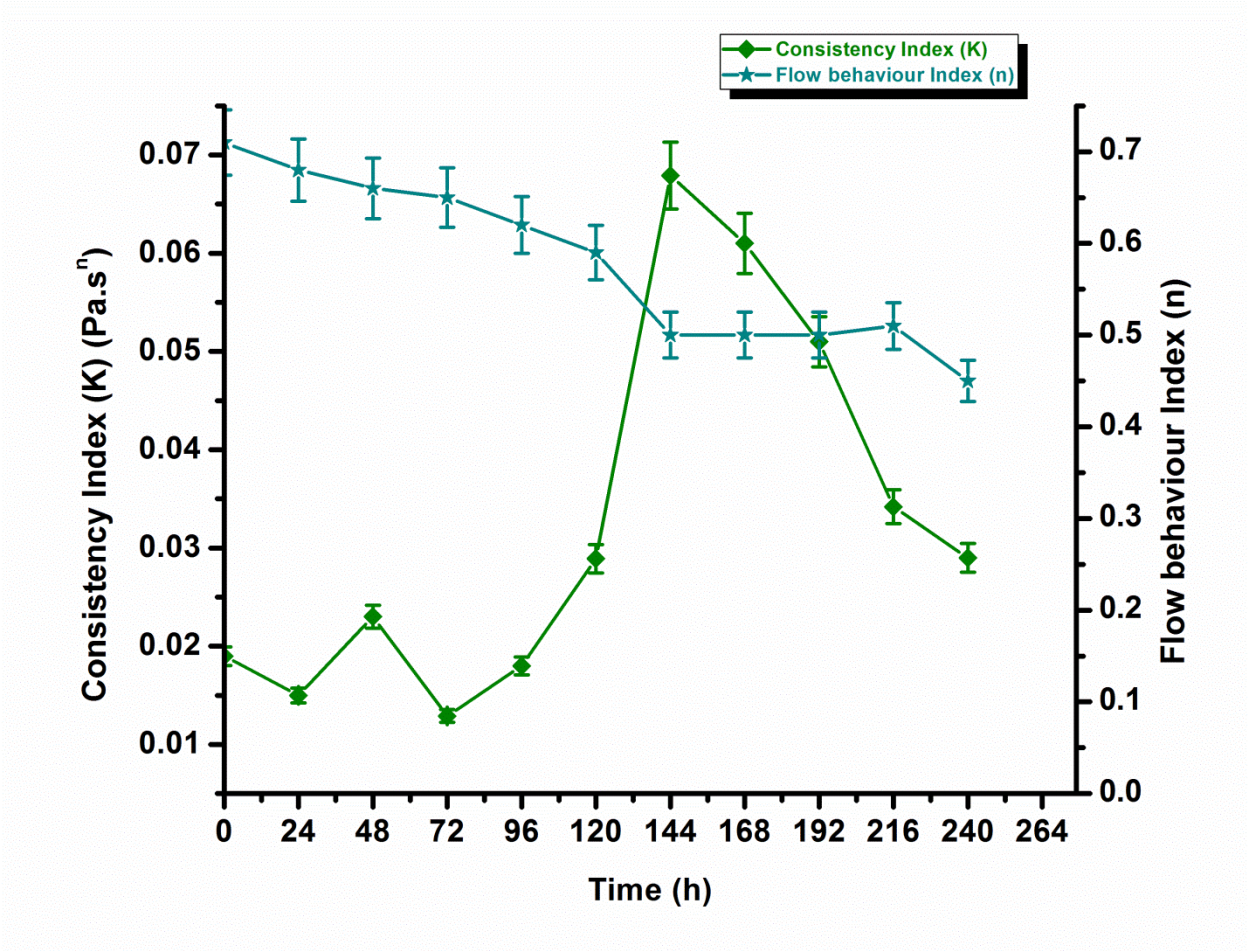

Figure 4.13. Change in consistency index (k) and flow behaviour index (n) with fermentation time

4.6 Oxygen utilization study

4.6.1 Variation of Dissolved Oxygen during Mycophenolic Acid Production

It was observed that during mycophenolic acid fermentation in stirred tank bioreactor, dissolved oxygen concentration gradually decreased with the increase in its time of fermentation process. Dissolved oxygen concentration was adjusted at the set point at fixed aeration rate of 2 vvm. It was observed that due to active cell growth during initial stage of fermentation until 120 h of cultivation, concentration of oxygen gradually dropped. Figure 4.14 represents the variation of dissolved oxygen during mycophenolic acid production.

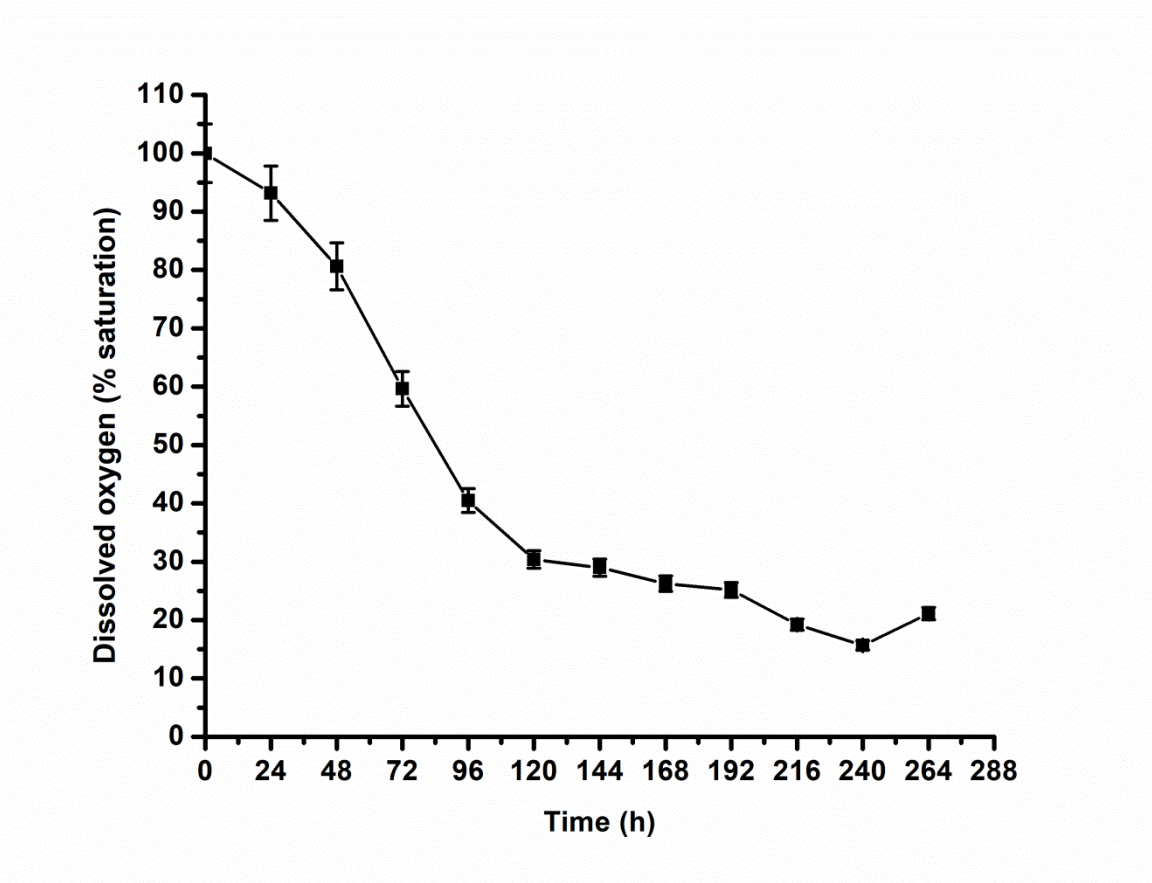

Figure 4.14. Variation of dissolved oxygen during mycophenolic acid production

A sharp decrease in dissolved oxygen concentration was observed up to 40 % at initial stage till 96 h and 15 % at the end of fermentation. This sharp decrease in dissolved oxygen concentration indicates the trophophase of fungal morphology. Oxygen concentration was maintained at 20 to 15 % at the final stage of fermentation using aeration.

4.6.2 Effect of Dissolved Oxygen Concentration on Mycophenolic Acid Production

The effect of dissolved oxygen concentration was studied on cell growth and mycophenolic acid production by *Penicillium brevicompactum* in stirred tank bioreactor. Different dissolved oxygen concentration 30%, 40%, and 50% of air saturation was used to study its effect on mycophenolic acid and cell growth. Regulation of dissolved oxygen concentration was done by adjusting the aeration speed. A piece of study was performed as control experiment without maintaining dissolved oxygen concentration. In control experiment, it was observed that dissolved oxygen concentration dropped gradually with active cell growth from the initial stage of culture and due to oxygen limitation thereafter cell grow slowly. Under control experiment, biomass was formed about 25.94 g/L and mycophenolic acid was produced 1.84 g/L.

When dissolved oxygen concentration was maintained 30% (Figure 4.15), plenty of intertangled mycelia occurred at initial stages to form clumped growth of *Penicillium brevicompactum* that varied largely. The dry cell weight concentration was observed 20.30 g/L (Figure 4.16) and mycophenolic acid concentration was found 1.20 g/L. Cell showed autolysis due to limitation of oxygen in culture broth.

At 40% dissolved oxygen concentration (Figure 4.15), it was observed that uniform size of pellets were formed that significantly increased the mechanism of mycophenolic acid production. At early stages cells grow exponentially till 120 h and

then enter into stationary phase and kept constant till last day of fermentation process with faster substrate depletion until 120 h. It was also observed that demand of oxygen was increased due to active cell growth up to 120 h of fermentation period. The maximum cell mass concentration was observed about 25.66 g/L (Figure 4.16) and maximum mycophenolic acid concentration was obtained 1.61 g/L.

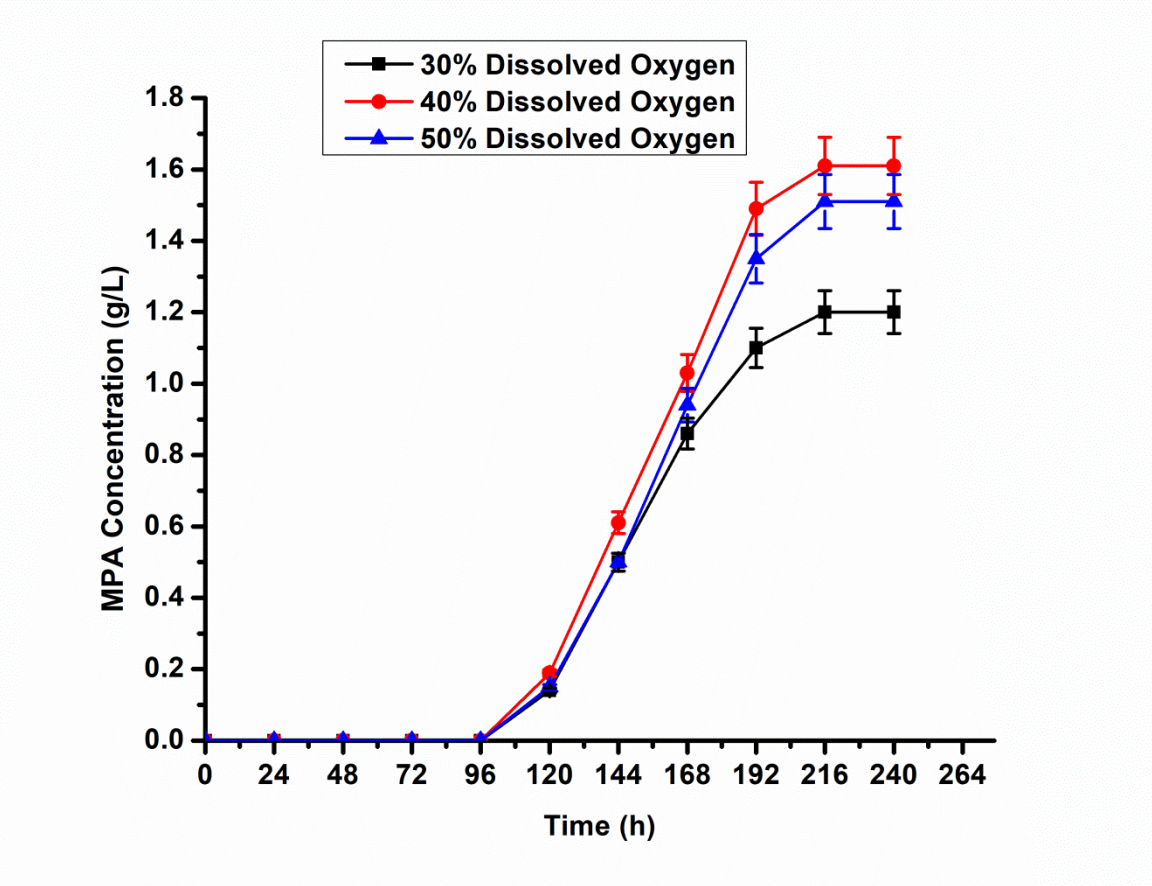

Figure 4.15. Effect of dissolved oxygen concentrations (30 % - 50 %) on mycophenolic acid concentration

When dissolved oxygen concentration was maintained at 50%, cells grew in uncontrolled manner due to no oxygen limitation occurred in culture broth and resulted in unfavourable morphological variations in the pellet formation. At the end of fermentation, pellets become hollow and size of the pellets were comparatively larger.

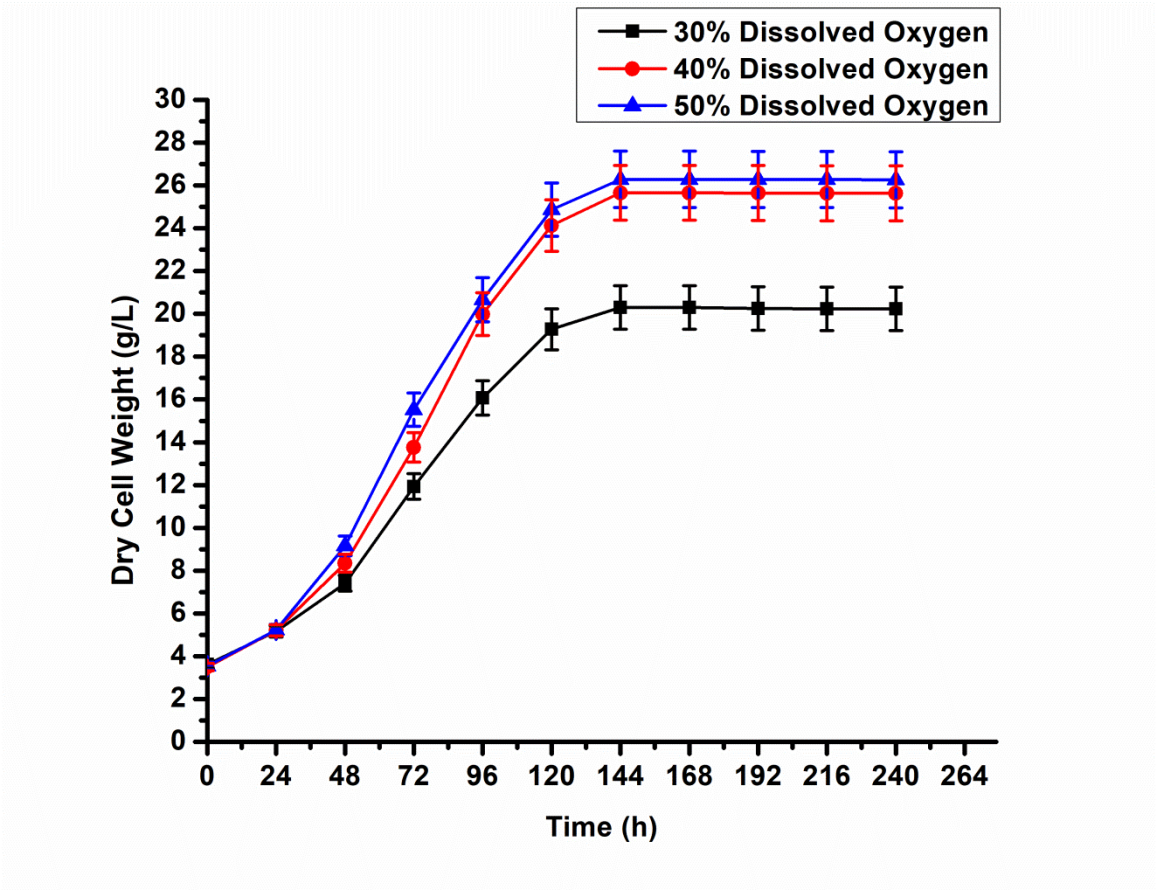

Figure 4.16. Effect of dissolved oxygen concentrations (30 % - 50 %) on biomass concentration

Mycophenolic acid production was started at after 120 h of cultivation, when cell growth enters into stationary phase. The results obtained during fermentation are suggests that proper mixing of nutrient due to aeration is important for production. The dissolved oxygen concentration cause an increased shear force exerted on cells and because of this increased shear force boundary layer on the cell surface becomes thin. It was studied that transfer of more oxygen molecules to the cells occurred because of increased shear force and thinning of boundary layer of cells.

The observations suggest that availability of oxygen has played a critically important role in mycophenolic acid production. It was observed that when dissolved

oxygen concentration increased in fermented broth mycophenolic acid production also increases [Higashiyama, et al., 1999]. The results suggested that adequate supply of dissolved oxygen without affecting pellet formation was essential for maximum mycophenolic acid production. High concentration of dissolved oxygen was beneficial to get maximum cell mass concentration.

4.6.3 Variation in Volumetric Oxygen Mass Transfer Coefficient (k_La) in Fermented Broth with Increasing Aeration rate

Batch fermentation was carried out for mycophenolic acid production in a 3.7 L stirred tank bioreactor for ten days. The oxygen transport phenomena was studied during fermentation process, from the gas phase through liquid phase and then to the microbial cells. The fall in concentration of dissolved oxygen in the fermented broth has been studied in terms of volumetric oxygen mass transfer coefficient (k_La). K_La values were experimentally determined in stirred tank bioreactor using dynamic gassing out method.

This work was performed without maintaining dissolved oxygen concentration as a control experiment, and it was observed that dissolved oxygen concentration dropped rapidly with active cell growth from the early stage of culture, and after that, cells grow slowly as a filamentous culture because of oxygen limitation. It can be seen in the figure 4.17 that dissolved oxygen levels remained at above 30% of the saturated dissolved oxygen concentration during the entire incubation period. This dissolved oxygen environment was necessary because it is well known that the oxygen uptake rate is significantly influenced by the dissolved oxygen concentration of the fermentation broth, especially when it is below a critical level, thus leading to reduced production of secondary metabolites [Ha, et al., 2018].

The volumetric oxygen transfer coefficient (k_La) was evaluated during the production of mycophenolic acid in batch fermentation using two aeration rates of 1 and 2 vvm and it was found that an increase in k_La and oxygen transfer rate as aeration rate increases. The k_La value was observed high (66.08 h^{-1} at 2 vvm) in the initial stage of the fermentation after that decreased due to an increase in viscosity. At 1 vvm aeration rate k_La value was observed about 51.50 h^{-1}, which was 22 % decrease than 2 vvm. In general, for fungal culture, k_La value decreased with viscosity. From the results, it was observed that k_La during late exponential phase can be decreases because of oxygen demand were high at the time of mycophenolic acid production. The decrease in k_La value probably results of the interaction between rigid pellets and oxygen bubbles [Shin, et al., 2013]. Figure 4.17 represents the variation in k_La values during the entire fermentation process.

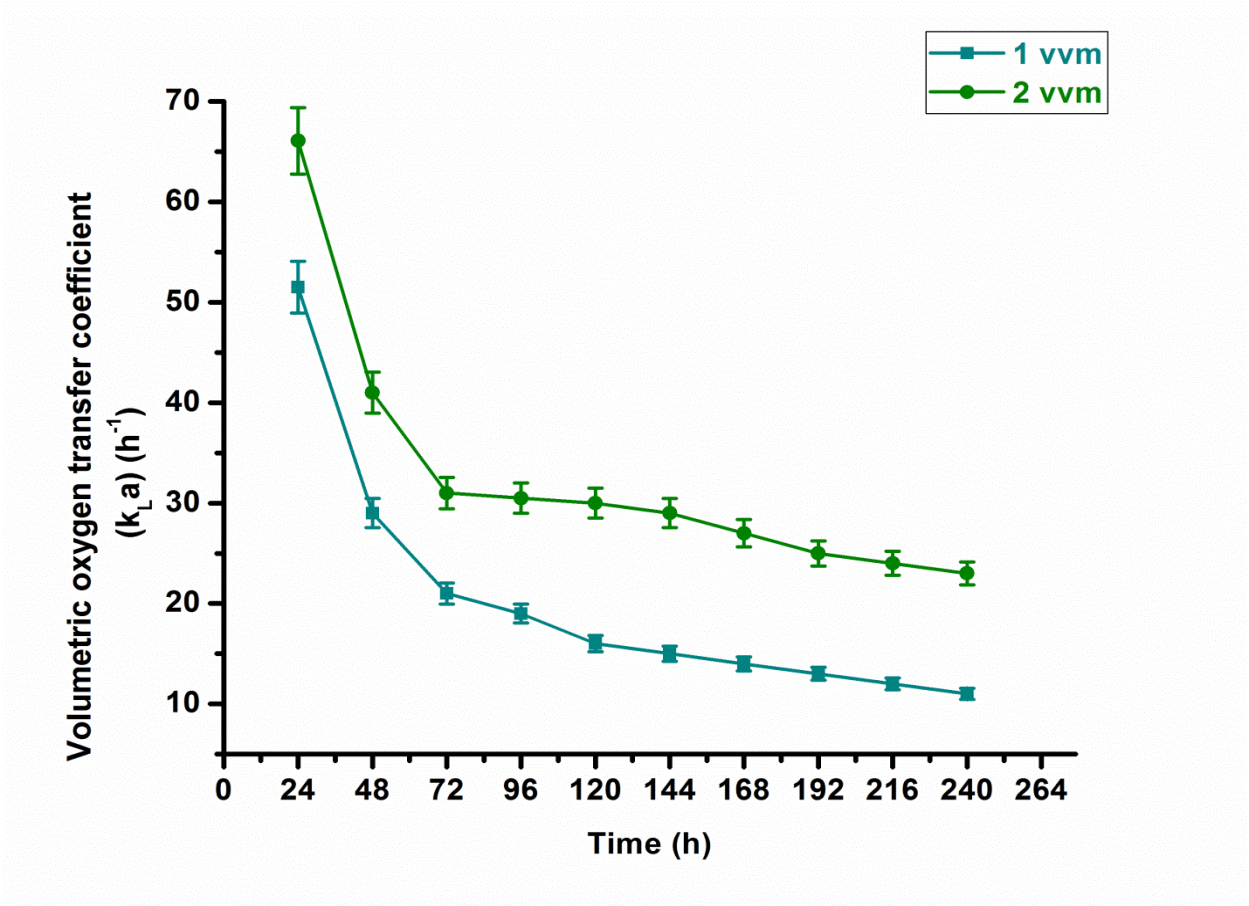

Figure 4.17. Effect of different aeration rate on k_La during mycophenolic acid production

4.7 Production Kinetics Studies of Mycophenolic Acid Production using Fed-batch Fermentation Process

Mycophenolic acid production shows non-growth associated product formation kinetics. In order to assess the productivity of MPA, the fed-batch cultivation was performed. 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 MPA. It was observed that the biomass concentration during the fed-batch fermentation process remained virtually constant and near its maximum value during the entire period due to the addition of the medium. In reference to the earlier batch fermentation experiments, depletion of glucose and the corresponding initiation of Idiophase in the fed-batch run were expected. The addition of the supplementary medium began after the initial fermentation time of 96 h. The feeding component and concentration were determined based on specific uptake rates of different nutrients and maintenance energy requirements. The maintenance energy was observed as 0.009 g substrate/ g cell mass/ h in batch fermentation. Glucose additions were made to 15 g/L at 96 h and 144 h of fermentation-based on this maintenance coefficient and substrate consumption profile. The time-course profiles of cell growth, glucose consumption, dissolved oxygen concentration, and mycophenolic acid production by *Penicillium brevicompactum* in fed-batch mode are shown in Figure 4.18. In the stationary period, i.e., 120 h to 288 h, the highest MPA production rate was observed. The measured maximum mycophenolic acid concentration was 1.91 g/L.

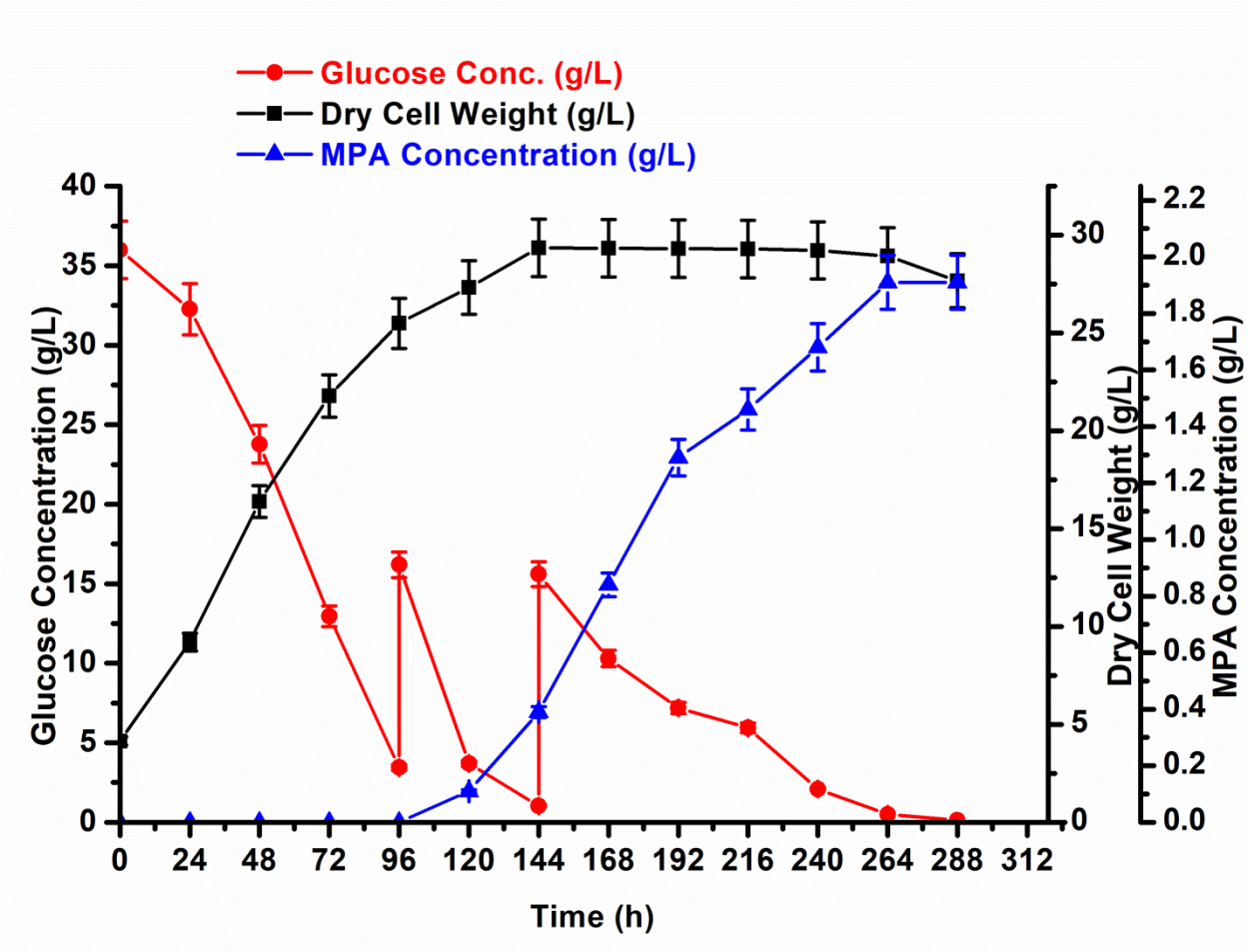

Figure 4.18. Time-course profiles of biomass growth, glucose consumption, dissolved oxygen concentration, and mycophenolic acid concentration by *Penicillium brevicompactum* in fed-batch process.

The time for feed starting was estimated by determining the glucose concentration in the medium. The fed batch production rates are higher than those of the conventional batch process. Intermittent addition of supplementary medium containing glucose resulted in a reduction in catabolite repression. Glucose tends to accumulate slowly in the reaction medium due to limited consumption rate. It was shown to be a much more advantageous strategy than the conventional batch process with glucose. It favoured maintenance of higher productivity during the process. The fed batch fermentation was done on the basis of maintenance energy coefficient. The higher the value of maintenance coefficient, the

more substrate goes towards maintaining the culture without contributing the cell growth [Dmytruk, et al., 2014; Patel, et al., 2018].

4.8 Study of Mycophenolic Acid Production using Continuous Mode of Cultivation

To study the productivity of MPA in the 3.7 L stirred tank bioreactor at a temperature of 28 °C, a continuous fermentation process was carried out. The working volume was 2.5 L in the continuous reactor for cultivation. 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 mycophenolic acid concentration. Initially, the process was run in batch mode for 72 hours and then switched to continuous mode, retaining aseptic conditions. The broth samples were taken every 24 h of interval and evaluated.

In order to verify the possibilities of improving the efficiency of continuous bioconversion, the effect of increasing the dilution rates were investigated during steady state growth in continuous culture. It may be explained that if the dilution rate is varied then the uptake rate of nutrients and production of metabolites also vary.

In stirred tank bioreactor, mycophenolic acid production profile using continuous fermentation mode was showed in Figure 4.19. Dilution rate variations from 0.01 to 0.03 h^{-1} were analyzed for 24 h each. The effect of the dilution rate on MPA production during steady-state growth in continuous culture was studied. Initially, the fermentation process was conducted as batch fermentation, after which when the microbial growth reached a steady state, the batch fermentation was transferred to continuous fermentation mode and after 72 h, and the substrate feeding was started for each and every dilution rate. The

optimum dilution rate was found to be 0.015 h^{-1} for the continuous MPA production process.

The efficient production of metabolites is possible by maximizing the rate of output under given conditions. Figure 4.19 indicates that a reduction in the mycophenolic acid concentration was found with rising dilution rates. The improved productivity of mycophenolic acid from dilution rate 0.01 to 0.015 h^{-1} was observed. A further rise in the rate of dilution has contributed to a decline in productivity. The mycophenolic acid concentration of culture broth decreased at a higher dilution rate than 0.015 h^{-1}. It might be due to the production of mycophenolic acid was a slow process. Also, it may be indicated that mycophenolic acid production follows non-growth associated production kinetics. The results suggested that mycophenolic acid's optimal production can be achieved at a dilution rate of 0.015 h^{-1} in stirred tank bioreactor, and mycophenolic acid concentration was observed to be 1.67 g/L. The optimal productivity of mycophenolic acid was observed to be 0.025 g/L/h. The outcome indicates that the dilution rate should be low to achieve a high mycophenolic acid production rate and yields. At a dilution rate of 0.03 h^{-1}, the cell washout limiting condition prevailed.

It has also been found that a keen deviation in substrate concentration in the fermentation broth occurs at the dilution rate near the washout condition. Continuous fermentation profile suggests that there is subsequent utilization of the unutilized substrate or partially formed products, which could be used for further mycophenolic acid production. In continuous culture, the overall bioreactor productivity was greater than in batch and fed-batch culture.

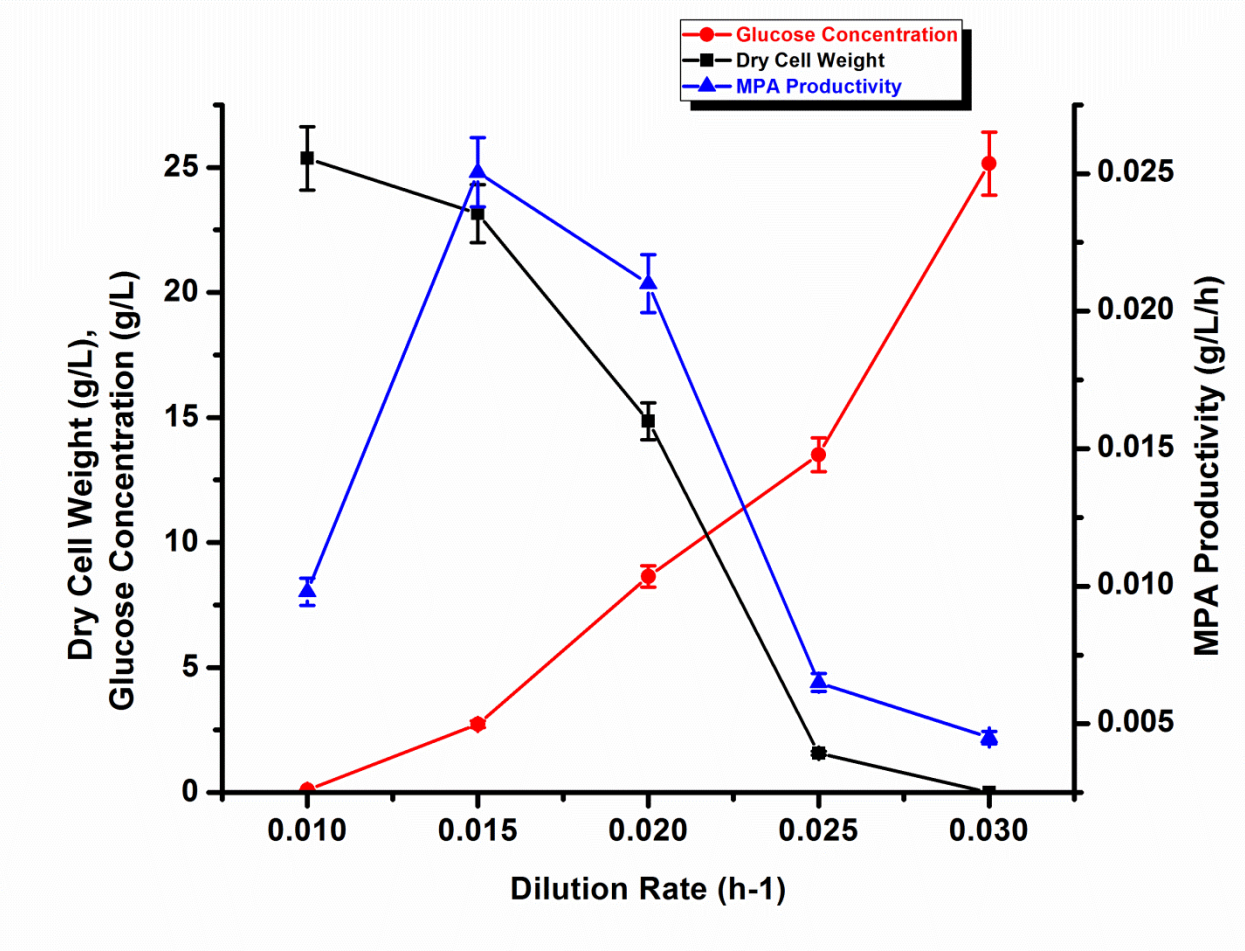

Figure 4.19. Dry cell weight, glucose concentration, and mycophenolic acid concentration during the continuous mode of fermentation of *Penicillium brevicompactum* at different dilution rates.

4.9 Comparison of the Batch, Fed-batch, and Continuous Fermentation Process for Mycophenolic Acid Production

The method leading to MPA production is sequential in the batch mode of mold cultivation, i.e., they exhibit separate physiological phases, i.e., the development period (Trophophase) followed by a production phase (Idiophase). Microbial growth was observed up to 120 h, followed by the production process of the MPA, which lasted until the end of fermentation.

The fed-batch analysis was carried out in a way that calculated the residual glucose levels for the feeding profile. The result obtained from fed-batch fermentation defined optimal production feeding conditions and shows how MPA production was successfully enhanced by first successive fermentation conditions for mycelia and secondary management of feeding conditions without catabolic repression, as may be seen in the feeding of glucose as a substrate.

The continuous mode can be proposed as superior regarding productivity over the other two approaches analysed with the comparative study of MPA production using various modes.

Mycophenolic acid concentration and productivity during MPA production in batch, fed-batch, and continuous cultivation modes are summarized in Table 4.2. Compared to other cultivation types, productivity was found to be the highest in the continuous cultivation process. In fed-batch fermentation, on the other hand, MPA production was observed to be highest.

Table 4.2. Mycophenolic acid concentration and productivity during a batch, fed-batch, and continuous fermentation in 3.7 L stirred tank bioreactor

S. No.	Cultivation Mode	MPA Concentration (g/L)	MPA Productivity (g/L/h)
1.	**Batch**	1.55	0.006
2.	**Fed Batch**	1.91	0.007
3.	**Continuous**	1.67	0.025

4.10 Extraction and Purification of Mycophenolic Acid

The submerged fermentation was carried out for 10 days, following which the crude fermented broth was filtered using a sintered glass vacuum filtration unit and the pH was adjusted to 2 with 2M 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.

4.10.1 Solvent extraction of Mycophenolic Acid from the Fermented Broth

The crude fermentation broth was taken for solvent extraction processes. Various solvents were used for the extraction process. The selection of various solvents was done on the basis of their polarity as defined below:

Ethanol > Isopropyl alcohol > Acetone > ethyl acetate.

MPA is observed to be insoluble in water. Further, the solubility of MPA was observed to increase with the polarity of the solvent. The extraction was carried out using different organic solvents to compare the extraction efficiency for MPA. 100 mL of broth (pH 5.5) was taken in 4 separate flasks and added 200 mL of Isopropyl alcohol, ethyl acetate, acetone and ethanol in each flask then stirred for 3 h at 28 °C and 200 rpm. HPLC confirmed the presence and concentration of MPA in the sample. Table 4.3 depicts the extraction efficiencies of various solvents used. Of all the solvents, Ethyl acetate exhibited the highest extraction efficiency, followed by acetone, isopropyl alcohol, and ethanol. Hence ethyl acetate was chosen for the further purification protocols owing to its high extraction efficiency.

Table 4.3. Extraction Efficiency of Solvents for Mycophenolic Acid

Solvent	Extraction efficiency (%)
Ethyl acetate	95.12
Acetone	89.38
Isopropyl alcohol	73.40
Ethanol	62.49

4.10.2 Purification of Mycophenolic Acid by Column Chromatography

Mycophenolic acid purification from fermented broth was done by using column chromatography technique. Different columns such as Alumina, Silica gel, and Kieselguhr (Diatomaceous Earth) columns were used for purification of mycophenolic acid. Glass columns of length 50 cm having an internal diameter of 3 cm were used, with a length to diameter ratio of 16.67. A flow rate of 2 mL/min was kept for all columns. The length, diameter, and the flow rate were kept similar in all cases. Column dimension is an important factor for separation.

A 100 mL extracted fermentation broth containing MPA was passed through Alumina column with ethyl acetate as the mobile phase. The ethyl acetate extract was concentrated in vacuum and applied to a column stacked with Alumina (mean particle size 45 µm). The column was initially equilibrated with 100 mL of ethyl acetate, to which the extracted broth was added and eluted with ethyl acetate. The eluted fractions were concentrated in vacuum and dissolved in methanol for HPLC. The isocratic elution

technique was used throughout the process. The analysis of the collected sample was done through the HPLC. Elution of MPA by the alumina column was obtained about 79.38%.

A similar protocol was followed when adding the extract to a prepared silica column (particle size 40-63 µm), with Ethyl Acetate as the mobile phase. The analysis of the collected sample was done through the HPLC. Elution of MPA in the silica column was obtained about 68.70%.

Similarly, 100 mL of extract containing MPA was passed through kieselguhr column (particle size 35 µm), with ethyl acetate as the mobile phase. The MPA was evaluated by using HPLC, and elution of MPA was observed to be 73.28%. Figure 4.20 shows the elution of MPA in different columns.

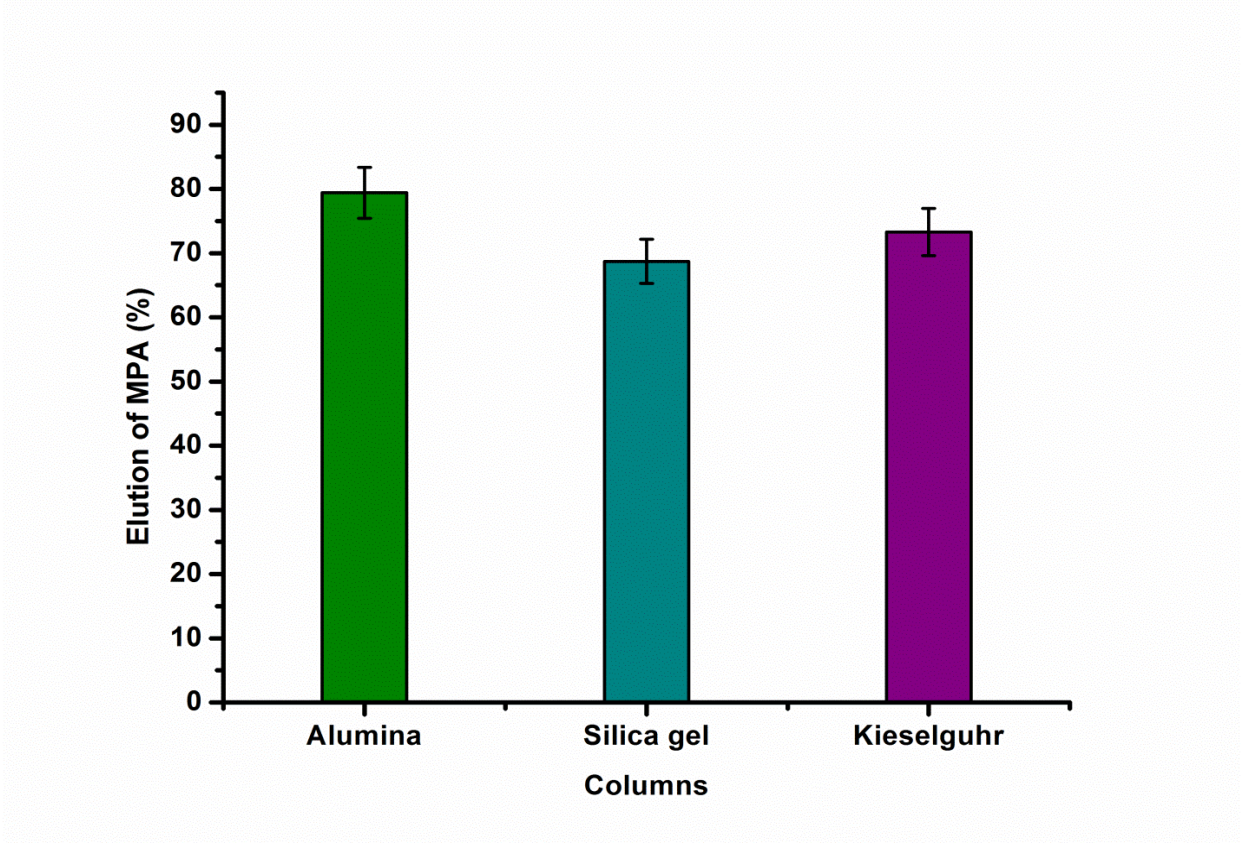

Figure 4.20. Elution of MPA in Alumina, Silica gel, and Kieselguhr columns.

Mycophenolic acid may be recovered from the solution by crystallization. Before crystallization, the MPA containing solution was concentrated by evaporation carried out under ambient or reduced pressure, or alternatively via heating at reflux temperature. Preferably, the solution was concentrated by distillation in a rotary vacuum evaporator at a temperature of 60 °C for 2 h. The solution was concentrated to the extent that cream-colored crystals along with oil-like droplets were found sticking to the glass vessel. Crystallization of mycophenolic acid from the extracted solution was preferably carried out by cooling. The crystallization from solvents such as ethyl acetate was done by cooling the solution to a temperature of about 0 °C to about -10 °C. The crystals were collected and dissolved in hot toluene. The clear solution was crystallized by cooling to about -5 °C. The MPA yield was confirmed by HPLC analysis.

4.10.3 Factors Affecting Mycophenolic Acid Purification in Column chromatography

Alumina column was used for further optimization of different factors affecting elution of MPA. Factors such as pH, the flow rate of the mobile phase, and the volume of eluent were used for the optimization process.

4.10.3.1 Effect of pH during Purification of Mycophenolic Acid

Experiments were conducted to evaluate the effect of pH in the purification of MPA. The fermented broth was adjusted in the pH range of 3 to 9 using H_2SO_4 and NaOH solutions and was passed through the standardized column of alumina. The eluents were concentrated and later sent for HPLC analysis. It was observed that when the pH was increased from 3 to 6, the MPA purification yield also increased and was observed to be maximum at pH 6.0. The alumina column yielded a maximum elution of MPA 79.38% at pH 6.0. The concentration of MPA in eluents at different pH is shown in Figure 4.21.

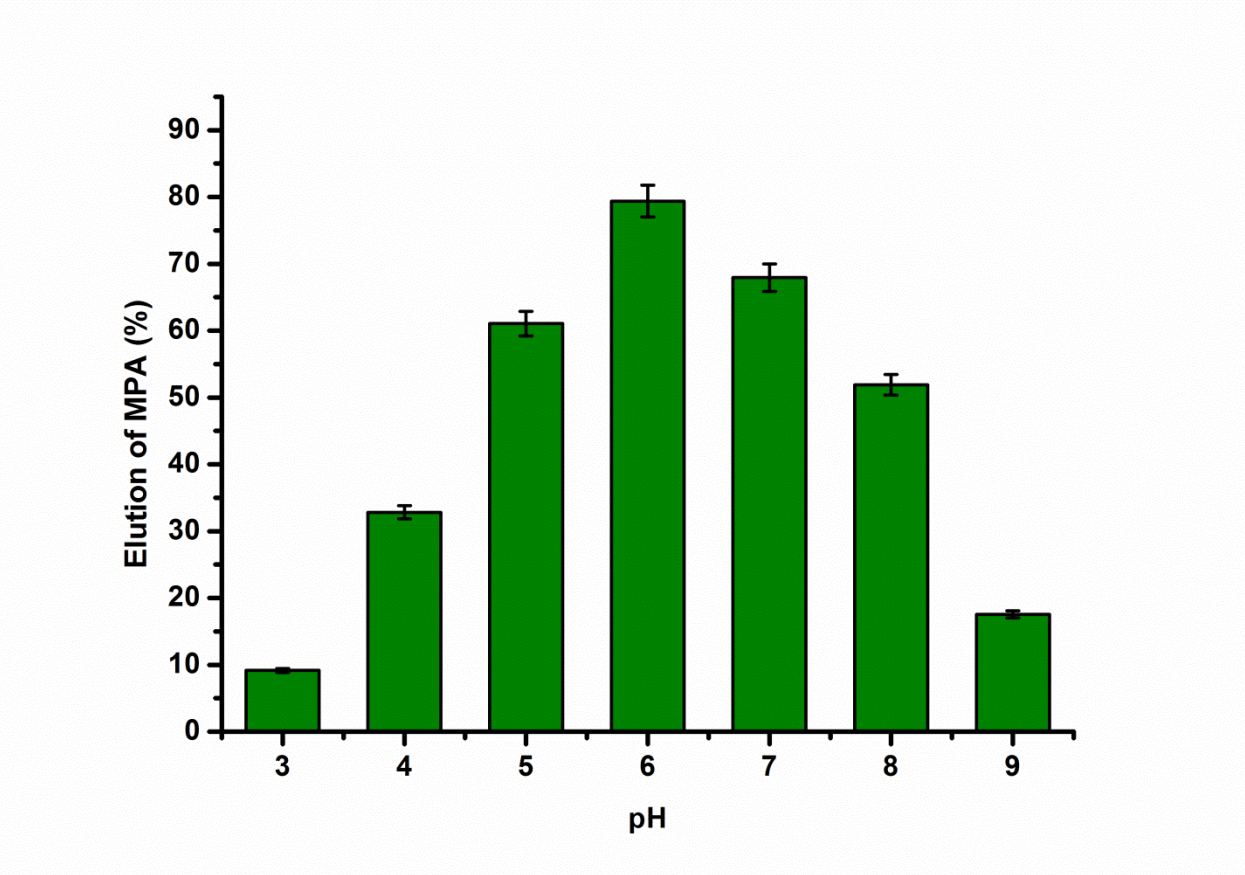

Figure 4.21. Effect of pH during purification of mycophenolic acid

4.10.3.2 Effect of Flow Rate during Purification of Mycophenolic Acid

The extracted fermentation broth was passed through the column at different flow rates, i.e., from 0.5 to 3.5 mL/min. Fractions were collected and analyzed by HPLC. It was observed that as the flow rate increased from 0.5 to 3.5 mL/min, the elution of MPA also increased up to 2 mL/min and found to be maximum at this flow rate. Elution of MPA was observed to be the highest, i.e., 77.09% when the flow rate was maintained at 2.0 mL/min. Elution of MPA through the column at different flow rates is shown in Figure 4.22.

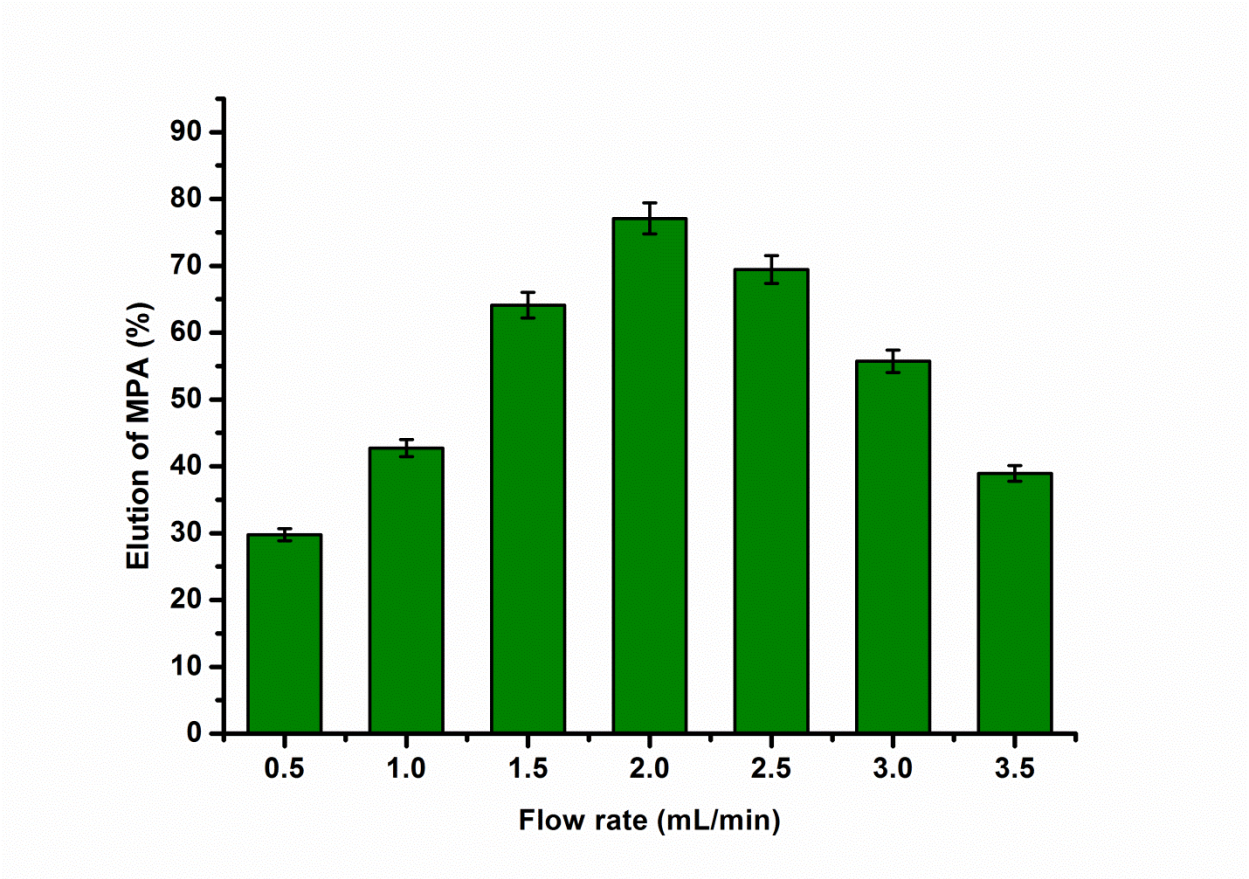

Figure 4.22. Effect of flow rate during purification of mycophenolic acid

4.10.3.3 Effect of Volume of the Eluent during Purification of Mycophenolic Acid

The extracted broth having varying volume was passed through the columns. Following this, the columns were washed with ethyl acetate and the fractions eluted using the same. The fractions were concentrated and sent for MPA detection via HPLC. The percent elution of MPA versus the volume of the eluent is plotted in Figure 4.23. The concentration evaluation through HPLC analysis indicated that when 150 mL of ethyl acetate was passed through the alumina column, the elution of MPA was recovered to be 75.57%.

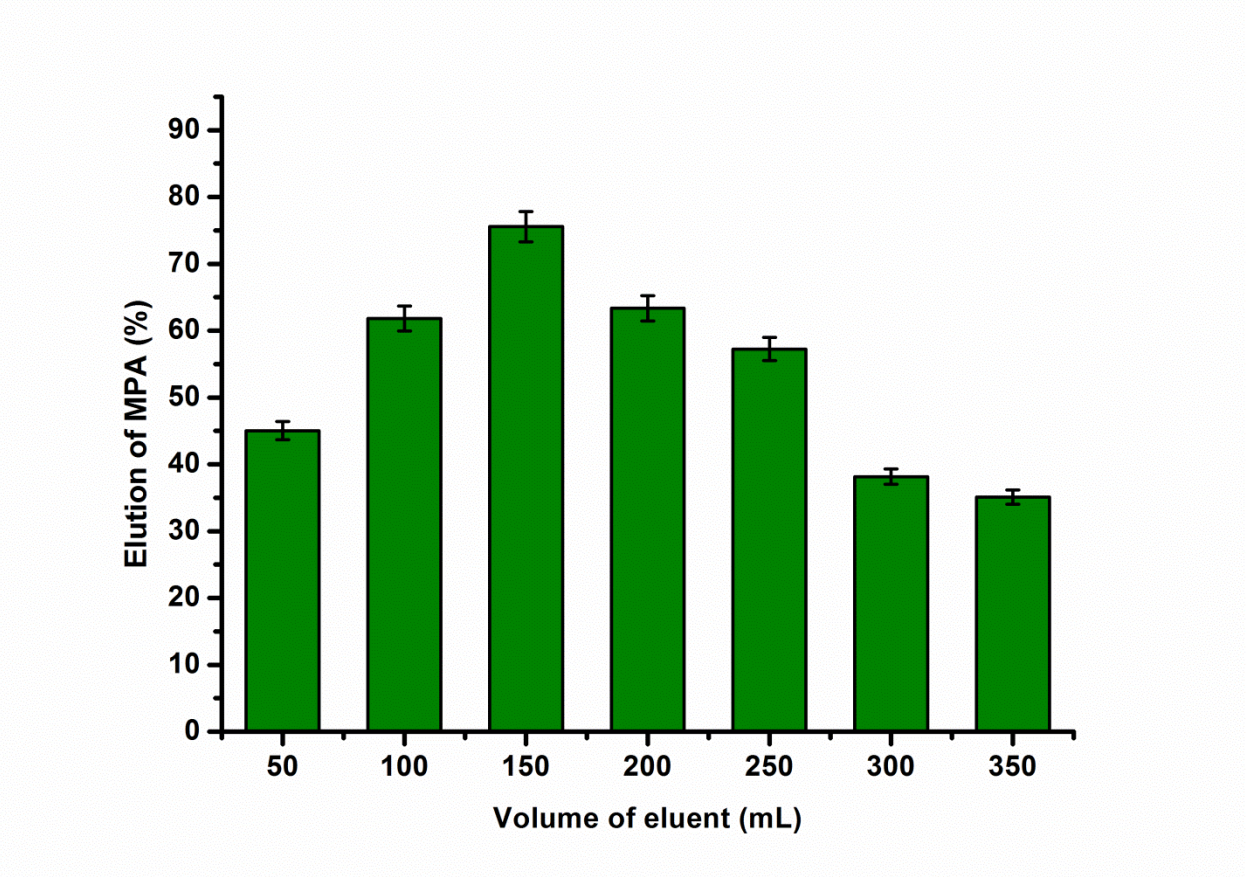

Figure 4.23. Effect of volume of eluent on purification of mycophenolic acid

4.11 Optimization of Mycophenolic Acid Purification by Response Surface Methodology

In the present study, a two-level three factors Central Composite Design (CCD) was employed for maximization of elution of MPA [Giovanni, 1983; Hill and Hunter, 1966]. The boundaries of these experimental parameters were selected on the basis of single parameter selection trials. Table 4.4 represents the level of the variables and design matrix to investigate this work.

Table 4.4 Selected parameters range for purification of mycophenolic acid

Variables	**-1**	**0**	**+1**
pH	5	6	7
Flow rate (mL/min)	1.5	2.0	2.5
Volume of eluent (mL)	100	150	200

Twenty experiments were carried out according to the CCD as shown in Table 4.5 were explained by the following second-order polynomial equation (Shukla & Mishra, 2013). Response results were analyzed using Minitab 16.0 software.

The t-test and P values were used to identify the effect of each factor on mycophenolic acid production as shown in Table 4.6. The application of multiple regression analysis methods yielded the following regression equation to the experimental data.

$$Y = -179.312 + 53.212A + 56.409B + 0.633C - 4.298A^2 - 14.612B^2 - 0.001C^2 + 0.475AB - 0.0147AC - 0.011BC$$

Where A is pH, B is flow rate, and C is the volume of eluent. Regression analysis of the experimental data showed that the pH of the eluent shows positive interactive studies with all the two factors. ANOVA of the quadratic equation for mycophenolic acid purification has been summarized in Table 4.6. The P-value of the model was <0.001, which predicts the model to be significant. The high values of the determination

coefficient (R^2 = 97.59%) and the adjusted determination coefficient (adjusted R^2= 95.42%) define the model to be significant [Koocheki, et al., 2009]. The response surface plots and contour plots in Figure 4.24A, 4.24B, 4.25A, 4.25B and 4.26A, 4.26B illustrate the effects of the pairwise combination of the three factors. The plots provide a visual indication of how any two factors interactively affected the response.

Table 4.5 CCD matrix employed for pH, Flow rate and Volume of Eluent independent variables

S. No.	pH	Flow rate (mL/min)	Volume of Eluent (mL)	Experimental value (%)	Predicted value (%)
1	5	2.5	100	69.7	70.58
2	6	1.5	150	80.21	81.21
3	6	2	150	84.12	84.39
4	6	2	150	84.01	84.39
5	7	2.5	200	70.89	71.18
6	6	2	150	83.92	84.42
7	6	2	150	83.6	84.39
8	6	2.5	150	82.64	80.27
9	7	2.5	100	72.86	73.27
10	6	2	200	80.49	80.04

11	5	1.5	100	71.38	71.42
12	5	1.5	200	73.54	73.46
13	7	2	150	80.19	80.46
14	6	2	150	84.12	84.39
15	6	2	150	83.91	84.39
16	5	2.5	200	70.69	71.46
17	7	1.5	200	72.79	72.24
18	6	2	100	80.97	80.06
19	7	1.5	100	73.59	73.15
20	5	2	150	81.37	79.73

4.11.1 ANOVA of Response Surface Plots

The fitted polynomial equation was expressed as three-dimensional surface plots to visualize the variation of the responses due to the interaction of the factors. ANOVA gives the value of the model and can explain whether this model adequately fits the variation observed in mycophenolic acid production with the designed parameter. The goodness of fit of the model was checked by determination coefficient (R^2). The combined effect of pH, flow rate, and volume of eluent for purification of MPA was performed. From all these interaction studies, it could be predicted that pH plays a vital role during the purification process [Bahry, et al., 2019; Bishai, et al., 2013; Kumar, et al., 2019; Patel, Patil, 2016]. Table 4.6 shows the ANOVA analysis of the RSM model.

Table 4.6 ANOVA analysis of the RSM model for purification of Mycophenolic Acid by *P. brevicompactum*

Source	DF[a]	Seq SS[b]	Adj SS[b]	Adj MS[c]	F	P
Regression	9	564.370	564.370	62.708	44.98	<0.001
Linear	3	3.563	3.563	1.188	0.85	0.497
Square	3	555.290	555.290	185.097	132.76	<0.001
Interaction	3	5.516	5.516	1.839	1.32	0.0322
Residual error	10	13.942	13.942	1.394		
Lack of fit	5	13.756	13.756	2.751	73.82	<0.001
Pure error	5	0.186	0.186	0.037		
Total	19	578.312				
R-Sq = 97.59%		**R- Sq (adj) = 95.42%**				

[a]degree of freedom, [b]sum of square, [c]mean of square

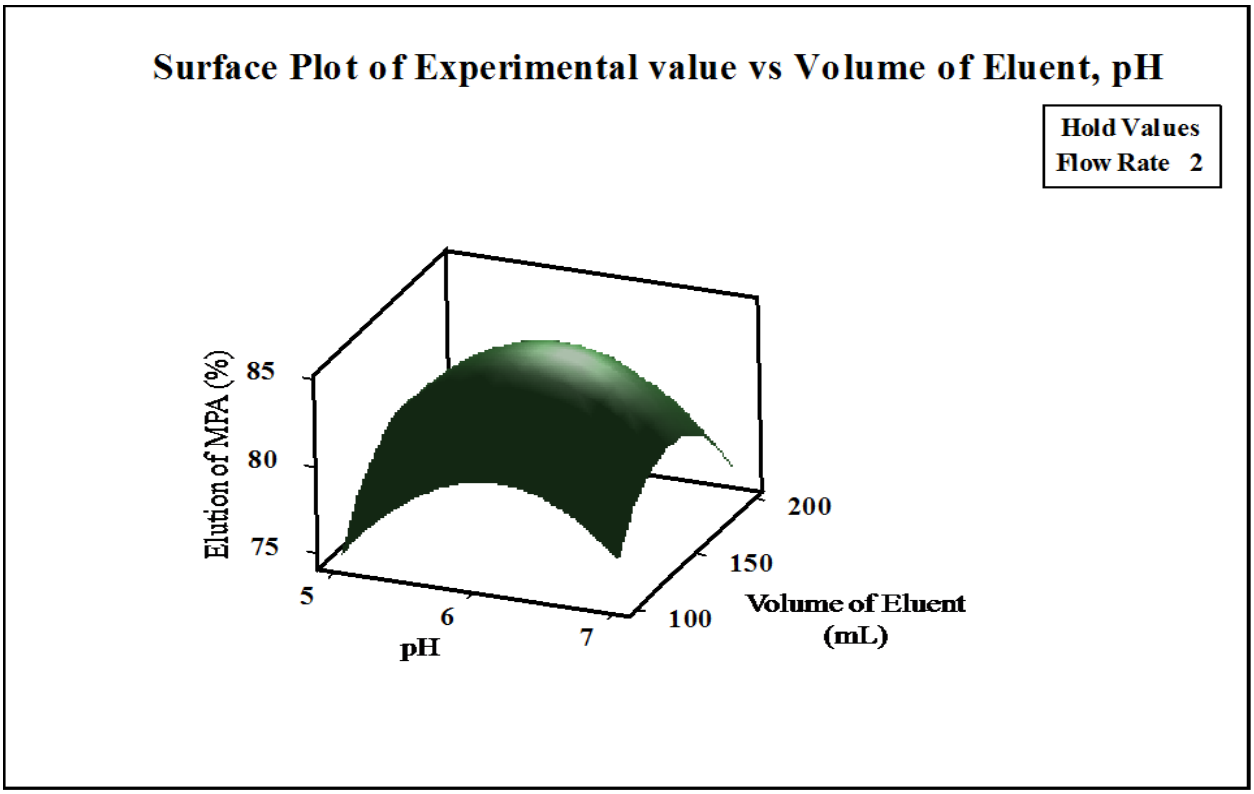

Figure 4.24A. Surface plot between pH and volume of eluent

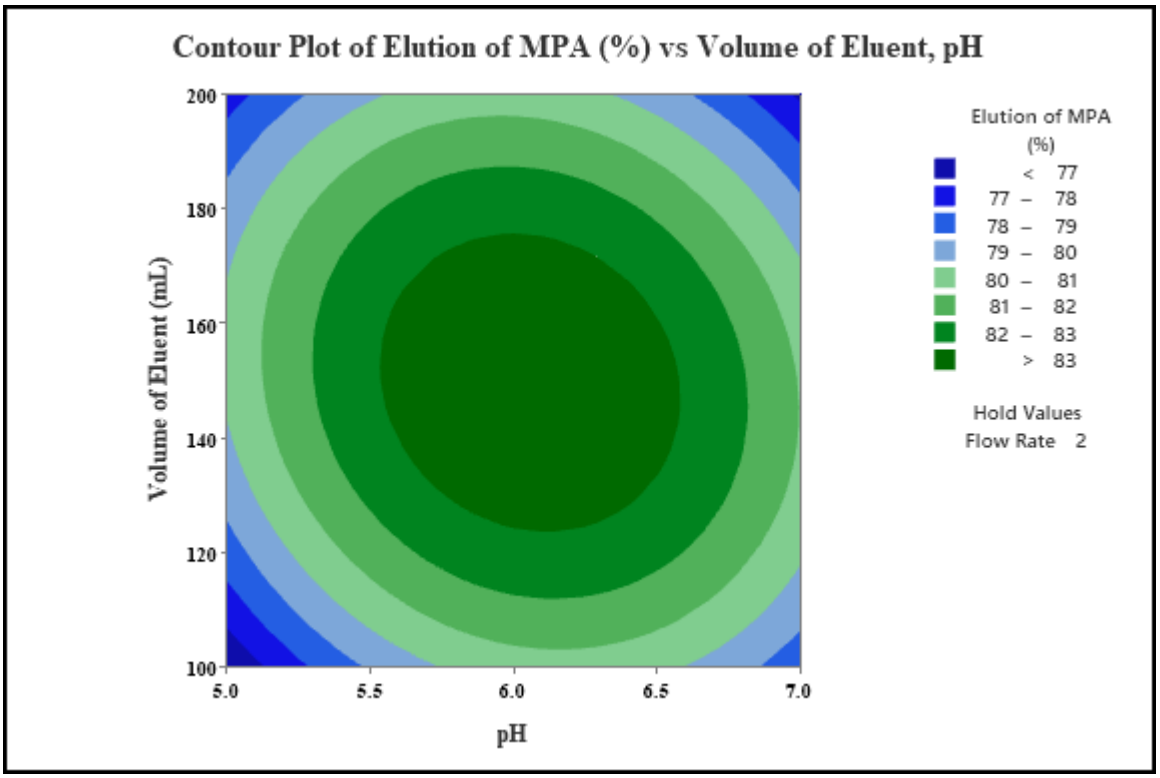

Figure 4.24B. Contour plot between pH and volume of eluent

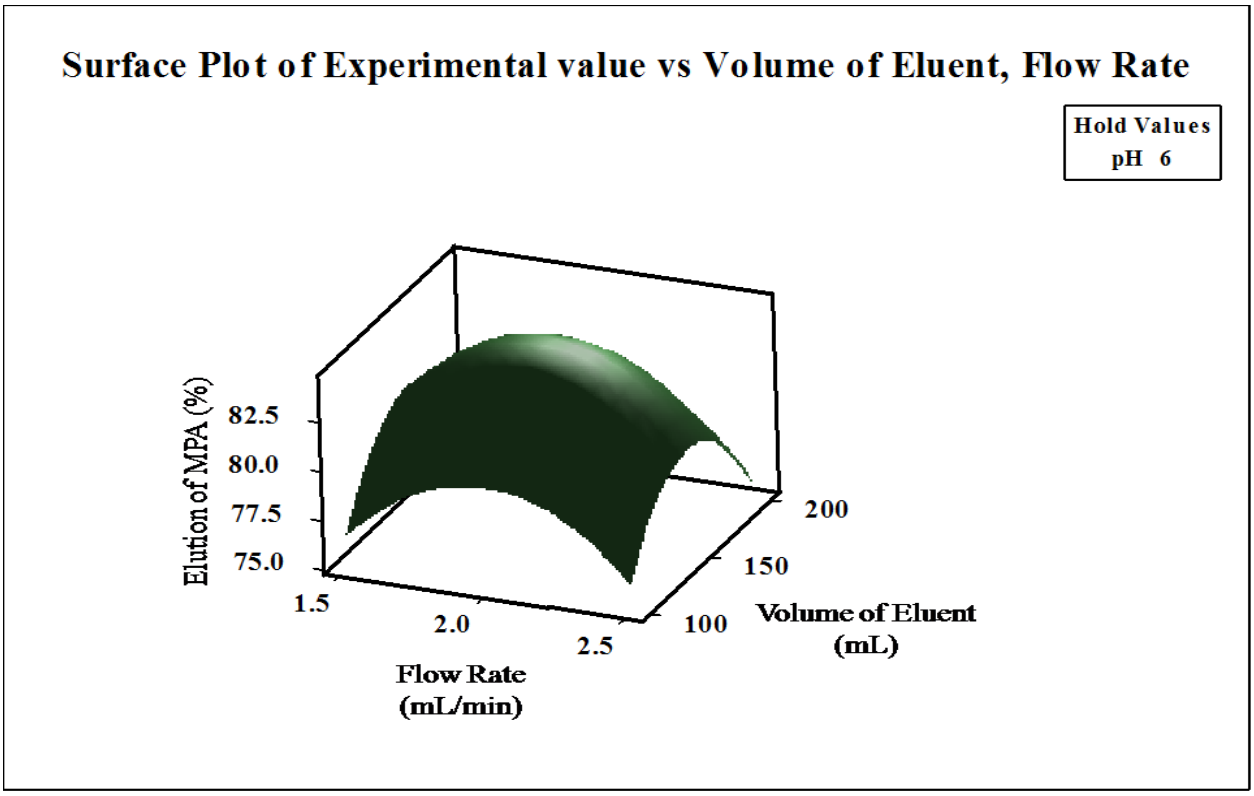

Figure 4.25A. Surface plot between flow rate and volume of eluent

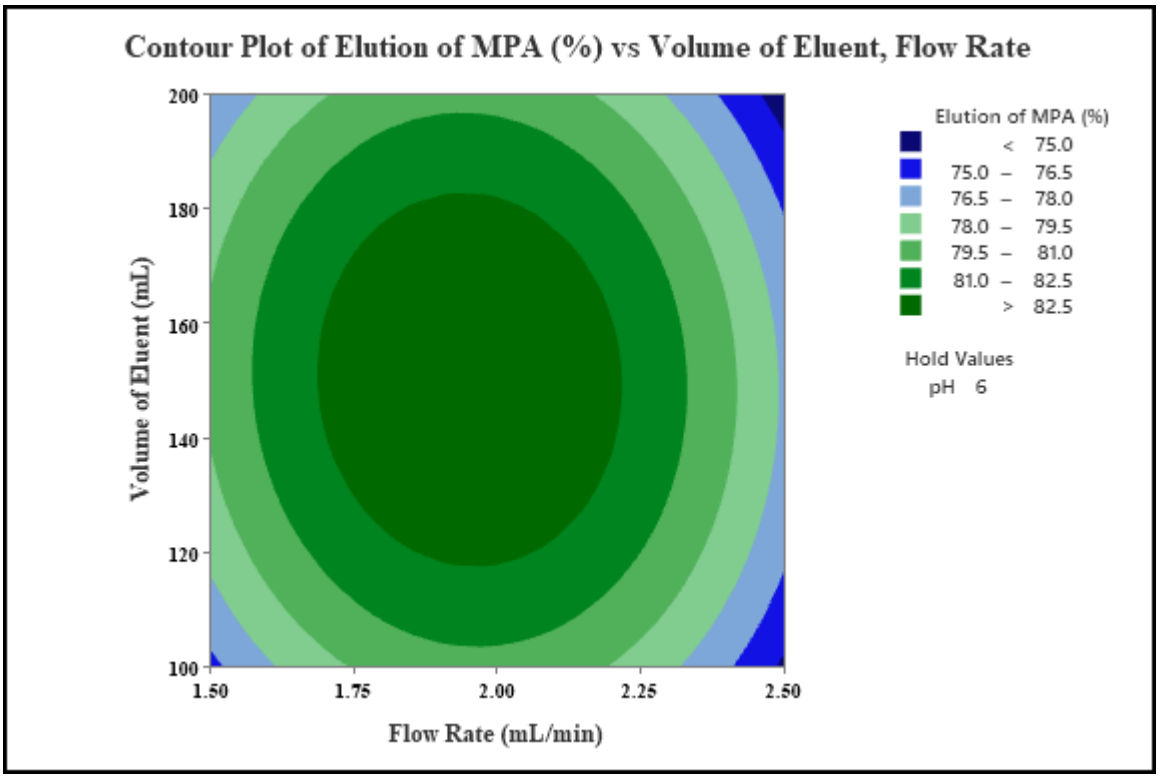

Figure 4.25B. Contour plot between flow rate and volume of eluent

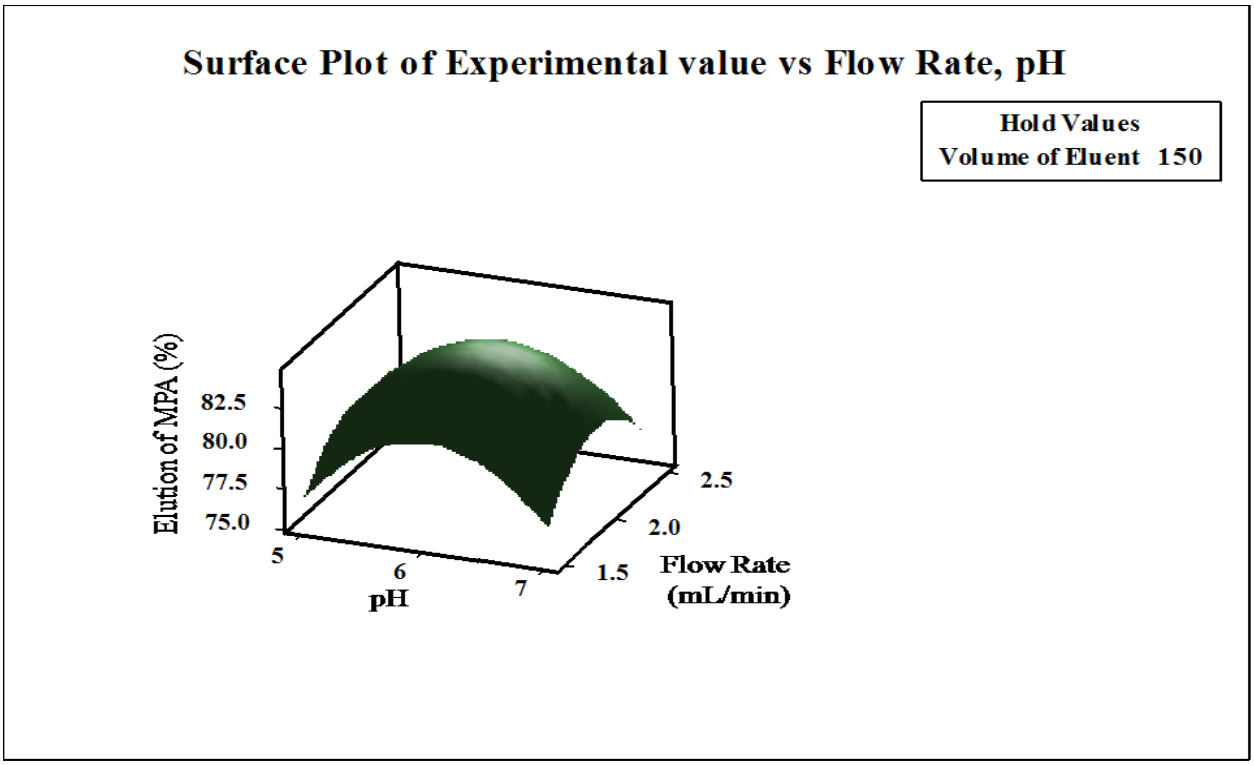

Figure 4.26A. Surface plot between pH and flow rate

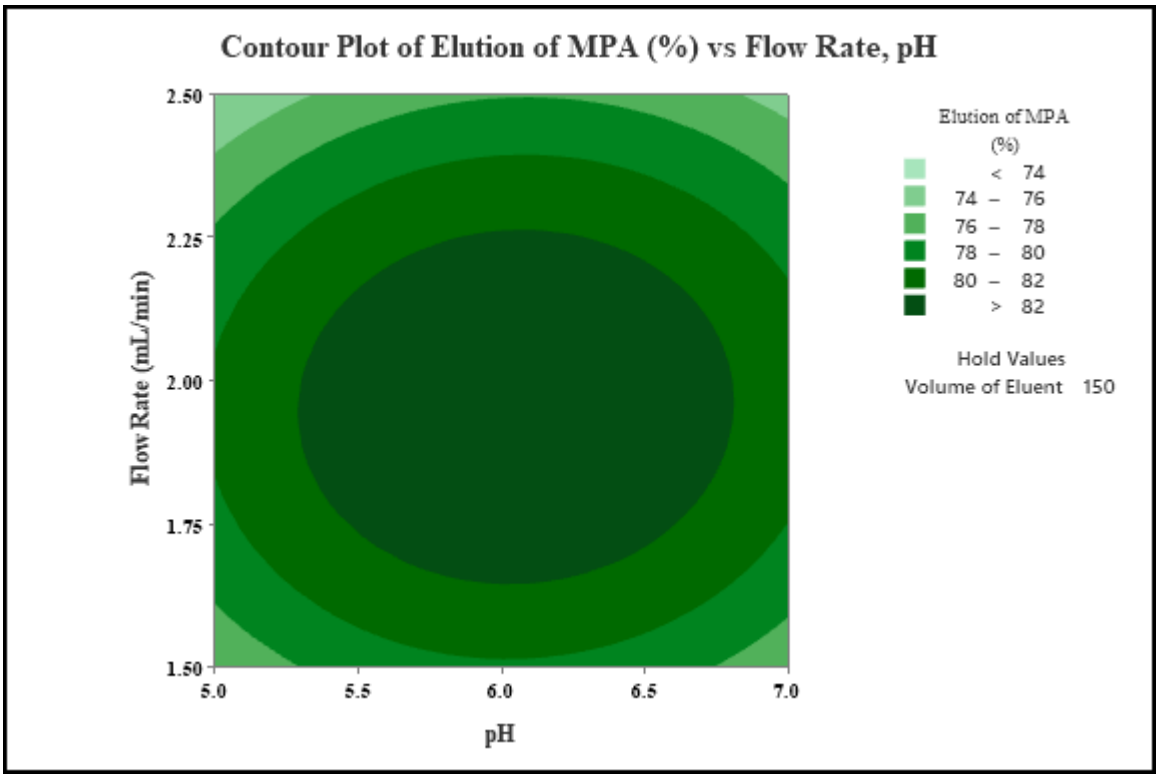

Figure 4.26B. Contour plot between pH and flow rate

The results of the ANOVA analysis gave the optimized value for MPA purification using Alumina column. Under pH 6.05, flow rate of 1.96 mL/min and volume of eluent 149.49 mL, the maximum predicted percentage of elution of MPA was 84.42%. To confirm the predicted response, experiments were conducted in triplicates. Experimentally, the maximum percentage of elution was found 84.12%, which was close to it. The result of this study could be used to design and enhance the mycophenolic acid production.

The optimization technique RSM was applied for optimization of purification process to enhance the yield of mycophenolic acid. The present study using RSM with CCD enabled us to find the importance of factors at different levels. The RSM, including an experimental design and ANOVA was an effective method for optimization of purification process. Regression analysis of the experimental data showed that the pH of the eluent shows positive interactive studies with all the two factors [Mohamed, et al., 2019; Patel, et al., 2020].

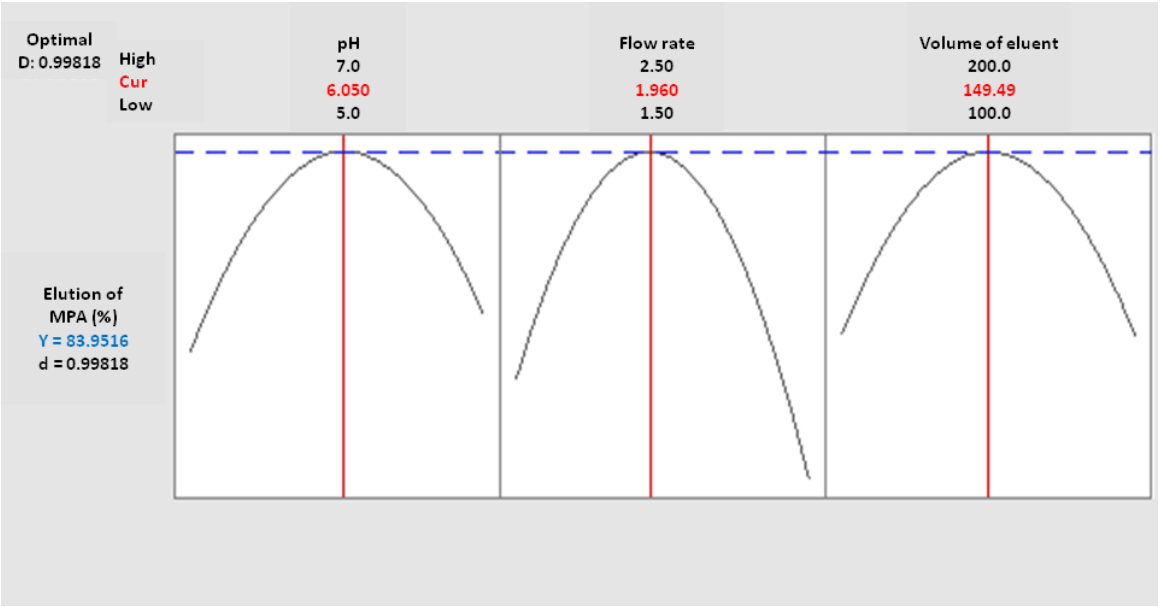

Figure 4.27. Optimum combinations of different parameters for mycophenolic acid purification

The optimum combination of different parameters for purification of mycophenolic acid obtained from contour and response surface plots for pH, flow rate and volume of eluent were 6.05, 1.96 mL/min, and 149.49 mL, respectively (Figure 4.27).

4.12 Quantitative analysis of purified sample using HPLC

The presence of MPA in fermented broth as well as in the purified sample was confirmed by HPLC. Matching of retention time of the purified sample with that of the standard MPA confirmed its presence in the sample. Some polar and non-polar impurities were present in the purified sample. HPLC chromatograms of standard Mycophenolic Acid (HIMEDIA) and purified Mycophenolic Acid was shown in Figure 4.28. HPLC analysis revealed that the purified MPA and authenticated purchased standard MPA (HIMEDIA) share similar chromatogram peak with similar retention time at approx. 6.8-7.5 min during the 15 min of run time (Figure 4.28).

Comparative study with mycophenolic acid produced from fermentation of *Penicillium brevicompactum* MTCC 549 and standard mycophenolic acid by HPLC, showed similar retention time.

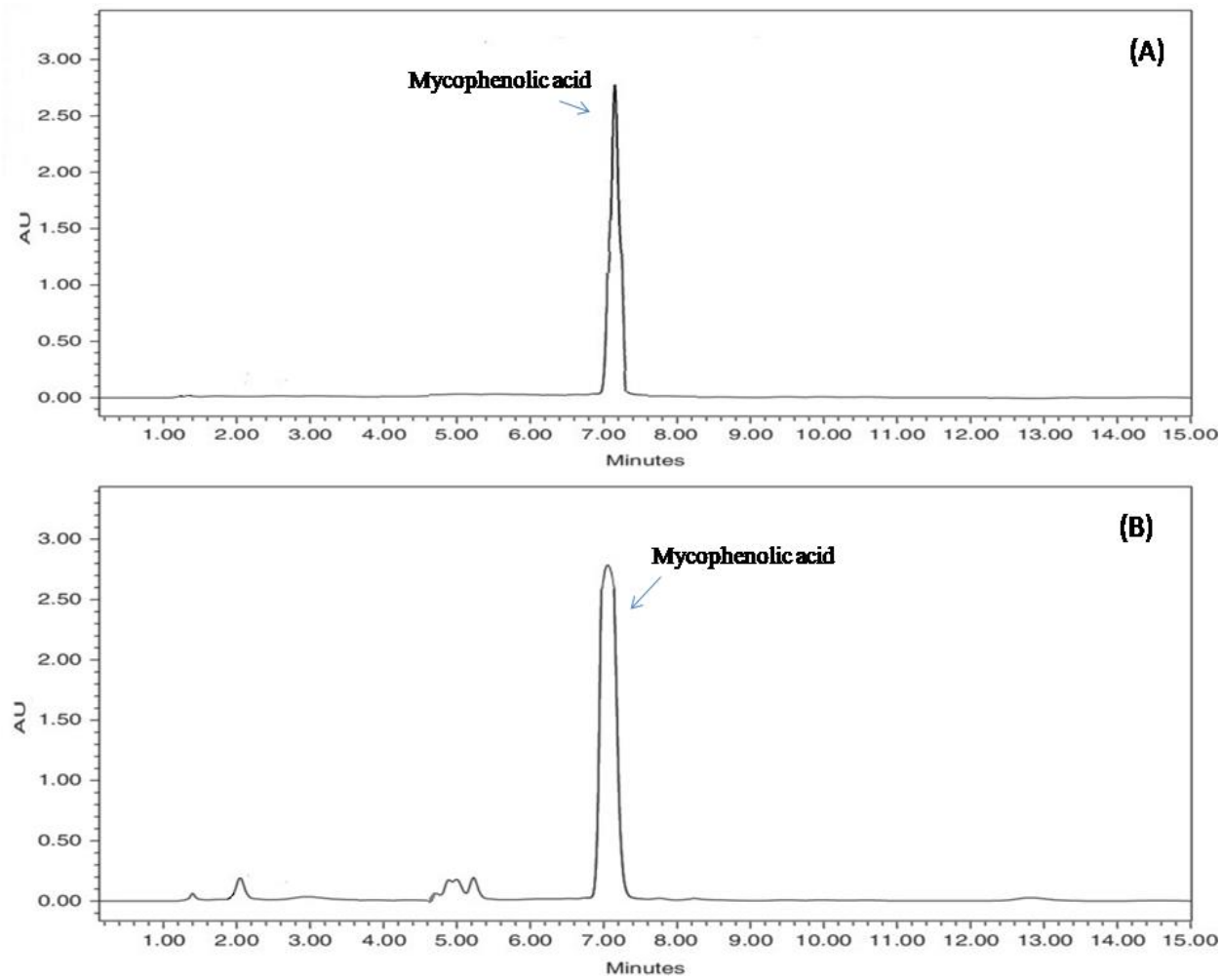

Figure 4.28. HPLC chromatogram of (A) standard mycophenolic acid (HIMEDIA) and (B) purified mycophenolic acid sample.

4.13 Qualitative analysis of purified sample using FTIR

The functional groups of MPA analyzed through FTIR were found to be similar to that of the standard MPA. Table 4.7 represents the absorption range of important functional groups present in mycophenolic acid. The standard mycophenolic acid shows a peak at 3415.31 cm^{-1} in the region 3200-3500 cm^{-1} which describe the O-H (alcohol) stretching and hydrogen bond present in the compound. The second peak at 2931.27 cm^{-1} (lies in the region 2500-3300 cm^{-1}), which describe the strong and broad O-H stretching of carboxylic group present in the structure. The peak at 1744.29 cm^{-1} and 1707.65 cm^{-1}

(lies in the region 1700-1750 cm^{-1}) represents the ester group. The band at 1624.25 cm^{-1} (lying in the region 1600-1680 cm^{-1}) denotes the C=C (alkene) double bond present in the mycophenolic acid structure. The band between 1440-1465 cm^{-1} represents the methyl group present in the mycophenolic acid. The band at 1206.25 cm^{-1} and 1075.12 cm^{-1} represents the phenol and ether group present in the mycophenolic acid structure. Similarly these bands are also present in the purified sample. Figure 4.30 define FTIR profile of the purified sample and standard mycophenolic acid (Figure 4.29).

Table 4.7 FTIR absorption range of important functional groups present in mycophenolic acid

Functional groups	**Absorption (cm^{-1})**
Phenolic group	3500 – 3200
Carboxylic group	3300 - 2500
Lactone ring	1750 - 1700
Alkene	1680 - 1600
Methyl group	1441 - 1465
Phenol group	1350 - 1250
Ether group	1100 - 1050

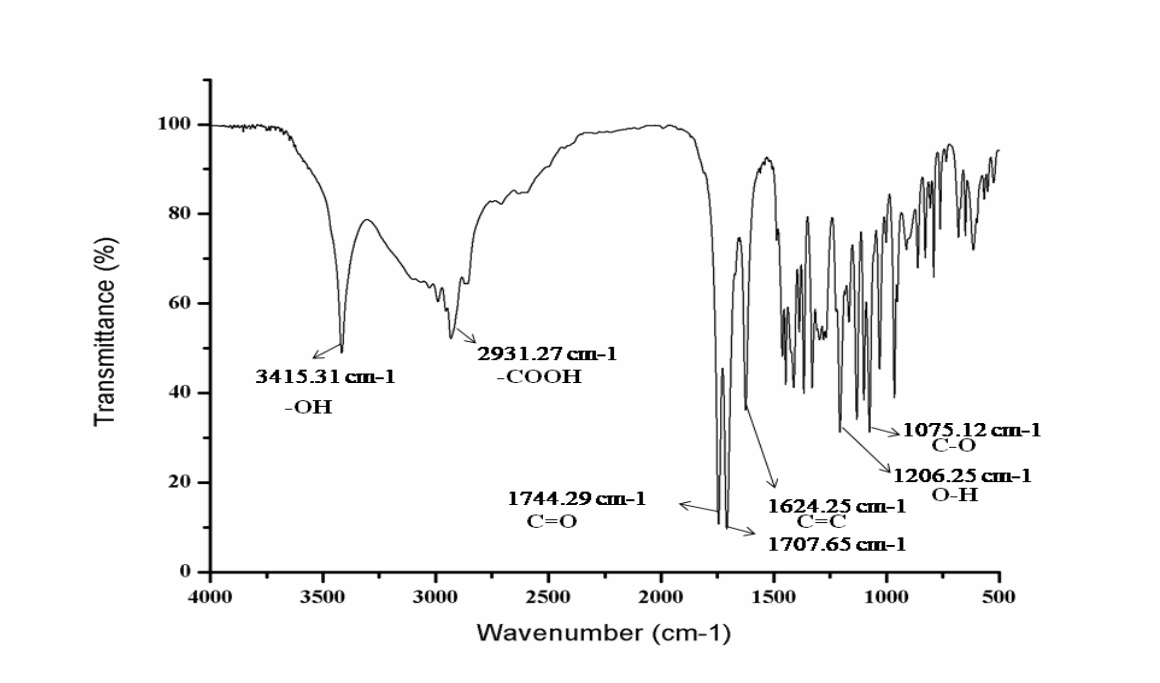

Figure 4.29. Fourier-transform infrared spectroscopy (FTIR) graph of standard mycophenolic acid

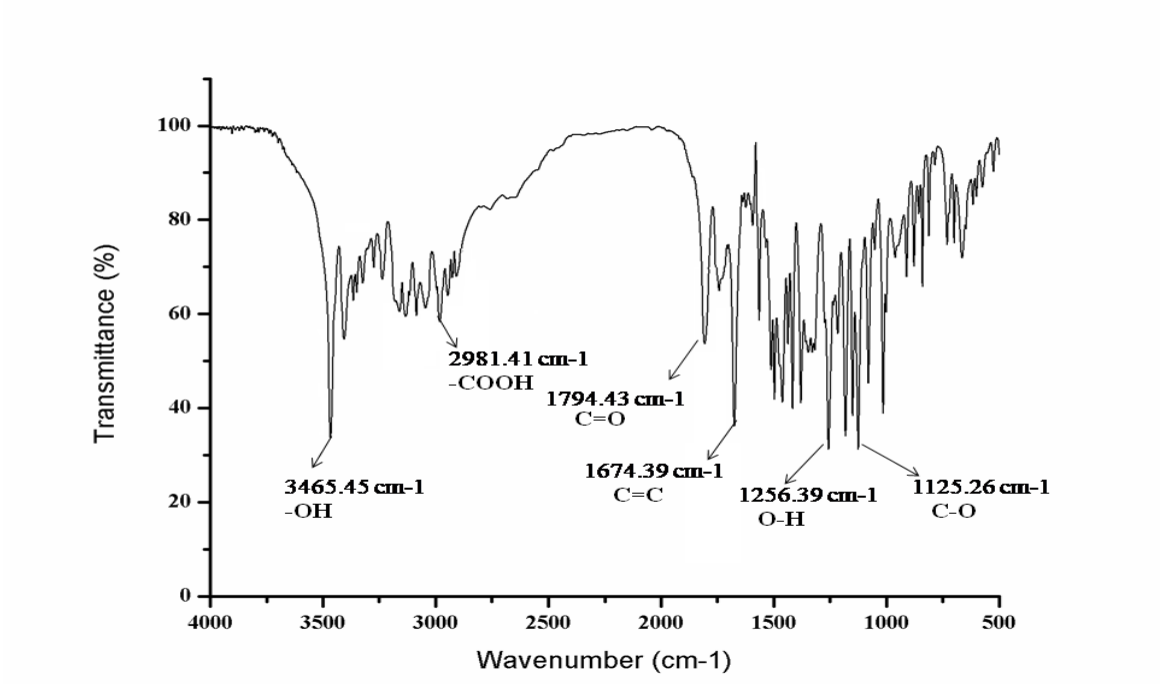

Figure 4.30. Fourier-transform infrared spectroscopy (FTIR) graph of mycophenolic acid sample