CHAPTER 5

Production of L-Glutaminase in Bioreactor and Its Kinetic Modeling

5.1. Introduction

The movement of a process from laboratory to commercial level is called as scale up. The scale up of bioprocess is divided into four sequential stages: aeration flask, lab scale bioreactor, pilot scale bioreactor, and commercial scale bioreactor. Lab-scale fermentation processes are typically used to screen microorganism, to optimize media and culture conditions, and to develop separation and purification methods. Pilot scale production in bioreactor is generally used to verify optimal large scale process conditions and to produce sufficient data for the appraisal of large scale production efficiency (Hochfeld, 2006). Common scale up parameters in batch-culture systems are generally based on tank geometry, oxygen supply, mixing effect and agitator power input. The frequently used scale-up method in aerobic fermentation process is based on the proper mixing of media and utilization of the oxygen mass transfer coefficient (Felse and Panda, 2000). Agitation is important for uniform mixing of the media components and maintaining optimum oxygen transfer rate within the fermenter (Kumdam et al., 2013). Lower agitation speed and higher agitation speed result reduction in cell growth and enzyme production may be due to heterogeneous mixing effects and higher sheer stress respectively. Oxygen shows diverse effects on product formation in aerobic fermentation process by influencing metabolic pathway and changing metabolic fluxes (Calik et al., 1998). The oxygen availability in the fermentation broth could be rate-limiting especially in high cell density culture of the fast-growing aerobes. Therefore, the scale-up success of such aerobic fermentation process depends on satisfying the oxygen demand all through the culture volume in the fermenter (Manolov, 1992; Shioya et al., 1999). The aeration rate in fermenter is generally dependent on critical concentration of dissolved oxygen. Below this critical

level, a small change in dissolved oxygen may cause a significant physiological alteration in cell metabolism and affect the product formation kinetic (Hwang et al., 1991; Plihon et al., 1996; Barberis and Segovia, 1997). The productivity of several bioactive compounds is influenced by varying dissolved oxygen concentration (Dick et al., 1994; Gavrilescu and Roman, 1994, Kempf et al., 1997). Oxygen uptake by growing microbial cell is the rate-limiting step in many aerobic fermentation processes and determined in term of oxygen transfer rate and volumetric oxygen transfer coefficient (K_La). It is essential to devise a scale-up strategy with optimum levels of agitation and aeration in bioreactor to give better yield of biochemical. This could minimize the production cost and optimize the cost-effectiveness for the overall production process (Feng et al., 2003).

Kinetic models are necessary for bioreactor design and scale up of fermentation process. Kinetic models enable bioengineers to understand, control and operate the fermentation process under optimized condition (Moser and Steiner, 1975). The development of kinetic models is based on comparison of assumed models with experimental data in order to obtain more relevant equations. These models help in evaluating the behavior of systems more rapidly than with laboratory experiments. Kinetic model for a microbial fermentation process can be expressed using two different mechanisms: structured and unstructured models (Sinclair and Kristiansen, 1987; Chen et al., 2004). Structured models are developed by considering some basic aspects of cell structure, function, and composition. However, unstructured models describe biomass and associated metabolic process by using knowledge of the experimental data.

In this study, the effects of aeration and agitation rates on L-glutaminase production from *Bacillus cereus* MTCC 1305 were investigated in bioreactor. The oxygen transfer parameters like volumetric transfer coefficient (K_La), oxygen uptake rate (OUR), and oxygen transfer rate (OTR) for this fermentation process were determined. Kinetic models were developed for cell growth, product formation and substrate consumption using Logistic equation, Luedeking-Piret equation and modified Luedeking-Piret equation respectively.

5.2. Materials and method

5.2.1. Production of L-glutaminase in fermenter

The production of L-glutaminase from *Bacillus cereus* MTCC 1305 was studied in 5litre fermenter (Scigenics Private Ltd, India) with 2litre production media (pH-7.5) containing sucrose (2.5g/l), peptone (2.5g/l), L-glutamine (5.0g/l), Na_2HPO_4 (6.0g/l), NaCl (0.5g/l) and $MgSO_4$ (0.5g/l). The fermenter was inoculated with 2% inoculum (2.13×10^5cell/ml, 10hrs age). Effect of different agitation speed (100, 200, 300 and 400rpm) on cell growth, L-glutaminase production, substrate consumption rate and % of saturation of dissolved oxygen (DO) in fermenter were studied. The concentration of dissolved oxygen was maintained above 20% throughout the fermentation process by adjusting air flow rate. Dissolved oxygen level was measured in % of saturation by DO probe of fermenter. Glass electrode immersed in the fermentation broth was used to measure pH of the medium. Effect of different aeration rate (1.0, 2.0, and 2.5vvm) was studied on microbial growth, glutaminase production, and % of saturation of dissolved oxygen. Samples from fermenter were drawn at regular intervals and analyzed for biomass and enzyme activity.

5.2.2. Estimation of cell biomass and sucrose concentration

Cell biomass in the fermentation broth was quantified by measuring dry cell weight. The cells were separated from the fermented broth by centrifugation at 4˚C, 10,000×g and washed twice with distilled water. The recovered biomass was dried to constant weight in an oven at 80˚C for 24hrs. Sucrose concentration in the broth during fermentation was estimated by phenol sulfuric acid method (Hansson and Phillips, 1981). 1ml phenol and 5ml sulfuric acid were added to 1 ml of cell free broth and cooled at room temperature. Absorbance of the solution was measured at 490 nm. The standard graph was plotted using different concentration (0.1, 0.2, 0.4, 0.6, 0.8, and 1.0mg/ml) of sucrose solution as shown in Fig5.1.

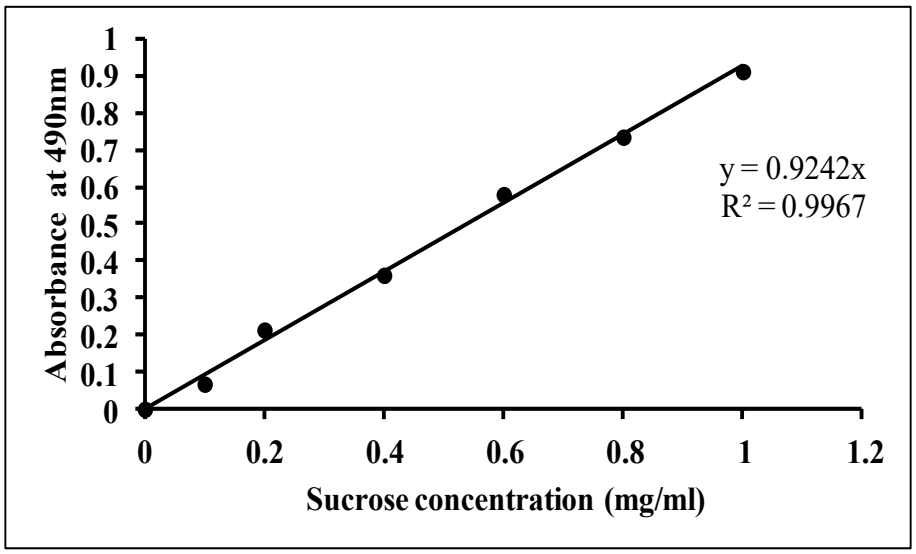


Fig.5.1. Standard graph for estimation of sucrose by phenol sulfuric acid estimation method

5.2.3. Determination of oxygen transfer characteristics

The value of oxygen uptake rate (OUR), oxygen transfer rate (OTR) and volumetric mass transfer coefficient (K_La) were determined using dynamic gassing-out method

(Gracia et al., 2000). Rate of change in dissolved oxygen concentration (dC_L/dt) was determined using the following mass balance equation:

$$dC_L/dt = OTR - OUR = K_La(C^* - C_L) - (QO_2.X) \ldots\ldots\ldots (5.1)$$

Where, C_L =Dissolved oxygen concentration in the liquid phase (mmol/l), C^* = Saturated dissolved oxygen concentration equilibrated to the gaseous oxygen partial pressure (mmol/l), K_La = Volumetric oxygen transfer coefficient (hr^{-1}), a = Gas/liquid interface area per liquid volume (cm^2cm^{-3}), QO_2 = Specific respiration rate (mmoles of oxygen/g-biomass/hr), X = Biomass concentration (g/l), OTR = Oxygen transfer rate, OUR = Oxygen uptake rate

5.2.4. Kinetic model for microbial cell growth

Some microbial fermentation processes did not follow the classical kinetic model of substrate-limited biomass growth and hence a Logistic equation was proposed as an alternative empirical function for sigmoid profile independent of substrate concentration (Rao et al., 2009). L-glutaminase from *Bacillus cereus* MTCC 1305 was produced during late exponential phase and hence logistic equation was proposed for this fermentation process. Cell growth rate of the microorganism was presented by the following equation:

$$dX/dt = \mu_m (1 - X/X_m) \ldots\ldots\ldots\ldots (5.2)$$

Where, dX/dt = Cell growth rate (g-cell/ hr), μ_m = Maximum specific growth rate (hr^{-1}), X =Biomass concentration (g), X_m=Maximum biomass concentration (g-cell/l).

At the beginning of the fermentation, when t = 0, the biomass concentration was given by the initial concentration value ($X = X_0$).The integrated form of equation 5.2 would give a sigmoid variation of *X* as a function of *t*, which may represent both an exponential and a stationary phase:

$$X_t = \frac{X_0 e^{\mu_m t}}{\left[1-\left(X_0/X_m\right)\left(1-e^{e^{\mu_m t}}\right)\right]} \ldots\ldots\ldots\ldots\ldots (5.3)$$

5.2.5. Kinetic model for Product formation

A suitable kinetic model was developed for product formation kinetic using the following Luedeking–Piret equation (Luedeking and Piret, 1959):

$$r_p = {}^{dp}/_{dt} = \alpha\left({}^{dx}/_{dt}\right) + \beta \ldots\ldots\ldots (5.4)$$

Where, r_p= Product formation rate, ${}^{dx}/_{dt}$= Cell growth rate, α =Growth associated constant, and β = Non growth associated constant.

The following equation 5.5 was obtained after integration of equation 5.4 and substituting value of X_t from equation 5.3:

$$P_t = P_0 + \alpha A_t + \beta B_t \ldots\ldots\ldots\ldots (5.5)$$

Where, P_0 = Product concentration at initial time (t=0), and P_t = Product concentration at any time (t),

$$A_t = X_0\left[\frac{e^{\mu_m t}}{1-\left({}^{X_0}/_{X_m}\right)(1-e^{\mu_m t})} - 1\right],\ B_t = \left({}^{X_m}/_{\mu_m}\right)\left[\ln 1 - \left({}^{X_0}/_{X_m}\right)(1 - e^{\mu_m t})\right]$$

The coefficients α and β in equation 5.5 were determined by plotting $[P_t - P_0] / B_t$ vs A_t / B_t which was a straight line with slope 'α' and intercept 'β' as shown in Fig5.2.

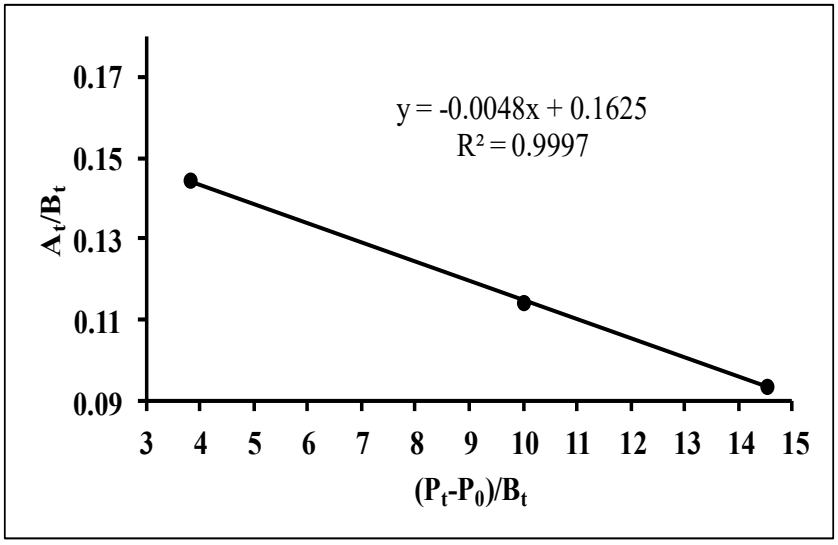


Fig.5.2. Graph between $[P_t - P_0] / B_t$ and A_t / B_t to determine the growth associated constant (α) and non growth associated constant (β)

5.2.6. Kinetic model for substrate consumption rate

Substrate was utilized by microorganism for their cell growth, product formation and cell maintenance. Kinetic model for the substrate consumption kinetics was developed using following modified Luedeking-Piret equation:

$$\frac{-dS}{dt} = \left(\frac{1}{Y_{X/s}}\right)\frac{dX}{dt} + \left(\frac{1}{Y_{P/s}}\right)\frac{dP}{dt} + m_s X \quad \text{.....(5.6)}$$

Where, $Y_{X/s}$ = Cell yield coefficient for substrate, $Y_{P/s}$ = Product yield coefficient for substrate, and m_s= Maintenance coefficient (g- substrate·g-cell hr^{-1})

The value of dx/dt from equation 5.2 was substituted in equation 5.6 and after integration of equation 5.6; the following expression was obtained for sucrose consumption:

$$-ds/dt = \left(1/Y_{X/s} + \alpha/Y_{P/s}\right)dX/dt + \left(\beta/Y_{P/s} + m_S\right)X \quad \text{.....(5.7)}$$

$$-ds/dt = \gamma\, dX/dt + \delta X \quad \text{.....(5.8)}$$

Where, $\gamma = \left(1/Y_{X/S} + \alpha/Y_{P/S}\right)$ and $\delta = \left(\beta/Y_{P/S} + m_s\right)$

At stationary phase, $dX/dt = 0$ and $X = X_m$ and after substituting these values in equation 5.8, the following equation was obtained:

$$\delta = \frac{\left[-\left(dS/dt\right)\right]}{X_m} \quad \text{.....(5.9)}$$

On integration of equation 5.8 by varying substrate concentration from S_0 to S with time, the following equation was obtained:

$$-\int_{S_0}^{S} dS = \gamma\, dX + \delta \int t dt \quad \text{.....(5.10)}$$

$$S = S_0 - \gamma C_t - \delta D_t \quad \text{.....(5.11)}$$

Where, S = Concentration of residual sucrose at time t, S_0= Concenration of sucrose at t=0,

$C_t = X_0 \left[\frac{e^{\mu_m t}}{1-\left(X_0/X_m\right)\left(1-e^{\mu_m t}\right)} - 1\right]$, $D_t = X_m/\mu_m \left[\ln 1 - X_m/\mu_m \left(1 - e^{\mu_m t}\right)\right]$

The coefficients γ and δ of equation 5.11 were determined by plotting $(S_t - S_0)/D_t$ versus C_t/D_t which was a straight line with slope 'γ' and intercept 'δ' as shown in Fig5.3.

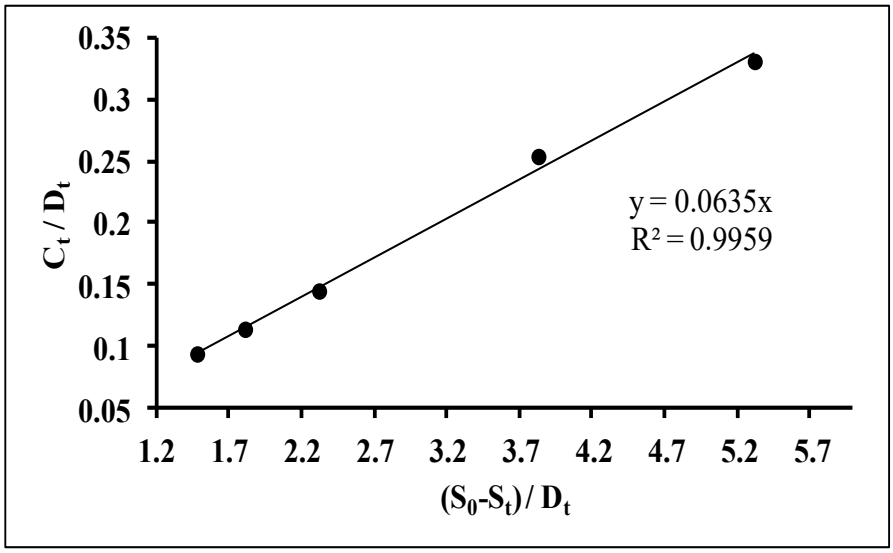


Fig.5.3. Graph between $(S-S_0)/D_t$ and C_t/D_t to determine γ and δ parameters

Linear regression using least square method was applied to fit the model to the experimental data (Wang et al., 2006). The deviation of experimental data from predicted data was estimated in term of residual sum of squares (RSS), which was evaluated using following equation:

$$RSS = \sum_{i=1}^{n}(y_i - \hat{y}_i)^2 \quad \text{.....(5.12)}$$

Where, n = Number of data points, y_i = Observed value, and $\hat{y}_i$= Fitted value

The performance of the model was evaluated in term of correlation coefficient (r), which was determined by following equation:

$$r = \sqrt{1 - \left[RSS / \{\sum_{i=1}^{n}(y_i - \bar{y})^2\}\right]} \quad \text{.....(5.13)}$$

Where, RSS= Residual sum of squares, y_i = Observed value, $\bar{y}$ = Mean value, n = Number of data points

The closeness of correlation coefficients (r) to 1 is an effective and practical measure of the validity of model prediction.

5.3. Results and Discussion

5.3.1. Optimization of Agitation speed and Aeration rate

Agitation rate is one of the indispensable parameter for proper oxygen transfer and homogeneous mixing of the nutrients in fermentation system. The growth profile showed that cell biomass of *Bacillus cereus* MTCC 1305 increased with increase in agitation rate and their maximum level was achieved at 300rpm (Fig5.4a). Similarly, production profile showed that production of L-glutaminase increased with increase in agitation rate up to 300rpm (Fig5.4b). The lower cell growth and low production of L-glutaminase was obtained at higher agitation speed (400rpm) which may be due to generation of rapid pressure fluctuation around the blade and of shear stress created by the blade tips of rushton turbine at high agitation speed (Toma et al., 1991; Nadeem et al., 2007; Jafari et al., 2007). Sucrose consumption profile in Fig5.4c showed that sucrose was utilized with high rate after exponential phase. The rate of consumption of sucrose was found almost similar at all agitation speed (100, 200, 300, and 400rpm). The effect of agitation speed on dissolved oxygen concentration is shown in Fig5.4d. Dissolved oxygen concentration was found to be maintained above 20% saturation upto exponential phase at all agitation speed and hence favored proper cell growth. During late exponential phase, the concentration of dissolved oxygen was maintained upto 20%saturation at higher agitation speed (300, 400rpm), whereas its saturation decreased below 20% at low agitation speed (100, 200rpm). At low concentration of dissolved oxygen (<20% saturation), microorganism could not be able to get sufficient amount of oxygen for their optimum cell growth (Ingle and Boyer, 1976; Atkinson and Mavituna, 1983; Milner et al., 1996; Okada and Iwamatu, 1997; Amanullah et al., 1998). These results showed that high agitation speed (300, 400rpm) would support better cell growth and higher production of L-glutaminase.

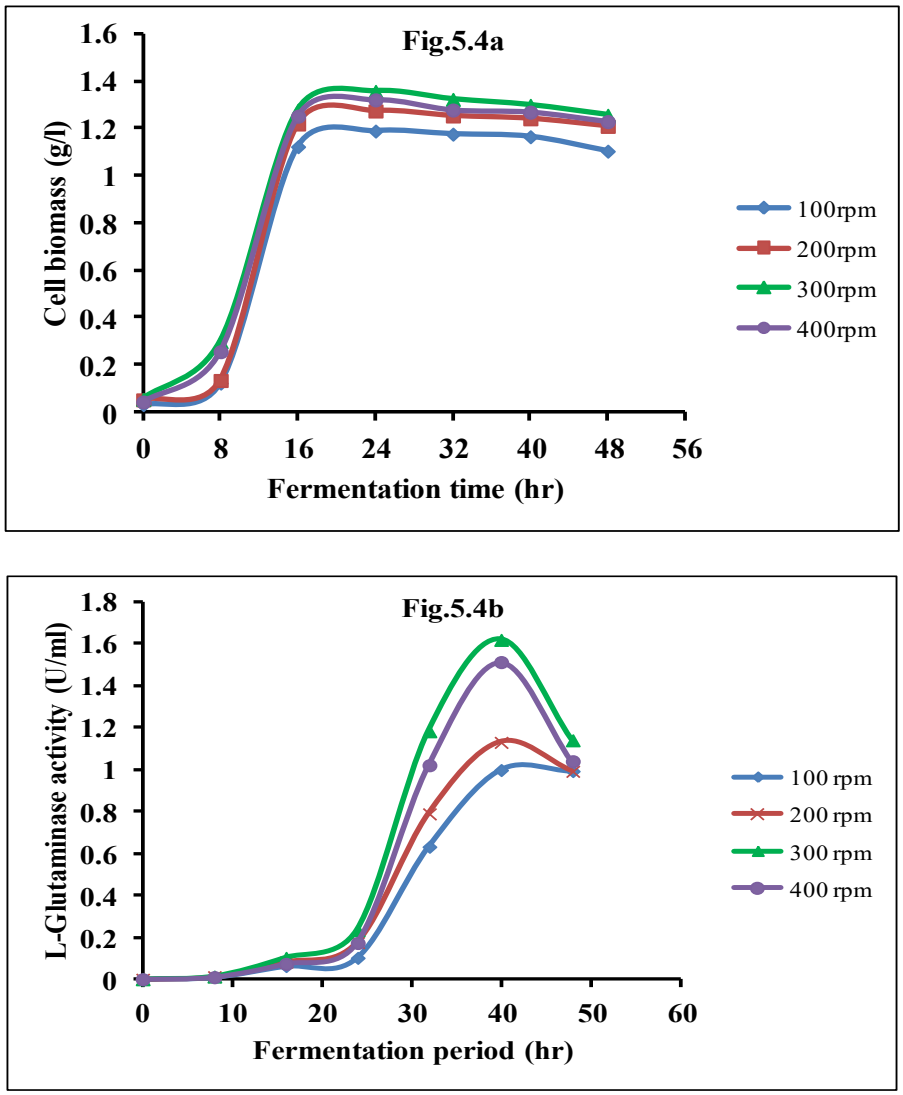
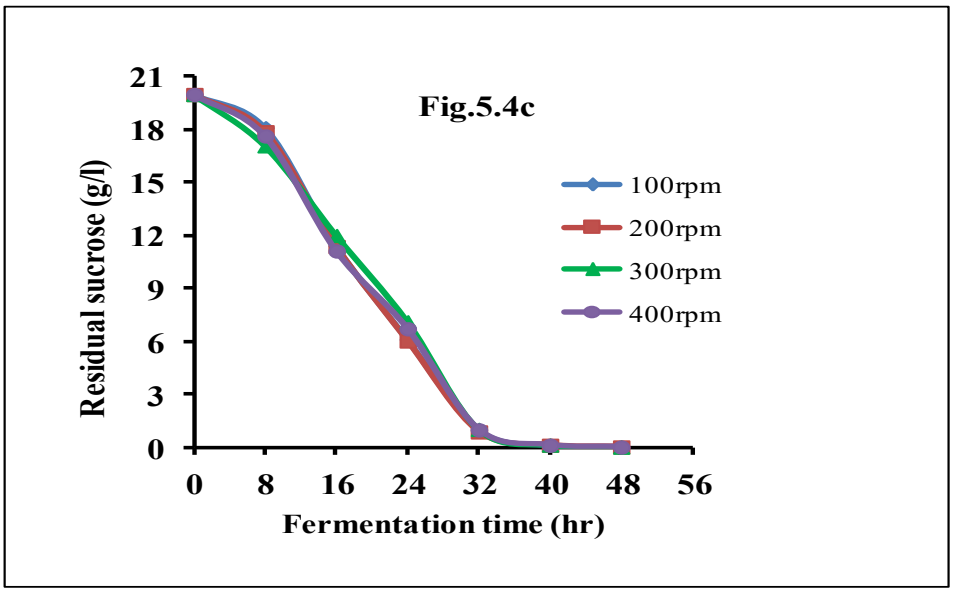


Fig.5.4. Effect of different agitation rates (100-400 rpm) on (a) Cell biomass (g/l); (b) activity of L-glutaminase from *Bacillus cereus* MTCC 1305 in 5litre fermenter



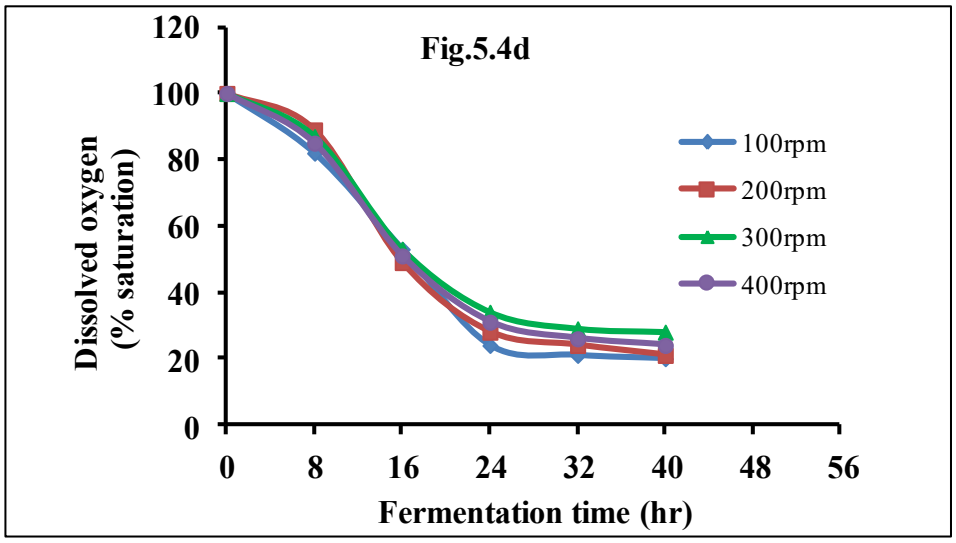
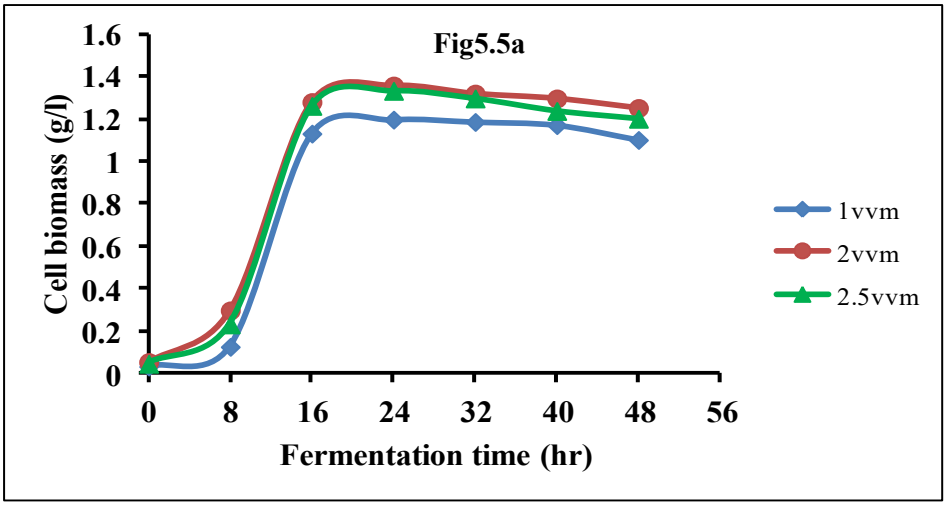
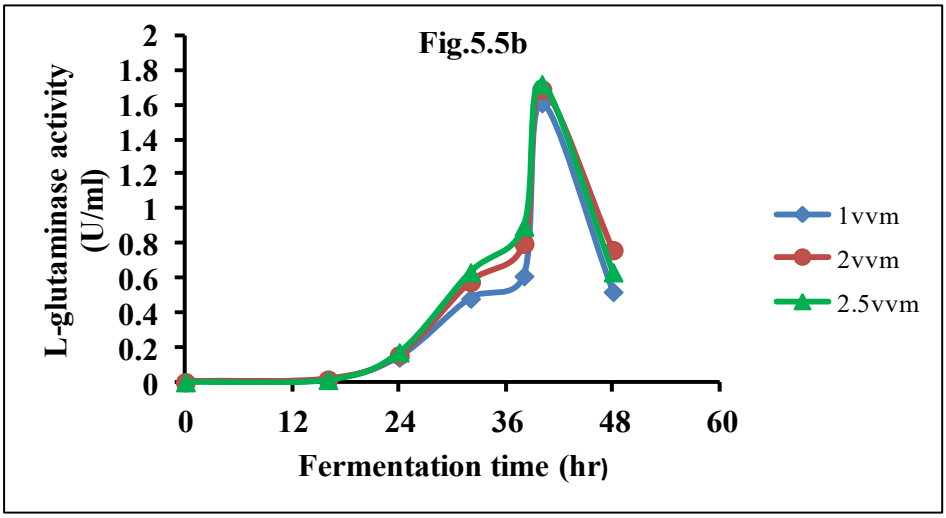


Fig.5.4. Effect of different agitation rates (100-400 rpm) on (c) Sucrose consumption (g/l); (d) Dissolved oxygen (% saturation) of L-glutaminase from *Bacillus cereus* MTCC 1305 in 5litre fermenter

The effects of aeration rates (1.0, 2.0 and 2.5vvm) on cell biomass, L-glutaminase production, and % of saturation of dissolved oxygen at optimized agitation speed of 300rpm are shown in Fig5.5a, 5.5b and 5.5c respectively. Cell biomass was varied with change in aeration rates and maximum cell biomass (4.89g/l) was obtained at 2vvm (Fig5.5a). Cell biomass decreased at higher aeration rate of 2.5vvm that might ascribe inappropriate transfer of oxygen in the growth medium. Impeller 'flooding' (Increase of air flow up in the vessel along the stirrer shaft at higher flow rates with low agitation speed) should be avoided, because an impeller surrounded by air column result in poor mixing, reduced air dispersion and diminished oxygen transfer rates (Doran, 1995). The improper air dispersion and nutrients mixing in the fermentation medium at higher aeration rates can reduce the growth of microorganism in the fermenter. The effect of different aeration rate on the production of L-glutaminase is shown in Fig5.5b. The maximum L-glutaminase activity was obtained at aeration rate of 2.0vvm. No significant variation in activity of L-glutaminase was observed at 2.5vvm but more power would be needed for a higher level of aeration (Papagianni et al., 1999; Feng et al., 2003). Therefore, the optimum aeration rate was selected as 2.0vvm for this fermentation process. The dissolved oxygen concentration profiles were significantly different under different aeration rates (Fig5.5c). At low aeration rate (1.0vvm), dissolved oxygen concentration decreased below 20% saturation after 24hrs and hence caused lower production of L-glutaminase. At higher aeration rate (2.0 and 2.5vvm), concentration of dissolved oxygen was maintained upto 20% saturation over the entire fermentation period of 40hrs and hence favored proper cell growth with higher production of L-glutaminase.





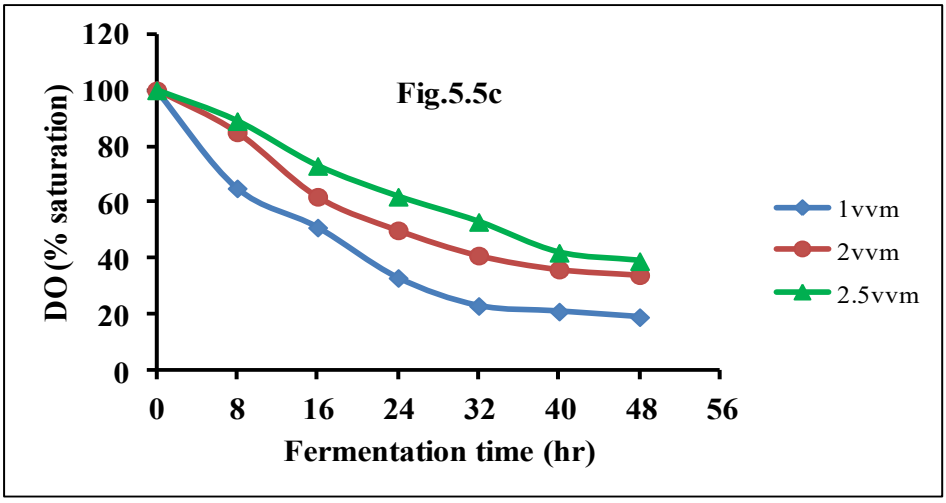


Fig.5.5. Effect of different aeration rates (2.0-2.5vvm) on (a) Cell biomass (g/l); (b) L-glutaminase activity (U/ml); (c) DO (% saturation) during the cultivation of *Bacillus cereus* MTCC 1305 in a 5litre fermenter

5.3.2. Determination of oxygen transfer characteristics and kinetics parameters

Dynamic gassing out method was applied to determine volumetric mass transfer coefficients (K_La), oxygen transfer rate (OTR) and oxygen uptake rate (OUR) during batch fermentation process at optimum agitation speed (300rpm) and aeration rate (2vvm). The values of OUR and OTR were determined from the graph plotted for % of saturation of dissolved oxygen versus fermentation time (Fig5.6). The decline in initial curve following air supply disruption showed the decrease in dissolved oxygen concentration in the fermenter due to oxygen consumption by the microorganisms for their growth (Fig5.6). Specific rate of oxygen consumption over a volume of liquid (QO_2X) was determined by measuring slope of the curve during the absence of air supply. Oxygen uptake rate (OUR) was determined as 3.478mmoldm^{-3}hr^{-1} using the following equation:

$$dC_L/dt = OUR = - (QO_2 . X) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (5.14)$$

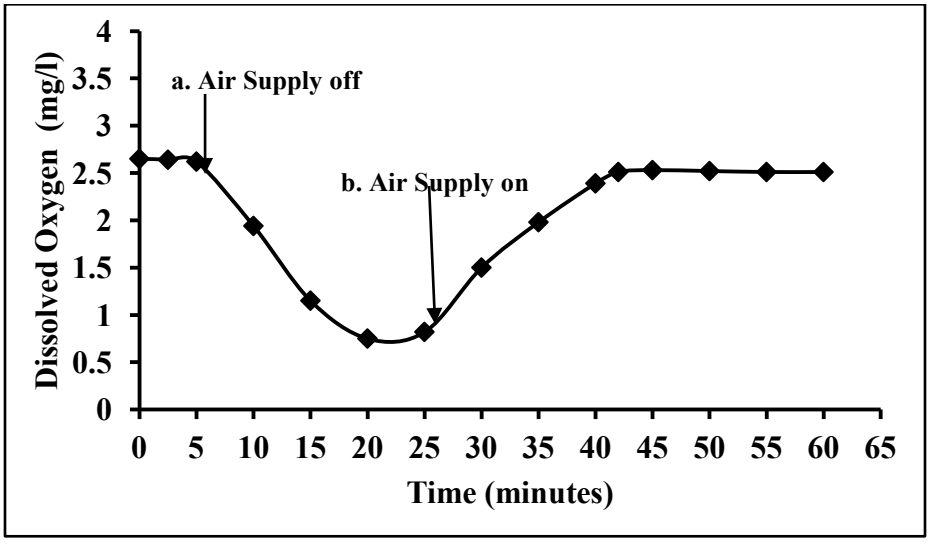


Fig.5.6. Time dependence of % of dissolved oxygen during fermentation of *Bacillus cereus* MTCC 1305 with slope of line between ↓a and ↓b as dC_L/dt = OUR and after ↓b as OTR = dC_L/dt + OUR

On resuming air supply (second phase of graph of Fig5.6), dissolved oxygen concentration (dC_L/dt) increased with fermentation time and OTR was determined as 2.096molm^{-3}hr^{-1} using following equation:

$$OTR = dC_L/dt + OUR \quad \text{.....(5.15)}$$

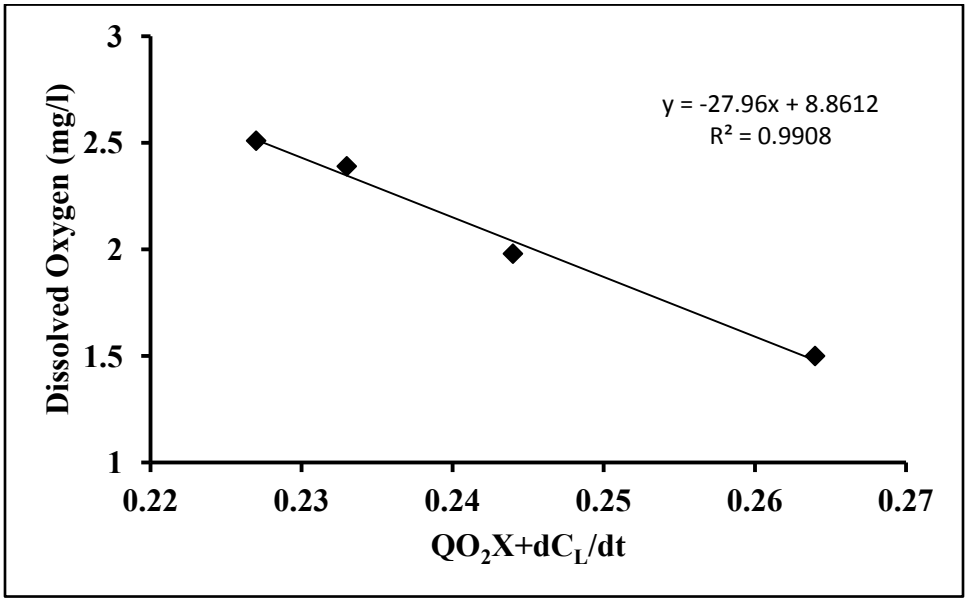


Fig.5.7. Determination of K_La for L-glutaminase production from *Bacillus cereus* MTCC 1305 using graph plotted between (QO_2X+dC_L/dt) versus DO concentration

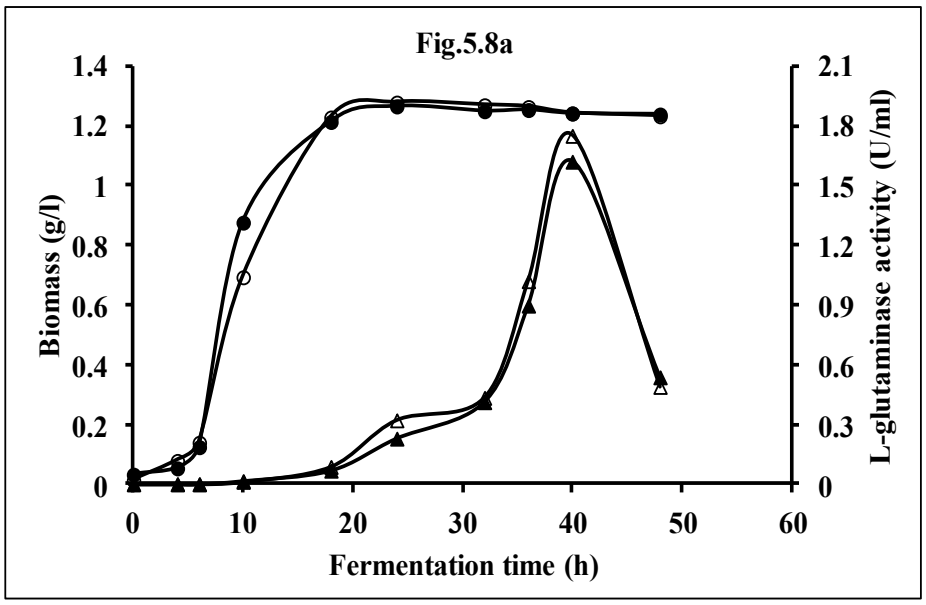
Volumetric mass transfer coefficient (K_La) for batch fermentation of L-glutaminase from *Bacillus cereus* MTCC 1305 was determined as 30.67hr^{-1} from reciprocal of slope of graph plotted between (QO_2X+dC_L/dt) versus DO concentration (Fig5.7). This finding is in agreement of K_La (32.5hr^{-1}) reported for L-glutaminase production from *Bacillus subtilis* RSP-GLU in lab scale fermenter (Satish and Prakasham, 2010). Yield coefficients viz., Yx/s, q_s, and Qs for substrate consumption rate were determined as 0.236 g-cell/g-sucrose, 0.102g-sucrose/g-cells/hr, 0.250g-sucrose/l/hr

respectively. Yield coefficients viz., Yp/s, Yp/x, $q_{p,}$ and Qp of product formation rates were determined as 0.034U/g-sucrose consumed, 0.143U/g-cells, 0.0034U/g-cells/hr, and 0.0084U/l/hr respectively.

5.3.3. Kinetic modelling of cell growth, product formation and substrate consumption

Various mathematical models were proposed for the kinetics of microbial growth and product formation (Ganzle et al., 1998; Richard and Margaritis, 2004). In case of extracellular microbial enzyme production, Logistic, Luedeking-Piret, and modified Luedeking-Piret equations were proposed to describe the time courses of cell growth, product formation and substrate consumption because these models were substantially more accurate than other models.

Bacillus cereus MTCC 1305 showed growth profile with a short lag phase of 4hrs, exponential phase of 20hrs and stationary phase up to 40hrs under optimized fermentation condition as shown in Fig.5.8a. The production profile of L-glutaminase from this strain showed that production of L-glutaminase started during exponential phase and maximum level was achieved in stationary phase (Fig5.8a). Substrate utilization graph in Fig5.8b showed that sucrose was almost consumed by the micro organisms during exponential growth phase.



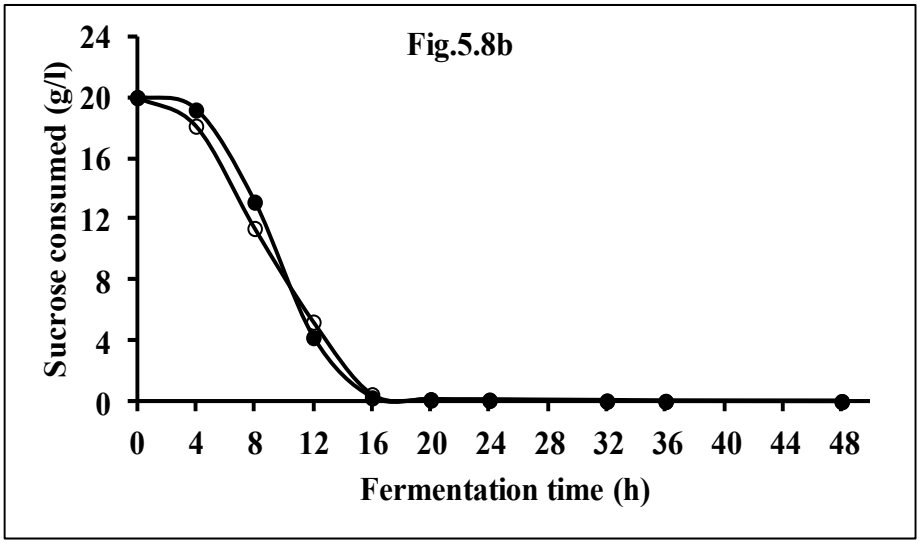


Fig.5.8. Time course of submerged fermentation of L-glutaminase from *Bacillus cereus* MTCC 1305 (a) Biomass profile of experimental data (●) and model predicted data (○), and activity of L-glutaminase profile of experimental data (▲) and model predicted data (△) (b) Sucrose consumption rate profile of experimental data (●) and model predicted data (○).

These kinetic studies showed that microbial cells entered the secondary metabolism process to produce L-glutaminase and did not follow the classical bacterial growth pattern. Logistic equation was applied to develop a kinetic model for cell growth and Mathematica software (version 4.0; Wolfram Research, Inc.) was used to analyze the

model parameters. The validity of model prediction was measured in term of the correlation coefficient (r). The experimental results for growth profile (Fig5.8a), production profile (Fig5.8a) and substrate utilization graph (Fig5.8b) showed good correlation with the proposed calibrated mathematical model with r value as 0.9594, 0.9062 and 0.9976 respectively. Maximum cell growth (X_m) was obtained as 1.278g/l from the model which was similar to experimental value of X_m (1.265g/l) as shown in Fig5.8a. Specific growth rate (μ_{max}) was obtained as 0.4912hr^{-1} which was similar to specific growth rate (μ_{max}=0.41hr^{-1}) reported for L-glutaminase from *Bacillus subtilis* (Chen et al., 2004; Satish and Prakasham, 2010). The activity of L-glutaminase was obtained maximum at fermentation period 40 hrs as shown in Fig5.8a. Luedeking-Piret equation was used to develop a kinetic model for L-glutaminase production. Growth associated constant (α) and non growth associated constant (β) were determined as 0.0048U/g-cell and 0.1625U/g-cell/hr respectively. The higher value of β may indicate that L-glutaminase production occurs throughout the stationary growth phase with a higher rate. The fermentation process of L-glutaminase showed that increase in biomass concentration was accompanied by decrease in residual sucrose concentration which may be due to utilization of sucrose for cell growth and cell maintenance. Modified Luedeking-Piret equation was used to develop a kinetic model for substrate consumption with different fermentation period and constant parameters γ, δ were determined as 0.0631g-sucrose/g-cell and 0.0013g-sucrose/g-cell/hr respectively. The higher value of γ indicates that sucrose is utilized at higher rate with growth of cell during exponential phase.

5.4. Conclusion

The maximum production of L-glutaminase from *Bacillus cereus* MTCC 1305 in 5litre fermenter was achieved at agitation speed 300rpm and aeration rate 2vvm. Dissolved oxygen concentration should be maintained above 20% saturation over the entire fermentation process. Kinetic models were developed using Logistic equation for cell growth, Luedeking Piret equation for L-glutaminase production and modified Luedeking Piret equation for sucrose utilization. Maximum production of L-glutaminase was found at 40hrs fermentation period with maximum specific growth rate (μ_{max}) as 0.4912hr^{-1}. L-glutaminase fermentation is non growth associated process with higher value of non-growth associated constant.