Optimization of Agitation and Aeration Rate for Maximum Production of L-Glutaminase by *Bacillus Cereus* MTCC 1305

Rathindra Mohan Banik and Priyanka Singh
Deaprtment of Biochemical Engineering, Institute of Technology,
Banaras Hindu University, Varanasi, India
*rmbanik@gmail.com

Abstract

Effects of agitation speed and aeration rate on the growth of *Bacillus cereus MTCC 1305*, production of L-glutaminase, sucrose consumption rate and % of saturation of dissolved oxygen were investigated in this study. Cell biomass and L-glutaminase activity was found to be higher with increase in agitation speed and aeration rates. Sucrose consumption rate was found maximum at 300 rpm and 2 vvm in exponential phase of growth. Dissolved oxygen concentration above 20% saturation was maintained consistently over the entire fermentation process at high agitation and aeration rate which results higher production of L-glutaminase. The optimum agitation and aeration parameters were obtained as 300 rpm and 2 vvm respectively. Volumetric mass transfer coefficient and oxygen transfer rate were determined as 30.67 h^{-1} and 2.096 (mol m^{-3} h^{-1}) respectively by dynamic gassing out method under optimized aeration and agitation condition in fermenter.

Key words - Agitation Speed, Aeration Rate, L-glutaminase, *Bacillus cereus MTCC 1305*, Volumetric Mass Transfer Coefficient, Oxygen Transfer Rate.

Introduction

Microbial growth and metabolite formation in bioreactor are greatly affected by nutrient media components and cultural condition like agitation, dissolved oxygen, temperature, inoculum density and incubation time (Gupta *et al.*, 2002). Oxygen shows diverse effects on product formation in aerobic fermentation process by influencing metabolic pathway and changing metabolic fluxes (Calik *et al*., 1998). It has been observed that the respiration rate of aerobic microbe is generally independent of dissolved oxygen above a certain critical level. However, below that level, a small change in the dissolved oxygen may cause a significant physiological alteration in cell metabolism (Hwang *et al.*, 1991). The dissolved oxygen concentration has been found to influence the productivity of several bioactive compounds (Dick *et al.*, 1994; Roman *et al.*, 1994; Kempf *et al.*, 1997). Oxygen could positively influence the product kinetics with increasing values by acting as a direct parameter of product formation (Plihon *et al.*, 1996). It has also been suggested that oxygen could enhance metabolite formation if the enzymatic reaction of product formation is strongly dependent on oxygen (Barberis *et al*., 1997). Supply of oxygen to the growing cell population is the rate-limiting step in many aerobic fermentation processes due to poor solubility of oxygen in the culture medium, which is determined by the oxygen transfer rate and is governed by the volumetric oxygen transfer coefficient ($K_L a$), one of the most important parameters in scaling-up aerobic fermentation processes. Agitation and aeration are considered as most critical parameters used for process scale-up and determination of productivity of the enzyme. It is

essential to devise a scale-up strategy with desired levels of agitation and aeration in the fermenter, which in turn give comparable or better yields relative to those obtained from shake flask studies. This could minimize the production cost and optimize the cost-effectiveness for the overall production process (Feng *et al*., 2003).

The current study focuses on developing a scale-up process in 5 L fermenter for glutaminase production from *Bacillus cereus MTCC 1305*. Glutaminase (glutamine aminohydrolase), which catalyzes the hydrolytic degradation of L-glutamine to L-glutamic acid and ammonia, is widely distributed from microorganisms to mammals. There has been renewed interest in this enzyme with respect to its use as a therapeutic agent, as a biosensor for detection of glutamic acid and glutamine, and in the food industry for flavor generation. It is a potent antileukaemic drug (Roberts *et al*., 1970; Pal *et al*., 1992) and a flavor-enhancing additive in the production of fermented foods (Sabu *et al*., 2000). Its commercial importance demands not only a search for newer and better yielding microbial strains, but also economically viable bioprocesses for its large-scale production. In microorganisms, glutaminases have been reported from many species including gram positive and gram-negative bacteria, yeasts, and fungi (Nandakumar *et al*., 1999, 2003). There are many reports on the screening of high L-glutaminase producing microorganisms and effect of nutrients on glutaminase production in submerged fermentation (Klein *et al*., 2002; Moriguchi *et al*., 1994; Weingand *et al*., 2003; Iyer *et al*., 2008; Chinae *et al*., 2001; Baskar *et al*., 2009) and solid state fermentation (Ashraf *et al*., 2009; Kashyap *et al*., 2002; Hesseltine *et al*., 1972; Prabhu *et al*., 1997) in shake flask studies. Little information is available about the optimum fermentation conditions in fermenter which is important for full-scale production of L-glutaminase (Iyer *et al*., 2009). In view of this, we have screened L-glutaminase producing strain, *Bacillus cereus MTCC 1305*, which is a potential industrial strain for L-glutaminase production because of its high yield, and its fermentation condition in shake flask has been studied. The objective of this study is to investigate the desired combination of aeration and agitation that would yield the highest L-glutaminase production by *B. cereus MTCC 1305* in a 5 L fermenter.

Materials and Method

Microorganism and culture maintenance conditions

The bacterium used throughout the study, *Bacillus cereus MTCC 1305*, was procured from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. This organism was grown in 50 ml media containing (g/l): beef extract 1.0, yeast extract 2.0, NaCl 5.0, peptone 5.0, and agar 15.0 (pH 7.0) at 35˚C. The organism was sub cultured every month and maintained at 4±1°C. The production of L-glutaminase has been studied in 100ml semi-synthetic medium (pH of 7.5) containing (g/l): glucose 2, $Na_2HPO_4 \cdot 2H_2O$ 6.0, KH_2PO_4 3, NaCl 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, $CaCl_2 \cdot 2H_2O$ 0.015 and peptone 2.0. The inoculum was prepared by adding a loop full of freshly prepared pure culture of slant into 50 ml growth media at 35ºC, 180 rpm for 10–12 h. Fermentation was carried out by adding 2% inoculum to 100ml production medium and incubating in orbital shaker at 35°C, 180 rpm for 40 h. Growth and production profile for culture was obtained after withdrawing samples at regular intervals and analyzed for biomass and glutaminase activity.

Production of L-glutaminase in fermenter

5 L fermenter (Scigenics Priivate Ltd, India) containing 2 L production medium (pH 7.5) was used to study the optimum aeration rate and agitation rate at 35 ± 1° C. The fermenter was inoculated with 2% (v/v) of inoculums and agitation was provided with a pair of six-bladed Rushton impellers (impeller

diameter was one third of the vessel diameter). Four side-walled equidistant baffle plates were used to prevent vortex formation. To study the effect of agitation speed, fermentations were carried out at different agitator speeds (100, 200, 300 and 400 rpm) and the dissolved oxygen (DO) was maintained above 20% by adjusting air flow rate. Glass electrode immersed in the fermentation broth was used to measure pH of media. Effect of different aeration rate (0.5, 1.0, 1.5, 2.0 and 2.5vvm) was studied on microbial growth, glutaminase production, sucrose consumption rate and % of saturation of dissolved oxygen. Samples from fermenter were drawn at regular intervals and analyzed for biomass and enzyme activity.

Estimation of cell biomass, L-glutaminase activity and sucrose remain in media

Cell biomass in the fermentation broth was quantified by dry-cell weight analysis and by measurement of the optical density of the broth. For dry weight determinations, the cells were recovered by centrifugation at 4°C, 8000 rpm and washed twice with distilled water. The recovered biomass was dried to constant weight in an oven at 80°C for 24 h. Absorbance was observed at 600 nm using a Shimadzu UV/Vis spectrophotometer 1700. Sucrose concentration in the broth during fermentation was estimated by phenol sulfuric acid method (Hanson, 1981). 1ml phenol and 5ml sulfuric acid were added to 1 ml of broth taken in test tube and cooled at room temperature. Absorbance for the solution was measured at 490nm The standard graph was prepared by using 0.1% of sucrose solution (Fig. 1).

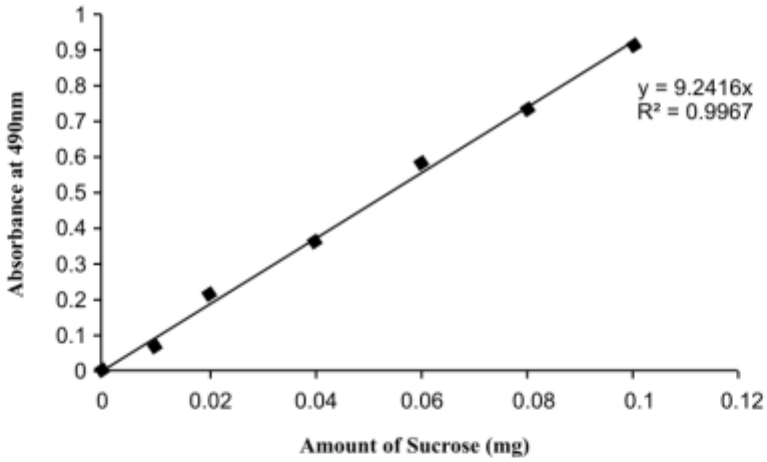

Figure 1: Standard curve for sucrose

Glutaminase activity was estimated by modified method of Imada (1973) in which reaction mixture (pH 7.5) containing 0.5 ml of crude extract of enzyme, 0.5 ml of 0.04 M L-glutamine solution, 0.5 ml of distilled water and 0.5 ml of 0.1 M phosphate buffer was incubated at 37˚C for 30 min. The reaction was terminated by the addition of 0.5 ml of 1.5 M Trichloroacetic acid to 0.1 ml of the reaction mixture. 3.7 ml of distill water was added to 0.1ml reaction mixture and then 0.2 ml of Nessler's reagent was added. The absorbance was measured at 450nm after two minutes. Reagent blank and substrate blank was also prepared subsequently. Standard graph using NH_4Cl (12×10^{-4}M) was plotted as the standard for computation of the concentration of ammonia (Fig. 2). One unit of glutaminase activity (Curthoys, 1995) was defined as enzyme required for deamination of 1.0 µmol of glutamine per min per ml of enzyme solution at pH 7.5, 35˚C under the above mentioned conditions.

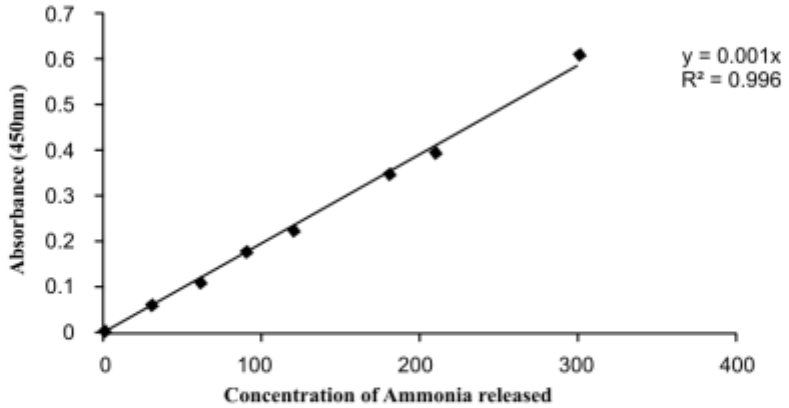

Figure 2: Standard curve for glutaminase assay by using Ammonium chloride

Determination of oxygen transfer characteristics and kinetics parameters

During cultivation, the OUR, OTR and K_La were determined by dynamic gassing-out techniques (Gracia, 2000 and Rainer, 1990). The dissolved oxygen changes (dC_L/dt) in batch fermentation were based on mass balance equation as given below:

$$dC_L/dt = OTR\text{-}OUR = K_La.(C^* - C_L) - (QO_2.X) \ldots\ldots\ldots\ldots\ldots\ldots 1$$

(μM)

Where, C_L =Dissolved oxygen concentration in the liquid phase (mmol l^{-1}) , C* = Saturated dissolved oxygen concentration equilibrated to the gaseous oxygen partial pressure (mmol l^{-1}), K_La = volumetric oxygen transfer coefficient (h^{-1}), QO_2 = Specific respiration rate (mmoles of oxygen g^{-1} biomass h^{-1}), X = Concentration of biomass (g/l), OTR = Oxygen transfer rate, OUR = Oxygen uptake rate.

OUR determination

The air supply was interrupted (OTR=0) at a certain time during fermentation for the determination of OUR. The dissolved oxygen tension (DOT) values decreased linearly with the cultivation time due to cellular respiration and slope of the graph (DOT vs. time) represented

OUR. Changes in the DOT per unit time were worked out according to the equation given below:

$$dC_L/dt = OUR = -(QO_2.X) \ldots\ldots\ldots\ldots\ldots\ldots 2$$

OTR determination

For OTR determination, the air supply was restarted, resulting in increase of DOT (%). OTR values were obtained by the following equation:

$$OTR = dC_L/dt + OUR \ldots\ldots\ldots\ldots\ldots\ldots 3$$

K_La determination

K_La_a value was determined by considering the final steady state of dissolved oxygen concentration (C_L') after re-oxygenation. When $C_L' = C_L$ and $dC_L/dt = 0$, the Eq.1 can be rearranged as given below:

$$QO_2.X = K_La.(C^* - C_L') \ldots\ldots\ldots\ldots\ldots\ldots 4$$

Substituting the value of QO2. X in Eq.1 with KLa.(C*- C_L') from the Eq.4:

$$dC_L/dt = K_La.(C_L' - C_L) \ldots\ldots\ldots\ldots\ldots\ldots 5$$

Assuming K_La is constant with time, the Eq.5 could be integrated in the time t_1 and t_2 using the integration rules. Then the equation for KLa is given as:

$$K_La = \ln((C_L'-C_{L1})/(C_L'-C_{L2}))/ t_2- t_1 \text{ ……...6}$$

The K_La value was calculated from slope by plotting $\ln(C_L'-C_{L1})/(C_L'-C_{L2})$ against time $(t_2 - t_1)$ according to Eq.6. The values of OUR, OTR and KLa were investigated at various aeration and agitation rates in the fermenter. L-glutaminase yield coefficients for substrate utilization and product formation rate were determined as follows:

$Y_{p/s}$: Enzyme produced (U)/g sucrose consumed

$Y_{p/x}$: Enzyme produced (U) /g cells

$Y_{x/s}$: g cells/g sucrose consumed

q_s : g sucrose consumed/g cells/h

Q_s : g sucrose consumed/L of cultivation medium/h

q_p : Enzyme produced (PU)/g cells/h

Q_p : Enzyme produced (U)/L of cultivation medium/h

Results and Discussion

Effect of agitation on growth and L-glutaminase production

Agitation rate is one of the indispensable parameter for proper oxygen transfer and homogeneous mixing of the nutrients in fermentation system. Therefore, the effects of four different agitation rates on cell growth, enzyme yield, sucrose consumption and dissolved oxygen, were shown in Fig. 3 (a-d). Cell biomass and production of L-glutaminase increased with increase in agitation rate. Maximum biomass and production of L-glutaminase were found as 3.9g/l and 1.619U/ml at 300rpm. The reduction of cell growth and L-glutaminase production at higher agitation rate may be due to sheer stress and heterogeneous mixing effects (Nadeem, 2007). High agitation rate particularly with high airflow rate used in this case could be toxic to the cells (Toma, 1991). Similarly, the maximum sucrose consumption was observed at 300 rpm (Fig. 3c), indicating a proportional relationship among substrate consumption, cell growth and enzyme yield.

The dissolved oxygen concentration profiles were different for the four levels of agitation (Fig. 3d). Dissolved oxygen concentrations could be consistently maintained at above 20% saturation over the entire fermentation process at 300 and 400 rpm. However, dissolved oxygen concentration was notably lower at 100 and 200 rpm and even reached <5% saturation at the beginning of the log-growth phase. The oxygen limitation resulted in a lower cell density and shorter log-growth phase at low agitation speed which results low production of enzyme. It has been reported that metabolism pathways might be changed with variation in critical dissolved oxygen concentration (Atkinson, 1983; Amanullah, 1998; Okada, 1997). It has been reported (Ingle, 1976) that when dissolved oxygen fell below 20%, an immediate decrease in enzyme biosynthesis would occur. It has been also proposed (Milner, 1996) that when dissolved oxygen concentration falls below a critical level, cell respiration could be shifted from dissolved oxygen to the gaseous form. A higher agitation speed increased the amount of dissolved oxygen and dispersion of macromolecules in the medium and result better growth and higher enzyme production. However, the shearing effect induced by the higher agitation speed on the cells and enzyme inactivation may contribute negatively towards cell growth and enzyme stability (Manolov, 1992; Shioya, 1999).

Oxygen limitation is thought to have contributed to the lower growth and enzyme activity observed at low agitation rate. The study indicated that 300 rpm was best for growth of *B.cereus* MTCC 1305 and production of L-glutaminase.

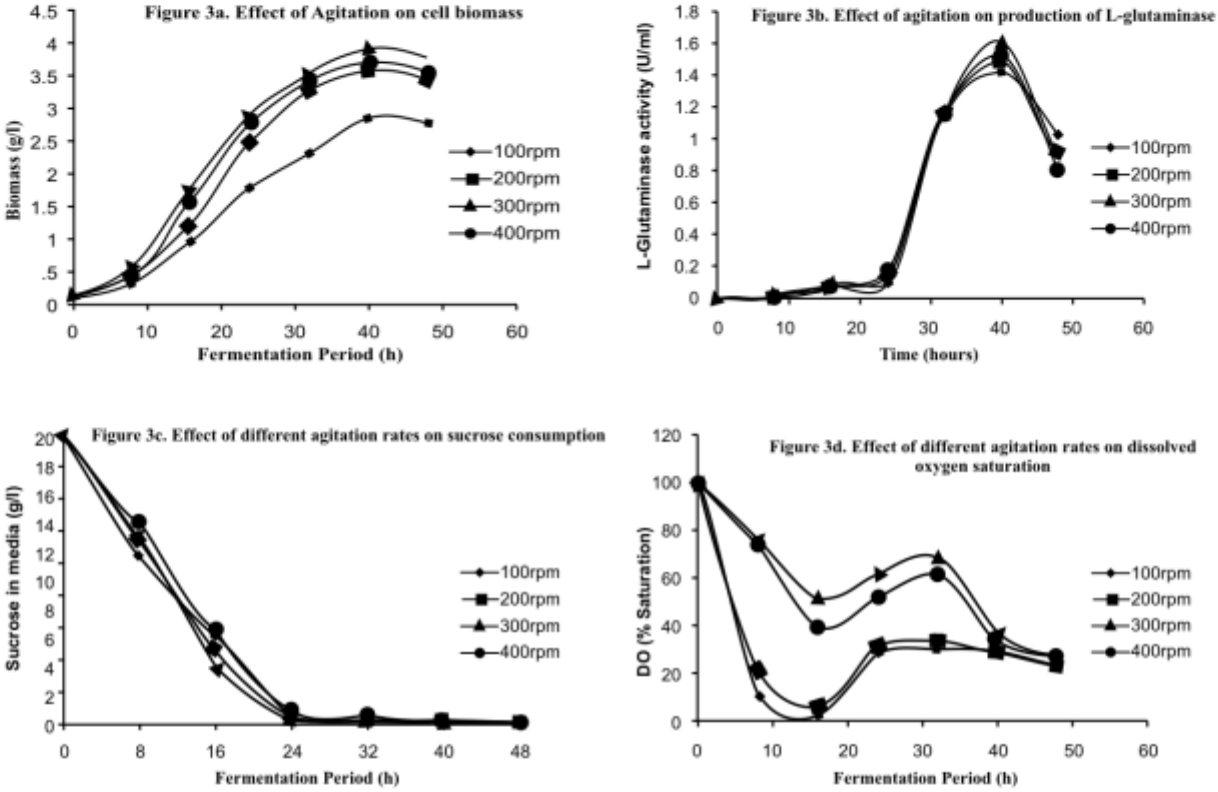

Figure 3: Effect of different agitation rates (100-400 rpm) a: Cell biomass (g/L); b: L-glutaminase activity (U/ml); c: Sucrose consumption (g/l); and d: DO (% sturation) during the cultivation of Bacillus cereus MTCC 1305 in a 2 L fermenter. The cultivation was carried out at 37˚C with a constant aeration rate of 1 vvm.

Effect of aeration rates on cell biomass and enzyme production

The effects of aeration rates from 0.5 vvm to 2.5 vvm on cell biomass (g/L), L-Glutaminase production (U/ ml), sucrose consumption and DO % during fermentation of *B. cereus MTCC 1305* at optimized agitation speed of 300 rpm are illustrated in Fig. 4 (a-d). Sampling was performed at 8 h interval for each aeration rate. The cell biomass values varied with change in aeration rates and maximum cell biomass (4.89g/l) was found at 2 vvm (Fig. 4a). This indicates that increase in aeration rates would yield a higher cell biomass. However, a decrease in cell biomass concentration was noted at 2.5 vvm aeration rate that might ascribe to 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, no longer contact the liquid properly, resulting in poor mixing, reduced air dispersion and diminished oxygen transfer rates (Doran , 1995) . These findings indicate that improper air dispersion and nutrients mixing in the fermentation medium at higher aeration rates can reduce the growth of microorganism in the fermenter. Similar pattern of increase or decrease in

L-glutaminase production with aeration rates was observed in Fig. 4b. The maximum activity of L-glutaminase had no significant difference at aeration rates of 2.0 and 2.5 vvm. Since more power would be needed for a higher level of aeration, 2.0 vvm was considered to be the optimum aeration rate in this study. Similar effects of high aeration rates on enzyme activity level in fermentation have been reported by some investigators (Papagianni, 1999). The total sucrose contents were also measured at each aeration rate at 8 h intervals and maximum consumption rate was found at 2 vvm in exponential phase of growth (Fig. 4c). However, no significant sucrose consumption was found during stationary growth phases of *B. cereus MTCC 1305.*

The dissolved oxygen concentration profiles were significantly different under different aeration rates (Fig. 4d). At a low aeration rate of 0.5 vvm and 1.0 vvm, dissolved oxygen concentration was between 10 and 20% saturation for most of the time. In contrast, dissolved oxygen concentrations were found to be above 20 and 60% saturation at the higher aeration rates of 1.5, 2.0 and 2.5 vvm, respectively. Oxygen limitation may be the reason for the lower cell mass and enzyme activity at the lower aeration rate. Thus, dissolved oxygen concentration above 20% saturation is necessary to satisfy oxygen demand of *B. cereus MTCC 1305* and to achieve a high level of L-glutaminase activity. These results indicated that aeration rate could influence dissolved oxygen concentration significantly, which in turn would affect cell growth, enzyme activity and substrate utilization.

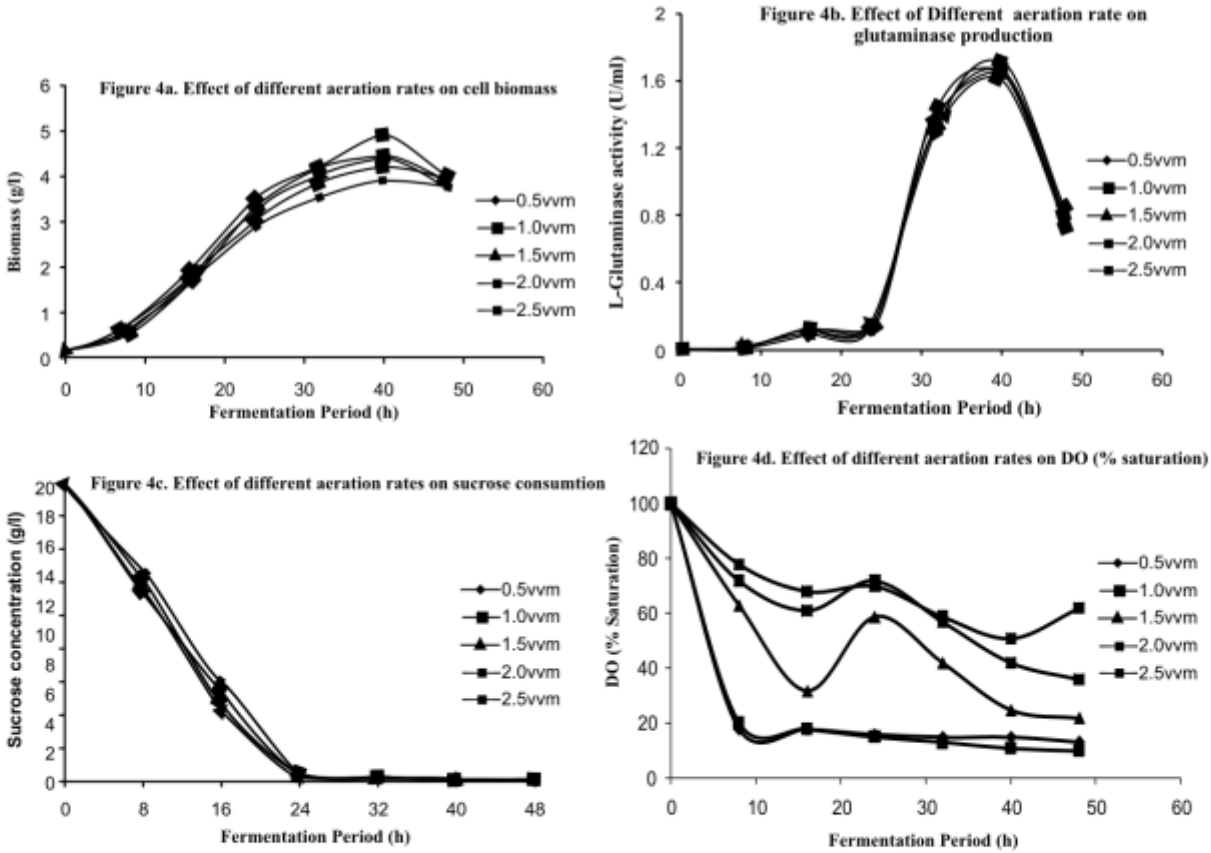

Figure 4: Effect of different aeration rates (0.5-2.5 vvm) on a: Cell biomass (g/L); **b:** L-glutaminase activity (U/mL); **c:** Sucrose consumption (g/L); **d:** DOT % during the cultivation of Bacillus cereus MTCC 1305 in a 2 L fermenter. The cultivation was carried out at 37˚C with a constant agitation rate of 300 rpm.

Determination of oxygen transfer characteristics and kinetics parameters

The dynamic gassing out method applied to find volumetric mass transfer coefficients (K_La), oxygen transfer rate (OTR) and oxygen uptake rate (OUR) during batch fermentation process at various aeration and agitation rates (Fig. 5). The decline in initial curve following air supply disruption showed the decrease in DOT in the fermenter due to oxygen consumption by the cells for their growth. The later part of the curve showed increase in DOT in the fermenter due to air supply being restarted while the oxygen was still consumed by the cells, indicating change in DOT (%) in the fermenter (dC_L/dt).

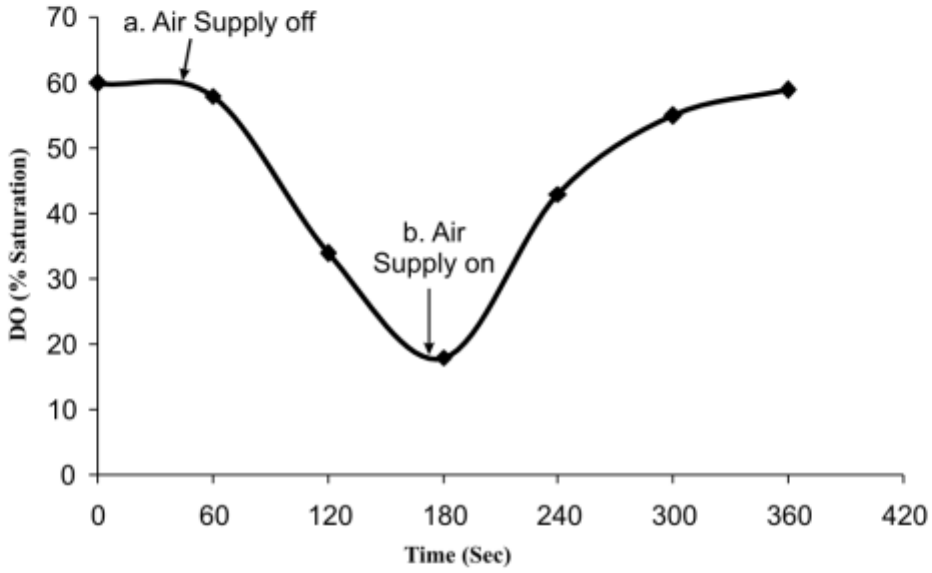

Figure 5: Time dependence of DOT (%) determined during ferementation of B.cereus MTCC 1305 at 2 vvm and 300 rpm in 5 L fermenter. The slope of line between ↓a and ↓b represents dC_L/dt = OUR and after ↓b OTR = dC_L/dt + OUR respectively.

The value of oxygen transfer rate can be measured from OUR and dC_L/dt determined by Eq.2. These parameters are important for the determination of K_La (Eq.6) and are widely used for the control of aeration and agitation rates in aerobic fermentation processes. The effects of various flow rates on volumetric mass transfer coefficients, oxygen transfer rates and operational fermentation parameters are summarized in Table 1. KLa and OTR values for batch fermentation of L-Glutaminase at optimum aeration rate of 2 vvm and agitation speed of 300 rpm were obtained as 30.67 h^{-1} and 2.096 (mol m^{-3} h^{-1}) respectively. Yield coefficients for substrate consumption rate and product formation rates were determined and values are shown in Table 1.

Table 1: Values of KLa, OUR, OTR and enzyme yield coefficients in a bioreactor at optimum agitation speed 300 rpm and aeration rate 2 vvm

KL_a (h^{-1})	OUR (mmole $dm^{-3}h^{-1}$)	OTR (mol m^{-3} h^{-1})	$Y_{p/s}$	$Y_{p/x}$	$Y_{x/s}$	q_s	Q_s	q_p	Q_p
30.67	3.478	2.096	0.034	0.143	0.236	0.102	0.250	0.0034	0.0084

Conclusion

The study on effects of agitation and aeration rate on microbial growth, enzyme production, dissolved oxygen and sucrose consumption can play an important role in improving microbial growth and enzyme production in batch fermenter. The correlation of volumetric mass transfer coefficient, oxygen transfer rate and yield coefficients could be used as an instructive tool in scale up process of enzyme production.

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