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Effect of Aeration and Agitation Rates on Alkaline Protease Production by *Bacillus licheniformis* UV-9 Mutant

[Havalandırma ve Çalkalama Hızının *Bacillus licheniformis* UV-9 Mutant Ürünü Olan Alkalen Proteaz Üretimine Etkisi]

[1]Muhammad Nadeem,

[1]Javed Iqbal Qazi,

[2]Shahjahan Baig

[1]Biotechnology and Food Research Center, PCSIR Laboratories Complex, Lahore-54600, Pakistan
[2]Department of Zoology, University of the Punjab, Lahore-54590, Pakistan

Yazışma Adresi
[Correspondence Address]

Muhammad Nadeem

Senior Scientific Officer,
Biotechnology and Food Resaerch Center,
PCSIR Laboratories Complex,
Lahore-54600, Pakistan
Tel: ++92-42-9230689/296
Fax: ++92-42-9231835
E-mail: mnadeempk@yahoo.com

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Purpose: The aim of the present study was to investigate the most suitable value of volumetric mass transfer coefficient for the maximum yield of alkaline protease by B. licheniformis UV-9.

Material and Methods: The effects of various aeration (0.5 to 2.5 vvm) and agitation rates (300-700 rpm) on cell growth, glucose consumption and protease production by B. licheniformis UV-9 were studied in a 2 L stirred tank bioreactor. Volumetric mass transfer coefficient values were measured by dynamics gassing-out techniques at each aeration and agitation rate.

Results and Conclusion: Maximum cell biomass (3.41 g/L) and protease yield (1270.20 PU/mL) were obtained at aeration rate of 2 vvm and agitation speed of 500 rpm. Volumetric mass transfer coefficient and oxygen transfer rate were found to be highly dependent on aeration and agitation rates. Their values in the fermented broth increased with the increase of aeration and/or agitation rates. However, the values of kinetic parameters were found maximum at 72 h-1 of volumetric mass transfer coefficient and 3.90 mol m-3 h-1 of oxygen transfer rate, corresponding to 2 vvm and 500 rpm. This correlation of volumetric mass transfer coefficient and yield coefficients would be used as an instructive tool in scale up process of protease production.

Key Words: Volumetric mass transfer coefficient, oxygen transfer rate, protease yield

Amaç: Çalışmada B. licheniformis UV-9 tarafından üretilen alkalen proteazın yüksek düzeyde eldesi için, hacimsel kütle transferi katsayısının en uygun olduğu değerlerin belirlenmesi amaçlanmıştır.

Yöntemler: Farklı havalandırma (0.5-2.5 vvm) ve çalkalama (300-700 rpm) koşullarının hücre büyümesine, glukoz tüketimine ve proteaz üretimine olan etkisi 2 L'lik biyoreaktörler kullanılarak incelenmiştir. Hacimsel kütle transfer katsayısı her bir havalandırma ve çalkalama koşulu için olmak üzere "dynamic gassing-out" tekniği kullanılarak ölçülmüştür.

Bulgular ve Sonuç: En fazla hücre kütlesi (3.41 g/L) ve proteaz verimi (1270.20 PU/mL) 2 vvm havalandırma ve 500 rpm çalkalama koşullarında elde edilmiştir. Hacimsel kütle transfer katsayısı ve oksijen aktarım hızının havalandırma ve çalkalamaya yüksek oranda bağlı olduğu bulunmuştur. Bu değerler fermente besi ortamında havalandırma ve çalkalama hızlarının artması ile parallel olarak artmaktadır. Bununla birlikte, 2 vvm havalandırma ve 500 rpm çalkalama uygulandığında, kinetik parametreler, 72 h-1 hacimsel kütle transfer katsayısı ve 3.90 mol m-3 h-1 oksijen aktarım hızı olmak üzere en yüksek değere ulaşmaktadır. Hacimsel kütle transfer katsayısı ve verim katsayıları arasındaki bu ilişki proteaz üretiminin arttırılması işleminde öğretici olarak kullanılabilir.

Anahtar Kelimeler: Hacimsel kütle transfer katsayısı, oksijen aktarım hızı, proteaz verimi

Introduction

Proteases constitute more than 65 % of the total commercial enzyme market (1). These enzymes have wide applications in several industrial sectors, particularly in the food, pharmaceutical, chemicals, detergent and leather processing industries (2). Among all proteases, alkaline proteases used as detergent additives are primarily indispensable in nature and account for 40 % of the total worldwide enzyme sales. The trend of alkaline protease exploitation in various industrial applications is expected to increase in near future (3,4). Microbial proteases can be produced from bacteria, fungi and yeasts using many processes like submerged fermentation and solid state fermentation (5,6). Among bacteria, the genus *Bacillus* is the most important group of industrial microorganisms that are, in fact, known as micro bioreactor within the bioreactor (7). The production of extracellular proteases by microorganisms in a bioreactor is greatly influenced by medium components and physical factors such as aeration, agitation, temperature, inoculum density, dissolved oxygen and incubation time (4,8,9). Oxygen has diverse effects on product formation in aerobic fermentation processes by influencing metabolic pathways of organisms (10). The dissolved oxygen (DO) concentration becomes a rate limiting factor in many aerobic fermentation processes due to poor solubility of the oxygen in the cultivating medium or due to high oxygen uptake rate (OUR) of fast growing microorganisms. Oxygen transfer rate (OTR) is the most important parameter, dependent on agitation speed and aeration rate in bioreactors, and plays a significant role in determining the productivity of fermentation process (11,12). The OTR could be affected by several factors such as geometry and characteristic of the vessel, liquid properties, dissipated energy in to the fluid, biocatalyst properties, medium concentration and morphology of the microorganisms (13). The availability of oxygen, determined by OTR, is also governed by the volumetric oxygen transfer coefficient (K_La) and the concentration of DO in the growth medium (14). The fixing of K_La values has been commonly used criterion for scale up of aerobic fermentation processes (15,16). The K_La values ensure a mass transfer capability of the process that can cope with oxygen demand of the culture and often serve to determine the efficiency of bioreactors and mixing devices as well as an important scale-up factor from small cultivation to large scale production (13). However, very little attention has been paid to study the role of aeration rate, agitation speed and K_La values in the production of extracellular protease production. Therefore, the aim of the present research work was to investigate the appropriate aeration and agitation rates for alkaline protease production in a stirred tank bioreactor by *Bacillus licheniformis* UV-9. The appropriate relationships among oxygen transfer rate (OTR), oxygen uptake rate OUR, volumetric mass coefficient (K_La) and yield coefficients of the alkaline protease were also established for further scale up study.

Materials And Methods

Microorganism

Bacillus licheniformis N-2 isolated from decaying organic soil (17) was mutated by UV irradiations. A UV treated strain *Bacillus licheniformis* UV-9 selected as a hyper-proteolytic mutant after extensive screening study (data not shown) was used for alkaline protease production in a 2 L lab scale bioreactor. *Bacillus licheniformis* UV-9 mutant was grown on nutrient agar (Oxoid) at 37 ^{o}C for 24 h and then preserved at 4 ^{o}C. The preserved culture was revived after every 4 weeks.

Inoculum preparation

Inoculum was prepared by transferring a loopful of 24 h old culture into 30 mL inoculum medium in 250 mL Erlenmeyer flask. The inoculum medium comprised of glucose 0.5 %, soybean meal (1 %), K_2HPO_4 (0.5 %), $MgSO_4.7H_2O$ (0.05 %), NaCl (0.05 %) and $CaCl_2.2H_2O$ (0.05 %). The pH of the inoculum medium was adjusted at 10 with 1 N HCl/NaOH before sterilization (121 ^{o}C, 15 min). The inoculated medium was incubated at 37 ^{o}C for 24 h in water bath shaker (Eyela, Japan) for the proliferation of bacteria up to 10^{8-10} CFU/mL.

Total viable count

Total viable count was determined according to the method described by Eaten *et al.* (18). The sample was diluted serially by transferring 1 mL of sample into 9 mL sterile Butterfield's phosphate buffer (pH 7.2). One mL of each dilution was poured into sterilized Petri plates, mixed properly with plate count agar (Oxoid) and allowed to settle down. After that, the plates were incubated at 35 ^{o}C for 48 h and then counted the bacterial colonies in each plate.

Estimation of dry cell biomass

The biomass content was determined by measuring the dry weight from a known amount of sample. The sample was centrifuged at 9000 x g for 10 min at 4 ^{o}C and then washed with sterilized physiological solution three times to remove the suspended particles. The washed sample was dried at 105 ^{o}C till the constant weight was obtained.

Bioreactor description and fermentation studies

The bioreactor studies were carried out in a 2 L lab scale bioreactor (B. Braun, Germany) with 1.5 L working volume. The fermentor is equipped with digitally controlled pH electrode, temperature probe, polographic DO electrode (Ingold, Switzerland) and two six-blade Rushton turbine impellers (5.2 cm diameter), fixed on the agitator shaft above 3.2 cm air sparger. The pH electrode was calibrated by using standard buffers (Fluka) at pH 7 and 9 prior to the sterilization of fermentor (121 ^{o}C for 20 min). However, the calibration of DO electrode was conducted after sterilization by sparging pure nitrogen gas

for 0 % reading following by the air until 100 % saturation was achieved. The foam was controlled manually by adding few drops of silicon based antifoam (Sigma) at the time of foaming.

Effect of different aeration and agitation rates on protease production

In the initial phase, fermentor was operated with 1.5 L of a growth medium to optimize the aeration and agitation rates for the maximum yield of alkaline protease. The growth medium for these experiments differed from earlier mentioned inoculum medium only in the concentration of glucose which was 1 % (w/v) instead of 0.5 % (w/v). The pH of the growth medium was adjusted at 10 with 1 N HCl/NaOH before sterilization at 121 ^{o}C for 20 min. Glucose was autoclaved separately (121 ^{o}C, 15 min) and mixed with the growth medium at the time of inoculation. Five aeration (0.5-2.5 vvm) and agitation (300-700 rpm) rates were studied by inoculating the fermentor with 30 mL of 24 h old inoculum. The inoculated fermentor broth was cultivated at 37 ^{o}C for 36 h and the samples were taken after every 3 h for the determination of cell biomass, glucose consumption and alkaline protease yield. The pH was kept constant at 10 with 2 N HCl/NaOH during entire fermentation process through auto-pH control system.

Effect of different aeration and agitation rates on OUR, OTR and K_La values

During cultivation, the OUR, OTR and K_La were determined by dynamic gassing-out techniques (15). The dissolved oxygen changes (dC_L/dt) in batch fermentation were based on mass balance equation as given below:

$$dC_L/dt = \text{OTR-OUR} = K_L a.\ (C_E - C_L) - (Qo_2.\ X) \qquad \text{Eq.1}$$

Where K_La. $(C_E - C_L)$ and $(Qo_2.\ X)$ are volumetric OTR and OUR respectively. C_E is the value of saturated oxygen in the fermentation medium, C_L is the value of dissolved oxygen concentration, Qo_2 is the specific respiration rate and X is cells concentration.

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:

$$dC_L/dt = \text{OUR} = -(Qo_2.\ X) \qquad \text{Eq.2}$$

OTR determination

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

$$\text{OTR} = dC_L/dt + \text{OUR} \qquad \text{Eq.3}$$

K_La determination

The K_La value was determined by considering the final steady state of dissolved oxygen concentration ($\acute{C}_L$) after re-oxygenation. When $\acute{C}_L = C_L$ and $dC_L/dt = 0$, the Eq.1 can be rearranged as given below:

$$Qo_2.\ X = K_L a.(C_E - \acute{C}_L) \qquad \text{Eq.4}$$

Substituting the value of $Qo_2.\ X$ in Eq.1 with $K_L a.(C_E - \acute{C}_L)$ from the Eq.4:

$$dC_L/dt = K_L a.(\acute{C}_L - C_L) \qquad \text{Eq.5}$$

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 K_La is given as:

$$K_L a = \ln[(\acute{C}_L - C_{L1})/(\acute{C}_L - C_{L2})]/\ t_2 - t_1 \qquad \text{Eq.6}$$

The K_La value was calculated from slope by plotting $\ln(\acute{C}_L - C_{L1})/(\acute{C}_L - C_{L2})$ against time $(t_2 - t_1)$ according to Eq.6. The values of OUR, OTR and K_La were investigated at various aeration and agitation rates in the fermentor.

Analytical methods

Determination of proteolytic activity

Alkaline protease activity was determined by using 1 % (w/v) casein solution as a substrate in 0.05 M glycine–NaOH buffer at pH 11 (19). The reaction mixture containing 2 mL of 1 % casein solution in 0.05 M Glycine-NaOH buffer having pH 11 and 1 mL of an enzyme solution were incubated at 60 ^{o}C for 15 min and the reaction was then stopped with the addition of 3 mL of 10 % trichloroacetic acid. After that the entire mixture was centrifuged at 9000 x g for 10 min at 4.0 ^{o}C and absorbance of the liberated tyrosine was measured with respect to sample blank at 280 nm. One proteolytic unit (PU) was defined as the amount of the enzyme that released 1 µg of tyrosine / mL/ min under the assay conditions.

Estimation of total protein contents

The total protein contents were measured according to the method of Lowry *et al.* (20) by using bovine serum albumin (BSA) as a reference standard.

Estimation of glucose contents

Total glucose contents were measured by the phenol-sulfuric acid method, as described by Dubois *et al.* (21). According to the method, 1 mL of 5 % aqueous phenol solution was added to 1 mL of sample solution and mixed properly. After that 5 mL of concentrated sulfuric acid was added and allowed to stand for 20 min for color development. The absorbance was then measured at 470 nm against blank to determine the concentration of glucose in the sample.

Chemicals used and statistical analysis

The chemicals used in this study were of analytical grade. Each experiment was carried out independently in triplicates and the standard deviation (SD) was calculated by using Microsoft Excel Program.

Results And Discussion

The role of oxygen in metabolism and the growth of aerobic microbes are well known. The enzyme production in aerobic microorganisms also depends on the availability of oxygen in the fermentation medium. Therefore, the effect of different aeration and agitation rates on enzyme yield, growth of microorganism, DOT (%), volumetric mass transfer coefficient (K_La) and oxygen transfer rate (OTR) were investigated in the fermentor by using *B. licheniformis* UV-9 as the producer organism.

Effect of aeration rates on DOT, cell biomass, enzyme production and substrate concentration

The effects of aeration rates from 0.5 vvm to 2.5 vvm on DOT (%), cell biomass (g/L), protease production (PU/mL) and glucose consumption (g/L) during fermentation of *B. licheniformis* UV-9 at constant agitation speed of 300 rpm are illustrated in Figure 1 (a-d). Sampling was performed at 3 h interval for each aeration rate. Figure 1a displays that DOT was dramatically reduced to 0 % at 9 h after inoculation at aeration rates of 0.5 vvm and 1 vvm. However, aeration rates of 2 vvm and 2.5 vvm maintained the appropriate DOT (for proper growth required >10 %) throughout the fermentation period. Similar pattern of increase in DOT with various aeration rates has also been reported by some other investigators (12,13). The cell biomass values also varied with change in aeration rates and maximum cell biomass appeared at a value of 2 vvm (Figure 1b). 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. It has been reported in an earlier investigation that higher flow rates with low agitation speed increased the air flow up in the vessel along the stirrer shaft. This phenomenon, known as impeller 'flooding' has been recommended to 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 (22). 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 fermentor. Similar pattern of

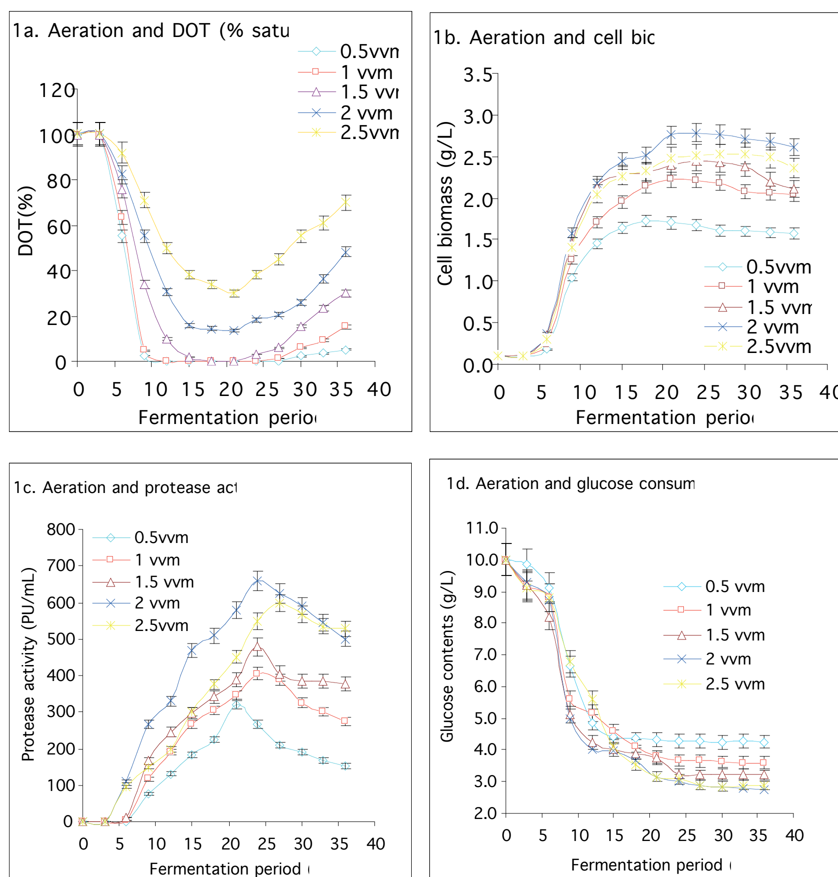

Figure 1. Effect of different aeration rates (0.5-2.5 vvm) on a: DOT %; b: Cell biomass (g/L); c: Protease activity (PU/mL) and d: Glucose consumption (g/L) during the cultivation of Bacillus licheniformis mutant UV-9 in a 2 L fermentor. The cultivation was carried out at 37 oC with a constant agitation rate of 300 rpm. Bars represented S.D.

increase or decrease in enzyme yield along with aeration rates appeared and are shown in Figure 1c. Maximum alkaline protease activity (657.88 PU/mL) was observed at 2 vvm aeration rate after 24 h incubation. Similar effects of high aeration rates on enzyme activity level in fermentation have been reported by some investigators (23,24). The total glucose contents were also measured at each aeration rate at 3 h intervals and maximum consumption rate was found at 2 vvm in exponential phase of growth (Figure 1d). However, no significant glucose consumption was found during lag and stationary growth phases of *B. licheniformis* UV-9. All these findings dictate a typical relationship among the aeration rates, DOT (%), cell growth and glucose consumption throughout the fermentation process. Since aeration rate of 2 vvm produced maximum yield of alkaline protease by *Bacillus licheniformis* UV-9 and therefore, it was selected for the subsequent study.

Effect of agitation rates on DOT level, cell biomass, enzyme production and substrate concentration

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 five different agitation rates on DOT (%), cell growth, enzyme yield and substrate consumption were studied at constant aeration of 2 vvm (Figure 2 (a-d)). Figure 2a demonstrates the effect of various agitation rates (300-700) on DOT (%). The DOT level increased with the increases in agitation speed. However, the optimal production of cell biomass (3.41 g/L) and the highest protease yield (1270.20 PU/mL) were obtained at stirring speed of 500 rpm. The higher agitations (600-700 rpm) reduced the cell growth and alkaline protease production due to sheer stress and heterogeneous mixing effects. These results showed more profound effect of agitation than aeration rates. Agitation affects both air bubble dispersion and mixing of nutrients during fermentation process. Similar effects of higher agitation rates on cell growth had been documented by some other researchers (25-27), too. A decrease in cell growth and enzyme production was also observed below 500 rpm resulting from incomplete mixing and/or oxygen transfer resistance at low agitation rates. Similarly, the maximum glucose consumption was observed at 2 vvm and 500 rpm (Figure 2d), indicating a proportional relationship among substrate consumption, cell growth and enzyme yield. The

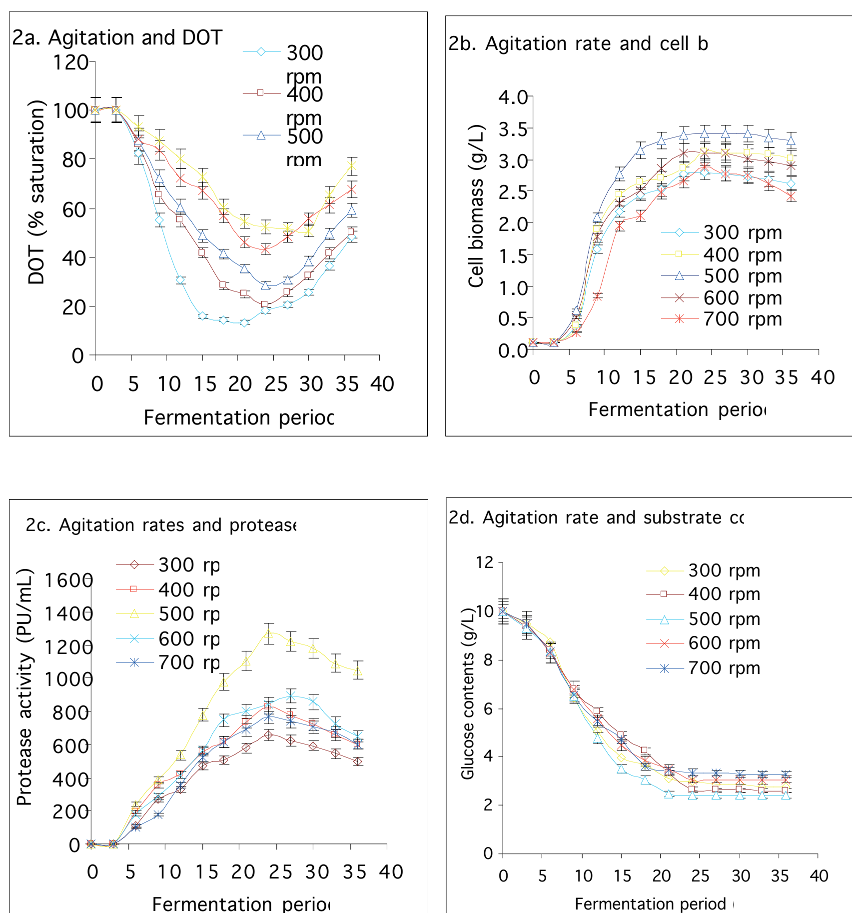

Figure 2. Effect of different agitation rates (300-700 rpm) a: DOT (%); b: Cell biomass (g/L); c: Protease activity (PU/mL); and d: Glucose consumption (g/L) during the cultivation of Bacillus licheniformis mutant UV-9 in a 2 L fermentor. The cultivation was carried out at 37 oC with a constant aeration rate of 2 vvm. Bars represented S.D.

extreme conditions may inhibit the growth and activity of microorganisms by blocking the nutrients transfer and their consumption by disturbing cell activities (13). All these efforts indicated that proper mixing is crucial for maximum production of microbial protease and cell biomass. This could be achieved by optimizing the both aeration and agitation rates for better oxygen transfer rate and product formation (10, 28, 29).

Effect of aeration rates on oxygen transfer characteristics and kinetics parameters

The dynamic gassing-out method was applied to find the 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 (Figure 3). The decline in initial curve following air supply disruption showed the decrease in DOT in the fermentor due to oxygen consumption by the cells for their growth. The later part of the curve showed increase in DOT in the fermentor due to air supply being restarted while the oxygen was still consumed by the cells, indicating change in DOT (%) in the fermentor (dC_L/dt). 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. The K_La value increased from 7.2 to 43.2 h^{-1} when the aeration rates were increased from 0.5 to 2.5 vvm. Similarly, OTR values increased from 1.628 to 2.60 mol m^{-3} h^{-1} as the aeration rates increased from 0.5 vvm to 2.5 vvm. The higher aeration rates inhibited cell growth by creating air dispersion problem in the fermentation system which in turn affected the cell biomass concentration in the fermentor. Oxygen is necessary for the growth of aerobic cultures but high aeration rates can disturb cell growth by damaging cells (30-33). The variation in oxygen transfer rates also affected the substrate consumption and product formation rates as it directly affected the cell growth and enzyme formation. Maximum glucose consumption ($Y_{x/s}$, g cells/g glucose and Q_s, g glucose /L/h) and product formation rates ($Y_{p/s}$, enzyme/g glucose; $Y_{p/x}$, enzyme/g cells; q_p, enzyme/g cells/h and Q_p, enzyme/L/h) were achieved at 2 vvm aeration having 31.68 h^{-1} K_La and 2.15 mol m^{-3} h^{-1} OTR values. However, a further increase in K_La value by increasing aeration rate (2.5vvm) decreased biomass and yield coefficients, indicating a non-linear relationship between K_La and productivity coefficients for alkaline protease production by *B. licheniformis* UV-9, contrary to the previous findings regarding enzyme production (34). The variation in results might be consequences of physiological differences between microorganisms, reflecting different oxygen requirements for their growth and other physiological functions

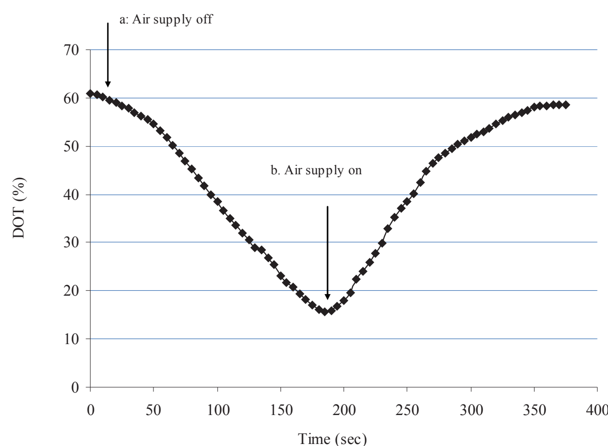

Figure 3. Time dependence of DOT (%) determined during ferementation of *B. licheniformis* UV-9 at 2 vvm and 500 rpm in 2 L fermentor. The slope of line between ↓a and ↓b represents dC_L/dt = OUR and after ↓b OTR = dC_L/dt + OUR respectively.

Effect of agitation rates on oxygen transfer characteristics and kinetics parameters

Oxygen transfer into the cells in aerobic fermentation processes strongly affects product formation by influencing metabolic pathways and changing metabolic fluxes (28). Therefore, oxygen supply into the medium becomes one of the decisive factors in scaling up of aerobic biosynthesis system. The effects of different agitation speeds (300-700 rpm) on oxygen transfer characteristics, cell biomass and productivity coefficients were investigated by maintaining constant working volume at 1.5 L, 2 vvm flow rate, 37 ^oC temperature and pH 10 (Table 2). The K_La and OTR values increased from 32.04 to 205.2 h^{-1} and 2.16 to 5.46 mol m^{-3} h^{-1}, respectively, when agitation rates were increased from 300 to700 rpm, indicating a proportional correlation among agitation rates, K_La and OTR values. The K_La value of *Monascus* sp. has been reported to increase gradually from 10.8 to 104.4 h^{-1} as rotational speed increases from 200 to 700 rpm (35). During kinetic studies among various agitation rates, maximum productivity coefficient values ($Y_{p/s}$, $Y_{p/x}$, q_p and Q_p) were obtained at 500 rpm having 72 h^{-1} K_La value (Table 2). However, lower and higher K_La values resulting from low and high agitation rates indicated reduction in protease productivity by *B. licheniformis* UV-9. This might happen that low agitation speed could cause improper mixing of nutrients whereas high agitation speed could mechanically damage microbial cells by exerting high sheer stress which ultimately affected protease yield. Current literature also pointed out that unsuitable agitation rates can cause a drastic reduction in protease production by *Bacillus* sp. (36-40). All these efforts indicated that there should be a balance between aeration and agitation rates for the optimum yield of cell

Table 1. Effect of different aeration rates on volumetric mass transfer coefficient (KLa), oxygen transfer rate (OTR) and protease yield coefficients of B. licheniformis UV-9 cultivated in a bioreactor

Air Flow rate (vvm)	K_La (h^{-1})	OTR (mol m^{-3} h^{-1})	$Y_{p/s}$, 1000	$Y_{p/x}$, 1000	$Y_{x/s}$	Q_s	$q_{p,1000}$	$Q_{p,1000}$
0.5	7.2	1.628	46.717	159.916	0.292	0.238	6.663	11.134
1	12.6	2.284	64.021	183.371	0.349	0.264	7.640	16.885
1.5	23.4	2.465	70.947	195.465	0.363	0.281	8.144	19.954
2	31.68	2.148	93.983	236.223	0.398	0.292	9.843	27.412
2.5	43.2	2.600	79.363	217.619	0.365	0.288	9.067	22.850

Agitation speed: 300 rpm; Temperature: 37 °C; pH: 10. $Y_{p/s}$: Enzyme produced (PU)/g glucose consumed; $Y_{p/x}$: Enzyme produced (PU) /g cells; $Y_{x/s}$: g cells/g glucose consumed; q_s(g/g cells/h): g glucose consumed/g cells/h; Q_s(g/L/h): g glucose consumed/L of cultivation medium/h; q_p (Enzyme/g/h): Enzyme produced (PU)/g cells/h; Q_p(Enzyme/L/h): Enzyme produced (PU)/L of cultivation medium/h.

Table 2. Effect of different agitation rates on volumetric mass transfer coefficient (K_La), oxygen transfer rate (OTR) and protease yield coefficients of *B. licheniformis* UV-9 cultivated in a bioreactor

Agitation speed(rpm)	K_La (h^{-1})	OTR (mol m^{-3} h^{-1})	$Y_{p/s}$, 1000	$Y_{p/x}$, 1000	$Y_{x/s}$	Q_s	$q_{p,1000}$	$Q_{p,1000}$
300	32.04	2.156	93.983	236.223	0.398	0.292	9.843	27.412
400	50.4	2.811	114.856	263.212	0.436	0.298	10.967	34.218
500	72.0	3.901	167.573	372.493	0.450	0.316	15.521	52.925
600	118.8	4.651	121.949	272.177	0.440	0.289	11.34	35.213
700	205.2	5.465	110.559	254.40	0.435	0.277	10.600	30.634

Aeration rate: 2 vvm; Temperature: 37 °C; pH: 10. $Y_{p/s}$: Enzyme produced (PU)/g glucose consumed; $Y_{p/x}$: Enzyme produced (PU)/g cells ; $Y_{x/s}$: g cells/g glucose consumed; q_s(g/g cells/h): g glucose consumed/g cells/h; Q_s(g /L/h): g glucose consumed/L of cultivation medium/h; q_p (Enzyme/g/h): Enzyme produced (PU)/g cells/h; Q_p(Enzyme/L/h): Enzyme produced (PU)/L of cultivation medium/h.

biomass and alkaline protease. Therefore, the K_La value of 72 h^{-1} with agitation speed of 500 rpm at aeration rate of 2 vvm provide an excellent balance between aeration and agitation rates for the maximum yield of alkaline protease produced by *B. licheniformis* UV-9.

Conclusions

The results obtained in these experiments indicated that cell growth and alkaline protease production by *B. licheniformis* UV-9 in stirred tank bioreactor are dependent on both aeration and agitation rates. At lower aeration and agitation rates, oxygen mass transfer was found to be limited, resulting in decreased cell biomass and protease productivity. However, the higher agitation rates (>500 rpm) also adversely affected the cell growth and protease production by exerting shear stress. The study of kinetic parameters revealed that maximum productivity of alkaline protease ($Y_{x/s}$) was obtained at 2 vvm and 500 rpm with K_La value of 72 h^{-1}. Negative effects on enzyme yield are observed at other K_La values under the influence of various aeration and agitation rates. These results suggested that a specific K_La value is very much crucial for the maximum productivity in microbial fermentation and it could only be achieved by appropriate combination of aeration and agitation. The optimum K_La value will be used as an informative tool to overcome the problem of difference in geometry and stirrer types among a variety of bioreactors during scale up study from lab scale fermentor to pilot scale fermentor.

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