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Effect of Aeration and Agitation Regimes on Lipase Production by Newly Isolated *Rhodotorula mucilaginosa*–MTCC 8737 in Stirred Tank Reactor Using Molasses as Sole Production Medium

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Abstract The influence of media and process parameters (aeration and agitation) on fermentation broth rheology and biomass formation has been studied in 1.5-l stirred tank reactor for lipase production using *Rhodotorula mucilaginosa* MTCC 8737. Molasses, as sole production medium, is used for lipase production by varying aeration (1, 2, and 3 vvm) and agitation speeds (100, 200, and 300 rpm). Maximum lipase activity of 72 U/ml was obtained during 96 h of fermentation at 2 vvm, 200 rpm, pH 7, and 25±2°C temperature. Lipase production kinetics with respect to dry cell weight of biomass showed $Y_{P/S}$ of 25.71 U/mg, specific product formation of 10.9 U/mg DC, and $Y_{X/S}$ 2.35 mg/mg. Maximum lipase activity (MC 2) of 56 U/ml was observed at 1% molasses, and a further increase in the molasses concentration of (%) 1.5 and 2 inhibited the product formation of lipase with 15 and 8.5 U/ml, respectively. The production kinetics of molasses media showed $Y_{P/X}$ was 14 U/mg DC, $Y_{P/S}$ 16 U/mg, and $Y_{X/S}$ 1.14 mg/mg during 96 h of bioreactor operation. The k_La values for all batches (MC 1–MC 4) at 96 h of fermentation were 32, 28, 21, and 19/h, and the |oxygen transfer rate were 54.4, 56, 35.7, and 17.29 mg/l h, respectively. Increase in molasses concentration resulted in decreased lipase activity by increase in viscosity of the fermentation broth.

Keywords Aeration · Agitation · Oxygen transfer rate · Lipase activity · Viscosity

Abbreviations

STR	stirred tank reactor
rpm	revolution per minute
vvm	volume of air per volume of media per minute
DO	dissolved oxygen
OTR	oxygen transfer rate
DCW	dry cell weight

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Nomenclature

k_La	volumetric liquid side oxygen mass transfer coefficient (per hour)
C_L	concentration of dissolved oxygen at any given time (mg/l)
C^*	saturated oxygen concentration in the media (mg/l)
$Y_{P/X}$	specific product formation (SPF) of lipase with respect to biomass (U/mg DC)
$Y_{X/S}$	yield of biomass with respect to substrate (mg/mg)
$Y_{P/S}$	yield of lipase with respect to substrate (U/mg)

Introduction

Lipases (triacylglycerol hydrolases, EC 3.1.1.3) have varied applications in industrial processes, such as in the production of biodiesel, detergents, cosmetics, pharmaceuticals, flavor enhancers, textiles, and foods [1–4]. Several microorganisms, such as *Candida rugosa*, *Candida antarctica*, *Burkholderia cepacia*, and *Pseudomonas alcaligenes*, can produce lipase efficiently and their lipases are commercially available [5, 6]. Yeast lipases are gaining much importance due to their high commercial demand [6]. Dimitris et al. [7] investigated the factors affecting the production of extracellular lipase by the yeast *Rhodotorula glutinis*. To meet the increasing demand of commercial exploitation of lipase, feasible methods capable of mass and cost-effective production of lipase from microorganisms should be developed and improved [8]. Microbial lipase fermentations are affected by the medium pH, temperature, medium composition, inoculation volume, aeration, and agitation and many other factors related to bioreactor design [9]. Alford and Smith [10] observed that the presence of air was essential for lipase production by *Staphylococcus aureus*. Lipase production by a Brazilian wild strain of *Yarrowia lipolytica* at different stirring speeds and airflow rates showed that the stirring speed had determined the pronounced effect of oxygen for lipase production [11]. It was reported earlier that dissolved oxygen (DO) concentration is not the intrinsic factor affecting rates and yields for lipase production by *Rhizopus arrhizus* [12]. In addition, in lipase production with *Aspergillus terreus*, Gulati et al. [13] verified that the modeling of aerobic fermentations is more appropriate for oxygen transfer rate (OTR) than in terms of DO levels, as previously suggested by Chen et al. [14] for *Acinetobacter radioresistens*. Mixing is important in the microbial synthesis of lipase enzyme in free-cell bioreactor and can be imparted by means of aeration and agitation. It is also largely dependent on higher oxygen mass transfer and lesser shear forces on microorganisms. For aerobic fermentation, oxygen transfer is a key variable and is a function of aeration and agitation. The agitation and aeration rates not only affect the productivity of the microbial process but also affect the overall energy requirement of the production process. Therefore, it is necessary to establish optimum combination of airflow and agitation for maximum yield [15]. As each industrial application requires specific properties of the enzyme, isolation of novel lipases from different microorganisms is at its front. A large number of microorganisms have been reported for lipase production, and at the same time, utilization of low cost substrate media has gained a lot of importance at industrial level due to overall reduction in production costs. Limitations of the industrial use of these enzymes have mainly been reduced owing to their high production costs [16]. The aim of present work was to analyze the effects of airflow rate and agitation rate on lipase production from a newly isolated yeast culture, *Rhodotorula mucilaginosa* MTCC 8737, using molasses as sole production medium. Experiments were conducted to analyze the effects of airflow rate and agitation rate on lipase production in a lab scale bioreactor to identify optimum combination of airflow and agitation parameters that

control lipase production. Further experiments were conducted to study the effect of molasses concentration on maximum lipase production and effects of viscosity on volumetric oxygen mass transfer coefficient. OTR in the aerobic fermentation process is also dependent on the broth rheology, primarily variations in the viscosity with the time of bioreactor operation.

Materials and Methods

Screening, Isolation, and Identification of Marine Yeasts

Marine soil samples near oil extraction platform inside Arabian Sea besides Mangalore port, Mangalore, India were collected and brought to the laboratory for screening of marine organisms. One gram of the marine soil was suspended in 100 ml of distilled water, and serial dilutions in the range of 10^{-1} to 10^{-7} were carried out. Media containing 3.0% malt extract, 3.0% yeast extract, 5.0% peptone, 10% glucose, 0.05% streptomycin sulfate (MYPG) were prepared, autoclaved, and dispensed into Petri plates along with 1 ml of sample in each plate (Pour plate method) and allowed to solidify. The plates were incubated for 48 h at 25±2 °C. Different colonies from the plates were transferred to plates containing 2.0% tributyrin and incubated at 25±2 °C for 3–4 days. The colonies that showed the highest zone were selected and further investigated.

The culture was identified as *R. mucilaginosa* (MTCC 8737) by its phenotypic characteristics and deposited in Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh India.

Cultivation of Marine Yeasts

Two loops of the cells of the purified strains were transferred to 50 ml of MYPG medium prepared in 250 ml Erlenmeyer flask and aerobically cultivated for 48 h. Cell culture was transferred to 100 ml of the production medium, which contained (in g/l) malt extract, 3; yeast extract, 3; peptone, 5; glucose, 10; and 1% olive oil as lipase inducer. The flasks were incubated for 120 h at 25±2 °C and 150 rpm.

Bioreactor Operation

Effect of Different Airflow and Agitation Rates on Lipase Production

The experiments were carried out in a lab scale 1.5-l bioreactor (B-Braun Biostat) with 1 l working volume, fixed with two-stage rushton type impeller of 50 mm diameter. In the initial phase, STR was operated to optimize airflow rate and agitation rate for the production of lipases. Three levels of airflow rates, 1, 2, and 3 vvm were studied, and at each airflow rate, three different agitation rates, such as 100, 200, and 300, were tested by inoculating the batch STR with 10% inoculum and cultivating for 5 days at 25±2 °C. Estimation of carbohydrates, biomass, and lipase activity were carried out at every 24-h interval.

Effect of Molasses Concentration on the Viscosity of Fermentation Broth

The optimum levels of airflow and agitation rates with respect to lipase production were determined in the first phase. Further four experiments, MC1, MC2, MC3, and MC4, were conducted in batch mode, at different initial concentrations of molasses in the levels of (in

%, *v*/*v*) 0.5, 1.0, 1.5, and 2.0, respectively, for the maximum production of lipase in the second phase of STR operation. The 2-day-old inoculum grown in 250-ml flask was used as seed to the reactor (10% of the working volume). Each batch was cultivated for 5 days at constant pH of 7±0.2 and temperature at 25±2 °C, and during the operation at every 24 h, lipase activity, carbohydrates, DO, viscosity, and volumetric oxygen mass transfer coefficient were estimated and recorded.

Determination of k_La and OTR in the Bioreactor

Determination of k_La by Dynamic Gassing Out Method

This method [15] is a simple one, based upon the dynamic oxygen balance in a batch culture, which has the following form.

$$\frac{dC_L}{dt} = k_La\left(C^* - C_L\right) - Q_{O_2}X \tag{1}$$

Where Q_{O_2} is the rate of oxygen consumption per unit mass of cells (Milli moles O_2/g h)

Rearranging Eq. 1 yields:

$$k_La\left(C^* - C_L\right) = Q_{O_2}X + \frac{dC_L}{dt} \tag{2}$$

The air supply is turned off at a certain time during fermentation, and the variation of C_L with time is followed with the aid of a DO probe. Since the term k_La (C^*-C_L) becomes zero when air is turned off, the C_L value decreases linearly with time according to Eq. 1. The slope of the C_L versus time curve yields a value for $Q_{O_2}X$. The air is then turned on, and the increase in DO with time, is followed. Having determined the $Q_{O_2}X$ value, C_L is plotted against $(Q_{O_2}X + dC_L/dt)$. The slope of this plot is equal to the reciprocal of k_La.

Determination of OTR in Batch Bioreactor Operation

OTR for stirred tank reactor [15] is given by following equation:

$$\mathrm{OTR} = k_La\left(C^* - C_L\right) \tag{3}$$

Kinetics evaluation Lipase activity kinetics [15], yield of biomass with respect to substrate ($Y_{X/S}$, mg/mg), yield of product with respect to substrate ($Y_{P/S}$, U/mg), and specific product formation of lipase (SPF, $Y_{P/X}$, U/mg) were calculated with respect to dry cell weight of biomass in the reactor for all batches for 96 h of operation.

Analytical Methods

Estimation of the biomass The yeast culture samples were centrifuged at 8,000 rpm for 10 min, and the pellet was weighed for the estimation of biomass.

Enzyme assay Enzyme activity in the supernatant of the culture was determined using *p*-nitrophenyl palmitate (pNPP) as the substrate [17]. A 30 mg of pNPP dissolved in 10 ml propanol-2-ol was emulsified in 90 ml 50-mM Tris–HCl buffer, pH 8.0, containing 400 mg Triton X-100 and 100 mg of gum Arabic. A 0.3-ml enzyme solution was mixed with 2.7 ml

of the pNPP containing emulsion, and the absorbance was measured spectrophotometrically at 410 nm. A blank was always used with buffer (50 mM Tris–HCl, pH 8.0) instead of enzyme solution. One unit was defined as the amount of enzyme that liberated 1 μmol *p*-nitrophenol per minute.

Protein estimation The protein content of the enzyme preparation was estimated by Lowry's method [18].

Carbohydrate estimation Total carbohydrate content was determined according to the phenol–sulfuric acid method [19].

Viscosity of the broth The viscosity of the broth sample was measured by using a Cannon–Fenske type viscometer (Fisher Scientific, Pittsburgh, PA, USA). All the data analyzed and presented was the average of triplicates.

DO estimation A polarographic type probe from Mettler Toledo was used, with >98% response in less than 90 s.

Results and Discussions

Optimization of Airflow Rates and Agitation Rates

The effect of temperature (in the range of 20–40 °C) on extracellular lipase production by newly isolated *R. mucilaginosa* MTCC-8737 in a shake flask was studied, and it was found that 25±2 °C of temperature is optimum for maximum lipase activity at 96 h (unpublished data). Batch STR was operated with 10% inoculum for 5 days at 25±2 °C and tested the effects of airflow rates and agitation rates on lipase production. Table 1 shows the lipase activity, biomass production, and carbohydrate utilization in STR under different cultivation conditions at 96 h of operation. Each batch of the STR has resulted in different levels of lipase activity. However, the trend of production and/or formation of lipase, carbohydrate utilization, and biomass growth (DCW, g/l) were similar in all batches irrespective of airflow and agitation rates. However, variations in lipase activity indicate the effect of airflow and agitation rates.

Maximum lipase activity of 72 U/ml (Table 1) was obtained at 200 rpm agitation speed and at 2 vvm airflow rate. During the cultivation period, lipase activity and biomass growth in batch STR has been found to be negatively affected by variations in agitation rates

Table 1 The lipase activity, biomass production and carbohydrate utilization in STR under different cultivation conditions at 96 h of operation.

Aeration	1 vvm			2 vvm			3 vvm		
Agitation	100	200	300	100	200	300	100	200	300
Carbohydrates (0 h)		9.7	9.7	9.8	9.7	9.8	9.9	9.8	9.7
Carbohydrates (96 h)	4	4.1	4	4.6	2.8	3.4	5	6.3	6.1
Biomass (96 h)	3.8	5.6	5	5.5	6.6	6.2	4.8	5.1	5.8
Lipase activity (96 h)	31.2	38.4	35	48	72	48.8	28.2	27	21.7

beyond 200 rpm, whereas at an agitation speed less than 200 rpm and at all airflow rates (1–3 vvm), lipase activity was in the range of 15–45 U/ml at the end of 96 h of reactor operation.

The trend of lipase activity was similar in all batches of STR operation, showing a decline in lipase concentration after 96 h (data not shown). Besides this, it is obvious from the given lipase activity, carbohydrate utilization, and biomass growth data that mixing was crucial for better oxygen and nutrient transfer rate during entire period of operation. However, mixing by means of only agitation was found to inhibit lipase activity at rpm above 200, whereas at all airflow rates, the lipase activity was high except at higher agitation rates.

Figure 1a–c shows the effect of airflow and agitation rates on lipase yield kinetics. Maximum $Y_{P/S}$ 25.71 U/mg (Fig. 2a), SPF of 10.9 U/mg DC (Fig. 2b), and $Y_{X/S}$ 2.35 mg/mg (Fig. 2-c) were recorded at 2 vvm and 200 rpm, during 96 h of bioreactor operation.

Mixing is important for maximal production of microbial lipase by optimizing the mixing rates in batch STR operation and can be imparted by means of both aeration and agitation for better oxygen mass transfer rate and for better product formation [11, 12, 14].

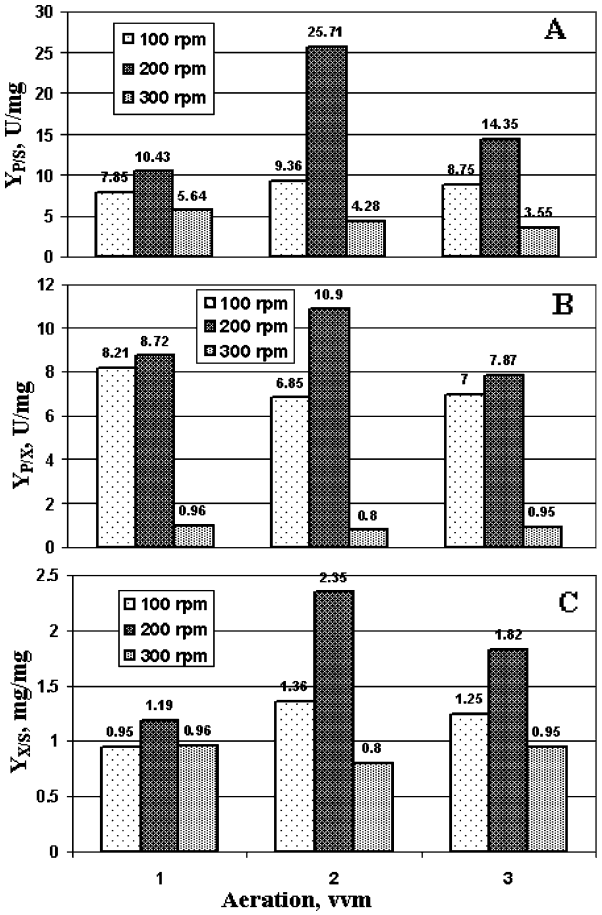

Fig. 1 Lipase production kinetics: effects of aeration and agitation (at the end of 96 h of bioreactor operation)

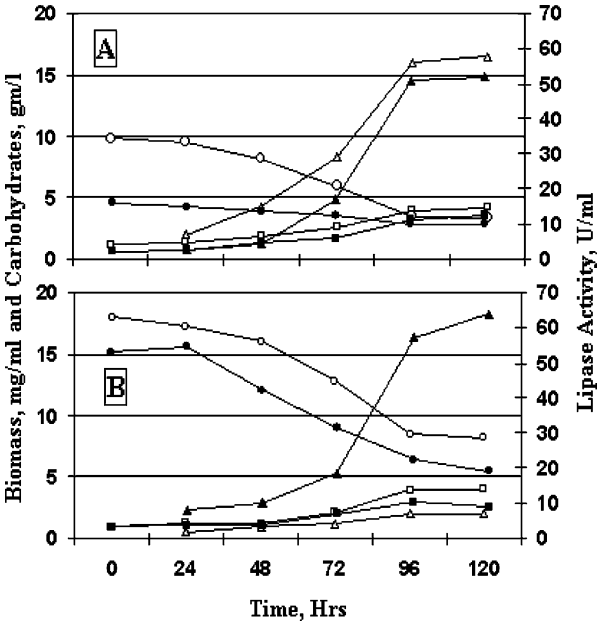

Fig. 2 Performance of STR at different initial molasses concentrations (%, *v/v*). **a** MC1 (0.5%)—*open square* biomass, *open circle* carbohydrates, *open triangle* lipase activity; MC2 (1.0%)—*filled square* biomass, *open circle* carbohydrates, *filled triangle* lipase activity. **b** MC3 (1.5%)—*open square* biomass, *open circle* carbohydrates, *open triangle* lipase activity; MC4 (2.0)—*filled square* biomass; *filled circle* carbohydrates, *filled triangle* lipase activity

Again, higher stirring rates may cause shear effects on microbial cells resulting in reduced biomass concentration. There should be an optimum balance between the aeration (oxygen tension) and agitation (shear) to have maximum cell growth and SPF. This could be achieved by optimizing the combinations of airflow and agitation rates that are important for maximal SPF. Higher shear rates will lead to breaking in cell wall, resulting in lower lipase production [20].

Lipase production was different at each combination of airflow and agitation rates in STR; therefore, these parameters could act as metabolism regulating parameters in the bioreactor for the maximum SPF. The calculation of SPF yields will help in determining the optimum combination of airflow and agitation rates especially in extracellular enzyme production in free-cell reactors [15]. The OTRs are dependent on aeration and agitation rates during the batch STR operation with free cells of *C. rugosa* [21] and *Thermus thermophilus* HB27 [22], which in turn results in maximum lipase yields. For large-scale systems, power consumption during aeration and agitation is a significant fraction of the total operating cost. To increase agitation rate, the power of the impeller motor must be increased [15].

Effect of Molasses Concentration on Lipase Production

Lab scale studies were carried out in 250-ml shake flaks (unpublished) to test the suitability of the sugarcane molasses as sole production medium for lipase production, which resulted in good lipase activity. Therefore, further experiments were conducted in batch STR at different molasses concentrations for lipase production using previously optimized agitation and airflow rates, i.e., 200 rpm and 2 vvm, respectively. The variations in DCW, carbohydrate utilization and lipase activity during the course of fermentation re shown in Fig. 2. It is evident from Fig. 2 that, during batch STR operation (MC 1-MC 4), the trend of carbohydrate utilization, biomass formation (DCW), and lipase activity was similar with maximum lipase activity during 96 h.

As the initial molasses concentration in the STR increases, the lipase activity rate decreases. Maximum lipase activity of 56 U/ml in MC 2 was observed at 1% molasses, and a further increase in the molasses at 1.5% and 2% in MC3 and MC4 inhibited the product formation rate by recording a low lipase activity of 15 and 8.5 U/ml, respectively, on the fourth day as shown in Fig. 3. Higher concentration of molasses, in MC 3 and MC 4 (Fig. 3), also inhibited the growth and biomass yields with a low DCW. The effect of molasses concentration on lipase production in batch STR could be best studied by calculating the production kinetics, i.e., $Y_{P/S}$, $Y_{X/S}$, and $Y_{P/X}$ for 96 h of operation. The results for all batches (MC 1–MC 4) are tabulated in Table 1. From Table 1, it is evident that maximum $Y_{P/X}$ was 14 U/mg DC, $Y_{P/S}$ was 16 U/mg, and $Y_{X/S}$ was 1.14 mg/mg in MC 2 during 96 h of bioreactor operation.

The increase in agitation rate produces higher shear stress in the broth, which may cause decrease in the growth of shear-sensitive microorganisms. Most of the industrial fermentation processes for lipases are sensitive for shear forces and at the same time requires proper oxygen mass transfer. The aeration and agitation altogether are used to enhance oxygen transfer from gas (air) to liquid medium for the optimal cell mass production or product formation [15, 23]. This could be due to lower availability of dissolved oxygen with low mixing rates. As dissolved oxygen is the rate-limiting factor because of its low solubility in the aqueous solutions, it affects the cell growth and yield of products in the aerobic fermentation [12, 14]. This could be done by optimizing the airflow and agitation rates simultaneously rather than optimizing individually.

Mass transfer from the gas to the liquid phase has been identified as an important rate limiting factor and is dependent on viscosity of the fermentation broth. The culture viscosity was found to be changing with increase in time of STR operational period throughout the cultivation, indicating that the initial concentration of molasses had significant effect on the culture viscosity and the fermentation broth followed non-Newtonian fluid rheology. The saturated initial viscosities and DO values of MC1, MC2,

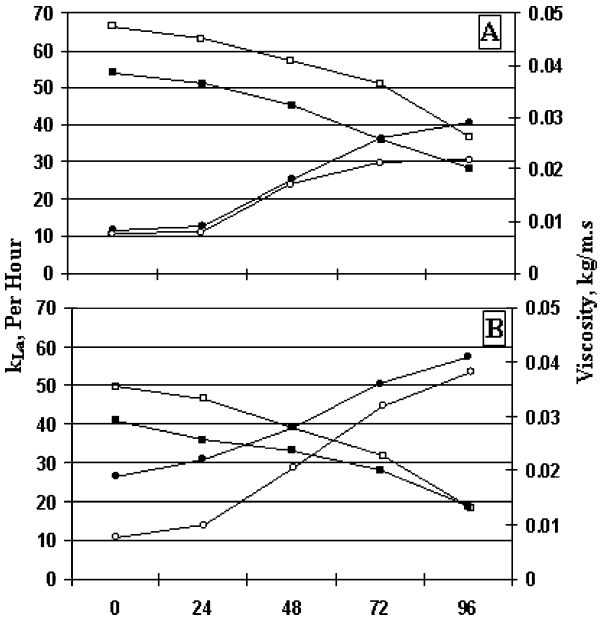

Fig. 3 Variation in volumetric oxygen mass transfer coefficient and viscosity with time during the operation of bioreactor. **a** MC1 (0.5%)—*open square* k_La, *open circle* viscosity; MC2 (1.0%)—*filled square* k_La; *filled circle* viscosity. **b** MC3 (1.5%)—*open square* k_La; *open circle* viscosity; MC4 (2.0%)—*filled square* k_La; *filled circle* viscosity

MC3, and MC4 was quantified at 25±2 °C and was found to be 0.0048, 0.0083, 0.0098, and 0.019 kg/m s and 4.8, 4.5, 4.1, and 3.8 mg/l, respectively. The data shows that, with the increase in initial viscosity of the fermentation broth, saturated dissolved oxygen concentration at 0 h was found to vary.

Figure 3 shows the effect of initial molasses concentration on viscosity and on the k_La during bioreactor operation. As the time of bioreactor operation increased, the viscosity also increased in all the four batches, which in turn affected the k_La of the system. The initial concentration of molasses also affected the viscosity of fermentation broth. From Fig. 3, it is evident that the effect of initial molasses concentration is significant on specific product formation rate and OTR. When the initial concentration of molasses varied in the levels of 0.5 (MC1), 1.0 (MC2), 1.5 (MC3), and 2.0 (MC4) % *v*/*v*, the corresponding viscosities were 0.0048, 0.0083, 0.0098, and 0.013 kg/m s, respectively, at zero hours. At the end of 96 h of operation, the viscosity of the batches, MC 1, MC 2, MC 3, and MC 4 were 0.0186, 0.029, 0.039, and 0.041 kg/ m s, respectively. As the viscosity of the fermentation broth increased with the time of bioreactor operation, the volumetric oxygen mass transfer coefficient was decreased (Fig. 3). The k_La values for MC 1, MC 2, MC 3, and MC 4 at the 24th hour were 58, 51, 41, and 36/h, and the values at 96 h was 32, 28, 21, and 19/h, respectively. The OTR at the end of 96 h of bioreactor operation was 54.4, 56, 35.7, 17.29 mg/l hr for MC 1, MC 2, MC 3, and MC 4, respectively (Table 2). As seen from the results, though the initial molasses concentration was affected by the OTR (MC 1–MC 4), the DO concentration of the fermentation broth in each batch was not reduced to below the critical oxygen concentration during fermentation.

This indicated that the initial concentration of molasses significantly affected the viscosity of the fermentation broth throughout the experimentation and in turn affected the volumetric oxygen mass transfer coefficient, ultimately resulting in lower lipase production rate. The change in viscosity of the system is because, during the production of extracellular enzymes in aerobic fermentation, formation of a water-soluble, viscous slime material containing D- and L-glutamic acid residues is common. This is because nitrogenous compounds undergo reaction with glutaminase enzyme action, which is quite common and widely distributed in microorganisms including bacteria, yeast, and fungi [24]. This glutaminase is responsible for even small amounts of glutamic acid produced during aerobic fermentation. Major genera of yeast reported to produce glutaminase and glutamic acid during aerobic fermentation includes *Candida scottii*, *Cryptococcus* sp., *Hansenula* [25], *Debaryomyces* sp. [26], *Saccharomyces cerevisiae* [27], *Cryptococcus albidus* [28], and *Cryptococcus nodaensis* [29].

The synthesis of even small amounts of PGA can be a problem in the fermentation industry, most notably in the production of microbial extracellular enzymes, where PGA accumulation causes increased viscosity of the fermentation broth, reduced enzyme yield, uncontrollable foaming, and complications in product recovery [30]. The reduction in the

Table 2 Lipase production kinetics: effects of initial molasses concentration (at the end of 96 h of bioreactor operation).

	Molasses (%, *v*/*v*)	$Y_{P/S}$ (U/mg)	$Y_{P/X}$ (U/mg)	$Y_{X/S}$ (mg/mg)	OTR (mg/l h)
MC1	0.5	4.45	3.94	1.12	54.4
MC2	1	16	14	1.14	56
MC3	1.5	2.5	5.27	0.47	35.7
MC4	2	0.8	1.74	0.45	17.29

k_La values was probably due to the reduction in the surface area of the bubbles caused by the viscous forces generated in the fermentation broth by the formation of PGA. With an increase in the viscosity, the resistances to the mass transfer increases. Hence, the viscosity effects are more significant in the production phase than in the initial 24 h of bioreactor operation [30]. Therefore, it could be concluded that the initial viscosity of the fermentation broth had less effects in the initial hours of operation. The effect of non-Newtonian fermentation broth viscosity on mass transfer of oxygen to the broth from bulk gas phase and then to culture has been gaining attention in bioprocess engineering studies, and it is an important factor at industrial level production.

Conclusion

Mixing is very crucial for the maximum productivity in microbial fermentations, and it could be achieved by means of aeration and agitation. However, agitation at higher stirring speeds may cause disruption of free cells in the reactor by shear forces and formation of vortex, which may result in poor mass transfer (oxygen/substrate). Therefore, it is important to provide optimum combination of aeration and agitation in free-cell batch bioreactor operation. From the results of present investigation, it could be concluded that agitation and aeration have a significant effect on lipase production. Designing a low-cost production medium is important for the economic production of lipase, and at the same time, the medium should not affect the productivity. Increase in molasses concentration resulted in decreased lipase production by increase in viscosity of the fermentation broth. Hence, it is important to identify suitable medium, both qualitatively and quantitatively for the efficient production of lipase in STR.

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