HIGH CELL DENSITY FERMENTATION OF *CANDIDA RUGOSA* ON PALM OIL FOR LIPASE PRODUCTION AND ITS MASS TRANSFER INVESTIGATION

MOHD NUR IKHMAL BIN SALEHMIN

DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2014

ABSTRACT

Extracellular lipase of the yeast *Candida rugosa* was produced via high cell density fed-batch fermentations using palm oil as the sole source of carbon and energy. Feeding strategies consisted of a pH-stat operation, foaming-dependent control and specific growth rate control in different experiments. Compared to foaming-dependent feeding and the pH-stat operation, the specific growth rate control of feeding proved to be the most successful. At the specific growth rate control set point of 0.05 h^{-1}, the final lipase activity in the culture broth was the highest at ~700 U L^{-1}. This was 2.6-fold higher than the final enzyme activity obtained at a specific growth rate control set point of 0.15 h^{-1}. The peak enzyme concentration achieved using the best foaming-dependent control of feeding was around 28% of the peak activity attained using the specific growth rate control of feeding at 0.05 h^{-1}. Similarly, the peak enzyme concentration attained using the pH-stat feeding operations was a mere 9% of the peak activity attained by specific growth rate control of feeding at a setpoint of 0.05 h^{-1}. The highest biomass specific productivity of the enzyme was 0.19 U g^{-1} h^{-1} for the fed-batch operation with the specific growth rate controlled at 0.05 h^{-1}. Fed-batch fermentations were performed in a 2-L stirred-tank bioreactor (30 °C, pH 7) with the dissolved oxygen level controlled at 30% of air saturation. Investigation on gas-liquid and liquid-liquid mass transfers by experimental and theoretical means was also carried out. The investigation was simulated in xanthan gum solution which resembles the viscosity of High Cell Density Fermentation (HCDF) at 1.68 mPa.s. Established correlations were successfully used to predict k_La value at different agitation rate ranging from 200-800 rpm for both oil and oxygen mass transfers under HCDF condition. However, the correlations

failed to predict k_La value at the highest agitation rate used (1000 rpm) for liquid-liquid mass transfer.

ABSTRAK

Lipase yang dihasilkan oleh yis *Candida rugosa* telah difermentasi melalui kultur berketumpatan tinggi dimana minyak kelapa sawit digunakan sebagai sumber karbon dan tenaga yang utama. Tiga strategi pemakanan nutrien yang berbeza iaitu, pH-stat, kebergantungan terhadap kejadian buih, dan pengawalan terhadap pengekalan kadar pertumbuhan tertentu telah dijalankan secara berasingan. Daripada tiga strategi pemakanan nutrient yang dijalankan, pengawalan terhadap pengekalan kadar pertumbuhan tertentu memberi hasil keputusan yang memberangsangkan. Sebanyak ~700 U L^{-1} aktiviti lipase telah dihasilkan melalui kaedah pengawalan makanan terhadap pengekalan kadar pertumbuhan tertentu pada μ_{set} 0.05 h^{-1}. Lipase yang dihasilkan adalah 2.6 kali ganda daripada aktiviti lipase yang dihasilkan pada μ_{set} 0.15 h^{-1}. Hanya 28% aktiviti lipase dihasilkan melalui teknik kebergantungan terhadap kejadian buih berbanding aktiviti lipase yang didapati melalui kaedah pengawalan makanan terhadap pengekalan kadar pertumbuhan tertentu pada μ_{set} 0.05 h^{-1}. Manakala aktiviti lipase yang dihasilkan melalui teknik pemakanan pH-stat hanya 9% berbanding aktiviti lipase yang diperolehi daripada kaedah pengawalan makanan terhadap pengekalan kadar pertumbuhan tertentu pada μ_{set} 0.05 h^{-1}. Nilai yang tertinggi untuk kadar produktiviti terhadap biomas tertentu pula adalah 0.19 U g^{-1} h^{-1} yang diperolehi melalui kaedah pengawalan makanan terhadap pengekalan kadar pertumbuhan tertentu pada μ_{set} 0.05 h^{-1}. Proses fermentasi kultur berketumpatan tinggi dilakukan di dalam 2-L tangki reaktor teraduk. Fermentasi dikawal pada (30 °C, pH 7) dan pengekalan paras oksigen pada 30% ketumpatan udara. Kajian ini juga melibatkan penentuan kadar pemindahan jisim gas kepada cecair dan cecair kepada cecair secara eksperimentasi dan melalui asas teori. Kajian dijalankan secara simulasi di dalam larutan gam xantham yang menyerupai kelikatan seperti kultur fermentasi berketumpatan tinggi

pada 1.68 mPa.s. Korelasi yang sedia ada telah berjaya meramalkan nilai k_La hampir kepada nilai yang didapati melalui eksperimentasi didalam julat kadar pengacauan antara 200-800 rpm untuk kedua-dua pemindahan jisim dibawah keadaan fermentasi kultur berketumpatan tinggi. Walaubagaimanapun, korelasi yang digunakan tidak dapat meramalkan nilai k_La pada kuasa pengadukan yang tertinggi (1000 rpm) bagi pemindahan jisim cecair kepada cecair.

ACKNOWLEDGEMENTS

I would like to acknowledge and extend my gratitude to my supervisor Assoc. Prof. Dr. Mohamad Suffian Bin Mohamad Annuar for his guidance and patient in guiding me to complete this project as well as giving me full attention in solving problems related to my study. His countless effort to help me over the years of my study is truly admired. I have learned a lot from his expertise especially in fermentation field but the knowledge I gain all this while was never enough and looking forward to dig more from him. I also would like to credit to the grant from IPPP University Malaya (PV036 2012A) for a full financial support for my project. From the bottom of my heart, I would like to extend my appreciation to Prof. Dr. Yusuf Chisti who was willing to collaborate and extensively revised the review paper I have published. Many thanks to all my colleagues for their support and assistance while I was conducting my research. I also owe many thanks to my parents, my wife, course mates and friends for their encouragement and understanding which help me in completing my study.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

Symbols

a	Interfacial area (m^{-1})
a_t	Specific total interfacial area (m^{-1})
c	Concentration of *p*NP
C_{AL}^{*}	Oxygen concentration at equilibrium ($\%O_2\ L^{-1}$)
dc	Different in concentration
d_e	Equivalent bubble diameter (m)
D_i	Diameter of impeller (m)
d_j	Droplet's diameter of a category (m)
$D_{mean.j}$	Average diameter of droplets from each category (m)
$D_{overall}$	Overall diameter (m)
D_{o2}	Diffusivity of oxygen in water ($m^2\ s^{-1}$)
D_{oil}	Diffusivity of palm oil in water ($m^2\ s^{-1}$)
dt	Difference in time
D_t	Diameter of tank (m)
D_{32}	Sauter mean diameter (m)
g	Gravity acceleration ($m^2 s^{-1}$)
k	Constant Eq.(14)
k_L	Liquid side mass transfer rate ($m\ s^{-1}$)
k_La	Volumetric mass transfer coefficient (s^{-1})
L	Length of light path (cm)
M	Molecular weight of H_2O (18 g $mole^{-1}$)
m	Constant Eq.(14)
n	Constant Eq.(14)
N_{Re}	Reynold's number
N	Agitation rate (rpm)
P	Pressure (atm)
P_m	Mixing Power (watt)
P_{mo}	Mixing Power for non-gassed systems (watt)
Q	Volumetric flow rate ($m^3 s^{-1}$)
qO_2	Specific oxygen uptake rate ($g^{-1}\ h^{-1}$)
Sc	Schmidt no. (Dimensionless)
T	Temperature (Kelvin)
t	Time (s)
V	Working volume (m^3)
$V_{m(o2)}$	Molar volume of oxygen (25.6 $cm^3\ g^{-1}$)
$V_{m(oil)}$	Molal volume of palm oil at normal boiling point ($m^3\ kmol^{-1}$)
V_s	Superficial velocity ($m\ s^{-1}$)
V_t	Terminal velocity ($m\ s^{-1}$)
X_a	Association factor 2.6 (H_2O)
X_{max}	Maximum biomass concentration (g L^{-1})
y	Total volume of reaction
z	Volume of sample
ε	Extinction coefficient of *p*NP

ρ_L	Density of liquids (continuos phase) (kg $m^{-1}s^{-1}$)
ρ_g	Density of gas (oxygen) (kg m^{-3})
μ_L	Viscosity of liquid (continuous phase) (kg m^{-1} s^{-1})
μ_g	Viscosity of the gas (dispersed phase) (kg $m^{-1}s^{-1}$)
ϑ	Kinematic viscosity of liquid ($m^2 s^{-1}$)
σ	Interfacial tension (kg s^{-2})
$\Delta\rho$	Density difference of two different phases present (kg m^{-3})
$\Delta\, pO_2\%$	Difference in oxygen level (%)
Δt	Difference in time taken (s)
ω	Agitation rate (rpm)
ϕ	Volume fraction of oil in the reaction mixture
θ_j	Mean area per total area of a category

Abbreviation

Abs	Absorbance
DO	Dissolved oxygen
MW	Molecular weight (g mol^{-1})
OTR	Oxygen transfer rate

LIST OF APPENDIX

CHAPTER 1

1.0 INTRODUCTION

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) catalyze the hydrolysis of ester bonds in triglyceride oils and other esters. Hydrolysis occurs in the presence of sufficient water. Synthesis of ester bonds is catalyzed in a low-water environment (Arroyo et al., 1999, Ducret et al., 1998, Gumel et al., 2011b, Kiran and Divakar, 2001, Klibanov, 1990, Klibanov, 1997, Sharma et al., 2001). Lipase catalysis is characterized by high enantio selectivity, regioselectivity and stereoselectivity (Chen et al., 1995, Ducret et al., 1998, Carstensen et al., 2011, Kodera et al., 1998, Therisod and Klibanov, 1987, Tsai and Dordick, 1996). In view of their versatility, lipases are widely used in processing of food, pharmaceuticals, leather, textiles, cosmetics, detergents and paper (Hasan et al., 2006, Schmidt-Dannert, 1999, Treichel et al., 2010, Vulfson, 1994) . Lipases function at oil-water interfaces. Such an interfaces is necessary for the active site of the enzyme to become exposed through conformational changes in the molecule (Domínguez de María et al., 2006, Reis et al., 2009, Sharma et al., 2001).

Among industrial enzymes, lipases rank third in sales after proteases and carbohydrate processing enzymes (Jaeger and Reetz, 1998). New applications of lipases keep emerging particularly in synthesis and they remain an active focus of research (Gumel et al., 2011a, Gumel et al., 2012, Gumel et al., 2011b, Pandey et al., 1999, Singh and Mukhopadhyay, 2012b). Although lipases occur widely in nature only the microbial lipases are mostly used commercially (Barros et al., 2010, Brownlee et al., 2010, Horne et al., 2009, Kurtovic et al., 2009, Mendes et al., 2012).

Microbial lipase can be produced inexpensively in bulk as microorganisms can be grown rapidly to a high concentration on inexpensive media. Recovery of lipases from

fermentation broth is easy in comparison with recovery from other sources. Many microorganisms have been selected to provide high titers of lipases (Treichel et al., 2010). Other microorganisms have been genetically modified or otherwise mutated to provide lipases with the desired characteristics (Gerritse et al., 1998; Schmidt-Dannert, 1999; Ferrer et al., 2001; Gaskin et al., 2001; Akoh et al., 2004; Dominguez de Maria et al., 2006; Schmidt et al., 2009). Lipases with improved thermostability (Haki and Rakshit, 2003), pH stability and specificity have been produced using recombinant microorganisms. Directed evolution has been used to improve lipases (Cherry and Fidantsef, 2003). Desirable lipases of higher animals may be produced in recombinant microorganisms. Recombinant lipases are widely used (Schmidt-Dannert et al., 1999; Ferrer et al., 2001; Olempska-Beer et al., 2006).

Microbial lipases (Gupta et al., 2004, Jaegar K. E, 1999, Rubin and Dennis, 1997, Salameh and Wiegel, 2007, Sharma et al., 2001, Singh and Mukhopadhyay, 2012a, Treichel et al., 2010, Contesini et al., 2010, Guncheva and Zhiryakova, 2011, Horchani et al., 2012, Joseph et al., 2008, Li and Zong, 2010, Rodrigues and Fernandez-Lafuente, 2010) and their applications (Contesini et al., 2010, Fan and Qian, 2010, Gumel et al., 2011b, Guncheva and Zhiryakova, 2011, Kobayashi, 2011, Yang et al., 2011) have been extensively reviewed in the literature, but their production by high-density fed-batch fermentations has received little attention. Table 1.1 (a and b) summarizes the diverse applications of lipases. Lipase catalyzed transesterification for the production of biodiesel from vegetable oils has been reviewed by (Bajaj et al., 2010) and (Bisen et al., 2010). Effects of surfactants on lipases have been discussed by (Delorme et al., 2011). Methods for improving the yield, activity and stability of lipases have been discussed by (Shu et al., 2010). Detection and characterization of lipases have been reviewed by (Hasan et al.,

2009). The use of immobilized lipases in bioreactors also has been discussed in the literature (Balcão et al., 1996, Malcata et al., 1992).

Here the study focused on the production of lipases by high cell density submerged culture in stirred tank reactor.

a) This study includes the implementation to optimize the effect of agitation rate on yeast growth, lipase production, and volumetric oxygen mass transfer coefficient *(k_La)* under high cell density fermentation;

b) This study compares three feeding strategies, i.e. the pH-stat operation, the foaming-dependent control of feeding and the specific growth rate control, in HCDF of the yeast *Candida rugosa* for producing lipases using palm oil as the sole source of carbon and energy;

c) Another aspect of the study was the determination of substrate mass transfer of gas-liquid and liquid-liquid by experimental and theoretical means. It is crucial to assess substrate mass transfer for the production of lipase particularly in HCDF which poses significant technical difficulties in terms of mass transfer.

Table 1.1 (a). Industrial application of lipases

Industry	Product	Application	References
Food industry	Dairy food	Infant formula milk scheme	(Osborn and Akoh, 2006)
		Acceleration of cheese ripening and its flavour development	(Kheadr et al., 2003)
	Cocoa butter	Trans and interesterification of fat in the production of delicate and inexpensive chocolate.	(Osborn and Akoh, 2006)
	Structured lipids	The inclusion of desired fatty acid through lipid modification for nutritional and therapeutic purposes	(Pszczola, 2001)
	Meat and fish	Lipase as fat remover from meat and fish product	(Kazlauskas and Bornscheuer, 1998)
	Beverage	Improve flavour and aroma	(Kazlauskas and Bornscheuer, 1998)
	Healthy foods	Reduced calorie and substituted fats	(Kanjilal et al., 1999, Akoh and Yee, 1997)
	Pharmaceutical	PUFAs are essential for normal synthesis of lipid membranes and prostaglandin	(Gill and Valivety, 1997, Belarbi et al., 2000)

Paper industry	Paper product	Lipases as pitch remover from the pulp	(Jaeger and Reetz, 1998)
Detergent industry	Lipolase Ultra, Lipoprime^{TM}, Lipex	Remove fat-containing stains	(Rathi et al., 2001)
Pharmaceutical industry	Anti inflammatory drugs , anticancer drugs, antiviral drug, antihypertensive drug, anti cholestrol drugs, anti-Alzheimer drug, and vitamin A.	Medicines	(Bonrath et al., 2002, Pandey, 2004)
Leather industry	Leather product	Liming and fat dispersion	(De Smet et al., 2001)
Cosmetic industry	Cosmetic products	Synthesis of isopropyl myristate (cosmetic ingredient)	(Yin et al., 2009, Verma et al., 2008)

Table 1.1 (b): Application of lipases in scientific researches

Application	Research	References
Lipases in organic synthesis	Used to catalyze a wide variety of chemo-, regio-, and stereoselective transformations	(Kazlauskas and Bornscheuer, 2008, Per and Karl, 2000, Byron Rubin, 1997)
	Use of lipases in the synthesis of enantiopure compounds	(Per and Karl, 2000)
	Esterification and transesterification are favored when there is little or no water present. However, hydrolysis is favored when there is excess of water.	(Klibanov, 1997)
	Lipase-catalyzed reactions in supercritical solvents	(Rantakylä et al., 1996, Turner et al., 2001, King et al., 2001)
	Hydrolysis of esters using lipase is commonly carried out in two-phase aqueous media	(Vaysse et al., 1997, Chatterjee et al., 2001)
Lipases in bioconversion in aqueous media	Hydrolysis of *p*-nitrophenyl palmitate (*pNPP*) in *n*-heptane by a lipase preparation of *Pseudomonas cepacia*	(Jaeger and Reetz, 1998)
	The enantioselective of lipases was greatly enhanced using mutagenesis method	(Bornscheuer, 2005, Gaskin et al., 2001)
	Novel lipase produced from *Burkholderia sp.* Could preferentially hydrolyze a bulky ester, *t*-butyl octanoate (TBO)	(Yeo et al., 1998)

Lipases in bioconversion in organic media	Lipases have been widely investigated for various non-aqueous biotransformations	(Klibanov, 1997, Therisod and Klibanov, 1987, Klibanov, 1990, Tsai and Dordick, 1996, Ducret et al., 1998, Dong et al., 1999, Kiran and Divakar, 2001)
Lipases in resolution of racemic acids and alcohols.	Racemic alcohols can also be resolved into enantiomerically pure forms by lipase-catalyzed transesterification.	(Klibanov, 1990)
	Influence of chiral corvones on selectivity of pure lipase-B from *Candida antartica*	(Arroyo M., 1995)
	Enhanced enantiospecificity of lipase catalysis in organic media induced by purification and catalyst engineering	(Tsai and Dordick, 1996)
	The importance of stereochemistry in the clinical pharmacokinetics of the 2-arylpropionic acid nonsteroidal anti-inflammatory drugs	(Hutt AJ., 1984)
	Optically pure profens was obtained through asymmetric chemical synthesis, catalytic kinetic resolution	(Van Dyck et al., 2001, Hutt AJ., 1984, Xin et al., 2001)
	2-phenoxy-1-propanol was resolved into its enantiomers using *Pseudomonas sp*. lipase by enantioselective transesterification	(Miyazawa et al., 1998)

	Lipases from *C. antarctica* and *R. miehei* catalyzed solvent-free thioesterification of fatty acids with long-chain thiols	(Weber et al., 1999)
	Solvent-free trans-thioesterification of fatty acid methyl esters with alkane thiols	(Weber et al., 1999)
Lipases in regioselective acylations	Production of monoacylated in anhydrous pyridine from triethyl carboxylates and various monosaccharides	(Therisod and Klibanov, 1987)
	Regioselective deacylation of preacylated methyl β- D-glucopyranoside using lipase from *A.niger*	(Chen et al., 1995)
	Regioselective deacetylation of preacetylated monosaccharide derivatives in 1,1,1-trichloroethane using a lipase modified with polyethylene glycol.	(Kodera et al., 1998)
Lipases in ester synthesis	Methyl and ethyl esters of long-chain acids have been used to enrich diesel fuels	(Packter, 1994)
	Esterification of lactic acid and alcohols using a lipase of *C. antarctica* in hexane	(From et al., 1997)
	Esterification of five positional isomers of acetylenic fatty acids (different chain lenghts) with *n*-butanol	(Lie Ken Jie and Xun, 1998)

	Optimum pre-equilibrium water activity value was necessary for obtaining a high rate of esterification of (*R,S*)-ibuprofen	(Arroyo et al., 1999)
	Esterification of sulcatol and fatty acids in toluene, catalyzed by *C. rugosa* lipase (CRL)	(Janssen et al., 1999)
	Lipase immobilized on silica and microemulsion-based organogels for ester synthesis	(Soni and Madamwar, 2001)
	A review on lipase mediated synthesis of sugar fatty acid esters	(Gumel et al., 2011b)
	Ultrasound assisted lipase catalyzed synthesis poly-6-hydroxanoate	(Gumel et al., 2012)
	Equi-volume blend of DMSO and *tert*-butanol with intermediate polarity proved to be most favorable for the esterification of D-glucose and decanoic acid	(Gumel et al., 2011a)
Lipases in oleochemical industry	Lipase catalyzed acidolysis, alcoholysis, and transesterification depend on type of subtrates used	(Balcão et al., 1996)

CHAPTER 2

2.1 High cell density fermentation

The volumetric productivity of constitutive enzymes such as lipases commonly depends directly on the concentration of the producing cells in the fermentation broth. This is because the volumetric productivity is a function of the cell density, or concentration in the broth, and the biomass specific productivity of the enzyme (Lee, 1996, Riesenberg and Guthke, 1999). Therefore, high cell density fermentation (HCDF) is useful in significantly improving the enzyme productivity relative to conventional batch fermentation that only attains a limited final biomass concentration. High cell density fermentations also allow elevated titers of the enzymes to be attained and this greatly reduces the expense of enzyme recovery and purification (Chisti, 2007).

A high cell density in a fermentation is normally achieved through a fed-batch operation (Lee, 1996). In a fed-batch operation, the growth limiting substrate is fed gradually so that substrate inhibition of growth is reduced, or prevented. For example, in *E. coli* fermentations, a glucose concentration of 50 g L^{-1} inhibits growth (Lee, 1996, Shiloach and Fass, 2005). High concentrations of other nutrients also inhibit growth. For example, ammonium at a concentration of 3 g L^{-1} inhibits growth in *E. coli* (Lee, 1996, Shiloach and Fass, 2005).

A fed-batch operation is nearly as simple as a batch operation and can be just as reliable (Bibila and Robinson, 1995). The design of the feeding strategy and its control are most influential in determining the productivity and the final titer in high cell density fermentation (Yamanè and Shimizu, 1984a, Akbari et al., 2010b, Lee et al., 1999, O'Connor et al., 2004). A variety of feeding strategies have been developed including the use of a

constant feeding rate, a stepwise increase of the feeding rate, and an exponentially increasing feeding rate (Lee, 1996, Shiloach and Fass, 2005). The feeding strategy may be modified during the course of a fed-batch fermentation to achieve different outcomes (Shojaosadati, 2008). The feeding may be controlled in response to online measured fermentation parameters (Lee, 1996).

High density fermentations were developed in the 1970s initially for the production of yeast biomass for use as single-cell protein. Subsequently, they have been used to produce a variety of recombinant proteins (Çelik and Çalık, 2012, Fieschko et al., 2004, Lee, 1996, Maghsoudi et al., 2012, Riesenberg et al., 1990, Shiloach and Fass, 2005, Yari et al., 2012, Yee and Blanch, 1992) as well as other products (Lee, 1996, Lee and Chang, 1995, Riesenberg and Guthke, 1999, Suzuki et al., 1987) in yeasts, bacteria, filamentous fungi and streptomyces. HCDF can attain exceptionally high concentrations of the biomass. For example, a final dry biomass concentration of 233 g $L^{-1}$was attained for *Methylobacterium extorquens* in a process for the production of poly-β-hydroxybutyric acid (PHB) (Suzuki et al., 1986). This fermentation was operated as a fed-batch with feeding controlled to maintain a constant concentration of dissolved oxygen (DO) in a DO-stat operation. The production of PHB was nearly 33 g L^{-1} d^{-1} (Suzuki et al., 1986).

An absolute upper limit on the attainable cell density is of course posed by the shape of the cells. The shape of the cells determines how closely they can pack together. This limit has been estimated to be about 200 g L^{-1} for *Escherichia coli* (Lee, 1996) although higher and lower estimates exist (Lee, 1996; Shiloach and Fass, 2005). At this concentration, the cells are packed as close together as possible to form a paste that does not flow (Lee, 1996). Obviously, the practical upper limit on the biomass concentration in a bioreactor is much lower than the limited dictated by space considerations. For high cell

density culture of *E. coli*, the maximum reported cell density in the broth is about 190 g L^{-1} (Shiloach and Fass, 2005). Although such high cell concentrations can be attained, exponential growth of *E. coli* in a high cell density fermentation becomes impossible to maintain once the biomass concentration is about 50 g L^{-1} (Shiloach and Fass, 2005). Subsequently, the biomass concentration increases but slowly.

Recombinant *E. coli* is being used to produce lipases on an experimental basis (Akbari et al., 2010a, Akbari et al., 2010b, Narayanan and Chou, 2009, Tang et al., 2000), but most industrial recombinant lipases are produced using nonenteric microorganisms.

A high concentration of cells in the bioreactor can be achieved also in a mode of operation known as perfusion culture (Voisard et al., 2003). In this operation, the cells are retained in the bioreactor by some type of a physical barrier, or through immobilization. Commonly, microfilter membranes are used for retaining cells (Carstensen et al., 2012, Chisti, 2007b). In perfusion culture, a cell-free product stream is continuously harvested from the bioreactor downstream of the retention device. This reduces or prevents the accumulation of inhibitory metabolites within the bioreactor. Although perfusion culture is widely used in production of monoclonal antibodies and other proteins from animal cells (Voisard et al., 2003) it has also been used for microbial products (Chang et al., 1994, Lee, 1996, Kim and Hou; 2006b; Pörtner and Märkl, 1998). It is expensive for large scale production of low-value products such industrial enzymes. Therefore, perfusion culture is not further discussed here.

High cell density fermentation has its drawbacks. A high biomass concentration of course does not always translate into a high productivity of the desired metabolite (Lee, 1996; Kheadr et al., 2003; Lee and Chang, 1995), as control of the culture conditions (e.g. dissolved oxygen concentration) can become difficult and this may adversely impact

production. Meeting the oxygen demand of a high density cell culture can be a challenge. Inhibitory metabolites can accumulate to high levels to adversely affect productivity of the target material (Lee, 1996). Removal of carbon dioxide becomes difficult as the cell density increases. Controlling the fermentation temperature may be a problem because of the high metabolic heat load. Mixing and other transport processes (oxygen transfer, carbon dioxide transfer, heat transfer) can be impeded by the high viscosity of a concentrated fermentation broth. These problems are discussed in the following sections and the strategies for mitigating them are outlined. The difficulties notwithstanding, the advantages of a high cell density fermentation can be quite attractive overall. High cell density fermentations can be cost effective, reduce capital outlay, reduce the size of the processing facility, simplify product recovery and reduce water consumption (no ref).

Fed-batch fermentations are certainly used in commercial production of microbial lipases, but not every fed-batch fermentation is a high cell density fermentation. High cell density fermentations appear promising for production of microbial lipases although there is limited information in this mode of operation relating directly to lipases (Adamitsch et al., 2003; Kim and Hou, 2006a).

2.2 Feeding strategies and their control

The feeding strategy is crucial to a successful operation of a HCDF process. The feeding strategy is used to control the cell growth and, to some extent, the formation of the product. Growth is typically controlled by limiting the supply of the carbon source. The aim is to minimize substrate inhibition, achieve rapid growth and obtain a high cell density while managing oxygen supply and carbon dioxide removal. The commonly used feeding strategies have been summarized by Lee, (1996).

The feeding methods may be classified into two main types: those without any kind of feedback control and those relying on some form of a feedback control mechanism. The latter type of feeding methods uses an online measured fermentation variable to control the feeding. The feeding methods not involving feedback control do not depend on any online measured information. The non-feedback feeding schemes include a constant rate feeding and an increasing rate feeding. In constant rate feeding, the nutrient feed rate is fixed at some preset value and, therefore, the specific growth rate progressively declines with time as the cell concentration increases and the rate of nutrient consumption increases. In increased rate feeding, the rate of feeding increases linearly, stepwise, or exponentially. Exponential feeding is generally intended to feed in such a way that the specific growth rate remains constant at some desired value. A model is used to calculate the required feeding rate (Lee, 1996; Lee and Rhee; 1993, Shiloach and Fass, 2005) as discussed later in this review.

Feeding methods involving feedback control can be quite diverse. For example, the substrate concentration measured online in the fermenter may be used to control the feeding. This of course requires a robust ability to measure the substrate concentration

online. Substrates such as oils and fatty acids that are used in some lipase fermentations are not easily measured online.

Online measurement is reliable for pH, temperature, agitation rate, carbon dioxide evolution rate (CER), oxygen uptake rate (OUR) and respiratory quotient (RQ) (Montesinos, 1994). Fermentation variables such as the biomass concentration and the concentration of the substrate can be estimated without being directly measured, by using online measured variables such as CER, OUR and RQ (Chattaway et al., 1992, Heinzle & Reuss, 1987, Von Schlien, 1995). Different levels of complexity are involved in these measurements.

Online measurement of extracellular lipolytic activity has been demonstrated (Valero, 1991), but the robustness of this method is questionable. In practice, lipases are almost never measured online during a fermentation. Although not useful of controlling a fed-batch operation, off line measurement of lipase activity in the filtrate of the fermentation broth is always required for assessing the productivity of the fermentation process. *In vitro* methods for quantifying the lipase activity have been discussed by Gilham and Lehner, (2005). An enzyme electrode for measuring the activity of lipases has been described by Ben Rejeb et al., (2007).

The feeding strategies developed for the other high cell density fermentations can be applied to production of microbial lipases (Lee et al., 1999). The feeding strategies vary in complexity, reliability and the expense of implementation. Constant rate feeding, exponential feeding (also known as specific growth rate control operation) and feeding methods based on measurement of dissolved oxygen concentration are among the easiest to implement and are reliable. The various feeding strategies are discussed in the following

sections. Irrespective the feeding scheme used, the fermenter must have the necessary space to accommodate the feed for the duration of the fermentation.

The development of a suitable feeding strategy requires a detailed understanding of how the components of the fermentation medium may affect the biochemistry of lipase production. For example, certain carbon sources may repress enzyme synthesis whereas some fatty acids and oils may induce production. If the production involves a recombinant microorganism, an understanding of the control of enzyme production at the gene level is necessary for implementing a successful production strategy.

2.3 Feeding without feedback control

2.3.1 Constant rate feeding

In constant-rate feeding, concentrated nutrients are fed at some pre-determined rate. As the cell population increases, the specific growth rate declines because the feeding rate is fixed but the rate of consumption increases with the increasing population (Lee, 1996, Shojaosadati, 2008). Constant rate feeding is of course extremely easy to implement and has been widely used in producing lipases. Generally, the final biomass concentration is lower in a constant rate feeding operation compared with the final biomass concentration in a specific growth rate controlled operation of the same duration. Fermentation conditions and outcomes for several reported constant rate feedings are shown in Table 2.1.

Table 2.1: Fed-batch fermentations and their operating conditions for lipase production

Microorganism	Operating conditions	Feeding mode	Biomass concentration	Enzyme titer
Candida rugosa (Gordillo et al., 1998a)	6L STR, 5 L working vol., pH 6.3, 500 rpm, 30^{o}C, 0.5–3 L min^{-1} air flow rate to maintain 20% dissolved O_2	Constant rate feeding	5.9 g L^{-1}	55 U mL^{-1}
Candida rugosa (Montesinos et al., 1996)	6L STR, controlled pH, 500 rpm, 30^{o}C, 0–5 L min^{-1} air flow rate to maintain 20% dissolved O_2	Constant rate feeding	N/A	45UmL^{-1}
Pseudomonas aeruginosa (Chartrain et al., 1993)	23L STR, 15 L working vol., pH 7, 600 rpm, 25^{o}C, 6 L min^{-1} air flow rate, 0.5 bar back pressure	Constant rate feeding	N/A	110 U mL^{-1}
Ophiostoma piceae (Tamerler and Keshavarz, 2000)	20L STR, 14 L working vol., 200-800 rpm to maintain 20% dissolved O_2, 26^{o}C, 9L inoculum	Constant rate feeding	N/A	254 UL^{-1}d^{-1}
Fusarium oxysporum (Tamerler and Keshavarz, 2000)	20L STR, 14 L working vol., 200-800 rpm to maintain 20% dissolved O_2, 26^{o}C, 9L inoculums	Constant rate feeding	N/A	224 UL^{-1}d^{-1}

Candida rugosa (Gordillo et al., 1998a)	6L STR, 5 L working vol., pH 6.3, 500 rpm, 30°C, 0.5–3 L min^{-1} air flow rate to maintain 20% dissolved O_2	Specific growth rate control	6.9 g L^{-1}	117 U mL^{-1}
Candida cylindracea (Kim and Hou, 2006a)	7.5 L STR, 3 L initial working vol., pH 6.3 controlled by 28% NH_4OH, 300-1000 rpm to maintain 30% dissolved O_2, 30°C	Specific growth rate control	90 g L^{-1}	23.7 U mL^{-1}
Candida rugosa (Montesinos et al., 1996)	6L STR, controlled pH, 500 rpm, 30°C, 0–5 L min^{-1} air flow rate to maintain 20% dissolved O_2	Specific growth rate control	-	45U mL^{-1}
Pichia pastoris expressing ROL (Resina et al., 2005)	5 L STR, 3.5 L initial working vol., pH 5.5 controlled by 5 M KOH, 800 rpm, 30% dissolved O_2, 30°C, 1.5-20 L min^{-1} air flow rate	Specific growth rate control	50.4 g L^{-1}	385 AU mL^{-1}
Pichia pastoris expressing ROL (Arnau et al., 2010)	5 L STR, 3.2 L initial working vol., pH 5.5 controlled by 5M KOH or NH_4OH 30% (v/v), 1000 rpm, 30% dissolved O_2, 30°C, 0.5-8 L min^{-1} air flow rate	Specific growth rate control	45.4 g L^{-1}	621.2 AU mL^{-1}

Pichia pastoris expressing *Candida rugosa* lipase (Zhao et al., 2008)	5, 30, 800L STR and 1.8, 10, 280L initial working volume, respectively. pH 5.5–6.5 controlled by 30% (v/v) NH_4OH. Agitation was varied to maintain 30% dissolved O_2. 10% (v/v) inoculums of initial working volume	Specific growth rate control	500 g L^{-1} (freshweight)	14,000 IU mL^{-1}
Schizosaccharomyces pombe (Ikeda et al., 2004)	5 L STR, 2.5 L initial working vol., pH 6 controlled by 25% NH_4, agitation was varied to maintain 1 ppm dissolved O_2, 30°C	Specific growth rate control	>50 g L^{-1}	16 U mL^{-1}
Brevibacterium linens (Adamitsch et al., 2003)	14 L STR, 10L working vol., pH 7, 800 rpm, 20% dissolved O_2 maintained by pure oxygen, 3 L min^{-1} gas flow rate 29°C, CO_2 in effluent gas was monitored	Transitional feeding	50 g L^{-1}	33UL^{-1}
Pseudomonas aeruginosa (Ito et al., 2001)	2 L STR, 900 ml initial working vol., Rushton turbine, 3 baffles, pH 7.4–7.6 maintained by 14% (v/v) NH_4 solution or 1 N HCL, 20% dissolved O_2 maintained by pure oxygen, 3 L min^{-1} gas flow rate, 29°C, CO_2 in effluent gas was monitored	Transitional feeding	30.2 g L^{-1}	96 IU mL^{-1}

Pichia pastoris expressing ROL (Minning et al., 2001)	5 L STR, 3.5 L initial working vol., pH 5.5 controlled by 2M NH_4OH, 500-900 rpm to maintain 20% dissolved O_2, 30^oC, 0.5-10 L min^{-1} air flow rate	Transitional feeding	N/A	12,888 $UL^{-1}h^{-1}$
Pichia pastoris expressing ROL (Surribas et al., 2007)	20 L STR, pH 5.5 controlled by 30% NH_4OH, 1000 rpm, 30^oC, 1-2 vvm air flow rate, foaming controlled by antifoam.	Transitional feeding	N/A	644 AU mL^{-1}
Yarrowia lipolytica (Fickers et al., 2009)	20 L STR, 15 L working vol., pH 6.5 ± 0.1 controlled by 4N NaOH or 4N H_3PO_4, 350 rpm, 29^oC t, 0.5 or 1 vvm air flow rate	Transitional feeding	1.7×10^9 cells mL^{-1}	3,044 U mL^{-1}

Simulations of *Candida rugosa* fermentations suggest that fed-batch operational strategies with a constant feeding rate can provide a higher final lipase activity compared with specific growth rate control feeding (Gordillo et al., 1998a, Gordillo et al., 1998b). In constant rate feeding at a low rate, the maximum final titer of lipase in the supernatant was 55 U m L^{-1} (Gordillo et al., 1998a, Gordillo et al., 1998b), but the lipase tended to accumulate within the cells at higher feeding rates. With a constant specific growth rate feeding strategy, lipase production was 10-fold greater compared with a batch operation (Gordillo M. A., 1998a). For the above specified titer of 55 U m L^{-1}, the substrate feeding rate was 1 g oleic acid h^{-1} (Gordillo et al., 1998a). Montesinos et al. (Montesinos J.L., 1996) reported an extracellular lipase titer of 46 U m L^{-1} at a feed rate of 0.4 g oleic acid h^{-1} in *C. rugosa* fermentation.

In constant rate feeding of the substrate, the lipase productivity is always better at a relatively low feeding rate as this ensures that the enzyme produced within the cell can be excreted so that the intracellular accumulation of the enzyme is kept at a minimum (Boareto et al., 2007, Gordillo et al., 1998, Montesinos et al., 1996). Too high a feeding rate leads to intracellular accumulation of the enzyme and inhibition of its production. Furthermore, too rapid an addition of the substrate increases oxygen demand but the oily substrates that are commonly used to induce the production of lipases tend to reduce the gas-liquid oxygen mass transfer coefficient. This combination of an increased oxygen demand and a reduced oxygen transfer coefficient starves the cells of oxygen.

Too low a substrate feeding rate also adversely affects the lipase titer because the low substrate concentration limits growth (Boareto et al., 2007). A maximum lipase activity of about 6×10^4 U L^{-1} was found at a feeding rate 2 g h^{-1} of oleic acid. Lower feed rates

reduced lipase titer and increasing the feeding rate to 3.5 g h^{-1} did not further increase the enzyme titer (Boareto et al., 2007).

The nature of the feed is another factor that influences the lipase productivity of a fermentation. For example, in lipase production by *Pseudomonas aeruginosa* in a fed-batch operation using a medium consisting of glucose and ammonium chloride, the optimum ratio of carbon to nitrogen has been found to be 4:13 (Chartrain et al., 1993). Using a feed with this optimal ratio and a feed rate of 0.3 g L^{-1} h^{-1} glucose and 0.142 g L^{-1} h^{-1} ammonium chloride, a lipase titer of 110 U m L^{-1} was attained (Chartrain et al., 1993). An optimal medium would of course depend on the microorganism being used for lipase production.

In 2L stirred tank bioreactors, fed-batch cultures of *Ophiostoma piceae* and *Fusarium oxysporum* afforded lipase productivities of 254 U L^{-1} d^{-1} (yield 73 U g^{-1} dry biomass) and 224 U $L^{-1}d^{-1}$ (yield 48 U g^{-1} dry biomass), respectively, (Tamerler and Keshavarz, 2000). During the fermentation, as much as 12.5 m L of rapeseed oil and 21 g of mycological peptone or 20.5 g of yeast extract were fed intermittently per liter between 72 and 144 h (Tamerler and Keshavarz, 2000). The fermentations were successfully scaled up to 20 L with comparable lipase productivity and yield.

Constant rate feeding of olive oil has been used in production of lipase from *Pseudomonas fluorescens* (Suzuki, 1988). As expected, the biomass concentration increased in direct proportion to the amount of the oil fed, but the production of the lipase activity was not directly proportional to the concentration of the biomass or the olive oil consumed.

2.3.2 Specific growth rate control

In principle, feeding at exponentially increasing rate can be used to control the specific growth rate at a constant level in a fed-batch fermentation. This method of feeding is known as specific growth rate control. The specific growth rate may be varied at different stages of the fermentation by changing the feed rate. Exponentially increasing feeding has been commonly used to control the specific growth rate at a relatively low constant level in many fed-batch lipase fermentations. Fermentation conditions and outcomes for several reported specific growth rate controlled feedings are shown in Table 2.1.

In specific growth rate control, the mass flow rate of the feed depends on the desired specific growth rate μ, as follows (Lee, 1996).

$$F_t = Q_t S_t = \left(\frac{\mu}{Y_{X/S}} + m\right) X_t V_t \tag{2.1}$$

In Equation (2.1), F_t is the mass flow rate of the feed at time t, Q_t is the volume flow rate of the feed at time t, S_t is the concentration of the growth limiting substrate in the feed at time t, $Y_{X/S}$ is the biomass yield on substrate, m is the maintenance coefficient, X_t is the biomass concentration in the fermenter at time t and V_t is the volume of the broth in the fermenter at time t. Equation (2.1) assumes a constant biomass yield on the growth limiting substrate and a constant value of the maintenance coefficient. During growth at a constant specific growth rate μ, the total amount of biomass in the fermenter, i.e. $X_t V_t$, increases with time as follows:

$$X_t V_t = V_0 X_0 e^{\mu t} \tag{2.2}$$

where V_0 is the initial volume in the fermenter and X_0 is the initial concentration of the biomass.

A substitution of Equation (2.2) in Equation (2.1), leads to the following feed rate equation:

$$F_t = \left(\frac{\mu}{Y_{X/S}} + m\right) X_0 V_0 e^{\mu t} \quad (2.3)$$

Feeding at a rate specified by Equation (2.3) should result in the growth being controlled at the specific growth rate μ. As the cell concentration increases, limitations of mass transfer and possible other factors could lead to a failure of the specific growth rate control strategy. The maintenance coefficient *m* is generally relatively small and has been commonly disregarded in calculations of the feeding rate (Kim, 2006). The yield coefficient $Y_{X/S}$ is generally taken to be a constant. Batch culture data are commonly used to determine $Y_{X/S}$ experimentally (Li et al., 2004, Resina et al., 2005).

Production of lipase by specific growth rate control has been widely reported (Arnau et al., 2010, Cos et al., 2005, Gordillo et al., 1998a, Kim, 2006, Li et al., 2004, Montesinos et al., 1996, Zhao et al., 2008). In lipase production from *C. rugosa*, an extracellular lipase activity of 117 U mL^{-1} could be achieved in 40 h (Gordillo et al., 1998). In lipase production from *Candida cylindracea* the highest enzyme productivity was attained at specific growth rate controlled at 0.08 h^{-1}, but control of the specific growth rate at a lower value of 0.02 h^{-1} helped in preventing the accumulation of oleic acid in the culture broth and a high titer of the extracellular lipase was attained (Kim and Hou, 2006). In lipase production using *Acinetobacter radioresistens* grown on Tween 80, control of the specific growth rate at an extremely low value of 0.016 h^{-1}, or a high value of 0.716 h^{-1}, reduced the extracellular lipase activity to vanishing levels (Li et al., 2004). Lipase production was best at a controlled specific growth rate of ~0.2 h^{-1} (Li et al., 2004). Using *C. rugosa* growing on oleic acid, Boareto et al.,(2007), achieved an extracellular lipase titer

of around 46 U mL^{-1} by controlling the specific growth rate at a low value of 0.06 h^{-1}. In view of these results, the optimal value of the controlled specific growth rate depends on the producer microorganism and possibly also on the carbon source being fed.

Specific growth rate control at relatively low values has been found suitable also in production of lipases in various recombinant microorganisms (Arnau et al., 2010, Cos et al., 2005). For example, in production of *Rhizopus oryzae* lipase (ROL) in *Pichia pastoris* growing on mixed substrates of methanol and sorbitol, the highest lipase activity was attained at a controlled specific growth rate of 0.01 h^{-1} (Arnau et al., 2010). Under these conditions, the maximum lipolytic activity was 621.2 AU mL^{-1} at a biomass concentration of 45.4 g L^{-1} (Arnau et al., 2010). Use of the rapidly assimilated methanol as the sole substrate reduced the biomass specific production rate of the enzyme. This was avoided by using sorbitol as a co-substrate (Arnau et al., 2010).

In production of *C. rugosa* lipase in a recombinant *P. pastoris*, a low controlled specific growth rate of 0.15 h^{-1} maximized the lipase activity at 14,000 IU mL^{-1} in a 800 L stirred tank fermenter (Zhao et al., 2008).

Use of a relatively low cell growth rate in fed-batch production of a human lipase in the yeast *Schizosaccharomyces pombe* enhanced the productivity of the extracellular enzyme (Ikeda et al., 2004). The slow growing cells were better able to adapt to the dynamic environment of the culture system (Ikeda et al., 2004). A control of the specific growth rate at a low level eased control of the dissolved oxygen concentration in the range 30 to 50% of air saturation value that was found to be optimal for production of the lipase (Ikeda et al., 2004).

2.3.3 Transitional feeding

In transitional feeding, the operational and feeding strategies are initially tailored to producing a sufficient amount of biomass and then the composition of the feed is altered to induce product formation. Feed transition, or switching of the feeds, may be based on time, or it may rely on some sort of a feedback signal from the online monitored culture. Transitional feeding has proved beneficial in production of lipases (Adamitsch et al., 2003, Fickers et al., 2009, Ito et al., 2001, Minning et al., 2001). Fermentation conditions and outcomes for several reported transitional feedings are shown in Table 2.1.

This feeding strategy was used in production of lipase from *Brevibacterium linens* (Adamitsch et al., 2003). A biomass concentration of up 50 g L^{-1} was attained in 60 h in an operation consisting of a batch phase followed by a fed-batch phase. The fed-batch operation consisted of two different feeding regimes: constant specific growth rate control at 0.12 h^{-1}followed by a constant rate feeding (Adamitsch et al., 2003). The volumetric productivity of the lipolytic activity was 7,300 U m L^{-1} h^{-1}.

A two-step process for lipase production from *P. aeruginosa* used a batch growth phase followed by a fed-batch enzyme production phase (Ito et al., 2001). In fed-batch operation, lipase production was induced by adding 5% (v/v) stearic acid to the fermenter 5 h after the residual glucose from the batch phase of operation had run out. Without induction, there was negligible production of lipase. Within 16 h of induction, corresponding to 33 h of culture, the lipase concentration reached 65 U mL^{-1} at a biomass concentration of 22 g L^{-1}. Stearic acid was fed again at a biomass concentration of 30 g L^{-1} and a biomass concentration of 48 g L^{-1}. A lipase activity of 96 U $mL^{-1}$was attained at the biomass concentration of 30 g L^{-1}, but the activity was reduced at the higher cell concentration of 48 g L^{-1} because of oxygen starvation (Ito et al., 2001).

Transitional feeding approaches for lipase production have generally used media optimized for growth in the biomass production phase and media tailored for enzyme production in the subsequent phase (Adamitsch et al., 2003, Fickers et al., 2009, Ito et al., 2001, Minning et al., 2001). In some case, in the production phase, the enzyme production was enhanced by controlling the concentration of repressors such as glucose (Fickers et al., 2009).

2.4 Feedback controlled feeding

In feedback controlled feeding, an online measured fermentation property is used to control the rate of feeding. The feedback control strategy is said to be indirect if the variable used to control the feeding is anything other than the measured concentration of the substrate in the fermenter. Direct feedback control relies on online measured concentration of the substrate in the fermenter to control the feeding.

2.4.1 Indirect feedback control

In this form of feeding, the feed rate is controlled by some online measured property of the fermentation other than the concentration of the substrate. Feeding may be controlled by the measured concentration of dissolved oxygen (DO), the pH, the carbon dioxide evolution rate (CER), the oxygen uptake rate (OUR), the respiratory quotient (RQ), the biomass concentration and metabolic heat generation rate (Biener et al., 2012; Gordillo et al., 1998a, Lee et al., 1999; Lee, 1996, Oh and Moo-Young, 1998).

The feeding scheme based on a DO-stat operation that relies on the online measured concentration of dissolved oxygen (DO) in the fermentation broth. As the substrate is depleted to a low level, the metabolic activity slows and the consumption of oxygen

reduces. This results in a rapid rise in concentration of dissolved oxygen. This signal is used to control the feeding so that the dissolved oxygen concentration remains at some preset value. A pH-stat operation that is similar in principle to the DO-stat, can be used to control feeding in some fermentations.

Carbon dioxide evolution rate (CER) is considered to be roughly proportional to the rate of consumption of the carbon source. For controlling the feeding, the CER is calculated online from mass spectrometric measurements of the carbon dioxide concentration in the gas leaving the fermenter. Similar measurements may be used to calculate the respiratory quotient online. Biomass concentration may be measured online by laser turbidimetry.

CER controlled feeding of olive oil has been used in production of lipase from *P. fluorescens* (Suzuki, 1988) and *C. rugosa* (Gordillo et al., 1998a). In *P. fluorescens* culture, once a biomass concentration of 60 g L^{-1} had been attained, controlling the feed rate at 30 g oil per mole of carbon dioxide evolved maximized lipase production (Suzuki, 1988). A lipase activity of 1980 U mL^{-1} was attained (Suzuki, 1988). Higher feeding rates of oil favored biomass growth over lipase production (Suzuki, 1988).

Online monitoring of CER and OUR have been used to detect the onset of the stationary phase in batch culture of *C. rugosa* prior to commencing constant rate feeding of oleic acid (Boareto et al., 2007).

In production of lipase by *A. radioresistens*, pH-stat feeding was found to approximate to exponential feeding in behavior and DO-stat feeding mimicked the constant rate feeding behavior (Li et al., 2004). This was because the specific growth rate intrinsically affected the lipase production rate when the dissolved oxygen level was controlled at 30%, or greater and the pH was in the range of 6.9–7.0. The DO-stat feeding was found to be a promising control method compared with the pH-stat feeding (Li et al.,

2005). The pH-stat feeding led to a rapid decline in the dissolved oxygen concentration because of a rapid increase in biomass concentration. In a different study, the DO-stat based feeding of methanol during production of a lipase afforded poor control and the methanol concentration exceeded the inhibitory level (Stratton & Meagher., 1998). This suggests that a suitable strategy for feedback controlled feeding depends on the microorganism and the nature of the feed.

Turbidity-dependent feeding was shown to be reliable in the production of lipase by *P. fluorescens* (Ishihara et al., 1989). Feeding of olive oil and Fe^{2+} was controlled in response to online measured turbidity. At the optimum specific consumption rate of olive oil of 0.2 g g^{-1} cells per h, the lipase titer was 5,600 U mL^{-1} (Ishihara et al., 1989). Use of online turbidity measurements to control the feeding in production of a recombinant human lipase in *Schizosaccharomyces pombe* has been reported (Ikeda et al., 2004).

In turbidity controlled feeding, the feed rate equation was as follows (Ikeda et al., 2004);

$$Q_t = \frac{q_s V_t X_t}{S_f} \tag{2.4}$$

where Q_t is the volumetric feed rate (L h^{-1}) at time t,q_s is the constant biomass specific substrate consumption rate (= 0.2 g g^{-1} h^{-1}), V_t (L) is the culture volume at time t, X_t (g L^{-1}) is the measured biomass concentration at time t, and S_f is the concentration of the substrate in the feed (g L^{-1}).

2.4.2 Direct feedback control

In direct feedback control, the online measured concentration of the growth limiting substrate in the bioreactor is used to control the rate of feeding. When the feed consists of triglyceride oils, as is common in production of lipases, the fatty acids released by lipase-mediated extracellular hydrolysis of the oil are measured to control the feeding. Fatty acids can be measured online using high-performance liquid chromatography (HPLC), gas chromatography (GC) and mass spectrometers (MS).

Various other carbon substrates that may be fed instead of triglycerides can also be measured online. For example, online gas chromatography has been used to control the concentration of methanol in production of *R. oryzae* lipase in *P. pastoris* (Minning et al., 2001). This method has proved better than the previously mentioned DO-stat based indirect feedback control of methanol feeding (Stratton J., 1998). An optimized methanol feeding strategy based on direct measurement of its concentration achieved a lipase productivity of 12,888 U L^{-1} h^{-1}, or 13.6-fold greater than was possible with the DO-stat based feeding (Minning et al., 2001).

The lack of online sensors for some analytes can be compensated by coupling some of the feasible online measurements to mathematical models in order to estimate in real time the concentration that cannot be measured directly. The model estimated concentration is then used to control the feeding. This approach was used in improving the production of *C. rugosa* lipase (Montesinos et al., 1996). Once a process model had been established and validated, it was used to simulate the batch, fed-batch and continuous culture operations for lipase production. For a fed-batch operation with a constant feeding rate of oleic acid, a feed rate of ~1 g h^{-1} was predicted to maximize the lipase activity at around 45 U m L^{-1} (Montesinos et al., 1996). A maximum lipase productivity of around 1.2 U m L^{-1} h^{-1} was

predicted at a constant feed rate of around 1.9 g h^{-1}. For specific growth rate controlled feeding, a maximum lipase productivity of around 0.85 U m L^{-1} h^{-1} was predicted at a specific growth rate of around 0.05 h^{-1} (Montesinos et al., 1996).

A combination of neural networks and phenomenological models with online measurements of carbon dioxide and substrate feed rate, has been used for predicting the concentrations of the lipase, the substrate and the biomass (Boareto et al., 2007). Mechanistic models for lipase production are discussed by Gordillo et al., (1998).

2.5 Oxygen transfer in lipase fermentation

Oxygen supply can be a major challenge in high cell density cultures because of a high demand for oxygen and a relatively viscous nature of the broth. The latter is a consequence of a high concentration of the biomass. The oxygen supply problem is further accentuated in lipase fermentations that commonly require water-immiscible substrates such as vegetable oils and fatty acids. These substrates can be essential for inducing the production of lipases, but coat the gas-liquid interface with a layer of oil to reduce the overall gas-liquid volumetric mass transfer coefficient, k_La. For example, adding palm oil to an aqueous broth has been found to reduce the oxygen mass transfer coefficient (Annuar et al., 2007, Sauid and Murthy, 2010). The effects of several different fermentation operational conditions on k_La and lipase production are shown in Table 2.2.

Table 2.2: The effects of different fermentation operational conditions on k_La in lipase production

Microbes	Substrate	Impeller type	Agitation speed	Aeration rate	k_La/DO	Lipase Titer
Acinetobacter Radioresistant (Chen et al., 2000)	Hexadecane 1% (v/v)	Rushton turbine	400 rpm	0.5 vvm	31 h^{-1}	0.25 U ml^{-1} h^{-1}
				1.0 vvm	49 h^{-1}	0.4 U ml^{-1} h^{-1}
				1.5 vvm	52 h^{-1}	0.45 U ml^{-1} h^{-1}
			500 rpm	0.5 vvm	68 h^{-1}	0.55 U ml^{-1} h^{-1}
				1.0 vvm	97 h^{-1}	0.8 U ml^{-1} h^{-1}
				1.5 vvm	122 h^{-1}	0.96 U ml^{-1} h^{-1}
Yarrowia lipolytica (Alonso et al., 2005)	Olive oil 1% (v/v)	Rushton turbine	100 rpm	0.8 vvm	8.5 ± 0.30 h^{-1}	960 U L^{-1}
			200 rpm	0.8 vvm	12.2 ± 1.02 h^{-1}	4680 U L^{-1}
			300 rpm	0.8 vvm	26.0 ± 0.64 h^{-1}	2390 U L^{-1}
			400 rpm	0.8 vvm	54.5 ± 0.51 h^{-1}	830 U L^{-1}
			200 rpm	0.8 vvm	12.2 ± 1.02 h^{-1}	4680 U L^{-1}
				1.7 vvm	21.0 ± 0.68 h^{-1}	4525 U L^{-1}
				2.5 vvm	25.5 ± 1.08 h^{-1}	2735 U L^{-1}
Rhizopus arrhizus (Elibol and Ozer, 2000)	PFC 20% (v/v)	Rushton turbine 6 flat blades	200 rpm	0.25 vvm	12 h^{-1}	0.124 U ml^{-1} h^{-1}
				0.5 vvm	28 h^{-1}	0.270 U ml^{-1} h^{-1}
				1.0 vvm	50 h^{-1}	0.370 U ml^{-1} h^{-1}
				1.5 vvm	60 h^{-1}	0.450 U ml^{-1} h^{-1}
			300 rpm	0.25 vvm	22 h^{-1}	0.23 U ml^{-1} h^{-1}
				0.5 vvm	68 h^{-1}	0.60 U ml^{-1} h^{-1}
				1.0 vvm	100 h^{-1}	0.80 U ml^{-1} h^{-1}
				1.5 vvm	118 h^{-1}	0.88 U ml^{-1} h^{-1}

Aspergillus terreus (Bajaj et al., 2010)	Corn oil 2% (v/v)	N/A	Controlled by agitation-aeration cascade	1–2 vvm	20% DO 40% DO	12000 U L^{-1} 12000 U L^{-1}
			250 rpm 300 rpm 350 rpm	1–2 vvm	20% DO	12300 U L^{-1} 14200 U L^{-1} 12000 U L^{-1}
Simulated system (pure water) (Sauid and Murthy, 2010)	Palm oil 5% (v/v) (Optimized oil fraction)	Rushton or Inter MIG	200 rpm	0.25 vvm 0.75 vvm 1.25 vvm	5 h^{-1} 7 h^{-1} 9 h^{-1}	N/A N/A N/A
			400 rpm	0.25 vvm 0.75 vvm 1.25 vvm	30 h^{-1} 35 h^{-1} 37 h^{-1}	N/A N/A N/A
Simulated system (xantham gum solution) (Sauid and Murthy, 2010)	Palm oil 5% (v/v)	(140 cP) Rushton	400 rpm	0.75 vvm	12 h^{-1}	N/A
		(290 cP) Inter MIG	400 rpm	0.75 vvm	10.5 h^{-1}	N/A

Simulated system (Pure water) (Amaral et al., 2008)	Olive oil (0.20 vol. Fraction)	Rushton Turbine (6 vertical blades)	160 rpm	1.0 L min^{-1}	9 h^{-1}	N/A
				1.5 L min^{-1}	10 h^{-1}	N/A
				2.0 L min^{-1}	12 h^{-1}	N/A
			250 rpm	1.0 L min^{-1}	13 h^{-1}	N/A
				1.5 L min^{-1}	17 h^{-1}	N/A
				2.0 L min^{-1}	17 h^{-1}	N/A
			350 rpm	1.0 L min^{-1}	27 h^{-1}	N/A
				1.5 L min^{-1}	28 h^{-1}	N/A
				2.0 L min^{-1}	30 h^{-1}	N/A

The oxygen requirement of a fermentation depends on the concentration of the biomass in the fermenter. Oxygen is commonly supplied by vigorous sparging of the culture broth with air. Mechanical agitation is used to further assist oxygen transfer. The oxygen transfer rate must match the oxygen demand, or the microorganisms will be starved of oxygen and this will adversely affect lipase productivity. Fermentations for producing lipases have generally used stirred tank fermenters. The oxygen transfer rate in a given stirred tank bioreactor depends on the stirring speed and the aeration rate. In a fermentation broth of given properties, the speed of agitation and the rate of aeration control the value of the overall volumetric oxygen mass transfer coefficient, $k_L a$. In view of its importance, the effects of oxygen transfer on lipase production have been investigated in various fermentations. In addition, in a lipase fermentation that uses water-immiscible oil as a substrate, the lipolysis of oil and the mass transfer of the dispersed carbon source is also influenced by agitation.

Water insoluble oxygen vectors such as perfluorocarbons have been used in lipase fermentations in attempts to enhance oxygen transfer (Amaral et al., 2006, Amaral et al., 2007, Elibol and Ozer, 2000). Perfluorocarbons tend to be chemically inert and, compared with water, can dissolved much more oxygen (Cho and Wang, 1988, Ju et al., 1991). Therefore, perfluorocarbons can provide a concentrated pool of oxygen for absorption by the aqueous fermentation broth.

Unlike most vegetable oils and fatty acids, perfluorocarbons tend to be much denser than water and form a pool at the bottom of the fermenter. The contents of this pool can be dispersed in the fermenter by intense mixing. Use of perfluorocarbons as oxygen carriers has been shown to improve lipase production by the yeast *Y. lipolytica* in a fermentation that used olive oil as the carbon source (Amaral et al., 2006). In the presence of 20% (v/v)

of perfluorocarbon, at an agitation rate of 250 rpm, the fermentation produced more lipase compared with control (Amaral et al., 2006). Increasing the volume fraction of perfluorocarbon to >20%, reduced the efficiency of dispersion and lipase production was reduced because of poor oxygen transfer (Amaral et al., 2006). At a perfluorocarbon level of 20%, reducing the agitation rate adversely affected the dispersion of the perfluorocarbon in the fermentation broth and this reduced oxygen transfer and lipase production. Under optimal production conditions, the lipase productivity was 48.5 ± 1.0 UL^{-1} h^{-1} compared with a productivity of 8.4 ± 0.3 U L^{-1} h^{-1} at an agitation speed of 160 rpm in the absence of perfluorocarbon (Amaral et al., 2006).

In a different study, the inclusion of 10% perfluorodecalin in the fermentation medium increased lipase production by *Rhizopus arrhizus* compared with control (no perfluorodecalin) (Elibol and Ozer, 2000).

Oxygen mass transfer coefficient k_La and hence the oxygen transfer rate generally depends more strongly on the speed of mechanical agitation compared with its dependence on the aeration rate (Figure 2.1). At a fixed agitation speed of 300 rpm increasing the dissolved oxygen level from 20 to 70% of air saturation only slightly improved cell growth and lipase production in *R. arrhizus* fermentation (Elibol and Ozer, 2000). On the other hand, at a fixed dissolved oxygen level of 20%, increasing in the agitation speed from 200 to 400 rpm significantly improved cell growth and lipase production (Elibol and Ozer, 2000). The productivity of lipase correlated directly with k_La (Elibol and Ozer, 2000). Only water soluble substrates were used.

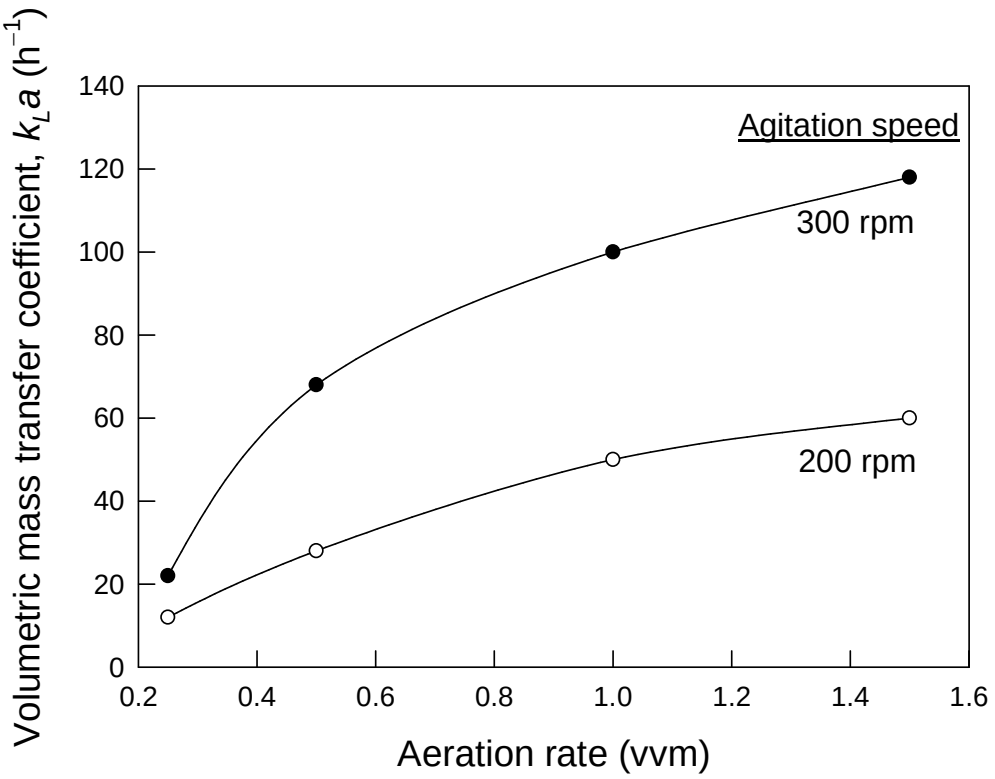

Figure 2.1: Dependence of the overall volumetric gas–liquid mass transfer coefficient (k_La) on aeration rate and the agitation speed of the impeller (a 6-bladed disc turbine, 6 cm in diameter) in *Rhizopus arrhizus* fermentation broth in a 1 L stirred tank fermenter operated at 30 °C. Adapted from Elibol & Ozer, (2000).

In production of lipase from *Yarrowia lipolytica* growing on olive oil, a maximum biomass concentration of 8.7 g L^{-1} and a lipase activity of 4680 U L^{-1} were attained at 240 h using an agitation speed of 200 rpm in combination with an aeration rate of 0.8 vvm (Alonso et al., 2005). Lower agitation speeds reduced oxygen transfer and lipase production. Increasing the agitation speed to 400 rpm dramatically reduced both the biomass concentration and the lipase activity. This behavior could be explained with reference to the unusual nature of the yeast *Y. lipolytica*. This yeast grows in a typical yeast-like morphology of spherical cells and also in the form of short mycelia. The mycelial

growth morphology appears to be adversely affected by the shear stress generated by intense agitation.

Similarly, in lipase production from the mycelial fungus *Aspergillus terreus*, too intense an agitation has been found to adversely affect the fermentation (Gulati et al., 2000). In this fermentation, lipase production was not greatly influenced by automatically controlling the dissolved oxygen level at values ranging from 20 to 40% of air saturation. An agitation-aeration cascade was used to control the dissolved oxygen level (Gulati et al., 2000, Bajaj et al., 2010). Lipase production was enhanced to 14,200 U L^{-1} by increasing the agitation speed to 300 rpm, but a further increase of speed to 400 rpm reduced lipase production. At the high agitation rate, fragmentation of the mycelium was observed due to excessive shear stress (Gulati et al., 2000)

Saponified palm kernel oil (SPKO) is a potential water-soluble substrate for lipase fermentations. In an aqueous medium, increasing the concentration of SPKO from 1to 5 g L^{-1} had not significant impact on k_La at an agitation speed of 600 rpm. A further increase in SPKO concentration to 10 g L^{-1} reduced the k_La by 50% compared with control (no SPKO) (Annuar et al., 2007). Although SPKO is a soluble substrate, it acts as a surfactant. An increase in concentration to 5 g L^{-1} likely negatively affected the k_L but increased a such that the two effects counteracted and the net effect on k_La was minor. As the concentration of SPKO was further increased to 10 g L^{-1}, k_L was reduced because of the surfactant molecules coating the gas bubbles (Moraveji et al., 2012, Suzuki et al., 1990, Kobayashi, 2011, Aiba S, 1973), but a did not change much and therefore k_La was reduced. Surfactants tend to reduce the mean bubble diameter and this too reduces k_L (Chisti and Moo-Young, 1987).

A limitation of oxygen supply is known to adversely affect lipase production in at least some microorganisms. In production of extracellular lipase by the yeast *C. cylindracea* growing on either olive oil or oleic acid in a 2 L stirred batch fermenter operated at an agitation speed of 500 rpm and an aeration rate of 1 vvm, a sufficiency of oxygen supply was assured only by supplementing the air with oxygen (Sokolovska et al., 1998). A dissolved oxygen level of 20% of oxygen saturation was recommended for lipase production. The maximum biomass concentration in the batch fermenter was about 5.5 g L^{-1}. *C. cylindracea* typically forms pseudohyphae and this morphology likely adversely impacted the gas-liquid mass transfer in this fermentation.

2.6 Concluding remarks

High cell density fed-batch fermentations have proven successful in production of extracellular lipases in bacteria, yeasts and mycelia fungi. The specific growth rate controlled- and constant rate feedings have been most commonly used as they are simple to implement and avoid the complexities of feedback controlled feeding. A suitable feeding strategy depends on the microorganism being used, the nature of the substrate and the need for a sufficient supply of oxygen. Depending on the microorganism and the substrate, the feeding rate should be such that the microbial growth rate remains at substantially less than the maximum possible specific growth rate. This appears to be necessary to allow the intracellularly produced lipases to be exported to the outside and reduces feedback inhibition of lipase production within the cell. Feeding may be designed to rapidly build up the biomass in a growth phase and a subsequent shift to the production phase involving slow growth on inducer substrates. Increasingly, recombinant lipases are being produced in preference to wild type enzymes. The use of recombinant microorganisms for lipase

production can greatly enhance enzyme productivity and yield compared with production in wild type species. No single clearly preferred universally applicable feeding method exists in view of the diverse microorganisms being used in lipase production.

CHAPTER 3

3.0 Materials and Methods

3.1 Yeast strain and maintenance medium

Candida rugosa ATCC 10571 was obtained from American Type Culture Collection (ATCC) and maintained on YM medium agar plate containing (per litre): yeast extract, 3.0 g; malt extract, 3.0 g; peptone, 5.0 g; glucose, 10.0 g; and agar, 15.0 g. The plate culture was incubated at 30 ^{o}C for three days to obtain full growth of yeast culture. From here, a plaque of agar was suspended in 10 ml potassium phosphate buffer and transferred to shake flasks containing YM medium without agar. The shake flask culture was then incubated for 24 hours at room temperature (25 – 27 ^{o} C). Then a loop full of this fresh culture was streaked onto empty YM agar plates to obtain single colony and to detect any possible contamination of the culture. After incubation for 2-3 days at 30 ^{o} C, a healthy single colony was transferred onto each of a number of vial containing 28% (v/v) glycerol and YM medium in the ratio 1:1 which was then stored at -20 ^{o}C. The frozen cell was used as stock culture and thawed at 37 ^{o}C prior to cultivation. After thawing, subsequent subculture of the cells onto YM agar plate was done to ensure the viability of the cells before it can be used for submerged fermentation.

3.2 *Shake-flasks cultivation*

Inocula were produced in a 250 mL shake flask containing 100 mL of the YM liquid medium. The preculture was incubated (180 rpm, 25–27 °C) for 28 h. Palm oil (1 mL) was then added and incubation was continued for a further 12 h at an increased agitation rate i.e 300 rpm. The cells were then harvested by centrifugation (7,650 *g*, 4 °C, 10 min) and rinsed twice, each time by resuspending in inoculum bottle which contained

100 mL of a saline solution (0.9% w/v NaCl). The harvested cells were aseptically transferred to the production medium in the bioreactor. The initial biomass concentration (X_0) in the freshly inoculated bioreactor was 0.2 – 0.3 g L^{-1}.

3.3 Production medium composition

The production medium used in the bioreactor contained (per L): 5.74 g K_2HPO_4, 3.7 g KH_2PO_4, 1.0 mL of a trace element solution (Lageveen et al. 1988), 10 mL of 0.1 M $MgSO_4 \cdot 7H_2O$, 2 g of $(NH_4)_2SO_4$, 4×10^{-4} g of inositol, 2×10^{-4}g of thiamine, 8×10^{-4}g of biotin and 10 mL of commercial palm oil as a sole source of carbon and energy. The solution of magnesium salt was sterilized separately by autoclaving (121 °C, 15 min). Solutions of trace elements and vitamins were filter sterilized. Palm oil was added nonsterile.

3.4 Fermenter set-up

A Biostat® B+ 2 L stirred tank bioreactor (Sartorius Stedim Biotech GmbH, Gottingen, Germany) equipped with jacketed vessel was used. The ratio of height-to-diameter of the vessel is approximately 2:1. The fermenter system also consists of six-bladed disc impellers and four baffles together with sparger ring for aeration. The diameter of the disc impeller and the width of the blades are 52 mm and 10.5 mm respectively. The length of shaft is 240 mm. The pH probe (Applisense), temperature probe (type Pt-100), partial oxygen probe (Mettler-Toledo, InPro 6800), and peristaltic pump were regularly checked and calibrated before every run for maximum accuracy. The parameters associated with these sensors were measured, monitored and controlled by a digital system and

displayed on controller interface screen. Those sensors are autoclavable and mounted along with the bioreactor during autoclaving which last for 15 minutes at 121^{o}C.

Partial oxygen level was maintained at 30% where a gas mixer was used to mix pure oxygen from a tank with filtered compressed air. The gas mixer is pre-installed in the STR controller. Pre-set pH of 7 during fermentation was balanced by feeding in 4N sulphuric acid H_2SO_4 and 25% (w/v) of ammonia solution (NH_4). Temperature was fixed at 30^{o}C which is the best condition for the cell growth. The working volume for batch cultivation was 1 liter containing 100 mL inoculum and 1% (v/v) palm oil. Equal working volume containing mentioned composition was prepared as start up culture for fed-batch cultivation in different experiment. After autoclaving, the STR was left to cool down by circulating cool water around jacketed vessel. Schematic diagram of the fermentation system is shown in Figure 3.1, 3.2, and 3.3.

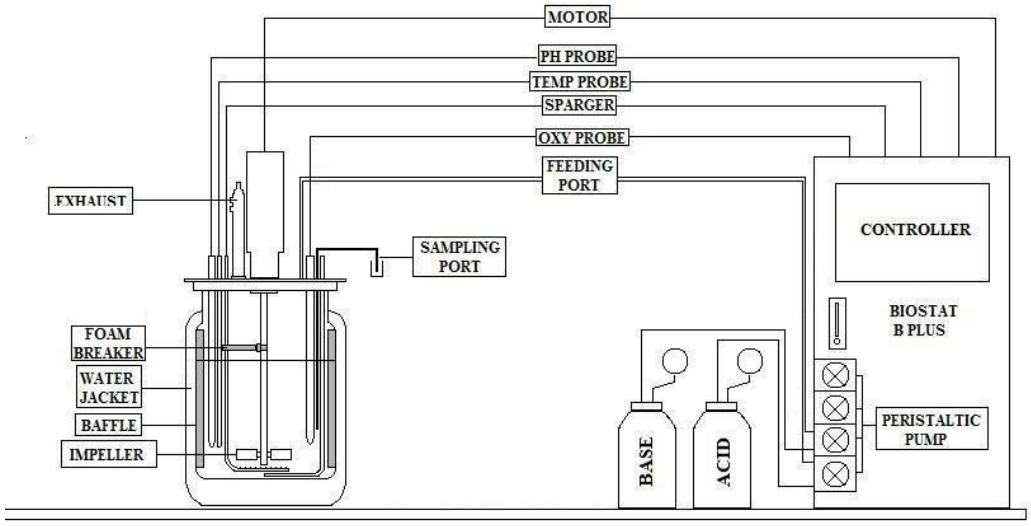

Figure 3.1: Schematic diagram of batch fermentation systems.

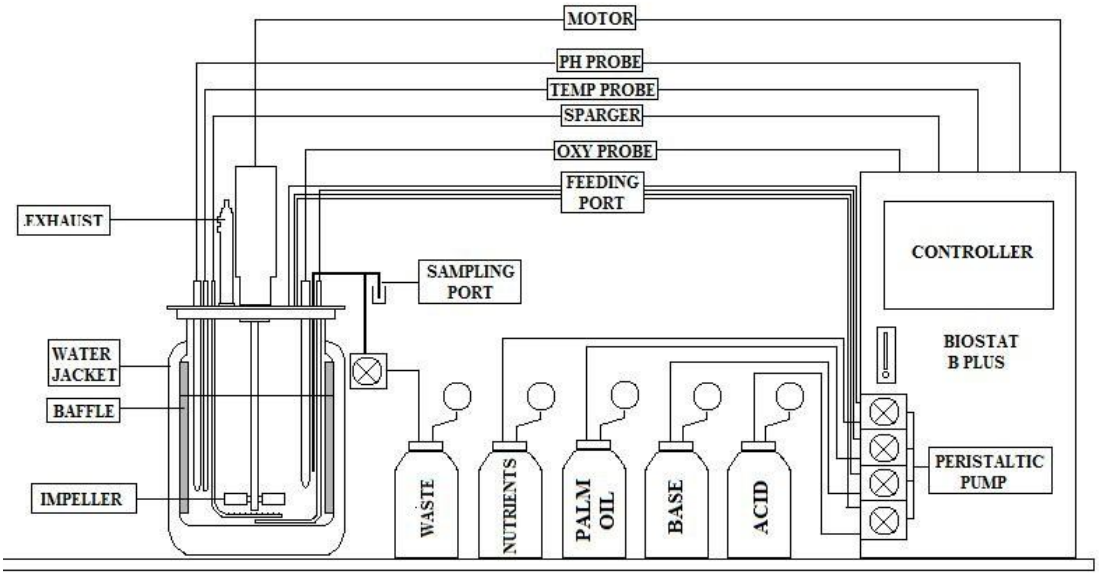

Figure 3.2: Schematic diagram of fed-batch fermentation systems (pH-stat and foaming dependent feeding).

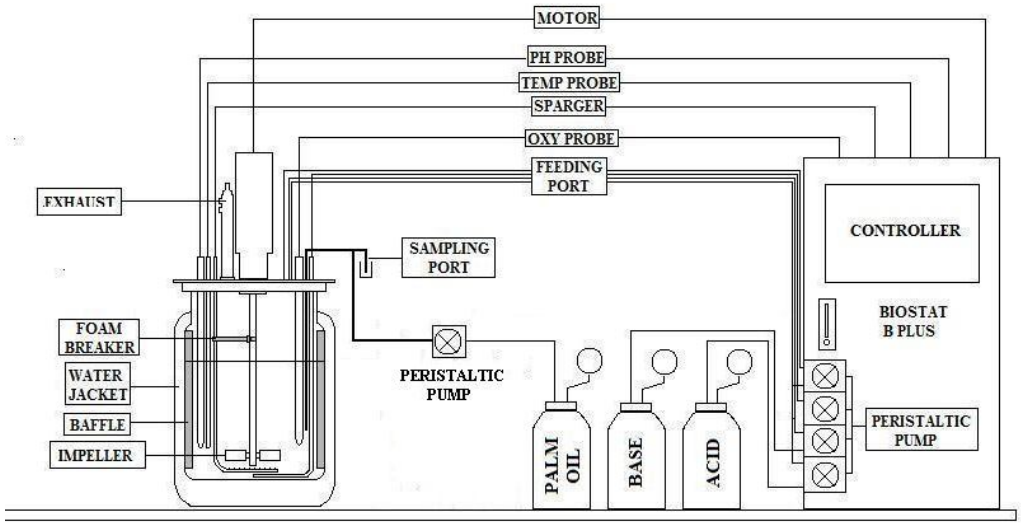

Figure 3.3: Schematic diagram of fed-batch fermentation system (Constant specific growth rate control feeding)

3.5 *Inoculation of yeast culture into bioreactor*

Inoculum which was placed temporarily in inoculum bottle was then connected to inoculum port on the STR through male and female coupling (Luer lock) in aseptic way. The inoculation was performed while the impeller was in motion and air was sparged into the reactor to avoid cell settlement. Then, 1% (v/v) palm oil was fed using peristaltic pump from the reservoir into bioreactor containing 1 litre production medium. The fermentation was started immediately after the yeast cells inoculation.

3.6 *Analytical method*

3.6.1 Biomass concentration measurement

Biomass concentration was estimated by spectrophotometric measurement of absorbance of the culture broth at a wavelength of 660 nm against a blank of 0.9% w/v solution of NaCl. A calibration curve was used to convert the absorbance to the biomass concentration as shown in Figure 3.4.

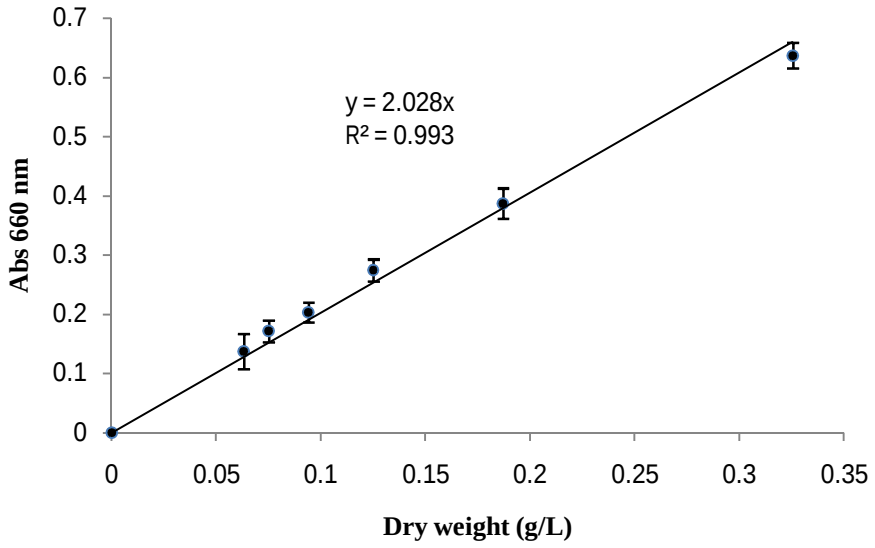

Figure 3.4 : Standard calibration of biomass concentration as a function of absorbance at 660 nm.

Estimation of biomass concentration was based on the relationship of $y = 2.028x$, where y is the absorbance (660 nm) after correction for the dilution and x (g L^{-1}) is the biomass concentration. The regression coefficient for the above equation was 0.993.

3.6.2 Lipase activity measurement.

The lipase activity was assayed in the cell-free culture supernatant by using a colorimetric method (Gupta et al. 2002). Thus, 4-nitrophenyl palmitate (NPP) was used as the substrate. The NPP solution was prepared by dissolving 30mg NPP in 10 mL of isopropanol. To this was added 90 mL of potassium phosphate buffer (50 mM, pH 7) solution containing sodium deoxycholate (207 mg) and acacia gum (100 mg). A 2.5 mL aliquot of the resulting turbid NPP solution was mixed with 50 μL of Triton X-100 and incubated at 50 °C for 5 min to yield a clear solution. The molar extinction coefficient of NPP hydrolysis product i.e. *para*-nitrophenol (*p*NP) at pH 7 after the addition of Triton X-100 was found to be $\varepsilon = 3.982 \times 10^3$ M^{-1} cm^{-1}. A 2 mL portion of this clear NPP solution was mixed with 1 mL of the culture supernatant in a thermostated cuvette (37 °C) and the hydrolysis of NPP was followed by continuously monitoring the spectrophotometric absorbance at 410 nm against a blank containing the heat deactivated enzyme (95 °C, 5 min). The determination of lipase activity was based on the Lambert-Beer law equation as follows;

$$Abs = \varepsilon\, c\, L \qquad (3.1)$$

where, *Abs* is the dimensionless quantity of optical density, ε is extinction coefficient of NPP solution at 410 nm, c is the concentration of NPP solution, and L is length of the light path.

Eq. (3.1) is defined and rearranged as follows;

$$\frac{dc}{dt} = \frac{Abs}{t} \times \frac{1}{\varepsilon} \times \frac{1}{L} \times \frac{y}{z} = \frac{\mu mol}{ml.min} = \frac{U}{ml} \quad (3.2)$$

Where, *dc/dt* is difference in PNP concentration as a function of difference in time, *t* is time (min), *y* is total volume of enzymatic reaction (mL), and *z* is volume of sample (mL). One unit of the enzyme activity was defined as the amount of enzyme needed to release 1 µmol of 4-nitrophenol from the substrate in 1 min under the above specified conditions.

3.6.3 Oxygen transfer coefficient measurement in stirred tank reactor.

The value of $k_L a$ in a gas-liquid contacting system is based on the dissociation of the liquid-side mass transfer coefficient (k_L) and the interfacial area (a) (Painmanakul et al., 2009). The $k_L a$ value is a key parameter in the characterization and design of industrial stirred or non-stirred gas–liquid reactors (Bouaifi et al., 2001). The main disadvantage of the calculation of $k_L a$ using static gassing out method is the slow response time of the electrode changes of oxygen concentration in the liquid. Response time is defined as the time required by the electrode to measure 63% of the global value of the change in the concentration of oxygen and it is related to the diffusion of oxygen through the membrane of the electrode. In order for the measured values to be sufficiently reliable, the response time of the electrode must be smaller or equal to $1/k_L a$ (Yamanè and Shimizu, 1984b). The form of equation used to measure mass transfer is given by Equation (3.3). Following derivation shown below, the $k_L a$ of the system can be determined by referring to the time when the ratio of C_L and C^* is 0.63 or 63%.

$$\frac{dC}{dt} = k_L a(C^* - C_L) \quad (3.3)$$

$$\frac{d\left(\frac{C_L}{C^*}\right)}{dt} = k_L a\left(1 - \frac{C_L}{C^*}\right)$$

$$\int_0^{C_L} \frac{d\left(\frac{C_L}{C^*}\right)}{\left(1-\frac{C_L}{C^*}\right)} = K_L a \int_0^t dt$$

$$-\ln\left[1 - \frac{C_L}{C^*}\right] = k_L a \bullet t$$

$$\ln\left[1 - \frac{C_L}{C^*}\right] = - k_L a \bullet t$$

$$e^{ln\left(1-\frac{C_L}{C^*}\right)} = e^{-K_L a \bullet t}$$

$$1 - \frac{C_L}{C^*} = e^{-K_L a \bullet t}$$

$$\frac{C_L}{C^*} = 1 - e^{-K_L a \bullet t}$$

If $t = \frac{1}{K_L a}$

$$\frac{C_L}{C^*} = 1 - e^{-1}$$

$$\frac{C_L}{C^*} = 1 - \frac{1}{e}$$

= 1- 0.368

= 0.632

An estimation of the response time of the oxygen electrode (Mettler-Toledo, InPro 6800) used throughout the experiments was made by measuring the variation of oxygen concentration after the electrode was moved from a saturated aqueous solution of nitrogen to a saturated aqueous solution of oxygen. The results indicate that 63% of oxygen saturation was reached at 30.8 (±3.0) s and 100% was reached at around 69.6 (±1.5) s. Thus, only values of $k_L a$ less than 0.0325 s^{-1} should be considered reliable when using this electrode.

During cultivation, dynamic method was employed to measure k_La where this method is based on an unsteady-state mass balance for oxygen as outlined by (Pouliot et al., 2000). The dynamic method makes use of the fate of the dissolved oxygen within the fermenter that is given by the following equation:

$$k_La = \frac{X_{\max .} qO_2}{C^*_{AL}} \tag{3.4}$$

Where,

$$qO_2 = \frac{\Delta\, pO_2\%}{(\Delta t)(X_{max})} \tag{3.5}$$

The specific oxygen uptake rate (OUR or $q\mathrm{O}_2$) and k_La were determined using the following procedure: aeration of the liquid phase (1 L per minute) was cut momentarily whereas the agitation was reduced from 400 or 600 or 800 rpm to 30 rpm in order to maintain cells in suspension.

The rate of decrease of dissolved oxygen is caused entirely by the OUR. This decrease is usually linear and the slope of the plot of C_L as a function of time provides a direct estimate of the oxygen uptake rate (Figure 3.5). The underlying hypothesis is that the rate of oxygen utilization is unaffected by the absence of air bubbling and lower dissolved oxygen concentration. Before the dissolved oxygen concentration reaches its critical lower limit, aeration and pre-set agitation were resumed and the dissolved oxygen concentration should returns to its initial level. Critical lower limit in this case was arbitrarily set at partial oxygen pressure ($p\mathrm{O}_2$) of 10%.

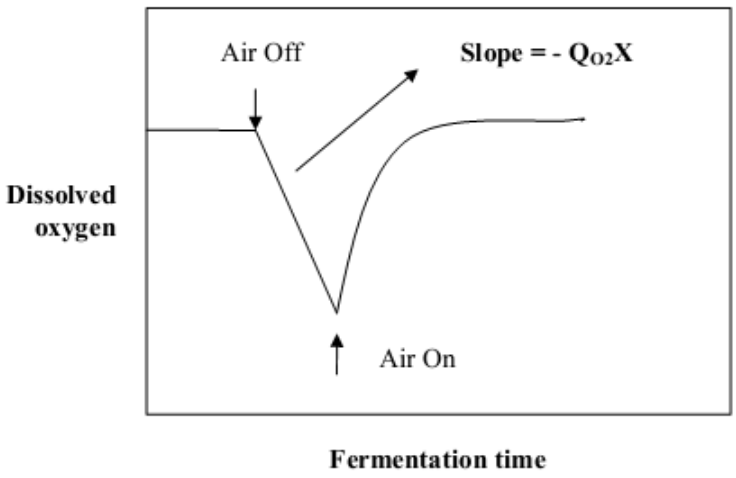

Figure 3.5: Determination of total oxygen consumption rate using the dynamic method.

3.6.4 Total protein concentration

The total protein in the culture supernatant was quantified using the Bradford method (Bradford, 1976).

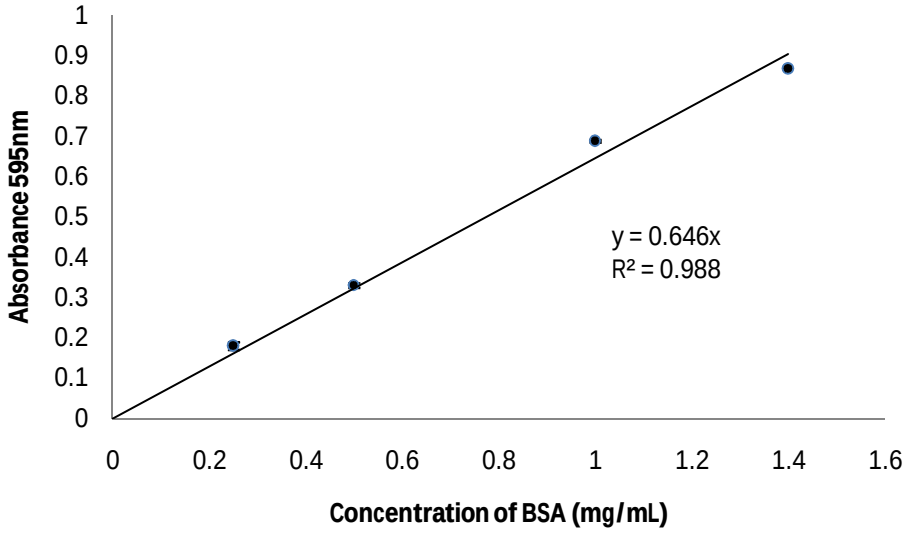

Figure 3.6: Standard calibration of Bovine serum albumin (BSA) concentration as a function of absorbance at 595 nm (standard deviation less than 5%).

Estimation of total protein concentration was based on the relationship of $y = 0.646x$, where y is the absorbance at 595 nm after correction for the dilution and x (g L^{-1}) is the concentration of BSA. The regression coefficient for the above equation was 0.988.

3.7 Cultivation in bioreactor

3.7.1 Batch fermentation

In batch cultivation, the study was mainly focused on the effects of agitation rate i.e., 400, 600 and 800 rpm, while other parameters including temperature (30 ^{o}C), pH (7), oxygen level (pO_2; 30%), inoculum concentration (0.2-0.3 g L^{-1}), working volume (1L), composition of the production medium and concentration of carbon source were kept constant. The concentration of the biomass and the lipase activity in the culture supernatant were measured via periodic sampling.

3.7.2 Fed-batch fermentation

Each fed-batch operation began as a batch operation as above and was switched to the fed-batch mode at 24 h when foaming was observed. Three feeding strategies were tested: a pH-stat operation, a foaming-dependent feeding and specific growth rate control feeding. Foaming was suppressed by adding palm oil with each feeding as an alternative to the commercial antifoam agents. All culture conditions were as previously specified for the batch operation. The agitation speed remained fixed at 600 rpm in all experiments.

3.7.2.1 pH-stat feeding.

In pH-stat feeding, the automatic pH control system of the batch operation was switched off. The limiting substrate was fed when the pH rose to above 7.0 indicating a depletion of the substrate as shown in Figure 4.4. For each feeding, 60 mL of the culture broth was pumped out of the bioreactor and replaced with a mixture of 50 mL of double-strength fresh production medium and 10 mL of palm oil so that the final volume of the broth in the bioreactor was 1 L.

3.7.2.2 Foaming-dependent feeding

In foaming-dependent feeding, the feeding was initiated in response to a massive accumulation of foam on the surface of the culture broth. This apparently occurred because the substrate (palm oil) was consumed and no longer able to provide its antifoaming action. A microscopic examination of the culture samples confirmed an absence of oil droplets whenever massive foaming occurred. Each time foaming occurred, 60 mL of the culture broth was withdrawn and replaced with an equal volume of the fresh medium as explained above for the pH-stat feeding operation.

3.7.2.3 Constant specific growth rate control feeding

For the specific growth rate controlled feeding (Montesinos et al. 1996; Gordillo et al. 1998; Li et al. 2004; Cos et al. 2005; Kim and Hou 2006; Zhao et al. 2008; Arnau et al. 2010), the feed rate F varied exponentially with time as calculated based on material balance equation on limiting substrate of palm oil as follows:

i. Substrate Feeding,

$$\frac{d(V.S)}{dt} = F\,.\,S_o - r_s\,.\,V \tag{3.6}$$

$$Y_{x/s} = \frac{r_x.V}{r_s.V} = \frac{\mu\,.\,x\,.\,V}{r_s\,.\,V} \tag{3.7}$$

where, V is working volume (L), S is substrate concentration, F is feeding rate (L h^{-1}), S_o is initial substrate concentration (g L^{-1}), x is biomass concentration (g L^{-1}), μ is specific growth rate (h^{-1}), $r_s.V$ is volumetric rate of substrate uptake (g h^{-1}), $r_x.V$ is volumetric rate of growth (g h^{-1}), and $Y_{x/s}$ is true biomass yield from substrate.

ii. Exponential growth

$$\frac{d(x\,.\,V)}{dt} = \mu\,.\,x\,.\,V \tag{3.8}$$

$$e^{ln}\left(\frac{X_1\,V_1}{X_o\,V_0}\right) = e^{\mu.t}$$

$$x\,.\,V = x_o\,.\,V_o\,.\,e^{\mu_{max}.t} \tag{3.9}$$

where, X_o is initial concentration of biomass (g L^{-1}), X_1 is biomass concentration at a specific time (g L^{-1}), V_o is initial working volume (L), V_1 is working volume at specific time (L), μ_{max} is maximum specific growth rate retrieved from batch cultivation.

Eq. (3.9) is substituted into Eq. (3.7):

$$Y_{x/s} = \frac{\mu\,.\,X_o.\,V_o.\,e^{\mu max\,.\,t}}{r_s\,.\,V}$$

And rearranged to;

$$r_s\,.\,V = \frac{\mu\,.\,X_o.\,V_o.\,e^{\mu max\,.\,t}}{Y_{x/s}} \tag{3.10}$$

The left hand side of Eq. (3.6) is defined as,

$$\frac{d(V\,.\,S)}{dt} = V\frac{ds}{dt} + S\frac{dV}{dt}$$

Since μ is constant, $\frac{ds}{dt} \approx 0$

$$\frac{d(V\,.\,S)}{dt} = S\frac{dV}{dt} = S.F \tag{3.11}$$

Finally, Eq. (3.10) and (3.11) is substituted into Eq. (3.6);

$$F.S = F.S_o - \frac{\mu . X_o . V_o . e^{\mu_{max} . t}}{Y_{x/s}} \quad (3.12)$$

$$\frac{\mu . X_o . V_o . e^{\mu_{max} . t}}{Y_{x/s}} = F.S_o - F.S$$

$$\frac{\mu . X_o . V_o . e^{\mu_{max} . t}}{Y_{x/s}} = F(S_o - S)$$

$$F = \frac{\mu . X_o . V_o . e^{\mu_{max} . t}}{Y_{x/s}(S_o - S)}$$

The feed rate *F* varied exponentially with time according to the following equation:

$$F = \frac{\mu . X_0 . V_0 . e^{\mu t}}{X - X_0} \quad (3.13)$$

In equation (3.13), μ is the desired constant specific growth rate. The following set point values of the constant specific growth rate (μ) were used in different experiments: 0.05, 0.10 and 0.15 h^{-1}. The initial working volume (V_0) was 1 L and the final volume was 1.5 L. X_0 was the initial concentration of the biomass and X was the biomass concentration at time *t*. A peristaltic pump (Masterflex model no. 7551-10) was controlled to provide the necessary feeding rate during the exponential feeding operation (Figure 3.7). The feed was palm oil, as a carbon source. Nitrogen was supplied via the ammonia solution that was used for pH control.

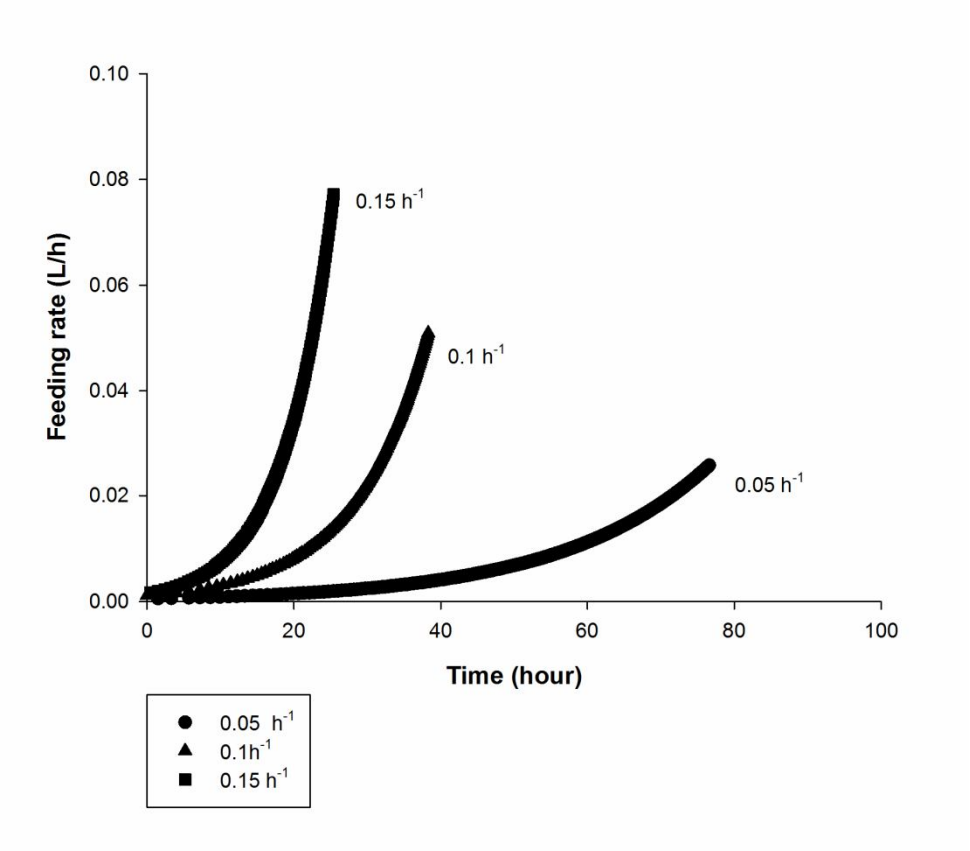

Figure 3.7: Feeding rate corresponded to each specific growth rate.

The calculated feeding rate was programmed into L/S® Computer-compatible Masterflex peristaltic pump (Model no. 7551-10) linked to a computer interface. The feeding of carbon source (palm oil) was initiated once the foaming was observed from the broth culture surface. Nitrogen source was supplied from ammonia solution that also acts as pH correction. The fermentation was run as batch system start-up culture and the feeding of palm oil was initiated at 24th hour when foaming was detected.

3.8 Mass transfer study under HCDF

Mass transfer plays a vital role in many reaction systems including high cell density fermentation which is formidably complex especially in term of substrates mass transfer. Since it is a major concern in bioprocess, two types of mass transfer were studied experimentally and theoretically as follows:

3.8.1 Experimental measurement of gas-liquid mass transfer

The measurement protocol for overall oxygen transfer coefficient (k_La) was described earlier. Biomass concentration at 58 g L^{-1} which produced from fed-batch fermentation was selected to measure k_La at different agitation rate (i.e 400, 600, and 800 rpm)

.

3.8.2 Theoretical measurement of gas-liquid mass transfer

Gas-liquid mass transfer coefficient (k_La) for each agitation rate (i.e. 400, 600, 800) was determined separately (k_L and a) using established correlation as proposed by Calderbank, (1958).

i. Power requirement

The power required by an impeller in a gas sparged system P_m is usually less than the power required by the impeller operating at the same speed in a gas-free liquids $P_{mo.}$ This effect appears partially due to the decrease in average density of the fluid being agitated. In sparged liquids, the uniformity of bulk mixing diminishes with increased agitation. The P_m for the flat-blade disk turbine can be calculated as follows (Nagata, 1975):

ii. Reynold's number

$$N_{Re_i} = \frac{\rho_L N D_i^2}{\mu_L} \tag{3.14}$$

iii. Mixing Power for non-gassed systems (watt)

$$P_{mo} = 6\rho_L N^3 D_i^5 \tag{3.15}$$

iv. Ratio of mixing power for gassed and non-gassed systems (watt)

$$log_{10}\frac{P_m}{P_{mo}} = -192\left(\frac{D_i}{D_t}\right)^{4.38}\left(\frac{D_i^2 N}{\nu}\right)^{0.115}\left(\frac{D_i N^2}{g}\right)^{1.96\left(\frac{D_i}{Dt}\right)}\left(\frac{Q}{N D_i^3}\right) \tag{3.16}$$

v. Sauter Mean diameter (D_{32}) (meter)

Calderbank (1958) correlated the Sauter-mean diameter for the gas-liquid dispersion in viscous liquid agitated by a flat-blade disk turbine impeller as follows:

$$D_{32} = 0.7\left[\frac{\sigma^{0.6}}{\left(P_m/V\right)^{0.4}\rho_L^{0.2}}\right]\left(\frac{\mu_L}{\mu_g}\right)^{0.1} \tag{3.17}$$

vi. Interfacial area (a_o)

Calderbank (1958) also correlated the interfacial areas for the gas-liquid dispersion agitated by a flat-blade disk turbine as follows:

$$a_o = 1.44\left[\frac{\left(P_m/V\right)^{0.4}\rho_L^{0.2}}{\sigma^{0.6}}\right]\left(\frac{V_s}{V_t}\right)^{0.5} \tag{3.18}$$

vii. Mass transfer coefficient (k_L)

Calderbank and Moo-Young (1959), separated k_La by measuring interfacial area and correlated mass-transfer coefficients in gas-liquid dispersions in mixing vessels, and sieve and sintered plate column

$$k_L S_c^{1/2} = 0.42 \left[\frac{\Delta\rho\mu_L g}{\rho_L^2}\right]^{1/3} \text{(Bubbles size } > 2.5\, mm) \quad (3.19)$$

$$k_L S_c^{2/3} = 0.31 \left[\frac{\Delta\rho\mu_L g}{\rho_L^2}\right]^{\frac{1}{3}} \text{(Bubbles size } < 2.5\, mm) \quad (3.20)$$

where,

$$Sc = \frac{\mu_L}{\rho_L D_{o2}}$$

Finally, volumetric mass transfer coefficient (k_La) value was obtained as a combination product of k_L and a.

3.8.3 Calculation of liquid-liquid mass transfer based on experimental determination of total interfacial area of the oil droplets

Liquid-liquid mass transfer was studied using an artificial dense culture where the solution of xanthan gum was prepared and its viscosity was identical as measured at 58 g L^{-1}. This biomass concentration represents a viscosity of 1.68 mPa.s. The viscosity measurement was done using SV-10 viscometer (Japan). All conditions practiced in batch condition were followed in this experimental study of mass transfer but the agitation rate was varied from 200 up to 1000 rpm at 200 rpm interval. The prepared xanthan gum solution was then added with 1% (v/v) palm oil and then mixing was initiated. Ten minutes after mixing the oil into the aqueous phase, a sample was drawn out using hollow plastic tube which was closed at one end. The sample was immediately placed on a microscopic glass slide for observation under microscope. The microscope (Motic BA 200) was

connected to an image analyzer *via* a mounted camera (Moticam 2300) and images of the oil droplets were captured using the camera. The captured image was then analyzed using Motic Image Plus 2.0 software. About 500 droplets were enlarged at 40x magnification and the radius as well as area of the droplets generated from each impeller geometry was determined. The droplets were then divided into eleven categories: droplets of diameter smaller than 50.99 μm, 51-100.99 μm, 101-150.99 μm, 151-200.99 μm, 201-250.99 μm, 251-300.99 μm, 301-350.99 μm, 351-400.99 μm, 400-450.99 μm, 451-500.99 μm and larger than 500 μm.

The total interfacial area (a_t) of the droplets captured by the microscope was determined using the following equations:

$$D_{mean.j} = \frac{\Sigma d_j^3}{\Sigma d_j^2} \tag{3.21}$$

$$D_{overall} = \Sigma\left(\frac{\theta_j}{D_{mean.j}}\right) \tag{3.22}$$

$$a_t = \frac{6\,\phi}{D_{overall}} \tag{3.23}$$

The specific interfacial area of oil droplet was determined using the method proposed by Mukataka et al., (1985). Sauter mean diameter (D_{32}) was simply determined using:

$$D_{overall} = \frac{6\emptyset}{a_t} \tag{3.24}$$

Finally, mass transfer coefficient (k_L) was determined using established correlation developed by Calderbank, (1958) according to droplets size which was experimentally measured.

i. $$k_L \mathrm{S_c}^{1/2} = 0.42 \left[\frac{\Delta\rho\mu_L g}{\rho_L^2}\right]^{1/3}$$ (Droplets size > 2.5 mm) (3.25)

ii. $$k_L \mathrm{S_c}^{2/3} = 0.31 \left[\frac{\Delta\rho\mu_L g}{\rho_L^2}\right]^{1/3}$$ (Droplets size < 2.5 mm) (3.26)

where,

$$Sc = \frac{\mu_L}{\rho_L D_{oil}}$$

Finally, volumetric mass transfer coefficient ($K_L a$) value was obtained from a product of k_L and a.

3.8.4 Calculation of liquid-liquid mass transfer based on theoretical determination of specific total interfacial area.

Under this study, Calderbank, (1958) suggested an equation which can be used to calculate the total specific interfacial area at various operating conditions as follows:

$$a_t = \frac{6\,\phi}{D_{overall}} = \frac{\alpha\,\omega^{\mathrm{m}}\,\phi\,T^k}{(1+\mathrm{n}\,\phi)} \quad (3.27)$$

Based on this equation, Al-Zuhair et al., (2003) developed a correlation for the total specific interfacial area as:

$$a_t = \frac{0.024\,\omega^{0.6}\,\phi\,T^{1.7}}{(1+3.0\,\phi)} \quad (3.28)$$

In developing this correlation, the researchers used Rushton turbine in STR, therefore this correlation is applicable since Rushton turbine was used throughout this study. Sauter mean diameter (D_{32}) was simply determined using Eq. (3.24).

Calderbank and Jones, (1961) have proposed a correlation for mass transfer to and from dispersion of low-density solid particles in agitated liquids which were designed to simulate mass transfer to microorganisms in fermenter. The correlation is as follows:

i. $k_L S_c^{1/2} = \frac{2D_{oil}}{D_{32}} + 0.42\left[\frac{\Delta\rho\mu_L g}{\rho_L^2}\right]^{1/3}$ (Droplet size > 2.5 mm) (3.29)

ii. $k_L S_c^{2/3} = \frac{2D_{oil}}{D_{32}} + 0.31\left[\frac{\Delta\rho\mu_L g}{\rho_L^2}\right]^{1/3}$ (Droplet size < 2.5 mm) (3.30)

where,

$$Sc = \frac{\mu_L}{\rho_L D_{oil}}$$

Since the diffusivity of palm oil in water is very low (2.76 x 10^{-10} m^2 s^{-1}), it is neglected and excluded from the correlation. Thus, Eq (3.29) and (3.30) reduced to Eq. (3.25) and (3.26), respectively. Finally, volumetric mass transfer coefficient (k_La) value was obtained as a product of k_L and a.

3.9 Statistical analysis

All quantitative measurements were conveyed as mean ± standard deviation (SD). Paired means from two samples were analyzed using Student's *t*-test and. Multiple means from a single factor were analyzed using one-way analysis of variance (ANOVA) and multiple comparison of means $p < 0.05$ was considered as statistically significant.

CHAPTER 4

4.0 Results and discussion

4.1 *Shake flask cultivation*

Shake flask cultures were used to identify the time required to achieve the peak biomass concentration for inoculation of the bioreactor. A maximum biomass concentration of 7.5 g L^{-1}) was achieved at 28 h on glucose as the sole carbon source (Figure 4.1). The maximum specific growth rate of the culture on glucose was 0.38 h^{-1}. At this point, palm oil (1%, v/v) was added and incubation was continued for a further 12 h to induce the production of lipase.

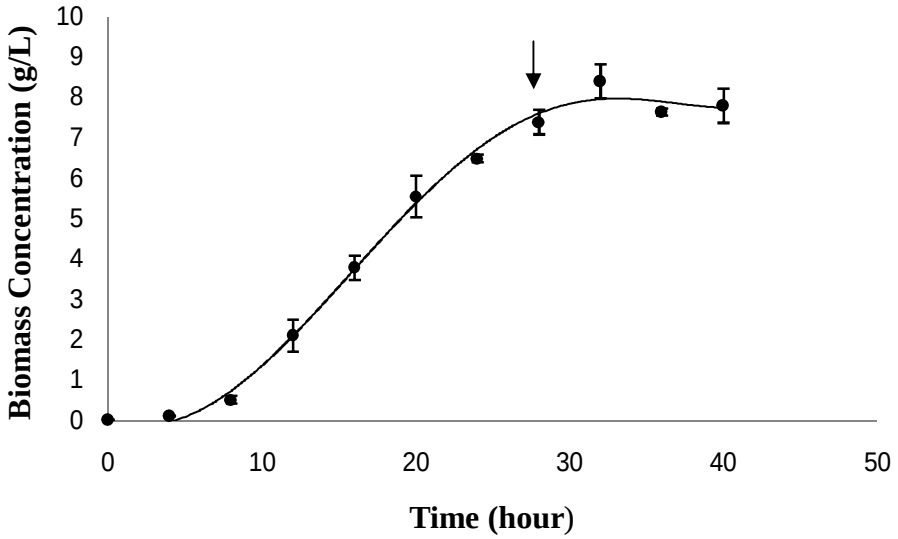

Figure 4.1**:** Inoculums preparation in shake flask cultivation. The arrow demarcates the initiation of oil induction.

4.2 Bioreactor cultivation

4.2.1 Batch fermentation

The batch fermentation profiles at various agitation speeds are shown in Figure 4.2 and the calculated values of the various fermentation parameters were provided in Table 4.1. Statistical analysis using one-way ANOVA demonstrated a significant difference for maximum biomass concentration at all agitation rates studied in the batch fermentation ($p < 0.05$). Similarly, maximum lipase activities detected at 600 and 800 rpm were significantly different ($p < 0.05$). Increasing the agitation speed clearly improved the specific biomass growth rate (Figure 4.2, Table 4.1) and shortened the time to achieve the peak biomass concentration (Figure 4.2). Increasing the agitation speed likely reduced the size of the oil droplets and enhanced the rate of extracellular hydrolysis of the oil to improve the carbon supply to the cells. This enhanced the growth rate. No lipase production was seen at the lowest agitation speed of 400 rpm (Figure 4.2). An agitation speed of 600 rpm resulted in the highest lipase productivity (Table 4.1). At 800 rpm, the lipase productivity was reduced to only 50% of the value at 600 rpm. Clearly, therefore, lipase production is highly sensitive to the speed of agitation. Too low an agitation speed limits lipase production possibly because the supply of the carbon source is limiting. On the other hand, too high an agitation rate results in a high specific growth rate, i.e. a non limiting supply of carbon, and this suppresses lipase production. A reduced lipase productivity has been commonly observed at high growth rates (Chartrain et al. 1993; Montesinos et al. 1996; Gordillo et al. 1998; Tamerler and Keshavarz 2000; Ikeda et al. 2004; Resina et al. 2005; Boareto et al. 2007; Zhao et al. 2008; Arnau et al. 2010). In specific growth rate controlled fermentations, Li et al. (2004) reported a reduced production of lipase at both low and high values of the specific growth rate.

In earlier studies, an increased production of lipases at high agitation speeds has been attributed to improved supply of oxygen (Elibol and Ozer 2000; Alonso et al. 2005), but this does not explain the observed behaviour in this study because the concentration of the dissolved oxygen always remained controlled at 30% of air saturation value and the oxygen transfer rate at 800 rpm could not have been less than at 600 rpm.

Different levels of lipase production by *C. rugosa* have been previously ascribed to the different prevailing morphological forms of the yeast in a fermentation (Ferrer et al. 2001). Little or no production of lipases is observed in cultures with dispersed ellipsoidal cells (Figure 4.3).Formation of elongated pseudohyphae is said to favor lipase production (Ferrer et al. 2001). In a fermentation, the morphology evolves from the ellipsoidal to pseudohyphal and back to ellipsoidal (Ferrer et al. 2001). These observations relating to morphology and lipase production were confirmed in the present study. The different morphologies observed are shown in Figure 4.3. At an agitation speed of 400 rpm when no lipase was produced (Figure 4.3a), the dispersed ellipsoidal cell morphology predominated (Figure 4.3b).

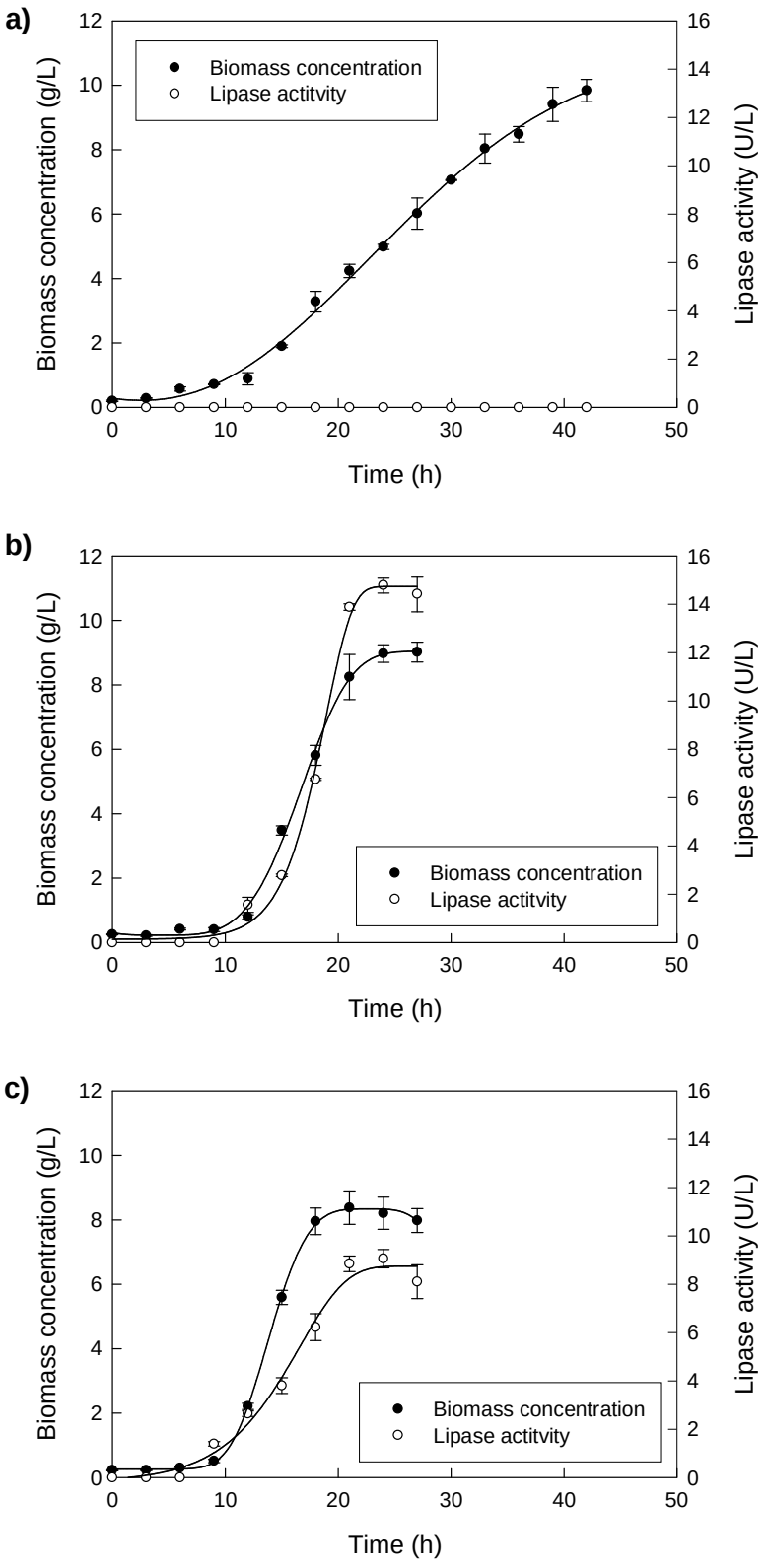

Figure 4.2**:** Batch fermentation profiles at agitation speeds of: (a) 400 rpm; (b) 600 rpm; and (c) 800 rpm.

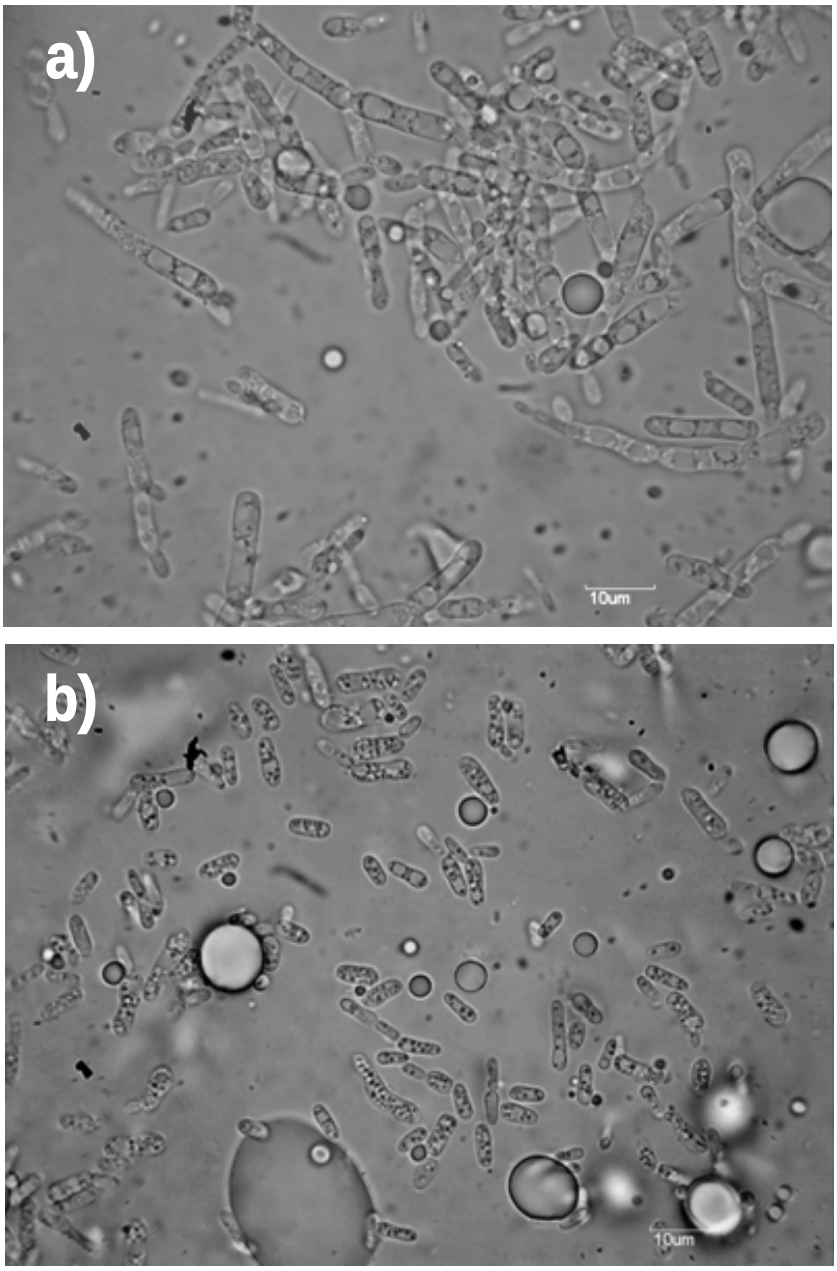

Figure 4.3**.** Cell morphology: a) the pseudohyphae morphology associated with an elevated production of lipase (photo taken at 36th h of cultivation in foaming-dependent feeding); and b) the dispersed ellipsoidal cells associated with poor production of lipase (photo taken at 30th h of cultivation in batch fermentation operated at 400 rpm).

4.2.2 *Fed-batch fermentation*

4.2.2.1 pH-stat feeding

The results of fed-batch operation with the pH-stat mode of feeding are shown in Figure 4.4. The agitation speed remained fixed at 600 rpm. Although both the peak biomass concentration and the lipase activity were high (Figure 4.4) compared with the batch cultures (Figure 4.2b), the pH-stat operation proved difficult to manage and could not be relied on for consistent results. After the first feeding demarcated by the arrow in Figure 4.4, the expected increase in pH was not observed to guide the next feeding. The pH-stat operation has previously been used for some other lipase fermentations (Gupta et al. 2004), but the specific nature of the substrate (palm oil in this case) may influence the reliability of this type of operation (Gupta et al. 2004). The final biomass specific lipase productivity (0.05 U g^{-1} h^{-1}; Table 4.1) was no better than in some of the batch fermentation (Table 4.1).

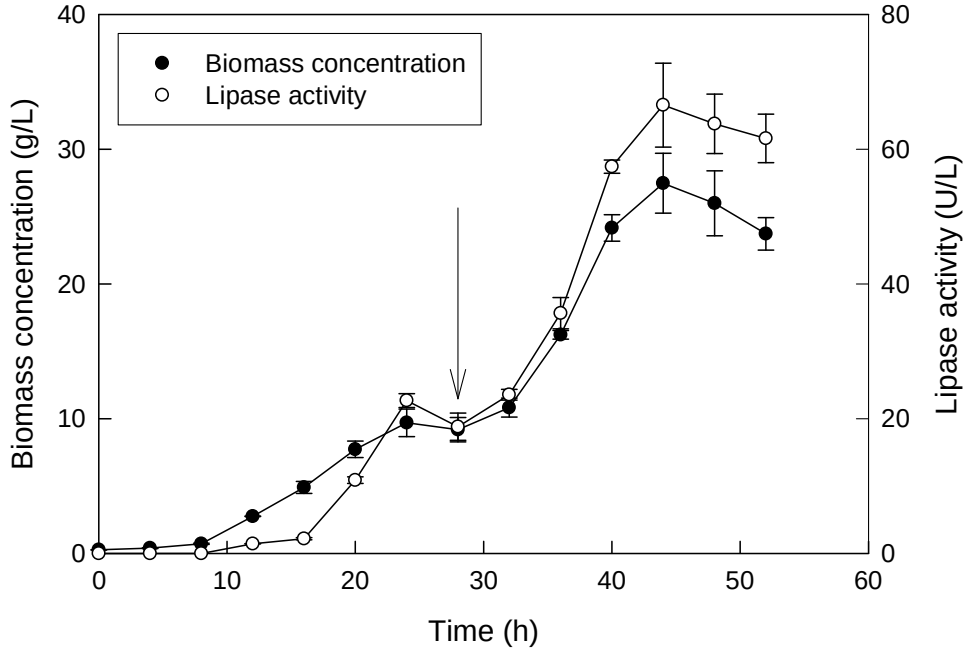

Figure 4.4**:** Fermentation profile of the pH-stat mode of feeding. The arrow demarcates the feeding event.

4.2.2.2 Foaming-dependent feeding

In foaming-dependent feeding, the feeding was intermittent in response to observations of foam on the surface of the fermentation broth. The fermentation profile with demarcations of the feeding events is shown in Figure 4.5. The fermentation was relatively well controlled as evidenced by the fairly smooth profiles of biomass growth and lipase activity (Figure 4.5). The peak biomass concentration was fairly high at 53 g L^{-1} as was the peak lipase activity (195 U L^{-1}) (Figure 4.5). The high lipase titer was simply a consequence of the high biomass concentration and the biomass-specific lipase productivity was only modestly better (0.09 U g^{-1} h^{-1}; Table 4.1) in comparison with that data for batch and pH-stat fed-batch fermentations (Table 4.1).

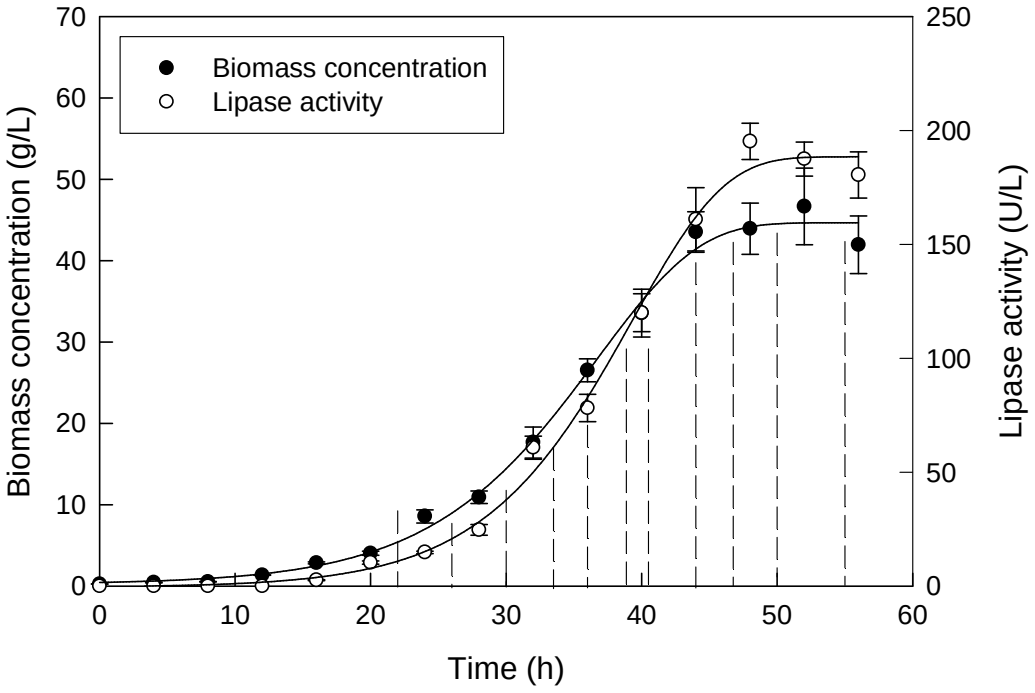

Figure 4.5: Fermentation profiles for the foam-dependent feeding operation at a fixed agitation speed of 600 rpm. The dotted line demarcate the specific feeding events.

4.2.2.3 Constant specific growth rate control feeding

This mode of operation was carried out with the specific growth rate control setpoint values of 0.05, 0.10 and 0.15 h^{-1} in different experiments. In all cases, the feeding of palm oil was initiated after 24 h of a batch phase. By this time, the palm oil added at the start of the batch operation had been consumed as signalled by the development of foam on the surface of the fermentation broth. The relevant fermentation profiles are shown in Figure 4.6 a–c.

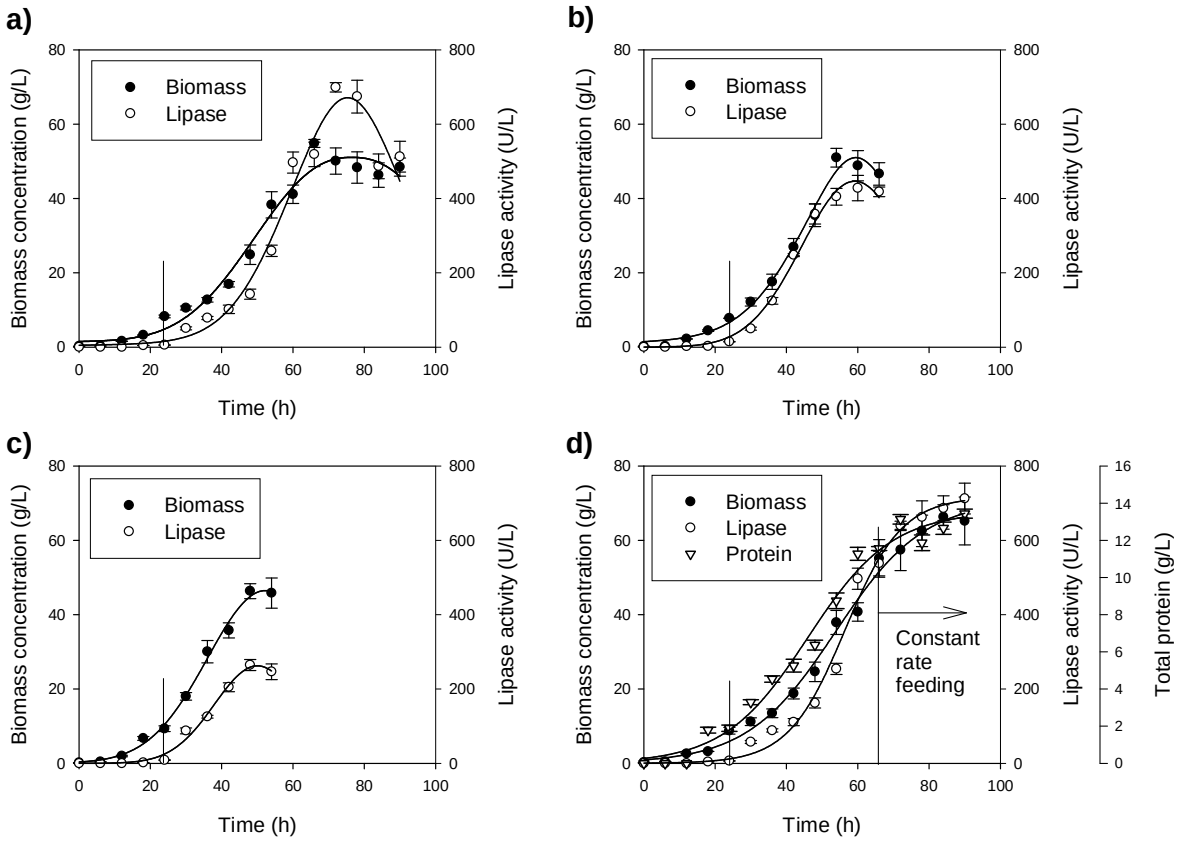

Figure 4.6**:** Fermentation profiles for constant specific growth rate control of feeding at the specific growth rate values of: a) 0.05 h^{-1}, b) 0.10 h^{-1}, c) 0.15h^{-1}; and d) 0.05 h^{-1} followed by a switch to a constant rate of feeding at 66 h.

The specific growth rate control at 0.05 h^{-1} resulted in an exceptionally high lipase titer of ~700 U L^{-1} (Table 4.1) compared with the titers obtained at higher values of controlled specific growth rates. An increase in the set point value of the specific growth rate resulted in a progressive reduction in the peak enzyme titer (Table 4.1). This was consistent with several earlier studies (Ikeda et al. 2004; Li et al. 2004; Cos et al. 2005; Kim and Hou 2006; Boareto et al. 2007; Zhao et al. 2008; Arnau et al. 2010) that reported a reduced production of extracellular protein (including lipase) with increasing specific growth rate in high cell density fed-batch fermentations involving various microorganisms. Evidently, an increased rate of growth results in metabolic resources being diverted from the production of lipase.

Although the changes in specific growth rate deeply affected the enzyme titer, their effect on the final biomass concentration was relatively modest and all fermentations (Figure 4.6 a–c) attained a peak biomass concentration of about 55–60 g L^{-1}. This was likely because nutrients other than the carbon source may have limited the growth. The growth may have been limited also by the adverse impact of the increased viscosity of the broth (~1.68 mPa s) on oxygen transfer to the cells.

As the fed-batch fermentation with the specific growth rate controlled at 0.05 h^{-1} was the most promising, it was repeated exactly as specified earlier, but the feeding was switched to a constant rate of 0.11 mL min^{-1} at the 66^{th} hour. The resulting fermentation profile is shown in Figure 4.6d. This mode of operation demonstrates a significant effect on the final biomass concentration (Figure 4.6d) relative to the data illustrated in Figure 4.6a (t-test; $p < 0.05$). However, no significant difference was observed for lipase activity when data from Figure 4.6a was compared with data from Figure 4.6d (t-test; $p > 0.05$). As a

result, a control of the specific growth rate at 0.05 h^{-1} without a subsequent phase of a constant-rate feeding was considered to be best overall for the production of lipase.

Batch fermentation				
Agitation rate (rpm)	**Specific growth rate (μ, h^{-1})**	**Maximum biomass concentration ($g\ L^{-1}$)**	**Maximum lipase activity ($U\ L^{-1}$)**	**Lipase productivity ($U\ g^{-1}\ h^{-1}$)**
400	0.14	9.8 ± 0.35 at 42nd h	ND	ND
600	0.23	9.0 ± 0.43 at 24th h	15 ± 0.31 at 24th h	0.07 ± 0.005
800	0.32	8.4 ± 0.44 at 21st h	9.1 ± 0.7 at 24th h	0.05 ± 0.003
Fed-batch fermentation (pH-stat feeding)				
600	NA	27.5 ± 2.85 at 44th h	66.5 ± 5.39 at 44th h	0.05 ± 0.003
Fed-batch fermentation (foaming-dependent feeding)				
600	0.14	46.7 ± 4.34 at 52nd h	195 ± 8.18 at 48th h	0.09 ± 0.006
Fed-batch fermentation (constant specific growth rate control)				
600	0.05	54.8 ± 3.06 at 66th h	699 ± 20.8 at 72nd h	0.19 ± 0.017
	0.10	51 ± 4.32 at 54th h	428 ± 15.6 at 60th h	0.15 ± 0.015
	0.15	46.3 ± 3.97 at 48th h	265 ± 21.2 at 48th h	0.12 ± 0.011
	0.05*	66.3 ± 4.17 at 84th h	713 ± 19.8 at 90th h	0.12 ± 0.013

* Feeding rate was switched to a constant value at 66 h; ND – not detectable; NA – not available.

Table 4.1: Kinetic parameters of batch and fed-batch fermentations

4.3 *Mass transfer study in HCDF*

4.3.1 *Experimental measurement of gas-liquid mass transfer in HCDC*

k_La value for gas-liquid mass transfer was measured experimentally and the results are presented in Table 4.2. Low agitation rate at 400 rpm exhibits k_La value lower than 600 rpm which can be attributed to agitation rate at 400 rpm was insufficient to break up the bubble to smaller ones and consequently resulted in relatively small interfacial area.

Table 4.2**:** Experimental value of k_La taken from fed-batch fermentation

Rpm	**Experimental value of (k_La)**
400	0.011 ± 0.002
600	0.027 ± 0.008
800	0.005 ± 0.001

As agitation rate increased to 600 rpm, k_La value was proportionally increased because the gas bubbles can be dispersed into smaller ones and this characteristic led to higher interfacial area. In addition, gas dispersion in stirred vessel takes place mainly in the immediate vicinity of the impeller. The present of gas in stirred liquid will be drawn into low-pressure cavities behind the stirrer blades. As the impeller speed increase, small gas bubbles are thrown out from the back of the cavities into the bulk liquid under the influence of dispersion processes that are not yet completely understood (Bruijn, 1976).

However, when the agitation rate was further increased to 800 rpm, the k_La value was unexpectedly decreased to a very low value about 0.005 s^{-1}. The possible explanation of this observation is the problems with very small and rigid bubbles generated by high impeller speed in viscous broth like in HCDF system. Even though no experimental data was collected for bubble diameter, it is relevant to take the calculated bubble diameter which was 1.98 mm at 800 rpm (Table 4.3) as a reference since the k_La value was well predicted using established correlation from Calderbank, (1958).

Table 4.3**:** Theoretical values of mass transfer parameter of gas-liquid mass transfer.

rpm	D_{32} **(mm)**	H	k_L **(m s^{-1})**	a **(m^{-1})**	k_La **(s^{-1})**
400	4.42	0.004	2.96 x 10^{-3}	4.9	0.015
600	2.76	0.005	2.96 x 10^{-3}	12.7	0.037
800	1.98	0.008	6.64 x 10^{-5}	24.7	0.002

D_{32}: Sauter mean diameter
H: Gas hold-up

In viscous condition, the bubbles take a long time to achieve homogenous gas dispersion. Furthermore, the surface of tiny bubbles behaviour which is lesser than 2-3 mm diameter would be dominated by the effects of surface tension. As a result, the bubbles behave as a rigid sphere with immobile surface and no internal gas circulation. A rigid bubble surface give lower k_L value and decreases with decreasing bubble diameter below 2-3 diameter. As a rule of thumb, relatively large bubbles are more favourable in viscous liquid as in HCDF broth (Doran, 1995).

4.3.2 *Theoretical determination of gas-liquid mass transfer*

The Sauter mean diameter was inversely proportional to impeller speed. On the other hand, the k_L of the system operated at 400 and 600 rpm was found to be similar. This can be explained in terms of the range of bubble size predicted from the correlation. Calderbank, (1958) has proposed that, for small bubbles less than 2.5 mm in diameter, the exponential dependency of molecular diffusivity on the mass-transfer coefficient in Eq. (3.20) is 2/3. Whereas, those bubbles with sizes larger than 2.5 mm in diameter, its exponential dependency of molecular diffusivity changes to ½ in Eq. (3.19). This indicates that the system was operated under different regime. This explained why k_L at 800 rpm was much smaller compared to the other agitation rates tested. As explained previously, high agitation rate will generate very small bubbles which behave as rigid spheres with immobile surface and no internal gas circulation. Another factor which influences k_La value in a system is gas hold up. The predicted gas hold up was increased as agitation increased and this must be due to the fact that small bubbles generated from intense agitation have correspondingly slow bubble-rise velocities. Consequently they stay for longer in the fermentation broth even well after their oxygen level was depleted. All data are presented in Table 4.3.

Overall, the correlation developed by Calderbank, (1958) was useful to predict volumetric oxygen transfer coefficient (k_La) in this study as the calculated values agreed quite well with those experimental values as shown in Table 4.4.

Table 4.4: Comparison of theoretical and Experimental value of gas-liquid mass transfer

rpm	k_La (s^{-1})	
	Theoretical value	**Experimental value**
400	0.007	0.011 ± 0.002
600	0.012	0.027 ± 0.008
800	0.004	0.005 ± 0.001

The calculations are shown in *Appendix A*.

4.3.3 *Calculation of liquid-liquid mass transfer based on experimental determination of total interfacial area of the oil droplets*

Interfacial area *a* was observed to be linear with increasing impeller speed (Figure 4.7) since it was able to shear the oil into tiny droplets and the average of droplets diameter range from 8.1 mm to 2.1 mm as shown in Table 4.5. An oil droplet image is shown in Figure 4.8.

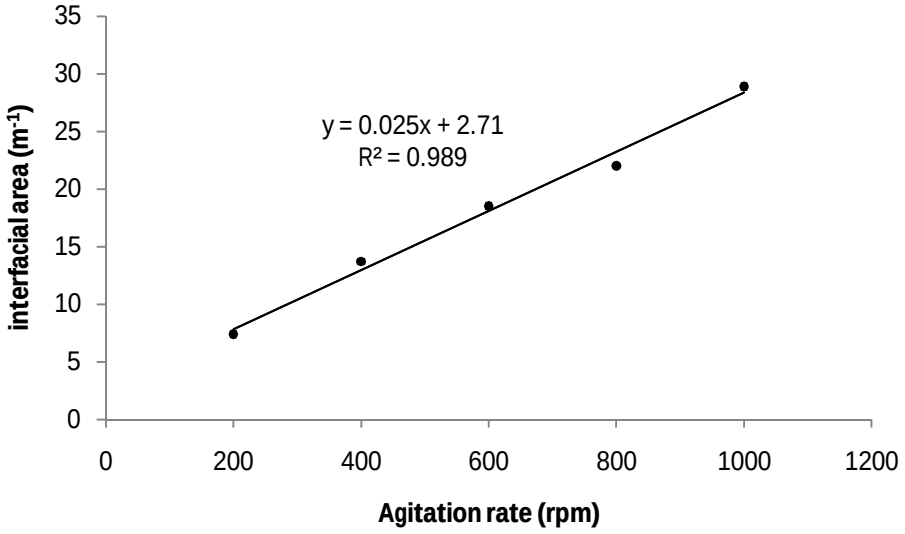

Figure 4.7**:** Linear relationship between interfacial area *a* and agitation rate (rpm**).**

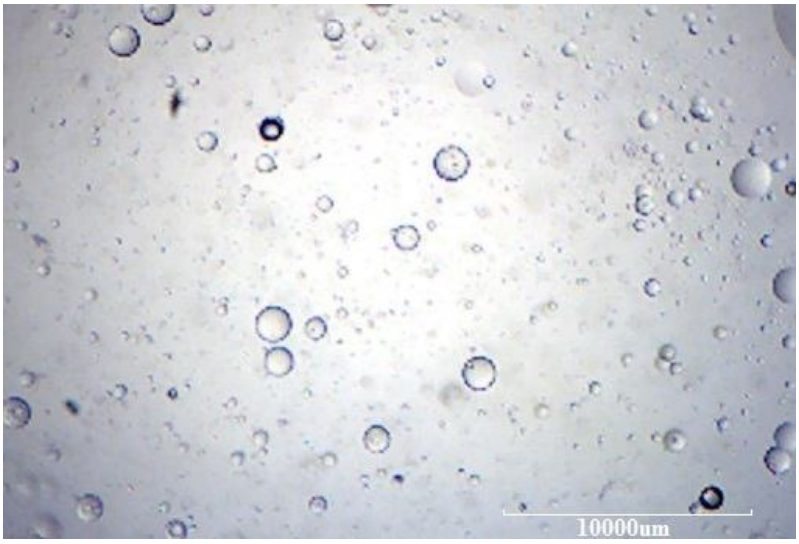

Figure 4.8: Oil droplet images at 40X magnification

The k_L value for all systems operated at 200, 400, 600, and 800 were identical since the average droplets size were measured larger than 2.5 mm in diameter and its exponential dependency of molecular diffusivity is similar (1/2). However the k_L value at 1000 rpm was lower compared to those calculated earlier. The reason for this is because the experimental droplet size was measured smaller than 2.5 mm. Thus, its exponential dependency of molecular diffusivity is bigger (2/3). Nevertheless, interfacial value was proportionally increased as the impeller speed was increased from 200 to 1000 rpm. As a result, the k_La value increased at agitation rate from 200 rpm to 800 rpm and decreased at 1000 rpm. Similar behaviour was also observed in a gas-liquid mass transfer study (Table 4.3) since the value of diffusivity of oil in water is negligible i.e. 2.76×10^{-10} m^2 s^{-1} as calculated in Appendix A. All experimental values are listed in Table 4.5.

Table 4.5**:** Experimental value of mass transfer parameter of liquid-liquid mass transfer

RPM	D_{32} **(mm)**	k_L **(m** s^{-1}**)**	a **(**m^{-1}**)**	k_La **(**s^{-1}**)**
200	8.1	6.18×10^{-5}	7.94	4.9×10^{-4}
400	4.4	6.18×10^{-5}	13.7	8.5×10^{-4}
600	3.2	6.18×10^{-5}	18.5	1.1×10^{-3}
800	2.7	6.18×10^{-5}	22	1.3×10^{-3}
1000	2.1	1.06×10^{-5}	28.9	3.1×10^{-4}

The calculations are shown in *Appendix A*.

4.3.4 *Calculation of liquid-liquid mass transfer based on theoretical determination of specific total interfacial area*

The average diameter of the droplets was expected to be inversely proportional to the agitation rate (Table 4.5). Since all the predicted average diameter of droplets was larger than 2.5 mm, similar correlation with exponential dependency of molecular diffusivity of 2/3 was employed. As a and k_L were inversely and directly proportional to the impeller speed, respectively, the product of it which is k_La may consequently decreased and increased as well. The correlations used in this study were able to predict the experimental data as presented in Table 4.6. For the observed effects of agitation on a and k_L (Table 4.6), earlier explanation is equally applicable in this case.

Table 4.6**:** Theoretical value of parameter in liquid-liquid mass transfer

RPM	D_{32} **(mm)**	k_L **(m s^{-1})**	a **(m^{-1})**	k_La **(s^{-1})**
200	7.5	6.18×10^{-5}	8	4.5×10^{-4}
400	5.0	6.18×10^{-5}	12	7.4×10^{-4}
600	3.9	6.18×10^{-5}	15.4	1.0×10^{-3}
800	3.3	6.18×10^{-5}	18.2	1.1×10^{-3}
1000	2.9	6.18×10^{-5}	20.9	1.3×10^{-3}

The correlation however failed to predict the k_La at 1000 rpm under HCDF as shown in Table 4.7.

Table 4.7**:** Comparison of k_La value between theoretical and experimental value

rpm	k_La **(s^{-1})**	
	Theoretical value	**Experimental value**
200	4.5×10^{-4}	4.9×10^{-4}
400	7.4×10^{-4}	8.5×10^{-4}
600	1.0×10^{-3}	1.1×10^{-3}
800	1.1×10^{-3}	1.3×10^{-3}
1000	1.3×10^{-3}	3.1×10^{-4}

The calculations are shown in *Appendix A*.

CHAPTER 5

5.0 Conclusions

Of the three feeding strategies tested, the specific growth rate control of feeding to achieve a relatively low value of the specific growth rate of 0.05 h^{-1} proved to be the best for achieving a high final titer of lipase (~700 U L^{-1}). For this mode of production, the biomass specific productivity of lipase was high at 0.19 U g^{-1} h^{-1}. The use of refined palm oil as a sole carbon source proved effective. Palm oil is a low-cost and abundantly available resource for producing lipases in regions such as Malaysia, Indonesia and Thailand. Using palm oil as the sole substrate obviated the need for chemical antifoams in the production of lipase.

Substrates mass transfer study was also carried out since it plays a major role in fermentation. The necessity of better substrate mass transfer is top priority particularly in HCDF. In this study, a number of established correlations were successfully used to predict k_La value at different agitation rates ranging from 200-1000 rpm for gas-liquid and liquid-liquid mass transfer under HCDF conditions.

It was found that linear relationship was observed between increased agitation rate (rpm) and k_La value for both mass transfers. However the experimental as well as theoretical k_La value for gas-liquid mass transfer was reduced at 800 rpm. For liquid-liquid system, the k_La value at the highest agitation rate used i.e. 1000 rpm determined through experimental and theoretical means did not agreed with each other. This showed that the correlations failed to predict k_La value at higher agitation rate such as 1000 rpm under HCDF.

APPENDIX A

1) Theoretical determination of gas-liquid mass transfer

i. The superficial gas velocity (V_s);

$$V_s = \frac{\text{Volumetric flow rate}}{\text{cross sectional area of vessel}} \quad (3.31)$$

Volumetric flow rate

$1\ \text{vvm} = 1\ \text{L min}^{-1}$

$$\left(\frac{1\ \text{L}}{\text{min}} \text{x} \frac{1\ \text{min}}{60\text{s}} \text{x} \frac{0.001\ \text{m}^3}{1\ \text{L}}\right)$$

$= 1.66 \text{ x } 10^{-5}\ \text{m}^3\ \text{s}^{-1}$

Cross sectional area of vessel

$= \pi r^2$

$= \pi \text{ x } (0.0656\text{m})^2$

$= 0.0135\ \text{m}^2$

The superficial gas velocity :

$$= \frac{1.66 \text{ x } 10^{-5}\ \text{m}^3\ \text{s}^{-1}}{0.0135\ \text{m}^2}$$

$= 1.23 \text{ x } 10^{-3}\ \text{m s}^{-1}$

ii. *Terminal velocity*

$$V_t = \frac{g d_e^2 (\rho_L - \rho_g)}{18\,\mu_L} \quad \text{(Stoke's law)} \tag{3.32}$$

iii. *Density of pure oxygen*: (3.33)

$$PV = n\,RT$$

$$n = \frac{mass}{(MW)}$$

$$PV = \left(\frac{mass}{(MW)}\right) RT$$

$$\frac{P\,(MW)}{RT} = \rho$$

$$\rho = \frac{(1\ atm)x(32\ g\ mol^{-1})}{(0.0821\ L\ atm\ mole^{-1}K^{-1})(303.15K)}$$

$= 1.29\ g\ L^{-1} = 1.29\ kg\ m^{-3}$

iv. *Diffusivity of oxygen into liquid medium.*

$$D_{o_2} = 7.4\ x\ 10^{-8} \left[\frac{T(X_a M)^{1/2}}{\mu_L\, V_{m(o_2)}^{0.6}}\right] = cm^{\,2}\, s^{-1} \tag{3.34}$$

$$D_{o_2} = 7.4\ x\ 10^{-8} \left[\frac{(303.15)(2.6\ x\ 18)^{1/2}}{(1.68\ cP)(25.6)^{0.6}}\right] = cm^{\,2}\, s^{-1}$$

$= 1.3 \times 10^{-5}\ cm^2\ s^{-1} = 1.3 \times 10^{-9}\ m^2\ s^{-1}$

Agitation rate: 400 rpm

i. Power requirement

$$N_{Re_i} = \frac{\rho N D_i^2}{\mu}$$

$$= \frac{(996\ kg\ m^{-3})(6.66\ r\ s^{-1})(0.052\ m)^2}{(1.68\ x\ 10^{-3}\ kg\ m^{-1}s^{-1})}$$

$$= \quad 10{,}676.55$$

The Reynolds number is much larger than 10000 above which the power number is constant at 6, thus,

$$P_{mo} = 6\rho N^3 D_i^5 = 6(996\ kg\ m^{-3})\ (6.66\ r\ s^{-1})^3\ (0.052\ m)^5 = 0.67\ W$$

The power required in the gas-sparged system:

$$log_{10}\ \frac{P_m}{P_{mo}} = -192\left(\frac{D_i}{D_t}\right)^{4.38}\left(\frac{D_i^2 N}{\nu}\right)^{0.115}\left(\frac{D_i N^2}{g}\right)^{1.96\left(\frac{D_i}{Dt}\right)}\left(\frac{Q}{ND_i^3}\right)$$

$$= -192\left(\frac{0.052}{0.1312}\right)^{4.38}\left(\frac{(0.052)^2(6.66)}{1.7\ x\ 10^{-6}}\right)^{0.115}\left(\frac{(0.052)(6.66)^2}{9.81}\right)^{1.96\left(\frac{0.052}{0.1312}\right)}\left(\frac{1.66\ x\ 10^{-5}}{(6.66)(0.052)^3}\right)$$

$log_{10}\ \frac{P_m}{0.67}$ = -0.055

$$P_m = 0.59\ W$$

ii. Sauter mean diameter

$$D_{32} = 0.7\left[\frac{\sigma^{0.6}}{(P_m/V)^{0.4}\ \rho_L^{0.2}}\right]\left(\frac{\mu_L}{\mu_g}\right)^{0.1}$$

$$D_{32} = 0.7\left[\frac{(0.07197)^{0.6}}{(0.59/0.001)^{0.4}\ (996)^{0.2}}\right]\left(\frac{1.68\ x\ 10^{-3}}{1.92\ x\ 10^{-5}}\right)^{0.1}$$

= 4.42 x 10^{-3} m = 4.42 mm

iii. *Terminal velocity (V_t)*

$$= \frac{(9.81)(4.42 \times 10^{-3})^2(996 - 1.29)}{18(1.68 \times 10^{-3})}$$

$$= 6.30 \text{ m s}^{-1}$$

iv. *Interfacial area*

$$a_o = 1.44\left[\frac{\left(P_m/V\right)^{0.4} \rho_c^{0.2}}{\sigma^{0.6}}\right]\left(\frac{V_s}{V_t}\right)^{0.5}$$

$$a_o = 1.44\left[\frac{\left(0.59/0.001\right)^{0.4}(996)^{0.2}}{0.07197^{0.6}}\right]\left(\frac{1.23\ x\ 10^{-3}}{6.3042}\right)^{0.5}$$

$$= 4.99 \text{ m}^{-1}$$

v. *Gas hold-up*

Since, $a_o = \frac{6H}{D_{32}}$

$$H = \frac{D_{32} \cdot a_o}{6}$$

$$= \frac{(4.42\ x\ 10^{-3})(4.99)}{6}$$

$$= 3.7\ x\ 10^{-3} = 0.004$$

vi. Volumetric mass transfer coefficient

Since average size of bubble is greater than 2.5 mm

$$k_L S_c^{1/2} = 0.42\left[\frac{\Delta\rho\mu_L g}{\rho_L^2}\right]^{1/3}$$

$$Sc = \frac{\mu_L}{\rho_L D_{o2}}$$

$$= \frac{(1.68\ x\ 10^{-3}\ \text{kg m}^{-1}\text{s}^{-1})}{(996\ \text{kg m}^{-3})\ \text{x}\ (1.3\ \text{x}\ 10^{-9}\ \text{m}^2\ \text{s}^{-1})}$$

$$= (1297.5)^{1/2}$$

$$= 36.02$$

$$k_L(36.02) = 0.42\left[\frac{\Delta\rho\mu_L g}{\rho_L^2}\right]^{1/3}$$

$$= 0.42\left[\frac{(996\ \text{kg m}^{-3} - 1.29\ \text{kg m}^{-3})\text{x}\ (1.68\ \text{x}\ 10^{-3}\ \text{kg m}^{-1}\text{s}^{-1})\text{x}\ (9.8\ \text{m s}^{-2})}{(996\ \text{kg m}^{-3})^2}\right]^{1/3}$$

$$k_L = \frac{0.107\ \text{m s}^{-1}}{36.02} = 2.96\ x\ 10^{-3}\ \text{m s}^{-1}$$

Therefore = $k_L\ x\ a$

= (2.96 x 10^{-3} m s^{-1}) x (4.99m^{-1})

= 0.015 s^{-1}

Agitation rate: 600 rpm

i. *Power requirement*

$$N_{Re_i} = \frac{\rho N D_i^2}{\mu}$$

$$= \frac{(996\ kg\ m^{-3})(10\ r\ s^{-1})(0.052\ m)^2}{(1.68\ x\ 10^{-3}\ kg\ m^{-1}s^{-1})}$$

$$= \quad 16030.85$$

The Reynolds number is much larger than 10000 hence, the power number is constant at 6, thus,

$$P_{mo} = 6\rho N^3 D_i^5 = 6(996\ kg\ m^{-3})\ (10\ r\ s^{-1})^3\ (0.052\ m)^5 = 2.27\ W$$

The power required in the gas-sparged system:

$$log_{10}\ \frac{P_m}{P_{mo}} = -192\left(\frac{D_i}{D_t}\right)^{4.38}\left(\frac{D_i^2 N}{\nu}\right)^{0.115}\left(\frac{D_i N^2}{g}\right)^{1.96\left(\frac{D_i}{Dt}\right)}\left(\frac{Q}{ND_i^3}\right)$$

$$= -192\left(\frac{0.052}{0.1312}\right)^{4.38}\left(\frac{(0.052)^2(10)}{1.7\ x\ 10^{-6}}\right)^{0.115}\left(\frac{(0.052)(10)^2}{9.81}\right)^{1.96\left(\frac{0.052}{0.1312}\right)}\left(\frac{1.66\ x\ 10^{-5}}{(10)(0.052)^3}\right)$$

$$= (-3.33)(3.042)(0.61074)(0.0118)$$

= -0.073

$log_{10}\ \frac{P_m}{2.27}$ = -0.073

$$P_m = 1.92\ W$$

ii. *Sauter mean diameter*

$$D_{32} = 0.7\left[\frac{\sigma^{0.6}}{(P_m/V)^{0.4}\,\rho_L^{0.2}}\right]\left(\frac{\mu_L}{\mu_g}\right)^{0.1}$$

$$D_{32} = 0.7\left[\frac{(0.07197)^{0.6}}{(1.92/0.001)^{0.4}\,(996)^{0.2}}\right]\left(\frac{1.68\,x\,10^{-3}}{1.92\,x\,10^{-5}}\right)^{0.1}$$

$= 2.76 \times 10^{-3}$ m = 2.76 mm

iii. *Terminal velocity (V_t)*

$$= \frac{(9.81)(2.76 \times 10^{-3})^2(996 - 1.29)}{18(1.68 \times 10^{-3})}$$

$= 2.458\ \text{m s}^{-1}$

iv. *Interfacial area*

$$a_o = 1.44\left[\frac{\left(P_m/V\right)^{0.4}\rho_c^{0.2}}{\sigma^{0.6}}\right]\left(\frac{V_s}{V_t}\right)^{0.5}$$

$$a_o = 1.44\left[\frac{(1.92/0.001)^{0.4}(996)^{0.2}}{0.07197^{0.6}}\right]\left(\frac{1.23\,x\,10^{-3}}{2.458}\right)^{0.5}$$

$= (1.44)(396.902)(0.022369775)$

$= 12.78\ \text{m}^{-1}$

v. *Gas hold-up*

Since, $a_o = \frac{6H}{D_{32}}$

$$H = \frac{D_{32}\,.\,a_o}{6} = \frac{(2.76\,x\,10^{-3})(12.78)}{6}$$

$$= 5.8\,x\,10^{-3} = 0.005$$

vi. Volumetric mass transfer coefficient

Since average size of bubble is greater than 2.5 mm

$$k_L S_c^{1/2} = 0.42 \left[\frac{\Delta\rho\mu_L g}{\rho_L^2}\right]^{1/3}$$

$$Sc = \frac{\mu_L}{\rho_L D_{o2}}$$

$$= \frac{(1.68\ x\ 10^{-3}\ \text{kg m}^{-1}\text{s}^{-1})}{(996\ \text{kg m}^{-3})\ \text{x}\ (1.3\ \text{x}\ 10^{-9}\ \text{m}^2\ \text{s}^{-1})}$$

$$= (1297.5)^{1/2}$$

$$= 36.02$$

$$k_L(36.02) = 0.42 \left[\frac{\Delta\rho\mu_L g}{\rho_L^2}\right]^{1/3}$$

$$= 0.42 \left[\frac{(996\ \text{kg m}^{-3} - 1.29\ \text{kg m}^{-3})\text{x}\ (1.68\ \text{x}\ 10^{-3}\ \text{kg m}^{-1}\text{s}^{-1})\text{x}\ (9.8\ \text{m s}^{-2})}{(996\ \text{kg m}^{-3})^2}\right]^{1/3}$$

$$k_L = \frac{0.107\ \text{m s}^{-1}}{36.02} = 2.96\ x\ 10^{-3}\ \text{m}^{-1}$$

Therefore = $k_L\ x\ a$

= $(2.96\ \text{x}\ 10^{-3}\ \text{m s}^{-1})\ \text{x}\ (12.78\ \text{m}^{-1})$

= $0.037\ \text{s}^{-1}$

Agitation rate: 800 rpm

i. Power requirement

$$N_{Re_i} = \frac{\rho N D_i^2}{\mu}$$

$$= \frac{(996\ kg\ m^{-3})(13.3\ r\ s^{-1})(0.052\ m)^2}{(1.68\ x\ 10^{-3}\ kg\ m^{-1}s^{-1})}$$

$$= \quad 21{,}321$$

The Reynolds number is much larger than 10000. Hence, the power number is constant at 6, thus,

$$P_{mo} = 6\rho N^3 D_i^5 = 6(996\ kg\ m^{-3})\ (13.3\ r\ s^{-1})^3\ (0.052\ m)^5 = 5.34\ W$$

The power required in the gas-sparged system:

$$log_{10}\ \frac{P_m}{P_{mo}} = -192\left(\frac{D_i}{D_t}\right)^{4.38}\left(\frac{D_i^2 N}{\nu}\right)^{0.115}\left(\frac{D_i N^2}{g}\right)^{1.96\left(\frac{D_i}{Dt}\right)}\left(\frac{Q}{ND_i^3}\right)$$

$$= -192\left(\frac{0.052}{0.1312}\right)^{4.38}\left(\frac{(0.052)^2(13.3)}{1.7\ x\ 10^{-6}}\right)^{0.115}\left(\frac{(0.052)(13.3)^2}{9.81}\right)^{1.96\left(\frac{0.052}{0.1312}\right)}\left(\frac{1.66\ x\ 10^{-5}}{(13.3)(0.052)^3}\right)$$

$log_{10}\ \frac{P_m}{5.34}$ = -0.088

$$P_m = 4.36\ W$$

ii. Sauter mean diameter

$$D_{32} = 0.7\left[\frac{\sigma^{0.6}}{(P_m/V)^{0.4}\ \rho_L^{0.2}}\right]\left(\frac{\mu_L}{\mu_g}\right)^{0.1}$$

$$D_{32} = 0.7\left[\frac{(0.07197)^{0.6}}{(4.36/0.001)^{0.4}\ (996)^{0.2}}\right]\left(\frac{1.68\ x\ 10^{-3}}{1.92\ x\ 10^{-5}}\right)^{0.1}$$

= 1.98 x 10^{-3} m = 1.98 mm

iii. *Terminal velocity (V_t)*

$$= \frac{(9.81)(1.98 \times 10^{-3})^2(996 - 1.29)}{18(1.68 \times 10^{-3})}$$

$$= 1.26 \text{ m s}^{-1}$$

iv. *Interfacial area*

$$a_o = 1.44\left[\frac{\left(P_m/V\right)^{0.4} \rho_c^{0.2}}{\sigma^{0.6}}\right]\left(\frac{V_s}{V_t}\right)^{0.5}$$

$$a_o = 1.44\left[\frac{\left(4.36/0.001\right)^{0.4}(996)^{0.2}}{0.07197^{0.6}}\right]\left(\frac{1.23\ x\ 10^{-3}}{1.26}\right)^{0.5}$$

$$= 24.74 \text{ m}^{-1}$$

v. *Gas hold-up*

Since, $a_o = \frac{6H}{D_{32}}$

$$H = \frac{D_{32} \cdot a_o}{6}$$

$$= \frac{(1.98\ x\ 10^{-3})(24.74)}{6}$$

$$= 8.16\ x\ 10^{-3} = 0.008$$

vi. Volumetric mass transfer coefficient

Since average size of bubble is less than 2.5 mm

$$k_L S_c^{2/3} = 0.31 \left[\frac{\Delta \rho \mu_L g}{\rho_L^2}\right]^{1/3}$$

$$Sc = \frac{\mu_L}{\rho_L D_{o2}}$$

$$= \frac{(1.68\ x\ 10^{-3}\ \text{kg m}^{-1}\text{s}^{-1})}{(996\ \text{kg m}^{-3})\ \text{x}\ (1.3\ \text{x}\ 10^{-9}\ \text{m}^2\ \text{s}^{-1})}$$

$$= (1297.5)^{2/3}$$

$$= 118.96$$

$$k_L(118.96) = 0.31 \left[\frac{\Delta \rho \mu_L g}{\rho_L^2}\right]^{1/3}$$

$$= 0.31 \left[\frac{(996\ \text{kg m}^{-3} - 1.29\ \text{kg m}^{-3})\text{x}\ (1.68\ \text{x}\ 10^{-3}\ \text{kg m}^{-1}\text{s}^{-1})\text{x}\ (9.8\ \text{m s}^{-2})}{(996\ \text{kg m}^{-3})^2}\right]^{1/3}$$

$$k_L = \frac{7.89\ x\ 10^{-3}\ \text{m s}^{-1}}{118.96} = 6.64\ x\ 10^{-5}$$

Therefore = $k_L\ x\ a$

= (6.64 x 10^{-5} m s^{-1}) x (24.74 m^{-1})

= 1.6 x 10^{-3} s^{-1} ≈ 0.002 s^{-1}

2) Calculation of liquid-liquid mass transfer based on experimental determination of total interfacial area of the oil droplets

i. Diffusivity of palm oil into liquid medium.

$$D_{oil} = 117.3\, x\, 10^{-18} \left[\frac{T(X_a M)^{1/2}}{\mu_L\, V_{m(O_2)}^{0.6}}\right] = m^2\, s^{-1} \qquad (3.35)$$

$$D_{oil} = 117.3 \times 10^{-18} \left[\frac{(303.15)(2.26 \times 18)^{1/2}}{(1.68 \times 10^{-3})(0.3037)^{0.6}}\right] = m^2\, s^{-1}$$

$$= 2.76 \times 10^{-10}\ m^2\, s^{-1}$$

ii. Mass transfer (k_L) for 200, 400, 600, and 800 rpm

Since D > 2.5 mm (obtained from experimental D_{32})

$$Sc = \frac{\mu_L}{\rho_L D_{oil}}$$

$$= \frac{1.68\, x\, 10^{-3}\ kg\, m^{-1} s^{-1}}{996\ kg\, m^{-3} \times\ 2.76 \times 10^{-10}\ m^2\, s^{-1}}$$

$$= 6111.4$$

$$K_L S_c^{1/2} = 0.42 \left[\frac{\Delta\rho \mu_L g}{\rho_L^2}\right]^{1/3}$$

$$= 0.42 \left[\frac{(996\ kg\, m^{-3} - 904.3\ kg\, m^{-3}) \times 1.68 \times 10^{-3}\ kg\, m^{-1} s^{-1} \times 9.8\ m\, s^{-2}}{992{,}016\ kg^2\, m^{-6}}\right]^{1/3}$$

$$= 0.42 \left[\frac{1.5097\ m^{-3}\, s^{-3}}{992016\ m^{-6}}\right]^{1/3}$$

$$= 4.831 \times 10^{-3}\ m\, s^{-1}$$

$$k_L = \frac{4.831 \times 10^{-3}\ m\, s^{-1}}{(6111.4)^{1/2}}$$

$$= 6.18 \times 10^{-5}\ m\, s^{-1}$$

iii. Mass transfer (k_L) for 1000 rpm

Since D < 2.5 mm (obtained from experimental D_{32})

$$Sc = \frac{\mu_L}{\rho_L D_{oil}}$$

$$= \frac{1.68\ x\ 10^{-3}\ \text{kg m}^{-1}\text{s}^{-1}}{996\ \text{kg m}^{-3}\ \text{x}\ 2.76\ \text{x}\ 10^{-10}\ \text{m}^2\ \text{s}^{-1}}$$

$$= 6111.4$$

$$K_L S_c^{2/3} = 0.31\left[\frac{\Delta\rho\mu_L g}{\rho_L^2}\right]^{1/3}$$

$$= 0.31\left[\frac{(996\ \text{kg m}^{-3} - 904.3\ \text{kg m}^{-3})\text{x}\ 1.68\ \text{x}\ 10^{-3}\ \text{kg m}^{-1}\text{s}^{-1}\ \text{x}\ 9.8\ \text{m s}^{-2}}{992{,}016\ \text{kg}^2\ \text{m}^{-6}}\right]^{1/3}$$

$$= 0.31\left[\frac{1.5097\ \text{m}^{-3}\ \text{s}^{-3}}{992016\ \text{m}^{-6}}\right]^{1/3}$$

$$= 3.565\ \text{x}\ 10^{-3}\ \text{m s}^{-1}$$

$$k_L = \frac{3.565\ \text{x}\ 10^{-3}\ \text{m s}^{-1}}{(6111.4)^{2/3}}$$

$$= 1.06\ \text{x}\ 10^{-5}\ \text{m s}^{-1}$$

3) *Calculation of liquid-liquid mass transfer based on theoretical determination of specific total interfacial area*

i. Mass transfer (k_L) for 200, 400, 600, 800 and 1000 rpm

Since D > 2.5 mm (obtained from experimental D_{32})

$$Sc = \frac{\mu_L}{\rho_L D_{oil}}$$

$$= \frac{1.68\ x\ 10^{-3}\ \text{kg m}^{-1}\text{s}^{-1}}{996\ \text{kg m}^{-3}\ \text{x}\ 2.76\ \text{x}\ 10^{-10}\ \text{m}^2\ \text{s}^{-1}}$$

$$= 6111.4$$

$$K_L S_c^{1/2} = 0.42\left[\frac{\Delta\rho\mu_L g}{\rho_L^2}\right]^{1/3}$$

$$= 0.42\left[\frac{(996\ \text{kg m}^{-3} - 904.3\ \text{kg m}^{-3})\text{x}\ 1.68\ \text{x}\ 10^{-3}\ \text{kg m}^{-1}\text{s}^{-1}\ \text{x}\ 9.8\ \text{m s}^{-2}}{992{,}016\ \text{kg}^2\ \text{m}^{-6}}\right]^{1/3}$$

$$= 0.42\left[\frac{1.5097\ \text{m}^{-3}\ \text{s}^{-3}}{992016\ \text{m}^{-6}}\right]^{1/3}$$

$$= 4.831\ \text{x}\ 10^{-3}\ \text{m s}^{-1}$$

$$k_L = \frac{4.831\ \text{x}\ 10^{-3}\ \text{m s}^{-1}}{(6111.4)^{1/2}}$$

$$= 6.18\ \text{x}\ 10^{-5}\ \text{m s}^{-1}$$

ii. *Agitation rate: 200 rpm*

a) Total interfacial area

$a_t = 0.024(3.33)^{0.6}\ (0.01)(303.15)^{1.7} / (1+3.0\ (0.01))$

$= 7.94\ m^{-1}$

b) Sauter mean diameter

$$D_{32} = \frac{6\emptyset}{a_t}$$

$$= \frac{6(0.01)}{7.94}$$

$= 7.55 \times 10^{-3}\ m = 7.5\ mm$

iii. *Agitation rate: 400 rpm*

a) Total interfacial area

$a_t = 0.024(6.66)^{0.6}\ (0.01)(303.15)^{1.7} / (1+3.0\ (0.01))$

$= 12.03\ m^{-1}$

b) Sauter mean diameter

$$D_{32} = \frac{6\emptyset}{a_t}$$

$$= \frac{6(0.01)}{12.03}$$

$= 4.98 \times 10^{-3}\ m = 4.98\ mm$

iv. *Agitation rate: 600 rpm*

a) Total interfacial area

$a_t = 0.024(10)^{0.6}\ (0.01)(303.15)^{1.7} / (1+3.0\ (0.01))$

$= 15.4\ m^{-1}$

b) Sauter mean diameter

$$D_{32} = \frac{6\emptyset}{a_t}$$

$$= \frac{6(0.01)}{15.4}$$

$= 3.89 \times 10^{-3}\ m = 3.89\ mm$

v. *Agitation rate: 800 rpm*

a) Total interfacial area

$a_t = 0.024(13.33)^{0.6}\ (0.01)(303.15)^{1.7} / (1+3.0\ (0.01))$

$= 18.24\ m^{-1}$

b) Sauter mean diameter

$$D_{32} = \frac{6\emptyset}{a_t}$$

$$= \frac{6(0.01)}{18.24}$$

$= 3.28 \times 10^{-3}\ m = 3.28\ mm$

vi. *Agitation rate: 1000 rpm*

a) Total interfacial area

$a_t = 0.024(16.66)^{0.6}\ (0.01)(303.15)^{1.7} / (1+3.0\ (0.01))$

$= 20.85\ m^{-1}$

b) Sauter mean diameter

$$D_{32} = \frac{6\emptyset}{a_t}$$

$$= \frac{6(0.01)}{20.85}$$

$$= 2.87 \times 10^{-3}\ m = 2.87\ mm$$

REFERENCES

ADAMITSCH, B. F., KARNER, F. & HAMPEL, W. A. 2003. High cell density cultivation of *Brevibacterium linens* and formation of proteinases and lipase. *Biotechnology Letters,* 25**,** 705-708.

AIBA S, H. A., MILLIS NF 1973. *Biochemical Engineering, 2nd edition,* University of Tokyo Press.

AKBARI, N., KHAJEH, K., GHAEMI, N. & SALEMI, Z. 2010a. Efficient refolding of recombinant lipase from *Escherichia coli* inclusion bodies by response surface methodology. *Protein expression and purification,* 70**,** 254-259.

AKBARI, N., KHAJEH, K., REZAIE, S., MIRDAMADI, S., SHAVANDI, M. & GHAEMI, N. 2010b. High-level expression of lipase in *Escherichia coli* and recovery of active recombinant enzyme through in vitro refolding. *Protein Expression and Purification,* 70**,** 75-80.

AKOH, C. C. & YEE, L. N. 1997. Enzymatic synthesis of position-specific low-calorie structured lipids. *Journal of the American Oil Chemists' Society,* 74**,** 1409-1413.

AL-ZUHAIR, S., HASAN, M. & RAMACHANDRAN, K. 2003. Kinetics of the enzymatic hydrolysis of palm oil by lipase. *Process Biochemistry,* 38**,** 1155-1163.

ALONSO, F. O. M., OLIVEIRA, E. B. L., DELLAMORA-ORTIZ, G. M. & PEREIRA-MEIRELLES, F. V. 2005. Improvement of lipase production at different stirring speeds and oxygen levels. *Brazilian Journal of Chemical Engineering,* 22**,** 9-18.

AMARAL, P., DE ALMEIDA, A., PEIXOTO, T., ROCHA-LEÃO, M., COUTINHO, J. & COELHO, M. 2007. Beneficial effects of enhanced aeration using perfluorodecalin in *Yarrowia lipolytica* cultures for lipase production. *World Journal of Microbiology and Biotechnology,* 23**,** 339-344.

AMARAL, P. F. F., FREIRE, M. G., ROCHA-LEÃO, M. H. M., MARRUCHO, I. M., COUTINHO, J. A. P. & COELHO, M. A. Z. 2008. Optimization of oxygen mass transfer in a multiphase bioreactor with perfluorodecalin as a second liquid phase. *Biotechnology and Bioengineering,* 99**,** 588-598.

AMARAL, P. F. F., ROCHA-LEÃO, M. H. M., MARRUCHO, I. M., COUTINHO, J. A. P. & COELHO, M. A. Z. 2006. Improving lipase production using a perfluorocarbon as oxygen carrier. *Journal of Chemical Technology & Biotechnology,* 81**,** 1368-1374.

ANNUAR, M., TAN, I., IBRAHIM, S. & RAMACHANDRAN, K. 2007. Production of Medium-Chain-Length Poly (3-hydroxyalkanoates) from Crude Fatty Acids Mixture by *Pseudomonas putida*. *Food and bioproducts processing,* 85**,** 104-119.

ARNAU, C., RAMON, R., CASAS, C. & VALERO, F. 2010. Optimization of the heterologous production of a *Rhizopus oryzae* lipase in Pichia pastoris system using

mixed substrates on controlled fed-batch bioprocess. *Enzyme and Microbial Technology,* 46**,** 494-500.

ARROYO, M., SÁNCHEZ-MONTERO, J. M. A. & SINISTERRA, J. V. 1999. Thermal stabilization of immobilized lipase B from *Candida antarctica* on different supports: Effect of water activity on enzymatic activity in organic media. *Enzyme and Microbial Technology,* 24**,** 3-12.

ARROYO M., S. J. 1995. Influence of chiral corvones on selectivity of pure lipase-B from *Candida antarctica. Biotechnology Letter,* 17**,** 30.

BAJAJ, A., LOHAN, P., JHA, P. N. & MEHROTRA, R. 2010. Biodiesel production through lipase catalyzed transesterification: An overview. *Journal of Molecular Catalysis B: Enzymatic,* 62**,** 9-14.

BALCÃO, V. M., PAIVA, A. L. & XAVIER MALCATA, F. 1996. Bioreactors with immobilized lipases: state of the art. *Enzyme and Microbial Technology,* 18**,** 392-416.

BARROS, M., FLEURI, L. & MACEDO, G. 2010. Seed lipases: sources, applications and properties-a review. *Brazilian Journal of Chemical Engineering,* 27**,** 15-29.

BELARBI, E.-H., MOLINA, E. & CHISTI, Y. 2000. A process for high yield and scaleable recovery of high purity eicosapentaenoic acid esters from microalgae and fish oil. *Process Biochemistry,* 35**,** 951-969.

BEN REJEB, I., ARDUINI, F., AMINE, A., GARGOURI, M. & PALLESCHI, G. 2007. Amperometric biosensor based on Prussian Blue-modified screen-printed electrode for lipase activity and triacylglycerol determination. *Analytica chimica acta,* 594**,** 1-8.

BIBILA, T. A. & ROBINSON, D. K. 1995. In pursuit of the optimal fed-batch process for monoclonal antibody production. *Biotechnology Progress,* 11**,** 1-13.

BIENER, R., STEINKAMPER, A. & HORN, T. 2012. Calorimetric control of the specific growth rate during fed-batch cultures of *Saccharomyces cerevisiae*. *Journal of Biotechnology,* 160**,** 195-201.

BISEN, P. S., SANODIYA, B. S., THAKUR, G. S., BAGHEL, R. K. & PRASAD, G. B. K. S. 2010. Biodiesel production with special emphasis on lipase-catalyzed transesterification. *Biotechnology Letters,* 32**,** 1019-1030.

BOARETO, A. J. M., SOUZA JR. M. B. D., VALERO F., AND VALDMAN B. 2007. A hybrid neural model (HNM) for the on-line monitoring of lipase production by *Candida rugosa*. *Journal of Chemical Technology Biotechnology,* 82**,** 319-327.

BONRATH, W., KARGE, R. & NETSCHER, T. 2002. Lipase-catalyzed transformations as key-steps in the large-scale preparation of vitamins. *Journal of Molecular Catalysis B: Enzymatic,* 19**,** 67-72.

BORNSCHEUER, U. T. 2005. Enzymes in Lipid Modification, 5 December 2005 ed.: Weinheim: Wiley-VCH, 200.

BOUAIFI, M., HEBRARD, G., BASTOUL, D. & ROUSTAN, M. 2001. A comparative study of gas hold-up, bubble size, interfacial area and mass transfer coefficients in stirred gas–liquid reactors and bubble columns. *Chemical Engineering and Processing: Process Intensification,* 40**,** 97-111.

BRADFORD, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry,* 72**,** 248-254.

BROWNLEE, I. A., FORSTER, D. J., WILCOX, M. D., DETTMAR, P. W., SEAL, C. J. & PEARSON, J. P. 2010. Physiological parameters governing the action of pancreatic lipase. *Nutrition research reviews,* 1**,** 1-9.

BRUIJN, W. 1976. Power consumption with aerated Rushton turbines. *Transactions of the Institution of Chemical Engineers*.52**,** 88-104.

BYRON RUBIN, E. A. D. 1997. Lipases, Part B: Enzyme Characterization and Utilization. *Methods in Enzymology,* vol. 286**,** 1 - 563.

CALDERBANK, P. 1958. Physical rate processes in industrial fermentation. Part I: The interfacial area in gas-liquid contacting with mechanical agitation. *Transactions of the Institution of Chemical Engineers* 36**,** 443-463.

CALDERBANK, P. & JONES, S. 1961. Physical Rate Processes in Industrial Fermentation Part III: Mass Transfer from Fluids to Solid Particles Suspended in Mixing Vessels. *Trans. Inst. Chem. Eng.,* 39**,** 363-368.

CALDERBANK, P. & MOO-YOUNG, M. 1959. The prediction of power consumption in the agitation of non-Newtonian fluids. *Transactions of the Institution of Chemical Engineers* 37**,** 26-33.

CARSTENSEN, F., APEL, A. & WESSLING, M. 2011. *In situ* product recovery: Submerged membranes vs. external loop membranes. *Journal of Membrane Science*.

CARSTENSEN, F., APEL, A. & WESSLING, M. 2012. In situ product recovery: Submerged membranes vs. external loop membranes. *Journal of Membrane Science,* 394–395**,** 1-36.

ÇELIK, E. & ÇALıK, P. 2012. Production of recombinant proteins by yeast cells. *Biotechnology Advances,* 30**,** 1108-1118.

CHANDRASEKHARAN, K. & CALDERBANK, P. 1981. Further observations on the scale-up of aerated mixing vessels. *Chemical Engineering Science,* 36**,** 818-823.

CHANG, H. N., YOO, I.-K. & KIM, B. S. 1994. High density cell culture by membrane-based cell recycle. *Biotechnology Advances,* 12**,** 467-487.

CHARTRAIN, M., MARCIN, C., KATZ, L., SALMON, P., BRIX, T., BUCKLAND, B. & GREASHAM, R. 1993. Enhancement of lipase production during fed-batch cultivation of *Pseudomonas aeruginosa* MB 5001. *Journal of Fermentation and Bioengineering,* 76**,** 487-492.

CHATTAWAY, T., DEMAIN, A.L., STEPHANOPOULUS, G., 1992. Use of various measurements for biomass estimation. *Biotechnology Progress,* 8**,** 81-84.

CHATTERJEE, T., CHATTERJEE, B. K. & BHATTACHARYYA, D. K. 2001. Study of lipase-catalyzed hydrolysis of some monoterpene esters. *Canada Journal of Microbioloyg,* 47**,** 397-403.

CHEN, H.-P., HSIAO, K.-F., WU, S.-H. & WANG, K.-T. 1995. Regioselectivity enhancement by partial purification of lipase from *Biotechnology Letters,* 17**,** 305-308.

CHEN, J. Y., WEN, C. M. & CHEN, T. L. 1999. Effect of oxygen transfer on lipase production by Acinetobacter radioresistens. *Biotechnology and Bioengineering,* 62**,** 311-316.

CHEN, J. Y., WEN, C. M. & CHEN, T. L. 2000. Effect of oxygen transfer on lipase production by *Acinetobacter radioresistens*. *Biotechnology and Bioengineering,* 62**,** 311-316.

CHERRY, J. R. & FIDANTSEF, A. L. 2003. Directed evolution of industrial enzymes: an update. *Current Opinion in Biotechnology,* 14**,** 438-443.

CHISTI, M. & MOO-YOUNG, M. 1987. Airlift reactors: characteristics, applications and design considerations. *Chemical Engineering Communications,* 60**,** 195-242.

CHISTI, Y. 2007. Principles of membrane separation processes. *In:* SUBRAMANIAM G, E. (ed.) *Bioseparation and bioprocessing: A handbook, second edition.* New York: Wiley-VCH.

CHO, M. H. & WANG, S. S. 1988. Enhancement of oxygen transfer in hybridoma cell culture by using a perfluorocarbon as an oxygen carrier. *Biotechnology Letters,* 10**,** 855-860.

CONTESINI, F. J., LOPES, D. B., MACEDO, G. A., NASCIMENTO, M. D. G. & CARVALHO, P. D. O. 2010. *Aspergillus sp*. lipase: Potential biocatalyst for industrial use. *Journal of Molecular Catalysis B: Enzymatic,* 67**,** 163-171.

COS, O., RESINA, D., FERRER, P., MONTESINOS, J. L. & VALERO, F. 2005. Heterologous production of *Rhizopus oryzae* lipase in *Pichia pastoris* using the alcohol oxidase and formaldehyde dehydrogenase promoters in batch and fed-batch cultures. *Biochemical Engineering Journal,* 26**,** 86-94.

DE SMET, B. L. A., PLUYTER, J. G. L. & JONES, L. A. 2001. Use of a lime soap dispersant in a detergent composition comprising lipase enzymes. EP Patent 0,662,119.

DELORME, V., DHOUIB, R., CANAAN, S., FOTIADU, F., CARRIÈRE, F. & CAVALIER, J.-F. 2011. Effects of surfactants on lipase structure, activity, and inhibition. *Pharmaceutical research,* 28**,** 1831-1842.

DOMÍNGUEZ DE MARÍA, P., SÁNCHEZ-MONTERO, J. M., SINISTERRA, J. V. & ALCÁNTARA, A. R. 2006. Understanding *Candida rugosa* lipases: An overview. *Biotechnology Advances,* 24**,** 180-196.

DONG, H., GAO, S., HAN, S.-P. & CAO, S.-G. 1999. Purification and characterization of a *Pseudomonas sp*. lipase and its properties in non-aqueous media. *Biotechnology and Applied Biochemistry,* 30**,** 251-256.

DORAN, P. M. 1995. *Bioprocess engineering principles,* Academic Press.

DUCRET, A., TRANI, M. & LORTIE, R. 1998. Lipase-catalyzed enantioselective esterification of ibuprofen in organic solvents under controlled water activity. *Enzyme and Microbial Technology,* 22**,** 212-216.

ELIBOL, M. & OZER, D. 2000. Influence of oxygen transfer on lipase production by *Rhizopus arrhizus*. *Process Biochemistry,* 36**,** 325-329.

FAN, Y. & QIAN, J. 2010. Lipase catalysis in ionic liquids/supercritical carbon dioxide and its applications. *Journal of Molecular Catalysis B: Enzymatic,* 66**,** 1-7.

FERRER, P., MONTESINOS, J. L., VALERO, F. & SOLÀ, C. 2001. Production of native and recombinant lipases by *Candida rugosa*. *Applied biochemistry and biotechnology,* 95**,** 221-255.

FICKERS, P., DESTAIN, J. & THONART, P. 2009. Improvement of *Yarrowia lipolytica* lipase production by fed-batch fermentation. *Journal of Basic Microbiology,* 49**,** 212-215.

FIESCHKO, J. C., EGAN, K. M., RITCH, T., KOSKI, R. A., JONES, M. & BITTER, G. A. 2004. Controlled expression and purification of human immune interferon from high-cell-density fermentations of *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering,* 29**,** 1113-1121.

FROM, M., ADLERCREUTZ, P. & MATTIASSON, B. 1997. Lipase catalyzed esterification of lactic acid. *Biotechnology Letters,* 19**,** 315-318.

GASKIN, D. J. H., ROMOJARO, A., TURNER, N. A., JENKINS, J. & VULFSON, E. N. 2001. Alteration of lipase chain length specificity in the hydrolysis of esters by random mutagenesis. *Biotechnology and Bioengineering,* 73**,** 433-441.

GERRITSE, G., HOMMES, R. W. & QUAX, W. J. 1998. Development of a lipase fermentation process that uses a recombinant *Pseudomonas alcaligenes* strain. *Applied and Environmental Microbiology,* 64**,** 2644-2651.

GILL, I. & VALIVETY, R. 1997. Polyunsaturated fatty acids, Part 1: Occurrence, biological activities and applications. *Trends in Biotechnology,* 15**,** 401.

GILHAM D, LEHNER R 2005. Techniques to measure lipase and esterase activity in vitro. *Methods* 36, 139-147.

GORDILLO M. A., S., A, SANCHEZ, A, VALERO, F., MOTESINOS, J. L., LAFUENTE, J., SOLA, C. 1998a. Enhancement of *candida rugosa* lipase production by using different control fed-batch operational strategies. *Biotechnology Bioengineering,* 60**,** 156-168.

GORDILLO M A, M. J. L., CASAS C, VALERO F, LAFUENTE J, SOLA C 1998b. Improving lipase production from *Candida rugosa* by a biochemical engineering approach. *Chemical and Physical Lipids,* 93**,** 131–142.

GULATI, R., SAXENA, R. & GUPTA, R. 2000. Fermentation and downstream processing of lipase from *Aspergillus terreus*. *Process Biochemistry,* 36**,** 149-155.

GUMEL, A., ANNUAR, M., HEIDELBERG, T. & CHISTI, Y. 2011a. Thermo-kinetics of lipase-catalyzed synthesis of 6-O-glucosyldecanoate. *Bioresource technology*.

GUMEL, A. M., ANNUAR, M. S. M., CHISTI, Y. & HEIDELBERG, T. 2012. Ultrasound assisted lipase catalyzed synthesis of poly-6-hydroxyhexanoate. *Ultrasonics sonochemistry,* 19**,** 659-667.

GUMEL, A. M., ANNUAR, M. S. M., HEIDELBERG, T. & CHISTI, Y. 2011b. Lipase mediated synthesis of sugar fatty acid esters. *Process Biochemistry,* 46**,** 2079-2090.

GUNCHEVA, M. & ZHIRYAKOVA, D. 2011. Catalytic properties and potential applications of *Bacillus* lipases. *Journal of Molecular Catalysis B: Enzymatic,* 68**,** 1-21.

GUPTA, N., RATHI, P. & GUPTA, R. 2002. Simplified para-nitrophenyl palmitate assay for lipases and esterases. *Analytical biochemistry,* 311**,** 98-9.

GUPTA, R., GUPTA, N. & RATHI, P. 2004. Bacterial lipases: an overview of production, purification and biochemical properties. *Applied Microbiology and Biotechnology,* 64**,** 763-781.

HASAN, F., SHAH, A. A. & HAMEED, A. 2006. Industrial applications of microbial lipases. *Enzyme and Microbial Technology,* 39**,** 235-251.

HASAN, F., SHAH, A. A. & HAMEED, A. 2009. Methods for detection and characterization of lipases: A comprehensive review. *Biotechnology Advances,* 27**,** 782-798.

HAKI, G. & RAKSHIT, S. 2003. Developments in industrially important thermostable enzymes: a review. *Bioresource technology,* 89**,** 17-34.

HEINZLE, E., REUSS, M 1987. Mass Spectrometry in Biotechnological process analysis and control. *Plenum, New York.*

HORCHANI, H., AISSA, I., OUERTANI, S., ZARAI, Z., GARGOURI, Y. & SAYARI, A. 2012. *Staphylococcal* lipases: Biotechnological applications. *Journal of Molecular Catalysis B: Enzymatic,* 76**,** 125-132.

HORNE, I., HARITOS, V. S. & OAKESHOTT, J. G. 2009. Comparative and functional genomics of lipases in holometabolous insects. *Insect biochemistry and molecular biology,* 39**,** 547-567.

HUTT AJ., C. J. 1984. The importance of stereochemistry in the clinical pharmacokinetics of the 2 arylpropionic acid nonsteroidal anti-inflammatory drugs. *Clinical Pharmacokinet,* 9**,** 3.

IKEDA, S., NIKAIDO, K., ARAKI, K., YOSHITAKE, A., KUMAGAI, H. & ISOAI, A. 2004. Production of recombinant human lysosomal acid lipase in *Schizosaccharomyces pombe*: Development of a fed-batch fermentation and purification process. *Journal of Bioscience and Bioengineering,* 98**,** 366-373.

ISHIHARA, K., SUZUKI, T., YAMANE, T. & SHIMIZU, S. 1989. Effective production of *Pseudomonas fluorescens*; lipase by semi-batch culture with turbidity-dependent automatic feeding of both olive oil and iron ion. *Applied Microbiology and Biotechnology,* 31**,** 45-48.

ITO, T., KIKUTA, H., NAGAMORI, E., HONDA, H., OGINO, H., ISHIKAWA, H. & KOBAYASHI, T. 2001. Lipase production in two-step fed-batch culture of organic solvent-tolerant *Pseudomonas aeruginosa* LST-03. *Journal of Bioscience and Bioengineering,* 91**,** 245-250.

JAEGAR K. E, D. B. W., REETZ M. T 1999. Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipases. *Annual Review of Microbiology* 53**,** 315-51.

JAEGER, K.-E. & REETZ, M. T. 1998. Microbial lipases form versatile tools for biotechnology. *Trends in Biotechnology,* 16**,** 396-403.

JANSSEN, A. E. M., SJURSNES, B. J., VAKUROV, A. V. & HALLING, P. J. 1999. Kinetics of lipase-catalyzed esterification in organic media: Correct model and solvent effects on parameters. *Enzyme and Microbial Technology,* 24**,** 463-470.

JOSEPH, B., RAMTEKE, P. W. & THOMAS, G. 2008. Cold active microbial lipases: some hot issues and recent developments. *Biotechnology Advances,* 26**,** 457-470.

JU, L. K., LEE, J. F. & ARMIGER, W. B. 1991. Enhancing oxygen transfer in bioreactors by perfluorocarbon emulsions. *Biotechnology Progress,* 7**,** 323-329.

KAMI, M., AHMAD, A. & BUSHRA, A. 2000. Studies On Minimum Agitation Speed For Liquid-Liquid Gas Dispersion In Mechanically Agitated Vessels. *Petroleum Refining and Petrochemical Based Industries in Eastern India.*, 116.

KAMIL, M., BUSHRA, A. & AHMAD, A. 2001. Minimum agitation speed for liquid–liquid–gas dispersion in mechanically agitated vessels. *Chemical Engineering and Processing: Process Intensification,* 40**,** 49-57.

KANJILAL, S., PRASAD, R. B., KAIMAL, T. N. & RAO, S. 1999. Synthesis and estimation of calorific value of a structured lipid-potential reduced calorie fat. *Lipids,* 34**,** 1045-1055.

KAZLAUSKAS, R. J. & BORNSCHEUER, U. T. 1998. Biotransformations with lipases. *Biotechnology Set***,** 37-191.

KAZLAUSKAS, R. J. & BORNSCHEUER, U. T. 2008. Biotransformations with Lipases. *Biotechnology*. Wiley-VCH Verlag GmbH.

KHEADR, E. E., VUILLEMARD, J. & EL-DEEB, S. 2003. Impact of liposome-encapsulated enzyme cocktails on cheddar cheese ripening. *Food research international,* 36**,** 241-252.

KIM, B. S. & HOU, C. T. 2006. Production of lipase by high cell density fed-batch culture of *Candida cylindracea*. *Bioprocess and Biosystem Engineering,* 29**,** 59-64.

KING, J., SNYDER, J., FRYKMAN, H. & NEESE, A. 2001. Sterol ester production using lipase-catalyzed reactions in supercritical carbon dioxide. *European Food Research and Technology,* 212**,** 566-569.

KIRAN, K. R. & DIVAKAR, S. 2001. Lipase catalyzed synthesis of organic acid esters of lactic acid in non-aqueous media. *Journal of Biotechnology,* 87**,** 109-121.

KLIBANOV, A. M. 1990. Asymmetric transformations catalyzed by enzymes in organic solvents. *Accounts of Chemical Research,* 23**,** 114-120.

KLIBANOV, A. M. 1997. Why are enzymes less active in organic solvents than in water? *Trends in Biotechnology,* 15**,** 97-101.

KOBAYASHI, T. 2011. Lipase-catalyzed syntheses of sugar esters in non-aqueous media. *Biotechnology Letters,* 33**,** 1911-1919.

KODERA, Y., SAKURAI, K., SATOH, Y., UEMURA, T., KANEDA, Y., NISHIMURA, H., HIROTO, M., MATSUSHIMA, A. & INADA, Y. 1998. Regioselective deacetylation of peracetylated monosaccharide derivatives by polyethylene glycol-modified lipase for the oligosaccharide synthesis. *Biotechnology Letters,* 20**,** 177-180.

KURTOVIC, I., MARSHALL, S. N., ZHAO, X. & SIMPSON, B. K. 2009. Lipases from mammals and fishes. *Reviews in Fisheries Science,* 17**,** 18-40.

LAGEVEEN, R. G., HUISMAN, G. W, PREUSTING, H, KETELAAR, P, EGGINK, G, WITHOLT, B. 1988. 1988. Formation of polysters by Pseudomonas oleovorans: effect of subtrates on formation and composition of poly-(R)-3-hydroxyalkanoates and poly-(R)-3-hydroxyalkenoates. *Applied and Environmental Microbiology,* 54**,** 2924-2932.

LEE, J., LEE, S. Y., PARK, S. & MIDDELBERG, A. P. J. 1999. Control of fed-batch fermentations. *Biotechnology Advances,* 17**,** 29-48.

LEE, S. Y. 1996. High cell-density culture of *Escherichia Coli*. *Trends in Biotechnology,* 14**,** 98-105.

LEE, S. Y. & CHANG, H. N. 1995. Production of poly (hydroxyalkanoic acid). *Advances in biochemical engineering/biotechnology,* 52**,** 27.

LEE, S. Y. & RHEE, J. S. 1993. Production and partial purification of a lipase from *Pseudomonas putida* 3SK. *Enzyme and Microbial Technology,* 15**,** 617-623.

LI, C.-Y., CHEN, S.-J., CHENG, C.-Y. & CHEN, T.-L. 2005. Production of *Acinetobacter radioresistens* lipase with repeated fed-batch culture. *Biochemical Engineering Journal,* 25**,** 195-199.

LI, C.-Y., CHENG, C.-Y. & CHEN, T.-L. 2004. Fed-batch production of lipase by *Acinetobacter radioresistens* using Tween 80 as the carbon source. *Biochemical Engineering Journal,* 19**,** 25-31.

LI, N. & ZONG, M.-H. 2010. Lipases from the genus *Penicillium*: Production, purification, characterization and applications. *Journal of Molecular Catalysis B: Enzymatic,* 66**,** 43-54.

LIE KEN JIE, M. & XUN, F. 1998. Studies of lipase-catalyzed esterification reactions of some acetylenic fatty acids. *Lipids,* 33**,** 71-75.

MAGHSOUDI, A., HOSSEINI, S., SHOJAOSADATI, S., VASHEGHANI-FARAHANI, E., NOSRATI, M. & BAHRAMI, A. 2012. A new methanol-feeding strategy for the improved production of β-galactosidase in high cell-density fed-batch cultures of *Pichia pastoris* Mut+ strains. *Biotechnology and Bioprocess Engineering,* 17**,** 76-83.

MALCATA, F. X., REYES, H. R., GARCIA, H. S., HILL, C. G. & AMUNDSON, C. H. 1992. Kinetics and mechanisms of reactions catalysed by immobilized lipases. *Enzyme and Microbial Technology,* 14**,** 426-446.

MENDES, A. A., OLIVEIRA, P. C. & DE CASTRO, H. F. 2012. Properties and biotechnological applications of porcine pancreatic lipase. *Journal of Molecular Catalysis B: Enzymatic,* 78**,** 119-134.

MINNING, S., SERRANO, A., FERRER, P., SOLÁ, C., SCHMID, R. D. & VALERO, F. 2001. Optimization of the high-level production of *Rhizopus oryzae* lipase in *Pichia pastoris*. *Journal of Biotechnology,* 86**,** 59-70.

MIYAZAWA, T., YUKAWA, T., UEJI, S., YANAGIHARA, R. & YAMADA, T. 1998. Resolution of 2-phenoxy-1-propanols by Pseudomonas sp. lipase-catalyzed highly enantioselective transesterification: influence of reaction conditions on the enantioselectivity toward primary alcohols. *Biotechnology Letters,* 20**,** 235-238.

MONTESINOS J.L., G. M. A., VALERO F., LAFUENTE J., SOLA C., VALDMAN B. 1996. Improvement of lipase productivity in bioprocesses using a structured mathematical model. *Journal of Biotechnology* 52**,** 207-218.

MONTESINOS, J. L., CAMPMAJO, C., IZA, J., VALERO, F., LAFUENTE, J., SOLA, C. 1994. Use of an intelligent system to monitor and control fermentation processes: Application to lipase production by *candida rugosa*. . *Process Control Quality,* 5**,** 237-244.

MORAVEJI, M. K., PASAND, M. M., DAVARNEJAD, R. & CHISTI, Y. 2012. Effects of surfactants on hydrodynamics and mass transfer in a split-cylinder airlift reactor. *The Canadian Journal of Chemical Engineering,* 90**,** 93-99.

MUKATAKA, S., KOBAYASHI, T. & TAKAHASHI, J. 1985. Kinetics of enzymatic hydrolysis of lipids in biphasic organic-aqueous systems. *Journal of fermentation technology,* 63**,** 461-466.

NAGATA, S. 1975. *Mixing: principles and applications*, Kodansha Tokyo, Japan.

NARAYANAN, N. & CHOU, C. P. 2009. Alleviation of proteolytic sensitivity to enhance recombinant lipase production in *Escherichia coli*. *Applied and Environmental Microbiology,* 75**,** 5424-5427.

O'CONNOR, G. M., SANCHEZ-RIERA, F. & COONEY, C. L. 2004. Design and evalution of control strategies for high cell density fermentations. *Biotechnology and Bioengineering,* 39**,** 293-304.

OH, G. & MOO-YOUNG, M. 1998. Automated fed-batch culture of recombinant *Saccharomyces cerevisiae* based on on-line monitored maximum substrate uptake rate. *Biochemical Engineering Journal,* 1**,** 211-217.

OLEMPSKA-BEER, Z. S., MERKER, R. I., DITTO, M. D. & DINOVI, M. J. 2006. Food-processing enzymes from recombinant microorganisms—a review. *Regulatory Toxicology and Pharmacology,* 45**,** 144-158.

OSBORN, H. & AKOH, C. 2006. Structured Lipids-Novel Fats with Medical, Nutraceutical, and Food Applications. *Comprehensive reviews in food science and food safety,* 1**,** 110-120.

PACKTER, N. M. 1994. Lipases — their structure, biochemistry and application: Edited by P Woolley and S B Petersen. pp 363. Cambridge University Press. 1994. £45. *Biochemical Education,* 22**,** 216-216.

PAINMANAKUL, P., WACHIRASAK, J., JAMNONGWONG, M. & HEBRARD, G. 2009. Theoretical Prediction of Volumetric Mass Transfer Coefficient (k_La) for Designing an Aeration Tank. *Engineering Journal,* 13**,** 13-28.

PANDEY, A. 2004. *Concise encyclopedia of bioresource technology*, CRC.

PANDEY, A., BENJAMIN, S., SOCCOL, C. R., NIGAM, P., KRIEGER, N. & SOCCOL, V. T. 1999. The realm of microbial lipases in biotechnology. *Biotechnology and Applied Biochemistry,* 29**,** 119-131.

PER, B. & KARL, H. 2000. Biocatalytic Synthesis of Enantiopure Compounds Using Lipases. *Stereoselective Biocatalysis.* CRC Press.

PÖRTNER, R. & MÄRKL, H. 1998. Dialysis cultures. *Applied Microbiology and Biotechnology,* 50**,** 403-414.

POULIOT, K., THIBAULT, J., GARNIER, A. & LEIVA, G. A. 2000. k_La evaluation during the course of fermentation using data reconciliation techniques. *Bioprocess Engineering,* 23**,** 565-573.

PSZCZOLA, D. E. 2001. Antioxidants: From preserving food quality to quality of life. *Food technology,* 55**,** 51-59.

RANTAKYLÄ, M., ALKIO, M. & AALTONEN, O. 1996. Stereospecific hydrolysis of 3-(4-methoxyphenyl)glycidic ester in supercritical carbon dioxide by immobilized lipase. *Biotechnology Letters,* 18**,** 1089-1094.

RATHI, P., SAXENA, R. & GUPTA, R. 2001. A novel alkaline lipase from *Burkholderia cepacia* for detergent formulation. *Process Biochemistry,* 37**,** 187-192.

REIS, P., HOLMBERG, K., WATZKE, H., LESER, M. & MILLER, R. 2009. Lipases at interfaces: a review. *Advances in colloid and interface science,* 147**,** 237-250.

RESINA, D., COS, O., FERRER, P. & VALERO, F. 2005. Developing high cell density fed-batch cultivation strategies for heterologous protein production in Pichia pastoris using the nitrogen source-regulated FLD1 Promoter. *Biotechnology Bioengineering,* 91**,** 760-7.

RIESENBERG, D. & GUTHKE, R. 1999. High-cell-density cultivation of microorganisms. *Applied Microbiology and Biotechnology,* 51**,** 422-430.

RIESENBERG, D., MENZEL, K., SCHULZ, V., SCHUMANN, K., VEITH, G., ZUBER, G. & KNORRE, W. A. 1990. High cell density fermentation of recombinant *Escherichia coli* expressing human interferon alpha 1. *Applied Microbiology and Biotechnology,* 34**,** 77-82.

RODRIGUES, R. C. & FERNANDEZ-LAFUENTE, R. 2010. Lipase from *Rhizomucor miehei* as a biocatalyst in fats and oils modification. *Journal of Molecular Catalysis B: Enzymatic,* 66**,** 15-32.

RUBIN, B. & DENNIS, E. A. 1997. Preface. *In:* BYRON RUBIN, E. A. D. (ed.) *Methods in Enzymology.* Academic Press.

SALAMEH, M. & WIEGEL, J. 2007. Lipases from extremophiles and potential for industrial applications. *Advances in applied microbiology,* 61**,** 253-283.

SALEHMIN M. N. I, ANNUAR M. S. M, CHISTI Y. 2013. High cell density fed-batch fermentations for lipase production: feeding strategies and oxygen transfer. *Bioprocess and Biosystems Engineering*.

SAUID, S. M. & MURTHY, V. 2010. Effect of Palm Oil on Oxygen Transfer in a Stirred Tank Bioreactor. *Journal of Applied Sciences(Faisalabad),* 10**,** 2745-2747.

SCHMIDT-DANNERT, C. 1999. Recombinant microbial lipases for biotechnological applications. *Bioorganic & medicinal chemistry,* 7**,** 2123-2130.

SHARMA, R., CHISTI, Y. & BANERJEE, U. C. 2001. Production, purification, characterization, and applications of lipases. *Biotechnology Advances,* 19**,** 627-662.

SHILOACH, J. & FASS, R. 2005. Growing *E. coli* to high cell density--A historical perspective on method development. *Biotechnology Advances,* 23**,** 345-357.

SHOJAOSADATI, S. A., MARJAN, S., KOLAEI, V., & FARNOUD, A. M. 2008. Recent advances in high cell density cultivation for production of recombinant protein. *Iran J Biotechnol,* 6**,** 63-84.

SHU, Z.-Y., JIANG, H., LIN, R.-F., JIANG, Y.-M., LIN, L. & HUANG, J.-Z. 2010. Technical methods to improve yield, activity and stability in the development of microbial lipases. *Journal of Molecular Catalysis B: Enzymatic,* 62**,** 1-8.

SINGH, A. K. & MUKHOPADHYAY, M. 2012a. Overview of fungal lipase: a review. *Applied biochemistry and biotechnology***,** 1-35.

SINGH, A. K. & MUKHOPADHYAY, M. 2012b. Overview of fungal lipase: a review. *Applied biochemistry and biotechnology,* 166**,** 486-520.

SINGH M, SINGH S, SINGH R. S, CHISTI Y., BANERJEE U. C. 2008. Transesterification of primary and secondary alcohols using *Pseudomonas aeruginosa* lipase. *Bioresource Technology* 99:2116-2120.

SOKOLOVSKA, I., ALBASI, C., RIBA, J.-P. & BÁLEŠ, V. 1998. Production of extracellular lipase by *Candida cylindracea* CBS 6330. *Bioprocess and Biosystems Engineering,* 19**,** 179-186.

SOUMANOU M. M, PERIGNON M., VILLENEUVE P. 2013. Lipase-catalyzed interesterification reactions for human milk fat substitutes production: A review. *European Journal of Lipid Science and Technology* 115:270-285.

SONI, K. & MADAMWAR, D. 2001. Ester synthesis by lipase immobilized on silica and microemulsion based organogels (MBGs). *Process Biochemistry,* 36**,** 607-611.

SPERANZA P., MACEDO G. A. 2012. Lipase-mediated production of specific lipids with improved biological and physicochemical properties. *Process Biochemistry* 47:1699-1706.

STRATTON J., C. V., MEAGHER M. 1998. High Cell-Density Fermentation. *Methods in Molecular Biology,* Volume 103**,** 107-120.

SURRIBAS, A., STAHN, R., MONTESINOS, J. L., ENFORS, S.-O., VALERO, F. & JAHIC, M. 2007. Production of a *Rhizopus oryzae* lipase from *Pichia pastoris* using alternative operational strategies. *Journal of Biotechnology,* 130**,** 291-299.

SUZUKI, T., MUSHIGA, Y., YAMANE, T., SHIMIZU, S. 1988. Mass production of lipase by fed-batch culture of Pseudomonas fluorescens. *Applied Microbiology And Biotechnology,* 27**,** 417-422.

SUZUKI, T., YAMANE, T. & SHIMIZU, S. 1986. Mass production of poly-β-hydroxybutyric acid by fed-batch culture with controlled carbon/nitrogen feeding. *Applied Microbiology and Biotechnology,* 24**,** 370-374.

SUZUKI, T., YAMANE, T. & SHIMIZU, S. 1987. Mass production of thiostrepton by fed-batch culture of *Streptomyces laurentii* with pH-stat modal feeding of multi-substrate. *Applied Microbiology and Biotechnology,* 25**,** 526-531.

SUZUKI, T., YAMANE, T. & SHIMIZU, S. 1990. Phenomenological Background and Some Preliminary Trials of Automated Substrate Supply in pH-Stat Modal Fed-Batch Culture Using a Setpoint of High Limit. *Journal of Fermentation and Bioengineering,* 69**,** 292-297.

TAMERLER, C. & KESHAVARZ, T. 2000. Lipolytic enzyme production in batch and fed-batch cultures of Ophiostoma piceae and Fusarium oxysporum. *Journal of Chemical Technology & Biotechnology,* 75**,** 785-790.

TANG, S. J., SUN, K. H., SUN, G. H., CHANG, T. Y. & LEE, G. C. 2000. Recombinant Expression of the *Candida rugosa* lip4 Lipase in *Escherichia coli*. *Protein expression and purification,* 20**,** 308-313.

THERISOD, M. & KLIBANOV, A. M. 1987. Regioselective acylation of secondary hydroxyl groups in sugars catalyzed by lipases in organic solvents. *Journal of the American Chemical Society,* 109**,** 3977-3981.

TREICHEL, H., DE OLIVEIRA, D., MAZUTTI, M., DI LUCCIO, M. & OLIVEIRA, J. 2010. A Review on Microbial Lipases Production. *Food and Bioprocess Technology,* 3**,** 182-196.

TSAI, S. W. & DORDICK, J. S. 1996. Extraordinary enantiospecificity of lipase catalysis in organic media induced by purification and catalyst engineering. *Biotechnology Bioengineer,* 52**,** 296-300.

TURNER, C., PERSSON, M., MATHIASSON, L., ADLERCREUTZ, P. & KING, J. W. 2001. Lipase-catalyzed reactions in organic and supercritical solvents: application to fat-soluble vitamin determination in milk powder and infant formula. *Enzyme and Microbial Technology*, 29**,** 111-121.

VALERO, F., POCH, M., SOLA, C., SANTOS-LAPA, R.A., LIMA. J.L., 1991. On line monitoring of lipase production in fermentation processes. *Biotechnol. Tech,* 5**,** 251-254.

VAN DYCK, S. M. O., LEMIÈRE, G. L. F., JONCKERS, T. H. M., DOMMISSE, R., PIETERS, L. & BUSS, V. 2001. Kinetic resolution of a dihydrobenzofuran-type neolignan by lipase-catalysed acetylation. *Tetrahedron: Asymmetry,* 12**,** 785-789.

VAYSSE, L., DUBREUCQ, E., PIRAT, J.-L. & GALZY, P. 1997. Fatty hydroxamic acid biosynthesis in aqueous medium in the presence of the lipase-acyltransferase from *Candida parapsilosis*. *Journal of Biotechnology,* 53**,** 41-46.

VERMA, M., CHAUHAN, G. & KANWAR, S. 2008. Enzymatic synthesis of isopropyl myristate using immobilized lipase from *Bacillus cereus* MTCC 8372. *Acta microbiologica et immunologica hungarica,* 55**,** 327-342.

VOISARD, D., MEUWLY, F., RUFFIEUX, P. A., BAER, G. & KADOURI, A. 2003. Potential of cell retention techniques for large-scale high-density perfusion culture of suspended mammalian cells. *Biotechnology and Bioengineering,* 82**,** 751-765.

VON SCHLIEN, R., FAGERVICK, K., SAXEN, B., RINGBOM, K., RYDSTROM, M 1995. Adaptive on-line model for aerobic *Saccharomyces cerevisiae* fermentation. *Biotechnology and Bioengineering,* 48**,** 631-638.

VULFSON, E. 1994. Industrial applications of lipases. *Lipases—their structure, biochemistry and application. Cambridge University Press, Cambridge, England***,** 271-288.

WEBER, N., KLEIN, E. & MUKHERJEE, K. D. 1999. Long-chain acyl thioesters prepared by solvent-free thioesterification and transthioesterification catalysed by microbial lipases. *Applied Microbiology and Biotechnology,* 51**,** 401-404.

XIN, J.-Y., LI, S.-B., XU, Y., CHUI, J.-R. & XIA, C.-G. 2001. Dynamic enzymatic resolution of Naproxen methyl ester in a membrane bioreactor. *Journal of Chemical Technology & Biotechnology,* 76**,** 579-585.

YAMANÈ, T. & SHIMIZU, S. 1984a. Fed-batch techniques in microbial processes. *Bioprocess Parameter Control.* Springer Berlin / Heidelberg.

YAMANÈ, T. & SHIMIZU, S. 1984b. Fed-batch techniques in microbial processes. *Bioprocess parameter control***,** 147-194.

YANG, Y., YU, Y., ZHANG, Y., LIU, C., SHI, W. & LI, Q. 2011. Lipase/esterase-catalyzed ring-opening polymerization: A green polyester synthesis technique. *Process Biochemistry,* 46**,** 1900-1908.

YARI, K., FATEMI, S. S.-A. & TAVALLAEI, M. 2012. High level expression of recombinant BoNT/A-Hc by high cell density cultivation of *Escherichia coli*. *Bioprocess and Biosystems Engineering,* 35**,** 407-414.

YEE, L. & BLANCH, H. 1992. Recombinant protein expression in high cell density fed-batch cultures of *Escherichia coli*. *Nature Biotechnology,* 10**,** 1550-1556.

YEO, S. H., NIHIRA, T. & YAMADA, Y. 1998. Screening and identification of a novel lipase from Burkholderia sp. YY62 which hydrolyzes t-butyl esters effectively. *Journal of General Applied Microbiology,* 44**,** 147-152.

YIN, C.-H., GAO, M. & LIU, J.-F. 2009. Study of enzymatic synthesis of isopropyl myristate with immobilized lipase. *China Surfactant Detergent & Cosmetics,* 6**,** 009.

ZHAO, W., WANG, J., DENG, R. & WANG, X. 2008. Scale-up fermentation of recombinant *Candida rugosa* lipase expressed in *Pichia pastoris* using the GAP promoter. *Journal of Industrial Microbiology & Biotechnology,* 35**,** 189-195.

LIST OF PUBLICATIONS

1. SALEHMIN M. N. I., ANNUAR M. S. M., & CHISTI Y., 2013. High cell density fed-batch fermentations for lipase production: feeding strategies and oxygen transfer. Bioprocess and Biosystems Engineering:1-17.
2. SALEHMIN M. N. I., ANNUAR, M. S. M. & CHISTI Y. 2014. High cell density fed-batch fermentation for the production of a microbial lipase. *Biochemical Engineering Journal*. 85, 8-14.
3. SALEHMIN M. N. I., ANNUAR M. S. M., ILHAM Z., 2013. **Gas-liquid and liquid-liquid mass transfers in yeast high cell density fermentation (under peer-reviewed by ISI Journal).**