MICROBIOREACTOR SYSTEM WITH INTEGRATED MIXING SCHEME, TEMPERATURE CONTROL AND OPTICAL DENSITY MEASUREMENT FOR FERMENTATION AND BIOCATALYSIS EXPERIMENTS.

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Alhamdulillah, thank GOD for His blessing that had made this project a blissful success

Especially dedicated to my lovely family, my Supervisor, and friends….

"Thanks for All Support and Encouragement"

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ABSTRACT

The objective of this project is to develop an online microbioreactor system with integrated mixing scheme, temperature control and optical density measurement for fermentation experiment. There are few methods of fabrication used in prototyping microbioreactor designed by using Inventor^{TM} software such as micromachining, soft lithography casting and 3D printing. The proposed microbioreactor platform has a working volume of 500-1500 μL and was fabricated from poly(methylmethacrylate) (PMMA) and poly(dimethylsiloxane) (PDMS) polymers. The reactor is equipped with independent on/off temperature controller and proportional-integral (PI) agitation rate controller. Furthermore, Beer's Lambert law was applied in on line optical density measurement at 600 nm with the use of fiber optics. Three different microbioreactors i.e. MBRv 1, MBRv 2 and MBRv 3 were fabricated to solve step by step of various technical aspects. Process control and automation were programmed by using LabVIEW^{TM} software (National Instruments) and implemented by using a data acquisition card (DAQ) for signal transmission. Experimental works were performed to evaluate the workability of each of the main reactor features such as (1) assessment of the measurement and control performance (mixing, temperature and optical density), (2) mixing quality and evaporation test and (3) proof-of-concept via starch hydrolysis and yeast fermentation to demonstrate the workability of the microbioreactor. Enzyme to substrate, *E/S* ratio, reaction temperature and stirring speed were varied to observe the impact of these reactor variables on the starch hydrolysis process. Results attained includes two main aspects i.e. enzyme catalysed reactions has been successfully performed using the microbioreactor and a low standard deviation (averaging at $\pm$ 0.03 mg·mL^{-1}) showed that all experiments were repeatable with error less than 5% of the mean value. In *S. cerevisae* fermentation, the microbioreactor ran stably for the entire length of operation which was nearly 40 hours with very minimal volume loss i.e. about 2.8 μL·hr^{-1} at 37^{o}C in every batch. The microbioreactor has the maximum oxygen transfer rate (OTR_{max}) of 16.6 mmol·L^{-1}·h^{-1} under the agitation rate of 300 rpm. Cell specific growth rate as high as 0.291 hr^{-1} was obtained in this condition. The experimental data in the microbioreactor operation was also reproducible in shake flask and bioreactor where comparable growth profiles were attained under a similar mixing time. All in all, it is anticipated that the microbioreactor fabricated in this project would be a potential substitute for shake flasks and/or microtiter plate as experimental tool to facilitate a high throughput bioprocessing experimental work.

ABSTRAK

Objektif projek ini adalah untuk membangunkan sistem mikrobioreaktor atas talian dengan menggabungkan skim pengadukan, kawalan suhu dan pengukuran ketumpatan optik untuk eksperimen fermentasi. Terdapat beberapa kaedah pembuatan yang digunakan dalam mikrobioreaktor yang direka dengan menggunakan perisian Inventor^{TM} iaitu pemesinan mikro, acuan litografi lembut dan percetakan 3D. Platform bagi mikrobioreaktor yang dicadangkan mempunyai isipadu kerja 500-1500 µL dan diperbuat daripada polimer poly(methylmethacrylate) (PMMA) dan poly(dimethylsiloxane) (PDMS). Reaktor ini dilengkapi dengan pengawal suhu buka/tutup dan pengawal kadar pengadukan berkadar-kamiran (PI). Tambahan pula, prinsip Beer Lambert diguna pakai pada pengukuran ketumpatan optik atas talian dan diperoleh dari pemancaran cahaya boleh dilihat pada 600 nm dengan menggunakan gentian optik. Tiga mikrobioreaktor berlainan iaitu MBRv 1, MBRv 2 and MBRv 3 telah difabrikasi untuk menyelesaikan pelbagai aspek teknikal langkah demi langkah. Proses kawalan dan automasi telah diprogramkan dengan menggunakan perisian LabVIEW^{TM} (National Instruments) dan dilaksanakan dengan menggunakan peranti pemerolehan data (DAQ) untuk penghantaran isyarat. Kerja-kerja ujikaji telah dijalankan untuk menilai kebolehkerjaan setiap ciri-ciri utama reaktor seperti (1) penilaian pengukuran dan prestasi kawalan (pengadukan, suhu dan ketumpatan optik), (2) kualiti pengadukan dan ujian penyejatan dan (3) bukti dari konsep melalui hidrolisis kanji dan penapaian yis untuk menunjukkan kebolehkerjaan mikrobioreaktor. Nisbah enzim kepada substrat, E/S, suhu tindak balas dan kelajuan pengaduk adalah berbeza-beza untuk memperhatikan kesan pemboleh ubah reaktor ke atas proses hydrolisis kanji. Keputusan yang diperolehi merangkumi dua aspek utama iaitu tindak balas kos rendah yang bermangkinkan enzim telah berjaya dijalankan menggunakan mikrobioreaktor dan sisihan piawai yang rendah (dengan purata ±0.03 $mg \cdot mL^{-1}$) menunjukkan bahawa semua eksperimen boleh diulangi dengan ralat kurang dari 5 % daripada nilai min. Dalam penapaian *S. cerevisae*, mikrobioreaktor berfungsi dengan stabil sepanjang hampir 40 jam operasi dengan kehilangan isipadu yang sangat minimum iaitu kira-kira 2.8 $\mu L \cdot hr^{-1}$ pada $37^{o}C$ dalam setiap kelompok. Mikrobioreaktor ini mempunyai kadar maksimum pemindahan oksigen (OTR_{max}) 16.6 $mmol \cdot L^{-1} \cdot h^{-1}$ di bawah kadar kelajuan pengaduk 300 rpm. Dalam keadaan ini, kadar spesifik pertumbuhan sel yang tinggi iaitu 0.291 hr^{-1} telah diperolehi. Data eksperimen dalam operasi mikrobioreaktor juga boleh diulang semula dalam kelalang goncang dan bioreaktor di mana profil perkembangan yang setara telah diperolehi di bawah masa adukan yang sama. Secara keseluruhannya, mikrobioreaktor yang telah direka dalam projek ini adalah dijangkakan akan menjadi pengganti berpotensi bagi kelalang goncang dan/atau plat mikrotiter sebagai alat eksperimen untuk memudahkan kerja-kerja eksperimen bioproses dengan murah.

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LIST OF ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
AC	Alternate current
atm	Atmosphere
CAD	Computer aided design
CNC	Computer numerical control
CO_2	Carbon Dioxide
Co^{2+}	Cobalt (II) ion
Cu^{2+}	Copper (II) ion
DAQ	Data Acquisition Card
DC	Direct current
D_i	Impeller diameter
DO	Dissolved oxygen
D_t	Vessel diameter
dV_r/dt	Volume change over time ($L.hr^{-1}$)
EC	Enzyme comission
E/S	Enzyme over substrate
Fe^{2+}	Iron (II) ion
h	height
H_i	Impeller height
HPLC	High Performance Liquid Chromatography
H_t	Vessel height

kcal	Kilocalories
$K_L a$	Volumetric mass transfer coefficient
LED	Light emitting diode
L_i	Impeller blade length
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic
DP	Degree of polymerization
M3	3 mm screw thread diameter
M2.5	2.5 mm screw thread diameter
MBRv	Microbioreactor version
meas.	Measurement
Mn^{2+}	Manganese (II) ion
N_2	Nitrogen
Ne'	Dimensionless power number
N_m	Measured agitation
N_{Re}	Reynold's number
N_{sp}	Set point agitation
O_2	Oxygen
OD	Optical density
OD_{600nm}	Optical density at 600 nm
OTR	Oxygen transfer rate
OTR_{max}	Maximum Oxygen transfer rate
OUR	Oxygen uptake rate
PCR	Polymerase chain reaction
PDMS	Polydimethylsiloxane

pH	Potential of Hyrogen ion
pH_o	Initial pH
pH_F	Final pH
P_i	Power input
PI	Proportional Integral
P_i/V_L	Volumetric power consumption
PMMA	Polymethylmethacrylate
Pt	Platinum
PVC	Polyvinylchloride
r	radius
RNA	Ribonucleic acid
subVI	Subsection of virtual instrument
S/V	Surface over volume
t_{90}	response time less than 90 seconds
T_m	Set point temperature
T_{sp}	Set point temperature
UV	Ultra violet
v/v	Volume per volume
w/v	Weight per volume
W_i	Impeller width

LIST OF SYMBOLS

Δ	Spacing between
Ø	Diameter
α/ β	Alpha or beta conformation of covalent bond
Ω	Ohm
λ	Path length
μ	Specific growth rate
$\mu L.hr^{-1}$	microliter per hour
Hz	Hertz
$Lmol^{-1}cm^{-1}$	Molar absorbtivity
M	Molarity ($molL^{-1}$)
mm Hg	milimeter of mercury
$mg \cdot L^{-1}$	miligram per Liter
MPa	Megapascal
N	Normality
Pa·s	Pascal second
rpm	Rotation per minute
$[S]_o$	Initial substrate concentration
Tg	Glass transition temperature (^oC)
U/g	Units of activity per gram

V	Voltages
W	Watt
$W.m^{-2}$	Watt per meter square

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CHAPTER 1

INTRODUCTION

1.1 Research Background

Fermentation process can be described as the production of specific bioproducts such as biomass, enzyme or metabolite with the use of living cell culture e.g. bacteria, yeast, fungi and other microbial life that utilizes carbon containing compounds (El-Mansi and Bryce, 2007). Generally, fermentation processes is performed under a controlled environment in which process temperature, pH and dissolved oxygen (DO) concentration level are often the controlled variables (Shuler and Kargi, 2002). A huge numbers of fermentation experiments are usually needed for fermentation process development. Shake flasks (or microtiter plates) and bench scale bioreactors are often used as an experimental tool to carry out these fermentation experiments in the lab scale. Shake flasks can easily operated in parallel at low volumes e.g. 66 flasks with 125 mL each in a typical two deck shaking incubator. It also possible to work at much lower volume i.e. down to 21.3 mL for 125 mL Erlenmeyer flask (Corning Inc, Tewksbury MA, USA) Recently, Sartorius AG Germany presents the SENSOLUX® tray, stand-alone version DCS09 shake flask that has been integrated with pH and dissolved oxygen optical sensors. This allows for the obtainment of online readings for the pH and DO level inside the flask under shaking conditions.

Bioreactors on contrary, offers a better parameters control i.e. compared to shake flaks (or microtiter plates). For example dissolved oxygen level, pH and temperature can all be tightly controlled during experimentation. Furthermore, bioreactor supports batch, continuous batch and fed batch mode of fermentation processes. However, a low numbers of experiment can be performed in a single run a typical 1.5 L lab scale bioreactor and consumes relatively large volume of medium (~ 0.5-1.5 L). This limits its usefulness for fermentation process development. In addition, conducting experiments with bench scale bioreactors is laborious due to the efforts required for preparation and cleaning of the reactor (Shuler and Kargi, 2002). It is also important to note here that both operations (i.e. shake flasks and bioreactors) require frequent sampling which increases risks of cells contamination. Clearly, there is a demand for an improved experimental tool for cells cultivation that could overcome these limitations.

Microbioreactor is a miniaturize scale bioreactor which is normally integrated with sensor for real time data, utilize polymer base (PMMA, PDMS and etc), disposable and no sampling possiblity due to very small working volume (Maharbiz *et al.*, 2004; Zanzotto *et al.*, 2004; Zhang *et al.*, 2006; Zainal Alam *et al.*, 2010; Schäpper *et al.*, 2010). It has been receiving increasing attention as it posses most general features of typical bench-scale bioreactor and retain the cost reducing advantages similarly to shake flasks or microtiter plate (Szita *et al.*, 2005). Designing such a microbioreactor system however, requires one to evaluate various design specifications. This include choice of operation, reactor operating feature and size, reactor mechanics, reactor fluidics, process control of physical parameters and detection methods for measuring the product concentration (Schäpper *et al.*,2009).

1.2 Statement of Problem

At laboratory scale, optimization of fermentation processes are generally performed using shake flasks and the bench-scale bioreactors. In the early fermentation process development, microbiologists often rely on parallel experimentation in shaken flasks to determine optimal medium composition and/or to screen for the suitable microbial strain (Betts and Baganz, 2006). In a more advance stage, where it is necessary to quantify important engineering parameters e.g. the oxygen transfer coefficient, k_La, volumetric power consumption, P_i/V_L, etc. and/or to assess the impact of fundamental engineering aspects such as mixing, heat transfer rate, etc. on the reactions, bench scale bioreactors are usually utilized (Stanbury *et al.*, 1999).

In general, shake flasks operations are much easier to set-up and they require low volumes of culture medium (~50-200 mL). Medium consumptions can be further reduced with the use of microtitre plates i.e. a miniature size shaken reactor with working volumes of few hundreds microlitre (100 - 200 µL) (Stanbury *et al.*, 1999). However, with this approach, experiments are normally carried out in batch mode and control of process parameters under shaking conditions is rather difficult. Bioreactor, on the other hand is a more sophisticated experimental tool. Culture conditions can be precisely controlled and it can be operated with a working volume as low as 180 mL ("Multifors 2" bioreactor system, Infors, Switzerland). Nevertheless, performing multiple fermentation experiments at few hundreds of millilitre per run is still relatively costly. Furthermore, extra laboratory work is usually needed for preparation and cleaning of the bioreactor (Schäpper *et al.*, 2009). In this respect, it is believed that microbioreactor platform could offer an attractive solution for this problem. Such a microbioreactor platform would inherit all the advantages of bioreactor system whilst maintaining the low cost operation of shake flask and/or microtiter plates.

1.3 Research Objectives

The aim of this study is to develop a microbioreactor system with integrated mixing scheme, temperature control and optical density measurement for fermentation and biocatalysis experiments.

1.4 Scope of Research

The followings are the scope of work for the project:

I. Design of the microbioreactor for fabrication was done using the Autodesk inventor™ engineering drawing software.
II. Fabrication of microbioreactor with working volume between 500 to 1500 µL.
III. Polymer substrates namely Polymethylmethacrylate (PMMA) and polydimethylsiloxane (PDMS) polymers were used as materials for fabrication.
IV. Mixing was provided by micro stirrer and agitation rate was controlled via PI controller algorithm.
V. Temperature was measured via miniature Pt 100 sensor. The heating element was implemented by using microheater and resistance wire.
VI. The microbioreactor was integrated with optical fibers for online measurement of cells optical density..
VII. Evaporation issue in micro scale operation was investigated and a suitable solution was proposed.
VIII. Aeration was introduced by pumping a humidified air to the surface of the reactor.

IX. A simple docking station for the microbioreactor was developed to integrate measurement and control of the microbioreactor. Additionally, the platform also supports the fluidics connection established.

X. Programs for measurement and control was written by using LabVIEW^{TM} software (National Instruments) and executed by using Natioal Instruments data acquisition card (DAQ) for signals transmission.

XI. Several tests were performed to evaluate the performance of microbioreactor system. These include assessment of the reliability of the mixing scheme and temperature control based on the accuracy, response time and settling time. Additionally, mixing test was performed to determine the mixing time by using fluorescent dye method.

XII. Three versions of microbioreactor were developed for various reasons; (1) MBRv 1 was to evaluate the mixing scheme and temperature control implemented, (2) MBRv 2 was to investigate the evaporation issue and the reactor capacity to facilitate biocatalysis process in batch mode, and (3) MBRv 3 was to evaluate the optical density measurement and to evaluate the reactor capacity to perform fermentation experiments in batch mode.

XIII. Starch hydrolysis and fermentation experiments were carried out to demonstrate the data comparability to the present fermentation instrument i.e. shake flasks and bench scale bioreactor. Moreover, sulphite method were carried out in microbioreactor, shake flasks and benchscale bioreactor to determine the maximum oxygen transfer rate.

1.5 Significance of Research

Microbioreactors can indeed be very useful for preliminary and advance studies on bioprocessing. Microbioreactor is simply a very small bioreactor system with working volume of less than 1 millilitre. Often, these types of reactors are made of polymers, and integrated with one or more microfluidic components to support the reactor operation (Szita *et al.,* 2005; Zhang *et al.,* 2006; Lee *et al.,* 2006; Schäpper *et*

al., 2010; Edlich *et al.,* 2010). Miniature size polymer-based reactors are disposable and thus, eliminate the need for cleaning of the reactor (Szita *et al.,* 2005; Zhang *et al.,* 2006; Lee *et al.,* 2006; Schäpper *et al.,* 2010; Edlich *et al.,* 2010). Moreover, non-invasive and disposable sensors can be integrated into such a microbioreactor platform for monitoring of essential fermentation variables e.g. temperature, pH, cell density and DO level (Schäpper *et al.,* 2009). The capacity of microbioreactors to facilitate fermentation experiments have been demonstrated in various lab scale cultivations especially using *S. cerevisae* (Szita *et al.,* 2005; Zhang *et al.,* 2006; Lee *et al.,* 2006) and *E. coli* microbial strains (Schäpper *et al.,* 2010; Edlich *et al.,* 2010). Development of microbioreactor has even evolved to a phase where results in bench scale bioreactor can be reproduced with microbioreactors (Zhang *et al.,* 2006; Lee *et al.,* 2006; Schäpper *et al.,* 2010; Edlich *et al.,* 2010).

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