DESIGN AND PERFORMANCE OF A MILLILITER RANGE BIOREACTOR PROTOTYPE FOR BIOPROCESS OPERATION

NOR AZYATI BINTI ABDUL MUTTALIB

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School of Chemical and Energy Engineering
Faculty of Engineering
Universiti Teknologi Malaysia

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Dedicated specially to my parents, family and friends...

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ABSTRACT

This study aimed to design a mililiter range bioreactor (MRB) prototype and evaluate the performance of this MRB using lactose (milk) hydrolysis enzymatic reaction. A scaling down approach was used in performing biocatalysis experiments using immobilized enzymes in a stirred MRB system, due to uneven distribution in packed bed reactor and cost effective way in enzyme application. Poly-methyl methacrylate polymer was utilized as raw material for fabrication of MRB vessel. Impellers were designed using computer-aided design software and fabricated using 3D printer. Online monitoring system of MRB was set-up via LabVIEW software. The MRB was integrated with agitation motor, heating element, inlet and outlet control system. The feasibility of MRB was evaluated through lactose hydrolysis reaction using immobilized β-galactosidase. The enzyme was immobilized on alginate beads and stirred in MRB system with a working volume between 12 to15 mL. The effects of temperature (27°C and 40°C), agitation speed (150, 250 and 300 rpm) and different types of impellers (T-shape, five-bladed turbine, paddle and edge beater blade impeller) on the glucose yield and rate of reaction were investigated to obtain an optimum lactose hydrolysis. The rate of reaction was calculated by measuring glucose production throughout the reaction. The sample was analyzed using glucose analyzer and high performance liquid chromatography. Performance of MRB was benchmarkedwith a bench-top stirred tank bioreactor (STR) system (volume of 250-450 mL). The bench-top STR used different types of impeller namely pitch blade turbine, Rushton turbine, marine propeller and pitch paddle. Kinetics study of the lactose hydrolysis was performed using Michaelis Menten model while kinetic adsorption model was used for immobilized β-galactosidase. Results showed that MRB with T-shape impeller system at temperature of 40°C and agitation speed of 150 rpm under batch operating mode was the best condition in achieving high yield of glucose. The rate of reaction increased about 25% w/v as the agitation speed increased from 150 rpm to 250 rpm. At constant agitation speed (250 rpm), rate of reaction increased double from 27°C to 40°C. MRB with T-shape impeller at 40°C and250 rpm was the best condition that resulted in the highest enzymatic rate of reaction of 0.23±0.03 mg/min. The result obtained showed that MRB can be utilized for lactose hydrolysis with 6% w/v more glucose production compared to bench-top STR. The kinetic adsorption models showed that all the samples were following Pseudo second order model. The Michaelis–Menten constants K_m and V_m for the immobilized enzyme have been determined at 0.07 mM and 3.22 mol ONP min^{-1} mg^{-1} enzyme, respectively. From this study, it can be concluded that the MRB has improved liquid-phase mass transfer and it is feasible to be used for lactose hydrolysis using stirred immobilized-enzyme beads system.

ABSTRAK

Kajian ini bertujuan untuk mereka bentuk prototaip bioreaktor berskala mililiter (MRB) dan menilai prestasi MRB menggunakan tindak balas enzimatik hidrolisis laktosa. Pendekatan penskalaan menurun telah digunakan dalam menjalankan eksperimen pemangkin-bio menggunakan enzim tidak bergerak dalam MRB yang diaduk untuk mengatasi pengagihan tidak sekata dalam reaktor turus terpadat dan kos enzim yang berkesan dalam peggunaan enzim. Polimer poli-metil metaakrilat digunakan sebagai bahan mentah untuk merekabentuk badan MRB. Pengaduk telah direka menggunakan perisian berbantu komputer dan direkabentuk menggunakan pencetak 3D dan sistem pemantauan dalam talian MRB telah dibuat menggunakan perisian LabVIEW. MRB telah dilengkapi dengan motor pengaduk, elemen pemanasan, sistem kawalan keluar dan masuk. Keberkesanan MRB telah dinilai melalui tindak balas hidrolisis laktosa menggunakan β-galactosidase yang tidak bergerak. Enzim-enzim telah dijerap pada manik alginat dan diaduk dalam MRB dengan isipadu bekerja antara 12 hingga 15 mL. Kesan suhu (27 °C dan 40 °C), kelajuan adukan (150, 250 dan 300 rpm) dan jenis pengaduk berbeza (bentuk T, lima bilah turbin, pendayung dan bilah pengaduk pemukul tepi) dalam penghasilan glukosa dan kadar tindak balas disiasat untuk mendapatkan hidrolisis laktosa optimum. Kadar tindak balas dikira dengan mengukur penghasilan glukosa sepanjang tindak balas.Sampel telah diuji menggunakan penganalisis glukosa dan kromatografi cecair prestasi tinggi. Prestasi MRB telah ditanda aras dengan sistem bioreaktor tangki berpengaduk atas bangku (STR) (isipadu antara 250-450 mL). STR menggunakan pelbagai jenis pengaduk iaitu turbin bilah pitch, turbin Rushton, kipas laut dan dayung pitch. Kinetik β-galactosidase dikaji dengan menggunakan model Michaelis Menten manakala model kinetik enzim pula menggunakan model penjerapan kinetik. Keputusan menunjukkan MRB dengan sistem pengaduk bentuk-T pada suhu 40°C dan kelajuan pengaduk 150 rpm di bawah mod operasi kelompok adalah keadaan terbaik dalam mencapai hasil glukosa yang tinggi. Kadar tindak balas meningkat kira-kira 25% w/v apabila kelajuan adukan meningkat dari 150 rpm hingga 250 rpm. Pada kelajuan adukan malar (250 rpm), kadar tindak balas meningkat dua kali ganda dari suhu 27°C hingga 40°C. MRB dengan pengaduk bentuk-T pada suhu 40°C dan 250 rpm adalah keadaan terbaik yang menghasilkan kadar tindak balas enzim tertinggi iaitu 0.23 ± 0.03 mg/min. Keputusan menunjukkan bahawa MRB boleh digunakan untuk hidrolisis laktosa dengan pengeluaran glukosa sebanyak 6% w/v lebih banyak berbanding dengan STR. Model kinetik menunjukkan bahawa semua sampel mengikuti model kinetik Pseudo tertib kedua. Pemalar Michaelis–Menten K_m and V_m untuk enzim tidak bergerak ditentukan masing-masing adalah pada 0.07 mM dan 3.22 mol ONP min^{-1} mg^{-1} enzim. Dari kajian ini dapat disimpulkan bahawa MRB meningkatkan pemindahan jisim fasa cecair dan ia boleh digunakan dalam hidrolisis laktosa menggunakan sistem manik-enzim yang tidak bergerak.

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LIST OF ABBREVIATION

MRB	-	mililitre range bioreactor
MB	-	miniature bioreactor
PBR	-	packed bed bioreactor
ABS	-	acrylonitrile butadiene styrene
3D	-	three dimension
CAD	-	computer aided design
STR	-	stirred tank bioreactor
MTZ	-	mass transfer zone
PEEK	-	poly(ether ether ketone)
DOT	-	dissolved oxygen
OD	-	optical density
CFD	-	computational fluid dynamic
MBR	-	miniaturebioreactor
MSBR	-	miniature stirrer tank bioreactor
MTP	-	microtiter plates
PMMA	-	poly-methylmethacrylate
PID	-	Proportional-Intergral-Derivative
UV	-	ultra violet
N_p	-	power number
P	-	power input
N	-	agitation rate

Re	-	Re
V	-	volume
D	-	diameter
BOD	-	biological oxygen demand
GOS	-	glucose
ONP	-	o-nitrophenol
HPLC	-	High performance liquid chromatography
PVDF	-	Polyvinylidene fluoride
Ca-Alginate	-	calcium alginate
Ca^{2+}	-	calcium ion
E. coli	-	Escherichia coli
PT100	-	platinum resistance thermometers
DC	-	direct current
C	-	center of the impeller
D	-	diameter of the impeller
W	-	width of the impeller
D_t	-	diameter of the bioreactor
H	-	height of bioreactor
HD	-	high definition
USB	-	universal serial bus
MCM-41	-	comersial mesoporous
A568	-	comersial resin
GA	-	Glucoamylase
Na Y	-	sodic forms of zeolites
APG	-	aminopropyl glass
SBP	-	Soybean peroxidase

MCP	-	metal ceramic powder
IMAC	-	metal immobilized affinity chromatographic
PVDF	-	polyvinylidene fluoride
TiO_2	-	titanium dioxide
Phe	-	phenylalanine
PKU	-	phenylketonuria
CNC	-	computer numerical control
DAQ	-	data acquisition
IMA	-	immobilized metal affinity
$CaCl_2$	-	calcium chloride
$CaCO_3$	-	calcium carbonate
P	-	Product
PHB	-	Poly-hydroxybutyrate
3D	-	three-dimensional
HAC-NaAC	-	acetic acid
HCl	-	hydrochlric acid
PBS	-	phosphate buffer saline
Tris–HCl	-	Tris hydrochloride
$NaHCO_3$	-	sodium bicarbonate
Na_2CO_3	-	sodium carbonate
NaOH	-	sodium hydoxide
L	-	length
$CaCl_2$	-	calcium chloride
ANOVA	-	analysis of variance
M	-	Marine propeller
R	-	Rushton turbine

LIST OF SYMBOL

W_i	-	width of impeller, cm
Dt	-	diameter of vessel, cm
Di	-	diameter of impeller, cm
H_L	-	height of vessel, cm
Hi	-	height from bottom of bioreactor to impeller, cm
$H_{bioreactor}$	-	height of bioreactor,cm
Dp	-	particle diameter
K_M	-	Michaelis-Menten constant
t_m	-	mixing time
V	-	velocity
N	-	speed of agitation
ρ	-	density
μ	-	dynamic viscosity
λ	-	scale of turbulence
ν	-	kinematic viscosity, kg/(s·m)
P	-	power
V	-	volume
Re	-	Reynold number
t	-	time
k	-	gelation constant
η	-	intrinsic viscosity

η_{sp}	-	specific viscosity
η_{rel}	-	relativity viscosity
X	-	enzyme conversion
k_{cat}	-	turnover number
V_{max}	-	maximum rate
q	-	amount of lactose adsorbed
C_e	-	concentration of lactose
k	-	maximum adsorption capacity
T	-	temperature
R	-	gas constant
ΔH	-	enthalphy
ΔS	-	entrophy
ΔG	-	Gibb energy
k_1	-	first-order adsorption rate constant
k_2	-	second-order adsorption rate constant
k_{Th}	-	Thomas rate constant
x	-	amount of adsorbent in the column
v	-	flow rate
V_{eff}	-	effluent volume
k_{AB}	-	kinetic constant
τ	-	time in required for 50% adsorbate breakthrough
q_e	-	amounts of glucose produce at equilibrium,(mg/g)
q_t	-	amounts of glucose produce at time,(mg/g)

C_0	-	glucose concentration, (mg/L)
C_t	-	glucose concentration at time t (mg /L)
Q	-	heat transfer coefficient
λ	-	thermal conductivity,W/mK
D	-	thermal diffusivity,m^2/s

CHAPTER 1

INTRODUCTION

1.1 Research Background

Bioreactor is a device that is manufactured specifically to facilitate various types of biological processes. These include microbial fermentation (Schäpper *et al.*, 2009), enzymatic reaction (Newman *et al.*, 2013), and biodiesel (Diao *et al.*, 2008). Contrary to microbial fermentation and cultivation of animal cells, experimentation pertaining to enzymatic reactions are very straight forward to be executed using a bioreactor. Enzymatic reactions are initiated simply by the addition of enzyme cocktails into the desired substrates and it does not have to be conducted under aseptic conditions. In enzymatic reactions, substrates are normally degraded (or in a more general term–'chopped-apart') into smaller compounds by catalytic action of the enzymes. Enzymes are very specific (tendency to act on specific substrates) and its rate of reaction is highly dependent on the environment condition such as pH, temperature, enzyme-to-substrates ratio, etc. (Mendes *et al.,* 2012). The use of enzymes in large scale is however limited by their high production cost and stability. In laboratory scale, often a small quantity of enzymes are used for research work and experiments are carried out typically using a microtiter plates platform (Nunes *et al.*, 2013). Since the working volume of a microtiter plate unit is very low (normally in few hundreds microliter), the cost of enzymes needed per experiment can be significantly reduced; thus, allowing for an extensive research work at affordable cost. Despite the low running cost, translating the experimental findings from a microtiter plates platform into a larger scale operation in a bioreactor device is difficult (Kumar *et al.*, 2004a). Although, the optimal environmental factors affecting the enzymatic reactions can be duplicated for larger scale operation, but the hydrodynamics of a bioreactorare completely different compared to microtiter plate (or shake flask). Furthermore, using microtiter plates would not allow one to explore the possibility of performing the enzymatic reactions using an advanced bioprocessing approach such as membrane systems or column packed with immobilized-enzymes.

In recent years, there have been a growing interest in the development of a miniature bioreactor system to facilitate biocatalyst processes (enzymatic reactions) (Kloke *et al.*, 2010). Miniature bioreactors are technically a direct copy of a classical bioreactor system. Literature shows that the working volume of a miniature bioreactor system (or a milliliter range bioreactor, MRB) is typically between 5 and 20 milliliters. The size of MRB is at least ten-fold larger than a microbioreactor system but still much smaller than a shake flask unit (50 mL). MRB system can be integrated with various sensors and actuators analogy to a standard bioreactor operation. This feature brings benefit to many researchers as a variety of biological experiments can be carried out inexpensively. Owing to MRB low running cost and small volumes, a high throughput experimental data can be obtained and most importantly, it offers the possibility for a direct scale-up to a larger scale bioreactor operation.

In industry, enzymes are often reused or recycled to reduce the operating cost. Popular methods for recycling of enzymes is either by immobilizing the enzymes on a suitable supports or performing the enzymatic reactions using a membrane bioreactor systems (Jochems *et al.*, 2011). Both methods have pros and cons. For instance, in an immobilized enzyme system, enzymes can be reused for a number of cycles, however mass transfer issue associated with a packed column bioreactor system hinders maximum productivity (Panesar *et al.*, 2011). As for a membrane bioreactor system, concentration polarization issue will have a negative impact on product separation and hence, may inhibit overall reaction yield (Sen *et al.*, 2011).

The aim of this project was to overcome the mass transfer limitation normally encountered in a packed column immobilized enzyme bioreactor system. Enzymes was immobilized on suitable beads/supports; however, instead of packing these immobilized enzymes in a column, the enzymes was stirred homogenously using a typical stirred tank bioreactor design; similar to a free-form enzyme system. The bioreactor size was scaled down to a milliliter range volumes in order to reduce the operation cost. The miniature bioreactor system designed for the work was equipped with the necessary stirring, pumping and temperature control capacity in order to accommodate the chosen enzymatic reactions. The work also emphasised on the mixing feature of the bioreactor in achieving a good mixing for the immobilized enzymes and the potential of applying such approach in industry as compared to the typical packed column bioreactor system.

1.2 Problem Statement

A free-form enzyme system is referred to a classical method in performing enzymatic reaction where enzymes are directly mixed with the substrates. In theory, this would warrant a good rate of reaction due to high contact times. However, the enzymes applied would not be possible to be reused – less cost effective system particularly in large scale operation. Due to this reason, many opt for an immobilized enzyme bioreactor system as one of the alternatives, since enzymes are immobilized and packed within the column, allowed for a lengthy operation and offered the possibility of reusing the enzymes for several times (or cycles) as long as the enzyme activity remains reasonably high. In a packed bed bioreactor (PBR), despite the advantages, there are few issues with the immobilized enzyme bioreactor system. These include uneven distribution of feed (substrates) in the column and difficulty in achieving a uniform temperature distribution throughout the reaction. Since there is no active mixing element that is present in a PBR, uniform heat distribution within the bioreactor is rather difficult to be achieved. This may lead to an undesirable temperature gradient between the central part and the side wall of the bioreactor if a thermostated water is being circulated in the jacketed layer of the bioreactor. Preheating the substrates before feeding it into the bioreactor is another option for maintaining a desirable working temperature.

As the work is to propose a bioreactor system that could overcome a mass transfer limitation of an immobilized-enzymes system, it also has to be cost effective. In this regards, the working volume was reduced down to milliliter range. Contrary to the small scale bioreactor system such as microtiter plates and/or shake flasks, mixing in a small volume bioreactor system is analogy to a typical bioreactor operation where mixing is achieved using an impeller system. Moreover, it is also difficult to integrate online monitoring features in microtiter plates and/or shake flasks operation platform because the whole unit is under a shaking condition. This is however not the case for a miniature bioreactor system. In brief, this work aimed to overcome the mass transfer limitation of immobilized-enzyme beads system using a milliliter range bioreactor equipped with control monitoring system.

1.3 Objectives of the Research

The main objectives of this study were as follows:

1) To design a milliliter range bioreactor (MRB) prototype for improved liquid-phase mass transfer of immobilized-enzyme beads system.
2) To analyze the effects of different design of impellers, heat transfer of heating element and online monitoring system of MRB.
3) To evaluate the usefulness of the MRB in a lactose hydrolysis reaction using a stirred immobilized-enzyme (β-galactosidase) beads system.

1.4 Scope of the Research

The following scopes were performed to achieve the objectives of this study:

1. Establishment of a milliliter range bioreactor(MRB)prototype with working volume of 15 mL. The bioreactor was integrated with basic features to perform enzymatic reactions. These include temperature control, stirring and pump to facilitate continuous bioreactor operation.

a) Fabrication of the MRB prototype using a poly-methyl methacrylate (PMMA) polymer to reduce the cost of fabrication.

b) Establishment of mixing mechanism for immobilized-enzyme beads system in a stirred bioreactor using a 3D printed impeller design where Acrylonitrile butadiene styrene (ABS) was used as material for the 3D printing.

c) Design of various types of impeller namely T-shape, five-bladed turbine, paddle and edge beater blade impeller by using Computer Aided Design (CAD) software.

d) Determination of the mixing times and evaluation of the mixing patterns for T-shape, five-bladed turbine, paddle and edge beater blade impeller designs at agitation rate of 150 rpm. A concentrated fluorescence dye was used as tracer for the mixing experiments.

e) Mixing experiments in bioreactor system with larger working volume (bench-top stirred tank bioreactor (STR)) (250 mL and 400 mL) using different types of impellers namely pitch blade turbine, Rushton turbine marine propeller and pitch paddle were conducted as control.

2. Evaluation of the performance of the MRB in carrying out lactosehydrolysis using immobilized β-galactosidase. The experiments were performed using different types of impeller (T-shape, five-bladed turbine, paddle and edge beater blade), agitation speed (150 rpm and 250 rpm) and at different temperature (27 ºC and 40 ºC). The kinetic reaction of lactose hydrolysis was monitored ased on glucose production.

a) Analysis of shape and morphology of the immobilized enzyme bead using portable digital microscope before and after reaction.

b) Investigation on the enzyme kinetic with the best parameter (T-shape impeller; 250 rpm agitation speed; 40 ºC) in MRB to verify the effectiveness of the design. The analysis of lactose and glucose was performed with glucose analyzer and high performance liquid chromatography (HPLC).
c) Comparison of the MRB (15 mL) performance with bench-top STR (250 mL and 450 mL) in terms of production of glucose from lactose hydrolysis using immobilized β-galactosidase.
d) Investigation of kinetic of adsorption in immobilized enzyme using two different kinetic models i.e., i) pseudo-first order and ii) pseudo-second-order to describe adsorption in the batch bioreactor (MRB and bench-top STR). The model was used to describe the nature of adsorption between the substrate and immobilized enzyme beads.
e) Determination of β-galactosidase activity at 40ºC using enzyme assay which was measured with spectrophotometer. Kinetic of β-galactosidase was calculated with Michaelis Menten model.

1.5 Significance of the Study

The design of MRB introduces a low cost bioreactor system.It is low cost because it is made of polymers and operated with only 15mLof substrate/enzyme per experiment. It is also easy to handle and the data obtained in the MRB are readily translated to a larger scale of operation. In microtiter plate operation or shake flasks, mixing is based on shaking principle. On the contrary, in MRB, mixing scheme analogy to an industrial scale bioreactor is implemented. In this manner, the hydrodynamics of the MRB can be assumed to be at least almost similar to what usually obtained in the larger bioreactor system. The proposed miniature bioreactor design has the capacity to change the mixing mechanism using various impeller designs to best fit any reaction in mind.

Meanwhile, an enzymatic reaction in MRB used a free form immobilized beads that will provide high contact time and optimum enzymatic reaction. Immobilize enzyme in MRB mimics the conventional bioreactor and could overcome limitations found in shake flasks and microtitre plate, by maximizing the enzyme activity. Especially with the 3D printer, it gives flexibility and composite drawing in designing various types of impellers. In addition, the miniature impeller from the 3D printer can provide favourable mixing properties as compared to the conventional impeller.

As a matter of fact, MRB has been shown to be applicable to perform lactose hydrolysis of milk utilizing immobilized-enzyme beads system in an MRB. The feasibility of MRB can be applied using other biocatalystsfor other hydrolysis reactions. Moreover, online control system could provide kinetic and efficiency of the reaction by manipulating; temperature, flow rate and agitation speed. MRB can be adopted and adapted as teaching material in laboratories and preliminary study in research and development by academia.

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