UNIVERSITI SAINS MALAYSIA

Laporan Akhir Projek Penyelidikan Jangka Pendek

A Stereospecific Biotransformation of Geraniol Citronellol with the Whole-Cell Saccharomyces Cerevisiae: Dynamic Modelling and Optimisation of the Reaction System

by

Dr. Mohamad Hekarl Uzir

Assoc. Prof. Dr. Mashitah Mat Don

2012

A Stereospecific Biotransformation of Geraniol Forming Citronellol with the Whole-Cell Saccharomyces cerevisiae: Dynamic Modelling and Optimisation of the Reaction System.

SHORT TERM FINAL REPORT

by
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LAPORAN AKHIR PROJEK PENYELIDIKAN JANGKA *PENDEK*

FINAL REPORT OF SHORT TERM RESEARCH PROJECT

Sila kemukakan laporan akhir ini melalui Jawatankuasa Penyelidikan di Pusat Pengajian dan Dekan/Pengarah/Ketua Jabatan kepada Pejabat Pelantar Penyelidikan

1. **Nama Ketua Penyelidik: MOHAMAD HEKARL UZIR (PhD.)**
Name of Research Leader

☐ Profesor Madya/ *Assoc. Prof.* ■ Dr./ *Dr.* ☐ Encik/Puan/Cik *Mr/Mrs/Ms*

2. **Pusat Tanggungjawab (PTJ): SCHOOL OF CHEMICAL ENGINEERING**
School/Department

3. **Nama Penyelidik Bersama: PROF. MADYA DR. MASHITAH MAT DON**

4. **Tajuk Projek:**
Title of Project **A Stereospecific Biotransformation of Geraniol Forming Citronellol with the Whole-Cell Saccharomyces cerevisiae: Dynamic Modelling and Optimisation of the Reaction System.**

5. **Ringkasan Penilaian**/*Summary of Assessment*:	Tidak Mencukupi *Inadequate*		Boleh Diterima *Acceptable*	Sangat Baik *Very Good*	
	1	2	3	4	5
i) **Pencapaian objektif projek:** *Achievement of project objectives*	☐	☐	☐	☑	☐
ii) **Kualiti output:** *Quality of outputs*	☐	☐	☐	☑	☐
iii) **Kualiti impak:** *Quality of impacts*	☐	☐	☑	☐	☐
iv) **Pemindahan teknologi/potensi pengkomersialan:** *Technology transfer/commercialization potential*	☐	☐	☐	☑	☐
v) **Kualiti dan usahasama :** *Quality and intensity of collaboration*	☐	☐	☑	☐	☐
vi) **Penilaian kepentingan secara keseluruhan:** *Overall assessment of benefits*	☐	☐	☑	☐	☐

6. Abstrak Penyelidikan
(Perlu disediakan di antara 100 - 200 perkataan di dalam **Bahasa Malaysia dan juga Bahasa Inggeris**. Abstrak ini akan dimuatkan dalam Laporan Tahunan Bahagian Penyelidikan & Inovasi sebagai satu cara untuk menyampaikan dapatan projek tuan/puan kepada pihak Universiti & masyarakat luar).

Abstract of Research
(An abstract of between 100 and 200 words must be prepared in Bahasa Malaysia and in English). This abstract will be included in the Annual Report of the Research and Innovation Section at a later date as a means of presenting the project findings of the researcher/s to the University and the community at large)

A mathematical model consisting of mass balance and mass transfer equations is presented to describe the biotransformation of monoterpene (geraniol) using whole cells of *Saccharomyces cerevisiae* in a continuous-closed-gas-loop bioreactor (CCGLB). The reliability of model simulations was tested using experimental data in the CCGLB at several substrate flow rates. Comparison between the model simulations with product profile at several substrate flow rates proves that the model is appropriate to describe the experimental results and simulate the bioreactor system. Sensitivity analysis from the model shows that the overall mass transfer coefficient of substrate (k_La) greatly affects the bioreactor performance. The model can later be used as a design tool to predict the k_La value of geraniol which is difficult to be measured experimentally.

7. Sila sediakan laporan teknikal lengkap yang menerangkan keseluruhan projek ini.
[Sila gunakan kertas berasingan]
Applicant are required to prepare a Comprehensive Technical Report explaning the project.
(This report must be appended separately)

Please refer attachement

Senaraikan kata kunci yang mencerminkan penyelidikan anda:
List the key words that reflects your research:

Bahasa Malaysia	*Bahasa Inggeris*
Biotransformasi	Biotransformation
Fermentasi	Fermentation
Pemisahan	Separation
Permodelan	Modelling

8. Output dan Faedah Projek
Output and Benefits of Project

(a) * **Penerbitan Jurnal**
Publication of Journals
(Sila nyatakan jenis, tajuk, pengarang/editor, tahun terbitan dan di mana telah diterbit/diserahkan)
(State type, title, author/editor, publication year and where it has been published/submitted)

(i) Aimi Aishah Ariffin, Mashitah Mat Don, **Mohamad Hekarl Uzir*, (2011)**, *Baker's Yeast-Mediated Biotransformation of Geraniol into Citronellol Using a Continuous-Closed-Gas-Loop Bioreactor (CCGLB) System.* Biochemical Engineering Journal. No. 1, Vol. 56, 219-224.

(ii) Aimi Aishah Ariffin, Mashitah Mat Don, **Mohamad Hekarl Uzir*, (2011)**, *The Feasibility of Growing Cells of* Saccharomyces cerevisiae *for Citronellol Production in a Continuous-Closed-Gas-Loop Bioreactor (CCGLB)*, Bioresource Technology, No. 19, Vol. 102, 9318-9320.

(b) **Faedah-faedah lain seperti perkembangan produk, pengkomersialan produk/pendaftaran paten atau impak kepada dasar dan masyarakat.**
State other benefits such as product development, product commercialisation/patent registration or impact on source and society.

The mathematical model can be used for other similar of gas-phase system.

* Sila berikan salinan/*Kindly provide copies*

(c) **Latihan Sumber Manusia**
Training in Human Resources

i) Pelajar Sarjana:
Graduates Students
(Perincikan nama, ijazah dan status)
(Provide names, degrees and status)

Name: **Aimi Aishah Arifin**
Degree: **Master (Chemical Engineering)**
Status: **Lecturer, Universiti Malaysia Terengganu.**

ii) Lain-lain:
Others ______________________

____

____

9. Peralatan yang Telah Dibeli:
Equipment that has been purchased

Modification of the existing rig for modeling purposes.

Tandatangan Penyelidik
Signature of Researcher

8 AUGUST 2012.

Tarikh
Date

Komen Jawatankuasa Penyelidikan Pusat Pengajian/Pusat
Comments by the Research Committees of Schools/Centres

Project successfully completed with excellent output in terms of publication.

TANDATANGAN PENGERUSI
JAWATANKUASA PENYELIDIKAN
PUSAT PENGAJIAN/PUSAT
Signature of Chairman
[Research Committee of School/Centre]

9/8/12
Tarikh
Date

Mathematical modeling and simulation for geraniol reduction mediated by *Saccharomyces cerevisiae* in a continuous-closed-gas-loop bioreactor (CCGLB).

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Abstract

A mathematical model consisting of mass balance and mass transfer equations is presented to describe the biotransformation of monoterpene (geraniol) using whole cells of *Saccharomyces cerevisiae* in a continuous-closed-gas-loop bioreactor (CCGLB). The reliability of model simulations was tested using experimental data in the CCGLB at several substrate flow rates. Comparison between the model simulations with product profiles at several substrate flow rates proves that the model is appropriate to describe the experimental results and simulate the bioreactor system. Sensitivity analysis from the model shows that the overall mass transfer coefficient of substrate (k_La) greatly affects the bioreactor performance. The model can later be used as a design tool to predict the k_La value of geraniol which is difficult to be measured experimentally.

Keywords: *Saccharomyces cerevisiae*; biotransformation; monoterpene; gas phase bioreactor; modeling.

1. Introduction

Biotransformation of organic compounds in non-conventional system has been extensively explored due to several weaknesses associated in conventional system such as low substrate solubility and substrate and/or product inhibition (Leon et al., 1998; Marques et al., 2010). Among them, biotransformation using gas phase system is emerging as a new technology especially in synthesizing gaseous and volatile organic compounds (VOCs). The system has been successfully employed in biotransformation of monoterpenes, drugs and VOCs. Both purified enzymes (Graber et al., 2003; Leonard et al., 2004) and whole-cell biocatalysts (Maugard et al., 2001; Goubet et al., 2002; Erable et al., 2005) have shown their capability to be incorporated within the system. The gas phase biotransformation possesses great advantages which include; better mass transfer between gas and liquid phases, eliminates substrate and/or product inhibition and also provides an *in-situ* product removal system.

The bioreactor used in this study is a continuous-closed-gas-loop bioreactor (CCGLB) as initially proposed by Steinig et al. (2000). In the CCGLB, substrate with high vapor pressure and high volatility was continuously recirculated in the bioreactor compartment. The substrate in gaseous form was transferred from its substrate reservoir into the bioreactor containing microorganism where it diffused and performed biological transformation. The work carried out by Steinig and co-workers dealt with the epoxide production using growing cells of *Pseudomonas oleovorans* as the biocatalyst. The study focused on the effect of substrate towards the growth of microorganism with the main concern on the modeling of *P. oleovorans* growth with the presence of 1, 7-octadiene. Recently, biotransformation of monoterpenes in a similar bioreactor configuration has also been reported by Pescheck et al. (2009). In their investigation, two types of *Penicillium* species have been tested for the biotransformation of α-pinene and (+)-limonene respectively, aiming to observe the toxicity effects of the monoterpenes towards the cells during the gas phase conversion. The gas loop has successfully eliminated the inhibitory effect of substrate to the growth of microorganism yielding high productivity. Yet, no investigation has been reported on modeling of the resting cell biotransformation in the CCGLB system, focusing on the effect of mass transfer coefficient of substrate to the overall process system. Process modeling and simulation could generate richer descriptions of the process in order to deduce the qualitative behavior of the reactor system (Bogusch and Marquardt, 1997).

The concept of the CCGLB system is similar to that of a gas-liquid reactor system in the manner that the gas is transferred into the liquid phase and *vice versa*. Prediction of the dynamic behavior in this multiphase reactor is usually more complex compared to that of a single phase reactor, since this system involves more than two component balances, and mass transfer at the gas and liquid interfaces need to be taken into account (Dunn et al., 2003). Basically, the kinetics of a reaction with a single substrate catalyzes by resting cell can be expressed based on the Michaelis-Menten (M-M) model (Goudar et al., 2004). This is due to the fact that the reaction is catalyzed by only a particular enzyme in the cell (Cinar et al., 2003). The M-M model can be complex if the cell has an inhibitory effect towards the substrate and/or product concentrations. Previous kinetic equations developed in the investigations of biotransformation using resting cells of *S. cerevisiae* described the inhibition effect of substrate concentration such as during the reduction of carbonyl compounds (C=O) (Buque-Taboada et al., 2005; Houng and Liau, 2006). However, little information is available on the kinetics of monoterpene alcohol (C=C) reduction by *S. cerevisiae*. Doig et al. (1998) have previously discussed the potential inhibition of geraniol to the yeast cell; however kinetic parameters which are important to describe the rate of reaction were not been clearly addressed.

The mass transfer model between gas and liquid phases is normally described by a two-film model, since it is rather simple and a convenient method (Parulekar and Amin, 1996; Maeda et al., 2005; Garcia-Ochoa and Gomez, 2009). An overall mass transfer coefficient is a key parameter in the two-film model and it is an important value in modeling the gas-liquid system.

The purpose of the present study was to conduct a preliminary investigation into the operation of the continuous-closed-gas-loop bioreactor. The CCGLB was used in the biotransformation of geraniol into citronellol using the whole cells of *Saccharomyces cerevisiae* as biocatalyst (Figure 1). Citronellol, which has a rose-like scent, is commercially used in food and cosmetic industries (Yadav and Lathi, 2004). Previously, a similar reaction model has already been carried out in the two-phase system (Gramatica et al, 1982; Doig et al, 1998). This work attempts to describe the CCGLB system by incorporating the mass transfer effect and later solves the model equations via numerical technique. Model simulations are then used to estimate the mass transfer coefficient of geraniol which is difficult to be measured experimentally due to no appropriate sensor available for the particular compound.

2. Materials and method

2.1 Biocatalyst and chemicals

Saccharomyces cerevisiae (baker's yeast type-II) was purchased from Sigma Chemical Co., USA. Standard geraniol (96%) and citronellol (99%), glucose anhydrous and hexadecane (99%) were purchased from Aldrich Chemical Co., USA. Potassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) were also purchased from the same company. All chemicals were of the highest purity.

2.2 Biomedium

Phosphate buffer solution was used as the biomedium for the biotransformation which comprised of 40 mM of KH_2PO_4 and K_2HPO_4. 10 g/L glucose was added as the main carbon source for the reaction. The pH of the biomedium was adjusted to pH 7 using 0.5 M HCL or 0.5 M KOH and was further steam sterilized at 121°C for 20 min prior to use.

2.3 Biotransformation of geraniol in a CCGLB

2.3.1 CCGLB configuration

The initial configuration of CCGLB system proposed by Steinig et. al (2000) was set-up with some modifications in order to fulfill the current biotransformation conditions. As shown in Figure 2, a complete CCGLB system mainly consists of three reservoirs; substrate, whole-cell and product. The reaction was carried out in a stirred-tank bioreactor, where glucose was supplied as co-substrate at the beginning of the process. The gaseous substrate was sparged continuously from the substrate reservoir **(1)** which subsequently flowed into the bioreactor vessel **(2)** (Labfors, Infors AG, Switzerland). The flow rate was adjusted using a flow meter which was already integrated into the bioreactor system. The gaseous geraniol further diffused into the liquid phase, which contained baker's yeast and formed the corresponding gaseous product of citronellol. The product then left the vessel via a condenser on top of the bioreactor in gaseous form and dissolved into a solvent, hexadecane, in the product reservoir **(3)**. The dissolved product in hexadecane was ready to be analyzed using a gas chromatography.

2.3.2 CCGLB set-up

200 mL pure liquid geraniol was placed in a 500 mL conical flask which acted as the substrate reservoir. The flask was tightly closed using a rubber stopper to avoid substrate and/or product losses. A PTFE-type tubing with a 10 mm outer diameter was used in all the connections.

The geraniol vapor was pumped into the bioreactor vessel through a sparger where the biocatalyst was readily available. Reaction was carried out in a 7.5 L bioreactor with 5 L working volume at 30°C. The pH of the biomedium was controlled via a pH probe (Mettler Toledo, Switzerland) and peristaltic pumps dosing acid of 3 M HCL and base of 3 M KOH. Dissolved oxygen (DO) was measured via a polarographic oxygen electrode (Mettler Toledo, Switzerland). The gaseous product leaving the condenser on top of the bioreactor was then dissolved in 200 mL hexadecane which acts as an extraction solvent. Samples were taken from the sampling port of the product reservoir at specific time intervals and were injected directly into a gas chromatograph for analysis.

Prior to each biotransformation, dissolved oxygen (DO) and pH probes were separately calibrated. The bioreactor with all the media components was steam sterilized at 121°C, 1 bar for 20 min. An antifoaming agent; polypropylene glycol 1200 (Sigma Aldrich), was also sterilized separately in a 250 mL multipurpose bottle. After cooling to room temperature, the bioreactor was connected to the motor, pH and DO cables and left to polarize for at least 6 hr. At the same time, the pure geraniol as well as the solvent (hexadecane) was placed in the reactant and product reservoirs, respectively.

2.4 Analytical methods

2.4.1 Gas chromatographic analysis

Samples were injected into a split injector of a gas chromatograph (Perkin Elmer, Clarus 500, USA) equipped with a flame ionization detector (FID). A Supelcowax^{10} capillary column (30 m x 0.25 mm i.d x 0.25 μm film thickness; France) was used for the chromatographic separation. The injector and the detector were kept at 200°C and 250°C respectively. The initial oven temperature of 160°C was held for 3 min and then ramped at a rate of 8°C/min to 230°C. Helium was used as the carrier gas and the flow rate in the column was maintained at 2.5 mL/min. Hydrogen and air were supplied to the FID at 30 and 300 mL/min respectively.

2.5 Model Development

The mathematical model of the CCGLB was developed by combining the substrate and product mass balances, mass transfer effect and reaction rate expression over the bioreactor system (Figure 3). The general model was simplified to the case of continuous operation with respect to the gaseous geraniol. The following simplifying assumptions have been made for the development of the model:

1) the composition of biomass is approximately constant under resting cell condition.
2) both phases were assumed to be perfectly mixed.
3) the internal pressure of the system was constant throughout the process.
4) all product formed was completely dissolved into the solvent.

2.5.1 Mass Balances

Geraniol and citronellol were both present in the CCGLB system in the form of gas and liquid phases. Therefore, mass balances of these components in each phase should be considered. Similar models with simplified equations were reported elsewhere (Levenspiel, 1999; Dunn et al., 2003).

Gas phase geraniol balance;

$$\mathrm{Ger}_{\mathrm{accumulation}} = \mathrm{Ger}_{\mathrm{inlet}} - \mathrm{Ger}_{\mathrm{outlet}} - \mathrm{Ger}_{\mathrm{transfer\ from\ gas\ to\ liquid\ phase}}$$

$$V_g \frac{dC_{G,g\ out}}{dt} = Q\left(C_{G,g\ in} - C_{G,g\ out}\right) - k_L a\left(C_{G,l}^{*} - C_{G,l}\right)V_l \qquad (1)$$

The equilibrium concentration of geraniol is given by Henry's law;

$$C_{G,l}^{*} = \frac{C_{G,g\ out}RT}{H} \qquad (2)$$

Substituting equation (2) into equation (1) and upon rearrangement gives;

$$\frac{dC_{G,g\ out}}{dt} = \frac{Q(C_{G,g\ in} - C_{G,g\ out})}{V_g} - \frac{k_L a\left[\frac{(C_{G,g\ out}RT)}{H} - C_{G,l}\right]V_l}{V_g} \qquad (3)$$

Liquid phase geraniol balance;

$$\mathrm{Ger}_{\mathrm{accumulation}} = \mathrm{Ger}_{\mathrm{transferred\ from\ gas\ phase}} - \mathrm{Ger}_{\mathrm{consumption\ in\ the\ biotransformation}}$$

$$V_l \frac{dC_{G,l}}{dt} = k_L a\left(C_{G,l}^{*} - C_{G,l}\right)V_l - rV_l \qquad (4)$$

Upon substituting equation (2) into equation (4) and further rearrangement gives;

$$\frac{dC_{G,l}}{dt} = k_L a\left(\frac{(C_{G,g\ out}RT)}{H} - C_{G,l}\right) - r \qquad (5)$$

Liquid phase citronellol balance;

$$\frac{dC_{C,l}}{dt} = r - k_L a\left(C_{C,l} - C_{C,l}^{*}\right) \qquad (6)$$

with

$$C_{C,l}^{*} = \frac{C_{C,g\ out}RT}{H}$$

hence;

$$\frac{dC_{C,l}}{dt} = r - k_L a\left(C_{C,l} - \frac{(C_{C,g\,out}RT)}{H}\right) \tag{7}$$

Gas phase citronellol balance;

$$V_g \frac{dC_{C,g\,out}}{dt} = k_L a\left(C_{C,l} - \frac{(C_{C,g\,out}RT)}{H}\right) - Q(C_{C,g\,out}) \tag{8}$$

Upon rearrangement of the above equation leads to the following equation;

$$\frac{dC_{C,g\,out}}{dt} = \frac{k_L a\left(C_{C,l} - \frac{(C_{C,g\,out}RT)}{H}\right)}{V_g} - \frac{Q(C_{C,g\,out})}{V_g} \tag{9}$$

where, $C_{G,g\,in}$ represents the concentration of gaseous geraniol at inlet, $C_{G,g\,out}$ and $C_{C,g\,out}$ are the concentrations of gaseous geraniol and citronellol at outlet, respectively, $C_{G,l}$ and $C_{C,l}$ are the concentrations of geraniol and citronellol in bulk liquid, respectively, Q is the gas volumetric flow rate, V_g and V_l give the volume of gas and liquid phase, k_La represents the overall volumetric mass transfer coefficient of geraniol, $C^*_{G,l}$ and $C^*_{C,l}$ are the equilibrium concentrations of geraniol and citronellol at interface, respectively, r is the rate of reaction, R is the ideal gas constant, T is the temperature and H is the Henry's coefficient of geraniol and citronellol.

4. Results and discussion

4.1 Estimation of kinetic parameters

A set of experiments in shake-flask culture was run to investigate the effect of geraniol concentration on the biotransformation rate in order to elucidate the kinetics of the baker's yeast as biocatalyst. The reaction was carried out at pH 7 and 30°C. The concentration of geraniol was varied from 3 to 20 g/L. As shown in Figure 4, the maximum reaction rate measured for citronellol increases with geraniol concentration. In the range tested, no inhibitory effect of geraniol was detected and the Michaelis-Menten constants, K_m and V_{max} for the resting cells of *S. cerevisiae* were determined. Initially, the parameters were estimated using a graphical method of Lineweaver-Burk plot and further optimized using POLYMATH® software based on a non-linear regression technique. Reciprocal initial rate versus reciprocal initial concentration of geraniol plot (Figure 5) gave the values of M-M constants K_m and V_{max} of 49 g/L and 0.03 g/L.hr.g_{cell}, respectively. The values were further optimized using POLYMATH® software. The values of kinetic constants and their coefficients R^2 obtained from both methods were shown in Table 1. Accordingly, the kinetic constants calculated from the POLYMATH® were used in the following simulation. Therefore, the rate equation for the reaction can be rewritten as follows:

$$r = -\frac{V_{max} C_{G,l}}{K_m + C_{G,l}} \tag{10}$$

while the citronellol formation is given as follows:

$$r = \frac{V_{max} C_{G,l}}{K_m + C_{G,l}} \tag{11}$$

where r is the rate of reaction (g/L.hr), V_{max} is the maximum rate of reaction (g/L.hr.g_{cell}), K_m is the Michaelis constant (g/L) and $C_{G,l}$ represents the concentration of geraniol in bulk liquid (g/L).

The results were contradictory to a study conducted by Doig et al. (1998) in the two-phase system who claimed that the strain was totally inhibited at 12 g/L geraniol concentration. The reason for this peculiar result is yet to be established. However, it may probably relate to the experimental configuration which is different. In the two phase system, the decrease in productivity does not only subjected to the elevated concentration of substrate, but may also cause by other factors, such as the solvent toxicity or insufficient phase volume ratio of organic to aqueous (V_{org}/V_{aq}). In fact, Li et al. (2007) in a similar report concluded that baker's yeast type-II was able to accept 2-octanone up to 500 mM with *n*-dodecane as the solvent which partly support the results of the current investigation.

4.2 Model simulation

The k_La value of volatile organic compound (VOC) of toluene was used as a basis since there is no available data of k_La for geraniol in the literature. The k_La of toluene was selected with the fact that it has low solubility in water with approximate Henry's coefficient of 0.03 L.atm/mol while geraniol and citronellol have the value of 0.06 L.atm/mol, which is close to that of toluene. It is worth noting that the k_La value for VOC was found to be lower than that of pure gaseous compounds such as oxygen at the same operating conditions (Bielefeldt and Stensel, 1999). This is due to the different physical nature between both compounds and thus, the selection of k_La value of VOC rather than the pure gaseous compound seems reasonable. Approximately, the k_La of VOCs reported in many wastewater treatment processes was below the value of 3×10^{-3} s^{-1} (10.8 hr^{-1}) (Reardon et al., 2000; Yonghong, 2005; Guillaume et al., 2010).

A full set of differential equations (3), (5), (7) and (9) with the incorporation of equations (10) and (11) was translated into MATLAB® software and later numerically solved using the embedded differential equation solver (ODE's) within the software. The numerical solutions generated were simulated with appropriate graphical representations. Such a simulation was undertaken in order to observe the implications of the changes in the defined parameters into the whole process system, which highly required in bioreactor design and optimization. The simulation was carried out using the data supplied in Table 2.

4.3 Model validation and application

One of the main concerns in mathematical modeling of the CCGLB is the determination of the unknown model parameter which is at this particular moment, difficult to be estimated using experimental procedure. The overall volumetric mass transfer coefficient (k_La) is a key parameter in modeling of a gas-liquid system. There is so far no available k_La data for gaseous geraniol reported in the literature since gaseous phase investigation is rather scarce. An estimation of k_La value of the gaseous geraniol using experimental technique is rather complex because there is no commercial electrodes available in the market especially to detect the presence of gaseous form of geraniol. Due to this particular reason, the model was designed in such a way that this parameter value could be estimated according to the given conditions.

As a starting point in this work, the value was used by a trial and error method. The initial guess of the value was tested within a range of 3 to 12 hr^{-1}. Experimental results of the biotransformation at different gas flow rates were used as a basis to predict the values of the k_La. At all given gas flow rates, the values were manipulated until the simulation and experimental results agreed between each other. From the trial and error technique, the k_La values at the gas flow rates of 4, 6 and 8 L/min were found to be 4, 7 and 10.8 hr^{-1}, respectively (Figure 6). The results from the simulations clearly proved that the gas flow rate has a positive relationship on the k_La value, where an increase in the initial gas flow rate tends to increase the value of k_La. This was previously discussed in many basic mass transfer studies that the value of k_La would be greatly increased by factors such as gas flow rate, agitation rate, type of impeller and the rheology of media (Stanbury and Whitaker, 1984; Scragg, 1991). The increase is generally contributed to the larger gas holdup at a higher inlet gas flow rate (Patel and Thibault, 2009). A high mass transfer rate contributed by a high k_La, further resulted in the increase of the overall productivity of the biotransformation process as can be seen from the same figure. The simulation also gives the trend of gaseous geraniol concentration with time at different flow rates which was not measured while carrying out the experimental work (Figure 7).

4.4 Model sensitivity

The model developed was used to investigate the dynamics of the citronellol concentration at different k_La values, while other conditions were kept constant. The simulation were performed with k_La values of 2, 4, 6 and 8 hr^{-1} at a fixed flow rate of 8 L/min. Figures 8 and 9 show the concentration profiles of product and substrate in the CCGLB system, respectively. As shown in Figure 8, the citronellol production increases with increasing k_La. The productivity was almost doubled with the increase of k_La from 2 to 8 hr^{-1}. A possible explanation behind this observation is that, when the k_La value is increased, the dissolved gaseous geraniol will approach more closely to the equilibrium concentration, $C^*_{G,l}$ (Wood et al., 1996). This will then increase the contact between the substrate and the cell. The calculated product formation is strongly affected by variations in the k_La, suggesting that this parameter should be estimated with adequate precision in order to enable more reliable simulations.

5. Conclusions

Kinetic studies and modeling of the CCGLB system for the reduction of geraniol to produce citronellol catalyzed by baker's yeast have been conducted. The reaction is found to follow the non-inhibitory Michaelis-Menten type equation with respect to geraniol concentration. A non-linear regression technique was used to determine the values of the kinetic constants. The values of V_{max} and K_m were estimated to be 0.015 $g/L.hr.g_{cell}$ and 17.9 g/L, respectively. The model has also been developed to simulate the biotransformation of gaseous geraniol at different flow rates. A good consistency between the experimental data and the theoretically predicted values verified that the proposed model could successfully simulate the dynamic behavior of the CCGLB system. The results from the simulations give the predicted values of mass transfer coefficient for geraniol (k_La) of 4, 7 and 10.8 hr^{-1} at substrate flow rates of 4, 6 and 8 L/min, respectively.

Acknowledgement

The author would like to thank USM for providing the Short-Term Grant (PJKIMIA/6035215) and subsequently (PJKIMIA/6035281) for financial support of this research.

Nomenclature

t	Time (hr)
S	Substrate concentration (g/L)
k_La	Overall mass transfer coefficient (hr^{-1})
H	Henry's coefficient (L.atm/mol)
$C_{G,g\ in}$	Concentration of geraniol in gas phase at bioreactor inlet (g/L)
$C_{G,g\ out}$	Concentration of geraniol in gas phase at bioreactor outlet (g/L)
$C_{C,g\ out}$	Concentration of citronellol in gas phase at bioreactor outlet (g/L)
$C_{G,l}$	Concentration of geraniol in bulk liquid (g/L)
$C_{C,l}$	Concentration of citronellol in bulk liquid (g/L)
Q	Gas flow rate (L/hr)
V_g	Volume of gas (L)
V_T	Volume of bioreactor (L)
V_l	Volume of liquid (L)
$C^*_{G,l}$	Equilibrium concentration of geraniol at interface (g/L)
$C^*_{C,l}$	Equilibrium concentration of citronellol at interface (g/L)
R	Ideal gas constant (L.atm/mol.K)
T	Temperature (K)
r	Rate of reaction ($g/L.hr.g_{cell}$)
V_{max}	Maximum rate of reaction ($g/L.hr.g_{cell}$)
K_m	Michaelis constant (g/L)

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Figures captions

Figure 1: Schematic representation of cofactor regeneration during a reduction of geraniol into citronellol by *S. cerevisiae* with glucose as hydrogen donor.

Figure 2: Schematic diagram of CCGLB system.

Figure 3: The boundary of CCGLB system used in the modeling work.

Figure 4: Initial rate of reactions of geraniol reduction by resting cells of *S. cerevisiae* at different substrate concentrations.

Figure 5: Reciprocal initial rate (r) versus reciprocal initial concentration of geraniol (S) for biotransformation of geraniol.

Figure 6: Comparison of experimental and simulated data for different initial gas flow rates. Symbols and lines represent experimental and simulated data, respectively.

Figure 7: Substrate concentration in the CCGLB predicted using the proposed model.

Figure 8: Citronellol concentration in CCGLB predicted using the proposed model at different k_La values.

Figure 9: Geraniol concentration in CCGLB predicted using the proposed model at different k_La values.

Tables captions

Table 1: Parameter constants

Table 2: Model parameters

Figure 1

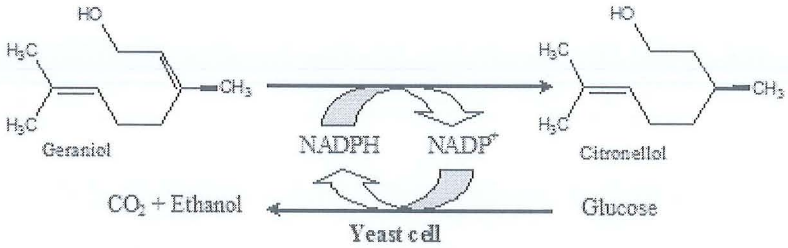
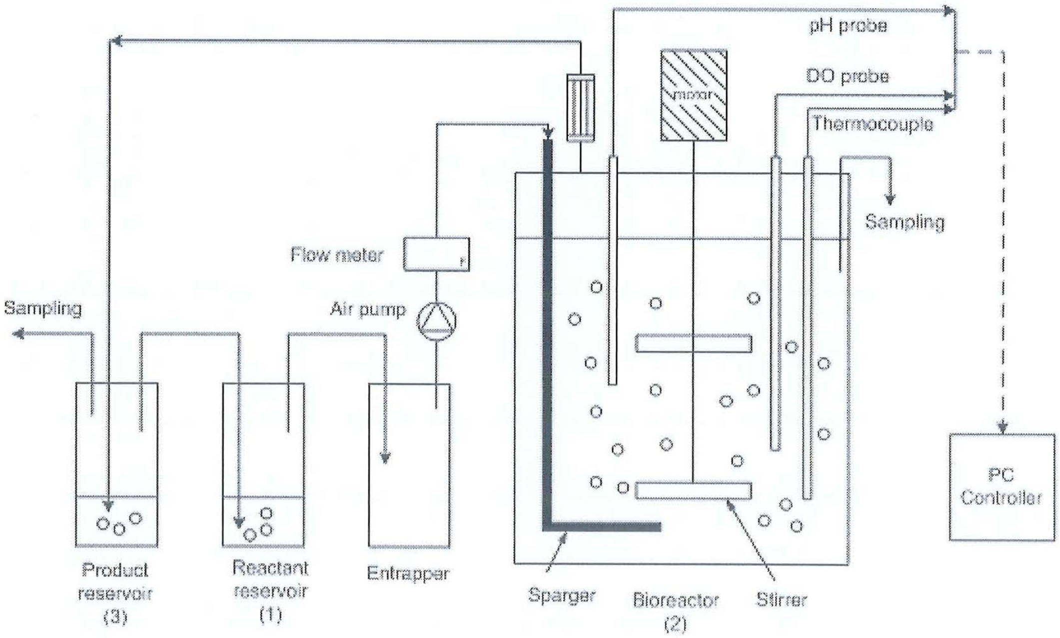


Figure 2



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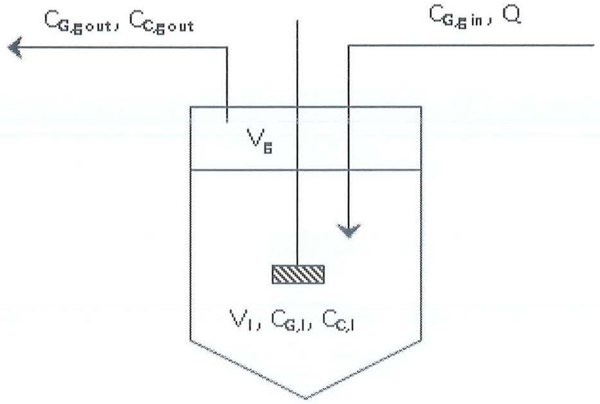

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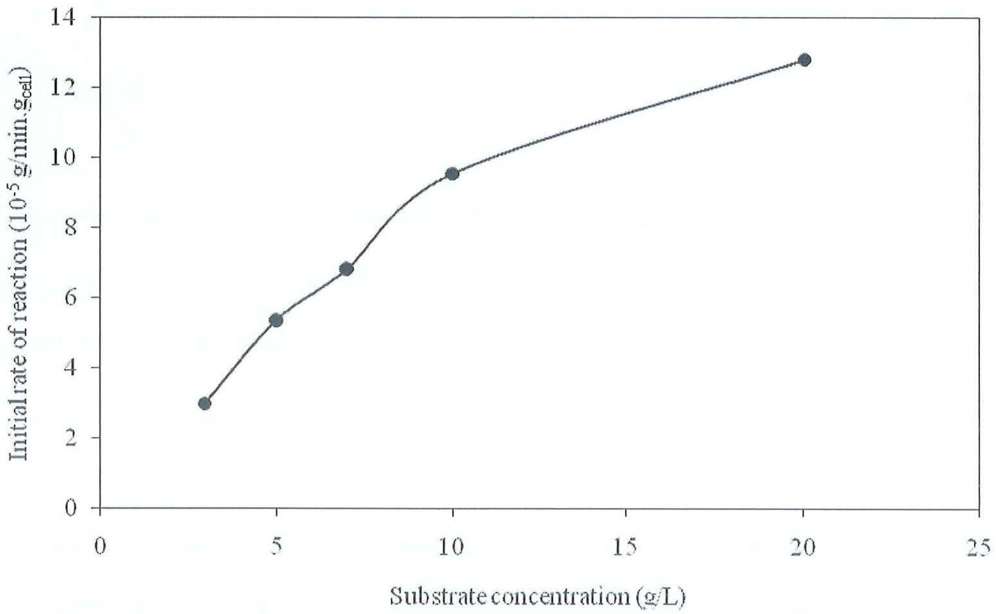


Figure 5

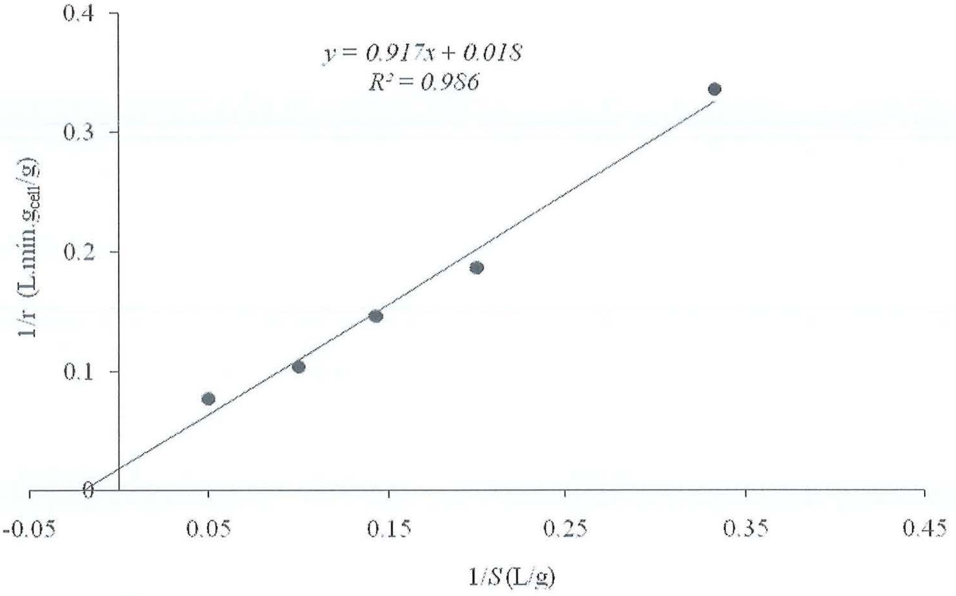


Figure 6

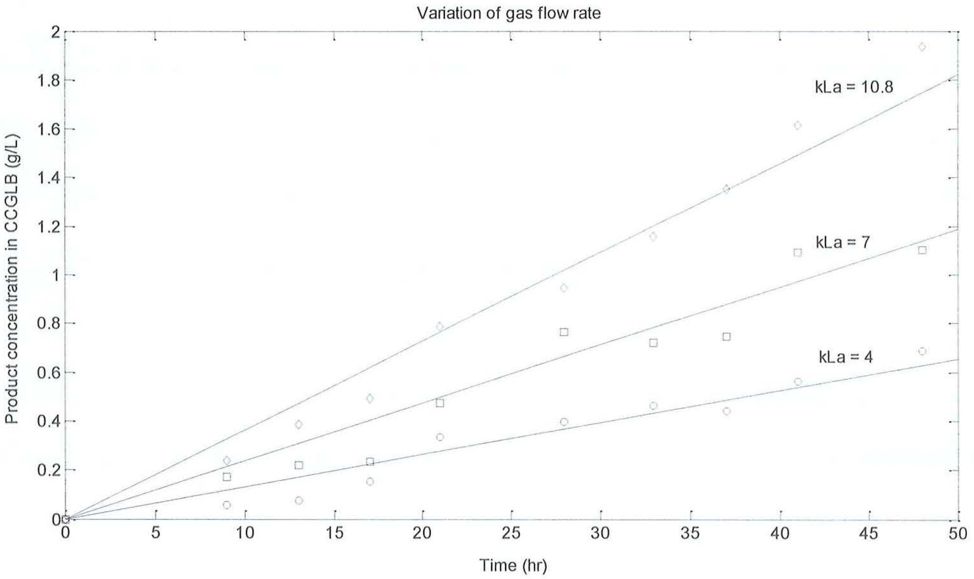
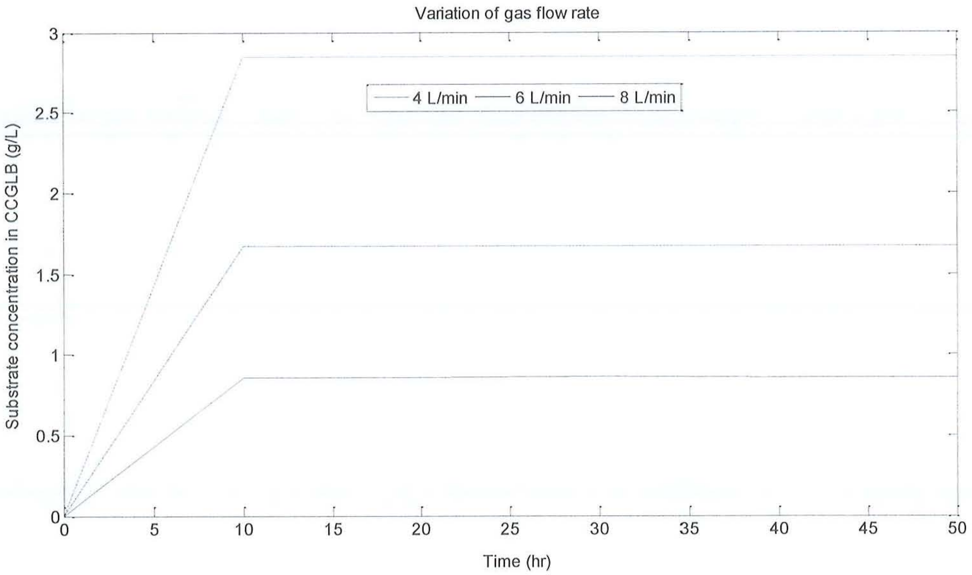


Figure 7



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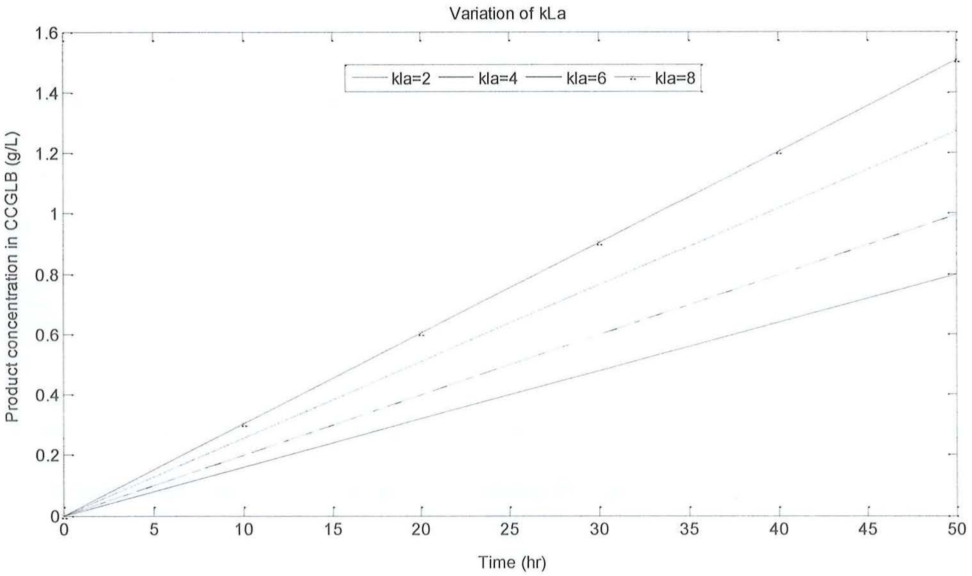

Figure 9

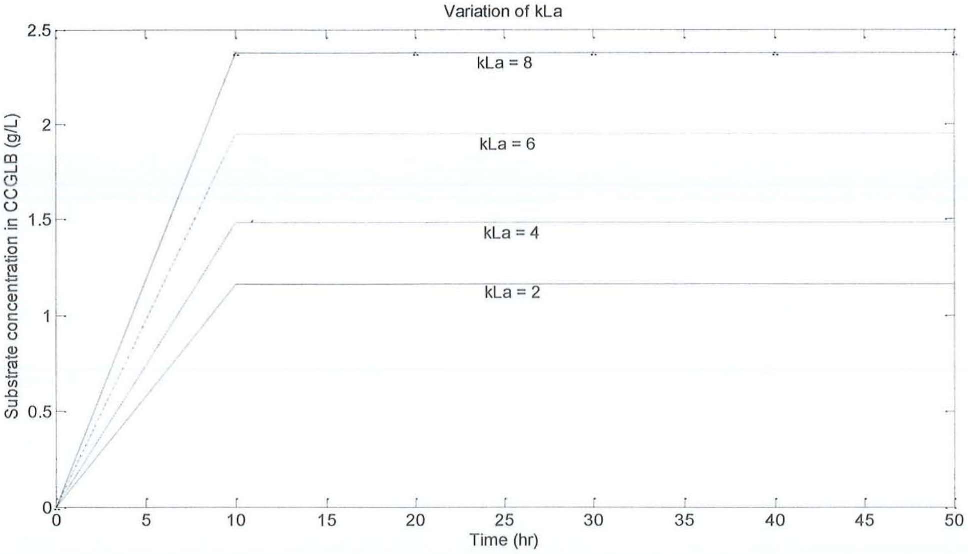
Variation of kLa
kLa = 8
kLa = 6
kLa = 4
kLa = 2
Substrate concentration in CCGLB (g/L)
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
30
35
40
45
50
Time (hr)

Table 1

Method	Kinetic parameters		R^2
	V_{max} (g/L.hr.g_{cell})	K_m (g/L)	
Lineweaver-Burk	0.03	49	0.986
Parameter estimation via optimization (POLYMATH®)	0.015	17.9	0.995

Table 2

Variable	Value	Unit
Initial concentration of gaseous geraniol, $C_{G,g\ in}$	6.2	g/L
Volume of bioreactor, V_T	7.5	L
Volume of liquid, V_l	5	L
Volume of gas, $V_g = V_T - V_l$	2.5	L
Henry's law coefficient, H	0.05	L.atm/mol
Gas constant, R	0.08206	L.atm/mol.K
Temperature, T	303	K
Michaelis constant, K_m	17.9	g/L
Maximum rate of reaction, V_{max}	0.015	g/L.hr.g_{cell}
Overall mass transfer coefficient of geraniol, k_La	3-12	hr^{-1}
Gas flow rate, Q	120 - 480	L/hr

JOURNAL PUBLICATION

Bioresource Technology 102 (2011) 9318–9320

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Short Communication

The feasibility of growing cells of *Saccharomyces cerevisiae* for citronellol production in a continuous-closed-gas-loop bioreactor (CCGLB)

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A R T I C L E I N F O

Article history:
Received 10 May 2011
Received in revised form 13 July 2011
Accepted 16 July 2011
Available online 22 July 2011

Keywords:
Biotransformation
Monoterpene
Saccharomyces cerevisiae
Continuous-closed-gas-loop bioreactor (CCGLB)
Reduction

A B S T R A C T

The present work aims to address the gas-phase biotransformation of geraniol into citronellol using growing cells of *Saccharomyces cerevisiae* (baker's yeast) in a continuous-closed-gas-loop bioreactor (CCGLB). This study revealed that the gaseous geraniol had a severe effect on the production of biomass during the growing cell biotransformation resulting in the decrease in the specific growth rate from 0.07 to 0.05 h^{-1}. The rate of reaction of the growing cell biotransformation was strongly affected by agitation and substrate flow rates. The highest citronellol concentration of 1.18 g/L and initial rate of reaction of 7.06×10^{-4} g/min g_{cell} were obtained at 500 rpm and 8 L/min, respectively.

1. Introduction

Whole-cell biotransformation has become one of the outstanding synthetic processes to produce enantiopure organic compounds especially for process that involved reduction reaction. Reduction reaction requires co-substrates for cofactor regeneration, preferentially performed with living cells as they provide an *in situ* cofactor regeneration system. The whole cells of *Saccharomyces cerevisiae* (baker's yeast) is the most common microorganisms used as a biocatalyst in reduction of organic compounds including drugs and monoterpenoids as it offers high redox capacity and generally much less expensive and easy to handle (Perles et al., 2008; Carballeira et al., 2009; Khor and Uzir, 2011).

Previous works carried out for biotransformation of geraniol using baker's yeast had dealt with the resting cells instead of the growing cells (Doig et al., 1998; Valadez-Blanco et al., 2008). The resting cell system separates between cell growth and biotransformation reaction and thus, avoids interference of the cell growth to the reaction (Wang et al., 2005). Furthermore, product isolation in the growing cell system is rather complex due to a mixture of fermentation medium with the product formed from the biotransformation itself (Kieslich et al., 1984).

The current research aimed at studying a gas-phase biotransformation of geraniol using the growing cells of baker's yeast in a continuous-closed-gas-loop bioreactor (CCGLB). The gas-phase system avoids direct contact between substrate, product and the reaction medium and therefore, the use of growing cells will not be directly influenced by the system. In addition, it promotes better diffusion and thus, eliminates mass transfer limitation between gas and liquid phases (Lamare and Legoy, 1993). Such a system has been successfully applied for fermentation (Taylor et al., 2010) as well as for whole-cell biotransformation (Maugard et al., 2001; Goubet et al., 2002; Pescheck et al., 2009).

2. Methods

2.1. CCGLB configuration

The configuration of CCGLB system initially proposed by Steinig et al. (2000) was set-up with some modifications in order to fulfill the current biotransformation conditions. The system mainly consists of three reservoirs; substrate, whole-cell and product as shown in Fig. 1. The gaseous substrate was sparged continuously from the substrate reservoir (1) containing 200 mL liquid geraniol (Aldrich Chemical Co., USA) and subsequently flowed into the 5 L bioreactor vessel (2) (Labfors, Infors AG, Switzerland) through an air pump (Gast Manufacturing Inc., USA). The flow rate was adjusted by using a flow meter which was already integrated into the bioreactor system. The gaseous substrate further diffused into the medium which contained baker's yeast (Sigma Chemical Co., USA) and formed the corresponding gaseous product, citronellol. The product then left the bioreactor vessel via a condenser mounted on top of the bioreactor and later dissolved into a

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doi:10.1016/j.biortech.2011.07.053

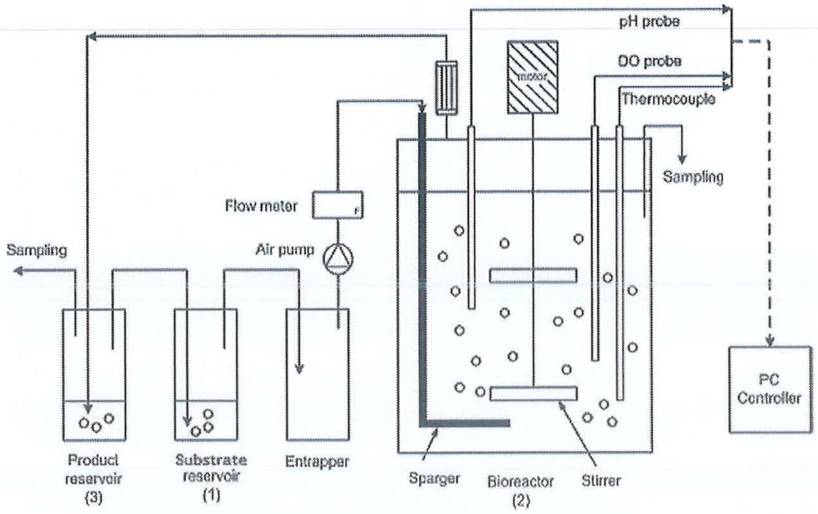

Fig. 1. Schematic diagram of the CCGLB system.

prepared 200 mL hexadecane (Aldrich Chemical Co., USA), which acted as a solvent in the product reservoir (3). The sample was ready to be analyzed using a gas chromatography.

2.2. Growing cell biotransformation

An anaerobic fermentation of yeast was carried out at pH 4 and 30 °C (optimized conditions). The composition of the media consisted of glucose (10 g/L), peptone (6 g/L) and yeast extract (3 g/L) (Fluka, USA). Using an aseptic technique, 1 g/L baker's yeast was added into the fermentation media. Biotransformation was carried out simultaneously by sparging gaseous substrate into the media for 36 h.

2.3. Analytical method

Biomass from the fermentation broth was measured using a spectrophotometer (Cecil 1000 series, UK) at 680 nm with appropriate dilution. The concentration of citronellol was measured using a gas chromatography (Perkin Elmer, Clarus 500, USA) equipped with a flame ionization detector (FID). A Supelcowax^{10} capillary column (30 m × 0.25 mm i.d × 0.25 μm film thickness; France) was used for the chromatographic separation. The injector and the detector were kept at 200 and 250 °C, respectively. The initial oven temperature of 160 °C was held for 3 min and then ramped at a rate of 8 °C/min to 230 °C. Helium was used as the carrier gas (Air Products, Malaysia) and the flow rate in the column was maintained at 2.5 mL/min. Hydrogen and air (Air Products, Malaysia) were supplied to the FID at 30 and 300 mL/min, respectively. Each sample was injected at least twice and the mean value was taken. An external standard method was used in sample analysis and calculations.

2.4. Determination of initial rate of reaction and specific activity

The initial rate of citronellol production was calculated for each batch experiment from plots of product accumulation against time (Doig et al., 1998). The ratio of citronellol produced per minute per gram of cell was measured to represent the corresponding initial rate of reaction. The specific activity of cells, (U/g_{cell}) or (μmol/min g_{cell}) was calculated from the ratio of the initial rate of reaction and molecular weight of citronellol (g/μmol).

3. Results and discussion

3.1. Effect of gaseous substrate (geraniol) on yeast growth

Fig. 2 represents the profile of yeast growth with and without the presence of gaseous geraniol. It shows that the growing cells were capable to perform the biotransformation in the CCGLB at which an accumulation of product was detected during the 36 h of reaction. About 0.77 g/L of total citronellol formation was obtained to give the initial rate of reaction and specific activity of 4.31×10^{-4} g/min g_{cell} and 2.76 U/g_{cell}, respectively. Growth of the cells however, showed a slight decline in the specific growth rate when the gaseous substrate started to accumulate in the fermentation medium. The cell growth without the presence of gaseous geraniol gives the specific growth rate of 0.07 h^{-1}, while with the presence of gaseous geraniol only gives 0.05 h^{-1}. This indicates that there was a competition of yeast cells to grow and simultaneously to catalyze the reduction reaction. Both processes directly compete for the same cofactor, which is regenerated in the glycolytic pathway (Perles et al., 2008). They may also compete for the same active sites of enzyme that responsible for the

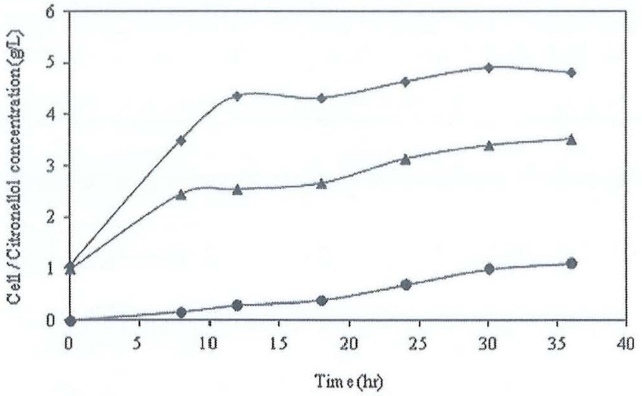

Fig. 2. Growth and biotransformation profiles during the course of geraniol reduction using growing cells of *S. cerevisiae* in CCGLB. The reaction was carried out for 36 h (conditions: pH 4, 30 °C, agitation rate of 350 rpm, substrate flow rate of 8 L/min, 10 g/L glucose, 1 g/L baker's yeast). '♦' Represents growth with free substrate (control); '▲' represents growth with biotransformation; '●' represents citronellol formation.

Table 1
Performance of growing cells of baker's yeast during biotransformation of geraniol in a CCGLB at different agitation and substrate flow rates.

Parameter	Value	Maximum product formation (g/L)	Initial rate of reaction (10^{-4} g/min g_{cell})	Specific activity (U/g_{cell})	Specific growth rate (h^{-1})
Agitation rate (rpm)	200	0.34	1.02	0.66	0.043
	350	0.77	4.31	2.76	0.056
	500	1.18	7.06	4.52	0.072
	800	0.85	5.03	4.14	0.063
Substrate flow rate (L/min)	4	0.56	1.82	1.16	0.052
	6	0.72	3.22	2.06	0.058
	8	1.18	7.06	4.52	0.072

reaction (Steinig et al., 2000). Even though the cell growth was severely affected by the substrate (geraniol), several strategies could be developed in order to increase the biomass production as well as the biotransformation productivity. The effects of agitation and substrate flow rates are believed to give a significant impact to both processes and they were further investigated in this study.

3.2. Effect of agitation rate

Study on the effect of agitation rate was carried out in order to identify the range of agitation rates that could promote a high specific growth rate as well as the rate of reaction in the CCGLB. A set of experiments was conducted by varying the stirrer speed between 200 and 800 rpm at a fixed substrate flow rate of 8 L/min, which is the maximum flow rate indicated on the flow meter. Table 1 shows the complete results from these experiments. By gradually increasing the agitation rate from 200 to 500 rpm, the biomass and citronellol production consequently increased, which implies that mechanical mixing helps to increase contacts between the cells and the nutrients in liquid media, and subsequently increased the cell metabolism and therefore the cell regeneration. Agitation rate at 800 rpm tends to decrease the specific growth rate as well as the initial rate of reaction with approximately 18% reduction of the total citronellol formation compared to that at 500 rpm. The decline in cell growth and citronellol productivity are probably due to the damage of one or more cellular component of yeast cells caused by the excessive mechanical stress (Saito et al., 2000; Bandaiphet and Prasertsan, 2006).

3.3. Effect of substrate flow rate

Substrate flow rate ranging from 4 to 8 L/min were investigated at constant agitation rate of 500 rpm. As indicated in Table 1, a lower substrate flow rate not only decreases the citronellol formation, but also the biomass production. Only 0.56 g/L citronellol and 3.52 g/L biomass were produced at the flow rate of 4 L/min at approximately a reduction of 47% and 14% respectively, than that determined at 8 L/min. The lower amount of citronellol produced at a lower substrate flow rate has led to a reduction in the initial rate of reaction. From these results, it can be deduced that, by accelerating the diffusion of geraniol into the aqueous phase seems to meet the demand of high biomass and citronellol production. The dispersion of the gaseous substrate may also help the movement of nutrients to diffuse from the liquid phase into the cellular compartment (Potumarthi et al., 2007; Garcia-Ochoa and Gomez, 2009).

4. Conclusions

A volatile compound of geraniol can be biologically transformed into citronellol by baker's yeast using the CCGLB system during its growth. Product formation was observed to be highly dependent on the cell growth. This information is believed to be the first encountered on such studies, since there are no previous investigations on biotransformation process during the exponential phase of cell growth. The growth of yeast was severely affected with the presence of substrate for biotransformation. However, strategies on varying parameters such as agitation and substrate flow rates were successfully introduced in order to enhance the overall productivity during the growing phase of a microorganism.

Acknowledgements

The authors would like to thank USM for providing the Short-Term Grants (PJKIMIA/6035215) and subsequently (PJKIMIA/6035281) to carry out the research work.

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Biochemical Engineering Journal 56 (2011) 219–224

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

Baker's yeast mediated biotransformation of geraniol into citronellol using a continuous-closed-gas-loop bioreactor (CCGLB) system

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ARTICLE INFO

Article history:
Received 30 September 2010
Received in revised form 2 June 2011
Accepted 3 July 2011
Available online 8 July 2011

Keywords:
Yeast
Biotransformations
In-situ product removal
Continuous-closed-gas-loop bioreactor (CCGLB)
Production kinetics
Heterogeneous biocatalysis

ABSTRACT

The kinetics of the biotransformation of geraniol into citronellol by the resting cells of *Saccharomyces cerevisiae* (baker's yeast type-II) was investigated in a continuous-closed-gas-loop bioreactor (CCGLB). Geraniol, which has high vapour pressure, high volatility and low solubility in aqueous medium, is highly suitable to be used in this system. Various operating parameters which affect the reduction reaction were investigated. The optimal conditions were; pH 7, agitation rate of 350 rpm, substrate flow rate of 8 L/min and glucose concentration of 50 g/L. The gas loop led to a maximum citronellol concentration of 2.38 g/L with the specific activity of 7.90 U/g_{cell}, which were apparently higher than that of the values obtained using conventional systems. The CCGLB system also has the advantage of in situ product removal and can be readily utilized in large-scale production.

1. Introduction

In recent years, biotransformation has become a popular method in organic compound synthesis for the production of enantiomerically pure compounds. Monoterpenoids are one of the most important starting materials in biotransformation and are widely exploited in the fine chemical sectors such as pharmaceuticals and food industries [1,2]. In the biotransformation field, asymmetric reduction reaction requires co-substrates for cofactor regeneration, preferentially performed with living cells as they provide an in situ cofactor regeneration system. The addition of expensive cofactors is necessary when dealing with an isolated enzyme system [3], which makes the choice of the system not practical. *Saccharomyces cerevisiae* (baker's yeast) is a known microorganism that is capable of reducing carbonyl and 'activated' carbon–carbon double bond compounds [4,5]. The selection of *S. cerevisiae* is favoured because it is easily available, cheap and has been proven to produce a high selectivity of product including monoterpenoids [6,7].

Conventionally, whole-cell biotransformation is carried out in an aqueous medium where the cells are most active. However, the substrate involved, which is usually organic, has low solubility in the aqueous phase which has the disadvantage of low volumetric productivity. Bioreactor design is one of the important strategies applied in biochemical engineering to increase the productivity of biotransformation. In the case of geraniol reduction by *S. cerevisiae*, very few effective bioreactor designs have been reported in the literature. Previously, there were reports on the biotransformation of geraniol by *S. cerevisiae* in a two-phase bioreactor system. However, the system suffered from substrate inhibition when geraniol reached a concentration higher than 0.3 g/L [8] and also formation of strong emulsion [9]. A membrane bioreactor was also used for the same purpose and the system was found to be successful in eliminating emulsion. Nonetheless, the yield of product was considerably low compared to that of the two-phase system [10]. The variation of *S. cerevisiae* performance in the bioreactor systems has led to the current study in order to investigate its feasibility in a continuous-closed-gas-loop bioreactor (CCGLB) system.

This work presents for the first time the biotransformation of geraniol, by resting cells of *S. cerevisiae* in a CCGLB system. In this study, geraniol which represents the substrate for biotransformation was supplied in gaseous form, taking advantage of the volatile nature of the compound. The gas phase system is believed to promote better diffusion and reduce mass transfer limitation in the liquid phase, resulting in an increase in overall productivity. In addition, in situ product removal is consequently developed. This could reduce a number of downstream processes and subsequently reduce the overall production costs especially on a large-scale production.

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doi:10.1016/j.bej.2011.07.001

. Materials and method

.1. Biocatalyst and chemicals

S. cerevisiae (baker's yeast type-II) as a source of biocatalyst vas obtained from Sigma Chemical Co., USA. Standard geraniol 96%) and citronellol (99%), glucose anhydrous and hexadecane 99%) were obtained from Aldrich Chemical Co., USA. Potassium ydrogen phosphate (K_2HPO_4) and potassium dihydrogen phoshate (KH_2PO_4) were also purchased from the same company. All hemicals were of the highest purity.

.2. Biomedium

Phosphate buffer solution was used as the medium for the bioransformation which comprised of 40 mM of KH_2PO_4 and K_2HPO_4. 0 g/L glucose was added as the main carbon source unless othervise stated. The pH of the medium was adjusted to the desired alue using 0.5 M HCL or 0.5 M KOH and was further steamterilized at 121 °C for 20 min prior to use.

.3. Biotransformation of geraniol in liquid phase

A preliminary work of the biotransformation was performed in 250 mL conical flask with 100 mL working volume. 10 g/L baker's east type-II was used to produce the corresponding enzyme. The lask was placed in an incubator shaker set at 30 °C and 150 rpm. ubstrate (20 g/L) was then added into the flask approximately 5 min after the inoculation. The reaction was left for 45 h and at pecific time intervals, 4 mL samples were withdrawn for optical lensity measurement as well as for product quantification.

.4. Biotransformation of geraniol in a ontinuous-closed-gas-loop bioreactor (CCGLB)

.4.1. CCGLB configuration

An initial configuration of the CCGLB system proposed by Steinig nd co-workers [11] was set up with some modifications in order o fulfill the current biotransformation conditions. As shown in ig. 1, a complete CCGLB system mainly consists of three reservoirs: ubstrate, whole-cell and product. Geraniol vapour was sparged ontinuously into a stirred-tank bioreactor. The vapour then left the eservoir **(1)** based on its vapour pressure and flowed into the air ump (Gast Manufacturing Inc., USA), which subsequently flowed nto the bioreactor vessel **(2)** (Labfors, Infors AG, Switzerland) hrough a sparger. The flow rate was adjusted using a flow meter vhich was already integrated into the bioreactor system. The eraniol vapour further diffused into the liquid phase containing east cells and converted into the liquid product (citronellol), which hen converted into vapour phase within the pressurized bioreactor essel. The gaseous citronellol then left the vessel via a condenser n top of the bioreactor and dissolved into a solvent, hexadecane n the product reservoir **(3)** and therefore, ready to be analyzed.

200 mL liquid geraniol was placed in a 500 mL conical flask vhich acted as the substrate reservoir **(1)**. The flask was tightly losed using a rubber stopper to avoid substrate and/or product osses. A PTFE-type tubing with a 10 mm outer diameter was used in ll the connections. Biotransformation process was carried out in a .5 L bioreactor with 5 L working volume. The pH of the biomedium vas controlled via a pH probe (Mettler Toledo, Switzerland) and eristaltic pumps dosing acid of 3 M HCL and base of 3 M KOH. Dissolved oxygen (DO) was measured via a polarographic oxygen lectrode (Mettler Toledo, Switzerland). The bioreactor was set-up o the desired temperature using circulated water in a doubleacketed system. 200 mL hexadecane was placed in the product eservoir **(3)**, which acted as an extraction solvent. Samples were taken from the product reservoir at regular intervals and were injected directly into a gas chromatograph for analysis. Samples from the fermentation broth were also harvested from the bioreactor outlet for optical density, glucose and ethanol concentrations measurements.

For the CCGLB experiments, the effects of pH, agitation rate, substrate flow rate and glucose concentration were examined. Experiments were conducted at pH values of 4, 7, 9 and 10. The corresponding agitation rate was controlled at the speeds of 200, 350 and 500 rpm, with the corresponding substrate flow rate adjusted to 2, 4, 6 and 8 L/min, while the glucose concentration was loaded at 10, 30 and 50 g/L, respectively. Experimental studies were carried out in duplicates and the results presented were the mean values of the data collected. The standard deviations for the experimental results were within ±5% of the mean values with 95% confident interval.

2.4.2. CCGLB set-up

Prior to each biotransformation, dissolved oxygen (DO) and pH probes were separately calibrated. Initially, the biomedium was prepared using similar compositions of the components as in the shake-flask procedure unless otherwise stated. The bioreactor with all the media components was steam-sterilized at 121 °C and pressure of 1 bar for 20 min. An antifoaming agent; polypropylene glycol 1200 (Sigma Aldrich), was also sterilized separately in a 250 mL multipurpose bottle. After cooling to room temperature, the pure geraniol as well as the solvent (hexadecane) was placed in the substrate and product reservoirs, respectively.

2.5. Analytical methods

2.5.1. Measurement of cell concentration

An optical method was developed in order to determine cell concentration during the reaction. At specific time intervals, approximately 2 mL samples were collected from the bioreactor and checked for its absorbance using a spectrophotometer (Cecil 1000 series, UK) at 680 nm with appropriate dilution. The cell concentration was determined by the correlation between the absorbance and dry weight of cell. A control sample was separately prepared. The effect of substrate dissolution towards the spectrophotometer reading was minimal and can be neglected.

2.5.2. Glucose analysis

Glucose concentration was measured using an assay of the dinitrosalicyclic colorimetric (DNS) method [12]. 1 mL of DNS reagent was added into 1 mL of glucose sample after centrifugation. Then the mixture was heated in boiling water for 5 min to develop a red-brown color. After cooling to room temperature, absorbance was recorded using a spectrophotometer with a wavelength set at 540 nm.

2.5.3. Ethanol analysis

Ethanol concentration was analyzed using a gas chromatography, HP 5890 series II (Hewlett-Packard, USA) equipped with a flame ionization detector (FID) with Porapak QS (Alltech Associates Inc., USA) 100/120 mesh. The injector and the detector were kept at 175 °C and 185 °C, respectively. Nitrogen was used as the carrier gas (Air Products, Malaysia). In all sample analyses, isopropanol was used as an internal standard [13].

2.5.4. Citronellol analysis

The concentration of citronellol in the reaction mixture was measured by gas chromatography (Perkin Elmer, Clarus 500, USA) equipped with a flame ionization detector (FID). A Supelcowax^{10} capillary column (30 m × 0.25 mm i.d × 0.25 μm film thickness;

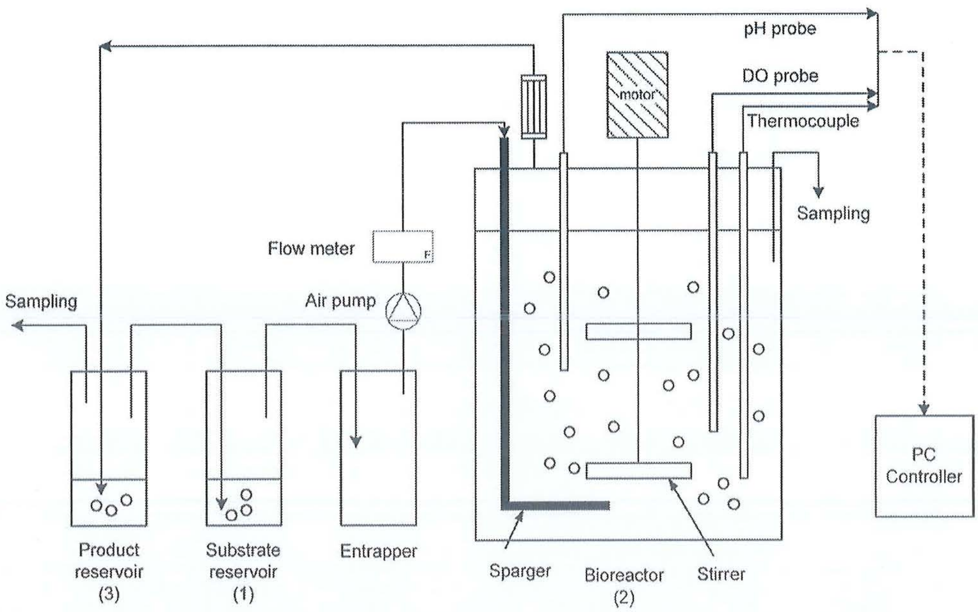

Fig. 1. Schematic diagram of the CCGLB system.

France) was used for the chromatographic separation. The injector and the detector were kept at 200 °C and 250 °C, respectively. The initial oven temperature of 160 °C was held for 3 min and then ramped at a rate of 8 °C/min to 230 °C. Helium was used as the carrier gas (Air Products, Malaysia) and the flow rate in the column was maintained at 2.5 mL/min. Hydrogen and air (Air Products, Malaysia) were supplied to the FID at 30 and 300 mL/min, respectively. Each sample was injected at least twice and the mean value was recorded. An external standard method was used for the calculations.

2.6. *Determination of initial rate of reaction and specific activity*

The initial rate of reaction was calculated from the ratio of citronellol produced per minute per gram of cell. The specific activity of cells, (U/g_{cell}) or (μmol/min g_{cell}) was calculated from the ratio of the initial rate of reaction and the molecular weight of citronellol (g/μmol).

2.7. *Determination of glucose consumption rate*

The rate of glucose consumption was calculated from the ratio of glucose consumed per minute as described in the literature [14].

3. Results and discussion

3.1. *Effect of initial pH*

A series of reactions were performed at various initial pH of biomedium. The reactions were carried out with 10 g/L of baker's yeast at 30 °C. The agitation and substrate flow rates were fixed at 200 rpm and 8 L/min, respectively. 10 g/L of glucose was used as the carbon source. The initial pH values which ranged from 4 to 10 were tested and the results of the initial reaction rates are displayed in Fig. 2. As observed from the experimental work, the maximum initial rate of reaction of 3.85×10^{-4} g/min g_{cell} was achieved when the biomedium pH was maintained at 7 to give the specific activity of 2.46 U/g_{cell}. At this condition, the final citronellol concentration detected was 0.60 g/L during 45 h of reaction. It was also observed that at a slightly alkaline pH of 9, the initial rate rose even higher (3.47×10^{-4} g/min g_{cell}) than that at pH 4 (2.64×10^{-4} g/min g_{cell}). However, at an alkaline pH of 10, a reverse trend was observed. Only 0.31 g/L citronellol was produced to give the lowest initial rate of 2.10×10^{-4} g/min g_{cell}. These results might be due to the hydrolysis of the substrate and the product under alkaline condition [15]. On the other hand, according to Pereira [16], the effect of pH on the reduction reaction is somehow related to the level of NADPH in the metabolic pathways during the glycolysis process. At high pH values, the level of NADPH was considerably low which was resulted from the low glycolysis rate during yeast metabolism.

3.2. *Effect of agitation rate*

Theoretically, for a bioprocess system which requires a transfer of gaseous compound into the liquid phase such as in an aerobic fermentation as well as in gas phase biotransformation, mixing within the bioreactor becomes a major factor in order to attain high mass transfer between the gas and liquid phases. This can be achieved by applying several techniques. One of them is providing an adequate agitation mechanism by varying the stirrer speed. It has been reported in many bioprocess studies that high agitation rate gives negative impact towards the cell structure and enzyme

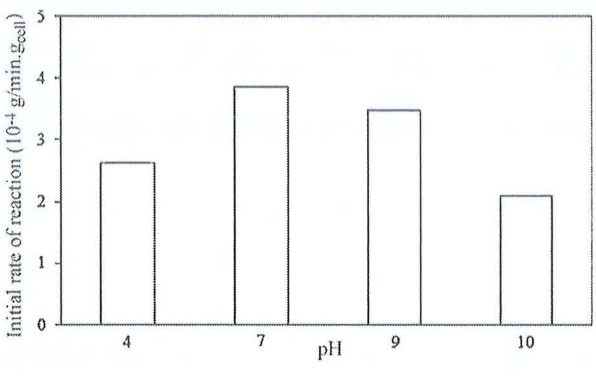

Fig. 2. Effect of pH on the initial reaction rate during the biotransformation of geraniol into citronellol by baker's yeast type-II in CCGLB system (conditions: 30 °C; agitation rate of 200 rpm; substrate flow rate of 8 L/min; 10 g/L of baker's yeast; 10 g/L of glucose).

able 1
ne initial reaction rates at different agitation rates and glucose concentrations.

Parameter	Value	Initial rate of reaction (10^{-4} g/min g_{cell})
Agitation rate (rpm)	200	3.85
	350	9.65
	500	0.73
Glucose concentration (g/L)	10	9.65
	30	11.23
	50	12.30

roduction due to the high shear stress, while low agitation rate auses insufficient mixing which in turn, led to the decrease in he overall productivity [17,18]. Therefore, the agitation rate needs o be optimized in order to ensure optimum productivity of the iotransformation.

The effect of agitation rate on the biotransformation of geranol in the CCGLB system was investigated in a range between 200 nd 500 rpm. The reactions were carried out with 10 g/L of baker's east at pH 7 and 30 °C. The substrate flow rate was fixed at 8 L/min vhile 10 g/L of glucose was used as the carbon source. The results rom Table 1 clearly show that when the agitation rate increases rom 200 to 350 rpm, an increase in the initial rate of reaction was btained. This is due to the fact that by increasing the agitation peed, the gaseous substrate reached the cell surface at a faster rate, hus decreasing the mass transport influence between the phases 19]. In the case of 200 rpm, cell sedimentation was clearly observed t the bottom of the vessel, which indicated an incomplete mixing vithin the bioreactor. This consequently led to the low biotransfornation rate. In this condition, only 0.60 g/L citronellol was formed, vhereas at 350 rpm, about 1.93 g/L citronellol was detected. Howver, a further increase in the stirrer speed from 350 to 500 rpm, nd hence the mixing rate of cell suspension, failed to increase he initial rate of reaction. A decline in the initial rate by 13-fold vas observed at 500 rpm with only 0.27 g/L citronellol produced ompared to that at 350 rpm. In this condition, the vigorous movenent of cells caused a turbulent environment in the fermentation broth with excess foaming appeared after approximately 12 h of eaction. This required the system to be shut-down. Surface active ubstances such as proteins, which have high surface activity and mphiphilic properties were reported as the foam-promoting compounds in the fermentation broth [20]. A small amount of foam can reate condition that may promote the lysis of cells which may n turn lead to an excessive amount of foam [21]. The decline in itronellol productivity was probably due to the damage of one or nore components of the yeast cells caused by excessive mechanical tress as well as the foaming effect. From this particular work, the best performance of the CCGLB system was obtained at 350 rpm vith the initial rate of reaction of 9.65×10^{-4} g/min g_{cell} and the pecific activity of 6.18 U/g_{cell}.

.3. Effect of substrate flow rate

In the CCGLB system, substrate in gaseous form was continuously sparged into the fermentation broth through a sparger. Besides the degree of agitation rate, mass transfer between the iquid and gas phases was also influenced by the substrate flow ate [22]. An initial hypothesis can generally be suggested regardng the effect of substrate flow rate. The higher flow of substrate vithin the bioreactor caused more substrate to come into contact vith the cells. Thus, there was an increase in the rate of citronellol production. In order to study the effect of substrate flow rate on the biotransformation rate, experiments were carried out at various substrate flow rates ranging from 2 to 8 L/min. 10 g/L of baker's yeast and 10 g/L of glucose were used in this work. Other experimental conditions were fixed at pH 7, 30 °C and 350 rpm.

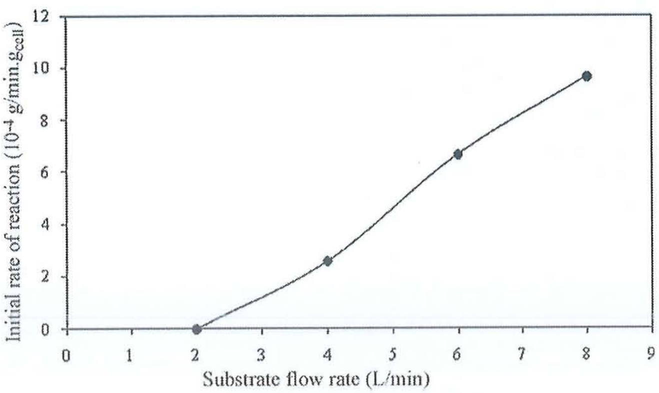

Fig. 3. Effect of substrate flow rate on the initial reaction rate during the biotransformation of geraniol into citronellol by baker's yeast type-II in CCGLB system (conditions: pH 7; 30 °C; agitation rate of 350 rpm; 10 g/L of baker's yeast; 10 g/L of glucose).

The results in Fig. 3 indicate that higher flow rates tend to give high initial rates. However, at 2 L/min and below, no trace of citronellol was detected in the product reservoir. At this condition, it was observed that only little bubbles were present from the sparger in the fermentation broth compared to the other stated conditions. This indicates that the substrate was incapable of dispersing into the fermentation broth at such flow rates, which resulted in undetectable reaction. As the flow rate was gradually increased from 4 to 8 L/min, the initial rate of reaction also significantly increased from 2.58×10^{-4} to 9.65×10^{-4} g/min g_{cell}. This can be explained, that high degree of flow rate increased the substrate concentration within the broth and therefore resulted in an increase of the frequency of contact between the substrate and the cell, which in turn enhanced the biotransformation rate. On the other hand, if the flow rate remained as low as 2 L/min, less interaction would be observed between the substrate and the cell. The maximum citronellol concentration and the specific activity at 8 L/min were determined as 1.93 g/L and 6.18 U/g_{cell}, respectively. It can be concluded from the results that the substrate flow rate gave a positive impact on the CCGLB system compared to the agitation effect. The increase in product formation promoted by the substrate flow rate suggests that the efficient dispersion of the substrate by diffusion in the biotransformation medium was a necessary prerequisite in order to obtain a high reaction rate.

A gas phase biotransformation is similar to that of an aerobic fermentation in the manner of the entering materials are modified or processed into the final biological materials. These processes have in common, fundamental principles of transport phenomena, particularly in terms of mass transfer mechanism of gaseous compound into the liquid phase. Theoretically, in an oxygen transferred system of a typical aerobic fermentation, the adequate transfer of oxygen could enhance the growth of cells as well as metabolites [23]. The process follows the principle of passive diffusion of molecules into the cell for metabolism as well as regeneration processes. A similar mechanism is believed to occur in the gaseous biotransformation process as well. This is accomplished when a sufficient amount of gaseous substrate diffuses into the cell, which consequently results in higher biotransformation productivity.

It is rather difficult to make a comparison between results obtained in this work to that from the current literatures because studies in this particular subject are scarce and if there are any, the results are presented in a different manner. For example, in solid/gas biotransformation systems, the main parameters investigated include; water activity and temperature instead of gaseous flow rate [24–26]. Similarly, biotransformation using a closed-gas-loop system as conducted by Pescheck and Steinig groups focused

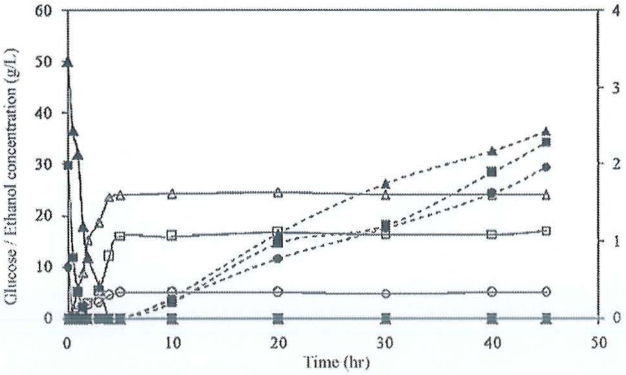

Fig. 4. Profiles of ethanol (solid lines, open markers), glucose (solid lines, closed markers) and citronellol (broken lines, closed markers) concentrations during the course of geraniol reduction by baker's yeast in CCGLB system at different initial glucose concentrations of 10 g/L (●,○), 30 g/L (■,□) and 50 g/L (▲,△). (Conditions: pH 7; 30 °C; agitation rate of 350 rpm; substrate flow rate of 8 L/min; 10 g/L baker's yeast.)

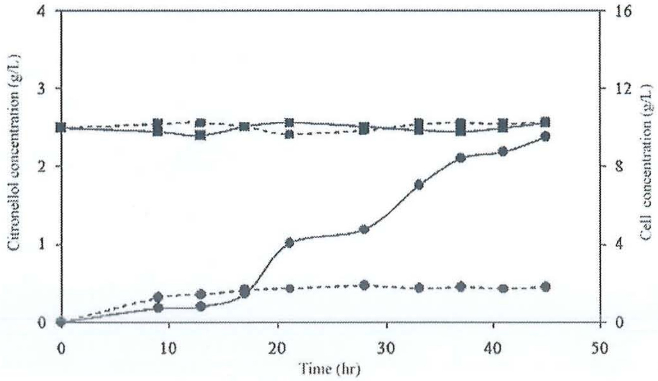

Fig. 5. Profiles of citronellol formation and cell concentration during the reduction of geraniol in liquid phase system (broken lines) and CCGLB system (solid lines). Symbols: (●) the citronellol concentration; (■) the cell concentration. Conditions for CCGLB: pH 7, 30 °C, 350 rpm, substrate flow rate of 8 L/min, 50 g/L glucose, 10 g/L baker's yeast. Conditions for liquid phase: pH 7, 30 °C, 150 rpm, 20 g/L geraniol, 50 g/L glucose, 10 g/L baker's yeast.

on the bioreactor design instead of optimizing the reaction conditions [11,27].

3.4. *Effect of glucose concentration*

In a microbial biotransformation, which involves reduction reaction, living cells either growing or resting, have an advantage of cofactor regeneration cycle within the cells. A reduction reaction by the whole cells requires NAD(H) and/or NADP(H) as cofactors, which are initially available in the cells. However, there is only a small amount of cofactors inside the cells and co-substrates such as glucose are necessary for recycling the cofactors through the metabolic pathways. Therefore, a sufficient amount of glucose should be supplied in order to achieve an optimum biotransformation rate. An extent of this parameter to the biotransformation rate was further investigated. Glucose concentrations ranging from 10 g/L to 50 g/L were selected and the performance of the resting cells of *S. cerevisiae* to catalyze the reduction of geraniol was monitored. Other experimental conditions were fixed at pH 7, 30 °C, agitation rate of 350 rpm and substrate flow rate of 8 L/min. Fig. 4 and Table 1 represent the results of these experiments. As shown in Table 1, the initial rate of reaction increased from 9.65×10^{-4} to 1.23×10^{-3} g/min g_{cell} when the glucose concentration was increased from 10 to 50 g/L. At 50 g/L glucose, the total production of citronellol was 2.38 g/L (Fig. 4) with the specific activity of 7.87 U/g_{cell} which was obtained within 45 h of reaction. At this particular level, 1.3-fold enhancement in the activity was achieved in comparison to that obtained with the lowest concentration of 10 g/L.

From the date plotted in Fig. 4, it was found that at the initial glucose concentration of 10 g/L, the rate of glucose consumption of 0.71 g/L min was determined. At 30 g/L, a longer time of 1 h was needed by the cells to metabolize the sugar with the glucose consumption rate obtained at 0.53 g/L min. However, with glucose concentration at 50 g/L, the compound was completely utilized within 4 h to give a rate of 0.21 g/L min. The glucose consumption showed high tendency of the cells to metabolize the supplied sugar for cellular activities including for use during the reduction of geraniol. Even though the biomedium was starved of glucose during the reaction, the product was still accumulated during the time course, indicated that the reaction had taken place. This can be explained by the accumulation of ethanol in the reaction medium which resulted from the glycolysis cycle in the yeast cells. A higher loading of glucose resulted in a higher amount of ethanol. According to Meitian and co-workers [28], when there were no carbohydrates such as glucose in the medium, ethanol would be used as the sole energy source in the reaction mediated by ethanol dehydrogenase in the yeast cells. The ethanol present would then be dehydrogenated to acetaldehyde and oxidized into acetic acid and carbon dioxide. At the same time, NAD^+ or/and $NAD(P)^+$ would be reduced to NADH and/or NAD(P)H by the oxidoreductase enzyme which would enhance the product yield.

An increase in glucose concentration is beneficial in some biotransformation processes by baker's yeast [29,30]. In other cases, the addition of the electron donor does not give any significant effect on the yield such as in ethylbenzoate reduction [31]. Nevertheless, it was reported that with the further extent of glucose concentration at certain levels, might result in the lowering of the biotransformation rate due to the accumulation of ethanol at high concentration levels. The increase of the amount of ethanol may inhibit the activity of the cells or the oxidoreductase enzyme for the reduction of the substrate [32,33]. In this present study, the increase in the amount of glucose tends to increase the production of citronellol. Therefore, it can be assumed that the ethanol effect was negligible during the reduction reaction as the residual ethanol in the liquid samples might be below the inhibition limits.

3.5. *Comparison of the CCGLB system to other biotransformation systems*

A preliminary study of geraniol reduction by *S. cerevisiae* was conducted in liquid phase system. The reactions were carried out with different initial concentrations of geraniol from 1 to 20 g/L. From these experiments, it was found that the higher substrate concentration resulted in the higher product formation and consequently an increase in the initial rate of reaction (data not shown). This system gave the maximum initial rate and specific activity of 1.28×10^{-4} g/min g_{cell} and 0.82 U/g_{cell}, respectively. As shown in Fig. 5, the cell concentration profiles give no significant effect to both systems, which indicates that there was no cell death within the reaction medium. However, it appears that the CCGLB system gives convincing results as the system achieved a 1.3-fold increase in the citronellol production over the shake-flask performance. Furthermore, the product continuously accumulates at the end of the reaction without any level-off behaviour compared to that of the liquid phase system. It can be considered that the transport of either geraniol into the cells and/or the citronellol out of the cells may be a factor that limits the biotransformation rate in the liquid phase system. The accumulation of those compounds inside the cells could block the active sites of the responsible enzymes. This hypothesis

able 2
omparison of citronellol production in the CCGLB with other biotransformation ystems.

Biotransformation system	Total product formation (g/L)	Cell activity (U/g_{cell})
Direct contact (shake-flask)[a]	0.46	0.82
Two-phase bioreactor [6]	N.A	0.05
Two-phase bioreactor [7]	N.A	0.30
Membrane bioreactor [6]	N.A	0.28
CCGLB	2.38	7.87

.A, not available.
[a] Preliminary study.

s supported by the article reported by Bishop and co-workers that aker's yeast possibly can retain essential oil in the cell cytoplasm 34]. From this result, it can be concluded that the CCGLB system pparently eliminates the mass transfer limitation. As a comparson, Table 2 summarizes the results from the literature using a imilar type of reaction system. The values apparently show that CGLB provides higher productivity compared to that of the twohase and membrane-based systems. Problems associated in the wo-phase system such as emulsion and substrate/product inhibiion [8] were completely resolved in the CCGLB. The reason may e attributed to better diffusion in the gas phase, thus eliminating nass transfer limitation within the cells. Furthermore, the increase n activity may be due to the increase in the solubility of the subtrate in gaseous form, therefore making it readily available to the ells for biotransformation [25,35].

. Conclusions

This present work is a comprehensive study on the reaction arameters influencing the biotransformation of geraniol in the CGLB system. The results show that this system could be applied n the biotransformation of monoterpenoids and might also be sed with other volatile organic compounds. The productivity of itronellol was highly dependent on the agitation and substrate low rates. It was also found that flow rate had the most prominent ffect on the system, as increasing the flow rate gave a positive mpact to the overall productivity. Product accumulation in the roduct reservoir appeared as a clear organic solution without any olid biocatalyst which eliminates the downstream processes such s centrifugation and filtration. This makes the CCGLB the best hoice if compared to other systems. It is believed that the data s important for a better comprehension of bioreduction since such nformation allows better control of the reduction reaction in a CGLB.

cknowledgements

The authors would like to thank USM for providing the hort-Term Grants (PJKIMIA/6035215) and subsequently PJKIMIA/6035281) to carry out the research work. Financial suport from the grants for A.A. Arifin persuing her MSc. Studentship s greatly acknowledged.

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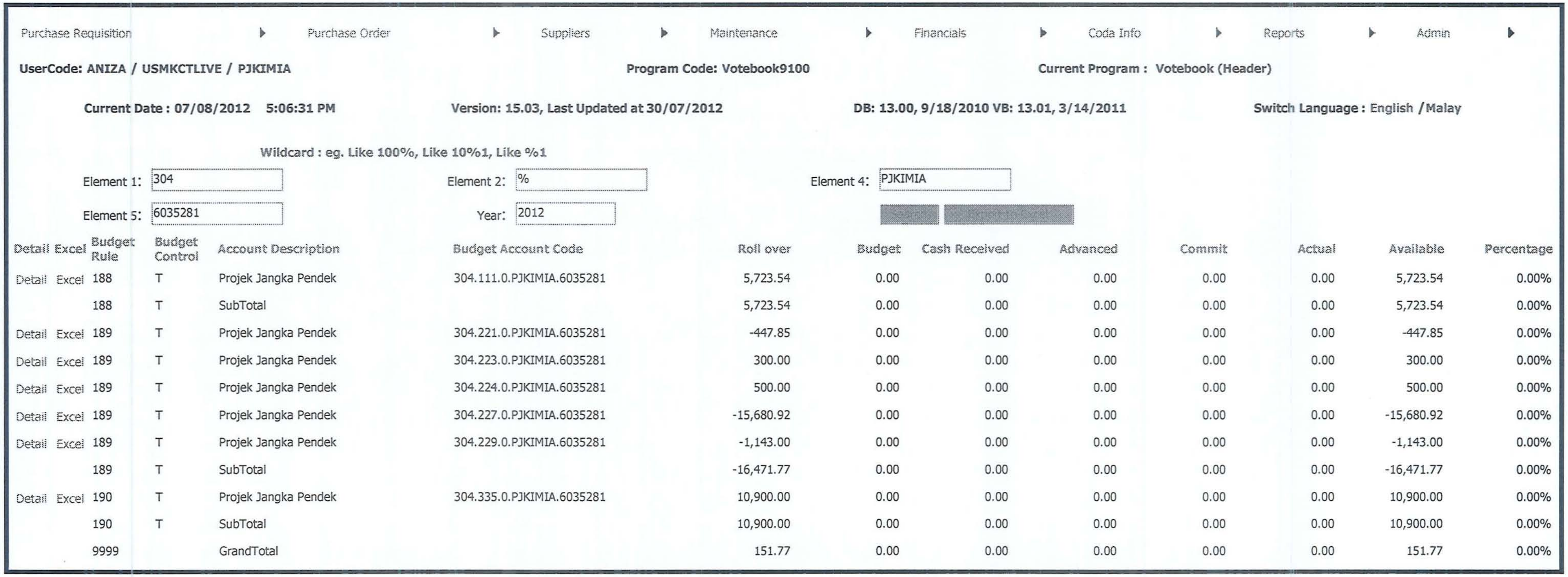
FINAL STATEMENT



UserCode: ANIZA / USMKCTLIVE / PJKIMIA **Program Code: Votebook9100** **Current Program : Votebook (Header)**

Current Date : 07/08/2012 5:06:31 PM **Version: 15.03, Last Updated at 30/07/2012** **DB: 13.00, 9/18/2010 VB: 13.01, 3/14/2011** **Switch Language :** English /Malay

Wildcard : eg. Like 100%, Like 10%1, Like %1

Element 1: 304
Element 2: %
Element 4: PJKIMIA
Element 5: 6035281
Year: 2012

Detail	Excel	Budget Rule	Budget Control	Account Description	Budget Account Code	Roll over	Budget	Cash Received	Advanced	Commit	Actual	Available	Percentage
Detail	Excel	188	T	Projek Jangka Pendek	304.111.0.PJKIMIA.6035281	5,723.54	0.00	0.00	0.00	0.00	0.00	5,723.54	0.00%
		188	T	SubTotal		5,723.54	0.00	0.00	0.00	0.00	0.00	5,723.54	0.00%
Detail	Excel	189	T	Projek Jangka Pendek	304.221.0.PJKIMIA.6035281	-447.85	0.00	0.00	0.00	0.00	0.00	-447.85	0.00%
Detail	Excel	189	T	Projek Jangka Pendek	304.223.0.PJKIMIA.6035281	300.00	0.00	0.00	0.00	0.00	0.00	300.00	0.00%
Detail	Excel	189	T	Projek Jangka Pendek	304.224.0.PJKIMIA.6035281	500.00	0.00	0.00	0.00	0.00	0.00	500.00	0.00%
Detail	Excel	189	T	Projek Jangka Pendek	304.227.0.PJKIMIA.6035281	-15,680.92	0.00	0.00	0.00	0.00	0.00	-15,680.92	0.00%
Detail	Excel	189	T	Projek Jangka Pendek	304.229.0.PJKIMIA.6035281	-1,143.00	0.00	0.00	0.00	0.00	0.00	-1,143.00	0.00%
		189	T	SubTotal		-16,471.77	0.00	0.00	0.00	0.00	0.00	-16,471.77	0.00%
Detail	Excel	190	T	Projek Jangka Pendek	304.335.0.PJKIMIA.6035281	10,900.00	0.00	0.00	0.00	0.00	0.00	10,900.00	0.00%
		190	T	SubTotal		10,900.00	0.00	0.00	0.00	0.00	0.00	10,900.00	0.00%
		9999		GrandTotal		151.77	0.00	0.00	0.00	0.00	0.00	151.77	0.00%