BIOTRANSFORMATI NG *SACCHAROMYCES CEREVISIAE*, *ASPERGILLUS NIGER*, *PSEUDOMONAS AERUGINOSA*, *ENTEROCOCCUS FAECALIS* AND *BACILLUS CEREUS*

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ABSTRACT

Benzyl acetone, a carboxylic derivative constitute a considerable percentage of the overall yield of essential oil extracted from *Aquilaria malaccensis* (agarwood) and thus, considered as one of the major constituents of agarwood oil. This study aimed at isolating benzyl acetone from essential oil of *A. malaccensis* using preparative GC (prep-GC); studying the fate of benzyl acetone when transformed using selected microorganisms commonly found in environment; developing efficient biotransformation system by optimizing parameters namely initial substrate and glucose concentration, initial pH and temperature of reaction. Essential oil of *A. malaccensis* was extracted using Microwave-Assisted Extraction (MAE) and preparative GC (prep-GC) was used to isolate benzyl acetone. Identity of isolated benzyl acetone was confirmed by comparing the peak to that of commercial benzyl acetone using GC-MS. The whole cells of *Saccharomyces cerevisiae*, *Aspergillus niger*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Bacillus cereus* were used for biotransformation of commercial benzyl acetone in this study. GC-MS was used to identify and quantify biotransformation product. Reduction of benzyl acetone to 4-phenyl-2-butanol occurred in all experiments using these five microorganisms. The optimum condition for the highest biotransformation activity using *S. cerevisiae*: 34.01 % conversion in 0.1 g/L benzyl acetone, 20 g/L glucose, pH 7.5 and 30°C; *A. niger*: 94.33 % conversion in 0.15 g/L benzyl acetone, 50 g/L glucose, pH 7.5 and 27°C; *P. aeruginosa*: 98.27 % conversion in 0.1 g/L benzyl acetone, 20 g/L glucose, pH 7.0 and 30°C; *E. faecalis*: 7.17 % conversion in 0.15 g/L benzyl acetone, 40 g/L glucose, pH 7.0 and 37°C; *B. cereus*: 9.84 % conversion in 0.15 g/L, 40 g/L glucose, pH 7.0 and 40°C. Biotransformation of benzyl acetone resulted in formation of 4-phenyl-2-butanol which is not naturally found in agarwood oil. *P. aeruginosa* was proven to be the most efficient biocatalyst for biotransformation of benzyl acetone to 4-phenyl-2-butanol, followed by *A. niger*, *S. cerevisiae*, *E. faecalis* and *B. cereus*.

ABSTRAK

Benzil aseton iaitu sejenis *carboxylic derivative* merupakan komponen yang merangkumi sebahagian besar daripada keseluruhan kandungan pati minyak yang diekstrak daripada *Aquilaria malaccensis* (agarwood). Kajian ini dijalankan dengan tujuan mengasingkan benzil aseton daripada minyak pati *A. malaccensis* menggunakan *preparative GC* (*prep-GC*); mengkaji hasil transformasi benzil aseton menggunakan mikroorganisma terpilih yang biasa didapati dalam persekitaran; membangun sistem biotransformasi yang cekap dengan mengoptimumkan parameter seperti konsentrasi asal substrat dan glukosa, pH dan suhu reaksi. Minyak pati *A. malaccensis* diekstrak menggunakan *Microwave-Assisted Extraction* (MAE) dan *prep-GC* digunakan untuk mengasingkan benzil aseton. Identiti benzil aseton terasing dikenalpasti melalui perbandingan dengan benzil aseton komersial menggunakan GC-MS. Keseluruhan sel *Saccharomyces cerevisiae*, *Aspergillus niger*, *Pseudomonas aeruginosa, Enterococcus faecalis* and *Bacillus cereus* telah digunakan untuk biotransformasi benzil aseton komersial dalam kajian ini. GC-MS digunakan bagi mengenalpasti produk biotransformasi yang terhasil. Kesemua eksperimen menggunakan kelima-lima mikoorganisma menunjukkan bahawa benzil aseton telah ditukarkan kepada bentuk 4-phenyl-2-butanol. Keadaan optimum dengan aktiviti tertinggi menggunakan *S. cerevisiae*: 34.01 % penukaran dalam 0.1 g/L benzyl acetone, 20 g/L glukosa, pH 7.5 dan 30°C; *A. niger*: 94.33 % penukaran dalam 0.15 g/L benzyl acetone, 50 g/L glukosa, pH 7.5 dan 27°C; *P. aeruginosa*: 98.27 % penukaran dalam 0.1 g/L benzyl acetone, 20 g/L glukosa, pH 7.0 dan 30°C; *E. faecalis*: 7.17 % penukaran dalam 0.15 g/L benzyl acetone, 40 g/L glukosa, pH 7.0 dan 37°C; *B. cereus*: 9.84 % penukaran dalam 0.15 g/L, 40 g/L glukosa, pH 7.0 dan 40°C. Biotransformasi benzil aceton menghasilkan 4-phenyl-2-butanol yang tidak wujud secara semulajadi dalam minyak pati gaharu. Kajian mendapati *P. aeruginosa* terbukti merupakan biopemangkin yang paling cekap untuk biotransformasi benzil aseton kepada 4-phenyl-2-butanol diikuti dengan *A. niger*, *S. cerevisiae*, *E. faecalis* and *B. cereus*.

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LIST OF SYMBOLS

<	Less Than
>	More Than
β	Beta
α	Alpha
δ	Delta
%	Percentage

LIST OF ABBREVIATIONS

GC	Gas Chromatography
MS	Mass Spectrometer
FID	Flame ionization detector
Prep-GC	Preparative Gas Chromatography
MAE	Microwave-Assisted Extraction
CET	Conventional Extraction Technique
HD	Hydrodistillation
MIC	Minimal Inhibitory Concentration
UV	Ultra violet
atm	Atmosphere
p	Pressure
h	Hour
min	Minutes
L	Liter
g	Gram
m	Meter
µl	Microliter
nm	Nanometer
M	Molarity
℃	Degree Celsius
pH	Hydrogen Ion Concentration
w/w	Weight by Weight
v/v	Volume by Volume
w/v	Weight by Volume

OD	Optical Density
NADH	Nicotinamide Adenine Dinucleotide Hydrogen
NAD(P)H	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NAD	Nicotinamide Adenine Dinucleotide
NAD(P)	Nicotinamide Adenine Dinucleotide Phosphate
GDP	Geranyl Diphosphate
GPP	Geranyl Pyrophosphate
ATP	Adenosine Triphosphate
AMP	Adenosine Monophosphate
Redox	Reduction/Oxidation
DNA	Deoxyribonucleic Acid
DNS	Dinitrosalicylic acid
RNA	Ribonucleic Acid
mRNA	Mitochondrial Ribonucleic Acid
m/z	mass-to-charge ratio
rpm	Revolutions per minute
FDA	Food and Drug Administration
NA	Nutrient Agar
ROS	Reactive oxygen species
AFB1	Aflatoxin B1
SC	Substrate control
CC	Culture control
RT	Room temperature
AHAS	Acetohydroxy acid synthase
NB	Nutrient Broth

PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth

CHAPTER 1

INTRODUCTION

1.1 RESEARCH BACKGROUND

Extraction of essential oil from the bark of *Aquilaria malaccensis* using hydrodistillation (HD) and microwave-assisted extraction (MAE) were compared for their yield and chemical composition. MAE was carried out at different power (W) and extraction time (h) to determine the best condition for high quality extraction. Benzyl acetone was isolated from essential oil of *A. malaccensis* using preparative gas chromatography (prep-GC) and subjected to gas chromatography mass spectrometry (GC-MS) analysis for comparison with commercial benzyl acetone.

The main part of this research would be on biotransformation of commercial benzyl acetone using whole-cells of selected microorganisms. Parameters, namely substrate and glucose concentration, pH and temperature were tested to determine the best condition for biotransformation of benzyl acetone. Biotransformation activity was measured based on the percentage of biotransformation products produced from initial substrate concentration. Identification and quantification was done using GC-MS analysis. Pattern seen in biotransformation activity was compared to that of residual glucose concentration to further understand the possible mechanism resulting in biotransformation of benzyl acetone.

1.2 PROBLEM STATEMENT

Agarwood oil has been prized for its unique smell thanks to complexity in its chemical profile. Researches have long been started to understand its formation by

giving focus on identifying marker compounds that contribute to the smell and possible mechanisms in trees that could have led to such formation. Lesser focus have been given on possibility that marker compounds might have formed as a result of chemical reaction of less complex compounds found in agarwood oil. Benzyl acetone is one of the simplest and earliest compounds in agarwood oil eluted on GC-MS that constitutes quite a major percentage of the total oil contents. Study have yet been done to link benzyl acetone and formation of marker compounds in agarwood oil.

Isolation of highly volatile compounds using conventional column chromatography such as benzyl acetone have been challenging and often resulted in loss of compound due to evaporation. Prep-GC promises better isolation of highly volatile compound with sharper resolution as compared to conventional method. Interaction of benzyl acetone with microorganisms commonly found in environment have not been studied so far. Such study is very important in order to learn if it is possible that interaction between these mircoorganisms and benzyl acetone can lead to formation of marker compounds in agarwood oil.

1.3 OBJECTIVES

This study was conducted with the aim to develop biotransformation system of benzyl acetone isolated from *A. malaccensis* oil using potential microorganisms. Several integrated objectives were constructed to achieve the main aim of this study and listed as follows:

1. To carry out isolation of benzyl acetone from *A. malaccensis* (Agarwood) essential oil using prep-GC technique.
2. To study the fate of benzyl acetone when transformed by microorganisms commonly found in the environment.
3. To develop an efficient biotransformation system by optimizing the parameters such as substrate concentration, glucose concentration, pH and temperature of the medium.

1.4 SCOPES OF STUDY

This study is an interdisciplinary, by which both principles of chemistry and microbiology are combined. The scopes involved:

1. Extraction of essential oil of *A. malaccensis* using hydrodistillation and MAE techniques.
2. Isolation of benzyl acetone from the oil using prep-GC; the peak of isolated benzyl acetone was compared to that of commercial benzyl acetone using GC-MS.
3. Use of commercial benzyl acetone as a substrate in biotransformation using microorganisms that have been screened beforehand for their ability to transform benzyl acetone.
4. Minimal inhibitory concentration (MIC) test on selected microorganisms to determine the suitable range of concentration of benzyl acetone to be used in experiments.
5. Optimization of parameters namely initial substrate and glucose concentration, pH and temperature of the medium to determine the best condition for efficient biotransformation of benzyl acetone.
6. Identification and quantification of biotransformation products using GC-MS.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION TO AGARWOOD

2.1.1 *Aquilaria* spp.

Aquilaria spp. belong to Thymelaeaceae family and commonly found in countries like Bhutan, India, Indonesia, Laos, Malaysia, Myanmar, Philippines, Singapore, Thailand and Vietnam. Among the species of *Aquilaria* that are commonly found in Malaysia are *A. malaccensis*, *A. beccariana*, *A. hirta* and *A. rostrata* (CITES, 2003); others, such as, *A. cumingiana*, *A. filarial*, *A. microcarpa*, *A. sinensis*, *A. crassna*, *A. bailonii* and *A. banaense* are among the most commonly found species in other countries (Barden *et al.*, 2002). According to The Plant List, 2010, there are at least 21 species of *Aquilaria* around the world. *Aquilaria* spp. populates wide range of area such as the rocky, sandy, calcareous, well-drained slopes and ridges and swamps. Highly valued for its fragrant resinous wood, some species of *Aquilaria* are traded in the form of wood, wood chips, powder and oil; this resinous wood is what known as agarwood, formed as result of plant defense mechanisms. However, the formation of agarwood is undetectable without cutting and splitting the tree, which has resulted in indiscriminate felling of Aquilaria tree and consequently, the drastic decline of its population.

2.1.2 *Aquilaria malaccensis*

A tree with a tall up to 40 m and diameter ranged from 1.5 to 2.5 m, *A. malaccensis* is among the most actively harvested and traded for its agarwood and this species is widely distributed in the mixed-forests of south and south east Asia. In Malaysia, except for the States of Kedah, it is found scattered throughout the Peninsular Malaysia and considered rare despite its good geographical coverage (Barden et al., 2002). Due to its drastic decline, *A. malaccensis* has been listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora, which obliges the member countries to issue CITES documents for any export activity that involves this plant species (CITES, 2003). Action has been taken by some of the member countries to keep the trading activities within the sustainable level. This include the extensive efforts in the cultivation of *A. malaccensis*, research for the production of artificial agarwood and the latest, development of technology to produce agarwood compounds using microbiological system.

2.1.3 Agarwood and its use

Agarwood or commonly known as Gaharu, has been widely used since the early time by Arab, Chinese, Indian and Southeast Asian community as incense, perfume and medicinal ingredients (Barden et al., 2002). It is believed that the formation of agarwood is due to the wounding or fungal infection on the tree that trigger the plant defense system; the specific mechanisms that involve in the formation however, is yet to be determined. The quality and quantity of resin produced by *Aquilaria* spp. is not consistent from one tree to the other and factors such as age of the tree and variations in the season, environment and genetic are believed to also contribute to such occurrence. Malaysia is one of the main producer of agarwood and has exported it to the destinations like Japan, Middle East and Taiwan.

2.1.4 Essential oil

Essential oil is defined as a volatile and aromatic liquid or semisolid which, generally constitute odorous principles of plants (Nor Azah et al., 2016). It is considered as a fragrant essence of plants in their purest, most concentrated state, most of which are

primarily composed of terpenes and respective oxygenated derivatives. Essential oil can be extracted from distillation of various parts of plants such as flowers, leaves, roots, barks, stems and wood. Distillation allows extraction of volatile and non-water soluble components of a plant that can be profiled using GC-MS and GC-FID. Apart from distillation, there are other techniques available for essential oil extraction such as, expression. Depending on the type and quality of starting material (plant), a different technique may be used.

Essential oil has been studied most from the viewpoint of its flavor and fragrance application. There is renewed interest in plant-derived essential oils, which are considered free from harmful synthetic additives. It is also an important component in religious and ritualistic occasion and believed to have healing power on human body, which made essential oil a subject of interest in medicinal application. Chinese has been using essential oil since ancient times along with the practice of acupuncture to treat various illness. Theoretically, pharmacological effects of essential oil are related to chemical changes that take place when the essential oil enters the bloodstream and react with hormones and enzymes.

2.1.5 Chemical constituents of agarwood oil

Previous work by Tajuddin and Yusoff, 2010 described the extraction of volatile oils from *A. malaccensis* by HD and analysis of its constituents using detailed gas chromatography-flame ionization detector (GC-FID) and gas chromatography/mass spectrometry (GC-MS) techniques. The study also showed that there were at least 31 fragrance-related compounds have been identified with benzyl acetone or benzyl acetone presented as one of the oil's major constituents (32.1%) along with jinkoh-eromol (6.5%) and α-guaiene (5.8%).

2.2 METHODS OF EXTRACTION

2.2.1 Hydrodistillation (HD)

History of distillation has first been recorded in the first century A.D during which the method is used for herbal extraction (Barden et al., 2002). Distillation of

essential oil has only begun around 1000 A.D when the Arab physician extracted the rose oil from rose petal. Since then, the study of chemical properties and development of a more refined distillation process have taken place among the Arab community. The European has only began producing essential oils in the 12th century. HD is one of the processes developed to serve the extraction of essential oil at laboratory and small industry scale. In laboratory, the Clevenger type apparatus is often an excellent tool, designed to improve the HD process. The sample, immersed in the water, is heated to boil for a certain period and the vaporized essential oil is condensed and trapped in a solvent.

The use of HD technique for the extraction of essential oil from *A. malaccensis* has long been popular and is an approved method for the quantification of essential oils (Nor Azah *et* al., 2016). The extraction may take from 12 to 24 hours to complete. The long extraction time, however, often consume a lot of energy and fuel which makes the whole process uneconomical. This slow and long process may not necessarily produce high quality oil since some of the essential volatile components in the oil may have been lost in the air. The thermal conduction and convection that take place in HD do not transfer the heat evenly to the whole sample, thus, making the extraction incomplete despite the long hours. The long contact with the direct source of heat could also burnt down the sample at the bottom part of the flask which will only cause further loss of essential chemical components.

2.2.2 Microwave-assisted extraction (MAE)

Extraction of essential oils from *Aquilaria* spp. has traditionally been done using HD method. Potential loss of volatile constituents, high energy use and long extraction time are among several notable drawbacks conferred by the adoption of this conventional method (Barden et al., 2002). Increase in the market demand for essential oils, on the other hand, is one of the chief factors that drive the industrial revolution in a way that development of an alternative technique that is rapid, sensitive, cost-effective and energy-efficient is highly desirable. Application of MAE has offered rectification to the problems confronted in HD by which, the former allows shorter extraction time, efficient energy use, improved extraction yield and reduced solvent consumption.

The use of MAE over HD for extraction of essential oils was proved to be advantageous by Jeyaratnam et al. (2016) in whose work, the comparison between these two methods was described. Comparison of these two methods was also demonstrated by Asghari et al., (2012) by which the constituents of the essential oils extracted from *Ferulago angulata* using both HD and MAE were compared. The result indicated that there is no significant difference observed between the constituents of essential oils extracted using both methods. Thus, it is appropriate to consider MAE as an alternative to conventional HD for generally, the former performs superiorly over the latter.

MAE is controlled by set of parameters that require characterization uniquely for each plant subjected to essential oil extraction. The extraction process in MAE is probably best described by Jeyaratnam et al. (2016) in whose work, the effects of operating conditions on the extraction of essential oil from *Cinnamomum cassia* by MAE was demonstrated. MAE has also been proven powerful as a tool in determining the accurate optimum values of experimental parameters as described in the extraction of pectin from waste *Citrullus lanatus* fruit rinds and owing to its special heating mechanisms, the interaction between variables is almost possible to evaluate with reduced number of experiments (Maran et al., 2013). These previous works have demonstrated MAE as a persuasive technique in helping the laboratory analyst to accomplish multiple quantitative sample extractions within minutes, with improved reproducibility.

The longer extraction time and high temperature used in the application of HD may not necessarily improve the quality of the essential oils and at some point could result in loss of the extract. Compared with HD, percentage of Z- β- Ocimene was found higher in the essential oils of *F. angulata* extracted using MAE despite the shorter extraction time confer by this modern technique (Asghari et al., 2012). The higher recovery of extract by MAE is granted by the complete disruption of the cells due to the high energy radiated from the microwave radiation, which is known for its ability to penetrate deep into biological materials. The shorter extraction time with better yield in application of MAE would make clear evidence in proving that this non-conventional method would be cost-effective and feasible for high productivity of essential oils, replacing the conventional use of HD.

2.3 TECHNIQUES OF CHEMICAL PROPERTIES ANALYSIS

2.3.1 Gas Chromatography (GC)

GC was described as an almost irreplaceable tool in the analysis of trace levels of components that may be contained in complex matrices (Seddik et al., 2017). Such complex matrices may include the materials derived from higher plants such as *A. malaccensis*. The use of GC for screening, identification and quantification of many groups of non-polar and semi-polar components was ardently stressed in the mentioned. The argument was based on the high separation power and a wide range of detectors employing various detecting principles conferred by GC. Generally, the steps involve in the analysis using GC include the introduction of the sample, followed by separation in the column, detection and identification as well as quantification.

Sample preparation is therefore, a critical step to determine the parameters of particular analytical method by which the matrix effect is reduced. This include the calibration of the method use as well as the optimization of the injection and separation system. Among the approaches that normally undertaken to calibrate the method use are the addition of standard or isotopically labelled internal standard and the use of matrix-matched standards to mask the active sites formed in GC system. Injection and separation system can be calibrated through the application of the appropriate injection technique, temperature and volume, liner size and its design, solvent expansion volume and column flow rate and its dimensions. Undertaking these steps will allow for a better analyte detection due to the reduced number of the active sites available for interactions and its duration. Quantification of the analyte will be made more accurate and is often done through a construction of calibrating curve using an external chemical standard (Seddik et al., 2017).

2.3.1.1 Gas Chromatography-Flame Ionization Detector (GC-FID)

A GC equipped with a FID works by detecting the components in the sample based on the measurable current produced by the charged particles in the gap between two electrodes in the detector (Grob and Grob, 1969). These charged particles are formed when the sample undergoes combustion in a hydrogen/synthetic air flame,

releasing ions and electrons. The current produced by these particles is relatively high to that of the pure carrier gas and the fuel gas flame; the information of the components in the sample is obtained based on the signal differential. Impurities such as hydrocarbon, moistures and oxygen could cause greater baseline noise; therefore, the sample preparation should include steps to reduce these interferences. Identification of the components in a sample analyzed by GC-FID is done using Kovats retention index system, which is based on the elution of a series of n-alkanes.

2.3.1.2 Gas Chromatography-Mass Spectroscopy (GC-MS)

The chemical composition of the essential oil of *A. malaccensis* has been extensively studied and assessed using GC-MS technique (Nor Azah et al., 2016). Depending on the origin, type and quality of *Aquilaria* sp., the chemical composition of its volatile components would vary. Benzyl acetone was one of the major components detected in the essential oil of *A. malaccensis* and the first to be eluted, according to the chromatogram produced. The use of GC-MS for identification and quantification of volatile component was extensively reviewed by Liu et al. (2009). The mass spectrum and retention time of agarwood oil extracted was checked using GC-MS and profiles of different samples were compared to determine the quality. Identification is normally done by comparing the peak of unknown to that of standard, commercially available in fine chemical market. Identical pattern in mass spectrum and retention time between these peaks will prove the identity.

2.4 METHODS OF ISOLATING A SINGLE COMPOUND

2.4.1 Preparative Gas Chromatography (Prep-GC)

Application of analytical instruments such as GC has long been ubiquitous and considered vital in analysis of compounds from various sample matrices which include essential oils from plants. The preparative works which covers fractionation of the mixtures and isolation of a singular component however, are rarely executed using prep-GC. This instrument is nevertheless, has been proven powerful as a tool for separation and isolation of organic compounds from complex mixtures without compromising its resolution quality. High-resolution isolation of compound performed by this instrument

is partly attributed to its enhanced column efficiency and the availability of different stationary-phase selectivity (Sciarrone et al., 2015). Prep-GC has so far been used to isolate sesquiterpenoids (Song et al., 2017), polycyclic aromatic hydrocarbons (Mandalakis and Gustafsoon, 2003), fatty acids, sterols, lipids, alkenes, alkanes (Eglinton et al., 1996), halogenated organic compounds (Holmstrand et al., 2006) and methoxylated polybrominated diphenyl ethers (Teuten et al., 2005).

Isolation of volatile compound such as monoterpenes by most of traditional methods is generally difficult, since such compounds could easily be lost through vaporization. Application of prep-GC on the other hand, allows the isolation of thermally and chemically unstable compounds which could be one of its major advantages over the conventional method (Sciarrone et al., 2015). Chrysanthemal, camphor, isolyratol, lyratol, chrysanthemol, chrysanthemyl acetate, fraganyl acetate, fraganol, and 2-isoprenyl-5-methylhexa-*trans*-3,5-dien-1-ol are among the reported monoterpenoids that have been successfully isolated from *Artemisia cana* ssp *viscidula* using prep-GC (Citoglu and Acikara, 2012). Previously, isolation of thermally unstable pheromone from German cockroach was accomplished using prep-GC with high recovery efficiency, owing to the use of cryogenic trapping (Nojima et al., 2004). Further improvement of the system that was carried out by simple modification of conventional GC and utilization of commercially available materials for trapping purposes has afforded >80 % trapping efficiency (Nojima et al., 2008).

Prep-GC is operating under a set of parameters that is similar to the conventional GC-MS. Despite the excellent performance shown by prep-GC, it is inevitable that its operating system requires an operator with strong knowledge on the instrumentation and refrigerant for cryogenic trapping. Previous studies have reported on the use of custom-made sample collection and desorption device to ensure efficient entrapment of the isolated compound in prep-GC system (Sciarrone et al., 2015). High recovery of the isolated compound by prep-GC has long been perceived as a result of the gradual temperature gradient along the collection trap (Brownlee and Silverstein, 1968 cited in Nojima et al., 2008); this theory is further supported by Grob and Grob (1969) cited in Nojima et al., (2008), describing the similar process in Grob splitless injection, by which the compounds are vaporized and condensed later to produce narrow band and sharp signals.

2.5 BENZYL ACETONE

Structure elucidation of benzyl acetone revealed that it is built up by 10 carbon atoms containing ketone group and thus, classified as a monoterpene ketone. The toxicity test carried out on benzyl acetone demonstrated that this compound exhibited pronounced effect against stored product insects (Yang et al., 2011). Benzyl acetone was also found to be one of the most abundant repellent and attractant compounds with sweet and flowery smell in *Nicotiana attenuata* (Kessler & Baldwin, 2006). Qi et al., (1998) demonstrated the production of benzyl acetone in *A. sinensis* upon infection by *Melanotus flavolivens* and its biotransformation into benzyl-2-propanol and benzyl carbinol, in association to plant protective mechanism.

Change of pattern in the release of benzyl acetone from plant has been described by Baldwin et al. (1997) and Kessler et al. (2010). Such naturally occurring mechanism was closely associated with the plant defense system, which works in favor of attracting the pollinators while at the same time avoiding the herbivores. On another note, Miyoshi et al. (2013) demonstrated the sedative effect of benzyl acetone by which it was explained through the reduction of locomotor activity of mice upon exposure to this compound. This claim may have done a great justice to the popular traditional Japanese culture of "listening to incense" since such practice has long been cherished for its relaxing effect on the mind and body.

2.6 BIOTRANSFORMATION AND MICROORGANISMS USED FOR BIOTRANSFORMATION

2.6.1 Biotransformation

Biotransformation is a chemical process where microorganism is used as a catalyst in the form of growing or resting cells. In some reactions, isolated enzymes are utilized as catalysts instead of whole-cell. Unlike traditional chemical synthesis, biotransformation involves series of reactions with high stereo- and regioselectivity, thus producing enantiomerically pure compounds (Cheng and Nian 2016). Initially, biotransformation was of pure academic interest, however some of the reactions are viable for industrial application. Considering the economic benefits, biotransformation

may be adopted as a functional part in chemical processes producing organic synthetic compounds.

Terpenes are found widely distributed in nature and usually existed as the constituents of essential oils. Mineralization of terpenes originated from higher plants by the soil microorganisms is common by which in such process the terpenes were subjected to oxidative metabolism, a crucial biochemical step in a naturally occurring carbon cycle. Monoterpenes however, particularly monoterpenoids are generally resistant to microbial degradation. Over the course of evolutionary events, some of the microorganisms have acquired the ability to degrade and live on these compounds. Several interesting rearrangements in the structure of monoterpenes achieved through biotransformation have been reported and in some of the reactions the monoterpenes were even ultimately degraded into carbon dioxide and water (Madyastha, 1984). Monoterpenes have recently gained a remarkable interest to be used as substrates for biotransformation because they are commercially available at low price. Currently, extensive efforts have been directed towards producing high valued fragrance compounds via microbial transformation of monoterpenes (Cheng and Nian 2016).

2.6.2 Cofactor regeneration in the whole cell biotransformation

Traditionally, the biotransformation is carried out using the whole cell as a biocatalyst. Advances in science and technology enable the isolation of enzymes which responsible in catalyzing certain chemical reactions from all kind of biological sources. The use of isolated enzyme in biotransformation is preferable to whole-cell biotransformation at the industrial level when there is limitation in substrate and product diffusion or occurrence of interferences such as side reactions caused by activity of other cellular enzymes (van Rossum et al., 2016).

However, some reactions involving more complex biocatalytic procedures are preferably conducted using whole-cell biotransformation system. Some of the enzyme reactions are cofactor dependent such as oxidation and reduction, where in these reactions, activity of oxidoreductases require NADH or NAD(P)H as cofactors. In whole-cell biotransformation system, these cofactors are continuously regenerated

through metabolic process. Biotransformation using isolated enzyme, on the other hand, require the addition of cofactor into the system which price can be very high.

2.6.3 Biotransformation of benzyl acetone

Recently, Cheng and Nian (2016) reported on enantioselective reduction of benzyl acetone into chiral 4-phenyl-2-butanol. Biotransformation of benzyl acetone was first reported by Qi et al., (1998). The biotransformation was carried out using fungus namely *Melanotus flavolivens* and the reaction has yielded mixture of 4-phenyl-2-butanol and phenylethyl alcohol. Based on the time course study, the formation of 4-phenyl-2-butanol was detected after 72 h or incubation whereas phenylethyl alcohol was detected after 96 h of incubation. The total reaction time was 240 h by which at the end of the experiment Benzyl acetone, 4-phenyl-2-butanol and phenylethyl alcohol were found in a percentage of 16 %, 35 % and 49 % respectively. A catabolic pathway was proposed and illustrated as follows:

Figure 2.1 shows that the carbonyl bond in benzyl acetone was first reduced to a single bond bearing hydroxyl group, forming 4-phenyl-2-butanol. According to Sofer and Martin (1987), the key enzymes capable of such catalytic activity are known as alcohol dehydrogenases. These enzymes comprise NADH- and NAD(P)H-dependent isozymes, catalyzing the oxidation of primary and secondary alcohols to aldehydes and ketones, as well as the reverse reaction. These reactions are also known as redox reaction. The carbon-carbon bond in 4-phenyl-2-butanol was then cleaved to form phenylethyl alcohol containing 8 carbons.

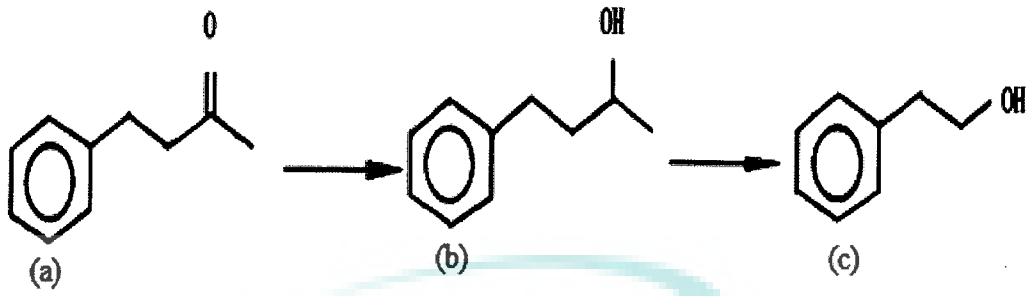

Figure 2.1: The proposed catabolic pathway in the biotransformation of Benzyl acetone (a) by *M. flavolivens* producing 4-phenyl-2butanol (b) and phenylethyl alcohol (c)

Source: Qi et al. (1998)

2.6.4 Biotransformation mediated by *Saccharomyces*

The fact that naturally, *S. cerevisiae* could not efficiently produce monoterpene due to the lack of monterpene synthases, is something noteworthy (Krivoruchko and Nielsen, 2015). However, *S. cerevisiae* possesses the ability to synthesize the phosphorylated form of geraniol, geranyl diphosphate (GDP) as an intermediate of farnesyl diphosphate synthesis, a key molecule in the isoprenoid pathway that leads to the synthesis of dolicils, ubiquinones and sterols. Widely known for its ability to carry out reduction activity, *S. cerevisiae* has been employed as a biocatalyst in many works that require reduction process to take place. Preparation of chiral alcohols through prochiral ketone reduction has been made possible with the use of *S. cerevisiae* as a biocatalyst, owing to remarkable specificity of its reductases toward monoterpenoid ketones; alcohol dehydrogenases are one of the several enzymes found to be responsible in carrying out such reaction involving carbonyl group.

2.6.5 Biotransformation mediated by *Aspergillus niger*

A. niger is categorized as black-spored species, prevalent in soil and typically associated with disease, namely black mold on fruits and vegetables. Researches on the use of *A. niger* for biotransformation of terpenoids have been extensively conducted as early as in 1960s. Among the enzymes which has been isolated from *A. niger* are amylases, proteases and lipases; these enzymes are of biotechnological importance and

mostly used in food production at industrial level (Parshikov and Sutherland, 2014). Although biotransformation of monoterpenes using *A. niger* has gained much interest recently, studies in this field are still limited in number especially when it comes to the monoterpene ketones and aldehydes. A study in the past showed that camphorquinone, a monoterpene ketone was readily reduced by *A. niger* into 2-*exo*-Hyroxy-epicamphor, 3-*endo*-Hydroxy-epicamphor and 3-*exo*-Hydroxy-epicamphor (Miyazawa et al., 1995).

2.6.6 Biotransformation mediated by *Pseudomonas*

P. aeruginosa may be considered as omnipresent since it is capable of living in diverse environmental conditions that include not only normal atmospheres but also hypoxic atmospheres. Owing to its availability in nature and ability to live on various types of substances, *P. aeruginosa* is therefore, has long been an interesting subject of the research, conducted particularly in the field of biotechnology. Among studies conducted on biotransformation of monoterpenes by *Pseudomonas* sp is the interesting transformation of limonene into α-terpineol (Esmaeili and Hashemi, 2011). Despite growing interest in the use of *Pseudomonas* for biotransformation, the number of studies conducted on the use of *P. aeruginosa* as a potential biocatalyst is still limited, especially in mediating the transformation of monoterpenes. Back in the 1960s, various species of *Pseudomonas* have been isolated from nature to be used as a biocatalyst for biotransformation of terpenes and their derivatives (Seubert, 1960).

2.6.7 Biotransformation mediated by *Enterococcus*

E. faecalis is probably among the most commonly studied species from the genus of *Enteroccoccus,* particularly for application in biotransformation. To the best of our knowledge, there is no report has been made yet on the use of *Enterococcus* sp. for the biotransformation of monoterpenes and therefore this thesis is probably the first to report the biotransformation of monoterpenoid, benzyl acetone using *E. faecalis*. *E. faecalis* is a Gram-negative bacteria, living in the mammals' gastrointestinal tract and capable of surviving under both aerobic and anaerobic conditions. The substantial interest in the study of *E. faecalis* especially in the medical field is probably attributed to its ability to tolerate extreme environments in gastrointestinal tract and resist highly toxic compounds such as bile salts (Ishibashi and Yamazaki, 2013), azo compounds

(Macwana et al., 2010; Lim et al., 2013; Madhuri and Girish, 2013; Kumar et al., 2013) and others.

2.6.8 Biotransformation mediated by *Bacillus*

The Gram-positive bacteria, *B. cereus* is found mostly dwelling in the soil environment and known as facultative anaerobe. This rod-shaped bacterium is also known for its peculiar ability to induce hemolysis and therefore can be easily differentiated from others when grown on blood agar. *Bacillus* sp. has recently become more significant in the field of biotransformation of organic compounds. However, there is still lack in number of published works when it comes to the use of *Bacillus* sp. for biotransformation of terpenes, particularly monoterpenes. (Liu and Rosazza, 1993).

CHAPTER 3

MATERIALS AND METHOD

3.1 OUTLINE OF METHODOLOGY

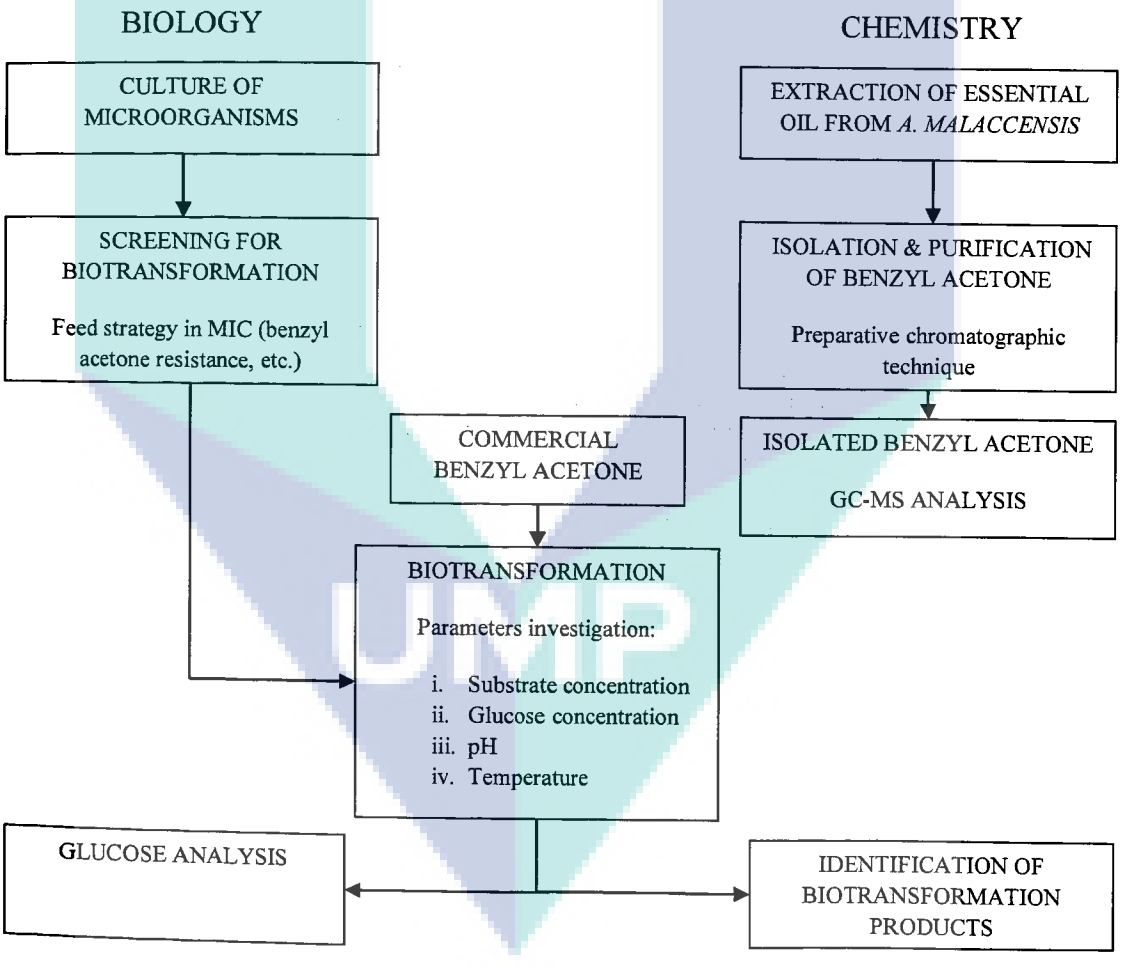

Figure 3.1: Flow chart of the overall experimental process.

3.2 INTRODUCTION

The research methodology was divided into two parts: chemistry and biology. The chemistry part included the steps taken in the specific methods for the sample preparation, extraction and analyses. The biology part included the steps taken in the specific methods for the culture preparation, biotransformation and analyses.

3.3 CHEMICALS

The chemicals used throughout the experiment were of at least, analytical grade as listed in Appendix A1.

3.4 SAMPLE PREPARATION

3.4.1 Sample collection

A. malaccensis was purchased from Mazlan Mohamed, who is a certified agarwood trader acknowledged by the Forestry Department, Malaysia and has grown the trees in Gua Musang, Kelantan Forest, Malaysia. Specimen (SK 2422/14) was sent for species identification and verified by Dr. Shamsul Khamis, Institute of Bioscience, Universiti Putra Malaysia, Malaysia. Preparation started with the collection of the high quality resin impregnated wood of *A. malaccensis*.

3.4.2 Drying

The barks of *A. malaccensis* were cut into chips and air dried. The chips were further cut into smaller pieces to increase the surface area and dried in a universal oven at 40 °C until the weight was constant.

3.4.3 Grinding

The dried wood chip was further milled to form ground wood with the sizes smaller than 1 mm and dried again at 40 °C in a universal oven, for three hours prior to storage in a sealed plastic bag at room temperature (RT) for later use.

3.4.4 Soaking

A sample of 100 g of ground wood was soaked in 1 L of double distilled water for 1 week at RT with a ratio of agarwood to water, equivalent to 1:10 (Tajuddin and Yusoff, 2010). The soaked agarwood would be subjected to extraction process.

3.5 EXTRACTION METHOD

To extract the essential oil from *A. malaccensis*, two methods were used, namely HD and MAE. HD was carried out based on the method outlined by Tajuddin and Yusoff (2010). The parameters tested were extraction time for both methods and microwave power for MAE.

3.5.1 Hydrodistillation (HD)

The soaked agarwood was transferred into 2 L round bottom flask and subjected to HD using Clevenger type-apparatus for 2, 4, 8, 12 and 24 h. 5 ml of n-hexane of analytical grade (99%) was used to contain the extracted oil. The extract was dried over anhydrous sodium sulphate (Na_2SO_4) and blown with a stream of industrial grade nitrogen (N_2) gas to remove the solvent. The extraction was performed in triplicate and the essential oils were stored in the amber vials with Teflon sealed cap at −4 °C until further analysis using GC-MS.

3.5.2 Microwave-assisted extraction (MAE)

Sample of 100 g soaked agarwood was transferred into 2 L round bottom flask and subjected to MAE which was performed using Clevenger type-apparatus, attached to a gravity microwave (MILESTONE, USA) with a maximum power of 800 W. The microwave was equipped with Easy-WAVE software that enables the control of time, temperature, pressure and power. Temperature was monitored by a shielded thermocouple (ATC-300) that was directly inserted into the sample container and by an external infrared (IR) sensor. Temperature was maintained by a feedback to the microwave power regulator. The MAE was conducted at 200, 300, 400, and 500 W for a period of 2, 3, 4, and 5 h. 5 mL of industrial grade n-hexane (99%) was used to

contain the extract. The solution was treated with Na_2SO_4 to eliminate the water content and carefully filtered afterwards. An empty vial was weighed before filled up with the extract (filtrate). The extract was blown with a stream of industrial grade nitrogen (N_2) gas to remove the solvent. The extraction was performed in triplicate and the essential oils were stored in the amber vials with Teflon sealed cap at −4 °C until further analysis using GC-MS. The results were reported in a manner of g of essential oil per 100 g of dried agarwood.

3.6 ANALYSIS TECHNIQUE

3.6.1 GC-MS

The samples of essential oil obtained through all experimental designs were analyzed using GC, equipped with MS (Agilent, USA), with a NON POLAR DB-1MS column (60 m x 0.25 mm, film thickness 0.25 µm). The system ran on the injection volume of 1 µl with split ratio 1:5 and helium as a carrier gas at 1.0 ml/min constant flow mode. The column temperature was programmed from 60 to 240 °C at 3 °C/min, with injector temperature set at 250°C. The mass detector operated in electron impact mode at 70 eV, with detector temperature 250°C. The mass spectra recorded in the range of 50-500 a.m.u.

3.6.2 GC-FID

Essential oil obtained through the best experimental design was analyzed using GC, equipped with FID (Agilent, USA), with a NON POLAR DB-1MS column (60 m x 0.25 mm, film thickness 0.25 µm). The purpose was to identify the area on the chromatogram for the isolation of a single compound using prep-GC. The system ran on the injection volume of 1 µl with split ratio 1:5 and helium as a carrier gas at 1.0 ml/min constant flow mode. The column temperature was programmed from 60 to 240 °C at 3 °C/min, with injector and detector temperature set at 250°C.

3.7 ISOLATION OF BENZYL ACETONE

3.7.1 Prep-GC

Isolation of Benzyl acetone from the essential oil of *A. malaccensis* was carried out using prep-GC (GERSTEL, USA) equipped with flame ionization detector (FID) and DB5 column (60 m x 0.25 mm, film thickness 0.25 μm). This system is endowed with three channels electronic pressure control module which purpose was to provide sufficient pressure to the Deans switch. The peak of interest eluted from the separation column outlet was isolated by micro-fluidic Deans switch, which also works to direct the flow to FID for chromatogram development and to the trapping capillary. The purity of the isolated peak and its identification was confirmed and verified using GC-MS.

3.7.2 Identity verification using GC-MS

The method described in 3.5.1 was applied for the verification of benzyl acetone identity by which, the peak of the isolated compound was compared to the standard solution of benzyl acetone.

3.8 CULTURE PREPARATION

3.8.1 Microorganisms

The microorganisms are choosed based on their availability and accessibility in the nature as mentioned in numerous works done in the past. Six bacteria: *Pseudomonas aeruginosa* (ATCC 1542), *Escherichia coli* (ATCC 10536), *Enterococcus faecalis* (ATCC 14506), *Bacillus subtilis* (ATCC 11774), *Bacillus cereus* (ATCC 1178) and *Staphylococcus aureus* (ATCC BAA-1026) and four fungi: *Saccharomyces cerevisiae* (ATCC 56763), *Aspergillus niger* (ATCC 16404), *Candida albicans* (ATCC 10231), *Trichoderma reesei* (ATCC 56763) were obtained from Culture Collection of Microbiology Laboratory, Universiti Malaysia Pahang.

3.8.2 Sterilization of glassware and plasticware

Glassware and plasticware used throughout the experiment were autoclaved at 121°C for 20 minutes.

3.8.3 Culture media preparation

Nutrient agar (NA) and Nutrient Broth (NB) were used for bacterial growth while Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB) were used for fungal growth. The pH was adjusted using 0.1 M NaOH and 0.1 M HCl. The agar plates were prepared as follows:

i. **NA**

1.0 L of NA was prepared by adding (g/L): peptone (10.0), beef extract (10.0) and NaCl (0.5) into 1.0 L of distilled water. Agar was added to the final concentration of 1.5%. The mixture was dissolved and the pH was adjusted to 7.0±0.2 and autoclaved at 121°C for 15 minutes.

ii. **NB**

1.0 L of NB was prepared by adding (g/L): peptone (10.0), beef extract (10.0) and NaCl (0.5), into 1.0 L of distilled water. The mixture was dissolved through heating. The pH was adjusted to 7.0±0.2 and autoclaved at 121°C for 15 minutes.

iii. **PDA**

36.0 g of PDA powder was weighed and added to 1.0 L of distilled water. The solution was heated with frequent agitation and boiled for 1 minute to dissolve the medium. The pH was adjusted to 7.0±0.2 and autoclaved at 121°C for 15 minutes.

iv. **PDB**

24.0 g of PDB powder was weighed and added to 1.0 L of distilled water. The solution was heated with frequent agitation and boiled for 1 minute to

dissolve the medium. The pH was adjusted to 7.0±0.2 and autoclaved at 121°C for 15 minutes.

3.8.4 Screening of microorganisms for biotransformation of benzyl acetone

The screening was conducted in 7 ml vial containing a total of 5 ml working volume of NB (bacteria) and PDB (fungi), added with 0.05 g/L commercial benzyl acetone (substrate) and the respective microorganisms. A culture control (CC) experiment was conducted using the same reaction mixture without the addition of the substrate to check the production of biotransformation product in each microorganism tested. The possibility of auto reduction of benzyl acetone was also tested by setting up a substrate control (PC) experiment using the same reaction mixture without the addition of the microorganism. The experiment was carried in incubator shaker at 37°C (bacteria) and 30°C (fungi), 150 rpm. The reaction using bacteria was left for 4 days while the reaction using fungi was left for 7 days; one vial was taken out per day and subjected to analysis using GC-MS. The results were compared to that of the controls.

3.8.5 Minimal Inhibitory Concentration (MIC) Test

Microorganisms with the ability to transform benzyl acetone were subjected to MIC. Feed strategy was used to determine the MIC of substrate on the selected microorganisms. The concentration benzyl acetone used was (g/L): 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2. The bacterial culture was subjected to incubation at 37°C for 1 day while the fungal culture was subjected to incubation at 30°C for 4 days. The control for each experiment was conducted using a broth media in the absence of microorganisms. Inhibition on bacterial culture was investigated by adding benzyl acetone of known concentrations into the broth containing bacterial culture whereas, inhibition on fungal culture was examined by growing fungi on agar, added with known concentrations of benzyl acetone; the former was checked using spectrophotometer while the latter was checked by measuring the diameter of the colony. This MIC test was conducted to determine the suitable working range of substrate concentration to be used in the biotransformation of Benzyl acetone using the selected microorganism.

3.9 BIOTRANSFORMATION

3.9.1 Biotransformation media preparation

Biotransformation was carried out in a complex medium specifically developed to support the growth of each microorganism used throughout the experiment. Extracts used in medium preparation were purchased from suppliers. The pH was adjusted using 0.1 M KOH and 0.1 M HCl. The specialized media were prepared as follows:

i. ***S. cerevisiae***

The Yeast Peptone Glucose medium was prepared, containing 5 g of yeast extract, 5 g of peptone and (0, 10, 20, 30, 40, 50) g of glucose in 1 L of distilled water. The pH was adjusted to 7.0±0.2 and autoclaved at 121°C for 15 minutes.

ii. ***A. niger***

The Malt Extract (ME) medium was prepared according to Demyttenarae and Willemen (1998) with some modifications which comprised of 5 g of peptone, 5 g of malt extract and (0, 10, 20, 30, 40, 50) g of glucose in 1 L of distilled water. The pH was adjusted to 7.0±0.2 and autoclaved at 121°C for 15 minutes.

iii. ***P. aeruginosa***

The medium was prepared according to Esmaeili and Hashemi (2011) with some modifications which comprised of 3 g of malt extract, 3 g of yeast extract, 5 g of peptone and (0, 10, 20, 30, 40, 50) g of glucose in 1 L of distilled water. The pH was adjusted to 7.0±0.2 and autoclaved at 121°C for 15 minutes.

iv. ***E. faecalis***

The medium was prepared according to Madhuri and Girish (2013) with some modifications which comprised of 1.5 g of yeast extract, 1.5 g of beef extract, 5 g of peptone, 5 g NaCl and (0, 10, 20, 30, 40, 50) g of

glucose. The pH was adjusted to 7.0±0.2 and autoclaved at 121°C for 15 minutes.

v. ***B. cereus***

The medium prepared was comprised of 5 g of yeast extract, 5 g of peptone, 5 g of NaCl, 5 g of K_2HPO_4 and (0, 10, 20, 30, 40, 50) g of glucose in 1 L of distilled water. The pH was adjusted to 7.0±0.2 and autoclaved at 121°C for 15 minutes.

3.9.2 Biotransformation of benzyl acetone in shake-flask culture

The 250 ml conical flasks were used for all experiments conducted with 100 ml of working volume of biotransformation media, unique for each microorganism. 1 ml of *S. cerevisiae*, *P. aeruginosa*, *E. faecalis* and *B. cereus* with optical density (OD) of 1.0 was aseptically inoculated to respective biotransformation media prior to incubation in incubator shaker, operated within the designated parameters. Inoculation of *A. niger* into the biotransformation medium on the other hand, was executed using 1 ml of spore suspension containing 1 x 10^8 spores/ ml. The induction of Benzyl acetone was performed in the middle of exponential phase respectively of each microorganism's growth. The reaction using *S. cerevisiae* and *A. niger* was left running for 7 days while the reaction using *P. aeruginosa*, *E. faecalis* and *B. cereus* was left for 4 days.

The parameters scrutinized are outlined as follows:

i. *S. cerevisiae*

Substrate concentration (g/L)	: 0.05, 0.1, 0.2, 0.4, 0.6, 0.8
Glucose concentration (g/L)	: 0, 10, 20, 30. 40. 50
Initial pH	: 6.5, 7.0, 7.5, 8.5, 9.0
Temperature (°C)	: 27, 30, 37, 40

ii. *A. niger*

Substrate concentration (g/L)	: 0.005, 0.05, 0.1, 0.15, 0.25, 0.4
Glucose concentration (g/L)	: 0, 10, 20, 30. 40. 50
Initial pH	: 6.5, 7.0, 7.5, 8.5, 9.0

Temperature (°C) : 27, 30, 37, 40

iii. *P. aeruginosa, E. faecalis and B. cereus*

Substrate concentration (g/L) : 0.005, 0.05, 0.1, 0.15, 0.25, 0.35

Glucose concentration (g/L) : 0, 10, 20, 30. 40. 50

Initial pH : 6.5, 7.0, 7.5, 8.5, 9.0

Temperature (°C) : 27, 30, 37, 40

3.9.2.1 Glucose analysis

Dinitrosalicylic (DNS) colorimetric method was adopted to determine glucose concentration by which the presence of reducing sugars is detected. Oxidation of glucose while simultaneously reducing 3,5-dinitro salicylic acid into 3-amino, 5-nitrosalicylic acid caused change in color of the assay. The intensity of the color is proportional to the amount of reducing sugar in the sample and to the OD read using spectrophotometer (Genesys™, USA).

3.9.2.1.1 Preparation of DNS reagent

DNS reagent was prepared in a dark room and stored in a dark Schott bottle. 40 g of NaOH pellets was dissolved in 200 ml of distilled water and the solution was left to cool down before the addition of 10 g of DNS and 2 g of phenol. The mixture was heated on the hotplate (FAVORIT, Malaysia) and stirred adequately using a magnetic stirrer to form a homogenous solution. Simultaneously, 300 g of potassium sodium tartrate was dissolved separately in 300 ml of distilled water and later, the two solutions were mixed together and cooled to RT. The DNS reagent was further diluted up to 1 L with distilled water and stored until further use.

3.9.2.1.2 Measurement of glucose level

Calculation of the glucose concentration in the sample tested was based on the calibration curve generated using known concentrations of glucose as illustrated in Appendix B3. 1 ml sample taken out from the reaction broth at certain time intervals was centrifuged at 6000 rpm, RT for 5 min to sediment the cells in the broth. The broth

was transferred into a new tube and added with 1 ml of DNS reagent. The mixture was boiled for 5 min and cooled down to RT before checked for its OD at a wavelength of 550 nm. Samples were diluted to fit the range of readable concentration by spectrophotometer.

3.9.2.2 Identification of chemical compound using GC-MS

4 ml was subjected to liquid-liquid extraction procedure which purpose was to extract the organic content which comprised of substrate and the corresponding biotransformation product from the reaction broth. The extraction was executed by adding n-hexane (3 x 2 ml) to the sample prior to homogenization using vortex mixer (FINEPCR, Korea) and centrifugation at 6000 rpm for 20 min in centrifuge (Hermle, Germany). The organic layer was further treated with anhydrous sodium sulphate and filtered using Whatman No. 1 filter paper. The filtrate was air-dried to remove solvent. Identification of the biotransformation product was carried by GC-MS using the same method described in 3.7.2 by which, the peak of the biotransformation products were compared to the standard of benzyl acetone and 4-phenyl-2-butanol.

3.9.2.3 Quantification of biotransformation product

The concentration of biotransformation product was calculated according to the equations obtained from the calibration curve constructed using the known concentrations of the benzyl acetone and 4-phenyl-2-butanol standards.

The same method described in 3.7.2 was used to identify the chemical compound produced by biotransformation of benzyl acetone by which, the peak of the biotransformation products were compared to the standard of benzyl acetone and 4-phenyl-2-butanol.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 INTRODUCTION

The oil extracts of *A. malaccensis* obtained through HD and MAE were compared for their yield and chemical composition based on the analysis carried out using GC-MS. The integrity of benzyl acetone isolated using prep-GC and its stability was carefully assessed. Also discussed is the capability of certain microorganisms to biotransform benzyl acetone and the optimum conditions that would best support the reaction. The efficiency of biotransformation was measured by the quantity of the products, growth of the microorganisms and the glucose consumption.

4.2 EXTRACTION OF ESSENTIAL OIL OF *A. MALACCENSIS*

4.2.1 Hydrodistillation (HD)

Figure 4.1 shows the increasing pattern in the yield of essential oil extracted from *A. malaccensis* by HD as the extraction time increased from 2 to 12 h by which the highest yield obtained was 0.2 % (w/w), after 12 h of extraction in this experiment. Further increase in extraction time was not necessary since the purpose was to observe the difference in extraction yield over time using HD to that of MAE.

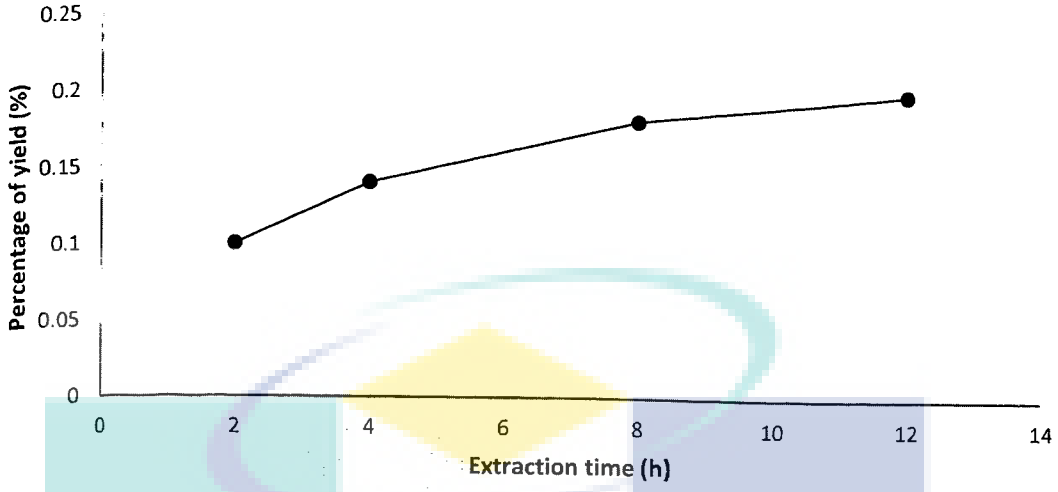

Figure 4.1: The yield of *A. malaccensis*' oil extracted by HD for a certain range of extraction time.

4.2.2 Microwave-Assisted Extraction (MAE)

The result in Figure 4.2 shows that at 200 to 500 W, the yield of essential of *A. malaccensis* rendered by MAE has generally increased with increase in extraction time from 2 to 5 h; the highest yield obtained was at 500 W and 5 h with a value of 0.24 % (w/w). A reverse pattern was observed when a higher power was applied which indicated the loss of the system's efficiency. A distinct increasing pattern can be seen in the percentage of yield obtained by MAE when the power increased from 200 to 500 W. The technique adopted for essential oil extraction indisputably influences the yield of extraction to a remarkable extent. Comparing to extraction yield rendered by HD as previously observed in Figure 4.1, it is apparent that MAE has granted a remarkable reduction in energy and time consumption which eventually allowed a cost-effective production.

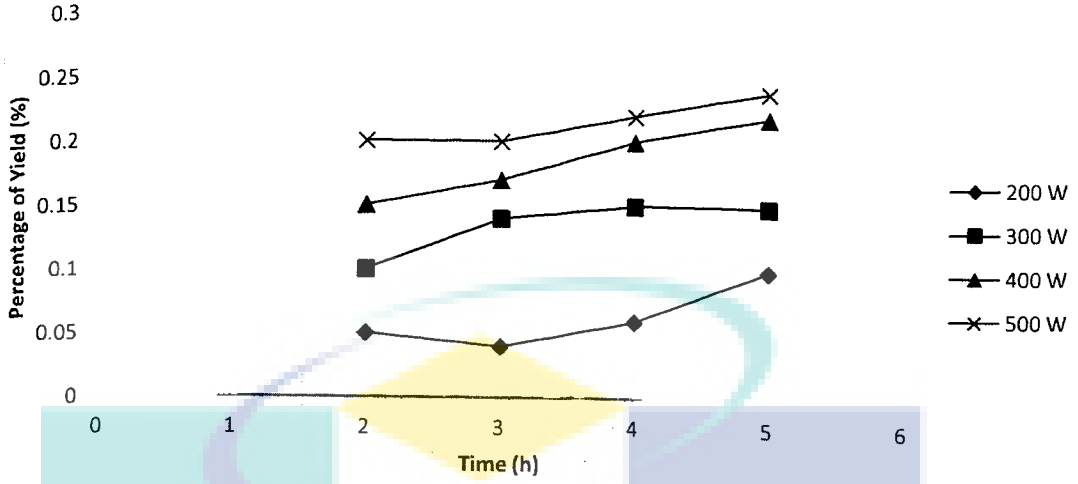

Figure 4.2: The yield of the essential oil of *A. malaccensis* obtained by MAE at different power and time.

Woody plant including *A. malaccensis* possesses complex cellular structure and arrangement which includes primary cell walls- embedding the growing and dividing cells as well as providing mechanical strength to the plant- and layers of secondary cell walls- encapsulating the mature cells and account for most of the carbohydrate in biomass ; extraction of essential oil from *A. malaccensis* is therefore achieved only by disrupting these cell walls which are typically protected by incorporated lignin. Yield of extraction is thus greatly affected by the level of cell walls disruption ensued during the extraction process.

The mechanism of heating involved during extraction of essential oil using MAE can be defined by the electromagnetic nature of the microwaves which operated under the influence of electric and magnetic field that worked perpendicular to each other. While heat conduction and convection in traditional HD could not afford a complete extraction of essential oil the use of MAE on the other hand granted a localized superheating by dipolar rotation and ionic conduction which effectively heated the entire sample simultaneously (Jeyaratnam et al., 2016). The mode of heat transfer in MAE allowed a complete disruption of the plant cells which eventually resulted in the release of the essential oil from the oil glands; the same result could not be produced using HD since the disruption of the plant cells were usually incomplete due to the limitation in transferring the heat to the whole sample.

Essential oil is usually distinguished by its color and scent; study of the essential oil's physical properties is therefore deemed important especially in determining its quality since the physical properties of an essential oil are firmly associated with its chemical profiles. The essential oil obtained from *A. malaccensis* in this study was of yellowish brown in color by which the result concurred to the earlier conjecture that the color of essential oil of *Aquilaria* spp. may vary from greenish brown to dark reddish brown (Nor Azah et al., 2008). Complex woody aroma released to the air during the extraction process which we deduced distinctively depicting agarwood aroma was faultlessly coming out of essential oil extracted from *A. malaccensis*. Similar peculiar spectrum of agarwood aroma emitted from essential oil of *Aquilaria* spp. has been reported multiple times in preceding studies (Nor Azah et al., 2008).

4.3 GC ANALYSIS OF ESSENTIAL OIL OF *A. MALACCENSIS* FROM HD AND MAE

Generally, it can be seen that MAE at 500 W gave a better recovery of chemical components compared to that of the lower power for all extraction time tested. The highest number of chemical components recovered through MAE was at 500 W for 5 hours of extraction which may be due to better cell disruption by MAE. The major compounds found in the extracts collected from MAE at the power of 200 – 300 W for 3 – 5 hours were common in profile to each other with slight variations in the percentage. The number of chemical compounds identified in the extracts seemed to generally increase with the increase in power and extraction time. MAE at 500 W for 5 hours has produced the best result with 49 compounds, the highest number found in comparison to the lower power and shorter extraction time tested. There were 17 chemical compounds that were found in the extracts from all designated experiments.

Based on the GC-MS analyses, it can be safely deduced that most of the chemical components in the essential oil of *A. malaccensis* was successfully retained by MAE in comparison to HD, considering the former gave out 49 compounds while the latter, only 42 compounds. It is important to note that such result was produced by HD only after 12 hours of extraction time while that of MAE only required 5 hours which significantly marked the reduction in extraction time by MAE without compromising the quality of the essential oil. The main compounds found in the essential oil extracted

through HD were benzyl acetone (7.0%), epoxybulsenene (6.5%), caryophyllene oxide (5.8%), guaiol (4.6%), 10-epi-γ-eudesmol (2.4%), agarospirol (9.0%), dehydrojinkoh-eremol (7.4%), and n-hexadecanoic acid (11.5%) which has pretty much concurred with previous finding (Tajuddin et al., 2013).

Despite the similarity observed between chemical composition of essential oil extracted by MAE and that of HD, distinction in distribution of the constituents was apparent. While adoption of MAE at 500 W for 5 hours in this study has allowed a relatively robust recovery of highly volatile benzyl acetone with a percentage of 11.5%, extraction using HD often resulted in small percentage of benzyl acetone recovered in agarwood oil (Tajuddin et al., 2013). Asghari et al. (2012) reported that a better percentage of major constituents of essential oil were obtained through the employment of MAE as opposed to HD. Conventional technique, particularly HD, often encountered difficulty in retaining compounds with high volatility due to inefficient control of temperature and long extraction period which sometimes can be up to several days. Longer extraction time and inefficient heating could lead to loss of volatile compounds through evaporation (Jeyaratnam et al., 2016).

4.4 ISOLATION OF BENZYL ACETONE USING PREP-GC

Benzyl acetone was isolated in this study, from essential oil of *A. malaccensis* using an advance technique known as prep-GC. GC-FID chromatogram of essential oil extracted from *A. malaccensis* was used as a basis for the collection of fraction containing benzyl acetone (Appendix B2). The fraction collected from prep-GC was subjected to analysis using GC-MS by which a single peak was produced, as shown in Figure 4.3. The peak was identified as benzyl acetone, in reference to the external chemical standard, ran on the GC-MS within the same parameter (Appendix B1 and B4). The result has proven that application of prep-GC could allow a direct transfer of retention times given that both prep-GC and analytical GC uses the same type of column and GC condition. The result has also demonstrated an excellent performance of prep-GC in isolating a single compound with high volatility which is hardly a case in conventional fractionating column.

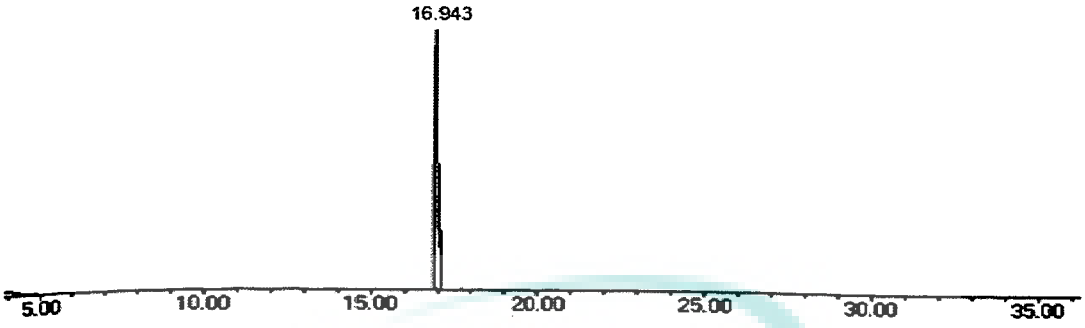

Figure 4.3: Chromatogram of benzyl acetone isolated from essential oil of *A. malaccensis*

Figure 4.4 shows mass spectrum of compound isolated in this study; the molecular ion (*m/z* 148) and base peak (*m/z* 105) of this compound was consistent with a $C1_{10}$ compound with a phenyl side loss. This analysis was done with a reference to a mass spectrum obtained from an external chemical standard and similar pattern was observed in both spectra. The result was also in consonance with a previous isolation of benzyl acetone carried out by Qi et al. (1998). The good quality of mass spectrum obtained in this study was most probably attributable to the good resolution of single compound produced by prep-GC. Previous studies reported on high rate of recovery permitted by prep-GC with a value more than 80% (Ball et al., 2012; Yang et al., 2011). The studies implied that a good quality mass spectrum could be produced with an adequate amount of target compound. Considering that benzyl acetone is a highly volatile compound, a good quality mass spectrum attained in this work demonstrated that a considerable yield of resolved benzyl acetone was successfully isolated using prep-GC. The amount, however, was not sufficient for further use in biotransformation experiment.

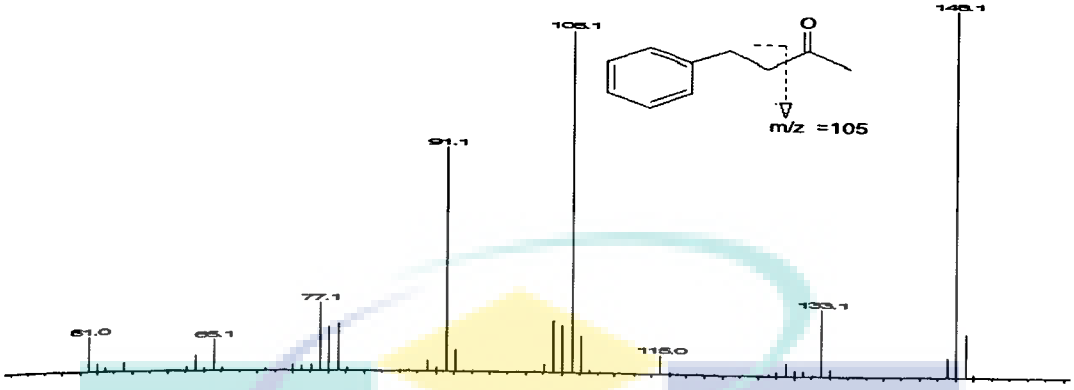

Figure 4.4: Mass spectrum of benzyl acetone isolated from essential oil of *A. malaccensis*

4.5 SCREENING OF MICROORGANISMS FOR BIOTRANSFORMATION OF BENZYL ACETONE

In this experiment, four fungal (*S. cerevisiae*, *A. niger*, *T. reesei* and *C. Albicans*) and six bacterial species (*P. aeruginosa*, *E. faecalis*, *B. cereus*, *B. subtilis*, *E. coli* and *S. aureus*) were screened for their ability to carry out biotransformation of benzyl acetone. Due to the insufficient amount of benzyl acetone isolated from the oil of *A. malaccensis*, a commercially available benzyl acetone was used in all experiments to study its fate after biotranformation. Benzyl acetone was incubated with fungal culture for seven days; incubation with bacterial culture was carried out for three days. Analysis of the results was conducted using GC-MS. Among all tested, two fungal (*S. cerevisiae* and *A. niger*) and three bacterial cultures (*P. aeruginosa*, *E. faecalis* and *B. cereus*) produced two prominent peaks on GC chromatogram following a certain period of incubation with benzyl acetone; the result, which is shown in Figure 4.5 indicated that benzyl acetone was successfully transformed into 4-phenyl-2-butanol by *P. aeruginosa*. Peaks with similar retention time were produced by *S. cerevisiae*, *A. niger*, *B. cereus* and *E. faecalis*.

The newly produced peak of biotransformation product was identified as 4-phenyl-2-butanol by GC-MS in reference to an external chemical standard (Appendix B5). The formation of 4-phenyl-2-butanol was checked whether or not it occurred

chemically under acidic condition or independently of biotransformation of benzyl acetone through substrate and culture control experiments. Analysis using GC-MS demonstrated that only one peak identified as benzyl acetone was produced when benzyl acetone was incubated in a sterile culture broth with a pH of 4.0 for seven days without the addition of microorganism. Likewise, 4-phenyl-2-butanol was not detected in fungal and bacterial cultures that were not added with benzyl acetone. The results have proven that the formation of 4-phenyl-2-butanol in this study occurred by biotransformation with benzyl acetone acted as a substrate.

There was relatively little study done on the potential industrial application of 4-phenyl-2-butanol, an analog to benzyl acetone. Despite the given circumstances, the importance of 4-phenyl-2-butanol to pharmaceutical industry was clearly outlined in a publication written by Liese et al. (2006). According to this study, 4-phenyl-2-butanol is a common substrate to the synthesis of antihypertensive agents such as labetalol and bufeniode and antiepileptic such as emepronium bromide.

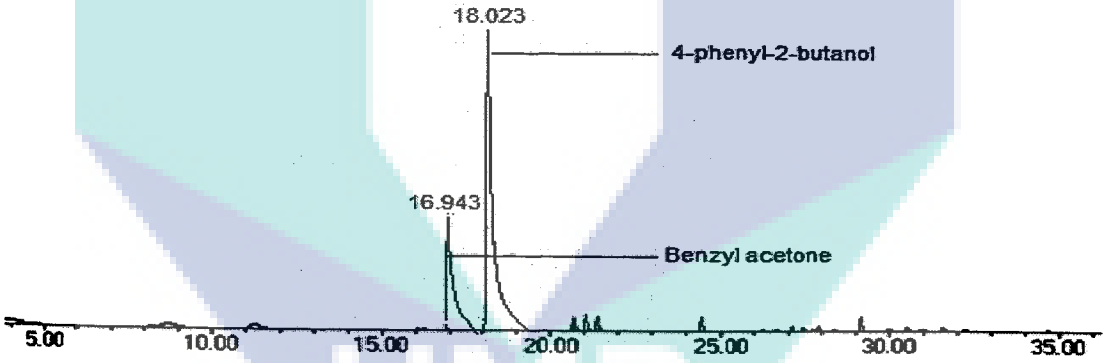

Figure 4.5: Chromatogram produced after biotransformation of benzyl acetone using *P. aeruginosa*, by which a new peak representing 4-phenyl-2-butanol was produced.

4.6 MINIMAL INHIBITORY CONCENTRATION (MIC) TEST

Astute observations on MIC of benzyl acetone towards cells of *S. cerevisiae*, *A. niger*, *P. aeruginosa*, *E. faecalis* and *B. cereus* were carried out and the result is summarized in Table 4.1. According to the result obtained, *S. cerevisiae* exhibited relatively high tolerance toward the toxicity of benzyl acetone compared to other microorganisms tested. The growth of *S. cerevisiae* was completely inhibited at 1.0 g/L

benzyl acetone while that of *A. niger* was at 0.8 g/L benzyl acetone. The growth of *P. aeruginosa*, *E. faecalis* and *B. cereus* were completely inhibited at 0.6 g/L benzyl acetone. The result, however, has in general demonstrated the strong susceptibility of all microorganisms tested towards the inhibitory effect of benzyl acetone since low concentration of benzyl acetone was adequate to completely inhibit the growth of these microorganisms.

Table 4.1: Minimal inhibitory concentration (MIC) of benzyl acetone against two fungal and 3 bacterial species

Microorganism	**Benzyl acetone Minimal Inhibitory Concentration (g/L)**
Fungi	
S. cerevisiae	1.0
A. niger	0.8
Gram –negative bacteria	
P. aeruginosa	0.6
Gram-positive bacteria	
E. faecalis	0.6
B. cereus	0.6

The level of destructive effect of antimicrobial agents differs in a way that they are dependent on their hydrophobicity and their partition in the cytoplasmic microbial membranes (Bardaji et al., 2016). The mechanism of action of antimicrobial agent is explained through the formation of hydrogen bonding between the hydrophobic compound and the membrane proteins, following the event of lipid bilayer partition. The study has also demonstrated a destruction of electron transport systems upon the exposure of microorganisms to high concentration of antimicrobial agents. Since electron transport systems are essential for cellular processes such as ATP generation,

impairment in these systems can therefore be detrimental to the cell metabolism as well as its growth.

The mechanism of action of antifungal was described by Prakash et al. (2015) by which disruption of plasma membrane permeability and mitochondrial dysfunction occurred in cells of *A. niger* upon exposure to antifungal agents. These irreversible alterations lead to increase in cell susceptibility toward oxidative damage by accumulated reactive oxygen species (ROS). The pathogenicity of *Aspergillus* spp. is often associated with the production of AFB1, a toxin considered as the most dangerous toxic metabolite among all classes of aflatoxin. When treated with a monoterpenoid known as nerol the synthesis of AFB1, suggesting vulnerability of *Aspergillus* sp. toward destructive effect of nerol. The inhibition may have occurred due to the formation of bonds with active enzymes which then lead to enzyme deactivation. Reduction in dry mycelium weight was also observed in the same experiment.

4.7 BIOTRANSFORMATION

4.7.1 Microbial reduction of benzyl acetone into 4-phenyl-2-butanol by *S. cerevisiae*, *A. niger*, *P. aeruginosa*, *E. Faecalis* and *B. cereus*

Based on the result obtained, all five microorganisms selected, namely, *S. cerevisiae*, *A. niger*, *P. aeruginosa*, *E. faecalis* and *B. cereus* have reduced benzyl acetone into 4-phenyl-2-butanol, a reaction of which normally attributable to the activity of reductases. The possible route of reaction was summarized and illustrated in Figure 4.6. Faber (2011) demonstrated the possibility that the orientation and fit of the molecules in the catalytic site of the monooxygenases, hydrogenases and reductases correspond to the positions of methyl and isopropyl groups relative to carbonyls group. This proposal may explain the similar reaction that had taken place during biotransformation of benzyl acetone using different microorganisms by which the same product, 4-phenyl-2-butanol was formed in all experiments conducted.

Respiration in microorganisms often associated with series of glucose breakdown through a process known as glycolysis; this first phase of respiration resulted in the production of CO_2 and H_2O as well as regeneration of high energy

electron carrying molecules, NADH. The second phase of respiration involves generation NADH and energy in the form of ATP through oxidation of acetate derived from carbon sources; NADH is used for various biochemical reactions and thus, its constant regeneration would positively trigger rapid biotransformation activity. In the absence of oxygen, only glycolysis occurs, indicating that fermentation has taken place. These cellular processes are nevertheless generating NADH and ATP, the entities that are essential for reduction of benzyl acetone to 4-phenyl-2-butanol by the whole cells of these microorganisms. Higher plants, algae and fungi generally possess the ability to produce monoterpenoids from the common substrate, geranyl pyrophosphate (GPP) It is generally accepted that all monoterpenes are produced through common C10 intermediate GDP.

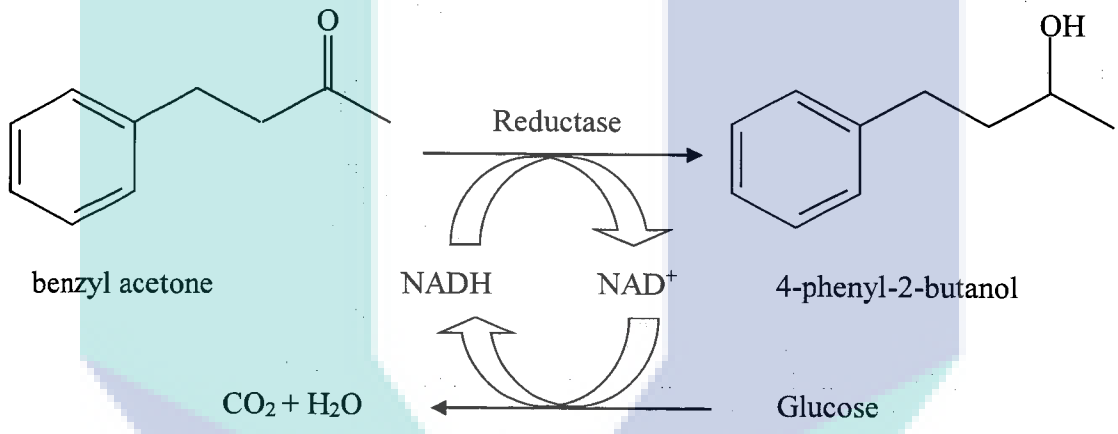

Figure 4.6: Summary of proposed mechanism involved during the reduction of benzyl acetone to 4-phenyl-2-butanol by microorganisms.

Source: Arifin et al. (2011)

4.7.2 Effect of initial substrate concentration on biotransformation

The effect of initial concentration of benzyl acetone (substrate) ranging from 0.05 to 0.8 g/L on the production of 4-phenyl-2-butanol is presented in Figure 4.7; the graph of time courses of 4-phenyl-2-butanol production generally shows an apparent decline in the percentage of 4-phenyl-2-butanol produced as the initial concentration of benzyl acetone increased and there was almost no production of 4-phenyl-2-butanol recorded at initial substrate concentration of 0.8 g/L throughout the experiment. The

highest percentage of conversion of benzyl acetone to 4-phenyl-2-butanol in this experiment was 16 %, achieved after 168 h, with the initial substrate concentration of 0.05 g/L. Glucose consumption has generally corresponded to biotransformation activity of *S. cerevisiae* as shown in Figure 4.8.

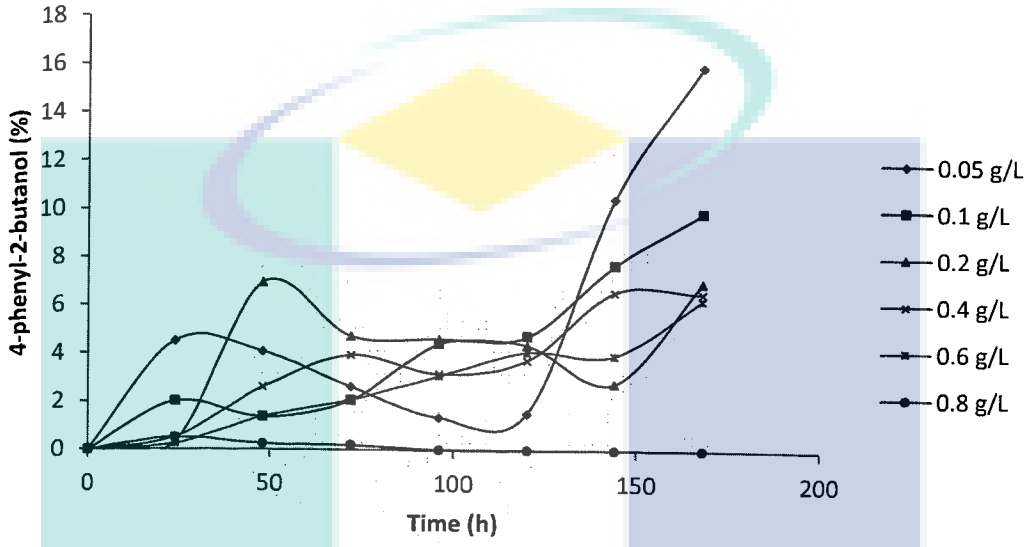

Figure 4.7: The product formation during biotransformation of benzyl acetone using *S. cerevisiae* at different initial substrate concentrations. The reaction was carried out in shake-flask culture for 168 h (Conditions: 10 g/L glucose, pH 7, 30°C, 150 rpm).

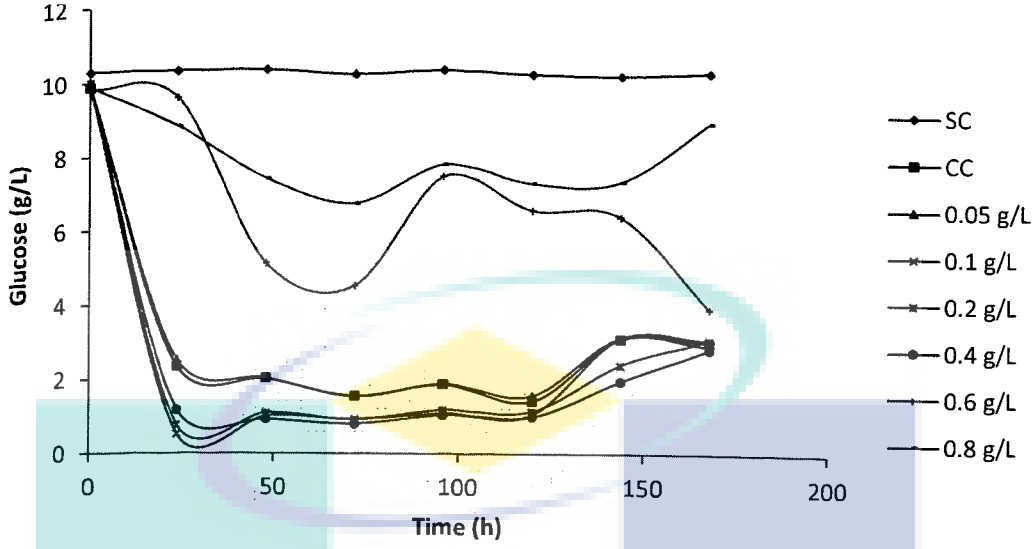

Figure 4.8: Glucose consumption during biotransformation of benzyl acetone using *S. cerevisiae* at different initial substrate concentrations. The reaction was carried out in shake-flask culture for 168 h (Conditions: 10 g/L glucose, pH 7, 30°C, 150 rpm).

The production of 4-phenyl-2-butanol from benzyl acetone by *A. niger* at different initial concentrations of substrate is shown in Figure 4.9. Increase in initial concentration of benzyl acetone has generally lowered the production of 4-phenyl-2-butanol by *A. niger*. It is important however, to point out that within 48 h of reaction time, the use of initial substrate concentrations ranged from 0.05 to 0.15 g/L has rendered more than 50 % conversion of benzyl acetone into 4-phenyl-2-butanol. As high as 95 % of benzyl acetone was converted into 4-phenyl-2-butanol after 72 h of reaction using 0.05 g/L of initial substrate concentration. In all tested concentrations, it can be seen that further incubation led to decrease in the level of 4-phenyl-2-butanol; such occurrence might have caused by further degradation of 4-phenyl-2-butanol to other degraded products or consumption by *A. niger*. The result in Figure 4.10 shows that as biotransformation activity increased, glucose consumption would increase too.

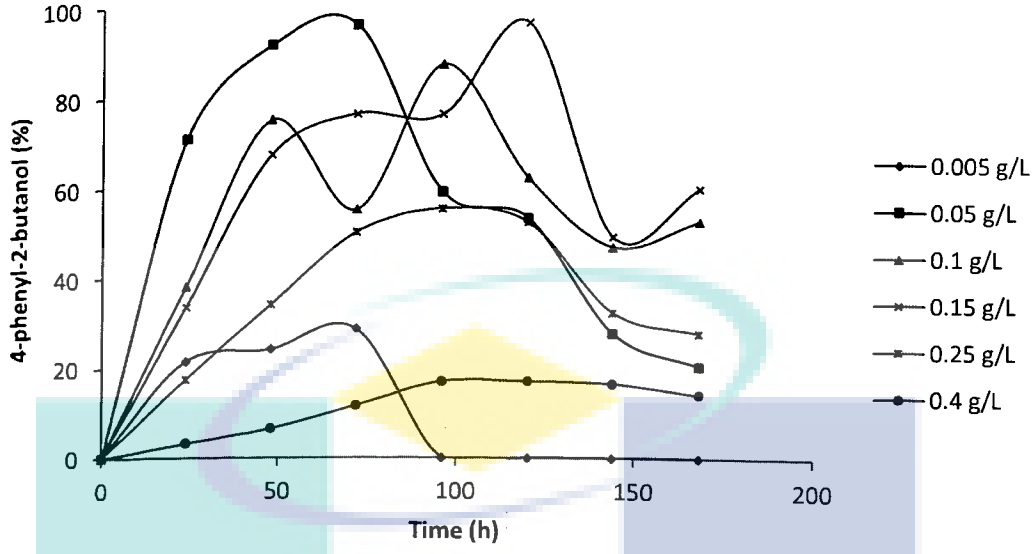

Figure 4.9: The product formation during biotransformation of benzyl acetone using *A. niger* at different initial substrate concentrations. The reaction was carried out in shake-flask culture for 168 h (Conditions: 10 g/L glucose, pH 7, 27°C, 150 rpm).

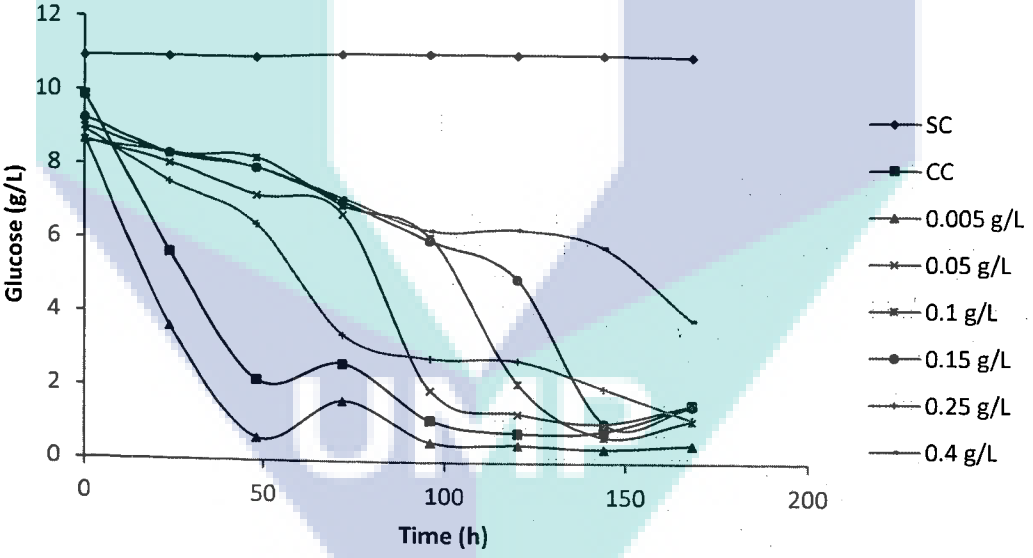

Figure 4.10: Glucose consumption during biotransformation of benzyl acetone using *A. niger* at different initial substrate concentrations. The reaction was carried out in shake-flask culture for 168 h (Conditions: 10 g/L glucose, pH 7, 27°C, 150 rpm).

Figure 4.11 shows that increase in concentration of benzyl acetone for biotransformation using *P. aeruginosa* has remarkably lowered its conversion into 4-phenyl-2-butanol with exception of the low production of 4-phenyl-2-butanol through

biotransformation using 0.005 g/L of benzyl acetone. The loss of biotransformation products through evaporation may have accounted for such observation. Remarkable difference was observed between the overall productions of 4-phenyl-2-butanol produced by biotransformation using 0.05 g/L of benzyl acetone and that of higher concentration. The highest percentage of benzyl acetone converted into 4-phenyl-2-butanol was, however, achieved after 72 h of reaction using 0.05 g/L with a value of 26%. Glucose consumption increased with increase in biotransformation activity of *P. aeruginosa* (Figure 4.12).

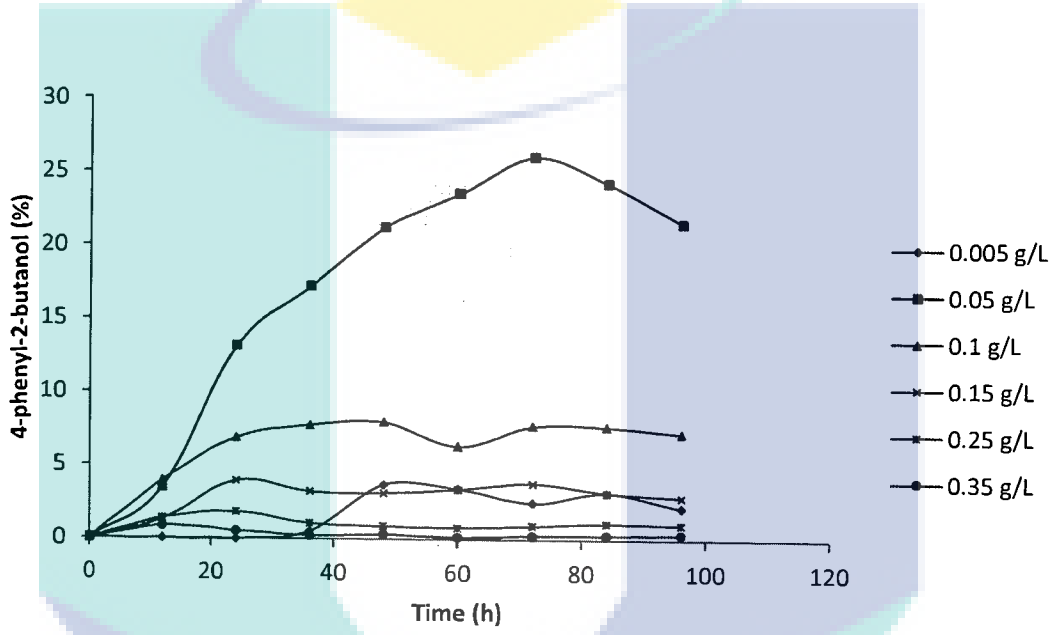

Figure 4.11: The product formation during biotransformation of benzyl acetone using *P. aeruginosa* at different initial substrate concentrations. The reaction was carried out in shake-flask culture for 96 h (Conditions: 10 g/L glucose, pH 7, 37°C, 150 rpm).

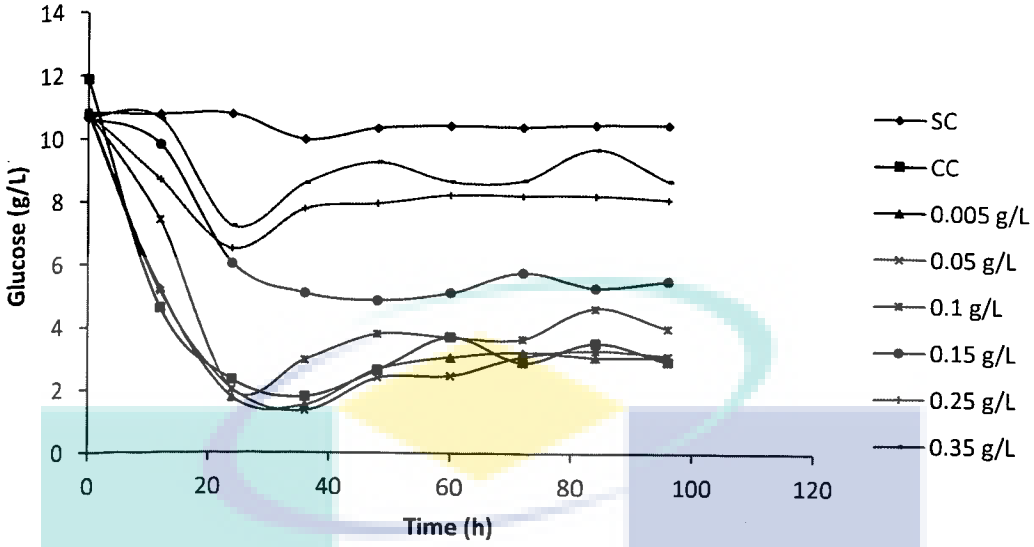

Figure 4.12: Glucose consumption during biotransformation of benzyl acetone using *P. aeruginosa* at different initial substrate concentrations. The reaction was carried out in shake-flask culture for 96 h (Conditions: 10 g/L glucose, pH 7, 37°C, 150 rpm).

Increase in initial concentration of benzyl acetone from 0.005 to 0.1 g/L has led to increase in its conversion into 4-phenyl-2-butanol in a biotransformation using *E. faecalis* as a biocatalyst. The result which is presented in Figure 4.13 has also demonstrated subtle variations in production of 4-phenyl-2-butanol as the initial concentration of benzyl acetone was further increased to 0.35 g/L. The highest percentage of benzyl acetone transformed into 4-phenyl-2-butanol in this biotransformation was about 6 %, ensued after 60 h of reaction using 0.25 g/L benzyl acetone. Decrease in residual glucose concentration beyond 60 h of reaction seemed quite of a random pattern since the result obtained did not correlate with the growth pattern obtained in an earlier experiment (Figure 4.14).

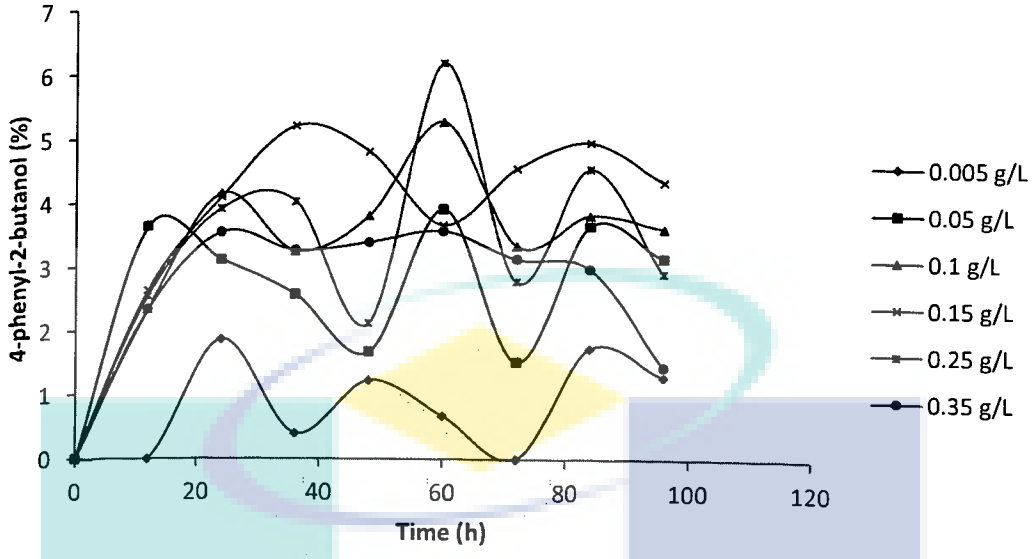

Figure 4.13: The product formation during biotransformation of benzyl acetone using *E. faecalis* at different initial substrate concentrations. The reaction was carried out in shake-flask culture for 96 h (Conditions: 10 g/L glucose, pH 7, 37°C, 150 rpm).

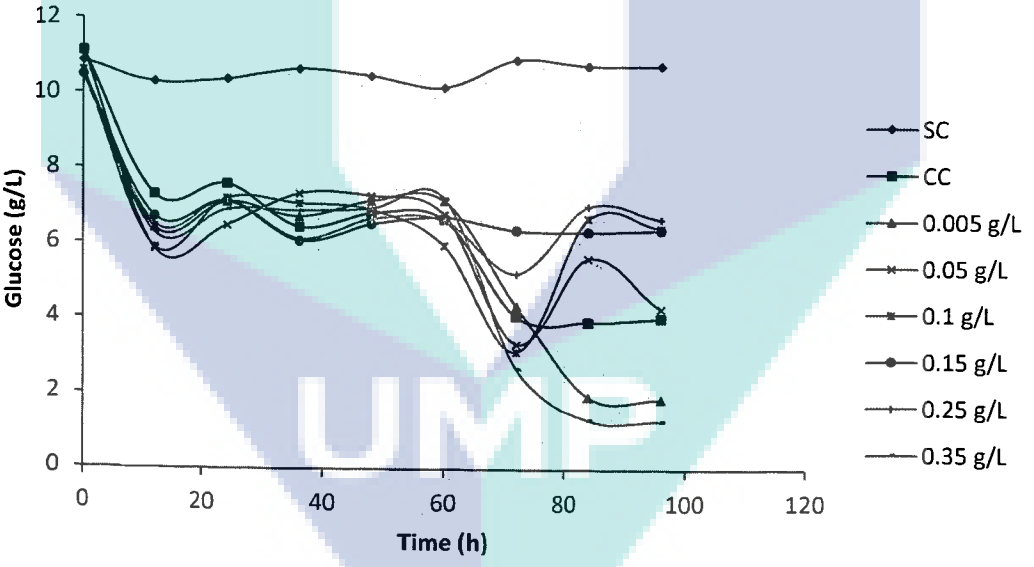

Figure 4.14: Glucose consumption during biotransformation of benzyl acetone using *E. faecalis* at different initial substrate concentrations. The reaction was carried out in shake-flask culture for 96 h (Conditions: 10 g/L glucose, pH 7, 37°C, 150 rpm).

Increase in initial concentration of benzyl acetone from 0.005 to 0.25 g/L has in overall boosted the production of 4-phenyl-2-butanol by *B. cereus* despite the lag observed within 24 h of reaction (Figure 4.15). Increase in initial concentration of

benzyl acetone to 0.35 g/L did not however, result in further upsurge in the production of 4-phenyl-2-butanol. On the contrary, low production of 4-phenyl-2-butanol was observed throughout the biotransformation except at the 36 h, by which the highest conversion of benzyl acetone to 4-phenyl-2-butanol was recorded with a value of 9%. Similar observation was seen in the biotransformation of puerarin by *B. cereus* by which the result has clearly demonstrated that biotransformation activity has decreased with increase in puerarin concentration. Toxicity of these substrates at high concentration toward the cells of *B. cereus* was probably the main cause contributing to decrease in biotransformation activity. As biotransformation activity increased, more glucose was consumed by *B. cereus* (Figure 4.16).

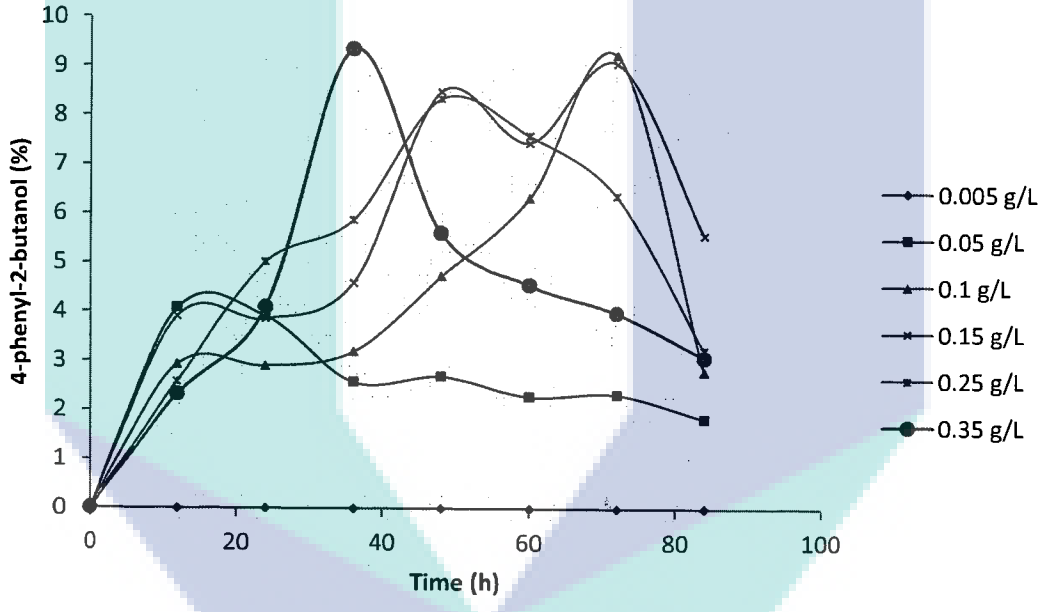

Figure 4.15: The product formation during biotransformation of benzyl acetone using *B. cereus* at different initial substrate concentrations. The reaction was carried out in shake-flask culture for 96 h (Conditions: 10 g/L glucose, pH 7, 37°C, 150 rpm).

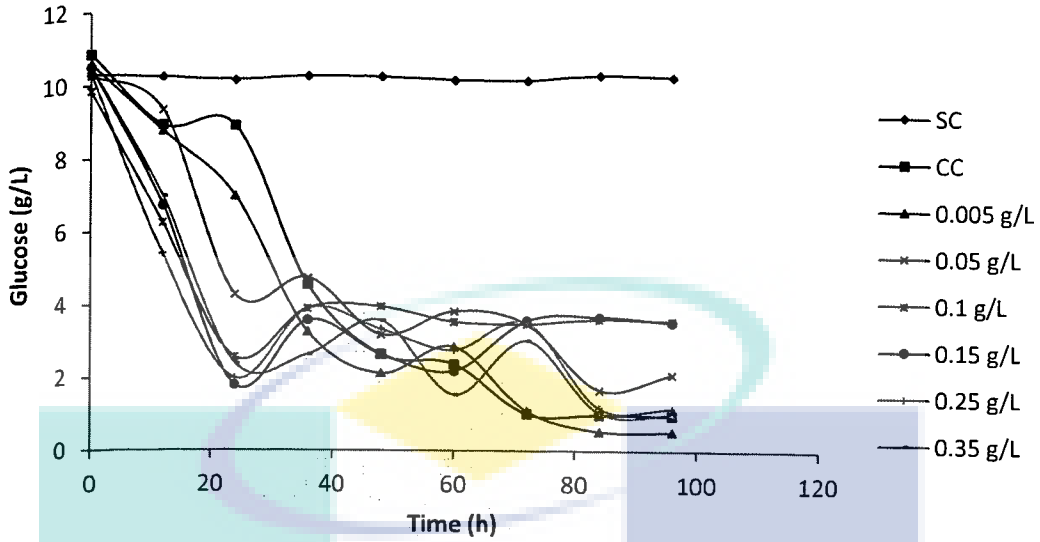

Figure 4.16: Glucose consumption during biotransformation of benzyl acetone using *B. cereus* at different initial substrate concentrations. The reaction was carried out in shake-flask culture for 96 h (Conditions: 10 g/L glucose, pH 7, 37°C, 150 rpm).

Rapid glucose consumption indicates rapid respiration and fermentation activities through which, molecules of ATP and NADH are generated. Rapid glucose consumption may have therefore, accounted for rapid conversion of benzyl acetone to 4-phenyl-2-butanol in these experiments considering such reaction would require high amount of energy (ATP) and cofactors (NADH). Higher residual glucose concentration measured at the end of the experiment using higher initial concentration of substrate indicated lesser activity of respiration and glucose fermentation which might have rendered the low formation of 4-phenyl-2-butanol.

Adamczyk et al. (2015), suggested the possibility of enzymes inhibition by high substrate concentration regardless of the nature of the biocatalyst used. The paper described inhibition of bioremediation enzymes and cellular metabolism activities due to the toxic effect exhibited by substrate at high concentration. Interestingly, Tian et al. (2013) opined that it is an induction of terpene transforming enzymes that plays major role in ensuring desirable biotransformation outcome rather than the adaptation mechanism of *A. niger* toward the biotransformation condition. The same explanation could be used to justify the low production of 4-phenyl-2-butanol at high initial benzyl acetone concentration

The study also added that, decrease in enzyme to substrate ratio at high concentration of substrate might have caused reduced degradation activity. Silva et al. (2012) was of the opinion that regardless of concentration used, the optimal substrate/enzyme ratio was the factor that influenced the reduction activity by *S. cerevisiae*. Therefore, there is possibility that biotransformation of benzyl acetone in this study was not directly related to the total substrate or enzyme concentration but rather the ratio between them.

Reduction in biotransformation activity at high initial concentration of substrate may at some point attributable to capacity of some substrates to denature protein (Lasori et al. 2016). Consequently, reduction in cell viability became apparent with increasing substrate concentration, as observed in this study. Toxicity of substrate or conversion product at high concentration toward membrane cells of microorganisms may also explain the result observed in this study, considering the use of whole cells for biotransformation. Maintenance of membrane-bound enzyme complexes involved in oxidative transformation of terpenes would also be inhibited under such condition. Reduction of membrane fluidity could have occurred in response to high concentration of benzyl acetone which then led to high unspecific membrane permeability and loss of membrane integrity, causing detrimental effect on the growth rate as well as biomass production of microorganisms.

Reduction in spore germination and germ tube elongation in *A.niger* with increasing concentration of monoterpene was also described by Tian et al. (2013). Chances are high that benzyl acetone, belonging to monoterpene group, may too exhibit such effect at high concentration. Suppression of mycelia growth could also have occurred at high initial substrate concentration (Bound et al. 2016). These may explain the lower glucose consumption by *A. niger* at high benzyl acetone concentration, as observed in this study. On the other hand, Hegazy et al. (2016) reported that despite inhibitory effect of monoterpene on sporulation of *A. niger* at higher concentration, monoterpene was completely utilized and converted into other forms. However, the idea of using low substrate concentration seems to be not economically feasible for industrial use. The use of ion exchange resins to absorb large quantity of substrate and slowly release it to the reaction solution may serve as a solution to such problem.

4.7.3 Effect of initial glucose concentration on biotransformation

Figure 4.17 shows an increasing pattern in the percentage of conversion by *S. cerevisiae* as the initial glucose concentration increased from 0 to 30 g/L; further increase in concentration has in general, led to decline in percentage of conversion. In the absence of glucose, it was difficult for *S. cerevisiae* to maintain biotransformation activity. The highest percentage of conversion was 26 %, a value achieved after 168 h of biotransformation using 30 g/L initial glucose concentration. The result conformed with the rapid glucose consumption observed within 24 h of biotransformation with 10 to 30 g/L initial glucose concentration; similar declining pattern was observed after 48 h of biotransformation using 40 g/L glucose; glucose level declined gradually in biotransformation using 50 g/L glucose, leaving remarkably high residual glucose concentration (Figure 4.18).

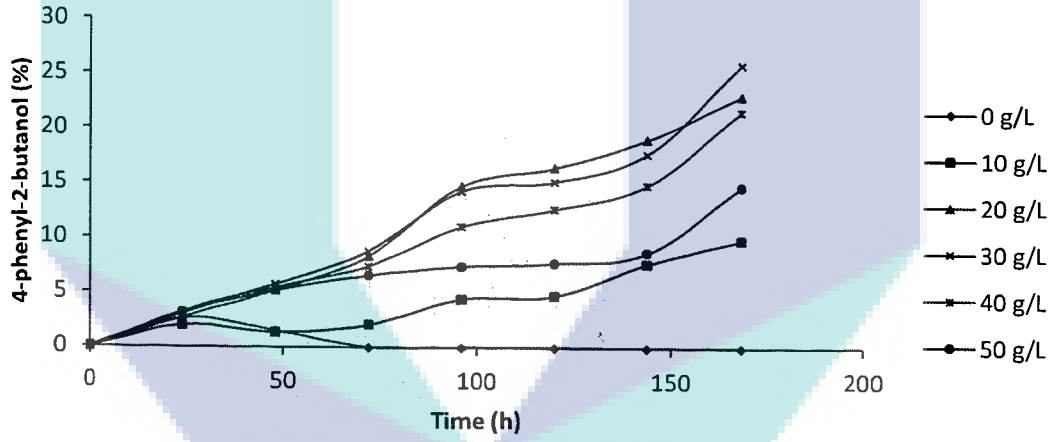

Figure 4.17: The product formation during biotransformation of benzyl acetone using *S. cerevisiae* at different initial glucose concentrations. The reaction was carried out in shake-flask culture for 168 h (Conditions: 0.1 g/L benzyl acetone, pH 7, 30°C, 150 rpm).

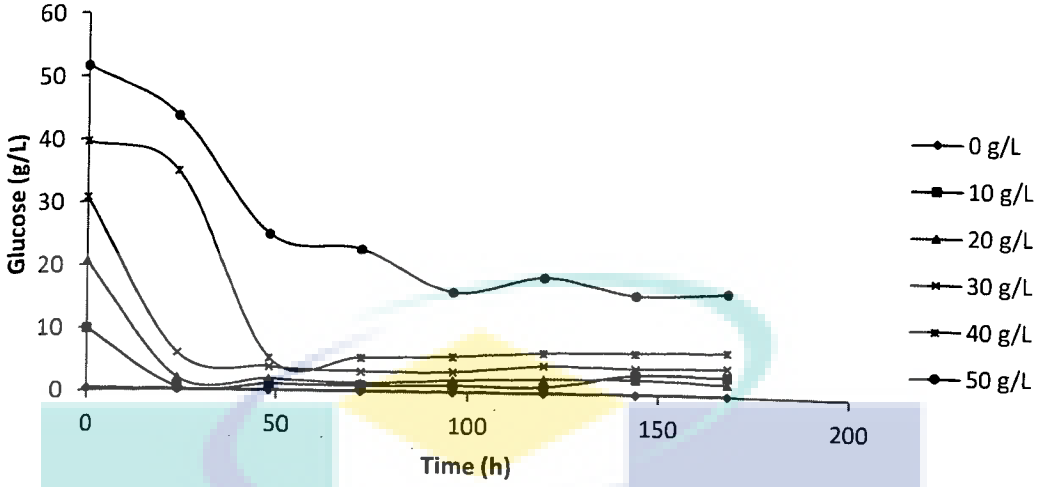

Figure 4.18: Glucose consumption during biotransformation of benzyl acetone using *S. cerevisiae* at different initial glucose concentrations. The reaction was carried out in shake-flask culture for 168 h (Conditions: 0.1 g/L benzyl acetone, pH 7, 30°C, 150 rpm).

Increase in initial glucose concentration ranged from 0 to 50 g/L has generally increased the percentage of conversion (Figure 4.19). There was hardly biotranformation activity by *A. niger* in the absence of glucose. Apart from the sudden drop in the percentage of 4-phenyl-2-butanol produced after 96 h of reaction using 20 g/L glucose, the results were in overall demonstrated that increase in the initial glucose concentration may help to prolong and maintain the production of 4-phenyl-2-butanol by *A. niger* up to a certain extent. Despite the pattern, the highest percentage of conversion (96 %) was achieved after 120 h of reaction with an initial glucose concentration of 10 g/L. Increase in initial glucose concentration has generally slower the consumption rate despite increased in biotransformation activity (Figure 4.20).

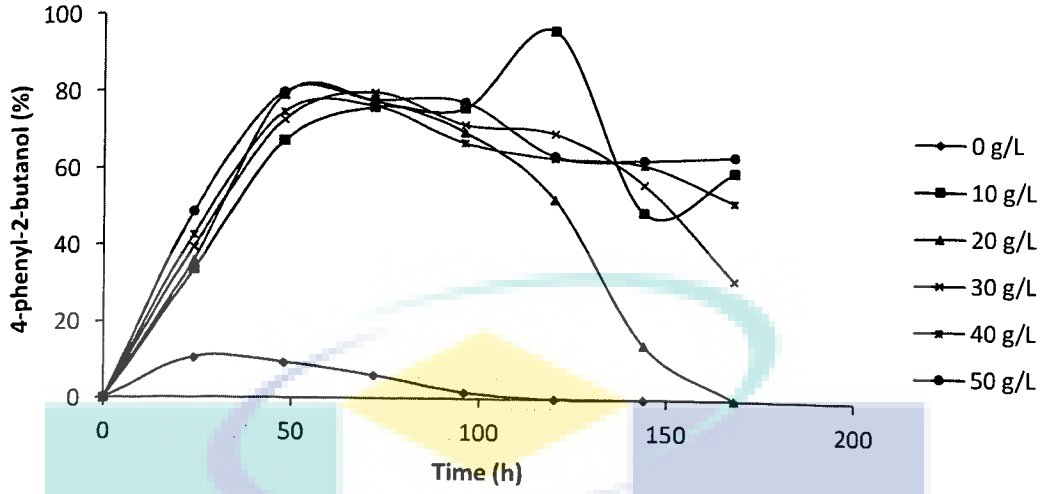

Figure 4.19: The product formation during biotransformation of benzyl acetone using *A. niger* at different initial glucose concentrations. The reaction was carried out in shake-flask culture for 168 h (Conditions: 0.15 g/L benzyl acetone, pH 7, 27°C, 150 rpm).

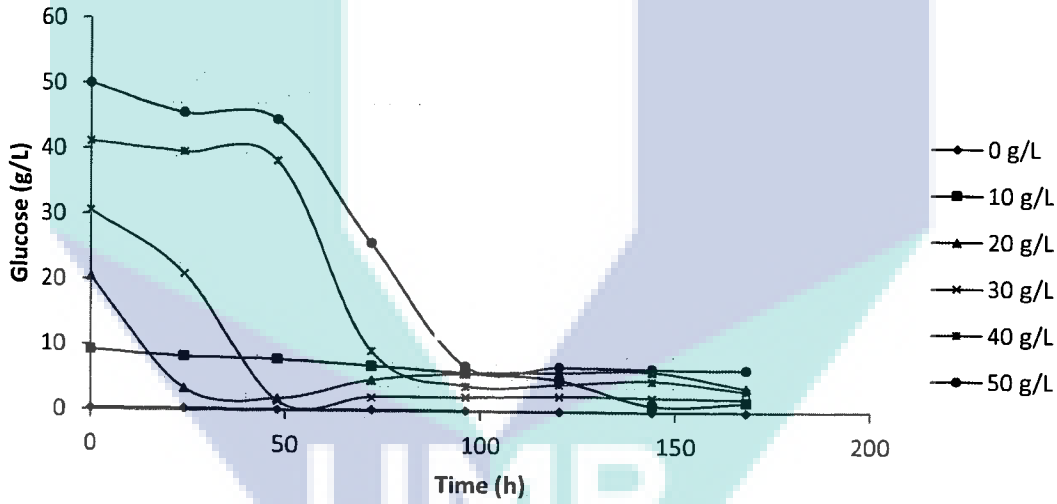

Figure 4.20: Glucose consumption during biotransformation of benzyl acetone using *A. niger* at different initial glucose concentrations. The reaction was carried out in shake-flask culture for 168 h (Conditions: 0.15 g/L benzyl acetone, pH 7, 27°C, 150 rpm).

Remarkable increase in the production of 4-phenyl-2-butanol by *P. aeruginosa* was achieved when the initial glucose concentration adjusted to 20 g/L; further increase caused only slight improvement in the yield (Figure 4.21). The fact that the highest conversion was achieved using 50 g/L initial glucose concentration however, is something noteworthy; the reaction has rendered 95% conversion of benzyl acetone to

4-phenyl-2-butanol within 24 h of reaction. The amount of glucose consumed by *P. aeruginosa* during biotransformation did not vary much when the reaction was carried out in different glucose concentrations (Figure 4.22).

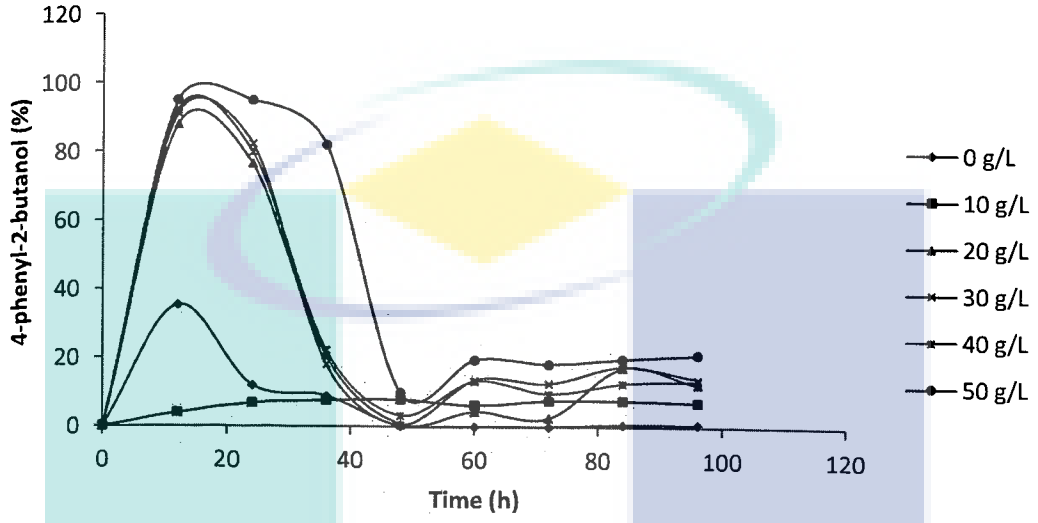

Figure 4.21: The product formation during biotransformation of benzyl acetone using *P. aeruginosa* at different initial glucose concentrations. The reaction was carried out in shake-flask culture for 96 h (Conditions: 0.1 g/L benzyl acetone, pH 7, 37°C, 150 rpm).

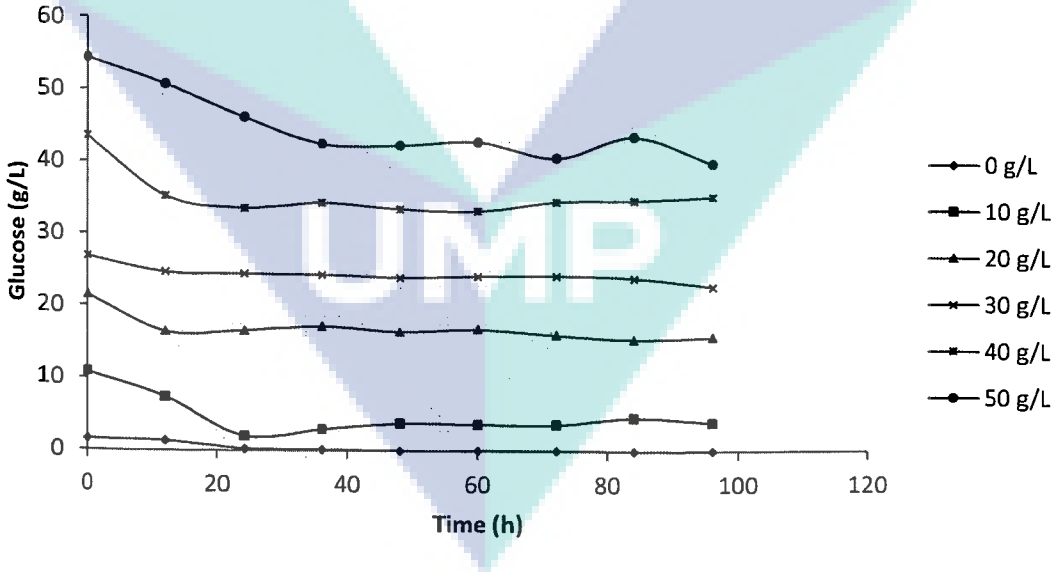

Figure 4.22: Glucose consumption during biotransformation of benzyl acetone using *P. aeruginosa* at different initial glucose concentrations. The reaction was carried out in shake-flask culture for 96 h (Conditions: 0.1 g/L benzyl acetone, pH 7, 37°C, 150 rpm).

The result presented in Figure 4.23 below demonstrated an increase in production of 4-phenyl-2-butanol as the initial glucose concentration increased from 0 to 40 g/L; improved in production of 4-phenyl-2-butanol was not observed when initial glucose concentration was further increased to 50 g/L. A substantial percentage of 4-phenyl-2-butanol produced in a biotransformation conducted in the absence of glucose may be something worthy of note since the result demonstrated a reduction of benzyl acetone by *E. faecalis* without the aid of glucose supply. The breakdown of internal glycogen may, at the moment, serve as the best explanation for such observation. The highest percentage of benzyl acetone transformed into 4-phenyl-2-butanol, a value which reached up to 7 %, was achieved after 12 h of biotransformation supplied with 40 g/L glucose. The pattern observed in reduction of glucose concentration during biotransformation was almost similar for all reactions supplied with different initial glucose concentration (Figure 4.24).

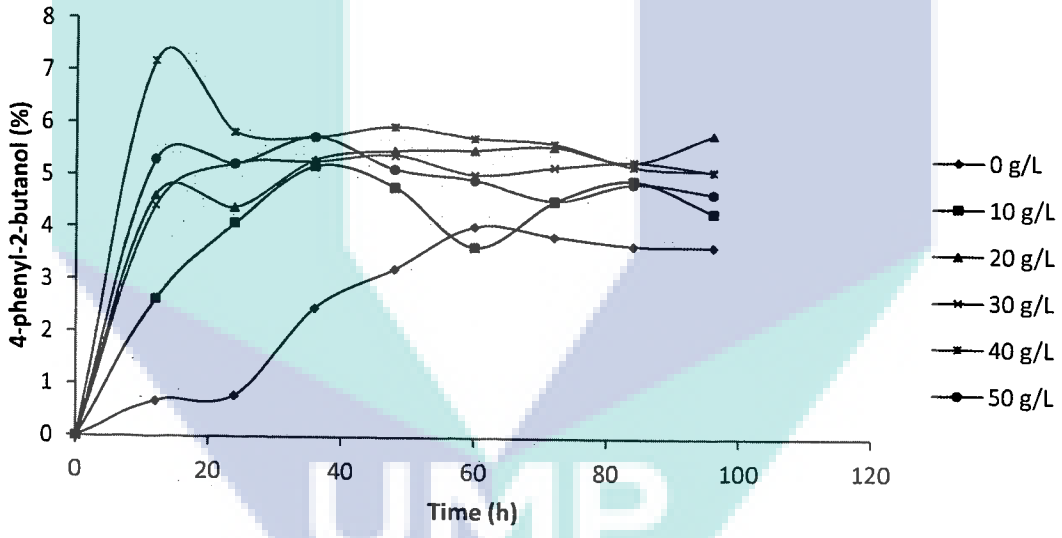

Figure 4.23: The product formation during biotransformation of benzyl acetone using *E. faecalis* at different initial glucose concentrations. The reaction was carried out in shake-flask culture for 96 h (Conditions: 0.15 g/L benzyl acetone, pH 7, 37°C, 150 rpm).

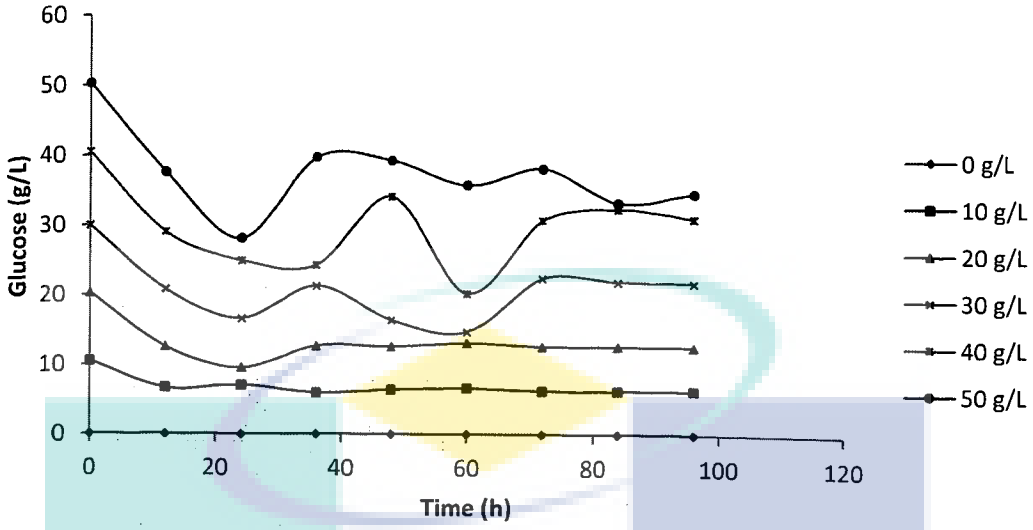

Figure 4.24: Glucose consumption during biotransformation of benzyl acetone using *E. faecalis* at different initial glucose concentrations. The reaction was carried out in shake-flask culture for 96 h (Conditions: 0.15 g/L benzyl acetone, pH 7, 37°C, 150 rpm).

Atypical effect of initial glucose concentration on biotransformation of benzyl acetone into 4-phenyl-2-butanol by *B. cereus* is presented in Figure 4.25 by which the highest production of 4-phenyl-2-butanol has for the most part occurred throughout the biotransformation carried out in the absence of glucose. Increase in the initial glucose concentration on the other hand, has generally resulted in decrease of overall production of 4-phenyl-2-butanol, saved for biotransformation carried out with 40 g/L glucose which has conferred considerably high conversion of benzyl acetone to 4-phenyl-2-butanol. The highest percentage of benzyl acetone transformed into 4-phenyl-2-butanol by *B. cereus*, the value of which has reached 11%, was achieved after 72 h of biotransformation carried out with 40 g/L glucose. Glucose residue found at the end of biotransformation in all tested conditions suggested that the glucose was not fully consumed during biotransformation, even when carried out with initial concentration of glucose as low as 10 g/L (Figure 4.26).

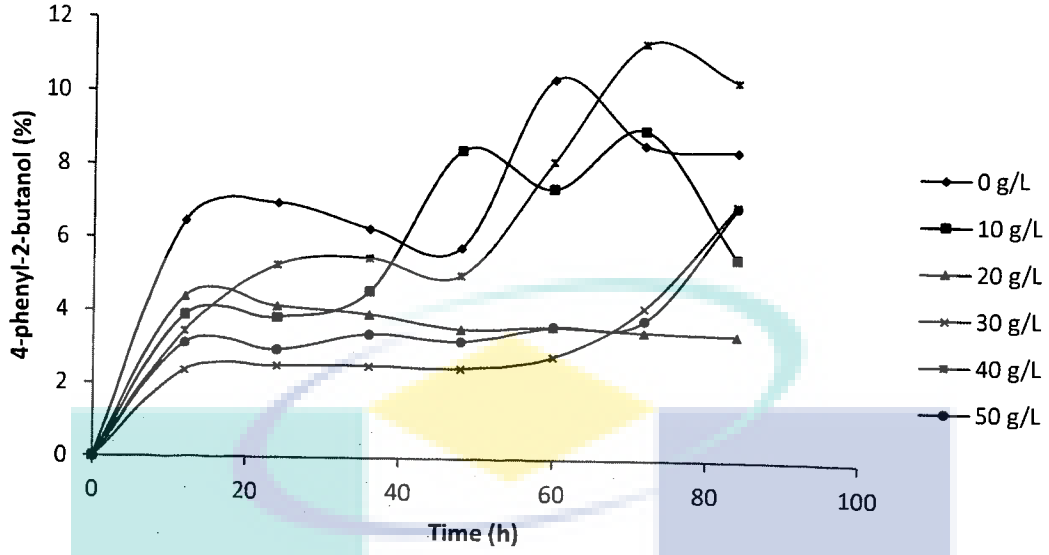

Figure 4.25: The product formation during biotransformation of benzyl acetone using *B. cereus* at different initial glucose concentrations. The reaction was carried out in shake-flask culture for 96 h (Conditions: 0.15 g/L benzyl acetone, pH 7, 37°C, 150 rpm).

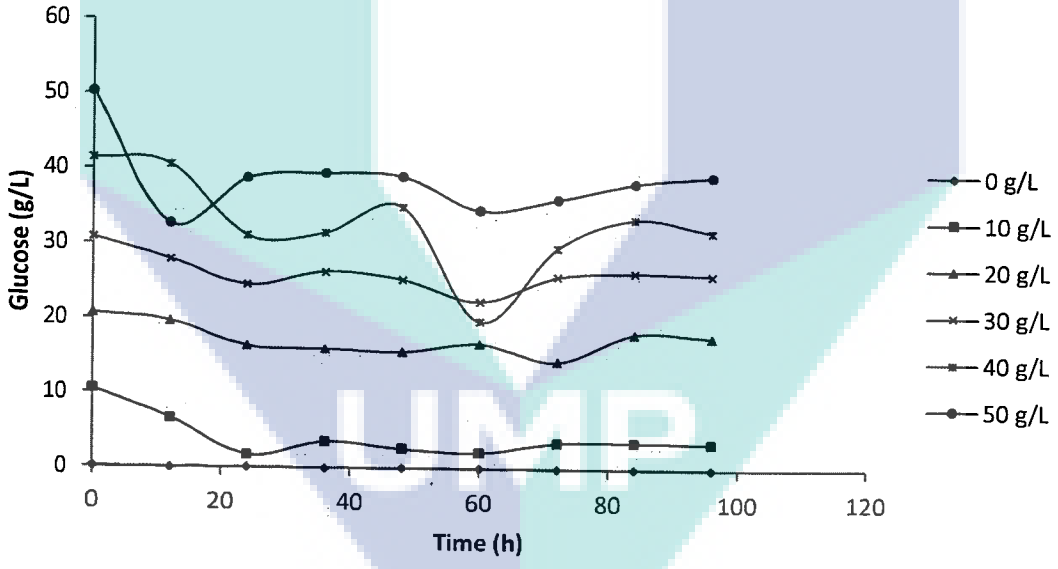

Figure 4.26: Glucose consumption during biotransformation of benzyl acetone using *B. cereus* at different initial glucose concentrations. The reaction was carried out in shake-flask culture for 96 h (Conditions: 0.15 g/L benzyl acetone, pH 7, 37°C, 150 rpm).

Interestingly, in the absence of glucose, biotransformation of benzyl acetone did take place in reaction using *E. faecalis* and *B. cereus*. It was probably attributable to breakdown of internal glycogen, a mechanism that produces energy to support reduction

of benzyl acetone. Another possible explanation might be the accumulation of ethanol through glycolysis, which in turn would be used as a sole carbon source, a reaction of which catalyzed by ethanol dehydrogenase (Arifin et al., 2011). Dehydrogenation of ethanol produced acetaldehyde that would further be oxidized into acetic acid and carbon dioxide. Hydrogen atom disengaged from ethanol would at the same time be used to reduce NADP to NADPH, a reaction catalyzed by oxidoreductase; these events might have consequently resulted in improved biotransformation activity. The use of accumulated ethanol as a carbon source may have contradicted other works reporting on inhibitory effect of ethanol on the growth and biotransformation activity by microorganisms. Utilization of ethanol as a sole carbon source in the absence of sugar is therefore possible only when the concentration of ethanol is below the inhibitory level.

As a powerful signaling molecule in yeast, concentration of glucose does impose pronounced effect on the growth of *S. cerevisiae*. At low glucose concentration, replicative and chronological longevity of *S. cerevisiae* is increased; high glucose concentration on the other hand, could result in oxidative damage on glycolytic enzymes. Improvement in replicative lifespan of cells of *S. cerevisiae* in low glucose concentration may be best explained by increase in respiratory rates which in turn, result in increase in regeneration of endogenous cofactors (van Rossum et al., 2016). It has been reported that the cells starved with glucose started to consume ethanol produced from former glucose catabolism, an event described as diauxic shift, similar to observation made in this study.

Considering its sugar fermenting nature, *S. cerevisiae* can live on various fermentable and non-fermentable carbon sources. Generation of energy in *S. cerevisiae* is mostly through glycolysis. Since sugar catabolism is related to oxygen availability, according to Pasteur Effect, rapid glycolysis occurs in growing cells under aerobic condition. Sugar catabolism in *S. cerevisiae* also greatly influenced by concentration of glucose available at a given time; excess in glucose concentration may induce respiratory fermentative metabolism, a respiratory route also known as Crabtree Effect. Study on *S. cerevisiae*'s protein expression profile has also revealed induction of proteins responsible for respiratory growth when glucose was restricted; mitochondrial ATP synthase, subunit alpha that produces mitochondrial ATP was one of the induced proteins. Interestingly, it was also found that in most occasions, glucose fermentation

was preferable than aerobic respiration even though aerobic respiration proved to be the most efficient pathway to support the growth of *S. cerevisiae*.

There was an evidence of glucose repression on some genes encoded the enzymes in *S. cerevisiae* (Kayikci and Nielsen 2015). Catabolism of slowly fermentable or non-fermentable carbon sources was repressed at transcriptional level in the presence of rapidly fermentable carbon sources such as glucose; genes controlling the Krebs cycle and electrons transport chain were among those repressed by excessive glucose. Years before, Jimenez-Marti et al. (2011) reported on higher expression of fifty-two genes by more than 2-fold in the presence of 20% glucose compared to 2% glucose; these genes exhibited glycerol metabolism and response to chemical inducement. Simultaneously, 242 genes exhibited an expression higher than 2-fold in the presence of 2% glucose than that of 20% glucose, a clear indication of their repression in high glucose concentration; genes involve in metabolism and generation of energy substrates such as genes related to ATP synthesis coupled with respiratory cycle were among those suppressed in high glucose concentration.

Similar repression was seen in a study conducted by Fontana et al. (2005) by which some of the enzymes were repressed in high sugar concentration. It was also found in the same study that change in glucose concentration could influence the aerobic and anaerobic route of metabolism through regulation of oxygen. Contradictory to this study, catabolic repression of pectinase production in high glucose concentration was demonstrated by Kazemi et al. (2016) despite the unaltered growth pattern and biomass production of *A. niger* when initial glucose concentration increased. Growth of *A. niger* in high glucose was found slower and the biomass production has remarkably reduced. Such condition may have triggered physiological and metabolic change in *A. niger*, causing the cells to utilize the glucose to produce fermentation products rather than biomass. The result obtained from the biotransformation of benzyl acetone by *A. niger* in this study has generally corroborated these past findings.

Many lactic acid bacteria metabolize citrate and pyruvate and both acids are generally co-metabolized with the fermentable sugar such as glucose. In *E. faecalis* however, citrate metabolism is hampered in the presence of glucose, indicating suppression of enzymes by glucose. Rea and Cogan (2003) demonstrated a proportional

relationship between glucose consumption and biomass production; the growth rate of *E. faecalis* on the other hand, was marginally affected by glucose. The same study has also reported on the stunted growth of *E. faecalis* when grown on citrate in the presence of high glucose concentration, alluding to the complete inhibition of citrate metabolism by glucose. The growth of *E. faecalis* was revived as well as the citrate metabolism upon the exhaustion of glucose supply. Another test conducted has shown that unlike citrate, pyruvate metabolism was not hindered in the presence of glucose, alluding to the fact that both substrates were co-metabolized by *E. faecalis*

In *A. niger*, glucose is metabolized to form citric acid; therefore, it is likely to find acidity level increase at the end of the biotransformation using *A. niger*. Carillo-Sancen et al. (2016) demonstrated low citric acid production at a concentration of glucose less than 10%; different dynamics in production of trehalose, glycerol, erythritol and mannitol by *A. niger* were also observed when glucose concentration was varied. Trehalose, which functions to prevent cell disruption, was produced during the growth phase of *A. niger* and the level remained throughout the incubation period when biotransformation was carried out in low glucose concentration; reduced activity of pyruvate carboxylase was also seen under the same condition which later led to reduction in citric acid production. This was exactly the case for biotransformation carried out in this study since all tested glucose concentrations were below 10%. The previously mentioned study also reported that high glucose concentration might have caused imbalance NADH regeneration since most of them were used in formation of glycolytic byproducts such as mannitol, as opposed to the backflux from mannitol to trehalose, observed in low glucose concentration.

This shift of glycolytic control was due to the loss of control at the fructose-6-phophate in high sugar concentration (Carillo-Sanchen et al., 2016). The mentioned study reported on the reduced size of mycelia clump and the absence of citric acid when *A. niger* was grown in media containing less than 2.5% glucose. Prolong in the lag phase was observed as the initial glucose concentration increased from 1 to 14%, indicating a mechanism of adaptation by cells of *A. niger*; considerable decrease in growth rate was, however, seen when *A. niger* was grown in 20% glucose. The same mechanism may have taken place in this study since similar effect was seen in the residual glucose concentration. Increase in initial glucose concentration has resulted in

slower glucose consumption within 48 h of reaction; the observation may serve as solid evidence supporting the previous argument on the possibility of adaptation mechanism of *A. niger* in high glucose concentration. The fact that the production of 4-phenyl-2-butanol in this study was hardly affected by changes in initial glucose concentration may suggest that the production was independent to the growth of *A. niger*.

Similar shift of glycolytic control may happen in glucose metabolism in *P. aeruginosa* which involves several complex pathways. Apparent inductive effect on the glucose enzymes was observed when glucose concentration was elevated from 6 to 8 mM (Ng and Dawes, 1973). Significant drop in citrate concentration was recorded at high glucose concentration which was thought to cause further increase in glucose enzymes activities. Among the key enzymes in this metabolism are glucose, glucose-6-phosphate, gluconate, and 6-phosphogluconate dehydrogenases; upregulation and downregulation of these enzymes depend on the concentration of glucose, citrate and their metabolic products. Overexpression of glucose dehydrogenases- enzymes responsible for oxidation of glucose to gluconate- in high glucose concentration was elaborated in the same study by which the amount of glucose oxidized by these enzymes is ten times higher than that of transported into the cell; overexpression of these enzymes is nonetheless least beneficial for a healthy growth of *P. aeruginosa* since no energy is yielded in that reaction.

Changing the initial glucose concentration affected the attachment of viable cells, according to Arutchelvi et al. (2011), by which the concentration of ATP in the biofilm was significantly altered when such changes were applied. It is essential to note that concentration of ATP in the biofilm is a clear indicator of active microbial biomass. Enhancement in rate and accumulation of biofilm on the surface of PP occurred with increase in initial concentration of glucose. Reduction of benzyl acetone is a process that requires a lot of energy in the form of ATP, increase in ATP generation at high glucose concentration could therefore, result in better conversion of benzyl acetone to 4-phenyl-2-butanol. Considering the resistance evinced by azo dyes to microbial degradation, it is natural to find that decolorization of Orange II requires rapid energy consumption and in such case, glucose serves as a suitable energy source for a rapid energy production. The same principle may be applied in biotransformation of benzyl acetone by *E. faecalis* in different glucose concentration since increase in 4-phenyl-2-

butanol formation was observed with increase in initial glucose concentration up to 40 g/L.

When grown in glucose concentrations that were lower than the adequate level, the growth of *E. faecalis* was, nonetheless, slowed down and reduction in biomass production would occur. Adequate level of glucose may not necessarily referring to the concentration of glucose alone since a healthy growth of *E. faecalis* can only be achieved by supplying the cells with a suitable proportion of carbon and nitrogen sources. Another study however, showed that significant increase in expression of 42 proteins in *E. faecalis* was observed in the event where a glucose supply has exhausted (Giard et al., 1997). This phenomenon was observed in both growing and resting cells of *E. faecalis*. Exhaustion of glucose supply might have triggered synthesis of proteins crucial for a long term survival of *E. faecalis* since the growth of *E. faecalis* was nonetheless observed in this study when glucose was not supplied, despite being of the poorest growth.

High level of reactive oxygen species (ROS) produced in high glucose concentration can cause damage to the cells; change in protein expression profile is therefore necessary to curb such dire situation. It was found that when grown in high glucose concentration, the growth of *S. cerevisiae* has slowed down during the initial phase of fermentation because part of their metabolisms was occupied with synthesis of compounds to resist the osmotic stress. Perhaps contradictory to the common belief that high glucose concentration gives rise to high production of ethanol, Reuter et al. (2016) demonstrated the down-regulation of adh1, a group of enzymes responsible for ethanol production. The down-regulation of these enzymes was most probably due to acceleration in the production of acidic compounds and glycerol, a mechanism to counteract high osmotic stress caused by high glucose concentration. Low level of ethanol was therefore produced in high glucose concentration since the production of acidic compounds and glycerol was of the higher priority at that moment.

4.7.4 Effect of initial pH on biotransformation

Preceding works have demonstrated that *S. cerevisiae* worked excellently under low pH condition in the range of pH 4.0 to 6.0 (Reuter et al., 2016). The results

obtained in this study, however, demonstrating otherwise as illustrated in Figure 4.27; production of 4-phenyl-2-butanol was generally the lowest when performed under the pH of 6.5 (slightly acidic). Generally, upsurge in the percentage of benzyl acetone converted to 4-phenyl-2-butanol was observed with the increase in the pH up to 7.5; the production, however, contracted when performed in the pH beyond 7.5. The highest percentage of 4-phenyl-2-butanol formed from benzyl acetone in this experiment was 23 %, achieved after 168 h of reaction, in a reaction with a pH of 7. Analysis on the residual glucose concentration indicated that the difference in glucose consumption by *S. cerevisiae* at different pH levels was of inconsequential (Figure 4.28).

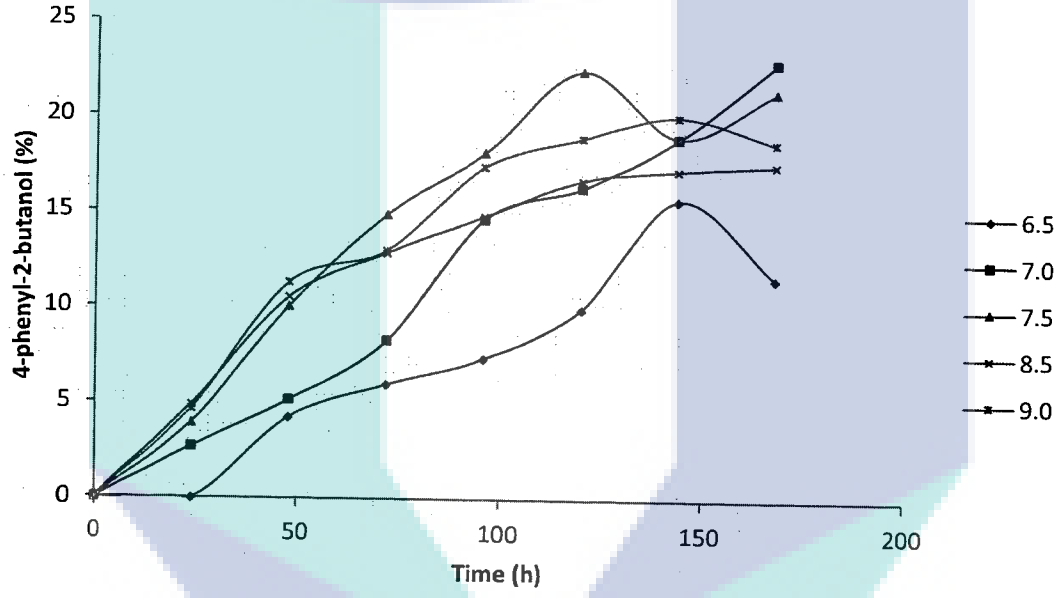

Figure 4.27: The product formation during biotransformation of benzyl acetone using *S. cerevisiae* at different initial pHs. The reaction was carried out in shake-flask culture for 168 h (Conditions: 0.1 g/L benzyl acetone, 20 g/L glucose, 30°C, 150 rpm).

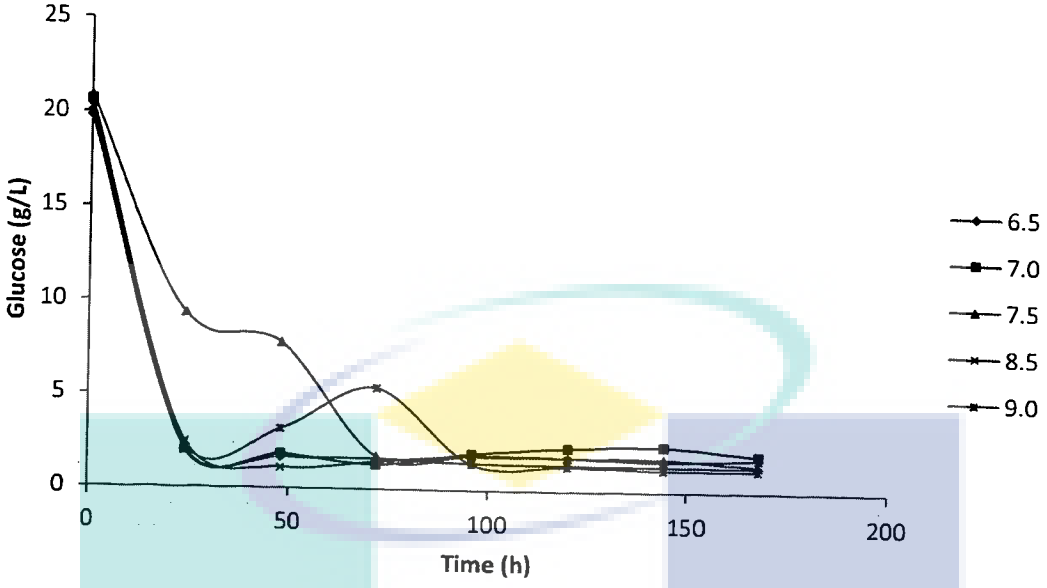

Figure 4.28: Glucose consumption during biotransformation of benzyl acetone using *S. cerevisiae* at different initial pHs. The reaction was carried out in shake-flask culture for 168 h (Conditions: 0.1 g/L benzyl acetone, 20 g/L glucose, 30°C, 150 rpm).

Transformation of benzyl acetone into 4-phenyl-2-butanol by *A. niger* seemed to be barely affected by changes in pH of reaction, the results of which illustrated in Figure 4.29. The highest percentage of benzyl acetone converted into 4-phenyl-2-butanol was achieved after 48 h of reaction carried out in a pH of 9 which value has reached 98%. It is interesting however, to note that for most of the times, biotransformation of benzyl acetone was sustainably performed by *A. niger* in a pH ranged from 6.5 to 7 and the quality of the performance declined as the pH rose higher. Effect of pH variations on biotransformation mediated by *A. niger* was described in several works (Do et al., 2013). Different optimum pH for biotransformation activity of *A. niger* were observed in different studies, indicating that such activities are enzymatically catalyzed and these enzymes were in turn require a specific range of pH to work optimally. No correspondence has generally been observed between biotransformation activity and glucose consumption when pH changed (Figure 4.30).

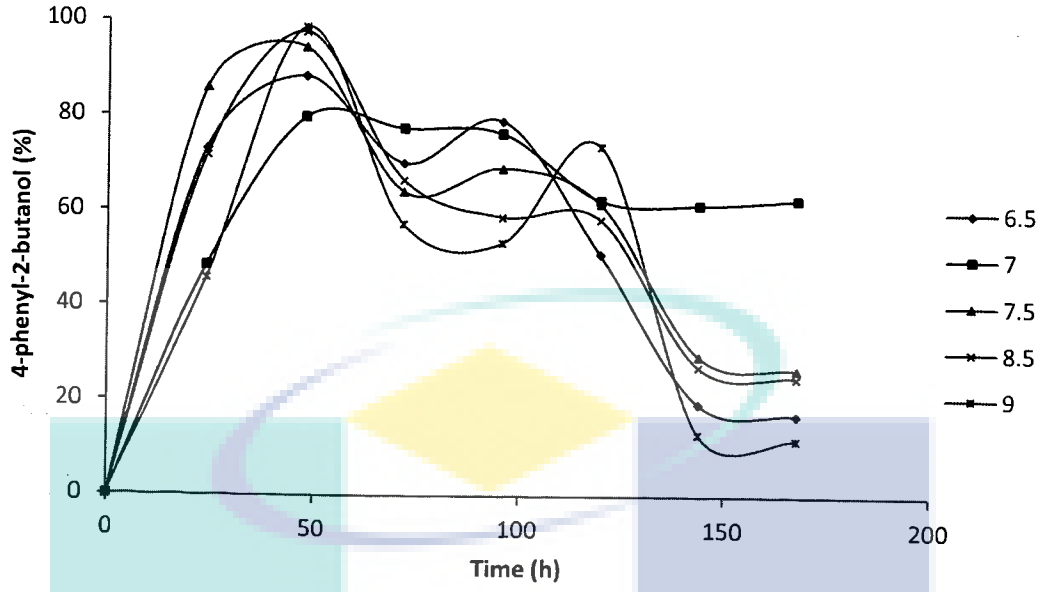

Figure 4.29: The product formation during biotransformation of benzyl acetone using *A. niger* at different initial pHs. The reaction was carried out in shake-flask culture for 168 h (Conditions: 0.15 g/L benzyl acetone, 50 g/L glucose, 27°C, 150 rpm).

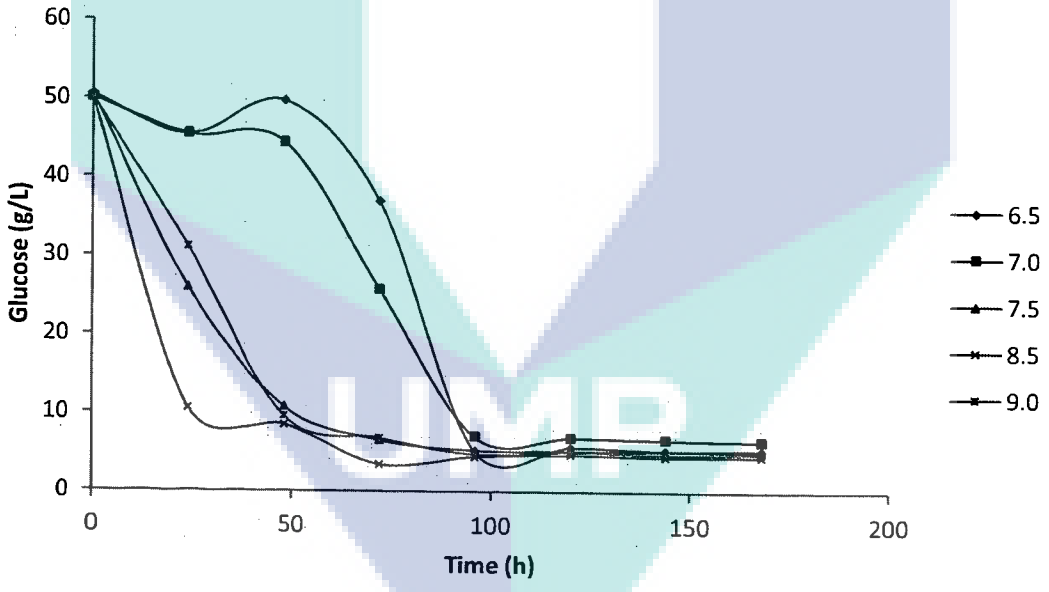

Figure 4.30: Glucose consumption during biotransformation of benzyl acetone using *A. niger* at different initial pHs. The reaction was carried out in shake-flask culture for 168 h (Conditions: 0.15 g/L benzyl acetone, 50 g/L glucose, 27°C, 150 rpm).

A considerable declining pattern was observed in production of 4-phenyl-2-butanol by *P. aeruginosa* as pH of biotransformation media was adjusted from 6.5 to 9.

The results shown in Figure 4.31 showed higher percentage of 4-phenyl-2-butanol produced when the biotransformation was carried out in reaction media with the pH ranged from 6.5 to 7; biotransformation carried out at higher pH ranged from 7.5 to 8.5 has yielded moderately lower production of 4-phenyl-2-butanol while that of pH 9 showed the poorest biotransformation activity by *P. aeruginosa*. The highest production achieved in this experiment was 88 % conversion of benzyl acetone into 4-phenyl-2-butanol after 12 h of incubation in a medium with a pH of 7. Analysis on residual glucose concentration has generally demonstrated that less glucose was consumed as pH increased; the result has conformed to biotransformation activity by *P. aeruginosa* (Figure 4.32).

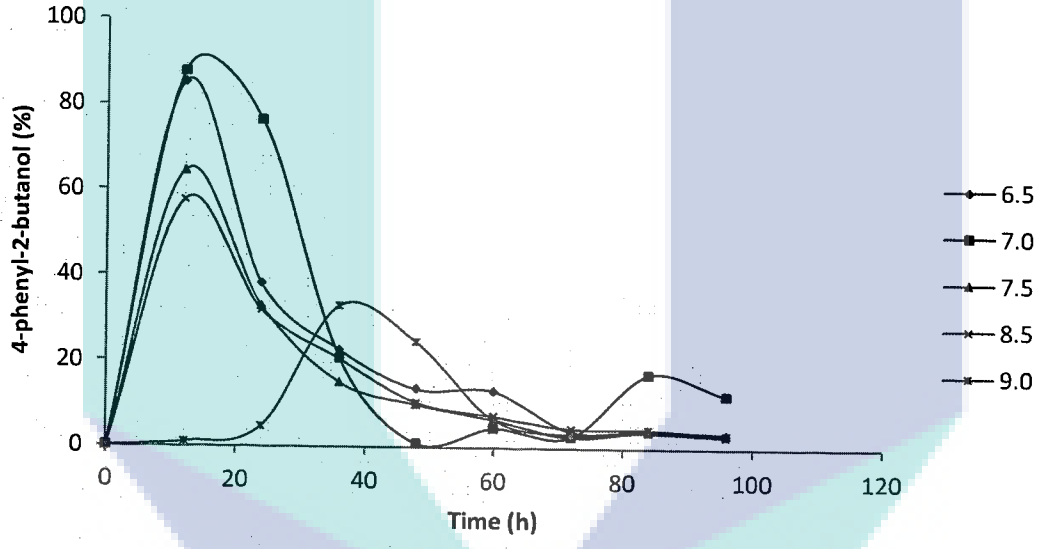

Figure 4.31: The product formation during biotransformation of benzyl acetone using *P. aeruginosa* at different initial pHs. The reaction was carried out in shake-flask culture for 96 h (Conditions: 0.1 g/L benzyl acetone, 20 g/L glucose, 37°C, 150 rpm).

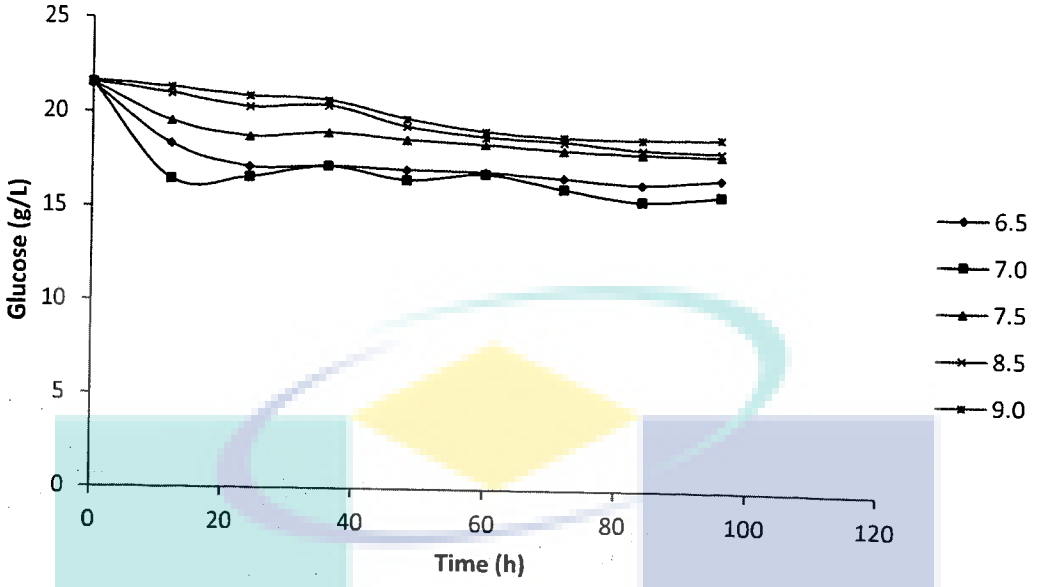

Figure 4.32: Glucose consumption during biotransformation of benzyl acetone using *P. aeruginosa* at different initial pHs. The reaction was carried out in shake-flask culture for 96 h (Conditions: 0.1 g/L benzyl acetone, 20 g/L glucose, 37°C, 150 rpm).

The result shown in Figure 4.33 demonstrates that maximum production of 4-phenyl-2-butanol was achieved in a biotransformation of benzyl acetone carried out at an initial pH of 7, with *E. faecalis* acted as a biocatalyst; the highest percentage of benzyl acetone converted into 4-phenyl-2-butanol was about 7%, achieved after 12 h of incubation. Substantial decline in the production of 4-phenyl-2-butanol was witnessed when biotransformation was carried out at an initial pH lower or higher than 7. The growth of *E. faecalis* on the other hand, did not vary significantly when the initial pH was set in the range of 6.5 to 8.5. Analysis on the residual glucose concentration has in overall shown that relatively high amount of glucose been consumed in a biotransformation carried out at an initial pH of 7 compared to other pH tested (Figure 4.34).

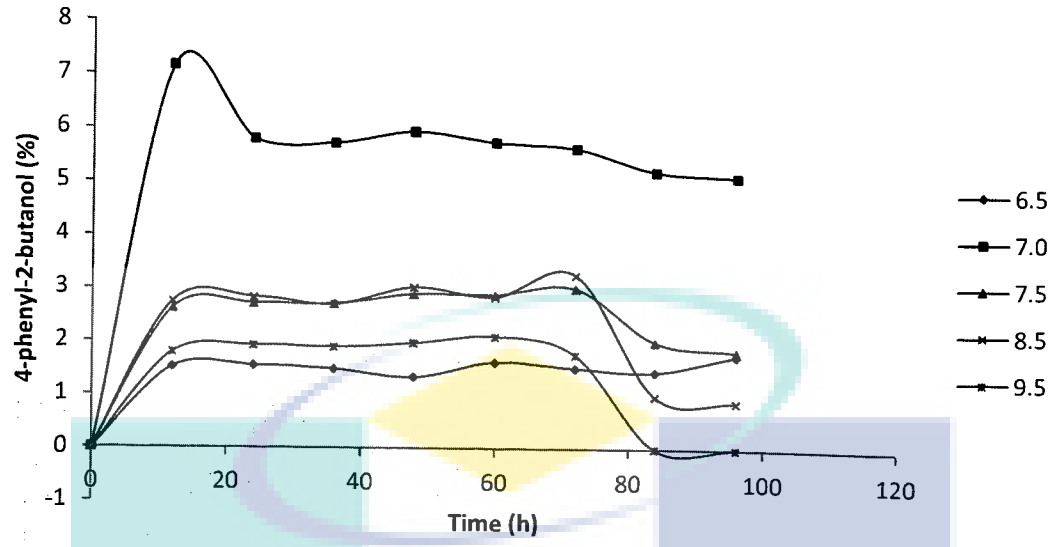

Figure 4.33: The product formation during biotransformation of benzyl acetone using *E. faecalis* at different initial pHs. The reaction was carried out in shake-flask culture for 96 h (Conditions: 0.15 g/L benzyl acetone, 40 g/L glucose, 37°C, 150 rpm).

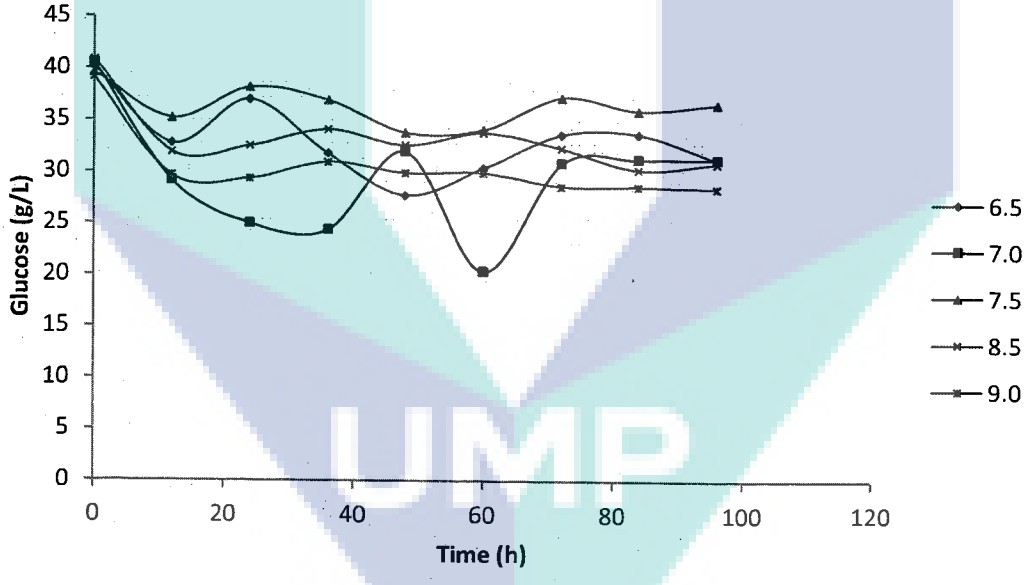

Figure 4.34: Glucose consumption during biotransformation of benzyl acetone using *E. faecalis* at different initial pHs. The reaction was carried out in shake-flask culture for 96 h (Conditions: 0.15 g/L benzyl acetone, 40 g/L glucose, 37°C, 150 rpm).

Production of 4-phenyl-2-butanol by biotransformation of benzyl acetone using *B. cereus* as a biocatalyst did vary with the change in initial pH as showed in Figure 4.35. Biotransformation of benzyl acetone by *B. cereus* was best performed in a reaction

with the initial pH of 7, according to the results presented; the production of 4-phenyl-2-butanol at this pH was of the highest after 24 h of reaction. The highest percentage of benzyl acetone transformed into 4-phenyl-2-butanol was 11 %, achieved after 72 h of biotransformation with the initial pH of 7. It is worthwhile to call the attention to the higher production of 4-phenyl-2-butanol recorded within 24 h of reaction when the biotransformation was carried out at a pH higher than 7. The production of 4-phenyl-2-butanol, however, started to recede over time; this observation was probably attributable to the rapid entry to stationary phase within this range of pH. The amount of glucose consumed by *B. cereus* during biotransformation did not vary much with changes in pH of the reaction (Figure 4.36).

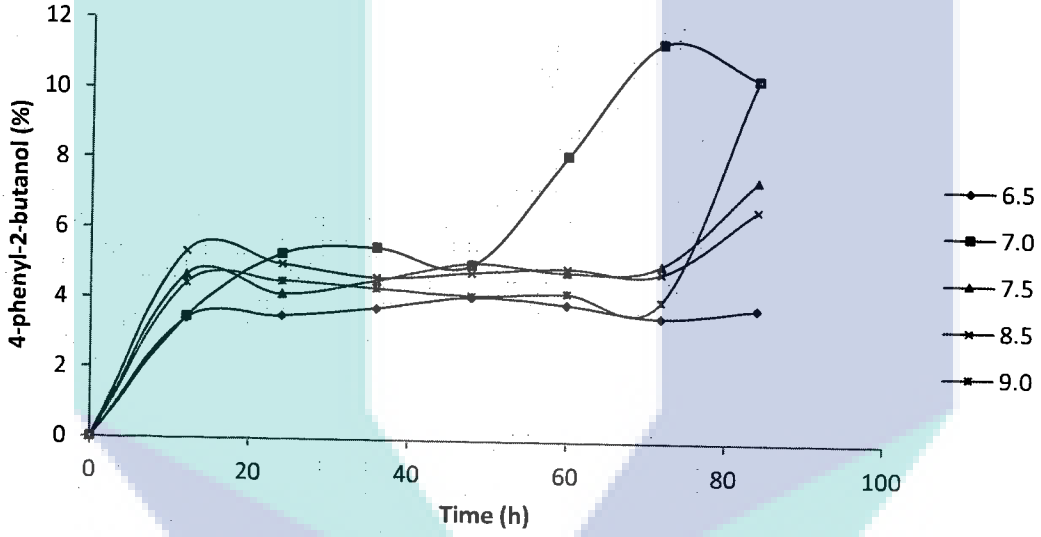

Figure 4.35: The product formation during biotransformation of benzyl acetone using *B. cereus* at different initial pHs. The reaction was carried out in shake-flask culture for 96 h (Conditions: 0.15 g/L benzyl acetone, 40 g/L glucose, 37°C, 150 rpm).

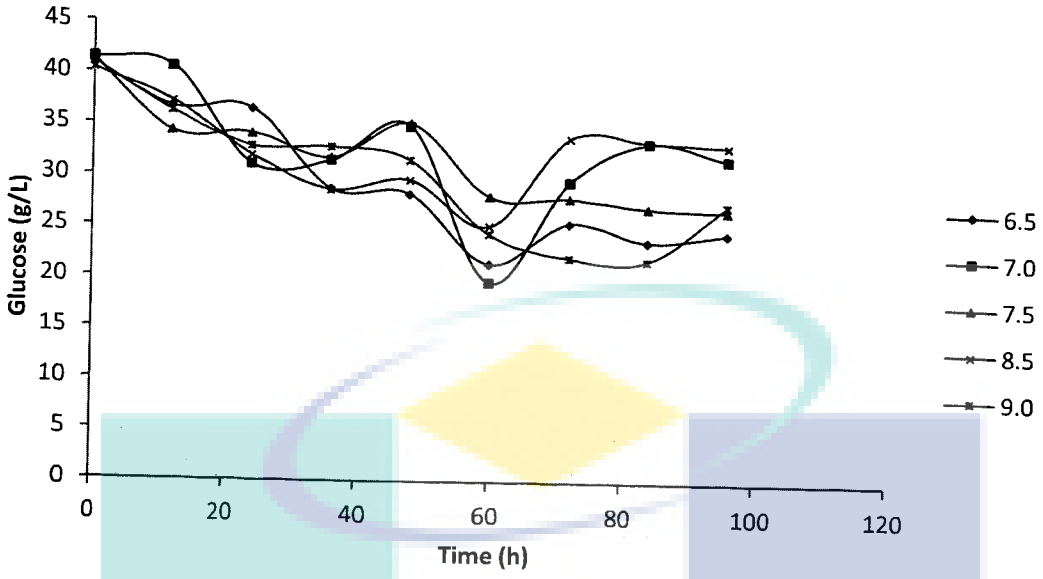

Figure 4.36: Glucose consumption during biotransformation of benzyl acetone using *B. cereus* at different initial pHs. The reaction was carried out in shake-flask culture for 96 h (Conditions: 0.15 g/L benzyl acetone, 40 g/L glucose, 37°C, 150 rpm).

A close association was observed between variations in enantioselectivity under different pH with the OH group produced through the reduction of ketone group to alcohol. Change in conformation of enzymes rendered by pH variations could be one of the factors that either slowed down or accelerate the rate of biotransformation. It is a general consensus that most enzymes activities are lost at extremely acidic or alkaline conditions (Abas et al., 2010). Acidity in internal medium has resulted in the activation of ATPase, a group of enzymes responsible in extruding protons to the exterior at the expense of hydrolysis of ATP to AMP. Constant hydrolysis of ATP caused accumulation of AMP which in turn, triggered the generation ATP through glycolytic pathway; the role of ATP/AMP in controlling the glycolytic pathway in *S. cerevisiae* was also elaborated by Arifin et al. (2011).

Change in external pH does influence the reduction of NAD(P)+,. It was shown that acidic exterior has resulted in the transport of protons to the interior of the cell; this mode of action was described as a counterbalance to the net negative charge in the cell interior. Reduction in the phosphorylation of glucose through pentose pathway was observed in the event of rapid ATP regeneration in the cells of *S. cerevisiae*, which then engendered less regeneration of cofactors. Arifin et al. (2011) reported pH 7 as an

optimum value for the growth of *S. cerevisiae* and its biotransformation activity. Low in activity of biotransformation was observed when the cells were grown in acidic condition; this occurrence might be due to the incompetence of the cells at being a good reducer of NADP(+) when grown in acidic environment. Despite an optimum performance seen at pH 7, a reverse trend was observed at pH 10, suggesting the possibility that the precursor and product being hydrolyzed under alkaline condition.

Carlin et al. (2010) described the mechanisms involved during the adaptation of *B. cereus* to low pH. Loss of acid-sensitive enzymes such as glycolytic enzymes happened when the cells of *B. cereus* were exposed to acidic environment. Reduction in cytoplasmic pH was held responsible for such observation. Since reduction process is strictly dependent on the availability of ATP and cofactor (NADH), loss of glycolytic enzymes activities could, therefore, affect the rate of reduction process. The low conversion of benzyl acetone to 4-phenyl-2-butanol at low pH may be well explained by such mechanisms. Lay et al. (2015) found that at low pH, the cells of *B. cereus* were more susceptible to heat compared to high pH; therefore, the growth and activity of *B. cereus* was found contracted at low pH. The heat sensitivity was most probably due to the damage of DNA at low pH, attributable to the reduction of divalent ions that aid in stabilizing the DNA molecules.

Glucose catabolism in *P. aeruginosa* is not limited by a single transport system and its accompanying phosphorylation step. Glucose can be catabolically broke down through alternative pathways which involve the action of the membrane-bound enzymes such as glucose dehydrogenase and gluconate dehydrogenase. These enzymes are responsible for extracellular direct oxidative pathway in *P. aeruginosa* through which the electron is directly transferred from sugar to the electron transport chain, leading to the generation of a proton motive force. Generation of proton motive force is closely associated with changes in pH gradient between the internal and external environments of the cell (Niepa et al., 2017). Since conformity and specificity of enzymes are marginally influenced by pH, most of enzymatic processes and transportation of various components across the cell membrane will be eventually affected.

While a study showed that the cellular integrity of *S. cerevisiae* was compromised at extreme pH of 2.5 and 12.0 (Olivero et al., 1982), another study

reported a range of pH from 2.75 to 4.25 held a vital role in supporting the growth of *S. cerevisiae* during wine fermentation (Heard and Fleet, 1988). Since biotransformation involves enzymatic activities, alteration in the ionic state of the precursor and enzymes with variation in pH may up to a certain extent affect the rate and yield of biotransformation. When enantioselectivity is a major concern, careful selection of the right pH is a matter of great importance in order to obtain desirable biotransformation products (Bubalo et al., 2015). Maximum activity of biotransformation by *S. cerevisiae* at pH 7.5 was observed in a work by Silva et al. (2012); production of citronellol from citral, mediated by *S. cerevisiae* has, on the other hand, reached its maximum level at pH 5.5 (Esmaeili et al., 2011).

On the other hand, the highest enantiomeric excess of 99.9% and conversion of 94.7% was obtained for the reduction of 1-(-4-bromophenyl)-ethanone to (R)-1-(-4-bromo-phenyl)-ethanol by *A. niger* when the cells were grown in a medium with a pH of 7 (Abas et al., 2010); low biotransformation enantioselectivity and yield were observed at pH lower or higher than 7 since alteration in the ionic state of precursor and enzymes occurred with changes in pH, leading to the change in enzymatic reactions. Changes in stereoselectivity may be attributable to simultaneous action of several enzymes with opposite stereoselectivity toward the same precursor.

Similar result was seen in Amaranth dye decolorization by *P. aeruginosa*, by which the decolorization was at its optimum level when the pH was set to 7 (Jadhav et al., 2013). The same was observed in AHAS activity in *P. aeruginosa* by which these enzymes worked optimally in a range of pH between 7 and 7.5; fell in activity was observed at a pH lower or higher than the optimum range (Cho et al., 2013). Chakraborty et al. (2016) deduced that the bacterial growth and degradation of precursor are immensely influenced by the concentration of hydrogen ion available in the culture medium. Enzymes such as protease were too found to be active at a pH of 7 and reduction in its activity at lower and higher pH indicated inactivation of these enzymes (Chia et al., 2008). Although sometimes the growth and enzyme activity did not change much in a certain range of pH but at larger scales with longer incubation times and more efficient aeration and agitation, the difference is expected to be significant.

An approximately parabolic trend was observed in a relationship between the growth rate of *A. niger* and pH; pH values close to optimum value resulted little changes in the growth rate of *A. niger* but significant decline was observed as the value close to acidic and alkaline growth limits (Carillo-Sancen et al., 2016).At different stages of life cycle, *A. niger* requires different pH to grow on. A pH higher than 5 is favorable to germination of spores of *A. niger* while the production of citric acid by *A. niger* is favored at pH lower than 2; while the production of citric acid is favorable in the latter, production of other organic acids is simultaneously inhibited. When *A. niger* was grown on a medium with a low pH, a group of dominant extracellular mycelium-bound invertase became active.

Another study reported the activity of xylanase under various pH values (Do et al., 2013); this study demonstrated maximum xylanase activity in media with a pH of 7. Active cellulase activity was on the other hand, observed in a range of pH between 4 and 4. Based on this finding, we may deduce that different enzymes and precursors react differently to changes in pH despite their similar origin. While the growth of *A. niger* exhibited differently at different pH, activities of enzymes were most of the time independent of its growth. The ability of *A. niger* to produce protons from the sparse nutrients available in saprophytic environments in order to achieve optimized metabolisms is however, noteworthy. This mode of mechanism was seen in acid production by *A. niger* by which the production did not stem from overflow metabolism but rather to fulfil the necessary proton production (Carillo-Sancen et al., 2016).

According to the results obtained in this study, it can be clearly seen that pH 7 has conferred better growth of *E. faecalis* and its glucose consumption. Perhaps rapid respiration as well as overall metabolism of *E. faecalis* at this pH is what have led to a better biotransformation activity compared to other conditions tested. The result was further supported by a previous study by which the growth of *E. faecalis* was detected in a broad range of pH between 5.0 and 9.6, with maximum growth recorded in pH ranged from 7 to 7.5 (Mate and Pathade, 2012). Compared to gram-negative bacteria, responses in *E. faecalis* toward changes in pH seem to be different. Unlike gram-negative bacteria, normal rate in protein synthesis may not be necessary in growing cells of *E. faecalis* in order to build tolerance against various pHs, according to Flahaut et al. (1997). The mentioned study has demonstrated that despite imperceptible change

recorded in *E. faecalis* growth with changes in pH, remarkable reduction in protein expression was observed at extreme pH. About 50% of proteins expressed under normal condition were not expressed when the cells of *E. faecalis* grown in media with high pH; a twofold increase in expression of some proteins was on the other hand, observed under alkaline stress.

Reduction in biotransformation activity of *B. cereus* at low pH was most probably attributable to increasing toxicity effect of the precursor and conversion product against *B. cereus* with decreasing pH (Ghati and Paul, 2016). Such observation may indicate low metabolism of *B. cereus* at low pH which could, in turn, result in low percentage of benzyl acetone transformed to 4-phenyl-2-butanol. *B. cereus* has generally been associated with foodborne illnesses. Since it is rare to find food commodities with high pH, the effect of such condition on the growth of *B. cereus* was, therefore, less studied. A published work by Garcia-Arribas and Kramer (1990) was among few studies reporting on the behavior of *B. cereus* when the cells were grown in alkaline condition. The study described a rapid cell division o *B. cereus* at pH 8.8 despite the extended lag phase observed under the same condition. The resistance of *B. cereus* toward higher pH may have contributed to the considerable percentage of benzyl acetone transformed to 4-phenyl-2-butanol. On the other hand, a recent study has demonstrated 25°C as an optimum temperature for decolorization of azo dye reactive black B, using *B. cereus* as a biocatalyst.

4.7.5 Effect of temperature on biotransformation

Conversion of benzyl acetone to 4-phenyl-2-butanol was remarkably high when the reaction was carried out at 30°C, a result of which clearly showed in Figure 4.37. Changes in temperature were mostly detected by biological thermosensors present in the polysaccharide membrane surface of bacteria (Pereira, 1994). Biochemical aspects of yeasts including *S. cerevisiae* were most of the times directly affected by temperature of fermentation (Heard and Fleet, 1988). The possibility that the optimum temperature for the growth of *S. cerevisiae* could be higher than 34°C was reported by Arroyo-Lopez et al. (2009) in which the author opined that at different pH, the optimum temperature could be different. The result has concurred with Torija et al. (2002), whose work has demonstrated faster fermentation activity by *S. cerevisiae* at temperatures higher than

34°C. Relatively slow consumption of glucose was observed at a temperature of 27°C within 96 h of reaction despite the rapid growth seen at this temperature (Figure 4.38).

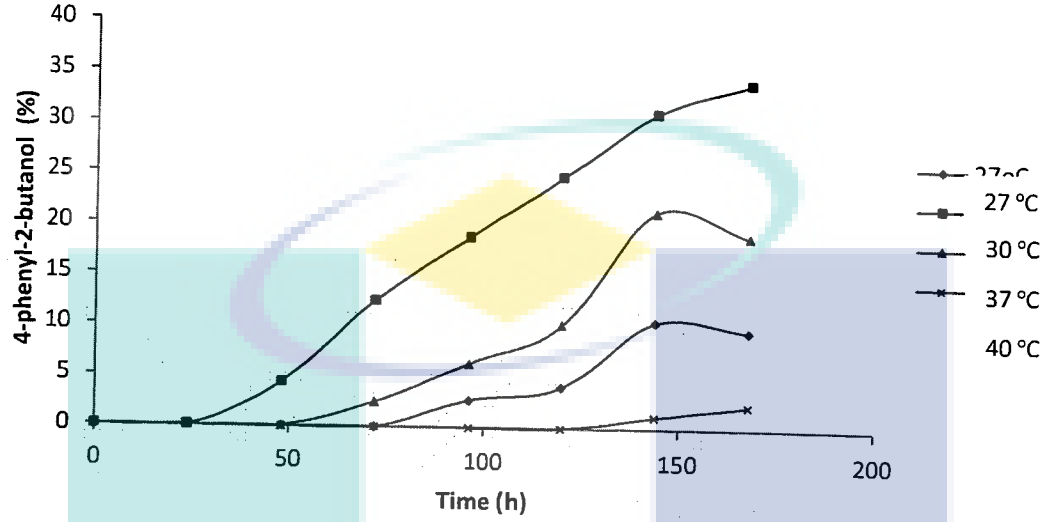

Figure 4.37: The product formation during biotransformation of benzyl acetone using *S. cerevisiae* at different temperatures. The reaction was carried out in shake-flask culture for 168 h (Conditions: 0.1 g/L benzyl acetone, pH 7.5, 150 rpm, 20 g/L glucose).

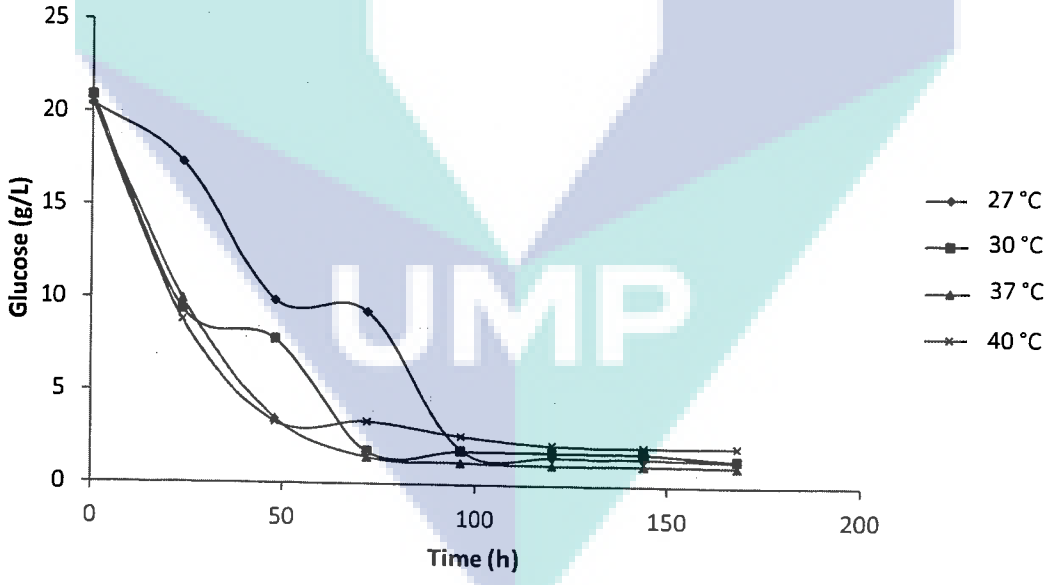

Figure 4.38: Glucose consumption during biotransformation of benzyl acetone using *S. cerevisiae* at different temperatures. The reaction was carried out in shake-flask culture for 168 h (Conditions: 0.1 g/L benzyl acetone, pH 7.5, 150 rpm, 20 g/L glucose).

Conversion of benzyl acetone into 4-phenyl-2-butanol by *A. niger* was considerably affected by temperature as shown in Figure 4.39. The percentage of benzyl acetone transformed into 4-phenyl-2-butanol in this biotransformation was in overall higher at the temperature of 27°C compared to other temperatures tested; the highest percentage recorded was 94 %, achieved after 48 h of reaction at 27°C. The production of 4-phenyl-2-butanol was remarkably low when the reaction was carried out at the temperature of 40°C. Notwithstanding the apparent effect of temperature demonstrated on the production of 4-phenyl-2-butanol, the pattern recorded in the glucose consumption during the biotransformation was on the other hand did not vary much between each of the temperatures tested (Figure 4.40).

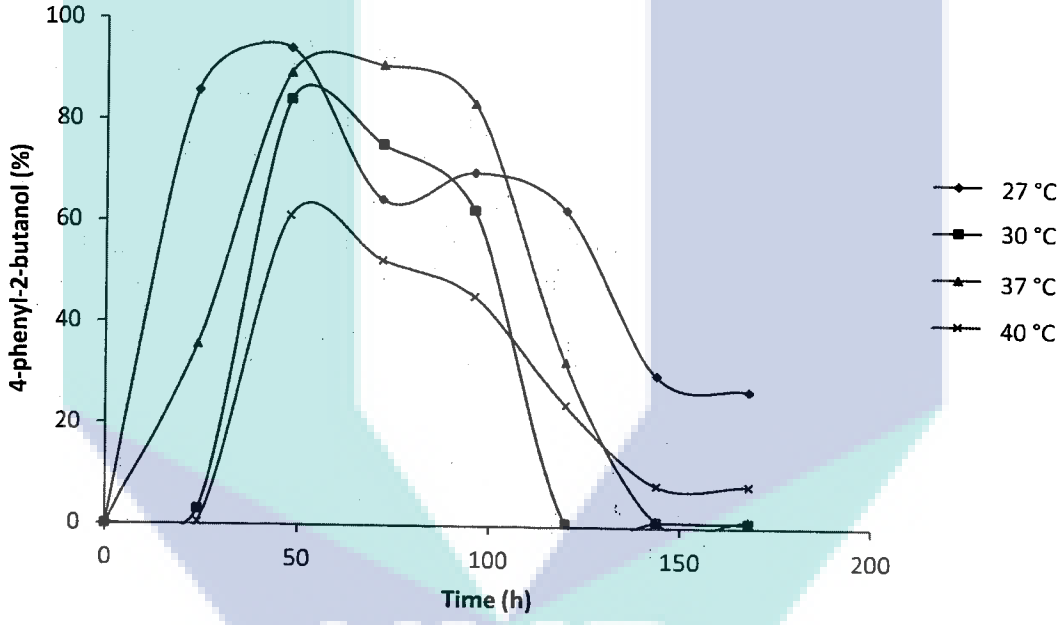

Figure 4.39: The product formation during biotransformation of benzyl acetone using *A. niger* at different temperatures. The reaction was carried out in shake-flask culture for 168 h (Conditions: 0.15 g/L benzyl acetone, 50 g/L glucose, pH 7.5, 150 rpm).

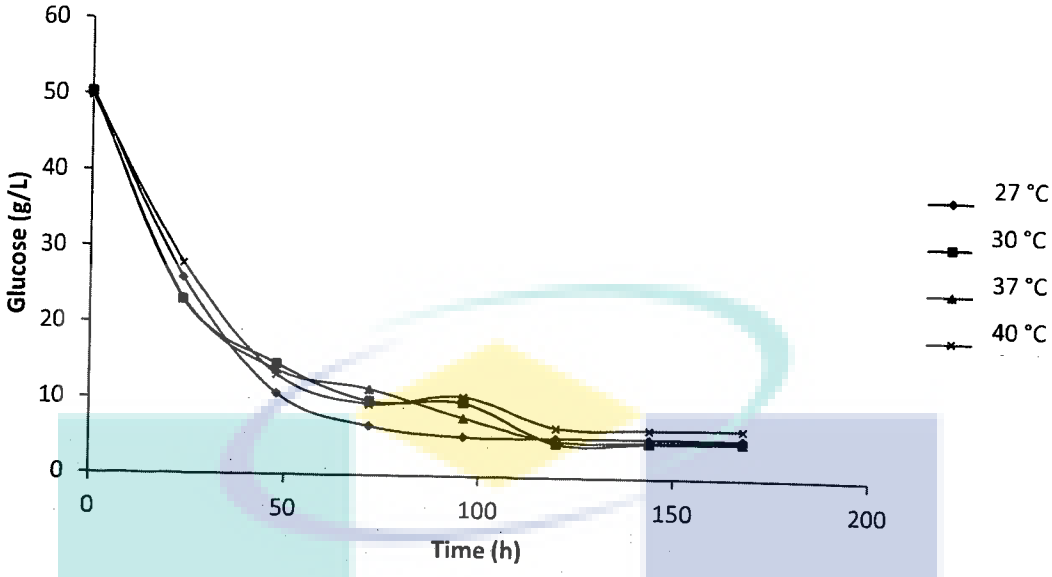

Figure 4.40: Glucose consumption during biotransformation of benzyl acetone using *A. niger* at different temperatures. The reaction was carried out in shake-flask culture for 168 h (Conditions: 0.15 g/L benzyl acetone, 50 g/L glucose, pH 7.5, 150 rpm).

The capacity of *P. aeruginosa* to carry out biotransformation of benzyl acetone was affected when changes in temperature were applied. Figure 4.41 showed that transformation of benzyl acetone into 4-phenyl-2-butanol by *P. aeruginosa* was best performed at the temperature of 30°C, with the highest percentage of conversion reached up to 98% after 12 h of incubation; slight decline in the production of 4-phenyl-2-butanol was observed as the temperature increased to 40°C. Biotransformation performed at 27 °C has proven to be of inferior quality since the production of 4-phenyl-2-butanol was remarkably the lowest among all conditions tested. Similar outcome was attained in a conversion of limonene to perillyl alcohol by *P. aeruginosa* by which the maximum yield was produced at 30°C; the fact that incubation at this temperature has conferred a considerable biomass production is something noteworthy (Chatterjee and Bhattacharyya, 2001). Incubation at different temperatures has in overall caused subtle changes in the amount of glucose consumed by *P. aeruginosa* during biotransformation (Figure 4.42).

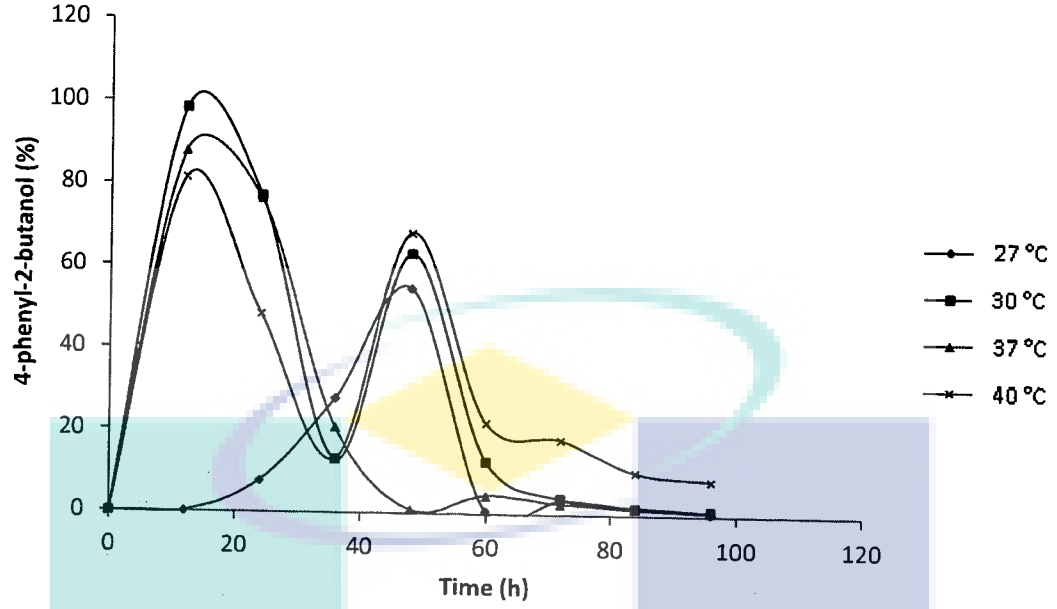

Figure 4.41: The product formation during biotransformation of benzyl acetone using *P. aeruginosa* at different temperatures. The reaction was carried out in shake-flask culture for 96 h (Conditions: 0.1 g/L benzyl acetone, 20 g/L glucose, pH 7, 150 rpm).

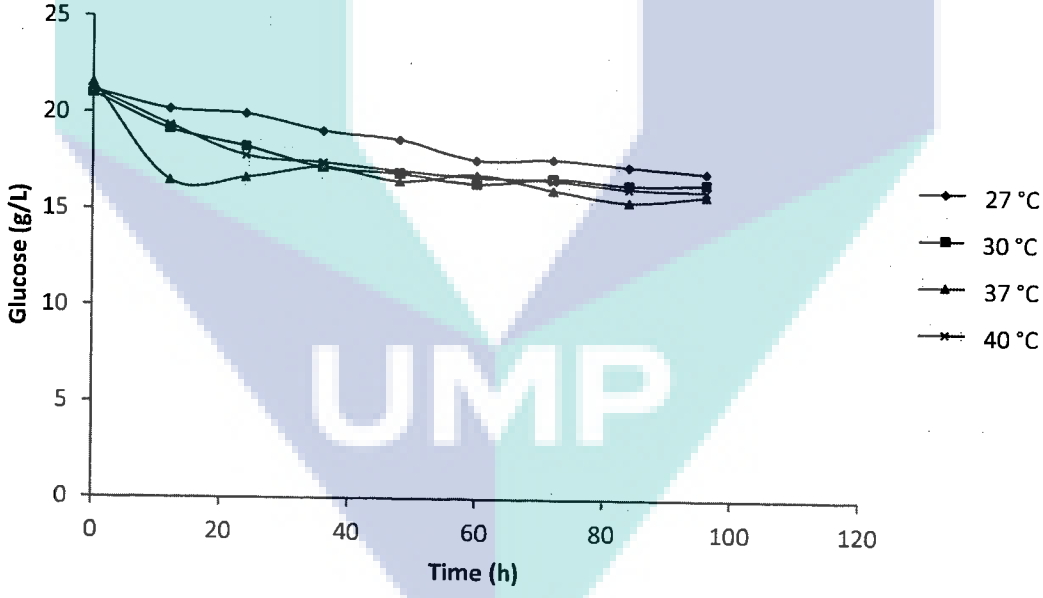

Figure 4.42: Glucose consumption during biotransformation of benzyl acetone using *P. aeruginosa* at different temperatures. The reaction was carried out in shake-flask culture for 96 h (Conditions: 0.1 g/L benzyl acetone, 20 g/L glucose, pH 7, 150 rpm).

Temperature does influence the production of 4-phenyl-2-butanol through biotransformation of benzyl acetone using *E. faecalis* as a biocatalyst, a deduction drawn based on the result presented in Figure 4.43. Relatively high percentage of benzyl acetone was reduced into 4-phenyl-2-butanol in a biotransformation at 37°C within 24 h of reaction compared to the production recorded at other temperatures. Between 40 to 60 h however, the production of 4-phenyl-2-butanol was relatively high in the reaction carried out at 27°C compared to other conditions tested; this result may imply that incubation at 27°C would slow down the biotransformation, but did not altogether inhibit this activity. Incubation at 40°C on the other hand, has evidently deteriorated biotransformation activity of *E. faecalis* and therefore, has resulted in a low production of 4-phenyl-2-butanol throughout the experimental time. The highest production was achieved after 72 h of incubation at 27°C, with 8 % of benzyl acetone transformed into 4-phenyl-2-butanol. Glucose consumption has generally corresponded to biotransformation activity of *E. faecalis* at different temperatures (Figure 4.44).

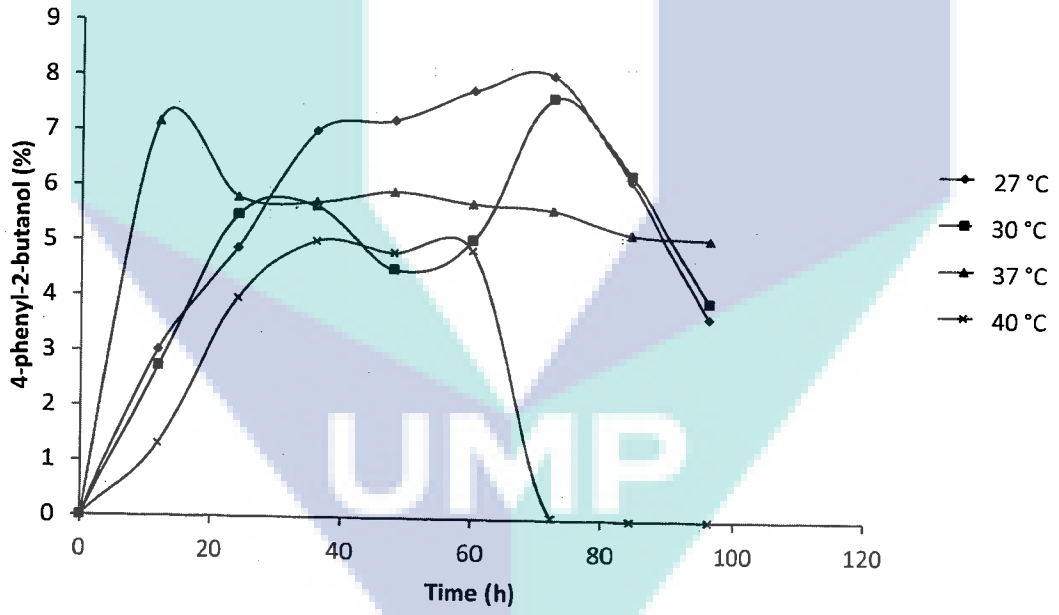

Figure 4.43: The product formation during biotransformation of benzyl acetone using *E. faecalis* at different temperatures. The reaction was carried out in shake-flask culture for 96 h (Conditions: 0.15 g/L benzyl acetone, 40 g/L glucose, pH 7, 150 rpm).

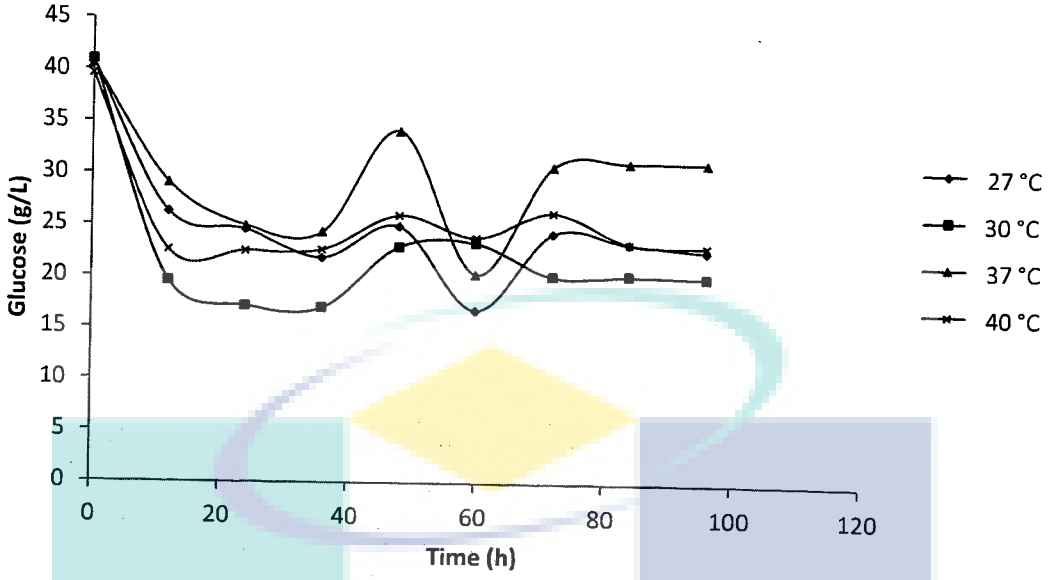

Figure 4.44: Glucose consumption during biotransformation of benzyl acetone using *E. faecalis* at different temperatures. The reaction was carried out in shake-flask culture for 96 h (Conditions: 0.15 g/L benzyl acetone, 40 g/L glucose, pH 7, 150 rpm).

Based on results displayed in Figure 4.45, it is evident that change in temperature has substantially affected the percentage of benzyl acetone transformed into 4-phenyl-2-butanol in a biotransformation carried out using *B. cereus*. Increase in temperature up to 37°C has resulted in increase in the production of 4-phenyl-2-butanol within 24 h of biotransformation. Beyond 24 h of reaction however, the production of 4-phenyl-2-butanol was generally highest in the reaction carried out at 40°C, despite the fluctuations observed. The result, therefore, demonstrated that increased in temperature has generally facilitated the conversion of benzyl acetone to 4-phenyl-2-butanol by *B. cereus*. A percentage of as high as 10% of benzyl acetone was converted into 4-phenyl-2-butanol by *B. cereus* after 72 h of biotransformation carried out at 40°C. Increase in biotransformation activity has generally resulted in increase in amount of glucose consumed by *B. cereus* (Figure 4.46).

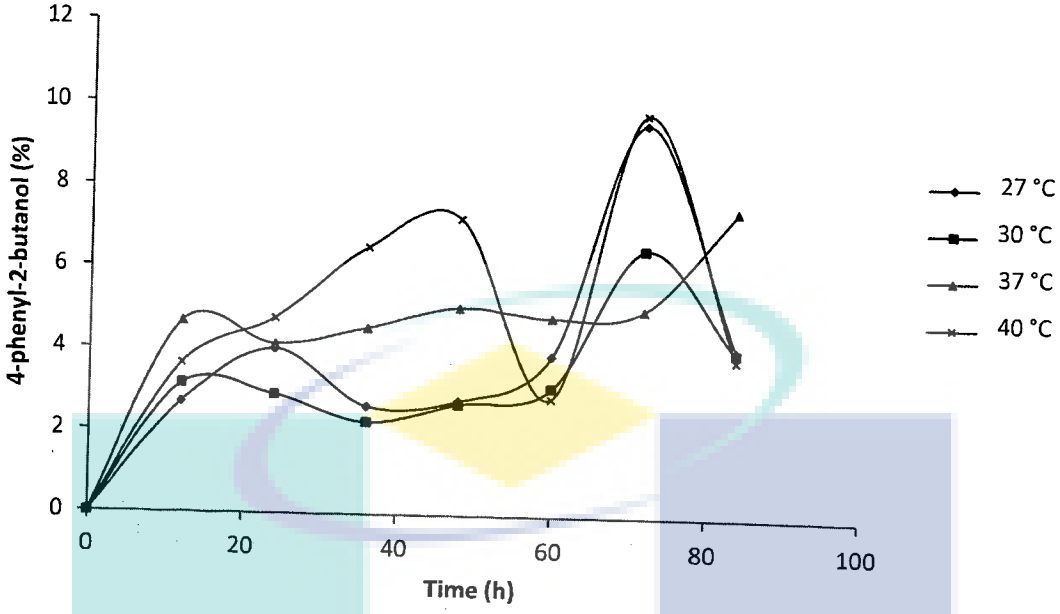

Figure 4.45: The product formation during biotransformation of benzyl acetone using *B. cereus* at different temperatures. The reaction was carried out in shake-flask culture for 96 h (Conditions: 0.15 g/L benzyl acetone, 40 g/L glucose, pH 7.5, 150 rpm).

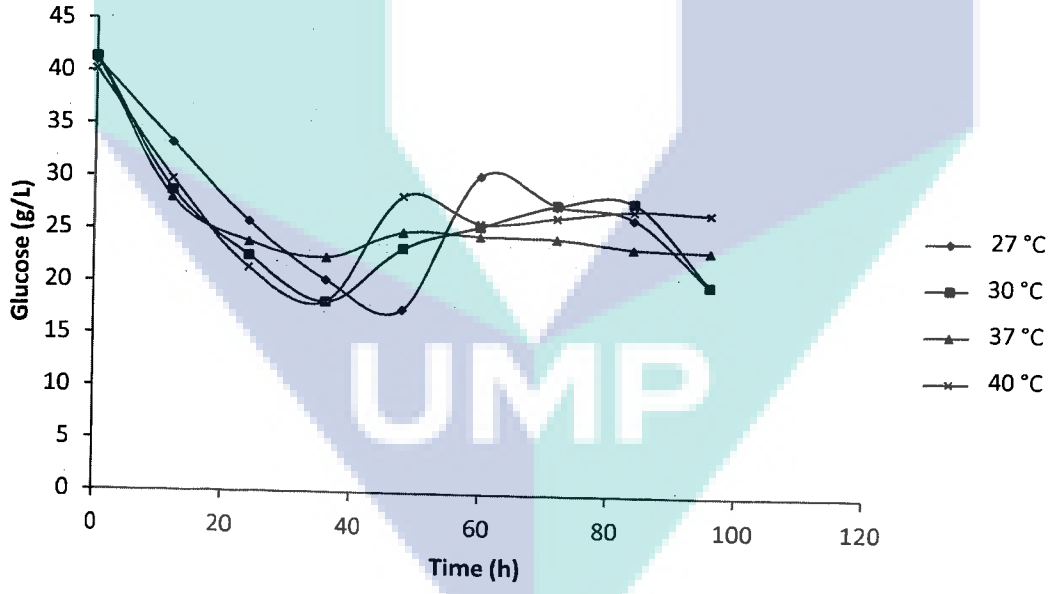

Figure 4.46: Glucose consumption during biotransformation of benzyl acetone using *B. cereus* at different temperatures. The reaction was carried out in shake-flask culture for 96 h (Conditions: 0.15 g/L benzyl acetone, 40 g/L glucose, pH 7.5, 150 rpm).

Compared to other species, *S. cerevisiae* stand a lesser chance of growing at low temperature which is probably due to the lack of ability to increase tolerance toward

ethanol (Gao and Fleet, 1988). Since *S. cerevisiae* metabolism is greatly influenced by temperature, the production of secondary metabolites such as glycerol, acetic acid, succinic acid, etc. are therefore affected too (Torija et al., 2002). Rapid production of acetic acid and glycerol might have inhibited the production of ethanol since less ethanol was produced as the temperature increased. Efficient sporulation of *S. cerevisiae* at temperature higher than 25°C is however, something worth a note since it concurred with the results obtained in this study.

The low conversion of benzyl acetone to 4-phenyl-2-butanol at low temperature was probably due to the reduction in the molecular transfer of the substrate across the cell membrane of microorganisms. According to Ayari et al. (2016), change in temperature has substantially resulted in modification of membrane fluidity. The modification took place by which the liquid fluid crystalline structure of the membrane has turned into a rigid gel state when the cells of *B. cereus* were exposed to low temperature; such changes, therefore, could affect the rate of molecular transfer of the substrate across the membrane. The relatively high conversion of benzyl acetone to 4-phenyl-2-butanol at high temperature, as seen in this study, was probably due to the adaptation mechanism of *B. cereus* to such condition. Change in protein expression for the survival of *B. cereus* at high temperature was also reported in the previously mentioned study.

It was further supported by earlier work by Chung et al. (1978) who demonstrated that as temperature decreased, increase in participation of pentose phosphate pathway as an alternative to glycolysis in glucose metabolism has occurred. The study also reported on the opposite effect observed with increasing temperature. As opposed to glycolysis, ATP is not generated but used in pentose phosphate pathway for the synthesis of ribose-5-phosphate and erythrose-4-phosphate. Such circumstances may, therefore, have accounted for slow biotransformation activity of *B. cereus* at low temperature since ATP required for reduction of benzyl acetone to 4-phenyl-2-butanol was less produced. Generation of NADH, however, does take place in pentose phosphate pathway, which has explained the increase in biotransformation activity at 27°C over time.

Alonso-del-real et al. (2017) showed different profiles of fermentation kinetics under different temperatures. While low temperatures conferred a slower growth of *S. cerevisiae*, high temperatures on the other hand, granted faster growth rate. Declining phase was, however, observed only at high temperatures as opposed to low temperatures, indicating that the cell viability decline with increase in temperature. Rapid glucose consumption by *S. cerevisiae* was reported when the cells were grown at low temperature since the high biomass was maintained throughout the reaction period. Higher mortality rate at high temperatures resulted in high residual glucose concentration at the end of reaction. High mortality rate might be due to the alteration in the structure of the membrane which would eventually decrease its functionality. While low temperatures seem feasible to cater the biomass production of *S. cerevisiae* delay in the fermentation is however not economically feasible from the industry point of view.

According to Contesini et al. (2010), maximum biomass production of *A. niger* was achieved at temperature of 30°C while Alborch et al. (2011) argued that temperature in a range of 25 to 40°C confers optimum condition for the growth of *A. niger*. Increase in temperature has led to marginal increase in the rate of biomass production but the cells entered declining phase earlier compared to that of lower temperature; the maximum enzyme activity was thus achieved in a relatively shorter time. At high temperature however, the cells are more susceptible to the action of antimicrobial agent due to the alteration of cell membrane composition and fluidity. Alteration of cell membrane may allow a better antimicrobial agent penetration into the cell. The growth of mycelia and production of enzymes may not necessarily correlated to each other. Germination of spores on the other hand took place rapidly at high temperature as demonstrated in the same study.

A close association between elevated temperature and oxidative stress events was demonstrated in several works (Li et al., 2008; O'Donnell et al., 2011). Both studies coincided with the opinion that the production of ROS occurs when cells of *A. niger* were exposed to thermal stress. The mechanism was further explained through the formation of superoxide in mitochondria upon electron leakage from respiratory chain which in turn led to a cascade of ROS production. Since it is generally proclaimed that the oxygen-derived free radicals are the chief source of the oxidative damage observed under thermal stress, respiratory enzymes and mitochondria are therefore especially

vulnerable to such damage. Irreversible oxidative damage such as protein carbonylation often leads to loss of protein function that will eventually cause destruction of microbial cells.

O'Donnell et al. (2011) conjectured the activation of alternative NADH dehydrogenases as a control mechanism to curb the production of ROS under thermal stress. High concentration of reductants such as NADH produced following the event of oxidative stress is what has triggered the activation of alternative NADH dehydrogenases. It is of common theory that conventional NADH dehydrogenases conferred an appropriate ratio of $NADH/NAD^+$ through respiratory activity. When the cells of *A. niger* grown aerobically, increase in respiratory activity allowed an adequate ATP generation and improved nutrient utilization. Alternative NADH dehydrogenases on the other hand, behave differently from the conventional NADH dehydrogenase by which the former lack the ability to support the formation of proton motive force across the cell membrane. Activity of ATP synthase, a group of enzymes responsible for ATP generation is substantially dependent on proton motive force; generation of ATP is therefore limited in the absence of this force. Since most of cellular processes and culture metabolism involve high energy consumption, lack of ATP in the cells could ultimately be detrimental to the fungal growth and biotransformation activity.

Alteration in the activity of enzyme is attributable to the strong influence that the temperature has on the molecular collision between the enzymes and substrates; rapid collisions lead to better rate of reactions (Alonso-del-Real et al., 2017). While higher temperature promotes acceleration in molecular collision between enzymes and substrates, denaturation of enzyme can also occur at high temperature. A study on the reduction of geraniol by *S. cerevisiae* has shown that temperatures in the range of 35 to 40°C were optimum for maximum reduction activity. The result was typical of enzymatic reactions but the fact that the reduction activity was favored at a broad range of temperature indicates that several enzymes might have involved in that reaction.

When activity of certain enzymes is concerned, different temperatures are required at different stages of fermentation in order to maximize the productivity of enzymes. Xylanase for instance, are mostly active at 28°C and it was observed that the high productivity of xylanase was accompanied by increase in biomass production; high

biomass production however, may not necessarily result in rapid productivity of enzymes. Contradictory to this finding, a much later study by Do et al. (2013) reported maximum activity of xylanase at 50°C. Acharya et al. (2008) reported maximum degradation of sawdust at 28°C through which xylanase was microbially produced by *E. faecalis*. Maximum biotransformation of citral by *A. niger* on the other hand, occurred at 27°C at which the optimum biomass production of *A. niger* was also conferred (Esmaeili et al., 2011).

Wu et al. (2012) demonstrated that temperature does affect bacterial genome expression; alteration in gene expressions was observed when the cells of *P. aeruginosa* were grown at different temperatures. Oxygen uptake and temperature influence the synthesis of enzyme either non-specifically or specifically; the former involve change in the rate of biochemical reactions in the cell dependently upon temperature alterations while the latter involve either induction or repression of enzymatic reactions. The effect of temperature was keenly observed in the same study whereby 37°C served as an optimum temperature for the growth of *P. aeruginosa* and 53% loss of enzymatic activity occurred at temperature higher than that. Also reported was the influence of temperature on extracellular enzymatic activity by which it was conjectured that temperature variations would cause alteration in physical properties of the cell membrane. Therefore, secretion of enzyme would be greatly affected by changes in temperature.

Dependence of both conversion yield and diastereoisomeric excess on the reaction temperature was also demonstrated in a work by Silva et al. (2012). Increase in conversion products was observed with increase in temperature; the yield has, however, started to decline as the temperature was further raised, suggesting the possibility that the stability of enzyme was compromised at higher temperature. In the mentioned study, the maximum conversion of citral to citronellol by *S. cerevisiae* was achieved at an optimum temperature of 27°C. When stereoselectivity is a major concern, the optimum temperature for high stereoisomeric excess may not be necessarily the same, as reported by (Bubalo et al., 2015). A study conducted by Deng et al. (2008) demonstrated that the optimum temperature for decolorization activity of *B. cereus* could be different, depending on the type of substrate. Decolorization of different type of dyes was conducted in the mentioned study by which the three dyes, namely, Acid Blue 25,

Malachite Green and Basic Blue X-GRRL were optimally decolorized at 37 – 45, 37 and 30°C, respectively. Decolorization was not observed at temperature below 20°C which indicated that inactivation of related enzymes has occurred.

The result obtained was also in harmony with a previous work, which has reported that the optimum temperature for maximum decolorization of Amaranth dye by *P. aeruginosa* was 30°C; further increase in temperature has resulted in the reduction of dye decolorization activity, according to Jadhav et al. (2013). Perhaps the stability of the substrate and product at a certain temperature is what has led to maximum enzyme activity and yield. This conjecture seems valid considering that the low temperature minimizes evaporation loss of volatile substrate and bioconversion products. Tremendous biomass production may influence bioconversion activity of *P. aeruginosa* since most of the time, enzyme activity is marginally affected by microbial growth. Chatterjee and Bhattacharrya (2001) explained that increase in the growth of *P. aeruginosa* at higher temperature, despite decrease in catalytic activity might be due to the enormous nutrients availability in the growth media.

4.8 COMPARISON OF BIOTRANSFORMATION EFFICIENCY

The results obtained from all experiments conducted in this study were compiled in Table 4.3. Comparing all five microorganisms tested, it can be clearly seen that the highest percentage of benzyl acetone converted to 4-phenyl-2-butanol was achieved by a biotransformation using *P. aeruginosa* as a biocatalyst; the result was achieved in a relatively short time compared to biotransformation carried out using other microorganisms. The second highest percentage of conversion was, on the other hand, achieved by a biotransformation carried out using *A. niger* with a reaction time of 48 h. While *S. cerevisiae* was frequently reported as an excellent agent used to reduce various monoterpenes, the result obtained in this study has proved otherwise. Nevertheless, a considerable amount of benzyl acetone was reduced to 4-phenyl-2-butanol by S. cerevisiae, albeit the longer reaction time. The weakest among all tested were *B. cereus* and *E. faecalis*, which were proven to be inefficient at transforming benzyl acetone, as far as economical practice is concerned.

Table 4.2: Summary of biotransformation conditions and yield of biotransformation.

Microorganism	Optimum conditions				Highest conversion (%)	Time of reaction (h)
	Initial substrate concentration (g/L)	Initial glucose concentration (g/L)	Initial pH	Temperature (°C)		
S. cerevisiae	0.1	20	7.5	30	34.01 ±0.55	168
A. niger	0.15	50	7.5	27	94.33±4.00	48
P. aeruginosa	0.1	20	7.0	30	98.27±2.52	12
E. faecalis	0.15	40	7.0	37	7.17± 0.36	12
B. cereus	0.15	40	7.5	40	9.84±0.35	72

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSION

Benzyl acetone was successfully transformed to 4-phenyl-2-butanol by *S. cerevisiae*, *A. niger*, *P. aeruginosa*, *E. faecalis* and *B. cereus*. Among all tested, *P. aeruginosa* and *A. niger* were proven to be the most efficient biocatalyst for biotransformation of benzyl acetone when carried out in following conditions:

i. *A. niger* : 0.15 g/L benzyl acetone, 50 g/L glucose, pH 7.5 and 27°C.

ii. *P. aeruginosa* : 0.1 g/L benzyl acetone, 20 g/L glucose, pH 7 and 30°C.

5.2 CONTRIBUTION

The use of microorganisms to produce a natural product of interest from a cheap source or substrate without endangering the natural resource was well highlighted in this study. Study of the factors affecting the biotransformation would allow future researcher to better comprehend the mechanisms that could possibly take place during the reaction. This study would serve as one of the fundamental blocks in paving the way to the production of expensive drugs, fragrances, flavors and other natural products using microorganisms.

5.3 RECOMMENDATIONS FOR FUTURE WORKS

Despite the successful transformation of benzyl acetone to 4-phenyl-2-butanol using *S. cerevisiae*, *A. niger*, *P. aeruginosa*, *E. faecalis* and *B. cereus*, there is still more to be done to further understand the mechanism that has taken place during such reaction. Among the recommendations are:

1) Investigation on the protein expression profile should be done to further explain the mechanism involve during biotransformation.

2) Expression of extracellular and intracellular enzyme during biotransformation should be compared to further understand the mechanism that takes place.

3) Isolation and identification of the enzymes potentially responsible for reduction of benzyl acetone to 4-phenyl-2-butanol.

4) Biotransformation using immobilized cell culture should be carried out and compared to that of living cell culture to check if other metabolic activities in the cell could affect the biotransformation activity.

5) A system with *in situ* product removal should be considered in the future to avoid interference of biotransformation product on the overall performance of biotransformation.

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APPENDIX A

LIST OF CHEMICALS

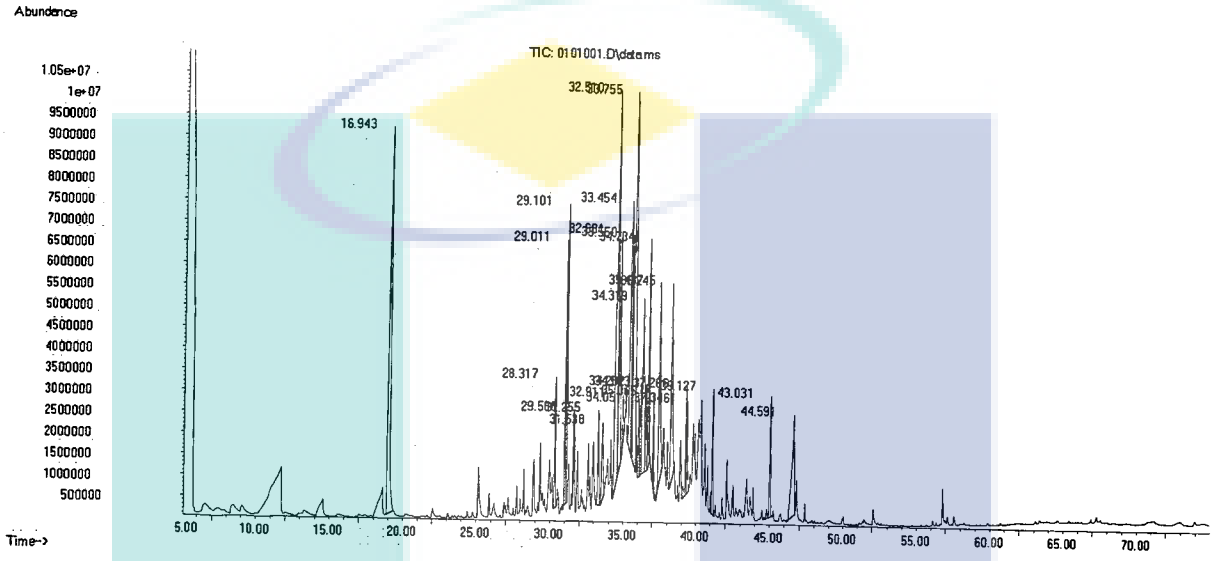
A1: List of chemicals used throughout the experiments

Chemicals	Supplier
n-Hexane (Chromatography grade)	Merck
Ethanol (Analytical grade)	Merck
Benzyl acetone (98%)	Merck
4-phenyl-2-butanol (98%)	Merck
Potassium hydroxide, KOH	Merck
Hydrochloric acid, HCl	Merck
Anhydrous Sulphate	Merck
Potassium hydrogen phosphate, K_2HPO_4	Merck
Sodium chloride (NaCl)	Merck
Sodium sulfate ($NaSO_4$)	Merck
3-5, dinitrosalicylic acid (DNS)	Merck
Sodium hydroxide, NaOH	Merck
Potassium sodium tartrate	Merck
Phenol	Merck
Potato dextrose agar	Oxoid
Nutrient agar	Oxoid
Potato dextrose broth	Oxoid
Nutrient broth	Oxoid
Beef extract	Sigma Aldrich
Yeast extract	Sigma Aldrich
Malt extract	Sigma Aldrich
Microbiological peptone	Merck
D-glucose	Merck

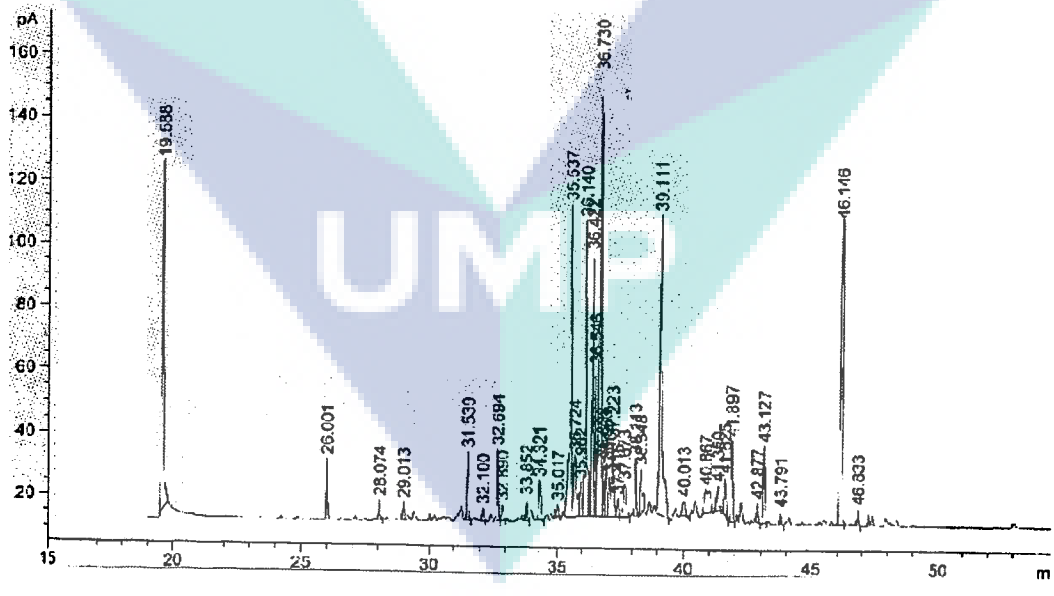
APPENDIX B

GC CHROMATOGRAMS

B1: GC-MS Chromatogram of essential oil of *A. malaccensis*



B2: GC-FID Chromatogram of essential oil of *A.malaccensis*



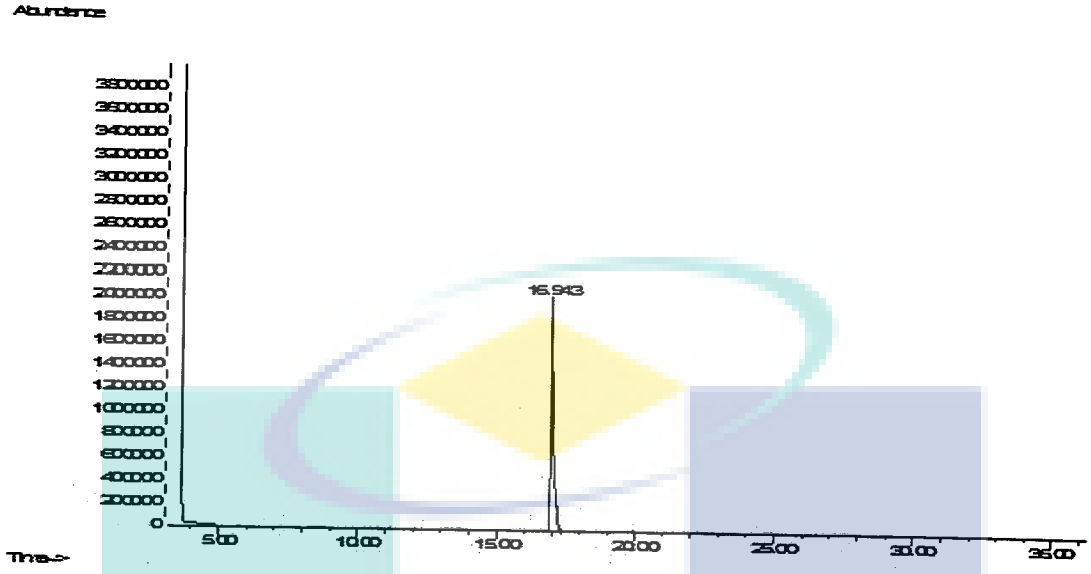
B3: Chemical composition of essential oil of *A. malaccensis* extracted through MAE and HD

Compounds	MAE at 200 W (h)				MAE at 300 W (h)				MAE at 400 W (h)				MAE at 500 W (h)				HD (h)
	2	3	4	5	2	3	4	5	2	3	4	5	2	3	4	5	12
Carboxylic acid and derivatives																	
Benzaldehyde																	
benzyl acetone (benzyl acetone)	8.6	9.5	9.3	11.2	6.7	9.2	7.9	7.4	6.8	9.1	9.1	9.4	5.9	0.9	7.6	0.3	
decanoic acid		1.3	1.4	1.2		1.2	0.9	0.9		1.5	1.2	1.4	0.7	0.1	0.2	11.5	7
Sesquiterpene hydrocarbons																0.2	0.1
β-Maaliene		0.1	0.4	0.3		0.4	0.3	0.3		0.4	0.3	0.4			0.2	0.2	0.3
α-guaiene						0.8	0.3	0.2		0.6	0.3	0.4			0.1	0.2	0.2
Aromadendrene																0.1	
Υ-gurjunene																0.1	0.3
β-agarofuran		1.3	1.4	1.4		1.4	1.1	1.1		1.4	1.2	1.3			0.2	0.1	1
α-selinene	1.8	0.3	0.2	0.3	1.9	0.3	0.2	0.2	0.4		0.2	0.2	0.4	0.1	0.3	0.3	0.2
α-muurolene									1.2								0.1
α-bulnesene		1.5	1.5	1.5		1.5	1.2	1.1		1.3	1.1	1.1	1.2	0.8	1.4	1.6	1.1
Oxygenated sesquiterpenes																	
α-elemol	2.2	0.5	0.5	0.4	2	0.6	0.4	0.4	1.4	0.8	0.5	0.6	1.2	0.3	0.7	0.9	0.4
α-agarofuran									0.4				0.4	0.3	0.6	0.6	0.8
Norketoagarofuran	1.5	1.2	1.2	1.3		1.2	1	0.8		1.1	0.9	1	1	0.2	0.1	0.2	0.3
Epoxybulsenene		9.1	8.9	9.2		8.12	7.5	7.1		8	6.3	6.8		2.4	3.6	3.4	6.5
Tetradecanal	1.5	1.1	1.6	1.5	1.4	1.1	1.2	1.4	1		1.2	1.4	1	0.8	1.3	1.3	1
caryophellene oxide	10.7	8.5	9.2	8.1	10.9	7.7	6.2	6.5	6.7	6.8	5.8	3.3	5.9	1.4	4.5	4.3	5.8
Guaiol	1.8	6.6	6.5	6.2	2	5.8	4.9	5.2	1.4	5.3	4.6	2.7	1.7	3.5	6	5.9	4.6
10-epi-Υ-eudesmol	1	3.3	3.4	3.1		3	2.7	2.8	0.6	3.1	2.5	1.9	0.9	6	2.9	2.8	2.4
Agarospirol	10.5	13.9	14.1	13.8	10.9	13.3	11	10.6	7.8	12.4	10.5	5.1	7.3	10.5	10.2	10.3	9
epi-α-cadinol	7.6	1.3	1.3	1.03	8.5	1.1	0.8	0.97	1.1	0.9	0.7	0.7	1	0.7	1	1.1	0.9
jinkoh-eremol	3.7	1.3	1.2	1.1	3.8	1.1	0.9	1	6.5	1	0.8	0.8	5.8	0.7	0.5	0.5	1
tridecanoic acid						1.3	1.1	1.2							0.6	0.8	
Kusunol		1.3	1.3	1.4		2	2.2	2.2		1.8	1.3	1.1		1.1	0.4	0.7	1.3
α-eudesmol				1.8			0.8	1.1						1.2	1.3	0.5	0.7
Bulnesol	1.5	1.5			1.6				1		3.3	2.2	1	0.8	1	0.9	0.9
dehydrojinkoh-eremol	2	10.8	7.9	8.5	2.2	9.7	9.3	9.7	1.5	5.2	8.2	8.5	1.7	5.5	4.2	2.6	7.4
epi-α-bisabolol																0.6	1.1
α-bisabolol											0.4					0.4	
selina-3,11-dien-9-one														0.4	1	0.8	0.7
Pentadecanal	11.9				13.5				9.8		0.7	0.4	9.8	1.7	1.7	1.7	1.3
Rotundone		0.6					0.8			5.4	0.3	1.1		0.7	0.5	1.7	1.8
selina-3,11-dien-9-ol				0.6			0.6	0.7	0.9				1.3	0.7	0.9	1.2	1.7
selina-4,11-dien-14-oic acid		2				1.6	3.7	3.5			1.4	4.1		1.1	1.2	0.7	1.7

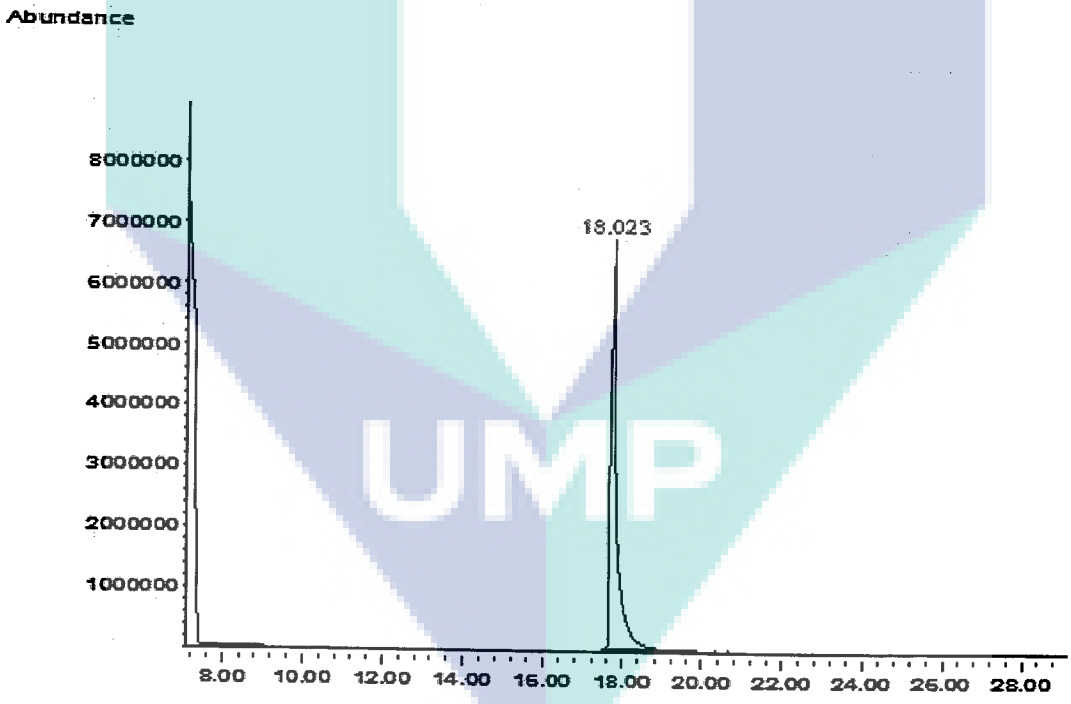
B3: Continued

Compounds	MAE at 200 W (h)				MAE at 300 W (h)				MAE at 400 W (h)				MAE at 500 W (h)				HD (h)
	2	3	4	5	2	3	4	5	2	3	4	5	2	3	4	5	12
selina-3,11-dien-14-al														2.3	2.9	4.7	2.3
9,11-eremophiladien-8-one		0.7	0.6	2		0.5	2.1	2.1	1.7		0.7	1.3	1.7		0.8	0.2	
selina-3,11-dien-14-ol		1.2	1.7			1	1.6	2	1.4	0.9	1	1.5	1.6	1.2	1.2	1.2	2.6
guaia-1(10),11-dien-9-one						0.6			1.1	1	1	1.1	1	1	0.8	0.5	1.6
selina-4,11-dien-14-al		1.8	1.9	2.1		2	1.8	1.8	1.5	2.3	2.1	2.3	1.4		0.4	0.4	0.3
guaia-1(10),11-dien-15-ol						0.7	0.7	0.6	0.6	0.7	0.7	0.9	1	0.8	0.5	0.4	0.4
selina-3,11-dien-14-oic acid									0.7				0.7	0.6	0.5	0.4	0.1
Sinenofuranol	2			0.4	2.4	0.4	0.2	0.3	1.8		0.5		1.7	0.3	0.4	0.4	
2-hexadecanone			0.4										0.4	0.2	0.5	0.4	0.3
Dihydrokaranone		0.4	2.1	2.7		1.9	2.4	2.3		2.4	2.6	2.8	1.7	0.2	0.5	0.9	2.1
Karanone	1.4	1.63	0.4	0.4	2	0.3	0.4	0.4	1.7	0.5	0.4	0.4		0.9	0.5	0.7	0.3
oxo-agarospirol									0.4		0.3		0.4	0.5	1.9	0.7	0.3
pentadecanoic acid														0.3	0.6	0.6	0.2
n-hexadecanoic acid	9.3	0.3	10.6	11.6	12.6	10.6	12.1	12.6	11.3	16.1	15.7	15.7	13	10.2	4.6	3	11.5
Others																	
1,5-diphenyl-2-pentene														0.2	0.3	0.3	
oleic acid														0.2	0.1	0.1	
Carboxylic acid and derivatives	8.6	10.8	10.7	12.4	6.7	10.4	8.8	8.3	6.8	10.6	10.3	10.8	6.1	1	7.8	12.1	7.1
Sesquiterpene hydrocarbons	1.8	3.2	3.5	3.5	1.9	4.4	3.1	2.9	1.6	3.7	3.1	3.4	1.6	0.9	2.18	2.6	3.2
Oxygenated sesquiterpenes	68.6	69	74.8	77.2	73.8	75.6	76.4	77.2	62.2	75.7	74.4	67.7	65.8	59.6	60.7	58.8	73.1
Others														0.4	0.4	0.4	
Total	79	83	89	93.1	82.4	90.4	88.3	88.5	70.7	90	87.8	81.9	74	61.9	83.3	73.9	83.4
% yield	0.05	0.04	0.06	0.1	0.1	0.14	0.15	0.15	0.15	0.17	0.2	0.22	0.2	0.2	0.22	0.24	0.2
Number of compounds	17	27	25	26	15	29	32	30	26	25	33	30	35	36	45	49	42

B4: GC-MS Chromatogram of standard peak of benzyl acetone



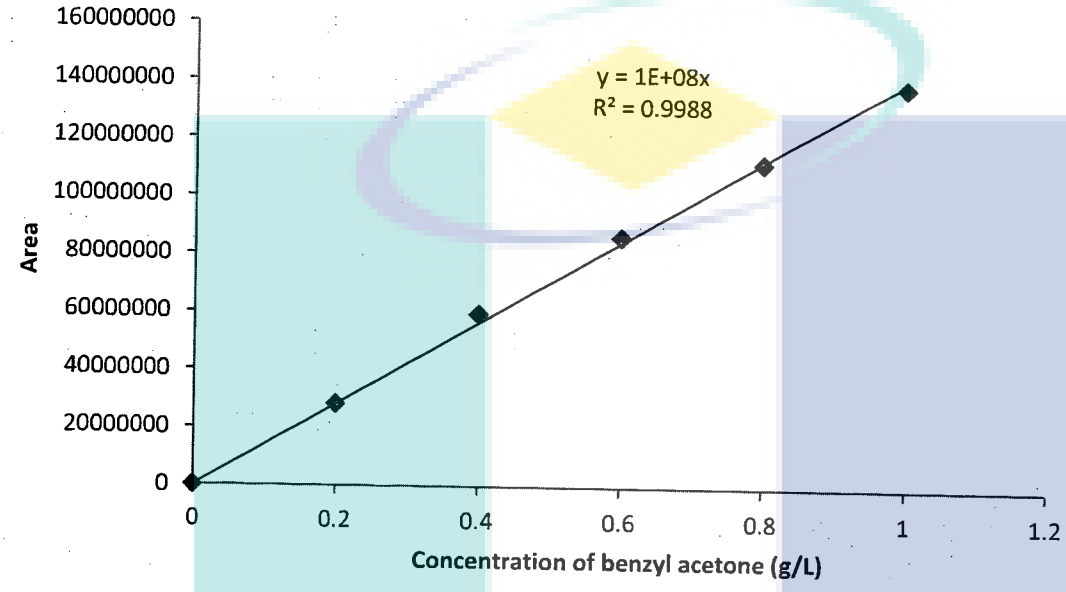
B5: GC-MS Chromatogram of standard peak of 4-phenyl-2-butanol



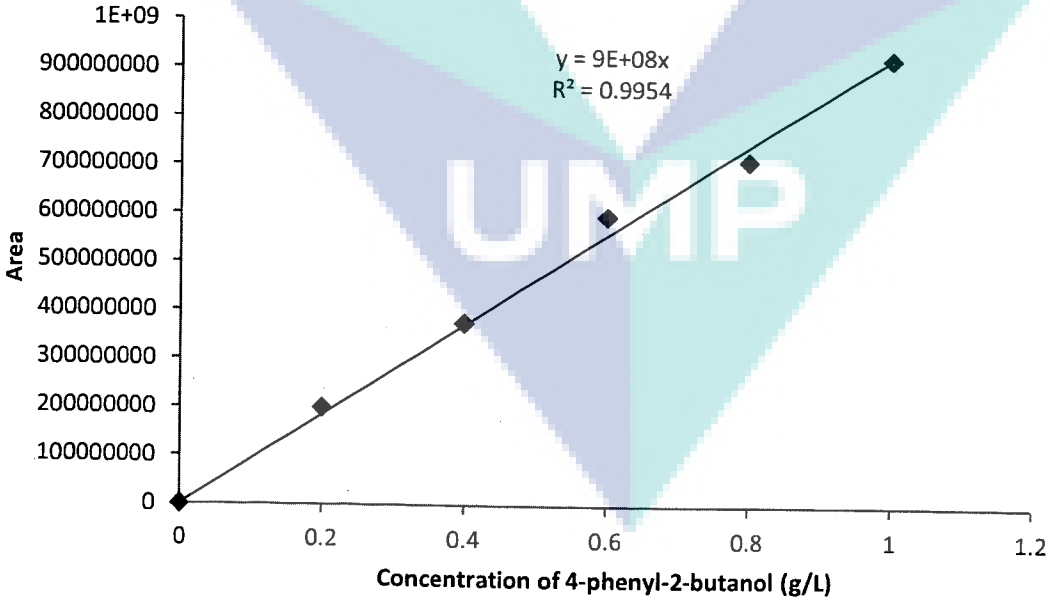
APPENDIX C

CALIBRATION CURVES

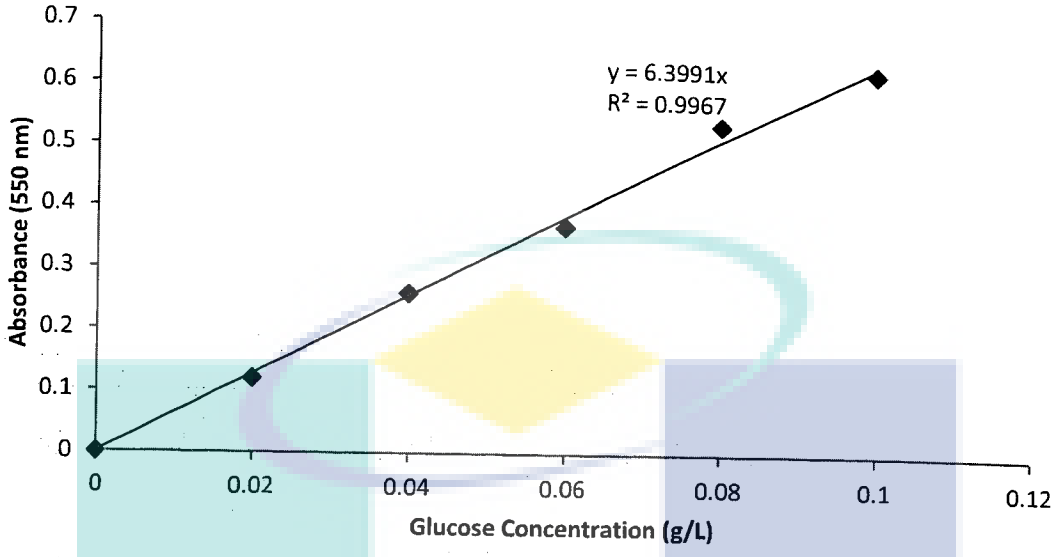
C1: Calibration curve of area against concentration of benzyl acetone



C2: Calibration curve of area against concentration of 4-phenyl-2-butanol



C3: Calibration curve of absorbance against concentration of glucose



APPENDIX D

MINIMAL INHIBITORY CONCENTRATION (MIC) TEST

D1: MIC of benzyl acetone against *S. cerevisiae*

Concentration (g/L)	Absorbance (600 nm)			Mean	Stdev
	1	2	3		
0.2	0.941	0.962	0.955	0.953	0.011
0.4	0.752	0.693	0.721	0.722	0.030
0.6	0.412	0.393	0.433	0.413	0.020
0.8	0.108	0.099	0.112	0.106	0.007
1.0	0	0	0	0.000	0.000
1.2	0	0	0	0.000	0.000

D2: MIC of benzyl acetone against *A. niger*

Concentration (g/L)	Diameter of colony (mm)			Mean	Stdev
	1	2	3		
0.2	88	85	83	85.3	2.5
0.4	64	62	66	64.0	2.0
0.6	56	55	53	54.7	1.5
0.8	0	0	0	0.0	0.0
1.0	0	0	0	0.0	0.0
1.2	0	0	0	0.0	0.0

D3: MIC of benzyl acetone against *P. aeruginosa*

Concentration (g/L)	Absorbance (600 nm)			Mean	Stdev
	1	2	3		
0.2	0.591	0.632	0.584	0.602	0.026
0.4	0.312	0.302	0.299	0.304	0.007
0.6	0	0	0	0	0
0.8	0	0	0	0	0
1.0	0	0	0	0	0
1.2	0	0	0	0	0

D4: MIC of benzyl acetone against *E. faecalis*

Concentration (g/L)	Absorbance (600 nm)			Mean	Stdev
	1	2	3		
0.2	0.578	0.581	0.542	0.567	0.022
0.4	0.506	0.487	0.473	0.489	0.017
0.6	0	0	0	0	0
0.8	0	0	0	0	0
1.0	0	0	0	0	0
1.2	0	0	0	0	0

D5: MIC of benzyl acetone against *B. cereus*

Concentration (g/L)	Absorbance (600 nm)			Mean	Stdev
	1	2	3		
0.2	1.104	1.113	1.009	1.075	0.058
0.4	0.982	0.975	0.903	0.953	0.044
0.6	0	0	0	0	0
0.8	0	0	0	0	0
1.0	0	0	0	0	0
1.2	0	0	0	0	0

APPENDIX E

LIST OF PUBLICATIONS

1. Norul, A.S., Nazira, M. and Tajuddin, S.N. 2015. Biotransformation of Benzyl acetone from Aquilaria malaccensis using microorganisms, *Australian Journal of Basic and Applied Sciences*. **9**[8]: 155-159.
2. Norul, A.S., Nazira, M., Tajuddin, S.N., Yusoff, M.M. 2013. Isolation of Benzyl acetone from the essential oil of *Aquilaria malaccensis* and its biotransformation using *Saccharomyces cerevisiae*, *1st International Scientific Symposium on Agarwood*, Universiti Putra Malaysia, Malaysia, 3-5 September 2013.
3. Norul, A.S., Tajuddin, S.N., Nazira, M., Lee, C.M. and Zareen, S. 2012. Production of phenylethyl alcohol from benzyl acetone isolated from *Aquilaria malaccensis* (Gaharu) oil using *Saccharomyces serevisiae*, *The 5th International Conference on Postgraduate Education*, Universiti Teknologi Malaysia, Malaysia, 18 -19 December 2012.
4. Norul, A.S., Tajuddin, S.N., Nazira, M. and Zareen, S. 2012. Isolation and screening of microorganisms with potential for biotransfomation of benzylacetone from *Aquilaria* (Gaharu) oil, *National Conference on Postgraduate Research 2012*, Universiti Malaysia Pahang, Malaysia, 7-9 September 2012.