ENZYMATIC ASSISTED CITRONELLA ESSENTIAL OIL EXTRACTION FROM *Cymbopogan winterianus*

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LIST OF SYMBOLS

A	Alpha (axial distance from centre point which makes the design rotatable)
β_0	Regression coefficients for the intercept coefficient
β_i	Regression coefficients for the linear coefficient
β_{ii}	Regression coefficients for the quadratic coefficient
β_{ij}	Regression coefficients for the interaction coefficient
°C	Celsius
Σ	Standard deviation
χ_i , χ_j	Coded independent variables
E	Residual associated to the experiments
G	Gram
hrs	Hour
K	Number of variable
M	Molar
mM	mili Molar
µm	micro meter
min	Minute
N	Number of measurements
rpm	Rotation per minute
V	Volume
v/v	Volume to volume ratio
v/w	Volume to weight ratio

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
Avg.	Average
BG	ß-glucosidase
CBH	Cellobiohydrolases
CCD	Central Composite Design
CO_2	Carbon dioxide
conc.	concentration
CV	Coefficient variation
DNA	Deoxyribonucleic acid
DoE	Design of Experiment
FP	Filter paper
FPU	Filter paper unit
EG	Endoglucanases
ET	Extraction time
GM	Growth medium
H+	Hydrogen ion
HMF	Hydroxymethyl furfural
H_2O	Dihydrogen oxide (water)
H_2SO_4	Sulfuric acid
ITS	Internal transcribed spacer
KBr	Potassium bromide
KOH	Kalium hydroxide

NA	Nutrient agar
n.a	not available
OH^-	Hydroxide ion
OPFB	Oil palm fruit bunch
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
RSM	Response Surface Methodology
RT	Reaction time
SSB	Sweat sorghum bagasse
SEM	Scanning Electron Microscope
U	Unit
Vs	Versus

ABSTRAK

Ekstraksi Pati Minyak Serai Wangi dari *Cymbopogan winterianus* dengan Bantuan Enzim

Minyak pati serai wangi kebiasaannya dihasilkan melalui proses penyulingan air atau stim dari akar serai wangi. Namun begitu, isipadu minyak yang dihasilkan secara umumnya agak rendah, iaitu di sekitar 0.22% - 1.0%. Keadaan ini membebankan para pengusaha minyak serai wangi untuk memenuhi permintaan tinggi terhadap penggunaan minyak tersebut. Oleh sebab itu, kajian ini dijalankan untuk meningkatkan kadar penghasilkan minyak dengan memperkenalkan penggunaan enzim mikrob yang didapati di sekitar akar serai wangi untuk diaplikasi dalam proses rawatan enzim sebelum proses pengekstrakan pati minyak. Pertama sekali, komposisi lignoselulosa akar serai wangi diselidiki bagi mengenalpasti jenis enzim yang sesuai untuk digunakan dalam proses rawatan awal akar serai wangi. Analisis komposisi kimia telah mengenalpasti komponen selulosa sebagai komponen terbanyak (38.21%±1.65), diikuti oleh hemiselulosa (30.49%±0.93), lignin (21.12%±1.86) and ekstraktif (4.97%±0.22). Sejumlah 31 daripada 52 spesis mikrob yang diasingkan telah dikenalpasti positif dalam penghasilan enzim selulase. Berdasarkan kepada indeks selulolitik yang memberangsangkan, Strain UniMAPF7, UniMAPF16, UniMAPF24, dan UniMAPF27 telah dipilih untuk penghasilan enzim mentah bagi ekstraksi pati minyak serai wangi pada skala makmal. Keputusan dari kajian analisis pertumbuhan mikrob dan kadar aktiviti enzim telah mencadangkan masa optima untuk proses penuaian enzim ialah pada hari ke 5 bagi UniMAPF7, hari ke 4 bagi UniMAPF24 dan hari ketiga bagi UniMAPF16 and UniMAPF27. Ekstraksi minyak dari akar yang telah dirawat dengan enzim semulajadi dari UniMAPF7 and UniMAPF24 menghasilkan peningkatan paling ketara masing–masing dengan pertambahan sebanyak 2.5 dan 1.7 kali ganda. Imej imbasan elektron mikroskop (SEM) bagi analisis morfologi akar yang telah dirawat membuktikan peningkatan keamatan liang-liang pori dan kerapuhan struktur fiber yang amat ketara. Analisa makroskop, mikroskop dan genomik telah mengenalpasti strain UniMAPF7*Aspergillus nomius* H5 strain (Nombor akses GenBank JF16646). Berdasarkan kepada parameter yang telah dikenalpasti, kaedah respons permukaan (RSM) berdasarkan reka bentuk komposit berpusat (CCD) telah diguna pakai untuk mengoptimumkan kondisi bagi penghasilan enzim selulase. Keadaan optimum untuk penghasil enzim sellulase adalah pada pH 4.73, suhu 31.7°C, dan kadar agitasi sebanyak 147rpm dengan aktiviti enzim maksimum sebanyak 0.2559U/ml. Hidrolisat enzim selulase yang optima kemudiannya digunakan untuk mengoptimumkan keadaan proses pra-rawatan enzim dan ekstraksi minyak pati. Di bawah keadaan optimum (perkadaran enzim, masa tindakbalas, masa ekstraksi), hasil minyak pati maksimum sebanyak 71.21 g/100 g substrat kering telah dicapai. Analisis varian (ANOVA) menunjukkan bahawa model dan semua parameter dianggap penting secara statistik pada 95% untuk kedua-dua kajian pengoptimuman menggunakan persamaan polinomial peringkat kedua. Selain itu, pengesahan model menunjukkan perkaitan yang rapat antara keputusan eksperimen dan ramalan respon. Oleh itu, model-model ini boleh digunakan dengan jayanya untuk mengenal pasti kombinasi yang berkesan daripada tiga faktor yang berbeza di dalam kedua-dua kajian pengoptimuman untuk meramalkan hasilan minyak pati dari akar serai wangi yang dirawat dengan enzim mikrob.

ABSTRACT

Enzymatic Assisted Citronella Essential Oil Extraction from *Cymbopogan winterianus*

The essential oil of Citronella is commonly extracted via typical steam distillation of citronella plan parts. However, the oil yield is generally low (0.22% - 1.0%), causing burden to many entrepreneurs to meet the strong demand. Therefore, the study was conducted to improve the yield of citronella oil recovery by introducing an optimised enzymatic assisted pre-treatment using crude microbial enzymes isolated from the soil surrounding the citronella roots. Firstly, the lignocellulosic components of dried citronella roots were investigated to evaluate the most abundance component for selection of enzymes useful for pre-treatment. The composition analysis has identified cellulose as the major lignocellulosic component (38.21%±1.65), followed by hemicellulose (30.49%±0.93), lignin (21.12%±1.86) and extractives (4.97%±0.22). 31 out of 52 isolated strains have been identified as positive cellulase producer. Based on the calculated cellulolytic indexes, 4 strains i.e; UniMAPF7, UniMAPF16, UniMAPF24, and UniMAPF27 were selected for application in the laboratory scale pre-treatment process. Results from the growth curve analysis and enzyme activity study suggested that the optimal time for enzyme harvesting occurred at day 5 for UniMAPF7, day 4 for UniMAPF24 and day 3 for UniMAPF16 and UniMAPF27. Laboratory scale oil extraction of pre-treated citronella root with crude enzymes of UniMAPF7 and UniMAPF24 yielded in a significant 2.5-fold and 1.7-fold increased in citronella oil recovery, respectively, when compared to the untreated citronella root as control. The scanning electron microscope images (SEM) of the corresponding pre-treated roots with crude UniMAPF7 cellulases illustrates an increased in pore intensity and the loosening structures of root fibers are clearly evident. Macroscopic, microscopic and genomic analysis of UniMAPF7 has identified the strain as *Aspergillus nomius* H5 strain (GenBank accession JF16646). With known parameters range, the Response surface methodology (RSM) based on Central Composite Design (CCD) was adopted to optimize the conditions for production of crude cellulase. The optimum conditions were found to be of pH 4.73, temperature of 31.7°C, and agitation rate of 147rpm with maximum FPase activity of 0.2559U/ml. The optimised crude cellulases were further used in the optimisation of pre-treatment conditions and oil extraction process. Under optimized conditions (enzyme ratio 15.88%v/v, reaction time of 8,57hrs, and extraction time of 6.51 hrs), a maximum oil yield of 4.7397 ml/100g dry roots was achieved. The Analysis of Variance (ANOVA) test revealed that the model and all independent parameters were considered statistically significant at 95% for both optimization studies using the second order polynomial equation. The model validation showed a good agreement between experimental results and the predicted responses. Therefore the models could be successfully used to identify the effective combinations of the three different factors in both optimization studies for predicting the oil yield from pre-treated citronella roots.

CHAPTER 1

INTRODUCTION

1.1 Overview

Cymbopogan winterianus, also known as serai wangi or citronella grass is native to Sri Lanka and Sounth India (Ahmed, 2005). It can also be found growing wild in most tropical countries such as Malaysia, Thailand and Indonesia. Recently, this plant has been planted commercially by enterprenours for its essential oil, commercially known as Citronella oil (Azmil *et al.*, 2005)

Citronella oil holds an important position in essential oil industry. The oil, which can be obtained from the leaves and roots of *Cymbopogan winterianus*, is regarded as one of the twenty most important essential oils found in the world trade (Lawrence, 1993). It contains unique chemical compounds such as citronellol, citronellal and geraniol which are widely used in perfumery, aromatherapy, detergent, cleaning compounds and other industrial products (Rosalinda & Tio, 2001). Apart from that, the oil has also being recognized as a potent insecticide and is widely used as the main ingredient in many insect repellent products (Pinheiro *et al.*, 2013)

The current world demand and price for Citronella oil is experiencing fluctuation due to proliferation of inexpensive synthetic isolates derived from turpentine oil and *Eucalyptus citriodara* oil (Katiyar, 2011). These synthetic isolates were generally cheaper in price, making them more preferable whenever the only criterion of choice is the price (Tiwari, 2010). However, the quality of natural citronella oil remains

superior compared to the synthetic one (Rosalinda & Tio 2001). Such property explains the strong demand exist for natural citronella oil as it is still the preferred choice for application in perfumery industry mainly because of its unique and stable properties which are vital in blending perfumes and compounding industrially important essences (Lawrence, 2004).

Presently, world production of citronella oil is approximately 5000 tonnes, valued at 20million USD per year (Tiwari, 2010). The major producing countries are China, Indonesia, Taiwan, Malaysia, Brazil, Ceylon, India, Guatemala, Argentina, Ecuador, Madagascar, and Mexico (Tiwari, 2010). The oil is commonly obtained via hydrodistillation or steam distillation of *C. nardus* plant parts, particularly the roots. However, these conventional methods of oil extraction often yielded a relatively low percentage of essential oil (0.22% - 1.0% of oil recovery), indirectly resulting shortage of worldwide supply (Cassel & Vargas, 2006; Wany *et al.*, 2013). Due to the increasing demand of the oil, countless research efforts have been made to improve the extraction yield of citronella oil; either by introducing sophisticated extraction techniques or by incorporating additional enzymatic or chemical pre-treatment steps prior to oil extraction processes.

Recently, the enzyme assisted extractions are gaining preference because the need for ecofriendly extraction technologies. This method also affects the possibility of greener chemistry as pressure mounts on trying to identify cleaner routes for the extraction of new compounds. Enzymatic pre-treatment has shown to successfully improved the extraction yield of cloves, celery, thyme (*Thymus capitatus* L.) and Rosemary (*Rosemarinus officinalis* L.) without altering the physical and chemical

characteristics of the oil (Munish *et al.*, 2012). In some cases like ginger and garlic, enzymatic pre-treatment have shown a 50% improvement in essential oil yield (Munish *et al.*, 2012). Interestingly, the enzymatic extraction of vanilla pod not only improved the oil yield, but also resulted in the production of superior quality of vanilla oil (Madava *et al.*, 2012). Despite the enzymatic assisted essential oil extractions on the aforementioned plant species were shown to be successful, its application on citronella oil extraction from *Cymbopogan winterianus* remains to be explored.

The limiting factor for an efficient oil extraction is due to the fact that the structures of essential oil producing cells are found embedded inside the lignocellulosic materials of the citronella root. Therefore, the propose method for this study is to enhance oil release by using biological degradation which involves enzymatic hydrolysis of the glycosidic linkages of the cellulose chains following simple mechanical treatment on the lignin polymer. This method was proven a success in enhancing oil recovery of other species. According to Munish *et al.*, (2012), enzymatic pre-treatment degrades the cell wall into simpler molecules, resulting in partial decomposition of lignocellulosic structure to facilitate the oil flow prior to extraction.

However, the enzyme-assisted extraction of bioactive compounds from plants (citronella roots) is subjected to few potential commercial limitations. Firstly, the use of commercial enzymes in industrial scale may be hampered by changes in environmental conditions such as pH, temperature and percentage of dissolved oxygen levels, which will eventually reduce the effectiveness of pre-treatment and would incur more cost for large-scale optimisation (Kalia *et al.*, 2001). Secondly, regardless of the high activity of the commercial enzymes, it's effectiveness for pre-treatment of lignocellulosic materials

is less convincing due to incomplete hydrolysis of plant cell wall (Munish *et al.*, 2012). On the onther hand, the high cost associated with purchasing commercial enzymes for pre-treatment of large volume of raw materials in the scale-up processes are often not practical. An expensive pre-treatment method is highly not prefereable since enterprenours are currently seeking for the most practical and cost effective pre-treatment process in order to maximise profit.

Alternatively, enzymatic pre-treatment using whole microbe seems promising since it incurs a much lower cost of production (Kalia *et al.*, 2001). There are a few established commercial enzymes producing microbes may be useful for assisting the extraction of citronella oil. Nevertheless, the use of these strains may not be successful as the oil was shown to possess strong antimicrobial properties as previously reported by Lee and Wendy, 2013. Various publications have also documented similar findings that showed the antimicrobial properties of citronella oil in inhibiting the growth of many organisms including the eight medically important *Candida* species (Silva *et al.*, 2008) and the five strains of *Propionibacterium acnes* (Luangnarumitchai *et al.*, 2007).

Nevertheless, microbes have evolved to develop resistance mechanisms upon exposure to any inhibitory compounds (Cloete, 2003). Considering this, it is speculated that the above limitation can be overcome by isolating microbes that lives surrounding the citronella roots (Pattnaik, 1996). Those microbes have been exposed to the antimicrobial properties of citronella oil. Therefore, the resistance mechanism is presumed to already been established, resulting in an improve tolerance towards the inhibitory affect of the citronella oil.

1.2 Problem Statements

The multitude usages of citronella oil have caused tremendous demand for its supply. The oil, obtained via typical steam distillation of citronella plan parts, however, often yielded a relatively low amount of oil volatiles. Alternatively, an eco-friendly technology of enzyme pre-treatment using commercial enzyme was proven successfull in improving the extraction yield in other plant species. This however, subjected to few limitations. The high cost associated with commercial enzyme is of greatest concern and the use of established enzyme producing microbes on the other hand could be hampered by the antimicrobial properties of the citronella oil. Considering this, the present investigation was intended to utilise the use of microbes isolated surrounding the citronella roots for production of crude microbial enzymes that would later be optimised it's production and it's suitability for pre-treatment process.