

UTILIZATION OF YEAST METABOLITES FOR BIOATTRACTION OF
COCKROACH

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*To my beloved family,
thank you for being there for me*

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ABSTRACT

Cockroaches are among the most persistent pests that thrive in protected locations all over the world. Cockroach control using insecticides and other chemicals are not desirable because they are toxic to organisms and the environment. Pests can develop resistance to the chemicals, and chemical raw materials are from unsustainable source. This study was aimed to identify the novel potential of locally isolated yeasts, namely *Pichia kudriavzevii* M12 and *Candida ethanolica* M2 in cockroach attraction. The yeasts were fermented in potato dextrose broth (PDB) up to a week and tested for bioattraction of cockroaches at six locations at the student residential halls at Universiti Teknologi Malaysia (Johor, Malaysia). Either the 1-day or 4-day fermented broth from the yeasts were placed as the baits on sticky trap overnight in order to attract cockroaches. Freeze drying on the fermented broth was carried out and the resultant powder was sprinkled on the cockroach trap and tested for cockroach attraction. PDB was used as control. The 4-day fermented PDB of *P. kudriavzevii* M12 was found to be the better cockroach attractant, which trapped the highest number of both nymphs and adult cockroaches (an average of 48 cockroaches per catch). Successive attraction was done consecutively at the same location after two weeks, which resulted in a decrease of almost 80% of the cockroach population at the studied location. The metabolites in the 4-day fermented PDB of *P. kudriavzevii* M12 were profiled by liquid chromatography-mass spectrometry (LC-MS/MS), thus revealing the presence of secondary metabolites from the yeast strain for cockroach attraction. A total of 44 exometabolites with diverse properties and structures were identified and many were intermediates and products of the central metabolic pathway such as lipids, carboxylic acids and esters. In conclusion, *P. kudriavzevii* M12 showed a great potential as an eco-friendly cockroach attractant and the attraction could be as a result of the metabolites produced.

ABSTRAK

Lipas adalah antara perosak yang paling sering ditemui di pelbagai lokasi yang dilindungi di seluruh dunia. Kawalan terhadap lipas menggunakan racun serangga dan bahan kimia lain tidaklah diterima disebabkan oleh ketoksikan kepada organisma dan persekitaran. Perosak boleh membangunkan rintangan kepada bahan kimia, dan bahan-bahan mentah kimia adalah dari sumber yang tidak mapan. Kajian ini bertujuan untuk mengenal pasti potensi baru yis tempatan terpencil, iaitu *Pichia kudriavzevii* M12 dan *Candida ethanolica* M2 sebagai tarikan lipas. Yis telah ditapai dalam *potato dextrose broth* (PDB) sehingga seminggu dan diuji untuk tarikan lipas di enam lokasi di kediaman pelajar di Universiti Teknologi Malaysia (Johor, Malaysia). Sampel yang telah ditapai selama 1 hari dan 4 hari diletakkan sebagai umpan perangkap yang lekit untuk menarik lipas selama semalaman. Kering beku sampel yang ditapai telah dijalankan dan serbuk yang terhasil dipercikkan pada perangkap lipas dan diuji untuk tarikan lipas. Eksperimen kawalan dijalankan menggunakan PDB sahaja. Sampel yang ditapai selama 4 hari oleh *P. kudriavzevii* M12 didapati menjadi umpan lipas yang lebih baik, yang berjaya memerangkap bilangan tertinggi anak dan lipas dewasa (purata 48 lipas setiap tangkapan). Tarikan dilakukan berturutan di lokasi yang sama selepas dua minggu, yang mengakibatkan pengurangan populasi lipas sebanyak hampir 80% di lokasi yang dikaji. Metabolit dalam sampel PDB yang ditapai pada hari ke-4 oleh *P. kudriavzevii* M12 telah dikajikan menggunakan kromatografi cecair-spektrometri jisim (LC-MS/MS) mendedahkan bahawa terdapatnya metabolit sekunder dari yis. Sebanyak 44 metabolit dengan ciri-ciri dan struktur yang pelbagai telah dikenalpasti dan sebahagiannya adalah perantara dan produk laluan metabolik pusat seperti lemak, asid karbosilik dan ester. Kesimpulannya, *P. kudriavzevii* M12 menunjukkan potensi yang besar sebagai umpan lipas yang mesra alam dan tarikan adalah disebabkan oleh metabolit yang dihasilkan.

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LIST OF SYMBOLS/ABBREVIATIONS/NOTATIONS/TERMINOLOGY

Bla g 1	-	Cockroach allergen
Bla g 2	-	Cockroach allergen
bp	-	Boiling point
°C	-	Degrees Celsius
DNA	-	Deoxyribonucleic acid
ESI	-	Electron Spray Ionization
g	-	gram
GC	-	Gas chromatography
HPLC	-	High performance liquid chromatography
LC	-	Liquid chromatography
LC-MS/MS	-	Liquid chromatography tandem mass spectrometry
MALDI	-	Matrix assisted laser desorption/ionization
mb	-	Milli bar
mmHg	-	Millimeters of mercury
MS	-	Mass spectroscopy
nm	-	nanometer
OD	-	Optical density
PDA	-	Potato dextrose agar
PDB	-	Potato dextrose broth
rpm	-	Revolutions per minute

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Cockroaches (order: *Blattaria*) are among the most persistent pests that thrive in protected locations all over the world. American, brown-banded, German and Oriental cockroaches are the four most common species in human habitats (Hahn and Ascerno, 2005). They can be found in boiler rooms, heated steam tunnels, floor drains, water heaters, bath tubs (Eggleston and Arruda, 2001), kitchen sink, wall cracks, underneath or inside cupboards, behind drawers, around pipes or conduits, behind window or door frames, in radio and TV cabinets (Koehler *et al.*, 2007).

Cockroaches are objectionable because they compete with humans for food, carry allergens in saliva, fecal material, secretions, cast skins, debris and dead bodies that can lead to asthma in humans (Eggleston and Arruda, 2001), harbor pathogenic bacteria, contaminate paper and fabrics, and impart repulsive stains and odours (Koehler *et al.*, 2007).

The life cycle of cockroach consists of egg, nymph and adult and they can live for one year. Nymphs are more than adults in natural populations (Hahn and Ascerno, 2005). Cockroaches are nocturnal and omnivorous and will eat anything though they can stay for about one month without food and for about two weeks without water. Cockroach population in an enclosure is governed by presence of food and water, height of location (for some species), humidity, temperature darkness (Eggleston and Arruda, 2001) and history of control measures (Wang and Bennett, 2006).

Trapping, mating disruptions, attracticides are used in cockroach control. Organophosphates, carbamates, pyrethrins, chlorinated hydrocarbons and inorganics are some of the pesticides used to control cockroaches (Eggleston and Arruda, 2001). Traps are relatively more effective in controlling cockroach (Rust *et al.*, 1999). Attractiveness of methylcyclohexyl n-alkanoates to the German cockroach was reported by Iida and co-workers (Iida *et al.*, 1981). Many chemical attractants used in commercial cockroach traps are injurious to humans and the environment (Quesada *et al.*, 2004). Pest resistance, toxicity and non-sustainable raw materials of pesticides are some of the factors behind the trend to replace chemical feedstock, processes and end products with bio-based alternatives.

Cockroaches emit pheromones for communication and other activities. Three semiochemicals from stale beer and peanut butter are capable of being used as cockroach bioattractants were identified by Karimifar and co-workers (Karimifar *et al.*, 2011). Foods such as bread, peanut butter, molasses and beer can act as cockroach attractants. Food baits are not used in commercial pest control strategies as they often turn out to be breeding grounds for pests (Cox and Collins, 2002). Many insects respond to chemicals released by same species or chemicals released by other organisms. Many plants release volatile chemicals that are able to affect other organisms such as insects. Semiochemicals are pheromones and allelochemicals, many of which are responsible for insect behaviors such as feeding and reproduction. They are mostly volatile metabolites that are involved in signaling and other functions (Maffei, 2010).

Metabolites are involved in metabolism either as intermediates or as the final products. Metabolism can be divided into anabolism in which simple molecules are used to build large, complex molecules and catabolism in which complex, large molecules are broken into simple molecules. Most of the time, each metabolite can serve more than one purpose (Kooijman and Segel, 2005). Most metabolites are within cells where they are utilized by enzymes in various biochemical reactions. Some of the metabolites have control functions. Metabolites include lysine, riboflavin (Damain, 1980), ethanol, acetic acid (Styger *et al.*, 2011). Two subgroups of metabolites are primary and secondary metabolites. Primary metabolites have physiological functions (Gika *et al.*, 2012). Secondary metabolites serve ecological

functions (Demain, 1998). Primary pathways normally yield few products but are present in many organisms. Secondary metabolic pathways yield many products but are present in few organisms. Antibiotics make up most of the 50 most important secondary metabolites (Demain, 1999).

Volatile metabolites are intermediates and final products of metabolism. A major feature of volatile metabolites is the ease of evaporation at normal temperature and pressure. They act over a wide range of distances, concentration and environment (Humphris *et al.*, 2002). Yeasts produce volatile metabolites which can be grouped into alcohols, acids, esters, ketones and phenols. Such volatiles include ethanol, 3-methyl-1-butanol, 3-hydroxy-2-butanone, ethyl propanoate, 2-phenyl-ethanol (Nout and Bartelt, 1998). Volatile metabolites are useful to both the organism producing them and other organisms. Volatiles are useful as flavors, fragrances (Berger, 2009), inhibitors (Ting *et al.*, 2010), and attractants (Rowan, 2011), as well as for communication (Maffei, 2010), identification and profiling (Frisvad *et al.*, 2007), and detection of contaminants (Berge *et al.*, 2011). Volatile metabolites are produced by plants, animals, microorganisms and through chemical synthesis. Yields from plants and animals are low compared to microbial sources (Vandamme, 2003). Plant cell cultures are not economically viable for producing volatile metabolites (Verpoorte *et al.*, 2002). Chemical (synthetic) volatile metabolites are relatively cheap with large market share but disadvantaged by perceived environmental toxicity and possible formation of racemic mixtures (Longo and Sanroman, 2006). Major advantages of microbial based production of volatile metabolites include low energy input, reduced emission of pollutants, easy purification and renewable raw materials (Chelmer *et al.*, 2006). Some volatile metabolites are produced through microbial fermentation of various substrates. Microbial volatile production depends on medium, species (Borjesson *et al.*, 1992), temperature, pH, initial aeration (Reddy and Reddy, 2011), culture age and volatiles from other microorganisms (Tirranen and Gitelson, 2006).

Yeasts are the most suitable source for microbial volatile production as they tolerate high sugar concentration, anaerobic growth, high salt concentration, resistance to inhibitors in the biomass (Nevoigt, 2008). An alcoholic fermentation takes place in the absence of oxygen and presence of yeasts, nutrient medium,

suitable pH and temperature. Alcohols, vitamins, hormones, antibiotics, enzymes, acids are made through fermentation. Yeast utilization of glucose can be through the fermentation of glucose, oxidation of glucose or oxidation of ethanol. *Saccharomyces cerevisiae* can completely ferment glucose, fructose, galactose, sucrose and maltose (Yoon *et al.*, 2003). Volatile metabolites are produced during bacterial and yeast fermentations but in an experiment, only volatile metabolites from yeast fermentation were able to attract insects (Nout and Bartelt, 1998).

The *Saccharomycetaceae* family has about 20 genera, which include *Saccharomyces*, *Candida* and *Pichia*. Members of the family reproduce by budding and live in environments rich in carbohydrates. *Saccharomyces cerevisiae* is the most important yeast as a model eukaryotic organism. *Saccharomyces cerevisiae* is used in baking, brewing and wine making. *Saccharomyces cerevisiae* and closely related yeasts dominate alcoholic fermentations as they are tolerant of high alcohol content and they also use the produced ethanol as feedstock (Piskur *et al.*, 2006).

Candida ethanolica was first isolated from industrial fodder yeast and can grow on ethanol as the only carbon source. *C. ethanolica* is one of the yeasts involved in *shubat* (a fermented camel milk) production (Shori, 2012), Ghanaian heaped cocoa bean fermentation (Daniel *et al.*, 2009), *mescal* (Mexican distilled beverage) (Valdez, 2011), sour cassava starch fermentation (Lacerda *et al.*, 2005).

Pichia kudriavzevii is synonymous with *Issatchenkia orientalis* based on molecular, biochemical and phenotypic characterization (Bhadra *et al.*, 2007). *Pichia kudriavzevii* was shown to degrade phytate in ‘*togwa*’, a cereal food in Tanzania (Hellstrom *et al.*, 2012), produce ethanol from alkali-treated rice straw (Oberoi *et al.*, 2012), ferment cotton stalks in a combined saccharification and fermentation process for ethanol production (Kaur *et al.*, 2012), part of *fen-daqu*, starter culture for Chinese liquor production (Zheng *et al.*, 2012), among the yeasts involved in ‘*tchapalo*’, a local beer in Cote d’Ivoire (N’guessan *et al.*, 2011) and among the yeasts involved in Ghanaian heap cocoa bean fermentation (Daniel *et al.*, 2009).

Media composition is important in yeast fermentation performance (Hahn-Hagerdal *et al.*, 2005). Agar plates and nutrient broth are used in most yeast fermentations. Among the common media for yeast fermentation are wort (Lodolo *et*

al., 2008), molasses (Alegre *et al.*, 2003) and potato dextrose broth (Kalyani *et al.*, 2000). Yeast stock cultures are normally kept at 4°C on potato dextrose agar (Lakshmanan and Radha, 2012). Most yeast utilize hexose sugars (monosaccharides and disaccharides). The media used can improve yield, increase biomass formation and decrease fermentation period (Chunkeng *et al.*, 2011). The ecology and profile of microorganisms during fermentation could be affected by the media used (Hsieh *et al.*, 2012). Potato dextrose broth is commonly used for the cultivation of yeasts and molds. Its low pH discourages bacterial growth (Condolab, 2012).

Chromatography is used to separate complex mixtures but it cannot readily identify the constituents of separated unknown mixtures. It is always desirable to couple separation processes to identification steps. Liquid chromatography (LC) and gas chromatography (GC) are two of the leading chromatographic methods. LC is favoured over GC for samples susceptible to degradation as LC does not involve derivatization step. In order to identify, LC is normally coupled to mass spectrometry (MS) that is in tandem. Liquid chromatography – tandem mass spectrometry (LC-MS/MS) was found to have lower limit of quantification, less costly to run, reduced preparation time, does not involve derivatization of sample, and reduced run time (Coles *et al.*, 2007). LC-MS/MS is best suited for the analysis of chemically and thermally unstable compounds (Johnson, 2005).

Downstream processing involves the recovery of the desired product from the fermentation broth. Removal of insolubles, product isolation, product purification and product polishing are the major groupings of the unit operations of fermentation products recovery. Low product concentration makes product recovery expensive (Schugerl, 2000).

Freeze drying (lyophilization) is a dehydration process in which solvent is removed through sublimation. It is used for the recovery of heat sensitive products such as volatile metabolites. Compared to air drying, the retention of volatile materials was more in the freeze dried sample (Krokida and Philippoules, 2006).

1.2 Statement of Problem

Cockroach control using insecticides and other chemicals are not desirable because they are toxic to organisms and the environment (Quesada *et al.*, 2004). Pests can develop resistance to the chemicals. In addition, chemicals are unsustainable as the raw materials of the chemicals are from petroleum. Hence, there is consumer demand for natural product based compounds.

On the other hand, foods as attractants favour all organisms thereby providing breeding grounds (Cox and Collins, 2002). Foods have limited range of effectiveness and are non-specific.

Biological based attractants from plants and animals are very low in yields (Vandamme, 2003). Extraction from plants is very difficult, depends on the season and some of the exotic plants have lost the ability to produce desired volatile metabolites (Dudareva and Pichersky, 2000).

Microorganisms produce volatile metabolites that can potentially act as insect attractants. Volatile metabolites from yeasts have been found to be better at attracting insects than volatile metabolites from bacteria (Nout and Bartelt, 1998).

Therefore, there is the need to test if volatile metabolites from yeast fermentation can attract cockroaches and the leading volatile metabolites responsible for the attraction. In this study, two locally isolated yeasts, namely *Candida ethanolica* M2 and *Pichia kudriavzevii* M12 were tested for their potential in bioattraction of domestic cockroaches and production of exometabolites.

1.3 Objectives of the Research

The specific objectives of this study were:

1. To determine the potential of yeast strains *C. ethanolica* M2 and *P. kudriavzevii* M12 in producing metabolites for bioattraction of domestic cockroaches
2. To compare the fresh and freeze-dried yeast culture supernatants in its efficiency in bioattraction of domestic cockroaches
3. To study the exometabolites of *P. kudriavzevii* M12

1.4 Scope of Research

This research work focused on the production of volatile metabolites from yeast strains *C. ethanolica* M2 and *P. kudriavzevii* M12 which were cultured in potato dextrose broth (PDB) up to a period of a week. The culture supernatant containing volatile metabolites were tested as bait of commercial trap for the attraction of domestic cockroaches at six different locations at the student residential halls in Universiti Teknologi Malaysia. Freeze-drying of the culture supernatant was attempted as a preservation technique and the efficiency of the freeze-dried metabolites in bioattraction of cockroach was determined. As the fermentation of *P. kudriavzevii* M12 was found to be significant, LC-MS/MS profiling was performed to determine the leading metabolites in the supernatant from the four-days fermentation broth.

1.5 Significance of Research

This research is significant as it has the potential to discover non-toxic, natural bait from yeasts which may replace the harmful chemical baits in domestic cockroach traps. In the future, the identified metabolites could be optimized for the production of cheap bait for domestic cockroach attraction.

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