IDENTIFICATION OF FUNGI ISOLATED FROM CLINICAL WASTES AND INACTIVATION OF FUNGAL SPORES BY USING SUPERCRITICAL CARBON DIOXIDE

EFAQ ALI NOMAN MOHAMMED

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by

EFAQ ALI NOMAN MOHAMMED

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DEDICATION

This thesis is dedicated to:

My beloved father: ALI NOMAN

Whose words of encouragement and push for tenacity ring in my ears

My beloved mother: For her endless love, support and encouragement.

Treasure, My lovely husband: ADEL GHEETHI

Who have always loved me unconditionally and whose good examples have taught me to work hard for the things that I aspire to achieve. I am truly thankful for having you in my life.

To my beloved daughters

HALA and JULIA

Both of you have been my best cheerleaders.

To my beloved younger brothers and sisters

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EFAQ ALI NOMAN

Eanm1984@gmail.com

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LIST OF ABBREVIATION

2-DE	Two-dimensional electrophoresis
a_w	Water activity
GC–MS	Gas Chromatography-Mass spectrometry
CM	Cell membrane
CLSM	Confocal Laser Scanning Microscopy
CW	Cell wall
CYA	Czapek Yeast Extract Agar
CZ	Czapek-Dox Agar
DS	Department of Statistics
EB	Ethidium bromide
IA	Invasive aspergillosis
IA	Invasive aspergillosis
ID	infectious doses
IFIs	Invasive fungal infections
MPa	Mega Pascal
MRSA	Methicillin-resistant *Staphylococcus* aureus
PCA	principal component analysis
PDA	Potato Dextrose Agar
PI	Propium Iodide
SC-CO_2	Supercritical carbon dioxide
SDA	Sabouraud dextrose agar
SEM	Scanning Electronic Microscopy
STAATT	State and territorial association on alternative treatment technologies

TEM	Transmission Electron Microscope
US. EPA	United State Environmental Protection Agency
V8A	V8 juice agar medium
VBNC	Available but non-culturable state
WHO	World Health Organisation

PENGENALPASTIAN KULAT DIPENCILKAN DARIPADA SISA KLINIKAL DAN PENYAHAKTIFAN SPORA KULAT DENGAN KARBON DIOKSIDA LAMPAU GENTING

ABSTRAK

Sisa klinikal dianggap sebagai sumber yang mendatangkan jangkitan kepada manusia disebabkan adanya mikroorganisma patogenik seperti bakteria, virus dan kulat. Untuk merawat bahan sisa klinikal, pembasmian kuman menggunakan bahan kimia dan pendedahan kepada sinaran rawatan sisa klinikal termasuk menginsinerasikan, biasa digunakan, namun kaedah ini tidak dapat membunuh patogen sepenuhnya. Membakar menerusi insinerasi bukan teknologi yang sesuai untuk merawat sisa klinikal disebabkan oleh pengeluaran bahan pencemar berbahaya termasuk dioksin dan furan yang tidak dapat dielakkan. Kajian ini dijalankan dengan mengenal pasti dan menyahaktifkan spesies kulat yang terdapat di dalam sisa klinikal yang diperoleh daripada Pusat Kesihatan USM (kampus utama). Penyahaktifan patogen (spora) dijalankan dengan menggunakan teknologi alternatif termaju bukan termal menggunakankarbon dioksida lampau genting (SC-CO_2) dalam rawatan sisa klinikal. Satu ratus lima puluh pencilan kulat dikenalpasti daripada 92 sampel sisa klinikal yang dikulturkan di atas media yang kaya dengan bahan sayuran (V8A). Pencilan kulat telah dijalankan menggunakan teknik spora tunggal dan dikenal pasti melalui kaedah fenotip. Ciri morfologi spora kulat dijalankan dengan menggunakan kaedah imbasan mikroskop elektron. Dari analisis fenotip, pencilan kulat diklasifikasikan ke dalam 8 genera dan 36 spesies. Antara kesemua pencilan, *Aspergillus* spp. di dalam seksyen Nigri (10%), *A. niger* (9.3%), *A. fumigatus* (8.7%),

P. simplicissium (8%), *A. tubingensis* (7.3%), *A. terreus var. terreus* (6.6%), *P. waksmanii* (6%) and *C. lunata* (6%) merupakan yang banyak ditemui. Dari pemerhatian yang dijalankan di antara kelima bahagian Pusat Sejahtera, sisa klinikal yang didapati dari makmal dignostik di Bahagian hematologi menunjukkan bilangan spesies kulat yang paling tinggi (29 spesies). Sisa klinikal seperti sarung tangan terpakai mempunyai bilangan spesies kulat (19 spesies) yang paling tinggi di kalangan 17 jenis sisa klinikal yang disarikan. Antara kesemua spesies kulat, *Aspergillus* spp. mempamerkan kadar pertumbuhan yang lebih tinggi pada suhu 37˚C daripada 28˚C, dan ini menunjukkan keupayaan kulat oportunis untuk mengenakan penyakit pada manusia. Penyahaktifan spora kulat menggunakan SC-CO_2 telah dikaji pada julat tekanan 30-40 MPa, suhu 35-75˚C dan masa 30-90 min dan nilai optimuma ditentukan dengan kaedah gerak balas permukaan (RSM). *A. niger* telah digunakan sebagai petunjuk biologi dengan kepekatan awal 6 log sebagaimana yang ditentukan dengan himotisometer dan teknik kultur (CFU g^{-1}). Kecekapan SC-CO_2 dalam menyahaktifkan spora kulat ditentukan berdasarkan penurunan log dan peratusan penyahaktifan. Penyahaktifan optimum diperolehi pada 35 MPa, 75 ˚C, dalam masa 90 min yang menunjukkan penurunan log yang diramalkan berbanding dengan yang dikaji adalah 5.93 dan 6 log masing-masing dengan 99,9999% nilai peratus penyahaktifan. Kulat yang telah dirawat dengan SC-CO_2 juga tidak menunjukkan kesan boleh hidup kembali setelah dikulturkan semula ke atas media. Pada tahap optimum SC-CO_2, spora kebanyakan spesies kulat yang paling biasa adalah dinyahaktifkan dengan sepenuhnya (6 pengurangan log). Penyahaktifan spora kulat dianalisakan di bawah mikroskop imbasan elektron, menunjukkan dengan rawatan SC-CO_2, dinding sel kulat musnah jika dibandingkan dengan autoklaf stim dan tidak ada aktiviti enzim. Esei plat yang dikaji menunjukkan bahawa spora kulat

yang telah dinyahaktifkan tidak menjanakan enzim luaran. Kesimpulan yang diperolehi adalah, bahan sisa klinikal mengandungi banyak kulat opportunis yang boleh menyebabkan beberapa penyakit di kalangan manusia. Kajian ini adalah signifikan dalam membuktikan penggunaan SC-CO_2 sebagai kaedah termaju bukan termal mampu menyahaktifkan species kulat yang terdapat di dalam sisa klinikal lantas mengurangkan risiko kesihatan semasa pengendalian sisa klinikal yang menjadikan ianya lebih selamat.

IDENTIFICATION OF FUNGI ISOLATED FROM CLINICAL WASTES AND INACTIVATION OF FUNGAL SPORES BY USING SUPERCRITICAL CARBON DIOXIDE

ABSTRACT

Clinical wastes are considered as a serious infectious source to humans due to the existence pathogenic microorganism such as bacteria, virus and fungi. For the treatment of clinical wastes, chemical disinfection and irradiations methods including UV are commonly used, however, these treatment methods are unable to destroy pathogens completely. Incineration is not a suitable technology for the treatment of clinical wastes due to the inevitable production of hazardous pollutants (dioxins and furans). The present study deals with the identification and inactivation of fungal species present in clinical waste samples collected from health care unit; the Wellness Centre of USM, Malaysia. The inactivation of the most common pathogens (fungal spore) were carried out using an advanced non-thermal alternative technology; supercritical carbon dioxide (SC-CO_2) for safe handling of clinical wastes. In this regards, 150 fungal isolates were identified from 92 clinical waste samples and cultured on V8A medium. The fungal isolates were purified using single spore technique and identified by phenotypic method. The morphological characterization of fungal spore were performed by using scanning electron microscope (SEM) analysis. On the basis of phenotypic analysis fungal isolates were classified in 8 genera and 36 species. Among all isolates, *Aspergillus* spp. in section Nigri (10%), *A. niger* (9.3%), *A. fumigatus* (8.7%), *P. simplicissium* (8%), *A. tubingensis* (7.3%), *A. terreus var. terreus* (6.6%), *P. waksmanii* (6%) and *C. lunata* (6%) were the most frequently found. It was also observed that among five sections

of the Wellness Centre, the clinical wastes collected from diagnostic labs of Haematology Section showed the highest numbers of fungal species (29). Glove wastes also demonstrated the highest number of fungal species (19) among seventeen types clinical wastes screened. Among all fungal species, *Aspergillus* spp. exhibited higher growth rate at 37˚C than at 28˚C, indicating the ability of these opportunistic fungi to cause diseases in human. The inactivation of fungal spores using SC-CO_2 was investigated at 30-40 MPa, 35-75 ˚C for a time period of 30-90 min and optimized by response surface methodology (RSM). *A. niger* was used as biological indicator with initial concentrations of 6 log as determined by haemocytometer counts and culture based technique. The efficiency of SC-CO_2 in the inactivation of fungal spores was determined in terms of log reduction and inactivation percentage. The optimal inactivation of spore was determined at 35 MPa, 75˚C in 90 min which showed the predicted and experimental reductions as 5.93 and 6 log, respectively with 99.9999% of inactivation. It was also found that after treatment the samples by SC-CO_2, no regrowth was observed. At the optimal condition of SC-CO_2, the spores of the most common fungal species were completely inactivated (6 log reduction). The attractions of the inactivated fungal spores were examined using SEM analysis which revealed that after SC-CO_2 treatment, the cell wall of fungal spore was completely damaged (burst) and deformed as compared to steam autoclaved and untreated ones. The plate assay study revealed that the inactivated fungi spores did not produce any extracellular enzyme. In conclusion, clinical wastes have high contents of opportunistic fungi, which might cause several diseases in human being. This study significant in proving that SC-CO_2 as an advanced non thermal method is capable in inactivating fungal species found in clinical wastes, hence reducing the health risks during the handling of these clinical waste thus making it safer.

CHAPTER ONE

INTRODUCTION

1.1 Fungi and clinical wastes

Fungi are eukaryotic organisms including yeasts and moulds. These organisms are classified in separate kingdom called Fungi. Yeasts are unicellular and reproduce by budding process. Moulds are multicellular filamentous organisms belong to the fungi group. The reproductions of filamentous fungi are by sexual and asexual spores. Fungi cause several serious diseases in humans, animals and plants. However, the opportunistic fungi, which are also called invasive fungal infections, are the most important for human health (van Burik and Magee 2001). The prevalence of fungal infections caused by opportunistic fungi has increased since the 1990s due to an intensive immunosuppressive chemotherapy, increasing of immune-compromised patients and the widespread use of broad-spectrum antibiotics (Beck-Sague and Jarvis 1993).

The expansions of communities and cities in the last two decades have led to an equal increase in the number of healthcare facilities and clinical wastes generated from these facilities. The amount of clinical wastes generated form healthcare facilities in Malaysia increased from 3,303 to 18,055 tonnes per year from 1997 to 2012. In 2012, there were 398 hospitals with 56,872 beds, thousands of clinics and hundreds of diagnostic, research and haemodialysis centres (Department of Statistics 2013a). The amount of clinical wastes generated from healthcare facilities expected to be 33,000 tonnes per year by 2020 due to the increasing of population and thus health establishments (Ambali et al. 2013).

Clinical wastes are defined as any waste containing blood or human body fluids generated from hospitals, medical centres, clinics, pharmaceutical or other related clinical activity. In general any wastes generated from healthcare facilities are managed as clinical wastes and represent a potential source of infection (WHO 2005). In Malaysia, Environmental Quality ACT 1974, Scheduled Wastes (Regulations 2009) has defined the clinical wastes in the following categories; pathogenic and clinical wastes generated from pathological Sections of hospitals, expired drugs containing toxic, harmful, carcinogenic or mutagenic (EQA 1974, 2011).

1.2 Risk caused by fungi in clinical wastes

Clinical wastes are considered one of the most important sources for the infection of human being due to the existence pathogenic microorganisms such as bacteria, virus and fungi (Saini et al. 2004; Park et al. 2009). However, fungi represent more importance than bacteria, since they could grow and multiply in clinical wastes without any intermediates as hosts. These characteristics are common in some bacteria. Moreover, fungi can grow at low levels of water activity (a_w) compared to bacteria (0.60-0.85 *vs.* 0.89-0.97 respectively). Another reason is fungi spread their external spores to the environment which has become harmful for human health (Jay 2000; Deacon 2005; Grant et al. 1989; Nielsen et al. 1999; WHO 2009).

The presence of fungi in the clinical wastes and environment has been reported by many investigators around the world. Neely and Orloff (2001) isolated *Fusarium* sp., *Mucor* sp., *Paecilomyces* sp., *Aspergillus* sp. from clinical wastes in USA. Vieira et al. (2010) identified *Alternaria* sp., *Aspergillus* spp., *Aureobasidium* sp., *Basipetospora* spp., *Cladosporium* spp., *Curvularia* spp., *Fusarium* spp., *Penicillium* spp., *Scopulariopsis* spp., and *Scytalidium* sp. in the dental healthcare facilities in

Brazil. Şahil and Otag (2013) recovered *Aspergillus* spp., *Acremonium* spp., *Alternaria* spp., *Cladosporium* spp. and *Fusarium* spp., from the clinical wastes in Turkey and revealed that these fungi survived for one year in the clinical wastes discharged into the environment.

In Malaysia, few studies have been conducted on the inventory of fungi in the wastes. *A. niger*, *A. fumigatus*, *Candida* sp., *Fusarium* sp., *Mucor* sp., *P. chrysosporium* and *T. harzianum* have been detected in palm oil mill effluents (POME) wastes (Kabashi et al 2006; Soleimaninanadegani and Manshad 2014; Bala et al. 2015). *Cryptococcus neoformans*, *Candida* spp. and *Pencillium marneffei* have been detected as the most common IFIs among the patients in Malaysia (Tamring et al. 2014). Abdul-Rahman et al. (2008) have indicated that *Candida* spp. were the most predominant in the genital, urine and blood culture specimens. However, these studies have not investigate those species in the disposed clinical wastes.

1.3 Treatment of clinical wastes

The clinical wastes have less organic contents compared to that in domestic refuse. However, the clinical wastes are responsible for more than 60% of the costs of the treatment processes by incinerations (Blenkharn 2005). In Malaysia, incineration is the main method of clinical wastes treatment. There are three main companies in the country for the handling and treatment of clinical wastes. These are Faber Medi-Serve Sdn Bhd (Perlis, Kedah, Penang, Perak, Sabah and Sarawak), Pantai Medivest Sdn Bhd (Negeri Sembilan, Melaka and Johor) and Radicare (M) Sdn Bhd (Wilayah Persekutuan Kuala Lumpur and Putrajaya, Selangor, Pahang, Kelantan and Terengganu) (Chuah et al. 2009; Khanehzaei et al. 2014). These companies collect the clinical wastes from the large hospitals daily in yellow containers, while smaller

hospitals that produce lesser amount of clinical wastes, the wastes are stored at -1 to -5˚C until collection every 3 days.

Several of the alternatives technologies have been suggested for the treatment of clinical wastes. These technologies including autoclave, irradiation, microwave and chemical disinfection were effective for inactivation of pathogenic organisms and have achieved 6 log reductions (U.S. EPA 1990; Salkin 2003; Lee et al. 2004; Da Silva et al. 2005; STAATT 2005; Shinee et al. 2008; Bendjoudi et al. 2009; Abd El-Salam 2010; Hossain et al. 2012).

The chemical disinfection and irradiations treatments of the clinical wastes are unable to completely damage of pathogens. Thus, the treated clinical wastes still contains heavily load of infectious agents due to weaknesses and disadvantages of these techniques (Tsakona et al. 2007; Nemathaga et al. 2008). Furthermore, these technologies create health hazard and environmental pollution rather than reducing infectious risk in the waste. This gap offered investigators a greater opportunity to explore the alternative technology is effective for the clinical wastes treatment and eco-friendly and this is the focus of this study.

The inactivation of yeast and moulds using SC-CO_2 in normal saline, distilled water, grape juice, barley grains and growth medium revealed that SC-CO_2 has significant capability to inactivation *Saccharomyces cerevisiae, A. niger, A. ochraceus, P. oxalicum* by 6 log reductions (Nakamura et al. 1994; Shimoda et al. 2002; Gunes et al. 2005; Park et al. 2013; Neagu et al. 2014). SC-CO_2 has found as an efficient tool for the sterilization of food and inactivating of microorganisms without any destruction of the nutritional contents (Balaban et al. 2001; Gunes et al. 2005). It

may be due to that SC-CO_2 depends on the diffusion of pressured CO_2 through the cell membrane of microorganism and not on temperature like autoclave (Zhang et al. 2006b). This characteristic makes SC-CO_2 advance compared to incineration, microwave and autoclave technologies for treating clinical wastes especially recyclable and reusable wastes.

1.4 Problem statement

Many fungi can be found in hospitals and other clinical facilities or laboratories, which form a composite in the wastes, generated from these premises. The clinical wastes present an appropriate and fertile environment for the reproduction of fungi due to the right temperature and proper nutrients as well as the adequate moisture and pH of the clinical wastes is favourable for their growth (U.S. EPA 1990). The fungal diversity in the clinical wastes have been investigated worldwide. In Malaysia, few information about the fungal contents in the clinical wastes is available. It was may be due to the fact that the amount of clinical wastes represent less than 3% of the total wastes (DS 2013a). Nevertheless, the importance of clinical wastes as one of the main sources for infectious agents warrants the needs to understand the fungal load in these wastes to facility choice the suitable treatment process and then protect human health. In the current study, the identification for the fungal inventory in the clinical wastes has investigated and this emphasizes the originality of the study.

The clinical wastes are treated by using incinerator, which convert it to ash and then dispose into landfill (Razali and Ishak 2010). However, incineration is not a suitable technology for the treatment of clinical wastes due to the inevitable production of hazardous pollutants including dioxins and furans (Blenkharn 2005; Coker et al. 2009; Ambali et al. 2013). This provides strong support for a move away from costly

destruction of clinical wastes by incineration. The new trends is to find a non-thermal technology of clinical wastes avoids the toxic by-products generated from utilization of the conventional technologies. As SC-CO_2 depends on the solubility and diffusion of pressure through the cell membrane of microorganism, it has an effective for the inactivation of microorganisms (Zhang et al. 2006a). SC-CO_2 is also nontoxic, inflammable and eco-friendly thus, has not negative effects on the human and environment compared to thermal or chemical treatments (Spilimbergo et al. 2002).

The efficiency of SC-CO_2 for inactivating of pathogenic bacteria in clinical wastes has been investigated which indicated that SC-CO_2 has inactivated *S. aureus* and *P. aeruginosa* by 6 log reductions and met the standards limits for level I recommended by STAATT (Banana 2013; Hossain 2013). However, up to date no study was reported regarding the inactivation of fungi in clinical waste samples. Therefore, this research focussed on the effectiveness of the SC-CO_2 to inactivate fungi found in clinical wastes and sought to prove if SC-CO_2 has the potential to be the initial step in treating the clinical wastes within the clinical premises.

1.5 Objectives

The general objective of present study is to explore the fungal contents in the clinical wastes. The present study focuses in the particularly to achieve the following aims:

1. To identify the fungal species recovered from the clinical waste samples based on phenotypic methods

2. To determine the efficiency of SC-CO_2 in the inactivation of the common fungal spores in clinical waste in comparison with steam autoclave.

3. To identify alterations on the morphology of SC-CO_2 treated spores and production of extracellular enzymes

1.6 Significance and scope of the study

The clinical wastes represent 3% of the total wastes in Malaysia. However, these wastes have high contents of pathogenic microorganisms. Therefore, raises the need to understand role of the clinical wastes as infectious agents for human and environment. It has been suggested that in order to minimize the amount of clinical wastes with inherent infectious risk, clinical waste management should incorporate waste minimization and additional segregation of wastes at source. Such initiatives can vastly reduce the volumes of clinical wastes sent for disposal; significant cost savings may accrue. Nonetheless, this does not circumvent the infectious nature and the risks borne by clinical wastes. In order to handle the infectious nature inherent in these pathogen contaminated wastes, proper inactivation treatment is deemed to be of great urgency. This study is one of the few studies if not the first study which addressed the fungal inventory of the clinical wastes and the inactivation of the fungi in these wastes in Malaysia.

The present study demonstrated that the high content of fungi in clinical wastes is a clear indicator for the biohazards of these wastes and the need to be treated before the final discharge into the environment. The identification of the fungal isolates was conducted using phenotypic method to study the similarity and differences between fungal species within each genus. The fungal content of clinical wastes depended of the type and generation source of these wastes indicating the role the segregation process of these wastes in the management of the clinical wastes. . The study revealed that the storage condition play an important role for the distribution of fungal spores into the air and suggested the storage of clinical should be less than 24 hrs before the final treatment to minimize the distribution of fungal spores into the surrounded environment.

The results in the current work confirmed the usefulness of SC-CO_2 as non-thermal alternative for safe handling of the clinical wastes. Besides avoiding the adverse effects of the incineration, the present treatment showed more advantages since this technique would be used for the raw clinical wastes without chemical additive as recommended by STAATT (2005) or primary treatment was required.

As, the fungal spore might be regrow after the inactivation processes, the inactivated spores were recovered on enriched medium (V8 agar) and moderately enriched medium (PDA) and incubated at the optimal condition for fungal growth (28˚C) for 10 days. No fungal growth was observed which indicated that the fungal spores have died and not only inactivated. Besides, since the fungal spores might produce cellulase and protease enzyme without germination, the bioassay for these enzyme and others (lipase and amylase) was assessed using plate assay technique. No enzyme production by inactivation spores was recorded compared to control (untreated) which has produced datable amount of these enzymes. These results confirmed that the fungal spores were dead. Then, as the fungal spore might be in an available but non-culturable state (VBNC) the integrity of cell wall and cell membrane was observed using SEM. The spore shape was completely damaged, cell wall and cell membrane was totally destroyed. These observations proved that the fungal cytoplasm was extracted and demonstrate that the spores have irreversible lost the ability to regrow even at the optimal conditions for fungal growth.

Finally, the irreversible loss of fungal growth is an evidence for the safe disposal of the clinical wastes, where the fungal spores would not represent any hazards on the human and environment.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

The selection an appropriate technology in the treatment of clinical wastes is crucial due to potential health risk associated with these wastes. Clinical wastes contain heavily loads of pathogenic microorganisms. The importance of these pathogens lie in their ability to cause severe diseases for human because they have already adopted with the human body as well as these pathogens have acquired resistance for several antibiotics that were used during the treatment process of the patients. Indeed, the pathogenic microorganisms in clinical wastes are same to that in the patient specimens; the clinical specimen is defined as a clinical waste just after the examination process in the diagnostic laboratories and dispose to the wastes basket.

The most common technologies applied for the sterilization or treatment of clinical wastes are the incineration, autoclave, irradiation and microwave. Moreover, the adverse environmental impact of these technologies which include the toxic by-products and detrimental effects on the human and environment have been extensively documented. Currently, the new trend is to use non-thermal sterilization technologies. Supercritical carbon dioxide (SC-CO_2) is one of these technologies because it is depend on the pressure and low temperature. It exhibited efficiency for inactivation of fungi in food and pharmaceutical products. The studies conducted on inactivation of pathogenic bacteria in clinical wastes have revealed that SC-CO_2 is effective for reduction of pathogenic bacteria; clinical wastes treated by SC-CO_2 have met the standards limits recommended by STAATT (2005) for safe disposal. Therefore, it is time to investigate SC-CO_2 as a non-thermal alternative technology for inactivation of

fungi in clinical wastes in order to safe handling and thus minimize the exposure for fungal spores associated with these wastes. In this chapter the fungal load of clinical wastes and the potential using SC-CO_2 for the inactivation of fungal spores in clinical wastes are viewed based on understand the inactivation mechanism of SC-CO_2 against fungi and bacteria that has been described by authors during the last years (Nakamura et al. 1994; Shimoda et al. 2002; Gunes et al. 2005; Park et al. 2013; Neagu et al. 2014).

2.2 Characteristics of fungi

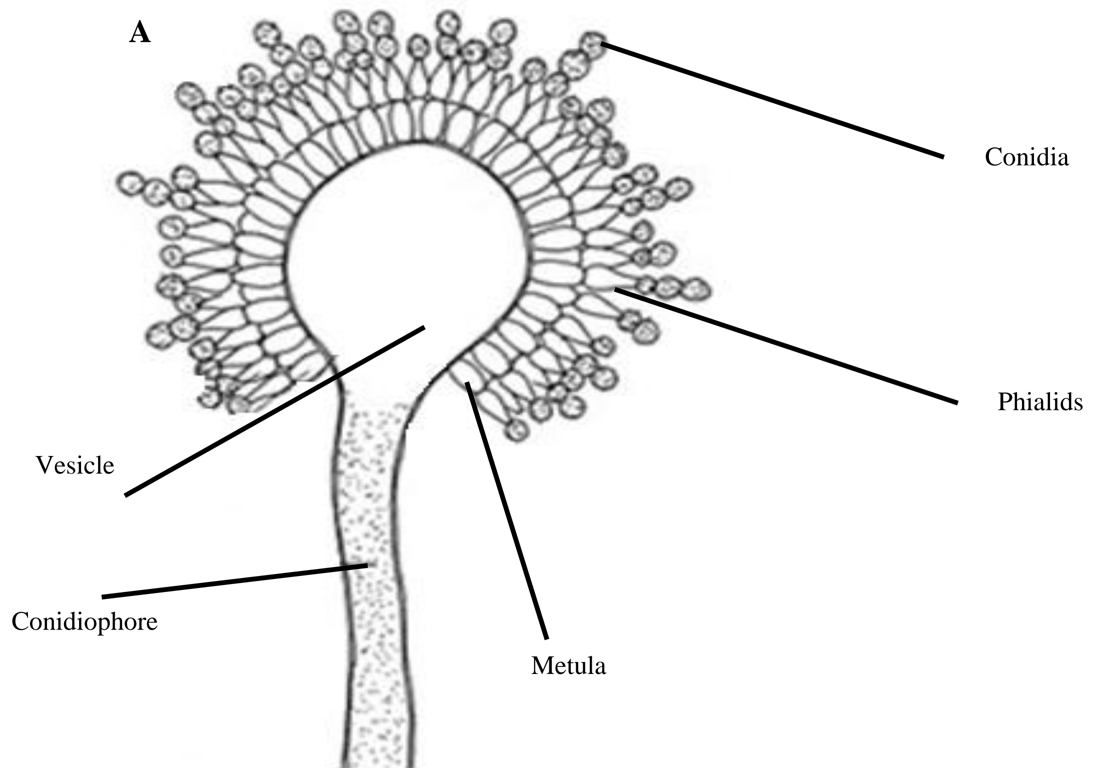
Fungi are eukaryotic organisms, which are composed of filaments called hyphae. Hyphae may contain internal cross walls, called septa. A mass of hyphae that is not a reproductive structure is called a mycelium. Hyphae represent the vegetative part of fungi, which contains nucleus, mitochondrion, ribosomes and all metabolic pathways of fungal cell. The hypha is surrounded by cell wall that protects fungus cell from changes in osmotic pressure and other environmental stresses. The cell wall is composed of macromolecules (chitosan, chitin, lipid, glucan, phospholipides), which contain amino, carboxyl, melanin, phosphate, hydroxides and sulphates groups (Fogarty and Tobin 1996; Bowman and Free 2006; Hardison and Brown 2012).

Fungi are typically non-motile, heterotrophic organisms and have quite simple nutritional requirements. Most of fungi are saprophytic organism which get the nutrient by breaking down dead organic material using hydrolysis enzymes such as lipase, cellulase, amylase and protease. Others are pathogenic for human such as *Blastomyces, Histoplasma, Coccidioides* and *Paracoccidioide* that named dimorphic or ‘endemic’ fungi, which grow as filamentous form at 25˚C and as yeasts form or spherules in host tissue when are incubated at 35˚C. Some of fungi are opportunistic

organisms that have a potential to cause secondary infection for human (Deacon 2005; Şahil and Otag 2013).

The fungi reproduce sexually and asexually, but the majority of fungi have asexually reproduction. Therefore, fungi represent a supreme example of spore-producing organisms (Deacon 2005). In asexual multiplication, the fungi produce conidia (spores) that are mounted on conidiophore. The spores are distributed into the environment and germinate to fungal mycelium.

The identification of fungi is performed using phenotypic and molecular techniques. In the phenotypic method, the identification depends on the culture and microscopic characteristics of the fungi, the diversity of the fungal conidiophores and spores in the shape and size are usefulness for the morphological identification of fungi (Kumara and Rawal 2008; Emine et al. 2010). In contrast with bacteria, fungi have high diversity in their microscopic morphology such as conidiophore and spores, these microscopic characteristics are useful to identify some of fungal isolates such *Aspergillus* sp. *Penicillium* sp., *Trichoderma* sp. *Curvularia* sp. and *Rhizopus* sp. to species level. In *Aspergillus* spp. the structure of conidiophore is very important for their identification (Robert et al. 2011; Silva et al. 2011). The conidiophores are originated from the mycelium, and terminate in a vesicle. The conidiophores structures depend on the fungal species (Figure 2.1 a,b).



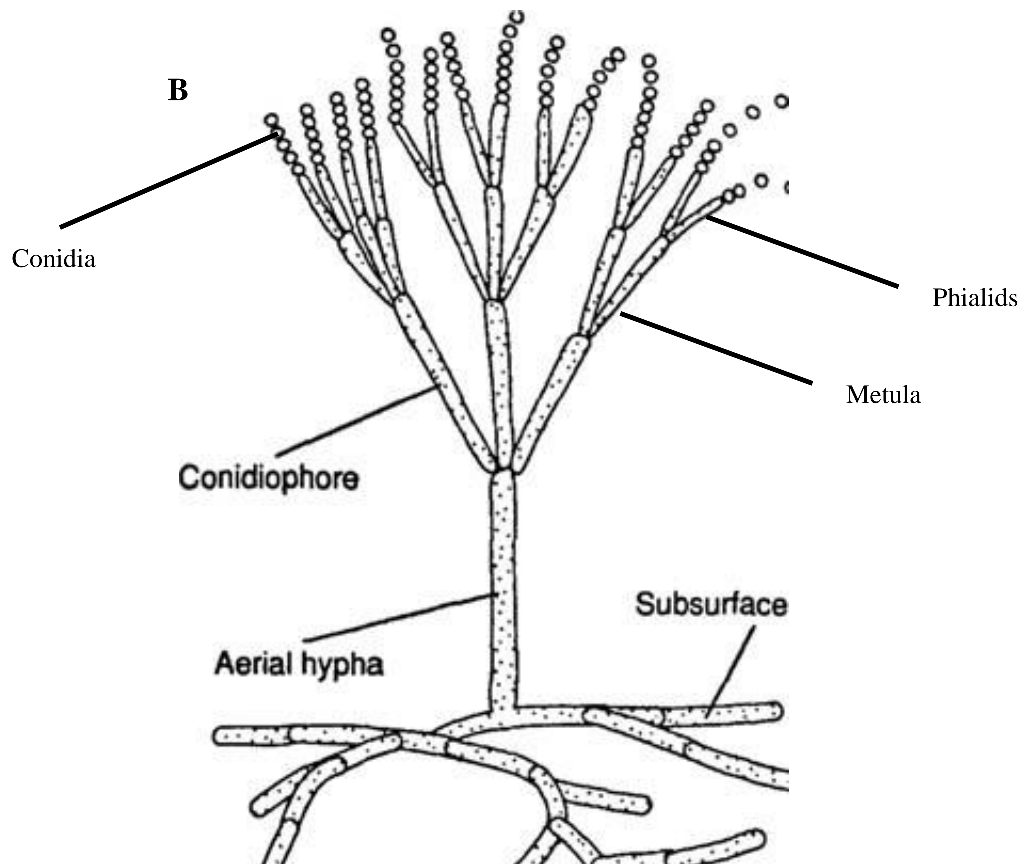


Figure 2.1 Conidiophore structure of fungi; A) *Aspergillus* spp. B) *Penicillium* spp.

The phialids (spore producing cells) which are located on the vesicle is also an important character for the identification. The phialids may be radiate head where they cover the entire vesicle surface or columnar head where they cover the vesicle surface partially. The phialids are attached to the vesicle directly (uniseriate) or via metula (biseriate) (Jensen et al. 2013).The spore shape and size as well as spore surface ornamentations also represent an important key for the phenotypic identification (Figure 2.2).

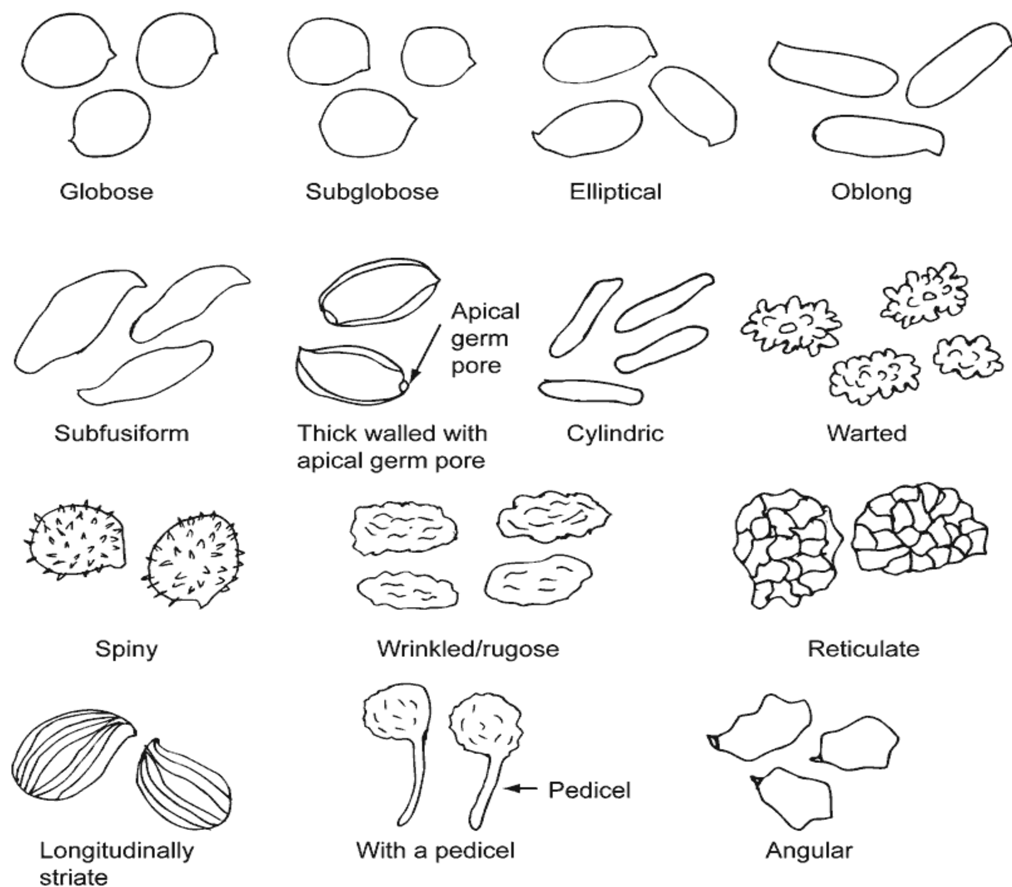


Figure 2.2 Fungal spores shape (CTR 2014)

The developments in the Scanning Electronic Microscopy (SEM), Fluorescent Microscopy and Flow Cytometry have enhanced the recognition of fine details of phenotypic taxonomic significance (Cole and Samson 1979; Guarro et al. 1999). The SEM has the ability to show the slight differences in the spore structure for classifying the fungal isolate to the varieties level. For instance, *C. lunata* has two varieties: *C. lunata* var. *aeria* with smooth conidia and stromata in culture; and *C. lunata* var. *lunata* with smooth to roughly conidia but without stromata in culture (Ellis 1971; Sivanesan 1987). These varieties are identified as *C. lunata* and *C. aeria* based on molecular analysis (Nakada et al. 1994; Cunha et al. 2013).

2.3 Clinical wastes in Malaysia

Clinical wastes are generally health wastes that are hazardous and represent a potential source of human infection. EPA (2009) identified the clinical wastes as any wastes contaminated with blood or human body fluids and generated from healthcare facilities during the treatment of patients or during research projects. The healthcare facilities include medical, dental, nursing, diagnostic laboratory, podiatry, pharmaceutical, tattooing, emergency, body piercing, services and blood banks. According to WHO (2005), human anatomical wastes, pathological wastes, diagnostic laboratory wastes, sharps, medicines and cytotoxic drugs, disposable solid wastes and liquid wastes (blood and body fluids) as well as chemical wastes could be classified as clinical wastes. The various types of clinical wastes are presented in Figure 2.3.

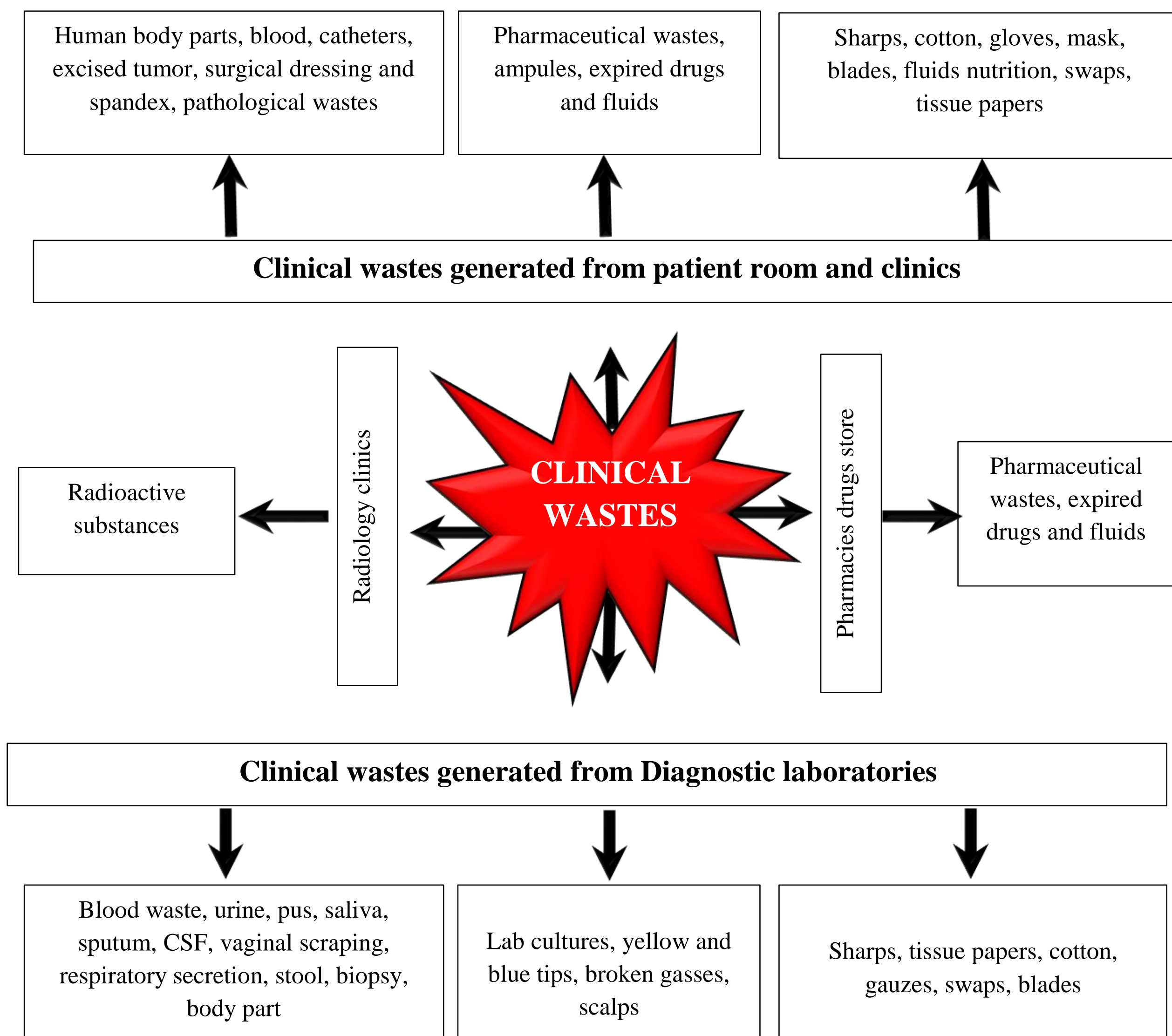


Figure 2.3 Clinical wastes generated from the healthcare facilities

The amount of clinical wastes generated from healthcare facilities have been increased during the recent years. Clinical wastes represent 12.5-69.3% of the total healthcare wastes, among them 10-25% are high-risk wastes (WHO 2005; Visvanathan 2006; Nemathaga et al. 2008; Shinee et al. 2008). WHO (2000) has estimated the quantities of clinical wastes generated from healthcare facilities to be between 0.5-3 kg/bed/day. In Malaysia, the average of healthcare waste generation is 1.9 kg/bed/day (Visvanathan 2006). This value is less than reported in North and South America and Western Europe, but higher than that estimated in Pakistan, Thailand, India and China (Figure 2.4). The variance found among these countries are a result of different level of economic strength and healthcare facilities, size and type of the medical institution and number of patient care (Johannessen 1997; WHO 2005; Da Silva et al. 2005; Visvanathan 2006; Marinkovic et al. 2008; Nemathaga et al. 2008; Cheng et al. 2009; Ruoyan et al. 2010).

As presented in Figure 2.4, it can be noted that the rate of clinical wastes generated from healthcare establishments depends on the level of developments of the country. Developed countries generate more clinical wastes at a higher rate than that of developing countries (Pruss et al. 1999). According to Halbwachs (1994), the health-care wastes generated from high-income countries ranged between 1.1 and 12.0 kg/person, while low-income countries produce between 0.5 and 3.0 kg/person.

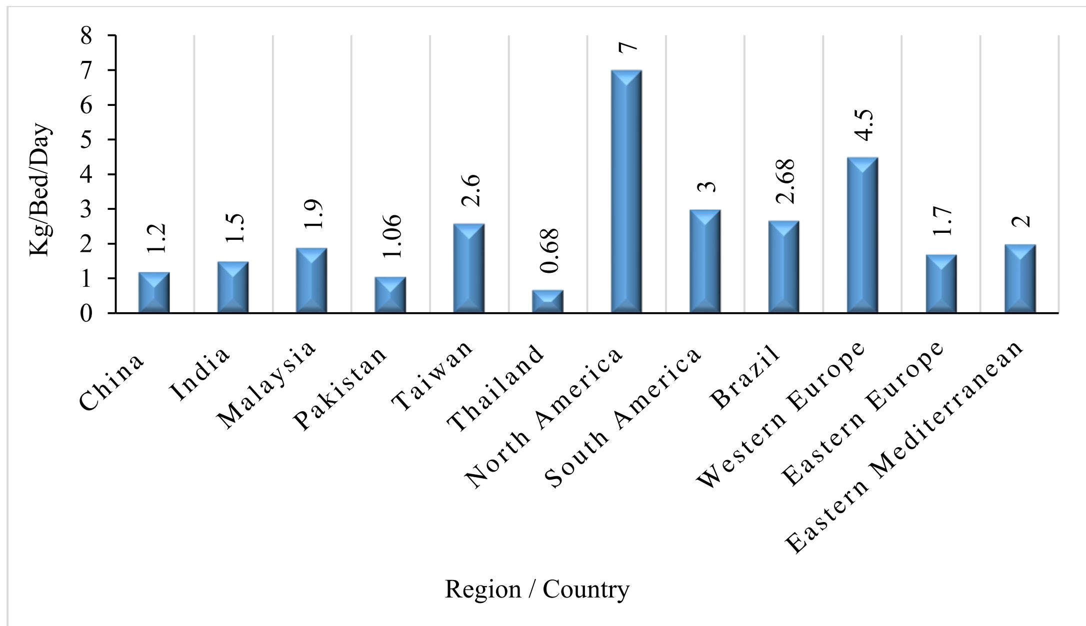


Figure 2.4 Estimated average healthcare waste generation in different region in the world (WHO 2005; Visvanathan 2006; Johannessen 1997; Da Silva et al. 2005; Cheng et al. 2009; Ruoyan et al. 2010).

The quantities of clinical wastes handled for the destruction by incineration in Malaysia during the period from 2006 to 2012 are shown in Figure 2.5. It was indicated that the quantities of these wastes increased by 37.1 %.

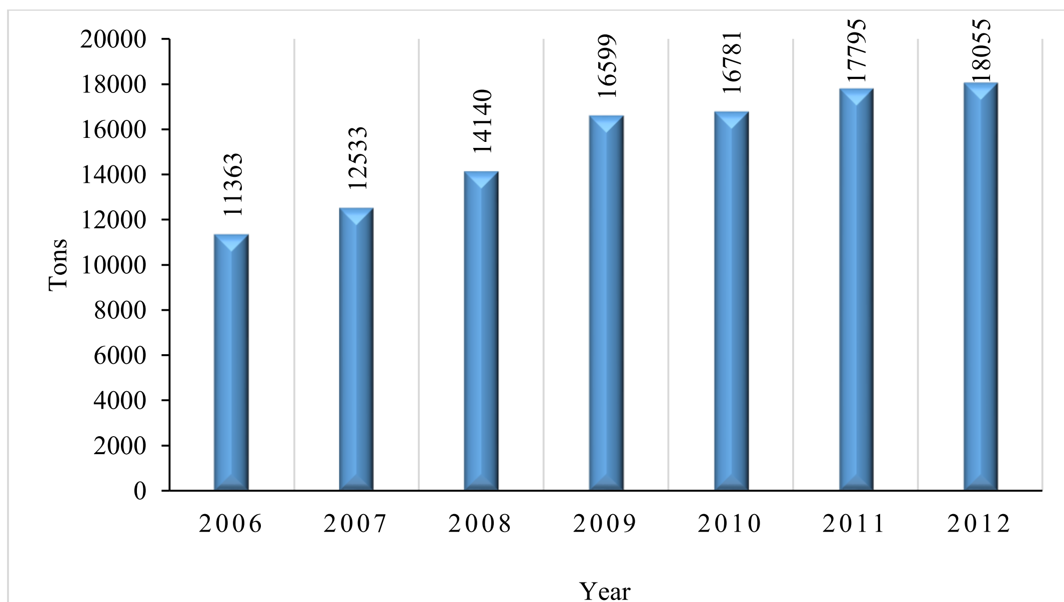


Figure 2.5 Quantity of clinical waste handled for destruction at incinerator Malaysia. Source; Department of Environment (Department of Statistics 2011, 2013a)

In 2012, Malaysia had more than 398 hospitals (147 public and 251 private hospitals) with 42707 *vs*. 14165 beds for public and private hospitals, respectively (DS 2013a). Based on the daily amount of clinical wastes generated in Malaysia (1.9 kg/bed/day), the estimated quantities of clinical wastes generated from public hospitals in 2012 were 29,617.546 tonnes/year. However, Department of Statistics in Malaysia (2013a) reported that only 18,055 tons have handled for destruction by incinerator (distributed for each state as depicted in Figure 2.6). It was due to that the maximum loading capacity for incinerators in Malaysia are 18,000 tons/year (Frost and Sullivan 2010).

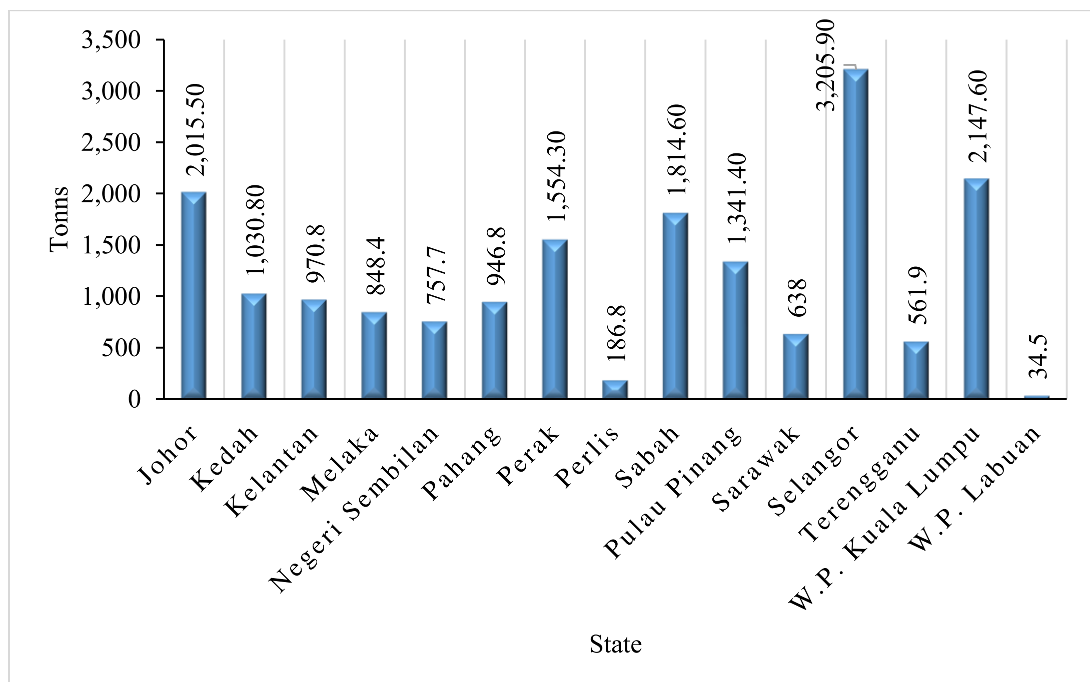


Figure 2.6 Quantity of clinical waste handled for destruction at incinerators by state, Malaysia, Source; Department of Environment (Department of Statistics 2013a)

The growing interest in controlling and monitoring clinical wastes aim to determine a safe and effective management of these wastes, in order to protect of humans and environment from pathogenic microorganisms associated with these

wastes (Alagoz and Kocasoy 2008; Marinkovic et al. 2008; Park et al. 2009; Wiafe et al. 2015; Khazaee et al. 2015). Several countries have adopted the regulations for the proper management of clinical wastes. In the UK, the Department of Health (2013) has published the guidelines for the management of healthcare waste. In the USA, EPA (2009) has regulated the organization of clinical wastes, including the storage, transport, treatment and disposal processes. In Australia, the legislation for collection, segregation and treatment of clinical waste has been in practice since the 1990s by the Ministry of Health (1998). In Turkey, the Ministry of Environment and Forestry (MEF), controls the management of healthcare wastes since 1993; the legislation includes the procedures for segregation, collection, transportation, storage of clinical wastes and the transportation to the final disposal area (MEF 2005).

In Malaysia, the regulations for clinical wastes management have started in 1989 (Razali and Ishak 2010; Ambali et al. 2013). These regulations are described in more detail in a new version of Environmental Quality ACT 1974, Scheduled Wastes, Regulations 2009 (EQA 1974, 2011). Three concession companies have been appointed by the government to handle and treat clinical wastes generated in public hospitals in Malaysia (Chuah et al. 2009), which use incineration for the final treatment of the clinical wastes; however, with increasing quantities of wastes, the needs for more incinerators to treat clinical wastes has also increased. In contrast, among the 150 incinerators constructed in New York State, USA, in 1990, 142 have been closed by 2000 due to the negative impacts on human health and the environment (Banana 2013).

Consequently, it is imperative that alternative technologies for treatment of clinical wastes, especially non-thermal sterilization technologies, be sought. In the last years, alternative technologies for treatment of clinical wastes have been ventured; for

instance, thermal treatment (microwaving, moist and dry heat, laser, infrared, plasma, gasification and pyrolysis), chemical disinfection (chlorination, ozonation, hydrogen peroxide and sodium hydroxide) and irradiation treatment (UV, Cobalt 60, electron beam) (STAATT 2005). In Malaysia, these technologies have not been applied at a large scale yet, and autoclaves are used in numerous hospitals as a primary treatment, while incineration is used as final treatment. Few studies have been conducted on the treatment of clinical wastes by autoclave, microwave and supercritical carbon dioxide at laboratory scale, which show the potential in applying these techniques at full scale (Banana 2013; Hossain 2013). However, these studies focused only on pathogenic bacteria, whereas clinical wastes contain several other pathogenic organisms including fungi and viruses. Therefore, more studies are necessary to accurate evaluate these techniques before full-scale application. In the United States, steam autoclave is used for treatment of clinical wastes, whereby there are more than 100 commercial autoclave plants for processing infectious waste (Hossain et al. 2011).

2.4 Health risks of clinical waste

Information regarding the microorganism presence in wastes generated by healthcare facilities can shed light on the level of serious potential of harm to the public and the environment (Sherman 2007). This is certainly critical and regulations imposed by countries consider this matter. The abatement of the risks has often been placed within the management as in the effective waste segregation or minimization of clinical waste that may reduce the biohazards risk of these wastes. The risks that are associated with the presence of microorganisms must, however, focus on the inactivation of these pathogens as soon as the wastes are generated. Effective clinical waste segregation or minimization may reduce the biohazards risk of these wastes,

however, the infectious agents to healthcare worker are still possible because of the infectious nature for these wastes. The health risk of clinical wastes that needs to be assessed is the presence of pathogenic microorganisms. As Park et al. (2009) aptly pointed out, infectious threat to healthcare worker is still possible because of the presence of the infectious pathogens in these wastes. Park et al. (2009) identified various types of pathogens, bacteria and fungi that are most commonly considered as health risks due to the ability of these organisms to multiply and persist for a long time in clinical wastes, while viruses cannot survive in clinical wastes without a host organism. The argument is congruent to Hall (1989) and Saini et al. (2004), who stressed that the environmental factors of clinical wastes such as presence of carbon and nitrogen source (sugars, protein, starch, fats and other compounds), and growth factors are suitable for growth and survival of bacteria and fungi. In the next section, the presence of bacteria and fungi in the clinical waste is reviewed.

2.4.1 Fungi in clinical wastes

The importance of fungi as infectious agents is well recognized worldwide. Fungi cause several serious diseases for human, of particular concern is the opportunistic pathogens including *Alternaria* spp., *Aspergillus* spp., *Cryptococcus neoformans*, *Candida* spp., *Fusarium* spp., *Mucorales*, *Paecilomyces* spp., and *Scedosporium* spp. which also called invasive fungal infections (IFIs) (Beck-Sague and Jarvis 1993; Sifuentes-Osornio et al. 2012; Tamring et al. 2014).

In Malaysia, *Cryptococcus neoformans*, *Candida* spp. and *Pencillium marneffei* are the most common species as IFIs. *A. niger, Curvularia* spp., *Fusarium* spp. and *P. lilacinus* have also detected in the laboratory diagnostic process (Tamring et al. 2014). Abdul-Rahman et al. (2008) have studied the distribution of *Candida* spp.

from 3837 clinical specimens in Hospital of USM during the period from 2001 to 2006. The study revealed that *Candida* spp. were predominant in genital specimens, urine specimens and blood culture specimens.

Aspergillus spp. are ubiquitous fungi and natural inhabitants of soil, water and organic vegetation and debris (Ryan 2004). The spores of *Aspergillus* spp. have the ability to survive for a long time in the environment and they have been isolated from dust produced during the course of hospital renovation (Araujo et al. 2006). *Aspergillus* can cause many diseases for human, *A. fumigatus*, *A. flavus*, *A. terreus*, *A. niger* and *A. nidulans* are the most common (Hogan et al. 1996; Macêdo et al. 2009; Şahil and Otag 2013). Invasive aspergillosis (IA) is the second most common cause of nosocomial fungal infections (Segal 2009). It has detected in 57.6% of immune-compromised patients (Pagano et al. 2011).

Penicillium spp. are the most common fungi in the environment, they have low pathogenicity for humans. However, in immune-compromised patients they can be virulent pathogens and can cause death (Oshikata et al. 2013). *Penicillium marneffei* has been recorded as the third most common opportunistic fungi among patients infected with human immunodeficiency virus in certain parts of Southeast Asia, India, and China (Duong 1996; Vanittanakom et al. 2006). Barcus et al. (2005) reported an intestinal invasion and disseminated disease associated with *Penicillium chrysogenum* in USA. *P. digitatum* has detected as infectious agent for human and caused the fatal pneumonia infection in Brazil (Oshikata et al. 2013).

The presence of fungi in the diagnostic specimens, lab cultures and others healthcare facilities are suspected to be associated with the contamination in clinical

wastes. Few studies have been published on the presence of fungi in clinical wastes, but these studies have revealed a wide range of fungi in clinical wastes. Neely and Orloff (2001) investigated survival of *Aspergillus* spp., *Fusarium* sp., a *Mucor* sp., and *Paecilomyces* sp. in hospital fabrics and plastics such as cotton, terry, blend, polyester, spandex, polyethylene and polyurethane in USA. *A. flavus, A. niger, A. fumigatus* and *A. terreus* can be lived more than 30 days on cotton, blend, polyster, polyethylene and polyurethane. *Fusarium* sp. survived for 10 days on cotton, blend, spandex and polyester and for more than 30 days on polyethylene, *Mucar* sp. persisted more than 20 days on all types of hospital fabrics and plastics except of terry (16 days), the maximum survival of *Paecilomyces* sp. has noted on spandex and polyurethane (11 days). Fungi also have the ability to live in the clinical wastes disposed into landfill, Oyeleke and Istifanus (2009) have studied the presence of fungi in the 24 hospital waste samples taken from different hospitals waste dumpsites and its surrounding soil in Nigeria. The isolated fungi included *A. niger* (34.48 *vs.* 44.44%), *A. flavus* (13.79 *vs.* 3.70%), *A. parasiticus* (0 *vs.* 7.41*%), A. versicolor* (3.45 *vs.* 3.45%), *A. nidulans* (0 *vs.*11.11%), *Penicillium rubrum* (6.86 *vs.* 3.70%), *Trichoderma roseum* (0 *vs.* 3.70%), *P. viricadum* (6.90 *vs.* 0%), *Rhizopus nigricans* (27.59 *vs.* 18.52%) and *Microsporum canis* (6.9 *vs.* 0%).

Fungi are saprophytic in origin, they produce external spores, which spread to air. Therefore, even if the clinical wastes generated from different sections of healthcare facilities are free from fungi. These wastes may expose to the fungal contamination during the storage period. It was due to the conditions of storage such as temperature, nutrients, moisture, pH are favourable for their growth (U.S. EPA 1990). The presence of airborne fungi in healthcare facilities has been reported. Vieira et al. (2010) evaluated airborne fungi in the Brazilian dental healthcare environment