



Selection of inactivation medium for fungal spores in clinical wastes by supercritical carbon dioxide

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Abstract

The present study aimed to select the best medium for inactivation of *Aspergillus fumigatus*, *Aspergillus* spp. in section Nigri, *A. niger*, *A. terreus* var. *terreus*, *A. tubingensis*, *Penicillium waksmanii*, *P. simplicissimum*, and *Aspergillus* sp. strain no. 145 spores in clinical wastes by using supercritical carbon dioxide (SC-CO₂). There were three types of solutions used including normal saline, seawater, distilled water, and physiological saline with 1% of methanol; each solution was tested at 5, 10, and 20 mL of the water contents. The experiments were conducted at the optimum operating parameters of supercritical carbon dioxide (30 MPa, 75 °C, 90 min). The results showed that the inactivation rate was more effective in distilled water with the presence of 1% methanol (6 log reductions). Meanwhile, the seawater decreases inactivation rate more than normal saline (4.5 vs. 5.1 log reduction). On the other hand, the experiments performed with different volumes of distilled water (5, 10, and 20 mL) indicated that *A. niger* spores were completely inactivated with 10 mL of distilled water. The inactivation rate of fungal spores decreased from 6 to 4.5 log as the amount of distilled water increased from 10 to 20 mL. The analysis for the spore morphology of *A. fumigatus* and *Aspergillus* spp. in section Nigri using scanning electron microscopy (SEM) has revealed the role of temperature and pressure in the SC-CO₂ in the destruction of the cell walls of the spores. It can be concluded that the distilled water represent the best medium for inactivation of fungal spores in the clinical solid wastes by SC-CO₂.

Keywords *Aspergillus* spp. in section Nigri · *A. Tubingensis* · Sea water · Log reduction · SC-CO₂ clinical wastes

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Introduction

Healthcare wastes are general terms used to define the wastes containing blood and infectious agents which are generated from healthcare facilities. In some references, these wastes are defined as clinical wastes, medical waste, bio-medical wastes, hospital wastes, health care waste, infectious waste, and hazards and bio-hazards wastes. However, this definition excludes the solid wastes which can be recycled or reused even after the treatment process. Moritz (1995) suggested that the disposable or waste terms should be used for non-recyclable materials, while the term of reusable or recyclable is used to define the solid materials which might be reused or recycle. Based on this definition, the sharps, cotton, and medical devices are defined as recyclable materials, while blood, body parts, chemicals, pharmaceuticals, and radioactive materials are wastes. In other meaning, it has to be mentioned that not all the wastes generated from the hospital were classified as clinical wastes. According to the Controlled Waste Regulations 1992 (S1588), the clinical wastes are defined as

any waste with human or animal tissue, excretions, blood, body fluids, excretions, swabs, syringes, and needles. These wastes were generated from the medical, dental, nursing, veterinary pharmaceutical or similar practice, investigation, treatment, care, teaching, or research centers. The clinical waste is classified as a type of the biohazard waste due to the heavily load of infectious agent which included *Staphylococcus aureus*, pathogenic strains of *E. coli*, *Salmonella* spp. *P. aeruginosa*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, and opportunistic fungi such as *Aspergillus niger*, *A. fumigatus*, *A. tubingensis*, *A. terreus* var. *terreus*, and *C. lunata* (Banana 2013; Noman et al. 2016a).

To date, several treatment technologies have been suggested in order to reduce the health risk associated with the presence of pathogenic fungi in the clinical wastes. Some of these technologies such as incineration, autoclave, irradiation, microwave, and chemical disinfection were effective for inactivation of pathogenic organisms and have achieved 6 log reductions (STAATT 2005; Efaq et al. 2015). However, Efaq et al. (2015, 2017) and Noman et al. (2016a, b) have revealed that the use of supercritical carbon dioxide (SC-CO₂) represents the best green and non-thermal alternative technology for the total inactivation of fungal spores in the clinical wastes. The studies suggested SC-CO₂ as an alternative technology based on the ability to total inactivation of *Aspergillus* spp. in section Nigri, *A. niger*, *A. fumigatus*, *A. tubingensis*, *A. terreus* var. *terreus*, *Aspergillus* sp. strain no. 145, *P. simplicissimum*, and *P. waksmanii* spores in the clinical wastes. The inactivation of the spores was irreversible process where no regrowth was recorded on the culture medium for the inactivated fungi. Nonetheless, the inactivation medium and the water contents are among the factors which influence the efficiency of the inactivation process by using SC-CO₂ (Kamihira et al. 1987; Dillow et al. 1999; Furukawa et al. 2009; Valverde et al. 2010). The selection of inactivation medium depends mainly on the presence or absence of the water and moisture. These studies indicated that the inactivation process was reduced or even impaired in low water content environments (Haas et al. 1989; Dillow et al. 1999). Furukawa et al. (2009) stated that *Geobacillus stearothermophilus* spores have more resistance to inactivation process in the low water content. However, Valverde et al. (2010) claimed that the inactivation of microorganisms by SC-CO₂ in the absence of water is faster because CO₂ was contacted directly with the microorganism cells; thus, the treatment period becomes shortened. It is due to the nature of the matrix and the presence of compounds in the inactivation medium as carbohydrates and fats play important role in protecting microorganisms from high-pressure CO₂ (Ferrentino et al. 2013). It has been reported that the presence of fat in suspending media might lead to reduce inactivation rate, due to decrease CO₂ penetration into cells by changing the structure of cell walls and membranes (Lin et al. 1994).

This study aims to determine the best medium and water content volume for inactivating *Aspergillus fumigatus*,

Aspergillus spp. in section Nigri, *A. niger*, *A. terreus* var. *terreus*, *A. tubingensis*, *Penicillium waksmanii*, *P. simplicissimum*, and *Aspergillus* sp. strain no. 145 spores in clinical wastes SC-CO₂. These fungi were investigated in distilled water as reported by Efaq et al. (2017). However, the inactivation process for these fungal spores in normal saline, seawater, and physiological saline with 1% of methanol has not been studied yet. The solutions were tested at three water volumes (5, 10, and 20 mL). In spite, one more study which need to be conducted is the effect of SC-CO₂ parameters on the mechanism of inactivation process. Overall, the role of pressure, temperature, and time in the destruction of fungal spores was investigated.

Materials and methods

Fungal strains

There were eight common strains of fungal investigated in the present study including *Aspergillus fumigatus*, *Aspergillus* spp. in section Nigri, *A. niger*, *A. terreus* var. *terreus*, *A. tubingensis*, *Penicillium waksmanii*, *P. simplicissimum*, and *Aspergillus* sp. strain no. 145 as reported by Noman et al. (2016a). The preparation of samples was by using methods described by Efaq et al. (2017).

Inactivation medium and process

Three types of solutions have been used in order to determine the most effective inactivation medium including normal saline, seawater, distilled water (5, 10, and 20 mL), and physiological saline supplemented with 1% of methanol. These solutions have different parameters; the normal saline plays an important role in the diffusion of CO₂ through the microbial cell wall, while the presence of salt contributes effectively in the inactivation process. In contrast, the presence of methanol accelerates the inactivation process due to the antimicrobial properties of the methanol. To date, there were no similar solutions that have been reported for being used as inactivation medium for fungal spores by SC-CO₂. Therefore, it was tested to choose the liquid solution required to achieve high inactivation rate of fungal spores in the clinical waste samples.

A fixed amount (10 g) of the sterilized clinical solid waste (Noman et al. 2016a, b) was prepared in four types of the solution medium to represent the typical loading conditions for SC-CO₂ sterilization vessel (for lab scale) as recommended by STAATT 2005 cited by Noman et al. (2016a, b). The samples were prepared in a separate experiment and each sample was transferred into a glass screw cap bottle (100 mL) containing three volume sizes (5, 10, and 20 mL) spore inoculum (10⁶ spores mL⁻¹ or g⁻¹ of waste sample). The sample was homogenized with 150 rpm for 10 min in a shaker. The sample was

transferred into a sterilized Biohazard autoclave bag and then into a SC-CO₂ sterilization vessel. The control samples were kept at room temperature (25 ± 2 °C) for the same period of the treatment process (90 min). The experiments for fungal spores inactivation were conducted at the optimum operating parameters of supercritical carbon dioxide (30 MPa, 75 °C, 90 min) as described by Noman et al. (2016b).

Effect of SC-CO₂ parameters on the inactivation process

In order to understand the role of temperature, time, and pressure in the inactivation process, *A. niger* was used as a model system as suggested by STAATT (2005) while 10 mL distilled water was used as an inactivation medium. The inactivation process was performed at different conditions as shown in Table 1.

The log reduction was determined based on final concentrations of fungal spores recovered from the treated samples on Sabouraud Dextrose Agar (SDA) medium by culture-based method (the colony-forming unit, CFU) using standard serial dilution spread plate method APHA (2005). The inactivation of fungal spores in clinical waste sample analysis using SC-CO₂ was calculated based on log reduction according to Eqs. 1, 2, 3, and 4 as described by STAATT (2005).

$$\text{Log IT} = \text{log IC} - \text{log NR} \quad (1)$$

$$\text{log NR} = \text{log IC} - \text{log IRC} \quad (2)$$

$$\text{Log reduction (Log Kill)} = \text{log IT} - \text{log RT} \quad (3)$$

$$\text{Inactivation percentage (\%)} = \frac{\text{IT} - \text{RT}}{\text{IC}} \times 100 \quad (4)$$

where

IC is initial numbers of fungal spores (10⁶ spore mL⁻¹ of suspension as determined by culture-based method).

NR is the number of fungal spores that were not recovered from a clinical waste sample before treatment (CFU g⁻¹ of clinical waste sample).

IT is initial number of fungal spores (CFU g⁻¹ of waste sample) introduced into clinical waste sample before treatment.

IRC is initial number of fungal spores recovered from a clinical waste sample before treatment by using culture-based method (CFU g⁻¹ of waste sample).

RT is the number of fungal spores (spore or CFU g⁻¹ of waste sample) recovered from a clinical waste sample treated by SC-CO₂.

Mechanism of inactivation process using SC-CO₂

The mechanism of inactivation process for *Aspergillus* spp. in section Nigri and *A. tubingensis* spores using SC-CO₂ was investigated. The spore shape and morphology were

Table 1 The experiments runs conducted to study the effect of SC-CO₂ parameters on inactivation of *A. niger* spores

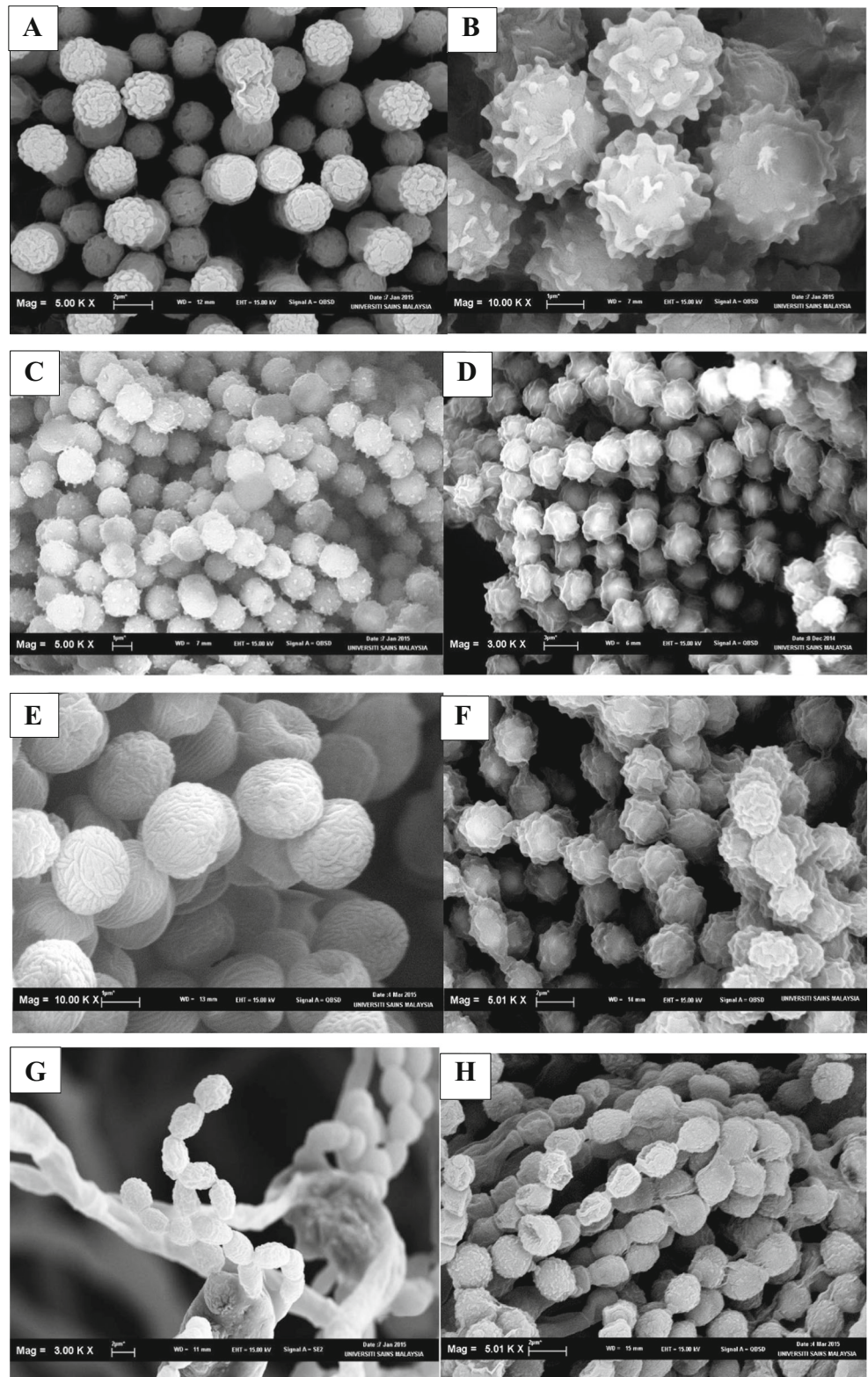
Block	No. treatment process	SC-CO ₂ parameters
A	1	35 MPa, 35 °C, 30 min
	2	40 MPa, 35 °C, 30 min
	3	30 MPa, 55 °C, 60 min
	4	35 MPa, 55 °C, 60 min
	5	40 MPa, 55 °C, 60 min
B	1	35 MPa, 35 °C, 60 min
	2	35 MPa, 55 °C, 60 min
	3	35 MPa, 75 °C, 60 min
C	1	35 MPa, 55 °C, 30 min
	2	35 MPa, 55 °C, 60 min
	3	35 MPa, 55 °C, 90 min

determined by using SEM analysis before and after the inactivation process. The spores of *Aspergillus* spp. in section Nigri and *A. tubingensis* spores were recovered from the inactivation medium using centrifugation at 4020×g for 20 min. The pellets of fungal spores were observed under the SEM according to the method described by Mazia et al. (1975). Briefly, the fixed spores were then mounted into SEM stub coated with gold powder with double-sided carbon tape and viewed using SEM (Zeiss Supra 50 VP, Germany).

Results and discussion

This study was conducted to investigate the effect of inactivation medium and water contents on the inactivation of *Aspergillus* spp. in section Nigri, *A. fumigatus*, *A. niger*, *A. terreus* var. *terreus*, *A. tubingensis*, *Aspergillus* sp. strain no. 145, *P. waksmanii*, and *P. simplicissimum* spores in the samples of clinical. The spores' morphologies for the investigated fungi are depicted in Fig. 1, which showed different shapes, surfaces, and ornamentation. The shape of the fungal spore is one of the most important factors which effect on the efficiency of SC-CO₂ in the inactivation process. Efaq et al. (2017) found that the spherical spore shape for *Aspergillus* spp. is exposed to the same effects resulted from the pressure and temperature. The cytoplasm contents are released, while the spore shrunk but the spore morphology still has the spherical shape. In contrast, the cytoplasm content of elongated shaped spores (such as *Penicillium* spp.) is extracted from the polar point which represents the weakest point in the elongated spores. Thus, different fungal species with different shapes, surfaces, and ornamentation were selected in this present study. On the other hand, the selection of species was determined based on a study by Noman et al. (2016a, b) which had indicated that those species are common in the clinical waste (Noman et al. 2016a, b).

Fig. 1 Scanning electron micrographs of fungal spores: **a** *Aspergillus* spp. in section Nigri ($\times 5000$); **b** *A. niger* ($\times 1000$); **c** *A. fumigatus* ($\times 5000$); **d** *A. tubingensis* ($\times 3000$); **e** *A. terreus* var. *terreus* ($\times 10,000$); **f** *Aspergillus* sp. strain no. 145 ($\times 5000$); **g** *P. simplicissimum* ($\times 3000$); **h** *P. waksmanii* ($\times 5001$)

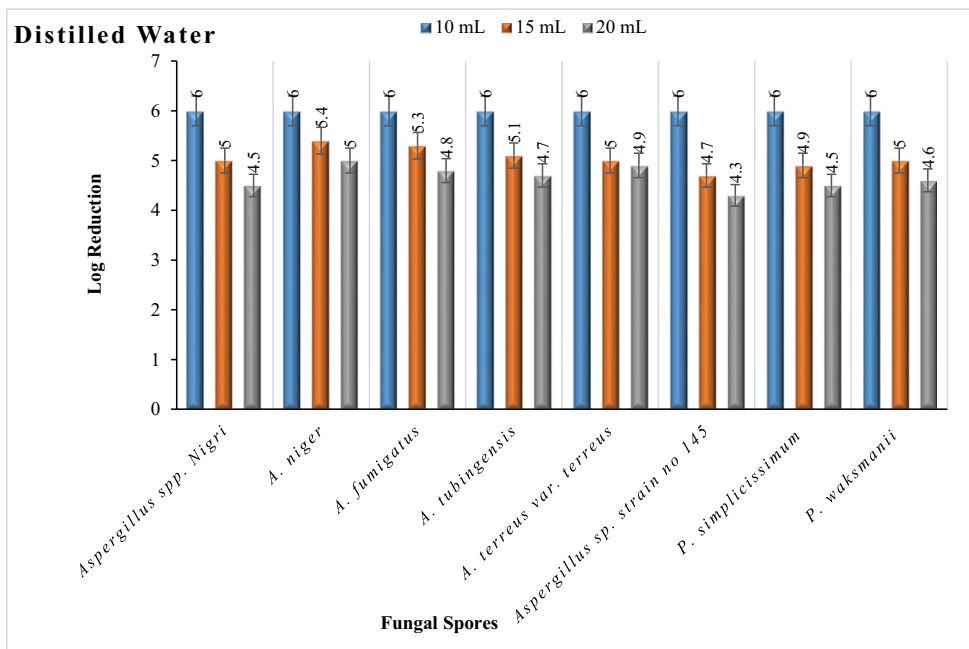


Selection of inactivation medium and water contents

The inactivation rate was more effective in distilled water (6 log reductions), in which the spores of the fungal species

investigated were completely inactivated (Fig. 2). Similar results were also recorded for the physiological saline with 1% of methanol (Fig. 3). In contrast, the maximum inactivation rates in the seawater medium and normal saline were 5.5 log₁₀ for *A. niger*

Fig. 2 Log reduction of fungal spores in distilled water using SC-CO₂. The inactivation process was conducted at 30 MPa, 75 °C, 90 min; the initial concentrations of the fungal spores were 10⁶ spore/mL. The control sample was kept at room temperature (25 ± 2 °C) for the same treatment period



(Fig. 4) and *Aspergillus* sp. strain no. 145 (Fig. 5) respectively. This could be due to the synergistic effect of distilled water which leads to swollen cell walls and membranes, thus making biological barriers more penetrable by CO₂ and positively influencing the sterilization process (Dillow et al. 1999). The presence salts in seawater will lead to shrinking of the fungal spore's cell wall and membrane due to the osmotic pressure and therefore will effect the penetration of CO₂ across cell wall and the inactivation rate. Debs-Louka et al. (1999) and

Garcia-Gonzalez et al. (2007) indicated that the presence of salts and sugars leads to reduce the water activity (*a_w*) of the medium and decreases the inactivation process of microorganisms. It has been mentioned in the literature that the constituents of the inactivation medium might effect on the efficiency of SC-CO₂ in the inactivation of fungal spores. For instance, the presence of carbohydrates, proteins, and fats might contribute in the protection of spores from the effect of high-pressure CO₂ (Ferrentino et al. 2013). Lin et al. (1994) has claimed that the presence of

Fig. 3 Log reduction of fungal spores in physiological saline with 1% of methanol using SC-CO₂. The inactivation process was conducted at 30 MPa, 75 °C, 90 min. The initial concentrations of the fungal spores were 10⁶ spore/mL. The control sample was kept at room temperature (25 ± 2 °C) for the same treatment period

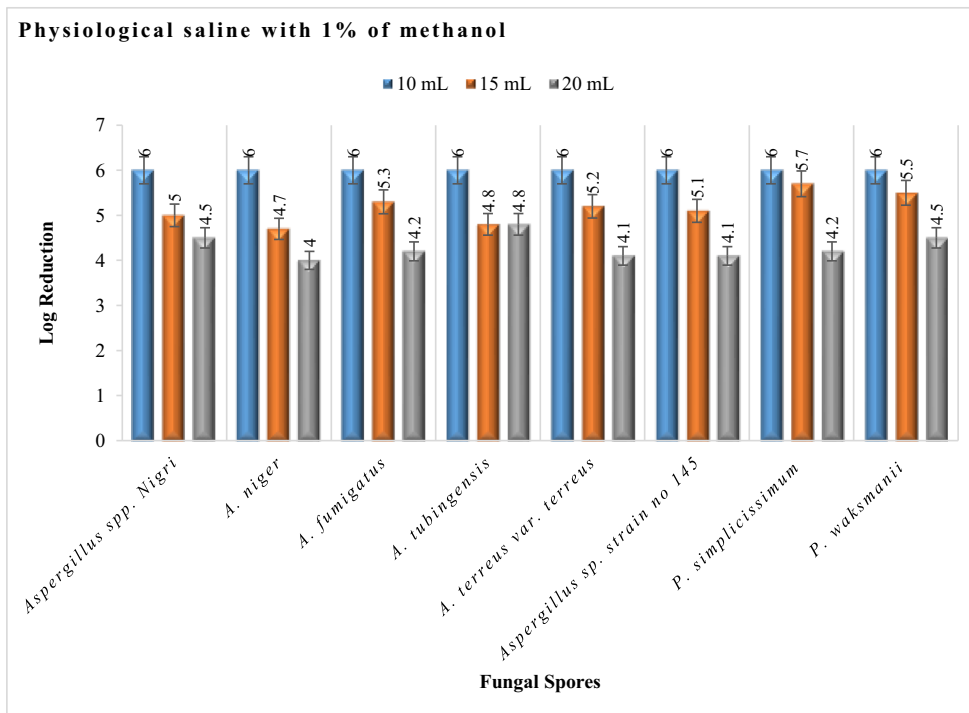
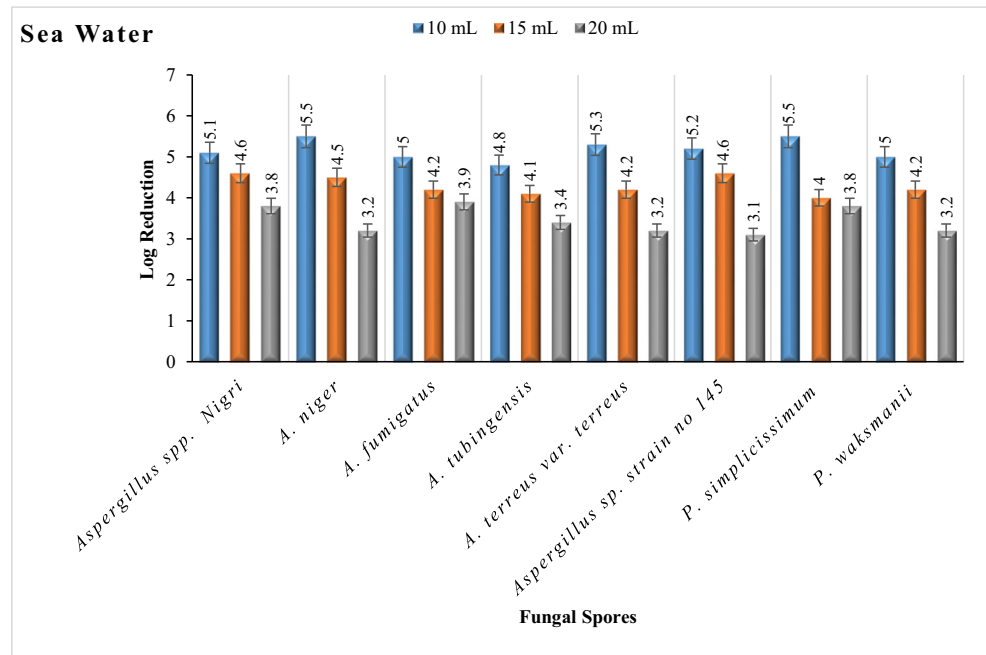


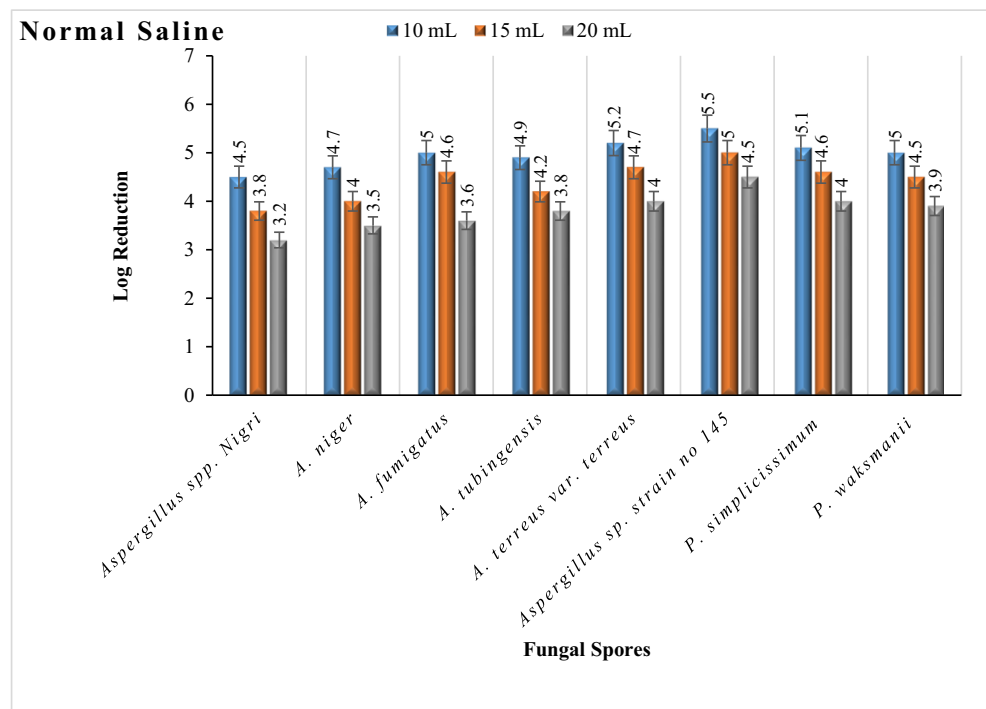
Fig. 4 Log reduction of fungal spores in seawater using SC-CO₂. The inactivation process was conducted at 30 MPa, 75 °C, 90 min. The initial concentrations of the fungal spores were 10⁶ spore/mL. The control sample was kept at room temperature (25 ± 2 °C) for the same treatment period



lipids reduces the penetration of CO₂ through the cell membrane into the cell cytoplasm and thus reduces the lethal effects of CO₂ which is the main mechanism for inactivation process. Moreover, the efficiency of inactivation medium by SC-CO₂ might increase by adding the polar co-solvent such as ethanol or acetic acid (Christensen and Kaufmann 1965). Kamihira et al. (1987) found that the log reduction of *A. niger* spores was 6 log by SC-CO₂ with the presence of ethanol while the maximum reduction was non-detectable in the free medium. Park et al.

(2012) have revealed that the adding of the ethanol with 16% into the inactivation medium enhanced completely the inactivation of *A. brassicicola* spores after 45 min at 15 MPa and 38 °C compared to 2 log reduction for control. *P. oxalicum* spores were completely inactivated at 10 MPa and 40 °C for 45 min with the presence of ethanol while similar inactivation was observed at 20 MPa, 45 °C for 25 min without ethanol (Park et al. 2013). In the present study, the results revealed that adding methanol into the inactivation medium has enhanced the log reduction to

Fig. 5 Log reduction of fungal spores in normal saline using SC-CO₂. The inactivation process was conducted at 30 MPa, 75 °C, 90 min. The initial concentrations of the fungal spores were 10⁶ spore/mL. The control sample was kept at room temperature (25 ± 2 °C) for the same treatment period



achieve 6 log for all fungal spores investigated. The efficiency of SC-CO₂ for inactivating of fungal spores in the presence of acids leads to increase the acidity of the inactivation medium and lowering the pH. The antimicrobial agent of methanol contributes effectively in the inactivation of fungal spores. However, STAATT (2005) has recommended that no chemicals or solvent should be added to clinical waste sample during the treatment by the alternative technologies which might be used as alternative for incineration.

Effect of water contents on the inactivation process

In order to determine the suitable moisture content, different volumes of DW were tested (5, 10, and 20 mL). The results indicated that the fungal spores were completely inactivated with 10 mL. The inactivation rate of fungal spores decreased from 6 to 4.5 log as the amount of water content increased from 5 to 20 mL of distilled water (Fig. 2). This might be due to the presence of low amount of water content; more CO₂ will be free and could penetrate the cell wall and thus increase the inactivation rate. These findings are inconsistent with those of previous studies which revealed that the inactivation process depends on the water content in the inactivation medium (Dillow et al. 1999). The studies conducted on *Geobacillus stearothermophilus* and *A. niger* spores revealed that both organisms exhibited a resistance to the inactivation process using SC-CO₂ while it was reduced by 6 log reduction with 90 and 2% of water content (Kamihira et al. 1987; Furukawa et al. 2009). However, Valverde et al. (2010) have claimed that the inactivation process of the spores SC-CO₂ was more efficient and fast in the absence of water. The same study had explained the findings based on the direct contact between CO₂ and the sample without the need for saturating the medium with CO₂. The inactivation process depends on the physical action of pressure in the destruction of cell wall of the spores and the chemical action for the acidic condition resulted from the reaction between CO₂ and water to generate H₂CO₃. The results have shown that the presence of a detectable amount of the water contents might enhance the inactivation process, while the mechanism action becomes less effective in the high amounts of the water contents. This explanation is consistent with that of the studies by Werner and Hotchkiss (2006) and Garcia-Gonzalez et al. (2007) who reported that the presence of moisture induces the inactivation rate as a result of external pH reduction, membrane cell swelling, permeability of cell membrane, and fast diffusion of CO₂ into the cell cytoplasm which cause intracellular disorders (Garcia-Gonzalez et al. 2007). The effect of media constituents on the sterilization process and inactivation of microbial cells is not limited to have an important role with SC-CO₂; it has also reported similar finding by steam autoclave with a different inactivation mechanism in comparison to SC-CO₂. Therefore, the examination of different inactivation medium is

necessary to determine the best solution should be used for achieving the highest inactivation rate of the fungal spores.

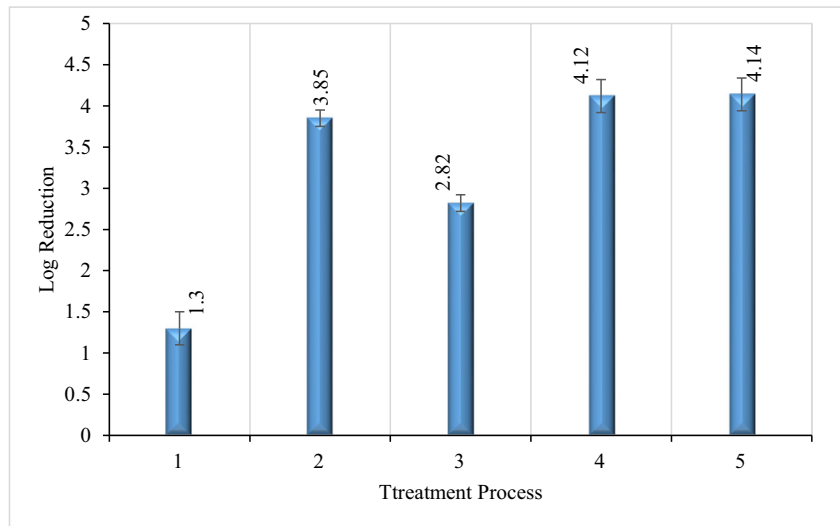
Effect of SC-CO₂ parameters on the inactivation of fungal spores

The findings on the effect of pressure, temperature, and time on inactivation of *A. niger* spores are depicted in Figs. 6, 7, and 8. It can be found that by using two treatments setup conducted at 75 °C for 90 min and two different pressures (30 and 40 MPa respectively), complete inactivation of *A. niger* spores was achieved (Fig. 6). Based on these results, the increasing of the pressure from 30 to 40 MPa has shown no contribution on the *A. niger* spores, since no more inactivation was achieved even after the pressure increased from 30 to 40 MPa. Similar findings were noted for the treatment performed at 55 °C for 60 min, where the increasing of pressure from 35 to 40 MPa has not correlated with the inactivation of fungal spores; the reductions were 4.12 and 4.14 log respectively. However, in the treatment process carried out at 55 °C for 60 min, the increase in pressure from 30 to 35 MPa associated with the reduction of *A. niger* spores from 2.82 to 4.12 log. The treatment processes conducted at 35 °C and 30 min had revealed that the increasing pressure from 35 to 40 MPa is associated with increasing the reduction of spores from 1.3 to 3.85 log (Fig. 6).

At 35 MPa, the increasing of the temperature from 35 to 55 °C correlated with more spores' reduction from 1.95 to 4.6 log. However, the over increasing of the temperature to 75 °C reduced the log reduction of the spores to 3.99 log (Fig. 7). These observations indicated that the pressure has more contribution at low temperature, while temperature has low contribution at high pressure. The finding recorded were in line with those of a previous study by Ballestra and Cuq (1998) who found that the increasing of temperature from 50 to 60 °C at 5 MPa correlated with low inactivation of *A. niger* spores. More explanations for the effect of pressure and temperature were reported by Calvo and Torres (2010), who claimed that the inactivation of bacterial cells takes place in two stages in a response to temperature. In the study, the increasing of temperature from 50 to 75 °C reduced the microbial lethality of CO₂ at 30 MPa (only 1.5 log reduction was achieved), while the log reduction has increased as the temperature was 80 °C (4.5 log reduction achieved).

On the other hand, the treatment processes conducted at a constant pressure (35 MPa) and temperature (55 °C) with two treatment periods (30 and 60 min) noted that the time has a real role in the inactivation of *A. niger* spores. The reductions recorded were 3.73 versus 4.6 log respectively. The over increasing of the time from 60 to 90 min correlated with high log reduction of the spores (4.6 vs. 5 log) (Fig. 8). In a previous study conducted by Haas et al. (1989), *P. roquefort* had reduced by 5 log at 5.4 MPa and 45 °C for 120 min while was totally reduced after 240 min with same temperature and pressure.

Fig. 6 Inactivation of *A. niger* spores in the clinical waste samples at different treatment conditions of SC-CO₂: (1) treatment process conducted at 35 MPa, 35 °C, 30 min; (2) treatment process conducted at 40 MPa, 35 °C, 30 min; (3) treatment process conducted at 30 MPa, 55 °C, 60 min; (4) treatment process conducted at 35 MPa, 55 °C, 60 min; (5) treatment process conducted at 40 MPa, 55 °C, 60 min



There are many of explanations which have been suggested by the authors in the literature regarding the effect of SC-CO₂ parameters (pressure, time, and temperature) on the inactivation of bacterial and fungal cells and spores. Park et al. (2012) and Park and Kim (2013) indicated that both pressure and temperature were effective in the reduction of *A. brassicicola* and *P. oxacilum*. However, Hossain (2013) claimed that pressure was the main factor for inactivation of *S. aureus*, *E. faecalis*, and *B. sphaericus* in the clinical solid wastes using SC-CO₂. It has to mention that the studies conducted by Park et al. (2012) and Park and Kim (2013) as well as by Hossain (2013) were performed on the vegetative cells of fungi and bacteria, while in the current work, the inactivation was carried out on the *A. niger* spores. The differences between the cell wall of the fungal cells and the spores contribute in the role of temperature, pressure, and time in the inactivation process. Bae et al. (2009) have claimed that the temperature has more influence on bacterial spores than

pressure. Similar findings were also reported in the current work; this might be related to the role of pressure which enhances the solubilization rate of CO₂ in water and thus facilitates the acidification and membrane expansion, while the role of temperature is to stimulate CO₂ diffusivity and increase the fluidity of cell membranes to make CO₂ penetration easier (Spilimbergo 2002).

Mechanism of inactivation process for the spores by SC-CO₂

The mechanism of inactivation process for the spores by SC-CO₂ was studied in two fungal species including *Aspergillus* in section Nigri (Fig. 9a, b) and *A. tubingensis* (Fig. 9c, d). The mechanism is explained based on the analysis of the spore morphology obtained from SEM images. The role of temperature in the inactivation of fungal spores in comparison to the role of pressure is the thermal revival of dormant spores, which does mean that the

Fig. 7 Inactivation of *A. niger* spores in the clinical waste samples at different treatment conditions of SC-CO₂: (1) treatment process conducted at 35 MPa, 35 °C, 60 min; (2) treatment process conducted at 35 MPa, 55 °C, 60 min; (3) treatment process conducted at 35 MPa, 75 °C, 60 min

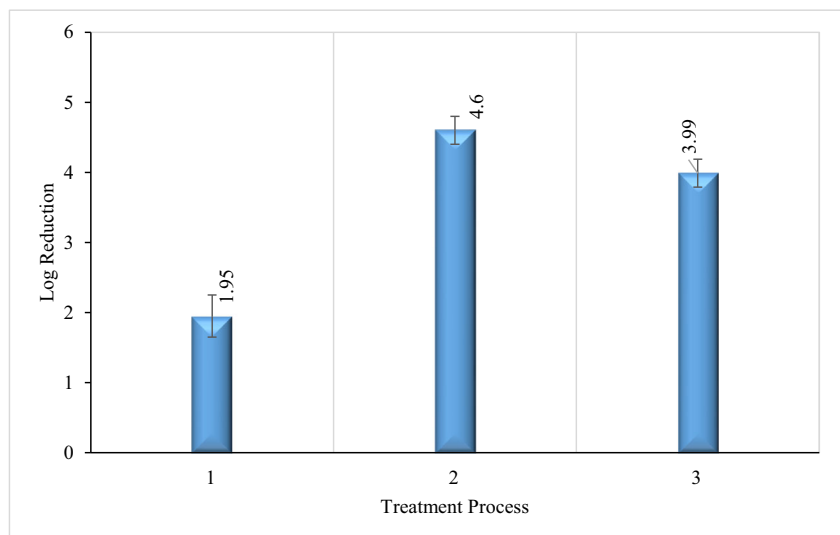
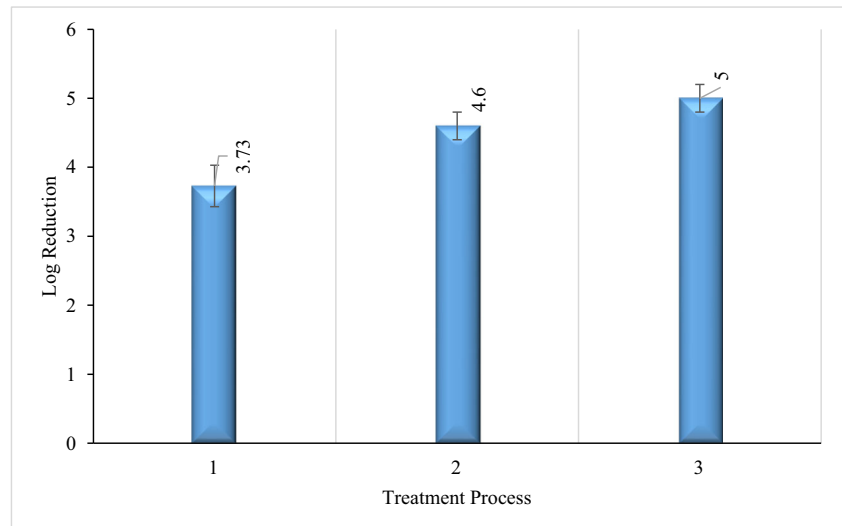


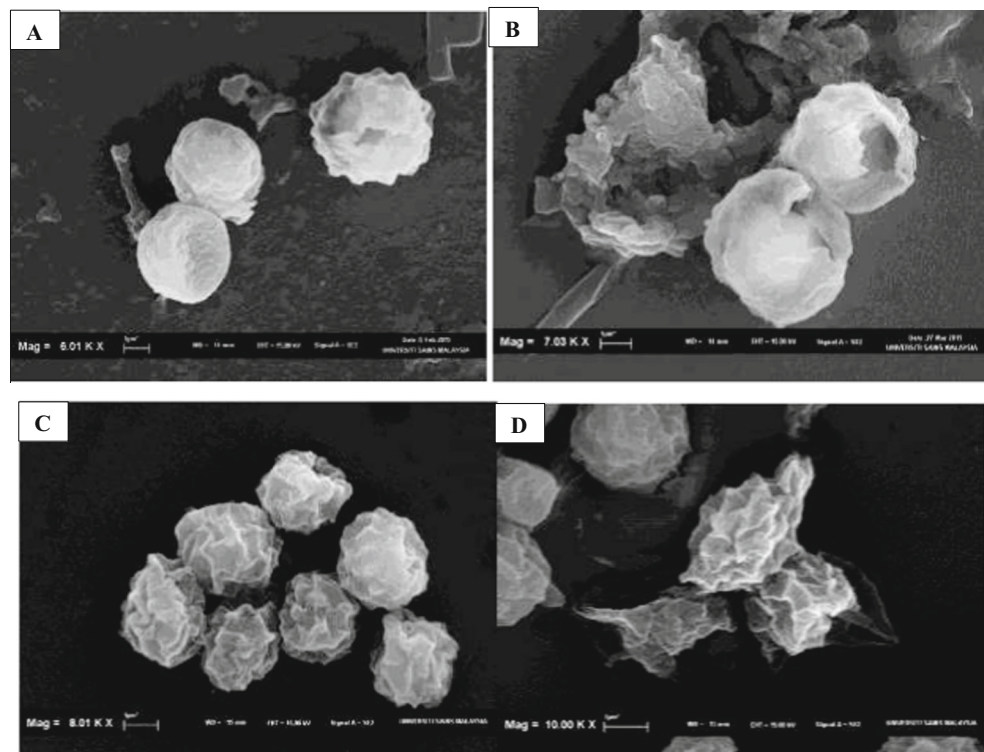
Fig. 8 Inactivation of *A. niger* spores in the clinical waste samples at different treatment conditions of SC-CO₂: (1) treatment process conducted at 35 MPa, 55 °C, 30 min; (2) treatment process conducted at 35 MPa, 55 °C, 60 min; (3) treatment process conducted at 35 MPa, 55 °C, 90 min



spore germination needs a heat shock to reactivate the germination enzymes (Keynan et al. 1964; Calvo and Torres 2010). This study showed that the treatment process conducted at 75 °C for 90 min was enough for reactivation of fungal spores to germinate. These findings indicate that the time is an associated factor with the temperature and pressure. Therefore, it explained that the role of each factor in the inactivation of fungal spores comprises the temperature, which is an inducible factor to revive the fungal spores, while the first stage of treatment period (45 min) is the time required for spore germination. The continuation of treatment

period for 90 min with high temperature leads to destruction of cell wall of the fungal spores, which facilitates the penetration of CO₂ more effectively due to the presence of high pressure. The accumulation of CO₂ in the spore leads to complete damage of the structure and this is a factor involved in breaking dormancy of fungal spores (Keynan et al. 1964; Chang et al. 1999; Kamil et al. 2011). The pressure and presence of metabolizable nutrient germination such as sugars have the ability to induce the fungal spore revival, but the revival process requires a very high pressure between 50 and 300 MPa combined with some heat, while the

Fig. 9 Scanning electron micrographs of inactivated spores: **a** *Aspergillus* spp. in section Nigri spores before treatment (control, × 7000); **b** *Aspergillus* spp. in section Nigri spores after treated using SC-CO₂ (35 MPa, 75 °C, 90 min) (× 7000); **c** *A. tubingensis* spores before treatment (control, × 800); **d** *A. tubingensis* spores after treated using SC-CO₂ (35 MPa, 75 °C, 90 min) (× 1000)



range of pressure used in this was between 30 and 40 MPa (Setlow 2003; Norton and Sun 2008). It has been demonstrated that the inactivation of fungal spores by SC-CO₂ is due to the change of thermodynamic state of the CO₂ in the response for temperature. It is because the gas like fluid of CO₂ in a supercritical state is due to the presence of temperature (Watanabe et al. 2003; Fomin et al. 2015). The completely inactivation of *A. niger* and *A. oryzae* at 140 MPa and –20 °C for 24 h has been reported by Hayakawa et al. (1998), which indicates that the hydrostatic action of pressure can effectively inactivate the microorganisms by high pressure. Therefore, the presence of temperature plays an important role to enhance the diffusion of CO₂ through the cell wall and cell membrane of the microorganism at moderate pressure as used in this study. SC-CO₂ enhances mass-transfer properties and thus removes vital constituents from the cell membrane of spores (Garcia-Gonzalez et al. 2007). Based on aforementioned, it can be indicated that the mechanism action of the SC-CO₂ in inactivating of fungal spores depends on the temperature used during SC-CO₂ treatment which acts to revive fungal spores and thus accelerate the CO₂ sporicidal effect. The adverse effect of the temperature becomes more influencing at low pH and long period. All of the SC-CO₂ parameters (pressure, temperature, and time) acts simultaneously to destruct the fungal spores (McDonnell 2007; Calvo and Torres 2010).

Conclusion

Among different inactivation media which might be used for enhancing the inactivation process of fungal spores, the distilled water and physiological saline with 1% methanol are the most efficient. Despite showing the effectiveness in inactivation process, the lowest and highest volume of water contents had reduced the effectiveness of inactivation process using SC-CO₂. The effect of temperature on the fungal spores is influenced by the function of protecting and regulating the internal environment of the membrane cell and changes it to become compromised, while the high pressure leads to spore bursting and released of the cytoplasm content. Increasing the treatment period facilitates the effectiveness of both pressure and temperature.

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