



Waterless sterilization of clinical solid waste using supercritical carbon dioxide: fungal spores inactivation mechanisms, optimization and artificial neural network models

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Abstract

Clinical solid waste contains pathogenic microorganisms and therefore requires effective sterilization prior to safe handling and disposal. This work deals with the inactivation of *Aspergillus niger*, *Aspergillus terreus var. terreus* and *Penicillium simplicissimum* spores in clinical solid waste using supercritical carbon dioxide (SC-CO₂) as a waterless sterilization technology. The artificial neural network (ANN) approach was used to study the behavior of the fungal spores for the pressure, temperature, time and initial fungal spore concentrations. The optimal operating parameters were 35 MPa, 35 °C, 100-min treatment time with 6 log₁₀ spores g⁻¹: the predicted and experimental results were 5.48 and 5.96 logs of *A. niger*, 5.56 and 5.96 logs of *A. terreus var. terreus*, 5.84 and 5.99 log reduction of *P. simplicissimum*. ANN analysis revealed that temperature, time and initial concentrations showed a greater influence on the inactivation of the fungal spore's inactivation. Inactivation results from destructing the cell wall, as evidenced by the lack of growth of fungi in the culture medium, indicating complete inactivation of the fungal spores. The findings of the present study would be useful in implementing the sustainable utilization of clinical solid waste materials.

Keywords Supercritical CO₂ · Waterless inactivation · *Aspergillus* · *Penicillium* · Clinical solid waste · Artificial neural network

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1 Introduction

Clinical or medical waste refers to the hospital or health-care facility waste. The main characteristic of these wastes is the presence of blood or fluids of the human body fluids [31]. Clinical wastes have the nutrients required for microbial growth. Noman et al. [18] performed a screening for fungal species in clinical solid waste. Among 92 samples, fungal strains were detected in 83.75%. The study identified 36 fungal species belonging to *Aspergillus terreus var. terreus*, *Aspergillus spp. section Nigri*, *Aspergillus fumigatus*, *Aspergillus niger*, *Penicillium waksmanii*, *Aspergillus tubingenensis*, *Penicillium simplicissimum* and *Curvularia lunata*. *Rhizopus spp.* detected in paper tissue and air of the clinical waste storage room. The main issue with the presence of fungi in clinical waste lies in their ability to multiply without the need for an intermediate host, and their spores are released to the air as airborne spores. In inactivation or disinfection of clinical solid waste, the spores of fungal strains represent a real hazard compared to bacteria, since

the fungi require less water activity (a_w) between 0.60 and 0.85, compared to bacteria, which require between 0.89 and 0.97 for growth [14, 27].

In previous research, we investigated the inactivation of fungus spores in solid clinical waste using supercritical carbon dioxide (SC-CO₂) as a non-thermal treatment technology. The inactivation process was optimized according to pressure, temperature, time, and inactivation mediums (normal salts, distilled water, marine and physiological salts contain 1% methanol) [7, 18, 19]. Studies have revealed an efficiency in reducing fungal spores. The main factors influencing process efficiency are pressure and temperature. The main limitation of the process consisted of the medium, water, which has a dual role: enhancing the inactivation process but also improving the regrowth of the inactivated fungal spore's regrowth if the inactivation is sub-lethal. The disposal of clinical solid waste with high moisture might effectively contribute effectively to fungal regrowth. Therefore, treated clinical solid waste should be subjected to water removal before final disposal into the environment. Therefore, an alternative method to efficiently achieve fungal inactivation might be to perform the SC-CO₂ treatment process in a waterless medium.

SC-CO₂ as a nonthermal sterilization technology was introduced several years ago as an alternative to thermal processes [10, 11] both in water and in waterless media [21]. The application of SC-CO₂ sterilization of clinical solid waste is still limited at the laboratory scale. STAATT [26] reported that alternative technologies for clinical solid waste treatment should have the ability to achieve 6 log reductions in fungal spores, meanwhile, they should be conducted without chemical additions. Furthermore, optimizing the inactivation process is crucial to identify the best process conditions and the maximum inactivation rate [11], so far, most of the optimization process has been performed using response surface methodology (RSM) in central composite design (CCD). However, an artificial neural network (ANN) may represent an alternative or additional tool to achieve a more precise optimization in terms of process variables, as it provides more information on the behavior of the spores during the inactivation process on large scales [1, 2]. Indeed, the current study aimed to use ANN since this model provide more information on the responses and sensitivity of fungal spores in the clinical wastes for the environmental factors during the inactivation process better than the RSM models which have been used to investigate the fungal response for a narrow range of each environmental factor. In this regard, the present study aimed to determine the feasibility of the SC-CO₂ inactivation process in *Aspergillus niger*, *A. terreus var. terreus* and *Penicillium simplicissimum* spores in waterless medium. The behavior of these spores during the inactivation process was investigated using ANN analysis, while the confirmation of total fungal spores was determined using

culture-based methods and microstructure of the morphology of the fungal spore morphology. ANN analysis for inactivation of the inactivation of the fungal spore by SC-CO₂ has not been investigated in previous studies in the literature that emphasize the novelty of the current work.

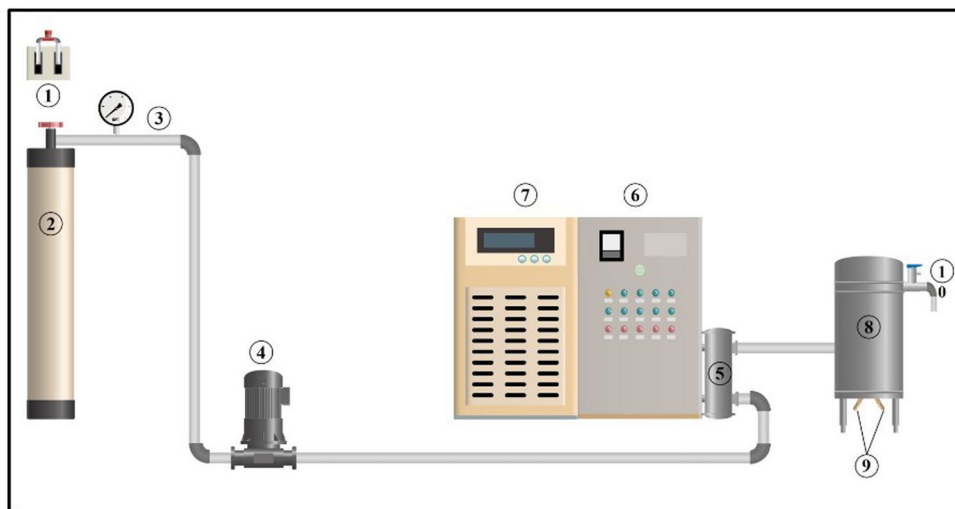
2 Materials and methods

2.1 Fungal strains, inoculation and preparation of samples

A. niger, *A. terreus var. terreus* and *P. simplicissimum* were obtained as pure culture from a previous study [18]. *A. niger* was used as a test microorganism as recommended by STAATT [26], while *A. terreus var. terreus*, and *P. simplicissimum* were used to validate the inactivation model. The fungal spores were subcultured in PDA media and incubated at 28°C for 4 days. Fungal spores were harvested by washing the surface of the culture plates with 10 mL of sterilized normal saline as described by Eissa et al. (2017). The spore inoculum was prepared in 10⁹ spore mL⁻¹ as determined using a hemocytometer. To prepare the clinical solid waste sample, three types of solid waste were selected including gloves, gauze and yellow tips. These wastes were chosen since they are made up of fabric and plastic that play an important role as a reservoir or vector for fungi in humans [8, 17]. A fixed weight (10 g) of the selected clinical solid waste samples was cut into small pieces (1 × 1 cm), sterilized by autoclave for safe handling and mixed with 100 mL of suspension of fungal spore's suspension (to obtain 10⁶ spores g⁻¹ of waste sample). To obtain waterless medium, the water was removed by filtration through gauze, and additionally the clinical solid waste sample was subjected to a freeze-dry system for 24 h to remove the water contents. The initial concentrations of fungal spores in the sample were checked using the stand method plating technique [3]. The sample was then placed in a sterilized biohazard autoclave bag with holes to allow CO₂ to penetrate and contact the fungal spores loaded on the clinical waste sample and then placed in the high-pressure SC-CO₂ sterilization reactor [7] before starting the experimental runs.

The SC-CO₂ apparatus used in the present study consisted of CO₂ cylinder gas (95% purity), sterilization reactor system (SRS), connecting valve (V01), chiller, cooler, CO₂ pump, decompression valve (V02 and V03), sterilization cell and safety valve (Fig. 1). Before adding clinical solid waste samples, the sterilization cells were disinfected with ethanol (70%) and closed when the SC-CO₂ reactors were turned on. The V₀₂ and V₀₃ were closed after the chiller system temperature reached 4 °C, while the CO₂ gas and the connecting valve (V01) were opened to allow CO₂ to be pumped into the sterilization cell. When the CO₂ pump is turned off, it is switched off, and the V₀₁ is still on during the inactivity

Fig. 1 Diagram of SC-CO₂ used in the current study; (1) power energy; (2) CO₂ cylinder gas; (3) connecting valve (V01); (4) sterilization reactor system; (5) chiller; (6) cooler; (7) CO₂ pump; (8) sterilization cell; (9) decompression valve (V02 and V03); (10) safety valve



period. At the end of each inactivation process, V₀₁ was closed, while V₀₂ and V₀₃ were slowly opened. The clinical solid waste sample was collected aseptically from the sterilization cell and subjected to microbial analysis.

2.2 Optimization of SC-CO₂ parameters

The central composite design (CCD) using Design Expert 6.0.11 software (Stat-Ease, Inc., Minneapolis, USA) was used to design the optimization process. CCD was selected to create a significantly better model as recommended by Mohamed et al. [16]. Four independent variables, pressure x_1 (20–35 MPa), temperature x_2 (35–90 °C), time x_3 (10–100 min) and initial fungal spore concentrations x_4 (3–6 log₁₀ spores g⁻¹) were considered with a total of 29 experimental runs. The maximum and minimum values for each independent factor are presented in S1. The inactivation procedure was the same as described in previous work [19]. Each data obtained is the mean value of at least three independent experimental runs.

2.3 Artificial neural network (ANN) model

The behavior of fungal spores for the independent factors was evaluated using the ANN model using JMP (statistical software). The developed model consisted of input layer that had four neurons (parameters) and represented by pressure x_1 (20–35 MPa), temperature x_2 (35–90 °C), time x_3 (10–100 min) and initial fungal spore concentrations x_4 (3–6 log₁₀ spores g⁻¹) (Fig. 2). The hidden layer (H) is the most efficient model of nine neurons. The output layer (O) consists of three neurons, which reflect the predicted reduction in the logs of fungi spores. The total of the experiments were 29 runs performed to build up the proposed ANN model according to Eq. (1).

$$f(x) = \frac{e^{2x} - 1}{e^{2x} + 1} \tag{1}$$

A total of 19 experimental runs, which represent 65.5% of the total runs, were used in the training process, while 10 runs (34.48%) were used in the testing phase (Table 1). Normalized data was used to reflect the experimental data (Eq. 2).

$$X_{\text{norm}} = 0.8 \times \left(\frac{x_i - x_{\text{min}}}{x_{\text{max}} - x_{\text{min}}} \right) - 0.1 \tag{2}$$

where, x_i represents the input or output value, x_{min} and x_{max} represent lowest and highest values. The efficiency of the inactivation process was determined based on the coefficient of correlation (R^2) that provided the experimental data fitting degree between the collected experimental data (y_{actual}) and the network output (y_{model}) (Eq. 3), while the standard error of mean (SD) was used to evaluate the goodness of the proposed model.

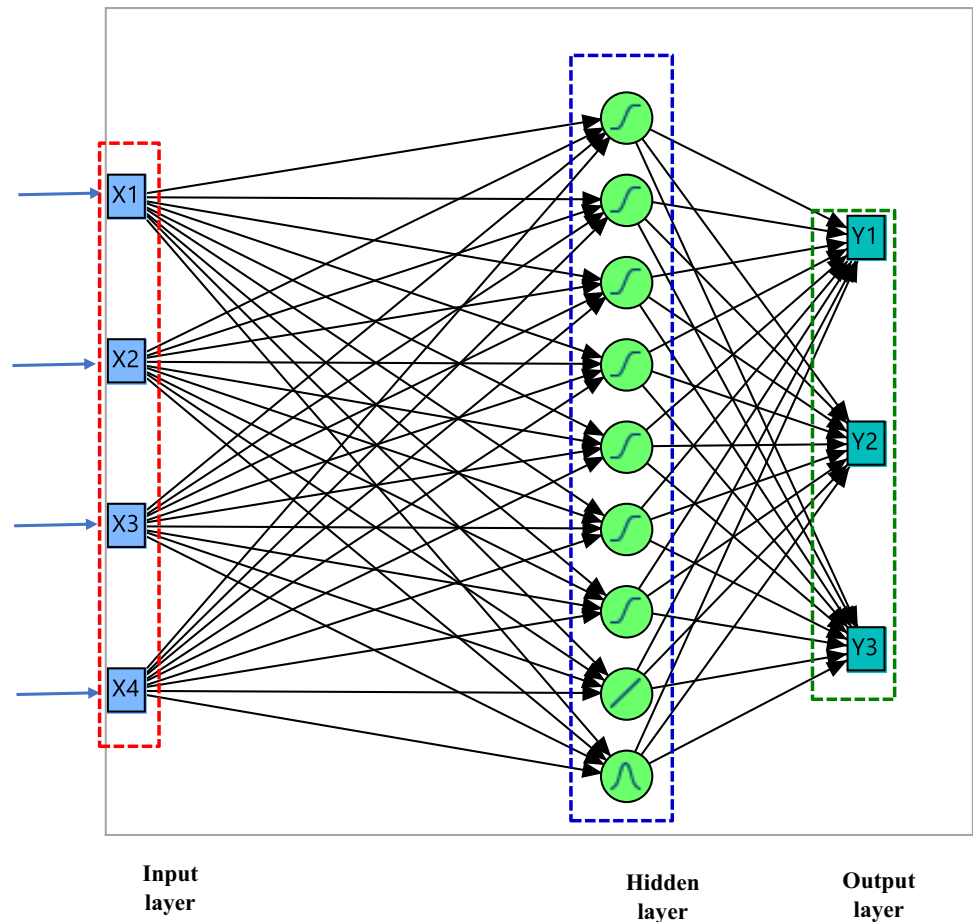
$$R^2 = \frac{\sum_{i=1}^n (y_{\text{actual } i} - y_{\text{model mean}})^2 - \sum_{i=1}^n (y_{\text{model}} - y_{\text{actual } i})}{\sum_{i=1}^n (y_{\text{actual } i} - y_{\text{model mean}})} \tag{3}$$

2.4 Evaluation of inactivation and regrowth of the fungal spore

The culture-based method (PDA agar medium) with standard plating technology was used to count fungal spores in raw and treated solid clinical waste samples. The reduction of logs of fungal spores is calculated using Eqs. (4–6) [26].

$$\text{LogIT} = \text{logIClogNR} \tag{4}$$

Fig. 2 Artificial neural network (ANN) model of fungal spores in the clinical solid wastes using SC-CO₂



$$\text{LogNR} = \log\text{IC} - \log\text{IRC} \quad (5)$$

$$\text{Log reduction}(\log \text{kill}) = \log\text{IT} - \log\text{RT} \quad (6)$$

where, IC is the concentrations of the fungal spores in the raw sample (10^{-3} – 10^6 spore g^{-1}); NR represents the fungal spores that are not recovered from the raw sample by the culture methods before treatment (CFU g^{-1}); IT represents the fungal spores (CFU g^{-1}) in the suspension; IRC represents the fungal spores in the sample before treatment (CFU g^{-1}); RT represents the fungal spores (CFU g^{-1}) recovered after the inactivation process. To test the inactivated fungal regrowth, a piece of the clinical solid waste sample (1 cm) was placed on a PDA surface of PDA and incubated at 28 °C for 10 days; the diameter of the colony diameter was measured daily.

2.5 Microstructure of the fungal spores

To explain the physical effects of SC-CO₂ on spores, spores from each strain of fungus were analyzed by SEM before and after inactivation. Fungal spores (control) before the inactivation process were prepared by

subculturing each fungal strain on PDA medium. After 4 days of culture, the surface of the media was washed with a fixed quantity of (10 mL) distilled water. The collected suspension was centrifuged for 10 min at 2000× g. The fungal spore pellets dried by flash freeze with liquid nitrogen and fixed for SEM analysis using ethanol (95%) and hexa-methyl-di-silazane (HMDS) as described by Mazia et al. [15]. The fixed spores were coated with gold powder and then analyzed by SEM (Zeiss Supra 50 VP, Germany). Inactivated fungal spores on the surface of the treated clinical solid waste were recovered by washing the treated sample with 10 mL of sterilized normal saline and subjected to centrifugation for the separation and then fixed as described by Mazia et al. [15] for SEM analysis.

3 Results and discussion

3.1 Optimization of the inactivation of the spores of fungi

Fungal inactivation optimization was performed both to achieve the target fungal reduction of 6 logarithmic

Table 1 Model validation using statistics indicators

	Training	Validation
<i>Aspergillus niger</i> (y_1)		
Rsquare	0.978076	0.9417401
RASE	0.1745305	0.34034
Mean abs dev	0.1460731	0.2773015
–Loglikelihood	– 6.207629	3.4112835
SSE	0.5787569	1.1583131
Sum freq	19	10
<i>A. terreus var. terreus</i> (y_2)		
Rsquare	0.9894356	0.9769625
RASE	0.1119998	0.2227461
Mean abs dev	0.0880015	0.1997011
–Loglikelihood	– 14.63607	– 0.82784
SSE	0.2383353	0.4961584
Sum freq	19	10
<i>Penicillium simplicissimum</i> (y_3)		
Rsquare	0.9925288	0.990583
RASE	0.0996285	0.1495332
Mean abs dev	0.0836216	0.1231088
–Loglikelihood	– 16.86	– 4.812984
SSE	0.1885909	0.2236017
Sum freq	19	10

reduction and to explain the interaction between independent factors and their effect on inactivation efficiency. The results revealed that the maximum reduction (5.47 log reduction) of *A. niger*, 5.66 log reduction of *A. terreus var. terreus* and 5.88 log reduction of *P. simplicissimum* spores were recorded after a process of 100-min process at 35 MPa, 35 °C with R^2 of 0.889. The main factors that influence process efficiency are pressure and time. It is worth comparing the present results with those of a previous study in which the inactivation process was conducted in a water medium: *A. tubingensis*, *A. niger*, *A. fumigatus*, *Aspergillus* sp. in the Nigri section, strain No 145 of *Aspergillus* sp. and *Aspergillus hortai*, as well as *P. simplicissimum*, were reduced by 6 log by SC-CO₂ at 35 MPa, 75 °C and 90 min [7, 19]. The main difference consists of the high pressure and lower temperature required in the waterless medium. Noman et al. [20] mentioned that higher pressure was required to achieve a 6 log reduction of the fungal spores in the water medium.

Table S2 presents the linear and quadratic regression coefficients for SC-CO₂-used fungus spore deactivation. It was noted that the pressure (x_1) exhibited a significant ($P < 0.05$) role ($P < 0.05$) in the reduction of *A. niger* and *A. terreus var. terreus* spores. In contrast, there is clearly a significant ($P < 0.001$) influence of the temperature (x_2) on the fungal inactivation. Furthermore, in fact, the treatment

time and initial concentrations caused a significant ($P < 0.05$) increase ($P < 0.05$) in the reduction of the fungal spores. Pressure and temperature showed a significant negative ($P < 0.05$) interaction in *A. niger*, *A. terreus var. terreus* spore reduction, which means that at high temperature a low pressure is required to achieve the maximum spore reduction, and vice versa. Pressure and time showed a synergistic effect on the inactivation of *A. terreus var. terreus* spores, while temperature and time have a synergistic effect on the inactivation of *P. simplicissimum* spores. Analysis of variance (ANOVA) highlighted that the investigated factors contributed significantly, by 87.76%, 95.66% and 98.17% in the inactivation of *A. niger* spores, *A. terreus var. terreus* spores and *P. simplicissimum* spores respectively (Table S3).

The linear and quadratic models for inactivating fungal spores in clinical solid waste by SC-CO₂ are given by Eqs. (7–9).

$$y_1 = - 2.43 + 0.08x_1 + 0.03x_2 - 0.11x_3 + 1.13x_4 - 0.001x_1x_2 + 0.0002x_1x_3 + 0.003x_1x_4 + 0.0001x_2x_3 + 0.0002x_2x_4 + 0.001x_3x_4 - 0.001x_1^2 - 0.0001x_2^2 + 0.0001x_3^2 - 0.04x_4^2 \tag{7}$$

$$y_2 = - 1.05 + 0.04x_1 + 0.02x_2 - 0.11x_3 + 0.99x_4 - 0.001x_1x_2 + 0.0004x_1x_3 + 0.004x_1x_4 + 0.0001x_2x_3 + 0.002x_2x_4 + 0.001x_3x_4 - 0.001x_1^2 - 0.0001x_2^2 + 0.0001x_3^2 - 0.02x_4^2 \tag{8}$$

$$y_3 = - 0.28 - 0.001x_1 + 0.01x_2 - 0.0001x_3 + 0.90x_4 - 0.001x_1x_2 + 0.0001x_1x_3 + 0.001x_1x_4 - 0.0001x_2x_3 + 0.002x_2x_4 + 0.0004x_3x_4 - 0.0001x_1^2 - 0.0001x_2^2 + 0.0001x_3^2 - 0.01x_4^2 \tag{9}$$

where y_1 is the response for the reduction of *A. niger*; y_2 *A. niger*, *A. terreus var. terreus* and y_3 is *P. simplicissimum*. The current work clearly demonstrates that the role of independent factors in the waterless medium differs from its role in the water medium, where lower pressure (20 MPa versus 35 MPa) was sufficient to achieve the reduction target of the fungal spore’s reduction (6 log reduction) [7]. Previous studies revealed that temperature and pressure were effective in reducing *Penicillium oxacilum* and *Alternaria brassicicola* by SC-CO₂ in water medium [22, 23], while Hossain et al. [11] claimed that pathogenic bacteria in clinical solid waste (water medium) were inactivated by SC-CO₂ mainly as a function of high pressure. In fact, the maximum temperature used in this study (90 °C) was shown to be not sufficient alone to achieve the total inactivation of the fungal spores in the absence of high pressure. For instance, on the basis of Eq. (7), the logarithmic reduction of *A. niger* at 0.1 MPa and 90 °C, for 100 min and with an initial concentration of 6 log₁₀ spores, was 1.34 logarithmic reduction. Jung et al. [12] claimed that the minimum temperature required to inactivate 99% of *Aspergillus versicolor* was 350 °C, while it was 400 °C for *Cladosporium cladosporioides* bioaerosols.

3.2 Artificial neural network (ANN) model analysis

The behavior of *A. niger* and *A. terreus var. terreus* and *P. simplicissimum* spores as a function of pressure x_1 (20–35 MPa), temperature x_2 (35–90 °C), time x_3 (10–100 min) and initial fungal spore concentrations x_4 ($3\text{--}6 \log_{10}$ spores g^{-1}) was investigated with ANN model analysis. Temperature (x_2) exhibited a greater influence on the reduction of *A. niger*, *A. terreus var. terreus*, and *P. simplicissimum* spores compared to the pressure x_1 , since the maximum reduction of fungal spores as a function of temperature reached 6 log reduction compared to 4 log reduction as a function of pressure (Fig. 3 A1,B1,C1). These findings indicate that fungal spores have a higher sensitivity to CO_2 at high temperature. Watanabe et al. [29] indicated that the inactivation of fungal spores takes place due to the thermodynamic state of CO_2 , where at high temperature, CO_2 is converted to a gas-like fluid and then becomes more effective in damage to the fungal spores. In comparison, the changes to the gaseous state as a response to the hydrostatic effect have a weaker effect on the fungal spores during the inactivation process. Efaq et al. [7] revealed that at high pressure (35 MPa) and temperature (75 °C), the inactivation of fungal spores (6 log reduction) in water medium was faster (60 min), compared to high pressure or high temperature alone, under which conditions the fungal spores were lower after 60 min and did not reach the target reduction target (6 log reduction). Specifically, Calvo and Torres [6] claimed that the sporicidal effect in the waterless medium was high even at low pressure, possibly due to the direct effect of CO_2 on fungal spores. Valverde et al. [28] mentioned that in the waterless SC- CO_2 medium, microorganism inactivation occurs faster due to direct contact between CO_2 and microbial cells. It can be explained that in the water medium, the pressure accelerates the diffusion of CO_2 through the surface of the water medium to reach the fungal spore surface, while the temperature increases CO_2 diffusion rate into the fungal spore. In the waterless medium, the role of pressure would be lower, since CO_2 could reach the surface of the fungal spore surface faster, while it still requires to diffuse CO_2 into the spore and achieve a high reduction especially at low temperature. It can be indicated that the effect of the temperature in SC- CO_2 is not related to the direct effect of the temperature on the fungal spores; rather, the high temperature changes the properties of the CO_2 gas to be more fungicidal compared with the gas at the low temperature.

The time x_3 exhibited no influence on the inactivation of *A. niger*, *A. terreus var. terreus*, as the log reduction was constant at different treatment times, while the pressure showed slight effects (log reduction increased from 4 to 4.5) in the inactivation of *P. simplicissimum* (Fig. 3 A2, A4, A6, B2, B4, B6C2, C4 and C6). The role of time in the inactivation process was not significant compared to the temperature. These findings are different from those reported in the inactivation process carried out in the water medium. Hossain et al. [11] claimed that pressure and time exhibited a stronger interaction compared to time and temperature in the inactivation of bacterial cells in clinical wastes. Noman et al. [20] indicated that the high availability of water content reduced the efficiency of SC- CO_2 in inactivating fungal spores in clinical wastes. This is because in the water medium, CO_2 must first saturate the water medium and then keep in contact with the microorganisms. However, Kamihira et al. [13] stated that the presence of a high water content (90%) increased the inactivation of *A. niger* compared to low water content (2%) where the spores were more resistant. The role of water in the inactivation process could be due to the diffusion of CO_2 through the cell membrane [30]. Nonetheless, it is known that CO_2 in a supercritical state has a gas-like diffusivity, thus a fast penetration through cell membrane in the dry medium.

The initial fungal spores x_4 have no interaction with the pressure, time and temperature. However, in all experiments, the inactivation rate increased significantly if the initial fungal spores increased (Fig. 3, A3, A5, B3, B5, B6, C3, C5 and C6). For instance, in the experimental run No 10, with the initial concentrations of $7.5 \log_{10}$ spores g^{-1} (out of the investigated range and used to test the validity of the model), the log reduction reached 7 logs. Shimoda et al. [25] indicated that the logarithmic reduction of *A. niger* reached 6.8 log with initial concentrations of $9 \log_{10}$ spore mL^{-1} .

The ANN model analysis developed new predictive equations for *A. niger* (y_1), *A. terreus var. terreus* (y_2) and *P. simplicissimum* spores (y_3) inactivation (Eqs. 10–12). These equations explain the behavior of the fungal spores for each factor (positive (+) or negative (-)). For example, in neuron 1, 2 and 6 ($\text{Tan}H_1, H_2$ and H_6), the pressure (x_1) has an effective role in the fungal inactivation, while the temperature exhibited an effective role in the neuron 1, 3, 4 ($\text{Tan}H_1, H_3$ and H_4). The predictive equations provided a new and significant insight into the effect of independent factors on fungal spore inactivation.

Aspergillus niger

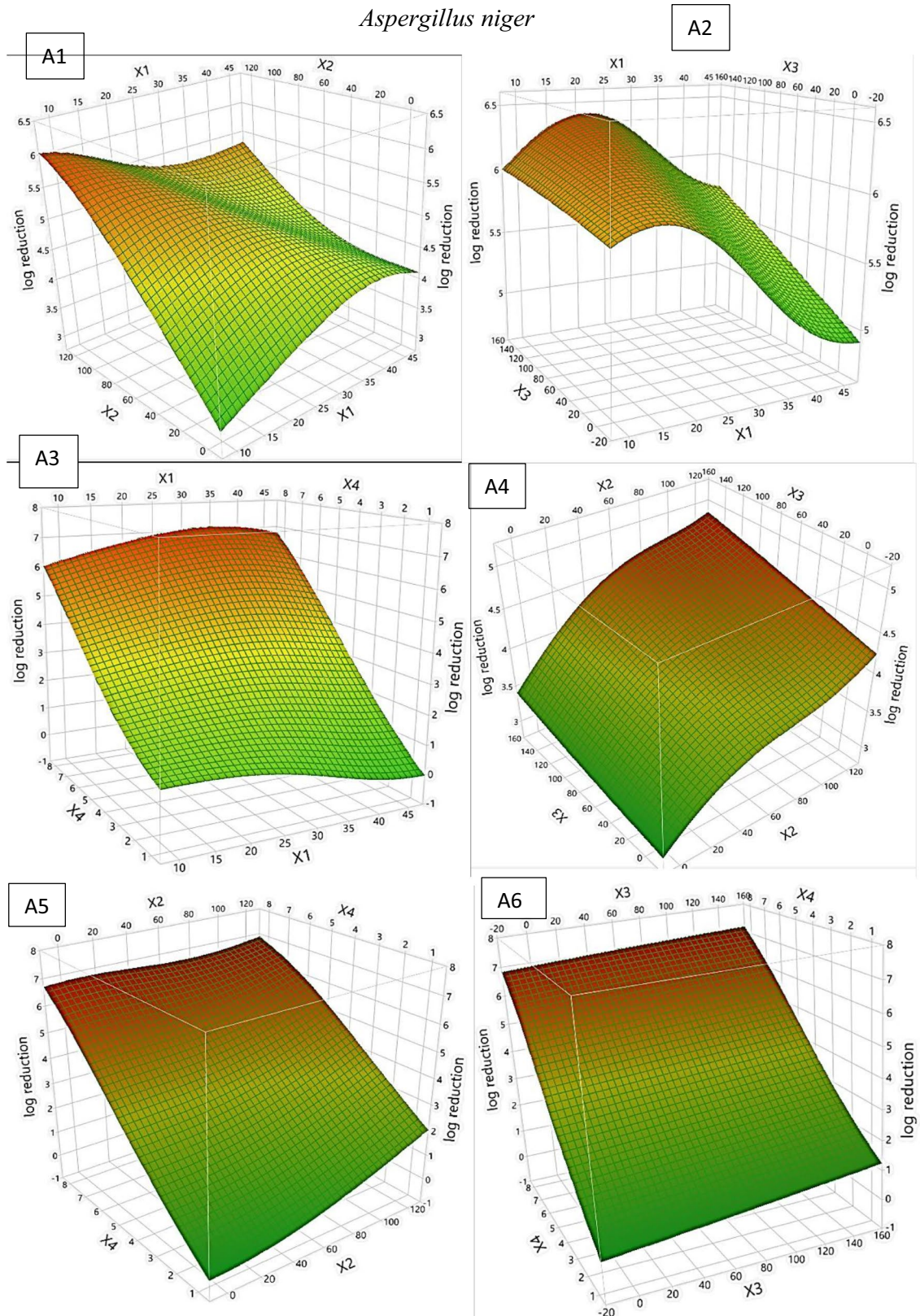


Fig. 3 Artificial neural network for fungal spore inactivation in the clinical solid wastes by SC-CO₂; pressure MPa (x_1); temperature (x_2); time (min) (x_3); initial fungal concentrations (\log_{10}) (x_4); y (log reduction)

A. terreus var. *terreus*

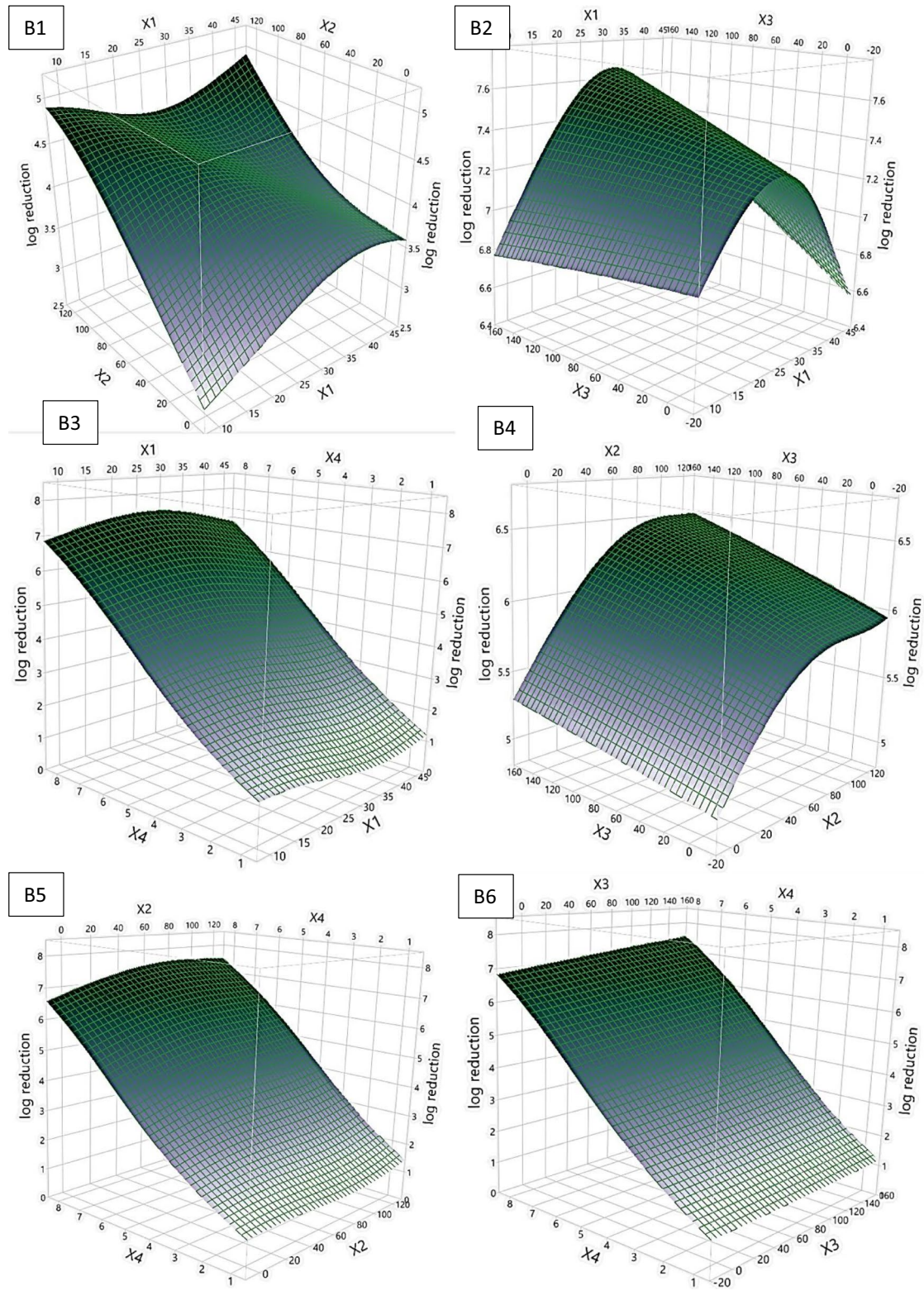


Fig. 3 (continued)

Penicillium simplicissimum

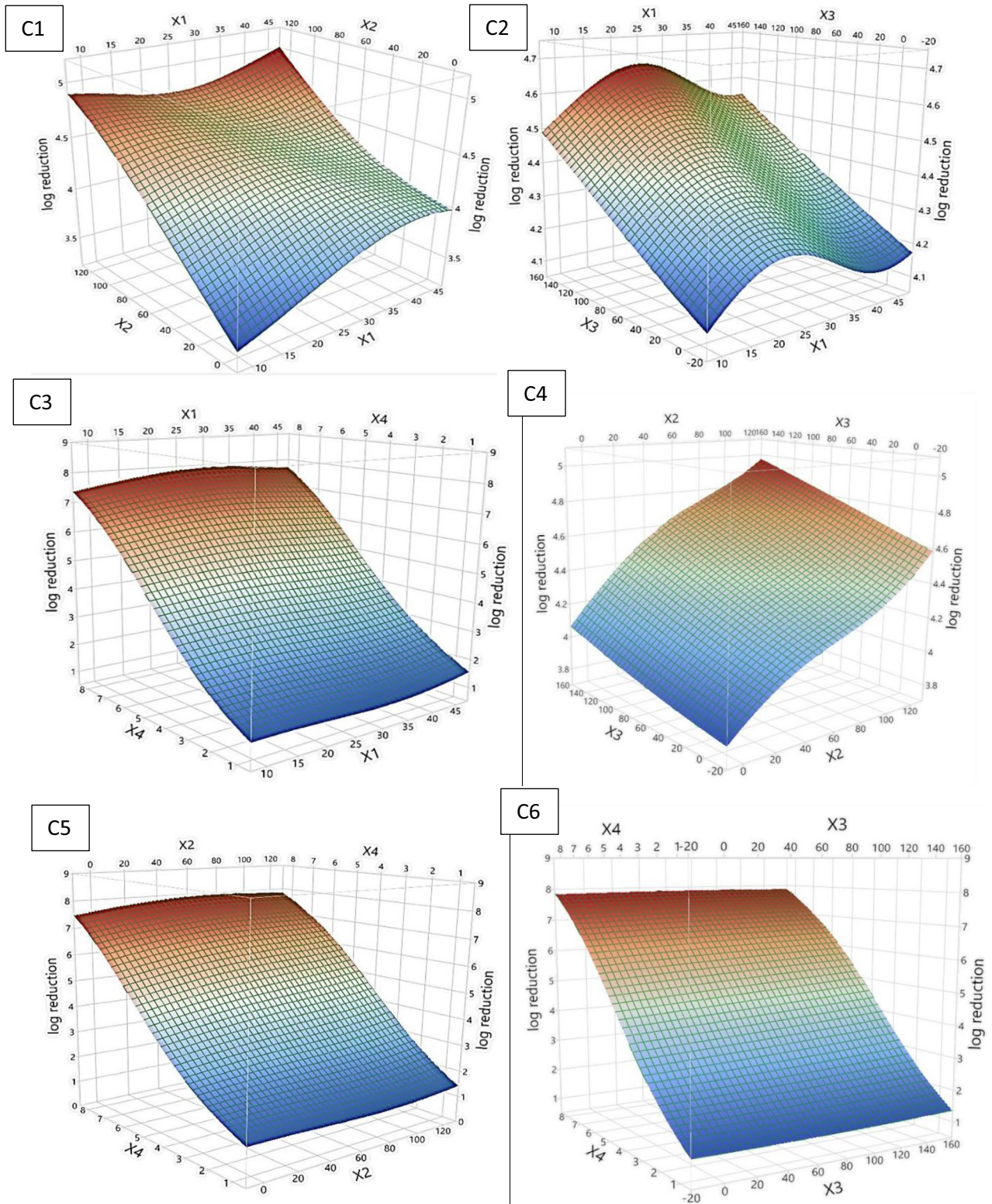


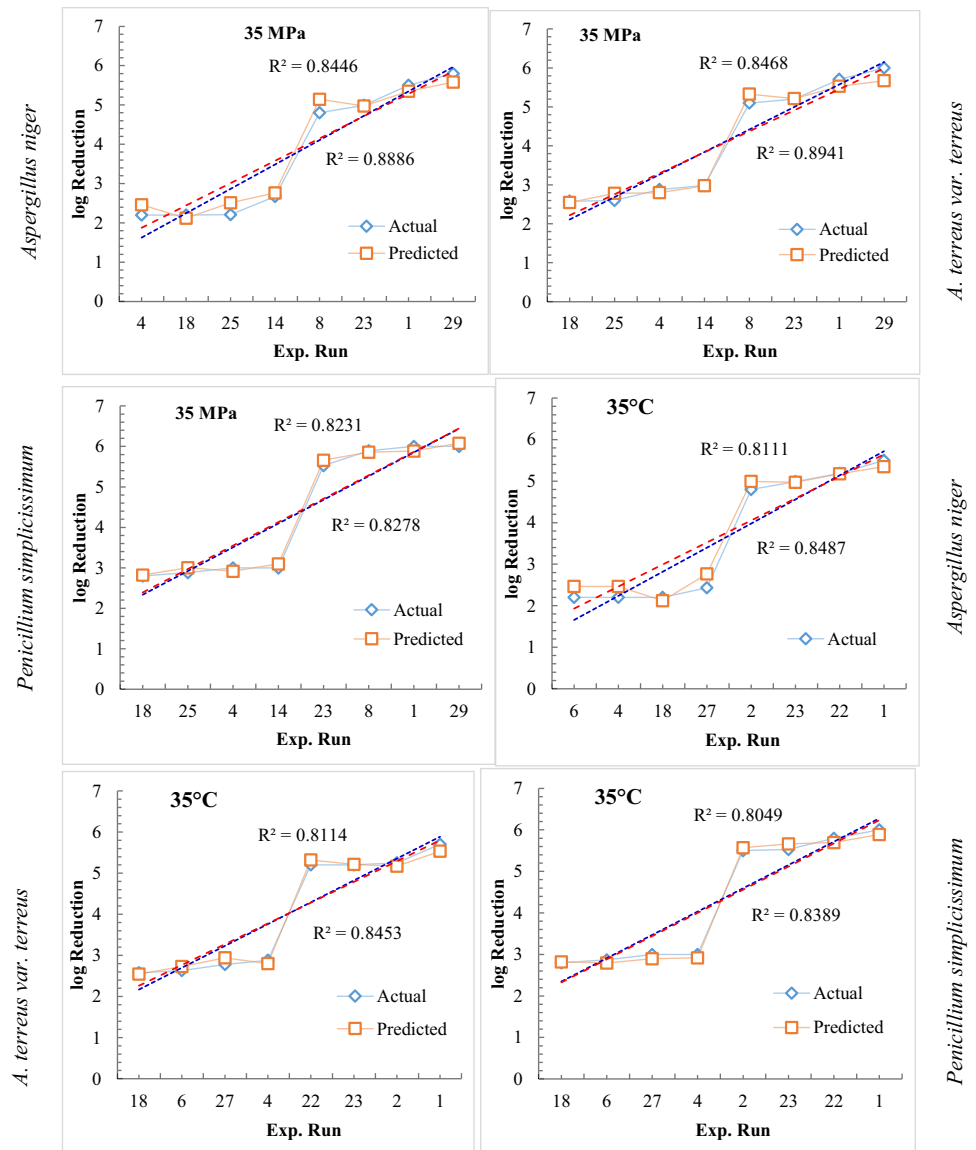
Fig. 3 (continued)

$$y_1 = 3.95 + \left(\begin{array}{l} 0.61 \times \text{TanH}_1(0.5(-0.204 + 0.007x_1 + 0.003x_2 + 0.002x_3 - 0.07x_4)) \\ -1.027 \times \text{TanH}_2(0.5(-1.40 + 0.048x_1 - 0.006x_2 - 0.003x_3 - 0.7101x_4)) \\ +0.928 \times \text{TanH}_3(0.5(-3.06 - 0.014x_1 + 0.006x_2 + 0.004x_3 + 0.576x_4)) \\ +1.47 \times \text{TanH}_4(0.5(0.219 - 0.016x_1 + 0.003x_2 + 0.004x_3 - 0.035x_4)) \\ +1.61 \times \text{TanH}_5(0.5(4.21 - 0.117x_1 - 0.027x_2 + 0.002x_3 + 0.235x_4)) \\ +1.1 \times \text{TanH}_6(0.5(-0.25 + 0.022x_1 - 0.001x_2 + 0.004x_3 - 0.146x_4)) \\ -4.86 \times \text{TanH}_7(0.5(2.30 - 0.034x_1 - 0.006x_2 + 0.003x_3 - 0.25x_4)) \\ -0.52(1.42 - 0.004x_1 - 0.009x_2 - 0.0002x_3 - 0.137x_4) \\ +0.185 \times \text{Exp}\left(-\left(0.5(-0.068 + 0.012x_1 + 0.000012x_2 + 0.0011x_3 - 0.072x_4)^2\right)\right) \end{array} \right) \quad (10)$$

$$y_2 = 4.147 + \left(\begin{array}{l} 0.129 \times \text{TanH}_1(0.5(-0.20 + 0.007x_1 + 0.003x_2 + 0.002x_3 - 0.07x_4)) \\ -0.238 \times \text{TanH}_2(0.5(-1.40 + 0.048x_1 - 0.006x_2 - 0.003x_3 - 0.101x_4)) \\ +1.37 \times \text{TanH}_3(0.5(-3.06 - 0.014x_1 + 0.006x_2 + 0.004x_3 + 0.576x_4)) \\ +1.41 \times \text{TanH}_4(0.5(0.219 - 0.016x_1 + 0.003x_2 + 0.004x_3 - 0.035x_4)) \\ +1.33 \times \text{TanH}_5(0.5(4.21 - 0.117x_1 - 0.027x_2 + 0.002x_3 + 0.253x_4)) \\ +1.1 \times \text{TanH}_6(0.5(-0.25 + 0.022x_1 - 0.001x_2 + 0.004x_3 - 0.146x_4)) \\ -2.95 \times \text{TanH}_7(0.5(2.30 - 0.034x_1 - 0.006x_2 + 0.003x_3 - 0.25x_4)) \\ -0.83(1.42 - 0.004x_1 - 0.009x_2 - 0.0002x_3 - 0.137x_4) \\ +0.014 \times \text{Exp}\left(-\left(0.5(-0.068 + 0.012x_1 + 0.000012x_2 + 0.0011x_3 - 0.072x_4)^2\right)\right) \end{array} \right) \quad (11)$$

$$y_3 = 4.54 + \left(\begin{array}{l} -0.29 \times \text{TanH}_1(0.5(-0.204 + 0.007x_1 + 0.003x_2 + 0.002x_3 - 0.07x_4)) \\ +0.373 \times \text{TanH}_2(0.5(-1.40 + 0.048x_1 - 0.006x_2 - 0.003x_3 - 0.101x_4)) \\ +2.27 \times \text{TanH}_3(0.5(-3.06 - 0.014x_1 + 0.006x_2 + 0.004x_3 + 0.576x_4)) \\ +0.142 \times \text{TanH}_4(0.5(0.219 - 0.016x_1 + 0.003x_2 + 0.004x_3 - 0.035x_4)) \\ +0.69 \times \text{TanH}_5(0.5(4.21 - 0.117x_1 - 0.027x_2 + 0.004x_3 + 0.235x_4)) \\ +272 \times \text{TanH}_6(0.5(-0.25 + 0.022x_1 - 0.001x_2 + 0.004x_3 - 0.146x_4)) \\ +0.27 \times \text{TanH}_7(0.5(2.30 - 0.034x_1 - 0.006x_2 + 0.003x_3 - 0.25x_4)) \\ -0.27(1.42 - 0.004x_1 - 0.009x_2 - 0.0002x_3 - 0.137x_4) \\ +0.037 \times \text{Exp}\left(-\left(0.5(-0.068 + 0.012x_1 + 0.000012x_2 + 0.0011x_3 - 0.072x_4)^2\right)\right) \end{array} \right) \quad (12)$$

Fig. 4 Sensitivity analysis of the inactivation process for independent factors at maximum and minimum value in the inactivation of fungal spores in the clinical wastes by SC-CO₂ (the experimental run number is available in Table S1)

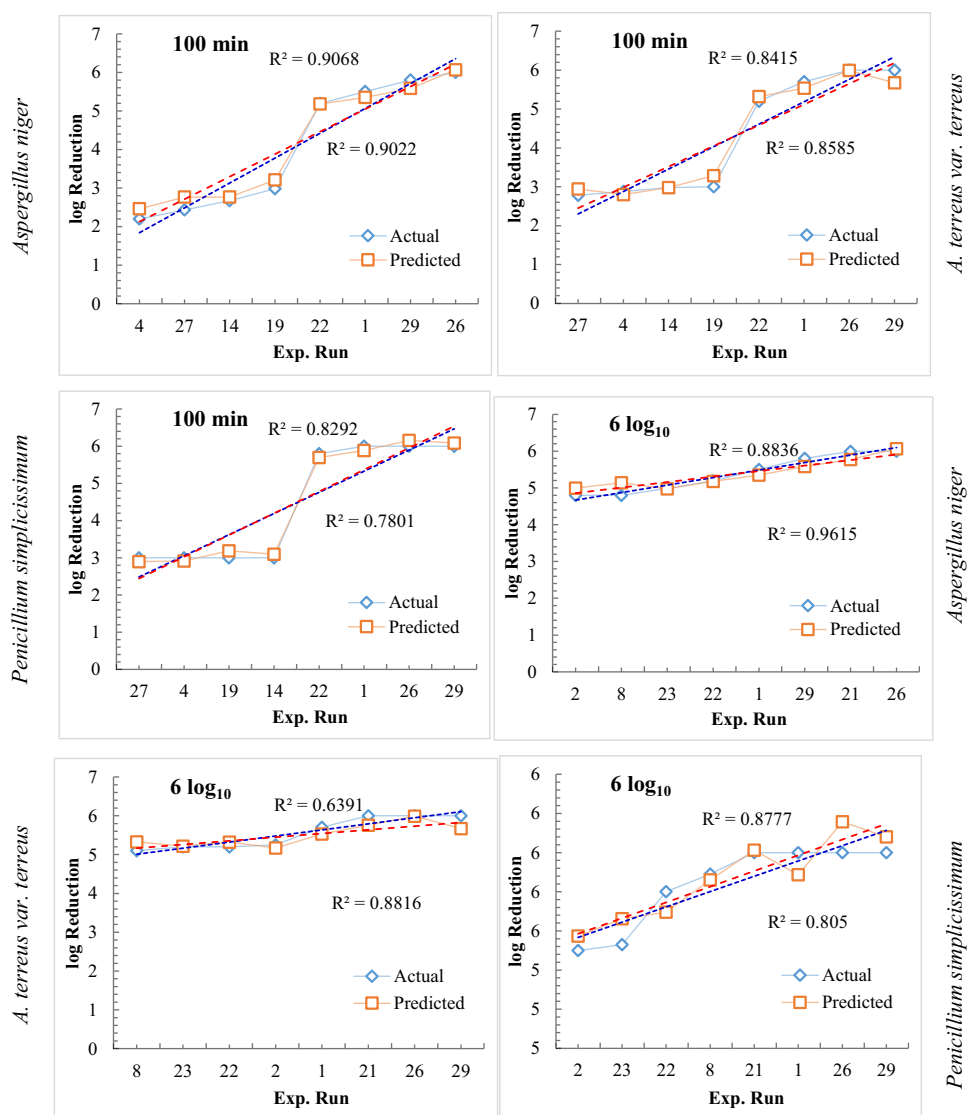


3.3 Sensitivity analysis of the fungal spores for inactivation

The accuracy, reliability and strength of Eqs. (10–12) were assessed based on the coefficients of predicted results and experimental results (Fig. 4). In the inactivation of *A. niger* at 35 MPa, the coefficient (R^2) was 0.8446 vs. 0.8886 respectively in the experimental and predicted results. Regarding the *A. terreus var terreus* spore, R^2 was 0.8468 vs. 0.8941 at 35 MPa, in the experimental and predicted results, respectively, as well as 0.8468 vs. 0.8941 at 35 MPa for the inactivation of *P. simplicissimum* spores in the experimental and predicted results, respectively. The variation between the coefficient (R^2) in the experimental results indicated that the fungal spores exhibited a different sensitivity at the same pressure but under different factors (Fig. 4). *A. niger* spores

showed a different sensitivity as a function of temperature (35 °C) compared to *A. terreus var. terreus* and *P. simplicissimum* spores (Fig. 4). Both *A. niger* and *A. terreus var. terreus* spores exhibited similar behavior at 100 min; these results indicated that the inactivation period for 100 min has no effects on the reductions of the fungal spore's reductions since the inactivation process takes place within a shorter time. In contrast, the *P. simplicissimum* spores exhibited more variation in their behavior after 100 min (Fig. 4). These differences might be related to the differences in cell wall structure differences between *Aspergillus* spp. and *Penicillium* sp. spores. The sensitivity of the fungal spores for the initial concentrations was unclear, since *A. niger* and *P. simplicissimum* have high variations in their behaviors at the low initial concentrations, while *A. terreus var. terreus*

Fig. 4 (continued)



spores exhibited high variation at high initial concentrations (Fig. 4).

3.4 Regrowth of inactivated fungal spores

The confirmation of the inactivation of the fungal spores as a function of the independent factors is validated using culture-based methods which reflect the influence of the factors. The suitability of treated clinical solid waste for final disposal is confirmed based on the ability of the treatment process to prevent fungal regrowth, which in function depends on the mechanism of inactivation and the level of destruction of fungal spores caused by SC-CO₂ [20]. The results of the inactivated fungal regrowth are depicted in Fig. 5. It was observed that fungal spores have grown in the culture media. However, the growth rate of the inactivated fungal spores was varied according to the parameters of the inactivation process. For example, in the case of *A.*

niger, the fungal spores inactivated at 35 MPa, at 35 °C, for 100 min (T1) exhibited slow growth after 5 days; the maximum colony diameter was 10 mm after 10 days, compared to the fungal spores inactivated at 20 MPa, 90 °C for 10 min (T 21) which occurred after 3 days and reached maximum growth (80 mm) after 10 days. Similar findings were also recorded for the *P. simplicissimum* spores, but the diameter of the colonies was smaller. These results revealed that the inactivation process might take place quickly. However, a long treatment process is necessary to destroy fungal spores and confirm the absence of regrowth. Furthermore, the results in T21 and T26 revealed that pressure has a long-term effect on fungal inactivation under sub-lethal conditions as the fungal spores exposed to high pressure for a long time grew slower than those exposed to high temperature for the same treatment time. The fungal inactivated under lethal conditions at 35 MPa for 100 min and at 90 °C did not re-regrow. Regrowth of inactivated

Fig. 5 Regrowth of fungal spores inactivated at sub-lethal treatment of SC-CO₂. The regrowth was determined based on the colony diameter on PDA during the incubation period from 1 to 10 days at 28 °C (the experimental run number is available in Table S1)

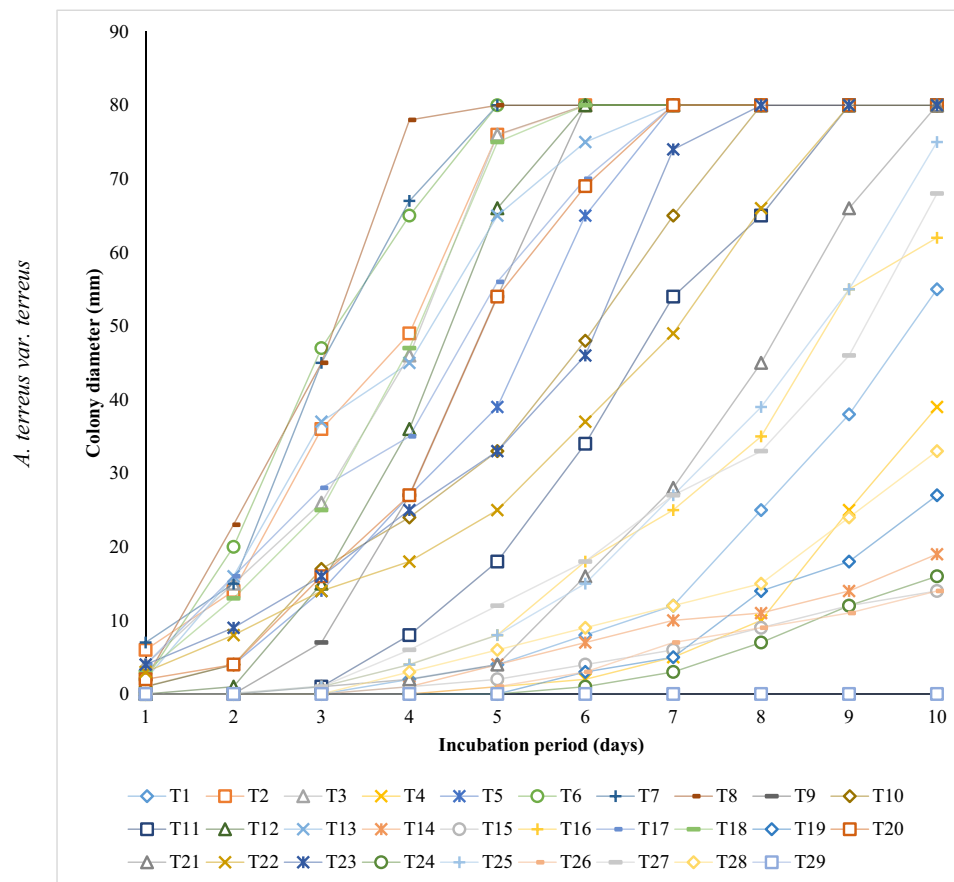
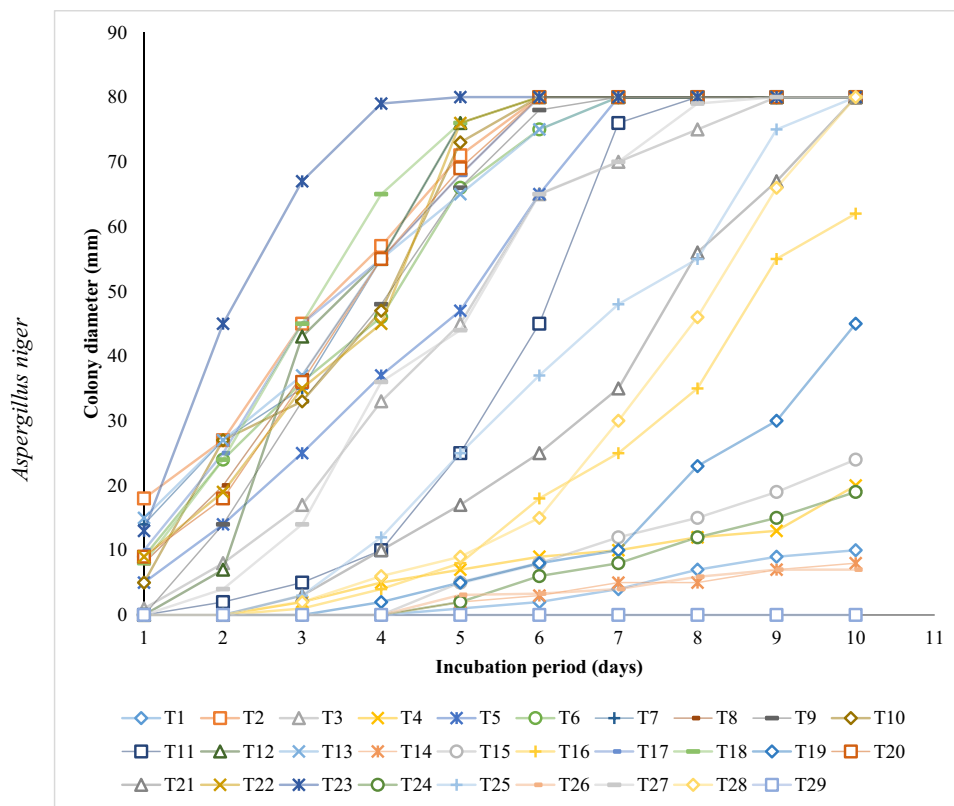
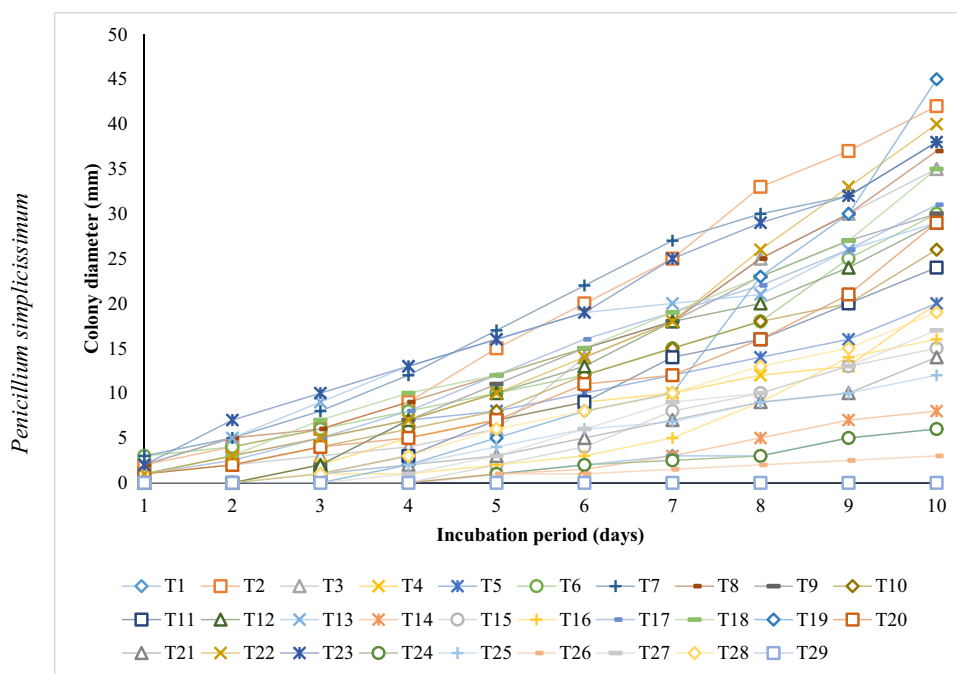


Fig. 5 (continued)



fungal spores under pressure has been reported in the literature. Caldwell [5] found that fungi inactivated at 1 MPa for 10 min were re-growing again. Robb [24] revealed that 50% of the fungal species (103 species) regrow after inactivation at 1 MPa, 25 °C for 7 days. However, the difference between those studies and the current work is in the type of gas used: O₂ was used in those studies, while CO₂ was used in the current work.

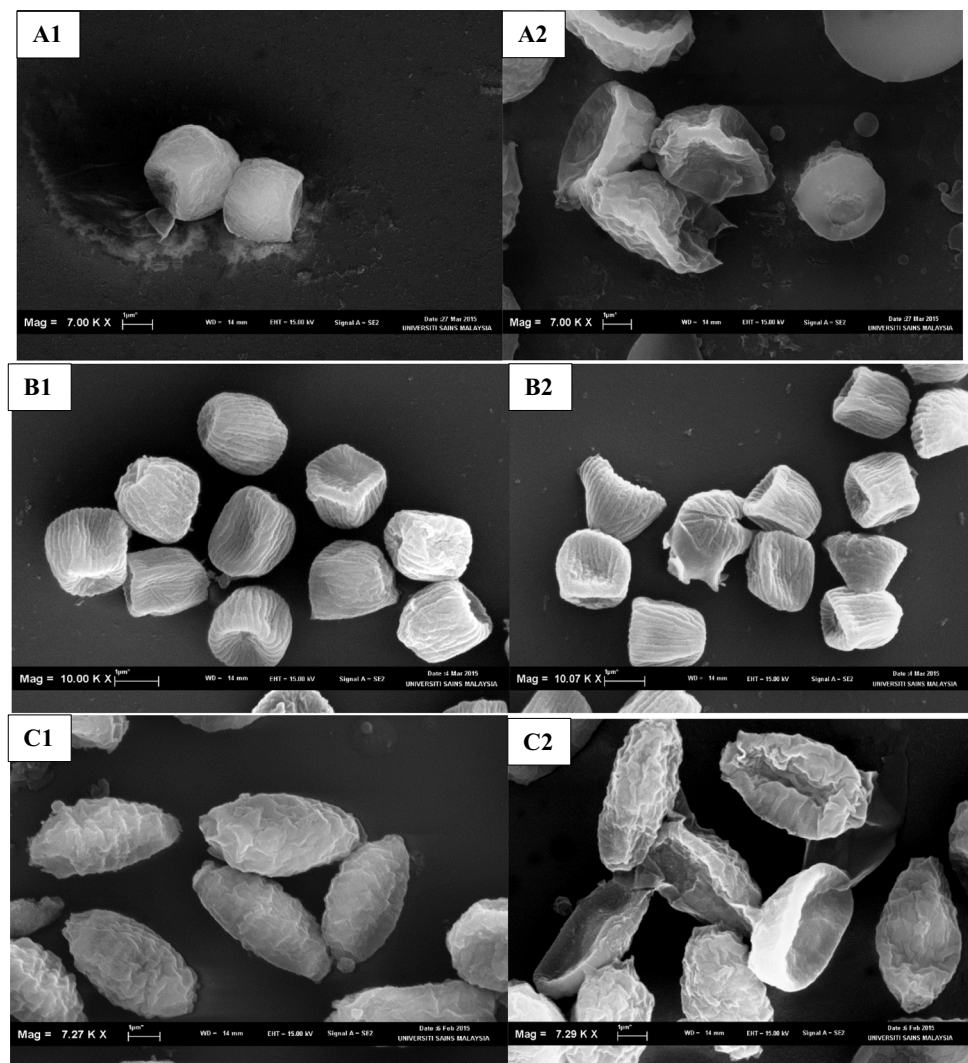
3.5 Inactivation mechanism

The microstructure of the inactivated fungal spore was analyzed using SEM to understand the mechanism of SC-CO₂ destruction that led to complete inactivation of the fungal spores. The results revealed that SC-CO₂ exhibited a strong effect on *A. niger* spores (Fig. 6), *A. terreus var. terreus* (Fig. 6) and *P. simplicissimum* (Fig. 6). The spore morphology has clearly changed after the inactivation process compared to the control (before treatment). The fungal spores were damaged and their shape deformed. The inability of fungal spores that were inactivated at the lethal conditions to regrow in PDA media might be an evidence of irreversible damage to fungal spores (Fig. 7). One of the main concerns in the inactivation of pathogens in waste is their ability to regrow after final disposal. SEM analysis of the surface of the spore revealed a destruction of the cell wall. Compared to a previous study [19, 20], in which the inactivation process was carried out with a high water content, it can be observed that the damage and destruction observed here were less evident than the one recorded before. In the

inactivation process conducted in the presence of water, the fungal spores are damaged as a result of the high diffusion of water into the spore cytoplasm. However, in the present work, no water content was used. Therefore, destruction of the fungal spore cell wall of fungal spores would be explained based on the principle of diffusion of molecules such as CO₂ through the cell wall of fungal spores. In normal conditions (before the treatment process), diffusion occurs as a function of thermodynamic phenomena and continues until equilibrium is reached [4], but without any effects on the morphological of the spore cell wall. Pressure contributes to the diffusion of CO₂ into the spore cytoplasm, while temperature accelerates the diffusion process. The high CO₂ diffusion into the spore cytoplasm leads to a high concentration in the spores and causes swelling of the spore cells; as a result, the spore is bursting, while the cytoplasm content releases in the environment. The behavior of fungal spores in the presence of SC-CO₂ differs from that of bacterial cells, where the lipophilic nature of CO₂ could help the penetration through the lipopolysaccharide (LPS) layers into the cytoplasm. In contrast, the fungal cell wall is composed of glucan, chitin and chitosan, with low phospholipid content with groups of groups of phospholipids with melanin, amino, phosphate, carboxyl, sulfates and hydroxides [9]. Therefore, since CO₂ is hydrophobic, the penetration of CO₂ through the fungal cell wall must take longer. However, high temperature might contribute to increased CO₂ diffusion into the fungal cytoplasm.

The findings of the present study reveal that SC-CO₂ is an effective method to sterilize clinical solid waste with

Fig. 6 Scanning electron micrographs of *A. niger* spores; A1) before inactivation process shows the normal spore shape without destruction of cell wall (7 k X); A2) *A. niger* spores inactivated by SC-CO₂ (20 MPa, 90 °C, 100 min) show the spore shape with wrinkles on the surface (7 k X); B1) *A. terreus var. terreus* spores before inactivation (10 k X); B2) inactivated *A. terreus var. terreus* spores (10 k X); C1) *P. simplicissimum* spores before inactivation (10 k X); C2) inactivated *P. simplicissimum* spores (10 K)



inactivation of microorganisms in clinical solid waste. Since SC-CO₂ operates at relatively low temperatures, heat sensitive waste materials will not degrade. Therefore, sterilized clinical solid waste could be recycled and reused. Therefore, the implementation of SC-CO₂ sterilization technology will promote sustainable utilization of clinical solid waste.

4 Conclusion

Inactivation of the fungal spore using SC-CO₂ in waterless inactivation exhibited high efficiency as a nonthermal alternative sterilization technology. The optimal operating parameters to achieve the target log reduction were obtained at 35 MPa, 35 °C after 100 min with an initial concentration

of 6 log₁₀ spores g⁻¹. Under this condition, the predicted and experimental value of inactivation was very close: 5.47 vs 5.96 log of *A. niger*, 5.56 vs 5.96 of *A. terreus var. terreus* and 5.84 vs. 5.99 log reduction of *P. simplicissimum* with $R^2=0.889$. ANN analysis revealed that fungal spores exhibited different behaviors for the inactivation considering the four different investigated factors (temperature, pressure, time and initial concentrations). Furthermore, it was shown that the exposure of the fungal spores to pressure for long period (100 min) effectively contributed effectively in the inhibition of fungal spore's regrowth. The inactivation mechanism probably concerns the diffusion of CO₂ through the cell wall of the fungal spore that led to irreversible damage to the fungal spores. Therefore, fungal spores do not regrow again in the culture medium.

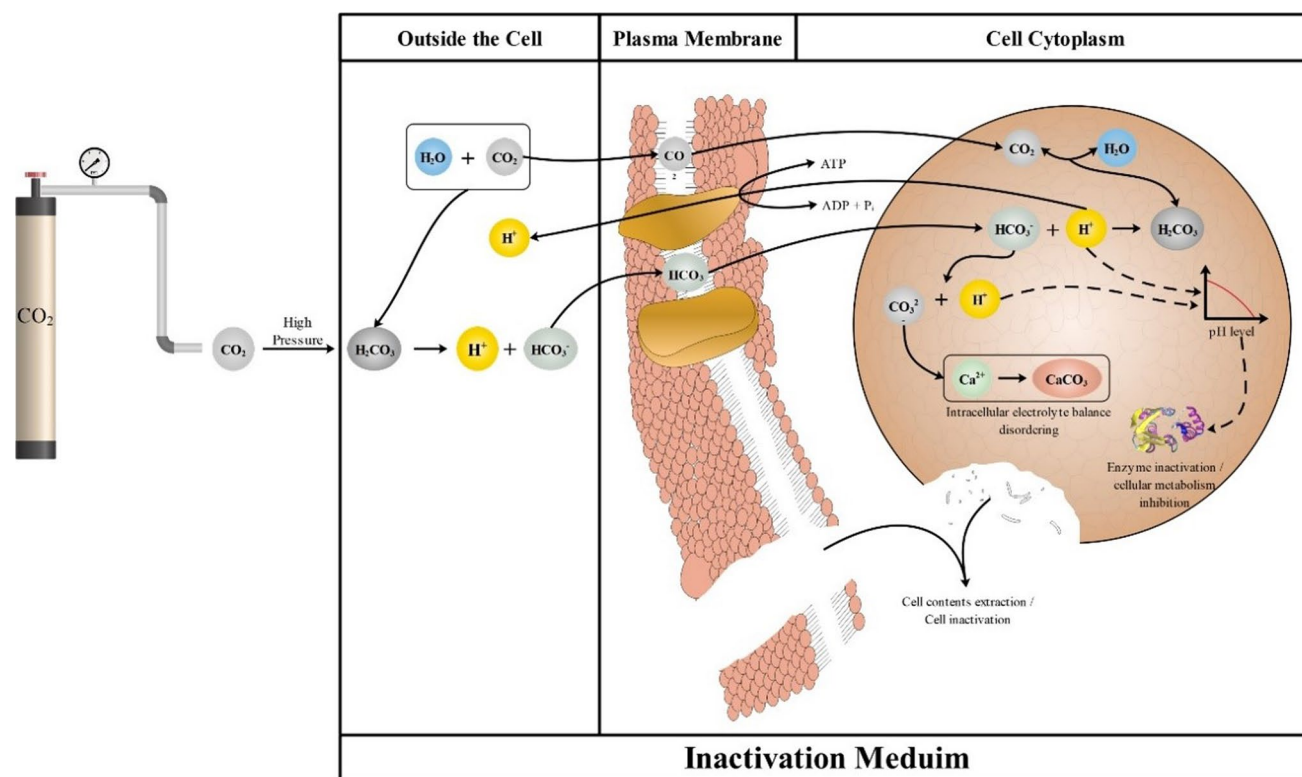


Fig. 7 Proposed mechanism of fungal spore inactivation

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Author contribution EN: methodology investigation and manuscript draft. AA: conceptualization, supervision, writing—review and editing. SS: writing—review and editing. MSH: writing—review and editing. RMSRM: writing—review and editing. NNNAR: conceptualization, supervision, writing—review and editing. MOAK: writing—conceptualization, supervision, writing—review and editing.

Data availability Not applicable.

Declarations

Ethics approval We confirmed that the current work is original for the authors and has not been published or under review in any journal.

Consent to participate All authors have read and contributed to this manuscript and agree on the submission.

Consent for publication All authors have read and contributed to this manuscript and agree on the publications.

Competing interests The authors declare no competing interests.

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