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Influence of substrate particle size and detention time on mycelium-colonized sawdust efficiency for removal of faecal bacteria from slaughterhouse wastewater in batch treatment

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Abstract. Mycofiltration is a recent cost-effective biotechnology that is still under development for wastewater treatment. The use of mycelium-colonised substrate for wastewater treatment in a batch system with delayed residence time and the effect of sawdust particles has not previously been considered. Slaughterhouse wastewater is discharged untreated in many developing countries majorly due to the high cost of existing conventional treatment systems. The effect of sawdust detention time and particle size on the removal efficiency of faecal bacteria from slaughterhouse wastewater by *Pleurotus ostreatus* mycelium was assessed in the lab using mycelium colonized with sawdust of particle sizes (0.6, 1.18, 2.36 mm and unsorted particle sizes) and under varying detention times (0, 12, 24, 36, 48, 60 and 72 hours) using batch treatment procedure. Hydrogen peroxide produced during mycelium colonisation of the sawdust was also evaluated. Oxidation-reduction potential (ORP) was measured during this study to determine the oxidative capacity in each treatment reactor. The removal efficiency of *Escherichia coli* ranged from -7.9 – 77.2 % and was the highest for mycofilter with 2.36 mm sawdust particle size at 72 hours detention time (0.7 log removal).

Salmonella spp. removal efficiency ranged from 0.66 – 71 % with the highest efficiency recorded in the system with 1.16 mm also at 72 hours (0.4 log removal). Mean hydrogen peroxide concentration ranged from 0.53±0.12 (unsorted inoculated) to 25.18±1.77 mg/l (1.18 mm, 72 hours). ORP values ranged from 5.0 ±2.2 mV (raw wastewater, 24 hours) to 232±55 mV (unsorted inoculated, 72 hours). The result of this study showed that substrate particle size and detention time have roles to play in the efficiency of mycofilters using batch treatment. The concentration of hydrogen peroxide was also influenced significantly by sawdust particle size. Therefore, there is a need to further study this system in a bid to optimize its ability to remove faecal bacteria from wastewater.

Keywords: batch treatment, colonized, faecal bacteria, mycelium, mycofilter

Introduction

Mycofiltration is a recent cost-effective biotechnology that is still under development for wastewater treatment. In order to increase its effectiveness in eliminating faecal bacteria, different factors have been considered. However, these factors though promising, have not fully addressed the challenges associated with mycofiltration. The application of mycofilters as columns in multiple series for faecal bacteria removal has been investigated (Olorunfemi *et al*., 2015: Taylor *et al.*, 2015: Shekhar *et al*., 2017:Vu *et al*.,2018). However, this system can be relatively expensive to set up, thus making it unsustainable as a viable alternative to conventional treatment systems. The use of mycelium-colonised substrate for wastewater treatment in a batch system with delayed residence time as in other disinfection treatment systems has been proposed (Martinez, 2016; Umstead, 2019). This is due to the proposition that the system, apart from being phagocytic to bacteria, can also produce antibacterial compounds that need time to react with the bacteria cells (Vu *et al*., 2018). However, a previous study on the application of mycofilters made with sawdust in batch treatment for bacterial elimination did not fully identify the necessary factors for improved removal of faecal bacteria. A recent study has reported the detection of a relatively high concentration of hydrogen peroxide in mycelium colonised sawdust submerged in water (Umstead, 2019). However, the study failed to demonstrate the correlation between faecal bacteria removal and hydrogen peroxide present during batch treatment using mycofiltration. Hydrogen peroxide is an enzymatic by-product of fungi colonisation of substrates (Eichlerová *et al.*, 2006).

It has been reported that sawdust promotes high fungi mycelium colonization (Pozdnyakova *et al.*, 2018). It is also readily available compared to other substrates used for fungus cultivation and pollutant removal in mycofiltration treatment systems. The application of mycelium colonised sawdust in batch treatment system for the removal of faecal bacteria has been reported to be not relatively efficient (Umstead, 2019). There is also a scarcity of adequate knowledge of the factors responsible for the removal process.

In addition, the use of faecal bacteria indicator *E. coli*, for quantifying the removal of faecal pathogens in mycofiltration treatment needs to be fully investigated. This is because pathogenic bacteria can enter a viable but not culturable (VBNC) state, which is a virulence strategy employed to evade the attack of antimicrobial compounds (Rogers, 2012).

Slaughterhouse effluents contain high levels of faecal pathogenic bacteria, which have been reported to be poorly removed by previously used conventional and biological treatment systems (Lawal *et al.*, 2018). The application of mycofiltration with delayed residence time to remove faecal bacteria has shown promise and can be efficient for their removal from slaughterhouse effluents (Martinez, 2016). This study was done to determine the effect of sawdust particle size in mycelium-colonised sawdust for the removal of faecal bacteria from slaughterhouse wastewater in a batch treatment system.

Materials and methods

Set up of experimental bioreactors

Mycofilters were prepared by inoculating the spawn of *Pleurotus ostreatus* into particle size separated autoclaved sawdust with particle sizes of 0.6, 1.18, and 2.36 and unsorted (with particle size range of 0.6-4.75 mm). The fungi spawn was purchased from Mycofarm and Synergy Ltd, Benin City. Sawdust sorting followed the process previously described by Osarenotor et al., 2021. The inoculated sawdust was then placed in the dark to allow for incubation at room temperature and was allowed to colonise for 20 days. After colonisation, the mycelium colonised sawdust was transferred into their respective treatment bioreactors.

The wastewater used in this study was obtained from a cattle slaughterhouse located in Edo state, South of Nigeria. The facility has no wastewater treatment unit. Hence the effluents from the facility are channelled into a nearby side drain from which the water flows directly into the nearby river which is approximately 100 meters away from the slaughterhouse. Samples were collected directly from the effluent pipe leading out of the facility. 50 L of wastewater was collected and immediately transported to the laboratory.

Before the commencement of the treatment experiment, the wastewater was kept in the refrigerator at 4°C after skimming and filtering to remove large floating matter and solids.

Treatment bioreactors were 500 ml Pyrex conical flasks. The experimental design included ten reactors. Each reactor was filled with 400 ml of wastewater. The reactors were separated into five treatment groups: two reactors each with mycofilter of sawdust of particle sizes; 0.6, 1.18 and 2.36 mm, two with mycofilter prepared from unsorted sawdust and two with sawdust only and two controls (blank reactors without sawdust nor mycofilter) Inoculated media were transferred into the reactor vessels and allowed to recolonize for three days. This was done to ensure that the fungi mycelium colonises the substrate evenly following the dislodgement during the transfer process. To each setup, 25 % mycofilter was introduced as recommended by Martinez, 2016, using this method 100 g of each treatment media which is 25 % of 400 ml was added. Each reactor was monitored for treated effluent after (0, 12, 24, 36, 48, 60 and 72 hours) detention time. Samples were collected from each reactor and analysed for pH, *E. coli*, *Salmonella* spp, total dissolved solids, electrical conductivity and extracellular hydrogen peroxide. The performance of the treatment reactors was assessed using mean removal efficiency and mean log removal *E. coli* and *Salmonella* spp.

Samples analysis

Faecal bacteria analysis

Raw wastewater, as well as treated effluents, were analysed for *E. coli* and *Salmonella* spp. All samples were serially diluted and tested in triplicate using the membrane filter technique. Diluted water samples were vacuum filtered using 0.45 µm filter pads and thereafter transferred to Petri dishes containing specific media. Eosine methylene blue agar (EMB) was used for *E. coli* detection and quantification, while Salmonella-SSA was used for *Salmonella* spp. The Petri dishes were then inverted and incubated for 24-48 hours at 24-48°C.

Analysis of physiochemical parameters

Total dissolved solids (TDS), electrical conductivity, oxidation-reduction potential (ORP) and pH of the wastewater samples were analysed using the multi-parameter meter following the standard protocol prescribed by the American Public Health Association (APHA), 2005.

Hydrogen peroxide determination

The concentration of hydrogen peroxide in the treatment reactors was determined using the spectrophotometric methods previously described by El Sayed and El-Sayed, 2020 with a slight modification.

Data analysis

All statistical analysis was performed using R version 3.2.4. Multiple analysis of variance (MANOVA) was applied to check if a significant difference exists between the *E. coli* as well as *Salmonella* log removal efficiency among the treatment units. The significant difference in hydrogen peroxide concentration and ORP was also checked. Pearson's correlation coefficient was used to determine the relationship between; *E. coli* as well as *Salmonella* log removal and hydrogen peroxide concentration, ORP and pH. The relationship between hydrogen peroxide and ORP was also determined. A possible correlation between faecal bacteria indicator *E. coli* and faecal pathogen *Salmonella* during this study was also determined. All P values were considered significant at a level of 0.05. Blue colours in the visual representation output imply positive correlation while red colours imply negative correlation. Dark colour is an indication of the intensity of the correlation, the darker the colour the stronger the correlation.

Results and discussion

Effect of sawdust particle size on removal efficiency

This study was carried out to investigate the influence sawdust particle size has on the performance of mycelium-colonised sawdust for faecal bacteria removal from wastewater. Mean *E. coli* removal efficiency ranged from -7.9 ± 2.34 (unsorted uninoculated) to 77.2 ± 1.5 % (2.36 mm, 72 hours) (Tab. 1). The mean removal efficiency was the highest in the reactor with mycofilter of sawdust particle size 2.36 mm at 72 hours detention time with corresponding *E. coli* log removal of 0.7 (Fig. 1). The removal of *E. coli* in this treatment was statistically significant. The significant removal recorded in this treatment could be related to the fact that antimicrobial compounds produced during the treatment process were sufficient to deactivate the *E. coli* cells. Another possibility may be that *E. coli* are pathogenic strains that are more prone to bactericidal action compared to the non-virulent strains which are employed as indicators of faecal pollution (Taylor *et al.*, 2015). The high removal efficiency recorded in this study is in line with the result obtained by Umstead, 2019, who also used sawdust as a substrate for removing *E. coli* from synthetic storm water. However, the highest mean removal efficiency recorded in this study was lower than the 99 % reported in their study. The reason for this may be that while this study used real slaughterhouse wastewater with a mean *E. coli* load of 101 x 10^6 cfu/100 ml, the previous study used synthetic wastewater with the mean initial bacteria load 1.94 x 10^4, which is 2 log lesser. As this is the first study using mycelium colonised sawdust for the treatment of real wastewater, there was no other basis for comparison apart from the related study by Umstead, 2019.

Statistical analysis revealed that detention time also had an effect on the removal efficiency of *E. coli* in this study. It showed that faecal pollutants were most significantly removed at 72 hours. This was contrary to the study by Umstead (2019), who reported no significant difference in removal efficiency with time. The reason for this disparity may be a result of the fact that the maximum detention time in their study was 48 hours. This time may not be enough for the antimicrobials to have inhibitory action on the bacteria. As it has been reported the contact time between antimicrobial agents from fungi and bacteria is needed for effective bacteriostatic or bactericidal action (Stamets, 2005).

Table 1. Mean *E. coli* removal efficiency in the batch treatment experiment (%)

Retention time (hours)	Sawdust particle size (mm)			Control treatment		
	0.6	1.18	2.36	Unsorted inoculated	Unsorted uninoculated	Untreated wastewater
10	9.9 ±1.2	13.8 ± 4.5	13.8 ± 2.5^{c}	11.8 ± 1.4	7.9 ± 2.6	0
12	12.8 ± 2.3	22.7 ± 2.1	36.6 ± 2.1^{c}	18.8 ± 3.6	3.9 ± 2.13	-2.97 ± 2.67
24	29.7 ± 2.6	34.6 ± 2.9	45.5 ± 3.5^{c}	23.7 ± 2.6	0.99 ± 2.14	-4.9 ± 4.56
36	24.7 ± 1.7	45.5 ± 1.8	49.5 ± 3.6^{c}	-11.8 ± 3.6	0.99 ± 1.56	34.6 ± 6.7
48	33.6 ± 2.6	54.4 ± 1.5	55.4 ± 2.1^{c}	46.5 ±2.6	- 7.9 ± 2.34	4.95 ± 1.2
60	47.5 ± 2.9	63.3 ± 1.5	66.3 ±2.5^{c}	55.4 ± 2.1	-5.94 ± 2.15	9.9 ± 1.3
72	51.4 ± 2.1^{a}	66.3 ± 3.2^{a}	77.2 ± 1.5^{b}	67.3 ± 4.6^{a}	-0.99 ±1.57 ^{a}	13.8 ± 3.4^{a}

*Values in rows and column with different superscripts are significantly different (P<0.05)

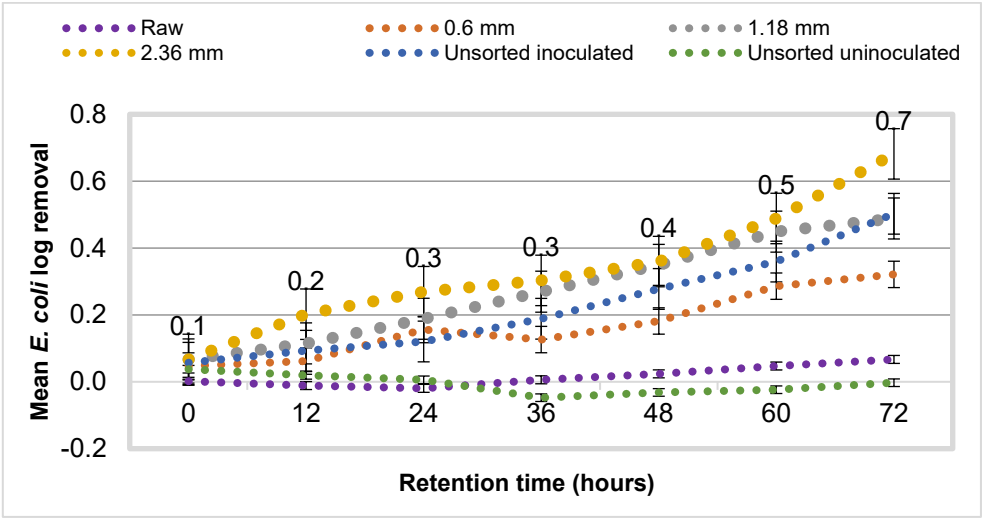

Figure 1. Mean *E. coli* log removal in the batch treatment experiment

Salmonella spp. removal

Mean *Salmonella* spp. removal efficiency in this experiment ranged from 0.66 ± 0.2 (control) to 71±3.8 % (1.18 mm, 72 hours) (Tab. 2) and was the highest in treatment with the sawdust particle size of 1.18 mm at 72 hours with a removal efficiency of 71 ±3.8 % with mean log removal of 0.4 (Fig. 2), however, this mean log removal was the same at 60 hours detention time.

Table 2. Mean *Salmonella* spp. removal efficiency in the batch removal experiment (%)

Retention time (hours)	Sawdust particle size (mm)			Control treatment		
	0.6	1.18	2.36	Unsorted inoculated	Unsorted uninoculated	Untreated wastewater
10	6.68 ± 2.34	12.7 ± 3.6[a]	10.7 ± 3.2	8.02 ± 4.3	5.3 ± 3.7	0
12	8.69 ± 3.4	18.7 ± 9.8[a]	26.08 ± 3.6	12.7 ± 3.2	2.67 ± 2.4	-2.0 ± 0.7
24	27.2 ± 2.1	29.2 ± 2.1[a]	29.2 ± 4.6	15.2 ± 5.7	-2.6 ± 0.7	-0.66 ± 0.2
36	36.9 ± 3.5	41.8 ±7.6[a]	34.14 ±7.2	24.3 ± 8.7	-10.4 ± 2.1	4.18 ± 1.1
48	44.1 ± 5.6	49.8 ± 3.2[a]	39.85 ± 3.1	37.7 ± 4.3	-7.1 ± 1.5	6.4 ± 1.4
60	53.4 ±4.6	59.9 ± 2.5[a]	48.3± 4.9	51.26 ± 6.7	-6.4 ± 0.4	7.94 ± 1.7
72	67.1 ± 7.6[a]	70.9 ± 3.8[b]	55.8 ± 3.8[a]	59.6 ± 4.7[a]	-1.50 ± 0.1[a]	12.8 ± 2.6[a]

*Values in rows and column with different superscripts are significantly different (P<0.05)

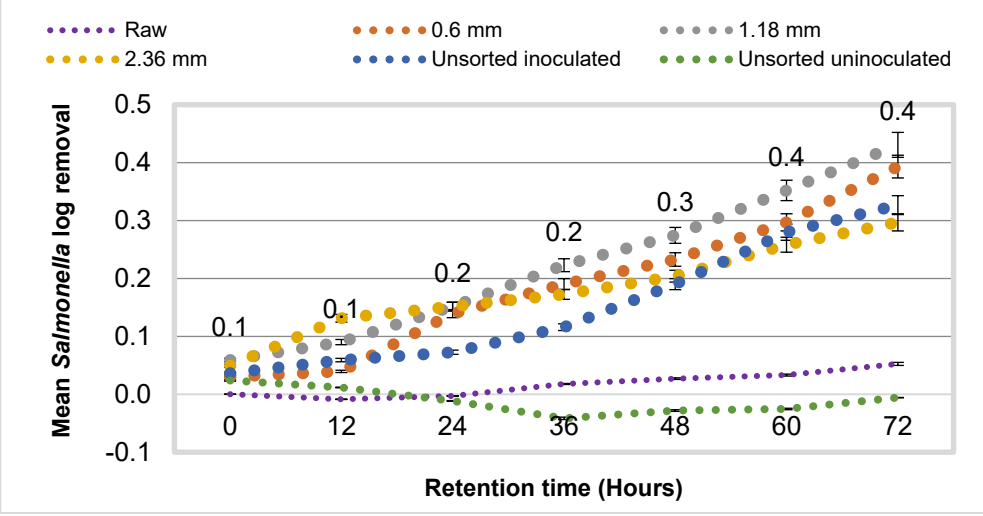

Figure 2. Mean *Salmonella* spp. log removal in the batch treatment experiment

Further statistical analysis, however, showed the mean log removal at 72 hours was significantly different from that at 60 hours ($P<0.05$). The high removal efficiency in this experimental treatment may be due to the high concentration of hydrogen peroxide detected in this treatment (see Tab. 3). Hydrogen peroxide is a potent disinfectant, therefore its presence in addition to other antimicrobials may be responsible for the significant removal efficiency recorded for *Salmonella* spp. *Salmonella* spp. is a more pathogenic bacteria compared to *E. coli* and may require a combination of antimicrobials to eliminate it (Chen *et al*., 2019). The attachment of pathogenic bacteria present in wastewater to organic matter can aid their ability to evade antimicrobial deactivation (Koivunen *et al*., 2003).

Table 3. Mean hydrogen peroxide concentration in batch removal experiment (mg/l)

Retention time (hours)	Sawdust Particle size (mm)			Control treatment		
	0.6	1.18	2.36	Unsorted inoculated	Unsorted uninoculated	Untreated wastewater
10	9.04 ± 0.4	7.97 ± 1.8	9.78 ± 1.1	2.58 ± 0.8	0	0
12	24.83 ± 1.8	25.18 ± 1.7	24.71 ± 1.8	2.46 ± 0.20	0	0
24	14.62 ±1.78	14.98 ± 1.8	14.51 ± 1.8	4.14 ± 1.63	0	0
36	7.82 ± 1.9	8.18 ± 1.7	7.70 ± 1.79	7.38 ± 1.79	0	0
48	4.42 ± 1.78	4.78 ± 1.7	4.90 ± 1.35	4.51 ± 1.67	0	0
60	2.45 ± 0.14	2.75 ± 0.20	2.41 ± 0.23	1.89 ± 0.46	0	0
72	0.99 ± 0.14	1.28 ± 0.20	0.94 ± 0.23	0.53 ± 0.12	0	0

Hydrogen peroxide quantification

The result of this study showed that hydrogen peroxide production during batch mycofiltration treatment is affected by the substrate particle size. Mean hydrogen peroxide concentration ranged from 0.53±0.12 (unsorted inoculated) to 25.18±1.77 mg/l (1.18 mm, 72 hours) (Tab. 3). Reactor treatment with sawdust of particle size 1.18 had a mean concentration of 25.18 ±177 mg/l which was the highest detected concentration in this study. Statistical analysis confirmed that the concentration of hydrogen peroxide was significantly different from other reactor treatments. The high concentration associated with this particle size may be due to the continuous high regeneration of hydrogen peroxide as the experiment progresses. As the sawdust was not used up completely, the mycelium could recolonize under an aqueous solution and produce more hydrogen peroxide. This is similar to the study by Umstaed, 2019 who reported that hydrogen peroxide production during mycelium colonization of sawdust under batch conditions is influenced by detention time and increases with time.

Effect of pH on bacteria removal

The mean pH during the treatment ranged from 1.5 ± 0.0 (unsorted inoculated at 72 hours) to 7.1± 0.1 (raw wastewater at 12 hours) (Tab. 4). The presence of various phenolic compounds during mycelium degradation of substrates is responsible for the low pH of the fungi colonised substrate. Low system pH is needed for the Fenton reaction and pollutant oxidation in the system. The relatively low pH in mycelium colonised treatments during this study are similar to the results of Vu et al.,2018, who reported low pH in broth cultures containing wood decaying fungi, *Flavodon flavus* and *Schizophyllum commune*. The mean pH value recorded in this study was however lower when compared to the mean values of 5-6 obtained in their study. This indicates that there is increased production of phenolic acids by fungi in the presence of substrates compared to artificial microbial growth media.

Table 4. Mean pH in batch removal experiment

Retention time (hours)	Sawdust Particle size (mm)			Control treatment		
	0.6	1.18	2.36	Unsorted inoculated	Unsorted uninoculated	Untreated wastewater
10	3.4 ± 0.0	2.7 ± 0.0	3.2 ± 0.0	2.4 ± 0.2	6.3 ± 0.0	6.4 ± 0.2
12	2.6 ± 0.1	3.0 ± 0.1	3.5 ± 0.1	2.2 ± 0.0	6.5 ± 0.0	7.1 ± 0.1
24	2.2 ± 0.16	2.6 ± 0.1	3.1 ± 0.1	1.8 ± 0.0	6.1 ± 0.0	6.7 ± 0.1
36	2.1 ± 0.1	2.5 ± 0.1	3.0 ± 0.1	1.7 ± 0.0	6.0 ± 0.0	6.6 ± 0.1
48	2.1 ± 0.1	2.5 ± 0.1	3.0 ± 0.1	1.7 ± 0.0	6.0 ± 0.0	6.5 ± 0.1
60	2.1 ± 0.1	2.5 ± 0.1	3.0 ± 0.1	1.7 ± 0.0	6.0 ± 0.0	6.5 ± 0.1
72	1.9 ± 0.1	2.2 ± 0.1	2.7 ± 0.1	1.5 ± 0.0	5.7 ± 0.0	6.3 ± 0.1

Influence of oxidation-reduction potential on removal of bacteria

ORP was measured during this study to determine the oxidative capacity of each treatment reactor. The result showed that mean ORP values ranged from 5.0 ±2.2 mV (raw wastewater, 24 hours) to 232±55 mV (unsorted inoculated, 72 hours) (Tab. 5). The ORP levels were relatively higher in the colonised sawdust compared to uncolonised sawdust. The high ORP is an indication of the active breakdown of hydrogen peroxide to hydroxyl radicals in the treatment systems. Hydroxyl radical generates high oxidative potential (Liu *et al*., 2020).

Correlation studies were done to determine the relationship between explanatory variables. The result revealed that there was no significant correlation between faecal bacteria indicator *E. coli* as well as *Salmonella* spp. and hydrogen peroxide (P>0.05) (Fig. 3).

Table 5. Mean ORP (mV) in batch removal experiment

Retention time (hours)	Sawdust Particle size (mm)			Control treatment		
	0.6	1.18	2.36	Unsorted inoculated	Unsorted uninoculated	Untreated wastewater
10	189.0 ± 14.5	211.3 ± 3.9	191.3 ± 4.6	232.0 ± 5.5	56.8 ± 22.3	21.0 ± 2.2
12	212.5 ± 1.3	198.8 ± 2.2	175.8 ± 1.7	234.0 ± 0.8	18.5 ± 9.3	9.0 ± 4.1
24	206.5 ± 1.3	192.8 ± 2.2	169.8 ± 1.7	228.0 ± 0.8	12.5 ± 9.3	5.0 ± 2.2
36	202.5 ± 1.3	188.8 ± 2.2	165.8 ± 1.7	224.0 ± 0.8	9.8 ± 7.8	2.5 ± 0.6
48	207.5 ± 1.3	193.8 ± 2.2	170.8 ± 1.7	229.0 ± 0.8	14.8 ± 7.8	7.5 ± 0.6
60	209.5 ± 1.3	195.8 ± 2.2	172.8 ± 1.7	231.0 ± 0.8	16.8 ± 7.8	9.5 ± 0.6
72	207.5 ± 1.3	193.8 ± 2.2	170.8 ± 1.7	229.0 ± 0.8	14.8 ± 7.8	7.5 ± 0.6

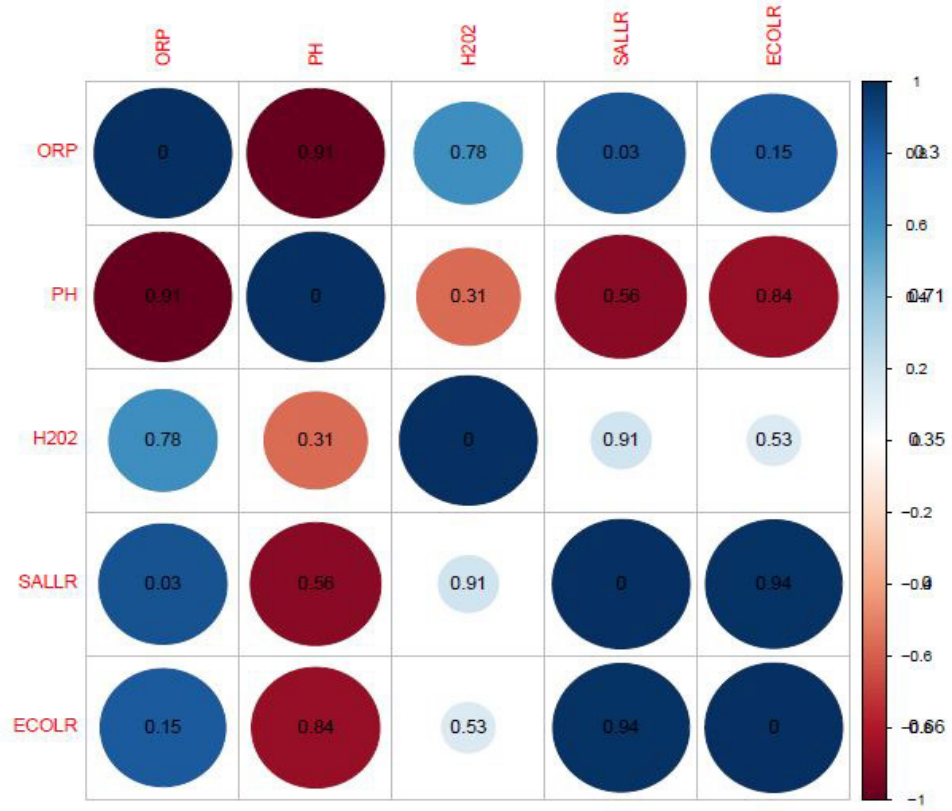

Figure 3. Visual representation of significant correlation among variables in batch treatment study

This observed non-significant correlation may be as a result of the the presence of antimicrobial compounds with bacteriostatic and bactericidal action. Phenolic compounds produced in fungal cultures have been demonstrated to have bactericidal actions against bacteria (Vu *et al*.,2018). The results are consistent with previous studies by Chen et al., 2019, who reported no significant correlation between *E. coli* removal and commercial hydrogen peroxide in their study using chemical Fenton for bacteria removal from wastewater. There was also no significant correlation between faecal indicator bacteria *E. coli* and *Salmonella* spp. during this study. This could imply that *E. coli* may not be the best indicator to establish the presence of faecal pathogens such as *Salmonella* spp. in mycofiltration studies. As previously reported, some *E. coli* strains can be subjected to a viable but not culturable state (VBNC) by some treatment systems and under that condition they may not be detectable while the actual pathogen is detected in high levels (George *et al*., 2002).

Further, the post-hoc test showed that the factors independent variables considered in this study (sawdust particle size and detention time) had an overall influence of 57 % and 74 % respectively on all dependent variables (*E. coli* and *Salmonella* spp. log removal, hydrogen peroxide concentration, pH and ORP) (Tab. 6). It also showed that the interaction between the two independent variables had an 80 % overall influence on all dependent variables. The individual size effect showed that treatment was significantly responsible for 82 and 99 % log removal in *E. coli* and *Salmonella* spp. respectively. Treatment time was also responsible for 73 and 99 % of the observed variation in *E. coli* and *Salmonella* spp. log removal respectively. Detention time was also responsible for 73 and 99 % of the observed variation in *E. coli* and *Salmonella* spp. log removal respectively.

Table 6. Estimated effect size table at 0.05 level for batch treatment experiment

Parameter	Particle size	Retention time	Particle size and retention time
Overall effect	57	74	80
E. coli log removal	82	73	60
Salmonella spp. log removal	99	99	99
Hydrogen peroxide	99	99	20
pH	99	94	84
Oxidation-reduction potential	99	56	65

Conclusion

The results of this study showed that particle size plays a role in the efficiency of mycelium-colonised sawdust used for the removal of faecal bacteria in batch treatment. While the removal of *E. coli* was most effective in treatment with sawdust of particle size 2.36, the removal of *Salmonella* spp. was the highest in the treatment system with sawdust of particle size 1.18 at 72 hours.

The study also showed that apart from sawdust particles which was the main factor considered in this study, detention time also had a significant influence on bacteria removal. This may be because antimicrobial compounds require some contact time to react with bacteria cell wall components before eventual killing of the whole bacterial cell. The concentration of hydrogen peroxide was also influenced significantly by sawdust particle size. The study showed that the removal of bacteria in batch treatment might also be influenced by phenolic compounds produced by fungi.

Therefore, there is a need to further study this system in a bid to optimize the production of hydrogen peroxide and other antimicrobial compounds that may be present for the large-scale removal of faecal bacteria from biological wastewater with a high load of these organisms. This can serve as a cheap and green eco-biotechnological way of slaughterhouse wastewater management.

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