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Enteropathogens survivor load in digestate from Two-phase mesophilic anaerobic digestion and Validation of hygienization regime

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ABSTRACT

The survival of potentially infectious enteropathogens in digestate from two-phase mesophilic anaerobic digestion (Two-phase MAD) technique raises concern on handling and application of the resulting digestate as far as hunan health and environment safety is concerned. In this research, temperature validation and hygienization potential of Two-phase MAD operated as a completely stirred-tank reactor with semi-continuous feeding was investigated. High enteropathogens survival up to 6.4 log *E. coli* and 6.1 log *Salmonella* Senftenberg was observed in digestate resulted from anaerobic digestion of potato peels artificially spiked with 8.7 log *Salmonella* Senftenberg and 8.8log *E. coli* simulating extra high pathogens contamination in biowaste used as feedstock. Even though an average decrease in pH by 2.3 units from the original value of 6.5 observed in hydrolysis reactor created unfavorable environment on the survival of both *E. coli* and *Salmonella* Senftenberg causing a 3.6 log and 3.8 log reduction, yet the alkaline condition in the methanation reactor pH 7.7 boosted the surviving rate by 1.3 log in *E. coli* and 1.2 log in *Salmonella* Senftenberg confirming the inefficiency of Two-phase MAD set-up at providing a completely hygienized digestate. This renders the biosafety of the resulting final digestate questionable and limit the possibility of farmapplication for nutrients recycling due to the involving environmental threat and health risks in case the digestate is to be used. Temperature-time combination at 65 °C for 30min was confirmed sufficient at completely elimination of *E. coli* and effecting >1 log cycle die-off equivalent to 90% die-off in *Salmonella* Senftenberg with z-Value of 11 ºC validating the temperature-time regime of 70 ºC for 60min proposed in European Commissions Regulation (EC) No. 208/2006.

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1. INTRODUCTION

So far, the prevalence, amplification and evolution of resistant pathogenic microorganisms strains in biowaste [1-3] has been globally researched, confirmed and well documented. While many studies recommend the use of biowaste as feedstock in anaerobic digestion as the best strategy in solving the challenge of managing incresingly solid waste, solve energy shortage crisis through biogas and solve soil infertility through land application of digestate, to most developing countries, commingled dumping of waste coupled with poor management applied to other waste streams such as wastewaters [4] biomedical waste [5, 6] and industrial generated wastes [7] is still a common practice therefore the major contributor of pathogens in the biowaste and the involving health risks [8].

Pathogens attraction and proliferation in biowaste is supported by the fact that biowaste contain high biodegradable organic matter, acceptable range of carbon and nitrogen, enough moisture, compromised pH with elevated proportion of both macro and micro-elements provide enough nutrients and conducive growing environment [9]. In that regards, it is apparently infered that achieving the latter benefit remain in limbo owing to the fact that achieving a complete pathogens inactivation in the digestate is questionable especially in biotreatments operated at mesophilic temperature range. However, to most biotreatment facilities, Two-phase MAD operated as co-phased of hydrolysis reactor in tandem to methanation reactor both phases operating at 30 ±7 ºC temperature range similar to the optimal microbial growth temperature is the most popular system in biowaste treatment being cartegorized as stable and cost-effective option given the less energy input required [10]. The survival possibility of potentially infectious pathogenic microorganisms in digestate produced from this technique raises concern on the safety aspect of both the biofacility operators during biotreatment process and to farmers during handling and application of the resulting digested as biofertilizer to cropland, gardens and direlict soil as soil conditioner which concequently pose a safety concern to the environment, food chain and general public [11-13].

Pasteurization which entails heating of material to temperature below boiling point and hold for some specified time has for many years remained the most commonly used method for hygienization in many biological treatment facilities in which a separate pasteurization unit is connected either before or after the MAD reactors set-up. However, numerous national proposed unharmonized temperature-time combination are in operation with no scientific proof and verified efficacy at producing pathogens free digestate. On the other hand, not all anaerobic digestion treatment facilities comply to the set pasteurization regimes due to the fact that an application of separate heat increase facilities operation costs and if not effectively monitored could affect the life of useful digesting microbes (the acidogens and methanogens), facilitate oxidation of methane. This study therefore investigated the hygienization potential of Two-phase MAD operated as continuous stirring tank reactor with semi-continuous feeding and validates the temperature-time combination necessary to induce a complete enteropathogens die-off in digestate for safer biological treatment, land application and general environment.

2. MATERIALS AND METHODS

2. 1. Feedstok for anaerobic digestion

Potato (*Solanum tuberosum*) peels were used as biowaste and feedstock for the Two-phase MAD. The peels were obtained from a restaurant dealing with preparation of potatoes based recipes (Kartoffelkiste, Spremberger Str. 37, 03046 Cottbus). To eliminate traces of background bacterial counts, once received, the peels were double cleaned using warm water. The fresh peels were then blended (Multipro compact blender) to minimize sample heterogeneity and reduce the size to 10-12 mm as recommended in Regulation (EC) No. 208/2006 and SANCO 7066/2010. Approximately 25g of the blended peels was weighed (Sartorius balance LP 4200S) precision 4200 ±0.1g and suspended in 500 mL capacity flasks containing 247.5 mL of sterilized water (pH 7.3 ±1.1; conductivity 514 µS/cm) prepared in-house as recommended elsewhere [14].

2. 2. Simulation of pathogens contamination in biowaste

Characteristics
Temperature = 37 ºC
pH = 6.5
Salinity = 1.1 ‰
Conductivity = 2.3 mS/cm
E. coli : 5.01×10^{8} CFU/g
Salmonella Senftenberg 6.31×10^{8} CFU/g

Figure 1. Enteropathogens spiked potato peels used as feedstock in Two-phase MAD reactor

Two indicator bacteria strains *Escherichia coli* (*E. coli*) and *Salmonella* Senftenberg (*Salmonella* Senftenberg) were used. *E. coli* was supplied from Leibniz Institute (Germany) while pure culture of *Salmonella* Senftenberg was supplied from The Institute of Bioanalysis, Environmental toxicology and Biotechnology (Germany). The choice for using *E. coli* and *Salmonella* Senftenberg was based on the fact that the two enteropathogens have specific and understandable detection methods, and that *Salmonella* Senftenberg has been named in EU 2000 working document as an indicator and surrogate microbe that reflects conditions to be met for inactivation of other heat-resistant bacterial species. Both bacterial strains were preserved

at 4 °C for the whole research period except during enrichment where strains were aseptically thawed at room temperature 23.2 ±1.8 °C. Prior to innoculation, the strains were aerobically enriched in 50 mL of Nutrient Broth (Carl Roth GmbH +Co. KG Schoemperlen, Karlsruhe) followed by incubation at 36 ºC to stationary phase as described in Brown [15]. Simulation of contaminated biowaste was prepared through artificial spiking of the blended potato peels (feedstock) (see Figure 1) with 2.5 mL of pre-enriched bacterial cocktail suspension to a load contamination of 8.7 log *E. coli* and 8.8 log *Salmonella* Senftenberg

2. 3. Set-up and operation of a Two-phase MAD system

A pilot-scale set-up of a Two-phase MAD operated with semi-continuous feeding using potato peels (23-25% TS) was used in a completely stirred-tank reactor model. In this set-up, hydrogenesis and methanogenesis process takes place in separate bioreactors. Two insulated plastic containers (high density polyethylene) having 70 L capacity each were used as bioreactors at 50 mL active volume. The organic loading rate (OLR) of 0.5 kgoTS/m^3.day proposed in the VDI-4630 (Association of Germany Engineers) [16] was maintained with an initial hydraulic retention time (HRT) of 25 days.

2. 4. Monitoring of hydrolysate and digestate quality and pathogens die-off in the bioreactors

The hydrolysis and methanation mesophilic anaerobic digestion system had been in operation for 15 days and 7 days respectively at working 37 ±2 ºC working temperature. Temperature and pH of hydrolysate (an effluent from hydrolysis reactor) and methanogenesis digestate (an effluent from methanogenesis reactor), biogas temperature, conductivity, salinity, biogas generation and total solids removal were analyzed on daily basis as part of the process monitoring activity. Samples for analysis of pathogens survivor in both hydrolysate and methanogenesis digestate were collected at least in an interval of 2 working days. The samples were serially diluted in 10-folds followed by triplicate plating of 100 µL of the hygienized slurry on both Endo agar and modified Brilliant Green (modified BG) agar. The petri-plates were incubated at 36 ºC for 24 hrs followed by enumeration of bacterial colonies on the following day. Temperatures in both slurry were monitored using automatic sensor inserted inside each bioreactor.

3. VALIDATION OF HYGIENIZATION TEMPERATURE-TIME REGIME

Pasteurization was used to effect hygienization in both hydrolysate and methanogenic digestate. A water-bath connected to a digitally controlled stirred water-bath and circulator (Grant Optima™ GD120) with both temperature and time displaying devices was used during hygienization process for heating and circulation of the heated water in the water-trough. The temperature of the thermostat was set at 2 °C higher than the desired hygienization temperature so as to accommodate heat loss due to water circulation in the pipes. The hygienization temperature in the sample as well as temperature of water in the water-trough was monitored by external thermometers. Two thermometers were used for recording the temperature. One of the thermometers was placed in the water-trough in order to control circulating water temperature while the other was for recording the attained temperature within the individual

flasks. The minimum and maximum hygienization temperatures were chosen to be 50 °C and 70 °C respectively. The 50 °C was selected as initial heat as per SANCO/7066/2010 based on empirical researches on anaerobic digestion reported from The Netherlands, Belgium and Germany and also the fact that this temperature is 4 °C higher than 46 °C reported as the maximum survivor temperature of *E. coli* [17, 18] which in this investigation represented the most heat sensitive pathogens.

The maximum temperature set of 70 °C was selected following the lethal temperature stated in European Commissions Regulation (EC) No. 208/2006. In each sample run, the hygienization countdown time commenced after the sample in individual flask has attained the targeted temperature at the specified hygienization holding time. The samples were taken for batch-pasteurization process at 50 ºC, 55 ºC, 60 ºC and 65 ºC using similar time of 30min, 60min and 24hrs. In this experiment the population of survived *E. coli* and *Salmonella* Senftenberg in the hydrolysate and digestate were used as the initial pathogens load to be inactivated. Researches including Funke-Whittle and Insam [19] concur that *Salmonella* Senftenberg is the most resistant specie to pasteurization compared to other enteropathogens.

Thus, in this experiment, the results derived from thermal reduction curves of *Salmonella* Senftenberg were used for the validation of the temperature-time combination that is effective at ensuring a complete die-off of the pathogens supposing that the temperature that is effective at effecting a complete elimination in *Salmonella* Senftenberg will be equally sufficient to completely eliminate the *E. coli*. Validation process was performed as described in Konieczka and Namiesnik [20]. Validation parameters were calculated based on analysis of variance for 5 replicates so as to maintain both accuracy and precision in results as recommended in Regulation (EC) No. 208/2006 and data were processed applying F_{max} - Hartley's test at a significance level $\alpha = 0.05$. Since the levels of bacteria die-off in the batch hygienization vessels differed, the calculations were therefore based on the values of coefficient of variation (CV) of the survived *Salmonella* Senftenberg colony units based on the three lowest hygienization temperatures of 50 ºC, 55 ºC and 60 ºC.

3. 1. Decimal reduction time (D-value) and bacterial survivor

The hygienization temperatures in which *Salmonella* Senftenberg survived were used in establishing the decimal reduction time (D-value). This is the time required to traverse 1-log cycle or effect a 90% die-off in pathogens from the pasteurized sample at a given temperature (Mazzola et al., 2003). Five replicates that were used in the investigation of hygienization experiment were used applying a first–order kinetic equation as stated in Murphy, et al. [21].

$$\frac{dN_t}{dt} = -kN_0$$

where:

N_t = viable *Salmonella* Senftenberg colony units at specific given time, k = the reaction rate constant in (1/min), N_0 = original population at the beginning of the experiment.

The counts of viable *Salmonella* Senftenberg colonies after hygienization were then plotted against residence time specified to be 0 min, 30 min and 60 min, and the D–value calculated therefore as a negative inverse of the slope of the best-fit-line for log-reduction in bacteria colonies against the sample residence time.

3. 2. Thermal Inactivation Kinetics (z-Value) and bacterial survivor

Thermal resistance constant, z-value which is the increase in temperature required to traverse 1-log cycle or effect a 90% inactivation was investigated in *Salmonella* Senftenberg population from the pasteurized sample at a given D-value. This was calculated as a negative inverse of the slope of the best-fit-line for log D-values obtained from the four selected temperatures against the varied temperature from bacterial incubation i.e., 36 ºC and pasteurization, i.e., 50 ºC, 55 ºC and 60 ºC applying the following equation proposed in Manas et al. [22].

$$\log_{10} D(T_t, T_g) = \log_{10} D(T_{50°c}, T_{36°c}) - T_t / Z$$

where:

$\log_{10}$ D(36 ºC; 50 ºC) is the theoretical decimal reduction time of *Salmonella* Senftenberg population density at the specified temperature, Tg = 36 ºC is the temperature used for enrichment of *Salmonella* Senftenberg culture Tt = is the treatment temperature i.e., 50 ºC and z = is the negative inverse of the slope.

In this experiment, the z-Value describes the influence of temperature on the decimal reduction time for *Salmonella* Senftenberg population. The logD-values versus three temperatures, i.e. 36 ºC, 50 ºC and 55 ºC were used to calculate the z-values.

4. RESULTS AND DISCUSION

4. 1. Digestate quality and pathogens die-off in Two-phase MAD

Characteristics
Temperature = 37 ºC
pH = 4.2
Salinity = 1.1-2.7 ‰
Conductivity = 2.3-5.5 mS/cm
E. coli : 1.1×10^5 CFU/g
Salmonella Senftenberg 7.9×10^4 CFU/g

Figure 2. Quality of hydrolysate from hydrolysis reactor used as feedstock for methanation reactor.

Characteristics
Temperature = 36.8 ºC
pH = 7.7
Salinity =5.6-7.3‰
Conductivity = 9.7-12.5 mS/cm
E. coli: 2.5×10^6 CFU/mL
Salmonella Senftenberg: 1.3×10^6 CFU/g

Figure 3. Quality of final digestate from methanation reactor.

Physico-chemical and bacteriological characteristics of the hydrolysate and digestate collected from the Two-phase mesophilic anaerobic digestion set-up were as shown in Figure 2 and 3 respectively. The hydrolysate was milky in colour with visible stachy granules both in suspension and some settled at the bottom of the sample collecting beaker an indicator of starch hydrolysis. The observed small incease in salinity and conductivity in hydrolysate was a result of hydrolysis of starch that exposed extracted minerals (both macro and micro-nutrients) present in the potato peels. The digestate from methanation reactor was deep-blackish in colour depicting the colur of innoculum used which equally influenced changes in both salinity and conductivity by an average of 4.5 units and 6.5 units respectively above the hydrolysate.

The pH values in both cases presented the ideal bacterial working ranges. In hydrolysate, the acidogens (facultative and obligatory anaerobic bacteria) cause fermentation of the starch and carbohyrate to form short-chain organic acids resulting into acidic condition. The acid formed during the hydrogenesis stage was responsible of the observed drop in pH of the hydrolysate from 6.5 to 4.2. The low pH formed affect enzymatic activity in microorganisms due to the fact that each microbe has its optimum pH for survive thus, the pathogens counts in hydrolysate that survived in such low pH decreased 10^3-folds in *E. coli* resulting into 1.1×10^5 CFU/g while *Salmonella* Senftenberg was highly affected by the observed pH drop causing 10^4-folds load decrease from the original spiked to 7.9×10^4 CFU/g. The rise in pH of the mathanogenic digestate induced favourable growth condition which resulted into an increase in *E. coli* by 10^1-fold while in the case of *Salmonella* Senftenberg a 10^2-folds increase was equally observed. As the concentration of fatty-acid in the hydrolysate increases gradually following the rate of fermentation [23, 24], so is the acid-adaptability of the pathogens. Acid adapted microbial cells tends to have increased resistant to pH changes [23].

This happens following increased cyclic fatt-acids and decrease the ratio of unsaturated fatty-acid to saturated fatty-acids in the bacteria cells that brings about a loss of fluidity in the bacterial cell membrane and therefore induced the resistance [25]. On the other hand, Jay et al., [26] observed that the presence of simple sugars in the order of sucrose > glucose > sorbitol >

fructose > glycerol in the digestate increases resistance to pathogens suspended therein. Therefore, high carbohydrate content of about 18% in potato peels out of which 89% is starch which is easily converted into simple sugars during fermentation could be the reason contributed much to the observed resistance and low pathogens die-off in the methanogenesis stage.

4. 2. Pathogens die-off at various temperature-time combination

The results in Table 1 present minimum and maximum colonies for *E. coli* and *Salmonella* Senftenberg survived at four different temperatures: 50 ºC, 55 ºC, 60 ºC and 65 ºC and three different holding time: 30 min, 60 min and 24 hrs. There exist threshold temperature-time combination to which every pathogen can be inactivated [27, 28]. Subjecting bacterial cells at heat above the threshold temperature denatures their enzymes and in the case of prolonged exposure to the temperature higher brings about a complete inactivation.

Table 1. Enteropathogens die-off (logCFU/mL) in digestate at varied temperature-time combinations

Temperature-Time regime	**Bacterial Survivor (logCFU/mL)**			
	Salmonella Senftenberg		*E. coli*	
	Min	Max	Min	Max
50 °C for 0 min	6.0	6.2	6.1	6.7
50 °C for 30 min	6.3	6.5	5.7	6.3
50 °C for 60 min	5.7	6.0	3.3	5.2
50 °C for 24 hrs	5.8	7.4	Ø	Ø
55 °C for 30 min	4.2	5.2	Ø	2.4
55 °C for 60 min	2.7	4.9	Ø	Ø
55 °C for 24 hrs	Ø	0.9	Ø	Ø
60 °C for 30 min	Ø	2.6	Ø	Ø
60 °C for 60 min	Ø	0.3	Ø	Ø
60 °C for 24 hrs	Ø	Ø	Ø	Ø
65 °C for 30 min	Ø	Ø	Ø	Ø
65 °C for 60 min	Ø	Ø	Ø	Ø
65 °C for 24 hrs	Ø	Ø	Ø	Ø

Ø = no growth of bacteria colony was observed, (n = 5)

The maximum enteropathogens survived the mesophilic anaerobic digestion process and therefore observed in the final digestate was 1.3×10^6 CFU/mL for *Salmonella* Senftenberg and 2.5×10^6 CFU/mL for *E. coli*. This initial population was therefore subjected to pasteurization in which an initial temperature of 50 °C was selected. It was observed that both pathogens survived the first initial pasteurization test for a temperature-time combination sets of 50 °C for 30min up to 60min of holding time. After 60min, there was still 6.31×10^5 CFU/g of *Salmonella* Senftenberg *and up to* 1.78×10^4 CFU/mL of *E. coli* in the digestate.

From a practical viewpoint, it was worth noting that there exists statistical significance difference ($p<0.05$) in the average heat resistance between *Salmonella* Senftenberg and *E.coli* treated at the same temperature and time combination as similarly reported in Mitscherlich and Marth (1984). At the onset of the 60 min, the population of *Salmonella* Senftenberg (average 6.1 log in the digestate) was not significantly affected ($p>0.05$) by hygienization heat treatment. *E. coli* population was significantly affected ($p<0.05$) by hygienization at the same temperature and holding times equally to what other previous studies [29] confirm.

Being heat sensitive microbe, *E. coli* was highly reduced by more than 2-folds after 60 min of pasteurization at 50 °C. While *Salmonella* Senftenberg was observed to surveve up to a pasteurization temperature of 60 °C for 60 min holding time, the observed complete elimination of *E. coli* from the suspension at 50 °C for 24 hrs confirmed its low resistance to heat.

4. 3. Effectiveness of batch hygienization on pathogens die-off at varied Temperature-Time regime

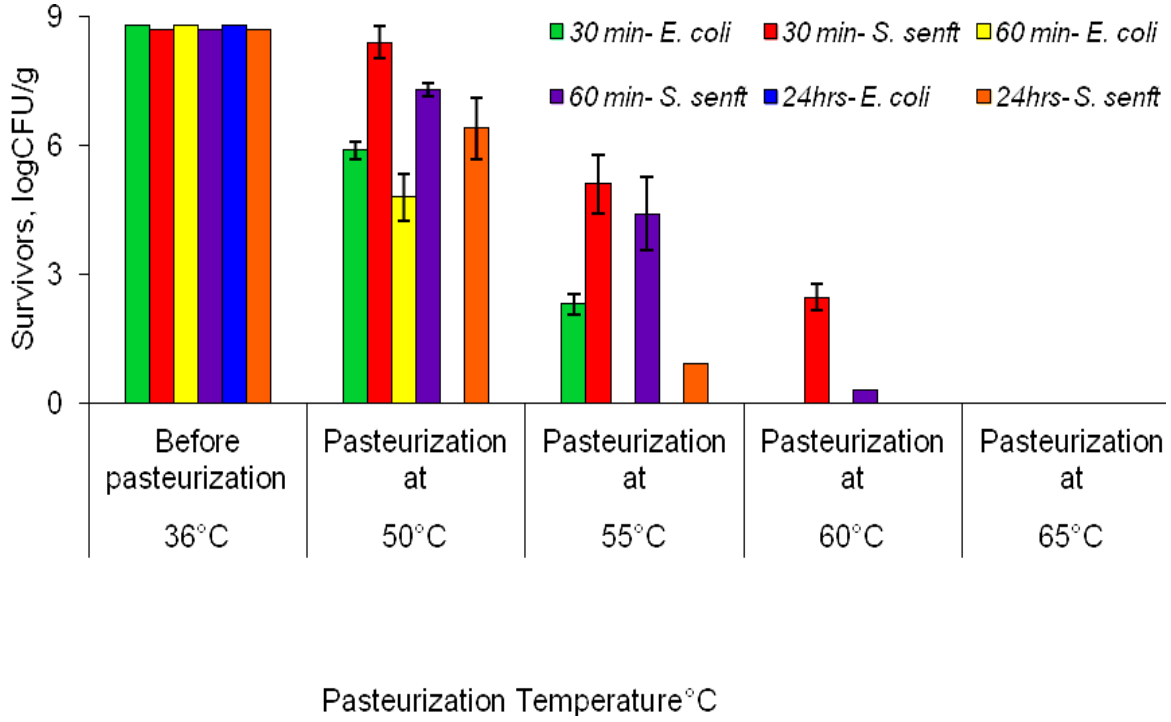


Figure 4. Mean log reduction of *E. coli* and *Salmonella* Senftenberg following batch hygienization at varied temperature-time regime

Confidence interval values for log reduction at 95% confidence level for *E. coli* and *Salmonella* Senftenberg survivor after hygienization process at various temperature and time combination are shown in Figure 4. The average results of independent batch hygienization (n = 5) shows reduction of the pathogens at various rate starting at 50 °C. At this initial minimum set temperature, the 30 min residence time was enough to cause about 2.8-3.1 log reduction in *E. coli* while for *Salmonella* Senftenberg only 0.2-0.5 log reduction was observed. *E coli* was even more affected following a prolonged holding time to 60 min in which the reduction reaching 3.5 to 5.5 logCFU/mL while that for *Salmonella* Senftenberg showed a die-off range of only 1.1-1.8 logCFU/mL.

Even at the longer retention of 24 hrs at 55 °C, the highest reduction in *S. senftenbergensis* only reached 1.3-3.3 log but a complete elimination was yet to be attained unlike in the case of *E. coli* which was completely inactivated during the 24 hrs sample holding time at even lower temperature of 50 °C. Sample hygienization at 55 °C and 30 min highly affected *E. coli* population as there was an average of only 2.1 logCFU/mL of the surviving cells. This demonstrated as great as >6.0 log die-off equivalent to 99.99% inactivation which is an acceptable level in conformity to the Regulation (EC) No. 208/2006 and DG ENV.C2/BZ/tb [28] as microbiologically safe in case the pasteurized material is to be used for purpose such as farmlands and/or application on pastures to be used for grazing with an exception to garden of raw eaten leafy vegetables. In other words, any biotreatment of biowaste contaminated with *E. coli* is capable at equally eliminating this enteric microorganism provided the temperature in the reactor is elevated to 55 °C and retained for at least 30 min without interruption.

Mitscherlich and Marth (1984) equally reported the survive time for *E. coli* to be 15 min at 54.5 °C. Addition of heating temperature above 55 °C confirmed the heat sensitivity of *E. coli* and there was no further resistance demonstrated or re-growth of *E. coli* in the other temperature-time combination further investigated. Hygienization at 55 °C for 30 min and 60 min though much reduced *Salmonella* Senftenberg up to the range of 3.6-5.3 log and 3.8-6.4 log respectively; there was still no complete elimination of this bacterium until when the hygienization time was prolonged up to 24 hrs. Major reduction in *Salmonella* Senftenberg was achieved following hygienization at 60 °C for 60 min in which the survived colonies were counted to be 0.3 log maximum in which 2 colony units were observed in one of the five samples pasteurized. Thereafter a 60 °C hygienization for 24 hrs was equally enough to ensure a complete inactivation of *Salmonella* Senftenberg. In this investigation, a hygienization process at 65 °C even at the lowest sample holding time of 30 min proved to be sufficient for providing a complete elimination of *Salmonella* Senftenberg. These results correlate well to the findings reported in Mitscherlich and Marth [30] in which the survival time of *Salmonella* Senftenberg in milk following heat treatments took more than 60 min at 54.4 °C, but the time was reduced to15min upon raising the temperature to 61.6 °C, and even more shorter as 2 min at 65.5 °C.

4. 4. Enteropathogens die-off in hydrolysate following post-biotreatment batch hygienization

The pathogens inactivation results expressed as confidence interval for 95% confidence for hygienization of hydrolysate and methanogenic digestate from the Two-phase MAD are presented in Figure 5 and 6 respectively. Regardless of the fact that equal volumes of bacteria suspension were added in both reactors, the pathogens density in hydrolysate was observed to be in the range of $\times 10^4$ CFU/mL and $\times 10^5$ CFU/mL for *Salmonella* Senftenberg and *E.coli*

respectively. This level of contamination was lower compared to the population of $x10^6$ CFU/mL observed in the methanogenic digestate for both *E. coli* and *Salmonella* Senftenberg. Several reasons could be responsible for the observed low population in the hydrogenesis reactor. Firstly it could be caused by acid nature of the suspended menstruum at low pH value of 4.7 following substrate fermentation. This pH is closer to the minimum reported pH for which the two enteropathogens can tolerate and regenerate, i.e. pH 4.4 for *E. coli* and pH 4.0 for *Salmonella* Senftenberg [31]. Secondly could be as results of H_2S toxicity in the hydrolysis reactor which was measured above 5000 ppm. Also the low C/N ratio in potato peel means higher nitrogen content which during fermentation process results into more NH_3 which is equally toxic to the microbial community in the reactor. Therefore, the combination of these factors together with microbial antagonism might have contributed to the observed difference in the initial pathogens population.

Despite the observed differences in initial bacteria densities in the hydrolysate and methanogenic digestate, the inactivation trends following batch-pasteurization was very similar. *Salmonella* Senftenberg continued to be the most resistant enteropathogen following this heat treatment. During post-treatment hygienization of the hydrolysate, no much of *Salmonella* Senftenberg inactivation was achieved at 50 °C for 30 min sample residence as there was only 0.1 log reduction from the initial bacterial population.

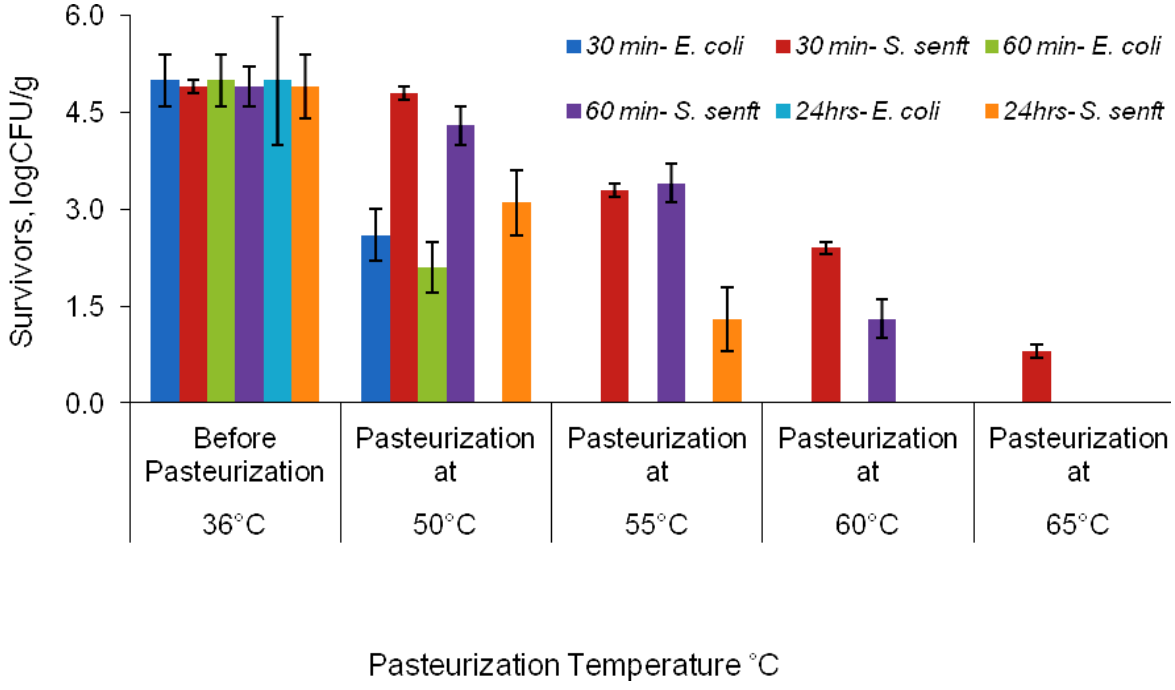


Figure 5. Mean log reduction of *E. coli* and *S. senftenbergensis* in acidogenic digestate of Two-phase MAD.

In *Salmonella* Senftenberg, a reduction of about 0.3 logCFU/mL was achieved at sample holding time of 60 min and even under prolonged residence time of 24hrs there was only 1.8 logCFU/mL reduction. *E. coli* being highly sensitive to heat treatment was highly affected

starting at 50 °C for 30 min as there was above 2.6 log die-off that increased even more to above 3.0 log following 60 min of sample holding time with no survivors after 24 hrs. Hygienization at 55 °C and 60 °C at 30 min were much effective for *E. coli* but not for *S. senftenbergensis* as there was still existing between 2.4 log to 3.3 log of the pathogen in the hydrolysate.

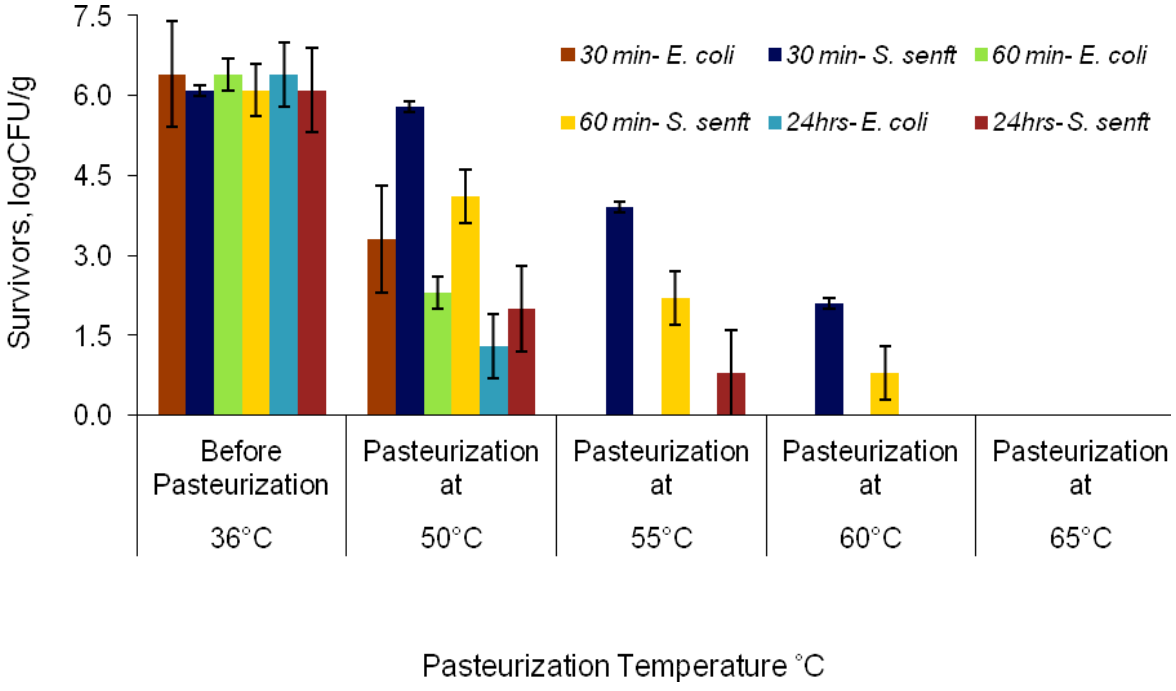


Figure 6. Mean log reduction of *E. coli* and *S. senftenbergensis* in digestate of Two-phase MAD.

On increased hygienization holding time to 60min, the massive reduction in *Salmonella* Senftenberg of above 3.6 logCFU/mL was achieved. While hygienization temperature of 60 °C for 60 min was sufficient to completely assure biosafety of the digestate in the case of *E. coli*. Surprisingly few population of about 20 CFU/mL *Salmonella* Senftenberg were observed after 55 °C hygienization for 24 hrs and it was observed during the hygienization of the hydrolysate that two petri-plates out of 5 incubated with sample from 65 °C for 30 min had 2 colonies each of *Salmonella* Senftenberg at 10^{-2} dilution level. The possible explanation for this observation could be as a result of the increase in resistance of the *Salmonella* Senftenberg cells following their adaptation to low pH. It is reported that acid-adapted bacteria cells develop higher cyclic unsaturated fatty acid up to 2-folds of the ratio of unsaturated fatty acid to that of saturated fatty acids [23], this change in fatty acids composition in the bacteria cells induces low membrane fluidity in bacteria cells hence becomes a factor behind the increase in heat resistance. Another possibility could be the fact that the hydrolysate was collected after 15 days of the anaerobic digestion process meaning that most of the bacteria cells whose regeneration time is between 5-16 days had reached stationary phase of growth. At this growth phase, bacteria are reported to be more resistance to both low pH and heat. The survived *Salmonella* Senftenberg validate the temperature-time regime of 70 °C at 60 min pasteurization residence time set by the

Regulation (EC) No. 208/2006 for effectively elimination of thermo-tolerance pathogenic microorganisms and assurance of biosecurity in digestate. Although the post-treatment batch hygienization results for final digestate was similar to those obtained during post-treatment batch hygienization of hydrolysate, there was one exception in which the hygienization temperature of 50 °C for 24hrs that showed to be effective at causing complete elimination of *E. coli*, its trend was not the same as in this experiment, yet there was about 1.3 logCFU/mL equivalent to 20 CFU/mL of *E. coli* in the suspending menstruum. Although the factor of induced tolerance could be the reason behind, the other possibility could be the growth of other *E. coli* species initially present in the cow manure slurry used as seeding for the inoculation of methanogenesis reactor. Similarly to the results observed in Ceustermans et al., [32], pasteurization at temperature >50 °C is confirmed to fully eliminate *E. coli* from the final digestate.

4. 5. Findings from temperature-time validation process

The calculated F_{max} was observed to be smaller than the critical Hartley's F_{maxo} for significance results at $\alpha = 0.05$ (see Table 2). This confirmed no significant differences ($p>0.05$) on the average die-off of *Salmonella* Senftenberg within-runs. Variation existed on the compared coefficient of variation (CV %) of *Salmonella* Senftenberg reduction $\log(N_o/N_t)$ between treatments at any given temperature and at every varied hygienization holding time. A negative linear relationships was obtained between the *Salmonella* Senftenberg survived against time of hygienization confirming a decreasing rate in bacteria population. D-Values for *Salmonella* Senftenberg in suspended menstruum at 50 °C, 55 °C and 60 °C were calculated from the lnear regression equations as negative inverse of the slope.

Table 2. Validation parameters for *Salmonella* Senftenberg following batch hygienization

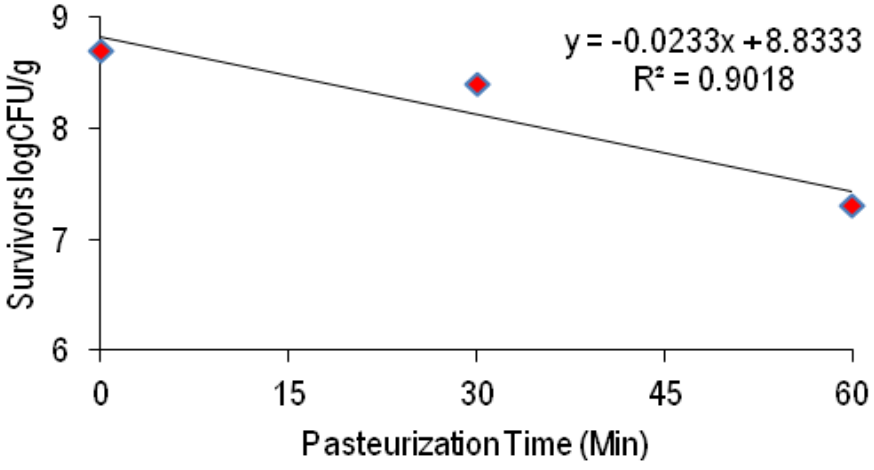
Treatment	**1**			**2**			**3**		
Sample size (n)	5	5	5	5	5	5	5	5	5
Temperature (°C)	50	50	50	55	55	55	60	60	60
Holding time (min)	0	30	60	0	30	60	0	30	60
Bacterial density (logCFU/mL)	8.7	8.4	7.3	8.7	5.8	4.4	8.7	2.5	0.3
Standard deviation	0.14	0.12	0.49	0.14	0.07	0.52	0.14	0.09	0.11
CV, %	1.6	1.4	6.7	1.6	1.2	11.8	1.6	3.6	36.7
F, *max*	4.8			9.8			22.9		
F, *maxo* (n = 5, df = 4, α = 0.05)	25.2			25.2			25.2		

As shown in Table 3, in order for the population of *Salmonella* Senftenberg to traverse 1log cycle reduction from the original population, pasteurization at 50 °C yield a $D_{50\,^{\circ}C}$-value of 44minutes, while that at 55 °C showed a $D_{55^{\circ}C}$-value of 14minutes and the one at 60 °C gave a $D_{60\,^{\circ}C}$-value of 7minutes so as to achieve the same hygienization effect with a high coleration coeficient r ≥0.95 between the survivors rate and time. This teperature-time regime required for 90% inactivation of *Salmonella* Senftenberg at 60 °C agrees well to the finding reported by other researcher [23] who reported $D_{63\,^{\circ}C}$–value of 6.27 minutes for *Salmonella* Senftenberg. As described elsewhere [33], a high value of validation parameters including strong correlation coefficient showed by high coefficient of determination (R^2 ≥0.90) confirmed linearity while high precision was infered by the small percent in relative standad deviation expressed as coefficient of variation to all D-value plots within runs (see Figure 7).

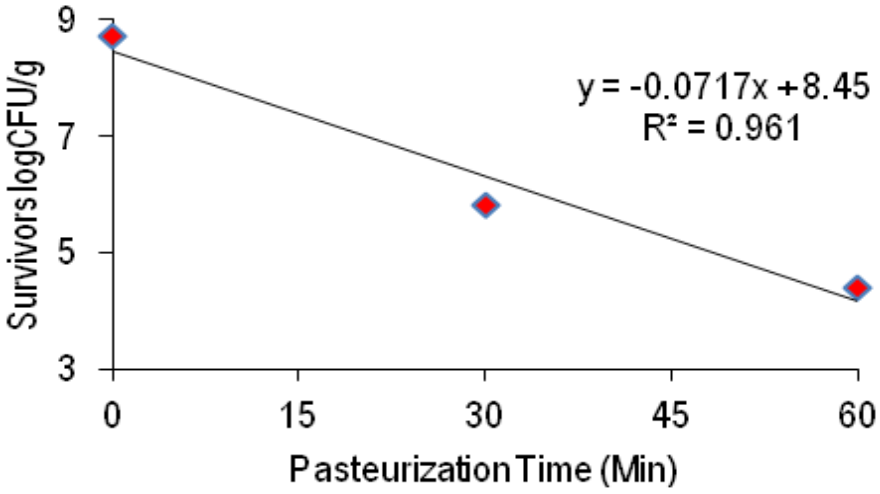
Right from the minimum temperature of 50 °C from the survivor curves, it was observed that the D-value for *Salmonella* Senftenberg was independent and did not change owing to the initial cells population. The results shows that it requires 44 min at the temperature of 50 °C for the population of *Salmonella* Senftenberg to be eliminated by 90%, compared to *E. coli* in which D-value graphically determined (Figure 8) shows that at the same minimum temperature of 50 °C, the time required is approximately 10 min.

Table 3. Regression equations and the D-values for *Salmonella* Senftenberg in suspended menstruum at various hygienization temperatures

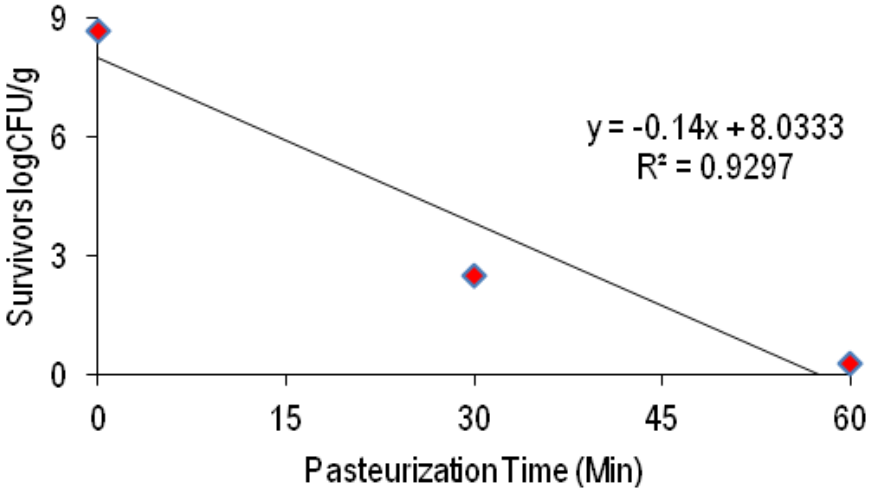
Temperature	Regression equation	Correlation coefficient (r)	D-Value (Min)
50 °C	y = -0.023x + 8.833	0.95	44
55 °C	y = -0.071x + 8.450	0.98	14
60 °C	y = -0.140x + 8.033	0.96	7



(a) $D_{50\,^{\circ}C}$ – value



(b) $D_{55\,^{\circ}C}$ – value



(c) $D_{60\,^{\circ}C}$ – value

Figure 7. Decimal reduction time (D-values) of *Salmonella* Senftenberg at varying pasteurization temperature

This confirms that at this same temperature, *Salmonella* Senftenberg requires 5-folds more of the D-value of *E. coli* for the same 1 log-cycle reduction of the initial bacteria population hence confirms the validation process for *Salmonella* Senftenberg on the thermal destruction (z-Value).

4. 6. Thermal destruction (z-Value) for *Salmonella* Senftenberg

z-Value refers to the temperature increase necessary to bring about a 1 log-cycle change in D-value during hygienization [34]. This temperature is normally calculated as the negative reciprocal of the slope of the thermal death (D-value) against temperature as shown in Figure 9 for *Salmonella* Senftenberg.

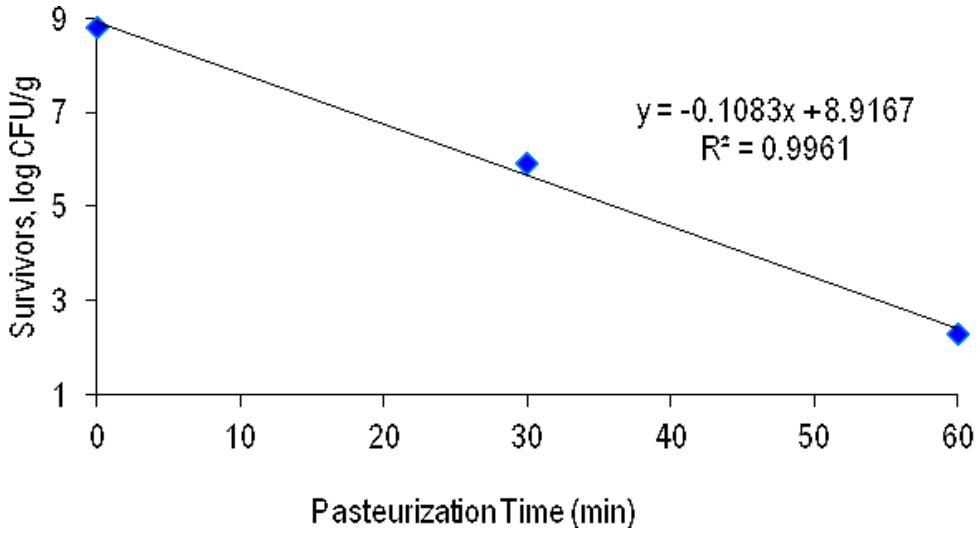


Figure 8. Decimal reduction time ($D_{50\,°C}$ - value) of *E. coli*

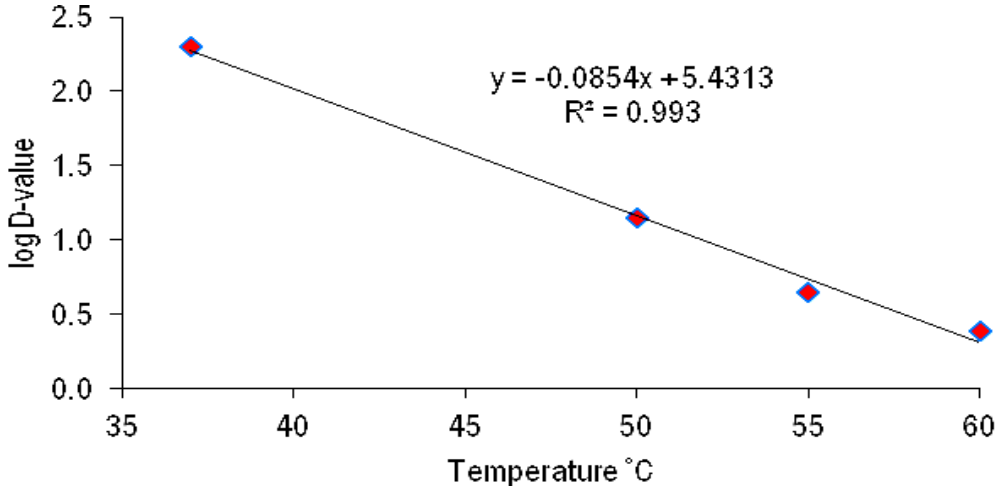


Figure 9. Thermal destruction z-Value for *Salmonella* Senftenberg

The log(D-value) against temperatures calculated from hydrolysate samples revealed an increase in resistance of microorganism to the different heating doze applied. The z-Value result in the graph above determined as the negative inverse of the slope of the regression equation equivalent effectiveness of hygienization in case various temperatures are to be assessed. In this case, since 300 min hygienization at 55 °C gave 90% elimination of *Salmonella* Senftenberg in a batch hygienization experiment, a z-value calculated gave three possibilities in which 3min at 75 °C, 65 °C at 30 min or 300 min at 55 °C could effect the same level of 90% die-off in *Salmonella* Senftenberg. The choice of temperature-time combination depends much on the digestate biosafety and the desired application of the final digestatewas therefore found to be 11 °C. From the conducted batch hygienization experiment, it took 6hrs for the process to

achieve 90% die-off in *Salmonella* Senftenberg at a hygienization temperature of 55 °C. Although it was observed that heating the suspending menstruum samples of potato peels at 65 °C for 30 min effectively eliminated both *E. coli* and *Salmonella* Senftenberg, hygienization of the hydrolysate required even higher temperature of 70 °C with 30 min holding time. Therefore, the z-value obtained is helpful in determination of the.

5. CONCLUSIONS

Unless external pasteurization of the final digested produced using mesophilic anaerobic digestion method is done, the biosafety of final digestate from Two-phase MAD is confirmed to be questionable. This is due to high levels of enteropathogens confirmed to have passed the Two-phase MAD system to the final digestate unaffected. Given the shorter residence time of digestate in the bioreactors, the presence of both *E. coli* and *Salmonella* Senftenberg in the final digestate confirm the inefficient of mesophilic anaerobic digestion at effecting self-hygienization.

The survived *Salmonella* Senftenberg cell in the final digestate at various temperature-time combination tested validates the temperature-time regime set by the EU Regulation (EC) No. 208/2006 of heat treatment of 70 °C with a holding time of 60 min for effectively elimination of the pathogens and assure biosafety in the resulting digestate. Eventhough hygienization may be costly in case where external heating energy has to be used, this research hereby recommend that if pasteurization is the chosen hygienization option, based on the validated temperature-time regime, the biotreatment facilities should abide on the temperature-time combination of not less than 70 °C for 30 min in biowaste confirmed to contain non-spore forming bacteria and to some places where there is scientific based evidence about the presence of more heat resistant microorganisms, then the temperature - time regime of 70 °C or higher and 60 min holding time should be used by every biotreatment facility using mesophilic anaerobic digestion technology.

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