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Original Research

Analysis of the Impact of Factors Affecting Survivability of Bacteria from the *Enterobacteriaceae* Family during Sewage Sludge Composting

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Abstract

The objective of the performed investigations was to assess the influence of the value of temperature, pH, gas emissions, and doses of BAF microbiological inoculum on the reduction or total elimination of bacteria from the *Enterobacteriaceae* family.

The performed experiment consisted of two stages. In the course of the first trial, sewage sludge was subjected to composting together with straw and sawdust in a cybernetic bioreactor. The experimental design included three treatments, two of which were inoculated twice with BAF inoculum: K1 – control, compost without inoculation; K2 – compost+1500 ml BAF; K3 – compost+750 ml BAF.

The BAF (Bacteria-Actinomycetes-Fungi) inoculum was designed at the Department of General and Environmental Microbiology of Poznań University of Life Sciences and was made up of bacterial strains, actinomycetes, and mould fungi isolated from mature compost.

The microbiological analyses applied in this experiment involved determination of total counts of bacteria belonging to the *Enterobacteriaceae* family using, for this purpose, Koch's plate method followed by biochemical tests confirming species membership of the above-mentioned bacteria.

In the second part of the experiment, bacteria from *Escherichia*, *Klebsiella*, and *Citrobacter* genera isolated from mature composts were subjected to the ring method with the aim of examining mutual interactions between the above-mentioned bacterial species and the BAF inoculum.

On the basis of the obtained research results, it was found that the recorded reduction in numbers of the discussed microorganisms was caused by physico-chemical factors of the composting process, especially by the inoculation of biowastes by the BAF biopreparation.

The performed *in vitro* analysis of interactions between the *Escherichia coli*, *Citrobacter freundii*, and *Klebsiella pneumoniae* strains and the applied BAF inoculum revealed considerable sensitivity of the above strains to the experimental biopreparation.

However, in *in vivo* conditions, *E. coli* bacteria exhibited resistance to the action of the above dose of the inoculum. Therefore, continuation of the above investigations appears necessary.

Keywords: *Enterobacteriaceae*, inoculum, sewage sludge, compost

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Introduction

Natural utilization of organic wastes, fully justified economically, brings with it a certain number of positive changes in the environment. However, it should also be remembered that huge quantities of organic matter and mineral components are introduced into the biological cycle, which, in conditions of deposition, may constitute a serious hazard for the environment [1].

Civilization and urban development of Poland contribute to the production of increasing quantities of sewage and sewage sludge [2]. It is evident from investigations of ecologists that there is not even one effective system of collection and utilization of communal wastes. Despite unquestionable improvement, Poland continues to experience an unfavorable situation regarding proper management of municipal wastes.

At the present time, deposition is the most popular method of sewage sludge management. Legal regulations of the European Union consider waste deposition as a necessary evil [3]. According to the act of 22 January 2010 (Directive from 2010, No. 22, Item 145) concerning the change of the Bill on Wastes (Regulation of the Minister of Economy and Labour from 7 September 2005 regarding criteria and procedures allowing waste deposition on dumps of various kinds – (Directive, No. 186, Item 1553) [4], sewage sludge not subjected to processing will not be allowed to be deposited. Therefore, it seems essential to develop effective methods of rendering sludge harmless in ways more friendly to the environment. One such method is the composting process that, according to Krzywy et al. [5], appears to be the most popular method of sludge stabilization to date.

Déportes et al. [6] maintain that, depending on the kind of composted wastes as well as the employed composting process technology, the obtained end-product – compost – may still contain compounds detrimental to the environment such as heavy metals, toxic organic compounds, or pathogenic microorganisms.

It is evident from a literature review that sewage sludge subjected to the composting process can contain pathogenic microorganisms, e.g. bacteria from the *Salmonella* genus [7-9], bacteria belonging to the *Enterobacteriaceae* family [10-13] as well as eggs of parasites from *Ascaris*, *Trichuris*, and *Toxocara* genera [14].

In order to prevent sanitary-epidemiological hazards associated with the application of wastes of animal origin, including those prepared on the basis of sewage sludge, a directive was introduced regulating the above issues: an order of the Minister of Agriculture and Rural Development regarding implementation of certain regulations of the Bill on Fertilizers and Fertilization [15].

It is clear from experiments carried out by Bustamante et al. [16] that the composting process of sewage sludge with some additives fails to reduce numbers of bacteria from the *Enterobacteriaceae* family; to the contrary, their numbers were found to have increased in mature compost.

On the other hand, Dröffner and Brinton [17] reported isolation of *Salmonella typhimurium* Q and *E. coli* B bacteria from industrial composts following a 59-day composting process despite application of the temperature of 60ºC.

Therefore, it appears from investigations of the above researchers that neither time nor temperature obtained during the composting process is among the major factors contributing to the elimination of pathogens from composts.

Turner et al. [18] suggest that such factors as moisture content of the material, release of ammonia, or activity of other microorganisms exerting an inhibitory effect on their development may contribute to the elimination of pathogens in composts.

It is evident from experiments carried out by Ichida et al. [19] that the application of microbiological inocula consisting of selected microorganisms resulted in complete destruction of pathogenic bacteria from, for example, the *Salmonella* genus.

Cotxarrera et al. [20] maintain that the introduction into composts of material containing selected inocula of saprophytic microorganisms leads to inhibition or complete eradication of pathogens. The above actions are based, among other things, on such processes as competition, antibiosis, parasitism, and predation.

The objective of the performed investigations was to assess the types of factors (chemical, physical, microbiological) that could affect reduction and elimination of bacteria from the *Enterobacteriaceae* family in composted sewage sludge.

Material and Methods

The experiment was established in 2009 in laboratory conditions. Investigations were carried out in four chambers of an isothermal bioreactor of 160 dm^3 each (Fig. 1), equipped with electronic sensors, making it possible to record some process parameters (temperature, carbon dioxide, ammonia). The construction of the employed bioreactor, in particular the thermal isolation used in it, created

Fig. 1. Schematic diagram of the 2-chamber bioreactor: 1. pump, 2. flow regulator, 3. flow meter, 4. isolated chamber, 5. drained liquids container, 6. composted mass, 7. sensors set, 8. air cooling system, 9. condensates container, 10. column of gases content analysis (NH_3, O_2, CO_2, CH_4, SH_2), 11. 16-channel recorder, 12. air pomp steering system (The construction – Instytute of Agricultural Engineering, University of Life Sciences in Poznań – dr hab. Jacek Dach).

Table 1. The contens of biowastes in composts.

Chamber	Sewage sludge	Straw	Sawdust	Water addition	Inoculum addition	C/N initial	C/N final
K1	45%	50%	5%	10,500 ml	-	17.05	13.00
K2	45%	50%	5%	9,000 ml	1500 ml	17.05	10.78
K3	45%	50%	5%	9,750 ml	750 ml	17.05	11.22

Table 2. The number of microorganisms (cfu·g^{-1} d.m) and eggs of parasites (piece·kg^{-1} d.m.) in biowastes used in the experiment.

Initial material	*Salmonella* spp.		*Enterobacteriaceae*		ATT	
	cfu*	SD**	cfu*	SD**	piece	SD**
Sewage sludge	0.00	0.00	88.05·105	7.01	0.00	0.00
Straw	0.00	0.00	0.00	0.00	0.00	0.00
Sawdust	0.00	0.00	0.11·103	0.001	0.00	0.00

*colony forming units
**Standard Deviation

conditions reflecting the course of the composting process in real conditions.

Materials for experiments were thoroughly mixed in a container proportionally to the weight share in relation to dry matter. The following materials were used to prepare the composting mixture: sewage sludge, straw (wheat), and sawdust. Their percentage weight shares were as follows: 45% sludge, 50% straw, and 5% sawdust (Table 1).

Table 2 presents microbiological properties of biowastes applied in the described experiments.

Materials composted in all chambers of the bioreactor were characterized by identical composition and were aerated by 4 l of air per minute. The experimental biowastes were inoculated with different doses of microbiological inoculum.

The microbiological vaccine used in the experiment was designed at the Department of General and Environmental Microbiology and was given the acronym BAF (Bacteria-Actinomycetes-Fungi). The developed biopreparation consisted of 15 strains of bacteria, 5 actinomycetes isolated from mature compost and the thermophilic fungus *Thermomyces lanuginosus*, which was isolated from composted material in a thermophilic phase. The above-mentioned strains were examined from the point of view of their proteolytic, ammonification, cellulolytic, amylolytic, and phospholytic activities. One millilitre of the applied biopreparation contained the following quantities of microorganisms: bacteria $1.76 \cdot 10^6$ cfu, actinomycetes $2.31 \cdot 10^3$ cfu, and molds $1.89 \cdot 10^2$.

The above inoculant was introduced into the composted materials in the amounts of 500 and 1000 ml at the initiation of the trial. The BAF inoculum was applied the second time at the termination of the thermophilic phase of the composting process, except that the thermophilic *Thermomyces lanuginosus* fungus was replaced by a mesophilic fungus from the *Trichoderma atroviride* species, which is characterized by strong cellulolytic properties.

At the beginning of the trial, the above inoculant was introduced according to the following pattern: K1 (control) – compost + 8000 ml tap water, K2 – compost + 7000 ml tap water + 1000 ml BAF vaccine, and K3 – compost + 7500 ml tap water + 500 ml BAF vaccine. Bearing in mind the declining volume of the composted biowastes, after the thermophilic phase of the composting process the BAF inoculum was applied at the level of 250 and 500 ml. The above biological preparation was introduced according to the following pattern: K1 (control) – compost + 2500 ml tap water, K2 – compost + 2000 ml tap water + 500 ml BAF inoculum, and K3 – compost + 2250 ml tap water + 250 ml BAF inoculum.

Compost samples indispensable for carrying out microbiological and biochemical analyses were collected in accordance with the Polish standard PN-Z-15011-1:1998 [21].

According to the adopted methodological assumptions of this study, the main determining moment of sample collection was the actual value of temperature prevailing in the composted biowastes (Fig. 2). The composting process was carried out for 118 days, and the compost collected on the last (5^{th}) date was considered mature and ripe.

Measurements of gas emissions were taken with the assistance of a set of gas sensors (measuring heads of type MG-72/NH_3 and MG-72/CO_2 of the company Alter-Polska) installed inside bioreactor chambers.

The first part of the experiment involved isolation from biowastes subjected to the composting process of colony forming units (cfu) of bacteria from the *Salmonella* genus and *Enterobacteriaceae* family using, for this purpose, Koch's plate method.

Salmonella spp. bacteria were determined on XLT 4 medium (Merck) after 18-24 hours at 35ºC [22]. In order to

make sure that the isolated bacteria were *Salmonella* spp., Polish Standard PN-Z-19000-1 [23] procedures were followed performing a confirming identification.

In order to determine numbers of bacteria from the *Enterobacteriaceae* family, the selective medium VRBD Agar (Merck) was used [24]. Plates were incubated at 37±1ºC for 18-24 hours. Isolated colonies were explanted onto nutrient agar (37ºC for 24 hours), and later on agar medium with glucose (37ºC for 24 hours). Colonies on nutrient agar were stained using Gram's method, which was followed by a rapid belt test for the identification of cytochrome oxidase presence.

Biochemical tests (EnteroPluri-Test) of the company Liofilchem were applied to identify species belonging to the *Enterobacteriaceae* family isolated from the examined composts on the day of establishment and termination of the experiment.

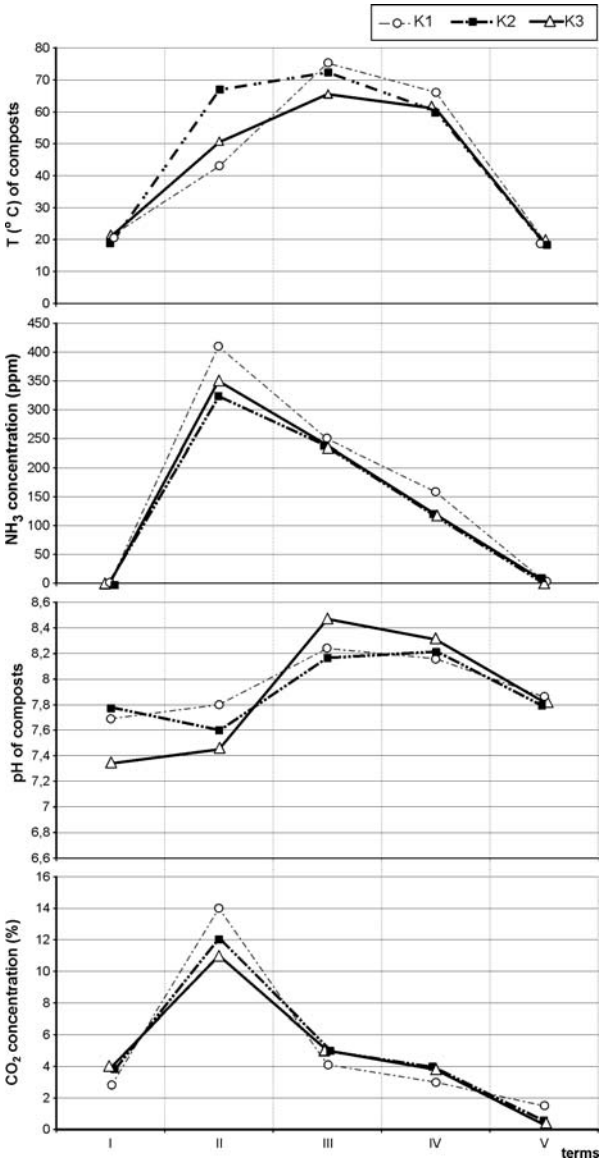

Fig. 2. Changes in temperature, pH, and NH_3 and CO_2 concentration in biowastes during the compositing process.

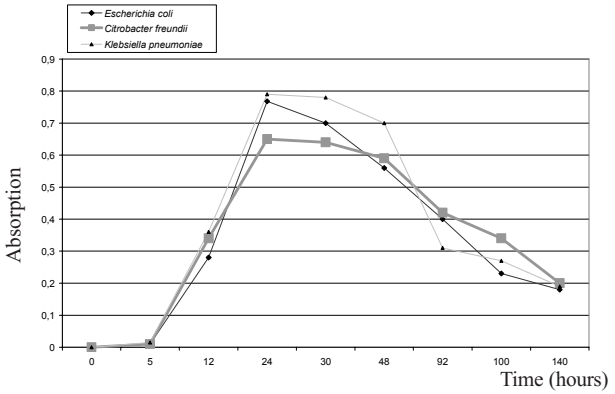

Fig. 3. Curve growth of microorganisms.

Eggs of parasites from *Ascaris* spp., *Trichuris* spp., and *Toxocara* spp. [14] were isolated from the sewage sludge used in the experiment with the assistance of a floating method.

The second part of the experiment consisted in determining types of interactions with the assistance of the well method [25], and the types of interactions between isolates of bacteria belonging to the *Enterobacteriaceae* family isolated from two combinations (K1 and K2) after 118 days of composting and the BAF inoculum.

For this purpose, a sterile broth medium was poured onto sterile Petri dishes and then three rings were placed aseptically on each plate. Sterile LB substrate [26] was poured onto the surface of the broth medium together with a 100-fold dilution of *E. coli*, *Citrobacter freundii*, and *Klebsiella pneumoniae* bacteria cell suspension isolated from mature compost (combinations K1 and K2).

Before initiation of the experiments, the employed bacterial strains were passed onto slants with fresh LB medium. In addition, on the basis of spectrophotometric analysis according to Pelczar [27], for each strain the culture growth curve was determined, which allowed determination of the moment at which the culture exhibited the greatest metabolic activity as well as the moment of its growth inhibition (Fig. 3).

Cultures of microorganisms forming the BAF inoculum were subjected to centrifugation at 19-23ºC at 1000 rpm (h=20 min.) and then, employing 5 replications, the impact of the precipitate containing cells of microorganisms and the supernatant on growth and development of the abovementioned bacteria from the *Enterobacteriaceae* family was investigated.

For this purpose, 0.05 ml each of the supernatant was added to two holes left after rings on one Petri dish and of cell precipitate on the other. The control was distilled water introduced to the third well. Plates were left for one hour to allow absorption of the suspension and then they were incubated at 37ºC for 18-24 hours. After incubation, the zone of *E. coli*, *Citrobacter freundii*, and *Klebsiella pneumoniae* bacteria growth inhibition was measured around respective wells and expressed in mm.

Statistical analyses employed in the experiment were performed using the program STATISTICA 8.0 [28].

Results and Discussion

In order to assess the sanitary condition of biowastes applied in the performed trial, they were subjected to analyses regarding the occurrence of bacteria from the *Salmonella* genus as well as helminth eggs of *Ascaris*, *Trichuris*, and *Toxocara* genera. The performed microbiological analyses of the sewage sludge and its structure-forming materials (straw, sawdust) revealed that they were free of such pathogens (Table 2).

In the case of the sewage sludge, the obtained results were satisfactory because the slightest amount of bacteria from the *Salmonella* genus or the presence of ATT would rule out the application of the sludge for agricultural purposes [29].

In addition, the biowastes used in the experiment were examined from the point of view of the presence of bacteria belonging to the *Enterobacteriaceae* family (Table 2). Identification of these bacteria in the applied materials appears very important because, apart from *Salmonella* spp., this family also comprises other potentially pathogenic microorganisms such as *E. coli*, *Proteus*, *Shigella*, and *Klebsiella*, which, according to Bustamante et al. [6], are considered faecal contamination indicators.

The current order of the Minister of Agriculture and Rural Development [26] fails to include the determination of numbers of the discussed microorganisms in sewage sludge intended for farming purposes, which raises some controversy.

It is evident from the data in Table 2 that the sewage sludge used in this trial was characterized by high contamination by the above-mentioned microorganisms, so it could pose a sanitary hazard.

According to Wolna-Maruwka [10], the composting process appears to be one of the methods of sewage sludge hygienization. High temperature obtained during the thermophilic phase of the process, pH changes, ammonia emission or presence of antibiotics manufactured, among others, by mould fungi or actinomycetes during the cooling phase, all contribute to the elimination of pathogens from composted biowastes [7, 18, 30].

According to Moore et al. [31], high temperature obtained in the composting process (55-65ºC) is a factor determining the process of sterilization of the composted material. Most pathogenic bacteria die under such conditions after several days, while some die as late as after several months. In the opinion of the above-mentioned authors, despite the rapid progress of molecular techniques for pathogen detection, bacteria in compost samples are the least studied of all and are much less understood in terms of their survival, persistence, and regeneration in compost.

Results from a study by Chroni et al. [32] show that the presence of *E. coli* in a compost heap, despite maintenance at 55ºC for 25 days, is a consequence of the re-contamination of the substrate in the centre of the pile by the cooler material from its outer parts during turning.

Because of sanitary contamination of the sewage sludge, it was subjected to the composting process together with structure-forming materials in order to eliminate bacteria belonging to the *Enterobacteriaceae* family.

Table 3. The number of *Enterobacteriaceae* in composts (cfu·10^2·g^{-1} d.m. of material).

Type of compost	Temperature of compost (ºC)	cfu·10^2·g^{-1} d.m. of compost	Standard Deviation (SD·10^2)
I date – beginning of experiment			
K1	21	3,091.09	90.91
K2	19	10,101.19	607.70
K3	21	1,201.00	74.34
$NIR_{0.05}$ = 1,937.97		$NIR_{0.01}$ = 2863.57	
II date – after 48 h (2 days)			
K1	43	0.03	0.008
K2	66.9	0.02	0.005
K3	50.5	0.02	0.001
$NIR_{0.05}$ = 0.02		$NIR_{0.01}$ = 0.03	
III date – after 96 h (4 days)			
K1	75.2	0.05	0.002
K2	72.5	0.23	0.01
K3	65.5	0.14	0.009
$NIR_{0.05}$ = 0.21		$NIR_{0.01}$ = 0.31	
IV date – after 386 h (16 days)			
K1	38	0.04	0.001
K2	37	0.1	0.002
K3	37.5	0.2	0.003
$NIR_{0.05}$ = 0.08		$NIR_{0.01}$ = 0.13	
V date – after 2832 h (118 days)			
K1	19.7	12.31	1.24
K2	18.9	5.31	0.24
K3	19	0.00	0.00
$NIR_{0.05}$ = 4.53		$NIR_{0.01}$ = 5.23	

It is clear from data presented in Table 3 that also on the 1st date of analysis the number of the discussed microorganisms in the composted biowastes (combinations K1-K3) was very high.

Analyzing the results from Table 3, it was concluded that at the moment of trial establishment (date 1), the highest number of the discussed bacteria occurred in biowastes composted in chamber K2. Furthermore, it was observed that the species composition of bacteria isolated from the analyzed composted materials was similar. In the majority of cases, the bacteria *Klebsiella* and *Serratia* dominated in the composted material (Table 4).

The composting process lasting 48 hours led to a temperature increase in the experimental biowastes (Fig. 2) and

Table 4. Species composition of the *Enterobacteriaceae* family in the material subjected to composting on the 1st date of analyses (prior to inoculum introduction).

K1	% share	K2	% share	K3	% share
Escherichia coli	5	*Escherichia coli*	10	*Escherichia coli*	8
Citrobacter freundii	7	*Citrobacter freundii*	8	*Citrobacter freundii*	8
Klebsiella pneumoniae	13	*Klebsiella pneumoniae*	19	*Klebsiella pneumoniae*	17
Klebsiella ozaenae	15	*Klebsiella ozaenae*	16	*Klebsiella ozaenae*	16
Pantoea agglomerans	5	*Serratia liquefaciens*	20	*Shigella sp.*	1
Serratia liquefaciens	12	*Hafnia alvei*	6	*Hafnia alvei*	9
Serratia odorifera	10	*Pantoea agglomerans*	9	*Yersinia pseudotuberculosis*	12
Serratia rubidaea	12	*Shigella sonnei*	1	*Serratia liquefaciens*	13
Serratia ficara	10	*Pantoea agglomerans*	9	*Serratia marcescens*	12
Enterobacter agglomernas	3	*Shigella flexeneri*	1	*Pantoea agglomerans*	2
Shigella boydii	1	*Yersinia aldovae*	1	*Enterobacter cloacae*	1
Enterobacter cloacae	5			*Proteus rettgeri*	1
Yersinia pseudotuberculosis	2				

Table 5. Pearson correlation coefficient between the number of *Enterobacteriacae* (cfu·10^2·g^{-1} d.m. of compost) and physico-chemical conditions.

Combination	Correlation Coefficient			
	T	pH	NH_3	CO_2
K1	-0.53	-0.61	-0.53	-0.26
K2	-0.6	-0.29	-0.52	-0.16
K3	-0.56	-0.59	-0.7	-0.12

higher ammonia emissions (Fig. 2), and caused changes in the reaction of materials (Fig. 2), and hence produced a rapid reduction in the numbers of bacteria from the *Enterobacteriaceae* family. The impact of the above-mentioned factors on changes in bacterial counts was confirmed statistically (Table 5). The change in dynamics of the discussed microorganisms was affected most strongly by the concentration of the released ammonia, then by the value of temperature, and finally by reaction changes of the composted materials. In addition, concentrations of CO_2 released from biowastes were not observed to affect changes in numbers of the discussed bacteria (Fig. 2).

Turner [18] maintains that temperature is one of the principal factors affecting pathogen reduction or elimination from composted biowastes.

It is evident from data presented in Fig. 2 that the thermophilic phase of the composting process was achieved already after 48 h (2nd date of analyses) in chambers K2 and K3. It can be presumed that the above phenomenon was caused by the inoculation of composts by the BAF inoculum.

Rapid heating of the composted wastes was also reported by Xi et al. [33] following the introduction into the bioreactor of different variants of inoculant consisting of *Trichoderma koningii*, *Streptomyces cellulosae*, bacteria from the *Bacillus* genus, and white-rot fungi.

Analyzing changes in temperature values in composted biowastes in our own investigations, it was observed that they occurred identically. Maximal temperature values, irrespective of the type of combination, were registered on the 3rd date of analyses, whereas from the 4th date onwards, the temperature in the chambers declined systematically.

The inhibiting impact of high temperatures on bacteria belonging to the *Enterobacteriaceae* family during the thermophilic phase was also observed by Hassen et al. [34].

According to Epstein [35], total reduction of *Escherichia coli* takes place at 60ºC already after one hour and the period of reduction shortens when the temperature is higher. On the other hand, Bustamanate et al. [16] claim that temperatures ranging from 50 to 60ºC obtained during biowaste composting failed to completely eliminate *Enterobacteriaceae* bacteria.

In the opinion of Chroni et al. [36] the rate of pathogen elimination from the composted material is affected by the

duration of thermophilic conditions. Studies conducted by the above-mentioned authors showed that despite the maintenance of 67ºC for 25 days, *E. coli* continued to be detected in the composted biowaste. As late as on the 57th day of the experiment, the population of *E. coli* declined to below the detection limit.

According to Sundberg et al. [37], the main factor affecting growth and development of microorganisms in composted materials is the pH value. Lei and VanderGheynst [38] maintain that pH value is one of the factors preconditioning the proper course of the composting process. It also results from a study by Chroni et al. [32] that the pH value of composted biowaste is significantly correlated with microbial growth and development, including *E. coli.* Excessively low pH in composted biowastes slows the process down and can result in its inhibition.

It is evident from data presented in Fig. 2 that, with the initiation of the composting process, pH values in all combinations increased to a level above 8 (date 3), and it decreased only after the termination of the thermophilic phase of this process.

Alongside temperature and pH values of the composted materials, another factor that can influence pathogen survivability in these substances is the concentration of released ammonia [19]. As evident from data presented in diagram 4, ammonia concentration measured at the outlet of chambers reached values of the order of 320-410 already after 48 hours of the composting process (date 2 of analyses). The highest level of this gas was then recorded in the control combination K1. Quantities of NH_3 released from composted materials decreased rapidly on consecutive dates of analyses.

On the other hand, Turner [18] reported that pathogen inactivation in composted materials may depend on the moisture content and the nature of the material.

In the opinion of Kim et al. [39] and Zhang et al. [40], indigenous microorganisms were a major factor for controlling pathogen growth in the compost. In a study by Kim et al. [41], the growth of *E. coli* O157:H7 in compost was negatively correlated with the population of indigenous microorganisms. The type of indigenous microorganisms, such as actinomycetes and fungi was critical for the suppression of *E. coli* O157:H7.

Analyzing the dynamics of *Enterobacteriaceae* development on successive dates of analysis, it was found that on the 2nd and 4th dates, numbers of the discussed microorganisms remained at a low level (Table 3) and the next increase of proliferation of these bacteria was reported on day 118 of the composting process (date 5), but only in combinations K1 and K2.

However, according to Tse-Dinh et al. [42] bacteria, similar to other organisms, possess mechanisms making it possible for them to survive unfavorable environmental conditions such as high temperatures. According to the above-mentioned researchers, at temperatures of 30-42ºC, thermo-resistant proteins are synthesized in *Escherichia coli* cells, allowing these mesophilic bacteria to become thermo-tolerant organisms capable of developing even at 52ºC.

Table 6. Species composition of the *Enterobacteriaceae* family in material composted for 188 days (date V of analyses – termination of experiment).

K1	% share	K2	% share	K3
Escherichia coli	30	*Escherichia coli*	100	-
Citrobacter freundii	40	-		-
Klebsiella pneumoniae	40	-		-

The objective of the performed investigations was to assess various factors (temperature, pH, gas emissions, and doses of microbiological inoculum) that could contribute to the reduction or complete elimination of *Enterobacteriaceae* family bacteria. Hence, on the basis of the obtained research results and performed statistical analysis, it was concluded that, apart from temperature and concentration of ammonia released from the composted biowastes, the main factor resulting in the decrease of numbers of the discussed bacteria was the addition of the BAF inoculum. The above opinion is confirmed by research results of Ichida et al. [19], according to whom the inoculation of biowastes with *Bacillus licheniformis* and *Streptomyces* sp. strains caused complete inactivation of *E. coli* and *Salmonella* sp. bacteria already on day 5 of the composting process.

However, it is evident from the data shown in Table 3 that complete elimination of *Enterobacteriaceae* in our own investigations occurred only in combination K3, to which a lower dose of the BAF preparation was introduced. In the remaining two combinations, a significant reduction in numbers of the discussed microorganisms was recorded.

It was found, on the basis of the performed confirmation biochemical tests (Table 6), that the bacteria isolated from combination K1 on the last date of analysis belonged to *Escherichia*, *Citrobacter*, and *Klebsiella* genera. On the other hand, from combination K2, in which the material was inoculated with a higher dose of inoculum, only microorganisms of the species *Escherichia coli* were isolated.

These observations corroborate the above conclusion that the inoculation of composted biowastes with a microbiological inoculum is, alongside temperature and ammonia concentration, the main factor affecting reduction of pathogen numbers.

Stachowiak et al. [34] maintain that the composted material is, firstly, an ideal feed base for microorganisms introduced to the inoculum, and secondly it is their natural carrier. However, according to Hoitink et al. [44], equally important are the time of inoculum application and the quantity applied. According to the above researchers, a single application of the inoculum before the thermophilic phase will not bring expected results because, if the microorganisms making up the inoculum do not produce spore forms, they will be destroyed during the thermophilic phase of the composting process.

It is possible that interactions of an antagonistic nature may take place between microorganisms introduced with the inoculum and pathogens occurring in the biowastes [19], as confirmed by the results of our own studies (Fig. 4). It is clear from data presented in this figure that the applied microbiological inoculum, used both as sediment and supernatant, exerted an inhibitory influence on the growth of bacteria from the *Enterobacteriaceae* family isolated from composts on the last date of analyses. In the case of *Citrobacter freundii* and *Klebsiella pneumoniae* bacteria, the inhibitory effect was stronger after the application of the sediment of cells constituting part of the BAF inoculum (Figs. 5 and 6). On the other hand, *E. coli* growth was inhibited more strongly after the application of the supernatant containing mainly metabolites (Fig. 7).

According to Ogawa et al. [45], organic acid manufactured microbiologically can inhibit growth of some *E. coli* strains and, additionally, exhibit capability for neutralization of toxins produced by these bacteria.

The inhibitory effect of organic acids on *E. coli* growth is due to the reduction in pH of the environment outside its optimal value.

Bagnicka et al. [46] claim that some microorganisms manufacture proteins that exhibit a cytotoxic effect in relation to other bacteria. According to Kunicki-Goldfinger [47], nisin provides an example of a short peptide that exhibits capabilities of inhibiting development of pathogenic microflora. In turn, a study by Randazzo et al. [48] revealed that e.g. bacteria found on plant-origin waste (e.g. genus *Lactococcus*) produce antimicrobial bacteriocins, which are a heterogeneous group of peptides and proteins of various molecular weight and composition.

In addition, the applied biopreparation also contained several actinomycetes isolated from mature compost. According to Dröffner et al. [49], antibiotic substances manufactured by microorganisms constitute a very important factor eliminating pathogenic microorganisms. Larski and Truszczyński [50] reported that bacteria belonging to the *Enterobacteriaceae* family are particularly sensitive to the streptomycin manufactured by actinomycetes. According to the above-mentioned researchers, the action of this aminoglycoside antibiotic involves disturbance of the genetic information reading of bacteria, synthesis of

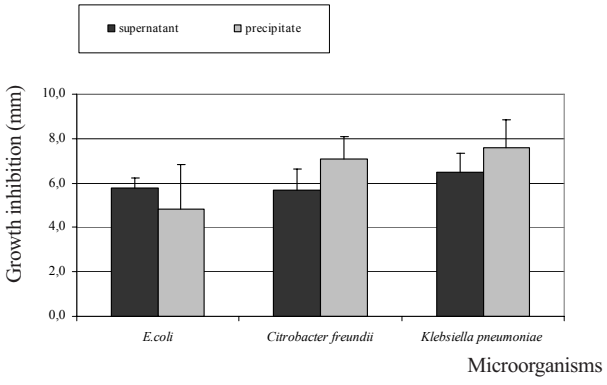

Fig. 4. Zones of interactions between microorganisms isolated from the compost and BAF inoculum.

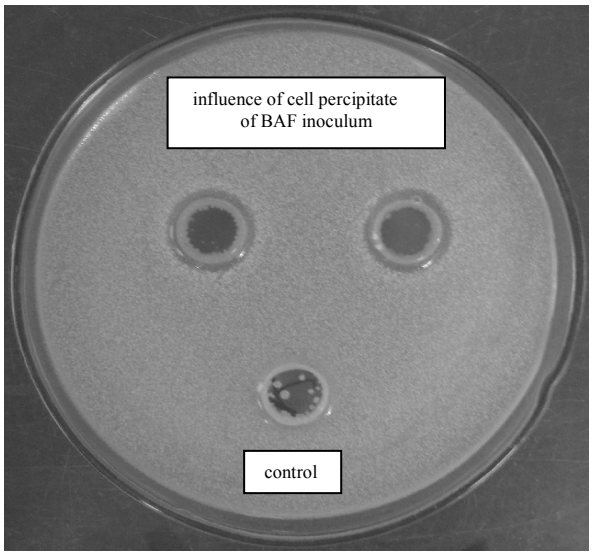

Fig. 5. Zones of growth inhibition of *Citrobacter freundii*.

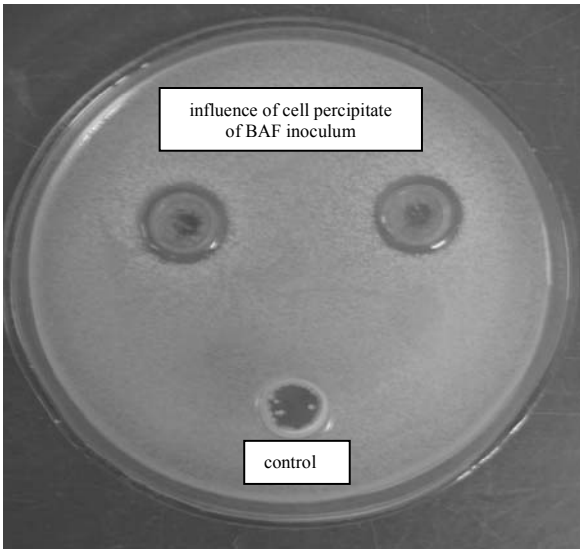

Fig. 6. Zones of growth inhibition of *Klebsiella*.

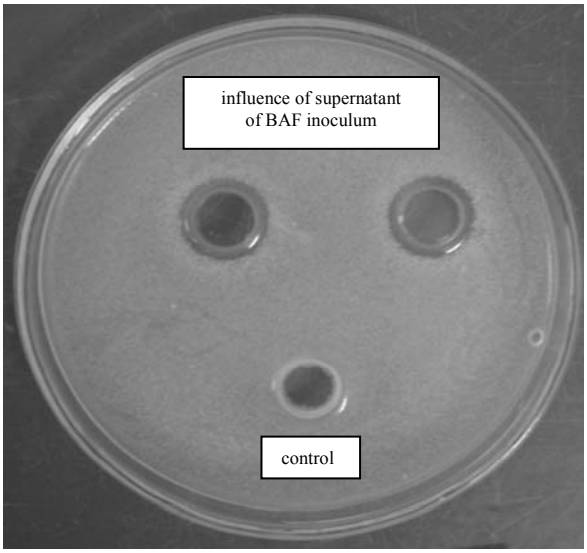

Fig. 7. Zones of growth inhibition of *E. coli*.

bacterial protein, or damage of the cytoplasmic membrane. It was found in a study by Patil et al. [51] that approximately 61% of bioactive metabolites in the environment are isolated from actinomycetes. In this respect a particularly significant role is ascribed to the genus *Streptomyces*, which has been found to contribute to the production of a broad range of antibiotics, i.e. aminoglycoside, anthracyclines, chloramphenicol, β-lactams, macrolides, and tetracyclines.

The applied inoculum contained, apart from proper bacteria and actinomycetes, the mould fungi *Thermomyces lanuginosus* and *Trichoderma atroviride*. The above mould fungi, because of their confirmed cellulolytic, amylolytic, and proteolytic properties (in particular *Thermomyces lanuginosus*), were first introduced in order to optimize the decomposition of organic matter of the composted biowastes [52]. Secondly, the inoculation of the biowastes with a biopreparation containing the above-mentioned microorganisms aimed at the reduction or complete elimination of bacteria from the *Enterobacteriaceae* family. It is quite clear from the literature on the subject [53] that the classical biocontrol mechanism involves competition between microorganisms. Mould fungi can, therefore, compete with pathogens both for ecological niches as well as for nutritive substances. The above-mentioned researcher maintains that the most frequent cause of death of microorganisms is the absence of nutritive substances in the substrate. In addition, it was demonstrated that *Trichoderma atroviride* exhibits biological activity in relation to Gram-negative bacteria, e.g. to sesquiterpene antibiotic or trichoviridin, to which *E. coli* is sensitive [54, 55].

Conclusions

1. It was found that only the lowest dose of the BAF inoculum (750 ml – combination K3) introduced to composted biowastes contributed to total elimination of bacteria from the family *Enterobacteriaceae*.
2. Despite the completed composting process, *Enterobacteriaceae* were still isolated from the control material (K1) and combination K2, to which 1500 ml of BAF inoculum was introduced, despite a temperature of over 70ºC generated in the above-mentioned combinations at the thermophilic stage of the composting process.
3. The calculated Pearson's linear correlation coefficient showed that among physico-chemical parameters of the composting process the reduction of the counts of *Enterobacteriaceae* in the analyzed composts (K1-K3) was connected most strongly with the emission of released ammonia, followed by the value of temperature, while it was influenced to the least degree by changes in substrate pH.
4. The greatest effect on the reduction of the number of the above-mentioned bacteria in the composted material, next to physico-chemical composting parameters, is ascribed to the applied BAF inoculum, which was confirmed by the ring method used in the analyses of interactions between microorganisms.
5. The presence of *E. coli* bacteria in ripe compost (K2), despite the application of the BAF inoculum, is controversial. It may only be assumed that a higher dose of BAF inoculum (1500 ml) introduced to the composted material contributed to a stronger decomposition of complex compounds to simple sugars, being a source substrate for *E. coli*.
6. Investigations concerning the effect of the microbiological inoculum on animal pathogen elimination should be continued. However, it seems important to determine the generic or species membership of microorganisms making up the biopreparation as well as the kind of cell metabolites produced by these microorganisms.

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