



Estimation of microbiological contamination of maize seeds using isothermal calorimetry

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Abstract

The storage of maize seed intended for industrial purposes in foil silo bags is associated with microbiological contamination of the material by bacteria and fungi. This results in the loss of a part of the raw material or its deterioration and causes financial losses. In this paper, the relationship between the number of microorganisms colonizing maize seeds as well as changes in heat flow has been proved. For this purpose, the heat flow and total metabolic heat emission values (total heat) were analyzed. Calorimetric measurements of seed samples were made using the TAM III isothermal calorimeter. The seed samples with the same heat flow curves shape were homogenized, and microbial DNA was isolated from them. The quantitative real-time polymerase chain reaction (qPCR) was performed for detecting the main group of microorganisms colonizing maize seeds. It has been shown that in the case of seeds less affected by *Bacillus* and fungi (including yeast), the heat flow (in the range of 0–360 min) has a falling shape, while for more infected it grows almost linearly. The more infected maize seeds also show significantly higher values of total heat emission. The described research can be used to quickly assess the degree of seed contamination.

Keywords *Bacillus* spp. · Bacteria · Fungi · Heat flow · *Zea mays*

Introduction

Long-term storage of maize seeds (intended for example for the production of bioethanol) in plastic silo bags creates problems of technological and economic nature. Microbial

contamination of the material by bacteria and fungi present on the seed coat negatively affects the fermentation processes and deterioration of the quality of the final product (e.g., presence of mycotoxins, ammonia, butyric acid). In addition, the reduction in the technological quality of seeds, and consequently the loss of a part of the raw material, causes significant financial losses. For this reason, monitoring the quality of the biological material taken is very important [1, 2].

Microbiological analysis of environmental samples based on microbial cultures (for example on Petri dishes) is time-consuming and their results are often ambiguous. A better alternative is molecular methods such as: DNA markers, metagenomics analysis, DNA barcoding, molecular phylogenetic or using PCR. However, the disadvantage of methods based on DNA analysis is the inability to recognize live cells from dead ones as well as the variable efficiency of DNA extraction. An additional aspect is the need to design primers and probes and optimize the hybridization conditions [3, 4]. For this reason, we have decided to use isothermal calorimetry to monitor the degree of microbial contamination of seeds, without the need of long-term and expensive analysis.

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The isothermal calorimetry can be successfully used to monitor the growth of microorganism community [5]. Recently, isothermal calorimetry has been proposed as an effective method of quantitative and qualitative measurement of bacterial contamination of water [6]. In these studies, it was shown that the application of calorimetry allows to detect bacterial contamination much faster than the application of traditional culture of microorganisms on the medium. Calorimetry has also been used successfully in medical research to quickly analyze bacterial infections of blood [7, 8]. This method has been applied in the measurement of microbial activity and monitoring of toxicity and degradation of organic compounds in soil as well as in other environmental studies [9–12]. In turn, in the food industry, the kinetics of the process of fermentation and spoilage of plant tissues have been repeatedly examined using isothermal calorimetry [13].

Most of the physicochemical and biological processes involved in the metabolism of living organisms are accompanied by the generation of heat. It is therefore an unavoidable by product also in the process of microbiome development on plant material [14, 15]. In addition, the formation of ordered spatial structures (related to the development of microorganism colonies) results in the release of Gibbs energy from the substrate, mainly as heat [9, 16]. Thanks to the recording of the heat flow by means of a calorimeter, it is possible to monitor the development of microorganisms (such as bacteria, fungi and yeast) in real time, without interference the structure of the analyzed material and the course of biological processes [6]. It is worth noting that new, multi-channel calorimeters allow for very accurate measurements (with an accuracy of approx. 0.02 °C) on many small samples at the same time and enable quantitative data interpretation, which makes calorimetry a good alternative to other microbiological analysis [9].

The aim of this experiment was to determine microbiological contamination of maize seeds based on the parameters describing heat production. For this purpose, the shape of heat flow curves and total metabolic heat emission values were analyzed as a function of microbiota abundance. After calorimetric measurements, the microbiome content on the seeds was determined based on the DNA amount of selected microorganism groups.

Materials and methods

Plant material

The research material was air-dry maize seeds (*Zea mays* L.), stored in foil silo bags on the area of the Ethanol Production Plant in Goświnowice (Poland). Samples for analysis

were collected using a probe enabling average harvest of seeds over a length of 1.5 m of the silo bag.

Calorimetric measurements

The metabolic activity of maize seeds was measured at 20 °C in a TAM III isothermal calorimeter equipped with TAM Assistant Software (TA instruments, Lindon, US). The thermostat temperature was set to 20 ± 10^{-6} °C. Seeds were stored before measurement in an air-conditioned room at 20 °C. About 4 g of maize seeds were put into the stainless-steel measuring ampoules of 4 cm³ capacity and tightly closed. The measurement was started 1 h after placing the sample in the calorimeter (full thermal stabilization of the sample). Then the heat flow in microwatts was recorded for 360 min. The total emission of metabolic heat in the sample ($\text{mJ g}_{\text{DW}}^{-1}$ —total heat) was obtained by integrating the surface under heat flow curve in a time interval of 0–360 min. The measurements were made on 18 seed samples from different silo bags. After calorimetric measurements, the samples were divided into two groups (9 samples each) according to the shape of heat flow curves. Within each group, the samples were combined at 3 and homogenized. As a consequence, the molecular analysis was made on 6 samples (3 for each group).

Molecular microbiological analysis

DNA isolation

DNA isolation was performed using the Soil DNA Purification Kit (EURx, Poland). Before isolation 10 g of maize seeds was grinding, next 100 mg samples were transferred to 2-mL tubes containing glass beads and lysis buffer and then homogenized in a TissueLiser LT (Qiagen, Germany). Cell lysis was carried out for 5 min at maximum speed. Further steps were taken in accordance with the instructions attached to the isolation kit.

Quantitative PCR

In this paper, the real-time PCR was used to determine the load of microorganisms in the analyzed samples. The qPCR in Taqman technique to assess the load of bacteria, performed using BAC338F and BAC805R primers, and the BAC516F probe [17]. For bacteria of the genus, *Lactobacillus* were performed using F_alllact_IS and R_alllact_IS primers and the P_alllact_IS probe [18]. Amount of total fungi ITS number were performed using FungiQuant-F and FungiQuant-R primers set, and FungiQuant-Prb probe with method determined by Liu et al. [19]. Load of *Pseudomonas* spp. were determined by method described by Hu et al. [20] used B2BF and B2BR3 primers for *phlD* gene.

For enumeration 16S rDNA copies gene for *Bacillus* spp. primers set 16SBACF and 16SBACR and method adapted from Mora et al. [21] were used. Reaction steps including: initial denaturation in 95 °C for 10 min and 40 cycles (95 °C for 15 s, 58 °C for 30 s, 72 °C for 50 s), after reaction tested of thermal curve were performed. Final concentration of primers were 200 nM.

Determination of the number of toxins-producing fungi of *Fusarium*, *Penicillium* and *Aspergillus* genera were performed with Tri5 forward and Tri5 reverse primers and probe for Tri5, rRNA forward, rRNA reverse primers and probe for rRNA and Pks forward Pks reverse and probe for Pks, respectively, in multiplex reaction. Method was performed according Vegi and Wolf-Hall [22]. Yeast were determined using qPCR in Sybr Green technique with YEASTF and YEASTR primers set and reaction was performed according to [23].

Appropriate amplicons of *Bacillus subtilis*, *Pseudomonas putida*, *Fusarium culmorum*, *Penicillium chrysogenum* and *Aspergillus niger* were ligated with plasmids (TOPO™ TA Cloning™ Kit, with pCR™ 2.1-TOPO™, Thermo Fisher Scientific) and served as standards for their respective domains. All reaction performed in volume 20 µL per sample, using a Maxima (Probe or SybrGreen) qPCR Master Mix 2x (Thermo Fisher Scientific, USA). The PCR efficiency of the reactions was between 0.96 and 1.01 (R^2 between 0.991 and 1). Estimation of gene copies was performed in “DNA Copy Number and Dilution Calculator” (ThermoFisher Scientific, <https://www.thermofisher.com/pl/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html>).

Statistical analysis

The results of the microbiome content were analyzed in the Statistica 12 program (Statsoft, Poland) using the one-way analysis of variance (ANOVA) and the Duncan’s post hoc test at the significance level of $p \leq 0.05$. In addition, principal component analysis (PCA) with Pearson’s correlation was performed, which shows the relationship between parameters (XLSTAT, Addinsoft, United States).

Results

Molecular methods based on DNA analysis are more reliable than classical methods (culture on microbiological media) because the counting of microorganism cells is done by counting marker (conserved) genes present in almost every microorganism cell. A pair of primers, i.e., short host complement sequences, is used for this. Primers can be

complementary to sequences at different taxonomic levels, which makes it possible to determine the overall charge of microorganisms or selected species and even pathogenic strains.

Molecular analysis allowed to distinguish samples with lower (LMC) and higher (HMC) microbiome content. Based on statistical results, it was found that number of gene copies of *Bacillus* spp. and total fungi for LMC samples were significantly lower than HMC samples set (Table 1). For example, content of *Bacillus* spp. was 12-fold higher in HMC than in LMC samples.

It has been shown that in the case of LMC the heat flow curves (in the range of 0–360 min) has a falling shape, i.e., for maize seeds less affected by *Bacillus* and fungi. The heat flow reached its maximum on average after about 90 min and then decreased. On the other hand, for HMC seeds heat flow curve grows almost linearly (Fig. 1). Moreover, the HMC seeds also show significantly higher total heat values (Fig. 1—insert).

The variability of biplot axes $F1$ and $F2$ in PCA was 70%. The dominant mass of variability reached $F1$ axis. The similarity of the HCM sample results was high; therefore, their location on the biplot was close to each other and coordinated in one quadrant (positively to the $F1$ and $F2$ axes). In general, the similarity of the LMC1 and LMC2 results was similar to each other and the coordination for the axes of both samples was negative for $F1$ and positive for $F2$, while the LMC3 sample was negatively coordinated for both axes. This indicates that part of the LMC3 results were partly different from the other LMCs but were still clearly different from HCM samples. Detailed PCA results have shown correlation ($r > 0.9$) between total heat value and *Bacillus* spp. content and average correlation with total fungi and yeast content (Fig. 2). However, these values (especially *Bacillus* spp. content and total heat) were high for all HMC samples, and generally low for LMC samples. It was also observed that the contents of total bacteria, *Lactobacillus* spp. and *Fusarium* were positively correlated with each other. The content of these groups of microorganisms was high in the LMC1 and LMC2 samples, but not in the LMC3 sample, which may indicate the influence of other factors on the obtained calorimetric parameters (Fig. 2). The content of *Pseudomonas* spp., and toxicogenic *Penicillium* spp. and *Aspergillus* spp. was below the limit of quantification (~ 50 gene copies).

Discussion

Comparison of the number of microorganisms (determined based on qPCR analysis) colonizing seeds with the efficiency of heat production proves that the shape of the heat flow curves depends on the amount and metabolic activity

Table 1 Number of gene copies of microorganisms in 1 g fresh mass of maize seeds, LMC—lower microbiome content, HMC—higher microbiome content

Sample name	Signal shape	Total bacteria	<i>Bacillus</i> spp.	<i>Lactobacillus</i> spp.	<i>Pseudomonas</i> spp.	Total fungi	<i>Fusarium</i> spp.	<i>Aspergillus</i> spp.	<i>Penicillium</i> spp.	Yeast
LMC	Declining	2.39 10 ^{10a}	5452 ^b	2.13 10 ^{9a}	< LQ*	4.14 10 ^{12b}	1.05 10 ^{3a}	< LQ	< LQ	7.43 10 ^{6a}
HMC	Increasing	1.49 10 ^{10a}	35197 ^a	1.67 10 ^{9a}	< LQ	8.81 10 ^{12a}	6.53 10 ^{4a}	< LQ	< LQ	1.26 10 ^{7a}

Values marked with different letters in the column differ significantly according to Duncan's test at $p \leq 0.05$, $n = 3$

* < LQ—below limit of quantification

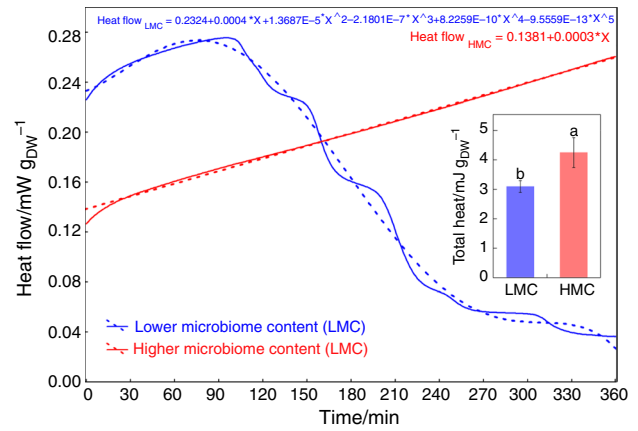


Fig. 1 The specific heat flow curves and total metabolic heat emission values (total heat—insert) of maize seeds with varying degrees of microbial contamination. Solid lines—the average value for 9 independent measurements for each line, dotted lines—fit curves, LMC—lower microbiome content, HMC—higher microbiome content

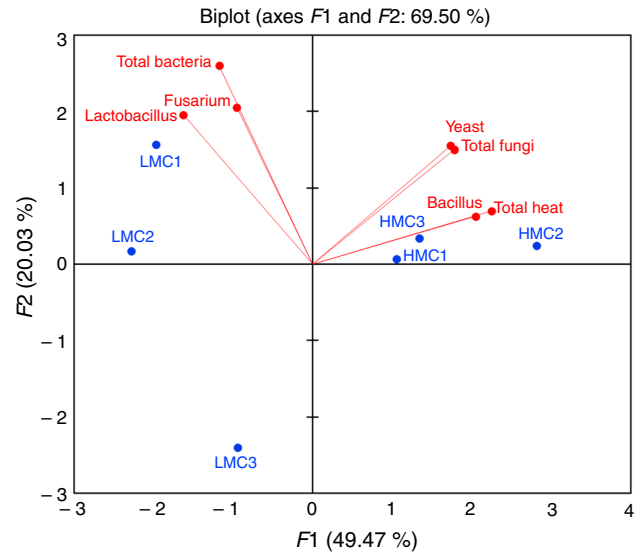


Fig. 2 Principal component analysis (PCA) of the bacterial communities colonizing maize seeds for lower microbiome content LMC and higher microbiome content HMC. LMC (1, 2, 3) and HMC (1, 2, 3) means individual replications for LMC and HMC samples

of the microbial community [14, 15]. The high total heat values in samples with a high microbiome content (mainly fungi and *Bacillus* spp.) are confirmed by experiments carried out on perishable food products, in which the development of microorganisms was accompanied by a heat flow increase [14, 24]. In these and other cases, the qualitative microbial analysis was not performed; however, the use of microbial growth inhibitors resulted in a decrease in total heat of samples. Similar conclusions can be drawn from analysis of sediments of water reservoirs

rich in anaerobic and aerobic microorganisms [25]. In this case, the non-specificity of the calorimetric method consists in the difficulty in distinguishing the metabolic heat of microorganisms from the heat of chemical reactions. A similar problem was observed in the experiment regarding the deterioration of moist tobacco products [26]. However, air-dry seeds are characterized by low metabolic activity. Also, during the quantitative determination of soil microorganisms, it was confirmed that the number of living cells of bacteria and fungi was strongly correlated with the recorded amount of heat [27]. It was proved that the increasing density of microorganisms in the soil resulted in a decrease in the heat production [12]. The low content of the microbiome in the sample makes the food base of microorganisms big. Therefore, as the time goes on, a rapid growth of microorganisms may take place. This would explain the visible falling shape of heat flow curves. At the same time, in samples with high microbiome content, the opposite trend was observed, suggesting a decrease in the number of living cells of microorganisms, and thus an increase in their metabolic activity. However, this hypothesis should be verified in further studies.

The results of this investigation clearly pointed out a longer activity of microbiota metabolism in samples with a high content of fungi (including yeast) and sporulating bacteria of the genus *Bacillus* spp. Mentioned process indicates the activity associated with the slow growth of spores *Bacillus* spp. on maize and utilization of nutrients such as starch or other high energy compounds. This phenomenon is very dynamic during the aerobic spoilage of silage. The quality of maize seeds after breaking anaerobic conditions undergoes some modifications conducted to deterioration of plant material. Qualitative changes mainly concern: the increase in the concentration of starch, consumption by aerobic microorganisms of volatile acids (mainly lactic acid, acetic acid and their formulas) as well as ethanol and their volatilization. In recent years, bacteria of the genus *Bacillus* or other spores and molds that are the main factor in the oxidative deterioration of maize silage are increasingly identified, which is associated with dynamic changes in heat release [28–30].

After additional tests on microbiological cultures on appropriate media (data not shown), it was possible to determine that despite the high number of gene copies (data from qPCR), the weak activity of molds and yeasts in all analyzed samples, with a large number of active sporulating bacteria was observed. This indicates a high content of these microorganisms in the earlier period (transport, storage or discontinuation of anaerobic conditions), which may explain the release of nutrients and the rapid development of the digestive bacteria responsible for heating and oxygen-based substrate deterioration.

Conclusions

In conclusion, the analysis of heat flow curves after 360 min measurements (and even after 180 min) allows to determine the degree of biological material contamination by bacteria *Bacillus* spp. and mold fungi. Isothermal calorimetry is therefore a promising method that can be used to quickly assess the degree of microbial infection of plant material, e.g., seeds. However, in vitro analysis with controlled inoculation of undesirable microorganisms is needed to initial establish calorimetric curves for key groups of microorganisms deteriorating the quality of plant material.

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