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Microbial Fermentation and Shelf Life of Potential Biotechnological Products Capable of Pesticide Degradation

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Abstract: The pesticide active ingredient azoxystrobin is widely used in agriculture and has negative effects for the environment and contained organisms. Bacterial strains have been reported to degrade azoxystrobin, but precise methodologies for producing and storing these strains as potential biotechnological products are lacking. The study focused on creating and optimising a non-sterile, small-scale microbial fermentation protocol to produce azoxystrobin-degrading products and to test their shelf life. By testing 14 variants and sampling at three production and two storage time points, the trial demonstrated the successful production and storage of microbial products capable of pesticide degradation. Various measurement parameters such as pH value and organic acids were used to monitor the quality of the microbial products during the production and storage. Further, we developed and validated qPCR assays to rapidly and specifically assess the concentration of the two azoxystrobin degrading strains, namely *Bacillus subtilis* strain MK101 and *Rhodococcus fascians* strain MK144. To ensure good specificity, the combination of two qPCR assays targeting two different genome regions was implemented for each strain. The study highlights the significant impact of media selection and bacterial inoculum quantity on the microbial product quality.

Keywords: pesticide-degrading bacteria; azoxystrobin; quantitative PCR; fermentation; shelf life

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1. Introduction

Azoxystrobin belongs to the strobilurin family, which contains the most widely used fungicides worldwide [1]. The fungicidal active substance azoxystrobin is used extensively due to its wide spectrum of action. It is used as a foliar spray, as well as for soil treatment, and is therefore regularly detected in different types of vegetables and fruits [2,3]. Azoxystrobin shows moderate to long persistence with a half-life (DT_{50}) of 279 days [4]. The octanol-water partition coefficient of azoxystrobin is log K_{ow} 2.5, which indicates that azoxystrobin has low water solubility. Regarding chemical safety, azoxystrobin is classified as acutely toxic and as an environmental hazard [5]. Eco-friendly approaches such as biodegradation of azoxystrobin by microorganisms has potential to reduce the concentration and thus the toxicity of the fungicide.

Only a limited number of azoxystrobin-degrading bacteria has been isolated so far from pesticide-contaminated soils, sludge from wastewater treatment systems, or from composing livestock excrements [6–10]. In a recent study, we isolated and characterised azoxystrobin-degrading bacteria from lettuce plants. Several strains, in particular, *Bacillus subtilis* strain MK101 and *Rhodococcus fascians* strain MK144, showed high degradation capacity in vitro and in greenhouse trials [11]. These strains are interesting for the development of products, which could be used to eliminate or reduce pesticide loads in agricultural or horticultural production. However, there is a notable lack of information regarding the production and upscaling conditions needed to produce these strains.

In such developments, it is crucial for the microbial product quality to ensure the proliferation and competitiveness of the strains during production. Further important monitoring parameters during fermentation are pH value, temperature, and the metabolism of the substrate to organic acids. Organic acids are naturally formed during fermentation and play a crucial role in regulating the pH value of fermented products. After the formulation production, microbial interactions within the formulation and the stability of the bacteria during storage become critical parameters.

Using cultivation-based techniques to test the presence of pesticide-degrading bacteria on selective agar plates or on liquid culture media is time-consuming and laborious. Molecular techniques for the detection of degrading genes within the genome of potential pesticide-degrading bacteria can harbour bias. For example, pesticide-degrading genes can be acquired by previously non-degrading microorganisms [12] or lost from degrading organisms [13]. Approaches for detecting and quantifying xenobiotic degrading strains in samples through (non-degrading) marker genes have been undertaken in the past [14].

The study ultimately aimed at developing methodologies for producing and storing the efficient azoxystrobin degraders, namely *Bacillus subtilis* strain MK101 and *Rhodococcus fascians* strain MK144, which we selected because of their high degradation capacities. Furthermore, the objective of this study was to develop, monitor, and optimise a small-scale fermentation system for the production of these strains. To achieve this, we tested 14 different variants, including two different initial bacterial concentrations, the combination of two strains as the starting inoculation, and various substrate sources for the fermentation process. The tested substrates included bio sugar cane molasses and bio glucose syrup, both widely used as substrates in microbial fermentation.

To allow for efficient monitoring of the fermentation and storage process, quantitative PCR (qPCR) assays were developed for both strains to rapidly and specifically detect them at various stages of the production process.

Other measurement parameters used to monitor the quality of the microbial products included, for example, the pH value, the temperature, the organic acids, and the viability of the bacteria.

2. Materials and Methods

2.1. Microbial Fermentation and Sampling for pH Value, Viability, qPCR Analysis, and Organic Acid Measurements

Bacillus subtilis strain MK101 and *Rhodococcus fascians* strain MK144 (stored as bacterial glycerol stocks at −80 °C) were streaked on 10% tryptic soy broth agar plates (Merck KGaA, Darmstadt, Germany). Plates were incubated for 48 h at 28 °C. Afterwards, single colonies were inoculated in 7 mL sterile 10% tryptic soy broth media in Duran test tubes 16 × 160 with straight rims (Duran Group, Mainz, Germany), closed with Labocap without a handle 15/16 mm (Schuett-Biotec GmbH, Göttingen, Germany), and incubated in a shaking incubator (Ovan Maxi OL30-ME, 180 rpm, 28 °C, overnight) at up to 1.5 OD_{600}. Bacteria were then re-inoculated in 700 mL 10% bio sugar cane molasses (lot# 41015258, August Töpfer & Co. KG, Hamburg, Germany) or 10% bio glucose syrup agenabon (lot# 3058, Agrana Stärke GmbH, Aschach an der Donau, Austria) twice autoclaved media (121 °C for 20 min) in 1 L bottles (Schott AG, Landshut, Germany). The flasks were incubated at 28 °C for 96 h and were shaken manually twice a day for 1 min. Afterwards, the bacteria were re-inoculated in different quantities, by using different dilutions and combinations in different media with a concentration of 5% (Table 1), prepared and autoclaved once at 105 °C for 20 min in 5 L canisters (Wolf Plastics Verpackungen GmbH, Kammern im Liesingtal, Austria), and closed with a screw cap (Rixius AG, Mannheim, Germany). Five biological replicates of each variant were made. A sterile defined culture medium was necessary to ensure the growth of only the intended strain. The inoculated canisters were placed in a non-sterile room with temperature monitoring and temperature control set to a temperature of a constant 38.5 °C to enable further bacterial growth. The canisters were shaken manually twice a day during the fermentation period of two weeks. Sampling for pH value, viability, and

qPCR analysis was performed at the beginning (0 d, T0), in the middle (7 d, T1), and at the end of the fermentation period (14 d, T2). The pH value was measured using a pH meter (Mettler-Toledo GmbH, Greifensee, Switzerland), and viability was analysed by cultivating dilution series on 10% tryptic soy broth agar plates. Sampling for gDNA extraction was performed in 2 mL tubes (duplicates), and the liquid samples were stored at −20 °C until further processing, as described below.

Table 1. Inoculation media and fermentation variants.

Variant Number	Inoculated Strain and Growth Media	Media for Fermentation Trial
	Cfu 1: 10^5 cfu/mL (3% of microbial suspension was added)	
01	*Bacillus subtilis* strain MK101 [1]	5% sugar cane molasses
02	*Rhodococcus fascians* strain MK144 [1]	5% sugar cane molasses
03	*Bacillus subtilis strain* MK101 [1] and *Rhodococcus fascians* strain MK144 [1]	5% sugar cane molasses
04	*Bacillus subtilis* strain MK101 [2]	5% glucose syrup agenabon
05	*Rhodococcus fascians* strain MK144 [2]	5% glucose syrup agenabon
06	*Bacillus subtilis* strain MK101 [2] and *Rhodococcus fascians* strain MK144 [2]	5% glucose syrup agenabon
	Cfu 2: 10^3 cfu/mL (3% of microbial suspension was added)	
07	*Bacillus subtilis* strain MK101 [1]	5% sugar cane molasses
08	*Rhodococcus fascians* strain MK144 [1]	5% sugar cane molasses
09	*Bacillus subtilis strain* MK101 [1] and *Rhodococcus fascians* strain MK144 [1]	5% sugar cane molasses
10	*Bacillus subtilis* strain MK101 [2]	5% glucose syrup agenabon
11	*Rhodococcus fascians* strain MK144 [2]	5% glucose syrup agenabon
12	*Bacillus subtilis* strain MK101 [2] and *Rhodococcus fascians* strain MK144 [2]	5% glucose syrup agenabon
	Uninoculated	
13	Uninoculated [1]	5% sugar cane molasses
14	Uninoculated [2]	5% glucose syrup agenabon

[1] 10% sugar cane molasses used as growth media. [2] 10% glucose syrup agenabon used as growth media.

Samples for the analysis of organic acids were taken in duplicates at the end of the fermentation period in 100 mL bottles (Greiner Packaging GmbH, Wartberg an der Krems, Austria) and closed using a screw cap (Sensoplast Packmitteltechnik GmbH, Oberhonnefeld-Gierend, Germany). Lactic- (Limit of Quantification < 0.024 mg/kg), acetic- (<0.024 mg/kg), propionic- (<0.024 mg/kg), iso-butyric- (<0.044 mg/kg), n-butyric- (<0.016 mg/kg), iso-valeric- (<0.009 mg/kg), and n-valeric- (<0.017 mg/kg) acid were analysed in an accredited laboratory [15].

Shelf Life of Potential Biotechnological Products and Sampling

For the storage trial, two 1 L bottles per variant were filled with fermentation products, closed using a screw cap, and stored for six weeks at 20 °C. Temperature measurements and recording during storage were conducted using an RC-5 temperature logger (Elitech Technology, Inc., San Jose, CA, USA). Sampling from closed 1 L bottles was performed after three weeks (T3) and six weeks (T4) of storage as described above. The measurement parameters included pH value, viability, qPCR analysis, and organic acids at the beginning of the storage trial (T2). Additionally, pH value, viability, and qPCR analysis were assessed after three weeks of storage (T3) and again after six weeks of storage (T4).

2.2. Quantitative PCR: qPCR Development and Assay Validation

For the primers and probes design, contigs of the whole genome sequences (BioProject number PRJNA886999) were matched with the NCBI nucleotide database (downloaded in January 2023) using a local installation of BLAST with the following parameters: e-value 1×10^{-5}, a maximum of 50 target sequences, and a maximum of 10 HSPs. A hit table

and graphic output were obtained to allow a visual inspection of the alignments. Regions of interest were selected based on the following criteria: at least three mismatches in a sequence of approximately 200 nucleotides. The online version of blastn https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed on 3 October 2022) was used to query for similar sequences and analyze the reproducibility of identified mismatches. Sequence segments with conserved mismatches were selected for primer design. PrimerQuestTM tool https://eu.idtdna.com/pages/tools/primerquest (accessed on 5 October 2022). was used for primer design by applying default settings Obtained primer and probe sequences were compared with the target region and related regions identified by BLAST using BioEdit v7.0.5.3 (Hall, 1999). If needed and possible, the primer and probe sequences were additionally modified manually to better exploit mismatches. The primers and TaqMan probes used for validation are shown in Table A1. All primers and probes were commercially synthesised by IDT Technologies (Integrated DNA Technologies, Leuven, Belgium).

The qPCR analyses for assay validation were performed using a cfx connect real-time PCR detection system (Bio-Rad Laboratories, Inc., Boulder, CO, USA). Each reaction was run in duplicate. The qPCR was performed in a 20 µL reaction mix containing 1 × Blue Probe qPCR Mix (Biozym Scientific GmbH, Hessisch Oldendorf, Germany), 400 nM of each primer, 200 nM probe, and 2 µL DNA template. In all cases, a non-template control (NTC) was included using 2 µL of DNAse free water instead of the DNA template. Standard curves from 10 ng to 10 fg were made using genomic DNA isolated from *Bacillus subtilis* strain MK101 (2.24×10^6–2.24 genome equivalents) and *Rhodococcus fascians* strain MK144 (1.6×10^6–1.6 genome equivalents). The estimation of genome equivalents was performed using a "Calculator for determining the number of copies of a template" (URI Genomics & Sequencing Center; https://cels.uri.edu/gsc/cndna.html accessed on 10 October 2022) based on the average genome sizes of targeted species published on the NCBI webpage (4.1348 Mb for *Bacillus subtilis* and 5.77407 Mb for *Rhodococcus fascians*; status January 2023). For method validation, the standard parameters of the qPCR master mix were initially used to assess their effectiveness and determine if further testing would be necessary. Cycling parameters were 2 min at 95 °C, 40 cycles of 5 s at 95 °C, and 25 s at 60 °C. The qPCR limit of detection (LOD) was determined based on the quantification cycle (Cq) of the last detectable standard. When NTC showed an amplification signal, the calculation of LOD was performed according to the formula Cq (LOD) = Cq (NTC) − 3 [16]. The samples with Cq values lower than Cq (LOD) were classified as positive, and samples with Cq values higher than Cq (LOD) were classified as non-determined. Amplification efficiencies were calculated from the slope of the standard curve (s) as $E = 10^{-1/s} - 1$ [17]. The reproducibility of the qPCR assays was assessed by determining intra-assay repeatability and inter-assay reproducibility. The coefficient of variation (CV) was calculated based on the Cq value. The validation of the developed qPCR methods was conducted with 40 *Bacillus* sp. isolates, 20 *Rhodococcus* sp. isolates, and environmental samples (selected from the previous study Table A2 [11]).

2.3. DNA Extraction

The gDNA of samples from the fermentation and shelf life trials was extracted using MagAttract® PowerSoil® Pro kit (Qiagen, Hilden, Germany) with Hamilton Microlab STAR Liquid Handling System (Hamilton Company, Reno, NV, USA).

In addition, to further check the specificity of the qPCR assay in the assessed matrix, gDNA was extracted from sugar cane molasses (two different batch numbers from August Töpfer & Co. KG, Hamburg, Germany and one batch number from Hansa Melasse, Bremen, Germany) and from microbial product samples (three different batch numbers of EM Active from Multikraft, Pichl/Wels, Austria).

2.4. qPCR Analysis

The qPCR assays were performed to quantify the number of pesticide-degrading bacteria. False-negative results can arise from the inhibition of the qPCR amplification

reaction from the sample matrix [18]. Therefore, we performed an inhibition test, as described by Gensberger et al., 2014, for samples from the fermentation system. Briefly, undiluted, 1:10 diluted and 1:50 diluted DNA samples were quantitatively analysed using qPCR assay targeting bacterial 16S rRNA gene [19].

The *B. subtilis* MK101 and *R. fascians* MK 144 qPCRs were performed as described above. The qPCR analysis cycling parameters were 2 min at 95 °C, 40 cycles of 5 s at 95 °C, and 25 s at 60 °C. In detail, we started with qPCR assay with code 91 for *Bacillus subtilis* strain MK101 detection and with qPCR assay with code 25 for *Rhodococcus fascians* strain MK144 detection. The second assays with codes 35 and 36 were only performed for the positive samples as confirmation (Table A1).

2.5. Statistical Analysis

Statistical analysis of results obtained from samples taken from the fermentation system and storage (pH values, organic acids, viability, and strain-specific qPCR results) was performed using the following software: Microsoft Excel (Office 2016), R-4.3.1 statistical environment, and RStudio version 2023.06.0. Each qPCR assay, viability, and pH dataset were analysed separately. The presence of outliers was accounted for by capping the values between the 5th and 95th percentiles. Variables with zero or nearly zero variance were removed, and any perfectly correlated or anti-correlated ones, if present, were grouped together. When multiple dependent variables were available, such as the pH data including the organic acids, the values were centered and scaled. Linear regression analysis, to compare the means of a single variable across different groups, was performed on qPCR and viability data using Analysis of Variance (ANOVA) following a visual inspection of model diagnostic plots. Estimated Marginal Means (EMMs) followed as post hoc analysis [20]. In EMMs pairwise comparisons, variant, time point, strain, inoculum, and medium were considered individually and in combination. The correlation between organic acids and pH values was examined (package psych; version: 2.4.6.26) [21] and a Permutational Multivariate Analysis of Variance (PERMANOVA) was applied to assess differences in multivariante data between groups (package vegan; version: 2.6-6.1) [22].

3. Results

3.1. Validation of qPCR Assays for Bacillus subtilis Strain MK101 and Rhodococcus fascians Strain MK144

For *Bacillus subtilis* strain MK101 and *Rhodococcus fascians* strain MK144 detection, the combination of two qPCR assays targeting two different regions of the genome was necessary to ensure good specificity (Table A1). The method validation demonstrated that the standard parameters of the qPCR master mix were effective; therefore, the number of cycles was set to 40. The validation showed that both efficiency and R^2 were within the expected range (Table A1). Although individual reference strains from the validation set (Table A2) were positive in individual qPCR assays, only *Bacillus subtilis* strain MK101 and *Rhodococcus fascians* strain MK144 were positive when both qPCR assays were used (cut-off SQ mean $\geq$ 10). Also, in the assay validation with the environmental samples (Table A2), some samples were positive in one qPCR assay but not detectable in another, ensuring clear differentiation. The six samples sampled in a greenhouse trial treated with *Bacillus subtilis* strain MK101 were clearly positive in both qPCR assays. Five out of six samples treated with *Rhodococcus fascians* strain MK144 were clearly positive in both qPCR assays (SQ mean $\geq$ 50). All samples expected to be negative showed a negative result in *Bacillus subtilis* strain MK101 detection. The batches of microbial products showed no amplification signal in strain-specific qPCR.

3.2. Microbial Behaviour during Fermentation and Storage

The inhibition test of the undiluted, 1:10 diluted and 1:50 diluted fermentation and storage samples showed that there was no matrix-induced inhibition in the qPCR amplification. *Bacillus subtilis* strain MK101 was detected in most of the samples; however, *Rhodococcus*

fascians strain MK144 was not detected in any of the samples. Therefore, variants two, five, eight, and 11 (Table 1), which had only *Rhodococcus fascians* strain MK144 as an inoculum, were excluded from the qPCR results evaluation. In the two uninoculated control variant numbers 13 and 14, *Bacillus subtilis* strain MK101 was not detected.

Both qPCR assays, coded 91 and 35, were used to detect *Bacillus subtilis* strain MK101 across different fermentation variants, and results revealed significant differences among them. Overall, the assays highlighted varying levels *of Bacillus subtilis* strain MK101, influenced by the type of medium and the amount of inoculum used (Figure 1).

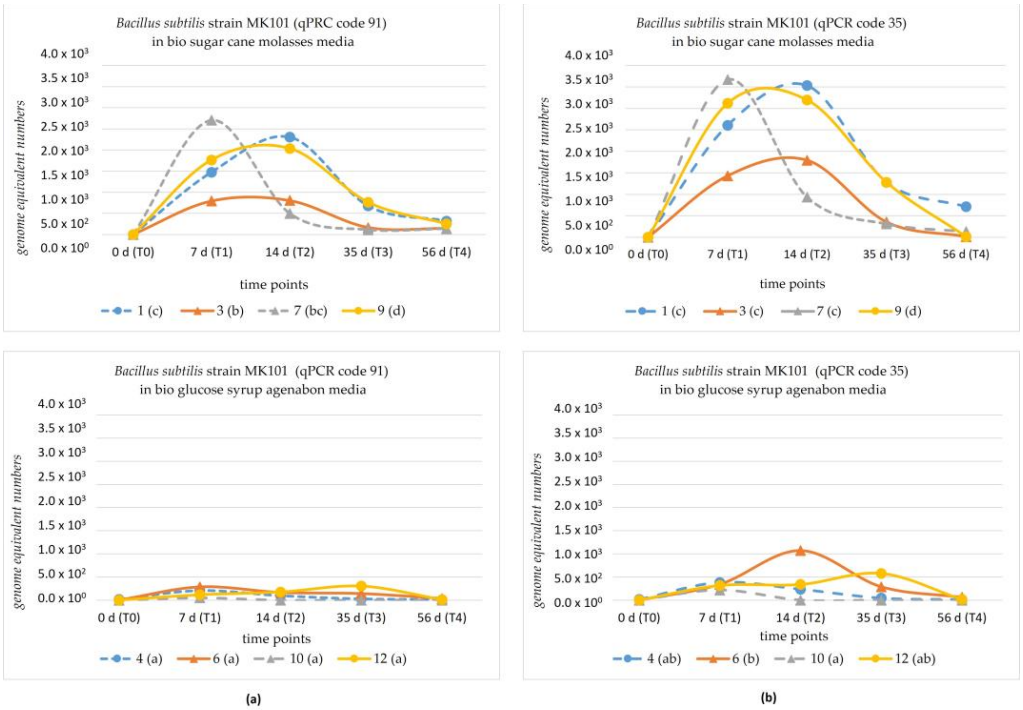

Figure 1. Genome equivalent numbers of *Bacillus subtilis* strain MK101 at the different time points. The lines show the different variants according to Table 1. Different letters in the variant name represent significant differences between measured qPCR values. The qPCR assays code 91 and code 35 were analysed separately: qPCR assay code 91 analysis (**a**); and qPCR assay code 35 analysis (**b**) (n = 5).

Statistical analysis of the qPCR results obtained with code 91 assay revealed five groups. The lowest amount of *Bacillus subtilis* strain MK101 was found in the variants fermented with glucose syrup, forming the first group a. Variant three, which combined strains with a high inoculum amount and fermented with sugar cane molasses media, formed the second group b. Variant seven, with an inoculation of 10^3 cfu/mL *Bacillus subtilis*, formed group bc. Variant one, with an inoculation of 10^5 cfu/mL *Bacillus subtilis*, formed group c. The highest genome equivalent numbers were observed in variant nine, with 10^3 cfu/mL of combined strains in sugar cane molasses, forming group d (Figure 1a).

In the statistical analysis of the subsequent qPCR assay with code 35, which confirmed the positive samples from assay 91, five groups were again identified. The first group a included variant ten with a low inoculum of 10^3 cfu/mL in glucose syrup. Variants four and 12 formed the second group ab, while variant six formed group b. Variants with bio sugar cane molasses media showed higher *Bacillus subtilis* genome equivalent values than variants fermented with glucose syrup. Variant three (inoculation of 10^5 cfu/mL combination of strains in sugar cane molasses), variant seven (single strain 10^3 cfu/mL *Bacillus subtilis*), and variant one (single strain 10^5 cfu/mL *Bacillus subtilis*) formed group c. The highest genome equivalent numbers in qPCR code 35 were found in variant nine, with 10^3 cfu/mL of combined strains in sugar cane molasses, forming group d (Figure 1b).

We also measured the viability of the bacteria at all time points during fermentation and storage by cultivating dilution series on 10% tryptic soy broth agar plates. Group a, which represented variants with low viability, was formed from variants 14, 13, two, seven, nine, six, eight, five, and three. Group b (variant 11) and group c (variants ten and one) showed middle viability. High viability was determined in variant 12 (group d) and the highest in variant four (group e) (Figure 2).

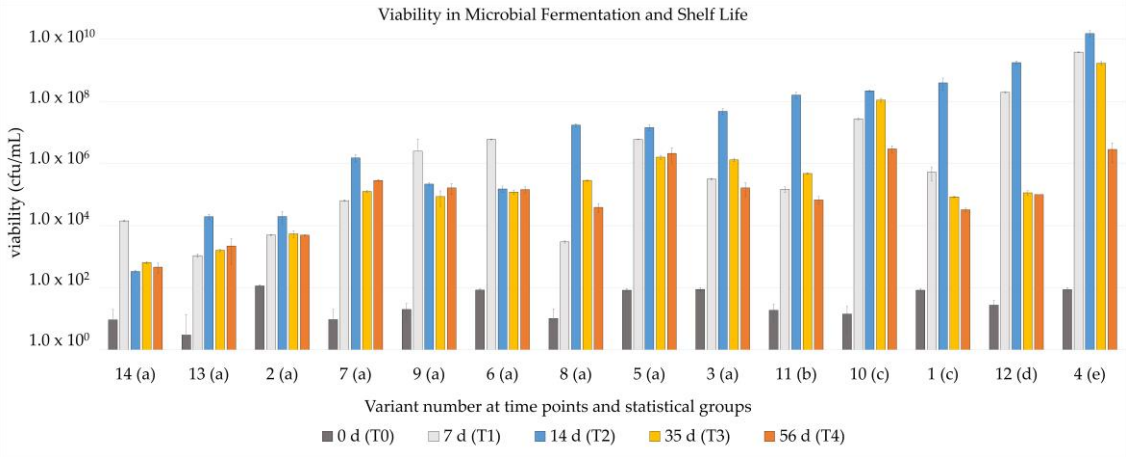

Figure 2. Viability in the 14 variants according to Table 1 at the five different time points in ascending order (n = 5). The numbers in parentheses following the variant numbers indicate the statistical groups of the measured values.

Upon closer examination of the differences between time points, we identified three statistically significant groups in the qPCR analysis. The start of fermentation (T0) and six weeks of storage (T4) formed group a, while three weeks of storage (T3) constituted group b. The middle (T1) and the end of fermentation (T2), which marked the start of the storage trial, showed the highest genome equivalent values and together formed group c.

In the viability analysis, four distinct groups were observed. The start of fermentation (T0) and six weeks of storage (T4) were grouped as a, and three weeks of storage (T3) formed group b. The middle of fermentation (T1) was classified as group c, and the end of fermentation (T2) formed group d. T1 and T2 ranked the highest according to both qPCR and cultivation results.

3.3. Monitoring Parameters during Fermentation and Storage

We measured the pH values in all five biological replicates of the 14 variants at all five time points (Table 2). For the ANOVA we included variant and time point in the first step and for the EMMs strain, inoculum and medium in the second step. All variables, excluding strain, were statistically different, starting already at the first time point. Concerning time points, T0 (group c), T1 (b) and the other time points together (T2, T3 and T4; group a) showed different groups. At T0 there were two different groups. Group a comprised variants with bio sugar cane molasses (variant numbers: 1, 2, 3, 7, 8, 9, 13) and group b variants with bio glucose syrup (variant numbers 4, 5, 6, 10, 11, 12, and 14). Over the observation period, the groups became more diverse and at the end of the storage trial variants seven and eight were group a, variants three and 13 were group ab, variant nine was group abc, variants one, two, 11, and six were variant bc, variant four was group cd and variant five, ten, 12, and 14 were group d. All variants belonging to group a were fermented with medium bio sugar cane molasses and all variants belonging to group d were fermented with medium bio glucose syrup agenabon. There were statistically significant differences in pH values related to the two different inoculum amounts 10^5 cfu/mL and 10^3 cfu/mL in the combination of two strains and with *Rhodococcus fascians* strain MK144, but not with single strain *Bacillus subtilis* strain MK101.

Table 2. Fermentation acids and pH values during fermentation and storage. The statistical significance was defined as $p < 0.001$.

Variant Number	pH Values at Different Time Points (n = 5)				
	0 d (T0)	**7 d (T1)**	**14 d (T2)**	**35 d (T3)**	**56 d (T4)**
1	5.85 ± 0.07	4.04 ± 0.07	3.59 ± 0.09	3.61 ± 0.08	3.61 ± 0.08
2	5.84 ± 0.07	4.06 ± 0.09	3.78 ± 0.24	3.64 ± 0.06	3.62 ± 0.10
3	5.82 ± 0.05	4.03 ± 0.05	3.50 ± 0.03	3.53 ± 0.03	3.53 ± 0.03
4	7.13 ± 0.09	4.11 ± 0.10	3.69 ± 0.19	3.70 ± 0.15	3.68 ± 0.14
5	7.10 ± 0.01	4.14 ± 0.10	3.76 ± 0.13	3.78 ± 0.11	3.78 ± 0.10
6	7.07 ± 0.07	4.16 ± 0.05	3.76 ± 0.20	3.72 ± 0.15	3.65 ± 0.09
7	5.82 ± 0.04	3.99 ± 0.05	3.45 ± 0.03	3.45 ± 0.04	3.45 ± 0.05
8	5.87 ± 0.07	3.97 ± 0.04	3.46 ± 0.01	3.45 ± 0.01	3.48 ± 0.01
9	5.8 ± 0-048	4.06 ± 0.04	3.51 ± 0.07	3.53 ± 0.08	3.56 ± 0.08
10	7.23 ± 0.32	4.18 ± 0.06	3.76 ± 0.10	3.79 ± 0.11	3.78 ± 0.09
11	7.04 ± 0.05	4.13 ± 0.08	3.59 ± 0.09	3.62 ± 0.08	3.63 ± 0.07
12	7.05 ± 0.03	4.15 ± 0.05	3.77 ± 0.15	3.83 ± 0.16	3.78 ± 0.13
13	5.86 ± 0.05	4.01 ± 0.04	3.51 ± 0.12	3.53 ± 0.12	3.54 ± 0.11
14	7.05 ± 0.03	4.16 ± 0.04	3,78 ± 0.11	3.82 ± 0.09	3.79 ± 0.08
	organic acids [mg/kg] at 14 d (T2) (n = 5)				
Variant number	Lactic acid	Acetic acid	n-butyric acid *		
1	8.52 ± 0.90	0.46 ± 0.18	1.48 ± 1.34		
2	6.59 ± 1.91	0.39 ± 0.10	2.00 ± 0.57		
3	9.39 ± 0.44	0.51 ± 0.03	2.09 ± 0.31		
4	1.37 ± 0.29	0.24 ± 0.07	0.22 ± 0.09		
5	1.24 ± 0.15	0.26 ± 0.05	0.05 ± 0.01		
6	1.30 ± 0.31	0.24 ± 0.09	0.05 ± 0.01		
7	10.41 ± 0.77	0.62 ± 0.13	2.75 ± 0.40		
8	10.00 ± 0.27	0.50 ± 0.07	2.37 ± 0.20		
9	9.11 ± 1.12	0.46 ± 0.11	1.88 ± 0.74		
10	1.19 ± 0.16	0.25 ± 0.03	<0.016		
11	1.50 ± 0.258	0.24 ± 0.06	0.13 ± 0.11		
12	1.21 ± 0.30	0.21 ± 0.06	<0.016		
13	9.35 ± 1.63	0.49 ± 0.12	1.87 ± 0.94		
14	1.10 ± 0.18	0.21 ± 0.04	<0.016		

* Propionic acid, iso-butyric acid, iso-valeric acid, and n-valeric acid were below the limit of quantification in all analysed samples.

We measured the organic acids in all five biological replicates of the 14 variants at the end of the fermentation period, which was time point two (Table 2). Lactic and acetic acids were present in all samples. In the n-butyric analysis, some samples were below the limit of quantification. Propionic acid, iso-butyric acid, iso-valeric acid and n-valeric acid were below the limit of quantification in all analysed samples. In organic acids analysis, significant differences between the variants were present. A high correlation between organic acids and pH values was identified at T2 by using PERMANOVA. In the pairwise comparisons, significant differences were present between some of the variants by analysing the indexes F value, p value and R^2. At the model split in the components (considering strain, medium, inoculum and organic acids), the strain and the inoculum amount did not play a role (no significant p-value). There was a significant difference, when considering different medium bio glucose syrup agenabon or bio sugar cane molasses. The p-value of the strain was significant in combination with the inoculum and the medium.

4. Discussion

Bioremediation, utilising microorganisms to degrade or remove pesticide residues from the environment, has emerged as a powerful technology. Microbial degradation of azoxystrobin has been reported by hydrolysis of the ester linkage under the catalysis of carboxylesterases [9] and by cleavage of the aromatic ring [6]. Recently, *Bacillus subtilis* strain

MK101 and *Rhodococcus fascians* strain MK144 were reported to degrade azoxystrobin [11]. However, there is a notable gap in the scientific literature regarding detailed protocols for the production and storage of bacterial strains as potential biotechnological products.

Overall, we were successful in the development and validation of a qPCR method for *Bacillus subtilis* strain MK101 and *Rhodococcus fascians strain* MK144 detection. These assays can be used in the future to verify the mentioned bacterial strains in the production and storage of microbial products. The combination of two qPCR assays targeting two different regions of the genome was necessary to ensure good specificity of detection. Using two qPCR assays targeting different genomic regions can be crucial for ensuring the specificity of detection due to several reasons like reduced cross-reactivity, flexibility in detection, compensation for genetic variability or confirmation of results [23]. Therefore, both assays should be used for detection. We recommend starting with qPCR assay with code 91 for *Bacillus subtilis* strain MK101 detection and with qPCR assay with code 25 for *Rhodococcus fascians* strain MK144 detection (Table A1). Furthermore, we recommend using the second assay (assays with codes 35 and 36) only for the positive samples as confirmation. The sample can only be considered positive if both assays give a positive result. This approach reduces the risk of false positives by confirming the target organism's presence through independent verification from two separate DNA regions. Reaction efficiency, sensitivity (the LOD of qPCR is a measure for this parameter) and specificity of the qPCR assay may vary due to various aspects such as sample complexity, DNA/RNA quality, target organisms or methodological variations [24]. The Cq cut-off value can be chosen depending on the situation. Therefore, we used LOD of $\geq$50 in qPCR assay validation for the detection of environmental samples treated with *Bacillus subtilis* strain MK101.

Further, we were successful in the microbial fermentation and storage of a product containing *Bacillus subtilis* strain MK101. In our fermentation experiments, we compared various media and notably observed that variants using bio sugar cane molasses allowed for better growth of *Bacillus subtilis* strain MK101 compared to those variants with bio glucose syrup agenabon. These findings are in line with the literature, substituting molasses sugars with glucose syrup has already been reported to reduce biomass yield [25]. The reported reasons are the absence of molasses buffering power and the lower nitrogen content of the glucose syrup. Spigno and co-workers (2009) proposed supplementing glucose syrup with a by-product of the corn-starch extraction process called corn steep liquor to compensate for the negative effects. However, for the growth of a *Bacillus subtilis*, the corn steep liquor was also reported to lack nutrients like nitrogen for good bacterial growth [26].

During our fermentation studies, we explored two different inoculation levels of bacteria. Interestingly, our findings indicated that variants with lower inoculation amounts of *Bacillus subtilis* strain MK101 lead to higher bacterial growth compared to variants with higher inoculation amounts. This phenomenon is a well-established fact and can be attributed to several factors, including reduced competition for nutrients and less initial stress on the microbial population, allowing for more efficient use and allocation of resources as the bacteria adopt to their environment [27]. Ensuring consistent inoculation throughout experiments is crucial, because studies have shown that the inoculation method itself can significantly influence the extent of bacterial growth [28]. These findings underscore the complexity of microbial dynamics in fermentation and growth processes and highlight the importance of optimising inoculation densities to achieve the best microbial performance under given conditions.

Our fermentation experiment spanned two weeks, after which the variants were bottled, and the storage trial began. The storage trial lasted six weeks. There were five sampling points during the experiment, at which samples were taken to compare bacterial numbers among other parameters. The sampling points were at the beginning (0 d, T0), in the middle (7 d, T1), and at the end (14 d, T2) of the fermentation period as well as after three (T3) and six (T4) weeks of storage. We observed the highest genome equivalents of *Bacillus subtilis* strain MK101 in qPCR analysis in the middle (T1) and the end (T2) of the fermentation, which was the start of the storage trial. The number of bacteria decreased

during storage of the fermented products, which is a common observation [29–31]. This reduction can be influenced by several factors, including the storage conditions and the initial microbial stability of the product. For instance, studies have shown that in fermented apple juice, the number of viable bacteria, such as *Lactobacillus* strains, decreases over a 30-day storage period at a temperature of 4 °C. The reduction in bacterial counts is typically accompanied by changes in pH and other physicochemical properties of the juice, which can affect the overall microbial viability [29]. However, this does not entirely apply to our statistical analysis of the pH values. Our analysis showed that the time points T2, T3, and T4 formed a statistical group in relation to the pH value, although they did not form one in relation to bacterial numbers. Similarly, a study on fermented milk and soymilk mixtures reported that viable bacterial counts tend to decline during storage, even though the product may maintain good sensory qualities and physical stability [30]. In another study of fermentation products involving *Bacillus subtilis*, it was observed that the numbers of these bacteria can decrease during storage. This decrease was influenced by several factors, including the storage temperature. Storage at room temperature could extend the shelf life of the product better than storage at low temperatures [30]. In our experiment, the samples were stored at room temperature (20 °C). These findings underscore the importance of optimising both fermentation and storage conditions to maintain the viability and quality of fermented products during storage.

The decline in detectable genome equivalents during storage may also indicate that *Bacillus subtilis* strain MK101 formed spores. *Bacillus subtilis* can form spores, which can survive in a dormant state for many years, maintaining the potential to revive under suitable conditions. This spore-forming capability is crucial for applications where long-term storage and stability are required. According to the literature, using qPCR analysis to measure *Bacillus subtilis* spores requires specific modifications due to the protective nature of the spore coat, which can inhibit DNA extraction necessary for qPCR analysis [32]. The ability of *Bacillus subtilis* strain MK101 to form spores should be investigated in future studies.

At every stage of fermentation and storage, we monitored bacterial viability by cultivating on tryptic soy broth agar plates. Our findings showed that the highest viability occurred in variants using glucose syrup, particularly with lower inoculation levels of the *Bacillus subtilis* strain MK101. Specifically, variant four demonstrated the most robust viability. In the qPCR analysis, the highest genome equivalent numbers were also observed with lower inoculation amounts but using bio sugar cane molasses as the medium. Upon further examination of the viability analysis across different time points, the grouping was consistent with the qPCR analysis at the start of fermentation and at three and six weeks of storage. The middle of the fermentation and the end of the fermentation revealed two distinct groups in viability analysis. The highest numbers concerning viability were achieved after the fermentation, which was slightly different to qPCR analysis, where during and after fermentation, the highest genome equivalent numbers were detected. Comparing microbial abundance measured by qPCR analysis and bacterial viability on agar plates is often used to give a comprehensive view of microbial communities, as noted in the microbiology research literature [33].

We measured pH values across all five biological replicates of the 14 variants at five time points during the fermentation and storage trial and performed statistical analyses. The statistical analysis of pH values indicated no significant differences attributable to the strain alone. Observed differences were only significant when interacting with other variables. For example, there were substantial pH differences between the used media. From T2 onwards, which marks the end of fermentation and the start of the storage trial, the pH value of all variants stabilised in the acidic range. However, the pH value in all variants using the bio sugar cane molasses medium was slightly more acidic compared to those using the bio glucose syrup agenabon medium. This demonstrates that the fermentation medium plays a crucial role in influencing the behaviour of bacteria during the fermentation process. Different components of the medium, including carbon and nitrogen sources, minerals, and growth factors, significantly affect the growth and metabolic activity of microorganisms,

thereby impacting the yield and quality of the fermented product. Based on production methods and formulations sugar cane molasses and bio glucose syrup agenabon have specific pH values. The pH value of sugar cane molasses is generally slightly acidic, whereas the pH of glucose syrup derived from corn typically ranges from mildly acidic to neutral. This difference in initial pH levels suggests that a longer fermentation duration may be required with certain bacterial strains to achieve the same pH level when using glucose syrup, compared to fermenting with molasses. The reason for this could be, on the one hand, the nutrient composition (molasses provides a richer nutrient environment compared to simple sugars, which can facilitate faster microbial growth and metabolism) and, on the other hand, the metabolic pathways of the bacteria. As described in studies, depending on strain and environmental conditions, sucrose present in molasses can more efficiently be metabolised compared to glucose [34]. Furthermore, solutions with high concentrations of glucose can create significant osmotic pressure, which might inhibit fermentation processes as cells expand energy to maintain osmotic balance instead of fermenting sugars. Molasses has been shown to be efficient in fermentation under various conditions, including high salinity that can mimic high osmotic pressures [35].

In our statistical analysis, we found a strong correlation between pH values and organic acid concentrations. A study demonstrates that the investigation of different organic acids, compared to those in our study, in the fermentation products can positively influence outcomes, such as enhancing phosphate solubilization in cucumber plants [36]. A study shows that vitamin B1 exhibits greater chemical stability in the presence of a higher concentration of inorganic acids [37]. There was a significant difference in organic acid production during the fermentation experiment when using different media bio glucose syrup agenabon or bio sugar cane molasses. Generally, fermentation products using bio sugar cane molasses exhibited higher levels of lactic acid, acetic acid, and n-butyric acid than products using bio glucose syrup agenabon as media. Optimised lactic acid production from sugarcane molasses as a carbon source compared to glucose under controlled fermentation conditions has already been reported for fermentation with the *Bacillus amyloliquefaciens* J2V2AA strain [38]. In future studies, it is crucial to examine additional metabolites and their stability, as numerous other factors beyond environmental conditions, medium composition or viability, as discussed in this paper, can significantly influence the final product. These factors include, for example, strain mutations and genetic drift [39], horizontal gene transfer, and the regulation of metabolic pathways [40].

In summary, we conclude that the here described microbial fermentation and storage protocols yielded a microbial product with a certain shelf life for the azoxystrobin-degrading *Bacillus subtilis* strain MK101. However, there is a need for additional studies to directly measure the bioremediation efficacy of the potential biotechnological products. This study demonstrates that the fermentation medium and the inoculation amount play a crucial role in influencing the behaviour of bacteria during the fermentation process and in storage. These findings aid in developing pesticide-degrading products and address the gap in methodologies for producing and storing bacterial strains as biotechnological products.

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Appendix A

Table A1. Oligonucleotide primers and probes used in this study (qPCR analysis) and efficiency and R^2 during validation qPCR assays.

Code	Name	Sequence (5′-3′)	Validation Efficiency (E) [%]	Validation R^2
	Bacillus subtilis strain MK101			
91	101F_91	AAACGAGGCAGCATCCAg	95.7	0.997
	101R_91	ACATTGCTTATCGGATGTATGGTAAT	107.1	0.997
	101P_91	ATGCTTTAGCTGAATCAATTCCGCTat	100.8	0.997
			104.4	0.993
35	101F_35	TCTGAACTCATAGGCTTTGG	103.5	0.997
	101R_35_mod	CAGTAAAAGCATTTGTGGTG	101.5	0.997
	101P_35	TGTCTAGTGCTTCTGCATCGGCTT	116.5	0.996
			98.7	0.999
	Rhodococcus fascians strain MK144			
25	144F_25_mod	GGGACCGGTGCTGCA	91.9	0.998
	144R_25_mod	CACAGCCGAGATCAGATCT	89.3	0.994
	144P_25_mod	TGCGCGCCACGATCTGACCGT	86.7	0.999
			86.1	0.993
36	144F_36	CACGTGAGCTGACGATCGAG	91.8	0.997
	144R_36_mod	CTTGTCCACGGCCACG	83.2	0.997
	144P_36_mod	tggaAGGGATGGAGATTCTGTACGC	87.4	0.999
			90.3	0.999

Table A2. List of bacteria that were used as reference strains and environmental samples used for qPCR assay validation. The gDNA of these samples was extracted in strict accordance with the instructions of the DNeasy PowerSoil Pro kit (Qiagen, Hilden, Germany).

***Bacillus subtilis* Strain MK101 Reference Strains**		***Rhodococcus fascians* Strain MK144 Reference Strains**	
Species	**Number**	**Species**	**Number**
Bacillus amyloliquefaciens FZB42 (*Bacillus velezensis* FZB42)	DSM 23117	*Rhodococcus ruber*	DSM 43338
Bacillus subtilis subsp. *subtilis*	DSM 1091	*Rhodococcus pyridinivorens*	DSM 44555
Bacillus turingiensis	DSM 5724	*Rhodococcus fascians*	DSM 20669
Bacillus turingiensis	DSM 2046	*Rhodococcus fascians*	DSM 43586
Bacillus altitudinis	DSM 21631	*Rhodococcus fascians*	DSM 45106
Bacillus amyloliquefaciens	DSM 7	*Rhodococcus* sp.	TA-R37
Bacillus atophaeus	DSM 7264	*Rhodococcus* sp.	TB-U1a
Bacillus firmus	DSM 12	*Rhodococcus* sp.	TB-U5
Bacillus flexus	DSM 1320	*Rhodococcus* sp.	TP-O25
Bacillus halotolerans	DSM 8802	*Rhodococcus* sp.	T2_12
Bacillus licheniformis	DSM 13	*Rhodococcus* sp.	H39_1
Bacillus mojavensis	DSM 9205	*Rhodococcus* sp.	Aga47_7
Bacillus pumilus	DSM 27	*Rhodococcus* sp.	LC1_14
Bacillus safensis	DSM 19292	*Rhodococcus* sp.	LC31_2
Bacillus sonorensis	DSM 13779	*Rhodococcus* sp.	F23_3

Table A2. *Cont.*

***Bacillus subtilis* Strain MK101 Reference Strains**		***Rhodococcus fascians* Strain MK144 Reference Strains**	
Species	**Number**	**Species**	**Number**
Bacillus subtilis subsp. *inaquosorum*	DSM 22148	*Rhodococcus* sp.	46
Bacillus subtilis subsp. *spizizenii*	DSM 15029	*Rhodococcus* sp.	Bpgp30
Bacillus subtilis subsp. *subtilis*	DSM 10	*Rhodococcus* sp.	IG-R-39
Bacillus vallismortis	DSM 11031	*Rhodococcus* sp.	IG-S-34
Bacillus xiamenensis	DSM 29883	*Rhodococcus* sp.	IG-S-76_3
Bacillus sp.	1190		
Bacillus sp.	1160	Environmental samples (three biological replicates)	
Bacillus sp.	1282	Lettuce leaf untreated; *B. subtilis* strain MK101 trial	
Bacillus sp.	1291	Soil untreated; *B. subtilis* strain MK101 trial	
Bacillus sp.	1116	Lettuce leaf treated with *B. subtilis* strain MK101	
Bacillus sp.	1359	Soil treated with *B. subtilis* strain MK101	
Bacillus sp.	1494	Lettuce leaf untreated; *R. fascians* strain MK144 trial	
Bacillus sp.	1513	Soil untreated; *R. fascians* strain MK144 trial	
Bacillus subtilis EKB010BA16	n.d.	Lettuce leaf treated with *R. fascians* strain MK144	
Bacillus thuringiensis	DSM 6018	Soil treated with *R. fascians* strain MK144	
Bacillus siamensis	DSM 25261	Sugar cane molasses: three different batch numbers	
Bacillus thuringiensis Berliner 1915	DSM 2046	Microbial product: three different batch numbers	
Bacillus simplex	DSM 1321		
Bacillus amyloliquefaciens strain AT-332	n.d.		
Bacillus velezensis GB03	n.d.		
Bacillus subtilis	DSM 1970		
Bacillus nakamurai	CCUG 68786		
Bacillus licheniformis	FMCH001		
Bacillus amyloliquefaciens strain 19b HAMBI	n.d.		
Bacillus velenzensis	RTI0301		

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