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Analysis of pectolytic enzymes and *Alternaria spp*. in fresh dill, mustard seeds, onions, and vinegar, and their influence on the softening of pickled cucumbers

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Abstract: Proteolytic enzymes or fungi have long been identified as causing softening of pickled cucumbers. As the softening of cucumbers occurs mainly in the pasteurized state, this study considers the hypothesis that vinegar and the added spices could be responsible for this softening by studying polygalacturonase (endo-/exo-PG), pectinesterase (PE), and pectin lyase, as well as *Alternaria spp*. found in the spices. Because of the high endo-PG activity found in dill, this spice emerged as a possible factor causing spoilage. Compared to dill, the enzyme activity in mustard seeds is eight times lower, and only low levels of enzymes or *Alternaria spp*. are present in onions or vinegar. Different harvest times and the associated degree of freshness of dill also played a crucial role regarding the endo-PG activity of up to 25.11 U/g (30°C, mature and very woody dill in late July) but of less than 9.00 U/g in fresh and soft green dill harvested in late June. A temperature of 80°C, held for 3 min, reduced the enzyme activity to less than 1.0 U/g. A final examination of cucumbers with a fixed quantity of mustard seeds, vinegar, and onions but with different variants of dill showed that the quantity of dill and the other ingredients added to the jars is not a potential factor leading to cucumber softening, which conflicts with the hypothesis of cucumber spoilage by vinegar and spices.

KEYWORDS
analytical methods, enzyme, fungi, spices & seasonings, viscosity

Practical Application: This work provides insights into the activity of various pectolytic enzymes and the load of *Alternaria spp*. in different ingredients used for pickle production. Based on these data and additional pasteurization experiments, this paper evaluates the influence of dill, onions, mustard seeds, and vinegar on cucumber softening.

1 | INTRODUCTION

Textural changes in pickled cucumbers, such as softening, are issues that have still not been resolved. Research from the last century attributed such changes to high pectolytic enzyme activities, especially those found in cucumber seeds and ripe fruits (Bell et al., 1951; Fabian et al., 1939), and more recent studies suggested that such changes

also result from storage conditions or salt concentrations (Maruvada & McFeeters, 2009).

Enzymes in cucumbers are active right up to the fermentation process. Therefore, they can destroy cell walls (Meurer & Gierschner, 1992). The cell wall consists of a primary and secondary wall that is stabilized by pectin in the middle lamella (Taiz & Zeiger, 2007). The degradation of pectin (complex polysaccharides high in galacturonic acid; Christiaens et al., 2015) is influenced by endogenous and exogenous enzymes. Two main groups of these pectolytic enzymes are depolymerases (polygalacturonase [PG], pectin lyase), and pectinesterases (PE) (Bell et al., 1950). PE deesterifies pectin (Duvetter et al., 2009; Micheli, 2001) and originates in phytopathogenic bacteria or fungi (Christiaens et al., 2015). Endo- and exo-PG hydrolyze glycosidic bonds and form oligogalacturonides and monogalacturonides and are present in fungi, bacteria, and yeast (Duvetter et al., 2009). Pectin lyase can be found in microorganisms (Yadav et al., 2009) and cleaves pectin using β-elimination (Duvetter et al., 2009).

The softening of cucumbers is caused mainly due to pectolytic enzymes after pasteurization and a few months of storage (Cho & Buescher, 2012; Voldřich et al., 2009) when fruit tissue is hydrolyzed by PG and cellulase activity (Voldřich et al., 2009). Temperatures and salt conditions are other possible factors (Maruvada & McFeeters, 2009). Blanching between 55°C and 75°C with 1.0 M NaCl causes decreased firmness of cucumbers during fermentation, while 0.3 M NaCl resulted in only small changes in texture. A temperature of 60°C, held for 10 min, led to lower PE activities while a loss of enzyme activity occurred at 70°C (Bell et al., 1951). Holding the temperature for only 6 min retained activity of 70.9% at 65°C (Maruvada & McFeeters, 2009) with inactivation of PE and PG at 79°C (Meurer & Gierschner, 1992).

High bacterial contamination of spices is also an important factor behind the deteriorating texture of fresh cucumbers (Fabian et al., 1939). Fungal diseases of cucurbits (powdery mildew, wilt, anthracnose) have a negative impact on plant health as well and can reduce yield (Paul et al., 2015). *Alternaria* species, which have already been detected in plant material, processed food, and soil (Paul et al., 2015; Pavón et al., 2010), form circular lesions on cucumbers and therefore cause losses due to its rapid spread (Vakalounakis, 1990). Previous research indicated a fungal origin for the softening of pickled cucumbers such as *Penicillium*, *Ascochyta*, *Fusarium*, *Cladosporium*, and *Alternaria* introduced via highly contaminated blossoms or the cucumbers themselves in the jars (Demain & Phaff, 1957; Etchells et al., 1958; Raymond et al., 1959). The ingredients for pickled cucumbers (dill, mustard seeds, and onions) can also be infected by *Alternaria spp*. and could be potential sources of the fungi in cucumbers (Boedo et al., 2012; Meena et al., 2010; Ramjegathesh & Ebenezar, 2012).

This study aimed to analyze possible causes for cucumber softening by analyzing the pectolytic enzyme activities and *Alternaria spp*. load in dill, mustard seeds, onions, and vinegar. First, the pure ingredients were quantified alone and examined for the heat stability of the pertinent enzymes. It was also tested if varying degrees of dill ripeness can cause a softening even after heating up to 80°C. After determining the decisive ingredients with high pectolytic or maybe fungal activities, cucumbers were pasteurized with variations of these critical spices in order to investigate their influence of them on the cucumbers themselves and the possible softening.

2 | MATERIALS AND METHODS

2.1 | Source of materials

To analyze the activity of pectolytic enzymes in the spices of pickled cucumbers, fresh dill, onions, mustard seeds, and vinegar were ordered from a cucumber-producing region in the east of Germany, Brandenburg (Spreewald), in August 2017. Two extracts of each spice were produced separately but originated from the same bulk sample. The mean values of both extracts are shown in Table 2. Mustard seeds were characterized by a yellow color with few black grains and were stored at room temperature (RT, 20°C, 60.0% humidity). Vinegar was stored in the refrigerator (4°C). Onions and dill were already cut, packaged in 100 g packs, and vacuum sealed before being stored at −20°C. Due to the fact that the dill harvested in 2017 was already woody and had a brown structure (Figure 1), renewed trials with dill were made in 2018. Dill harvested in late June 2018 was characterized by a green color and soft structure. Dill samples procured in late July appeared already brown in color and were almost completely withered. These samples were also prepared and stored at −20°C.

2.2 | Experimental design of this study

This study is divided into several parts to investigate the possibility of cucumber softening by dill, mustard seeds, onions, and vinegar and to identify a possible correlation between enzymes and high *Alternaria* loads. In the first investigation, the enzymes which are normally found in cucumbers (endo-/exo-PG, PE, and pectin lyases) are examined for their presence and quantity in the named spices. The *Alternaria* load on these spices was also analyzed. Heat measurements up to 80°C were performed to determine residual enzyme activity, while the last investigation concludes with the pasteurization of cucumbers

FIGURE 1 Samples of the dill variants from different harvest months and years: (a) August 2017, (b) late June 2018, (c) mid-July 2018, (d) late July 2018, and (e) commercially dried

with these spices in their commercial weight but with different dill variants. A storage period of three months and the quantities of enzymes measured in the pickled cucumbers should provide a definitive result about the correlation between the spices used and fruit softening.

2.3 | Sample preparation for measuring the enzyme activity

Samples were prepared as performed by Meurer and Gierschner (1992) to determine the enzyme activity of endo- and exo-PG, PE, and pectin lyase. Therefore, dill and onions were prepared using ultrafiltration, whereas mustard seeds underwent ammonium sulfate precipitation. Vinegar, however, was simply buffered up to pH 6.0 with 5.0 and 0.5 M NaOH. Chemicals for preparations and measurements were purchased from Carl Roth (Carl Roth GmbH & Co.KG, Karlsruhe, Germany) and Sigma-Aldrich (Sigma-Aldrich, Corp., St. Louis, MO, USA).

2.3.1 | Sample preparation with ultrafiltration

100.0 g of frozen dill/onions were mixed (GM 200, Retsch GmbH, Haan, Germany) and homogenized (IKA T 25 Ultra-Turrax®, IKA®-Werke GmbH & Co.KG, Staufen, Germany) with 1.0 M NaCl (100 ml) and saponine (0.1 g) for 1 min each. The dill samples were also enriched with 0.5 M NaCl to solubilize PE and PG (Patel & Phaff, 1960). After subsequent stirring for 2 h (4°C), the liquid was squeezed through a nylon mesh and centrifuged for 25 min (3200 rpm, 4°C; Sigma Laborzentrifugen GmbH, Osterode, Germany) before filtration through a paper filter and ultrafiltration (VivaFlow 200 Membrane 10.000 MWCO PES, Sartorius AG, Göttingen, Germany). The concentrated sample was left in a dialysis solution (0.01 M acetate buffer pH 5.6; 0.1 M NaCl; 0.001 M sodium azide) overnight before being centrifuged (20 min, 5000 rpm, 4°C) and filtered, followed by measurement of the final volume (Table 1).

2.3.2 | Sample preparation with ammonium sulfate

Mustard seeds were crushed for 30 s using a universal mill (IKA® M20 universal mill, IKA®-Werke GmbH & Co.KG) and homogenized for 1 min with 0.025 g saponine. 37.0 ml hexane was added and stirred for 15 min at RT, and the mixture was centrifuged for 10 min (3500 rpm, 4°C). This procedure was repeated two times with removal of the hexane after each centrifugation step followed by a third centrifugation for 20 min. Subsequently, hexane was pipetted in again and stirred with deionized water and NaCl (100 ml/9.38 g) for 2 h in an ice bath. After filtering, centrifuging (25 min), repipetting, and discarding the solid/emulsion phase, 30.0% ammonium sulfate was added to the liquid, stirred (30 min), and centrifuged again. This process was also repeated with 80.0% ammonium sulfate; only the solid phase was subsequently mixed with 50 ml of the dialysis preparation and placed in a tube in dialysis solution overnight (4°C). Finally, the solution was centrifuged (5000 rpm, 4°C), and its volume was determined (Table 1).

2.4 | Tests for measuring pectolytic enzyme activities

2.4.1 | Polygalacturonase viscosity test

Cucumber endo-PG has optimum activity at pH 5.6 and can be determined viscometrically (McFeeters et al., 1980; Meurer & Gierschner, 1992). 200 ml of a 0.12 M citrate-phosphate buffer with pH 5.5 was stirred with 0.8 g of 65.0%–72.0% esterified pectin from citrus fruits (Carl Roth GmbH & Co.KG), cooled to RT, and filtered through a G2 frit (Schott AG, Mainz, Germany). The substrate solution (buffer and pectin C) was adjusted to 30°C, and 12.5 ml of the solution was mixed with 2.5 ml of the test sample and placed into an Ubbelohde viscometer (type 53210/I K = 0.01, Schott AG). Starting the measurement after 3 min (t_0), two light barriers were passed before the procedure was repeated after 10/15 min (t_1) (Formula 1).

TABLE 1 Relevant data for the sample preparation

Variant	Raw data for sample preparation		
	Raw weight sample [g]	Volume after ultrafiltration/ammonium sulfate precipitation [ml]	Concentration factor
Dill no. 1	100.29	36.5	2.75
Dill no. 2	69.84	29.0	2.41
Dill—late June	97.29	75.0	1.30
Dill—mid-July	93.09	63.5	1.47
Dill—late July	100.48	74.5	1.35
Dried dill	100.71	71.5	1.41
Mustard seeds no. 1	25.00	57.0	0.44
Mustard seeds no. 2	25.00	70.0	0.36
Onions no. 1	100.29	51.5	1.95
Onions no. 2	100.25	32.0	3.13

TABLE 2 Activity of pectolytic enzymes in dill, mustard seeds, onions, and vinegar at 30°C

Enzyme	Enzyme activity of spices and vinegar			
	Dill [U/g]	Mustard seeds [U/g]	Onions [U/g]	Vinegar [U/ml]
Endo-PG	9.53 ± 0.15	1.11 ± 0.21	0.15 ± 0.02	0.08 ± 0.04
Exo-PG (x 10^{-4})	18.25 ± 0.21	19.15 ± 3.61	0.95 ± 0.21	1.00 ± 0.00
PE	10.45 ± 1.25	0.73 ± 0.05	0.22 ± 0.03	0.05 ± 0.02
Pectin lyases	ΔE = 0.009	nd	ΔE = 0.001	nd

Note: The results are presented as mean values ± standard deviation derived from two extracts with three repetitions each.
Abbreviation: nd, not detectable.

$$\frac{U}{g} = \left(\frac{t_w}{t_1 - t_w} - \frac{t_w}{t_0 - t_w}\right) \times \frac{1000}{[\text{min}]} \times \frac{[\text{ml}]\,\text{Total enzyme volume}}{[\text{g}]\,\text{Sample weight of the raw material}} \quad (1)$$

t_w represents the blank value of water and min the testing time of 10/15 min.

In the literature, enzyme activities are often presented as units per ml. In this paper, all activities are described as units per gram of the tested ingredients by multiplying the values of $\frac{\text{U}}{\text{ml}}$ with the volume of the concentrated enzyme extract and subsequently dividing by the raw weight of the test sample.

2.4.2 | Polygalacturonase photometrical test

Exo-PG activity can be measured photometrically (UV-3100 PC, VWR International, LLC, Radnor, USA) using a method slightly different from Honda et al. (1982) to analyze reducing carbohydrates. 0.25 ml of desalinated and diluted sample (represented as "[ml] enzyme extract" and based on the dilution of 3.5 ml bidistilled water to 2.5 ml enzyme extract, shown as $\frac{3.5}{2.5}$) was placed in test tubes with 0.5 ml of acetate buffer (0.2 M, pH 5.0 with 0.4 mM $CaCl_2$) and 0.25 ml of 0.1% sodium salt of polygalacturonic acid (substrate). Blank values for the buffer (B_{buffer}), substrate ($B_{\text{substrate}}$), and the enzyme (B_{enzyme}) were prepared with 0.25 ml of distilled water instead of the specific buffer.

$$\textbf{Total blank value}\; B = \left(B_{enzyme} + B_{substrate}\right) - B_{buffer} \quad (2)$$

After resting in a water bath at 30°C for 180 min (incubation time), 2 ml of 0.2 M boric acid-borax buffer and 1 ml of 1.0% 2-cyanoacetamide were added. Finally, the samples were heated to 100°C (11 min) and cooled to RT before being measured at 276 nm within 1 h using a 1 cm quartz cell (Hellma GmbH & Co.KG, Müllheim, Germany). To set up the calibration curve, 0.01 M galacturonic acid was diluted to 50.0 µmol/L to 200.0 µmol/L. 0.5 ml of the dilutions were mixed with 0.5 ml of 0.2 M acetic buffer, 1 ml 1.0% 2-cyanoacetamide, and 2 ml boric acid-borax buffer. A

calibration curve was created after reheating (11 min) and cooling. To determine the enzyme activity of exo-PG, the linear equation must first be calculated:

$$y = mx + b \tag{3}$$

$$\frac{U}{g} = \frac{\left(\frac{\Delta E - b}{m} \times \frac{1}{1000 \times [\text{min}]\ \text{Incubation time} \times [\text{ml}]\ \text{Enzyme extract}} \times \frac{3.5}{2.5}\right) \times [\text{ml}]\ \text{Total enzyme volume}}{[\text{g}]\ \text{Sample weight of the raw material}} \tag{4}$$

ΔE was calculated by subtracting the measured extinction and the total blank value.

2.4.3 | Pectinesterase test

150 ml distilled water and 30 ml of 1 M NaCl were mixed and heated to 60°C before adding and dissolving 1 g of citrus pectin (65.0%−72.0% degree of methyl esterification; Carl Roth GmbH & Co.KG). The mixture was cooled to RT, the pH was adjusted to 6.5 with 0.3 M NaOH, and the volume was made up to 200 ml. The mixture was then heated to 30°C again. 10 ml of the pectin solution was placed into a double-walled beaker and mixed with 1 ml of the enzyme extract for 6 min. In the first 2 min, it was necessary to increase the pH to 7.0 using 0.01 M NaOH before the pH was held constant by titrating with 0.01 M NaOH throughout the testing time of 4 min. The total volume of the enzyme extract and the weight of the raw material were also part of the calculation of PE activity:

$$\frac{U}{g} = \left(\frac{[\text{ml}]\ 0.01\ \text{M NaOH} \times [\text{ml}]\ \text{Pectin solution}}{[\text{min}]\ \text{Time} \times [\text{ml}]\ \text{Enzyme extract}}\right) \times \left(\frac{[\text{ml}]\ \text{Total enzyme volume}}{[\text{g}]\ \text{Sample weight of the raw material}}\right) \tag{5}$$

Fresh dill samples were diluted 1:10 because of their high quantity of PE.

2.4.4 | Pectin lyase test

100 ml of 0.1 M acetate buffer and 0.2% pectin from apples (degree of methyl esterification: >20.0%) were mixed and adjusted to 30°C. 0.6 ml of desalinated, temperature-adjusted enzyme extract and 2.5 ml of the buffer solution were mixed and measured at 235 nm from 0 to 180 s and for up to 15 min (in steps of 5 min).

2.5 | Sample preparation and measurement for the detection of molds

The samples were taken each from a thoroughly mixed batch of dill, onion, vinegar, or mustard.

2.5.1 | DNA extraction

DNA extraction from dill, onion, mustard seeds, and vinegar samples (each $n = 3$) was performed with NucleoSpin® Soil Kit (Macherey-Nagel GmbH & Co.KG, Düren, Germany) and the contained lysis buffer SL1. The sample weight for extraction was 350.0 ± 1.0 mg each for onion and dill, 203.0 ± 1.0 mg for mustard seeds, and 500 ml for vinegar. Three mustard seed samples with a total weight of 1000.0 ± 1.0 mg each were divided into five partial samples each for DNA extraction and the DNA was recombined at the end. Dividing the samples was necessary because of the strong gelation of the crushed mustard seeds. All samples were ground with a plunger in the reaction vessel and incubated with lysis buffer at RT for 2 h in a rotary shaker (Enviro-Genie, Scientific Industries, Inc., NY, USA). Collected bulk DNA samples (45 µL) were stored at 4°C until further analysis. DNA quality was analyzed by NanoDrop (ThermoFisher Scientific, Inc., Waltham, MA, USA).

2.5.2 | Real-time PCR

Bulk DNA samples were used to quantify *Alternaria spp*. using real-time PCR (Applied Biosystems®, QuantStudioTM 12K Flex, Waltham, MA, USA). The specific primer pair Alt-F 5′-tct ttt gcg tac ttc ttg ttt cct t-3′ (forward primer) and Alt-R 5 ′-tta ctg acg ctg att gca att aca-3′ (reverse primer) and probe Alt-prt 5′-tgg gtt cgc cca cta gga ca-3′ (Biomers.net GmbH, Ulm, Germany) were used. The composition of the real-time PCR reaction with a 20 µl reaction volume was as follows: 5× HOT FIREPol® Probe GC qPCR Mix (solis Biodyne, Tartu, EST) in a final concentration of 150 nM of each primer, 330 nM of the probe, and 1 µl DNA extract. The real-time PCR cycles took place under the following conditions: Initial denaturation at 95°C for 10 min, 45 cycles at 95°C for 15 s, followed by annealing/extension at 64°C for 45 s. All samples were tested under these conditions. The concentration of *Alternaria spp*. (*Alternaria*

spp./g) of each sample was determined using a dilution series of defined DNA concentration extracted from isolated *Alternaria* plates, which was always treated in parallel. The serial dilution for quantification was gradually reduced by factor of 10 (7 times). Fluorescence intensity over time was determined using the Ct value with QuantStudio™ 12K Flex Software v1.2. The values were always related to the initial weight.

Total fungal DNA was amplified using the highly conserved fungal rRNA gene primers (ITS1F cttggtcatttagaggaagtaa and ITS4 tcctccgcttattgatatgc) previously described (Gardes & Bruns, 1993; White et al., 1990). The total fungal DNA in a sample was quantified by SYBR green fluorescence qPCR (Applied Biosystems®, QuantStudioTM 12K Flex) using an external standard curve. Real-time PCRs (20 µl) contained 5 µl of template DNA qPCR HRM-mix (3 mM $MgCl_2$, Solis BioDyne, Tartu, Estonia), 100 nM of ITS1F primer, and 500 nM ITS4 primer. The real-time PCR thermal protocol consisted of an initial 15 min denaturation step at 95°C, 32 amplification cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 60 s, and a final extension step of 72°C for 10 min.

2.6 | Further trials with dill containing high amounts of pectolytic enzymes and molds

2.6.1 | Temperature and harvest trials

Fresh dill samples with different harvest dates in 2018 and high levels of pectolytic enzymes and *Alternaria spp*. were each subjected to heating tests of 60°C, 70°C, and 80°C for 3 min ($n = 3$) to check the stability of enzyme activities after heating and the influence of harvest time.

2.6.2 | Analysis of pectolytic enzymes in pickled cucumbers with different variants of dill

To analyze the possible softening of pickled cucumbers due to different variants of dill (fresh, dried, or without dill), dill was ordered with cucumbers (mixture of Platina and Liszt; size 6/9 cm), pre-cut onion slices, mustard seeds, and vinegar from the same region in Brandenburg. Cucumbers were prepared according to a pack-out ratio of 56:44 (346 ± 11 g cucumbers: 275 ± 1 ml vinegar) with the addition of an average of 6.05 g of onions and 1.48 g of mustard seeds. 1.06 g of dried dill (first variant) and 1.05 g of fresh dill (second variant) were also added in a duplicate test, while the third variant contained no dill. The ingredients were placed in sterilized jars with volumes of 720 ml (diameter: 95.5 mm; height: 132.5 mm) and pasteurized in a water bath at 85°C for 20 min before they were slowly cooled with tap water and stored at RT. The activity of the corresponding enzyme was measured after storage for three months (RT, 20°C, 60.0% humidity) and was derived from three measurements from two different cucumber jars.

2.7 | Statistical methods

Three trials of two extracts per ingredient were conducted for each enzymatic test to determine the pectolytic enzyme activities of spices and vinegar. The values for the real-time PCR were performed in triplicate. Results were presented as mean ± standard deviation and analyzed by analysis of variance (ANOVA). Tukey-HSD test was performed for multiple comparisons. SPSS package (SPSS 26.0 for Windows, SPSS Inc, Chicago, USA) software was used for data analysis.

3 | RESULTS AND DISCUSSION

3.1 | Analysis of pectolytic enzyme activities

3.1.1 | Endo-PG activity in dill, mustard seeds, onions, and vinegar

The activity of endo-PG found in spices and vinegar is shown in Table 2. High activity of endo-PG could be detected in pure dill with 9.53 ± 0.15 U/g after an incubation period of 3 min at 30°C. Temperatures of up to 60°C, 70°C, and 80°C (Table 3) resulted in an 86.4% reduction of activity at 60°C to 1.30 ± 0.04 U/g ($p < 0.05$) and a loss of activity at 70°C and 80°C. More than 80 years ago, Fabian et al. (1939) noted that the bacterial content of highly contaminated, unsterilized spices and dill weed is an important factor in the softening of pickled peaches and pears and identified a link between endo-PG activities in higher plants and declining fruit texture. This softening is mainly caused by enzymes of the anaerobic metabolism in fermented fruits (Duvetter et al., 2009; Meurer & Gierschner, 1992). Therefore, the quantity of endo-PG determined in pure and unsterilized dill had to be tested with fermented cucumbers to make an appropriate statement about the softening effect of dill.

Compared to the pectolytic activity of dill, mustard seeds were associated with an activity of approximately 1.11 ± 0.21 U/g, which is almost nine times lower. Onions, however, had an enzyme activity of less than 0.20 U/g and for vinegar the values were close to zero and therefore probably without softening potential.

TABLE 3 Endo-PG and PE activity in dill after an incubation time of 3 min at temperatures of 30°C, 60°C, 70°C, and 80°C and their residual enzyme activity compared to 30°C (%)

	Endo-PG [U/g]		PE [U/g]	
Temperature [°C]	Total enzyme activity (U/g)	Residual enzyme activity (%)	Total enzyme activity (U/g)	Residual enzyme activity (%)
30	9.53 ± 0.15[a]	100.00	10.45 ± 1.25[a a]	100.00
60	1.30 ± 0.04[b]	13.62	1.19 ± 0.30[b]	11.36
70	nd[c]	–	0.05 ± 0.02[b]	0.45
80	nd[c]	–	0.04 ± 0.01[b]	0.36

The results of the heating trials are presented as mean values ± standard deviation derived from two extracts with three repetitions each. Different lowercase letters in the same column indicate significant differences ($p < 0.05$).

Abbreviation: nd, not detectable.

[a-c]Mean values having a different lowercase letter within a column differ ($p < 0.05$).

TABLE 4 Activity of pectolytic enzymes in pickled cucumbers with different variants of dill after a storage period of three months and humidity of 60.0%

	Enzyme activities of pickled cucumbers with different variants of dill		
Variant of dill	Endo-PG [U/g]	Exo-PG [U/g]	PE [U/g]
Dried dill	0.084 ± 0.008[a]	nd	0.026 ± 0.010[a]
Fresh dill	0.046 ± 0.004[b]	$<1 \times 10^{-3}$	0.016 ± 0.005[a]
Without dill	0.098 ± 0.011[a]	nd	0.029 ± 0.015[a]

The results are presented as mean values ± standard deviation derived from two extracts with three repetitions each. Different lowercase letters in the same column indicate significant differences ($p < 0.05$).

Abbreviation: nd, not detectable.

[a-b]Mean values having a different lowercase letter within a column differ ($p < 0.05$).

3.1.2 | PE activity in dill, mustard seeds, onions, and vinegar

The highest level of PE could be found in dill at pH 7.0 with an average amount of approximately 10.45 ± 1.25 U/g. In contrast, mustard seeds showed only a negligible activity of 0.73 ± 0.05 U/g (Table 2). Low levels of activity were detected in the samples of vinegar or onions, as well (0.05 ± 0.02 U/ml vinegar, 0.22 ± 0.03 U/g onions). Much lower results for onions were found by Misekow and Fabian (1953) with a PE activity of 0.11×10^{-2} to 0.16×10^{-2} U/ml in garlic obtained with a final concentration of 0.15 M NaCl, pH 7.5 (30°C), and a 0.4% pectin solution. Heating a dill extract to 60°C resulted in a residual enzyme activity of 11.4% with a final activity of 1.19 U/g, while temperatures of 70°C and 80°C led to activities of less than 0.5% when the holding time was 3 min. The findings of Meurer and Gierschner (1992) were similar with residual enzyme activity of 1.5% after blanching at 79°C. Chang and Shiau (1986) noticed a reduction to 8.0% after blanching cucumber slices at 80°C for 5 min. Maruvada and McFeeters (2009) achieved a residual enzyme activity of 71.0%, which is more than six times higher for low salt cucumbers blanched at 65°C.

3.1.3 | Exo-PG and pectin lyase activity in dill, mustard seeds, onions, and vinegar

Unlike the results of Pressey and Avants (1975) with exo-PG activities between 0.29 and 0.87 U/ml for dialyzed solutions of cucumbers of different weights (55.0 to 230.0 g), enzyme activities in this study of between 1.9×10^{-3} U/g in mustard seeds and 1.0×10^{-4} U/g in onions ($< 1.0 \times 10^{-2}$ U/ml for all samples) could not be verified as meaningful.

While cucumbers have an optimum pH of 5.0 for exo-PG and there is no inhibition of this enzyme if the maximum addition of $CaCl_2$ is 0.4 mM (Meurer & Gierschner, 1992), trials in this study (acetate buffer pH 5.0; 0.4 mM $CaCl_2$) showed only small detectable amounts. These values may also be subjected to limits of variations and measurement inaccuracies. Based on the work of Saltveit and McFeeters (1980) who identified high PG activity at pH 6.2 for ripe fruits and pH 4.6 for crude fruits, further trials with spices at pH 6.2 would be recommended to confirm exo-PG activities. Although according to Pressey and Avants (1975), the exo-PG can cause softening in cucumbers by pectin degradation, the activities detected in these trials are probably too low for softening to occur. Almost undetectable levels of enzyme activity were also shown for pectin lyases.

TABLE 5 Amount of *Alternaria* spp. in dill, mustard seeds, and onions

Sample	*Alternaria spp.* (genomes/g sample [$n = 3$])
Dill	3 907 ± 382
Mustard seeds	7 405 ± 3 455
Onions	36 ± 10

3.2 | Quantification of *Alternaria spp.* in dill, mustard seeds, onions, and vinegar

For the quantitative determination of *Alternaria spp*. load of ingredients, DNA extracts of dill, mustard seeds, onions, and vinegar were analyzed using real-time PCR. Mustard seeds had the highest *Alternaria spp*. contamination with levels that were about twice as high as those of fresh dill (Tables 5 and 6). In contrast, freshly cut onion samples were only slightly contaminated with *Alternaria spp*. No DNA could be isolated from the vinegar due to its high acidity, which is why in this case an analysis was not possible.

A study conducted in Bangladesh analyzing the prevalence of fungi on mustard seeds showed that among seven fungi genera, *Alternaria spp*. were the third most common with regional variations (Saleh et al., 2003). A study in Nepal also documented a pathogen incidence of 42.5% *Alternaria spp*. on mustard seeds after seven days' incubation (Rijal et al., 2020). The present study showed an increased *Alternaria* occurrence of 58.2% on average in mustard seeds. Despite the high natural heterogeneity of fungal contamination of the single samples, the percentage of *Alternaria* level in the samples remained stable.

The diverse load could also lead to different levels of enzyme input into the cucumber jar. Should fungal contamination lead to increased enzyme values, such contamination could no longer be detected in the product based on the enzyme measurements. A correlation between fungal contamination and enzyme values in the product is therefore not clear.

The *Alternaria* prevalence on dill showed lower standard deviations. The high pathogen incidence correlates with the high proven enzyme activity. *Alternaria* leaf and umbel blight is the most damaging disease of dill (Khalequzzaman, 2020). In addition, this aromatic, annual, herbaceous plant is difficult to clean for processing because of its finely structured leaves. Transfer of pathogens on dill into cucumber jars would therefore be entirely conceivable. Heat treatment is sufficient to inactivate the enzymes, as shown here. Consequently, enzyme-producing fungi are also inhibited by the preservation process.

The low occurrence of *Alternaria spp*. on onions is reflected in the low enzyme activity. Several *Alternaria* species are responsible for leaf blight disease in onions, mainly affecting the above-ground parts of the onion (Ramjegathesh & Ebenezar, 2012). Nevertheless, disease symptoms such as purple and brownish blotches caused by *Alternaria* also appear on the onion bulb (Bock, 1964). The onions used here were samples for direct use in processing and did not show any symptoms of disease, so low *Alternaria* exposure was expected. Importation of the fungus via the onion ingredient into the cucumber jars can be prevented by paying attention to symptoms of disease.

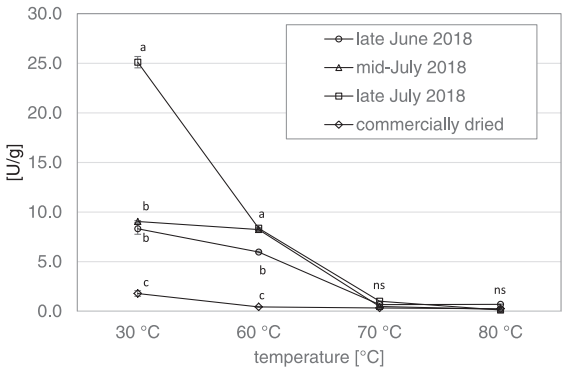

FIGURE 2 Influence of temperatures of 30°C to 60°C, 70°C, and 80°C on the enzyme activity of endo-PG (*y* axis) for varying harvest dates of dill (late June to late July 2018). Temperatures were held for 3 min at the selected temperature. Bars represent the standard deviations. Different lowercase letters on bars within same temperature indicate significant differences ($p < 0.05$). Non-significant differences are represented by ns

3.3 | Evidence of pectolytic enzymes in variations of sample quality, temperature, and pickled cucumbers

3.3.1 | Analysis of pectolytic activities in different harvested and temperature-adjusted dill variants

High amounts of *Alternaria spp*. and enzyme activities were found in the dill sample from 2017 and also in the second trial with fresh dill samples in 2018 and a harvest period of 2 weeks between late June and mid-July (Figure 2). Like the results from 2017, dill samples from 2018 had enzyme activities between 8.00 and 9.00 U/g at 30°C while dill collected in late July diverged, with endo-PG activities of 25.11 ± 0.57 U/g. This sample showed distinctive features with large decreases in enzyme activity of 8.37 ± 0.07 U/g at 60°C. The other two samples had a slightly lower decrease with values of 5.97 ± 0.08 U/g (late June) and 8.24 ± 0.11 U/g (mid-July) at the same temperature. According to the findings of Maruvada and McFeeters

TABLE 6 Quantity of *Alternaria* spp. and fungi in mustard seeds

	Molds (genomes/ g sample)		
Mustard seeds	***Alternaria* ($n = 3$)**	**Fungi ($n = 3$)**	**Percentage of *Alternaria* of fungi [%]**
Sample 1	11 356 ± 2 805	19 373 ± 3 568	58.6
Sample 2	4 950 ± 1 052	9 446 ± 2 237	52.4
Sample 3	5 909 ± 1 585	9 287 ± 1 948	63.6

(2009) regarding the softening of salt cucumbers, temperatures of more than 79°C inactivated the enzymes PG and PE almost completely. Furthermore, a higher enzyme activity obtained with a harvest date in July corresponded to Etchells et al. (1973) and an increasing microbial population.

Compared to commercially dried dill, levels less than 2.00 U/g were significantly lower at 30°C ($p < 0.05$) and a reduction of 75.4% to 0.44 ± 0.05 U/g was achieved at 60°C and of more than 80.0% at 70°C and 80°C.

3.3.2 | Evidence of pectolytic activities in pickled cucumbers with variations of dill

After obtaining high enzyme levels for endo-PG and PE in fresh dill, trials with pickled cucumbers were performed with a selection of dill variants (fresh, dried, without dill) and a storage period of 3 months. The quantities of the other ingredients were not changed based on a common recipe. While pectin lyases were not tested, no exo-PG activity was found (Table 4). Based on low activities, which can be also caused by measurement inaccuracies, the presence of PE was negligible with amounts less than 3.0×10^{-2} U/g. This is much less than the approximately 1.80 to 2.30 U/g determined in the authors' own investigations of fresh cucumbers which did not form part of this study. While fresh dill samples showed high enzyme activities of endo-PG, the stored cucumbers with different variants of dill could not reach those values. Cucumbers with dried dill had endo-PG levels of <0.10 U/g, which could be caused by the bacteriological status of the dill weed as determined more than 80 years ago by Fabian et al. (1939). Certainly, the values of cucumbers with fresh dill, and the sample without it, were in a similar range that could be explained by bacteria on mustard seeds or onions, too. Additionally, mustard inhibits bacterial growth by 5.0% (Fabian et al., 1939). According to the texture data which were measured using TA.XT plus and completed before the extracts were prepared, no significant textural differences ($p > 0.05$) were seen in pickled cucumbers, either. The firmness was in a similar range between 2.96 (dried), 3.10 (fresh), and 3.24 N/mm (without dill) with no difference regarding detectable enzymes. An amount of 1.0 g dill per jar compared to the typical amounts of mustard seeds, onions, and vinegar is obviously too little to cause softening of the cucumbers due to pectin degradation.

4 | CONCLUSION

Analyzing the pectolytic and fungal activities of spices and vinegar for pickled cucumbers, pure dill showed higher enzyme activities but smaller levels of *Alternaria spp*. than mustard seeds. Endo-PG in particular could be found in every stage of maturity with the highest activity of more than 20.00 U/g measured in woody and withered dill. However, since withered dill would usually no longer be used in industry, this stage of maturity can be disregarded. Nevertheless, the pure dill sample used for pickled cucumbers in August 2017 exhibited a high amount of endo-PG and PE, whereas vinegar, onions, and mustard seeds that were used for the same sample had only small amounts of PG, PE, and pectin lyases.

The quantity of *Alternaria spp*. found in mustard seeds was very inhomogeneous with high peaks within the analyzed batch, which explains the high standard deviation.

Since heating to 80°C (3 min) caused a decrease in the level of enzymes, conventional pasteurization at 80°C could result in almost complete enzyme inactivation. In addition to this, pickled cucumbers contain only a small amount of dill, so the commonly used quantities and qualities of dill would probably not soften cucumbers on their own.

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AUTHOR CONTRIBUTIONS

Sabrina Scharf: Conceptualization; data curation; investigation; visualization; writing—original draft. Anne-Katrin

Kersten: Data curation; investigation; writing—supporting draft. Peter Lentzsch: Funding acquisition; project administration; supervision. Peter Meurer: Funding acquisition; methodology; project administration; supervision. S. Scharf not only completed the research of pectolytic enzymes in spices and vinegar but also in pickled cucumbers. A.-K. Kersten analyzed and interpreted the data of *Alternaria*.

CONFLICTS OF INTEREST

The funder had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript or in the decision to publish the results. The authors of this paper do not have any conflicts of interest.

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