THE THESES OF THE PHD DISSERTATION

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Budapest

2025

EVALUATION OF PATULIN PRODUCTION BY *ASPERGILLUS* STRAINS FROM HUNGARIAN APPLE-GROWING REGIONS AND YEAST-BASED DEGRADATION STRATEGIES

DOI: 10.54598/007330

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2025

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1 INTRODUCTION

Apples are among the most widely cultivated and consumed fruits worldwide, with deep cultural and economic significance. In Hungary, they represent the most-produced fruit, yet maintaining orchards free from hazards remains a challenge. Environmental factors, soil conditions, and exposure to pests and pathogens affect yield and quality, while fungal contamination and mycotoxin accumulation are critical threats during storage and post-harvest handling (Zhong et al., 2018). Fungal pathogens such as *Venturia inaequalis*, *Podosphaera leucotricha*, *Colletotrichum* spp., *Penicillium expansum*, and *Botrytis cinerea* not only reduce fruit quality but can also produce toxic metabolites, leading to economic losses and health concerns (Patriarca, 2019)

Among apple-associated mycotoxins, patulin (PAT) is the most alarming. Produced predominantly by *P. expansum* but also reported in *Aspergillus* species, PAT is stable and persists in processed apple products such as juices and purées, raising significant food safety concerns. Although regulatory limits exist, repeated findings of contamination emphasize the need for improved control measures. Conventional fungicide-based strategies, while effective, face limitations due to fungal resistance, environmental impacts, and consumer rejection, underscoring the importance of sustainable alternatives (Moake et al., 2005).

Biocontrol agents, particularly yeasts, have emerged as promising tools to inhibit toxigenic molds and degrade patulin. Certain strains act through competition for nutrients, secretion of antifungal compounds, and enzymatic mechanisms that degrade or adsorb the toxin, thereby contributing to food safety (Castoria et al., 2005). However, gaps remain regarding the contribution of *Aspergillus* species to patulin contamination and the identification of the most effective yeast strains for its mitigation. Addressing these gaps requires investigating fungal diversity in apples, assessing their genetic potential for patulin production, and evaluating environmental influences such as pH, temperature, and humidity. By inhibiting fungal growth and detoxifying patulin, yeasts represent an eco-friendly alternative to synthetic fungicides, offering significant potential for sustainable apple production and post-harvest management.

This study investigates fungal contamination and mycotoxin production in Hungarian apples, focusing on *Aspergillus* species and their contribution to patulin contamination. The main objectives were to isolate and characterize *Aspergillus* species from Hungarian apples, assess their patulin-producing potential while identifying the genetic and environmental factors influencing toxin synthesis, and screen yeast strains for antagonistic activity against patulin-producing *Aspergillus*, including their ability to degrade or bind patulin. By combining fungal characterization with biocontrol evaluation, this research supports the development of eco-friendly strategies for maintaining apple quality, improving food safety, and promoting sustainable post-harvest management.

2 MATERIAL AND METHODS

2.1 Isolation and identification of molds

In 2022, forty apple samples from seven regions of northwestern Hungary were collected, representing different cultivation methods and totaling 120 fruits. Samples were processed using peptone water and plated on malt extract agar (MEA), chosen for species differentiation and patulin production. After seven days of incubation at 37 °C, colonies were counted, grouped by morphology, purified, and preserved, with additional biomass prepared for molecular analysis. Colony, hyphae, and spore features were examined microscopically, allowing classification into *Aspergillus*, *Alternaria*, *Penicillium*, and others. DNA was extracted, the ITS region amplified by PCR, and sequences compared with databases to identify fungal communities, focusing on patulin-producing *Aspergillus*.

2.2 Restriction enzyme screening for mold identification using RFLP- PCR

Reference strains of *Aspergillus* spp. from the Hungarian National Collection, along with isolates obtained from Hungarian apples, were cultured in malt extract broth under controlled conditions, and DNA was extracted using the Geneaid® kit with mechanical disruption. The ITS and β-tubulin regions were amplified by PCR, purified, and subsequently digested with the restriction enzymes XhoI, KpnI, AlwI, HpaII, and AluI, selected through *in silico* analysis of gene sequences. Digested products were separated by agarose gel electrophoresis, generating species-specific RFLP profiles. These profiles were compared with reference sequences and established patterns, allowing reliable confirmation and more precise identification of the *Aspergillus* isolates associated with Hungarian apples.

2.3 Occurrence of patulin-related genes and mycotoxin production

The presence of the *idh* gene, a marker for patulin biosynthesis, was assessed in *Aspergillus* and *Penicillium* isolates by PCR amplification using primers described by Paterson et al. (2003), yielding an expected ~600 bp fragment. To confirm toxin production, isolates were cultured in malt extract broth at 35 °C for 7 days with shaking, and patulin was extracted for thin layer chromatography (TLC). Ten microliters of each extract were applied to silica gel plates, developed in solvent, treated with MBTH, heated, and patulin visualized as a yellow-orange spot under UV light.

2.4 Influence of environmental factors on patulin production

The influence of physicochemical parameters on patulin biosynthesis was evaluated using *Aspergillus clavatus* B9/6 grown in 100% commercial apple juice. Twenty milliliters of juice were dispensed into sterile Erlenmeyer flasks, and the pH was adjusted to 2.5, 4.0, or 5.5 before inoculation with 1 mL of a spore suspension (10^7 spores/mL). Cultures were incubated at 25 °C or 35 °C for 7 days without agitation. Following incubation, cultures were centrifuged, filtered through 0.22 µm membranes and the filtrates were stored at –20 °C for subsequent patulin extraction and quantification by HPLC.

2.5 Yeast-based biocontrol of *A. clavatus* B9/6

A wide collection of yeast strains was screened for their ability to inhibit *Aspergillus clavatus* B9/6. Initial assays on agar included commercial strains, several *Saccharomyces* isolates, and 13 *Wickerhamomyces anomalus* strains, with inhibition assessed after 48 h and 7 days. A secondary assay focused on *W. anomalus*, streaked in parallel with *A. clavatus* for direct interaction, identifying it as the most promising antagonist.

The best-performing *W. anomalus* strains (01499, 01655, 0961, and 0170) were then tested in apple juice for patulin degradation. Strains 01499 and 01655 were selected for kinetic studies, monitoring patulin over 48 h by HPLC. Further co-cultivation showed that these strains not only inhibited *A. clavatus* but also reduced patulin during 15 days of incubation, highlighting their dual biocontrol and detoxification role.

To clarify mechanisms, *W. anomalus* 01499 was examined *in vivo* on apples and through enzyme- and adsorption-based assays. Yeast inoculation before or with the mold limited fungal growth, while *in vitro* tests showed intracellular and extracellular enzymes degraded patulin, and adsorption assays confirmed binding. These results emphasize the potential of *W. anomalus*, particularly strain 01499, as a biocontrol agent against fungal contamination and patulin.

2.6 Patulin extraction

Samples containing yeasts or molds were centrifuged (4,000 rpm, 10 min), and 4 mL of supernatant was extracted with 2 mL dichloromethane. After shaking in the dark for 20 min, 1 mL of the organic phase was centrifuged (14,000 rpm, 1 min), and 0.5 mL was transferred, evaporated at 40 °C, and re-dissolved in 1 mL of 10% acetonitrile.

2.7 High-performance liquid chromatography (HPLC) method

Patulin was quantified by HPLC under isothermal, isocratic conditions using a C18 column, with acetonitrile–water (10:90, v/v) as the mobile phase at 0.3 mL/min, and UV detection at 276 nm.

2.8 Statistical evaluation

All experiments were done triplicates. Statistical analyses were performed using STATISTICA software, with significance set at $p < 0.05$. Differences between groups were assessed using one-way ANOVA followed by t-test where applicable.

3 RESULTS AND DISCUSSION

3.1 Mold isolation and identification

From 40 apple samples representing 28 cultivars, 7 locations, and 5 cultivation methods, a total of 183 mold strains were isolated. Based on morphology, they were grouped into *Alternaria* (67 isolates), *Aspergillus* (45), *Penicillium* (13), and other molds.

Aspergillus, Alternaria, and *Penicillium* are filamentous fungi with distinct traits. Microscopically, *Aspergillus* shows a vesicle at the tip of its conidiophore producing chains of conidia, *Alternaria* has branched hyphae with beaded conidia featuring multiple septa, and *Penicillium* forms brush-like conidiophores resembling a paintbrush. Colony colors also differ: *Aspergillus* is usually green or yellow, *Alternaria* dark brown to black, and *Penicillium* blue-green with a velvety or powdery texture (Amalaradjou & Venkitanarayanan, 2008)

Although this study focuses on patulin, which is primarily produced by *Penicillium* species, both *Alternaria* and *Aspergillus* also can produce mycotoxins. *Alternaria* is known to produce alternariol (AOH) and tenuazonic acid (TeA), which have been associated with cytotoxic and genotoxic effects, posing potential health risks. *Penicillium*, on the other hand, beside patulin, produces a variety of mycotoxins, including ochratoxin A (OTA) and citrinin, both of which can be

harmful to human and animal health, with OTA being particularly concerning due to its nephrotoxic and carcinogenic properties (Bacha et al., 2023; Navale et al., 2021).

The highest number of isolates was found in Debrecen-Pallag with integrated farming (47 in total, 6.7 per apple), followed by Újfehértó (6.3 per apple), where *Alternaria* was not dominant. The lowest number was found in Lövőpetri, with only 27 colonies from 14 cultivars, averaging two per apple, an unexpected result given their bio-based cultivation without chemical fungicides. A possible explanation for the low fungal presence is the cultivation of more resistant apple cultivars, which may naturally inhibit mold growth. Additionally, factors such as favorable environmental conditions during the growing season, lower initial fungal contamination, proper orchard management practices, or the presence of beneficial microbial communities could have contributed to this reduced fungal load.

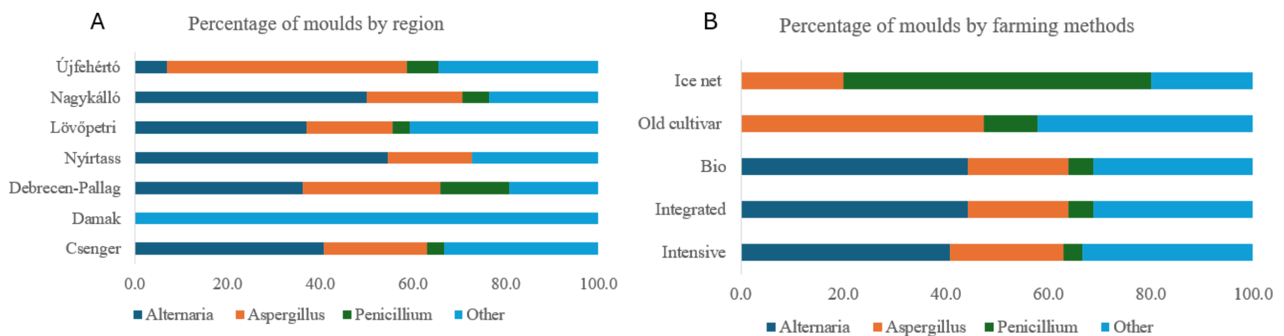

Figure 1 - Percentage of contamination by *Aspergillus, Alternaria, Penicillium,* and other molds for A - location and B - farming method

In Figure 1, the percentage occurrences of the four mold clusters are shown for each location (Fig. 1A) and farming method (Fig. 1B). Due to the higher temperature used for mold isolation, the number of samples containing *Penicillium* was low, with only 10 different apples presenting it. This result was expected as the temperature used partially inhibited the development of this genus (Gougouli & Koutsoumanis, 2010). These results also draw attention to *Penicillium* spp. that are able to grow at elevated temperatures.

Location possesses a higher influence on the distribution of mold genera than farming method, as "ice-net" and "old cultivars" results showed a different trend compared to results of other farming methods, both located in the Újfehértó region. Despite the use of higher temperature, *Aspergillus* species dominated only for the region of Újfehértó with approximately 50% of the isolates belonging to the genus. Four of the seven locations assessed: Csenger, Debrecen-Pallag, Nyírtass, and Nagykálló, were dominated by *Alternaria* species. This result agrees with the findings of other studies, where *Alternaria* was also the predominant genus when isolating potential mycotoxin producers from apples in Ontario, Canada (Soliman et al., 2015). No significant trends were observed for apples of the same cultivar. It is necessary to highlight that the composition of microbiota is multifactorial, and not exclusively dependent on the environmental factors but also anthropogenic effects, as the use of pesticides or other agrichemicals, can implement changes to the biodiversity in the short and long term. No difference in mold counts between bio and non-bio farming methods was observed, the sum

of colonies was slightly higher for the bio apples, the most common molds belonged to the cluster named Other followed by *Alternaria* and *Aspergillus*.

3.2 Occurrence of patulin-related genes and mycotoxin production

3.2.1 Detection of the patulin gene in *Aspergillus* and *Penicillium* strains

The *idh* gene is a marker of potential patulin production, though some strains may not produce the toxin despite carrying it (Varga et al., 2003). Among all *Aspergillus* isolates, only strain B9/6 from Golden Reinders apples in Debrecen-Pallag tested positive (Figure 2).

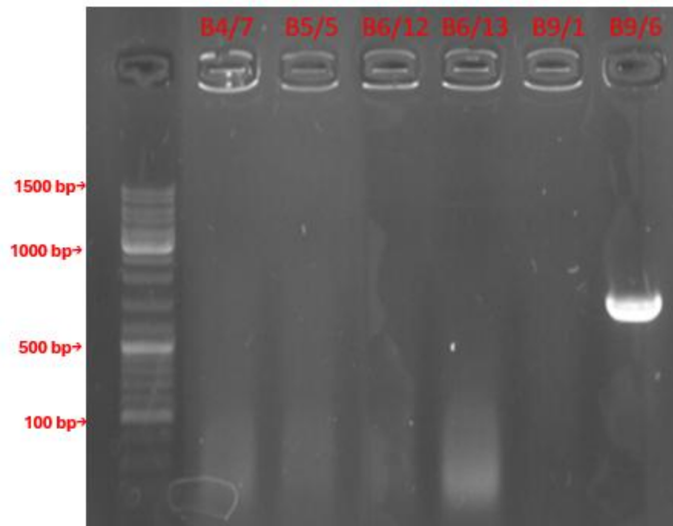

Figure 2 - Presence and absence of the *idh* gene in *Aspergillus* isolates B4/7, B5/5, B6/12, B6/13, and B9/6

The presence of the gene is proved by the bright band located between 600 and 500 bp.

Figure 3 shows the morphology of the *Aspergillus clavatus* B9/6 strain on malt extract agar medium and its microscopic image.

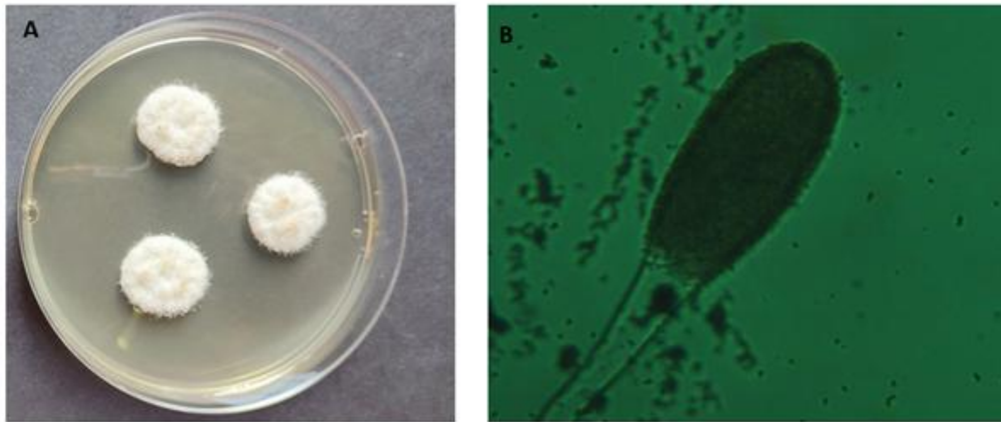

Figure 3 - *Aspergillus clavatus* B9/6 colony morphology on malt extract agar (A) and its microscopic image 100x (B)

Based on morphological characteristics and ITS sequencing, this strain was identified as *Aspergillus clavatus.* This species is characterized by large-sized, elongated, club-shaped vesicles as it is shown in Figure 3. The isolated molds belonging to the genus *Penicillium* were also tested for the presence of the *idh* gene. The results are shown in Figure 4.

Figure 4 - Presence and absence of the *idh* gene in *Penicillium* isolates. From left to right: ladder, no template control, positive control (*A. clavatus* B9/6), B4/5, B6/13, B10/6, B12/9, B13/7, B14/2, B14/19, B15/4, B21/6, B27/2, B30/4, B38/3, and B38/10

Unlike in case of *Aspergillus* spp., of the *Penicillium* isolates more than one strain presented bands in the gel, however, only for B10/6 had the band the expected size of the *idh* gene (500-600 bp). Other works have already reported longer sequences of this gene, these codes can be found under NCBI GenBank: AF006680.1 and DQ084388.1 (Gaucher and Fedeshko, 1997; White and Dobson, 2005). Although isolate B10/6 was morphologically characterized as part of the *Penicillium* cluster, the ITS sequencing results showed that this strain should be classified as *Talaromyces pinophilus.* The high similarity between these two genera is well documented, and till recent years *Talaromyces* spp. were classified as a sexual state from the genera *Penicillium* (Yilmaz et al., 2014).

Figure 5 shows the morphology of the *Talaromyces pinophilus* B10/6 strain on Malt Extract Agar plate and its microscopic image.

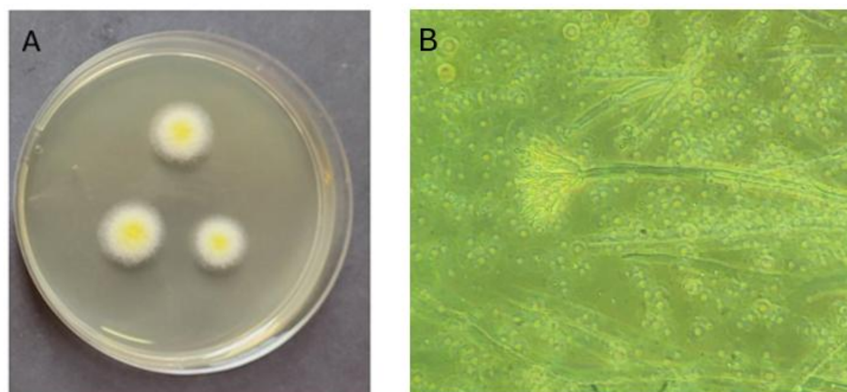

Figure 5 - *Talaromyces pinophilus* B10/6 colony morphology on malt extract agar (A) and its microscopic image 400x (B)

As positive *idh* gene results do not guarantee mycotoxin production, further assays were required. Confirming PCR results, the only *Aspergillus* strain producing patulin was *A. clavatus* B9/6 (Figure 6), indicating its ability to synthesize the toxin under the tested conditions. The sample also showed a brighter spot than the control, suggesting patulin content above 50 ppm.

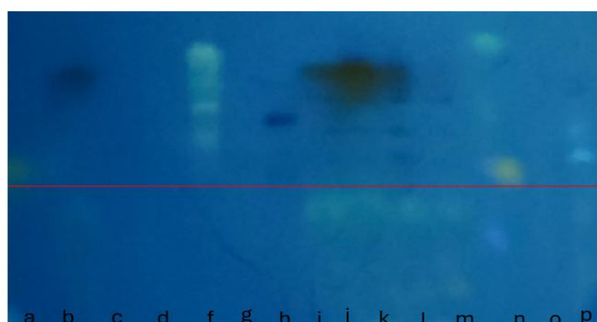

Figure 6 - Thin layer chromatography for patulin detection – a - patulin standard 50 ppm; b - patulin standard 5 ppm; c -B1/2; d - B1/8; e - B4/7; f - B5/6; g - B6/7; h - B6/12; i - B6/12a; j -B6/13; k - B7/5; l - B7/6; m - B9/1; n - B9/6; o - B10/4; p -B10/5; the red line indicates the band height for patulin

For the *Penicillium*-like group, according to the TLC tests, B10/6 was the only strain producing patulin under the studied conditions as shown in Figure 7, confirming the results obtained in the *idh* PCR assay.

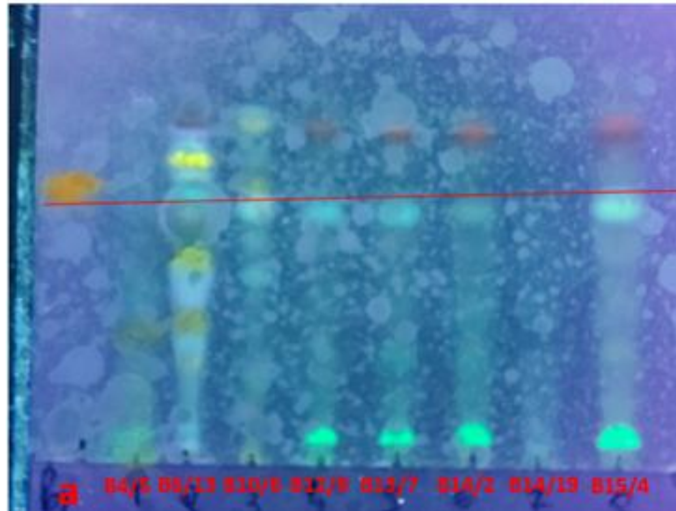

Figure 7 - Thin layer chromatography for patulin detection. a - patulin standard 50 ppm, isolates B4/5, B6/13, B10/6, B12/9, B13/7, B14/2, B14/19, B15/4. The red line indicates the band height for patulin.

3.2.2 Molecular microbiological identification of selected mold isolates

While morphological analysis can help differentiate fungal genera, molecular identification is required for precise species determination. Based on colony and microscopic characteristics, isolates were grouped, and representatives were sequenced. The 45 *Aspergillus* isolates were identified as *A. fumigatus* (28), *A. flavus* (15), *A. nomius* (1), and *A. clavatus* (1). The detection of *A. nomius* is noteworthy as, to our knowledge, this is the first report of its occurrence in Hungary from a crop. This aflatoxin-producing species is typically associated with warm, humid environments and soil (Kurtzman et al., 1987). The morphology of this strain is presented in Figure 8, in different culture media cultivated for 7 days at 25 °C.

Aspergillus nomius B39/1

Figure 8 - Colony morphology of *Aspergillus nomius* B39/1 on different culture media

To confirm the aflatoxin production of *A. nomius* B39/1, a TLC test was performed using standard mixture of aflatoxin B1 and G1. The result is presented in Figure 9.

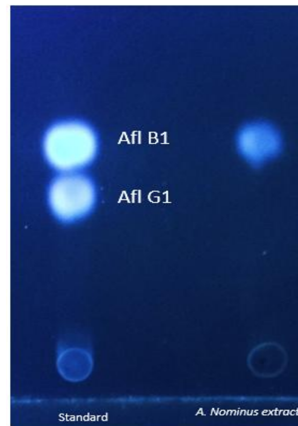

Figure 9 – Thin layer chromatography for aflatoxin production of *A. nomius* B39/1

The *A. nomius* B39/1 strain isolated from apple grown in Újfehértó, cultivated with ice-net was able to produce aflatoxin B1 but did not produce aflatoxin G1.

3.3 PCR-RFLP method development for rapid identification of *Aspergillus* species

3.3.1 Restriction enzyme screening for mold identification (RFLP-PCR)

This study highlighted the need for a faster method to distinguish high numbers of *Aspergillus* species, with PCR-RFLP providing a rapid and reliable approach. The technique amplifies target DNA and digests it with restriction enzymes to generate species-specific patterns (Nasri et al., 2015). β-tubulin sequences of target strains were retrieved from NCBI, analyzed with APE software for enzyme selection, and two strains per species were sequenced (β-tubulin and ITS) to confirm identity before digestion.

With KpnI, no differences were observed, although both *A. flavus* isolates produced two bands instead of one, likely reflecting variability in the β-tubulin gene. Using XhoI, *A. fumigatus*, *A. clavatus*, and *A. ochraceus* showed distinct patterns, whereas *A. niger*, *A. parasiticus*, and *A. flavus* shared the same, indicating that these enzymes are not suitable for species differentiation.

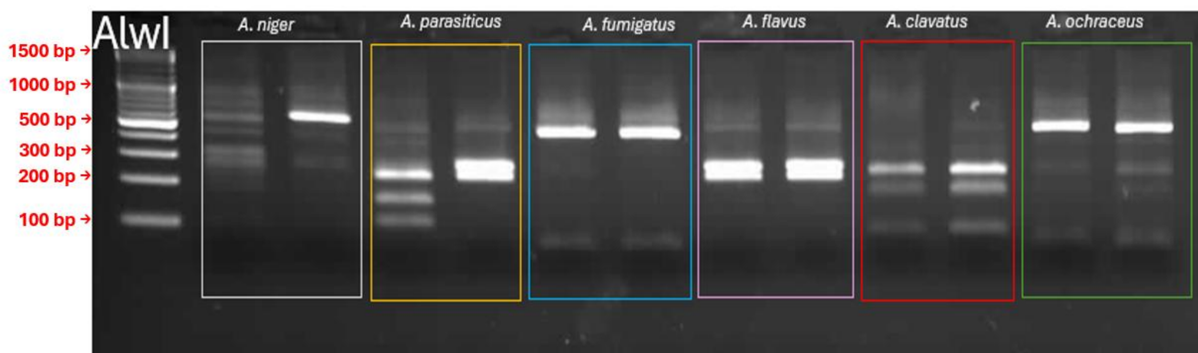

Figure 10 - Fragment profile of *β*-tubulin gene from *Aspergillus* spp. digested with AlwI enzyme

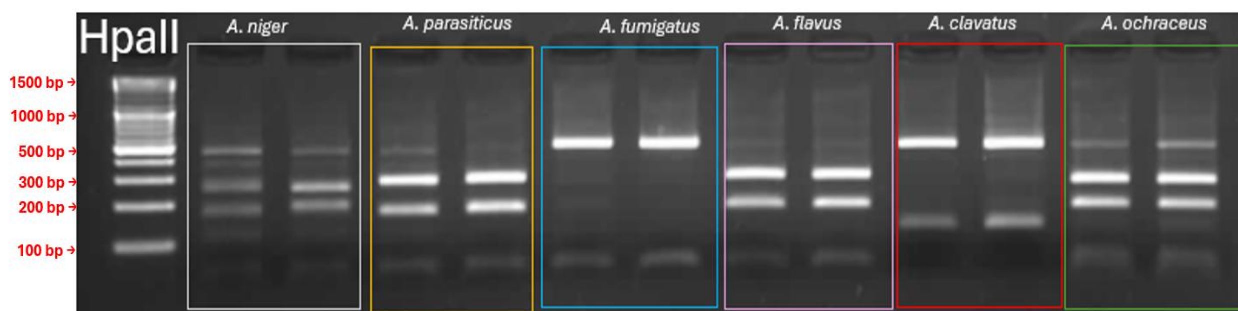

Figure 11 - Fragment profile of β-tubulin gene from *Aspergillus* spp. digested with HpaII enzyme

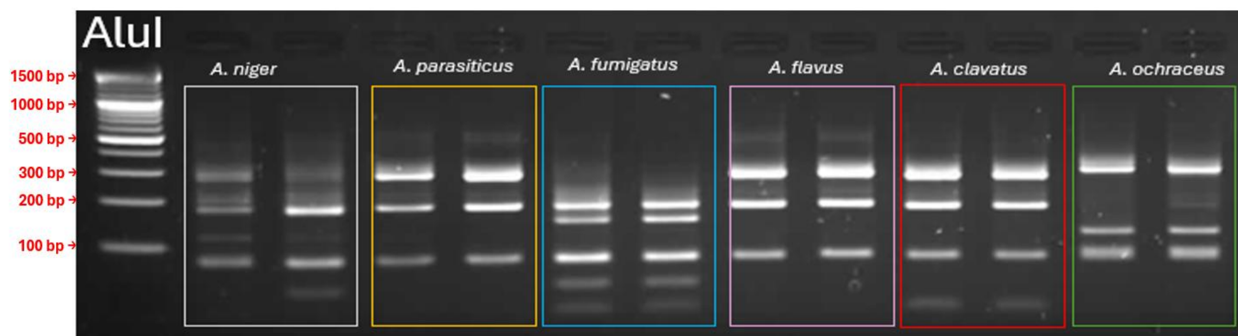

Figure 12 - Fragment profile of β-tubulin gene from *Aspergillus* spp. digested with AluI enzyme

Restriction digestion with, AlwI, HpaII and AluI (Figures 10, 11 and 12, respectively) produced distinct profiles among *Aspergillus*, though AlwI yielded few bands, often a single fragment, limiting its value. *A. parasiticus* also showed strain variability, reducing reproducibility, unlike earlier reports of AlwI as effective (Nasri et al., 2015).

AluI produced clear profiles, uniquely separating *A. fumigatus*, *A. clavatus*, *A. niger*, and *A. ochraceus*, while *A. parasiticus* and *A. flavus* overlapped. Adjusting electrophoresis could enhance its power. HpaII gave the best results, clearly distinguishing *A. fumigatus*, *A. parasiticus*, and *A. clavatus*; even when *A. parasiticus* and *A. flavus* appeared similar, higher gel concentration improved separation, confirming HpaII's strong potential.

3.3.2 RFLP-PCR analysis of mold isolates from apples

After the initial screening, the two enzymes with the best potential, AluI and HpaII, were applied to a larger group of samples, previously isolated from Hungarian apples and morphologically classified, to assess their applicability to environmental samples from agricultural sources. The results are presented in Figures 13 and 14.

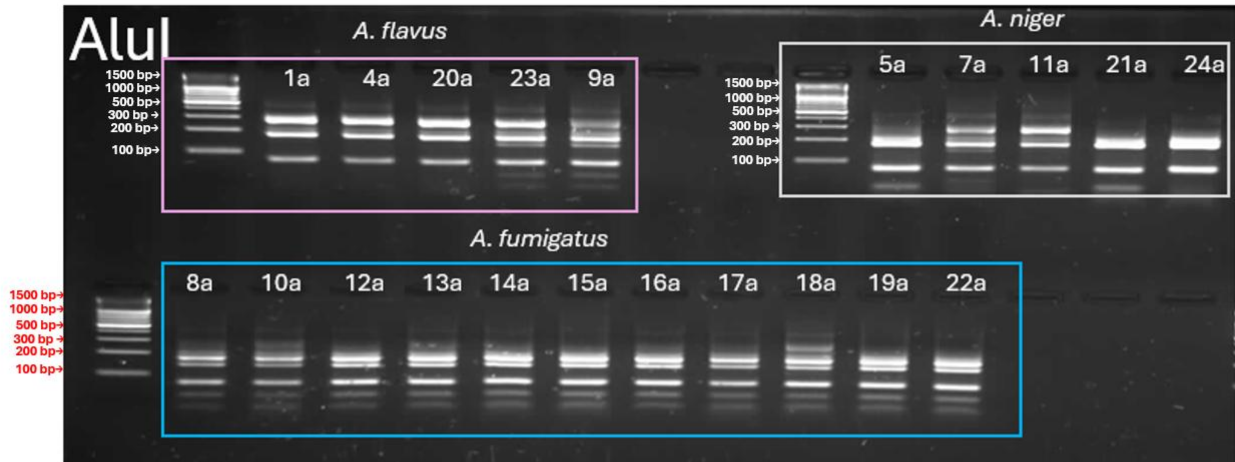

Figure 13 - Fragment profile of β-tubulin gene digested with AluI enzyme for environmental samples

Samples were grouped by morphology. All *A. fumigatus* isolates matched the standard, with only minor band shifts from electrophoresis conditions, and ITS sequencing of 22a confirmed correct clustering. *A. flavus* samples showed the three characteristic strong bands, though 23a and 9a had two faint extras, likely reflecting variability or method limits; still, band positions matched the standard, confirming reliability. For *A. niger*, extra bands appeared in 7a and 11a, while ITS sequencing of 5a showed similarity to both *A. niger* and *A. tubingensis*, closely related Nigri section species (Kizis et al., 2014). AluI effectively distinguished Nigri from other agriculturally relevant *Aspergillus*, underscoring its value as a broader identification tool.

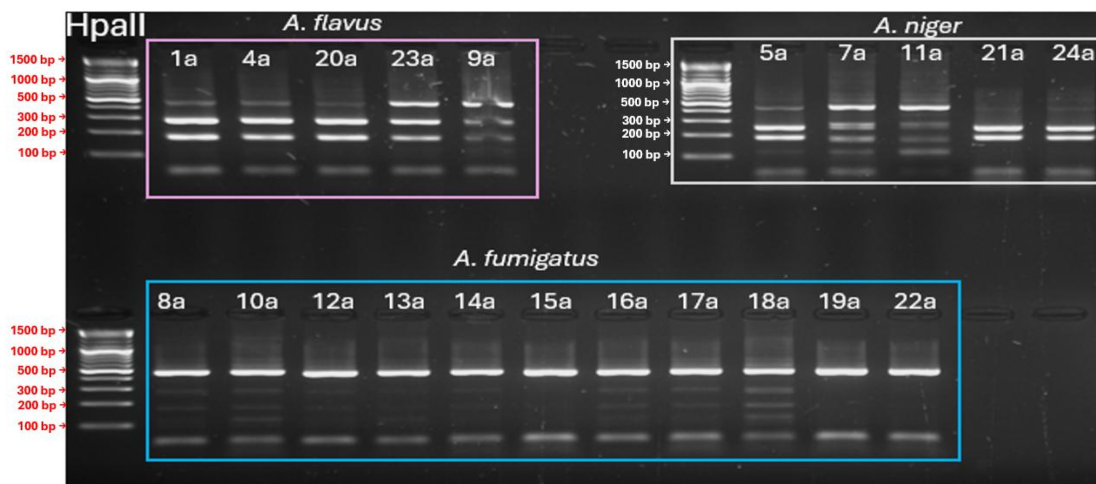

Figure 14- Fragment profile of β-tubulin gene digested with HpaII enzyme for environmental samples

In *A. flavus*, unlike with AluI, all samples showed nearly identical HpaII patterns, with 23a and 9a having a brighter ~500 bp band, possibly due to partial digestion or slight differences in PCR

product concentration. For *A. niger*, samples 7a, 11a, and 5a had similar profiles, though 5a's first band was weaker, consistent with possible incomplete digestion or intraspecific polymorphisms affecting cleavage patterns. *A. fumigatus* samples were the most uniform, showing a bright ~500 bp band and a second prominent band below 100 bp. These results highlight the complexity of digestion profiles and the need to account for intraspecific variability when selecting restriction enzymes.

3.4 Effect of pH and temperature on patulin production by *A. clavatus* B9/6

At both 25 °C and 35 °C, pH strongly influenced fungal growth and morphology. At pH 2.5, growth was almost absent at 35 °C and, although colonies developed at 25 °C, no sporulation occurred. In contrast, abundant sporulation was observed at pH 4 and 5.5 at 25 °C, consistent with the higher patulin levels detected under these conditions.

Patulin production by *Aspergillus clavatus* peaked at 25 °C with 2,754 ± 462 ppm at pH 4 and 6,061 ± 756 ppm at pH 5.5, confirming this as the most favorable condition for toxin synthesis. At 35 °C, toxin levels were minimal, with only 3.6 ± 1 ppm detected at pH 4, while at pH 2.5 patulin remained below quantification. Such findings emphasize the risk of storing apple juice (pH 3.5–4.5) near 25 °C, conditions that favor both mold growth and patulin synthesis, whereas higher temperatures or stronger acidification can significantly reduce contamination.

3.5 Yeast-based biocontrol of *A. clavatus* B9/6

3.5.1 Screening of yeast strains for antifungal activity

Yeast applications are widely studied, with *Saccharomyces* spp. broadly used in food industries and recognized as GRAS. Their patulin detoxification ability is known, e.g., *S. cerevisiae* CITCC 93161 degraded 100% of 100 mg/L PAT *via* endoenzymes (M. Li et al., 2018). Here, twenty commercial yeast products, mainly *S. cerevisiae* for beer, wine, and cider, were tested against *A. clavatus* B9/6. Despite their market availability, none inhibited mold growth, which overtook yeast colonies. Similar results with *Saccharomyces* from the NCAIM confirmed lack of antifungal activity under the tested conditions.

Therefore, assays focused on *W. anomalus* (formerly *Pichia anomala*), a GRAS species with documented biocontrol against several fungi, mainly *via* VOCs and sometimes killer toxins (Godana et al., 2024). All 13 *W. anomalus* strains from NCAIM inhibited *A. clavatus* B9/6, though effectiveness varied. To better distinguish them, a second assay assessed their ability to restrict mold spread from unevenly distributed spores. The most effective strains are shown in Figure 15.

Figure 15 – Inhibition screening of *A. clavatus* B9/6 using different strains of *W. anomalus* by parallel streaks inoculation

In this assay, greater variation was observed among *W. anomalus* strains. Strains 0213, 00717, and 00545 behaved similarly to the saline control, allowing *A. clavatus* to spread across the plate despite visible yeast growth lines. Strains 0459, 00935, and 0367 limited mold spread but did not fully prevent it. The best inhibition was achieved by strains 01499, 0961, 0170, and 01655, where no colonies were detected beyond the yeast growth area; these were selected for further testing.

3.5.2 Patulin degradation by *Wickerhamomyces anomalus* strains

As the aim was to find strains that could both inhibit mold growth and degrade patulin, a degradation assay was performed with *W. anomalus* 01499, 01655, 0961, and 0170 in apple juice. The use of a real food matrix was based on findings by Ma et al. (2023), who reported that degradation efficiency and time can differ between culture media and fruit juices due to their more complex composition. Here, the four strains were tested for their ability to reduce 10 ppm patulin after 24 h incubation (Figure 16).

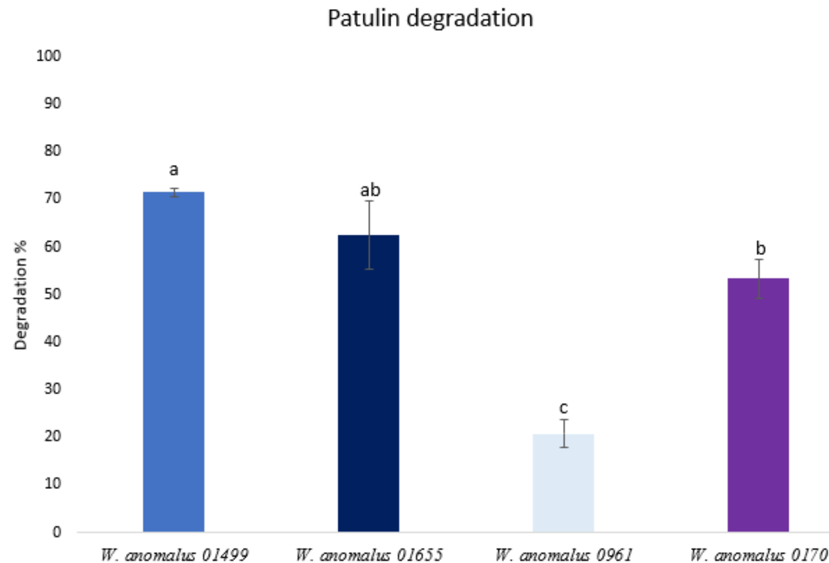

Figure 16 – Patulin degradation by different strains of *W. anomalus* (Superscripts a, b and c represent statistically significant differences based on Tukey's test ($p < 0.05$))

W. anomalus 01499 showed the highest patulin degradation (71% in 24 h), followed by 01655 (62%), 0170 (53%), and 0961 (20%). These values are comparable to previous reports, such as *Hannaella sinensis* degrading ~70% in pear juice (Ma et al., 2023), *Lactobacillus pentosus* DSM 20314 achieving 53% in apple juice (Lai et al., 2022). As 01499 and 01655 showed statistically similar results, both were selected for the next assay.

3.5.3 Kinetics of patulin degradation by *W. anomalus* 01499 and 01655

The kinetics of PAT degradation were determined for *W. anomalus* strains 01499 and 01655. Since complete degradation was not achieved within 24 hours, the incubation period was extended to 48 hours to assess whether a longer duration would enhance the degradation process. Figure 17 illustrates the variations in toxin concentration over time when incubated with viable yeast cells.

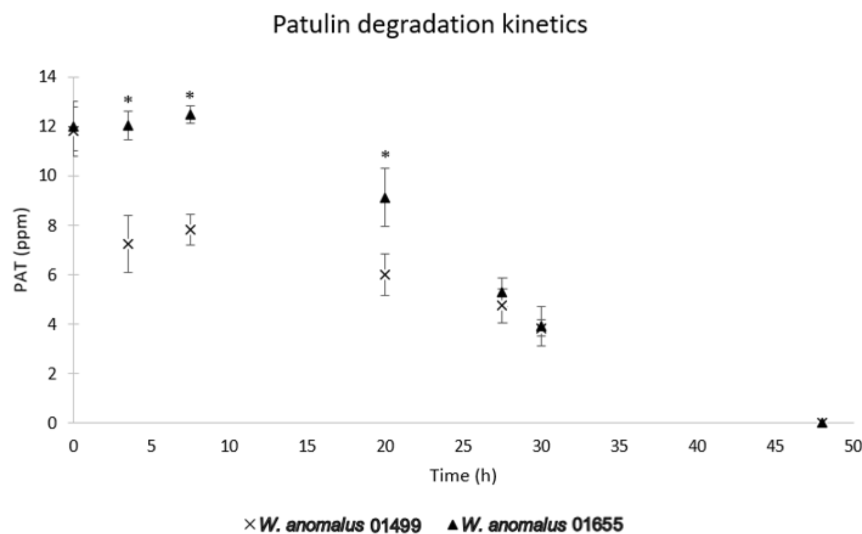

Figure 17 - Patulin degradation kinetics by *Wickerhamomyces anomalus* 01499 and 01655 in apple juice at 25 °C. Symbols represent mean ± SD (n = 3), *indicates $p < 0.05$ between strains at the same time point (Tukey's test)

Both strains completely degraded patulin within 48 h, but with different profiles. *W. anomalus* 01655 showed no significant change in the first 10 h, followed by a steady decline from 20 h to 48 h. *W. anomalus* 01499 reduced patulin by ~35% in the first 3.5 h, stabilized until 10 h, then decreased gradually, reaching full degradation by 48 h. This suggests 01499 adapts faster to the medium, even in the presence of patulin. PAT degradation efficiency can also be influenced by temperature, as shown with *L. casei* YZU01, which performed best at 37 °C (Zheng et al., 2022). Optimizing environmental conditions and studying metabolic pathways may further enhance the bioremediation potential of *W. anomalus* 01499.

3.5.4 Co-cultivation of *W. anomalus* 01499 and 01655 with *A. clavatus* B9/6

Since *Wickerhamomyces anomalus* 01499 and 01655 inhibited *Aspergillus clavatus* B9/6 and degraded patulin in apple juice, they were further evaluated in co-cultivation assays with mold spores under identical starting concentrations. After 15 days, the control showed normal fungal growth with

mycelial spheres and spore lines, whereas yeast cultures remained cloudy and suppressed mold development; only strain 01655 showed a few mycelial spheres, but at much lower levels than the control.

Extended incubation tested whether *A. clavatus* could outcompete the yeasts, as sometimes occurs in fermentation (Hymery et al., 2014), but no microbial shift was observed. Fungal growth remained suppressed, and patulin was undetectable in yeast co-cultures, while the control accumulated 21,304 ± 515 ppm of the toxin. This level was higher than in the environmental parameter assays, likely due to the longer incubation at 25 °C and pH 3.6. Overall, strains 01499 and 01655 effectively colonized the medium, limited mold survival, and prevented toxin synthesis, confirming their potential as biocontrol agents against patulin contamination.

3.5.5 *In vivo* evaluation of *A. clavatus* B9/6 inhibition using *W. anomalus* 01499

To evaluate the potential of the yeast *W. anomalus* to inhibit *A. clavatus* B9/6 in actual fruits, tests were performed inoculating both microorganisms at different times and together beside a negative control injected only with water and a positive control in which only *A. clavatus* was injected. The results are presented in Figure 18.

Figure 18- *In vivo* assay of inhibition of *A. clavatus* B9/6 using *W. anomalus* 01499. A - Negative control, B- Positive control, C – *W. anomalus* 01499 injected 24 h before *A. clavatus* B9/6, D - *W. anomalus* 01499 injected at the same day as *A. clavatus* B9/6, E - *A. clavatus* B9/6 injected 24 h before *W. anomalus* 01499

When *W. anomalus* 01499 was inoculated 24 h before *A. clavatus* B9/6, almost no infection symptoms appeared, indicating strong protection. Simultaneous inoculation caused minor initial infection, but it did not spread or reach the apple core. In the positive control, fruit decay was evident, with dark lesions and mycelial growth at the center. When the mold was inoculated 24 h before the yeast, symptoms were more severe, including dark spots and small holes near injection points. This suggests the mold had already established infection, weakening tissue and increasing susceptibility. A similar effect was reported with *Rhodosporidium paludigenum* against *P. expansum*, where higher yeast concentration enhanced mold virulence and patulin levels (Zhu et al., 2015), indicating the need to test different yeast doses. In the negative control, only the needle mark was visible, with no microbial growth.

3.5.6 Intracellular and extracellular enzymatic degradation of patulin and PAT binding capacity of *W. anomalus* 01499

This study evaluated patulin degradation by *Wickerhamomyces anomalus* 01499 through extracellular, intracellular, and cell wall mechanisms. Exoenzymes secreted after 24 h showed no activity, indicating the absence of effective extracellular detoxification under the tested conditions, consistent with results reported for *Hannaella sinensis* (Ma et al., 2023). Cell wall binding contributed to an 11% reduction in patulin. The strongest effect was observed with intracellular enzymes, which reduced patulin levels by 33%, confirming that active enzymatic degradation plays a dominant role. Together, these findings suggest that *W. anomalus* detoxifies patulin mainly through intracellular pathways, with cell wall binding offering a complementary but limited contribution. These findings indicate that optimizing factors such as pH, temperature, nutrient availability, and exposure time could enhance enzyme production and PAT removal.

4 CONCLUSION AND RECOMMENDATIONS

This study investigated the mold population on Hungarian apples, focusing on potential patulin-producing *Aspergillus* species, a group less studied in this context than *Penicillium*. From 183 isolates collected across seven locations and various farming systems, 67 belonged to *Alternaria*, 45 to *Aspergillus*, and 13 to *Penicillium*, with the rest classified as other genera. Geographical location influenced genus distribution more than farming method. *Aspergillus* dominated only in Újfehértó (~50% of isolates), while *Alternaria* prevailed in most other locations.

Species identification revealed *A. fumigatus* (28), *A. flavus* (15), *A. clavatus* (1), and *A. nomius* (1), the latter reported here for the first time in Hungarian crops and known for aflatoxin B1 production. Only *A. clavatus* B9/6 carried the patulin gene, confirmed by TLC assays, and was selected for further tests. A rapid RFLP method using HpaII restriction enzyme successfully distinguished the six studied *Aspergillus* species. Environmental assays showed that *A. clavatus* B9/6 produced high amounts of patulin at 25 °C, peaking at 6,061 ppm at pH 5.5, while production fell sharply at 35 °C and was absent at pH 2.5. In biocontrol tests, *Saccharomyces* spp. showed no inhibition, but all 13 *Wickerhamomyces anomalus* strains from NCAIM inhibited *A. clavatus* to some extent. The best results came from strains 01499, 0961, 0170, and 01655.

In patulin degradation assays, 01499 achieved 71% reduction in 24 h, 01655 62%, 0170 53%, and 0961 20%. Kinetics showed 01655 degrading completely after 48 h (no change in first 10 h), while 01499 reduced patulin by 35% in 3.5 h, then more gradually to full degradation. Co-cultivation in apple juice prevented fungal growth and patulin formation. *In vivo* apple assays confirmed strong protection when 01499 was applied 24 h before inoculation, moderate protection with simultaneous inoculation, and severe symptoms when the mold was applied first. Mechanism studies showed no extracellular enzymatic activity; cell wall binding accounted for 11% reduction, while intracellular enzymes achieved 33%, indicating a major role for endoenzymes in detoxification.

Overall, *W. anomalus* strains, especially 01499 and 01655, demonstrated strong potential for inhibiting *A. clavatus* growth and degrading patulin, supporting their use as biocontrol agents in post-harvest disease management and food safety.

Suggestions:

- Investigate *A. clavatus* B9/6 patulin production at lower temperatures
- Test *W. anomalus* against other mycotoxin-producing molds
- Identify and optimize enzymes responsible for patulin degradation
- Develop a fermented apple juice beverage using *W. anomalus*

5 NEW SCIENTIFIC RESULTS

- First report of *Aspergillus nomius* in Hungarian crops, expanding the known range of this aflatoxin B1–producing species.
- First identification of patulin-producing *A. clavatus* in Hungarian apples, revealing a previously unrecognized contamination source.
- Development of an HpaII-based restriction fragment length polymorphism (RFLP) method, enabling efficient differentiation of six mycotoxigenic *Aspergillus* species.
- Screening of 13 *Wickerhamomyces anomalus* strains showed consistent antifungal activity against *A. clavatus* B9/6, with four strains showing strong inhibition.
- *W. anomalus* 01499 achieved complete patulin degradation within 48 h — the first report of this detoxification ability in the species.
- *In vivo* assays confirmed that pre-inoculation of apples with *W. anomalus* 01499 prevented infection, while delayed treatment increased severity, emphasizing the importance of timing.
- Patulin detoxification occurred *via* cell wall binding and intracellular enzymes, with no extracellular activity detected, offering new insight into the mechanism.
- *W. anomalus* displayed dual functionality in food safety, both inhibiting fungal growth and degrading patulin, supporting its use as a sustainable post-harvest biocontrol agent

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