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## Reducing patulin levels in apple juice by fermentation with *Saccharomyces cerevisiae*

Reducering av patulinhalter i äppelmust genom fermentering med  
*Saccharomyces cerevisiae*

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**Omfattning:** 15 hp

**Nivå och fördjupning:** G2E

**Kurstitel:** Kandidatarbete i biologi

**Kurskod:** EX0493

**Program/utbildning:** Hortonom

**Examen:** Kandidatexamen i biologi

**Ämne:** Biologi (EX0493)

**Utgivningsort:** Alnarp

**Utgivningsmånad och -år:** juni 2015

**Omslagsbild:** Oscar Håkansson

**Elektronisk publicering:** <http://stud.epsilon.slu.se>

**Nyckelord:** patulin, *Penicillium expansum*, apple juice, fermentation, HPLC, *Saccharomyces cerevisiae*, yeast

SLU, Sveriges lantbruksuniversitet

Fakulteten för landskapsarkitektur, trädgårds- och växtproduktionsvetenskap

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## **Abstract**

Patulin is a mycotoxin that can be found in many apple products all over the world. It has been well-known for many decades and is nowadays one of the most regulated mycotoxins in food. Today when fruit and vegetables are traded internationally more than ever before, the quality of the goods might fall victim for careless storage during logistics. Still, with natural resources becoming scarce, we need to take care of what we got in our reach. Maybe at some point in the future we might have to find a usage for a shipment of apple juice contaminated with patulin. In this study, we focus on finding ways to decrease the amount of patulin in apple juice. By inoculation apples with the mold *Penicillium expansum*, 3 grades of decay were produced in the fruits. Diameters of lesion that the infection reached were sorted into 25 mm, 35 mm and 45 mm. Using a household fruit juicer these 3 grades of infected apples were juiced separately, to produce 3 juices containing different amounts of patulin. Having these 3 juices, using 5 treatments found in commercial juicing businesses (unfermented, pasteurized, fermented with commercial yeast strains Safale S-04 or Safale US-05, or spontaneously fermented by wild *Saccharomyces cerevisiae*) could prove if there were any process that would reduce the patulin concentration. Using a common extraction method and a HPLC analysis with a UV diode array detector at 276 nm, it was concluded that both the commercial yeasts lowered the concentration significantly if compared to the unfermented and the pasteurized. A significant difference in patulin concentration was concluded between the 3 degrees of lesion, where the highest grade of decay (45 mm lesion diameter) had the highest levels of patulin in its juice and the lowest grade of decay (25 mm lesion diameter) resulted in the lowest patulin concentration.

## **Acknowledgements**

I would like to thank my supervisor Marie Olsson for being so very supportive to my project. Also I would like to thank Ibrahim Tahir for helping me out with apples and *Penicillium expansum* spore suspension, Karl-Erik Gustavsson for helping me out with the HPLC-unit for more than 2 day in a row, and Staffan Andersson for helping me out with the computer statistical programs and general guidance in the laboratory.

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## Introduction

Patulin, 4-Hydroxy-4H-furo 3,2-C-pyran-2(6H)-one, is a commonly found mycotoxin among stored fruits and berries. It is normally produced by different fungi species within the genera of *Penicillium* and *Aspergillus* (Varga et al., 2008). Among all these species the mold *Penicillium expansum* is considered the most important microorganism to produce patulin during storage of apples (Barkai-Golan, 2008). In 2004, patulin was the most regulated mycotoxin in the world, as there were around 160 regulations in some 50 countries at the time (van Egmond and Jonker, 2004). Today it is well known by people in fruit business, and both occurrence and effects of patulin have been studied by scientist for many years. It can be encountered all over the world and has due to its frequent appearance become a part of the quality index in processed apple products (Rovira et al. 1993). Even though patulin is commonly spoiling products made from apples and pears (Barkai-Golan, 2008), traces of it have also been found in fruit juices from tropical fruits, like pineapple and lychee (Lee et al., 2014).

The EU commission has classified patulin as harmful to humans (European Commission, 1996). The amount of patulin ( $\mu\text{g}/\text{kg}$ ) that is allowed in food products may vary depending on which food that is contaminated. In the EU there is a limit of 50  $\mu\text{g}$  patulin/kg apple juice or apple cider. If the product is targeted for kids, the limit is 10  $\mu\text{g}$  patulin/kg apple product (European Commission, 2006).

A number of different techniques have been tried to reduce the amount of patulin in apple juice, but most of them are considered problematic due to either their loss in juice quality or too high processing costs (de Souza et al., 2008). Even though pasteurization is known to affect the patulin content in apple juice, it cannot be regarded as a complete reducing factor (Welke et al., 2009; Wheeler et al., 1987). There are also many known methods for determination of patulin in fruit juice. The most common machinery used today is the HPLC-unit with UV detection or photodiode array detection (Sabino, 2008; Shephard and Leggott, 2000).

Today it is well known that fermentation with yeast can decrease the levels of patulin in apple juice (Moss and Long, 2002; Sumbu et al., 1983). At first, scientists realized that they could not find any traces of patulin in alcoholic beverages made from apple juice. And later

on, in one of the early experiments in the subject, Stinson et al. (1978) used 8 commercial strains of *Saccharomyces cerevisiae*, all of which was used for fermenting different apple juice products. First, they manually added patulin at a rate of 15 mg/liter to the apple juice. Their result show that 6 of the strains where degrading the added patulin to less than, at the time, detectable limit at 50 µg/liter, and that all 8 strains did reduce the added patulin by over 99 % in the juice (Stinson et al. 1978). Studies have also shown that the patulin content does not descend as much if the fermentation with *Saccharomyces cerevisiae* is done aerobically (Moss and Long, 2002).

## **Objective**

This study will evaluate if fermentation with *Saccharomyces cerevisiae* would lower different concentrations of patulin in apple juice to beneath the limit of patulin allowed in the EU.

## **Material and Methods**

### Apparatus:

Scale, HPLC, laboratory shaker, centrifuge (Beckman LE-80K Ultracentrifuge)

### Laboratory equipment:

Pipettes, micropipettes, Erlenmeyer flasks 200-300 ml, polypropylene centrifuge tubes 5-15 ml, Eppendorf tubes 1.5 ml, glass measuring cups 25-500 ml

### Reagents:

#### *Chemicals:*

Patulin, 1-Hexanol, ethyl acetate/n-Hexane 60/40, ethyl acetate/acetic acid 50/50 were purchased from Sigma-Aldrich, NaCl, Na<sub>2</sub>SO<sub>4</sub>, Millipore H<sub>2</sub>O (GenPure Pro UV/UF - TOC system), acidulated water (pH 4.0), HPLC solvents: Solvent A, 80% (0.1% HCOOH) and Solvent B, 20% (0.1% HCOOH in acetonitrile)

#### *Apples:*

Apple cultivar Santana was used in this experiment. The apples were grown organically in Kivik (Sweden) and had been picked in September 2014 and stored in a cold storage room at 2°C and RH = >90%. When this experiment was performed they had already been in cold storage for approximately 5 months.

*Penicillium expansum* spore suspension:

The *Penicillium expansum* spore suspension had been cultivated according to the method of Tahir et al. (2009). See Appendix, page 20.

Yeast, *Saccharomyces cerevisiae*:

Two commercial dry yeast strains were used. Both Safale S-04 and Safale US-05 (Lesaffre: Fermentis, France) are commonly used when making beer, especially ales. Each package was, according to the instructions, designed to fit 20-30 litres of wort. This product contained  $> 6 \times 10^9$  yeast cells/g (Fermentis, 2012). A calculation was made to estimate how much yeast was needed per sample. The samples contained approximately 100 ml each.

$$\frac{11.5g}{20l} = 0.575 g/l$$
$$0.575 g/l \rightarrow 0.0575g/100 ml$$

To avoid other contaminating microorganisms to grow the calculated yeast weight was doubled just to ensure that the added *Saccharomyces cerevisiae* would establish itself fast enough.

$$0.0575 g * 2 = 0.115 g$$

Therefore, per each 100 ml juice sample, 0.115 grams of yeast was added.

## Principle

The concept of this project was to control the size of the lesions caused by *Penicillium expansum* infection in the apples. By producing 3 different grades of decay in the fruits, and then extracting juice from these grades individually, there would be a chance to evaluate how 5 different treatments, for each degree of decay would affect patulin concentration in apple juice. The 5 treatments analyzed were:

- Unfermented juice
- Pasteurized
- Fermented with *Saccharomyces cerevisiae* strain: Safale S-04
- Fermented with *Saccharomyces cerevisiae* strain: Safale US-05
- Spontaneous fermented; Wild yeast (wild *Saccharomyces cerevisiae*)

Except the mentioned treatments, the resulting bottom sediment from Safale S-04 and Safale US-05 were to be analyzed as well.

The grades of decays were differentiated by measure the diameter of the lesion on the apples. The 3 different grades of decay used were:

- 25 mm lesion
- 35 mm lesion
- 45 mm lesion

The experiment was performed in 6 steps:

1. Inoculate the apples with *Penicillium expansum*
2. Pressing the apples for juice
3. Treating the juice in different ways
4. Extract the patulin from the juice samples using 2 different methods
5. Analyze the patulin content with a HPLC
6. Collect data and analyses statistically

### Step 1: Inoculation of apples

Approximately 200 apples of the same size was washed in distilled water. After being dried with napkins, a small strip was marked on the peel with a red marker pen. In the middle of the strip a tiny hole was made, 2-3 millimeters deep, with a pipette tip (Eppendorf). With the hole facing upwards, the apples were inoculated with 20 µl of the prepared *Penicillium expansum* spore suspension. After 5 minutes of absorption, the apples were placed in two layers in plastic fruit-storage boxes still placed with the hole facing upwards. The apples were left at 21°C without exposure of direct sunlight.

At day 6 the diameter of the infected lesion had reached 25 millimeters in most of the apples. At that point 60 apples were transferred to a cold storage room, at 2°C and RH = >90%. The next day (day 7) a new batch of 60 apples were moved to the cold storage room, now with a lesion size of 35 millimeters. During day 8 and 9, 24 resp. 36 apples with the lesion size of 45 millimeters was transferred to cold storage.



## Step 2: Pressing

From the 200 inoculated apples, 180 apples were picked out for pressing. Only apples that had not been damaged or attracted a secondary pathogen during the cold storage were chosen. Apple juice was obtained by using a household juice-extractor (Bosch MES3000 Whole Fruit Juicer, 700 Watt). To avoid pseudo replicate and achieving a triplicate sample for each treatment, apples were juiced in 9 separate batches. Using abbreviations for each triplicate sample, a scheme was made to prevent any possible mistakes that could be done during the juicing session (Table 1). Three control samples with 4 apples pressed in each were made from apples that were not inoculated with *Penicillium expansum*.

Apples were pressed in one batch (20 apples) at a time. During extraction, foam in the juice was built up due to the turbulence from the centrifuge. This was removed after about 1 minute when all had risen to the surface. It was very important that this step was executed quickly to avoid too much oxidation of the apple juice. After all the foam and floating particles were gone, the clear juice was poured into 2\*50 ml plastic tubes with screw caps. Approximately 100 ml was collected for each sample. All samples were quickly put on ice, except three samples, with the wild yeast ones, which instead were poured directly into 200 ml Erlenmeyer flasks.

With 3 degrees of decay and 5 treatments, there were in total  $3 \times 5 = 15$  different samples to account for. Using the setup in Table 1, shortenings were compiled to represent each sample.

**Table 1: Scheme for displaying the juice treatments and the different degrees of lesion. Note that each abbreviation represents a triplicate sample.**

Treatment	Degree of lesion		
	25 mm	35 mm	45 mm
Unfermented	25U	35U	45U
Pasteurized	25P	35P	45P
Safale S-04	25SaS	35SaS	45SaS
Safale US-05	25SaUS	35SaUS	45SaUS
Wild yeast	25W	35W	45W

With 3 degrees of decay and 5 treatments, there were in total 15 different samples to account for. Also, since bottom sediment were to be analyzed in Safale S-04 and Safale-US-05 at lesion size 45 mm, 2 more treatments were added to the other 15. So in the end there were 17 triplicate samples analyzed.

### Step 3: Juice treatments

#### *Storage*

All samples, except the wild fermented, were placed in a freezer with the temperature -20°C. The wild fermented were directly after juice pressing put in 200 ml Erlenmeyer flasks with cotton stuffed down the bottle neck. 24 hours later all of the frozen samples, except the unfermented treatment, were taken out to be prepared in different ways. During the storage in the freezer, some of the juice samples seemed to deposit cloudy fractions. Later on, when extracting the juices, only the clear fraction was taken to avoid the risk of bringing solid particles into the HPLC analysis.

#### *Pasteurization*

Using a water bath, the pasteurization was done by being submerging the plastic tubes by  $\frac{3}{4}$  of their length into 78°C water. Each 30 seconds the samples were shaken. By making a hole in the cap of a plastic tube filled with water, the temperature could be measured with a thermometer in the liquid in the tube. When the temperature in the test tube turned 72°C the samples were allowed to stay for 15 seconds, and after that they were quickly removed to a sink with cold water to cool off. When the samples were cool enough to be hold by hand, they were put in the freezer at -20°C.

#### *Fermentation*

All fermenting was done using 200 ml Erlenmeyer flasks with tightly squeezed cotton stuffed in the opening. To the Safale S-04 and Safale US-05 samples, the calculated weight (0.115 g) of respective yeast was added per 100 ml sample. The temperature was stable at 21°C and they were placed out of reach from sunlight. Day 7 after pressing, 7 ml was transferred from the wild fermented samples to a 10 ml centrifuge tube with cap. These were quickly put in the freezer at -20°C. The next day, 7 ml of juice from each sample of Safale S-04 and Safale

US-05 were transferred under the same premises as the wild fermented the day before. Though, this time there were also taken 3.5 ml of the bottom sediment from respective samples at 45 mm lesion; Samples 45SaS and 45SaUS. The bottom sediment was put in 10 ml centrifuge tubes and put in the freezer along with all the other samples.

#### Step 4: Patulin extraction

Two types of extraction methods were tried out:

- *1-Hexanol extraction* from Abu-Bakar et al. (2014)
- *Ethyl acetate/n-Hexane extraction* from Arranz et al. (2005)

##### *1-Hexanol extraction*

A NaCl solution was made with the concentration 20 % w/v (weight/volume). For each sample, 8.0 ml of NaCl solution was transferred to a 15 ml plastic centrifuge tube. 400 µl of the clear juice was added with a pipette. The mixture was diluted with distilled water to 10 ml (1.6 ml H<sub>2</sub>O). 400 µl of 1-hexanol was added, and the tube was put on a vortex at full speed for 45 seconds. After the vortex session, the samples were centrifuged at 4000 rpm for 5 min (Beckman LE-80K Ultracentrifuge). When separation was done, 150 µl of the surfactant, the organic solvent, was transferred to HPLC vials with inserts (500 µl). The vials were kept in a freezer at -20°C until the HPLC analysis.

##### *Ethyl acetate/ n-Hexane extraction*

5 g of Na<sub>2</sub>SO<sub>4</sub> was added to a 15 ml centrifuge tube with cap. To that, 3 ml ethyl acetate/n-hexane 60/40 solution and 3 ml juice sample was applied. The tubes were vigorously shaken while standing upright on a laboratory shaking table for 10 minutes, and then centrifuged at 4000 rpm for 10 minutes. For each sample, 1000 µl of the supernatant was removed to an Eppendorf tube, and then 50 µl of ethyl acetate/acetic acid 50/50 solution was added. The liquid in the Eppendorf tubes were dried out simultaneously by a stream of nitrogen gas (N<sub>2</sub>) at 37 °C. When all the liquid in the samples had evaporated, the remaining residue was diluted with 100 µl of acidulated water, pH 4. The diluted samples in the Eppendorf tubes were briefly vibrated for 5 seconds on a vortex and centrifuged at 5000 rpm in 2 min. 75 µl of each sample was transferred to an individual HPLC vial with insert (500 µl).

### Step 5: HPLC-analysis

An Agilent 1100 machine with binary pump was used. The column used was Phenomenex Synergi™ 4u Hydro (25cm\*6mm\*4µm; Phenomenex, USA). The flow rate was 1.0 ml/min, and injection volume 10.00 µl. Spectrometer used was an Agilent 1100, with a Diode Array Detector (Agilent Technologies, USA) and detection was made at UV-wavelength 276 nm.

Patulin standard solutions were provided for calibration of the HPLC run, making a standard curve. These had been made by diluting a stock solution of patulin (Sigma-Aldrich, USA) with acetonitrile to two different concentrations, 1µg/ml and 10µg/ml. By injecting these standard solutions in varying volumes, 5 different amounts of patulin could be analyzed. To be able to calibrate the whole HPLC run, 3 sessions of patulin standards were put in with 3 vials each and analyzed along all the juice samples. The first 3 vials of standard solutions were put to be analyzed in the beginning of the HPLC run. After 27 juice samples vials, 3 vials with standard solution were placed in line, and after 27 juice samples again, 3 standards operated as last comparing units. In total there were 63 samples, in which 9 of them were standard solutions.

### Step 6: Collecting data and statistic analyzing

By using the given areas from the patulin standard solutions a calibration curve with a linear function was made in Microsoft Excel 2010. In each juice samples the area at 276 nm was multiplied with the factor, calculated from the standard curve, to get the patulin content in µg/µl. Using the given equation from the method by Arranz et al. (2005), the patulin content in the juice extracts could be converted to the actual patulin content in the juices in µg/liter.

Mean value and standard deviation was calculated for unfermented, pasteurized, Safale S-04 and Safale US-05. For these 4 treatments statistical analyzes were made using SAS System. LSD tests with ANOVA-tables were used to demonstrate possible differences between lesion diameter and treatments.

## Results

### Difference in extraction method

The 1-hexanol extracts did not show any peaks on the chromatogram at 276 nm at all. No data could be collected from this extraction, and therefore this method had to be excluded from being statistically analyzed.

The ethyl acetate/ n-Hexane extraction gave distinct peaks on the chromatogram. Because of that, these values were used for all the calculations in Excel and SAS.

### Calibration curve

Figure 1 shows the calibration done with the patulin standard solutions during the HPCL run.

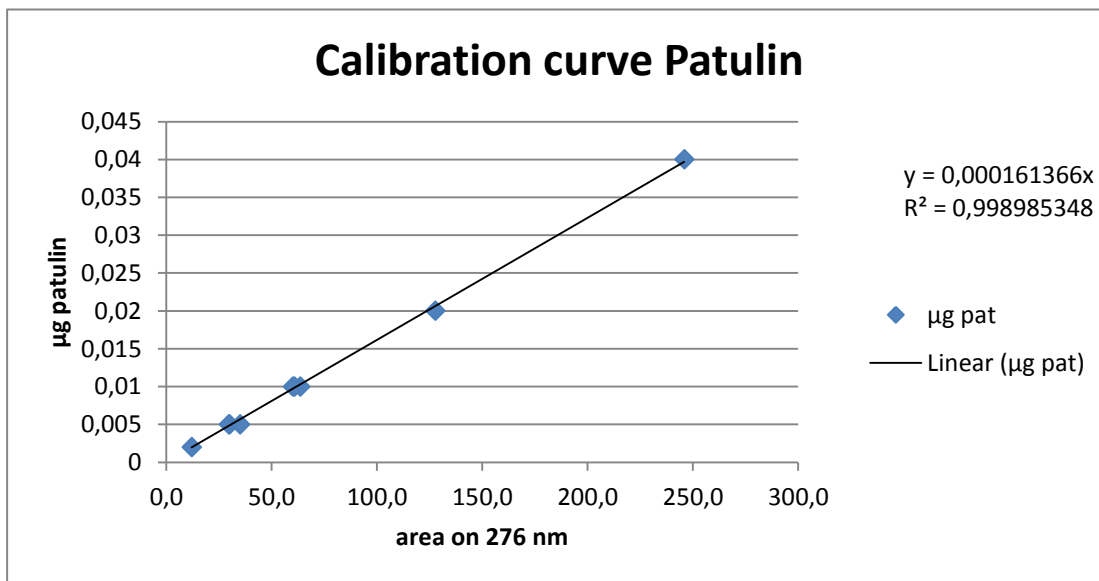


Figure 1: This graph shows the linear calibration curve and the function that was calculated in Microsoft Excel 2010. Amounts of patulin calibrated with (µg): 0.002, 0.005, 0.01, 0.02, 0.04.

### Patulin content: In juice samples

Significant differences in patulin content could be detected between all the mean values of the unfermented samples (25U, 35U, 45U). In the pasteurized samples significant difference was found between 45P and 35P/25P, but no significant difference between 35P and 25P.

No significant difference could be seen between any of the fermented samples, Safale S-04 and Safale US-05. 25U and 25P did get partially grouped together with the fermented samples.

Outliers were considered too misleading and were therefore not included when calculating the mean value and standard deviation within each triplicate sample. When excluding Wild yeast, Sediment S-04 and Sediment US-05, differences of patulin content in both degree of lesion and treatment could be made more visible. See Appendix Table 3.

**Table 2: Mean value and following standard deviation of patulin ( $\mu\text{g}/\text{liter}$ ) in unfermented, pasteurized, Safale S-04 and Safale US-05. Again, note that the outliers were not included when calculating mean for each triplicate sample. See Appendix: Table 3 for values used in calculations.**

	Unferm.	Pasteur.	Safale S-04	Safale US-05
25 mm	394,73	357,16	39,47	37,22
Stan D:	115,05	192,21	24,85	38,30
35 mm	1475,78	661,01	43,32	45,79
Stan D:	217,36	49,60	28,47	17,55
45 mm	3451,83	1969,14	41,96	91,13
Stan D:	708,33	446,27	24,12	37,26

Means with the same letter are not significantly different.

t	Grouping	Mean	N	sample
	A	3451.8	2	45U
	B	1969.1	3	45P
	C	1475.8	2	35U
	D	661.0	3	35P
E	D	394.7	3	25U
E	D	357.2	2	25P
E		91.1	3	45SaUS
E		45.8	3	35SaUS
E		43.3	3	35SaS
E		42.0	3	45SaS
E		39.5	3	25SaS
E		37.2	3	25SaUS

Figure 2: Results from the ANOVA. In the column “sample”, the labels represent the shortenings compiled from Table 1.

Patulin content: Difference between lesion diameters

Significant difference in patulin content could be found between all degree of lesion when comparing all samples from unfermented, pasteurized, Safale S-04 and Safale US-05 together.

Means with the same letter are not significantly different.

t	Grouping	Mean	N	DoL
	A	1200.94	11	45mm
	B	472.90	11	35mm
	C	193.51	11	25mm

Figure 3: Statistic analyzing done on difference in lesion diameter, and DoL= Diameter of Lesion

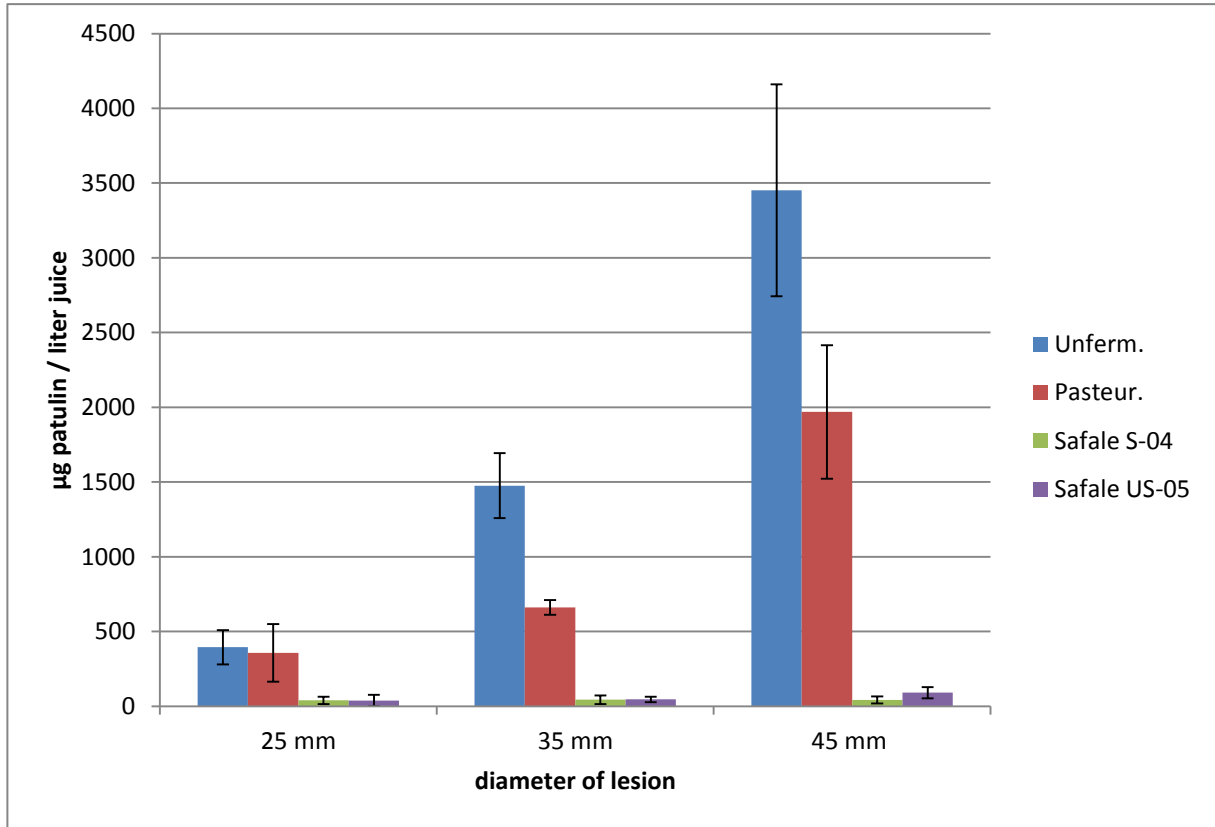


Figure 4: Mean value and standard deviation of patulin ( $\mu\text{g/liter}$ ) in unfermented, pasteurized, Safale S-04 and Safale US-05.



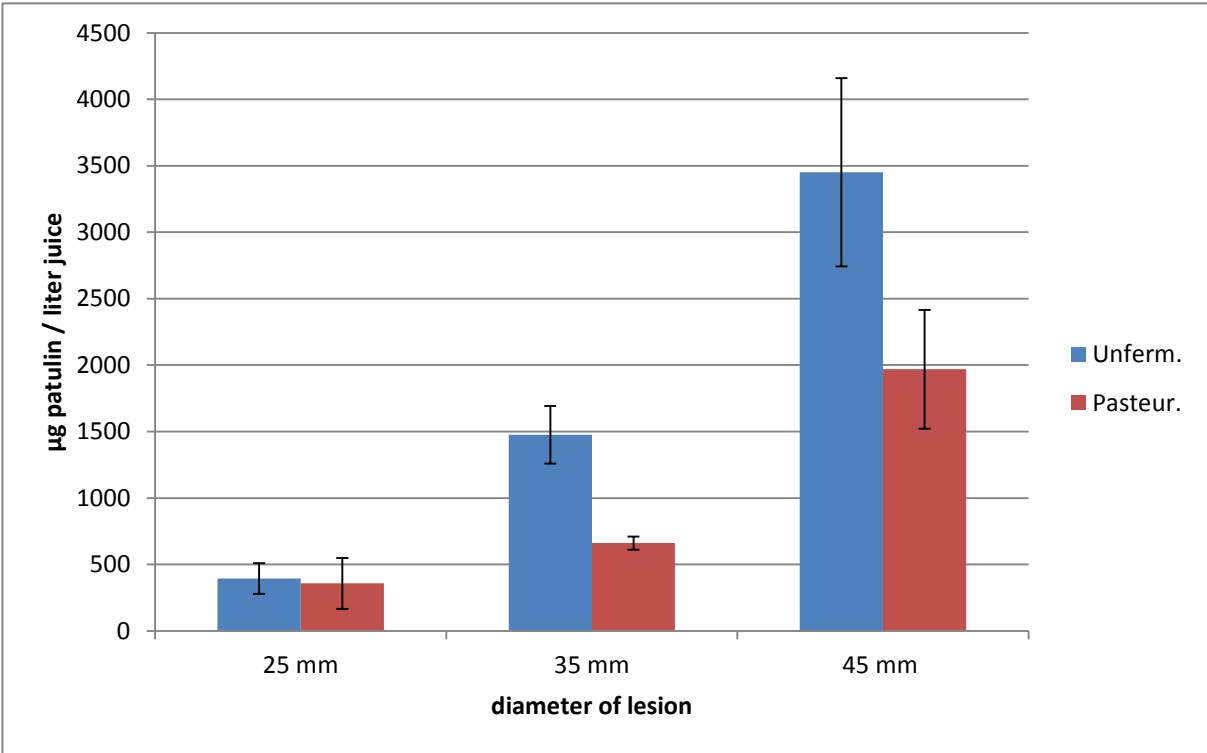


Figure 5: Mean value and standard deviation of patulin ( $\mu\text{g/liter}$ ) in the unfermented and pasteurized apple juice

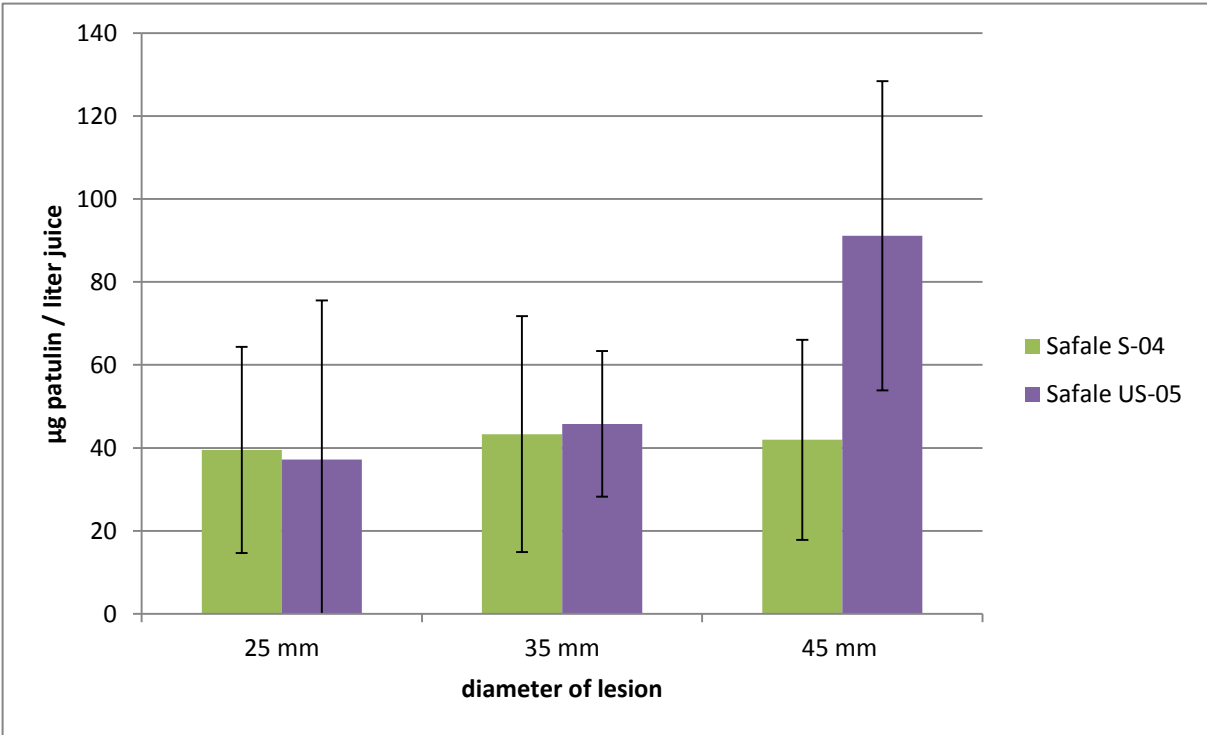


Figure 6: Mean value and standard deviation of patulin ( $\mu\text{g/liter}$ ) in juice with Safale S-04 and Safale US-05.

Patulin content: Difference between treatments

A significant difference among the treatments was found between all but the two fermented ones, Safale S-04 and Safale US-05. See Figure 8.

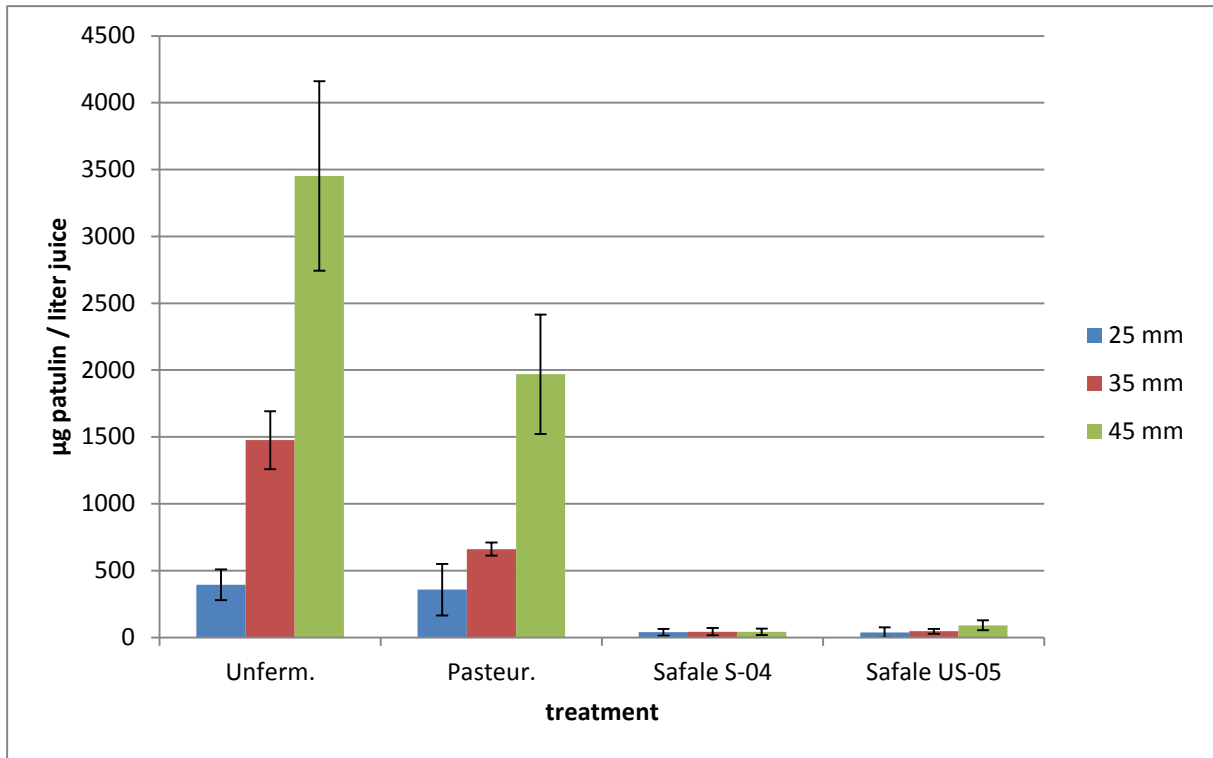


Figure 7: Mean value and standard deviation of patulin (µg/liter) in lesion diameter 25 mm, 35 mm and 45 mm.

Means with the same letter are not significantly different.

t Grouping	Mean	N	Treatment
A	1577.1	7	Unferm
B	1075.6	8	Pasteur
C	58.0	9	Saf US-05
C	41.6	9	Saf S-04

Figure 8: Calculations from the ANOVA table.

## Discussion

The result shows that there was a significant difference between the patulin concentration when the apple juice was fermented with *Saccharomyces cerevisiae*. This confirms the results from earlier studies in the subject that all agreed on this phenomenon (Stinson et al., 1978; Moss & Long, 2002; Coelho et al., 2008; Harwig et al., 1973). No significant difference in patulin reduction could be found between the two fermented samples, Safale S-04 and Safale US-05. This corresponds directly to the results from Stinson et al. (1978), where only small differences were noticed when different strains of yeast had fermented in patulin contaminated apple juice. Safale S-04 actually reached below 50 µg/liter, which would have made legal to sell as apple cider in the EU. Pasteurization of the apple juice also showed a significant difference in patulin concentration in comparison to the unfermented. This can be confirmed by the results from Wheeler et al., 1987 and Welke et al., 2009.

Looking at the differences depending on diameter of lesion, there was seen a significant difference between patulin content in all diameters of lesion within all groups (unfermented, pasteurized, Safale S-04 and Safale US-05) when patulin content were merged in respective diameter of lesion. Especially the difference in patulin content of the unfermented juices between the different lesion diameters can conclude that there is a significant difference between all three of them. Since the unfermented juice represents the most unchanged treatment, it could be assumed to be the “natural” control group for all the treatments that contained patulin. Bandoh et al. (2009) states that patulin is nearly only present in the obviously infected lesion on the apple. Therefore, it would sound natural that the larger the lesion area, the higher concentrations of patulin in the apple.

Looking at Table 3 in Appendix, it can be concluded that the wild fermentation had by far the highest concentrations of patulin. A reason for this could be that the expected spontaneous fermentation from wild *Saccharomyces cerevisiae* had not occurred. Instead, the *Penicillium expansum* had formed a green and white mold colony covering the surface of the juice. This whole treatment must be considered a “fault” in the experiment, since it did not set out to do as it was expected.

In the bottom sediments from the two fermented treatments of Safale S-04 and Safale US-05 it could not be found any notable amounts of patulin. This corresponds to the thesis that patulin is being degraded by *Saccharomyces cerevisiae* (Moss and Long, 2002).

To be able to more accurately quantify the amount of patulin in apple juice that is known to be heavily contaminated, either patulin standard solutions of higher concentration or diluted juice extracts could be used. In this study there could be some doubts about how accurate the calculated values for the wild fermented samples are, especially because the calibration curve was made from patulin standard solutions that were absorbing much less than the higher amounts from the other samples. Although, despite a potential misleading factor for multiplying the area at 276 nm, we still get an impression on the differences in patulin content between all the samples, and sometimes that is enough to tell us what has occurred.

The 1-Hexanol extraction did not show any fluctuations on the UV-detection chromatogram. In retrospect, there are some speculations if the ratio between 1-Hexanol/juice sample and the NaCl-solution was too diluted, so that there was not enough patulin to be extracted. This method was way faster to go through with, so it would be interesting to see if there are any possibilities to enhance the current method to perform better. Also, there are many different columns for the HPLC that could be applied and experimented with.

Even if juices and ciders made with apples that had a *Penicillium expansum* infection are found, it is very seldom that the product would have a smell from the mold that was present before pressing the apples. Errampalli (2004) describes the scent of *Penicillium expansum* as a “musty odour”. Some people believe it smells more like “earth cellar”. This might actually be an important point in the future if there is found a method to remove extreme amounts of patulin from apple juice; will it still be drinkable due to the taste from the *Penicillium* mold?

## ***Conclusions***

The results suggest that fermentation with *Saccharomyces cerevisiae* will reduce the levels of patulin present in the apple juice. Even though all the concentrations of patulin in the apple juice from the inoculated apples were relatively high, Safale S-04 managed to reduce the patulin levels to beneath the maximum limit allowed in the EU in all of the lesion diameters.

## References

- Abu-Bakar, N. B., Makahleh, A., & Saad, B. (2014). Vortex-assisted liquid–liquid microextraction coupled with high performance liquid chromatography for the determination of furfurals and patulin in fruit juices. *Talanta*, *120*, ss. 47-54.
- Arranz, I., Derbyshire, M., Kroeger, K., Mischke, C., Stroka, J., & Anklam, E. (2005). Liquid chromatographic method for quantitation of patulin at 10 ng/mL in apple-based products intended for infants: Interlaboratory study. *Journal of AOAC International*, *88*(2), ss. 518-525
- Bandoh, S., Takeuchi, M., Ohsawa, K., Higashihara, K., Kawamoto, Y., & Goto, T. (2009). Patulin distribution in decayed apple and its reduction. *International Biodeterioration & Biodegradation*, *63*(4), ss. 379-382.
- Barkai-Golan, R. (2008). Penicillium mycotoxins | : Barkai-Golan, Rivka, and Pastor, Nachman, eds. *Mycotoxins in Fruits and Vegetables*. Burlington, MA, USA: Academic Press, 2008. ss. 153-183
- Coelho, A. R., Celli, M. G., Ono, E. Y. S., Hoffmann, F. L., Pagnocca, F. C., Garcia, S., Sabino, M., Harada, K. I., Wosiacki, G., & Hirooka, E. Y. (2008). Patulin biodegradation using *Pichia ohmeri* and *Saccharomyces cerevisiae*. *World Mycotoxin Journal*, *1*, ss. 325-331.
- van Egmond, H.P. and Jonker, M.A. (2004) Regulations and Limits for Mycotoxins in Fruits and Vegetables, | : Barkai-Golan, Rivka, and Pastor, Nachman, eds. *Mycotoxins in Fruits and Vegetables*. Burlington, MA, USA: Academic Press, 2008, ss.45-74
- Errampalli, D. (2004). Effect of fludioxonil on germination and growth of *Penicillium expansum* and decay in apple cvs. Empire and Gala. *Crop Protection*, *23*(9), ss. 811-817.
- European Commission. (2006). Commission Regulation (EC) 1881/2006 of 19 December 2006. Official Journal of the European Communities, L364. Available from: <http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02006R1881-20140701&from=EN> [2015-05-25]
- European Commission (1996) *Reports of the Scientific Committee for Food* (Thirty-fifth series) [http://ec.europa.eu/food/fs/sc/scf/reports/scf\\_reports\\_35.pdf](http://ec.europa.eu/food/fs/sc/scf/reports/scf_reports_35.pdf) [2015-05-25]
- Fermentis, (2012). *Safale S-04*. [online] Available from: [http://www.fermentis.com/wp-content/uploads/2012/02/SFA\\_S04.pdf](http://www.fermentis.com/wp-content/uploads/2012/02/SFA_S04.pdf) [2015-05-25]
- Harwig, J., Scott, P. M., Kennedy, B. P. C., & Chen, Y. K. (1973). Disappearance of patulin from apple juice fermented by *Saccharomyces* spp. *Canadian Institute of Food Technology journal*.
- Lee, T. P., Sakai, R., Manaf, N. A., Rodhi, A. M., & Saad, B. (2014). High performance liquid chromatography method for the determination of patulin and 5-hydroxymethylfurfural in fruit juices marketed in Malaysia. *Food Control*, *38*, ss. 142-149.

M. O. Moss & M. T. Long (2002) Fate of patulin in the presence of the yeast *Saccharomyces cerevisiae*, *Food Additives & Contaminants*, 19:4, ss. 387-399, DOI: [10.1080/02652030110091163](https://doi.org/10.1080/02652030110091163)

Rovira, R., Ribera, F., Sanchis, V. and Canela, R. (1993). Improvements in the quantitation of patulin in apple juice by highperformance liquid chromatography. *J. Agric. Food Chem.* 41, ss. 214– 216.

Sabino, M. (2008). Detection and determination of patulin in fruits and fruit products. . I: Barkai-Golan, Rivka, and Pastor, Nachman, eds. *Mycotoxins in Fruits and Vegetables*. Burlington, MA, USA: Academic Press, 2008, ss. 261-270.

Shephard, G. S., & Leggott, N. L. (2000). Chromatographic determination of the mycotoxin patulin in fruit and fruit juices. *Journal of Chromatography A*, 882(1), ss. 17-22.

de Souza Sant'Ana, A., Rosenthal, A., & de Massaguer, P. R. (2008). The fate of patulin in apple juice processing: a review. *Food Research International*, 41(5), ss. 441-453.

Stinson, E.E, Osman, S.F., Huhtanen, C.N. and Bills, D.D. (1978) Disappearance of patulin during alcoholic fermentation of apple juice. *Appl. Environ. Microbiol.* 36, ss. 620-622

Sumbu, Z. L., Thonart, P., & Bechet, J. (1983). Action of patulin on a yeast. *Applied and environmental microbiology*, 45(1), ss. 110-115.

Tahir, I. I., Johansson, E., and Olsson, M. E. (2009). Improvement of apple quality and storability by a combination of heat treatment and controlled atmosphere storage. *HortScience*, 44(6), ss. 1648-1654.

Varga, J., Houbraken, J., Samson R.A., and Frisvad, J.C. (2008). Molecular diversity of *Aspergillus* and *Penicillium* species on Fruits and Vegetables. I: Barkai-Golan, Rivka, and Pastor, Nachman, eds. *Mycotoxins in Fruits and Vegetables*. Burlington, MA, USA: Academic Press, 2008, ss. 206-208

Welke, J. E., Hoeltz, M., Dottori, H. A., & Noll, I. B. (2009). Effect of processing stages of apple juice concentrate on patulin levels. *Food Control*, 20(1), ss. 48-52.

Wheeler, J. L., Harrison, M. A., & Koehler, P. E. (1987). Presence and stability of patulin in pasteurized apple cider. *Journal of Food Science*, 52(2), ss. 479-480.

## Appendix

### Penicillium expansum spore suspension (Method from Tahir et al., 2009):

The *Penicillium expansum* strain had been isolated from apples that been infected naturally. The strain was cultivated in petri dishes with potato dextrose agar (PDA) at 4°C. After 10 days the conidia were harvested from the surface and dissolved in 5 ml distilled water containing 0.05% Tween 80. To remove mycelia, the suspension was filtered through cheese cloth. The final spore concentration was adjusted by a hemocytometer to reach 10<sup>5</sup> conidia/ml.

**Table 3: Content of patulin µg/liter juice in the different treatments and lesion areas. Red cells mark the “outliers”.**

	Unferm.	Pasteur.	Safale S-04	Safale US-05	Wild yeast	Sediment S-04	Sediment US-05
25 mm	510,08	3660,17	42,20	81,42	24722,24	~~	~~
25 mm	394,13	221,25	13,38	13,99	17283,43	~~	~~
25 mm	279,98	493,07	62,84	16,25	20565,77	~~	~~
35 mm	1629,47	614,19	36,72	65,06	28971,97	~~	~~
35 mm	1322,08	712,98	74,51	30,73	24092,43	~~	~~
35 mm	11,42	655,86	18,74	41,58	26875,02	~~	~~
45 mm	63,56	1477,31	32,00	93,68	35178,43	51,13	55,16
45 mm	2950,96	2348,23	24,41	52,66	27229,87	42,92	70,01
45 mm	3952,69	2081,88	69,46	127,05	28239,05	43,01	186,81
Controls		25,79	21,29	235,40			