Patulin Degradation by Yeast Protein Extract

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Abstract

The mycotoxin patulin, produced by a number of fungi, most prominently *Penicillium expansum*, has proven problematic for the apple industry due to contamination of apple juice and apple cider. Presently, techniques to control patulin accumulation have proven increasingly ineffective due to the presence of antifungal resistant strains of mold, stability of patulin during thermal processing, and conflicting data on the efficacy of other treatments. However, fermented apple products such as hard ciders and apple cider vinegars are devoid of patulin. Fermentation with yeast resulted in complete degradation of patulin, possibly due to enzymatic degradation by yeast enzymes. Patulin has also been shown to be susceptible to adduct formation with free thiol containing molecules such as glutathione, which is naturally present in yeast cells. Limited studies have also looked at patulin adsorptivity onto the call walls of yeast. Degradation of patulin is, therefore, hypothesized to be caused by multiple mechanisms mainly caused by yeast proteins/enzymes.

To assess the loss of patulin by protein extracted from yeast (*Rhodosporidium kratochvilovae* strain 62-121), patulin extraction methods were compared to determine the optimal method for patulin extraction from protein rich environments. The effect of boiling to halt any possible enzymatic degradation on total patulin loss was assessed by comparing patulin recovery to that of samples placed on ice after the assay. Yeast growth was optimized for the production of patulin-degrading protein extracts by surveying days of growth and subsequent storage at 4°C. Additionally, free thiol group reactivity with patulin was assessed upon incubation with protein extract, cysteine, and glutathione. Liquid chromatography and mass spectrometry (LC/MS) was used to detect patulin degradation products. Potential enzymatic activity was assessed by comparing the degradation activity of different protein extracts from yeast. Finally, patulin loss due to adsorption to inactivated yeast cell walls was determined.

The use of acid and salt to precipitate the protein before patulin extraction resulted in the best patulin recovery from protein rich media, and an additional extraction following a modified AOAC method allowed for removal of excess salt without sacrificing patulin recovery. The use of boiling to denature the protein after the assay resulted in 10% higher patulin loss than when the samples were placed on ice, presumably due to adduct formation with thiol groups. Growing yeast for 6 days at room temperature was deemed adequate to obtain optimal patulin degradation; and subsequent incubation of the yeast at 4°C did not impair the patulin degradation activity. Yeast protein extracts were found to be inconsistent with respect to patulin degradation activity, nevertheless patulin degradation activity (up to 100% patulin) was observed in several batches. Patulin incubated with cysteine showed signs of free thiol blockage in both samples of protein extract and pure cysteine. Patulin incubated with glutathione was degraded at both pH 7 and 3.7, and one patulin-glutathione adduct (462 m/z) was identified via LC/MS. Lyophilized yeast cells demonstrated patulin adsorption capabilities after incubation at 30°C for 20 min.

Observed results confirm that patulin can be degraded by the protein extract from yeast. The exact mechanism of patulin degradation by protein extracts remains unclear, yet it appears to be either enzymatic or chemical through thiol adduct formation. Our results indicated that the mechanism is a combination of the two. This research offers insight into possible patulin degradation mechanisms, and can give direction in applying this new method of patulin control in an industrial setting.

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1. Review of Literature

1.1. Introduction and Objectives

The mycotoxin patulin, produced by several species of fungi, mainly *Penicillium expansum*, is primarily an issue in apples that have been stored for later use in processed foods, such as purees, juices, and ciders. Patulin contamination in apple products can have significant economic ramifications, mainly attributed to recalls. For instance, in 2010 a Minnesota apple orchard, Pepin Heights, had to issue a major recall on apple cider, which resulted in a profound economic loss. In addition, patulin contamination has limited the potential for international transportation and trade of apples and apple products. Limiting the international trade of apples and apple products is a major inconvenience since the US demand for apple products surpasses its production.

Nationally and globally, strict limits exist for the concentration of patulin in products such as apple juice and apple cider due to the adverse health effects associated with it. Among these risks are cancer and cell damage. Traditionally, the use of antifungal agents was employed to control patulin-producing fungi. Until recently, this method was successful; however, a gradual decrease in the effectiveness of antifungal agents was observed, suggesting that resistant strains have developed. Additionally, traditional processing methods such as pasteurization and clarification have only showed marginal success in reducing patulin contamination, and have not lead to a definitive solution to the problem. Therefore, it is essential to identify a new method of control for this natural toxin. Certain strains of yeast have been discovered to have the potential for degrading patulin enzymatically, thus offering a possible solution to the problem.

With this in mind, two objectives were identified to best address these quality and procedural issues in as quick, precise, and economical manner as possible:

1. Optimize the extraction of proteins/enzymes from a yeast strain that demonstrated patulin degradation capabilities.

2. Elucidate the mechanism of patulin degradation by protein extracted from yeast.

1.2. Apple Production and Processing

The United States is among the leading global apple producers. While a bulk of the apples grown in the States are intended for sale as whole fruit, much are intended to undergo processing such as canning, freezing, drying, and juicing. Apples that sustained damage through mechanical picking, windfall, and insect infestation are subjected to processing. Between 1980 and 2010, nearly 60 million tons of apples were processed (Figure 1), half of which were used to make apple juice and apple cider (USDA, 2012).

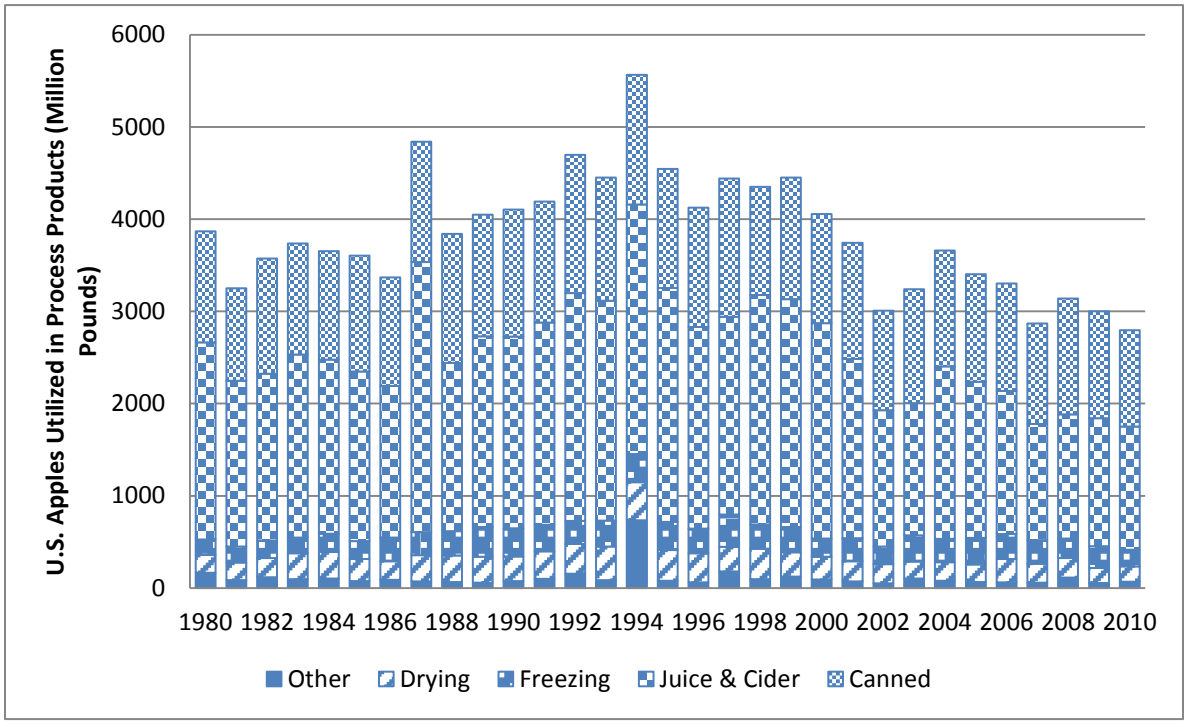

Figure 1: U.S. apple use in processed apple products between 1980 and 2010. (USDA, 2012)

1.2.1. Apple Juice and Apple Cider Processing

It is not uncommon for apples intended for juice and cider production to be subjected to extended storage conditions in between harvests. Storage

conditions include refrigeration (as low as 0°C and up to 95% relative humidity), possibly with a modified atmospheric conditions (2-3% oxygen and 1-4% carbon dioxide). Apples are normally stored for up to 9 months, until the next year's apple harvest is available. Specific storage conditions and the length of storage can affect the quality of the apples.

For the production of apple juice, apples are ground with the assistance of pectinase enzymes (both naturally occurring in the apple and commercially added) to help free the juice for pressing. The milled apple chunks are pressed, resulting in a cloudy slurry, which can be partially clarified by running the juice through a metal screen to remove large particulates. The partially clarified juice is treated with pectinases and cellulases to remove suspended fiber from the solution for both aesthetic and processing reasons. The enzymes break down polysaccharide chains, such as pectin and hemicelluloses, thus decreasing the juice viscosity, allowing for easier filtration. The clarified apple juice is then pasteurized at 88°C for 25 to 30 sec, and then packaged.

Apple cider production is similar to that of apple juice. However, cider production usually lacks some of the clarification steps (such as the enzyme treatment), allowing the solution to remain as a cloudy slurry. Likewise, pasteurization may also be omitted at the discretion of the producer. However if the sweet cider is intended to be fermented into hard cider, it is generally pasteurized to ensure there is no other microbial growth to compete with the yeast inoculum.

For the processing of hard cider, yeast cultures and sugar syrups are added to the apple juice. Sulfites are also generally added to a concentration of 50-150 ppm to inhibit the growth of undesirable microorganisms. The inoculated juice is stored in an anaerobic environment at 50-60°F to allow fermentation to progress. After the mono and disaccharides have been converted to alcohol (that may take weeks to months based on the batch size and the initial amount of yeast inoculation) the fermentation process is complete and the mixture is transferred to a separate container to age (at the discretion of the producer) apart from the

initial yeast inoculum or is immediately bottled. To produce cider vinegar, the fully fermented hard cider is inoculated with species of *Acetobacter* and allowed to grow in aerobic conditions. This allows for the conversion of ethanol to acetic acid.

1.2.2. Apple Contamination with Fungi

Contamination of the apples with fungi, including patulin-producing fungi, can occur in two main parts of the production process. During harvest, spores can easily contaminate the apples, particularly if they are picked from the ground. Mold spores from patulin-producing fungi are commonly found in the soil. Jackson et al. (2003) showed that apples harvested directly from the tree were devoid of the characteristic fungal growth, while those picked from the ground showed some patulin-producing fungal growth. The prevalence of patulin-producing fungi is especially common in fruit bearing lesions from machinery and insect damage. On the other hand, a cross-contamination effect can occur if the holding containers for the apples are not washed regularly. In this later case, mold spores can remain on the sides of the holding container, causing later batches to be contaminated. Mold growth will progress during storage leading to excessive production of patulin.

In ideal situations, these moldy apples, or at least the contaminated portions of them, would be removed before processing. This would reduce the amount of patulin that could be present on the fruit or in the processed products. However, some studies suggested that patulin can penetrate 1 to 2 cm into the fruit, thus removing the moldy part of the apple will not eliminate the risk of patulin contamination entirely (Taniwaki et al, 1992; Rychlik and Schieberle, 2001). Additionally, the extra personnel or equipment required to screen for unsanitary apples can be more than the processing plant is capable of supplying. For this reason some producers are finding it difficult to keep patulin at a reasonable level in compliance with the maximum allowable limit set for liquid apple products.

1.3. Patulin

Patulin (also known as 4-hydroxy-4H-furo [3,2c] pyran-2 [6H]-one) is a small (MW = 154.12 $g*mol^{-1}$) compound (Figure 2) produced by a number of mold species such as *Penicillium griseofulvum*, *Penecillium roqueforti*, *Alternaria alternate*, and *Bysochlamis nivea*, but most commonly *Penicillium expansum*. This later mold, commonly referred to as blue mold, is found in the environment, primarily in the soil, making it difficult to control due to the nature of harvested apples (Jackson et al, 2003).

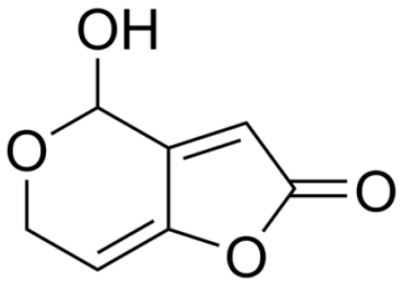

Figure 2: Patulin chemical structure. Molecular weight 154.12 Da.

Evidence suggests that patulin, which acts as an antimicrobial agent, is produced when the mold is stressed by a limitation on cell growth, such as diminishing nitrogen content (Grootwassink and Gaucher, 1980). The mold is thus eliminating the competition posed by other microbial strains. There is further evidence that patulin may be produced under other environmental stresses such as less than optimal O_2 and CO_2 conditions (Sommer et al, 1974).

Originally, patulin was recognized for its antimicrobial property. Patulin is capable of inhibiting over 75 different bacterial species, of both Gram positive and gram negative varieties (Ciegler et al, 1971). Thus it was believed to have medicinal properties based off of a limited number of studies (Korzybski et al, 1967; Raistrick et al, 1943). Patulin was prescribed for patients suffering from nasal congestion and as a treatment option for the common cold (Medical Research Council, 1944).

However, more recent studies found that patulin is not only toxic to bacteria, but to larger animals as well. Between 1944 and 2002, a large number of studies have been conducted on the toxicity of patulin. Proposed symptoms of patulin

poisoning are diverse and can include agitation, convulsions, dysponea, pulmonary congestion, edema, hyperemia, GI tract distension (Escoula et al, 1977; Hayes et al, 1979), nausea (Walker and Wiesner, 1944), epithelial cell degradation, intestinal hemorrhage, intestinal inflammation (McKinley and Carlton, 1980; McKinley et al, 1982; Mahfoud et al, 2002), and ulceration (Escoula et al, 1977; Hayes et al, 1979; McKinley and Carlton, 1980; McKinley et al, 1982; Mahfoud et al, 2002). Likewise, more chronic symptoms have been identified, and are believed to be genotoxic (Mayer and Legaror, 1969; Korte, 1980; Thust et al, 1982; Lee and Roschenthaler, 1986; Roll et al, 1990; Hopkins, 1993; Pfriffer et al, 1998), neurotoxic (Hopkins, 1993), immunotoxic, (Hopkins, 1993; Wichmann et al, 2002), immunosuppressive (Wichmann et al, 2002), teratogeneic (Ciegler et al, 1976; Roll et al, 1990), and/or carcinogenic in nature (Dickens and Jones, 1961; Oswald et al, 1978). Some cellular effects are believed to include plasma membrane disruption (Riley and Showker, 1991; Mahfoud et al, 2002), protein synthesis inhibition (Hatez and Gaye, 1978; Miura et al, 1993; Arafat and Musa, 1995), transcription disruption, translation disruption (Moule and Hatey, 1977; Arafat et al, 1985; Lee and Roschenthaler, 1987), DNA synthesis inhibition (Cooray et al, 1982), Na-coupled amino acid transcription inhibition (Ueno et al, 1976), Interferon-γ production inhibition (Wichmann et al, 2002), RNA polymerase inhibition (Moule and Hatey, 1977), aminoacyl-tRNA synthesis inhibition (Arafat et al, 1985), Na-K ATPase inhibition (Phillips and Hayes, 1977, 1978; Riley and Showker, 1991), muscle aldolase inhibition (Ashoor and Chu, 1973), urease inhibition (Reiss, 1977), loss of free glutathione (Burghardt et al, 1992; Barhoumi and Burghardt, 1996), protein crosslink formation (Fliege and Metzler, 1999), and protein prenylation inhibition (Miura et al, 1993). From this evidence, it is clear that the health risks associated with patulin consumption are great enough to warrant its control.

1.3.1. Patulin Allowable Levels

In light of the observed health effects, the U.S. Food and Drug Administration has set a limit for patulin contamination at 50 µg/L of apple juice and cider (USDA 2004). The World Health Organization has placed a limit on maximum daily intake of 0.4 µg/kg of bodyweight (WHO, 1995). Several countries have set more stringent limits at 25 to 35 µg/L (van Egmond, 1989). However, according to a 2001 FDA report, the “no observed adverse effect level” (NOAEL) was found to be 43 µg/kg bodyweight per day (FDA, 2001).

1.3.2. Patulin Analysis and Quantification

Currently there are a number of official methods in place for the detection and quantification of patulin in both clear and cloudy apple products. These include a qualitative thin-layer chromatography method that is able to detect the presence of patulin down to 20 µg/L (AOAC Method 974.18), and two quantitative liquid chromatography methods with limits of detection as low as 20 µg/L (with standard error values between 10-20%) and 25 µg/L (with standard error values between 8-36%) (AOAC Method 995.10, AOAC Method 2000.02, respectively). Patulin quantification by LC/MS has also been carried out, with limits of detection as low as 0.2 µg/L (Takino et al, 2003).

1.3.3. Patulin Control

Methods for patulin control have been widely investigated. Studies looking at the production of patulin and patulin stability over a range of thermal treatments, storage methods, pH levels, extraction techniques, and processing conditions have been researched.

1.3.3.1. Fungal Control

The use of benzimidazole fungicides had been an effective method in the control of the patulin-producing fungi from the 1970s to the 1990s (Rosenberger, 2003). However in more recent years, the emergence of fungicide resistant strains has left producers searching for other control methods of patulin-producing fungi.

One standard control method used in industry prior to processing is a washing step. The efficacy of postharvest washing of apples using both potable water and 100-200 ppm chlorine have shown varied results, with patulin levels being decreased by 20-100% (Jackson et al, 2003). While this offers a potentially beneficial tool in the prevention of patulin buildup, it has not been proved reliable in dealing with the problem. Much of the inconsistency with postharvest washing of apples is that it only addresses the toxin on the surface of the fruit, leaving any on the interior untouched.

Temperature-controlled storage has shown marginal promise in the delay of fungal growth by 0-50% for 7 weeks at temperatures ranging from 0 to 30°C. However, patulin content was found to increase by as much as 800% at the cooler temperatures when compared to the room temperature (30°C) samples over the same time period and temperature range (Sommer et al, 1974). Paster et al (1995) were in agreement, showing that patulin production was either increased or not significantly different at cooler temperatures ranging from 3 to 17°C. In the same study, temperatures between 0-25°C were able to decrease patulin accumulation by 60-100% in 2 strains tested (*P. expansum* NRRL 6069 and CBS 481.84) yet induced production in a third strain (*P. expansum* NRRL 2034). While this may offer some protection from visible mold growth on the apple, the environmental stress on the mold that does grow appears to promote the toxin's production. Thus while cosmetically being sound, it is seemingly detrimental from a food safety standpoint.

Combined, these aforementioned fungal control methods offer some options to control patulin accumulation, however their inconsistent effects render them inadequate measures of control. The only other alternatives that are currently

being employed are Good Manufacturing Practices (GMPs) along with a strong Hazard Analysis and Critical Control Point (HACCP) plan. While GMPs and a HACCP plan can reduce the amount of patulin introduced into the processing line, they lack the ability to deal with patulin accumulation once fungal contamination during storage has occurred.

1.3.3.2. Controlled Atmospheric Storage

Controlled atmospheric storage can reduce the amount of mold growth and apple decay from patulin-producing fungal species. Reduced mold growth was found under low levels of O_2 and CO_2 (3% and 3% respectively), and ultra-low levels (3% and 1% respectively) of the same gasses (Morales et al, 2008). Using 1% CO_2 and 3% O_2 caused a delay in the onset of mold growth that corresponded to patulin levels below detection (to as low as 4 µg/mL) (Lovett et al, 1975). Storage of apples at 3% CO_2 and 2% O_2 was found to lower patulin accumulation by approximately 30% in 3 patulin-producing fungal strains surveyed (all of which *P. expansum*) while an increased O_2 content (up to 20%) was found to offer no change (Paster et al, 1995).

Like refrigerated storage, one of the primary limitations of controlled atmosphere storage is that the decreased mold growth does not always correspond to decreased patulin content. The efficacy of the technique is heavily dependent on the particular strain of patulin-producing fungi involved. In a study by Lovett et al (1975), 2 strains of *P. expansum* were tested for their ability to grow in controlled atmosphere conditions (1% CO2, 3% O2, and 96% N), it was found that while patulin production was prevented in both rotten and sound tissue in one strain (*P. expansum* 1071), the controlled conditions were not able to fully prevent patulin accumulation (an average of 0.5 µg/mL was observed) in the rotten apple tissue of the other strain (*P. expansum* NRRL 973) after 14 weeks of incubation. While controlled atmosphere storage was generally able to reduce the amount of

patulin in the samples, it offers a tool but not a solution to the problem of patulin producing molds due to the variability that comes from each strain.

1.3.3.3. Processing Effect on Patulin Degradation

Under alkaline conditions, patulin is prone to degradation and was shown to have a perceived half life of 55 and 2.6 days in buffer solutions at pH 6 and pH 8, respectively at 25°C (Brackett and Marth, 1979). Unfortunately, the pH of apple juice and cider falls well below this range. Patulin's stability during storage has been shown to be optimal at acidic pH levels, particularly around the pH of apple juice and apple cider (3.35-4.0) (Lovett and Peeler, 1973). At these standard pH ranges, patulin has been proven to be fairly stable not only under storage at room temperature, but also at pasteurization temperatures and times which range from 160°F for 6 sec, to 180°F for 0.3 sec (FDA, 2010).

In some extreme heating conditions, patulin has been shown to be prone to degrade at acidic pH ranges. A 50% decrease in patulin was observed in apple juice after heating for 20 minutes at 80°C (Scott and Somers, 1968). A similar study found a 60% decrease in patulin content after 2 successive pasteurization treatments of 80°C for 20 min each (Raiola et al, 2012). A patulin decrease of only 15% was observed in apple juice after 10 min of heating at 90°C (Wheeler et al, 1987). Furthermore, heating whole apple products to 38°C for 4 days was adequate for a reduction of patulin producing spores to the point where no signs of decay (and by extension patulin production) were evident after 6 months of storage at 1°C (Conway et al, 1999). However, heating for 20 min at 80°C and 4 days at 38°C are processing parameters that exceeds that of traditional pasteurization of apple juice. Such parameters can lead to a greater financial burden on the part of the producer, and also cause a decrease in desirable sensory characteristics.

1.3.3.4. Chemical Modification

Another avenue explored for dealing with patulin is through chemical modification. Treatment with ammonia (0.03 M solution heated to 120°C for 15 min) or oxidation via potassium permanganate (0.12 M in alkaline conditions for 3 h) have both shown capabilities for degradation of patulin in aqueous solutions (Fremy et al, 1995). However, these treatments have also rendered the product unfit for human consumption (Ellis et al, 1980) and would only be reasonable methods of dealing with the toxin in a waste stream.

Sulfur dioxide is another compound that has been found to degrade patulin (Pohland and Allen, 1970; Aytac and Acer, 1994). Up to 90% patulin reduction was observed (from 77 ppm) in one study with a sulfur dioxide content of 2000 ppm (Burroughs, 1977). Another study observed a 50% decrease in patulin content at 100 ppm sulfur dioxide after 15 min (initial patulin concentration was 25 ppm) (Ough and Corison, 1980). A third study suggested that patulin degradation by sulfur dioxide is dependent on the pH of the medium, where the more favorable conditions are in alkaline environments (Steiner et al, 1999). The effectiveness of sulfur dioxide varies from study to study, bringing into question the reliability of this method. Furthermore, the maximum allowable limit for sulfur dioxide in food is set at 200 ppm which limits the effectiveness of this strategy; it is also undesirable from a labeling standpoint.

1.3.3.5. Miscellaneous Processing Techniques

A number of other processing options have been explored for decreasing patulin content. Centrifugation at 6,551 x g of the apple juice can reduce the patulin content by 89% (from 2 ppm), yet can make the precipitate layer too toxic for use thereafter (Bissessur et al, 2001). Likewise, depectinizing, clarifying, and vacuum filtering have been shown to decrease patulin levels by 39% through the interaction of patulin with processing aids (such as depectinizing agents) added

to the juice that are removed before packaging (Acar et al, 1998). Also, the use of activated charcoal can decrease patulin content by 41% through adsorption. However it also tends to adsorb other phenols and decrease the clarity of the juice, thus making it an undesirable option (Artik et al, 2001). All of these methods offer a separate technique for dealing with patulin, which may be used together to greatly reduce patulin levels, yet are also limited by factors such as specialized equipment that make them not feasible for industrial use.

1.3.3.6. Patulin Degradation by Biological Agents (Yeast)

Patulin is either absent or found in reduced levels in fermented products. After only 5 days of fermentation in the production of beer, 0.125 ppm of patulin was completely degraded (Inoue et al, 2013). Majerus and Kölb (2008) found a 60% decrease in patulin content after only 4 days of wine production. Another study investigated the loss of naturally occurring patulin in samples of grape must (for wine production) with actively fermenting yeast and found over a 60% loss in patulin compared to the unfermented samples (Majerus et al, 2008).

Additionally, there are a number of studies that investigated the fermentative strain of yeast in relation to patulin loss. Harwig et al (1973) noted complete patulin loss during apple juice fermentation after 7 days for *Saccharomyces cerevisiae* (Y-99) and 11 days for *S. ellipsoideus* (DAVIS #522). Burroughs (1977) showed over 90% of patulin loss over the course of a 3 day incubation at 25°C with 2 strains of *Saccharomyces cerevisiae* (from 67.8 µg/mL). A study investigating 8 yeast strains used in commercial production of apple wine and hard apple cider showed undetectable levels of patulin after 10-14 days incubation at 23-25°C (from 15 µg/mL) in 6 of the strains, and the remaining 2 showed over a 99.5% reduction (Stinson et al, 1978). Similarly, *Rhodotorula glutinis*, *Cryptococcus laurentii*, *Saccharomyces cerevisiae*, and *Aureobasidium pullulans* species have been shown to reduce high patulin levels (100-500 µg/mL) by as much as 90% over the course of a 10 day incubation at 23°C

(Castoria et al., 2005). *Pichia ohmeri* strain 158 was found capable of decreasing levels of detectable patulin from 8.92 µg/mL down to 0.02 µg/mL in 5 days at 25°C, and below the limit of detection in 15 days (Coelho et al, 2008). In a recent study, 6 strains of yeast (*P. ohmeri* and *R. kratochvilovae*) showed between 20-70% patulin loss over a 72 h incubation period at 24°C (from an initial patulin content of 3 µg/mL) (Linghu, 2013). While the degree to which patulin is reduced through yeast fermentation differs, the fact that it consistently causes reduction in patulin offers a promising control tool.

1.4. Challenges of Patulin Degradation by Yeast

While the use of yeast to degrade patulin can be an effective control method, there are a number of challenges associated with this technique. Fermentation changes the chemical makeup of the product through the production of metabolites (such as alcohol and acetic acid) that are undesirable for apple juice and apple cider given the target market tends to be minors. The production of these metabolites would also pose a problem from a labeling standpoint (as in the case of alcohol-production), and a sensory one as well due to overall compositional changes.

Additionally, due to the antifungal properties of patulin, using the active yeast cells as a control mechanism might not be a feasible option. As patulin levels increase, yeast growth becomes increasingly impaired, and at high concentrations growth is not even possible (Sumbu et al, 1983). One study conducted on 3 strains of *P. ohmeri* and 3 *R. kratochvilovae* showed that in patulin concentrations greater than 10 µg/mL, yeast growth begins to be impaired (Linghu, 2013). While 10 µg/mL translates to a contamination level (10,000 µg/L) far above the legal maximum limit of patulin (50 µg/L), the decreased efficacy of yeast growth still remains a concern. When the biological control capabilities are considered in conjunction with the undesirable metabolites produced during yeast growth, an alternate method of patulin degradation is deemed necessary.

1.5. Potential Enzymatic Degradation of Patulin

Characterization of the patulin degrading capabilities of yeast has yet to be thoroughly elucidated beyond the point of the degree of patulin loss. Castoria et al (2011) noted a constant rate over time of patulin loss using *Rhodosporidium kratochvilovae* strain LS11. The authors showed the formation of a proposed patulin degradation product, desoxypatulinic acid, at a rate proportional to the rate of patulin loss (Castoria et al, 2011). The production of a metabolite could be explained by enzymatic degradation occurring during yeast fermentation.

By extracting the potential patulin degrading enzyme (or enzymes) from the yeast and using them as a processing tool, the challenges associated with the use of active yeast are circumvented. Enzymatic degradation of patulin could be a feasible approach to control patulin levels in apple juice and apple cider without incurring the negative effects associated with yeast metabolite production Likewise, stunted yeast growth from patulin's antimicrobial effects would be avoided entirely.

In order to prove that the degradation of patulin by yeast is enzymatic, it is essential to achieve protein extraction from yeast cells in a manner that maintains enzyme activity. Extraction would then be followed by a series of enzyme kinetics experiments to determine the nature of the patulin loss.

1.6. Industrial Application of a Patulin Degrading Enzyme

At this point, preliminary work has been done using protein extracts from yeasts that have proved capable of degrading patulin. These active protein extracts showed up to 100% loss of patulin around physiological pH values (pH 7), and up to 58% loss at the pH of apple juice and apple cider (pH 3.7) at 30°C for 20 min

(from 5 µg/mL patulin) (Linghu, 2013). Further investigation needs to be carried out in an effort to understand the mechanism of patulin degradation.

A proposed industry application for utilizing a patulin-degrading enzyme would be to flow the apple juice or apple cider product over an immobilized enzyme-bound surface, and thus break down the patulin with minimal treatment. At this point, in the processing line it would be possible to alter the temperature for optimum enzyme activity. It would be less feasible to change the pH or some of the substrates involved in the process due to the sensory or labeling changes that would be required, thus compatibility of the enzyme with the low pH is a requirement. This method would also be an economical option as there would be a minimal equipment cost involved in this treatment.

1.7. Alternative Loss of Patulin Through Sulfhydryl Adduct Formation

One alternative explanation provided for the loss of patulin upon thermal treatment at physiological pH values is the possibility of an adduct formation with free sulfhydryl groups. Fliege and Metzler (1999) showed that patulin can bind to and ultimately form a number of degradation products when reacted with free sulfhydryl groups from cysteine (Figure 3). The presence of free cysteine in the rumen negated the detrimental effects of patulin in an *in vitro* study (Morgavi et al, 2003). Another study investigated the mechanism for inactivation of aminoacyl-tRNA synthesases by patulin and found that nearly 100% of the lost activity could be regained with the addition of thiol-group reducing agents (Arafat et al, 1985). Additionally, another study showed a decrease in patulin content in solutions of cysteine, glutathione, and whole wheat bread flour, up to 99%, 96%, and 86%, respectively, after 8 days of storage at room temperature (from 1541.2 µg/mL, pH 5) (Reiss, 1976).

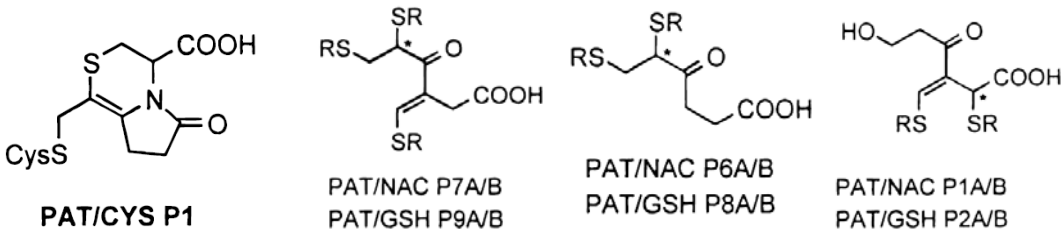

Figure 3: Proposed patulin (PAT) degradation products when reacted with cysteine (CYS), glutathione (GSH), and N-acetyl-L-cysteine (NAC) (Fliege et al, 1999)

1.8. Yeast Cell Wall Potential Reactivity

Within the polysaccharide matrix that makes up the cell walls of yeast cells are cell wall proteins that are primarily held together through disulfide bonds and non-covalent interactions (Pitarch et al, 2008). Post-diauxic yeast cells have been shown to feature markedly less permeable cell walls with six-fold higher content of disulfide bonds (Klis et al, 2006).

Patulin loss has been observed in inactivated yeast cell suspensions in apple juice (*S. cerevisiae* strain YS3) during incubation at 29°C for 24 h (Guo et al, 2012). In this study, patulin contents between 50 and 500 µg/mL were tested using 1 g/40 mL inactivated yeast powder. Between 40 and 80% patulin loss was recorded at pH 4 after 24 h. Another study on the release of patulin from inactivated yeast cell suspension showed that washing the cells with ethanol resulted in up to 36% recovery of patulin that was not initially extracted during the standard extraction method (AOAC method 2000.02). The binding of patulin to the yeast cells was not explored much further, and it was proposed based on the findings that patulin interact with the yeast cell walls in part through weak, non-covalent interaction. Other, stronger interactions could have taken place since patulin was deemed inextractable after the wash treatment; these interactions could include thiol-adduct formation from surface sulfhydryl groups. If this is the

case, an adsorptive process (similar to that of activated charcoal treatment) could be an option with little to no detrimental effect to sensory characteristics.

A separate study looked at the adsorptivity of patulin to cell wall components of lactic acid bacteria. Up to 80% of patulin was removed from apple juice (pH 4) by 0.1 g/mL lyophilized LAB cells incubated at 30°C for 24 h, from an initial concentration of 100 µg/L (Hatab et al, 2012). A concentration dependent relationship between patulin and the percent reduction was noted. At 150 µg/L a maximum of 50% reduction was seen, and at 200 µg/L less than 25% reduction was observed. The number of potential binding sites on the surface bacterial cells was thought to be the limiting factor.

Together, these studies suggest a relationship between cell wall components of microbial patulin decontamination and the absence of patulin in fermented products. If the mechanism of patulin loss is connected to a binding mechanism to the cell walls, the patulin loss is likely connected to the removal of the mycotoxin along with the fermentation agent (in the case of cider – the yeast).

1.9. Conclusion

In light of the presence of antifungal resistant strains of patulin producing mold species, it is necessary to explore other avenues for control of the toxin. Likewise, other control methods currently in place have fallen short of preventing contamination and toxin accumulation, and recalls still take place. Limited studies suggest that patulin degradation by yeast is enzymatic, which may offer an alternative method for control during processing. Furthermore, evidence suggests that the degradation may be chemical and patulin is merely removed from the solution along with the yeast when fermentation is completed. In either case, elucidation of the mechanism of patulin loss during yeast fermentation could lend valuable insight into future work.

Thus far, patulin extraction techniques have only focused on media that are low in protein (similar to apple juice) and thus the AOAC method was adequate. To test the theory that interaction with a protein is involved in the degradation by yeast, a method must be developed that ensures optimal patulin extraction from protein rich media to accurately quantify the amount of patulin degraded.

A method for the production of crude protein extracts from yeast that can actively degrade/bind to patulin needs to be developed. Currently, methods for patulin degradation involving yeast have only focused on whole yeast cells, active or inactivated through lyophilization. Data suggested that yeast cell walls may have patulin binding capabilities, and intracellular peptides such as glutathione are able to degrade or bind patulin as well. However, an attempt to extract the peptides or proteins responsible for the degradation, thus taking the intact yeast cell out of the picture, has yet to be attempted.

More than one mechanism is likely responsible for the degradation or irreversible binding of patulin in samples of crude protein extract. By exploring the reactivity of non-enzymatic biological components that potentially have some bearing on patulin loss (such as molecules consisting of free thiol groups like glutathione), valuable information can be obtained for the characterization of the overall patulin loss.

2. Materials and Methods

2.1. Materials and Sources

2.1.1. Yeast Strain

R. kratochvilova (62-121) was purchased from the Phaff Yeast collection, University of California, Davis. The strain was isolated from J.F.T. Spencer of *Campanula rotundifolia* flower. This species was chosen based on research by Castoria et al (2005). The specific strain was chosen based on prior work done to screen several strains of the same yeast for both patulin degrading capabilities and growth rate (Linghu, 2013). The strains were kept as a frozen stock a -80°C, from which all future inoculations were taken.

2.1.2. Yeast Growth Media

Two culture media were utilized for yeast growth; yeast peptone dextrose (YPD) and Lilly-Barnett (LB) based on procedures outlined by Castoria et al. (2005) and Coelho et al (2008).

The YPD media was utilized for its nutrient-rich characteristics. It typically contains 1% yeast extract, 2% peptone, and 2% dextrose (w/v). When mixed with 1.8% agar, the YPD media was plated in Petri dishes to supply optimal starting growth conditions for the yeast.

The LB media was intended to be a more simple growth media that experimentally gave the greatest ease of patulin extraction and quantification via HPLC analysis. This media contained 10 g D-glucose, 2.0 g L-asparagine, 1.0 g KH_2PO_4, 0.5 g $MgSO_4$-$7H_2O$, 0.01 mg $FeSO_4$-$7H_2O$, 8.7 mg $ZnSO_4$-$7H_2O$, 3.0 mg $MnSO_4$-H_2O, 0.1 mg biotin, and 0.1 mg thiamine (per Liter) (Castoria et al., 2011). Due to the trace amounts of Biotin (0.4 µM), Thiamine (0.3 µM), and $FeSO_4$-$7H_2O$ (0.04 µM), 1 mg/mL stock solution(s) were individually prepared

and the required aliquots were added to the media mixture. Each liter of sterilized LB media was then separated into 100 mL aliquots for yeast suspension growth.

Alternatively, all constituents of the growth media were weighed out and dissolved freshly before preparation due to decreasing yeast growth as the age of the frozen stock solution increased. Over time this was deemed the better option as it gave optimal yeast growth for protein extraction.

2.1.3. Chemicals and Solvents

Chemicals for the protein extraction from yeast were obtained as a kit (CelLutic Y Plus Kit, CYP1-1KT) from Sigma-Aldrich (St. Louis, MO). This kit included the reaction buffer, 1M Dithiothreitol (DTT) solution, Lyticase, extraction buffer, Triton X-100, and a protease inhibitor cocktail. Additional lyophilized lyticase enzyme (L2524-50KU) was also ordered from Sigma-Aldrich, dissolved in reaction buffer to a dilution of 17.25 KU/mL of buffer, and frozen in 300 µL aliquots. A reaction mixture of the reaction buffer and DDT solution was made up to a concentration of 30 µM DTT. Additionally, beta-nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH) (N1630), patulin (P1639-5MG), ethyl acetate, sodium sulfate, L-asparagine (A4159), biotin (B4639), thiamine (39499), potassium phosphate (60220), iron sulfate heptahydrate (F7002-250G), zinc sulfate heptahydrate (Z0251-100G), manganese sulfate monohydrate (M8179), phosphoric acid (452289-250ML), sodium acetate (S2889-250G), bovine serum albumin (BSA) (A2153), L-glutathione reduced (G6529), L-cysteine (168149), 5,5'-dithiobes(2-nitrobenzoic acid) (D218200), and ethanol (99%) were obtained from Sigma-Aldrich. The yeast extract (212750), peptone (211677), and agar (214530) were purchased from Becton, Dickinson and Company (Sparks, MD). Dextrose (4912-12) was obtained from Avantor Performance Materials, Inc. (Phillipsburg, NJ). Coomassie brilliant blue (BP100-25), sodium chloride, and high performance liquid chromatography (HPLC) grade acetic acid (A38-212) and acetonitrile (A998-4) were purchased from Fisher Scientific (Fair Lawn, NJ).

Magnesium sulfate (2500-01) was obtained from J.T. Baker Inc. (Phillipsburg, NJ). A patulin stock solution was prepared by dissolving the entirety of the powdered toxin in 5 mL of ethanol while in the reagent bottle (to a concentration of 1 mg/mL). This was transferred to an Erlenmeyer flask and diluted to 25 mL with ethanol to achieve a final concentration of 0.2 mg/mL. The patulin stock solution was stored in 1 mL aliquots in HPLC vials at -20°C.

2.2. Methods

2.2.1. Yeast Growth

Frozen stock solution (stored at -80°C) of *R. kratochvilova* (62-121) was used to inoculate the YPD agar plates by heating an inoculation loop over a flame and collecting a drop of the stock yeast solution. The yeast stock solution was then streaked on the plate and allowed to grow at 30°C for 1 to 2 days.

Colonies were randomly chosen from 3 different colony forming units on the Petri dish using a sterilized inoculation loop and added to 100 mL of sterile LB media in 250 mL Erlenmeyer flasks. In total, 2-3 flasks were inoculated in this manner and the yeast suspension was allowed to grow for 2-6 days on a shaking platform set to 100 rpm at room temperature (23°C) until the optical density (OD), measured at 600 nm, was ≥1 (Dunn and Wobbe, 2001). All OD values were measured using a Varian Cary 50 spectrophotometer (Walnut Creek, CA).

Once the OD_{600} of the primary suspension reached the target value, fresh 100 mL aliquots of LB media were inoculated to achieve an OD_{600} value of 0.1. Inoculation to an OD_{600} value of 0.1 was chosen based on methods outlined by Dunn and Wobbe (2001). This was achieved by calculating the volume of the primary suspension using the following equation:

$$\left(\frac{0.1\ OD\ Units}{mL}\right) 100\ mL = \left(\frac{x\ OD\ Units}{mL}\right) y\ mL$$

Based on previous data, a patulin concentration of 0.25 µg/250 µL did not inhibit yeast growth (Linghu, 2013). Thus in an effort to encourage the patulin degradation mechanism in the growing yeast cells, 100 µg of patulin stock solution (200 µg/mL in ethanol) was added to each flask before inoculation. The suspension was allowed to grow on a shaking platform set at 100 rpm at temperature for 2-8 days until the suspension became a salmon-pink, at which point the protein was either immediately extracted or stored under refrigerated conditions until extraction (up to 8 days).

2.2.2. Protein Extraction Using the CelLytic Y Plus Kit

The contents of 3-5 flasks were pooled and transferred to pre-weighed 250 mL plastic, screw cap bottles and centrifuged at 4000 x g for 5 min at 4°C. The supernatant was discarded and the tubes re-weighed to find the weight of the yeast pellet. The pellet was suspended in 4-6 volumes of cold DDW (w/v) and transferred to pre-weighed 50 mL plastic centrifuge tubes. The tubes were centrifuged at 5000 x g for 5 min at 4°C. The supernatant was discarded and the weight of the washed yeast cells was obtained. The pellets were then suspended in 1-2 volumes of the reaction buffer-DTT solution (w/v). The starting OD of the solution was measured at 800 nm by combining 10 µL of the suspended yeast cells with 990 µL of DDW. Lyticase was added at 5000 u/g yeast cells. Once the lyticase was added, the sample was incubated at 37°C for 3 h with gentle shaking. After incubation, the OD_{800} was measured by removing 10 µL from the sample on the shaker, and combining it with 990 µL, then measuring the absorbance at 800 nm as before. Due to the nature of *R. kratochvilova* (62-121), incubation step was considered complete if the OD_{800} had dropped by 10-20% of the initial value. If this was not the case the sample was incubated for another 30 min. The tubes were centrifuged at 1500 x g for 5 min at 4°C, the supernatant was discarded, and the tubes were weighed to find the weight of the lysed

spheroplasts which were the yeast cells with perforated cell walls that were ready for protein extraction.

The lysed spheroplasts were then suspended in 2 volumes (w/v) of extraction buffer containing 1% Triton X-100. The suspended yeast cells were then incubated for 15 min at 4°C while stirring. The spheroplasts were then homogenized (PRO 200 homogenizer, Double Insulated, 120V, AC 6=50-60 Hz, 1.15 A, 5000-30000 RPM, PRO Scientific Inc., Munroe, CT) for 1 min on ice. Additionally, the sphereoplasts were sonicated at 115 wats for 3-4 cycles of 30 sec with a 1 min cooling period in between each cycle (VCW130, 130 W, 20 kHz, Sonics & Materials Inc., Newton CT). The cells were then centrifuged at 12,000 x g for 15 min at 4°C and the supernatant was transferred to a clean chilled test tube. The protein content of the extract was quantified using the Bradford Assay, and the extract was aliquoted into 300-500 µL aliquots, and stored at -70°C.

2.2.3. Bradford Assay for Protein Quantification

The Bradford Protein Assay was used to quantify the protein content of the yeast extracts following the method outlined by Bradford (1976). Six BSA standards were prepared in triplicate to concentrations of 0, 2.5, 5, 10, 20, and 40 µg/2.04mL. This was done using a BSA standard solution (1 µg/µL) and bringing the volume to 40 µL. A set of 3 samples consisting of 10 µL of the protein extract and 30 µL DDW were prepared. To each vial, 2 mL of Bradford reagent (100 mg Coomassie Brilliant Blue R-250, 50 mL 95% ethanol, and 100 mL 85% (w/v) phosphoric acid were diluted to 1 L and filtered through Whatman filter paper) was added, bringing the total volume to 2.04 mL, and allowed to sit at room temperature (23°C) for 10 min. Absorbance was then measured at 595 nm and the protein concentration was measured in µg/10µL. A calibration curve can be found in Appendix B (Figures 18-24).

2.2.4. Patulin Degradation Assay Using Crude Protein Extract

Several protein extracts, obtained from yeast grown for different lengths of time, were tested for patulin degradation capabilities. Frozen protein extract was thawed on ice and an aliquot was added to a microcentrifuge tube. The volume of the aliquot (consistently 12.5 µg of protein) was based on the protein concentration of the extract. Additionally, 20 µL of 0.021 M NADPH solution was added to each tube, followed by the addition of acetate buffer (0.01 M, pH 3.7) to a final volume of 200 µL. This reaction parameter was chosen to mimic the pH of apple juice and apple cider. Additionally, the acidic pH was chosen to discourage patulin adduct formation with sulfhydryl groups. Each sample was inoculated with 50 µL of patulin standard solution for a final concentration of 5 ppm and immediately placed in a 30°C water bath. Sample mixtures were incubated undisturbed for 0 and 20 min and then ethyl acetate was added to halt the reaction. All samples were placed on ice before the patulin extraction was carried out. All sample mixtures were prepared for analysis in triplicate.

Protein controls were prepared by first boiling the sample mixture without the patulin for 5 min, cooling the samples on ice, and then carrying out the assay. Patulin controls were prepared by omitting the protein from the sample mixture and making up the excess volume with additional acetate buffer. The patulin stock solution was used to mimic 100% extraction and account for variability between stock solutions.

To test whether a final boiling step to terminate the reaction had accelerated the adduct formation with free sulfhydryl groups, the reaction was carried out without the boil step. Instead, the protein was precipitated out of solution using salt and acid as will be discussed in sections 2.2.7 and 2.2.9.

2.2.5. Patulin Degradation Assay Using Lyophilized Yeast Cells

Yeast cells were grown as outlined in section 2.2.1. and the flasks were pooled and washed as outlined in 2.2.2. However, after the cells were washed with DDW, the pelleted cells were lyophilized. The lyophilized yeast powder was dissolved in acetate buffer (pH 3.7) to a final concentration of 1 g/L and 0.01 g/L, and 125 µL was added to microcentrifuge tubes. To each sample, 20 µL 0.021 M NADPH and 50 µL patulin standard solution (5 µg/mL total) were added. The final volume was brought to 250 µL using acetate buffer. The final protein concentration was 5000 or 50 µg/mL. The samples were then incubated at 30°C for 20 min. The patulin was extracted 3 times with ethyl acetate as outlined in section 2.2.9. without the use of sodium carbonate and sodium sulfate. The samples were analyzed via HPLC analysis as outlined in section 2.2.10 in triplicate.

2.2.6. Patulin Extraction Using the AOAC Method

To each microcentrifuge tube, 1 mL of ethyl acetate was added and vortexed for 5 sec. The samples were centrifuged at 1000 x g for 5 min and 800 µL of the top (ethyl acetate) layer was transferred to a clean glass vial. Another 1 mL of ethyl acetate was added, the mixture was centrifuged, and a full 1 mL was removed and added to the previous extract. This procedure was repeated once more to obtain a final sample volume of 2.8 mL. The samples were cleaned up using 80 µL of sodium carbonate (1.5% w/v) and 70 mg of anhydrous sodium sulfate, then were vortexed for 5 sec. The samples were then centrifuged at 1000 x g for 5 min, and the supernatant was transferred to clean glass vials where they were kept under refrigerated conditions until analysis was performed (2.6 mL total).

2.2.7. Patulin Extraction Using Acid and Salt

A method to increase the patulin extracted from protein rich solutions was desired to more accurately assess patulin loss. As such, the use of acid and salt

were used to precipitate the protein out of solution, allowing for a greater degree of protein extraction (Castoria et al, 2011). After the patulin assay was carried out, 2.4 µL of 2N HCl was added to the sample mixture to drop the solution to pH 2 and was saturated with NaCl (until salt crystals are visible on the bottom of the microcentrifuge tube). After this, patulin extraction was carried out as outlined in 2.2.6.

2.2.8. Patulin Extraction with a Water Rinse

Patulin was extracted as outlined in section 2.2.7. This was followed by a 1 mL DDW wash to remove the salt dissolved in the ethyl acetate. The samples were centrifuged at 1000 x g for 5 min and 2.2 mL (to avoid taking up any of the solution at the interface) of the ethyl acetate layer was transferred to a clean vial. Finally, 70 mg anhydrous sodium sulfate was added, the mixture was vortex for 5 sec, and 2 mL of the ethyl acetate layer were transferred to a clean glass vial.

2.2.9. Patulin Extraction Combining the AOAC Method and Acid and Salt

The samples were initially prepared as outlined in 2.2.7, using the modified AOAC treatment that utilized acid and salt to precipitate the proteins out of solution. The samples were then dried down under a stream of nitrogen gas, and reconstituted using 250 µL of acetate buffer (pH 3.7). The extraction then followed the procedure as listed in 2.2.6 without the sodium carbonate rinse and the sodium sulfate treatment to remove some of the salt from sample.

2.2.10. Free Thiol Group Assay

One of the pathways cited in literature for chemical patulin degradation is through binding to free thiol groups, as can be found on cysteine. Samples, in triplicate,

of cysteine and lyophilized protein extract were dissolved individually in acetate buffer (pH 3.7) (to obtain a final concentration of 12.5 µg/mL for each sample in 1 mL total) and used in place of the crude protein extract and reacted with patulin (5 µg/mL) as outlined in section 2.2.4. Patulin was then extracted and analyzed following the methods outlined in sections 2.2.9. and 2.2.12. However, the aqueous layer was collected and lyophilized. Free sulfhydryl groups in the lyophilized samples were determined following the method outlined by Manderson, Hardman, and Lawrence (1999). Samples were each dissolved in 1 mL tris buffer (pH 8) and 10 µL DNTB solution (4 mg/mL), vortexed, and allowed to sit at room temperature for 15 min, at which point they were analyzed via spectrophotometric analysis at 412nm. The absorbance values were compared to a standard curve prepared using cysteine standards (Appendix C, Figure 25).

2.2.11. Glutathione Degradation of Patulin

To deduce whether patulin degradation was caused by glutathione extracted from the yeast cell, an assay was performed to see the potential degradation capabilities of glutathione at pH 7 and 3.7. Standard solutions of glutathione (1 mg/mL) were prepared using DDW and acetate buffer (pH 3.7). Solutions were incubated in triplicate with 5 µg/mL patulin, at 5:1, and 10:1 glutathione to patulin ratio (w/w). Samples were incubated at 30°C for 20 minutes and immediately placed on ice or boiled for 5 min before being placed on ice. An additional sample consisting of a 1:1 (w/w) ratio of glutathione to patulin was incubated at 37°C for 7 days in an effort to detect patulin degradation products as was previously reported by Schebb, Faber, and Maul (2009). Preparation for HPLC analysis was carried out using the method outlined in section 2.2.12.

2.2.12. Patulin Analysis Via HPLC

Extracted patulin samples in ethyl acetate were dried down under a nitrogen stream and reconstituted with 1 mL 1% acetic acid (pH 4). The samples were vortexed for 5 sec and passed through a 0.45 µm syringe filter. The samples were then placed in HPLC vials for analysis.

All analysis was carried out on 2 HPLC systems, both manufactured by Shimadzu Scientific Instruments. The first utilized 2 dual reciprocating plunger pumps (LC-20AT), a HPLC system controller (CBM-20A), a UV-VIS detector (monitoring at 276 nm) (SPD-20A), and a column oven (CTO-20A). The second utilized the same model of pumps, system controller, and column oven, with the addition of an auto injector (SIL-10AF), and a photo diode array detector unit (monitoring the wavelength range 220-350 nm) (SPD-M20A) instead of the UV-VIS detector. The column used was a 250 mm x 4.6 mm i.d., 5 µm, YMC-Pack ODS-AM-12S RP P-C18 column. A 20 mm x 4 mm guard column of the same material (YMC-Pack ODS AM) was used. The column was kept at 38°C during the analytical run.

A linear HPLC gradient at a flow rate of 1.2 mL/min was used. Solvent A was DDW, and solvent B was acetonitrile, with both containing 0.1% (v/v) glacial acetic acid. The initial concentration was 10% solvent B, which was held constant for 10 min. The concentration was then linearly increased to 50% over the course of 5 min, held constant for 5 min, and then decreased to 10% in 2 min. The concentration was then held at 10% for 13 min before the next sample could be analyzed. Absorption was monitored at 276 nm. Patulin content was quantified using a standard curve.

2.2.13. Patulin Standard Curve Preparation

Patulin standard solutions were prepared in triplicate at concentrations of 0.1, 0.5, 1, 2.5, 5, and 7.5 ppm. Standard solutions were prepared using 200 µg/mL patulin stock solution dried down under a nitrogen stream and solublized in 1 mL

1% acetic acid solution (pH 4). Samples were analyzed via HPLC using the method outlined in 2.2.10. A standard curve was generated using peak areas analyzed with Shimadzu software (version 7.4). A standard curve can be found in Appendix A (Figure 17).

2.2.14. Glutathione Adduct Detection Via LC-MS

Samples were prepared for HPLC analysis as outlined in section 2.2.12. Separation was carried out using a Shimadzu HPLC unit consisting of a column oven (CT0-10A), a low pressure gradient valve (FCV-10AL), high performance pump (LC-10AD), a UV-Vis detector (SPD-10AV), and system controller (SCL-10Avp). The column was the same as that used in section 2.2.12. Samples were analyzed on a Waters Micromass ZQ mass detector using electrospray ionization coupled with a quadrupole mass analyzer operating in positive ion mode. The m/z values monitored were 155, 177, 179, 194, 233, 287, 308, 315, 444, 462, 476, 484, 516, 530, 560, 613, and 615 which correlated to known patulin-glutathione degradation products and product fragments (Schebb et al, 2009). A range of 110-700 m/z was also monitored over the course of 1 sec, with 0.1 sec interscan delay. The parameters used were a capillary voltage of 4.2 kV, a cone voltage of 15 V, source temperature set to 140°C, resolution temperature set to 400°C, desolvation flow rate set at 600 L/hr, and cone flow rate set at 100 L/hr. Separation was achieved using a gradient of 99.9:01 DDW:acetic acid (solvent A) acetonitrile:acetic acid (solvent B). The flow rate was set to 0.5 mL/min at 5% solvent B for 30 min.

2.2.15. Statistical Analysis

Analysis of variance (ANOVA) was carried out using SPSS. The level of significance (P-value) was set to 0.05; separation of the means were carried out using the Tukey-Kramer honestly significant difference test.

3. Results and Discussion

3.1. Effect of Patulin Extraction Methods on Patulin Recovery

Patulin extraction via the AOAC method in protein-rich solutions was inadequate in accurately quantifying patulin content (Figure 4). The use of acid and salt to precipitate the protein yielded the highest recovery of patulin from samples treated with the protein extract. However, percent recovery of patulin from patulin control samples was not significantly different ($P \leq 0.05$) when following the acid and salt method from that observed when the AOAC method was followed. The use of a DDW rinse after the acid and salt treatment resulted in the lowest recovery of patulin. Percent patulin recovery from both the patulin control and the protein control obtained after the acid and salt extraction method were not significantly ($P \leq 0.05$) different from that obtained when the acid and salt method and the AOAC method were used in tandem.

While the AOAC method yielded the highest percent recovery of patulin from the patulin control samples, it was suboptimal in quantifying patulin in solutions high in protein. The `AOAC method's low yield is attributed to the fact that the method was designed for extracting patulin from a media that is primarily water and carbohydrates, such as apple juice. Being a partially non-polar compound, patulin interacts with the hydrophobic moiety of the protein making it hard to be released into the extraction solvent. Precipitating the protein with salt and acid resulted in an effective release of patulin into the extraction solvent, and therefore enhanced recovery. However, the excess salt present in the ethyl acetate layer resulted in increased back pressure on the HPLC system, which lead to mechanical complications. The DDW wash removed the excess salt, but greatly impaired patulin recovery, making it a poor choice for accurate quantification. The low patulin recovery from the method utilizing a DDW rinse step is likely due to the amphiphilic nature of patulin, causing much of it to be lost in the aqueous layer of the DDW wash before analysis. A combination of the acid

and salt precipitation of the proteins in tandem with the AOAC method resulted in the best patulin recovery while negating the harmful side effects associated with the acid and salt extraction method. The salt dissolved in the ethyl acetate from the initial extraction remained in the aqueous layer of the second extraction. Accordingly, the combined method of acid and salt precipitation of the proteins in tandem with the AOAC method was chosen as the ideal method of patulin extraction for the study.

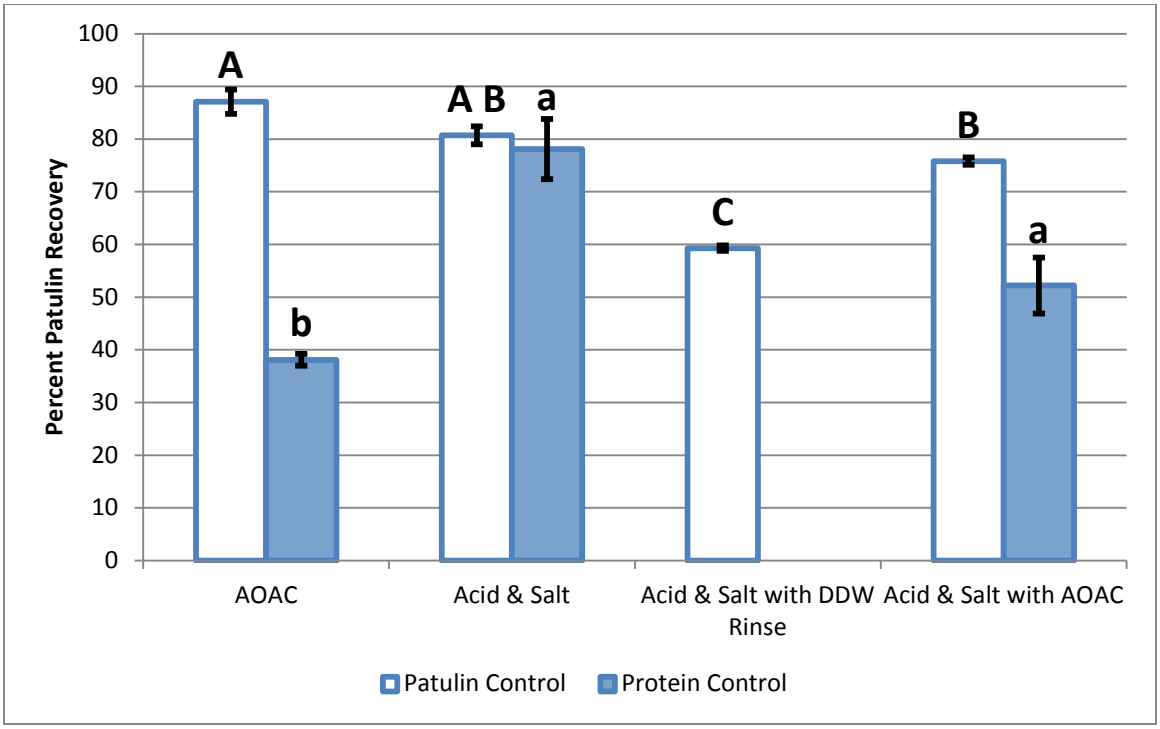

Figure 4: Average percent patulin recovery (n=2-6) in samples extracted using the AOAC method, acid and salt, acid and salt washed with DDW, and acid and salt followed by the AOAC method utilizing 62.5 µg crude protein extract and 5 ppm patulin without incubation. Bars with same capital letter represent patulin control recovery values that are not significantly different. Bars with the same lower case letter represent patulin recovery from protein control values that are not significantly different. Difference was determined based on the Tukey-Kramer honestly significant difference test ($P \leq 0.05$).

3.2. Effect of Yeast Growth Days and Storage Time on Patulin Degradation Activity

Protein extracted from yeast samples after incubation at room temperature showed increasing patulin degrading activity up to 6 days of growth, after which there was no statistically significant ($P \leq 0.05$) change in the patulin degrading activity (Figure 5). Protein extracted from yeast incubated at room temperature followed by storage at 4°C showed equal or greater patulin degrading activity when compared to that extracted from yeast grown only at room temperature for the same number of days. Protein extracted from yeast grown at room temperature for 4, 6, and 8 days and subsequently stored at 4°C showed patulin degrading activity not significantly different than that of the protein extracted from yeast grown for 6 and 8 days at room temperature. For the yeast grown at room temperature for 2 and 4 days, additional storage at 4°C resulted in protein extracts with significantly ($P \leq 0.05$) higher patulin degrading activity. The observed increase in activity indirectly confirmed that additional days of incubation (even at the lower temperature) resulted in continued growth of the yeast.

Based on these observations, protein extraction after 6 days of yeast growth at room temperature was determined to be sufficient to achieve optimal patulin degradation by the protein extracts. Subsequent storage of the yeast suspensions at 4°C was acceptable without any repercussions to the quality of the protein extracted.

Stinson et al (1978) found that yeast (*S. cerevisiae*) fermentation at room temperature for up to 14 days was capable of decreasing patulin content up to 99.5% (from 15 µg/mL). Strains of *R. glutinis*, *C. laurentii*, *S. cerevisiae*, and *A. pullulans* were found to decrease high levels of patulin (100-500 µg/mL) up to 90% after 10 days of incubation at room temperature (Castoria et al, 2005). Another study found that *P. ohmeri* strain 158 was capable of almost complete patulin loss (from 8.92 µg/mL) after 5 days of incubation at room temperature

(Coelho et al, 2008). Finally, *R. kratochvilovae* (62-121) resulted in 70% patulin reduction (from 3 µg/mL) after 3 days of incubation at room temperature (Linghu, 2013). Through optimizing the yeast growth methodology, the likelihood of extracting active proteins capable of degrading patulin consistently is increased.

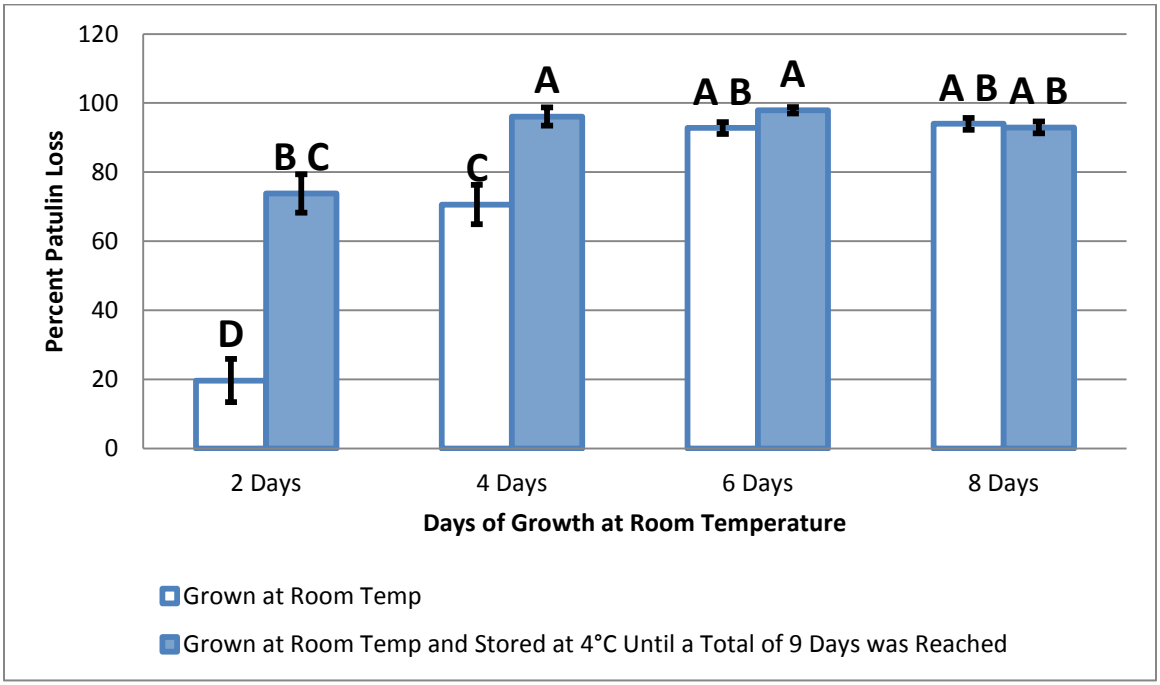

Figure 5. Percent patulin loss upon incubation of patulin (5 µg/mL) for 20 min at 30°C with protein extracts (30 µg/mL) from yeast grown for 2-8 days at room temperature, or being stored for additional days at 4°C until a total of 9 days have passed since the initial inoculation (i.e. 2 days of growth at room temperature with 7 days of storage at 4°C, 4 days of growth at room temperature and 5 days storage at 4°C, 6 days of growth at room temperature and 3 days storage at 4°C, and 8 days of growth at room temperature and 1 day of storage at 4°C). Error bars represent standard error (n=2-5). Bars with the same uppercase letter represent means that are not significantly different. Significant difference was assessed based on the Tukey-Kramer honestly significant difference test ($P \leq 0.05$).

3.3. Effect of Boiling to Terminate the Potential Enzymatic Reaction on Patulin Degradation

The patulin assay was run with protein extracts followed by boiling for 5 min or by placing on ice to test if boiling further affects patulin loss via chemical degradation pathways. Boiling was intended to terminate potential enzymatic degradation of patulin by denaturing the enzyme. Previous studies have looked at patulin stability under thermal processing conditions and have reported 15 to 80% decrease in patulin concentration in apple juice (Scott and Somers, 1968; Wheeler et al, 1987; Raiola et al, 2012). However, much of the decrease in patulin (up to 80%) was observed under more extreme heating conditions such as 80°C for 20 min and 90°C for 10 min (Scott and Somers, 1968; Wheeler et al, 1987). The acidic pH was chosen to mimic conditions similar to apple juice and apple cider, and to discourage chemical degradation believed to be favored at alkaline pH values, namely SH-adduct formation (Fliege and Metzler, 1999). While ideally the acidic pH would inhibit chemical degradation of patulin, the elevated temperature seemed to have driven the reaction forward regardless (Figure 6).

Samples of protein extract and patulin that were boiled showed a significantly ($P \leq 0.05$) higher percent patulin loss than those placed on ice at the end of the designated reaction time. Boiling the samples caused additional patulin loss beyond what was observed from the 20 min incubation at 30°C (Figure 6). A significantly higher ($P \leq 0.05$) percent patulin loss was noted in the samples incubated for 0 min when boiled than when directly put on ice. The differences observed in samples that were boiled compared to those placed on ice could be attributed to patulin adduct formation assuming that sulfhydryl groups are present in the protein extract. Schebb et al (2009) identified several patulin-glutathione adducts when patulin was incubated with glutathione (thiol rich) for up to 48 hours at pH 7.4. Likewise, Fliege and Metzler (1999) noted several patulin

degradation products, and possible product intermediates, after 250 hours of incubation at pH 7.4 of patulin with glutathione at a ratio of 1:5. Both of the aforementioned studies only investigated patulin-glutathione adduct formation at neutral pH, and after days of incubation.

Despite the fact that adduct formation is discouraged at acidic pH, our results showed that boiling might have driven the reaction forward resulting in 15% additional patulin loss when compared to non-boiled samples. However, the percent patulin loss was not as pronounced as was observed at pH 7 (Linghu, 2013). In the study by Lingu (2013), patulin loss was close to 100% in samples of patulin and protein extract from *R.* kratochvilovae (62-121) boiled at pH 7.

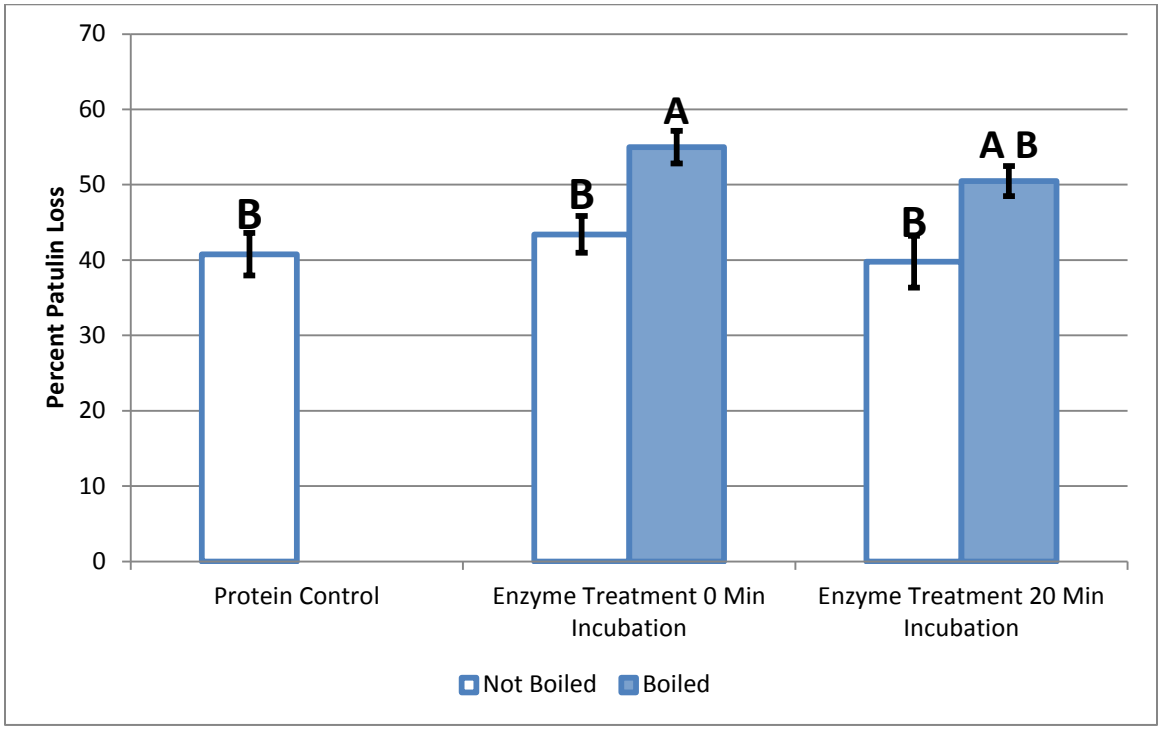

Figure 6. Percent patulin loss upon incubation of protein extracts from yeast (30 µg/mL) with patulin (5 µg/mL) for 20 min at 30°C. Reaction was terminated by either boiling the sample, or placing it on ice followed by an ethyl acetate extraction. Error bars represent standard error values (n=2). Bars with the same uppercase letter represents means that are not significantly different. Significant

difference was assessed based on the Tukey-Kramer honestly significant difference test ($P \leq 0.05$).

The increased degradation of patulin under boiled conditions is in agreement with findings by Scott and Somers (1968), Raiola et al (2012), and Wheeler et al (1987) who reported up to 80% patulin degradation in apple juice during high heat treatments (80-90°C). In comparison to their findings, patulin degradation attributed to the high heat treatment (boiling in this case) was marginally higher, yet statistically significant, compared to the samples that were not boiled. From these observations it was deemed appropriate to forego boiling the samples to terminate the potential enzymatic degradation of patulin and instead place them on ice to stop the reaction. Foregoing the boiling step would eliminate chemical degradation pathways in order to more accurately characterize any potential enzymatic degradation.

3.4. Patulin Reduction Due to Free Thiol Groups

Free thiol groups in both the protein extract and pure cysteine were measured to assess the formation of patulin adducts. Cysteine was used to measure the loss in what would be an ideal environment with a plentiful supply of free thiol groups. The free thiol groups of both the protein extract and cysteine were measured before and after incubation with patulin for 20 min at 30°C. Despite the fact that patulin's reactivity with free thiol groups at acidic pH is not favored, evidence of thiol group blockage was seen (Figure 7), without additional boiling. Close to 50% reduction in free thiol content of the protein extract over the course of the assay was observed. This decrease in free thiol groups of the protein extract (4.7 µM) corresponded to a theoretical drop in patulin content (from 5 ppm) of 0.72 µg/mL (14.4%) based on the molar mass of patulin (Equation 1) and the fact that one thiol group can react with one molecule of patulin (Figure 3). When the patulin extract was analyzed via HPLC, content dropped by 1.04 µg/mL; an amount comparable to the calculated theoretical drop. By comparison, the loss in

thiol groups from the samples reacted with cysteine corresponds to a theoretical patulin loss of 0.97 µg/mL; compared to an actual decrease of 0.37 µg/mL.

Equation 1:

$$Theoretical\ Patulin\ Drop\ (\mu g/mL) = \frac{decrease\ in\ \mu mol\ thiol\ groups}{1000\ mL} \times \frac{154.12\ g\ patulin}{mol\ patulin}$$

There was significant (P≤0.05) thiol blockage in both the patulin samples incubated with cysteine and those incubated with the freeze dried protein. The loss of free sulfhydryl groups was most pronounced in the samples incubated with cysteine. However, the patulin loss did not follow the same trend observed for the patulin incubated with the freeze dried protein, which showed a significantly higher percent of patulin loss after incubation at 30°C for 20 min (Figure 9).

The significantly higher (P≤0.05) loss in thiol group content and lower patulin loss of samples incubated with cysteine can be attributed to multiple sulfhydryl adduct formation during incubation. The reaction of one patulin molecule with multiple thiol groups is more likely with unsterically hindered molecules such as cysteine, explaining the greater loss of thiol groups. Schebb et al (2009) identified several products from 1 to 3 glutathione (a tripeptide possessing a cysteine residue) molecules reacting with a single molecule of patulin (Figure 9).

Reiss (1976) observed almost complete patulin loss after 8 days of incubation with cysteine (121 µg/mL) at pH 5 and room temperature (1000:1 cysteine to patulin on a molar basis). The previous study did not, however, measure free thiol groups, and accordingly did not distinguish between loss due to thiol adduct formation and loss due to patulin degradation over time given the pH and temperature of incubation. Our results follow a similar trend but go a step further and note the loss of thiol groups in relation to the loss of patulin. Furthermore, the lower pH level (3.7 versus 5), shorter reaction time (20 min versus 8 days), and

lower cysteine amount (333:1 cysteine to patulin on a molar basis) contributed to the lower patulin loss observed in our study.

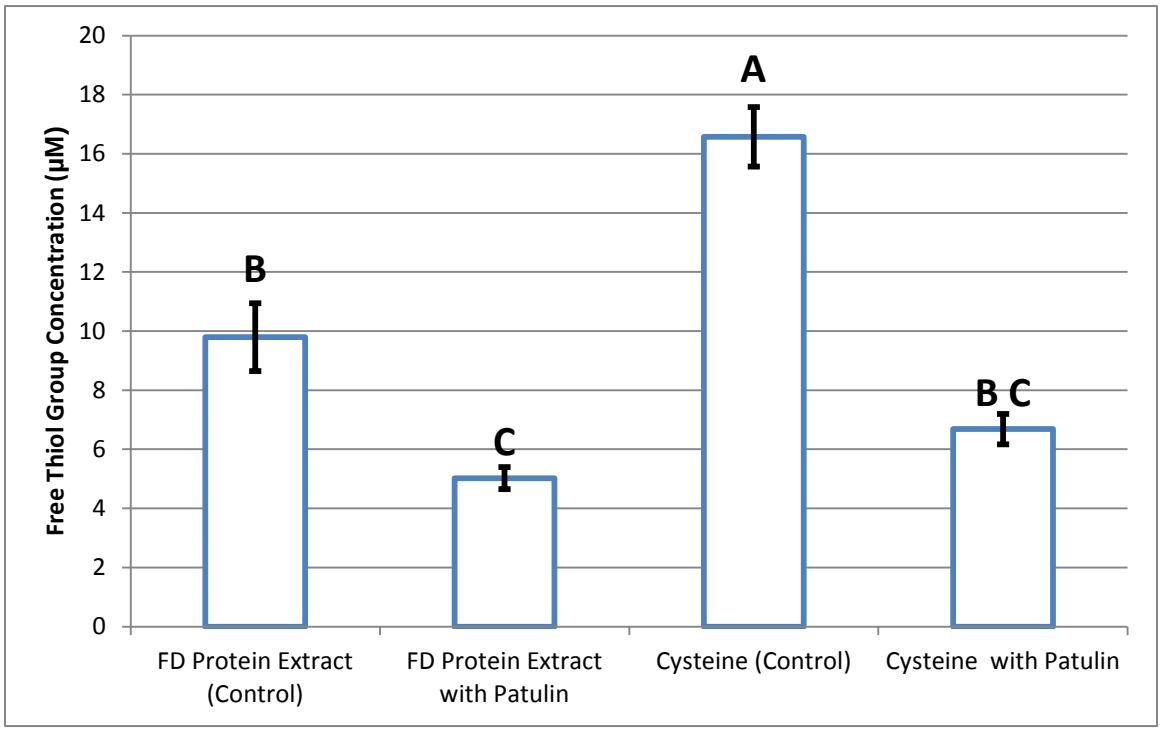

Figure 7. Free thiol group content of samples of freeze dried (FD) protein extract and cysteine (12.5 µg/mL) after incubation with and without patulin (5 µg/mL) at 30°C for 20 min. Bars with the same capital letter represent means that are not significantly different (P≤0.05). Error bars were determined based on standard error values. Significant difference was assessed based on the Tukey-Kramer honestly significant difference test.

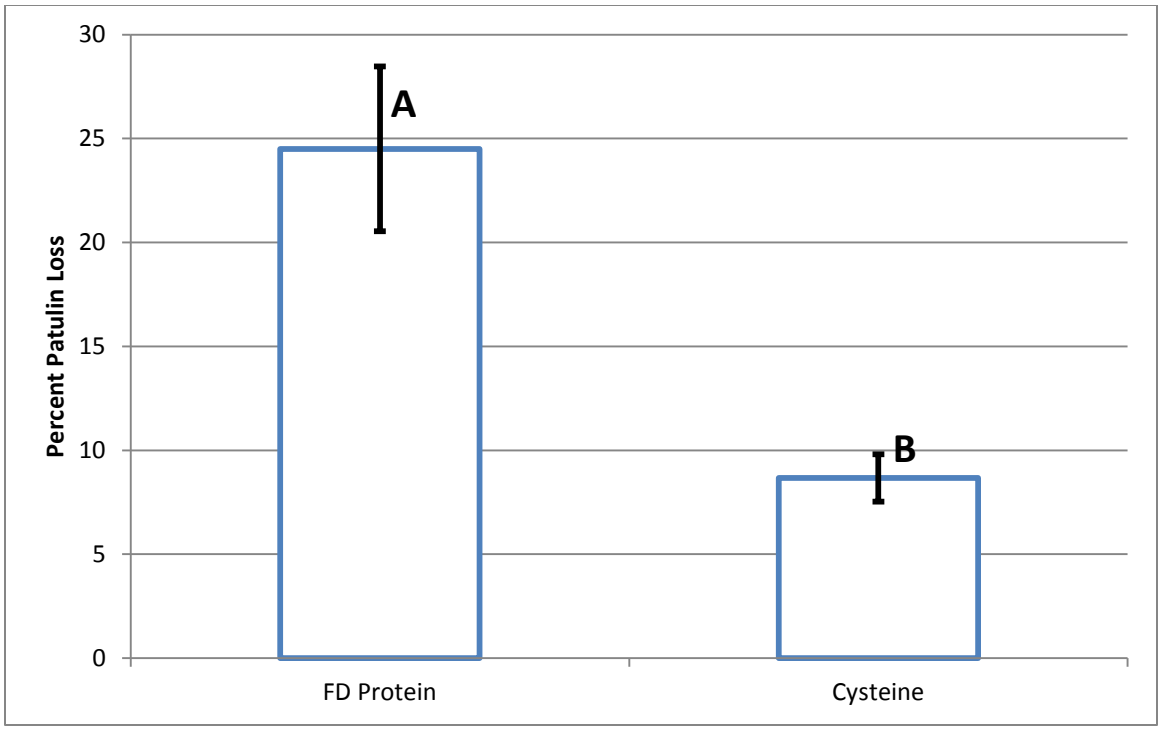

Figure 8. Percent patulin loss from samples incubated with cysteine (12.5 µg/mL) and freeze dried (FD) protein extract (12.5 µg/mL) after incubation with patulin (5 µg/mL) at 30°C for 20 min. Bars with the same capital letter represent means that are not significantly different (P≤0.05). Error bars were determined based on standard error values (n=3). Significant difference was assessed based on the Tukey-Kramer honestly significant difference test.

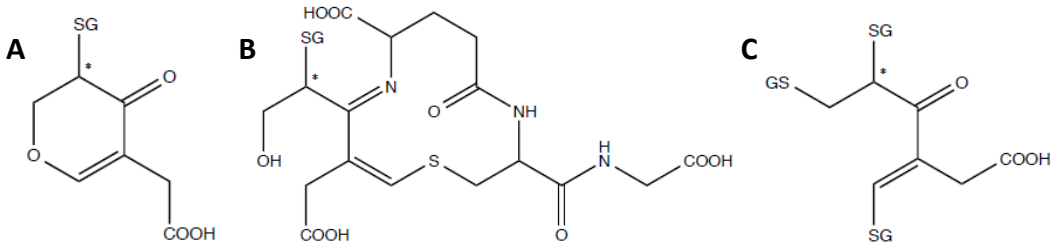

Figure 9. Patulin-glutathione adduct products with A) 1; B) 2; and C) 3 glutathione molecules per patulin molecule (Schebb et al, 2009)

3.5. Patulin Reduction Due to Glutathione

Patulin incubated with glutathione has shown highest degradation at pH 7 for both samples incubated at 30°C for 20 min and those boiled after incubation. Samples of glutathione incubated with patulin in ratios of 5:1 and 10:1 (w/w) at pH 7 showed significantly higher (P≤0.05) patulin loss than those incubated at pH 3.7. As discussed in section 3.5., this is expected from literature values due to the prevalence of adduct formation under alkaline pH conditions. At pH 3.7 there was no significant difference between the patulin controls and those treated with glutathione, with the exception of the boiled sample of 10:1 glutathione to patulin ratio (Figure 10) that resulted in 20% patulin loss.

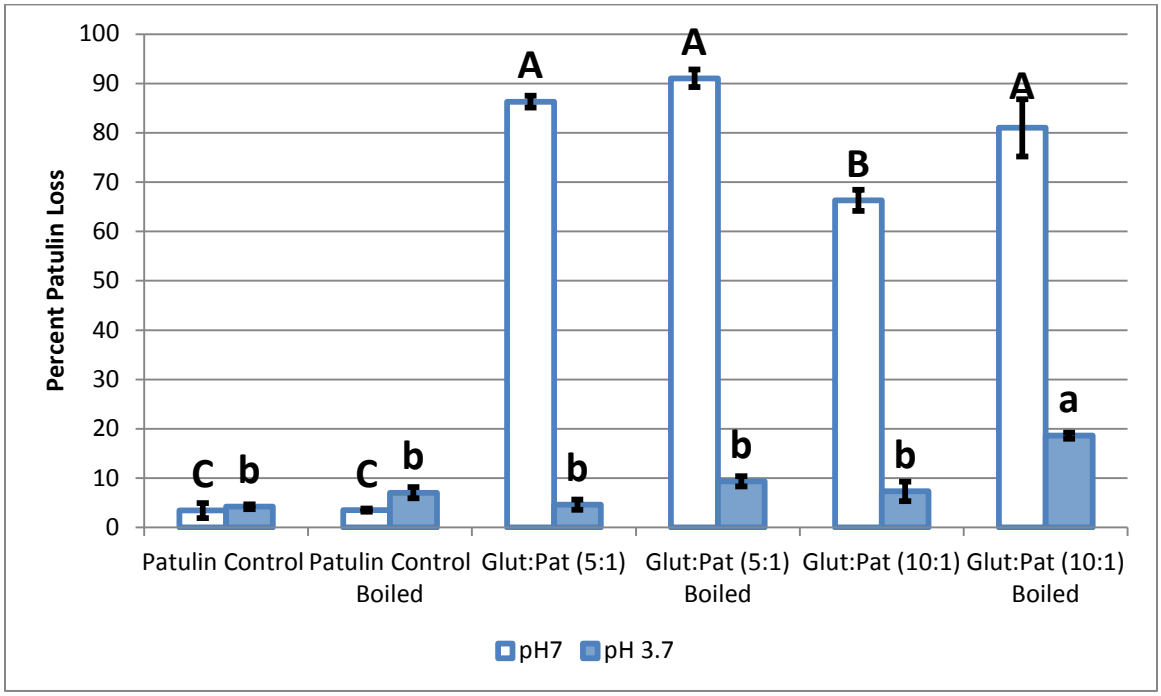

Figure 10. Percent patulin loss upon incubation of patulin (5 µg/mL) at 30°C for 20 min with glutathione (25 and 50 µg/mL) at w:w ratios of 1:5 and 1:10 (patulin:glutathione) with and without boiling for 5 min. Bars with the same capital letter represent means that are not significantly different (P≤0.05) amongst pH 7 samples. Bars with the same lower case letter represent means that are not significantly different amongst pH 3.7 samples. Error bars were determined based on standard error values (n=3). Significant difference was assessed based on the Tukey-Kramer honestly significant difference test.

The high reactivity of glutathione both with and without boiling the sample before analysis was expected at pH 7. Lingu (2013) found that there was large loss in patulin (up to 100%) in samples incubated with crude protein extract from *R. kratochvilovae* (62-121) at neutral pH. In the previous study, loss was assumed to be the result of adduct formation with free thiol groups such as those found on glutathione (a cellular component that is found in yeast). The patulin reduction observed at pH 3.7 (compared to that observed at pH 7) explain the observation of the previous study when patulin degradation at pH 3.7 was only 56% upon boiling. The higher percent loss observed previously at pH 3.7 compared to our results could be attributed to more than one mechanism for degradation by the protein extract.

At pH 3.7, only the sample boiled with glutathione at a 1:10 ratio showed significantly higher patulin loss. This evidence of glutathione's ability to degrade patulin at acidic pH, where the thiol adduct formation is supposedly discouraged, might explain some of the degradation activity (at least 20%) of the protein extracts. Glutathione has been linked to detoxification mechanisms in yeast cells at acidic pH (Thorsen et al, 2012). In the previous study, glutathione excreted from yeast cells bound to arsenic, decontaminating the extracellular environment. Linghu's (2013) found that incubation with NADPH caused a greater degree of patulin loss which could be explained by the presence of glutathione reductase. Glutathione reductase is an enzyme found in the same environment as glutathione that helps cleave disulfide bonds between glutathione molecules with the use of NADPH (Carlberg and Mannervik, 1977). In this case, the NADPH would allow for the release of more glutathione, increasing the amount in solution capable of forming adducts with patulin.

The reaction with glutathione in this study gave rise to several additional chromatographic peaks (retention time 2-5 min), but only in samples incubated in excess of the normal 20 min assay (in this case 1 week) (Figure 11). The appearance of the additional peaks indicated the possible production of patulin-

glutathione adducts possessing different degrees of hydrophobicity than the native patulin molecule. In samples treated for the allotted 20 min at 30°C, despite a high degree of patulin loss, no peaks other than the patulin peak was observed (Figure 26, Appendix K). It was not until the samples were analyzed via LC-MS that one patulin-glutathione product was identified (Figure 12). The particular peak identified was only observed through MS analysis, while the concurrent UV analysis at 276 nm was devoid of the corresponding peak. The absent peak at 276 nm can be explained by either the low concentration of the product, or the structural binding of patulin to glutathione that may have caused a configuration change that resulted in a product with no UV absorptivity at 276 nm. Evidence for the later hypothesis is that the glutathione, however plentiful it was in the sample, was never observed at 276 nm during HPLC analysis.

Three main molecular ions were identified in the MS scan, a patulin peak (155 m/z) (in conjunction with a patulin-sodium adduct, 179 m/z), a glutathione peak (308 m/z) (which bore traces of glutathione-glutathione complexes from both disulfide bond formation, 613 m/z, and non-covalent linkages, 615 m/z), and a patulin-glutathione adduct from the addition of 1 glutathione molecule to 1 patulin molecule (462 m/z). Schebb et al (2009) observed this same product along with several others from the addition of 1, 2, or 3 glutathione molecules reacting with a single patulin molecule at pH 7 (Figure 9). The authors were able to identify a greater number of products through use of MS-MS analysis, elucidating several structural components from the various products observed. Identification of more products was achieved due to the longer incubation times employed. Peak isolation was also achieved by the authors using a low flow rate (50 µL/min) and a biochemical detection instrument (a fluorescent detection method based on glutathione-S-transferase inhibition) in conjunction with the HPLC system, allowing for more sensitive detection of the patulin-degradation products.

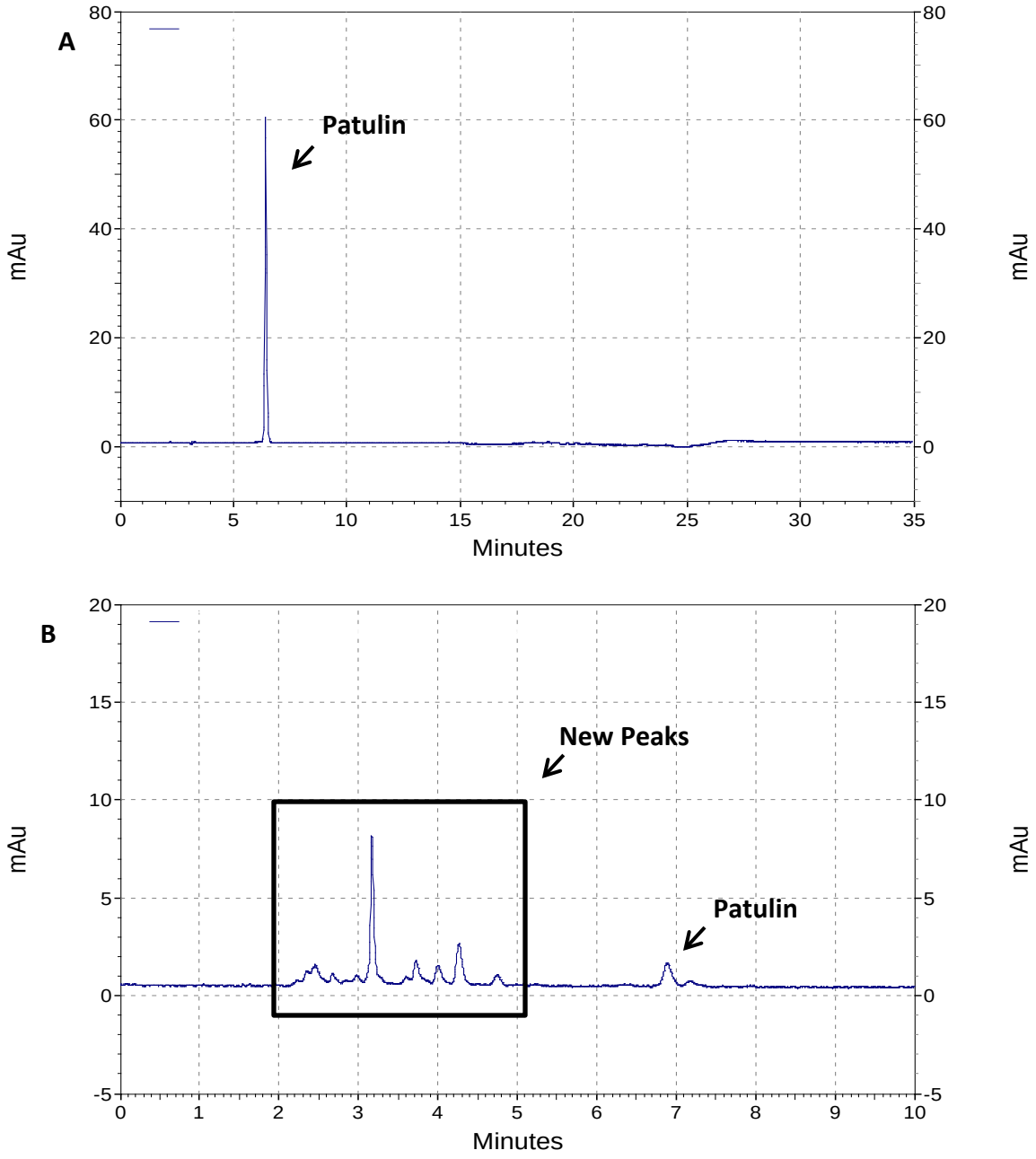

Figure 11. HPLC chromatograms of A) patulin control (5 µg/mL) incubated for 20 min at 30°C; B) Glutatione (25 µg/mL) incubated with patulin (5 µg/mL) for 7 days at 37°C, noting the greatly reduced patulin peak and the appearance of several small peaks between 2 and 5 min.

Further analysis of patulin incubated with glutathione at pH 3.7 was carried out, yet yielded no appreciable molecular ions due to the limited reactivity of patulin

with free thiol groups at acidic pH, and by extension limited product formation (Figure 13). Despite the incomplete product identification under both conditions, our results demonstrated that patulin degradation can be initiated by glutathione at both pH 7 and 3.7, and the identified patulin-glutathione degradation product strongly supports one method potentially utilized by yeast *in vivo* to degrade patulin.

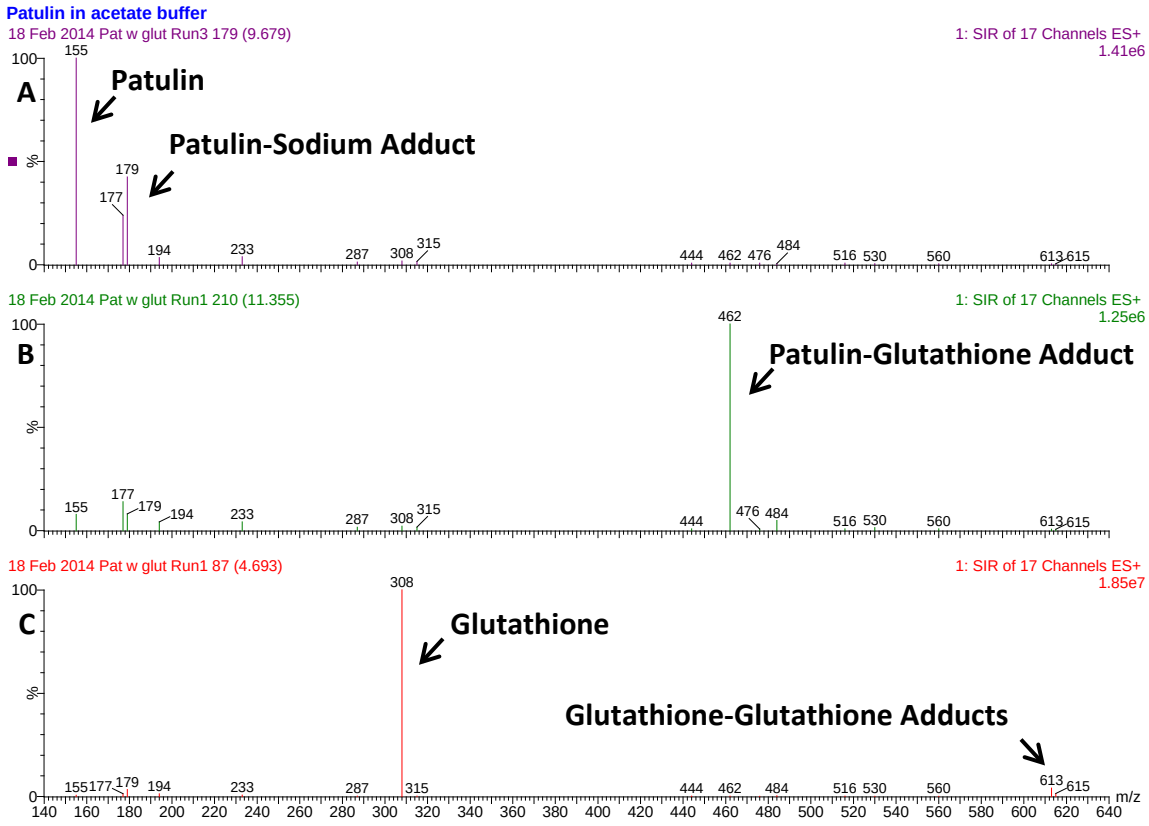

Figure 12: Mass spectra (in positive ion mode) of patulin (5 µg/mL) incubated with glutathione (50 µg/mL) at 30°C for 20 min, and then boiled. A) Patulin (155 m/z) and patulin-sodium adducts (177/179 m/z); B) Patulin-glutathione adduct (462 m/z); C) Glutathione (308 m/z) and trace amounts of glutathione-glutathione coupling though disulfide bond formation (613 m/z) and glutathione-glutathione coupling through non-covalent bond (615 m/z).

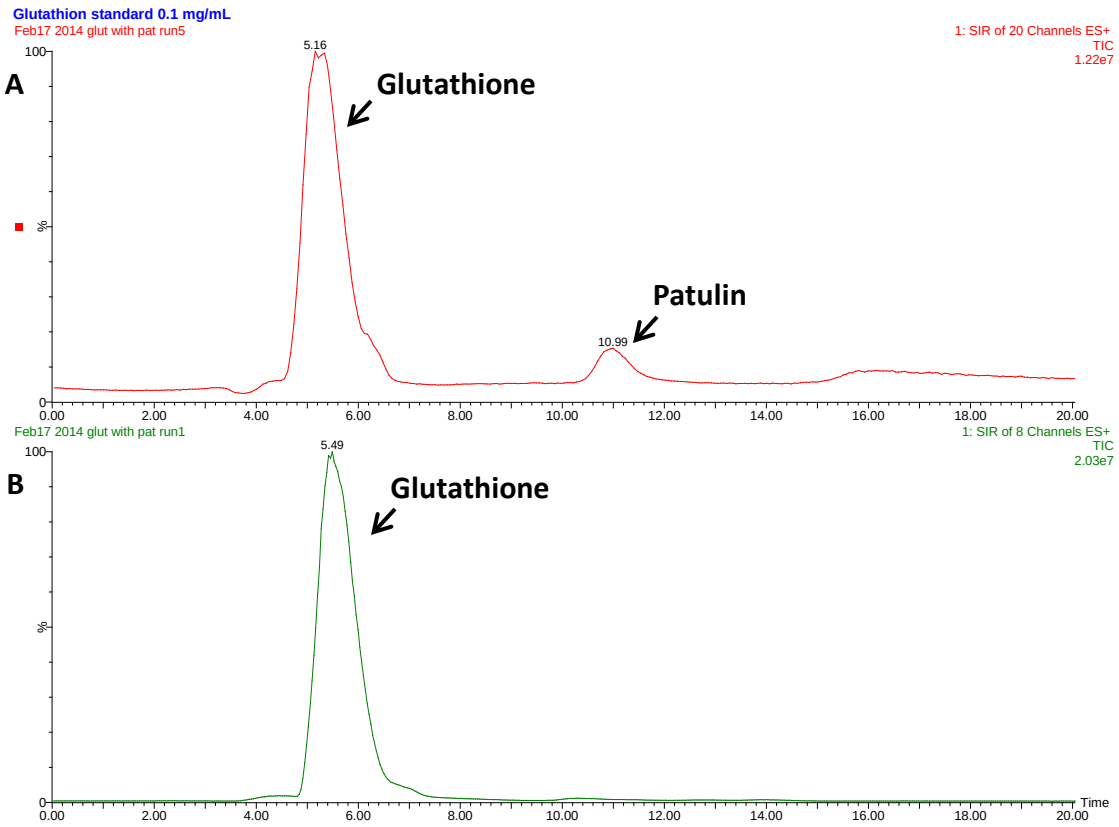

Figure 13: Mass spectra of patulin (5 µg/mL) incubated at pH 3.7 with glutathione, A) 25 µg/mL and B) 50 µg/mL for 20 min at 30°C. Chromatograms show the loss of patulin (eluting at 11 min) as the glutathione concentration increased without the observation of a degradation product.

3.6. Patulin Degradation Activity of Crude Protein Extract: Is it Enzymatic?

Throughout the duration of the work (over two years), yeast had been grown and the protein extracted several times, generating widely different results of patulin degrading activity (Table 1). Of the different protein extracts, several have shown promise for patulin-degrading activity. Of the protein extracts that exhibited patulin degrading activity, some, such as the protein extracted on July 23, 2013 resulted in greater than 90% reduction. The protein extracted on June 16, 2013,

however, showed no significant reduction in patulin. On the other hand, the protein extracted on June 6, 2012 showed moderate patulin degradation activity. Between the protein extracts, there is no discernable trend pertaining to the activity and the amount of yeast cells grown, nor the concentration of the protein extract.

Table 1: Protein extraction dates, weight of yeast cells, protein concentration of the extract, and patulin degradation activity.

Extraction Date	Yeast Weight (g/100 mL LB)	Protein Concentration (µg/µL)	Protein Activity
June 6, 2012	1.28	1.56	Moderate[1]
Sept. 27, 2012	2.07	0.39	Moderate[1]
Feb. 5, 2013	--	0.58	Yes[2]
May 20, 2013	1.58	2.28	Limited[3]
June 16, 2013	1.69	--	No[4]
June 26, 2013	0.15; 0.54; 0.86; 0.82	1.57; 0.76; 0.24; --	Yes[2]
July 9, 2013	0.26; 0.47; 1.25; 0.81	1.51;1.32; 0.98; 1.1	Yes[2]
July 23, 2013	8.76	0.71	Yes[2]
Aug. 13, 2013	--	0.98	Limited[3]
Oct. 1, 2013	--	1.3	Limited[3]
Oct. 25, 2013	1.15	0.97	No[4]
Nov. 12, 2013	1.42	0.71	No[4]
Nov. 19, 2013	1.54	0.78	No[4]

[1] Moderate signifies 50-75% degradation

[2] Yes signifies 75% reduction in patulin content or greater

[3] Limited signifies between 25-50% patulin reduction after treatment

[4] No signifies Less than 25% reduction after treatment with the active protein

*Protein were deemed inactive if the patulin loss was observed to be indistinguishable in the non-denatured protein treatments compared to the control.

For each protein extract, patulin loss was assessed using patulin incubated with active protein (protein extract not boiled prior to the assay) for 0 and 20 min (enzyme treatments) and inactivated protein (protein extract boiled to denature

the protein prior to running the assay) for 0 and/or 20 min (protein control). Incubation occurred at 30°C without subsequent boiling. For protein control, the protein extract was boiled prior to the addition of patulin to denature any potential patulin-degrading enzyme(s) present in the extract. Incubation of patulin with active and inactivated protein allowed for the prediction of three possible mechanisms of patulin degradation (Figure 14).

The extract from June 6, 2012 showed significantly higher ($P \leq 0.05$) percent patulin loss when patulin was incubated with active protein for 20 min compared to 0 min. The higher patulin loss in the incubated sample with active protein suggested that patulin degradation could have been enzymatic. However, the percent loss was only around 40% overall.

The extract from June 26, 2013 showed no significant ($P \leq 0.05$) difference between either sample with the active protein, yet significantly more patulin loss than the protein control that had been incubated. The significantly lower ($P \leq 0.05$) loss in the protein control suggests that the active protein plays a role in the patulin loss to a greater degree than the denatured one. It had previously been established that patulin can react with free thiol groups in acidic solution, however it is possible that the boiling step used to denature the protein created some disulfide bond formation. With fewer thiol groups available to interact with patulin, chemical degradation may be impaired. By comparison, the active protein would be comparatively optimal for the chemical degradation of patulin, presumably through thiol adduct formation. Another degradation mechanism could be enzymatic, yet this cannot be confirmed since no significant difference was seen in patulin loss between zero and 20 min incubation of patulin with the active protein.

The extract obtained on July 23, 2013 showed significantly ($P \leq 0.05$) higher patulin loss (100%) in the active protein sample incubated for 20 min than both the incubated and non-incubated protein controls. Enzymatic degradation could also be assumed in this case. There was a trend showing that incubation of

patulin with the active protein for 20 minutes resulted in a greater patulin loss than that incubated for zero min, however, not statistically significant. The high degree of patulin loss in all samples regardless of whether the protein was previously boiled, suggested that chemical degradation by thiol-adduct formation could have contributed partially to patulin loss, at least 20% as shown in section 3.5. Results indicated that both chemical and enzymatic reactions occurred together, thus making up the overall greater degree of patulin loss.

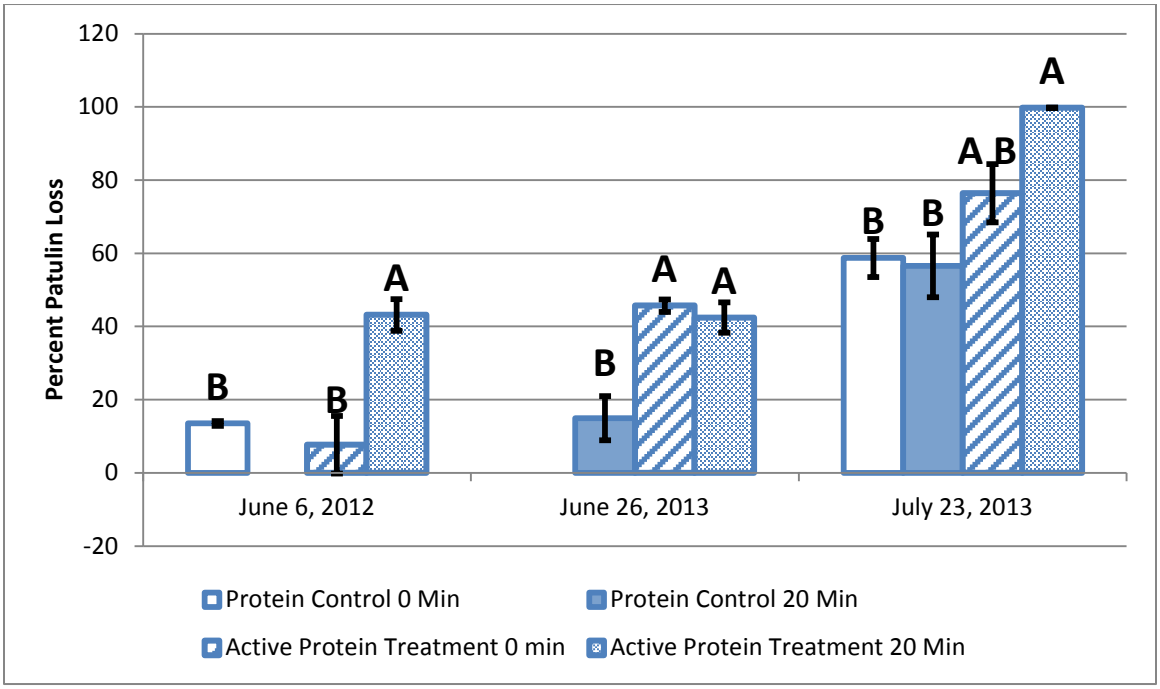

Figure 14. Percent patulin loss obtained with different protein extracts (12.5 µg/mL) incubated with patulin (5 µg/mL) for 20 min at 30°C. Bars with the same capital letter represent means that are not significantly different (P≤0.05) within the same batch of protein extract (n=2 to 5). Error bars were determined based on standard error values. Significant difference was assessed based on the Tukey-Kramer honestly significant difference test.

The diverse range of patulin degradation activity is a cause for concern. The ultimate goal of performing a study on enzyme/reaction kinetics could not be realized due to this variability (Figure 15). Some extracts showed some patulin loss, such as those extracted from June 6, 2012 to June 6, 2013. Others showed

substantial patulin degrading activity as seen between July 9, 2013 and July 23, 2013. Finally other extracts, like the one from October 25, 2013 showed virtually no patulin degrading activity. Due to this variability it has not been possible to perform the kinetics work needed to confirm enzymatic degradation activity of the protein extract from yeast.

Aguilar-Uscanga and François (2003) found variations in cell wall mass, enzyme lysis susceptibility, and cell wall polysaccharide content based on growth media, temperature, pH, and carbohydrate source. Their study offered some insight on a possible sources of variation. The pH of the media, growth media, and carbohydrate source remained constant amongst all protein extracts, and should not have contributed to the differentiation. However, temperature variations were shown to cause as much as a 50% increase in mannan cell wall polysaccharides and decreased the cells susceptibility to lysis at higher temperatures. If the temperature of the growth environment changes throughout the year, due to variation in heating/cooling of the labs, it is likely that the physiological changes in the yeast would lead not only to different protein concentration in the extracts, but to different protein constitutions entirely.

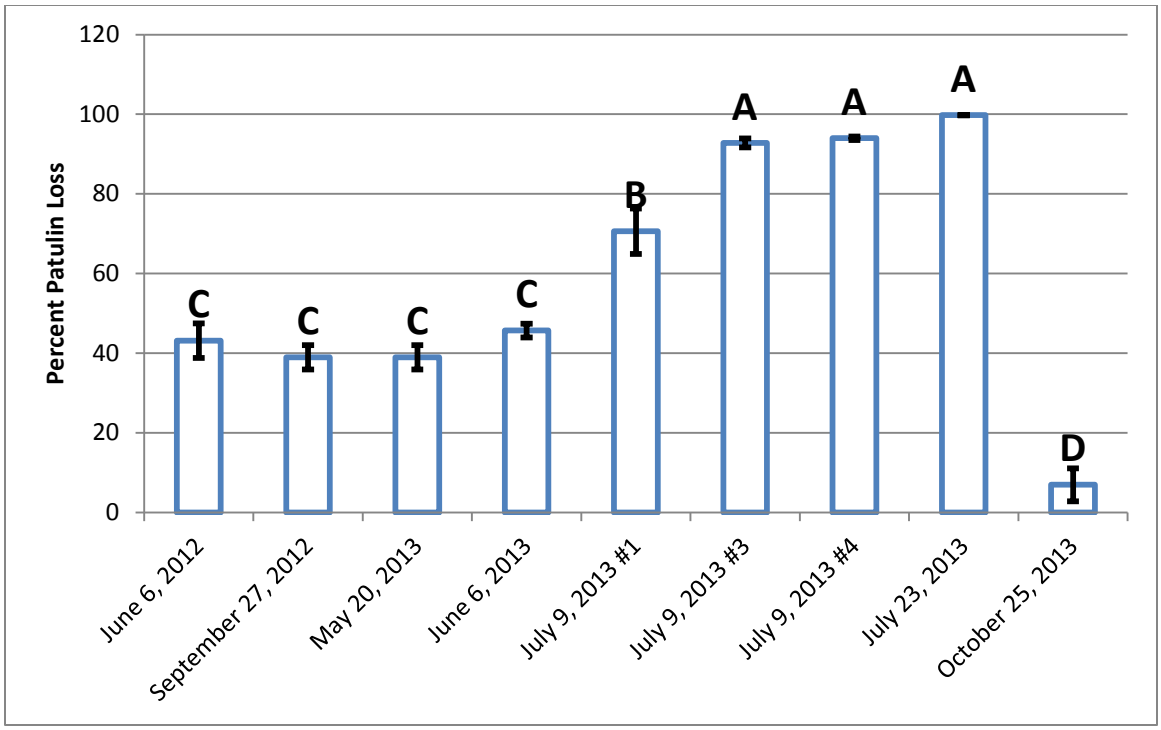

Figure 15. Percent patulin loss obtained from different protein extracts (12.5 µg/mL) incubated with patulin (5 µg/mL), incubated for 20 min at 37°C. Bars with the same capital letter represent means that are not significantly different (P≤0.05) amongst samples (n=2 to 5). Error bars were determined based on standard error values. Significant difference was assessed based on the Tukey-Kramer honestly significant difference test.

3.7. Patulin Reduction Utilizing Lyophilized Yeast Powder

Lyophilized yeast cultures incubated with patulin showed a modest increase in percent patulin loss over time. Specifically, 5 mg of yeast cell powder showed a significantly (P≤0.05) higher percent patulin loss than 50 µg of yeast cell powder incubated for 20 min at 30°C (Figure 16).

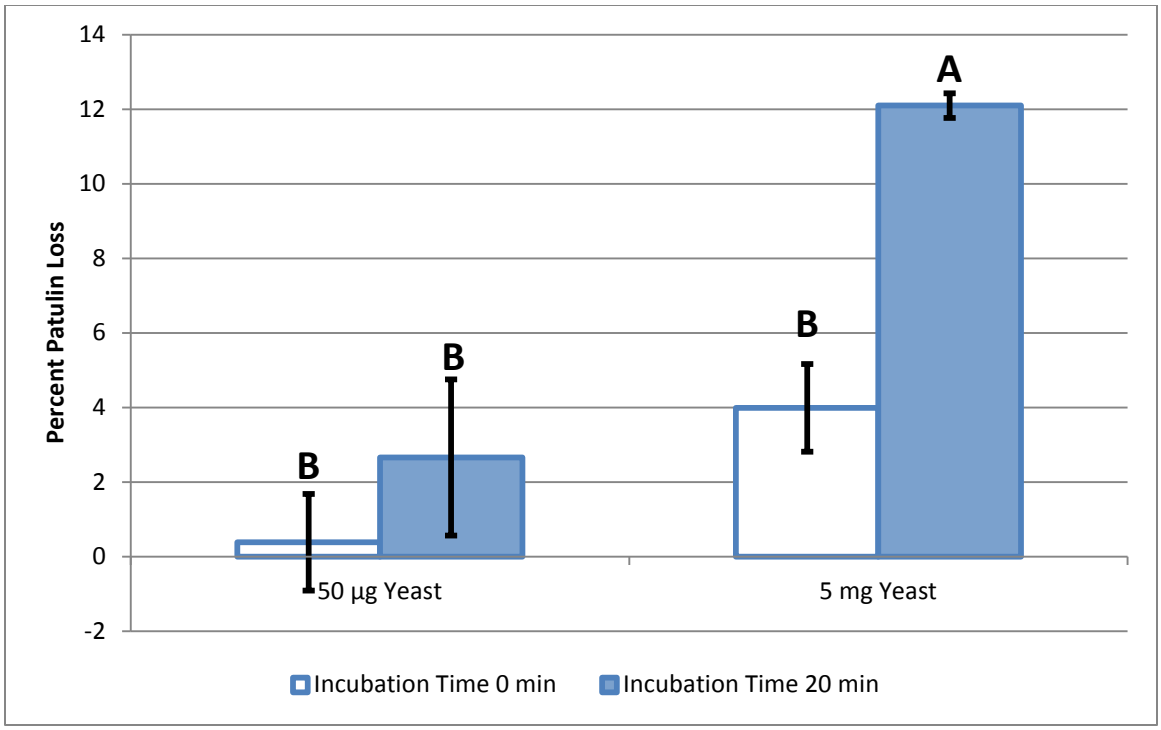

Figure 16. Percent patulin loss upon incubation of freeze dried yeast cells (50 µg to 5 mg) with patulin (5 µg/mL) at 30°C for 20 min. Bars with the same capital letter represent means that are not significantly different (P≤0.05). Error bars were determined based on standard error values (n=2 or 3). Significant difference was assessed based on the Tukey-Kramer honestly significant difference test.

The observed patulin loss upon incubation with inactivated yeast cells followed the trend observed by Guo et al (2012) who found that inactivated yeast cultures (*S. cerevisiae* YS3) (25 mg/mL) were capable of adsorbing patulin (100 µg/mL down to 40 µg/mL) onto the cell walls over the course of 24 h at room temperature. The authors also noted a sharp initial rate (12% reduction) of patulin loss in the initial inoculation with lyophilized yeast cells, which became more gradual in the hours of incubation that followed. While there only appears to be a trend (yet not a major one) using the lyophilized *R. kratochvilovae* (62-121), the observed loss in patulin suggested that a component of the cell wall is capable of removing patulin from the solution. It is believed that with longer

incubation times (>20 min) greater patulin loss would be observed. The implications of this observation is that the mechanism for patulin removal from fermented apple products could be connected to adsorption onto the yeast cell walls, with the toxin being removed with the intact yeast cells after fermentation. Therefore, in addition to chemical and possible enzymatic degradation by yeast, our observations indicated a third mechanism; binding to the yeast cell walls.

4. Conclusions, Implications, and Recommendations

The use of acid and salt to precipitate the protein from the reaction mixture solution before extraction of patulin with ethyl acetate, followed by a second round of extraction with ethyl acetate proved to be the best method for maximum recovery of patulin from high and low protein solutions. An efficient extraction method is beneficial for future research focused on alternative methods of removing patulin from apple juice and apple cider, as it will allow for accurate patulin quantification.

The use of boiling to terminate a possible enzymatic reaction for patulin degradation was found to initiate chemical degradation, presumably through adduct formation with free thiol groups. Instead, the samples were placed on ice to slow the reaction rate until ethyl acetate extraction was carried out, allowing for a more accurate measure of the degradation that occurred during the incubation at 30°C.

Glutathione and cysteine were both capable of decreasing the patulin content, even at pH 3.7. Thus patulin degradation by yeast could be partially attributed to chemical degradation. Chemical degradation via formation of sulfhydryl adducts provide insight for a new patulin control approach for apple juice and cider with the use of GRAS additives without the need to use compounds like sulfites which have limited allowable usage.

Evidence of possible enzymatic degradation was seen in some, but not all, of the protein extracts collected over the course of the study. High levels of patulin loss over a relatively short period of time (20 min versus days) suggested that at least some of the protein extracts had high patulin degradation activity in their non-denatured state. Without a consistent supply of active protein extracts kinetic work cannot be carried out to confirm or deny the enzymatic degradation of patulin by yeast protein extracts.

At this point, 3 possible reaction mechanisms for patulin degradation via yeast protein extract have been revealed. The enzymatic potential of the protein extract has inconsistently been demonstrated, yet if confirmed reproducibly, would be a powerful tool to degrade patulin continuously. The chemical degradation of patulin via free thiol groups can potentially be utilized through the use of GRAS additives, such as glutathione. The mechanical adsorption potential of yeast cell walls could lead to a new strategy of dealing with patulin, such as flowing the apple juice and cider over an immobilized substrate to adsorb the excess toxin.

For further work to be conducted a consistent supply of active protein extract must be collected for a kinetics study to be carried out. Isolation of the protein would aid in elucidation of the patulin degradation mechanism as the mixture of proteins currently utilized can potentially cause all three of the aforementioned modes of patulin loss.

When the mechanism is elucidated, further steps can be carried out to apply it to processing techniques currently in place. Utilization of an immobilized substrate to either bind or degrade patulin would offer a convenient treatment step to deal with the toxin on the processing line. The use of additives (such as glutathione) could similarly offer industrial potential for removing high levels of patulin from apple juice and apple cider. This project offers insight into the possibility of patulin degradation via yeast protein extract, and the potential role the proteins can play in control of the mycotoxin.

5. Comprehensive Bibliography

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Appendix A: Calibration Curves for Patulin Quantification

The figures shown in this appendix are the calibration curves utilized to determine the line equations for patulin concentration in relation to peak area at 276 nm of patulin standards. The line equation was used to quantify patulin concentration in samples after analysis.

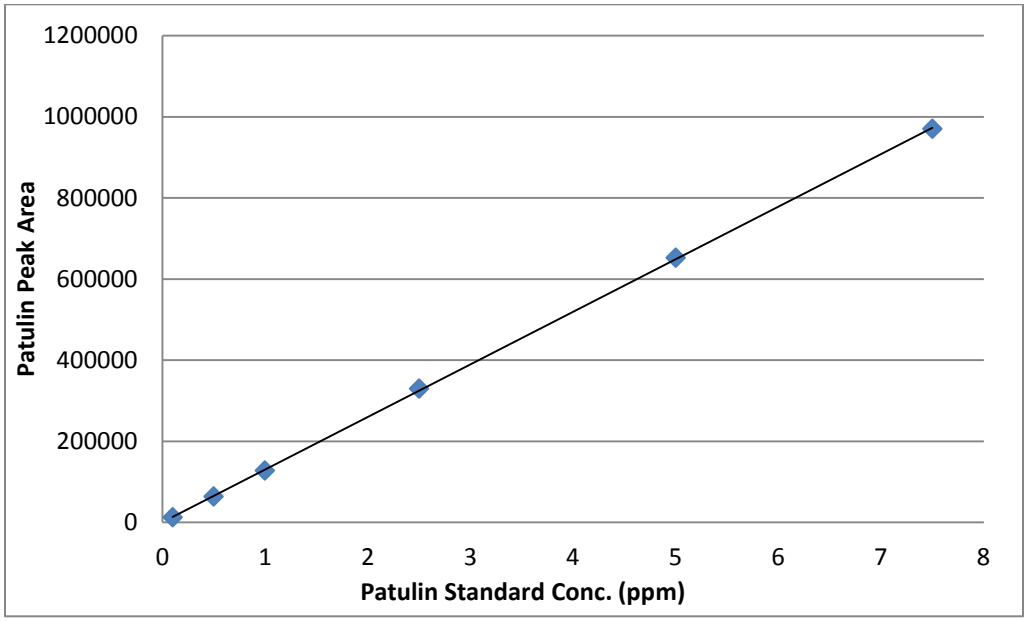

Figure 17: Calibration curve for the peak area of the patulin peak, measured at 276 nm. The line equation was obtained by performing simple linear regression analysis of the data, giving the equation: $y = 129707x + 515.23$ with an R^2 value of 0.9999.

Appendix B: Calibration Curves for the Bradord Protein assay

The figures shown in this appendix are some of the calibration curves utilized to determine the protein content of the protein extracts from yeast based on absorbance at 595 nm. Points on the standard curve are relative to bovine serum albumin (BSA) reacted with Bradford Reagent. The line equation was used to quantify protein concentration of protein extracts which determined the volume utilized for the patulin degradation assays.

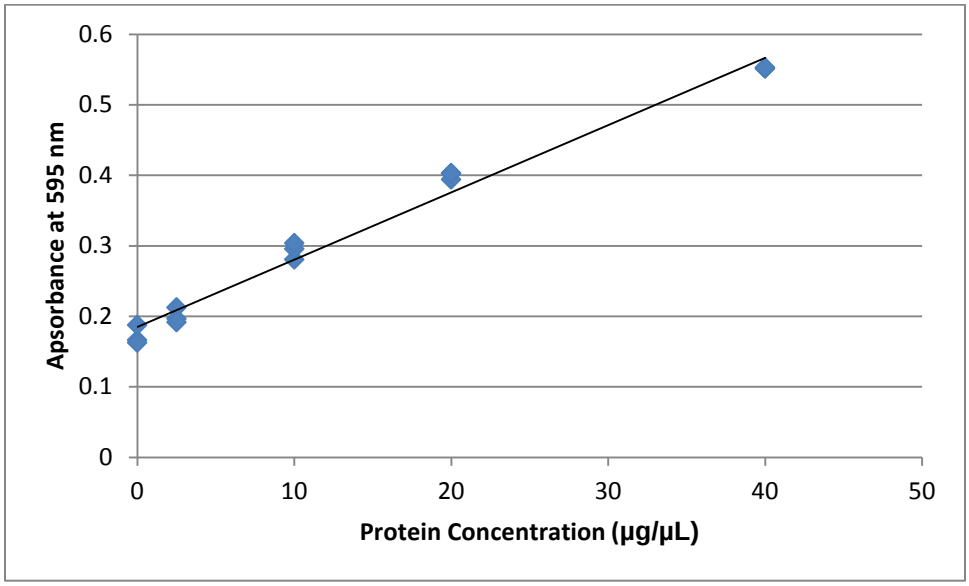

Figure 18: Calibration curve for the protein concentration of the protein extract from yeast based on the Bradford Assay, measured at 595 nm using a BSA standard curve. The line equation was obtained by performing simple linear regression analysis of the data, giving the equation: y = 0.0095x + 0.1851 with an R^2 value of 0.9845.

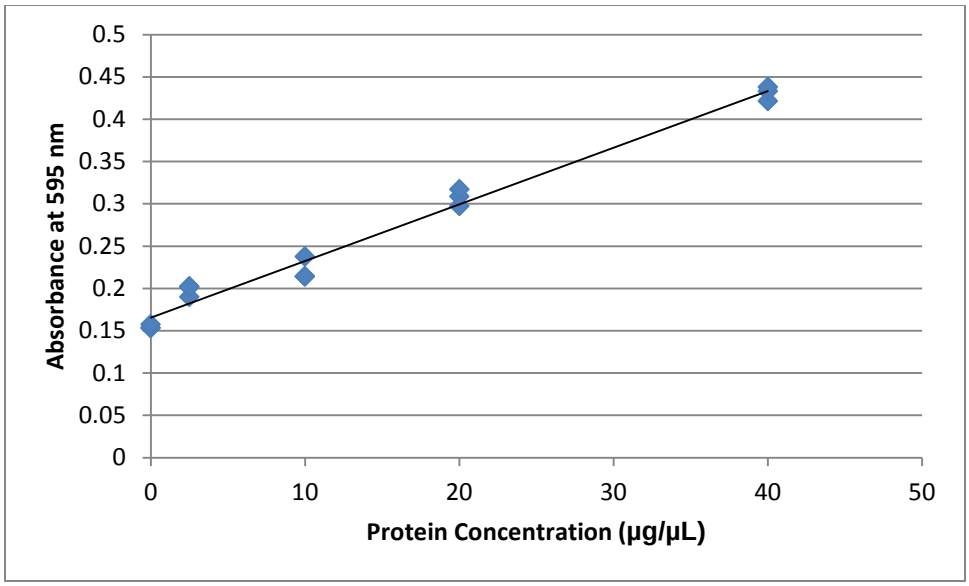

Figure 19: Calibration curve for the protein concentration of the protein extract from yeast based on the Bradford Assay, measured at 595 nm using a BSA standard curve. The line equation was obtained by performing simple linear regression analysis of the data, giving the equation: $y = 0.0067x + 0.1654$ with an R^2 value of 0.9829.

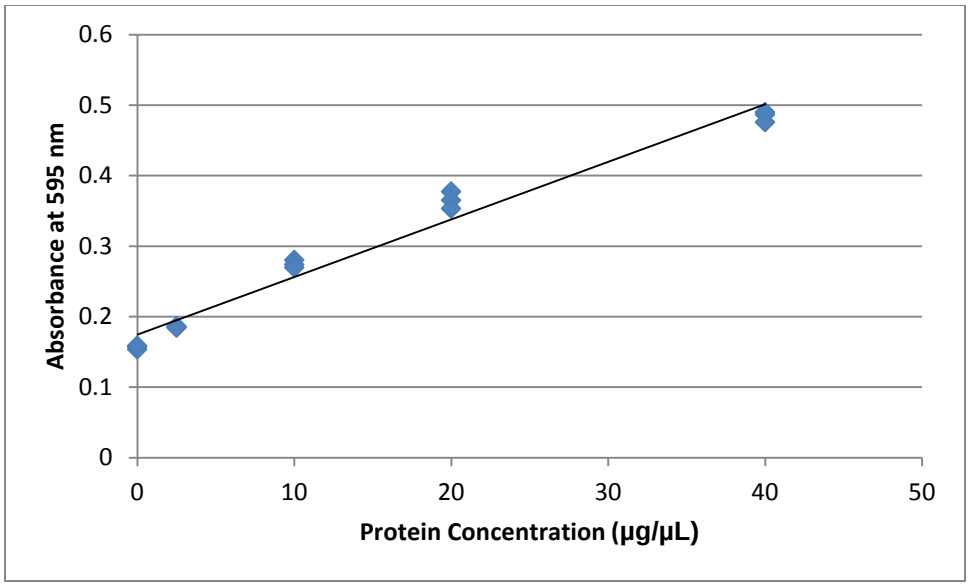

Figure 20: Calibration curve for the protein concentration of the protein extract from yeast based on the Bradford Assay, measured at 595 nm using a BSA standard curve. The line equation was obtained by performing simple linear regression analysis of the data, giving the equation: $y = 0.0082x + 0.1744$ with an R^2 value of 0.9730.

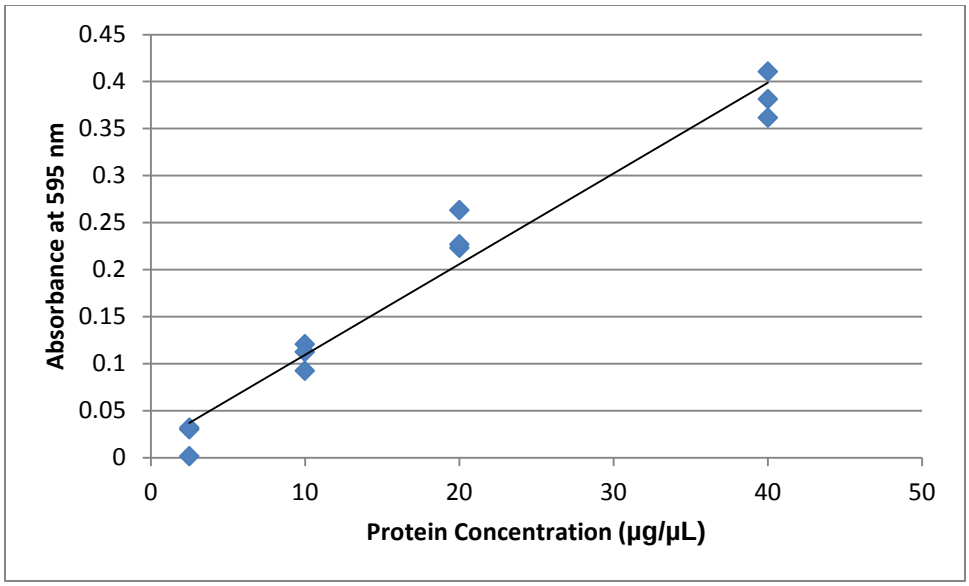

Figure 21: Calibration curve for the protein concentration of the protein extract from yeast based on the Bradford Assay, measured at 595 nm using a BSA standard curve. The line equation was obtained by performing simple linear regression analysis of the data, giving the equation: $y = 0.0096x + 0.0129$ with an R^2 value of 0.9667.

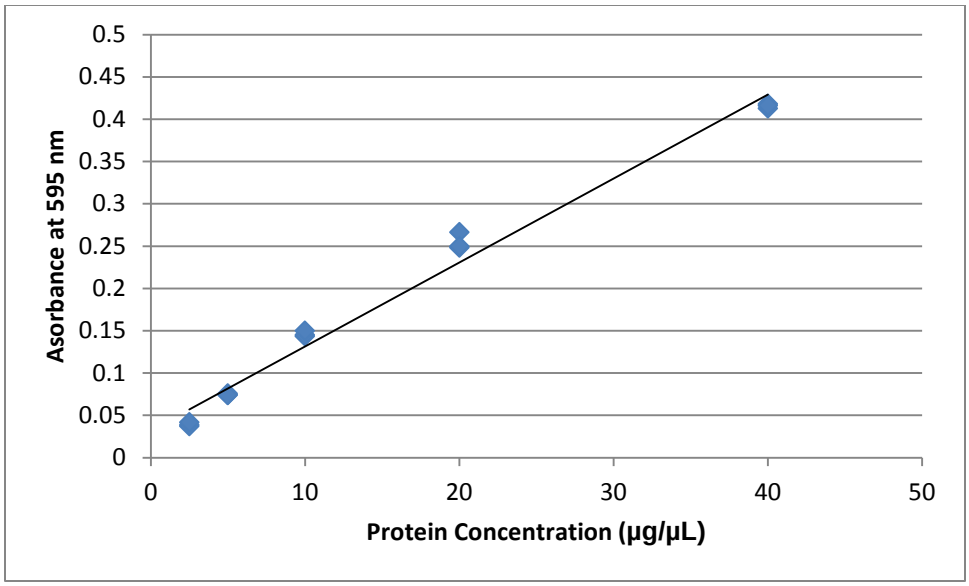

Figure 22: Calibration curve for the protein concentration of the protein extract from yeast based on the Bradford Assay, measured at 595 nm using a BSA standard curve. The line equation was obtained by performing simple linear regression analysis of the data, giving the equation: $y = 0.0099x + 0.032$ with an R^2 value of 0.9846.

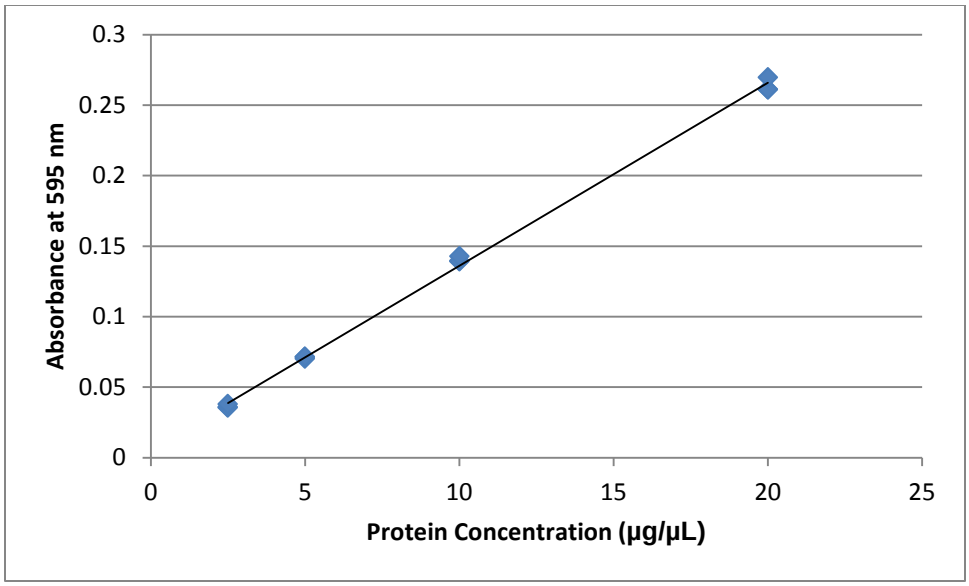

Figure 23: Calibration curve for the protein concentration of the protein extract from yeast based on the Bradford Assay, measured at 595 nm using a BSA standard curve. The line equation was obtained by performing simple linear regression analysis of the data, giving the equation: $y = 0.013x + 0.0063$ with an R^2 value of 0.9983.

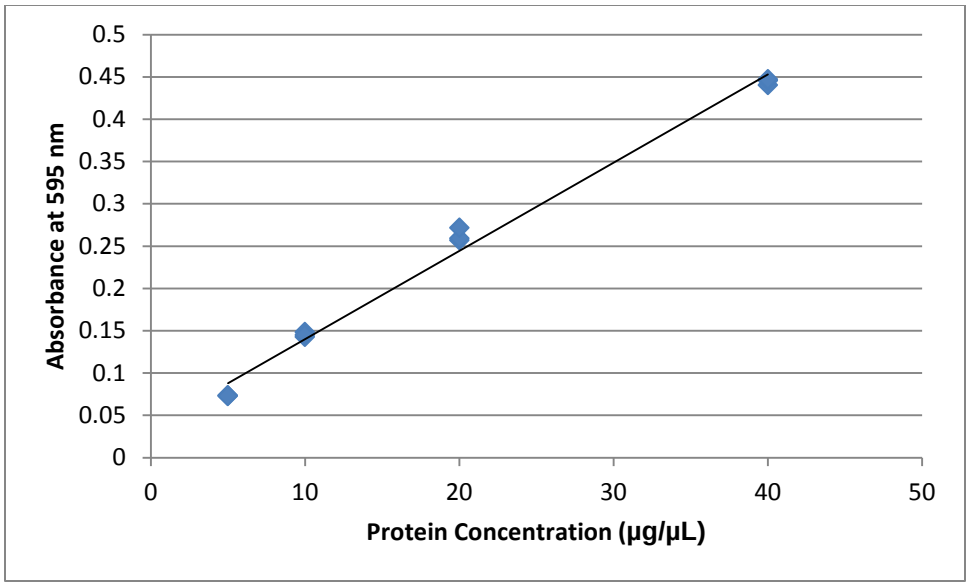

Figure 24: Calibration curve for the protein concentration of the protein extract from yeast based on the Bradford Assay, measured at 595 nm using a BSA standard curve. The line equation was obtained by performing simple linear regression analysis of the data, giving the equation: $y = 0.0104 + 0.0358$ with an R^2 value of 0.991.

Appendix C: Calibration Curve for Free Thiol Group Quantification

The figure shown in this appendix is the calibration curve utilized to determine the line equation for free thiol group quantification based on the absorbance at 412 nm for cysteine standard solutions reacted with Elman's reagent. The line equation was used to quantify free thiol group concentration in samples after analysis.

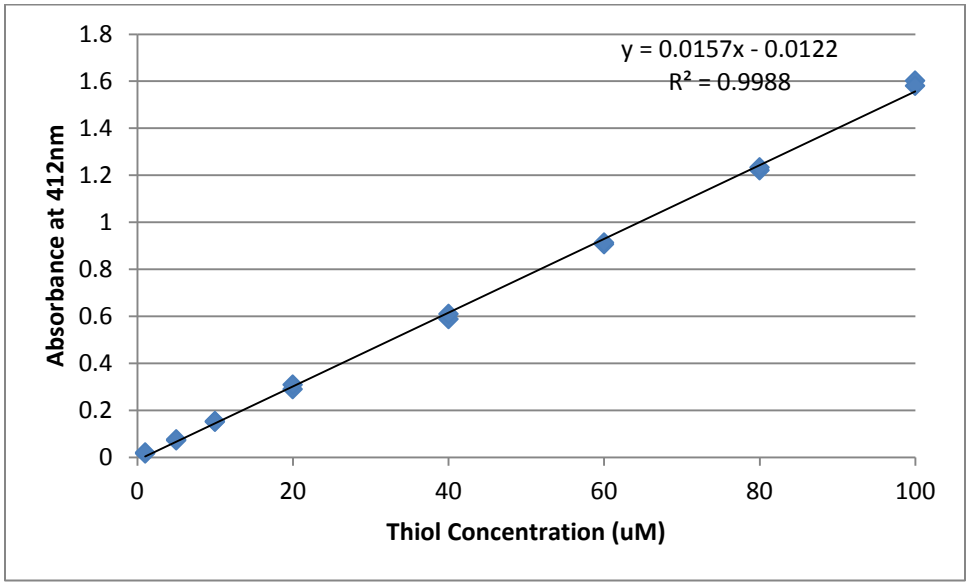

Figure 25: Calibration curve for the absorbance measured at 412 nm and the concentration of cysteine standard solutions. The line equation was obtained by performing simple linear regression analysis of the data, giving the equation: $y = 0.0157x - 0.0122$ with an R^2 value of 0.9988.

Appendix D: Analysis of Variance Table for Determination of the Effect of Extraction Method on Patulin Recovery.

Table 2. ANOVA of percent patulin recovery in patulin control and protein control from different patulin extraction methods.

Treatment	Dependent Variable	Source of Variation	Degrees of Freedom	Mean Squares	F-Value	Significance (P ≤ 0.05)
Patulin Control	Patulin Recovery	Patulin Extraction Method	3	282.625	70.281	0.000
		Error	5	4.021		
Protein Control	Patulin Recovery	Patulin Extraction Method	2	1664.018	36.722	0.000
		Error	6	45.314		

Appendix E: Analysis of Variance Table for the Determination of the Effect Growth Time and Storage Conditions have on the Patulin Degradation Activity of Protein Extracts from Yeast.

Table 3. ANOVA of percent patulin loss in samples incubated with protein extract from yeast grown for 2-8 days with and without additional storage at 4°C.

pH	Dependent Variable	Source of Variation	Degrees of Freedom	Mean Squares	F-Value	Significance (P ≤ 0.05)
3.7	Patulin Loss	Days of Yeast Growth	7	3322.153	39.966	0.000
		Error	27	83.125		

Appendix F: Analysis of Variance Table for Determination of the Effect Boiling has on Patulin Loss.

Table 4. ANOVA of percent patulin loss in samples boiled to stop the reaction and placed on ice.

pH	Dependent Variable	Source of Variation	Degrees of Freedom	Mean Squares	F-Value	Significance (P ≤ 0.05)
3.7	Patulin Loss	Reaction Termination Method	4	216.316	6.258	0.002
		Error	20	34.569		

Appendix G: Analysis of Variance Table for Determination of the Loss of Free Thiol Groups in Samples of Protein Extract and Cysteine Incubated with Patulin.

Table 5. ANOVA of percent patulin loss from protein extracts from yeast in samples treated with glutathione at pH 7 and 3.7 incubated at 30°C for 20 min.

Measure-ment	Dependent Variable	Source of Variation	Degrees of Freedom	Mean Squares	F-Value	Significance (P ≤ 0.05)
Free Thiol Groups	Thiol Conc.	Protein Source	3	78.034	49.003	0.000
		Error	7	1.592		
Patulin Loss	Patulin Loss	Protein Source	1	375.641	14.734	0.018
		Error	4	25.495		

Appendix H: Analysis of Variance Table for Determination of the Effect of Glutathione and pH on Patulin Loss.

Table 6. ANOVA of percent patulin loss in samples treated with glutathione at pH 7 and 3.7 incubated at 30°C for 20 min.

pH	Dependent Variable	Source of Variation	Degrees of Freedom	Mean Squares	F-Value	Significance (P ≤ 0.05)
7	Patulin Loss	Glutathione Treatment	5	5037.052	221.952	0.000
		Error	12	22.694		
3.7	Patulin Loss	Glutathione Treatment	5			
		Error	12	83.923	20.775	0.000

Appendix I: Analysis of Variance Tables for the Comparison of Different Protein Extracts that Show Patulin Loss in Some Manner, or Inconsistency Between Protein Extracts.

Table 7. ANOVA of percent patulin loss from protein extracts extracted from yeast grown of different dates showing some patulin degrading activity after incubation at pH 3.7 for 20 min at 30°C.

Extraction Date	Dependent Variable	Source of Variation	Degrees of Freedom	Mean Squares	F-Value	Significance (P ≤ 0.05)
June 6, 2012	Patulin Loss	Protein Extraction Date	2	723.174	13.437	0.032
		Error	3	53.819		
June 26, 2013	Patulin Loss	Protein Extraction Date	2	732.025	11.259	0.023
		Error	4	65.016		
July 23, 2013	Patulin Loss	Protein Extraction Date	3	1747.964	7.997	0.002
		Error	15	218.581		

Table 8. ANOVA of percent patulin loss from protein extracts from different batches of yeast incubated with patulin for 20 min at 30°C demonstrating the variability in the patulin loss.

pH	Dependent Variable	Source of Variation	Degrees of Freedom	Mean Squares	F-Value	Significance (P ≤ 0.05)
3.7	Patulin Loss	Protein Extraction Date	8	3177.175	65.010	0.000
		Error	19	48.872		

Appendix J: Analysis of Variance Table for the Determination of the Effect Lyophilized Yeast Cells have on Patulin Loss

Table 9. ANOVA of percent patulin loss from patulin incubated with freeze dried yeast powder at 30°C for 20 min.

pH	Dependent Variable	Source of Variation	Degrees of Freedom	Mean Squares	F-Value	Significance (P ≤ 0.05)
3.7	Patulin Loss	Freeze Dried Yeast Treatment	3	58.536	9.113	0.008
		Error	7	6.424		

Appendix K: Chromatogram of Patulin Reacted with Glutathione Depicting the Presence of Patulin and no Distinct Peaks for Glutathione.

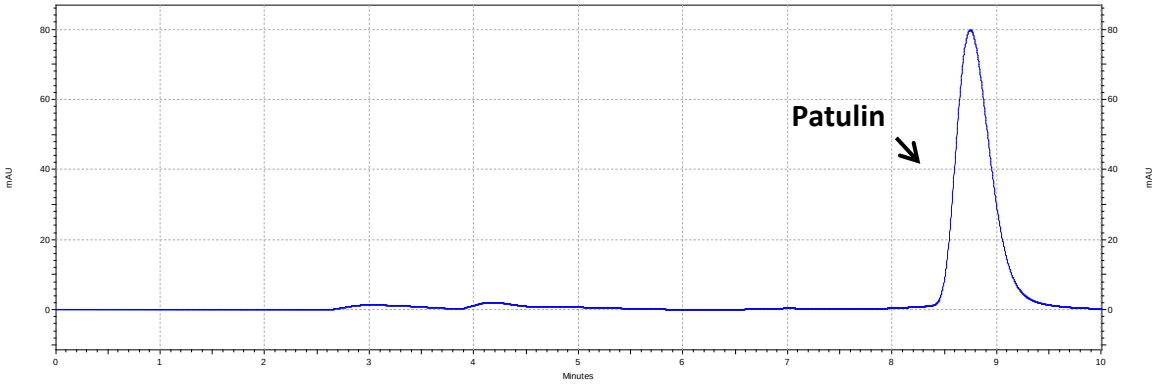

Figure 26: Chromatogram at 276 nm of patulin (5 µg/mL) and glutathione (50 µg/mL) incubated at 30°C for 20 min at pH 7.