INHIBITION OF *FUSARIUM* GROWTH AND TRICHOTHECENE ACCUMULATION IN GRAIN BY ANTIFUNGAL COMPOUNDS FROM LACTIC ACID BACTERIA

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Inhibition of *Fusarium* growth and trichothecene accumulation in grain by antifungal compounds from lactic acid bacteria

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Fusarium head blight (FHB) is a widely occurring plant disease, which is caused by fungi in the genus *Fusarium*. FHB leads to mycotoxin accumulation on grain, which causes food safety risk and economic loss. In addition to chemical treatments, biological strategies, like application of lactic acid bacteria (LAB), could be useful in preventing and/or eradicating mycotoxigenic *Fusarium* growth and mycotoxin production.

After comparision of the anti-*Fusarium* activities by a microdilution assay against *Fusarium graminearum* 08/RG/BF/51, *Lactobacillus rhamnosus* VT1 was found to have the highest anti-*Fusarium* activity. Response surface methodology (RSM) was employed to optimize the incubation conditions for the production of cell-free *Lactobacillus* culture supernatant (CFLCS) from the strain. The best combination included 34ºC, 55 hours, and shaking at 170 rpm for production of CFLCS from *L. rhamnosus* VT1. Under these incubation conditions, a 10% cell-free culture of *Lactobacillus rhamnosus* VT1 inhibited 83.7% of the *Fusarium* growth on microplate. MIC value of the CFLCS with a 10^4 conidia /well inoculum concentration is 18%.

To identify the mechanisms of anti-*Fusarium* activity, a stepwise regression, with α to enter = 0.15 and α to remove = 0.15, was performed to analyze the data of the RSM design. It was indicated that pH, total acidity, and 3-phenyllactic acid were the most important factors and could be used to explain 39.2% variation of the anti-*Fusarium* activity. In addition, proteinaceous compounds might be important due to the possible synergistic effect in the CFLCS.

CFLCS applied directly to grain not only prevented *Fusarium* growth, but also changed mycotoxin accumulation. *Fusarium* growth was inhibited completely by a 50%

concentration (V/V) of the CFLCS applied on rice media after 14 days incubation, and almost no mycotoxins were detected. Concentrations of 15%, 30% and 50% of CFLCS as steeping water inhibited *Fusarium* growth and mycotoxin accumulation on barley in the malting process. Almost no mycotoxins were detected in the samples treated by 50% CFLCS. However, the germination ability of the barley samples was inhibited.

In general, the CFLCS showed potential effective anti-*Fusarium* activity. However, the strategies of application of the CFLCS on grain should be further investigated.

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GENERAL INTRODUCTION

Mycotoxins, which are toxic secondary metabolites produced by fungi, have come to the attention of researchers since the 1960's. Aflatoxins, fumonisins, ochratoxins, patulin, trichothecenes, and zearalenone are the six most frequently occurring classes of mycotoxins in food systems. Mycotoxins cause a wide range of effects including carcinogenic and immunotoxic effects, which are food safety concerns. Mycotoxins also cause great economic loss worldwide.

Fusarium head blight, FHB, is a widely occurring grain plant disease, which is caused by fungi of the genus *Fusarium*. The most commonly encountered *Fusarium* mycotoxins in cereal crops, the trichothecenes, include deoxynivalenol (DON), 3-acetyl-4-deoxynivalenol (3-DON), 15-acetyl-4-deoxynivalenol (15-DON), nivalenol (NIV), 4-acetyl-nivalenol (FX), and T-2 (Brul and Coote, 1999). *Fusarium* mycotoxins may lead to technical problems in the food industry, which can have negative effects on quality of food products (Champeil et al., 2004). Also, trichothecene intoxication results in symptoms including vomiting, reduction in food consumption, skin irritation, diarrhea, multiple hemorrhage, and immunosuppression (Champeil et al., 2004; Parent-Massin, 2004). Trichothecenes are heat-stable structures and cannot be decomposed by typical processes of food production. Due to concerns about the effects of DON and other trichothecenes in food, the United States have determined the maximum DON advisory levels for cereals and maize-based food and feed (FDA, 2010).

In order to reduce the food safety risk and economic loss, biological inhibition through biological antagonists, which include food microorganisms, are employed to decrease the trichothecenes in grain. For an example, a microorganism, Biomin® BBSH

797 by BIOMIN GmbH, is one of the first-ever European Union (EU) authorized biological intervention products to biodegrade trichothecenes in animal feed made by *Fusarium* contaminated grains. It showed significant reduction of DON on sows and dairy cows as well as T-2 reduction on growing broilers with addition Biomin® BBSH 797 in animal feed (Biomin, 2014). However, this product is still not available in the U.S and Canada.

Among the potential biological antagonists, lactic acid bacteria (LAB) have been widely used to preserve food throughout human history. LAB are the most common microorganisms in bio-preservation research and application. LAB are widely used in fermented food production. Some products of LAB fermentation are used as natural biological food preservatives in food processing to help improve the shelf life of many foods such as lactic acid, nisin, etc. Also, LAB were reported to control mold growth with strong antimicrobial properties. Recently, LAB were associated with removal of trichothecenes *in vitro* (Niderkorn et al., 2006; Fuchs et al., 2008). Using antifungal activity of LAB to inhibit growth of *Fusarium* and other fungi have been investigated by different scientists. Many LAB strains of the genus *Lactobacillus* have been frequently reported to have antifungal activity, including *L. coryniformis* (Magnusson et al., 2003), *L. plantarum* (Magnusson et al., 2003; Laitila et al., 2002). *L. paracasei subsp. tolerans* (Hassan and Bullerman, 2008), *L. rhamnosus* (Stiles et al., 2002), *L. acidophilus*, *L. amylovorus*, *L. brevis*, and *L. coryniformis subsp. coryniformis* (De Muynck et al., 2004). These strains inhibit the growth of many different yeast and fungi, including various species of *Aspergillus*, *Penicillium*, and *Fusarium*.

Major bio-active compounds, which are associated with the antimicrobial activity of LAB, have been reported, including organic acids, such as lactic acid, benzoic acid, acetic acid, propionic acid, sorbic adic, formic acid acid, and 3-phenyllactic acid (Niku-Paavola et al., 1999; Brul and Coote, 1999; Rizzello et al., 2011; Corsetti et al., 1998;). A synergistic effect among a combination of *Lactobacillus plantarum* MiLAB 393 produced active compounds: cyclo (L-Phe-L-Pro), cyclo (L-Phe-*trans*-4-OH-L-Pro), and 3-phenyllactic acid has been suggested (Sjögren et al., 2002).

Therefore, the previous research has shown that LAB could be a potential approach to reduce the level of mycotoxins in grain. However, the mechanism of the active compounds is still unclear, and the application of the active compounds on mycotoxin reduction on grains has not been reported.

The general goal of this research is to test whether *Fusarium* trichothecene accumulation in cereals may be reduced by the inhibition of mycotoxin synthesis through the action of LAB strains associated with antifungal activity. The aim of this research then is to discover the most powerful bio-active compounds in LAB cultures and to partially explore the mechanism of the antifungal activity to help understand and enhance the bio-activity. The validation of the LAB culture in a barley malting process, was performed. This information will help instruct food technologists to avoid *Fusarium* sp. growth and mycotoxin accumulation by using various food processing technologies or adding different bio-active mixtures with *Fusarium* activity during processings.

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HYPOTHESIS AND OBJECTIVES

Hypothesis

Fusarium trichothecene accumulation in barley may be reduced by inhibiting the synthesis through the combination of metabolites produced by lactic acid bacteria**.**

Objectives

1. To develop an efficient method to screen LAB strains with the ability to reduce trichothecene accumulation.
2. To develop of a culture system to yield a high level of anti-*Fusarium* activity from the selected LAB strain.
3. To characterize of the cell free culture *Lactobacillus* supernatant (CFLCS) using a combination of statistical and experimental methods.
4. To validate the anti-*Fusarium* activity of CFLCS on rice culture and in the barley malting process.

LITERATURE REVIEW

Fusarium Head Blight (FHB)

Fusarium Head Blight (FHB) is a widely occurring grain plant disease around the world, which is caused by fungal infection before harvest. FHB has been reported to contaminate many small grain plant species, including maize, durum wheat, oats, wheat, and barley. The first recorded FHB outbreak associated with significant wheat yield loss was reported in 1891 and later defined as wheat scab (Arthur, 1891). FHB epidemics occurred in Canada, the U.S., Europe, Africa, and Asian countries including Korea, China and Japan (McMullen et al., 1997; Chen et al., 2000; Li et al., 2002; Marasas et al., 1977). In the 1990s, several severe FHB outbreaks occurred in North America. This led to a multidisciplinary approach to solve the issue and included the fields such as microbiology, plant pathology, plant breeding, plant genetics, plant metabolism, and toxicology.

FHB significantly reduces crop yield by reduction of grain kernel weight and decreases seed germinative energy. Usually, FHB infected wheat kernels are smaller, lighter and more wrinkled than normal kernels due to the degradation of proteins in grain, and with some white or pink mycelium sometimes produced by the fungus (Schwarz, 2003; Champeil et al., 2004). Various research work conducted by optical and electron microscope techniques found that endosperm cell walls in FHB infected wheat kernels were extensively degraded, and the proteins matrix disappeared, which resulted in over-modification of starch granules (Bechtel et al., 1985; Nightingale et al., 1999). Growing evidence indicates that FHB is the most serious concern for small grain cereals (Wagacha and Muthomi, 2007).

FHB Related Mycotoxins

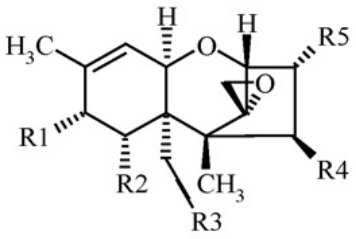
FHB results in the accumulation of mycotoxins, which are the secondary metabolites of pathogenic fungi in the infected crops. Most commonly, FHB is caused by a complex of two genera of pathogenic fungi: *Microdochium* and *Fusarium*. This includes one *Microdochium* species, *Microdochium nivale* and five *Fusarium* species, *F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. poae* and *F. triticum*. Mycotoxins that are produced in crops have only been produced by *Fusarium*, of which the most common mycotoxin-produced fungi species are *Fusarium graminearum* and *Fusarium culmorum* (Champeil et al., 2004).

Among the mycotoxins produced by *Fusarium* species, trichothecenes; including deoxynivalenol (DON), 3-acetyl-4-deoxynivalenol (3-DON), 15-acetyl-4-deoxynivalenol (15-DON), nivalenol (NIV), T2 toxin, and 4-acetyl-nivalenol (FX) are the most commonly found in cereal crops (Brul and Coote, 1999). *Zearalenone* and *Fumonisins* are also frequently associated with *Fusarium* infection in FHB disease (Champeil et al., 2004). Due to the chemical and thermal stability, mycotoxins are a considerable food safety concern (Bechtel et al., 1985; Seltz et al., 1985; Boutigny et al., 2008; Hazel et al., 2004).

The Structure and Property of Trichothecenes

As the major group of mycotoxins associated with FHB, the group of trichothecenes has a stable common chemical structure: a tetracyclic, sesquiterpenoid 12, 13-epoxytrichothec-9-ene ring system. Various functional radicals attached to the ring system divide trichothecenes into four groups: type A, B, C, and D trichothecenes. Comparatively, type A and B, such as T-2, NIV, and DON, are the most toxic and popular

(Voyksner et al., 1987; Yoshizawa et al., 1980; Sudakin, 2003). The structural differences between type A and type B are the substituitions on R1 and R2 of the ring system (Figure 1; Ueno, 1983).



	R1	R2	R3	R4	R5
Type A					
HT-2	O-isovaleryl	H	O-acetyl	OH	OH
T-2	O-isovaleryl	H	O-acetyl	O-acetyl	OH
Type B					
Nivalenol (NIV)	=O	OH	OH	OH	OH
Desoxynivalenol (DON)	=O	OH	OH	H	OH
3-AcetylDON (3-AcDON)	=O	OH	OH	H	O-acetyl
15-AcetylDON (15AcDON)	=O	OH	O-acetyl	H	OH

Figure 1. Structures of type A and B trichothecenes (Ueno, 1983).

The differences make type A trichothecenes act on the initiation of protein elongation, while type B trichothecenes act on the elongation and termination steps of protein synthesis (McLaughlin et al., 1977). Generally, type A trichothecenes have been found to be more toxic than type B trichothecenes. Cytotoxicity can be measured using MTT-cleavage test with cell line BHK-21, which is sensitive to trichothecene. The midpoint cytotoxicity values 1.6 ng/ml for T-2 toxin and 112 ng/ml for DON (Rotter et al., 1993). It was concluded that type A trichothecenes including T-2 toxin and HT-2 toxin are more acutely toxic while type B trichothecenes showed more chronic toxicoses (Rotter et

al., 1996). However, nivalenol (an OH- in R4), as one of type B trichothecenes, has been reported to be 10 times more toxic than DON (an H- in R4) (Ueno, 1985).

Furthermore, type B trichothecenes are relatively heat-stable molecules, which make elimination of this type of mycotoxin impossible by thermal processing of foods (Hazel and Patel, 2004). Therefore, reducing trichothecenes in plants before and during processing by inhibition of trichothecene accumulation is a better strategy to lower the food intoxication risk caused by FHB disease.

Safety and Economic Concern Caused by FHB

The commonly known acute intoxication symptoms of FHB related mycotoxins on animals and humans include food/feed refusal, vomiting, reduction in food consumption, skin irritation, diarrhea, multiple hemorrhage, and immunosuppression (Wu et al., 2010; D'Mello et al., 1999; Rafai et al., 1995; Conkova et al., 2003; Borutova et al., 2008; Konigs eet al., 2008). For chronic toxicity, there is strong evidence that some mycotoxins such as T-2, HT-2, fumonisins, and zearalenone, are associated with carcinogenicity (Zinedine et al., 2007; Van der Fels-Klerx and Stratakou., 2010; Ayed-Boussema et al., 2007; Kushiro et al., 2006).

Yield loss and quality reduction caused by mycotoxins, due to FHB infection, has resulted in considerable economic losses within the agricultural industry. Nganje et al. (2004) estimated the economic loss caused by in hard red spring wheat, soft red winter wheat, durum wheat, and barley by FHB during 1998 to 2001 to be $871 million for direct production and price losses and $1.8 billion for the secondary economic losses .

Legislation and Regulation of DON

DON, one of type B trichothecenes and a potent inhibiter of protein synthesis, is the most abundant naturally occurring trichothecene produced by FHB pathogens (Eudes et al., 2001). Occurrence of DON have been frequently reported in North America and Europe since it was first reported in corn in 1982 (Thiel et al., 1982). Although the toxicity of DON is less than other trichothecenes such as T-2 and HT-2, DON is commonly and widely detected in cereal grains and processed foods including malts and beer (Hussein and Brasel, 2001; Creppy, 2002). Therefore, DON is usually regulated as the representative trichothecene in many countries. Considering the serious effects of DON on health and economics, at least 37 countries have specific regulatory levels for DON ranging from 0.3 to 2 mg/kg (FAOUN, 2003).

As for the worldwide limits for DON in wheat (flour) and other cereals, the lowest limit is 0.3 mg/kg in Cuba, while the highest limit is 2 mg/kg in Bulgaria and Canada; the most frequently occurring limit in 19 countries is 0.75 mg/kg (FAOUN, 2003). The updated maximum levels for deoxynivalenol in cereals and cereal-based infant food products are 1 and 0.2 mg/kg, respectively, for most of countries in 2013 (FAOUN, 2013). The US Food and Drug Administration (FDA) advises DON at 1 mg/kg in finished wheat products for human consumption. The concentrations of DON in grains and grain by-products destined for animals ranges varies from 5 to 30 mg/kg with limited percentage blending in animal feed (FDA, 2010).

Factors Affecting DON Accumulation in Grain

DON accumulation in grain is significantly affected by environmental factors. Moisture and temperature are the most important variables. Usually, cold and wet weather favor mycotoxin production during the growing season. Although little is known about the exact influence of weather on mycotoxin production, there are still some hints obtained from previous research. By a statistical analysis of the DON levels in Norwegian cereals from 1988 to 1996, Langseth and Elen (1997) found a strong positive correlation between the precipitation in July in growing season and the mean DON content. Dry spring accelerates the susceptibility to *Fusarium* infection of grains and late harvest may increase the DON contamination of the crops as well (Langseth and Elen, 1997). Another statistical analysis performed by Kriss et al. indicated that the environmental variables including humidity and temperature influence mycotoxin production significantly on the basis of significant semi-partial correlations. Those influences were non-predicted because of the high variability in the relationships (Kriss et al., 2012).

An excellent model was established by Bondalapati et al. (2012) to predict the risk of DON accumulation for barley on the basis of data from the mid-west states in the United States from 2005 to 2009. The verification of the model by data in 2010 suggested that FHB is strongly associated with DON accumulation in the barley and the weather conditions could be used to predict the DON accumulation risks (Bondalapati et al., 2012). Harvest data also is a significant factor in determining DON level given the favorable weather condition (Langseth and Elen, 1997; Pageau et al., 2009). In general, weather factors especially temperature and humidity affect the DON level in harvested grains. The most favorable temperature is between 15 to 30 ºC with a relative humidity higher than

90% for extended periods of time (Schmale and Bergstrom, 2003). However, how the weather factors work on the accumulation of DON during growing season has not been elucidated completely.

Factors Affected DON Accumulation in Culture

DON production by *Fusarium* during infection is much higher than axenic growth in culture (Gardiner et al., 2009), which makes the understanding of DON production critical to regulation and management of mycotoxins including DON in grains. In order to better understand DON accumulation in grain development, DON production by *Fusarium* in culture was investigated regarding the variables including strain, culture medium, incubation temperature, and incubation time (Gilbert, et al., 2002; Duverger et al., 2011; Jiao et al., 2008; Ryu and Bullerman, 1999; Gardiner et al., 2009). Gilbert et al. (2002) identified 15 Canadian isolates of *F. graminerum* and found their abilities to produce mycotoxins ranged between 0.2 and 249 μg/g. Their results indicated that among all the same species of *Fusarium*, the high diversity was an important factor affecting DON production (Gilbert, et al., 2002).

Rice was found to be a more favorable substrate for DON production, but the culture condition (liquid or solid culture) did not show any effect on mycotoxin production including DON and ZEN (Duverger et al., 2011). Twelve carbon sources were compared for the influences on DON production in liquid cultures, where sucrose, 1-kestose and nystose were found to be the carbon sources with highest DON production under the same incubation conditions. The research indicated that DON production was not regulated by

carbon catabolite repression and sucrose may trigger the expression of *Tri* 5 gene and induce the DON production (Jiao et al., 2008).

Production of DON and ZEN was also significantly affected by temperature and incubation time. Low cycling temperature induced higher mycotoxin production while higher cycling temperature led to highest amounts of fungal growth measured by free ergosterol (Ryu and Bullerman, 1999). The pH value, as an incubation condition, was demonstrated to have significant impact on DON production. Although *Fusarium* growth was better at neutral or higher pH (5-10), low extracellular pH is necessary for DON production in *Fusarium graminearum*. Once pH was greater than 4.0, DON production was decreased (Gardiner et al., 2009). Furthermore, the low extracellular pH promoted the DON production, which could be attributed to the enhancement of the expression of the *Tri* 5 gene through the combination of the low pH and amines (Gardiner et al., 2009). A number of compounds including magnesium ion, phenolic acids, hydrogen peroxide, etc., also affected the DON production in culture, both positively or negatively (Pinson-Gadais et al., 2008; Boutigny et al., 2008; Ponts et al., 2006). Generally, DON production was influenced by various factors in culture including carbon source, strains, and incubation conditions. These factors should be considered when investigating the regulation of DON production.

Relationship between FHB, *Fusarium* Biomass and DON

The relationship between FHB severity and DON production has been widely investigated. Paul et al. (2005) employed meta-analysis to quantitatively review the relationship between FHB intensity and DON production. The results of analysis of 163

strains indicated DON production and FHB were positively correlated, and *Fusarium*-damaged kernels had the strongest relationship with DON production with a correlation coefficient at 0.73. Also, DON production was correlated with field disease severity and diseased-head severity. Significant, positive, and linear relationships between FHB severity and DON production in wheat cultivars were also demonstrated (Hernandez et al., 2012; Salas et al., 1999). However, some researchers obtained different conclusions. They thought the current information could not prove the positive relationship between FHB severity and DON production (Mesterházy et al. 1999; Snijders 2004). The contrast results indicated the uncertainty of the association between FHB disease and mycotoxin accumulation.

The correlation between *Fusarium* biomass and DON production is controversial. Duverger et al. (2011) used ergosterol quantification to measure the fungal biomass in rice culture, and they found there was no correlation between mycotoxin, including DON production, by *F. graminearum* strain in rice culture and the *Fusarium* biomass (Duverger et al., 2011). In research done by Gilbert et al. (2002), there was no correlation among FHB severity, DON production and *Fusarium* biomass measured by ergosterol production in rice culture. However, the amount of DON produced by *F. graminearum* was concluded to be positively correlated with *Fusarium* biomass measured by real-time PCR in small grains (Wegulo, 2012). Positive relationship between *F. graminearum* biomass measured by ergosterol and DON produced by *F. graminearum* in Canadian hard and soft wheat was also demonstrated via statistical analysis (Abramson et al., 1998). The various research results reflect the complication of the relationship between *Fusarium* biomass and DON.

Reduction Methods for FHB Associated Mycotoxins

Strategies that could be used to reduce the toxic effect of mycotoxins include prevention of mycotoxin accumulation and detoxification of preformed mycotoxins in food matrices. Prevention strategies focus on the reduction of mycotoxin contamination via inhibition of mycotoxin formation during the growth, harvesting, storage and processing. Development of FHB resistant wheat and barley varieties is a strategy to deal with FHB issue. For example, 'Quest' barley is a spring, six-rowed, malting barley released by the Minnesota Agricultural Experiment Station in 2010, which has been approved by American Malting barley Association on the basis of its improved resistance to *Fusarium* head blight. Quest has shown about 40% less DON, when compared to the historically important cultivar 'Robust' (Smith, 2013). The complexity of the relationship between FHB growth and DON, however, production makes the breeding FHB resistance grain varieties more difficult (Boutigny et al., 2008).

Detoxification is the reduction of accumulated mycotoxins in food matrices. Approaches used to reduce the mycotoxins produced by FHB could be classified into three categories: physical, chemical, and biological. Chemical approaches, such as using chlorine and aqueous sodium at specific concentration to treat FHB infected corn, were demonstrated to be effective methods to obtain more than 85% DON reduction (Young, 1986). Physical approaches, such as thermal treatment, was indicated to get rid of 50-90% of the DON produced by FHB pathogens in wheat (Young et al., 1986). The effect of aqueous sodium was also demonstrated to be effective on wheat (Young et al., 1986). However, due to the natural preferences of consumers, the chemical approaches are not prefered in the food industry. Also, physical approaches are not economically feasible at

large scale and may affect the characteristic of food matrices during food processing process.

Therefore, biological approaches have attracted more researchers as a potential approach to solve the problem of mycotoxin accumulation in FHB infected grains. Biological agents are a group of microorganisms with the abilities of lowering the severity level of FHB and reducing the accumulation of mycotoxins produced by the pathogens associated with FHB. The reported potential biological agents include bacteria belonging to *Bacillus subtilies*, *Pseudomonas fluorescens* (Sato et al., 1999; Khan and Doohan, 2009), and *Brevibacillus* sp. BRC263 and *Streptomyces* sp. BRC87B (Palazzini et al., 2007); yeasts belonging to *Cryptococcus*, *Rhodotorula, Sporobolomyces* (Sato et al., 1999; da Luz, 2000; Schisler et al., 2006); and fungi belonging to *Trichoderma* (Fernandez, 1992), and *Clonostachys rosea* (Xue et al., 2008). Most of the above studies were done pre-harvest under greenhouse and/or field conditions.

Antifungal Activity Associated with Lactic Acid Bacteria (LAB)

With the increasing demand for natural products in food markets, bio-preservation, which means extension of storage time and enhancement of food safety by microorganisms and/or their metabolites (Stiles, 1996), has been a research topic since the end of the last century. LAB have been widely used to preserve food throughout human history. For instance, LABs have been used as starter cultures for brewing and cheese production. Presently, LABs are also the most common microorganisms in bio-preservation research and application. LABs are used to preserve foods by removing fermentable carbohydrates, consuming oxygen, producing organic acids, and decreasing the pH of the food matrices.

furthermore, specific inhibitors produced by various LAB strains, such as nisin and bacteriocins, could improve the preservative effect (Ganzle, 2009).

In recent studies, the organisms of the genus *Lactobacillus* was frequently reported to have antifungal activity. Various species of *Lactobacillus* strains show different antifungal activities against molds, including *Aspergillus, Penicillium, Fusarium* and yeast. For instance, Magnusson et al. (2003) investigated more than 1200 isolates of lactic acid bacteria from numerous environments and found that *Lactobacillus coryniformis* and *Lactobacillus plantarum* had the strongest inhibition activities against several molds, including *Aspergillus fumigates*, *A. nidulans* and *F. sporotrichioides* (Magnusson et al., 2003). Hassan and Bullerman (2008) reported that *Lactobacillus paracasei subsp.tolerans*, which was isolated from a traditional sourdough bread culture, inhibited the *F. proliferatum* and *F. graminearum* species grown in liquid culture. The antifungal activity was associated with organic acids and low pH (Hassan and Bullerman, 2008). The *Lactobacillus plantarum* strain VTT E-78076 and VTT E-79098 were confirmed to have potential antifungal properties against *Fusarium* species in culture (Laitila et al., 2002). *Lactobacillus rhamnosus* was found to have a possible synergistic action in combination with sodium acetate, which is consistent with the results that sodium acetate has antifungal properties and may promote the antifungal activity of LAB culture (Stiles et al., 2002). In another study, 17 LAB strains and three commericial probiotic cultures were screened for antifungal activity. Among these LAB strains, *Lactobacillus acidophilus* LMG 9433, *Lactobacillus amylovorus* DSM 20532, *Lactobacillus brevis* LMG 6906, *Lactobacillus coryniformis subsp.coryniformis* LMG 9196, were found to have promising antifungal activity; and the antifungal metabolites produced were pH-dependent (De Muynck et al.,

2004). Furthermore, *Lactobacillus casei subsp. rhamnosus* inhibited mold growth on wood-based building materials, which widens the spectrum of molds that are inhibited by LAB (Yang and Clausen, 2005).

In Vitro Removal of Trichothecenes and Other Mycotoxins by Lactic Acid Bacteria

Lactic acid bacteria, LAB, are commonly used food microorganisms. As natural antagonists, LABs have become bio-preservatives in food due to their antimicrobial activity. They are believed to be harmless and beneficial to human health. El-Nezami et al. (1998a) reported that both *L. rhamnosus* strain GG and LC-705 effectively removed aflatoxin B_1 in wheat system. The processes of removal were rapid, and the amount of aflatoxin removed depended on temperature and bacterial concentration.

In related work, it was observed that after treating with hydrochloric acid, the binding ability of bacterial pellets were enhanced; heat treatment had a positive effect except treating with autoclaving or boiling at 100°C in a water bath (El-Nezami et al., 1998b). Later, El-Nezami et al. (2002) investigated the removal ability of the two strains on *Fusarium* toxins, including DON, 3-AcDON, NIV, and T-2. Their research indicated that the *L. rhamnosus* strain GG was more efficient than strain LC-705 in removing the toxins from wheat system.

Niderkorn et al. (2006) described the abilities of twenty-nine LAB strains, including *L. rhamnosus* strain GG, to remove DON, fumonisins B1 and B2 (FB1, FB2) in subacidic medium. Removal was up to 55% for DON by *Lactobacillus delbruekii* ssp. *bulgaricus* while the removal by *L. rhamnosus* strain GG was around 54% (Niderkorn et al., 2006). The most common point in these studies was that the possible mechanism was

binding because no degradation products were detected. In order to test the relationship between reducing toxin concentration and toxin property reductions of ochratoxin A, micronucleus assays (MCN) were employed to measure growth of human derived hepatoma cell line before and after removal of ochratoxin A by *Lactobacillus acidophilus* (Fuchs et al., 2008). More than 30% reduction of ochratoxin A induced MCN formation indicating that LAB was able to detoxify the toxins (Fuchs et al., 2008).

Agar Diffusion Assay Associated with Antifungal Activity

The agar diffusion method has been frequently used to test the antifungal activity associated with potential antifungal compounds, including bacteria, organic acids, and proteinaceous compounds (Gerez et al., 2009; Rizzello et al., 2011; Yang et al., 2011; Wang et al., 2012). In many studies, sterile filter paper disks were involved in the agar diffusion method. Usually, 10 μl of a potential antifungal compound solution was spotted on the sterile filter paper disks (0.5 cm diameter), and three paper disks were placed on the surface of potato dextrose agar (PDA) which was already inoculated with the fungus after the mycelia had developed. Zones of inhibition were measured until the mycelia growth overlaid the negative control paper disk (Rizzello et al., 2011).

Another type of agar diffusion is the incorporation of the media and the potential antifungal compounds at serial concentrations and inoculation of the mixture at the center surface of agar with test fungi. The diametrical difference in perpendicular directions of the growth zone between the control and the sample were measured after incubation for several days to determine the antifungal activity (Wang et al., 2012). Finally, the well-diffusion assay is also a common agar diffusion method used to test the antifungal activity

associated with lactic acid bacteria (Corsetti et al., 1998). Fungal conidia were mixed with agar first, then, wells with a specific diameter were punched out from the agar, and the potential bio-active compounds were added and allowed to diffuse from the wells. The inhibition zones were compared after incubation (Yang et al., 2011). The using of agar diffusion assay were different due to the various experimental objectives.

Bio-active Compounds Associated with Antifungal Activity of LAB Strains

Most of the bio-active compounds associated with the antimicrobial activity of LAB are organic acids, including lactic, benzoic, propionic, sorbic, and formic acids (Niku-Paavola et al., 1999; Brul & Coote, 1999; Rizzello et al., 2011; Corsetti et al., 1998). Niku-Paavola et al. (1999) reported that 1% lactic acid can inhibit the growth of *Fusarium avenaceum* VTT D-80147 by 10-15%, while other compounds such as benzoic acid or methylhydantoin at a concentration of 10 ppm inhibited the growth of the strain by 10-15% (Niku-Paavola et al., 1999). When combined with benzoic aicd and methylhydantoin, 1% lactic acid inhibited 100% growth of *Pantoea agglomerans* VTT E-90396 and 20% growth of *Fusarium avenaceum* VTT D-80147 (Niku-Paavola et al., 1999). Benzeneacetic acid and 2-propenyl ester produced by LAB were reported to have antifungal activity by Wang et al. (2012), which was the first report of these two new antifungal substances, which are produced by lactic acid bacteria (Wang et al., 2012).

PLA is another frequently reported bio-active compound synthesized by LAB (Lavermicocca et al., 2000; Lavermicocca et al., 2003; Gerez et al., 2009; Prema et al., 2010; Ndagano et al., 2011). A *Lactobacillus plantarum* strain was demonstrated to produce antifungal compounds including PLA, lactic and acetic acids. The PLA was

verified as a fungistatic compared by the NMR spectrum of the antifungal compound with the commercial 3-PLA (Prema et al., 2010). Acetic acid and PLA were identified as the major antifuanl compounds in three *Lactobacillus* spp, which included *L. plantarum*, *L. reuter*, and *L. breviswere* (Gerez et al., 2009). In the antifungal research performed by Lavermicocca et al. (2003), PLA and 4-hydroxy-PLA were suggested as the novel antifungal compounds via chromatographic and spectroscopic analyses. Their subsequent research work evaluated the antifungal ability of PLA and found that 7.5 ml/ml PLA in buffered medium at pH 4 could inhibit 50 to 92.4% fungal growth for all of the tested strains including *Aspergillus*, *Penicillium*, and *Fusarium*. Furthermore, PLA was confirmed as an antifungal compound with the concentration of 0.56 mM in *Lactobacillus plantarum* VE 56 culture (Ndagano et al., 2011). It was higher than the concentration of PLA from *L. plantarum* CRL 778, 0.26 mM (Dallagnol et al., 2011). In general, PLA has been identified as an effective antifungal compound produced by many *Lactobacillus* spp.

Yang and Chang (2010) isolated 3,6-bis(2-methylpropyl)-2,5-piperazinedion from *Lactobacillus plantarum* AF1, which was isolated from kimchi and had a molecular weight of 226 units. Later, the same team claimed the effective bio-active compound produced by the same strain was δ-dodecalactone with a molecular weight of 198.3 units (Yang et al., 2011). The antifungal compounds from *Lactobacillus casei* AST18 were confirmed to be cyclo-(Leu-Pro), 2,6-diphenyl-piperidine, and 5,10-diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-a;1',2'-d] pyrazine (Li et al., 2012).

More research during the past decades has demonstrated that there is a synergistic effect among combinations of organic acids or organic acids combined with proteinaceous compounds. A mixture of acetic, caproic, formic, propionic, butyric and n-valeric acids

was concluded to be the active antifungal compounds from a strain belonging to *Lactobacilllus* spp, and the organic acids worked as a synergistic system (Corsetti et al., 1998). Strom et al. (2002) reported weak synergistic effects among *Lactobacillus plantarum* MiLAB 393 produced active compounds: cyclo (L-Phe-L-Pro), cyclo (L-Phe-*trans*-4-OH-L-Pro), and 3-phenyllactic acid. This explains the broad spectrum antifungal ability against food- and feed-borne filamentous fungi and yeasts by this LAB strain (Strom et al., 2002). The antifungal activity of *Lactobacillus casei* AST18 was demonstrated to have a synergistic effect with lactic acid and the cyclopeptides mentioned above (Li et al., 2012).

According to the research reviewed, the mechanism of antifungal activity of lactic acid bacteria is very complicated. Most probably, the antifungal activity is due to combinations of various antifungal compounds found in the specific culture. The utilization of the antifungal activity depends on the knowledge regarding the mechanism of antifungal activity at a certain level.

Identification of Bio-active Compounds in Lactic Acid Bacterial Cultures

The antifungal compounds from *Lactobacillus* culture are a group of mixed bio-active compounds, which may consist of organic acids, proteinaceous compounds, or other unusual organic compounds. In order narrow down the categories of antifungal compounds, the common starts are the sensitivity tests to pH, proteolytic enzymes, and temperature (Gourama, 1997; Falguni et al., 2009; Li et al., 2012; Ndagano et al., 2011). This information is very valuable to determine if the potential antifungal compounds are proteinaceous compounds. For example, the antifungal compounds from *Lactobacillus*

casei AST18 was found to be heat- and pH-sensitive and not stable to the treatment of trypsin and pepsin. In Gourama's (1997) research, the cell free supernatants were found to be sensitive to proteolytic enzymes and to high temperature, which indicates that the antifungal compounds most probably were proteinaceous compounds.

Molecular weight estimation is another approach to determine the basic chemical property of the potential antifungal compounds. Fractionation of cell-free supernatants via ultra filtration with various size molecular weight cutoff filters, was used to test the antifungal activity of each filtrate was tested. The possible molecular weight of the potential antifungal compounds could be determined in a small range (Yang and Clausen, 2005; Rizzello et al., 2011). Cell free supernatants from *Lactobacillus* culture were fractionated with 10-, 3-, and 1-kDa molecular weight cutoff filters and then tested for the antifungal activity. The 1-kDa molecular weight was confirmed with strong antifungal activity, suggesting the apparent molecular weight of the effective antifungal compounds from the initial *Lactobacillus* culture should be less than 1k Da (Yang and Clausen, 2005). Dialysis was also widely used to estimate the molecular weight. The molecular weight of the antifungal compound from *Lactobacillus brevis* NCDC 02 was found to range from 1- to 5-kDa via dialyzing treated culture supernatants (Falguni et al., 2009).

Further identification has relied on chromatographic and spectrometric analysis. HPLC has been commonly used to test for organic acids from LAB culture. Usually, the standard organic acids are required to compare the retention time and peak area of the test samples. For example, lactic and phenyllactic acids from *Lactobacillus plantarum*, *Lactobacillus reuteri*, and *Lactobacillus brevis* were evaluated using an ion-exclusion Aminex 87 H column with a UV (210 nm) detector in a HPLC system (Gerez et al., 2009).

Moreover, although C-18 was also used to test organic acids, the combination of Aminex 87 H column and UV detector seems to be a powerful tool to test organic acids in the antifungal compounds research area with various inorganic salt solution at low pH as mobile phase (Wang et al., 2012; Rizzello et al., 2011; Dallagnol et al., 2011; Ndagano et al, 2011). HPLC was also combined with mass spectrometry (MS) to verify the target antifungal compounds (Ndagano et al, 2011; Wang et al., 2012).

Gas chromatography/mass spectrometry (GC/MS) is also widely used to identify the antifungal compounds from LAB. Li et al. (2012) used semi-preparative HPLC to prepare samples and then performed GC/MS analysis to determine the structure of the main unknown substances obtained from HPLC. The cyclopeptides structures of the substances were confirmed (Li et al., 2012). Antifungal δ-dodecalactone from *Lactobacillus plantarum* AF1 was also determined by GC/MS (Yang et al., 2011). In addition, phenyllactic and 4-hydroxy-phenyllactic acids were identified from *Lactobacillus plantarum* 21B (Lavermicocca et al, 2000).

Nuclear magnetic resonance (NMR) was employed to determine the structure of the antifungal compounds. Prema et al. (2010) used NMR to compare commercial 3-phenyllactic acid with the 3-phenyllactic acid produced by a *Lactobacillus plantarum* strain which was isolated from grass silage. It was confirmed to be the same structure as the commercial standard (Prema et al., 2010). Strom et al. (2002) used NMR to determine the structure of cyclic dipeptides cyclo(L-Phe-L-Pro) and cyclo(L-Phe-trans-4-OH-L-Pro) by comparing with commercial cyclo(L-Phe-L-Pro). Therefore, NMR could be used to confirm the structure of the antifungal compounds on the basis of known target compounds including organic acids and proteinaceous compounds.

Micro Dilution Assay for Antifungal Activity

Compared with the agar diffusion assay, the microdilution assay has gained increasing interest in the past decade. Presently, the microdilution assay, has been widely used to test the antifungal activity of the potential antifungal compounds with less experimental time, lower cost as well as objective interpretation of results (Riesselman et al,. 2000). Usually, micro dilution assays have been developed on the basis of standard multi-well plates (Gerez et al., 2009). The media, potential antifungal compounds as well as the fungal strain conidia were transferred to each well. The optical densities before and after incubation were recorded to compare the fungal growth (Oliva et al., 2003; Salah et al., 2003). The most common wavelengths used to compare optical fungal growth range from 580 to 620 nm (Dallagnol et al., 2011; Gerez et al., 2009).

The Problem of FHB Infected Barley in Malting and Brewing

During malting and mashing process, various starch- and protein-degrading enzymes such as alpha-amylase, beta-amylase, limit-dextrinase, and proteinases work together to breakdown starch to fermentable sugars and total protein to polypeptides and amino acids, which will be converted to alcohol and flavor compounds during yeast fermentation. However, FHB-infected barley receives additional enzymes such as beta-glucanase, xylanase and proteinase activities from *Fusarium* that significantly affect barley quality, resulting in poor malting performance and poor wort and beer quality as well. Infection of barley with species of *Fusarium* may also be responsible for beer gushing due to mycotoxins produced by *Fusarium* (Schwarz et al.， 2002).

A comparative study regarding the fate of DON from FHB-infected barley to beer showed that DON levels elevated during the mashing process, which might be due to the gradual solubilzation of DON from malt to wort. They also found DON was stable after wort boiling for 90 min and DON level in the finished was similar to that in malt, therefore yeast fermentation did not show any affect on DON level (Niessen and Donhauser, 1993). The fate of DON and other *Fusarium* mycotoxins during malting and brewing of naturally infected barley was investigated, and it was concluded that DON from FHB-infected barley can carry over to the finial beer product (Schwarz et al., 1995).

The Application of Bio-control Agents in Malting for FHB

The malting and brewing industries are still struggling with the FHB problem and DON levels in barley malt. As for malting companies, barley procurement plays one of the most important roles in controlling DON level in the finished malt; although good malting practice can reduce *Fusarium* growth to some extent during steeping and germination. Physical treatments such as using a barley washer and increasing overflow time are believed to reduce *Fusarium* spores by cleaning barley kernels. In the past, chemical treatment including formaldehyde with hot water, hypochlorite, and mercuric chloride showed powerful effects on *Fusarium* reduction in barley (Kottapalli and Wolf-Hall, 2008 Medina et al., 2006; Ramakrishna et al., 1991). However, many of these practices may affect product quality, or result in severe side reactions, and may be not be as effective as expected. Furthermore, the demand for safe and natural food products by consumers has made these practices out of the question.

Researchers have already tried various strategies on biological control of FHB and fungal growth in malting process. A starter culture of yeast strain belonging to *Geotrichum sp* was applied in the first steep in malting process at lab scale. The results indicated that the *Fusarium* growth were completely inhibited and the finished malts were mycotoxin free compared to the control sample which showed significant contamination (Boivin and Malanda, 1997).

Laitila et al. (2007) investigated the effect of a yeast strain *Pichia anomala* on reduction of *Fusarium* growth and mycotoxin production. The addition of the strain into steeping water was demonstrated to be useful to restrict *Fusarium* growth as well as prevent beer gushing. Combination of a *Lactobacillus plantarum* strain and the yeast strain may help (Laitila et al., 2007). Another yeast strain, *Geotrichum candidum*, was found to effectively reduce the final concentration of T-2 toxin by 93% in beer when it was added into the steeping process (Gastelum-Martinez et al., 2012). Yeasts are still the major microbes used in the field of biological control of FHB and DON production.

The bio-active compounds made by microbes were also studied for *Fusarium* growth inhibition in the malting process. AFP, an antifungal protein from fungal strain belonging to *Aspergillus giganteus* was applied into steeping process to test its capacity as a bio-control agent. It appeared that the reduction of *Fusarium* growth was close to 50% whereas the DON production was 33% lower than the control sample. The quality analysis also suggested that there was no significant difference between the control sample and the treated barley malts (Barakat et al., 2010).

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SCREENING OF LACTIC ACID BACTERIA FOR ANTI-FUSARIUM ACTIVITY AND OPTIMIZATION OF INCUBATION CONDITIONS

Abstract

Anti-*Fusarium* activities of lactic acid bacteria (LAB), *Lactobacillus plantarum* 299V, *Lactobacillus plantarum* NRRL-4496, and *Lactobacillus rhamnosus* VT1, were determined by a microdilution assay against *Fusarium graminearum* 08/RG/BF/51. A cell-free *Lactobacillus* culture supernatant (CFLCS) of *L. rhamnosus* VT1 showed the highest anti-*Fusarium* activity.

Response surface methodology (RSM) was employed to optimize the incubation conditions for production of CFLCS. A Box-Behnken factorial design was used to investigate the effects of incubation time, shaking speed and incubation temperature. The results suggested that an incubation temperature at 34ºC, a shaking speed at 170 rpm, and an incubation time of 55 hours were the best combination for production of CFLCS from *L. rhamnosus* VT1. Under these incubation conditions, a 10% CFLCS of *Lactobacillus rhamnosus* VT1 was predicted to inhibit the growth of *Fusarium graminearum* by 75.6% *in vitro*, and inhibited 83.7% of the *Fusarium in vitro*. This study demonstrated that the CFLCS of *Lactobacillus rhamnosus* VT1 was an effective anti-*Fusarium* mixture.

Introduction

Fusarium graminearum is the predominant pathogenic microorganism that causes *Fusarium* head blight (FHB), a widely occurring plant disease of cereal crops. FHB reduces the yield and quality of grain. In addition, *Fusarium graminearum* produces

trichothecenes mycotoxins. The most commonly encountered *F. graminearum* trichothecenes in cereal crops are deoxynivalenol (DON), 3-acetyl-4-deoxynivalenol (3-DON), 15-acetyl-4-deoxynivalenol (15-DON), nivalenol (NIV), and 4-acetyl-nivalenol (FX) (Brul and Coote, 1999). Mycotoxins may lead to safety and quality problems in the food industry, such as negative effects on quality of beer and bread (Champeil et al., 2004). Trichothecenes can lead to symptoms including vomiting, reduction in food consumption, skin irritation, diarrhea, multiple hemorrhage, and immunosuppression (Champeil et al., 2004; Parent-Massin, D, 2004). Furthermore, it is known that these trichothecenes are heat-stable and cannot be eliminated by regular processes of food processing (El-Nezami et al, 1998a). The major trichothecene, DON, is reported to be stable up to 170 °C at neutral to acidic pH (Wolf and Bullerman, 1998). In general, FHB results in food safety risks and economic losses. Therefore, discovery of new approaches that inhibit the growth of *Fusarium* and reducing of mycotoxin production in plants and food products has been an important research objective.

Biological intervention strategies could replace the traditional synthetic fungicides in preventing and/or eradicating mycotoxigenic *Fusarium* growth and mycotoxin production. For an example, as the only commercial microorganism, Biomin® BBSH 797 ®, has been authorized by EU for reduction of trichothecene levels caused by *Fusarium* in animal feed (Biomin, 2014).

Lactic acid bacteria (LAB) have been widely used to preserve food throughout human history. Presently, LAB are also the most common microorganisms in bio-preservation research and application. LAB are thought to preserve foods by removing fermentable carbohydrates, consuming oxygen, and producing organic acids, which

decrease the pH value in food matrices; furthermore, specific inhibitors produced by various LAB strains could also be the reason for the improved preservative effect (Ganzle, 2009). The LAB strains of the genus *Lactobacillus* frequently are reported to have antifungal activity, and include *L. coryniformis* (Magnusson et al., 2003), *L. plantarum* (Magnusson et al., 2003; Laitila et al., 2002). *L. paracasei subsp.tolerans* (Hassan and Bullerman, 2008), *L. rhamnosus* (Stiles et al., 2002), *L. acidophilus*, *L. amylovorus*, *L. brevis*, and *L. coryniformis subsp.coryniformis* (De Muynck et al., 2004). These strains inhibit the growth of many different yeast and fungi, such as various species of *Aspergillus*, *Penicillium*, and *Fusarium*. Because *Lactobacillus* species have been widely reported to be associated with antifungal activity, it is possible that metabolites produced by *Lactobacillus* may inhibit the growth of *Fusarium* and reduce the accumulation of *Fusarium*-produced trichothecenes.

Previously, the most commonly used assay to test the antifungal activity of potential antifungal compounds was the agar diffusion assay. Various agar diffusion assays include sterile paper disk, well in agar, and direct diffusion in agar, whereby the antifungal activity of compounds are determined by the size of inhibition zone produced (Rizzello et al., 2011; Wang et al., 2012; Gerez et al., 2009; Yang et al., 2011). Thus, a challenge with agar diffusion assays is the subjective interpretation of results. The microdilution assay, has been widely used to test the antifungal activity of the potential antifungal compounds with less experimental time, lower cost, as well as more objective interpretation of results (Riesselman et al,. 2000).

The objective of this study was to screen lactic acid bacteria with the highest anti-*Fusarium* activity among *Lactobacillus plantarum* 299V*, Lactobacillus plantarum* NRRL-

4496, and *Lactobacillus rhamnosus* VT1. A statistical model using Response Surface Methodology (RSM) was further employed to optimize the incubation conditions for the selected LAB strain.

Material and Methods

Fungal strain and lactic acid bacteria strains

F. graminearum 08/RG/BF/51 was used in this study. This culture was provided by Dr. Rubella S. Goswami in the department of Plant Pathology at North Dakota State University and is known to be a strong producer of DON.

Lactobacillus plantarum 299V, *Lactobacillus plantarum* NRRL-4496, and *Lactobacillus rhamnosus* VT1 were the LAB used in the study. *Lactobacillus plantarum* 299V and *Lactobacillus rhamnosus* VT1 were from the Food Science and Technology Department at the University of Nebraska (Stiles et al., 2002). *Lactobacillus plantarum* NRRL-4496 was from the USDA ARS Culture Collection (Peoria, IL).

Preparation of F. graminearum inoculum suspension

Carboxymethyl cellulose (CMC) broth was used for conidia production by *F. graminearum* 08/RG/BF/51 strain. This broth contained 0.5 g NH_4NO_3, 0.5 g KH_2PO_4, 0.25 g $MgSO_4*7H_2O$, 0.5 g yeast extract, 7.5 g carboxymethyl cellulose, and 500 ml distilled water (Cappellini and Peterson, 1965). The culture was incubated with shaking at a speed of 250 rpm at room temperature for 14 days. The culture was filtered through sterile cheese cloth and centrifuged at 16,300×g for 40 minutes. The pellet was collected

and resuspended in 50 ml of 15% sterilized glycerol solution. The concentration of the *Fusarium* conidia was determined using a hemacytometer.

Preparation of LAB culture supernatant

All LAB strains were grown separately in MRS broth (Difco; Sparks, MD) at 35ºC for 24 or 48 h. The cultures were centrifuged at 6,160×g for 10 min and the supernatant was filtered through a sterile 0.2 µm syringe filter (Pall Life Sciences; Port Washington, NY). The cell free LAB culture supernatant (CFLCS) was stored at 4ºC.

Microdilution assay

A microdilution assay was developed with standardized 96-well microplates to investigate the anti-*Fusarium* activity of CFLCS. For screening of LAB strains, the proportion of the CFCLS was 50% while the proportion of the CFLCS for optimization of incubation conditions was 10%. One hundred microliters of CFLCS, 80 µl of concentrated GYEP broth containing 2.5 g/L peptone, 2.5 g/L yeast extract, 25 g/L dextrose, and per L distilled water (Zhang, 2006), as well as 20 µl of *F. graminearum* conidia suspension containing 10^4 conidia were dispensed into each well (n=5) of the 96 will microplate (Figure 2) to test the antifungal activity of the lab culture suspension. One hundred microliters of CFLCS, 80 µl of concentrated GYEP broth, and 20 µl of sterile water were dispensed into each well (n=3) as a sample blank (Lavermicocca et al, 2003). One hundred microliters of sterile water, 80 µl of concentrated GYEP broth, and 20 µl of conidia suspension containing 10^4 spores were dispensed into each well (n=8) as a positive control while 120 µl of sterile water and 80 µl of concentrated media were added into each well

(n=8) as the negative control. The control and blank wells on microplates were used to compare the fungal growth within different experimental series.

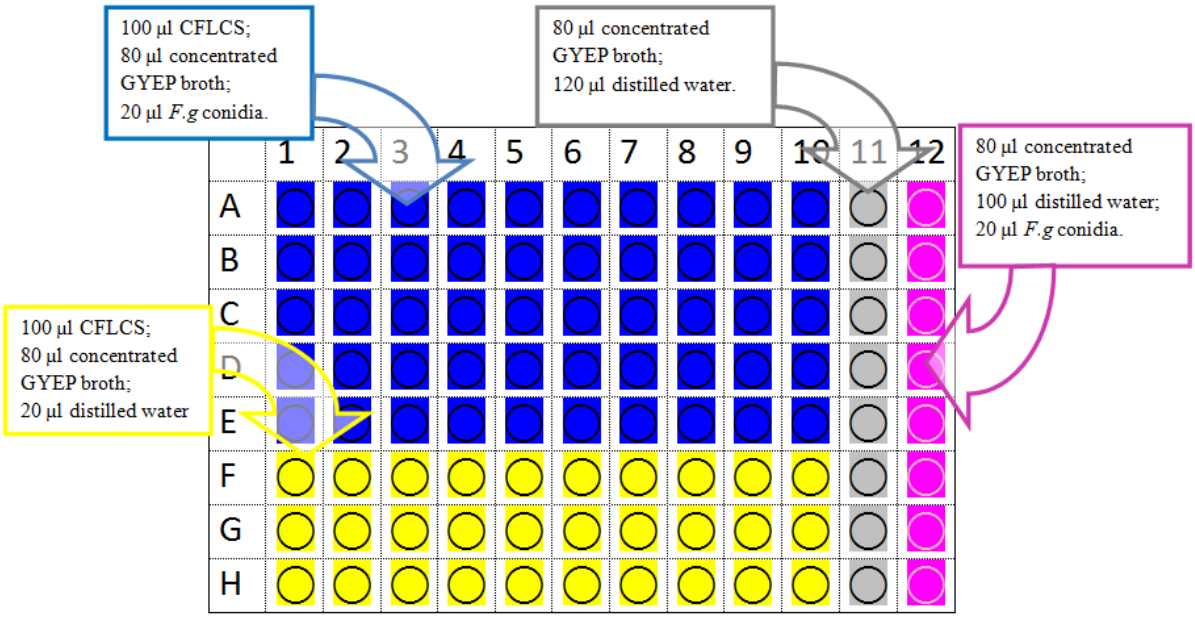

Figure 2. The well layout of microplates for the microdilution assay.

The optical density (OD) 580 nm was determined at 0, 24, 48, and 72 hours of incubation via a microplate photometer (Biotek; Highland Park, WI). The growth was evaluated by measuring the OD value of each well (Oliva et al., 2003; Salah et al., 2003). The fungal growth in sample wells were compared to the fungal growth in the wells with MRS broth substituting for the CFLCS. The anti-*Fusarium* activity of the CFLCS was calculated as *Fusarium* inhibition percentage by the following formula (Kouassi et al., 2012):

$$\text{The \% } Fusarium \text{ inhibition} = \left[1 - \frac{sample\ readings\ at\ 72nd\ hr - sample\ blank\ readings\ at\ 72\ hr}{MRS\ readings\ at\ 72nd\ hr - \ MRS\ blank\ readings\ at\ 72\ hr}\right] * 100$$

In order to obtain a wider range of *Fusarium* inhibition percentage, when operating the experiments of optimization of incubation conditions, the CFLCS was diluted fivefold before dispensed into wells and led to a 10% final concentration of CFLCS in each well.

Experimental design for screening

Incubation time is a potential factor to affect the anti-*Fusarium* activity of LAB strains (Batish et al., 1997; De Muynck et al., 2004; Bianchini, 2010). Therefore, incubation time for the three LAB strains was involved in screening of LAB for anti-*Fusarium* activity. Multiple LAB strains and incubation time in this study were evaluated (Table 1).

Table 1. Combination of the LAB strain and incubation time of the CFLCS samples.

Sample name	Strain	Incubation time
STA1	*Lactobacillus plantarum* NRRL-4496	24 hours
STA2	*Lactobacillus plantarum* NRRL-4496	48 hours
STB1	*Lactobacillus plantarum* 299V,	24 hours
STB2	*Lactobacillus plantarum* 299V,	48 hours
STC1	*Lactobacillus rhamnosus* VT1	24 hours
STC2	*Lactobacillus rhamnosus* VT1	48 hours

Determination of minimal inhibitory concentration (MIC) value of the CFLCS

To obtain more information regarding the CFLCS (produced by *Lactobacillus rhamnosus* VT1 via the optimized incubation condition), a minimal inhibitory

concentration (MIC) value, the lowest concentration of the CFLCS where no growth of *Fusarium* could be observed, was determined (Lind et al., 2005). The MIC value of the CFLCS was determined in quintuplicate by the microdilution assay developed in this study. CFLCS at various concentrations, 10%, 12%, 14%, 16%, 18%, 20%, were added into the microplate wells with media and conidia. The same concentrations of MRS broth were used as controls.

Experimental design for incubation condition

Response surface methodology (RSM) was used to select the best incubation conditions for improving anti-*Fusarium* inhibition rate. RSM is a collection of mathematical and statistical techniques and uses quantitative data to give predictive multivariate equations and analyzes optimum conditions of factors for the best solution. The RSM has been widely used for optimization of fermentation parameters for various final products (Li et al., 2007a,b). Therefore, in order to achieve the highest anti-*Fusarium* activity of the CFLCS, RSM was employed to optimize the incubation conditions in this study including temperature, shaking speed, and time.

Box-Behnken factorial design, which is a widely used RSM design, was chosen to investigate the incubation conditions for *Lactobacillus rhamnosus* VT1. This design included three factors and three levels, as well as three replicates at the center point (Li et al., 2005). The coded values of the variables in the experimental design (Table 2) and design matrix (Table 3) with 15 trials are shown in below, respectively.

Table 2. The coded values of the variables used in central composite rotary design.

Independent variables	Level		
	-1	0	1
Incubation temperature	30	35	40
Shaking speed (rpm)	0	100	200
Incubation time (h)	24	48	72

Table 3. Box-Behnken experiment design matrix.

Trial #	Variables			Trial #	Variables		
	X_1 (Temp, ºC)	X_2 (Speed, rpm)	X_3 (Time, h)		X_1 (Temp, ºC)	X_2 (Speed, rpm)	X_3 (Time, h)
1	30	0	48	2	30	200	48
3	40	0	48	4	40	200	48
5	35	0	24	6	35	0	72
7	35	200	24	8	35	200	72
9	30	100	24	10	40	100	24
11	30	100	72	12	40	100	72
13	35	100	48	14	35	100	48
15	35	100	48				

This methodology allows the modeling of a second order equation that describes the regression process. Anti-*Fusarium* inhibition rate was analyzed by a multiple regression through the least squares method to fit the following equation:

$$Y = A_0 + \sum A_i X_i + \sum A_{ii} X_i^2 + \sum A_{ij} X_i X_j$$

where Y is the predicted response variable (inhibition rate); A_0 is the constant coefficient, A_i, A_{ii}, A_{ij} are interaction coefficients of the linear, quadratic, and second order terms of the model, respectively; and X_i, X_j (i =1, 3; j =1, 3, i ≠ j) represent the independent variables (incubation conditions) in the form of coded values. In general, the coefficient of determination, R^2, was used to evaluate the accuracy and reliability of the polynomial model. In the current study, we carried out each experimental design in triplicate (n=3).

Statistical analysis

The desired goals of the current study were to select the maximum *Fusarium* inhibition rate by optimizing several incubation conditions. The Design-Expert® 8.0 software (demo version; Stat-Ease Inc., Minneapolis, USA) is a powerful Windows®-based program to optimize process parameters to achieve best performance. Numerical optimization can be performed in this program to find maximum desirability for tens of responses simultaneously. Therefore, it was chosen for the current study to analyze the data and to estimate the coefficient of the regression equation.

The regression analysis and analysis of variance (ANOVA) were conducted using the response surface module of the Design-Expert® 8.0 package to fit second order polynomial equations for the response variable, *Fusarium* inhibition rate. The quality of fit of the polynomial model was expressed by the coefficient of determination, R^2, and the adjusted coefficient of determination, $R\text{adj}^2$, and statistical significance was checked by the Fisher's F-test. Canonical analysis was used to predict the optimal incubation condition of screened *Lactobacillus* strain.

Results and Discussion

Screening LAB, based on the anti-Fusarium activity by microdilution assay

Three LAB strains, *Lactobacillus plantarum* 299V*, Lactobacillus plantarum* NRRL-4496*,* and *Lactobacillus rhamnosus* VT1, were investigated in this study. Considering the incubation time may affect the anti-*Fusarium* activity of the LAB culture due to the effect on production of anti-*Fusarium* compounds (Batish et al., 1997; De Muynck et al., 2004; Bianchini, 2010), the combination of the LAB strains and incubation time were also investigated in this study. Six CFLCS samples were obtained and compared for their anti-*Fusarium* activity with MRS broth as a negative control. The anti-*Fusarium* activities of the various CFLCS are shown in Figure 3.

In this study, anti-*Fusarium* activity was described by the inhibition rate of the CFLCS. The higher inhibition rate represents the lower *Fusarium* growth in the wells on the microplate. The CFLCS samples had various anti-*Fusarium* activities (Figure 3). The lowest inhibition rate among the six samples was 85.6%, with 50% CFLCS produced by *L. plantarum* 299V after a 24-hr incubation. The ANOVA analysis indicated that the anti-*Fusarium* activities could be categorized into four significantly different groups, which means the dependent variables, LAB strain and incubation time, affected the independent variable, the inhibition rate.

Furthermore, considering the effect of incubation time on the inhibition rate within the same strain, the incubation time affected the inhibition rate more obviously (Figure 3). As for *L. plantarum* 299V and *L. rhamnosus* VT1, the anti-*F*usarium activity of the CFLCS increased with increasing incubation time; however, *L. plantarum* NRRL-4496

produced opposite results where the anti-*Fusarium* activity of the corresponding CFLCS decreased with extended incubation time.

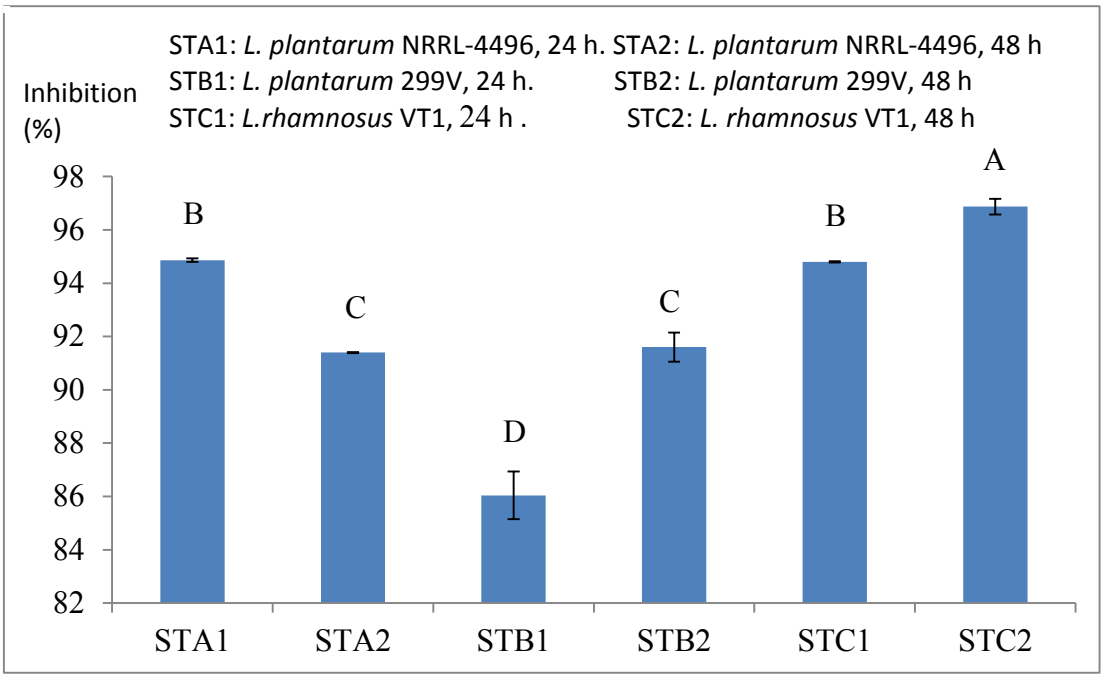

Figure 3. The effects of LAB strain and incubation time on the *Fusarium* inhibition. means with standard deviations (n=3).

In general, STC2, which was produced by *L. rhamnosus* VT1 after 48 hours incubation, had significantly higher anti-*Fusarium* activity compared to the others. Therefore, *L. rhamnosus* VT1 was selected as the target strain for further research.

Optimization of incubation conditions for anti-Fusarium activity by RSM

Regression models of response

In the current study, RSM based on the Box-Behnken design was used to investigate the effects of incubation conditions on the antifungal inhibition rate and optimize the best conditions. Fifteen experimental runs with the combination of three factors in terms of incubation temperature, shaking speed and incubation were carried out (Table 2). According to the Box-Behnken experiment design matrix, treatment runs 13-15 were repeated three times as the center points in the design and to estimate of the error of the model (Table 3). The experimental responses for the 15 runs are shown in Table 4, which suggests that there was a considerable variation in the antifungal inhibition rate relying on the three independent variables.

Table 4. The inhibition rate values of the CFLCS following Box-Behnken design.

Trial number	Inhibition Rate (%)	Trial number	Inhibition Rate (%)
1	50.26	2	61.97
3	61.74	4	56.00
5	44.80	6	51.87
7	48.66	8	69.00
9	23.66	10	55.93
11	56.28	12	41.58
13	73.00	14	74.00
15	74.40		

The maximum inhibition rate (74.4%) was achieved in run # 15, while the minimum inhibition rate (23.66%) was observed in run # 9. By simply comparing the two runs, the results clearly indicated the dependent variables, a given shaking speed, the changes of incubation temperature and time significantly resulted in the dramatic change in the inhibition rate. The variances for the response of antifungal inhibition rate was analyzed by the Fisher's statistical test (Table 5).

Table 5. Analysis of variance for the response of antifungal inhibition rate[a].

Source	Degree of freedom	Sum of squares	Mean square	*F*-value	*P*>*F*
Linear	3	418.27	-	12.05	0.0100
Quadratic	3	0.57	-	43.89	0.0005
Crossproduct	3	0.25	-	19.35	0.0035
Total model	9	0.98	-	25.10	0.0012
Total error	5	57.86	11.57		-

[a]Coefficient of variation (CV) = 6.05; coefficient determination (R^2) = 0.978; correlation coefficient (R) = 0.989

A very low probability value (($P_{model}>F$) = 0.0012) and the associated *F*-value (25.10) suggests that the model is highly significant. The second order model was found to be well adjusted to the experimental data by judging the analysis of variance (*F*-test). Coefficient of variance (CV) was calculated from the ratio of standard deviation and mean and also indicates the degree of precision with which the treatments were compared. In

general, the lower the CV, the higher the relative reliability of the estimate (Li et al., 2007c). The lower value of CV (6.05) indicates a greater reliability of the experiments performed (Table 5) in the model (Li et al., 2007b). The coefficient of determination (R^2) is the square of the correlation between the constructed predictor and the response variable and measures the global fit of the model. In statistical models, the coefficient of determination (R^2) is commonly used to predict future outcomes based on other related information. The determination coefficient (R^2) implies that the sample variation of 97.8% for antifungal inhibition rate is attributed to the three independent variables, and only about 2.2% of the total variation cannot be explained by the RSM model. Generally, if R^2 value is greater than 0.9, this means a very satisfactory model performance (Li et al., 2007a). Finally, the quadratic model was selected in this optimization study due to the significances in both linear and quadratic terms at the 1% level.

The Student *t*-distribution and the corresponding *P*-value, along with the parameter estimate indicate significant differences existed (Table 6). The *P*-values are used as a tool to check the significance of each of the coefficients, and also help us better understand the pattern of the mutual interactions between various variables (Li et al., 2007d). A smaller *P*-value indicates a larger significance of the corresponding coefficient.

The parameter estimates and the corresponding *P*-values (Table 6) indicates that among the independent variables including incubation temperature and shaking speed, incubation time had an individual significant effect on inhibition rate. Positive coefficients for all three independent variables indicated a linear effect to increase inhibition rate. The quadric terms of incubation temperature and incubation time also showed a significant effect but with negative coefficients to decrease inhibition rate. One interaction between

incubation temperature and incubation time was also found to contribute to the response at a significant level.

Table 6. The least-square fit and parameters (significance of regression coefficient).

Model term	Degree of freedom	Estimate	Standard Error	*t* value	*P*> | *t* |
Intercept	1	-845.14	91.06	-9.28	0.0002*
X_1	1	41.88	5.02	8.34	0.0004*
X_2	1	0.34	0.13	2.66	0.0451*
X_3	1	6.30	0.58	10.80	0.0001*
X_1^2	1	-0.51	0.071	-7.21	0.0008*
X_1X_2	1	-0.0087	0.0034	-2.56	0.0504
X_2^2	1	-0.00035	0.00018	-2.00	0.1017
X_1X_3	1	-0.098	0.014	-6.90	0.0011*
X_2X_3	1	0.0014	0.00071	1.95	0.1086
X_3^2	1	-0.029	0.0031	-9.42	0.0002*

* Significant at 5% level (P<0.05)

By applying multiple regression analysis on the experimental data, the following second-order polynomial equation was used to estimate the relationship between antifungal inhibition rate and three incubation factors by only considering the significant terms and is shown below:

[Inhibition Rate]

$$= 41.88 \times [\text{Temp}] + 0.34 \times [\text{Speed}] + 6.3 \times [\text{Time}] - 0.51 \times [\text{Temp}]2$$

$$- 0.098 \times [\text{Temp}] \times [\text{Time}] - 0.29 \times [\text{Time}]2 - 845.14$$

Comparison of observed and predicted antifungal incubation rate

The observed inhibition rate (the response) versus those predicted values generated from the empirical model clearly indicates that the predicted inhibition rate data from the empirical model was in good agreement with the observed ones in the range of the investigated variables (Figure 4).

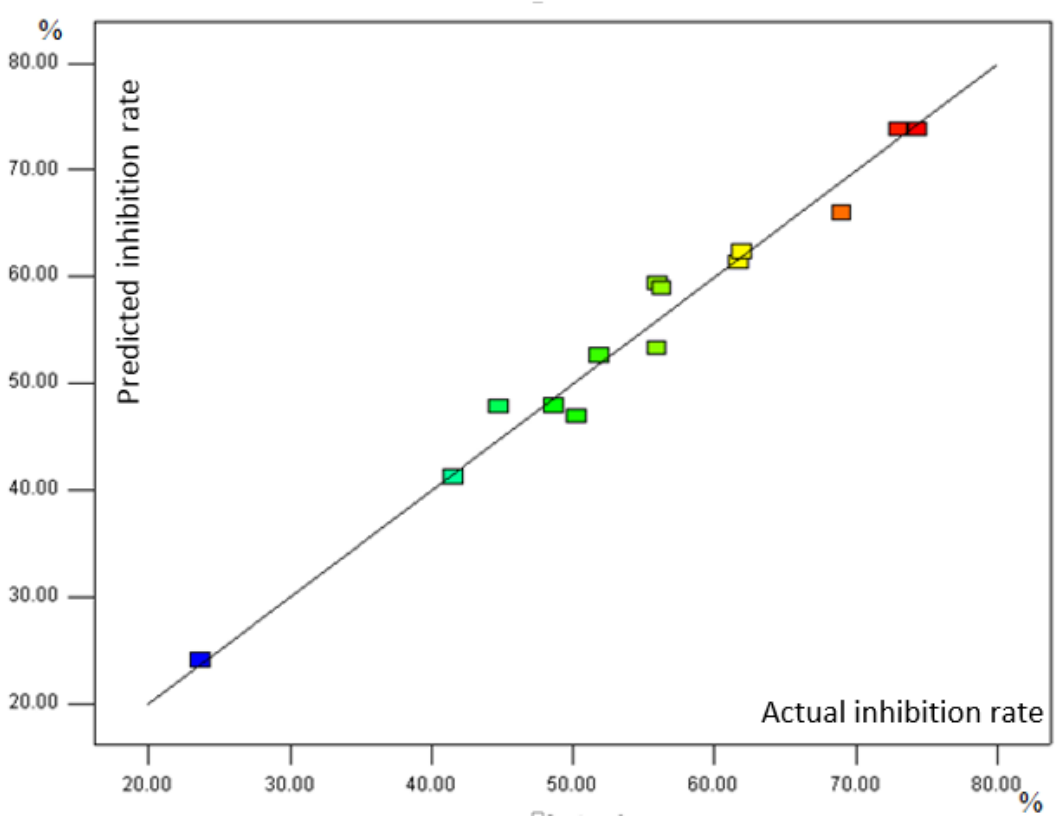

Figure 4. Observed inhibition rate versus the predicted inhibition rate under optimum incubation conditions.

Before estimating the best conditions of the model, it is of necessity to check the fitted model to ensure that it provides an adequate approximation to the real system. By constructing a normal probability plot of the residuals, a check was made for the normality assumption (Figure 5). The straight line observed in the residual plots satisfies the normality assumption as the residuals plot approximated along a straight line. Therefore, we believe that the empirical model is adequate to describe the inhibition rate by response surface.

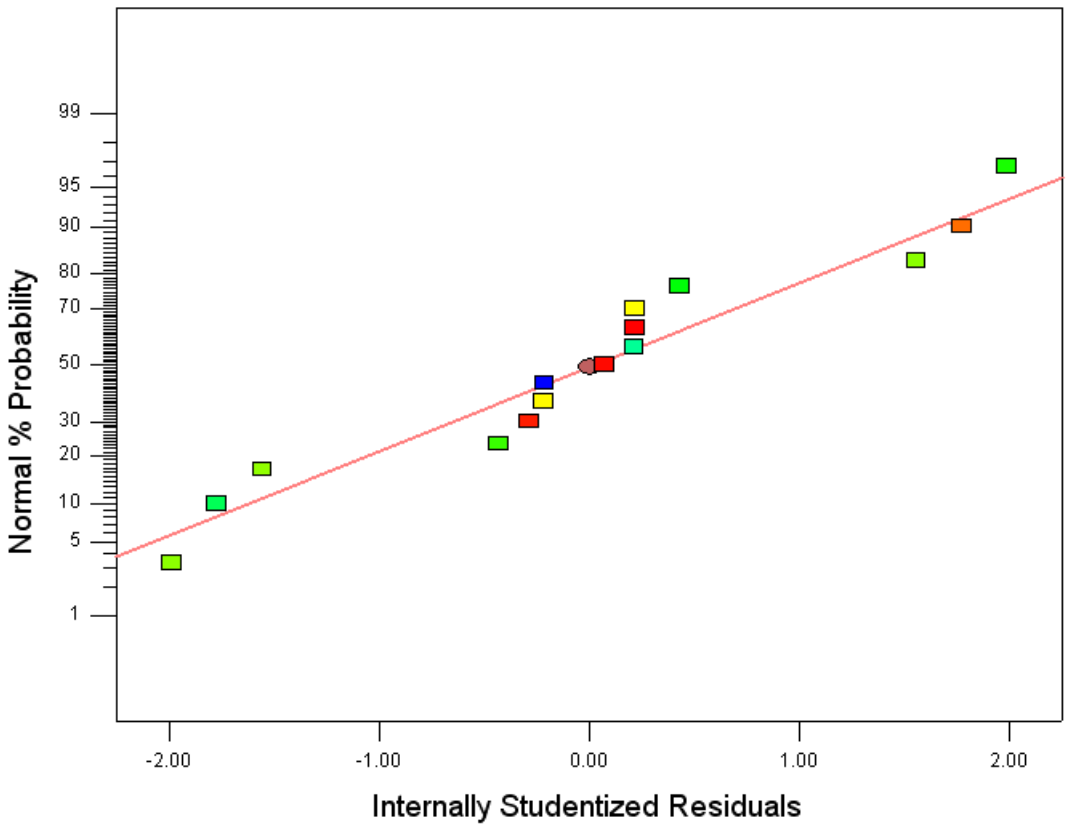

Figure 5. Normal probability of internally studentized residuals.

Localization of optimum condition

The fitted response surface 3D plots and their corresponding contour plots for the inhibition rate by the above model are shown in Figure 6, 7, and 8, respectively.

At relative high shaking speed (above 100 rpm), the antifungal inhibition rate increased gradually with increasing incubation temperature (Figure 6) . However, with low incubation temperature range, there was no significant effect of shaking speed on the inhibition rate. The analysis of Figure 6 revealed that the optimal ranges of incubation temperature and shaking speed for antifungal inhibition rate were 34.6-35.7ºC and 140-155 rpm, respectively. By solving the inverse matrix using the Design-Expert software, the maximum predicted inhibition rate of 74.6% was obtained.

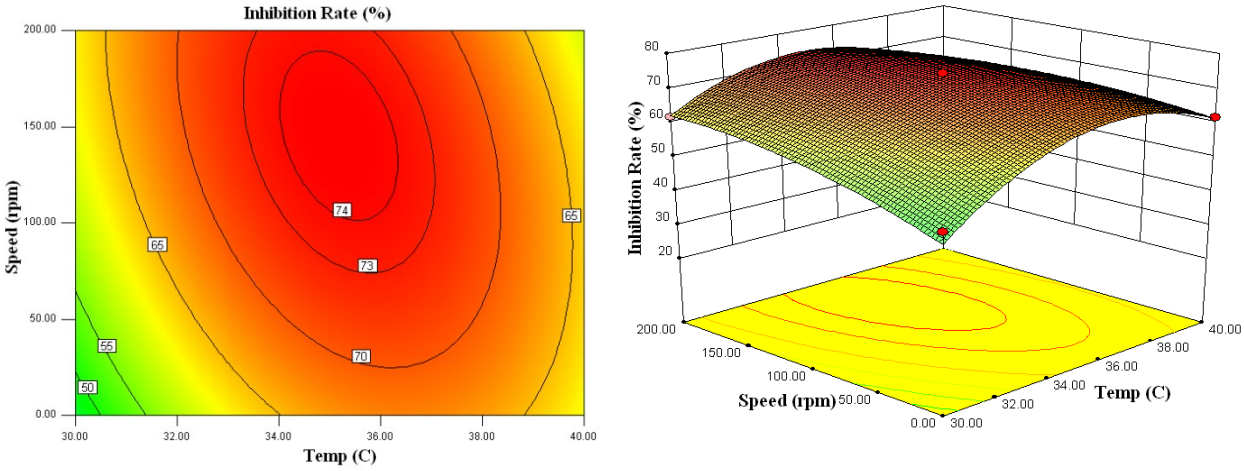

Figure 6. Response surface plot and contour plot of the combined effects of incubation temperature and shaking speed for the inhibition rate.

The effect of incubation temperature and incubation time on the inhibition rate indicated both incubation temperature and incubation time at the middle range was beneficial to improvement of antifungal inhibition rate (Figure 7).

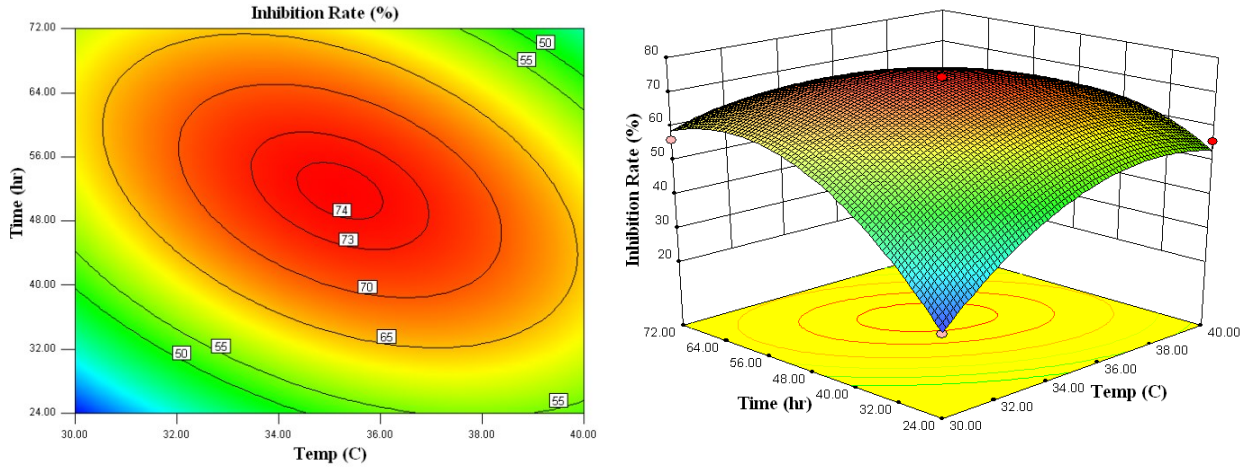

Figure 7. Response surface plot and contour plot of the combined effects of incubation temperature and incubation time for the inhibition rate.

The maximum inhibition rate could be obtained in the range of incubation temperature 34.7-35.6^{o}C, and incubation time 49-54 hours.The effects of shaking speed and incubation time on the inhibition rate, while incubation temperature was kept at its middle levels (35^{o}C) indicated that at relative high shaking speed, extending incubation time to certain extent significantly improved antifungal inhibition rate (Figure 8).

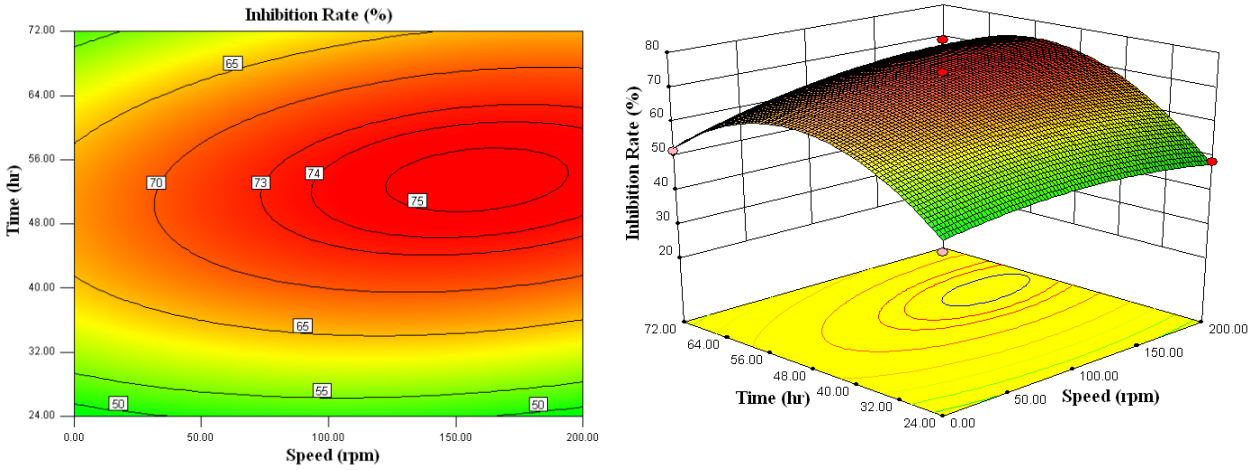

Figure 8. Response surface plot and contour plot of the combined effects of shaking speed and incubation time for the inhibition rate.

The optimal ranges of incubation time and shaking speed for antifungal inhibition rate were 52-54 h and 147-168 rpm, respectively. By solving the inverse matrix using the Design-Expert software, the maximum predicted inhibition rate of 75.4% was obtained.

Prediction and validation of the model

The statistical optimal values of variables were obtained when moving along the major and minor axis of the contour and the response at the center point yields maximum inhibition rate (Cui et al., 2009). These observations were also verified from canonical analysis of response surface. The canonical analysis also confirmed that the maximum point is a stationary point and revealed a minimum region, best optimal conditions, for the model. The stationary point presenting a maximum incubation rate had the following critical values: incubation temperature = 34.3^{o}C, shaking speed =170 rpm, and incubation time = 55 h. Under these incubation conditions, the predicted antifungal incubation rate of a 10% CFLCS of *Lactobacillus rhamnosus* VT1 was 75.6% *in vitro*. Further experiment indicated that 83.7% of the *Fusariumin in vitro* was inhibited by a 10% CFLCS of *Lactobacillus rhamnosus* VT1 for these incubation conditions. This study demonstrated that the CFLCS of *Lactobacillus rhamnosus* VT1 was an effective anti-*Fusarium* mixture.

MIC value of the CFLCS

MIC value, the lowest concentration where no *Fusarium* growth could be observed in the microplate wells, was detected for the CFLCS. Under the specific conditions, MIC value of the CFLCS obtained in this study was 18%, which means that once the CFLCS

reaches a concentration of 18% in the microplate well, there will be no *Fusarium* growth with a 10^4 conidia /well inoculum concentration.

Conclusions

In comparisons with other strains, *Lactobacillus rhamnosus* VT1 was selected as the target strain for further study due to its higher anti-*Fusarium* activity. RSM design with three factors and three levels as well as triplicates at the center point was conducted to optimize the incubation conditions of this strain. The results of the optimization experiments suggested that incubation temperature at 34ºC, shaking speed at 170 rpm, and incubation time of 55 hours were the best combination condition for production of antifungal compounds from *Lactobacillus rhamnosus* VT1 culture. Under this combinations, the % *Fusarium* inhibition due to CFLCS of *Lactobacillus rhamnosus* VT1 at a concentration of 10% was predicted to be 75.6 % *in vitro* and verified to be 83.7% of the *Fusariumin in vitro* with these optimized incubation conditions. With a 10^4 conidia /well inoculum concentration, MIC value of the CFLCS obtained in this study was 18%. CFLCS obtained in this study was an effective anti-*Fusarium* bio-active system and could be used as an inhibitor in the further study.

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ANALYSIS AND IDENTIFICATION OF THE BIO-ACTIVE COMPONENTS IN CELL FREE CULTURE SUPERNATANT FROM LACTOBACILLUS RHAMNOSUS VT1

Abstract

Identification of the bio-activie compounds in cell free *Lactobacillus* culture supernatant (CFLCS) could improve the understanding and enhancement of the mechanisms of anti-*Fusarium* activity. In order to identify the bio-active components of CFLCS, a stepwise regression, with α to enter = 0.15 and α to remove = 0.15, was performed to evaluate the significance of the factors on anti-*Fusarium* activity of CFLCS. The regression model was indicated that pH, total acidity, and 3-phenyllactic acid were the most important factors, which were statistically significant. Effects of heat treatments showed that the anti-*Fusarium* activity of the CFLCS was mostly lost by the heat treatment at 121ºC for 15 min and partially lost by the treatment at 100ºC for 15 min. Sensitivity test of the CFLCS to proteolytic enzymes indicated that proteinaceous compounds in the CFLCS were responsible for 5-10% of anti-*Fusarium* activity.

Introduction

Fusarium head blight (FHB) is a widely occurring plant disease predominantly caused by a fungi called *Fusarium graminearum*. FHB leads to a reduction in the yield and quality of grain. Trichothecenes, a group of heat-stable mycotoxins produced by *Fusarium graminearum*, result in some technical problems in the food industry such as negative effects on quality of beer and bread (Champeil et al., 2004; El-Nezami et al, 1998a). In

addition, trichothecenes are important food safety concerns (Champeil et al., 2004; Parent-Massin, D, 2004).

New biological approaches for inhibition of *Fusarium* growth and reduction of trichothecenes are of interest. Only one biological intervention strategy was authorized by the European Union (EU) as far as to reduce trichothecenes in feed which involves in *Eubacterium* sp. BBSH 797 (Biomin, 2014). Among the potential biological approaches, lactic acid bacteria (LAB), are commonly known for production of antimicrobial compounds, including organic acids, hydrogen peroxide, diacetyl, CO_2 and bacteriocins (Corsetti et al., 1998). Due to their GRAS (generally recognized as safe) status (Magnusson et al., 2003; Brosnan et al., 2012), LAB are promoted as a high priority strategy for inhibition of *Fusarium* growth and reduction of trichothecenes.

The major bio-active compounds associated with the antimicrobial activity of LAB are organic acids, including lactic, benzoid, acetic, propionic, sorbic, formic, and 3-phenyllactic acids (Niku-Paavola et al., 1999; Brul and Coote, 1999; Rizzello et al., 2011; Corsetti et al., 1998). Also, a synergistic effect among a combination of *Lactobacillus plantarum* MiLAB 393 produced active compounds: cyclo (L-Phe-L-Pro), cyclo (L-Phe-*trans*-4-OH-L-Pro), and 3-phenyllactic acid has been identified (Sjögren et al., 2002).

In previous research (1st part of this dissertation), a LAB strain, *Lactobacillus rhamnosus* VT1, was found to produce cell-free culture *Lactobacillus* supernatant (CFCLS) with high anti-*Fusarium* activity. After optimization of the incubation conditions for this *Lactobacillus* strain, anti-*Fusarium* activity of the CFLCS was confirmed. Furthermore, in order to improve anti-*Fusarium* activity of the CFLCS and understand the mechanism, it is necessary to investigate the bio-active components of the CFLCS.

The objective of this study was to investigate the components of the CFLCS to determine which components play the most significant role in anti-*Fusarium* activity. To determine the potential bio-active compounds in the CFLCS, detection methods for lactic acid, acetic acid, and 3-phenyllactic acid were developed to analyze the CFLCS samples obtained from previous research (1st part of this dissertation). Besides the levels of these organic acids, pH, and total acidity as well as the biomass of the LAB culture before centrifugation and filtration were also measured. Stepwise regression was employed to determine the significant factors for the anti- *Fusarium* activity of the CFLCS. Sensitivity of the CFLCS to heat treatment and proteolytic enzymes were also tested.

Materials and Methods

Fungal strain and Lactobacillus strain

F. graminearum 08/RG/BF/51 was used in this study. This culture was provided by Dr. Rubella S. Goswami in the department of Plant Pathology at North Dakota State University and is known to be a strong producer of DON.

Lactobacillus rhamnosus VT1, which was from the Food Science and Technology Department at the University of Nebraska (Stiles et al., 2002), was used in this study to obtain the CFLCS with anti-*Fusarium* activity.

Preparation of inoculum suspension

Carboxymethyl cellulose (CMC) broth was used for conidia production by *F. graminearum* 08/RG/BF/51 strain. This broth contained 0.5 g NH_4NO_3, 0.5 g KH_2PO_4, 0.25 g $MgSO_4*7H_2O$, 0.5 g yeast extract, 7.5 g Carboxymethyl cellulose, and 500 ml

distilled water (Cappellini and Peterson, 1965). The culture was incubated at room temperature with shaking at 250 rpm for 14 days. The culture was filtered through sterile cheese cloth and centrifuged at 16,300×g for 40 minutes. The pellet was collected and resuspended in 50 ml of sterile, 15% glycerol solution. The concentration of the *Fusarium* conidia was determined using a hemacytometer.

Preparation of CFLCS

All *Lactobacillus* strains were grown separately in MRS broth (Difco; Sparks, MD) at 35ºC for 24 or 48 h. The cultures were centrifuged at 6,160×g for 10 min and the supernatant was filtered through a sterile 0.2 µm syringe filter (Pall Life Sciences; Port Washington, NY). The cell free LAB culture supernatant (CFLCS) was stored at 4ºC.

Microdilution assay

A standardized 96-well micro-plate assay was developed to investigate the anti-*Fusarium* activity of the CFLCS (Figure 9). Twenty µl of CFLCS, 80 µl of concentrated GYEP broth containing 2.5 g/L peptone, 2.5 g/L yeast extract, 25 g/L dextrose, and 1L distilled water (Zhang, 2006), 80 µl of sterile distilled water and 20 µl of *F. graminearum* conidia suspension containing 10^4 conidia were dispensed into each well (n=5) of the 96 well microplate to test the antifungal activity of the lab culture suspension, while 20 µl of LAB culture supernatant, 80 µl of concentrated GYEP broth, and 100 µl of sterile distilled water were dispensed into each well (n=3) as blanks (Lavermicocca et al, 2003). One hundred microliters of sterile water, 80 µl of concentrated GYEP broth, and 20 µl of conidia suspension containing 10^4 spores were dispensed into each well (n=8) as a positive

controls while 120 µl of sterile water and 80 µl of concentrated media were added into each well (n=8) as a negative control.

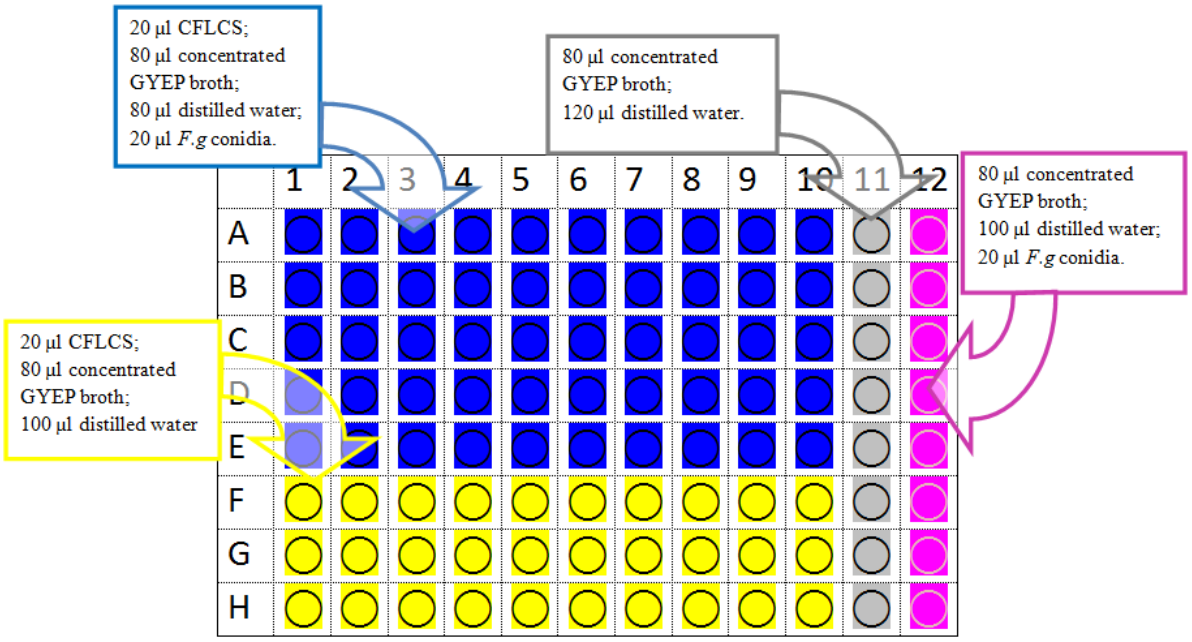

Figure 9. The layout of wells on microplate for the microdilution assay.

The optical densities (OD) were read at 0, 24, 48, and 72 hours of incubation via microplate photometer at 580 nm, and growth was evaluated (Oliva et al., 2003; Salah et al., 2003). Considering the probable effect of MRS broth on the fungal growth, the fungal growth in sample wells were compared to the fungal growth in the wells with MRS broth substituted for the CFLCS. The % *Fusarium* inhibition of the CFLCS was calculated by the following formula (Kouassi et al., 2012):

The % $Fusarium$ inhibition

$$= \left[1 - \frac{sample\ readings\ at\ 72nd\ hr - sample\ blank\ readings\ at\ 72\ hr}{MRS\ readings\ at\ 72nd\ hr - \ MRS\ blank\ readings\ at\ 72\ hr}\right] * 100$$

Determination of lactic acid and acetic acid in the CFLCS

The lactic acid and acetic acid levels in the CFLCS were detected using a Waters HPLC system 2695 (Milford, MA) with a photodiode array (PDA) detector. All *Lactobacillus* cultures were centrifuged at 6,160×g for 10 min, diluted with sterile distilled water (culture supernatant: sterile distilled water = 1:39) , and filtered through a sterile 0.2 μm syringe filter (Pall Life Sciences; Port Washington, NY) to be ready for analysis. MRS broth was treated with same method as a negative control. The HPLC column was a C18 column (Synergic 4μ Hydro-RP 80A, 150×4.6 mm, 3.5 μm). The mobile phase used was 0.02 mM KH_2PO_4/water solution which was adjusted to pH 2.55 by 0.1 M H_3PO_4. The mobile phase flow rate was 0.6 ml/min. Injection volume was 20 μl. The detection wavelength was 220 nm. Commercial lactic acid (85%) and acetic acid (Sigma-Aldrich; St.Louis, MO) were used as a standard.

Determination of 3-phenyllactic acid in the CFLCS

The 3-phenyllactic acid concentration in the CFLCS was measured by HPLC, which was operated on an Agilent 1200 series HPLC (Santa Clara, CA) with a UV detector. All *Lactobacillus* cultures were centrifuged at 6,160×g for 10 min, and filtered through a sterile 0.2 μm syringe filter (Pall Life Sciences; Port Washington, NY) to be ready for analysis. MRS broth was treated with same method as control. The columns included a C18 column (Zorbax Eclipse-XDB, 4.6×75 mm, 3.5 μm) and a guard column (Zorbax Eclipse-XDB, 4.6×12.5 mm, 5 μm). The system was flushed by a gradient mobile phase as follows: (1) 0–3 min, 75% A and 5% B; (2) 4–6 min , 50% A and 50% B; (3) 8–12 min, 100% B; (4) 14–15 min, 75% A and 25% B, where A was 0.1% acetic acid and B

was 0.1% acetic acid in acetonitrile (Mallinckrodt Baker). The flow rate was 0.8 ml/min. Injection volume was 20 µl. The detection wavelength was 210 nm. Commercial 3-phenyllactic acid (Sigma-Aldrich; St.Louis, MO) was used as a standard.

Determination of biomass of the Lactobacillus rhamnosus* VT1 *culture

The incubated *Lactobacillus rhamnosus* VT1 culture was mixed evenly by vortex vibrations. The culture (1 ml) was transferred to a screw-cap tube with 9 ml sterile distilled water and mixed. Then 5 ml of the diluted culture was transferred to another screw-cap tube with 5 ml sterile distilled water. After mixing completely, 2-3 ml of the diluted culture was transferred to a cuvette. MRS broth was treated with same method as control. The optical density of the diluted culture was read at 620 nm.

Determination of total acidity of the CFLCS

Two milliliter of the CFLCS was transferred to a 125-ml flask. Ten milliliter of distilled water and three drops of phenolphthalein indicator were added to the flask. A standard 0.1 mol/L NaOH solution was used to titrate the mixture to a persistent pink color. The volume of NaOH to achieve the pink color was recorded to calculate the total acidity (Downes and Ito, 2001). According to the concentration of NaOH used and the volume of CFLCS tested, the formula used to calculate the total acidity (% lactic acid) was:

$$\%\ \text{lactic acid} = \frac{(\text{ml NaOH}) \times 0.1 \times 0.09 \times 100}{2}$$

Sensitivity of the CFLCS to proteolytic enzymes

In order to test the occurrence of the proteinaceous compounds in the CFLCS, the CFLCS, which had an initial pH value of 3.95, was adjusted with 1 M NaOH solution to pH 4, pH 7 and 8, and then treated with proteolytic enzymes (Ndagano et al., 2011). Three proteolytic enzymes were employed: pronase (pH 4), proteinase K (pH 7), and trypsin (pH 8). All of enzymes were dissolved in phosphate or citric acid buffer at a concentration of 10 mg/mL. The final concentration of the enzymes in CFLCS was 1 mg/ml. The mixtures of CFLCS and enzyme were incubated at 30ºC for 1 h. A 5-min denaturation of proteolytic enzymes was performed after incubation for all of the samples by incubation at 60ºC. The treated samples were stored at 4ºC. All of sample preparation experiments were repeated in triplicates. Then the anti-*Fuasarium* activities of these treated samples were tested by the microdilution assay.

Sensitivity of the CFLCS to temperature

In order to test the temperature sensitivity of the CFLCS, four CFLCS samples were incubated in a water bath at 30, 60, and 80ºC for 30 min and heated in a boiled water bath for 15 min, respectively. A fifth CFLCS sample was autoclaved at 121ºC for 15 min (Todorov et al., 2011; Hassan and Bullerman, 2008; Falguni et al., 2009). All sample preparations experiments were repeated three times. All treated samples were tested for anti-*Fuasarium* activities by the microdilution assay.

Statistical analysis

In order to evaluate all factors and to consider all reasonable regression models for anti-*Fusarium* activity, a stepwise regression was performed to analyze the data of the samples obtained from the RSM design performed in previous research. Stepwise regression is widely used among the variable selection methods,. The procedure of stepwise selection is as follows: (1) a simple linear regression model of each independent variable is fitted with the smallest sum of squares due to error (SSE); (2) After a new independent variable is added to the model, all variables are tested for significance to determine if any variable should be deleted; (3) The procedure continues until all variables are either added or deleted to the model on the basis of setting significant level (Weisberg, 2005). In this study, all calculations regarding stepwise regression were done by SAS Enterprise Guide 4.3. Proc REG was used to run a stepwise regression, with α to enter and remove equal to 0.15. In this analysis, stepwise regression was used to evaluate how much variability could be explained by each independent variable including lactic acid, pH, biomass, acetic acid, total acidity, and 3-phenyllactic acid, for the dependent variable, inhibition rate.

Results and Discussion

Stepwise analysis for the factors that may affect anti-Fusarium activity associated with the CFLCS

Lactobacillus rhamnosus VT1, was screened due to its high anti-*Fusarium* activity among the group of LAB strains tested (1st part of this dissertation). Later, the incubation

conditions of the strain were optimized via a RSM design, which indicated that the anti-*Fusarium* activity of 83.7% could be achieved with a CFLCS concentration of 10%. However, the bio-active components in the CFLCS were unknown.

The potential antifungal compounds produced by LAB may include organic acids, such as lactic, benzoid, acetic, propionic, sorbic, formic, and 3-phenyllactic acids ((Niku-Paavola et al., 1999; Brul and Coote, 1999; Rizzello et al., 2011; Corsetti et al., 1998; Strom et al., 2002; Prema et al., 2010). Also, it has been indicated that there is a synergistic effect of combinations of *Lactobacillus plantarum* MiLAB 393 produced active compounds including cyclo (L-Phe-L-Pro), cyclo (L-Phe-*trans*-4-OH-L-Pro) and 3-phenyllactic acid (Sjögren et al., 2002).

In this study, an independent variable selection was performed to obtain a regression model, which could explain the dependent variable reasonably, and to obtain a model which could as accurately as possible describe, predict, and control the dependent variable. Among all dependent variables involved in the model, PLA was the first one to enter the model while pH and total acidity were also tested to be significant in the model (Table 7).

Lactic acid level, acetic acid level, and biomass were not significant to the dependent variable, inhibition rate, or the anti-*Fusarium* activity of the CFLCS. Although the three significant variables could be taken out after stepwise selection, the model only could be used to explain 39.19% of variation of the anti-Fusarium activity. It means only 39.19% change of the dependent variable could be explained by the model which composed by the the three variables, PLA levels, pH, and Total acidity.

Table 7. Summary of Stepwise Selection for the RSM design data.

Step	Variable Entered	Variable Removed	# of Vars In	Partial R^2	Model R^2	C(p)	F Value	Pr > F
1	PLA*	-	1	0.2539	0.2539	2.7985	9.53	0.0045
2	pH	-	2	0.0805	0.3343	1.6927	3.26	0.0820
3	Total_acidity	-	3	0.0575	0.3919	1.4722	2.46	0.1289

*PLA: 3-phenyllactic acid

SAS software was used to check the fitness of the model resulting in the the fit diagnostics for the dependent variable of the model (Figure 10).

The residuals from the predicted value are mostly distributed randomly in the entire area (Figure 10-a). A new normal distribution of the residuals along quantile while no obvious outlier could be observed (Figure 10-b). The comparisons between the real experimental value and the predicted value of the inhibition rate was determined (Figure 10-c). The data points are mostly normal distributed around the trend line, which means the model obtained from the stepwise analysis is good to use for predicting the inhibition rate. However, Figure 10-d, the figure of Percentage vs. Residual, which should be normally distributed, shows a right shifting trend and a short tail. Also, it is apparent that fit-mean and residual were not normal distributed with proportion less according to the bended trend line (Figure 10-e).

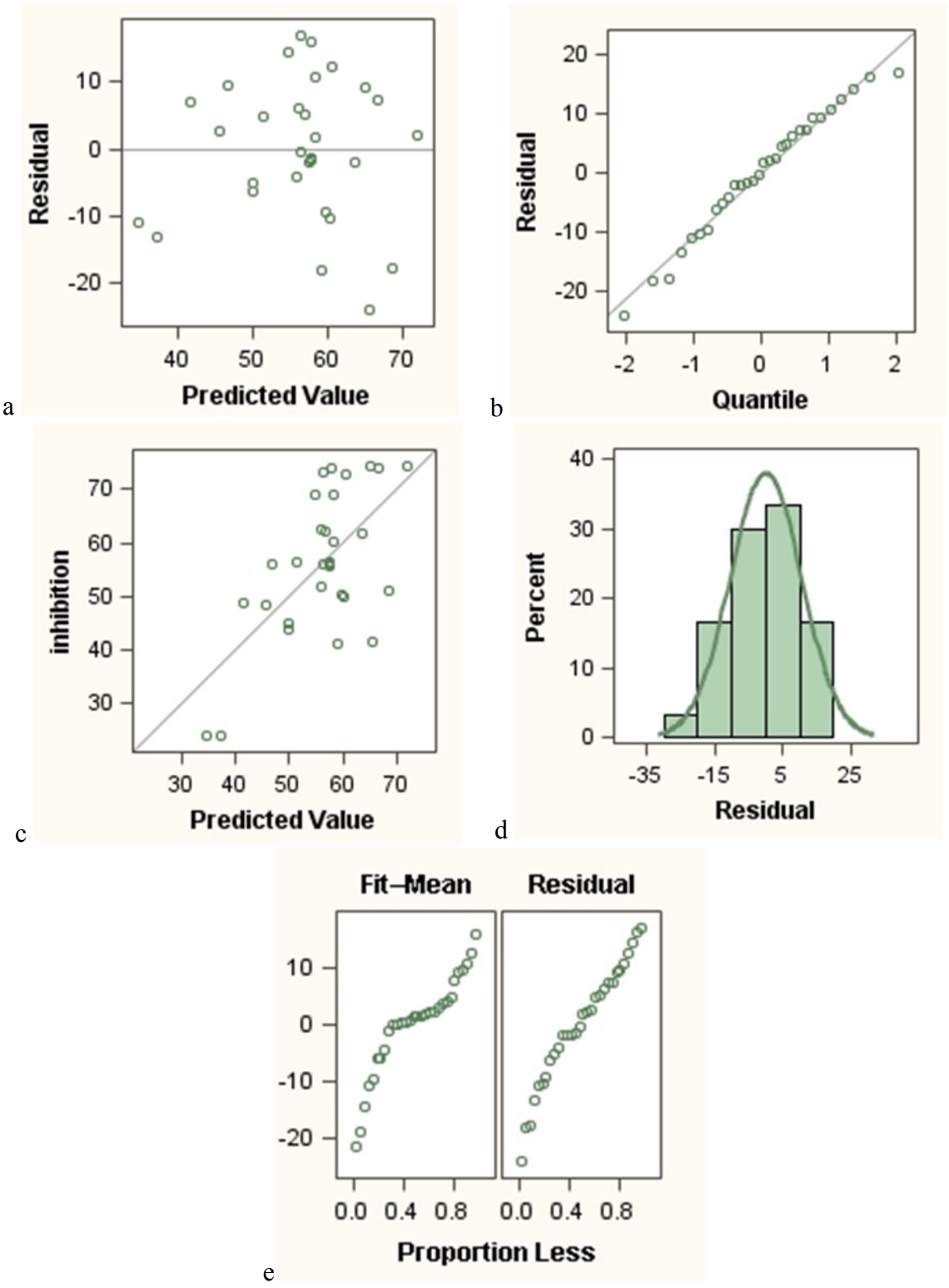

Figure 10. Fit diagnostical charts for *Fusarium* inhibition rate.

In general, stepwise selection has clarified that PLA, pH, and total acidity are the three significant factors for anti-*Fusarium* activity of the CFLCS. Also, although PLA level is low compared to other organic acids detected, it was the most important compound for anti-*Fusarium* activity. The model provided valuable information for identification of the anti-*Fusarium* compounds in the CFLCS. However, the fit diagnostic for anti-*Fusarium* activity suggested that the model is not a perfect one to predict the anti-*Fusarium* activity of the CFLCS. In other words, the hidden information indicated that there may be other important bio-active compounds in the CFLCS besides PLA. Although lactic acid and acetic acid are not significant factors for the anti-*Fusarium* activity, which is not consistent with some research that lactic acid is a bio-active compound in *Lactobacillus* culture; the other organic acids and other acidic compounds in the CFLCS would also be responsible for anti-*Fusarium* activity.

Comparison of anti-Fusarium activity of the CFLCS as well as the individual and mixtures of chemicals in the CFLCS

In order to have more information regarding the possible bio-active components of the CFLCS, the anti-*Fusarium* activities of the components of CFLCS were compared with the CFLCS. Lactic acid (LA), acetic acid (AA), 3-phenyllactic acid (PLA), and the mixtures of lactic acid and acetic acid (LA & AA), lactic acid and 3-phenyllactic acid (LA & PLA), acetic acid and 3-phenyllactic acid (AA & PLA) as well as lactic acid, acetic acid, and 3-phenyllactic acid (LA, AA & PLA) at the same concentrations as their concentrations in the CFLCS were tested for anti-Fusarium activities and compared to the CFLCS (Figure 11).

In order to describe the relative anti-*Fusarium* activities of the samples more clearly, the anti-*Fusarium* activities of the various samples were normalized where the value of the CFLCS was adjusted to 100%. All of organic acids and mixures showed various anti-*Fusarium* activities. LA had the highest anti-*Fusarium* activity among the individual organic acid samples while the mixture of lactic acid, acetic acid, and 3-phenyllactic acid had the highest anti-*Fusarium* activity among all four mixtures.

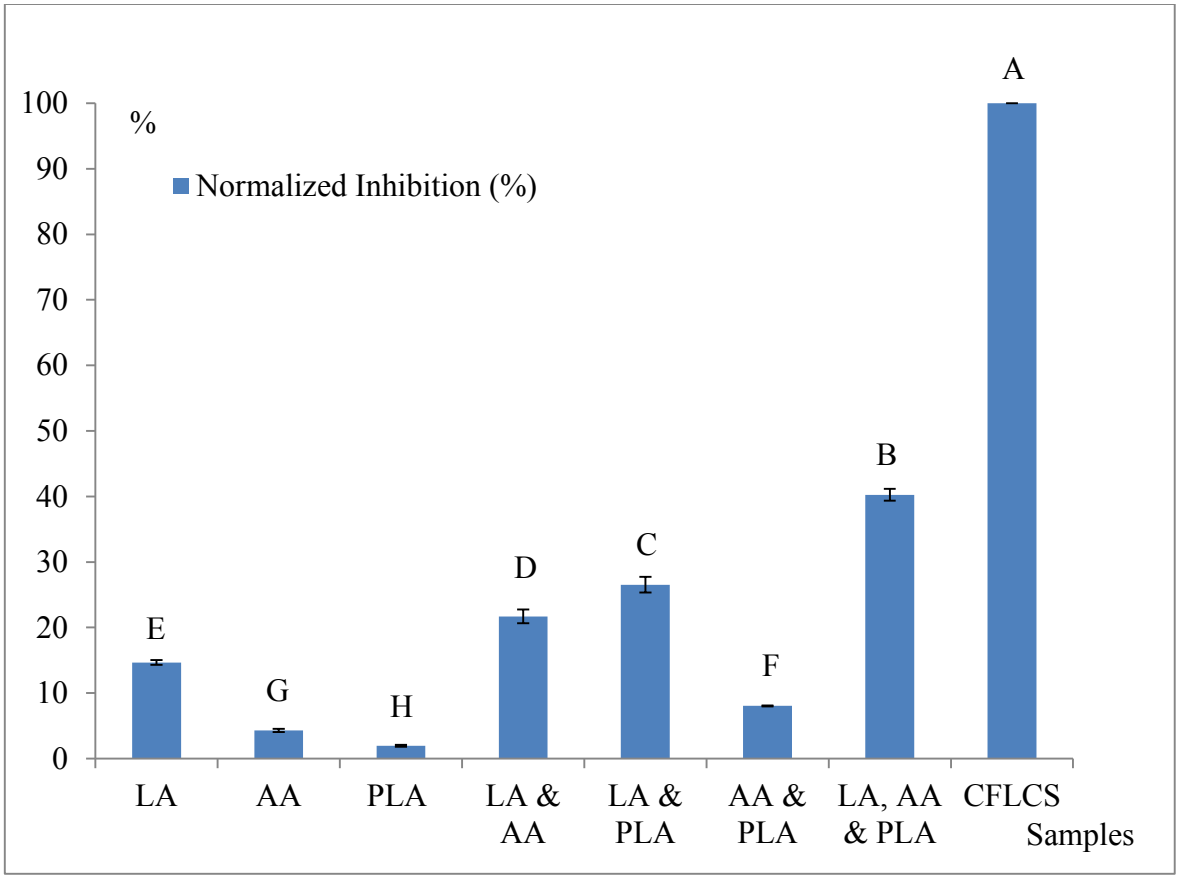

Figure 11. Comparision of anti-*Fusarium* activity of individual and mixed compounds in the CFLCS and the orginal CFLCS. Means with standard deviations (n=3).

Although it was demonstrated that PLA plays an important role in the CFLCS via the stepwise selection, it did not have high anti-*Fusarium* activity at the very low

concentration in the CFLCS (44.9 ± 1.0 μg/ml). On the other hand, the higher anti-*Fusarium* activity of lactic acid and acetic acid might be due to a concentration (2.31 % for lactic acid, 0.78% for acetic acid) effect. The results of the mixtures also indicated that the combination of the individual organic acids improved the anti-*Fusarium* activities.

Sensitivity of the CFLCS to temperature and proteolytic enzymes

In addition to organic acids, antifungal activity associated with LAB was also reported for proteinaceous compounds, such as nisin, cyclo (L-Phe-L-Pro), cyclo (L-Phe-*trans*-4-OH-L-Pro), lactocin NK24 (Noonpakdee et al., 2003; Sjögren et al., 2002). Compared to organic acids, proteinaceous compounds are more sensitive to temperature. Therefore, sensitivity of the CFLCS to temperature was tested in this study (Table 8).

Table 8. Sensitivity of the CFLCS to heat treatments.

Temperature (ºC)	Treated Time (min)	Relative Anti-*Fusarium* activity (%)	Statistical significant difference
30	30	99.74	A
60	30	95.69	B
80	30	82.45	C
100	30	46.53	D
121	15	15.81	E

There is almost no effect on anti-*Fusarium* activity after heating the CFLCS at 30ºC for 30 min (Table2-2). Most of anti-*Fusarium* activity was lost after autoclaving at 121ºC for 15 min while partial anti-*Fusarium* activity was lost after heating at 100ºC for 30 min. The results indicated that temperature sensitive compounds in the CFLCS affect its anti-*Fusarium* activity.

In order to investigate further if there is any proteinaceous compound in the CLFCS, sensitivity of the CFLCS to proteolytic enzymes was tested in this study. Previously, the concentration of the CFLCS in the microdilution assay was 10% for the RSM design. However, in this experiment, concentration at 10% was too low to detect the anti-*Fusarium* activity of the CFLCS sample with higher pH. In a preliminary experiment for sensitivity of CFLCS to proteolytic enzymes, it was found that the anti-*Fusarium* activity of the CFLCS decreased quickly with increasing pH. *Fusarium* grew very well in microplate wells with 10% concentration CFLCS samples at pH 8. The final readings also indicated that these conditions could not inhibit any *Fusarium* growth. Therefore, a higher concentration of 15% was employed as the concentration of the CFLCS added in the wells to test the anti-*Fusarium* activity.

The anti-*Fusarium* activities of the CFLCS and treated CFLCS demonstrate a pH effect (Figure 12). Blank means the original CFLCS at pH 3.95. pH4, pH7, and pH 8 blank represent the adjusted CFLCS with pH equal to 4, 7, and 8, respectively. pH4, pH7, and pH 8 sample are the CFLCS treated with different proteolytic enzymes.

According to Figure 12, pH affects the anti-*Fusarium* activity of the CFLCS: the higher pH, the lower the anti-*Fusarium* activity. The pH value of the original CFLCS is close to 4, which was also reflected by the similar anti-*Fusarium* activity of the two

samples. After the pH of the CFLCS was adjusted to 7, anti-*Fusarium* activity of the CFLCS was decreased quickly even without the treatment of proteolytic enzyme. It was apparent that the change from pH 4 to pH 7 affected anti-*Fusarium* activity much more than the treatment of proteolytic enzymes. This was consistent with the results in stepwise selection, which indicated that pH is a significant factor to the anti-*Fusarium* activity of the CFLCS. The effect of pH also indicated that undissociated forms of organic acids may play a more important role in anti-*Fusarium* activity of the CFLCS than the dissociated forms (Presser et al., 1997).

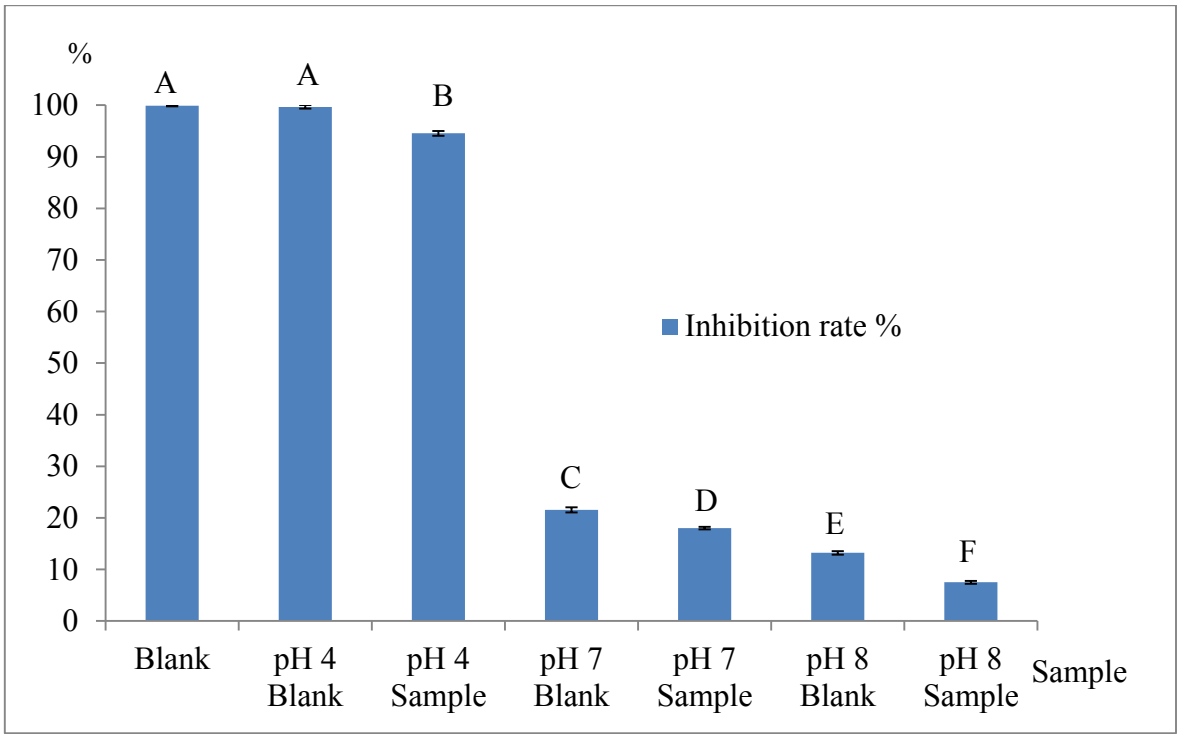

Figure 12. Sensitivity of the CFLCS to proteolytic enzymes. Means with standard deviations (n=3).

Similar to the effect of pH adjustments, after hydrolization by various proteolytic enzymes, the anti-*Fusarium* activity of the CFLCS decreased. The anti-*Fusarium* activity reductions caused by pronase (pH 4), proteinase K (pH 7), and trypsin (pH 8), which were

5.1%, 3.6%, and 5.7%, respectives, were much lower than the percentage reductions caused by pH change along. This indicated that pH may play a more important role in CFLCS than proteinaceous compounds responsible for anti-*Fusarium* activity. Also, the reduction percentage caused by different proteolytic enzyme suggested that the proteinaceous compounds are mixtures of compounds with various isoelectric points (pI). Furthermore, these preoteinaceous compounds in the CFLCS probably include more acidic amino acids than neutral and basic amino acids.

Conclusions

A stepwise regression, with α to enter = 0.15 and α to remove = 0.15, was performed to evaluate the significance of lactic acid, pH, biomass, acetic acid, total acidity, and 3-phenyllactic acid on anti-*Fusarium* activity of the CFLCS. The results demonstrated that pH, total acidity, and 3-phenyllactic acid were statistically significant to the regression model and could be used to explain 39.19% variation of the anti-*Fusarium* activity.

According to the model, the anti-*Fusarium* activity of individuals and mixtures of lactic acid, acetic acid, 3-phenyllactic acid, at same concentration with the CFLCS were compared with the original CFLCS. The results indicated that the anti-*Fusarium* activities of the three organic acid individualls and mixtures were obviously lower than the CFLCS. Effects of heat treatments also showed that the anti-*Fusarium* activity of the CFLCS was mostly lost by the heat treatment at 121ºC for 15 min and partially lost by the treatment at 100ºC for 15 min.

In order to test the sensitivity of the CFLCS to proteolytic enzymes, the CFLCS was treated by pronase (pH 4), proteinase K (pH 7), and trypsin (pH 8), and the anti-

Fusarium activities of the treated CFLCS were compared. It was clarified that the proteinaceous compounds in the CFLCS only were responsible for 5-10% of anti-*Fusarium* activity.

In general, the anti-*Fusarium* activity of the CFLCS could not just be explained by the major organic acids, lactic acid and acetic acid. Proteinaceous compounds are unknown, but they may serve an important role due to the possible synergistic effect between organic acids and proteineous compounds.

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EFFECTS OF LACTOBACILLUS RHAMNOSUS VT1 CULTURE SUPERNATANT ON FUSARIUM GRAMINEARUM GROWTH AND MYCOTOXIN PRODUCTION IN CULTURE AND BARLEY MALT

Abstract

Cell free *Lactobacillus* culture supernatant (CFLCS) was demonstrated to inhibit *Fusarium graminearum* growth in glucose-yeast extract-peptone (GYEP) broth and homemade potato dextrose agar (HPDA). The CFLCS at concentrations of 30% and 40% applied in rice culture inhibited the growth of *F. graminearum,* but increased mycotoxin concentrations in rice culture. At a concentration at 50% CFLCS, *F. graminearum* growth was inhibited completely and no mycotoxins were detected.

As replacements of steeping water during the malting process, the CFLCS concentrations of 30% and 50% significantly reduced the *F. graminearum* growth and mycotoxin accumulations in naturally infected barley. However, the germinative abilities of the barley samples were inhibited.

Introduction

In the United States, *Fusarium graminearum* is considered the predominant causative agent of *Fusarium* head blight (FHB) in barley (Mathre, 1997). FHB occurred frequently in the Upper Midwest region of Minnesota, North Dakota, and South Dakota during the 1990's and led to reduced crop yield as well as quality (Salas et al., 1999; Schwarz et al., 1995). FHB resulted in the accumulation of mycotoxins as well, which are the toxic secondary metabolites of the pathogenic fungi in the infected crops. Among the

mycotoxins produced by *Fusarium* species, trichothecenes, especially deoxynivalenol (DON), are the most commonly found mycotoxins in cereal crops (Brul and Coote, 1999). It is known that adverse effects on human and animal health can occur when mycotoxins enter food and feed chains (Champeil et al., 2004).

The increasing demand for natural food products and food ingredients has triggered the research on biological intervention strategies in preventing and/or eradicating mycotoxigenic *Fusarium* growth and mycotoxin production. Among all potential biological strategies, lactic acid bacteria (LAB) are considered as a high priority strategy for inhibition of *Fusarium* growth and reduction of trichothecenes due to their GRAS (generally recognized as safe) status (Magnusson et al., 2003; Brosnan et al., 2012).

Although there is no certain relationship between the growth of *Fusarium* and the accumulation of trichothecenes (Mesterházy et al. 1999; Snijders 2004), one possible strategy that could be applied in malting and brewing is to reduce the trichothecene accumulation during the malting process via prevention of the *Fusarium* growth.

In previous research (1st part of the dissertation), a LAB strain, *Lactobacillus rhamnosus* VT1, was screened for inhibition of growth of *Fusarium graminearum* via a microdilution assay. After optimization of the incubation conditions for this *Lactobacillus* strain, anti-*Fusarium* activity of the cell free *Lactobacillus rhamnosus* VT1 culture supernatant (CFLCS) was confirmed and increased. The objective of this study was to investigate the effect of the CFLCS on *F. graminearum* growth and DON accumulation. CFLCS was applied in glucose-yeast extract-peptone (GYEP) broth and homemade potato dextrose agar (HPDA) to test the effect on growth of *F. graminearum*. Also, CFLCS was applied in rice culture to test the effect of the CFLCS on *F. graminearum* growth and DON

accumulation. On the basis of the result of application on rice, the anti-*Fusarium* activity and DON-reducing ability of the CFLCS was tested on barley during the malting process.

Materials and Methods

Grains

Long-grain white rice used for culture medium was purchased at a local grocery store. Barley used for malting in this study included varieties Stellar and Pinnacle, which were grown in North Dakota during 2012 and provided by the Department of Plant Sciences at North Dakota State University. Barley was infected with FHB.

Fungal culture and lactic acid bacterium strains

The *Fusarium* strain used in this study was *F. graminearum* 08/RG/BF/51, which was provided by the department of Plant Pathology at North Dakota State University, and is known as a strong DON producer.

Lactobacillus rhamnosus VT1, which was from the Food Science and Technology Department at the University of Nebraska, was used in this study to obtain the CFLCS with anti-*Fusarium* activity (Stiles et al., 2002).

Preparation of inoculum suspension

CMC broth was used for conidia production by *F. graminearum* 08/RG/BF/51. This broth contained 0.5 g NH_4NO_3, 0.5 g KH_2PO_4, 0.25 g $MgSO_4*7H_2O$, 0.5 g yeast extract, 7.5 g carboxymethyl cellulose (CMC), and 500 ml distilled water (Cappellini and Peterson, 1965). The culture was incubated with shaking at a speed of 250 rpm at room

temperature (~25ºC) for 14 days. Then the culture was filtered through sterile cheese cloth and centrifuged at 16,300Xg for 40 minutes. The pellet was collected and resuspended in 50 ml of 15% sterile glycerol solution. The concentration of the *Fusarium* conidia was determined using a hemocytometer.

Preparation of CFLCS

The *L. rhamnosus* VT1 was grown separately in MRS broth (Difco; Sparks, MD) at 35ºC for 24 and 48 h. The cultures were centrifuged at 6,160Xg for 10 min and the supernatant was filtered through a sterile 0.2 µm syringe filter (Pall Life Sciences; Port Washington, NY). The cell free *Lactobacillus* culture supernatant (CFLCS) was stored at 4ºC.

Application of the CFLCS in liquid culture

0.8 ml of concentrated GYEP media (2.5 g/L peptone, 2.5 g/L yeast extract, and 25 g/L dextrose), and 0.2 ml of conidia suspension containing 10^5 conidia were added to 20 ml screwe capped glass tubes in duplicate. One milliliter of diluted CFLCS in water solution at concentrations of 20%, 30%, 40%, and 50% was added to the tubes, respectively. The tubes were incubated at 25ºC for two days. *Fusarium* growth was determined visually. A clear solution was recorded as no growth, and a turbid solution was recorded as growth. Photographs were taken on first four days to record the growth of *Fusarium*. The experiment was conducted in triplicates and repeated twice.

Application of the CFLCS on homemade potato dextrose agar (HPDA)

Homemade potato dextrose agar was prepared according to the method set by Nelson et al. (1983). Once the poured plates were cooled and the agar was solidified, a sterile burette was used to dig wells in the agar. Then, 40 µl of CFLCS at concentrations of 100% and 50% were transferred to the wells, respectively. MRS broth was used as the blank. Each well was inoculated with 10 µl of conidia suspension at a concentration of 105 conidia /ml. Then the plates were incubated at 25ºC with natural light for seven days. Photographs were taken on first four days to record the growth of *Fusarium*. The experiment was performed in triplicate and repeated twice.

Application of the CFLCS in rice culture

Application of the CFLCS in rice culture utilized the culture method of Wolf-Hall and Bullerman (1998). After the rice medium with the moisture content at 40% in jars were ready for use, 12.5 ml of the solution of sterile MRS broth and CFLCS were placed on the surface of the 25 g rice in each jar evenly to obtain the final moisture content at 60% for the control samples. The CFLCS concentration investigated in this study were based on the preliminary study and were 30, 40, and 50% CFLCS/ water dilution, respectively. MRS broth was employed as control to compare the anti-*Fusarium* activity of the CFLCS in rice culture. The jars were shaken for 30 seconds in order to distribute the liquids. Then the jars were left to equilabrate for 24 hr. *F. graminearum* 08/RG/BF/S1 conidia (1 ml) at a concentration of 10^6 conidia per 1ml in 15% sterile glycerol solution was transferred to each jar, while 1 ml of 15% sterile glycerol solution was added to blank jars. The jars were shaken for 30 seconds in order to distribute the liquids. The blank, control and sample jars

were repeated in triplicates. The jars were then incubated at room temperature (~25ºC) for 14 days with a 12 hr/day fluorescent light cycle. On the first two days, the jars were shaken by hand for 30 seconds to ensure even growth and prevent clumping. The experiments were conducted in triplicates and repeated twice.

Application of the CFLCS on barley malting process

A standard malting protocol, including a 34-hour steeping process and regular 4-day germination, was used in this study. Based on the dry weight of two barley samples, 22.20 g Stellar and 22.24 g Pinnacle, were weighed and put in 16 oz. mason jars, in triplicates. 75 ml steeping solution with CFLCS at 15%, 30%, or 50% were added to the mason jars. The concentration of the CFLCS was determined by the results of the application of CFLCS in rice culture. After six hours of steeping, barley samples were drained and given a two-hour air rest. Then fresh steeping solution at the appropriate concentrations was added to the samples in the mason jars. The steeping and air rest steps were repeated four times. After the fourth air rest, fresh steeping solution was added for a two-hour final steep. Then the barley samples were dried and weighed to 35.78 g via adjustment with distilled water. Then the barley samples were kilned (Karababa et al., 1993). Barley samples were kilned using a 24-hr schedule in which the temperature was sequentially ramped from 49 to 85°C. The details were as follows: 49°C for 10 hr, raise to 54° C with 30-min ramp rate; 54°C for 4hr, raise to 60°C with 30-min ramp rate; 60°C for 3 hr, raise to 68°C with 30-min ramp rate; 68°C for 2 hr; raise to 85°C with 40-min ramp rate for final 3 hr. The same concentration of MRS broth was used as a control in this study. The experiment was conducted in triplicate. The final samples were stored at 4 ºC.

Quantification of F. graminearum 08/RG/BF/S1 in rice culture via real-time PCR

Real-time PCR was employed to quantify the amount of *F. graminearum* 08/RG/BF/S1 growth in rice culture (Boddeda, 2006). The fungal DNA of rice culture was extracted via Quick DNA kit following manufacturer's instructions. The extracted DNA was stored at -18 ºC. After DNA extraction, the purity of the obtained DNA was measured by Nanodrop. For real-time PCR, 1 µl of *Fusarium*Tri-5 forward primer, 1 µl of *Fusarium*Tri-5 rear primer, 1 µl of extracted fungal DNA, 9.5 µl sterile Nano pure water, and 12.5 µl Sybr green mixture were put in each well. The reactions were carried out on an iCycler iQ real-time PCR machine (Bio-Rad; Hercules, CA). The experimental cycle for the real-time PCR is shown in Table 9. Each reaction was performed in triplicate.

Table 9. Real-time PCR protocol.

Cycles	Repeat times	Steps	Temperature (ºC)	Time
Cycle 1	1	Step 1	95.0	03:00
Cycle 2	40	Step 1	95.0	00:30
		Step 2	60.0	00:30
		Step 3	72.0	00:30
Cycle 3	1	Step 1	72.0	05:00
Cycle 4	1	Step 1	95.0	01:00
Cycle 5	1	Step 1	55.0	01:00
Cycle 6	80	Step 1	55.0ºC	00:10
Cycle 7	1	Step 1	4.0	HOLD

Analysis of deoxynivalenol in rice culture and barley samples

Levels of mycotoxins including deoxynivalenol (DON), zeralenone (ZEA), and 3-acetyl-deoxynivalenol (3-ADON) were detected on GC-ECD by James Gillespie in Malting Barley Quality lab in the Department of Plant Sciences at North Dakota State University (NDSU) (Tacke and Casper, 1996). The limit of quantitation is 0.4 ppm.

Analysis of Fusarium biomass in barley samples

Levels of *Fusarium* biomass in barley samples were measured via quantitative real-time PCR by Dr. Robert Brueggeman in the Barley Diseases Lab in the Department of Plant Pathology at using method of Sarlin et al. (2006) NDSU. The fungal DNA was isolated via a DNeasy® Plant Mini kit (Qiagen Inc., Valencia, CA, USA) following the manufactures standard procedure. For realtime PCR, 10 µl Taqman probe powermix (BIO-RAD, Hercules, CA, USA), 5 pmol TMTrif primer, 5 pmol TMTrir primer, 5 pmol of the TMTrip Taqman probe and 2.5 µl of each sample DNA as template were put in each well. TMTrif (CAGCAGMTRCTCAAGGTAGACCC) and TMTrir (AACTGTAYACRACCATGCCAAC) were used as the Tri5 primer sequences in this method (Halstensen et al., 2006). Q-PCR was operated in a CFX96 Real-Time System thermocycler (BIO-RAD, Hercules, CA, USA). The reaction procedure was 2 min 45 sec at 95 °C followed by 40 cycles, each consisting of 15 s denaturing at 95 ºC, 45 s annealing and extension at 59.5 ºC. Two replicates of each qPCR reaction.

Results and Discussion

Application of the CFLCS in liquid culture

In previous research, the CFLCS of *Lactobacillus rhamnosus* VT1 was demonstrated to have anti-*Fusarium* activity. Before testing the potential anti-*Fusarium* activity of the CFLCS of *Lactobacillus rhamnosus* VT1 in grains, the effect of its anti-*Fusarium* ability in liquid culture was evaluated. The visual difference of *Fusarium* mycelium growth in the samples with various concentrations of the CFLCS applied after two days incubation is apparent (Figure 13).

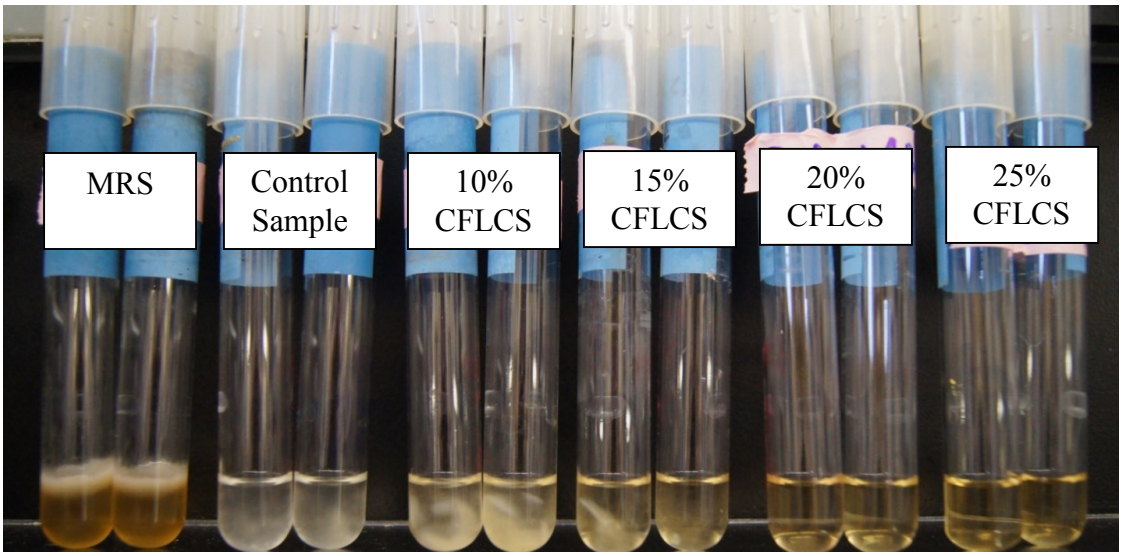

Figure 13. The growth difference of *F. graminearum* in GYEP broth with various concentration of the CFLCS applied after two days of incubation.

The investigated concentration of the CFLCS included 0, 10, 15, 20, and 25%. The tubes labeled MRS were the MRS control sample, which used 50% MRS broth to substitute the CFLCS in other tubes while water was used to substitute the CFLCS in the

tubes labeled Control. The objective of the study is to compare the anti-*Fusarium* activity of the CFLCS that from the MRS culture incubated with *Lactobacillus rhamnosus* VT1 with initial MRS broth.

According to the Figure 13, no visible mycelium growth was observed in the tube with the CFLCS concentration equal and greater than 20%. After one week, there was still no *Fusarium* growth in the tube with the CFLCS concentration equal to or greater than 20% according to the visible observation.

Application of the CFLCS on HPD agar

The application of the CFLCS on HPDA was also tested. Figure 14 shows the effects of the different concentrations of CFLCS on inhibition of *Fusarium* growth after four days of incubation. The same amount of mixture of culture, water, and *Fusarium* conidia were diffused in each well on HPDA in triplicate. *Fusarium* growth around the wells with the CFLCS was visibly reduced, indicating that the *Fusarium* growth at the first four days was decreased by the CFLCS. The results indicated that the CFLCS could reduce the growth of the *Fusarium* strain. Since the regular germination process in barley malting process is four days, the results indicated the CFLCS might be an effective strategy for inhibition of *Fusarium* growth during the malting process due to the four days inhibition on *Fusarium* growth.

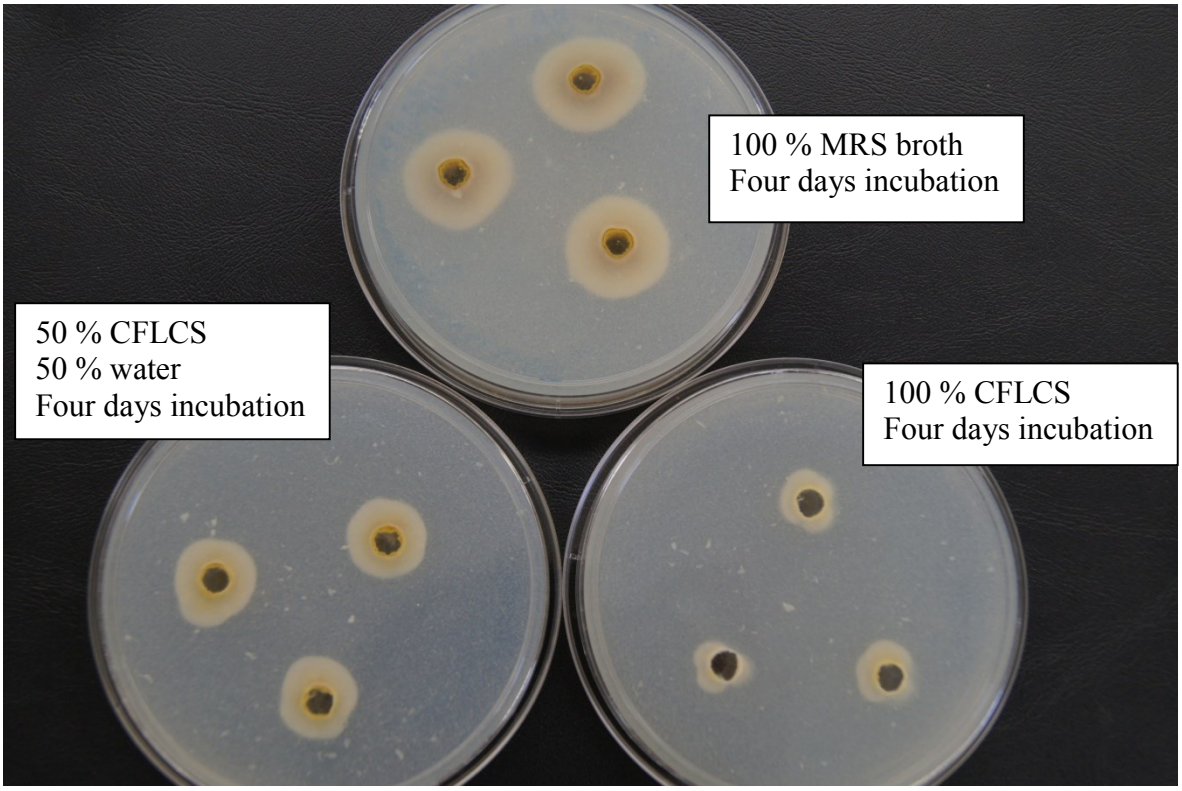

Figure 14. The inhibition effect of the CFLCS on *Fusarium graminearum* growth on home made potato agar.

Application of the CFLCS in rice culture

Autoclaved rice was used as the medium to test the effect of the CFLCS on DON reduction after 14 days incubation. The effect of three concentrations of the CFLCS on the *Fusarium* growth was investigated. The *Fusaium* growth in various rice medium samples after 3, 4, 5, 6 and 14 days incubation indicated different anti-*Fusarium* activity of the CFLCS (Figure 15).

Figure 15. Visible *Fusarium graminearum* growth in rice culture treated with different concentrations of CFLCS after 3, 4, 5, 6 and 14 days of incubation. Blank: rice media without *F. graminearum*; Control: rice media with *F. graminearum*; MRS: rice media with *F. graminearum* plus extra 50% MRS broth; LAB30: rice media with *F. graminearum* plus extra 30% CFLCS; LAB40: rice media with *F. graminearum* plus extra 40% CFLCS; LAB50: Rice media with *F. graminearum* plus extra 50% CFLCS.

As shown in Figure 15, MRS broth did not inhibit the growth of *Fusarium* in rice culture; while the anti-*Fusarium* activity in rice culture depended on the concentration of CFLCS applied. For instance, it was observed that the *Fusaium* mycelium and yellow/red pigments on the control and MRS samples after three days incubation while there was little mycelium in the rice media with 30% CFLCS. After five days incubation, the *Fusarium* mycelium started to grow in the rice medium with 40% CFLCS. At the end of the 14-day

incubation, no visible *Fusarium* growth was observed on the rice medium with 50% CFLCS. Therefore, the observed results are generally consistent with the results from in the liquid medium, which indicated that the CFLCS was able to inhibit the growth of *Fusarium*.

On the basis of biosynthetic pathway of trichothecenes in *Fusarium* species, the *Tri* 5 gene encodes trichodiene synthase that catalyzes the cyclisation of farnesyl pyrophosphate to trichodiene (Proctor et al. 1996; Brown et al. 2001). Therefore, the amount of the *Tri* 5 gene is commonly used to represent the *Fusarium* biomass. Boddeda (2006) developed a real-time PCR method to quantify the *Tri*5 gene in trichothecene producing *Fusarium* species in cereal grains and the method was verified to be a reliable and quick method compared with the conventional PCR. The levels of *Fusarium* Tri-5 gene and mycotoxins including DON, zearalenone (ZEA), and 3-acetyl-4-deoxynivalenol (3A-DON) for treated and control samples after two weeks incubation supports the activity of the 50% CFLCS (Figure 16).

The highest mycotoxin levels occurred in the sample with 40% CFLCS applied (Figure 16). Mycotoxins were also produced in the sample with water applied and the sample with 30% CFLCS applied. The mycotoxin concentration in the treatment with MRS applied was very low while the treatment with 50% CFLCS applied had almost no mycotoxin. The treatment with MRS broth applied had the highest *Fusarium* biomass level. The treatment with 50% CFLCS applied had the lowest *Tri 5* concentration compared with other samples.

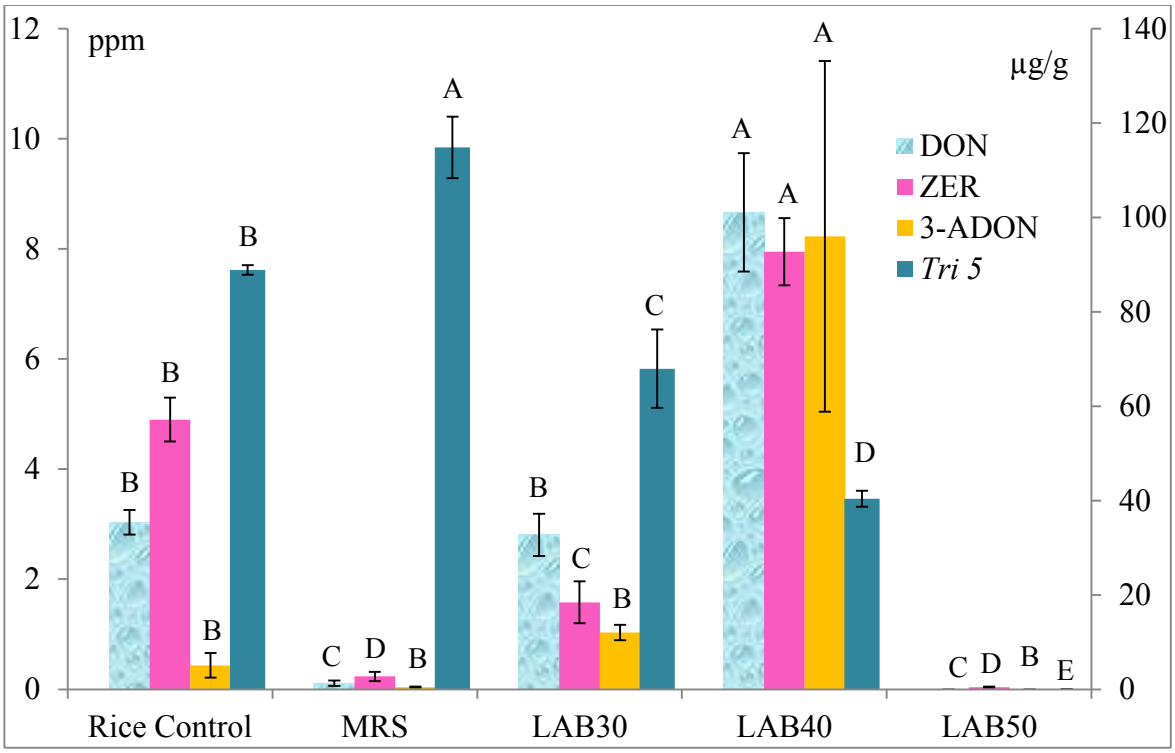

Figure 16. The DON, ZEA, 3-ADON level, and *Fusarium* Tri-5 DNA amount in rice culture after 14 of days of incubation. Means with standard deviations (n=3); different letters indicate statistically significant differences.

The MRS broth treatment had the highest *Fusarium* growth, but relatively low levels of mycotoxin production. The possible reason is the nutritional ingredients in MRS broth encouraged the growth of fungi without stressors that would promote mycotoxin production. The CFLCS, at a 40% concentration, inhibited more than half of *Fusarium* growth on rice culture; however, the unfavorable condition for *Fusarium* growth, may induce mycotoxin production. Only when the *Fusarium* growth was inhibited completely by 50% CFLCS was the mycotoxin production on rice effectively reduced.

In general, the application of the CFLCS on rice indicated that if the growth of *Fusarium* could not be inhibited effectively, the mycotoxin production might be a bigger

problem due to the stressful growth environment for *Fusarium* strains. This observsation supports that anti-fungal research should not focus only on the effects on fungal growth of potential antifungal compounds.

Application of the CFLCS on barley

In order to test the anti-*Fusarium* activity of the CFLCS on barley, a special steeping process was employed with replacement of steeping water by a specific concentration of CFLCS. MRS broth was used as the control. After the 34-hour steeping process, the steeped barley grains were germinated through a regular four-day germination process. The treated malt and control samples indicated that different *Fusarium* inhibition (Figure 17).

There was no visual *Fusarium* growth or barley seed germination at 50% CFLCS. However, the negative impact of the CFLCS on the barley malting process was apparent. Compared to the MRS treated samples, the germination of the CFLCS treated samples was inhibited completely, even at the lowest concentration of the CLFCS. Some unknown compounds from CFLCS apparently inhibited the barley embryo, leading to the failure of barley seed germination.

Treatment	Concentration (%)	Pinnacle	Stellar
CFLCS	50		
MRS	50		
CFLCS	30		
MRS	30		
CFLCS	15		
MRS	15		

Figure 17. Visible *Fusarium* growth after malting naturally FHB infected barley.

Figure 18, 19, 20 and 21 summarize the *Tri* 5 gene amount, DON, 3A-DON, and ZEA concentrations of the finished barley malt samples. T-grouping was utilized to compare the differences of the measurement levels of the combinations.

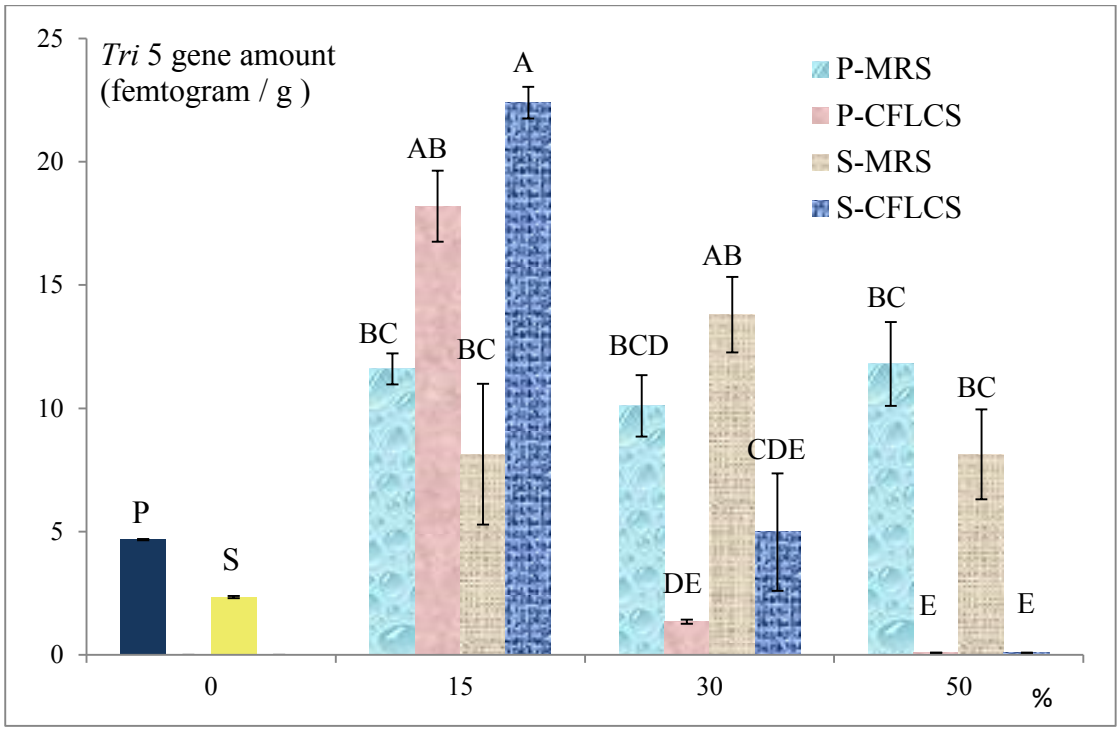

Figure 18. The *Tri* 5 gene amount in the MRS and CFLCS treated barley samples. Means with standard deviations (n=3); letters indicate statistically significant differences.
P: Pinnacle barley sample; S: Stellar barley sample;
P-MRS: Pinnacle barley sample steeping with MRS solution;
P-CFLCS: Pinnacle barley sample steeping with CFLCS solution;
S-MRS: Stellar barley sample steeping with MRS solution;
S-CFLCS: Stellar barley sample steeping with CFLCS solution.

As described earlier in this paper, the barley samples used in this study were naturally infected by FHB. The levels of *Tri* 5 gene amount and mycotoxins of the original barley sample are included in the figures. The *Tri* 5 gene amounts in Pinnacle barley sample were higher than Stellar while the mycotoxin levels in Pinnacle barley sample were

lower or equal to Stellar. This indicated that the relationship between *Fusarium* biomass and mycotoxins produced including DON, 3A-DON, and ZEA (Figures 3-7, 8, and 9,) were not positively correlated, which is consistent with the research findings by Duverger et al. (2011).

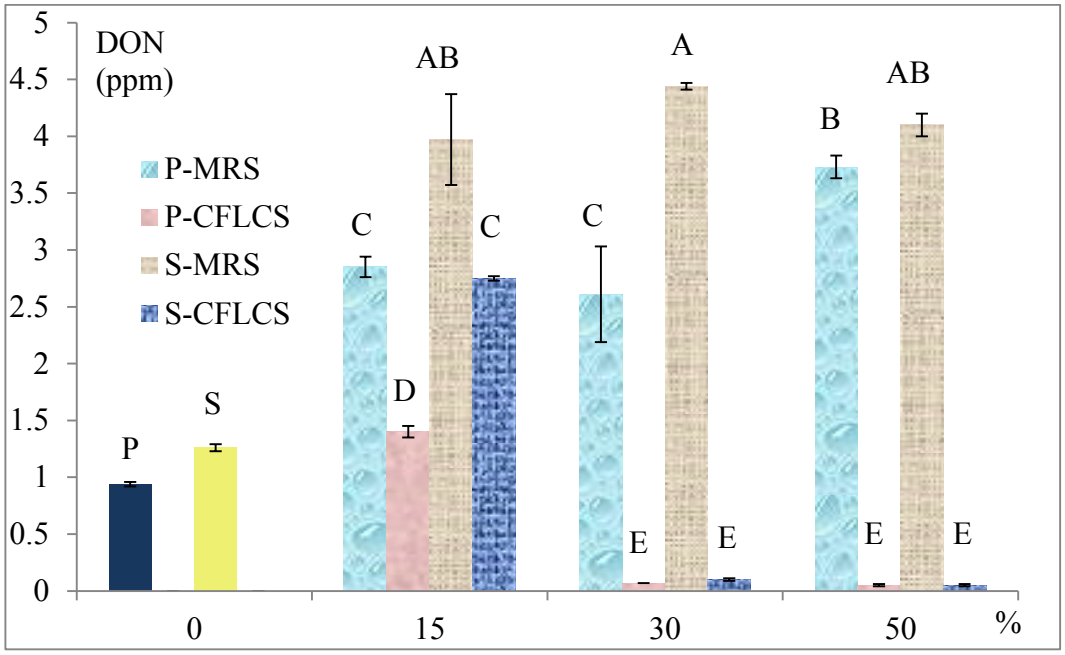

Figure 19. The DON level in the MRS and CFLCS treated samples. Means with standard deviations (n=3); letters indicate statistically significant differences.
P: Pinnacle barley sample; S: Stellar barley sample;
P-MRS: Pinnacle barley sample steeping with MRS solution;
P-CFLCS: Pinnacle barley sample steeping with CFLCS solution;
S-MRS: Stellar barley sample steeping with MRS solution;
S-CFLCS: Stellar barley sample steeping with CFLCS solution.

Although 15% CFLCS favored the growth of *Fusarium*, 30% CFLCS had inhibition activity on the growth of *Fusarium*. Between the two barley varieties, Pinnacle and Stellar, the inhibitory effects of the CFLCS were stronger on Stellar barley sample. These differences were not visible according to the germinated samples (Figure 17). For both varieties, the 15% CFLCS treated barley sample had the highest *Tri* 5 gene amount.

The treatment affected the DON concentration significantly (Figure 19). Fifteen percent CFLCS led to DON production increasing while the CFLCS with higher concentration had very strong DON reduction ability. The 30% CFLCS could significantly decrease the DON level in final malting products.

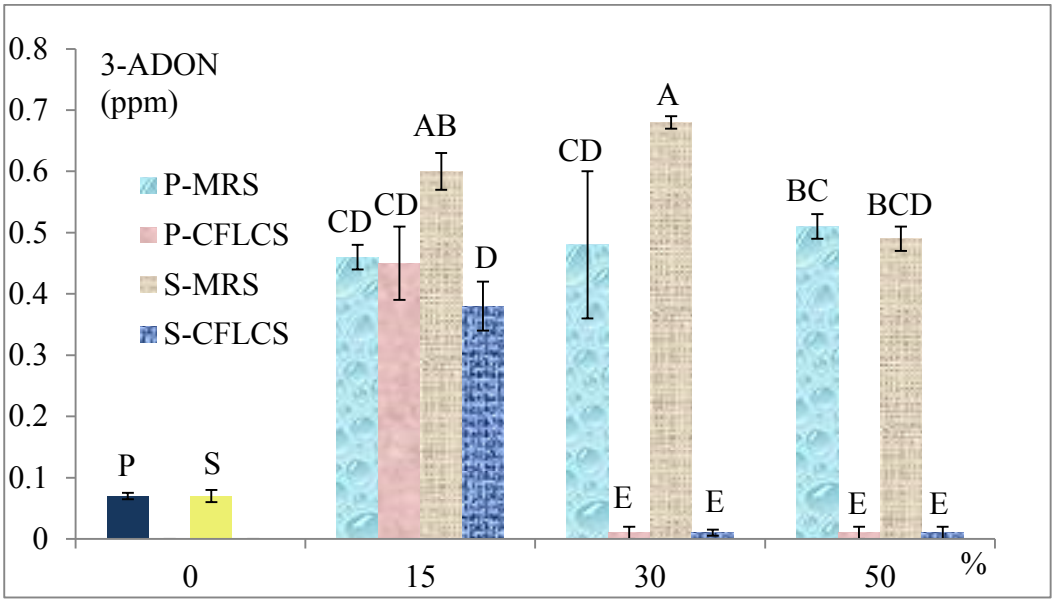

Figure 20. The 3-ADON level in the MRS and CFLCS treated samples. Means with standard deviations (n=3); letters indicate statistically significant differences.
P: Pinnacle barley sample; S: Stellar barley sample;
P-MRS: Pinnacle barley sample steeping with MRS solution;
P-CFLCS: Pinnacle barley sample steeping with CFLCS solution;
S-MRS: Stellar barley sample steeping with MRS solution;
S-CFLCS: Stellar barley sample steeping with CFLCS solution.

Similar effects were observed for the other mycotoxins produced in the barley samples including 3A-DON (Figure 20) and ZEA (Figure 21). The effect of the CFCLS on inhibition of ZEA was not as clear as those on DON and 3A-DON, and even the difference between the 15% CFLCS and 50% CFLCS was not significant. This may be due to the limitation of quantification (0.4 ppm) at the low levels detected.

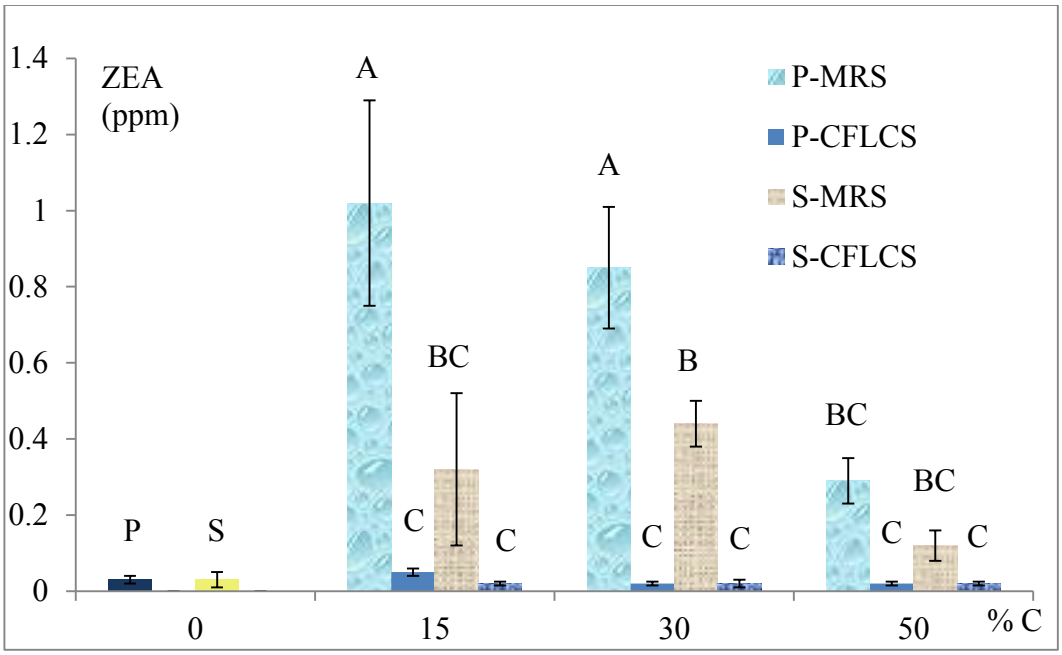

Figure 21. The ZEA level in the MRS and CFLCS treated samples. Means with standard deviations (n=3); letters indicate statistically significant differences.
P: Pinnacle barley sample; S: Stellar barley sample;
P-MRS: Pinnacle barley sample steeping with MRS solution;
P-CFLCS: Pinnacle barley sample steeping with CFLCS solution;
S-MRS: Stellar barley sample steeping with MRS solution;
S-CFLCS: Stellar barley sample steeping with CFLCS solution.

In general, the CFLCS had demonstrated to have strong inhibitory ability on both *Fusarium* growth and mycotoxin accumulation. However, the CFLCS impacted the barley seed germinative capability.

Conclusions

Preliminary experiments were performed to investigate the anti-*Fusarium* activity of the CFLCS in both culture. A concentration of 20% of CFLCS in GYEP broth inhibited *Fusarium* growth completely with an inoculum concentration at 5×10^4 conidia /ml media. An agar well diffusion assay also showed that 20 µl CFLCS inhibited growth.

Concentrations of CFLCS at 30% and 40% of the CFLCS applied in rice culture inhibited the growth of *Fusarium*; however, the mycotoxin levels in rice culture increased at these CFLCS concentrations. A concentration of 50% of the CFLCS inhibited *Fusarium* growth completely and almost no mycotoxins were detected.

The CFLCS dilutions at concentrations of 15%, 30% and 50% were used as steeping water to investigate the effect of the CFLCS on naturally infected barley during the malting process. Although the CFLCS could reduce the *Fusarium* growth and mycotoxin accumulation when the concentration of the CFLCS was equal to or greater than 30%, the germinative ability of the barley samples was negatively affected.

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GENERAL CONCLUSIONS

Lactobacillus rhamnosus VT1 was selected as the target strain due to its higher anti-*Fusarium* activity. The optimized incubation conditions of the strain for highest anti-*Fusarium* activity was obtained via a RSM design with three factors and three levels as well as triplicates at the center point: the incubation temperature at 34ºC, the shaking speed at 170 rpm, and the incubation time of 55 hours. Under this condition, the % *Fusarium* inhibition due to CFLCS of *Lactobacillus rhamnosus* VT1 at a concentration of 10% was predicted to be 75.60 % *in vitro* and verified to be 83.7% of the *Fusariumin in vitro* with these optimized incubation conditions.

A stepwise regression, with α to enter = 0.15 and α to remove = 0.15, was performed to evaluate the significance of lactic acid, pH, biomass, acetic acid, total acidity, and 3-phenyllactic acid on anti-*Fusarium* activity of the CFLCS. The results demonstrated that pH, total acidity, and 3-phenyllactic acid were statistically significant to the regression model and could be used to explain 39.19% variation of the anti-*Fusarium* activity.

It is indicated that the anti-*Fusarium* activity of the CFLCS could not just be explained by the major organic acids, lactic acid and acetic acid. 3-phenyllactic acid is most probably a critical bio-active compound among all of the components in the CFLCS in spite of the low concentration. Proteinaceous compounds were not identified, but they may serve a more important role due to the possible synergistic effect between organic acids and proteineous compounds.

Concentrations of CFLCS at 30%, 40% of the CFLCS applied in rice culture inhibited the growth of *Fusarium*; however, the mycotoxin levels in rice culture increased at these CFLCS concentrations. At a concentration at 50% of the CFLCS, *Fusarium*

growth was inhibited completely and no mycotoxins were detected. The CFLCS dilutions at concentrations of 15%, 30% and 50% were used as steeping water to investigate the effect of the CFLCS on naturally infected barley during the malting process. Although the CFLCS could reduce the *Fusarium* growth and mycotoxin accumulation when the concentration of the CFLCS was equal to or greater than 30%, the germinative ability of the barley samples was negatively affected.

In general, a combination of the metabolites produced by *Lactobacillus rhamnosus* VT1 at certain condition could inhibit the growth of *Fusarium* and reduce the level of *Fusarium* trichothecenes during malting process.

FUTURE RESEARCH

Due to the negative effect on germination capability of the treated barley samples, although the CFLCS with high bio-activity had the inhibition of *Fusarium* growth and mycotoxin accumulation, the strategies of application of the CFLCS on cereal grains should be intensively investigated in the future. Finding out the pertinent strategies for various food matrices, the CFLCS would be a useful natural anti-*Fusarium* and mycotoxin-reduction agent.

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