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# Effects of Fermentation by Yeast and Amylolytic Lactic Acid Bacteria on Grain Sorghum Protein Content and Digestibility

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Effects of Fermentation by Yeast and Amylolytic Lactic Acid Bacteria on Grain Sorghum Protein Content and Digestibility

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**ABSTRACT**

Grain sorghum is an underutilized crop despite many advantages to its cultivation. A drought-tolerant plant with many pest-resistant cultivars, its applications are limited by problems with nutrient availability, particularly protein digestibility. Digestibility of grain sorghum protein is reduced by antinutritional compounds in the grain like tannins and phytates, and by moist-heat cooking. Some of these concerns can be mitigated by how the grain is processed. Fermentation is one processing method that can improve digestibility. Fermentation can also concentrate protein in a substrate. In this experiment, grain sorghum was subjected to different treatments and used as a substrate for yeast fermentation. Two species of yeast were tested; common baker's yeast (*Saccharomyces cerevisiae*), and an amylolytic strain (*Lipomyces kononenkoae*). Effects of pasteurization or sterilization of the substrate, nitrogen supplementation, amyloglucosidase addition, and co-culture with an amylolytic lactic acid bacteria *Lactobacillus amylovorous* were examined. After 48 hours of incubation, baker's yeast samples treated with enzyme increased in crude protein, from 9% in unfermented grain to approximately 27% after treatment. Nitrogen supplementation accelerates protein enrichment and is a significant factor at 24 hours of fermentation. Pepsin digestibility of fermented samples was improved compared to thermally processed controls. Phytates increased in concentrated high-protein samples, but the ratio of phytate to protein was reduced by both yeasts. Both types of yeast increased pepsin digestibility of sorghum protein compared to thermally processed control samples. *L. kononenkoae* reduced phytates in the substrate, but did not enrich protein content. The lactic co-culture had no significant effect on measured responses, but decreased incidence of spoilage and contamination of the fermentation samples. A fermentation with baker's yeast can concentrate sorghum protein in a substrate if the grain starch is hydrolyzed first, and can increase digestibility as well.

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# CHAPTER 1: INTRODUCTION, OBJECTIVES, AND LITERATURE REVIEW

## INTRODUCTION

Grain sorghum is a staple food for people and their livestock all over the world. It is of particular importance to human nutrition in arid parts of Africa and Asia, as it is better suited to dry climates than other cereal crops. Globally, the United States is the top producer of grain sorghum, with much of the crop exported for animal feed applications (Kumar et. al., 2011). Sorghum is considered an important dietary protein source despite being relatively low in protein. Like many cereals, it is deficient in lysine and methionine as well (Belton and Taylor, 2004). Additionally, the presence of antinutritional factors interferes with digestion and absorption of the protein that the grain does contain (Weaver, Hamaker and Axtell, 1998). Cooking also reduces protein digestibility through various mechanisms including protein crosslinking and amino acid racemization, both of which make the protein resistant to digestive enzymes of monogastric organisms (Duodu et al., 2003). These issues limit the extent to which sorghum can be used in feed applications; though grain sorghum contains roughly the same amount of protein as maize, the digestibility of sorghum protein is much lower (Duodu et al., 2003).

Because of its dietary importance and potential for expanded applications, much work has been done on improving the availability of protein in grain sorghum. Cultivars with low or no tannin content generally have better protein digestibility than high-tannin grain, but at the cost of reduced pest resistance which increases losses in the field. Breeding for cultivars with higher protein is another approach, however increased protein in the grain does not necessarily translate into more protein metabolized by the consumer (Axtell et al., 1981). Post-harvest processing of the grain can be used to improve protein quality and digestibility. Chemical and physical

processing such as alkali treatment and de-hulling can decrease tannins and phytates; removal of these compounds correlates with higher protein digestibility (Chavan et al., 1979).

Fermentation is another way of increasing protein digestibility. Many traditional preparation methods for sorghum foods involve a fermentation step, and studies of these methods show improvement in nutrition over non-fermented methods (Axtell et al., 1981). Often the bacteria indigenous to the microbiome of the grain are enough to facilitate the fermentation (Chavan, Chavan, and Kadam 1988) but the process can be controlled and improved by inoculation with a specific organism (Oboh, 2006). Lactic fermentations are commonly employed in making porridges, breads, and other sorghum food products; studies of these fermented foods have shown higher digestibility and improved amino acid profile compared to foods prepared from unfermented grain. (Hassan and El Tinay 1995; Correia et al., 2005) However, these fermentations do not change the total protein content of the grain food product.

There are many other benefits to lactic acid fermentation. Increased vitamin content and mineral bioavailability have been observed in fermented foods (Kazanas and Fields, 1981; Pyo, Lee and Lee, 2005; Leroy and De Vuyst, 2004). Lactic acid bacteria also contribute to shelf life and food safety by inhibiting growth of many types of spoilage and pathogenic organisms. This occurs not only because of the reduced pH as lactic acid accumulates, but can also result from the production of antimicrobial compounds called bacteriocins. These compounds have been shown to inhibit contamination by *Listeria* and *Salmonella* species in fermented food products (Ashenafi, 1991; Makras et al., 2006).

Fermentation also has been used to increase protein contents of plant matter, generally for feed applications. Organisms used in these processes are generally fungi, particularly in solid-substrate applications. Molds have the advantage of rapid growth, which means faster

carbohydrate respiration and accumulation of biomass. However, production of mycotoxins is a concern when using mold, either by the desired organism or contamination by another. Yeasts are also used in protein production applications. Yeast biomass can be used directly as a protein supplement (Winkler et al., 2011; Maylin, Cervantes, and Beames, 1984), or as part of a fermentation byproduct as with distiller's grain (Ferreira et al., 2010; Øverland et al., 2013). Many common yeasts can metabolize glucose as a carbon source; this sugar can be obtained by breaking down starch, as with malting in beer processing. Some yeasts are amylolytic, and can produce enzymes to degrade the starch directly. Such organisms may still require processing of the substrate to function efficiently. Yeasts also require other vitamins and minerals to thrive, in some protein-enrichment studies these nutrients are provided using yeast extract, a common ingredient in yeast growth media formulas. Yeast extract is also a good nitrogen source, but an expensive one. Replacement of yeast extract with other nitrogen sources such as urea or ammonium salts have proved successful in some studies (Rosma and Cheong, 2007).

Development of an inexpensive processing method that could preserve or improve the digestibility of protein in sorghum grain could provide several benefits. Increasing the content of digestible protein in the grain would make it more useful in food or feed applications. Increase in demand for sorghum could allow commercial farmers to shift production to sorghum during drought conditions and still have a profitable harvest if other more water-intensive crops fail. The goal of this research is to determine conditions for a fermentation process to enrich and improve protein content in grain sorghum.

## RESEARCH OBJECTIVES

The overall purpose of this study is to increase protein content and quality of grain sorghum by a fermentation process using yeast. Preliminary studies with baker's yeast and

sorghum meal have demonstrated that a statistically significant increase in percent protein can be achieved with the *Saccharomyces cerevisiae* available to consumers if the grain has been treated enzymatically to convert the starch into sugars. However, literature has shown increases in percent protein with other species of yeasts and different substrate treatments. This study will compare commercial active dry baker's yeast to an amylolytic strain of yeast, *Lipomyces kononenkoae*, under different substrate treatment conditions, to determine which of the factors tested have the most effect on protein content in the final fermented grain product.

Digestibility is also important in assessing the quality of protein in a food or feed product. The amount of crude protein in sorghum grain is similar to that of other grains, but the digestibility of sorghum protein is lower. This is a result of the structure of the grain, as well as antinutrient compounds present in the grain. A fermentation process that could increase both the quantity and digestibility of sorghum grain would expand the range of potential food and feed applications for this crop.

**Objective 1**: Examine the effects of thermal and enzyme treatments, nitrogen supplementation, and bacterial co-culture on the final percent protein in yeast-fermented sorghum

**Objective 2**: Compare the nutritional quality of sorghum fermented by different species of yeast in terms of in vitro protein digestibility and phytate content.

## LITERATURE REVIEW

### Sorghum Cultivation and Use

Sorghum is an important crop in many parts of the world. Indigenous to Africa, sorghum has been cultivated in a wide range of locations and climates in Asia, Australia and the Americas as well as its native habitat. There are different varieties of sorghum grown for several applications; many consumers are familiar with sorghum syrup, which is extracted from the

sugar-rich stalks of sweet sorghum cultivars. There are leafy varieties grown for livestock forages; some fast-growing cultivars have also been examined as feedstock for biofuel production (Zhao et al, 2009; Schittenhelm and Schroetter, 2014). Grain sorghum is grown primarily as a food source for humans and livestock. Currently the top producing nations are the United States, Mexico, Nigeria, India, and Ethiopia (FAOSTAT 2015).

There is recent interest in bioethanol production from grain sorghum (Wu et al. 2007; Dyartanti et al. 2015). However, food and feed are still the most common applications for the grain. Grain sorghum can be made into many food products, including porridge, breads, tortillas, beverages, and a snack similar to popcorn (Anglani, 1998). There is also increasing interest in sorghum as an ingredient in gluten-free products (Pineli et al., 2015; de Meo et al., 2011). In the United States, grain sorghum is used primarily for animal feed applications. Sorghum grain has been examined as a low-cost replacement for corn or other grains in feed formulations. Sorghum has been studied in feed applications for dairy and beef cattle (Delgado-Elorduy et al. 2002; Wood et al. 2011), poultry (Selle et al., 2010), swine (Lizardo, Peiniau, and Aumaitre, 1995), and fish (Alderolu et al., 2009) with varying degrees of success.

**Benefits of Grain Sorghum Use**

Grain sorghum is a very water efficient crop. Native to arid desert regions, it is well-adapted to dry climates can often be grown without any irrigation and still produce a harvest. Various cultivars have been developed for optimized performance in warmer or cooler climates and different patterns of water availability including rainy seasons and irrigation. Careful cultivation has increased yield without requiring more land area, production volumes have been maintained even in places where land area used for sorghum cultivation has decreased (Kumar et. al., 2011; FAOSTAT 2015). Cultivars can be selected for resistance to insect and bird damage

as well, with tannins and seed hardness contributing to reduced losses caused by these pests (Kumar et al., 2011). Though other grain crops such as maize and wheat have surpassed sorghum in its native regions for economic reasons, sorghum remains an important source of energy particularly when heat and drought reduce yields of these other crops.

**Challenges to Grain Sorghum Use**

While there are many advantages to cultivation of grain sorghum, there are limitations to its practical application. The hard seed and high tannin contents that confer pest resistance to some cultivars of the grain also make them unpalatable to livestock, and difficult to metabolize nutrients for both people and animals. One major reason other cereals are preferred over sorghum for many applications is the relatively low digestibility of sorghum grain. The percentage of crude protein sorghum contains is similar to that of other grains, but the digestibility of that protein is lower by comparison. And, unlike many other grains, the digestibility decreases significantly upon cooking (Duodu et al., 2002). Low digestibility of nutrients slows growth in livestock, which makes sorghum less attractive for feed applications. Studies comparing sorghum grain to maize have shown sorghum to result in reduced weight gain or weight loss by poultry (Harms et al., 1958), swine (Sotak-Peper et al., 2015) and fish (Allan et al., 2000). Low protein digestibility also contributes to malnutrition in humans who depend on sorghum as a primary food source (Wong et al., 2009).

One contributing factor to the low digestibility of sorghum grain is presence of anti-nutritional compounds in the grain, particularly tannins and phytates. While many species of sorghum grown in the United States are low-tannin or tannin-free sorghum cultivars, many of the more drought-tolerant and pest-resistant cultivars widely grown in other parts of the world are high in tannins. Sorghum tannins help protect the grain from insect and bird damage, and reduce

other types of loss in the field (Price et al., 1979). However, these tannins have antinutritional properties in humans and livestock. In addition to being unpalatable, which reduces overall food intake, tannin compounds can bind to protein and inhibit enzymatic digestion of the nutrient (Hassan and El Tinay, 1995). Even non-tannin sorghum cultivars contain phytate compounds, which also bind protein and interfere with protein digestion (Osman, 2004; Duodu et al., 2003).

Another factor which contributes to reduced protein digestibility is heat processing of the grain. The grain must be cooked for human consumption, and is often subjected to some thermal treatment for use as animal feed, such as steam-flaking. Such wet-heat processing has been shown to reduce protein digestibility (Duodu et al., 2001). The mechanisms behind this are not completely understood, but may include formation of protein-starch complexes, as well as cross-linking of proteins in general and of sulfur-containing amino acids in particular. Changes in protein structure that result from these reactions can result in increased resistance to the typical protease enzymes found in monogastric organisms' digestive systems (Duodu et al., 2003). Wet-heat applications could also possibly cause isomerization of amino acids from L to D isomers, which would reduce their bioavailability as well as their digestibility (Duodu et al., 2003). These adverse effects of heat treatment are less pronounced or even absent in dry-heat processing, such as extrusion (Duodu et al., 2001; de Mesa-Stonestreet, Alavi, and Gwirtz, 2012)

Improving sorghum protein availability is of great concern; though most of its calories come from carbohydrates, the grain is considered to be more important as a source of dietary protein. It is most vital in areas where other types of protein are difficult to access, so there has been extensive research into mitigating the effects of anti-nutritional factors and optimizing the digestibility of the amino acids it contains (Duodu et al., 2003). Sorghum, like many other grains, is known to be deficient in lysine (Virupaksha and Sastry, 1968), so efficient and cost-effective

ways to supplement the amino acids it lacks have also been studied. Blending sorghum grain with other available plant proteins has been one way of supplementing needed protein in sorghum foods (Chavan, Chavan and Kadam, 1988). Fermentation has also been employed as a means of improving availability of protein from sorghum (Correia et al., 2005).

**Fermentation Process Applications**

Fermentation has been used as a food processing method throughout human history, for various reasons. The wild fermentations that traditionally resulted in foods like cheese, tempeh and sauerkraut have been gradually refined into processes that today produce a myriad of food and pharmaceutical products as well as industrial chemicals. Fermentation is an efficient way of generating complex molecules that would be time-consuming, expensive, and difficult (if even possible) to synthesize by other means. Fermentation can also remove undesirable compounds as the fermenting organisms metabolize the substrate. Many organisms can be used in fermentation processes, including yeasts, molds, algae, bacteria and mixed cultures.

In food, fermentation has several applications. Traditional fermentation methods were used to make food safe to eat, to preserve it for storage, or to make foods taste better. Many fermentation organisms produce organic acids, peroxides, and antimicrobial chemicals that inhibit pathogens and other organisms from colonizing the food product (Bourdichon et al., 2012; Chang and Chang, 2010). Fermenting perishable foods can extend their shelf lives by reducing pH or making the food otherwise inhospitable to spoilage organisms; fermentation effects can help make other preservation methods such as drying or curing more effective (Ross, Morgan and Hill 2010). Fermentation can impart desirable sensory characteristics as well.

Fermentation has been found to reduce antinutritional factors and toxic compounds and can increase vitamin and antioxidant content (Caplice and Fitzgerald, 1999; Juan and Chou,

2010). Enzymatic processes involved in fermentations increase digestibility of nutrients, so they are easier to metabolize by the intended consumer, an effect has been seen in both human food and livestock feed preparations (Gibson, Perlas and Hotz, 2006; Kiers et al., 2000; Feng et al., 2007). Fermentation can change molecular structures in foods, for instance by hydrolysis of carbohydrates or proteins, which can result in improvements in nutritional quality.

**Protein Enrichment by Fermentation**

Fermentation processes can be used to increase protein content in foods in addition to increasing protein quality. Bacterial fermentation can be used to generate proteinaceous biomass from a carbon-rich fluid substrate, such as a sugar solution (Wang et al., 2013), industrial processing byproduct (Bough et al. 1972), or even methane gas (Bothe et al., 2002). However, most bacterial protein must be separated from the growth medium for use as an ingredient, and separation processes are often expensive. Fungal fermentations are generally more efficient, and have a wider range of applications (Nasseri et al., 2011). Fungal fermentations can use a variety of substrates, including semi-solid, solid-state, and solid substrate fermentations with water activities too low for bacterial culture. Co-cultures and mixed cultures with many types of organisms are commonly used for protein production, and are often more effective at protein accumulation than monoculture processes (Fields, Tantratian, and Baldwin, 1991; Cojho et al., 1993; Tesfaw and Assefa 2014)

Yeast fermentation can increase both content and digestibility of proteins in plant foods, and often yield a product with acceptable sensory qualities (Khetarpaul and Chauhan, 1990). Yeast fermentation processes have been demonstrated to double the protein contents in sweet potato and cassava processing wastes (Yang, 1988; Oboh, 2006), and triple the protein in potato and cactus process wastes (Gélinas and Barrette 2007; Araújo et al., 2005). A fermentation of

sorghum with yeast to generate ethanol yielded a distillers grain byproduct that was 44% protein (Horn, du Preez and Kilian, 1992). The goal of this work was to convert all of the starch in the grain into ethanol, a process that took up to 200 hours. A more modest increase in protein could be achieved in a shorter amount of time.

Factors that can affect the amount of protein increased by yeast fermentation include inoculation rate, type of carbon source, and micronutrient availability. Compared to bacteria, yeast are tolerant of a wider range of temperatures and pH, and can survive much lower water activities and moisture contents; varying these conditions appears to have less of an effect on growth compared to the amount of yeast added initially (Araújo et al., 2005). Inoculum sizes used in literature range from 3.6 mg dry yeast per gram (0.36%) of substrate weight (Hong et al., 1989) to 15 percent of the substrate weight (Araújo et al., 2005) in fresh yeast, which contains about 70% moisture. Fresh yeast is approximately 40% crude protein on a dry basis, so an inoculation rate of 15 grams of fresh yeast per 100 g would immediately increase the total protein content by about 2 percent. However, the increase in percent protein at such an inoculation rate was seen to be much greater than that 2 percent, indicating that the increase is not merely an artifact of the added yeast. Increased inoculum size also shortens the lag time in the yeast growth cycle, reducing the total incubation time (Horn, du Preez and Kilian, 1992). However, such large quantities of yeast may not be feasible for a cost effective process.

**Applications of Fermentation to Sorghum**

There are several types of fermentation processes that use sorghum as a substrate. Sorghum is used for the production of ethanol for both biofuel and consumption in beverages. Sweet sorghum and sorghum cultivars developed specifically for cellulosic biomass are generally used over grain sorghum for bioethanol applications (Mamma et al., 1996), but

fermentation of grain sorghum has been studied for this purpose as well. The distillers grain from sorghum ethanol fermentation has been shown to be nutritionally similar to other distillers grain byproducts (Al-Suwaiegh et al., 2002). Fermentation of sorghum grain has been used to increase digestibility in both food and feed applications. Fermentation in traditional preparations of foods, like porridges and flatbreads, has been shown to increase digestibility of protein and starch, reduce phytate and tannins, and increase bioavailability of iron (Kazanas and Fields, 1981; Taylor and Taylor, 2002; Osman, 2004). As a feed ingredient, fermenting sorghum can result in better outcomes compared to using unfermented grain (Aderolu, Kuton and Odu-Onikosi, 2009).

# CHAPTER 2: PRELIMINARY STUDIES AND PARAMETER SELECTION

## INTRODUCTION

Preliminary studies were conducted to determine what substrate conditions would support fermentation, which organisms should be used for fermentation, and how cultures for the organisms used would be maintained and propagated. Treatment parameters were selected over a series of experiments, which demonstrated their effect on the sorghum meal substrate. Initially, the study was on the effects of lactic fermentation of sorghum, and only inoculation with *L. amylovorous* was studied. However, the desired information on how the treatments affected the sorghum substrate were still relevant. Here, the effects of different pH control methods, nitrogen supplementation, enzyme treatments, and thermal treatments were studied. Different yeast strains were tested for use in this substrate system as well. The results of these studies helped establish the treatments parameters used in the main experiment.

## EXPERIMENT OVERVIEW

Grain sorghum was ground and mixed with water to make a slurry for the fermentation substrate. The substrate was subjected to different treatments including thermal processing, enzyme treatment, pH control, nitrogen supplementation or any combination of those. Substrates were inoculated with lactobacillus, yeasts, or co-cultures of both types of organism. Control samples without inoculation were prepared for these treatments as well. Samples and were incubated for up to 96 hours, with samples collected at intervals throughout the duration of the run. Substrate samples were tested for pH, lactic acid, and crude protein, to monitor for microbial growth and any occurrence of protein enrichment.

PRELIMINARY STUDIES

## pH control

Fermentation by lactobacillus and yeast both result in reduced pH of the fermentation substrate. As the name suggests, lactic acid bacteria are quite efficient at producing lactic acid, and though these bacteria are acid tolerant they can generate enough to inhibit their own growth. While the main fermentation products of *Saccharomyces cerevisiae* are carbon dioxide and ethanol, other compounds can be produced, particularly from complex substrate systems. Many of these minor products are acidic, including malate, pyruvate and several amino acid degradation byproducts (Postma et. al., 1989; Zelle et. al., 2008).

Changes in pH can affect the progress of the fermentation. Microorganisms and enzymes have optimum pH ranges for best performance, as well as pH limits that will result in death or deactivation if exceeded. In these experiments, various methods of pH control with different compounds were tested. Calcium carbonate ($CaCO_3$), ammonium carbonate ($(NH_3)_2CO_3$), ammonium sulfate ($(NH_3)_2SO_4$), and solutions of sodium hydroxide (NaOH) were tested as means of raising acidic pH during fermentation.

During initial trials pH was adjusted every few hours using $CaCO_3$, $(NH_3)_2CO_3$, or NaOH. The $CaCO_3$ was rarely effective enough to use alone; the quantity required for even a modest change in pH was very large compared to the total amount of substrate. Instead, it was often combined with NaOH or $(NH_3)_2CO_3$, with the stronger basic compounds used for gross pH adjustments, and the $CaCO_3$ used to stabilize that pH and maintain it for longer periods.

In studies on lactic acid production, it was shown that the pH could be held within a given desirable range by adding the pH adjustment compounds directly to the substrate before

inoculation (Hofvendahl and Hahn-Hägerdal 2000; Yang et. Al., 2015). The presence of $CaCO_3$ in the substrate would bind lactic acid continuously as it is generated to form calcium lactate, which is mostly insoluble in aqueous solution. The lactate salt would precipitate from the substrate as it formed, and the pH would decrease much more slowly. However, in practice, the lactobacillus often failed to grow when the pH was modulated in advance. Addition of $(NH_3)_2CO_3$ with the $CaCO_3$ resulted in very high pH of the substrate; adjusting the pH to neutral with a solution of hydrochloric acid before inoculation did not result in any bacterial growth. Samples subjected to this treatment showed no lactic acid production. Substrate treated with $(NH_3)_2SO_4$ and $CaCO_3$ did show growth and lactic acid production, but $(NH_3)_2SO_4$ is mildly acidic and does not raise pH of the substrate. The pH of substrate treated with $(NH_3)_2SO_4$ started low and continued to fall throughout the fermentation unless $CaCO_3$ was also present. Even samples that showed some growth were very slow to ferment, with no lactic acid detected until 66 hours of incubation. Pre-emptive pH control was not feasible for this system, in subsequent experiments pH control was not applied until after microbial growth was established.

When the scope of the experiment shifted to protein enrichment by yeast, pH control became less critical to the result. The yeast appeared to tolerate a low pH, so the main concern was for the enzymes added. The amyloglucosidase chosen to hydrolyze starch into sugars has an optimum pH range between 4.5 and 4.8. For subsequent experiments pH was monitored early in the fermentation and adjusted if necessary. After several trials it was determined that pH should be checked 8 to 9 hours after inoculation to allow sufficient growth of the organisms before adjustments were made, and any sample with a pH below 4.5 at that time would be adjusted to between 4.5 and 5 with $CaCO_3$ and a NaOH solution. Addition of $CaCO_3$ was not to exceed 1.5g for a 40g sorghum substrate, and any further adjustment required was performed with the NaOH

solution. Subsequent experiments used $CaCO_3$ and NaOH exclusively for adjusting the pH of the samples.

**Nitrogen supplementation**

During the pH adjustment work with *L. amylovorous*, it was noted that substrate samples adjusted with ammonium carbonate and ammonium sulfate showed slightly more lactic acid production than those treated with other compounds; this was true even though ammonium sulfate is acidic and did very little to control the pH of the substrate. Literature on the matter revealed that these compounds are readily metabolized by microorganisms as a nitrogen source (Belasco, 1954; Nancib et. al., 2001). Experiments were conducted to separate the effects of nitrogen supplementation from those of pH control. Ammonium carbonate was eliminated from the study for its effects on the pH of the substrate. Ammonium sulfate was studied as a nitrogen source since it had the least effect on pH, as well as superior material handling properties such as the absence of dust and ammonia fumes. Nitrogen addition increased lactic acid production; measurement of the acid was used to monitor bacterial growth.

Though nitrogen supplementation was having an effect on the growth of the lactobacillus, it was not affecting the total protein in the substrate. It was hypothesized that a more effective nitrogen supplement could promote enough growth to increase the substrate protein. Organic nitrogen sources like urea, corn steep liquor, and yeast extract were considered; these are good nitrogen sources but costly, their use could prove prohibitively expensive for most processes. Yeast extract in particular is used as a supplement because not only does it provide nitrogen, but other trace nutrients as well. One way to take advantage of these benefits is to blend yeast extract with a less expensive nitrogen source. This was the approach used in a 2007 study by Rosma and Cheong, which found that a 1:4 w/w blend of yeast extract and ammonium sulfate performed as

well as pure yeast extract in terms of lactic acid produced by the fermentation. The same ratio of yeast extract and ammonium sulfate was used to supplement a lactic fermentation at a rate of 5% of the dry grain weight in the substrate. This resulted in a modest increase in lactic acid production, but no change in percent protein. The lactobacillus were growing well, but not to an extent that their accumulated biomass could have any effect on the percentage of protein in the substrate.

It was determined that the lactobacillus used here could not increase the amount of protein in the substrate. Literature on protein enrichment of complex substrates often use fungi rather than bacteria (Durand and Chereau, 1988; Yang 1988; Araújo et. al., 2005). In light of this fact, a fermentation was conducted with baker's yeast instead of, and in co-culture with, the *L. amylovorous* used in prior experiments. Initially the substrate tested was raw sorghum meal mixed with sterile deionized water, and treatments included addition of a saccharification enzyme (amyloglucosidase) and the addition of a 3:1 blend of ammonium sulfate and yeast extract, at an addition rate of 3% of the dry grain. Tests for crude protein showed an increase in the fermented samples, from 12% (dry basis) in the unfermented grain to about 16% in the fermented grain after 48 hours. A similar experiment was performed on thermally processed substrate, there crude protein increased to between 23% and 29% after 48 hours of incubation. The method by which the crude protein was determined showed the amounts of ammonia nitrogen left in the supplemented samples. It was noted that there was an excess of ammonia left in the samples after fermentation was mostly complete, an indication that more nitrogen was being added than the organisms were able to metabolize. This finding prompted a reduction of the nitrogen addition rate in the proposed work, to 2% of the dry grain weight in the substrate.

**Enzyme selection**

A test was run to study the activity of amyloglucosidase with and without amylase. The amylase used was a solution of thermostable α-amylase from *Bacillus lichenformis* (18,000 U/ml), and the amyloglucosidase was produced by *Aspergillus niger* (321 U/ml); both enzymes were purchased from Sigma-Aldrich Co. (St. Louis, MO). Four sets of grain samples were prepared; raw (R), cooked (C), cooked with amylase (A), and cooked with amylase and amyloglucosidase (AG). A 1:1 w/v ratio of grain to water was used. Addition rates of the enzymes were 5.33 µl amylase per gram sorghum, and 8.0 µl amyloglucosidase per gram sorghum in the substrate. Each of these were prepared in pairs, with and without a nitrogen supplement, which was added at 5% of the dry grain weight. The thermal treatment applied was to place the sample flasks in a water bath maintained at 86°C for 90 minutes; the time and temperature selected were the optimum conditions for the thermostable α-amylase to act on starch. The same treatment was applied to all the cooked samples including the ones without enzyme treatment. After the thermal treatment, the samples were cooled to 40°C. The AG samples were adjusted to a pH of 4.5, from an initial pH of about 6.3, using a 2 N solution of HCl. Amyloglucosidase was added to the pH-adjusted samples, and the samples were held at 55°C for another 30 minutes; the other 3 samples were held at the incubation temperature of 37°C. At the end of the enzymatic treatment, the pH of the AG samples was returned to the initial pH of about 6.3 using a 4 N solution of NaOH. These were equilibrated to 37°C also. The lactobacillus inoculum was prepared and each sample received $3.8*10^8$ cells. These samples were incubated at 37°C for 72 hours, with samples collected for nitrogen testing at 0, 24, and 72 hours, and for lactic acid by HPLC more frequently. The pH of each sample was taken at each sampling and adjusted to about 5 with $CaCO_3$ if it fell below 4.5.

Samples were inoculated with *Lactobacillus amylovorous* to see how the treatments affected the growth of the organism. After incubation, lactic acid was present in all samples except the amylase/amyloglucosidase treatment. The reason why the lactic bacteria did not grow in these samples was not determined, but it is possible that the pH adjustment raised the salinity of the samples above a concentration that the organism could survive. Lactobacillus become stressed at sodium chloride levels exceeding 10 g/L in part because of reduced water activity; this effect is exacerbated by high sugar concentrations (Neysens, Messens, and De Vuyst, 2003) Reducing the pH to 4.5 for the treatment using 2 N HCl, and raising it back to the original pH of 6.3 using 4 N NaOH added an equivalent to 10 g salt per liter of water in the substrate, which already had a high solid content. The sugars generated by the enzymes combined with the excess salt could have been enough to inhibit the growth of the *L. amylovorous* in these samples. These samples showed signs of microbial growth eventually, but had a high pH for most of the incubation period. Toward the end of the 72 hour incubation the pH dropped below 4, indicating some acid-forming organism was growing in the substrate. However, HPLC analysis showed no lactic acid, indicating that the organisms growing were not the *Lactobacillus* originally inoculated.

Because of the difficulty of pH adjustment and potential for poor growth as a result, another test was conducted using only amyloglucosidase, with no pH adjustment for the enzyme treatment. The enzyme works best at a pH range of 4.5 to 4.8, and should be more effective in conjunction with amylase, which will break the starch into smaller dextrins with more ends exposed for the amyloglucosidase to attack. It was hypothesized that the enzyme would be more effective at hydrolyzing starch into fermentable sugars when the amylolytic lactobacillus was present, as *L. amylovorous* would release amylases into the substrate to degrade the starch, and

reduce the pH as well. The test was conducted without thermal treatment, sorghum meal was mixed with water 1:1 (w/v) with treatments administered as in Table 1:

**Table 1: Crude protein in raw sorghum meal substrate by treatment**

| Treatment | avg. % protein |
|---|---|
| ground sorghum control | 12.05[a] |
| ground sorghum/yeast control | 12.23[a] |
| ground sorghum/yeast/nitrogen control | 13.45[abc] |
| t=0 yeast only | 12.68[a] |
| t=0 yeast/lactic co-culture | 13.06[ab] |
| t=6 yeast/lactic co-culture | 12.78[a] |
| t=0 yeast/nitrogen | 13.07[ab] |
| t=0 yeast/nitrogen/lactic co-culture | 13.57[abc] |
| t=6 yeast/amyloglucosidase | 15.22[cd] |
| t=6 yeast/amyloglucosidase/lactic co-culture | 14.92[bcd] |
| t=6 yeast/amyloglucosidase/nitrogen | 16.23[d] |
| t=0 yeast/amyloglucosidase/nitrogen | 16.39[d] |
| t=6 yeast/amyloglucosidase/nitrogen/lactic co-culture | 15.63[d] |
| t=0 yeast/amyloglucosidase/nitrogen/lactic co-culture | 16.50[d] |

These samples were inoculated with dry *Saccharomyces cerevisiae* baker's yeast (Best Choice Traditional Active Dry Yeast, AWG Brands, Kansas City, KS) granules at a rate of 0.5% w/w of the dry grain meal, either at the start of the incubation, or 6 hours later. The delayed inoculation was to determine whether there was any advantage to allowing sugars to be produced before the yeast were added to the substrate. Inoculated samples were incubated for 72 hours, including the 6 hour delay. The samples were tested for crude protein at 48 hours of incubation.

Though it was expected that the samples with the acidity and amylases conferred by the lactic bacteria would result in more sugars generated, there did not appear to be much difference in the final results. Amyloglucosidase samples with the lactic co-culture did not exhibit more fermentation than the amyloglucosidase samples without it, and delaying the inoculation did not make a difference in final percent protein. Samples with amyloglucosidase and the lactic co-culture were visibly liquefied, as were samples treated with amyloglucosidase but no co-culture.

Protein enrichment occurred in all samples with amyloglucosidase added, and none of the samples without it, regardless of which other treatments were also applied.

**Thermal treatment**

The previous test was conducted without thermal treatment, but amylolytic enzymes are generally more effective against gelatinized starch. Initially, when only *L. amylovorous* fermentation was being studied, it appeared that raw grain was an acceptable substrate. This organism produces enzymes that are capable of hydrolyzing raw starch, so lactic acid was produced in similar concentrations whether the substrate was thermally processed or not, particularly when the fermentation was permitted to run for several days. Making the starch easier to hydrolyze by gelatinizing it with a thermal treatment did not appear to make a difference, possibly because the inoculation rates used were the same; if the bacteria were already metabolizing as much of the carbohydrate as that number of bacteria could consume, increasing the availability of the starch without increasing the amount of bacteria would not be likely to change the amount of lactic acid produced.

For a protein enrichment by baker's yeast, which is not amylolytic, the amount of growth depends on availability of fermentable sugars. The amyloglucosidase enzyme is much more effective at hydrolyzing gelatinized starch, so cooking the starch should increase the amount of sugar available to the yeast, which would potentially yield higher protein enrichment. A thermal process should be able to increase the amount of starch hydrolyzed by the amyloglucosidase.

To test this hypothesis and determine an appropriate grain/water ratio to use in a thermally processed substrate, four different slurries were prepared in duplicate. The 1:1 ratio of grain to water used for the raw samples would not be a feasible moisture level for a thermally processed sample, so different grain to water ratios were tested as illustrated in Table 2. The

water ratios used were (in weight of dry grain to volume water) 2:3, 1:2, 1:3, and 1:4, each with 20 grams of sorghum meal and 0.6 grams of the ammonium sulfate/yeast extract nitrogen supplement. Sterilized slurry samples were autoclave sterilized in a Tuttnauer model 3870 ELVC vertical steam sterilizer (Tuttnauer USA, Hauppauge, NY, USA) at 121°C for 15 minutes. Pasteurized slurry samples were processed in a VWR Model 1227 water bath (VWR, Radnor, PA, USA) at 86°C for 90 minutes, with 20 rpm shaking to maintain water circulation. After processing, the samples were cooled to room temperature for addition of amyloglucosidase and the lactic co-culture. The samples were then stirred and inoculated with dry baker's yeast granules. Incubation was conducted at 37°C for up to 96 hours, with samples taken at 24-hour intervals. Crude protein was tested initially and after 48 hours of incubation. The grain/water ratio with the highest protein enrichment was chosen for the main experiment.

Liquefaction of the substrate was apparent in all samples when pH was adjusted after 8 hours, a phenomenon not observed with raw grain substrate. Bottles with lower water-to-grain ratios had firm lumps of substrate floating in them, and sterilized samples had more solid chunks of substrate than pasteurized samples. Better mixing at the start of fermentation to break up the large pieces could prevent this by allowing the enzyme to contact more of the substrate. All thermally processed samples increased in percent protein. The 1:3 w/v ratio resulted in high

**Table 2: Crude protein results by thermal treatment**

| Treatment | Grain-to-water ratio (w/v) | initial % CP | 48-hour % CP |
|---|---|---|---|
| Pasteurized | 2:3 | 11.49 | 24.74 |
| Pasteurized | 1:2 | 10.76 | 23.70 |
| Pasteurized | 1:3 | 10.4 | 29.68 |
| Pasteurized | 1:4 | 11.62 | 28.77 |
| Sterilized | 2:3 | 10.85 | 22.50 |
| Sterilized | 1:2 | 10.94 | 28.05 |
| Sterilized | 1:3 | 11.74 | 28.85 |
| Sterilized | 1:4 | 11.04 | 28.53 |

protein for both thermal treatments as seen in the result in Table 2, this ratio was used in the main experiment.

**Co-culture organism**

The co-culture organism was selected for its ability to metabolize starch, demonstrated in preliminary studies as well as literature (Nakamura, 1981). *Lactobacillus amylovorous* has been used to decrease viscosity of grain slurries, which occurs as it secretes extracellular amylases into the substrate. Yeast and lactic acid bacteria compete for the same nutrients in a fermentation system, and have been shown to inhibit growth of each other in mixed culture. Because of this, lactobacillus are often considered a contaminant in ethanol fermentation processes (Narendranath et. al., 1997). However, ethanol production was not the goal of this work, and test runs showed that baker's yeast and *L. amylovorous* were able to grow in the same substrate without evidence of serious growth inhibition for either organism. For the yeast, substrate weight losses were similar between samples with and without the lactic co-culture, which would only occur if the yeast were metabolizing sugars at similar rates in both sample treatments. Decreases in pH of the co-culture samples were comparable to the pH reduction using only *L. amylovorous* for lactic fermentation, suggesting the bacterial growth and subsequent lactic acid production were similar in both systems.

**Amylolytic yeast selection**

The previous tests were performed exclusively using baker's yeast and *L. amylovorous*. However, one objective of the proposed research was to determine whether an amylolytic yeast could be used to metabolize starch directly for protein enrichment of the sorghum substrate, without the need for the enzymatic treatment required by normal *Saccharomyces cerevisiae*. To

select an amylolytic organism, yeasts documented to have the ability to produce amylases and glucoamylases were requested from the United States Department of Agriculture's Agricultural Research Service (USDA ARS) Culture Collection. Five strains with this capability were available; *Pseudozyma tsukubaensis* NRRL Y-7792, *Piskurozyma capsuligena* NRRL Y-6355, *Saccharomycopsis fibuligera* NRRL Y-1062, *Schwanniomyces occidentalis* var. *occidentalis* NRRL Y-2477, and *Lipomyces kononenkoae* NRRL Y-11553. The cultures arrived as lyophilized pellets in glass vials; pellets were aseptically transferred to cryovials with 0.6 ml sterile YM broth; each vial was streaked to its own YM agar plate and grown for approximately 3 days at between 25°C and 28°C. The vials containing the original cultures were frozen at -80°C with 30% v/v of a sterile 87% glycerol solution for long-term storage.

After the initial incubation, one isolated colony from each agar plate was streaked to new YM plates and grown for 24 hours. From each plate, 0.02 grams of cells were collected with a sterile loop and placed into Erlenmeyer flasks containing 50 ml of YM broth. The flasks were incubated in a MaxQ 4450 orbital shaker (ThermoScientific, Waltham, MA, USA) at 250 rpm and 27°C for 9 hours, then 18 ml of fresh YM was added to each flask. Shaking was increased to 275 rpm to ensure aeration, and incubation was continued for another 9 hours for 18 hours total. Cells were counted with a hemocytometer to choose the inoculum volume, between 15 and 30 ml of broth. The inoculum was transferred to centrifuge tubes and centrifuged in and Allegra X-22R (Beckman Coulter, Brea, CA, USA) at 1450 g for 10 minutes.

To test the yeasts, a commercial food-grade white sorghum (Pleasant Hill Grain, Hampton, NE, USA) was ground into meal, sieved through #18 mesh, and mixed with sterile water in a 1:3 w/v ratio and pasteurized at 86°C for 90 minutes. Each bottle was inoculated with one yeast species and incubated at 35°C for 48 hours. Weights of each bottle were taken initially,

after 24 hours of incubation, and after 48 hours of incubation. The organism associated with the most weight loss, *Lipomyces kononenkoae*, was selected for the research experiment.

**Table 3: Substrate weight reduction by each amylolytic yeast**

| Species | grams lost |
|---|---|
| *Lipomyces kononenkoae* | 4.95 |
| *Schwanniomyces occidentalis occidentalis* | 1.94 |
| *Piskurozyma capsuligena* | 0.90 |
| *Pseudozyma tsukubaensis* | 1.47 |
| *Saccharomycopsis fibuligera* | 2.23 |

**Propagation of yeast inoculum**

Throughout the proposed research, cultures of the fermentation organisms had to be maintained and propagated for inoculating test samples. To maintain consistency, both the *Saccharomyces cerevisiae* baker's yeast and the *Lipomyces kononenkoae* cultures were streaked to separate YM agar plates. Colonies harvested from these plates were streaked or spread onto new plates to grow cells for the inoculum. Initially, it was intended that the test substrates would be inoculated with the same cell counts for both species of yeast. However, the *L. kononenkoae* cells are much smaller than the baker's yeast cells, and the disparity in cellular mass added to the substrates could have resulted in an incorrectly perceived advantage in efficiency for the larger yeast. Adding the same cell mass was also considered, but the smaller yeast species was also more difficult to cultivate and would require several agar plates to grow enough colonies to match the amount of baker's yeast used in the other preliminary experiments. Instead, it was decided that volumes of YM broth medium would be inoculated with the same weight of cells, and incubated for the same amount of time. The broth inoculation rate used was 0.02 grams of live yeast cells for 50 milliliters of media. At the end of the incubation, the same volume of broth would be used per test sample for each type of yeast.

Initially it seemed that both yeasts had comparable growth requirements; each had been grown in shaker flasks of YM broth for 18 hours, near room temperature. However, in the first trial with both yeast species, while the baker's yeast flourished none of the *L. kononenkoae* samples showed any signs of growth of the inoculated species. The propagation method was revisited, and it was found that the incubation temperature for the inoculation broth had been raised to 32°C, from a temperature range of 25°C to 27°C. In the next trial run the temperature was reduced to the original temperature.

The next inoculum was grown for a substrate weight loss study. New YM agar spread plates were grown for each yeast species, resulting in a dense lawn of cells of each yeast. Two 500ml Erlenmeyer flasks containing 100ml aliquots of YM broth were inoculated with 0.04 grams of cells for each yeast, for 200ml of inoculated broth per yeast species. The flasks were incubated at 26°C with 250-rpm rotary shaking for 18 hours; 36ml of fresh sterile YM broth was added to each flask after 9 hours of incubation. After 18 hours, cell counts for both yeasts were on the order of $10^6$ cells. This was a normal count for baker's yeast, but at least one order of magnitude low for the *L. kononenkoae*. The samples were inoculated anyway, with the volumes increased to make up for the low *L. kononenkoae* count. Agar plates were streaked from each flask and incubated at room temperature while the fermentation ran. The baker's yeast samples grew well, but the *L. kononenkoae* showed no signs of growth in the fermentation substrate. There was no growth of *L. kononenkoae* on the agar plate streaked from the inoculum.

At this point a study on the cultivation of *L. kononenkoae* was conducted. Colonies were harvested from older plates that had been stored in the refrigerator (2-8°C) to determine if age of the colonies influenced the growth in the broth medium. One plate was had been incubated for 43 hours before refrigeration, and the other plate had been incubated 69 hours. Two 250 ml

Erlenmeyer flasks containing 50 ml YM broth were inoculated with 0.02 g of cells from each plate. Spread plates were inoculated from each plate as well. Broth cell counts were taken at 8.5 and 18 hours. Both flasks appeared to have good growth, with the broth inoculated from the 43-hour old plate showing higher counts. The spread plate inoculated from the 43-hour old plate was incubated for 45 hours, and substrate was prepared for another experimental run. Cells from the new plate were harvested to inoculate 3 flasks of fresh YM broth; baker's yeast was cultured from a spread plate grown at the same time the *L. kononenkoae* plate was started. The flasks were incubated for the same amount of time at the same temperature as the cultivation study. After 18 hours, the baker's yeast had good counts; the broth in the *L. kononenkoae* flasks was clear and showed no signs of growth. The substrate had not been hydrated yet, and was saved for a later date. The *L. kononenkoae* flasks were left shaking in the incubator; several hours later one of the three flasks showed signs of growth. Examination of the broth under a microscope showed *L. kononenkoae* cells, and the flask emitted the characteristic odor of *L. kononenkoae* fermentation. Neither of the other two *L. kononenkoae* flasks showed any activity.

After this inoculum failed to grow, the original plates were reexamined. The first plate, which grew successfully, had sparse, isolated colonies, while the second plate was covered in a dense lawn of growth. It appeared that overcrowding on the plate was inhibiting the cells' growth, even after they were removed from the plate and placed in new media. Baker's yeast appeared to have no such difficulty growing in densely populated conditions. Subsequent propagation plates were streaked with diluted broth, and any plate without well-isolated colonies was discarded and not used to prepare the inoculum.

Substrate was prepared for another run. Broth was inoculated from several plates with well-isolated colonies for both yeast species. Yeast cell counts were taken after 10 and 18 hours

of incubation at 25°C with constant shaking. After 18 hours, the counts for *L. kononenkoae* had fallen slightly, from $6.9x10^7$ to $6.5x10^7$ cells, but these counts were still considered sufficient for inoculation. The substrate was inoculated and the fermentation was conducted under the same conditions as the amylolytic yeast test (35°C, 72 hours, pH adjustment at 9 hours). Growth was detected, but not to the extent that it was during the preliminary test- the most weight lost from any substrate treatment was not very different than the pasteurized control.

At this point it was suspected that the drop in cell counts was not a result of error in counting or variation in the sample collected, but of actual cell death. This would imply that the cells were past the exponential growth phase, and even the stationary phase as they were beginning to die. A test was conducted to determine the optimum incubation time for *L. kononenkoae*. A 500 ml Erlenmeyer flask with 150 ml sterile YM broth was inoculated with 0.06 grams of cells scraped from appropriately isolated colonies. The flask was incubated at 25°C with shaking. Cell counts were taken at 6, 9, 12, and 15 hours, at which point the counts began to drop. Prepared substrate samples were inoculated with cells from 15 ml of broth at 7, 9, 11, 13, and 15 hours of incubation. The inoculated samples were incubated for 72 hours, with samples collected at 48 and 72 hours. Sample bottle weights were recorded every 24 hours.

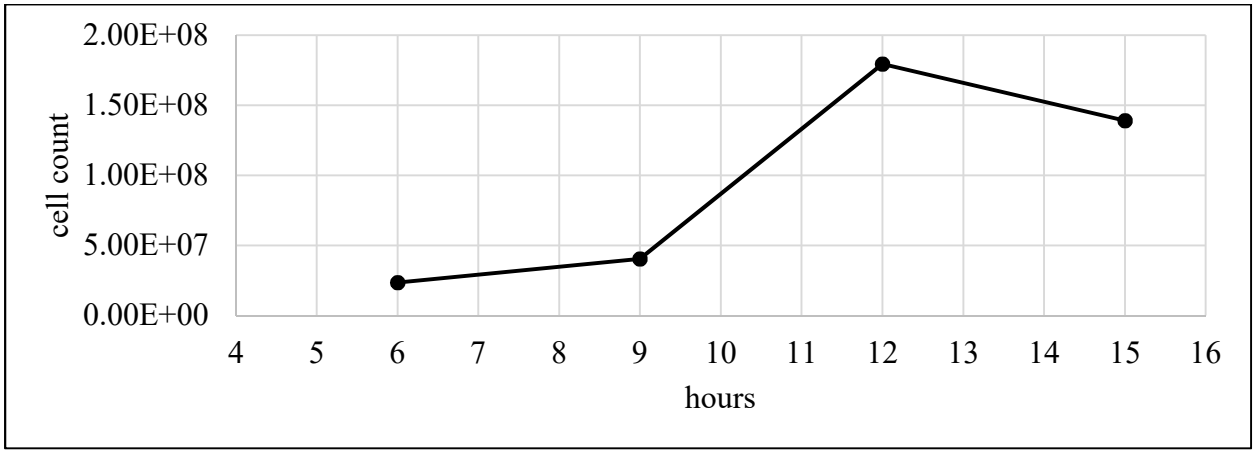


**Figure 1: *L. kononenkoae* cell counts**

The sample inoculated with 9-hour broth showed the most substrate weight loss after both 48 and 72 hours of incubation, as seen in Table 4. All subsequent *L. kononenkoae* inoculation broth cultures were incubated for 9 hours instead of 18 hours. Baker's yeast incubation time was not altered.

**Table 4: Substrate weight loss by inoculum incubation time**

| Inoculum age (hours) | 48 hour loss (g) | 72 hour loss (g) |
|---|---|---|
| 7 | 0.55 | 0.79 |
| 9 | 2.99 | 4.31 |
| 11 | 0.82 | 1.28 |
| 13 | 1.02 | 2.55 |
| 15 | 1.85 | 2.99 |

**Lactic acid analysis**

Though the method was not used in the main research experiment, much of the preliminary work used high-pressure liquid chromatography (HPLC) to analyze lactic acid. Lactic acid production was used to monitor the progress of fermentation by *L. amylovorous*, and many of the decisions around thermal processing, pH control, and nitrogen supplementation were based on these results. The HPLC method used to analyze for lactic acid was high-performance size exclusion chromatography (HPSEC) with a refractive index (RI) detector. The HPSEC-RI system was a Waters (Milford, MA, USA) which included a 515 HPLC pump and 2410 refractive index detector. The column used was a 150 x 7.80 mm Rezex ROA organic acid column (Phenomenex Inc., Torrance CA, USA), and the mobile phase was an aqueous 0.005 N $H_2SO_4$ solution. The sample preparation for this method was both rapid and simple; the fermented samples were extracted 1:10 in sterile deionized water and filtered through a 45µm syringe filter, 50 µl of this filtrate was injected into the column. This convenient method could quantify simple sugars, dextrins, and carbonate compounds as well as lactic acid when

appropriate standards were used. Degradation of starch in to dextrins and accumulation or consumption of sugars could be monitored concurrently with lactic acid production.

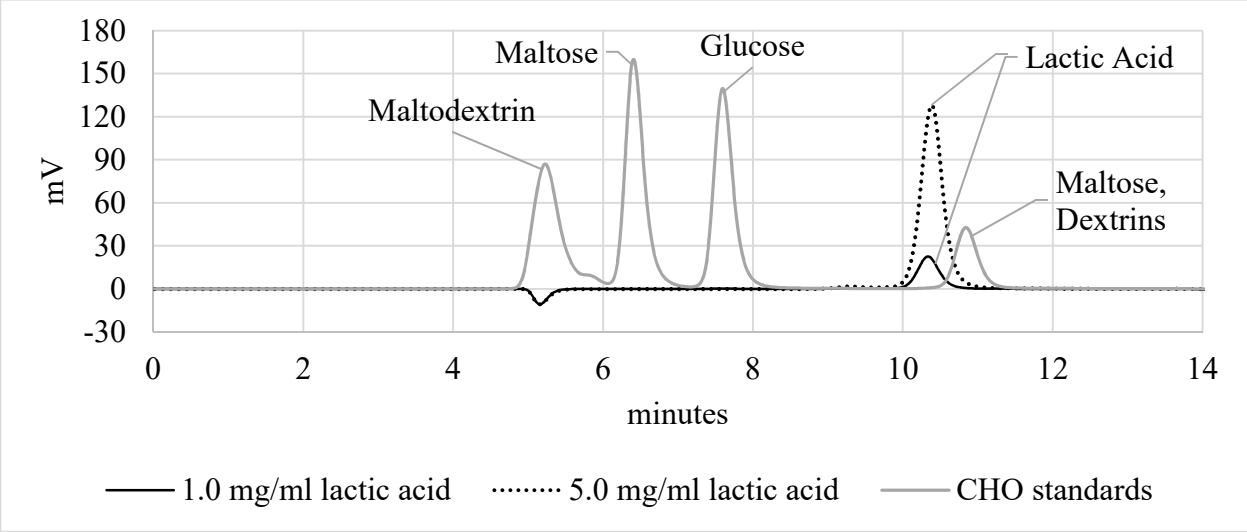


**Figure 2: Chromatograms of glucose, maltose, maltodextrin, and lactic acid standards.**

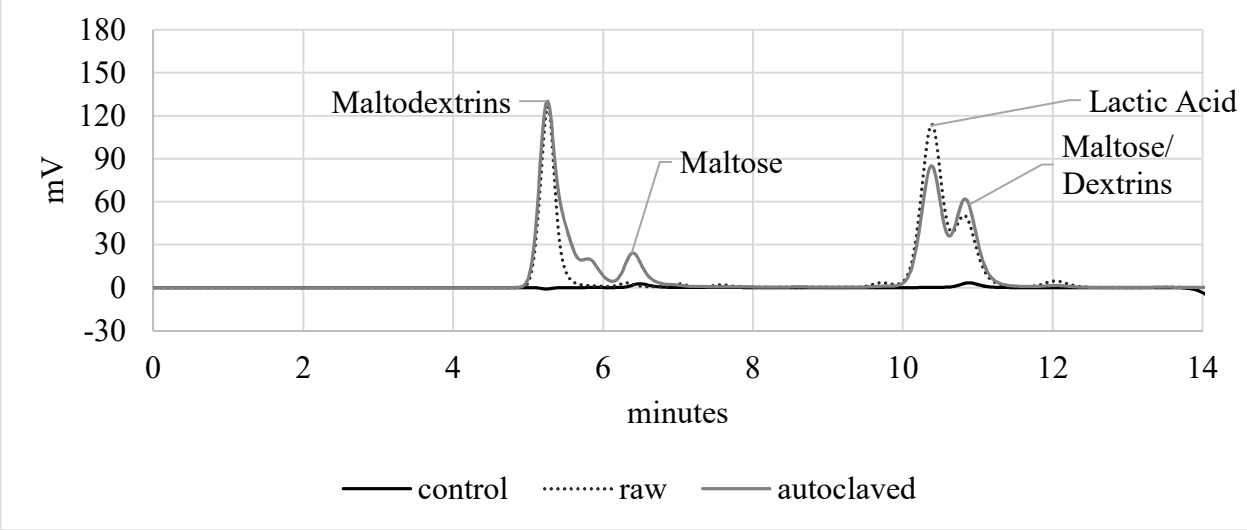


**Figure 3: Chromatograms of raw and autoclaved sorghum slurries fermented with *L. amylovorous* compared to unfermented sorghum control.**

PARAMETER SELECTION SUMMARY

Based on the results of the preceding trials, the following conditions were selected for the research experiments: the pH of the fermentation samples was checked after 9 hours of fermentation, if the pH fell below 4.5 pH adjustment was performed with calcium carbonate. Nitrogen was supplemented by a 3:1 w/w blend of ammonium sulfate and yeast extract, at a rate of 2% w/w of the dry grain in the substrate. Amyloglucosidase from *Aspergillus niger* (321 U/ml, Sigma-Aldrich) was added at a rate of 0.8% v/w of dry grain. Thermal treatments used were pasteurization at 86°C and sterilization at 121°C, the appropriate grain/water ratio for thermal treatments applied was 1:3 w/v. At least $10^8$ cells of *Lactobacillus amylovorous* were added to the co-cultured sample flasks. Fermentation yeasts selected were *Lipomyces kononenkoae* and baker's yeast (*S. cerevisiae)*. Fermentation temperature was 37°C during the preliminary tests; this was the optimum temperature for *L. amylovorous* growth. However, yeasts tend grow better at lower temperatures, so temperature was reduced to 35°C for fermentations during the main research experiment as well as studies on substrate weight and effect of yeast cultivation method. Fermentations were initially run for 96 hours, but very little change in substrate mass occurs after 72 hours. Analyses were conducted on samples incubated for 48 hours, so the incubation time was reduced to 72 hours for the research experiment.

**Table 5: Summary of experimental fermentation conditions**

| Fermentation conditions | |
|---|---|
| Nitrogen supplement | 3:1 ammonium sulfate/yeast extract; 2% of dry substrate weight |
| Thermal treatment | Pasteurization 86°C/90 minutes; Sterilization 121°C/15 minutes |
| Enzyme treatment | Amyloglucosidase from *Aspergillus niger*, 8µl/g dry substrate |
| Co-culture organism | *Lactobacillus amylovorous*, $1x10^8$ cells per sample |
| Yeast species | *Saccharomyces cerevisiae* (baker's yeast); *Lipomyces kononenkoae* |
| Fermentation temperature | 35°C |
| pH adjustment | Add $CaCO_3$ if pH falls below 4.5 |

## CHAPTER 3: OBJECTIVE 1 - PROTEIN ENRICHMENT FERMENTATION OF GRAIN SORGHUM

INTRODUCTION

The overall goal of this work is to develop a fermentation method that can improve both the content and quality of protein in grain sorghum. To determine the conditions required for such a process to be successful, critical parameters must be identified. The preliminary studies showed that enrichment will occur when a nitrogen supplemented, thermally processed, and enzyme treated substrate is inoculated with yeast and lactic acid bacteria. The goal of this experiment is to separate the effects of each treatment factor and determine which ones are the most important for protein enrichment of sorghum.

**Objective 1:** Examine the effects of thermal and enzyme treatments, nitrogen supplementation, and bacterial co-culture on the final percent protein in yeast-fermented sorghum

MATERIALS AND METHODS

### Culture Propagation and Inoculum Preparation

*Lactobacillus propagation*

Cultures of *Lactobacillus amylovorous* NRRL B-4540 obtained from the USDA Agricultural Research Service (ARS) were maintained frozen in deMan, Rogosa and Sharpe (MRS) broth (EMD, Gibbstown NJ, USA) with 15% v/v of an 80-86% sterile glycerol solution in 1ml aliquots at -80°C. The frozen vials were revived in 50 ml MRS broth per 2 ml culture and stored in 4 ml aliquots at -20°C. The 4 ml vials were grown in MRS broth to use for the inoculum within 2 weeks of freezing.

Approximately 48 hours before the fermentation run, two 4-ml vials of frozen *L. amylovorous* were thawed and placed into two 250ml Erlenmeyer flasks containing 100 ml of

prepared sterile MRS broth. The flasks were incubated at 37°C with 150 rpm rotary shaking for 18 hours. After 18 hours, a 1/100 dilution of the culture medium was prepared using two 9 ml 0.1% peptone blanks, a drop of the diluted medium was placed on a hemocytometer to count the cells. Once the count was obtained, aliquots of the volume required for at least $10^8$ cells were frozen in centrifuge tubes until the fermentation was performed. The frozen tubes were thawed in cool water, centrifuged and decanted, then the cells were suspended in PBS buffer immediately prior to inoculation.

*Baker's yeast propagation*

To maintain consistency with the amylolytic yeast samples, dry yeast granules (AWG Brands, Kansas City, KS) were suspended in Difco Yeast Mold (YM) broth (Becton, Dickinson and Co., Sparks, MD, USA) and incubated at 35°C. This broth was used to streak YM agar plates, which were incubated at 27°C for 24 hours. Isolated colonies from those plates were collected, suspended in sterile water, and spread on YM agar plates and incubated to grow lawns of cells. These cells were used to grow the fermentation inoculum, or to streak new plates for the next run.

Approximately 3 days prior to the fermentation run, a new YM agar plate was streaked with isolated yeast colonies from a previously grown plate. This plate was incubated for at least 43 hours, at which point there was enough growth to collect 0.02 g of cells per 50 ml YM broth. Cultures were usually 0.06 g in 150 ml YM or 0.08 g in 200 ml YM; this was the maximum volume used in a single flask to ensure sufficient aeration. The inoculated YM broth was incubated between 25°C and 28°C with rotary shaking between 250 and 300 rpm in a MaxQ 4450 incubator for 18 hours. At the end of the incubation, the cells were diluted 1/20 and 1/200 in aqueous 0.1% sterile peptone (Amresco, Solon, OH, USA). The cells were counted under an

AO Model L150 microscope (American Optical, Buffalo, NY, USA) using a Bright Line hemocytometer (Hausser Scientific, Horsham, PA, USA). The volume of broth required to supply approximately $1x10^8$ cells was calculated, usually between 15 and 30 ml per sample. The broth was mixed well and distributed between 16 sterile disposable centrifuge tubes (VWR LLC, Radnor, PA, USA) and centrifuged at 1200 g for 10 minutes. The broth media was decanted and the cells were re-suspended in 3 ml sterile deionized water by vortex mixing. The suspension of cells in sterile water was used for inoculating samples.

*Lipomyces kononenkoae propagation*

The amylolytic yeast was obtained from the USDA ARS Culture Collection. Culture method for *L. kononenkoae* was similar to that used for the baker's yeast, with the notable exceptions that cells harvested from agar plates to inoculate the culture broth had to be from well-isolated colonies, and the incubation period was reduced to 9 hours for the last two attempts. The target cell count was $1x10^9$ in 15-30 ml broth, and the centrifuged cultures were decanted to approximately 5ml and mixed by vortex to re-suspend the cells in this reduced volume of the original growth media, instead of being suspended in water.

**Fermentation Process**

*Substrate preparation*

A single lot of food-grade, USDA organic, white grain sorghum (Pleasant Hill Grain, Hampton, NE, USA) was used in this experiment. The grain was ground in a Sunbeam Mr. Coffee grinder (Jarden Consumer Solutions, Boca Raton, FL, USA) and sieved through #18 mesh to yield particle sizes of 1mm or less. Forty grams of grain were weighed into sterile glass media bottles labeled 1 through 34 for the 32 treatments, pasteurized control, and sterilized control samples. The samples that received nitrogen supplement had the ammonium sulfate and

yeast extract mixed into the dry sorghum meal. Sterile deionized water was added to each bottle, and thermal treatment was applied immediately. Sterilized treatment bottles were autoclaved while the pasteurized treatment bottles were placed in an approximately 90°C water bath. The pasteurization time was measured from when the bath and samples equilibrated to 86°C, this temperature was maintained for 90 minutes. The pasteurized and sterilized samples were cooled to room temperature in a water bath while the yeast and lactobacillus were prepared for inoculation.

*Batch Fermentation*

Once the thermally-processed substrate cooled to room temperature, amyloglucosidase was added to bottles designated for enzyme treatment. The centrifuged *Lactobacillus* cultures had the MRS broth decanted, and the cells were re-suspended in 2 ml of PBS buffer and added to the co-culture treatment samples. The prepared yeast inoculum was added to the substrate. A summary of the treatment combinations used is listed in Table 6. The initial weight of each bottle was recorded, then the inoculated media bottles were placed in a 35°C water bath. One pasteurized control and one sterilized control sample of uninoculated grain slurry was placed in the bath with each run. After 9 hours of incubation, the bottles were removed from the bath, dried, and weighed again. The pH of each bottle was taken, and any pH below 4.5 was adjusted to around 5 with calcium carbonate (Alfa Aesar, Ward Hill, MD, USA). After pH adjustment was complete, all samples were weighed again and returned to the bath. Weights and/or samples were taken at 24-hour intervals. Samples collected were dried in an oven between 55°C and 70°C, transferred to sterile containers and held at room temperature until chemical analyses were conducted.

**Table 6: Experimental design by treatment**

| Sample | Yeast species | Nitrogen supplement | Thermal treatment | Enzyme addition | Lactic co-culture |
|---|---|---|---|---|---|
| 1 | *Saccharomyces cerevisiae* | Yes | 86°C | Yes | Yes |
| 2 | *Saccharomyces cerevisiae* | Yes | 86°C | Yes | No |
| 3 | *Saccharomyces cerevisiae* | Yes | 86°C | No | Yes |
| 4 | *Saccharomyces cerevisiae* | Yes | 86°C | No | No |
| 5 | *Saccharomyces cerevisiae* | Yes | 121°C | Yes | Yes |
| 6 | *Saccharomyces cerevisiae* | Yes | 121°C | Yes | No |
| 7 | *Saccharomyces cerevisiae* | Yes | 121°C | No | Yes |
| 8 | *Saccharomyces cerevisiae* | Yes | 121°C | No | No |
| 9 | *Saccharomyces cerevisiae* | No | 86°C | Yes | Yes |
| 10 | *Saccharomyces cerevisiae* | No | 86°C | Yes | No |
| 11 | *Saccharomyces cerevisiae* | No | 86°C | No | Yes |
| 12 | *Saccharomyces cerevisiae* | No | 86°C | No | No |
| 13 | *Saccharomyces cerevisiae* | No | 121°C | Yes | Yes |
| 14 | *Saccharomyces cerevisiae* | No | 121°C | Yes | No |
| 15 | *Saccharomyces cerevisiae* | No | 121°C | No | Yes |
| 16 | *Saccharomyces cerevisiae* | No | 121°C | No | No |
| 17 | *Lipomyces kononenkoae* | Yes | 86°C | Yes | Yes |
| 18 | *Lipomyces kononenkoae* | Yes | 86°C | Yes | No |
| 19 | *Lipomyces kononenkoae* | Yes | 86°C | No | Yes |
| 20 | *Lipomyces kononenkoae* | Yes | 86°C | No | No |
| 21 | *Lipomyces kononenkoae* | Yes | 121°C | Yes | Yes |
| 22 | *Lipomyces kononenkoae* | Yes | 121°C | Yes | No |
| 23 | *Lipomyces kononenkoae* | Yes | 121°C | No | Yes |
| 24 | *Lipomyces kononenkoae* | Yes | 121°C | No | No |
| 25 | *Lipomyces kononenkoae* | No | 86°C | Yes | Yes |
| 26 | *Lipomyces kononenkoae* | No | 86°C | Yes | No |
| 27 | *Lipomyces kononenkoae* | No | 86°C | No | Yes |
| 28 | *Lipomyces kononenkoae* | No | 86°C | No | No |
| 29 | *Lipomyces kononenkoae* | No | 121°C | Yes | Yes |
| 30 | *Lipomyces kononenkoae* | No | 121°C | Yes | No |
| 31 | *Lipomyces kononenkoae* | No | 121°C | No | Yes |
| 32 | *Lipomyces kononenkoae* | No | 121°C | No | No |

**Weight loss study**

The mechanism by which protein is concentrated as a result of fermentation is removal of carbohydrate from the substrate as the yeast metabolize sugars into carbon dioxide and water vapor. As such, a relationship between the decrease in mass of the substrate and the final protein

content could be expected. To determine whether this was the case, bottles of substrate with and without nitrogen supplementation were prepared for each yeast species and inoculated. The baker's yeast samples had amyloglucosidase added at the same rate used in the main experiment, 8 µl enzyme per gram of dry grain. The *L. kononenkoae* samples had no enzyme added, as in the preliminary screening study. These bottles were weighed and sampled at shorter intervals, with weights recorded at 0, 8, 18, 24, 30, 48, 71, and 99 hours, and protein analyses conducted at 0, 18, 30, and 48 hours.

**Crude protein analysis**

Samples collected during the run were dried in an oven between 55°C and 70°C, and crushed with a metal spoonula or ground with a mortar and pestle to prepare them for analyses. Dried samples were analyzed for percent moisture using an Omnimark µ-wave moisture analyzer (Sartorius Corporation, Bohemia, NY, USA), and prepared samples and extracts were tested for total and ammonia nitrogen at the Altheimer Laboratory in Fayetteville, AR. Total nitrogen was assayed by combustion using an Elementar Rapid N III unit (Elementar Analysensysteme GmbH, Langenselbold, Germany). Ammonia nitrogen was assayed as follows: one gram of sample was extracted into 30 ml of a 2 N solution of potassium chloride by mixing the samples and KCl solution in sterile plastic centrifuge tubes. The tubes were placed on a shaker at 180 rpm for 15 minutes, then the extract was filtered through #4 Whatman qualitative filter paper (GE Healthcare, Buckinghamshire, UK) into glass vials with screw caps. The filtered extract was analyzed for ammonia nitrogen content by Skalar autoanalyzer (Skalar Analytical, Breda, The Netherlands). Crude protein was calculated from the difference between total and ammonia nitrogen using a factor of 6.25, which is appropriate for calculating sorghum protein (Tontisirin and others, 2003).

**Statistical analysis**

Statistical analyses were performed using SAS 9.4 software (SAS Institute Inc., Cary, NC, USA). Effects of individual treatments and treatment interactions on crude protein were determined using analysis of variance (ANOVA), and means were compared with Fisher's least significant difference (LSD) test.

## RESULTS AND DISCUSSION

**Crude protein analysis**

After 48 hours of fermentation, only samples inoculated with baker's yeast and treated with amyloglucosidase exhibited any crude protein increase (Figure 4). Percent protein in samples with just one of these factors but not the other were not significantly different from the uninoculated control samples. No significant protein enrichment was observed in any sample inoculated with *L. kononenkoae* regardless of what other treatments were applied. Substrate mass

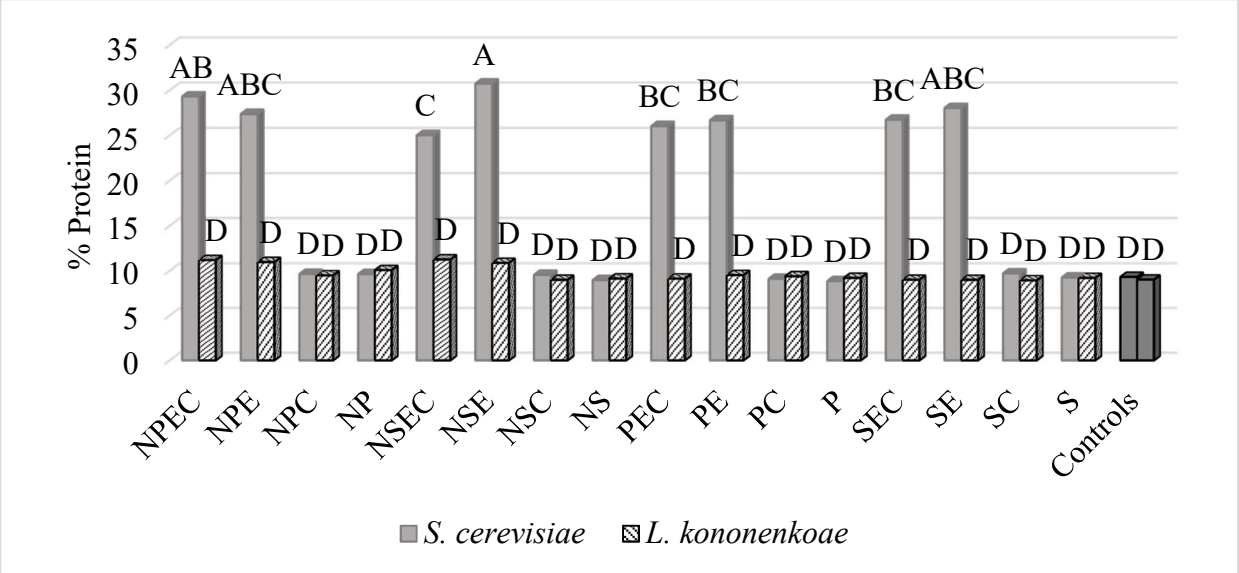


**Figure 4: Crude protein by yeast species after 48 hours of fermentation, compared to pasteurized and sterilized controls. N- Nitrogen supplement; P- Pasteurized; S- Sterilized; E- Enzyme treatment; C- Co-culture**

loss was observed with *L. kononenkoae* fermentation in the preliminary tests, but did not occur in any of the trials during the experiment, despite improvements to the culture method. Effects for each factor and their interactions are displayed in the analysis of variance (Table 7), only yeast type and enzyme addition have significant effect.

**Table 7: Significance of each treatment on percent crude protein at 48 hours of incubation. Y- yeast species, N- nitrogen supplement, T- thermal treatment, E- enzyme, C- co-culture. Significant factors (P<0.0001) are Y, E, and Y*E**

| Source | DF | Anova SS | Mean Square | F Value | Pr > F |
|---|---|---|---|---|---|
| Y | 1 | 1819.36248 | 1819.36248 | 321.42 | <.0001 |
| N | 1 | 0.138928 | 0.138928 | 0.02 | 0.876 |
| Y*N | 1 | 1.471635 | 1.471635 | 0.26 | 0.6119 |
| T | 1 | 19.618608 | 19.618608 | 3.47 | 0.0672 |
| Y*T | 1 | 0.730108 | 0.730108 | 0.13 | 0.7207 |
| N*T | 1 | 2.449287 | 2.449287 | 0.43 | 0.513 |
| Y*N*T | 1 | 1.823811 | 1.823811 | 0.32 | 0.5723 |
| E | 1 | 2158.122176 | 2158.122176 | 381.26 | <.0001 |
| Y*E | 1 | 1801.93874 | 1801.93874 | 318.34 | <.0001 |
| N*E | 1 | 0.410555 | 0.410555 | 0.07 | 0.7886 |
| Y*N*E | 1 | 0.007776 | 0.007776 | 0 | 0.9705 |
| T*E | 1 | 10.943101 | 10.943101 | 1.93 | 0.1692 |
| Y*T*E | 1 | 0.657035 | 0.657035 | 0.12 | 0.7344 |
| N*T*E | 1 | 0.013824 | 0.013824 | 0 | 0.9607 |
| Y*N*T*E | 1 | 0.729759 | 0.729759 | 0.13 | 0.7207 |
| C | 1 | 2.30764 | 2.30764 | 0.41 | 0.5254 |
| Y*C | 1 | 1.353275 | 1.353275 | 0.24 | 0.6265 |
| N*C | 1 | 4.532704 | 4.532704 | 0.8 | 0.3742 |
| Y*N*C | 1 | 6.091345 | 6.091345 | 1.08 | 0.3035 |
| T*C | 1 | 0.237009 | 0.237009 | 0.04 | 0.8385 |
| Y*T*C | 1 | 0.496513 | 0.496513 | 0.09 | 0.7681 |
| N*T*C | 1 | 3.369752 | 3.369752 | 0.6 | 0.4432 |
| Y*N*T*C | 1 | 5.012376 | 5.012376 | 0.89 | 0.3502 |
| E*C | 1 | 3.365257 | 3.365257 | 0.59 | 0.4435 |
| Y*E*C | 1 | 6.140817 | 6.140817 | 1.08 | 0.3015 |
| N*E*C | 1 | 6.842676 | 6.842676 | 1.21 | 0.2757 |
| Y*N*E*C | 1 | 8.799126 | 8.799126 | 1.55 | 0.217 |
| T*E*C | 1 | 0.000963 | 0.000963 | 0 | 0.9896 |
| Y*T*E*C | 1 | 1.039584 | 1.039584 | 0.18 | 0.6697 |
| N*T*E*C | 1 | 6.745841 | 6.745841 | 1.19 | 0.2791 |
| Y*N*T*E*C | 1 | 3.627815 | 3.627815 | 0.64 | 0.4263 |

Literature on protein enrichment fermentation using *L. kononenkoae* exists, but the organism almost always appears in co-culture with a non-amylolytic yeast (Horn, du Preez, and Kilian, 1992; Horn, du Preez and Lategan, 1988). It seems the predominant use of this organism is as an enzyme source. In the 1992 study, fermentation of a 10% sorghum slurry with a co-culture of *L. kononenkoae* and *Candida utilis* yielded a final crude protein content of 43.7 percent, but a fermentation using *L. kononenkoae* alone was not attempted. The low percent solids in the substrate was used to facilitate continuous stirring for oxygenation, which results in higher protein content. Stirring was not possible above 20% solids, so no higher percent solids were used.

Lactic co-culture, thermal treatment, and nitrogen supplement did not have any significant effect at 48 hours. However, based on mass lost in the test samples, addition of the nitrogen supplement did have an effect on the fermentation rate. The maximum amount of substrate mass lost was reached 24 to 30 hours earlier in samples with nitrogen supplementation, suggesting an effect on crude protein as well. Nitrogen supplementation has been shown to increase the rate and amount of protein accumulation in enrichment fermentation processes (Yang, 1988; Correia, Magalhães, and Macêdo, 2007).

The mechanism by which protein enrichment occurs from plant material is by the removal of non-protein substrate by the fermentation organism (Araújo et. al, 2005). The organism must be able to convert the carbon portion of the substrate into a substance that will leave the system, specifically $CO_2$ or water vapor, while retaining any proteinaceous mass. The biomass of the organisms themselves may have little effect on the total protein of the substrate, instead the amount of carbohydrate they can consume and release from the substrate will change the protein content. A study of ethanol fermentation from sorghum grain (Horn, du Preez, and

Killian 1992) yielded a high-protein distillers grain co-product. After complete conversion of starch to ethanol, the substrate was 43.2% protein. Initially, the substrate was 1000 kg of grain containing 120 kg protein, with 8.5 kg of protein from the yeast that was added. After all starch was consumed there was 300 kg of grain biomass left, which still contained 120 kg protein, and 9.5 kg of yeast protein. Almost 93% of the protein in was originally present in the grain, the increase in yeast biomass accounted for 0.77% of the total protein. A fermentation of sorghum specifically for protein enrichment resulted in a 30-fold increase in protein from yeast biomass, but not all starch was consumed, and the total protein in the substrate was still about 43% at the end of the fermentation (Horn, du Preez, and Killian 1992).

The amount of carbohydrate consumed was dependent on how much starch was converted to fermentable sugars. The amyloglucosidase was very effective at hydrolyzing the starch into sugars, and the baker's yeast was very effective at consuming those sugars. Despite a reported ability of *L. kononenkoae* to produce extracellular amylases capable of complete hydrolysis of starch (Spencer-Martins and van Uden, 1979; Ramachandran, Pretorius and Otero, 2005), the organism did not consume much carbohydrate in the substrate. There was no difference between starch only gelatinized by the thermal process, and starch hydrolyzed into sugars by the addition of amyloglucosidase. The fact that no significant protein enrichment occurred in either case implies that enzyme production by the yeast was not the limiting factor for enrichment to occur.

**Weight loss study**

As the fermentation progresses, the substrate mass decreases, and percent protein increases as a result. A plot of the weight lost in substrates treated with each yeast species shows a faster rate of loss in substrates with nitrogen added (Figure 5). Total weight loss was greater in

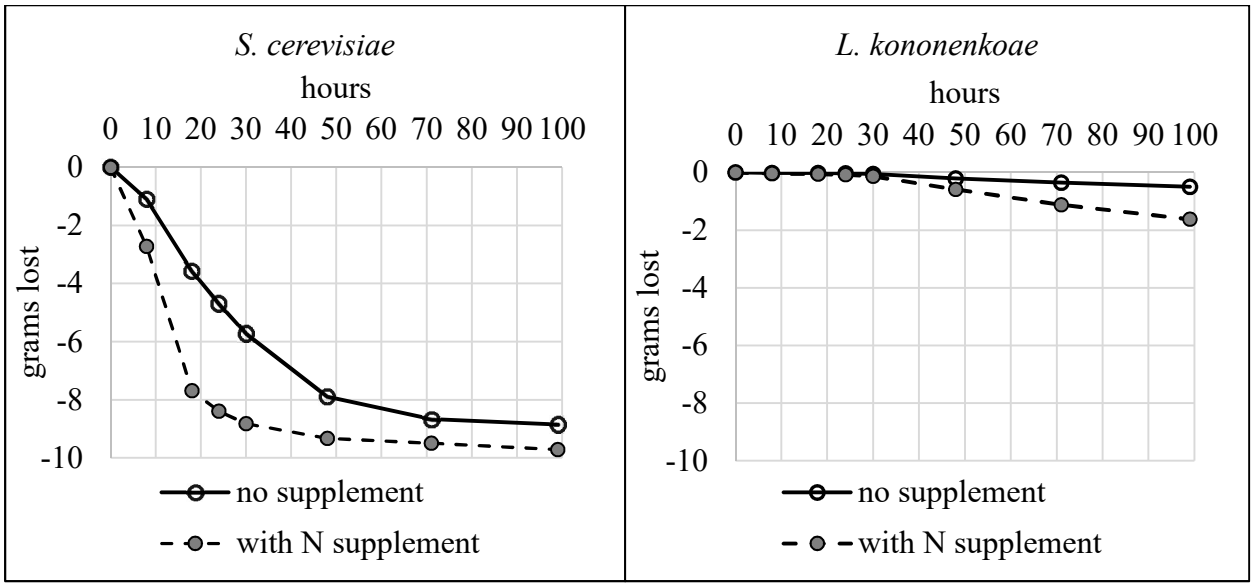


**Figure 5: Substrate weight losses over time for both yeast species**

the baker's yeast samples than in *L. kononenkoae* samples. Select samples from this study were analyzed for crude protein. The percent protein was calculated and plotted against the amount of substrate weight loss for each type of yeast. Percent crude protein was plotted against substrate weight loss (Figure 6). A linear correlation between weight loss and crude protein was observed for the baker's yeast samples, with an $R^2$ value of 0.98. No correlation was seen for the *L. kononenkoae* samples, possible since no statistically significant protein accumulation occurred in

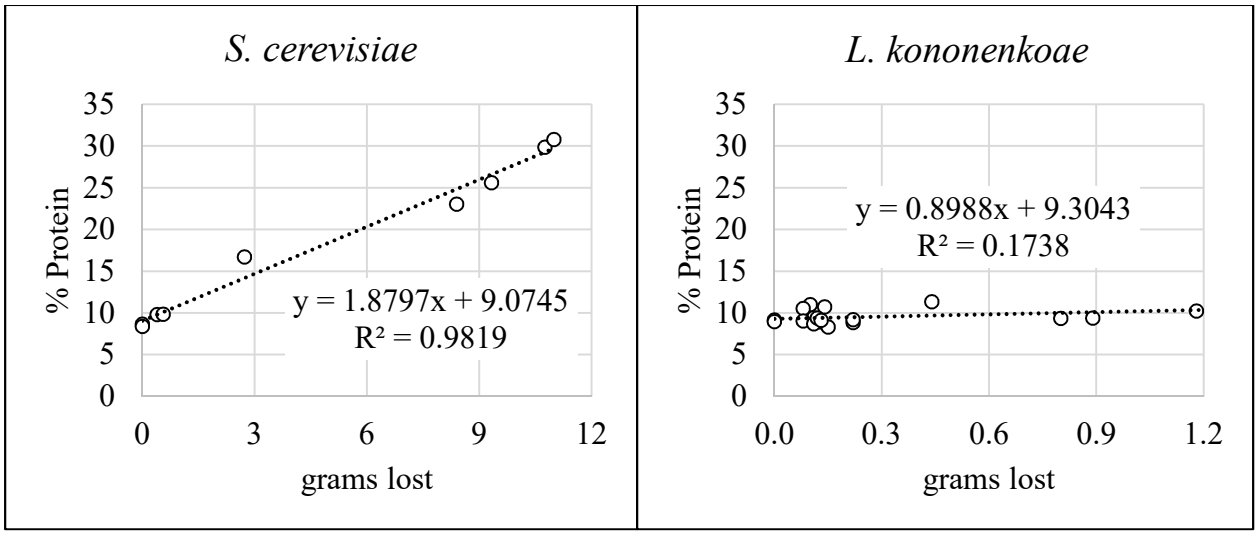


**Figure 6: Correlations between weight loss and percent crude protein in fermented samples**

any of these samples. The *L. kononenkoae* inoculum for this test initially failed to grow, so the samples were re-inoculated with freshly grown cells 18 hours later. Some weight loss occurred after the second inoculation, but did not show correlation to the protein content. The *L. kononenkoae* sample with nitrogen supplement did lose slightly more substrate mass than the sample without it, though this was not enough to make a significant difference in the amount of protein in the substrate.

Crude protein content analyses on the experimental samples was conducted after 48 hours of fermentation; to check the validity of the linear correlation, the equation was applied to the weight lost from the baker's yeast samples at 48 hours. The calculated values were plotted against the analytical values in to determine whether they were similar. The plot exhibited a linear correlation with an $R^2$ value of 0.976 (Figure 7). Thus, it is reasonable to assume the correlation can predict protein content from substrate weight losses that result from baker's yeast fermentation.

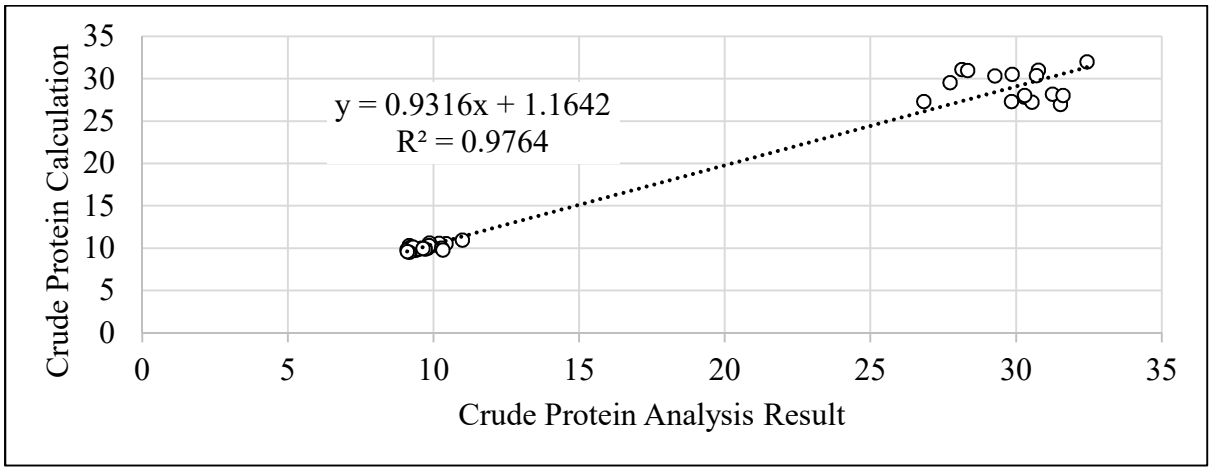


**Figure 7: Correlation between analytical results and calculated values for crude protein in baker's yeast fermentation samples after 48 hours of incubation**

## Treatment effects

*Nitrogen supplementation*

After 48 hours of fermentation, there was no significant difference in protein content between samples with the nitrogen supplement added and without it. However, in the baker's yeast samples it was apparent that weight loss occurred much more quickly in the nitrogen supplemented samples. The linear relationship found between weight loss and protein content was used to estimate the percent protein in the samples after 24 hours. The calculated values show a significant difference in protein between samples with nitrogen treatment and their untreated counterparts. This suggests that nitrogen availability is an important factor for fermentation efficiency, and has a significant effect on protein content early in the process.

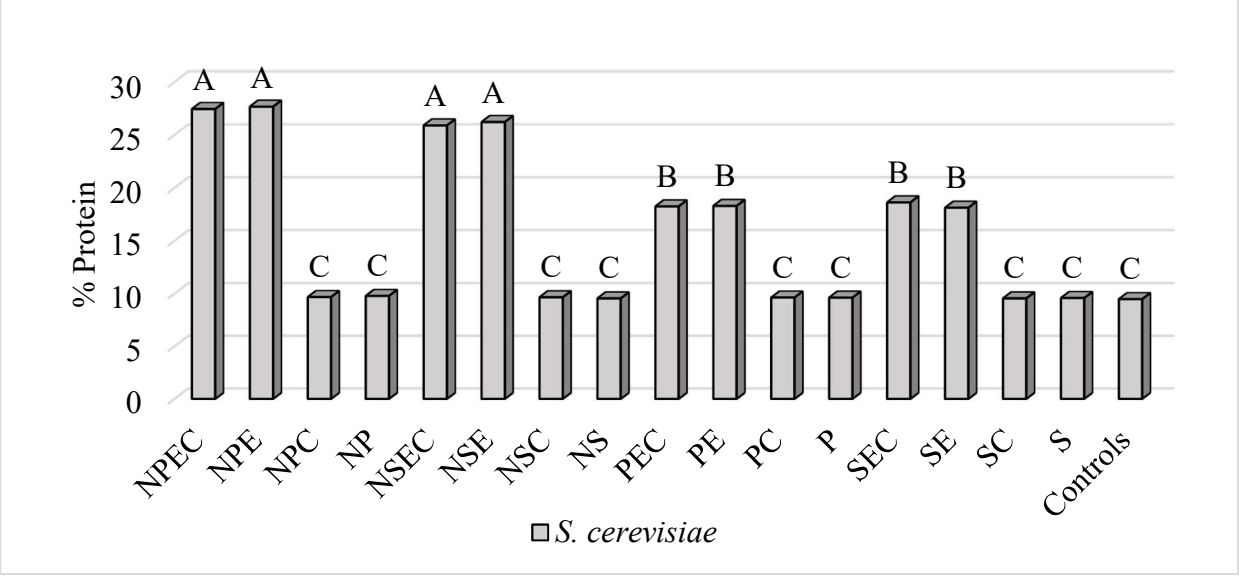


**Figure 8: Protein in baker's yeast samples calculated from weight lost after 24 hours of incubation. N- Nitrogen supplement; P- Pasteurized; S- Sterilized; E- Enzyme treatment; C- Co-culture**

The nitrogen source in the supplement used in this work was predominantly ammonium sulfate. Ammonia nitrogen was assayed as part of the crude protein determination, and this information can also give some insight on the fate of the nitrogen supplement in this system. The

nitrogen supplementation rate was reduced from the amount used in the preliminary study because high levels of ammonia nitrogen were detected in the fermented substrate, even in samples that displayed a lot of growth. Nitrogen concentrations that are too high can inhibit growth of some organisms under certain circumstances, (Rajagopal, Massé, and Singh, 2013) so the supplement was reduced form 3% to 2% for the main experiment.

The amount of ammonia nitrogen in preliminary samples with the 3% supplementation rate increased after fermentation. The increase was most likely a result of the entire substrate being concentrated as carbohydrate was consumed. However, at the 2% supplementation rate, ammonia nitrogen decreased in samples where protein enrichment occurred, even as the substrate became concentrated. Presence of ammonia nitrogen in inoculated samples that did not undergo protein enrichment shows that the ammonia nitrogen does not evaporate or get removed by other mechanisms during the fermentation, drying, or other steps in the process. This suggests that the fermentation organisms were in fact metabolizing the nitrogen, and doing so more effectively at the lower supplementation rate. Both the 3% and the 2% supplementation resulted in the same amount of protein enrichment, to between 25% and 30% protein for thermally

**Table 8: Typical examples of residual ammonia nitrogen in Baker's yeast samples. *'unfermented' samples were either not inoculated, initial time, or no amylase added. **'fermented' samples were incubated for 48 hours**

| Substrate treatment / nitrogen supplement rate | unfermented* NH4-N (mg/g) | fermented** NH4-N (mg/g) |
|---|---|---|
| Raw, co-culture / 3% | 4.314 | 4.474 |
| Raw / 3% | 4.395 | 5.178 |
| Pasteurized, co-culture / 3% N | 4.030 | 4.525 |
| Sterilized, co-culture / 3% N | 3.451 | 5.351 |
| Pasteurized, co-culture / 2% N | 2.537 | 0.568 |
| Sterilized, co-culture / 2% N | 2.613 | 1.247 |
| Pasteurized / 2% N | 2.814 | 0.503 |
| Sterilized / 2% N | 2.730 | 0.501 |
| Pasteurized Control / 0% N | 0.038 | 0.051 |
| Sterilized Control / 0% N | 0.047 | 0.021 |

processed samples. A study on nitrogen effects on wine yeast growth and sugar consumption reached a similar conclusion; nitrogen supplied in excess of what the yeast will metabolize will not have any effect on growth (Taillandier et. al., 2007). That study also found that a large excess in nitrogen could reduce the rate of sugar consumption by the yeast, which would slow the rate of protein concentration in a protein enrichment fermentation. This would imply that the lower supplementation amount is more efficient for this protein enrichment fermentation process.

*Effect of thermal processing*

The thermal process method had no significant effect on final crude protein. The purpose of the thermal treatments was to gelatinize the starch, and reduce microbial load of the substrate to prevent competition from the natural flora of the grain. One of the two factors that had an effect on crude protein was enzyme treatment; if the enzyme was more effective with one of the thermal treatments it could result in differences in the protein content. The fact that there was no difference suggests that the enzyme treatments were similarly effective on both types of thermally treated substrate. A similar outcome was obtained in a study on ethanol production from corn starch (Shigechi et. al., 2004); a strain of Saccharomyces cerevisiae engineered to express amylolytic enzymes produced the same amount of ethanol from starch cooked at 80°C as it did from starch cooked at 120°C. The specific temperature at which the starch is gelatinized is not as important as long as it is above the minimum gelatinization temperature for the starch, and the length of time at the temperature is sufficient for gelatinization to occur.

The thermal process also did not appear to have a direct effect on growth of the fermentation organisms themselves; protein enrichment was not significantly different between sterile and unsterile substrate. Any competition from the grain's natural flora did not affect the amount of substrate consumed by the yeast. The physical condition of the substrate after thermal

processing did not appear to have an effect on microbial growth. The pasteurized substrate remained soft and easily stirred after thermal processing, while the sterilization process completely solidified the grain slurry. Despite the rheological differences that resulted from the thermal processes at the initial time, both enzymatic and microbial action resulted in at least partial liquefaction of both types of substrate by the time pH adjustment was performed.

*Effect of co-culture organism*

Presence of *L. amylovorous* had no significant effects on crude protein content. The amylases secreted by *L. amylovorous* did result in some degree of starch liquefaction, which was more readily observed in samples without amyloglucosidase treatment. However, the activity of the enzymes produced by the bacteria did not have any detectable effect on the effectiveness of the added enzyme, and there was no evidence of increased fermentation rates in samples with co-culture added. If more sugars were being formed, no difference would be seen if there were not enough yeast to utilize them. The exogenous amylases produced by the co-culture also had no effect on the *L. kononenkoae* fermentations, which did not appear to be promoted or inhibited by the presence of the co-culture. Lactobacillus has been documented to reduce ethanol production by inhibiting yeast growth (Narendranath et. al., 1997), but any growth inhibition that may have occurred was not enough to reduce the amount of protein enrichment by the baker's yeast.

## CONCLUSION

Of the five treatments tested, the combined effect of baker's yeast and amyloglucosidase was the only factor that increased final crude protein content. Samples with one of those treatments applied but not the other had no significant increase in protein. Inoculation with *L. kononenkoae* did not result in any significant protein enrichment. Type of yeast and availability

of fermentable sugars are the most important factors in protein enrichment, nitrogen supplementation can accelerate the rate at which protein is enriched in the substrate.

## CHAPTER 4: OBJECTIVE 2- EFFECTS OF FERMENTATION ON PROTEIN DIGESTIBILITY AND PHYTATE CONTENT OF GRAIN SORGHUM

### INTRODUCTION

In addition to protein enrichment, fermentation has other effects on the substrate. Low digestibility of sorghum protein is one of the problems that limit its applications, and fermentation is one way to improve digestibility. Phytate compounds in grain can reduce digestibility of protein and other nutrients, so removal of this antinutrient is one mechanism by which digestibility increases. A fermentation that could improve digestibility as well as increase protein could increase the utility of this grain. The next set of analyses was conducted to determine fermentation effects on *in vitro* digestibility and phytate content in the substrate.

**Objective 2**: Compare the nutritional quality of sorghum fermented by different species of yeast in terms of in vitro protein digestibility and phytate content.

### MATERIALS AND METHODS

#### Percent digestibility

Percent digestibility was analyzed *in vitro* using two enzymatic methods: a multi-enzyme pH-drop method based on one developed by Hsu et. al. in 1977, and a standard method for pepsin digestibility modified from AOAC method 971.09. The pepsin method was performed by the Tyson Foods, Inc. Food Safety and Research Laboratory in Springdale, AR.

For the multienzyme method, the dried and ground samples were weighed into small glass beakers and a 1% NaCl solution was added. The amount of sample used was determined by the amount of protein in the sample, to achieve a slurry concentration with 6.25 mg protein per ml of the NaCl solution. The method was tested at the original sample size of 50 ml of the NaCl solution, and a reduced sample size which used only 15 ml of NaCl solution. Casein from bovine

milk (Sigma Aldrich Co., St. Louis, MO, USA) was used as a standard. The casein powder as received was approximately 93% protein, so amounts tested were 0.336 g in 50 ml of 1% NaCl, and 0.1008 g in 15 ml of the NaCl solution. The multienzyme solution was prepared fresh immediately prior to each test. Enzymes used were trypsin, chymotrypsin, and peptidase (Sigma Aldrich). Solid enzymes were dissolved into 1% NaCl at 1.6 mg trypsin/ml, 3.1 mg chymotrypsin/ml, and 1.3 mg peptidase/ml of the solution. The pH of the enzyme solution was adjusted to 8.0, and the enzyme solution was kept in an ice bath for the duration of the test. The pH of all samples and standards were adjusted to 8.0 immediately prior to analysis. Beakers were placed in a 37°C water bath with constant stirring while the pH was adjusted to 8.0, once the desired pH stabilized, the multienzyme solution was added at 10% of the sample volume (5 ml for 50 ml samples or 1.5 ml fir 15 ml samples). The resulting pH change after the enzyme addition was recorded over a period of 10 minutes. Percent digestibility was calculated by the applying the following equation:

$$\% \text{ digestibility} = 210.46 - 18.10 \times (\text{pH at 10 minutes})$$

The test was started when the initial pH stabilized at $8.0 \pm 0.01$, and the final pH was standardized by taking the absolute difference and subtracting it from 8.0 before applying the calculation.

Pepsin digestibility was determined as follows: solid pepsin enzyme was dissolved in 0.075M HCl to make three different concentrations of acid pepsin solutions. A 0.20 percent solution, a 0.020 percent solution, and a 0.002 percent solution (w/v) were prepared and used for this test. One gram of the dried, ground sample was weighed into filter paper for each test. The samples were extracted with petroleum ether to remove and quantify any fat by Soxhlet method, using a Gerhardt Soxtherm unit (C. Gerhardt GmbH & Co., Königswinter, Germany). The

defatted samples were transferred to 250 ml Erlenmeyer flasks and mixed with 150 ml of acid pepsin solution. The flasks were covered with foil and digested by incubation in a shaking water bath set to 45°C and 100 rpm for 16 hours. The digested samples were vacuum filtered through Whatman #541 filter paper on Buchner funnels and washed with several aliquots of DI water to remove the acid. The filter papers were transferred to Kjeldahl tubes to analyze the undigested residue for nitrogen using the Kjeldahl distillation method. The original samples were assayed for protein using a LECO Nitrogen Analyzer (LECO Corporation, Saint Joseph, MI, USA), and the percent digestibility was calculated from the difference in nitrogen between the original and pepsin-digested samples, using the following equation:

$$\text{Pepsin digestibility} = 100 \text{ x } \frac{(\text{Original \%N}) - (\text{Digested \%N})}{(\text{Original \%N})}$$

The digestibility was calculated separately for each concentration of pepsin used.

**Phytate Determination**

Phytate content was analyzed using the colorimetric method described by Haug and Lantzsch in 1983. Standards were prepared from solid phytic acid sodium salt hydrate (Sigma Aldrich) over a range of 3 to 30 µg/ml. To maintain an acidity of 0.2 N HCl for all standards, an aqueous stock solution of 300 µg/ml phytic acid was diluted 1:1 in 0.4 N HCl, and all standards were prepared by diluting this solution in volumes of 0.2 N HCl. A solution was prepared by dissolving 0.1 gram of solid of ammonium ferric sulfate (Acros Organics, Geel, Belgium) in 50 ml 0.2 N HCl, and diluting to 500 ml with deionized water. The indicator solution was made by dissolving 2.5 grams of 2,2' bipyridine (TCI America, Portland, OR, USA) and 2.5 ml of thioglycolic acid (TCI America) in 250 ml deionized water.

Approximately 0.04 grams of each sample were extracted into 10 milliliters of 0.2 N HCl, by rotary shaking in 50 ml Erlenmeyer flasks at 250 rpm for 2 hours at 29°C. After

extraction, 0.5 ml of each sample extract or standard were mixed with 1 ml of the ferric ammonium sulfate solution in 13x100 mm glass tubes with screw caps and boiled for 30 minutes. The samples were cooled in an ice bath for 15 minutes, then brought to room temperature. There are two methods to complete this analysis, and both were attempted. In the first, the 2 ml of the 2,2'-bipyridine/ thioglycolic acid indicator solution is added directly to the cooled tubes, mixed, and transferred to a disposable cuvette to read immediately. In the second method, the glass tubes were placed inside plastic centrifuge tubes and centrifuged at 3000 g for 30 minutes. One milliliter of the supernatant was mixed with 1.5 ml of the 2,2'-bipyridine/ thioglycolic acid indicator solution, which was then transferred to a cuvette. The absorbance was read at 519nm using a UV-1700 PharmaSpec spectrophotometer (Shimadzu, Columbia, MD, USA) for both methods. Phytate in the samples was quantified against a phytic acid standard curve.

**Statistical analysis**

Statistical analyses were performed using SAS 9.4 software (SAS Institute Inc., Cary, NC, USA). ANOVA with Fisher's LSD test was used to compare means and determine differences in phytate and multienzyme digestibility of select samples.

## RESULTS AND DISCUSSION

**Percent digestibility**

Two methods were used to determine percent digestibility. Pepsin digestibility is a standard method for animal feeds, the use of multiple concentrations of pepsin increase the sensitivity of the test (Johnston and Coon, 1979). The multi-enzyme method was designed to better predict digestion in human models (Hsu et. al., 1977). Four baker's yeast-fermented

samples and two *L. kononenkoae*-fermented samples were evaluated for pepsin digestibility; the same four baker's yeast samples were evaluated with the multi-enzyme assay.

The pepsin digestibility test yielded results similar to literature values for sorghum digestibility (Duodu et. al., 2003). The pepsin digestibility test was performed on composite samples because of limited sample availability, so the values obtained represent an average for the samples. According to the test method, low concentrations of pepsin give better correlation to *in vivo* digestibility, with the lowest concentration used here showing a strong correlation to *in vivo* lysine digestibility. Thermal processing appeared to reduce digestibility, with sterilized samples generally less digestible than pasteurized samples. Digestibility was increased by fermentation with both types of yeast compared to thermally processed controls, a result seen in yeast fermentations of other grains (Khetarpaul and Chauhan, 1990). Pasteurized samples fermented with baker's yeast were as digestible as raw grain, with sterilized baker's yeast samples slightly lower. Sterilized *L. kononenkoae* samples had slightly higher digestibility than sterilized baker's yeast samples. Natural fermentation was noted in the pasteurized control

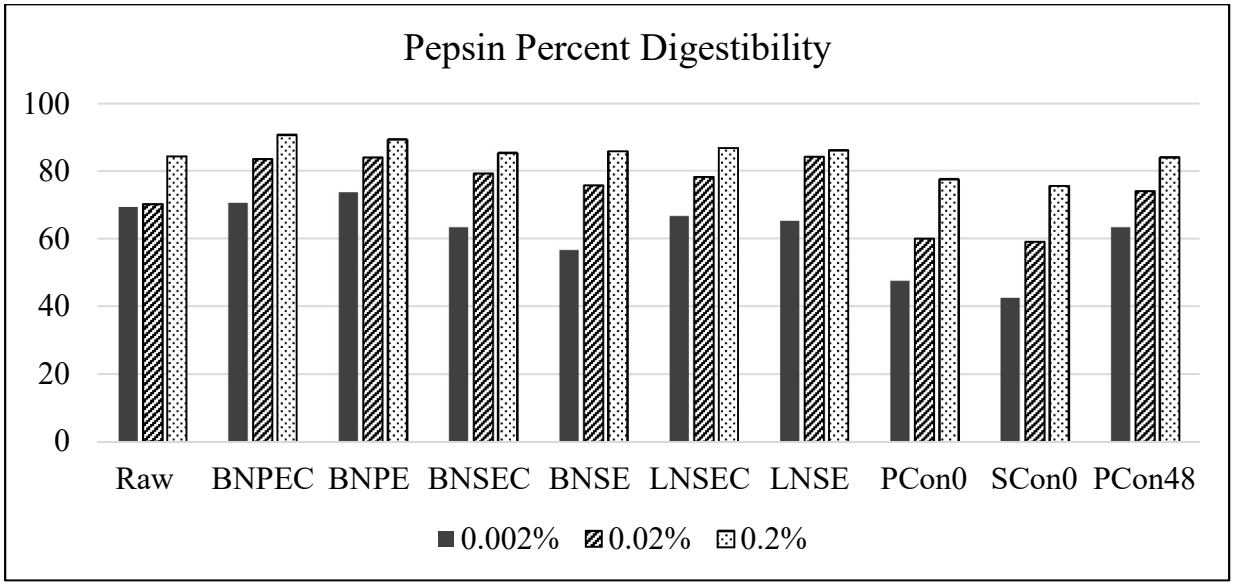


**Figure 9: Pepsin digestibility of select samples at 3 pepsin concentrations. B- Baker's yeast; L- *L. kononenkoae*; N- Nitrogen supplement; P- Pasteurized; S- Sterilized; E- Enzyme; C- Co-culture**

sample, an expected result of the non-sterile condition. This fermentation appears to have increased the digestibility compared to the initial condition of that sample, as seen in studies on natural fermentations of sorghum and green gram (Chavan, Chavan, and Kadam, 1988) and pearl millet (Elyas et. al., 2002).

The multi-enzyme test resulted in questionable values for percent digestibility. The average results for the test samples were similar to those obtained using the pepsin assay, but the standards and controls gave both inconsistent and unexpected results. The digestibility of the thermally processed sorghum controls at the initial time were not significantly different from the raw control or fermented samples. The digestibility of both thermally processed control samples was higher at the initial time than they were after 48 hours, despite evidence of natural fermentation (from wild flora in the pasteurized samples, or contamination of sterile samples)

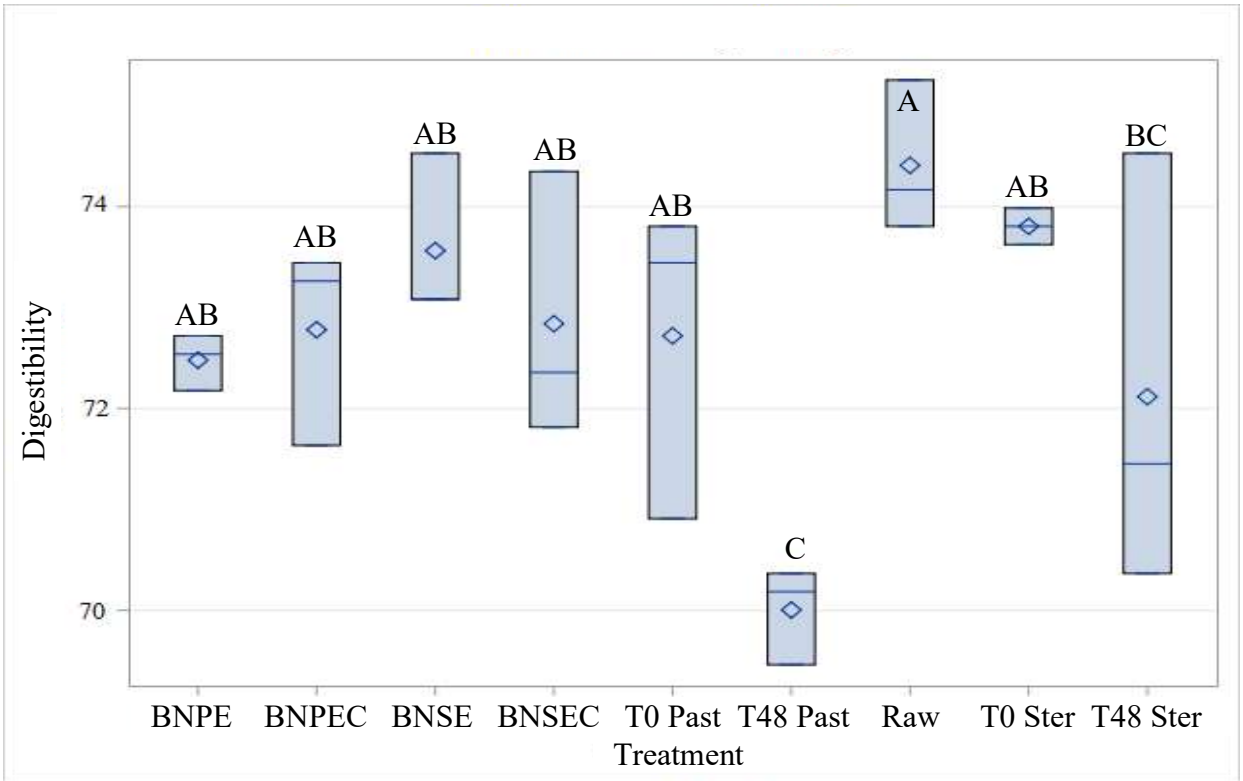


**Figure 10: Multi-enzyme digestibility of baker's yeast (B) and unfermented (Pasteurized, Raw, and Sterilized) samples at initial condition or 48 hours of incubation. N- Nitrogen supplement; P- Pasteurized; S- Sterilized; E- Enzyme; C- Co-culture**

occurring in the sample bottles. The digestibility for the casein standard used to verify the test yielded inconsistent results as well, with the pH drop varying from 1.12 to 1.38 for trials run on different days. This amount of variation in the pH should not occur for the same material. Similar variations were seen in the test samples, not necessarily during the same trials in which the casein standards were off. The pH meter was calibrated with fresh standards prior to each run, and verified against those same standards during and at the end of the run, but the sample variability persisted. One potential source of error was the time the sample spent stirring in the 37°C bath before the enzyme was added, which was not specified by the method. It is also unknown if additives like the ammonium sulfate used in the nitrogen supplement could potentially interfere with the method, no literature could be found on this matter.

**Phytate determination**

Microorganisms can use phytases to degrade phytates in plant materials to obtain phosphorus (Sreeramulu et. al., 1996; Türk, Carlsson, and Sandberg, 1996). This is one mechanism by which phytate concentrations in plant matter are reduced as a result of fermentation. In one study, fermentation of millet by yeasts and lactic acid bacteria resulted in a 50% reduction in phytic acid after 36 hours (Elyas et. al., 2002). A study of phytate in whole grain bread found that an acidic pH caused by the presence of organic acids in the dough can increase the activity of phytase, and baker's yeast phytases were most active at a pH of 4.5 (Türk, Carlsson, and Sandberg, 1996). However, in this experiment, the baker's yeast samples with the highest protein enrichment actually had higher phytate levels than the unfermented control samples (Figure 11). This was most likely a result of substrate concentration, as there is not a mechanism for phytates to be produced in the system. The carbohydrate reduction that resulted in higher percent protein in these samples would concentrate other components present

in the grain substrate. For a protein enrichment fermentation, simply evaluating the phytate content would give an incomplete view of the effect of the fermentation process.

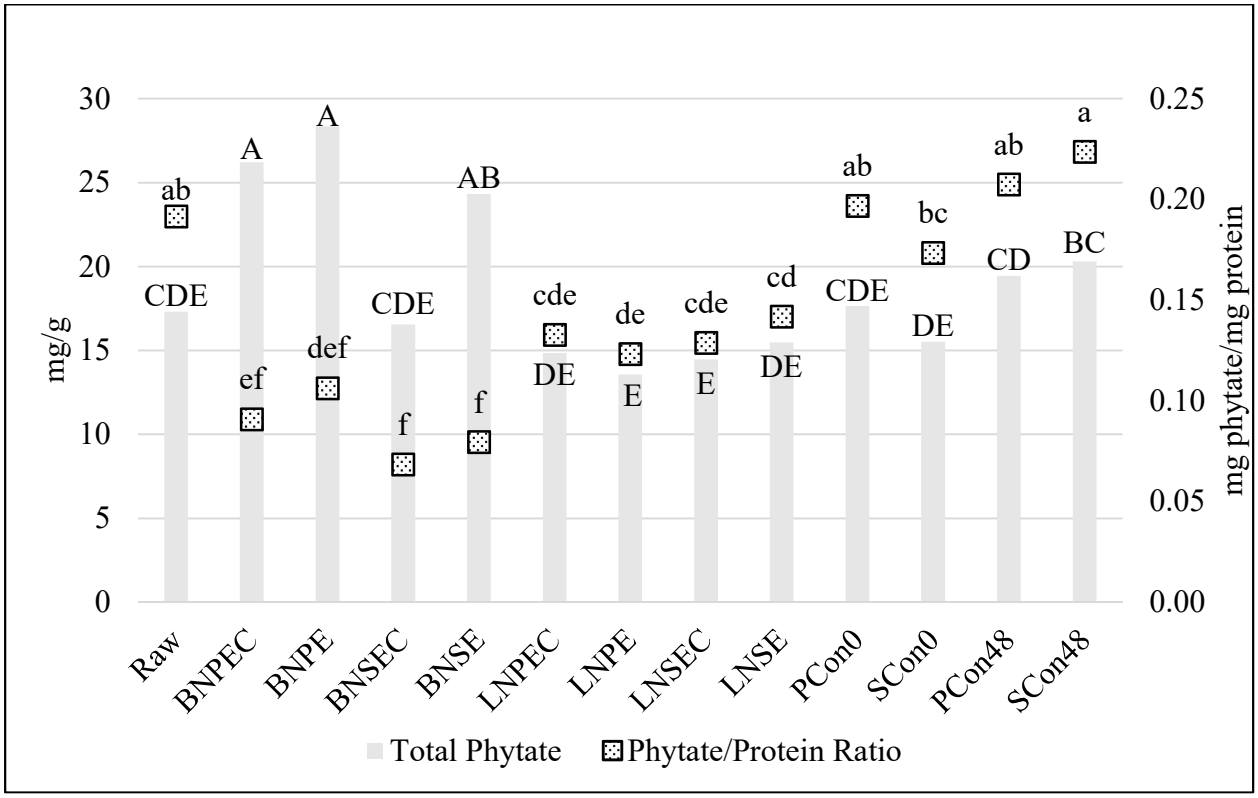


**Figure 11: Total phytate and phytate/protein ratio for select samples. B- Baker's yeast; L- *L. kononenkoae*; N- Nitrogen supplement; P- Pasteurized; S- Sterilized; E- Enzyme; C- Co-culture**

Phytate is considered an antinutrient because it binds to proteins, making them resistant to digestive enzymes (Osman, 2004). Thus, reducing the amount of phytate relative to the amount of protein should increase protein digestibility, even if the total phytate does not decrease. The amount of phytate was compared to the amount of protein in the samples. The phytate/protein ratio was lower in the fermented samples than the control samples, as seen in Figure 11. For baker's yeast, the ratio of phytate to protein is much lower than in the control samples, though with the concentration effect it is unclear if the reduction is merely an artifact of the high percent protein, or if the fermentation activity actually removed phytate from the substrate. The low protein samples baker's yeast samples were not tested, because it was not

certain that fermentation by the baker's yeast had occurred in these samples. There were *L. kononenkoae* samples that showed growth despite the lack of protein concentration; these samples were tested for phytate. These samples exhibited reduced phytate/protein ratios compared to the control samples, even without protein enrichment. This shows that the fermentation did reduce phytate content.

## CONCLUSION

Digestibility of sorghum grain was reduced by thermal treatments, but the protein enrichment fermentation described was demonstrated to mitigate decreases in pepsin digestibility. Some fermented samples approached or exceeded the percent pepsin digestibility of raw grain. Fermentation using *L. kononenkoae* also increased pepsin digestibility despite the fact that the fermentation did not result in protein enrichment. The multi-enzyme test attempted was not found to be suitable for this substrate. Phytate reduction was demonstrated in the *L. kononenkoae* fermentation, and implied in the baker's yeast fermentation, though the absolute phytate increased as the substrate became concentrated. Decreases in the ratio of phytate to protein may account for some of the positive effect on protein digestibility of the grain sorghum substrate.

# CHAPTER 5: CONCLUSIONS AND OPPORTUNITIES FOR FUTURE RESEARCH

Baker's yeast significantly increased percent protein of the sorghum substrate if amyloglucosidase was added; it has no amylolytic capability but is very efficient at metabolizing simple sugars if they are made available. Furthermore, the digestibility of the protein was improved by fermentation, each organism used increased pepsin digestibility compared to thermally processed control samples. The combined effect of the increase in protein content and digestibility results in over 20% digestible protein in the pasteurized samples fermented with baker's yeast, even at the lowest concentration of pepsin.

**Table 9: Percent digestible protein in sorghum samples at different pepsin concentrations. B- Baker's yeast; L- *L. kononenkoae*; N- Nitrogen supplement; P- Pasteurized; S- Sterilized; E- Enzyme; C- Co-culture**

| Sample | % Crude Protein | Digestible protein at 0.002% pepsin | Digestible protein at 0.02% pepsin | Digestible protein at 0.2% pepsin |
|---|---|---|---|---|
| Raw | 9.035 | 6.27 | 6.35 | 7.62 |
| Pasteurized | 9.257 | 4.40 | 5.55 | 7.17 |
| Sterilized | 8.91 | 3.79 | 5.26 | 6.74 |
| BNPEC | 29.312 | 20.67 | 24.47 | 26.55 |
| BNPE | 27.369 | 20.15 | 22.98 | 24.42 |
| BNSEC | 25.022 | 15.84 | 19.82 | 21.34 |
| BNSE | 30.736 | 17.38 | 23.27 | 26.37 |
| LNSEC | 11.261 | 7.51 | 8.80 | 9.76 |
| LNSE | 10.875 | 7.09 | 9.15 | 9.36 |

The *S. cerevisiae* used commercially and sold to consumers for baking is a particularly robust organism. There is evidence that species in this genus of yeast has been in use by humans for food production for 2500 years, and beverage production considerably longer (Mortimer, 2000), and is well adapted to these applications. It is capable of growing at a wide range of temperatures and does not appear to suffer from temperature variation or overcrowding issues that were observed when using *L. kononenkoae*. It is also generally recognized as safe (GRAS)

for human consumption, and is already used as a protein source in animal feed applications (Winkler et. al., 2001; Lim, Li, and Klesius, 2011). These attributes make this yeast a good candidate for continued research in protein enrichment.

The protein increase observed in this study could likely be improved by altering the fermentation process. Baker's yeast grows most efficiently under aerobic conditions, but the batch system employed here is not the most effective for oxygen transfer, especially once the samples liquefy from enzymatic activity. Oxygen-limited systems favor ethanol production over cell growth (Strohm and Dale, 1961), better oxygenation results in more carbohydrate consumption, reduced ethanol production, and higher biomass accumulation (Novak et. al., 2007). A process with constant stirring or air bubbled through the substrate could result in higher protein accumulation, or shorter fermentation time as a result of more aerobic activity.

Enzyme use in the system could be optimized as well. The amyloglucosidase addition rate in this experiment was in excess of literature values for sorghum starch hydrolysis (du Preez et. al., 1985) and could most likely be reduced for this system. Enzymes are one of the most expensive components in this process, the fermentation would be more economically feasible if the quantity of enzyme could be decreased. Some work has been done to eliminate enzyme addition completely through the use of *S. cerevisiae* that can metabolize starch (Ogden and Tubb, 1984). Genetic modification of *S. cerevisiae* by insertion of genes for amylase production has been attempted, with limited success in direct starch conversion (Eksteen et. al., 2003). However, this could sacrifice some of the utility as a food ingredient if the modified yeast did not retain GRAS status.

Though use of *L. kononenkoae* did not result in effective protein concentration, there are other amylolytic organisms that could be used for this purpose. Of the organisms screened for

this research, *Saccharomycopsis fibuligera* was very effective at liquefaction of the substrate, and is known to make both amylase and amyloglucosidase enzymes. However, the main product of the fermentation is the sugar trehalose (Chi et. al., 2009), which remains in the substrate; no protein concentration occurs. Combining this yeast with an organism capable of metabolizing that sugar into carbon dioxide and water could yield an effective protein concentration process. Co-culture may be more effective than monoculture for this application.

This work has shown the potential for improvement of sorghum grain through yeast fermentation. Not only can the protein digestibility be improved, and the antinutrient phytate decreased, but the percentage of protein can increase significantly though fermentation. Though the process reduces the total grain mass, the increase in protein could yield a more valuable product than the original grain. One potential application of the work here could be to test the effect of yeast fermentation on the types of tannin sorghum commonly grown in other areas of the world. Development of an efficient fermentation process that could increase both protein digestibility and content in these grains could improve the nutritional quality and utility of this staple crop.

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## APPENDIX 1: OTHER EFFECTS OF FERMENTATION

While the main objectives of this study were to determine the effects of five test factors on three responses (crude protein, percent digestibility, and phytate content) there were other interesting observations on the effects of the treatments. Qualitative effects of fermentation observed are discussed below. Although thermal treatment and lactic co-culture did not have an effect on protein enrichment or phytate content, they did have an obvious effect on organoleptic qualities of the fermented grain. Sterilization and lactic fermentation reduced incidence of spoilage and other signs of contamination. These effects were not in the scope of the main experiment, but would be worthwhile considerations for any future work.

*Effect of thermal processing*

The parameters chosen for the thermal process were meant to capture the extremes of potential thermal processes. The standard sterilization method is a relatively harsh process, which subjects the substrate to very high temperatures and pressures in a moist environment. These are the conditions documented to have negative effects of sorghum digestibility (Duodu et. al., 2003). The sterilization was compared to a pasteurization conducted at a relatively low temperature, to determine whether reducing the thermal processing temperature had an effect, particularly on digestibility of the sorghum protein.

A notable difference was seen between thermal processes in pepsin digestibility, but more testing would be needed to confirm its significance. Type of thermal process did not appear to make a difference in phytate content. The thermal process did have qualitative effects on the fermentation. As pasteurized samples are not sterile, evidence of activity by other organisms was observed in most of these, particularly in samples without the lactic co-culture. Gray, red, purple, and yellow discoloration was often observed, occasionally slime formation was seen as well.

Pasteurized samples also tended to develop rotten or rancid odors; these were less prevalent in sterilized samples. Signs of spoilage did occur in some sterilized substrates, most likely as a result of contamination during sampling or pH adjustment, but there were far fewer instances of discoloration or off odors in the sterilized substrate bottles. Organoleptic qualities do become important in food and feed applications, this would be another factor that would need to be considered in choosing an appropriate thermal process. The milder process, which preserves digestibility, could be used with other antimicrobial treatments to maintain quality.

*Lipomyces kononenkoae fermentation*

*L. kononenkoae* has been used in fermentation studies for nutrient enrichment. As the genus name suggests, the organism generates fatty acids as a fermentation product. It also metabolizes carbohydrates into carbon dioxide, and produces amylases to consume starch, which makes it a candidate for protein enrichment of starchy substrates. This organism has appeared in a few such studies, but seems to be primarily used for the extracellular amylase it produces rather than as the primary fermentation organism; it occasionally appears as a co-culture organism (Horn et. al., 1992). In the work described here, the exponential phase of growth occurred between 8 and 12 hours at 25°C, and a 9-hour incubation was used to prepare the inoculum for the experiments. Cell counts fell after 15 hours, and the viability of the cells was severely diminished by 18 hours. However, in literature, incubations for *L. kononenkoae* cultures ranged between 20 and 24 hours (Horn et. al., 1992; Wang et. al., 2011). Even if the cells did begin to die off, the amylolytic enzymes they produced would remain functional in the broth media such that a second organism would have access to sugars freed by the activity of those enzymes. In the work described here, *L. kononenkoae* is used for fermentation directly. Compared to unfermented grain, slight protein enrichment did occur, but the amylolytic yeast was not

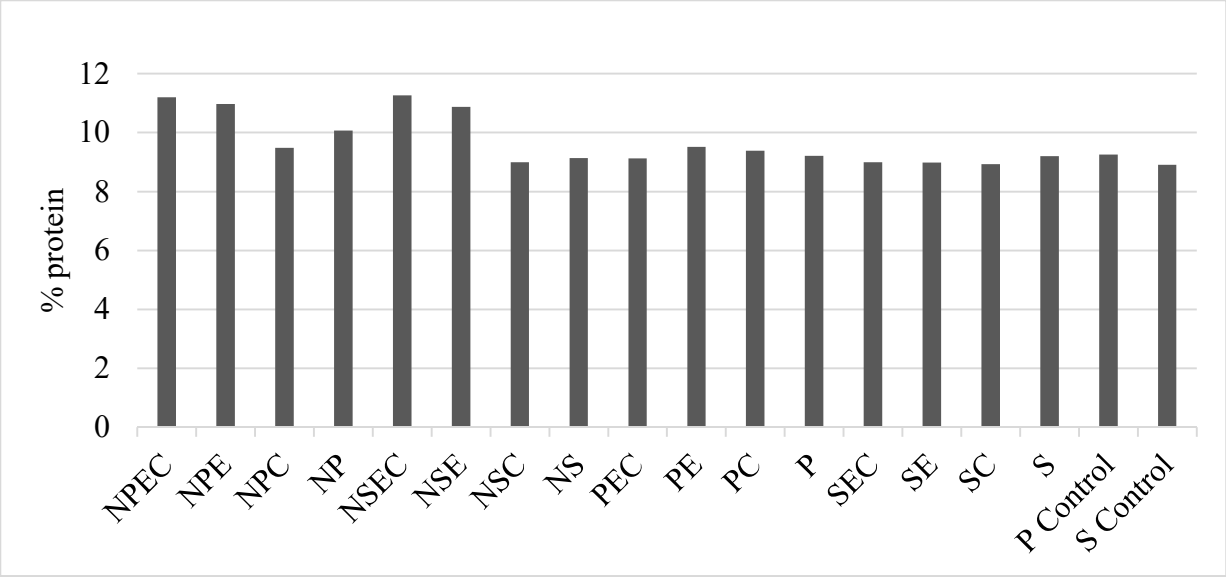


**Figure 12: Percent protein in *L. kononenkoae*-fermented samples at 48 hours of incubation. N- Nitrogen supplement; P- Pasteurized; S- Sterilized; E- Enzyme; C- Co-culture**

effective at concentrating the protein in sorghum grain compared to the baker's yeast with enzyme addition. When only *L. kononenkoae* samples are considered, the samples follow a similar pattern to the baker's yeast samples, with enzyme treatment and nitrogen addition having an influence on percent protein.

One possible reason this yeast did not perform as well as expected is that it is difficult to cultivate; it has a narrow temperature range for optimum growth, and will reach the stationary phase rapidly with low cell counts at temperatures above 29°C. Growth of the organism is also inhibited by crowded conditions. Cells collected from a continuous lawn on an agar plate will have an extended lag phase and slow growth, it may take up to 20 hours before any turbidity is observed in the growth medium. The cells are very small, and relatively high counts do not yield much cellular mass compared to other yeast species. Attempting to increase the counts by increasing the inoculum may result in inhibited growth, or no observable growth in the media.

Fermentation temperature may have also had an effect on the performance of *L. kononenkoae* in this case. The temperature in this experiment was fixed at 35°C and not

considered a factor in the experimental design. It was determined that incubation temperature had little effect on the performance of the baker's yeast in preliminary studies, an observation also seen in other fermentations using *S. cerevisiae* for protein enrichment (Araújo et al, 2005). The temperature selected for the study was between the optimum growth temperature for the yeasts and that of the lactic co-culture organism; the optimum temperatures for the enzymes to function was also considered, but such temperatures (40°C to 60°C depending on type and source of the amylase) would be too high for the organisms to survive. A specific study of the effect of temperature on *L. kononenkoae* may have been helpful in determining what temperature to use for this experiment, but it did appear that *L. kononenkoae* would grow at 37°C during the preliminary screening for amylolytic yeasts. It also appeared to grow at 35°C during the propagation experiment to determine the incubation time.

*Effect of co-culture organism*

Presence of *L. amylovorous* appeared to have no significant effects on the investigated results. There were not significant differences in protein content or phytate concentration between samples with the co-culture and samples without it, and its presence in the fermentation samples did not have an obvious effect on digestibility.

Though the amylolytic lactic co-culture did not improve the final protein content, other benefits were observed. The yeast appeared to tolerate the presence of *L. amylovorous* well enough that there was no decrease in protein in co-culture treated samples compared to samples without the co-culture. The presence of the co-culture effectively reduced contamination by other organisms, either present in the pasteurized substrate or introduced into the sterilized substrate by sampling or pH adjustment. Samples without the co-culture were more likely to exhibit poor organoleptic qualities such as foul odors, off colors, slime formation, and other evidence of

spoilage. By contrast, the samples with *L. amylovorous* added generally had no offensive odors and could even be described as pleasant. Though mold can grow at the reduced pH induced by lactic fermentation, samples with the *L. amylovorous* co-culture did not have as many instances of observable mold contamination as samples only inoculated with yeast.

## APPENDIX 2: PHYTATE RESULT TABLE

**Table 10: Phytates in control and select test samples. N- Nitrogen supplement; P- Pasteurized; S- Sterilized; E- Enzyme treatment; C- Co-culture.**

| Treatment | | mg/g Phytate | | mg Phytate / mg Protein | |
|---|---|---|---|---|---|
| Raw Control | | 17.31 | CDE | 0.192 | AB |
| Bakers yeast | NPEC | 26.20 | A | 0.091 | EF |
| | NPE | 28.35 | A | 0.106 | DEF |
| | NSEC | 16.54 | C | 0.068 | F |
| | NSE | 24.32 | AB | 0.080 | F |
| L. kononenkoae | NPEC | 14.85 | E | 0.133 | CDE |
| | NPE | 13.58 | E | 0.123 | DE |
| | NSEC | 14.47 | DE | 0.129 | CDE |
| | NSE | 15.49 | DE | 0.142 | CD |
| Thermally Processed Controls | P 0 | 17.64 | CDE | 0.197 | AB |
| | S 0 | 15.51 | DE | 0.173 | BC |
| | P 48 | 19.44 | CD | 0.207 | AB |
| | S 48 | 20.31 | BC | 0.224 | A |

## APPENDIX 3: PROTEIN AND FAT ANALYSIS FROM TYSON FOOD SAFETY AND RESEARCH LABORATORY

**Table 11: Protein by combustion (AOAC 992.15; 990.03) and fat by Rapid Soxhlet petroleum ether extraction (AOAC 2003.05; AOAC 991.36). B- Baker's Yeast, L- *L. kononenkoae*, N- nitrogen, P- pasteurized, S- sterilized, E- enzyme, C- co-culture**

| Sample | Treatments | % Protein | % Fat |
|---|---|---|---|
| Raw Control | None | 7.54 | 2.26 |
| 1 | B, N, P, E, C | 26.50 | 5.34 |
| 2 | B, N, P, E | 28.44 | 5.94 |
| 5 | B, N, S, E, C | 25.85 | 4.92 |
| 6 | B, N, S, E | 29.07 | 6.26 |
| 21 | L, N, S, E, C | 10.29 | 1.20 |
| 22 | L, N, S, E, | 9.91 | 1.78 |
| 33 initial | P | 7.51 | 1.19 |
| 34 initial | S | 7.93 | 1.53 |
| 33 48 hours | P | 8.16 | 2.09 |