Title
Conversion of Almond Hulls into Protein-Enriched Animal Feed Supplements

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Conversion of Almond Hulls into Protein-Enriched Animal Feed Supplements

By

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Abstract

Almond hulls are a byproduct of the almond industry and are typically used as cattle feed. They have a high fiber content but low protein content. Due to downsizing in the dairy industry and an increasing almond industry in California, demand for the hulls as dairy feed has decreased. However, poultry and aquatic industries are pursuing new materials for protein-rich feed supplements. Using a solid-state fermentation (SSF) conversion process, the hulls were used as substrate for filamentous fungi which increased the protein content of the hulls and resulting fermentation residue to the appropriate levels for animal feed supplements. The first objective in converting the hulls into protein-rich fungal biomass was to identify the optimal fungal strain for biomass production using the hulls as the substrate. The second objective was to optimize the SSF process using the optimal fungi identified through the first objective. After screening various fungi, *Myceliophthora thermophila* was identified as the optimal fungi for protein production capable of producing fermentation reside with a maximum crude protein content of 18.10%.

Fermentation conditions for key parameters were optimized. The optimized fermentation condition for the *M. thermophila* strain was almond hull particle size (75-250 μm), fermentation time (96 hours), and solid-loading (6.90% w/w). The fermentation residue produced contained 18.10% crude protein and 6.25% crude fat. The protein yield was 0.20 g crude protein/g carbohydrate consumed. The residue had good *in vitro* digestibility (80.37 ± 7.06%) and contained a complete amino acid profile. The fermentation temperature and relative humidity were kept constant at 48-50°C with relative humidity controlled at 70-90%. Therefore, the *M. thermophila* fermentation residue could potentially serve as a protein-rich supplement for poultry feed supplements. However, the fiber content of the residue is too high for direct use as an

aquatic feed supplement. Additional procedures are needed to extract the protein from the fermentation residue for aquatic feeds.

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1. Introduction

1.1. Almond Hulls and their Nutritional Content

California produces nearly 80% of the world's almond supply [1]. During almond processing and drying, almond hulls are obtained as an agricultural byproduct of the almond industry. They surround and protect the shells encasing the almond kernels [2]. After almonds are harvested, the hulls are removed from the nuts that are to be sold and consumed [3]. In California, 4.031 billion pounds of hulls were produced during the 2019/2020 crop year [1]. These hulls are a low-quality crop residue meaning they are low in protein but high in fiber [4]. They are traditionally sold and used as an important dairy feedstuff in California [3], [5]. Dairy cattle are ruminants allowing them to break down and digest the tough lignocellulosic material of the hulls. The hulls also contain high levels of soluble sugars, which can be easily fermented in the rumen of the cattle [3].

Almond hulls are composed of soluble sugars (21-25%), cellulose (9-16%), hemicellulose (7-10%), lignin (4-15%), pectin (4-6%), fat (1-2%), ash (6-13%), and sugar alcohols such as inositol (2-2.5%) and sorbitol (3-5%) on a dry weight basis [6]. However, they also have a low protein content (4-6%) in addition to a high fiber content (30-40%), preventing them from being used directly as a feedstuff for animals other than cattle.

1.2. Research Background and Motivation

The number of dairy operations in the United States has been steadily decreasing over the past 15 to 20 years [7]. Dairy farmers are struggling to maintain their operations due to the global oversupply of milk leading to depressed milk prices [8]. These are extremely difficult conditions for dairy farmers, prompting them to sell their cows and leave the industry [7]. Since 2003, over half of these dairy operations have been lost, leaving the current number of operations around

32,000 [7]. California is the largest milk-producing state in the U.S. and was reported to have 1,335 licensed dairy herds in 2018 [7], [9]. However, that number decreased to 1,255 in 2019 following the downward trend of dairy operations in the country [9]. Along with the decline in licensed dairy operations, milk production per cow has increased about 10.6% from 2010 [10]. This also leads to a lower number of milk cows needed. Almonds hulls are traditionally sold to these California dairies as they are close to the almond facilities and lower transportation costs [11].

The downsizing of the dairy industry in California has lowered the need for almond hulls as dairy feedstuff and bedding. However, the almond industry continues to grow, resulting in increased production of the almond hull byproduct. This results in an excess amount of hulls and reduces their price [12]. In 2017, almond hulls were selling for about $60 per ton, whereas the price was almost double at around $120 per ton in 2012 [11]. Alternative uses for the almond hulls need to be identified to turn this waste stream into value-added products. Thus, expanding agricultural industries were explored and assessed for their need of alternative raw materials to support their growing operations. Poultry and aquatic industries are actively seeking alternative raw materials for protein-rich feed supplements to support their continued production growth [13], [14].

1.3. Chicken and Fish Feed Industries as Alternative Markets

The U.S. has the world's largest poultry industry [15]. Americans are consuming more poultry than both beef and pork. The U.S. is also the second-largest exporter of poultry, exporting around 18% of the total poultry produced [15]. In 2014, 8.54 billion broilers were produced [16]. In 2018, this number increased to 9.04 billion broilers produced [15]. Poultry

production in the U.S. continues to steadily increase along with an increased need for feed sources.

Broilers consume approximately 15 lbs of feed over their lifetime [17]. Based on the broiler production in the U.S. in 2018, 135.6 billion lbs of feed were needed to support broiler production. Chicken feed costs reported by farmers in the northwestern part of the country are approximately $700 per ton [18]. Using the reported price of chicken feed, it costs approximately $47.46 billion to supply the feed needed for broiler production in 2018. Chicken feed makes up the largest portion of production costs for farmers.

In 2017, 170 million tons of fish were produced worldwide through aquaculture. Fish feed makes up 30-60% of these production costs [13]. The most expensive part of the fish feed is fishmeal. Fishmeal is the protein supplement within the feed that is typically made from small, pelagic fish [13]. Due to increases in ocean water temperatures, the reproductive cycle of these fish is affected and makes them more difficult to fish [13]. The scarcity of pelagic fish for fishmeal and their increased demand have greatly increased the price of fishmeal. Fishmeal price has increased from $350 per ton in 2000 to $1,600 per ton in 2017 [13]. In 2015, 4,731,000 tons of fishmeal were produced worldwide, while production within the U.S. was about 263,000 tons [19]. Based on the price of fishmeal in 2017, it costs about $7.57 billion to supply the needed amount of fishmeal for worldwide fish production and $420.80 million for U.S. production in 2015. Fish feed costs, like chicken feed costs, are the greatest expense for farmers.

Both chicken and fish feed markets are actively seeking alternative raw materials for protein feed sources. Almond hulls are a viable source of raw organic material if the protein content could be increased to 15% which is suitable for both chicken and fish feed supplements [20], [21]. A method needed to be identified to increase the protein content of the almond hulls

to make them suitable for the growing need of chicken feed supplements. The extracted protein could also be used as aquatic feed supplements [13].

1.4. Fungal Conversion

Filamentous fungi have a high crude protein content of about 40-50% and are classified as a microbial-based protein source [13]. These fungi are capable of growing on numerous organic wastes and producing valued products such as enzymes through their biological conversion processes [13]. Some filamentous fungi produce cellulases and ligninases to help breakdown tough lignocellulosic material and release cellulose from both hemicellulose and lignin, increasing the free sugars available for the fungi to consume [22]. Fungal biomass has also been shown to have a desirable amino acid profile compared to fishmeal and soybean meal used for poultry [23]–[25]. Therefore, fungal conversion using the almond hull as the organic waste substrate was proposed as a promising method to increase the protein content of the hulls.

Almond hulls were predicted to be suitable as a substrate for filamentous fungi as they have a high amount of soluble sugars readily available for fungal consumption [6]. They are also composed of lignocellulosic material, which fungi are capable of breaking down with produced enzymes to increase the availability of free sugars [13]. Additionally, using a fungal conversion method for protein production does not compete with human food sources and makes use of a waste stream that would otherwise be discarded [13]. Fungal conversion of a substrate can be performed using different types of fermentation processes, including solid-state fermentation (SSF) and submerged fermentation (SmF). Different fungal species will also vary in protein content, biomass composition, and amino acid profiles. Thus, it is important to choose a fermentation process and fungal strain that will produce optimal results. For the present study,

SSF was used with different fungal species and almond hulls to produce high protein fermentation residue.

1.5. Solid-State Fermentation

Solid-state fermentation (SSF) is a conversion process where microorganisms are grown on solid substrates with little to no free water [4], [26]. In some cases, SSF is capable of providing economic and environmental benefits over the more commonly used submerged fermentation (SmF). SmF requires liquid media and constant agitation, which can lead to high production costs [27]. With SSF, there are smaller volumes of effluents, and no agitation is needed [4]. This allows for more natural cultivation of the fungi as it would grow in nature and, in some cases, also promotes higher production of enzymes that help break down the lignocellulosic material [28]. SSF of fungal cultures compared to SmF has been reported to produce a higher titer of crude enzymes [27]. There is also increased aeration compared to SmF [4].

Overall, SSF has lower reactor costs, lower energy requirements, lower water consumption, and smaller reactor size [4]. However, unique heat and mass transfer challenges can occur in SSF. Lignocellulosic material has poor conductivity and makes heat transfer challenging through a solid matrix [29]. The heat produced by the microorganism's metabolism during fermentation can also lead to spatial temperature gradients within the reactor [29]. Homogeneity in the SSF reactors is also difficult to achieve due to the lack of agitation and water loss from high fermentation temperatures and microbial consumption [26]. Humidity control is necessary for a successful SSF process to combat water loss from evaporation and control the capillary water supply [26]. Capillary water is the major form of water present in SSF located in the capillary space of the substrate and is available for direct use by microorganisms [26]. Thus,

it is important to make sure this water supply is kept constant to aid in the transfer of nutrients, heat transfer, and homogeneity of the cultures [26].

1.6. Thermophilic Fungi for Fungal Conversion

Fungal species for the proposed study were identified and selected using a few main criteria based on the almond hull composition and SSF process. Fungal species were selected based on requirements that they were thermophilic, cellulolytic, pectinolytic, and wild-type strains. Thermophilic filamentous fungi have several benefits during SSF. They are able to grow at higher temperatures which reduces the risks for contamination by foreign microorganisms as well as lowers the cooling costs for the reactor [30]. Thermophilic fungi also tend to have shorter fermentation times due to their faster metabolism [30]. Decreased fermentation times help to lower production costs and increase productivity.

The fungal species is desirable to be cellulolytic, which means that they produce cellulase enzymes capable of degrading cellulose [31]. Cellulose needs to be broken down for the fungi to access the glucose and take advantage of the lignocellulosic material provided by the almond hulls [31]. These cellulases may also be more thermostable in thermophilic fungi and function optimally at higher temperatures [31]. Fungal strains also are desirable to be pectinolytic producing pectinase enzymes to catalyze the significant portion of pectin (4-6%) available in the hulls [31]. The target was to select fungi capable of utilizing all sources of carbon in the hulls.

Lastly, selected fungi needed to be wild-type strains without any genetic modifications to avoid potential roadblocks on the way to marketing and commercialization of the fungal fermentation residue [32]. When genetically modified organisms are used in feeds they can be subject to additional labeling policies, safety assessments, and require molecular characterization of the feed [32]. Thus, wild-type fungal strains were targeted solely to prevent producers and

consumers from any barriers during and after production. Based on these criteria, three fungi were selected and screened in the proposed study: *Myceliophthora thermophila*, *Thermothielavioides terrestris*, and *Thermoascus aurantiacus*. All three fungi were thermophilic, cellulolytic, pectinolytic, and wild-type strains [33]–[35]. The fungi are all capable of secreting high levels of cellulases, xylanases, pectinases, and other industrial enzymes.

SSF with the use of filamentous fungi has been studied using different lignocellulosic materials, including wheat straw and sugar beet pulp [29], [35]. In a study using sugar-beet pulp and various thermophilic fungi in a SSF process, the crude protein concentration of the pulp was increased to around 14% [30]. The most successful thermophilic fungal strains cultured on sugar beet pulp were found to be *T. aurantiacus* and *M. thermophila* with the highest rate of growth and highest protein increases in the fermentation biomass [30]. Along with these two strains, *Chaetomium cellulolyticum* has been used with SSF and wheat straw resulting in a fermentation biomass crude protein content of 16-18% [36].

1.7. Research Objective and Hypothesis

Almond hull protein content needs to be increased to provide a suitable chicken feed supplement. This protein could also be extracted for use as fish feed supplements. A conversion process was proposed that uses the almond hulls as a substrate for thermophilic, filamentous fungi to produce a high protein fungal fermentation residue through a SSF process. The resulting fungal fermentation residue will be used as chicken feed supplements while the extracted protein could be used as aquatic feed supplements as the fiber content may be too high for direct use as a fish feed. The filamentous fungi are a microbial based protein source with a high protein content (40-50%) and desirable amino acid profile which are predicted to produce fermentation residue

with comparable protein content and amino acid profiles of current animal feed supplements [13].

The main objective of the present study was to evaluate the feasibility of converting the almond hulls to protein-rich fungal biomass and the suitability of the fermentation residue for use as poultry and aquatic feed supplements *in vitro*. A SSF process was proposed using thermophilic, filamentous fungi to increase the protein content of the almond hulls to around 15% [20], [21]. It was hypothesized that by providing nutrients and fungal spores, filamentous fungi would be capable of growing on the almond hulls. This process would lower the fiber content of the hull fermentation residue and increases the protein content to about 15% allowing it to be suitable for chicken and fish feed supplements.

The first objective in converting the almond hulls into protein-rich fungal biomass and use of the fermentation residue for suitable use as chicken and fish feed supplements was to identify the optimal fungal strain for biomass production using the almond hull as the substrate. The objective was carried out using the following strategies:

1) Screen fungal strains in a SmF process.
2) Screen fungal strains in SSF process.
3) Analyze fermentation residue to crude protein.
4) Perform compositional analysis of fermentation residue.

The second objective was to optimize the SSF process using the optimal fungi identified through the first objective. Within this objective, optimal fermentation conditions were developed. The fungal fermentation residue composition was assessed to ensure it had the appropriate nutrient values needed to be a chicken feed supplement. Fermentation parameters including almond hull particle size, fermentation time, solid-loading, and inoculum amount were

optimized. Pretreatment of the almond hulls was hypothesized to be an important step to help break down the lignocellulosic material of the hulls and increase the free sugars available for the fungi to consume. Fermentation temperature and relative humidity were consistently controlled. The objective was carried out using the following strategies:

1) SSF with optimal fungi and varying almond hull particle size ranges.
2) SSF with optimal fungi over 168 hours (7 days).
3) SSF with optimal fungi and different solid-loading levels.
4) SSF with optimal fungi and different fungal inoculum amounts.

2. Materials and Methods

2.1. Microorganisms

Myceliophthora thermophila (DSMZ 1807) and *Thermoascus aurantiacus* (DSMZ 1831) fungal strains were purchased from Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures. *Thermothielavioides terrestris* (NRRL 8126) was purchased from the Northern Regional Research Laboratory (NRRL) collection. Frozen glycerol stocks of spores were stored at -80°C.

2.2. Inoculum

Fungal seed cultures were grown on 40 ml Vogel's media agar flasks containing 1X Vogel's minimal media (pH 5.8) [38], sucrose (20 g/L), and 3-3.5% agar. After 7-10 days of cultivation at 50°C, spores were harvested by adding 10 ml of sterile DI water and gently scraping the surface of the agar to release the spores. The spore-containing liquid was filtered using a sterile cheese cloth to remove mycelia. All experimental flasks were inoculated with spore suspension for a final OD_{600} of 0.05.

2.3. Preparation of mechanically pretreated almond hulls

Dried almond hulls from the 2019 harvest year were obtained from Dr. Zhongli Pan's lab at UC Davis. Almond hulls were of mixed California varieties and were dried at temperatures ranging from 40-90°C. Dried almond hulls were mechanically pretreated using an electric grain grinder mill and then subjected to a sieving procedure using a vibratory sieve shaker. Almond hulls were classified into four different particle size ranges: <75 μm, 75-250 μm, 250-425 μm, and 425-1180 μm.

2.4. Compositional analysis of raw almond hulls

The composition of the raw almond hulls was needed to determine the percentages of soluble sugars (fermentable and non-fermentable), structural sugars, lignin, protein, and ash. Warm water extraction was performed first to determine the amount of soluble sugars and sugar alcohols (glucose, sucrose, fructose, xylose, inositol, sorbitol). Almond hulls with a particle size of <75 μm were used for the analysis. 1 g of the almond hull and 10 mL of DI water was weighed and put into 15 mL Falcon tubes. Samples were performed in triplicate. The tubes were secured in a rotating mixer and incubated at 50°C for 5 hours. Samples were taken every hour by centrifuging the tubes at 6,500 rpm for 20 minutes and pipetting out the supernatant. The liquid volume was replaced with an equivalent amount of water before resuming the extraction procedure. The supernatant was stored in the refrigerator until analysis by high performance liquid chromatography (HPLC).

The almond hull filter cake (insoluble material after warm water extraction) was collected and dried at 105°C for 24 hours. The weight of the dried material was recorded and then used for compositional analysis following the National Renewable Energy Laboratory (NREL) standard biomass laboratory analytical procedure, "Determination of Structural Carbohydrates and Lignin

in Biomass" [39]. Structural sugars, acid-insoluble and acid-soluble lignin, and ash content were determined through this two-step hydrolysis procedure.

Almond hull filter cake samples of 300 mg were used for each analysis and placed in pressure tubes. Acid hydrolysis with 72% H_2SO_4 was performed to fractionate the residue into quantifiable forms [39]. Pressure tubes were put in a 30°C water bath for 60 minutes and stirred every 5-10 minutes. After the 60 minute hydrolysis, the acid was diluted with 84.00 mL of DI water, and Teflon caps were screwed on securely. Pressure tubes were autoclaved at 121°C for 1 hour. Tubes were allowed to cool to room temperature before removing the caps.

Autoclaved hydrolysates were vacuumed filtered through pre-weighed filtering crucibles. Filtering crucibles were placed in the 550°C furnace for 4 hours and allowed to cool in a desiccator before being weighed. The filtrate was immediately used for lignin analysis measured on a UV-Vis spectrophotometer (Synergy 4, BioTek Instruments, Winooski, VT, USA) with a wavelength of 320 nm ($\varepsilon_{320} = 30\ L\ g^{-1}cm^{-1}$). The filtrate was stored in the refrigerator until analysis by HPLC.

HPLC equipped with a refraction index detector (RID) manufactured by Shimadzu was used to measure all sugar concentrations. The supernatant from the warm water extraction was analyzed for sucrose, glucose, xylose, fructose, and inositol concentrations. The filtrate from the two-step hydrolysis was analyzed for glucose, xylose, galactose, arabinose, mannose, and galacturonic acid concentrations. A CarboSep CHO 87P column was used with water as the mobile phase to measure all sugar concentrations except for galacturonic acid. The mobile phase flow rate was 0.5 ml/min, and the column temperature was sustained at 85°C. Galacturonic acid concentration was also determined with HPLC, but using a Coregel ION 300 column with a

mobile phase of 5 mM H_2SO_4. The mobile phase flow rate was 0.5 ml/min, and the column temperature was sustained at 60°C.

Raw numerical HPLC results were compiled and analyzed through Microsoft Excel. Data tables and graphs were produced in Excel. Error bars represent the standard deviation of triplicate results.

2.5. Submerged fermentation experiments

Submerged fermentation experiments were carried out in 250 mL Erlenmeyer flasks with 2 g of the almond hull, 10 mL of 5X Vogel's media, and 40 mL of DI water for a total volume of 50 mL. The substrate was wetted in the flasks with the DI water prior to autoclaving at 121°C for 30 minutes. After autoclaving, the 5X Vogel's media was added, followed by fungal inoculum for a final OD_{600} of 0.05. Flasks were shaken at 200 rpm in a shaking incubator and kept at a temperature of 50°C for all fermentations.

2.6. Solid-state fermentation experiments

Solid-state fermentation experiments were carried out in 250 mL Erlenmeyer flasks with 1.50 g almond hull for a 10% (w/w) solid loading. The substrate was wetted in the flasks with 8.5 mL of DI water and autoclaved at 121°C for 30 minutes. After autoclaving, 5 mL of sterile 10X Vogel's minimal media was added to the flasks, followed by fungal inoculum for a final OD_{600} of 0.05. The temperature for all fermentations was 48-50°C. The relative humidity was kept in a range of 70-90% by using an Inkbird Dual Stage Humidity controller [40] and the combination of a one and two-gallon humidifier.

2.7. Fermentation residue harvesting, drying, and storage

Fermentation flasks were harvested in triplicate (three biological replicates) on their respective days, followed by washing and drying the fermentation residue. For SmF flasks,

contents were poured directly into 50 mL Falcon centrifuge tubes and centrifuged at 6000 rpm for 20 minutes. For SSF flasks, 100 mL of DI water was first added to the flasks and shaken at 200 rpm for 10 minutes before being poured into the centrifuge tubes. The supernatant was then filtered through pre-weighed Buchner ceramic funnels (60 mm diameter) with Whatman No. 3 filter paper. The fermentation residue was washed with a total of 200 mL DI water and centrifuged after each wash. The resulting supernatant was filtered after each centrifuge round.

After filtering, the fermentation residue was dried in the funnels at 70°C overnight (~16 hours). Funnels were placed in a desiccator to cool and then weighed to calculate the amount of fermentation residue. The fermentation residue was removed from the filter paper, ground with a mortar and pestle set, and stored in 15 mL Falcon tubes at room temperature.

2.8. Crude protein content analysis

The crude protein content of all fermentation residue samples (except those from the optimization experiments of initial fungal inoculum and solid-loading) was determined using a nitrogen combustion method known as the Dumas method. Samples were analyzed for total nitrogen using a Vario Max Cube Elemental Analyzer in Dr. Juliana Maria Leite Nobrega de Moura Bell's lab at UC Davis and a LECO FP-528 analyzer through the UC Davis analytical lab [41]. Fermentation residue samples of ~0.2 g were used for each analysis. Crude protein content was calculated by taking the resulting total nitrogen percentage and multiplying it by a protein conversion factor of 6.25, a constant based on the nitrogen content of proteins being 16% [42]. The following equation was used:

$$\%\ Crude\ Protein\ =\ \%\ nitrogen\ in\ sample\ \times\ 6.25$$

Crude protein content was also determined using a spectrophotometric adapted to accommodate the amount of nitrogen present in the samples [43]. The total nitrogen results were

converted to crude protein using the same protein factor as the Dumas method of 6.25. The adapted method utilized a nitrogen persulfate oxidation reaction and a reduction reaction where amines were added to form a color compound detected spectrophotometrically. The nitrogen content was determined using a standard nitrogen curve.

A 5X oxidizing solution was prepared by dissolving 49.5 g potassium peroxodisulfate ($K_2S_2O_8$) in 1 L of 0.5225 M sodium hydroxide (NaOH) solution. Serum bottles were used for the oxidizing reaction, and each contained 90 mL of 5X oxidizing solution. The total volume was then brought to 100 mL with varying amounts of sample and water. Digestibility samples contained 1.6 mL of supernatant and 8.4 mL of DI water. Fermentation residue samples contained 10 mL of DI water and 0.02 g of fermentation residue. Reagent blanks were prepared the same way as the fermentation residue samples but without the samples. Calibration standards contained 10 mL of the corresponding nitrogen dilution. All serum bottles were then autoclaved at 121°C for 90 minutes. After autoclaving, the bottles were allowed to cool to room temperature.

A total nitrogen mix reagent with boric acid was prepared for the colorimetric reaction. The total nitrogen mix contained 200 mL of a vanadium chloride solution, 40 mL of a sulfanilamide solution, and 40 mL of an amine solution. The vanadium chloride solution was prepared by dissolving 1.6 g of vanadium (III) chloride (VCl_3) into 170 mL of DI water and 16.8 mL of 37% hydrochloric acid (HCl). The solution was then brought to a total volume of 200 mL. The sulfanilamide solution was prepared by dissolving 1.0 g of sulfanilamide ($NH_2 \cdot C_6H_6 \cdot SO_2 \cdot NH_2$) into 85 mL of DI water and 14.5 mL 37% hydrochloric acid (HCl). The amine solution was prepared by dissolving 0.07 g of N-(1-naphtyl)-ethylenediamine dihydrochloride ($C_{10}H_7NHCH_2 \cdot CH_2 \cdot NH_2 \cdot 2HCl$) in 100 mL of DI water. 1 mL of 17 g/L boric

acid (H_3BO_3) solution was then added to every 100 mL of the total nitrogen mix reagent. Screw cap test tubes were used for the colorimetric reaction, each containing 10 mL of total nitrogen mix and 50 µL of oxidized sample. The tubes were then incubated in a 45°C water bath for an hour. The tubes were removed and allowed to cool before analyzing samples on the spectrophotometer.

Samples were measured on a UV-Vis spectrophotometer (Synergy 4, BioTek Instruments, Winooski, VT, USA) with a wavelength of 545 nm. The calibration curve produced during the nitrogen analysis was made using five dilutions of a 140 µg/mL N (10 µmol/mL N) total nitrogen standard. The nitrogen standard was prepared by dissolving 0.1862 g of disodium ethylenediaminetetraacetate dihydrate (Na_2-EDTA) in DI water and bringing the total volume to 100 mL. The total nitrogen concentration of the samples was calculated by the calibration curve.

2.9. Compositional analysis of fermentation residue

Compositional analysis of the fermentation residue was performed using the NREL standard biomass laboratory analytical procedure, "Determination of Structural Carbohydrates and Lignin in Biomass" [39] found in section 2.4 in the Materials and Methods. Structural sugars, acid-insoluble and acid-soluble lignin, and ash content were determined through this two-step hydrolysis procedure. The procedure was adjusted to accommodate for smaller sample sizes of 100 mg of fermentation sample. Volumes of acid and water for dilutions were adjusted accordingly. Glucose, xylose, galactose, arabinose, mannose, and galacturonic acid concentrations were measured by HPLC following the method in Section 2.4.

Raw numerical HPLC results were compiled and analyzed through Microsoft Excel. Data tables and graphs were produced in Excel. Error bars represent the standard deviation of triplicate results.

2.10. *In vitro* Protein Digestibility

The *in vitro* protein digestibility of the fermentation residue was determined using pepsin and pancreatin enzyme systems [37]. Fermentation residue samples (50 mg protein equivalent) were weighed out and put into 50 mL Falcon tubes. Digestibility studies were performed in duplicate. 7.5 mL of 0.1 N hydrochloric (HCl) solution containing 1.0 mg of pepsin enzyme was added to each tube and incubated for 3 hours at 37°C. The tubes were gently shaken every 30 minutes. The samples were then neutralized with 3.75 mL of 0.2 N sodium hydroxide (NaOH). 3.75 mL of phosphate buffer (0.1 M and pH 8.0) solution containing 2.5 mg of pancreatin enzyme was added to each sample, followed by the addition of 0.5 mL toluene to prevent microbial growth. The samples were incubated for 24 hours at 37°C. An enzyme blank was also prepared the same way but without the fermentation residue.

After the incubation period, 5 ml of 10% trichloroacetic acid (TCA) solution was added to each tube to inactive the enzyme and to precipitate the undigested protein [37]. The volumes were then made up to 25 mL with DI water and centrifuged at 3,214 x g for 20 minutes. The TCA soluble protein at 0 hours was also determined using fermentation residue samples (50 mg equivalent) and the same amount of 10% TCA solution. The final volume was brought up to 25 mL using DI water. The samples were shaken at 200 rpm for 30 minutes and then centrifuged the same way as the digested samples. The resulting supernatant from all tubes was determined for total nitrogen content by the spectrophotometric method as described in section 2.8 of the Materials and Methods [43].

The percent protein digestibility was calculated using the following equation:

$$\%\ Protein\ Digestibility = \frac{protein\ in\ supernatant - protein\ in\ enzyme\ blank}{protein\ in\ sample - TCA\ soluble\ protein\ at\ 0\ hours} \times 100$$

2.11. Amino acid analysis

Amino acid analysis of the fermentation residue was performed by the UC Davis Genome Center in the Molecular Structure Facility. Fermentation residue samples of ~10 mg were submitted for each analysis. Amino acids were quantified by HPLC using the Hitachi L-8800 amino acid analyzer [44]. This instrument utilizes a sodium citrate buffer system and ion-exchange chromatography to separate the amino acids. All essential and non-essential amino acids were quantified: arginine, glycine, histidine, isoleucine, leucine, lysine, methionine, cysteine, phenylalanine, tyrosine, threonine, tryptophan, valine, asparagine, serine, glutamine, proline, alanine, and taurine. The amino acid results were provided on a % (w/w) basis. The amino acid profile values were presented on a percent of protein basis. The % (w/w) of each amino acid was divided by the total percent of protein represented by the following equation:

$$Amino\ Acid\ Percentage\ (\%\ of\ protein) = \frac{amino\ acid\ \%\ (w/w)}{\%\ protein}$$

2.12. Crude Fat Analysis

Crude fat analysis of the fermentation residue was performed by the UC Davis analytical lab. Fermentation residue samples of ~3 g were submitted for each analysis. The crude fat was quantified using the Randall modification of the standard Soxhlet extraction after an initial water extraction [45].

2.13. Calculation of real protein yield and productivity

The protein yields of the fungal fermentations were calculated on a grams of protein produced per grams of carbohydrate consumed basis. Carbohydrates include the structural carbohydrates quantified through the two-step hydrolysis procedure including cellulose, xylan, galactan, mannan, and galacturonic acid. The following equation was used:

$$Protein\ yield\ = \frac{X}{\Delta C_{carbohydrate\ consumed}}$$

where

X is the crude protein amount in the fermentation residue (g) and

$\Delta C_{arbohydrate\ consumed}$ is the carbohydrate consumed during the fungal fermentation (g).

The protein amount was calculated by multiplying the fermentation residue weight in grams by the crude protein percent and dividing by 100 as follows:

$$X = Fermentation\ residue\ amount(g) \times protein\ content\ (\%)$$

The total carbohydrate consumed during fermentation was calculated by subtracting the amount of structural carbohydrates left in the fermentation residue from the initial structural carbohydrates found in the almond hulls. It is assumed that all the soluble sugars are consumed during fermentation since cellulolytic microorganisms will usually not produce cellulase or pectinase when soluble sugars are present [46]. The following equation was used:

$$\Delta C_{carbohydrate\ consumed} = (S_{initial\ structural\ carbohydrate} - S_{fermentation\ resiude\ structural\ carbohydrate}) + S_{soluble\ sugar}$$

$$Carbohydrate\ Conversion\ Percentage\ (\%) = \left(\frac{\Delta C_{carbohydrate\ consumed}}{S_{soluble\ sugar} + S_{initial\ structural\ carbohydrate}}\right) x\ 100$$

2.14. Statistical analysis

One-way ANOVA tests were performed to determine if differences existed among the means of measured parameters using SAS 9.4 statistical software. The statistical significance level was set at $P<0.05$. Measurement replications are included in the figure captions.

3. Results and discussion

3.1. Compositional analysis of raw almond hulls

The composition of the raw almond hulls used in this study was needed to determine the percentages of soluble sugars (fermentable and non-fermentable), structural sugars, lignin, protein, and ash. The total composition is included in Table 3.1.1. The almond hull used in this study had a soluble sugar content of 16.70% and structural sugar content of 38.23% (Table 3.1.1). The protein and ash contents were 4.71% and 2.04%, respectively (Table 3.1.1). The lignin content was 19.94% (Table 1.1). The unidentified fraction of the hulls was less than 20% (Table 3.1.1).

Table 3.1 The composition of the almond hulls used in the study is presented in the table. Standard errors are listed in parentheses next to each subcomponent. The composition was done on a dry matter basis. Moisture content of the hulls was 4.5%.

Compositional Analysis of Almond Hulls Used in This Study				
	Dry Hulls (%)		**Soluble (%)**	**Insoluble (%)**
Soluble Sugars	16.70		16.70	
Glucose		7.63 (0.06)	7.63	
Sucrose		0.59 (0.04)	0.59	
Fructose		7.10 (0.08)	7.10	
Xylose		0.3 (0.06)	0.30	
Inositol		1.08 (0.02)	1.08	
Total Sugars	16.70			
Ash		2.04 (0.04)		2.04
Cellulose		14.60 (0.86)		14.60
Galacturonic Acid		12.03 (1.05)		12.03
Xylan		5.07 (0.76)		5.07
Galactan		1.87 (0.52)		1.87
Arabinan		3.94 (0.24)		3.94
Mannan		0.71 (0.10)		0.71
Lignin		19.94 (0.34)	1.27	18.67
Protein		4.71 (0.09)		
Unknown	18.37			
Total	100.00		17.98	58.94

3.2. Screening of fungal strains using a submerged fermentation process with hulls

Submerged fermentation (SmF) was initially used to screen fungal strains for protein production on almond hulls. SmF and SSF each provide unique benefits for fungal cultivation, but SmF is more easily controlled compared to SSF, which requires relative humidity control to prevent water loss [26]. SmF also requires agitation of the liquid culture and has better mass and heat transfer as compared to SSF [47]. *M. thermophila* (MT), *T. terrestris* (TT), and *T. aurantiacus* (TA) fungal strains were screened for biomass production through a SmF process by comparing their fermentation residue crude protein content and protein yields. Fungi were cultivated on 2 g of almond hulls with a particle size of <75 μm. The cultures were carried out in flasks at 50°C for 48, 72, and 96 hours, as described in Materials and methods.

Figure 3.2A represents the SmF residue crude protein percent for *M. thermophila* (MT), *T. terrestris* (TT), and *T. aurantiacus* (TA) at 48, 72, and 96 hours. The maximum crude protein content of 26.38% for *M. thermophila* and 22.35% for *T. aurantiacus* were obtained at 48 hours. The maximum crude protein content of 28.03% for *T. terrestris* was obtained at 96 hours. There was a significant difference ($P<0.05$) in the crude protein content maximums of *M. thermophila* and *T. terrestris*, but it took 48 hours longer to reach the maximum protein content for *T. terrestris*. *T. aurantiacus* had a significantly lower ($P<0.05$) maximum crude protein content than both *M. thermophila* and *T. terrestris*.

Figure 3.2B represents the SmF protein yield for *M. thermophila* (MT), *T. terrestris* (TT), and *T. aurantiacus* (TA) at 48, 72, and 96 hours. The maximum protein yields of 0.22 g crude protein/g carbohydrate consumed with *M. thermophila* and 0.19 g crude protein/g carbohydrate consumed with *T. aurantiacus* were obtained at 48 hours, while the maximum for *T. terrestris* was 0.15 g crude protein/g carbohydrate consumed at 72 hours. There was a significant

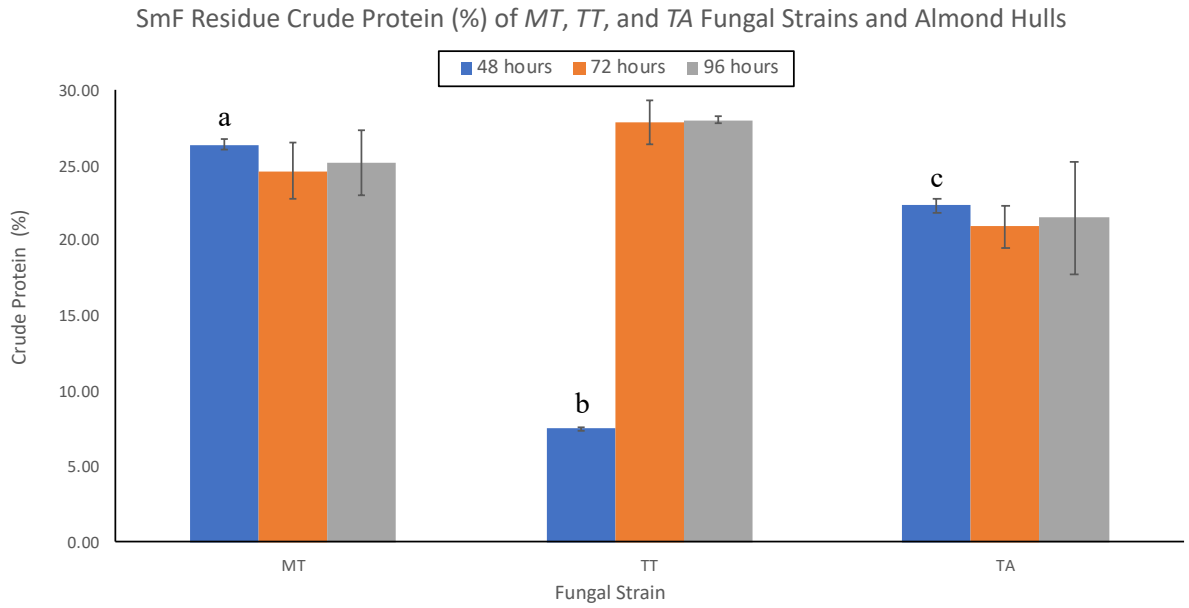
difference ($P<0.05$) between the maximum protein yield of *M. thermophila* and the other two fungi. The maximum protein yield on a per gram of almond hull basis of 0.094 g crude protein/g almond hull with *M. thermophila* and 0.081 g crude protein/g almond hull with *T. aurantiacus* were obtained at 48 hours while the maximum for *T. terrestris* was 0.072 g crude protein/g almond hull at 72 hours. Thus, *M. thermophila* was identified as the optimal fungal strain in SmF.

Fermentation residue weights and total carbohydrates consumed during the fermentations were also important measurements to assess the productivity of the fungi. The fermentation residue weights are represented in Figure 3.2C for all fungi at each time point. The time at which the maximum fermentation residue was attained for each fungi closely correlated with the time at which the maximum crude protein percent was achieved. Maximum fermentation residue amounts of 0.72 g and 0.73 g were achieved at 48 hours for *M. thermophila* and *T. aurantiacus*, respectively. The maximum fermentation residue weight of 0.52 g for *T. terrestris* was achieved at 72 hours whereas the maximum crude protein percent was achieved at 96 hours. However, there was no significant difference ($P>0.05$) for fermentation residue weights at 72 and 96 hours for *T. terrestris*.

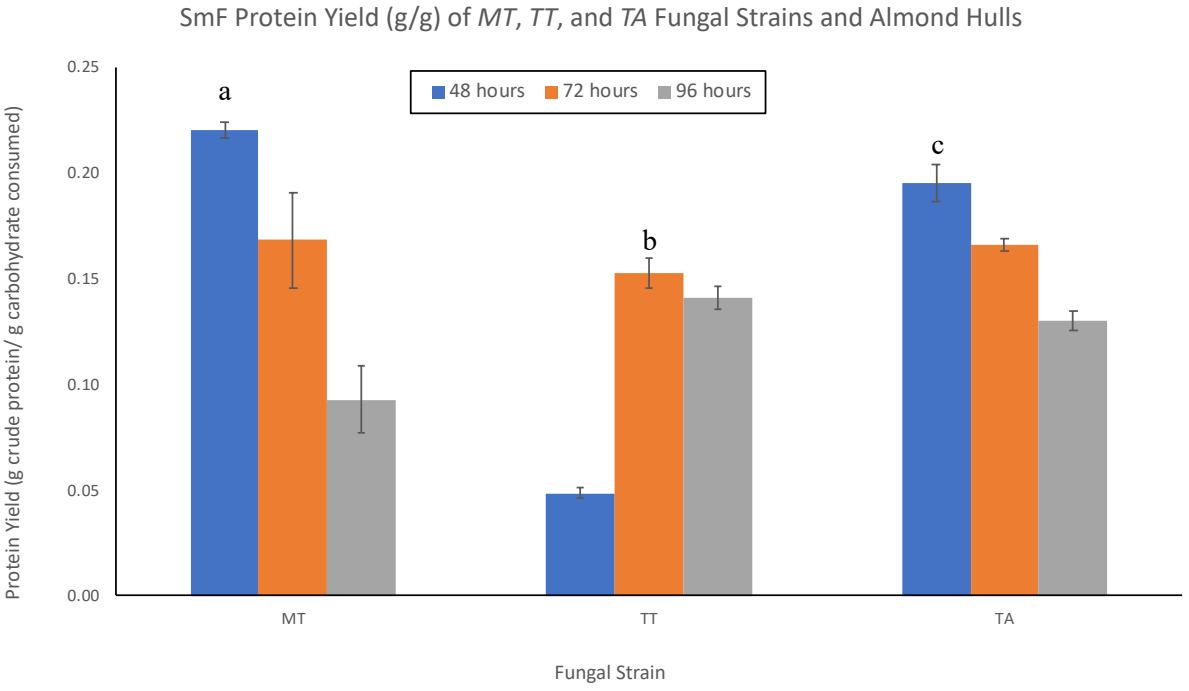
The total carbohydrate consumed during the fermentations are represented in Figure 3.2D for the three fungi at all time points. The total carbohydrate consumed at each harvest time point was similar for *M. thermophila* and *T. terrestris*. However, the total carbohydrate consumed for *T. aurantiacus* at each time point was consistently lower than that of the other two fungi. At 96 hours, the total carbohydrate consumed was 0.98 g, 0.97 g, and 0.92 g for *M. thermophila, T. terrestris,* and *T. aurantiacus*, respectively. The carbohydrate conversion percent at 96 hours was 93.15%, 92.29%, and 88.14% for *M. thermophila, T. terrestris,* and *T. aurantiacus*, respectively.

The amounts of carbohydrate consumed and utilized during fermentation for *M. thermophila* and T. *terrestris* supports their superior protein productivity results compared to *T. aurantiacus*.

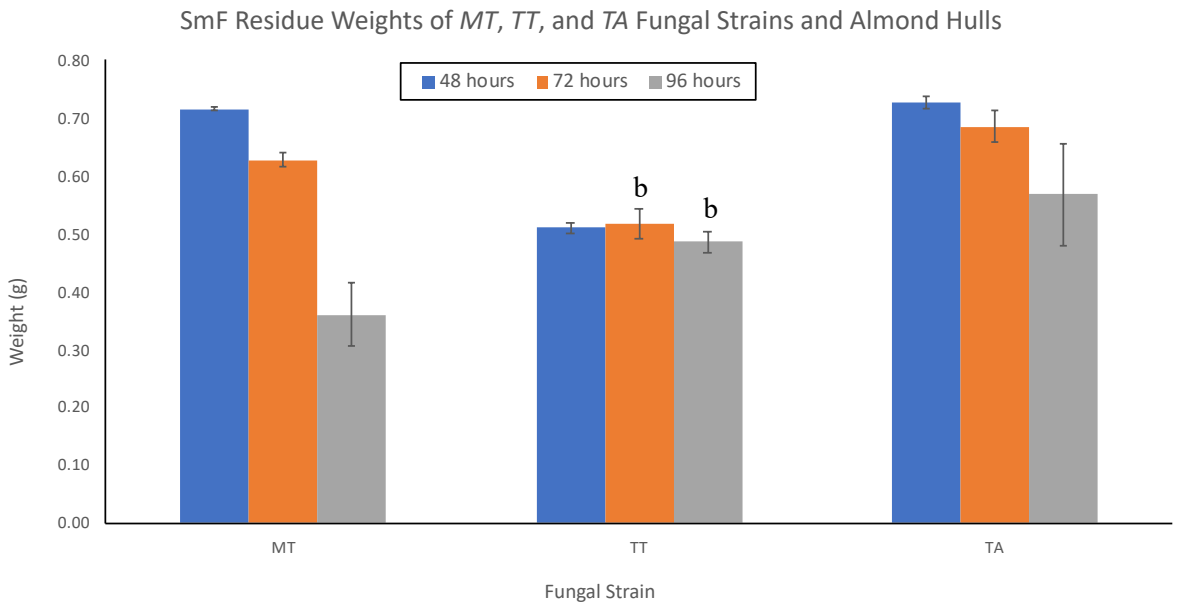
A)


SmF Residue Crude Protein (%) of *MT*, *TT*, and *TA* Fungal Strains and Almond Hulls
48 hours
72 hours
96 hours
Crude Protein (%)
30.00
25.00
20.00
15.00
10.00
5.00
0.00
a
b
c
MT
TT
TA
Fungal Strain

B)


SmF Protein Yield (g/g) of *MT*, *TT*, and *TA* Fungal Strains and Almond Hulls
48 hours
72 hours
96 hours
Protein Yield (g crude protein/ g carbohydrate consumed)
0.25
0.20
0.15
0.10
0.05
0.00
a
b
c
MT
TT
TA
Fungal Strain

C)



D)

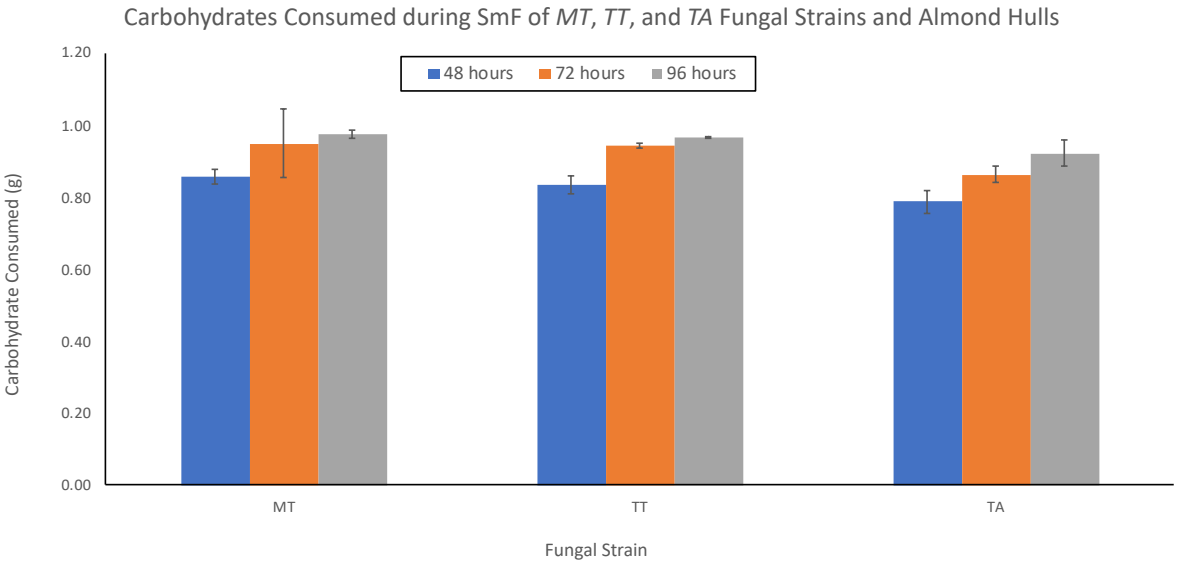


Figure 3.2.1 Screening of fungal strains through SmF of *M. thermophila* (MT), *T. terrestris* (TT), and *T. aurantiacus* (TA) on almond hulls at 48, 72, and 96 hours comparing A) fermentation residue crude protein (%) B) protein yield (g crude protein/ g carbohydrate consumed) C) fermentation residue weight (g) and D) carbohydrate consumed during the fermentation (g). Error bars represent one standard deviation (n = 3 fermentations). Identical letters indicate no statistically significant difference between means.

3.3. Screening of fungal strains using a solid-state fermentation process with hulls

After identifying the optimal fungi for protein production in SmF, it was important to investigate if the same fungi would be optimal in a SSF process. *M. thermophila* (MT), *T.*

terrestris (TT), and *T. aurantiacus* (TA) fungal strains were screened again for biomass production through a SSF process by comparing their fermentation residue crude protein content and protein yields. Fungi were cultivated on 1.50 g of the almond hull with a particle size of <75 μm for a 10% (w/w) solid loading. The cultures were carried out in flasks at 48-50°C for 72, 96, and 120 hours, as described in Materials and methods.

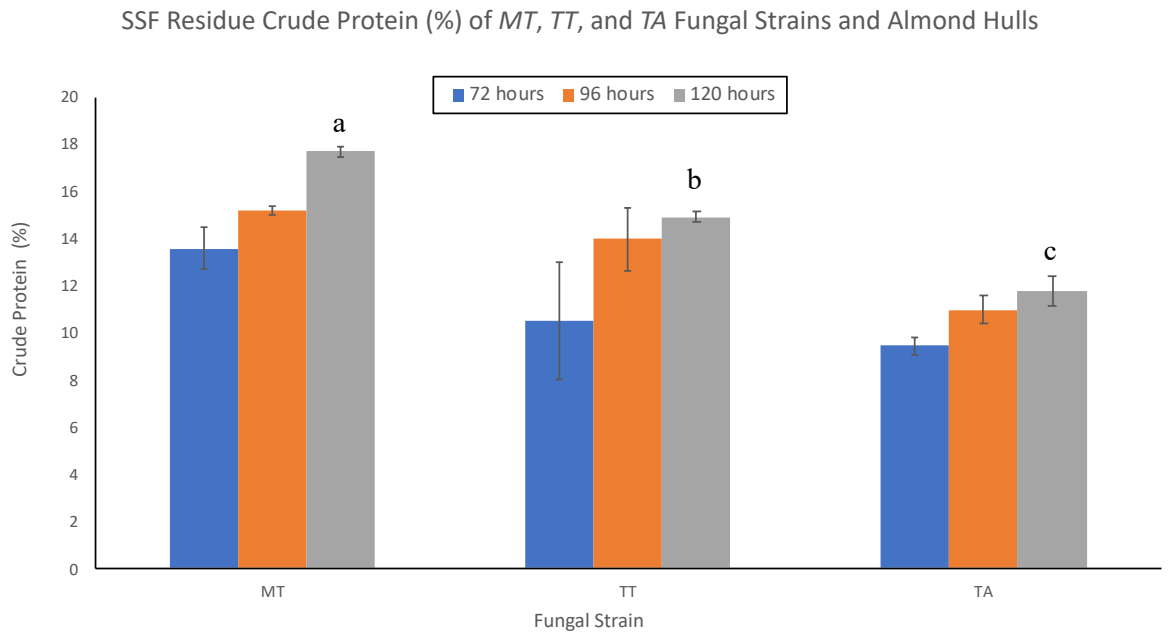
Figure 3.3A represents the SSF residue crude protein percent for *M. thermophila* (MT), *T. terrestris* (TT), and *T. aurantiacus* (TA) at 72, 96, and 120 hours. The maximum crude protein contents for *M. thermophila*, *T. terrestris,* and *T. aurantiacus* were 17.69%, 14.92%, and 11.77%, respectively. All maximum values were obtained at 120 hours. *M. thermophila* had a significantly higher ($P<0.05$) maximum protein content compared to both *T. terrestris* and *T. aurantiacus*. The crude protein content of the fermentation residue produced by *M. thermophila* was about 16% higher than that of *T. terrestris*. *T. aurantiacus* had a significantly lower ($P<0.05$) maximum crude protein content than both *M. thermophila* and *T. terrestris* just as in SmF.

Figure 3.3B represents the SSF protein yield for *M. thermophila* (MT), *T. terrestris* (TT), and *T. aurantiacus* (TA) at 72, 96, and 120 hours. The maximum protein yields for *M. thermophila*, *T. terrestris,* and *T. aurantiacus* were 0.21 g crude protein/g carbohydrate consumed, 0.17 g crude protein/g carbohydrate consumed, and 0.12 g crude protein/g carbohydrate consumed, respectively. All maximum values were obtained at 120 hours. There was a significant difference ($P<0.05$) between the maximum protein yield of *M. thermophila* and the other two fungi. The maximum protein yields on a per gram of almond hull basis for *M. thermophila*, *T. terrestris,* and *T. aurantiacus* were 0.084 g crude protein/g almond hull, 0.068 g crude protein/g almond hull, and 0.047 g crude protein/g almond hull, respectively. All maximum values were also obtained at 120 hours. Thus, *M. thermophila* was identified as the

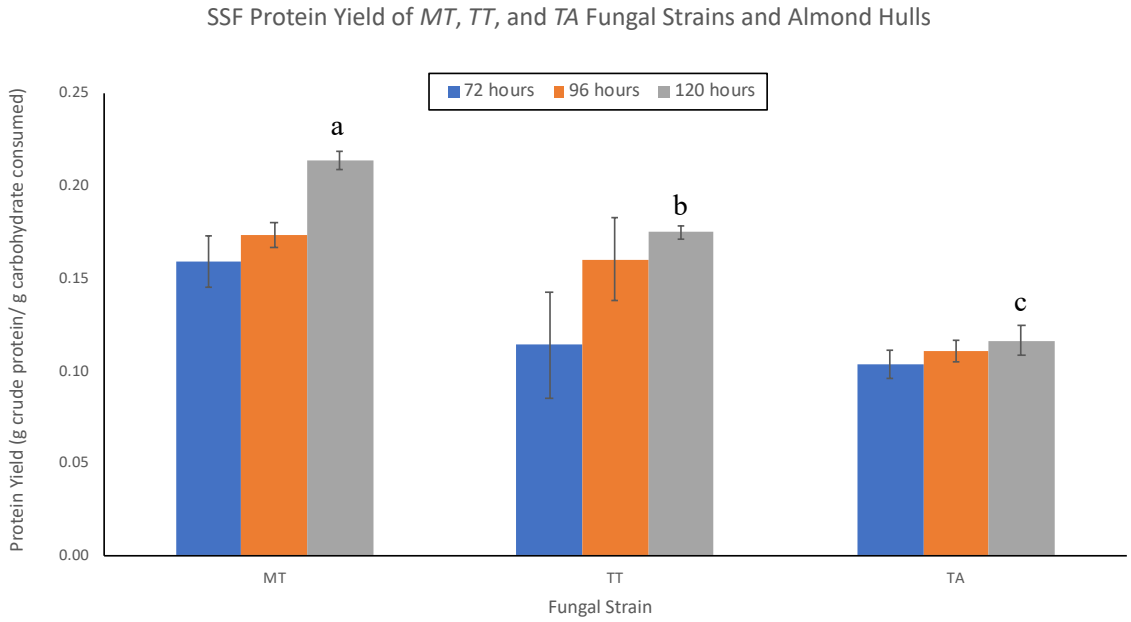
optimal fungal strain for SSF on almond hulls. *M. thermophila* had the highest crude protein content and protein yield in SSF as well as the highest protein yield in SmF. Therefore, *M. thermophila* was identified and selected as the fungi to optimize in the SSF process on almond hulls to further enhance protein production.

Fermentation residue weights and total carbohydrate consumed during the fermentations are also represented in Figures 3.3C and 3.3D for all fungi at each time point. Just as in SmF, the time at which the maximum fermentation residue was attained for each fungi closely correlated with the time at which the maximum crude protein percent was achieved. Maximum fermentation residue amounts of 0.71 g, and 0.68 g were achieved at 120 hours for *M. thermophila* and *T. terrestris*, respectively. The maximum fermentation residue weight of 0.61 g for *T. aurantiacus* was achieved at 72 hours, whereas the maximum crude protein percent was achieved at 120 hours. However, the fermentation residue weights for *T. aurantiacus* at all time points were similar with no significant difference ($P>0.05$). The total carbohydrate consumed at each harvest time point in SSF was similar for all three fungi. At 120 hours, the total carbohydrate consumed was 0.59 g, 0.58 g, and 0.61 g for *M. thermophila, T. terrestris,* and *T. aurantiacus*, respectively. The carbohydrate conversion percent at 120 hours was 76.29%, 75.25%, and 78.85% for *M. thermophila, T. terrestris,* and *T. aurantiacus*, respectively.

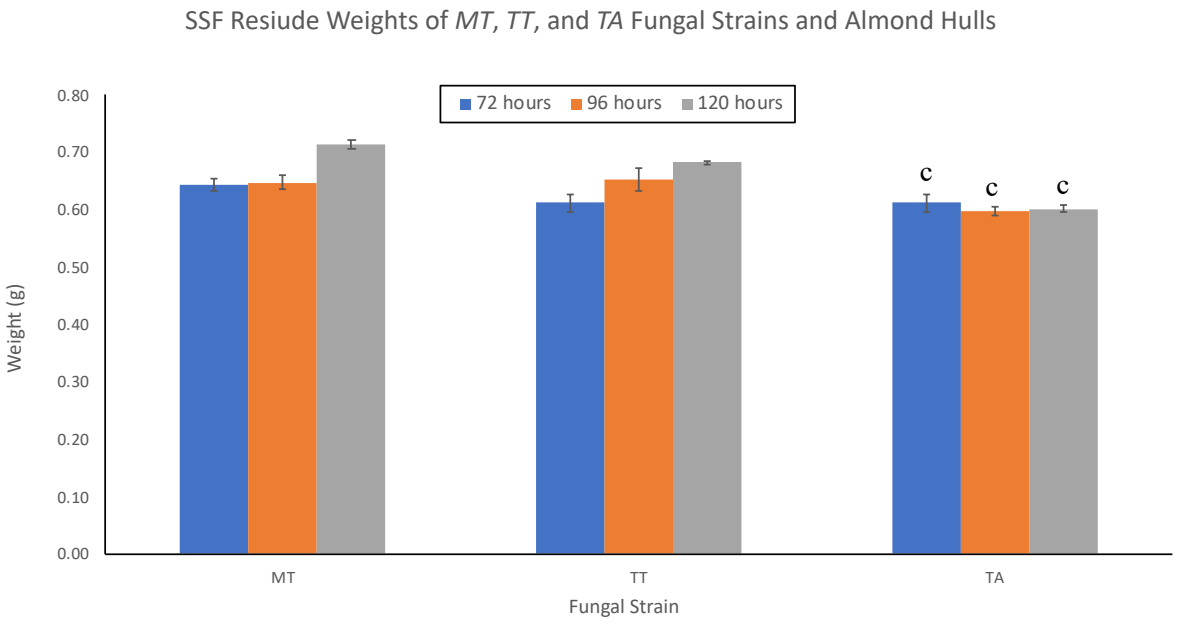
A)


SSF Residue Crude Protein (%) of *MT*, *TT*, and *TA* Fungal Strains and Almond Hulls
72 hours
96 hours
120 hours
Crude Protein (%)
20
18
16
14
12
10
8
6
4
2
0
a
b
c
MT
TT
TA
Fungal Strain

B)


SSF Protein Yield of *MT*, *TT*, and *TA* Fungal Strains and Almond Hulls
72 hours
96 hours
120 hours
Protein Yield (g crude protein/ g carbohydrate consumed)
0.25
0.20
0.15
0.10
0.05
0.00
a
b
c
MT
TT
TA
Fungal Strain

C)



D)

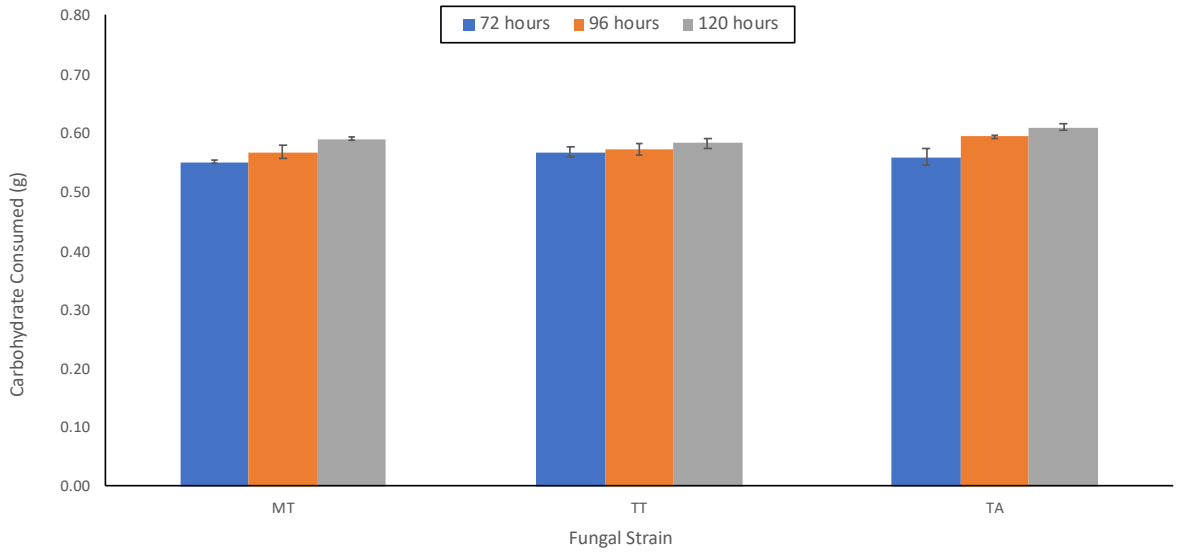


Figure 3.3.1 Screening of fungal strains through SSF of *M. thermophila* (MT) , *T. terrestris* (TT), and *T. aurantiacus* (TA) on almond hulls at 72, 96, and 120 hours comparing A) fermentation residue crude protein (%) B) protein yield (g crude protein/ g carbohydrate consumed) C) fermentation residue weight (g) and D) carbohydrate consumed during the fermentation (g). Error bars represent one standard deviation (n = 3 fermentations). Identical letters indicate no statistically significant difference between means.

3.4. Mechanical pretreatment of the hulls improves protein production

In the SSF process, the particle size of the substrate plays an important role in microbial growth and protein production [48]. Particle size impacts both the specific surface area and inter-particular porosity of the solid substrate [48]. Typically, smaller particle sizes allow for increased access to the substrate, but have lower inter-particular porosity while larger particle sizes allow for less access to the substrate, but have higher inter-particular porosity [48]. Thus, the optimal particle size for protein production was investigated using hulls of various sizes. *M. thermophila* was cultivated through solid-state fermentations on almond hulls of varying particle size ranges: <75 μm, 75-250 μm, 250-425 μm, and 425-1180 μm. The cultures were carried out in flasks at 48-50°C for 96 hours using 1.50 g of almond hull for a 10% (w/w) solid loading, as described in Materials and methods.

Figure 3.4 shows the fermentation residue weights (g), total carbohydrate consumed during fermentation (g), protein yield (g crude protein/g carbohydrate consumed), and crude protein (%) of the fermentation residue for each particle size range. The maximum crude protein content, protein yield, and protein yield on a per gram of almond hull basis was 19.79%, 0.23 g crude protein/g carbohydrate consumed, and 0.083 g crude protein/g almond hull were obtained on the almond hull with a particle size of 75-250 μm. The maximum total carbohydrate consumed during the fermentation of 0.55 g with a carbohydrate conversion percent maximum of 75.68% was also obtained on the almond hull particle size of 75-250 μm. When the particle size was increased or decreased from this optimal range, the crude protein percent ($P<0.05$) was significantly reduced. There was no significant difference ($P>0.05$) in crude protein percent between particle sizes of <75 μm and 250-425 μm. However, there was significant decrease

(P<0.05) in crude protein percent between the particle size range of 425-1180 µm and all other particle size ranges.

The optimal particle size range of 75-250 µm was identified and used for the remaining optimization studies. This range signifies a balance between the specific surface area and inter-particular porosity to support optimal microbial growth and resulting protein production. This particle size range may also provide good aeration opportunities to support optimal fungal growth [48].

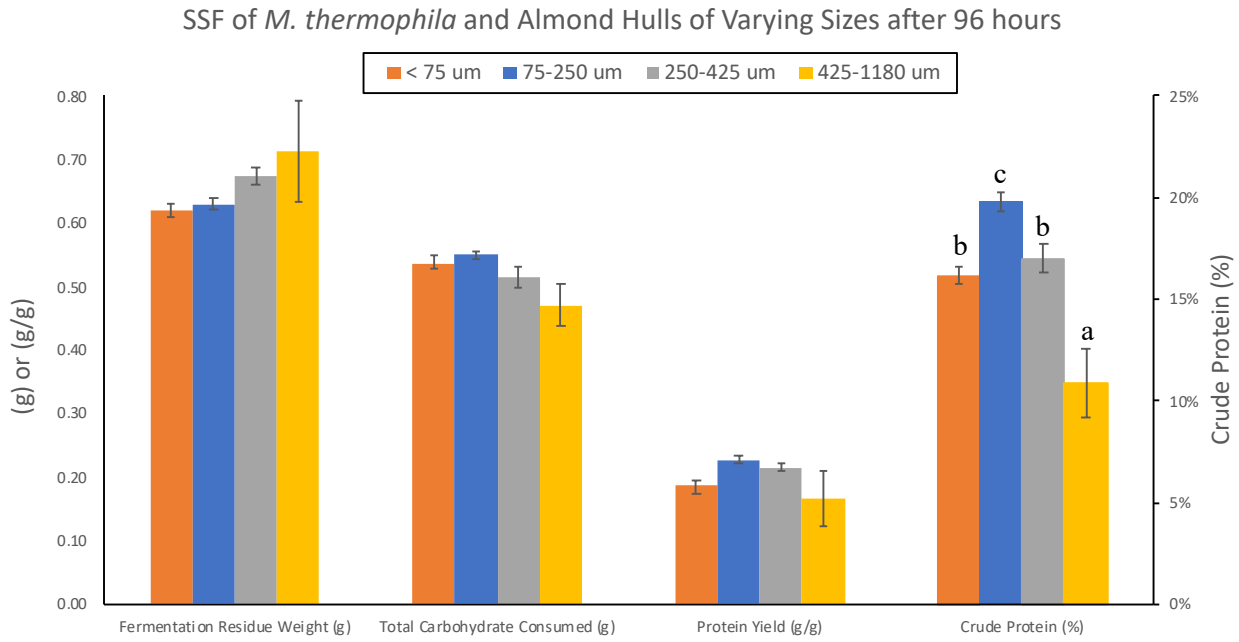


Figure 3.4.1 Fermentation residue characteristics and fermentation activity results from SSF of *M. thermophila* on almond hulls of varying particle sizes. Fermentation residue weight (g), total carbohydrate consumed (g), protein yield (g crude protein/g carbohydrate consumed), and crude protein (%). Error bars represent one standard deviation (n = 3 fermentations). Identical letters indicate no statistically significant difference between means.

3.5. Optimal harvest time determined for most productive strain

Culture time was another critical factor of the fermentation process to optimize to achieve the highest protein production with the shortest culture time. Shorter fermentation times help to

lower production costs and increase productivity [30]. Thus, the optimal fermentation time for protein production was investigated over a period of 168 hours. *M. thermophila* was cultivated through SSF on almond hulls with a particle size range of 75-250 μm. The cultures were carried out in flasks at 48-50°C using 1.50 g of almond hull for a 10% (w/w) solid loading, as described in Materials and methods. Flasks were harvested at 72, 96, 120, 144, and 168 hours in triplicate.

Figure 3.5 shows the fermentation residue weights (g), total carbohydrate consumed during fermentation (g), protein yield (g crude protein/g carbohydrate consumed), and crude protein (%) of the fermentation residue for each fermentation time point. The maximum crude protein content, protein yield, and protein yield on a per gram of almond hull basis was 18.10%, 0.20 g crude protein/g carbohydrate consumed, and 0.078 g crude protein/g almond hull, respectively. All were obtained at 96 hours.

Fermentation residue weights and total carbohydrate consumed during the fermentations are also represented in Figure 3.5. Fermentation residue amounts were very similar from 72 to 144 hours at about 0.64-0.66 g ($P>0.05$). However, there was a significant decrease to 0.60 g of fermentation residue at 168 hours ($P<0.05$). The total carbohydrate consumed showed an upward trend over the 168 hours. The maximum total carbohydrate consumed was 0.60 g with a maximum carbohydrate conversion percent of 76.04% at 168 hours ($P<0.05$) while the total carbohydrate consumed at 96 hours was 0.57 g with a carbohydrate conversion percent of 73.01%. The optimal fermentation time was determined to be 96 hours when the maximum crude protein and protein yields were achieved.

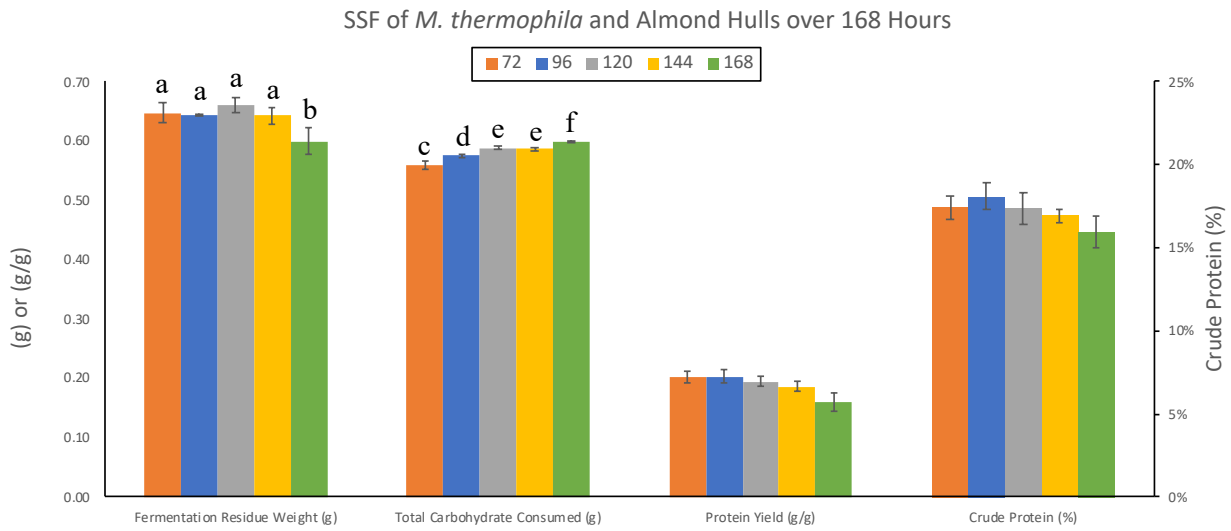


Figure 3.5.1 Fermentation residue characteristics and fermentation activity results from SSF of *M. thermophila* on almond hulls over 168 hours. Fermentation residue weight (g), total carbohydrate consumed (g), protein yield (g crude protein/g carbohydrate consumed), and crude protein (%). Error bars represent one standard deviation (n = 3 fermentations). Identical letters indicate no statistically significant difference between means.

3.6. Optimization of initial fungal inoculum and solid-loading for protein production in solid-state fermentation

After optimization of the almond hull particle size as substrate and fermentation time of the SSF process, the percent solid-loading and initial fungal inoculum amounts were optimized. [49]. Percent solid-loading refers to the initial amount of almond hull used in each fermentation on a w/w basis. Initial fungal inoculum is the initial amount of fungal spore suspension added to each fermentation. This method allowed for the identification of significant factors and how they impact the dependent variable. Crude protein content was selected as the dependent variable for these studies.

The optimal solid-loading percent (w/w) for protein production was investigated using different amounts of almond hull: 1.00 g (6.90% w/w), 1.50 g (10% w/w), 1.75 g (11.48% w/w), and 2.00 g (12.90% w/w). In a separate experiment, the optimal initial fungal inoculum amount for protein production was investigated using different final OD_{600} levels: 0.05, 0.20, 0.40, and

0.50. For both experiments, *M. thermophila* was cultivated through SSF on the almond hulls in flasks at 48-50°C for the optimal fermentation time of 96 hours, as described in Materials and methods.

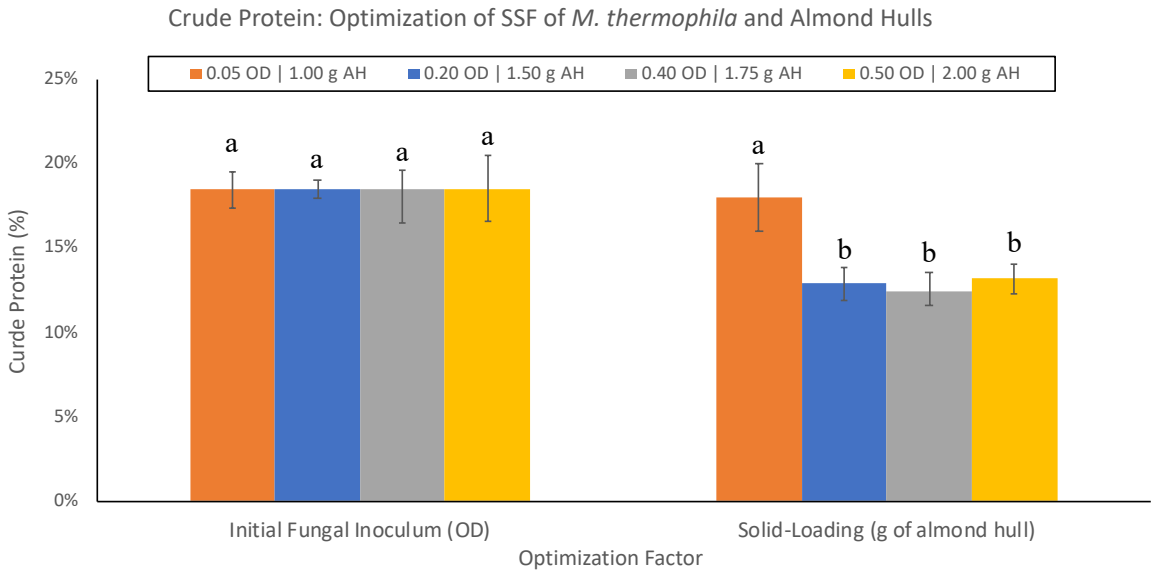
Figure 3.6A shows the fermentation residue crude protein (%) from the experiments optimizing solid-loading level and initial fungal inoculum amount. For the experiment optimizing solid-loading, the maximum crude protein content of 17.99% was obtained using 1.0 g of almond hull correlating to a 6.90% (w/w) solid-loading ($P<0.05$). When the solid-loading was increased higher than 6.90% (w/w), the crude protein content significantly decreased ($P<0.05$). The fermentation residue amount represented in Figure 3.6B increased as the solid-loading level increased. Thus, as the solid-loading level is increased past the optimal level of 6.90%, the protein production slows and results in a lower crude protein content.

For the experiment optimizing initial fungal inoculum amount, the maximum crude protein content of 18.50% was obtained with an initial OD_{600} of 0.50. However, there was no significant difference in the crude protein content ($P>0.05$) between any of the initial fungal inoculum levels. The fermentation residue amount represented in Figure 3.6B did not increase or decrease significantly with increasing levels of initial fungal inoculum ($P>0.05$). Within the studied range, increasing the initial fungal inoculum amount did not improve the crude protein content of the fermentation residue.

It was found that solid-loading level was a significant factor in the SSF process with lower solid-loading levels eliciting higher crude protein contents. However, the initial fungal inoculum level was not a significant factor as it produced no significant increase in crude protein content as the level was increased. However, it is important to remember all fermentation residue

was harvested at 96 hours and the inoculum level could have had an impact on the growth rate of the fungi.

A)



B)

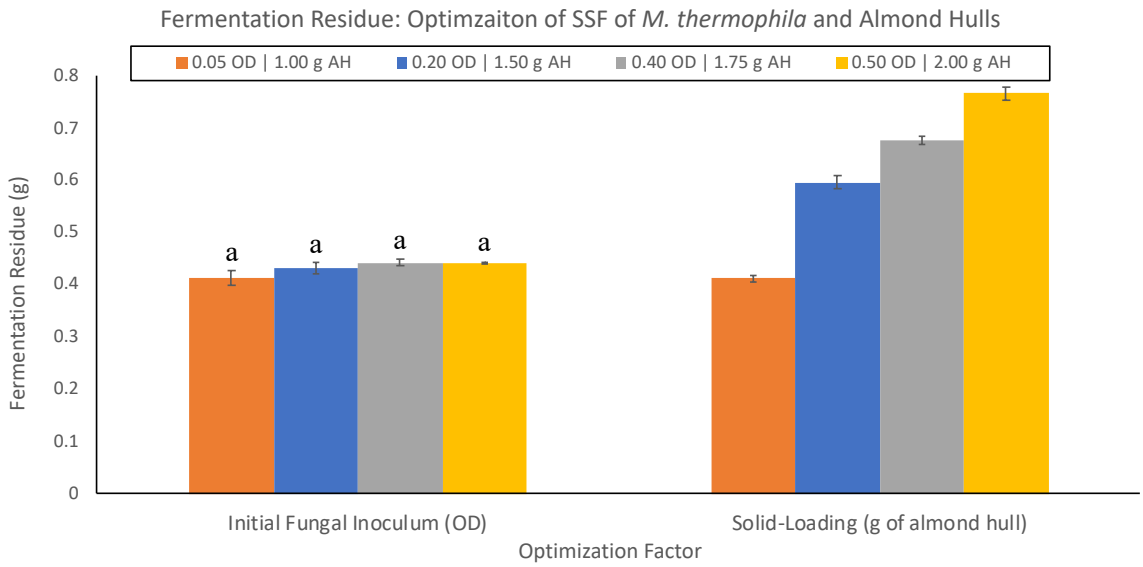


Figure 3.6.1. Optimization of initial fungal inoculum and solid-loading in SSF of *M. thermophila* on almond hulls for 96 hours. Fermentation residue A) crude protein (%) and B) amount (g) results are displayed at each level for both factors. Initial fungal inoculum (OD_{600}) levels were: 0.05, 0.20, 0.40, and 0.50. Solid-loading levels varied by amount of almond hull (g): 1.00, 1.50, 1.75, and 2.00 g. Error bars represent one standard deviation (n = 3 fermentations). Identical letters indicate no statistically significant difference between means.

3.7. *In vitro* digestibility of the fermentation residue as a feed supplement

Protein digestibility is an important measurement to assess the quality of both poultry and aquatic feeds. Digestible protein is the fraction of protein that is digested and absorbed by the animal in amino acid form [50]. The *in vitro* digestibility assay is a method to evaluate the protein quality which utilizes digestive enzymes that imitate the animal's digestive system. The *in vitro* digestibility was determined for the fermentation residue harvested at 120 hours for all three fungi: *M. thermophila*, *T. terrestris*, and *T. aurantiacus*. Samples were tested in duplicate.

The *in vitro* protein digestibility for *M. thermophila*, *T. terrestris*, and *T. aurantiacus* was 80.37 ± 7.06%, 89.70 ± 8.35%, and 47.14% ± 7.24%, respectively. Both *M. thermophila* and *T. terrestris* had good *in vitro* protein digestibility which were comparable to fishmeal with a relative protein digestibility of 78.07% [51]. Soybean meal, a protein supplement for poultry, also had a comparable relative protein digestibility of 76.08% [51]. There was no significant difference ($P>0.05$) in the percent digestibility of the fermentation residue between *M. thermophila* and *T. terrestris*. However, *T. aurantiacus* had a significantly lower ($P<0.05$) protein digestibility compared to both fermentation residues produced by *M. thermophila* and *T. terrestris*.

3.8. Amino acid profile of fermentation residue

Along with digestibility, quality protein sources for both chicken and fish feed are determined by their amino acid composition [13]. Thus, it was important to analyze the amino acid composition of the fungal protein to determine if the amino acid profiles were complete and contained all the essential amino acids. The amino acid profiles are provided in Table 3.9.1 for all three fungal proteins: *M. thermophila* (MT), *T. terrestris* (TT), and *T. aurantiacus* (TA). The profiles were compared to the desired amino acid profiles of both poultry and fish feed on a

percent of protein basis [23]–[25]. All amino acids were within or above the needed poultry and fish feed ranges. The fungal protein also contained a good amount of lysine (~6% on protein basis) which is one of the most important amino acids in feed.

Table 3.2 Amino acid profiles for *M. thermophila* (MT), *T. terrestris* (TT), and *T. aurantiacus* (TA) on a percent of protein basis.

	Values (% of protein)				
Amino Acids	**MT**	**TT**	**TA**	**Fish Requirements**	**Poultry Requirements**
Arginine*	6.78	6.54	5.39	4.0-6.0	2.8-3.5
Glycine	5.01	5.10	5.10	---	5.00
Histidine*	2.95	2.79	3.06	1.5-2.5	1.5-2.0
Isoleucine*	6.19	6.45	7.73	2.2-3.0	2.5-3.8
Leucine*	9.83	9.05	11.22	3.3-3.9	7.0-7.5
Lysine*	6.59	6.64	5.54	4.0-5.0	5.0-5.5
Methionine*	2.26	2.41	2.19	2.0-3.0**	3.5-3.8**
Cysteine*	1.28	1.25	1.60	2.0-3.0**	3.5-3.8**
Phenylalanine*	5.01	4.81	5.25	5.0-6.0	6.0-8.0***
Tyrosine	3.44	3.08	3.35	---	6.0-8.0***
Threonine*	5.01	5.29	4.66	2.0-5.0	2.8-3.5
Tryptophan*	2.95	1.73	3.64	0.5-1.0	0.75-1.0
Valine*	5.90	6.16	5.69	2.5-4.0	4.0-4.3
Asparagine/Aspartic Acid	9.73	9.72	9.48	---	---
Serine	5.01	5.20	5.25	---	---
Glutamine/Glutamic Acid	11.60	12.03	10.50	---	---
Proline	4.72	5.29	4.96	---	---
Alanine	5.70	6.45	5.39	---	---
Taurine	0.00	0.00	0.00	---	---
Total	**100.00**	**100.00**	**100.00**	---	---

*Essential amino acid
** Includes methionine and cysteine
***Includes phenylalanine and tyrosine

3.9. Crude fat analysis of the fermentation residue

Crude fat content is another important factor for the assessment of the fermentation residue as a high-quality feed supplement for poultry and aquatic industries. Crude fat analysis

was performed on the fermentation residue harvested at 120 hours for all three fungi: *M. thermophila*, *T. terrestris*, and *T. aurantiacus*. Samples had only one replicate due to the large amount of solids needed.

The crude fat percent for *M. thermophila*, *T. terrestris*, and *T. aurantiacus* was 6.25%, 2.20%, and 5.58%, respectively. The values were similar to those found in soybean meal used for poultry and fishmeal [52], [53].

3.10. Compilation of compositional analysis of fermentation residues

Compositional analysis was performed on the fermentation residue harvested at 120 hours with an almond hull particle size of 75-250 μm for all three fungi: *M. thermophila*, *T. terrestris*, and *T. aurantiacus*. The cellulose composition percent for *M. thermophila*, *T. terrestris*, and *T. aurantiacus* was 18.28%, 19.18%, and 18.83%, respectively. The hemicellulose composition percent for *M. thermophila*, *T. terrestris*, and *T. aurantiacus* was 13.54%, 17.06%, and 17.53%, respectively. The ash percentages for *M. thermophila*, *T. terrestris*, and *T. aurantiacus* were all low at 1.08%, 2.75%, and 2.53%, respectively. The lignin percentages for *M. thermophila*, *T. terrestris*, and *T. aurantiacus* were similar at 44.65%, 41.58%, and 47.35%, respectively.

For all three fungi, the crude protein content of the hulls was increased. However, *M. thermophila* had the highest crude protein and fat percentages. It also had fermentation residue with the lowest percentage of cellulose, hemicellulose, and ash. The low ash content is an attractive aspect for both poultry and fish feed supplements as it can interfere with the way nutrients are absorbed intestinally [13]. The ash content for poultry feed cannot be higher than 16% whereas ash content ranges from around 5-25% in fish feed protein sources [25]. However,

the high fiber content of the fermentation residue is not suitable for direct use as aquatic feed supplements [13].

Table 3.3 Composition of the fermentation residues for *M. thermophila* (MT), *T. terrestris* (TT), and *T. aurantiacus* (TA) including crude protein (%), crude fat (%), cellulose (%), hemicellulose (%), lignin (%), and ash (%). Standard errors are listed in parentheses next to each subcomponent. Crude fat samples had one replicate. Crude proteins samples for *T. terrestris,* and *T. aurantiacus* were run in single replicates.

Fungi	Crude Protein [%]	Crude Fat [%]	Cellulose [%]	Hemicellulose [%]	Lignin [%]	Ash [%]
MT	17.35 (0.77)	6.25	18.28 (0.40)	13.54 (0.20)	44.65 (1.18)	1.08 (0.95)
TT	16.25	2.20	19.18 (0.83)	17.06 (1.63)	42.09 (1.53)	2.75 (1.48)
TA	12.44	5.58	18.83 (0.89)	17.53 (2.49)	47.35 (0.57)	2.53 (1.06)

4. Conclusion

M. thermophila was the optimal fungi for protein production using a SSF process with almond hulls. The fermentation conditions optimized for the SSF process were particle size the of the hulls, fermentation time, solid-loading, and initial fungal inoculum. The optimal fermentation conditions were found to be a particle size range of 75-250 μm, fermentation time of 96 hours, and a solid-loading of 6.90% (w/w). The fermentation temperature and relative humidity were kept constant at 48-50°C with relative humidity controlled at 70-90%. The initial fungal inoculum level was not a significant factor in the studied SSF process.

M. thermophila was capable of producing fermentation reside with a maximum crude protein content of 18.10% and 6.25% crude fat. The protein yield was 0.20 g crude protein/g carbohydrate consumed. The fermentation residue had good *in vitro* digestibility (80.37 ± 7.06%) and a complete amino acid profile. Therefore, the *M. thermophila* fermentation residue could potentially serve as a protein-rich supplement for poultry supplements. The protein could also be extracted with additional procedures for use as aquatic feed supplements.

5. Future work

The SSF process using thermophilic, filamentous fungi was capable of increasing the protein content of the almond hulls to above 15% which is suitable for chicken feed supplements. Important parameters of the fermentation were also optimized including substrate particle size, solid-loading level, and initial fungal inoculum. However, further improvements in the crude protein content and protein yields could potentially be achieved with additional optimization work. Optimization of the temperature, relative humidity, and nitrogen source could allow for higher protein production and shorten fermentation times.

The media components could also be explored and optimized through response surface methodology allowing for statistical modeling and optimization of multiple components. Future work could also include investigating the addition of Tween 80 during the SSF process [54]. The Tween 80 acts as a surfactant during fermentation and has been shown to enhance the production of cellulases during SSF with thermophilic fungi [54]. Additionally, the SSF process could be scaled up to larger shake flasks and then tray bed reactors to test the scalability of the process for industrial feed production purposes.

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Appendix 1- Supporting Data for Figures & Sections

A1.1 Mean and Standard Error (S.D.) for SmF Fungal Data for Section 3.2.

Table 5.1 Fermentation residue crude protein percent (%) data corresponding to Figure 3.2.1A.

	48 (h)		72 (h)		96 (h)	
Fungal Strain	**Mean**	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**
MT	26.38	0.36	24.65	1.85	25.16	2.17
TT	7.51	0.16	27.87	1.45	28.03	0.26
TA	22.35	0.46	20.95	1.38	21.54	3.75

Table 5.2 Protein yield (g crude protein/g carbohydrate consumed) data corresponding to Figure 3.2.1B.

	48 (h)		72 (h)		96 (h)	
Fungal Strain	**Mean**	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**
MT	0.22	0.00	0.17	0.02	0.09	0.02
TT	0.05	0.00	0.15	0.01	0.14	0.01
TA	0.19	0.01	0.17	0.00	0.13	0.00

Table 5.3 Fermentation residue weight (g) data corresponding to Figure 3.2.1C.

	48 (h)		72 (h)		96 (h)	
Fungal Strain	**Mean**	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**
MT	0.72	0.00	0.63	0.01	0.36	0.05
TT	0.51	0.01	0.52	0.03	0.49	0.02
TA	0.73	0.01	0.69	0.03	0.57	0.09

Table 5.4 Carbohydrates consumed during fermentation (g) data corresponding to Figure 3.2.1D.

	48 (h)		72 (h)		96 (h)	
Fungal Strain	**Mean**	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**
MT	0.86	0.02	0.95	0.09	0.98	0.01
TT	0.79	0.03	0.94	0.01	0.97	0.00
TA	0.83	0.03	0.86	0.02	0.92	0.03

Table 5.5 Protein yield (g crude protein/g almond hull) data corresponding to section 3.2.

	48 (h)		72 (h)		96 (h)	
Fungal Strain	**Mean**	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**
MT	0.094	0.002	0.051	0.041	0.045	0.007
TT	0.019	0.000	0.072	0.003	0.068	0.003
TA	0.081	0.003	0.072	0.002	0.060	0.002

Table 5.6 Carbohydrate conversion percentage (%) data corresponding to Section 3.2.

	48 (h)		72 (h)		96 (h)	
Fungal Strain	**Mean**	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**
MT	81.88	1.92	90.73	8.92	93.15	1.10
TT	79.63	2.51	89.93	0.63	92.29	0.26
TA	75.21	2.92	82.48	2.06	88.14	3.28

A1.2 Mean and Standard Error (S.D.) for SSF Fungal Data for Section 3.3.

Table 5.7 Fermentation residue crude protein percent (%) data corresponding to Figure 3.3.1A.

	72 (h)		96 (h)		120 (h)	
Fungal Strain	**Mean**	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**
MT	13.58	0.90	15.19	0.17	17.69	0.23
TT	10.52	2.49	13.98	1.34	14.92	0.25
TA	9.44	0.39	10.96	0.60	11.77	0.63

Table 5.8 Protein yield (g crude protein/g carbohydrate consumed) data corresponding to Figure 3.3.1B.

	72 (h)		96 (h)		120 (h)	
Fungal Strain	**Mean**	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**
MT	0.16	0.01	0.17	0.01	0.21	0.01
TT	0.11	0.03	0.16	0.02	0.17	0.00
TA	0.10	0.01	0.11	0.01	0.12	0.01

Table 5.9 Fermentation residue weight (g) data corresponding to Figure 3.3.1C.

	72 (h)		96 (h)		120 (h)	
Fungal Strain	**Mean**	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**
MT	0.64	0.01	0.65	0.01	0.71	0.01
TT	0.61	0.02	0.65	0.02	0.68	0.00
TA	0.61	0.02	0.60	0.01	0.60	0.01

Table 5.10 Carbohydrates consumed during fermentation (g) data corresponding to Figure 3.3.1D.

	72 (h)		96 (h)		120 (h)	
Fungal Strain	**Mean**	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**
MT	0.55	0.00	0.57	0.01	0.59	0.00
TT	0.57	0.01	0.57	0.01	0.58	0.01
TA	0.56	0.01	0.59	0.00	0.61	0.01

Table 5.11 Protein yield (g crude protein/g almond hull) data corresponding to section 3.3.

	72 (h)		96 (h)		120 (h)	
Fungal Strain	**Mean**	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**
MT	0.058	0.005	0.065	0.001	0.084	0.002
TT	0.043	0.010	0.061	0.008	0.068	0.002
TA	0.038	0.003	0.044	0.002	0.047	0.003

Table 5.12 Carbohydrate conversion percentage (%) data corresponding to Section 3.3.

	72 (h)		96 (h)		120 (h)	
Fungal Strain	**Mean**	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**
MT	71.21	0.44	73.42	1.59	76.29	0.38
TT	73.42	1.04	73.95	1.28	75.25	1.25
TA	72.24	1.74	76.78	0.32	78.85	0.82

A1.3 Mean and Standard Error (S.D.) for Mechanical Pretreatment of the Hulls to Improve Protein Production in SSF Data for Section 3.4.

Table 5.13 Fermentation residue crude protein percent (%), total carbohydrate consumed (g), protein yield (g crude protein/g carbohydrate consumed), and crude protein (%) data corresponding to Figure 3.4.1.

	Fermentation Residue Weight (g)		Total Carbohydrate Consumed (g)		Protein Yield (g crude protein/g carbohydrate consumed)		Crude Protein (%)	
Particle Size (um)	**Mean**	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**
< 75	0.62	0.01	0.54	0.01	0.19	0.01	16.15	0.41
75-250	0.63	0.01	0.55	0.01	0.23	0.01	19.79	0.46
250-425	0.67	0.01	0.51	0.02	0.22	0.00	16.98	0.70
425-1180	0.71	0.08	0.47	0.03	0.17	0.04	10.90	1.69

Table 5.14 Protein yield (g crude protein/g almond hull) and carbohydrate conversion (%) data corresponding to Section 3.4.

	Protein Yield (g crude protein/g almond hull)		Carbohydrate Conversion (%)	
Particle Size (um)	**Mean**	**S.D.**	**Mean**	**S.D.**
< 75	0.067	0.003	73.97	1.70
75-250	0.083	0.002	75.68	0.79
250-425	0.076	0.004	70.79	2.29
425-1180	0.052	0.014	64.86	4.68

A1.4 Mean and Standard Error (S.D.) for Optimal Harvest Time Determined for *M. thermophia* in SSF Data for Section 3.5.

Table 5.15 Fermentation residue crude protein percent (%), total carbohydrate consumed (g), protein yield (g crude protein/g carbohydrate consumed), and crude protein (%) data corresponding to Figure 3.5.1.

	Fermentation Residue Weight (g)		Total Carbohydrate Consumed (g)		Protein Yield (g crude protein/g carbohydrate consumed)		Crude Protein (%)	
Particle Size (um)	**Mean**	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**
72	0.65	0.02	0.56	0.01	0.20	0.01	17.42	0.73
96	0.64	0.00	0.57	0.00	0.20	0.01	18.10	0.83

120	0.66	0.01	0.59	0.00	0.19	0.01	17.35	0.77
144	0.64	0.01	0.59	0.00	0.19	0.01	16.92	0.38
168	0.60	0.02	0.60	0.00	0.16	0.02	15.94	0.92

Table 5.16 Protein yield (g crude protein/g almond hull) and carbohydrate conversion (%) data corresponding to Section 3.5.

	Protein Yield (g crude protein/g almond hull)		**Carbohydrate Conversion (%)**	
Particle Size (um)	**Mean**	**S.D.**	**Mean**	**S.D.**
72	0.075	0.004	71.13	0.81
96	0.078	0.003	73.01	0.42
120	0.076	0.003	74.73	0.41
144	0.073	0.003	74.49	0.39
168	0.064	0.006	76.04	0.33

A1.5 Mean and Standard Error (S.D.) for Optimization of Initial Fungal Inoculum and Solid-Loading for *M. thermophia* in SSF Data for Section 3.6.

Table 5.17 Crude protein percent (%) and fermentation residue weight (g) data corresponding to Figure 3.6.1A&B.

	Crude Protein (%)		**Fermentation Residue (g)**	
OD (600 nm)	**Mean**	**S.D.**	**Mean**	**S.D.**
0.05	18.44	0.01	0.41	0.01
0.20	18.46	0.01	0.43	0.01
0.40	18.44	0.01	0.44	0.01
0.50	18.50	0.02	0.44	0.00

Table 5.18 Crude protein percent (%) and fermentation residue weight (g) data corresponding to Figure 3.6.1A&B.

	Crude Protein (%)		**Fermentation Residue (g)**	
Solid-Loading (g almond hull)	**Mean**	**S.D.**	**Mean**	**S.D.**
1.00	17.99	0.02	0.41	0.01
1.50	12.88	0.01	0.60	0.01
1.75	12.46	0.01	0.68	0.01
2.00	13.15	0.01	0.77	0.01

A1.6 Mean and Standard Error (S.D.) for *In vitro* Digestibility of the fermentation residue Data for Section 3.7.

Table 5.19 *In vitro* digestibility percent (%) of the SSF residue data corresponding to Section 3.7.

	Digestibility (%)	
Fungal Strain	**Mean**	**S.D.**
MT	80.37	7.06
TT	89.70	8.35
TA	47.14	7.24