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Exploring Bioprocessing Technologies for Diverse Industrial Application of Canola

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EXPLORING BIOPROCESSING TECHNOLOGIES FOR DIVERSE INDUSTRIAL APPLICATION OF CANOLA

BY

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This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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I dedicate this dissertation to my incredible mother (Wedad Albasha), lovely wife (Sara Shetiwi), wonderful children (Khaled, Wedad and Wassim), supportive brothers, especially (Dr. Khaled Alhomodi), very kind sisters, especially (Eman Alhomodi), supportive brothers-in-law especially (Dr. Nasser Aljarallah), and wonderful father/mother-in-law (Khaled Shetiwi and Asma Qatan) for their unlimited support, and prayers to achieve my academic goal.

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ABSTRACT

EXPLORING BIOPROCESSING TECHNOLOGIES FOR DIVERSE INDUSTRIAL APPLICATION OF CANOLA

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Globally, canola is the second largest oilseed crop after soybean, which is processed for the commercial production of high value oil. The industrial processing steps for canola oil extraction include preprocessing (cleaning, pressing, flaking, and cooking), mechanical pressing, and/or organic solvents. This process results in large quantities of protein-rich (~ 40% dry basis) meal as a co-product. The meal is used partially in animal diet (<30% inclusion) due to high levels of antinutritional factors (ANFs) such as high fibers, phytic acid, and glucosinolates, and low metabolizable energy. Thus, this research was designed to examine various bioprocessing technologies (i.e., traditional sprouting, solid state fermentation, submerged state fermentation, co-culture fermentation, mild pretreatments, and their combinations) for possible advancement in canola use. Canola seed sprouting for 6-day period led to an increase in protein content and a reduction in ANFs and oil content of sprouts compared to ungerminated seed. Subsequent submerged state fermentation of 6-day old sprouts using three different strains (*Aureobasidium pullulans*, *Trichoderma reesei* and *Neurospora crassa*) further increased protein content and lowered ANFs. Solid-state fermentation of 144 h old sprouts using *A. pullulans, N. crassa and T. reesei* enhanced the protein content and reduced ANFs of sprouts. Sprouting canola seed for three days helped in hull removal, leading to high protein meal accompanied by low fiber and phytic acid level. Three-day seed sprouting had no effect on oil yield, but free fatty acid content was

higher compared to seed oil. Co-culture fermentation of HECM under solid state process showed maximum reduction in fiber content with co-culture of *A. pullulans* and *N. crassa* while a combination of *A. pullulans* and *T. reesei* promoted the highest GLS and phytic acid reductions compared to other combinations, which indicated the advantage of co-culture inoculation over monoculture in terms of ANFs reduction. Mild pretreatment of HECM using deionized water resulted in washed HECM (WHECM) with lower soluble sugars and GLS compared to untreated HECM, whereas protein and amino acid were concentrated due to the removal of soluble components. WHECM compared to HECM showed higher protein digestibility when fed to rainbow trout. Subsequent mono- and co-culture fermentation of HECM and WHECM under submerged process resulted in higher protein and amino acid content and lower ANFs levels compared to uninoculated controls. The results of cellulase, endoglucanase and β-glucosidase activity indicated the crucial role of used substrates, fungi, fermentation modes (solid state/submerged stated) and inoculation methods (mono-/co-culture) on enzyme activities.

Chapter 1. Introduction

Global population is in the rise with the estimated earth population of 10 billion by 2050 (Kusiak et al., 2022). While significant world population is already having a limited access to enough nutritious and sustainable foods, this predicted population growth is expected to worsen this issue even further. Additionally, global food production system seems to be greatly impacted by the various factors (environmental, urbanization, climate change, etc.) highlighting the need of sustainable food production system. The healthy human diet is comprised of the significant amount of food proteins and therefore the demand of animal protein is expected to increase by 13% in 2026 (OECD-FAO, 2017). For example, the annual average rate of global food fish consumption in 2017 increased by 3.1% since 1961 while animal protein foods increased by 2.1% yearly (FAO, 2020). One way to tackle this problem is to develop the alternative proteins of plant origins to replace the animal proteins in the diets of humans and animals. The proteins of plant origins are sustainable resource and have potential to provide the solution to the growing global food demand.

Canola meal (CM), a byproduct of canola seed oil extraction, is known to be a nutrient rich commodity with a minimum protein content of 40% on dry basis (CCC, 2019). Although CM is abundant and estimated to be double the amount of produced oil (Östbring et al., 2020) with attractive protein content for feed formulation, its inclusion in animal diet is restricted to ~30%, which lower its market value (Newkirk, 2009b). Presence of antinutritional factors (ANFs) such as glucosinolates (GLS) and phytic acid, besides high fiber content hinders the full utilization of CM. Broken-down products of GLS have been reported to lead to low feed intake, iodine efficiency and enlargement of liver (Tripathi and

Mishra, 2007) while phytic acid complexes with minerals and proteins, thereby declining their bioavailability (Francis et al., 2001). The high fiber content and low nutrient density is attributed to the seed hull because there is no industrial scale dehulling process for canola seed.

In general, several studies have been reported on the improvement of protein content and the reduction of the ANFs in various plant-based protein using different bioprocessing technologies such as sprouting (Yıltırak et al., 2021; Ohanenye et al., 2020; Bhardwaj and Hamama, 2007) and fermentation (Sun et al., 2021; Olukomaiya et al., 2021). Seed sprouts (plant shoot and its appendages) contains high level of nutritious components (proteins, carbohydrates, vitamins, and minerals) and low level of ANFs compared to unsprouted seed (Santos et al., 2020; Park et al., 2019; Benincasa et al., 2019). Due to that, sprouts have become a part of human diet and their demand have increased (Benincasa et al., 2019; Abdel-Aty et al., 2019; Santos et al., 2020). Besides, sprouts have been included in livestock diets for their nutritional values.

Fermentation in both modes (i.e., solid and submerged state fermentations) have been applied for a long time for various applications (Pandey et al., 2008). Solid state fermentation (SSF) is a process that is performed under low moisture environments. It is favored for mainly fungi and molds due to low water activity requirement (Chilakamarry et al., 2022). SSF is cost effective because of no need to long drying process (Intasit et al., 2020). However, SSF can have some challenges such as controlling pH, moisture, and temperature (Hölker et al., 2004). In contrast, submerged state fermentation (SmF) is where microbes are cultured in nutrient liquid media (Kim and Han, 2014). The high-water level in SmF helps in homogenizing substrate mixture and controlling process. However, the

drawback of SmF is the generation of high wastewater in downstream processing that raises the processing cost (Krishania et al., 2018).

Fermentation has been shown to reduce the ANFs and upgrade the nutritional values of different plant-based protein products such as cereal grains, pulses and oilseeds (Baldwin et al., 2019; Simon et al., 2017; Nkhata et al., 2018; Frias et al., 2017). Beside the ability to reduce undesirable components during fermentation, the used microorganisms can serve as single cell protein (SCP) to the product output. SCP can be named as microbial protein, bioprotein or biomass (Muhammad et al., 2016) that can provide 10 to 80% protein besides other nutrients such as carbohydrates, fats, minerals and vitamins (Jones et al., 2020). Many of these microorganisms can be exploited in feed and food processing industry as they are generally recognized as safe (GRAS) status (Wang et al., 2005b).

Fermentation using co-culture of microorganisms have been applied to produce traditional food such as cheese, yogurt, and pickles (Bader et al., 2010; Taniguchi and Tanaka, 2004), antifungal secondary metabolites (Li et al., 2020), bioethanol (Naseeruddin et al., 2021), enzymes (Anh et al., 2021), and biodegradation of food waste (Roslan et al., 2021). Co-culture fermentation provide high fermentation efficiency when microbes synergistically secrete enzymes that degrade different substrates (Chen, 2011). Compared to monoculture, the substrate metabolization in co-culture fermentation can occur by a combined metabolic activity of two different microorganisms (Bader et al., 2010). It was reported that a co-culture of yeast and bacteria enhanced the protein and starch digestibility of fermented pearl millet sprouts (Khetarpaul and Chauhan, 1990b).

Since bioprocessing technologies have potential provide high value products, our goal is to employ different processing technologies which includes traditional sprouting process combined with microbial process, microbial fermentation using different modes of fermentations, different microbes, and inoculation types to develop optimal and economical process to advance the canola utilization for various industrial applications. Our first study (chapter 3) is focused on determining the effects of seed sprouting followed by submerged state fermentation on the nutritional composition of canola seed sprouts. Because the results of sprouting followed by submerged state fermentation were promising, we wanted to explore the possibility of achieving similar results in shorter time frame. Hence, we conducted second study (chapter 4) where our focus was to assess the impact of sprouting period on the composition of canola seeds on daily basis (up to 6 days) and solid-state fermentation on 6-day old sprout composition. While we conducted our two previous studies focusing on the sprouting, two questions arose, which are: 1) how does sprouting affect the oil composition of the canola, which is a larger commercial commodity and 2) what impacts it will have on the nutrient composition of the meal post defatting. Simultaneously, it was observed that sprouting could potentially be used as a dehulling process to remove the outer coat of the seeds prior to the oil extraction. Hence, the third study (chapter 5) was designed to determine the impact of sprouting on oil and meal composition and the potential of sprouting as dehulling process. In the fourth study (chapter 6), our focus was on determining the effect of co-culture fermentation on the nutritional composition of hexane extracted canola meal (HECM). Because co-culture fermentation was successful in improving the nutrient composition of HECM with respect to the ANFs, but fiber reduction was still low, our fifth study (chapter 7), was designed to determine the

combined effect of mild pretreatment prior to co-culture fermentation on the nutritional characteristics of HECM and nutrient digestibility of washed HECM (WHECM) in Rainbow trout. Because all our previous studies on microbial fermentation of CM (raw and processed) with different strains indicated that same microbes could yield different outcomes in terms of protein solubilization and ANFs reduction indicating different level of enzymatic activities, we designed our sixth study (chapter 8) to determine the activities of enzymes (cellulase, endoglucanase, and β-glucosidase) produced by three fungal strains cultivated on three differently processed canola substrates HECM, WHECM, and canola seed sprout meal (CSM) in solid- and submerged state fermentations.

Chapter 2. Literature Review

2.1. Oilseeds

Oilseeds are cultivated for edible use or industrial application of their oil content (Rathnakumar and Sujatha, 2022), whereas solids left after oil extraction can be exploited in various food and non-food industrial applications (Nevara et al., 2022). Various types of oilseeds are grown based on the industrial purpose of production and the climate of the region. Some of the major oilseeds include sunflower, soybeans, rapeseed, cotton, sesame, etc. (Rathnakumar and Sujatha, 2022).

2.2. Global Production of Oilseeds

Global production of oilseeds in the year 2021/22 is estimated at 629.16 million metric tons (Table 2.1) (USDA, 2021). Soybean annual production top the list in the USDA report (2021) with the estimated production of 384.42 million metric tons. Five years data reported by USDA shows that there is an increase in the total oilseed production as compared to the previous years (19/20 and 20/21). Rapeseed is the second largest oilseed crop after soybeans although it is reported to have reduced production by 4.12 million tons in 2021/22 compared to 2020/21.

Table 2.1: World production of major oilseeds (million metric tons)

Oilseeds	2017/18	2018/19	2019/20	2020/21	2021/22
Copra	5.76	5.79	5.66	5.56	5.82
Cottonseed	45.09	43.10	44.42	41.22	43.32
Palm kernel	18.66	19.44	19.29	19.06	19.93
Peanut	47.12	47.00	48.41	49.66	50.53
Rapeseed	75.07	72.60	69.08	72.29	68.17
Soybean	344.19	361.33	339.73	363.27	384.42
Sunflower seed	48.01	50.66	53.92	50.07	56.97
Total	583.90	599.93	580.51	601.14	629.16

(USDA, 2021)

2.3. Oilseeds Classification

2.3.1. Legumes

Legumes are generally described by their unusual flower structure, podded fruit (Graham and Vance, 2003) and the capability of most species to build a symbiotic association in their root nodules with bacteria called *Rhizobia* (Etesami, 2022). Legumes contain main dietary grain legumes such as cowpea, bean, pigeon pea, lentil, chickpea, mung bean, adzuki bean, pea, and faba bean (Juárez-Chairez et al., 2022). Legumes play important role in world trade economics due to their uses as human feed, animal feed and various commercial application such as pharmaceutical products, paints, chemicals, etc. (Singh et al., 2007).

2.3.2. Brassicaceae Family

Brassicaceae family contains more than 300 genera and 3000 species (Daun, 2011). The genus *Brassica*, containing about 37 species (Cartea et al., 2011), is a commercially important genus for its various uses as food, oilseed crops, and ornamental plants (Vaughn and Berhow, 2005). Oilseed plants of the genus *Brassica* give many advantages when cultivated as cover crops. These advantages involve stabilization and improvement of soil health, avoidance of erosion and control of allelopathic weeds through breakdown products of glucosinolate hydrolysis. These factors have sparked interest in *Brassicas* growing as either cover crops or seed crops for edible oil production (Haramoto and Gallandt, 2004).

2.4. Canola

Canola is a descendant of rapeseed (*Brassica napus* and *Brassica campestris*/*Brassica rapa*) grown using standard techniques of plant breeding to gain low content of erucic acid (< 2%) in the oil fraction and low levels of glucosinolates (< 30

μmol/g) in the meal fraction. The name canola was given for (Canadian oil) to distinguish it from rapeseed. Other countries, especially in Europe, give the term "double-zero rapeseed" to specify seed, oil, and meal quality having low erucic acid and glucosinolates (CCC, 2019).

Brassica napus (canola) belongs to the genus *Brassica* having bright yellow flowers (Bonnardeaux, 2007), and it is featured as one of the most economically important oilseed plants among other species (Scarth and Tang, 2006). In Europe, winter canola varieties are typically seeded (Rakow, 2012). In the fall, winter canola is cultivated and then harvested in the following summer. Varieties of winter canola can tolerate a high cold temperature, however, still freeze when dry soils and rapid variations in temperature come together (Brown et al., 2008). Usually, yield of winter canola varieties outweighs spring varieties by 20-30%. In addition, winter crops provide larger seeds containing higher oil content (Brown et al., 2008) and yield almost double compared to the spring type (Rakow, 2004). In contrast, spring canola varieties are typically grown in the United States, Canada, India, and parts of China (Rakow, 2012). In the early spring, the varieties of spring canola are seeded, and then late in summer are harvested (Brown et al., 2008).

2.4.1. Canola Seed Physical Characteristics

Canola seeds have a size that ranges between 1.5 and 2.5 mm in diameter (Çalışır et al., 2005) and varies in color depending on species. *B. napus* exhibits a black or reddish color, *B. rapa* has a black or yellow color and *B. juncea* shows a yellow color (Newkirk and Daun, 2011). Furthermore, seeds can come in three different shapes (oblong, spherical or slightly flattened laterally) (Barthet and Daun, 2011). As compared to other oilseeds, canola seeds are smaller in size; therefore, seed weight is greatly influenced by the moisture

content of the seed. A thousand seed mass is used to measure the canola seed weight that typically ranges between 2.5 and 4.6 g/1000 seeds (Ayton, 2014). Rapeseeds consist of three main parts (Figure 2.1) (Borisjuk et al., 2013), which are embryo that contains (cotyledon, hypocotyl and radicle), endosperm and seed coat that encloses the embryo and the endosperm (Borisjuk et al., 2013).

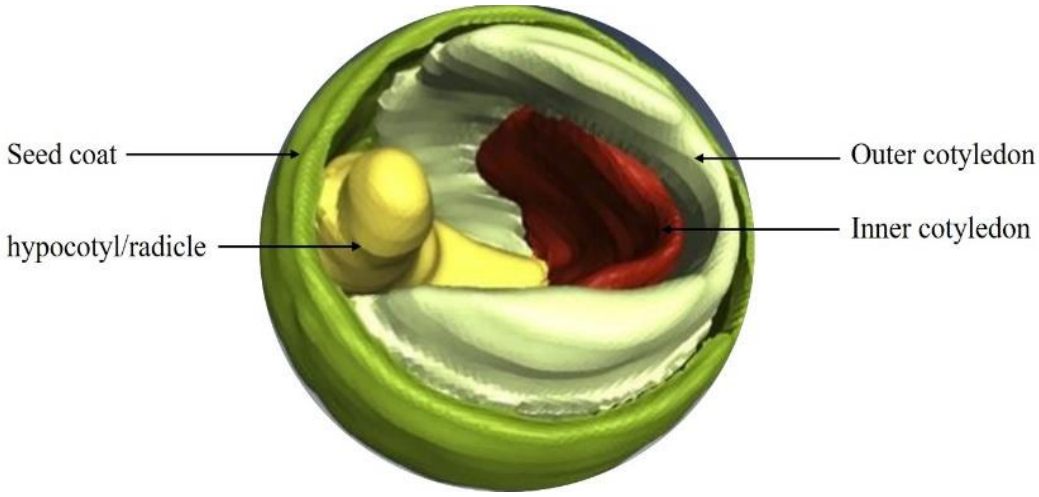

Figure 2.1: A cut-away three-dimensional model of the seed (Borisjuk et al., 2013)

2.4.2. Canola Seed Composition

Canola seed composition varies based on cultivar, climate condition during seed maturation time and sulfur content in the seed (Aider and Barbana, 2011). Cotyledon represents about 25% protein and 55% oil content in comparison with the entire seed and seed coat that contains protein and non-protein such as glucosinolates and tri-amine compounds (Wanasundara et al., 2012).

2.5. Canola Seed Oil Extraction

Canola seeds are not consumed as whole seeds but are initially conveyed to a processing facility to extract the oil from the seeds. The separation of oil from canola seed involves many different steps (Drying and handling, seed cleaning and preparation,

extraction, and processing of oil). There are also various methods of extracting the oil, including: cold press, expeller and solvent extraction (CCC, 2019).

2.5.1. Solvent Oil Extraction (SOE)

Solvent extraction is the most efficient method of extracting oil commercially, leaving around 2 to 4% of the residual oil in the meal (Figure 2.2) (CCC, 2015). This type of extraction uses two phases to extract high levels of oil. The seeds normally undergo a pre-conditioning process prior to oil extraction, which usually incorporates seed cleaning, pre-conditioning and flaking (Newkirk, 2009a). The oil then is removed from preconditioned flakes mechanically by screw press at 100-120 °C to generate a seed cake comprised of about 20% oil (Spragg and Mailer, 2007). The resulting seed cake is then exposed to solvent extraction using hexane and then goes through a desolventizer-toaster at 100-115 °C for 30 minutes to remove hexane from the meal (Spragg and Mailer, 2007; Newkirk, 2009a).

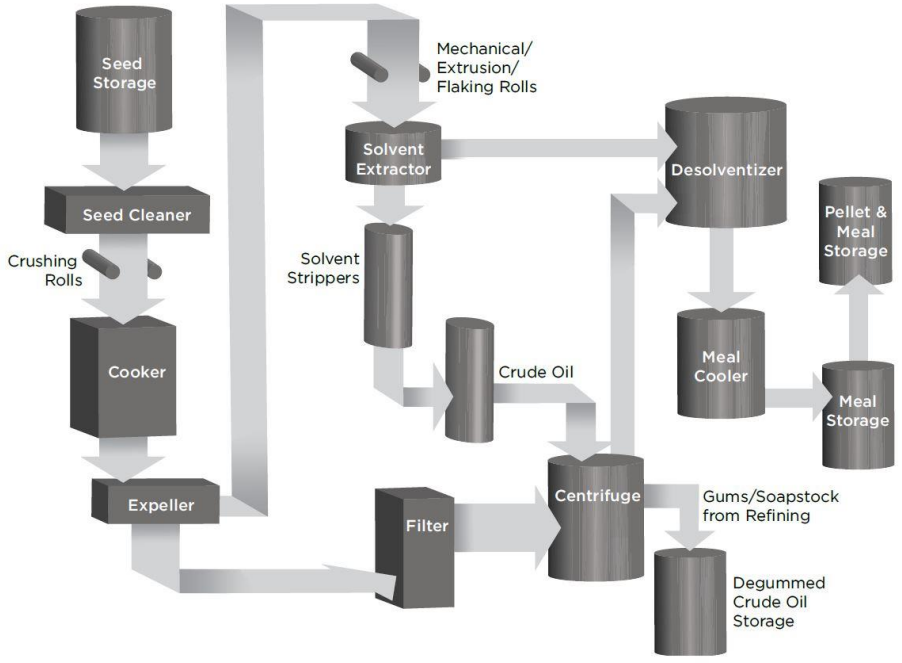

Figure 2.2: Pre-conditioning and solvent extraction process (CCC, 2015)

2.5.1.1. Advantages and Disadvantages of SOE

Even though hexane solvent extraction process provides meals with less 4% oil (Spragg and Mailer, 2007) and decreases glucosinolates content by ~53%, high temperature applied during toasting steps can have adverse effects on the amino acid digestibility (Newkirk et al., 2003) and reduce meal palatability that could be as a result of products stemming from Maillard reactions (Newkirk and Classen, 2002). In contrary, solvent extraction without a toasting step showed high amino acid digestibility in broiler chickens (Newkirk et al., 2003).

2.5.2. Expeller/Double Pressing Oil Extraction

Expeller pressing is termed as well double pressing. It has a similar pre-conditioning stage to solvent extraction process (Newkirk, 2009a). However, desolventization, toasting, drying, and cooling steps are not included. Figure 2.3 illustrates the applied screw press in expeller and cold press as well (Leming and Lember, 2005b). The screw press can be fed with seeds that are pressed via rotating screw; thus, oil can flow throw mesh at the bottom. Due to the exclusion of solvent step, the meal is expelled twice to extract additional oil (Newkirk, 2009a). A double pressing extraction provides a meal with 8-11% oil.

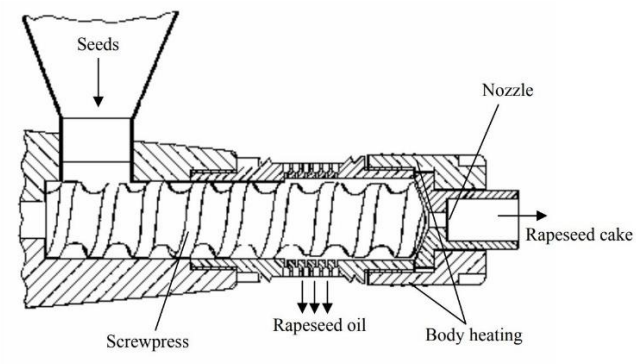

Figure 2.3: Diagram of mechanical screw press for double press and cold press oil (Leming and Lember, 2005b)

2.5.2.1. Advantages and Disadvantages of Expeller/Double Pressing

In expeller extraction, residual oil adds higher metabolizable, digestible and net energy content to the meal compared to solvent extracted meal. In double pressing, heat level of meal reaches up to 160 °C when passing through the press with preserved protein meal quality due to low moisture content and short duration. However, meal needs cooling down quickly after expelling, otherwise protein quality deteriorates (CCC, 2015).

2.5.3. Cold Press Oil Extraction

Unlike in solvent extraction and expeller pressing process, preconditioning stage is not included in cold pressing process before oil extraction. Cold pressed and expeller extraction share similar mechanical separation as shown previously (Figure 2.3). Seeds go through a series of screw presses comprised of a rotating screw shaft inside a cylindrical tube (Newkirk, 2009a). In the process, friction and pressure are generated to crush the seeds. The friction generated during the brief period the seed cake is passing through the expeller raises the temperature to less than 65°C (Spragg and Mailer, 2007). Extracted oil flows through a mesh at the bottom of the barrel for collection. In a cold press extraction, 50-70% oil is extracted from seeds (Seneviratne et al., 2011) and 11-20% oil stays in the meal (Leming and Lember, 2005a).

2.5.3.1. Advantages and Disadvantages of Cold Press

Canola oil extracted by cold press is named virgin oil. This oil is considered an organic and natural food. Besides, its price usually is higher than solvent and double press extracted oil (Daun et al., 2015). However, this extraction method still requires more improvements to increase its efficiency (Szydłowska-Czerniak et al., 2010).

2.5.4. Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) is known as well in different terms such as dense gas extraction and supercritical gas extraction. The concept of this method is to apply heat and pressure to work around the critical point of the fluid. Supercritical fluids is more diffusible and less dense and viscous than used conventional organic solvents (Díaz-Reinoso et al., 2006). However, the properties of the supercritical fluids can be changeable based on pressure and temperature (Bozan and Temelli, 2002). This technique has been investigated as a substitution for conventional oil extraction (King and List, 1996). Carbon dioxide (CO_2) is commonly used in SFE as solvent due to low required temperature (30.1 °C) and pressure (7.39 MPa) to reach its critical point (Uquiche et al., 2012). Supercritical CO_2 extraction was used to extract oil from soybean, cottonseed, and corn germ (Friedrich and Pryde, 1984). In addition, it was used to extract bioactive compounds from a waste byproduct stemming from vegetable oil industries (Asl and Niazmand, 2020).

2.5.4.1. Advantages and Disadvantages of SFE

The advantages of using SFE over liquid organic solvents are the lower density and higher diffusivity giving higher solvent power and better transport characteristic, which resulting in higher mass transfer rate (Patel et al., 2011). SEF extracts better taste and scent substances from plant and herbs (Díaz-Reinoso et al., 2006).The benefit of using carbon dioxide (CO_2) as solvent in SFE is due to its attributes such as (nonflammable, nontoxic, Eco-friendly, highly abundant, inexpensive, appropriate to extract heat labile substrates, and easily removable from extracted compounds (Díaz-Reinoso et al., 2006). However, high costs of high-pressure equipment (Friedrich et al., 1982) and batch extraction (Friedrich and Pryde, 1984) are considered drawbacks of SEF.

2.6. Canola Oil

The global canola oil production was 31.7 million tons in 2018, and it is expected to reach 44.4 million tons by 2024 (IMARC, 2019). Edible oils and fats consist mainly of triacylglycerols (TAGs) comprising of three fatty acid molecules bound to the glycerol molecule. TAGs basically make up 94.5 to 99% of canola oil (Przybylski et al., 2005). Tocopherols, phytosterols, phenolics and pigments constitute the non-triacylglycerol fractions. Canola oil is considered as one of the healthiest edible oils among other vegetable oils due to its healthy fatty acid profile and has a variety of uses such as frying and food ingredients. On average, traditional canola oils consist of 60% oleic acid, 20% linoleic acid, 10% linolenic acid, 7% saturated fats (palmitic acid and stearic acid), and less than 2% erucic acid (Bocianowski et al., 2012).

2.6.1. Oleic Acid

Oleic acid is a monounsaturated fatty acid comprised of 18 carbon atom chain with one double bond. It is a dominant fraction making up 60% of canola oil (Ayton, 2014). Due to the oleic acid ability to maintain resistance to oxidation, high level of oleic acid (>70%) is desired by food manufacturing industry applications where high temperature is applied during the production process (Aukema and Campbell, 2011). Canola oil containing high level of oleic acid and low level of linolenic acid is preferred for fried food products with better food quality such as food flavor, texture, and stability (Matthäus, 2006). In addition, food product is formulated with high oleic canola oils for longer shelf life during storage (DeBonte et al., 2012).

2.6.2. Linoleic Acid

Linoleic acid is a polyunsaturated fatty acid consist of 18 carbon atom chain with two double bonds. It comes second after oleic acid and constitutes 20% of canola oil (Ayton, 2014). It is an essential omega-6 fatty acid (CCC, 2020). Oil with linoleic acid between 23 and 37% showed a better flavor stability in fried food (Warner et al., 1997). In addition, low level of linoleic acid <20% in oil is desirable in manufacture for shelf stable foods (DeBonte et al., 2012).

2.6.3. Linolenic Acid

Linolenic acid is an essential omega-3 fatty acid (CCC, 2020). It is a polyunsaturated fatty acid contains 18 carbon atom chain with three double bonds. It makes up 10% of canola oil (Ayton, 2014). However, frying performance of oil was improved when canola oil contained low level of linolenic acid. Deep fat frying using oil with more than 1.1% linolenic acid led to higher fishy and paint odors (Carre et al., 2003). Reducing linolenic acid content in canola oil have been found to provide higher frying stability (Warner and Mounts, 1993). Thus, decreasing the linolenic acid level in canola and other major oils has been targeted by plant breeders to enhance the oxidative stability and avoid partial hydrogenation (DeBonte et al., 2012).

2.6.4. Erucic Acid

Erucic acid is a monounsaturated fatty acid having 22 carbon atom chain with one double bond (Ayton, 2014). High level of erucic acid has detrimental effects on human. It was found to be in charge of generating cardio pathological changes (Clandinin and Yamashiro, 1982). Therefore, erucic acid content was decreased by genetic alterations through traditional plant breeding program to present at less than 2% in canola oil (CCC,

2019). However, based on oil ultimate use, high level of erucic acid in oil is targeted by different industrial manufactures in the production of various products such as nylon, inks, paints, coatings and high pressure grease (Aukema and Campbell, 2011).

2.7. Canola Oil as Fuel Source

The global need for fossil fuel substitutions has led to exploitation of oil to produce biofuels (Hollister et al., 2013). Various types of oilseed such as soybean, canola, rapeseed, palm and sunflower are utilized for biodiesel production (Moser, 2010; Sarwar et al., 2013). Biodiesel is generated from oils and fats through transesterification process, a chemical reaction in which alcohol (for example methanol) reacts with oil in the existence of a catalyst. As a result, free fatty acid ester and glycerol forms (Gunstone, 2004). Biodiesel in the US is created from soybean oil, whereas the European Union depends on canola oil as source for biodiesel production. The interest of using canola oil in biodiesel production is due to seed possession of high oil content and low level of saturated fatty acid (Ayton, 2014).

2.8. Canola Meal

Canola meal is the solid portion remained after oil extraction. Canola meal is the second widely used source of protein for animal feed after soybean meal. The composition of canola meal varies depending on the oil extraction method used (cold press, expeller press and solvent extraction). Use of solvents in oil extraction removes oil up to 95% from the press cake, whereas using expeller press leaves 8 to 15% oil in the meal (Seneviratne et al., 2010; Spragg and Mailer, 2007). Even though solvent extraction meal has less than 5% oil, most processors put up to 2% of the gums (phospholipids removed from crude oil by the addition of a mild organic acid) back to the meal (Ayton, 2014), which increase the

fat content and energy value. The gum also decreases the amount of dust within dry meals (Spragg and Mailer, 2007).

2.8.1. Canola Meal Proximate Composition

The proximate composition of canola meal is shown in Table 2.2, which can vary depending on various factors. Various factors such as cultivar types, environmental conditions during the growing period, harvest conditions, storage, and seed/meal processing can lead to different meal compositions (Bell and Keith, 1991). Growing conditions (dry climate) provide canola seed with high levels of protein and low oil content (Spragg and Mailer, 2007). Different canola oil extraction processing conditions have shown to affect canola meal quality (CCC, 2019). Applied heat, desolventizing and toasting steps lower protein digestibility (Newkirk and Classen, 2002). Different degree of heat applied during oil extraction process can reduce lysine, a heat sensitive amino acid (Newkirk, 2009b).

Table 2.2: Composition of solvent extracted canola meal

Component	12% moisture basis
Crude protein (N × 6.25), %	36.9
Ether extract, %	2.81
Oleic acid, %	1.74
Linoleic acid, %	0.56
Linolenic acid, %	0.24
Ash, %	6.42
Calcium, %	0.67
Phosphorus, %	1.03
Total dietary fiber %	33.6
Acid detergent fiber, %	16.3
Neutral detergent fiber, %	25.5
Sinapine, %	0.88
Phytic acid, %	2.02
Glucosinolates, μmol/g	3.14

(CCC, 2019)

Canola meal also contains complex carbohydrate matrix (fiber and non-fiber component) (Table 2.3). The fiber fraction contains lignin with linked polyphenols, cellulose, lignin, glycoprotein, and non-cellulosic polysaccharides which comprise mainly of pectic substances (Slominski and Campbell, 1990). The non-fiber fraction presents as monosaccharides (fructose and glucose), disaccharides (sucrose), oligosaccharides, and starch (CCC, 2019). The variability of carbohydrate content in canola meal as well can be affected by growth condition, genetic distinctions, and method of analysis (Bell, 1993). In terms of minerals and vitamins, canola meal has essential minerals and vitamin content that is higher than soybean meal (Wickramasuriya et al., 2015). Canola meal produced in Canada contains 3.2% fat content which is higher than other countries because of the gum added back to the meal during oil refining (CCC, 2019).

Table 2.3: Carbohydrate components of solvent extracted canola meal

Component %	12% moisture basis
Non-starch polysaccharides	20.15
Cellulose	7.65
Non-cellulosic polysaccharides	12.5
Glycoprotein	4.30
Lignin and polyphenols	8.68
Lignin	5.82
Monosaccharides (Fructose and glucose)	1.55
Disaccharides (Sucrose)	5.58
Oligosaccharides	2.23
Starch	0.43

(CCC, 2019)

2.8.2. Canola Meal in Feed Applications

Canola and rapeseed meals are widely included in animal feed. They come second after soybean meal as most traded protein ingredient in the world (CCC, 2019). They have been included in ruminant, swine, poultry and aquaculture diets. Ravichandiran et al. (2008) reported that 5-month-old calves consumed 1.10 kg of canola meal, similar quantity

to control diet 1.08 kg when glucosinolate level of canola meals was less than 20 µmol/g. In the same study, feed uptake reduced to 0.76 g when claves were fed with rapeseed meal containing glucosinolates over 100 µmol/g. In a comparison meal study, using four different protein supplement in diary diets (high protein dried distillers grains, soybean meal, canola meal, and dried distillers grains with soluble) showed no differences in dry matter intake, milk production, feed efficiency, and energy-corrected milk yield (Christen et al., 2010). In swine diet, 20% substitution of soybean meal in a diets with raw canola meal and extruded canola meal with low, medium or high extruder intensity showed no significant difference in average daily feed intake, average daily gain and a gain to feed ratio (Heyer et al., 2018). CM have been fed to different types of poultry (broilers, turkeys, ducks, goose, and quail) at different concentration, but it has a lower energy value compared to soybean meal (CCC, 2019). CM have been included in aquaculture diets as alternative vegetable-based protein to fish meal. However, CM inclusion in fish diets is still limited due to the presence of antinutritional factors that need to be considered when formulating the fish diet with CM. Collins et al. (2012) reported that formulating fish diet with CM had a negative effect on rainbow trout growth, but no significant effect was noticed when using canola protein concentrate.

2.8.3. Canola Meal Protein

Canola meal contains about 35-45% protein with balanced amino acids, which makes it a good ingredient for animal feed (Newkirk, 2009b). Based on the protein solubility, canola proteins are categorized into four group: soluble in water (albumins), salt solution (globulins), alkaline solution (glutelin), and alcohol (prolamins) (Aider and Barbana, 2011). In addition, canola meal possesses minor proteins (trypsin inhibitors,

thionins and lipid transfer proteins) (Bérot et al., 2005). Napin, a 2S albumin with molecular weight (12.5-14.5 kDa) has good foaming properties (Schmidt et al., 2004). Cruciferin, a 12S globulin with molecular weight (300-310 kDa) has gelling properties (Ghodsvali et al., 2005). Both Napin and cruciferin are the major family of storage protein in Canola. They make up 20 and 60% of the total protein content, respectively (Barthet and Daun, 2011). Prolamins presents exclusively as oleosin, the structural proteins linked to the oil bodies (Mieth et al., 1983). Oleosins are the predominant oil body proteins followed by steroleosins and caleosins (Tzen, 2012).

2.8.4. Canola Protein Concentrate and Isolate

Protein concentrates are obtained by extracting non-protein fractions such as sugars and antinutritional factors from original meals (Moure et al., 2006), whereas protein isolates are prepared by protein extraction and subsequent precipitation (Chmielewska et al., 2020). The reduction of canola seed hull, sugars, phenolics and glucosinolates concentrates the protein level up to 70% (Wanasundara, 2011). Protein extraction with the removal of undesirable non-protein fraction provides protein isolate over 90%. Protein isolate preparation has been reported using alkaline extraction followed by acid precipitation (Ivanova et al., 2017), protein micellation method (PMM) (NCBI, 2022), and membrane technology (Ghodsvali et al., 2005). However, protein yield and properties highly depend on protein extraction methods. Commercially canola protein isolates (>90% protein) as food grade products presents under several names Puratein®, Supertein™, Burcon Nutrascience, Isolexx™ and CanolaPRO™ (Wanasundara et al., 2016). Puratein® and Supertein™ are comprised of >80% cruciferin and >80% napin, respectively.

Isolexx™ consists of 30-35% albumins and 60-65% globulin while CanolaPRO has about 40-65% cruciferins and 35-60% napins (Chmielewska et al., 2020).

2.8.5. Canola Protein in Food Applications

Due to the high nutritional value and properties of canola proteins, many studies have been reported over the past decades to incorporate canola protein in food commodities as substitute for animal-derived proteins. Various characteristics of rapeseed protein isolate give different applications such as food foaming, gelling agent, thickener, emulsifier, and binder, besides as protein supplement (Chmielewska et al., 2020). Von Der Haar et al. (2014) reported that rapeseed protein concentrate can replace up to 100% of the eggs in pound cakes but with the possibility of the presence of little darker color in the final product. In the same study, preparation of mayonnaises with rapeseed protein instead of eggs did not show any difference in product firmness after preparation, but the product became firm after storage compared to reference. In terms of sausages preparation, the maximum rapeseed protein inclusion was 2%, over than that can lead to off flavor and color change. The canola protein isolate Puratein® (cruciferin) and Supertein™ (napin) can be included up to 2% in bakery products such as bread, croissant, cakes, etc. In addition, they can be included up to 10% in fruit and vegetable-based juices (FDA, 2010). The rapeseed protein isolate (CanolaPRO™) can be incorporated into dairy products, beverages, medical nutrition, sport nutrition, and bakery products up to 5, 5, 10, 10 and 5%, respectively (FDA, 2016).

2.8.6. Canola Protein Properties

Canola protein has characteristics that make them suitable for human nutrition. It has well balance amino acid compositions and higher protein efficiency (2.64) compared

to soybean (2.19) (Delisle et al., 1984). Canola proteins share some properties with those of casein and have better protein properties compared to other plant proteins such as wheat, pea, and soybean (Ghodsvali et al., 2005). The differences among primary, secondary, and tertiary structures of canola protein fractions give them different properties and functionalities (Wanasundara et al., 2012). Canola protein isolate shows a comparable emulsification capacity to egg proteins (Yoshie-Stark et al., 2008). Canola protein can be use in dressing, meat and baked products for its good emulsifying, gelling and binding properties (Mejia et al., 2009). The effectiveness of canola meal extracts is restricted by the existence of antinutritional factors (glucosinolates, phenols, and phytates) that affect protein isolate utilization (Aider and Barbana, 2011). However, the advances in separation technology has successfully brought filters with lower cost and higher efficiency (Gesan-Guiziou, 2017). Applied filtration process has been shown to lower or remove antinutrient factors from the rapeseed protein dispersion (Tzeng et al., 1988; Von Der Haar et al., 2014). Thus, rapeseed protein has the potential to be included as protein with high quality and functional properties in human food (Dong et al., 2011). However, the various isoelectric points and molecular weights of canola proteins are another problem leading to a reduction in the yield and efficiency of protein extraction (Aachary and Thiyam, 2012). Thus, canola protein fractions can be extracted based on their solubility and corresponding sedimentation coefficient in Svedberg units (Aider and Barbana, 2011).

2.8.6.1. Solubility

Protein solubility is a crucial property affecting other properties such as gelling, foaming, emulsification, and water/oil holding capacity (Wanasundara et al., 2016). Generally, canola meal protein extraction has been done as a whole protein rather than a

fraction using methods such as alkaline extraction or salting out with NaCl (Tan et al., 2011a). In addition, specific canola protein fractions can be categorized based on protein fraction solubility in solution (Aider and Barbana, 2011). The two major protein fractions in canola (cruciferin and napin) have different solubility level under various pH, temperature, and salt concentration (Wanasundara et al., 2012). Cruciferin and napin, respectively showed isoelectric pH of 7.2 (Schwenke et al., 1981) and 11 (Crouch and Sussex, 1981) where the net charge is zero. Both cruciferin and napin can be soluble above pH of 5.5, but napin can exhibit wider pH solubility (2 to 10) due to its amino acid composition. Cruciferin was found not to be soluble between pH 2 and 3 because of its interaction with other chemical compounds (Wanasundara et al., 2016). In addition, canola protein processing can reduce protein solubility. Khattab and Arntfield (2009) reported that exposing canola meal protein to heat treatment (dry roasting and water boiling) resulted in 29.07 and 25.61% decline, respectively in solubility. The change of protein structure due to heat treatment could expose the hydrophobic sides, thereby lowering protein solubility (Aider and Barbana, 2011). Klockeman et al. (1997) reported that extraction conditions can be a reason for lower canola protein solubility.

2.8.6.2. Gelation

Gelation is an important functional feature in food applications such as edible film production (Akbari and Wu, 2016). When proteins are exposed to heat, they partially unfold leading to aggregation and gel-network formation (Zheng et al., 1993). The stabilization of protein gel network can be attributed to hydrophobic interactions, hydrogen bonding and covalent bonding cross links such as S-S bonds (Damodaran, 2008). All different proteins can form a gel, but gel strength can vary. Minimal protein concentration

required to generate a gel can determine the ability of protein gelling formation (Moure et al., 2006). Various factors can affect gel formation such thermal stability, molecular size, pH, temperature, and present salts (Doi, 1993). Most canola protein gel formation studies focus on heat-induced gelation (Wanasundara et al., 2016). Cruciferin exhibits stronger heat set gel formation under high pH compared to low pH (Léger and Arntfield, 1993). Napin as well has a gelation property, but it can only form a weak gel (Tan et al., 2014b). Napin resists gel formation between pH 4 and 8 (Folawiyo and Apenten, 1997) as a result of unfolding at low pH (Krzyzaniak et al., 1998). Both factors (protein concentration and pH) affect the maximum gelation temperature of cruciferin and napin. Schwenke et al. (1998) stated that the maximum gelation temperature for 12.5% dispersions of cruciferin near pH of 7 was 72 °C, whereas the gelation temperature of napin fraction at the same pH was at 95 °C. Canola protein gelation properties can be enhanced by adding polysaccharides (Tan et al., 2011a). Uruakpa and Arntfield (2006) reported that gels stemming from a mixture of canola protein isolate (CPI) and κ-carrageenan generated better networks with less sensitivity to environmental factors compared to CPI solely.

2.8.6.3. Foaming Capacity/Stability

Proteins forming stable foams play an important role in various food applications. Foams present as two-phase systems comprised of air bubbles encapsulated by lamellar phase, a thin continuous liquid layer. Foam structure (Air bubble size, distribution, and uniformity) can affect food properties (Zayas, 2012). Canola proteins foaming properties have been evaluated based on their foaming capacity and foaming stability. Foaming capacity shows the ability of protein to generate foam particles while foaming stability exhibits the duration formed foams stay stable prior to collapsing (Kinsella, 1981). In a

comparison study among raw, roasted and water-boiled meals (canola, soybean, and flaxseed meals), canola meal was the best in foaming properties compared to other meals. However, heat treatment showed a significant reduction in foaming capacity and stability of all meals (Khattab and Arntfield, 2009). In terms of canola protein isolates, defatted canola meals showed better foaming capacity and stability than their acid precipitated protein isolates (Aluko and McIntosh, 2001). Pedroche et al. (2004) found out that foaming capacity of *Brassica carinata* defatted meal was better in comparison to its protein isolates at different pH (10,11, and 12).

2.8.6.4. Emulsification

Emulsification is the process where the emulsifying agent lowers the interfacial tension of two immiscible liquids (oil and water) in a system, thereby forming stable oil-water interfaces (Moure et al., 2006). Proteins play an important role as emulsifying agents in many food applications such as salad dressings and mayonnaise (Aider and Barbana, 2011) and non-food application such as cosmetic products (Campbell et al., 2016). Emulsion properties of meal and proteins are expressed as emulsion activity index (EAI), emulsifying capacity (EC) and emulsifying stability (ES). EAI and EC measure the ability of protein to create an emulsion, whereas EC measures the ability of an emulsion to resist changes over a specific time (Aluko and McIntosh, 2001). Many studies have been done on evaluating canola meal and protein emulsifying properties (Tan et al., 2011a). Khattab and Arntfield (2009) reported that both raw and heat-treated canola meal (roasted and boiled) showed better emulsion capacity than raw and heat-treated soybean and flaxseed meals. In addition, they found that heat treatments reduced the emulsifying capacity of all meal compared to raw meals. Defatted *Brassica juncea* meal and its protein concentrates

showed higher emulsifying activity index compared to *Sinapis alba*. This was attributed to the high-molecular mass polypeptide possessed by S. alba (Aluko et al., 2005). Tan et al. (2014a) examined and compared the emulsifying capacity among canola protein globulin fraction, albumin fraction, canola protein isolate and commercial soy protein isolate. The researchers showed that at different pH (4, 7 and 9), canola protein globulin fraction possessed the highest emulsifying capacity compared to other fractions.

2.9. Antinutritional Factors in Canola

While canola meal accommodates approximately 70 to 80% of soybean meal protein levels, it is usually sold at about 60 to 75% of its value (Ayton, 2014). Factors affecting the quality of canola meal include higher fiber level and the presence of glucosinolates and phytic acid (Wu and Muir, 2008).

2.9.1. Fiber

Canola meal fiber content including pentosans, cellulose, and lignin contribute to 30% of the meal. The high fiber level is attributed to the presence of seed hulls that are not removed prior to oil extraction (Newkirk, 2009a). The concentration of crude fiber, neutral detergent fiber (NDF), and acid detergent fiber (ADF) in canola meal varies based on canola varieties, seed composition, and meal processing (Spragg and Mailer, 2007; Newkirk, 2009a). NDF represents the primary cell wall component (cellulose, hemicellulose, lignin, insoluble ash and cutin) that is insoluble in neutral detergent solution, whereas ADF comprises (cellulose, lignin, insoluble ash, cutin, but not hemicellulose) that are insoluble in weak acid (Ayton, 2014).

The high fiber content reduces the digestibility of canola meal unlike soybean meal that contains less fiber and higher digestibility (Mailer et al., 2008b). High fiber content

entraps the digestible nutrients by inhibiting enzymatic degradation and besides decreases the gut transit time (Brunson et al., 1997). Thus, canola meal has low nutritional value when is used as monogastric feed (Bell, 1993). Landero et al. (2011) reported that increasing inclusion of canola meal lowered gross energy, digestible energy, dry matter, and crude protein in diet tested on 220 weaned pigs. Hilton and Slinger (1986) stated that canola meal has low digestibility due to high fiber content and cannot be a substitute for soybean meal or fish meal in diets for young rainbow trout (*Salmo gairdneri*).

2.9.2. Glucosinolates

Glucosinolates (GLS) are plant secondary metabolites called β-thioglucoside N-hydroxysulfates. GLS can be categorized into three groups based on the amino acid precursor (aliphatic, aromatic and indolic glucosinolates) (Sønderby et al., 2010). GLS itself is not toxic, but its hydrolysis by enzyme myrosinase present in the seed or the microflora of an animal guts results in bioactive breakdown toxic products (Vaughn and Berhow, 2005). Both Glucosinolates and the enzyme myrosinase are located separately in canola (Rask et al., 2000). The enzyme myrosinase breaks the glucose from glucosinolates which leads to the production of toxic products such as nitriles, isothiocyanates, and thiocyanates that plants use as self-defense (Figure 2.4) (Halkier and Gershenzon, 2006). Upon consumption by animals, GLS breakdown products can negatively interfere with thyroid and lead to goiters due to iodine deficiency (Burel et al., 2001). High levels of glucosinolates have been shown to raise death in poultry, along with lower egg production and egg weight (Tripathi and Mishra, 2007). In addition, the high level can lower the palatability of the meal (Bonnardeaux, 2007).

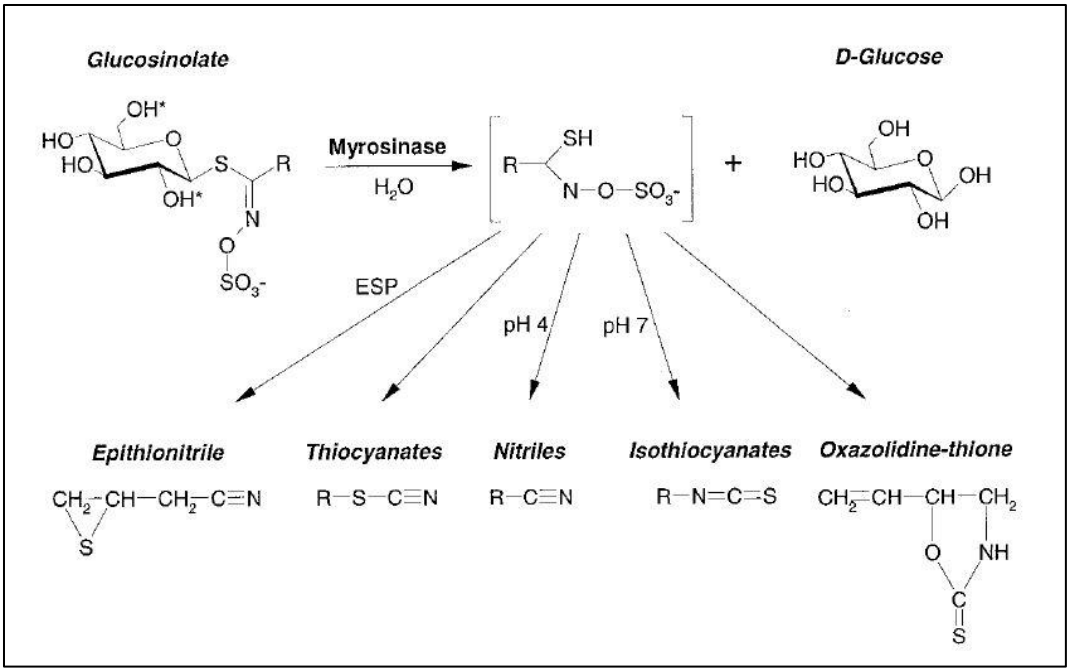

Figure 2.4: The structure of glucosinolates and breakdown products (Rask et al., 2000).

2.9.3. Phytic Acid

Phytic acid is the major storage structure of phosphorus that builds up in seed during development to maturation in many oilseeds (Pandey et al., 2001; Lott et al., 2000). Phytic acid is also called Myo-inositol (1,2,3,4,5,6) hexakisphosphate consisting of 6 groups of phosphates connected to the inositol ring (Figure 2.5). It constitutes about 85% of the total phosphorous in rapeseeds (Maison, 2014). Its negatively charged makes it a strong chelating agent that binds to proteins and metallic cations, thereby reducing their bioavailability and subsequently causing mineral deficiency (Shivanna and Venkateswaran, 2014). It is unmetabolizable in monogastric animals that lack phytase, the digestive enzyme of phytic acid (Pandey et al., 2001; Spier et al., 2008), which leads to the accumulation of phosphorous in manure, thereby excreting into the soil (Chen et al., 2014) and later causes eutrophication of fresh water and seawater (Vats et al., 2005).

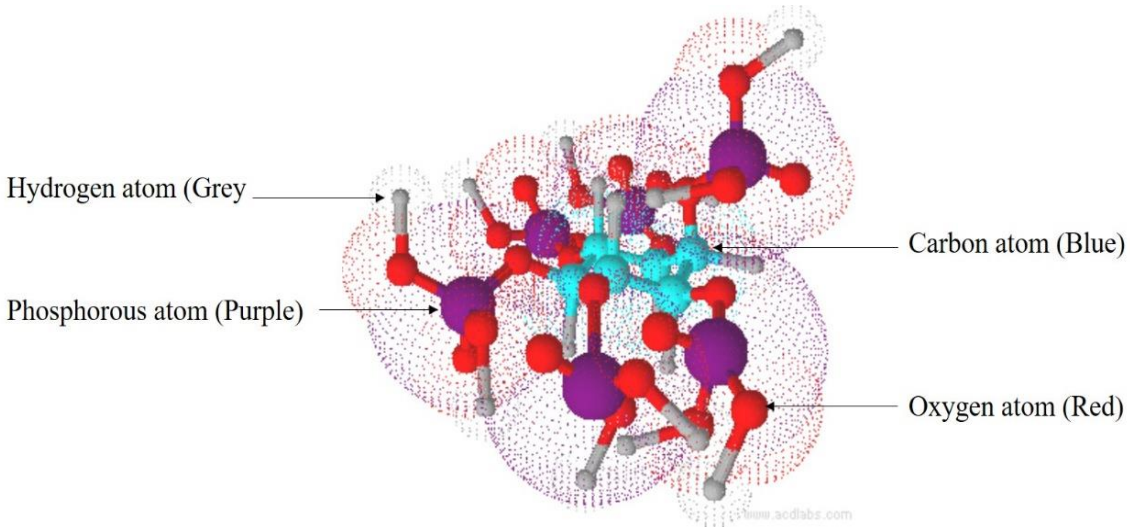

Figure 2.5: Myo-inositol (1,2,3,4,5,6) hexakisphosphate in boat formation illustrating the 5 equatorial and the 1 axial group (Bohn et al., 2008)

2.10. Process Develop to Maximize the Canola Utilization

2.10.1. Canola Seed Dehulling

Canola meal is composed of 38-43% protein and 13% crude fiber (Shahidi, 1990), which limits the canola meal inclusion in animal feed formulation (Leslie et al., 1973). The high fiber content in canola meal is attributed to the presence of outer coat (hull) in the seed during oil extraction. Removing hulls from canola seed can enhance the oil and meal quality and provide suitable products for human consumption (Ohlson, 1992). Canola seed hull constitutes 10.5 to 20% of seed weight while hulls make up 20-30% of oil free meal (Bell, 1993; Mohamadzadeh et al., 2009). The difficulty of seed dehulling stems from the tightly adherence of hull to endosperm and embryo (Sosulski and Zadernowski, 1981). Several dehulling process have been proposed and reported. In France during the 1980s, the industrial scale dehulling was attempted, but few years later the dehulling process was ceased due to substantial oil loss in hull fraction and finite market interest in dehulled meal (Carré et al., 2016). Reichert et al. (1986) came up with a tangential abrasive dehulling device that comprises of an abrasive horizontally rotating disk and a stationary lid with

some grain cups on the rotating disk. However, the process requires seed pre-conditioning to optimize hull removal (Thakor et al., 1995). Attempts to dehull rapeseed prior to oil extraction is scarce because of oil loss during the dehulling process (Khajali and Slominski, 2012). In contrast, several studies have been done on partly separating hulls from oil free meal using sieve (Mejicanos, 2015), sieve followed by air classification (Diosady et al., 1986), or just air classification (King and Dietz, 1987). Air classification is (tail-end dehulling method) depending on the distinctions in density between hull and cotyledon fraction. Based on size and density, a low-fiber, light-particle fraction, and a high-fiber, heavy-particle fraction are partially split up by means of air streams (Mejicanos and Nyachoti, 2018). However, air classification is less applicable to rapeseed due to high fat materials leading to agglomeration of solvent extracted meal (Santos et al., 2014b; Hansen et al., 2017). Thus, further investigations are required to advance dehulling process that improves nutritional values of canola meal for the inclusion in feed and food formulation.

2.10.2. Canola Seed Sprouting

Sprout, a young shoot emerged from plant seeds, is considered as a great source for micronutrients and macronutrients. It has been included in diets for a long time as nutritious and healthy food in many civilizations. A thousand of year ago, Egyptian and Chinese ancients consumed sprouts for healing and rejuvenation (Azulay, 1997). Today in the United State and other Western countries, sprout becomes a favored healthy food. Many studies have shown the composition of sprouts as a nutritionally complete food (Chung et al., 1989; Bhardwaj and Hamama, 2007). In addition, sprouts have a role in the prevention of many diseases because of their possession of antioxidant, antigenotoxic, anticancer, and antibiotic compounds (Marton et al., 2010). The consumption of the raw *Brassica* sprouts

have been shown to contribute to the inhibition and treatment of diseases due to owning bioactive compounds (Chung et al., 1989; Bhardwaj and Hamama, 2007). In particular, canola sprout contains around 25.1 % protein, 27.3 % oil and 10% crude fiber on a dry basis, and it has a variety of high mineral levels compared to radish, alfalfa, brussels and mung bean sprouts (Bhardwaj and Hamama, 2009b). However, factors such as light, moisture, temperature, duration of germination, cultivars, and analytical methods leads to variations in composition of sprout (Bau et al., 1997). Dawood et al. (2013) reported that soaking and germination of two varieties of canola seed (*Brassica napus*/*juncea*) resulted in a reduction in oil content. Bhardwaj and Hamama (2009a) claimed that sprouting seed of four canola cultivars grown at three locations in Virginia showed various differences on oil content and fatty acid composition in sprouts. Barthet and Daun (2005) showed that canola sprout had lower oil content and higher protein and free fatty acid compared to unsprouted seed. In addition, canola sprout had lower oil level but higher tocopherol content compared to intact seed (Zhang et al., 2007).

2.10.3. Microbial Fermentation Process

The utilization of microorganisms as ingredient or a substitute for protein-rich foods seems unacceptable to some communities, but the fact is that using microbes as food can be ideal to sustain the global future food scarcity. People have been consuming either intentionally or unintentionally the microbial biomass producing alcoholic beverages, cheese, yogurt, and soya sauce. Many species of microorganisms can grow on inexpensive waste materials and subsequently provide high microbial protein as single cell protein (SCP) (Tusé and Miller, 1984) with fats, carbohydrates, nucleic acids, vitamins, and minerals (Jamal et al., 2008). Based on substrate type, fungi and bacteria can provide true

protein ranges (30-70%) and (50-83%), respectively (Ravindra, 2000). Both fungi and bacteria have advantages and disadvantages when used as single cell proteins (Table 2.4). Thus, not all microbes can be used in food and feed applications due to the risk of presence of unwanted compounds that can be produced during fermentation process such as toxin. Therefore, the microorganisms in our research were selected based on their capability of producing various hydrolytic enzymes and their status as generally recognized as safe (GRAS) in the food processing industry.

Table 2.4: Advantage and disadvantages of fungi and bacteria as single cell protein

Fungi	
Advantages	**Disadvantages**
- Easy to harvest due to their size.[1] - Low in nucleic acid.[1] - High in lysine content.[1] - Growth at acidic pH.[1] - Growth on wide range of substrate.[2] - Growth on lignocellulosics (filamentous fungi).[2]	- Lower growth rates than bacteria.[2] - Lower protein content.[1] - Low methionine content.[1] - Presence of mycotoxin in many species (filamentous fungi).[2]
Bacteria	
Advantages	**Disadvantages**
- High growth rates.[1] - Growth on wide range of substrate.[2] - High in protein content.[1]	- Difficult to harvest and costly due to their size.[1] - High in nucleic acid.[1] - Presence of endotoxin in gram negative bacteria.[2] - Easy to be contaminated.[1]

Nasseri et al. (2011)[1]
Singh (1998)[2]

2.10.3.1. Aureobasidium pullulans

Aureobadidium pullulans a yeast-like fungus is frequently residing on the phyllosphere and carposphere of vegetables and fruit crops (Barata et al., 2012). *A. pullulans* can be found in three different physiological states (elongated branched septate filaments, large chlamydospores, and smaller elliptical yeast-like cells) (Chi et al., 2009).

In addition, it has the capability to form multiple budding cells unlike the traditional yeast cells (Zalar et al., 2008). *A. pullulans* colony color changes based on culture ages (yellow, cream, light pink or light brown, and black when chlamydospore forms) (Chi et al., 2009). *A. pullulans* is known for an extracellular and unbranched homopolysaccharide production called pullulan comprised of α-(1→6) linkages of α-(1→4)-linked maltotriose units (Seo et al., 2004). Pullulan is widely applied in food processing and preservation (Farris et al., 2014). In terms of enzyme production, *A. pullulans* has been reported to produce beneficial industrial enzymes, such as β-glucosidase, mannanases, cellulases, lipases, proteases, amylases, and xylanases (Bozoudi and Tsaltas, 2018). These various enzyme produced by *A. pullulans* can be beneficial to bio-convert the agricultural residues into single cell protein (Kudanga and Mwenje, 2005) that can be used as an ingredient or a substitute for protein-rich foods (Bozoudi and Tsaltas, 2018).

2.10.3.2. Neurospora crassa

Neurospora a red bread mold fungus was first reported as contaminant destroying bread in French bakeries in 1843 (Shear and Dodge, 1927). Later based on the complete sexual cycle, a red mold fungus was reclassified as new genus *Neurospora*. It includes many heterothallic and homothallic species (Perkins and Turner, 1988). It was exploited as a model organism for its high growth rate, haploid genetics, a short life cycle, and minimal growth requirements until the bacterium *Escherichia coli* took over as a new model organism in genetic studies. However, the species *Neurospora crassa* has still been used as a model for complicated eukaryotic systems (Roche et al., 2014). In addition, *N. crassa* has been grown on different agricultural wastes for enzyme and bioethanol production due to its ability to produce various hydrolytic enzymes such as endoglucanase, exoglucanase,

β-glucosidase, xylanase and β-xylosidase (Dogaris et al., 2009). Xiros et al. (2008) reported the production of cellulolytic and hemicellulolytic enzymes by *N. crassa* grown on optimized medium of brewing and wheat straw. Rath et al. (2011) reported the production of ethanol 20g/kg of acid pretreated sugarcane bagasse by *N. crassa* in 5 days. Co-culture of *N. crassa* and *Lactobacillus plantarum* was found to increase the amino acid content 37.4% on fermented oil tea seed cake. Besides, feeding hens with fermented oil tea seed cake improved the egg production ratio from 65.71 to 80.10% (Liu et al., 2016).

2.10.3.3. Aspergillus niger

Aspergillus niger is an aerobic filamentous fungus present in nature on organic matter, soil, litter, and decayed plant material. It can grow at a wide range of temperature (6-47 °C) and pH (1.4-9.8) (Schuster et al., 2002). It can be distinguished from other *Aspergillus* species through the formation of carbon black or very dark brown spores (Raper and Fennell, 1965). It has a high growth rate on culture media where it forms black colored colonies (Gautam et al., 2011). Industrially, *A. niger* is the most versatile filamentous fungal strain able to produce various compounds such as acids, enzymes, proteins, and medicinal drugs. Many companies worldwide have been using *A. niger* to produce citric acid, glucoamylase, lactoferrin, β-galactosidase, glucose oxidase, hemicellulase, protease, phytase, chymosin, arabinase, asparaginase, catalase, cellulase, lactoferrin, lipase, pectinase, protease, xylanase, inulinase, and glucosidase (Cairns et al., 2018). Various studies have used *A. niger* to improve nutritional values of plant protein sources by fermentation process. Jannathulla et al. (2017a) reported that growing *A. niger* on different plant protein sources led to a reduction in antinutritional factors and fiber level with enhancement in major essential nutrients. Shi et al. (2015) stated that performing solid

state fermentation using *A. niger* reduced total glucosinolate content in rapeseed cake by 76.89%. Yabaya and Ado (2008) claimed the ability of *A. niger* grown on banana peels as substrates to produce mycelia protein.

2.10.3.4. Trichoderma reesei

Trichoderma reesei is mesophilic filamentous ascomycetes fungus that was first isolated from US Army tools on the Solomon Islands during the 2nd World War (Bischof et al., 2016). Initially, *T. reesei* was recognized as *Trichoderma viride* and called QM6a, but because of noticed differences between the two species, QM6a was changed to *T. reesei* in honor of Elwyn T. Reese, the Natick laboratory researcher (Simmons, 1977). *T. reesei* showed the ability to secrete extracellular cocktail of lignocellulosic enzymes. In terms of cellulase activity, *T. reesei* produces enzymes (endoglucanases and exoglucanases) acting together to reduce cellulose to cellobiose (Aubert et al., 1988; Saloheimo et al., 1994). Another enzyme is β-glucosidases involved in hydrolyzing cellobiose to glucose (Barnett et al., 1991). *T. reesei* secretes swollenin, protein that disrupts crystalline cellulose structures, thereby increasing the accessibility of polysaccharides to hydrolysis (Saloheimo et al., 2002). In addition, *T. reesei* produces hemicellulosic enzymes such as xylanase and mannanase that split the xylan and mannan main chains of hemicellulose. It also produces acetyl xylan esterase, β-glucuronidase, and arabinofuranosidase, β-xylosidase, β-galactosidases (Foreman et al., 2003). Industrially, *T. reesei* is widely used for cellulase production (Qian et al., 2016). In addition, it has been invested as single cell protein (SCP) grown on different low value substrates. Ghanem et al. (1991) reported that *T. reesei* provided a protein production of 34% when cultivating on beet-pulp compared to control. Another researcher claimed that reduction in lignin and increase in protein content

achieved by *T. reesei* grown on rice straw pulp in solid state fermentation (Zaki and Said, 2018).

2.11. Types of Fermentation

2.11.1. Solid State Fermentation

Solid-state fermentation (SSF) has been defined as the bioprocessing of solid substrates implemented in presence of low water content that is adequate to back up the growth of fermentative microbes and their metabolic activities. The potential of SSF is to provide an environment that simulates the natural environment of microorganisms from where they are originally adapted (Nigam and Pandey, 2009). Generally, filamentous fungi and yeast are considered the most appropriate microbes for solid state incubation since they require lower water activity (0.5-0.6 a_w) compared to bacteria (0.8-0.9 a_w) (Thomas et al., 2013). SSF process is suitable when using agro-industrial residues and by-product as feedstock because it increases the commercial and nutritional value of them instead of disposing them and consequently causing environmental contamination (Nigam and Pandey, 2009). In addition, SSF process is beneficial because it does not require long drying process and big vessels as submerged fermentation process, thus it reduces the industrial processing costs (Smits et al., 1993). However, mass transfer and controlling the pH, temperature and moisture are still the drawbacks of SSF (Hölker et al., 2004).

2.11.2. Submerged State Fermentation

Submerged fermentation (SmF) is a process where microorganisms are cultivated in excessive nutritional liquid medium (Kim and Han, 2014). It is widely used to produce bioproducts in industry using specific strains of microorganisms. Excessive water used in SmF system is advantageous because it increases the homogeneity of the mixture and

aeration during bioprocessing (Chicatto et al., 2014) , and facilitates the sterilization and controlling process (Costa et al., 2008). In addition, SmF provides ease separation process to remove undesirable water soluble products (Sindelar, 2014). However, the disadvantage of SmF is that the high water needed in up-stream processing resulting in high wastewater present in downstream processing, which increases the processing cost. The addition of antifoam agents to control the foam stemming from rigorous mixing raises the pollution load (Krishania et al., 2018).

2.11.3. Co-Culture Fermentation

Co-culture fermentation are widely applied to produce secondary metabolites, ferment food, and degrade various substrates. The interaction among used microorganisms can be through direct cell to cell communications or by signal substances produced by microorganisms, which attaches to cell surface proteins during fermentation (Chen, 2011). Co-culture incubation plays an important role in degrading different substrates when used microorganisms work synergistically to produce a specific set of enzymes (Stoilova and Krastanov, 2008). The inoculation with two or more fungi functioning synergistically leads to higher enzyme production, thereby breaking down a variety of agricultural substrates (Jahangeer et al., 2005). *Aspergillus* is known to efficiently produce β-glucosidase (Raza et al., 2011) and *Trichoderma* is a producer of endo and exo-glucanase, but not β-glucosidase (Wang et al., 2012). Thus, combining the two strains in fermentation system can generate multi-complex cellulase matrix. Stoilova and Krastanov (2008) reported that the co-culture of (*A. niger* and *Fusarium moniliforme*) and (*Trametes versicolor* and *A. niger*) grown on wheat bran, oats straw and beetroot press led to 8.4-fold increase in laccase over monoculture. Juwaied et al. (2010) claimed that inoculating wastepaper with co-

culture of *A. niger* and *T. viride* (ratio 1:1) increased the cellulase up to 2.4-fold and 3.7-fold over monoculture of *A. niger* and *T. viride,* respectively.

2.12. Enzyme

High energy and chemicals, required for many chemical transformation processes (Hagen, 2015), cause a production of environmental harmful by-products due to non-specificity (Buchholz et al., 2012). Thus, global industries seek alternative technologies that provide desirable products with less resource inputs and impact on the environment. Enzymes, proteins generated by living organisms, catalyze biochemical reactions in vivo and in vitro (Jegannathan and Nielsen, 2013). Unlike chemical-based processes, enzymes are highly selective with high reaction rates and low impact on the eco-system. Besides, enzymes are implemented in mild working conditions and consume less energy and materials (Jegannathan and Nielsen, 2013).

2.12.1. Cellulolytic Enzymes

Among various types of enzymes, cellulase is one of the most important enzymes that catalyzes cellulolytic materials. Cellulase are composed of three enzymes (Exoglucanase, endoglucanase and β-glucosidase) that synergistically simplify cellulose (Figure 2.6) into oligosaccharides and glucose units (Imran et al., 2016). Exoglucanase cleaves single glucose unit from the end of cellulose chain. Endoglucanase randomly cleaves internal β-1-4, linkages while β-glucosidase hydrolyzes cellobiose into glucose (Sharada et al., 2013).

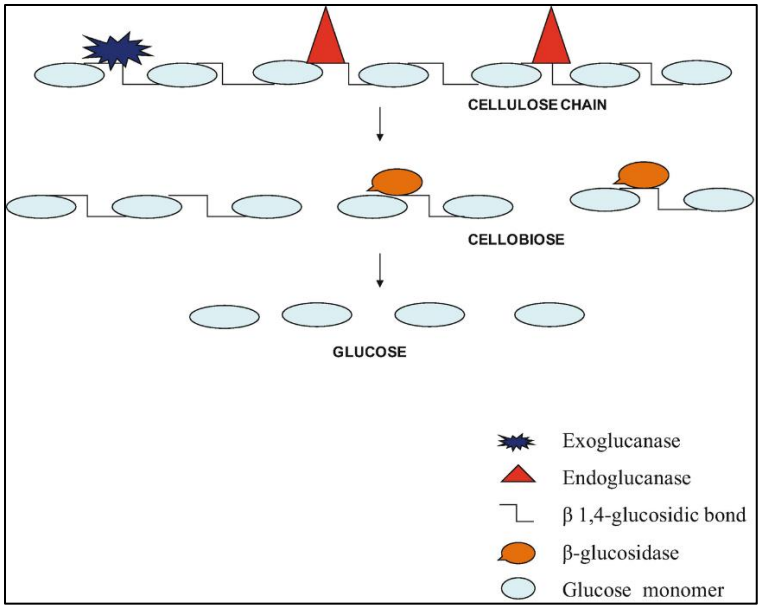

Figure 2.6: Cellulose hydrolysis by cellulase system (Mishra et al., 2019)

2.12.1.1. Cellulase Activity

Cellulase activity can be measured as total cellulase activities or by measuring three types of cellulolytic enzymes (exoglucanase, endoglucanase and β-glucosidase). For total cellulase activity, insoluble pure substrates such as Whatman No 1 filter paper, microcrystalline cellulose, cotton linter, etc. are used to evaluate the activity (Zhang et al., 2006). The most common assay used for the determination of total cellulase activity is filter paper assay (FPA) recommended by International Union of Pure and Applied Chemistry (IUPAC) (Ghose, 1987). This assay is based on fixed amount of released glucose (2.0 mg) from 50 mg filter paper within 60 min incubation in 50 °C water bath. The released glucose (reducing glucose) in this assay is determined using dinitrosalicylic acid (DNS). However, the released glucose during the hydrolysis is not correlated with the amount of added enzyme due to the use of solid heterogeneous substate (Zhang et al., 2009). For endoglucanase, it can be measured using water soluble celluloses such as carboxymethyl cellulose (CMC) and hydroxyethyl cellulose. Endoglucanase assay (CMCase) using CMC

as a substrate and DNS as reducing sugar detector is recommended by IUPAC. This assay is based on specific amount of liberated reducing glucose (0.5 mg) within 30 min water bath incubation at 50 °C. In terms of β-glucosidase activity, soluble substrate such as cellobiose, *P*-nitrophenyl-β-D-glucosides, cellodextrins, etc. are used to determine the activity. Among the different substates, using cellobiose assay is recommended by IUPAC and is based on a fixed amount of released glucose (1.0 mg) from cellobiose within 30 min water bath incubation at 50 °C. Unlike FPA, the reducing sugar is measured using HPLC or commercial kits such as glucose oxidase, glucose hexose kinase and glucose-6 phosphate dehydrogenase kits (Zhang et al., 2009). Unlike β-glucosidase and endoglucanase, exoglucanase does not have specific substrate which makes it difficult to determine its activity. This is due to the ability of exoglucanase to liberate both glucose and cellobiose from the end of cellulose chain and the presence of other cellulases that interfere during the reaction. Thus, the measurement of exoglucanase activity required a purified exoglucanase, thereby Avicel can be used as substrate (Zhang and Lynd, 2004; Zhang et al., 2009).

Chapter 3. Composition of Canola Seed Sprouts Fermented by *Aureobasidium pullulans, Neurospora crassa,* and *Trichoderma reesei* under Submerged-State Fermentation [†]

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3.1. Abstract

Seed sprouts are nutrient-rich components; and their use in human and animal diets have increased in recent times. Microbial fermentation of seed sprouts not only improves the nutrient digestibility but also elevates the nutritional characteristics by lowering the level of antinutritional factors (ANFs). This study evaluates the effect of sprouting followed by fungal fermentation on the nutritional characteristics of canola seed sprouts. Canola seeds were soaked overnight and sprouted over a 6-day period. Dried and ground seed sprouts were subjected to submerged fermentation for 5 days using three fungal strains (*Aureobasidium pullulans, Trichoderma reesei, Neurospora crassa*). Results showed an improvement in proteins and reduction in ANFs of sprouts when compared to seeds. Fermentation with *T. reesei* resulted in higher protein content, while *N. crassa* fermented sprouts exhibited slight reduction in crude fiber. Sprouting prior to fermentation helped in enhancing the protein solubility of canola seed and reducing the overall fermentation duration. Based on these findings it could be concluded that sprouting prior to fermentation can potentially lead to the production of nutritionally enriched canola based high protein ingredient. However, further research needs to be conducted to develop the mechanism for minimizing the oil loss during seed sprouting process.

3.2. Introduction

Canola is one of the major oilseed crops in the world ranking second only to soybean. It contains high levels of oil (40-45%) and protein (~25%), and large quantities of canola seeds are processed annually for oil production. For instance, ~152K tons of canola seeds alone were crushed in November 2019 with the co-production of ~85K tons of protein-rich meal (USDA, 2020). While canola oil market continues to grow rapidly due to its use in food and other industrial applications, canola meal (CM) remains as one of the underutilized agricultural commodities. Despite being high in a protein (35-45% dry basis) with excellent nutritional qualities, CM's use is only limited to livestock feed as a protein resource that too at low inclusion rate (Newkirk, 2009b). Primary factors hindering the complete utilization of CM are the presence of antinutritional factors [glucosinolates (GLS), high nondigestible fiber, and phytic acid] accompanied by low protein digestibility (Montoya and Leterme, 2010). Some of these issues have been addressed with the help of advanced technologies including, a) use of modern breeding techniques to develop canola varieties with a low level of GLS (Beszterda and Nogala-Kałucka, 2019; Rai et al., 2019) ; b) application of pretreatments (physical, chemical, biological) and fungal fermentation to improve the nutrition of CM (Croat et al., 2017). However, high fiber and low digestibility of CM proteins continue to be the challenge (Beszterda and Nogala-Kałucka, 2019; Rai et al., 2019); therefore, further research is needed to explore alternative avenues for advanced utilization of CM.

Seed sprouts (young shoots including the seed portions) are known to have intrinsic nutritional qualities as compared to their unsprouted seed counterparts, containing a high level of nutrients (essential and non-essential nutrients such as proteins, carbohydrates,

vitamins and minerals) and low levels of anti-nutritional factors (Benincasa et al., 2019; Park et al., 2019; Santos et al., 2020). Due to their desirable nutritional characteristics, sprouts have been a part of human culinary for very long time, and lately their global demand is surging upward (Abdel-Aty et al., 2019; Benincasa et al., 2019; Santos et al., 2020). Similarly, there are references of feeding sprouted grains to animals as feed ingredients to enhance overall livestock health, high birth rates, better nutrient digestibility, and palatability (Gebremedhin, 2015; Mattioli et al., 2016; Reed et al., 2005). Even though some reports exist on canola seed sprouting, the primary focus of these studies has been to understand the impact of sprouting on oil quality and characteristics (Bhardwaj and Hamama, 2009a; Barthet and Daun, 2005; Chung et al., 1989; Dawood et al., 2013). The oil characteristics of canola sprouting seed are promising with no loss in essential fatty acids, and only a slight reduction in total oil content (Bhardwaj and Hamama, 2009a; Barthet and Daun, 2005; Dawood et al., 2013). However, knowledge on canola sprout composition is scarce, and only study that examined the proximate composition and digestibility of canola seed sprouts showed no improvement in the nutritional quality of canola post- sprouting (Chung et al., 1989). Therefore, this study was designed to assess the composition of canola seed sprouts and to apply fungal fermentation to upgrade the nutritional qualities of canola seed sprouts.

Microbial fermentation (bacteria and/or fungi) on various types of cereal sprouts is found to be beneficial in improving the overall nutritional composition. For example, Khetarpaul and Chauhan (1990b) showed a significant improvement in starch and protein digestibility during co-culture fermentation of the pearl millet sprouts using yeasts and bacteria. Similarly, fermentation of red cabbage sprouts by lactic acid bacteria

(*Lactobacillus plantarum* ATCC 8014, *Lactobacillus acidophilus* NCFM, Danisco, *and Lactobacillus rhamnosus* GG E522 (ATCC 53103) led to a significant increase of antioxidant functionalities compared with those of their unfermented counterparts (Hunaefi et al., 2013). Likewise, three GRAS (generally regarded as safe) approved fungal strains [*Aureobasidium pullulans* (Y-2311-1), *Neurospora crassa*, and *Trichoderma reesei*] have been used to enhance oilseed meal utilization: including canola meal, sorghum hominy, and soybean meal (Croat et al., 2016b; Zahler et al., 2018; Baldwin et al., 2019).

Overall, rising consumer interest in seed sprouts for healthy living, potential health-benefits to sprout-fed livestock, with no adverse effects on canola oil quality, and the anticipated promising outcomes post-fermentation of sprouts led to the plan of this study. Our goal was to determine the effects of seed sprouting followed by fungal fermentation on the nutritional composition of canola seed sprouts. The major nutritional composition factors including crude protein, fibers (crude, acid detergent, neutral detergent), phytic acid, total soluble sugars, and GLS content were determined before and after fermentation. Results from this study should be helpful in creating the new market opportunities for canola as a novel food and feed ingredient.

3.3. Materials and Methods

3.3.1. Canola Seed Sprouting

Canola seeds were obtained from ADM/Specialty Commodities LLC (Fargo, ND). Five hundred grams of canola seeds were soaked in tap water at room temperature (~22 °C). After 24 h of soaking, seeds were transferred into plastic Tupperware and covered partially with semi-transparent lids to ensure adequate air ventilation. The Tupperware were sprayed once daily with water to keep seeds moist during the sprouting period (6

days). At the end of the sprouting, the sprouts were dried in an oven at 60 °C for 48 h, and total weight before and after drying was recorded. Dry seed sprouts were milled into fine powder using a coffee grinder (Krups, Solingen, Germany) and were stored at room temperature in plastic bags.

3.3.2. Cultures, Maintenance, and Inoculum Preparation

Three microbial strains (*Aureobasidium pullulans* (Y-2311-1), *Neurospora crassa*, and *Trichoderma reesei*) were acquired from the National Center for Agricultural Utilization Research (Peoria, IL). The potato dextrose agar (PDA) stock cultures of each strain were stored in the refrigerator and sub-cultured at monthly intervals. One hundred mL working volume of a glucose yeast extract (GYE) medium comprised of 5 % glucose and 0.5 % of yeast extract with optimal pH for each microbe were prepared in 250 mL Erlenmeyer flasks. After autoclaving the media, an 8 mm microbial colony plug of agar growth was transferred into GYE media, then covered with a foam plug and aluminum foil. The flasks were then incubated for 48 h at 30 °C in rotary shaker at 150 rpm.

3.3.3. Submerged Fungal Fermentation

Erlenmeyer flasks (250 mL) with a working volume of 100 mL at a 10% solid loading rate (SLR) of dry weight canola seed sprouts were prepared. The pH of the media was adjusted to the optimal level for each microbe, for instance *A. pullulans* was adjusted to 3.0 ± 0.1 with 5 M sulfuric acid, whereas *T. reesei* and *N. crassa* pH was adjusted to the range of 5.0 - 5.5. Flasks were foam plugged and covered with aluminum foil during autoclave sterilization (121 °C for 20 min). The flasks were then inoculated with 1 mL of a 48-h culture of *A. pullulans*, *N. crassa*, and *T. reesei* separately. Flasks were incubated at 30 °C at 150 rpm for 120 h. Three replicates were daily collected, pH was monitored, and

sample was centrifuged at 10,000 rpm for 10 min to separate solid and liquid fractions. Liquid sample (15 mL) was used for determining the total soluble sugar content, while solid fraction was oven dried at 80 °C for 24 h. The dried sample was then ground into a fine powder using coffee grinder and used for further analysis of protein, fiber, phytic acid, and GLS composition.

3.3.4. Analytical Assays

3.3.4.1. Moisture Content

The moisture content of the sample was determined by drying approximately 2 g of samples in a hot air oven set at 80 °C for 24 h.

3.3.4.2. Crude Proteins

Approximately ~0.2500 g of dried and finely ground sample was used for protein analysis. Samples were uploaded onto a LECO model FP528 (St. Joseph, MI, USA) and the total nitrogen content measured (AOAC Method 990.03) (Horwitz and Latimer, 2006) and a conversion factor of 6.25 was used to determine crude protein content (%).

3.3.4.3. Total Glucosinolate (GLS) Content

Total GLS content of different samples were measured according to the method of (Berhow et al., 2013). This method is a modified HPLC method developed by Betz and Fox (1994), and using reverse phase HPLC with UV detection to quantify the GLS content. The extract was run on a Shimadzu (Columbia, MD) HPLC System (two LC 20AD pumps; SIL 20A autoinjector; DGU 20As degasser; SPD-20A UV-VIS detector; and a CBM-20A communication BUS module) running under the Shimadzu LC solutions Version 1.25 software. The column was a C18 Inertsil reverse phase column (250 mm X 4.6 mm; RP C-18, ODS-3, 5u; GL Sciences, Torrance, CA). The GLS were detected by monitoring at 237

nm. The initial mobile phase conditions were 12% methanol/88% aqueous 0.005 M tetrabutylammonium bisulfate (TBS) at a flow rate of 1 mL.min-1. After injection of 15 µl of sample, the initial conditions were held for 2 min, and then up to 35% methanol over another 20 min, then to 50% methanol over another 20 min. then up to 100% methanol over another 10 min. Standards, prepared in this lab or purchased, were run to determine retention times and extinction coefficients. The presence of glucosinolates were confirmed by negative ion mode analysis on an Thermo Electron LTQ Oribitrap Discovery Mass Spectrometer (a linear ion trap MS, coupled to a high precision electrostatic ion trap MS) with an electrospray ionization (ESI) source, and a Thermo Scientific ACCELA series HPLC system, all running under Thermo Scientific Xcalibur 2.1.0.1140 LC-MS software.

3.3.4.4. Phytic Acid Content

Phytic acid was determined according to the Megazyme Phytic acid/Total phosphorus kit (Megazyme, 2017). One-gram sample was mixed with 20 mL HCl of 0.66 M concentration in a 50 mL glass container. Flasks were covered with foil and stirred vigorously in a shaker (SCILOGEX MS-M-S10) for overnight at room temperature (~22 °C). One mL of the extract was then transferred into a 1.5 mL microtube and centrifuged at 13,000 rpm. for 10 min. The extract supernatant (0.5 mL) was transferred to a new tube and neutralized with 0.5 mL of NaOH. Controls were run with oat flour powder. The kit contains prepared phytase and alkaline phosphatase along with the buffers for the reaction. The reaction is conducted at 40 ℃ with phytase for 10 min and alkaline phosphatase for 15 min. The enzyme reaction is stopped by the addition of trichloroacetic acid 50% w/v. Phosphorus is quantified using a colorimetric determination. Ascorbic acid/sulfuric acid and ammonium molybdate mixed in a 5:1 ratio creates the color reagent. One ml of the

sample is mixed with the color reagent and incubated for 1 hour at 40 ℃. Reactions are read on a microplate reader at 655 nm. Replicates for each sample were run individually.

3.3.4.5. Fiber

Fiber analysis [crude fiber (CF), neutral detergent fiber (NDF), and acid detergent fiber (ADF)] was conducted by Agricultural Experiment Station Chemical Laboratories at the University of Missouri-Columbia.

3.3.4.6. Total Soluble sugars

Total soluble sugars of raw canola seeds, and seed sprouts were determined according to the previously described method of (Croat et al., 2016b). Briefly, ~1 g of dried sample was mixed with 9 mL of deionized water and stored at 4 °C. After 24 h, slurry was separated into solid and liquid fraction by centrifuging at 10,000 rpm for 10 min. Solid fraction was discarded and supernatant was poured into a 2-mL microcentrifuge tube and frozen overnight. Thawed sample was centrifuged a second time at 10,000 rpm for 10 min to remove any particulates, and the liquid fraction was syringe filtered through a 0.22-µm filter and into a high-performance liquid chromatography (HPLC) vial. Liquid samples from submerged fermentation were processed by boiling for 10 min to inactivate the fungal cultures. Samples were then centrifuged at 10,000 g for 10 min and afterwards process was repeated exactly as described above for raw seed and seed sprouts. The processed samples were then eluted using mobile phase (50 ppm EDTA in deionized water) at a flow rate of 0.5 mL/min and column temperature of 85 °C in a HPLC system (Agilent Technologies, Santa Calara, CA, USA) equipped with a refractive index detector (Model G1362A) and a waters size-exclusion chromatography column (SugarPak column I with pre-column module, Waters Corporation, Milford, MA, USA). Different concentrations of sugars

(stachyose, raffinose, glucose, galactose, galactose-mannose, and arabinose at purity level of 99.9%) were prepared to construct the calibration curve of concentration vs. HPLC area as previously established by (Karunanithy et al., 2012).

3.3.5. Statistical Analysis

In this study, the effects of different fungal strains on total protein level, crude fiber, ADF, NDF, total GLS, and phytic acid contents were determined using a combination of Microsoft® Excel® for Office 365 MSO 64-bit and R version 1.2.5001 (Boston, MA). The statistically significant differences among and within group variances determined by one-way ANOVA at $p \leq 0.05$ level of significance.

3.4. Results and Discussion

3.4.1. Proximate Composition of Canola Seed and Seed Sprouts

Daily observations were made throughout the sprouting period of 6 days. The early emergence of the root and shoot was noted. The soaked canola seeds started to germinate within 24 h of sprouting period. This observation contrasted with that of previous findings of Chung et al. (1989) who reported the emergence of root and shoot between the third and fourth days of sprouting. Presoaking of canola seeds in water (~12 h) likely accelerated the sprouting process as compared to the dry seeds used by Chung et al. (1989).

The proximate analysis results of canola seed and seed sprout indicated that significant change occurred in the composition of seed during sprouting (Table 3.1). The increase in moisture content of soaked and germinated canola seeds (79.52%, db) compared with the dry seeds (6.5%, db) was due to the high-water uptake by the cells during germination (Nonogaki et al., 2010).The rate of water uptake during sprouting varies depending on the seed types; however, an increase in moisture during sprouting has

been consistently reported on many seed types including cowpea (Devi et al., 2015), soybeans (Murugkar, 2014), and Mungbean (Shah et al., 2011). The total oil content of the seed sprouts (25.6%, db) was reduced significantly compared to that of unsprouted seeds (40.3%, db) (Table 3.1). Similar results were reported in previous studies on canola seed (Chung et al., 1989; Barthet and Daun, 2005; Dawood et al., 2013). Dawood et al. (2013) reported a reduction in oil content of eight varieties of canola seeds as well as a decline in fatty acid composition of two canola varieties due to soaking and sprouting. Depletion in the oil content of oilseeds is mostly attributed to the energy production by catabolic activities of the seeds during sprouting process (Kornberg and Beevers, 1957; Mostafa et al., 1987; Onimawo and Asugo, 2004).

Similar to the results of this study, an increase in protein content during sprouting period has been shown in a wide range of seeds (Kornberg and Beevers, 1957; Mostafa et al., 1987; Onimawo and Asugo, 2004) including canola seeds (Barthet and Daun, 2005). An improvement in protein content of the seeds during sprouting has been partially attributed to the synthesis of enzyme proteases and/or due to the overall compositional change from the formation of new cell organelles during germination (Stoddart et al., 1973; Simon, 1984; Bau et al., 1997; Megat Rusydi et al., 2011).

We found no significant change in the fiber content (CF, ADF, NDF) of seed sprouts as compared to unsprouted seeds. These results corroborated the previous findings of Chung et al. (1989) reported no change in fiber content of barley seed sprouts as compared to unsprouted seed counterparts. Likewise, Uppal and Bains (2012) demonstrated an increase in crude fiber content of cowpea by 20 to 24% after sprouting. These differences in fiber content of the seed sprouts may be explained by the fact that

change in fiber content of the seed sprouts also vary significantly between the seed grain types (chickpeas, beans, oilseeds, etc.) and the germination conditions (sprouting time, temperature, etc.).

Sprouting is reported to be highly effective process for reducing the phytic acid level in wide range of seed grains such as rice, maize, millet, sorghum, wheat (Azeke et al., 2011), and pulses (Patterson et al., 2017). Our study also revealed a significant reduction in the phytic acid content (64.7%) of the canola seed sprouts as compared to unsprouted seeds. These results are in close agreement with those of Fouad and Rehab (2015), who reported the reduction of the phytate content of lentils by 73.76% on the 6th day of sprouting. It is well documented in the literature that plant seed uses phytate as a source of the inorganic phosphate and therefore phytase activity tends to increase during the sprouting period leading to the reduction in the level of phytic acid content (Azeke et al., 2011; Kumar et al., 2010; Ou et al., 2011; Sung et al., 2005).

The regulatory guidelines for total glucosinolate content require GLS levels to below 30 µmol/g for rape seed (*Brassica rapus*) to be labeled as "canola" (Canola Standards and Regulations, 2005). Total GLS content of canola seed in this study is relatively lower than the mostly reported GLS contents of canola seeds. For example, Mailer et al. (2008b) found GLS levels ranging from 9 to 169 µmol/g in different cultivars of *Brassica napus.* Several factors such as cultivar types, maturity level can influence the levels of the GLS content in the seeds (Mailer et al., 2008b; Vale et al., 2015; Elahi et al., 2016). Though there are not enough reports available on the GLS content of canola seed sprouts, according to literature, *Brassicaceae* family is known to synthesize GLS as a secondary metabolite product during the sprouting period (Elahi et al., 2016), hence an

increase in GLS post- sprouting would be anticipated. A study by Vale et al. (2015) found an increase in GLS content of the four different types of the *Brassica* sprouts (red cabbage, broccoli, galega kale, penca cabbage) grown at different photoperiod conditions. The total GLS content of the canola seed remained unaltered post-6 days of sprouting (1.30 vs1.65 µmol/g). However, total GLS followed an upward trend post-sprouting.

Mono- and oligosaccharides such as glucose, stachyose and raffinose was measured in the seed and seed sprouts (Table 3.1). There was no significant change in the total sugar concentration (sum of sugars) of seed sprouts when compared to the seed. However, sugar composition of seed sprouts showed significantly high level of raffinose and low level of stachyose, and glucose as compared to that of seed. Sugar composition of the seed sprout can vary significantly depending on the seed type (Barthet and Daun, 2005). For instance, decrease in sucrose level was reported for the cottonseed and soybeans during germination while mung beans germination led to a sucrose increase (Kuo et al., 1988).

Table 3.1: Proximate composition of canola seed and canola seed sprouts

Analysis, dry basis	**Seed**	**Seed sprouts (6 d)**
Dry matter, %	93.5±0.02[a]	20.48±1.5[b]
Oil content, %	40.6±0.3[a]	25.6±1.7[b]
Crude protein, %	25.7±0.12[a]	29.9±1.7[b]
Crude fiber, %	12.1±0.13[a]	11.1±0.65[a]
Acid detergent fiber, %	18.7±0.67[a]	16.6±0.53[b]
Neutral detergent fiber, %	21.9±0.86[a]	20.8±0.54[a]
Phytic acid, g/100g	1.7±0.04[a]	0.6±0.08[b]
GLS, µmol/g	1.3±0.26[a]	1.65±0.15[a]
Total soluble sugars, mg/mL	4.29±0.02[a]	4.05±0.16[a]
Stachyose, mg/mL	1.29±0.01[a]	0.08±0.07[b]
Raffinose, mg/mL	1.23±0.03[b]	2.56±0.36[a]
Glucose, mg/mL	1.77±0.02[a]	1.41±0.17[b]
Ash, %	3.8±0.12[a]	7.6±0.03[b]

Mean ± standard deviation, significant differences ($p \leq 0.05$) among seed and seed sprouts for each analysis is indicated by letter superscripts.

3.4.2. Characteristics of Fermented Canola Seed Sprouts

Canola seed sprouts were subjected to submerged fungal fermentation using three different fungal strains. Submerged fermentation is well-adapted commercial technology for processing of various types of feedstocks. This process uses large volumes of water to achieve homogenous mixing and high efficiency output, but an unintended side-effect of this process is the possible loss of soluble proteins in the liquid fraction due to degradation of peptides during microbial enzymatic activity. Hence, post-fermentation of seed sprouts, the fermented slurry was separated into a solid and liquid fraction by using centrifugation separation. Solids were further analyzed for total proteins, fibers, phytic acid, and GLS content, whereas liquid fraction was evaluated for its total protein content. Results from these flask trials are presented in the section below.

3.4.2.1. Crude Protein Content

Table 3.2 and 3.3 show the protein content of uninoculated control and fermented seed sprouts on solid, and liquid fractions, respectively. There was no change in the percent solid recovery of uninoculated controls, and fermented seed sprouts with the resulting average total solid recoveries of ~71 and ~22%, respectively for solid and liquid fractions (Table 3.2 and 3.3). Though there was no significant change in the amounts of solid recoveries during several days of incubation, a decreasing trend was observed with solid and liquid fractions of *N. crassa* and *T. reesei* fermented sprouts, respectively.

The protein content of solid fraction of uninoculated control (28.1%, Table 3.2) at 0 h was lower than the seed sprouts (29.9%, Table 3.1), while high protein content were present in the liquid fractions (41.1%, Table 3.3) at the beginning of the incubation. Our results indicate that sprouting results in the breakdown of high molecular weight proteins

into soluble short-chain peptides/and free amino nitrogen with more polar groups. It is also well documented in the literature that protein solubility is improved during germination of lentils and cereal grains (Chung et al., 1989; Ghumman et al., 2016). The protein data of the solid fraction showed that fermentation of seed sprouts with *T. reesei* improved the protein content to the highest level (31.5%) followed by *N. crassa* (29.5%) (Table 3.2). *T. reesei* is widely known for its ability to produce cellulase enzymes, hence it was anticipated to exhibit high protein content due to conversion of cellular carbohydrates into cell protein mass (Croat et al., 2016b; El-Shishtawy et al., 2015; Singhania et al., 2013; Zahler et al., 2018). However, *A. pullulans* exhibited the lowest protein levels throughout the incubation period. Our observation that the protein content of *A. pullulan* fermented sprouts was lower than the uninoculated control suggesting further hydrolysis of proteins into shorter-chain peptides occurred during *A. pullulan* fermentation, which ultimately led to an increase in protein content in the liquid fraction (up to 44%) (Table 3.3). *A. pullulan* is known to produce proteases resulting in the protein solubilization. Similar results have been previously reported by Zahler et al. (2018) on sorghum hominy. Extended periods of fermentation did not have significant impact on overall protein content. For example, there was no significant increase in protein content of solid fractions after 72 h of incubation, however, protein solubility continued to increase as fermentation duration was extended beyond 72 h for *A. pullulans* and *N. crassa* with the exception of *T. reesei* (Table 3.3). Interestingly, the protein content of *T. reesei* fermented sprouts in the liquid fractions started to decrease significantly after 48 h of incubation (Table 3.3). A study by Zahler et al. (2018) also reported a decline in protein content of liquid fraction after 72 h fermentation of sorghum hominy with *T. reesei*. Achieving the maximum protein content in shortest

possible time with the maximum retention of proteins in the solid fraction is economically viable. Seed sprouting prior to fermentation can potentially benefit the overall efficiency of the fermentation process.

Table 3.2: Protein content and total solid recovery data of fermented solid fractions of canola seed sprouts

Sample	Microbes	Protein content in solid fraction (%, dry basis)					
		Fungal fermentation (h)					
		0	24	48	72	96	120
Canola seed sprouts	Control	28.1±0.39 $^{a/A}$ (7.14)	28.7±0.31 $^{a/AB}$ (7.08)	28.5±0.28 $^{a/A}$ (7.22)	28.4±0.05 $^{a/C}$ (6.87)	28.3±0.45 $^{a/C}$ (6.82)	28.2±0.24 $^{a/B}$ (6.93)
	A. pullulans	27.1±0.14 $^{c/B}$ (8.13)	27.3±0.14 $^{bc/C}$ (7.01)	27.7±0.25 $^{ab/A}$ (7.23)	27.7±0.12 $^{ab/D}$ (6.66)	27.8±0.11 $^{ab/C}$ (6.94)	28.1±0.37 $^{a/B}$ (6.73)
	T. reesei	26.4±0.27 $^{b/B}$ (7.18)	29.2±0.24 $^{a/A}$ (7.05)	29.5±1.46 $^{a/A}$ (7.24)	30.6±0.18 $^{a/A}$ (7.67)	31.4±0.13 $^{a/A}$ (7.48)	31.5±0.24 $^{a/A}$ (7.08)
	N. crassa	27.3±0.49 $^{b/AB}$ (7.11)	28.6±0.23 $^{ab/B}$ (6.92)	29.0±0.45 $^{ab/A}$ (6.67)	29.6±0.18 $^{a/B}$ (6.51)	29.8±0.33 $^{a/B}$ (6.38)	29.5±1.33 $^{a/B}$ (6.18)

Mean ± standard deviation. Three replicates. Uppercase letters signify significance among groups for each incubation time at ($p \leq 0.05$). Lowercase letters signify significance among incubation time for each fungus at ($p \leq 0.05$). Control is uninoculated. Solid recovery data (g) is presented in parenthesis for every fermentation hour.

Table 3.3: Protein content and total solid recovery data of fermented liquid fractions of canola seed sprouts

Sample	Microbes	Protein content in liquid fraction (%, dry basis)					
		Fungal fermentation (h)					
		0	24	48	72	96	120
Canola seed sprouts	Control	41.1±0.96 $^{a/A}$ (2.34)	38.7±0.13 $^{ab/A}$ (2.35)	38.2±0.79 $^{b/B}$ (2.20)	37.8±0.70 $^{b/BC}$ (2.49)	39.0±0.23 $^{ab/C}$ (2.57)	37.7±0.41 $^{b/AB}$ (2.44)
	A. pullulans	36.0±0.64 $^{d/B}$ (1.52)	36.9±0.45 $^{cd/B}$ (2.35)	39.6±0.11 $^{bc/A}$ (2.18)	41.6±1.33 $^{ab/A}$ (2.14)	44.0±0.30 $^{a/A}$ (2.01)	42.9±1.14 $^{a/A}$ (2.18)
	T. reesei	39.7±0.86 $^{a/A}$ (2.31)	38.5±0.16 $^{ab/A}$ (1.39)	37.3±0.18 $^{bc/B}$ (1.34)	36.3±0.55 $^{cd/C}$ (1.14)	34.7±0.23 $^{d/D}$ (1.22)	34.9±0.86 $^{d/B}$ (1.55)
	N. crassa	41.5±1.04 $^{a/A}$ (2.28)	39.1±0.55 $^{a/A}$ (2.32)	39.6±0.35 $^{a/A}$ (2.32)	40.0±0.67 $^{a/AB}$ (2.36)	40.3±0.41 $^{a/B}$ (2.34)	42.9±3.84 $^{a/A}$ (2.05)

Mean ± standard deviation. Three replicates. Uppercase letters signify significance among groups for each incubation time at ($p \leq 0.05$). Lowercase letters signify significance among incubation time for each fungus at ($p \leq 0.05$). Control is uninoculated. Solid recovery data (g) is presented in parenthesis for every fermentation hour.

3.4.2.2. Fiber Content (Crude, ADF, NDF)

The mixing of solid substrates with sufficient water followed by autoclaving are the two major preprocessing steps during submerged fermentation. At the end of the fermentation, centrifugation-based separation step is applied, which removes the water and concentrates insoluble fractions like fibers (CF, ADF, NDF). Therefore, an increase in fiber contents of the uninoculated control (Figure 3.1a) as compared to the seed sprouts (Table 3.1) may be attributed to a "concentration effect". Increase in fermentation duration did not have any effect on the fiber contents of uninoculated control (Figure 3.1a). As seen in the Figures (3.1b, 3.1c, 3.1d), fungal fermentation of seed sprouts resulted in high concentrations of fibers (CF, ADF, NDF) as compared to seed sprouts. The minimal fiber hydrolysis during fungal fermentation has been reported with canola meals (hexane extracted and cold pressed) and sorghum hominy (Croat et al., 2016b; Zahler et al., 2018). Production of fiber degrading enzymes at low rates due to the presence of high level of mono-, di-, and oligo-saccharides could be the one possible reason for the lack of sufficient fiber degradation (Sukumaran et al., 2005). Total fiber amount was calculated by dividing the final dry weight following incubation (see Table 3.2) by the initial dry weight (10 g) and multiplying by fiber concentrations. Results show that total amount of fibers in the fermented product varied depending on the fungal strains used. The total amount of fiber of *A. pullulans* fermented sprout were similar to that of uninoculated control. While during *T. reesei* fermentation, total fiber amount remained same as control for 96 h but towards the end of incubation (120 h) total fiber amounts were found to be slightly higher than uninoculated control. The CF content of *N. crassa* fermented sprouts was slightly reduced at 120 h as compared to that of uninoculated control but ADF and NDF amount remained

unaffected, in contrast to the results shown in *A. pullulans* and *T. reesei* fermented sprouts. *N. crassa* is known to grow on varieties of substrate and produce cellulase and hemicellulase, which could be one reason there was some level of fiber reduction achieved with this strain (Xiros et al., 2008).

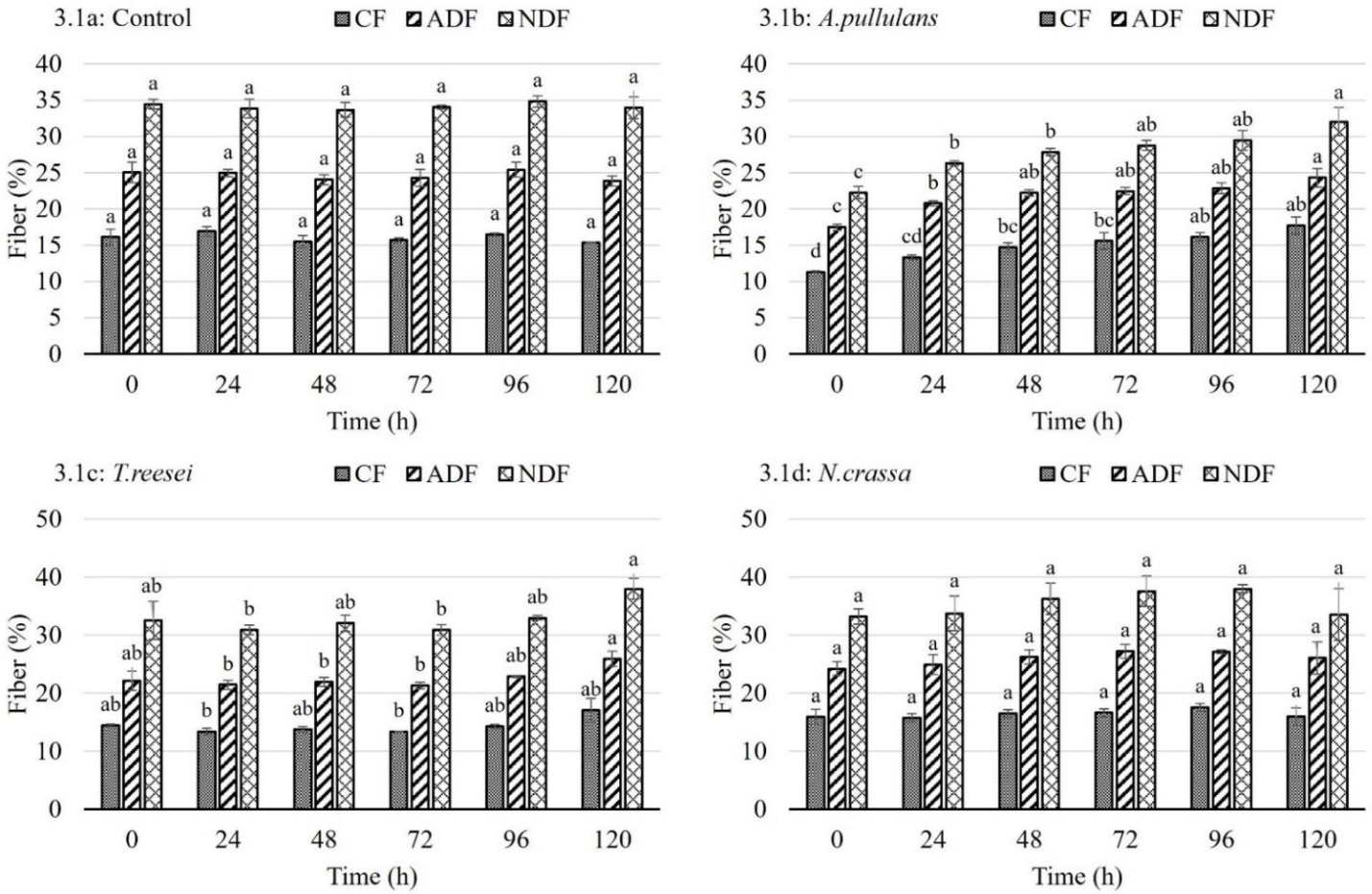

Figure 3.1: Crude fiber, ADF and NDF content of uninoculated control and fermented samples; 3.1a: uninoculated control; 3.1b: *A. pullulans*; 3.1c: *T. reesei*; 3.1d: *N. crassa.*

3.4.2.3. Phytic Acid Content

Phytic acid content as determined for uninoculated control and 120 h fermented sprouts are presented in Figure 3.2. Due to the activation of phytase enzyme during sprouting significantly low level of phytic acid was present in the seed sprouts (Table 3.1). Which was further reduced to 0.25 (uninoculated control) from 0.63 g/100g (seed sprouts) post-heat sterilization and 120 h of incubation. Heat treatment is known to cause loss in total phytate. According to Reale et al. (2007) a total phytate loss occurred when heat applied to cereal flours prior to lactic acid fermentation. Among the three fungal strains that were tested *A. pullulans* exhibited the highest reduction in phytic acid 0.23 g/100g, whereas *T. reesei* did not show any change. *N. crassa* led to an insignificant increase on total phytic acid 0.35 g/100 g. Reduction of phytic acid depends on microbial abilities to produce phytase.

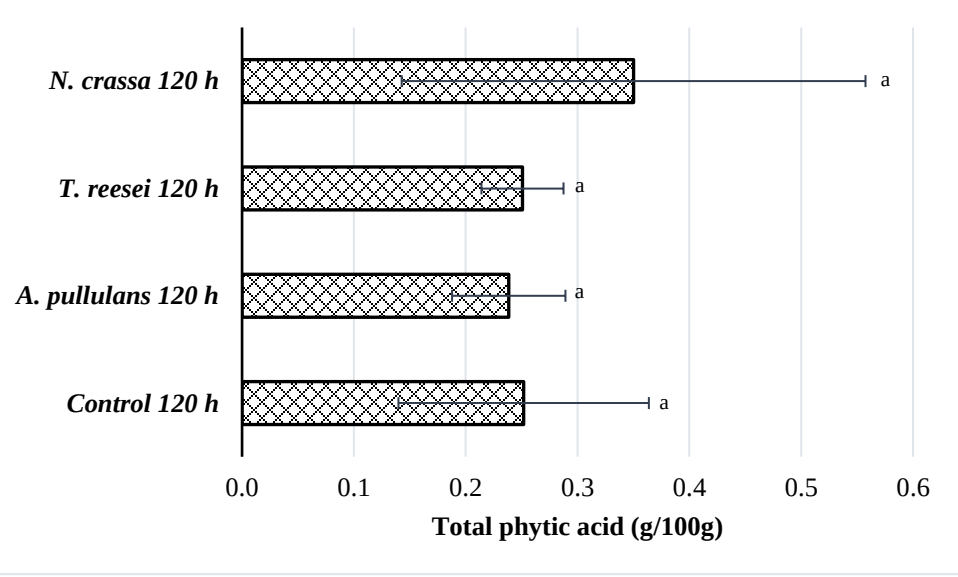

Figure 3.2: Phytic acid content of uninoculated control and fermented samples at 120 h of incubation.

3.4.2.4. Total Glucosinolate Content (GLS)

GLS content of the canola seed sprouts decreased significantly from 1.65 µmol/g (Table 3.1) to less than 0.5 µmol/g after heat sterilization and oven drying (Table 3.4). Loss of GLS content due to the breakdown of GLS into a volatile component during heat treatment is well documented (Halkier and Gershenzon, 2006; Croat et al., 2016b). Clearly there was some degradation in GLS content of seed sprouts post-fermentation with three different fungal strains, *A. pullulans* exhibiting the maximal reduction in 120 h. However, due to the presence of very low level of GLS in the starting material, there was no significant different among fungal strains. Similarly, fungal fermentation time did not have any significant impact on the residual GLS content of the fermented sprouts. Previous studies have shown that various microbes, including *T. reesei, A. pullulans, N. crassa*, are able to degrade GLS content of oilseed meals (Croat et al., 2016b; Simon et al., 2017)

Table 3.4: Total glucosinolate content of fermented canola seed sprouts

Sample	Microbes	Total glucosinolates (µmol/g), dry basis					
		Fungal fermentation (h)					
		0	24	48	72	96	120
Canola seed sprouts	Control	0.56±0.02 a/B	0.66±0.06 a/AB	0.70±0.06 a/A	0.58±0.04 a/AB	0.47±0.09 a/AB	0.59±0.02 a/A
	A. pullulans	0.82±0.06 a/A	0.39±0.05 b/C	0.35±0.06 b/B	0.35±0.18 b/B	0.27±0.02 b/B	0.23±0.07 b/A
	T. reesei	0.52±0.02 a/B	0.81±0.07 a/A	0.69±0.09 a/A	0.77±0.02 a/A	0.79±0.33 a/A	0.43±0.16 a/A
	N. crassa	0.45±0.06 a/B	0.56±0.03 a/B	0.62±0.02 a/A	0.61±0.05 a/AB	0.53±0.10 a/AB	1.09±0.70 a/A

Mean ± standard deviation. Three replicates. Uppercase letters signify significance among groups for each incubation time at ($p \leq 0.05$). Lowercase letters signify significance among incubation time for each fungus at ($p \leq 0.05$). Control is uninoculated.

3.4.2.5. Total Soluble Sugars

Total soluble sugars (sum of mono- and oligo- saccharides) content as determined for uninoculated control and three fungal strains at different time periods of fermentation are shown in (Figure 3.3). As expected, total soluble sugar content of uninoculated control was consistent throughout the incubation period (0 to 120 h). Total soluble sugars for all fungal treated samples showed a declining pattern over time. Which could be attributed to the fungal utilization of carbohydrate as energy source (Olukomaiya et al., 2020a). Among all the strains tested, no detectable amounts of sugars were present by the 72 h of fermentation for *N. crassa*, while significant reduction in total soluble sugar contents of *A. pullulans* (96.6%) and *T. reesei* (75.9%) was observed by 120 h of incubation as compared to 0 h. Our results were consistent with previous results by Croat et al. (2017), who reported total soluble sugar reduction ranged from 88.5-94.6% in submerged fermented hexane extracted canola meal using *Fusarium venenatum*, *A. pullulans*, and *T. reesei*.

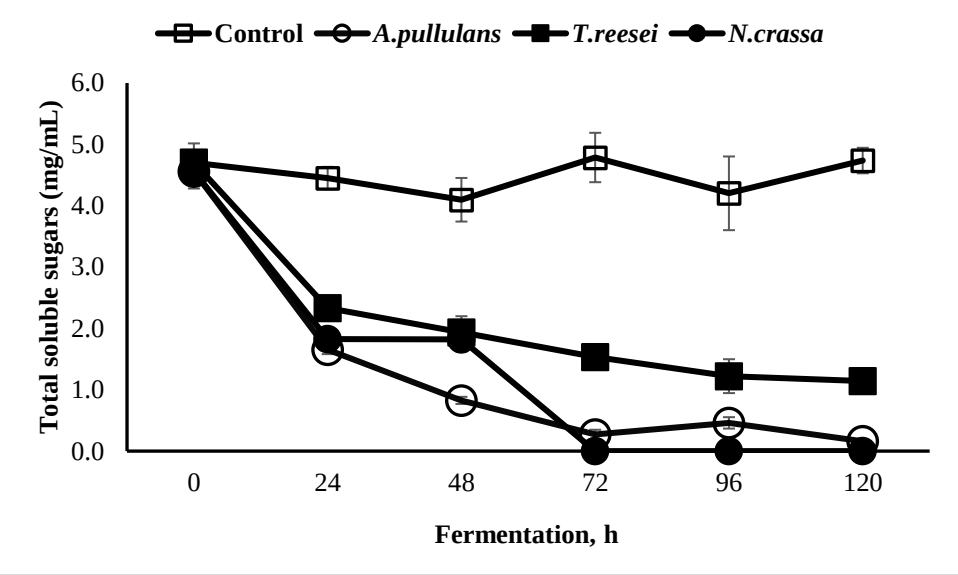

Figure 3.3: Total soluble sugar content of uninoculated control and fermented samples at different time periods of fermentation.

3.5. Conclusion

The experiments presented in this study were designed to determine the proximate composition of canola seed sprouts, and to explore the effects of fungal fermentation of canola sprouts to further improve its nutritional characteristics. Results showed that after 6 days of sprouting, there was a significant loss in the total oil content and while total protein content was improved. The other nutritional characteristics of sprout were found to be enhanced by the reduction of phytic acid and fiber content, and only slight increase in GLS content. Fungal fermentation of seed sprouts further improved the measured nutritional characteristics as total fiber amount was reduced with *N. crassa* and the total protein content was increased with *T. reesei* fermented sprouts. Interestingly, we noticed that fermentation process with sprouts tend to be more robust than other oilseed meals suggesting overall high process efficiency. These results demonstrated that canola seed sprouting when combined with the fungal fermentation, resulted in generally improved nutritional characteristics of canola as a feed ingredient with a relatively short fermentation period. However, process parameters such as sprouting conditions (days, moisture, light, etc.) and fermentation mechanism need to be fully optimized to understand the full potential of these processes. Additional research is currently underway to determine the nutritional characteristics of defatted canola seed sprouts. This line of research on improving the nutritional quality of canola as a feed ingredient could lead to the further enhancement and diversification of canola market.

Chapter 4. Daily Development of Nutritional Composition of Canola Sprouts Followed by Solid-State Fungal Fermentation [†]

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4.1. Abstract

Sprouting is a beneficial way to increase the nutritional value of the original seeds. Besides, fungal fermentation of sprouts can further improve sprouts composition by reducing antinutritional factors and concentrating protein content. Thus, this study characterized the daily nutritional changes in canola sprouts and further evaluated the effect of fungal fermentation on 144 h sprouts under solid state fermentation conditions. Sprouting process resulted in high moisture containing sprouts (75.3%) due to water uptake by seeds. The oil content of sprouts (27.2% at 144 h) was significantly ($p \leq 0.05$) reduced when compared to raw seeds (39.6%). Likewise, phytic acid, crude fiber, acid detergent fiber, and neutral detergent fibers were reduced by 49.7, 32.8, 19.7, and 16.6%, respectively when compared to raw seeds. There was significant increase in protein and total soluble sugar contents of sprouts, and glucosinolates also increased from 1.3 to 3.5 μmol/g post sprouting. Fungal fermentation with *Neurospora crassa* resulted in the highest protein increase (32.8%). Heat-sterilization reduced glucosinolates by 38.8% and a further reduction (4.0%) was obtained by fermentation with *Trichoderma reesei.* A reduction in phytic acid content of 81.4, 45.8 and 10.2% was achieved by fermentation with *N. crassa*, *T. reesei*, and *Aureobasidium pullulans,* respectively. Total soluble sugars were reduced by 3.3 mg/mL post heat-sterilization, and fungal fermentation led to the further reduction

of total soluble sugars, but total fibers were found to be concentrated post fermentation. These results highlight the enhancement of nutritional values of sprouted seeds and further fermented sprouts compared to ungerminated seeds and unfermented sprouts, respectively.

4.2. Introduction

Rapeseed contributes to 13% of the global supply of food grade vegetable oil (Raymer, 2002) and is ranked as the second largest cultivated oilseed crop in the world (Östbring et al., 2020). Canola seed is a genetically modified breed of Canadian rapeseed, developed by plant breeding techniques with an oil fatty acid composition of <2% erucic acid and defatted seed meal that contains <30 μmol/g glucosinolates. When canola is processed for oil extraction, a large quantity of defatted meal is generated as a co-product. The meal constitutes high level of protein [34.5 to 38.4% (wet basis) depending on seed varieties (*B. napus, B. rapa, B. juncea*)] (Tan et al., 2011b) and has a well-balanced amino acid profile and a high protein efficiency ratio (3.29) (Khattab and Arntfield, 2009). Although canola meal protein composition is desirable for animal feed applications (Mailer et al., 2008a) or human nutrition (Khattab and Arntfield, 2009), it still remains underutilized. The presence of antinutritional factors (ANFs) such as (glucosinolates, phytic acid and high fiber content) that lower the digestible energy content (Khajali and Slominski, 2012) is one of the major factors limiting its use.

Sprouting and fermentation processes are known to improve the nutritional characteristics of food as compared to their unsprouted and unfermented counterparts (Nkhata et al., 2018). Sprouting is the method of soaking and incubating seeds under optimal growth conditions (temperature, light, etc.) until they germinate and begin to grow. The activation of endogenous enzymes during sprouting leads to the degradation of antinutritional factors. Some of the examples could include, phytic acid reduction due to phytase activity, and an increase in protein digestibility due to proteases (Ghavidel and Prakash, 2007; Onyango et al., 2013; Rasane et al., 2015). Fermentation is a process where

microorganisms enzymatically alter the food matrix (Karovičová and Kohajdova, 2007) resulting in enhanced bio-accessibility and bio-availability of nutrients (Hotz and Gibson, 2007). Several studies have shown further improvement in sprout composition when sprouts were used as substrates for microbial fermentations. Maejima et al. (2011) observed an increase in antioxidant activity of fermented buckwheat sprouts. Hunaefi et al. (2013) reported that fermenting red cabbage sprouts using three types of lactic acid bacteria increased antioxidant activity and decreased the total phenolic compounds, anthocyanins, and L-ascorbic acid.

The present study was designed and conducted with the goal of understanding the impact of sprouting durations on the nutrient composition of canola, and possible changes in the sprout composition that might occur due to fungal fermentation. Microbial fermentation can be performed in submerged fermentation (SmF) (liquid medium) or solid-state fermentation (SSF) (solid medium). SSF has become popular due to the production of higher yields with better characteristics compared to SmF (Pandey, 2003). Besides, SSF does not require excessive water that needs further downstream processing as in SmF. In addition, SSF more closely mimics the natural habitat of cultivated fungi, which enhances the growth and production, hence, SSF was used for fermenting canola sprouts.

The specific goal of the study was to determine if sprouting followed by fermentation would be beneficial in improving the canola meal composition for its advanced utilization. Hence, the objectives of the study were, a) to evaluate the daily nutrient composition of canola sprouts (day 1 to 6); and b) to determine the effect of solid-state fermentation on the nutrient composition of 6 days old canola sprout using three generally recognized as safe (GRAS) fungal strains: 1) *Aureobasidium pullulans*, 2)

Trichoderma reesei, and 3) *Neurospora crassa*. Besides being GRAS, these fungi were previously reported to be effective in improving the nutritional composition of canola (Alhomodi et al., 2021a; Alhomodi et al., 2021b; Croat et al., 2016a) due to their abilities to produce multi enzymes (Chi et al., 2009; Wen et al., 2005); hence these three fungi were selected for the present study.

4.3. Materials and Methods

4.3.1. Process for Canola Seed Sprouting

A bulk quantity (100 kg) of canola seeds was obtained from ADM/Specialty Commodities LLC (Fargo, North Dakota, United States). Two different set of sprouting experiments were conducted for SSF and daily compositional observation of sprouts. For daily nutritional sprout observation, seven batches of 100 g of canola seeds were weighted out from 100 kg seed container and then soaked separately in tap water at room temperature (~22 °C). After 24 h of soaking, seeds were transferred into sealed plastic containers and covered partially with semi-transparent lids to ensure enough air ventilation. The container contents were sprayed twice daily with tap water to keep seeds moist during sprouting time. Sprouts were harvested daily, starting with 24 h soaked seed and ending with 144-h sprouts. Each batch was dried in an oven at 60 °C for 24 h, and total weight before and after drying was recorded. Dry batches were milled into fine powder using a Krups coffee grinder (Solingen, Germany) and were stored at room temperature in plastic Ziploc bags. Sprouts for SSF were prepared using the same protocol as described above for the daily compositional observation, except for sample size. Five hundred grams of seeds were soaked in tap water for 24 h and then germinated for 6-day periods. All experiments were conducted in triplicate.

4.3.2. Microbial Inoculum Preparation

Aureobasidium pullulans, Trichoderma reesei, and *Neurospora crassa* were obtained from the National Center for Agricultural Utilization Research (Peoria, IL). Each strain was stored in the refrigerator and sub-cultured at monthly intervals. Eight mm plug of a microbial colony grown on potato dextrose agar (PDA) was transferred into 250 mL Erlenmeyer flask prepared with sterile 100 mL medium of 5.0% glucose, 0.5% yeast extract, and optimal pH of 3.0 ± 0.1, 5.0 ± 0.1, and 5.0 ± 0.1 for *A. pullulans*, *T. reesei* and *N. crassa,* respectively. The flasks then were incubated for 48 h at 30 °C in rotary shaker at 150 rpm.

4.3.3. Solid State Microbial Fermentation of Canola Sprouts

Dried and ground 6-day old canola sprouts (100 g) was placed into a 500 ml Erlenmeyer flasks and equal amount of water was added to achieve solid loading rate of 50% on dry weight basis. Prepared flasks were then adjusted for optimal pH of 3.0 ± 0.1, 5.0 ± 0.1, and 5.0 ± 0.1 for *A. pullulans*, *T. reesei* and *N. crassa,* respectively. Flasks covered with foam plugs and aluminum foil were autoclaved at 121 °C for 20 min. The flasks were then inoculated with 10 ml of a 48h culture of *A. pullulans, T. reesei,* and *N. crassa*. Additionally, uninoculated flasks were also used as control. All flasks with final moisture content of 60% were incubated at 30 °C for 168 h in a static mode. Triplicate flasks of each microbe and uninoculated control were harvested, and pH measured at 168 h. Fungal colonization were observed daily. All recovered solids were oven dried (80 °C for 24 h) and ground for residual sugar, total protein, fiber, phytic acid, and total glucosinolate analyses.

4.3.4. Analytical Assays

4.3.4.1. Fresh Canola Sprout Yield

Daily fresh sprouts starting with overnight soaked seed were weighed and expressed as fresh yield in grams. The sprouts were then dried at 60 °C for 24 h.

4.3.4.2. Moisture Content of Samples

All samples were analyzed for their moisture content by drying around 2 g of samples at 80 °C for 24 h using a hot air oven (AACC, 2010).

4.3.4.3. Determination of Crude Fat

Crude fat was analyzed by Agricultural Experiment Station Chemical Laboratories at the University of Missouri-Columbia using ether extraction, AOAC Official Method 920.39 (A) (Padmore, 1990).

4.3.4.4. Determination of Crude Protein Content

About 0.2490~0.2500 g of each sample was prepared for protein analysis in duplicate. Samples were uploaded onto a LECO model FP528 (St. Joseph, MI, USA), and the total nitrogen gas content emerged after sample combustion was measured (AOAC Method 990.03) (Horwitz and Latimer, 2006). The nitrogen content of the samples was used to calculate the protein percentage using a conversion factor of 6.25.

4.3.4.5. Determination of Total Glucosinolate Content

Total GLS content of different samples were measured according to Berhow et al. (2013), a modification of the HPLC method developed by Betz and Fox (1994), to quantify the GLS content. The methanol extracts of the dried sprouts were run on a Shimadzu (Columbia, MD) HPLC System (two LC 20AD pumps; SIL 20A autoinjector; DGU 20As degasser; SPD-20A UV-VIS detector; and a CBM-20A communication BUS module)

running under the Shimadzu LC solutions Version 1.25 software. The column was a C18 Inertsil reverse phase column (250.0 mm X 4.6 mm; RP C-18, ODS-3, 5u; GL Sciences, Torrance, CA). The GLS were detected by monitoring at 237 nm. The initial mobile phase conditions were 12% methanol/88% aqueous 0.005 M tetrabutylammonium bisulfate (TBS) at a flow rate of 1 mL.min-1. After injection of 15 µl of sample, the initial conditions were held for 2 min, then up to 35% methanol over another 20 min, then to 50% methanol over another 20 min and then up to 100% methanol over another 10 min. Standards, prepared in this lab or purchased, were run to determine retention times and extinction coefficients. The presence of glucosinolates were confirmed by negative ion mode analysis on an Thermo Electron LTQ Oribitrap Discovery Mass Spectrometer (a linear ion trap MS, coupled to a high precision electrostatic ion trap MS) with an electrospray ionization (ESI) source, and a Thermo Scientific ACCELA series HPLC system, all running under Thermo Scientific Xcalibur 2.1.0.1140 LC-MS software as needed.

4.3.4.6. Determination of Phytic Acid Content

Phytic acid was measured as phosphorous released by phytase and alkaline phosphatase using the Megazyme Phytic acid/Total phosphorus kit (Megazyme, 2017). The method includes acid extraction of inositol phosphates and further treatment by phytase and alkaline phosphatase to free phosphates. Then, a modified colorimetric method is used to measure the total released phosphate that is given as grams of phosphorus per 100 g of sample.

4.3.4.7. Determination of Fiber Content

Crude fiber (CF), neutral detergent fiber (NDF) and acid detergent fiber (ADF) content for fermented canola sprout meal were determined by Agricultural Experiment

Station Chemical Laboratories at the University of Missouri-Columbia. AOAC official method 978.10 2006 (AOAC, 2006d) was used for CF. NDF was conducted according to JAOAC v. 56, 1352-1356, 1973 (Holst, 1973), while ADF was tested using AOAC 973.18 (A-D) 2006 official method (AOAC, 2006c). For daily sprout CF, NDF and ADF determination samples were submitted to Midwest Laboratories Inc., (Omaha, NE). For CF analysis the official method AOCS Ba 6a-05 (AOCS, 2005) was used, whereas ANKOM Tech. method (ANKOM, 2006) was used for NDF and ADF.

4.3.4.8. Determination of Total Soluble Sugar Content

Total soluble sugars were determined according to previous reported method by (Croat et al., 2016a). In 9 mL deionized water, 1 g of each 24 h dried sample was added, mixed, and refrigerated at 4 °C overnight for solubilization. Next, the sample was centrifuged at 10,000 rpm for 10 min and the subsequent generated supernatant was transferred into 2 mL microcentrifuge tube and frozen for 24 h. Thereafter, supernatant was thawed and centrifuged at 10,000 rpm for 10 min to ensure supernatant purification. Besides, supernatant was further filtered into a high-performance liquid chromatography (HPLC) vial using a 0.2-µm filter connected to a 5 mL syringe. HPLC system (Agilent Technologies, Santa Calara, CA, USA) equipped with a refractive index detector (Model G1362A), and a waters size-exclusion chromatography column (SugarPak column I with pre-column module, Waters Corporation, Milford, MA, USA) at a flow rate of 0.5 mL/min (50 ppm EDTA in deionized water) and column temperature of 85 °C was used for processed sugar sample analysis. Several concentrations of (stachyose, raffinose, glucose, galactose, galactose-mannose, and arabinose) at 99.9% purity were used to build

calibration curve of concentration vs. HPLC area as previously reported (Karunanithy et al., 2012).

4.3.4.9. Determination of Ash Content

Ash content was conducted by combusting 5 g of each sample at 575 °C for 7 h using Thermolyne F1730 Heat-treat oven [serial No. 15547; Volts 230; cycles 60; phase 1] (Sluiter et al., 2005).

4.3.5. Statistical Analysis

Microsoft® Excel® for Office 365 MSO 64-bit and R version 1.2.5001 (Boston, MA) were used in the statistical analysis. The statistically significant differences among variances determined by one-way ANOVA at $p \leq 0.05$ level of significance.

4.4. Results and Discussion

4.4.1. SSF of Canola Sprouts

The 6-day old canola sprout substrate was fermented for 168 h under SSF condition using three strains, *A. pullulans*, *N. crassa* and *T. reesei* besides uninoculated control. The increase in crude protein post fermentation ranged from 3.7 to 9.8% compared to raw sprouts (Figure 4.1). *N. crassa* exhibited the highest protein level by 9.8% followed by *T. reesei* (9.3%) and *A. pullulans* (5.4%). This increase in protein could be derived from the loss of dry matter (Osman, 2011), changes in the ratio of other component due to the fungal nutrient consumption during fermentation (Baldwin et al., 2019), the extracellular fungal enzymatic secretion (proteins) for nutrient degradation (Oseni and Akindahunsi, 2011) and or the increase in microbial cells (Ojokoh et al., 2013). Similarly, Inyang and Zakari (2008) reported slightly higher increase in protein (20% protein increase in sprouted and naturally fermented pearl millet) as compared to what was found in our study. This could have been

due to the removal of hulls from pearl millet sprout prior to fermentation unlike in our study where hulls were kept intact during fermentation. Ongol et al. (2013) reported that fermentation of two 72 h germinated varieties of maize (Zm 607 and Tamira Pool A9) resulted in protein increase of 12.1% in Zm 607 and 12.1% in Tamira Pool.

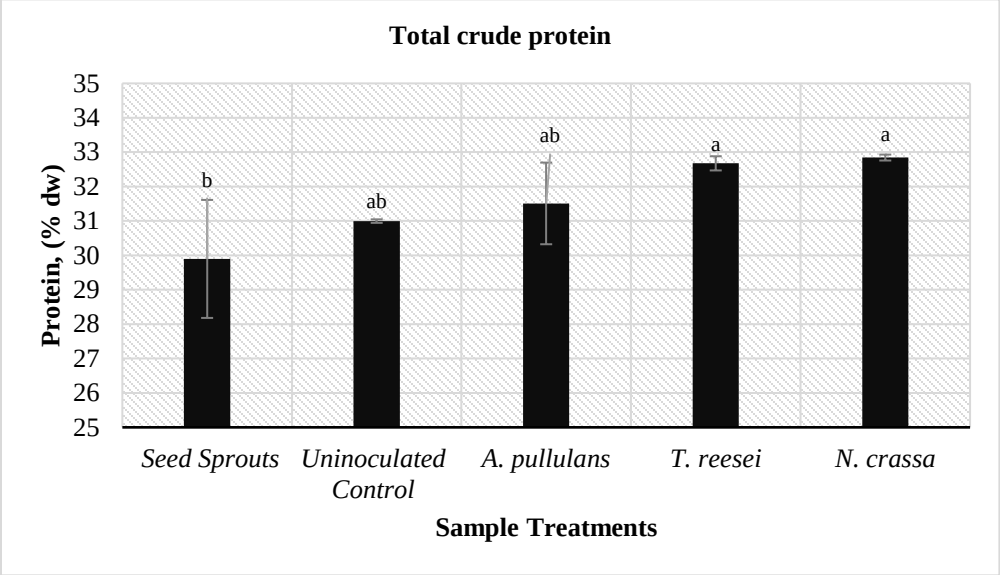

Figure 4.1: Total protein content of fermented canola sprouts at 168 h. Letters signify significance among groups at ($p \leq 0.05$); control is uninoculated.

Total GLS (Figure 4.2) in uninoculated control showed a significant drop in total GLS (38.8%) as compared to the seed sprouts. This may be due to low thermal stabilities of GLS (Oerlemans et al., 2006), which leads to degradation during heat sterilization and further oven drying. Similarly, Croat et al. (2016a) reported 65.5% GLS reduction in autoclaved and dried samples of canola meal compared to the raw meal. Comparing to uninoculated control, *T. reesei* further reduced the total GLS by 4.0%. The reduction can be attributed to fungal exploitation of sugar and sulfur moieties of GLS (Vig and Walia, 2001). The GLS reduction indicates the ability of *T. reesei* to produce myrosinase that catalyzes the GLS into glucose and thiohydroximate-o-sulfonate (Sakorn et al., 1999). This result is in agreement with Croat et al. (2016a) who reported a decline in total GLS of

fermented canola meal by *T. reesei* and other fungi. Sakorn et al. (1999) reported the production of myrosinase by *Aspergillus* sp. NR~4201when was cultivated in GLS (sinigrin) medium, whereas no myrosinase in sinigrin free medium was detected. Unlike *T. reesei*, *A. pullulans* and *N. crassa* caused an increase in total GLS by 0.22 and 0.86 µmol/g, respectively. This increase may be derived from the fungal alternations of physical properties of fermented meal, which in return increases the extractability of GLS (Ye et al., 2019). Besides, it could be the lack of myrosinase activity by *A. pullulans* and *N. crassa* leading them to consume other carbon sources. According to Sakorn et al. (1999) *Aspergillus* NR-4201 was only the one consuming GLS (sinigrin) completely in three days among other three fungi grown on sinigrin glucose plates for 7 days. This can demonstrate that the preferability of GLS hydrolysis can vary among fungi, especially when different carbon sources (carbohydrates) are made available to microbes.

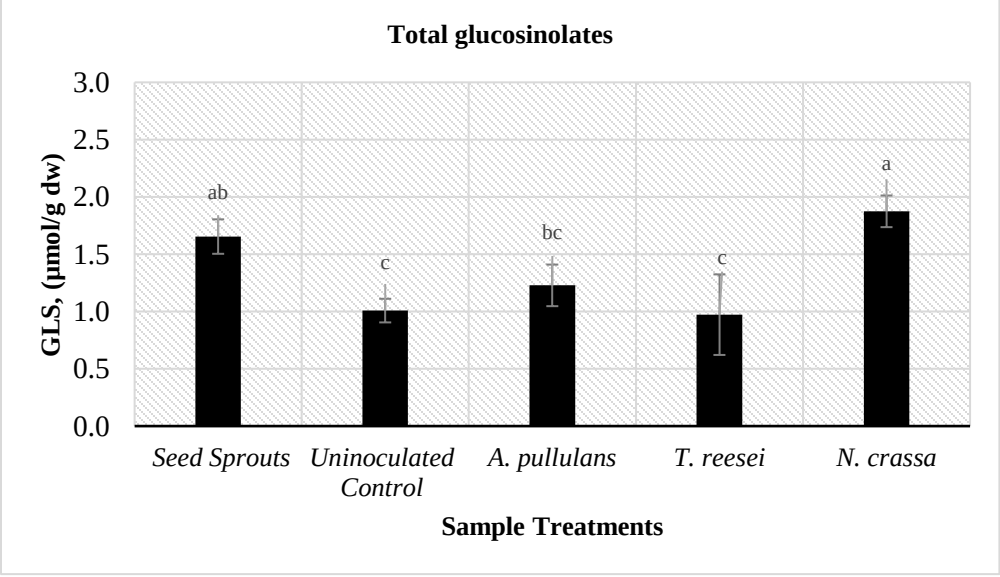

Figure 4.2: Total glucosinolate content of fermented canola sprouts at 168 h. Letters signify significance among groups at ($p \leq 0.05$); control is uninoculated.

There was no change in the total phytic acid content (0.63 g/100 g) of seed sprouts and uninoculated control. Except for *A. pullulans* fermented sprouts, there was a significant

decline in total phytic acid content of sprouts post fermentation (Figure 4.3). Specifically, *N. crassa* showed the maximum reduction in phytic acid content (0.11 g/100 g) followed by *T. reesei* (0.32 g/100 g) and *A. pullulans* (0.53 g/100g), when compared to uninoculated control (0.59 g/100g). The reduction of phytic acid in fermented sprout meal is indicative of phytase activity of fungal inoculums during SSF. Egounlety and Aworh (2003) reported that fermentation of soybean for 36 h by *Rhizopus oligosporus* reduced phytic acid content (0.88%) as compared to raw meal (1.27%). Similar results were obtained by El-Batal and Karem (2001) who reported a phytic acid decline in fermented rapeseed meal using *Aspergillus niger*. Ibrahim et al. (2002) reported that fermentation of pretreated cowpeas by *Rhizopus oligosporus* and *Lactobacillus plantarum* reduced phytic acid content to 2.79 and 2.39 g/100 g, respectively compared to raw meal phytic acid content of 4.54 g/100 g.

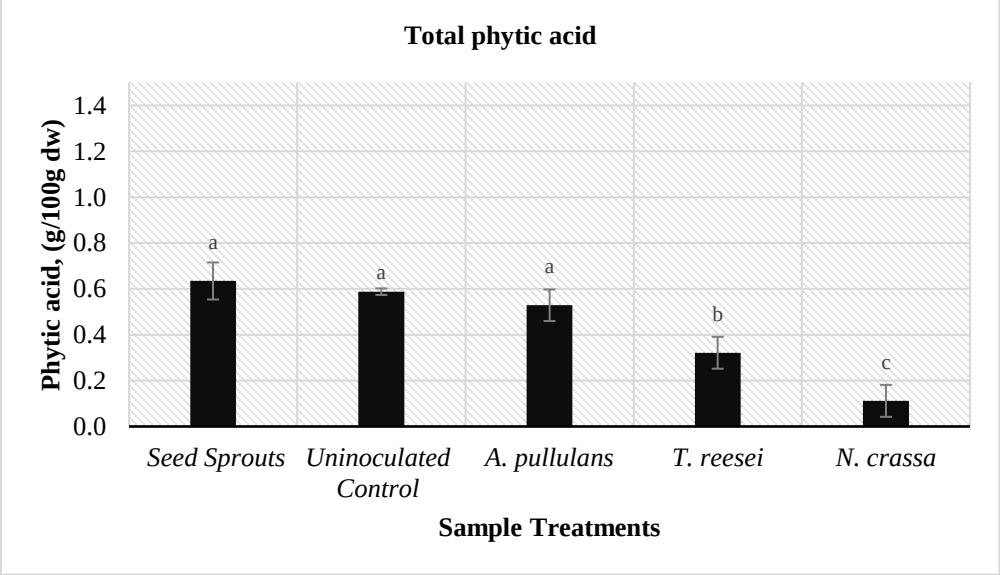

Figure 4.3: Total phytic acid content of fermented canola sprouts at 168 h. Letters signify significance among groups at ($p \leq 0.05$); control is un-inoculated.

Total soluble sugars of raw sprouts, uninoculated control and fermented samples are presented in Figure 4.4. Heat-sterilization of uninoculated control caused 3.3 mg/mL reduction in total soluble sugars compared to raw sprouts. This phenomenon was seen as

well by Croat et al. (2016b) who stated a 2.7-5.5% sugar reduction of autoclaved canola meal compared to raw meal. This might be attributed to Maillard reactions, where a condensation of amino group on protein with carbonyl group on reducing sugars occurs (Lund and Ray, 2017). In addition, heat-sterilization of sugars with other nutrients can trigger its degradation (Wang and Hsiao, 1995). A reduction of oligosaccharides (raffinose and stachyose) was reported in soaked and cooked red beans at 77.0 and ~99.3 °C, respectively (Nyombaire et al., 2007). Three major categories of carbohydrates (soluble, insoluble, fiber) are present in the canola, where soluble sugars could make up to 10% of the oil-free weight and constituting sugars like sucrose (3.9 to 9.8%), raffinose (0.3 to 2.6%), stachyose (0.8 to 1.6%), fructose (0.1 to 0.5%) and glucose (0.1 to 0.4%) at varying level (Barthet and Daun, 2011). *A. pullulans* exhibited the highest total soluble sugar reduction by 29.9% compared to uninoculated control. Baldwin et al. (2019) reported full consumption of monosaccharides (glucose and fructose) present in soybean meal by *Aureobasidium pullulans* within 24 h of incubation. *T. reesei* and *N. crassa* showed a different trend, where an increase in total soluble sugars by 0.2 and 0.8 mg/mL, respectively was observed. This trend of increase in sugars reflects the enzymatic ability of both strains to break down complex carbohydrates into oligosaccharides and simple sugars, thereby increasing the total soluble sugar levels during fermentation.

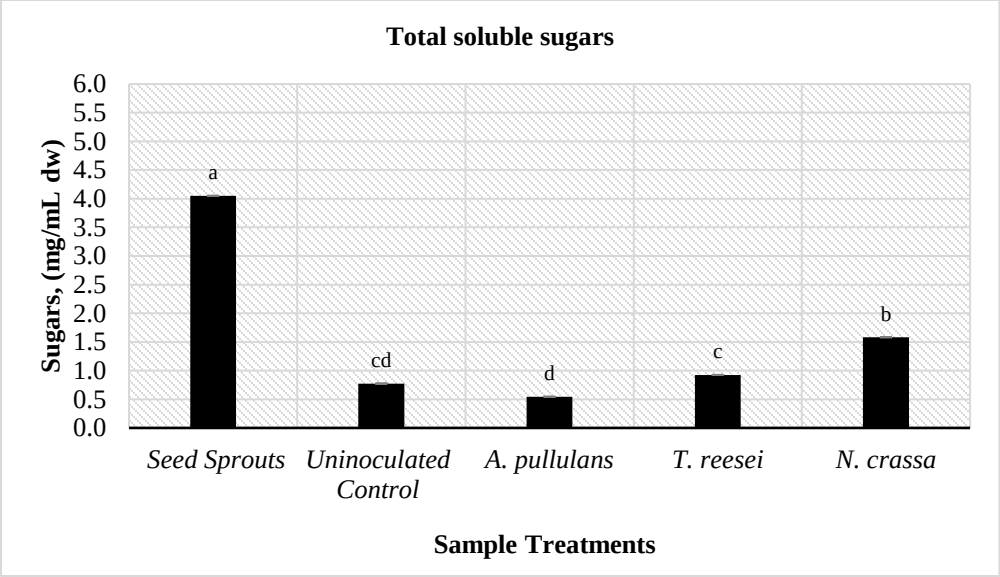

Figure 4.4: Total soluble sugar content of fermented canola sprouts at 168 h. Letters signify significance among groups at ($p \leq 0.05$); control is uninoculated.

Total fibers (CF, ADF and NDF) and total solid recoveries are shown in Table 4.1. There were significant losses in total solid recoveries as compared to uninoculated control. Among three strains tested *A. pullulans* resulted in minimal loss of solids with resulting solid recoveries of (93.8 g) followed by *T. reesei* (91.7 g), and *N. crassa* (91.6 g). Compared to seed sprouts, fiber results (CF, ADF and NDF) of uninoculated control and three fungal strains showed a slight increase in fibers. These increases may be attributed to dry matter loss as seen in Table 4.1. *A. pullulans* reduced CF and NDF by 6.1 and 4.2%, respectively while increased ADF by 7.4% compared to uninoculated control. *N. crassa* and *T. reesei* increased all fiber fractions (CF, ADF, and NDF) compared to uninoculated control. The possible reason for this increase may be justified by the fungal metabolization of easily digestible sprout components leading to the concentration of other components. Similar results were reported by Croat et al. (2016a) who noted an increase in acid detergent fiber and neutral detergent fiber of hexane extracted and cold pressed canola meal

fermented by *Pichia kudriavzevii, Fusarium venenatum, Mucor circinelloides,* and three strains of *A. pullulans*.

Table 4.1: Total fibers (CF, ADF and NDF) and total solid recoveries of solid-state fermented canola sprouts at 168 h

Process parameters	Total fiber (dry basis)			
	Fungal fermentation at 168 (h)			
	TSR (g)	CF (%)	ADF (%)	NDF (%)
Control	97.8±0.59[a]	12.2±0.94[a]	18.6±0.08[bc]	22.6±0.79[a]
A. pullulans	93.8±0.35[b]	11.5±1.11[a]	20.0±0.73[ab]	21.6±0.89[a]
T. reesei	91.7±0.48[c]	13.1±1.00[a]	21.3±1.11[a]	26.0±2.80[a]
N. crassa	91.6±0.54[c]	12.9±1.11[a]	19.9±0.65[ab]	23.7±3.20[a]

Mean ± standard deviation. Letters signify significance among groups for each incubation time. Control is uninoculated. TSR: total solid recovery; CF: crude fiber; ADF: acid detergent fiber; NDF: neutral detergent fiber.

4.4.2. Daily Nutritional Changes of Canola Seed Sprouts

Daily nutrient composition changes of canola seed sprouts in terms of dry matter, crude oil, crude protein, phytic acid, GLS, total soluble sugars, ash, and fibers (CF, ADF and NDF) were observed starting from raw seeds, soaked seeds and daily germination intervals up to 144 h. Significant amount of water uptake occurred during the overnight soaking of canola seeds, which led to an increase in moisture content of raw seeds from 7.1% to 56.3% in soaked seeds (Table 4.2). This rapid increase in moisture content of soaked seeds is due to imbibition of water to rehydrate cell matrices (Nonogaki et al., 2010). As sprouting progressed, sprouts moisture also continued to increase from 56.7% (24 h) to 75.3% (144 h) demonstrating a linear upward trend of moisture vs sprouting duration. The possible explanation for daily moisture increase is due to formation of new cells requiring hydration (Nonogaki et al., 2010). Our results concurred with Masood et al. (2014) who reported a daily increase in moisture content of germinated mung bean and chickpea.

Crude oil content of soaked seeds (44.2%) was found to be higher than the raw seeds (39.6%), and oil content of sprouts remained unchanged up until 48 h of sprouting (Table 4.2). The oil content of sprouts started to drop after 72 h of sprouting (39.5%), and a gradual drop proceeded until last day of sprouting (144 h) with resulting oil content of 27.2%. Oil reduction is due to the metabolization of nonpolar lipid by sprouts for energy source (Barthet and Daun, 2005). Our results concur with Bhardwaj and Hamama (2007); Hamama and Bhardwaj (2011); Dawood et al. (2013) findings in terms of crude oil reduction, whereas contradicting them with respect to the increase in crude fat of soaked seeds. It is known that seed imbibition causes damages to cell membrane besides other organelles that later undergo to cellular restoration with sprouting time (Nonogaki et al., 2010). Based on the aforesaid fact, we assume that observed high oil content may be due to the solute leakage of inorganic ions, sugars, amino acids, proteins, or organic acid during seed rehydration (Nonogaki et al., 2010). Our total soluble sugar data (Table 4.2) showed 54.8% reduction in total soluble sugars post 24 h soaking, which could be due to the loss of total soluble sugars at that phase leading to the concentration of other components such as the oil fraction. Similarly, an increase in lipid content of sorghum seeds was reported to be 4-fold due to soaking, and seed sprouts had about 2.5 fold increase in fat contents as compared to raw seeds Obizoba and Atii (1991) .The lipid increase in their study was attributed to the synthesis of fatty acids.

The crude protein content of the canola seed was increased during soaking and throughout the sprouting period (Table 4.2). During soaking and initial phase of sprouting (up to 48 h) crude protein content of canola seeds increased by ~6.9% when compared to the raw canola seeds. The protein content only slightly increased from 48 h (24.6%) until

the end of sprouting time (26.3%). This increase can be attributed to the synthesis of new proteins during sprout development (Nonogaki et al., 2010) or due to changes in protein ratio to other components. A gradual increase in protein content of mung bean varieties with an increase in sprouting period have been previously reported Shah et al. (2011). Similarly, Masood et al. (2014) reported an increment in protein content of mung bean from 23.5 to 30.5% and chickpea from 17.8 to 23.4% when germinated for 120 h.

Phytic acid content of raw seeds decreased after soaking by 19.4% (Table 4.2). The level of phytic acid continued to drop with sprouting time and reached maximal reduction of 49.7% at 144 h of sprouting, when compared to raw seeds. Phytic acid reduction after soaking can be attributed to the leaching of phytate ions during imbibition (Kaur et al., 2020). Further decrease of phytic acid during germination is caused by the increase of phytase activity (Reddy et al., 1989). Frias et al. (2005) reported that 4-day germination of cruciferous seeds (radish seeds, small radish seeds, rapeseed and white mustard seeds) resulted in 50.0% phytic acid reduction, except small radish seeds that showed 77.0% reduction under dark condition. Kaur et al. (2020) reported that soaking rice bean for 18 h led to 43.1% reduction in phytic acid while 60.3% reduction was seen in 72 h germinated sprouts. Ibrahim et al. (2002) reported that soaking cowpeas for different intervals (8 h, 12 h, and 16 h) led to a phytic acid reduction.

Total GLS levels of soaked seeds and sprouts showed higher values than raw seeds (Table 4.2). Comparing to raw seeds, soaked seeds exhibit 7.0% increase in GLS. With sprouting time, GLS further increased from 1.5 to 3.5 µmol/g. Although there was sudden spike in total GLS content of sprouts at 72 h of sprouting, we believe that this was not indicative of any significant alternations in GLS composition. Rather it could have been

the consequence of several factors. For instance, one possibility could be the differences in seed viability during sprouting process as individual set-up was used for sprouting experiments at each time interval. Another reason may be due to the efficiency of GLS extractability and detectability during the analysis because of the low levels of these glucosinolates in canola seeds. Bellostas et al. (2007) reported an increase in total GLS of red cabbage from seed to 4-day old sprouts, while an increase was seen in savoy cabbage and cauliflower from day 4 to day 7. Total GLS of rapeseed sprouts grown in light showed an increase in day 1 and 2 compared to the raw seeds followed by a decrease in the levels of GLS with sprouting time (Ciska et al., 2008).

Soaking and sprouting had a significant effect on total soluble sugars (primarily a sum of stachyose, raffinose, glucose, and galactose) (Table 4.2). Soaked seeds showed a reduction by 54.8% compared to the raw seeds. Germination from 24 h to 144 h exhibited an upward increase in the total soluble sugars. Sprouts grown for 144 h showed 86.7% increase in total soluble sugars compared to ungerminated seeds. The initial sugar reduction can be attributed to the leakage of soluble sugars during imbibition due to seed organelles and membrane damages (Nonogaki et al., 2010). In contrast, the total soluble sugars increase can be as a results of the conversion of free fatty acid produced during lipolytic hydrolysis to carbohydrates by metabolic pathways with sprouting time (Black and Bewley, 1983). Similar results were obtained by Balasaraswathi and Sadasivam (1997) who reported total soluble sugar reduction in the first two days of sunflower seed (*Helianthus annuus*) germination followed by an increase up to 5 days. Ibrahim et al. (2002) reported that soaking cowpeas for 16 h led to a raffinose and stachyose reduction by 100.0 and 83.8%, respectively. On the same line, Mulimani et al. (1997) reported that

soaking soybean for 16 h reduced stachyose by 44.8% and raffinose by 80.3%. A study on the effect of germination on carbohydrates of pearl millet showed a significant increase in total soluble sugars, reducing sugars and non-reducing sugars after 24 h germination (Khetarpaul and Chauhan, 1990a). Obizoba and Atii (1991) reported a significant increase in reducing sugars of sorghum sprouts compared to ungerminated seeds.

Ash content of raw seeds insignificantly increased after imbibition and sprouting (Table 4.2). The highest ash level was seen at 144 h sprouting time with 4.0% compared to raw seed 3.7%. This increase can be explained by the incremental reduction of the mineral chelator (phytic acid) (Table 4.2) which led to the liberation and concentration of minerals. Similar results were obtained by Fouad and Rehab (2015) who reported gradual ash content increases in lentil spouts germinated for 3, 4, 5, and 6 days. Sun Lim et al. (2001) reported a rise in the ash content of buckwheat seed grown for 6, 7, and 8 days.

Table 4.2: Daily nutrient changes of canola sprouts

Samples	Analysis, dry basis						
	DM	**CO**	**CP**	**PA**	**GLS**	**TSS**	**Ash**
RS	92.9^{a}	39.6±0.42^{b}	23.1±0.36^{f}	1.8±0.05^{a}	1.3±0.08^{c}	4.3±0.02^{d}	3.7±0.17^{a}
SS	43.7^{c}	44.2±0.05^{a}	24.6±0.01^{e}	1.4±0.01^{b}	1.4±0.10^{c}	1.9±0.43^{f}	3.8±0.05^{a}
24h-S	43.3^{d}	44.9±0.15^{a}	24.6±0.03^{e}	1.4±0.01^{b}	1.5±0.15^{c}	2.8±0.04^{e}	3.8±0.02^{a}
48h-S	47.0^{b}	44.1±0.01^{a}	24.9±0.09^{de}	1.2±0.01^{c}	1.5±0.06^{c}	6.5±0.33^{c}	3.8±0.11^{a}
72h-S	38.7^{e}	39.5±0.44^{b}	25.3±0.08^{cd}	1.1±0.01^{cd}	2.3±0.03^{b}	6.5±0.06^{c}	3.9±0.11^{a}
96h-S	37.3^{f}	38.1±0.19^{c}	25.5±0.08^{bc}	1.1±0.01^{d}	1.9±0.10^{b}	7.3±0.02^{b}	4.0±0.05^{a}
120h-S	29.3^{g}	32.1±0.02^{d}	26.1±0.11^{ab}	0.9±0.01^{e}	1.9±0.26^{b}	7.9±0.03^{a}	4.0±0.01^{a}
144h-S	24.7^{h}	27.2±0.11^{e}	26.3±0.15^{a}	0.9±0.01^{e}	3.5±0.11^{a}	8.0±0.16^{a}	4.0±0.04^{a}

Mean ± standard deviation. Letters signify significance among raw seed, soaked seed and sprout germination times at ($p \leq 0.05$). RS: raw seed; SS: soaked seeds; S: sprouts; DM: dry matter %; CO: crude oil %; CP: crude protein; PA: phytic acid (g/100g); GLS: total glucosinolates (µmol/g); TSS: total soluble sugars (mg/mL).

CF, ADF, and NDF increased post-imbibition by 49.2, 6.7, and 15.2%, respectively (Figure 4.5). This increase can be attributed to a concentration factor due to the leakage of soluble components during seed rehydration (Abu El Gasim et al., 2008) that causes a temporary membrane damage (Nonogaki et al., 2010). After soaking, total fiber (CF, ADF, and NDF) started decreasing with sprouting time, and the last day of sprouting (144 h) exhibited a decline by 32.8% for CF, 19.7% for ADF, and 16.6% for NDF compared to raw seed. Ongol et al. (2013) reported 25.0 and 23.0% reduction in fiber content of 72 h germinated maize varieties (Zm 607 and Tamira Pool A9), respectively. Khalil et al. (2007) reported that sprouting two types of chickpeas (kabuli and desi) for (24, 48, 72 and 96 h) had insignificant effect on crude fiber. Shah et al. (2011) reported a crude fiber increase in mung bean seed sprouts (Ramzan variety) at 24 h germination followed by a reduction after 48 h then again increase with sprouting time. Different studies showed an increase in crude fiber of germinated seeds compared to ungerminated seeds such as mung bean, chickpea (Masood et al., 2014), and barley (Chung et al., 1989).

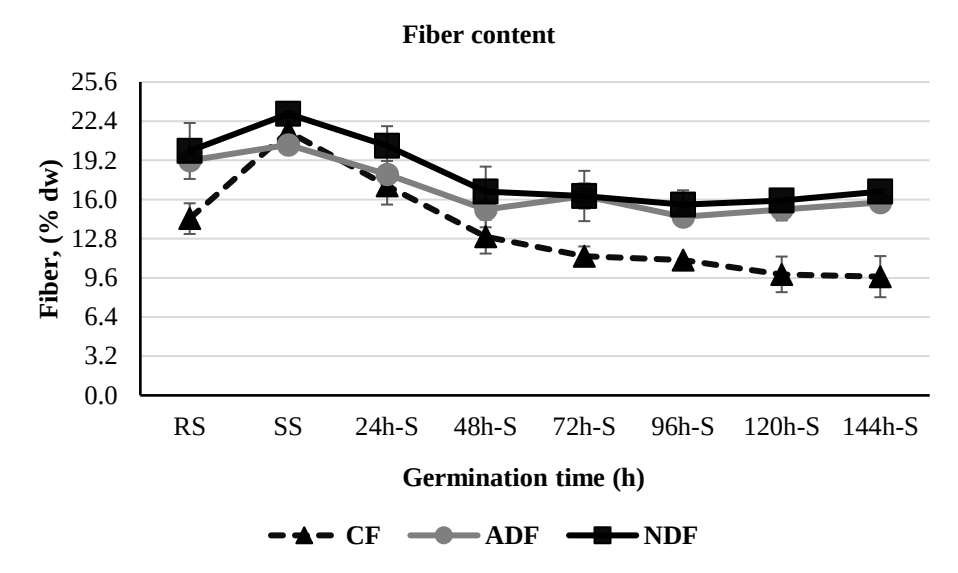

Figure 4.5: Fiber content of daily sprouting canola seeds up to 6 days. RS: raw seeds; SS: soaked seeds; S: sprouts; CF: crude fiber %; ADF: acid detergent fiber %; NDF: neutral detergent fiber %.

4.5. Conclusion

Canola sprouts at different germination period showed various changes in the nutrient composition compared to ungerminated seeds. This knowledge of nutritional change of sprout composition showed the potential of selecting the optimal sprouting interval for desirable sprout characteristics. Further sprout compositional improvement can be achieved by, 1) Optimizing germination conditions like temperature, light, moisture, seed type, etc., and 2) Mechanically separating hulls from sprout. Further fungal fermentation to 6-day canola sprout substrate under SSF enhanced canola sprout nutrient composition and aided in the removal of undesirable anti-nutritional components. For further sprout compositional enhancement, co-culture fermentation process can be applied.

Chapter 5. Meal Nutritional Characteristics and Oil Profile of Sprouted, Dehulled, and Solvent- Extracted Canola [†]

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5.1. Abstract

BACKGROUND: Canola meal has limited utilization in feed and food applications due to the presence of antinutritional factors and high fiber content. Thus, this study used three-day canola seed sprouting followed by hull removal to improve the nutritional quality of canola as a feed and food ingredient to further enhance and diversify canola market.

RESULT: Seed sprouting, and hull removal process resulted in 63.2% sprouts, 29.3% mix fractions (MF) (hulls, ungerminated seed, and delayed sprouts), and 8.1% mass loss during sprouting. Fresh sprouts and MF were dried, ground, and defatted to compare the obtained meals and oils with their counterparts of raw seed. Defatted sprouts (DFSP) resulted in a reduction of 46.2% in crude fiber, 34.3% reduction in acid detergent fiber, and 43.4% reduction in neutral detergent fiber as compared to defatted raw seed (DFSE). DFSP had a 10.1% higher protein content and a 5.9% increase in total amino acid content with higher essential amino acids as compared to DFSE. Total soluble sugars, phytic acid content, and ash content were lowered by 5.5, 25.9, and 5.5%, respectively in DFSP, whereas the total glucosinolate content was higher in DFSP (13.1 μmol/g) than DFSE (8.8 μmol/g). Sprouts and MF had an oil content of 38.4 and 9.6%, respectively as compared to raw seed (34.5%).

CONCULUSION: Sprouting and hull removal of canola seed can potentially provide nutritive meal for food and feed applications.

5.2. Introduction

Rapeseed/canola (*Brassica sp.*) is an important oilseed crop consisting of outer seed hull (13-20%), and internal seed kernel (80-87%) (Aider and Barbana, 2011). It contains high level of oil (40-45%), and protein (~25%) with excellent nutritional quality (Aider and Barbana, 2011; Mitaru et al., 1984). The industrial processing of canola involves preprocessing (cleaning, pressing, flaking, and cooking), mechanical pressing, and/or organic solvents to recover the oil. This process generates large quantities of protein-rich [~ 40% dry basis (db)] meal as a co-product (CCC, 2019). The meal is a valuable source of proteins, but it contains high levels of antinutritional factors (ANFs) such as high fibers, phytic acid, and glucosinolates, and low metabolizable energy (Croat et al., 2017). Therefore, it is partially included in the animal diets. Several research attempts have been made to remove ANFs by applying different pre- and post-processing (Olukomaiya et al., 2020c; Alhomodi et al., 2021a; Thakor et al., 1995; Hansen et al., 2017). The interest is mainly related to the removal of ANFs to enrich the canola meal proteins for its industrial application. Microbial fermentation of the meal potentially reduces the significant amount of ANFs except high fibers, which is still a challenge (Croat et al., 2016a). High fiber in canola is largely contributed to the hull fraction (~65% fiber, db) of the seed. Thus, dehulling the seeds to remove the fibrous envelope to obtain low fiber meal could potentially offer a solution to the problem. Dehulling operation in canola however has been difficult to implement in the industrial process due to the significant loss of the proteins and oils in the hulls (Mohamadzadeh et al., 2009). These problems have mainly occurred due to the small seed size and close association of hulls with the kernels. Hence, dehulling mechanisms such as front-and tail-end dehulling, and hydrothermal pretreatment have not

moved beyond research (Rempel et al., 2020). While an efficient dehulling mechanism is still lacking for the small sized seeds, growing global demand for the plant-based proteins has surged the research focus in canola seeds with the aim of advancing its industrial use. Few studies have used traditional sprouting process to elevate the level of key nutrients (essential and non-essential nutrients such as proteins, carbohydrates, vitamins, and minerals) and lower the levels of ANFs in canola (Bhardwaj and Hamama, 2009b; Bhardwaj and Hamama, 2009a; Bhardwaj and Hamama, 2007; Barthet and Daun, 2005; Chung et al., 1989).

The consumption of raw *brassica* sprouts has been touted for their health benefits owing to high level of bioactive compounds (Kristal and Lampe, 2002). Literatures available on the canola seed sprouting revealed that proximate composition of six-day canola sprouts is affected by duration of sprouting, and cultivar types (Bhardwaj and Hamama, 2009a; Bhardwaj and Hamama, 2009b; Bhardwaj and Hamama, 2007). Considering the advantages that sprouting offers, we previously conducted series of experiments where sprouted canola seeds were fermented with a set of microbes to produce nutrient enriched meals (Alhomodi et al., 2021c; Alhomodi et al., 2021b). Some key observations made during our previous studies led us to conceptualize this study. We noticed that during sprouting process, seed coat gets loosened, which could be easily removed by applying external force to separate hulls from kernels. This would lead to the dehulled sprouts which could either be consumed directly as nutrient source or subjected to further oil extraction to produce the defatted protein-rich meal. The nutritive value of the protein- rich meal and its applicability may create new markets for the canola industry. Though there are few reports that indicate that extraction of oil from dehulled seeds yield

superior oil (Koubaa et al., 2016) as compared to traditionally extracted canola oil, the combined effect of sprouting and dehulling on the oil and meal composition is currently lacking and should therefore be investigated.

In this study, canola seeds were sprouted at the minimal sprouting time, and hulls were removed manually to produce the dehulled sprouts. Dehulled sprouts and full fat seeds were subjected to solvent extraction to remove oil. The effect of sprouting and dehulling on the oil yield, oil quality, and meal proximate composition were assessed and compared with the unsprouted seeds.

5.3. Materials and Methods

Canola seeds were obtained from ADM/Specialty Commodities LLC (Fargo, ND). Seeds were stored in a sealed container at room temperature until use.

5.3.1. Canola Seed Sprouting and Hull Removal

Fifty grams of canola seeds were mixed with 800 mL of deionized (DI) water and kept at room temperature (~22 °C) for soaking. After 24 h of soaking, seeds were transferred into 1 L round wide-mouth Pyrex glass bottles. Bottles were covered with cheese cloths to ensure adequate ventilation. The seeds in the bottles were kept moist by adding water (20 mL) twice daily (at the interval of 12 h), and access water was drained. Bottles were kept at room temperature for 3 days. After 72 h of sprouting, the sprouts were poured into a bucket (18.9 L, 25 cm width × 36 cm length) and water was added directly from the tap. During water addition, hulls were detached from the seed endosperm and floated on the top. The floating hull fractions were then removed by using strainer, whereas partially attached hulls to the sprouts were separated by hand agitation. Due to the difficulty of separating the unsprouted seeds and the hull fraction, they were combined, and called

mixed fraction (MF). All fractions were dried in an oven at 50 °C for 24 h, and total weight before and after drying was recorded (Dawood et al., 2013). Dry sprouts and MF were separately milled into fine powder using a coffee grinder and were stored at room temperature in plastic Ziploc bags. Graphical details of canola seed sprouting followed by manual hull removal are presented in the Figure 5.1. To ensure adequate quantity of sprouted and dehulled material, sprouting and dehulling experiments were conducted in 100 small batches (50 g seeds/batch experiment) to produce 5 kgs of each fraction. Upon completion of experiments, all the sprouts and hull fractions were combined separately to produce large quantities of dehulled sprouts and mixed fractions. These two fractions were then submitted to oil extraction via pilot scale processing.

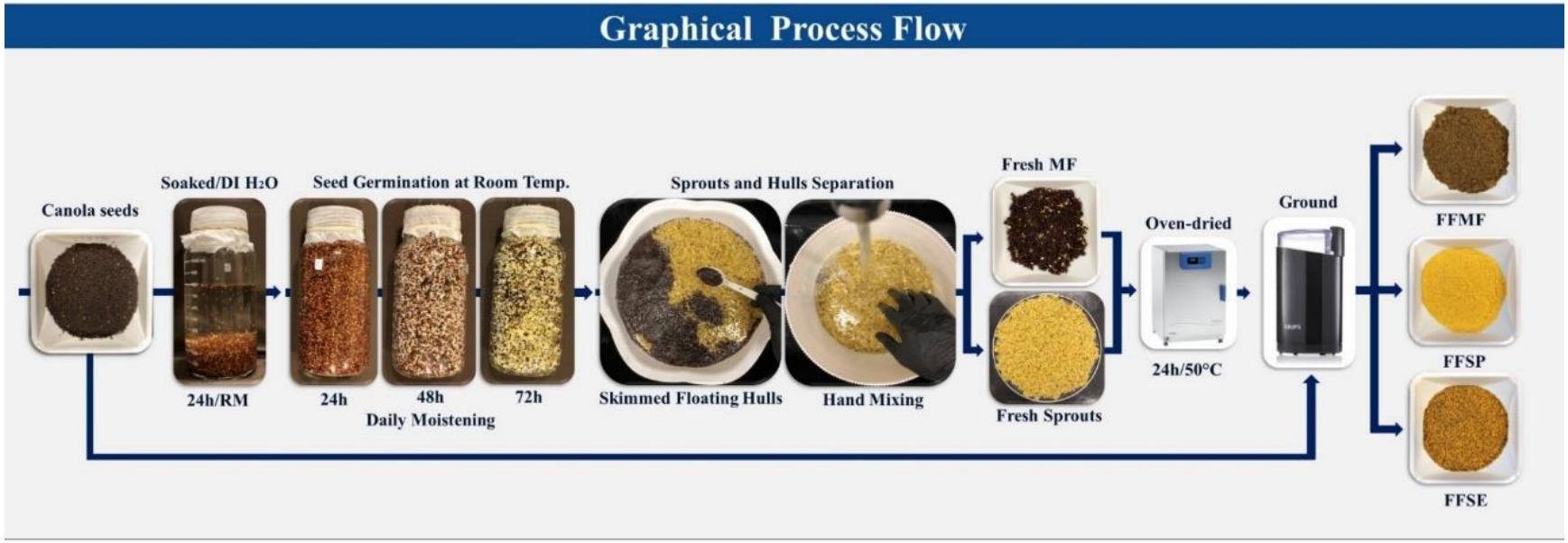

Figure 5.1: Graphical representation of process flow. FFSE: full fat seeds; FFSP: full fat sprouts; FFMF: full fat mixed fraction; MF: mixed fraction.

5.3.2. Oil Extraction

A pilot scale solvent extraction unit (model FT29, Armfield Ltd, Hampshire, England) was used to defat the canola samples. Three canola samples (raw canola seeds, dehulled sprouts, and mix fraction) were defatted separately. During the defatting process, the sample (~2 kg) were put in a 0.5 mm meshed bag and loaded into the extractor vessel. Hexane was added in the vessel to submerge the sample in the bag. The extraction content was then heated to 55-60 °C and allowed at that temperature range for 30 min. The miscella (hexane and oil) was drained and a fresh hexane was added again, and the process was repeated twice. After the second extraction step, the solvent was removed from defatted meal by using a vacuum oven at 60 °C and pressure of 300 mmHg for 8 h. These experiments were conducted in duplicate. Overall process flow used in this study is presented in the Figure 5.2.

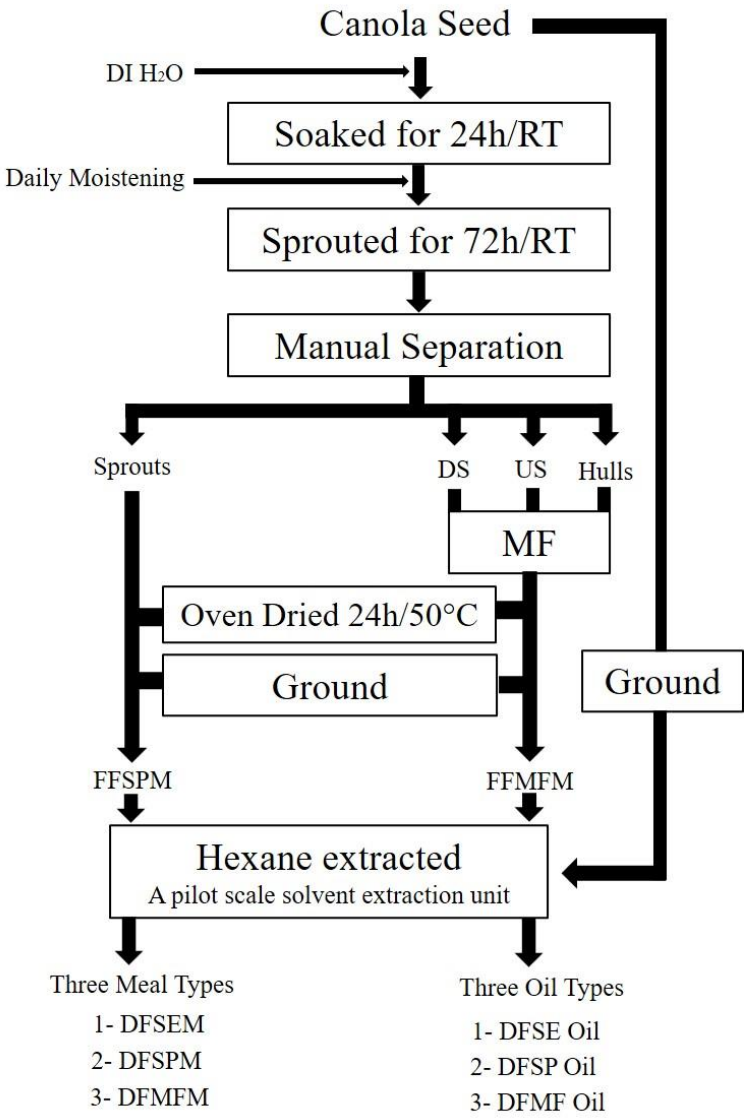

Figure 5.2: Overall process flow of canola seed sprouting and oil extraction. DI, deionized water; RT, room temperature; MF: mixed fraction; FFSPM: full fat sprout meal; FFMFM: full fat mixed fraction meal; DFSEM: defatted seed meal; DFSPM: defatted sprout meal; DFMFM: defatted mixed fraction meal.

5.3.3. Analytical Assays

5.3.3.1. Oil Content

The oil content of the samples before and after defatting was analyzed using an accelerated solvent extractor, ASE 200 (Dionex Sunnyvale, CA) where the oil in 7-9 g samples were extracted and quantified. The ASE 200 used hexane and was set at 100 °C,

6.7 MPa, 5 min heat time, 10 min static, 100% flush volume, 60 s purge time, and three static cycles. This analysis was conducted in duplicate for each sample.

5.3.3.2. Oil Profile and Free Fatty Acid Content

Oil profile was analyzed by Agricultural Experiment Station Chemical Laboratories at the University of Missouri-Columbia. AOAC Official Method 996.06 [Analysis of methyl esters by Capillary GLC] (AOAC, 2002), AOCS Official Method Ce 2-66 [Preparation of Methyl Esters of Fatty Acids] (AOCS, 1998), and AOAC Official Method 969.33 [Oils and Fat, Boron Trifluoride method] (AOAC, 2000) was used for determination of fatty acid profile while AOCS Official Method Ca 5a-40 (AOCS, 1997) was used for free fatty acid content. For fatty acid methyl esters (FAME) measurement, Supelco® 37 Component FAME Mix was used as standard [(Sigma Chemical cat. No. CRM47885, certified reference material, TraceCERT®, in dichloromethane (varied conc.), and an ampule of 1 mL]. These analyses were conducted in triplicate.

5.3.3.3. Crude Proteins

A LECO model FP528 (St. Joseph, MI, USA) was used to determine the total nitrogen content of samples. Around 0.2490~0.2500 g of dried ground sample was loaded onto LECO instrument in triplicates and the total nitrogen content generated from samples combustion was measured (AOAC Method 990.03) (Horwitz and Latimer, 2006). Then, the nitrogen content of the samples was used to calculate the protein percentage by using a conversion factor of 6.25.

5.3.3.4. Amino Acid Profile

Complete amino acid analysis was determined by Agricultural Experiment Station Chemical Laboratories at the University of Missouri-Columbia using AOAC Official

Method 982.30 E([a,b,c]), chp. 45.3.05, 2006 (AOAC, 2006a) and AOAC Official Method 988.15, chp. 45.4.04, 2006 (AOAC, 2006b). These analyses were conducted in triplicate.

5.3.3.5. Fiber Content

Fiber analysis [crude fiber (CF), neutral detergent fiber (NDF), and acid detergent fiber (ADF)] was conducted by Agricultural Experiment Station Chemical Laboratories at the University of Missouri-Columbia. CF was tested according to AOAC official method 978.10 2006 (AOAC, 2006d). NDF was conducted according to JAOAC v. 56, 1352-1356, 1973 (Holst, 1973), while ADF was tested using AOAC 973.18 (A-D) 2006 official method (AOAC, 2006c). These analyses were conducted in triplicate.

5.3.3.6. Total Soluble Sugars

Sugar samples were prepared according to previously reported method by Croat et al. (2016a). First soluble sugars were extracted by mixing one gram of dried sample with 9 mL of DI H_2O. The mixture was then stored at 4 °C for 24 h. Then, supernatant acquired by centrifugation at 10,000 ×g for 10 min, was poured into a 2 mL microcentrifuge tube and frozen overnight. After that, samples were thawed and followed by centrifugation at 10,000 ×g for 10 min to remove any impurities. The soluble sugars in the supernatant was quantified by HPLC system (Agilent Technologies, Santa Calara, CA, USA) equipped with a refractive index detector (Model G1362A), and a waters size-exclusion chromatography column (SugarPak column I with pre-column module, Waters Corporation, Milford, MA, USA) at a flow rate of 0.5 mL/min (50 ppm EDTA in deionized water) and column temperature of 85 °C. Sugar standards were prepared at various concentrations of (stachyose, raffinose, glucose, galactose, galactose-mannose, and arabinose) at 99.9% purity to build calibration curve of concentration vs. HPLC peak area as previously

reported by Karunanithy et al. (2012). The sum of the soluble sugars was presented as total soluble sugars. These analyses were conducted in triplicate for each sample.

5.3.3.7. Phytic Acid Content

Phytic acid content was quantified according to the Megazyme Phytic acid/Total phosphorus kit (Megazyme, 2017). In brief, the method includes acid extraction and enzyme treatment (phytase and alkaline phosphatase) to liberate phosphates form myo-inositol phosphate. The released phosphate is then determined by a modified colorimetric method and expressed as grams of phosphorus per 100 g of sample material. These analyses were conducted in triplicate.

5.3.3.8. Total Glucosinolates (GLS)

GLS levels were determined according to Berhow et al. (2013), using reverse phase HPLC to measure the intact GLS level, using a modified HPLC method developed by Betz and Fox (1994). The meal (~3 g) was extracted with methanol in Soxhlet extractors for 24 h. The resulting methanol extracts were diluted with a small amount of water, rotoevaporated to remove the methanol, and the resulting aqueous was then freeze-dried. The freeze-dried extracts in 0.25 g sets of triplicates were resuspended in 1 mL of water to which 2 mL of methanol was added to precipitate proteins and salts. The extracts were then filtered through 0.45 µm nylon 66 filters. The extract was run on a Shimadzu (Columbia, MD) HPLC System (two LC 20AD pumps; SIL 20A autoinjector; DGU 20As degasser; SPD-20A UV-VIS detector; and a CBM-20A communication BUS module) running under the Shimadzu LC solutions Version 1.25 software. The column was a C18 Inertsil reverse phase column (250 mm × 4.6 mm; RP C-18, ODS-3, 5µ; GL Sciences, Torrance, CA). The GLS were detected by monitoring at 237 nm. The initial mobile phase conditions were

12% methanol/88% aqueous 0.005 M tetrabutylammonium bisulfate (TBS) at a flow rate of 1 mL min^{-1}. After injection of 15 µL of sample, the initial conditions were held for 2 min, and then up to 35% methanol over another 20 min, then to 50% methanol over another 20 min. then up to 100% methanol over another 10 min. Standards, prepared in this lab or purchased, were run to determine retention times and extinction coefficients. The presence of glucosinolates were confirmed by negative ion mode analysis on a Thermo Electron LTQ Orbitrap Discovery Mass Spectrometer (a linear ion trap MS, coupled to a high precision electrostatic ion trap MS) with an electrospray ionization (ESI) source, and a Thermo Scientific ACCELA series HPLC system, all running under Thermo Scientific Xcalibur 2.1.0.1140 LC-MS software as needed. The standards used for GLS measurement were purchased from Extrasynthese (Genay Cedex France). This analysis was conducted in triplicate.

5.3.3.9. Moisture and Ash Content

The moisture content of the sample was defined by hot air oven drying of ~2 g of samples at (80 °C) for 24 h (AACC, 2010). Meanwhile, ash content was measured by combusting 5 g of each sample at 575 °C for 7 h using Thermolyne F1730 Heat-treat oven [serial No. 15547; Volts 230; cycles 60; phase 1] (Sluiter et al., 2005). These analyses were conducted in triplicate.

5.3.4. Statistical Analysis

Microsoft® Excel® for Office 365 MSO 64-bit and R version 1.2.5001 (Boston, MA) were used in the statistical analysis. The statistically significant differences among and within group variances determined by one-way ANOVA at $p \leq 0.05$ level of significance.

5.4. Results and Discussion

5.4.1. Oil Content and Composition

After the sprouting period, hulls were separated manually from sprouts by using hand and gentle agitation in water. We observed that there were about 1% of the seeds that did not germinate, and some seeds took little longer than majority of seeds to sprout, labelled as "delayed sprouts" herein. Delayed sprouts generally retained the hulls and made it difficult to completely remove them, resulting in two sprout fractions, 1) dehulled sprouts; 2) mixed fraction, consisting primarily of hulls, ungerminated seeds, and delayed sprouts. The mass distribution of dehulled sprouts, and MF was found to be 63.17% and 29.34%, respectively, whereas 8.14% was lost during the sprouting and dehulling mechanism.

Total oil contents of raw canola seed, dehulled sprouts, and MF are shown in Table 5.1. Dehulled sprouts had significantly high level of oil as compared to raw seed meal. This increase in oil content of the sprouts is due to removal of hulls from sprouts which led to the concentration of oil in sprouts. Because MF consisted of ungerminated seeds and delayed sprouts in addition to hulls, there was a significant amount of oil present (9.64%). The short sprouting time (72 h) limited the reduction of oil content that was seen in previous canola seed sprouting studies (Dawood et al., 2013; Bhardwaj and Hamama, 2009a; Barthet and Daun, 2005). A study conducted by Zhang et al. (2004) on the germination and seedling growth of canola seed under illuminated and dark environments showed that oil content did not change up to 2 days of sprouting. In our sprouting process, oil content stayed stable up to 3 days, which positively indicated that the hydrolysis speed of oil can be affected by growth conditions such as temperature, light, moisture, and sprouting time.

Sprouting, however had significant impact on the free fatty acid content of the resulting oils. For example, oil extracted from sprouts and MF had significantly higher levels of free fatty acid (FFA) as compared to the raw seed oil (1.02%) (Table 5.1). This FFA increase is attributed to lipolytic enzymes causing the hydrolysis of triacylglyceride to FFA during sprouting (Barthet and Daun, 2005). The sprout harvesting time was initiated at 3 days of sprouting where the hulls were manually separated from sprouts. According to Trelease and Doman (1984), lipid degradation proceeds slowly up to 2 days post imbibition, then rapid degradation occurs from 3 to 8 days. Barthet and Daun (2005) reported that FFA of 48 h canola sprouts was higher than raw seed, but still within the industry guidelines (FFA <1%). Thus, early harvesting sprout (1~2 days) where the activity of lipolytic enzymes is still low would be effective with little change in the nutritional composition. Alternatively, high FFA containing oil could either be processed to remove FFA prior to its use in food application or could also be used for other industrial applications such as energy source in animal diets, and biodiesel production (NCBI, 2022; Plascencia et al., 1999; Haas, 2005).

The effect of sprouting process on the oil profile is shown in Table 5.1. Total saturated fatty acid (SFA) of sprout oil decreased by 1.9% compared to raw seed oil. Fatty acid (C14:0) 0.07%, (C20:0) 0.66%, (C22:0) 0.44%, (C23:0) 0.01%, (C24:0) 0.23% in sprout oil were slightly higher than raw seed oil 0.06, 0.65, 0.36, 0.00 and 0.19%, respectively. Fatty acid (C16:0) 4.38% and (C18:0) 1.68% in sprout oil were lower compared to raw seed oil 4.54 and 1.82%, respectively. In terms of monosaturated fatty acid, sprout oil increased by 0.47% compared to raw seed oil. Sprout oil showed a slight increase in elaidic acid (C18:1n9t) 0.03%, oleic acid (C18:1) 58.50%, and gondoic acid

(C20:1n9) 1.12% compared to raw seed oil 0.02, 57.22 and 1.07%, successively. A reduction was noticed in (C16:1n7) 0.26%, nervonic acid (C24:1n9) 0.08%, cis-vaccenic acid (C18:1n7c) 2.55% compared to raw seed oil 0.28, 0.08 and 2.55%, respectively. Regarding total polyunsaturated fatty acid, sprout oil exhibited 4.4% increase compared to raw seed oil. Fatty acid (C22:2n6) 0.03%, linoleic acid (C18:2n6) 20.27%, (C20:3n3) 0.01%, arachidonic acid (C20:4n6) 0.04%, EPA (C20:5n3) 0.03% a-linolenic acid (C18:3n3) were marginally higher than raw seed oil 0.01, 19.27, 0.00%, 0.03, 0.00 and 7.47%, consecutively. The three-day sprouting process did not have a major effect on the oil profile. The slight reduction in SFA accompanied by an slight increase in unsaturated fatty acids is attributed to the chemical dehydrogenation reactions that successively alters saturated fatty acid into unsaturated fatty acid with the same number of carbons during sprouting, [for example, Stearic acid (C18:0) → oleic acid (C:18:1) → linoleic acid (C18:2) → linolenic acid (C18:3)] (Assa et al., 2010). Our results were in contradiction with Dawood et al. (2013) who reported increases in saturated fatty acids (3 folds increase) and decreases in unsaturated fatty acid (12.5%) of canola seed (low erucic acid variety) after soaking and sprouting. According to Barthet and Daun (2005), there is no difference in fatty acid composition of oil extracted from canola sprouts grown on wet paper towels for 48 h as compared to raw seed. This indicates that an optimal sprouting process can limit the degradation of oil constituents.

Table 5.1: Oil analysis of canola seed, sprout, and mixed fractions

Oil Analysis	Seed fractions		
	Seed oil	**Sprout oil**	**MF oil**
Oil content of meal (%, db)	34.50±0.16^{B}	38.43±0.16^{A}	9.64±0.05^{C}
Free Fatty Acid (%, as is)	1.02±0.07^{C}	17.85±0.15^{A}	13.09±0.13^{B}
Oil profile (%)	Fat > 98.5	Fat > 98.5	Fat > 98.5
C14:0	0.06±0.00^{A}	0.07±0.00^{A}	0.08±0.01^{A}
C15:0	0.03±0.01^{B}	0.03±0.00^{B}	0.05±0.00^{A}
C16:0	4.54±0.14^{B}	4.38±0.02^{B}	5.33±0.16^{A}
C17:0	0.05±0.01^{AB}	0.05±0.00^{B}	0.06±0.00^{A}
C18:0	1.82±0.05^{B}	1.68±0.02^{C}	1.93±0.02^{A}
C20:0	0.65±0.02^{A}	0.66±0.01^{A}	0.65±0.01^{A}
C21:0	0.05±0.01^{A}	0.05±0.00^{A}	0.03±0.00^{B}
C22:0	0.36±0.01^{B}	0.44±0.00^{A}	0.39±0.01^{AB}
C23:0	0.00±0.00^{A}	0.01±0.00^{A}	0.01±0.00^{A}
C24:0	0.19±0.01^{A}	0.23±0.01^{A}	0.23±0.01^{A}
Total SFA	7.75±0.23	7.6±0.15	8.76±0.17
C16:1n7	0.28±0.01^{B}	0.26±0.00^{B}	0.54±0.02^{A}
C17:1n7	0.06±0.00	0.06±0.00	0.06±0.00
Elaidic acid [C18:1n9t]	0.02±0.00^{B}	0.03±0.00^{AB}	0.04±0.00^{A}
Oleic acid [C18:1]	57.22±1.83^{A}	58.50±0.21^{A}	52.96±0.70^{B}
Cis-vaccenic acid [C18:1n7c]	3.51±0.12^{B}	2.55±0.19^{C}	5.73±0.12^{A}
Gondoic acid [C20:1n9	1.07±0.03^{A}	1.12±0.01^{A}	0.98±0.01^{B}
Nervonic acid [C24:1n9]	0.15±0.01^{A}	0.08±0.10^{A}	0.13±0.09^{A}
Erucic acid [C22:1n9]	0.02±0.00	0.02±0.00	0.02±0.00
Total MUSFA	62.33±1.98	62.62±0.14	60.46±0.71
C20:2	0.08±0.01^{A}	0.08±0.00^{A}	0.08±0.00^{A}
C22:2n6	0.01±0.01^{A}	0.03±0.00^{A}	0.03±0.00^{A}
Linoleic [C18:2n6]	19.91±0.64^{B}	20.27±0.01^{AB}	21.39±0.26^{A}
g-Linolenic [C18:3n6]	0.04±0.00	0.04±0.00	0.04±0.00
a-Linolenic [C18:3n3]	7.47±0.25^{B}	8.25±0.01^{A}	6.90±0.07^{C}
C20:3n3	0.00±0.00^{A}	0.01±0.00^{A}	0.01±0.00^{A}
Arachidonic [C20:4n6]	0.03±0.00^{A}	0.04±0.00^{A}	0.04±0.00^{A}
EPA [C20:5n3]	0.00±0.00^{A}	0.03±0.00^{A}	0.03±0.00^{A}
Total PUFA	27.54±0.88	28.75±0.01	28.52±0.33

Letters indicate a significant difference among different fractions at $p \leq 0.05$. Measurement for (C14:1n5), (C15:1n5), (Linoelaidic [C18:2n6t]), (Stearidonic [C18:4n3]), (Homo-g-linolenic [C20:3n6]), (C20:4n3), (C21:5n3), (Clupanodonic [C22:5n3]), (DHA [C22:6n3]) were zero for all seed fractions. MF: mixed fractions (hulls+ungerminated seed+delayed sprouts); SFA: saturated fatty acid; MUSFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid.

5.4.2. Protein Content and Amino Acid Profile

The sprouting and dehulling process generated two fractions: 1) sprouts (~0.63 sprouts/g seeds), and 2) MF (~0.3 MF/g seeds). Protein content as measured prior to defatting showed that sprouting led to an increase in protein content of the canola seeds by 10.1% when compared to raw full fat seed (FFSE) (Figure 5.3). Full fat MF (FFMF) also had significant amount of protein present (21.46%, db); however, protein content was lower than that of FFSE and FFSP as bulk of this fraction was primarily hull (Figure 5.1). As expected, protein content of all three substrates further increased upon oil removal. Two possible reasons for this could be the emission of non-protein nutrients as carbon dioxide due to carbohydrate metabolization, or concentration effects of dehulling (removal of protein poor hull fraction). These protein measurements are consistent with previously reported increases in protein levels in canola sprouts (Barthet and Daun, 2005), and germinated and dehulled rice (Moongngarm and Saetung, 2010).

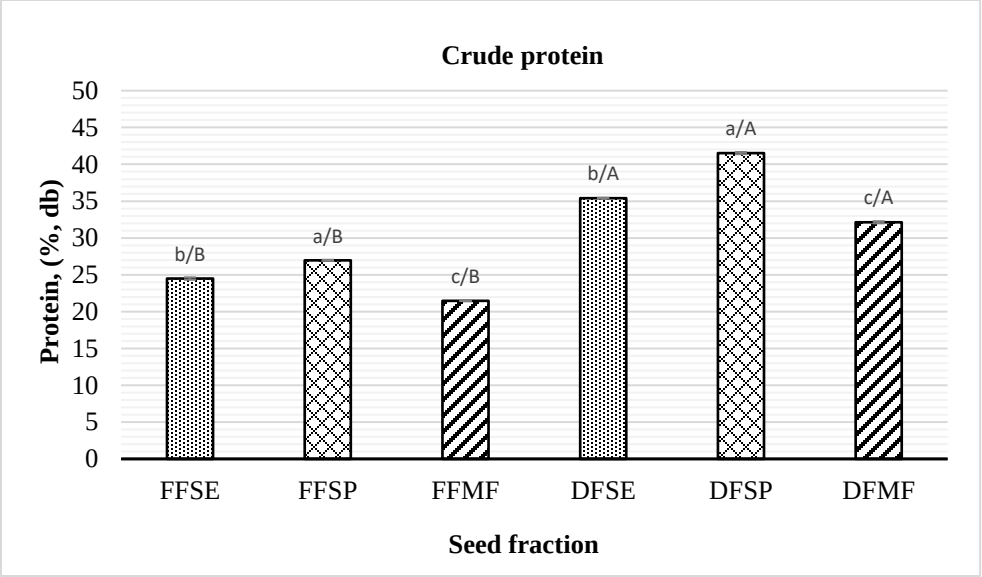

Figure 5.3: Total crude protein of full fat and defatted canola seed, sprouts, and hull fraction. Uppercase letters: comparison of significance difference between the full fat and defatted fractions (FFSE vs DFSE; FFSP vs DFSP, FFMF vs DFMF) at $p \leq 0.05$. Lowercase letters: comparison of significance difference among full fat fractions (FFSE, FFSP and FFMF) and defatted fractions (DFSE, DFSP, and DFMF) at $p \leq 0.05$. FFSE: full fat seeds; FFSP: full fat sprouts; FFMF: full fat mixed fractions; DFSE: defatted seed; DFSP: defatted sprouts; DFMF: defatted mixed fractions.

Amino acid profiles of DFSE and DFSP revealed that sprouting led to an increase in total amino acids of canola meal by 5.9% when compared to DFSE (Table 5.2). Interestingly, all essential amino acid of DFSP (Threonine, valine, methionine, isoleucine, leucine, phenylalanine, lysine, and histidine) increased by 13.9, 22.2, 2.9, 17.4, 14.5, 16.4, 12.7 and 10.4%, respectively compared to DFSE, except for tryptophan that was constant. Likewise, other DFSP amino acids (Taurine, aspartic acid, serine, alanine, tyrosine, ornithine) exhibited an increase by 10.0, 23.6, 10.8, 27.5, 22.4 and 50.0%, respectively while hydroxylysine was 0.44% compared to DFSE (0.07%). However, DFSP showed a reduction in hydroxyproline, glutamic acid, proline, lanthionine, glycine, cysteine, and arginine by 12.5, 15.6, 9.4, 63.6, 2.9, 30.0 and 5.1%, respectively compared to DFSE. During sprouting process, DI water was the only supplement for sprout growth, thus the absence of external nitrogen source during sprouting likely resulted in the breakdown of

reserve proteins for the interconversion and utilization of amino acids (Boulter and Barber, 1963). Therefore, the noticed reduction and increase of amino acids can be attributed to the biosynthesis of new amino acids during sprouting. These results showed the positive effect of canola seed sprouting on total amino acid composition, especially the essential amino acids that cannot be synthesized in the human body (Akram et al., 2011). Similar results were obtained by Chiou et al. (1997) who reported an increase in the total free amino acid of peanut kernels with sprouting time. Wang et al. (2005a) reported that sprouting of peanut kernels for 9 days resulted in a significant increase in total free amino acids. Hung et al. (2012) reported an increase in the total free amino acid of 48 h-germinated wheat compared to ungerminated wheat.

Table 5.2: Amino acid profile of defatted canola seed and sprouts (%, dry basis)

Amino Acid profile	DFSE	DFSP
Taurine [a]	0.10±0.02^{A}	0.11±0.01^{A}
Hydroxyproline	0.32±0.04^{A}	0.28±0.02^{A}
Aspartic Acid	2.33±0.01^{B}	2.88±0.02^{A}
Threonine [b]	1.44±0.01^{B}	1.64±0.03^{A}
Serine	1.20±0.01^{B}	1.33±0.07^{A}
Glutamic Acid	5.70±0.05^{A}	4.81±0.07^{B}
Proline	2.02±0.04^{A}	1.83±0.03^{B}
Lanthionine [a]	0.11±0.01^{A}	0.04±0.01^{B}
Glycine	1.71±0.01^{A}	1.66±0.01^{B}
Alanine	1.49±0.01^{B}	1.90±0.02^{A}
Cysteine	0.89±0.00 ^{A}	0.62±0.00^{B}
Valine [b]	1.80±0.01^{B}	2.20±0.02^{A}
Methionine [b]	0.70±0.01^{A}	0.72±0.01^{A}
Isoleucine [b]	1.44±0.01^{B}	1.69±0.02^{A}
Leucine [b]	2.35±0.02^{B}	2.69±0.02^{A}
Tyrosine	0.98±0.01^{B}	1.20±0.01^{A}
Phenylalanine [b]	1.40±0.01^{B}	1.63±0.01^{A}
Hydroxylysine	0.07±0.00^{B}	0.44±0.01^{A}
Ornithine [a]	0.02±0.00^{B}	0.03±0.00^{A}
Lysine[#]	2.13±0.01^{B}	2.40±0.02^{A}
Histidine [b]	0.96±0.01^{B}	1.06±0.00^{A}
Arginine	2.17±0.02^{A}	2.06±0.01^{B}
Tryptophan [b]	0.42±0.02^{A}	0.42±0.01^{A}
Total	31.76±0.10^{B}	33.65±0.20^{A}

Uppercase letters indicate a significant difference among different fractions at $p \leq 0.05$. [a] Non-proteinogenic amino acid; [b] Essential amino acids for humans. DFSE: defatted seed; DFSP: defatted sprouts

5.4.3. Fiber Content and Ash Content

Crude fiber (CF), acid detergent fiber (ADF), and neutral detergent fiber (NDF) of FFSE, DFSE, FFSP, DFSP, FFMF and DFMF are presented in Table 5.3. All sprout hulls removed at the end of sprouting process was present in the MF, hence MF reflects the high value of CF, ADF, and NDF before and after defatting. Separation of hulls from sprouts was done by skimming the surface floating hulls after dumping grown sprouts into a water

filled bucket without applying any mechanical agitation, which resulted in the presence of residual number of hulls in the sprouts. Even though FFSP and DFSP contained some residual hulls, they showed a high reduction in CF, ADF, and NDF compared to FFSE and DFSE. FFSP had lower CF, ADF, and NDF by 56.3, 59.5 and 56.7%, respectively compared to FFSE. Similarly, DFSP had less CF, ADF, and NDF by 46.2, 34.3 and 43.4%, successively compared to DFSE. The sprouting of canola seed enabled the removal of hulls to a great extent. However, applying mechanical agitation or separator is required to further purify sprouts from hulls, thereby providing sprout meal containing low level of fiber. Our previous study (Alhomodi et al., 2021c) showed that CF, ADF, and NDF decreased with sprouting time, which indicates that CF, ADF, and NDF of FFSP and DFSP in this study were due to the presence of hulls. This is in a good agreement with Ongol et al. (2013) who noticed 25 and 23% fiber reduction in two germinated maize varieties (Zm 607 and Tamira pool A9) for 72 h, respectively.

In terms of ash content (Table 5.3), a marginal reduction in FFSP (3.70%) and DFSP (5.47%) was noticed compared to FFSE (3.78%) and DFSE (5.55%). FFMF (3.62%) and DFMF (5.23%) had slight further decrease compared to other fractions. This slight reduction compared to raw seed can be attributed to mineral infiltration during imbibition and sprouting (Ghavidel and Prakash, 2007). Similar ash reduction was previously reported in germinated green gram, cowpea, lentil, chickpea (Ghavidel and Prakash, 2007) and barley (Youssef et al., 2013).

Table 5.3: Fiber and ash content of full fat and defatted canola seed, sprout, and hull

Fraction	Ash	Fiber (%, dry basis)		
		CF	ADF	NDF
FFSE	3.78±0.11$^{a/B}$	12.09±0.13$^{b/B}$	18.69±0.67$^{b/B}$	21.85±0.86$^{b/B}$
FFSP	3.70±0.03$^{a/B}$	5.28±0.05$^{c/B}$	7.57±0.13$^{c/B}$	9.46±0.08$^{c/B}$
FFMF	3.62±0.07$^{a/B}$	16.23±0.08$^{a/B}$	27.67±0.30$^{a/B}$	30.81±0.23$^{a/B}$
DFSE	5.55±0.04$^{a/A}$	15.13±0.15$^{b/A}$	23.35±0.29$^{b/A}$	29.63±1.86$^{b/A}$
DFSP	5.47±0.02$^{a/A}$	8.14±0.11$^{c/A}$	15.35±0.88$^{c/A}$	16.78±0.24$^{c/A}$
DFMF	5.23±0.09$^{b/A}$	25.30±0.16$^{a/A}$	42.75±0.15$^{a/A}$	47.57±1.18$^{a/A}$

Uppercase letters indicate a significance between full fat and defatted within fraction. Lowercase letters indicate a significance among fractions for each full fat and defatted. CF: crude fiber; ADF: acid detergent fiber; NDF: neutral detergent fiber; FFSE: full fat seed; DFSE: defatted seed; FFSP: full fat sprout; DFSP: defatted sprout; FFMF: full fat hulls, ungerminated seed, and delayed sprouts; DFMF: defatted hull, ungerminated seed and delayed sprouts.

5.4.4. Phytic Acid Content

Phytic acid content of FFSP and DFSP decreased by 28.8 and 25.9% compared to FFSE and DESE, respectively (Figure 5.4). FFMF (0.89 g/100 g) and DFMF (1.32 g/100 g) showed lower phytic content compared to other fractions (sprouts and seed). Phytic acid was reduced during sprouting owing to increasing of phytase activity (Centeno et al., 2001; Larsson and Sandberg, 1992). Similarly, phytic acid reduction was reported in germinated rice (Moongngarm and Saetung, 2010), green gram, cowpea, lentil, chickpea (Ghavidel and Prakash, 2007) and oat seed (Tian et al., 2010).

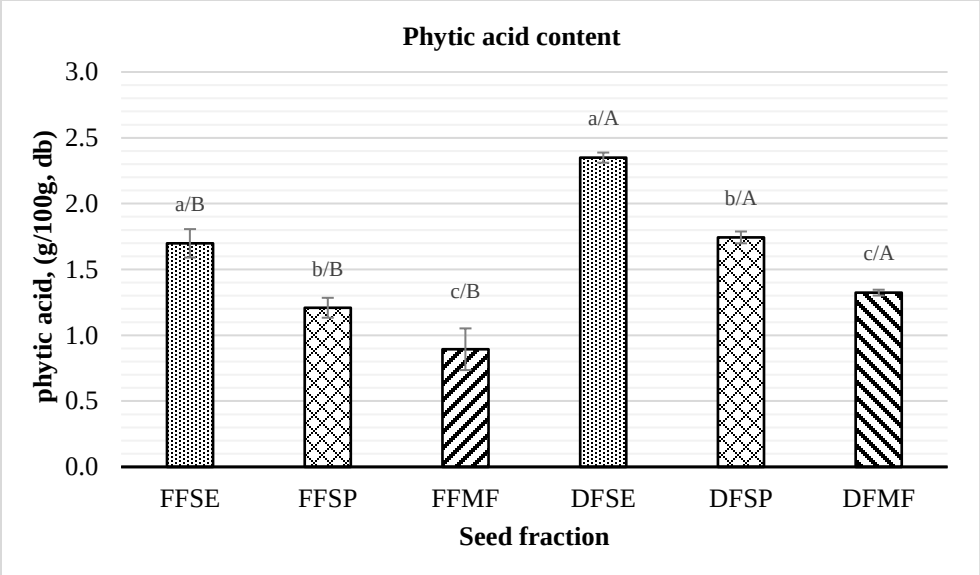

Figure 5.4: Phytic acid content of full fat and defatted canola seed, sprout, and hull fractions. Uppercase letters: comparison of significance difference between the full fat and defatted fractions (FFSE vs DFSE; FFSP vs DFSP, FFMF vs DFMF) at $p \leq 0.05$. Lowercase letters: comparison of significance difference among full fat fractions (FFSE, FFSP and FFMF) and defatted fractions (DFSE, DFSP, and DFMF) at $p \leq 0.05$. FFSE: full fat seeds; FFSP: full fat sprouts; FFMF: full fat mixed fractions; DFSE: defatted seed; DFSP: defatted sprouts; DFMF: defatted mixed fractions.

5.4.5. Total Soluble Sugars

Total soluble sugars (stachyose, raffinose, arabinose, galactose-mannose, and glucose) of FFSE, DFSE, FFSP, DFSP, FFMF, DFMF are shown in Figure 5.5. FFSP and DFSP had lower soluble sugar content by 27.5 and 5.5% compared to FFSE and DFSE, respectively. This soluble sugar reduction can be attributed to the leakage of soluble sugar during imbibition (Nonogaki et al., 2010) or the utilization of sugars as carbon source during sprouting (Abrahamsen and Sudia, 1966). The amount of soluble sugar present in FFMF and DFMF came from the ungerminated seeds and delayed sprouts that was added to hull fractions. Mubarak (2005) reported total carbohydrate reduction in dehulled, soaked and germinated mung bean seed. Parameswaran and Sadasivam (1994) reported a decrease in total soluble sugar during the sprouting of proso millet (*Panicum miliaceum*).

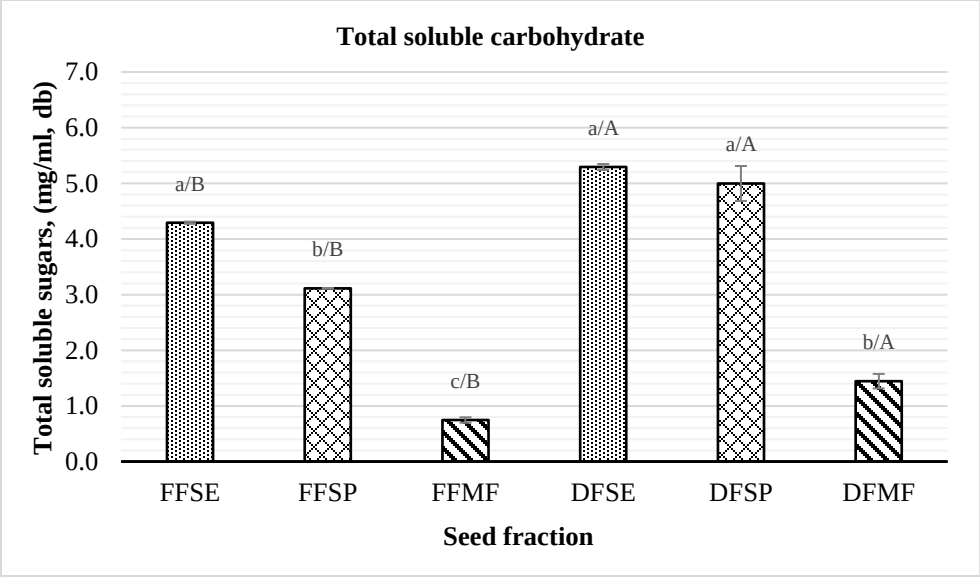

Figure 5.5: Total soluble sugar content of full fat and defatted canola seed, sprout, and hull fractions. Uppercase letters: comparison of significance difference between the full fat and defatted fractions (FFSE vs DFSE; FFSP vs DFSP, FFMF vs DFMF) at $p \leq 0.05$. Lowercase letters: comparison of significance difference among full fat fractions (FFSE, FFSP and FFMF) and defatted fractions (DFSE, DFSP, and DFMF) at $p \leq 0.05$. FFSE: full fat seeds; FFSP: full fat sprouts; FFMF: full fat mixed fractions; DFSE: defatted seed; DFSP: defatted sprouts; DFMF: defatted mixed fractions.

5.4.6. Total Glucosinolates (GLS)

Figure 5.6 shows the total glucosinolates, which is the sum of progotrin, gluconapolieferin, gluconapin, hydroxyglucobrassicin, glucobrassicanapin, and gluconasturtiin. Total GLS level in FFSP (11.8 µmol/g) and DFSP (13.1 µmol/g) was higher than FFSE (10.1 µmol/g) and DFSE (8.8 µmol/g), respectively. FFMF (5.0 µmol/g) and DFMF (5.5 µmol/g) showed lower GLS content compared to other fractions due to the combination of ungerminated seed and delayed sprouts. The GLS increase in sprouts can be as a result of the biosynthesis of GLS during sprouting beside concentration effects due to the removal of hulls. It is important to note that though sprouting increased the GLS, increase was insignificant as it was lower than the GLS limit set for canola meal (30 µmol/g)(CCC, 2019). Andini et al. (2019) reported that biosynthesis of GLS was induced in three plant species (*Brassuca napus*, and *B. juncea* and *Sinapis alba*) by sprouting.

Another study by Bellostas et al. (2007) showed an increase in the total GLS concentration of red cabbage from germination to 4 day sprouting time. Overall, our results indicate that applicability of the proposed process would largely be governed by the end use of the product. For example, sprouting followed by dehulling greatly influenced the reduction of major ANFs like fibers and phytic acid, but simultaneously increased the GLS content. While low fiber meal is anticipated to have wider industrial applications, but depending on its end use, GLS may have to be either further reduced by the fermentation for animal feed (Alhomodi et al., 2021c; Alhomodi et al., 2021b) or extracted and used in therapeutic applications (Melrose, 2019).

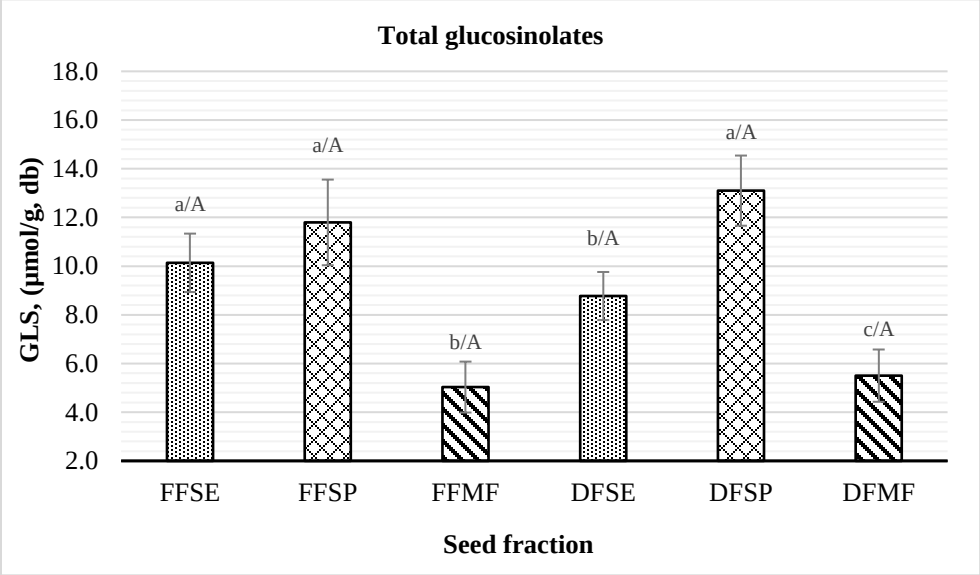

Figure 5.6: Total glucosinolate content of full fat and defatted canola seed, sprout, and hull fractions. Uppercase letters: comparison of significance difference between the full fat and defatted fractions (FFSE vs DFSE; FFSP vs DFSP, FFMF vs DFMF) at $p \leq 0.05$. Lowercase letters: comparison of significance difference among full fat fractions (FFSE, FFSP and FFMF) and defatted fractions (DFSE, DFSP, and DFMF) at $p \leq 0.05$. FFSE: full fat seeds; FFSP: full fat sprouts; FFMF: full fat mixed fractions; DFSE: defatted seed; DFSP: defatted sprouts; DFMF: defatted mixed fractions.

5.5. Conclusion

The present study shows that sprouting and hull removal of canola seed can potentially provide nutritive meal for food and feed applications. Oil was not affected significantly during 3-day sprouting, but oil quality in terms of free fatty acid content was high. Some challenges experienced while conducting this experiment were (i) delay in some seed to germinate, (ii) manually separating the hull from the kernels before 3-day sprouting time. Thus, for intact sprout oil profile and meal with low fiber content, we propose that further study should consider, (i) selecting good quality canola seed with high viability; (ii) optimizing sprouting time; (iii) applying mechanical hull separation; (iv) ensuring optimal growth conditions.

Chapter 6. Application of Cocultures of Fungal Mycelium During Solid-State Fermentation of Canola Meal for Potential Feed Application [†]

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6.1. Abstract

In this study, the mono-, bi-, and tri-cultivation of *Aureobasidium pullulans*, *Neurospora crassa* and *Trichoderma reesei* in solid state fermentation were applied to improve the nutritional values of hexane extracted canola meal (HECM) along with the reduction of antinutritional factors for animal feed applications. Static fermentation trials of 50% moisture content HECM were conducted in 500 mL Erlenmeyer flasks for 168 h at 30 °C. The results showed that fungal cultivation had positive effects on the level of protein, fiber and, glucosinolates (GLS). Monoculture of *N. crassa* exhibited the highest protein level of 49%. The combination of *A. pullulans* and *N. crassa* provided the highest reduction of crude fiber (CF), acid detergent fiber (ADF) and neutral detergent fiber (NDF) by 21.9, 1.7 and 9.1%, respectively. Bi-culture of *A. pullulans* and *T. reesei* resulted in the best GLS reduction by 81.3% (0.3 vs 1.6 µmol g^{-1} GLS of uninoculated control). These results indicate that each fungal strain possess different enzymatic ability and selectively can work with other fungi in synergistic relationship for better fungal conversion of canola meal.

6.2. Introduction

Solid-state fermentation (SSF) is generally termed when microorganisms are grown on solid substrates at low moisture content (Lonsane et al., 1985). SSF, however, requires the adequate moisture to enhance microbial growth and metabolic activity (Thomas et al., 2013). Many industrial products such as pigments, organic acids, biopolymers, enzymes and biosurfactants were developed by applying SSF techniques (Thomas et al., 2013). Fungal SSF is considered to be highly efficient as it simulates the natural environment for fungi (Couto and Sanromán, 2006) and therefore fungal mycelia can penetrate and grow on the solid substrates (Ramachandran et al., 2004). SSF also reduces the potential of bacterial contamination because of low moisture content (Pandey et al., 2000; Singhania et al., 2009). Commercially, SSF costs less and requires smaller incubation vessels in contrast to submerged bioprocessing methods (Smits et al., 1993). However, controlling the temperature, moisture, and pH in SSF is a major drawback (Hölker et al., 2004).

In a natural environment, many bioconversions are mediated by mixed microbial cultures (Chen, 2011), for example in forest soils, compost piles, and in the aerobic and anaerobic zones of water (Bader et al., 2010). Mixed microbial cultures have been used successfully in commercial scale, fermentations such as in wastewater treatment plants, for soil remediation, in biogas production, and in the production of traditional foods such as cheese, yoghurt, pickles and in alcoholic beverage production (Bader et al., 2010; Taniguchi and Tanaka, 2004). Khetarpaul and Chauhan (1990b) observed a significant increase in protein and starch digestibility of fermented pearl millet sprouts after co-culturing with yeast and bacteria. Santos et al. (2014a) noticed that strains used in peanut-

soy milk fermentation were more efficient in the use of available carbohydrates and release of metabolites in co-cultured fermentations as compared to single cultured fermentations.

SSF has previously been reported to be used successfully for converting agricultural by-products into higher value products. Substrates used for fungal fermentations and resulted in upgraded nutritional profiles include soybean meal, wheat straw, food waste, industrial wastewater (Pensupa et al., 2013) and canola meal (Croat et al., 2016a; Croat et al., 2016b; Croat et al., 2017).

Canola meal (CM) is a by-product of canola seed oil extraction and contains up to 50% protein on a dry basis (Kimber and McGregor, 1995). CM despite being nutrient enriched commodity (Mariscal-Landín and de Souza, 2008), it remains as one of the most underutilized by-products of the oil processing industry. Only a fraction of produced CM goes into animal feeds (Montoya and Leterme, 2010) as presence of antinutrients such as phenolics, glucosinolates (GLS), phytates, and high fiber content makes this product undesirable (Wu and Muir, 2008; Yoshie-Stark et al., 2008). Research on additional processing and modification of CM has been ongoing. For example, Croat et al., (2016a;2016b; 2017) used combined effect of pretreatment technologies and fungal mechanism to enhance CM composition with low level of ANFs and high level of proteins. Fungal enzymes aid in degrading antinutritional factors and fibers and usually increase protein content. However, high fiber content in CM is still a challenge and further investigation is required to widen its utilization.

Based on insights from commercial and natural systems, we examined the potential of applying co-culture fungal incubation to determine effects on the measurable nutritional value of CM. Fungi and yeast have been regarded as the most appropriate microorganisms

for SSF due to their lower water activity requirement (Thomas et al., 2013). In this study, three fungal strains were evaluated in SSF systems: 1) xylan-degrading fungus *Aureobasidium pullulans* (Y-2311-1) (Chi et al., 2009), 2) lignocellulose-degrading fungus *Neurospora crassa*, (Znameroski et al., 2012), and 3) cellulolytic-degrading fungus *Trichoderma reesei* (Wen et al., 2005). The objectives of the study were: a) to determine if the fungal strains can be grown synergistically on the CM; b) to determine the best combination of fungal strains to achieve full potential of SSF; c) to determine the composition of CM in terms of crude proteins, crude fiber (CF), acid detergent fiber (ADF) neutral detergent fiber (NDF), GLS, phytic acid and total soluble sugars.

6.3. Materials and Methods

6.3.1. Preparation of Feedstocks

Hexane-extracted canola meal (HECM) was obtained from ADM/Specialty Commodities LLC (Fargo, ND). HECM was ground using a coffee grinder prior to use and then stored in sealed buckets at room temperature (~23 °C) throughout the trial period. Dry weight analysis of HECM was conducted by drying ~2 g of ground meal in triplicate at 80 °C for 48 h using a drying oven.

6.3.2. Preparation of Inoculum for Single and Co-culture Fermentation

Three different fungi *Aureobasidium pullulans* (NRRL-Y-2311-1), *Trichoderma reesei* (NRRL-3653), *Neurospora crassa* were acquired from the National Center for Agricultural Utilization Research (Peoria, IL). The potato dextrose agar (PDA) stock culture of each fungal strain was stored in the refrigerator and sub-cultured monthly. The inoculum of each fungus was prepared by transferring 8 mm plug of 5-day fungal colony grown on PDA into autoclaved 250 mL Erlenmeyer flasks containing 100 mL working

volume of a glucose yeast extract (GYE) medium consisting of 5% glucose and 0.5% yeast extract with pH of 5.5. The flasks were then incubated for 48 h at 30 °C in rotary shaker at 150 rpm.

6.3.3. Examination of Interactions among Used Fungi in Co-culture Systems

Dual interactions of all possible combination of used fungal species were tested on PDA media to screen for antagonism. A PDA plate was divided into two, and a three 8-mm disc of actively growing mycelium for each fungus was place into each half, while a single cell fungus (*A. pullulans*) was swapped instead. Plates were then sealed with parafilm and incubated for 7 days at 30 °C. The morphological growth pattern of these fungal strains on the PDA plates were observed daily.

6.3.4. Preparation of Solid-State Media

SSF was conducted in 500 mL Erlenmeyer flasks with a working volume of 80 mL. The solid loading rate (SLR) of 50% dry weight of HECM and 50% moisture was used during SSF. The pH was adjusted to 5.5±1.0 with 5 M sulfuric acid. Flasks were then covered with foam plugs and aluminum foil and were autoclaved at 121 °C for 20 min.

6.3.5. Single Culture Fermentations

Prepared flasks (6) of HECM were inoculated with 4 mL of a 48-h culture of *A. pullulans* of which three flasks were withdrawn immediately for 0 h sample processing, whereas remaining three inoculated flasks were incubated statically at 30 °C for 168 h. These incubation conditions were chosen based on the previous study on canola meal fermentation by Croat et al., (2016a). Daily fungal colonization was monitored visual observation to determine any potential contamination as well as to ensure fungal growth during the incubation process. The pH of 0 h and 168 h of fermented and unfermented

control flasks were recorded, and the solid residues were collected and then dried for residual sugar, crude protein, CF, ADF, NDF, phytic acid and GLS analyses. The exact process as described was also used with *N. crassa* and *T. reesei* inocula. Control (uninoculated) trials were also conducted in triplicate using the same process but without inoculation.

6.3.6. Co-culture Fermentations

Flasks for coculturing were prepared according to the method described in the section 2.3. Prepared flasks of HECM were inoculated in triplicate with four combination of fungi (1) *A. pullulans+ T. reesei* (2) *A. pullulans+ N. crassa*, (3) *T. reesei+ N. crassa* (4) *A. pullulans+ N. crassa+ T. reesei)*. For the combination 1 to 3, 4 mL of inoculum containing 2 mL each of 48-h culture of respective fungal strain was used. Whereas for the combination 4, total of 6 mL inoculum containing 2 mL each of 48 h culture of *A. pullulans*, *N. crassa*, and *T. reesei* was used. Flasks were subsequently incubated statically at 30 °C for 168 h, and three 0 h replicates for each fungus were collected. Thereafter, all flasks treated as mentioned before for analysis.

6.3.7. Crude Protein Content Determination

A LECO model FP528 (St. Joseph, MI, USA) was used for protein analysis. Approximately 0.2 g of dried and finely ground samples was uploaded onto a machine and the total nitrogen gas level emerged after samples were combusted was measured (AOAC Method 990.03) (Horwitz and Latimer, 2006). A conversion factor of 6.25 was used to calculate the protein percentage from the nitrogen content of the samples. Moisture content of the sample was adjusted to determine the protein on dry weight basis. All the analysis on proteins were duplicated.

6.3.8. Total Glucosinolate (GLS) Analysis

GLS content of different samples were measured by HPLC according to the method of (Berhow et al., 2013) using a modified method developed by Betz and Fox (1994). The extract was run on a Shimadzu (Columbia, MD) HPLC System (two LC 20AD pumps; SIL 20A autoinjector; DGU 20As degasser; SPD-20A UV-VIS detector; and a CBM-20A communication BUS module) running under the Shimadzu LC solutions Version 1.25 software. The column was a C18 Inertsil reverse phase column (250.0 mm X 4.6 mm; RP C-18, ODS-3, 5u; GL Sciences, Torrance, CA). The GLS were detected by monitoring at 237 nm. The initial mobile phase conditions were 12% methanol/88% aqueous 0.005 M tetrabutylammonium bisulfate (TBS) at a flow rate of 1 mL.min-1. After injection of 15 µl of sample, the initial conditions were held for 2 min, and then up to 35% methanol over another 20 min, then to 50% methanol over another 20 min. then up to 100% methanol over another 10 min. Standards, prepared in this lab or purchased, were run to determine retention times and extinction coefficients. The presence of glucosinolates were confirmed as needed by negative ion mode analysis on an Thermo Electron LTQ Oribitrap Discovery Mass Spectrometer (a linear ion trap MS, coupled to a high precision electrostatic ion trap MS) with an electrospray ionization (ESI) source, and a Thermo Scientific ACCELA series HPLC system, all running under Thermo Scientific Xcalibur 2.1.0.1140 LC-MS software.

6.3.9. Phytic Acid Analysis

Phytic acid was determined according to the Megazyme Phytic acid/Total phosphorus kit (Megazyme, 2017). One-gram sample was mixed with 20 mL HCl of 0.66 M concentration in a 50 mL glass container. Flasks were covered with foil and stirred vigorously in a shaker (SCILOGEX MS-M-S10) for overnight at room temperature (~22

°C). One mL of the extract was transferred into a 1.5 mL microtube and centrifuged at 13,000 rpm for 10 min. 0.5 mL of the extract supernatant was transferred to a new tube and neutralized with 0.5 mL of (0.75 M) NaOH. A control was run using oat flour powder as a substrate. The kit comes with prepared phytase and alkaline phosphatase along with the buffers for the reaction. The reaction was conducted at 40 ℃ with phytase for 10 min and alkaline phosphatase for 15 min. The enzyme reaction was stopped by the addition of trichloroacetic acid 50% w/v. Phosphorus was quantified using a colorimetric determination using ascorbic acid/sulfuric acid and ammonium molybdate mixed in a 5:1 ratio to create the color reagent. One ml of the sample was mixed with the color reagent and incubated for 1 hour at 40 ℃. Reactions were read on a microplate reader at 655 nm.

6.3.10. Fiber

Fiber analysis [crude fiber (CF), neutral detergent fiber (NDF), and acid detergent fiber (ADF)] was conducted by Agricultural Experiment Station Chemical Laboratories at the University of Missouri-Columbia. CF was tested according to AOAC official method 978.10 2006. NDF was conducted according to JAOAC v. 56, 1352-1356, 1973, while ADF was tested using AOAC 973.18 (A-D) 2006 official method.

6.3.11. Total Soluble Sugar Analysis

Total soluble sugars of hexane extracted meal before and after fermentation were determined based on method illustrated by (Croat et al., 2016a). One g of dried sample was blended with 9 mL of deionized water and stored at 4 °C for 24 h. Thereafter, supernatant was obtained by centrifugation at 10,000 rpm for 10 min, then poured into a 2-mL microcentrifuge tube and frozen overnight. Next, samples were thawed and followed by centrifugation at 10,000 rpm for 10 min to eliminate any impurities. Supernatant was

filtered into a high-performance liquid chromatography (HPLC) vial using a 0.22-μm filter attached to a syringe. A HPLC system (Agilent Technologies, Santa Calara, CA, USA) equipped with a refractive index detector (Model G1362A) and a waters size-exclusion chromatography column (SugarPak column I with pre-column module, Waters Corporation, Milford, MA, USA) was used (mobile phase [50 ppm EDTA in deionized water], flow rate of 0.5 mL/min, column temperature of 85 °C). Sugar standards of stachyose, raffinose, glucose, galactose, galactose-mannose, and arabinose were prepared at various concentration to set up calibration curve of concentration vs. HPLC area as previously described by (Karunanithy et al., 2012).

6.3.12. Statistical Analysis

The effect of single and co-culture fermentation on total protein content, crude fiber, ADF, NDF, total GLS, total soluble sugars, phytic acid level were analyzed using a combination of Microsoft® Excel® and R version 1.2.5001. The differences between the means were analyzed using one-way ANOVA and considered significant when $p \leq 0.05$. Results were expressed as means with standard deviation.

6.4. Results and Discussion

6.4.1. Crude Protein Content of Fermented HECM

Figure 6.1 presents the results of the analysis for the protein levels of raw HECM, uninoculated control, and fermented HECM (mono-, bi-, and tri- fungal inoculum combinations). The protein content of the HECM increased significantly from 42.1 to 44.5% after autoclaving and incubation for 168 h (Figure 6.1). Among three fungal strains used during monoculture fermentation, *N. crassa* showed the highest protein levels at (49.9%) followed by *A. pullulans* and *T. reesei* with respective protein levels of 47.4 and

44.7%. These results were in contrast to the results of Croat et al. (2016a) who reported 23% increase in protein content of *T. reesei* fermented HECM. This difference can be attributed in part to the different properties of the substrates used in these studies such as proximate composition, agronomic differences, particle size, water holding capacity, and available surface area. It has been shown that the levels of hemicellulases and cellulases in the fungal cultures are inducible and influenced by the nature of the substrate used in the fermentation (Xiros et al., 2008). The researchers elucidated the differences among the studied fungi in terms of enzyme production and subsequent modified protein levels. The results of co-culture fermentation using bi-and tri-fungal inoculums showed no further improvement in the protein levels of HECM as compared to monoculture fermented HECM (Figure 6.1). Bi-fungal inoculum containing *A. pullulans* and *N. crassa* was found to be the best combination to produce the highest protein level of 49.6%. *T. reesei* in combination with *A. pullulans* and/or *N. crassa* did not yield higher protein levels. Overall, however, the total protein levels in monocultured *N. crassa*, bi-cultured *A. pullulans* + *N. crassa*, and tri-cultured *A. pullulans* + *N. crassa* + *T. reesei* treatments increased significantly ($p \leq 0.05$) by 18.5, 17.8 and 10.7%, respectively when compared to unfermented HECM. In dual culture fermentation, these results indicated that *N. crassa* consistently performed well individually and in combination with other strains. *T. reesei* cultures produced the lowest protein level irrespective of mono-or co-culture conditions. For example, the protein level dropped by 4.8 and 4.9%, respectively for *N. crassa* and *A. pullulans* when they were combined with *T. reesei.* Unlike *A. pullulans* and *N. crassa*, the loss of dry matter was low with individual inoculation of *T. reesei.* The complete degradation of biomass requires the complementary interactions of different enzymes.

Lack of a critical enzymes could substantially slow down the hydrolysis process, resulting in the low biomass production. Therefore, co-cultivation of fungi is often used as an effective approach to solve enzyme deficiency problems (Ahamad and Vermette, 2008). *Trichoderma* derived cellulases are usually deficient in β-glucosidase (Wen et al., 2005), while *N. crassa* have high β-glucosidase activity (Macris et al., 1989), and *A. pullulans* are known to be high in xylanases (Ohta et al., 2010), cellulases (Leite et al., 2007), and proteases (Chi et al., 2007). Therefore, when *T. reesei* was co-cultivated either with *N. crassa* or *N. crassa* and *A. pullulans*, there was no accumulation of soluble sugars (Figure 6.2, discussed later), which indicates the complete hydrolysis and subsequent fermentation of sugars into high protein biomass highlighting the importance of co-culture fermentation. Olukomaiya et al. (2020b) reported that solid state fermentation of CM with *Aspergillus sojae, Aspergillus ficuum* and their co-cultures for 7 days resulted in protein increase by 4.6, 0.8 and 1.2%, respectively compared to unfermented CM. Hu et al. (2016) observed a 5.9% increase in crude protein content of fermented rapeseed meal by *Bacillus subtilis, Candida utilis* and *Enterococcus faecalis*. The increase in crude protein may stem from an increase in mycoprotein content (Hölker et al., 2004) or as a result of a decline in dry matter content due to the utilization of substrate by microorganisms as carbon and energy source (Olukomaiya et al., 2019).

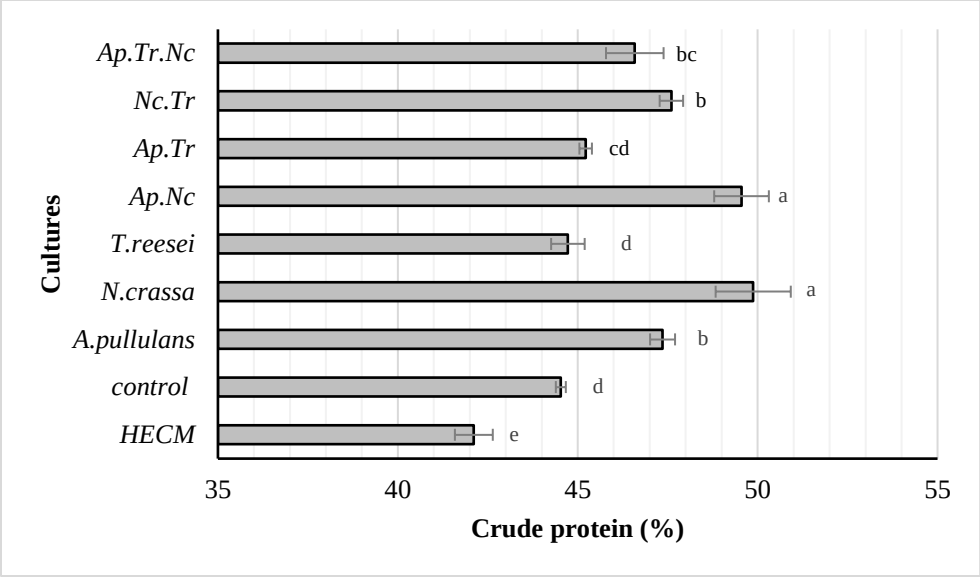

Figure 6.1: Crude protein level for HECM and 168h fermented samples. Significant differences ($p \leq 0.05$) among different treatments are indicated by letters. Control is uninoculated. HECM: hexane extracted canola meal, Ap: *Aureobasidium pullulans*, Nc: *Neurospora crassa*, Tr: *Trichoderma reesei*.

6.4.2. Antinutritional Components

The total solid recovery (TSR), crude fiber (CF), acid detergent fiber (ADF), neutral detergent fiber (NDF), total glucosinolates (GLS) and phytic acid (PA) are given in Table 6.1. The TSR after autoclaving and incubation for 168 h was about 97.8%. But the fermentation of HECM either with mono- or co-cultures led to the significant loss in total solids (6.8 to 16.5%) (Table 6.1). The highest solid loss was seen with mixed culture of *A. pullulans* with *N. crassa* with a loss of 16.5% solids. It was noticed the presence of *N. crassa* individually, or in combination with other fungi, led to a decline in the dry matter compared to other individual fungi or combinations. This loss in total solids can be attributed to the fungal metabolization of carbohydrates into carbon dioxide (Tabacco et al., 2011).

Comparing with uninoculated controls, the highest CF, ADF, and NDF level reductions in monocultures were achieved by *N. crassa* at 19.5, 1.3 and 14.5%,

respectively. In dual culture, the combination of *A. pullulans* and *N. crassa* exhibited the highest drop by 21.9, 1.7 and 9.1% as compared to the other fugal combinations. Tri-cultures of *A. pullulans*, *N. crassa* and *T. reesei* showed a decline of 3.1% in the CF and 1.2% in the NDF but not in the ADF. *N. crassa* by itself was the best overall in reducing CF, ADF and NDF levels, and when it was used in combination with *A. pullulans*, a further insignificant reduction was achieved in CF (2.9%) and ADF (0.6%) but not in NDF. Nuraini and Mahata (2015) reported a decrease in crude fiber, NDF and ADF in fermented durian and tofu waste mixture by *Phanerochaete chrysosporium* and *N. crassa.* Although *T. reesei* is known to produce many hydrolytic enzymes (Li et al., 2013), low levels of fiber degradation were anticipated. The presence of high amounts of soluble sugars could be one of the limiting factors for fungal degradation of the insoluble fiber components (Amanullah et al., 2014). Castillo et al. (1994) reported that the co-culture of *T. reesei* and *A. niger* must be inoculated sequentially because of the specific requirements for the growth rates and enzyme production rates between them. Lio and Wang (2012) noticed a lower enzyme activity in some fungal combinations compared to single fungus inoculation when fermenting soybean fiber. Duenas et al. (1995) stated that the combination of *T. reesei* with *Aspergillus phoenicis* did not enhance fiber content reduction due to the production of reducing sugars by *T. reesei* via cellulose hydrolysis that may accelerate the growth of *Aspergillus*.

Autoclaving media at 121 °C for 15 min led to a significant reduction in GLS. Aqueous thermal treatments are known to degrade or volatilize GLS (Halkier and Gershenzon, 2006; Jensen et al., 1995). GLS levels in uninoculated controls compared to the untreated HECM showed a drop in GLS levels by 56.8%. Further microbial conversion

(mono-, bi-, and tri- cultures) compared to uninoculated control showed an additional drop in GLS levels by 25, 81.3 and 43.8%, respectively. In monocultures, *A. pullulans* exhibited the highest reduction by 25%, while co-cultures (*A. pullulans* and *T. reesei*) resulted in a total GLS reduction by 81.3% as compared to uninoculated control. Jannathulla et al. (2017b) reported a 41.7% GLS reduction on agricultural residues by *A. niger*. GLS reduction caused primarily by microbial utilization of glucose and sulfur molecules during fermentation (Shi et al., 2015).

Phytic acid level was not affected by the autoclave treatment (HECM vs uninoculated control) as shown in Table 6.1. During mono- and co-culture fermentation of HECM, level of phytic acid increased significantly (2.7-3.4 g/100g) except for in the co-cultures of (*A. pullulans* + *T. reesei*). It is likely that the utilization or the changes in other nutrients such as carbohydrates resulted in concentrating phytic acid level. Additionally, studies have shown the effectiveness of phytase enzymes to be optimal at pH range of 4.0 to 6.0 (Zhao et al., 2017; Lei and Porres, 2003), but in our study final pH was recorded to be at the higher end (7.1 to 8.0). This could be another hindering factor in phytic acid degradation during fungal fermentation. Maintaining pH during fermentation could potentially enhance the phytic acid degradation.

Table 6.1: TSR, CF, ADF, NDF, GLS and PA of fermented canola meal after 7 days solid state fermentation

Parameters	TSR (%)	CF (%)	ADF (%)	NDF (%)	GLS (µmol/g)	PA (g/100g)
HECM	40±0.00^{a}	12.8±0.8^{a}	20.0±0.04^{a}	24.9±1.09^{a}	3.7±0.07^{a}	2.6±0.10^{c}
Control	97.8±1.17^{ab}	12.8± 0.27^{a}	17.5±0.57^{bc}	24.2±0.52^{a}	1.6±0.36^{bc}	2.6±0.02^{c}
A. pullulans	91.3±0.98^{cd}	14.0± 0.44^{a}	20.5±0.08^{a}	24.9±0.24^{a}	1.2±0.09^{bc}	3.4±0.11^{a}
N. crassa	86.3±0.82^{de}	10.3±0.07^{b}	17.3±1.20^{bc}	20.7±0.76^{b}	2.3±0.64^{b}	3.3±0.02^{a}
T. reesei	93.3±0.12^{bc}	13.9±0.41^{a}	20.5±0.33^{a}	24.0±0.15^{a}	2.2±1.18^{b}	3.1±0.16^{a}
Ap+Nc	83.5±0.31^{e}	10.0±0.19^{b}	17.2±0.92^{c}	22.0±0.58^{b}	1.9±0.13^{b}	2.9±0.01^{b}
Ap+Tr	88±1.53^{cde}	12.9±0.32^{a}	20.3±0.86^{a}	24.4±0.28^{a}	0.3±0.02^{c}	2.6±0.04^{bc}
Nc+Tr	87±0.04^{de}	13.3±1.50^{a}	19.1±0.33^{ab}	24.5±0.47^{a}	1.5±0.02^{bc}	2.7±0.13^{bc}
Ap+Tr+Nc	86.5±1.08^{de}	12.4±0.38^{a}	20.3±0.48^{a}	23.9±1.17^{a}	0.9±0.30^{bc}	2.7±0.05^{bc}

Mean ± standard deviation. Significant differences ($p \leq 0.05$) among different treatments for each analysis is indicated by letter superscripts. Control is uninoculated. HECM: hexane extracted canola meal; TSR: total solid recovery; CF: crude fiber; ADF: acid detergent fiber; NDF: neutral detergent fiber; GLS: total glucosinolates; PA: phytic acid.

6.4.3. Total Soluble Sugars of Raw and Fermented HECM

Total soluble sugars (Figure 6.2) represent the sum of mono- and oligo- saccharides (stachyose, raffinose, arabinose, galactose-mannose, and glucose). Comparing to untreated HECM, the uninoculated control showed a little drop in total soluble sugars (1.1 mg/mL), which could be stemmed from degradation of sugars in the presence of other nutrients during heat sterilization prior to fungal inoculation (Wang and Hsiao, 1995). Nyombaire et al. (2007) claimed that soaking (77 °C) and cooking (~99.3 °C) of red kidney bean resulted in diminishing oligosaccharides (stachyose and raffinose). As compared to uninoculated control, fungal fermentation of HECM with monocultures of *A. pullulans* and *N. crassa* exhibited a further reduction in total soluble sugars by 74.7 and 60.7%, respectively. Additionally, cultivation of HECM with these two fungi led to a significant reduction in total soluble sugars (91.6%) as compared to the uninoculated control. Our results corroborated with the previous findings of Croat et al. (2016a) who reported a 50-95% drop in total soluble sugars of fermented hexane extracted and cold pressed canola meal using

filamentous fungi including *A. pullulans* and *T. reesei*. In contrast to the findings of Croat et al., (2016), inoculation of HECM with *T. reesei* increased total soluble sugars by 33.9% as compared to uninoculated control. Similarly, co-cultivation of *T. reesei* either with *N. crassa* or *A. pullulans* showed the similar pattern with no change in total soluble sugars when compared to the uninoculated control. The higher total soluble sugars with *T. reesei* can be attributed to the ability of the fungus to produce cellulolytic enzymes (Peterson and Nevalainen, 2012) which in turn releases more soluble sugars during fermentation. Additionally, presence of significant amounts of soluble sugars in the meal at the end of fermentation suggest that *T. reesei* was not able to fully utilize soluble sugars, and this could be why low levels of proteins were recorded with *T. reesei* fermented meal (Figure 6.1). Only residual amounts of soluble sugars were present (<1 mg/mL) when *T. reesei* was cultivated either with *N. crassa* or *N. crassa* and *A. pullulans* combined. These results clearly demonstrated that soluble sugars are released and utilized more efficiently when *T. reesei* is co-cultivated with either *N. crassa* or *N. crassa* and *A. pullulans* as compared to using monoculture of *T. reesei*.

A study is being currently underway to determine if full potential of *T. reesei* in combination with other microbes can be achieved by optimizing the process parameters including sequential co-fermentation using different microbes.

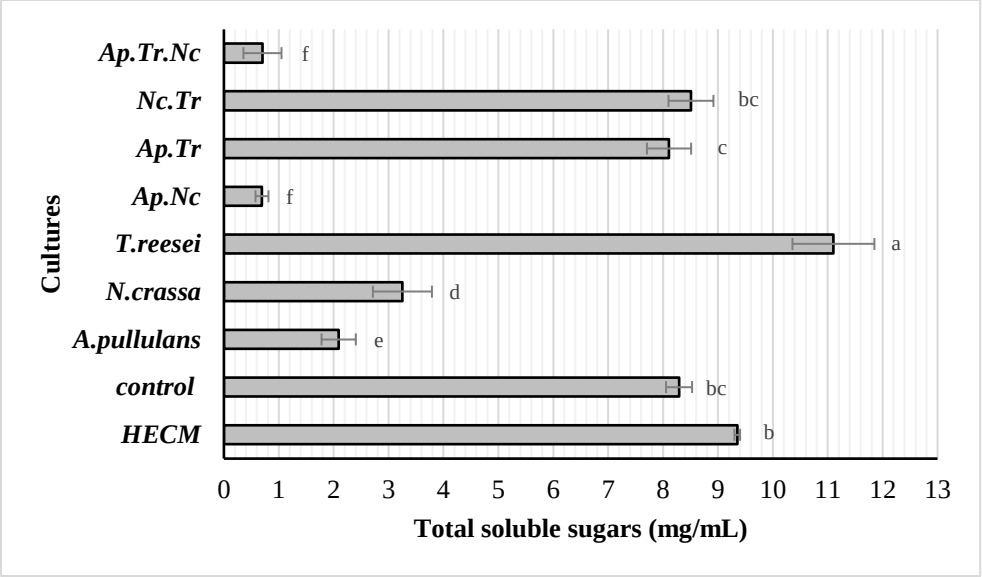

Figure 6.2: Total soluble sugars for HECM and 168 h fermented samples. Significant differences ($p \leq 0.05$) among different treatments are indicated by letters. Control is uninoculated. HECM: hexane extracted canola meal, Ap: *Aureobasidium pullulans*, Nc: *Neurospora crassa*, Tr: *Trichoderma reesei*.

6.5. Conclusions

Overall, co-culture solid phase fermentations of HECM were found to be effective in improving the nutritional characteristics of HECM as shown by an increase in protein content and decreases in levels of several ANFs. Co-culture of *A. pullulans* and *N. crassa* was found to be the most effective at reducing fiber content (CF and ADF) likely due to synergistic growth effects. Similarly, combination of *A. pullulans* with *T. reesei* resulted in higher levels of reduction of GLS. All strains in individual and co-cultures resulted in higher protein contents as compared to uninoculated control. This study demonstrates the importance of ensuring the presence of synergistic relationship among selective fungi for better improving the nutritional characteristics of HECM.

Chapter 7. Combined Effect of Mild Pretreatment and Fungal Fermentation on Nutritional Characteristics of Canola Meal and Nutrient Digestibility of Processed Canola Meal in rainbow trout [†]

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7.1. Abstract

The effects of mild pretreatment and fungal fermentation on the nutritional characteristics of hexane extracted canola meal (HECM) was examined. HECM is a by-product of canola oil extraction process and has restricted use in animal diets due to presence of high levels of antinutritional factors (ANFs) and limited digestibility resulting in low nutrient availability. In this study, HECM was washed with water at different solid loading rates (SLR) to remove soluble ANFs. The wash process removed significant amounts of soluble sugars and glucosinolates from HECM, yielding a meal with higher levels of proteins when compared to raw HECM. The digestibility test conducted on rainbow trout with the feed that had 30% of fish meal replaced with washed HECM showed slight improvement with no statistical significance in protein digestibility when compared to HECM. Fermentation of washed HECM and raw HECM with mono- and co-cultures of *Aureobasidium pullulans*, *Neurospora crassa* and *Aspergillus niger* resulted in higher protein and amino acid content when compared to the uninoculated controls. Additionally, different strains under mono- and co-culture fermentations exhibited varied reductions in the ANFs. Overall, these findings suggested that pretreatment with mild water washing and/or fermentation enhances the nutritional value of HECM.

7.2. Introduction

Canola meal (CM) is the solid material that remains after canola seed is processed for oil extraction (Olukomaiya et al., 2020c). CM can be up to 67% of the seed by weight (Östbring et al., 2020), and contains a minimum of 40% protein on dry basis. It is widely exploited in animal diets (ruminant, swine, poultry and fish) and is ranked second after soybean as one of the most traded protein feed ingredients (CCC, 2019). However, CM has several antinutritional factors (ANFs) such as non-digestible oligosaccharides and non-starch polysaccharides, glucosinolates (GLS), and phytic acid, which limits the amount of CM that can be included in feed formulations (Wu and Muir, 2008; Yoshie-Stark et al., 2008).

These ANFs have various negative effects on different animal species. Complex carbohydrates elevate gut viscosity and lower nutrient digestion, while high fiber content dilutes nutrient density (Booth et al., 2001), and reducing availability (Mustafa et al., 1996). ANFs negatively impact animal growth and performance (Ramesh et al., 2006). The GLS and/or their breakdown products cause low feed intake, iodine deficiency, as well as enlargement of the liver, kidney, and the thyroid (Tripathi and Mishra, 2007). The highly negatively charged phytates forms complexes with minerals, thereby reducing their bioavailability (Francis et al., 2001).

Several methods have been developed to reduce or eliminate ANFs such as enzymatic pretreatments (Hussain et al., 2016), thermal extrusion, dilute acid and alkaline pretreatments (Croat et al., 2017), tail end dehulling (Hansen et al., 2017) and microbial fermentation (Alhomodi et al., 2021a; Croat et al., 2016b). All these processes have been

reported to reduce ANFs, however, the benefits are offset by higher processing costs, a net loss of protein, and/ or incomplete reduction of ANFs.

The GLS content in CM is low (<30 µmol/g) and its variable composition (CCC, 2019) may not impact meal palatability compared to other *Brassica* species defatted seed meals such as carinata that is high in sinigrin (Ban et al., 2017; Warwick et al., 2006). Therefore, ANFs in CM generally impact nutrient utilization, especially in monogastric animals, but not nutrient intake.

Microbial fermentation shows perhaps the most potential for improving CM nutritional composition by reducing ANFs (Alhomodi et al., 2021a), but the presence of high levels of simple sugars in CM limit the effect of fermentation on the complex polysaccharides. The depletion of simple carbohydrates would enable the microbes to have a more profound effect on the other complex components such as fibers. The soluble simple carbohydrates could be removed by washing the CM before fermentation. Although washing concentrates fiber of the pre-fermented meal, its overall effect on digestibility in fish is positive (Kasiga et al., 2021). In addition to lowering ANFs and increasing protein, fermented meals have also been reported to enhance some nonspecific immune responses in some fish species (Dossou et al., 2018; Zhang et al., 2020).

Washed and fermented CM that is high in protein and amino acids, and low in soluble sugars, would be ideal for use in carnivorous fish diets. This is because such diets are high in protein due to the high amino acid requirements of these species (NRC, 2011). Carnivorous fish also generally have a low ability to utilize high amounts of soluble carbohydrates and their diets rarely contain more than 20% carbohydrate (Wilson, 1994). Rainbow trout (*Oncorhynchus mykiss*) was the second most produced fish species in the

US in 2018 (USDA-NASS, 2019). To meet the digestible protein content of 38% (NRC, 2011), the crude protein contents are usually greater than 40% in the diet. Considering the carnivorous nature of rainbow trout, it is more efficient at utilizing protein and lipid for nutrients and energy (Gatlin III, 2010). Therefore, plant meals that are low in carbohydrate would be ideal for use in its diets.

In this study we evaluated the effects of washing hexane extracted canola meal (HECM) with water to remove undesirable components, followed by fungal fermentation on both washed solid fraction and supernatant using various combinations of three different microbes. To achieve the homogenous fungal growth via agitation during incubation, submerged fermentation was chosen over solid state fermentation. The fungal cultures used in this study are generally regarded as safe and are previously reported to enhance the nutritional composition of canola meal: *Aureobasidium pullulans*, *Neurospora crassa* (Alhomodi et al., 2021a) and *Aspergillus niger* (Shi et al., 2015). The objectives of the study were to determine: (1) the effect of washing on meal composition, (2) the effect of washing on mono- and co-culture fermentation, (3) the effect of washing on the digestibility of protein of HECM in rainbow trout.

7.3. Materials and Methods

7.3.1. Canola Meal Preparation

HECM was received from ADM/Specialty Commodities LLC (Fargo, North Dakota, USA). The ground meal (Krups Coffee Grinder, Solingen, Germany) was stored in sealed buckets at room temperature (~23 °C). Dry weight determination of HECM was done by drying ~2 g of ground meal in triplicate at 80 °C for 24 h using a drying oven (AACC, 2010).

7.3.2. Canola Meal Washing Process

Details of canola meal processing is provided in the Figure 7.1. Briefly, HECM was mixed in 100 mL of deionized water at room temperature (~23 °C) for 2 min, using a stainless-steel spatula. The homogeneous mixture was centrifuged at 10,000 g for 10 min at room temperature. The solid fraction was oven dried (80 °C/48 h) for proximate analysis. Portions of the supernatant were oven dried (80 °C/48 h) for crude protein and GLS analyses, while total soluble sugars and phytic acid were analyzed directly on the supernatant. Both fractions of 20% washed meal were used for fungal fermentation, and solid fractions were moisture adjusted prior to fermentation (Figure 7.1).

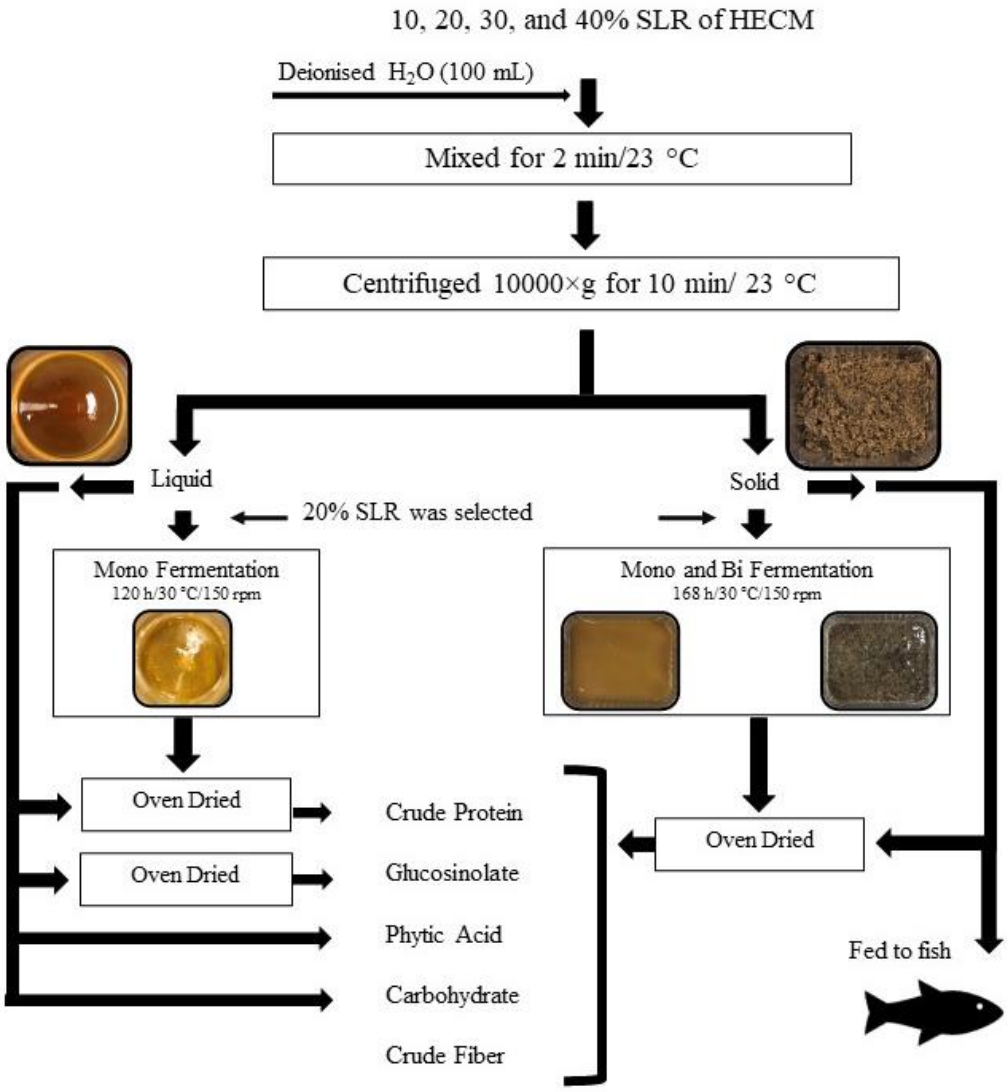

Figure 7.1: Overall process flow of mild wash of hexane extracted canola meal followed by fungal fermentation. HECM: hexane extracted canola meal; SLR: solid loading rate.

7.3.3. Fungal Inoculum Preparation for Mono- and Co-culture Fermentation

Fungal strains, *Aureobasidium pullulans* (NRRL-Y-2311-1), *Aspergillus niger*, and *Neurospora crassa* were obtained from the National Center for Agricultural Utilization Research (Peoria, Illinois, USA). The fungal strains were stored in the refrigerator and sub-cultured monthly on potato dextrose agar (PDA). For inoculum preparation, 8 mm plug from 5-day fungal colony grown on PDA was transferred into sterile glucose yeast extract

(GYE) media (100 mL working volume of 5% glucose and 0.5% yeast extract) with pH of 5.5. Thereafter, flasks were incubated for 48 h at 30 °C in rotary shaker at 150 rpm.

7.3.4. Fungal Interactions in Co-culture Systems

Prior to fungal fermentation experiments, fungal strains were tested for any potential antagonistic behavior by plating them together (combination of co-cultures) on a potato dextrose agar (PDA). Two 8-mm disc of actively growing mycelium for each fungus was placed into each half of PDA medium, whereas a single cell fungus (*A. pullulans*) inoculum was spread on PDA. Plates were then sealed with parafilm and incubated for 7 days at 30 °C. The morphological growth pattern of these fungal strains on the PDA plates were observed daily. The assurance of two fungal growth in co-culture systems was determined by monitoring morphological growth and inhibition zone in the inoculated plates.

7.3.5. Submerged Fermentation

7.3.5.1. Fermentation of Raw and Washed Solid Fractions of Canola Meal

Submerged fermentation (SmF) was conducted in 500 mL Erlenmeyer flasks with a working volume of 200 mL The amount of deionized water was added based on the final moisture content of washed HECM (solid fraction) to have a solid loading rate of 20% on dry basis (db). The pH of the slurry was 5.5. All flasks were autoclaved (121 °C for 20 min) and then inoculated with 2 mL of a 48-h culture of *A. pullulans*, *N. crassa* and *A. niger* for monoculture fermentation and 1 mL of each fungal strain for co-culture incubation. All flasks were incubated at 30 °C at 150 rpm for 168 h. At the end of fermentation, pH was measured, and samples were dried at 80 °C for 48 h using a drying oven. Sets of similar experiments were conducted with the raw HECM. Also uninoculated

control was conducted with washed solids and raw HECM. All experiments were conducted in triplicate.

7.3.5.2. Fermentation of Supernatant Recovered from Washing Process

SmF was performed in 250 mL Erlenmeyer flasks with a working volume of 100 mL of the liquid obtained from washed HECM. The pH of the supernatant was ~5.5 and no further adjustment was made. All flasks were autoclaved (121 °C for 20 min) and then inoculated with 1 mL of a 48-h culture of *A. pullulans*, *N. crassa* and *A. niger* for monoculture fermentation for 120 h. All flasks then were processed as mentioned above in section 7.3.5.1. All experiments were performed in triplicate.

7.3.6. Diet Formulation and Manufacturing for the Digestibility Study

A reference diet (Table 7.1) was formulated for rainbow trout, and it contained ~44% crude protein and 16% lipid. The reference diet contained 40% animal meals including 20% fishmeal. Test diets were formulated by replacing 30% of the reference diet with the test ingredient. A single big batch of the reference blend without chromic oxide was made and portions used in test diets. The weighed dry ingredients were ground to particles less than 0.5 mm with a Fitzpatrick comminutor mill (Elmhurst, IL) and then blended with supplemental oil in a double action Leland 100DA70 food mixer (Fort Worth, TX). All diets contained 0.75% chromic oxide as an inert marker. The reference blend, test ingredient, chromic oxide and water (~30% moisture) were mixed in a Hobart HL200 mixer (Troy, OH) for about 10 mins. All diets were cold pressed (Hobart 4146 grinder), and the pellets (3 mm in diameter) were air dried for 24 h using a fan. The diets were stored at -20 °C, pending use. Protein and chromium were analyzed using AOAC 2006 methods 972.43 and 990.08, respectively (AOAC, 2006e).

Table 7.1: Formulation of the reference diet used in apparent digestibility studies

Ingredient	Inclusion (%)
Menhaden fishmeal[1]	20.0
Porcine blood meal[2]	3.0
Poultry byproduct meal[3]	15.0
Feather meal (hydrolyzed)[4]	2.0
Whole cleaned wheat[5]	23.0
Yellow whole corn[6]	7.5
Wheat gluten[7]	12.0
Vitamin premix[8]	1.7
Trace minerals[8]	1.5
Stay C[9]	0.3
Choline chloride 60%[10]	0.3
Lysine[11]	1.0
Tryptophan[11]	0.1
Methionine[12]	0.5
Betaine[13]	0.5
Taurine[14]	0.5
Menhaden fish oil[1]	6.5
Soybean oil[15]	3.6
Lecithin[16]	1.0

[1]Omega protein, Houston, Tx; [2]Mason city byproducts Inc, Mason city, IA; [3]Tyson Foods Inc., Springdale, AR; Consumer supply, Sioux city, IA; [e]Prairie Ag Partners, Lake Preston, SD; [6]Rancher's choice, North Sioux city, SD; [7]Cargill, Minneapolis, MN; [8]Nutriblend, Neosho, MO; [9]DSM Jiangshan Pharmaceutical Co. Ltd, Jingjiang, Jiangsu, China; [10]Biochem Corporation, New Hampton, NY; [11]Anjinomoto Animal Nutrition group, Chicago, IL; [12]Adiddeo Blue Star Company, North Point Parkway, GA; [13]Jiangyin, Huachang Food Additives, Jiangyin Jiangsu, China; [14]Pure Bulk, Rosenburg, OR [15]South Dakota Soybean Processors, Volga, SD; [16]Solae LLC, St. Louis, MO.

7.3.7. Digestibility Fish Culture System and Fecal Collection

Two recirculating aquaculture systems (RAS) were used, and each RAS consisted of 30 or 32 tanks of 110 L each. This study was part of a large digestibility study of five meals. Six rainbow trout ranging from 300-350 g were stocked into each of the 27 tanks of each RAS and the remaining 3 or 5 tanks were not used. Each replicate was made of three tanks, and three replicates were used per treatment. Each RAS had 4 or 5 tanks of each

treatment, and tanks were randomly assigned a treatment diet within a given RAS. Dissolved oxygen, temperature and pH were monitored daily using a YSI Professional Plus (Yellow Springs, OH), and they averaged 9.48±0.12 mg/L, 15.99±0.09 °C and 7.37±0.03, respectively for RAS1; and 9.44±0.07 mg/L, 15.29 ±0.08 °C and 7.22±0.02, respectively for RAS2. Unionized ammonia and nitrite were monitored once a week and were 0.002 and 0.063 mg/L, respectively for RAS1, and 0.002 and 0.035 mg/L, respectively for RAS2. All fish were fed a commercial diet for one week and test diets for another week, before fecal stripping (Glencross et al., 2007). Fecal stripping occurred ~8 h after the morning feeding. Feces from three tanks were pooled into a single replicate and the fecal samples were stored at -20 °C. After freezing, the samples were freeze-dried using a Labconco Freezone 2.5 freeze dryer (Kansas City, MO) for 48 hours.

7.3.8. Analytical Assays

7.3.8.1. Crude Protein

Crude protein was determined by combusting ~0.2500 g of each sample using a LECO model FP528 (St. Joseph, MI, USA) (Horwitz and Latimer, 2006). The total emitted nitrogen gas was then multiplied using a conversion factor of 6.25 to obtain the percentage of protein.

7.3.8.2. Total Glucosinolates (GLS)

Intact GLS were extracted from 0.25 g of meal with 3 mL of methanol by sonication for 30 min and allowed to stand overnight at room temperature. The extracted GLS in methanol (15 µL) were then filtered through a 0.45 µm nylon 66 filter and analyzed by high performance liquid chromatographic (HPLC) separation on a Shimadzu (Columbia,

MD, USA) HPLC System using a method developed by Betz and Fox (1994), with slight modifications.

7.3.8.3. Phytic Acid

Phytic acid assay kit (K-PHYT, Megazyme, Bray, Ireland) was used to determine the phytic content in both liquid and solid samples (Megazyme, 2017). Briefly, phytic acid of samples (liquid and solid) were acid extracted followed by enzymatic treatments (phytase and alkaline phosphatase) to free phosphates. Then, a modified colorimetric method was used to determine the total released phosphate that is given as grams of phosphorus per 100 g of solid samples and grams of phosphorus per 100 mL of liquid samples.

7.3.8.4. Crude Fiber

Crude fiber (CF) was analyzed by Agricultural Experiment Station Chemical Laboratories at the University of Missouri-Columbia, using AOAC (2006) method 978.10. (AOAC, 2006d).

7.3.8.5. Total Soluble Sugars

Total soluble sugar content was determined by HPLC system (Agilent Technologies, Santa Calara, CA, USA) equipped with a refractive index detector (Model G1362A), and a waters size-exclusion chromatography column (SugarPak column I with pre-column module, Waters Corporation, Milford, MA, USA) at a flow rate of 0.5 mL/min (50 ppm EDTA in deionized water) and column temperature of 85 °C Different concentrations of (stachyose, raffinose, glucose, galactose, galactose-mannose, and arabinose) at 99.9% purity as sugar standards were prepared to set up calibration curve of concentration vs. HPLC peak area as previously reported by (Karunanithy et al., 2012).

Sugar samples from solids and liquids were prepared using methods described in (Croat et al., 2016a) and (Croat et al., 2017), respectively.

7.3.8.6. Calculation of Apparent Digestibility Coefficients (ADCs)

Protein and chromium contents of the feces were analyzed using methods described previously. Chromic oxide in the ingested diets was assumed to be inert, indigestible, and passed through the digestive tract at the same rate as the dietary nutrients (Austreng et al., 2000). Chromic oxide was also assumed to be homogenously distributed in the diets and the feces. Protein ADCs were calculated according to NRC (2011) equations:

$$ADC_{ref\ and\ feed} = 1 - \frac{\text{Chromium in feed}}{\text{Chromium in feces}} \times \frac{\text{protein content of feces}}{\text{protein content of feed}}$$

$$ADC_{ingred} = ADC_{test\ diet} + [(ADC_{test\ diet} + ADC_{ref\ diet}) \times (0.7 \times D_{ref} / 0.3 \times D_{ingred})]$$

where D_{ref} is the percentage of protein of the reference diet, and D_{ingred} is the percentage of protein of the test ingredient.

7.3.9. Statistical Analysis

R version 1.2.5001 (Boston, MA, USA) was used for the statistical analysis. All data were analyzed using one-way ANOVA except for the digestibility data that was analyzed using a two sample T-test. $p \leq 0.05$ was used as the level of significance.

7.4. Results and Discussion

7.4.1. Composition of Washed HECM

Solid recovery (SR), supernatant recovered, crude protein (CP), crude fiber (CF), phytic acid (PA), total glucosinolates (GLS), and total soluble sugars (TSS) for washed HECM at different solid loading rates (SLRs) compared to the raw counterpart are shown in Table 7.2. An increase in SLR from 10 to 40% gradually decreased the SR in the supernatant (16.0% for 10% SLR vs. 13.5, 10.0, and 8.8%, respectively for 20, 30, and

40% SLR) suggesting the low mass transfer at high SLRs. A high amount of water was retained at the higher level of SLRs (Table 7.2) indicating the high-water adsorption characteristics of HECM. It was previously reported that high fiber content in canola meal (Aider and Barbana, 2011) holds water ~4 times its dry weight (Khattab and Arntfield, 2009).

CPs of washed HECM at 10, 20, 30 and 40% were 45.9, 43.9, 43.5, and 43.2%, respectively higher than that of the raw HECM (41.2%). CP increases were due to mass transfer of soluble components into supernatant. The protein recovered in the supernatant at 10, 20, 30, and 40% SLR were 12.4, 12.1, 9.4, and 9.1%, respectively. This protein loss from the CM was due to protein solubility during washing process. CM proteins are classified based on their solubility in alkaline solution (glutelin's), water (albumins), alcohol (prolamins), and salt solution (globulins) (Tan et al., 2011a). Protein solubility of the CM is dependent on several factors such as pH, temperatures, and salt levels (Wanasundara et al., 2016). In this study, deionized water at room temperature was used in washing process, and the pH of the solubilized CM was 5.5. It is estimated that the protein loss in supernatant was largely the water-soluble albumins, and some non-protein nitrogen containing compounds.

When the meal was washed at 10, 20, 30 and 40% SLRs, the CF were 14.1, 14.1, 13.5, and 13.3%, respectively compared to raw HECM (11.1%). CM contains a substantial amount of insoluble fiber, due to the presence of the seed hulls which are not removed during oil processing (CCC, 2019). The observed high CF could be interpreted as the concentration of CF due to the removal of soluble components during washing process.

Comparison of the PA content of HECM (2.73 g/100g), and the washed meals at 10, 20, 30 and 40% SLRs resulted in PA content of 12.5, 6.6, 2.9, and 2.9%, respectively. This increase was likely due to mass transfer of other meal components into supernatant, leading to higher PA concentrations. Washing at 10, 20, 30, and 40% SLRs barely solubilized PA into the supernatant. The PA content in the supernatant ranged between 0.02 to 0.09 g/100g. This low PA could be due to the alteration of PA into an insoluble form during washing process. PA is known to possess a metal ion chelating ability because of its chemical structure allowing it to have a strong affinity with cations (Graf and Eaton, 1990). Because HECM contains several cations such as copper, iron, manganese, zinc and calcium (CCC, 2019), PA chelates such cations, resulting in the water-insoluble phytate salts (Matsuno and Fujimura, 2014).

Total GLS in SR were lower by 54.7, 44.2, 47.9 and 42.8% compared to HECM when meal was washed at 10, 20, 30, and 40% SLRs, respectively. The supernatant at different SLRs had no detectable GLS even though there were GLS reductions in SRs after washing. This is because HECM contained very low levels of these glucosinolates and the presence of other interfering components in the supernatant that affected GLS extractability and detectability by HPLC.

Compared to HECM, the total soluble sugars and oligosaccharides (stachyose, raffinose, glucose, galactose-mannose, and arabinose) declined significantly by 77.0, 71.4, 40.2, and 39.4% in the SRs at 10, 20, 30, and 40% SLRs, respectively. These results showed more sugar removal at lower SLRs, but in terms of supernatant, the total soluble sugars was higher at 40% SLR (1.88 g) compared to 30% (1.79 g), 20% (1.79 g), and 10% (1.40 g) SLRs.

Table 7.2: Proximate composition of hexane extracted canola meal (HECM) and washed HECM

Analysis, dry basis	HECM	Washed HECM at different solid loading rates (SLR)							
		10%		20%		30%		40%	
		Solid	Liquid	Solid	Liquid	Solid	Liquid	Solid	Liquid
SR, g	NA	8.1 ± 0.12^{d}	1.6 ± 0.06^{C}	17.3 ± 0.06^{c}	2.7 ± 0.06^{B}	26.8 ± 0.06^{b}	3.0 ± 0.15^{B}	36.0 ± 0.32^{a}	3.5 ± 0.25^{A}
Supernatant, mL	NA	NA	77.7 ± 0.58^{A}	NA	57.0 ± 0.00^{B}	NA	43.7 ± 2.08^{C}	NA	39.0 ± 1.00^{D}
CP, %	41.2 ± 0.85^{c}	45.9 ± 0.36^{a}	32.0 ± 0.51^{D}	43.9 ± 0.25^{b}	37.0 ± 0.17^{C}	43.5 ± 0.63^{b}	38.8 ± 0.19^{B}	43.2 ± 0.14^{b}	42.7 ± 0.17^{A}
CF, %	11.1 ± 0.14^{c}	14.1 ± 0.52^{a}	NA	14.1 ± 0.16^{ab}	NA	13.5 ± 0.35^{ab}	NA	13.3 ± 0.28^{b}	NA
PA, g/100g	2.73 ± 0.02^{c}	3.07 ± 0.02^{a}	$0.04\pm0.00^{BC*}$	2.91 ± 0.01^{b}	$0.02\pm0.02^{C*}$	2.81 ± 0.09^{bc}	$0.06\pm0.02^{AB*}$	2.81 ± 0.01^{bc}	$0.09\pm0.01^{A*}$
GLS, µmol/g	2.11 ± 0.16^{a}	1.18 ± 0.06^{b}	Und	1.36 ± 0.03^{b}	Und	1.23 ± 0.13^{b}	Und	1.34 ± 0.00^{b}	Und
TSS, mg/mL	13.2 ± 0.22^{a}	3.75 ± 0.61^{b}	$1.40\pm0.03^{B+}$	4.37 ± 0.93^{b}	$1.79\pm0.13^{A+}$	8.83 ± 1.14^{ab}	$1.79\pm0.08^{A+}$	8.89 ± 4.06^{ab}	$1.88\pm0.09^{A+}$

Mean ± standard deviation, significant differences ($p\leq0.05$) among different wash concentrations for each analysis is indicated by upper and small case letter superscripts for liquid and solid, respectively. SR: solid recovered; NA: not applicable; CP: crude protein; CF: crude fiber; PA: phytic acid; GLS: total glucosinolates; Und: undetectable; TSS: total soluble sugars; HECM: hexane extracted canola meal; *: g/100 mL; +: g.

7.4.2. Amino Acid of Raw and Washed HECM

Washing at 20 and 30% SLRs led to higher total amino acid concentrations at 38.82 and 38.39%, respectively compared to HECM (36.35%) (Table 7.3). In terms of single amino acids, there were no significant differences in threonine, valine, methionine, isoleucine, leucine, phenylalanine, and lysine concentrations in the 20% and 30% SLRs, but the concentrations of these amino acids were higher than those in HECM, except for lysine that did not change. At 20% SLR, histidine (1.09%) was significantly higher than both HECM (1.05%) and 30% SLR (1.07%) while tryptophan at the 30% SLR (0.43%) was lower than HECM (0.46%) and 20% SLR (0.46%).

Table 7.3: Amino acid of hexane extracted canola meal (HECM) and washed HECM

Amino Acid	HECM	Washed HECM at different concentrations	
		20%	30%
Threonine	1.65 ± 0.01^{B}	1.82 ± 0.01^{A}	1.80 ± 0.01^{A}
Valine	2.06 ± 0.01^{B}	2.29 ± 0.01^{A}	2.26 ± 0.01^{A}
Methionine	0.75 ± 0.00^{B}	0.82 ± 0.01^{A}	0.81 ± 0.00^{A}
Isoleucine	1.61 ± 0.00^{B}	1.78 ± 0.02^{A}	1.76 ± 0.01^{A}
Leucine	2.67 ± 0.02^{B}	2.91 ± 0.00^{A}	2.89 ± 0.03^{A}
Phenylalanine	1.55 ± 0.01^{B}	1.70 ± 0.01^{A}	1.68 ± 0.01^{A}
Lysine	2.22 ± 0.02^{A}	2.31 ± 0.01^{A}	2.20 ± 0.04^{A}
Histidine	1.05 ± 0.01^{B}	1.09 ± 0.00^{A}	1.07 ± 0.01^{B}
Tryptophan	0.46 ± 0.00^{A}	0.46 ± 0.01^{A}	0.43 ± 0.01^{B}
Total*	36.35 ± 0.30^{B}	38.82 ± 0.11^{A}	38.39 ± 0.25^{A}

Mean ± standard deviation, significant differences ($p\leq0.05$) among different group for each amino acid are indicated by letter superscripts. HECM: hexane extracted canola meal; *: essential and nonessential amino acid.

7.4.3. Fermentation of Raw and Washed HECM

SR, CP, CF, PA, GLS, and TSS of fermented raw HECM (RM) and fermented washed HECM (WM) in mono- and co-culture incubation are shown in Table 7.4. SR of RM and WM post autoclaving were 90.5 and 91.0%, respectively. Mono- and co-culture

fermentation showed further reduction in SR in both RM and WM. Compared to uninoculated control, the monoculture of RM with *N. crassa* had the highest dry matter loss (DML) of 12.2% followed by *A. pullulans* (8.8%) and *A. niger* (5.0%). In co-cultures of RM, the co-cultures of (*A. pullulans* + *N. crassa*) showed the highest DML of 13.3%, whereas *N. crassa* + *A. niger*, and *A. pullulans* + *A. niger* showed a reduction of 11.0 and 9.9%, respectively compared to uninoculated control. The observed DML could be due to fungal metabolization of carbohydrates into carbon dioxide (Tabacco et al., 2011). Washing HECM at 20% followed by fermentation resulted in 23 to 32.5% DML. In monoculture of WM, *A. niger* presented the highest DML of 20.9% followed by *N. crassa* (11.5%) and *A. pullulans* (10.4%). In co-culture of WM, *A. pullulans* + *N. crassa* showed the highest DML of 17.0% followed by the co-culture of *N. crassa* + *A. niger* (15.9%) and *A. pullulans* + *A. niger* (10.4%), compared to uninoculated control. This higher DML seen in WM compared to RM in both mono- and co-culture incubations could be due to fungal metabolism of complex carbohydrates in washed meal due to the lack of available soluble carbohydrates as a result of washing process as discussed above.

Compared to uninoculated control in monoculture of RM, *N. crassa* yielded the highest CP of 45.8% compared to other fungi, *A. niger* (45.4%) and *A. pullulans* (43.3%). In co-culture of RM, a combination of *A. pullulans* + *N. crassa* was the highest in CP (47.4%) followed by *N. crassa* + *A. niger* (46.0%) and *A. pullulans* + *A. niger* (44.0%). The protein content increase could be due to loss of other dry matter components (Osman, 2011) or due to fungal consumption of other non-protein components (Baldwin et al., 2019). Several studies have reported protein increases in fermented canola meal in solid and submerged fungal fermentations (Alhomodi et al., 2021a; Aljubori et al., 2017; Croat

et al., 2016b). In WM, monoculture of *N. crassa* resulted in the highest CP of 47.3%, followed by *A. niger* (44.7%) and *A. pullulans* (42.9%) compared to the uninoculated control (42.7%). While in co-culture of WM, the combination of *A. pullulans* + *N. crassa* yielded the highest CP of 46.3% compared to other co-cultures *N. crassa* + *A. niger* (44.3%) and *A. pullulans* + *A. niger* (43.2%). The co-culture of *A. pullulans* + *N. crassa* yielded the highest CP among other cultures (mono and co) in RM, but in WM, the monoculture of *N. crassa* was the best in increasing CP compared to other mono-/co-cultures. The observed difference in protein concentration between RM and WM when same cultures systems were used may be attributed to differences in fungal behavior due to the alteration of substrate properties post washing. According to Croat et al. (2017), the protein content of HECM fermented by *Fusarium venenatum*, *Trichoderma reesei*, and *Aureobasidium pullulans* changed when HECM was subjected to different pretreatments (dilute acid/alkaline, hot water, and extrusion). That suggests the changes of substrate matrix can affect fungal growth and metabolism.

CF in monoculture of RM were decreased by 10.7% (*A. niger*) and 13.6% (*N. crassa*), however *A. pullulans* showed a 2.5% increase due to mass balance change. In co-culture of RM, *A. pullulans* + *N. crassa* was the best giving a CF reduction of 22.2% followed by *N. crassa* + *A. niger* (5.3%), while the combination of *A. pullulans* + *A. niger* exhibited an increase of 6.2%. When *A. pullulans* was cultured with *A. niger*, an increase in fiber was noticed. This is probably due to high growth rate of *A. pullulans* as compared to *A. niger* in submerged fermentation. According to Duenas et al. (1995) a co-culture of *Trichoderma reesei* + *Aspergillus phoenicis* did not improve fiber degradation, due to the release of reducing sugar by *T. reesei* that accelerated *A. phoenicis* growth. However, the

combination of *A. pullulans* with *N. crassa* showed better CF reduction, which indicated the compatible growth of these two fungi. Alhomodi et al. (2021a) reported that a co-culture of *A. pullulans* with *N. crassa* had the highest decreases in crude fiber, acid detergent fiber, and neutral detergent fiber in canola meal of 21.9, 1.7, 9.1%, respectively. In mono- and co-culture of WM, a similar reduction pattern was noticed in terms of fungi, but *A. niger* reduced the most CF among monocultures. This low CF reduction in WM indicates the need for longer incubation times for fungi to secrete cellulosic enzymes that synergistically hydrolyze the complex carbohydrate into simple sugars or medium optimization (temperature, inoculum size, pH, etc.) for higher fiber metabolization.

The PA content was reduced by all tested fungi in mono- and co-culture of RM and WM compared to their uninoculated controls. In monoculture of RM, *A. niger* showed the highest reduction by 48.9% followed by *N. crassa* (19.1%) and *A. pullulans* (4.3%). While in co-culture, the highest reduction was seen with a combination of *N. crassa* + *A. niger* (21.3%) followed by *A. pullulans* + *N. crassa* (19.1%) and *A. pullulans* + *A. niger* (6.4%) compared to uninoculated control. This reduction in PA indicates the fungal production of phytase during fermentation. These results concurred with El-Batal and Karem (2001) who reported a reduction of PA content in rapeseed meal fermented by *Aspergillus niger*. Egounlety and Aworh (2003) as well reported an achievement of 0.88% phytic acid reduction in fermented soybean meal for 36 h using *Rhizopus oligocarpous*. In monoculture of WM, *A. niger* again was the best in reducing PA by 15.5%, followed by *N. crassa* (3.4%) and *A. pullulans* (1.7%). In co-culture of WM, the co-culture of *A. pullulans* + *N. crassa* showed a PA decline of 12.1%, followed by *N. crassa* + *A. niger* (10.3%) and *A. pullulans* + *A. niger* (5.2%) compared to uninoculated control. Despite the reduction of PA in WM,

fungi in RM showed higher PA reduction. According to Ebune et al. (1995) glucose concentration can affect the increase of biomass growth, production of phytase and rate of PA hydrolysis. This can explain the higher phytase production in RM than WM that has lower soluble sugars.

Compared to uninoculated control, in monoculture RM, fermentation with *A. pullulans* provided higher GLS reduction (48.9%) followed by *N. crassa* (13.3%), whereas *A. niger* concentrated GLS by (17.8%). This increase could be attributed to concentration effect. In co-culture of RM, the combination of *A. pullulans* + *A. niger* showed the highest decline of 57.8%, followed by *A. pullulans* + *N. crass* (37.8%), whereas *N. crassa* + *A. niger* showed an increase of 4.4% compared to uninoculated control. This reduction can be a result of fungal consumption of sugar and sulfur moieties of GLS (Vig and Walia, 2001). These results are in line with Croat et al. (2016a) who reported a GLS reduction of fermented canola meal using different fungal strains. Unlike *N. crassa* (2.7 µmol/g), the monoculture of WM with *A. pullulans* (1.6 µmol/g) and *A. niger* (2.2 µmol/g), reduced GLS compared to uninoculated control (2.3 µmol/g). In co-culture of WM, *A. pullulans* + *A. niger* exhibited a concentrated GLS (5.7 µmol/g) unlike *A. pullulans* + *N. crassa* and *N. crassa* + *A. niger* that lowered GLS content by 9.5 and 16.7%, respectively compared to uninoculated control.

In the monoculture of RM, *N. crassa* utilized all soluble sugars (100%), whereas *A. niger* and *A. pullulans* reduced the total soluble sugars by 86.4 and 77.7%, respectively compared to uninoculated control. In co-culture of RM, the combination of *N. crassa* + *A. niger* and *A. pullulans* + *N. crassa* exhibited 100% reduction in total soluble sugars while *A. pullulans* + *A. niger* showed a decrease by 87.4% compared to uninoculated control.

Interestingly, all mono- and co-cultures of WM reduced total soluble sugars by 100% compared to uninoculated control.

Table 7.4: Fermentation of hexane extracted canola meal (HECM) and washed HECM

	Parameter	Analyses, dry basis					
		SR, g	**CP, %**	**CF, %**	**PA, g/100g**	**GLS, µmol/g**	**TSS, mg/mL**
HECM	**Control**	18.1±0.10^{a}	42.0±0.34^{e}	13.4±0.48^{a}	2.6±0.02^{b}	2.5±0.10^{ab}	5.7±2.60^{a}
	A. pullulans	16.5±0.06^{c}	43.3±0.61^{de}	15.1±0.67^{a}	2.7±0.01^{a}	1.4±0.85^{c}	1.4±0.94^{b}
	N. crassa	15.9±0.23^{de}	45.8±0.81^{b}	13.2±1.80^{a}	2.4±0.03^{d}	2.5±0.26^{ab}	00.0±0.00^{b}
	A. niger	17.2±0.21^{b}	45.4±0.20^{bc}	12.6±0.75^{a}	1.4±0.01^{f}	3.1±0.23^{a}	0.8±0.11^{b}
	A.p + N.c	15.7±0.12^{e}	47.4±0.76^{a}	12.1±1.31^{a}	2.4±0.01^{c}	1.8±0.18^{bc}	00.0±0.00^{b}
	A.p + A.n	16.3±0.15^{cd}	44.0±0.44^{cd}	15.8±0.44^{a}	2.7±0.02^{a}	1.2±0.16^{c}	0.8±0.33^{b}
	N.c + A.n	16.1±0.26^{cde}	46.0±0.35^{ab}	14.3±3.11^{a}	2.3±0.01^{e}	2.9±0.20^{a}	00.0±0.00^{b}
Washed HECM	**Control**	18.2±0.00^{a}	42.7±0.52^{d}	14.2±0.28^{c}	3.2±0.02^{c}	2.3±0.07^{bc}	5.7±1.63^{a}
	A. pullulans	16.3±0.21^{ab}	42.9±0.23^{d}	16.4±0.44^{a}	3.5±0.02^{a}	1.6±0.38^{c}	00.0±0.00^{b}
	N. crassa	16.1±0.69^{b}	47.3±0.31^{a}	14.7±0.59^{bc}	3.5±0.04^{a}	2.7±0.20^{b}	00.0±0.00^{b}
	A. niger	14.4±0.61^{b}	44.7±0.42^{c}	15.7±0.27^{ab}	3.4±0.02^{ab}	2.2±0.50^{bc}	00.0±0.00^{b}
	A.p + N.c	15.1±0.56^{b}	46.3±0.17^{b}	15.4±0.48^{abc}	3.4±0.03^{ab}	2.5±0.18^{b}	00.0±0.00^{b}
	A.p + A.n	16.3±1.21^{ab}	43.2±0.29^{d}	16.4±0.77^{a}	3.4±0.01^{b}	5.7±0.17^{a}	00.0±0.00^{b}
	N.c + A.n	15.3±0.85^{b}	44.3±0.29^{c}	15.4±0.35^{abc}	3.4±0.01^{a}	2.3±0.05^{bc}	00.0±0.00^{b}

Mean ± standard deviation, significant differences ($p \leq 0.05$) among different culture for each analysis is indicated by letter superscripts. SR: solid recovery; CP: crude protein; CF: crude fiber; PA: phytic acid; GLS: total glucosinolates; TSS: total soluble sugars; HECM: hexane extracted canola meal.

7.4.4. Amino acid of Fermented Raw and Washed HECM

Tables 7.5 and 7.6 show the essential amino acids of RM and WM, respectively in mono- and co-culture incubation. The total amino acids in mono- and co-culture of RM were higher than in the uninoculated control (Table 5). *A. pullulans* was the highest at 39.98% as individual, whereas in co-culture, the combination of *A. pullulans* + *N. crassa* was the best at (40.99%). In terms of individual amino acids, *A. pullulans* showed an increase in all amino acids compared to uninoculated control while other mono- and co-culture systems showed various increases and decreases in different amino acids as compared to uninoculated control. This can be explained by fungal conversion of some amino acids into others based on fungal needs. According to Je et al. (2005), fermentation of oyster (*Crassostrea gigas*) sauce at different fermentation times resulted in an increase in the amino acid composition.

The total amino acid in mono- and co-culture of WM, were lower than uninoculated control, except for the individual incubation by *N. crassa* (40.54%) and for the combination of *A. pullulans* + *N. crassa* (39.61%). *N. crassa* showed higher individual amino acid concentrations compared to uninoculated control except for histidine. *A. pullulans* + *N. crassa* was the best among other fungal combinations in amino acid increase but with lower histidine and tryptophan compared to uninoculated control. Compared to amino acid concentration of RM, WM showed lower concentration in total and individual amino acid in both mono- and co-culture incubation. This may be due to fungal metabolism of amino acids as nutrient sources due to the low soluble sugars in the washed meal.

Table 7.5: Amino acids of fermented hexane extracted canola meal for 168 h

Amino acid	Fungi						
	Control	***A. pullulans***	***N. crassa***	***A. niger***	***A.p + N.c***	***A.p + A.n***	***N.c + A.n***
Threonine	1.72±0.01^{d}	1.96±0.00^{a}	1.89±0.00^{bc}	1.75±0.03^{d}	1.97±0.02^{a}	1.95±0.03^{ab}	1.86±0.01^{c}
Valine	2.20±0.00^{d}	2.37±0.01^{c}	2.52±0.01^{ab}	2.34±0.04^{c}	2.57±0.02^{a}	2.32±0.00^{c}	2.48±0.00^{b}
Methionine	0.79±0.01^{b}	0.80±0.01^{ab}	0.83±0.01^{a}	0.70±0.01^{d}	0.82±0.00^{a}	0.75±0.01^{c}	0.81±0.01^{ab}
Isoleucine	1.70±0.01^{d}	1.84±0.01^{bc}	1.92±0.00^{a}	1.72±0.04^{d}	1.95±0.03^{a}	1.77±0.01^{cd}	1.88±0.01^{ab}
Leucine	2.82±0.01^{d}	3.06±0.02^{b}	3.14±0.01^{b}	2.79±0.04^{d}	3.24±0.04^{a}	2.95±0.02^{c}	3.08±0.01^{b}
Phenylalanine	1.65±0.01^{d}	1.80±0.01^{b}	1.78±0.01^{bc}	1.65±0.02^{d}	1.87±0.01^{a}	1.74±0.02^{c}	1.77±0.01^{bc}
Lysine	2.23±0.01^{ab}	2.27±0.03^{a}	2.31±0.02^{a}	2.09±0.02^{c}	2.29±0.01^{a}	2.16±0.01^{bc}	2.26±0.04^{a}
Histidine	1.07±0.01^{ab}	1.11±0.01^{a}	0.97±0.01^{cd}	0.97±0.02^{cd}	1.01±0.01^{bcd}	1.03±0.01^{bc}	0.95±0.03^{d}
Tryptophan	0.40±0.01^{bc}	0.46±0.01^{a}	0.41±0.01^{b}	0.35±0.01^{c}	0.43±0.01^{ab}	0.41±0.03^{ab}	0.42±0.00^{ab}
Total*	37.74±0.01^{d}	39.98±0.05^{b}	39.47±0.14^{bc}	37.90±0.29^{d}	40.99±0.12^{a}	38.98±0.15^{c}	39.06±0.11^{c}

Mean ± standard deviation, significant differences ($p \leq 0.05$) among different group for each amino acid are indicated by small case letter superscripts. *A.p*: *A. pullulans*; *N.c*: *N. crassa*; *A.n*: *A. niger*; *: essential and non-essential amino acid.

Table 7.6: Amino acids of fermented 20% washed hexane extracted canola meal for 168 h

Amino acid	Fungi						
	Control	***A. pullulans***	***N. crassa***	***A. niger***	***A.p + N.c***	***A.p + A.n***	***N.c + A.n***
Threonine	1.83±0.01^{bc}	1.64±0.01^{e}	1.89±0.01^{a}	1.54±0.01^{f}	1.85±0.01^{ab}	1.80±0.01^{c}	1.71±0.00^{d}
Valine	2.29±0.06^{b}	2.31±0.03^{b}	2.55±0.00^{a}	2.23±0.00^{b}	2.49±0.01^{a}	2.33±0.03^{b}	2.33±0.01^{b}
Methionine	0.82±0.02^{a}	0.82±0.00^{a}	0.83±0.01^{a}	0.77±0.01^{bc}	0.83±0.01^{a}	0.74±0.01^{c}	0.80±0.01^{ab}
Isoleucine	1.78±0.04^{b}	1.66±0.03^{c}	1.97±0.01^{a}	1.65±0.01^{c}	1.89±0.01^{a}	1.73±0.02^{bc}	1.76±0.00^{c}
Leucine	2.97±0.04^{b}	2.89±0.02^{c}	3.18±0.01^{a}	2.68±0.00^{d}	3.16±0.00^{a}	2.88±0.02^{c}	2.82±0.00^{c}
Phenylalanine	1.72±0.03^{b}	1.90±0.01^{a}	1.92±0.01^{a}	1.64±0.01^{c}	1.89±0.00^{a}	1.75±0.01^{b}	1.72±0.01^{b}
Lysine	2.41±0.06^{b}	2.69±0.02^{a}	2.63±0.02^{a}	2.03±0.01^{d}	2.67±0.00^{a}	2.29±0.02^{c}	2.22±0.00^{c}
Histidine	1.13±0.03^{a}	0.87±0.01^{d}	1.07±0.00^{b}	0.80±0.00^{e}	0.94±0.01^{c}	0.78±0.01^{e}	0.87±0.00^{d}
Tryptophan	0.43±0.02^{ab}	0.41±0.00^{abc}	0.45±0.00^{a}	0.38±0.01^{c}	0.40±0.00^{bc}	0.37±0.01^{c}	0.43±0.01^{ab}
Total*	39.05±0.88^{b}	35.19±0.16^{c}	40.54±0.30^{ca}	32.53±0.14^{d}	39.61±0.04^{ab}	35.67±0.11^{c}	35.98±0.08^{c}

Mean ± standard deviation, significant differences ($p \leq 0.05$) among different group for each amino acid are indicated by small case letter superscripts. *A.p*: *A. pullulans*; *N.c*: *N. crassa*; *A.n*: *A. niger*; *: essential and non-essential amino acid.

7.4.5. Protein Content of Fermented Supernatant

The fermentation of recovered supernatant resulted in more concentrated protein compared to uninoculated control (Figure 7.2). *A. pullulans* showed the highest protein content (48.4%) followed *N. crassa* (44.1%) and *A. niger* (44.0%) compared to uninoculated control (36.1%). This increase in protein content was attributed to conversion of soluble sugars into cell protein mass (Zahler et al., 2018).

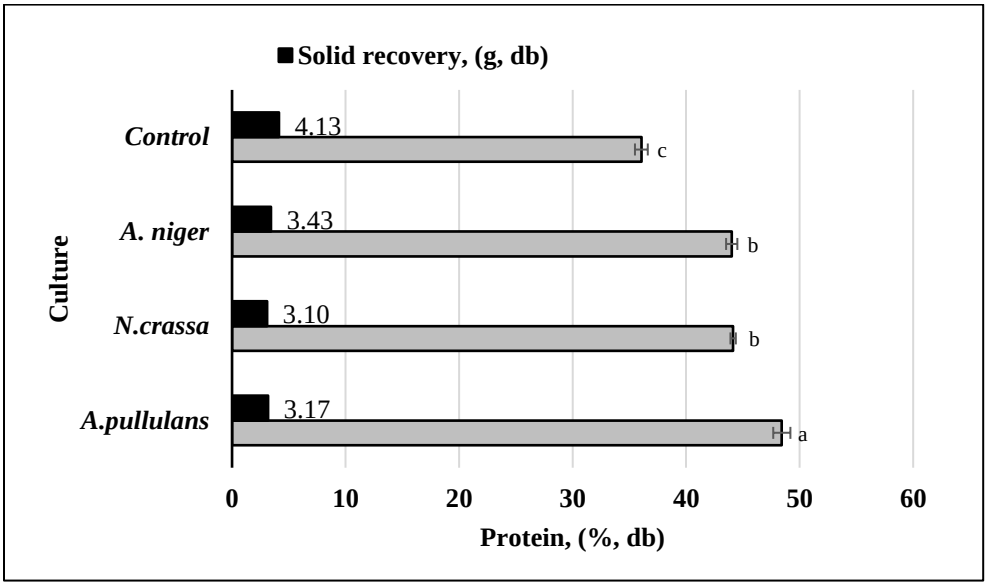

Figure 7.2: Protein content of the supernatant after 120 h of fermentation with different microbes. Significant differences ($p \leq 0.05$) among different cultures are indicated by letter.

7.4.6. Protein Digestibility

The protein ADC of washed canola (86.1%, Table 7.7) was similar to that of raw canola (81.0%), partly because one of its replicates was much lower than the other two. The protein ADC of raw canola is higher than what was reported by Barrows and Sealey (2015) (75%), but lower than for Mwachireya et al. (1999) (88.1%) and Cheng and Hardy (2002) (94.8%). However, the differences in protein ADCs of canola meal reported above cannot be explained by differences in the diet manufacturing process (Cheng and Hardy,

2003; Booth et al., 2000) because the diet used by (Barrows and Sealey, 2015) was cook-extruded, unlike diets in other studies that were cold pressed. Earlier studies reported that washing improved protein (White, 2017) and amino acid (Kasiga et al., 2021) ADCs of diets containing soybean meal and carinata meal, respectively; subsequently resulting in improvement in fish growth (Kasiga et al., 2021; White, 2017). However, in these studies, the meals were washed twice, and a lower solid loading rate was used. These two factors ensure more removal of solubles containing some ANFs that likely reduce nutrient digestibility.

Table 7.7: Apparent protein digestibility coefficients (ADC, %) of hexane extracted canola meal (HECM) and washed HECM

Meal	ADCs (%)
HECM	80.98±3.77
Washed HECM	86.09±5.07
P. value	0.4638

7.5. Conclusion

This study was designed to explore the effects of mild water washing pretreatment and mono-/co-culture fermentation on the ANFs of HECM and its protein digestibility to diversify its market utilization. A mild washing step improved the nutritional characteristics of HECM by removing significant amounts of soluble sugars, while concentrating protein and amino acid content. Though mild wash of HECM slightly improved protein digestibility when it was fed to rainbow trout as compared to untreated HECM, improvement was statistically insignificant. Fungal fermentation of washed HECM and untreated HECM further improved their nutritional characteristics. Mono- and co-culture fermentations improved protein and total amino acid levels as compared to the

uninoculated controls. The reduction of GLS, phytic acid, crude fiber, and soluble sugar level was achieved by using fungi in mono- and co-culture fermentations. A short water wash followed by fungal fermentation improves the nutritional characteristics of HECM providing a protein rich feed ingredient with significantly low level of ANFs. Further optimization of pretreatment process (temperature, pH, mixing time, etc.) and fungal fermentation growth conditions (inoculum size, pH, temperature, etc.) can help improve HECM as a feed ingredient. Currently additional research is underway to optimize the process and determine the protein digestibility of fermented HECM on fish.

Chapter 8. Estimation of Cellulase Production by *Aureobasidium pullulans*, *Neurospora crassa*, and *Trichoderma reesei* during Solid and Submerged State Fermentation of Raw and Processed Canola Meal †

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8.1. Abstract

In this study, cellulase, endoglucanase, and β-glucosidase activity produced by three fungal strains (*Aureobasidium pullulans*, *Neurospora crassa* and *Trichoderma reesei*) were determined on three differently processed canola substrates [hexane extracted canola meal (HECM), washed HECM (WHECM), and canola seed sprout meal (CSM)] in mono- and co-culture fermentation under solid- and submerged- state processes. Results showed that the co-culture inoculation yielded better results as compared to the monocultures except for *T. reesei* which yielded the highest β-glucosidase activity in WHECM (440.02 U/g). Co-cultures of *A. pullulans* + *N. crassa* had the highest cellulase activity in CSM (1.89 FPU/g), and WHECM (1.22 FPU/g); and the maximal endoglucanase activity at (HECM, 2.67 U/g), (CSM, 3.69 U/g) and (WHECM, 3.21 U/g). These results highlight the impact of various conditions on microbial enzymatic activities, and the need of strategically designed fermentation process for optimal cellulase activities to achieve maximal fiber degradation in canola meal.

8.2. Introduction

Canola meal (CM), a protein-rich (~42, dry basis) by-product of canola oil extraction process (Alhomodi et al., 2021a), is used mostly as an animal feed supplement in swine, poultry, ruminant or aquaculture. It is produced in large volumes and has potential to be a food grade protein (Wanasundara et al., 2017; Wanasundara et al., 2016). However, there are no reports on industrial use of this material as a source of food protein ingredients, which indicates the lack of efficient technologies for the extraction of CM proteins. Additionally, the use of CM in feed is also limited at low levels (10 to 30% inclusion rates) due to the presence of undesirable components [glucosinolates (GLS), phytic acid, high fiber contents, etc.] (Wu and Muir, 2008; Yoshie-Stark et al., 2008). Therefore, majority of CM remains unused and hence considered as the low-priced commodity. In recent times, the demand of plant-based proteins in animal and human diet has increased, and therefore wide range of technologies have been explored to improve the quality and the subsequent use of CM proteins (Alhomodi et al., 2021c; Ismail et al., 2020; Croat et al., 2017). The successful outcomes of these attempts are expected to not only increase the price and value of CM but would also become an alternative to soybean meal protein which continues to dominate the plant protein market. Among all, fermentation technology has a potential opportunity to improve the nutritional quality of CM. Reportedly, fermentation of CM with filamentous fungi effectively reduces the majority of antinutritional factors (ANFs) to low levels except fiber (Alhomodi et al., 2021a; Croat et al., 2016a; Croat et al., 2016b). High fiber is attributed to mostly seed hulls as hulls are kept intact during oil extraction process of canola (CCC, 2019). The lignocellulosic nature of the fiber was difficult to degrade even

with the application of pretreatment (hot water, mild chemical pretreatment, extrusion, etc.) prior to fungal fermentation (Croat et al., 2017).

Moreover, the extent of fiber degradation of CM during fermentation varies greatly among the published studies. According to Croat et al. (2016a), solid state fermentation (SSF) of raw and processed CM using different microorganisms including *Aureobasidium pullulans* and *Trichoderma reesei* showed no fiber reduction, while Alhomodi et al. (2021a) reported 28.9 and 33.3% fiber reduction in CM by *Neurospora crassa* and the co-culture of *A. pullulans* and *N. crassa* under SSF, respectively. Another study by Alhomodi et al. (2022b), showed different fiber reduction of raw CM and washed CM fermented by *A. pullulans, N. crassa* and *Aspergillus niger* and their co-cultures under submerged fermentation (SmF). According to (Alhomodi et al., 2021c), SSF of canola sprouts showed 6.1% decrease in crude fiber by *A. pullulans* while *N. crassa* and *T. reesei* exhibited no fiber reduction. But when similar fungi were grown on canola sprouts under SmF, *N. crassa* showed 6.6% fiber reduction while no fiber decrease was seen by *A. pullulans* and *T. reesei* (Alhomodi et al., 2021b). All these observations led to the assumption that cellulolytic activities of the tested microorganisms were likely governed by various factors (substrate type, fermentation conditions, etc.), and therefore same microbe yielded different degree of fiber reduction. The conversion of fibers into simple sugars during fermentation would require the microorganisms to produce three different enzymes that are collectively named as cellulases (exoglucanase, endoglucanase, and β-glucosidase), and lack of any one of them would result in the partial or no degradation of fibers. Hence, estimating the cellulase production during fermentation of CM under various processing conditions would generate

the knowledge that can be implemented to advance fermentation technology needed for upgrading the CM.

The present work determines the cellulase activities (cellulase, endoglucanase, and β-glucosidase) of three fungal strains (*A. pullulans* NRRL-Y-2311-1, *N. crassa* NRRL-2332 and *T. reesei* ATCC 24449) cultivated on three differently processed canola substrates [hexane extracted canola meal (HECM), washed HECM, and canola seed sprout meal (CSM)] under solid- and submerged- state fermentations, putting special emphasis on fermentation as an efficient technology for fiber degradation. Hence, the objective of this study was to determine the fungal enzymatic activities on (a) three different canola substrates; (b) two different fermentation systems (solid vs submerged); (c) monoculture process of *A. pullulans*, *N. crassa* and *T. reesei*; and (d) co-culture process of (*A. pullulans* + *N. crassa*). Co-culture of *A. pullulans* and *N. crassa* was only tested as these microorganisms were found to be the ideal combination for CM fiber reduction as compared to their combination with *T. reesei* (Alhomodi et al., 2021a). All the microorganisms used in this study are generally regarded as safe (GRAS).

8.3. Materials and Methods

8.3.1. Chemicals

The Pierce™ Coomassie Plus (Bradford) Assay Kit and Amplex® Red Glucose/Glucose Oxidase Assay Kit were purchased from Thermo Fisher Scientific Inc. (Waltham, Massachusetts, USA).

8.3.2. Preparation of Different Canola Substrates

8.3.2.1. Hexane Extracted Canola Meal (HECM)

HECM was received from ADM/Specialty Commodities LLC (Fargo, North Dakota, USA). HECM was ground using Krups coffee grinder (Solingen, Germany) and stored in air-tight container at room temperature (~22 °C) until use.

8.3.2.2. Washed Canola Meal

In 200 mL centrifuge bottle, 20 g of HECM was mixed in 100 mL deionized water at room temperature (~22 °C) for 2 min using a spatula. The slurry was centrifuged at 10,000 g for 10 min at 23 °C. Total amount of solid fraction as recovered was weighed and oven dried (60 °C/24 h) while liquid fraction was discarded. The dried solid was ground and stored at room temperature (~22 °C) in a tightly sealed container. CM was washed in triplicate.

8.3.2.3. Canola Seed Sprout Meal Preparation

Canola seeds were obtained from ADM/Specialty Commodities LLC (Fargo, North Dakota, USA) and stored in a sealed container at room temperature (~22 °C) throughout experiment period. Details of CSM preparation can be found in previous work by Alhomodi et. al. (2022a). Briefly, canola seed (50 g) were soaked in water (800 mL) at room temperature (~22 °C) for 24 h. At the end of soaking, excess water was drained, and seeds were placed in a 1 L round wide-mouth Pyrex glass bottles and covered with cheesecloth. The bottles were then incubated at room temperature (~22 °C) for 72 h with periodic moisture addition during incubation. At the end of sprouting period, hulls were removed partially using water and then dried in an oven at 50 °C for 24 h. The dried sprouts then were ground using Krups coffee grinder (Solingen, Germany) and defatted via a pilot

scale solvent extraction unit (model FT29, Armfield Ltd, Hampshire, England). The obtained defatted canola sprout meal was stored in sealed buckets at room temperature (~22 °C). Multiple set of sprouting experiments were conducted to produce 2 kg of CSM and mixed prior to defatting CSM for further use as substrate for fungal fermentation.

8.3.3. Inoculum Preparation for Mono- and Bi-culture Incubation

Fungal strains, *Aureobasidium pullulans* (NRRL-Y-2311-1), *Neurospora crassa*, (NRRL-2332) and *Trichoderma reesei* (ATCC 24449) were acquired from the National Center for Agricultural Utilization Research (Peoria, Illinois, USA). Eight mm plug of 5-day fungal colonies grown on potato dextrose agar were transferred into 100 mL defined medium (GYE) (5% glucose and 0.5% yeast extract). The inoculated GYEs were used as inocula after 48 h incubation in rotary shaker (30 °C/150 rpm).

8.3.4. Submerged Fermentation of HECM, Washed HECM, and Canola Seed Sprout Meal

Submerged fermentation (SmF) was performed in 250 mL Erlenmeyer flasks with a working volume of 100 mL and solid loading rate of 10% on dry basis. The pH was adjusted to (~5.5). All flasks were autoclaved (121 °C for 20 min) and then inoculated with 1 mL (48-h) culture of respective microbe. Whereas for the co-culture (*A. pullulans* + *N. crassa*), 0.5 mL of each microbe was used as the inoculum. The co-cultures of *T. reesei* either with *A. pullulans* or *N. crassa* were excluded from this study due to the low levels of fiber reduction with these combinations as compared to *A. pullulans* and *N. crassa* (Alhomodi et al., 2021a). All flasks in triplicates were incubated in rotary shaker at 30 °C /150 rpm for 120 h.

8.3.5. Solid State Fermentation of HECM, Washed HECM, and Canola Seed Sprout Meal

Solid state fermentation (SSF) was conducted in 250 mL wide mouth Erlenmeyer flasks at 1:1 solid to water ratio on w/w basis (10 g solids mixed with 10 mL of water) and pH of ~5.5. All flasks were heat-sterilized (121 °C/20 min). Monoculture flasks were inoculated with 1 mL of 48 h inoculum, whereas co-culture flasks (*A. pullulans* + *N. crassa*) were inoculated with 0.5 mL of 48 h cultures of respective microbes. SSF was continued for 168 h at 30 °C in a static incubator (Lab-line 3526 orbital incubator shaker).

8.3.6. Preparation of Crude Enzyme Extract for Enzyme Assays

There is slight difference in the way crude enzyme extract were prepared for submerged- and solid-state fermentations. For instance, at the end of SmF, the whole content of flask was centrifuged at 10,000 g for 10 min at 4 °C. The supernatant recovered was used as crude enzyme for enzyme assays, whereas the solids recovered were dried at 80 °C for 48 h using an oven. While at the end of SSF, 50 mL of 0.05 M citrate buffer was added to fermented meal and then placed in rotary shaker at 30 °C/200 rpm for 30 min. Thereafter, the contents of flasks were processed as mentioned above in SmF, and the supernatant recovered was used as crude enzyme.

8.3.7. Analytical Assays

8.3.7.1. Determination of Protein Content

Crude protein for solid was determined using a LECO model FP528 (St. Joseph, MI, USA). Firstly, the total emitted nitrogen gas of combusted samples (0.2490~0.2500g) was measured and used for determining total protein content by using a conversion factor

of 6.25. While the protein content of the supernatant was quantified using Pierce™ Coomassie Plus (Bradford) Assay Kit.

8.3.7.2. Determination of Enzyme Activity

8.3.7.2.1 Cellulase Activity

Filter paper assay (FPA) (Ghose, 1987) was used to measure cellulase activity. A 1.0×6.0 cm Whatman No. 1 paper strip was prepared as substrate for testing the cellulase activity present in crude enzyme. In a test tube, a rolled filter paper strip, 1.0 mL of 0.05 M citrate buffer, and 0.5 mL of tested crude enzyme were added together. Then, the tube was incubated in water bath at 50 °C for exactly 60 min. After incubation, 3 mL of dinitrosalicylic acid (DNS) (Miller, 1959) was added to test samples, blanks, controls and standards. All tube samples were then placed in boil bath for 5 min followed by ice bath to stop the reaction. After that 0.2 mL of each sample was diluted into 2.5 mL of deionized water (dH_2O) and then 0.3 mL of each sample was placed into 96 well plate. The absorbance of samples was measured at 540 nm using synergy 2 MicroPlate Reader.

To determine the activity of endoglucanase in obtained crude enzyme, the endoglucanase (CMCase) assay was used as described by Ghose (1987). In a test tube, 0.5 mL of 2% carboxymethyl cellulose solution and 0.5 mL of tested enzyme were mixed and followed by water bath incubation at 50 °C for 30 min. After incubation, all tubes (test samples, blanks, controls, and standards) followed same procedures mentioned in cellulase assay.

β-glucosidase activity was measured using a Ghose (1987) method. A test tube with 1.0 mL of 0.015 M cellobiose and 1.0 mL 0.05 M citrate buffer was incubated in water bath at 50 °C for 30 min. At the end of incubation, all tubes (test samples, blanks, controls,

and standards) were placed in boiling water bath to stop the reaction. The reducing sugar in the reaction then was measured using Amplex® Red Glucose/Glucose Oxidase Assay Kit.

In all above enzyme assays, the enzyme activity (U) was defined as the amount of enzyme required to release 1 µmol of glucose per minute under each assay condition.

8.3.8. Statistical Analysis

Microsoft® Excel® for Office 365 MSO 64-bit and R version 1.2.5001 (Boston, MA) were used in the statistical analysis. The statistically significant differences among variances determined by one-way ANOVA at $p \leq 0.05$ level of significance.

8.4. Results and Discussions

8.4.1. Cellulase Activity under Submerged State Fermentation

Table 8.1 shows the enzymatic activity (cellulase, CMCase, and β-glucosidase) of single and mixed cultures of fungi on different CM substrates (HECM, CSM and WHECM) during 120 h of submerged incubation. CM contains significant level of soluble proteins and sugars, and therefore enzyme assay (which essentially measures the free glucose present in the extract) of uninoculated control gave the false positive enzyme activity. To determine the true enzyme activity of microbes in the CM, enzyme activity of fermented samples was reported by subtracting the enzyme activity of uninoculated control.

In HECM, *N. crassa* showed the highest cellulase activity (0.26 FPU/g) followed by *A. pullulans* (0.24 FPU/g) while *T. reesei* and co-culture of *A. pullulans* + *N. crassa* showed the least cellulase activity of 0.19 FPU/g (Table 8.1). When fungi were grown on CSM, *A. pullulans* exhibited maximal cellulase activity (0.32 FPU/g) followed by co-

culture of *A. pullulans* + *N. crassa* (0.31 FPU/g), but *N. crassa* alone did not produce cellulase, and *T. reesei* had minimal cellulase activity (0.08 FPU/g). Reportedly, pure cellulose is a strong inducer of cellulases (Bischof et al., 2013), thus high fiber containing substrates can be better suited for the cellulase production. As majority of the hull fractions were removed from the CSM during processing, it is primarily composed of low levels of insoluble fibers and high level of soluble sugars (Alhomodi et al., 2022a), which likely are the contributing factors for the low levels of cellulases in CSM. Unlike *N. crassa* forming dispersed mycelia and *T. reesei* forming dispersed pellet in submerged fermentation, *A. pullulans* being single celled yeast, has homogenous growth, and more access to the substrates during orbital shaking incubation, thereby inducing cellulase production. In WHECM, *N. crassa* presented the highest cellulase level (0.80 FPU/g) followed by *A. pullulans* (0.34 FPU/g), *A. pullulans* + *N. crassa* (0.17 FPU/g) and *T. reesei* (0.14 FPU/g). The high levels of cellulases in WHECM compared to HECM and CSM can be attributed to the composition of WHECM, where fibers were concentrated due to the removal of soluble components including sugars (Alhomodi et al., 2022b). For CMCase activity, *A. pullulans* exhibited the maximal activity of 2.27, 3.17 and 3.09 U/g in HECM, CSM and WHECM, respectively compared to other microbial cultures. Thus, *A. pullulans* cultivated on CSM would be the best for endoglucanase production. For β-glucosidase activity, the co-culture of *A. pullulans* and *N. crassa* showed the highest activity at 302.56 U/g in HECM, subsequent by *A. pullulans* at 87.36 U/g, whereas *N. crassa* and *T. reesei* had activity of 2.64 and 2.46 U/g, respectively. In CSM, the β-glucosidase activity was at the highest with the co-culture of *A. pullulans* + *N. crassa* (229.32 U/g) followed by *A. pullulans* (60.37 U/g), *N. crassa* (27.54 U/g) and *T. reesei* (24.16 U/g). The highest β-

glucosidase activity in WHECM was achieved by the co-culture of *A. pullulans* + *N. crassa* at 177.18 U/g followed by *A. pullulans* (156.45 U/g), *N. crassa* (58.36 U/g) and *T. reesei* (43.88 U/g). Among all canola processed substrates and cultures, the co-culture of *A. pullulans* + *N. crassa* and HECM were the best combination of cultures and growth medium for β-glucosidase production. These observations led to the conclusion that single cell fungi such as *A. pullulans* can perform better in terms of enzymatic production over filamentous fungi in submerged fermentation, besides the structural and or compositional characteristics of the substrates can greatly influence the cellulase induction during fermentation.

Table 8.1: Enzyme activity of mono- and co-culture under submerged fermentation

Enzymes	Processed substrate	Culture			
		A. pullulans	***N. crassa***	***T. reesei***	***A.p+N.c***
Cellulase (FPU/g)	HECM	0.24 ± 0.12^{aA}	0.26 ± 0.05^{aAB}	0.19 ± 0.04^{aA}	0.19 ± 0.32^{aA}
	CSM	0.32 ± 0.10^{aA}	0.00 ± 0.00^{cB}	0.08 ± 0.07^{bcA}	0.31 ± 0.13^{abA}
	WHECM	0.34 ± 0.05^{aA}	0.80 ± 0.54^{aA}	0.14 ± 0.07^{aA}	0.17 ± 0.16^{aA}
CMCase (units/g)	HECM	2.27 ± 0.43^{aB}	0.67 ± 0.42^{bB}	0.62 ± 0.08^{bB}	0.54 ± 0.05^{bB}
	CSM	3.17 ± 0.22^{aA}	1.08 ± 0.40^{bAB}	1.27 ± 0.11^{bA}	1.22 ± 0.18^{bA}
	WHECM	3.09 ± 0.33^{aAB}	2.17 ± 0.74^{abA}	1.20 ± 0.18^{bcA}	0.45 ± 0.01^{cB}
β-glucosidase (units/g)	HECM	87.36 ± 2.00^{bB}	2.64 ± 0.38^{cB}	2.46 ± 1.82^{cB}	302.56 ± 0.17^{aA}
	CSM	60.37 ± 9.05^{aC}	27.54 ± 18.61^{aAB}	24.16 ± 13.07^{aAB}	229.32 ± 20.33^{aB}
	WHECM	156.45 ± 5.73^{aA}	58.36 ± 9.57^{bcA}	43.88 ± 10.24^{cA}	177.18 ± 4.12^{abC}

Mean ± standard deviation. Lowercase letters signify significance among cultures for each substrate at ($p\leq0.05$). Uppercase letters signify significance among substrates for each fungus at ($p\leq0.05$). HECM: hexane extracted canola meal; CSM: canola sprout meal; WHECM: washed hexane extracted canola meal; *A.p+N.c*: *A. pullulans* + *N. crassa*.

8.4.2. Protein Content in Liquid and Solid Fraction under Submerged State Fermentation

Unlike reportedly used cellulosic substrates fortified with minerals and inorganic nitrogen for cellulase production, HECM, WHECM and CSM are protein rich substrates with protein content of 42.86, 43.81 and 43.44%, respectively. The protein content of those canola derived substrates has been reported to further increase when subjected to microbial

fermentation due to microbial metabolism of carbohydrates and removal of soluble components (Alhomodi et al., 2021a; Alhomodi et al., 2021b; Alhomodi et al., 2021c).

Figures 8.1 and 8.2 show the protein content of the liquid and solid fraction separated by centrifugation from mono- and co-fermented processed substrates (HECM, CSM and WHECM), respectively under submerged incubation. In HECM (Figure 8.1), the co-culture of *A. pullulans* + *N. crassa* showed the higher protein content in the liquid fraction (19.49 mg/g) compared to *A. pullulans* (13.98 mg/g), *N. crassa* (2.30 mg/g) and *T. reesei* (1.11 mg/g). In return, the protein content of HECM in solid fraction (Figure 8.2) showed *T. reesei* with the highest protein content (48.6%) followed by co-culture of *A. pullulans* + *N. crassa* (46.9%) and *N. crassa* (46.3%) while *A. pullulans* had least protein at 40.5%. For protein content of CSM in liquid fraction (Figure 8.1), *A. pullulans*, *N. crassa* and co-culture of *A. pullulans* + *N. crassa* showed close protein solubility at 14.25, 13.82, and 14.10 mg/g, respectively, whereas *T. reesei* presented the least protein at 9.05 mg/g. In contrast, *T. reesei* revealed maximal protein at 50.2% followed by *N. crassa*, co-culture (*A. pullulans* + *N. crassa*) and *A. pullulans* at 50.0, 49.0 and 45.6%, respectively in the solid fraction (Figure 8.2). The protein content of liquid fraction in WHECM (Figure 8.1) was at the maximal (26.21 mg/g) with *A. pullulans* followed by co-culture of *A. pullulans* + *N. crassa* (21.86 mg/g), *N. crassa* (14.82 mg/g) and *T. reesei* (10.68 mg/g). While in solid fraction of WHECM (Figure 8.2), *T. reesei* again showed the highest protein at 49.2% subsequent by *N. crassa*, co-culture (*A. pullulans* + *N. crassa*) and *A. pullulans* with protein level of 48.4, 46.6 and 41.6%, consecutively. These results indicate the solubilization of protein during fermentation can differ based on substrates and microbes used. According to Zahler et al. (2018) the protein content of supernatant recovered by centrifugation from

fermented sorghum hominy at 120 h was lower with *T. reesei* compared to *N. crassa* and *A. pullulans*, whereas *T. reesei* had higher protein content in solid fraction compared to other fungi. Similarly, the separation of liquid from solid fraction of 120 h fermented canola seed sprout showed lower protein in liquid but higher in solid with *T. reesei* compared to other microbes (Alhomodi et al., 2021b).

Regardless the level of protein present in the liquid fraction used as crude enzyme, there was no positive correlation between enzyme activity and protein content when comparing protein level (Figure 8.1) with enzymatic activity (Table 8.1). Nevertheless, this study is focused on cellulolytic enzymes, it is critical to highlight the other possible enzymes that can play role in fermentation process such as proteases. The high soluble protein present in liquid fraction indicates the high protease activity, which might reduce the cellulase activity. According to Qian et al. (2019) a rapid decline in cellulase activity of *Trichoderma reesei* was seen at late cultivation stages (>120 h) when using corn steep liquor as nitrogen source compared to $(NH_4)_2SO_4$. The researchers attributed the cellulase reduction to the high protease activity. Thus, using substrate with suitable protein level, hydrolyzed protein, or non-protein nitrogen sources could avoid this phenomenon.

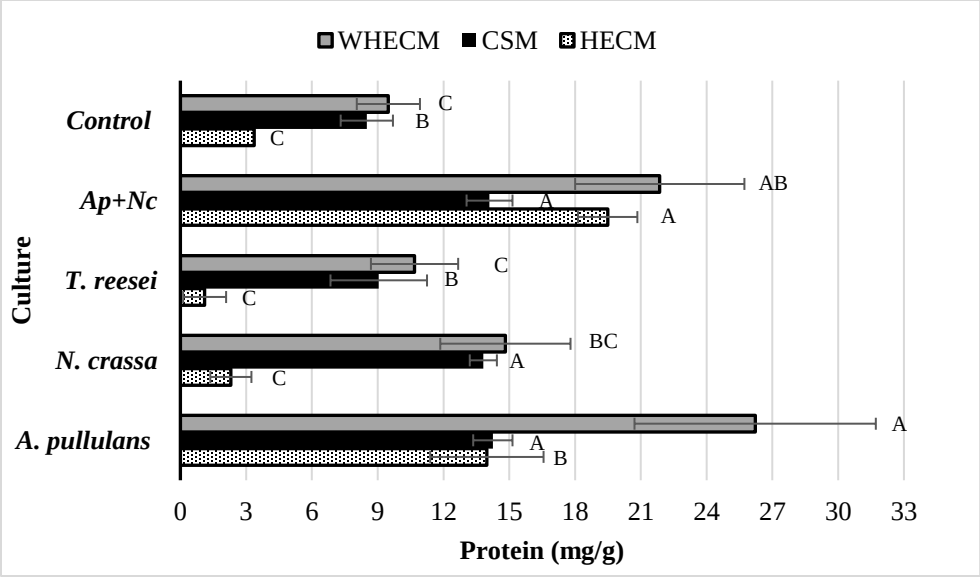

Figure 8.1: Protein content in the supernatant of mono- and co-culture under submerged fermentation. Uppercase letters signify significance among cultures for each substrate at ($p \leq 0.05$). *A.p+N.c*: *A. pullulans* + *N. crassa*.

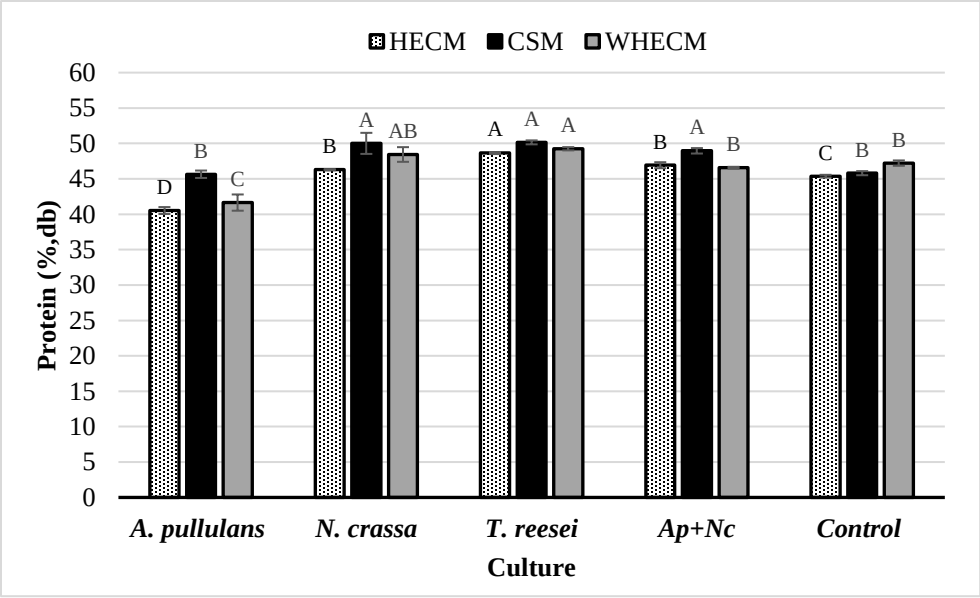

Figure 8.2: Crude protein in the pellet of mono- and co-culture under submerged fermentation. Uppercase letters signify significance among cultures for each substrate at ($p \leq 0.05$). *A.p+N.c*: *A. pullulans* + *N. crassa*.

8.4.3. Enzyme Activity under Solid State Fermentation

Table 8.2 shows the enzymatic activity (cellulase, endoglucanase, and β-glucosidase) of mono- and co-culture in HECM, CSM and WHECM under solid state

incubation. The highest cellulase activity in HECM was seen with *N. crassa* at 0.82 FPU/g followed by its incubation with *A. pullulans* at 0.73 FPU/g while *A. pullulans* alone and *T. reesei* showed lower activity at 0.10 and 0.07 FPU/g, respectively. In CSM, co-culture of *A. pullulans* + *N. crassa* produced higher cellulase at 1.89 FPU/g as compared to individual fungi (*N. crassa*, 1.44 FPU/g), (*A. pullulans*, 0.28 FPU/g) and (*T. reesei*, 0.12 FPU/g). Cellulase activity of co-culture of *A. pullulans* + *N. crassa* again showed the maximal in WHECM at 1.22 FPU/g, whereas *N. crassa* alone came second with 0.89 FPU/g followed by *A. pullulans* (0.19 FPU/g) and *T. reesei* (0.17 FPU/g). Compared to *N. crassa* cellulase production in SmF (Table 8.1), *N. crassa* showed better cellulase activity in SSF (Table 8.2), which indicates the effect of fermentation system on fungal performance. In addition, co-culture of *A. pullulans* + *N. crassa* showed higher cellulases in all processed canola substrates in SSF (Table 8.2) compared to SmF (Table 8.1). Ensuring the ability of co-cultured fungi to perform well in both fermentation modes (SmF and SSF) could help in better synergistic relationship, thereby higher cellulase. The low level of cellulase seen in SmF (Table 8.1) was attributed to morphological growth of *N. crassa* (dispersed mycelia) compared to *A. pullulans* (single cell). However, the better performance of co-culture in SSF is due the ability of both fungi to grow on solid substrate. Especially, *A. pullulans* (yeast like fungi) that can be adapted to both fermentation modes because of its ability to show different morphological growth based on cultivation modes (septate filaments, large chlamydospores, and yeast-like cell) (Chi et al., 2009). For endoglucanase, co-culture of *A. pullulans* and *N. crassa* presented the highest CMCase in (HECM, 2.67 U/g) followed by *A. pullulans* (2.24 U/g), *N. crassa* (1.89 U/g) and *T. reesei* (1.78 U/g). Similarly, co-culture of *A. pullulans* + *N. crass* produced maximal CMCase in CSM at 3.69 U/g,

subsequent by *A. pullulans*, *N. crassa* and *T. reesei* at 2.70, 2.47 and 1.08 U/g, respectively. Again, co-culture of *A. pullulans* and *N. crassa* had highest endoglucanase activity (3.21 U/g) in WHECM, while lower activities were achieved by *N. crassa* (1.80 U/g), *A. pullulans* (1.62 U/g) and *T. reesei* (1.35 U/g). These data highlighted the importance of co-culture of *A. pullulans* + *N. crassa* in producing higher endoglucanase compared to monoculture even when grown on different processed substates. It is reported that the high sugar level causes feedback inhibition of cellulolytic enzyme (Bader et al., 2010), thus the high seen level of endoglucanase might indicate the high rate of sugar consumption by fungi which shows the advantage of co-culture in preventing the accumulation of simple sugar. For β-glucosidase activity, the co-culture of *A. pullulans* + *N. crassa* exhibited much higher activity at 180.74 U/g in HECM compared to *A. pullulans* (94.64 U/g), *N. crassa* (18.09 U/g) and *T. reesei* (3.02 U/g). Similarly, in CSM, the co-culture of (*A. pullulans* + *N. crassa*) showed superiority in β-glucosidase activity at 220.58 U/g compared to monoculture (*A. pullulans*, 128.00 U/g), (*N. crassa*, 190.84 U/g) and (*T. reesei*, 203.72 U/g). But in WHECM, the highest β-glucosidase activity was achieved with *T. reesei* at 440.02 U/g followed by co-culture (*A. pullulans* + *N. crassa*), *A. pullulans*, and *N. crassa* at 348.57, 332.63 and 216.64 U/g, respectively. WHECM showed better suitability for β-glucosidase production with all cultures compared to other canola processed substrates. The removal of soluble sugars during mild pretreatment in WHECM could be a reason of forcing the fungi to produce β-glucosidase to cleave cellobiose, liberated by the combined action of endoglucanase and exoglucanase on cellulose (Foreman et al., 2003), into glucose for growth. In comparison to previous reports (Table 8.3), the high detectable enzymatic activity (cellulase, endoglucanase and β-glucosidase) can be attributed to the substrate

composition and microbes used. It is evident from the literature that the enzyme activity is largely governed by the substrate composition and microbe used. Unlike reportedly used low protein lignocellulosic substrates supported by inorganic nitrogen prior to fermentation, canola derived substrates (WHECM, CSM, and HECM) are nutrient rich substrates with high organic protein content and mixture of carbohydrates (poly-, oligo- and mono-saccharides), that can provide a complete set of nutrients for microbial growth and induce the cellulolytic enzymes. According to Brijwani et al. (2010), *T. reesei* (ATCC 26921) produced 6.55 FPU/g, which was higher than the used *T. reesei* (ATCC 24449) in this study; however, authors indicated that *T. reesei* (ATCC 26921) produces 1.5-2.0 times higher cellulase on cellulose medium compared to strain *T. reesei* (ATCC 24449) used in this study. According to Chakraborty et al. (2016), the carbon and nitrogen sources had significant effect on cellulase, endoglucanase and β-glucosidase activity of *Trichoderma sp.* RCK65. In their study, the maximum cellulase (41.09 U/g substrate), endoglucanase (149.64 U/g substrate) and β-glucosidase (106.27 U/g substrate) by *Trichoderma sp*. RCK65 was achieved when the wheat bran was supported by 2.4% soybean meal (soybean oil process byproduct) as organic nitrogen source. Similarly, Vieira et al. (2021) reported maximum cellulase activity (FPase 2.45 U/mL; CMCase, 6.86 U/mL) after 60 h when soybean meal was used as a non-synthetic nitrogen sources. However, the present study used 100% canola processed substates (WHECM, CSM, and HECM), which may explain the high enzymatic activities as compared to the reported values during SSF.

Table 8.2: Enzyme activity of mono- and co-culture under solid state fermentation

Enzymes	Processed substrate	Culture			
		A. pullulans	***N. crassa***	***T. reesei***	***A.p+N.c***
Cellulase (FPU/g)	**HECM**	0.10 ± 0.04^{bB}	0.82 ± 0.28^{aB}	0.07 ± 0.01^{bA}	0.73 ± 0.08^{aC}
	CSM	0.28 ± 0.06^{cA}	1.44 ± 0.20^{bA}	0.12 ± 0.04^{cA}	1.89 ± 0.17^{aA}
	WHECM	0.19 ± 0.05^{bAB}	0.89 ± 0.18^{aAB}	0.17 ± 0.08^{bA}	1.22 ± 0.21^{aB}
CMCase (U/g)	**HECM**	2.24 ± 0.30^{aB}	1.89 ± 0.08^{aB}	1.78 ± 0.59^{aA}	2.67 ± 0.14^{aB}
	CSM	2.70 ± 0.05^{bA}	2.47 ± 0.23^{bA}	1.08 ± 0.31^{cA}	3.69 ± 0.24^{aA}
	WHECM	1.62 ± 0.05^{bC}	1.80 ± 0.26^{bB}	1.35 ± 0.07^{bA}	3.21 ± 0.22^{aA}
β-glucosidase (U/g)	**HECM**	94.64 ± 1.89^{dB}	18.09 ± 1.12^{cB}	3.02 ± 1.11^{bC}	180.74 ± 19.82^{aA}
	CSM	128.00 ± 3.24^{bB}	190.84 ± 12.24^{abA}	203.72 ± 6.28^{aB}	220.58 ± 8.65^{abA}
	WHECM	332.63 ± 5.99^{abA}	216.64 ± 17.23^{bA}	440.02 ± 6.18^{aA}	348.57 ± 15.27^{abA}

Mean ± standard deviation. Lowercase letters signify significance among cultures for each substrate at ($p\leq0.05$). Uppercase letters signify significance among substrates for each fungus at ($p\leq0.05$). HECM: hexane extracted canola meal; CSM: canola sprout meal; WHECM: washed hexane extracted canola meal; *A.p+N.c*: *A. pullulans + N. crassa*.

Table 8.3: Comparison of cellulase productions on different substrates and microbes

Substrate	Microbe	Parameters	Cellulase	Beta	Endo	Reference
Boiled bagasse syrup + wheat bran based	*T. reesei* #NCIM 1186	SSF, 10 days	5.07 IU/mL	-	-	(Verma and Kumar, 2020)
Boiled bagasse syrup + wheat bran based	*N. crassa* #NCIM 1021	SSF, 10 days	2.41 IU/mL	-	-	(Verma and Kumar, 2020)
Luffa cylindrica peel waste + Normal basal salt media	*T. reesei* #NCIM 1186	SSF, 6 days	4.0 IU/mL	-	-	(Verma et al., 2018)
Soybean hulls and wheat bran in a 4:1 ratio	*T. reesei* #ATCC 26921	SSF, 4 days	6.55 FPU/g	6.30 U/g	60.17 U/g	(Brijwani et al., 2010)
Oil palm frond leaves+ Mendel medium	*Trichoderma asperellum* UC1	SSF, 7 days	26.03 U/g	130.09 IU/g	136.16 IU/g	(Ezeilo et al., 2019)
Wheat bran + 2.4% soybean meal	*Trichoderma sp* RCK65	SSF, 3 days	41.09 U/g	106.27 U/g	149.64 U/g	(Chakraborty et al., 2016)
Wheat bran + mineral salt solution	*Inonotus obliquus*	SSF, 7 days	3.16 IU/g	2.53 IU/g	27.15 IU/g	(Xu et al., 2018)
Sugarcane bagasse	*A. pullulans* LB83	SmF, 3 days	2.27 U/mL	-	7.42 U/mL	(Vieira et al., 2021)
Washed hexane extracted canola meal	*A. pullulans*	SSF, 7 days	0.19 FPU/g	332.63 U/g	1.62 U/g	Present study
Washed hexane extracted canola meal	*N. crassa*	SSF, 7 days	0.89 FPU/g	216.64 U/g	1.80 U/g	Present study
Washed hexane extracted canola meal	*T. reesei*	SSF, 7 days	0.17 FPU/g	440.02 U/g	1.35 U/g	Present study

Beta: β-glucosidase; Endo; endoglucanase. -: not reported.

8.4.4. Protein Content in Liquid and Solid Fraction under Solid State Fermentation

Figures 8.3 and 8.4 present the protein content in liquid (crude enzyme by 50 mL of 0.05 M citrate buffer) and solid of mono- and co- fermented processed substrates (HECM, CSM, and WHECM), respectively under solid state fermentation. The co-culture of *A. pullulans* + *N. crassa* solubilized more protein at 27.75 mg/g than monoculture of *A. pullulans* (11.26 mg/g), *N. crassa* (12.57 mg/g) and *T. reesei* (2.09 mg/g) in HECM (Figure 8.3). In solid fraction of HECM (Figure 8.4), co-culture of *A. pullulans* + *N. crassa* showed maximal protein of 44.2% while *A. pullulans* came second with 42.3% followed by *T. reesei* (41.1%) and *N. crassa* (40.5%). Co-culture of *A. pullulans* + *N. crassa* again presented higher protein content 26.89 mg/g in CSM (Figure 8.3) compared to *A. pullulans* (13.30 mg/g), *N. crassa* (20.38 mg/g) and *T. reesei* (7.62 mg/g). While in the solid fraction of CSM (Figure 8.4), *N. crassa* had the highest protein (56.9%) followed by co-culture of *A. pullulans* + *N. crassa* (54.8%), *T. reesei* (47.9%) and *A. pullulans* (46.2%). In liquid fraction of WHECM (Figure 8.3), co-culture of *A. pullulans* + *N. crassa* exhibited the highest protein as well at 29.00 mg/g followed by *N. crassa*, *A. pullulans* and *T. reesei* at 20.07, 12.68 and 11.20 mg/g, respectively, but in the solid fraction (Figure 8.4), co-culture of *A. pullulans* + *N. crassa* exhibited maximal protein level (47.2%) followed by *N. crassa* (46.8%), *A. pullulans* (44.5%) and *T. reesei* (42.8%). Unlike submerged fermentation, the high protein seen in solid fractions is due to the dry matter loss during solid state fermentation where fungi metabolize carbohydrates into carbon dioxide (Tabacco et al., 2011). Whereas the high protein presents in liquid is attributed to solubilization of protein because of fungal proteases and low amount of buffer used (50 mL) for protein extraction, which led to concentrated protein in the obtained liquid.

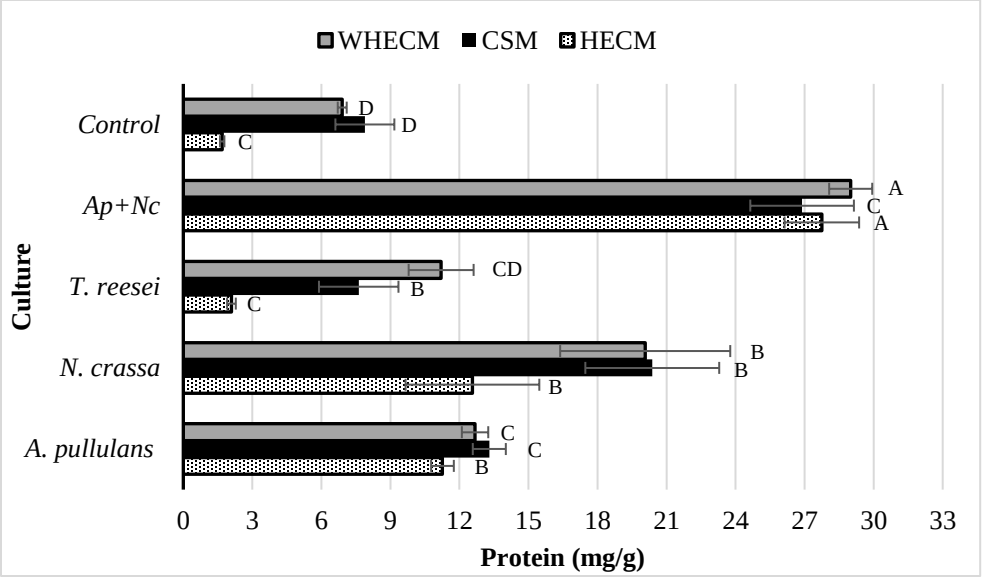

Figure 8.3: Protein content in the supernatant of mono- and co-culture under solid state fermentation. Uppercase letters signify significance among cultures for each substrate at ($p \leq 0.05$). *A.p+N.c*: *A. pullulans* + *N. crassa*.

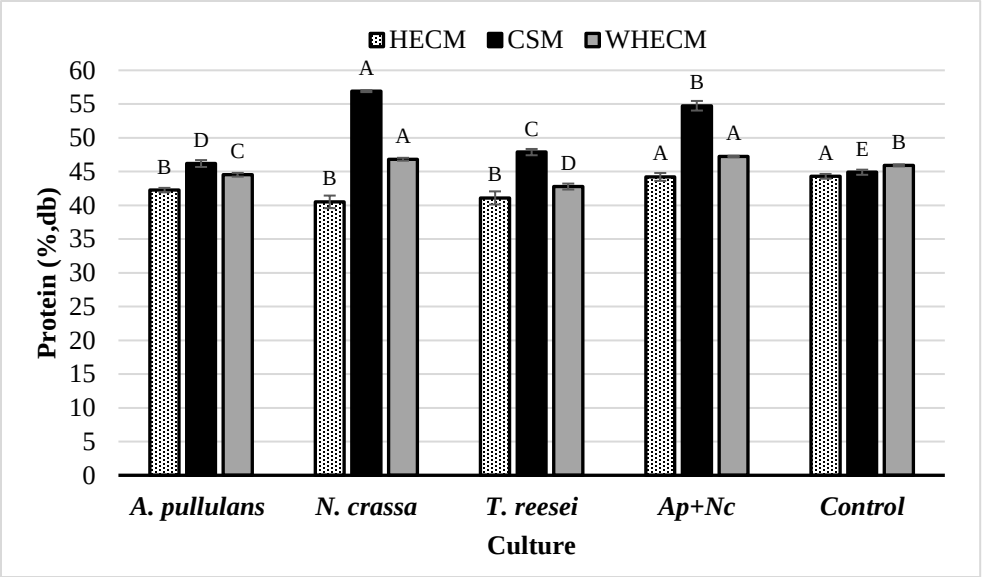

Figure 8.4: Crude protein in the pellet of mono- and co-culture under solid state fermentation. Uppercase letters signify significance among cultures for each substrate at ($p \leq 0.05$). *A.p+N.c*: *A. pullulans* + *N. crassa*.

8.5. Conclusion

This study indicates that fungi (yeast and filamentous), substrates, fermentation systems (solid and submerged state processes), and type of inoculation (mono- and co-cultures) can play crucial role in affecting cellulase, endoglucanase and β-glucosidase enzyme production. Cellulase activities were low during the CM fermentation by using fungi for short duration (5 to 7 days). Therefore, to achieve the high level of fiber degradation in CM, fermentation process needs to be strategically designed for maximal cellulase activities by carefully selecting the key parameters such as microbial strains, submerged- or solid-state fermentations, and mono- or co-culture inoculations.

Chapter 9. Summary and Future Studies

Canola is an important oil seed crop providing seeds with about 44% oil that is extracted for use as one of the healthiest edible oils. Processing canola seed for oil extraction results in the production of a byproduct called canola meal (CM) that has around 42% protein on dry basis and is used for animal feed (CCC, 2019). CM has high fiber content attributed to seed hull not removed prior to oil extraction (CCC, 2019) and contains antinutritional factors (such as glucosinolates, phytic acid, and phenolic). Therefore, only ~30% of CM can be included in animal diet (Newkirk, 2009b). Thus, we aimed to develop a process to diversify the use of canola via this dissertation. Throughout this dissertation, sets of experiment were conducted on canola substrates, where various technologies (sprouting, fermentation, pretreatments) were either applied individually or in combination to upgrade the meal quality for its wider application. For instance, the traditional seed sprouting process followed by submerged and solid-state fermentation; mono- and co-culture solid state fermentation of hexane extracted CM (HECM); and mild pretreatment of HECM followed by mono and co-culture submerged incubation were applied. Besides, the cellulolytic enzyme activity (cellulase, β-glucosidase, and endoglucanase) were estimated under all meal treatments and parameters.

Seed sprouts are known to enhance the level of essential and non-essential nutrients (proteins, carbohydrates, vitamins, and minerals) and reduce the antinutritional factors compared to unsprouted seed (Benincasa et al., 2019; Park et al., 2019; Santos et al., 2020). Daily observations in compositional changes of sprouted canola seed starting from soaking up to 144 h incubation provided insight into selecting the optimal sprouting intervals for desirable sprout characteristics for its final use. Harvesting canola seed sprout at 72 h

helped in removing seed hull; conserving oil content and oil profile but with undesirable free fatty acid increase; and providing meal with high protein and amino acid and significantly low levels of fibers and other ANFs such as phytic acid. When canola seed sprout was harvested at 144 h, oil content and phytic acid reduced while soluble sugars, protein, moisture and glucosinolates (GLS) increased. These data highlight the benefit of seed sprouting as process to boost the canola desirable compositions. But long sprouting intervals need to be avoided to conserve seed oil profile and yield. Thus, it is advisable to optimize sprouting time to be less than three days to prevent the hydrolysis of oil thereby avoiding the increase of free fatty acid content. In addition, at sprout harvesting time, it was noticed some delayed sprout seeds that were difficult to dehull or separate from other sprout hulls. Thus, it is suggested to select good quality canola seed for sprouting and apply mechanical hull separation for efficient hull removal.

Microbial fermentation have been shown to improve the composition of different seed sprouts such as pearl millet sprouts (Khetarpaul and Chauhan, 1990b), red cabbage sprouts (Hunaefi et al., 2013) and buckwheat sprouts (Maejima et al., 2011). Fermentation of 144 h canola seed sprouts using three different generally regarded as safe fungal strains (*Aureobasidium pullulans*, *Neurospora crassa* and *Trichoderma reesei*) under submerged and solid-state incubations further improved the sprout composition by concentrating protein and reducing ANFs compared to uninoculated controls. However, fermentation was performed on full fat sprouts as an attempt to provide full fat fermented meal as feed ingredient. Thus, the removal of fat fraction prior to or after fermentation would further concentrate protein content and show the effect of fermentation on oil composition. For more efficient fermentation process and for lower fermentation cost, growth conditions

such as pH, temperature, solid loading rate, etc. are proposed to be optimized to obtain similar or better outcome at minimal fermentation time.

Co-culture fermentation have been used to produce many products such as chemicals, food additives, antibiotics, enzymes, microbial biomass, etc. (Bader et al., 2010). Co-culture solid state fermentations of hexane extracted canola meal (HECM) enhanced the nutritional characteristics of HECM by increasing protein content and decreasing levels of several ANFs. Mild water washing pretreatment of HECM helped in removing significant amounts of GLS and soluble sugars while concentrating protein and amino acid content. In addition, mild washed HECM (WHECM) had higher protein digestibility compared to untreated HECM when fed to rainbow trout. Mono- and co-culture fermentation of HECM and WHECM under submerged process further improved protein and total amino acid levels compared to uninoculated controls besides the reduction of GLS, phytic acid, crude fiber, and soluble sugar levels. However, for effective exploitation of co-culture fermentation and lower processing cost, optimization of growth conditions, subsequential inoculation, and co-culture between fungi and bacteria can be tested to further improve meal compositions and decrease fermentation time. Also, it is suggested to test the digestibility and palatability of fermented CM on desirable animals to ensure the feasibility of the fermented product as feed ingredient prior to scaling up the process.

From these previous studies data, it was noticed that same microbes yielded different outcomes in terms of ANF reductions when they were gown on different canola substrates and conditions. Which indicated the effect of media and growth factors on microbial enzyme activity. Microbial enzymes have been used for a long time in various

industries such as food, chemicals, pharmaceuticals, etc. due to their cost effectiveness and ecofriendly properties (Singh et al., 2016). Specifically, cellulolytic enzymes have been exploited in bioconversion of complex carbohydrates into simple sugar and produced from fermented lignocellulose (Siqueira et al., 2020). Mono- and co-culture fermentation under solid- and submerged- state processes of three differently processed canola substrates (HECM, WHECM and canola seed sprout meal) resulted in various activities of cellulase, β-glucosidase and endoglucanase which indicated the importance of preprocessing conditions, microbial selections, and fermentation systems on microbial enzymatic activities. In addition to aforementioned factors, growth conditions such as pH, temperature, medium size, inoculum size, etc. can be examined at deferent levels to further understand and evaluate their effect on fermentation process, enzymatic activity, and final product. Further investigation on protease, phytase and myrosinase activity of fermented CM would provide a complete insight into the correlation between enzyme activity and CM targeted compounds.

Overall, the obtained data in this study indicates the advantages of using traditional seed sprouting, mono-/co- fermentation under solid and submerged processes, and mild water washing pretreatment on providing nutritive canola meals with high desirable nutrients (protein, amino acid) and low ANFs (fibers, GLS, phytic acid, soluble sugars) for potential expanded use of canola. In addition, the enzymatic activity data denotes tested fungi can produce different level of enzymes based on growth conditions and used substrates. The findings of this study lead us to some future work suggestions, which are:

1. Studying the effect of full fat and defatted dehulled canola sprout meal diet on growth and body composition of fed animals.

2. Evaluating the protein extractability and functional properties of canola seed sprout for potential inclusion in human food.
3. Evaluating the protein extractability and functionality of fermented CM.

Chapter 10. Literature Cited

AACC, I. (2010). Approved methods of analysis: In method 44.20.01 moisture- air-oven Method. *AACC International, 44.20.01*(11th Ed), St. Paul, MN, USA.

Aachary, A. A., & Thiyam, U. (2012). A pursuit of the functional nutritional and bioactive properties of canola proteins and peptides. *Critical reviews in food science and nutrition, 52*(11), 965-979.

Abdel-Aty, A. M., Salama, W. H., Fahmy, A. S., & Mohamed, S. A. (2019). Impact of germination on antioxidant capacity of garden cress: New calculation for determination of total antioxidant activity. *Scientia Horticulturae, 246*, 155-160.

Abrahamsen, M., & Sudia, T. (1966). Studies on the soluble carbohydrates and carbohydrate precursors in germinating soybean seed. *American journal of botany, 53*(2), 108-114.

Abu El Gasim, A. Y., Mohammed, M. A., & Baker, A. A. A. (2008). Effect of soaking, sprouting and cooking on chemical composition, bioavailability of minerals and in vitro protein digestibility of roselle (Hibiscus sabdariffa L.) seed. *Pakistan Journal of Nutrition, 7*(1), 50-56.

Aider, M., & Barbana, C. (2011). Canola proteins: composition, extraction, functional properties, bioactivity, applications as a food ingredient and allergenicity–A practical and critical review. *Trends in food science & technology, 22*(1), 21-39.

Akbari, A., & Wu, J. (2016). Cruciferin nanoparticles: Preparation, characterization and their potential application in delivery of bioactive compounds. *Food Hydrocolloids, 54*, 107-118.

Akram, M., Asif, H., Uzair, M., Akhtar, N., Madni, A., Shah, S. A., & Hasan, Z. (2011). Amino acids: A review article. *Journal of medicinal plants research, 5*(17).

Alhomodi, A. F., Berhow, M., Gibbons, W. R., Monono, E., & Karki, B. (2022a). Meal nutritional characteristics and oil profile of sprouted, dehulled, and solvent-extracted canola. *Journal of the Science of Food and Agriculture*. doi:https://doi.org/10.1002/jsfa.11794

Alhomodi, A. F., Kasiga, T., Berhow, M., Brown, M. L., Gibbons, W. R., & Karki, B. (2022b). Combined effect of mild pretreatment and fungal fermentation on nutritional characteristics of canola meal and nutrient digestibility of processed canola meal in rainbow trout. *Food and Bioproducts Processing, 133*, 57-66.

Alhomodi, A. F., Zavadil, A., Berhow, M., Gibbons, W. R., & Karki, B. (2021a). Application of cocultures of fungal mycelium during solid-state fermentation of canola meal for potential feed application. *Journal of the American Oil Chemists' Society, 98*(5), 509-517. doi:https://doi.org/10.1002/aocs.12479

Alhomodi, A. F., Zavadil, A., Berhow, M., Gibbons, W. R., & Karki, B. (2021b). Composition of canola seed sprouts fermented by Aureobasidium pullulans, Neurospora crassa, and Trichoderma reesei under submerged-state fermentation. *Food and Bioproducts Processing, 126*, 256-264. doi:https://doi.org/10.1016/j.fbp.2021.01.008

Alhomodi, A. F., Zavadil, A., Berhow, M., Gibbons, W. R., & Karki, B. (2021c). Daily development of nutritional composition of canola sprouts followed by solid-state fungal fermentation. *Food and Bioprocess Technology, 14*, 1673–1683. doi:https://doi.org/10.1007/s11947-021-02667-2

Aljubori, A., Idrus, Z., Soleimani, A. F., Abdullah, N., & Juan Boo, L. (2017). Response of broiler chickens to dietary inclusion of fermented canola meal under heat stress condition. *Italian Journal of Animal Science, 16*(4), 546-551.

Aluko, R. E., & McIntosh, T. (2001). Polypeptide profile and functional properties of defatted meals and protein isolates of canola seeds. *Journal of the Science of Food and Agriculture, 81*(4), 391-396.

Aluko, R. E., McIntosh, T., & Katepa-Mupondwa, F. (2005). Comparative study of the polypeptide profiles and functional properties of Sinapis alba and Brassica juncea seed meals and protein concentrates. *Journal of the Science of Food and Agriculture, 85*(11), 1931-1937.

Amanullah, S., Kim, D., Lee, H., Joo, Y., Kim, S., & Kim, S. (2014). Effects of microbial additives on chemical composition and fermentation characteristics of barley silage. *Asian-Australasian journal of animal sciences, 27*(4), 511.

Andini, S., Dekker, P., Gruppen, H., Araya-Cloutier, C., & Vincken, J.-P. (2019). Modulation of glucosinolate composition in Brassicaceae seeds by germination and fungal elicitation. *Journal of agricultural and food chemistry, 67*(46), 12770-12779.

Anh, H. T. H., Shahsavari, E., Bott, N. J., & Ball, A. S. (2021). Application of co-culture technology to enhance protease production by two halophilic bacteria, Marinirhabdus sp. and Marinobacter hydrocarbonoclasticus. *Molecules, 26*(11), 3141.

ANKOM. (2006). Acid detergent fiber and neutral detergent fiber in feeds - filter bag technique (for A2000 and A2000I). Retrieved from https://www.ankom.com/analytical-methods-support/fiber-analyzer-a2000

AOAC. (2000). Official method 969.33. fatty acids in oils and fats, preparation of methyl esters boron trifluoride method. *AOAC INTERNATIONAL*, Arlington, Virginia.

AOAC. (2002). Official method 996.06 fat (total, saturated, and unsaturated) in foods. *AOAC INTERNATIONAL*, Gaithersburg, Maryland, USA.

AOAC. (2006a). Official method 982.30 E (a, b, c), chp, 45.3. 05. *Association of Official Analytical Chemists*, Arlington, Virginia.

AOAC. (2006b). Official method 988.15 , chp. 45.4.04. *Association of Official Analytical Chemists*, Arlington, Virginia.

AOAC. (2006c). Official methods 973.18 (A-D) 18th Ed. *AOAC INTERNATIONAL*, Gaithersburg, Maryland, USA.

AOAC. (2006d). Official methods 978.10. 18th Ed. *AOAC INTERNATIONAL*, Gaithersburg, Maryland, USA.

AOAC. (2006e). Official methods of analysis (methods 972.43 and 990.08). *Association of Official Analytical Chemists*, Arlington, Virginia.

AOCS. (1997). Official method Ca 5a-40: free fatty acids. *American Oil Chemists' Society*, Champaign, IL, USA.

AOCS. (1998). Official method Ce 2-66: preparation of methyl esters of fatty acids. *American Oil Chemists' Society*, Champaign, IL, USA.

AOCS. (2005). Official method Ba 6a-05, official methods and recommended practices of the AOCS. *American Oil Chemists' Society*, Champaign, IL, USA.

Asl, P. J., & Niazmand, R. (2020). Modelling and simulation of supercritical CO2 extraction of bioactive compounds from vegetable oil waste. *Food and Bioproducts Processing, 122*, 311-321.

Assa, R. R., Konan, J. K., Prades, A., Nemlin, J., & Koffi, E. (2010). Physicochemical characteristics of kernel during fruit maturation of four coconut cultivars (Cocos nucifera L.). *African Journal of Biotechnology, 9*(14), 2136-2144.

Aubert, J.-P., Beguin, P., & Millet, J. (1988). Biochemistry and genetics of cellulose degradation.

Aukema, H., & Campbell, L. (2011). Oil nutrition and utilization. In *Canola* (pp. 245-280): Elsevier.

Austreng, E., Storebakken, T., Thomassen, M. S., Refstie, S., & Thomassen, Y. (2000). Evaluation of selected trivalent metal oxides as inert markers used to estimate apparent digestibility in salmonids. *Aquaculture, 188*(1-2), 65-78.

Ayton, J. (2014). Variability of quality traits in canola seed, oil and meal: a review. *New South Wales Department of Primary Industries. New South Wales, Australia*.

Azeke, M. A., Egielewa, S. J., Eigbogbo, M. U., & Ihimire, I. G. (2011). Effect of germination on the phytase activity, phytate and total phosphorus contents of rice (Oryza sativa), maize (Zea mays), millet (Panicum miliaceum), sorghum (Sorghum bicolor) and wheat (Triticum aestivum). *Journal of food science and technology, 48*(6), 724-729.

Azulay, S. (1997). Sprouts-the forgotten food (part 1). An overview of sprouts and nutrition. *San Diego Earth Times (SDET) Health and diet. San Diego, CA*, 1-5.

Bader, J., Mast-Gerlach, E., Popović, M. K., Bajpai, R., & Stahl, U. (2010). Relevance of microbial coculture fermentations in biotechnology. *Journal of applied microbiology, 109*(2), 371-387.

Balasaraswathi, R., & Sadasivam, S. (1997). Changes in oil, sugars and nitrogenous components during germination of sunflower seeds, Helianthus annuus. *Plant foods for human nutrition, 51*(1), 71-77.

Baldwin, E. L., Karki, B., Zahler, J. D., Rinehart, M., & Gibbons, W. R. (2019). Submerged vs. solid-state conversion of soybean meal into a high protein feed using Aureobasidium pullulans. *Journal of the American Oil Chemists' Society, 96*(9), 989-998.

Ban, Y., Khan, N. A., & Yu, P. (2017). Nutritional and metabolic characteristics of Brassica carinata co-products from biofuel processing in dairy cows. *Journal of agricultural and food chemistry, 65*(29), 5994-6001.

Barata, A., Malfeito-Ferreira, M., & Loureiro, V. (2012). The microbial ecology of wine grape berries. *International Journal of Food Microbiology, 153*(3), 243-259.

Barnett, C. C., Berka, R. M., & Fowler, T. (1991). Cloning and amplification of the gene encoding an extracellular β-glucosidase from Trichoderma reesei: evidence for improved rates of saccharification of cellulosic substrates. *Bio/technology, 9*(6), 562-567.

Barrows, F. T., & Sealey, W. M. (2015). Feed ingredients and the reduction of dependence on marine harvested products for aquaculture feeds. *Derived from https://makeway.org/wp-content/uploads/2015/12/Barrows_Sealey_White_Paper_on_Feed_Ingredients.pdf*.

Barthet, V. J., & Daun, J. K. (2005). Effect of sprouting on the quality and composition of canola seed and oil. *Journal of the American Oil Chemists' Society, 82*(7), 511-517.

Barthet, V. J., & Daun, J. K. (2011). Seed morphology, composition, and quality. In *Canola* (pp. 119-162): Elsevier.

Bau, H. M., Villaume, C., Nicolas, J. P., & Méjean, L. (1997). Effect of germination on chemical composition, biochemical constituents and antinutritional factors of soya bean (Glycine max) seeds. *Journal of the Science of Food and Agriculture, 73*(1), 1-9.

Bell, J. (1993). Factors affecting the nutritional value of canola meal: a review. *Canadian Journal of Animal Science, 73*(4), 689-697.

Bell, J., & Keith, M. (1991). A survey of variation in the chemical composition of commercial canola meal produced in Western Canadian crushing plants. *Canadian Journal of Animal Science, 71*(2), 469-480.

Bellostas, N., Kachlicki, P., Sørensen, J. C., & Sørensen, H. (2007). Glucosinolate profiling of seeds and sprouts of B. oleracea varieties used for food. *Scientia Horticulturae, 114*(4), 234-242.

Benincasa, P., Falcinelli, B., Lutts, S., Stagnari, F., & Galieni, A. (2019). Sprouted grains: A comprehensive review. *Nutrients, 11*(2), 421.

Berhow, M. A., Polat, U., Glinski, J. A., Glensk, M., Vaughn, S. F., Isbell, T., . . . Gardner, C. (2013). Optimized analysis and quantification of glucosinolates from Camelina sativa seeds by reverse-phase liquid chromatography. *Industrial Crops and Products, 43*, 119-125.

Bérot, S., Compoint, J., Larré, C., Malabat, C., & Guéguen, J. (2005). Large scale purification of rapeseed proteins (Brassica napus L.). *Journal of Chromatography B, 818*(1), 35-42.

Beszterda, M., & Nogala-Kałucka, M. (2019). Current research developments on the processing and improvement of the nutritional quality of rapeseed (Brassica napus L.). *European Journal of Lipid Science and Technology, 121*(5), 1800045.

Betz, J., & Fox, W. (1994). High-performance liquid chromatographic determination of glucosinolates in Brassica vegetables. In. Washington, DC: ACS Publications.

Bhardwaj, H. L., & Hamama, A. A. (2007). Yield and nutritional quality of canola sprouts. *HortScience, 42*(7), 1656-1658.

Bhardwaj, H. L., & Hamama, A. A. (2009a). Cultivar and growing location effects on oil content and fatty acids in canola sprouts. *HortScience, 44*(6), 1628-1631.

Bhardwaj, H. L., & Hamama, A. A. (2009b). Effect of cultivar and growing location on the mineral composition of canola sprouts. *HortScience, 44*(2), 508-511.

Bischof, R., Fourtis, L., Limbeck, A., Gamauf, C., Seiboth, B., & Kubicek, C. P. (2013). Comparative analysis of the Trichoderma reesei transcriptome during growth on the cellulase inducing substrates wheat straw and lactose. *Biotechnology for Biofuels, 6*(1), 1-14.

Bischof, R. H., Ramoni, J., & Seiboth, B. (2016). Cellulases and beyond: the first 70 years of the enzyme producer Trichoderma reesei. *Microbial cell factories, 15*(1), 106.

Black, M., & Bewley, J. D. (1983). Physiology and biochemistry of seeds in relation to germination.

Bocianowski, J., Mikołajczyk, K., & Bartkowiak-Broda, I. (2012). Determination of fatty acid composition in seed oil of rapeseed (Brassica napus L.) by mutated alleles of the FAD3 desaturase genes. *Journal of applied genetics, 53*(1), 27-30.

Bohn, L., Meyer, A. S., & Rasmussen, S. K. (2008). Phytate: impact on environment and human nutrition. A challenge for molecular breeding. *Journal of Zhejiang University Science B, 9*(3), 165-191.

Bonnardeaux, J. (2007). Uses for canola meal. *Department of Agriculture and Food*.

Booth, M. A., Allan, G. L., Frances, J., & Parkinson, S. (2001). Replacement of fish meal in diets for Australian silver perch, Bidyanus bidyanus: IV. Effects of dehulling and protein concentration on digestibility of grain legumes. *Aquaculture, 196*(1-2), 67-85.

Booth, M. A., Allan, G. L., & Warner-Smith, R. (2000). Effects of grinding, steam conditioning and extrusion of a practical diet on digestibility and weight gain of silver perch, Bidyanus bidyanus. *Aquaculture, 182*(3-4), 287-299.

Borisjuk, L., Neuberger, T., Schwender, J., Heinzel, N., Sunderhaus, S., Fuchs, J., . . . Denolf, P. (2013). Seed architecture shapes embryo metabolism in oilseed rape. *The Plant Cell, 25*(5), 1625-1640.

Boulter, D., & Barber, J. (1963). Amino-acid metabolism in germinating seeds of vicia faba L. in relation to their biology. *New phytologist, 62*(3), 301-316.

Bozan, B., & Temelli, F. (2002). Supercritical CO2 extraction of flaxseed. *Journal of the American Oil Chemists' Society, 79*(3), 231-235.

Bozoudi, D., & Tsaltas, D. (2018). The multiple and versatile roles of Aureobasidium pullulans in the vitivinicultural sector. *Fermentation, 4*(4), 85.

Brijwani, K., Oberoi, H. S., & Vadlani, P. V. (2010). Production of a cellulolytic enzyme system in mixed-culture solid-state fermentation of soybean hulls supplemented with wheat bran. *Process biochemistry, 45*(1), 120-128.

Brown, J., Davis, J., Lauver, M., & Wysocki, D. (2008). Canola growers' manual. *US Canola Association*, 1-71.

Brunson, J., Romaire, R., & Reigh, R. (1997). Apparent digestibility of selected ingredients in diets for white shrimp Penaeus setiferus L. *Aquaculture Nutrition, 3*(1), 9-16.

Buchholz, K., Kasche, V., & Bornscheuer, U. T. (2012). *Biocatalysts and enzyme technology*: John Wiley & Sons.

Burel, C., Boujard, T., Kaushik, S., Boeuf, G., Mol, K., Van der Geyten, S., . . . Quérat, B. (2001). Effects of rapeseed meal-glucosinolates on thyroid metabolism and feed utilization in rainbow trout. *General and Comparative Endocrinology, 124*(3), 343-358.

Cairns, T. C., Nai, C., & Meyer, V. (2018). How a fungus shapes biotechnology: 100 years of Aspergillus niger research. *Fungal biology and biotechnology, 5*(1), 13.

Çalışır, S., Marakoğlu, T., Öğüt, H., & Öztürk, Ö. (2005). Physical properties of rapeseed (Brassica napus oleifera L.). *Journal of Food Engineering, 69*(1), 61-66.

Campbell, L., Rempel, C. B., & Wanasundara, J. P. (2016). Canola/rapeseed protein: Future opportunities and directions—Workshop proceedings of IRC 2015. In: Multidisciplinary Digital Publishing Institute.

Carré, P., Citeau, M., Robin, G., & Estorges, M. (2016). Hull content and chemical composition of whole seeds, hulls and germs in cultivars of rapeseed (Brassica napus). *OCL, 23*(3), A302.

Carre, P., Dartenuc, C., Evrard, J., Judde, A., Labalette, F., Raoux, R., & Renard, M. (2003). *Frying stability of rapeseed oils with modified fatty acid composition.* Paper presented at the Proceedings of 11th International Rapeseed Congress.

Cartea, M. E., Lema, M., Francisco, M., & Velasco, P. (2011). Basic information on vegetable Brassica crops. *Genetics, genomics and breeding of vegetable Brassicas*, 1-33.

Castillo, M. R., Gutierrez-Correa, M., Linden, J. C., & Tengerdy, R. P. (1994). Mixed culture solid substrate fermentation for cellulolytic enzyme production. *Biotechnology letters, 16*(9), 967-972.

CCC. (2015). Feed industry guide • 5th edition. Canola Council of Canada, Winnipeg, MB, Canada. Retrieved from https://www.canolacouncil.org/media/516716/2015_canola_meal_feed_industry_guide.pdf

CCC. (2019). Feed industry guide • 6th edition. Canola Council of Canada, Winnipeg, Canada. Retrieved from http://canolamazing.com/wordpress/wp-content/uploads/2019/10/2019-Canola-Meal-Feed-Guide-Web.pdf

CCC. (2020). Health benefits of canola oil. Canola Council of Canada, Winnipeg, MB. Retrieved from https://www.canolacouncil.org/oil-and-meal/canola-oil/health-benefits-of-canola-oil/

Centeno, C., Viveros, A., Brenes, A., Canales, R., Lozano, A., & de la Cuadra, C. (2001). Effect of several germination conditions on total P, phytate P, phytase, and acid phosphatase activities and inositol phosphate esters in rye and barley. *Journal of agricultural and food chemistry, 49*(7), 3208-3215.

Chakraborty, S., Gupta, R., Jain, K. K., & Kuhad, R. C. (2016). Cost-effective production of cellulose hydrolysing enzymes from Trichoderma sp. RCK65 under SSF and its evaluation in saccharification of cellulosic substrates. *Bioprocess and biosystems engineering, 39*(11), 1659-1670.

Chen, L., Vadlani, P. V., & Madl, R. L. (2014). High-efficiency removal of phytic acid in soy meal using two-stage temperature-induced Aspergillus oryzae solid-state fermentation. *Journal of the Science of Food and Agriculture, 94*(1), 113-118.

Chen, Y. (2011). Development and application of co-culture for ethanol production by co-fermentation of glucose and xylose: a systematic review. *Journal of industrial microbiology & biotechnology, 38*(5), 581-597.

Cheng, Z., & Hardy, R. (2002). Effect of microbial phytase on apparent nutrient digestibility of barley, canola meal, wheat and wheat middlings, measured in vivo using rainbow trout (Oncorhynchus mykiss). *Aquaculture Nutrition, 8*(4), 271-277.

Cheng, Z. J., & Hardy, R. W. (2003). Effects of extrusion and expelling processing, and microbial phytase supplementation on apparent digestibility coefficients of nutrients in full-fat soybeans for rainbow trout (Oncorhynchus mykiss). *Aquaculture, 218*(1-4), 501-514.

Chi, Z., Wang, F., Chi, Z., Yue, L., Liu, G., & Zhang, T. (2009). Bioproducts from Aureobasidium pullulans, a biotechnologically important yeast. *Applied microbiology and biotechnology, 82*(5), 793-804.

Chicatto, J., Costa, A., Nunes, H., Helm, C., & Tavares, L. (2014). Evaluation of hollocelulase production by Lentinula edodes (Berk.) Pegler during the submerged fermentation growth using RSM. *Brazilian Journal of Biology, 74*, 243-250.

Chilakamarry, C. R., Sakinah, A. M., Zularisam, A., Sirohi, R., Khilji, I. A., Ahmad, N., & Pandey, A. (2022). Advances in solid-state fermentation for bioconversion of agricultural wastes to value-added products: Opportunities and challenges. *Bioresource technology, 343*, 126065.

Chiou, R. Y.-Y., Ku, K.-L., & Chen, W.-L. (1997). Compositional characterization of peanut kernels after subjection to various germination times. *Journal of agricultural and food chemistry, 45*(8), 3060-3064.

Chmielewska, A., Kozłowska, M., Rachwał, D., Wnukowski, P., Amarowicz, R., Nebesny, E., & Rosicka-Kaczmarek, J. (2020). Canola/rapeseed protein–nutritional value, functionality and food application: a review. *Critical reviews in food science and nutrition*, 1-21.

Christen, K., Schingoethe, D., Kalscheur, K., Hippen, A., Karges, K., & Gibson, M. (2010). Response of lactating dairy cows to high protein distillers grains or 3 other protein supplements. *Journal of dairy science, 93*(5), 2095-2104.

Chung, T., Nwokolo, E., & Sim, J. (1989). Compositional and digestibility changes in sprouted barley and canola seeds. *Plant foods for human nutrition, 39*(3), 267-278.

Ciska, E., Honke, J., & Kozłowska, H. (2008). Effect of light conditions on the contents of glucosinolates in germinating seeds of white mustard, red radish, white radish, and rapeseed. *Journal of agricultural and food chemistry, 56*(19), 9087-9093.

Clandinin, M. T., & Yamashiro, S. (1982). Dietary factors affecting the incidence of dietary fat-induced myocardial lesions. *The Journal of Nutrition, 112*(4), 825-828.

Collins, S. A., Desai, A. R., Mansfield, G. S., Hill, J. E., Van Kessel, A. G., & Drew, M. D. (2012). The effect of increasing inclusion rates of soybean, pea and canola meals and their protein concentrates on the growth of rainbow trout: concepts in diet formulation and experimental design for ingredient evaluation. *Aquaculture, 344*, 90-99.

Costa, A. M., Ribeiro, W. X., Kato, E., Monteiro, A. R. G., & Peralta, R. M. (2008). Production of tannase by Aspergillus tamarii in submerged cultures. *Brazilian Archives of Biology and Technology, 51*(2), 399-404.

Couto, S. R., & Sanromán, M. A. (2006). Application of solid-state fermentation to food industry—a review. *Journal of Food Engineering, 76*(3), 291-302.

Croat, J., Karki, B., Berhow, M., Iten, L., Muthukumarappan, K., & Gibbons, W. (2017). Utilizing pretreatment and fungal incubation to enhance the nutritional value of canola meal. *Journal of applied microbiology, 123*(2), 362-371.

Croat, J. R., Berhow, M., Karki, B., Muthukumarappan, K., & Gibbons, W. R. (2016a). Conversion of canola meal into a high-protein feed additive via solid-state fungal incubation process. *Journal of the American Oil Chemists' Society, 93*(4), 499-507.

Croat, J. R., Gibbons, W. R., Berhow, M., Karki, B., & Muthukumarappan, K. (2016b). Enhancing the nutritional value of canola (Brassica napus) meal using a submerged fungal incubation process. *Journal of Food Research, 5*(5), 1-10.

Crouch, M. L., & Sussex, I. M. (1981). Development and storage-protein synthesis in Brassica napus L. embryos in vivo and in vitro. *Planta, 153*(1), 64-74.

Damodaran, S. (2008). *Amino acids, peptides and proteins* (Vol. 4): CRC Press: Boca Raton, FL.

Daun, J. K. (2011). Origin, distribution, and production. In *Canola* (pp. 1-27): Elsevier.

Daun, J. K., Eskin, M. N., & Hickling, D. (2015). *Canola: chemistry, production, processing, and utilization*: Elsevier.

Dawood, M., Sadak, M., Reyad, B., El-Sayed, A., & El-Gayar, S. (2013). Changes in chemical composition during germination of some canola varieties changes in oil content and fatty acid composition. *Scientia Agriculturae, 2*(3), 77-82.

DeBonte, L., Iassonova, D., Liu, L., & Loh, W. (2012). Commercialization of high oleic canola oils. *Lipid Technology, 24*(8), 175-177.

Delisle, J., Amiot, J., Goulet, G., Simard, C., Brisson, G., & Jones, J. (1984). Nutritive value of protein fractions extracted from soybean, rapeseed and wheat flours in the rat. *Plant foods for human nutrition, 34*(4), 243-251.

Devi, C. B., Kushwaha, A., & Kumar, A. (2015). Sprouting characteristics and associated changes in nutritional composition of cowpea (Vigna unguiculata). *Journal of food science and technology, 52*(10), 6821-6827.

Díaz-Reinoso, B., Moure, A., Domínguez, H., & Parajó, J. C. (2006). Supercritical CO2 extraction and purification of compounds with antioxidant activity. *Journal of agricultural and food chemistry, 54*(7), 2441-2469.

Diosady, L., Rubin, L., Tar, C., & Etkin, B. (1986). Air classification of rapeseed meal using the Tervel separator. *The Canadian Journal of Chemical Engineering, 64*(5), 768-774.

Dogaris, I., Vakontios, G., Kalogeris, E., Mamma, D., & Kekos, D. (2009). Induction of cellulases and hemicellulases from Neurospora crassa under solid-state cultivation for bioconversion of sorghum bagasse into ethanol. *Industrial Crops and Products, 29*(2-3), 404-411.

Doi, E. (1993). Gels and gelling of globular proteins. *Trends in food science & technology, 4*(1), 1-5.

Dong, X. Y., Guo, L. L., Wei, F., Li, J. F., Jiang, M. L., Li, G. M., . . . Chen, H. (2011). Some characteristics and functional properties of rapeseed protein prepared by ultrasonication, ultrafiltration and isoelectric precipitation. *Journal of the Science of Food and Agriculture, 91*(8), 1488-1498.

Dossou, S., Koshio, S., Ishikawa, M., Yokoyama, S., Dawood, M. A., El Basuini, M. F., . . . Olivier, A. (2018). Effect of partial replacement of fish meal by fermented rapeseed meal on growth, immune response and oxidative condition of red sea bream juvenile, Pagrus major. *Aquaculture, 490*, 228-235.

Duenas, R., Tengerdy, R., & Gutierrez-Correa, M. (1995). Cellulase production by mixed fungi in solid-substrate fermentation of bagasse. *World Journal of Microbiology and Biotechnology, 11*(3), 333-337.

Ebune, A., Al-Asheh, S., & Duvnjak, Z. (1995). Effects of phosphate, surfactants and glucose on phytase production and hydrolysis of phytic acid in canola meal by Aspergillus ficuum during solid-state fermentation. *Bioresource technology, 54*(3), 241-247.

Egounlety, M., & Aworh, O. (2003). Effect of soaking, dehulling, cooking and fermentation with Rhizopus oligosporus on the oligosaccharides, trypsin inhibitor, phytic acid and tannins of soybean (Glycine max Merr.), cowpea (Vigna unguiculata L. Walp) and groundbean (Macrotyloma geocarpa Harms). *Journal of Food Engineering, 56*(2-3), 249-254.

El-Batal, A., & Karem, H. A. (2001). Phytase production and phytic acid reduction in rapeseed meal by Aspergillus niger during solid state fermentation. *Food Research International, 34*(8), 715-720.

El-Shishtawy, R. M., Mohamed, S. A., Asiri, A. M., Abu-bakr, M. G., Ibrahim, I. H., & Al-Talhi, H. A. (2015). Saccharification and hydrolytic enzyme production of alkali pre-treated wheat bran by Trichoderma virens under solid state fermentation. *BMC biotechnology, 15*(1), 1-13.

Elahi, N., Duncan, R. W., & Stasolla, C. (2016). Modification of oil and glucosinolate content in canola seeds with altered expression of Brassica napus LEAFY COTYLEDON1. *Plant physiology and Biochemistry, 100*, 52-63.

Etesami, H. (2022). Root nodules of legumes: a suitable ecological niche for isolating non-rhizobial bacteria with biotechnological potential in agriculture. *Current Research in Biotechnology*.

Ezeilo, U. R., Lee, C. T., Huyop, F., Zakaria, I. I., & Wahab, R. A. (2019). Raw oil palm frond leaves as cost-effective substrate for cellulase and xylanase productions by Trichoderma asperellum UC1 under solid-state fermentation. *Journal of Environmental Management, 243*, 206-217.

FAO. (2020). *State of World Fisheries and Aquaculture 2020. Sustainability in action*. Rome, Italy.

Farris, S., Unalan, I. U., Introzzi, L., Fuentes-Alventosa, J. M., & Cozzolino, C. A. (2014). Pullulan-based films and coatings for food packaging: Present applications, emerging opportunities, and future challenges. *Journal of Applied Polymer Science, 131*(13).

FDA. (2010). GRAS notice No. 327. GRAS notification for cruciferin-rich and napin-rich protein isolates derived from canola/rapeseed. U.S. Food and Drug Administration. Retrieved from https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=327&sort=GRN_No&order=DESC&startrow=1&type=basic&search=327

FDA. (2016). GRAS notice No. 683. The safety and the generally recognized as safe (GRAS) status of the proposed uses of canola/rapeseed protein isolate in human food. U.S. Food and Drug Administration. Retrieved from https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=683&sort=GRN_No&order=DESC&startrow=1&type=basic&search=683

Folawiyo, Y., & Apenten, R. O. (1997). The effect of heat-and acid-treatment on the structure of rapeseed albumin (napin). *Food chemistry, 58*(3), 237-243.

Foreman, P. K., Brown, D., Dankmeyer, L., Dean, R., Diener, S., Dunn-Coleman, N. S., . . . Kelley, A. S. (2003). Transcriptional regulation of biomass-degrading enzymes in the filamentous fungus Trichoderma reesei. *Journal of Biological Chemistry, 278*(34), 31988-31997.

Fouad, A. A., & Rehab, F. (2015). Effect of germination time on proximate analysis, bioactive compounds and antioxidant activity of lentil (Lens culinaris Medik.) sprouts. *Acta scientiarum polonorum. Technologia alimentaria, 14*(3).

Francis, G., Makkar, H. P., & Becker, K. (2001). Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture, 199*(3-4), 197-227.

Frias, J., Penas, E., & Martinez-Villaluenga, C. (2017). Fermented pulses in nutrition and health promotion. *Fermented foods in health and disease prevention*, 385-416.

Frias, J., Zieliński, H., Piskuła, M. K., Kozłowska, H., & Vidal-Valverde, C. (2005). Inositol phosphate content and trypsin inhibitor activity in ready-to-eat cruciferous sprouts. *Food chemistry, 93*(2), 331-336.

Friedrich, J., List, G., & Heakin, A. (1982). Petroleum-free extraction of oil from soybeans with supercritical CO 2. *Journal of the American Oil Chemists' Society, 59*(7), 288-292.

Friedrich, J., & Pryde, E. (1984). Supercritical CO2 extraction of lipid-bearing materials and characterization of the products. *Journal of the American Oil Chemists' Society, 61*(2), 223-228.

Gatlin III, D. (2010). Principles of fish nutrition. Southern Regional Aquaculture Center (SRAC) Publication No. 5003. In. Stoneville, MS.

Gautam, A. K., Sharma, S., Avasthi, S., & Bhadauria, R. (2011). Diversity, pathogenicity and toxicology of A. niger: an important spoilage fungi. *Research Journal of Microbiology, 6*(3), 270-280.

Gebremedhin, W. K. (2015). Nutritional benefit and economic value of feeding hydroponically grown maize and barley fodder for Konkan Kanyal goats. *IOSR J. Agric. Vet. Sci, 8*, 24-30.

Gesan-Guiziou, G. (2017). Dairy industry and animal products processing applications. In.

Ghanem, K., El-Refai, A., & El-Gazaerly, M. (1991). Protein-enriched feedstuff from beet pulp. *World Journal of Microbiology and Biotechnology, 7*(3), 365-371.

Ghavidel, R. A., & Prakash, J. (2007). The impact of germination and dehulling on nutrients, antinutrients, in vitro iron and calcium bioavailability and in vitro starch and protein digestibility of some legume seeds. *LWT-Food Science and Technology, 40*(7), 1292-1299.

Ghodsvali, A., Khodaparast, M. H., Vosoughi, M., & Diosady, L. (2005). Preparation of canola protein materials using membrane technology and evaluation of meals functional properties. *Food Research International, 38*(2), 223-231.

Ghose, T. (1987). Measurement of cellulase activities. *Pure and applied Chemistry, 59*(2), 257-268.

Ghumman, A., Kaur, A., & Singh, N. (2016). Impact of germination on flour, protein and starch characteristics of lentil (Lens culinari) and horsegram (Macrotyloma uniflorum L.) lines. *LWT-Food Science and Technology, 65*, 137-144.

Glencross, B. D., Booth, M., & Allan, G. L. (2007). A feed is only as good as its ingredients–a review of ingredient evaluation strategies for aquaculture feeds. *Aquaculture Nutrition, 13*(1), 17-34.

Graf, E., & Eaton, J. W. (1990). Antioxidant functions of phytic acid. *Free Radical Biology and Medicine, 8*(1), 61-69.

Graham, P. H., & Vance, C. P. (2003). Legumes: importance and constraints to greater use. *Plant physiology, 131*(3), 872-877.

Gunstone, F. D. (2004). *Rapeseed and canola oil: production, processing, properties and uses*: CRC Press.

Haas, M. J. (2005). Improving the economics of biodiesel production through the use of low value lipids as feedstocks: vegetable oil soapstock. *Fuel processing technology, 86*(10), 1087-1096.

Hagen, J. (2015). *Industrial catalysis: a practical approach*: John Wiley & Sons.

Halkier, B. A., & Gershenzon, J. (2006). Biology and biochemistry of glucosinolates. *Annu. Rev. Plant Biol., 57*, 303-333.

Hamama, A. A., & Bhardwaj, H. L. (2011). Characterization of total and individual sterols in canola sprouts. *Journal of the American Oil Chemists' Society, 88*(3), 361-366.

Hansen, J. Ø., Skrede, A., Mydland, L. T., & Øverland, M. (2017). Fractionation of rapeseed meal by milling, sieving and air classification—Effect on crude protein, amino acids and fiber content and digestibility. *Animal Feed Science and Technology, 230*, 143-153.

Haramoto, E. R., & Gallandt, E. R. (2004). Brassica cover cropping for weed management: A review. *Renewable agriculture and food systems, 19*(4), 187-198.

Heyer, C., Wang, L., Beltranena, E., & Zijlstra, R. (2018). 327 growth performance ofweaned pigs fed raw or extruded canola meal with low to high extrusion intensity. *Journal of animal science, 96*(suppl_2), 176-176.

Hilton, J., & Slinger, S. (1986). Digestibility and utilization of canola meal in practical-type diets for rainbow trout (Salmo gairdneri). *Canadian Journal of Fisheries and Aquatic Sciences, 43*(6), 1149-1155.

Hölker, U., Höfer, M., & Lenz, J. (2004). Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. *Applied microbiology and biotechnology, 64*(2), 175-186.

Hollister, E. B., Hu, P., Wang, A. S., Hons, F. M., & Gentry, T. J. (2013). Differential impacts of brassicaceous and nonbrassicaceous oilseed meals on soil bacterial and fungal communities. *FEMS microbiology ecology, 83*(3), 632-641.

Holst, D. O. (1973). Holst filtration apparatus for Van Soest detergent fiber analyses. *Journal of the Association of Official Analytical Chemists, 56*(6), 1352-1356.

Horwitz, W., & Latimer, G. (2006). AOAC official method 990.03, protein (crude) in animal feed, combustion method. *Official Methods of Analysis of AOAC International*, 1-30.

Hotz, C., & Gibson, R. S. (2007). Traditional food-processing and preparation practices to enhance the bioavailability of micronutrients in plant-based diets. *The Journal of Nutrition, 137*(4), 1097-1100.

Hu, Y., Wang, Y., Li, A., Wang, Z., Zhang, X., Yun, T., . . . Yin, Y. (2016). Effects of fermented rapeseed meal on antioxidant functions, serum biochemical parameters and intestinal morphology in broilers. *Food and Agricultural Immunology, 27*(2), 182-193.

Hunaefi, D., Gruda, N., Riedel, H., Akumo, D. N., Saw, N. M. M. T., & Smetanska, I. (2013). Improvement of antioxidant activities in red cabbage sprouts by lactic acid bacterial fermentation. *Food biotechnology, 27*(4), 279-302.

Hung, P. V., Maeda, T., Yamamoto, S., & Morita, N. (2012). Effects of germination on nutritional composition of waxy wheat. *Journal of the Science of Food and Agriculture, 92*(3), 667-672.

Hussain, S., Afzal, M., Nasir, S., Javid, A., Makhdoom, S., Jabeen, F., . . . Shah, S. (2016). Efficacy of phytase supplementation in improving mineral digestibility in Labeo rohita fingerlings fed on canola meal-based diets. *Iranian Journal of Fisheries Sciences, 15*(2), 645-661.

Ibrahim, S., Habiba, R., Shatta, A., & Embaby, H. (2002). Effect of soaking, germination, cooking and fermentation on antinutritional factors in cowpeas. *Food/Nahrung, 46*(2), 92-95.

IMARC. (2019). Canola oil market: Global industry trends, share, size, growth, opportunity and forecast 2019-2024.

Imran, M., Anwar, Z., Irshad, M., Asad, M. J., & Ashfaq, H. (2016). Cellulase production from species of fungi and bacteria from agricultural wastes and its utilization in industry: a review. *Advances in Enzyme Research, 4*(2), 44-55.

Intasit, R., Cheirsilp, B., Louhasakul, Y., Boonsawang, P., Chaiprapat, S., & Yeesang, J. (2020). Valorization of palm biomass wastes for biodiesel feedstock and clean solid biofuel through non-sterile repeated solid-state fermentation. *Bioresource technology, 298*, 122551.

Inyang, C., & Zakari, U. (2008). Effect of germination and fermentation of pearl millet on proximate, chemical and sensory properties of instant "Fura"-a Nigerian cereal food. *Pakistan Journal of Nutrition, 7*(1), 9-12.

Ismail, B. P., Senaratne-Lenagala, L., Stube, A., & Brackenridge, A. (2020). Protein demand: Review of plant and animal proteins used in alternative protein product development and production. *Animal Frontiers, 10*(4), 53-63.

Ivanova, P., Kalaydzhiev, H., Rustad, T., Silva, C. L., & Chalova, V. I. (2017). Comparative biochemical profile of protein-rich products obtained from industrial rapeseed meal. *Emirates Journal of Food and Agriculture*, 170-178.

Jahangeer, S., Khan, N., Jahangeer, S., Sohail, M., Shahzad, S., Ahmad, A., & Khan, S. A. (2005). Screening and characterization of fungal cellulases isolated from the native environmental source. *Pakistan Journal of Botany, 37*(3), 739.

Jamal, P., Alam, M., & Salleh, N. (2008). Media optimization for bioproteins production from cheaper carbon source. *Journal of Engineering Science and Technology, 3*(2), 124-130.

Jannathulla, R., Dayal, J. S., Vasanthakumar, D., Ambasankar, K., & Muralidhar, M. (2017a). Effect of fermentation methods on amino acids, fiber fractions and anti-nutritional factors in different plant protein sources and essential amino acid index for Penaeus vannamei Boone, 1931. *Indian Journal of Fisheries, 64*(2), 40-47.

Jannathulla, R., Syama Dayal, J., Vasanthakumar, D., Ambasankar, K., & Muralidhar, M. (2017b). Effect of fermentation methods on amino acids, fiber fractions and anti-nutritional factors in different plant protein sources and essential amino acid index for Penaeus vannamei Boone, 1931. *Indian Journal of Fisheries, 64*(2), 40-47.

Je, J.-Y., Park, P.-J., Jung, W.-K., & Kim, S.-K. (2005). Amino acid changes in fermented oyster (Crassostrea gigas) sauce with different fermentation periods. *Food chemistry, 91*(1), 15-18.

Jegannathan, K. R., & Nielsen, P. H. (2013). Environmental assessment of enzyme use in industrial production–a literature review. *Journal of cleaner production, 42*, 228-240.

Jensen, S. K., Liu, Y.-G., & Eggum, B. (1995). The effect of heat treatment on glucosinolates and nutritional value of rapeseed meal in rats. *Animal Feed Science and Technology, 53*(1), 17-28.

Jones, S. W., Karpol, A., Friedman, S., Maru, B. T., & Tracy, B. P. (2020). Recent advances in single cell protein use as a feed ingredient in aquaculture. *Current Opinion in Biotechnology, 61*, 189-197.

Juárez-Chairez, M. F., Meza-Márquez, O. G., Márquez-Flores, Y. K., & Jiménez-Martínez, C. (2022). Potential anti-inflammatory effects of legumes-A review. *British Journal of Nutrition*, 1-36.

Juwaied, A. A., Adnan, S., & Al-Amiery, A. (2010). Production cellulase by different co-culture of Aspergillus niger and Tricoderma viride from waste paper. *Journal of Yeast and Fungal Research, 1*(6), 108-111.

Karovičová, Z. K.-J., & Kohajdova, J. (2007). Fermentation of cereals for specific purpose. *Journal of Food and Nutrition Research, 46*(2), 51-57.

Karunanithy, C., Karuppuchamy, V., Muthukumarappan, K., & Gibbons, W. R. (2012). Selection of enzyme combination, dose, and temperature for hydrolysis of soybean white flakes. *Industrial Biotechnology, 8*(5), 309-317.

Kasiga, T., White, B. M., Gibbons, W. R., & Brown, M. L. (2021). Amino acid availability of processed Brassica carinata meals in hybrid striped bass Morone chrysops♀ x M. saxatilis♂. *Aquaculture Reports, 19*, 100597.

Kaur, D., Dhawan, K., Rasane, P., Singh, J., Kaur, S., Gurumayum, S., . . . Kumar, V. (2020). Effect of different pre-treatments on antinutrients and antioxidants of rice bean (Vigna umbellata). *Acta Universitatis Cibiniensis. Series E: Food Technology, 24*(1), 25-38.

Khajali, F., & Slominski, B. (2012). Factors that affect the nutritive value of canola meal for poultry. *Poultry science, 91*(10), 2564-2575.

Khalil, A. W., Zeb, A., Mahmood, F., Tariq, S., Khattak, A. B., & Shah, H. (2007). Comparison of sprout quality characteristics of desi and kabuli type chickpea cultivars (Cicer arietinum L.). *LWT-Food Science and Technology, 40*(6), 937-945.

Khattab, R., & Arntfield, S. (2009). Functional properties of raw and processed canola meal. *LWT-Food Science and Technology, 42*(6), 1119-1124.

Khetarpaul, N., & Chauhan, B. (1990a). Effect of germination and fermentation on available carhohydrate content of pearl millet. *Food chemistry, 38*(1), 21-26.

Khetarpaul, N., & Chauhan, B. (1990b). Effect of germination and fermentation on in vitro starch and protein digestibility of pearl millet. *Journal of Food Science, 55*(3), 883-884.

Kim, D., & Han, G. D. (2014). Fermented rice bran attenuates oxidative stress. In *Wheat and rice in disease prevention and health* (pp. 467-480): Elsevier.

Kimber, D., & McGregor, D. (1995). *Brassica oilseeds: production and utilization*: Cab international.

King, J. W., & List, G. R. (1996). *Supercritical fluid technology in oil and lipid chemistry*: The American Oil Chemists Society.

King, R., & Dietz, H. (1987). Air classification of rapeseed meal. *Cereal Chem, 64*(6), 411-413.

Kinsella, J. E. (1981). Functional properties of proteins: possible relationships between structure and function in foams. *Food chemistry, 7*(4), 273-288.

Klockeman, D. M., Toledo, R., & Sims, K. A. (1997). Isolation and characterization of defatted canola meal protein. *Journal of agricultural and food chemistry, 45*(10), 3867-3870.

Kornberg, H., & Beevers, H. (1957). The glyoxylate cycle as a stage in the conversion of fat to carbohydrate in castor beans. *Biochimica et biophysica acta, 26*(3), 531-537.

Koubaa, M., Mhemdi, H., & Vorobiev, E. (2016). Influence of canola seed dehulling on the oil recovery by cold pressing and supercritical CO2 extraction. *Journal of Food Engineering, 182*, 18-25.

Krishania, M., Sindhu, R., Binod, P., Ahluwalia, V., Kumar, V., Sangwan, R. S., & Pandey, A. (2018). Design of bioreactors in solid-state fermentation. In *Current Developments in Biotechnology and Bioengineering* (pp. 83-96): Elsevier.

Kristal, A. R., & Lampe, J. W. (2002). Brassica vegetables and prostate cancer risk: a review of the epidemiological evidence. *Nutrition and cancer, 42*(1), 1-9.

Krzyzaniak, A., Burova, T., Haertlé, T., & Barciszewski, J. (1998). The structure and properties of napin-seed storage protein from rape (Brassica napus L.). *Food/Nahrung, 42*(03-04), 201-204.

Kudanga, T., & Mwenje, E. (2005). Extracellular cellulase production by tropical isolates of Aureobasidium pullulans. *Canadian journal of microbiology, 51*(9), 773-776.

Kumar, V., Sinha, A. K., Makkar, H. P., & Becker, K. (2010). Dietary roles of phytate and phytase in human nutrition: A review. *Food chemistry, 120*(4), 945-959.

Kuo, T. M., VanMiddlesworth, J. F., & Wolf, W. J. (1988). Content of raffinose oligosaccharides and sucrose in various plant seeds. *Journal of agricultural and food chemistry, 36*(1), 32-36.

Kusiak, M., Oleszczuk, P., & Jośko, I. (2022). Cross-examination of engineered nanomaterials in crop production: Application and related implications. *Journal of hazardous materials, 424*, 127374.

Landero, J., Beltranena, E., Cervantes, M., Morales, A., & Zijlstra, R. (2011). The effect of feeding solvent-extracted canola meal on growth performance and diet nutrient digestibility in weaned pigs. *Animal Feed Science and Technology, 170*(1-2), 136-140.

Larsson, M., & Sandberg, A. S. (1992). Phytate reduction in oats during malting. *Journal of Food Science, 57*(4), 994-997.

Léger, L. W., & Arntfield, S. D. (1993). Thermal gelation of the 12S canola globulin. *Journal of the American Oil Chemists' Society, 70*(9), 853-861.

Lei, X. G., & Porres, J. M. (2003). Phytase enzymology, applications, and biotechnology. *Biotechnology letters, 25*(21), 1787-1794.

Leming, R., & Lember, A. (2005a). Chemical composition of expeller-extracted and cold-pressed rapeseed cake. *Agraarteadus, 16*, 103-109.

Leming, R., & Lember, A. (2005b). Chemical composition of expeller-extracted and cold-pressed rapeseed cake. *Agraarteadus, 16*(2), 103-109.

Leslie, A., Hurnik, J., & Summers, J. (1973). Effects of color on consumption of broiler diets containing rapeseed meal and rapeseed. *Canadian Journal of Animal Science, 53*(2), 365-369.

Li, C., Yang, Z., Zhang, R. H. C., Zhang, D., Chen, S., & Ma, L. (2013). Effect of pH on cellulase production and morphology of Trichoderma reesei and the application in cellulosic material hydrolysis. *Journal of biotechnology, 168*(4), 470-477.

Li, T., Tang, J., Karuppiah, V., Li, Y., Xu, N., & Chen, J. (2020). Co-culture of Trichoderma atroviride SG3403 and Bacillus subtilis 22 improves the production of antifungal secondary metabolites. *Biological Control, 140*, 104122.

Lio, J., & Wang, T. (2012). Solid-state fermentation of soybean and corn processing coproducts for potential feed improvement. *Journal of agricultural and food chemistry, 60*(31), 7702-7709.

Liu, P., Li, J., & Deng, Z. (2016). Bio-transformation of agri-food wastes by newly isolated Neurospora crassa and Lactobacillus plantarum for egg production. *Poultry science, 95*(3), 684-693.

Lonsane, B., Ghildyal, N., Budiatman, S., & Ramakrishna, S. (1985). Engineering aspects of solid state fermentation. *Enzyme and Microbial Technology, 7*(6), 258-265.

Lott, J. N., Ockenden, I., Raboy, V., & Batten, G. D. (2000). Phytic acid and phosphorus in crop seeds and fruits: a global estimate. *Seed Science Research, 10*(1), 11-33.

Lund, M. N., & Ray, C. A. (2017). Control of maillard reactions in foods: Strategies and chemical mechanisms. *Journal of agricultural and food chemistry, 65*(23), 4537-4552.

Maejima, Y., Nakatsugawa, H., Ichida, D., Maejima, M., Aoyagi, Y., Maoka, T., & Etoh, H. (2011). Functional compounds in fermented buckwheat sprouts. *Bioscience, biotechnology, and biochemistry, 75*(9), 1708-1712.

Mailer, R. J., McFadden, A., Ayton, J., & Redden, B. (2008a). Anti-nutritional components, fibre, sinapine and glucosinolate content, in australian canola (Brassica napus L.) meal. *Journal of the American Oil Chemists' Society, 85*(10), 937-944.

Mailer, R. J., McFadden, A., Ayton, J., & Redden, B. (2008b). Anti-nutritional components, fibre, sinapine and glucosinolate content, in Australian canola (Brassica napus L.) meal. *Journal of the American Oil Chemists' Society, 85*(10), 937-944.

Maison, T. (2014). *Evaluation of the nutritional value of canola meal, 00-rapeseed meal, and 00-rapeseed expellers fed to pigs*. University of Illinois at Urbana-Champaign,

Mariscal-Landín, G., & de Souza, T. R. (2008). Ileal digestibility of protein and amino acids from canola meal in weaned piglets and growing pigs. *Livestock Science, 116*(1-3), 53-62.

Marton, M., Mandoki, Z., Csapo-Kiss, Z., & Csapo, J. (2010). The role of sprouts in human nutrition. A review. *Acta Univ. Sapientiae, 3*, 81-117.

Masood, T., Shah, H., & Zeb, A. (2014). Effect of sprouting time on proximate composition and ascorbic acid level of mung bean (Vigna radiate L.) and chickpea (Cicer Arietinum L.) seeds. *The Journal of Animal & Plant Sciences, 24*(3), 850-859.

Matsuno, K., & Fujimura, T. (2014). Induction of phytic acid synthesis by abscisic acid in suspension-cultured cells of rice. *Plant Science, 217*, 152-157.

Matthäus, B. (2006). Utilization of high-oleic rapeseed oil for deep-fat frying of French fries compared to other commonly used edible oils. *European Journal of Lipid Science and Technology, 108*(3), 200-211.

Mattioli, S., Dal Bosco, A., Martino, M., Ruggeri, S., Marconi, O., Sileoni, V., . . . Benincasa, P. (2016). Alfalfa and flax sprouts supplementation enriches the content of bioactive compounds and lowers the cholesterol in hen egg. *Journal of Functional Foods, 22*, 454-462.

Megat Rusydi, M., Noraliza, C., Azrina, A., & Zulkhairi, A. (2011). Nutritional changes in germinated legumes and rice varieties. *International Food Research Journal, 18*(2).

Megazyme. (2017). Phytic acid (phytate)/Total phosphorus. Retrieved from https://www.megazyme.com/documents/Booklet/K-PHYT_DATA.pdf

Mejia, L. A., Korgaonkar, C. K., Schweizer, M., Chengelis, C., Novilla, M., Ziemer, E., . . . Empie, M. (2009). A 13-week dietary toxicity study in rats of a napin-rich canola protein Isolate. *Regulatory Toxicology and Pharmacology, 55*(3), 394-402.

Mejicanos, G., & Nyachoti, C. (2018). Effect of tail-end dehulling of canola meal on apparent and standardized ileal digestibility of amino acids when fed to growing pigs. *Animal Feed Science and Technology, 243*, 102-111.

Mejicanos, G. A. (2015). Tail-end dehulling of canola meal: Chemical composition and nutritive value of dehulled meal for broiler chickens and weaned pigs.

Melrose, J. (2019). The Glucosinolates: A sulphur glucoside family of mustard anti-tumour and antimicrobial phytochemicals of potential therapeutic application. *Biomedicines, 7*(3), 62.

Mieth, G., Schwenke, K. D., Raab, B., & Bruckner, J. (1983). Rapeseed: constituents and protein products Part I. Composition and properties of proteins and glucosinolates. *Food/Nahrung, 27*(7), 675-697.

Miller, G. L. (1959). Glucose assay by dinitrosalicylic colorimetric method. *Analytical Chemistry, 31*, 426.

Mishra, S., Goyal, D., Kumar, A., & Dantu, P. K. (2019). Biotechnological applications of β-glucosidases in biomass degradation. In *Recent Advancement in White Biotechnology Through Fungi* (pp. 257-281): Springer.

Mitaru, B., Blair, R., Reichert, R., & Roe, W. (1984). Dark and yellow rapeseed hulls, soybean hulls and a purified fiber source: Their effects on dry matter, energy, protein and amino acid digestibilities in cannulated pigs. *Journal of animal science, 59*(6), 1510-1518.

Mohamadzadeh, J., Sadeghi-Mahoonak, A., Yaghbani, M., & Aalami, M. (2009). Effect of hydrothermal pretreatment of canola seeds on dehulling efficiency and oil quality. *World J. Dairy Food Sci, 4*(1), 14-18.

Montoya, C. A., & Leterme, P. (2010). Validation of the net energy content of canola meal and full-fat canola seeds in growing pigs. *Canadian Journal of Animal Science, 90*(2), 213-219.

Moongngarm, A., & Saetung, N. (2010). Comparison of chemical compositions and bioactive compounds of germinated rough rice and brown rice. *Food chemistry, 122*(3), 782-788.

Moser, B. R. (2010). Camelina (Camelina sativa L.) oil as a biofuels feedstock: Golden opportunity or false hope? *Lipid Technology, 22*(12), 270-273.

Mostafa, M., Rahma, E., & Rady, A. (1987). Chemical and nutritional changes in soybean during germination. *Food chemistry, 23*(4), 257-275.

Moure, A., Sineiro, J., Domínguez, H., & Parajó, J. C. (2006). Functionality of oilseed protein products: a review. *Food Research International, 39*(9), 945-963.

Mubarak, A. (2005). Nutritional composition and antinutritional factors of mung bean seeds (Phaseolus aureus) as affected by some home traditional processes. *Food chemistry, 89*(4), 489-495.

Muhammad, S., Iqra, Y., Murtaza, M. A., Iqra, F., & Shehwar, S. (2016). Single cell proteins: a novel value added food product. *Pakistan Journal of Food Sciences, 26*(4), 211-217.

Mulimani, V., Thippeswamy, S., & Ramalingam, S. (1997). Enzymatic degradation of oligosaccharides in soybean flours. *Food chemistry, 59*(2), 279-282.

Murugkar, D. A. (2014). Effect of sprouting of soybean on the chemical composition and quality of soymilk and tofu. *Journal of food science and technology, 51*(5), 915-921.

Mustafa, A., Christensen, D., & McKinnon, J. (1996). Chemical characterization and nutrient availability of high and low fiber canola meal. *Canadian Journal of Animal Science, 76*(4), 579-586.

Mwachireya, S., Beames, R., Higgs, D., & Dosanjh, B. (1999). Digestibility of canola protein products derived from the physical, enzymatic and chemical processing of commercial canola meal in rainbow trout Oncorhynchus mykiss (Walbaum) held in fresh water. *Aquaculture Nutrition, 5*, 73–82.

Naseeruddin, S., Desai, S., & Rao, L. V. (2021). Co-culture of Saccharomyces cerevisiae (VS3) and Pichia stipitis (NCIM 3498) enhances bioethanol yield from concentrated Prosopis juliflora hydrolysate. *3 Biotech, 11*(1), 1-12.

Nasseri, A., Rasoul-Amini, S., Morowvat, M., & Ghasemi, Y. (2011). Single cell protein: production and process. *American Journal of food technology, 6*(2), 103-116.

NCBI. (2022). PubChem Patent Summary for US-6251460-B1, Refining of vegetable oil. Retrieved from https://pubchem.ncbi.nlm.nih.gov/patent/US-6251460-B1

Nevara, G. A., Giwa Ibrahim, S. a., Syed Muhammad, S. K., Zawawi, N., Mustapha, N. A., & Karim, R. (2022). Oilseed meals into foods: an approach for the valorization of oilseed by-products. *Critical reviews in food science and nutrition*, 1-14.

Newkirk, R. (2009a). Canola meal feed industry guide. *Canadian International Grains Institute*.

Newkirk, R. (2009b). Canola meal feed industry guide 4th edition. *Canola Council of Canada, Winnipeg, Manitoba*, 1-47.

Newkirk, R., & Classen, H. (2002). The effects of toasting canola meal on body weight, feed conversion efficiency, and mortality in broiler chickens. *Poultry science, 81*(6), 815-825.

Newkirk, R., Classen, H., Scott, T., & Edney, M. (2003). The digestibility and content of amino acids in toasted and non-toasted canola meals. *Canadian Journal of Animal Science, 83*(1), 131-139.

Newkirk, R., & Daun, J. (2011). Canola: Chemistry, production, processing and utilization. *Biotech Business Week, 288*.

Nigam, P. S.-N., & Pandey, A. (2009). *Biotechnology for agro-industrial residues utilisation: utilisation of agro-residues*: Springer Science & Business Media.

Nkhata, S. G., Ayua, E., Kamau, E. H., & Shingiro, J. B. (2018). Fermentation and germination improve nutritional value of cereals and legumes through activation of endogenous enzymes. *Food Science & Nutrition, 6*(8), 2446-2458.

Nonogaki, H., Bassel, G. W., & Bewley, J. D. (2010). Germination—still a mystery. *Plant Science, 179*(6), 574-581.

NRC. (2011). Nutrient requirements of warm water fishes and shrimp. National Academy Press, Washington, D.C. *National Research Council*.

Nuraini, A. D., & Mahata, M. E. (2015). Improving the nutrient quality of durian (Durio zibethinus) fruit waste through fermentation by using Phanerochaete

chrysosporium and Neurospora crassa for poultry diet. *Int. J. Poult. Sci*, *14*, 354-358.

Nyombaire, G., Siddiq, M., & Dolan, K. (2007). Effect of soaking and cooking on the oligosaccharides and lectins in red kidney beans (Phaseolus vulgaris L.). *ANNUAL REPORT-BEAN IMPROVEMENT COOPERATIVE*, *50*, 31.

Obizoba, I. C., & Atii, J. (1991). Effect of soaking, sprouting, fermentation and cooking on nutrient composition and some anti-nutritional factors of sorghum (Guinesia) seeds. *Plant foods for human nutrition*, *41*(3), 203-212.

OECD-FAO. (2017). *OECD-FAO Agricultural Outlook 2017-2026*: OECD/Food Agriculture Organization of the United Nations.

Oerlemans, K., Barrett, D. M., Suades, C. B., Verkerk, R., & Dekker, M. (2006). Thermal degradation of glucosinolates in red cabbage. *Food chemistry*, *95*(1), 19-29.

Ohanenye, I. C., Tsopmo, A., Ejike, C. E., & Udenigwe, C. C. (2020). Germination as a bioprocess for enhancing the quality and nutritional prospects of legume proteins. *Trends in food science & technology*, *101*, 213-222.

Ohlson, R. (1992). Modern processing of rapeseed. *Journal of the American Oil Chemists' Society*, *69*(3), 195-198.

Ojokoh, A., Daramola, M., & Oluoti, O. (2013). Effect of fermentation on nutrient and anti-nutrient composition of breadfruit (Treculia africana) and cowpea (Vigna unguiculata) blend flours. *African Journal of Agricultural Research*, *8*(27), 3566-3570.

Olukomaiya, O., Fernando, C., Mereddy, R., Li, X., & Sultanbawa, Y. (2019). Solid-state fermented plant protein sources in the diets of broiler chickens: A review. *Animal Nutrition*, *5*(4), 319-330.

Olukomaiya, O. O., Adiamo, O. Q., Fernando, W. C., Mereddy, R., Li, X., & Sultanbawa, Y. (2020a). Effect of solid-state fermentation on proximate composition, anti-nutritional factor, microbiological and functional properties of lupin flour. *Food chemistry*, *315*, 126238.

Olukomaiya, O. O., Fernando, W. C., Mereddy, R., Li, X., & Sultanbawa, Y. (2020b). Solid-state fermentation of canola meal with Aspergillus sojae, Aspergillus ficuum and their co-cultures: Effects on physicochemical, microbiological and functional properties. *LWT*, *127*, 109362.

Olukomaiya, O. O., Fernando, W. C., Mereddy, R., Li, X., & Sultanbawa, Y. (2020c). Solid-state fermentation of canola meal with Aspergillus sojae, Aspergillus ficuum and their co-cultures: Effects on physicochemical, microbiological and functional properties. *LWT*, 109362.

Olukomaiya, O. O., Pan, L., Zhang, D., Mereddy, R., Sultanbawa, Y., & Li, X. (2021). Performance and ileal amino acid digestibility in broilers fed diets containing solid-state fermented and enzyme-supplemented canola meals. *Animal Feed Science and Technology*, *275*, 114876.

Ongol, M. P., Niyonzima, E., Gisanura, I., & Vasanthakaalam, H. (2013). Effect of germination and fermentation on nutrients in maize flour. *Pakistan Journal of Food Sciences*, *23*(4), 183-188.

Onimawo, I., & Asugo, S. (2004). Effects of germination on the nutrient content and functional properties of pigeon pea flour. *Journal of Food Science and Technology-Mysore*, *41*(2), 170-174.

Onyango, C., Ochanda, S., Mwasaru, M., Ochieng, J., Mathooko, F. M., & Kinyuru, J. (2013). Effects of malting and fermentation on anti-nutrient reduction and protein digestibility of red sorghum, white sorghum and pearl millet. *Journal of Food Research, 2*(1), 41.

Oseni, O., & Akindahunsi, A. (2011). Some phytochemical properties and effect of fermentation on the seed of Jatropha curcas L. *American Journal of food technology, 6*(2), 158-165.

Osman, M. A. (2011). Effect of traditional fermentation process on the nutrient and antinutrient contents of pearl millet during preparation of Lohoh. *Journal of the Saudi Society of Agricultural Sciences, 10*(1), 1-6.

Östbring, K., Malmqvist, E., Nilsson, K., Rosenlind, I., & Rayner, M. (2020). The effects of oil extraction methods on recovery yield and emulsifying properties of proteins from rapeseed meal and press cake. *Foods, 9*(1), 19.

Ou, K., Cheng, Y., Xing, Y., Lin, L., Nout, R., & Liang, J. (2011). Phytase activity in brown rice during steeping and sprouting. *Journal of food science and technology, 48*(5), 598-603.

Padmore, J. (1990). Animal feed-AOAC official method 920.39–Fat (crude) or ether extract in animal feed. *Official Methods of Analysis, 15th ed.; Helrich, K., Ed*, 79.

Pandey, A. (2003). Solid-state fermentation. *Biochemical Engineering Journal, 13*(2-3), 81-84.

Pandey, A., Soccol, C. R., & Larroche, C. (2008). *Current developments in solid-state fermentation*: Springer Science & Business Media.

Pandey, A., Soccol, C. R., & Mitchell, D. (2000). New developments in solid state fermentation: I-bioprocesses and products. *Process biochemistry, 35*(10), 1153-1169.

Pandey, A., Szakacs, G., Soccol, C. R., Rodriguez-Leon, J. A., & Soccol, V. T. (2001). Production, purification and properties of microbial phytases. *Bioresource technology, 77*(3), 203-214.

Parameswaran, K. P., & Sadasivam, S. (1994). Changes in the carbohydrates and nitrogenous components during germination of proso millet, Panicum miliaceum. *Plant foods for human nutrition, 45*(2), 97-102.

Park, C. H., Kim, N. S., Park, J. S., Lee, S. Y., Lee, J.-W., & Park, S. U. (2019). Effects of light-emitting diodes on the accumulation of glucosinolates and phenolic compounds in sprouting canola (Brassica napus L.). *Foods, 8*(2), 76.

Patel, R. N., Bandyopadhyay, S., & Ganesh, A. (2011). A simple model for super critical fluid extraction of bio oils from biomass. *Energy conversion and management, 52*(1), 652-657.

Patterson, C. A., Curran, J., & Der, T. (2017). Effect of processing on antinutrient compounds in pulses. *Cereal Chemistry, 94*(1), 2-10.

Pedroche, J., Yust, M., Lqari, H., Girón-Calle, J., Alaiz, M., Vioque, J., & Millán, F. (2004). Brassica carinata protein isolates: chemical composition, protein characterization and improvement of functional properties by protein hydrolysis. *Food chemistry, 88*(3), 337-346.

Pensupa, N., Jin, M., Kokolski, M., Archer, D. B., & Du, C. (2013). A solid state fungal fermentation-based strategy for the hydrolysis of wheat straw. *Bioresource technology, 149*, 261-267.

Perkins, D. D., & Turner, B. C. (1988). Neurospora from natural populations: toward the population biology of a haploid eukaryote. *Experimental Mycology, 12*(2), 91-131.

Peterson, R., & Nevalainen, H. (2012). Trichoderma reesei RUT-C30–thirty years of strain improvement. *Microbiology, 158*(1), 58-68.

Plascencia, A., Estrada, M., & Zinn, R. (1999). Influence of free fatty acid content on the feeding value of yellow grease in finishing diets for feedlot cattle. *Journal of animal science, 77*(10), 2603-2609.

Przybylski, R., Mag, T., Eskin, N., & McDonald, B. (2005). Canola oil. *Bailey's industrial oil and fat products*.

Qian, Y., Zhong, L., Hou, Y., Qu, Y., & Zhong, Y. (2016). Characterization and strain improvement of a hypercellulytic variant, Trichoderma reesei SN1, by genetic engineering for optimized cellulase production in biomass conversion improvement. *Frontiers in microbiology, 7*, 1349.

Qian, Y., Zhong, L., Sun, Y., Sun, N., Zhang, L., Liu, W., . . . Zhong, Y. (2019). Enhancement of cellulase production in Trichoderma reesei via disruption of multiple protease genes identified by comparative secretomics. *Frontiers in microbiology, 10*, 2784.

Rai, S., Bawa, V., Dar, Z. A., Sofi, N., Mahdi, S., & Qureshi, A. M. I. (2019). Use of modern molecular biology and biotechnology tools to improve the quality value of oilseed Brassicas. In *Quality Breeding in Field Crops* (pp. 255-266): Springer.

Rakow, G. (2004). Species origin and economic importance of Brassica. In *Brassica* (pp. 3-11): Springer.

Rakow, G. (2012). Classical genetics and traditional breeding. *Genetics, Genomics and Breeding of Oilseed Brassicas*, 73-84.

Ramachandran, S., Patel, A. K., Nampoothiri, K. M., Francis, F., Nagy, V., Szakacs, G., & Pandey, A. (2004). Coconut oil cake—a potential raw material for the production of α-amylase. *Bioresource technology, 93*(2), 169-174.

Ramesh, K., Devegowda, G., & Khosravinia, H. (2006). Effects of enzyme addition to broiler diets containing varying levels of double zero rapeseed meal. *Asian-Australasian journal of animal sciences, 19*(9), 1354-1360.

Raper, K. B., & Fennell, D. I. (1965). The genus Aspergillus. *The genus Aspergillus.*

Rasane, P., Jha, A., Kumar, A., & Sharma, N. (2015). Reduction in phytic acid content and enhancement of antioxidant properties of nutricereals by processing for developing a fermented baby food. *Journal of food science and technology, 52*(6), 3219-3234.

Rask, L., Andréasson, E., Ekbom, B., Eriksson, S., Pontoppidan, B., & Meijer, J. (2000). Myrosinase: gene family evolution and herbivore defense in Brassicaceae. *Plant molecular biology, 42*(1), 93-114.

Rath, S., Jena, S., & Prabhu, M. V. (2011). Direct ethanol production by pretreatment of lignocellulosic biomass using Neurospora crassa. *Global Journal of Applied Agricultural Research, 1*(1), 33-41.

Rathnakumar, A., & Sujatha, M. (2022). Breeding major oilseed crops: Prospects and future research needs. In *Accelerated Plant Breeding, Volume 4* (pp. 1-40): Springer.

Ravichandiran, S., Sharma, K., Dutta, N., Patianaik, A., Chauhan, J., Agnihotri, A., & Kumar, A. (2008). Performance of crossbred calves on supplements containing

soybean meal or rapeseed-mustard cake with varying glucosinolate levels. *Indian Journal of Animal Sciences (India)*.

Ravindra, P. (2000). Value-added food: Single cell protein. *Biotechnology advances, 18*(6), 459-479.

Raymer, P. L. (2002). Canola: an emerging oilseed crop. *Trends in new crops and new uses, 1*, 122-126.

Raza, F., Raza, N. A., Hameed, U., Haq, I., & Maryam, I. (2011). Solid state fermentation for the production of β-glucosidase by co-culture of Aspergillus niger and A. oryzae. *Pak. J. Bot, 43*(1), 75-83.

Reale, A., Konietzny, U., Coppola, R., Sorrentino, E., & Greiner, R. (2007). The importance of lactic acid bacteria for phytate degradation during cereal dough fermentation. *Journal of agricultural and food chemistry, 55*(8), 2993-2997.

Reddy, N., Pierson, M., Sathe, S., & Salunkhe, D. (1989). Influence of processing technologies on phytate. *Phytates in Cereals and Legumes*, 111-135.

Reed, J., Bauer, M., Loe, E., Caton, J., & Lardy, G. (2005). Effects of processing on feeding value of sprouted barley and sprouted durum wheat in growing and finishing diets for beef cattle. *The Professional Animal Scientist, 21*(1), 7-12.

Reichert, R., Tyler, R., York, A., Schwab, D., & Tatarynovich, J. (1986). Description of a production model of the tangential abrasive dehulling device and its application to breeders' samples. *Cereal Chemistry, 63*(3), 201-207.

Rempel, C., Li, X., Geng, X., Liu, Q., & Zhang, Y. (2020). Manufacture of defatted canola meal with enhanced nutritive composition by air classification on an industrial scale. *Journal of the Science of Food and Agriculture, 100*(2), 764-774.

Roche, C. M., Loros, J. J., McCluskey, K., & Glass, N. L. (2014). Neurospora crassa: looking back and looking forward at a model microbe. *American journal of botany, 101*(12), 2022-2035.

Roslan, M. A. M., Jefri, N. Q. U. A., Ramlee, N., Rahman, N. A. A., Chong, N. H. H., Bunawan, H., . . . Razali, H. (2021). Enhancing food waste biodegradation rate in a food waste biodigester with the synergistic action of hydrolase-producing Bacillus paralicheniformis GRA2 and Bacillus velezensis TAP5 co-culture inoculation. *Saudi Journal of Biological Sciences, 28*(5), 3001-3012.

Sakorn, P., Rakariyatham, N., Niamsup, H., & Kovitaya, P. (1999). Sinigrin degradation by Aspergillus sp. NR-4201 in liquid culture. *Science Asia, 25*(4), 189-194.

Saloheimo, A., Henrissat, B., Hoffrén, A. M., Teleman, O., & Penttilä, M. (1994). A novel, small endoglucanase gene, egl5, from Trichoderma reesei isolated by expression in yeast. *Molecular microbiology, 13*(2), 219-228.

Saloheimo, M., Paloheimo, M., Hakola, S., Pere, J., Swanson, B., Nyyssönen, E., . . . Penttilä, M. (2002). Swollenin, a Trichoderma reesei protein with sequence similarity to the plant expansins, exhibits disruption activity on cellulosic materials. *European Journal of Biochemistry, 269*(17), 4202-4211.

Santos, C., Silva, B., MP Valente, L., Gruber, S., & W Vasconcelos, M. (2020). The effect of sprouting in lentil (Lens culinaris) nutritional and microbiological profile. *Foods, 9*(4), 400.

Santos, C. C. A. d. A., Libeck, B. d. S., & Schwan, R. F. (2014a). Co-culture fermentation of peanut-soy milk for the development of a novel functional beverage. *International Journal of Food Microbiology, 186*, 32-41.

Santos, R. d. S., Muruci, L. N. M., Santos, L. O., Antoniassi, R., da Silva, J. P. L., & Damaso, M. C. T. (2014b). Characterization of different oil soapstocks and their application in the lipase production by Aspergillus niger under solid state fermentation. *Embrapa Agroindústria de Alimentos-Artigo em periódico indexado (ALICE)*.

Sarwar, M. F., Sarwar, M. H., Sarwar, M., Qadri, N. A., & Moghal, S. (2013). The role of oilseeds nutrition in human health: A critical review. *Journal of Cereals and oilseeds*, *4*(8), 97-100.

Scarth, R., & Tang, J. (2006). Modification of Brassica oil using conventional and transgenic approaches. *Crop science*, *46*(3), 1225-1236.

Schmidt, I., Renard, D., Rondeau, D., Richomme, P., Popineau, Y., & Axelos, M. A.-V. (2004). Detailed physicochemical characterization of the 2S storage protein from rape (Brassica napus L.). *Journal of agricultural and food chemistry, 52*(19), 5995-6001.

Schuster, E., Dunn-Coleman, N., Frisvad, J., & Van Dijck, P. (2002). On the safety of Aspergillus niger–a review. *Applied microbiology and biotechnology, 59*(4-5), 426-435.

Schwenke, K., Raab, B., Linow, K. J., Pahtz, W., & Uhlig, J. (1981). Isolation of the 12 s globulin from rapeseed (brassica napus l.) and characterization as a "neutral" protein on seed proteins. Part 13. *Food/Nahrung*, *25*(3), 271-280.

Schwenke, K. D., Dahme, A., & Wolter, T. (1998). Heat-induced gelation of rapeseed proteins: Effect of protein interaction and acetylation. *Journal of the American Oil Chemists' Society, 75*(1), 83-87.

Seneviratne, R., Beltranena, E., Newkirk, R., Goonewardene, L., & Zijlstra, R. (2011). Processing conditions affect nutrient digestibility of cold-pressed canola cake for grower pigs. *Journal of animal science, 89*(8), 2452-2461.

Seneviratne, R., Young, M., Beltranena, E., Goonewardene, L., Newkirk, R., & Zijlstra, R. (2010). The nutritional value of expeller-pressed canola meal for grower-finisher pigs. *Journal of animal science, 88*(6), 2073-2083.

Seo, H.-P., Son, C.-W., Chung, C.-H., Jung, D.-I., Kim, S.-K., Gross, R. A., . . . Lee, J.-W. (2004). Production of high molecular weight pullulan by Aureobasidium pullulans HP-2001 with soybean pomace as a nitrogen source. *Bioresource technology, 95*(3), 293-299.

Shah, S. A., Zeb, A., Masood, T., Noreen, N., Abbas, S. J., Samiullah, M., . . . Muhammad, A. (2011). Effects of sprouting time on biochemical and nutritional qualities of Mungbean varieties. *African Journal of Agricultural Research, 6*(22), 5091-5098.

Shahidi, F. (1990). *Canola and rapeseed: production, chemistry, nutrition, and processing technology*: Springer Science & Business Media.

Sharada, R., Venkateswarlu, G., Venkateshwar, S., & Rao, M. A. (2013). Production of cellulase-A review. *International Journal of Pharmaceutical, Chemical & Biological Sciences, 3*(4).

Shear, C. L., & Dodge, B. O. (1927). *Life histories and heterothallism of the red bread-mold fungi of the Monilia sitophila group*: US Government Printing Office.

Shi, C., He, J., Yu, J., Yu, B., Huang, Z., Mao, X., . . . Chen, D. (2015). Solid state fermentation of rapeseed cake with Aspergillus niger for degrading glucosinolates

and upgrading nutritional value. *Journal of animal science and biotechnology, 6*(1), 13.

Shivanna, G. B., & Venkateswaran, G. (2014). Phytase production by Aspergillus niger CFR 335 and Aspergillus ficuum SGA 01 through submerged and solid-state fermentation. *The Scientific World Journal, 2014*.

Simmons, E. (1977). *Classification of some cellulase-producing Trichoderma species.* Paper presented at the Second International Mycological Congress, Abstracts.

Simon, E. (1984). Early events in germination, DR Murray (ed.) Seed physiology, vol. 2, germination and reserve mobilization. In: Academic Press, Orlando, FL.

Simon, J., Wootton, S., Johnson, T., Karki, B., Zahler, J., Baldwin, E., & Gibbons, W. (2017). Solid state fermentation of carinata (Brassica carinata) meal using various fungal strains to produce a protein-rich product for feed application. *J Microb Biochem Technol, 9*, 577-582.

Sindelar, S. C. (2014). Utilization of soybean products as fish-meal protein replacements in Yellow Perch Perca flavescens feeds.

Singh, B. D. (1998). *Biotechnolog* (B.Sc. ed.). New Delhi: Kalyani

Singh, R., Chung, G., & Nelson, R. L. (2007). Landmark research in legumes. *Genome, 50*(6), 525-537.

Singh, R., Kumar, M., Mittal, A., & Mehta, P. K. (2016). Microbial enzymes: industrial progress in 21st century. *3 Biotech, 6*(2), 1-15.

Singhania, R. R., Patel, A. K., Soccol, C. R., & Pandey, A. (2009). Recent advances in solid-state fermentation. *Biochemical Engineering Journal, 44*(1), 13-18.

Singhania, R. R., Patel, A. K., Sukumaran, R. K., Larroche, C., & Pandey, A. (2013). Role and significance of beta-glucosidases in the hydrolysis of cellulose for bioethanol production. *Bioresource technology, 127*, 500-507.

Siqueira, J. G. W., Rodrigues, C., de Souza Vandenberghe, L. P., Woiciechowski, A. L., & Soccol, C. R. (2020). Current advances in on-site cellulase production and application on lignocellulosic biomass conversion to biofuels: a review. *Biomass and bioenergy, 132*, 105419.

Slominski, B. A., & Campbell, L. D. (1990). Non-starch polysaccharides of canola meal: quantification, digestibility in poultry and potential benefit of dietary enzyme supplementation. *Journal of the Science of Food and Agriculture, 53*(2), 175-184.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., & Templeton, D. (2005). *Determination of ash in biomass: Laboratory Analytical Procedure (LAP)*. Retrieved from Golden, Colorado, USA: https://www.nrel.gov/docs/gen/fy08/42622.pdf

Smits, J., Knol, W., & Bol, J. (1993). Glucosinolate degradation by Aspergillus clavatus and Fusarium oxysporum in liquid and solid-state fermentation. *Applied microbiology and biotechnology, 38*(5), 696-701.

Sønderby, I. E., Geu-Flores, F., & Halkier, B. A. (2010). Biosynthesis of glucosinolates–gene discovery and beyond. *Trends in plant science, 15*(5), 283-290.

Sosulski, F., & Zadernowski, R. (1981). Fractionation of rapeseed meal into hour and hull components. *Journal of the American Oil Chemist's Society, 58*(2), 96-98.

Spier, M. R., Greiner, R., Rodriguez-León, J. A., Woiciechowski, A. L., Pandey, A., Soccol, V. T., & Soccol, C. R. (2008). Phytase production using citric pulp and

other residues of the agroindustry in SSF by fungal isolates. *Food Technology and Biotechnology, 46*(2), 178-182.

Spragg, J., & Mailer, R. (2007). Canola meal value chain quality improvement. *Canola meal value chain quality improvement.*

Stoddart, J., Thomas, H., & Robertson, A. (1973). Protein synthesis patterns in barley embryos during germination. *Planta, 112*(4), 309-321.

Stoilova, I., & Krastanov, A. (2008). Overproduction of laccase and pectinase by microbial associations in solid substrate fermentation. *Applied biochemistry and biotechnology, 149*(1), 45-51.

Sukumaran, R. K., Singhania, R. R., & Pandey, A. (2005). Microbial cellulases-production, applications and challenges.

Sun Lim, K., Young Koo, S., Jong Jin HWANGl, S., Han Sun, H., & PARK, C. H. (2001). Development and utilization of buckwheat sprouts as functional vegetables. *Fagopyrum, 18*(49), 4.

Sun, X., Devi, N. D., Urriola, P. E., Tiffany, D. G., Jang, J.-C., Shurson, G. G., & Hu, B. (2021). Feeding value improvement of corn-ethanol co-product and soybean hull by fungal fermentation: Fiber degradation and digestibility improvement. *Food and Bioproducts Processing, 130*, 143-153.

Sung, H., Shin, H., Ha, J., Lai, H.-L., Cheng, K.-J., & Lee, J. (2005). Effect of germination temperature on characteristics of phytase production from barley. *Bioresource technology, 96*(11), 1297-1303.

Szydłowska-Czerniak, A., Karlovits, G., Hellner, G., Dianoczki, C., & Szłyk, E. (2010). Effect of enzymatic and hydrothermal treatments of rapeseeds on quality of the pressed rapeseed oils: Part I: Antioxidant capacity and antioxidant content. *Process biochemistry, 45*(1), 7-17.

Tabacco, E., Righi, F., Quarantelli, A., & Borreani, G. (2011). Dry matter and nutritional losses during aerobic deterioration of corn and sorghum silages as influenced by different lactic acid bacteria inocula. *Journal of dairy science, 94*(3), 1409-1419.

Tan, S. H., Mailer, R. J., Blanchard, C. L., & Agboola, S. O. (2011a). Canola proteins for human consumption: extraction, profile, and functional properties. *Journal of Food Science, 76*(1), R16-R28.

Tan, S. H., Mailer, R. J., Blanchard, C. L., & Agboola, S. O. (2011b). Extraction and characterization of protein fractions from Australian canola meals. *Food Research International, 44*(4), 1075-1082.

Tan, S. H., Mailer, R. J., Blanchard, C. L., & Agboola, S. O. (2014a). Emulsifying properties of proteins extracted from australian canola meal. *LWT-Food Science and Technology, 57*(1), 376-382.

Tan, S. H., Mailer, R. J., Blanchard, C. L., Agboola, S. O., & Day, L. (2014b). Gelling properties of protein fractions and protein isolate extracted from australian canola meal. *Food Research International, 62*, 819-828.

Taniguchi, M., & Tanaka, T. (2004). Clarification of interactions among microorganisms and development of co-culture system for production of useful substances. In *Recent Progress of Biochemical and Biomedical Engineering in Japan I* (pp. 35-62): Springer.

Thakor, N. J., Sokhansanj, S., McGregor, I., & McCurdy, S. (1995). Dehulling of canola by hydrothermal treatments. *Journal of the American Oil Chemists' Society, 72*(5), 597-602.

Thomas, L., Larroche, C., & Pandey, A. (2013). Current developments in solid-state fermentation. *Biochemical Engineering Journal, 81*, 146-161.

Tian, B., Xie, B., Shi, J., Wu, J., Cai, Y., Xu, T., . . . Deng, Q. (2010). Physicochemical changes of oat seeds during germination. *Food chemistry, 119*(3), 1195-1200.

Trelease, R. N., & Doman, D. C. (1984). Mobilization of oil and wax reserves. In *Germination and Reserve Mobilization* (pp. 201-245): Academic Press.

Tripathi, M., & Mishra, A. (2007). Glucosinolates in animal nutrition: A review. *Animal Feed Science and Technology, 132*(1-2), 1-27.

Tusé, D., & Miller, M. W. (1984). Single-cell protein: Current status and future prospects. *Critical Reviews in Food Science & Nutrition, 19*(4), 273-325.

Tzen, J. T. (2012). Integral proteins in plant oil bodies. *International Scholarly Research Notices, 2012*.

Tzeng, Y., Diosady, L., & Rubin, L. (1988). Preparation of rapeseed protein isolates using ultrafiltration, precipitation and diafiltration. *Canadian Institute of Food Science and Technology Journal, 21*(4), 419-424.

Uppal, V., & Bains, K. (2012). Effect of germination periods and hydrothermal treatments on in vitro protein and starch digestibility of germinated legumes. *Journal of food science and technology, 49*(2), 184-191.

Uquiche, E., Romero, V., Ortiz, J., & Del Valle, J. (2012). Extraction of oil and minor lipids from cold-press rapeseed cake with supercritical CO2. *Brazilian journal of chemical engineering, 29*(3), 585-598.

Uruakpa, F. O., & Arntfield, S. (2006). Network formation of canola protein–κ-carrageenan mixtures as affected by salts, urea and dithiothreitol. *LWT-Food Science and Technology, 39*(8), 939-946.

USDA-NASS. (2019). 2018 Census of Aquaculture. United States Department of Agriculture, National Agricultural Statistics Service. Washington, D. C. *Food biotechnology*.

USDA. (2020). Fats and oils: Oilseed crushings, production, consumption and stocks. Retrieved from https://www.nass.usda.gov/Publications/Todays_Reports/reports/cafo0120.pdf

USDA. (2021). *Oilseeds: World markets and trade* Retrieved from USA: https://downloads.usda.library.cornell.edu/usda-esmis/files/tx31qh68h/x346f3139/d217rn256/oilseeds.pdf

Vale, A., Santos, J., Brito, N., Fernandes, D., Rosa, E., & Oliveira, M. B. P. (2015). Evaluating the impact of sprouting conditions on the glucosinolate content of Brassica oleracea sprouts. *Phytochemistry, 115*, 252-260.

Vats, P., Bhattacharyya, M. S., & Banerjee, U. C. (2005). Use of phytases (myo-inositolhexakisphosphate phosphohydrolases) for combatting environmental pollution: a biological approach. *Critical Reviews in Environmental Science and Technology, 35*(5), 469-486.

Vaughn, S. F., & Berhow, M. A. (2005). Glucosinolate hydrolysis products from various plant sources: pH effects, isolation, and purification. *Industrial Crops and Products, 21*(2), 193-202.

Verma, N., & Kumar, V. (2020). Impact of process parameters and plant polysaccharide hydrolysates in cellulase production by Trichoderma reesei and Neurospora crassa under wheat bran based solid state fermentation. *Biotechnology Reports, 25*, e00416.

Verma, N., Kumar, V., & Bansal, M. C. (2018). Utility of Luffa cylindrica and Litchi chinensis peel, an agricultural waste biomass in cellulase production by Trichoderma reesei under solid state cultivation. *Biocatalysis and agricultural biotechnology, 16*, 483-492.

Vieira, M. M., Kadoguchi, E., Segato, F., da Silva, S. S., & Chandel, A. K. (2021). Production of cellulases by Aureobasidium pullulans LB83: optimization, characterization, and hydrolytic potential for the production of cellulosic sugars. *Preparative Biochemistry & Biotechnology, 51*(2), 153-163.

Vig, A. P., & Walia, A. (2001). Beneficial effects of Rhizopus oligosporus fermentation on reduction of glucosinolates, fibre and phytic acid in rapeseed (Brassica napus) meal. *Bioresource technology, 78*(3), 309-312.

Von Der Haar, D., Müller, K., Bader-Mittermaier, S., & Eisner, P. (2014). Rapeseed proteins–Production methods and possible application ranges. *OCL, 21*(1), D104.

Wanasundara, J., Tan, S., Alashi, A., Pudel, F., & Blanchard, C. (2017). Proteins from canola/rapeseed: Current status. In *Sustainable protein sources* (pp. 285-304): Elsevier.

Wanasundara, J. P. (2011). Proteins of Brassicaceae oilseeds and their potential as a plant protein source. *Critical reviews in food science and nutrition, 51*(7), 635-677.

Wanasundara, J. P., Abeysekara, S. J., McIntosh, T. C., & Falk, K. C. (2012). Solubility differences of major storage proteins of Brassicaceae oilseeds. *Journal of the American Oil Chemists' Society, 89*(5), 869-881.

Wanasundara, J. P., McIntosh, T. C., Perera, S. P., Withana-Gamage, T. S., & Mitra, P. (2016). Canola/rapeseed protein-functionality and nutrition. *OCL, 23*(4), D407.

Wang, C., Wu, G., Chen, C., & Chen, S. (2012). High production of β-glucosidase by Aspergillus niger on corncob. *Applied biochemistry and biotechnology, 168*(1), 58-67.

Wang, K.-H., Lai, Y.-H., Chang, J.-C., Ko, T.-F., Shyu, S.-L., & Chiou, R. Y.-Y. (2005a). Germination of peanut kernels to enhance resveratrol biosynthesis and prepare sprouts as a functional vegetable. *Journal of agricultural and food chemistry, 53*(2), 242-246.

Wang, L., Ridgway, D., Gu, T., & Moo-Young, M. (2005b). Bioprocessing strategies to improve heterologous protein production in filamentous fungal fermentations. *Biotechnology advances, 23*(2), 115-129.

Wang, X. J., & Hsiao, K. C. (1995). Sugar degradation during autoclaving: effects of duration and solution volume on breakdown of glucose. *Physiologia Plantarum, 94*(3), 415-418.

Warner, K., & Mounts, T. (1993). Frying stability of soybean and canola oils with modified fatty acid compositions. *Journal of the American Oil Chemists' Society, 70*(10), 983-988.

Warner, K., Orr, P., & Glynn, M. (1997). Effect of fatty acid composition of oils on flavor and stability of fried foods. *Journal of the American Oil Chemists' Society, 74*(4), 347-356.

Warwick, S., Gugel, R., McDonald, T., & Falk, K. (2006). Genetic variation of Ethiopian mustard (Brassica carinata A. Braun) germplasm in western Canada. *Genetic Resources and Crop Evolution, 53*(2), 297-312.

Wen, Z., Liao, W., & Chen, S. (2005). Production of cellulase by Trichoderma reesei from dairy manure. *Bioresource technology, 96*(4), 491-499.

White, B. M. (2017). *An evaluation of nitrogen supplementation and processed soy fractions on the performance of cultured fishes*: South Dakota State University.

Wickramasuriya, S. S., Yi, Y.-J., Yoo, J., Kang, N. K., & Heo, J. M. (2015). A review of canola meal as an alternative feed ingredient for ducks. *Journal of animal science and technology, 57*(1), 29.

Wilson, R. (1994). Utilization of dietary carbohydrate by fish. *Aquaculture, 124*(1-4), 67-80.

Wu, J., & Muir, A. (2008). Comparative structural, emulsifying, and biological properties of 2 major canola proteins, cruciferin and napin. *Journal of Food Science, 73*(3), C210-C216.

Xiros, C., Topakas, E., Katapodis, P., & Christakopoulos, P. (2008). Hydrolysis and fermentation of brewer's spent grain by Neurospora crassa. *Bioresource technology, 99*(13), 5427-5435.

Xu, X., Lin, M., Zang, Q., & Shi, S. (2018). Solid state bioconversion of lignocellulosic residues by Inonotus obliquus for production of cellulolytic enzymes and saccharification. *Bioresource technology, 247*, 88-95.

Yabaya, A., & Ado, S. (2008). Mycelial protein production by Aspergillus niger using banana peels. *Science World Journal, 3*(4).

Ye, J.-H., Huang, L.-Y., Terefe, N. S., & Augustin, M. A. (2019). Fermentation-based biotransformation of glucosinolates, phenolics and sugars in retorted broccoli puree by lactic acid bacteria. *Food chemistry, 286*, 616-623.

Yıltırak, S. l., Kocadağlı, T., Çelik, E. E., Özkaynak Kanmaz, E., & Gökmen, V. (2021). Effects of sprouting and fermentation on free ssparagine and eeducing sugars in wheat, einkorn, oat, rye, barley, and buckwheat and on acrylamide and 5-hydroxymethylfurfural formation during heating. *Journal of agricultural and food chemistry, 69*(32), 9419-9433.

Yoshie-Stark, Y., Wada, Y., & Wäsche, A. (2008). Chemical composition, functional properties, and bioactivities of rapeseed protein isolates. *Food chemistry, 107*(1), 32-39.

Youssef, M. K. E., El-Fishawy, F., Ramadan, E., & Abd El-Rahman, A. (2013). Nutritional assessment of barley, talbina and their germinated products. *Sci. J. Crop Sci, 2*, 8-19.

Zahler, J. D., Karki, B., & Gibbons, W. R. (2018). Microbial processing to increase the nutritional value of sorghum mill feed (Milo Hominy). *Journal of the American Oil Chemists' Society, 95*(11), 1445-1452.

Zaki, M., & Said, S. (2018). *Trichoderma Reesei single cell protein production from rice straw pulp in solid state fermentation.* Paper presented at the IOP Conf Ser Mater Sci Eng.

Zalar, P., Gostinčar, C., De Hoog, G., Uršič, V., Sudhadham, M., & Gunde-Cimerman, N. (2008). Redefinition of Aureobasidium pullulans and its varieties. *Studies in mycology, 61*, 21-38.

Zayas, J. F. (2012). *Functionality of proteins in food*: Springer science & business media.

Zhang, H., Vasanthan, T., & Wettasinghe, M. (2004). Dry matter, lipids, and proteins of canola seeds as affected by germination and seedling growth under illuminated and dark environments. *Journal of agricultural and food chemistry, 52*(26), 8001-8005.

Zhang, H., Vasanthan, T., & Wettasinghe, M. (2007). Enrichment of tocopherols and phytosterols in canola oil during seed germination. *Journal of agricultural and food chemistry, 55*(2), 355-359.

Zhang, X., Sun, Z., Cai, J., Wang, J., Wang, G., Zhu, Z., & Cao, F. (2020). Effects of dietary fish meal replacement by fermented moringa (Moringa oleifera Lam.) leaves on growth performance, nonspecific immunity and disease resistance against Aeromonas hydrophila in juvenile gibel carp (Carassius auratus gibelio var. CAS III). *Fish & Shellfish Immunology, 102*, 430-439.

Zhang, Y.-H. P., Himmel, M. E., & Mielenz, J. R. (2006). Outlook for cellulase improvement: screening and selection strategies. *Biotechnology advances, 24*(5), 452-481.

Zhang, Y. H., Hong, J., & Ye, X. (2009). *Cellulase assays*. In Vol. 581. (pp. 213–231). doi:https://doi.org/10.1007/978-1-60761-214-8_14

Zhang, Y. H. P., & Lynd, L. R. (2004). Toward an aggregated understanding of enzymatic hydrolysis of cellulose: noncomplexed cellulase systems. *Biotechnology and Bioengineering, 88*(7), 797-824.

Zhao, H.-M., Guo, X.-N., & Zhu, K.-X. (2017). Impact of solid state fermentation on nutritional, physical and flavor properties of wheat bran. *Food chemistry, 217*, 28-36.

Zheng, B.-A., Matsumura, Y., & Mori, T. (1993). Relationship between the thermal denaturation and gelling properties of legumin from broad beans. *Bioscience, biotechnology, and biochemistry, 57*(7), 1087-1090.

Znameroski, E. A., Coradetti, S. T., Roche, C. M., Tsai, J. C., Iavarone, A. T., Cate, J. H., & Glass, N. L. (2012). Induction of lignocellulose-degrading enzymes in Neurospora crassa by cellodextrins. *Proceedings of the National Academy of Sciences, 109*(16), 6012-6017.