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INCREASING THE NUTRITIONAL VALUE OF CANOLA MEAL VIA FUNGAL BIOPROCESSING

BY

JASON R. CROAT

A thesis proposal in partial fulfillment of the requirements for the

Master of Science

Major in Biological Sciences

Specialization in Microbiology

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2015

INCREASING THE NUTRITIONAL VALUE OF CANOLA MEAL VIA FUNGAL BIOPROCESSING

This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis 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 thesis to my family; Michael, Vicky, Joshua, and Brandon; who have always encouraged me to follow my dreams. This would not have been possible without their love and support.

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ABBREVIATIONS

ADF - acid detergent fiber

CP - cold pressed

dw - dry weight

GLS - glucosinolate

GRAS - generally recognized as safe

GYE - glucose yeast extract

HE - hexane extracted

lc-ms - liquid chromatography-mass spectrometry

NDF - neutral detergent fiber

NPN - non-protein nitrogen

PDA - potato dextrose agar

q-tof - quadrupole time-of-flight

rpm - revolutions per minute

RS - residual sugar

rp-hplc - reverse phase high performance liquid chromatography

SLR - solid loading rate

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ABSTRACT

INCREASING THE NUTRITIONAL VALUE OF CANOLA MEAL VIA FUNGAL BIOPROCESSING

JASON R. CROAT

2015

The main limitation of meals from canola and other *Brassica* spp. is the presence of glucosinolates (GLS), which are anti-nutritional and can even be toxic at high ingestion levels. Furthermore, large amounts of GLS can reduce palatability for livestock and thus reduce intake and growth rates. For this reason canola was bred to contain lower levels of GLS (< 30 μmol/g) and erucic acid (< 2%). However, feed inclusion rates are still limited to ~30%, and this reduces the value of canola meal. The goal of this thesis was to optimize a pretreatment and fungal conversion process to enhance the nutritional value of canola meal. Various combinations of physical/chemical pretreatments, fungal cultures, and incubation methods were investigated to metabolize GLS into cell mass, CO_2, or other non-toxic components. These treatments also served to hydrolyze canola meal fiber into carbohydrates which were then metabolized by the fungi into single cell protein.

Solid-state incubation conditions were initially tested, since filamentous fungi are well adapted to grow at lower moisture levels, and this would potentially reduce contamination issues with bacteria. Flask trials were performed using 50% moisture, hexane extracted (HE) or cold pressed (CP) canola meal, with incubation for 168 h at 30°C. On HE canola meal *Trichoderma reesei* (NRRL-3653) achieved the greatest increase in protein content (23%), while having the lowest residual levels of sugar (8%

w/w) and GLS (0.4 µM/g). On CP canola meal *T. reesei* (NRRL-3653), *Aureobasidium pullulans* (NRRL-58522), and *A. pullulans* (NRRL-Y-2311-1) resulted in the greatest improvement in protein content (22.9, 16.9 and 15.4%, respectively), while reducing total GLS content from 60.6 µM/g to 1.0, 3.2 and 10.7 µM/g, respectively. GLS levels were reduced to 65.5 and 50.7% by thermal treatments while solid-state microbial conversion further reduced GLS up to 99 and 98% in HE and CP canola, respectively. Fiber levels increased due to the concentration effect of removing oligosaccharides and GLS.

Submerged incubation conditions were also tested, as this approach is more commonly used in industry due to easier material handling and process control. Flask trials were performed using 10% moisture content HE or CP canola meal, with incubation for 168 h at 30ºC while being agitated at 150 rpm. Canola meals were either subjected directly to submerged incubation with the fungal strains, or were first saccharified with a cellulase enzyme cocktail and then incubated with the fungi. *Aureobasidium pullulans* (Y-2311-1), *Fusarium venenatum* and *Trichoderma reesei* resulted in the greatest improvements in protein levels in HE canola meal, at 21.0, 23.8, and 34.8%, respectively. These fungi reduced total GLS content to 2.7, 7.4, and 4.9 µM/g, respectively, while residual sugar levels ranged from 0.8-1.6% w/w. In trials with CP canola meal, the same three fungi increased protein levels by 24.6, 35.2, and 37.3%, and final GLS levels to 6.5, 4.0, and 4.7 µM/g, respectively, while residual sugar levels ranged from 0.3-1.0 % w/w. *P. kudriavzevii* was the only fungi able to significantly reduce ADF in both saccharified HE and CP canola meal, representing a reduction of 6.5 and 9.6%, respectively. Similar to solid-state incubation, most cases resulted in an increase of fiber levels due to the concentration effect of removing oligosaccharides and GLS.

Due to the lack of fiber hydrolysis in the trials described above, HE and CP canola meal were subjected to various pretreatments (extrusion, hot water cook, dilute acid, and dilute alkali) to determine if fibers could be made more susceptible to enzymatic hydrolysis. Following pretreatment, canola samples were subjected directly to submerged incubation with the fungal strains (*A. pullulans* Y-2311-1, *F. venenatum* NRRL-26139, and *T. reesei* NRRL-3653). The combination of extrusion pretreatment followed by incubation with *T. reesei* resulted in the greatest overall improvement to HE canola meal, increasing protein to 51.5%, while reducing NDF, GLS, and residual sugars to 18.6%, 17.2 μM/g, and 5% w/w, respectively. Extrusion pretreatment and incubation with *F. venenatum* performed the best with CP canola meal, resulting in 54.4% protein while reducing NDF, GLS, and residual sugars to 11.6%, 6.7 μM/g, and 3.8% w/w, respectively.

Chapter I - Literature Review

1.1 Oilseed Production

1.1.1 Types of Oilseeds

On a global basis, the major oilseed crops are soybean, sunflower, rapeseed, cotton, and peanuts (Ash 2012; Sarwar et al. 2013). Oilseeds are primarily grown for their oil content for either edible or industrial applications (Ash 2012), although the remaining meals are valuable livestock feeds. The type of oilseed planted depends on the purpose of production (oil, meal, or as cover crops) (Haramoto and Gallandt 2007), and the climatic conditions in the region.

1.1.1.1 Leguminous

Legumes are broadly defined by their unusual flower structure, podded fruit, and the ability of 88% of the species to form nodules with *Rhizobia* spp. bacteria (Graham and Vance 2003). The primary dietary legumes include soybean, pea, chickpea, broad bean, pigeon pea, cowpea, and lentil (Graham and Vance 2003). Soybeans and peanuts provide more than 35% of the world's processed vegetable oil (Graham and Vance 2003). The soybean is a legume crop native to Eastern Asia (Medic et al. 2014). Its production has expanded worldwide due to its high levels of protein and oil that help accommodate food and fuel needs of the growing world population. Soybeans are the most dominant oilseed in the U.S. Most soybeans are planted as a row crop in May and early June then harvested in late September and October (Ash 2012).

As mentioned above, legumes such as soybeans, develop root organs (nodules) after micro-symbiont induction to conduct biological nitrogen fixation (Gresshoff et al. 2015). *Rhizobia* are gram-negative soil bacteria that infect root tissues of the legume and induce the formation of the nitrogen fixing nodules (Ardourel et al. 1994). *Rhizobia* penetrates the host via root hairs and stimulates cell wall growth to entrap the *Rhizobia* in the nodule that is formed (Ardourel et al. 1994). To establish symbiosis, the plant supplies the carbon source for the energy-dependent reduction of dinitrogen and protects the oxygen-sensitive nitrogenase enzyme (Stougaard 2000). In exchange, the *Rhizobia* provides fixed nitrogen to the legume (Vance 2001).

It is well known that soil organic carbon and nitrogen are important to sustain soil quality and promote soil production (Al-Kaisi et al. 2005). Implementing crop rotations can be used to effectively manage soil carbon and nitrogen levels. Soil nitrogen levels increase when nitrogen-fixing legumes are included in rotation crops (Riedell et al. 2009). About 200 million ton of nitrogen are added to the biosphere annually by symbiotic nitrogen fixation of legumes (Graham and Vance 2003). Thus, the symbiosis between the legume and soil *Rhizobium*-type bacteria can reduce the need for nitrogen fertilizer (Gresshoff et al. 2015). Other benefits of rotation crops include improved soil structure, increased soil organic matter levels, increased water use efficiency, enhanced mycorrhizal associations, and increased crop yields (Riedell et al. 2009).

1.1.1.2 *Brassicaceae* Family:

The *Brassica* genus of oilseed plants is part of the Brassicaceae family, which is an economically important family containing many food and oilseed crops, as well as ornamental plants and noxious weeds. The Brassicaceae family, also known as the crucifer family, is characterized by the presence of a group of secondary compounds called glucosinolates (Vaughn and Berhow 2005). The term ‘rapeseed’ may refer to both high and low glucosinolate/erucic acid varieties used for edible and industrial applications (Brown et al. 2008). Some areas of the world where canola varieties are less widely used continue to use the term ‘rapeseed’ for all related varieties (Ash 2012).

Brassica genus oilseed plants provide several benefits when grown as cover crops. These benefits include maintaining and improving soil quality, preventing erosion, and allelopathic weed control via glucosinolate hydrolysis products (Haramoto and Gallandt 2007). These reasons have increased the interest in growing *Brassicas*, both as cover crops and seed crops harvested for oil production (Haramoto and Gallandt 2007). Rapeseed can be cultivated in cooler agricultural regions and as a winter crop in temperate locations (Pospiši et al. 2007).

Brassica napus (canola) is a bright yellow flowering member of the *Brassica* genus (Bonnardeaux 2007) and is the most commercially important oilseed crop in this genus. Winter canola varieties are generally planted in Europe, Ukraine, Russia, and parts of China (Ash 2012). Winter canola is planted in the fall and harvested the following summer (Brown et al. 2008). Winter canola varieties have a strong cold tolerance, but can still freeze out with the combination of dry soils and rapid temperature fluctuations (Brown et al. 2008). Winter canola varieties typically yield 20-30% more than spring varieties (Ash 2012). Winter crops also tend to have larger seeds with higher oil content than spring crops (Brown et al. 2008). Spring canola varieties are generally planted in parts of China, India, Canada, and the United States (Ash 2012). Spring canola varieties are primarily planted early spring and harvested in late summer (Brown et al. 2008). Spring crops mature as early at 85 days after planting, depending on the variety and weather conditions (Ash 2012). There is a small timeframe when harvesting canola to avoid immature seeds or seed shatter loss. Generally, producers will swath the plant then combine to collect the seed when the moisture content is safe to store (Ash 2012).

Non-food oilseeds such as carinata and camelina are being developed to serve as sources on non-edible oil for fuel/chemical production, as well as rotational crops. They will provide another cash crop to farmers, and enhance agricultural sustainability by diversifying crop rotations to break weed and pest cycles (Cardone et al. 2003). These crops produce high yields of long-chain oils that can be converted into jet fuel and diesel by existing technologies (Cardone et al. 2003).

Brassica carianta (carinata) is a native plant of the Ethiopian highlands and also belongs to the *Brassica* genus (Bouaid et al. 2009). Research has shown that carinata can adapt and have higher production in adverse conditions (clay- and sandy-type soils and in semi-arid temperate climates) when compared with canola (Cardone et al. 2003). Optimal regions for carinata production include the southern prairies of Canada and the northern plains of the United States, as well as countries including Spain and Italy (Bouaid et al. 2009). Carinata contains significantly higher levels of undesirable glucosinolates and erucic acid when compared to the closely related canola (Alemayehu and Becker 2002), making it more ideal for non-food applications. Carinata’s oil profile has been optimized for use in the biofuel industry, specifically bio-jet fuel. Because carianta is being optimized for industrial use and is not a food crop, more genetic work has been focused to improve the oil content in carinata rather than reducing glucosinolate content (Alemayehu and Becker 2002). The high level of glucosinolates in carinata meal limits inclusion levels in animal feeds (Cardone et al. 2003).

Camelina sativa (camelina) is a member of the Brassicaceae family and is commonly known as gold-of-pleasure of false flax (Moser 2010). Camelina has lower water, pesticide, and fertilizer requirements than other traditional oilseed crops, such as rapeseed/canola and soybeans (Moser 2010). Camelina is a short-season crop (85-100 days) that is well adapted in temperate climate zones (Grady and Nleya 2010). Camelina is traditionally grown in Europe and Asia (Moser 2010), but has also been introduced into the lower 48 states of continental United States as well as Alaska and Canada (Grady and Nleya 2010). Camelina oil can be used in both edible and industrial products (Fleenor 2011). Camelina is particularly attractive as an alternative feedstock for biodiesel production as a result of its low cost versus commodity oils (Moser 2010). The resulting camelina meal contains ~40% protein and a moderately low glucosinolate content (Grady and Nleya 2010). Similar to soybean and canola meal, camelina meal can be fed in livestock diets at limited inclusion rates up to 10% (Grady and Nleya 2010).

1.1.2 Global Production

1.1.2.1 Global Production Trends of Major Oilseeds

The USDA projected 2014/15 global oilseed production at 535.6 million tons (USDA 2015a). The global production of soybeans reached 318.25 million metric tons in 2014/2015 (Table 1.1), up from 117 million metric tons in 1992/1993, representing a 63% increase in production (Medic et al. 2014). Again, the term ‘rapeseed’ refers to both high and low glucosinolate/erucic acid varieties, which includes edible variety canola (Brown et al. 2008). Canola has rapidly expanded over the past 40 years, rising from the sixth largest oilseed crop to the second largest (Ash 2012). The United States and Brazil have remained the top oilseed producers from 2011-2015, representing 22% and 18% of global production, respectively (Table 1.2).

Table 1.1: Global production of major oilseeds (million metric tons) (USDA 2015a)

	2011/12	2012/13	2013/14	2014/15
Copra	5.59	5.79	5.43	5.43
Cottonseed	48.26	46.26	45.93	44.47
Palm Kernel	13.86	14.91	15.79	16.35
Peanut	38.47	40.48	41.16	39.32
Rapeseed	61.57	63.69	71.38	71.71
Soybean	240.43	268.82	283.25	318.25
Sunflower	39.69	36.02	42.87	40.03
Total	447.87	475.96	505.82	535.57

Table 1.2: Global production of major oilseeds[1] by country (million metric tons) (USDA 2015a)

	2011/12	2012/13	2013/14	2014/15
United States	92.44	93.32	99.02	117.17
Brazil	70.24	84.76	90.24	97.62
Argentina	44.82	53.68	57.02	63.78
China	59.60	59.79	58.89	57.56
India	37.11	37.52	36.80	35.43
Other	143.67	146.89	163.86	164.02
Total	447.87	475.96	505.82	535.57

[1] Major oilseeds include copra, cottonseed, palm kernel, peanut, rapeseed, soybean, and sunflower

Global production of soybeans has greatly increased due to bioengineering technologies (Ash 2012). The U.S. is the largest producer of soybeans, representing a 34% of global production in 2014/15 (Table 1.3). Brazil and Argentina follow the U.S. in production, representing 30 and 19%, respectively (Table 1.3).

Table 1.3: Soybean production by country (thousand metric tons) (USDA 2015a)

	2011/12	2012/13	2013/14	2014/15
United States	84,291	82,791	91,389	108,014
Brazil	66,500	82,000	86,700	94,500
Argentina	40,100	49,300	53,500	59,500
China	14,485	13,050	12,200	12,350
India	11,700	12,200	9,500	9,800
Paraguay	4,043	8,202	8,200	8,500
Canada	4,467	5,086	5,359	6,050
Other	14,841	16,195	16,405	19,539
Total	240,427	268,824	283,253	318,253

Global production of rapeseed has remained steady from 2012/13-2014/15 (Table 1.4). However, interest in using non-edible varieties as an alternative feedstock for advanced biofuels may spark rapid growth (Cardone et al. 2003). The European Union, China, and Canada are the top three global producers of rapeseed, representing 37, 23, and 12%, respectively.

Table 1.4: Rapeseed production by country (thousand metric tons) (USDA 2015a)

	2012/13	2013/14	2014/15
China	6,579	6,500	6,223
India	2,500	2,450	2,500
Canada	3,050	3,060	3,080
Japan	1,054	1,075	1,075
European Union	9,946	10,251	9,975
Other	3,464	3,863	3,872
Total	26,593	27,199	26,725

Global production of canola is concentrated in areas with dry weather (18-20 inches precipitation per year) and shorter growing seasons (85-125 days) (Brown et al. 2008; Ash 2012). Winter canola is generally planted in Europe, Ukraine, Russia, and parts of China while spring canola varieties are primarily planted in parts of China, India, Canada, and the United States (Ash 2012). Spring varieties of canola may mature as quickly as 85 days after planting. Production trends in Canada are much larger and have a significant impact on production and processing of canola in the U.S. (Ash 2012). Canada produces more than half of the world's canola seed, meal, and oil while continuing to expand (Ash 2012).

1.1.2.2 Production Trends of Soybeans and Canola in the United States

Table 1.5 provides the United States' production of soybeans and canola from 1991-2014. The acres of soybeans and canola have increased by 30 and 90%, respectively, showing no displacement of acreage for either oilseed. Yield trends have shown general improvements over time due to bioengineering of desired traits and improved seeding practices (Ash 2012).

Table 1.5: U.S. production of soybeans and canola from 1991-2014 (USDA 2015b)

	Acres Harvested		Production in bushels	
Year	Soybeans	Canola	Soybeans	Canola[1]
1991	58,011,000	147,000	1,986,539,000	3,822,000
1992	58,233,000	112,000	2,190,354,000	2,880,740
1993	57,307,000	187,000	1,869,718,000	5,049,000
1994	60,809,000	340,000	2,514,869,000	8,948,800
1995	61,544,000	429,000	2,174,254,000	10,968,940
1996	63,349,000	347,000	2,380,274,000	9,610,420
1997	69,110,000	631,000	2,688,750,000	15,614,200
1998	70,441,000	1,076,000	2,741,014,000	31,156,000
1999	72,446,000	1,044,000	2,653,758,000	27,273,600
2000	72,408,000	1,498,000	2,757,810,000	39,966,200
2001	72,975,000	1,455,000	2,890,682,000	39,970,300
2002	72,497,000	1,281,000	2,756,147,000	30,668,400
2003	72,476,000	1,068,000	2,453,845,000	30,245,000
2004	73,958,000	828,000	3,123,790,000	26,790,600
2005	71,251,000	1,114,000	3,068,342,000	31,619,700
2006	74,602,000	1,021,000	3,196,726,000	27,886,240
2007	64,146,000	1,155,500	2,677,117,000	28,614,680
2008	74,681,000	989,000	2,967,007,000	28,901,280
2009	76,372,000	808,000	3,360,931,000	29,295,600
2010	76,610,000	1,430,700	3,331,306,000	48,952,560
2011	73,776,000	1,033,000	3,097,179,000	30,560,200
2012	76,144,000	1,717,900	3,042,044,000	47,832,200
2013	76,253,000	1,264,500	3,357,984,000	44,210,100
2014	83,061,000	1,555,700	3,968,823,000	50,219,900

[1] Assuming 50 pounds of canola per bushel

1.1.2.2.1 Soybean Trend Data

Large-scale soybean production did not take place until the 20th century in the U.S. but has expanded rapidly (Ash 2012). Currently, soybeans are the second most planted field crop in the U.S. and account for 90% of domestic oilseed production (Ash 2012). More than 80% of U.S. soybean production is concentrated in the upper Midwest (Ash 2012).

1.1.2.2.2 Canola Trend Data

The U.S. portion of total global canola production remains small, but is growing in the Northern Plains (Ash 2012). Approximately 90% of U.S. canola production takes place in North Dakota, with smaller amounts in Colorado, Idaho, Minnesota, Montana, and South Dakota (Ash 2012). The U.S. Department of Agriculture's National Agricultural Statistics Service estimated 2014 U.S. canola crop at 2.52 billion pounds harvested from over 1.55 million acres (US Canola 2014).

1.2 Oilseed Crushing & Extraction

Oilseeds are typically not sold directly to consumers but are initially transported to a processing facility to separate the oil from the meal (Ash 2012). This process is commonly referred to as "crushing," and it can be accomplished by three main methods: solvent extraction, expeller, and cold pressing (Spragg and Mailer 2007). The extracted oil is the most valuable crush component and provides the majority of the processing facilities' revenue (Ash 2012). Thus oil extraction yield and efficiency are important aspects to this process. The top five countries in crushing capacity included China, United States, the European Union, Argentina, and Brazil, representing 68% of total global production (Table 1.6). China is the global leader in oilseed crushing, representing 26% of the market in 2014/15 (USDA 2015a).

Table 1.6: Global crushing production of major oilseeds by country (million metric tons) (USDA 2015a)

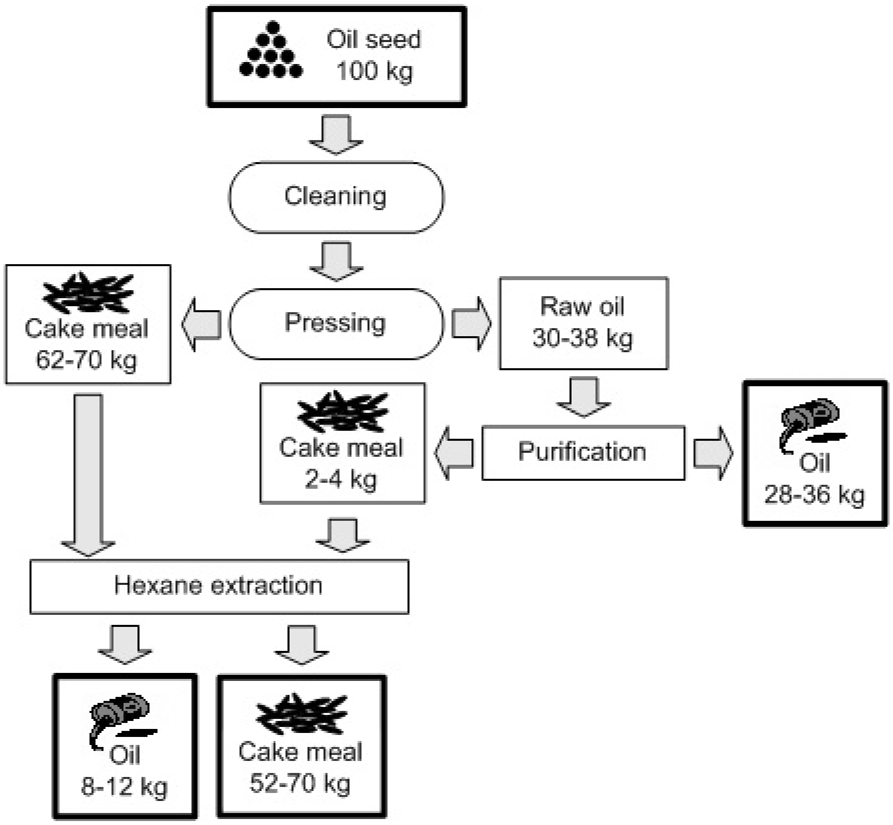
	2011/12	2012/13	2013/14	2014/15
China	96.29	102.64	107.63	111.68
United States	50.32	50.23	51.47	53.61
Argentina	39.95	36.25	38.77	42.63
European Union	41.06	42.07	45.33	46.11
Brazil	41.17	37.71	39.73	41.97
India	28.59	29.30	28.70	27.48
Russia	11.96	11.05	13.85	13.75
Ukraine	10.28	9.20	12.28	11.48
Indonesia	8.35	8.99	9.66	10.29
Canada	8.41	8.27	8.52	8.68
Mexico	5.86	5.70	6.08	6.50
Pakistan	5.60	5.30	5.58	6.27
Malaysia	4.99	5.34	5.53	5.54
Japan	4.35	4.38	4.40	4.45
Paraguay	1.07	3.17	3.49	3.81
Other	36.93	37.67	38.50	40.63
Total	395.16	397.24	419.49	434.85

1.2.1 Solvent Extraction

Hexane extraction is the most common and capital-intensive oil extraction method used commercially. This extraction process utilizes two steps to extract high percentages of oil (Fig. 1). Before oil extraction, the seeds typically go through a pre-conditioning process, which usually includes seed cleaning, de-hulling, seed pre-conditioning, and flaking (Newkirk 2009). Pre-conditioned flakes are then mechanically pressed at 100-120°C to produce a seed cake containing approximately 20% oil (Spragg and Mailer 2007). The pressed seed cake is then subjected to solvent extraction using hexane, and then undergoes desolventizing and toasting of the meal at 100-115°C for 30 minutes (Spragg and Mailer 2007; Newkirk 2009).

Figure 1.1: Pre-conditioning and process diagram used for solvent oil extraction

(Baquero et al. 2011)


Oil seed
100 kg
Cleaning
Pressing
Cake meal
62-70 kg
Raw oil
30-38 kg
Cake meal
2-4 kg
Purification
Oil
28-36 kg
Hexane extraction
Oil
8-12 kg
Cake meal
52-70 kg

1.2.1.1 Advantages and Disadvantages

Hexane extraction recovers over 96% of the oil, and thereby also results in meals containing the highest percentage of protein (Spragg and Mailer 2007). The high temperatures used for desolventizing and toasting the meal to remove the solvent (Newkirk 2009) can eliminate volatile anti-nutritional factors, such as glucosinolates (Jensen et al. 1995). Studies have shown that the desolventizing and toasting steps of hexane extraction reduced glucosinolate levels by ~40% (Newkirk et al. 2003). However, this additional heat treatment can reduce the digestibility of some amino acids, particularly lysine (Anderson-Hafermann et al. 1993, Newkirk 2009). Non-toasted canola meal contains higher levels of digestible amino acids than conventional toasted canola meal, therefore affecting palatability (Newkirk et al. 2003). Processing at a maximum of 100°C during the desolventizing and toasting process can significantly increase lysine digestibility (Newkirk 2009). Jensen et al. evaluated multiple toasting times to compare glucosinolate reduction, protein solubility, and true digestibilities of rapeseed meal in rats (Table 1.7.) (Jensen et al. 1995). This study had also shown a reduction of volatile anti-nutritional factors (glucosinolates) up to 95% however, protein solubility also decreased from 85 to 40% over 120 minutes (Table 1.7).

Table 1.7: Effect of 100°C toasting time on glucosinolates and nutritional value of rapeseed meal (Jensen et al. 1995)

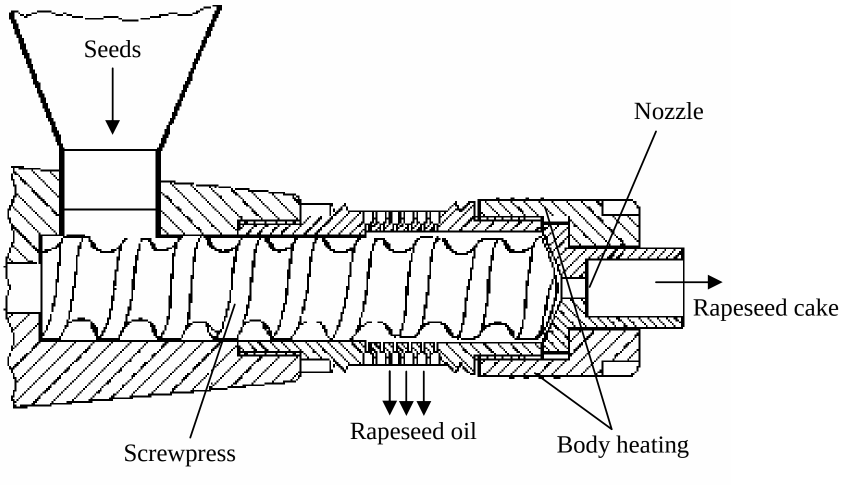
Time (min)	GLS Reduction	Protein Solubility	True Digestibility
0		85%	77%
15	24%	81%	74%
30	46%	61%	72%
60	70%	52%	73%
120	95%	40%	71%

The higher processing heat may oxidize the oil and damage antioxidants, decreasing the stability and value of the oil (Wanasundara and Shahidi 1994). Chemical solvents can be hazardous to workers while chemical residues left in the oil (up to 25 ppm) can be toxic to the end consumer. The United States Environmental Protection Agency has listed n-hexane among 187 hazardous air pollutants due to its toxic nature (Uquiche et al. 2012).

1.2.2 Expeller

Up to the point of solvent extraction, the expeller process is similar to the traditional solvent extraction process (Newkirk 2009). Expeller pressing uses a similar pre-conditioning step while excluding the use of solvents, desolventization, toasting and drying/cooling stages (Newkirk 2009). Figure 1.2 represents the mechanical screw press typically used for both expeller and cold pressed oil extraction. In expeller extraction, seeds are heat conditioned than passed directly through a series of screw presses consisting of a rotating screw shaft inside a cylindrical barrel (Newkirk 2009). A cage or mesh at the bottom of the barrel allows oil to seep out for collection. The expeller press is used to extract ~75% of the canola seed oil (Spragg and Mailer 2007; Seneviratne et al. 2010), resulting with a residual oil content of 10 to 15% in the meal (Leming and Lember 2005). Seeds can also be crushed using a double expeller process, thus expelling the seed twice to extract oil. The resulting meal has an oil content ranging from 8-11% (Newkirk 2009).

Figure 1.2: Mechanical screw press diagram used for expeller and cold pressed oil extraction (Leming and Lember 2005)



1.2.2.1 Advantages and Disadvantages

Similar to solvent extraction, expeller extraction applies significant levels of heat to the seed during the process. Expeller extraction can generate meal temperatures up to

135°C for a brief period while passing through the press (Spragg and Mailer 2007). The residual oil in the meal has a higher metabolizable, digestible, and net energy content than traditional solvent extracted meal (Newkirk 2009).

Double pressing of the canola seed increases the oil extraction yield compared to traditional expelling. However, the meal is subjected to potential heat effects due to the friction generated during the extraction process. Meal temperatures may reach up to 160°C, but the protein quality is generally preserved due to low moisture content and short heat duration (Newkirk 2009). As mentioned above, high processing temperatures can eliminate volatile anti-nutritional factors, such as glucosinolates. The reduction of total glucosinolates using the expeller process would be less than solvent extraction but more than cold pressing. The temperatures reached in this process may lead to some loss of antioxidant potency.

1.2.3 Cold Pressing

The cold pressing process does not have a pre-conditioning step prior to oil extraction. Again, Figure 1.2 represents the mechanical screw press typically used for expeller and cold pressed extraction. Seeds pass directly through a series of screw presses consisting of a rotating screw shaft inside a cylindrical barrel (Newkirk 2009). This process produces pressure and frictional forces to pulverize the seeds (Spragg and Mailer 2007). While the mechanical process is similar, expeller extraction temperatures reach up to 135°C, cold pressing is significantly lower at 65°C (Spragg and Mailer 2007). A cage or mesh at the bottom of the barrel allows oil to seep out for collection. Cold pressing of the oilseeds usually remove 50-70% of the seed oil content (Newkirk 2009; Seneviratne et al. 2011) while the resulting meal contains 11-20% residual oil (Leming and Lember 2005; Spragg and Mailer 2007).

1.2.3.1 Advantages and Disadvantages

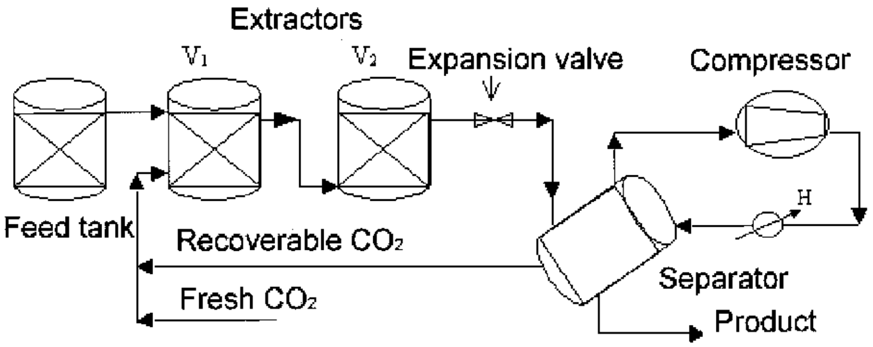
Cold pressing produces higher quality oil with better color and flavor. For these reasons, interest in improving oil recovery by cold pressing has increased (Tuberoso et al. 2007). The oil produced from this process is also known as virgin oil and is in demand by consumers of organic and natural foods, and usually sells for a higher price than solvent extracted oil (Maison 2013). Cold pressing involves low initial and operating costs when compared to solvent extraction (Szydłowska-Czerniak et al. 2010). However, this method is relatively low in efficiency, thus requiring improvements (Szydłowska-Czerniak et al. 2010).

1.2.4 Alternative Oil Recovery Methods (Supercritical CO_2)

The first commercially successful use of supercritical fluid extraction was performed in 1978 for the decaffeination of green coffee beans, soon followed by the extraction of hop flavors (Raventos et al. 2002). Both applications have given rise to numerous variations, which have also developed on an industrial scale. Supercritical fluid technologies may be an alternative to current oil extraction methods. Supercritical fluids are often called dense gases where the gas used is above its critical temperature and pressure (Friedrich et al. 1982). A gas, at or above its critical temperature, increases in density when compressed. Therefore, the supercritical gas has the density of a liquid while maintaining the diffusivity of a gas (Friedrich et al. 1982). CO_2 is the solvent most commonly used in supercritical fluid extraction processes because it has a relatively low critical temperature (31.1°C) and moderate critical pressure (7.39 MPa) (Uquiche et al. 2012).

Figure 1.3 outlines a batch supercritical extraction process. The supercritical fluid extraction process consists of two essential steps: extraction and separation (Raventos et al. 2002). The material to be extracted is first placed in an extractor with supercritical fluid at specific pressure and temperature conditions (Raventos et al. 2002). Solid materials must be extracted in a batchwise process. After extraction, the fluid and extracted material are passed through a separator, and by reducing temperature and/or changing temperature the dissolving power of the supercritical fluid is reduced and the separation of the compound occurs (Raventos et al. 2002).

Figure 1.3: Batchwise supercritical extraction process diagram (Raventos et al. 2002)



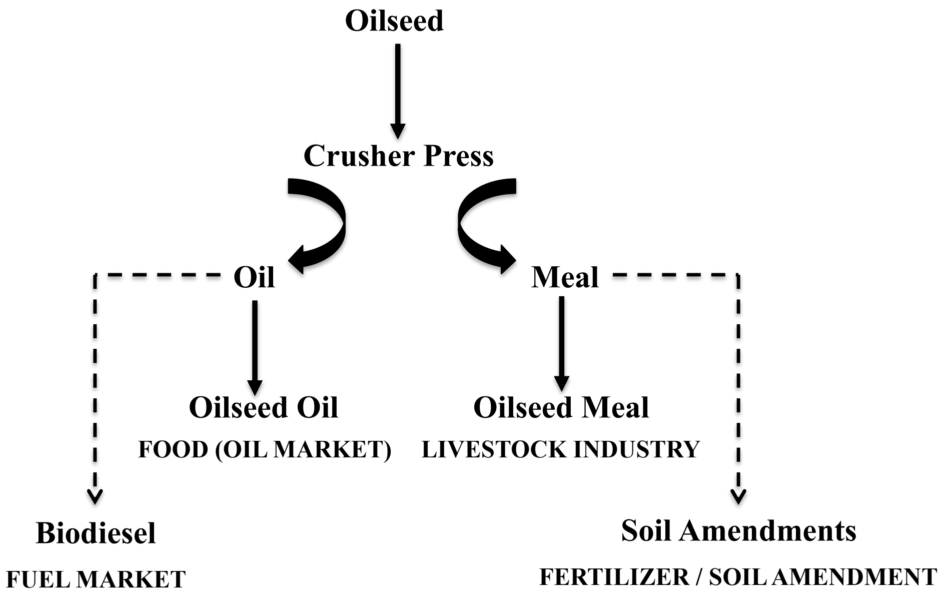
1.2.4.2 Advantages and Disadvantages

Supercritical fluid technologies have been recognized for over 100 years but commercial applications have been slow developing, likely due to sophisticated and expensive high pressure equipment needed for the process (Friedrich et al. 1982). The liquid-like density of a supercritical fluid solvent provides high solvent power, whereas the gas-like diffusivity creates excellent transport properties, which enhances the mass transfer rate as compared to liquid organic solvents (Patel et al. 2011). Therefore this method will have oil recovery efficiencies similar to solvent extraction. Additional benefits from supercritical fluid processing include improved color, odor, functionality, and elimination of residual enzymatic activity (King et al. 2001). Supercritical CO_2 is an ideal solvent because it is a non-toxic, non-explosive, cheap, and recyclable resource that can be easily removed from extracted products (Friedrich et al. 1982; Uquiche et al. 2012). Supercritical CO_2 can be processed at lower temperatures; therefore the nutrients in the oil will not be oxidized to produce a more superior product for the consumer.

1.3 Oilseed Utilization

Figure 1.4 outlines the products produced from oilseed processing and the potential markets of these products. Oilseeds are generally grown as a source of edible oil or for biodiesel/jet fuel production (Singh and Singh 2010). Following oil extraction, the remaining meal is used as a protein source for livestock or as a fertilizer/soil amendment. The uses of oilseed products depend on composition and economics.

Figure 1.4: General utilization flowchart of oilseed processing and markets


Oilseed
Crusher Press
Oil
Meal
Oilseed Oil
FOOD (OIL MARKET)
Oilseed Meal
LIVESTOCK INDUSTRY
Biodiesel
FUEL MARKET
Soil Amendments
FERTILIZER / SOIL AMENDMENT

1.3.1 Oilseed Oil

Oilseed oils are predominately used in the food or biofuel markets. Food markets consume 80% of vegetable oils on a global basis, with the industrial and biodiesel markets far behind (Rosillo-Calle et al. 2009). Currently palm, soybean, and canola oil represent the top three oil commodities worldwide (Pospiši et al. 2007). The range of oil contained in soybeans is about 20% while rapeseeds like canola can contain over 40% oil (Aider and Barbana 2011; Sarwar et al. 2013). Rapeseeds, in general, have undergone intensive breeding over the last fifteen years to modify the fatty acid composition of the oil (Pospiši et al. 2007). Rapeseed cultivars have high concentrations of erucic acid while canola cultivars are low in erucic acid (Brown et al. 2008). High concentrations of erucic acid can have toxic effects on the heart at high enough doses.

1.3.1.1 Food Uses

In the U.S., canola and soybean oils are used in frying and baking applications, and as ingredients in salad dressings, margarine, and a variety of other products (Ash 2012). Canola oil is one of the most important vegetable oils due to its high content of both omega-3 (α-linolenic acid) and omega-6 (linoleic acid) fatty acids (Tuberoso et al. 2007). Canola oil has much higher amounts of linoleic acid (8-12%) when compared to other vegetable oils, such as soybean, sunflower, olive, and corn, which contain 8.0, 0.2, 0.8, and 0.7%, respectively (Wanasundara and Shahidi 1994). The high level of oleic acid, the low content of saturated fatty acids, and the presence of linoleic and linolenic acids provide rapeseed oils unique nutritive value and efficiency in preventing cardiovascular diseases (Pospiši et al. 2007; Ash 2012).

Both low-linolenic and high-linolenic canola oil have the additional benefit of longer shelf life than other oils used in the frying industry (Brown et al. 2008). Table 1.8 compares the fatty acid composition of soybean and canola oil. Canola oil contains approximately half the saturated fatty acids (SAT) when compared to soybean oil. Canola oil also contains approximately three times the amount of monounsaturated fatty acids (MUFA) and half the amount of polyunsaturated fatty acids (PUFA). The American Heart Association dietary guidelines recommend a diet that provides up to 15% MUFA and 10% PUFA, so the higher concentrations of MUFA in canola oil can be beneficial (Kris-Etherton 1999).

Table 1.8: Fatty acid composition of soybean and canola oil (Przybylski et al. 2013)

Fatty Acids	Soybean	Canola
SAT (%w/w)	15.5	7.3
MUFA (%w/w)	22.7	62.4
PUFA (%w/w)	60.3	28.3
Total Tocopherols (µg/g)	1,877	837

The seeds of oil crops, particularly those containing high percentages of polyunsaturated fatty acids are thought to be rich in antioxidants (Peschel et al. 2007). Canola oil contains high amounts of bioactive compounds, such as polyphenols, phytosterols, tocopherols, and other antioxidants (Szydłowska-Czerniak et al. 2010). Soybean oil contains over twice the amount of tocopherols than canola oil (Table 1.8). Antioxidants can play an important role in the prevention and treatment of chronic diseases, such as heart disease, neurodegenerative disease, aging, cancer, and rheumatoid arthritis (Richards et al. 2008; Laoretani et al. 2014)

The global increase in vegetable oil production (Table 1.9) has helped to gradually replace animal fats in foods. Vegetable oil production has increased by 10%

from 2011/12 to 2014/15. Vegetable oils are preferred over animal fats because of their higher content of unsaturated fatty acids, while solid animal fats contain more saturated fatty acids (Arif et al. 2012). Dietary unsaturated fatty acids are very effective in lowering blood cholesterol and may be important in preventing coronary heart disease (Rowghani et al. 2007). The increased availability of vegetables oils allows consumers to take advantage of their proven beneficial components and promote healthier diets (Kris-Etherton 2003). Canola oil is the second most abundant source of edible oil (Aider and Barbana 2011). This variety of rapeseed was developed in Canada during the early 1970s to create a variety more suitable for consumption (Bell 1993). Plant breeders were able to do this by creating a low erucic acid and low glucosinolate variety, which was registered under the name "canola" (Ash 2012). The FDA granted canola oil Generally Recognized as Safe (GRAS) status in January 1, 1985 (Ash 2012). Between 1999-2009, canola oil represented 13-16 percent of world vegetable oil production (Ash 2012).

Table 1.9: Global vegetable oil production from major oilseeds (million metric tons) (USDA 2015a)

	2011/12	2012/13	2013/14	2014/15
Coconut	3.43	3.65	3.38	3.35
Cottonseed	5.27	5.25	5.18	5.17
Olive	3.45	2.45	3.14	2.33
Palm	52.58	56.59	59.54	61.65
Palm Kernel	6.17	6.58	6.98	7.27
Peanut	5.30	5.51	5.64	5.59
Rapeseed	24.10	24.90	26.59	27.20
Soybean	42.74	43.09	45.00	47.84
Sunflowerseed	14.74	13.26	15.96	15.10
Total	157.78	161.27	171.41	175.51

1.3.1.2 Fuel Uses

Demand for alternative fuels has sparked interest in the production of biofuels from oil-rich seeds (Hollister et al. 2013). Canola, rapeseed, palm, soybean, and sunflower are of high interest for bio-diesel production due to their high oil content (Moser 2010; Sarwar et al. 2013). Biodiesel made from oils with low saturated fats have improved cold flow properties, while low polyunsaturated fats have lower nitrous oxide emissions (Brown et al. 2008). Regional availability would certainly dictate which feedstock oil to be utilized for liquid fuel production. Oils extracted from food sources, such as canola and soybeans, would only be economical for biofuels if there were an over-abundance of supply. Production of soybean oil is still increasing, primarily due to biodiesel production in the U.S. and South America (Rosillo-Calle et al. 2009). According to the National Biodiesel Board, soybean oil represented 53% of the feedstock utilized for biodiesel production in 2013, followed by recycled oils (13%), animal fats (11%), distillers corn oil (10%), canola oil (7%), and palm oil (6%) (National Biodiesel Board 2015).

Carinata’s oil profile has been optimized for use in the biofuel industry, specifically bio-jet fuel. Carinata contains longer chained oils that result in higher yields of jet fuel. Rapeseed oils high in erucic oil, such as carinata, have significantly higher lubricity when compared to other vegetable oils (Brown et al. 2008). Carinata may be considered as an alternative feedstock for biodiesel by considering its low input crop conditions and similar performance versus conventional biodiesel production from soybeans (Cardone et al. 2003).

1.3.2 Oilseed Meal

The use of plant proteins in livestock diets has long been a major industrial sector (Booth et al. 2001). Oilseed meals that have served as cost-effective feed ingredients include soybean, cotton seed, sunflower, canola, and flax meals, and have been used in ruminant, swine, poultry, aquaculture, and companion animal diets (Sarwar et al. 2013). The protein content of defatted oilseed meal depends on the seed but generally ranges between 35 to 60%. Many of the oilseed meals contain anti-nutritional factors such as oligosaccharides, trypsin inhibitors, phytic acid, and tannins, and may have low protein solubility, which can limit feed and food applications (Moure et al. 2006).

Soybean and canola meals are the most abundantly available and dominate the livestock market over the other oilseed meal types (Table 1.10). In the marketing year 2008/2009, global canola meal production was 30.8 million metric tons, versus 151.6 million for soybean meal (Ash 2012). In 2014/15, soybean and rapeseed meal represented 69 and 14% of the major protein meals produced on a global basis. On a worldwide basis, canola meal is second only to soybean meal for use as a feed (Newkirk 2009).

Table 1.10: Global production of major protein meals (million metric tons) (USDA 2015a)

	2011/12	2012/13	2013/14	2014/15
Copra	1.83	1.94	1.81	1.79
Cottonseed	15.84	15.77	15.68	15.58
Fish	4.18	4.37	4.14	4.29
Palm Kernel	7.27	7.83	8.35	8.65
Peanut	6.47	6.75	6.90	6.86
Rapeseed	35.69	36.90	39.41	40.24
Soybean	180.49	181.27	189.47	202.63
Sunflowerseed	15.66	14.02	16.79	15.96
Total	267.43	268.86	282.54	296.00

1.3.2.1 Soybean Meal (digestibility and performance)

Soybean meal is the main protein source for ruminants and monogastic livestock because of its high palatability, high protein content, and amino acid profile (Booth et al. 2001; Chen 2013). Generally, soybean meal contains 44-49% protein (Cromwell 2008; Ash 2012). Soybean meal contains approximately 20% more protein and 75% less crude fiber than canola meal while gross energy is relatively the same (Table 1.11). Soybean meal protein is high in lysine (6.5%) but low in sulfur-containing amino acids, with methionine being the most limiting (Cromwell 2008; Chen 2013).

Table 1.11: Chemical composition and gross energy values of soybean meal and canola meal (Bell 1993)

Component	Soybean Meal	Canola Meal
Moisture (%)	10.00	8.50
Crude protein (%)	48.10	38.29
Ether extract (%)	0.70	3.59
Acid detergent fiber (%)	5.00	17.47
Neutral detergent fiber (%)	7.10	21.54
Crude fiber (%)	3.40	12.01
Gross energy (MJ kg-1)	20.07	18.64
Minerals		
Phosphorous (%)	0.65	1.03
Calcium (%)	0.30	0.64
Postassium (%)	2.11	1.24
Magnesium (%)	0.29	0.52
Sulfur (%)	0.42	0.86
Sodium (%)	-	0.70
Boron (%)	-	2.10
Copper (ug g^{-1})	23.00	5.80
Iron (ug g-1)	140.00	144.00
Manganese (ug g^{-1})	31.00	50.10
Molybdenum (ug g^{-1})	-	1.40
Selenium (ug^{-1})	0.10	1.12
Zinc (ug^{-1})	52.00	69.40
Vitamins (mg kg^{-1})		
Vitamin E (alpha-tocopherol)	2.40	14.50
Pantothenic acid	16.30	9.50
Niacin	28.00	160.00
Choline	2609.00	6700.00
Ribflaven	2.90	5.80
Biotin	0.32	1.07
Folic acid	0.60	2.30
Pyridoxine	6.00	7.20
Thiamin	6.00	5.20

1.3.2.1.1 Limitations Caused by Anti-nutritional Factors

Anti-nutritional factors contained in soybean meal can limit inclusion rates in young monogastic livestock (Chen 2013). Protease inhibitors, such as trypsin and chymotrypsin, account for approximately 6% of soybean protein (L'Hocine and Boye 2007). The presence of trypsin inhibitors in soybean meal are responsible for growth depression by reducing proteolysis and excessive fecal loss of pancreatic enzymes rich in sulfur-containing amino acids (Chen 2013). Trypsin inhibitors account for 30-50% of the growth inhibition effect (Denter et al. 1998). Soybean meal can be heat treated to inactivate trypsin inhibitors. However, soybean varieties that contain less trypsin inhibitors may provide a reduced feed cost due to the energy saved from the heat treatment (Goebel 2010).

The oligosaccharide composition in soybean meal is also a common anti-nutritional factor. Stachyose and raffinose represent of 4 to 6% dry matter in soybean meal (Goebel 2010). These oligosaccharides can cause gas production, diarrhea, and animal discomfort in non-ruminants (Rackis 1975). This is due to the inability of the small intestine to digest these oligosaccharides from the lack of the enzyme alpha-galactosidase (Goebel 2010).

1.3.2.1.2 Levels of Usage In Various Livestock Types

Globally, soybean meal accounts for nearly 69% of all protein sources while representing 92% of the total oilseeds used for animal feeds (Cromwell 2008). The low concentration of sulfur-containing amino acids (methionine and cysteine) and threonine limit the nutritional value of soybean meal in poultry and swine feeds (Medic et al. 2014). Approximately 48% of soybean meal is used in poultry feeds, 26% in swine feeds, 12% in beef cattle feeds, 9% in dairy feeds, 3% in fish feeds, and 2% in pet feeds (Cromwell 2008). Growing pigs may be fed inclusion rates up to 30% soybean meal, however weanling pigs should be restricted to less than 20% due to lower tolerance to oligosaccharides and antigens in the meal (Stein 2012). The average soybean meal inclusion rates range from 20-35% in starter and 15-28% in grower poultry feeds (Mitchell).

1.3.2.2 Canola Meal

Canola meal accounts for 60% of the canola seed (Newkirk et al. 2003). Oil extracted canola meal generally contains 35-36% protein, 12% crude fiber, and a high content of minerals and vitamins (Khattab and Arntfield 2009). As mentioned above, canola meal contains approximately 20% less protein and 75% more crude fiber than soybean meal, while gross energy is relatively the same (Table 1.11). The lysine content of canola meal protein is approximately 5.8%, which is also less than soybean meal (Cromwell 2008). Canola meal represented 12.4% of the world protein meal production in 2004/2005, at 207 million metric tons (Khattab and Arntfield 2009). Canola meal contains less digestible energy and protein than soybean meal, but over three times as much fiber (Bell 1993). Canola meal also contains glucosinolates, which were previously described as limiting inclusion levels in livestock feeds (Bell 1993). However, canola meal can be an economical protein source for animals that do not have high energy or lysine requirements (Ash 2012).

1.3.2.2.1 Limitations Caused by Anti-nutritional Factors

The main limitation of meals from *Brassica* spp. is the presence of glucosinolates (GLS), which are anti-nutritional and can even be toxic at high ingestion levels (Tripathi and Mishra 2007). When consumed, the breakdown products of GLS can cause deleterious effects on the thyroid, and ultimately cause goiters from iodine deficiency (Burel et al. 2001). Furthermore, large amounts of glucosinolates can reduce palatability for livestock and thus reduce intake and growth rates (Bonnardeaux 2007). For this reason canola was bred to contain lower levels of GLS and erucic acid (Newkirk 2009). Canola is characterized as containing less than 2% erucic acid in the oil and < 30 umol/g glucosinolates (Bonnardeaux 2007; Newkirk 2009). However, feed inclusion rates are still limited to ~30%, and this reduces the value of canola meal (Newkirk 2009). The relatively low digestible and metabolizable energy of canola meal are associated with the high level of fiber (Bell 1993).

1.3.2.2.2 Levels of Usage In Various Livestock Types

Globally, canola meal accounts for nearly 13% of all protein sources used in animal feeds (Cromwell 2008). Industrial rapeseed is high in erucic acid (>45%), which can be mildly toxic to animals, especially poultry (Bonnardeaux 2007). Erucic acid levels beyond 0.605% in diets can cause growth depression, reduce feed intake, and efficiency of growing chicks (Bonnardeaux 2007). Canola meal has the benefit over traditional rapeseed meal of being low in erucic acid (<2%) (Newkirk 2009).

There has been much interest in utilizing rapeseed meal to replace soybean meal in ruminant and monogastric feeds (Lomascolo et al. 2012). Canola meal is primarily fed in cattle and pig rations. The majority of canola meal in the U.S. is fed to dairy cattle because the high fat content enhances milk production (Ash 2012). There is currently no recommended maximum inclusion level for calves, beef, or dairy cattle due to the ability of the rumen breaking down the carbohydrate and fiber fractions in canola meal (Newkirk 2009). Supplementing canola meal from 7.5-22.5% in growing pig rations did decrease digestible and net energy because of higher fiber concentrations, but had no negative effects on growth performance (Montoya and Leterme 2010; Seneviratne et al.

2010). Increased dietary inclusions of canola meal with higher concentrations of residual oil could reduce pork quality (Seneviratne et al. 2010). Canola oil is rich in unsaturated fatty acids, which may soften the carcass fat (Rowghani et al. 2007). Thus expeller and cold pressed canola meal inclusion rates should be targeted to achieve targeted growth rates and market demands (Seneviratne et al. 2010).

Poultry and aquaculture feeds can also use canola meal as a protein source, but this is limited due to the high fiber content and low palatability of canola meal, and the distance between canola growing regions and feeding operations for these species (Ash 2012). De-hulling and extrusion processes improve both digestibility and nutritional value of canola meal fed to silver perch by removing anti-nutrients, such as glucosinolates, fiber, and phytic acid (Booth et al. 2001; Allan and Booth 2004). Breeding advances have reduced the glucosinolate content of canola meal. Therefore, the rations for broilers and laying hens can now contain 20% of canola meal without producing any adverse effects (Khajali and Slominski 2012). Ongoing breeding advances have been focused on crop disease and pest resistance.

1.3.3 Soil Amendment and Fertilizer

Biologically based treatments using organic-residue amendments can be used as an alternative to broad-spectrum biocides to manage soilborne plant pathogens (Mazzola et al. 2007). Members of the *Brassicaceae* family contain glucosionlates that are hydrolyzed to form compounds toxic to a variety of soil-borne organisms, including weeds (Haramoto and Gallandt 2007). Depending on glucosinolate levels, rapeseed meal has the potential for use as a bio-fumigant to replace chemical fumigants, such as methyl bromide, which are being banned due to environmental concerns (Bonnardeaux 2007).

Isothiocyanates, a glucosinolate hydrolysis product, has been identified as the bioactive compound responsible for suppressing weeds and plant pathogens (Mazzola et al. 2007; Hollister et al. 2013). It has been shown that isothiocyantes degrade rapidly (99% within 24-72 h); however, the amended soil retains the ability to suppress weeds and pathogens for longer periods of time, up to several weeks post-amendment (Mazzola et al. 2007). This may be due to altering the microbial community in the treated soil.

Soilborne disease pathogens and nematodes may be suppressed, however beneficial organisms, such as nitrifying bacteria, may also be eliminated during treatment with glucosinolates (Bonnardeaux 2007; Mazzola et al. 2007). Glucosinolate breakdown products may alter seed germination and plant growth, but these products have a generally short lifetime. Further field trials need to be completed to determine the long-term effects on the soil ecosystem when oilseed meals are applied as a bio-fumigant.

Canola and other rapeseed meals can also be used to provide soil with nitrogen and other nutrients needed for plant growth (Wang et al. 2012). Additionally, applying oilseed meals would increase the levels of easily decomposable carbon in the soil. However, this value only equates to $100 per ton (Bonnardeaux 2007), which is far below the value of the meals in feed applications (Bell 1993). Scale-up of non-food rapeseed varieties for biofuel applications may serve as an ideal source of meals for soil amendments (Wang et al. 2012).

1.4 Industrial Oilseed Meal Processing

The composition of oilseed meal can be manipulated by various industrial methods to produce higher value products. Traditional processing of oilseed protein has involved physico-chemical and thermal treatments to remove undesirable components and produce protein concentrates or isolates. These processes alter the nutritional value, anti-nutritional factors, and functional aspects of the final product (Moure et al. 2006). More recently, biological and enzymatic approaches have been used to process oilseed meals (Sindelar 2014).

1.4.1 Current Physical and Chemical Methods

Protein concentrates and isolates are “purified” by separating the carbohydrates from the proteins (Stein 2012). Consequently, both of these processes are able to dramatically increase the protein content of the treated meal. Protein concentrates and isolates are generally used in diets fed to weanling pigs because they do not elicit antigenic responses, as do non-processed protein sources (Stein 2012).

1.4.1.1 Protein Concentrate

Protein concentrates are becoming increasingly popular in specialty feeds such as pet food, milk replacers, creep feed, and aquaculture feed mainly as a replacement for fish meal, which is becoming increasingly expensive (Swick 2007). Protein concentrates are manufactured by removing non-proteic components, mainly soluble minerals, carbohydrates, low molecular weight nitrogen compounds, and anti-nutritional factors from full fat or defatted meals (Moure et al. 2006). The removal of carbohydrate components had shown improvements in both digestibility and nutritional value of oilseed meals (Booth et al. 2001; Allan and Booth 2004). In the traditional protein concentrate process, the meal is passed through an ethanol extractor, which removes some carbohydrates and anti-nutritional factors while concentrating protein and fiber (Swick 2007). The material is then neutralized (pH 6.5-7), dried, and milled to produce a product containing at least 65% on a dry basis (Lusas and Riaz 1995; Stein et al. 2008).

In 2006, MCN Bioproducts Inc. patented a mechanical process to concentrate and purify canola proteins (Newkirk et al. 2006). This process is similar to methods currently used to produce soy protein concentrate and isolate, and achieves a product with up to 80% protein by weight dry matter (Newkirk et al. 2009). Unfortunately, the multiple separation steps of this process are expensive and result in a low protein yield, since proteins also fractionate into lower value co-products. The result is an expensive product similar to soy protein isolate that is more suitable for use in human foods. In 2012 Bunge acquired MCN BioProducts to commercialize this technology for high value protein applications.

1.4.1.2 Protein Isolate

Protein isolates were initially created for use in extrusion, meal processing, baking, and baby food applications (Lusas and Riaz 1995). Protein isolate contains approximately 80% protein on a dry basis and is the most purified product in the processing industry (Sindelar 2014). Initially, the meal is finely milled. The protein is then solubilized at pH 6.8-10 at 27-66°C using sodium hydroxide and other alkaline agents approved for food use (Lusas and Riaz 1995). The protein solution is separated by centrifugation while the solids are sold as a byproduct (Lusas and Riaz 1995). The protein solution is then acidified to pH 4.5, using hydrochloric or phosphoric acid, and the protein is precipitated as a curd (Lusas and Riaz 1995). The curd is then concentrated by centrifugation and either neutralized (pH 6.5-7) or spray dried in its acidic form (Lusas and Riaz 1995).

1.4.2 Current Biological and Enzymatic Methods

Biological and enzymatic methods can alternatively concentrate protein by hydrolzing carbohydrates, phytic acid, and allergenic proteins, and making the protein fraction more digestible (Swick 2007). Additionally, hydrolysis can reduce peptide length of the protein fraction to increase digestibility (Swick 2007). Biological and enzymatic may also serve as a method to break down fiber fractions in animal feeds by utilizing naturally produced and industrial enzymes, respectively.

1.4.2.1 Biological Methods

There has been increasing emphasis on creating novel microbial approaches to convert cheap agro-residues into value-added animal feeds. Biological processing can be used to enhance oilseed meals by utilizing metabolic diversity of microorganisms to reduce carbohydrates, fiber, and anti-nutritional factors, while increasing protein levels. Fungal single-cell protein could also improve the amino acid profile of the resulting feeds. The protein content of bioprocessed products typically range from 55-65% and are lower than protein concentrate and isolate products (Swick 2007). However, bioprocessing eliminates the need for alcohol washing solvents, thus significantly reducing processing costs (Sindelar 2014).

Nutraferma (Dakota Dunes, SD) is a biotechnology company that utilizes a proprietary solid-state fermentation process to produce high-value proteins from soybean meal. Nutraferma's NF8**™** product is produced using *Pediococcus pentosaceus* and *Bacillus subtilis*. Another product, PepSoyGen**™**, is produced using *Aspergillus oryzae* and *Bacillus subtilis*. PepSoyGen**™** increases the protein content of soybean meal to ~59% on a dry basis and is low in dietary fiber (<3%) (Barnes et al. 2014). In addition, the microbial species in PepSoyGen™ remain viable in the final product, which can provide probiotic effects (Barnes et al. 2014). Preliminary studies have indicated that PepSoyGen™ could replace at least 60% of fish meal in Rainbow Trout diets with no decrease in rearing performance (Barnes et al. 2014).

Prairie AquaTech (Brookings, SD) was founded in 2012 as an animal health and nutrition company focused on the use of agricultural commodity products and by-products to produce higher value products via fungal bioprocessing. Prairie AquaTech has successfully developed high protein fish meal replacement products from other plant-based materials, including soybean meal and dried distillers grain. The product originating from soybean meal has been branded ME-PRO™ and is currently being scaled-up for commercialization. Prairie AquaTech's patent pending process has been able to increase the protein content of soybean meal to ~65% on a dry basis while depleting all anti-nutritional factors.

1.4.2.2 Enzymatic Methods

Enzymes occur in all living organisms and are used to catalyze bio-chemical reactions that are needed to support life. Many enzymes currently used in bioprocessing are derived from recombinant microorganisms to develop and manufacture enzymes with improved properties. This allows efficient production of enzymes that are free of other undesired enzymes or microbial metabolites.

Hamlet Protein A/S (Horsens, Denmark) has developed a patented process using enzymatic treatment of soybean meal using a proprietary blend of enzymes (Goebel 2010). In Hamlet's process, soybean meal is blended with water and enzymes to hydrolyze carbohydrate polymers into simple sugars. Enzymes are then inactivated, solids are recovered, dried, and milled (Goebel 2010). This novel bioconversion process reduces all natural anti-nutritional factors in soybean meal (antigens, trypsin inhibitors, oligosaccharides, and phytic acid) to a safe level for animal consumption. Hamlet's HP 300 product (56% crude protein) is ideal for use in swine, aquaculture, and pet feed. Swine feeding trials have reported higher crude protein and amino acid digestibility as well as improved average daily gain (Goebel 2010).

1.5 **Characteristics of *Brassica* Oilseed Meals**

A major factor that affects the composition of canola meal is the growing environment of the plant (Newkirk 2009; Barthet and Daun 2011). Variables such as soil moisture, temperature, and harvest time can affect the composition of the seed, and ultimately the meal (Barthet and Daun 2011). Canola produced in cool, wet weather results in seeds having a greater concentration of oil than when the crop is grown in hot and dry weather (Barthet and Daun 2011). The method of crushing (solvent extraction, hot expeller, or cold pressing) and the specific conditions used in the crushing process obviously affect the composition of the meal.

1.5.1 **Hexane Extraction vs Expeller vs Cold Pressing**

The goal of oilseed crushing is to maximize the oil recovery because the oil is the most profitable product. Table 1 .12 shows meal composition resulting from the three most common crushing methods. Hexane extraction is the most effective method of removing oil from oilseeds and produces a more consistent end product (Spragg and Mailer 2007). Hexane and expeller extraction processes apply significantly higher levels of heat compared to cold pressing, and therefore the level of heat damage to or thermal denaturation of proteins in these meals is higher (Spragg and Mailer 2007). However, the higher processing temperatures can help degrade or volatilize anti-nutritional factors (Newkirk et al. 2003; Allan and Booth 2004). A study by Newkirk et al. (2003) found that cold pressed canola meal contained higher levels of digestible amino acids than hexane extracted meal, but that the cold pressed meal also contained higher levels of anti-nutritional factors and residual oil.

Table 1.12: Nutrient content comparison of canola meal via common oil extraction methods (Beltranena 2014)

Component (%)	Solvent Extracted	Expeller	Cold Pressed
Moisture	10.5	9.6	12.7
Protein	38.1	31.4	25.8
Available Lysine	2.0	1.6	1.4
SID Lysine (g/kg)	15.0	13.2	12.0
Fat	2.7	10.5	20.2
Crude Fiber	7.4	8.3	5.8
NDF	27.4	18.8	15.3
ADF	19.8	15.0	11.5
Phosphorus	1.1	1.0	0.9

1.5.2 Oil Content

Hexane extracted canola meal generally contains less than 5% residual oil while expeller and cold pressed meals contain 10-15 and 11-20% residual oil, respectively (Leming and Lember 2005; Spragg and Mailer 2007). Higher residual oil content in canola meal provides a higher bulk density. Residual oil also has an effect upon the digestible energy content when fed to livestock. Assuming the nutritional content of the meal is adequate for the livestock species, a greater residual oil content can provide an economic benefit to producers (Beltranena 2014). The greater residual oil content in expeller and cold pressed canola meal co-products provide more dietary energy and less amino acids than those in solvent-extracted canola meal (Seneviratne et al. 2010). Higher concentrations of oil can provide the extra calories needed to increase weight gain and reduce days to market for nursery and grower pigs while increasing milk production and reduce body tissue loss for nursing sows (Beltranena 2014). The linoleic acid content within canola meal is a function of the residual oil content, with solvent extracted meals containing less linoleic acid relative to expeller and cold pressed meals (Spragg and Mailer 2007). High heat from solvent extraction and expeller processing can have a negative effect on the natural antioxidants in the oil (Nicoli et al. 1997), unlike cold pressing.

1.5.3 **Protein Content**

Canola seeds contain ~17-26% protein before oil extraction (Aider and Barbana 2011). The crude protein content of canola meal is positively correlated with the canola seed protein content and negatively correlated with the canola seed oil content (Spragg and Mailer 2007). The protein content of canola meal is also affected by the method of oilseed crushing, with meal from the hexane extraction process generally at ~38% protein, compared to ~31% from expeller processes and ~26% from cold pressing (Table 1.12). Overheating will cause the binding of amino acids with carbohydrates causing reduced digestibility of the amino acids, especially lysine (Cromwell 2008). Thus heating should be carefully monitored during crushing.

1.5.4 **Fiber Content**

The concentration of crude fiber, ADF, and NDF in canola meal ranges from 5.8-7.4%, 11.5-19.8%, and 15.3-27.4% in solvent extracted, expeller, and cold pressed canola meals, respectively (Table 1.12). While these values differ between various sources, the trends stay the same. The fiber content is generally similar for both solvent and expeller meals (Spragg and Mailer 2007) while cold pressed meals contain a lower fiber content due to the higher concentration of residual oil. Fiber including cellulose, pentosans, and lignin from cell walls is mainly present in the hulls of canola that remain in the meal after processing (Mailer et al. 2008; Newkirk 2009). Canola meal has relatively poor digestibility when compared to other food sources, such as soybean meal, due to its higher fiber content (Mailer et al. 2008). This reduces the feed value of canola meal, especially monogastrics (Bell 1993). Excessive fiber in monogastric diets may also lead to a decrease in feed utilization by obstructing digestive enzymes and diluting nutrient density (Booth et al. 2001). Thus lower fiber levels may provide an increase in digestible energy for monogastrics (Spragg and Mailer 2007). Breeding programs are underway to create higher protein varieties that contain less fiber.

1.5.5 Oligosaccharide Content

The concentration of soluble carbohydrates in mature canola seeds is approximately 10% on an oil-free dry weight, consisting of 3.9-9.8% sucrose, 0.3-2.6% raffinose, 0.8-1.6% stachyose, 0.1-0.5% fructose, and 0.1-0.4% glucose (Barthet and Daun 2011). The concentration of hemicellulose is approximately 3%, cellulose ranges from 4-5%, and starch is 1% (Maison 2013). Table 1.13 lists the major carbohydrates in canola meal (oil-free, dry matter). Differences in oil crushing and extraction procedures influence the concentration of carbohydrates in canola meal (Bell 1993; Newkirk et al. 2003). Extraction efficiency and moisture content during processing may influence the carbohydrate levels due to a concentration effect (Spragg and Mailer 2007). In extraction methods using high processing heat (solvent extraction and expeller), amino acids may form complexes with carbohydrates, rendering them unavailable for metabolism (Spragg and Mailer 2007). Spragg and Mailer (2007) had found similar carbohyrate levels for both solvent and expeller meals.

Table 1.13: Major carbohydrate components and component sugars of the non-starch polysaccharides in canola meal (Bell 1993)

Component	% (db)	Component	% (db)
Cellulose	4.9	Sugars from NSP	
Oligosaccharides	2.5	Rhamnose	0.2
Sucrose	7.7	Fucose	0.2
Starch	2.5	Arabinose	4.5
Non-starch polysaccharides (NSP)	17.9	Xylose	1.6
Soluble NSP	1.5	Mannose	0.4
Insoluble NSP	16.4	Galactose	1.7
		Glucose	5.0
		Uronic acid	4.3

1.5.5.1 Problems Caused

Oligosaccharides are a common anti-nutritional factor in canola meal. The raffinose family of oligosaccharides, including stachyose and verbascose, cause gas production, diarrhea, and discomfort in monogastrics, which can negatively affect livestock performance (Rackis 1975). Monogastrics lack the enzyme alpha-galactosidase (Goebel 2010), and therefore these oligosaccharides pass into the lower intestinal tract where they are fermented by anaerobic bacteria into hydrogen, carbon dioxide, and small amounts of methane (Rackis 1975). Supplementing dietary alpha-galactosidase can help alleviate these adverse effects and improve nutrient digestion. Ruminants are able to utilize these oligosaccharides due to rumen microbes.

1.5.6 Phytic Acid Content

Phytic acid is the main storage form of phosphorus in many cereal grains, legumes, and oilseeds (Pandey et al. 2001). Phytic acid chelates various metals and proteins, decreasing the bioavailability of proteins and nutritionally important minerals such as calcium, magnesium, phosphorus, zinc, and iron (Nair and Duvnjak 1991; Spier et al. 2008; Shivanna and Venkateswaran 2014). Utilization of bound phosphorus, therefore, requires hydrolysis of phytic acid by the enzyme phytase (Vig and Walia 2001, Spier et al. 2008). Several strains of bacteria, yeasts, and fungi have been used for phytase production, but *Aspergillus niger* and *Aspergillus ficuum* have most commonly been used for commercial production (Pandey et al. 2001). Some physical methods of reducing phytic acid levels include extrusion and dehulling (Booth et al. 2001; Allan and Booth 2004).

Approximately 85% of total phosphorus in canola and rapeseed products is present as phytic acid; therefore, the digestibility of phosphorus in these products is ~25-30% (Maison 2013). Although high in phytate, canola meal is also one of the richest sources of non-phytate phosphorus. Canola meal contains 0.38% non-phytate phosphorus, compared to 0.28, 0.23, 0.09, 0.26, 0.07, and 0.13% for soybean meal, cottonseed meal, wheat, wheat bran, corn, and barley, respectively (Khajali and Slominski 2012). Canola meal has an estimated bioavailability of 30-50% of the total phosphorus level (Newkirk 2009). The concentration of phosphorus is minimally affected by the process used for oil recovery, as solvent extraction had only showed an increase of 0.2% compared to cold pressing (Table 1.12).

1.5.6.1 Problems Caused

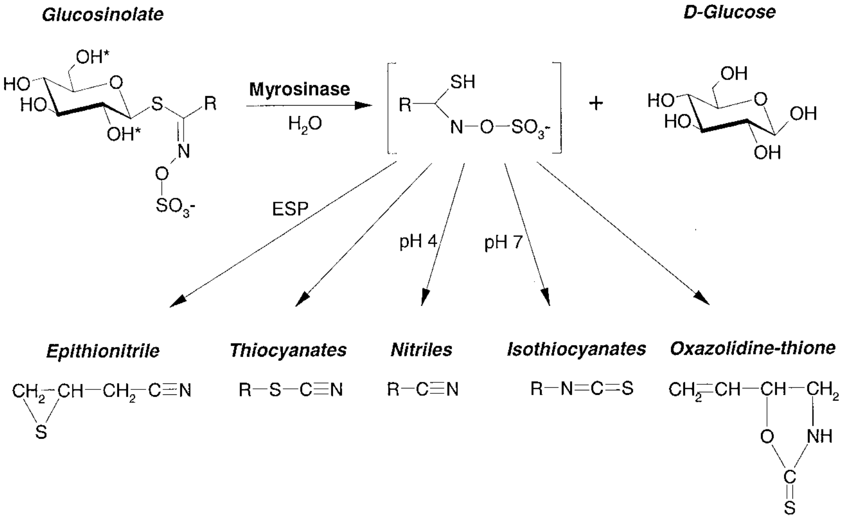
Monogastics lack the digestive enzyme phytase making them unable to metabolize phosphorus bound by phytic acid (Pandey et al. 2001; Spier et al. 2008). Furthermore, phytic acid influences protein and important mineral digestion of monogastric animals (Chen et al. 2013). The unabsorbed phytate passes through the gastrointestinal tract of monogastric animals, elevating phosphorus levels in manure (Chen et al. 2013). Environmental pollution due to high-phosphorus manure has intensified phytase research (Pandey et al. 2001).

Addition of microbial phytase into monogastric diets can increase phosphorus availability and enhance amino acid digestibility (Newkirk 2009). In contrast, the rumen microflora of ruminants produce phytase. Microbial bioprocessing of other feeds is another alternative reduce phytic acid levels (Nair and Duvnjak 1991; Spier et al. 2008). One study showed that *Aspergillus niger* and *Aspergillus ficuum* were capable of producing maximum levels of phytase of 60.6 units per gram of dry substrate (U/gds) and 38 U/gds, respectively, via submerged and solid-state fermentation (Shivanna and Venkateswaran 2014). Another study had showed a 95% reduction in phytic acid by *Rhizopus oligosporus* via solid-state fermentation (Nair and Duvnjak 1991).

1.5.7 Glucosinolate Content

Glucosinolates are a class of organic anions that can be hydrolyzed (non-enzymatically or enzymatically by the enzyme myrosinase) to produce multiple toxic compounds (Vaughn and Berhow 2005). GLS and the enzyme myrosinase are compartmentally stored separately in *Brassica* spp. (Rask et al. 2000). Upon disruption of plant tissues, myrosinase cleaves glucose from GLS, which in turn is converted into toxic compounds such as nitriles, thiocyanates, and isothiocyanates depending on pH (Fig. 1.5). This self-defense mechanism evolved to reduce animal and insect browsing of the plant (Halkier and Gershenzon 2006).

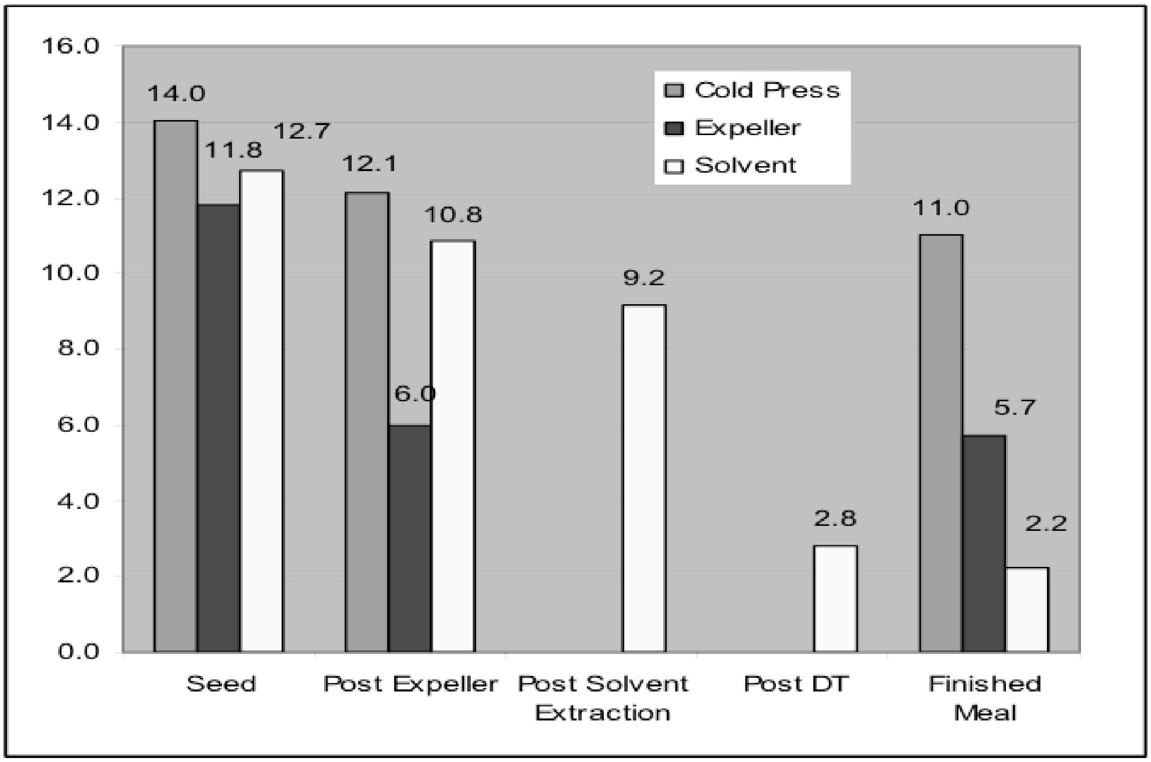
Figure 1.5: General structure of glucosinlates and enzymatic degradation products (Rask, et al., 2000)


Glucosinolate
D-Glucose
OH*
HO
HO
O
S
R
OH*
N
O
SO3-
Myrosinase
H2O
R
SH
N–O–SO3-
+
OH
HO
HO
O
OH
OH
ESP
pH 4
pH 7
Epithionitrile
Thiocyanates
Nitriles
Isothiocyanates
Oxazolidine-thione
CH2–CH–CH2–C≡N
S
R–S–C≡N
R–C≡N
R–N=C=S
CH2=CH–CH–CH2
O
NH
C
S

Canola was bred to contain low levels of erucic acid (<2%) in oil and glucosinolates (<30 μmol/g) in defatted meal (Newkirk 2009). There are approximately 120 different types of glucosinolates that share a similar chemical structure, but have varying R groups derived from one of eight amino acids (Halkier and Gershenzon 2006, Sonderby et al. 2010, Berhow et al. 2013). Glucosinolates can be divided into three groups according to the amino acid precursor: 1) aliphatic glucosinolates, derived from Ala, Leu, Ile, Val, and Met; 2) benzenic glucosinolates, derived from Phe or Tyr; and 3) indolic glucosinolates, derived from Trp (Sonderby et al. 2010).

Glucosinolates are heat labile, therefore the total glucosinolate content declines with increasing levels of processing heat (Spragg and Mailer 2007). Thus hexane extracted meals would have less glucosinolates than that of expeller and cold pressed meals (Fig. 1.6).

Figure 1.6: Glucosinolate content of seed, in-process, and finished canola meal (µmoles/g) (Spragg and Mailer 2007)



1.5.7.1 Problems Caused

Glucosinolates are anti-nutritional and can even be toxic at high ingestion levels (Tripathi and Mishra 2007). Table 1.14 presents the biological effects of glucosinolate consumed at various ranges by several livestock species. When consumed, the breakdown products of GLS can cause deleterious effects on the thyroid, and ultimately cause goiters from iodine deficiency (Burel et al. 2001). Furthermore, large amounts of glucosinolates can affect growth rates by making meal less palatable to livestock (Bonnardeaux 2007).

Table 1.14: Biological effects of glucosinolates on livestock (Tripathi and Mishra 2007)

Animal	Total Glucosinolate (μmol/g/diet)	Effect on animals
Pig	0.16-0.78	No adverse effect during growth, pregnacy and lactation
	1.3	Reduced gain during finishing period
	1.3-2.79	Reduced feed intake and growth
	2.2	No adverse effect during growth period
	7.0	Severe growth depression
	9.0-10.0	Induced iodine deficiency, hypothyroidism, reduced bone and serum zinc content and alkaline phosphatase activity
Poultry	0.9	No adverse effect on intake and growth
	2.3-8.18	No adverse effect on weight gain
	4.6	Reduced feed intake by 0.09%
	5.4-11.6	No adverse effect on intake and gain
	7.6-15.3	Severe growth depression
	34.0	Severe growth depression
Calves	1.2-2.4	No adverse effect on thyroid and liver function
Steers	10.0-15.0	No detrimental effect on growth and feed conversion
Cow	11.0	Induced iodine deficiency
	11.7-24.3	Depressed feed intake and milk production
	≥23.0	Reduced intake and milk production
	31.0	Thyroid disturbance and depressed fertility
Sheep	1.2-1.6	Reduced plasma levels of estradiol provoked reproductive disturbance
	1.2-2.2	Weight loss during lactation
	<4.22	No adverse effect on lamb performance
	≥4.22	Induced iodine deficiency and influenced thyroid weight and histology in lambs
	15.0	Reduced growth in lambs
	17.5	No effect on intake but increaed thyroid weight in lamb
	33.0	Growth depression in lamb
Fish	2.18	Reduced growth by 0.15 level
	19.3	Severe growth depression and thyroid disturbances

1.6 Proposed Bioprocessing of Oilseed Meals

Attempts to upgrade the nutritional value of canola meal have involved the reduction of glucosinolates and fiber by plant breeding, steam stripping, and solvent extraction/leaching. Unfortunately, all of these processes have major drawbacks such as loss of proteins, incomplete reduction in glucosinolate/fiber levels, high cost, and lack of commercial feasibility (Vig and Walia 2001). As an alternative to physical methods, researchers have shown that various microbes are able to degrade GLS and metabolize the resulting glucose and sulfur moieties. For example, Vig and Walia (2001) observed that *Rhizopus oligosporus* reduced GLS and their byproducts during fungal incubation. Similarly, Rakariyatham and Sakorn (2002) reported the complete degradation of GLS after 60-96 h using solid-state fermentation with *Aspergillus* sp.

Microbial bioprocessing has the potential to metabolize GSL and toxic breakdown products while converting oilseed meal fiber and carbohydrates into cell mass to improve the protein quality and availability. Fungi are widely used in the fermentation industry and are a principal source of extracellular enzymes (Wang et al. 2005) that may enhance the hydrolysis of the fiber fraction in oilseed meals. Higher protein levels and digestibility, along with reduced levels of GSL and fiber may enable higher inclusion levels in livestock rations.

1.6.1 Submerged Incubation

Submerged incubation has been defined as processing in the presence of excess water, and has been a proven large-scale process due to easier material handling and process control (Singhania et al. 2010). In contrast to solid-state incubation, submerged incubation has the advantage of being a more homogenous mixture while allowing improved streamlining and standardization of processing (Chicatto et al. 2014). Some microorganisms require high moisture content and flowability to increase aeration during bioprocessing. Gunashree and Venkateswaran (2008) It has also been shown that addition of surfactants may enhance fungal pelletization to increase extra cellular enzyme synthesis in submerged incubation processes (Gunashree and Venkateswaran 2008). Submerged incubation also has the potential to eliminate water-soluble anti-nutritional factors during the separation of process mash and water (Sindelar 2014).

1.6.2 Solid-state Incubation

Solid-state incubation has been defined as the processing of solid substrates that contain minimal water (Pandey et al. 2000). In these processes, microbial activity occurs in the thin water film on the surface of substrate particles. For many types of fungi, solid-state incubation more closely replicates the natural environment to which fungi are adapted (Couto and Sanromán 2006). Filamentous fungi can grow to significant extent in the absence of free water (Singhania, Sukumaran et al. 2010). Research has shown that fungal mycelia can effectively penetrate solid substrate agro-industrial residues (Ramachandran et al. 2004). The low water content of the solid-state environment enhances fungal cell adhesion, which is fundamentally related to growth on and within solid substrates (Singhania et al. 2010). In this process, the solid substrate not only supplies the fungi nutrients but also serves as an anchorage for microbial cells (Sathya et al. 2010). Solid-state conditions also limit bacterial contamination due to the reduced water activity (Pandey et al. 2000; Singhania et al. 2009). Lower drying costs and the ability to use smaller incubation vessels, compared to submerged incubation processing, can help minimize industrial processing costs (Smits et al. 1993). The main disadvantages of solid-state incubation are mass transfer and control of temperature, pH and moisture (Holker et al. 2004).

1.7 Fungal Strains

Several fungal strains were chosen based on their ability to produce single-cell protein and hydrolytic enzymes (Wang et al. 2005). Fungi are ideal organisms to use for production of single-cell protein, as the biomass contains all of the essential amino acids (Moore and Chiu 2001). The protein content of fungal single-cell protein typically ranges from 20-30% (Moore and Chiu 2001), but some species may reach up to 50% dry matter crude protein (Steen 2014). Many fungal species also have GRAS status in the food and food processing industry (Wang et al. 2005).

Fungi are able to produce a large variety of enzymes, most of which are only produced in small amounts and are involved in cellular processes (Andrade et al. 2002). Extracellular enzymes are usually capable of digesting insoluble nutrients such as cellulose, protein, and starch, and the digested parts are transported into the cell to be used as growth nutrients (Andrade et al. 2002). Extracellular enzymes produced by some of these fungal strains would benefit the incubation process by breaking down fiber into carbohydrates that can be easily metabolized by the fungus. A disadvantage to this process would be using the optimum pH or temperature of the fungal strain, rather than the optimum parameters of the enzymes. Thus the enzymes may or may not perform optimally at the parameters used for optimum cell growth.

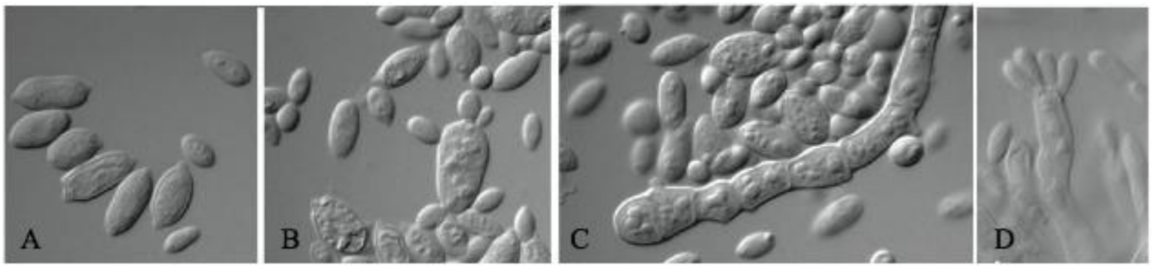
1.7.1 Yeast-like Fungi

1.7.1.1 *Aureobasidium pullulans*

Aureobasidium pullulans is a yeast-like fungus also known as black yeast. Strains of *A. pullulans* can grow in three distinctive forms (Fig. 1.7), which includes elongated branched septate filaments, large chlamydospores, and smaller elliptical yeast-like cells

(Chi et al. 2009). While traditional yeast cells can only produce one budding cell at a time, *A. pullulans* has the ability to produce multiple budding cells and filament chains (Zalar et al. 2008). Colonies begin to grow as yellow, cream, light pink, or light brown, but they become black at later stages of growth due to chlamydospore formation, as well as melanin production (Chi et al. 2009).

Figure 1.7: Cellular morphologies of *Aureobasidium pullulans* (Zalar et al. 2008)



A. Unicellular; B. Multiple buds; C. Filamentous; D. Multiple buds from filamentous

A. pullulans is utilized for the production of the biopolymer pullulan. Pullulan, an exopolysaccharide, is a linear homopolymer composed of maltotriose subunits interconnected with α-1,6 glucosidic linkages (Prasongsuk et al. 2007). The Hayashibara Company, Japan, first began commercial production of pullulan in 1976 (Leathers 2003). Pullulan can be used in various applications in the food manufacturing and pharmaceutical industry as a biodegradable coating, adhesive additive, flocculating agent, and environmental remediation agent (Chen et al. 2014).

Han et al. (1976) achieved a cell mass titer of 1.5 g/L with a crude protein content of 42.6% with ryegrass straw hydrolysate. West and Strohfus (2001) reached a maximum cell mass titer of ~20 g/L with a 5% sucrose solution under sterile conditions. Similarly, Singh et al. (2012) achieved a maximum cell mass titer of 21 g/L with basal media containing additional carbon and nitrogen sources under sterile conditions. ~~S~~everal strains of *A. pullulans* are also known to produce amylase, cellulase, xylanase, and single-cell protein (Leathers 2003; Kudanga and Mwenje 2005; Chi et al. 2009). Cellulases produced by *A. pullulans* have the potential to convert fiber fractions of agro-residues into single-cell protein, thus enhancing the nutritional value (Kudanga and Mwenje 2005). Enzymes and pullulan from *A. pullulans* are listed as GRAS products, ensuring this strain is safe for the food processing industry (Olempska-Beer et al. 2006; Prajapati et al. 2013)

1.7.1.2 ***Pichia kudriavzevii***

Pichia kudriavzevii is a unicellular yeast that is ubiquitous in nature, being found in the soil, fruits, and various fermented beverages (Eureka Brewing 2014). *P. kudriavzevii* is mainly associated with food spoilage, and causes surface biofilms in low pH products (Eureka Brewing 2014). *P. kudriavzevii* reproduction occurs like traditional yeast, asexually by multilateral budding. *Pichia* is telemorphic and can form hat shaped, hemispherical, round ascrospores, and cluster of two cells during reproduction. Unicellular yeasts, such as *P. kudriavzevii,* do not produce as much cell mass as filamentous fungi, which may affect the protein production. Revah-Moiseev and Carroad (1981) produced a cell mass with 45% crude protein with an enzymatic hydrolysate of shellfish chitin waste. Toivari et al. (2013) produced a cell mass titer of 15.3 g/L in sterile media containing yeast extract, peptone, glucose, and xylose to produce xylonate.

P. kudriavzevii has potential probiotic effects that may be beneficial to livestock meal bioprocessing (Ogunremi 2015). *P. kudriavzevii* also has the potential to produce phytase, an enzyme that hydrolyzes insoluble phytic acid to release soluble phosphorus. The soluble phosphorus would then be available for uptake in human and animal diets

(Chan et al. 2012). *P. kudriavzevii* has also shown excellent antibacterial activity against several pathogens, and can be utilized as a food preservative and biocontrol for fermentation industries (Bajaj et al. 2013).

1.7.2 Filamentous Fungi

1.7.2.1 *Fusarium venenatum*

F. venenatum is a filamentous fungus that was first isolated from a soil sample in the United Kingdom and was given the designation *F. graminearum* A3/5 (Olempska-Beer et al. 2006). This strain was subsequently reclassifed as *F. venenatum* based on its morphological, molecular, and mycotoxin data (Olempska-Beer et al. 2006). In 2001, the FDA reviewed a GRAS notice for the xylanse enzyme produced from *F. venenatum* containing the xylanse gene from *Thermomyces lanuginosus* (Olempska-Beer et al. 2006). The *F. venenatum* strain used for xylanase expression is a descendant of the wild-type strain A3/5 (Olempska-Beer et al. 2006).

F. venenatum mycelium has been utilized as a source of mycoprotein for human consumption in the United Kingdom under the trade name ‘Quorn’ since 1985 (Olempska-Beer et al. 2006). Twelve years of research was completed to ensure the safety of this organism (also a potential plant pathogen) before testing the product in the European market (Wiebe 2002). *F. venenatum* myco-protein contains approximately 44% protein on a dry basis (Wiebe 2002). To produce Quorn, glucose is provided as the carbon source and ammonium as the nitrogen source, both in levels of excess (Wiebe 2002). Both temperature (28-30°C) and pH (6.0) are controlled during this process and a specific growth rate of 0.17-0.20 h^{-1} and biomass titer ~15 g/L can be produced (Wiebe 2002). Quorn is one of the few successful fermentor-grown fungal foods on the market

(Moore and Chiu 2001). In 2001, the FDA reviewed a GRAS notice of *F. venenatum* production of mycoprotein and was accepted as safe for use in food as a meat replacer in the United States (Wiebe 2002; Olempska-Beer et al. 2006). Studies have shown that *F. venenatum* is capable of producing mycotoxins, however their production can be avoided by controlling fermentation conditions (Wiebe 2002). *F. venenatum* has been proven as an established and safe source of fungal protein, as long as mycotoxin production is avoided.

1.7.2.2 *Trichoderma reesei*

T. reesei is a filamentous fungus that belongs to a group of metabolically versatile aerobic mesophilic fungi (Nevalainen et al. 1994). *T. reesei* is common in soil in all climatic zones and are particularly prevalent in humid, mixed hardwood forests (Nevalainen et al. 1994). *T. reesei* was first isolated from the Solomon Islands during World War II (1944) because of its degradation of canvas and garments of the US army (Olempska-Beer et al. 2006). All strains of *T. reesei* used in industry were derived from the original isolate.

Ghanem (1992) produced a fungal cell mass with 49.3% crude protein using beet pulp as a substrate. He et al. (2014) produced a cell mass titer of 25.4 g/L from cane molasses medium. *T. reesei* fungal cell mass is also GRAS listed (Seiboth et al. 2011). Many *Trichoderma* species are well-known for their capacity to produce enzymes that hydrolyze cellulose and hemicellulose (Muthuvelayudham and Viruthagiri 2006). In 1999, the FDA confirmed the GRAS status of the cellulase enzyme from a non-pathogenic and non-toxicogenic strain of *T. reesei* (Olempska-Beer et al. 2006). Cellulases from *T. reesei* have been used safely in food, animal feed, and pharmaceuticals since the 1960s (Nevalainen et al. 1994). Major food applications of *Trichoderma* cellulases include baking, malting, and grain alcohol production (Olempska-Beer et al. 2006). Solid-state fermentation of agro-industrial residues using *T. reesei* has shown to be an economical method for cellulase production (Pandey et al. 1999; Xia and Cen 1999; Latifian et al. 2007). The combination of metabolic diversity and extracellular enzyme production would make *T. reesei* an ideal fungal strain to hydrolyze and convert oligosaccharide and fiber fractions into single-cell protein.

1.7.2.3 *Mucor circinelloides*

Mucor circinelloides is a dimorphic fungus that belongs to the Zygomycete class (Nordberg et al. 2014). Members of the genus *Mucor* occur typically as saprophytes in soil, but have been utilized in the food and fermented beverage industry (Andrade et al. 2002). This fungus has the capacity of accumulating high levels of lipids in its mycelium, has good biomass production during submerged batch cultivation in bioreactors, is able to use a wide range of carbon sources, and can grow in industrial stirred-tank fermentors (Nordberg et al. 2014). *Mucor* biomass could be an alternative feedstock in biodiesel production by utilizing microbial oil accumulated pellets formed during cell growth (Xia et al. 2011). *M. circinelloides* is a GRAS listed organism (Ratledge, 2013). There is currently no literature available on the protein content of *M. circinelloides.*

M. circinelloides has been determined to be a cellulolytic fungus and has the ability to convert various cellulose substrates to glucose. One study had found that *M. circinelloides* was able to produce a complete cellulase enzyme system, which includes endoglucanase, cellobiohydrolase, and β-glucosidase (Saha 2004). Another study observed protease enzyme production from several agro-industrial residues using *M.*

circinelloides (Sathya et al. 2010). Proteases produced by this fungi possess high milk-clotting activity and low proteolytic activity, making them ideal substitutes for the cheese industry (Andrade et al. 2002). *M. circinelloides* may benefit bioprocessing of agro-residues from cellulase production and increasing digestibility from proteolytic activity.

Chapter II - Introduction

The demand for food, and especially protein, is increasing along with the increase in the global human population (Steen 2014). By the year 2050, the global human population is expected to reach 9 billion (World Bank 2013), which will require a >70% increase in food production compared to today (FAO 2009). Fish are an important source of protein for a large percentage of the population (Steen 2014), and this trend is expected to continue. Fish and shellfish currently, represent 16% of all animal protein consumed on a global basis (World Bank 2013).

Due to increasing demand for fish and a finite supply of wild caught fish, the majority (70%) of marine fish stocks are exploited, over-exploited, or depleted (FAO 2014). This has led to rapid growth in aquaculture as a means to fill the gap between demand and wild caught fish resources. The worldwide aquaculture industry has sustained a 9% annual growth rate for the last three decades (FAO 2014). Aquaculture is also the most efficient means of increasing animal protein production due to the much higher feed conversion efficiency of fish compared to traditional livestock (Kaushik and Seiliez 2010). Fish have a feed conversion efficiency of 1:1 compared to poultry at 2:1, swine at 4:1, and cattle at 8:1 (National Research Council 2000; Brown et al. 2001; FAO 2006).

The rapid growth of aquaculture has resulted in an equally large increase in demand for fish meal, which is the primary protein source used in aquaculture diets (Olsen and Hasan 2012). Fish meal and other animal protein byproducts (blood meal, poultry meal, bone meal, etc) have traditionally been used in feed applications where highly concentrated and digestible proteins are needed (e.g. aquaculture, weaning diets, etc.) (Booth et al. 2001; Goebel 2010). Fish meal is derived from the waste stream of fish processing (whole fish, fish remains, or other fish by-products such as heads, tails, bones, and other offals) and from the harvest of small pelagic fish, particularly anchoveta species (FAO 2014).

Unfortunately, decades of over-harvest of marine species used to produce fish meal have capped annual global fish meal production. Fish meal production peaked in 1994 at 30.2 million tons while dropping to 16.3 million tons in 2012 (FAO 2014). Meanwhile, growth of aquaculture and other competing uses of fish meal have caused prices to exceed $2,000/ton, and this has adversely affected profitability (World Bank 2014). Thus, efforts are being made to investigate alternative protein and energy sources (Booth et al. 2001) to replace fish meal with more sustainable and economical plant-based proteins (Barnes et al. 2014).

The principal plant-based protein sources that have been investigated as fish meal replacements include soybean meal (Barnes et al. 2014), soy protein concentrates (Stein et al. 2008), corn distillers' grains (Boucher et al. 2009), and alternative oilseed meals (Allan and Booth 2004). The main challenges in plant-based protein sources include the presence of anti-nutritional factors (e.g., indigestible oligosaccharides and fibers, trypsin inhibitors, saponins, glucosinolates, etc), non-optimal amino acid profiles, and/or low protein digestibility (Sindelar 2014). These compositional issues have limited the amount of plant-based proteins that can replace fish meal to less than 60% (Barnes et al. 2014; Von Eschen 2014).

Single cell protein is another alternative protein source that could be used in applications where highly digestible protein is required. Fungi are ideal organisms to use for production of single-cell protein, as the biomass contains all of the essential amino acids (Moore and Chiu 2001). In addition, single cell protein can be produced from different residual streams derived from industry (Pandey et al. 1999). This provides the possibility to have inexpensive production from renewable and sustainable feedstocks. The protein content of fungal single-cell protein typically ranges from 20-30% (Moore and Chiu 2001), but some species may reach up to 50% dry matter crude protein (Steen 2014). Many fungal species also have generally recognized as safe (GRAS) status in the food and food processing industry (Wang et al. 2005).

In 2011, Gibbons and Brown (2012) combined the ideas of using filamentous fungi to metabolize the anti-nutritional factors in plant based proteins with the production of single celled protein to create a highly digestible, high protein feed to replace fish meal. Development work on this idea has primarily focused on enhancement of soybean meal and corn distillers' grains (Sindelar 2014; Von Eschen 2014). Microbial processed soybean meal has been successfully used to replace 100% of the fish meal in yellow perch diets (Gibbons and Brown 2012). The base product contains ~65% protein on a dry basis prior to diet formulation (Gibbons and Brown 2012). The process developed by Gibbons and Brown is being rapidly commercialized by Prairie AquaTech (Brookings, SD) under a NSF SBIR Phase II award.

Another feedstock that could be potentially improved using this technology is canola meal. Canola production has rapidly expanded over the past 40 years, rising from the sixth largest oilseed crop to the second largest (Ash 2012). On a worldwide basis, canola meal is second only to soybean meal for use as a feed (Newkirk 2009). Oil extracted canola meal generally contains 35-36% protein, 12% crude fiber, and a high content of minerals and vitamins (Khattab and Arntfield 2009). Unfortunately, the lysine content of canola meal protein is ~5.8%, which is also less than soybean meal (Cromwell 2008). Canola meal also contains less digestible energy and protein than soybean meal, but over three times as much fiber (Bell 1993).

The main limitation of meals from canola and other *Brassica* spp. is the presence of glucosinolates (GLS), which are anti-nutritional and can even be toxic at high ingestion levels (Tripathi and Mishra 2007). When consumed, the breakdown products of GLS can cause deleterious effects on the thyroid, and ultimately cause goiters from iodine deficiency (Burel et al. 2001). Furthermore, large amounts of GLS can reduce palatability for livestock and thus reduce intake and growth rates (Bonnardeaux 2007). For this reason canola was bred to contain lower levels of GLS and erucic acid (Newkirk 2009). Canola is characterized as containing less than 2% erucic acid in the oil and < 30 umol/g GLS (Bonnardeaux 2007, Newkirk 2009). However, feed inclusion rates are still limited to ~30%, and this reduces the value of canola meal (Newkirk 2009). Canola meal is typically sold at a 30% discount to soybean meal (47% protein), but only 90% of this differential can be explained by protein levels. The remaining discount is due to the 10-15% lower digestibility of canola meal and to the presence of GLS, which are toxic secondary metabolites found in almost all Brassicales.

To increase the nutritional value of canola meal, solid-state and submerged fungal incubation methods were used to screen the performance of seven metabolically diverse fungal strains. For many types of filamentous fungi, solid-state incubation more closely replicates the natural environment (absence of free water) to which fungi are adapted (Couto and Sanromán 2006). Lower drying costs and the ability to use smaller incubation vessels, compared to submerged incubation processing, can help minimize industrial processing costs (Smits et al. 1993). However, the main disadvantages of solid-state incubation are mass transfer and control of temperature, pH and moisture (Holker et al. 2004). Submerged incubation has been defined as processing in the presence of excess water, and has been a proven large-scale process due to easier material handling, process control, and improved standardization (Singhania et al. 2010; Chicatto et al. 2014). Solid-state incubation results are provided in Chapter III, while submerged incubation results are in Chapter IV. Based on this work, three fungal strains were down-selected and used to evaluate four pretreatment methods to enhance breakdown of the fiber fraction. Pretreatment methods included extrusion, hot water cook, dilute acid, and dilute alkali. Following pretreatment, submerged fungal incubation was used to evaluate effects on product composition. These results are provided in Chapter V.

Chapter III - Conversion of canola meal into a high protein feed additive via solid-state fungal incubation process

Abstract

The study goal was to determine the optimal fungal culture to reduce glucosinolates (GLS), fiber, and residual sugars while increasing the protein content and nutritional value of canola meal. Solid-state incubation conditions were used to enhance filamentous growth of the fungi. Flask trials were performed using 50% moisture content hexane extracted (HE) or cold pressed (CP) canola meal, with incubation for 168 h at 30ºC. On HE canola meal *Trichoderma reesei* (NRRL-3653) achieved the greatest increase in protein content (23%), while having the lowest residual levels of sugar (8% w/w) and GLS (0.4 μM/g). On CP canola meal *Trichoderma reesei* (NRRL-3653), *A. pullulans* (NRRL-58522), and *A. pullulans* (NRRL-Y-2311-1) resulted in the greatest improvement in protein content (22.9, 16.9 and 15.4%, respectively), while reducing total GLS content from 60.6 μM/g to 1.0, 3.2 and 10.7 μM/g, respectively. HE and CP canola meal GLS levels were reduced to 65.5 and 50.7% by thermal treatments while solid-state microbial conversion further reduced GLS up to 99 and 98%, respectively. Fiber levels increased due to the concentration effect of removing oligosaccharides and GLS.

3.1. Introduction

Canola (*Brassica napus*) is grown widely in Canada and the northern U.S. as a source of edible oil or for biodiesel/jet fuel production. The U.S. Department of Agriculture's National Agricultural Statistics Service estimated the 2014 US canola crop at 2.52 billion pounds harvested over 1.55 million acres (US Canola Association 2014). Following oil extraction, the remaining meal is used as a protein source for livestock. On a worldwide basis, canola meal is second only to soybean meal for use as a feed (Newkirk 2009). There has been much interest in utilizing rapeseed meal to replace soybean meal in ruminant and monogastric feeds (Lomascolo et al. 2012). However, a limitation of meals from *Brassica* spp. is the presence of GLS, which are anti-nutritional and can even be toxic at high ingestion levels (Tripathi and Mishra 2007).

GLS and the enzyme myrosinase are compartmentally stored separately in *Brassica* spp. (Rask et al. 2000). Upon disruption of plant tissues, myrosinase cleaves glucose from GLS, and the intermediate is then converted into toxic compounds such as nitriles, thiocyanates, and isothiocyanates, depending on pH (Kliebenstain et al. 2005). This self-defense mechanism evolved to reduce animal and insect browsing of the plant (Halkier and Gershenzon 2006). When consumed, a few of these toxic breakdown products can cause deleterious effects on the thyroid, and ultimately cause goiters from iodine deficiency (Burel et al. 2001). For this reason canola was bred to contain lower levels of GLS (Newkirk 2009). However, feed inclusion rates are still limited to ~30%, and this reduces the value of canola meal (Newkirk 2009). Canola meal would be more competitive in the marketplace if it had more digestible energy, more protein, and less GLS (Bell 1993).

One approach to improving the composition of canola meal is use of physical fractionation methods to remove anti-nutritional factors from the protein. In 2006, MCN Bioproducts Inc. patented a mechanical process to concentrate and purify canola proteins (Newkirk et al. 2006). This process is similar to methods currently used to produce soy protein concentrate and isolate, and achieves a product with up to 80% protein by weight dry matter (Newkirk et al. 2009). Unfortunately, the multiple separation steps of this process are expensive and result in a low protein yield, since proteins also fractionate into lower value co-products. The result is an expensive product similar to soy protein isolate that is more suitable for use in human foods. In 2012 Bunge acquired MCN BioProducts to commercialize this technology for high value protein applications.

To generate a less expensive canola protein concentrate, this work was completed to take advantage of the metabolic diversity of fungi to convert canola fiber and carbohydrates into protein-rich cell biomass, while simultaneously degrading GLS and the breakdown products. We hypothesized that this process would generate a more digestible product with enhanced nutritional value to a range of aquaculture and other livestock species. Based on their ability to produce cellulose degrading enzymes, filamentous fungi selected for initial evaluation included *Trichoderma reesei*, *Fusarium venenatum, Mucor circinelloides,* and *Aureobasidium pullulans. T.reesei* (Seiboth et al. 2011), *F. venenatum* (Wiebe 2002), and *Mucor circinelloides* (Ratledge 2013) are listed GRAS strains, while enzymes and pullulan from *A. pullulans* are GRAS products (Olempska-Beer 2006; Prajapathi et al. 2013). The yeast *Pichia kudriavzevii* was also included due to its potential probiotic effects (Ogunremi et al. 2015). Since the cold press and hexane extraction are the two commercialized methods of oil extraction, meals from both processes were evaluated.

A single-step, solid-state incubation process was used in this research because it replicates the natural environment to which fungi are adapted (Pandey et al. 2000; Couto and Sanromán 2006). Research has shown that fungal mycelia can effectively penetrate solid substrate agro-industrial residues (Ramachandran et al. 2014). Solid-state conditions also limit bacterial contamination due to the reduced water activity (Pandey et al. 2000;

Singhania et al. 2009). Lower drying costs and the ability to use smaller incubation vessels, compared to submerged incubation processing, can help minimize industrial processing costs (Smits et al. 1993). The main disadvantages of solid-state incubation are mass transfer and control of temperature, pH and moisture (Holker et al. 2004).

3.2. Materials and Methods

3.2.1. Feedstocks and Preparation

HE canola meal was obtained from North Dakota State University (Fargo, ND, USA), while CP canola meal was obtained from Agrisoma Biosciences (Ottawa, Ontario, Canada). Both HE and CP meals were milled through a 2 mm screen via knife mill prior to use, and were stored at room temperature in sealed buckets throughout the duration of experimentation. Dry weight (dw) analysis was conducted by drying ~5 grams of canola meal at 80ºC in a drying oven for at least 48 h. Proximate analysis was conducted following AOAC protocols by SGS (Brookings, SD, USA) and Table 1 provides the composition of each feedstock.

3.2.2. Cultures, Maintenance, and Inoculum Preparation

Aureobasidium pullulans (NRRL-58522), *A. pullulans* (NRRL-42023), *A. pullulans* (NRRL-Y-2311-1), *Trichoderma reesei* (NRRL-3653) and *Fusarium venenatum* (NRRL-26139) were obtained from the National Center for Agricultural Utilization Research (Peoria, IL). *Pichia kudriavzevii* and *Mucor circinelloides* were isolated as contaminates from prior trials, and were identified by ARS-USDA (Peoria, IL, USA) using 16s RNA analysis from methods developed by O’Donnell (2000). Short-term maintenance cultures were stored on Potato Dextrose Agar (PDA) plates and slants at 4 degrees Celsius (ºC). Lyophilization was used for long-term storage. Inocula for all experiments were prepared by transferring isolated colonies or a square section of agar growth (filamentous fungi) into glucose yeast extract (GYE) medium consisting of 5% glucose and 0.5% yeast extract. The flasks used for inocula growth consisted of 100 milliliter (ml) GYE working volume in 250 ml Erlenmeyer flasks, covered with a foam plug and aluminum foil. Cultures were incubated for ~72 hours (h) at 30ºC in a rotary shaker at 150 revolutions per minute (rpm).

3.2.3. Solid State Trials

Solid-state trials were conducted in 500 ml Erlenmeyer flasks with 100 g of 50% moisture content canola meal (hexane extracted vs cold pressed). The pH of individual flask contents was adjusted with 10 N sulfuric acid to the optimum pH for each organism. The pH for *Aureobasidium*, *Pichia*, and *Mucor* cultures was adjusted to 3 with 10N sulfuric acid, while pH 5-5.5 was used for *Trichoderma* and *Fusarium*. Flasks were covered with foam plugs and aluminum foil and were then autoclaved at 121ºC for 20 min. Flasks were inoculated with 10 ml of 72 h inoculum cultures, then incubated statically at 30ºC for 168 h. Visual subjective rating of colonization percentages on surfaces were conducted daily. Following incubation the solids were recovered, the pH was measured, and the solids were then dried and analyzed for carbohydrates, protein, fiber, and GLS.

3.2.4. Analytical Methods

3.2.4.1. Residual Sugars

After 168 h incubation, the pH of each sample was measured (Oakton pH Spear). The solids were then dried for 2 d at 80ºC. One g of each dried sample was removed and mixed with 9 ml DIH$_2$O, then allowed to solubilize at 4ºC overnight. This solution was then centrifuged at 10,000 rpm for 10 min and the supernatant was then poured into a 2 ml microcentrifuge tube and frozen overnight. After thawing, the supernatant was centrifuged a second time at 10,000 rpm for 10 min to remove any precipitants, and this supernatant was then filtered through a 0.2 μm filter and into a HPLC vial. A Waters size-exclusion chromatography column (SugarPak column I with pre-column module, Waters Corporation, Milford, MA, USA) and high performance liquid chromatography system (Agilent Technologies, Santa Calara, CA, USA) equipped with refractive index detector (Model G1362A) were used to measure the sugars. The sugars were eluted using a de-ionized water as mobile phase at flow rate of 0.5ml/min and column temperature of 80ºC. Sugars quantified included arabinose, galactose, glucose, raffinose, stachyose, and sucrose.

3.2.4.2. Total protein

Approximately 5 g of sample was used for protein and GLS analysis. Protein was quantified using a LECO model FP528 (St. Joseph, MI, USA) to combust the sample and to measure the total nitrogen gas content in the sample. Protein percentage was then calculated from the nitrogen content of the sample using a conversion factor of 6.25.

3.2.4.3. Glucosinolates

Individual GLS (gluconapin, glucotropealin, and sinigrin) were confirmed to be present by quadrupole time-of-flight (q-tof) liquid chromatography-mass spectrometry (LC-MS) and quantified using reverse phase high performance liquid chromatography (RP-HPLC) (Berhow et al. 2013). For GLS quantification, a modification of a high-performance liquid chromatography (HPLC) method developed by Betz and Fox (1994) was used. 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 LCsolutions Version 1.25 software. The column a C_{18} Inertsil reverse phase column (250 mm X 4.6 mm; RP C-18, ODS-3, 5u; with a Metaguard guard column; Varian, Torrance, CA). The GLS were detected by monitoring at 237 nm. Initial mobile phase conditions were 12% methanol/88% aqueous 0.005M tetrabutylammonium bisulfate (TBS) at a flow rate of 1 ml/min. After injection of 15 ul of sample, the initial conditions were held for 2 min, and then up to 35% methanol over another 20 minutes, then to 50% methanol over another 20 minutes then up to 100% methanol over another 10 minutes.

3.2.4.4. Fiber

Fiber analysis was completed as Neutral Detergent Fiber (NDF) and Acid Detergent fiber (ADF). NDF is a method commonly used for animal feed analysis to determine the amount of lignin, hemicellulose and cellulose while ADF represents the least digestible fiber fraction of animal feed including lignin, cellulose, silica but not hemicellulose. NDF and ADF analysis were completed by Midwest Laboratories (Omaha, NE, USA) using ANKOM Technology (Macedon, NY, USA) filter bag methods.

3.3. Results and Discussion

Seven fungal strains were grown on HE vs CP canola meal using a solid state incubation process. These trials were done in shake flasks, where mixing and mass transfer are limiting factors. However, these non-optimized trials were simply meant to quickly down-select for the best microbe for each type of canola meal. Other investigators have previously used a similar solid-state incubation process to quickly assess phytase activity of various strains of bacteria, yeasts and fungi when grown on canola and other rapeseed meals (Saha 2004; Bhargav et al. 2008).

3.3.1. Compositional analysis

As listed in Table 3.1, the composition of HE and CP meals were different in terms of the fat and fiber content. We were concerned that GLS in both feedstocks (42.8-60.6 μM/g), or the higher oil content of CP canola meal (Table 3.1), might be inhibitory. It is known that GLS can inhibit some types of microbes (Bogar et al. 2003), although we anticipated that the fungi tested herein could actually metabolize these compounds. We also postulated that canola oil might be inhibitory, as it is known that high oil concentrations can reduce microbial growth (Bock et al. 2007; Bednarek et al. 2009). Cold pressing typically remove only 75-85% of canola seed oil, while solvent extraction removes 96%+ (Haron et al. 2013).

Table 3.1 Proximate analysis of hexane extracted and cold pressed canola meal before and after processing with *A. pullulans* (Y-2311-1)[1]

	Hexane Extracted		**Cold Pressed**	
Analysis (% w/w, db)[2]	Raw	Processed	Raw	Processed
Dry Matter	93.5	96.1	93.7	96.9
Crude Ash	7.5	8.7	6.8	7.2
Crude Fat	5.4	5.7	17.1	18.6
Crude Fiber	12.4	13.5	7.3	7.9
Crude Protein	36.1	41.3	38.6	44.6

[1] The data in Table 1 represents composite samples of three replicates. This was necessary to have sufficient material for the proximate analysis.

[2] Proximate analysis was completed as a percentage weight per weight (% w/w) on a dry basis (db).

3.3.2. Fungal growth rates

When the microbial strains were cultivated in those meals, a different trend of colonization was observed. Figures 3.1a and 3.1b show the percent surface colonization for each strain during incubation of HE and CP canola meal, respectively. While these visual ratings were subjective, they do provide an indication of relative growth rates on the two feedstocks. Figure 1a shows that *A. pullulans* (NRRL-Y-2311-1) and *F. venenatum* grew the most rapidly on HE canola meal, achieving 100% colonization in 72 h. *P. kudriavzevii* grew the slowest, only achieving 20% colonization in 168 h. Figure 1b shows that *A. pullulans* (NRRL-Y-2311-1), *F. venenatum*, and *T. reesei* grew the most rapidly on CP canola meal, achieving 100% colonization in 72 h. *P. kudriavzevii* again grew the slowest, only achieving 30% colonization in 168 h. Therefore the higher oil content of CP canola meal did not affect growth of *A. pullulans* (NRRL-Y-2311-1) and *F. venenatum*, and actually stimulated growth of *T. reesei*. The extra oil also improved the growth of *A. pullulans* (NRRL-42023) and *M. circinelloides*, perhaps by providing an additional carbon and energy source (Spragg and Mailer 2007). The slower colonization rate and extent of *P. kudriavzevii* on both feedstocks was expected, as it is a single celled yeast that does not grow in a filamentous morphology. It was included in these trials because it frequently occurs as a contaminant in larger scale trials where we are processing plant-based protein meals in submerged conditions. In broth culture *P. kudriavzevii* exhibits a much faster growth rate of >0.30 h^{-1} compared to the filamentous fungi (Salihu et al. 2012).

Figure 3.1a Surface colonization of various fungi ± SD on hexane extracted canola meal

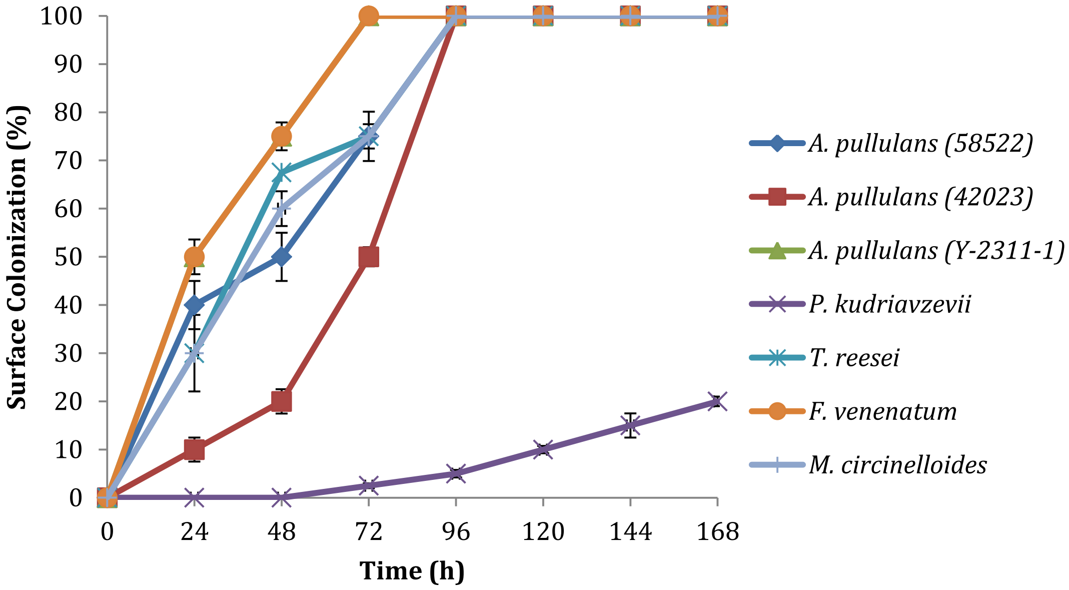
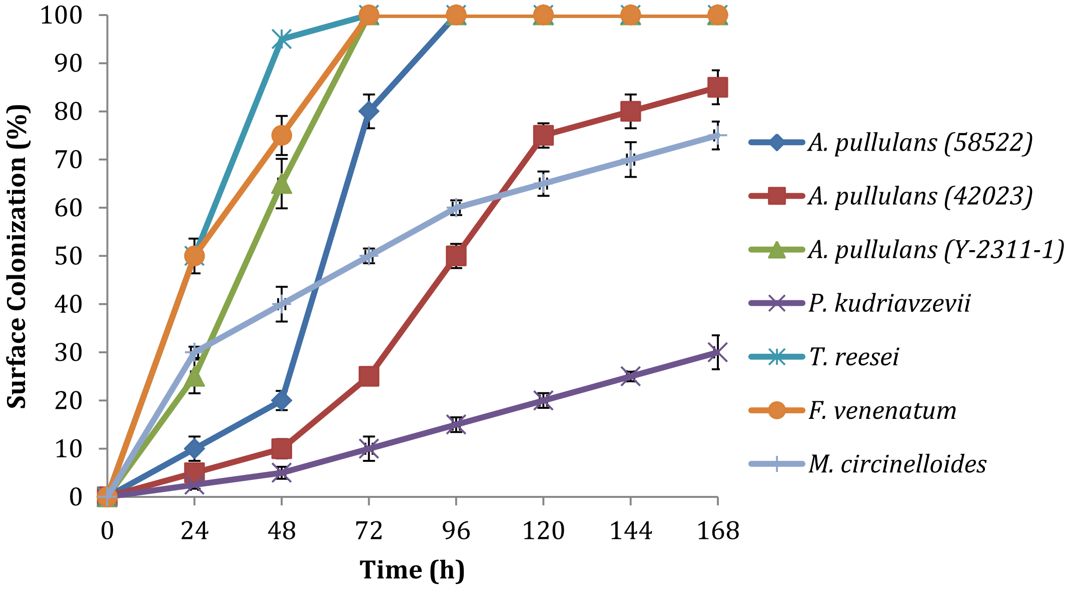


Figure 3.1b Surface colonization of various fungi ± SD on cold pressed canola meal



3.3.3. pH change over fungal incubation

Table 3.2 shows the initial versus final pH levels for these trials. The pH in the un-inoculated controls stayed stable throughout 168 h incubation, and the pH in trials with *A. pullulans* (NRRL-58522), *A. pullulans* (NRRL-42023), and *P. kudriavzevii* increased by less than 1 pH unit. The pH increased from 3.0 to 3.9-4.9 in the *M. circincelloides* trials, however studies have shown this fungus has a broad pH range of 3.0-8.0 (Toivari et al. 2013). In trials with *A. pullulans* (NRRL-Y-2311-1), *T. reesei*, and *F. venenatum*, the pH also rose, in some cases to slightly above the optimal range. Nevertheless, these strains still exhibited the most rapid colonization rates in both HE and CP canola meals.

In most cases the pH rose to a higher level in HE canola meal compared to CP meal. Due to the fact that these solid-state trials were not mixed or sampled until the end of incubation, it was not possible to adjust pH during incubation. However it is possible that the increased pH might have affected fungal metabolism, and hence protein production. Therefore, subsequent optimization studies that will be conducted in a paddle-type reactor will include pH control.

Table 3.2 Initial versus final pH ± SD of hexane extracted and cold pressed canola meal

Fungal Culture	Optimal pH Range	Initial pH HE Canola	Final pH HE Canola	Initial pH CP Canola	Final pH CP Canola
Control	--	3.2±0.0	3.2±0.0	2.9±0.1	3.0±0.1
***A. pullulans* (NRRL-58522)**	3.0-5.0[1]	3.0±0.0	3.8±0.5	3.1±0.1	3.9±0.4
***A. pullulans* (NRRL-42023)**	3.0-5.0[1]	3.1±0.0	3.7±0.1	3.0±0.0	3.0±0.4
***A. pullulans* (NRRL-Y-2311-1)**	3.0-5.0[1]	3.2±0.2	6.2±0.4	3.1±0.1	4.9±0.6
P. kudriavzevii	3.0[2]	3.0±0.0	3.7±0.3	3.0±0.1	3.1±0.2
***T. reesei* (NRRL-3653)**	4.0-6.0[3]	5.0±0.0	7.7±0.1	5.0±0.1	7.1±0.3
***F. venenatum* (NRRL- 26139)**	4.5-6.0[4]	5.1±0.3	7.4±0.4	5.1±0.1	6.2±0.7
M. circinelloides	3.0-6.0[5]	3.1±0.1	4.9±0.7	3.1±0.1	3.9±0.8

[1] (Chen et al. 2014)
[2] (Toivari et al. 2013)
[3] (Li et al. 2013)
[4] (Gordon et al. 2000)
[5] (Saha 2004)

3.3.4. Residual sugars

Residual sugars represent the combined levels of arabinose, galactose, glucose, raffinose, stachyose, and sucrose. Between 50-95% of sugars present in the HE and CP meals were utilized by the fungi during incubation, resulting in residual sugar levels of 0.9-8.4 % w/w. *A. pullulans* (NRRL-Y-2311-1) and *F. venenatum* exhibited the lowest residual sugar levels on both substrates, while *M. circincelloides* and *T. reesei* had the highest final levels of residual sugars. In the case of *T. reesei*, the higher than optimal final pH levels might explain why sugars were not more completely consumed (Li et al.

2013). As we have shown with our previous work with soybean meal (Brown and Gibbons 2014), optimization of process conditions (such as mixing and nitrogen supplementation) will substantially increase protein levels and further reduce residual sugars. Other studies have also noted that nitrogen supplementation boosts conversion of sugars into cell mass, thereby increasing protein and reducing residual sugar levels (Bertolin et al. 2003; Membrillo et al. 2008).

3.3.5. Total protein

Figures 3.2a and 3.2b present the maximum protein levels and residual sugar levels in HE and CP canola meals, respectively, for the un-inoculated control versus the various fungi. Protein levels increased from 36.1% in hexane extracted meal to 39.7-44.4% after solid-state microbial conversion, representing relative improvements of ~10-23%. Protein levels increased from 38.6% in CP meal to 42.2-47.5% after solid-state microbial conversion (relative improvements of ~9-23%). *T. reesei* achieved the highest protein levels for both substrates, while *P. kudriavzevii* exhibited the lowest protein enhancement of all strains. *T. reesei* is known to produce many hydrolytic enzymes (Li et al. 2013), and was expected to provide the greatest conversion of fiber and oligosaccharides into cell mass. As a single-celled yeast, *P. kudriavzevii* does not produce cellulase enzymes and was therefore anticipated to result in the lowest protein improvement. The final protein levels for all other fungal strains were relatively similar, at 40-41% in HE canola meal and 43-45% protein in CP canola meal.

Figure 3.2a Maximal protein and residual sugar levels ± SD of hexane extracted canola meal

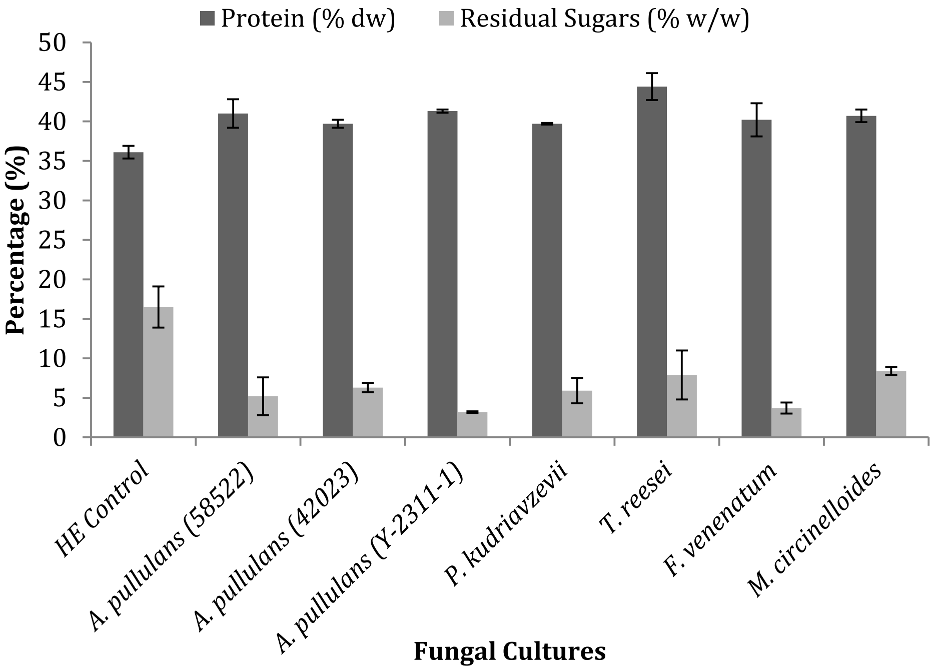
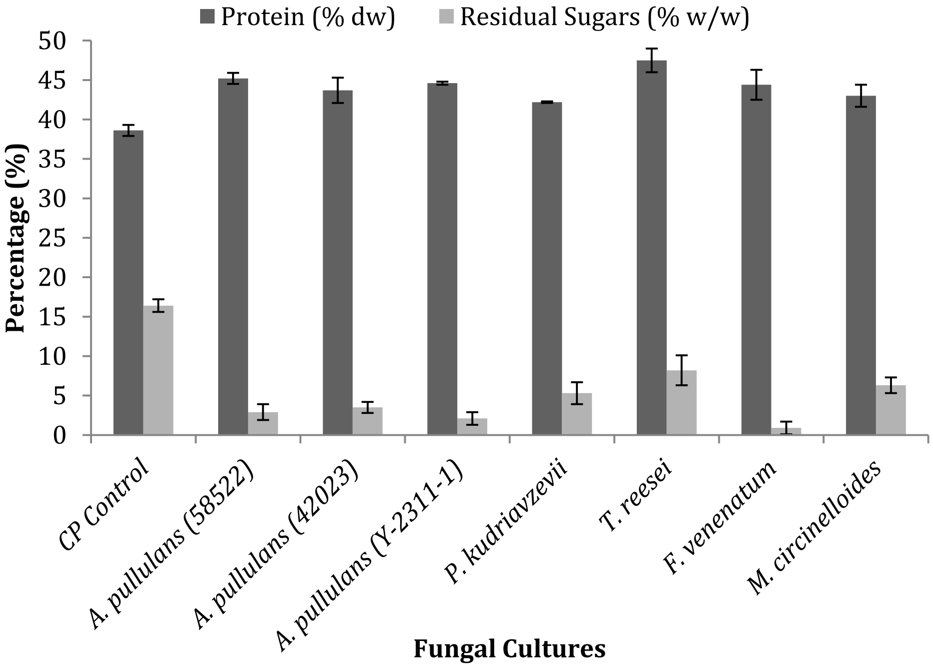


Figure 3.2b Maximal protein and residual sugar levels ± SD of cold pressed canola meal



3.3.6. Glucosinolates

Table 3.3 lists GLS and fiber (ADF and NDF) levels for the raw HE and CP canola meals, and for the meals following incubation (un-inoculated control vs the various fungal cultures). GLS levels were reduced from 42.8 μM/g in raw HE meal to 14.8 μM/g after the thermal treatments in the control (autoclave sterilization and final drying), representing a 65.5% reduction. This was presumed due to the conversion of some of the GLS to volatile breakdown products (Halkier and Gershenzon 2006). Solid-state microbial conversion further reduced GLS content to 0.4-13.3 μM/g, representing a total reduction of 69-99%. Similarly, GLS levels in raw CP meal were reduced from 60.6 μM/g to 29.9 μM/g due to the thermal steps of the conversion process (reduction of 50.7%). Again, solid-state microbial conversion further reduced GLS content to 1.0-28.73 μM/g (total reduction of 53-98%). In future trials with the best fungi we will seek to quantify GLS breakdown products.

T. reesei (NRRL-3653) exhibited the greatest reduction in GLS levels, likely due to its robust capability for producing extracellular enzymes (Li et al. 2013). Several of the *A. pullulans* strains were next most effective. Previous studies have shown that various microbes, including *Aspergillus* sp. and *Rhizopus oligosporus*, are able to degrade GLS and metabolize the resulting glucose and sulfur moieties (Rakariyatham and Sakorn 2002; Vig and Walia 2001). The complete degradation of GLS was achieved after 60-96 h using solid-state fermentation with *Aspergillus* sp. (Rakariyatham and Sakorn 2002). As expected due to its minimal production of extracellular hydrolytic enzymes, *P. kudriavzevii* resulted in the least reduction in GLS. There was a large standard deviation in GLS levels for CP canola processed with *A. pullulans* (NRRL-42023). In some instances, the quantification of total GLS can be problematic using HPLC chromatography due to low baselines levels. This gives a much higher standard deviation value for the replicates as the numbers can vary at these low levels.

3.3.7. Fiber

Fiber levels (Table 3.3) actually increased during the fungal incubation process as a result of the "concentration effect" as sugars and GLS were metabolized, along with the apparent lack of any substantial fiber hydrolysis due to cellulase activity. We have previously shown that feedstock pretreatment increases the susceptibility of fibers to hydrolysis (Karki et al. 2013), and that optimizing the fungal incubation conditions will also enhance cellulase production and activity. This will be evaluated in future studies. The resulting sugars would then be available for conversion into additional cell mass and protein.

Table 3.3 Reduction of total glucosinolates and fiber ± SD during solid-state fungal incubation

	Glucosinolates				Fiber			
	Hexane Extracted		Cold Pressed		Hexane Extracted		Cold Pressed	
Fungal Culture	Total GLS (µM/g)	Reduction (%)[1]	Total GLS (µM/g)	Reduction (%)[1]	ADF (%)	NDF (%)	ADF (%)	NDF (%)
Raw Meal	42.8±1.3	--	60.6±2.5	--	19.9±0.2	23.1±0.3	11.5±0.5	15.0±0.3
Process Control	14.8±1.2	65.5±2.7	29.9±0.8	50.7±1.3	17.2±0.5	20.4±0.6	9.7±0.4	11.9±0.6
***A. pullulans* (NRRL-58522)**	4.5±1.0	89.4±2.4	3.2±2.5	94.8±4.1	17.7±1.3	21.3±0.7	10.2±1.0	12.9±0.2
***A. pullulans* (NRRL-42023)**	2.6±1.5	94.0±3.5	15.1±14.1	83.4±21.9	18.0±0.6	20.7±0.7	10.5±0.8	14.1±0.9
***A. pullulans* (NRRL-Y-2311-1)**	3.9±1.0	90.9±2.2	10.7±2.7	82.4±4.4	22.4±0.7	25.8±0.7	12.4±0.7	15.4±0.2
P. kudriavzevii	13.3±1.1	68.9±2.5	28.7±0.8	52.6±1.3	18.7±0.7	22.3±0.6	10.6±0.1	12.6±0.5
***T. reesei* (NRRL-3653)**	0.4±0.0	99.1±0.1	1.0±0.2	98.3±0.3	19.1±0.4	23.6±0.8	11.0±0.9	13.5±0.7
***F. venenatum* (NRRL- 26139)**	5.7±1.6	86.7±3.8	10.9±4.0	82.1±6.6	22.5±0.7	28.9±0.5	11.6±1.2	14.6±1.2
M. circinelloides	10.4±1.0	75.8±2.2	25.4±2.7	58.1±4.4	20.1±0.8	23.4±0.5	11.1±0.8	13.3±0.7

[1] GLS reduction from raw canola meal

3.3.8. Protein Yield

Table 3.4 provides a summary of the dry matter yield and total amount of protein achieved in these trials. While the composition (protein, GLS and fiber) of the microbially converted canola meal is important, the product yield (especially protein) is also important. Based on dry matter yield and protein concentration, one can calculate the total protein content in the product, and on this basis, *T. reesei* performed the best for both HE and CP canola meal. *M. circinelloides* was the second best, while *P. kudriavzevii* was next, due to the high yield. Several of the filamentous fungi that produced relatively high protein levels actually yielded less total protein due to their reduced dry matter yields.

Table 3.4 Dry matter yield and protein ± SD from solid-state incubation of canola meal

Fungal Culture	**Hexane Extracted**			**Cold Pressed**		
	Dry Matter Yield (%)	Protein (%, dw)	Total Protein (g)	Dry Matter Yield (%)	Protein (%, dw)	Total Protein (g)
Control	100.3±0.3	36.1±0.8	18.1±0.1	100.7±0.4	38.6±0.7	19.4±0.1
***A. pullulans* (NRRL-58522)**	95.2±3.2	41.0±1.8	19.2±0.4	92.0±1.2	45.2±0.7	20.7±0.3
***A. pullulans* (NRRL-42023)**	97.3±1.3	39.7±0.5	19.2±0.1	88.5±3.4	43.7±1.6	19.3±0.7
***A. pullulans* (NRRL-Y-2311-1)**	91.0±1.2	41.3±0.2	18.8±0.3	90.6±1.6	44.6±0.2	20.3±0.4
P. kudriavzevii	100.0±1.2	39.7±0.1	19.9±0.2	98.0±1.4	42.2±0.1	20.5±0.0
***T. reesei* (NRRL-3653)**	91.9±1.8	44.4±1.7	20.6±0.0	91.2±1.7	47.5±1.5	21.5±0.4
***F. venenatum* (NRRL- 26139)**	92.5±5.0	40.2±2.1	18.3±1.2	91.5±3.1	44.4±1.9	20.1±0.8
M. circinelloides	96.9±1.0	40.7±0.8	19.7±0.0	97.4±1.0	43.0±1.4	20.9±0.0

3.4. Conclusions

Solid-state incubation with various fungal strains enhanced the nutritional composition of canola meal. *T. reesei* (NRRL-3653), *A. pullulans* (NRRL-58522), and *A. pullulans* (Y-2311-1) resulted in the greatest improvement in protein content, exhibiting maximum protein increases of 22.9, 16.9 and 15.4%, respectively, in solid-state incubated CP canola meal. This treatment also resulted in the reduction of the total GLS content to the greatest extent, ranging from 89.4-99.1 and 82.4-98.3% in HE and CP canola meal, respectively. Fiber levels were increased due to a "concentration effect" as sugars and GLS were metabolized.

Chapter IV - Enhancing the nutritional value of *Brassica napus* meal using a submerged fungal incubation process

Abstract

The aim of this study was to determine the optimal fungal culture to increase the nutritional value of canola meal so it could be used at higher feed inclusion rates, and for a broad range of monogastrics, including fish. Submerged incubation conditions were used to evaluate performance of seven fungal cultures in hexane extracted (HE) and cold pressed (CP) canola meal. *Aureobasidium pullulans* (Y-2311-1), *Fusarium venenatum* and *Trichoderma reesei* resulted in the greatest improvements in protein levels in HE canola meal, at 21.0, 23.8, and 34.8%, respectively. These fungi reduced total glucosinolate (GLS) content to 2.7, 7.4, and 4.9 µM/g, respectively, while residual sugar levels ranged from 0.8-1.6% w/w. In trials with CP canola meal, the same three fungi increased protein levels by 24.6, 35.2, and 37.3%, and final GLS levels to 6.5, 4.0, and 4.7 µM/g, respectively. Additionally, residual sugar levels were reduced to 0.3-1.0 % w/w.

4.1. Introduction

Canola (*Brassica napus*) is grown widely in Canada and the northern United States and is the second most abundant source of edible oil (Aider and Barbana 2011). Canola meal is also the second most abundant protein source for livestock feed, behind soybean meal (Newkirk 2009). The abundance and lower price of canola meal have driven interest in replacing soybean meal in ruminant and monogastric feeds (Lomascolo et al. 2012). On a cost per Kg of protein basis, canola protein is typically valued at 80-85% the value of soybean meal because it contains less gross energy, less protein, and over three times as much fiber. Canola also contains GLS that can have anti-nutritional effects on livestock. However, due to its lower cost it may be an economical protein source for animals that do not have high energy or lysine requirements (Bell 1993).

The presence of GLS in canola meal limits inclusion levels in livestock diets, as they can be toxic when consumed at high levels (Tripathi and Mishra 2007). GLS and the enzyme myrosinase are compartmentally stored separately in *Brassica* spp. (Rask et al. 2000). Upon mechanical disruption or other stresses on plant tissues, myrosinase cleaves glucose from GLS, which produces toxic compounds such as nitriles, thiocyanates, and isothiocyanates. This self-defense mechanism evolved to reduce animal and insect browsing of the plant (Halkier and Gershenzon 2006). When consumed, these toxic breakdown products can cause deleterious effects on the thyroid, and ultimately cause goiters from iodine deficiency (Burel et al. 2001). For this reason canola was bred to contain lower levels of GLS and erucic acid (Newkirk 2009). However, feed inclusion rates are still limited to ~30%, and this reduces the value of canola meal (Newkirk 2009).

MCN Bioproducts Inc. patented a process to fractionate high value protein concentrates from solvent and non-solvent expelled canola meal (Newkirk et al. 2006; Newkirk et al. 2009). These protein concentrates contained greater than 60% protein, no detectable phytic acid, and less than 5 μM/g of total GLS. However, this process utilizes multiple separation steps, which can be expensive and result in a relatively low protein yield in the primary marketed fraction. Bunge licensed this technology in 2012.

In contrast to mechanical separation to isolate protein, the metabolic diversity of fungi may be exploited to convert canola carbohydrates into protein-rich, single celled protein, and thereby produce a less expensive canola protein concentrate. In addition, fungal bioprocessing has been shown to significantly reduce GLS levels (Croat et al.

2015). We hypothesized that this process would generate a more digestible product with enhanced nutritional value to a range of aquaculture and other livestock species. Fungi selected for initial evaluation included *Aurobasidium pullulans, Trichoderma reesei*, *Fusarium venenatum, Pichia kudriavzevii, and Mucor circinelloides.* Several of these fungi are known to produce cellulose degrading enzymes. Both HE and CP canola meals were evaluated with a submerged incubation process, which allowed for better activity of cellulolytic enzymes.

4.2. Materials and Methods

4.2.1. Feedstocks and Preparation

HE canola meal was obtained from North Dakota State University (Fargo, ND, USA), while CP canola meal was obtained from Agrisoma Biosciences (Ottawa, Ontario, Canada). Both HE and CP meals were milled through a 2 mm screen via knife mill prior to use, and were stored at room temperature in sealed bucket throughout the duration of experimentation. Dry weight (dw) analysis was conducted by drying ~5 grams of canola meal at 80 degrees Celsius (°C) in a drying oven for at least 48 h.

4.2.2. Cultures, Maintenance, and Inoculum Preparation

A. pullulans (NRRL-58522), *A. pullulans* (NRRL-42023), *A. pullulans* (NRRL-Y-2311-1), *T. reesei* (NRRL-3653) and *F. venenatum* (NRRL-26139) were obtained from the National Center for Agricultural Utilization Research (Peoria, IL, USA). *P. kudriavzevii* and *M. circinelloides* were isolated as contaminates from prior trials, and were identified by ARS-USDA (Peoria, IL, USA) using 15s RNA analysis. Short-term maintenance cultures were stored on Potato Dextrose Agar plates and slants at 4°C. Lyophilization was used for long-term storage. Inocula for all experiments was prepared by transferring isolated colonies or a square section of agar growth (filamentous fungi) into glucose yeast extract (GYE) medium consisting of 5% glucose and 0.5% yeast extract. The pH for *Aureobasidium*, *Pichia*, and *Mucor* cultures was adjusted to 3 with 10N sulfuric acid, while pH 5-5.5 was used for *T. reesei* and *F. venenatum*. GYE flasks consisted of 100 milliliter (ml) working volume in 250 ml Erlenmeyer flasks, covered with a foam plug and aluminum foil. Cultures were incubated for ~72 hours (h) at 30°C in a rotary shaker at 150 revolutions per minute.

4.2.3. Experimental Procedures

Submerged trials were conducted in 1 L Erlenmeyer flasks with 500 ml total volume at 10% solid loading rate (SLR) dry weight canola meal. Flasks were covered with foam plugs and aluminum foil. For trials to be subjected to an initial saccharification step, 10 N sulfuric acid was used to adjust the initial pH to 5 (this is the optimal pH level for the commercial cellulase and hemicellulase enzymes used). For trials lacking the saccharification step, the pH was adjusted to the levels indicated previously for specific microbes. Flasks were then autoclaved at 121°C for 20 min. For saccharification trials, 0.052 mL CTec2 and 0.138 mL HTec2 (Novozymes, Franklinton, NC, USA) were added, and flasks were incubated at 50°C and 150 RPM for 24 h. Following saccharification, the pH was adjusted (if necessary) for the specific microbes and the slurry was cooled to 30°C. Saccharification and non-saccharification trials were inoculated with 5 ml of a 72 h culture of the appropriate organism and incubated at 30°C at 150 RPM for 168 h. Daily samples of ~50 ml were collected and used to monitor pH, cell counts, carbohydrates, protein, fiber, and GLS as described later. At the end of incubation the slurry was dried for 2 d at 80°C.

4.2.4. Analytical Methods

4.2.4.1. Total Protein

The pH of each sample was measured (Oakton 110 series pH meter). Forty-five ml of each sample was dried for 2 d at 80°C. Approximately 0.5 g of sample was used for protein analysis in duplicate. Protein was quantified using a LECO model FP528 (St. Joseph, MI, USA) to combust the sample and to measure the total nitrogen gas content in the sample. Protein percentage was then calculated from the nitrogen content of the sample using a conversion factor of 6.25. An additional 0.25 g of sample was dried at 80°C for 48 h to determine the dry matter of protein samples.

4.2.4.2. Residual Sugars

HPLC was used to measure residual sugars using five ml of sample supernatant. Samples were boiled for 10 min to ensure the fungal culture and/or saccharification enzymes were inactivated. Samples were then centrifuged at 10,000 RPM for 10 min, and the supernatant was poured into 2 ml microcentrifuge tubes and frozen overnight. The supernatant was then thawed re-centrifuged at 10,000 RPM for 10 min to remove any precipitants. The final supernatant was then filtered through a 0.2 micrometer (μm) filter and into a HPLC vial and frozen until analysis. A Waters size-exclusion chromatography column (SugarPak column I with pre-column module, Waters Corporation, Milford, MA, USA) and high performance liquid chromatography system (Agilent Technologies, Santa Calara, CA, USA) equipped with refractive index detector (Model G1362A) were used to measure the sugars. The sugars were eluted using a de-ionized water as mobile phase at flow rate of 0.5mL/min and column temperature of 80°C. Sugars to be quantified included arabinose, galactose, glucose, raffinose, stachyose, and sucrose.

4.2.4.3. Glucosinolates

Approximately 3 g of dried sample was used for GLS analysis. Individual GLS were confirmed to be present by quadrupole time-of-flight liquid chromatography-mass spectrometry and quantified using reverse phase high performance liquid chromatography (Berhow et al. 2013). For GLS quantitation, a modification of a high-performance liquid chromatography (HPLC) method developed by Betz and Fox (1994) was used. 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 LCsolutions Version 1.25 software. The column a C_{18} Inertsil reverse phase column (250 mm X 4.6 mm; RP C-18, ODS-3, 5u; with a Metaguard guard column; Varian, Torrance, CA). The glucosinolates were detected by monitoring at 237 nm. The initial mobile phase conditions were 12% methanol/88% aqueous 0.005M tetrabutylammonium bisulfate (TBS) at a flow rate of 1 ml/min. After injection of 15 ul of sample, the initial conditions were held for 2 min, and then up to 35% methanol over another 20 minutes, then to 50% methanol over another 20 minutes then up to 100% methanol over another 10 minutes.

4.2.4.4. Fiber

Fiber analysis was completed as Neutral Detergent Fiber (NDF) and Acid Detergent fiber (ADF). NDF is a method commonly used for animal feed analysis to determine the amount of lignin, hemicellulose and cellulose while ADF represents the least digestible fiber fraction of animal feed including lignin, cellulose, silica but not hemicellulose. NDF and ADF analysis were completed by Midwest Laboratories

(Omaha, NE, USA) using ANKOM Technology (Macedon, NY, USA) filter bag methods.

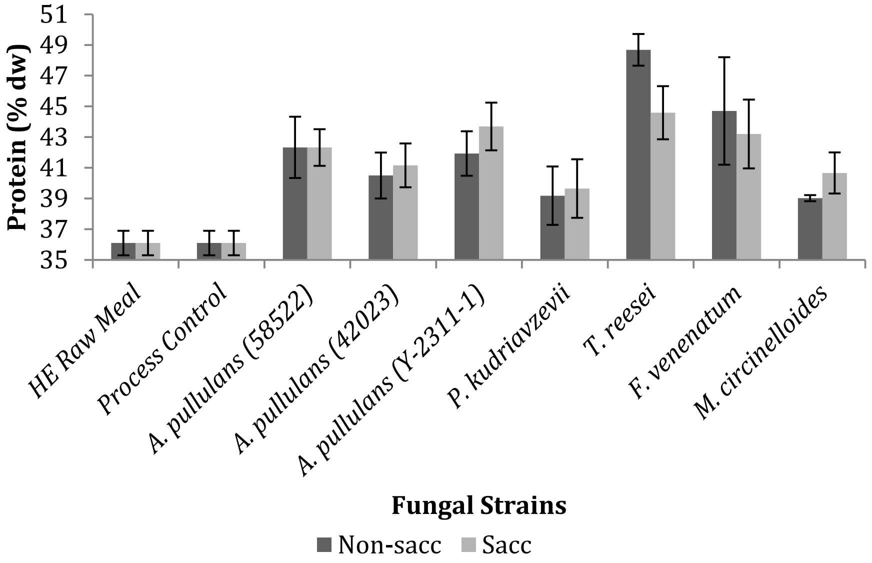
4.3. Results and Discussion

Seven fungal strains were grown on HE vs CP canola meal using a submerged incubation process. The fungi were tested both on raw (non-saccharified) and saccharified meal slurries using commercial cellulases to enhance fiber breakdown. These trials were done in shake flasks, where mixing and mass transfer are limiting factors. However, these non-optimized trials were meant to quickly down-select the best microbe for each type of canola meal. Other investigators have previously used a similar submerged incubation process to quickly assess phytase activity of various strains of bacteria, yeasts and fungi when grown on canola and oilseed meals (Nair and Duvnjak 1991).

4.3.1. Total Protein

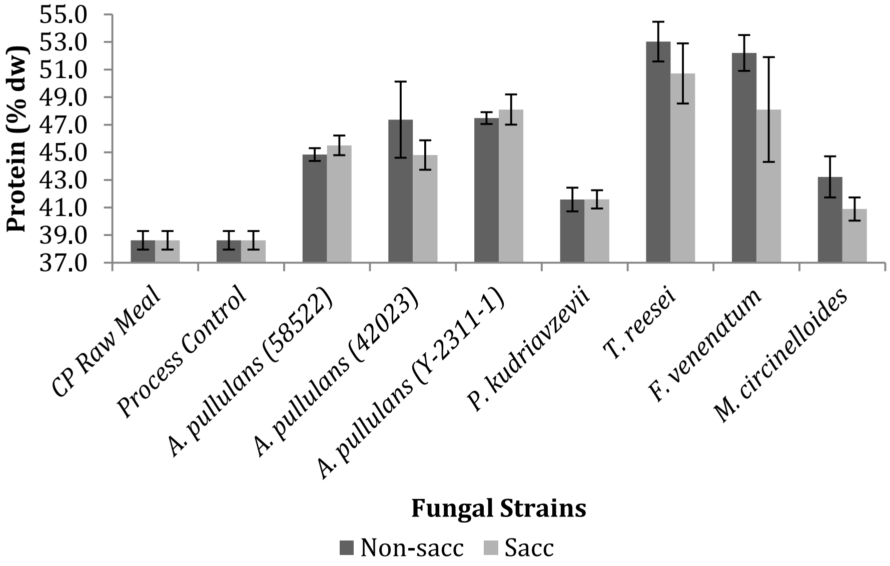
Figures 4.1a and 4.1b present the maximum protein levels in HE and CP canola meals, respectively, for raw meal and un-inoculated controls versus the various fungi, both under non-saccharified and saccharified conditions. As expected, protein levels for the un-inoculated controls were similar to the raw meals. In HE meal, protein levels increased from 36.1% in the raw meal to 39.0-48.7% after the fungal conversion process (relative improvements of ~8.0-34.9%) (Fig. 4.1a). The *M. circinelloides* trial was the only one in which an enzymatic hydrolysis step prior to inoculation proved beneficial. In the case of *T. reesei*, the non-saccharified trial actually resulted in higher protein titers. We had anticipated that saccharification would have a significant positive effect on fiber hydrolysis, and subsequently protein levels. It could be that canola fibers require pretreatment to increase susceptibility to enzymatic hydrolysis (Gattinger 1991; Yaun 2014). In future work we will investigate various pretreatment methods to make canola fibers more susceptible to hydrolysis by the fungal enzymes, thus releasing more sugar for conversion into single celled protein.

Figure 4.1a. Maximal protein levels ± SD of HE canola meal following submerged fungal incubation



In the CP canola meal (Fig. 4.1b) the protein level in the un-inoculated control was 38.6%, and rose to 40.9-53.0% after microbial conversion, representing relative improvements of ~6.0-37.3%. CP canola meal was ~3% higher in protein than HE meal and following incubation, protein levels were ~2-8% higher in CP canola meal trials compared to HE meal for each pair of fungi. HE is a more effective method of removing oil from canola seed, however this process applies significantly higher levels of heat, which may denature or degrade some protein (Spragg and Mailer 2007). We observed that the enzymatic hydrolysis step prior to inoculation did not significantly affect protein levels for all the fungi tested. Thus for un-pretreated canola meal, there was no benefit to adding cellulolytic enzymes.

Figure 4.1b. Maximal protein levels ± SD of CP canola meal following submerged fungal incubation



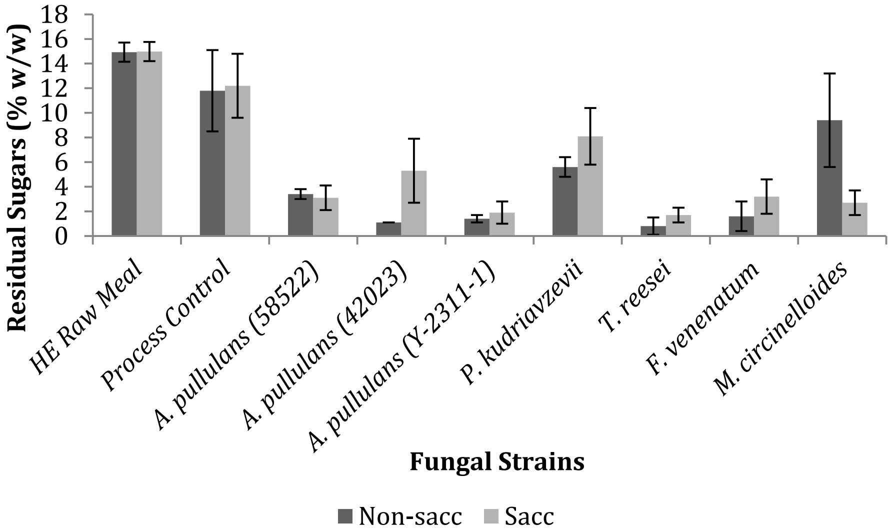
T. reesei achieved the highest protein levels for both substrates, while *P. kudriavzevii* exhibited the lowest protein enhancement. *T. reesei* is known to produce many hydrolytic enzymes (Li el al. 2013), and was expected to provide the greatest conversion of fiber and oligosaccharides into cell mass. As a single-celled yeast, *P. kudriavzevii* does not produce cellulase enzymes and was therefore anticipated to result in the lowest protein improvement. The final protein levels for all other fungal strains were relatively similar, at 40-45% in HE canola meal and 43-52% protein in cold pressed canola meal.

4.3.2. Residual Sugars

Arabinose, galactose, glucose, raffinose, stachyose, and sucrose were measured throughout incubation via HPLC. For simplicity, the final levels of these sugars were combined and are presented as residual sugars in Figures 4.2a and 4.2b for HE and CP canola meal, respectively. The total residual sugar concentrations decreased slightly (2.7-5.5%) from the raw meals compared to the process controls. Nyombaire et al. (2007) found that a pre-soaking and 80°C cook was sufficient to hydrolyze oligosaccharides such as raffinose and stachyose. Autoclaving the 10% SLR canola slurries may have achieved a similar effect, thereby reducing the raffinose and stachyose concentrations.

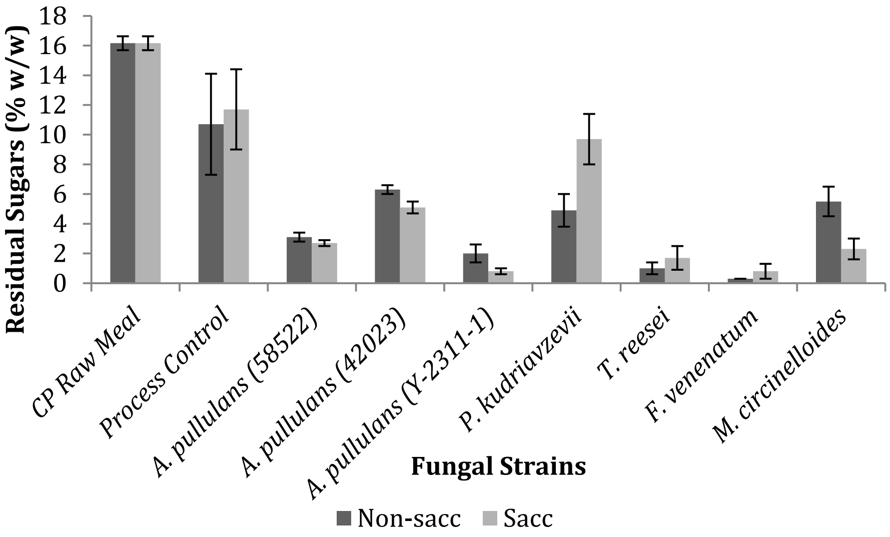
In non-saccharified HE meal (Fig. 4.2a) between 37.0-94.6% of sugars present were used by the fungi during incubation, resulting in residual sugar levels of 0.8-9.4%. Similarly, 39.0-88.6% of sugars present in saccharified HE meal was utilized by the fungi, resulting in residual sugar levels of 1.7-9.1 %. *T. reesei* exhibited the lowest residual sugar levels on both non-saccharified and saccarified HE meals, while *M. circinelloides* and *P. kudriavzevii* had the highest final levels in non-saccharified and saccharified trials, respectively. *M. circinelloides* did show a benefit from saccharfication, showing a significant drop in residual sugars from 9.4 to 2.7% w/w when compared to non-saccharification.

Figure 4.2a. Residual sugar levels ± SD of HE canola meal following submerged fungal incubation



In non-saccharified CP meal (Fig. 4.2b) between 61.0-98.1% of sugars present were metabolized by the fungi during incubation, decreasing residual sugar levels to 0.3-6.3%. Similarly, 40.0-95.0% of sugars present in saccharified CP meal were metabolized by the fungi during incubation, decreasing residual sugar levels to 0.8-9.7%. *F. venenatum* and *T. reesei* exhibited the lowest residual sugar levels on both non-saccharified and saccarified CP meal, while *A. pullulans* (NRRL-42023) and *P. kudriavzevii* had the highest final levels in non-saccharified and saccharified material, respectively. Saccharification significantly reduced residual sugars in trials with *M. circinelloides* and *A. pullulans* (NRRL-42023) when compared to non-saccharification trials.

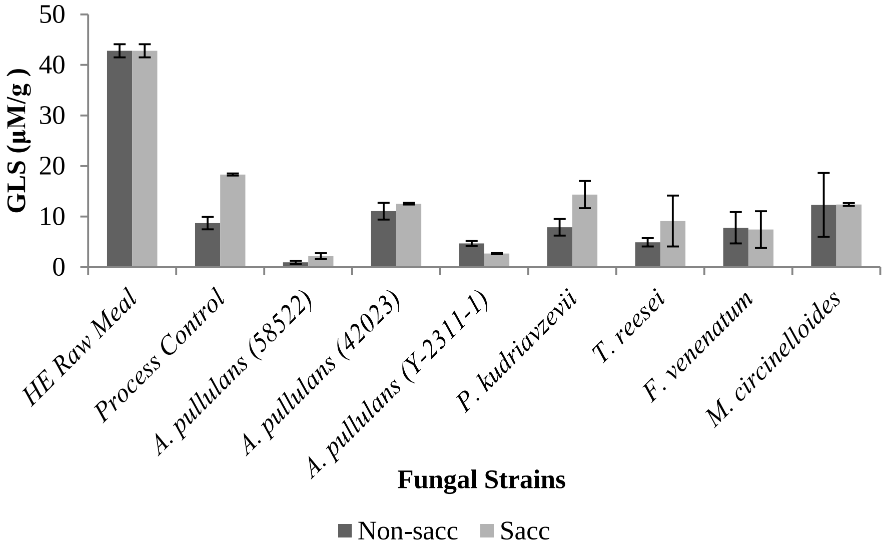
Figure 4.2b. Residual sugar levels ± SD of CP canola meal following submerged fungal incubation



4.3.3. Glucosinolates

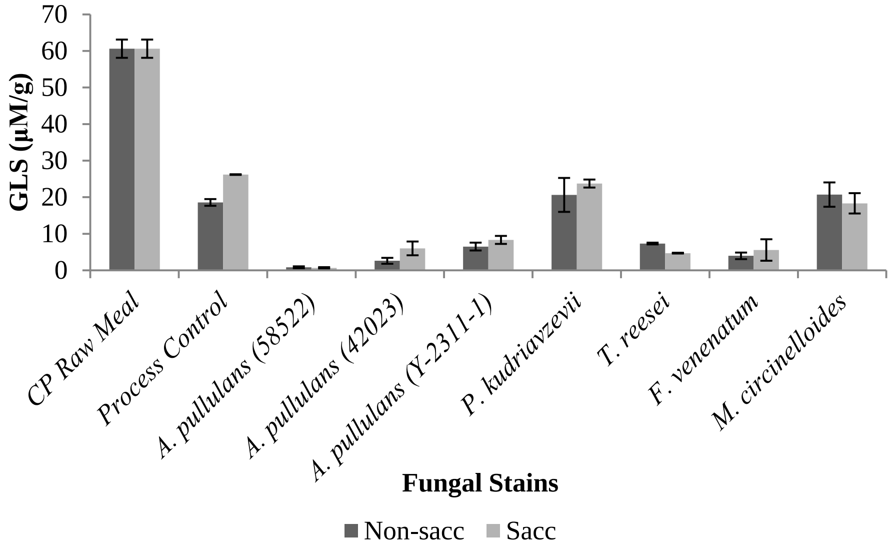
Figures 4.3a and 4.3b show GLS levels for the HE and CP canola meal trials, respectively. GLS levels were reduced from 42.8 µM/g in raw HE meal to 8.7 µM/g (non-saccharified) and 18.3 µM/g (saccharified) in the un-inoculated process controls. This represents 79.6 and 57.2% reductions, respectively, and was presumed due to the conversion of some of the GLS into volatile breakdown products (Halkier and Gershenzon 2006). Newkirk et al. (2003) also noted that high processing heat can be used to remove volatile anti-nutritional factors, however this can also denature proteins. Submerged microbial conversion further reduced GLS content to 1.0-14.4 µM/g, representing a total reduction of 66.5-97.8%.

Figure 4.3a. Reduction of total GLS ± SD following sterilization submerged fungal incubation in HE canola meal



GLS levels in raw CP meal (60.6 µM/g) were higher than in HE meal (42.8 µM/g) since the former does not include the high temperature de-solventizing step which can eliminate GLS. Treatment of the CP meal with the autoclaving and drying steps in the process control reduced GLS levels to 18.6 and 26.2 µM/g, respectively in non-saccharified and saccharified trials (reduction of 69.4 and 56.8%), respectively. Again, submerged microbial conversion further reduced GLS content to 0.7-23.7 µM/g (total reduction of 60.8-98.9%).

Figure 4.3b. Reduction of total GLS ± SD sterilization submerged fungal incubation in CP canola meal



Overall, *A. pullulans* (NRRL-58522) caused the greatest reduction in GLS levels in both HE and CP canola meals (ranging from 94.5-98.9%), likely due to its robust capability for producing extracellular enzymes (Kudanga and Mwenje 2005). *A. pullulans* (NRRL-Y-2311-1) was also very effective in reducing GLS concentrations (ranging from 86.3-93.7%), followed by *F. venenatum* (81.8-93.5%) and *T. reesei* (78.7-92.2%). Previous studies have shown that various microbes are able to degrade GLS and metabolize the resulting glucose and sulfur moieties. For example, Vig and Walia (2001) observed that *Rhizopus oligosporus* reduced GLS and their byproducts during fungal incubation of *Brassica napus* meal. Similarly, Rakariyatham and Sakorn (2002) reported the complete degradation of GLS after 60-96 h using solid-state fermentation of *Brassica juncea* with *Aspergillus* sp. In the work reported herein, *P. kudriavzevii* and *M. circincelloides* resulted in the least reduction in GLS, as expected due to minimal production of extracellular hydrolytic enzymes when compared to the other fungi tested.

4.3.4. Fiber

Table 4.1 provides the ADF and NDF fiber levels of raw, process control, and treated canola meals. In general, most fiber levels were statistically similar to the raw meal, indicating that the conversion process had minimal effects on fiber levels. The only trial to show a statistically significant reduction in ADF in HE meal was *P. kudriavzevii*, while trials with *A. pullulans* (Y-2311-1), *P. kudriavzevii*, *T. reesei*, *F. venenatum*, and *M. circincelloides* all statistically reduced ADF and/or NDF fiber levels in CP canola meal (Table 4.1). Thus the cellulase producing fungi were effective in hydrolyzing fiber in CP canola meal, however did not show similar results in HE canola meal. A possible explanation for the reduced fiber degradation in HE canola meal is that the heating steps of the hexane extraction process may have reduced the susceptibility of the fibers to subsequent enzymatic hydrolysis. Also, the enzyme cocktail used in the saccharification trials were not optimized for canola fiber, and this may provide a future opportunity to enhance fiber degradation. In some cases the conversion process actually resulted in a concentration of fibers, caused by the removal of sugars and GLS. Trials with *A. pullulans* (58522), *A. pullulans* (Y-2311-1), *F. venenenatum*, and *M. circinelloides* all increased fiber levels in HE canola meal, while *A. pullulans* (58522) and *A. pullulans* (42023) treatments both increased ADF and/or NDF fiber levels in CP canola meal (Table 4.1). Karki et al. (2013) had shown that feedstock pretreatment increases the susceptibility of fibers to hydrolysis, and that optimizing the fungal incubation conditions will also enhance cellulase production and activity. The resulting sugars would then be available for conversion into additional cell mass and protein. This will be evaluated in future studies using extrusion, hot cook, dilute acid, and dilute alkali pretreatments.

Table 4.1. Fiber reduction of non-saccharified and saccharified canola meal ± SD during submerged fungal incubation

	Hexane Extracted				Cold Pressed			
	Non-Saccharified		Saccharified		Non-Saccharified		Saccharified	
Fungal Culture	ADF (%)	NDF (%)	ADF (%)	NDF (%)	ADF (%)	NDF (%)	ADF (%)	NDF (%)
Raw Meal	19.9±0.2	23.1±0.3	19.9±0.2	23.1±0.3	11.5±0.5	15.0±0.3	11.5±0.5	15.0±0.3
Process Control	18.7±0.3	22.0±0.8	23.0±1.1	29.0±1.8	9.5±0.6	12.4±0.8	14.8±1.4	16.1±1.6
A. pullulans (NRRL-58522)	22.0±1.6[2]	29.1±0.8[2]	20.6±2.4	25.2±4.6	12.1±1.0	16.8±0.7[2]	11.6±1.1	15.9±0.6
A. pullulans (NRRL-42023)	20.4±1.5	24.3±1.3	19.4±2.2	22.6±0.5	12.4±0.4	16.9±0.4[2]	11.2±1.1	15.1±0.3
A. pullulans (NRRL-Y-2311-1)	22.3±0.9[2]	24.5±0.7[2]	21.2±1.4	24.0±0.9	13.6±2.1	14.8±2.4	11.0±0.9	12.6±1.0[1]
P. kudriavzevii	19.7±1.6	23.1±1.9	18.6±0.3[1]	22.7±1.9	11.0±0.4	13.5±0.3[1]	10.4±0.4[1]	12.4±0.5[1]
T. reesei (NRRL-3653)	19.9±3.1	22.5±4.0	19.8±0.8	26.4±3.3	7.6±0.8[1]	10.1±0.6[1]	8.1±1.2[1]	10.8±2.0[1]
F. venenatum (NRRL- 26139)	21.3±2.4	26.7±2.6[2]	20.8±2.2	26.9±0.7[2]	7.6±0.9[1]	10.7±1.2[1]	10.7±2.5	12.9±3.0
M. circinelloides	21.0±0.5[2]	25.9±1.2	19.6±1.2	22.6±1.0	10.2±0.9	12.8±0.9[1]	10.8±0.6	15.6±1.3

[1] Indicates fiber level was statistically lower than raw meal

[2] Indicates fiber level was statistically higher than raw meal

4.4. Conclusions

Submerged incubation with various fungal strains improved the nutritional content of canola meal. *T. reesei* (NRRL-3653), *F. venenatum* (NRRL-26139), and *A. pullulans* (Y-2311-1) resulted in the greatest improvement in protein content in HE canola meal (34.8, 23.8, and 21.0%), respectively, while reducing total GLS and residual sugar content by 82.6-93.7% and 89.3-94.6%. In trials with CP canola meal, the same three fungi increased protein levels to the greatest extent (37.3, 35.2, and 24.6%), respectively, while reducing total GLS and residual sugar content by 89.3-93.5% and 93.8-98.1%.

Chapter VI - Utilizing pretreatment and fungal incubation to enhance the nutritional value of canola meal

Abstract

The objective of this study was to determine the optimal pretreatment and fungal strain to reduce glucosinolates (GLS), fiber, and residual sugars while increasing the nutritional value of canola meal. Submerged incubation conditions were used to evaluate four pretreatment methods (extrusion, hot water cook, dilute acid, and dilute alkali) and three fungal cultures (*Aureobasidium pullulans* Y-2311-1, *Fusarium venenatum* NRRL-26139, and *Trichoderma reesei* NRRL-3653) in hexane extracted (HE) and cold pressed (CP) canola meal. The combination of extrusion pretreatment followed by incubation with *T. reesei* resulted in the greatest overall improvement to HE canola meal, increasing protein to 51.5%, while reducing NDF, GLS, and residual sugars to 18.6%, 17.2 μM/g, and 5% w/w, respectively. Extrusion pretreatment and incubation with *F. venenatum* performed the best with CP canola meal, resulting in 54.4% protein while reducing NDF, GLS, and residual sugars to 11.6%, 6.7 μM/g, and 3.8% w/w, respectively.

5.1. Introduction

The use of plant proteins to replace animal-based protein sources (such as fish, blood, and bone meals) in livestock diets has become a recent priority due to economic and regulatory issues (Booth et al. 2001). The growth of aquaculture and other competing uses of fish meal have caused prices to exceed $2,000/ton (World Bank 2014). Furthermore, there have been increasing concerns with potential transmission of infective agents due to the practice of feeding livestock with animal byproducts (European Food Safety Authority 1997; Crump et al. 2002).

Canola meal has been recognized as a highly concentrated protein source with a well-balanced amino acid profile (Mailer et al. 2008; Seneviratne et al. 2010). Canola is the second most abundant plant protein source for livestock feed, behind soybean meal (Newkirk 2009). The abundance and lower price of canola meal have driven interest in replacing soybean meal in ruminant and monogastric feeds (Lomascolo et al. 2012). However, the presence of GLS and fiber in canola meal has limited inclusion of this meal in livestock diets (Montoya and Leterme 2010). GLS are anti-nutritional factors present in *Brassica* spp. meals, and can be toxic when consumed at high levels (Tripathi and Mishra 2007). Canola meal also contains less gross energy, less protein, and over three times as much fiber when compared to soybean meal (Bell 1993; Liu et al. 2014). Excessive fiber in monogastric animal diets may also lead to a decrease in feed utilization by obstructing digestive enzymes and diluting nutrient density (Booth et al. 2001). Canola meal would be more competitive in the market if it contained more digestible energy, more protein, and less GLS.

We have previously demonstrated that the metabolic diversity of fungi can be exploited to convert canola carbohydrates into protein-rich single celled protein, and thereby produce a less expensive canola protein concentrate. *Aureobasidium pullulans* (NRRL-58522), *A. pullulans* (NRRL-42023), *A. pullulans* (NRRL-Y-2311-1), *Pichia kudriavzevii*, *Mucor circinelloides*, *Trichoderma reesei* (NRRL-3653), and *Fusarium venenatum* (NRRL-26139) were evaluated under solid-state and submerged incubation conditions (Croat et al. 2015b; Croat et al. 2015c). Solid-state incubation with *T. reesei*, *A. pullulans* (NRRL-58522), and *A. pullulans* (NRRL-Y-2311-1) resulted in the greatest improvement in protein content (23.1, 13.6 and 14.5%, respectively) on HE canola meal, while also resulting in the greatest improvement in protein content of CP canola meal (22.9, 16.9 and 15.4%, respectively) (Croat et al. 2015b). The same fungi reduced the total GLS content to the greatest extent, ranging from 89.4-99.1 and 82.4-98.3% reductions in HE and CP canola meal, respectively (Croat et al. 2015b). Submerged incubation with *A. pullulans* (Y-2311-1), *F. venenatum* and *T. reesei* resulted in the greatest improvements in protein levels in HE canola meal, at 21.0, 23.8, and 34.8%, while increasing protein levels of CP canola meal by 24.6, 35.2, and 37.3%, respectively (Croat et al. 2015c). The same fungi reduced the total GLS content to the greatest extent, ranging from 82.6-93.7% and 89.3-93.5% in HE and CP canola meal, respectively (Croat et al. 2015c). Unfortunately, fiber levels were not reduced, and actually increased in some cases due to the concentration factor of removing oligosaccharides, sugars, and GLS.

To further improve this process, we hypothesized that pretreatment would make canola fibers more susceptible to hydrolysis by the fungal enzymes, thus releasing more sugar for conversion into single celled protein. This could potentially result in even higher protein levels and lower fiber levels, creating a more digestible product with enhanced nutritional value to a range of aquaculture and other livestock species. Numerous biomass pretreatment approaches have been assessed over the past four decades to enhance the susceptibility of lignocellulosic biomass for subsequent enzymatic hydrolysis (Hendriks and Zeeman 2009). In this paper we report on the use of these four pretreatment methods on both hexane extracted and cold pressed canola meals, followed by microbial conversion with *A. pullulans* (Y-2311-1), *F. venenatum* (NRRL-26139) and *T. reesei* (NRRL-3653).

Thermal extrusion has long been utilized in the animal feed industry to destroy anti-nutritional factors and improve digestibility, nutrient availability, and palatability of animal feeds (Liang et al. 2002; Allan and Booth 2004). The interaction of screw speed, moisture content, and bore temperature can reduce GLS and fiber concentrations, resulting in an improvement in protein digestibility (Liang et al. 2002). Extrusion has also been widely evaluated as a pretreatment for lignocellulosic ethanol processes (Zheng and Rehmann 2014). For example, Ahmed et al. (2014) reduced the crude fiber of canola meal by ~21% utilizing thermal extrusion.

Treating biomass with hot water at 150-180°C solubilizes hemicelluloses and then lignin (Hendriks and Zeeman 2009). Hemicellulose hydrolysis promotes the formation of acids that catalyze further hydrolysis (Hendriks and Zeeman 2009). Hot water pretreatment does not require additional chemicals that add to process costs from neutralizing and/or recovery (Mosier et al. 2006). Mosier et al. (2006) and Zhou et al. (2010) have both achieved ~90% glucose yields from the hydrolysis of corn stover using hot water pretreatment at 190 and 210°C, respectively.

Dilute acid pretreatment solubilizes hemicellulose into fermentable sugars, especially under strong acid conditions, however temperature needs to be closely monitored to avoid production of compounds such as furfural and hydroxymethyl furfural that can inhibit fermentation organisms. (Saha et al. 2005). Karki et al. (2011) achieved glucose yields of 98 and 84% from the hydrolysis of dilute acid pretreated wheatgrass and switchgrass, respectively.

Dilute alkali pretreatment swells biomass, making it more accessible for enzymes and microbes (Hendriks and Zeeman 2009). However, this pretreatment often results in the conversion of hemicellulose into degradation products that can inhibit fermentation microbes (Hendriks and Zeeman 2009). Karki et al. (2011) achieved glucose yields of 70.4 and 70.7% from the hydrolysis of dilute alkali pretreated wheatgrass and switchgrass, respectively. Pryor et al. (2012) achieved glucose yields greater than 85% from the hydrolysis of dilute alkali pretreated switchgrass.

5.2. Materials and Methods

5.2.1. Feedstocks and Preparation

HE canola meal was obtained from North Dakota State University (Fargo, ND, USA), while CP canola meal was obtained from Agrisoma Biosciences (Ottawa, Ontario, Canada). Both HE and CP meals were milled through a 2 mm screen via knife mill prior to use, and were stored at room temperature in sealed bucket throughout the duration of experimentation. Dry weight (dw) analysis was conducted by drying ~5 grams of canola meal at 80°C in a drying oven for at least 48 h.

5.2.2. Cultures, Maintenance, and Inoculum Preparation

A. pullulans NRRL-Y-2311-1, *T. reesei* NRRL-3653 and *F. venenatum* NRRL-26139 were obtained from the National Center for Agricultural Utilization Research (Peoria, IL, USA). Short-term maintenance cultures were stored on Potato Dextrose Agar plates and slants at 4°C. Lyophilization was used for long-term storage. Inocula for all experiments were prepared by transferring isolated colonies or a square section of agar growth (filamentous fungi) into glucose yeast extract (GYE) medium consisting of 5% glucose and 0.5% yeast extract. Flasks to grow inocula consisted of 100 ml GYE medium in 250 ml Erlenmeyer flasks, covered with a foam plug and aluminum foil. Cultures were incubated for ~72 hours (h) at 30°C in a rotary shaker at 150 revolutions per minute.

5.2.3. Pretreatments

Extrusion was conducted under conditions previously determined as optimal (Kaur and Muthukumarappan, 2015), which included a barrel temperature of 80°C, and a screw speed of 50 or 100 rpm for CP and HE canola meals, respectively. Extrusion was completed at meal moisture contents of 4.6 and 7.3% for CP and HE canola meals, respectively. Extruded material was stored in a sealed bucket until trials were completed. Hot water cook, dilute acid, and dilute alkali pretreatments were conducted on homogenized, 15% w/w (dm) slurries of CP and HE canola meals at 160°C for 20 min using a stainless steel steam jacketed reactor tube in 8-10 l batch-wise increments. Dilute acid pretreatment also incorporated 0.5% w/w sulfuric acid, while dilute alkali pretreatment used a 4% w/w ammonia concentration (using 30% ammonium hydroxide). Following pretreatment these slurries were frozen for storage. Prior to use, slurries were thawed, re-homogenized, and evenly dispensed into flasks for various trials.

5.2.4. Experimental Procedures

Submerged fungal incubation trials were conducted in 1 L Erlenmeyer flasks with 500 ml total volume at 15% SLR dry weight canola meal. The pH for *A. pullulans* was adjusted to 3 with 10N sulfuric acid, while pH 5-5.5 was used for *T. reesei* and *F. venenatum*. Flasks were covered with foam plugs and aluminum foil. Flasks were then autoclaved at 121°C for 20 min. Trials were inoculated with 10 ml of a 72 h culture of the appropriate organism and incubated at 30°C at 150 RPM for 168 h. Daily samples of ~50 ml were collected and used to monitor pH, cell counts, carbohydrates, protein, fiber, and GLS as described later. At the end of incubation the slurry was dried at 80°C for 2 d.

5.2.5. Analytical Methods

5.2.5.1. Total Protein

The pH of each sample was measured with an Oakton 110 series pH meter. Forty-five ml of each sample was dried for 2 d at 80°C. Approximately 0.5 g of sample was used for protein analysis in duplicate. Protein was quantified using a LECO model FP528 (St. Joseph, MI, USA) to combust the sample and to measure the total nitrogen (N) gas content in the sample. Protein percentage was then calculated from the N content of the sample using a conversion factor of 6.25. An additional 0.25 g of sample was dried at 80°C for 48 h to determine the dry matter of protein samples. Dilute alkali samples were also assayed for non-protein nitrogen (NPN) levels due to the high content of residual ammonia. NPN analysis was completed by Midwest Laboratories (Omaha, NE, USA) using AOAC method 941.04.

5.2.5.2. Residual Sugars

HPLC was used to measure residual sugars. Samples were boiled for 10 min to ensure the fungal cultures were inactivated. Samples were then centrifuged at 10,000 RPM for 10 min, and the supernatant was poured into 2 ml microcentrifuge tubes and frozen overnight. The supernatant was then thawed re-centrifuged at 10,000 RPM for 10 min to remove any precipitants. The final supernatant was then filtered through a 0.2 micrometer (μm) filter and into a HPLC vial and frozen until analysis. A Waters size-exclusion chromatography column (SugarPak column I with pre-column module, Waters Corporation, Milford, MA, USA) and high performance liquid chromatography system (Agilent Technologies, Santa Calara, CA, USA) equipped with refractive index detector (Model G1362A) were used to measure the sugars. The sugars were eluted using a de-ionized water as mobile phase at flow rate of 0.5mL/min and column temperature of 80°C. Sugars to be quantified included arabinose, galactose, glucose, raffinose, stachyose, and sucrose.

5.2.5.3. Glucosinolates

Approximately 3 g of dried samples were used for GLS analysis. Individual GLS were confirmed to be present by quadrupole time-of-flight liquid chromatography-mass spectrometry and quantified using reverse phase high performance liquid chromatography (Berhow et al. 2013). For GLS quantitation, a modification of a high-performance liquid chromatography (HPLC) method developed by Betz and Fox (1994) was used. 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 LCsolutions Version 1.25 software. The column a C_{18} Inertsil reverse phase column (250 mm X 4.6 mm; RP C-18, ODS-3, 5u; with a Metaguard guard column; Varian, Torrance, CA). The glucosinolates were detected by monitoring at 237 nm. The initial mobile phase conditions were 12% methanol/88% aqueous 0.005M tetrabutylammonium bisulfate at a flow rate of 1 ml/min. After injection of 15 µl of sample, the initial conditions were held for 2 min, and then up to 35% methanol over another 20 minutes, then to 50% methanol over another 20 minutes then up to 100% methanol over another 10 minutes.

5.2.5.4. Fiber

Fiber analysis was completed as Neutral Detergent Fiber (NDF) and Acid Detergent fiber (ADF). NDF is a method commonly used for animal feed analysis to determine the amount of lignin, hemicellulose and cellulose while ADF represents the least digestible fiber fraction of animal feed including lignin, cellulose, silica but not hemicellulose (Udén et al., 2005). NDF is presented in this work for simplicity and to include hemicellulose into the total fiber fraction. NDF and ADF analysis were completed by Midwest Laboratories (Omaha, NE, USA) using ANKOM Technology (Macedon, NY, USA) filter bag methods.

5.3. Results and Discussion

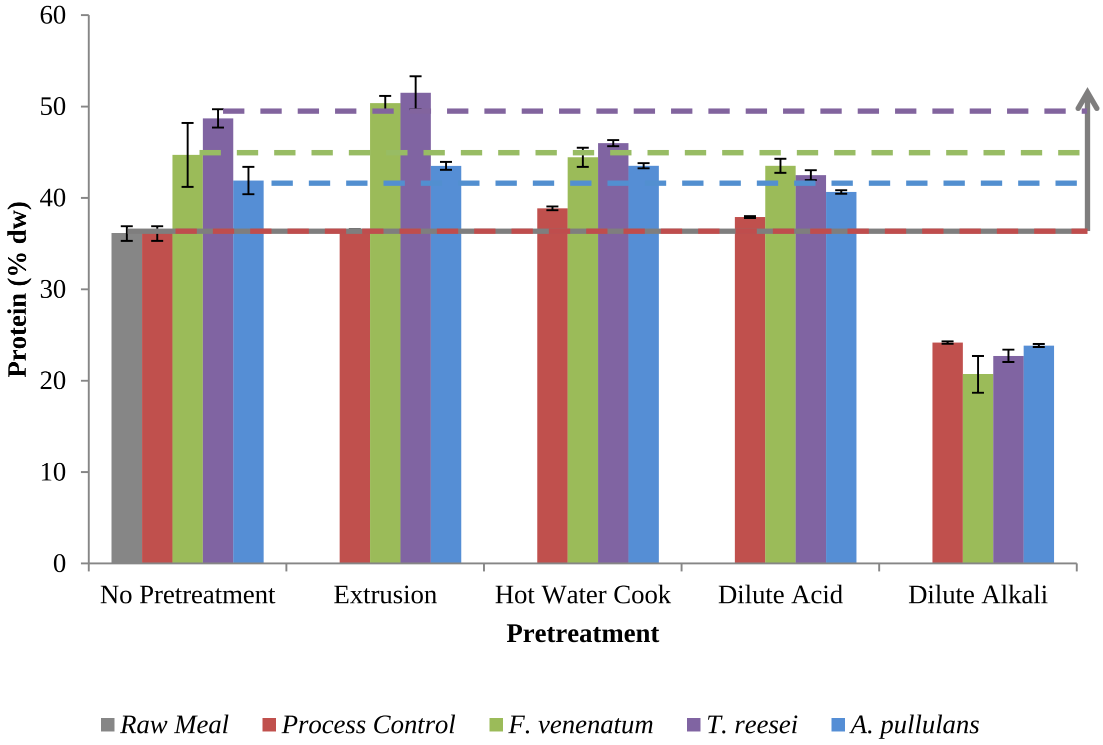
Three fungal strains were grown on pretreated and non-pretreated HE versus CP canola meal using a submerged incubation process. These trials were done in shake flasks, where mixing and mass transfer are limiting factors. However, these non-optimized trials were meant to down-select the best pretreatment method and fungus for each type of canola meal

5.3.1. Total Protein

Figures 5.1a and 5.1b represent the maximum protein levels achieved during the various treatments with HE and CP canola meal, respectfully. The initial protein level of the raw, un-pretreated meals is provided, along with process control samples, which were processed identically to the other treatments within each series, except that they were not inoculated with fungi. Hence the process controls represent the effects of the pretreatment, autoclaving, and drying steps.

As shown in Figure 5.1a, extrusion pretreatment by itself did not affect protein levels, and very slight increases were observed for the process controls in the hot water cook and dilute acid pretreatments. However, the dilute alkali pretreatment resulted in a large reduction in true protein, dropping the levels from ~36% to ~25% in the respective process controls. The most likely explanation is that the alkali treatment cleaved ammonia groups from amino acids. Kaye et al., (2004) noted that alkaline hydrolysis can initiate random breaking of ~40% of peptide bonds in protein, while some amino acids (arginine, asparagine, glutamine, and serine) are destroyed and others are racemized. Dilute alkali pretreatment may also cause other adverse effects on the nutritional quality of livestock meals. De Groot and Slump (1969) found that severe alkali treatment at pH 12.2 resulted in the formation of the amino acid derivative lysinoalanine, which contributes to decreased cysteine and lysine content, along with decreased net protein utilization. Bell et al. (1981) evaluated alkali treated *Brassica hirta* and *B. juncea* and found a 15-20% reduced lysine availability when fed to swine.

Figure 5.1a. Maximal protein levels ± SD in HE canola meal following submerged fungal incubation



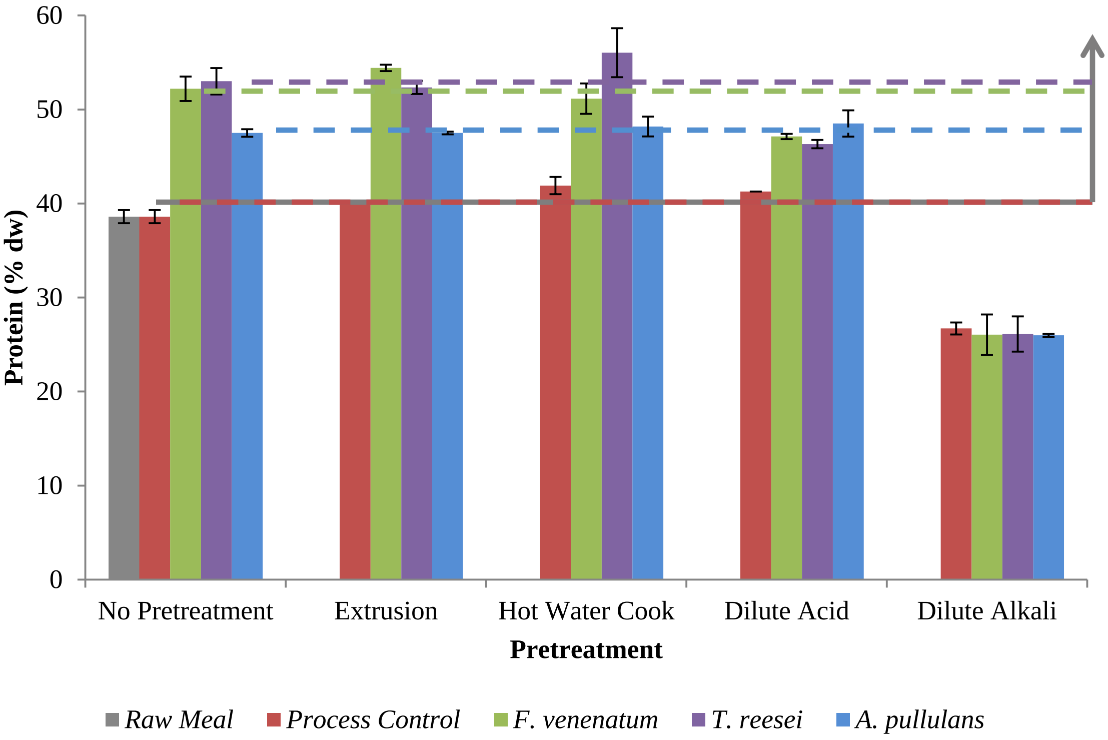
The dilute alkali pretreatment used a 4% w/w ammonia concentration, and because the final meal following pretreatment and incubation contained high levels of ammonia, all these samples were assayed for NPN levels. The NPN value was then subtracted from the LECO nitrogen analyzer data so that true protein content could be calculated. For example, in the case of HE canola meal, the LECO N content (12.7%) was subtracted from the NPN content (8.8%), resulting in a nitrogen level of 3.9%, which converts into a protein content of 24.17%.

In a previous study that evaluated seven fungal strains on non-pretreated HE canola meal, protein levels increased from 36.1% in raw meal to 41.9-48.7% after incubation with the three best fungi (*T. reesei* > *F. venenatum* > *A. pullulans*) (Croat et al., 2015c). This represented relative improvements of ~16.1-34.8%. Similar results were observed herein with the un-pretreated, extrusion pretreated, and how water cook pretreated HE canola meal, with *T. reesei* achieving the highest protein level, although it was not statistically different from *F. venenatum*.

Overall, extrusion was the most effective pretreatment for HE canola meal, achieving protein levels of 51.5, 50.4, and 43.5% for *T. reesei*, *F. venenatum*, and *A. pullulan,* respectively. Hot water cook and dilute acid pretreatments were relatively similar to the un-pretreated control, however, dilute alkali pretreatment reduced protein levels to 20.7-24.2, representing a protein loss of ~33.0-42.7%. Dilute alkali was also the only pretreatment in which subsequent fungal incubation did not increase the protein content compared to the process control. The enhanced degradation of fiber from dilute alkali pretreatment may have produced inhibitory compounds that could have prevented fungal single-cell protein production (Haque et al., 2012). Gossett el al. (1982) concluded that alkaline treated lignin at a concentration of 1 g/l was inhibitory to microorganisms. Canola meal may contain up to 10.4% lignin and polyphenols (Khajali and Slominski, 2012), which can be solubilized and form microbial inhibitory compounds, such as furfural and hydroxyfurfural (Hendriks and Zeeman, 2008).

Figure 5.1b shows similar data for pretreatments of the CP canola meal. Again, extrusion pretreatment by itself did not affect protein level, while the process controls in the hot water cook and dilute acid pretreatments showed very slight gains in protein. The dilute alkali pretreatment once again resulted in a significant loss of protein, due to the reasons mentioned previously.

Figure 5.1b. Maximal protein levels ± SD in CP canola meal following submerged fungal incubation



In our previous study we also evaluated fungal performance on non-pretreated CP canola (38.6% protein), and protein levels increased to 47.5-53.0% with the same three fungi (*T. reesei* > *F. venenatum* > *A. pullulans*), representing relative improvements of ~23.0-37.3% (Croat et al. 2015c). Figure 5.1b shows similar performance improvements by the three fungi in this study on the same un-pretreated feedstock. For the unpretreated, extrusion, and hot water cook pretreated samples, *T. reesei* and *F. venenatum* performed better than *A. pullulans*. However, unlike the trials with HE canola meal where extrusion was clearly the best pretreatment (Fig. 5.1a), both hot water cook and extrusion resulted in similar performance, which was not much different than the un-pretreated control. Dilute acid pretreatment slightly reduced protein levels, while dilute alkali pretreated caused a significant reduction in protein levels due to the reasons postulated earlier.

In summary, *T. reesei* achieved the highest protein levels for both substrates, while *A. pullulans* exhibited the lowest protein enhancement. *T. reesei* is known to produce many hydrolytic enzymes (Li, et al. 2013), and was expected to provide the greatest conversion of fiber and oligosaccharides into cell mass (i.e. protein). Extrusion of HE canola meal was the only pretreatment to show a consistent boost in protein levels following incubation with the three fungi. Dilute alkali pretreatment resulted in a significant loss of protein in all cases. Maximal protein levels for the other pretreatments were similar to the un-pretreated control.

5.3.2. Residual Sugars

Figure 5.2a provides the total residual sugar levels following pretreatment and fungal incubation of HE canola meal. The residual sugar content represents combined levels of arabinose, galactose, glucose, raffinose, stachyose, and sucrose. The un-inoculated process controls showed that the hot water cook (18.2%) and dilute acid pretreatments (18.6%) increased residual sugar levels compared to the un-pretreated control (14.9%). These results suggest that these pretreatments hydrolyzed oligosaccharides such as pectin into shorter chain carbohydrates including arabinose, galactose, glucose, xylose, and mannose (Garna et al., 2004). Garna et al. (2004) also found that acid concentration, temperature, and hydrolysis time commonly used for the hydrolysis of pectin vary from 1-2 M, 100-121°C, and 2-3 h, respectively. Extrusion pretreatment did not affect residual sugar levels, while dilute alkali pretreatment resulted in a decrease in residual sugar content at 12%. Monosaccharides, such as glucose, galactose, and mannose, are rapidly destroyed by the hot aqueous alkaline solution used with dilute alkali pretreatment (Kaye et al., 2004).

Following fungal incubation, the non-pretreated HE canola meal samples showed the lowest residual sugar content, ranging from 0.8, 1.4, and 1.7% with *T. reesei*, *A. pullulans*, and *F. venenatum*, respectively. For the pretreated samples, *A. pullulans* depleted sugar levels to the greatest extent, with the exception of the dilute alkali trial. In comparing the four pretreatments, extrusion and hot water cook had the lowest residual sugar titers, while dilute alkali pretreatment had the highest.

Figure 5.2a. Reduction of total residual sugar levels ± SD from raw HE canola meal by pretreatment and submerged fungal incubation

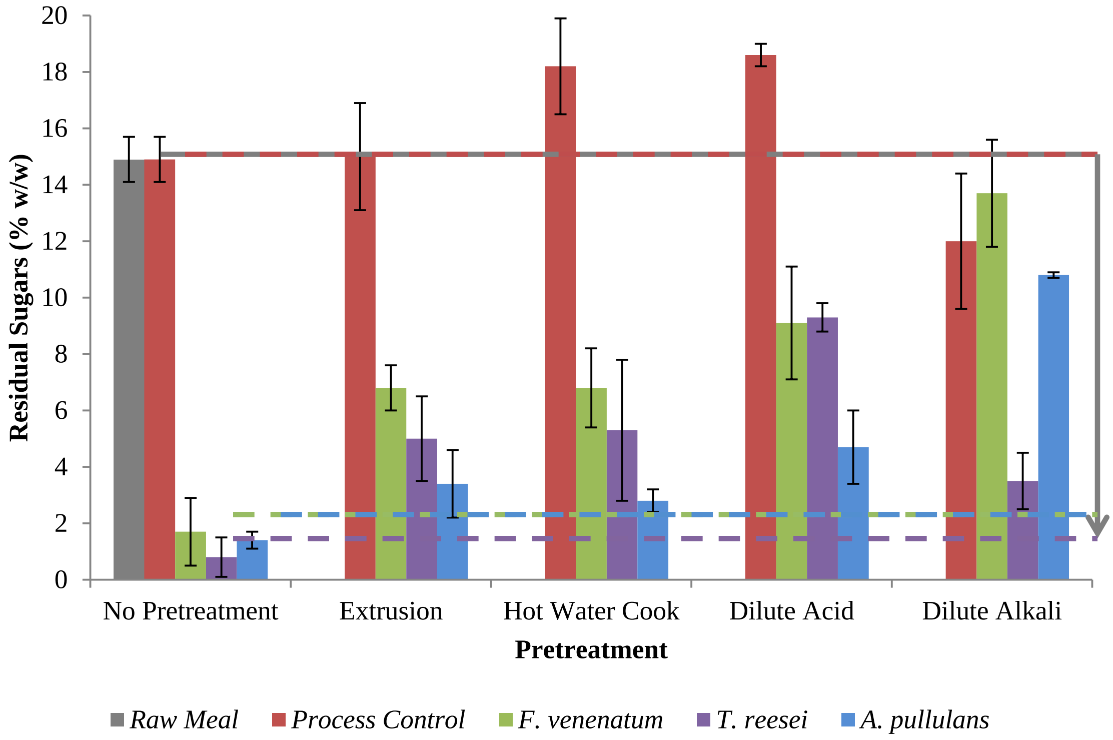
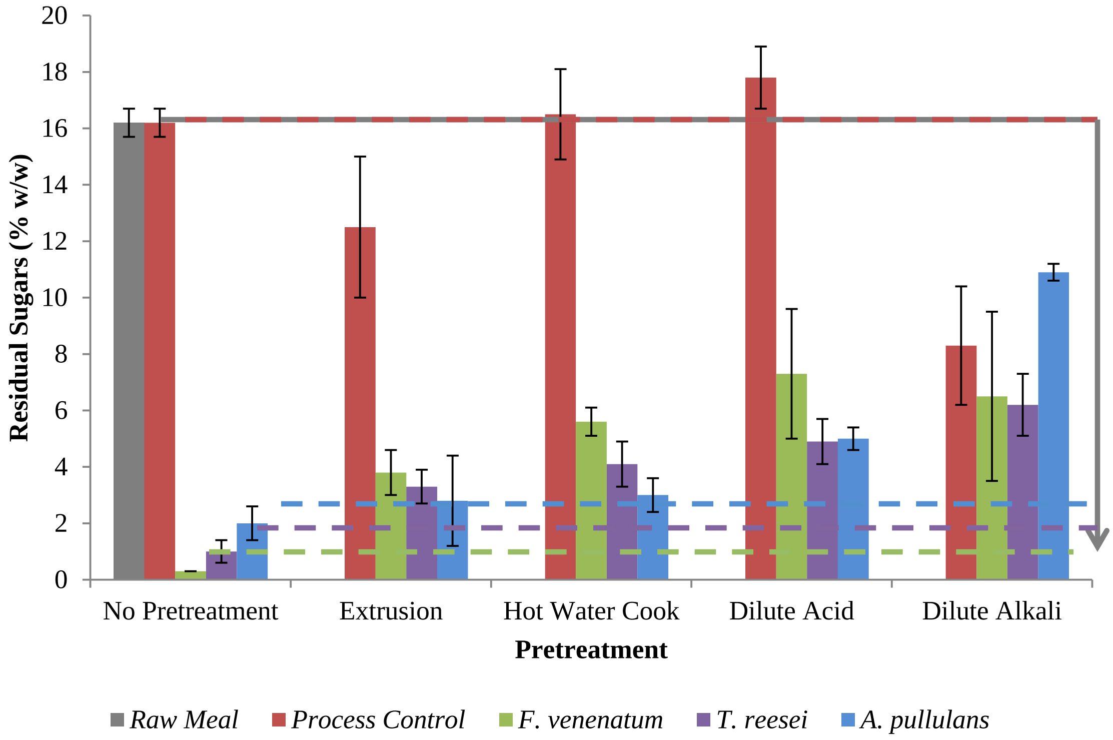


Figure 5.2b provides the total residual sugar levels following pretreatment and incubation of CP canola meal. In the un-inoculated process controls, the dilute acid pretreatment showed a slightly higher sugar level (17.8%) compared to the un-pretreated control (16.2%), while the hot water cook pretreatment was similar. Both the extrusion (12.5%) and dilute alkali (8.3%) pretreatments showed decreased residual sugar contents. In general, the remaining comparisons were similar to those noted in Figure 5.2a for HE canola meal. The non-pretreated CP canola meal showed the lowest residual sugar levels following incubation, at 0.3, 1.0, and 2.0% for *F. venenatum*, *T. reesei*, and *A. pullulans*, respectively. Of the pretreated trials, extrusion showed the lowest residual sugar levels after fungal incubation, while dilute alkali pretreatment was the highest.

Figure 5.2b. Reduction of total residual sugar levels ± SD from raw CP canola meal by pretreatment and submerged fungal incubation



5.3.3. Fiber

Figures 5.3a and 5.3b provide the neutral detergent fiber (NDF) levels following pretreatment and incubation of HE and CP canola meals, respectively. Dilute alkali was the most effective pretreatment in reducing NDF levels, achieving 54.5 and 64.7% reductions in the un-inoculated process controls compared to the raw HE and CP canola meals, respectively. Haque et al. (2012) achieved a maximum removal of lignin and hemicellulose at 84.8 and 79.5%, respectively, using 2% sodium hydroxide dilute alkali pretreatment of barley straw. Similarly, McIntosh and Vancov (2010) achieved a maximum removal of lignin and hemicellulose at 77.3 and 90%, respectively, using 2% sodium hydroxide dilute alkali pretreatment of sorghum straw. The other less obvious trend was that the hot water cook pretreatment resulted in a slight increase in NDF levels, perhaps as a result of solubilizing other components (eg. oligosaccharides) and thus “concentrating” NDF.

There were no strong trends regarding the effects of the fungal incubation process, but trials with *T. reesei* and *F. venenatum* typically had lower residual fiber levels than *A. pullulans*. This is consistent with the greater cellulase production capabilities of these two filamentous fungi. However, fiber levels were not reduced to the levels we anticipated. This may have been due to the presence of simple sugars into the latter stages of incubation (Fig. 5.2a and 5.2b), which would have repressed cellulase production. Addition of external cellulases will be explored in future trials. In some instances (Fig. 5.3a and 5.3b), fiber levels actually increased during the fungal incubation process as a result of the “concentration effect” as sugars and GLS were metabolized and protein levels increased (Fig. 5.1a and 5.1b).

Figure 5.3a. NDF fiber levels ± SD in HE canola meal following submerged fungal incubation

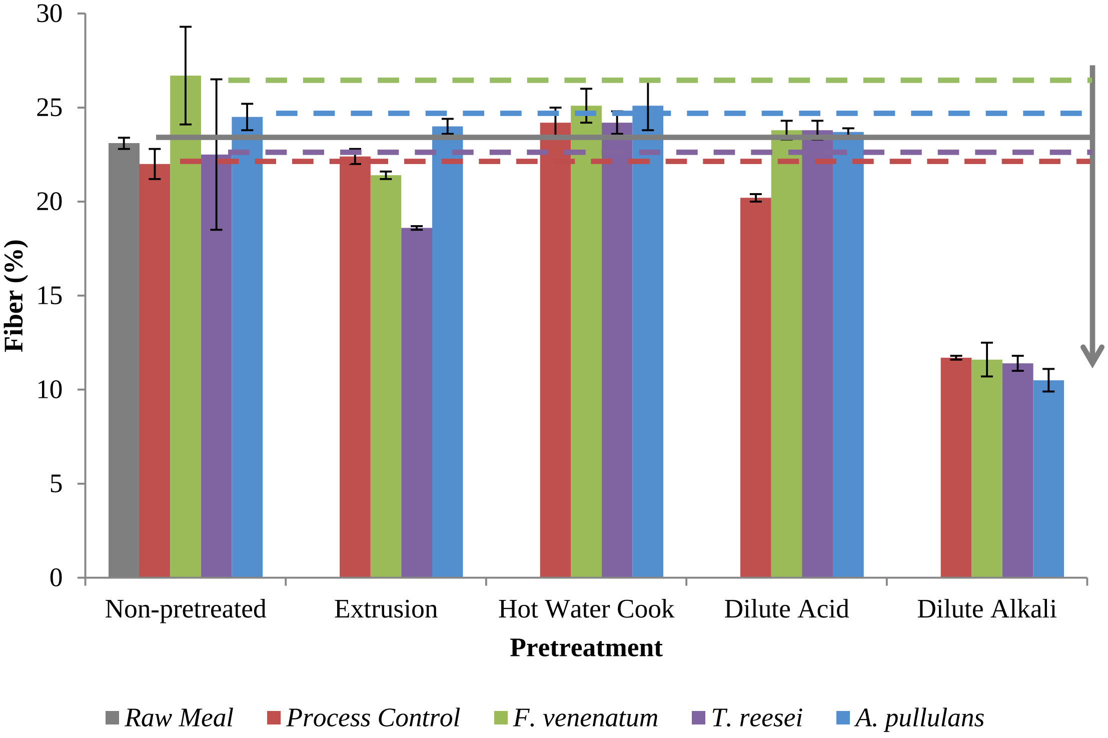
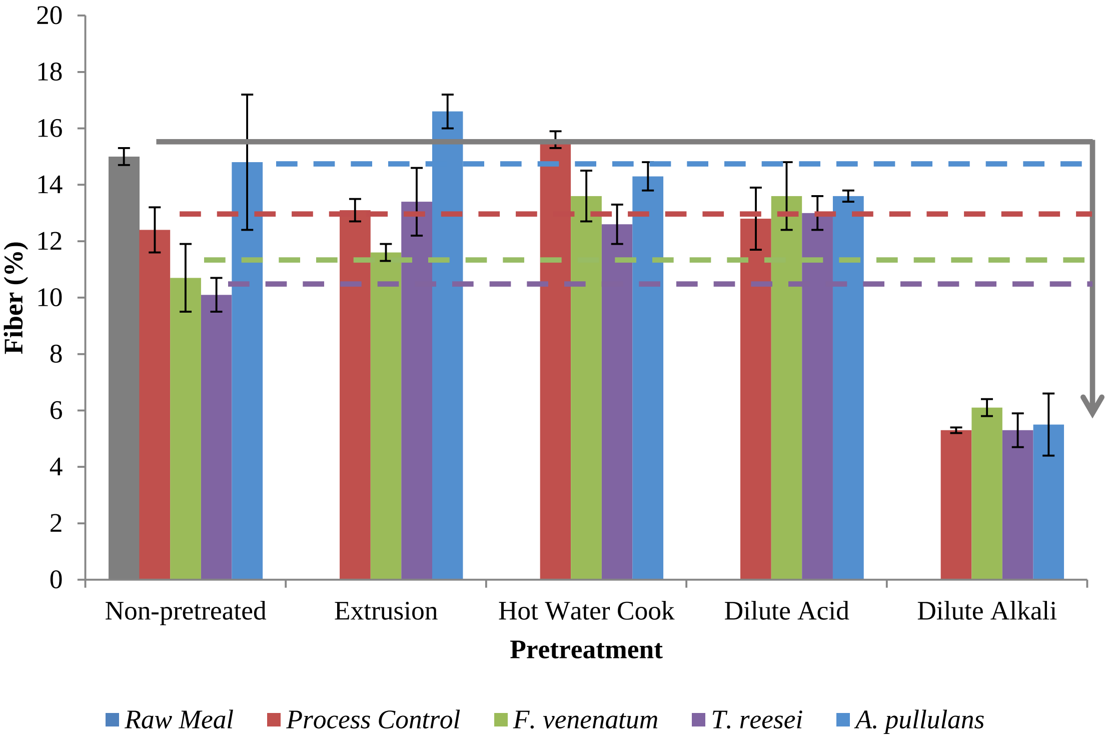


Figure 5.3b. NDF fiber levels ± SD in CP canola meal following submerged fungal incubation



5.3.4. Glucosinolates

Figures 5.4a and 5.4b show GLS levels following pretreatment and fungal incubation of HE and CP canola meals, respectively. The un-inoculated process controls for the non-pretreated feedstocks showed that the autoclave and final drying steps of the process degraded and/or volatilized significant amounts of GLS (Jensen et al., 1995; Halkier and Gershenzon, 2006; Croat et al., 2015b). In HE meal, GLS levels were reduced from 42.8 µM/g in raw meal to 8.7 µM/g after these treatments, representing a 79.7% reduction. Similarly, GLS levels in raw CP meal were reduced from 60.6 µM/g to 18.6 µM/g due to the thermal steps of the conversion process (reduction of 69.3%). CP processing uses significantly lower processing temperatures than HE processing, which explains the higher starting GLS content in CP versus HE canola meals (Newkirk et al., 2003; Spragg and Mailer, 2007).

Dilute alkali was the only pretreatment that reduced total GLS content to lower levels than non-pretreated meals in un-inoculated process controls of HE and CP canola meal (dilute alkali < non-pretreatment < hot cook < extrusion < dilute acid) (Fig. 5.4a and 5.4b). Surprisingly, GLS levels in the process controls in the extrusion, hot water cook, and dilute acid pretreatments were higher than the corresponding non-pretreated trials. The enzyme myrosinase is temperature sensitive, however it is very pressure stabile, which implies that high activity can still be retained after pressure treatment (Van Eylen et al. 2006).

Submerged microbial conversion further reduced GLS content in almost all cases to below that of the corresponding un-inoculated process control, however there was no consistent pattern in the most effective fungi. In our prior studies (Croat et al. 2015b) *T. reesei* exhibited the greatest reduction in GLS levels, likely due to its robust capability for producing extracellular enzymes (Li et al., 2013). Other researchers have shown that various microbes are able to degrade GLS and metabolize the resulting glucose and sulfur moieties. For example, Vig and Walia (2001) observed that *Rhizopus oligosporus* reduced GLS and their byproducts during fungal incubation. Similarly, Rakariyatham and Sakorn (2002) reported the complete degradation of GLS after 60-96 h using solid-state fermentation with *Aspergillus* sp.

Figure 5.4a. Reduction of total GLS levels ± SD from raw HE canola meal by pretreatment and submerged fungal incubation

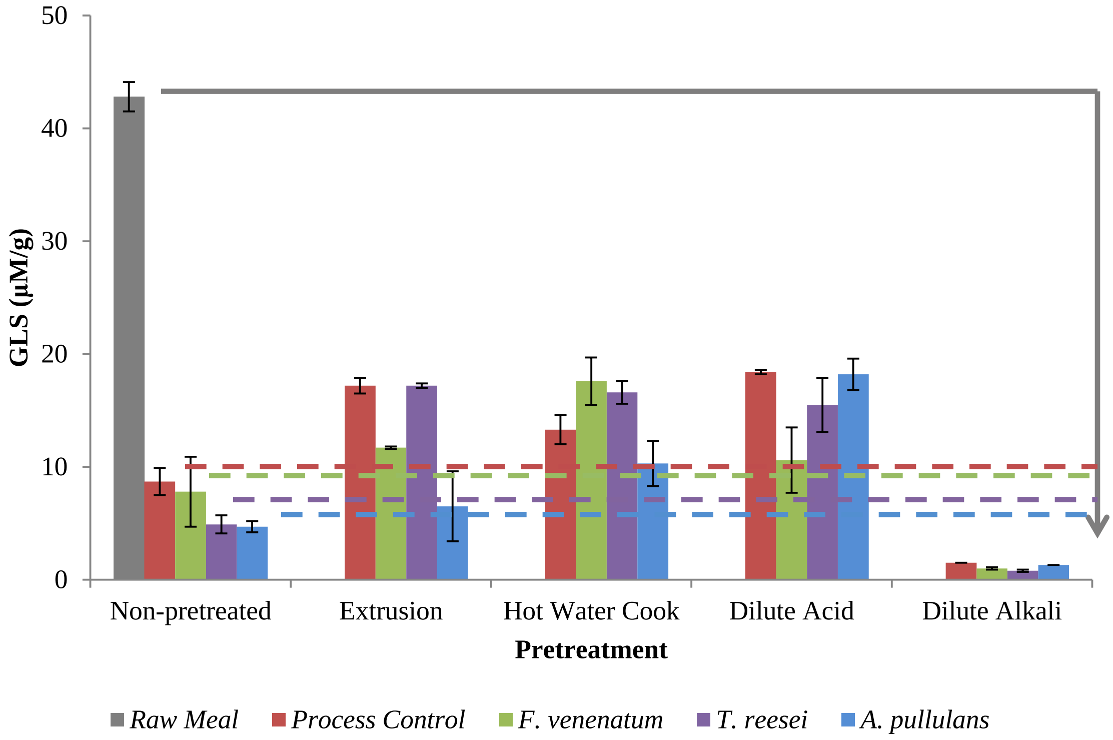
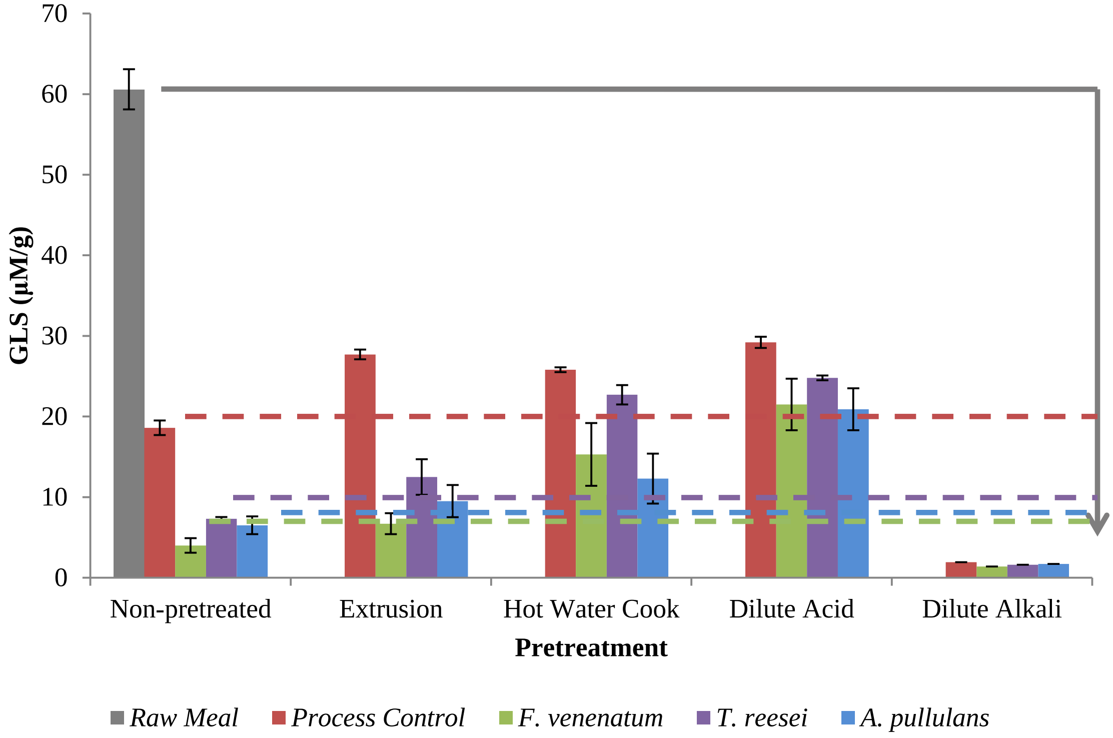


Figure 5.4b. Reduction of total GLS levels ± SD from raw CP canola meal by pretreatment and submerged fungal incubation



5.4. Conclusions

Pretreatment, followed by submerged fungal incubation, improved the nutritional content of canola meal. Extrusion and *T. reesei* (NRRL-3653) incubation resulted in the greatest overall improvement to HE canola meal, increasing protein to 51.5% and reducing NDF, GLS, and residual sugars to 18.6%, 17.2 µM/g, and 5% w/w, respectively. Extrusion and *F. venenatum* (NRRL-26139) incubation performed the best with CP canola meal, resulting in 54.4% protein while reducing NDF, GLS, and residual sugars to 11.6%, 6.7 µM/g, and 3.8% w/w, respectively. Further trials will be completed in bioreactors to produce a more controlled environment and process control.

Chapter VI – Summary and Conclusions

Canola production has rapidly expanded over the past 40 years, rising from the sixth largest oilseed crop to the second largest (Ash 2012). On a worldwide basis, canola meal is second only to soybean meal for use as a feed (Newkirk 2009). De-fatted canola meal generally contains 35-36% protein, 12% crude fiber, and a high content of minerals and vitamins (Khattab and Arntfield 2009). Canola meal contains less digestible energy and protein than soybean meal, but over three times as much fiber (Bell 1993).

The main limitation of meals from canola and other *Brassica* spp. is the presence of glucosinolates (GLS), which are anti-nutritional and can even be toxic at high ingestion levels (Tripathi and Mishra 2007). Furthermore, large amounts of GLS can reduce palatability for livestock and thus reduce intake and growth rates (Bonnardeaux 2007). For this reason canola was bred to contain less than 30 μmol/g GLS (Newkirk 2009; Bonnardeaux 2007). However, feed inclusion rates are still limited to ~30%, and this reduces the value of canola meal (Newkirk 2009). To increase the nutritional value of canola meal, pretreatment and solid-state versus submerged fungal incubation methods were used to screen the performance of seven metabolically diverse fungal strains.

For many types of filamentous fungi, solid-state incubation more closely replicates the natural environment (absence of free water) to which fungi are adapted (Couto and Sanromán 2006). Lower drying costs and the ability to use smaller incubation vessels, compared to submerged incubation processing, can help minimize industrial processing costs (Smits et al. 1993). However, the main disadvantages of solid-state incubation are mass transfer and control of temperature, pH and moisture (Holker et al. 2004).

Solid-state incubation with various fungal strains enhanced the nutritional composition of canola meal (Chapter III). *T. reesei* (NRRL-3653), *A. pullulans* (NRRL-58522), and *A. pullulans* (Y-2311-1) resulted in the greatest improvement in protein content, exhibiting maximum protein increases of 22.9, 16.9 and 15.4%, respectively, in solid-state incubated CP canola meal. These treatments also resulted in the reduction of total GLS content to the greatest extent, ranging from 89.4-99.1% and 82.4-98.3% in HE and CP canola meal, respectively. Fiber levels were increased due to a “concentration effect” as sugars and GLS were metabolized.

Submerged incubation has been defined as processing in the presence of excess water, and has been a proven large-scale process due to easier material handling, process control, and improved standardization (Singhania et al. 2010; Chicatto et al. 2014). Submerged incubation with various fungal strains improved the nutritional content of canola meal (Chapter VI). *T. reesei* (NRRL-3653), *F. venenatum* (NRRL-26139), and *A. pullulans* (Y-2311-1) resulted in the greatest improvement in protein content in HE canola meal (34.8, 23.8, and 21.0%), respectively, while reducing total GLS and residual sugar content by 82.6-93.7% and 89.3-94.6%. In trials with CP canola meal, the same three fungi increased protein levels to the greatest extent (37.3, 35.2, and 24.6%), respectively, while reducing total GLS and residual sugar content by 89.3-93.5% and 93.8-98.1%.

Based on this work, three fungal strains were down-selected and used to evaluate four pretreatment methods to enhance breakdown of the fiber fraction (Chapter V). Pretreatment methods included extrusion, hot water cook, dilute acid, and dilute alkali. Pretreatment, followed by submerged fungal incubation, improved the nutritional content of canola meal. Extrusion and *T. reesei* (NRRL-3653) incubation resulted in the greatest overall improvement to HE canola meal, increasing protein to 51.5% and reducing NDF, GLS, and residual sugars to 18.6%, 17.2 μM/g, and 5% w/w, respectively. Extrusion and *F. venenatum* (NRRL-26139) incubation performed the best with CP canola meal, resulting in 54.4% protein while reducing NDF, GLS, and residual sugars to 11.6%, 6.7 μM/g, and 3.8% w/w, respectively.

Future trials should be conducted in bioreactors under more controlled conditions of aeration and agitation, where better mass transfer can be achieved. Research into alternative pretreatments should also be evaluated, as well as the use of a separate saccharification step using pectinase and cellulase enzymes. Once sufficient fiber conversion into simple sugars is achieved, nitrogen supplementation during fungal incubation should be studied to determine if protein levels can be further increased. Converted canola meal should then be used in fish feeding trials to determine palatability, digestibility, and growth performance.

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