INVESTIGATION OF SOLID-STATE FERMENTATION TO ENHANCE THE NUTRITIONAL VALUE OF COLD-PRESSED AND HEXANE-EXTRACTED CANOLA MEALS, AND THE FUNCTIONAL PROPERTIES OF EXTRACTED CANOLA PROTEIN

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Saskatoon

By

Chenghao (Charles) Li

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Dean
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University of Saskatchewan
116 Thorvaldson Building, 110 Science Place
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ABSTRACT

Canola meal is the byproduct of the canola oil extraction process and is rich in protein (30%-45%), total dietary fibre (~33%), B-vitamins and essential minerals. However, its utilization in animal feed and food industry is limited by the presence of anti-nutritional factors especially phytic acid and total phenolic content. The overall goal of this thesis was to apply solid-state fermentation (SSF) using *Aspergillus niger* NRRL 334 and *Aspergillus oryzae* NRRL 5590 to hexane-extracted (HE) and cold-pressed (CP) canola meal to study the effect on the reduction of antinutrients and functionalities of extracted canola protein products. In study 1, a significant reduction of phytic acid (~80%) was found after SSF, whereas a decrease in TPC from 2.7-3.1 to ~1.0 mg (gallic acid equivalent)/ g dry meal (~65% to 81% reduction) was reported. Oil content of CP meal decreased from ~12% to 9%. An increase in crude protein level was observed upon SSF with both strains (from ~36% to ~40%) while minor changes were found for ash content. In study 2, functionality of protein products extracted from fermented meals using salt-extraction (SE) and alkaline extraction-isoelectric precipitation (AE-IP) were tested. Protein products obtained using SE showed high protein content (~95%), while products extracted using AE-IP showed much lower protein content (56.5% to 85%). After SSF, solubility of protein products decreased at pH 3 but increased at pH 7. Increases were found in water and oil holding capacities of protein products after SSF. All protein products displayed emulsifying activity index values ranging from 5.6 to 21.1 m^2/g and emulsion stability index values from 1.1 to 4.5 min. All canola protein products showed a foaming capacity ranging from 154.4% to 480.0% with foaming stability ranging from 68.0% to 89.0%. Overall, SSF using *Aspergillus niger* NRRL 334 and *A. oryzae* NRRL 5590 has showed the potential to improve the nutritional value of both CP and HE meals by increasing the protein content while reducing the levels of phytic acid and total phenolic compounds. After 72-hour SSF, both beneficial and adverse impacts were found on functionality of protein products extracted from fermented meals.

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ABBREVIATIONS

AE-IP	Alkaline extraction-isoelectric precipitation method
CP	Cold-pressed canola meal
CPI	Canola protein isolate
EAI	Emulsifying activity index
ESI	Emulsifying stability index
FC	Foaming capacity
FS	Foaming stability
GAE	Gallic acid equivalents
GI	Gastrointestinal
GLS	Glucosinolates
HE	Hexane-extracted canola meal
NDF	Neutral detergent fibre
OHC	Oil holding capacity
PAC	Phytic acid content
SDS	Sodium dodecyl sulphate
SE	Salt extraction-dialysis method
SSF	Solid-state fermentation
TPC	Total phenolic content
WHC	Water holding capacity

1. OVERVIEW

1.1 Introduction

Canola was firstly introduced in Canada in 1979 to identify rapeseed varieties that produce oil with low erucic acid (< 2%) and glucosinolates (GLS) (< 30 μmol/g dry meal) contents (Canola Council of Canada, 1990). Since then, Canada has become a world leader in the large-scale production of canola (Kimber *et al.*, 1995). According to the Canola Council of Canada, the yield of canola was more than 20 million tonnes in 2018 (decreased to 18 million tonnes in 2020), which largely contributes to the worldwide canola industry (Canola Council of Canada, 2020). After oil extraction, approximately 60% of canola seed weight separates into meal (Newkirk *et al.*, 2021). The leftover meal is rich in protein (30%-45%) and total dietary fibre (~33%) and is typically used for animal feed markets (Canola Council of Canada 2009). Canola meal has a favorable pattern of essential amino acids and as such, could be used as a feedstock for further protein processing. It also has other advantages including low cost and good environmental sustainability attributes to contribute to the economic value and industrial interest of canola meal. Canola proteins also display excellent functionality attributes as protein ingredients which can be used in a range of food applications. Although canola protein has favorable biological value and balanced amino acid composition, its use for animal feed and further for human food is limited by the high total dietary fibre content (~33%) and the presence of anti-nutritional factors such as GLS, phytic acid, polyphenols and sinapine (Croat *et al.*, 2017). Besides the anti-nutritional factors, the proteins of canola meal are less soluble and difficult to recover because of the process-induced changes during oil extraction processes and possible interactions between protein and antinutrients.

Solid-state fermentation (SSF) is one of the most common fermentation methods whereby there is nearly no free water in the solid substrate, making it viable for certain fungi to grow. Recently, researchers have employed SSF with fungi such as *Aspergillus*, *Rhizopus*, *Trichoderma*, and *Fusarium* for canola meal and achieved a significant reduction in GLS and GLS breakdown products (thiooxazolidones), phytic acid, and neutral detergent fibre (NDF) levels with an enhancement in protein content. The magnitude of the decline of antinutrients (GLS) and increase

In protein content depend on the conditions of SSF including types of ferment strains, water activity (Aw), temperature, substrate oxygen concentration, pH and time. Among them, types of ferment strains, temperature, and fermentation time play a more important role than others due to the low moisture content of SSF (Couto and Sanromán, 2006). Therefore, it could be possible to use SSF as a pretreatment to the canola meal to reduce levels of the anti-nutritional factors and to improve protein recovery rates and modify the functional quality such as solubility, and emulsifying, foaming and oil/water holding ability. As such, it is important to understand the effects of SSF conditions on the ability to reduce the level of antinutrients and to increase protein recovery from canola meal.

The risk of adverse health effects including non-communicable diseases (*e.g.*, stroke and cardiovascular diseases) because of a high consumption of protein-rich animal food, such as processed meat or red meat has become a significant consumer concern in consuming animal products (Abete *et al.*, 2014). These diseases may be related to the components of animal-based foods, such as saturated fatty acids, potential carcinogens and atherogenic methionine metabolite homocysteine (Micha *et al.*, 2010). Thus, plant proteins have drawn special attention due to their low cost, high sustainability, potential health benefits as well as their positive environmental impact and comparable functionality and digestion compared to animal protein (Ahnen *et al.*, 2019). Among plants, rapeseed (*Brassica napus*) is considered as an important crop for its high-quality oil and agro-industrial by-product, constituted of approximately 30% to 45% of proteins, which is known as canola meal. However, the remaining protein is mainly used for feed and non-food applications, or even regarded as waste (Leip *et al.*, 2014). With the continuous growth of world population, more protein is demanded for human consumption and animal feed. More options on protein resources and the diverse utilization of protein are therefore required. The use of by-products such as canola meal as a protein resource can provide more protein options.

Within this research, cold-pressed (CP) and hexane-extracted (HE) canola meals will undergo a pre-treatment of SSF with *Aspergillus niger* NRRL 334 or *A. oryzae* NRRL 5590 prior to alkaline extraction-isoelectric precipitation (AE-IP) or salt-extraction-dialysis (SE) to produce canola protein products. To identify the optimal fermentation conditions, the effects of fungal strain type and fermentation time will be studied to screen for the nutritional value of canola meal (*i.e.*, reduction in phytic acid and polyphenol compounds and increase in crude protein content). Degree of protein hydrolysis (DH) and pH will also be determined to study the fermentation process.

Canola proteins of the meal produced using optimized SSF conditions for each strain and meal will be processed by two different methods: AE-IP and SE and then will be characterized for their crude protein content and functionality including protein (nitrogen) solubility, water/oil holding capacity, foaming (foaming capacity and stability) and emulsifying (emulsification activity and stability indices) properties.

1.2. Objectives

Specific objectives include:

1) To examine the effects of SSF conditions (types of fungi strains and canola meals and fermentation time) on composition (increase in certain nutrients and reduction in antinutrients) of CP and HE canola meals.
2) To examine the effects of SSF on protein isolation production using AE-IP and SE methods. Canola meal fermented with optimal SSF conditions will be used for protein isolation.
3) To characterize the selected functional properties of canola protein products produced from fermented meals by AE-IP and SE.

1.3. Hypotheses

The following hypotheses were tested in this research:

1) SSF with *Aspergillus niger* NRRL 334 and *Aspergillus oryzae* NRRL 5590 under controlled moisture and temperature conditions will be able to improve the nutritional value of canola meal available from cold-press processes and hexane-extraction process by increasing the protein content while reducing the content of antinutrients namely, phytic acid and phenolic compounds.
2) By increasing the fermentation time with the above-mentioned organisms under SSF, canola meal with better quality (higher in protein content and lower in antinutrients content) can be produced.
3) Canola protein products obtained using AE-IP and SE methods from different meals (unfermented and fermented under different conditions) will show significant differences in the functional properties including protein (nitrogen) solubility across various pH, water/oil holding capacity, emulsifying and foaming properties.

2. LITERATURE REVIEW

2.1. Canola meal processing

Generally, Canadian canola seed contains ~45% of oil (Tetteh, *et al.*, 2019). Hexane-extraction, the conventional process for extracting canola oil involves thermal treatment at ~110°C followed by extraction with a non-polar solvent (most commonly hexane) at ~60°C for approximately 1 h followed by desolventization, which leads to a high yield of oil but can be detrimental to the macromolecules within the meal, especially the proteins (Koubaa *et al.*, 2016; Kalaydzhiev *et al.*, 2019). Cold pressing extraction refers to a process where no solvent is applied and the process only involves mechanically crushing the seed at low temperature to collect the oil (Febrianto *et al.*, 2011). During the pressing step, seeds are directed into the mechanical press where the temperature shortly rises to 50-60°C. The cold pressing process therefore leaves CP meal containing protein and other components in a less disturbed form, allowing for better utilization in animal feed and human food. Cold pressing has important industrial advantages *i.e.*, high nutritional value, low energy consumption and low equipment cost. Unfortunately, the oil extraction rate of cold pressing is not optimal (Xie *et al.*, 2019) compared to the hexane-extraction process and leaves a higher level of residual oil in CP meal.

Both the chemical composition and nutritional value of canola meal can be influenced by many different factors that are closely related to the processing technology and conditions (*e.g.*, type of oil press, temperature and humidity during the extraction process). Leming *et al.* (2005) studied the chemical composition of canola meal obtained from expeller pressing at 60ºC; cold pressing. According to their results, CP canola meal contained crude protein (30.6%), non-protein compounds (28.2%) and crude fibre (11.2%) but had high levels of crude fat (17.8%). The higher energy content of the CP meal than HE meal is mainly due to its high oil content. There were no significant differences in crude ash, calcium and phosphorus content between CP and HE meals.

2.2. Chemical composition

The composition of canola varies depending on its varieties and growing conditions. In general, Canadian canola seed contains high amounts of oil (~45%) and proteins (~30%) with less than 2% of erucic acid in the oil and less than 30 µmol/g of GLS in oil-free meal (Slominski *et al.*, 2012, Ghazani and Marangoni, 2013). The oil is characterized by its low content of saturated fatty acids. Canola meal, the by-product of canola oil extraction, has ~40% of protein and a relatively high content of minerals and vitamins compared to other plant-based meal such as soy (Khattab and Arntfield, 2009). A moisture content of 11.4%, a crude ash content of 6.3% and a nitrogen-free extract content of 43.3% was also reported (Khattab & Arntfield, 2009). The major protein constituents of canola protein are seed storage proteins napin and cruciferin, and also a smaller amount of structural protein, oleosins. Napin is a 2*S* (*S* is a Svedberg Unit) albumin protein, whereas cruciferin is a 12*S* globulin-type protein that represent 20% and 60% of the total protein content of mature seeds, respectively (Wu and Muir., 2008). Napin is a small protein of low molecular weight (12.5-14.5 kDa), characterized by basic nature caused by the high level of amidation of amino acids and possesses good foaming properties because of its low molecular weight (Schmidt *et al.,* 2004). Cruciferin is a hexameric protein with a high molecular weight of 300~310 kDa and is known to have good gelling properties. As for oleosin, it is a structural protein associated with oil bodies. Other minor proteins in canola meal include thionins, trypsin inhibitors and lipid transfer proteins (Bérot *et al.,* 2005).

Canola protein is considered as an attractive protein source due to the balanced amino acid profile. The amino acid composition of canola meal is nutritionally balanced especially with relatively higher levels of lysine and sulfur-containing amino acids (especially methionine, cysteine) compared to other plant proteins (Aider *et al.*, 2011). Among them, lysine is still considered as the first limiting amino acid and also the most temperature sensitive, partaking in several reactions such as the Maillard reaction (Newkirk *et al.*, 2003). In addition, the 3.0% to 4.0% sulfur-containing amino acid content in canola meal is closer to the requirements of the Food and Agriculture Organization (FAO) for humans' consumption compared to any other vegetable protein available (Ohlson and Anjou 1979). Besides, canola meal also shows a higher protein efficiency ratio (2.64) than that of soybean (2.19) (Delisle *et al.*, 1984). As mentioned above, there are two major fractions of protein in canola. They showed differences in amino acid profile. Chabanon *et al.* (2007) studied the amino acid profile of napin and cruciferin. Napin isolate showed

lower contents of isoleucine, phenylalanine, tyrosine and sulphur-containing amino acid (methionine and cysteine), while higher levels of lysine, valine and glutamate compared with the cruciferin isolate.

2.3. Antinutritional factors

2.3.1. Antinutrients in canola meals

Although canola meal has a favorable biological value and nutritionally balanced amino acid composition, further application is limited by the high fibre content (~32%) and the presence of anti-nutritional factors such as GLS (18 to 30 μmol/g meal), phenolic compounds (~5 mg gallic acid equivalents (GAE)/ g dry meal) and phytic acid (5%-7%). The impact of these components can lead to undesirable properties of canola meal including relatively inferior physicochemical properties, poor digestibility, objectionable colour, and bad taste (Wu and Muir 2008). Challenges such as protein extraction from biomass and antinutritional factors also arise. The interactions between proteins and certain compounds as phytic acid, fibres, and polyphenols can result in difficulties in protein extraction and a possible loss of the protein functionalities (Serraino and Thompson, 1984; Wanasundara, 2011). In addition, those antinutrients can also bring toxic effects and undesirable colour of the extracted canola protein (Tan *et al.*, 2011).

Phenolic compounds (mainly sinapic acid) are major antinutrients found in canola meal with levels of 17.7 g/kg seeds in *Brassica napus* (Ismail *et al.*, 1981) and ~5 mg GAE/ g dry meal in common canola meal. This level is almost 30-times higher than that found in soybean meal (Shahidi and Naczk, 1992). They have been considered as inhibitors of enzymes and protein digestion, as well as interfere with iron within the gastrointestinal (GI) tract, limiting its absorption. They also deliver a bitter or astringent taste to the meal (Naczk *et al.*, 1998). Phytic acid (5%-7%) is the second largest amount of the antinutrients found in canola meal. They associate with protein, starch, and fibre to form matrices and thereby reduce their digestibility. In addition, they reduce the bioavailability of minerals mainly by chelation with divalent cations (Serraino *et al.*, 1984; Das *et al.*, 2013). Phytic acid can range from 5% to 7% in canola meal depending on the variety and oil extraction process used (Embaby *et al.*, 2010). The level of GLS in canola meal ranges between 18 to 30 μmol/g oil-free meal and has been shown to have anti-nutritional or toxic effects in animal studies (Lücke *et al.*, 2019; Topps, 1992). Thus, it is necessary to further process canola meal to

minimize the level or eliminate these phytochemicals to make the meal more suitable for animal and even human consumption.

2.3.2. Reduction strategies of antinutrients

In order to improve the value and extend the utilization of canola meal, several methods including physical treatment (during processing or induce extra process) and chemical solvent extraction have been used to reduce the levels of antinutrients over the last decades.

2.3.2.1 Physical treatment

Physical treatment including heat, steam and pressure has been used to reduce the antinutrients in canola meals at an early stage due to the close connection between physical treatment and parameters of oil extraction process. Jensen *et al.* (1995) studied the effect of heat treatment on GLS and nutritional value of several commercial canola meal samples (from Denmark, Germany and China). Decreases in total content of GLS of 24%, 46%, 70% and 95% were found after toasting at 100°C for 15 min, 30 min, 60 min and 120 min, respectively. However, an adverse impact was reported on amino acid content including decrease of total lysine content from 5.93 to 4.91 g per 16 g nitrogen (N) and 12% loss of cysteine after 120-min toasting, while other amino acids remained relatively unchanged. A linear decrease in protein solubility was also found from 85% to 81%, 61%, 52% and 40% after the respective toasting times ($p < 0.05$). Heat treatment is a fast and cheap method to remove the GLS, but it could denature the canola protein and lead to a loss of certain amino acids. In addition, the effect of heat treatment on other antinutrients still remains unclear and the parameters including temperature and time for different antinutrients and meals needs to be optimized.

Gu *et al.* (2011) evaluated the steam explosion with different steam pressures (1.0, 1.2, 1.4, 1.6, 1.8, 2.4, 3.2 and 4.0 MPa) and different treatment times (30, 60, 90, 120, 150 and 180 s) as a detoxification method for rapeseed meals. A reduction rate of 99% on GLS could be achieved within 180 s when the steam pressure was increased to 1.6 Mpa or higher. The GLS breakdown products including isothiocyanates, oxazolidinethiones and nitriles, were also decreased by 97, 93 and 59%, respectively. However, lysine, arginine, aspartate, cysteine, methionine and tryptophan contents were dropped by 21%, 24%, 14%, 13%, 17% and 8%, respectively. Similar to heat

treatment, steam explosion is a fast and simple method, but has adverse impacts on amino acids. In addition, whether other antinutrients can be reduced by steam is uncertain.

2.3.2.1 Solvent-extraction

Solvent-extraction is one of the most common methods used to remove or extract certain components from materials due to its low cost, simplicity and availability in industry. Dietz *et al.* (1991) introduced the aqueous extraction to remove GLS from defatted broken rapeseeds. Results indicated that ~96% of the GLS could be removed with a seed/water ratio of 1:20 and an extraction time of 60 min in a single batch, while 98% of GLS could be removed by cross-current extraction over three stages at a seed/water ratio of 1:10. However, they also reported a crude protein loss of 8.6% during cross-current extraction. According to their conclusions, ethanol treatment of the rapeseed meal efficiently reduced phenols (no data reported) and GLS contents, while decreasing protein levels. In terms of the loss of amino acids, the processing also resulted in a decrease of lysine, glycine and glutamate contents of approximately 1 g/ 100 g protein. In addition, the effect of aqueous extraction on other antinutrients was not included.

Das *et al.* (2013) compared the efficacy of different solvent mixtures containing acetone or methanol, pure or combined with water or an acid (hydrochloric, acetic, perchloric, trichloroacetic, phosphoric), for removing antinutrients (phenolic compounds, tannins, GLS, allyl isothiocyanates, and phytates) from canola meal. Canola meal was mixed with solvent mixture at a 1:10 ratio (w/v) and allowed continuous stirring at 200 rpm for 2 h under 25°C. After centrifugation, the supernatant was then collected for analysis. The authors suggested a 0.2% $HClO_4$-containing mixture (1:1, v/v) could achieve the optimal result for extraction of the antinutrients (polyphenols, phytates, allyl isothiocyanates) from canola meal. However, the effect of different solvent systems on certain antinutrients remains unclear and the effect on other components such as protein and amino acids was not studied.

Recently, Kalaydzhiev *et al.* (2020) studied the influence of ethanol pre-treatment of the rapeseed meal on the nutritional value of canola meal (contents of antinutrients and amino acid composition) and protein extractability. The ethanol treatment was achieved by continuous stirring meal with 75% aqueous ethanol solution at a meal to solvent ratio of 1:3 (w/v) for 30 min at room temperature (four times). Results showed a significant reduction ($p < 0.05$) on both phenolics (from 0.5% to 0.18%) and GLS (from 85 to 5 µmol/g) contents after treatment. The protein content was

increased from 37.4% to 42.3% while the lipid concentration dropped from 1.9% to 1.1%. They also reported a reduction in lysine content after the ethanol treatment of canola meal. As for protein extractability, protein yield decreased from 26.4% to 23.6% after ethanol treatment on meal, while a higher decrease was found from 47.8% to 26.4% after ethanol treatment on the rape seeds. The results indicated the ability of ethanol treatment to remove the phenols and GLS from commercial canola meal. However, the treatment also brought adverse impacts on amino acid content and protein solubility, which affects the protein extraction process.

Overall, heat and steam methods were proved to be able to reduce the antinutrients especially GLS to desired level (< 2% in final product). Properties such as low cost, timesaving, simplicity and availability in industry make them suitable for industrial use. However, more studies are required to better understand the effect of heat and steam explosion on antinutrients besides GLS. Also, the adverse impact on protein and amino acid caused by heat and pressure limited the utilization of these two methods. As for solvent-extraction, a higher reduction rate on antinutrients and fat was reported compared to simple physical treatment. It has become one of the most common methods to detoxify canola meal. But the unavoidable denaturation of protein could lead to difficulty in further extraction and utilization of canola protein. A more effective method with less adverse impact on protein is therefore needed.

2.4. Solid-state fermentation

2.4.1. SSF and use of SSF for oilseed meals

In order to remove the antinutrients while leaving the rest of the compounds undisturbed as much as possible, the SSF process was introduced to canola meals. Fermentation is the process accomplished by the metabolism of microorganisms that catalyze nutrients, synthesize secondary metabolites, and complete other physiological activities under anaerobic or aerobic conditions. Solid-state is one of the most common fermentation methods whereby there is nearly no free water in the solid substrate. The solid-state is preferred to the natural habitat of certain filamentous fungi (*e.g.*, *Aspergillus, Rhizopus, Trichoderma,* and *Fusarium*). The main advantage of SSF is a sufficient supply of oxygen. In industrial production, SSF is energy-saving and has less organic wastewater with higher product yield than submerged-type fermentation. However, there are some disadvantages of SSF compared to submerged-type or liquid-state fermentation. For instance, heat

transfer efficiency in SSF is low, the parameters are difficult to monitor and control, and the design and amplification of bioreactors are complicated.

Recently, SSF has been applied to canola meal to improve the quality and extraction rate of canola protein. Pal and Walia (2001) used *Rhizopus oligosporus* as the ferment culture to produce a high-protein product from HE canola meal. The results showed a significant decline on contents of glucosinalates (~43%), thiooxazolidones (~31 %), phytic acid (~42%), and fibre (~26%) after 10-day SSF. In addition, a ~65% increase was reported for crude protein. Lücke *et al.* (2019) obtained similar results using SSF with *Rhizopus microsporus* on cold-pressed canola meal with approximately 14% oil left. SSF was achieved under the conditions of 90%–95% relative humidity and 32°C for 30 to 48 h. A significant ($p < 0.05$) reduction on the contents of neutral detergent fibre was reported from 19.1% to 8.9%, while an increase of crude protein (10.6%) and oil content (10.7%) was found. In their experiments, GLS contents were reduced from 32.1 to 27.5 μmol/g dry meal, by 14.3%. In addition, a reduction on polyphenols from 1586 to 1170 GAE / 100 g (26.2%) was found.

Shi *et al.* (2015) used *Aspergillus niger* to ferment HE canola meal and detected the levels of crude protein, GLS, trichloroacetic acid soluble protein (TCA-SP), neutral detergent fibre (NDF) and amino acid in fermented canola cake after 72 h of incubation at 34°C. After SSF, a 77% decrease in GLS was reported. TCA-SP, crude protein and ether extract contents were increased by 104%, 23% and 24%, respectively. In addition, the contents of NDF and phytic acid declined by 9% and 45%, respectively. Total amino acids and essential amino acids contents as well as *in vitro* digestibility of canola meal were improved significantly. Moreover, the enzyme activity of endoglucanase, xylanase, acid protease and phytase were also increased during SSF. Fibre levels increased due to the concentration effect of removing oligosaccharides and GLS.

Croat *et al.* (2016) determined the optimal ferment culture among *Aureobasidium pullulans* NRRL-58522, *A. pullulans* NRRL-42023, *A. pullulans* NRRL-Y-2311-1, *Tricho-derma reesei* NRRL-3653, and *Fusarium venenatum* NRRL-26139 in SSF of HE or CP canola meal to increase the protein content while reducing GLS. SSF was achieved under the conditions of 50% moisture content and 30°C for 168 h. According to the results, *Trichoderma reesei* (NRRL-3653), *Aspergillus pullulans* (NRRL-58522), and *A. pullulans* (NRRL-Y-2311-1) improved protein content by 22.9%, 16.9% and 15.4 % (d.b.) respectively, while reducing the total GLS content from 60.6 to 1.0, 3.2 and 10.7 μmol/g dry meal, respectively. After SSF, a significantly higher

content of dry matter yield was reported in HE meal compared to CP meal, which is mainly due to the high oil residual in CP meal. Slightly higher levels of protein content and yield were found in CP meal after SSF. This may indicate the adverse impact of high temperature (~110°C) and other extreme processing conditions on canola protein during the hexane-extrication and desolventization.

These results indicate that the SSF offers an effective approach to improving the nutritional quality of rapeseed proteins by removing antinutrients (*e.g.*, GLS and phytic acid). Among the various factors affecting SSF, besides the microorganisms involved in fermenting the substrate, water activity (A_w), temperature, substrate oxygen concentration, pH and time are important considerations. In addition, some pre-treatments have been proved to show the ability to enhance the effects of SSF on canola meal fermentation including extrusion processing, hot water cooking, addition of phytase, dilute acid and dilute alkali (Croat *et al.*, 2017; Rodrigues *et al.*, 2017).

2.4.2. Parameters affecting SSF

2.4.2.1 Water activity

Water activity (A_w) relates to the content of available water for microorganisms. In general, bacteria require an $A_w > 0.85$, for yeast > 0.80-0.90 and for fungi > 0.60-0.70. For aerobic fungi, the water film tension becomes the main limiting factor affecting mycelial extension due to the low rate of oxygen diffusion in water, which is only 1/200,000 of that in air (Chen *et al.*, 2013). The increase in water content will hinder mycelial stretching through the pores of the substrate. In addition to meeting the physiological requirements of the microbes, the water content plays an important role in the variation of the three-phase structure related to water retention, water permeability and thermal conductivity.

2.4.2.2. Fermentation temperature

The fermentation temperature also affects microbial growth, metabolism and spore germination of microorganisms. Because of poor heat conductivity and accumulation of metabolic heat in the material and decreased porosity during fermentation, gas convection is restricted (Chen *et al.*, 2013). Heat buildup becomes the typical effect of temperature on SSF due to microorganism metabolism. Previous studies showed that the major resistance to heat transfer in SSF was low

conduction efficiency (Sargantanis *et al.*, 1993). Moisturizing is a common measure of temperature control. Routine operations (*e.g.*, forced ventilation, jacket cooling) can also help solve these problems. Evaporative cooling is one of the main SSF temperature control measures. It can take away 60%–80% of calories from the substrate. The evaporative cooling rate can be adjusted by regulating the forced ventilation airflow rate and the water content of the medium. In general, the temperature gradient of the medium can be reduced by increasing the forced ventilation airflow rate. It is difficult to maintain the temperature at an ideal range in a large-scale SSF system, thus, coupling of ventilation, temperature, and humidity is usually a control measure used in large-scale SSF (Lenz *et al.*, 2004).

2.4.2.3. Substrate oxygen concentration

Oxygen is a key factor affecting microbial growth and metabolites production in SSF (Oostra *et al.*, 2001). Oxygen consumption rate and carbon dioxide production are applied to assess the state of the SSF process. However, the values and assessments can vary for different microorganisms. Gowthaman *et al.* (1993) studied the impact of gas concentration gradient on average enzyme activity in a packing bed bioreactor. The results showed that the gas concentration gradient can be eliminated, and the ability of mass transfer can be enhanced by forced ventilation, which indicated an increase in enzyme activity. Ghildyal *et al.* (1994) studied the impact of the gas concentration gradient on average enzyme activity in a tray solid-state fermentation bioreactor by *Aspergillus niger*. The results showed that the difference between O_2 and CO_2 concentration gradients was obvious, which significantly affected the product yield. With gradient increasing, the yield decreased. Oostra *et al.* (2001) concluded the intra-particle oxygen diffusion combined with moisture can also affect the SSF. Results showed that the optimal oxygen transfer in SSF depended on the available interfacial gas–liquid surface area and the thickness of the wet fungal layer. It is suggested that the moisture content of the substrates affects both above parameters and, therefore, plays an important role in oxygen transfer in SSF.

2.4.2.4. pH

The pH value also plays an important role in SSF. However, it is difficult to measure and control the pH values during the fermentation in the whole system. Because of the low water

content of the substrate, the pH values are difficult to determine by conventional detection. In many fermentation processes, the pH values change. Materials with a buffering capacity are often used as a substrate to eliminate the adverse effects of the changing pH values. For example, nitrogen-containing inorganic salts (such as urea) are sometimes used as nitrogen sources to offset the fermentation process pH variation (Sargantanis *et al.*, 1993). Jiang *et al.* (2012) studied a rapid method to determine pH in SSF of wheat straw by FT-NIR spectroscopy and efficient wavelengths selection. Liu *et al.* (2013) detected variable pH in SSF process by FT-NIR spectroscopy.

2.4.2.5. Fermentation time

Fermentation time also plays an important role in SSF. Results from the study of Croat *et al.* (2016) showed the impact of fermentation time on surface colonization of various fungi (*Aureobasidium pullulans* NRRL-58522, *A. pullulans* NRRL-42023, *A. pullulans* NRRL-Y-2311-1, *Tricho-derma reesei* NRRL-3653 and *Fusarium venenatum* NRRL-26139) during SSF on both cold-pressed and hexane-extracted canola meal. In general, a period of 3 or 4 days is required to achieve the desired results (*e.g.*, strain growth, enzyme activity, residual antinutrients, and protein content). However, the fermentation time also depends on the type of strains and other conditions mentioned above. Thus, specific testing is advised in order to determine the best ferment time.

2.5. Extraction of canola protein

According to previous studies, canola protein isolates are isolated from other components including seed coats (mainly crude fibre fraction) and alcohol solubles (sugars, GLS and some phenolics) from defatted meal, to give an enriched protein content up to ~70% (Wanasundara, 2011). Protein isolate formation (>90% protein on a moisture-free weight basis) involves further removing most of the unwanted non-protein components (*e.g.*, fibre, residual oil, antinutrients) to achieve a highly pure form. Depending on the method of protein extraction employed, the final product could vary in terms of the protein content, fractions, and extent of interaction with non-protein components. In general, alkali extraction and isoelectric precipitation (AE-IP), salt extraction-dialysis (SE), protein micellation method (PMM) and low pH extraction combined with membrane separation have been used for canola protein isolates extraction (Tan *et al.*, 2011; Wanasundara, 2011).

2.5.1. Alkali extraction-isoelectric precipitation

Canola proteins become soluble when the pH value is away from its isoelectric point due to charge repulsion, while aggregation occurs at their isoelectric point. Thus, canola protein isolate (CPI) can be prepared from defatted meal using alkaline extraction (diluted NaOH solution) followed by isoelectric precipitation (AE-IP), centrifugation, neutralization, and then spray dried or freeze dried. The use of alkali produces strong conditions (pH 11 to 12) that can lead to a high nitrogen solubility and protein extraction rate from the canola meal (Wanasundara, 2011). During commercial canola oil processing, high temperatures and exposure to non-polar solvent have been reported to result in a set of undesirable reactions such as protein denaturation, which can affect the extraction of canola protein (Moure *et al.,* 2006; Pedroche *et al.,* 2004). In addition, several factors including pH, temperature and solid to liquid ratio can also lead to different results. Thus, it is essential to control the steps and parameters during AE-IP of canola protein. According to previous studies shown in Table 1.1, a pH value between 11 and 12.5 and a solid to liquid (S/L) ratio of 5 to 10 is preferred during alkaline extraction under temperatures ranging from 55 to 75°C to obtain higher yields of canola protein. These parameters were chosen according to preliminary studies, with the highest protein extraction yield achieved at pH 12.5 and 75°C and S/L ratio of 5.14 (Rodrigues *et al.,* 2012). An optimal protein extraction yield (72.1%) was achieved with alkaline extraction at pH 12.5 and 75°C for 60 min and S/L ratio of 10 after phytase pre-treatment, whereas a yield of only 51.3% was previously obtained with alkaline extraction as a unique step as shown in Table 1.1.

Table 1.1 Comparison of alkaline extraction-isoelectric precipitation (AE-IP) procedures of canola protein isolate in different studies.

	Aluko *et al.* (2005)	Pedroche *et al.* (2004)	Rodrigues *et al.* (2012)	Souza *et al.* (2016)
Alkaline extraction	A 1:10 (w/v) of meal 0.1 N NaOH solution ratio, stirred at 23℃ for 20 min; then filtration.	A 1:10 (w/v) of meal 0.2% NaOH solution ratio at pH 11, stirred for 1 h, twice.	A 1:5 (w/v) of meal and water ratio and stirred at pH 12.5 and 75℃ for 90 min.	A 1:6 (w/v) of meal and water ratio and stirred at pH 12.5 and 75℃, after the addition of phytase.
Centrifugation	10000 × *g* for 30 min at 8◦C.	8000 rpm for 25 min.	1520 × *g* for 15 min.	1520 × *g* for 15 min.
Drying	Freeze-dry.	Freeze-dry.	Freeze-dry.	Freeze-dry.
Extraction yield	n.d.*	n.d.*	51.3%	72.1%

*n.d.: not determined.

2.5.2. Salt extraction-dialysis

Salt extraction-dialysis (SE) is one of the most common methods to prepare canola proteins recently due to the high final protein content. Protein extraction with salt solutions have been used to prepare CPI from defatted meals due to the high solubility (> 90%) of canola proteins in salt solution (Cheung *et al.,* 2014). Cruciferin, a salt-soluble globulin protein (12*S*, MW ~300 kDa) dominates the canola proteins (~60%) while a smaller protein fraction napin (1.7-2*S*, MW ~14–17 kDa) only consists of ~20% of canola protein (Hoglund *et al.*, 1992, Chabanon *et al.*, 2007, Cheung *et al.*, 2014). In addition, low concentration salt solutions enhance protein solubility due to the "salting-in" effect. The interactions between ions in the solution and the protein charges weaken the protein–protein interactions and increase the protein solubility by limiting protein aggregation.

Several studies as shown in Table 1.2 have proved the applicability of SE to obtain CPI with a high protein content over 90%. Klassen *et al.* (2011) used salt extraction followed by dialysis to remove salt and small molecules to obtain canola protein isolates with a composition of 95.0% of protein (w.b., %N × 5.70), 0.7% of moisture, 0.3% of lipid, 2.3% of ash and 1.7% of carbohydrate. Karaca *et al.* (2011) showed that CPI with a crude protein content of 93.1% (w.b., %N × 6.25) can be obtained by SE. A similar method was used by Chang *et al.* (2015) who obtained a CPI with a crude protein content of 99.1% (w.b., %N × 6.25). In the study of Cheung *et al.* (2014), a modified method without dialysis based on the patent of Murray *et al.* (1980) was used to prepare a cruciferin-rich CPI with a crude protein content of 90.3% (w.b., %N × 6.25). Details of above studies were shown in Table 1.2.

Table 1.2. Comparison of salt extraction-dialysis procedures of canola protein isolate in different studies.

	Klassen *et al.* (2011)	Cheung *et al.* (2014)	Chang *et al.* (2015)	Karaca *et al.* (2011)
Salt extraction	Defatted canola meal : 0.1 M NaCl solution at pH 7 (Tris–HCl buffer) at 1:10 (w/v) under continuous stirring for 2 h at room temperature.	Defatted canola : 0.2 M NaCl solution at pH 5.8–6.3 at 1:10 (w/v) under constant stirring (500 rpm) for 90 min at room temperature.	Defatted canola : 0.1 M NaCl at pH 7 (0.05 M Tris–HCl buffer) at 1:10 (w/v) under continuous stirring for 2 h at room temperature.	Defatted canola : 0.2 M NaCl solution at pH 5.8–6.3 at 1:10 (w/v) under constant stirring (500 rpm) for 90 min at room temperature.
Supernatant recovery centrifugation	18,600 × *g* for 1 h at 4°C twice.	17,700 × *g* for 20 min at 4°C. Vacuum filtration (Whatman No. 1 filter paper).	3000 × *g* for 1 h. Filtration (No. 1 Whatman filter paper).	17,700 × *g* for 20 min at 4°C. Vacuum filtration (Whatman No. 1 filter paper).
Dialysis	Spectro/Por® tubing, 6–8 kDa cut off, at 4°C for 48 h.	Dilution to 10× volume and settle overnight. Spectro/Por® tubing, 6–8 kDa cut off, at 4°C for 48 h.	Spectro/Por® tubing, 6–8 kDa cut off, at 4°C for 72 h.	Dilution to 10× volume and settle overnight. Spectro/Por® tubing, 6–8 kDa cut off, at 4°C for 48 h.
Centrifugation	18,600 × *g* for 2 h at 4°C.	Desalted protein micelles were directly collected.	3000 × *g* for 1 h twice.	Desalted protein micelles were directly collected.
Drying	Freeze-drying.	Freeze-drying.	Freeze-drying.	Freeze-drying.
Protein content	95.0 % (w.b., %N × 5.70).	90.3% (w.b., %N × 6.25)	99.1% (w.b., %N × 6.25)	93.1% (w.b., %N × 6.25)

2.7. Functionalities of canola protein isolates

Over the past decades, continuous efforts are being made to optimize extraction methods and improve canola proteins functionalities for its potential applications in the food industry. Moreover, several factors (high temperature, pressure and chemical factors) during the oil extraction underwent by the meal can cause heat denaturation of the protein, which further affects the functionality. In addition, SSF can lead to possible positive or negative impacts in canola protein extraction and functionality performance as an ingredient or product. Thus, it is important to study the functionality of canola protein from unfermented and SSF fermented meals.

2.7.1. Solubility

For food applications, protein solubility is an important parameter that influences other functional attributes (emulsifying, foaming, and gelling properties). Factors including protein structure (charge and hydrophobicity, isoelectric point) along with solution pH, temperature and salts (type and concentration) can affect the solubility (Cheung *et al.*, 2015). Solubility of a cruciferin-rich CPI prepared by Cheung *et al.* (2014) was examined over a pH range (3.0, 5.0 and 9.0) and NaCl concentration (0, 50 and 100 mM), and was the lowest at pH 5.0 and 7.0 without NaCl (<20 %), however greatly improved (>80 %) when pH shifted to 3.0 and 9.0 or NaCl was added. Wanasundara *et al.* (2012) mentioned the two main canola proteins, cruciferin and napin, have different solubility behavior. For instance, napin was more soluble between pH 2 and 5 than cruciferin, due to their different isoelectric points, estimated for cruciferin at pH 5-7 (Schwenke *et al.*, 1981) and for napin at pH 11 (Crouch and Sussex, 1981). Cheung *et al.* (2014) studied the impact of pH and NaCl on the solubility of napin protein isolate (NPI). Solubility was determined to be between 93.4% and 100% and was similar at pH 3.0 (98.2%) and pH 5.0 (99.2%), however was slightly reduced at pH 7.0 (96.3%). Solubility of NPI was also similar at NaCl levels of 0 mM (96.8%) and 100 mM (96.5%), however were completely soluble with the addition of 50 mM NaCl (salting-in effect). Although significant differences were found among the treatments (pH, $p < 0.001$; NaCl concentration, $p < 0.05$), their associated interaction was not significant different ($p > 0.05$) and solubility for all NPI solutions remained high (~93.3%).

2.7.2. Emulsifying properties

Canola proteins are surface-active agents that are capable of stabilizing oil-in-water emulsions. The emulsion is a dispersion of oil droplets within a continuous aqueous phase, formed after the input of mechanical energy with the presence of an emulsifier. Several studies reported on the feasibility of using canola proteins in emulsion-type foods. In general, emulsion activity index (EAI), emulsion stability index (ESI), emulsion capacity (EC), creaming stability and average droplet size are used to determine and compare the emulsifying properties of different proteins. Karaca *et al.* (2011) determined the emulsifying properties of both alkaline extracted and salt extracted CPI including EC, EAI, ESI. Emulsions were prepared with 20% canola oil (w/w) and 1% protein (w/w). Both EC and EAI for CPI produced by salt extraction (528.9 g oil/g protein and 35.1 m^2/g, respectively) were greater than the alkaline extracted isolate (502.2 g oil/g protein and 15.0 m^2/g, respectively), while ESI values measured for both production methods were similar (~ 10.5–15.5 min). Tan *et al.* (2014) studied the emulsifying properties of alkaline extracted CPI, napin- and cruciferin-rich fractions, and found that both individual proteins were capable of stabilizing emulsions at pH 4.0, 7.0 and 9.0. The individual isolates exhibited higher EC (184–331.2 g oil/g protein) than a mixed protein isolate obtained by alkali extraction and precipitation at pH 4 (92–174 g oil/g protein) or a commercial soy protein isolate (92–276 g oil/g protein). Cheung *et al.* (2014) studied the impact of pH (3.0, 5.0, and 7.0) and NaCl concentration (0, 50, and 100 mM) on the emulsifying properties of CPI. Surface hydrophobicity was the lowest for CPI at pH 7.0 with 100 mM NaCl, and highest at pH 3.0 without NaCl. Interfacial tension was lowest at 10-11 mN/m for pH 5.0 with 0 mM NaCl and pH 7.0 with 50/100 mM NaCl, while under all other conditions interfacial tension was higher (> 15 mN/m). As NaCl levels increased from 0 to 100 mM, EAI stayed stable at pH 3.0 (18.8 and 19.4 m^2/g), whereas decreased from 21.1 to 12.8 m^2/g at pH 5.0 and decreased from 14.9 to 5.2 m^2/g at pH 7. ESI was reduced with the addition of NaCl from ~15.7 to ~11.6, and ~12.0 min at 50, and 100 mM NaCl concentration, respectively.

2.7.3. Foaming

Similar to emulsions, foams are mixtures of two immiscible phases with gases and water representing the dispersed and continuous phases, respectively. Protein-based foams are used in the food industry such as meringues, mousses, beer and whipped desserts. An energy input (*i.e.*, whipping, sparging, pouring) is required to form foams. After whipping, proteins tend to migrate

to the gas-water interface due to the amphiphilic nature with hydrophobic amino acids towards the gas phase while hydrophilic ones towards the water phase, and then form a gel-like film surrounding the gas which resists against rupturing (Cheung *et al.*, 2014). This film is connected by adjacent proteins to create a cage-like network with entrapped gas to constitute the foam structure. Foam capacity (FC) is related to properties of the protein such as surface hydrophobicity, conformation/flexibility, size and level of denaturation. Foam stability (FS) is typically best at a pH near the pI of the protein, where repulsive electrostatic forces are minimum. More viscous protein solutions tend to produce more stable foams, as liquid drainage from the protein cage-like network is reduced. Pedroche *et al.* (2014) studied the foaming properties of canola isolates and reported a FC of 280% at pH 10. A much lower result was found by (Galves et al., 2019) using a canola protein concentrate (66% protein content), where the FC was 35%, 32% and 162% at pH 3, 5 and 7 respectively with a relatively low FS of < 60% for all pHs.

2.7.4. Water holding capacity

Water holding capacity (WHC) is defined as the amount of water that can be absorbed by one gram of protein. It significantly affects the ability of ingredients to absorb and retain water, which can improve the water binding capacity of the food product, enhance its flavour retention, improve its mouthfeel and adjust the moisture of food products. Pedroche *et al.* (2014) reported a WHC of 1.0 g/g protein for canola protein isolate. Aider and Barbana. (2011) compared the WHC of acid and alkaline extracted canola proteins and found the alkaline extracted canola protein showed higher WHC than that of acid extraction. They concluded that the WHC is mainly determined by the amount of hydrophilic binding sites of proteins (types of proteins), which is able to bind more water. They also found that the defatting process had no effect ($p > 0.05$) on WHC of canola protein.

2.7.5. Oil holding capacity

The oil holding capacity (OHC) is defined as the ability to absorb and entrap oils, which is important in food applications because fat acts as a flavour retainer, a consistency trait and an enhancer of mouthfeel (Tan *et al.*, 2011b). In general, the oil holding capacity depends on several properties, such as type of protein, powder particle size, surface tension and hydrophobicity.

Generally, proteins with higher amounts of exposed hydrophobic sites on the surface tend to absorb a greater amount of oil per gram protein. An OHC of ~2.4 g/g protein was reported by Galves *et al.* (2019) using a canola protein concentrate. The result was in agreement with Pedroche *et al.* (2014) who reported an OHC of ~2.5 g/g protein for CPI. In general, the OHC is negatively correlated with WHC (Ghodsvali *et al.*, 2005; Aider and Barbana., 2011). In addition, the OHC can be modified by different treatments, such as heat treatments, which are associated with the denaturation of proteins and exposure of non-polar residues buried in the interior of the protein molecules (Kinsella & Melachouris, 1976).

2.7.6. Gelling ability

In general, all proteins can form a gel under the right conditions but vary in gel strength and structure. The gelling abilities at high temperatures can be determined by the least gelation concentration, which indicates the minimal protein concentration required to produce a gel that is strong enough not to slide down the walls of an inverted tube (Moure *et al.*, 2006). In addition, the gelation of proteins increases with molecular weight (size) because large molecules can form extensive networks by cross-linking in three dimensions (Oakenfull *et al.*, 1997). The protein gel network is stabilized mainly through hydrogen-bonding, hydrophobic interactions and covalent cross-links such as disulfide bonds (Damodaran, 2008). Kim *et al.* (2016a) studied the impact of pH on the gelling properties of CPI and compared it to commercial soy protein isolates (SPI). CPI could not form a gel at pH 3.0, whereas gels were formed with the onset temperature of 68, 79, and 79°C at pH 5.0, 7.0, and 9.0, respectively. They found that CPI performed a better gel and stronger networks than SPI with the presence of weaker inter- and intramolecular junction zones.

3. Effect of solid-state fermentation on the composition of cold-pressed and hexane-extracted canola meals.

Abstract

Canola meal, a by-product and valuable material from industrial oil-extraction processing, deserves particular attention due to its high protein and carbohydrate (fibre) content. The effect of solid-state fermentation, including strains (*Aspergillus niger* NRRL 334 and *Aspergillus oryzae* NRRL 5590) and fermentation time (24, 48 and 72 h), were found to have different influences on the composition of both cold-pressed (CP) and hexane-extracted (HE) canola meals. In this study, changes to the composition of both canola meals were examined after a controlled solid-state fermentation pre-treatment. An increase of protein content of both CP and HE meals was reported (from ~36% to ~40%) while minor changes were found for other components. Oil content of CP meal decreased from ~12% to 9% after fermentation, which may help during protein extraction for obtaining high protein ingredients. A significant reduction (~80%) of phytic acid content was found after fermentation using *Aspergillus niger* NRRL 334 and *Aspergillus oryzae* NRRL 5590 on both CP and HE meal, whereas all fermented samples showed a decrease in total phenolic content from 2.7-3.1 to ~1.0 mg GAE/ g DM (~65% reduction) with an exception for HE (*A. niger*) sample which had a greater decrease from 3.1 to 0.6 mg GAE/ g DM (~81% reduction). These changes indicated the potential and availability of SSF using *A. niger* and *A oryzae* in modification of canola meal composition and further improving the nutritional value by increasing the protein content while decreasing the content of antinutrients.

3.1. Introduction

SSF is a common fermentation method whereby there is nearly no free water in the solid substrate, making it acceptable for fungi to grow. Compared to chemical or physical processes, SSF showed some advantages including higher product concentration, higher product recovery, simple equipment requirements, less wastewater output, lower capital investment, and lower plant operation cost (El-Batal and Abdel, 2001). Recently, researchers have employed SSF with fungi strains of *Aspergillus*, *Rhizopus*, *Neurospora, Trichoderma*, and *Fusarium* (Croat *et al.*, 2017, Simon *et al.*, 2017 and Lücke *et al.*, 2019) for canola meal and achieved a significant reduction in GLS (Shi *et al.*, 2015), GLS breakdown products such as thiooxazolidones, phytic acid (El-Batal *et al.*, 2001), total phenolic compounds and neutral detergent fibre (NDF, Pal *et al.*, 2001) levels with an enhancement in protein content.

Ebune *et al.* (1995) studied the effect of SSF conditions (moisture content of media, inoculum age and homogenization) using *Aspergillus ficuum* NRRL 3135 on the production of phytase and reduction of phytic acid content of canola meal. As a result, optimum conditions were reported as: 64% of media moisture content with an inoculum age between 2 and 5 days with homogenization. Under optimal conditions, they found a total reduction (> 99%) of phytic acid could be achieved after 4-d SSF. Later, El-Batal and Abdel (2001) studied the effect of media moisture content, addition of glucose, phosphate, some surfactants and gamma irradiation on the production of phytase and reduction of phytic acid in canola meal fermented using SSF with *Aspergillus niger*. Optimum results included a 60% of media moisture content, 6% of glucose concentrations in solid state culture (SSC), and phosphate concentration of 0.5 mg per SSC system. The addition of surfactants Tween 20, Tween 40, Tween 60, Tween 80 as well as oleic acid increased the phytase production. Gamma irradiation at a dose level at 1 kGy also increased the amount of phytase. Pal and Walia. (2001) employed SSF using *Rhizopus oligosporus* to canola meal and achieved a reduction in the contents of glucosinolates, thiooxazolidones, phytic acid and crude fibre by 43.1%, 34%, 42.4% and 25.5%. An increase in nitrogen and protein contents of the meal was also reported. Croat *et al.* (2016) applied SSF with *Aureobasidium pullulans* (NRRL 58522), *A. pullulans* (NRRL 42023), *A. pullulans* (NRRL-Y-2311-1), *Trichoderma reesei* (NRRL 3653), and *Fusarium venenatum* (NRRL 26139) to thermal pre-treated hexane-extracted (HE) and cold-pressed (CP) canola meal and achieved a glucosinolates reduction up to 99% and 98% for

both meals. Later, they combined an extrusion pretreatment with SSF (*F. venenatum*) which resulted in a greater reduction in NDF, glucosinolates and residual sugars to 11.6%, 6.7 μmol/g and 3.8% (w/w), respectively (Croat *et al.*, 2017). Simon *et al.* (2017) employed *Neurospora crassa* NRRL 2332 as SSF culture and achieved a complete reduction of GLS in HE and CP meals with a concomitant increase in crude protein content

These studies showed the applicability of SSF in the production of a protein-enriched canola meal with low antinutrients content that has great potential for feed and food application. The magnitude of the decline of antinutrients and increases in protein content depend on the conditions of SSF including types of ferment strains, water activity (A_w), temperature, substrate oxygen concentration, pH and time. Thus, it is important to understand the effects of SSF on the changes of canola meal composition to further utilize this processing method.

In this study, SSF was used as a pre-treatment to improve the nutritional value of canola meals. The effects of SSF on two types of canola meal substrates (cold-pressed and hexane-extracted), at different fermentation times (0, 24, 48 and 72 h), with two types of fungal strains (*Aspergillus niger* NRRL 334 and *A. oryzae* NRRL 5590, both have Generally Regarded as Safe or GRAS status) were examined. It was hypothesized that SSF would produce a more useful canola meal with enhanced nutritional value (increase in protein content and decrease in antinutrients) for livestock species and possible food applications.

3.2. Materials and methods

3.2.1. Materials

Commercial cold-pressed meal was obtained from Pleasant Valley Oil Mills (Clive, AB, Canada). Commercial hexane-extracted meal was gifted from Bunge Canada (Harobe, MB). Fungal strains *Aspergillus niger* NRRL 334 and *A. oryzae* NRRL 5590 were GRAS status obtained from Agricultural Research Service (ARS) Culture Collection (NRRL, Peoria, Illinois, US). All media, reagents and chemicals used were analytical grade and obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada).

3.2.2. Sample preparation

3.2.2.1. Preparation of fungi spore suspensions

Both *Aspergillus niger* NRRL 334 and *A. oryzae* NRRL 5590 were incubated on potato dextrose agar (PDA) plates at 30°C for 7 d. Spore suspensions were prepared using deionized water, followed by spore counting using direct microscopy counting (Leica, Model 97 S6E, Wetzlar, Germany) with a hemocytometer (Bright-Line, Horsham, PA, USA). Spore suspensions were prepared before inoculation.

3.2.2.2. Solid-state fermentation

HE and CP canola meals (200 g of each) were fermented with *Aspergillus niger* NRRL 334 and *A. oryzae* NRRL 5590 spore suspensions. Both *Aspergillus* strain spore suspensions were standardized to a spore concentration of 10^7 colony forming units (CFU) to apply per gram of meal prior and used as the starter culture for fermentation according to previous studies (Shi *et al.*, 2015; Croat *et al.*, 2016 and 2017). The canola meal, spore suspension and deionized water were mixed at speed 5 for 3 min using a commercial stand mixer (Pro 600, KitchenAid, US) before spreading out thinly (< 1.5 cm) and evenly onto a stainless-steel sheet pan. Fermentation was started at 50% moisture content (w/w), at 30°C over a 72-h period in an Isotemp incubator (Fisher Scientific, Model 650D, Waltham, MA, USA). Mill-Q water was added according to the weight loss each day to maintain the moisture content. Samples (~ 50 g) were collected on random spots on each batch at the time of initial inoculation 24, 48 and 72 h, freeze-dried and then saved for later analysis. To stop the fermentation, the meals were frozen at –20°C right after fermentation until further freeze-drying. Both dried powders were stored at 4°C until further testing.

3.2.3. Physicochemical and composition analysis

3.2.3.1. pH determination

The pH value was determined by measuring the pH of fermented and unfermented meal slurry using a pH meter. The slurries were prepared by mixing 1 g of dried meal and Milli-Q water at 1:10 (w/v) ratio and allowed to stir for 30 min at room temperature (21-23°C). Measurements were made in triplicate on each of the replicate batches (n = 3) and reported as the mean ± one standard deviation.

3.2.3.2. Degree of protein hydrolysis

The protein hydrolysis was measured as the released free amino groups using 2,4,6-trinitrobenze sulfonic acid (TNBS) method according to Adler-Nissen (1979) and Jung *et al.* (2005). In brief, freeze-dried meal containing 1 g of protein (corrected by protein content) was added to distilled water, followed by stirring for 30 min at room temperature (21-23℃). The mixture was centrifuged (Sorvall RC-6 Plus centrifuge, Thermo Scientific, Asheville, NC, USA) at 6000 × g for 15 min at room temperature and the supernatant was carefully collected. Then, 250 µL of the supernatant was added to 2 mL of 20 mM sodium phosphate buffer at pH 7.8 followed by the addition of 2 mL of 0.01% TNBS solution. After mixing with a votex for 15 s, the mixture was placed in a water bath at 50℃ for 1 h covered by aluminium foil to protect the samples from lights. The reaction was stopped by adding 4 mL of 0.1 N HCl and allowed to cool for 10 min. The absorbance value of the reaction mixture was recorded at 340 nm using a spectrophotometer (Genesys 10S UV-VIS, Thermo Scientific, Madison, WI, USA). The total acid hydrolysis was also measured as part of the degrees of hydrolysis calculation according to methods of Adler-Nissen (1979) and Jung *et al.* (2005). The total acid hydrolysis was determined by adding 24 mg of sample (corrected by protein content) to a screw cap pyrex tube with 15 mL of 6.0 N HCl. The tubes were then incubated at room temperature (21-23ºC) for 20 h. The dispersion was then adjusted to pH 7.0 with 2 M NaOH, followed by filtration through Whatman Grade 3 filter paper. Then 250 µL of above sample was added to 2.00 mL of 1% SDS solution in buffer. This was followed by the addition of 250 µL of above mixed solution to 2.00 mL of 10 mM sodium phosphate buffer at pH 7.8. This mixed solution was performed in triplicate and analyzed by the previous method above. The sample blank was prepared by adjusting a solution of 6.0 N NaOH and 6.0 N HCl to obtain a pH of 7.0. A 1.5 mM glycine solution was used to create a standard curve to calculate the α-NH_2-glycine equivalents of each sample and used in following equations to calculate the degree of protein hydrolysis:

$$h = (h_t - h_c) \times DF \quad (3.1)$$

$$DH = \left(\frac{h}{h_{tot}}\right) \times 100\% \quad (3.2)$$

where h is the yield of hydrolysis equivalents of α-NH_2-glycine equivalents, h_t is the mM concentration of α-NH_2-glycine equivalents, h_c is the mM concentration of α-NH_2-

glycine equivalent at the time of initial inoculation before microorganism was added and DF is the dilution factor. Measurements were made in triplicate on each of the replicate batches (n = 3) and reported as the mean ± one standard deviation.

3.2.3.3. Proximate composition analysis

Crude protein was determined using the combustion nitrogen analyzer (FP-628 CAN, LECO Corp., Saint Joseph, MI, AACCI method 46-30.01) with a nitrogen conversion factor of 6.25. Ash was determined using 2 g of canola meal (CM) in a muffle furnace at 600°C for a 2-h period according to AOAC method 943.05 (AOAC 2005). Crude lipid was determined using the Goldfisch apparatus according to AOAC method 920.39 (AOAC 2005). Measurements were made in triplicate on each of the replicate batches (n = 3) and reported as the mean ± one standard deviation.

3.2.3.4. Anti-nutritional components: phytic acid and total phenolic content

Phytic acid content (PAC) was determined using the phytic acid assay kit (Megazyme Inc., Sydney, Australia). All measurements were performed in triplicate (n = 3) and reported as percentage of phytic acid on a dry weight basis.

Total phenolic content (TPC) was determined using the Folin-Ciocalteu method according to Olukomaiya *et al.* (2020) with some modification. In brief, 0.5 g of freeze-dried sample was extracted using 5 mL of 80% (v/v) methanol (1:10, w/v) for 15 min (stirring at 500 rpm at room temperature). The supernatant was carefully collected after centrifugation at 5000 × *g* for 10 min and filtered with a Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, United Kingdom) at room temperature. The remaining pellet was extracted using 80% (v/v) methanol again, followed by above centrifugation and filtration to obtain an aqueous phenolic extract for analysis. An aliquot of 0.1 mL of extract was mixed with 2.5 mL of 10% (v/v) Folin-Ciocalteu reagent. After a period of 2-min reaction time, 2.5 mL of 7.5% (w/w) sodium carbonate was immediately added and allowed to incubate in the dark for 30 min at room temperature. Absorbance was recorded at 750 nm using a spectrophotometer (Genesys 10S UV-VIS, Thermo Scientific, Madison, WI, USA). Gallic acid (reagent grade) was used as the standard. All

measurements were done by triplicate independent assays (n = 3) and reported as milligrams of gallic acid equivalents (GAE) per gram of dry meal (mean ± standard deviation).

3.2.3.5. Statistical analysis

Fermentation was made in triplicate using a separate plate and spore suspension (n = 3). A three-way analysis of variance (ANOVA) was used to study the statistical differences in composition and anti-nutritional factors as a function of meal type, fungal strains and fermentation time with a significance level of $p = 0.05$. A post-hoc Tukey's test (multiple comparison procedures) was used to detect statistical differences in fermentation time. All statistics were performed using the SPSS Version 28.0 software (IBM Corp. NY, IL, USA).

3.3. Results and discussion

3.3.1. Proximate composition, pH and degree of protein hydrolysis

HE and CP canola meals were fermented with *Aspergillus niger* NRRL 334 and *Aspergillus oryzae* NRRL 5590 over a 72-h period. After 48 h of fermentation, significant hyphae were observed, and spores started to form on the surface after 72 h of fermentation as shown in Figure 3.1 a-d. The pH for all fermentation experiments increased from ~pH 6 to pH 7-8 after the 72-h fermentation as shown in Table 1a. In contrast to the result of the present study, Olukomaiya *et al.* (2020) found a reduction in pH value from 6.26 to 6.13 after SSF for 7 days using *Aspergillus ficuum*, *Aspergillus sojae* and co-culture *Lactobacillus salivarius* on autoclaved solvent-extracted canola meal. The authors described this to be caused by an increase in organic acid during SSF especially when using *Lactobacillus salivarius*. Aljuobori *et al.* (2017) also reported a relatively stable value or a decrease in pH value when using *Lactobacillus salivarius*. The differences in results may be explained by the canola meal, fungi strains, fermentation conditions and methods of analyses used in these studies in comparison to the present study. In addition, the organic acids could be used as nutrients by *Aspergillus niger* and *A. oryzae* during SSF. Protein hydrolysis measured as the degree of hydrolysis (DH) increased from 15%-30% (24 h) to ~40% (48 h) and remained at 45%-50% after 72 h as shown in Table 1b. For the first two days, the DH increased quite fast due to the large surface area that is exposed to oxygen, which helped the hyphae to grow. Proteins were most likely hydrolyzed partially by proteases synthesized by fungal cells, which

resulted in the increase of the DH by releasing free amino acids. However, the relatively compact nature of the lower layers of particles of the fermenting meal significantly lowered the speed of growth because the fungi could not easily penetrate to the bottom of the solid substrate.

Changes to the proximate composition were similar for both strains fermented with CP and HE meals. As shown in Table 1a, crude protein was increased from ~34% (w/w) to ~37% (w/w) (9%-11% increase) after 72 h of fermentation regardless of the strains used for CP meals. However, protein levels remained relatively constant, ranging between 39%-41% (w/w) in HE meal during SSF. The concentration effect contributed to the increase of levels of crude protein and ash. The carbohydrates, including sucrose and fibre, acted as a carbon source for fungi to grow and carbon dioxide (CO_2) was produced as a result, which concentrated the levels of remaining compounds. With different or similar materials and strains, Wang *et al.* (2012) reported a 27% increase in crude protein content using composite strains with the presence of *Candida tropicalis* CICIM Y0079(T). Results also agreed with Plaipetch & Yakupitiyage (2014), who found a 9% of increase in crude protein in *Saccharomyces cerevisiae* yeast-fermented canola meal. Jessika *et al.* (2017) reported an increase of crude protein from 42% to 44%-50% (d.b.) in fermented HE meal and a ~14% of increase in crude protein content of fermented CP meal depending on the strains (*Aureobasidium pullulans* NRRL-58522 3.0, *Aureobasidium pullulans* NRRL-Y-2311-1, *Trichoderma reesei* NRRL 3653, *Fusarium venenatum* NRRL 26139, *Paecilomyces variotti* NRRL 1115, *Rhizopus microspores* var. *oligosporus* NRRL 2710, *Neurospora crassa* NRRL 2332, *Mucor circinelloides* and *Pichia kudriavzeii*) used. Increases in nitrogen contents of 11.6%, 23.1%, 34.2%, 47.6% and 65.4% after 1, 2, 5, 8 and 10 days of incubation, respectively, were reported by Pal and Walia (2001). Compared to HE meal, fermented CP meal had a lower original crude protein content ($p < 0.05$) due to the high oil content even after a 72-h period of SSF. The presence of high levels of residual oil in CP meal can be inhibitory to fungal growth and could lead to the result similar to Simon *et al.* (2017). They introduced and compared several strains (*Aureobasidium pullulans* NRRL-58522 3.0, *Aureobasidium pullulans* NRRL-Y-2311-1, *Trichoderma reesei* NRRL 3653, *Fusarium venenatum* NRRL 26139, *Paecilomyces variotti* NRRL 1115, *Rhizopus microspores* var. *oligosporus* NRRL 2710, *Neurospora crassa* NRRL 2332, *Mucor circinelloides* and *Pichia kudriavzeii*) when used as SSF cultures in HE and CP canola meals and reached a conclusion that fungal strains preferred HE meal compared to CP meal due to the low level of residual oil in the HE meal. The hypothesis for increased protein levels was mainly due to the concentration effect.

Fungi could also convert canola protein or amino acids into fungi protein or the chitin of fungi cell walls as non-protein nitrogen, which could result from the conversion of oligosaccharides and fibre to cell mass by fungi during fermentation (Simon *et al.*, 2017). A similar hypothesis was presented by Rozan *et al.* (1996) as the loss of dry matter at the expense of fermentable sugars (sucrose, oligosaccharides and fibre as carbon source) during fermentation by microorganisms, which are optimal sources of proteins, that led to an increase in nitrogen (crude protein). In contrast, crude lipid levels decreased from ~12% to ~9% (w/w) for CP meals with fermentation, whereas contents in HE meals decreased from ~3% to ~1% (Table 2b). A slight increase of ash content for the CP was found from ~6% to ~7% (w/w) after 72 h fermentation, whereas ash level of HE meals increased from ~8% to ~10% (w/w) (Table 2c).

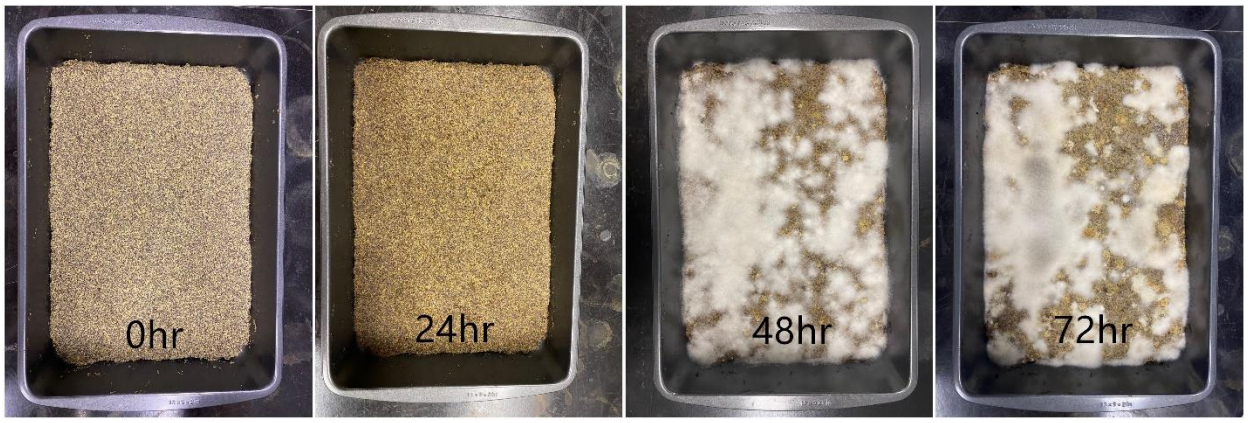

Figure 3.1 a. CP + *A. niger* NRRL 334

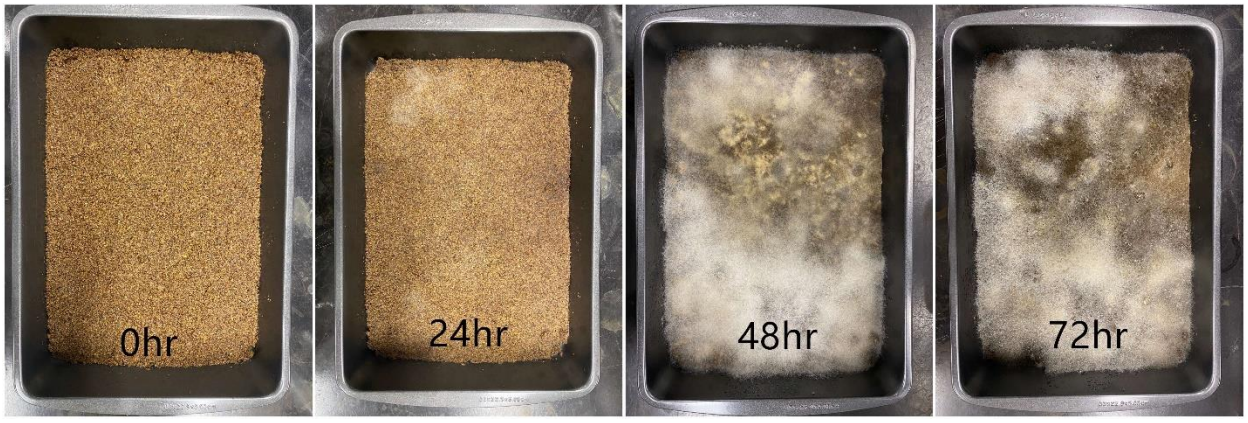

Figure 3.1 b. HE + *A. niger* NRRL 334

Figure 3.1 c. CP + *A. oryzae* NRRL 5590

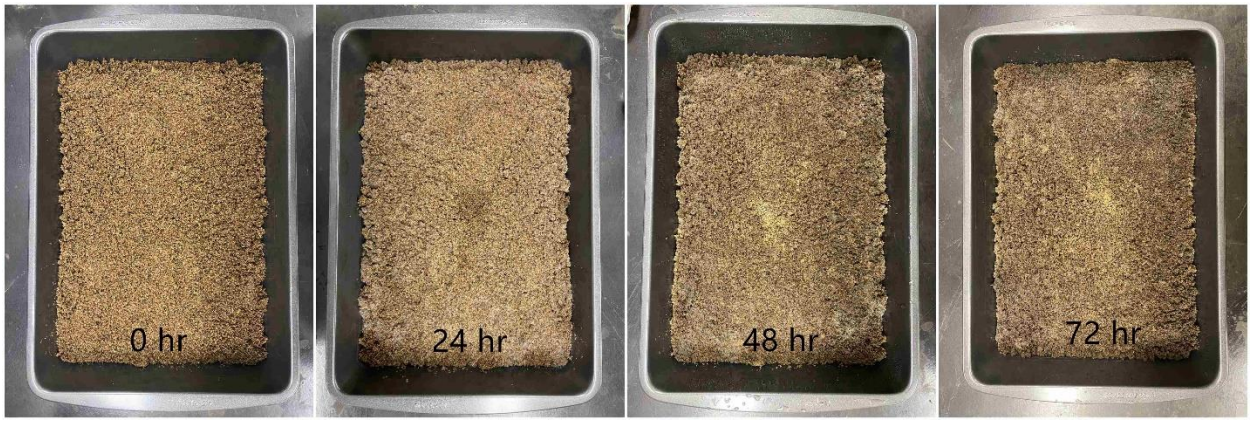

Figure 3.1 d. HE + *A. oryzae* NRRL 5590

Figure 3.1 a-d. Cold-pressed and hexane-extracted canola meals fermented with *Aspergillus niger* NRRL 334 and *A. oryzae* NRRL 5590.

Table 3.1. The pH value (a) and degree of protein hydrolysis (%, b) of fermented and unfermented control hexane-extracted (HE) and cold-pressed (CP) canola meals. Data was reported as mean ± one standard deviation (n = 3). Significant difference exists between data with different letters as a function of fermentation time ($p < 0.05$).

	Fermentation time (h)			
	0 h	24 h	48 h	72 h
a. pH				
CP – *A. niger* NRRL 334	5.9 ± 0.1^{a}	6.1 ± 0.3^{a}	7.0 ± 0.1^{b}	6.9 ± 0.2^{b}
CP – *A. oryzae* NRRL 5590	5.9 ± 0.1^{a}	6.6 ± 0.2^{a}	8.2 ± 0.1^{a}	8.2 ± 0.1^{b}
HE – *A. niger* NRRL 334	6.0 ± 0.2^{a}	6.8 ± 0.3^{b}	8.1 ± 0.2^{c}	8.2 ± 0.1^{c}
HE– *A. oryzae* NRRL 5590	6.0 ± 0.2^{a}	6.6 ± 0.2^{b}	8.2 ± 0.1^{c}	8.2 ± 0.1^{d}
b. DH (%, d.b.)				
CP – *A. niger* NRRL 334	0	30.1 ± 1.2^{a}	45.0 ± 1.0^{b}	46.4 ± 0.7^{b}
CP – *A. oryzae* NRRL 5590	0	28.3 ± 1.0^{a}	36.9 ± 1.0^{b}	45.1 ± 0.8^{c}
HE – *A. niger* NRRL 334	0	23.4 ± 0.9^{a}	41.9 ± 1.0^{b}	52.3 ± 2.5^{c}
HE– *A. oryzae* NRRL 5590	0	15.8 ± 1.7^{a}	43.7 ± 0.1^{b}	45.7 ± 0.4^{b}

Table 3.2. Crude protein, lipid and ash content of fermented and control hexane-extracted and cold-pressed canola meals. Data was reported as mean ± one standard deviation (n = 3). Significant difference exists between data with different letters as a function of fermentation time ($p < 0.05$).

	Fermentation time (h)			
	0 h	24 h	48 h	72 h
a. Crude protein (%, d.b.)				
CP – *A. niger* NRRL 334	33.9 ± 0.7^{a}	34.2 ± 1.0^{a}	36.5 ± 3.2^{a}	38.4 ± 3.3^{b}
CP – *A. oryzae* NRRL 5590	33.9 ± 0.7^{a}	34.3 ± 0.7^{a}	36.4 ± 2.1^{b}	36.1 ± 2.4^{b}
HE – *A. niger* NRRL 334	39.7 ± 0.1^{a}	40.3 ± 0.2^{ab}	41.5 ± 0.4^{b}	41.5 ± 1.0^{b}
HE– *A. oryzae* NRRL 5590	39.7 ± 0.1^{a}	40.6 ± 2.0^{a}	41.1 ± 1.8^{a}	40.7 ± 1.2^{a}
b. Crude lipid (%, d.b.)				
CP – *A. niger* NRRL 334	12.3 ± 0.2^{a}	12.7 ± 0.3^{a}	10.7 ± 0.7^{b}	9.5 ± 0.4^{c}
CP – *A. oryzae* NRRL 5590	12.6 ± 0.2^{a}	11.1 ± 2.0^{ab}	10.3 ± 1.8^{b}	8.9 ± 2.1^{c}
HE – *A. niger* NRRL 334	2.4 ± 0.4^{a}	1.6 ± 0.2^{b}	1.4 ± 0.2^{b}	0.9 ± 0.1^{c}
HE– *A. oryzae* NRRL 5590	2.5 ± 0.2^{a}	2.2 ± 0.2^{a}	1.2 ± 0.4^{b}	0.9 ± 0.1^{b}
c. Ash (%, d.b.)				
CP – *A. niger* NRRL 334	5.9 ± 0.1^{a}	6.6 ± 0.4a	7.0 ± 0.4^{a}	7.5 ± 0.8^{b}
CP – *A. oryzae* NRRL 5590	5.9 ± 0.2^{a}	6.6 ± 0.2^{ab}	7.2 ± 0.2^{bc}	7.4 ± 0.3^{c}
HE – *A. niger* NRRL 334	7.9 ± 0.2^{a}	8.7 ± 0.4^{a}	9.6 ± 0.6^{a}	9.9 ± 0.5^{b}
HE– *A. oryzae* NRRL 5590	7.8 ± 0.9^{a}	8.4 ± 0.5^{a}	9.7 ± 0.2^{b}	10.0 ± 0.3^{b}

3.3.2 Contents of phytic acid and total phenolic compounds

As for antinutrients, all fermented samples showed a decrease in PAC and TPC as shown in Table 3. For instance, PAC of CP meals decreased from 5.9% to ~1 % (w/w, 74%-85% reduction) with 72 h of fermentation, whereas for HE meals, PAC decreased from 5.9% to ~1.4 % (w/w, ~76% reduction). The reduction of phytic acid was achieved by the phytase synthesized by *A. niger* and *A. oryzae* during SSF. All fermented samples showed a decrease in TPC from 2.7-3.1 to ~1.0 mg GAE/ g DM (~65% reduction) with an exception for HE (*A. niger*) sample which had a greater decrease from 3.1 to 0.6 mg GAE/ g DM (~81% reduction). Overall, the reduction in PAC and TPC was correlated with fungal growth, indicating the high oil residual in CP meal was still available for *A. niger* NRRL 334 and *A oryzae* NRRL 5590 to grow without affecting the reduction on antinutrients (phytic acid and phenol compounds).

Lower reductions of phytic acid and total phenolic content were reported by Olukomaiya *et al.* (2020) where a ~20% reduction in PAC and ~17% reduction in TPC was acheived using *Aspergillus sojae* ATCC 9362 and *A. ficuum* ATCC 66876 on solvent-extracted canola meal at 45% moisture content and 30℃ for 7 days. Vig and Walia (2001) also reported a similar result that a maximum decrease (42.4%) of PAC occurred after 10-d fermentation using *Rhizopus oligosporus* with a water: rapeseed meal ratio of 1:3 at 25℃. The reduction of phytic acid by SSF is in agreement with studies of Nair and Duvnjak (1991) and Woods (1999). According to these two investigator groups, the reduction of PAC could be due to the production of phytase by fungi which results in the breakdown of phytic acid. Olukomaiya *et al.*, (2020) indicated the potential of SSF in increasing the activity of native phytase to hydrolyze the insoluble organic antinutritional complexes or a promising phytate-degrading ability of the *Aspergillus* strains (*A. niger, A. oryzae, A. sojae* and *A. ficuum*). The degradation of antinutritional compounds in canola meals might require multiple enzymes. Thus, low reduction of the antinutritional factors in previous studies could be explained by the lack of certain enzymes for specific antinutritional compounds when using different ferment strains. Most of the authors mentioned SSF with a single strain may have difficulty in hydrolyzing the diverse chemical compounds that antinutrients are complexed with due to the lack of specific enzyme or insufficient level of enzyme produced by the microorganism.

Table 3.3. Phytic acid (PAC) and total phenolic content (TPC) of fermented and control hexane-extracted and cold-pressed canola meals. Data was reported as mean ± one standard deviation (n = 3). Significant difference exists between data with different letters as a function of fermentation time ($p < 0.05$).

	Fermentation time (h)			
	0 h	24 h	48 h	72 h
a. PAC (%, d.b.)				
CP – *A. niger* NRRL 334	5.9 ± 0.1^{a}	2.8 ± 0.3^{b}	1.1 ± 0.9^{c}	0.9 ± 0.7^{d}
CP – *A. oryzae* NRRL 5590	5.9 ± 0.2^{a}	2.4 ± 0.2^{b}	1.9 ± 0.1^{c}	1.6 ± 0.1^{d}
HE – *A. niger* NRRL 334	5.9 ± 0.2^{a}	2.4 ± 0.3^{b}	1.9 ± 0.3^{c}	1.5 ± 0.8^{d}
HE – *A. oryzae* NRRL 5590	5.8 ± 0.1^{a}	1.9 ± 0.3^{b}	1.9 ± 0.1^{b}	1.3 ± 0.5^{c}
b. TPC (mg GAE/ g DM, d,b.)				
CP – *A. niger* NRRL 334	2.7 ± 0.1^{a}	1.9 ± 0.1^{b}	1.3 ± 0.1^{c}	1.0 ± 0.2^{d}
CP – *A. oryzae* NRRL 5590	2.7 ± 0.1^{a}	1.3 ± 0.3^{b}	1.2 ± 0.1^{c}	1.0 ± 0.1^{d}
HE – *A. niger* NRRL 334	3.1 ± 0.0^{a}	1.3 ± 0.1^{b}	0.7 ± 0.1^{c}	0.6 ± 0.0^{d}
HE– *A. oryzae* NRRL 5590	3.1 ± 0.0^{a}	2.0 ± 0.1^{b}	1.2 ± 0.1^{c}	1.0 ± 0.0^{d}

*Values = mean ± one standard deviation. Different letters in a row represents a significant difference at the level of $p < 0.05$.

PAC: phytic acid content; TPC: total phenolic content.

mg GAE/ g DM: milligram gallic acid equivalents (GAE) per gram of dry sample.

3.4. Conclusions

SSF using *Aspergillus niger* NRRL 334 and *Aspergillus oryzae* NRRL 5590 can be applied on both CP and HE canola meal to modify the composition respectively. Enzymes from fungal cells played critical roles during the fermentation processing including lipase, proteinase and phytase. The longer fermentation time (0, 24, 48, 72 h) can result in a higher reduction in crude fat of CP meal (from 13% to 9%), and PAC (74% to 85%) and TPC (65% to 81%) of both CP and HE meals, while a higher increase in crude protein content (from ~33% to ~37%) of CP meal and higher degree of protein hydrolysis (reached to ~50% after 72 h). These changes were correlated with the fermentation time and an optimal result was found at the time of 72 h.

As for the fungal strains *A. niger* NRRL 334 and *A oryzae* NRRL 5590, similar results were found during the fermentation processing. However, *A. niger* NRRL 334 showed better performance to reduce the level of PAC of CP meal and TPC in HE meal. Similar results were found between CP and HE meals with the exception of still a higher crude lipid content (~9%) in CP meal. Both of the strains were unable to decrease the crude fat content to further lower levels (~2%) to achieve a defatting process without extra chemicals and extraction procedure. In addition, a higher level of crude protein (~40%) was found in HE meal compared to CP meal, which is mainly due to the original high oil content (~12%) in CP meal. Besides, the high residual oil in CP meal was still available for *A. niger* NRRL 334 and *A oryzae* NRRL 5590 growth without significantly affecting the reduction of the antinutrients (phytic acid and phenol compounds).

3.5. Linkage to the next study

Over the past few decades, SSF with various fungal strains under different conditions has been investigated for canola meal to modify meal chemical composition. These studies have not been extended to obtain protein isolates from the fermented canola meals or investigated whether these compositional modifications affect the functional properties that canola proteins can provide. One drawback for rapeseed/canola meal protein isolates production is the low protein recovery (approximately 20%) which makes it not favored in industry (Chabanon *et al.*, 2007). To increase the value and market integration of canola meal as a source of plant proteins, a greater understanding of the relationships between modifications of SSF and the functionalities of the proteins of the fermented substrate is needed. Thus, the effect of SSF on the functional properties of canola protein ingredients has become an important part of this study to further understand the potential utilization of canola meal in the food industry. As the results reported in study 1, a protein content of 36-41% was found after a period of 72-h fungal SSF in both CP and HE canola meals. It was hypothesized that the SSF may be able to improve the canola protein isolation process due to the compositional changes of CP and HE meals reported in study 1. As indicated by DH values, meal proteins may be partially disintegrated, released from their complexation with other molecules, therefore improvements in solubility and extractability are expected. In addition, a reduction of oil content was found after SSF (~13% to ~9%) in CP meal, which may also help the protein extraction process. The next study will investigate protein extractability and selected functional properties of extracted proteins of SSF fermented CP and HE meal in comparison with unfermented counterpart meals.

4. Use of fermented canola meals for protein isolation, and assessment of selected functional properties of resulting products

Abstract

Canola meal has gained significant industrial interest due to its highly functional proteins and well-balanced amino acid profile. The effects of solid-state fermentation (SSF) with *Aspergillus niger* NRRL 334 or *Aspergillus oryzae* NRRL 5590 on protein extraction of both cold-pressed (CP) and hexane-extracted (HE) canola meals and functionalities of extracted canola protein products were studied. Fermented canola meals obtained were used in protein isolate production based on two processes: alkaline extraction-isoelectric precipitation (AE-IP) and salt extraction-diaysis (SE), followed by characterization of their functional properties. All SE products had high protein content (~95) than AE-IP products (~56% to ~86%). For both meals regardless of fermented or not, protein solubility for most AE-IP protein products showed lower values at pHs 3, 5 and 7 than the SE products. SSF decreased the solubility at pH 3 and 5 but increased the solubility at pH 7 depending on the type of meal and strain used. Both water/oil holding capacity (WHC/OHC) values increased after SSF. For the type of strains, *A. oryzae* was preferred to improve the WHC of both AE-IP and SE while *A. niger* was better for OHC values. All protein products displayed emulsifying properties with emulsifying activity index (EAI) values ranging from 5.6 to 21.1 m^2/g and relatively low emulsion stability index (ESI) values from 1.1 to 4.5 min. As for foaming properties, all canola protein products showed a high foaming capacity (FC) ranging from 154.4% to 480.0% with foaming stability (FS) ranging from 68.0% to 89.0%. After SSF, both beneficial and adverse impacts were found on foaming and emulsifying properties. In addition, CP products had better foaming properties than HE products. The results indicated the potential of SSF for improving the foaming properties at pH 7 of protein products extracted from *A.niger* fermented HE meal. For type of meals, CP products had better functionality than HE while HE showed higher increases in functionality. Both *A. niger* and *A. oryzae* showed the ability to modify the functionality of products. For extraction method, SE products tended to show better functionality compared to AE-IP products.

4.1. Introduction

Canola meal, the by-product of canola oil extraction process, is gaining tremendous interest in the food industry due to its high protein content (35%-45%) and the well-balanced amino acid profile of canola protein. The use of canola meal as an alternative protein source can provide more proteins options to the food industry. Canola proteins are dominated by two major proteins, a salt-soluble globulin protein (cruciferin, 12*S*, MW ~300 kDa) and a smaller water-soluble albumin (napin, 1.7-2*S*, MW ~14–17 kDa). It is generally known that the amino acid composition of canola meal is well balanced with a relatively high protein efficiency ratio of 2.64 and therefore can be used for human nutrition (Aider *et al.*, 2011). In recent research, canola protein has presented a high solubility, foaming capacity and stability and comparable or moderate emulsifying properties compared to other plant proteins such as soybean and pea protein (Wu and Muir, 2008; Khattab and Arntfield, 2009; Tan *et al.*, 2011a; Cheung., 2014; Chang *et al.*, 2015). These characteristics make canola protein a potential ingredient for food industry.

According to previous studies, several methods including alkaline extraction-isoelectric precipitation (AE-IP), salt extraction-dialysis (SE), protein micellation method (PMM), low pH extraction combined with membrane separation and ultrafiltration (UF) have been widely used for extraction of canola protein isolates (Tan *et al.*, 2011; Wanasundara, 2011). However, results on the structural composition and functionality of canola proteins can vary significantly due to the difference of extraction methods (Hoglund *et al.*, 1992; Wu and Muir, 2008; Karaca *et al.*, 2001). In addition, protein extraction from canola is especially difficult due to the differences in protein fractions (widely differing isoelectric points from pH 4 to 11 and molecular weights from 13 to 320 kDa) and the presence of antinutrients (GLS, phytic acid and polyphenols), pigments and fibre in the canola meal (Wu and Muir, 2008). It is expected that pre-treatment and specific purification process may improve meal protein solubility and consequently protein extractability.

In this study, fermented canola meals were used in the wet fractionation process to produce protein products. Two wet extraction processes: 1) alkaline extraction-isoelectric precipitation (AE-IP); and 2) salt extraction-dialysis (SE) were employed. The resulting protein products were characterized for selected nutritional and functional properties and compared with the products of meals that were not pre-treated. We hypothesize that a SSF pre-treatment has the potential to improve the protein extractability because of reduction in antinutrients and oil ae well as partial protein hydrolysis. It is hypothesized that the microbes may loosen protein-carbohydrate (CHO)

interactions leading to easier extraction, even greater protein yields and modify the functionality. In study 1, a ~45% degree of protein hydrolysis was found in both CP and HE meals after 72-hour fermentation using *Aspergillus. niger* NRRL 334 and *A. oryzae* NRRL 5590. It is expected that SSF could modify the functional properties of the protein products extracted using AE-IP and SE.

4.2. Material and methods

4.2.1. Material

Canola meals (CP and HE) of 150 g were fermented separately with *Aspergillus niger* NRRL 334 and *A. oryzae* NRRL 5590 (each at 10^7 spores/g meal) for three days (72 h) while moisture was maintained at 50% and temperature at 30°C. Meal after fermentation was used for protein extraction using both AE-IP and SE methods on the same day to obtain canola protein products. All the reagents and chemicals used were analytical grade.

4.2.2. Preparation of canola protein products

4.2.2.1. Alkaline extraction-isoelectric precipitation products

Fermented (72 h) CP (without defatting) and HE meals (100 g) were extracted using alkaline solution at a 1:10 (w/v, meal: water) ratio, at pH 11 (adjusted using 2 N NaOH solution) for a 1-h period at room temperature (21-23°C) according to Aider and Barbana (2011). The suspension was then centrifuged (4500 × *g* at 4°C for 15 min) and the supernatant was vacuum filtered with No. 1 Whatman filter paper (Whatman International Ltd., Maidstone, England) to remove the insolubles and any floating particles. The pH of the supernatant was then adjusted to pH 5 by adding 2 N HCl solution to precipitate the protein. Precipitated protein was collected as a pellet by centrifugation at 4500 × *g* at 4°C for 15 min, followed by freeze drying. The crude protein content was determined using micro-Kjeldahl according to modified AOAC 960.52 method with a conversion factor of 6.25.

4.2.2.2. Salt extraction-dialysis products

Canola protein products were also prepared from fermented (after 72 h) CP (without defatting) and HE canola meal according to Klassen *et al.* (2011) and Chang *et al.* (2015) with minor modifications. In brief, 0.05 M Tris–HCl buffer (pH = 7.0) with 0.1 M NaCl was mixed with fermented canola meal at a 1:10 (w/v) ratio and stirred at 500 rpm (IKAMAG RET-G, Janke

& Kunkel GmbH & Co. KG, IKA-Labortechnik, Germany) for 2 h under room temperature (21-23℃). The supernatant was then collected using a centrifuge (Sorvall RC Plus Superspeed Centrifuge, Thermo Fisher Scientific, Asheville NC, USA) at 6000 × *g* for 30 min, followed by vacuum filtration with No.1 Whatman filter paper. Protein extract so obtained was dialysed to remove NaCl and other small molecules using Spectra/Por molecular porous membrane tubing (6–8 kDa cut off, Spectrum Medical Industries, Inc., USA) at 4 ℃ for 72 h against fresh distilled water (distilled water was refreshed twice a day). The dialyzed protein solution was then centrifuged at 3000 × *g* for 30 min at 4℃ to collect the pellet, followed by freeze drying to obtain dried products. The protein content was determined using micro-Kjeldahl according to modified AOAC 960.52 method with a conversion factor of 6.25.

4.2.2.3. Protein product controls

Canola protein product controls were extracted directly from unfermented CP and HE meals (without defatting process) using both AE-IP and SE methods. In addition, a defatted CP meal was also prepared as a control to study the effect of residual oil on protein extractability. In brief, unfermented CP meal was defatted with a meal : hexane ratio of 1:3 (w/v) over a 1.5-h period of stirring at room temperature, followed by vacuum filtration with No. 1 Whatman filter paper to recover defatted solids. Above defatting process was repeated twice to reach an oil content of < 2% and the resulting meal was used as unfermented defatted CP control. Defatted canola protein product control (DCP) was then extracted from above defatted CP meal using both AE-IP and SE methods. Three canola protein product controls were prepared from HE, CP and defatted CP meals using both AE-IP and SE method.

4.2.3. Functionality analysis

4.2.3.1. Solubility

Protein solubility was studied as a function of pH (3, 5 and 7) according to Karaca *et al.* (2014) and Chang *et al.* (2015) with minor modifications. In brief, 20 mL of 0.25% (w/w) protein (corrected by protein content) solutions at a certain pH were prepared and allowed to stir for 1 h at room temperature (21-23℃). For each sample, the protein solution was then transferred to a 15 mL centrifuge tube and centrifuged at 4,180 × *g* for 10 min at room temperature. Supernatant was carefully collected, followed by Bradford method to determine the protein content. In detail, 50

µL of supernatant was added to 1.5 mL centrifuge tubes and 1.5 mL of Coomassie dye (Thermo Fisher Scientific) was added. The mixture was vortexed for 10 s at max speed (speed 10) then left for 5 min. Absorbance of samples was measured at 595 nm using a Genesys 20 UV-visible spectrophotometer (Thermo Scientific, Madison, WI, USA), and Milli-Q water was used as a blank while 50 µL of 0.5 NaOH and 1.5 mL of Coomassie dye was used as the sample blank. Different levels of BSA (bovine serum albumin) were used to create a standard curve. Total protein content was determined by dissolving protein samples in NaOH solution. In brief, 20 mg of protein sample was dissolved in 20 mL of 0.5 N NaOH solution and allowed to stir for 1 h at room temperature (21–23°C), followed by centrifugation at 12,100 × *g* for 30 min. The supernatant was carefully collected, and total protein content was determined using the above Bradford method. Protein solubility (%) was determined by dividing the protein content (N% × 6.25) within the solutions at different pH by the total protein in the sample, multiplied by 100 %. All measurements were reported as the mean ± one standard deviation (n = 3).

4.2.3.2. Water and oil holding capacity

Water/oil holding capacity (WHC/OHC) was determined according to Stone *et al.* (2015). In brief, 0.5 g of protein (corrected by protein content) was mixed with 5.0 g of water/oil in a 50 mL screw cap centrifuge tube. Mixtures were then vortexed for 10 s every 5 min for a total period of 30 min, followed by centrifugation at 1000 × *g* for 15 min. The remaining pellet was weighed after carefully decanting the supernatant. Water/oil holding capacity was calculated by dividing the weight gained by the original sample weight (w/w). All measurements were reported as the mean ± one standard deviation (n = 3).

4.2.3.3. Emulsifying properties

Emulsifying activity index (EAI, m^2/g) and stability index (ESI, min) were determined according to Pearce and Kinsella (1978) and Cheung *et al.* (2014). In brief, 0.25% (w/v) protein solutions (corrected by protein content) were prepared as a function of pH (3, 5 and 7) and allowed to stir at room temperature (21-23°C) overnight. For each emulsion preparation, 5.0 g of protein solution was mixed with 5.0 g of canola oil, followed by homogenization using an Omni Macro Homogenizer (Omni International, Marietta, GA, USA) with a 20 mm saw tooth generating probe positioned at the oil–water interface at speed 4 (~7,200 rpm) for 5 min. Immediately after homogenization, 50 µL of the emulsion from the bottom of the tube was carefully pipetted and

added to 7.5 mL of 0.1 % (w/v) sodium dodecyl sulphate (SDS), followed by vortexing for 10 s. The absorbance of the diluted emulsion samples was determined using a Genesys 20 UV-visible spectrophotometer (Thermo Scientific, Madison, WI, USA) at 500 nm with plastic cuvettes (1 cm path length). A second absorbance reading was taken from the dilution after 10 min. EAI and ESI was calculated by following equations (1 and 2).

$$EAI\ (\frac{m^2}{g}) = \frac{2 \times 2.203 \times A_0 \times N}{c \times \varphi \times 10000} \quad (4.1)$$

$$ESI\ (min) = \frac{A_0}{\Delta A} \times t \quad (4.2)$$

where, A_0 is the absorbance of the diluted emulsion immediately after homogenization, N is the dilution factor, c is the weight of protein per volume (g/mL), φ is the oil volume fraction of the emulsion, and ΔA is the difference in absorbance between 0 and 10 min ($A_0 - A_{10}$) and t is the time interval (10 min). All measurements were reported as the mean ± one standard deviation (n =3).

4.2.3.4. Foaming properties

Foaming properties were determined according to Liu *et al.* (2010) and Stone *et al.* (2015). In brief, 1.0% (w/w) protein solution (corrected by protein content) was prepared as a function of pH (3, 5 and 7) and allowed to stir overnight at 4°C. For each protein solution, 15 mL (V_{li}) was transferred into a 400 mL high form glass beaker (inner diameter = 69 mm; height = 127 mm; as measured by a digital caliper) and foamed using an Omni Macro homogenizer (Omni International, Marietta, GA, USA) with a 20 mm saw tooth generating probe for a total of 5 min at speed 4 (~7200 rpm). The foam was immediately transferred to a 50- or 100-mL graduated cylinder (inner diameter = 26 mm) after homogenization. Foam volume was recorded at time zero (V_{fi}) and after 30 min at room temperature (V_{ft}). Foaming capacity (FC) and foaming stability (FS) were determined using following equations, respectively.

$$\%FC = \frac{V_{fi}}{V_{li}} \times 100\% \quad (4.3)$$

$$\%FS = \frac{V_{ft}}{V_{fi}} \times 100\% \quad (4.4)$$

where V_{fi} is the volume of foam immediately after homogenization and V_{ft} is the volume of foam remaining after 30 min.

4.2.3.5. Statistical analysis

All protein products were prepared in triplicate on separate fermented meals (n = 3). The results were reported as mean ± one standard deviation. A two-way analysis of variance (ANOVA) was used to study the statistical differences in protein functionality using Tukey's test with a significance level of $p = 0.05$. A simple Pearson correlation was used to describe the relationship between protein functional properties. All statistics were performed using the IBM SPSS Version 28.0 software (IBM Corp. NY, IL, USA).

4.3. Results and discussion

4.3.1. Protein content of canola protein products

Protein products extracted using AE-IP from unfermented HE and unfermented and non-defatted CP meals had protein levels of 86.5% and 68.9%, respectively. After the defatting process, 81.6% protein content was achieved from CP meal indicating that the high oil content negatively influenced protein extraction using AE-IP method. With fermentation, canola protein products obtained from CP meal fermented with *Aspergillus niger* NRRL 334 and *A. oryzae* NRRL 5590 had protein levels of 62.7% and 57.9%, respectively. The protein products from HE meals fermented with *A. niger* and *A. oryzae* had protein levels of 56.3% and 58.4%, respectively. Both CP and HE meals when fermented with either of the fungal strains gave products with lower protein enrichment upon AE-IP.

In contrast, all SE canola protein products had protein levels higher than 90%. Overall, SE method resulted in higher protein levels for all unfermented and fermented canola protein products than those produced by AE-IP. Therefore, the hypothesis was confirmed that protein levels of produced isolates could be different depending on the extraction method. The lower protein content found in the AE-IP CPI may indicate the heterogeneous nature of canola proteins (cruciferin and napin), which are proved to have different isoelectric points that complicate their precipitation (Wu and Muir, 2008).

Findings suggested an opposite result from the original hypothesis that SSF could help improve the protein extraction process. It is hypothesized that fermentation may lead to partial hydrolysis of the protein leading to increased surface hydrophobicity (decrease in protein solubility) and stronger protein-lipid interactions. The high degree of hydrolysis may also lead to a larger

number of peptides, which could be easily lost during centrifugation processing. Also, the large number of hyphae produced by fungi may act like 'glue' to connect the solid substrates tightly, which affected the protein extraction by limiting the soluble protein dissolving into the surrounding aquatic system. After SSF, the degradation of fibre might release more insoluble or soluble carbohydrates (polysaccharides or starch) that made it difficult to separate the protein from the complex (protein-carbohydrate interaction). In addition, oil could largely affect the protein extraction process (Krause *et al.*, 2001), which may be the case in CP meals. SSF was not able to reduce the lipid contents to a point (1-2%) where extraction could be improved.

4.3.2. Protein solubility

Solubility is one of the most important physicochemical properties of proteins, as it is generally related to other functional properties which may affect the rheological, hydrodynamic, and surface activity characteristics. Thus, during development and analysis of new protein ingredients, solubility is always the first functional property to be determined (Zayas., 1997). As shown in Table 4.2, the AE-IP CP control resulted in a significantly higher ($p < 0.05$) solubility at pH 3 where it was 88.9% soluble compared to the defatted CP control and HE control (76.2%, 81.4% respectively). Similar to at pH 3, the AE-IP CP control (53.5%) showed higher solubility than defatted CP control (36.9%) and HE control (2.0%) at pH 5, while HE control (70.0%) had a higher solubility than CP control (27.7%) and defatted CP control (37.8%) at pH 7. As for SE controls, higher solubility was found for CP (94.0%), defatted CP (97.1%) and HE (94.6%) controls at pH 3 compared to AE-IP controls. At pH 5, higher solubility was found for SE HE control (29.4%) compared to AE-IP, whereas it was lower than the CP control (37.7%) and similar to the defatted CP control (37.3%). Both SE CP (51.8%) and defatted CP control (49.3%) had higher solubility than AE-IP CP controls at pH 7 with the exception of SE HE control (43.2%) being lower than AE-IP HE control. Overall, SE products showed higher solubility at pH 3 and 7. Stone *et al.* (2015) also found a lower solubility of canola protein products obtained from AE-IP compared to SE process. Similar solubility values were found for both CP and defatted CP controls at all pHs and tended to be higher than HE control.

During SSF, protein was partially hydrolyzed, which led to the exposure of hydrophobic groups that used to be buried inside the protein molecules. Thus, canola protein products prepared from fermented meals tended to show a decreased trend in solubility at pH 3, 5 and 7 compared to control products. At pH 3, all AE-IP and SE controls showed higher solubility than products from

fermented meals. However, the solubility of products from *A. oryzae* fermented meals was significantly higher than products from *A. niger* fermented meals. This indicated *A. oryzae* was able to keep the solubility (pH 3) at a relatively higher level than *A. niger* after SSF. In other words, using *A. oryzae* may be favorable to obtain a more soluble protein product at pH 3. After fermentation, all samples showed significant decreases ($p < 0.05$) in solubility and resulted in low solubility at pH 5 ranging from 4.5% to 11.8%. Increases in solubility of AE-IP HE control were - found from 2.0% to 11.8% and 5.8% at pH 5 when fermented using *A. niger* and *A. oryzae*, respectively. According to a previous study on canola protein, a 6.2 isoelectric point and lower solubility at pH 5 (~37%) and 7 (~23%) were reported in untreated canola protein products (Chang *et al.*, 2015). The results indicated that SSF processing decreased the protein solubility at pH 5 which may be due to a change in the isoelectric point (close to pH 5) by partial hydrolysis of the protein causing the shift in solubility with pH value.

At pH 7, the solubility of AE-IP canola protein products showed significantly lower ($p < 0.05$) solubility than SE products obtained from control meals with the exceptions of HE control (70%) and HE product from *A. niger* fermented meal (70%). Unlike at pH 3 and 5, SSF showed the potential ability to improve the protein solubility at pH 7. For instance, the AE-IP CP product from *A. niger* fermented meal showed a significant ($p < 0.05$) increase in solubility (47.5%) compared to both AE-IP CP and defatted CP control. In addition, the same level of solubility (70.0%) was found for AE-IP HE control and HE product from *A. niger* fermented meal as shown in Table. 4.2However, A decrease in solubility ($p < 0.05$) was found for protein products from *A. oryzae* fermented CP and HE meals compared to controls, which indicated the *A. niger* was preferred to maintain or increase the solubility of AE-IP protein products at pH 7. Opposite results were found for SE protein products, products from *A. oryzae* fermented meals (90.7% for *A. oryzae* CP and 55.6% for *A. oryzae* HE) resulted in higher solubility values compared to the controls and products obtained from control meals ($p < 0.05$). An increase in the solubility of SE HE product from *A. niger* fermented meal (48.5%) was also observed compared to HE control ($p < 0.05$). However, a decrease in solubility was reported for SE CP product from *A. niger* fermented meal (37.2%) compared to controls. When extracting protein via SE, both *A. niger* and *A. oryzae* can be acceptable inoculums for meals that could maintain or increase the protein solubility at pH 7. Whereas *A. niger* might be a better culture choice for AE-IP products at pH 7.

Above results may indicate the differences between AE-IP and SE canola protein (protein fraction, the percentage of napin and cruciferin) and the hydrolysis mechanism of *A. niger* and *A. oryzae* including possible different proteinase, length of peptides, structure of hydrolyzed protein and synthesized metabolism. Protein products extracted using SE method showed higher solubility than AE-IP products mainly due to the high protein content, possible difference in protein fraction and possible interaction of protein and carbohydrate in AE-IP products. In addition, protein products extracted form CP meal showed higher solubility than products from HE meal. Protein denaturation during hexane-extraction (heat treatment) could explain the solubility reduction. Generally, protein solubility is highly related to the presence of hydrophobic and hydrophilic residues. Heat treatment could result in the exposure of hydrophobic groups, which contribute to the reduction in protein solubility (Khattab and Arntfield, 2009). In other words, the partial denaturation of protein can alternate the balance of protein hydrophobicity/hydrophilicity, which further affects the solubility (Moure, Sineiro, Dominguez, & Parajo, 2006).

Table 4.1. Protein content (%, d.b.) of alkaline extraction-isoelectric precipitation (AE-IP) and salt extraction-dialysis (SE) protein products prepared from fermented and unfermented (control) cold-pressed (CP) and hexane-extracted (HE) canola meals. Data was reported as mean ± one standard deviation across each extraction process (n = 3). Significant difference exists between data with different letters in a row ($p < 0.05$).

Protein isolation process	Cold-pressed meal				Hexane-extracted meal		
	Unfermented		Fermented		Unfermented	Fermented	
	No defatting	With defatting*	*A. niger*	*A. oryzae*	Control	*A. niger*	*A. oryzae*
AEIP	68.9 ± 0.3^{c}	81.6 ± 1.1^{b}	62.7 ± 0.2^{d}	57.9 ± 0.2^{f}	86.5 ± 0.2^{a}	56.5 ± 0.2^{g}	58.5 ± 0.2^{e}
SE	99.6 ± 1.0^{a}	96.5 ± 1.3^{d}	93.3 ± 2.2^{f}	99.0 ± 1.2^{b}	97.7 ± 3.3^{c}	95.9 ± 2.3^{e}	95.8 ± 4.2^{e}

* Defatted CP control: canola protein products extracted from defatted CP meal using AE-IP or SE.

Table 4.2. Protein (nitrogen-based) solubility (%, d.b.) of protein products obtained from alkaline extraction-isoelectric precipitation (AE-IP) and salt extraction-dialysis (SE) from fermented and unfermented (control) canola meals. Data was reported as mean ± one standard deviation (n = 3). Significant difference exists between data with different letters across each pH ($p < 0.05$).

Protein product	AE-IP product (solubility, %)			Salt-extracted product (solubility, %)		
	pH 3	pH 5	pH 7	pH 3	pH 5	pH 7
CP control	88.9 ± 3.7^{a}	53.5 ± 2.6^{a}	27.7 ± 2.2^{d}	94.0 ± 0.4^{a}	37.7 ± 0.2^{a}	51.8 ± 1.1^{bc}
DCP contro.*l	76.2 ± 3.6^{cd}	36.9 ± 2.1^{b}	37.8 ± 2.4^{c}	97.1 ± 0.5^{a}	37.3 ± 0.8^{a}	49.3 ± 0.8^{c}
CP *A. niger*	25.9 ± 1.9^{f}	5.9 ± 0.9^{d}	47.5 ± 2.9^{b}	21.3 ± 1.6^{e}	4.5 ± 0.2^{d}	37.2 ± 0.1^{e}
CP *A. oryzae*	72.7 ± 1.2^{d}	7.5 ± 0.5^{cd}	25.6 ± 0.9^{d}	83.4 ± 0.2^{b}	6.6 ± 0.4^{c}	90.7 ± 3.8^{a}
HE control	81.4 ± 0.9^{b}	2.0 ± 0.9^{e}	70.0 ± 1.4^{a}	94.6 ± 1.7^{a}	29.4 ± 2.3^{b}	43.2 ± 1.1^{d}
HE *A. niger*	10.1 ± 1.0^{g}	11.8 ± 1.0^{c}	70.0 ± 1.1^{a}	43.8 ± 0.9^{d}	5.9 ± 0.4^{c}	48.5 ± 1.6^{c}
HE *A. oryzae*	53.0 ± 1.1^{e}	5.8 ± 0.4^{d}	38.5 ± 3.2^{c}	49.8 ± 0.7^{c}	6.5 ± 0.3^{c}	55.6 ± 2.7^{b}

* DCP control: canola protein control prepared from defatted unfermented CP meal using AE-IP or SE.

4.3.3. Water holding capacity

The ability of protein to absorb and retain water is critical in stabilizing the structure, improving flavour retention and mouth feel and reducing moisture loss of food products (Khattab and Arntfield, 2009). As shown in Table 4.3a, AE-IP CP control protein products showed higher WHC (2.1 g/g) than the products of HE control (1.8 g/g), which was possibly due to the thermal and chemical changes of HE protein which occurred during the solvent extraction processing, leading to the increase in protein-protein aggregation and limiting the protein-water or protein-oil interactions. However, the value of defatted CP control (2.7 g/g) was higher than that of non-defatted ones (2.1 g/g), which was mainly due to the presence of residual oil that impacted the hydrophilic ability. Similar results were found in SE controls that defatted CP control (2.1 g/g) showed higher WHC value than HE (1.3 g/g) and non-defatted CP controls (1.1 g/g) as shown in Table 4.3b.

After a period of 72-h fermentation of the source meal, a significant increase ($p < 0.05$) in WHC of AE-IP products was found. WHC increased from 2.1 g/g (CP control) and 2.7 g/g (non-defatted CP control) to 3.1 g/g (*A. niger* fermented) and 3.0 g/g (*A. oryzae* fermented). No significant difference ($p > 0.05$) was found between strains for AE-IP CP products. In addition, *A. oryzae* showed a higher increase in WHC of AE-IP HE products (3.2 g/g) than *A. niger*. (2.9 g/g). Similar results were found for SE HE products as SSF increases WHC values from 1.3 g/g to 2.3 g/g (*A. niger*) and 2.7 g/g (*A. oryzae*). The partial hydrolysis of protein during the fermentation process may change the compact protein structure and exposes more hydrophilic and hydrophobic sites (additional binding sites available for water), which made it easier for protein to adsorb and hold water (Kinsella, 1982). In contrast, the WHC values of SE CP products remained stable ($p > 0.05$) after SSF using *A. niger* (1.3 g/g) and *A. oryzae* (1.4 g/g) compared to non-defatted CP control (1.1 g/g) and decreased when compared to defatted CP control (2.1 g/g). For the type of strains, *A. oryzae* was preferred to improve the WHC of both AE-IP and SE HE product. In addition, all HE products showed higher increases in WHC than CP products.

Overall, defatted CP controls showed higher WHC values than non-defatted CP controls and HE controls. As for the difference between AE-IP and SE products, AE-IP protein products showed slightly higher WHC value than SE ones. This was possibly due to the lower protein level of AE-IP products, which led to a larger sample amount of AE-IP products used for testing than SE products (corrected by protein content). The non-protein compounds in AE-IP products such

as possible soluble fibre and polysaccharides may contribute to the ability of water absorbing and binding. Thus, it is important to take the possible contained fibre and polysaccharides into consideration. The presence of these compounds can enhance the overall water holding capacity of canola products (Aider and Barbana, 2011).

4.3.4. Oil holding capacity

Fats act as a flavour carrier and mouthfeel enhancer, so the ability of protein to absorb and retain oil is important in food formulations (Kinsella, 1982). Fat adsorption of protein mainly depends on the physical entrapment of oil through a capillary-attraction process (Kinsella, 1982). As shown in Table 4.3a, AE-IP CP control showed a higher OHC value (2.9 g/g) than the HE control (2.3 g/g), possibly due to the thermal and chemical changes of protein which occurred during the solvent extraction processing, which led to the increase in protein-protein aggregation and limited the protein-water or protein- oil interactions. Similar results were observed for the products obtained using SE method in Table 4.3b. CP control had a higher OHC value (3.2 g/g) than the products of HE control (2.4 g/g). The OHC value of AE-IP defatted CP control (2.4 g/g) was lower than that of non-defatted CP control (2.9 g/g), whereas SE defatted CP control (3.9 g/g) had a higher OHC value than CP non-defatted control (3.2 g/g).

After 72-h SSF, OHC of AE-IP CP products increased to 2.8 g/g (both *A. niger* and *A. oryzae*) compared to defatted CP control (2.4 g/g, $p < 0.05$), while remained stable when compared to non-defatting CP control (2.9 g/g, $p > 0.05$). AE-IP HE products also showed an increase ($p < 0.05$) from 2.3 g/g to 3.1 (*A. niger*) and 3.3 g/g (*A. oryzae*). As for SE products, an increase in OHC was found for both AE-IP and SE protein products compared to controls ($p < 0.05$). For SE CP products, OHC values increased from 3.2 (CP control) and 3.9 g/g (defatted CP control) to 4.6 (*A. niger*) and 4.7 g/g (*A. oryzae*), while increased from 2.4 g/g (HE control) to 4.5 (*A. niger*) and 3.9 g/g (*A. oryzae*) for HE products. The partial hydrolysis of protein during fermentation process may change the compact protein structure and exposes more hydrophilic and hydrophobic sites, which made it easier for protein to adsorb and hold oil. Above results were agreed by Kinsella. (1982) that the increase in fat absorption could be associated with the hydrolysis and denaturation of proteins due to the exposure of the non-polar residue buried inside the protein molecules. For the type of strains, there is no significant difference ($p > 0.05$) between *A. niger* and *A. oryzae*

except for SE HE products, where *A. niger* was preferred to increase the OHC of the products. Similar to WHC, higher increase was found in HE meal compared with CP meal after SSF.

Different from WHC results, canola protein products obtained using SE showed better OHC compared to those prepared using AE-IP. It may indicate that a higher level of protein (~95% for SE products while ~60% to 80% for AE-IP products) is preferred to obtain protein products with better OHC. It could also be concluded that the proteins of SE might be more hydrophilic than those from AE-IP therefore high protein solubility in SE products. The lower solubility and greater OHC suggest increased hydrophobic nature of the protein recovered from AE-IP process.

4.3.5. Emulsifying properties

Emulsifying properties are critical functional properties of food proteins. Several factors can affect the emulsifying properties including the type of protein, concentration, pH, ionic strength, and viscosity of the system. Many chemical and physical factors are involved in the formation, stability, and textural properties of protein–oil–water emulsions (Chang *et al.*, 2015). Emulsifying activity index (EAI) is a measurement of interfacial area coated by protein during the formation of an emulsion and acts as a good predictor for protein surface activity, whereas the emulsifying stability index (ESI) provides a measurement to exam the stability of the diluted emulsion over a fixed period of time (Karaca *et al.*, 2011).

As shown in Table 4.4a, AE-IP HE control had higher EAI value (13.2 m^2/g) than the CP control (11.4 m^2/g) and defatted CP control (8.3 m^2/g) at pH 3, which may indicate a higher hydrophobicity value of HE products compared to CP controls at pH 3. Similarly for pH 7, the higher EAI value was found for HE control (18.3 m^2/g) than CP control (11.7 m^2/g) and defatted CP control (11.6 m^2/g) at pH 3. As for pH 5, defatted CP control (5.0 m^2/g) had higher EAI value than CP control (4.1 m^2/g, $p > 0.05$) and HE control (3.0 m^2/g, $p < 0.05$). As for SE products, defatted CP control (11.0 at pH 3, 9.4 at pH 5 and 14.5 m^2/g at pH 7) showed the highest EAI value compared to CP (5.6 at pH 3, 5.8 at pH 5 and 12.9 m^2/g at pH 7) and HE control (5.9 at pH 3, 6.8 at pH 5 and 13.9 m^2/g at pH 7) at pH 3, 5 and 7. The difference between AE-IP and SE products may be due to the different protein fractions and nature of products extracted using AE-IP and SE. As for pH, EAI values tended to be low at pH 5 ranging from 1.3 to 5.5 m^2/g due to the low protein solubility. Higher EAI values were found at pH 7 compared to pH 3 for all the protein

products obtained from unfermented control meals, which was possibly related to the higher solubility at pH 7 than pH 3.

During SSF, the partial hydrolysis of protein loosens the compact protein structure and exposes more hydrophobic sites buried inside the protein molecule. A higher hydrophobic ity value can be achieved. Townsend and Nakai (1983) suggested a positive correlation between hydrophobicity and emulsifying properties of proteins. It is agreed by our results that canola products extracted from fermented meals showed better or at least unchanged emulsifying properties compared to controls. For AE-IP products, EAI values increased to 13.8 (*A. niger*) and 21.1 m^2/g (*A. oryzae*) at pH 3 for the CP products ($p < 0.05$). In addition, only *A. oryzae* improved EAI value of HE product (17.6 m^2/g), while a decrease (8.5 m^2/g) was found using *A. niger* at pH 3. When pH increased to 7, both *A. niger* and *A. oryzae* could help improve or remain the EAI values of AE-IP products. In detail, the EAI values of AE-IP CP products extracted from *A. niger* and *A. oryzae* fermented CP meals significantly increased ($p < 0.05$) to 25.8 and 27.6 m^2/g, respectively. Similar as AE-IP HE products, the EAI values were significantly increased to 18.0 and 25.5 m^2/g when fermented using *A. niger* and *A. oryzae*. The EAI results of AE-IP products at pH 5 remained relatively low ranging from 1.3 to 5.5 m^2/g for both AE-IP CP and HE products regardless of fermented or not due to the low protein solubility (pH 5). In addition, low ESI was reported for all protein products and ranged from 1.1 to 4.5 min. For SE products, *A. niger* and *A. oryzae* improved the EAI values of CP products at pH 3 to 8.5 (*A. niger*) and 9.7 m^2/g (*A. oryzae*) compared to CP control (5.6 m^2/g), however they were lower than the defatted CP control (11.0 m^2/g). Increases in SE HE product at pH 3 were also reported after SSF as 8.5 (*A. niger*) and 11.3 m^2/g (*A. oryzae*) compared to HE control (5.9 m^2/g). However, SSF decreased the EAI values of the CP and HE products to 1.8 (CP, *A. niger*), 4.6 (CP, *A. oryzae*), 4.1 (HE, *A. niger*) and 3.0 m^2/g (HE, *A. oryzae*) at pH 5. At pH 7, decreases in EAI values of SE CP and HE products were also reported to 12.2 (CP, *A. niger*), 14.9 (CP, *A. oryzae*), 11.4 (HE, *A. niger*) and 7.6 m^2/g (HE, *A. oryzae*). For type of meals, protein products extracted from HE meals tend to have slightly better EAI values than those from CP meals due to the higher hydrophobicity of HE products. As for type of strains, *A. oryzae* was preferred to modify the EAI values of canola protein products. Higher levels of increases in AE-IP products and decreases in SE products were found using *A. oryzae*.

The above EAI and ESI results reported were lower than those of previous studies by Cheung *et al.* (2014) and Chang *et al.* (2015). Cheung *et al.* (2014) suggested that high solubility was positively correlated with the ESI values due to the additional protein precipitation and adherence to the viscoelastic film surrounding the droplets. Lower solubility was reported compared to previous studies on canola protein, which may result in a reduction in ESI values. In contrast, Kinsella & Melachouris (1976) explained that a high protein solubility and high fat-adsorption capacity were positively correlated with the ability of emulsifying (form and stabilize emulsions). Wu and Muir. (2008) and Cheung *et al.* (2014) examined the emulsifying properties of the two major canola proteins cruciferin and napin. It has been reported that the emulsion prepared with cruciferin showed a significantly higher specific surface area and a lower particle size than that of napin. The study reported by Wu and Muir (2008) indicated that the presence of napin could detrimentally affect the emulsion stability of canola protein isolates (Wu & Muir, 2008). The differences may be a result of canola cultivar, preparation of canola meal (oil extraction method) and protein products (extraction methods and conditions) and method of determination employed. These differences might result in different fractions and levels of protein that further affected the emulsifying properties.

Table 4.3 Water/oil holding capacity (WHC) (g/g) of protein products obtained from alkaline extraction-isoelectric precipitation (AE-IP, 6a) and salt extraction-dialysis (SE, 6b) from fermented and unfermented (control) canola meals. Data was reported as mean ± one standard deviation (n = 3). Significant difference exists between data with different letters in a row ($p < 0.05$).

a) AE-IP protein products

Property	Cold-pressed meal				Hexane-extracted meal		
	Unfermented		Fermented		Unfermented	Fermented	
	No defatting	With defatted*	*A. niger*	*A. oryzae*	Control	*A. niger*	*A. oryzae*
WHC	2.1 ± 0.0^{d}	2.7 ± 0.1^{c}	3.1 ± 0.1^{ab}	3.0 ± 0.0^{b}	1.8 ± 0.0^{e}	2.9 ± 0.1^{b}	3.2 ± 0.0^{a}
OHC	2.9 ± 0.0^{b}	2.4 ± 0.1^{c}	2.8 ± 0.1^{b}	2.8 ± 0.1^{b}	2.3 ± 0.1^{c}	3.1 ± 0.1^{ab}	3.3 ± 0.1^{a}

b) SE protein products

Property	Cold-pressed meal				Hexane-extracted meal		
	Unfermented		Fermented		Unfermented	Fermented	
	No defatting	With defatted*	*A. niger*	*A. oryzae*	Control	*A. niger*	*A. oryzae*
WHC	1.1 ± 0.1^{c}	2.1 ± 0.1^{b}	1.3 ± 0.1^{c}	1.4 ± 0.1^{c}	1.3 ± 0.1^{c}	2.3 ± 0.1^{b}	2.7 ± 0.0^{a}
OHC	3.2 ± 0.1^{d}	3.9 ± 0.1^{c}	4.6 ± 0.1^{a}	4.7 ± 0.0^{a}	2.4 ± 0.1^{e}	4.5 ± 0.0^{b}	3.9 ± 0.1^{c}

* Defatted CP control: canola protein product extracted from defatted cold-press meal using AE-IP or SE.

Table 4.4 (a, b). Emulsifying activity index (EAI, m^2/g) and stability index (ESI, min) of protein products obtained from alkaline extraction-isoelectric precipitation (AE-IP, a) and salt extraction-dialysis (SE, b) of fermented and unfermented (control) canola meals. Data was reported as mean ± one standard deviation (n = 3). Significant difference exists between data with different letters across each pH ($p < 0.05$).

a) AE-IP protein products

AE-IP Protein product	EAI, m^2/g			ESI, min		
	pH 3	pH 5	pH 7	pH 3	pH 5	pH 7
CP control	11.4 ± 0.9^{cd}	4.1 ± 0.3^{a}	11.7 ± 1.5^{c}	4.5 ± 1.4^{ab}	1.4 ± 0.1^{ab}	1.2 ± 0.1^{b}
DCP control*	8.3 ± 0.6^{d}	5.0 ± 0.4^{a}	11.6 ± 1.7^{c}	1.5 ± 0.1^{ab}	1.6 ± 0.1^{ab}	1.4 ± 0.2^{b}
CP *A. niger*	13.8 ± 0.7^{c}	2.1 ± 0.3^{bc}	25.8 ± 1.9^{a}	4.8 ± 2.9^{a}	1.1 ± 0.1^{b}	1.4 ± 0.2^{b}
CP *A. oryzae*	21.1 ± 1.4^{a}	5.5 ± 0.1^{a}	27.6 ± 1.8^{a}	1.8 ± 0.3^{ab}	1.5 ± 0.1^{ab}	1.6 ± 0.1^{ab}
HE control	13.2 ± 1.6^{c}	3.0 ± 0.5^{b}	18.3 ± 1.6^{b}	3.1 ± 0.6^{ab}	2.2 ± 0.7^{a}	2.0 ± 0.3^{a}
HE *A. niger*	8.5 ± 0.8^{d}	1.3 ± 0.1^{c}	18.0 ± 1.9^{b}	1.6 ± 0.4^{ab}	1.3 ± 0.2^{b}	1.3 ± 0.2^{b}
HE *A. oryzae*	17.6 ± 1.9^{b}	4.8 ± 0.6^{a}	25.5 ± 2.5^{a}	1.2 ± 0.1^{b}	1.3 ± 0.1^{b}	1.2 ± 0.2^{b}

b) SE protein products

SE protein product	EAI, m^2/g			ESI, min		
	pH 3	pH 5	pH 7	pH 3	pH 5	pH 7
CP control	5.6 ± 1.1^{c}	5.8 ± 0.5^{bc}	12.9 ± 0.5^{b}	1.7 ± 0.2^{b}	1.2 ± 0.1^{d}	3.4 ± 0.3^{a}
DCP control*	11.0 ± 0.4^{a}	9.4 ± 0.2^{a}	14.5 ± 0.4^{a}	1.2 ± 0.0^{b}	1.2 ± 0.0^{d}	1.6 ± 0.1^{b}
CP *A. niger*	8.5 ± 0.5^{b}	1.8 ± 0.2^{d}	12.2 ± 0.7^{b}	4.2 ± 1.0^{a}	2.8 ± 0.4^{a}	1.8 ± 0.2^{b}
CP *A. oryzae*	9.7 ± 0.4^{ab}	4.6 ± 0.5^{c}	14.9 ± 0.3^{a}	1.2 ± 0.0^{b}	1.2 ± 0.1^{d}	1.7 ± 0.1^{b}
HE control	5.9 ± 0.5^{c}	6.8 ± 0.4^{b}	13.9 ± 0.7^{ab}	1.6 ± 0.2^{b}	2.4 ± 0.3^{ab}	4.3 ± 1.3^{a}
HE *A. niger*	8.5 ± 0.5^{b}	4.1 ± 0.6^{cd}	11.4 ± 0.4^{b}	1.3 ± 0.1^{b}	1.9 ± 0.1^{bc}	1.5 ± 0.1^{b}
HE *A. oryzae*	11.3 ± 0.9^{a}	3.0 ± 0.3^{d}	7.6 ± 0.8^{c}	1.2 ± 0.1^{b}	1.4 ± 0.2^{cd}	1.2 ± 0.0^{b}

* DCP control: canola protein product extracted from defatted cold-press meal using AE-IP or SE.

4.3.6. Foaming properties

The foaming properties of proteins are important in the production of a variety of foods (Hettiarachchy *et al.*, 2012). Foaming properties can be defined as two parts, the ability to form a two-phase system consisting of air cells surrounded by a thin continuous liquid layer (the lamellar phase) and the ability to stabilize the foams. All canola protein products in this study, regardless of the source of meal or fermentation treatment showed a foaming capacity (FC) from 131.1 to 480.0 % and moderate foaming stability (FS) ranging from 68.0% to 89.0% at pH 3, 5 and 7.

For controls, defatted AE-IP CP control (306.7% at pH 3 and 243.8% at pH 7) had higher FC than CP (224.4% pH 3 and 165% at pH 7) and HE control (244.4% at pH 3 and 154.4% at pH 7) possibly due to the residual oil in CP control and low protein content (~60%) of both CP and HE control. As for SE products, CP control had higher foaming capacity (322.2%) than defatted CP (246.7%) and HE (266.7%) control at pH 3. At pH 7, SE defatted CP control showed the highest FC in all products as 480.0 %, higher than SE CP (464.4%) and HE (145.6%) control. At pH 5, only CP (241.1% for AE-IP and 211.1% for SE) and defatted CP control (131.1% for AE-IP and 222.2% for SE) showed the ability to form stable foams for both AE-IP and SE products. However, the poor solubility at pH 3 still had adverse impact on both FC and FS compared to the values at pH 3 and pH 7. Besides, the high temperatures, exposure to hexane during oil extraction may be the reason that could explain the loss of foaming ability of HE control at pH 5. In addition, SE products showed significantly higher FC values than AE-IP. This might be due to the higher levels of protein in SE products (> 93%) than AE-IP products (< 80%) and the possible difference in protein fractions (napin and cruciferin).

For AE-IP products, only AE-IP CP protein products by *A. oryzae* showed the ability to form foams (228.9 %) with a low FS of 33.0% at pH 3 after SSF. At pH 7, all AE-IP products from fermented showed foaming properties as 230.7% (CP, *A. niger*), 180.0% (CP, *A. oryzae*) 244.2% (HE, *A. niger*) and 136.7% (HE, *A. oryzae*) with comparable FC compared to controls ranging from 72.0 to 84.6%. AE-IP HE sample pre-treated using *A. niger* even showed significantly higher FC value (244.2%) compared to HE control (154.4%). As for SE products, all samples were able to form foams at pH 3 and 7. In detail, FC values of CP products from fermented meals were decreased to 196.5% (*A. niger* at pH 3), 175.6% (*A. oryzae* at pH 3), 191.1% (*A. niger* at pH 7), and 153.3% (*A. oryzae* at pH 7) compared to CP control at pH 3 and 7. For HE products, decreases were also found for products at pH 3, as 195.6% (*A. niger*) and 135.6% (*A. oryzae*), while

significant increases were reported at pH 7 as 306.7% (*A. niger*) and 155.6% (*A. oryzae*) compared to HE control (266.7% at pH 3 and 155.6% at pH 7). In addition, the FS values decreased to ~52% for all products from fermented meals at pH 3, while they remained stable, at 77.6%-88.4%, at pH 7. At pH 5, none of the products extracted from fermented meal showed the ability to form stable foams. All proteins mentioned above with poor foaming properties at pH 3 and 5 could only form weak foams with large bubbles which disappeared within 5 min. This was possibly due to the extremely low protein solubility (< 10% for protein from fermented meals) of protein from fermented meals at pH 5. The SSF was found to decrease the solubility of canola protein products to <10% at pH 5, which could have adverse impacts on further foaming properties. Overall, protein products prepared from fermented HE meals using both extraction methods showed better FC and FS than the HE controls, while the opposite results were found in CP canola meal products.

The above results indicate the potential of SSF for improving the foaming properties at pH 7 of protein products extracted from HE meal especially when using *A. niger*. In other words, *A. niger* was preferred to provide protein products with better foaming properties compared to *A. oryzae*. However, the SSF tends to decrease the foaming properties of CP products by partially denaturing the protein during fermentation. Although the FC and FS values decreased after SSF, proteins extracted from fermented CP meal displayed acceptable foaming properties compared to other plant proteins like soybean, pea and lentil (~200%) (Chabanon *et al.*, 2007 and Stone *et al.*, 2015). However, they still could not produce comparable protein products similar to the ones from unfermented CP meal.

Table 4.5 (a, b). Foaming capacity (%) and stability (%) (as a function of pH 3, 5 and 7) of alkaline extraction-isoelectric precipitation (AE-IP, a) and salt-extraction dialysis (SE, b) canola concentrates and isolates from different fermented and control canola meals. Data was reported as mean ± one standard deviation (n = 3). Significant difference exists between data with different letters across each pH ($p < 0.05$).

a) AE-IP protein products

AEIP protein product	Foaming capacity (%)			Foam stability 30-min (%)		
	pH 3	pH 5	pH 7	pH 3	pH 5	pH 7
CP control	224.4 ± 20.4^{c}	241.1 ± 16.4^{a}	165.0 ± 5.2^{bc}	93.0 ± 2.3^{a}	83.2 ± 5.5^{a}	68.0 ± 1.4^{d}
DCP control	306.7 ± 20.0^{a}	131.1 ± 7.7^{b}	243.8 ± 8.1^{a}	87.0 ± 1.9^{b}	32.3 ± 4.2^{b}	78.9 ± 0.6^{abc}
CP *A. niger*	/	/	230.7 ± 6.8^{a}	/	/	82.3 ± 3.1^{ab}
CP *A. oryzae*	228.9 ± 3.8^{c}	/	180.0 ± 3.3^{b}	33.0 ± 1.1^{c}	/	84.6 ± 4.0^{a}
HE control	244.4 ± 10.2^{bc}	/	154.4 ± 6.9^{cd}	17.3 ± 1.6^{d}	/	75.2 ± 3.8^{bcd}
HE *A. niger*	/	/	244.2 ± 12.4^{a}	/	/	72.0 ± 3.0^{cd}
HE *A. oryzae*	/	/	136.7 ± 8.8^{d}	/	/	79.6 ± 3.0^{ab}

b) SE protein products

SE protein product	Foaming capacity (%)			Foam stability 30-min (%)		
	pH 3	pH 5	pH 7	pH 3	pH 5	pH 7
CP control	322.2 ± 7.7^{a}	211.1 ± 20.4^{b}	480.0 ± 11.5^{a}	87.2 ± 1.5^{a}	87.2 ± 4.0^{a}	83.8 ± 0.7^{ab}
DCP control	246.7 ± 17.6^{b}	222.2 ± 7.7^{a}	464.4 ± 20.4^{b}	84.2 ± 0.4^{a}	87.4 ± 4.2^{a}	89.0 ± 2.0^{a}
CP *A. niger*	196.5 ± 6.6^{c}	/	191.1 ± 13.9^{d}	54.6 ± 0.1^{c}	/	88.4 ± 1.2^{a}
CP *A. oryzae*	175.6 ± 10.2^{c}	/	153.3 ± 8.8^{e}	51.0 ± 1.1^{c}	/	89.0 ± 4.3^{a}
HE control	266.7 ± 6.7^{b}	/	145.6 ± 10.2^{e}	53.4 ± 4.9^{c}	/	77.2 ± 5.4^{b}
HE *A. niger*	195.6 ± 13.9^{c}	/	306.7 ± 17.6^{c}	51.8 ± 2.0^{c}	/	86.4 ± 4.6^{ab}
HE *A. oryzae*	135.6 ± 10.2^{d}	/	155.6 ± 3.8^{de}	51.3 ± 1.4^{d}	/	77.6 ± 2.9^{ab}

AE-IP: alkaline extraction-isoelectric precipitation; SE: salt extraction-dialysis. DCP: unfermented defatted CPI control prepared from defatted unfermented cold-press meal using AE-IP or SE. /: no obvious foams were found, or foams disappeared in 5 min.

4.4. Conclusions

After a 72-hour period of SSF, significant differences were found in all canola protein functionalities compared to the controls (protein extracted from unfermented meal). All functionalities decreased to a varying degree in different treatments (fungal strains and meal types) with the exception of water/oil holding capacity showing stable or increased results. The two types of fungal strains showed different effects on the functionality of canola protein products extracted from fermented meals, which indicated the importance of strain selection on SSF. The differences between functionality of canola proteins extracted with alkaline extraction-isoelectric precipitation (AE-IP) and salt-extraction dialysis (SE) methods were mainly due to the complexity of canola protein fractions (different isoelectric points and molecular weights). With SE method, this problem could be relatively overcome and canola proteins with better functionality (solubility, foaming and emulsifying properties) could be extracted. In addition, canola proteins extracted from cold-pressed meal showed better functionality than those from hexane-extracted meal. The major reason can be explained by the processing conditions (high temperature and pressure) during oil extraction, which lead to extensive denaturation of HE meal proteins resulting in poorer functionality compared to proteins from CP meal. The partial hydrolysis of protein during the fermentation process may change the compact protein structure and expose more hydrophilic and hydrophobic sites (additional binding sites available for water) buried inside the protein molecule, which may decrease the solubility of canola proteins while increasing the ability of protein to adsorb and hold water/oil (Kinsella, 1982). In addition, the released short peptides may also have an impact on protein functionality such as a reduction in emulsion activity and stability (Kristinsson & Rasco, 2000). More studies on the mechanism of SSF and the relationship between SSF and physicochemical functionality of canola protein are required for a better understanding of the effect of SSF on canola proteins, which can further extend the utilization of SSF and canola protein.

5. OVERALL DISCUSSIONS

CP and HE canola meals are the converting substrate used in the two studies. They are from two different oil extraction processes practiced for canola seed. According to composition analysis, CP meal contained ~36% of protein and ~12% oil, while HE meal contained ~39% of protein and 2% of oil. After a SSF treatment with *Aspergillus niger* NRRL 334 and *A. oryzae* NRRL 5590, both CP and HE canola meals showed a change in the contents of phytic acid, total phenolic compounds, oil and protein. An increase in crude protein content was found in all samples, regardless of meal type or the ferment fungal strain. This is mainly due to the concentration effect that carbon dioxide was produced from fermentable carbohydrates (sucrose, oligosaccharides and sugars of fibre fraction as carbon source) during the growth and metabolic activities of fungi and lost, which resulted in an increase in nitrogen (crude protein) concentration. During SSF, fungi could also convert canola protein into fungi protein or the chitin of fungi cell walls as non-protein nitrogen (Jessika *et al.*, 2017) as mycelial growth progressed during fermentation. A relatively high degree (~45%) of protein hydrolysis was found in both CP and HE meals after SSF and the resulting peptides and amino acids were included in nitrogen-based protein content. A significant reduction in crude fat content in CP meal, decrease in phytic acid and total phenolic compounds were also reported after SSF. The high residual oil content in the starting CP meal was available for *A. niger* NRRL 334 and *A oryzae* NRRL 5590 without significantly affecting the growth and allowed meal composition modification with a reduction in oil and antinutritive phytic acid and total phenolic compounds. Enzymes secreted from fungal cells include lipases, proteases and phytases and played a critical role during the fermentation processing. In addition, a longer fermentation time (72 h) resulted in a higher reduction of crude fat in CP meal (from 13% to 9%), phytic acid (PAC, 74% to 85%) and total phenol content (TPC, 65%-81%) compared to 24 and 48 h. The higher increase in crude protein content of CP meal, from ~33% to ~37%, was in part due to the reduction of lipid content. CP meal also recorded a higher degree of protein hydrolysis reaching ~50% after 72 h. The above changes were correlated with the fermentation time and an optimal result was found at the time of 72 h.

As for the two fungal strains, *A. niger* NRRL 334 and *A oryzae* NRRL 5590, had similar effects during the fermentation. However, *A. niger* NRRL 334 showed a better performance in reducing the level of PAC of CP meal and TPC in HE meal indicating the canola meal system is more favored by *A. niger* NRRL 334 for its growth and concomitant modification of meal composition. During SSF, the strain *A. oryzae* NRRL 5590 produces neutral and alkaline proteases, amylases (α-amylase and glucoamylase), glutaminase, metallopeptidases, carboxypeptidases and tyrosinase (Liang *et al.*, 2009). In contrast, the *A. oryzae* may have limited ability to digest insoluble cellulose or raw and granular starch due to lack of glycosyl hydrolases with a cellulose- and starch- binding domain (Fedorova *et al.*, 2005). Whereas, α-amylase, arabinofuranosidase, catalase, glucoamylase, glucose oxidase, pectin esterases, phospholipase A_2, phytase, and xylanase activities are always related to *A. niger* (Pariza and Johnson., 2001; Frost and Moss., 1987), which help the growth in canola meal. Although enzymes to break down proteins and structural carbohydrates are reported for both, *A. niger* NRRL 334 and *A oryzae* NRRL 5590, lack of specific enzymes (*e.g.*, lipases) to hydrolyze the oil is significant when CP meal was utilized as fermenting substrate. Thus, both the strains were proven to be unable to decrease the crude fat content to desired low levels (~2%) similar to the levels achieved in a chemical defatting process. Several secondary metabolites, which may be considered as food additives or pharmaceuticals such as citric acid and azaphilones (add colour and as antioxidants in food) are produced by fermentation with *A. niger* and *A oryzae*. These metabolites include pigments, acids and other organic compounds, which may result in changes of colour, flavour, smell and pH of canola meal during fermentation (Frisvad *et al.*, 2018). In addition, previous studies have reported that culture used in SSF often enhances the enzyme production, especially amylases and proteases (Narahara *et al.*, 1982) and explains the high degree of protein hydrolysis (~45%) observed in fermented canola meals of the present study.

Above results indicate the potential of SSF to add nutritional value to canola meal by converting carbohydrates of the fibre fraction into a protein-rich biomass while simultaneously reducing the contents of antinutrients. Therefore, SSF could be used as a potential pre-treatment for both CP and HE meals to generate a more digestible product with enhanced nutritional value for animal feed and even for the food industry.

The protein products obtained from CP and HE canola meals upon 72-h SSF exhibited significant differences in the functionalities compared to the protein extracted from respective

unfermented meal, regardless of the ferment stain involved. All measured functional properties showed lower values except for water/oil holding capacity with unchanged or increased levels. The changes of these properties were to a varying degree depending on the fungal strain and meal type. The partial hydrolysis of protein during fermentation process as indicated by DH may change the compact protein structure and expose more hydrophilic and hydrophobic sites making additional binding sites available to interact which made it easier for protein to adsorb and hold water/oil (Kinsella, 1982). Canola proteins of CP meal showed significantly ($p < 0.05$) different values for the functional properties than those obtained from HE meal. The major reason can be the high temperature and pressure conditions that HE meal is exposed to during the oil extraction process and desolventization which can lead to denaturation of protein resulting in low values for functional properties compared to canola proteins from CP meal.

In addition, the functionality of canola proteins extracted from fermented meals showed a difference with the type of fungal strain (*A. niger* NRRL 334 and *A oryzae* NRRL 5590) used for SSF indicating the importance of strain selection on SSF. The reason is mainly due to the differences in growth conditions in the fermenting system that relates to enzyme production between *A. niger* NRRL 334 and *A oryzae* NRRL 5590.

Protein products extracted using AE-IP and SE on the same canola meal resulted in differences in functionality. The conditions employed in these two processes were different, therefore the two major proteins of canola meal, napin and cruciferin, may have been in different levels in the products obtained because of their differences in solubility properties at different pH and salt levels. Different levels of napin and cruciferin may be the primary difference of canola products obtained from AE-IP and SE which affected the functional properties. Napin (~pH 11) and cruciferin (pH 5-7) have different isoelectric points (Tan, 2011a). In addition, a significantly lower protein solubility of AE-IP products was reported at pH 3 and 7. In the process of AE-IP, more cruciferin may be recovered in the protein products and lead to a cruciferin-rich product. However, the pH manipulation is not involved for SE products. This may be one reason that protein products of AE-IP had low solubility at pH 3 and 7 comparted to SE protein. However, SE method resulted in a much lower protein yield.

Accordant to Vioque *et al.* (2000), canola protein products extracted using AE-IP have poor emulsifying properties. Townsend and Nakai (1983) suggested a positive correlation between hydrophobicity and emulsifying properties of proteins. During SSF, the partial hydrolysis of

protein may loosen the compact protein structure and expose more hydrophobic sites buried inside the protein molecule. According to the present study, canola proteins extracted from fermented meals showed higher or unchanged emulsifying properties compared to controls obtained from unfermented meals. Canola protein products extracted by AE-IP showed higher EAI than those extracted using SE. The variation in protein composition of CPI from the two extraction methods may have also contributed to this observed difference. Chang *et al.* (2015) suggested that the presence of napin is likely related to the low emulsifying ability of canola proteins due to its strongly basic nature (isoelectric point > 10). The presence of basic amino acids in napin could interrupt the electrostatic interactions between protein molecules (Schwenke 1994), which may lead to deteriorated effect on emulsions. The possible different contents of napin and cruciferin in the protein isolates obtained by AE-IP and SE methods may affect the emulsifying properties. In addition, ~45% DH was found after 72-h SSF. Xue *et al.* (2009) studied the effect of DH on emulsifying properties of canola protein. The emulsifying properties showed an increase up to 5% DH, however, a continued increase of DH up to 15% and higher could lead to a loss of the EAI and ESI. The reduction in emulsion activity and stability has been explained to be due to the presence of short peptides from protein hydrolysis, which are less favored in interfacial tension reduction (Kristinsson and Rasco, 2000).

As for the foaming properties, all samples showed a significant decrease in both FC and FS, while complete loss of foaming ability was observed for some protein products from fermented meals. Solubility of protein is one of the most important factors that contributes to foaming properties, higher solubility is related to higher foaming properties (Aluko and McIntosh 2001). For FS, the ability of proteins holding water in the film surrounding the air particle is critical (Kinsella *et al.*, 1985). Therefore, the solubility differences of constituent proteins in the AE-IP and SE products may be one of the possible explanations for SE proteins showing higher FC and FS values than AE-IP proteins. The differences of protein composition (contents of napin and cruciferin) relates to the differences in the conditions used in extraction methods. A significant ($p < 0.05$) decrease in solubility was found in canola protein products of fermented meals which may have led to poor foaming properties.

According to the study of Xue *et al.* (2009), functional properties (foaming capacity and the emulsifying properties) of canola proteins were improved by the hydrolysis process at low DH (5%-15%). In addition, hydrolyzed napin (albumin) isolate tends to present better functional

properties than hydrolyzed cruciferin (globulin) isolate except for foaming stability and fat adsorption. The difference in the hydrophilic and polar nature between napin and cruciferin isolates might be one of the possible reasons (Chabanon *et al.*, 2007) which could further affect the functionality of extracted canola protein. Thus, it can be suggested that a limited and controlled SSF may help to improve the functional properties of canola protein. However, more studies are needed to better understand the effect of SSF on functional properties of canola protein.

The studies presented in this thesis highlight the potential of SSF to improve the nutritional value of canola meal and also to modify functionalities of the extracted proteins. As mentioned above, the well-balanced amino acid profile of canola protein and comparable functionality to the other plant proteins provide the potential of canola as a plant protein source. However, canola proteins have limitations in its colour and flavour like most vegetable proteins. Thus, it would be an important point to improve these attributes to further expand the spectrum of applications of canola proteins in feed and food industries. With certain pre-treatment such as heat or SSF and optimized extraction methods it is possible to eliminate the antinutritional factors and objectionable taste and colour of the canola proteins, which are major limitation for extending their use, particularly in human consumption. The functional properties of canola proteins from unfermented meals were interesting and could be applied to certain food formulations. The solubility and foaming properties of SE products from fermented meals appear to be the most promising at certain pH values. However, protein modifications to further improve emulsifying capacity of these proteins may be needed to extend the potential applications in emulsion-type formulations. In study 2, the effect of SSF on canola protein functionality was studied. An increase in emulsifying properties was reported in canola protein products. However, there is a lack of knowledge on mechanism of SSF, therefore the relationship between SSF and physicochemical properties of canola protein remain incomplete. As for the nutritional value, canola proteins could compensate the deficiency or lack of certain amino acids such as sulfur-containing AAs (methionine and cysteine) in other plant proteins and therefore may be a suitable protein for blending to optimize essential amino acids. Overall, this thesis provides a reference for the utilization of SSF on canola meals or other plant-based materials and prediction of possible results according to the fermentation conditions for future studies. As for protein extractability, there was no significant evidence in this study to prove the ability of SSF to improve the protein extraction

process. More research is needed to understand the detailed effect of SSF on protein extractability and mechanism during the fermentation process to make better use of SSF on canola meals.

6. OVERALL CONCLUSIONS

Studies were conducted to investigate the effect of SSF of CP and HE canola meals with *A. niger* and *A. oryzae* on the composition and the protein products obtained from the fermented meals. Results of the investigations showed the potential of SSF to modify the composition of both CP and HE canola meals using *A. niger* and *A. oryzae*. The changes of composition included increase in levels of protein, reduction in phytic acid and total phenolic compounds and decrease in crude lipid content. A positive correlation between fermentation time and the levels of change (increase or decrease) of selected compositional parameters was observed for both meals and both ferment strains. An optimal increase in protein content (by ~33% to ~37% for CP meal) and reduction on PAC (by 74% to 85%), TPC (by 65% to 81%) and crude lipid content (from 13% to 9% for CP meal) were found at 72-h fermentation time. In addition, a degree of protein hydrolysis of ~50% was reported after 72-h SSF for all samples. There is a significant difference in composition between HE and CP control meals, which further led to significantly different results in final composition of fermented CP and HE meals. In terms of fermentation time and meal types, a significant difference in modification of meal composition was found between meals fermented with *A. niger* and *A. oryzae*.

According to above results, the hypothesis 1 that SSF could improve the nutritional value of HE and CP meals using *A. niger* and *A. oryzae* was possible. SSF improved the nutritional value of HE and CP meals by increasing the levels of protein while decreasing the levels of the antinutrients phytic acid and total phenolic compounds. In addition, the hypothesis 2 that the conditions including fermentation time, types of meals and strains could affect the results of SSF was true. An optimal result was found at 72-h fermentation time. CP meal was more favored due to a higher increase in protein content and reduction on PAC compared to HE meal. As for the two strains, similar results were found when used to ferment the same meal.

The investigations conducted under study 2 showed significant changes in functional properties of both CP and HE canola protein products obtained using AE-IP and SE methods from fermented meals with *A. niger* and *A. oryzae*. The functional properties that changed due to SSF

included protein (nitrogen) solubility, foaming properties (FC and FS values) and emulsifying properties (EAI and ESI values) at pH 3, 5 and 7, plus WHC and OHC at original sample pH value. Canola protein products obtained from AE-IP showed significant differences in protein content and functionality compared to SE products. Products extracted using SE tended to show higher functionality values than products extracted using AE-IP, except for WHC and EAI at pH 3.

A significant ($p < 0.05$) difference in changes of functionality of protein products was seen between the two types of fungal strains (*A. niger* and *A. oryzae*) and types of meals (CP and HE). CP meal was favored for changes in WHC, OHC, EAI and solubility at pH 7, while HE was favored for the changes in foaming at pH 7. Depending on the desired functional property to be modified in a particular meal, the ferment strain can be selected.

According to above results, the hypothesis 3 that there would be significant differences in the functional properties of protein products, including protein (nitrogen) solubility across various pH, water/oil holding capacity, emulsifying and foaming properties, based on protein extraction method was possible and available. In addition, differences in functionality of protein extracted were found based on the type of meals (CP and HE) and strains (*A. niger* and *A. oryzae*) used in SSF.

Overall, biotransformation or pre-treatment using SSF on CP and HE meals using *A. niger* strain and *A. oryzae* strain could provide canola meals with higher nutritional value, which may provide more opportunities for canola meal. The ability of SSF to modify functional properties of canola meal protein could extend a new direction of canola meal utilization.

7. FUTURE RESEARCH

Further studies on the modification of canola meal composition by SSF will be very useful for the canola industry. Investigating growth parameters for *A. niger* NRRL 334 and *A oryzae* NRRL 5590 including pH, moisture content, temperature, culture composition (single vs mixed culture), can be studied to optimize the SSF procedure for both CP and HE canola meals. In addition, studying suitable GRAS fungal stains that can better utilize lipids in CP meal will be essential to improve uses of the meal. CP meal protein already showed better potential than HE meal. Scaling up of the fermentation process is required for validating the use for both canola and plant protein industries.

Assessment of the effect of SSF on canola protein, recovery rate and yield and a more detailed functional property assessment is needed. The understanding of the relationship between the physicochemical characteristics and the functions of protein hydrolysis due to SSF can lead to broader utilization of canola proteins. In addition, the relationship between the functionality of hydrolyzed canola protein and peptides generated during hydrolysis needs further investigation. In other words, the exact mechanism of how SSF or enzymatic hydrolysis on canola protein functionality modification remains unclear. The effect of SSF on the quality of final products including texture, appearance (colour), flavour and consumer acceptance should also be considered. Studies also reported the presence of potential bioactive peptides in canola protein hydrolysates, which can be used in functional foods in the prevention and/or treatment of hypertension (Marczak *et al.*, 2003). These peptides could be used as nutritional additives in cell culture media to improve cell growth (Farges-Haddani *et al.*, 2006). Xue *et al.* (2009) later studied the possible conversion of canola proteins into functionally active ingredients for food applications. These studies showed the potential of canola proteins to be used as a source for economical production of bioactive peptides for nutraceutical applications. Investigation of the bioactivity of canola protein is therefore highly relevant to the canola industry. This line of study remains relatively unexplored and could lead to interesting discoveries.

Aluko and McIntosh (2005) partially replaced egg yolk with unmodified or enzymatically modified canola proteins in mayonnaise preparations. They proved the ability of canola protein to be used as emulsifier to stabilize the mayonnaise emulsion and showed that canola protein has the potential to be a suitable ingredient in the formulation of food emulsions. This is an example where further studies can be directed to extend the usage of canola proteins in the food industry. It is expected that future research will focus on developing improved extraction technology and pre-treatment such as enzymatic hydrolysis brought out by fermentation of canola meal or protein, in order to modify and enhance the functional properties of canola proteins required in applications.

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